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Abstract

Biotin-Containing Enzymes from Brassica napus and Arabidopsis thaliana.

By Jonathan E. Markham

Seven biotin-containing proteins were detected in different stages of developing *Brassica napus* seeds with M_rs of 220, 150, 76, 71, 65 35 and 30kDa. The 220 kDa protein is Type I acetyl-CoA carboxylase. The 35kDa protein was detected in purified *Brassica napus* chloroplasts and identified as a subunit (BCCP) of a multi-subunit, dissociable (Type II) acetyl-CoA carboxylase. The BCCP subunit was regulated in a pattern consistent with that of lipid accumulation and was differentially expressed as multiple isoforms when detected by 2-D Western blots and RT-PCR. Antibodies against the BCCP subunit detected both BCCP and the 30kDa proteins suggesting that they are related. Evidence shows that the 76kDa protein is β-methylcrotonyl-CoA carboxylase and the sequence of the cDNA from *Arabidopsis thaliana* is reported. The remaining 150, 71 and 65kDa proteins were postulated to be geranyl-CoA carboxylase, pyruvate carboxylase and the SBP65 storage protein respectively.

The biotin carboxylase subunit of Type II acetyl-CoA carboxylase was cloned from *Brassica napus* and the sequence is reported. Antibodies against overexpressed biotin carboxylase protein detected a 48kDa protein in extracts from *Brassica napus* seeds which was regulated in a pattern similar to the BCCP subunit. Evidence was also presented to indicate that these two subunit may be able to interact *in vitro*.

A cDNA encoding a biotinylated protein isolated from an *Arabidopsis* library was sequenced. This revealed that no conserved biotinylation domain was present and over-expression of the protein demonstrated that the biotinylation was an artefact. The cDNA clone encodes for a DNA helicase as it shows 63.7% identity to the Human Bloom's syndrome protein and the protein sequence shows 38% identity with the human recQ. This represents the first reported cloning of a DNA helicase from plants.



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Biotin-Containing Enzymes from *Brassica napus* and *Arabidopsis thaliana.*

By

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A thesis submitted for the degree of Doctor of Philosophy at the University of Durham

Department of Biological Sciences
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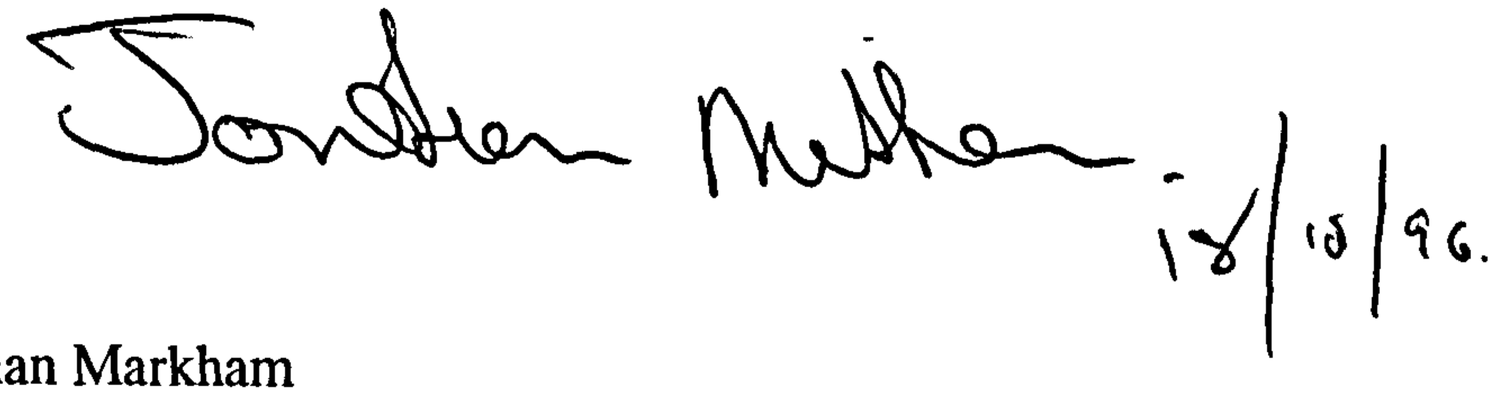
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Declaration

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Jonathan Markham

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Abbreviations

Abbreviations were avoided wherever possible but some abbreviations for frequently used terms were used to aid the reader and are listed below. Abbreviations for biological terms are defined at their first occurrence.

ADP	Adenosine diphosphate
Amp	Ampicillin
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
BCIP	5-Bromo-4-Chloro-3-Indolyl Phosphate
BSA	Bovine Serum Albumin, fraction V
cpm	Counts per minute
cDNA	Complementary DNA
Cm	Chloramphenicol
CoA	Coenzyme A
daf	Days after flowering
ddH_2O	Distilled, deionised water (18.2 MΩ electrical resistance at room temperature)
dsDNA	Double stranded DNA
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease I
dNTP	Deoxyribonucleoside triphosphate
DTT	Dithiothreitol

EDTA	Ethylenediaminetetraacetic acid
HEPES	N-[2-Hydroxyethyl]piperazine-N'-[2-ethane-sulphonic acid])
hnRNA	Heteronuclear RNA
HRPO	Horse radish peroxidase
IPTG	Isopropylthiogalactopyranoside
kan	Kanamycin
kDa	Kilo daltons
MES	2-[N-morpholino]ethanesulphonic acid
MCS	Multiple Cloning Site
MOPS	3-[N-morpholino]propanesulphonic acid
mRNA	Messenger RNA
NADH	β-Nicotinamide adenine dinucleotide, reduced form
NBT	Nitro Blue Tetrazolium
OD_{600}	Optical density (absorbance) at wavelength 600 nm
ORF	Open Reading Frame
PAGE	Polyacrylamide gel electrophoresis
PBS(-T)	Phosphate Buffered Saline (with 0.05% Tween)
PCR	Polymerase Chain Reaction
p.f.u.	Plaque forming units
P_i	Inorganic phosphate
pI	Isoelectric point
PMSF	Phenylmethylsulphonyl fluoride

polyA$^+$	Poly adenylated
rpm	Revolutions per minute
RNase	Ribonuclease, pancreatic
SDS	Sodium dodecyl sulphate
ssDNA	Single stranded DNA
TEMED	N,N,N',N'-tetramethylethylene diamine
Tet	Tetracycline
Tm	Annealing temperature
Tris	Tris-(hydroxymethyl)-methylamine-[2-amino-2-(hydroxymethyl)-propane-1,3-diol (tris)]
tRNA	Transfer ribonucleic acid
U	Unit of enzyme activity
UTR	Un-translated region
UV	Ultraviolet
var.	Variety
v/v	Volume for volume
v/w	Volume for weight
w/w	Weight for weight
X-gal	5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside

Chapter 1: Introduction

1.1 Biotin

Biotin was discovered in 1935 as an essential growth factor for yeast (Wood & Barden, 1977). It is a small molecule of 244.3 M_r. The absolute structure of biotin was resolved by X-ray crystallography (Trotter & Hamilton, 1966) and is shown in Figure 1.1.

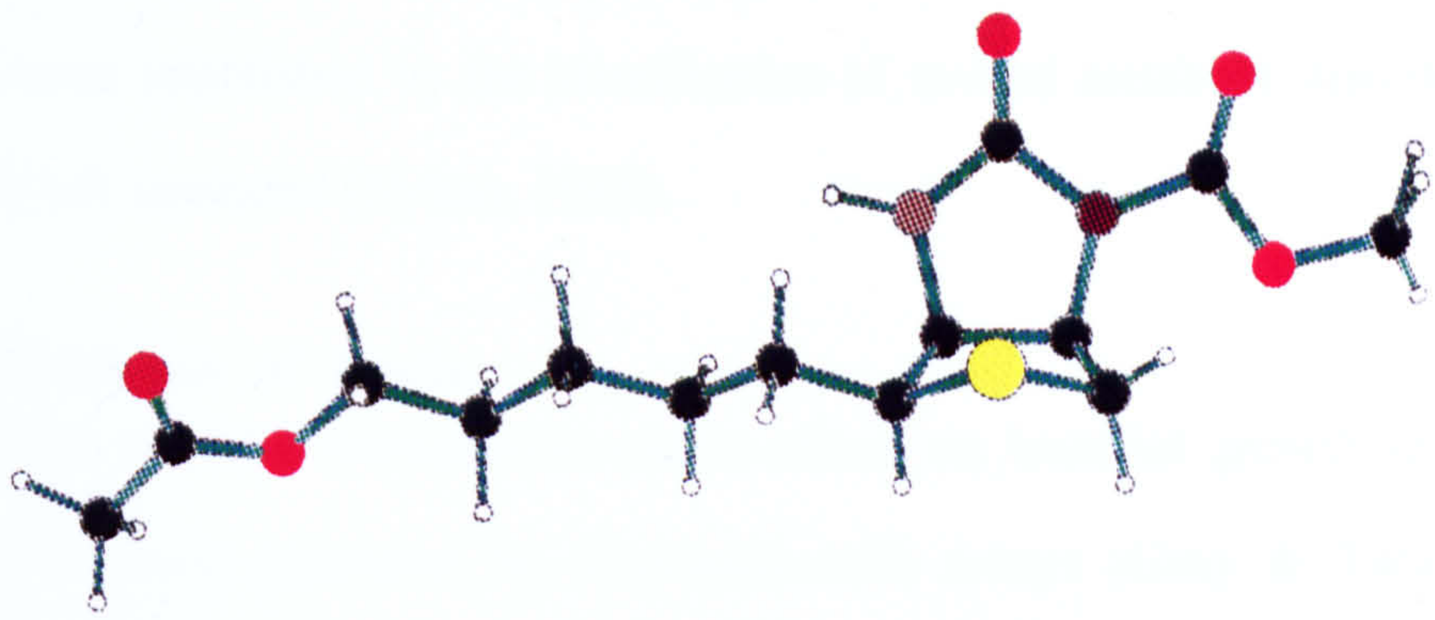


Figure 1.1 The structure of N1' methoxycarbonyl biotinyl acetate (Stallings & DeTitta, 1985).

The atoms are colour coded black, carbon; red, oxygen; yellow, sulphur and white, hydrogen.

1.1.1 Requirement for biotin

All organisms require biotin but it is only synthesised by bacteria and plants. The demonstration that biotin was required by bacteria was established by the isolation of mutants of *E.coli* which were unable to synthesise their own biotin and were dependent upon external biotin in the culture media for growth (Gray & Tatum, 1944). That plant cells need biotin was demonstrated by a mutant of *Arabidopsis thaliana* which causes defective embryo development (Shellhammer & Meinke, 1990). Development was arrested at a critical stage in embryogenesis at which time no biotin was detectable. The mutant phenotype was specifically rescued when grown in the presence of biotin. Presumably this mutant was deficient in one of the enzymes required for biotin biosynthesis in plants. Mammals are not normally deficient in biotin as it is synthesised by endogenous gut bacteria and absorbed through the

intestinal wall. This symbiosis can be interrupted by feeding large quantities of a protein which specifically binds biotin, avidin (Bitsch *et al.*, 1985). This led to a biotin deficiency disease. It was demonstrated that biotin was required for growth by several cultured mammalian cells lines, including HeLa cells, fibroblasts and BHK cells (Dakshinamurti *et al.*, 1985). In the absence of biotin, growth of cells in culture was arrested and viability is reduced. This appeared to be separate from the role of biotin in identified essential metabolic enzymes and could be linked to a regulatory protein within the cell nucleus. That humans have an absolute requirement for dietary biotin has been established by the identification of several metabolic disorders which are lethal if left untreated (Nyhan, 1985).

1.1.2 Synthesis of biotin

Early work on bacterial mutagenesis and its effects on bacterial growth involved the isolation of mutants generated by irradiation with x-rays (Gray & Tatum, 1944). Among the first to be isolated and characterised was *E.coli*-58, a mutant that required biotin for growth. Since then, many biotin requiring mutants have been isolated to help establish the biotin biosynthetic pathway shown in Figure 1.2.

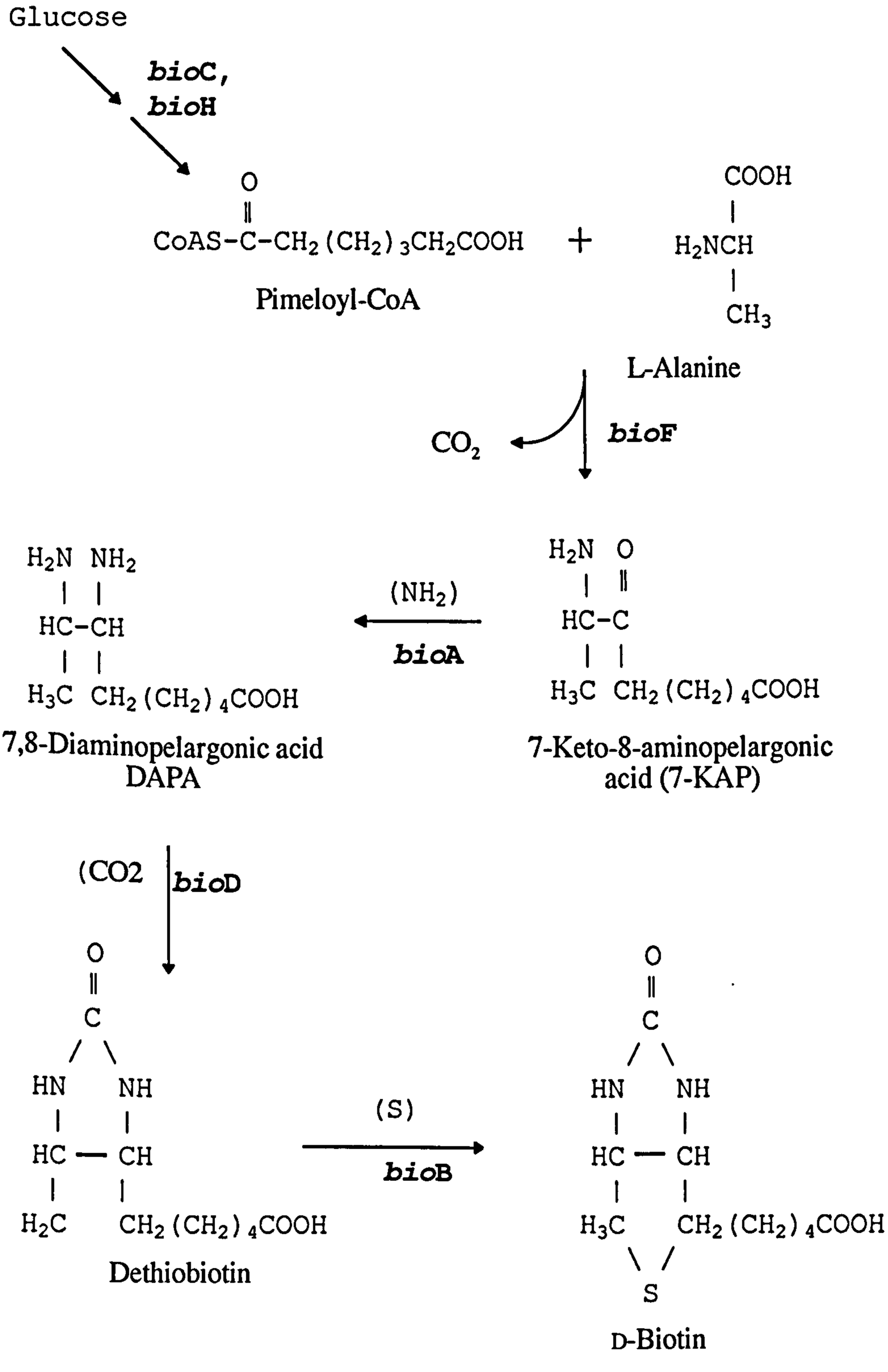


Figure 1.2 Biotin biosynthetic pathway in *E.coli*. The *bio* operon genes are indicated in bold.

1.1.3 The *bio* operon

The *bio* operon is amongst the most extensively studied of any vitamin due to the isolation of mutants for the biosynthetic and regulatory enzymes (Eisenberg, 1985). A map of the genes on the *E.coli* chromosome is shown in Figure 1.3. Although intensively studied, the last step in biotin biosynthesis remains elusive as the nature of the sulphur donor remains unknown, although it has been suggested that it may be from cysteine or methionine (Frappier & Marquet, 1981; DeMoll *et al.*, 1984). There have been reports of a sulphur containing intermediate (Salib *et al.*, 1979) but this has yet to be corroborated.

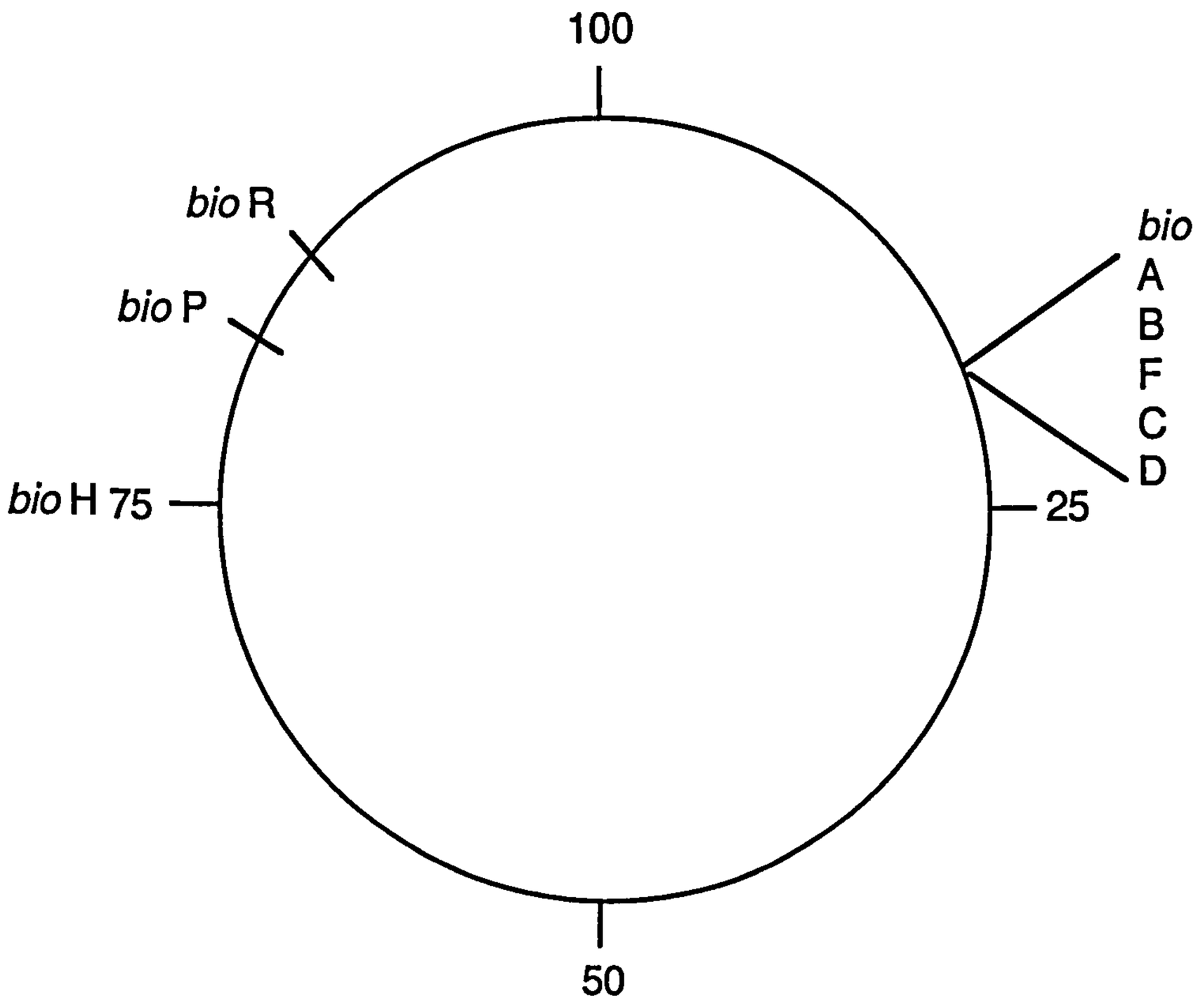


Figure 1.3 Map of the biotin genes on the *Escherichia coli* chromosome.

The biotin locus is situated on the *E.coli* chromosome next to well characterised loci. These occur in order the *gal*, λ *att*, *bio* on the *E.coli* chromosome(Eisenberg, 1985). The close proximity to the λ *att* enabled the biotin marker to be transducible and hence permitted the mapping of the operon to 17.5 minutes on the *E.coli* chromosome. Complementation showed the genes to be in the order A B F C D, as shown in

Figure 1.3, and this was confirmed by sequencing of the entire operon (Otsuka *et al.*, 1988). Also mapped but unlinked loci were *bio* H, *bio* P a permeability gene and *bio* R a regulatory gene. *bio* R has been characterised as the biotin holoenzyme synthetase which is a bi-functional enzyme in *E.coli* (Eisenberg *et al.*, 1982). The enzyme activates biotin in the presence of ATP to form D-biotinyl 5'-AMP, an activated form of biotin. This activated form of biotin can either be transferred to a specific lysine on the apo-biotin carboxyl carrier protein or remain bound to the biotin holoenzyme synthetase protein, which in turn enables it to bind to the operator site and inhibit transcription of the *bio* operon. The biosynthetic pathway, shown in Figure 1.2, appears to be common to both bacteria and plants (Baldet *et al.*, 1993b) although there is more evidence to suggest the presence of a thiol-intermeadiate in the biosynthesis of biotin from dethiobiotin in plants that in *E.coli*.

1.1.4 Biotin as a cofactor

Biotin is used as a cofactor by a small number of enzymes with a diverse set of metabolic functions. The biotin moiety is covalently attached to the ε-amino group of a specific lysine residue by its free carboxyl group. The attachment requires energy and this is provided by activating biotin with ATP to form biotinyl-5'-AMP. The enzyme holoenzyme synthetase is responsible for recognising the correct lysine and attaching biotin to it. This enzyme has recently been characterised from *Pisum sativum* (Tissot *et al.*, 1996) but there are no indications as to whether this protein has regulatory properties similar to the *E.coli* protein.

1.1.5 Biotin binding proteins

The discovery of biotin is closely linked to the discovery of a protein which binds biotin, avidin. Avidin is a minor constituent of egg white and is able to induce nutritional deficiency in rats by forming an extremely stable non-covalent complex with what was subsequently proven to be the vitamin biotin (Gyorgy, 1954). The biological role of avidin appears to be as a scavenger for biotin, inhibiting microbial growth. This is possible through its extraordinarily high affinity for biotin. The

properties of avidin and those of a remarkably similar protein streptavidin from the micro-organism *Streptomyces avidinii* are summarised in Table 1.1.

Property	*Avidin*	*Streptavidin*
Amino acid residues	128	125-127
Subunit size		
From sequence	15,600	13,400
SDS gels	16,400	14,500
Subunits		
K_d biotin (M) (pH7, 25°C)	0.6×10^{-15}	4×10^{-14}
$t_{1/2}$ (days)	200	2.9

Table 1.1 Properties of the biotin binding proteins avidin and streptavidin (Green, 1990).

Avidin and streptavidin are by no means the only biotin-binding proteins, there is a lower affinity egg-yolk biotin-binding protein with a Km of $\sim 10^{-12}$ (Bayers & Wilcheck, 1990), a biotin binding protein in human and rat serum of 43kDa (Dakshinamurti *et al.*, 1985) and a monoclonal antibody (Dakshinamurti & Rector,1990) with a relatively low Km of $\sim 10^{-4}$. Avidin has been extensively characterised due initially to its very high affinity for biotin (Green, 1975). Increased interest was brought about by the discovery of the coenzyme function of biotin in CO_2 transfer (Wakil *et al.*, 1958) and by the subsequent use of avidin to identify biotin-containing enzymes.

1.2 The enzymes

There are nine known enzymes that use biotin as a cofactor. The common principle of the reaction catalysed is that CO_2 is transferred from a donor to an acceptor using the biotin moiety as carrier. The known enzymes can be divided into three classes; 1) carboxylases, enzymes which fix CO_2 from HCO_3^- to an acceptor molecule; 2) transcarboxylases, enzymes which transfer CO_2 between a donor and an acceptor; and 3) decarboxylases, enzymes which remove a carboxyl group as CO_2. The known biotin carboxylases are listed in Table 1.2.

Carboxylases	*Organisms*	*CO_2 donor*	*CO_2 acceptor*
Acetyl-CoA carboxylase	Animals[1], bacteria[1], plants[4] and yeast[12]	HCO_3^-	Acetyl-CoA
Geranyl-CoA carboxylase	*Citronella*[11], plants[2]	HCO_3^-	Geranyl-CoA
Methylcrotonyl-CoA carboxylase	Plants[15], animals[1], and bacteria[7].	HCO_3^-	Methylcrotonyl-CoA
Propionyl-CoA carboxylase	Animals[1], *Rhodococcus*[8].	HCO_3^-	Propionyl-CoA
Pyruvate carboxylase	Animals[10], fungi[14], plants[15], yeast[12]	HCO_3^-	Pyruvate
Decarboxylases	*Organims*	*CO_2 donor*	
Methylmalonyl-CoA decarboxylase	*Micrococcus lactilyticus*[3]	Methylmalonyl-CoA	
Oxalate decarboxylase	*Pseudomonas ovalis*[6], *Micrococcus lysodeklicus*[5].	oxaloacetate	
Urea carboxylase (hydrolysing)	*Saccharomyces cerevisiae*[9], *chlorella*[9]	Urea	
Transcarboxylases	*Organisms*	*CO2 donor*	*CO2 acceptor*
Transcarboxylase	*Propionibacterium shermanii*[13]	Methylmalonyl-CoA	Pyruvate

Table 1.2 Known biotin carboxylases and their source.

References [1] Alberts & Vagelos, 1972; [2] Caffrey *et al.*, 1995; [3] Galivan & Allen, 1968; [4] Hatch & Stumpf, 1961; [5] Herbert, 1955; [6] Horton & Kornberg, 1964; [7] Knappe *et al.*, 1961; [8] Nagy *et al.*, 1992; [9] Roon & Levenberg, 1968; [10] Scrutton & Young, 1972; [11] Seubert *et al.*, 1963; [12] Sumper & Riepertinger, 1972; [13] Swick & Wood, 1960; [14] Tozo *et al.*, 1992; [15] Wurtele & Nikolau, 1990

1.2.1 Reaction outline

The mechanism of reaction for the biotin dependent carboxylases has been deciphered (Knappe, 1970; Scrutton & Young, 1972; Alberts & Vagelos, 1972; Wood & Barden, 1977; Knowles, 1989). The reaction schemes for the three classes of carboxylases are summarised as follows;

<u>Class I - carboxylases</u>

$$enz-biotin + ATP + HCO_3^- \overset{Mg(II)}{\Longleftrightarrow} enz-biotin-CO_2^- + ADP + P_i + H^+$$

$$enz-biotin-CO_2^- + R-H \Longleftrightarrow enz-biotin + R-CO_2^-$$

Biotin is carboxylàted in the first step in an ATP dependent fashion using bicarbonate as the donor. In the second step, the carboxyl group is transferred to an acceptor such as acetyl-CoA, β-methylcrotonyl-CoA, geranyl-CoA, propionyl-CoA, pyruvate and urea.

<u>Class II - decarboxylases</u>

$$enz-biotin + R-CO_2^- \Longleftrightarrow enz-biotin-CO_2^- + R-H$$

$$enz-biotin-CO_2^- + 2(Na^+)_{in} \Longleftrightarrow enz-biotin + HCO_3^- + 2(Na^+)_{out}$$

Class II enzymes are a specialist group of decarboxylases found in anaerobes which mediate sodium transport coupled to the decarboxylation of β-keto acids and their thioesters such as oxaloacetate, methylmalonyl-CoA and glutaconyl-CoA

<u>Class III - transcarboxylase</u>

$$enz-biotin + oxalacetate \Longleftrightarrow enz-biotin-CO_2^- + pyruvate$$

$$enz-biotin-CO_2^- + propionyl-CoA \Longleftrightarrow enz-biotin + methylmalonyl-CoA$$

The final class contains one enzyme, transcarboxylase, that couples two carbon carboxylations.

Notwithstanding the varied nature of the substrates, the reaction schemes are all comparable and require a similar arrangement of reaction. Each class requires an

enzyme, or a component of the enzyme, to carboxylate the biotin moiety. The enzyme responsible for this reaction is the biotin carboxylase which is able to carboxylate free biotin in the presence of ATP, Mg(II) and HCO_3^- (Guchhait *et al.*, 1974). *In vivo* the biotin carboxylase acts upon the biotin carboxyl carrier protein subunit / domain of the enzyme to carboxylate the biotin. The specificity of the complete enzyme for the CO_2 donor is a result of this subunit. The carboxy-biotin intermediate is stable enough that it can be isolated (Scrutton & Young, 1972). The second reaction is the transfer of CO_2 from the biotin carboxyl carrier protein to the acceptor which requires no further energy input. This reaction is catalysed by the carboxyltransferase subunit, and this in turn determines the specificity of the enzyme for the CO_2 acceptor.

1.2.2 Mechanism of reaction

The mechanism of pyruvate carboxylase from mammalian sources has been deciphered (Scrutton & Young, 1972).

$$\text{enz-biotin} + \text{MgATP}^{2-} + \text{HCO}_3^- \overset{Me^{2+},\,Me^{+}}{\Longleftrightarrow} \text{enz-biotin-CO}_2^- + \text{MgADP}^- + P_i \quad (1)$$

$$\text{enz-biotin-CO}_2^- + \text{pyruvate} \Longleftrightarrow \text{enz-biotin} + \text{oxalacetate} \quad (2)$$

Product inhibition studies have shown that the reaction mechanism is two site Ping-Pong Bi Bi Uni Uni in which the two partial reactions (1) and (2), occur at separate catalytic sites as shown in Figure 1.4. This might be expected for the *E.coli* enzyme acetyl-CoA carboxylase which is readily dissociable into subunits with separate activities (Alberts & Vagelos, 1972; Guchhait *et al.*, 1974). Pyruvate carboxylase has been isolated from a number of animals (Wood & Barden, 1977) and the enzyme consists of a single polypeptide. In this case the two separate catalytic activities (biotin carboxylase and carboxyltransferase) are located on the same polypeptide with the biotin moiety communicating between them.

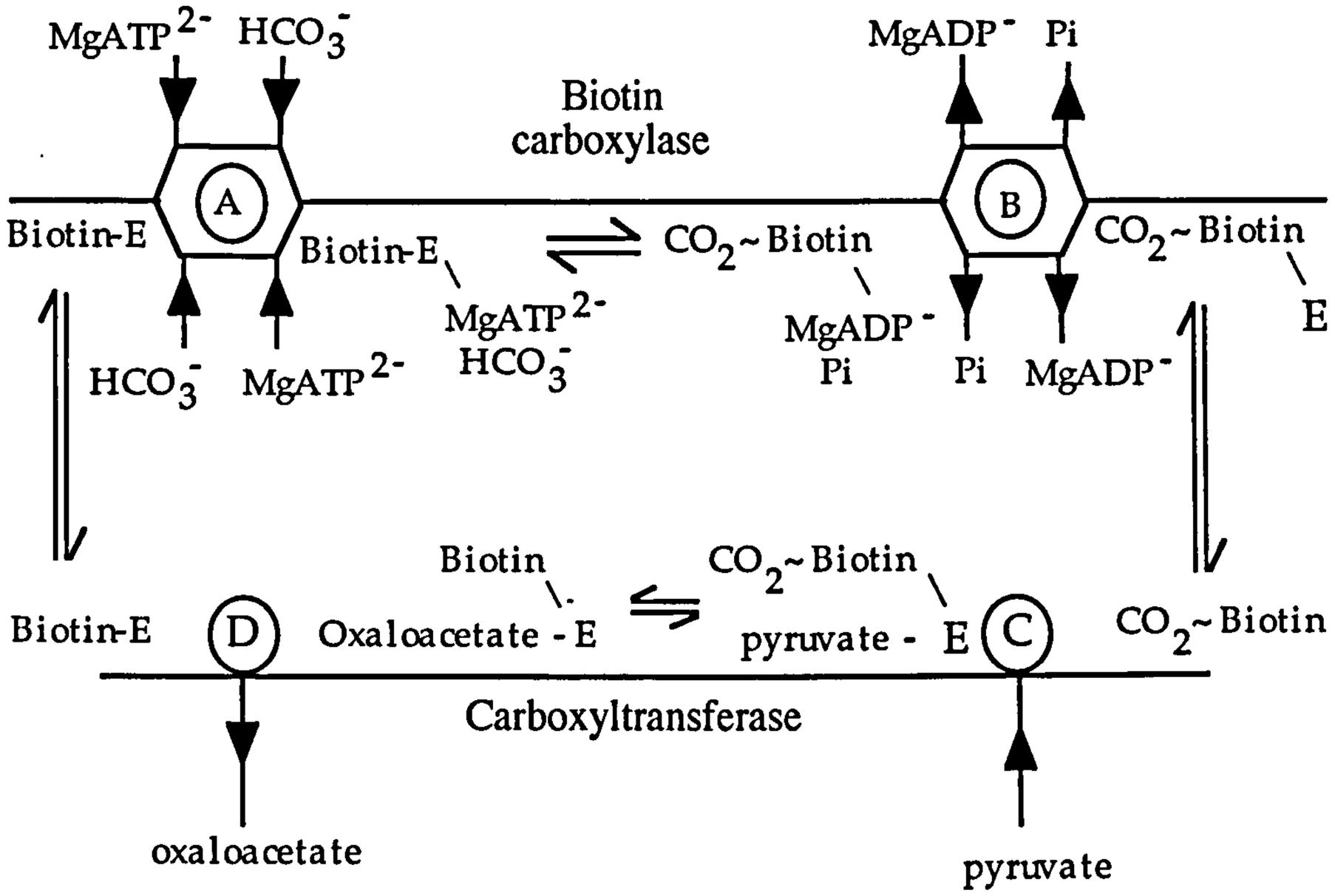


Figure 1.4 A kinetic scheme describing the reaction catalysed by pyruvate carboxylase.

Evidence suggests that this mechanism maybe applicable to all biotin enzymes (Scrutton & Young, 1972).

The two reactions A→B and C→D occur at the two reaction sites biotin carboxylase and carboxyltransferase respectively. The reaction from B→C and from D→A involves the isomerisation of the enzyme in which the biotin residue moves between catalytic sites. The isomerisation is possibly a result of the difference between the carboxylated and non-carboxylated state of the biotin.

1.2.3 The carboxylation of biotin

The structure of N1' methoxycarbonylbiotinyl acetate is shown in Figure 1.1. It has been demonstrated that biotin is involved in CO_2 transfer in acetyl-CoA carboxylase (Wakil *et al.*, 1958), and isolation of carboxybiotin intermediates has demonstrated conclusively that biotin is the carrier of CO_2 in such reactions (Scrutton & Young, 1972; Knowles, 1989). The isolation of biotin from the biotin carboxylase reaction showed that biotin is carboxylated at the N_1 position. The precise mechanism of how this occurs has been carefully reviewed (Knowles, 1989) and the tentative summary reached is shown in Figure 1.5.

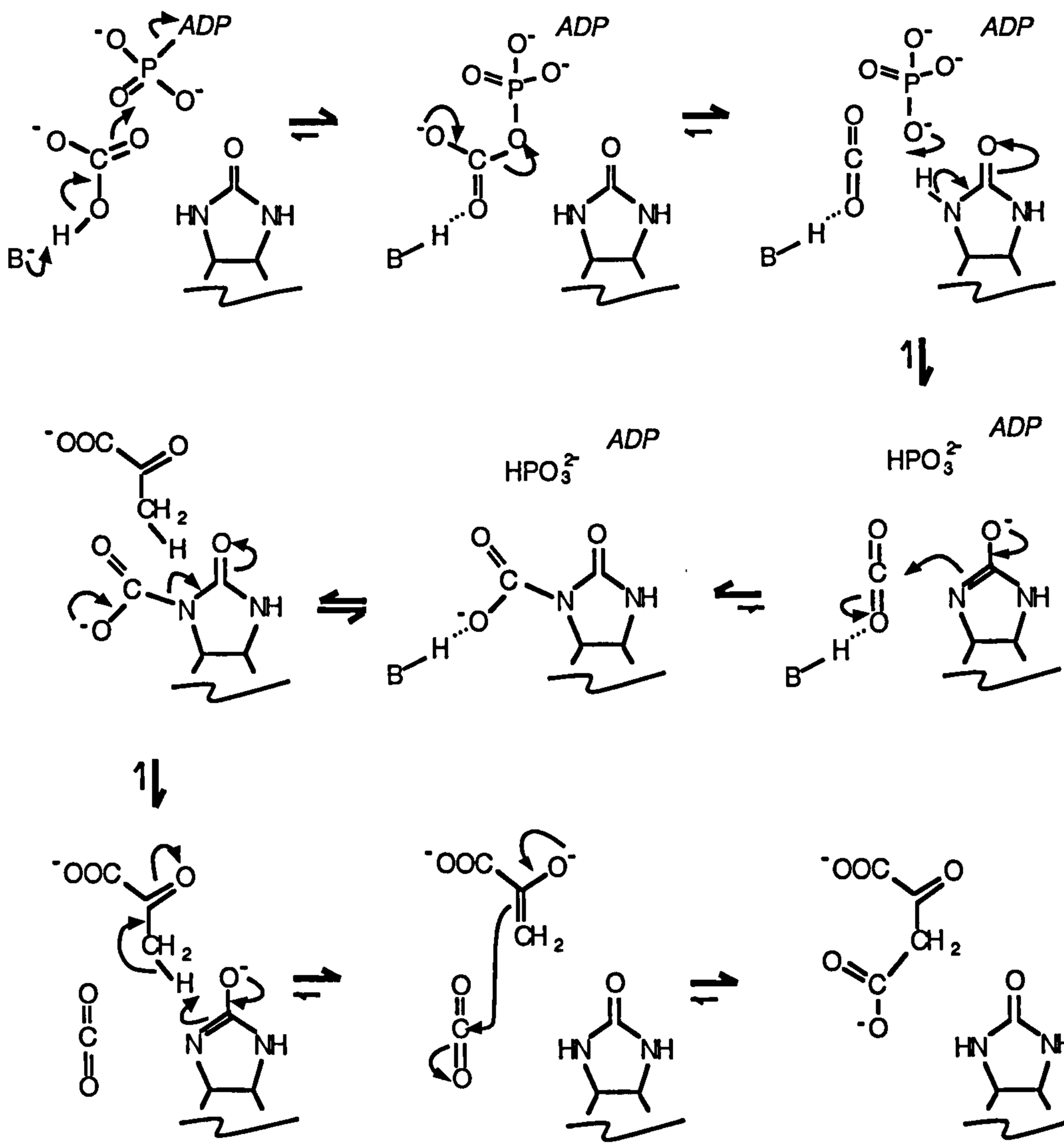


Figure 1.5 Mechanistic pathway for the biotin dependent carboxylases (Knowles, 1989).

The figure shows the carboxylation of pyruvate but the reaction is probably similar for any CO_2 donor or acceptor. At both steps, the localised production of CO_2 as the electrophile is proposed.

Biotin acts as a CO_2 carrier as shown in Figure 1.5. Its role is to act as a nucleophile in the biotin carboxylase step and the carboxyltransferase step and as a carrier of CO_2 between the two active sites. The role of the sulphur atom in the thiolane ring of biotin is not explained by this mechanism as only the ureido ring is featured. That the sulphur group is important is demonstrated by the fact that although *O*-heterobiotin (in which oxygen replaces sulphur) can be incorporated into transcarboxylase, the enzyme is inactive (Knowles, 1989). Speculation suggests that the role of the thiolane ring may be to stabilise the carboxybiotin intermediate to prevent immediate

decarboxylation before the biotin isomerises from the biotin carboxylase catalytic site to the carboxyltransferase site. This would account for the long half-life of carboxybiotin, >100 minutes at pH8.

Biotin, its structure and function in biotin-containing enzymes, has been intensively studied and the essential elements of the reaction unravelled. Some aspects of the reaction remain speculative and will probably only be resolved by characterisation of a biotin-containing enzyme at atomic resolution. What is clear, is that biotin-containing enzymes all work by a similar mechanism and must therefore have features in common around the catalytic sites.

1.2.4 The structure of biotin-containing enzymes

Enzymes that utilise biotin as a cofactor have three structural properties. An active site for each of the half reactions and an element that links the two sites by allowing the carboxybiotin to isomerise between the two. In the simplest of cases, acetyl-CoA carboxylase from *E.coli*, the enzyme consists of four subunits. The enzyme is readily dissociable into an ATP-dependent biotin carboxylase that performs the first half reaction, an acetyl-CoA carboxyltransferase which catalyses the second half reaction and a carboxyl carrier protein which contains the covalently attached biotin that shuttles the carboxyl group between the two active sites (Guchhait *et al.*, 1974).

Enzymes from other organisms have a similar makeup in that they consist of three recognisable subunits. The main difference is that in certain cases these subunits are to be found fused into a single polypeptide. These single polypeptides contain areas distinguishable as the two catalytic sites and the biotin carboxyl carrier domain. A diagram showing the range of fusions is shown in Figure 1.6

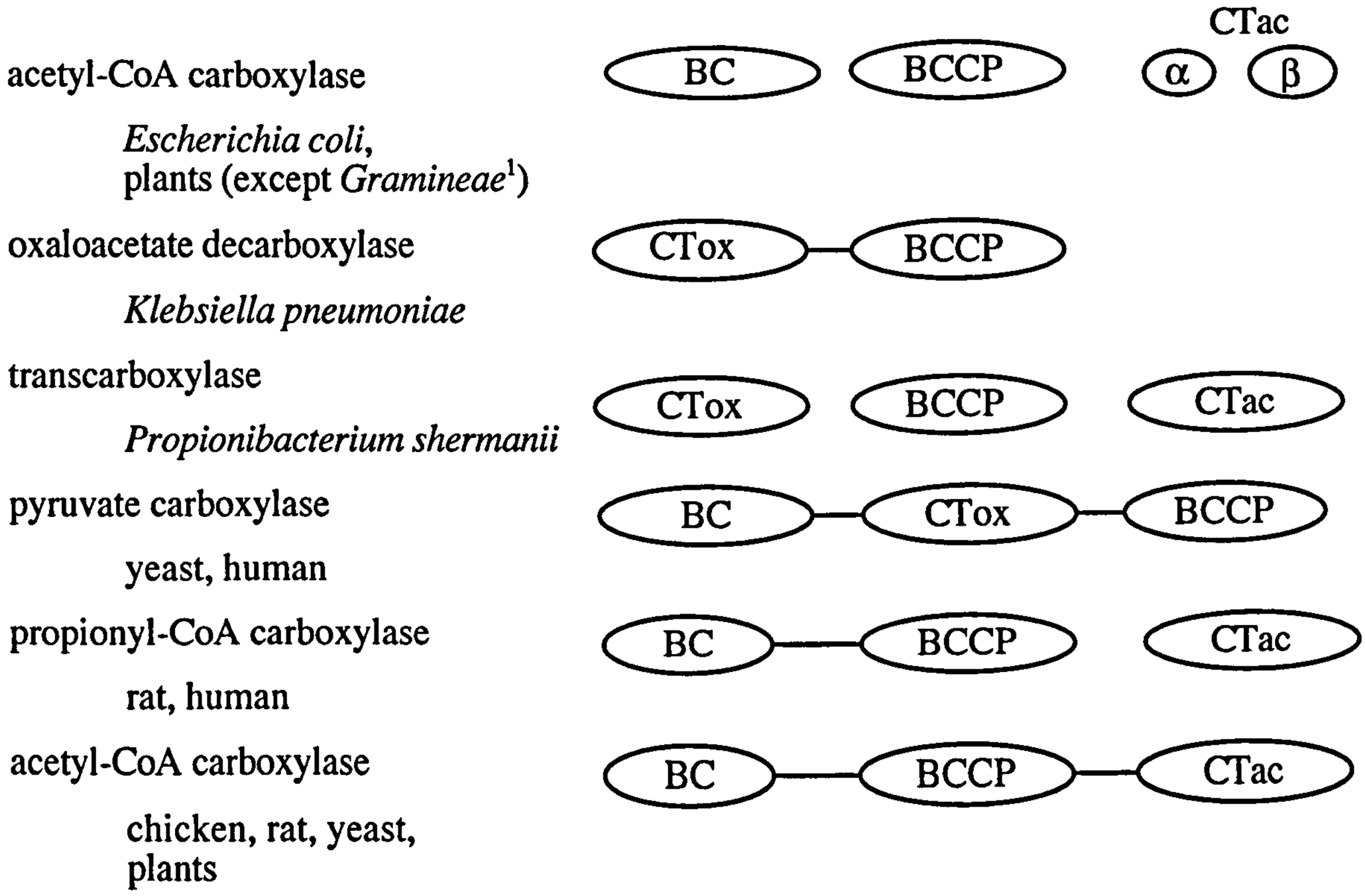


Figure 1.6 Schematic diagram for the domain and / or subunit structures of the biotin-dependent carboxylases (Toh *et al.*, 1993).

Abbreviations in the diagram are as follows; BC, biotin carboxylase; BCCP, biotin carboxyl carrier protein; CTac, carboxyltransferase to acyl-CoAs; CTox, carboxyltransferase to oxaloacetate. References; [1] Konishi *et al.*, 1996.

The variation in tertiary structure of these enzymes hides the amazing similarity in the primary structure. The most highly conserved region is the biotin attachment site. This subunit has been sequenced from several sources and a variety of enzymes, these are aligned in Figure 1.7 around the biocytin (ε-biotinylated lysine) residue.

```
P.shermanii Transcarboxylase          GQtvlVLEAMKMEteinaptdGkV
Oxalate decarboxylase                 GevllILEAMKMEteiraaqaGtV
Chicken acetyl-CoA carboxylase        GQcfaeIEVMKMvmtltagesGcI
E.coli acetyl-CoA carboxylase         GntlcIVEAMKMmnqieadksGtV
yeast pyruvate carboxylase            GQpvaVLsAMKMemiisspsdGqV
human pyruvate carboxylase            GQplcVLsAMKMEtvvtspmeGtV
sheep pyruvate carboxylase             gqplVLsAMKMEtvvtspvtegV
rat pyruvate carboxylase               gqplVLsAMKMEtvvtapr
human propionyl-CoA carboxylase       GQeicVIEAMKMqnsmtagktGtV
tomato biotinyl peptide               GQpvlVLEAMKMEhvvkapanGyV
```

Figure 1.7 Alignment of the biotinylation sites of ten different known biotin-containing enzymes (Samols *et al.*, 1988).

The bold **K** represents the biotinylated lysine. Letters in capitals are conserved or represent a >60% consensus. Letters in italics represent conservative substitutions.

The conservation of residues around the biotinylation site could be for two reasons, to allow recognition of the correct lysine by biotin holoenzyme synthetase, or because the residues are required for the correct functioning of biotin as a carrier of CO_2. Several reports have addressed this question with mutations at the residues around the biotinylation site not necessarily affecting biotinylation, but reducing catalytic activity (Shenoy *et al.*, 1988; Shenoy *et al.*, 1992; Shenoy *et al.*, 1993). Identification of peptides which are biotinylated but share little or no homology with other functional biotin enzymes, demonstrates conclusively that the conservation around the biotinylated lysine is for enzyme activity and not for recognition by the biotin holoenzyme synthetase (Shatz, 1993; Duval *et al.*, 1994).

1.2.5 Identification of biotin-containing proteins

Biotin-containing proteins can be detected either by labelling with [^{3}H]biotin (Chandler & Ballard, 1986), by modified western blotting using [^{125}I] labelled streptavidin (Nikolau *et al.*, 1985) or by gel shift assays using a streptavidin-protein complex in electrophoresis (Elborough *et al.*, 1994a). Biotinylated proteins have been detected using tritium labelled biotin by growing mammalian cells in culture in the presence of [^{3}H]-biotin. Cell proteins labelled with tritium labelled biotin were detected by separation by SDS / PAGE and fluorography, presumably the only labelled proteins will be biotin-containing ones. Using a modified Western blotting technique total protein extracts from any source can be assessed for biotin-containing proteins. This technique assumes that the streptavidin only recognises the biotin molecule on western blots. Streptavidin shift assays detect biotin proteins by the change in mobility when a streptavidin binds to the biotinylated protein. The streptavidin can be used labelled or unlabelled to detect proteins (Elborough *et al.*, 1994a).

The major biotinylated proteins in 3T3-L1 cells detected by cell labelling with [3H]biotin have a M_w of 220, 120, 75 and 72kDa (Chandler and Ballard, 1986) with many more minor biotinylated bands detected within the mitochondria. Carrot embryos have been shown to contain biotinylated proteins of 210, 140, 73, 50 39 and 34kDa by detection with [^{125}I]streptavidin. Some of these proteins have been

identified. Clearly these proteins must represent, amongst others, the activities acetyl-CoA carboxylase, methylcrotonyl-CoA carboxylase, propionyl-CoA carboxylase and pyruvate carboxylase present in animal and plant cells.

1.2.6 The role of biotin-containing enzymes in cell metabolism

That biotin is an essential requirement for the growth of eukaryotic cells should not be surprising when the varied and important nature of their role in cellular metabolism is reviewed. Figure 1.8 shows a summary of the known eukaryotic enzymes.

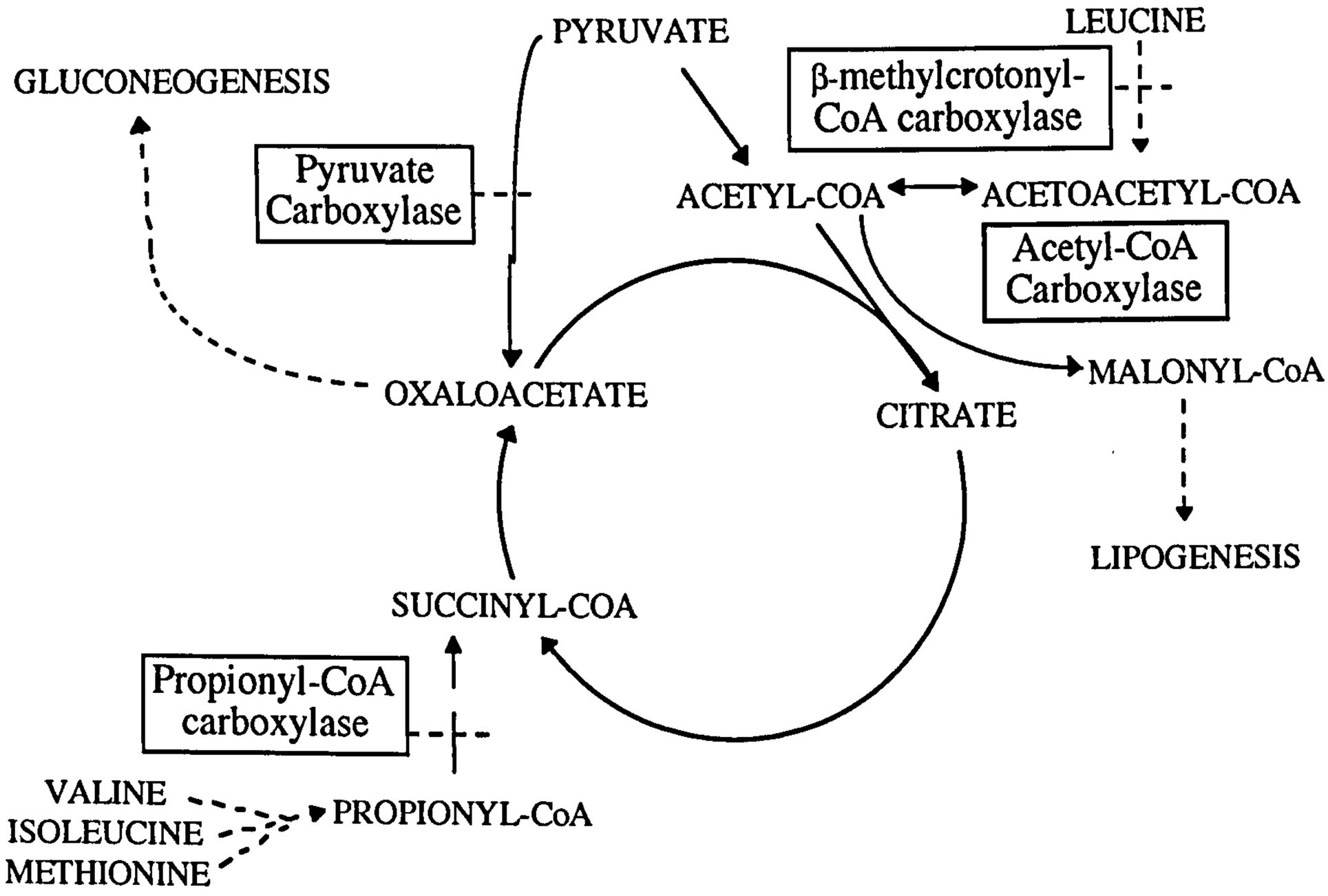


Figure 1.8 Biotin carboxylases in eukaryotic cellular metabolism (Dakshinamurti *et al.*, 1985; Wurtele & Nikolau, 1990)

Acetyl-CoA carboxylase and pyruvate carboxylase are key enzymes in the anabolic pathways of lipogenesis and gluconeogenesis respectively (Wakil, 1958; Utter & Keech, 1960 & 1963). β-methylcrotonyl-CoA carboxylase and propionyl-CoA carboxylase catalyse catabolic reactions in the degradation of amino acids (Alberts & Vagelos, 1972). As a result of their essential contribution, a comprehension of the molecular and biochemical arrangement of these enzymes will provide a more detailed understanding of cellular metabolism

1.3 Acetyl-CoA carboxylase (E.C. 6.4.1.2)

Acetyl-CoA carboxylase (ACCase) catalyses the biotin-dependent carboxylation of acetyl-CoA to form malonyl-CoA in the two step reaction:

$$\text{enz-biotin} + \text{ATP} + \text{HCO}_3^- \Longleftrightarrow \text{enz-biotin-CO}_2^- + \text{ADP} + \text{P}_i$$

$$\underline{\text{enz-bioitn-CO}_2^- + \text{CH}_3\text{CO-SCoA} \Longleftrightarrow {}^-\text{OOCCH}_2\text{CO-SCoA} + \text{enz-biotin}}$$

$$\text{ATP} + \text{HCO}_3^- + \text{CH}_3\text{CO-SCoA} \overset{\text{Me}^{2+},\ \text{enz-biotin}}{\Longleftrightarrow} {}^-\text{OOCCH}_2\text{CO-SCoA} + \text{ADP} + \text{P}_i$$

This reaction is the first committed step in the biosynthesis of fatty acids, providing the essential substrate for fatty acid synthetase (Harwood, 1988). Consequently, it has been the subject of intense study for several decades from animal, bacterial, plant and yeast sources (Alberts & Vagelos, 1972; Iritani, 1992; Rock & Cronan, 1996; Harwood, 1996).

1.3.1 Acetyl-CoA carboxylase from animals

In animals, ACCase has been purified from avian liver, rat epididimal adipose tissue, bovine adipose tissue, rat liver, rat mammary gland (Alberts & Vagelos, 1972; Ahmad & Ahmad, 1985) and chicken liver (Beaty & Lane, 1982). The rat mammary gland acetyl-CoA carboxylase has been shown to be made up of possibly non-identical subunits of around 210,000 M_r (Ahmad & Ahmad, 1985). Purified chicken liver ACCase has a M_r of 225,000. These subunits are associated together to form a native complex of four subunits. A unique feature of the animal ACCase is its strong activation by citrate and its down regulation by phosphorylation. The activation of ACCase by citrate causes polymerisation of the native protomer structures into filaments. The avian liver enzyme has a protomeric molecular weight of 410kDa but in the presence of citrate the enzyme polymerises into particles with a molecular weight of 4 to 8 million, made up of 10-20 protomers (Alberts & Vagelos, 1972). These can be seen under the electron microscope and have been measured at 0.2-0.5μm in length. This polymerisation of the protein protomer corresponds to a 7-8 fold activation of the rat mammary gland enzyme (Ahmad & Ahmad, 1985). Measurement of the rates of

half-reactions showed that the effects of polymerisation were mostly a result of an increase in the carboxyltransferase, second half-reaction. Evidence suggests that sulphydryl groups might play a role in the self-association of ACCase by citrate. Phosphorylation of rat mammary gland ACCase led to a >3 fold loss in specific activity (Ahmad & Ahmad, 1985).

This strict regulation of acetyl-CoA carboxylase in animals is in concordance with its role as the first step toward lipid biosynthesis. When intermediates of the citric acid cycle are not metabolised away by respiration, and glucose concentrations are high due to consumption, acetyl-CoA carboxylase is activated to produce malonyl-CoA and lipogenesis results.

1.3.2 Yeast acetyl-CoA carboxylase

Yeast mutants defective in acetyl-CoA carboxylase have been isolated as auxotrophs for long-chain fatty acids (Mishina *et al.*, 1980; Roggenkamp *et al.*, 1980). In accordance with reports from vertebrate sources, the yeast enzyme has a subunit M_r of 265,000 (Witters & Watts, 1990), and is active as a tetrameric complex (Obermayer & Lynen, 1976). The enzyme is phosphorylated and consequently inactivated by protein kinases (Witters & Watts, 1990). Disruption of the *acc*1 gene in yeast leads to an initial 50% decrease in mRNA due to disruption of one allele (Haßlacher, *et al.*, 1993). After sporulation of the diploid disruptants, spores containing the disrupted *acc*1 allele failed to grow, even when the media was supplemented with long chain fatty acids, indicating that acetyl-CoA carboxylase is somehow essential for growth, possibly for a process other that *de novo* fatty acid biosynthesis.

Expression of ACCase in yeast is down regulated 3-fold by the inclusion of phospholipid precursors such as inositol and choline in the growth media (Haßlacher, *et al.*, 1993). ACCase expression is also down regulated in the presence of 0.01% palmitic acid and the regulatory factors inositol-2-phosphate and inositol-4-phosphate providing evidence that the key step of fatty acid biosynthesis is regulated in conjunction with phospholipid synthesis.

1.3.3 *Escherichia coli* acetyl-CoA carboxylase

The acetyl-CoA carboxylase from *E.coli* is amongst the most well studied of all biotin-containing enzymes to date. It is the only biotin-containing enzyme in *E.coli* making biochemical studies easier (Li & Cronan, 1993). Early purification attempts showed that the enzyme was readily dissociable into two fractions, E_a and E_b. which were necessary for enzyme activity (Alberts & Vagelos, 1972). It was also demonstrated that if these two components were separated and enzyme activity was lost, it could also be regained by recombining the two fractions. E_a was shown to contain biotin and E_b was shown to contain no biotin. Fraction E_a was able to incorporate $H^{14}CO_3^-$ into high molecular weight protein, stochiometrically with the amount of biotin present in E_a. Proof that these two fractions contained the separate activities for the two half reactions was acquired by first making carboxybiotin with fraction E_a, isolating the carboxybiotin protein and incubating this with E_b and acetyl-CoA. The subsequent formation of malonyl-CoA demonstrated that acetyl-CoA carboxylase from *E.coli* contained at least two separate proteins.

Disc gel electrophoresis of E_a showed that this faction contained two proteins, a biotin carboxylase and a biotin carboxyl carrier protein. Gel filtration of the E_b fraction showed a subunit molecular weight of 90kDa but SDS / PAGE of the E_b fraction revealed a minimal molecular weight of 45-50kDa suggesting that this fraction was also made up of a dimer of two proteins (Alberts & Vagelos, 1972).

Work since 1972 has progressed such that all the genes encoding for the subunits of acetyl-CoA carboxylase in *E.coli* have been identified (Muramatsu & Mizuno, 1989; Kondo *et al.*, 1991; Li & Cronan, 1992a & 1992b). The molecular characteristics are summarised in Table 1.3

Subunit	*Gene name*	*Position in E.coli genome*	*Calculated size*	*Size on SDS /PAGE*
BCCP	*fab*E (*acc*B)	72min	16,668	22,500
BC	*fab*G (*acc*C)	72min	49,320	51,000
CT α	*acc*A	4.3min	35,114	35,000
CT β	*acc*D (*ded*B)	50min	33,188	31,500

Table 1.3 Summary of the characteristics of the genes and protein subunits of *E.coli* acetyl-CoA carboxylase.

Table 1.3 shows some interesting characteristics of the *E.coli* acetyl-CoA carboxylase. The most notable observation is that the size of BCCP on SDS/PAGE gels (22.5kDa) is much larger than the calculated size (16.6kDa). This is explained by the peculiarity of the amino acid sequence between residues 34 and 101 (Figure 1.9) where nearly half the residues are alanine or proline. Similar protein sequences are known to cause

```
1                                                              50
MDIRKIKKLI ELVEESGISE LEISEGEESV RISRAAPAAS FPVMQQAYAA

51                                                             100
 PMMQQPAQSN AAAPATVPSM EAPAAAEISG HIVRSPMVGT FYRTPSPDAK

101                                                            150
  AFIEVGQKVN VGDTLCIVEA MKMMNQIEAD KSGTVKAILV ESGQPVEFDE

151    156
  PLVVIE
```

Figure 1.9 Amino acid sequence of the *E.coli* BCCP.

The conserved biotinylation sequence in underlined.

aberrant molecular weights on SDS / PAGE (Miles *et al.*, 1988; Radford *et al.*, 1989; Easom *et al.*, 1989).

The genes for BC and BCCP in *E.coli* map together to 72min on the genome. Sequencing has revealed that the two genes are separated from each other by 10bp of DNA (Kondo *et al.*, 1991; Li & Cronan, 1992a). This suggests that the genes are cotranscribed and analysis of mRNA showed this to be the case (Li & Cronan, 1992a). BCCP and BC are known to function and purify together as a complex (Alberts & Vagelos, 1972; Nenortas & Beckett, 1996), so presumably co-transcription

ensures stochiometric production of the subunits and could facilitate efficient formation of the complex. The closeness of the genes would suggest that a gene fusion could feasibly be created to form a single protein with both functionalities, although a simple deletion of the 10bp between them would give a BCCP-BC fusion and of the fusions where BC is next to BCCP the order is BC-BCCP.

The previously unknown ORF *ded*B */usg* has now been identified as CT-β (Li & Cronan, 1992b) and renamed *acc*D. Table 1.3 shows that the calculated size of CT is 33,188 yet SDS / PAGE of the pure subunit shows a size of 31.5kDa. This was due to proteolytic cleavage of the first 15 residues during isolation.

The bacterial ACCase has not been demonstrated to be affected by citrate or phosphorylation. Instead it has been demonstrated that the level of expression of ACCase directly correlates to the rate of cellular growth (Li & Cronan, 1993).

1.3.4 Plant acetyl-CoA carboxylase

Initial studies on acetyl-CoA carboxylase claimed to have proved the existence of a multisubunit, prokaryotic type of acetyl-CoA carboxylase in isolated chloroplasts of spinach (Kannangara & Stumpf, 1972, 1973). The initial observation was that [1-^{14}C] acetate was readily incorporated into fatty acids by intact chloroplasts of spinach and lettuce in the presence of bicarbonate, ATP, coenzyme A and light. In contrast neither acetate nor acetyl-CoA could be incorporated into fatty acids by disrupted chloroplasts, but this could be overcome by using [1-^{14}C] malonyl-CoA as substrate in the presence of *E.coli* ACP, DPNH and TNPH (Brooks & Stumpf, 1966). It was shown that disrupted spinach chloroplasts could incorporate [^{14}C] bicarbonate into an acid labile protein dependent upon ATP or light and Mn^{2+} and was inhibited by avidin (Kannangara & Stumpf 1972). The conversion of [^{14}C] bicarbonate and acetyl-CoA to labelled malonyl-CoA could be recovered in disrupted chloroplasts by the addition of the *E.coli* E_b fraction (see previous section). Experiments showed that there was a heat stable inhibitor of the E_b fraction. It was interpreted that this was the reason for low incorporation of [1-^{14}C] acetate in disrupted chloroplasts, the transcarboxylase

subunit of a multisubunit prokaryotic type acetyl-CoA carboxylase was being inhibited. The inhibitor was not identified and presumably does not act in intact chloroplasts.

The BCCP protein was located by incubating purified chloroplasts with [^{14}C] bicarbonate and fractionating the chloroplasts into lamellae, grana and stroma. Most of the labelled protein was located attached to the lamellae and grana, was acid labile and was identified as was identified as carboxylated biotin (Kannangara & Stumpf, 1973). This protein was later identified as having a M_r of 21,000 by polyacrylamide disc gel electrophoresis in barley chloroplast membranes (Kannangara & Jensen, 1975).

Purification of plant acetyl-CoA carboxylase has been attempted many times from many sources. Initial attempts at purification of the enzyme from wheat germ yielded five different proteins which could catalyse a number of exchange reactions between malonyl-CoA-^{14}C-acetyl-CoA and between ATP-$^{32}P_i$ (Heinstein and Stumpf, 1969). Another purification ten years later revealed that the situation was still complex (Nielsen *et al.*, 1979). ACCase was purified 634 fold from wheat germ, electrophoresis in denaturing gels revealed a complex picture, major bands were 135, 84, 76, 56, 45 and 21.5kDa in size. It was postulated that these bands could be a result of a multi-subunit enzyme with the subunits in varying combinations. The question of proteolysis was addressed but no positive conclusions could be reached as to whether the large number of bands purified were a result of proteolysis or not.

A year later ACCase was purified 2,180-fold from what germ (Egin-Bühler *et al.*, 1980). The purified enzyme consisted of two band with a M_w of 240 and 98kDa. It was demonstrated that only the larger protein contained biotin by avidin-shift techniques. It was not ruled out that the smaller protein was a contaminant or a proteolytic product.

Several other purifications of the plant acetyl-CoA carboxylase followed and a summary is shown in Table 1.4.

Source Material	*Subunit(s) size kDa*	*Reference*
Wheat germ	135, 84, 76, 56, 45 and 21.5	Nielsen et al., 1979
Parsley cell culture	210, 105	Egin-Bühler et al., 1980
Wheat germ	240, 98	Egin-Bühler et al., 1980
Parsley cell culture	220	Egin-Bühler & Ebel 1983
Maize leaf	60	Nikolau & Hawke, 1984
Oilseed rape seed	220	Hellyer et al., 1886 Slabas et al., 1986
Maize leaf	227, 219	Elgi *et al.*, 1993
Maize leaf	230	Ashton *et al.*, 1994
Wheat germ	220	Elborough *et al.*, 1994a

Table 1.4 Purifications of acetyl-CoA carboxylase from various sources and the observed M_r.

With the development of purification technology and protease inhibitors it has been possible to purify the acetyl-CoA carboxylase from parsley, maize, wheat germ and oilseed rape to homogeneity. The procedures are usually rapid to prevent as much proteolysis as possible and use affinity techniques.

The purification of maize acetyl-CoA carboxylase has enabled the production of antibodies which in turn, through the screening of an expression library, have enabled the isolation of a cDNA clone (Ashton *et al.*, 1994). Genes encoding acetyl-CoA carboxylase have now been cloned from a number of sources including oilseed rape (Elborough *et al.*, 1994b), maize (Ashton *et al.*, 1994), wheat (Elborough *et al.*, 1994a; Gornicki *et al.*, 1994), alfalfa (Shorrosh *et al.*, 1994) and *Arabidopsis* (Roesler *et al.*, 1994; Yanai *et al.*, 1995). The size of the predicted proteins from all these cloned cDNAs are all of an M_r between 210,000 and 250,000. The primary structure of the protein reveals that all three enzymatic functions, the biotin carboxylase, the biotin carboxyl carrier protein and the carboxyltransferase, are to be found on the one polypeptide (Elborough *et al.*, 1994b). The native enzyme would appear to be a dimer with a M_r of about 5×10^5 (Gornicki & Haselkorn, 1993).

It would appear that acetyl-CoA carboxylase in plants is similar to other eukaryotic type ACCases in rat and chicken (see Figure 1.6), and that the previous reports of a

multi-subunit ACCase in chloroplasts of spinach were erroneous. This was thought to be the case because; a) purification of ACCase produced a large polypeptide which contained ACCase activity; b) this polypeptide was known to be extremely labile and so reports of smaller enzymes were dismissed as proteolytic artefacts. What was assumed in the literature was that; 1) maize and wheat would have the same ACCases as spinach and, 2) smaller biotinylated protein must be proteolytic degradation products of the large form. What this failed to answer was the original problem, why don't ruptured chloroplasts fix [1-^{14}C] acetate into fatty acids, and how come the system can be recovered by the addition of the *E.coli* carboxyltransferase subunit? (Kannangara & Stumpf, 1972).

It was reported in 1991 (Smith *et al.*, 1991) that an unknown ORF in the chloroplast genome of pea had homology to *ded*B, which was at that time an ORF with unknown function in *E.coli*, the protein product of which was thought to be involved in folate metabolism (Nonet *et al.*, 1987; Bognar *et al.*, 1987). Part of this ORF, entitled ORF587, had been previously identified and designated *zfp*A as it has homology with conserved zinc-finger proteins (Sasaki *et al.*, 1989). Also noted was the homology with rat propionyl-CoA carboxylase β-subunit, but nothing further was commented other than it may have functional similarity. The β-subunit of rat propionyl-CoA carboxylase catalyses the transfer of CO_2 from the biotinylated α-subunit to propionyl-CoA (Kraus *et al.*, 1986). As the function of *ded*B was unknown, the conclusion was vague, suggesting that ORF587 may be involved in C_1 metabolism (Smith *et al.*, 1991).

The carboxyltransferase β-subunit from *E.coli* was purified and the amino acid sequence determined the following year (Li & Cronan, 1992b). The amino acid sequence of the carboxyltransferase β-subunit matched the predicted amino acid sequence from *ded*B. Subsequently, ORF587 from pea chloroplast genome and the corresponding ORFs from liverwort, tobacco and rice were identified as a subunit of a biotin-dependent carboxylase (Li & Cronan, 1992c). Antibodies to the protein

product of ORF587 (*acc*D) detected a protein of 87kDa on SDS / PAGE (Sasaki *et al.*, 1993). The protein was detectable in a fraction of lysed chloroplasts washed with 1M NaCl. In the presence of the antibody, ACCase activity was reduced by some 80%. The antibody was also used to immunoprecipitate the protein and any protein that were complexed with it. A 35kDa biotin-containing protein was precipitated with the *acc*D protein and detected by streptavidin probing along with a 91kDa protein. Other proteins below 50kDa could not be discerned from the background of precipitated proteins.

The realisation of a multisubunit prokaryotic Type II form of acetyl-CoA carboxylase provided a molecular answer to the sensitivity of plants from the family *Gramineae* to aryloxyphenoxypropionate herbicides (Konishi & Sasaki, 1994; Alban *et al.*, 1994). All higher plants examined except the *Gramineae* contain the *acc*D gene, and presumably the Type II ACCase (Konishi *et al.*, 1996). In plants such as wheat and rice there is a Type I ACCase present in the chloroplasts (Konishi & Sasaki, 1994), the *acc*D gene in such plants is severely truncated and probably not expressed(Li & Cronan, 1992c). The strong inhibition of Type I ACCase by aryloxyphenoxypropionic acid herbicides has been demonstrated (Alban *et al.*, 1994) whereas the activity of the Type II ACCase is not affected. Hence, the *Gramineae* which do not contain the resistant Type II ACCase are sensitive to these herbicides.

The genes for the four subunits which make up the Type II ACCase have all recently been cloned. The subunits are as follows; Biotin Carboxylase (BC), nuclear encoded, 58.4kDa (tobacco, Shorrosh *et al.*, 1995); Biotin Carboxyl Carrier Protein, nuclear encoded, 35kDa (oilseed rape, Elborough *et al.*, 1996), 38kDa (*Arabidopsis*, Choi *et al.*, 1995); CarboxylTransferase α-subunit, nuclear encoded 91kDa (pea, Shorrosh *et al.*, 1996); CarboxylTransferase β-subunit, chloroplast encoded, 87kDa (pea, Sasaki *et al.*, 1993). All the subunits are translocated into the chloroplast and presumably assembled there as a complete, active enzyme.

The mechanism of regulation of acetyl-CoA carboxylase in plants is unknown. Certainly the levels of enzyme activity increase during lipogenesis in developing embryos of *Brassica napus* (Turnham & Northcote, 1983). These workers observed that enzyme activity increases before lipid biosynthesis and declines rapidly with the decline in rate of lipid formation. Western and northern analysis of the two forms of acetyl-CoA carboxylase in *Brassica napus* indicates a change in the levels of mRNA and protein during embryogenesis, for both Type I and Type II ACCases, consistent with the rise and fall of ACCase (Elborough *et al.*, 1994b & 1996). What regulates this change and the role of the two forms of ACCase in *Brassica napus* is unknown. It has been demonstrated that in barley and maize leaves ACCase exerts strong flux control over lipid biosynthesis (Page *et al.*, 1994), but these tissues lack a Type II acetyl-CoA carboxylase. In contrast, the activity of acetyl-CoA carboxylase in developing embryos of *Brassica napus* has been shown to decline relative to the rate of lipid biosynthesis throughout development (Kang *et al.*, 1994). The method used in this study was quite different than in previous studies, using a plastid isolation medium to extract the proteins. Presumably therefore the Type II ACCase would have been isolated in intact or partially intact chloroplasts, preventing disassociation of the subunits. The problem with assaying the two forms of the enzyme together is that Type I ACCase has been shown to require Triton X-100 for extraction (Hellyer *et al.*, 1986), whereas this will rupture the chloroplasts and allow disassociation of the Type II enzyme which leads to a loss of activity (Kannangara & Stumpf, 1972). This negative correlation between the rate of lipid biosynthesis and the rate of ACCase activity when extracted under high osmotic strength conditions, implies regulation of the Type I and Type II ACCases are quite different. Further characterisation of the molecular mechanisms which regulate Type II ACCase expression and activity are clearly needed to reach an understanding of this enzymes role during lipid formation in developing embryos of *Brassica napus*.

1.4 β-Methylcrotonyl-CoA Carboxylase (E.C. 6.4.1.4)

β-Methylcrotonyl-CoA carboxylase (MCCase) catalyses the biotin-dependent carboxylation of β-methylcrotonyl-CoA to form β-methylglutaconyl-CoA in the two step reaction:

$$\text{enz - biotin} + HCO_3^- + \text{ATP} \overset{Mg^{2+}}{\Longleftrightarrow} \text{enz - biotin - } CO_2^- + \text{ADP} + P_i$$

$$\underline{\text{enz - biotin - CO} + (CH_3)_2CCHCO\text{ - SCoA} \Longleftrightarrow {}^-OOCCH_2(CH_3)CCHCO\text{ - SCoA} + \text{enz - biotin}}$$

$$\text{ATP} + HCO_3^- + (CH_3)_2CCHCO\text{ - SCoA} \overset{Mg^{2+},\ \text{enz-biotin}}{\Longleftrightarrow} {}^-OOCCH_2(CH_3)CCHCO\text{ - SCoA} + \text{ADP} + P_i$$

Before 1990 β-Methylcrotonyl-CoA Carboxylase had only been studied in animal and bacterial systems where it was thought to be involved in leucine degradation (Alberts & Vagelos, 1972). Increased interest in the plant enzyme has occurred since its identification (Wurtele & Nikolau, 1990).

1.4.1 Animal β-Methylcrotonyl-CoA Carboxylase

The initial characterisation of β-methylcrotonyl-CoA carboxylase was in rat liver preparations which were shown to be capable of converting leucine to acetoacetate and acetyl-CoA (Alberts & Vagelos, 1972). $H^{14}CO_3^-$ was shown to be stochiometrically incorporated into the carboxyl carbon of acetoacetate. The enzyme responsible was eventually determined to be β-methylcrotonyl-CoA carboxylase. This was shown to be a biotin-containing enzyme as mitochondria from biotin deficient rats were unable to convert isovaleric acid or β-methylcrotonic acid to acetoacetic acid (Alberts & Vagelos, 1972). The enzyme was proposed to form part of the leucine degradation pathway in Figure 1.10.

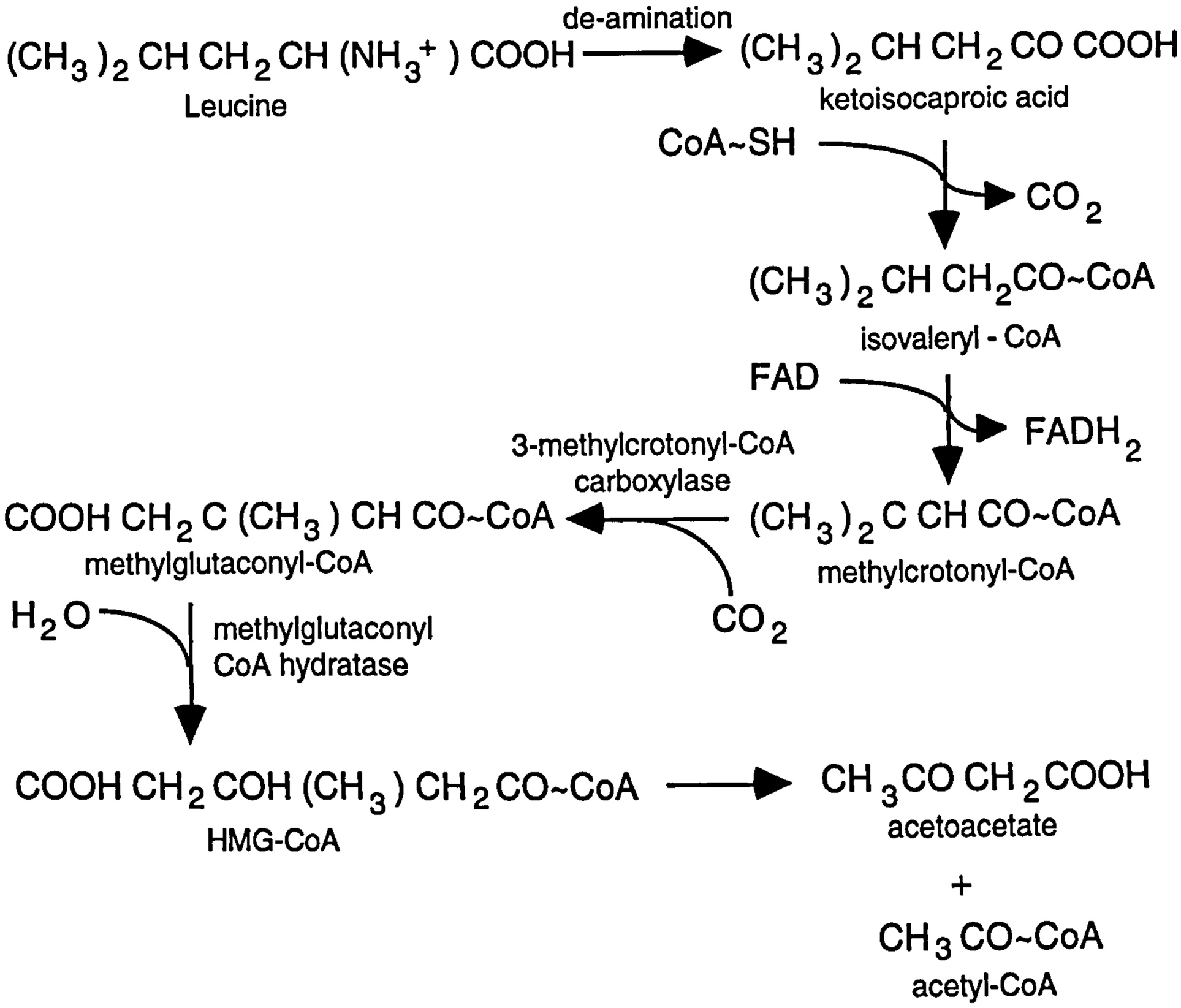


Figure 1.10 Pathway of leucine degradation in animals (Moss & Lane, 1972).

MCCase has been purified from bovine kidney (Lau *et al.*, 1980), pig kidney (Lau & Fall, 1981), and rat liver (Oei & Robinson, 1985). The bovine kidney enzyme is a multimeric complex composed of two subunits with a combined native M_r of 835,000. The biotinylated subunit has a M_r of 73,500 whilst the non-biotinylated subunit has a M_r of 61,000.

The vital role MCCase has to play in human metabolism is exemplified by the identification of an inherited metabolic disorder, 3-methylcrotonylglycinuria (Gompertz *et al.*, 1973; Finnie *et al.*, 1976; Weyler *et al.*, 1977). Patients with the rare genetic disorder are deficient in MCCase and which results in an organic acidaemia, which can prove fatal due to increased levels of 3-hydroxyvaleric acid in the blood.

1.4.2 Bacterial β-Methylcrotonyl-CoA Carboxylase

β-methylcrotonyl-CoA carboxylase is only known in a few species of bacteria but has been purified from *Achromobacter* (Schiele *et al.*, 1975) and *Pseudomonas citronellolis* (Hector & Fall, 1976). The enzyme has the same structure in bacteria as observed in animals. The subunits are 96kDa and 78 kDa in *Achromobacter* and 68kDa and 62kDa in *Pseudomonas*. The heavier protein was biotinylated in both cases. The regulation of the enzyme in these species has not been investigated but it is presumed to be induced in response to growth on special substrates.

1.4.3 Plant β-Methylcrotonyl-CoA Carboxylase

There has been much interest in the biotin-containing enzyme β-methylcrotonyl-CoA carboxylase since it was first discovered in plant species (Wurtele & Nikolau, 1990). β-methylcrotonyl-CoA carboxylase activity was demonstrated in extracts of carrot embryos, oilseed rape seeds, maize leaves and sunflower embryos by the incorporation of $H^{14}CO_3^-$ into acid stable products when using β-methylcrotonyl-CoA as a substrate.

That plants require the mechanisms to degrade leucine was demonstrated by work with sucrose starved plants (Genix *et al.*, 1990). It was demonstrated that a decrease in total cell protein occurred once all the carbohydrate pools had been used up, presumably due to macroautophagy, with a parallel increase in amino acids. This suggests that plants are able to catabolise amino acids for respiration. In order to catabolise leucine MCCase would be required.

It has also been postulated that MCCase may be part of the mevalonate biosynthesis pathway (Gray, 1987). This is important because the source of mevalonate for biosynthesis of isopentenylpyrophosphate is presently unclear. Figure 1.11 shows the cycle proposed to covert mevalonate into HMG-CoA or vice versa.

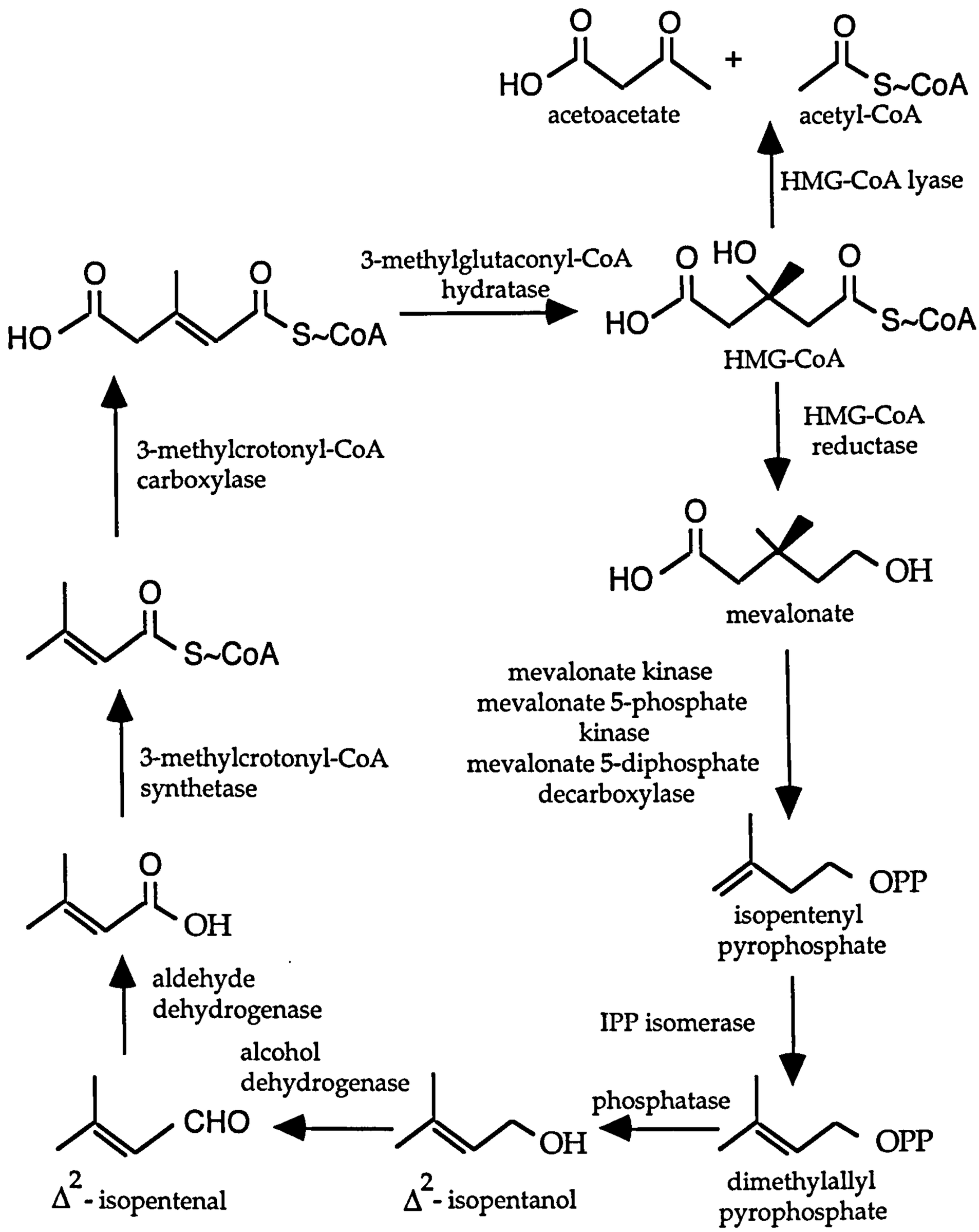


Figure 1.11 The "mevalonate shunt" (Gray, 1987).

Most of the reaction steps are readily reversible except for HMG-CoA reductase.

The mevalonate shunt has been proposed to function in plants to account for the incorporation of mevalonate into non-isoprenoid in the presence of the HMG-CoA reductase inhibitor mevinolin (Edmond & Popjack, 1974; Nes & Bach, 1985).

MCCase was detected in purified mitochondria from pea leaves (Baldet *et al.*, 1992). A single biotinylated polypeptide with a M_r of 76,000 was detected in purified plant mitochondria from pea leaves and assumed therefore to be the biotinylated subunit of MCCase. This was later purified to homogeneity along with another non-biotinylated subunit of M_r 54,000 (Alban *et al.*, 1993). The subunit sizes are similar to the purified subunit from bacterial and animal sources with the same subunit being biotinylated, the large one. MCCase has also been purified from *Daucus carota* (carrot, Chen, *et al.*, 1993) and *Solanum tuberosum* (potato, Alban *et al.*, 1993). The M_r of the large biotinylated subunits and small subunits were shown to be 78,000 and 65,000, and 74,000 and 53,000 respectively.

The genes encoding MCCase were cloned from tomato (Wang *et al.*, 1994), by screening a tomato cDNA library with a mistakenly isolated cDNA which contained within its sequence the conserved biotinylation sequence AMKM, and was biotinylated when expressed in *E.coli* (Hoffman *et al.*, 1987). A gene also encoding for MCCase was isolated from soybean using degenerate oligos designed against the biotinylation sequence (Song *et al.*, 1994). The gene from *Arabidopsis thaliana* has also been isolated (Weaver *et al.*, 1995). Two genomic clones were isolated from tomato suggesting that MCCase has two isoforms in this species.

More recent work has focused on the regulation of MCCase. MCCase activity was found to be ten fold greater in roots than in leaves yet the steady state levels of the MCCase protein and mRNA were approximately the same in both tissues (Wang *et al.*, 1995). It was shown that this was due to a difference in the biotinylation status of the enzyme in roots and leaves. Thus the lower activity of MCCase in leaves was associated with a larger pool of non-biotinylated apoenzyme. This was not due to a lack of biotin as a large pool of free biotin was found in leaves, even though it has been reported that no biotin could be detected in purified mitochondria from pea (Baldet *et al.*, 1993). It was concluded that this was a mechanism to regulate the activity of MCCase in leaves.

Levels of β-methylcrotonyl-CoA carboxylase activity have been measured in various tissues in maize plants (Clauss *et al.*, 1993). Levels were seen to be greater in old tissues compared to young tissues (quaternary leaf compared to primary leaf, bottom of leaf compared to tip). MCCase was also shown to be induced by putting plants in the dark for 3-7days (Clauss *et al.*, 1996).

Levels of MCCase have also been shown to increase in response to carbohydrate starvation but not simply to an increase in exogenous leucine (Aubert *et al.*, 1996). This indicates that leucine is not responsible for induction of MCCase but is triggered by an unknown controlling factor associated with macroautophagic process.

Consequently, it appears as though, β-methylcrotonyl-CoA carboxylase is an enzyme involved in catabolic processes, most likely to be the degradation of leucine as in animals. The MCCase is more likely to have a specialist role in plants as under stable conditions plants are not likely to have a significant requirement for leucine. Antisense constructs are probably the best way of deciphering the role of this enzyme in plants as this could show if, under normal circumstances, an extremely reduced level of MCCase had any effect. If not it would then be interesting to assess the plants response under conditions of carbohydrate starvation.

1.5 Propionyl-CoA carboxylase (E.C. 6.4.1.3)

Propionyl-CoA carboxylase (PCCase) catalyses the biotin-dependent carboxylation of propionyl-CoA to form *S*-methylmalonyl-CoA in the two step reaction;

$$\text{enz - biotin} + \text{ATP} + \text{HCO}_3^- \overset{Mg^{2+}}{\Longleftrightarrow} \text{enz - biotin - CO}_2^- + \text{ADP} + \text{P}_i$$

$$\text{enz - biotin - CO}_2^- + \text{CH}_3\text{CH}_2\text{CO - SCoA} \Longleftrightarrow {}^-\text{OOC(CH}_3\text{)CHCO - SCoA} + \text{enz - biotin}$$

$$\text{ATP} + \text{HCO}_3^- + \text{CH}_3\text{CH}_2\text{CO - SCoA} \overset{Mg^{2+},\ \text{enz-biotin}}{\Longleftrightarrow} {}^-\text{OOC(CH}_3\text{)CHCO - SCoA} + \text{ADP} + \text{P}_i$$

Propionic acid arises from the β-oxidation of odd-numbered fatty acids and from the degradation of branched-chain aliphatic amino acids (Alberts & Vagelos, 1972).

1.5.1 Animal Propionyl-CoA carboxylase

Propionyl-CoA carboxylase functions in a pathway which converts propionic acid to succinate, enabling it to be broken down by the tricarboxylic acid cycle (Figure 1.12)

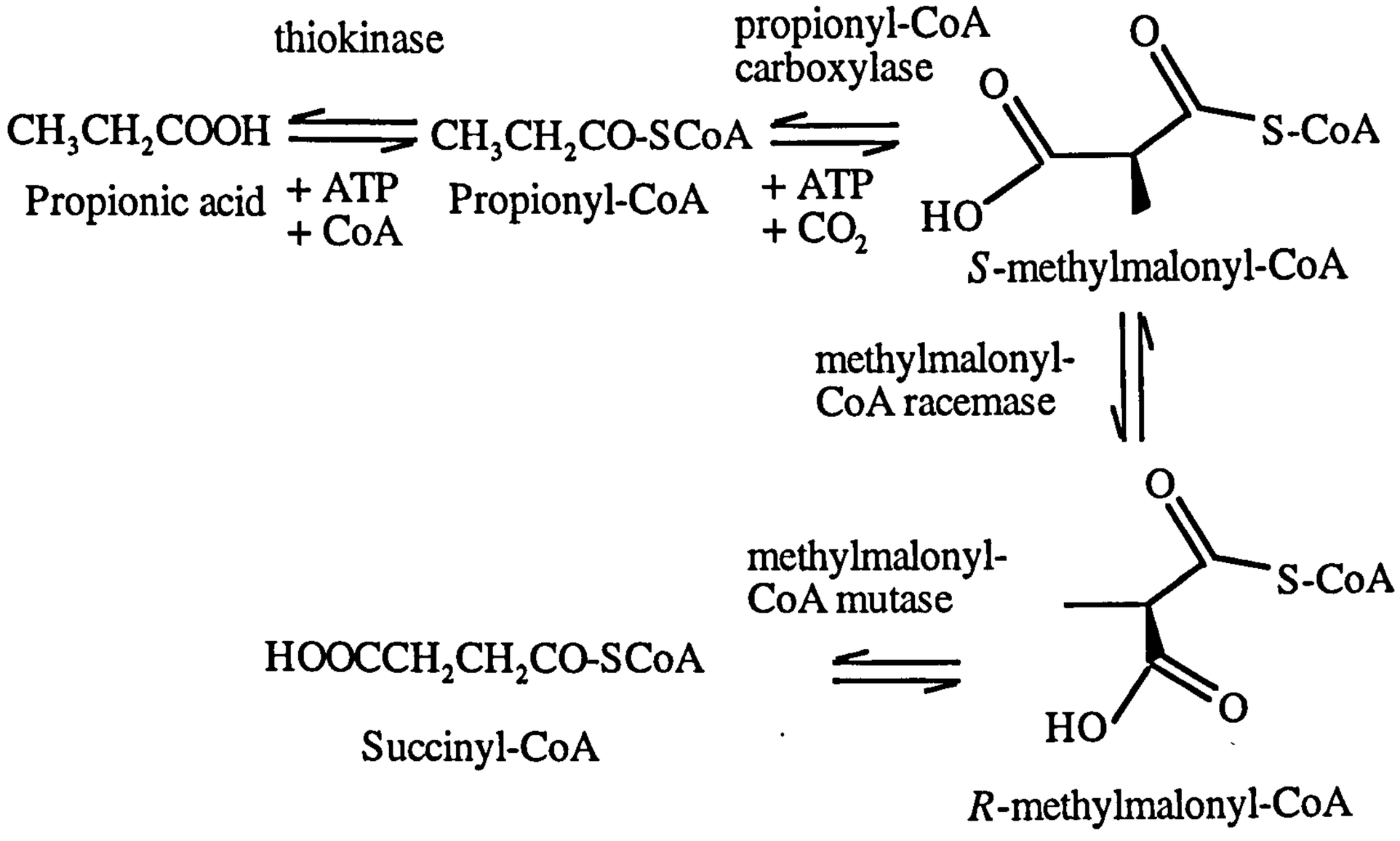


Figure 1.12 Pathway for propionic acid metabolism in animal tissues (Moss & Lane, 1972).

Mitochondria from the livers of biotin-deficient rats were shown to have a greatly reduced capacity to metabolise propionate, implicating the involvement of a biotin-containing enzyme in propionate metabolism (Alberts & Vagelos, 1972). The enzyme

has been purified from rat liver (Oei & Robinson, 1985), human liver (Gravel *et al.*, 1980; Kalousek *et al.*, 1980) and bovine kidney (Lau *et al.*, 1979). In all cases the enzyme has been shown to be made up of two non-identical subunits with a M_r of 72,000 - 75,000 for the biotinylated α-subunits and a M_r of 56,000-60,000 for the small non-biotinylated β-subunit, depending upon the species. The enzyme from human liver consists of an α and β-subunit in a stochiometric combination. The genes for both of these subunits have been isolated and sequenced (Lamwonah *et al.*, 1986; Lamwonah *et al.*, 1987).

1.5.2 Bacterial and Fungal Propionyl-CoA Carboxylase

There is little variation in the structure of the enzyme between animal and prokaryotic sources. PCCase has been purified from *Mycobacterium smegmatis* and been shown to have a biotinylated α-subunit of 64,000 M_r and a smaller β-subunit of 57,000 M_r (Haase *et al.*, 1982). The enzyme has also been characterised in *Rhodosporillum rubrum* (Knight, 1962), *Streptomyces coelicolor* (Bramwell *et al.*, 1996), *Streptomyces erythreus* (Hunaiti *et al.*, 1982) and *Rhodococcus* sp. (Nagy *et al.*, 1992). The enzyme from *R. rubrum* is able to carboxylate acetyl-CoA as well as propionyl-CoA albeit at a reduced rate (Alberts & Vagelos, 1972). The enzyme is of special interest in bacteria as the product of the reaction methylmalonyl-CoA, is not always fed into the TCA cycle but is used as a precursor for mycobacterial multimethyl-branched mycocerosic acids (Rainwater & Kolattukudy, 1985) and for polyketide antibiotics such as erythromycin (Bevitt *et al.*, 1992). It has been suggested that in some mycobacteria, a single acyl-CoA carboxylase may be responsible for both ACCase and PCCase activities involved in fatty-acid biosynthesis (Wheeler *et al.*, 1992).

1.5.3 Plant Propionyl-CoA Carboxylase

The enzyme associated with propionyl-CoA carboxylase activity has not been identified in plants. Crude extracts of plant material have been shown to contain propionyl-CoA carboxylase activity but as yet a single protein responsible for the total amount of activity measured has not been identified (Wurtele & Nikolau, 1990). It

has been conclusively demonstrated that acetyl-CoA carboxylase from parsley cells can carboxylate a variety of substrates at various rates, acetyl-CoA carboxylase, 100%; propionyl-CoA carboxylase, 60% and butyryl-CoA carboxylase, 15% (Egin-Bühler & Ebel, 1983). This provides evidence that the PCCase activity measured in plant extracts may be associated with the Type I acetyl-CoA carboxylase in parsley. In a study on the various levels of plant acyl-CoA carboxylases, patterns of PCCase activity closely followed ACCase activity in all but one instance, in which seven day old, light grown, barley seedlings were placed in the dark for periods up to seven days and enzyme activities measured (Clauss *et al.*, 1993). The level of PCCase in such plants peaked after five days in the dark whereas ACCase activity peaked after six days in the dark. This apparent difference in activities could be explained by there being a separate PCCase and ACCase enzyme. It could also be explained by there being more than one isoform of ACCase with differing activities towards propionyl-CoA. Two forms of acetyl-CoA carboxylase have been characterised in maize leaves, although whether they have differing specificities towards PCCase remains to be seen (Herbert *et al.*, 1994). Specific herbicides known to inhibit ACCase activity have also been shown to completely block PCCase activity in extracts of barley (Motel *et al.*, 1993).

Substantial evidence, refuting the possibility of a separate PCCase enzyme comes from studying its role in metabolism. The pathway for degradation of branched chain amino acids shown in Figure 1.22 probably does not operate in plants as the final enzyme, *R*-methylmalonyl-CoA mutase, is a vitamin B_{12} requiring enzyme. It has been shown that a modified β-oxidation pathway exists in plants which is able to catabolise propionate (Giovanelli & Stumpf, 1958). The modified β-oxidation or α-oxidation pathway is shown in Figure 1.13.

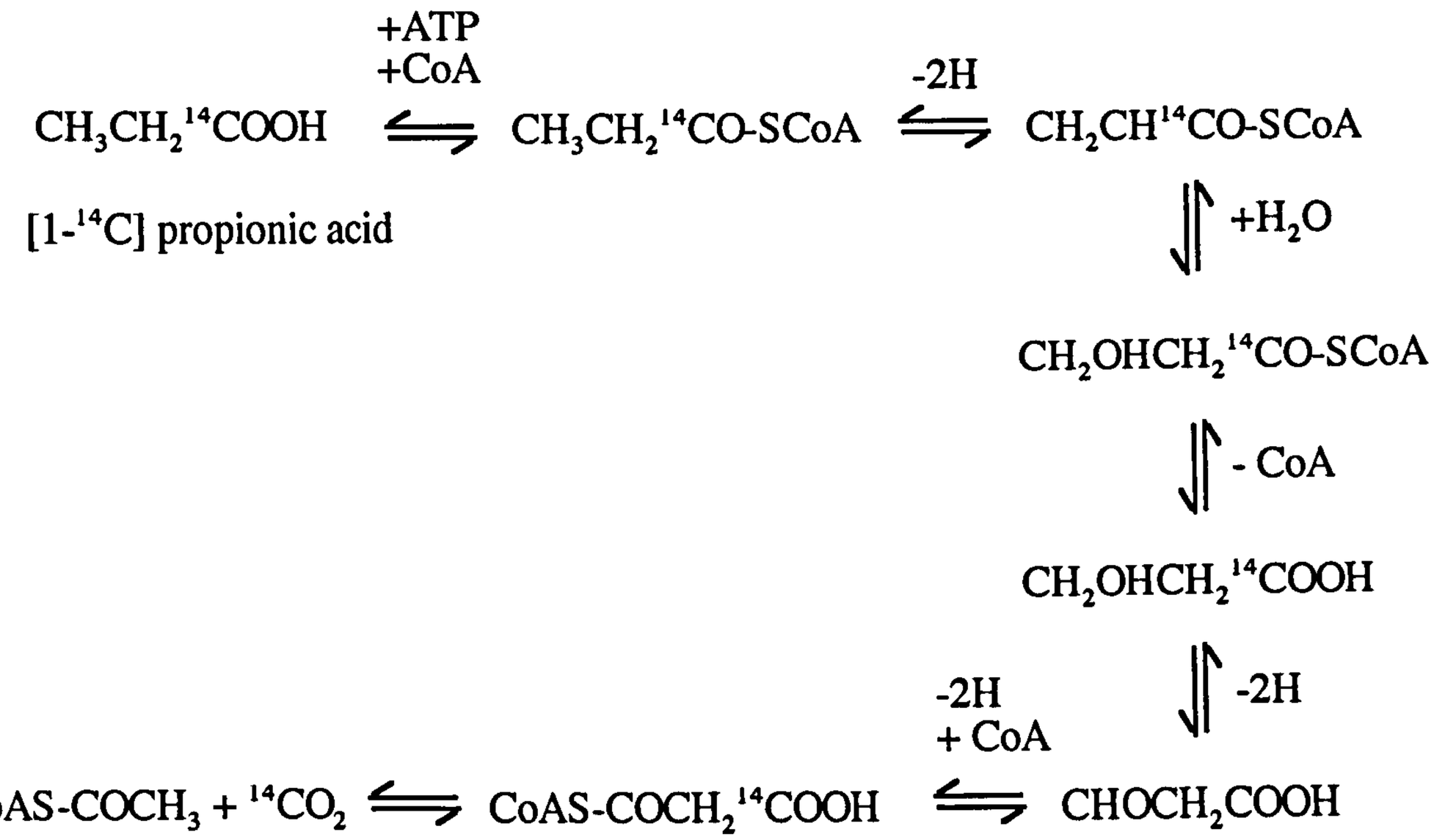


Figure 1.13 Modified β-oxidation or α-oxidation pathway (Giovanelli & Stumpf, 1958).

[1-^{14}C] propionic acid fed to germinating peanut released the 1-^{14}C carbon first, suggesting the modified pathway.

Although there have been reports of B_{12} dependent enzymes in potato tubers (Poston, 1978) it has been demonstrated that the initial product from propionate metabolism in leaves of lima bean is 3-hydroxypropionate (CH_2OHCH_2COOH) (Hanlarkar *et al.*, 1988). Assays for B_{12} in tissues of the lima bean plant showed none detectable in the leaves or stems, but some could be detected in the roots, 46pg/g dry weight (Hanlarkar *et al.*, 1988). It seems most likely that the detected B_{12} in plant tissue is a result of contaminating bacteria in the soil, possibly present on the surface or absorbed into the tissues. This possibility had not been assessed. Whether plants use such B_{12} for catalytic activity is unknown as no B_{12} dependent enzymes have been cloned or purified from a plant source.

As a result, it seems likely that plants do not posses a separate propionyl-CoA carboxylase with a specific metabolic function. If PCCase did exist in a catabolic role it would most likely be present in the mitochondria. It has been reported that the measurable PCCase activity in plants is associated with chloroplasts (Clauss *et al.*, 1993) and that purified mitochondria contain no detectable PCCase activity (Baldet *et al.*, 1992). This suggests PCCase in plants is a side reaction of the Type I ACCase.

1.6 Pyruvate Carboxylase (E.C. 6.4.1.1)

Pyruvate carboxylase (PCase) catalyses the biotin-dependent carboxylation of pyruvate to form oxaloacetic acid in the two step reaction;

$$\text{enz-biotin} + \text{ATP} + \text{HCO}_3^- \overset{Me^{2+}}{\Longleftrightarrow} \text{enz-biotin-CO}_2^- + \text{ADP} + \text{P}_i$$

$$\underline{\text{enz-biotin-CO}_2^- + \text{pyruvate} \Longleftrightarrow \text{enz-biotin} + \text{oxaloacetate}}$$

$$\text{ATP} + \text{HCO}_3^- + \text{pyruvate} \overset{Me^{2+},\ \text{enz-biotin}}{\Longleftrightarrow} \text{oxaloacetate} + \text{ADP} + \text{P}_i$$

Pyruvate carboxylase has been identified in a variety of plant, animal, microbial and fungal species (Wallace, 1985; Wurtele & Nikolau, 1990). It has been intensively studied in many species due to its central role in directing carbon flux (Scrutton & Young, 1972, Keech & Wallace, 1985, Osmani *et al.*, 1985). Known pyruvate carboxylases can be classified into two distinct types according to their structure and response to metabolic effectors (Osmani *et al.*, 1985).

1) Type I; Found in various animals, *Arthrobacter globiformis*, *Bacillus stearothermophilius*, and *Aspergillus nidulans*. The enzyme has a native M_r of 440,000 - 520,000 and is a tetramer made from four identical subunits of M_r 110,000 - 130,000. The native enzyme contains 4 moles biotin / mole as well as four moles of Mn^{2+} / mole (in *S.cerevisiae* the Mn^{2+} is replaced by Zn^{2+}). The enzyme is activated by acyl-CoA

2) Type II A multisubunit type enzyme found in *Pseudomonas citronellolis*, *Azobacter vinelandii* and *Leptosphaeria michotii* (Tozo *et al.*, 1992). These enzymes are not stimulated by acyl-CoA. The native M_r is 454,000 consisting of subunits of 65,000 and 54,000. Only the larger chain contains biotin and they combine as an $\alpha_4\beta_4$ complex

1.6.1 Animal pyruvate carboxylase

PCase has been identified in most mammalian tissues but is especially high (milligram / gram fresh weight of protein) in the liver, kidney cortex and adipose tissue (Scrutton

& Young, 1972). Sources of tissue for purification of the enzyme include sheep liver (Attwood *et al.*, 1986), chicken liver (Attwood & Graneri, 1991) and rat liver (Oei & Robinson, 1985; Thampy *et al.* 1988). The enzyme has been localised to the mitochondria (Utter & Keech, 1960) in chicken liver and has been characterised as a Type I enzyme. Pyruvate carboxylase has been implicated in a variety of metabolic pathways; (a) gluconeogenesis (Utter & Keech, 1960 & 1963), (b) anapleurotic synthesis of oxaloacetate (Kornberg, 1966) and (c) glycerogenesis and lipogenesis (Hanson & Ballard, 1967). The role of PCase in animal tissues is summarised in Figure 1.14.

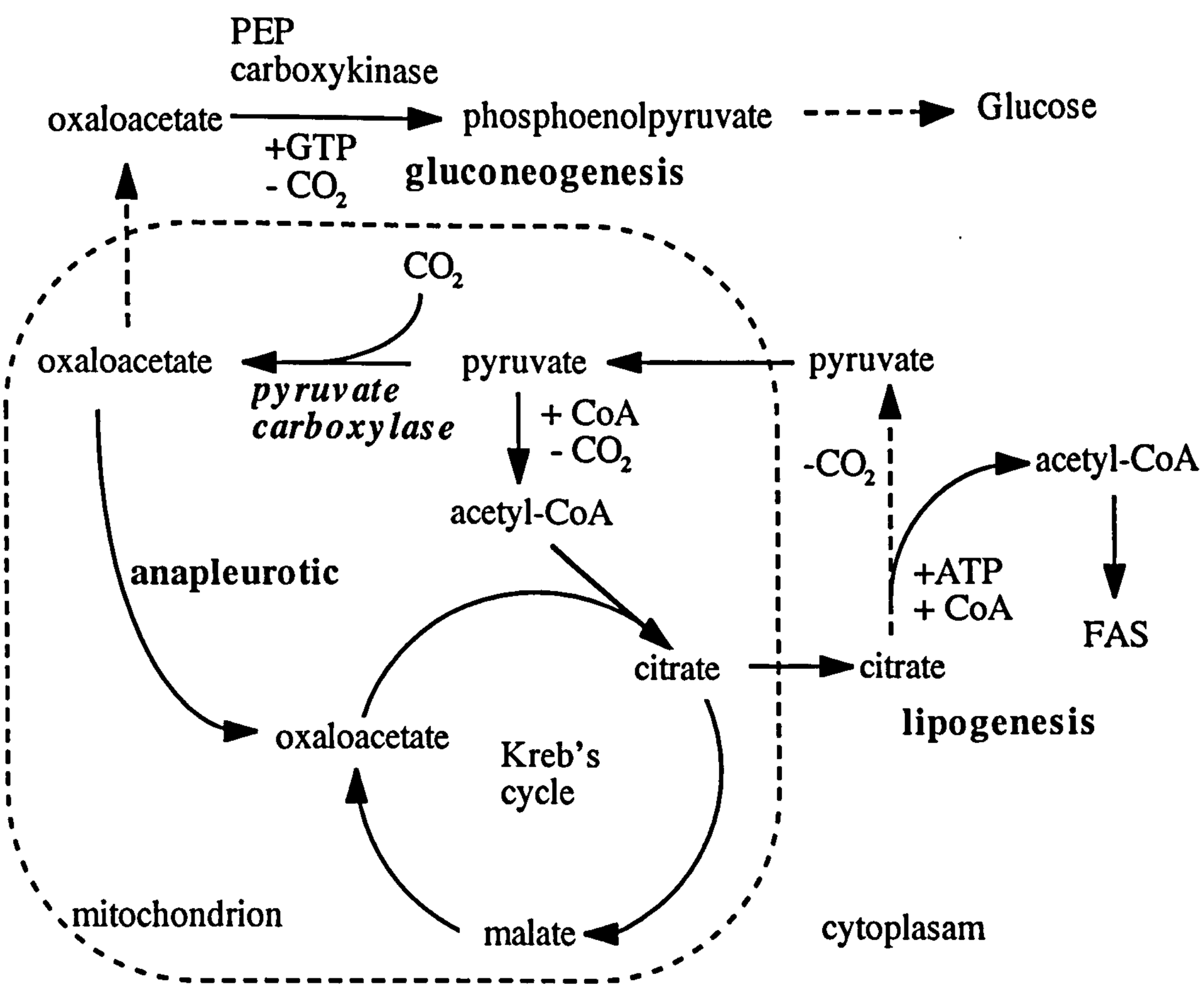


Figure 1.14 Carbohydrate metabolism in mammalian mitochondria with particular reference to pyruvate carboxylase. Oxaloacetate, the end product of the PCase reaction, may be used to 1) supplement Kreb's cycle when intermediates are low (**anapleurotic**); 2) synthesise glucose in a reversal of glycolysis (**gluconeogenesis**); or 3) provide substrate for FAS by export to the cytoplasm and degradation to pyruvate and acetyl-CoA (**lipogenesis**). Oxaloacetate is not able to cross the mitochondrial membrane so must be converted to citrate, malate or aspartamate, transported across and converted back to oxaloacetate in the cytoplasm. This transfer is shown as a series of dashes to indicate more than one step is involved.

As PCase is implicated in several metabolic pathways it is inevitable that the enzyme should be highly regulated. The most potent effector of PCase activity is acyl-CoA. In chicken liver, pyruvate carboxylase is all but inactive in the absence of acetyl-CoA but activity increases one thousand fold in saturating levels of acetyl-CoA (Scrutton & Young, 1972). Pyruvate carboxylase from chicken liver is inhibited by L-glutamate (Scrutton, 1978). These properties enable the enzyme to respond to the metabolic conditions. As glycolysis produces acetyl-CoA this should be incorporated into the TCA cycle and metabolised away. If the intermediates in the TCA cycle are limiting then the rate of production of acetyl-CoA by glycolysis may exceed the rate at which it can be metabolised. In such a case the levels of acetyl-CoA will rise and pyruvate carboxylase will be activated. The product of the PCase reaction is an intermediate in the TCA cycle so the levels of TCA cycle intermediates will rise, more acetyl-CoA will be metabolised and the levels will decrease, deactivating PCase accordingly (Kornberg, 1966). This represents the anaplerotic function of PCase. In instances where the TCA cycle is unable to metabolise the acetyl-CoA then levels will remain high keeping PCase active. In these instances, the oxaloacetate produced is exported to the cytoplasm and used for synthesis of gluconeogenesis (Utter & Keech, 1960 & 1963). Clearly gluconeogenesis and glycolysis do not operate simultaneously or the result would be a futile cycle so the carbon source of pyruvate in this case is not glucose. The inhibitory effect of L-glutamate may have an influence here as normal *in vitro* levels of L-glutamate are inhibitory, but under gluconeogenic conditions the levels of L-glutamate decrease to a non-inhibitory level (Scrutton, 1978).

PCase contributes to the synthesis of fatty acids by shuttling acetyl-CoA out of the mitochondria to the cytoplasm where the components of the fatty acid synthesis complex are located (Hanson & Ballard, 1967). Pyruvate is converted to oxaloacetate by pyruvate carboxylase, this in turn combines with acetyl-CoA to form citrate. Citrate is exported to the cytoplasm where it is broken down to acetyl-CoA and oxaloacetate. The acetyl-CoA is converted to malonyl-CoA by ACCase and used by the fatty acid synthetase complex for fatty acid biosynthesis. The oxaloacetate is

converted to malate, decarboxylated back to pyruvate which is transported back into the cytoplasm to continue the cycle.

1.6.2 Fungal Pyruvate Carboxylase

Pyruvate carboxylase has been studied in *Leptosphaeria michotii* (Tozo *et al.*, 1992), *Saccharomyces cerevisiae*, *Neurospora crassa*, *Rhizopus arrhizus*, and a variety of species of *Aspergillus* (Osmani *et al* 1985). The fungal pyruvate carboxylases studied to date are very similar to the mammalian and avian form of the enzyme except for the enzyme from *Leptosphaeria michotii* which has a Type II structure (discussed in section 1.6.3). Of the others the subunit size and structure are very similar, the native M_r is 500,000, the subunit size is M_r 125,000 - 135,000 and the native enzyme consists of four subunits. Whereas mammalian pyruvate carboxylase is located in the mitochondrion, fungal pyruvate carboxylase is located in the cytosol. The enzyme is activated by acetyl-CoA but not as strongly as the mammalian or avian enzyme and fungal PCase is also activated by long chain acyl-CoAs unlike the mammalian enzyme. L-aspartate or 2-oxoglutarate serves as the regulatory inhibitor of PCase in fungi, not L-glutamate. The difference in regulatory properties reflects the different metabolic arrangement in fungi.

The structure of the enzyme in filamentous fungi and yeast has been examined under the electron microscope (Osmani *et al.*, 1985; Rohde *et al.*, 1986). A tetrameric structure is observed, consistent with the number of subunits found in the native enzyme. The subunits are arranged into a tetrahedron-like configuration consisting of two pairs of identical subunits located in two different planes which are orthogonal to each other, very similar to the vertebrate enzyme (Mayer *et al.*, 1980)

1.6.3 Type II (Bacterial) Pyruvate Carboxylases

Type II pyruvate carboxylases have been detected in *Azobacter vinelandii* (Scrutton & Taylor, 1974), *Pseudomonas citronellolis* (Cohen *et al.*, 1979) and the fungi *Leptosphaeria michotii* (Tozo *et al.*, 1992). They are classified by their activity independent of metabolic effectors.

The structure of Type II pyruvate carboxylases studied is different to the Type I form, the enzyme has a native M_r of 440,000 from *L.michotii* and approximately 500,000 from *P.citronellolis*. The enzyme is made up from two dissociable subunits with a M_r of 60,000 and 50,000 in *L.michotii* and 65,000 and 54,000 in *P.citronellolis* the larger subunit being the biotin-containing one. In both cases the subunits combine in an $\alpha_4\beta_4$ structure. Electron micrographs of the purified protein from *P.citronellolis* show that the enzyme may have a tetramer structure not unlike the arrangement seen for the Type I enzyme in *Saccharomyces cerevisiae* (Cohen *et al.*, 1979).

The regulation of Type II acetyl-CoA carboxylase occurs by altering the level of enzyme, in *L.michotii* at least(Tozo *et al.*, 1992). This is the only known mechanism of enzyme regulation for this form of pyruvate carboxylase as all metabolites investigated to date, other than oxaloacetate (the reaction product), have no effect on the rate of reaction (Scrutton & Taylor, 1974). In *A.vinelandii* and *P.citronellolis* the enzyme is induced by growth on both succinate and pyruvate or lactate (Scrutton & Taylor, 1974).

1.6.4 Plant pyruvate carboxylase

Plants contain a detectable pyruvate carboxylase activity (Wurtele & Nikolau, 1990). Other than that nothing else is known about this enzyme in plants but some speculation is possible concerning a possible role. Gluconeogenesis in plants occurs during photosynthesis in the chloroplasts of green plants. The only time plants are required to synthesis glucose by mechanisms other than photosynthesis, is during the germination of oilseeds, and in this case acetyl-CoA derived from fatty acids is converted to glucose via the glyoxylate cycle. Lipogenesis in plant tissue occurs

within chloroplasts. Although the actual source of acetyl-CoA for lipid biosynthesis is undetermined, chloroplasts contain all the necessary enzymes required for generation of acetyl-CoA and pyruvate carboxylase is not implicated (Harwood, 1988).

In animals pyruvate carboxylase also has an anaplerotic role in supplementing the TCA cycle with intermediates (Kornberg, 1966). If pyruvate carboxylase were located in the mitochondria of plants it would be possible that it could carry out this process. Purified mitochondria from pea leaf contain only one detectable biotinylated protein MCCase (Baldet *et al.*, 1992), which suggests that pyruvate carboxylase is not present in the mitochondria of this tissue at least.

Pyruvate carboxylase has not been isolated or cloned from plants, and it seems to be redundant as most of its functions in animals are superseded by the biosynthetic pathways of the chloroplast in plants.

1.7 The Aims of this Research

Biotin-containing enzymes are important because the reactions that they catalyse are associated with vital metabolic pathways. Accordingly, these enzymes have been well characterised from animal sources but scant attention has been paid to the variety of biotin-containing enzymes in plants and their function in plant metabolism.

Commercially, the most important of the biotin-containing enzymes in plants is acetyl-CoA carboxylase due to its involvement in lipid biosynthesis. Oil biosynthesis is of major commercial importance due to the variety of commercial applications to which they are put such as soaps and detergents, cooking oils, margarine, solvents and high temperature lubricants (Knauf, 1987). A knowledge of how malonyl-CoA required for oil biosynthesis is formed, is therefore of much commercial value. Acetyl-CoA carboxylase is of particular interest as genetic engineering allows the production of new products in plants, particularly polyhydroxybutyrate (PHB). PHB is a valuable short hydrocarbon synthesised from acetyl-CoA, which can be used as a building block for bio-degradable plastics and is therefore commercially very desirable (Poirier *et al.*, 1992; Nawrath *et al.*, 1994). If the activity of acetyl-CoA carboxylase can be reduced in such plants an increase in the yield of PHB may be expected. To redirect metabolic flux in such a way demands that an in depth knowledge of the enzyme involved, in this case acetyl-CoA carboxylase, be obtained.

The recent (re)discovery of a prokaryotic type acetyl-CoA carboxylase in plants such as *B.napus* demonstrates the need for research into this enzyme in commercially important oilseed crops.

The other known enzymes, pyruvate carboxylase, methylcrotonyl-CoA carboxylase and propionyl-CoA carboxylases, while maybe of less commercial interest now are nonetheless important enzymes in carbon metabolism. Whilst their function remains in doubt it is not possible to say with certainty how carbon is metabolised in plant tissues and how these enzymes may direct flux. Pyruvate carboxylase for example,

has such a vital role to play in animals that one wonders what function it must have in plants where carbon metabolism is so different.

Whilst all biotin-containing proteins are similar in that they contain biotin, they are involved in such a wide variety of processes that it may seem extraordinary for one project to focus on all of them at once. But because they all contain biotin they can all be studied at once just as easily as in isolation. The properties of biotin and the proteins that bind it, avidin and streptavidin, have made it a system of choice for many detection and purification procedures (see Wilchek & Bayer, 1990 for applications of avidin-biotin technology). The use of streptavidin to directly detect biotinylated proteins on western blots from plant tissue has been demonstrated (Nikolau *et al.*, 1985). Biotin-containing proteins can also be directly isolated by affinity chromatography using avidin-monomer columns (Henrickson *et al.*, 1979). The biotin moiety common to all these enzymes therefore acts a handle by which these proteins may be detected and purified.

The aims of this research was to characterise the biotinylated proteins present in the commercially important agricultural crop, *Brassica napus* (oilseed rape). The objectives were as follows. (1) Demonstrate the presence of biotin-containing enzymes in *Brassica napus*. This could be done by a) enzyme assays or b) detection on Western blots with labelled streptavidin (Nikolau *et al.*, 1985). This would indicate how many biotin-containing enzyme activities were present in *Brassica napus* and how many biotin-containing proteins could also be detected. Comparison of the M_rs of biotinylated proteins in *B.napus* with known biotin-containing protein from other organisms would help to reveal possible similarities giving a clue to their function.

(2) Analysis of cDNA clones encoding biotinylated proteins. Genes could be isolated by, a)screening an expression library with labelled streptavidin (Collins *et al.*, 1987; Hoffman *et al.*, 1987; Wang *et al.*, 1993), or by b) purification of biotin-containing proteins using a combination of standard chromatography techniques and avidin-

monomer affinity chromatography, and sequencing the protein or raising antibodies, both of which provide tools which can be used for isolation of genes. Isolation of genetic material for biotin-containing enzymes would provide a powerful tool for analysis of these genes. Northern and southern data would provide information on the expression of the mRNA and the number of genes present, suggesting how many isoforms might be expressed. Overexpression of proteins in *E.coli* is a powerful method for obtaining large quantities of material, possibly for the raising of specific antisera or for physical analysis of the proteins. Overexpression of a cDNA encoding for a biotin-containing protein in *E.coli* is likely to produce a biotinylated protein (Cronan, 1990). This could be used for functional studies of these proteins.

Characterisation of the isolated genes or proteins for biotin-containing enzymes would provide information on the function of these protein *in vivo* by showing what tissues and at what stage of development they were expressed in, where the proteins were located within a cell, what the physical characteristics of the proteins were, and how this might correspond to the metabolic processes in the tissue at that stage. Information derived from these studies and others like them should prove useful in deciphering a metabolic role and significance for the biotin-containing proteins in *Brassica napus*.

Chapter 2: Materials and Methods

Most procedures were based upon the methods of Sambrook *et al.*, 1989 or Ausubel *et al.*, 1994. Protocols derived from elsewhere have been referenced appropriately.

2.1 Materials

2.1.1 Reagents and Supplies

All materials were purchased from Sigma Chemical Company, Sigma-Aldrich Company Ltd., Fancy Road, Poole, Dorset, BH12 4QH, UK, or BDH Chemicals Ltd., Poole, Dorset, BH15 1TD, except for those listed below.

[-^{32}P] dCTP, [^{125}I]-streptavidin, [U-^{14}C]-L-leucine, [^{35}S]-methionine and [^{14}C]-bicarbonate were from Amersham International Plc., Amersham Place, Little Chalfont, Amersham, Buckinghamshire, HP7 9NA.

Agar, Bacto-tryptone, bacto-yeast and casamino acids were from DIFCO laboratories, PO Box 14B, Central Avenue, West Molesey, Surrey, KT8 2SE.

Ampicillin and Tetracycline were from NBL Gene Sciences Ltd., South Nelson Industrial Estate, Cramlington, Northumberland, NE23 9HL.

AmpliWax™ PCR Gem-100 was from Perkin Elmer Ltd., Post Office Lane, Beaconsfield, Buckinghamshire, HP9 1QA.

AquaPhenol™ was from Appligene, Pinetree Centre, Durham Road, Birtley, Chester-le-Street, Co. Durham, DH3 2TD, UK.

Chromatography media (Mono Q, Blue Sepharose FF, Superose-12 HR 10/30) were from Pharmacia Biotech, 23 Grosvenor Road, St. Albans, Hertfordshire, AL1 3AW, and used on a Pharmacia HiLoad of FPLC chromatography system.

Colony / Plaque Screen™ Hybridisation Transfer Membrane were from NEN® Research Products, Du Pont (UK) Ltd., Diagnostics & Biotechnology Systems, Wedgwood Way, Stevenage, Hertfordshire, SG1 4QN.

Developer and fixer was from H.A. West [X-Ray] Ltd., 41 Watson Crescent, Edinburgh, EH11 1ES.

DNA (custom synthesis) was by Perkin Elmer Applied Biosystems, Beechwood Science Park, Park North, Warrington, Cheshire.

Double-stranded Nested Deletion Kit was from Pharmacia Biotech, 23 Grosvenor Road, St. Albans, Hertfordshire, AL1 3AW

ECL detection reagents were from Amersham International Plc., Amersham Place, Little Chalfont, Amersham, Buckinghamshire, HP7 9NA.

Electrophoresis pure Acrylamide, Ammonium persulphate, Bis-acrylamide, Glycine, SDS, TEMED and Tris base, were from BIO-RAD Laboratories LTD., Bio-Rad, Maylands Avenue, Hemel Hempstead, Hertfordshire, HP2 7TD.

Ficoll 400 was from Pharmacia Biotech, 23 Grosvenor Road, St. Albans, Hertfordshire, AL1 3AW

GeneAmp™ Thin Walled Reaction Tubes were from Perkin Elmer Ltd., Post Office Lane, Beaconsfield, Buckinghamshire, HP9 1QA

IPTG, X-gal and DTT were from Melford Laboratories Ltd., Chelsworth, Ipswich, Suffolk, IP7 7LE.

Hybond C extra, Hybond N and Hybond N+ were from Amersham International Plc., Amersham Place, Little Chalfont, Amersham, Buckinghamshire, HP7 9NA.

Miracloth was from Calbiochem corporation, La Jolla, CA 92037, USA.

Minisart 0.2µm filters (sterile) were from Sartorius GmbH, Postfach, D-3400, Göttingen, Germany.

NHS-LC-biotin was from Pierce & Warriner (UK) Limited, 44 Upper Northgate Street, Chester, CH1 4EF.

Pharmalytes 5/8 and 3/10 were from Pharmacia Biotech, 23 Grosvenor Road, St. Albans, Hertfordshire, AL1 3AW

QIAGEN-Tip 100 were from Qiagen Ltd. Unit 1, Tillingbourne Court, Dorking Business Park, Dorking, Surrey, RH4 1HJ, UK.

QIAEX® II Gel Extraction Kit was from Qiagen Ltd. Unit 1, Tillingbourne Court, Dorking Business Park, Dorking, Surrey, RH4 1HJ, UK.

*redi*prime DNA labelling system was from Amersham International Plc., Amersham Place, Little Chalfont, Amersham, Buckinghamshire, HP7 9NA.

Restriction Endonucleases and buffers were from Boehringer Mannheim UK (Diagnostics and Biochemicals) Ltd., Bell Lane, Lewes, East Sussex, BN7 1LG, UK

Streptavidin : Horse Radish Peroxidase conjugate, Sheep anti-rabbit IgG : alkaline phosphatase conjugate and Donkey anti-sheep IgG : alkaline phosphatase conjugate were from Serotec Ltd., 22 Bankside, Station Approach, Kidlington, Oxford OX5 1JE, UK.

Subcloning Efficiency DH5α™ Competent Cells were from Life Technologies, 3 Fountain Drive, Inchinnan Business Park, Paisley, PA4 9RF.

SUPERSCRIPT™ Preamplification System for First Strand cDNA Synthesis was from Life Technologies, 3 Fountain Drive, Inchinnan Business Park, Paisley, PA4 9RF.

T4 DNA Ligase + buffer was from Promega Ltd., Delta House, Enterprise Rd, Chilworth Research Centre, Southampton, SO16 7NS.

TNT® Coupled Reticulocyte Lysate System was from Promega Ltd., Delta House, Enterprise Rd, Chilworth Research Centre, Southampton, SO16 7NS.

UltraPure Solution dNTPs were from Pharmacia Biotech, 23 Grosvenor Road, St. Albans, Hertfordshire, AL1 3AW.

Ultrafree®-MC filters were from Millipore (UK) Limited, The Boulevard, Blackmoor Lane, Watford, Hertfordshire, WD1 8YW.

Vent™ DNA Polymerase was from New England Biolabs (UK), Ltd., 67 Knowl Piece, Wilbury Way, Hitchin, Hertfordshire, SG4 OTY, England.

Wizard™ Miniprep DNA purification system was from Promega Ltd., Delta House, Enterprise Rd, Chilworth Research Centre, Southampton, SO16 7NS.

X-Ray film was from Fuji Photo Film [UK] Ltd., Fuji Film House, 125 Finchley Road, Swiss Cottage, London, NW3 6JH.

2.1.2 Phage strains

Bacteriophage λZAP II (Short *et al.*, 1988) used in this study were a gift from Dr. A.S.N Reddy, University of Colorado. VCSM13, ExAssist™ phage were used for *in vivo* excision from λZAP II (Stratagene, La Jolla, CA 92037, USA.).

2.1.3 Bacterial Strains

The bacterial strains used in this study were all *Escherichia coli* and are listed in Table 2.1.

Bacterial Strain	*Genotype*	*Source and Reference*
BL21(λDE3)	F^- *ompT [lon] hsdS*$_B$ (r_B^- m_B^-) *gal* (λ*c* I*ts*857 *ind*1 *S*am7 *nin*5 *lac*UV5-T7 gene*1*)	Lab glycerol, Studier & Moffat, 1986.
DH5α	*supE*44 Δ*lac*U169 (ϕ80 *lacZ*ΔM15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1	Lab glycerol, Hanahan, 1983.
SOLR™	e14$^-$(McrA$^-$) Δ(*mcrCB-hsdSMR-mrr*)171 *sbcC recB recJ uvrC umuC::Tn5* (Kan^r) *lac gyrA*96 *relA*1 *thi-1 endA*1 λ^R [F' *proAB lacI*^{q}*Z*Δ*M*15] Su$^-$ (nonsuppressing)	Stratagene, Hay & Short, 1992
TB1	F^- *ara* Δ(*lac-proAB*) *rpsL* (Str^r) [ϕ80 d*lac*Δ(*lacZ*)*M*15] *thi hsdR* (r_K^- m_K^-)	New England Biolabs
XL1-Blue	*supE*44 *hsdR*17 *recA*1 *endA*1 *gyrA*46 *thi relA*1 *lac*$^-$	Lab glycerol, Bullock *et al.*, 1984.
Y1090	Δ*lac*U169 Δ*lon araD*139 *strA supF mcrA trpC22::*Tn*10*(Tet^r) [pMC9 Amp^r Tet^r]	Lab glycerol, Young & Davis, 1983.

Table 2.1 Bacterial strains employed in this study

2.1.4 Plasmids

Plasmids used in this study were maintained in *E.coli* DH5α. The plasmids and their prominent features are shown in Table 2.2.

Plasmid	*Remarks*	*Source and Reference*
pBluescript™ SK -	Amp^r, excised from λZAP II	Lab Stocks, Short *et al.*, 1988
pET11a	Amp^r, T7 promoter, *lac*, rbs	Novagen Inc. 597 Science Drive, Madison WI 53711
pET23(+)	Amp^r, T7 promoter	Novagen
pET24a	Kan^r, T7 promoter, *lac*, rbs	Novagen
pMAL-c2	Amp^r, p*tac* promoter,	New England Biolabs (UK), Ltd., 67 Knowl Piece, Wilbury Way, Hitchin, Hertfordshire, SG4 OTY, England
pT7groE	Cm^r, *groE* operon, T7 promoter	Dr. G. Lorimer, Kalbach & Gatenby, 1993

Table 2.2 Plasmid employed throughout this research. Rbs - ribosome binding site.

2.1.5 Seed Material

Three varieties of *Brassica napus* seed material were used throughout, Falcon, Jet neuf and Westar. Seeds material was collected removed from freshly harvested pods and either immediately frozen in liquid N_2 or the embryos dissected out from the testa and frozen. Embryos were stored in liquid N_2 and seed material was stored at -80°C.

2.1.6 Buffers and Stock Solutions

10 × SDS running buffer

10g SDS

144g glycine

30.3g Tris base

in 1L ddH_2O. The pH of 1 × running buffer should be 8.3.

20 × SSPE
175.3g NaCl

27.6g $NaH_2PO_4 \cdot H_2O$

40ml 0.5M EDTA (pH8.0)

per litre ddH_2O. The pH was adjusted to 7.4 with NaOH.

20 × SSC
175.3g NaCl

88.2g Na.citrate

per litre ddH_2O. The pH was adjusted to 7.0 with HCl.

5× Native sample buffer
3.7g Tris base

63g glycerol

0.025g bromophenol blue

in 100ml ddH_2O. The pH was adjusted to 6.8 with HCl.

5× SDS sample buffer
2.5g SDS

1.25g DTT

0.0125g bromophenol blue

0.95g Tris base

12.5ml glycerol

in 25ml ddH_2O. The pH was adjusted to 6.8 and the buffer was aliquoted into 1ml aliquots which were stored at -20°C.

50 × Denhardt's solution
5g Ficoll 400

5g polyvinylpyrrolidone

5g BSA fraction V

per 500ml. The solution was filtered through 0.22μm filter and stored as aliquots at -20°C.

6× DNA loading dye
0.25% (w/v) bromophenol blue

0.25% (w/v) xylene cyanol

15% Ficoll 400

60mM EDTA

in ddH_2O. The pH was adjusted to 8.0 with NaOH.

Denaturing solution
87.7g NaCl

20.0g NaOH

per litre ddH_2O.

Electrotransfer buffer
14.4g glycine

3.03g Tris base

0.19g SDS

200ml methanol

in 1L ddH_2O.

Hybridisation solution
50% formamide

5 × SSPE

2 × Denhardt's solution

0.1% SDS

100µg/ml denatured, fragmented DNA

Neutralisation solution
87.7g NaCl

121.14g Tris base

per litre ddH_2O. The pH was adjusted to 7.4 with HCl.

Phosphate Buffered Saline (Tween) (PBS-(T))
8g NaCl

0.2g KCl

1.44g Na_2HPO_4

0.24g KH_2PO_4

in 1L ddH_2O. The pH was adjusted to 7.4 if necessary with NaOH or HCl. PBS-T contained 0.05% Tween-20.

100mM PMSF
0.35g PMSF

per 20ml of propan-2-ol. Diluted 100 fold in required buffer immediately before use.

Pre-hybridisation solution
50% formamide

5 × SSPE

5 × Denhardt's solution

0.1% SDS

200µg/ml denatured, fragmented salmon sperm DNA

Pre-washing solution
5 × SSC

0.5% SDS

1mM EDTA (pH8.0)

TE buffer pH8.0
10mM Tris-HCl, pH8.0

1mM EDTA

Tris/acetate (TAE) 50 × stock
242g Tris base

57.1ml glacial acetic acid

100ml 0.5M EDTA (pH8.0)

per litre ddH_2O.

2.1.7 Growth media

All growth media described below could be used as liquid media, or could be solidified by the addition of 1.5% agar or 0.7% agarose.

LB (Luria-Bertani)

10g bacto-tryptone

5g bacto-yeast extract

10g NaCl

per litre ddH_2O. The pH was adjusted to 7.5 with NaOH.

M9 MM

200ml 5 × M9 salts (see below)

20ml 20% glucose

in 1L ddH_2O.

5 × M9 salts were made by combining 42.5g $Na_2HPO_4 \cdot 2H_2O$, 15g KH_2PO_4, 2.5g NaCl and 5.0g NH_4Cl per litre. M9MM media was made by adding M9 salts and glucose to 780ml of sterile ddH_2O < 50°C.

NZYCM

10g NZ amine (Casein enzymatic hydrolysate)

5g NaCl

5g bacto-yeast extract

1g casamino acids

2g $MgSO_4 \cdot 7H_2O$

per litre ddH_2O. The pH was adjusted to 7.0 with NaOH.

SOB

20g bacto-tryptone

5g bacto-yeast extract

0.5g NaCl

The contents were dissolved in 980ml of ddH_2O to which 10ml of 250mM KCl were added and the pH adjusted to pH7.0 with 1N NaOH. Five millilitres of a sterile solution of 2M $MgCl_2$ was added per litre just before use.

SOC

SOC was made from SOB + 20mM glucose. Twenty millilitres of a filter sterilised solution of 1M glucose was added when the SOB solution had been autoclaved and had cooled to < 60°C.

TB (Terrific Broth)

12g bacto-tryptone

24g bacto-yeast extract

4ml of glycerol

per 900ml of ddH_2O.

After autoclaving 100ml of a sterile solution of 0.17M KH_2PO_4, 0.72M K_2HPO_4 was added when the solution had cooled to < 60°C.

2.1.8 Antibiotics

Ampicillin (Amp)

A stock solution was made in ddH_2O at a concentration of 50mg/ml, sterilised by filtration and stored at -20°C. Routinely it was used at a concentration of 50µg/ml. Under conditions where a high plasmid stability was required, i.e. during expression from pET vectors, it was used at twice the concentration. Ampicillin was added at 200µg/ml to TB as growth times are extended in this media. Ampicillin affects bacterial cell wall synthesis. The resistance gene *bla* encodes for β-lactamase which cleaves the lactam ring of the antibiotic (Sambrook *et al.*, 1989). β-lactamase is excreted from the bacteria, and therefore hydrolyses the antibiotic in the culture media. The levels of antibiotic are depleted as growth continues so a higher concentration of starting antibiotic in cultures using TB, ensures only bacteria containing the *bla* gene will grow to a high density.

Chloramphenicol (Cm)

A stock solution was made in 96% (v/v) ethanol at a concentration of 34mg/ml and stored at -20°C. A concentration of 50µg/ml was routinely used. The resistance gene Cm^r encodes an enzyme chloramphenicol acetyltransferase. Chloramphenicol binds the 50S ribosomal subunit inhibiting protein synthesis (Sambrook *et al.*, 1989). The acetylated derivatives made by chloramphenicol acetyltransferase in the presence of acetyl-CoA are unable to bind ribosomes.

Kanamycin (Kan)

A stock solution was made in ddH_2O at a concentration of 10mg/ml, sterilised by filtration and stored at -20°C. The antibiotic was used in cultures at a concentration of 25µg/ml. Kanamycin resistance is conferred by the gene *kan* which encodes a protein with aminoglycoside phosphotransferase activity. Kanamycin is a aminoglycoside compound which binds to ribosomal components inhibiting protein synthesis. Phosphorylation prevents the transport of the antibiotic into the cell (Sambrook *et al.*, 1989).

Rifampicin

A stock solution of 20mg/ml in methanol was made and stored in the dark at -20°C for up to 2 weeks. A concentration of 200µg/ml was used to inhibit bacterial transcription during expression from the T7 promoter. Rifampicin inhibits mRNA synthesis by binding to DNA-dependent RNA polymerase. The T7 DNA-dependent RNA polymerase is not affected by rifampicin (Ausubel *et al.*, 1994).

Tetracycline (Tet)

A stock solution of 12.5mg/ml was made in 96% (v/v) ethanol and stored in the dark at -20°C. A final concentration of 12.5µg/ml was used. Tetracycline binds to the 30S subunit of the ribosome and inhibits binding of the tRNA-amino acid complexes. The *tet* resistance gene encodes a membrane associated protein that prevents the antibiotic from entering the cell (Sambrook *et al.*, 1989).

2.2 Bacteriological methods

2.2.1 Working practices

A sterile working practice was maintained throughout. All media and materials used in experimental work involving bacterial cultures were sterile. Solutions that were not able to be sterilised by autoclaving were filter sterilised by passing the solution through a Sartorius Minisart 0.2μm nitrocellulose filter. Other solutions were sterilised by autoclaving for 15 minutes at 15p.s.i, 123°C.

All glassware and plastic-ware used in the maintenance of cultures or the manipulation of DNA was autoclaved before use. All disposable plastic-ware used for manipulation of RNA was taken from fresh, unopened packaging and autoclaved to prevent RNase contamination. Examination gloves were worn when handling DNA or RNA.

All bottle necks were flamed in a roaring Bunsen flame before and after use and caps were replaced on all containers as soon as possible. Culture plates were sterilised after pouring by flaming the surface of the molten agar medium with a roaring Bunsen flame.

2.2.2 Maintenance of Stock Cultures

Stains harbouring no plasmids or selectable markers were cultured in Luria-Bertani media or on Luria-Bertani agar plates without antibiotics. Strains containing plasmids or selectable markers were grown in medium under selective conditions. Strains were kept indefinitely in 15% (v/v) glycerol at -80°C. For glycerol stocks, a 5ml LB culture was inoculated with a single colony from a LB agar plate and incubated at 37°C overnight with shaking. A 0.85ml aliquot of bacterial culture was combined with 0.15ml of sterile glycerol in a screw-cap microfuge tube, and mixed by vortexing for 30 seconds. The tube was labelled and snap-frozen in liquid N_2. Tubes were transferred to a -80°C freezer for long term storage.

2.2.3 Measurement of growth - bacteria

Bacteria grown in liquid culture were monitored for growth by measurement of the optical density at 600nm. From each culture, 1ml samples were taken at the appropriate times and placed in a 1ml disposable cuvette. The absorbance at 600nm was measured in a spectrophotometer blanked against ddH_2O (which has the same OD_{600} as LB).

2.2.4 Measurement of growth - Phage Titration

A serial dilution was created by taking 10µl of phage suspension and diluting with 90µl SM buffer (5.8g/L NaCl, 2.0g/L $MgSO_4$, 50ml/L 1M Tris-HCl pH7.5, 5ml/L 2% (w/v) gelatine) in a series until a dilution of 10^8 was reached. After the addition of 100µl of appropriate log-phase host cells to the above dilution, phage and host cells were incubated at 37°C for 20 minutes to allow infection. The bacteria and diluted phage were transferred to a 15ml sterile polypropylene centrifuge tube and 3ml of NZYCM agarose at 42°C was added and mixed. The molten NZYCM agarose was immediately poured onto the surface of a NZYCM agar plate and left to set. The plates were incubated inverted, at 37°C overnight. The phage concentration in plaque forming units (p.f.u) could be calculated by the formula p.f.u./ml = plaques/plate × 1/dilution × 100.

2.2.5 Growth of plating bacteria.

A 50ml NZYCM culture in a 250ml flask containing 0.2% maltose and 12.5µg/ml tetracycline was inoculated with a single colony of *E.coli* XL1-Blue bacteria from a freshly streaked LBtet plate. The culture was grown to mid log phase (4-6 hours, OD_{600} 0.5-1.0) at 37°C with shaking (220rpm). Lambda bacteriophage infect *E.coli* through the maltose receptor in the cell wall so the presence of maltose in the growth media aids in infection by increasing the number of receptors present in the cell wall. Mid-log phase bacteria are used to reduce the number of dead bacteria present in the culture. If phage bind to a dead bacterium, they cannot propagate. Cells were harvested by centrifugation at 3,000 × g for 10 minutes at 4°C. The pellet was re-suspended 4ml of sterile 10mM $MgSO_4$ and stored at 4°C for up to 1 week.

2.2.6 Expression of cloned genes in λZAP II.

Phage were plated as described in section 2.3.6 at a dilution suitable to give 100 - 200 plaques per 70mm petri dish. After plating of the phage and the NZYCM agarose had set, the infected plates were incubated at 42°C for 3.5 hours. A 70mm nitrocellulose filter was soaked in 10mM IPTG for 5 minutes. Using blunt ended forceps the membrane was transferred to a filter paper and allowed to air-dry. Plates with bacteria and forming plaques (approximately 3.5hrs growth at 42°C) were removed from the incubator and quickly overlayed with the IPTG soaked filter before being returned to a 37°C incubator for a further 4 hours growth.

Plates were removed from the 37°C incubator and stored at 4°C for 30 minutes to cool the NZYCM agarose and prevent it peeling away with the filter. Pre-punched holes in the filter were marked on the reverse of the plate with a marker pen, and the filter carefully lifted off. Proteins expressed by the phage were detected with an appropriate antibody or streptavidin-HRPO.

2.2.7 Amplification of λZAP II phage.

Stocks of phage with too low a titre were "amplified" to produce a stock with a higher titre. Phage were plated as described in section 2.3.6 at a dilution suitable to give approximately 500 plaques per 70mm petri dish. Plaques were grown overnight at 37°C and transferred to 4°C to stop further growth. Phage were removed from the plate by placing the plate on a rotary platform at 4°C and covering the top agarose with 5ml of SM buffer. Plates were incubated for ≥2 hours before the SM buffer was pipetted off the surface of the plate into a sterile 15ml polypropylene centrifuge tube. The solution was spun at 3,000 × g for 15 minutes to pellet the bacteria and the supernatant was decanted into another tube to which 20µl of chloroform was added to kill any remaining bacteria and preserve the bacteriophage. Phage were stored in this state indefinitely, although the titre dropped with time.

2.2.8 *In vivo* excision from λZAP II phage.

The bacterial strain *E.coli* SOLR™ was used for excision of pSK- from λZAP II phage. Bacteria were grown in NZYCM + 0.2% maltose + 25μg/ml kanamycin to mid-log phase. Of this culture, 200μl was combined with 200μl of bacteriophage (≥ 1x 10^5 p.f.u./ml in order to have a 100 × excess of phage) and 1μl of ExAssist™ helper phage. After incubation at 37°C for 15 minutes, 3ml of SOC was added and the culture was grown at 37°C for 3 hours. The culture was heated to 70°C for 20 minutes to kill the bacteria. The ExAssist™ phage create a pBluescript™ SK- M13 ssDNA filamentous phage from the λZAP II vector and this survives the 70°C incubation. The culture was spun at 3,000 × g for 10 minutes to remove bacterial debris. The supernatant containing the ssDNA M13 phagemids was transferred to a fresh tube.

Of the *E.coli* SOLR™ bacteria prepared earlier, 200μl was placed in a microfuge tube with 20μl of ssDNA M13 phagemids. After incubation at 37°C for 10 minutes the culture was plated out on LB plates containing 50mg/ml ampicillin. The advantage of using the *E.coli* SOLR™ strain is that the ExAssist™ helper phage are unable to propagate in this host and therefore there are no problems with subsequent colonies being infected with helper phage. Ampicillin is the selectable marker present on the pBluescript™ plasmid and therefore, bacteria infected by the M13 phage would be resistant.

2.2.9 Expression of cloned proteins in lambda lysogens

Lysogens were generated in the *lon* protease deficient strain *E.coli* Y1090 (Mahy, 1985). A single colony from a freshly streaked LB + 50μg/ml ampicillin agar plate containing colonies of *E.coli* Y1090, was used to inoculate a 5ml LB + 50μg/ml ampicillin culture. The culture was grown to OD_{600} of 0.4 at 30°C. A 1ml aliquot of culture was diluted to 10ml with LB, 200μl of this would contain roughly 6.4 × 10^6 cells, this was transferred to a sterile 15ml tube. The cells were infected with a 100-200 fold multiplicity of phage (i.e. 10μl of plate lysate with a titre of 10^7p.f.u/ml) for

20 minutes at room temperature. The sample was diluted to 10ml with LB. A 15µl aliquot was taken from this dilution and diluted again to 10ml. Of this, 200µl was spread on a LB + 50µg/ml ampicillin plate and grown overnight at 30°C. Twenty colonies were replica plated onto two LB + 50µg/ml ampicillin on a numbered grid. One plate (the master) was grown at 30°C overnight, whilst the other was grown at 42°C.

Colonies which grow at 30°C but not at 42°C were assumed to contain an integrated copy of the lambda phage. Under conditions of heat stress, the integrated lambda phage excises itself and enters lytic mode, killing the infected *E.coli* and thereby preventing growth.

Expression from lambda lysogens was induced by growing the lysogen at 30°C to OD_{600} of 0.5 in LB + 50µg/ml ampicillin, causing excision of the lambda phage by heat shock at 43°C for 20 minutes in a shaking water bath, and then adding IPTG to cause expression from the *lac*Z promoter. Bacteria were grown for ≥1hour at 37°C before they were rapidly cooled on ice. Further growth at this stage leads to complete lysis of the culture.

Protein were extracted from the induced lysogens by pelleting the cells and re-suspending in 2/25th volumes of 50mM Tris-HCl pH7.5, 1mM EDTA, 1mM PMSF, 5mM DTT. Cells were lysed by three rounds of freeze-thaw lysis in a ethanol-dry ice bath. Lysozyme was added to 0.5mg/ml from a 10mg/ml fresh stock in ddH_2O and the bacteria incubated on ice for 15 minutes. The solution was made 1M with respect to NaCl (from a 5M stock) and placed on a rotary shaker for 15 minutes at 4°C. The cell debris was pelleted by centrifugation at 12,000 × g at 4°C and the supernatant taken as a crude bacterial protein extract containing the cloned recombinant protein. An aliquot of the sample was diluted 1:4 with ddH_2O and analysed by SDS / PAGE.

2.3 Biochemical Methods

2.3.1 Preparation of dialysis tubing

Dialysis tubing was cut into pieces of a convenient length (300mm). The tubing was boiled in ≥300ml of 1mM EDTA (pH8.0), 2% (w/v) sodium bicarbonate for 10 minutes. The tubing was rinsed thoroughly in distilled water and boiled again in 1mM EDTA (pH8.0) for 10 minutes. The tubing was rinsed once more in distilled water before being stored at 4°C submerged in 20% ethanol. Once boiled, dialysis tubing was always handled with gloves. Before use, dialysis tubing was washed thoroughly with distilled water.

2.3.2 Preparation of a total protein extract from *Brassica napus*.

Previous methods have used a simple extraction procedure based on extraction into 1 × SDS sample buffer (Fowler, 1996). This procedure, whilst sufficient for 1-D gels was not suitable for 2-D quantification, so to standardise the protein on 2-D and 1-D gels the following method was used for analysis of protein by both techniques.

Tissue samples of *Brassica napus* were frozen in liquid N_2 or removed from -80°C storage and cooled in N_2. A mortar and pestle was also cooled with liquid N_2. The tissue to be extracted was placed into the cooled mortar which was approximately one half filled with liquid N_2. The tissue was ground gently at first to prevent spillage. A more vigorous grinding was possible as the amount of liquid N_2 decreased. Grinding was continued until no liquid N_2 was left and the tissue was a fine powder, at which stage it was transferred to a pre-weighed microfuge tube and weighed. The amount of tissue present was calculated. All the ground tissue was transferred to a 15ml polypropylene centrifuge tube without spillage and 10 volumes of Plant Extract Buffer (20mM Hepes-NaOH pH7.5, 1mM EDTA, 0.5mM DTT, 1mM benzamidine-HCl, 5mM 6-aminocaproic acid, 1mM PMSF, 0.5% Triton X-100, 10% (v/v) glycerol) was added. The material was homogenised, on ice, for 30 seconds using a T8 ULTRA TURRAX with a S8N-5G dispersing element attached.

The extract was spun at 12,000 × g at 4°C for separate the lipid layer and the cell debris from the protein supernatant. As much of the protein supernatant was removed as possible and spun at 200,000 × g at 4°C for 1.5 hours. This centrifugation run pelleted the membranes and smaller cell debris. The clarified protein supernatant was carefully pipetted from beneath the small lipid layer. Fractions of 100µl were precipitated with methanol / chloroform and stored at -20°C. Proteins stored in this form were stable for a period of 1 year or more.

2.3.3 Isolation of chloroplasts from *Brassica napus*.

Chloroplasts were isolated from young leaves of oilseed rape, using a 40% percoll cushion (Mills & Joy, 1980). Plants were grown in a growth room under ambient conditions (16°C day temperature, 12°C night, 16 hour light period 1500 µE/m^2/sec). After 4 weeks, plants were placed in a dark room at 20°C for 48 hours to destarch the leaves. After the dark period, leaves were harvested and illuminated in a tray of ice water. Illumination increases the yield of intact chloroplasts but the leaves are kept cold to prevent starch synthesis occurring. After 30 minutes illumination, the leaves were removed and the mid-rib cut out. All the following steps were performed in a cold-room and as quickly as possible.

The leaves were cut into 2 cm^2 pieces with a sharp knife. In a square, 500ml perspex tube, 30g of rape leaf material was combined with 300ml of Buffer A (340mM sorbitol, 15mM MES-KOH pH6.3, 5mM $MgCl_2$, 5mM sodium ascorbate) and homogenised for 2 × 10 seconds with a polytron homogeniser with a PTA 20S attachment at 75% full speed. The brei was filtered through 4 layers of muslin with 1 layer of miracloth and again through 4 layer of muslin with 1 layer of absorbent cotton wool. The filtrate was divided equally between 8 polycarbonate 50ml centrifuge tubes and spun in a fixed angle Beckman JA20 rotor at 4,000 rpm. Centrifugation continued for 30 seconds as soon as the rotor had reached 4,000 rpm before stopping the rotor with full brake. The supernatant was discarded and the pellet washed by swirling 1 ml of Buffer A gently over the pellet. The wash was discarded.

The pellet was re-suspended in 2.5ml of Buffer A by gently running buffer over the pellet with a disposable plastic bulb end pipette. The re-suspended pellets were layered carefully onto 4 × 7.5ml of 40% percoll (v/v) in Buffer A in a 50ml polycarbonate centrifuge tube. This was spun in a Sorval HP4 swing out rotor at 2,500 × g for 3 minutes and then brought to rest without braking. The pellet represented intact chloroplasts. The supernatant was pipetted away without contaminating the pellet, and the pellet was washed twice with 1ml of Buffer B (340mM sorbitol, 50mM Hepes-KOH pH7.6, 2mM EDTA, 1mM $MnCl_2$, 1mM $MgCl_2$, 0.2% fat free BSA). The pellet was re-suspended in 2ml of Buffer B by gently running buffer over the pellet with a disposable plastic bulb end pipette. The re-suspended pellets were combined in a polycarbonate centrifuge tube and diluted with Buffer B to 20ml. The chloroplasts were finally pelleted again by centrifuging in a fixed angle Beckman JA20 rotor at 4,000 rpm. Centrifugation continued for 30 seconds as soon as the rotor had reached 4,000 rpm before stopping the rotor with full brake. This removes remaining percoll from the chloroplasts. The final pellet was re-suspended in a minimal volume of Buffer B (≤0.5ml) and stored on ice. Aliquots of 50µl were snap frozen in liquid N_2 and stored at -80°C for subsequent protein analysis.

2.3.4 Isolation of biotin-containing proteins with avidin-agarose

Isolation of biotin-containing proteins with avidin-agarose has been reported previously (Turnham & Northcote, 1983; Wang *et al.*, 1994). These methods used tetravalent avidin to bind the proteins as opposed to monovalent (Henrickson *et al.*, 1979). Proteins have to be eluted from tetravalent avidin-agarose by boiling in 2% SDS.

The required tissue was weighed and ground in a pre-cooled mortar with a chilled pestle until it was a fine powder. The pulverised tissue was placed in a 15ml tube and 2 volumes (w/v) of extract buffer was added (100mM Tris-HCl pH7.0, 5mM EDTA, 10mM DTT, 2% (w/v) SDS). The tissue was homogenised for 30 seconds with a T8 ULTRA TURRAX with a S8N-5G dispersing element attached. Proteins were

solubilised and denatured by heating in a tube for 15 minutes in a vigorously boiling water bath. The cell debris was removed by centrifuging for 15 minutes at 12,000 × g. The supernatant was recovered and diluted to 50ml with 100mM Tris-HCl pH7.0, 5mM EDTA. The supernatant was applied at a rate of 0.5ml/min to a 3ml tetravalent avidin-agarose column pre-equilibrated in 100mM Tris-HCl pH7.0, 5mM EDTA. When the extract had all been loaded onto the column 250mls of wash buffer (100mM Tris-HCl pH7.0, 5mM EDTA, 0.2% SDS) was passed through. Proteins were eluted from the washed matrix by removing the matrix from the column into a 15ml tube, adding 3 ml of column elutions buffer (100mM Tris-HCl pH7.0, 5mM EDTA, 2% SDS) and heating in a boiling water bath for 5 minutes. The supernatant was recovered by centrifuging at 4,000 × g for 10 minutes and removing the supernatant.

2.3.5 Methanol / Chloroform precipitation of proteins

This is a quantitative technique for recovery of protein from small to medium samples (Wessel & Flügge, 1984), ≤5ml per 50 ml tube. Protein can be recovered from very dilute samples. This technique was useful for desalting samples before analysis or for concentrating protein solutions. The protein is denatured during the process so it was usually used as an analytical method.

To one volume of sample four volumes of methanol were added. The two were mixed and one volume of chloroform was added. The mixture was vortexed thoroughly for 10 seconds and three volumes of ddH_2O were added. The solution was vortexed again for 10 seconds and spun at ≥2,000 × g for 10 minutes to resolve the phases. The protein precipitate was formed at the interphase. The upper aqueous phase was discarded by aspiration, and three volumes of methanol were added. The solution was vortexed for 10 seconds to mix the solution and spun at ≥2,000 × g for 10 minutes to pellet the protein. The supernatant was aspirated away without disturbing the pellet and the pellet dried under vacuum. The dried pellet could be re-suspended in 1 × SDS sample buffer and run directly on acrylamide gels.

2.3.6 Bradford protein assay

Protein concentration was determined using the method of Bradford, 1976. Coomassie G-250 absorbs strongly at 595nm when bound to protein under acidic conditions. The reagents were bought from BIO-RAD in a ready to use solution (BIO-RAD protein assay). Assays were performed in a microtitre plate and read at 595nm with a microplate reader.

A 10mg/ml solution of BSA was used as a standard. A serial 5-fold dilution of BSA in a total volume of 200µl was made in a microtitre plate along with a 5-fold serial dilution of the samples to be measured. Fifty microlitres of protein assay reagent (BIO-RAD) was added to each well and mixed. The assay was left to stand for 5 minutes and the absorbance read on a microplate reader at 595nm. The results were plotted on a graph of protein concentration / dilution against Abs_{595}. The protein concentration for each sample was calculated from a point in the graph where the correlation between Abs_{695} and protein concentration / dilution was linear.

2.3.7 SDS Polyacrylamide gel electrophoresis for separation of proteins.

The buffer system of Laemmli, 1970 was used in conjunction with BIO-RAD's mini PROTEAN II vertical electrophoresis equipment. Gels were 70 × 100 × 0.75 mm in size unless otherwise stated. The gels were discontinuous, routinely a 12% resolving gel and a 5 mm 4% stacking gel. BIO-RAD electrophoresis pure reagents were used throughout.

The resolving gel consisted of 37.5:1 acrylamide : bis-acrylamide in 0.375M Tris-HCl pH8.8, 0.1% SDS, 0.05% ammonium persulphate (from fresh 10% stock) and 0.05% TEMED. The solution was degassed for ≥15 minutes by stirring in a beaker under vacuum, before addition of ammonium persulphate and TEMED. After degassing the catalyst was added, the solution swirled gently in the beaker to mix the catalyst, and the resolving gel solution poured into the gel former to not more than 2.5cm from the top. The top of the gel was gently overlaid with 150µl of water saturated *n*-butanol, to ensure a level surface. The resolving gel was left to set at room temperature

(approx. 1 hour) after which the *n*-butanol was washed away with ddH_2O The stacking gel was made up using 37.5 : 1 acrylamide : bis-acrylamide in 0.125M Tris-HCl pH6.8, 0.1% SDS, 0.05% ammonium persulphate and 0.1% TEMED. The stacking gel solution was also degassed before addition of the catalysts. The stacking gel was poured on top of the resolving gel and a gel comb former inserted to create wells. The stacking gel was allowed to set at room temperature. When the stacker was set, the gel comb former was removed and any remaining unpolymerised acrylamide was washed away with ddH_2O water.

Protein samples were prepared for electrophoresis making them 1 × with respect to SDS sample buffer by dilution from a 5 × or 2 × stock. The samples boiled for 2 minutes, cooled, and spun for a few seconds in a microfuge before being applied to the gel. Molecular weight markers were always included on the gel (Appendix B).

The gels were electrophoresed 1 × SDS running buffer at 20mA per gel, for approximately 1 hour until the dye front reached the bottom of the gel.

Proteins were stained by covering with approximately 50ml of Coomassie I solution (25% propan-2-ol, 10% acetic acid, 0.025% w/v Coomassie R-250), and microwaving on medium heat for 1 minute by which time the solution was approximately 60°C. The gel was allowed to cool in the Coomassie I solution on a rotary platform for >15 minutes. The Coomassie I solution was discarded and replaced with Coomassie II (10% propan-2-ol, 10% acetic acid, 0.003% w/v Coomassie R-250). The microwaving and cooling procedure was repeated. The Coomassie II solution was replaced with Coomassie III (10% acetic acid, 0.003% Coomassie R-250) and the microwaving procedure repeated again. After cooling the Coomassie III solution was finally replaced with destaining solution (10% glycerol, 1% acetic acid) and microwaved once more including a piece of sponge in with the gel to soak up the excess Coomassie.

Gels were dried down for a permanent record with a Hoefer Scientific Instruments gel dryer.

2.3.8 Gradient gels.

In this study, 5-15% denaturing gels and 5-20% native have been used. These were made using a gradient maker, a peristaltic pump and the BIO-RAD multicaster. This enabled loading of multiple gels simultaneously from the bottom to make comparable even gradients.

Gradient gels were made by mixing two solutions during pouring of the gel, a light solution (5% (w/v) acrylamide) and a heavy solution (15 or 20% (w/v) acrylamide). The multicaster was assembled according to the manufacturer's instructions using 1mm spacers. The gradient maker was set up as shown in Fig. 2.1, and the outlet from the mixing chamber attached to a peristaltic pump. The other end of the pump was attached to the inlet at the bottom of the multicaster. Six 1mm, 100mm × 75mm gradient gels required 18ml of each solution (36ml total) allowing for the gaps in the multicasting chamber.

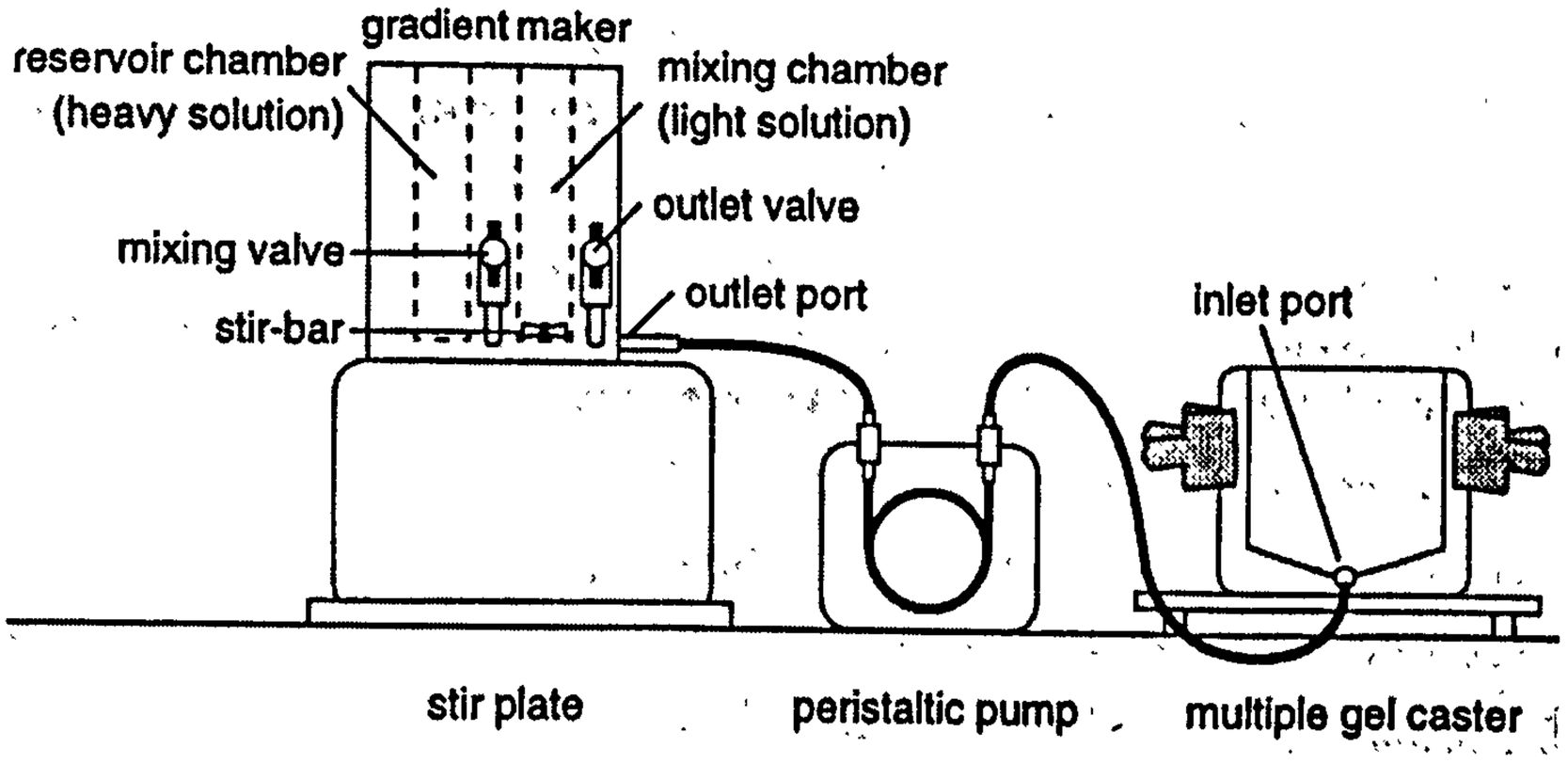


Figure 2.1 Set up of the gradient maker and gel multicaster to enable casting of multiple gradient gels.

The heavy solution was made up of 37.5:1 acrylamide : bis-acrylamide in 15% sucrose, 0.375M Tris-HCl pH8.8, 0.1%SDS, 0.05% ammonium persulphate (from fresh 10% stock) and 0.05% TEMED. This was placed in the reservoir chamber with the valve connecting the two chambers in the closed position. The valve was opened slightly to allow air trapped in the connecting tube between the two chambers to

escape. The light solution consisted of 37.5:1 acrylamide : bis-acrylamide in 0.375M Tris-HCl pH8.8, 0.1% SDS, 0.05% ammonium persulphate (from fresh 10% stock) and 0.05% TEMED and was placed in the mixing chamber. This was placed in the mixing chamber with a 5mm stir bar and positioned above a magnetic stirrer. The valve connecting the two chambers was opened and the magnetic stirrer started. The outlet from the mixing chamber was opened at the solution pumped using the peristaltic pump into the multicasting chamber at about 3ml/min. When nearly all the acrylamide solution had gone from the gradient maker, the pump was stopped and the valve connecting the two chambers closed. Plug solution (0.125M Tris-HCl pH8.8, 50% (w/v) sucrose, 0.001% (w/v) bromophenol blue) was added to the mixing chamber, approximately 5ml, and the pump restarted. Plug solution was pumped into the multicaster until it reached the bottom of the plates. The top of the gels were overlaid with 150µl of water saturated *n*-butanol and left to set at room temperature for two hours. When the gels had set the inlet tube was disconnected and the *n*-butanol washed from the top of the gels.

The stacking gel was prepared as described in the previous section and poured onto the top of each gel in the multicaster and a comb former inserted. When the stacker was set, the gel comb former was removed and any remaining unpolymerised acrylamide was washed away with ddH_2O water.

Native 5-20% acrylamide gels were cast and run as for denaturing gels but with the omission of SDS from the gel resolving and stacking solutions and the running buffer. Samples were diluted with 1/5th volume of 5× Native sample buffer. Gels were run and stained in the same way as previously described (section 2.4.7).

2.3.9 Preparative gel electrophoresis

The model 491 Prep Cell from BIO-RAD was used to purify insoluble proteins. The apparatus was assembled according to the manufacturer's instructions. The optimal % acrylamide for each run was calculated on the basis of the graph supplied by the manufacturer and the M_r of the protein. The length of the gels cast depended upon the

amount of protein to be purified but was a minimum of 60mm. The separating gel was made from 37.5 : 1 acrylamide : bis-acrylamide in 0.375M Tris-HCl pH8.8, 0.025% ammonium persulphate (from fresh 10% stock), and 0.025% TEMED. The monomer solution was degassed for 15 minutes by placing in a beaker with stirring under vacuum. The catalyst was added to the monomer and mixed by gentle swirling. The monomer solution was poured into the assembled casting apparatus to the required length, and overlayed with 500µl of *n*-butanol. The gel was left to set at room temperature but with water flowing through the cooling core to prevent a substantial temperature gradient arising. After 2 hours the *n*-butanol was replaced with 0.375M Tris-HCl pH8.8 and the gel was left at room temperature overnight.

A 10mm 4% acrylamide stacker was cast on top of the resolving gel the following morning. The stacker consisted of 37.5 : 1 acrylamide : bis-acrylamide, 0.125M Tris-HCl pH6.8, 0.05% ammonium persulphate and 0.05% TEMED. The overlay buffer on top of the resolving gel was poured off and replaced with enough stacking solution to make a 10mm stacker. The stacker was overlaid with 500µl of *n*-butanol and left to polymerise for 2 hours at room temperature. When the stacker had set, the *n*-butanol was washed away with ddH_2O and the gel apparatus was assembled.

The elution buffer chamber was filled with 1 × SDS running buffer. The protein to be purified was solubilised in ≤2ml of 1 × SDS sample buffer, centrifuged at top speed in a microfuge for 2 minutes and loaded onto the top of the stacker using a length of capillary tubing and a syringe. The gel was run at 12W constant power until the dye front reached the bottom of the gel.

The elution pump was switched on at a rate of 0.5ml/min and fractions were collected automatically every 10 minutes. Fractions were collected for the time recommended by the manufacturer, but usually for ≥3.5 hours (21 fraction) for a 6cm gel.

2.3.10 Isoelectric focusing

Isoelectric focusing was carried out in the Mini-Protean II Tube Cell from BIO-RAD using the buffer system of O'Farrell, 1975.

Capillary tubes were placed in the casting tube sealed at one end with Parafilm. The capillary tubes were filled with First Dimension gel monomer solution (9.2M urea, 4% (w/v) 37.5:1 acrylamide : bis-acrylamide, 2.0% de-ionised Triton X-100, 1.6% Pharmalyte 5/8, 0.4% Pharmalyte 3/10, 0.01% (w/v) ammonium persulphate (from fresh 10% stock), 0.1% TEMED), by using a needle and syringe and injecting the monomer solution between the capillary tubes so they filled from the bottom upwards. The casting tube was sealed at the other end, placed vertically in a test-tube rack and left to set overnight.

Capillary tubes with set First Dimension gel inside, were removed from the casting tube, cleaned and assembled in the Tube Cell module according to the manufacturer's instructions. The upper chamber was filled with approximately 120ml of degassed 20mM NaOH and the lower chamber with approximately 800ml of degassed 10mM H_3PO_4. Air was expelled from the top of the capillary tubes with a Hamilton syringe, and the tubes pre-electrophoresed for 10 minutes at 200V, 15 minutes at 300V and 15 minutes at 400V. After pre-electrophoresis the upper and lower chamber buffers were replaced with fresh buffer.

The samples to be isoelectrically focused were prepared by methanol / chloroform precipitation and re-suspension in First Dimension sample buffer (9.5M urea, 2.0% de-ionised Triton X-100, 5% β-mercaptoethanol, 1.6% Pharmalyte 5/8, 0.4% Pharmalyte 3/10). The sample was loaded into the sample reservoir with a Hamilton syringe, not more than 50µl being loaded in one tube. The sample was overlayed with 40µl of First Dimension sample overlay buffer (9M urea, 0.8% Pharmalyte 5/8, 0.2% Pharmalyte 3/10, 0.005% bromophenol blue). The apparatus was reconnected to the power supply and run at 500V for 10 minutes and 750V for 3.5 hours.

After the run, the capillary tubes were removed, labelled and stored at -20°C until required or used immediately. The acrylamide gel was removed from the capillary tube, bottom first, using the gel ejector as recommended by the manufacturer. The gel was extruded onto a piece of Parafilm laboratory film and carefully slid off the film between the glass plates of a 1mm 5-15% SDS / PAGE gel cast using a preparative comb (1 small well, 1 large well). The gel tubes were normally placed with the top (negative end) nearest the M_r markers. The gel tube was covered with 250µl of 1 × SDS sample buffer and allowed to equilibrate for 5 minutes before the second dimension gel was run as previously described.

2.3.11 Western blotting

Proteins for analysis by Western blotting were separated by SDS / PAGE but the gel was not stained. The transfer of protein to Hybond C extra was done in a Trans-Blot® electrophoretic Transfer Cell (BIO-RAD) according to the manufacturer's instructions.

The SDS/PAGE gel to be blotted and a piece of Hybond C extra, cut 100mm ×75mm were soaked in 100ml of Transfer buffer for 10 minutes. Six pieces of filter paper also 100mm × 75mm were soaked in the buffer with the nylon gauze supplied by the manufacturer. The gel holder was opened and placed in a tray clear side down. The gel sandwich was assembled placing the components on top of each other in the following order; nylon gauze, 3 pieces of filter paper, Hybond C extra, SDS/PAGE gel, 3 pieces of filter paper and a second piece of nylon gauze. Air bubbles were removed after each layer was added using a wetted plastic roller. The cassette was closed and inserted into the holder such that the clear side faced the anode (clear - clear, grey - grey). Transfer was usually performed at 40mA for 16 hours with stirring. After transfer the membrane was stained with Ponceau-S (0.1% (w/v) Ponceau-S, 1% acetic acid) to establish efficient transfer of proteins. The position of M_r markers were marked on the membrane was marked with a pencil.

The membrane was placed in a Techne Hybridisation Tube in a Techne Hybridiser HB-1 at 4°C. The membrane was washed with 20ml of PBS-T to remove the Ponceau-S stain and then blocked for 2 hours in 3% (w/v) BSA in PBS-T to block the remaining protein binding sites on the membrane. The membrane was briefly washed in 20ml of PBS-T before the tube was stood on end on a paper towel for 5 minutes to allow excess liquid to drain off. Ten millilitres of 1% (w/v) BSA in PBS-T was pipetted into the tube and the antibody added to the required dilution (10µl for A 1:1000 dilution). The primary antibody was incubated for 2 hours after which the membrane was washed for 30 minutes with three changes of 20ml of PBS-T. After the final wash, the tube was left to drain on a paper towel again for 5 minutes. Another 10 ml of 1% BSA in PBS-T was added to the tube and 5µl of the appropriate secondary antibody was added. The blot was incubated for another 2 hours after which the membrane was washed for 30 minutes with three changes of 20ml of PBS-T. After the final wash, the tube was allowed to drain on a paper towel.

2.3.12 Detection of antibody : enzyme conjugates

Alkaline Phosphatase

<u>On solid supports</u>

A BCIP/NBT buffered substrate tablet was dissolved in 10ml of ddH_2O and poured into a 130mm petri dish. The membrane was placed in this solution for 5 minutes and the image allowed to develop. The reaction was stopped by rinsing the filter in ddH_2O. The filters were air dried and stored in the dark. A deep purple to black band indicated the presence of an immunoreactive protein in the original gel.

<u>In solution</u>

A tablet containing 5mg *p*-nitrophenolphosphate (SIGMA), was dissolved in 5ml of 10% (v/v) diethanolamine-HCl pH9.8. One hundred microlitres was placed in individual wells on a microtitre plate and enzyme activity detected by measuring the increase in absorbance at 405nm with a microtitre plate reader.

Horse Radish Peroxidase

Colorimetric detection

Horse radish peroxidase was detected using the substrate diaminobenzidine (DAB). The detection solution (100mM Tris-HCl pH7.5, 0.08% (w/v) DAB, 0.16% (w/v) $NiCl_2$, 0.009% (w/w) H_2O_2) was prepared and 50mls poured into a glass dish with the membrane. The reaction was allowed to develop for 5 minutes at which point the reaction was stopped by rinsing the filter in ddH_2O. The membranes were air dried and stored in the dark. A brown colour indicated the presence of immunoreactive protein in the original gel.

Enhanced Chemiluminescence detection (ECL)

ECL uses the enzyme horse radish peroxidase to oxidise the chemical luminol in the presence of H_2O_2. Once luminol has been oxidised, is exists in an excited state which decays to a more stable state by the emission of a light particle.

ECL was used to detect bound streptavidin-HRPO conjugate to biotinylated proteins. The principle is the same as for Western blotting. Proteins to be detected were bound to a nitrocellulose support (Hybond C) and the remaining protein binding sites on the filter blocked with a solution of 3% (w/v) BSA in PBS-T. The blocking solution was replaced with 10ml of 1% (w/v) BSA after 2 hours. Streptavidin-HRPO was added to 1:1000 dilution and the filter incubated for a further 2 hours after which time it was washed with three changes of PBS-T over 30 minutes.

After washing, detection was performed using the ECL kit (Amersham) following the manufacturer's instructions. Excess buffer was dried from the membrane with a filter paper and the membrane was incubated in an equal mixture of solutions 1 and 2 for 1 minute. Excess detection reagent was removed by blotting the membrane on a filter paper before it was wrapped in SaranWrap and exposed to X-ray film for 15 seconds to 2 minutes, depending on the strength of the signal. Exposed films were developed as described in section 2.5.18.

2.3.13 Expression of heterologous protein in *Escherichia coli*

The following precautions were taken when expressing proteins in *E.coli* from plasmid sources. A suitable strain was transformed with the required plasmid and the bacterial colonies allowed to develop for no more than 16 hours. Primary transformants were picked with a sterile toothpick and suspended in 200µl of LB containing the appropriate antibiotic (ampicillin was used at 200µg/ml) and 15% (v/v) glycerol. These "seed stocks" were frozen in liquid N_2 and stored at -80°C until required.

A single seed stock was used to inoculate a culture from 50-1000ml containing the appropriate antibiotic (ampicillin was used at 100µg/ml) and the culture was shaken at 220rpm at 37°C. The culture was grown to an OD_{600} of 1.0 at which point an aliquot of cells was removed (uninduced cells) and the culture was made 1mM with IPTG (100mM stock in ddH_2O, filter sterilised). Growth was continued for up to 3 hours when the bacteria were harvested by centrifugation.

2.3.14 Isolation of Inclusion bodies

Cells were collected from a 50ml culture by centrifugation at 3,000 × g for 10 minutes in a 50ml tube. The supernatant was discarded and the bacteria were re-suspended in 1/10th of the culture volume, in this case 5ml, of 50mM Tris-HCl pH8.0, 2mM EDTA. Lysozyme was added to 100µg/ml from a freshly made stock (10mg/ml in 50mM Tris-HCl pH8.0, 2mM EDTA). The tube with bacteria was left on ice for 20 minutes after which time Triton X-100 was added to 0.1% and the tube was incubated at 37°C with shaking for 15 minutes. The bacterial lysate was then sonicated with a sonicating tip at full power and maximum amplitude for two 10 second pulses after which time the solution lost its viscosity. The bacterial lysate was then centrifuged in a Beckman centrifuge at 12,000 × g for 15 minutes at 4°C. The supernatant after centrifugation contained soluble *E.coli* proteins whilst the pellet represented a crude inclusion body preparation. The inclusion bodies could be purified further by washing with various detergents or urea.

2.3.15 Production of polyclonal antisera in rabbits

Female New Zealand white rabbits were bought from a local supplier and maintained for 1 week prior to experimentation to allow acclimatisation. Rabbits were immunised with one antigen to yield a polyclonal antibody response (Catty, 1988).

A pre-immune bleed was taken 10 days before the primary injection. Between 200 and 500μg of antigen was made 1 × with PBS from a 10 × stock and combined with and equal volume of Freund's complete adjuvant. The adjuvant was added to the aqueous protein drop-wise with vigorous vortexing in a 50ml tube. When all the adjuvant had been added, the emulsion was sonicated for 20 seconds at maximum power to disperse any oil or water droplets. Emulsion was stored at -20°C until required. The rabbit was injected intramuscularly in one thigh and intradermally at six locations on the back with 2ml of the emulsion.

After 40 days, the first booster injection was given. This time 100-200μg of protein was made 1 × with PBS and combined with an equal volume of Freund's incomplete adjuvant in the same way as before. The rabbit was injected with 1ml of adjuvant intramuscularly in the other thigh. After 10-14 days the rabbit was test bled, the blood was coagulated at 37°C for 1 hour, the clot pulled away from the sides of the tube with a sterile inoculation stick, and the clot left to retract at 4°C overnight. The serum was poured away from the clot and any blood cells were centrifuged out of solution at 300 × g for 30 minutes. The supernatant was stored at 4°C with 0.02% NaN_3. Aliquots were taken to determine the specificity and titre on Western blots. Further boosts were given as required and test bleeds taken. When the titre and specificity of the antibodies was sufficiently high, the rabbit was terminated by cardiac puncture. Blood was allowed to clot at 37°C and red cells were pelleted by centrifugation at 300 × g for 30 minutes. The serum supernatant was made 0.02% with respect to NaN_3 and frozen in 10ml aliquots at -80°C.

2.3.16 Gel filtration chromatography of protein samples.

Size exclusion or gel filtration chromatography is able to separate protein molecules according to their native subunit size. Small molecules are able to enter the molecular sieve like structure of the beads whereas larger molecules are unable to. Larger molecules pass through quicker than smaller molecules and are separated as a result. The column used was a PHARMACIA pre-packed Superose-12 HR 10/30, which will separate proteins with M_r 1,000 to 300,000.

The superose-12 column was pre-equilibrated with 50mM Tris-HCl pH8.0, 2mM EDTA, 100mM NaCl at 0.5ml/min. A UV lamp at 280nm was set to zero against this buffer. For each sample to be analysed, 500µg of protein was loaded onto the top on the 24ml column in a total volume of 500µl of 50mM Tris-HCl pH8.0, 2mM EDTA, 100mM NaCl. Fractions of 1ml in volume were collected as soon as the protein was applied and proteins started to elute in fraction six.

2.3.17 Enzyme-linked immunosorbent assays

ELISAs used to quickly detect biotinylated protein in many samples, such as from a column, using affinity purified anti-biotin antibodies. The direct plate ELISA technique was used (Catty, 1988) in which protein are absorbed onto the plate surface and proteins directly detected with the antibody.

Proteins in a volume of 100µl in a 50mM Tris-HCl buffer pH8.0 were absorbed onto Nunc ImmunoAssay Plates overnight at 4°C. Unbound proteins were removed from each well by draining over a sink and the wells were washed 1 × with PBS-T. Non-specific binding of the antibody was prevented by blocking the wells with 300µl of 1% BSA in PBS-T for 2hrs at room temperature. The wells were drained once more and banged dry on paper towel. The affinity purified anti-biotin antibody was diluted to 1:2000 in PBS and 150µl was added to each well. The antibody was incubated for 2hrs at RT after which time the wells were drained, washed 3 × with PBS-T and banged dry on a paper towel. The primary antibody was detected by 150µl of donkey anti-sheep IgG : alkaline phosphatase conjugate at a 1:6000 dilution in PBS for 2hrs at

RT. The wells were drained once more and washed 3 × with PBS-T. Enzyme activity was detected by adding 100µl of 1mg/ml *p*-nitrophenolphosphate in 10% diethanolamine pH9.5, incubating for 1hr at 37°C and measuring the Abs_{450} with a BIO-RAD microplate reader.

2.4 Genetic Manipulations

2.4.1 Preparation of plasmid DNA

Plasmid DNA was prepared by the alkaline lysis method. Large scale and plasmid minipreps were performed, using 50ml and 5ml or 10ml cultures respectively.

A - Low grade mini-plasmid preps

Plasmid DNA which was required for transformation or restriction analysis was routinely prepared. In a 1ml microfuge tube, 1ml of culture from a 5ml overnight culture in LB media containing 50μg/ml ampicillin was spun in a microfuge for 5 minutes to harvest the cells. The pellet was re-suspended in 100μl of 25mM Tris-HCl, pH8.0, 10mM EDTA, 50mM glucose and stored on ice for 5 minutes. Two hundred microlitres of a freshly prepared solution of 0.2M NaOH, 1% (w/v) SDS was added to lyse the cells. The tube contents were mixed by inversion (not vortexing) to lyse all the cells but at the same time to prevent shearing of genomic DNA. The cells were stored on ice until the solution clarified or for not longer than 5 minutes after which 150μl of an ice cold solution of 3M NaAc, 5M Ac was added (made by combining 60ml of 5M KAc and 11.5ml of glacial acetic acid per 100ml). The tube contents were mixed by gentle inversion until the two phases were no longer visible, causing precipitation of the SDS and cell wall fragments along with chromosomal DNA. The tube was stored for 5 minutes on ice before being centrifuged for 10 minutes at top speed in a microfuge. The supernatant was decanted into a fresh microfuge tube and an equal volume of phenol : chloroform pH8.0 (1:1) added. The contents were vortexed 10 seconds and spun for 10 minutes at top speed in a microfuge to resolve the two phases. The aqueous phase (upper) was pipetted into a fresh microfuge tube. The extraction procedure was repeated with 1) chloroform : isoamyl alcohol (24:1) and 2) chloroform. These organic extractions remove protein contaminants from the DNA. After the final extraction, DNA was precipitated by the addition of 2 volumes of 96% (v/v) ethanol. The tube was incubated on ice for 30 minutes or at -20°C for 15 minutes to encourage precipitation of DNA. The DNA was recovered by centrifugation at top speed in a microfuge at room temperature. The

precipitated DNA was washed with 500µl of 70% (v/v) ethanol and dried under vacuum. The pellet was dissolved in TE buffer containing 50µg/ml DNase free RNase.

DNA was stored at -20°C until required.

B - Promega Wizard™ Minipreps

When high grade plasmid DNA was required for DNA sequencing, subcloning or PCR, it was prepared by the Promega Wizard™ Miniprep system. This system uses alkaline lysis as in the low grade prep but proteins are removed from the DNA by binding the DNA to a resin and washing the proteins away. DNA is recovered in a low salt buffer or ddH_2O.

A 10ml overnight cell culture of LB + 50µg/ml ampicillin was harvested by centrifugation in a sterile 15ml polypropylene centrifuge tube using a swing out rotor. The supernatant was decanted and the pellet allowed to drain upside-down in a paper towel to drain excess media. The cell pellet was re-suspended in 300µl of 50mM Tris-HCl, pH7.5, 10mM EDTA, 100µg/ml RNase by pipetting up and down. The re-suspended cells were transferred to a 1.5ml microfuge tube. Cells were lysed by the addition of 300µl of 0.2M NaOH, 1% SDS and inverting until the suspension clarified (not more than 5 minutes). After lysis 300µl of 1.32M KAc pH4.8 was added and the phases were mixed by inversion. The cell lysate was centrifuged for 10 minutes and the supernatant divided equally (450µl each tube) between two fresh microfuge tubes. Five hundred microlitres of Wizard™ Miniprep DNA purification resin was pipetted into each tube, mixed and left to stand at room temperature for 5 minutes. This ensures complete binding of the DNA to the resin.

The resin was recovered from the mix by vacuum filtration using the Vac-Man™ manifold. A Minicolumn was attached to each Luer-Lok® of the manifold and a 5ml syringe barrel inserted into the Minicolumn. The resin / bacterial supernatant mix was pipetted from both microfuge tubes into a single syringe / Minicolumn assembly. The Luer-Lok® was opened a vacuum applied to pull the resin / lysate mix into the

Minicolumn. When all the sample had passed into the column 2 × 2ml of wash buffer (90mM NaCl, 9mM Tris-HCl, pH7.5, 2.25mM EDTA, 55% ethanol) was passed through to wash away contaminants. The resin trapped in the Minicolumn was cleared of wash buffer by attaching it to a microfuge tube with the lid detached and centrifuging for 1 minute in a microfuge. The dried Minicolumn was transferred to another microfuge tube with the lid detached. DNA was eluted from the resin in the Minicolumn by the addition of 100µl of ddH_2O preheated to 70°C. The column was allowed to rest 1 minute at room temperature before the DNA solution was eluted from the column by centrifuging in a microfuge for 1 minute.

DNA prepared in this way was routinely 200-250µg/ml (total yield >20µg) and could be used directly for DNA sequencing. DNA was not eluted into TE buffer as this affected the quality of the sequence obtained due to the presence of EDTA.

C - Qiagen Plasmid Midi preparations

When large amounts of plasmid DNA were required, DNA was prepared using a QIAGEN-Tip 100 from a 50ml culture according to the manufacturer's instructions. The bacteria are lysed by alkaline lysis, but the DNA is isolated by batch ion-exchange chromatography on a small column.

A 50ml overnight LB + 50µg/ml ampicillin culture was centrifuged in a 50ml polypropylene sterile centrifuge tube for 15 minutes at 3,000 × g at 4°C. The bacterial pellet was re-suspended in 4ml of 50mM Tris-HCl, pH8.0, 10mM EDTA, 100µg/ml RNase A and transferred to a Nalgene 50ml polypropylene centrifuge tube. 4ml of freshly prepared solution of 0.2M NaOH, 1% (w/v) SDS was added and mixed by inversion until the suspension clarified (not more than 5 minutes). When the suspension had clarified 4ml of 2.55M KAc, pH4.8 was added and mixed immediately but gently by inversion. The bacterial lysate was spun at >20,000 × g for 30 minutes after which the supernatant was decanted into a fresh 50ml tube.

A fresh QIAGEN-Tip 100 was pre-equilibrated with 3ml of 750mM NaCl, 50mM MOPS pH 7.0, 15% (v/v) ethanol, 0.15% Triton X-100 by gravity flow. The

bacterial supernatant from the >20,000 × g centrifugation was applied to the top of the QIAGEN-Tip 100 and allowed to flow through by gravity flow. The flow through was discarded. The QIAGEN-Tip 100 was washed with 2 × 5ml of 1.0M NaCl, 50mM MOPS pH7.0, 15% ethanol by gravity flow. The flow through was discarded. The DNA was eluted from the QIAGEN-Tip 100 by applying 5ml of 1.25M NaCl, 50mM MOPS pH 8.2, 15% ethanol and allowing it to flow into a fresh sterile 15ml polypropylene centrifuge tube. The DNA is eluted in this buffer by the high pH and higher NaCl concentration. When the buffer had all flowed through, the remaining buffer was eluted from the QIAGEN-Tip by pushing a plunger from a 10ml syringe into the top of the QIAGEN-Tip. The DNA was precipitated from the buffer by adding 3.5ml of propan-2-ol. The precipitate was collected by centrifugation at 12,000 × g at 4°C for 30 minutes. The DNA was washed with 2ml of 70% ethanol and dried under vacuum. The DNA was re-suspended in 500µl of TE buffer

2.4.2 DNA sequencing

DNA prepared by Promega Wizard™ Minipreps was sequenced using an Applied Biosystems (ABI) 373 DNA sequencer, which uses the Sanger method of dideoxy-mediated chain termination. Automated sequencing was performed by Ms. J Bartley, Department of Biological Sciences, University of Durham. For each sequencing run, 11µl of DNA was supplied in ddH_2O at a concentration of 200-250ng/µl. Sequencing was usually from the forward (-21M13) or reverse (M13) primers which are located on either side of the MCS in pSK- (see Appendix A). Other primers were synthesised as required.

2.4.3 DNA Sequence Analysis

Primary DNA sequence analysis was carried out by entering the DNA sequence into the DNAStrider program (Marck, 1988) assembling the complementary strand and analysing restriction sites, creating graphical maps of the sequenced fragments, comparing sequences by matrices, identification of open reading frames and translation of hypothetical reading frames, as described in the manual. Database searches were performed using the Daresbury BBSRC SEQNET facility and analysis using the

University of Wisconsin GCG (Devereux *et al.*, 1984) was performed. Additionally, this package was used to create alignment of multiple DNA sequences and to create phylogenetic trees. The LaserGene suite of programs from DNAstar (Clewley, 1995) was used for sequence project management, multiple protein alignments, predictions of protein characteristics such as pI and M_r and construction of phylogenetic trees.

2.4.4 Custom DNA synthesis

DNA required for PCR or sequencing primers was either synthesised by Mr. J Gilroy, Dept. Biological Sciences, University of Durham using an Applied Biosystems ([ABI], Warrington) 381A DNA Synthesiser using the standard protocol, or were purchased from Perkin-Elmer Applied Biosystems Ltd.

2.4.5 CTAB(Cetyltrimethylammonium bromide) Plant DNA Extraction (Medium to Large Scale)

Genomic DNA preparations were made from developing leaf material. This gives the best yield and purity of DNA because the total number of cells in the leave of *Brassica napus* and *Arabidopsis thaliana* , is established early in development followed by expansion and elongation. Therefore the amount of DNA in the starting material per g of fresh weight is highest in younger leaves. The CTAB isolation procedure was used (Murray & Thompson, 1980), with modifications by Janice Keller (DNAP, 6701 San Pablo Avenue, Oakland, CA 94608, U.S.A) and Ian Bancroft (Department of Molecular genetics, the Cambridge Laboratory, The John Innes Centre for Plant Sciences Research, Colnoy Lane, Norwich, NR4 7UJ, England).

Two to 5 g of leaf material were ground to a very fine powder using a liquid N_2 cooled mortar and pestle. This was added to 25ml of CTAB buffer (140mM sorbitol, 220mM Tris-HCl pH8, 22mM EDTA, 800mM NaCl, 1% V. Lauroyl Sarkosyl, 0.8% cetyltrimethylammonium bromide (CTAB)) in a 50ml polypropylene tube. The tube and contents were incubated at 65°C for 20 minutes., with occasional vigorous shaking. After 20 minutes the tube was cooled to hand warm before adding 10ml of chloroform. This was shaken well before being placed on an end-over-end wheel for 20 minutes. After shaking the tube was centrifuged for 5 minutes at 3,000rpm for 5

minutes to resolve the organic and aqueous phases. The aqueous phase was transferred to a fresh tube and 17ml of isopropanol was added. This was mixed gently and left on ice for 10 minutes to allow the DNA to precipitate. The precipitate was collected by centrifuging for 5 minutes at 3,000 rpm. The supernatant was discarded and any remaining liquid on the sides of the tube removed with tissue. The pellet was re-dissolved in 4ml of TE buffer by gentle pipetting with a 1ml tip with the narrow end cut back 5mm with a sterile razor blade to increase the aperture. This prevents shearing of the DNA whilst handling. 4ml of sterile 4M Li Acetate was added to the re-dissolved pellet and left on ice for 20 minutes. The precipitated RNA was collected by centrifuging for 5 minutes at 3,000rpm. The supernatant was transferred to a fresh tube, 16ml of ethanol added and left on ice for 20 minutes to allow the DNA to precipitate. The DNA was collected by centrifuging at 10,000 rpm for 10min. The supernatant was drained off, the sides of the tube dried with tissue and the DNA re-dissolved in 0.9ml of TE buffer. 100µl of sterile 3M NaAc was added and the DNA divided into 2 × 500µl aliquots in microfuge tubes. The DNA was then cleaned further by successive extractions with equal volumes of phenol, phenol / chloroform (1:1) and then chloroform. The DNA was precipitated again by addition of 2 volumes of ethanol and incubating on ice for 5 minutes. After centrifuging at full speed in a microfuge for 10 minutes the supernatant was discarded taking care not to disturb the pellet. The pellet was spun again and further liquid removed. Using a fine, drawn Pasteur pipette as much remaining liquid as possible was removed but the pellet was not dried. The pellet was re-suspended in 500µl of TE buffer.

2.4.6 Digestion of DNA with restriction enzymes

DNA was incubated with the chosen restriction enzyme(s) in the buffer recommended by the manufacturer. Where double digests were required, a buffer was chosen which gave maximum activity for both enzymes as determined by the manufacturer. Digests were carried at the optimum temperature for the enzyme recommended by the

manufacturer, for >2 hours with 1U of enzyme per μg of DNA, usually in a total volume of 20μl.

2.4.7 DNA Molecular Weight Markers

Only lambda Pst I markers were used and these were prepared by digesting 50μg of lambda DNA (Pharmacia), with 50U of Pst I in Boehringer SureCut Buffer H in a total volume of 250μl, at 37°C overnight. After digestion, 50μl of 6× DNA loading dye was added with 200μl of ddH_2O and mixed thoroughly. The mix was aliquoted into 100μl fractions and stored at -20°C. An equivalent of 0.5 or 1μg (5 or 10μl) was loaded onto agarose gels as size standards (Appendix B).

2.4.8 Separation of DNA fragments by agarose gel electrophoresis

Gel electrophoresis was performed in Scotlab electrophoresis tanks. Gels were made of the appropriate concentration of agarose depending on the size of fragment to be analysed.

% (w/v) agarose in gel	*Efficient range of separation of linear DNA molecules kilo base pairs*
0.7	0.8-10
0.9	0.5-7
1.2	0.4-6
1.5	0.2-3
2.0	0.1-2

Table 2.3 Percentage agarose required for efficient separation of linear DNA molecules.

Normally 0.8% agarose gels were used as most DNA fragments were >500bp. Agarose was dissolved in 1 × TAE buffer by heating to boiling in a microwave. The solution was cooled to hand warm and ethidium bromide was added to 0.5μg/ml. The gels were poured in a gel mould with the required comb inserted and allowed to set. Large gels were 150mm long × 11cm wide, smaller gels were 60mm long × 110mm or 50mm wide. Running buffer consisted of 1 × TAE.

DNA samples were prepared by the addition of 1/6th volume of 6× DNA loading dye (1μl per 5μl sample). Once the gel had set it was submerged in running buffer so that

it was just covered and the comb was removed. DNA samples were pipetted into the wells and the gels run at 100V until the bromophenol blue had migrated about 2/3 distance along the gel. DNA was viewed by illumination with UV light and a permanent record made using a gel documentation system (BIO-RAD).

2.4.9 Southern blotting

At least 10μg of genomic DNA was digested in 50μl with 10U of a frequent cutting enzyme at 37°C overnight. An additional 5U of enzyme was added the following morning and left for a further 3 hours to ensure complete digestion.

DNA samples were made 2 × with DNA loading dye and loaded into the wells of a 0.7% agarose 150mm × 110mm Scotlab gel. DNA samples were electrophoresed through the agarose at 25V overnight. The gel was photographed under UV light and soaked or 15 minutes in 0.1N HCl, this acid treatment nicks DNA, ensuring that larger strands are transferred more efficiently. The gel was washed twice in ddH_2O and the DNA denatured by soaking in enough denaturing buffer to completely cover the gel for 45 minutes on a rotary platform. The denaturing solution was poured away and the gel rinsed twice in ddH_2O before being neutralised in 2 changes of neutralising buffer over a 45 minute period. When the gel was fully neutralised, it was rinsed in 10 × SSC. The gel mould was wrapped width-ways in a 400mm × 150mm strip of Whatmann 3MM filter paper, and placed upside-down in a glass dish containing 200ml of 10 × SSC so the ends of the filter paper were immersed in the 10 × SSC solution. The neutralised gel was placed on the filter paper covered gel mould. A 150mm × 110mm piece of Hybond N+ was laid over the top of the gel. A large piece of SaranWrap with a 150mm × 110mm hole but in the middle was orientated over the glass dish so the hole sat over the gel and protected the filter paper and 10 × SSC solution. Three pieces of 150mm × 110mm Whatmann 3MM paper were laid on top of the membrane followed by two absorbent nappies. A weight of 0.5-1kg was placed on top of the nappies and the transfer left overnight. The 10 × SSC was drawn up through the gel into the nappies overnight, drawing the DNA through onto the

membrane. The DNA was fixed to the membrane by placing it between 2 sheets of filter paper and baking for 1.5 to 2 hours.

2.4.10 Isolation of DNA fragments from agarose gels.

The commercially available QIAEX® Gel Extraction Kit (QIAGEN) was used for DNA fragment isolation according the instruction manual but with the following modifications for maximum recovery.

DNA digests were run on the appropriate % agarose gel and viewed under long wave UV light (302nm) for as short a time as possible to prevent DNA damage. The band to be isolated was excised from the gel with a clean razor blade and trimmed to the minimum size possible. The gel slice was placed in a pre-weighed microfuge tube and re-weighed. It was assumed at 100mg of agarose gel was equivalent to 100µl. For fragments between 100bp and 4kbp 3volumes of buffer QX1 was added. For fragments >4kbp, 3 volumes of QX1 and 2 volumes of sterile ddH_2O were added. 10µl of 3M NaAc was added to lower the pH, this modification suggested by the manufacturer lowers the pH increasing the binding of DNA to the glass resin. 10µl of thoroughly vortexed QIAEX suspension was added and the microfuge tube incubated at 50°C for 10 minutes, during which time the tube was vortexed every 2 minutes to keep the QIAEX particles in suspension. The QX1 buffer contains sodium perchlorate which disrupts the binding of the agarose gel. The QIAEX resin consists of silica particles which bind DNA under conditions of high ionic strength.

The QIAEX particles were recovered by centrifugation for 30 seconds in a microfuge and the supernatant was removed with a pipette. The pellet was washed with 500µl of QX1 by vortexing and spun again for 30 seconds. This step removed residual agarose from the pellet. The pellet was washed twice with 500µl of buffer PE, this step removes any remaining salt contaminants. The final pellet was not air dried as recommended by the manufacturer. Instead, all the supernatant was removed from the pellet and the sides of the tube with a Pasteur pipette drawn out to a fine end in a Bunsen flame. The still wet pellet was re-suspended in 20µl of TE by vortexing.

The sample was left for 5 minutes at room temperature to allow the DNA to elute. For DNA fragments >4kbp the sample was placed at 50°C for 10 minutes. The DNA was recovered by centrifuging the tube for 30 seconds in a microfuge and carefully removing the supernatant without disturbing the pellet. A further 20µl of TE was added to the pellet and recovered as before. These two low ionic-strength elutions from the QIAEX particles ensure that all the DNA has been eluted. The 40µl of TE was combined in a fresh microfuge tube and dried for 30 minutes in a Jouan RC10.22 Rotary Vacuum Evaporator at 50°C. This step evaporated all the buffer and any traces of ethanol contamination away leaving a dry DNA / buffer pellet. The pellet was re-suspended in 20µl of ddH_2O and 1µl run on an agarose gel to check the recovery.

2.4.11 DNA ligations

DNA fragments with either cohesive of blunt termini were mixed together in a molar ratio of 3:1 to 1:1 insert : vector (to a final concentration of approximately 50µg/ml) and 1µl of 10 × T4 DNA ligase buffer was added. DNA Ligase Buffer was bought from Promega and aliquoted upon first use into 10µl aliquots which were stored at -20°C and used only once. This was to preserve the ATP which is sensitive to repeated freeze-thawing. 3U of T4 DNA ligase were added and the reaction mixture made up to 10µl. The mixture was incubated at 16°C overnight before 5µl was used directly for transformation competent *E.coli* cells.

2.4.12 Polymerase Chain Reaction for amplification of DNA

PCR was carried out using thermostable DNA polymerases based upon the method of Saiki *et al.*, 1988.

Vent® DNA Polymerase was used throughout the research with ThermoPol Reaction Buffer (1 × 20mM Tris-HCl pH 8.8 @ 25°C, 10mM KCl, 10mM $(NH_4)_2SO_4$, 2mM $MgCl_2$, 0.1% Triton X-100) purchased from New England Biolabs. Buffers and enzymes were stored at -20°C and thawed on ice when required. Vent® DNA Polymerase was used due to its proof reading capability, ensuring that the amplified product has the same sequence as the original DNA template. This is important if the

product is to be used for expression or sequencing as errors introduced by TAQ polymerase (about 1 every 17,000bp) would affect the result. Vent® DNA Polymerase also has the useful property of making 95% of product end blunt, instead of the overhanging A produced by TAQ, enabling simple subcloning of reaction products.

PCR reactions unless otherwise stated contained 1 × ThermoPol Reaction Buffer, 400μM dNTPs, 0.4 μM each primer, 10pg-1ng plasmid DNA template and 1U of Vent® DNA Polymerase. The reactions were carried out in 250μl microfuge tubes or GeneAmp Thin-Walled Reaction Tubes. Evaporation was reduced during thermal cycling by placing mineral oil on the surface of the reaction mix or by the use of AmpliWax™ PCR Gems.

Reactions were hot started by the addition of the enzyme through the oil layer when the reactions were at 94°C, unless using AmpliWax™ PCR Gems. When using AmpliWax™ PCR Gems hot start was facilitated by placing the required amount of dNTPs and primers for the complete reaction in 1 × ThermoPol Reaction buffer in the bottom of a tube with an AmpliWax™ PCR Gem. The tube was heated to 60°C for 1 minute to melt the an AmpliWax™ PCR Gem and cooled to room temperature to allow the wax to seal the contents of the tube in the bottom. The required amount of DNA template and Vent® DNA Polymerase were placed above the wax layer in 1 × ThermoPol Reaction buffer. Thermal cycling was started as normal without interruption. The first step in thermal cycling, denaturing at 94°C, melts the wax layer allowing the contents of the tube to mix. Hot start is especially important when using Vent® DNA Polymerase as its proof reading ability means it has a 3'-5' DNase activity. If primers are allowed access to Vent® DNA Polymerase in the absence of template or low [dNTP] the enzyme may partially remove bases from the end of primers. These shortened primers can produce non-specific products.

Thermal cycling was normally carried out over 30 cycles of 94°C 1 minute, Tm°C 1 minute, 74°C (1 × anticipated product length in kilo base pairs) minute, on a DNA

Thermal Cycler 480. Vent® DNA Polymerase has a slightly higher temperature for greatest activity than TAQ, this helps to prevent formation of non-specific products.

2.4.13 Purification of DNA in solution

These methods make use of the different partitioning properties of DNA and contaminants such as proteins in organic and aqueous phases.

Phenol : Chloroform Extraction of DNA

AquaPhenol™ was processed according to the manufacturer's instructions to buffer the phenol to pH8.0. Phenol : chloroform was prepared by mixing buffered AquaPhenol™ with chloroform in a 1:1 ratio (v/v), and stored under buffer pH8.0. An equal volume of buffered phenol : chloroform was added to the DNA solution and gently emulsified. The emulsion was separated into two phases by centrifugation 12,000 × g for 5 minutes and the aqueous phase was transferred to a fresh tube. The extraction protein could be repeated until no precipitate at the interphase was formed. As all the phenol is not removed from the aqueous phase by centrifugation, the sample must be further extracted with chloroform.

Chloroform : Isoamyl alcohol Extraction of DNA

Chloroform : Isoamyl alcohol was prepared by mixing 24 parts chloroform with 1 part isoamyl alcohol. An equal volume of chloroform : isoamyl alcohol 24:1 (v/v) was added to the DNA solution and mixed gently by shaking. The phases were separated by centrifugation for 2 minutes at 12,000 × g and the upper aqueous phase pipetted into a fresh tube. An equal volume of chloroform was added, mixed thoroughly and the phases separated by centrifugation at 12,000 × g for 2 minutes. Not all the chloroform is removed from the aqueous phase by centrifugation, so the DNA must be precipitated by alcohol precipitation.

DNA Precipitation from Aqueous Solutions

DNA could be precipitated with isopropanol of with ethanol in the presence of sodium or ammonium acetate. For isopropanol precipitation, 0.7 volumes of isopropanol were added to the DNA solution and then immediately centrifuged to 30 minutes at

12,000 × g at 4°C. The supernatant was aspirated or pipetted away and the pellet washed with 1ml of ice cold 70% ethanol. After further centrifugation for 1 minute the supernatant was aspirated of and dried in a Jouan MR18.22 rotary evaporator under vacuum. For ethanol precipitation, 1/5th volume of 10M NH_4Ac or 1/10th volume of 3M NaAc was added to the DNA solution followed by 2 volumes of ethanol. Tubes were left on ice for 15 minutes or at -20°C for 15 minutes to allow the DNA to precipitate after which the DNA was pelleted by centrifugation at 12,000 × g for 15minutes. Pellets were washed and dried as described from isopropanol precipitations. Generally, the addition of monovalent anions could be omitted if the DNA was already in a high salt buffer.

2.4.14 Methods for introducing plasmids into *Escherichia coli*

E.coli was transformed in three different ways depending upon the efficiency required.

Low efficiency transformation with $CaCl_2$

This methods was used when relatively large amounts of DNA were available and a particular bacterial strain (*E.coli* BL21(λDE3) or TB1) was required for transformation.

A fresh LB plate was streaked with the required strain and grown overnight at 37°C. A single colony was picked from the plate with a flamed platinum wire and used to inoculated a 5ml culture of LB. The culture was grown overnight at 37°C with shaking. 200µl of the overnight culture was used to inoculate a fresh 5ml LB culture and this was grown for a further 2 hours. The culture was transferred to a 15ml sterile polypropylene centrifuge tube and the bacteria harvested by centrifugation at 3,000 × g for 10 minutes. The supernatant was discarded and the bacteria were re-suspended in 500µl of ice-cold $CaCl_2$. The bacteria were left on ice for 10 minutes after which time they were harvested by centrifugation, the supernatant removed and the bacteria were re-suspended in 200µl of ice-cold $CaCl_2$.

After 5 minutes on ice the bacteria were transferred into a tube containing 5µl of DNA and mixed by pipetting up and down. The bacteria / DNA mix was left on ice for 30

minutes to allow the cells to bind the DNA. The DNA was introduced into the competent cells by heat shock. The bacteria / DNA mix was placed in a 42°C water bath for 90 seconds and then transferred back onto ice for 2-3 minutes. 800µl of SOC pre-warmed to 37°C was added and the tubes incubated at 37°C for 1 hour with occasionally shaking. The cells were plated out on selective LB agar.

Where blue / white selection of transformants was possible, 40µl of X-gal (2% (w/v) in dimethylformamide) and 10µl of 100mM IPTG were spread on to the surface of the LB plate before plating the bacteria.

High efficiency transformation with commercial E.coli DH5α

Subcloning Efficiency DH5α™ Competent Cells were bought from Life Technologies and transformed according to the manufacturers instructions. Cells were plated onto selective LB agar. These were used when maximum efficiency was required, such as when difficult ligations were performed.

High efficiency transformations with Frozen Competent E.coli.

When commercial cells were not available, stocks of frozen competent cells were used. These were generated by the method of Hanahan (1983) described in Sambrook *et al.*, 1989.

A fresh SOB plate was streaked with *E.coli* DH5α from a glycerol and grown overnight at 37°C. A single colony was picked from the plate with a flamed platinum wire and used to inoculate 50ml of SOB containing 20mM $MgSO_4$ in a 250ml baffled flask. The cells were grown to an OD_{600} of 0.5. The culture was transferred to a 50ml sterile polypropylene centrifuge tube and the cells harvested by centrifugation at 3,000 × g for 10 minutes at 4°C. The media was decanted from the cells and the tube left inverted on a paper towel to allow excess media to drain away. Cells were re-suspended in 20ml of ice cold FSB and stored on ice for 10 minutes. Cells were recovered by centrifugation at 3,000 × g for 10 minutes and re-suspended in 4ml of

ice-cold FSB. 140µl of DMSO[1] was added to the re-suspended cells and mixed gently by swirling. The cells were incubated on ice for 15 minutes after which time an additional 140µl of DMSO[1] was added and mixed by gentle swirling. The cells were rapidly dispensed into 200µl aliquots and snap frozen in liquid N_2. Aliquots were stored at -80°C until required.

When needed, an aliquot of cells was removed from the -80°C freezer, thawed and placed on ice for 10 minutes. The aliquot was transferred to a fresh microfuge tube containing 5µl of a DNA ligation or other DNA and mixed by gentle pipetting. The cells were incubated on ice for 30 minutes. The DNA was introduced into the cells by heat shock. The tubes were transferred to a circulating water bath at 42°C for 90 seconds after which they were immediately transferred to an ice bucket and allowed to cool for 1 -2 minutes. Eight hundred microlitres of SOC warmed to 37°C was added to the cells, which were then placed in at shaking incubator at 37°C for 45 minutes. This time allows the cells to recover and express the antibiotic resistance marker. An appropriate volume (usually 200µl) was plated onto LB agar plus the selective antibiotic and left to grow inverted in a 37°C incubator overnight.

Where blue / white selection of transformants was possible, 40µl of X-gal (2% (w/v) in dimethylformamide) and 10µl of 100mM IPTG were spread on to the surface of the LB plate before plating the bacteria.

2.4.15 Radiolabelling of DNA fragments

DNA was labelled using the *redi*prime DNA labelling system from Amersham. The DNA to be labelled was diluted to 45µl with ddH_2O and heated for 5 minutes in a boiling water bath to denature the strands. The contents of the tube were transferred to a *redi*prime tube and the contents reconstituted by flicking the tube until the blue dye was evenly distributed. Five microlutres of [^{32}P]dCTP ≥300Ci/mmol was added to the tube and mixed by pipetting. The contents of the tube were collected at the bottom

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[1]DMSO was bought fresh in a sealed bottle from the supplier and upon opening immediately aliquoted and frozen at -70°C to prevent oxidation. Oxidation products of DMSO inhibit transformation.

of the tube by brief centrifugation and the reaction was incubated at 37°C for 15 minutes.

The reaction was stopped by the addition of 5µl of reaction stop mix (20mM EDTA pH8.0, 2mg/ml blue dextran, 0.02mg/ml xylene cyanol). The DNA probe was purified with a BIO-RAD BioSpin Chromatography column containing Bio-Gel P-6 polyacrylamide gel, which has an exclusion limit equivalent to approximately 5 base pairs. The column was centrifuged at 1,100 × g in a swing out rotor for 2 minutes to remove the storage buffer. The reaction mix containing the radiolabelled DNA fragment, was applied to the centre of the top of the column, and centrifuged at 1,100 × g for 4 minutes. The purified DNA was recovered from the collection tube at the bottom of the column.

The DNA probe was boiled for 5 minutes before use.

2.4.16 Colony hybridisation

Using sterile toothpicks colonies were picked and streaked on replica plates. The master plate had a numbered grid marked on the bottom. The replica plate was overlayed with a 70mm nitrocellulose filter (Colony / Plaque Screen™ Hybridisation Transfer Membrane) with an identical grid drawn on and numbered with a pencil. Colonies were streaked on identical numbered positions on the two grids. The plates were incubated at 37° overnight.

The master plate was stored at 4°C. The nitrocellulose filter was peeled from the replica plate and placed colony side up on a 10% SDS saturated piece of Whatmann 3MM filter paper for 3 minutes. The nitrocellulose was transferred to a similar piece of Whatmann 3MM filter paper soaked in denaturing buffer for 5 minutes, followed by 2 × 5 minutes on a paper soaked with neutralisation buffer. The nitrocellulose filter was finally transferred to a sheet of 3MM paper saturated with 2 × SSC and left for 5minutes. Filters were let to dry at room temperature on a dry sheet of 3MM paper before being baked at 80°C in a vacuum oven for 1-2hours.

2.4.17 Hybridisation of radiolabelled DNA to blots

Hybridisation was performed in a Techne Hybridisation Tube in a Techne Hybridiser HB-1.

For colony hybridisation filters, were wetted in 2 × SSC. Filters were pre-washed in 25ml of pre-washing solution at 50°C for 30 minutes. Bacterial debris was carefully scraped from the surface of the filter to reduce background signal with a spatula. The filters were incubated in 25ml of pre-hybridisation solution for ≥2 hours at 42°C. The pre-hybridisation solution was poured away and replaced with 10ml of hybridisation solution to which the radiolabelled DNA probe was added. The denatured radiolabelled probe was hybridised for the filter overnight at 42°C. The filters were washed twice in 50ml of 2 × SSC, 0.1% SDS at room temperature for 10 minutes, then washed twice in 50ml 1 × SSC, 0.1% SDS for 1 hour at 60°C. A final wash in 0.2 × SSC, 0.1% SDS at 65°C reduced background to a minimum.

For Southern and Northern hybridisation, filters were wetted in 2 × SSC and soaked in 25ml of pre-hybridisation solution for ≥2 hours at 42°C. The pre-hybridisation solution was poured away and replaced with 10ml of hybridisation solution to which the radiolabelled DNA probe was added. The denatured radiolabelled probe was hybridised for the filter overnight at 42°C. The filters were washed twice in 50ml of 2 × SSC, 0.1% SDS at room temperature for 10 minutes, then washed twice in 50ml 1 × SSC, 0.1% SDS for 1 hour at 60°C. For maximum stringency, filters were finally washed in 0.1 × SSC, 0.1% SDS at 60°C for 30 minutes.

2.4.18 Detection of radioactivity on solid supports

Two methods were used to detect radioactivity on a solid support such as a membrane or in a gel, X-ray film or phosphor imaging. X-ray film was pre-flashed to increase the sensitivity (Laskey & Mills, 1975) and film cassettes with intensifying screens were used. Exposure was carried out at -180°C to reduce background signal and exposed films were developed with Ilford Phenisol developer for 2-5 minutes and fixed with Kodak Unifix fixer for 2 minutes.

A Model GS-525 Molecular Imager® (BIO-RAD) was used throughout this study for analysis of radioactivity bound to solid supports. The Storage Phosphor Imaging System provides an enhanced sensitivity over X-ray film of approximately 10-fold. Samples and images were all processed according to the manufacturer's recommendations and images were scanned at the highest resolution available. A High Sensitivity screen was used with ^{14}C and ^{35}S samples as they are weaker emitters of radioactivity. Images were stored and processed by a Macintosh Quadra 800 computer and printed at 600dpi.

2.4.19 Exo III / S1 nuclease deletion of DNA.

The Exo III / S1 nuclease kit is a convenient system for producing unidirectional nested deletions in a DNA insert allowing sequence data to be determined for internal sequence. The Exo III nuclease will digest 3' termini from blunt and 5' protruding termini, but not 3' single strand termini. Deletions of an insert in the pBluescript™ vector proceeded according to the manufacturer's recommendations, using only the reagents supplied with the kit.

The plasmid construct was double digested to completion with two restriction enzymes to generate a 5'-protruding or blunt end with one enzyme and a 3'-protruding termini with the other. The 5'-protruding termini is between the sequence to be deleted and the 3'-terminus. The DNA was phenol : chloroform extracted, ethanol precipitated and dissolved in TE buffer.

Alternatively, if a suitable 3'-protruding terminus could not be made by restriction, a 5'-terminus was protected by end filling with thio-dNTPs. The 5' terminus to be protected was located 5' to the DNA sequence to be digested, leaving a suitable restriction site in between for digestion to create a susceptible 5' terminus. Five micrograms of the DNA to be blocked was digested with the first enzyme. The DNA was recovered into TE from an agarose gel and the ends blocked by combining, DNA (3μg), 1μl of Klenow 10 × buffer, 1μl of dNTPαS and 0.05U of Klenow in 10μl with

ddH_2O. The reaction was incubated at 37°C for 15 minutes. The DNA was recovered by ethanol precipitation and re-suspended in 20µl of TE.

The Exo III reactions were done in 75mM NaCl at 37°C which corresponds to a deletion rate of approximately 120bp/min. Double strength Exo III buffer (150mM NaCl) was prepared by combining 8µl 6× Exo III buffer (75mM NaCl), 10µl 0.3M NaCl, 6µl ddH_2O.

The Exo III reactions were set up as follows;

double digested or dNTPαS protected DNA	2µg
2 × Exo III buffer (150mM NaCl)	20µl
Exonuclease III	1µl
ddH_2O to	40µl

Before the reaction was started by the addition of the Exo III nuclease, the reaction mix was incubated at 37°C and a 2µl aliquot was removed and placed on ice as the zero time point. The enzyme was added and the reaction incubated at 37°C. Aliquots were removed at appropriate time points (usually 90 seconds which corresponds to approximately 150bp deletions) and placed in a microfuge tube on ice, with 3µl of S1 nuclease buffer (1 × S1 nuclease buffer, 1/100 dilution of S1 nuclease) which prevents Exo III activity. When all time points had been collected, the tubes were incubated together at room temperature for 30 minutes for the S1 nuclease to digest the other strand of DNA. After 30 minutes, 1µl of S1 nuclease stop solution was added to each time point and the reactions heated to 65°C for 10 minutes. Three microlitres of each time point was run on an agarose gel to check the deletion had proceeded.

Deleted DNA produced by the Exo III / S1 nucleases was self-ligated in the presence of 6% PEG and T4 DNA ligase and transformed into *E.coli* DH5α cells. Transformants were screened for the presence of the required size of deletion.

Chapter 3: Identification of the biotin-containing proteins in *Brassica napus*.

3.1 Introduction

The presence of biotin-containing proteins has been demonstrated in all cells examined to date (Moss & Lane, 1971; Alberts & Vagelos, 1972; Scrutton & Young 1972; Wood & Barden, 1977; Dakshinamurti & Bhagavan, 1985b). The distribution and number of biotin-containing proteins to be found in the cells of different species is not the same. Only one biotinylated protein was detected in *Escherichia coli*, acetyl-CoA carboxylase, when the cells were labelled with [^{3}H]-biotin (Li & Cronan, 1993). In contrast to this simple bacterium, rat and human cells grown in culture and labelled with [^{3}H]-biotin revealed four different biotin-containing proteins (Chandler & Ballard, 1985). These proteins were acetyl-CoA carboxylase (ACCase), pyruvate carboxylase (PCase), propionyl-CoA carboxylase (PCCase) and methylcrotonyl-CoA carboxylase (MCCase). Similar experiments with mouse 3T3-L1 cells revealed more than twenty additional labelled proteins of unknown function (Chandler & Ballard, 1986). Some of these were demonstrated to be proteolytic fragments of known biotin-containing proteins, but others were shown not to be proteolytic fragments and may represent as yet unknown biotin-containing proteins.

It has also been demonstrated that plant cells possess multiple biotin-containing proteins (Wurtele & Nikolau, 1990). Embryos of *Daucus carota* (carrot) have been shown to contain biotinylated proteins with M_r values of 210, 140, 73, 50, 39 and 24kDa. This tissue also contained enzyme activities for the known biotin dependent enzymes acetyl-CoA carboxylase, pyruvate carboxylase, propionyl-CoA carboxylase and methylcrotonyl-CoA carboxylase. Suggested functions based on comparisons of M_r with known biotinylated proteins were; 210kDa protein as ACCase, 140kDa protein as PCase, 73 kDa protein as either methylcrotonyl-CoA carboxylase or propionyl-CoA carboxylase or both (Wurtele & Nikolau, 1990). The function of the other three biotin-containing protein in carrot embryos have not been identified.

Previous to this work, only one biotin-containing protein had been identified in *Brassica napus*. This was a 220kDa protein with acetyl-CoA carboxylase activity (Hellyer *et al.*, 1986). Other biotin-containing proteins had been inferred by purification with avidin-agarose (Turnham & Northcote, 1983). Multiple bands were purified from total protein extracts of embryos 22 days after pollination by simple incubation of a total protein extract with avidin-agarose. Proteins were recovered from the avidin-agarose by denaturing with SDS. The only bands noted by the authors were the 220kDa ACCase and a doublet of about 48kDa M_r. Other bands were purified but not noted, these had an approximate M_r of 29, 34, 66 and 75kDa.

3.2 Aims

The aims of the research presented in this chapter were to describe the identification and characterisation of biotinylated proteins in *Brassica napus*. This would be done by; a) detecting enzyme activity in radiometric assays and b) detecting biotinylated proteins on Western blots. These experiments would reveal how many biotin-dependent activities were present, and how many biotinylated proteins of different M_r could be detected in total protein extracts of *Brassica napus*. It would be attempted to assign a function to each of the detected biotinylated proteins by comparison of the M_rs with known biotinylated proteins from other sources.

In this way, a better understanding of the role these important proteins have to play in *Brassica napus*, might be gained.

3.3 Detection of biotin-dependent carboxylase activities in Brassica napus embryos.

The identity of several biotin dependent enzymes from prokaryotes and animal systems has already been discussed, these enzymes have a simple, sensitive radiometric assay for their activity. The presence of the known biotin-dependent carboxylases could be confirmed by an assay for the individual activities present in protein extracts of *Brassica napus*. The method described by Wurtele & Nikolau 1990, was used without any adaptations, to confirm the presence of biotin-dependent activities in our seven year old, -80°C freezer stored maturing seeds of *Brassica napus*. Previous work had cited only very low levels of pyruvate carboxylase activity in *Brassica napus* (Wurtele & Nikolau, 1990). These levels might have been expected because the reactions were not done in the presence of acetyl-CoA which is an absolute requirement for significant levels of pyruvate carboxylase activity in mammals (Scrutton & Young, 1972).

3.3.1 Preparation of crude extract

A crude developing seed extract was prepared in the same way as described by Wurtele & Nikolau 1990. One gram of frozen seed material was ground to a fine powder in liquid N_2 with a mortar and pestle and homogenised with 3 ml of extraction buffer (50mM Tris-HCl pH7.5, 10mM β-mercaptoethanol, 1mM EDTA, 1mM PMSF and 1% PVP) using a T8 ULTRA TURRAX with a S8N-5G dispersing element attached. This was spun at 10,000 × g for 10 minutes. The supernatant from between the oil layer and the pellet was taken, filtered through a glass wool filter and applied to a Bio-Rad EconoPac P6 cartridge pre-equilibrated with extract buffer -PVP. The cartridge was pre-equilibrated by passing 30ml of extract buffer -PVP through at 1ml /min with a peristaltic pump. The sample was applied slowly using a syringe. The first 1.5ml to elute from the EconoPac column was discarded and the subsequent 3ml of effluent collected and used as a total protein extract for assays.

3.3.2 Assays

Assays were performed in a ground glass tube in a total volume of 200µl. Twenty five microlitres of crude extract (containing 20µg total protein by Bradford Assay against BSA standard), was assayed for specific enzyme activity by incorporation of [^{14}C] HCO_3^- into acid stable products over 30 minutes. As the assays were carried out exactly as previously described, (Wurtele & Nikolau, 1990) the linearity of the assay over time and with protein content was not assessed. The incorporation of radioactivity had been reported as being linear up to 30 minutes incubation time and with up to 30µg of total protein (Wurtele & Nikolau, 1990). Two times assay mix was prepared in a ground glass tube (100mM Tris-HCl pH8.0, 11.2mM $MgCl_2$, 11.2mM ATP pH7.0, 4.0mM DTT, 188mM KCl, 12.2mM $NaH^{14}CO_3$ (16µCi/µmol), and kept on ice until required. Substrates were prepared as 6mM stocks (final 0.3mM) and NADH as a 67mM stock (final 3mM). The assays were pre-incubated by addition of components except substrate and incubating for 5 minutes at 30°C. The reaction was started by addition of substrate.

The reactions were terminated after 30 minutes incubation time at 30°C, by the addition of 100µl of 6N HCl. The addition of HCl drops the pH to around pH 1, preventing further enzyme activity. At the same time, the remaining bicarbonate is converted to carbon dioxide gas. The reaction was vortexed and left to stand at room temperature for 5 minutes to allow the radioactive gasses to disperse. Half of the total (150µl) was put into a plastic scintillation vial and lypholised in a Jouan SpeedVac overnight. After desiccation the dried pellet was solubilised in 300µl ddH_2O and 3ml of scintillant. The amount of radioactivity incorporated was measured by a liquid scintillation counter.

Substrate	*+ extract*	*-extract*	*Total incorporation*
Acetyl-CoA	271	43	228
Propionyl-CoA	280	75	205
β-methylcrotonyl-CoA	15046	100	15306
pyruvate	4955	127	4828
pyruvate + acetyl-CoA	4492	145	4347

Table 3.1 cpm incorporated into acid stable products by a total protein extract from developing rape seed. Assays were repeated without protein extract to monitor background incorporation.

The results reported here showed that the freezer stored material contained biotin-dependent carboxylase activities, albeit at different levels to previous reports, and that PCase activity was not increased by the addition of 300μM acetyl-CoA. To identify the biotin-containing subunits of these enzymes, Western blots of protein extracts from *Brassica napus* would be probed with biotin detecting reagents such as streptavidin, and the number of biotin-containing proteins assessed.

3.4 Detection of biotinylated proteins in B. napus

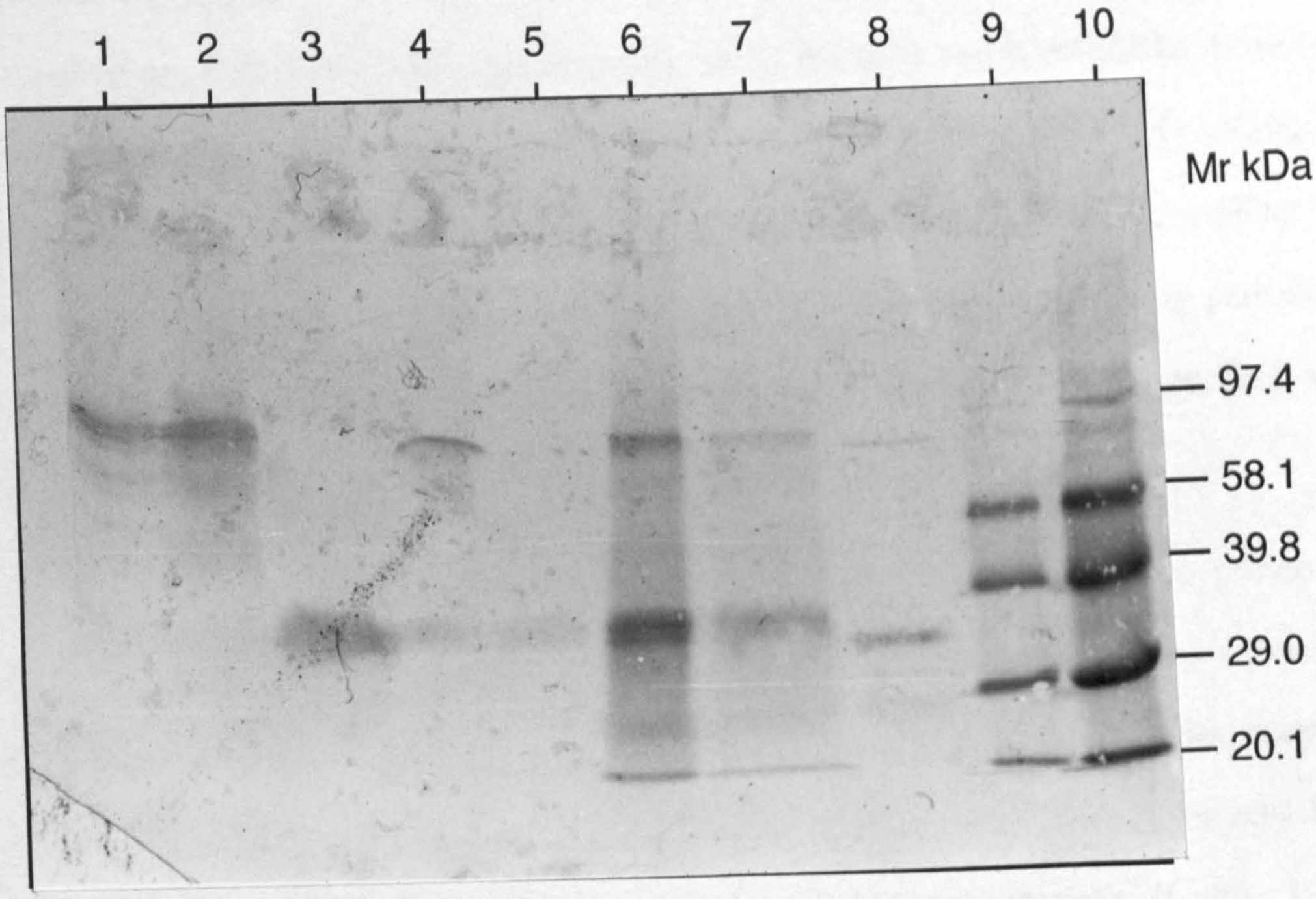
Previously, ^{125}I labelled streptavidin has been used to examine the number of biotinylated proteins found in crude extracts of *Daucus carota* (carrot, Nikolau, *et al.*, 1985; Wurtele & Nikolau, 1990). In order to see how many biotinylated proteins could be detected in total protein extracts from *B. napus*, a similar experiment was performed using a streptavidin : horse radish peroxidase conjugate. The detection properties of the streptavidin : horse radish peroxidase conjugate would be similar to those of ^{125}I labelled streptavidin as the molecule should bind specifically to biotin. The streptavidin : horse radish peroxidase conjugate was detected using diaminobenzidine which becomes insoluble when oxidised, leaving a coloured band on the membrane.

One gram of *Brassica napus* tissue (either seed, root or leaf) was ground in a mortar and pestle pre-cooled with liquid N_2. The ground tissue was added to 10 volumes (w/v) of boiling 2% SDS and allowed to boil for 5 minutes. After 5 minutes the solution was cooled on ice and spun at full speed in a microfuge for 10 minutes. The supernatant was aliquoted into 100µl aliquots and stored at -80°C. Twenty microlitres of 5 × SDS sample buffer was added to a thawed 100µl aliquot, boiled for 1 minute and 25µl was loaded onto a 10% acrylamide gel. SDS / PAGE and Western blotting was carried out as described in section 2.4.11, except blotting was done at 100V for 1-2 hours. The membrane was incubated in PBS containing 1% haemoglobin for 16 hours. The blocking buffer was replaced with 15ml of fresh PBS containing 1% haemoglobin and 5µl of streptavidin : horse radish peroxidase conjugate was pipetted into the tube. The membrane was left incubating at room temperature in a Techne Hybridisation Oven for 3 hours after which time it was washed with 2 × 20ml of PBS for 10 minutes each wash. The blot was developed with diaminobenzidine (DAB) and hydrogen peroxide in a colorimetric reaction.

Figure 3.1 shows a photograph of the Western blot. A doublet of about 80kDa was detected in the seed extract shown in Lane 1 of Figure 3.1, but no other bands were detected. This 80kDa band can also been seen in a crude chloroplast prep from leaf (lane 4), and in avidin-agarose purified proteins (purification described in section 2.4.4) from rape seed (lanes 6 and 7). A similar band at a slightly lower M_r is apparent in *Arabidopsis* cell extract (Lane 8). Another diffuse band or doublet was detected at 36kDa in leaf tissue (lane 3) and pure chloroplasts (lane 5). This band was also purified by avidin-agarose (lanes 6 and 7) and a similar but slightly lower M_r protein is detected in *Arabidopsis* cell extract (lane 8). As many more proteins, six in total, had been detected in protein extracts of *Daucus carota* (carrot, Wurtele & Nikolau, 1990), further investigations were carried out to improve the quality of the Western blots and the sensitivity of detection.

Figure 3.1 Western blot of various *Brassica napus* tissues showing biotinylated proteins detected with Streptavidin-HRPO.

The lanes contain total protein extracts from the following; Lane 1, Seed (var. Cobra); Lane 2, germinating seeds; Lane 3, Leaf; Lane 4, Chloroplast prep, crude pellet; Lane 5, Chloroplast pre, 40% percoll purified pellet; Lane 6, Avidin-agarose isolated proteins from seed × 5; Lane 7, Avidin-agarose isolated proteins from seed × 1; Lane 8, *Arabidopsis thaliana* suspension culture, whole cell extract (gift from John Gilroy, Department of Biological Sciences, University of Durham); Lane 9, biotinylated molecular weight markers, 1µl; Lane 10, biotinylated molecular weight markers, 10µl. Preparation of protein extracts are described in the text, preparation of chloroplasts and avidin-agarose purified proteins are described in Materials & Methods. The Western blot was developed with diaminobenzidine and hydrogen peroxide.


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10
Mr kDa
97.4
58.1
39.8
29.0
20.1

3.5 Anti-biotin antisera

It became apparent that there were several problems associated with the continued use of streptavidin-HRPO. It was very difficult to get consistent results using streptavidin-HRPO as a probe, partially due to the fact that the properties of the enzyme changed when frozen and did not give consistent results over time. Both of these made for a bad probe, especially if accurate qualitative or quantitative results were required. In order to overcome these difficulties it was decided to raise anti-biotin antibodies in sheep and rabbit which should have many benefits over the commercial preparations of streptavidin. 1) Once the antibodies had been raised there would be enough serum for years of work, so in the long run it would be more cost effective. 2) There were a large number of commercially available anti-IgG conjugates which would give an amplification step over the use of streptavidin, making the antibodies more sensitive. 3) Antibodies are usually stable for very long periods of time, are able to accommodate a variety of conditions and can also be purified very simply to remove background problems.

The production of polyclonal antibodies raised against biotin has been previously described (Berger, 1975). Biotin is a small hapten, so in order to use it as an antigen it had to be linked to a carrier protein before injection. Keyhole Limpet Haemocyanin (KLH) was used as a carrier as it is extremely antigenic, being of extremely large molecular weight (>2MDa) widely used and available commercially (Catty, 1988). Also available commercially is a biotinylation reagent, sulfosuccinimidyl-6(biotinamide)hexanoate (NHS-LC-biotin) (PIERCE) which was used according to manufacturers instructions to biotinylate 10mg of KLH for production of antibodies.

3.5.1 Biotinylation of KLH

Ten milligrams of Keyhole Limpet Haemocyanin was dissolved in 9ml of 100mM $NaHCO_3$, pH8.0, and stirred for 4 hours. During this time the pH was maintained at pH 8.0 by monitoring the pH with a pH meter and adding 0.1N HCl whenever the pH dropped below 7.5. To remove any possible amine salts present in the dissolved KLH preparation the 9ml was loaded into prepared dialysis tubing (10kDa molecular weight cut-off) and dialysed against 1L of 100mM $NaHCO_3$, pH8.0 for 16 hours in a flask with stirring. This was important as NHS-LC-biotin attaches to free amine groups on the protein, therefore any amine salts in solution will react with the NHS-LC-biotin preventing it from attaching to the KLH. A 3M solution of NHS-LC-biotin was made up by dissolving 1.63mgs in 1ml of DMSO. The NHS-LC-biotin solution in DMSO was added to the 9ml of KLH solution expunged from the dialysis tubing, and left stirring at room temperature for 1 hour. To remove unreacted NHS-LC-biotin and to buffer exchange the product the solution was dialysed against 2 x 1L of PBS, pH7.4 for 24 hours. The protein was used to immunise two rabbits and a sheep, by Zeneca Seeds according to their own protocol, as part of an ongoing joint project with our laboratory. Six months later the terminal bleed from 2 rabbits and 1 sheep were returned. The antibody titre and specificity was assessed by probing a Western blot of successive lanes of SDS 7 protein markers and biotinylated SDS markers . Four dilutions of antibody were chosen for titre, 1:250, 1:500, 1:1000 and 1:2000. The secondary antibody used was donkey anti-sheep IgG : HRPO conjugate. The Western blots were subsequently developed with diaminobenzidine and hydrogen peroxide. The results of the titration gave clear evidence that the antibodies were specific from all animals and that the titre was high enough for detection on Western blots (1:1000). The best results were obtained from the sheep serum, of which greater than 500ml of serum was obtained. These antibodies were then used to probe Western blots of total protein extracts from *B.napus* embryos in a developmental series.

3.6 Western blots of total protein from B. napus

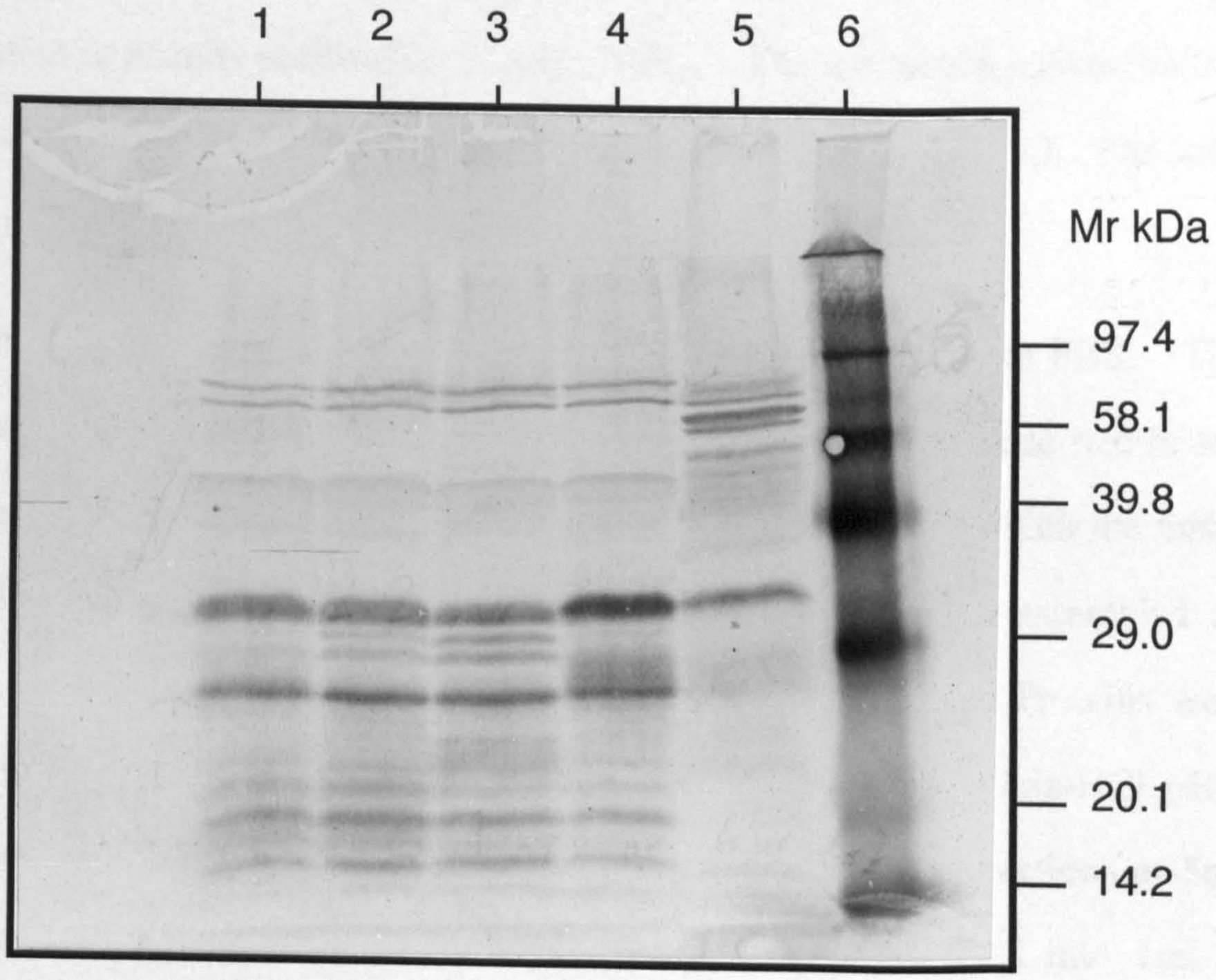
A series of Western blots were done to determine the number and nature of biotinylated proteins present in the embryos of *B. napus*. The preparation of material and nature of the prepared antibody / antisera varied in each of them to give a final blot which is more representative of the number of biotinylated proteins to be found in *Brassica napus*.

3.6.1 Aqueous extract probed with anti-biotin serum

The proteins from 5 embryos were extracted by grinding with liquid N_2 in a pre-cooled mortar and pestle. Embryos were used as opposed to whole seeds, because it was hoped that they would give a better protein extract due to the removal of the testa. Embryos were stored in liquid N_2 after harvest which should prevent proteolytic degradation of proteins during storage. The material was added to 0.5ml of "R" buffer (50mM Tris-HCl pH 7.5, 1mM EDTA, 0.5mM DTT, 10% glycerol and 1mM PMSF) and the material homogenised with a T8 ULTRA TURRAX fitted with a S8N-5G dispersing element in a 15ml polypropylene centrifuge tube. The extract was then spun at 10,000 × g for 30 minutes and the supernatant from between the lipid layer and the cell debris removed. Twenty microlitres of extract was run on two duplicate 12% acrylamide gels. The proteins in one gel were stained with Coomassie R-250 and the proteins in the other transferred to nitrocellulose. The Western blot was probed with sheep anti-biotin serum, a donkey anti-sheep IgG : alkaline phosphatase conjugate was used as a secondary antibody and the immunoreactive proteins detected with BCIP / NBT. The resulting Western blot (Figure 3.2) shows a number of bands some of which are undoubtedly biotin-containing proteins. The only known biotinylated protein in *Brassica napus*, the 220kDa ACCase was not detected. This is because the extraction buffer did not contain Triton X-100 which has been shown to be required for solubilisation of the 220kDa ACCase protein. Other bands that were detected included a doublet around 80kDa and a 36kDa band, both previously observed in Figure 3.1. Many lower molecular weight bands were observed of unknown identity as well as a 65kDa protein unique to late stage embryos (Figure 3.2, lane 5, 68daf)

Figure 3.2 Western blot of total protein extract from *Brassica napus* showing the bands detected with anti-biotin serum from sheep.

The lanes consist of equal loadings of material (in number of embryos of starting material) from staged embryo protein extracts. The lanes contain protein from the following staged embryos; Lane 1, 38daf; Lane 2, 44daf; Lane 3, 54daf; Lane 4, 57daf; Lane 5, 68daf. Lane 6 contains 5µl of biotinylated markers. The Western blot was probed with anti-sheep IgG : alkaline phosphatase conjugate as a secondary antibody, and developed with BCIP / NBT.


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Mr kDa
97.4
58.1
39.8
29.0
20.1
14.2

3.6.2 Affinity purified anti-biotin antibodies

The previous Western blot in Figure 3.2 presents some initial problems in that there are a large number of bands present between 30 and 14.3kDa and a band at 50kDa, which are probably not all biotin-containing-bands, but represent the background of antibodies in the serum which unfortunately pick up proteins in the total protein of rape seed. This background made it impossible to say with certainty that the detected proteins were biotin containing proteins as the antisera was not 100% specific. In order to be sure that the detected proteins are biotin-containing proteins it was necessary to remove this background. Background can be removed from antisera by absorption or affinity purification (Catty, 1988). The anti-biotin antibodies from our sheep serum were affinity purified on a biotin-agarose matrix (SIGMA). The anti-biotin IgG purification was performed as follows.

Five millilitres of sheep anti-biotin serum was diluted to 50ml with PBS. This was incubated in a glass bottle with 5ml packed volume of biotin-agarose free in solution, on a rotary platform. This was left agitating for 16 hours after which the matrix was poured into a column and allowed to settle. The column was assembled and the matrix washed with 100ml of Tris-HCl pH7.5 at 2.5ml / min. Proteins were then eluted from the matrix using 20ml each of, a) 500mM NaCl in Tris-HCl pH7.5, b) 100mM glycine pH2.5 and c) 100mM triethylamine pH11.5. Fractions of 5ml were collected in each case and those at extreme pHs were collected into 1ml of 1M Tris-HCl pH8.0 to immediately neutralise the acid or base, and mixed. The fractions eluted at each stage which showed high absorbance at 280nm, (about 2 whole fractions of 5ml) were pooled and dialysed against 2L of PBS overnight. The proteins eluted at each stage were tested for anti-biotin specificity by dot blotting 1µl of biotinylated protein markers onto nitrocellulose and developing with the eluted proteins followed by donkey anti-sheep IgG : alkaline phosphatase. Only those proteins eluted with 100mM glycine produced a positive reaction, so these were taken as the affinity purified anti-biotin sheep IgGs.

These affinity purified anti-biotin antibodies were subsequently used to probe a Western blot of the same protein extracts used for Figure 3.2. The Western blot shows that some of the background bands have been eliminated, significantly a band previously observed at around 50kDa in Figure 3.2. Importantly though, because the antibodies have been purified by binding to a biotin-agarose column, it is possible to say that the bands being detected are biotin-containing and not background.

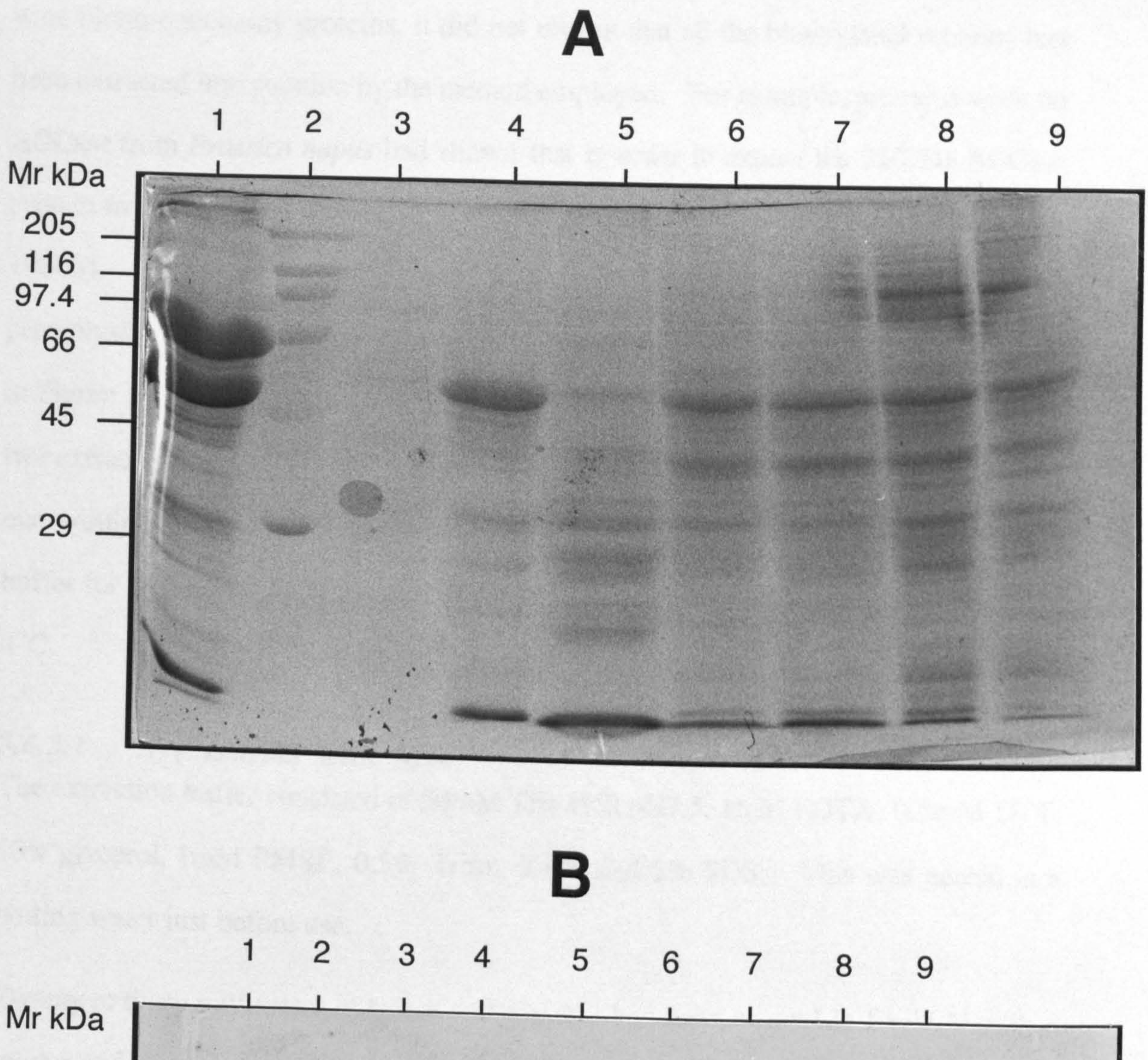
The problem remaining now was that the proteins detected may have been proteolytic degradation products. Although the embryos were stored in liquid N_2 the proteolytic degradation could have occurred during the extraction procedure. Also there are clearly problems with the extraction of all the biotinylated protein from the source material as not all the known biotinylated proteins are visible on these blots, specifically the 220kDa ACCase. This is because the extract was not done with Triton X-100 as previously mentioned. Now that the antibodies had been improved to the extent that it was possible to say with more certainty that the detected bands were biotin-containing, it was necessary to improve the nature of the extract to ensure that all the biotin-containing proteins were extracted and not degraded by proteases during the extraction process.

Figure 3.3 A Coomassie stained 12% acrylamide gel of proteins run for Western blotting.

The protein extracts from embryo material are the same as those prepared for Figure 3.2, but some extra tissues were run on this gel. The lanes contain the following; Lane 1, protein from purified chloroplasts; Lane 2, molecular weight markers (SDS-6H in the Coomassie stained gel, biotinylated markers on the Western blot); Lane 3, root total proteins; Lane 4, leaf total proteins. Lanes 5-9 are all embryo protein extracts from different harvesting times as follows; Lane 5, 68daf; Lane 6, 57daf; Lane 7, 54daf; Lane 8, 44daf; Lane 9, 38daf

Figure 3.3 B Western blot showing the effect of affinity purifying the anti-biotin antibodies raised in sheep.

A duplicate gel of the gel shown in Figure 3.3 A was run except lane 2 contained biotinylated molecular weight markers. The gel was not stained but proteins were transferred to nitrocellulose. The Western blot was probed with affinity purified anti-biotin antibodies and with donkey anti-sheep IgG : alkaline phosphatase conjugate as a secondary antibody. The Western blot was developed with BCIP/NBT.


A
1 2 3 4 5 6 7 8 9
Mr kDa
205
116
97.4
66
45
29
B
1 2 3 4 5 6 7 8 9
Mr kDa
97.4
58.1
39.8
29.0
20.1
14.2

3.6.3 Preparation of total protein from *Brassica napus* embryos

Whilst the Western blot in Figure 3.3 ensured that all the proteins detected actually were biotin-containing proteins, it did not ensure that all the biotinylated proteins had been extracted into solution by the method employed. For example, previous work on ACCase from *Brassica napus* had shown that in order to extract the 220kDa ACCase protein from seeds of *B. napus* it was necessary to include Triton-X100 (Hellyer *et al.*, 1986b). The previous extraction procedure employed did not guard against proteolysis of the proteins, and this could have produced the variety of bands present in Figure 3.3. In order to try and negate these criticisms it was undertaken to produce two extracts from the same seed samples at the same time using the same buffer, but one would containing 2% SDS and the crude homogenate would be boiled in this buffer for 20 minutes. The other would contain no SDS and would be extracted at 0-4°C.

3.6.3.1 A (Extract with SDS)

The extraction buffer consisted of 50mM Tris-HCl pH7.5, 1mM EDTA, 0.5mM DTT, 10% glycerol, 1mM PMSF, 0.5% Triton-X100 and 2% SDS. This was heated in a boiling water just before use.

Twenty to thirty milligrams of frozen material that had been ground in liquid $N_2$with a mortar and pestle, was placed in a chilled 15ml polypropylene centrifuge tube and kept in liquid N_2. Just before use the tube was removed from the liquid N_2 and warmed till the material began to thaw, at which point 10 volumes (v/w) of the boiling extract buffer were added, the tube vortexed to suspend the material, and returned to the boiling water bath for 10 minutes. After this time had elapsed the sample was homogenised with a T8 ULTRA TURRAX fitted with a S8N-5G dispersing element for 30 seconds at full speed before being returned to the boiling water bath for a further 10 minutes. After this time the extract was chilled on ice and spun at 13,000 × g for 10 minutes at 4°C. The entire fat layer and supernatant was removed, made 0.5 × SDS sample buffer, boiled for 2 minutes and then spun at full speed in a microfuge at room temperature for 3 minutes. A Hamilton syringe was inserted through the fat

layer, soluble protein draw into the syringe and 25µl loaded onto a 12% SDS / PAGE gel.

3.6.3.2 B (without SDS)

The extraction buffer consisted of 50mM Tris-HCl pH7.5, 1mM EDTA, 0.5mM DTT, 10% glycerol, 1mM PMSF and 0.5% Triton-X100. This was chilled on ice before use and all subsequent steps were done on ice.

Twenty to thirty milligrams of frozen material that had been ground in liquid $N_2$with a mortar and pestle, was placed in a chilled 15ml polypropylene centrifuge tube and kept in liquid N_2. Just before use the tube was removed from the liquid N_2 and warmed till the material began to thaw, at which point 10 volumes (v/w) of ice cold extraction buffer were added and the material was homogenised vigorously with a T8 ULTRA TURRAX fitted with a S8N-5G dispersing element in a ice water bath for 1-2 minutes. The homogenate was spun at 13,000 x g for 10 minutes at 4°C. The entire fat layer and supernatant was removed, made 1x SDS sample buffer, boiled for 2 minutes and then spun at full speed in a microfuge at room temperature for 3 minutes. A Hamilton syringe was inserted through the fat layer, soluble protein draw into the syringe and 25µl loaded onto a 12% SDS / PAGE gel.

3.6.4 Developmental Western blots

The above extraction procedures were used to produce two sets of identical total protein extracts from developing seeds of *Brassica napus* var. Falcon. The stages extracted were; 38daf, 44daf, 47daf, 58daf, 61daf, and 70daf Figure 3.4 shows the results of these Western blots probed with the affinity purified anti-biotin antibody, and donkey anti-sheep IgG : alkaline phosphatase, developed with BCIP / NBT. A 220kDa protein can be seen in these Westerns due to the use of Triton X-100 in the extraction buffer which solubilises the large form of ACCase. By extracting the proteins in this way, the variety of proteins <29kDa observed in Figure 3.2 and 3.3 have been reduced, suggesting that they were proteolytic degradation products of a larger protein.

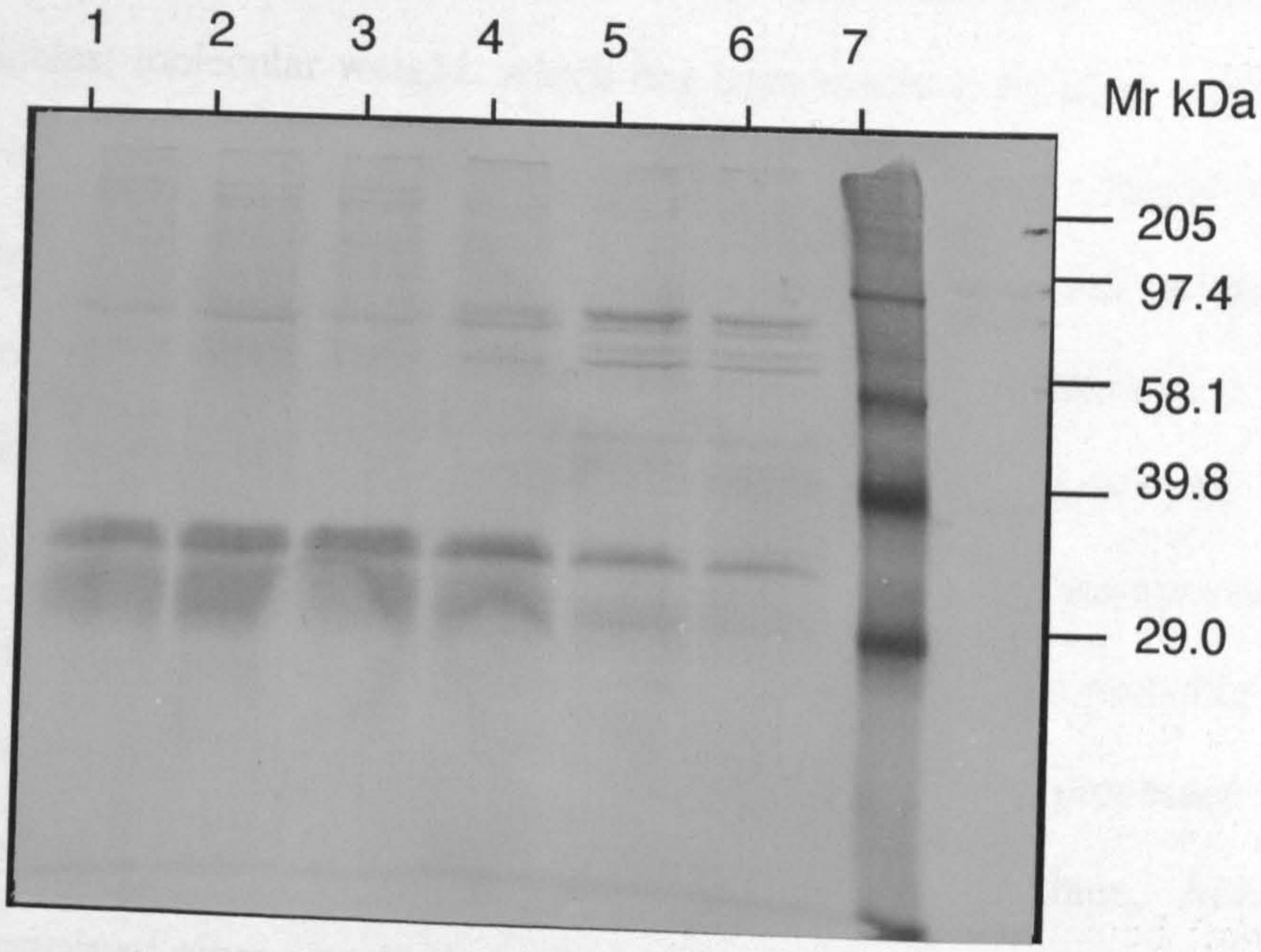
Figure 3.4 A Developmental Western blots of total protein from *Brassica napus* developing embryos extracted by method A.

Proteins were extracted from seeds harvested at a known number of days after flowering by method A (see section 3.6.3.1). Exactly the same amount of extract (per weight of starting material) was loaded onto each 12% SDS/PAGE gel, blotted onto nitrocellulose and probed with anti-biotin antibodies, detected with donkey anti-sheep IgG : alkaline phosphatase conjugate and developed with BCIP/NBT. The lanes show the following protein extracts; Lane 1, 38daf; Lane 2, 44daf; Lane 3, 47daf; Lane 4, 58daf; Lane 5, 61daf; Lane 6, 70daf; Lane 7, biotinylated molecular weight markers. Lane 8, (not marked on diagram) contained SDS-6H markers, these were stained with Ponceau-S before the blot was processed and their position marked with pencil.

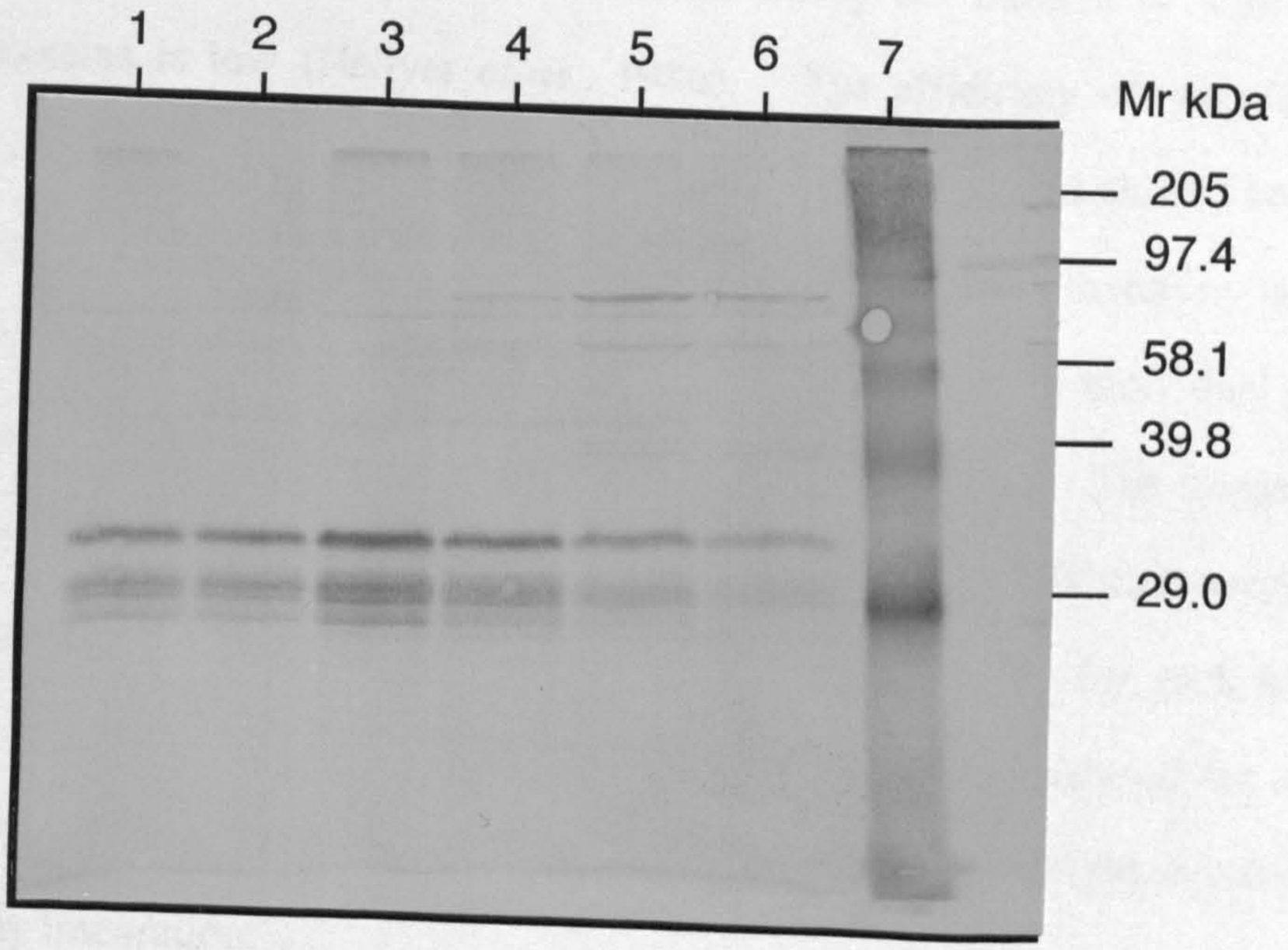
Figure 3.4 B Developmental Western blots of total protein from *Brassica napus* developing embryos extracted by method B.

Proteins were extracted from seeds harvested at a known number of days after flowering by method B (see section 3.6.3.2). The lanes contain protein extracted from the same tissue as shown in Figure 3.4 B. The Western blot was processed with the same reagents in the same tube as the one shown in Figure 3.4 A.

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Mr kDa
205
97.4
58.1
39.8
29.0

B


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7
Mr kDa
205
97.4
58.1
39.8
29.0

3.7 Quantification of the levels of expression of biotinylated protein in rape seeds during embryogenesis.

Figure 3.4 clearly shows that there are a variety of biotinylated proteins in rape seed and that their levels vary during embryogenesis. A photograph of a Western blot by itself does not easily reveal all of its data without further analysis. Figure 3.4 presents three variables; molecular weight, which has been resolved by SDS PAGE; temporal variation of expression, which has been investigated by analysis staged extracts; and finally intensity of signal or level of protein expression. In order to investigate the levels of expression further and present the results of this Western in a quantitative way the Western blot was scanned with a BIO-RAD Imaging Densitometer Model GS-690. The quantification of these results requires the following assumptions. 1) That all proteins were equally solubilised in all tissue extracts, this was probable because all extracts containing a similar ratio of material : buffer and were processed identically. 2) That the proteins have all entered the 12% gel equally in each lane. Although some fractions contained more protein than others due to an increase in the amount of protein in the material, none of the lanes were overloaded so all proteins should have entered the gel equally in each lane. 3) That transfer from gel to membrane was the same for all proteins. It has been reported that the efficiency of transfer of high molecular weight proteins is low (Hellyer *et al.*, 1986). The efficiency of transfer may be different between high and low molecular weight proteins, but it should be the same for the same protein in each lane. The quantification is therefore only semi-quantitative in that it only reflects the changes in the levels of an individual protein, it does not represent actual amounts present in the starting material. The image from the densitometer was processed with BIO-RAD's Molecular Analyst® software according to their instruction manual to produce an optical density profile for each lane in the Western blot. Peaks (corresponding to protein bands) were analysed for molecular weight (using the biotinylated markers in lane 7 as molecular weight standards), and intensity by integration.

The resulting data has been plotted on bar graphs in Figure 3.5.A and Figure 3.5.B corresponding to each of the Western blots A and B in Figure 3.4. There was some discrepancy between the calculated M_r for each protein. The M_rs were calculated by the Molecular Analyst® software using the biotinylated markers as standards. Even so, the highest M_r protein on Figure 3.5 A does correspond to the highest M_r protein on Figure 3.5 B.

Due to the reasons discussed above it was not possible to compare different proteins with one another. It was possible to compare the levels of a single protein with the levels detected in different tissues and at different times throughout embryogenesis, assuming that there is no change in the % of protein extracted by the extraction process. The data shows that the proteins detected at 31 and 35 kDa are highly induced during embryogenesis, peaking at 47daf. Other proteins at 61kDa and 76 kDa (Figure 3.5 A) are induced later in embryogenesis. The levels of the 71kDa protein vary probably due to the detection limits of the densitometer, but are essentially the same at 44 daf as at 70daf. These observations shows that there are three types of biotinylated proteins in embryos of *Brassica napus*, those that are induced to mid-embryogenesis (220kDa, 35kDa, 31-29kDa proteins), those that remain relatively constant throughout embryogenesis (71kDa protein) and those that are induced late in embryogenesis (61kDa and 76kDa proteins).

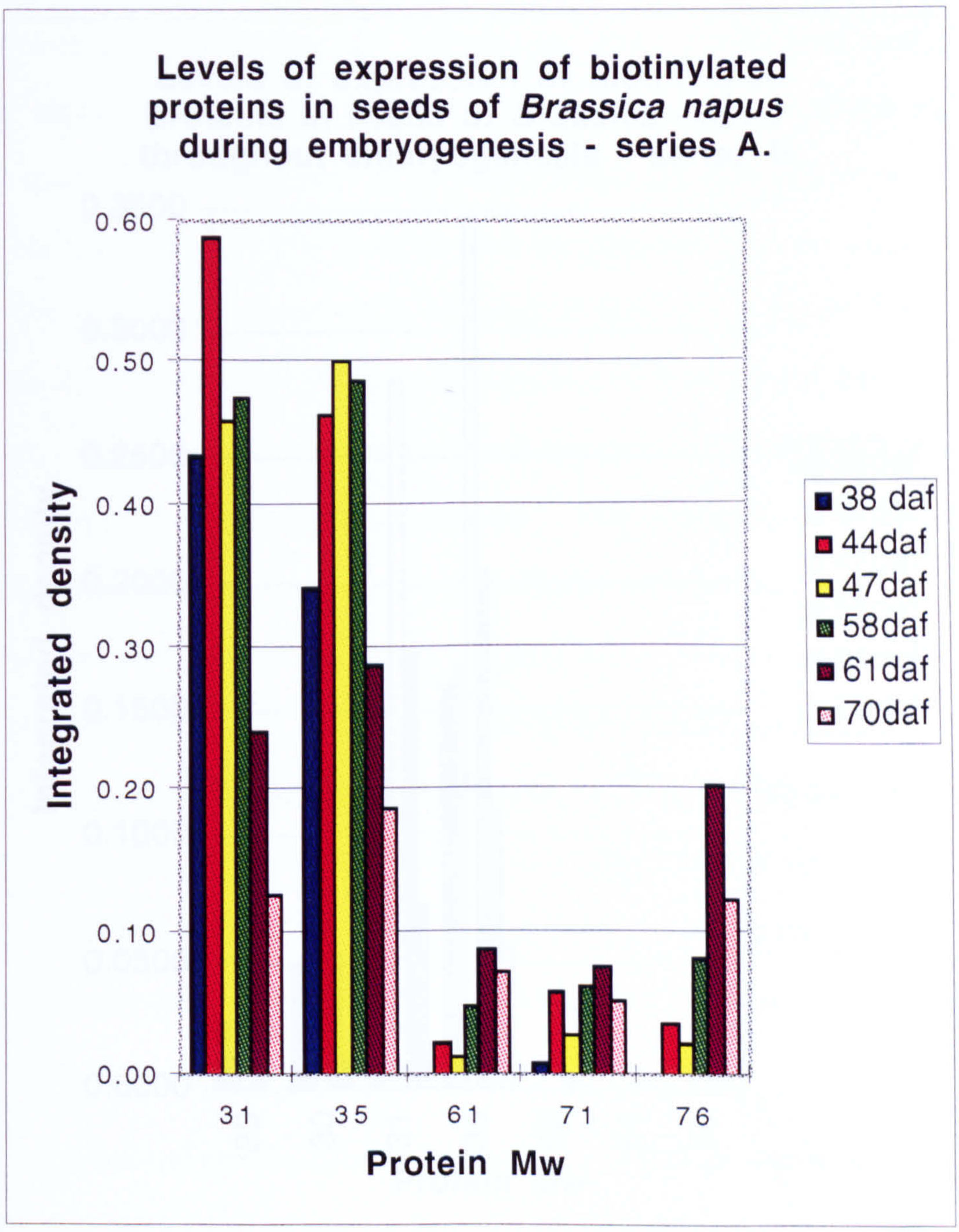


Figure 3.5 A Bar graph showing levels of expression of biotinylated proteins throughout embryogenesis in *Brassica napus*.

Proteins were extracted from maturing seeds according to protocol A and the Western is shown in Figure 3.4 A. The x-axis shows the molecular weight for proteins detected by the BIO-RAD Imaging Densitometer and calculated from the molecular weight standards. The y-axis shows the total integrated density measured. The columns for each molecular weight represent a different harvesting time for extraction and are coloured accordingly.

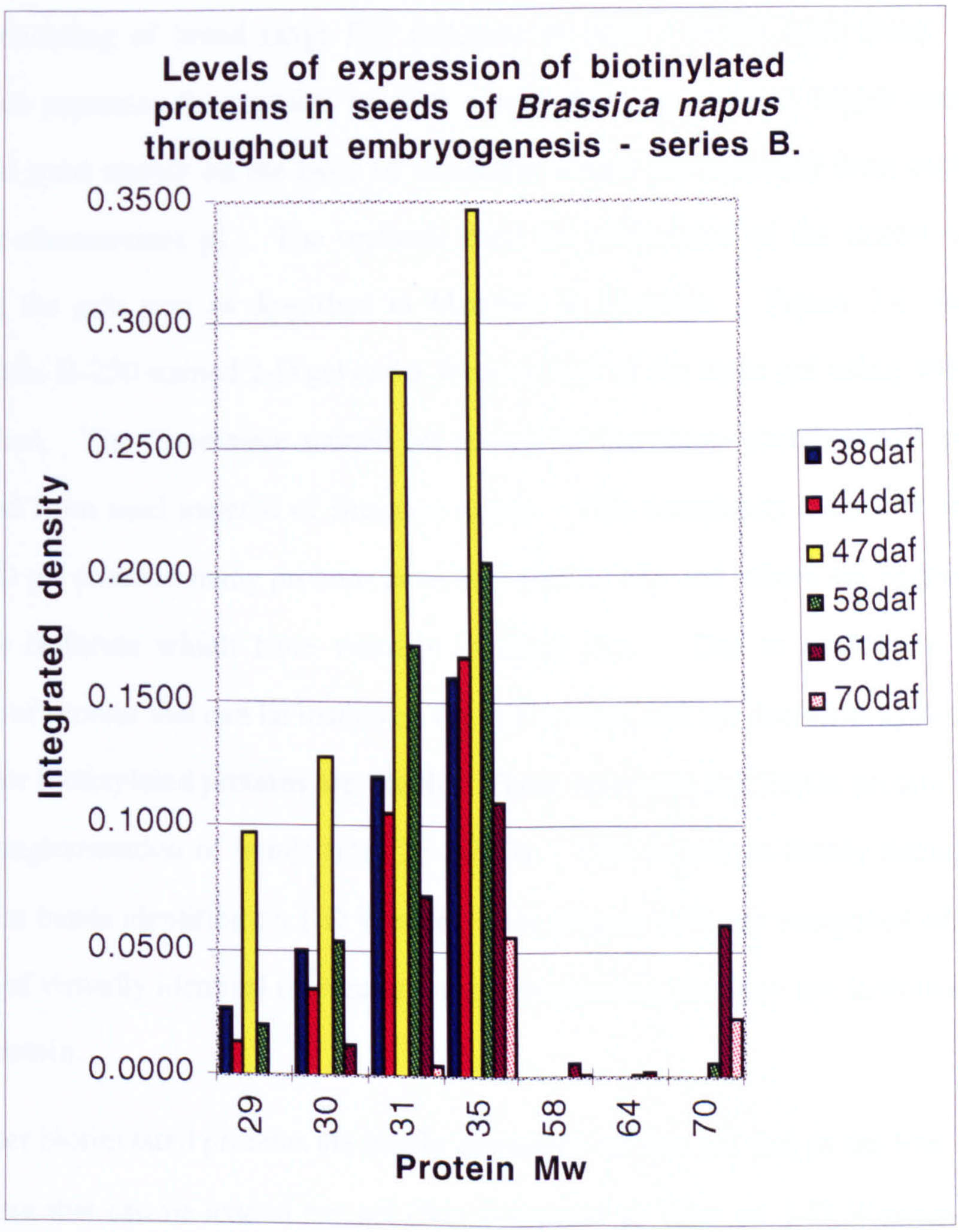


Figure 3.5 **B** Bar graph showing levels of expression of biotinylated proteins throughout embryogenesis in *Brassica napus*.

Proteins were extracted from maturing seeds according to protocol B and the Western is shown in Figure 3.4 B. The x-axis shows the molecular weight for proteins detected by the BIO-RAD Imaging Densitometer and calculated from the molecular weight standards. The y-axis shows the total integrated density measured. The columns for each molecular weight represent a different harvesting time for extraction and are coloured accordingly.

3.8 2-D analysis of biotinylated proteins

To build a final picture of the variety of biotinylated proteins in *Brassica napus* 2-D blots consisting of broad range IEF followed by SDS / PAGE were done. This technique separates the proteins in such a way that any proteins which cannot be resolved apart merely on the basis of molecular weight are separated from each other by their characteristic pI. The methods used for preparation of the extract and for running the gels was as described in Materials & Methods. Figure 3.6 shows a Coomassie R-250 stained 2-D gel and a Western blot of the same gel using anti-biotin antibodies. The Coomassie stained gel shows the enormous complexity of proteins extracted from seed material of *Brassica napus*. This complexity cannot be revealed by a 1-D gel alone as many proteins have very similar M_rs and others are expressed as multiple isoforms which have virtually identical M_rs. Due to constraints on the amount of protein that can be loaded on the IEF system and the detection system, only the major biotinylated proteins are visible. These represent the 35kDa protein and the other conglomeration of bands from 25-30kDa. This technique clearly demonstrates that these bands identified on 1-D Western blots (Figure 3.4) are composed of several protein of virtually identical molecular weight suggesting that they are isoforms of the same protein.

The other biotinylated proteins are barely detectable on this gel due to the low amount of protein that can be loaded per gel (5µg compared to 20µg on 1-D Western blots). The 76kDa protein is just visible at the negative end of the isoelectric dimension but has not focused well, presumably because it's pI is outside the pH gradient of the gel.

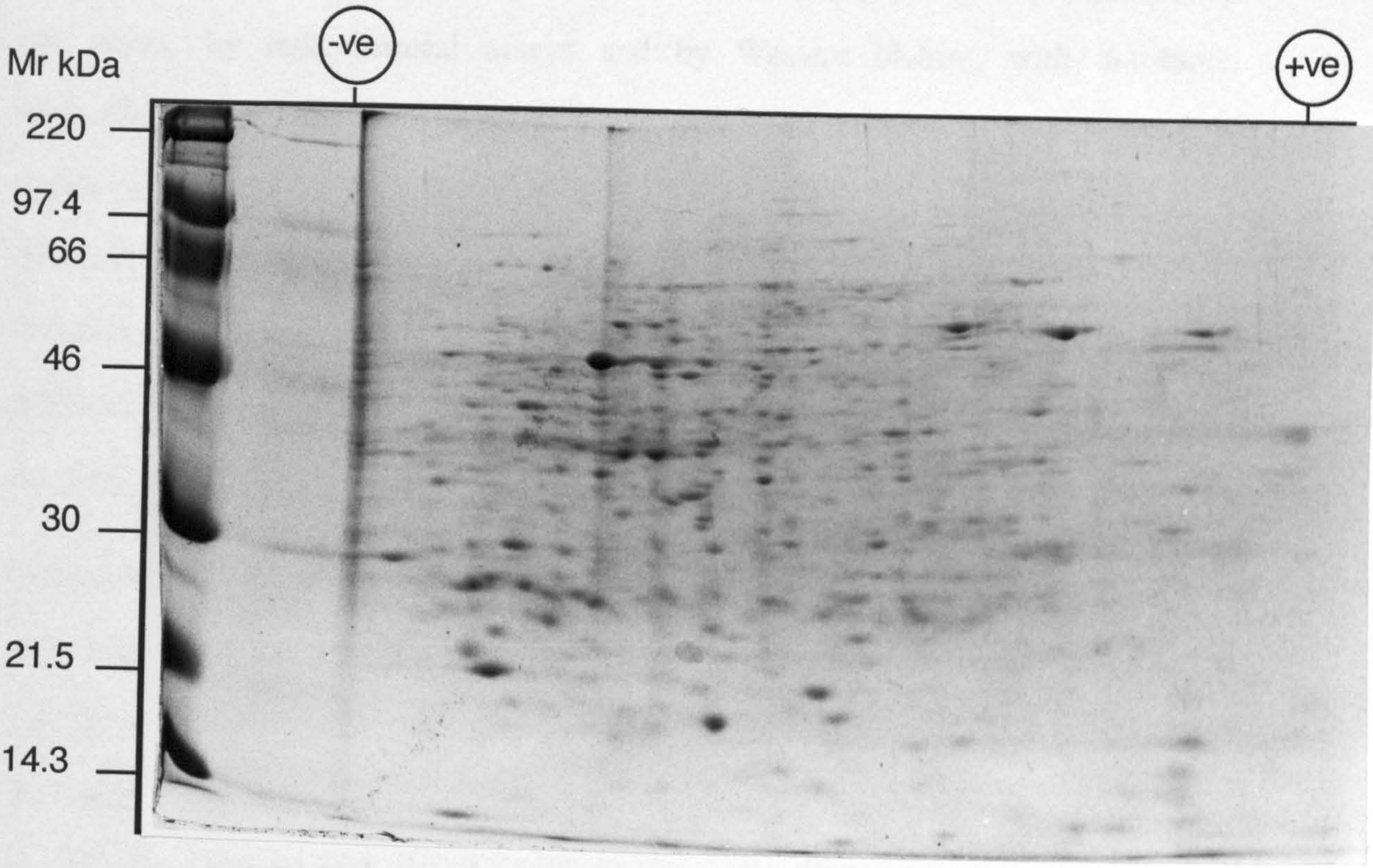
Figure 3.6 A 2-D analysis of total protein extract from *Brassica napus.*

A Coomassie stained gel after isoelectric focusing of proteins and then SDS / PAGE on a 12% gel. Proteins were extracted from embryos 44daf

Figure 3.6 **B** 2-D Western blot of total proteins from *Brassica napus.*

A duplicate gel to the one shown in Figure 3.6 A was blotted on to nitrocellulose and probed with affinity purified anti-biotin antibodies as previously described.

A


-ve
+ve
Mr kDa
220
97.4
66
46
30
21.5
14.3

B


+ve
-ve
Mr kDa
97.4
58.1
39.8
29.0
20.1
14.3

3.9 Discussion

In this chapter the biotin-containing enzymes in *Brassica napus* have been examined in two ways, by radiochemical assays and by Western blotting with anti-biotin antibodies. The enzyme activities for acetyl-CoA carboxylase, propionyl-CoA carboxylase, methylcrotonyl-CoA carboxylase and pyruvate carboxylase have all been detected. Identification of biotin-containing proteins using anti-biotin antibodies has revealed that *Brassica napus* contains multiple biotin-containing proteins which are differentially expressed in tissues throughout embryogenesis, some as multiple isoforms.

Initially enzyme assays were done to determine which of the known enzyme activities were present. ACCase, MCCase, PCCase and PCase activities were detected in a total protein extract of stored rape seeds. As the assays were essentially a repeat of the experiments previously reported (Wurtele & Nikolau, 1990). The linearity of the assays was assumed to be the same as previously reported and was therefore not determined. The assays reveal that MCCase is active and stable when stored under these conditions, as is pyruvate carboxylase. The amount of radioactivity incorporated into acid stable products when using β-methylcrotonyl-CoA as a substrate was of the same order of magnitude as previously reported, but the levels incorporated when using pyruvate as substrate were substantially greater. Nonetheless, activity was detected for each of the known plant biotin-dependent carboxylases, confirming previous observations (Wurtele & Nikolau, 1990) and demonstrating that our seed material contained these enzymes.

The measured level of propionyl-CoA carboxylase activity was of the same order of magnitude as ACCase activity. This would infer that the Type I ACCase (the 220kDa, single polypeptide ACCase), which is known to have a side activity towards propionyl-CoA (Egin-Bühler, 1983) is responsible for PCCase activity and most if not all of the ACCase activity. This suggests that the Type II (prokaryotic, multi-subunit form) ACCase activity was reduced, which could account for the large difference in

ACCase activity reported here compared to the previous report (Wurtele & Nikolau, 1990). A reduction in Type II ACCase could have been due to an instability of the multisubunit complex during long term storage or to the presence of inhibitors in the seed material. Type II ACCase has been shown to be susceptible to inhibition by an unknown compound when purified chloroplasts are lysed (Kannangara & Stumpf, 1972). Type II ACCase may have been stabilised by extraction into a high osmotic strength medium which has been reported to give higher levels of ACCase activity (Kang *et al.*, 1994) but this was not investigated.

Pyruvate carboxylase activity from *Brassica napus* var. Jet neuf was not induced by acetyl-CoA (Table 3.1), which suggests that pyruvate carboxylase in this organism is a Type II enzyme. Previous reports on pyruvate carboxylase failed to determine whether pyruvate carboxylase was a Type I (activity dependent on acetyl-CoA) or a Type II enzyme (activity not affected by metabolic intermediates) (Wurtele & Nikolau, 1990). The assays reported here show that in the presence of 300μM acetyl-CoA pyruvate carboxylase activity was not increased. Typical concentrations required for a 50% activation of Type II PCases are 2μM for the enzyme from chicken liver and 92μM for the enzyme from *S.cerevisiae* (Scrutton & Young, 1972). The only other known Type II PCase from a eukaryotic source is present in the cytosol not in the mitochondrion (Tozo *et al.*, 1992). The mitochondria of *Brassica napus* have not been examined for PCase activity nor has the number of biotinylated proteins contained within been examined. Mitochondria from *Pisum sativum* (pea) have been isolated and examined and were found to contain only one biotinylated protein, that of MCCase (Baldet *et al.*, 1992). It seems possible that pyruvate carboxylase in higher plants is a Type II enzyme located in the cytosol, similar to the case in fungi (Tozo *et al.*, 1992).

The demonstration of ACCase, MCCase, PCCase and PCase in total protein extracts of *B.napus* embryos confirms previous reports of multiple biotin-containing enzymes in higher plants (Wurtele & Nikolau, 1990). Previous reports have not revealed how

many biotin-containing proteins are present in *Brassica napus*. Using anti-biotin antibodies the number of biotinylated proteins has been assessed in developing embryos, leaf and root tissues. The anti-biotin antibodies have provided an excellent means of detecting biotin-containing proteins, and the affinity purification of them has since improved their specificity to the point where it is possible to say, with near certainty, that the bands detected are biotinylated proteins. Their distinct advantage over streptavidin is that they are more stable, more cost effective, give a much sharper concise band, and by using a secondary antibody conjugated to alkaline phosphatase a double amplification of the signal can be achieved. Using this detection system 10 proteins of distinct molecular weight have been detected in total protein extracts of *Brassica napus* seed material of 220 kDa, 150 kDa, 76 kDa, 71kDa, 65kDa, 61 kDa, 35kDa, 31kDa, 30kDa and 29kDa.. Several of these bands are made up of multiple isoforms as detected by IEF and SDS / PAGE.

The Western blots displayed in Figure 3.4 present a conclusive picture of the status of biotinylated proteins in developing seeds of *Brassica napus*. The only previously characterised biotinylated protein from *B. napus* was the 220kDa acetyl-CoA carboxylase (Slabas *et al.*, 1986). This protein can be seen on these blots which, unlike previous extraction procedures, used 0.5% Triton-X100 in the extraction protocol. This has previously been reported as a requirement for the 220kDa acetyl-CoA carboxylase to be solubilised (Hellyer *et al.*, 1986). Presumably this is either a result of the proteins large size or as a result of it being associated with biological membranes.

Densitometric analysis of the levels of these various proteins throughout embryogenesis shows that the major detected biotinylated protein is the 35kDa protein (Figure 3.5.B). Figure 3.5.A does not show the 31kDa, 30kDa and 29kDa bands as three separate bands. This is due to the high percentage of SDS in the sample. In such cases the SDS is thought to aggregate into micelles which, being negatively charged, run into the gel and affect the resolution of low molecular weight proteins.

There are some key differences between the two Western blots resulting from the difference in extraction procedures in 3.6.3.1 A and B, notably the levels of extracted protein (notice that the maximum peak in Figure 3.5.A is near a density of 0.6 and in Figure 3.5.B the maximum peak is 0.35). This is why in Figure 3.4.B the higher molecular weight bands are barely visible, whereas in Figure 3.4.A they can seen in more detail. This is due in part to the low efficiency of transfer of high molecular weight proteins (Hellyer *et al.*, 1986). This distorts the accuracy of the Western blot when comparing the level of high molecular weight proteins with low molecular weight proteins. The general pattern of expression of the other bands is the same in both Western blots. The dominant protein of 35kDa is induced to a maximum level at 47daf, in correlation with lipid biosynthesis, and fall to a lower level than at 38daf by the time the seeds are mature, >61daf The proteins below this molecular weight i.e. 29-31kDa, also follow this pattern of expression. This would suggest that these proteins are related to the 35kDa band, possibly as degradation products present *in vivo* or as truncated isoforms of that protein. It is interesting to note that in Figure 3.3 Lane 1 (proteins from isolated chloroplasts) only one biotinylated protein is visible. This has a molecular weight of 35kDa, no smaller 29-31kDa bands are visible nor is the 220kDa acetyl-CoA carboxylase. Taken at face value this can only mean that the 35kDa protein is part of acetyl-CoA carboxylase simply because no other biotinylated proteins are present and plastids must contain an acetyl-CoA carboxylase enzyme as they are the site for lipid biosynthesis (Weaire & Keckwick, 1975). This observation would seem to be in contradiction to previous reports that acetyl-CoA carboxylase is a 220kDa protein, both in leaf and in seed tissue. There are of course other possibilities to be considered, the protein could be some other biotin-containing protein in plastids or a proteolytic degradation product. It has been reported that the 220kDa acetyl-CoA carboxylase from *B. napus* was susceptible to proteolytic degradation, but that this could be reduced by rapid isolation (less than 9 hours) and the inclusion of PMSF in the isolation buffers (Hellyer *et al.*, 1986). The chloroplasts used in Figure 3.3, lane 1 were isolated from fresh tissue within an hour, as were the

proteins for the Western blots in Figure 3.4 which would seem to discount the proteolysis argument. The exact identity of the 35kDa protein in chloroplasts of oilseed rape has recently been discerned as the biotin carboxyl carrier protein (Elborough *et al.*, 1996), a subunit of a prokaryotic form of acetyl-CoA carboxylase. Interestingly, this protein certainly seems to be expressed as multiple isoforms. The 2-D blot in Figure 3.6 clearly shows at least three proteins separated by isoelectric point with a M_r of 35kDa. How these proteins are related to each other is an area for further research. The relationship between the biotinylated 35kDa protein and the 29kDa, 30kDa and 31kDa proteins also remains to be discerned. Their pattern of expression indicates an involvement with lipid biosynthesis as they are clearly regulated to coincide with lipid biosynthesis in seeds (mid embryogenesis).

The other major biotinylated proteins in seeds of *Brassica napus* would appear to not be linked to lipid biosynthesis as they are induced late in seed development, after lipid biosynthesis would have occurred and when the seed would be starting to mature and desiccate. This suggests they may have a role in catabolism as opposed to anabolism like the 35kDa protein. Although these proteins have not been identified directly in *Brassica napus*, similar proteins have been identified in other plants including *Arabidopsis*.

The 76kDa protein is probably MCCase which has been detected in carrot, M_r 78,000 (Chen, *et al.*, 1993), potato, M_r 74,000 (Alban *et al.*, 1993), soybean, M_r 85,000 (Song, *et al.*, 1994), tomato, M_r 76,000 (Wang *et al.*, 1994), *Arabidopsis*, M_r 78,000 (Weaver *et al.*, 1995) and mitochondria of pea, M_r 76,000 (Alban *et al.*, 1993). This protein has a role in leucine catabolism which could be associated with the desiccation of the seed and the degradation of cell proteins. This is consistent with the increased amounts of protein detected in late stage embryos. This protein is also detected in rape leaf. The identity of the 71kDa protein below it is not known. It is possible that both of these are MCCase and one isoform is specifically expressed in late embryos of

oilseed rape. Two genes for MCCase have been isolated from tomato (Wang *et al.*, 1994).

The protein band at approximately 61 kDa in Figure 3.4 B may be the equivalent of the biotinylated storage protein detected in pea (Duval *et al.*, 1994). This protein was specifically induced late in embryogenesis and had a molecular weight of 65,000 on SDS / PAGE. Other bands visible at around 50 and 65kDa are of unknown origin and may be proteolytic degradation products as the 65 kDa band is visible on in Figure 3.4 A, but not B where the extract was done into hot SDS.

In conclusion, activity of biotin-dependent carboxylases has been demonstrated in maturing seeds of *Brassica napus*. It has also been demonstrated that seeds of *Brassica napus* contain multiple biotin-containing proteins which are differentially expressed throughout embryogenesis and in different tissues. The identity of several of these biotinylated proteins has already been deciphered, but others remain as yet unidentified. This presents a complicated picture for these enzymes some of which are undoubtedly central to plant biochemistry.

Chapter 4: Characterisation of novel biotinylated proteins of *Arabidopsis thaliana*.

4.1 Introduction

A possible method for directly isolating cDNAs which encode for biotinylated proteins would be to screen an expression library with a biotin detecting reagent such as avidin or anti-biotin antibodies.

It has been demonstrated that when screening expression libraries with a biotinylated primary probe and avidin or streptavidin as a secondary probe, positive plaques may be isolated which will still record positive even when the primary probe is omitted (Collins *et al.*, 1987 Wang *et al.*, 1993). These artefacts are expressing phage which encode biotinylated proteins. One such protein has been isolated and sequenced (Hoffman *et al.*, 1987).

Dr. A.S.N. Reddy from the University of Colorado had been screening an expression library using biotinylated calmodulin as a primary probe and avidin conjugated to horse radish peroxidase as a secondary probe, in order to detect calmodulin binding proteins. The discovery of phage amongst his positives which were able to react to the avidin conjugate without first incubating with biotinylated calmodulin suggested that these clones did not encode calmodulin binding proteins, but probably encoded for biotinylated proteins. In a collaboration, these cDNAs were given to Professor Slabas to determine the nature of these unknown clones which possibly encoded for biotinylated proteins.

4.2 Aims

Sixteen cDNA clones had been isolated which were able to react with the avidin-horse radish peroxidase conjugate directly without first being incubated with biotinylated calmodulin. The aim of the collaboration was to identify which clones were unique, to determine the primary sequence of unique cDNA clones to facilitate identification, and finally to characterise the biotinylated proteins encoded.

4.3 Restriction analysis of cDNA clones

Several of the clones had already been partially sequenced and a restriction map determined for those sequenced. Table 4.1 summarises the identification system used for the clones, how many there were, and what was known about them upon reception. The clones were identified by a number and the prefix pBC was added meaning "plasmid Biotin Clone".

Clone identification	*Plasmid rescued*	*Restriction map*	*Sequenced forward*	*Sequenced reverse*
pBC 1.1	No			
pBC 1.2	Yes	Yes	Yes	No
pBC 1.3	No			
pBC 1.4	No			
pBC 1.5	No			
pBC 1.6	No			
pBC 2.1	No			
pBC 2.2	No			
pBC 2.3	No			
pBC 2.4	Yes	Yes	No	No
pBC 2.5	Yes	Yes	Yes	Yes
pBC 2.6	No			
pBC 3.2	Yes	Yes	Yes	No
pBC 3.3	No			
pBC 3.4	No			
pBC 3.5	No			

Table 4.1 Status upon receiving the biotin-containing clones.

All clones were isolated to plaque purity before receiving them, by directly screening with avidin conjugated to alkaline phosphatase. Sequencing was done with M13 forward and reverse primers.

The sequences in the forward direction provided with the clones, were analysed using DNAStrider. DNA matrices of the sequences showed that they had areas of identical sequence. Figure 4.1 and Figure 4.2 show where these sequences have similarity.

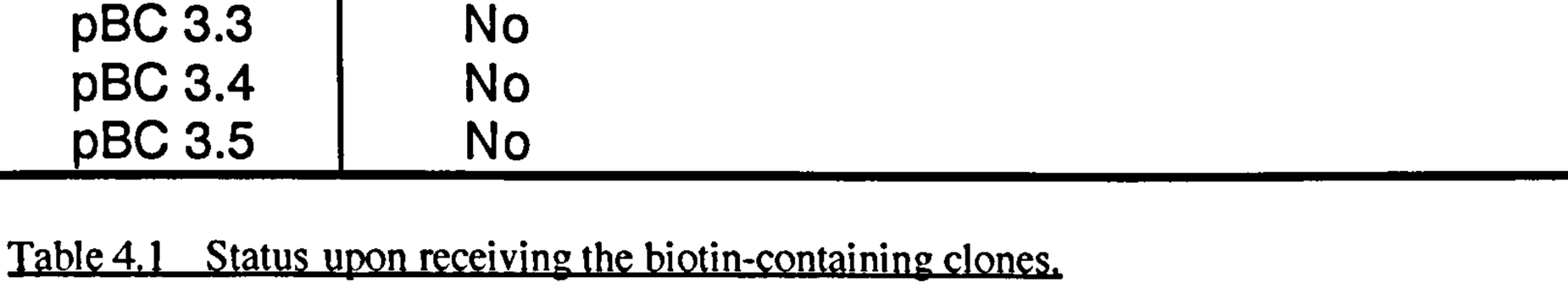


Figure 4.1 Graphical alignment map of pBC clones previously sequenced in the forward direction.

Alignments were made using the DNASTAR SEQMAN program.

```
1                                                                   50
1.2  ATCAATTTTA TTTTATGGAA ATGAACACCC GTCTACAGGT CGAACATCCT
3.2  .......... .......... .......... .......... ..........
2.5  .......... .......... .......... .......... ..........

51                                                                 100
1.2  GTAACTGAGA TGATTGTTGG TCAAGATCTT GTGGAATGGC AAATTCGGGT
3.2  .......... .......... .......... .......... .........T
2.5  .......... .......... .......... .......... ..........

     101                                                           150
1.2  TGCCAATGGG GAACCTCTCC CCTTAAGCCA ATCCGAGGTG CCAATGTCAG
3.2  TGGCTTAAGG GGAGAGGTTC CCCATTGGCA ACCCGAGGTG CCAATGTCAG
2.5  .......... .......... .......... .......... ..........

     151                                                           200
1.2  GTCATGCCTT TGAGGCCAGG ATATATGCTG AAAACGTTCC AAAAGGATTT
3.2  GTCATGCCTT TGAGGCCAGG ATATATGCTG AAAACGTTCC AAAAGGATTT
2.5  .......... .......... .......... .......... ..........

     201                                                           250
1.2  CTTCCTGCAA CTGGGGTCCT CAATCATTAT CGCCCTGTTG CAGTCTCACC
3.2  CTTCCTGCAA CTGGGGTCCT CAATCATTAT CGCCCTGTTG CAGTCTCACC
2.5  .......... .......... .......... .......... ..........

     251                                                           300
1.2  ATCAGTTCGG GTTGAAACTG GAGTTGAGCA AGGAGACACT GTTAGCATGC
3.2  ATCAGTTCGG GTTGAAACTG GAGTTGAGCA AGGAGACACT GTTAGCATGC
2.5  .......... .......... .......... .......... ..........

     301                                                           350
1.2  ACTATGATCC TATGATTGCA AAGCTTGTTG TCTGGGGAGG TAATCG....
3.2  ACTATGATCC TATGATTGCA AAGCTTGTTG TCTGGGGAGG TAATCGTGGC
2.5  .......... .......... .......... .......... .........GC

     351                                                           400
1.2  .......... .......... .......... .......... ..........
3.2  GAAGCTTTAG TGAAACTGAA GGATTGCTTG TCTAACTTTC AGGTAGCAGG
2.5  GAAGCTTTAG TGAAACTGAA GGATTGCTTG TCTAACTTTC AGGTAGCAGG

     401                                                           450
1.2  .......... .......... .......... .......... ..........
3.2  TGTACCTACG AACATAAATT TCCTTCAAAA ACTTGCTAGT CATAAGGAGT
2.5  TGTACCTACG AACATAAATT TCCTTCAAAA ACTTGCTAGT CATAAGGAGT

     451                                                           500
1.2  .......... .......... .......... .......... ..........
3.2  TTG ...... .......... .......... .......... ..........
2.5  TTGCAGTGGG CAATGTAGAA ACTCATTTTA TTGAGCACCA TAAAAGTGAT

     501                                                           550
1.2  .......... .......... .......... .......... ..........
3.2  .......... .......... .......... .......... ..........
2.5  CTATTTGCTG ACGAAAGCAA TCCAGCTCAA CAGAAGTGGC ATACAAGGCA

     551                                                           600
1.2  .......... .......... .......... .......... ..........
3.2  .......... .......... .......... .......... ..........
2.5  GTCAAGCATA GTGCAGCATT GGTGGCTGCT TGTATCTCCA CAATCGAGCA

     601                                  633
1.2  .......... .......... .......... ...
3.2  .......... .......... .......... ...
2.5  TTCTACTTGG AATGAAAGTA ATCATGGGAA AGT
```

Figure 4.2 Alignment of the sequences obtained by sequencing from the forward primer of pSK- for pBC1.2, pBC3.2 and pBC2.5 using the PILEUP program from the Wisconsin Package GCG.

The clones that has already been sequenced, had also been analysed by restriction mapping. Figure 4.3 is a graphical illustration of the restriction map for the sequenced clones. The far left EcoR1 site and the far right Xho I site are the cloning sites.

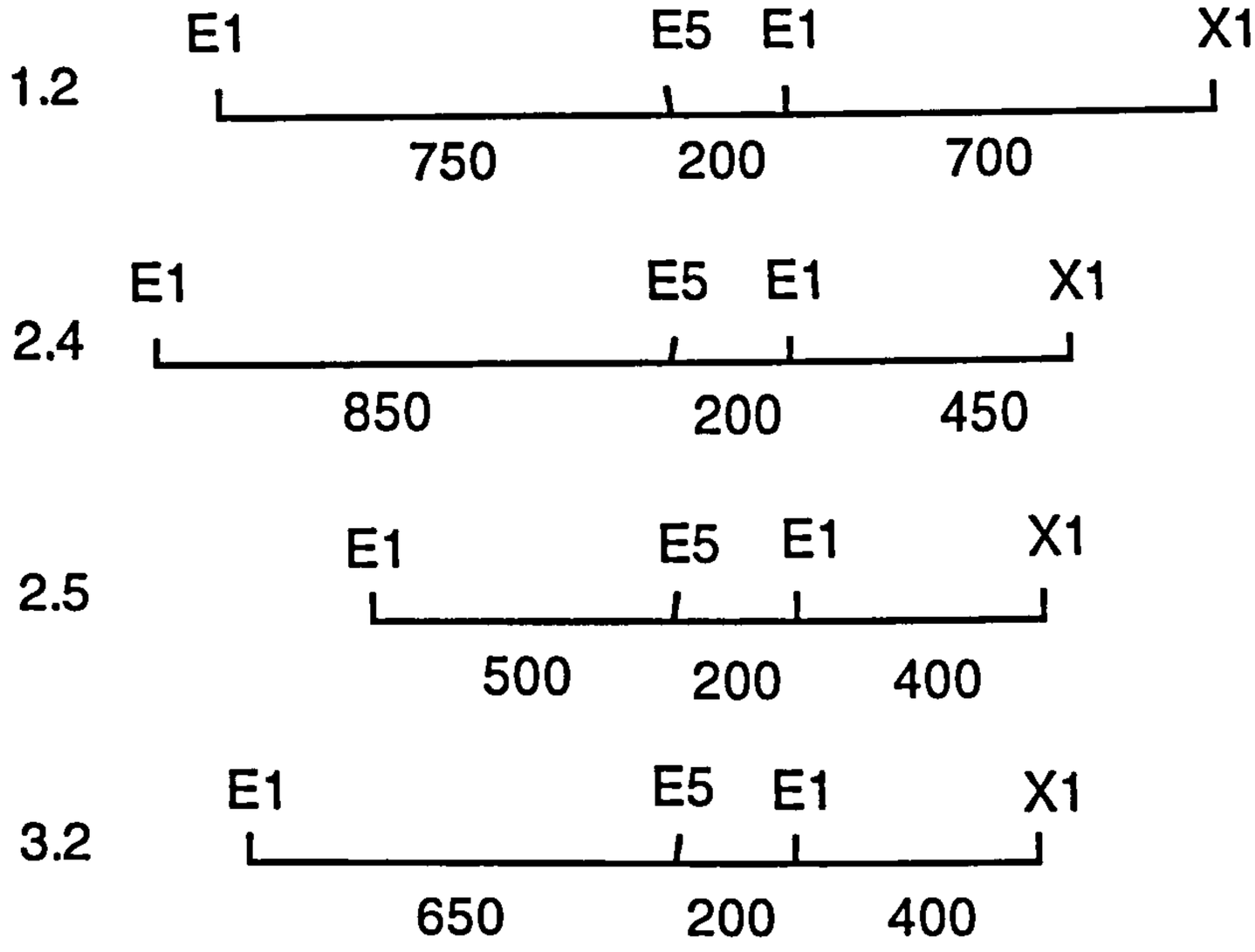


Figure 4.3 Graphical illustration of the restriction maps of clones previously analysed.

The 200bp EcoR1-EcoRV fragment is characteristic of this class of clone. The symbols represent; E1, Eco RI; E5, Eco RV; X1, Xho 1.

The sequence data shows that these clones are similar if not identical. In order to ascertain whether any of the remaining clones were the same as the three already sequenced, a restriction map of all the clones was made. The purified phage were amplified to $>10^9$pfu/ml and plasmid rescued. Bacteria transformed with the rescued plasmids were grown up in liquid culture overnight and plasmid DNA isolated from the cells by the mini-prep isolation method. Approximately 1 - 2µg of plasmid DNA from each preparation was digested with 1U each of Eco RI, Eco RV and Xho I and 37°C for 4 hours. The digested plasmids were separated by electrophoresis in agarose gels as shown in Figure 4.4. A characteristic of the clones examined to date was a 200bp Eco R1 - Eco RV fragment in the middle of the clones. This provided a marker for identification of similar clones.

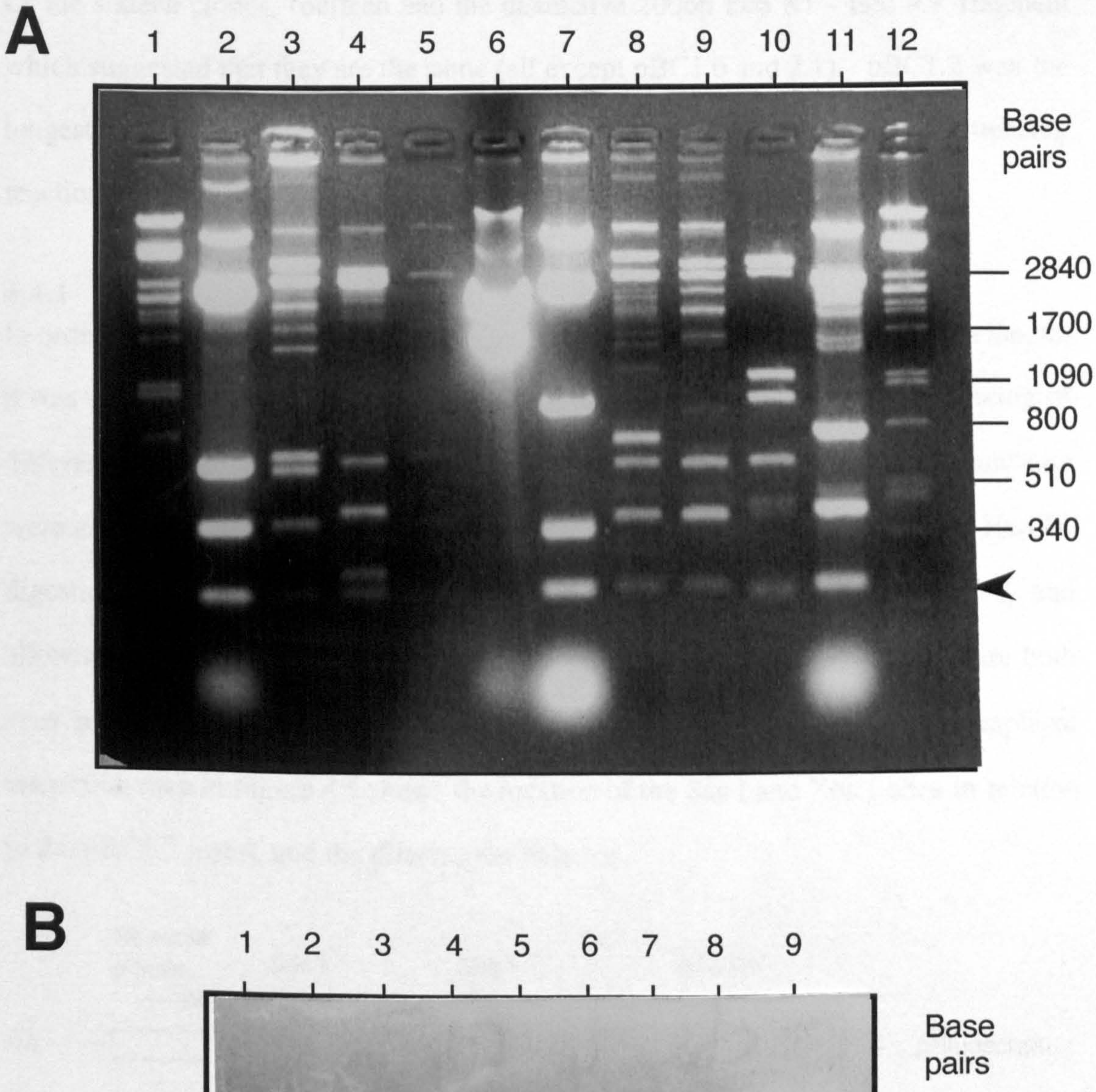
Figure 4.4 A and B showed that every isolated clone, except pBC1.6 and 2.1 (Lanes 4 and 5 in Figure 4.4 B) contained a 200bp fragment when cut with Eco RI, Eco RV and Xho I. The possibility that this band could be from a different class of clones with two restriction sites for the same enzyme 200bp apart, was eliminated by digesting all the plasmids with Xho I, Eco RI and Eco RV separately (data not shown). The 200bp band was not produced in any of these digests and was only produced when Eco RI and Eco RV were included together in the digest. This implied that most of the clones were the same. The clones were therefore separable into two classes based upon their restriction patterns, those that produced a 200bp band when cut with Eco RI and Eco RV, and those that did not. Those that did contain a 200bp Eco RI - Eco RV fragment were analysed first as they made up the majority of the isolated clones.

Figure 4.4 A Analysis of pBC plasmids cut with Eco RI, Eco RV and Xho I separated on a 1% agarose gel.

Diagnostic of the class of clones previously identified is the 200bp Eco RI - Eco RV band. Lanes show restriction digests of the following; Lane 2, pBC 1.1; Lane 3, pBC 1.3; Lane 4, pBC 1.4; Lane 5, pBC 1.5; Lane 6, pBC 2.1; Lane 7, pBC 2.2; Lane 8, pBC 2.3; Lane 9, pBC 2.6; Lane 10, pBC 3.4; Lane 11, pBC 3.5. Lanes 1 and 12 contain 1μg of λPst I markers. The arrow marks the position of the diagnostic 200bp band.

Figure 4.4 B Analysis of pBC plasmids cut with Eco RI, Eco RV and Xho I separated on a 0.7% agarose gel.

Lanes show restriction digests of the following; Lane 1, pBC1.1; Lane 2, pBC1.3; Lane 3, pBC1.5; Lane 4, pBC1.6; Lane 5, pBC2.1; Lane 6, pBC3.2; Lane 7, pBC3.3. Lane 9 contains 1μg of λPst I markers. The arrow marks the position of the diagnostic 200bp band.


A
1 2 3 4 5 6 7 8 9 10 11 12
Base pairs
2840
1700
1090
800
510
340
B
1 2 3 4 5 6 7 8 9
Base pairs
1160
800
510
340

4.4 Sequencing of pBC1.2

Of the sixteen clones, fourteen had the distinctive 200bp Eco R1 - Eco RV fragment which suggested that they are the same (all except pBC1.6 and 2.1). pBC1.2 was the longest clone of this class and was therefore used as a template for sequencing reactions in order to determine the full sequence.

4.4.1 Nested deletions

In order to sequence the whole of pBC1.2 which was approximately 1700bp in length, it was necessary to create a set of nested deletions which would allow sequencing of different parts within the clone. A combination of Exo III nuclease and S1 nuclease were used to create deletions. The strategy used was to protect the DNA from Exo III digestion in the undesired direction (into the vector) by restriction with Sac I, and allowing deletions to proceed into the insert by restriction with Xba I. These are both sites present in the multiple cloning site of pBluescript™ SK-. The graphical restriction map in Figure 4.5 shows the location of the Sac I and Xba I sites in relation to the pBC1.2 insert, and the direction of deletion.

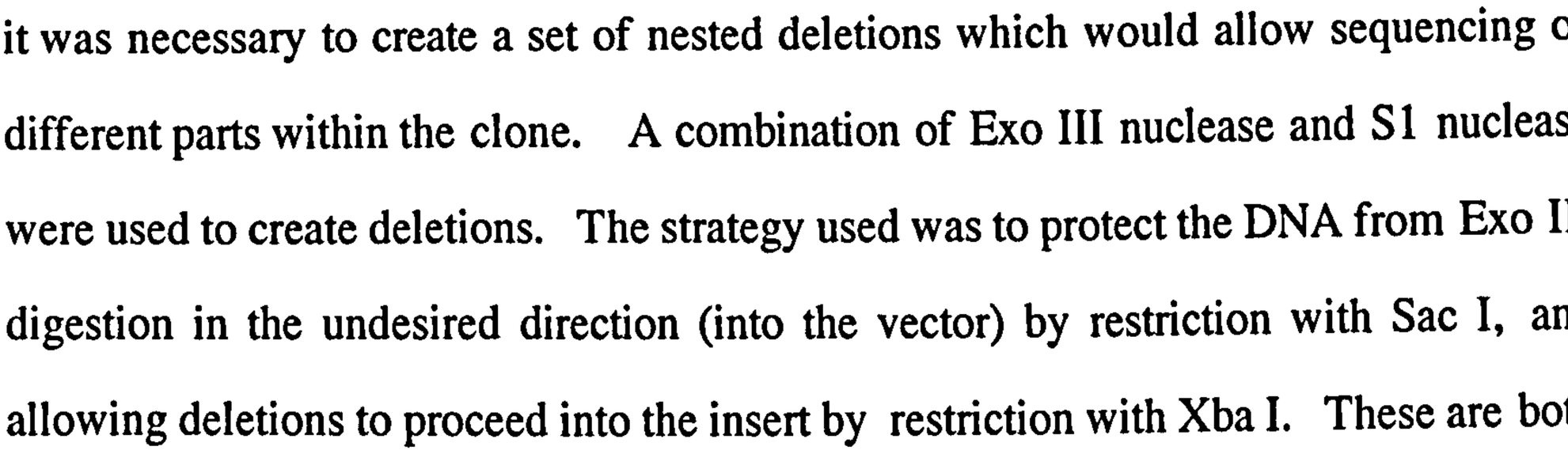


Figure 4.5 Position of restriction sites used to allow directional deletion of pBC1.2.

The Sac I and Xba I sites are located in the plasmid MCS up from the Eco RI sites used for insertion of the cDNA. The arrows show the direction that deletion will occur due to the fact that Sac I presents a 3' overhang which prevents Exo III digestion, whereas Xba I presents a 5' overhang which will allow deletion. Both enzyme sites are absent from the 1.2 sequence. The Eco RI site marks the start of the insert.

A set of nested deletions was created by digesting 3μg of pBC1.2 DNA with 3U each of Sac I and Xho I for 16 hours at 37°C. Deletions were then made with the PHARMACIA Nested Deletions Kit. Nested deletions were analysed for the extent of

deletion of DNA by transforming the deleted and re-ligated DNA into *E.coli* DH5α cells and preparing plasmid DNA from colonies obtained by the mini-prep method. The size of the insert was analysed by restriction of the DNA obtained with Xho I which would linearise the plasmid DNA and separating the linearised plasmids on an agarose gel. The size of the complete linearised plasmid in such a digest would have been approximately 4,600bp. A sequence of linearised plasmids which were less than 4,600bp were sequenced to build up data from various positions throughout the clone.

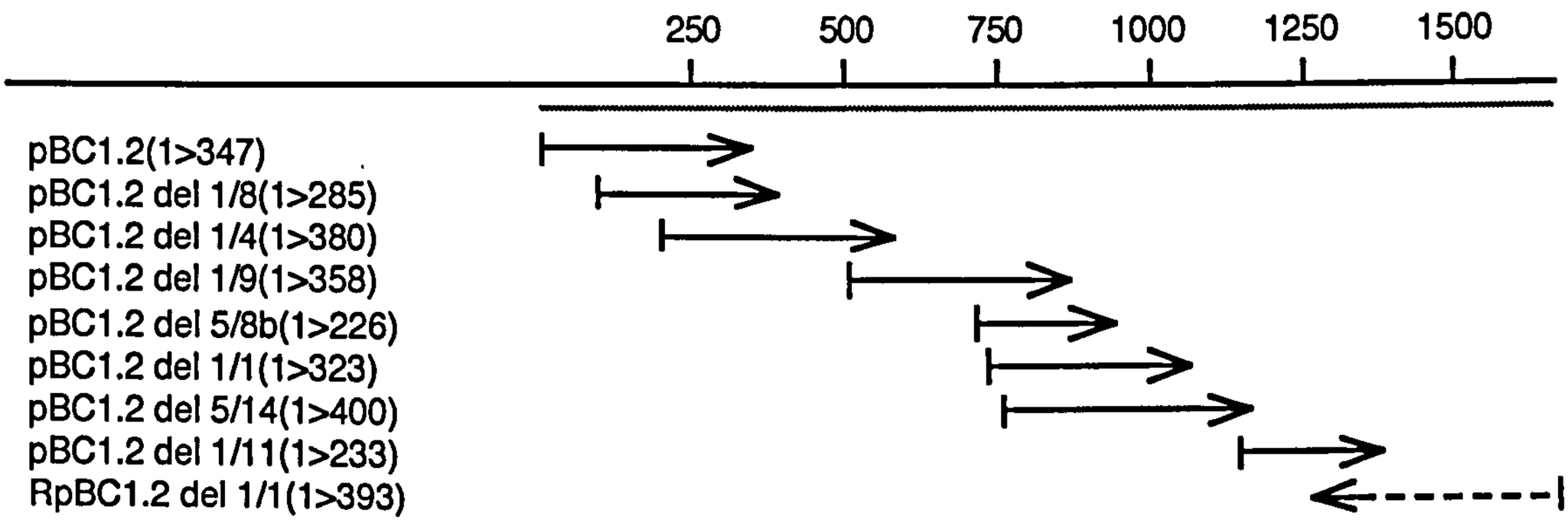


Figure 4.6 Sequence strategy for pBC1.2.

Various deletions were sequenced from the reverse primer of SK - into the deleted region of the 1.2 insert. The final 300bp were obtained by sequencing the complementary strand using the M13 forward primer. Overlapping sequence was assembled into a final sequence using the DNASTAR SEQMAN program. The figure shows the deletion identity on the left. The insert sequence starts from a vertical bar (|) and continues to the end of the arrow.

The full sequence of pBC1.2 compiled by the DNAStar Seqman program form overlapping clones in one direction only. The full sequence and the deduced protein sequence is presented in Figure 4.7. The DNA was not sequenced in both directions as the initial sequence results were used to search Genbank. These results are presented in Figure 4.8 and show that the sequence for this cDNA had just been deposited in the database. Previous searches with the partial sequence had not picked up anything with strong homology.

4.4.2 Sequence of pBC1.2

```
1/1                                                 31/11
*AT CAA TTT TAT TTT ATG GAA ATG AAC ACC CGT CTA CAG GTC GAA CAT CCT GTA ACT GAG
 X   Q   F   Y   F   M   E   M   N   T   R   L   Q   V   E   H   P   V   T   E
61/21                                               91/31
ATG ATT GTT GGT CAA GAT CTT GTG GAA TGG CAA ATT CGG GTT GCC AAT GGG GAA CCT CTC
 M   I   V   G   Q   D   L   V   E   W   Q   I   R   V   A   N   G   E   P   L
121/41                                              151/51
CCC TTA AGC CAA TCC GAG GTG CCA ATG TCA GGT CAT GCC TTT GAG GCC AGG ATA TAT GCT
 P   L   S   Q   S   E   V   P   M   S   G   H   A   F   E   A   R   I   Y   A
181/61                                              211/71
GAA AAC GTT CCA AAA GGA TTT CTT CCT GCA ACT GGG GTC CTC AAT CAT TAT CGC CCT GTT
 E   N   V   P   K   G   F   L   P   A   T   G   V   L   N   H   Y   R   P   V
241/81                                              271/91
GCA GTC TCA CCA TCA GTT CGG GTT GAA ACT GGA GTT GAG CAA GGA GAC ACT GTT AGC ATG
 A   V   S   P   S   V   R   V   E   T   G   V   E   Q   G   D   T   V   S   M
301/101                                             331/111
CAC TAT GAT CCT ATG ATT GCA AAG CTT GTT GTC TGG GGA GGT AAT CGT GGC GAA GCT TTA
 H   Y   D   P   M   I   A   K   L   V   V   W   G   G   N   R   G   E   A   L
361/121                                             391/131
GTG AAA CTG AAG GAT TGC TTG TCT AAC TTT CAG GTA GCA GGT GTT CCT ACG AAC ATA AAT
 V   K   L   K   D   C   L   S   N   F   Q   V   A   G   V   P   T   N   I   N
421/141                                             451/151
TTC CTT CAA AAA CTT GCT AGT CAT AAG GAG TTT GCA GTG GGC AAT GTA GAA ACT CAT TTT
 F   L   Q   K   L   A   S   H   K   E   F   A   V   G   N   V   E   T   H   F
481/161                                             511/171
ATT GAG CAC CAT AAA AGT GAT CTA TTT GCT GAC GAA AGC AAT CCA GCT GCA ACA GAA GTG
 I   E   H   H   K   S   D   L   F   A   D   E   S   N   P   A   A   T   E   V
541/181                                             571/191
GCA TAC AAG GCA GTC AAG CAT AGT GCA GCA TTG GTG GCT GCT TGT ATC TCC ACA ATC GAG
 A   Y   K   A   V   K   H   S   A   A   L   V   A   A   C   I   S   T   I   E
601/201                                             631/211
CAT TCT ACT TGG AAT GAA AGT AAT CAT GGG AAA GTT CCA TCG ATA TGG TAT TCG AAT CCT
 H   S   T   W   N   E   S   N   H   G   K   V   P   S   I   W   Y   S   N   P
661/221                                             691/231
CCT TTT AGG GTC CAT CAT GAA GCC AAA CAA ACC ATT GAG CTA GAA TGG AAT AAT GAA TGC
 P   F   R   V   H   H   E   A   K   Q   T   I   E   L   E   W   N   N   E   C
721/241                                             751/251
GAG GGA ACT GGC TCT AAC CTC ATA TCA CTC GGT GTA AGA TAT CAA CCA GAT GGA AGC TAT
 E   G   T   G   S   N   L   I   S   L   G   V   R   Y   Q   P   D   G   S   Y
781/261                                             811/271
CTC ATT GAG GAA GGC AAT GAT TCT CCA AGT TTA GAA CTC AGA GTA ACA CGA GCA GGA AAG
 L   I   E   E   G   N   D   S   P   S   L   E   L   R   V   T   R   A   G   K
841/281                                             871/291
TGC GAT TTT AGA GTT GAA GCG GCC GGA TTG AGC ATG AGT GTT AGT CTA GCT GCG TAC TTG
 C   D   F   R   V   E   A   A   G   L   S   M   S   V   S   L   A   A   Y   L
901/301                                             931/311
AAG GAT GGT TAT AAG CAT ATC CAT ATA TGG CAT GGT TCA GAA CAC CAT CAG TTC AAG CAG
 K   D   G   Y   K   H   I   H   I   W   H   G   S   E   H   H   Q   F   K   Q
961/321                                             991/331
AAG GTA GGA ATC GAA TTC TCT GAA GAT GAA GAA GGT GTC CAA CAC AGA ACC AGC TCC GAA
 K   V   G   I   E   F   S   E   D   E   E   G   V   Q   H   R   T   S   S   E
1021/341                                            1051/351
ACA TCA TCA CAC CCT CCA GGA ACC ATT GTG GCC CCC ATG GCT GGT TTG GTA GTC AAG GTC
 T   S   S   H   P   P   G   T   I   V   A   P   M   A   G   L   V   V   K   V
1081/361                                            1111/371
CTT GTC GAA AAC GAA GCC AAA GTA GAT CAA GGT CAA CCT ATA TTA GTC CTA GAG GCA ATG
 L   V   E   N   E   A   K   V   D   Q   G   Q   P   I   L   V   L   E   A   M
1141/381                                            1171/391
AAG ATG GAG CAC GTT GTG AAA GCA CCA TCC TCT GGA AGC ATA CAG GAC CTC AAA GTT AAG
 K   M   E   H   V   V   K   A   P   S   S   G   S   I   Q   D   L   K   V   K
1201/401                                            1231/411
GGC CAA CAG GTT TCA GAT GGC AGT GCT CTA TTC AGG ATC AAA GGG TAA ATA ACA CGA TTT
 G   Q   Q   V   S   D   G   S   A   L   F   R   I   K   G   *
GCG ATT GAT CAA CCA TTC GCT GGT TAC GCG AAA CCT GAA CCT AAT AAG CAT TTG GGT GTG
TAT ATA AAC CGA AAT CTT ACA AAA GAT AAA TAA ATC CAA AGC TTT ATA AAT GTT TTT ACT
GCC TCT ATA TAG ATC TGT GCG GCT AGA GAT TGA AGG TAA CTT CTT AGT AAT TCG AAA TGG
TGA TTA CAT TAA TAT TCA AGA TAG TCC GAG CCA TTT CTT GAA TAC TAA ACC AGC TTC TCT
CTT CTT GAG CTG AGA CTC GCT CTG AAC TTT TGG AGC TTT GCC TTT ATA AGC AGT CTT GTG
ATA CTT CAA GAA GAG CAT CCG ATA CTT GTA CTT ATC AAC AAA CAT ATT TTT TTT CTC CAT
CCA AAA AAA AAA AAA AAA AAA
```

Figure 4.7 Entire sequence of pBC1.2. The conserved biotinylation site EAMKM is underlined.

```
DE   Arabidopsis thaliana 3-methylcrotonyl-CoA carboxylase precursor
SCORES:            99.5% identity in 634 base pair overlap

                                                      10        20        30
        pBC1.2                                      ATCAATTTTATTTTATGGAAATGAACACCC
                                                    ||||||||||||||||||||||||||||||
        at1253 CGGTGGAATTTATAGTTGACACCGAGTCTGATCAATTTTATTTTATGGAAATGAACACCC
                   880       890       900       910       920       930
                       40        50        60        70        80        90
        pBC1.2 GTCTACAGGTCGAACATCCTGTAACTGAGATGATTGTTGGTCAAGATCTTGTGGAATGGC
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
        at1253 GTCTACAGGTCGAACATCCTGTAACTGAGATGATTGTTGGTCAAGATCTTGTGGAATGGC
                   940       950       960       970       980       990
                      100       110       120       130       140       150
        pBC1.2 AAATTCGGGTTGCCAATGGGGAACCTCTCCCCTTAAGCCAATCCGAGGTGCCAATGTCAG
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
        at1253 AAATTCGGGTTGCCAATGGGGAACCTCTCCCCTTAAGCCAATCCGAGGTGCCAATGTCAG
                  1000      1010      1020      1030      1040      1050
                      160       170       180       190       200       210
        pBC1.2 GTCATGCCTTTGAGGCCAGGATATATGCTGAAAACGTTCCAAAAGGATTTCTTCCTGCAA
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
        at1253 GTCATGCCTTTGAGGCCAGGATATATGCTGAAAACGTTCCAAAAGGATTTCTTCCTGCAA
                  1060      1070      1080      1090      1100      1110
                      220       230       240       250       260       270
        pBC1.2 CTGGGGTCCTCAATCATTATCGCCCTGTTGCAGTCTCACCATCAGTTCGGGTTGAAACTG
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
        at1253 CTGGGGTCCTCAATCATTATCGCCCTGTTGCAGTCTCACCATCAGTTCGGGTTGAAACTG
                  1120      1130      1140      1150      1160      1170
                      280       290       300       310       320       330
        pBC1.2 GAGTTGAGCAAGGAGACACTGTTAGCATGCACTATGATCCTATGATTGCAAAGCTTGTTG
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
        at1253 GAGTTGAGCAAGGAGACACTGTTAGCATGCACTATGATCCTATGATTGCAAAGCTTGTTG
                  1180      1190      1200      1210      1220      1230
                      340       350       360       370       380       390
        pBC1.2 TCTGGGGAGGTAATCGTGGCGAAGCTTTAGTGAAACTGAAGGATTGCTTGTCTAACTTTC
               ||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
        at1253 TCTTGGGAGGTAATCGTGGCGAAGCTTTAGTGAAACTGAAGGATTGCTTGTCTAACTTTC
                  1240      1250      1260      1270      1280      1290
                      400       410       420       430       440       450
        pBC1.2 AGGTAGCAGGTGTACCTACGAACATAAATTTCCTTCAAAAACTTGCTAGTCATAAGGAGT
               |||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||
        at1253 AGGTAGCAGGTGTTCCTACGAACATAAATTTCCTTCAAAAACTTGCTAGTCATAAGGAGT
                  1300      1310      1320      1330      1340      1350
                      460       470       480       490       500       510
        pBC1.2 TTGCAGTGGGCAATGTAGAAACTCATTTTATTGAGCACCATAAAAGTGATCTATTTGCTG
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
        at1253 TTGCAGTGGGCAATGTAGAAACTCATTTTATTGAGCACCATAAAAGTGATCTATTTGCTG
                  1360      1370      1380      1390      1400      1410
                      520       530       540       550       560       569
        pBC1.2 ACGAAAGCAATCCAGCT-CAACAGAAGTGGCATACAAGGCAGTCAAGCATAGTGCAGCAT
               ||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
        at1253 ACGAAAGCAATCCAGCTGCAACAGAAGTGGCATACAAGGCAGTCAAGCATAGTGCAGCAT
                  1420      1430      1440      1450      1460      1470
             570       580       590       600       610       620       629
        pBC1.2 TGGTGGCTGCTTGTATCTCCACAATCGAGCATTCTACTTGGAATGAAAGTAATCATGGGA
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
        at1253 TGGTGGCTGCTTGTATCTCCACAATCGAGCATTCTACTTGGAATGAAAGTAATCATGGGA
                     1480      1490      1500      1510      1520      1530
              630
         pBC1.2 AAGT
                ||||
        at1253 AAGTTCCATCGATATGGTATTCGAATCCTCCTTTTAGGGTCCATCATGAAGCCAAACAAA
                     1540      1550      1560      1570      1580      1590
```

Figure 4.8 Results from searching Genbank with pBC1.2.

The DNA of pBC1.2 was used to search Genbank using the FASTA program, which is part of the Wisconsin Package GCG. The highest scoring result, with a sequence similarity of 99.5% is shown.

The full sequence of pBC1.2 was used to search a database, Genbank using the FASTA program, part of the Wisconsin package GCG. The results of the database search (Figure 4.8) show that this gene has already been identified as methylcrotonyl-CoA carboxylase in *Arabidopsis thaliana.* The first reported cloning of a plant (tomato, Wang et al 1994) methylcrotonyl-CoA carboxylase was publicised in the same month in which the sequencing of the *Arabidopsis* clone above was started. Whilst others have sequenced the Arabidopsis cDNA for MCC (Weaver *et al.*, 1995), the sequence published in this thesis was obtained concurrently and independently.

4.5 Southern analysis of MCCase

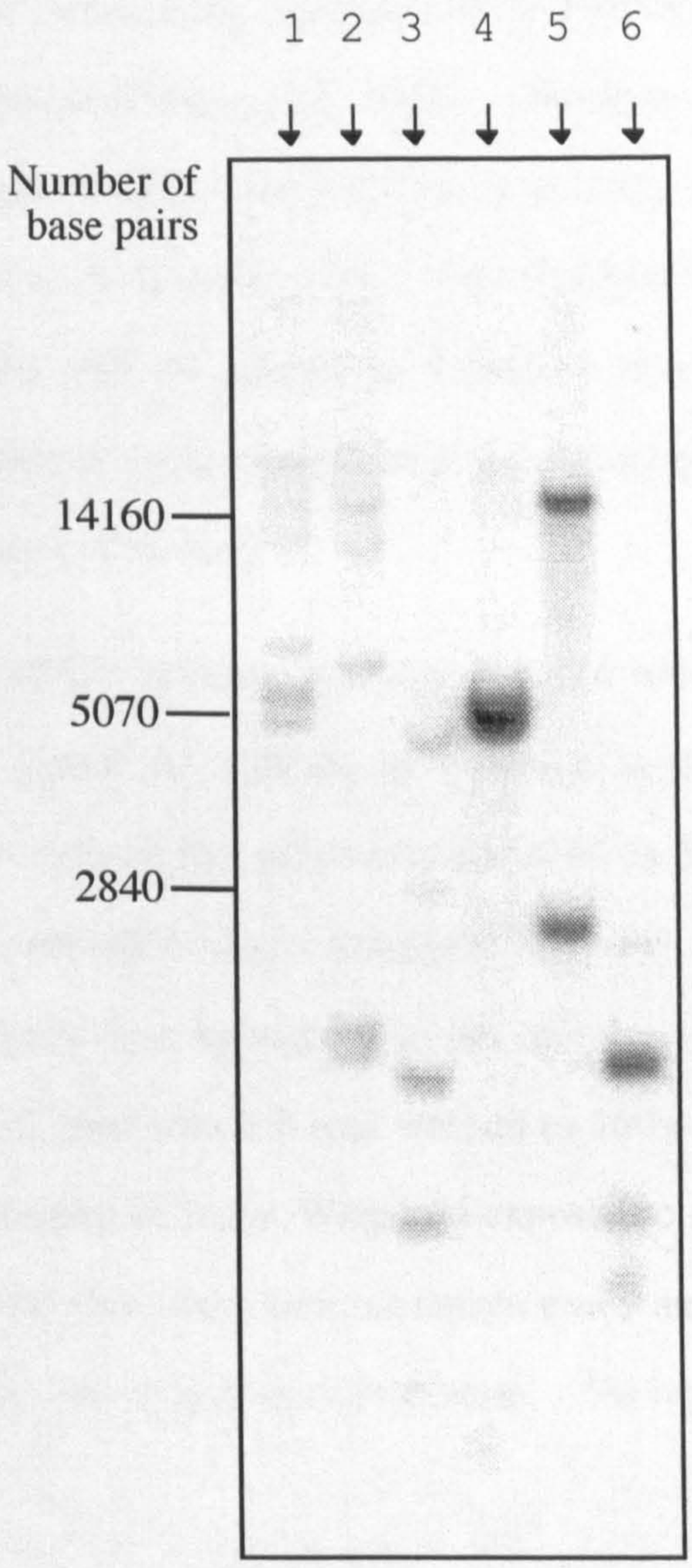
The cDNA insert of pBC1.2 (MCCase) was used to investigate the number of gene(s) encoding MCCase in *Brassica napus* and *Arabidopsis thaliana.* This would reveal whether there were multiple genes for MCCase in either species indicating whether there were possible isoforms of MCCase.

Genomic DNA was prepared from young leaves of *Brassica napus* and leaves of *Arabidopsis thaliana.* Southern blots of *B.napus* and *A.thaliana* DNA digests were made using three different restriction enzymes that cut genomic DNA frequently. A probe was prepared by digesting 2μg of pBC1.2 DNA with Pst I and Xho I. Neither of these cut internally in the pBC1.2 insert. Xho I is the 3' cloning site and Pst I is 6bp away from the Eco RI 5' cloning site in the pSK- MCS (Eco RI could not be used to cut out the insert as it also cuts internally). This produces a 1,700bp fragment containing the pBC1.2 insert (MCCase clone), the adapter sequences used in making the library and 6bp of pBluescript™ DNA. The additional DNA fragments are unlikely to produce any bands in southern analysis as they are so small. The DNA fragment was isolated by running the digest on a 0.7% agarose gel and isolating the 1700bp fragment. Of the isolated fragment, 50ng was labelled with [^{32}Pα]-dCTP and hybridised to the southern blot for 12 hours. The blot was washed with 0.1 × SCC, 0.1% SDS at 65°C, wrapped in SaranWrap and exposed to a phosphor imaging screen for 16 hours after which time the screen was scanned at maximum resolution. The image is shown in Figure 4.9.

The Southern blot shows two hybridising bands in lane 5, the *Arabidopsis* Eco RV digest, implying that there are two genes in this organism. Lane 4 looks as though it contains two bands very close to each other and lane 6 shows one strong band and many smaller bands. This is consistent with there being two or more genes as the sequence reported previously in this study for pBC1.2 (MCCase) contains three Hind III restriction sites. The probe hybridises to four individual bands in *Brassica napus* genomic DNA digested with Hind III or Eco RI implying that there are four copies of this gene in this organism, which is consistent with the tetraploid organisation of this genome.

Figure 4.9 Southern blot of *Brassica napus* and *Arabidopsis thaliana* genomic DNA using pBC1.2 (MCCase) DNA as a probe.

Twenty micrograms of *Brassica napus* and 10μg *Arabidopsis thaliana* genomic DNA were used for each digest. Lane 1-3, *Brassica napus* ; Lane 4-6, *Arabidopsis thaliana*. Lane 1+4 Eco RI, Lane 2+5 Eco RV, Lane 3+6 Hind III. The probe was hybridised with the blot for 12 hours in an equivalent of 5 × SSC. After hybridisation the blot was washed with 0.1 × SSC at 65°C before cooling, washing again with 2× SSC and sealing in SaranWrap. The probed blot was exposed to a phosphor imaging screen for 16 hours and scanned at maximum resolution.


1 2 3 4 5 6
Number of base pairs
14160
5070
2840

4.6 Northern Analysis of MCCase

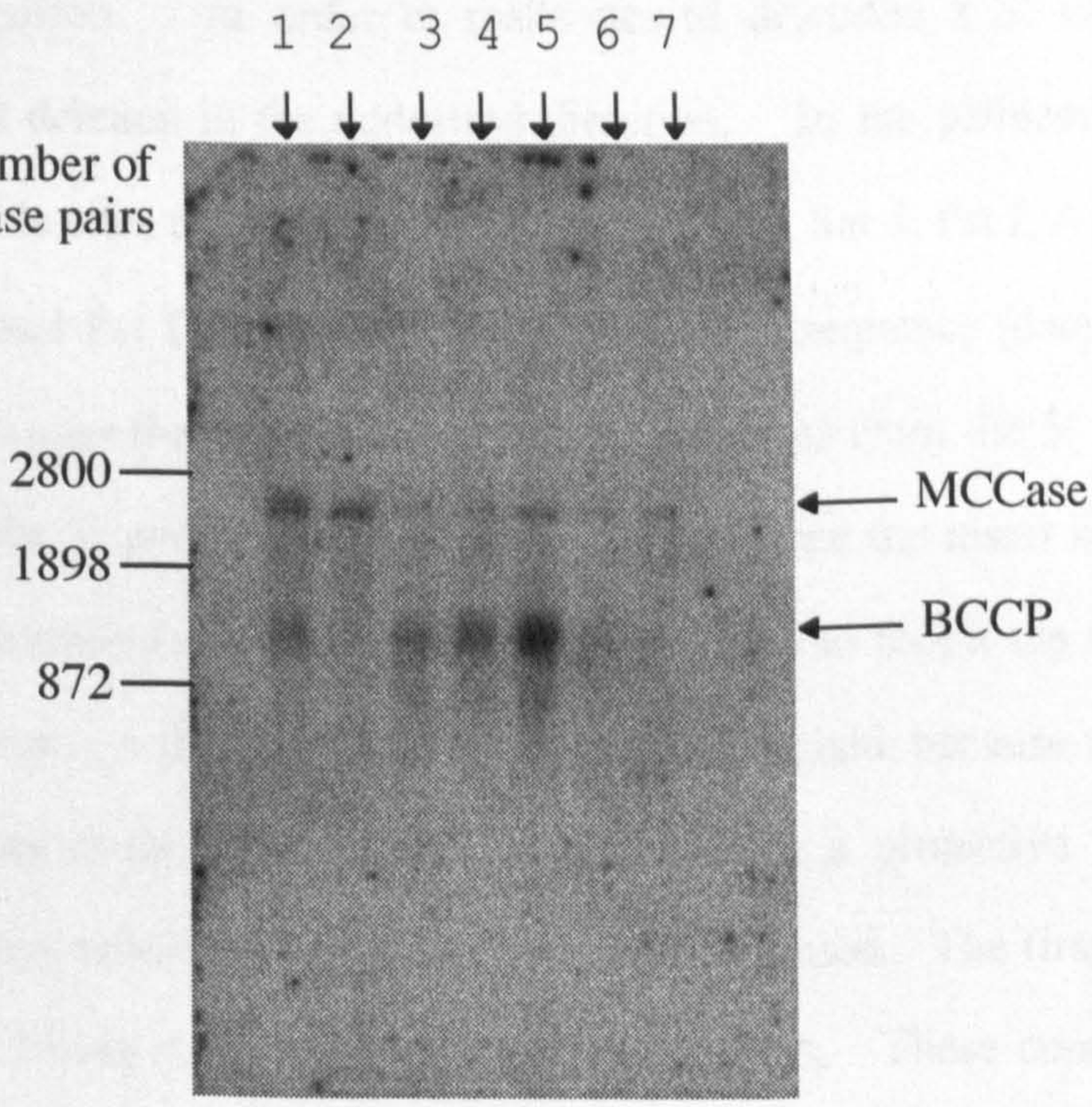
The cDNA insert of pBC1.2 (MCCase) was also used to investigate the expression of the mRNA for MCCase in tissues of *Brassica napus*. This would reveal the pattern of expression of the mRNA for this gene providing information on it function. It should also be possible to correlate the expression of the mRNA with observed changes is the activity of this enzyme (Clauss *et al.*, 1993). Northern analysis would also revel the size of a full length transcript for MCCase in *Brassica napus*, which in turn would enable an estimation of the protein M_r. This predicted protein M_r should aid in the identification of the MCCase protein on anti-biotin Western blots of *Brassica napus* tissues. The pattern of expression of the protein could possibly be correlated with the pattern of expression of the mRNA

The probe prepared for Southern analysis was also used to probe a northern blot of *Brassica napus* poly A^+ mRNA in different tissues and throughout embryo development. A northern blot previously prepared by Russell Swinhoe, University of Durham, Department of Biological Sciences, was used. Each lane had 1µg of poly A^+ mRNA. The probe was hybridised to the blot for 12 hours in 6 × SSPE, 50% formamide at 42°C after which it was washed to 100% stringency. The processed Northern was wrapped in Saran Wrap and exposed to a BIO-RAD phosphor imaging screen for 16 hours after which time the screen was scanned with a GS-525 Molecular Imager system (BIO-RAD) at 100µm resolution. The image is shown in Figure 4.9.

The northern blot shows that expression of the pBC1.2 (MCCase) mRNA is highest in root and then leaf tissues. Expression of the mRNA during embryogenesis is confined to mid-late stages. This is consistent with the role of MCCase in leucine catabolism (Aubert *et al.*, 1996) and the detection of MCCase activity only in older more mature tissues (Clauss *et al.*, 1993). The gene length of 2600bp suggests a possible protein product of 88kDa (allowing 200bp for 3' and 5' UTRs, the M_r is calculated by dividing the number of base pairs by 3 and multiplying by 110, which is the average weight of one amino acid). MCCase is located in the mitochondria (Baldet *et al.*, 1992) so a transit peptide might be expected of about 6-10kDa. This correlates with an identified 76kDa protein on Western blots of *Brassica napus* embryo and leaf tissue (this study, chapter 3). This protein was induced during late embryogenesis which correlates with the observed levels of mRNA in late embryo tissues.

Figure 4.10 Northern blot of *Brassica napus* poly A$^+$ mRNA probed with a partial cDNA (pBC1.2 insert) for MCCase.

All lanes contain approximately 1μg of poly A$^+$ mRNA. Lanes contain as follows; Lane 1, Root; Lane 2, Leaf; Lanes 3 - 7 embryo; (Lanes 3, 38daf; Lanes 4, 44daf; Lanes 5, 49daf; Lanes 6, 58daf; Lanes 7, 63daf). The probe was hybridised to the blot for 12 hours in 5 × SSPE, 50% formamide at 42°C before washing with 0.1 × SSC, 0.1% SDS at 42°C and sealing in SaranWrap. The blot was exposed to a phosphor imaging screen for 16 hours and scanned with a GS-525 Molecular Imager system (BIO-RAD) at 100μm resolution. The complete coding length of methylcrotonyl-CoA carboxylase is approximately 2600bp. The Northern was previously used by another worker with a gene probe specific for a lipid biosynthetic gene. The Northern was not stripped before re-use to prevent loss of RNA. The previous worker's band was 1,100bp and is marked BCCP. β-methylcrotonyl-CoA carboxylase is labelled MCCase.


1 2 3 4 5 6 7
Number of base pairs
2800
1898
872
MCCase
BCCP

4.7 Sequencing of pBC1.6

Of the sixteen clones received, all except two were identical. These two, pBC1.6 and pBC2.1 were characterised further. Initial sequencing from the reverse primer showed that these two clones were identical at the 5' end of the cDNA so, pBC1.6 was chosen for sequencing as it appeared to be the longer of the two (1,800bp vs. 1,600bp respectively).

4.7.1 Nested deletions of pBC1.6

In order to sequence the 1800bp that made up the insert of pBC1.6 a set of nested deletions were required. In order to make nested deletions a 3' overhang was required to prevent deletion in the undesired direction. In the pBluescript™ vector there were 4 possible sites that could create a 3' overhang, Sac I, Pst I, Apa I and Kpn I. Of these Sac I and Pst I cut pBC1.6 within the insert sequence (data not shown). These were the two sites that would enable a set of deletions from the 5' end, so a set was created from the 3' end using Apa I and Xho I. Once the insert had been fully sequenced using deletion from the 3' end, it was necessary to repeat the sequencing in the opposite direction. A different strategy had to be designed, because there were no suitable unique sites at the 5' end which would provide a protective 3' overhang. There were two ways around this problem and both were used. The first was to make a protected end by filling in a 5' overhang with thio-dNTPs. These contain a sulphur group at the α phosphate position. This made the bases resistant to cleavage by Exo III nuclease. In this case a Not I digest was made upstream from the 5' Eco RI site, filled in with thio-dNTPs and a blunt Sma I cut made subsequently to provide a blunt end for Exo III deletion in the correct direction. The restriction digests used to create these two sets of nested deletions are shown in Figure 4.11.

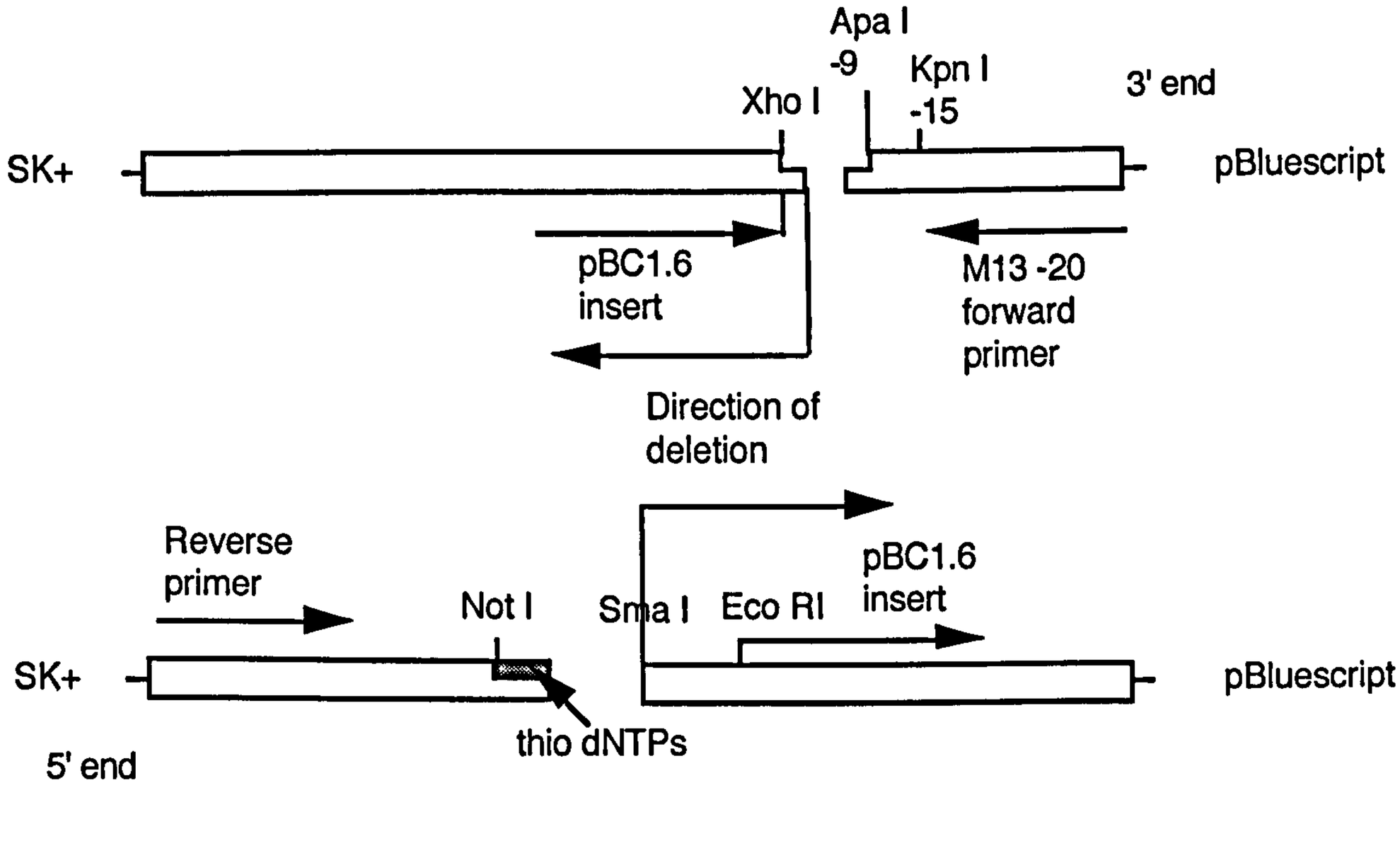


Figure 4.11 The position of restriction sites which allowed directional deletion of pBC1.6.

In the top diagram Apa I provided a 3' overhang to protect the pBluescript™ DNA from deletion by the Exo III nuclease. The Xho I site provided a 5' overhang which allowed the Exo III to delete the pBC1.6 insert from the 3' end. In the lower diagram a Not I digest was made first and then filled with thio-dNTPs. This protected the DNA from deletion with Exo III. To then make a 5' end available for Exo III deletion a Sma I digest was made, which allowed the Exo III nuclease to delete the pBC1.6 insert from the 5' end.

The second way to get around the problem of no suitable restriction sites to create a set of nested deletions, was to make sequencing primers using the sequence data from the other strand. In this way, a set of overlapping sequences were complied which made up the complete sequence of pBC1.6. Figure 4.12 shows how these were arranged and Figure 4.13 gives the entire sequence.



Figure 4.12 Sequencing strategy for pBC1.6.

Sequence starts from vertical bar (|) and continues to end of arrow. Sequencing from the 3' end is shown as dashed arrows, sequencing from the 5' end as solid arrows. The names of individual deletions are given on the left. pBC1.6 P105(1-6) are the six individual primers designed for sequencing.

```
  1 GAGG ATG AAG AGG ATC AAG AAA ACT CCC TGG CAA AGA AAA ACA GTT CAA ATG GGA AAG AAT 61
  1      [1]M   K   R   I   K   K   T   P   W   Q   R   K   T   V   Q   M   G   K   N 19
 62 TGT CAG AAG CGT ATC TGG AAG ATG AAA CTG ACA TTT TCC AGA GTG TTG ATG ATT GGG ATG 121
 20  C   Q   K   R   I   W   K   M   K   L   T   F   S   R   V   L   M   I   G   M  39
122 TGG CTT GTG GCG AGT TTT GCG CAA TGC CTT CTT GTG AAC TCC TGG AAA TAC CTG TCC CTT 181
 40  W   L   V   A   S   F   A   Q   C   L   L   V   N   S   W   K   Y   L   S   L  59
182 CTG AAA AGC AAA AGG ATT TAG AAGGACTGACAATCATATACGTTCCTACAAGAAAGGAGAGTGTCAACATTGC 254
 60  L   K   S   K   R   I   *                                                      66
255 TAAATATCTTTGTGGGGTTGGACTGAAAGCTGCAGCTTATA ATG CAT CGG TAT GGA CTG ATA AAT CTT    322
  1                                           [2]M   H   R   Y   G   L   I   N   L    9
323 CTT ACT TTT TCC TGC TAT AAG AAG CCC TTT GCA CTG TTT TTT AGG AAT GAA ATT TGT CAA 382
 10  L   T   F   S   C   Y   K   K   P   F   A   L   F   F   R   N   E   I   C   Q  29
383 CCT TTG TTA ACT TGG TTG TAG AAATTTTGTTTCCAATTTCTG ATG GAA AAA TTT TGT ATT CCA   445
 30  P   L   L   T   W   L   *                       [3]M   E   K   F   C   I   P    7
446 AAT TAA GAGCTGTTCTTTTTAAATATTGGT ATG GAT GTA TGA ACAGTTTGTTAAGTTTCACAGTTGCCAAAAA 518
  8  N   *                               [4]M   D   V   *                           4
519 AACATCTCAGACAAGTGCACCAGGACTTTCACGATAATAAGCTGCAGGTTGTTGTTGCCACAATTGCGTTTGGA ATG  595
  1                                                                           [5]M    1
596 GGA ATT GAC AAG AAA AAT GTT CGG AAA ATC ATC CAT TAT GGT TGG CCG CAG AGC TTG GAA 655
  2  G   I   D   K   K   N   V   R   K   I   I   H   Y   G   W   P   Q   S   L   E  21
656 GCA TAC TAC CAA GAA GCT GGT CGA GCT GGA AGA GAT GGC GAA TTG GCT GAG TGT GTG CTC 715
 22  A   Y   Y   Q   E   A   G   R   A   G   R   D   G   E   L   A   E   C   V   L  41
716 TAT GCT GAT CTA TCG AGA GCA CCA ACG CTT TTG CCA AGC CGT AGG AGC AAA GAA CAG ACA 775
 42  Y   A   D   L   S   R   A   P   T   L   L   P   S   R   R   S   K   E   Q   T  61
776 GAG CAA GCA TAC AAG ATG CTA TCT GAC TGT TTC AGA TAT GGA ATG AAC ACT TCA CAA TGT 835
 62  E   Q   A   Y   K   M   L   S   D   C   F   R   Y   G   M   N   T   S   Q   C  81
836 CGG GCC AAA ATA CTT GTG GAG TAC TTT GGG GAG GAA TTT AGT TCC AAA AAG TGT AAC TCA 895
 82  R   A   K   I   L   V   E   Y   F   G   E   E   F   S   S   K   K   C   N   S 101
896 TGT GAT GTT TGC ACT GAA GGG CCT CCG GAG CTA GTA GAT GTA CGA GAG GAA GCT AAT CTT 955
102  C   D   V   C   T   E   G   P   P   E   L   V   D   V   R   E   E   A   N   L 121
956 TTG TTT CAA GTA ATC ACT GCT TTT CAT GTA GAC AAT GAC TCT GAG CAT GCA CCA TAT GAA 1015
122  L   F   Q   V   I   T   A   F   H   V   D   N   D   S   E   H   A   P   Y   E 141
1016 GAT TAT GGA CTC GGC AAC AGC AAA CAA AAT AAA TTA TCC CAC AAG CCA AAT CTT CTG TTC 1075
142  D   Y   G   L   G   N   S   K   Q   N   K   L   S   H   K   P   N   L   L   F 161
1076 TTC ATC AGT AAA ATC AGG GAA CAG TGT GAA AAA TTC AAG GAA ACA GAT TAT CTT TGG TGG 1135
162  F   I   S   K   I   R   E   Q   C   E   K   F   K   E   T   D   Y   L   W   W 181
1136 AAA GGT CTT GCC CGT ATC ATG GAA GCC GAA GGA TAC ATC AAA GAG ATG GAT AAC AAG GAT 1195
182  K   G   L   A   R   I   M   E   A   E   G   Y   I   K   E   M   D   N   K   D 201
1196 CGT CGA GTT GAG ATT AAA TTC ATA CAA CCA ACA GAA AAA GGG AAG AAA CAA CTA GAT TTC 1255
202  R   R   V   E   I   K   F   I   Q   P   T   E   K   G   K   K   Q   L   D   F 221
1256 CAA GAT GAC AAA CCA CTC TAT GTT TAT CCA GAA GCC GAC ATG CTA CTT TCG TTG AAA CAA 1315
222  Q   D   D   K   P   L   Y   V   Y   P   E   A   D   M   L   L   S   L   K   Q 241
1316 GAT AGA ACA TAC AGT GGA TTC TCT GAA TGG GGA AAA GGA TGG GCA GAT CCA GAG ATC CGG 1375
242  D   R   T   Y   S   G   F   S   E   W   G   K   G   W   A   D   P   E   I   R 261
1376 CGC CAG CGG TTA GAG AGA AGA GAA AGA AAG CCT CGA AGA GAA AGG AAA CCC CGA AAA AAA 1435
262  R   Q   R   L   E   R   R   E   R   K   P   R   R   E   R   K   P   R   K   K 281
1436 CGA ACA CGA GGA CGT TCT TCG AAG AAG TTA CAT CCA TGG AGA AGC AAA GAA TAA GAATCTCA 1497
282  R   T   R   G   R   S   S   K   K   L   H   P   W   R   S   K   E   *           299
1498 ACCCTCTTTAAGCTCCTTCTTTGCTCTGAGTCCCATCTTGAGGCATAGATACCGCGCCATCGAGAAGCTGAGGCTTGTAA 1577
1578 ACGAGGCTTTGGTAACGGCGGAAGAGAAGGTAACAAGGCTTCAAGAACGTC ATG ATG TGA TTCTCAAAGAGAT   1650
  1                                                     [6]M   M   *                   3
1651 ATG TTC TTA TTA TCT CGT TAA CACCGAGCTCC ATG AAG CTT TGG TTT CAT CTC GTG CAG CCA 1712
   1 [7]M   F   L   L   S   R   *           [8]M   K   L   W   F   H   L   V   Q   P  10
1713 TTA ATG ATG CTT CTT CAT TTG TCA TGG AGC TTA GAA GAT TAC AGT TGA ATATCTTAAACTCTCT 1776
  11  L   M   M   L   L   H   L   S   W   S   L   E   D   Y   S   *                  26
1777 TTCTTAATCACATTTTTTGTACTTATTACACTAATTAAAACCAGAGTTTGGGT                          1829
```

Figure 4.13 Complete sequence of pBC1.6.

Possible open reading frames are translated. Open reading frames start with at ATG (M) and end at TAA, TAG or TGA (*).

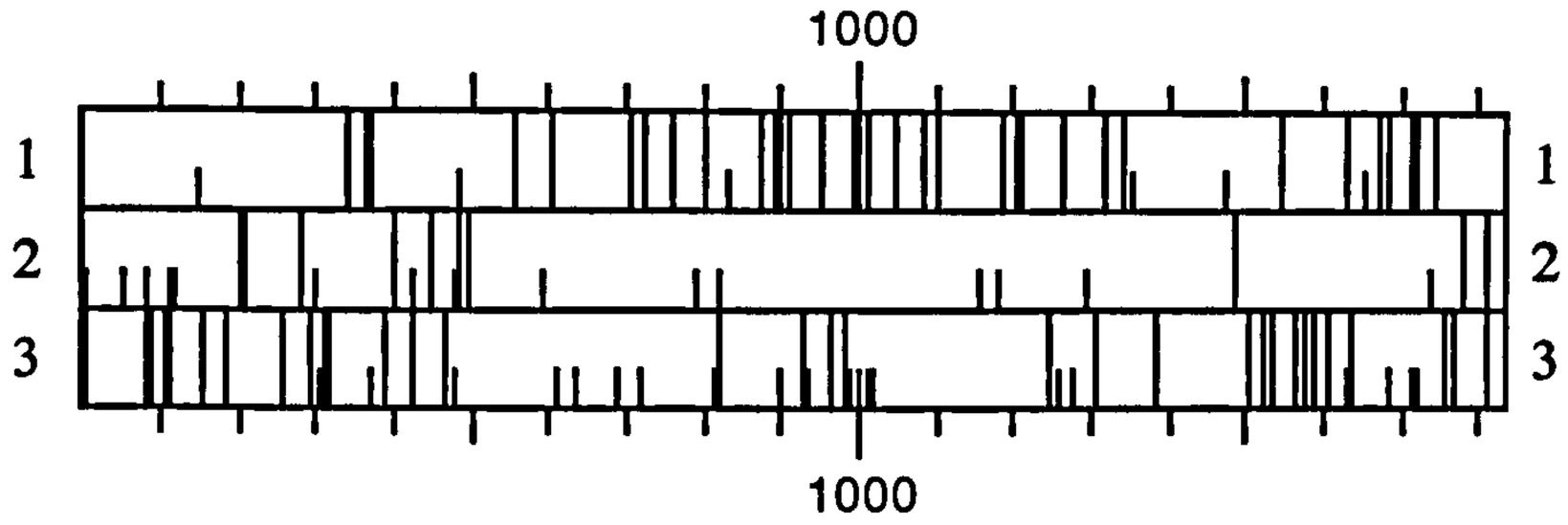


Figure 4.14 Open reading frames of pBC1.6.

Half lines show ATG start codons and full lines show stop codons. Stop and start codons are shown in all three reading frames.

The full sequence of pBC1.6 is shown in Figure 4.13. Translation of the sequence shows that there were no continuous open reading frames throughout the sequence, and this can be seen in Figure 4.14. This could have been for several reasons. It was possible that there was an artefact in the sequence. Alternatively, during the making of the library, two cDNAs could have been ligated together to produce this fusion of two genes. This is a recognised problem when creating libraries. If this were the case, it could be expected that there would be a poly A^+ region in the spurious region around 300-500bp over 12bp in length, representing the poly A tail of the fused gene. This would be a reasonable assumption as the library was made from poly T primed cDNA. No such region was in evidence however. Another possibility was that there were errors in the sequencing or errors had been introduced by the reverse transcriptase. For this to be the case there would have to of been 3 errors within the 200bp region between 300-500bp. The error rates of the reverse transcriptase and TAQ polymerase used for sequencing, were considerably lower than this rate, although this did not exclude the possibility. A final possibility was the existence of an intron. These have been known to occur in cDNA libraries, representing the cDNA copy of a hnRNA which had failed to be or had not finished being processed. Indeed, it was possible that the processing was correct and finished and the intron was not meant to be spliced out at all. This would cause a change in the protein product, possibly giving it a different function.

4.8 Identification of intron sequence in pBC1.6

4.8.1 Identification of conserved intron boundaries

If the spurious region of pBC1.6 between 300 and 500bp was an intron then it should have certain features which make it identifiable as such. It should have been present in genomic DNA and have conserved intron / exon boundaries. Should the section between the conserved boundaries be removed at the conserved splice sites a complete open reading frame should then be obtained. The sequence on either side of the 300 to 500bp region was compared to the consensus of known intron /exon boundaries of *Arabidopsis thaliana* . The results are shown in Figure 4.15.

5' slice site

A	119	164	268	51	|	3	7	300	250	100	114	141
C	114	145	70	19	|	2	3	17	74	29	68	81
G	78	72	38	334	|	430	6	48	22	229	42	56
T	132	62	67	39	|	8	427	78	97	85	219	165
Consensus	**A**/C/T	A/C	A	**G**	|	**G**	**T**	**A**	A	**G**	T	T/**A**
pBC1.6	^{300}**A**	T	C	**G**	|	**G**	**T**	**A**	T	**G**	G	^{310}**A**

3' splice site

A	87	92	74	147	24	428	8	|	112	96	120	117
C	64	60	54	27	272	3	2	|	53	64	67	72
G	85	69	38	155	8	7	430	|	221	83	113	144
T	207	22	277	114	139	5	3	|	57	200	143	110
Consensus	T	A	T	G/A/T	C/T	**A**	**G**	|	G/A	**T**	T/A/G	G/AT
pBC1.6	^{559}G	C	**T**	**G**	**C**	**A**	**G**	|	**G**	**T**	**T**	^{569}**G**

Figure 4.15 Intron / exon boundaries of *Arabidopsis thaliana* .

The 5' splice site is at the top and the 3' splice site at the bottom. The table shows the occurrence of certain bases in the intron exon boundaries in relation to the splice site marked (|). The consensus sequence is made up of the highest scoring base for that position. Where the scores are roughly equal, the two or three bases are shown in the sequence with the highest scoring base first. Where the consensus matches identified sequence in the pBC1.6 clone the bases have been marked in bold. The numbers next to the bases in the pBC1.6 sequence denote the base number in the sequence presented in Figure 4.12.

As previously mentioned, if these intron / exon boundaries were correct, then removing the sequence from between these sites should have produced a single open reading frame. The results of removing the sequence between the putative splice sites are shown in Figure 4.16.

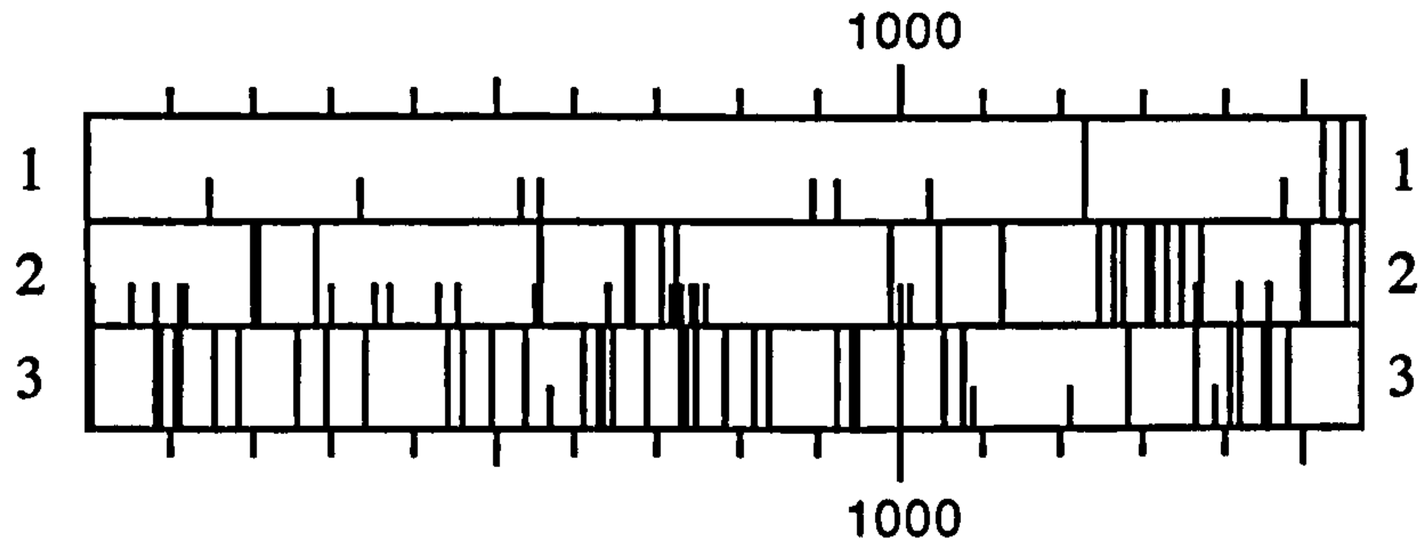


Figure 4.16 Open reading frame map of pBC1.6 with the putative intron removed.

The DNA sequence inbwteen the intron / exon boundaires was removed and the result is a single open reading frame 1,200bp in length.

4.8.2 PCR of intron from genomic DNA

The second criteria for identification of an intron was that it should be present in genomic DNA. In order to test this, two primer pairs were made which matched regions on either side of the intron sequence. They were designed to have a high Tm by making them >25bp in length so that a high specificity could be obtained in the PCR from genomic DNA.

PCR was carried out with the following primers;

^{248} 5'-ACATTGCTAAATATCTTTGTGGGGTTGG-3' ^{275} Primer 1078; Tm 60.4

^{561} 5'-AGCTTATTATCGTGAAGTCCTGGTGC-3' ^{535} Primer 1079 Tm 59.1

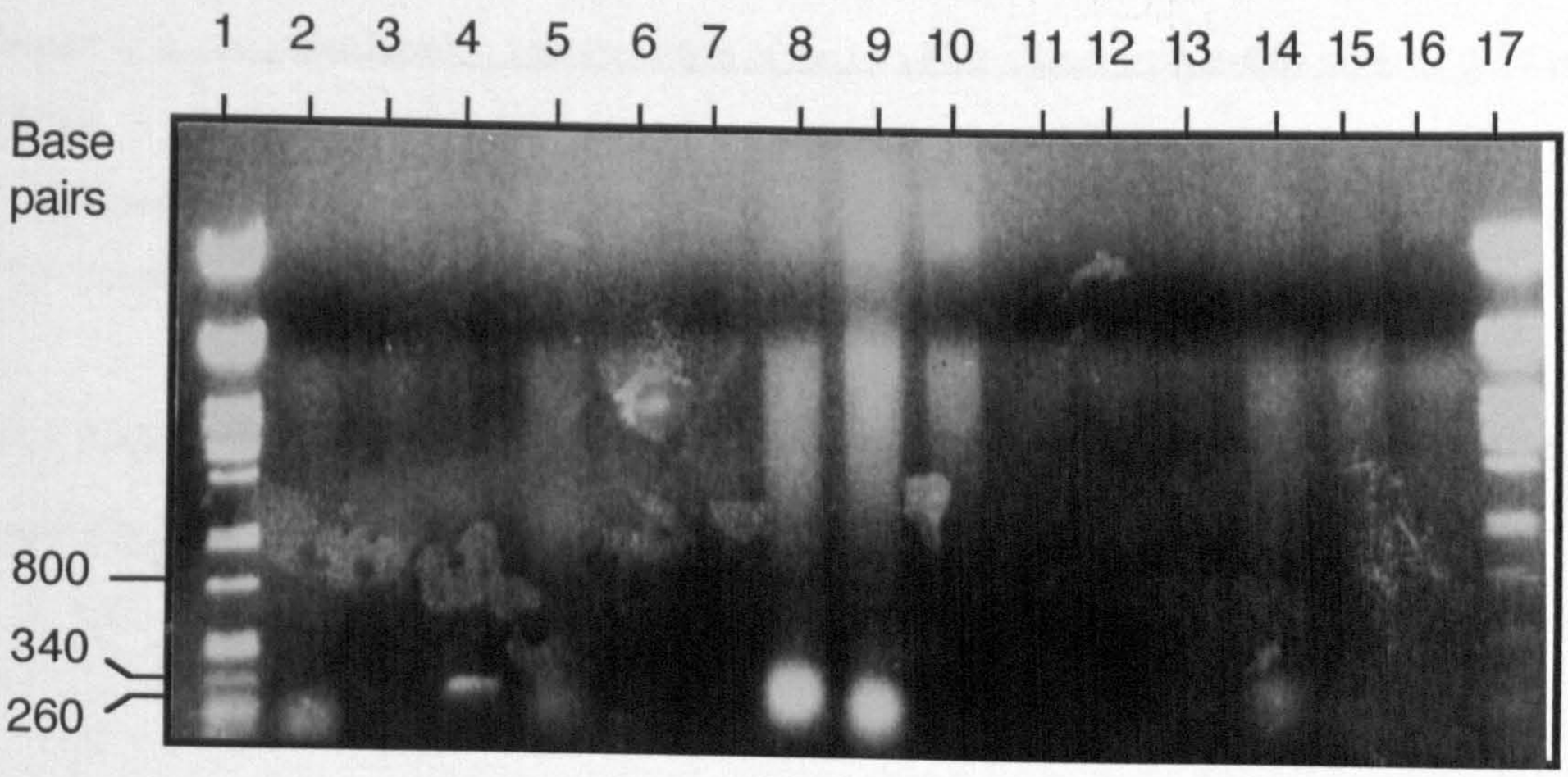
These primers were used to produce a product 314bp in length over 30 cycles of 94°C, 1 minute; 60°C, 1 minute; 74°C, 30 seconds; with the thermostable Vent ® DNA polymerase. As expected, the PCR product was around 300bp in length, Figure4.17. Only one reaction produced a fragment of the predicted size (lane 4 on the gel). This was using 2mM $MgSO_4$ and 1µg of genomic DNA as template. Lanes 8 and 9 appear to contain some amplified DNA, but because it was not a single tight band it was not analysed further. The products were probably a result of non-specific primer binding under high Mg^{2+} conditions.

Figure 4.17 PCR products amplified from *Arabidopsis thaliana* genomic DNA with primers 1078 and 1079 separated on a 0.8% agarose gel.

Primers 1078 and 1079 were used in a PCR reaction with varying amounts of $MgSO_4$ and genomic DNA template. The reaction was run over 30 cycles of 94°C 1 minute, 60°C 1 minute, 74°C 30 seconds. Of the complete reaction, 10µl was run on a 0.8% agarose gel stained with ethidium bromide and viewed under UV light.

The lanes contain PCR reactions run with the following amounts of Mg^{2+} and template; Lanes 2-4, all 2mM $MgSO_4$; Lane 2, 100ng DNA; Lane 3, 500ng DNA; Lane 4, 1µg DNA; Lanes 5-7, all 5mM $MgSO_4$; Lane 5, 100ng DNA; Lane 6, 500ng DNA; Lane 7, 1µg DNA; Lanes 8-10 all 10mM $MgSO_4$; Lane 8, 100ng DNA; Lane 9, 500ng DNA; Lane 10, 1µg DNA

The following lanes contained PCR reaction controls with only one primer to show that the product is produced only when both primers are present; Lanes 11-16, all 2mM $MgSO_4$ Lane 11, primer 1078 only, 100ng DNA; Lane 12, primer 1078 only, 500ng; Lane 13, primer 1078 only, 1µg DNA; $MgSO_4$ Lane 14, primer 1079 only 100ng DNA; Lane 15, primer 1079 only, 500ng; Lane 16, primer 1079 only, 1µg DNA. Lanes 1 and 17 contain 1µg of λ Pst I markers.


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
Base pairs
800
340
260

The PCR fragment shown in Figure 4.17, Lane 4, was isolated from an agarose gel and cloned into pBluescript™ SK+ cut with Eco RV, and sequenced in the forward and reverse orientation. A clone from each of two separate PCR reactions was sequenced in the forward and reverse orientation. Figure 4.18 shows the sequence location and direction of the two clones when compared to pBC1.6.

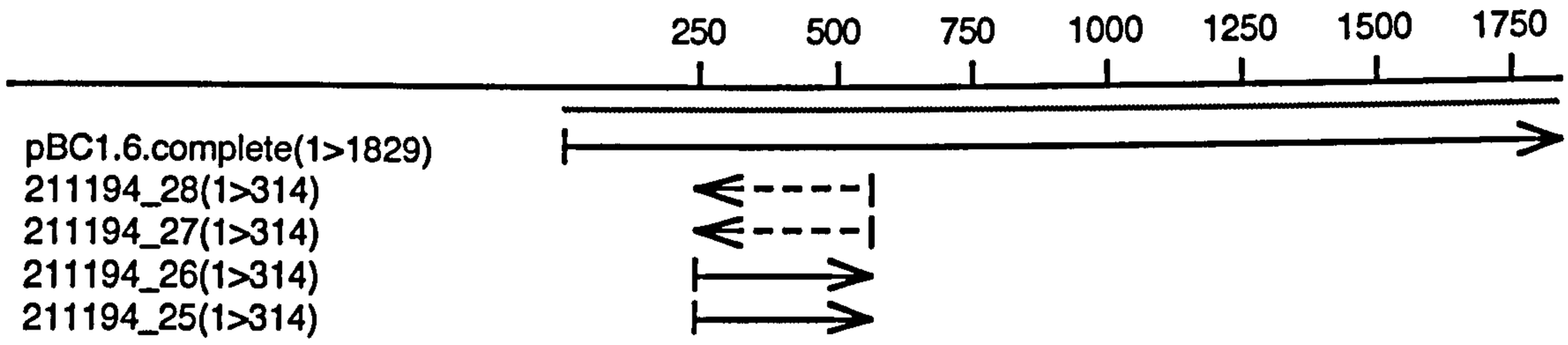


Figure 4.18 Sequence location and orientation of the two PCR clones generated by primers 1078 and 1079.

PCR was across the putative intron region of pBC1.6 and genomic *Arabidopsis* DNA was used as a PCR template.

The sequences of the PCR clones were used to create a consensus sequence. This was aligned with the sequence of pBC1.6 as shown in Figure 4.19.

```
                201                                                  250
pBC1.6      AGAAGGACTG ACAATCATAT ACGTTCCTAC AAGAAAGGAG AGTGTCAACA
gen1.6      .......... .......... .......... .......... .......ACA

                251                                                  300
pBC1.6      TTGCTAAATA TCTTTGTGGG GTTGGACTGA AAGCTGCAGC TTATAATGCA
gen1.6       TTGCTAAATA TCTTTGTGGG GTTGGACTGA AAGCTGCAGC TTATAATGCA

                301                                                  350
pBC1.6      TCGGTATGGA CTGATAAATC TTCTTACTTT TTCCTGCTAT AAGAAGCCCT
gen1.6       TCGGTATGGA CTGATAAATC TTCTTACTTT TTCCTGCTAT AAGAAGCCCT

                351                                                  400
pBC1.6      TTGCACTGTT TTTTAGGAAT GAAATTTGTC AACCTTTGTT AACTTGGTTG
gen1.6       TTGCACTGTT TTTTAGGAAT GAAATTTGTC AACCTTTGTT AACTTGGTTG

                401                                                  450
pBC1.6      TAGAAATTTT GTTTCCAATT TCTGATGGAA AAATTTTGTA TTCCAAATTA
gen1.6       TAGAAATTTT GTTTCCAATT TCTGATGGAA AAATTTTGTA TTCCAAATTA

                451                                                  500
pBC1.6      AGAGCTGTTC TTTTTAAATA TTGGTATGGA TGTATGAACA GTTTGTTAAG
gen1.6       AGAGCTGTTC TTTTTAAATA TTGGTATGGA TGTATGAACA GTTTGTTAAG

                501                                                  550
pBC1.6      TTTCACAGTT GCCAAAAAAA CATCTCAGAC AAGTGCACCA GGACTTTCAC
gen1.6       TTTCACAGTT GCCAAAAAAA CATCTCAGAC AAGTGCACCA GGACTTTCAC

                551                                                  600
pBC1.6      GATAATAAGC TGCAGGTTGT TGTTGCCACA ATTGCGTTTG GAATGGGAAT
gen1.6       GATAATAAGC T......... .......... .......... ..........
```

Figure 4.19 DNA alignment of the consensus sequence generated by sequencing the PCR product of primer 1078 and 1079 (gen1.6), and pBC1.6.

The two sequences are identical. However, pBC1.6 represents a cDNA sequence and gen1.6 represents a PCR product from genomic DNA. The putative identified intron is underlined.

These results confirmed that the spurious region of DNA between bases 300 and 500 in Figure 4.14 was an intron, although the identified splice sites are not definite as they have only been identified by consensus and not by actual comparison between processed cDNA and genomic DNA.

4.9 Identification of a biotinylation motif

pBC1.6 was isolated from a cDNA library by screening with a probe specific for biotin. In this way a clone for a known biotin-containing protein, β-methylcrotonyl-CoA carboxylase was isolated and a conserved biotinylation motif identified. Examination of the sequence data for pBC1.6 showed that no conserved biotinylation motif could be distinguished. Figure 4.20 below shows an alignment of known biotinylated proteins including the pBC1.2 protein as *A. thaliana* MCC. The consensus sequence is shown below.

Human PC	G	Q	P	L	C	V	L	S	A	M	**K**	M	E	T	V	V	T	S	P	M	E
Sheep PC		G	Q	P	L	V	L	S	A	M	**K**	M	E	T	V	V	T	S	P	V	T
S.cerevisiae PC	G	Q	P	V	A	V	L	S	A	M	**K**	M	E	M	I	I	S	S	P	S	D
Human PCC	G	Q	E	I	C	V	I	E	A	M	**K**	M	Q	N	S	M	T	A	G	K	T
Rat PCC	G	Q	E	I	C	V	I	E	A	M	**K**	M	Q	N	S	M	T	A	G	K	M
Chicken ACC	G	Q	C	F	A	E	I	E	V	M	**K**	M	V	M	T	L	T	A	G	E	S
Maize ACC	D	V	P	Y	A	E	V	E	V	M	**K**	M	C	M	P	L	L	S	P	A	S
C.cryptica ACC	G	Q	P	Y	V	E	V	E	A	M	**K**	M	I	M	P	I	K	A	T	E	S
S.cerevisiae ACC	G	Q	P	Y	A	E	I	E	V	M	**K**	M	Q	M	P	L	V	S	Q	E	N
E.coli ACC	G	N	T	L	C	I	V	E	A	M	**K**	M	M	N	Q	I	E	A	D	K	S
S.mutans ACC	N	Q	P	L	M	I	L	E	A	M	**K**	M	E	N	E	I	V	A	G	M	A
Anabeana ACC	G	Q	T	V	C	I	I	E	A	M	**K**	L	M	N	E	I	E	A	D	V	S
P.aeruginosa ACC	G	D	I	R	C	T	A	E	A	M	**K**	M	M	N	H	I	E	A	E	V	S
Alfalfa ACC	D	T	P	Y	A	E	V	E	V	M	**K**	M	C	M	P	L	L	S	P	A	S
A.thaliana ACC	D	T	P	Y	A	E	V	E	V	M	**K**	M	C	M	P	L	L	S	P	A	S
B.napus ACCI	D	M	P	Y	A	E	V	E	V	M	**K**	M	C	M	P	L	L	S	P	A	S
B.napus ACCII	G	Q	V	V	C	I	I	E	A	M	**K**	L	M	N	E	I	E	A	E	K	S
Tomato peptide	G	Q	P	V	L	V	L	E	A	M	**K**	M	E	H	V	V	K	A	P	A	N
P.shermanii TC	G	Q	T	V	L	V	L	E	A	M	**K**	M	E	T	E	I	N	A	P	T	D
K.pneumoniae ODC	G	E	V	L	L	I	L	E	A	M	**K**	M	E	T	E	I	R	A	A	Q	A
S.typhimurium ODC	G	D	V	L	L	I	L	E	A	M	**K**	M	E	T	E	I	R	A	A	Q	A
Urea amidolyase	G	Q	G	L	L	I	I	E	A	M	**K**	A	E	M	I	I	S	A	P	K	S
A.thaliana MCC	G	Q	P	I	L	V	L	E	A	M	**K**	M	E	H	V	V	K	A	P	S	S
				I		V						Q		V							
		E		V		I	I		V			C			L		S				
Consensus	G	Q		L		L	L	E	A	M	**K**	M	E			I		A			
SBP65	E	D	F	G	G	V	R	D	M	G	**K**	F	Q	M	E	S	K	G	G	N	K
Position(-)	10	9	8	7	6	5	4	3	2	1	0	1	2	3	4	5	6	7	8	9	10	(+)

Figure 4.20 Biotinylation domains of several biotin enzymes

The diagram is adapted from Duval *et al.* 1994 to include some important, recently sequenced plant biotin enzymes including pBC1.2 as *A.thaliana* MCC. The consensus biotinylation site is shown beneath these conserved protein sites. SBP65 is the protein reported by Duval *et al.* 1994 in which it was demonstrated that the biotin-containing seed storage protein of apparent M_r 65kDa was biotinylated at a non-conserved site shown above. Alignment and numbering are relative to the biocytin (bold K).

SBP65 represented the first reported finding of a protein biotinylated at a non-conserved site. The protein sequence of pBC1.6 is given in Figure 4.21.

```
ORF1    1 EDEEDQENSL AKKNSSNGKE LSEAYLEDET DIFQSVDDWD VACGEFCAMP 50

ORF1   51 SCELLEIPVP SEKQKDLEGL TIIYVPTRKE SVNIAKYLCG VGLKAAAYNA 100

ORF1  101 SVWTDKSSYF FLL*                                        113

ORF 2 168 VSQLPKKHLR QVHQDFHDNK LQVVVATIAF GMGIDKKNVR KIIHYGWPQS 217

ORF 2 218 LEAYYQEAGR AGRDGELAEC VLYADLSRAP TLLPSRRSKE QTEQAYKMLS 267

ORF 2 268 DCFRYGMNTS QCRAKILVEY FGEEFSSKKC NSCDVCTEGP PELVDVREEA 317

ORF 2 318 NLLFQVITAF HVDNDSEHAP YEDYGLGNSK QNKLSHKPNL LFFISKIREQ 367

ORF 2 368 CEKFKETDYL WWKGLARIME AEGYIKEMDN KDRRVEIKFI QPTEKGKKQL 417

ORF 2 418 DFQDDKPLYV YPEADMLLSL KQDRTYSGFS EWGKGWADPE IRRQRLERRE 467

ORF 2 468 RKPRRERKPR KKRTRGRSSK KLHPWRSKE*                       496
```

Figure 4.21 Translation of the two major open reading frames in pBC1.6.

The first open reading frame was thought to have been expressed as a fusion protein with lacZ. The second open reading frame could not have been expressed as a fusion protein, so it would have had to initiate it's own translation, the 1st methionine in the second ORF is double underlined. The closet sequence to the consensus biotinylation site shown in Figure 4.20 has been underlined. The lysine residues have been highlighted in bold for ease of location.

All possible open reading frames of pBC1.6 were examined but none of them contained the conserved sequence. The nearest sequence was in ORF 2 at amino acid Lys-265 but there is no evidence to suggest that this sequence is being translated as it is not fused to the lacZ protein so would have to initiate transcription from a different start codon. No similarity was found to the novel SPB65 protein either, nor to the biotin binding site contained within its sequence. As a result, it became necessary to positively confirm that the protein product of pBC1.6 was indeed biotinylated.

4.9.1 λZAP II phage pBC1.6 expresses a biotinylated protein.

The plaque pure phage designated pBC1.2 and pBC1.6 were plated separately as on 70mm petri dishes. A third plaque pure clone which encoded a hydratase gene which is not biotinylated, was used as a negative control. Plaques were grown to produce pin-prick sized plaques before being induced to express their cloned gene by laying marked, IPTG soaked filters on top and continuing growth for a further 4 hours. The filters were taken and washed with a boiling solution of 2% SDS to remove the *E. coli* cell debris and reduce background. The washed filters were then blocked in 20ml of 1% Haemoglobin in PBS for 2 hours before the solution was replaced with 10ml of 1% Haemoglobin in PBS and 5µl of streptavidin-HRPO was added. The filters were left to incubate for a further 2 hours before being washed 3 × 20 minutes in 20ml of PBS. The filters were then developed with the chemiluminescence kit ECL (AMERSHAM) and exposed to X-ray film. The positive result for pBC1.6 is shown in Figure 4.22. The pBC1.2 clone produced a similar positive result as would be expected, seeing as this encodes a known biotinylated protein. The hydratase clone produced a negative result as this is a non-biotinylated protein.

A control experiment, in which 1mM biotin was included in the incubation medium with the streptavidin-HRPO produced no signal. This demonstrates that the pBC1.6 clone specifically produces a biotinylated protein and that the streptavidin is not binding to the pBC1.6 protein by itself.

To confirm that there was a novel protein being produced by the λZAPII pBC1.6 clone, lambda lysogens were made using the pBC1.6 phage in *E. coli* Y1090 cells. The lysogens were grown to log phase at 30°C and then induced by heat shock and addition of IPTG to 1mM. The cultures were then grown for a further 1 hour before rapid chilling and harvesting to prevent lysis of the bacteria by the phage. The Western blot in Fig4.22 B shows lanes of *E.coli* Y1090 cells next to lanes of *E.coli* Y1090 lysogens containing the pBC1.6 phage. The western has been probed with anti-biotin antibodies, donkey anti-sheep IgG : alkaline phosphatase conjugate and

developed with BCIP/NBT. In the lanes of induced lysogen a 29kDa biotinylated protein can be seen (Figure 4.22 B, Lanes 2 and 4).

E.coli DH5α cells containing the pBC1.6 plasmid were grown in 50ml LB + 50μg / ml ampicillin from a single colony on a plate, to OD_{600} 0.5. Expression from the *lacZ* promoter was induced by addition of IPTG and the cells were grown for a further 2 hours. The cells were harvested by centrifugation at 3,000 × g, an aliquot made 1 × with SDS sample buffer and run on a 12% acrylamide gel. Proteins were blotted onto nitrocellulose and detected with anti-biotin antibodies (Figure 4.22 B). No expression of the novel biotinylated protein produced by the lysogens could be detected. This could be because the expression of the protein was very low or because an element of the bacteriophage, was required to cause expression such as a phage promoter or polymerase. This might be possible as several promoters are present on the bacteriophage genome and bacteriophage polymerases by be able to initiate transcriptions from different sites than bacterial ones.

Figure 4.22 A Detection of biotinylated protein produced by the clone pBC1.6 λZAPII phage.

pBC1.6 phage were grown up on a plate and overlayed with a nitrocellulose filter soaked in 1mM IPTG. The induced proteins were detected with streptavidin-HRPO and ECL.

Figure 4.22 B Expression of pBC1.6 protein product from λZAPII lysogens and from pBluescript™.

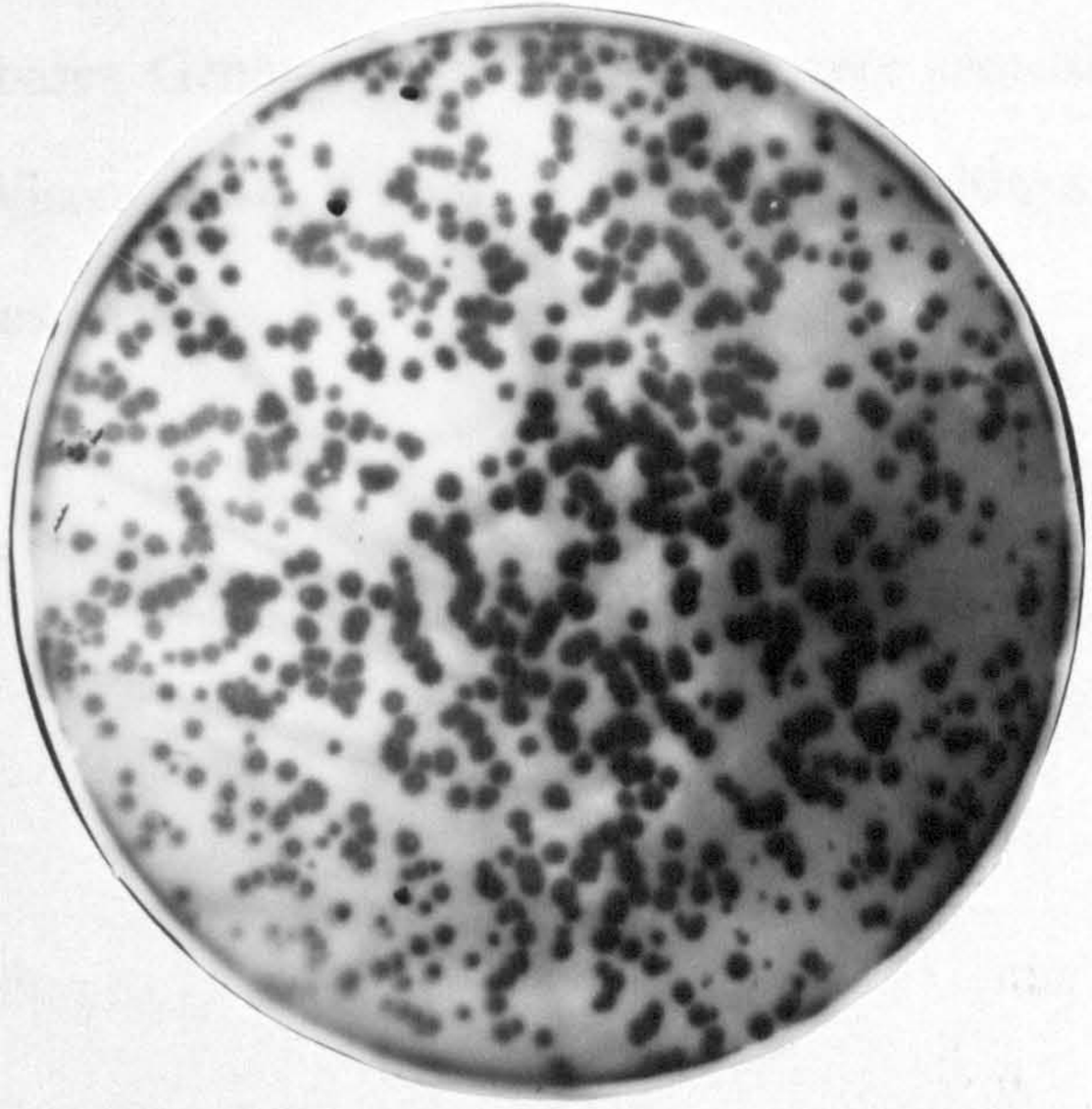
E.coli Y1090 that contained pBC1.6 λZAPII in lysogenic cycle were induced into lytic mode by heat shock at 42°C and expression from the *lacZ* promoter induced by the addition of IPTG to 1mM. Protein samples from the induced lysogens were taken after 1hours growth and analysed by Western blotting with anti-biotin antibodies.

The lanes contain the following samples. Lane 1, uninduced *E.coli* Y1090 cells; Lane 2, induced *E.coli* Y1090 pBC1.6 lysogen; Lane 3, induced *E.coli* Y1090 cells; Lane 4, induced *E.coli* Y1090 pBC1.6 lysogen; Lane 9, biotinylated Mw markers. The arrow marks the position of the detected biotinyalted protein in *E.coli* lysogens.

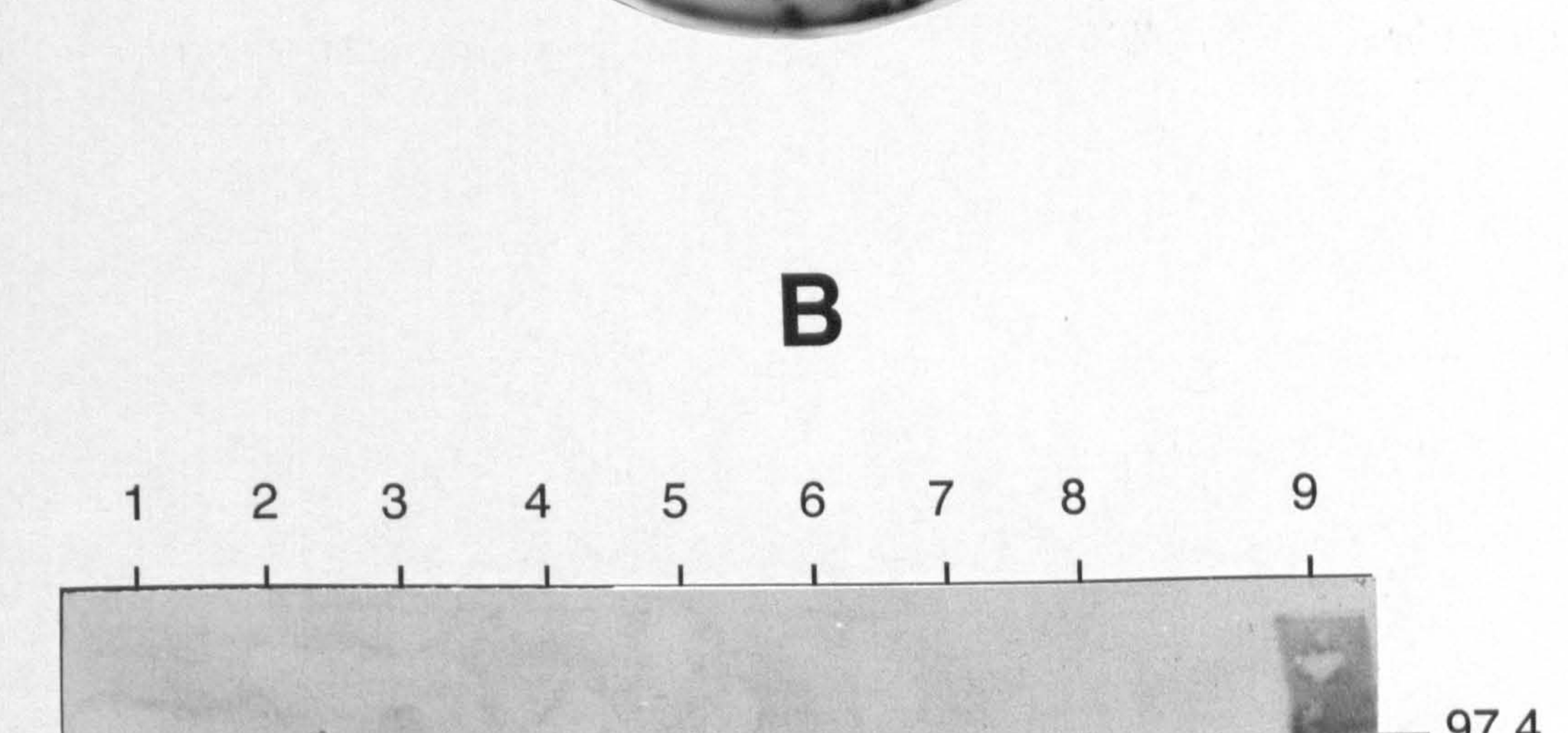
E.coli DH5α cells containing the pBC1.6 plasmid were grown in culture and expression from the *lacZ* promoter induced by the addition of IPTG to 1mM. After 2hours growth proteins were extracted from the bacteria into 1 × SDS sample buffer and analysed by Western blotting with anti-biotin antibodies.

The lanes contain the following samples. Lane 4, uninduced *E.coli* DH5α cells; Lane 5, uninduced *E.coli* DH5α with pBC1.6; Lane 6, induced *E.coli* DH5α cells; Lane 7, induced *E.coli* DH5α with pBC1.6; Lane 9, biotinylated protein markers.

A



B



4.10 Database searches with pBC1.6

Very often, proteins with similar functionalities have similar amino acid sequences and hence DNA sequences. This is how pBC1.2 was identified as β-methylcrotonyl-CoA carboxylase. In order to try and obtain information on the function of pBC1.6 therefore, the databases Genbank and SwissProt were searched using the FASTA program from the Wisconsin GCG package with default settings. The top scores in the database searches are shown in Figure 4.23.

A - search with complete 1829bp of pBC1.6 of Genbank.

em_pr:hs3981710 U39817 Human Bloom's syndrome protein (BLM) mRNA, complete cds. 63.7% identity in 270bp overlap Opt: 290

em_fun:spac2g11 Z54354 *S.pombe* chromosome I cosmid c2G11. DEAH-box; helicase. 53.8% identity in 665bp overlap Opt 278

em_pr:hsrecq L36140 *Homo sapiens* (clone 1311) DNA helicase Q1, partial cds. 52.5% identity in 746bp overlap Opt 260

B - search with pBC1.6 with intron sequence removed of Genbank.

em_ba:hi32756 U32756 *Haemophilus influenzae* narP, lysA, recQ, pro S, plsC genes. 55.1% identity in 539bp overlap Opt 268

em_ba:hihi0728 L45367 *Haemophilus influenzae* Rd DNA-dependent ATPase, DNA helicase (recQ) gene. 55.1% identity in 539bp overlap Opt 268

em_pr:hs3981710 U39817 Human Bloom's Syndrome protein (BLM) mRNA, complete cds. 63.8% identity in 232bp overlap Opt 265

C - search with translation of gene 5 (fifth translated ORF in Figure 4.13) of SwissProt

yabc_schpo Q09811 Putative ATP-dependent DNA Helicase C2G11.12
37.5% identity in 126aa overlap. Opt 255

sgs1_yeast P35187 Helicase SGS1 (helicase TPS1)
30.2% identity in 162aa overlap. Opt 255

recq_ecoli P15043 ATP-dependent DNA Helicase recQ (EC 3.6.1.-)
34.1% identity in 129aa overlap Opt 209

D - search with translation of pBC1.6 without intron of SwissProt

yabc_schpo Q09811 Putative ATP-dependent DNA Helicase C2G11.12
38.5% identity in 135aa overlap. Opt 293

recq_human P46063 ATP-dependent DNA Helicase Q1
38.0% identity in 137aa overlap Opt 225

Figure 4.23 Results from searches of Genbank and SwissProt using pBC1.6

THe databases were searched using the FASTA program which is part of the Wisconsin Package GCG. Results are displayed with highest optimal scoring and sequence identity first. cds. coding sequence; Opt, optimal score; aa, amino acids

The best scores from the searches of Genbank and SwissProt are 290 for Human Bloom's Syndrome (BLM) mRNA and 293 for a putative ATP-dependent DNA Helicase respectively. The other significant scores are all DNA helicases. The Bloom's Syndrome gene product has high homology to the recQ helicases, a subfamily of DExH box containing DNA and RNA helicases (Ellis *et al.* 1995, Watt & Hickson, 1996). Figure 4.24 shows the results when these sequences are aligned together with a 125 amino acid fragment of pBC1.6 with intron removed. The large gap that occurs at amino acid 101 in the pBC1.6 alignment coincides with the putative intron splice site. It is possible therefore that these proposed splice sites are incorrect and that the mature pBC1.6 protein would have encoded amino acids in that region. The possible sequence of amino acids that could encode that region should the intron / exon boundaries be incorrect have been inserted below. The over-lined regions represent the seven conserved helicase domains (Ellis *et al.* 1995).

```
                                                                  II
                                                          ERKLLARFVI BLM
                                                          EARRFTRIAV REQL
                                                          ADGKLARIVV SGS1
                                                          AHWNPVLIAV recQ
                                                                 III
                                                          PRVQKDILTQ BLM
                                                          NHVLTDAQKI REQL
                                                          EQVRMDIIHN SGS1
                                                          DTTRQDIVRL recQ
                                                                                  -
                                                          GYYQESGRAG RDGEISHCLL BLM
                                                          NYYQESGRAG RDDMKADCIL REQL
                                                          GYYQETGRAG RDGNYSYCIT SGS1
                                                          SYYQETGRAG RDGLPAEAML recQ
                                                          AYYQEAGRAG RDGELAECVL pBC1.6


                                                          NLYSMVHYCE NITECRRIQL BLM
                                                          KLYEMVSYCQ NISKSRRVLM REQL
                                                          KLQQVMAYCD NCTDCRRKLV SGS1
                                                          KLNAMGAFAE AQT-CRRLVL recQ
                                                          NTSQCRAKIL pBC1.6
```

Figure 4.24 Alignment of the highest scoring hits from the FASTA search results.

The four highest scoring hits in Figure 4.23 were aligned using the MEGALIGN computer program (DNAStar). All protein sequences are of the recQ family of DExH box-containing helicases. BLM, Bloom's Syndrome protein; REQL, Human recQL; SGS1, S.cerevisiae SG; and recQ, *Escherichia coli* recQ. Dashes indicate gaps inserted by the program when aligned with the protein fragment from translated pBC1.6 without intron, amino acids 72 to 197. Red characters indicate 5 consensus amino acids, blue characters 4 and green characters only 3 matching amino acids. Over-lined sequences mark the five of the seven helicase domains (Ellis *et al.* 1995). The DExH conserved sequence is in domain II. The clone pBC1.6 was not long enough to compare into this domain. The amino acids below amino acid 101 in pBC1.6 represent the possible amino acids that could be encoded if the proposed splice sites in Figure 4.15 were incorrect. 5' shows the amino acids after the proposed 5' splice site and 3' shows the possible amino acids before the 3' splice site.

4.11 Southern Analysis of pBC1.6

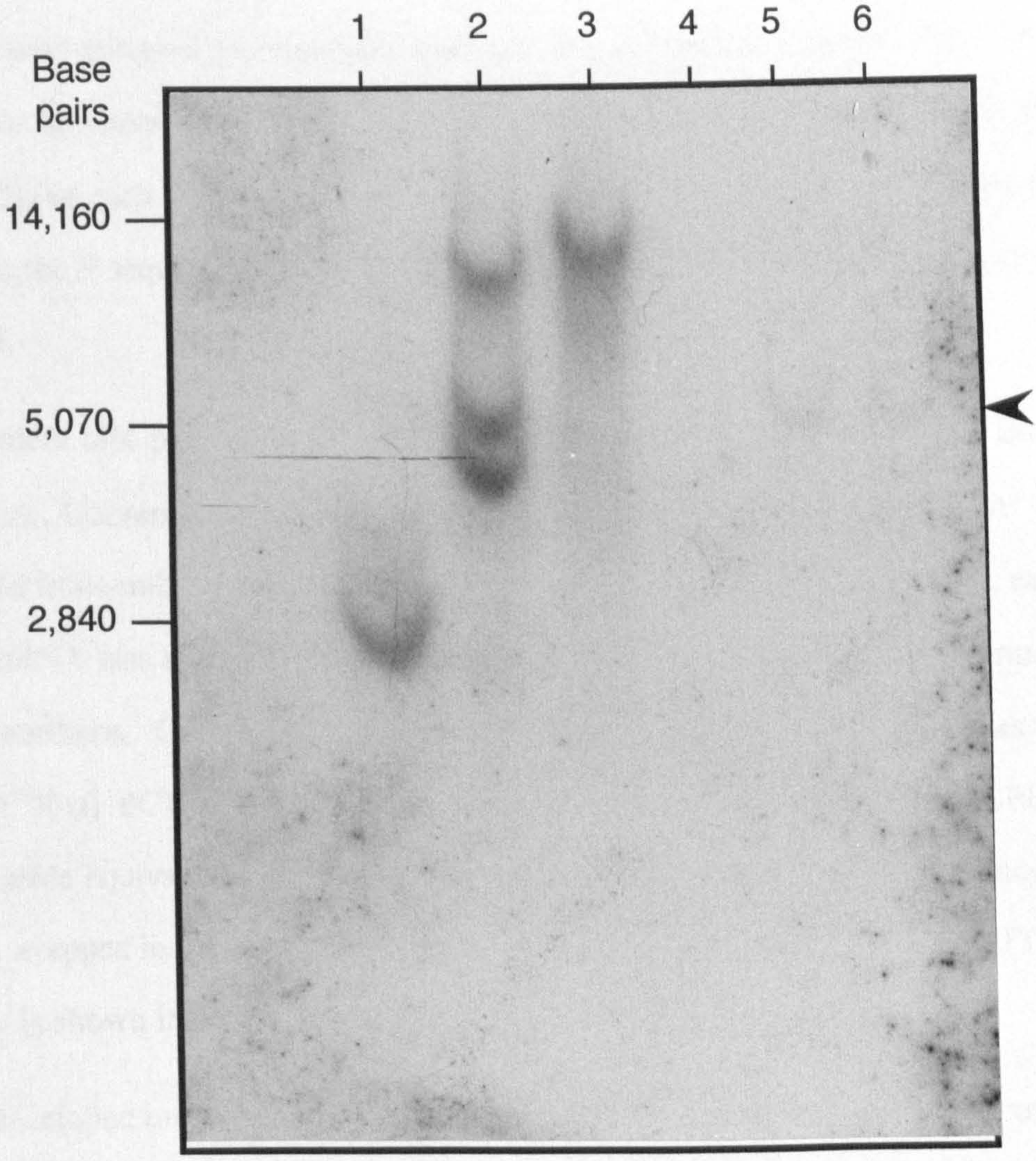
The cDNA sequence of pBC1.6 was used to probe a Southern blot of fragmented genomic DNA from *A.thaliana* and *B.napus*. This would indicate how many copies of the gene for the pBC1.6 cDNA were present in the genomes of these species.

Genomic DNA was prepared from leaf tissue of *A.thaliana* var. Columbia and *B.napus* var. Jet neuf. Southern blots of *Brassica napus* and *Arabidopsis thaliana* DNA digests were made with three different frequently cutting enzymes. A probe for southern analysis was made by cutting 2 μg of pBC1.6 DNA with Eco RI and Xho I. These restriction enzymes both cut at the cloning sites and not internally to produce a 1,800bp fragment as shown in Figure 4.4. The DNA was isolated from a 0.7% agarose gel and 90ng was labelled with [^{32}Pα]-dCTP. The southern blot was pre-incubated and hybridised with the probe for 6 hours before being washed and exposed to X-ray film. The image developed is shown in Figure 4.25.

One hybridising band is just visible in lanes 5 and 6, implying the existence of one copy of this gene in *Arabidopsis*. Lane 2 shows 3 bands which might suggest the existence of 3 genes in *Brassica napus* but Lanes 1 and 3 show only 1 band. As *Brassica napus* is a tetraploid genome we would expect twice as many genes as in *Arabidopsis* so there are probably 2 genes in *Brassica napus*.

Figure 4.25 Southern blot of *Brassica napus* and *Arabidopsis thaliana* genomic DNA using pBC1.6 DNA as a probe.

Ten microgram each of *Brassica napus* and *Arabidopsis thaliana* genomic DNA were run per lane. Lane 1-3, *Brassica napus* ; Lane 4-6, *Arabidopsis thaliana* . Lane 1+4 Eco RI, Lane 2+5 Bam HI, Lane 3+6 Hind III. The probe was hybridised to the blot for 6 hours in an equivalent of 5× SSC. The blot was washed with 2 × SSC, 0.1% SDS at 65°C before cooling, washing with 2 × SSC and sealing in SaranWrap. The Southern was exposed to pre-flashed X-ray film overnight at -80°C and developed the subsequent day. The arrow marks the faint *Arabidopsis* band.


1
2
3
4
5
6
Base pairs
14,160
5,070
2,840

4.12 Northern Analysis of pBC1.6

The cloned cDNA in pBC1.6 was used to probe a northern blot of polyA$^+$ mRNA isolated from various tissues of *Brassica napus*. This would reveal the expression of the mRNA for pBC1.6 in these tissues and provide information of the size of a full length mRNA in *Brassica napus* indicating how much of the cDNA had been cloned and sequenced

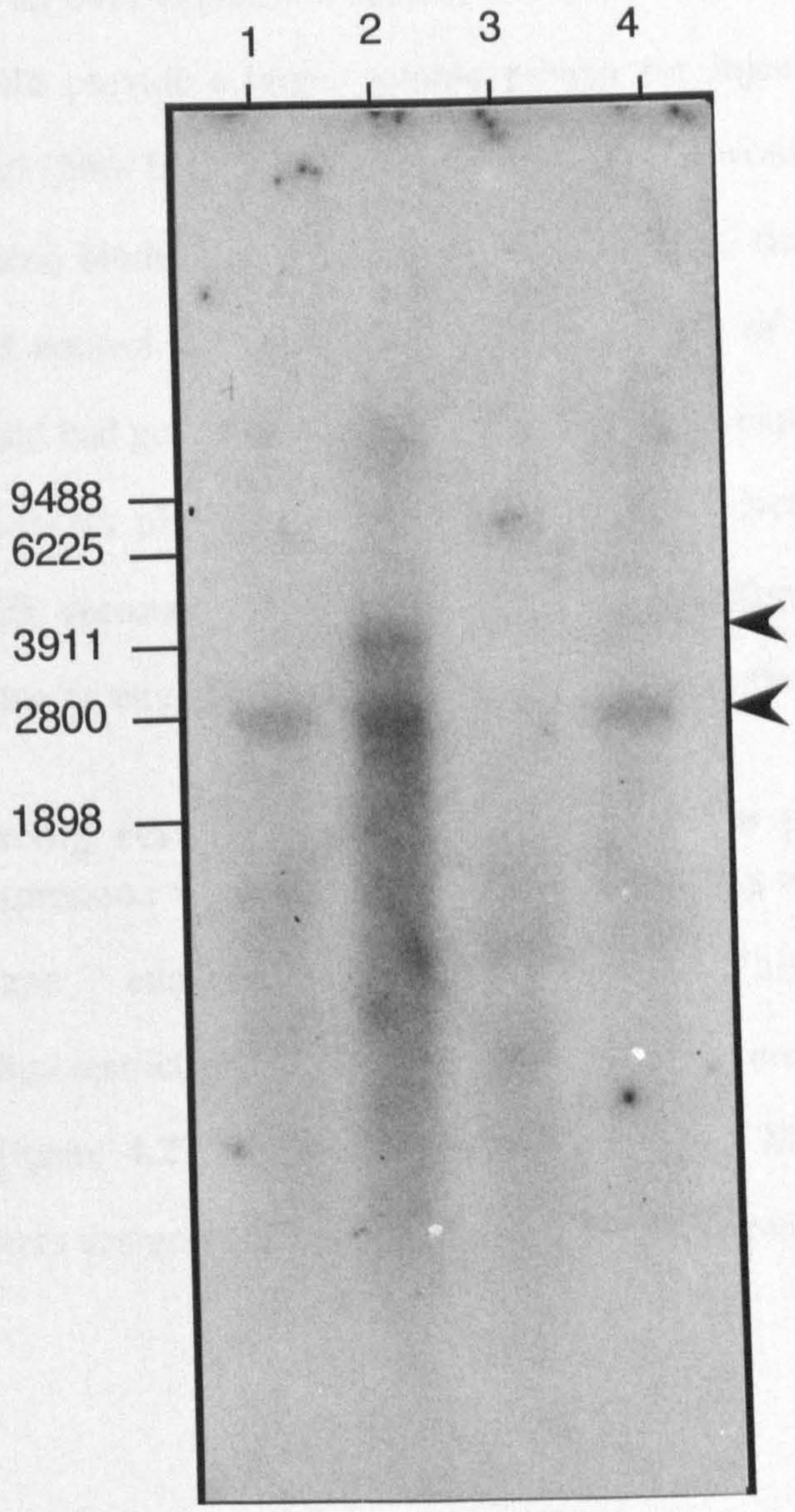
The probe prepared for Northern analysis was substantially shorter than the probe used for Southern analysis. A Bst XI and Xho I digest was done on 2µg of pBC1.6 using 2U of each enzyme, to produce a fragment of 1100bp in length. This did not contain the 5' sequence or the putative intron sequence as Bst XI cuts 664bp from the 5' end.

A northern blot previously prepared by Russell Swinhoe, Department of Biological Sciences, University of Durham, was used. Each lane had 1µg of poly A$^+$ mRNA isolated from embryos of *Brassica* napus var. Westar at two different stages, early and late. mRNA was also isolated from roots and leaves of *Brassica napus* and run on the same northern. Of the isolated pBC1.6 Bst XI - Xho I fragment, 70ng was labelled with [^{32}P-α] dCTP. The probe was hybridised overnight in 6 × SSPE, 50% formamide equivalent at 42°C. The northern was washed to 80% stringency before being wrapped in Saran Wrap and exposed to X-ray film for 3 weeks at -80°C. The image is shown in Figure 4.26.

The developed image shows that pBC1.6 is expressed in rape embryo and root, but no expression was detected in leaf. The size of the transcript is 2,800bp suggesting that we have sequenced over half of the cDNA for this mRNA. Also evident, is a transcript of a larger number of base pairs in older embryos. The nature of this larger transcript is unknown, it could be a similar gene which is expressed only in late stage embryos. It is also possible that it could be a unprocessed mRNA in which an intron or some introns have not been spliced. This may explain why the original pBC1.6 clone was isolated with an intron sequence in the reading frame.

Figure 4.26 Northern blot of pBC1.6 using *Brassica napus* poly A^+ mRNA.

All lanes contain approximately 2μg of poly A^+ mRNA. Lanes contain polyA^+ mRNA isolated from the following tissues; Lane 1, embryo stage A+B (early to mid, Slabas 1995); Lane 2, embryo stage C (late, Slabas 1995); Lane 3, Leaf; Lane 4, Root. The probe was hybridised to the blot for 16 hours in 5 × SSPE, 50% formamide at 42°C before washing with 2 × SSC at 42°C and sealing in SaranWrap. The blot was exposed to a pre-flashed X-ray film for 3 weeks at -80°C and developed. The DNA standards were cut from the agarose gel before blotting and the number of base pairs is shown alongside. The calculated size of the bands marked by the arrows are 2800bp and 3900bp.


1
2
3
4
9488
6225
3911
2800
1898

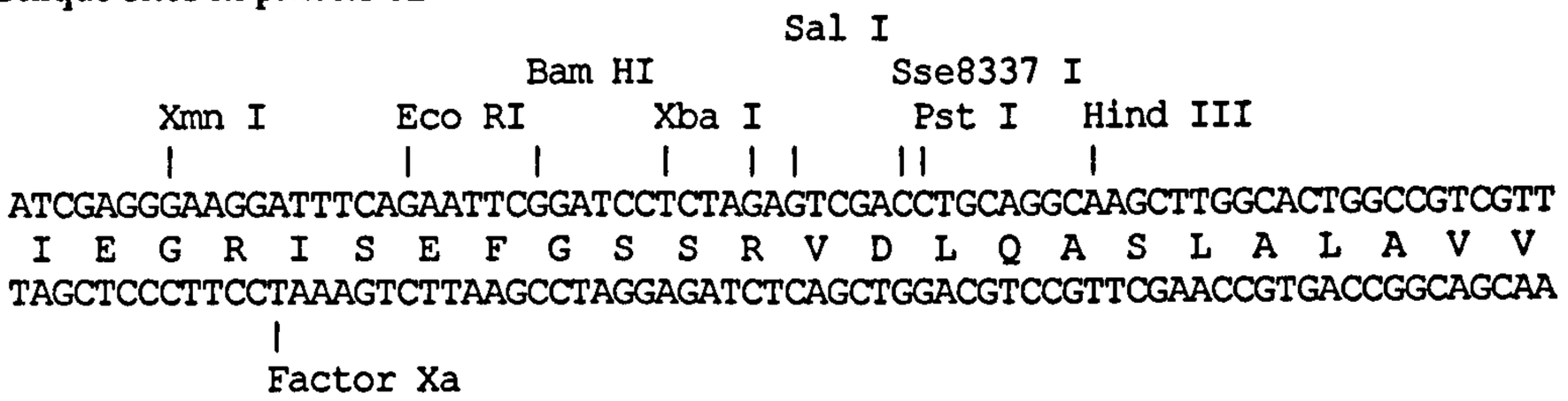
4.13 Overexpression of pBC1.6 as a fusion protein

Heterologous expression studies were carried out with pBC1.6 in order to learn two things. 1) Was an over-expressed protein biotinylated, if so, it could be purified and sequenced to determine the biotinylation site. 2) An over-expressed protein could be used to raise antibodies which are powerful analytical tools for studying proteins. With these is mind, an over-expression system was used which would facilitate easy purification and could provide a large, soluble protein for injection. The vector chosen was pMAL-c2 (NEW ENGLAND BIOLABS). This vector would produce a fusion protein with a maltose binding protein (not present in plant tissues), from a p*tac* promoter under *lac*I control. This was not the first choice of vector. Previous workers in the lab had had good results from NOVAGEN's pET expression system, but this could not be used for pBC1.6 as the sequence has both Nco I and Nde I sites internally, which pET vectors required for 5' ligation. Therefore, it would not have been possible to clone an engineered 5' Nco I or Nde I site into the pET vector system.

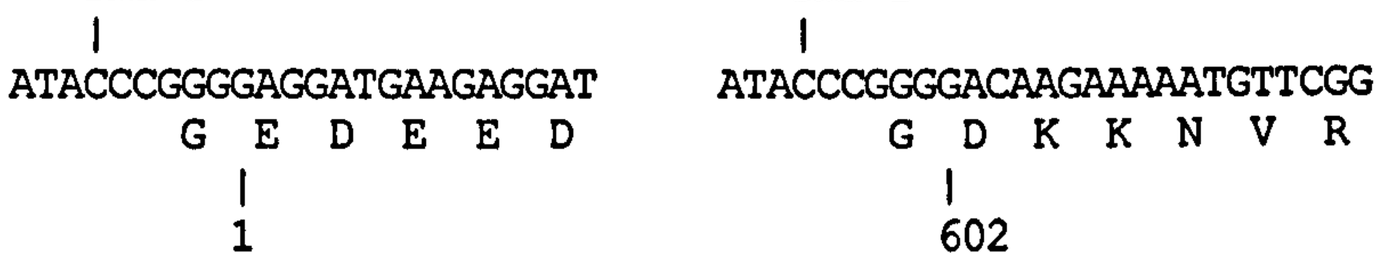
4.13.1 Engineering restriction sites for cloning into pMAL-c2

Cloning into the expression vector pMAL required engineering of restriction sites, in frame, into the 5' and 3' end of the open reading frame. This was done by PCR using primers that had restriction sites engineered into them, to enable in-frame cloning into pMAL-c2. Figure 4.27 shows the unique sites in the MCS of the pMAL-c2 vector and the primers designed to create restriction sites for cloning.

Unique sites in pMAL-c2



Primers for engineering restriction sites



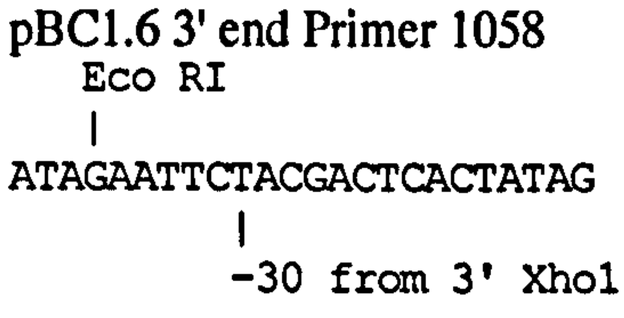


Figure 4.27 Unique sites in the pMAL-c2 polycloning site.

The protein translation is shown with the Factor-Xa proteolytic cleavage site. The primers are designed to hybridise to pBC1.6 DNA at their 3' end with a tail containing a restriction site at the 5' end. When PCR was performed with the above primers, a new DNA fragment with restriction sites at either end was produced which, when cut with Sma I and Eco RI could be ligated into the pMAL-c2 vector cut with Xmn I and Eco RI. The result would be to produce a fusion protein between the maltose binding protein in the pMAL-c2 vector and the pBC1.6 open reading frames. The primer at the 5' end would fuse the initial 1st open reading frame to the maltose binding protein. The internal primer would fuse the 2nd open reading frame to the maltose binding protein from base 602 (after the putative intron). The 3' end primer was located 30 base pairs past the Xho1 cloning site in the pBluescript™ vector in both cases the open reading frames would come to a stop at their own stop codons.

4.13.2 Construction of pMAL-1.6 expression vector

PCR was performed with primer pairs 1057 + 1058 and 200pg of pBC1.6 DNA to produce a 1914bp fragment, and with primer pairs 1106 + 1058 to produce a truncated 1336bp fragment. The PCR reactions with primers 1057 + 1058 were phenol / chloroform extracted and ethanol precipitated to produce a pure DNA pellet. This was re-suspended in 10µl of ddH_2O and cut with Eco RI and Sma I to produce the restricted DNA. The restricted PCR fragment was run on a 1% agarose gel and the

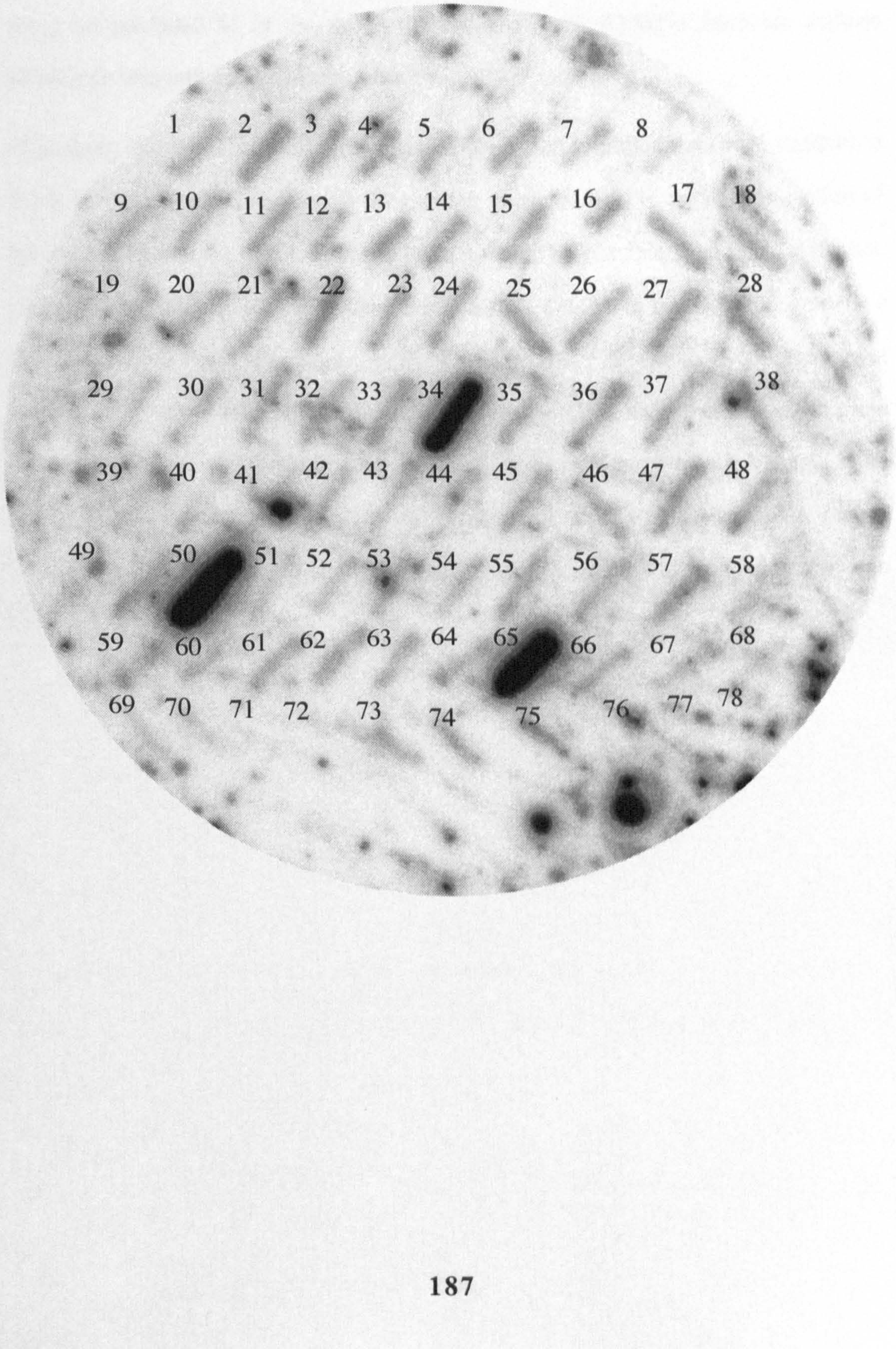
fragment isolated. This was ligated into pMAL-c2 cut with Xmn I and Eco RI, with T4 DNA ligase. The pMAL-c2 vector contains a β-galactosidase protein fused to the maltose binding protein. If a insert is made into the polycloning site the *lacZ* fusion is disrupted. This enables blue / white selection when plasmids are transformed into *E.coli* DH5α and plated on IPTG / X-gal plates. To confirm that the pMAL-1.6 ligation products contained the correct ligation product, 78 colonies resulting from the ligation of Xmn I / Eco RI cut pMAL-c2 and the Sma I / Eco RI cut PCR fragment from the PCR reaction with primers 1057 and 1058, were replica plated as follows; 1, Master plate LBamp; 2, X-gal/IPTG, blue white selection; 3, Hybond N filter on LBamp plate. The plates were marked into 70, 0.5cm^2 squares and a colony picked from the transformation plate and streaked onto each of the three replica plates. The colonies were allowed to grow overnight at 37°C.

The Hybond N filter was then screened with the radioactive probe of pBC1.6 made for southern analysis, to look for colonies that contained an insert. The colonies were lysed in by laying the filter on a pool of 2% SDS for 5 minutes. The filter was then processed in the same way as southern blots, except a pre-hybridisation wash with 0.1% SDS was done to remove bacterial debris baked onto the filter. The filter was hybridised to the probe for 4 hours at 65°C, 5 × SSC equivalent and washed with 0.1% SDS 0.2 × SSC at 65°C before wrapping in SaranWrap and exposing to a phosphor imaging screen for 16 hours. The image was scanned in using a BIO-RAD Molecular Imager at 100μm resolution and is shown in Figure 4.28.

This showed that colonies 34, 50 and 65 were positive. These were all white colonies when grown in the presence of X-gal and IPTG. These results indicated that these colonies contained plasmids with the pBC1.6 insert in them.

Figure 4.28 Analysis of pMAL transformants for pBC1.6 insert by hybridisation with radioactive probe.

Colonies were replica plated onto Hybond-N on LBamp and grown overnight. The colonies were lysed on 2% SDS before the DNA was fixed to the filter. Colonies were screened by hybridising with the pBC1.6 probe used previously for Southern analysis. The probe was hybridised to the filter for 4 hours at 42°C in 50% formamide, 5 × SSC equivalent before washing with 0.1% SDS, 0.2 × SSC, wrapping in SaranWrap and exposing to a phosphor imaging screen for 16 hours. The image was scanned at 100μm resolution. Colonies 34, 50 and 65 are positive.


1 2 3 4 5 6 7 8
9 10 11 12 13 14 15 16 17 18
19 20 21 22 23 24 25 26 27 28
29 30 31 32 33 34 35 36 37 38
39 40 41 42 43 44 45 46 47 48
49 50 51 52 53 54 55 56 57 58
59 60 61 62 63 64 65 66 67 68
69 70 71 72 73 74 75 76 77 78

The direction of insertion for each of the three positives 34, 50 and 65 was checked using restriction digestion with Nde I and Pvu II. Both these enzymes cut within the insert and the vector to produce a predictable restriction pattern. If the insert was somehow ligated in the reverse orientation a characteristic restriction pattern would be observed. In all three cases the correct restriction pattern was seen and the expression vector was used for further analysis. This construct was named pMAL-1.6(55), 55 being the predicted M_r of the expressed fusion protein, 42.5kDa from the maltose binding protein and the remainder from the pBC1.6 insert.

PCR using primers 1106 and 1058 consistently failed to produce a single product of the desired size in PCR reaction under a variety of conditions. Careful examination of the DNA sequence for pBC1.6 revealed that it would be possible to produce a ligation of pBC1.6 into pMAL-c2 using the restriction sites present in pBC1.6 and produce a translational fusion. There were two Pst I sites in pBC1.6, one at 284bp and one at 560bp. The second Pst I site at 560bp would cut the DNA such that if it was ligated into pMAL-c2 cut with Pst I then it would produce an in-frame fusion. A single Hind III site was to be found in pBC1.6 at 1686bp, past the final stop codon of the second large open reading frame, so this was used for the 3' restriction site. This is shown in Figure 4.29.

```
                              Pst I
                              |
pBC1.6 551     GATAATAAGCTGCA GGTTGTTGTT 574
                D  N  K  L   Q  V  V  V
pBC1.6 551     CTATTATTCG ACGTCCAACAAGTT 574

                              Pst I
                              |
 pMAL-c2 2710  AGAGTCGACCTGCA GGCAAGCTTG 2733
                R  V  D  L   Q  A  S  L
 pMAL-c2 2710  TCTCAGCTGG ACGTCCGTTCGAAC 2733

                              Pst I
                              |
pMAL-1.6(80) 2710 AGAGTCGACCTGCA GGTTGTTGTT 2733
                   R  V  D  L   Q  V  V  V
pMAL-1.6(80) 2710 TCTCAGCTG GACGTCCAACAACAA 2733
```

Figure 4.29 Diagram showing the restriction sites present in pBC1.6 and pMAL-c2.

By cutting both plasmids with Pst I (Pst I cuts DNA at ctgcag before the final g) and ligating one to the other a translational fusion was produced called pMAL-1.6(80). The position in the protein can be recognised by the characteristic VVV sequence which occurs at amino acid 103 in Figure 4.24. The 3' end ligation of Hind III sites is not shown as it does not have to be in frame. Indeed a stop codon occurs 199bp before the Hind III restriction site.

Two micrograms of pBC1.6 and pMAL-c2 were incubated separately in a dual digestion with 2U of Pst I and Hind III for 4 hours. The fragments were separated on a 1% agarose gel and isolated. The isolated fragments were ligated together using T4 DNA ligase and transformed into *E.coli* DH5α cells for analysis. The transformants were analysed in the same way as before, by replica plating and hybridisation with a radioactive probe. The direction of insertion was checked by digestion with Nde I and again all positives were in the correct direction. This construct was designated pMAL-1.6(80).

4.13.3 Expression of pMAL-1.6(80) fusion protein

The construct pMAL-1.6(80) was transformed into the strain for expression *E.coli* TB1 by treatment with cold $CaCl_2$. Seed stocks were made of the transformed bacteria and kept at -80°C until required. A single seed stock was used to inoculate a 50ml flask of LB+100μg/ml ampicillin and was grown with shaking (200rpm) at 37°C until the optical density at 600nm reached 1.0. A 1ml aliquot was removed for pre-induction analysis. The cultures were then induced by making then 0.3mM IPTG.

After a further 2 hours growth the cells were harvested by centrifugation in 50ml polypropylene centrifuge tubes at 3000 × g. Cells were re-suspended in 5ml of 50mM Tris-HCl pH8.0, 2mM EDTA a 100µl aliquot removed and the remainder frozen for future use.

The 1ml pre-induction aliquot was spun down in a microfuge and re-suspended in 100µl of 1× SDS sample buffer. This was boiled for 2 minutes to lyse the cells and release the proteins. After cooling the lysed cells were spun for 1 minute in a microfuge and 10µl loaded onto a 12% acrylamide gel. The 100µl aliquot of the post-induction cells was made 1× SDS sample buffer by the addition of 25µl of 5× SDS sample buffer and boiled for 2 minutes. After cooling the cells were spun for 1 minute in a microfuge and 10µl was loaded onto a 12% acrylamide gel for analysis. The results of these expression experiments are shown in Figure4.30.

The expression results shown in Figure 4.30 show that a protein of around the predicted molecular weight (80kDa) is being expressed by the cells containing the pMAL-1.6(80) vector construct. The Western blot of the same proteins also shows that the product is not biotinylated. The only band visible on the western is the 22kDa subunit of acetyl-CoA carboxylase found in *E. coli.* Similar experiments were performed with the pMAL-1.6(55) construct which produced an overexpressed protein of the predicted molecular weight but again, Western blots showed the product was not biotinylated. This meant it was not possible to sequence the biotinylated protein to determine the biotinylation site.

Figure 4.30 A Coomassie R-250 stained 12% gel of *E.coli* TB1 cells harbouring pMAL-1.6(80).

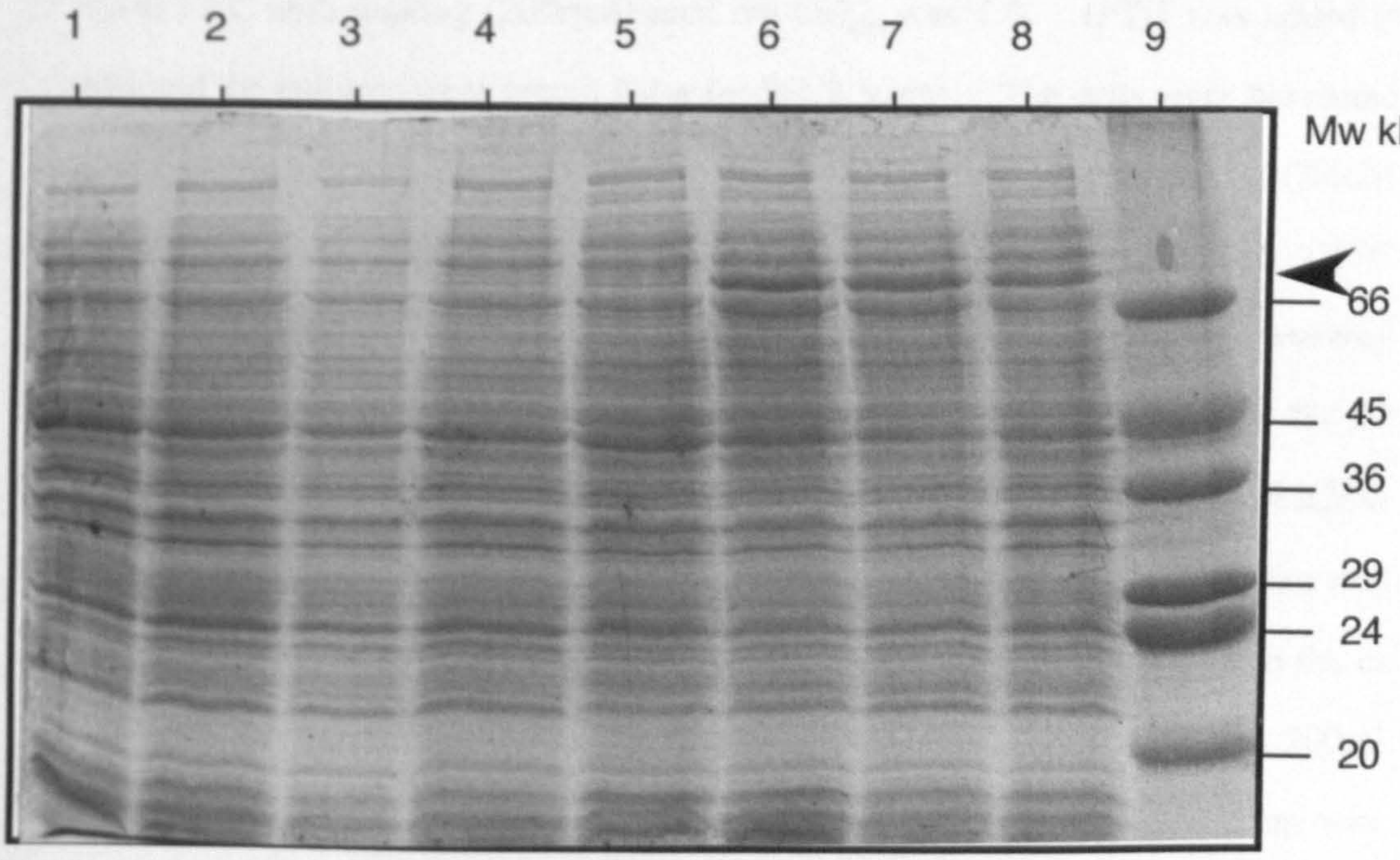
Cells were grown in LB to mid-log phase, made 0.3mM IPTG and grown for another 2 hours after which time the cells were harvested. Whole cells from uninduced and induced time points were boiled in 1× SDS sample buffer to lyse the cells and release the proteins.

The lanes contain the following; Lanes 1+ 5; *E.coli* TB1 cells (no plasmid, grown without ampicillin in culture) ; Lanes 2+6, pMAL-1.6(80) number 1; Lanes 3+7, pMAL-1.6(80) number 2; Lanes 4+8, pMAL-1.6(80) number 23; Lane 9; Mw markers (SDS7 or biotinylated markers). Lanes 1-4 are uninduced cells, Lanes 5-8 are induced cells 2 hours after induction. The overexpressed protein can be seen above the 66kDa Mw marker in lanes 6-8 next to the arrow.

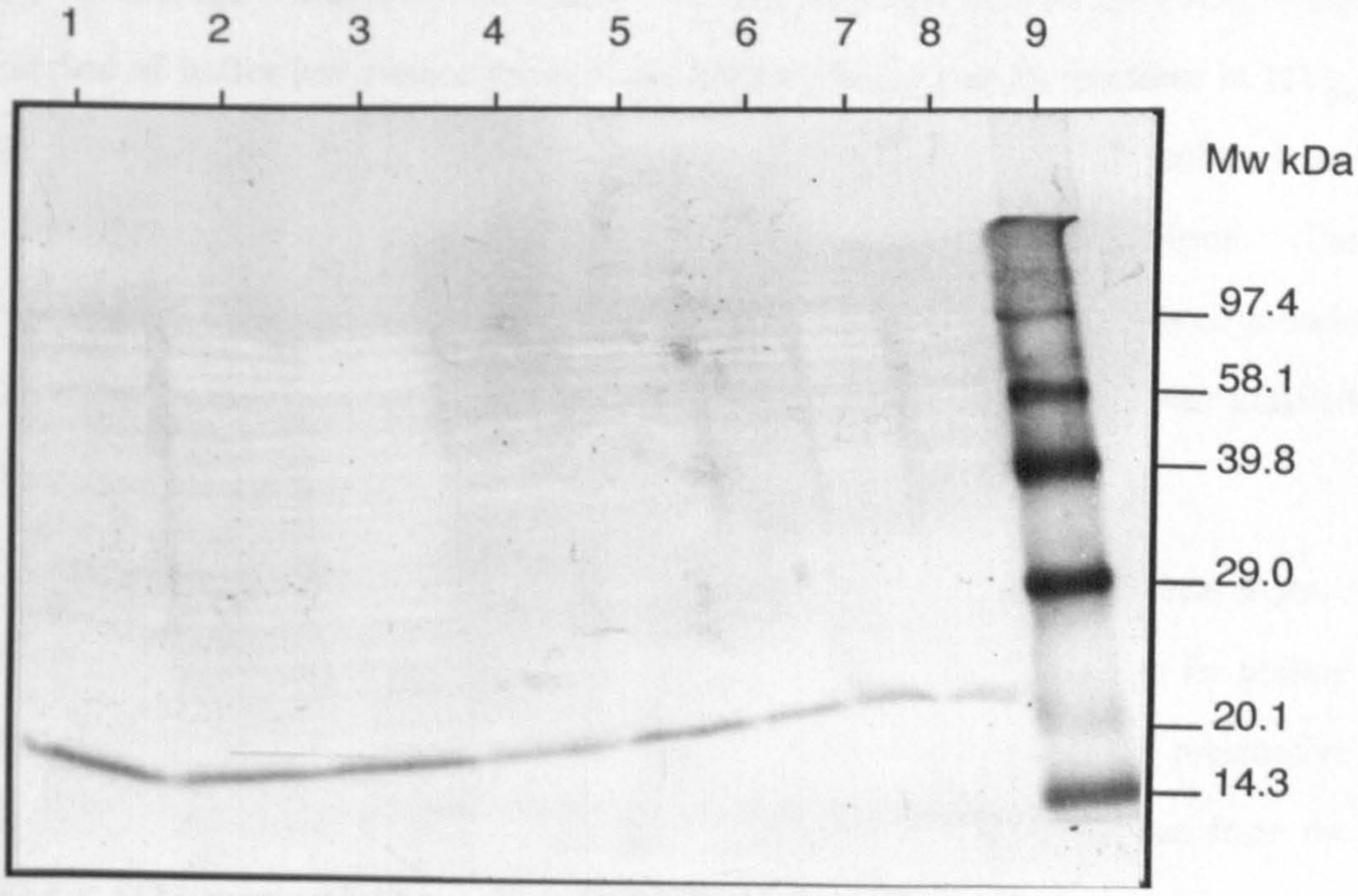
Figure 4.30 B Western blot with anti-biotin antibodies of duplicate gel to that shown in Figure 4.30 A.

A 12% gel was run as for A but the proteins were not stained but transferred to nitrocellulose. Proteins were detected with affinity purified anti-biotin antibodies. The secondary antibody was donkey anti-sheep IgG : alkaline phosphatase conjugate and the Western blot was developed with BCIP/NBT. Lanes contain the same as in A. The only visible detected band is the *E.coli* BCCP protein (22kDa).

A


1
2
3
4
5
6
7
8
9
Mw kDa
66
45
36
29
24
20

B


1
2
3
4
5
6
7
8
9
Mw kDa
97.4
58.1
39.8
29.0
20.1
14.3

4.13.4 Purification of the pMAL-1.6(80) protein for antibody production

Two times 1L of LB+100μg/ml ampicillin were inoculated with a seed stock and grown at 37°C with shaking (200rpm) until the OD_{600} was 1.0. IPTG was added to 0.3mM and the cultures were grown for a further 2 hours. The cells were harvested by centrifugation at 3,000 × g and re-suspended in 2 × 50ml of column buffer (20mM Tris-HCl pH7.4, 1mM EDTA, 200mM NaCl). The cells were frozen at -20°C overnight to weaken the cells. After thawing the cells were broken by passing through an automated French press. The broken cells were spun at 40,000 × g for 30 minutes to remove cell debris and the supernatant filtered through a Millipore 0.22μm filter. A 10ml column of amylose resin was poured into a 2.5 × 10cm column and washed with 100ml of column buffer at a flow rate of 1ml/min after which time the in line UV_{280} lamp was set to zero. The bacterial protein extract was diluted with 300ml of column buffer and applied to the column at a rate of 1ml/min. The column was washed with 100ml of column buffer at 1ml / min or until the UV_{280} trace returned to near zero, before proteins were eluted with column buffer + 10mM maltose. As soon as the maltose buffer was applied to the top of the column, a fraction collector was started to take 5ml fractions of the elutate. Proteins were eluted from the column after about 5ml of buffer had passed through the column judging by the increase in UV_{280} absorbance. The protein in the fractions eluted from the amylose column had precipitated out of solution and the fractions appeared opaque white in colour. The protein in these fractions was analysed by SDS /PAGE. Both the precipitated protein and the soluble proteins were of insufficient purity for injection. Protein was purified further by preparative gel electrophoresis.

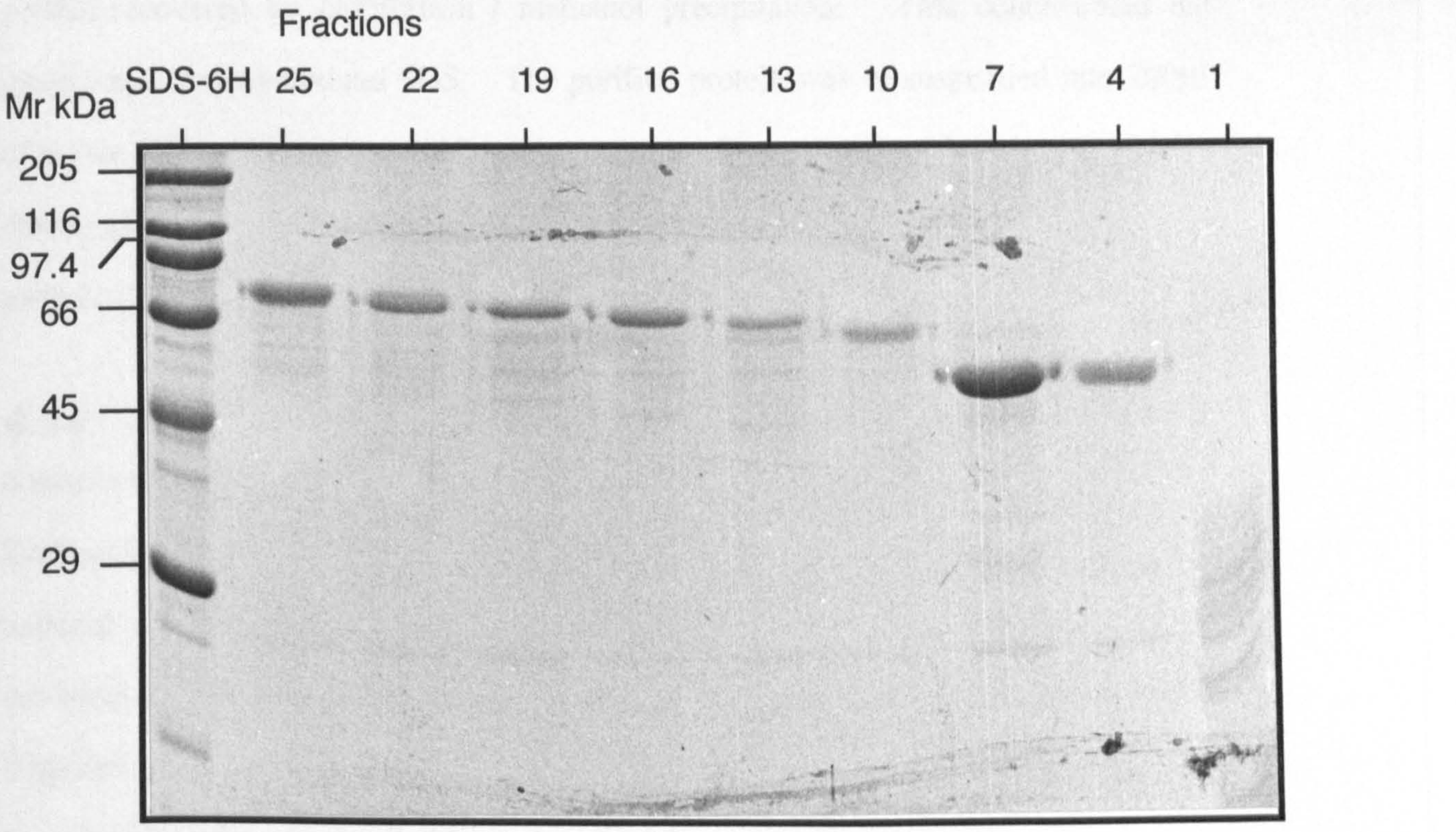
Protein eluted from the amylose column was pooled and precipitated by chloroform / methanol. The protein was re-suspended in 2ml of 1 × SDS sample buffer by boiling and vortexing. The protein was loaded onto the top of a 120mm, 7.5% preparative continuous elution gel and run at 10W constant power. Proteins were eluted from the gel in 1 × SDS running buffer. Five millilitre fractions were collected when the dye

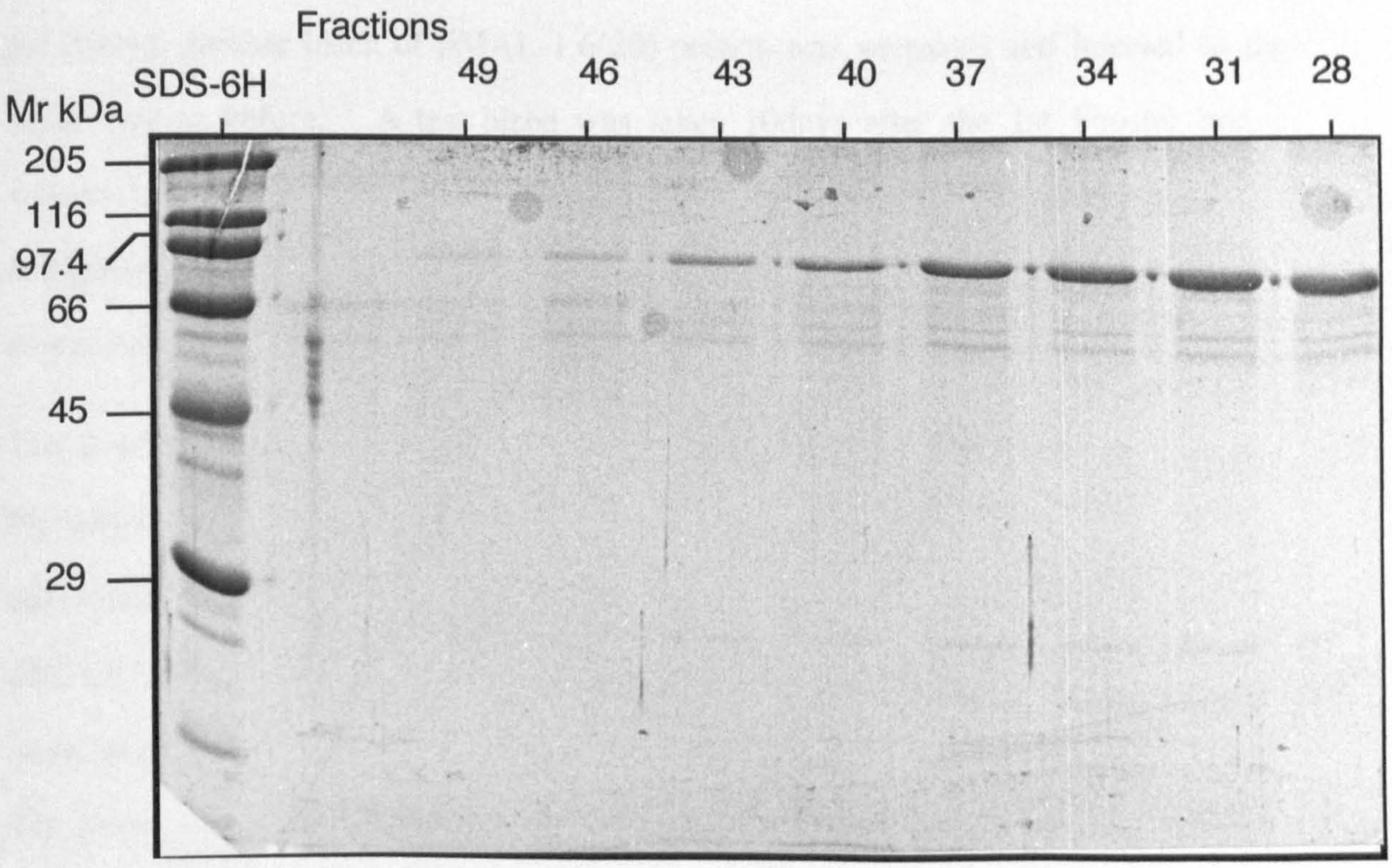
front had eluted from the gel. Every 3rd fraction was analysed for protein content by SDS/PAGE on a 12% acrylamide gel (Figure 4.31).

The gels show the stages in elution of proteins from the preparative gel. Fraction 1 contains many low M_r contaminant proteins which contaminated the elutate from the amylose column. The main contaminant from the amylose column was separated away from the 80kDa protein and is visible in Fractions 4 and 7. As this protein also bound to the amylose column, it was probably a proteolytic fragment of the pMAL-1.6(80) protein product.

Figure 4.31 Fractions eluted from a 7.5% preparative gel and analysed on a 12% acrylamide gel.

Proteins eluted from the amylose column were precipitated and dissolved in 2ml of 1 × SDS sample buffer. This was loaded onto a 120mm, 7.5% preparative gel and run at 10W constant power in the BIO-RAD 491 Prep cell. Every 3rd fraction collected from the gel was analysed by SDS/PAGE for protein content. 100µl of each fraction was chloroform / methanol precipitated and re-suspended in 1 × SDS sample buffer. This was run on a 12% gel and stained with Coomassie R-250.


Fractions
SDS-6H
25
22
19
16
13
10
7
4
1
Mr kDa
205
116
97.4
66
45
29


Fractions
SDS-6H
49
46
43
40
37
34
31
28
Mr kDa
205
116
97.4
66
45
29

Protein fractions containing the purified protein (fractions 25-37) were pooled and the protein recovered by chloroform / methanol precipitation. This concentrated the protein and removed bound SDS. The purified protein was re-suspended into 200µl of sterile PBS and stored at -20°C until required. By using a combination of affinity chromatography and preparative gel electrophoresis, approximately 500µg of the pMAL-1.6(80) protein product has been purified to >95% purity.

4.14 Anti-pMAL-1.6(80) protein antisera.

Antisera was produced to the purified pMAL-1.6(80) protein by injection into a New Zealand White rabbit. A day before the antigen was required for injection, the frozen material was thawed and emulsified with initially an equal volume of Freund's complete adjuvant (FCA). The material was loaded into 2 × 1ml syringes with a 23gauge needle and injected intramuscularly to the rabbit. The rabbit followed a course of injections as detailed in section 2.4.15

The first rabbit died 7 days after the first booster was given due to unknown causes, no test bleed had been taken other than the pre-immune. A second rabbit was purchased, another batch of pMAL-1.6(80) protein was prepared and injected in the same way as before. A test bleed was taken 10days after the 1st booster and a western on total protein extracted from *Brassica napus* is shown using these antibodies in Figure 4.32. Unfortunately no further bleeds were taken because the rabbit was put down 3 days later due as it continued to refuse food.

The Western shown in Figure 4.32 A showed that a dominant band was detected by the antisera at about 50kDa. This was probably not the pMAL-1.6(80) protein equivalent as the detected protein is very highly expressed in leaf, whilst no mRNA for pBC1.6 could be detected in this tissue. The detected protein had approximately the same M_r as the rubisco protein (50kDa). No other obvious bands are in evidence. The proteins detected at around 29kDa were probably the background picked up by antibodies in the serum and not those raised to pMAL-1.6(80) as they are of a much lower M_r than expected, based on the size of the mRNA detected on northern blots.

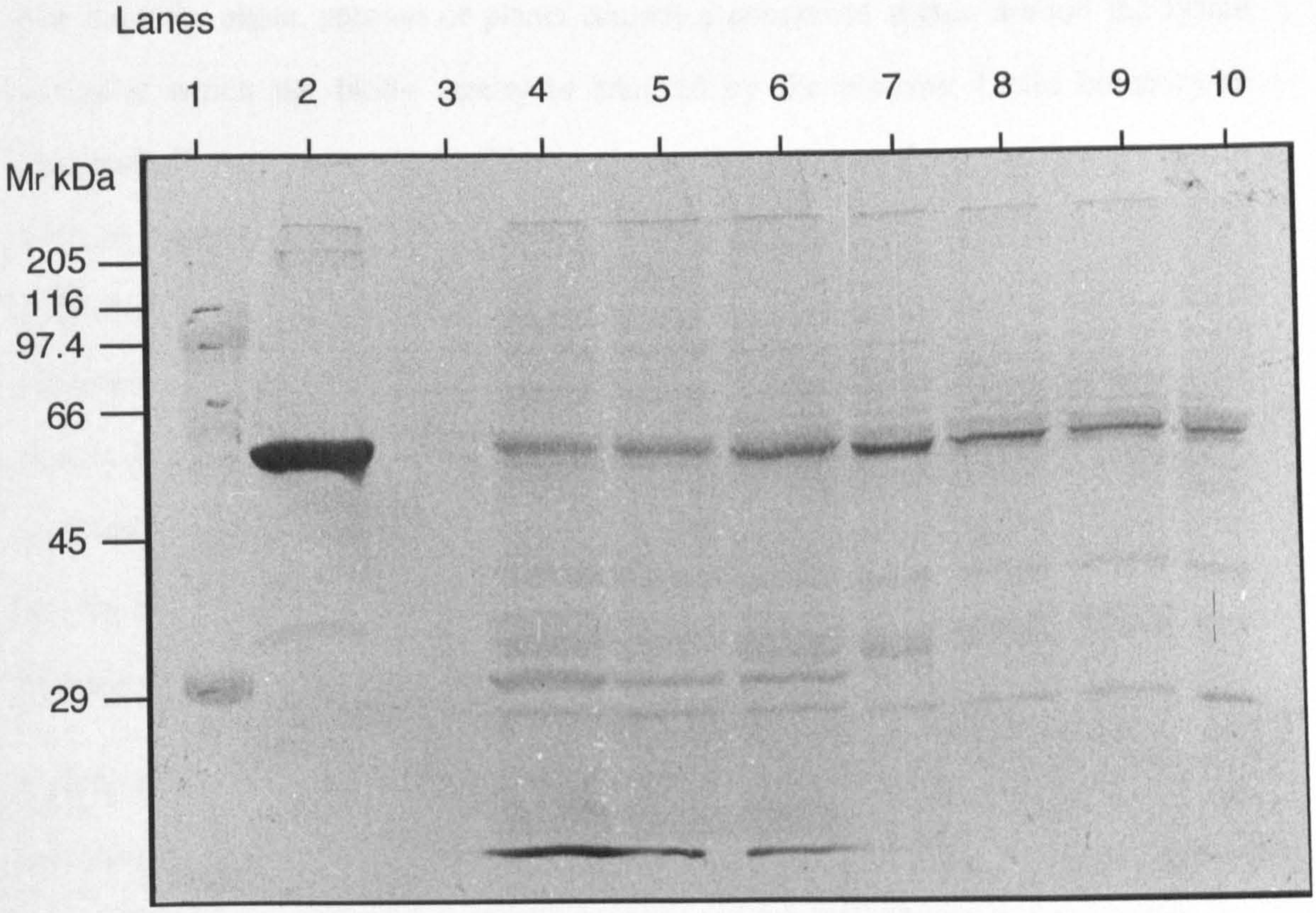
Figure 4.32 **A** Western blot with anti-pMAL-1.6(80) antisera of total protein extracts from *Brassica napus* leaf, root and developing seed material.

Extracts were prepared as described in section 2.4.2. Samples were re-suspended in 100µl of 1 × SDS sample buffer by boiling for 2 minutes and vortexing for 10 minutes. Equal loadings of 25µl were applied to each lane. The lanes contain total protein extracts from developing embryos, lanes 1-7; root, lane 8; leaf lane 9; and M_r markers lane 10. The developing embryo extracts were from embryos 38, 44, 47, 51, 56, 61 and 70 daf. Samples were electrophoresed on a 12% acrylamide gel and transferred to nitrocellulose membrane. Proteins were detected with 1:1000 dilution of the rabbit anti-pMAL-1.69(80) sera. The secondary antibody was sheep anti-rabbit IgG : alkaline phosphatase conjugate and the image was developed with BCIP/NBT.

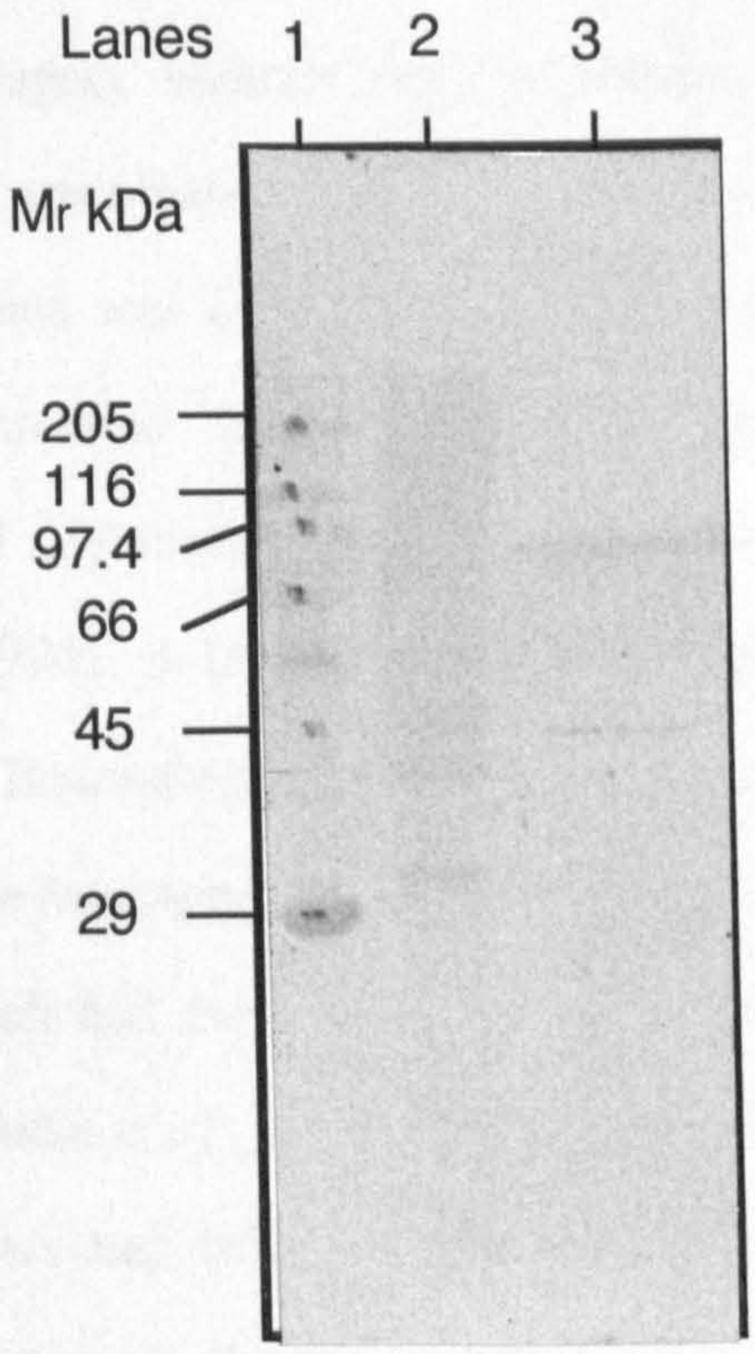
Figure 4.32 **B** Western blot with pre-immune serum from the rabbit used for pMAL-1.6(80) antibodies.

A bleed was taken from the rabbit 14 days before the initial injection. A total protein extract prepared as described in section 2.4.2 from embryos 49daf was run on a 12% gel (Lane 2) with SDS-6H M_r markers (Lane 1). Lane 3 contains 1µl of the purified pMAL-1.6(80) protein which was injected. Proteins were transferred to nitrocellulose and detected with a 1:500 dilution of the sera. The secondary antibody was sheep anti-rabbit : alkaline phosphatase conjugate and the image was developed with BCIP/NBT.

A


Lanes
1
2
3
4
5
6
7
8
9
10
Mr kDa
205
116
97.4
66
45
29

B


Lanes
1
2
3
Mr kDa
205
116
97.4
66
45
29

4.15 Discussion

With only one exception all biotin-containing enzymes sequenced to date, whether from bacteria, algae, animals or plants contain a conserved region around the lysine residue at which the biotin moiety is attached by the enzyme, biotin holoenzyme synthetase (Duval *et al.*, 1994). This means that expression of a heterologous protein in *E.coli* which would be biotinylated in its host organism, can be biotinylated by the biotin holoenzyme synthetase of *E.coli* (Cronan, 1989). This has led to "false" positives for some researchers when screening cDNA libraries with a biotinylated primary probes which they have later detected with avidin or streptavidin (Collins *et al.*, 1987, Hoffman *et al.*, 1987, Wang *et al.*, 1994). For researchers hoping to identify biotin-containing enzymes this represents a possible method to easily clone the relevant genes.

A series of biotin-positive plaques were obtained by Dr. Reddy and co-workers, when screening an *A.thaliana* inflorescence library for calmodulin binding proteins. The primary probe used for screening the library was biotinylated calmodulin which was detected with avidin linked to horse radish peroxidase. These clones reacted directly with the secondary detecting reagent without first incubating with biotinylated calmodulin. When sequence data was obtained from the 3' end of one of these clones the conserved biotinylation sequence was seen, providing good evidence that these clones encoded biotin-containing proteins. pBC1.2 has been sequenced in its entirety and, although previously identified as β-methylcrotonyl-CoA carboxylase shortly after our work began (Wang *et al.*, 1994), it is conclusive proof that biotin-containing enzymes can be cloned this way. Interestingly, the original isolation of the cDNA for β-methylcrotonyl-CoA carboxylase from tomato (Wang *et al.*, 1994), was by screening a cDNA library with a clone which had previously isolated by mistake when using avidin as a secondary probe (Hoffman *et al.*, 1987). The southern blot of MCCase on restriction digests of *Brassica napus* and *Arabidopsis thaliana* genomic DNA suggest that there are two genes for MCCase in *A. thaliana* and four genes in *B. napus*. It is not known how these two genes are expressed or the pattern of expression of isoforms

in either species. This is indicative of there being two different genes with separate functions in *A. thaliana.* As the *B. napus* genome is tetraploid, it would be expected for there to be double the number of gene seen in *A. thaliana.* This is consistent with the observation of two genes in a southern blot of tomato genomic DNA (Wang *et al.*, 1994), and their isolation of two different genomic clones from tomato. Northern analysis of β-methylcrotonyl-CoA carboxylase shows that levels of mRNA encoding this enzyme are low in developing embryos in comparison to leaf and root material. This observation is consistent with the observations in which the activity of MCCase was measured in various plant tissues (Clauss *et al.*, 1993). It was found that MCCase has high activity in older and dark grown tissues compared to young and growing tissues in the light. The northern blot in Figure 4.10 shows that expression of the mRNA for MCCase is higher at the end of embryogenesis that at the beginning, which may suggest a role for MCCase in senescent tissues in the breakdown of leucine perhaps or for cycling of mevalonate (Clauss *et al.* 1993).

pBC1.6 was isolated by the same techniques as pBC1.2. The plaques were found to be positive when screened with avidin-HRPO alone without an intermediary. It has been further demonstrated that the phage containing pBC1.6 produce a biotinylated protein by detection of the plaques with streptavidin-HRPO, and also by the use of lysogens. By generating lysogens it was possible to exactly locate the protein being produced by the phage through western blotting and detection with anti-biotin antisera (Figure 4.22). These showed conclusively that a novel biotinylated protein was produced in the bacteria and that it had a M_r of 29kDa.

The usual method of detection of proteins from lysogens is to either examine a Coomassie stained gel for a different protein pattern or, if the level of expression is too low for detection, to use anti-β-galactosidase antibodies to look for expression of the a lacZ fusion protein, created by in frame cloning of the cDNA. In the case of pBC1.6 this was not done because a lacZ fusion protein was not able to be expressed as could be seen from DNA sequencing (Figure 4.33)

```
1/1                                                 31/11
ATG ACC ATG ATT ACG CCA AGC TCG AAA TTA ACC CTC ACT AAA GGG AAC AAA AGC TGG AGC
M   T   M   I   T   P   S   S   K   L   T   L   T   K   G   N   K   S   W   S
61/21                                               91/31
TCC ACC GCG GTG GCG GCC GCT CTA GAA CTA GTG GAT CCC CCG GGC TGC AGG AAT TCG GCA
S   T   A   V   A   A   A   L   E   L   V   D   P   P   G   C   R   N   S   A
121/41
CGA GGA GGA TGA
R   G   G   *
```

Figure 4.33 DNA sequence of the β-galactosidase gene in pSK- expressed from *lacZ* promoter.

The underscored sequenced is the pBC1.6 insert. The first M is the start codon of β-galactosidase found in pSK- and the sequence in lower case represents the insert sequence used in construction of the library.

As the Figure 4.33 shows, a stop codon occurs in the pBC1.6 insert almost immediately. This has been confirmed by sequencing several times. If a novel protein is being expressed from pBC1.6 then, it must be being translated from an initiation codon other than the expected one of lacZ. Whilst this cannot be excluded as a possibility, there is no data to show this to be the case other than the fact that a unique biotinylated protein is seen in Westerns of lysogens containing pBC1.6. All possible open reading frames in both directions of pBC1.6 were searched for any resemblance to a biotinylation motif but no consensus sequence was found. The nearest candidate was found in ORF 2 as shown in Figure 4.21. When this ORF was expressed as a fusion protein with the maltose binding protein in a pMAL vector, the expressed protein was not biotinylated. The biotinylated protein product is only expressed in lysogens of pBC1.6 and not when the pBluescript™ plasmid containing pBC1.6 is induced in *E.coli* DH5α cells. This suggests the biotinylated gene is not under the control of the lacZ promoter. It is possible that the biotinylated gene product is translated from a mRNA made under the control of the λ P_I or P_L promoters which

initiate transcription in the opposite direction to P_{lac}. There are no open reading frames in the reverse orientation of sufficient size to generate a protein with a predicted M_r close to the observed protein. Several attempts were made at purifying the lysogen protein but these were unsuccessful because the level of expressed protein was so low (results not shown). In order to try and boost expression any proteins which were translated from their own unique initiation codons, the entire coding sequence of pBC1.6 was inserted into a pET23+ vector (NOVAGEN) in the forward direction. Unlike other pET vectors, pET23 does not provide an initiation of translation codon or a ribosome binding site. These must be provided by the inserted sequence, which they would be if a protein was being expressed from an unknown ORF in pBC1.6. Experiments revealed no expression of a biotinylated protein from this system (data not shown). As a result, the biotinylated protein product which led to the cloning of pBC1.6 has not been identified.

Why pBC1.6 phage produces a biotinylated product is probably of little relevance to the functionality of the gene it contains. It has been shown that the conserved EAMKM site is not required for biotinylation (Shenoy *et al*, 1988). The high degree of conservation around the biocytin residue in biotin-containing enzymes is more likely to be associated with the activity of the enzyme, than simply for recognition by biotin holoenzyme synthetase (Brocklehurst & Perham, 1993). If either of the two flanking methionine residues are dispensed with the lysine is still biotinylated *in vivo* but the enzymatic activity is lost (Shenoy *et al.*, 1988). Conversely, if residues +30 amino acids past the lysine to be biotinylated are mutated in the biotin subunit of transcarboxylase, the protein product is not biotinylated (Murtif & Samols, 1987). It has been demonstrated that a variety of peptides can be biotinylated by the *E. coli* biotin holoenzyme synthetase (Schatz, 1993). It is possible therefore that a segment of a recombinant protein could contain a domain which the *E. coli* biotin holoenzyme synthetase is able to recognise as a substrate, but that the complete protein may not serve as a substrate for biotinylation. The novel biotinylated protein expressed in *E.coli* BL21(λDE3) cells, encoded by the phage from which pBC1.6 was derived, has

a M_r of 29,000. The only ORF in pBC1.6 long enough to encode such a protein is ORF2 shown in Figure 4.21 in which a potential biotinylation site has been identified. This site is close to the amino terminus end of the predicted protein sequence. When this ORF is expressed as a fusion protein from a pMAL vector though, a biotinylated product is not seen. This could be as a result of changes in the primary structure or even the secondary structure when the fusion protein is present, preventing the biotin holoenzyme synthetase from recognising the site.

Southern analysis of pBC1.6 shows that the cDNA has homology to a plant gene as it hybridises to bands in southern blots of *A.thaliana* and *B napus* genomic DNA. This demonstrates that the gene is of plant origin and not a contaminant from another library. The southern blot indicates that there is only one copy of this gene in *A thaliana* and two copies in *B.napus*. The gene is transcribed as demonstrated by northern analysis of expression of mRNA in leaf, root and embryo tissues. The level of transcription would seem to be at a low level as an exposure time of 3 weeks was required to get a good image on X-ray film. No signal was seen in leaf tissues, but root and embryo tissues showed a low level of transcript of 2,800bp. In late stage embryos, a second band can be seen at a higher number of base pairs (3,900bp). This band could be a different gene which is expressed solely in late stage embryos and has high homology to pBC1.6 or it could be a product of differential splicing. The pBC1.6 cDNA was probably isolated with an intron sequence still intact. This could have been because it was an unprocessed hnRNA or it could have been a result of differential splicing to produce an early termination of translation and a truncated protein with possibly a different function. Further analysis of the gene by cloning a genomic or a full length cDNA could provide the answer.

Repeated attempts were made to isolate new clones for pBC1.6 from *B.napus* and *A.thaliana* libraries using pBC1.6 as a DNA probe, but with consistent failure. This disappointing result may have been because; a) the libraries used were all amplified libraries in which low level transcripts can be lost due to differential amplification or b) the gene was originally isolated from a library of poly A^+ mRNA extracted from

inflorescence of *A.thaliana.* The original library was not re-screened. These problems could be overcome by making a primary library from *Arabidopsis thaliana* or by obtaining a sample of the original library neither of which were as other work on biotin-containing proteins was more pressing and relevant to this study.

The antisera to pMAL-1.6(80) was particularly disappointing. It was used to probe a Western blot of *Brassica napus* proteins, but the major protein detected is most likely not the pBC1.6 equivalent, but rubisco hence its large expression in leaf. (Figure 3.3 A shows a Coomassie stained gel of purified chloroplast proteins, the rubisco protein makes up the majority of protein in this lane and runs at about 50kDa.) This protein was not detected by a 1:500 dilution of the pre-immune. Why the antisera detected rubisco is not known, but it is probably a result of the animals diet and the massive boost to the immune system caused by the adjuvant (FCA). It is not possible to assign any of the detected bands to pMAL-1.6(80) as it would be expected that the antigen being injected would produce the highest titre antibodies which has not happened in this case. Further injections and hence an increase in the titre of the pMAL-1.6(80) antibody were not possible as both animals died after booster injections for unknown reasons. If the animal had raised high titre antibodies against rubisco then it is possible that it had an immune response to its diet and stopped eating as a result. Animals bought from the same supplier, kept under the same conditions, injected with the same batch of Freund's adjuvants with antigens prepared in this laboratory have not died prematurely. Whether the actual antigen, pMAL-1.6(80) protein was responsible is not known. Other workers in this laboratory have used the pMAL system to successfully raise antibodies so the only variable in the system is the protein itself. Whether the pMAL-1.6(80) protein causes an auto-immune response or an allergic reaction is not known.

The exact function of pBC1.6 is still unknown, but the homology to known recQ DExH DNA helicases is striking in that the areas of significant homology are in the conserved helicase domains IV, V and VI present in all members of the SF2 super-family of putative DNA helicases (Gorbalenya *et al.*, 1989). When the functionality

of two non-identical proteins is thought to be the same or similar, it is often the case that the areas responsible for the activity or function of the protein are conserved. For example it has been demonstrated that all biotin-containing enzymes required the conserved biotinylation site for activity so one could predict that the sequence of an unknown biotin dependent enzyme would contain the conserved biotinylation site. In the case of DExH box DNA helicases, the structure of the proteins are highly conserved in seven identifiable regions in every case. As pBC1.6 contains the last three of these sequences, in order and with good homology to the known sequences it seems sensible to suggest that this protein should have functional similarity to the known sequences.

To date no DNA helicases have been sequenced from a plant source although a recent report described the isolation of a 68kDa protein from pea chloroplasts which has DNA helicase activity in the 3' - 5' direction (Tuteja *et al.*, 1996). The size of the mRNA on northern blots of 2800bp is of sufficient length to encode a protein of 68kDa, although it is currently not known if pBC1.6 encodes for this isolated helicase.

Chapter 5: Expression of multiple forms of Biotin Carboxyl Carrier Protein in *Brassica napus*.

5.1 Introduction

Brassica napus is an important oilseed crop, agriculturally and commercially, thus it has become the target of genetic manipulation experiments to try and exploit the oils it produces (Knauf, 1987). In order to understand and alter lipid biosynthesis in *Brassica napus*, it is important to gain an understanding of the molecular mechanisms involved in fatty acid biosynthesis and the regulation of this pathway (Slabas *et al.*, 1995). Acetyl-CoA carboxylase has been targeted for intensive study because it provides the substrate for fatty acid biosynthesis, and is probably a regulatory enzyme of the pathway as is the case in animal systems where it comes under hormonal control by phosphorylation (Postbeittenmiller *et al.*, 1991).

Acetyl-CoA carboxylase has been purified from *Brassica napus* and found to be a single 220kDa polypeptide (Hellyer *et al.*, 1986; Slabas *et al.*, 1986). Partial cDNAs for this enzyme were subsequently isolated and sequenced (Elborough *et al.*, 1994b). Evidence also accumulated which pointed to a multi-subunit form of the enzyme in higher plants (Sasaki *et al.*, 1993). A DNA sequence in the chloroplast genome was identified as having homology to the β-CT subunit of acetyl-CoA carboxylase in *E.coli* (Li & Cronan, 1992c). Other subunits were identified by their homology to known *E.coli* and algal sequences as they appeared in EST databases Several projects world-wide are focusing on sequencing random cDNAs or expressed sequence tags (ESTs) from important oilseed species such as castor (van de Loo *et al.*, 1995) and *Arabidopsis* (Newman *et al.*, 1994). Comparison of these ESTs with known DNA sequences can aid in revealing their identity. ESTs identified as having homology to subunits of ACCase from *E.coli*, have been used to clone the BC subunit of ACCase from tobacco (Shorrosh, 1995) and the BCCP subunit from *Arabidopsis* (Choi *et al.*, 1995) and *Brassica napus* (Elborough *et al.*, 1996).

The BCCP from *B.napus* is encoded for by a multigene family of at least six individual genes (Elborough *et al.*, 1996). *Brassica napus* is a tetraploid genome which means it has two sets of chromosomes (Bennet & Smith, 1976), so four different isoforms might be expected, presumably two from each parent *Brassica oloraceae* and *Brassica campestris*. The existence of two other isoforms suggests that for some reason it is important for the BCCP to be expressed as multiple isoforms. Reasons might include different subcellular localisation or a different subunit structures which could combine with other subunits of the enzyme in different ways to create a complete enzyme with altered catalytic properties.

5.2 Aims

The aims of this part of the study were to characterise the biotin carboxyl carrier protein (BCCP) in *Brassica napus*. This would involve the raising of specific anti-BCCP antibodies. Although highly specific anti-biotin antibodies were available, anti-BCCP antibodies were required to demonstrate that the genes previously cloned (Elborough *et al.*, 1996) encoded for protein in extracts from *B.napus* embryos. BCCP had been identified as a 35kDa protein by detection with anti-biotin antibodies in chloroplasts and total protein extracts of *B.napus* embryos (Elborough *et al.*, 1996). Speculation in section 3.9 of this study, about the series of biotinylated proteins beneath the 35kDa band (see Figure 3.4 B and 3.5 B), included the suggestion that they could represent truncated isoforms of BCCP or proteolytic degradation products. If anti-BCCP antisera cross reacted with these proteins it would provide evidence that they were either a truncated form of BCCP or derived from the full length protein. The antisera would almost certainly detect a truncated form because even though some antigenic determinants would have been removed in a truncated form, others would still be present. As the nature of the antisera would be polyclonal, it would recognise many different determinants throughout the whole protein.

Characterisation would also address the expression of isoforms. Other proteins involved in lipid biosynthesis have been shown to exist as multiple isoforms (Fawcett

et al., 1994) which are differentially expressed. Using affinity purified anti-biotin antibodies it would be possible to asses the levels of expression of different isoforms of the 35kDa biotinylated protein thought to be BCCP, throughout development. This would not reveal the identity of any differentially expressed isoforms, as the antibody would not be specific for any individual isoform but would detect them all. The expression of the individual mRNAs for the different isoforms would be studied by RT-PCR using isoform specific primers. The expression pattern of the mRNA for individual isoforms would be compared to the expression pattern of protein isoforms in an attempt to decipher whether any isoforms were differentially expressed and, if they were, which isoforms they were. These studies would perhaps enable the identification of an isoform of BCCP which was specifically expressed in embryo or leaf tissue or at a particular time during embryogenesis. In this case, by identification of the gene and cloning of its genomic counterpart one would be able to isolate and study a tissue / development specific promoter.

5.3 Analysis of BCCP genes.

The cDNAs encoding the BCCP isoforms from *Brassica napus* were isolated and sequenced by Dr. Robert Winz in this laboratory (Elborough *et al.*, 1996). To facilitate further work on the analysis of BCCP isoforms an initial analysis of the cDNAs encoding BCCP was required. This analysis would entail computer alignments and phylogenetic analysis to try and establish which isoforms were the closest relatives. Multiple copies of an allele can be generated by gene duplication (Hattori *et al.*, 1996) so presumably all BCCP isoforms had a common ancestor. As the sequence of any ancestral gene is unknown, it was only possible to compare the present day sequences for similarities. Only two of the cDNAs isolated for BCCP encoded a full length transcript, BP4 and BP6 (Elborough *et al.*, 1996). Figure 5.1 shows the amino acid alignment of the six genes, the biotinylation site, translation start site and the putative transit peptide cleavage. Translation start site, biotinylation site and the transit peptide cleavage site were deduced by Dr. Kieran Elborough, Department of Biological Sciences, University of Durham by comparison to known sequences.

```
     1                                                            50
bp4 .......... .......... .ISSVST[M]AS LSVPYAKISA PNRRVGSIPG
bp6 KTSLSTISSS SCFLGYIVKV HFHPIST[M]AS LSVPYAKISA PNRRVESIPG

                    Transit peptide
     51             Cleavage site                                100
bp1 .......... ..........▼ .......... .........S EGKDENSSKD

bp4 RTRWQPQLNG VSFPSDVSQ N QSTIWRLRAT TNEVVSNSTP VTNGGCLNGN
bp6 IRRQPQPSGI SFHVSHVSQT  QSTIWRLRAT TNEVVSNSTP VTNGGCLNGN
                        ▲
     101                                                          150
bp1 PSSSTDLATE ESISEFLTQV TTLVKLVDSR DIVELQLKQL DCELVIRKKE
bp3 .......... ...KELTLIL VLESKQMASS FWNIAYQQAM RSGVFVAKVY
bp4 VKSNVP..ES AKLSNFMAKV SGLLKLVDSR DIVELELKQL DCEIVIRKKE
bp6 VKTNVPPEPA AELSDFISKV SGLLKLVDSR DIVELELKQL DCEIVIRKR.
bp7 .......... .......... .......... .......... DCEIVIRKKE

     151                                                          200
bp1 ALPQPESPAQ YVMMQQPNQS SYVQSVA... ...PPSAPAA SPAPSTPASS
bp3 SFLHITTNYL AFPAYAFGPS SMMPPPPMAG LPMPPSPPVS LPA...PSSA
bp4 ALQQQPT... ..PPPAPVYH SM..ASPMAG LQMAPSQPVA PPPFSLVLSA
bp6 ....KLT... ..AATAPVYH SMMPPPPMEG LPMPPSPPVS PPA...PSSA
bp7 ALQQQQP... ..APPAPVYH S.MPPPPTAG FPMPPSQPVA PPA.STPSSA

     201                                                          250
bp1 PTHLH..YSS XSSLPTVKSP MA.GTFYRSP GPGEPPFIKV GDKVQKGQVL
bp2 .......... .......... .GLQEF...A AAGEPPFIKV GDKVQKGQVL
bp3 PATEKPATAP SSSHPPLKSP MA.GTFYRSP GPGEPPFVKV GDKVQKGQVV
bp4 PETAKPVTPP SSFTSSTQES YGLGTFYRSP GPGEPPFVKV GDKVQKGQVV
bp6 PATANTATAP SSSHPPLKSP MA.GTFYRSP GPGEPPFVKV GDKVQKGQVV
bp7 PATEKPATAP ASSDPPLKSP MA.GTFYRSP GPGEPPFVKV GDKVQKGQVV

     251  *                                                  292
bp1 CIVE[AMKLMN] EIESDN.GTV VDIVAE.MQP VSLDTPLFVV QP
bp2 CIVE[AMKLMN] EIESDQTGTV VDIVAEDGKP VSLDTPLFVV QP
bp3 CIIE[AMKLMN] EIEAEKSGTI TELLAEDGKP VSVDTPLFTI VP
bp4 CIIE[AMKLMN] EIEAEKSGTI TELLAEDGKP VSVDTPLFTI VP
bp6 CIIE[AMKLMN] EIEAEKSGTI TELLAEDGKP VSVDTPLFTI AP
bp7 CIIE[AMKLMN] EIEAEKSGTI TELLAEDGKP VSVDTPLFTI VP
    Biotinylation site
```

Figure 5.1 ORF-derived amino acid sequences of the *B.napus* BCCP cDNA clones (from Elborough *et al.*, 1996).

The translation start site and the biotinylation site are boxed. The transit peptide cleavage site has been marked with arrows.

BP 4 and 6 were the longest cDNAs were obtained and were determined to be full length by northern analysis (Elborough *et al.*, 1996). The alignment of the protein sequences shows that all the BCCP genes have high homology at the 3' end, especially around the biotinylation site which was identified by its conserved sequence. It is evident that there are differences between the isoforms and that initially they roughly fall into two groups of BP1/2 and BP 3/4/6/7.

The DNA sequences were also aligned for the six isoforms using the PILEUP program from the Wisconsin GCG package. This would be necessary to identify regions for 3' UTR analysis in future experiments. Analysis of the sequences was performed to create a unrooted phylogenetic tree shown in Figure 5.2.

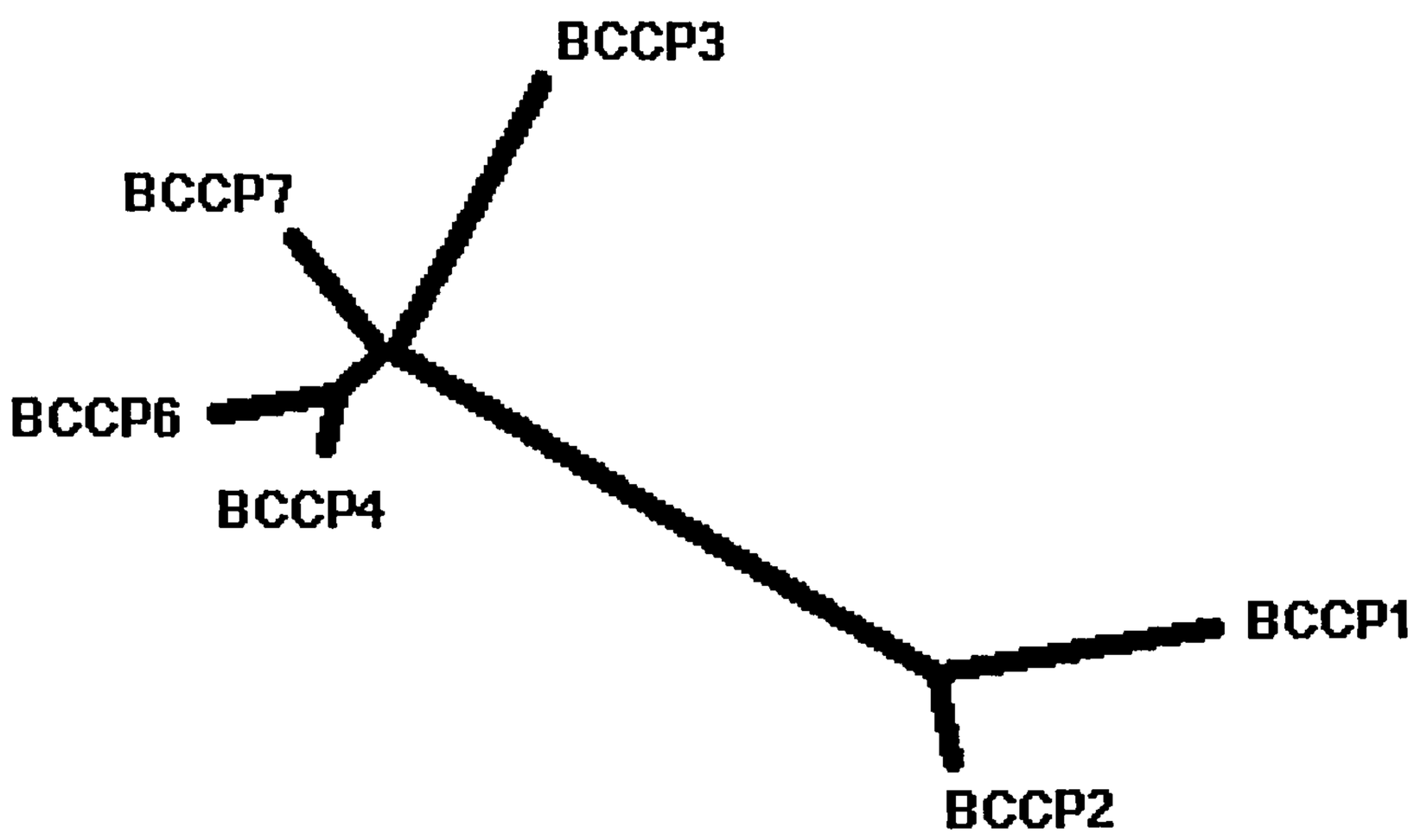


Figure 5.2 Unrooted phylogenetic tree of the BCCP DNA sequences.

The tree was created using the NEIGHBOUR program from the Wisconsin GCG package. The result clearly shows the two groups into which the BCCP isoforms are divided.

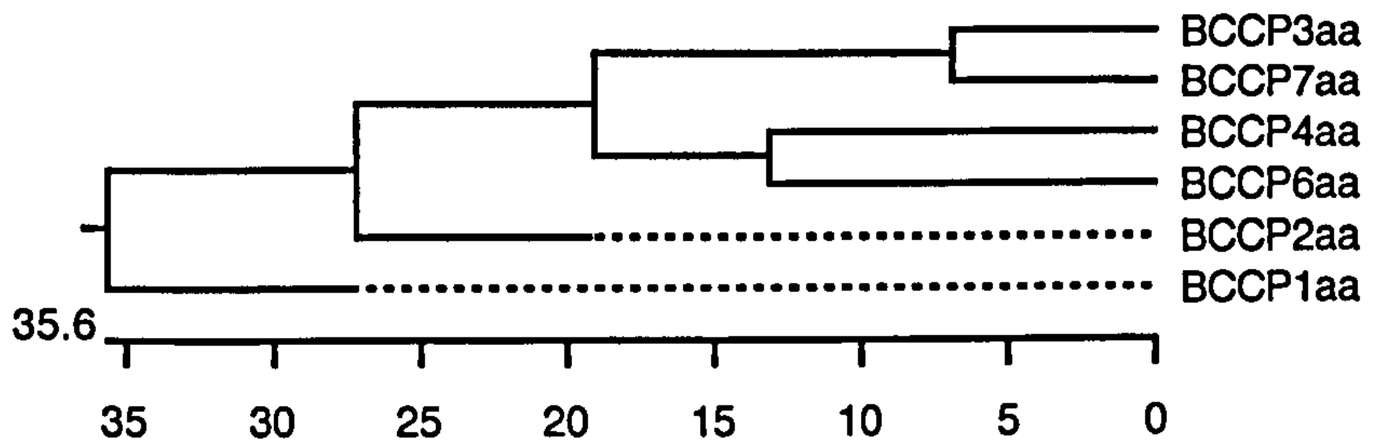


Figure 5.3 Phylogenetic tree of BCCP isoforms

The tree was created using the MEGALIGN program from the DNASTAR package. Alignments were of protein sequences.

The rooted tree in Figure 5.3 presents a different picture of the way the isoforms are linked to each other. The results have to be viewed with some caution as the cDNAs obtained were not full length for all of the clones, except BCCP 4 and 6. This is why in this tree, BP2 which is a very short bit of sequence has not been shown as being divergent from BP1 as in Figure 5.2 The data used to produce Figure 5.3 is presented in Figure 5.4 to show the sequence similarities (top right) and sequence distances (bottom left) of the isoforms

Percentage similarity

	BP1	*BP2*	*BP3*	*BP4*	*BP6*	*BP7*	
BP1		72.1	36.6	43.8	44.8	48.6	BP1
BP2	16.7		64.7	64.7	64.7	64.7	BP2
BP3	54.6	34.3		52.0	66.3	75.0	BP3
BP4	50.8	35.3	41.6		71.3	74.3	BP4
BP6	50.0	35.3	41.7	26.1		77.1	BP6
BP7	53.0	35.3	13.5	34.0	34.7		BP7
	BP1	*BP2*	*BP3*	*BP4*	*BP6*	*BP7*	

Percentage divergence

Table 5.1 Table showing the homology between the BCCP isoforms.

The table shows sequence similarities, top right triangle, and sequence divergence, bottom left triangle, as a percentage. Analysis was done with predicted protein sequence from the BCCP clones.

The table shows how the isoforms can be subdivided into groups. BP1 and BP2 are 72.1% similar but only 16.7% divergent, all other sequences are >50% divergent from

BP1. BP7 and BP3 are 75% similar with 13.5% divergence, although BP6 shows greater similarity to BP7 than BP3 it is 34.7% divergent which implies that BP3 is a closer relative than BP6. BP4 and BP6 are 71.3% similar and 26.1% divergent.

This analysis shows that the genes for BP4 and BP6 are very similar. Other genes are more divergent from these two with varying degrees of similarity to each other. The possible value of this analysis is that it demonstrates which genes are the most closely related, implying that they have diverged from a common ancestor more recently than other genes. Genetic analysis has been used previously to asses the molecular evolution of biotin dependent carboxylases (Toh *et al.*, 1993). Genetic analysis of the BP genes shows that BP 4and 6 are closely related as are BP 3 and 7. BP 1 and 2 are shown as being closely related in Figure 5.2 but the very short length of sequence available for this gene makes a final conclusion difficult. Genes that are very similar to each other might be expected to have originated from a different parent where they carried out a similar function. It must also be noted that there may be more isoforms of BCCP which were not cloned in the original screening. It could be that there are eight copies of this gene in *B.napus*, four from each parent at two loci. Analysis of the isoforms in *Brassica napus* by 2-D Western blotting should reveal how many isoforms of BCCP can be detected.

To study the expression of isoforms by 2-D Western blotting, an antibody to the desired protein must be available. An anti-biotin antibody had been raised (this study, section 3.5) and could be affinity purified to improve specificity. This antibody detected a 35kDa protein in protein from purified chloroplasts, which was predicted to be the BCCP (this study, Figure 3.3, Elborough *et al.*, 1996). To confirm that the cloned cDNAs encoded for this biotinylated protein, a specific anti-BCCP antibody was required. This would demonstrate that a) the 35kDa biotinylated protein was encoded for by the cloned cDNAs and, b) this protein could be detected by an anti-biotin antibody.

5.4 Overexpression of B.napus BCCP.

For the production of antibodies to a single protein in a rabbit it is recommended that at least 200μg of purified protein be available, although less can be used (Catty, 1988). This protein could be isolated from tissue of *Brassica napus* or, by cloning the isolated genes into a suitable vector, the protein could be highly expressed in a heterologous system, such as *E.coli* and purified from there. Isolation of proteins from source material is usually difficult, time consuming and uses large amounts of tissue. In this case the tissue would be mid-stage seeds which are expensive and difficult to obtain in large quantities and the final yield of protein may not have been enough to raise antibodies. It was considered quicker and easier to use a pET vector (NOVAGEN) to overexpress the protein in *E.coli* and purify the protein from there. Purification from a recombinant source would be aided by the fact that the protein can be expressed up to and exceeding 50% of the total protein, whereas in the source material even when highly expressed in mid stage embryos, BCCP would probably make up less that 1% of the total protein. Additionally, expression of the protein in an isolated system such as *E.coli* would allow further studies on the subunit interactions of components of Type II ACCase.

5.4.1 Construction of pET vector

pET 11a was chosen for overexpression of BCCP. This vector uses the T7 promoter under *lac*I control and initiates translation from an Nde I cloning site. Nde I contains an ATG start codon. The cDNA isolated in pBP4 was chosen for overexpression because it was a full length sequence and did not contain any internal restriction sites that would interfere with the cloning procedure. To clone the coding region from pBP4 into pET11a in frame two PCR primers were designed which would engineer an Nde I site to the 5' end of the BP4 insert and a Bam HI site to the 3' end. These could be used to ligate the sequence into pET11a as shown in Figure 5.4.

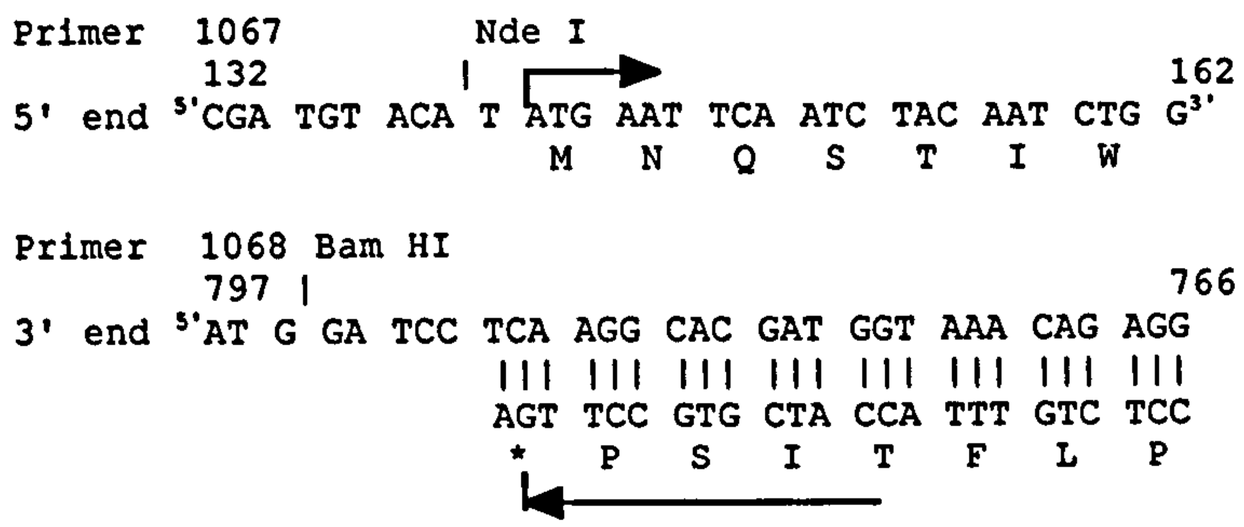


Figure 5.4 Primers designed to allow overexpression of BCCP4.

The primers were designed to engineer restriction sites for cloning of pBP4 into pET11a. The diagram shows the ATG start codon in primer 1067 and the TGA stop codon in primer 1068.

The primers 1067 and 1068 were designed to amplify a fragment of 665 base pairs, which encodes the BCCP4 protein without the predicted transit peptide. The amplification reaction was run for 30 cycles of 94°C 1 minute, 50°C 1 minute and 74°C 1 minute from 2ng of pBP4 DNA, after which 10µl was analysed on a 1% agarose gel (Figure 5.5 A, Lane 4). This shows a PCR product of the predicted size only (665 base pairs, boxed), in this lane.

As the PCR reaction produced a product of the expected size (665 base pairs), the remainder was extracted with phenol: chloroform (1:1) followed by extraction with chloroform. This removed the proteins from the DNA and eliminated traces of mineral oil from the reaction. The remaining DNA in the aqueous phase was ethanol precipitated, washed with 70% ethanol and the pellet dried in a Jouan SpeedVac for 10 minutes. The resulting pellet was re-suspended in 11µl of ddH_2O water, 1µl was run on a 1% agarose gel to check that the was DNA recovered and the resulting concentration (data not shown). The remaining 10µl was digested in a total volume of 20µl with Nde I and Bam HI in Boehringer Mannheim SureCut Buffer B at 37°C overnight. A separate digest of 2µg of pET11a with Bam HI and Nde I was also incubated for the same time. After the incubation time the digested DNA was separated by electrophoresis on a 1% agarose gel. The desired fragments, Nde I / Bam HI cut pET11a and the 665 base pairs PCR fragment similarly digested, were isolated from the gel (Figure 5.5 A, Lanes 2 and 4).

These two fragments were ligated together in calculated molar ratios of 3:1, 1:1 and 1:3 using 3U of T4 DNA Ligase in a volume of 10μl using the manufacturers supplied buffer at 14°C for 24hrs. The 10μl of each ligation was transformed into 200μl of competent *E.coli* DH5α, plated onto LBamp and left to grow at 37°C overnight. Colonies obtained were analysed for the presence of an insert by digestion with Nco I which should have been removed and should not therefore cut the DNA, and also by digestion with Nde I and Bam HI which should cut out the 66 base pairs insert. The results are shown in Figure 5.5 B.

The results show that a fragment of the expected size was amplified by the primers designed for expression of the BCCP subunit of Type II ACCase. This fragment was directionally cloned into pET11a as demonstrated by the digestion of the recovered plasmids with Nde I and Bam HI. This should enable expression of BCCP protein in *E.coli.*

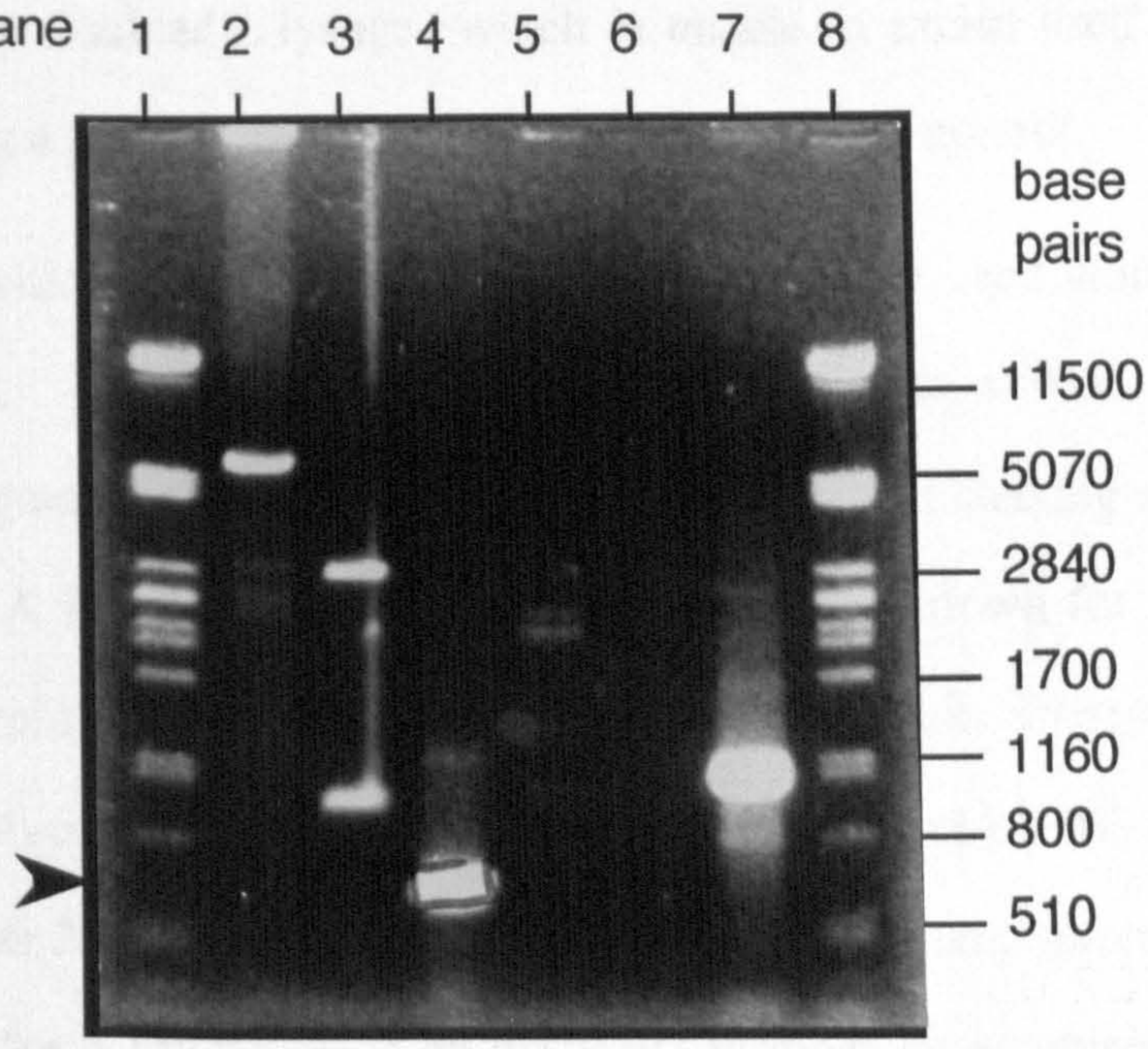
Figure 5.5 A A 1.0% agarose gel showing fragments required to construct the vector pETBP4.

The lanes contain the following; Lane 2, pET11a digested with Nde I & Bam HI; Lane 3, pBP4 digested with Eco RI & Xho I, the smaller fragment is the complete pBP4 insert; Lane 4, PCR products using primers 1067 & 1068 from pBP4; Lane 5, PCR using primer 1067 alone from pBP4; Lane 6 PCR using primer 1068 alone from pBP4; Lane 7 PCR with T3 and T7 primers from pBP4, these primers amplify the complete pBP4 insert; Lane 1 + 8 , 1 μg of λ Pst I markers.

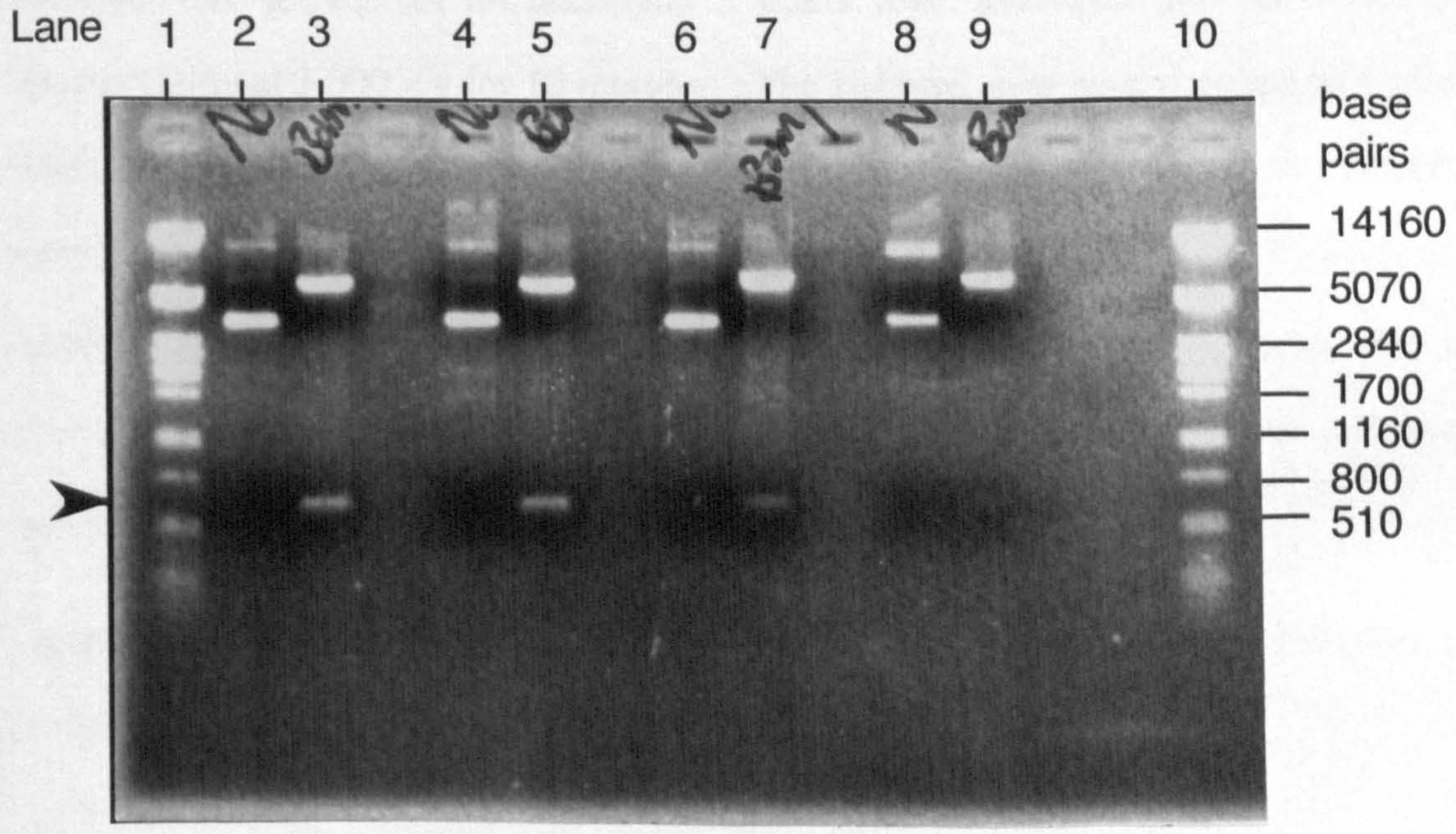
Figure 5.5 B A 0.8% agarose gel showing restriction digests of pETBP4 plasmid constructs.

Four plasmid constructs made from the ligation of pET11a and the pBP4 665 base pairs PCR fragment were analysed by restriction analysis. Lanes contain the following plasmids (numbered by colony identified); Lanes 2+3, construct pETBP4.7; Lanes 4+5, construct pETBP4.12; Lanes 6+7, pETBP4.13; Lanes 8+9, construct pETBP4.16. The gel shows the fragments obtained when plasmid DNA was digested as follows; Lanes 2, 4, 6 and 8 are Nco I digests; Lanes 3, 5, 7 and 9 are Nde I / Bam HI digests. Lanes 1+ 10 contain 1 μg of λ Pst I markers each.

A


Lane
1
2
3
4
5
6
7
8
base pairs
11500
5070
2840
1700
1160
800
510

B


Lane
1
2
3
4
5
6
7
8
9
10
base pairs
14160
5070
2840
1700
1160
800
510

5.4.2 Expression of BCCP4 from pETBP4.

The constructs created and shown in Figure 5.5 B were analysed for expression of BCCP in *E.coli*. The plasmids were transformed into the host *E.coli* BL21(λDE3) which harbours a disabled λ lysogen which is unable to excise itself and enter lytic mode, containing a gene for T7 DNA Polymerase under *lac*I control.

Transformants which grew on LBamp were used to create seed stocks which were stored at -80°C. One seed stock was used to inoculate a 50ml culture of LB containing 100μg/ml ampicillin and was grown at 37°C with shaking at 220rpm to an OD_{600} of 1.0. A 1ml sample of cells was removed, spun down for 1 minute at full speed in a microfuge, the supernatant removed and the cells frozen at -20°C until required for analysis. The remaining media was made 1mM IPTG and allowed to grow for a further 3hrs. IPTG induces expression from the *lacZ* promoter controlling transcription of the T7 polymerase on the λDE3 lysogen by combining with the lacI protein and preventing it binding to the DNA repressor site. This allows expression of the T7 polymerase message and translation of the protein. The T7 polymerase protein acts on the T7 promoter found in the pET plasmids and transcribes the cloned cDNA.

Bacteria were grown for an additional 3 hours after induction and harvested by centrifugation at 3,000 × g for 10 minutes. The bacteria were re-suspended in 5ml of 50mM Tris-HCl pH8.0, 2mM EDTA and frozen at -20°C. Proteins from the bacteria were analysed by SDS / PAGE. The pellet of uninduced cells was thawed and re-suspended in 100μl 1 × SDS sample buffer. Eighty microlitres of the induced cells were combined with 20μl of 5 × SDS sample buffer. Both samples were heated for 2 minutes in a boiling water bath and loaded onto a 12% acrylamide gel.

Figure 5.6 A shows the cell samples before and after induction. The overexpressed protein is visible next to the arrow.

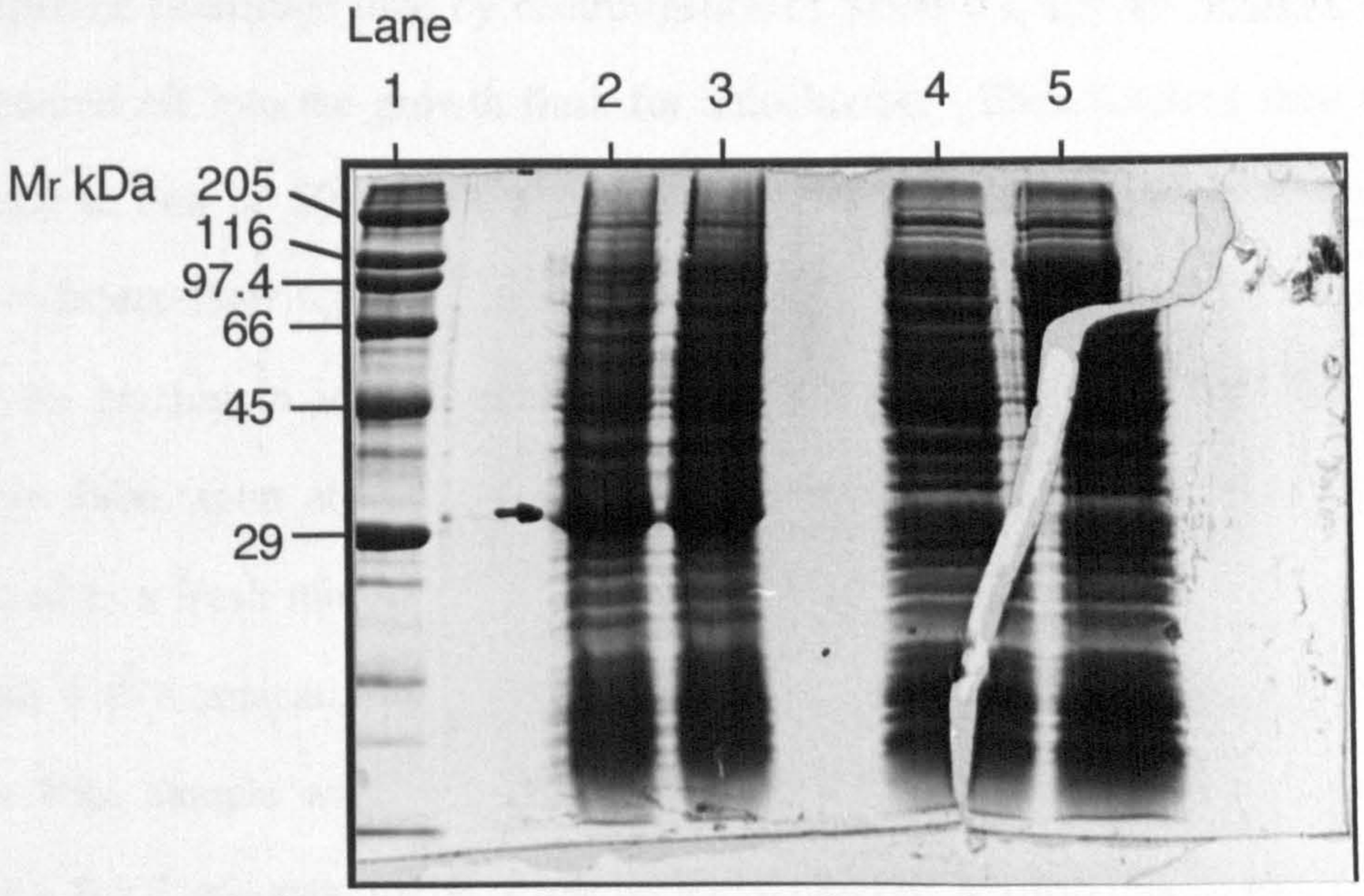
Figure 5.6 A Coomassie stained 12% acrylamide gel showing the expression of BCCP from pETBP4.

E.coli BL21(λDE3) cells harbouring pETBP4 were grown up in a 50ml culture of LB + 100μg/ml ampicillin to OD_{600} of 1.0. An aliquot of uninduced cells was harvested and frozen for analysis. The remaining culture was made 1mM IPTG and gown for a further 3 hours. Equal amounts of uninduced (Lanes 4 + 5) and induced cell (Lanes 2+3) were loaded per lane. Lane 1 contains SDS-6H protein standards, 2μg each protein. The overexpressed protein is visible next to the arrow marked on the gel.

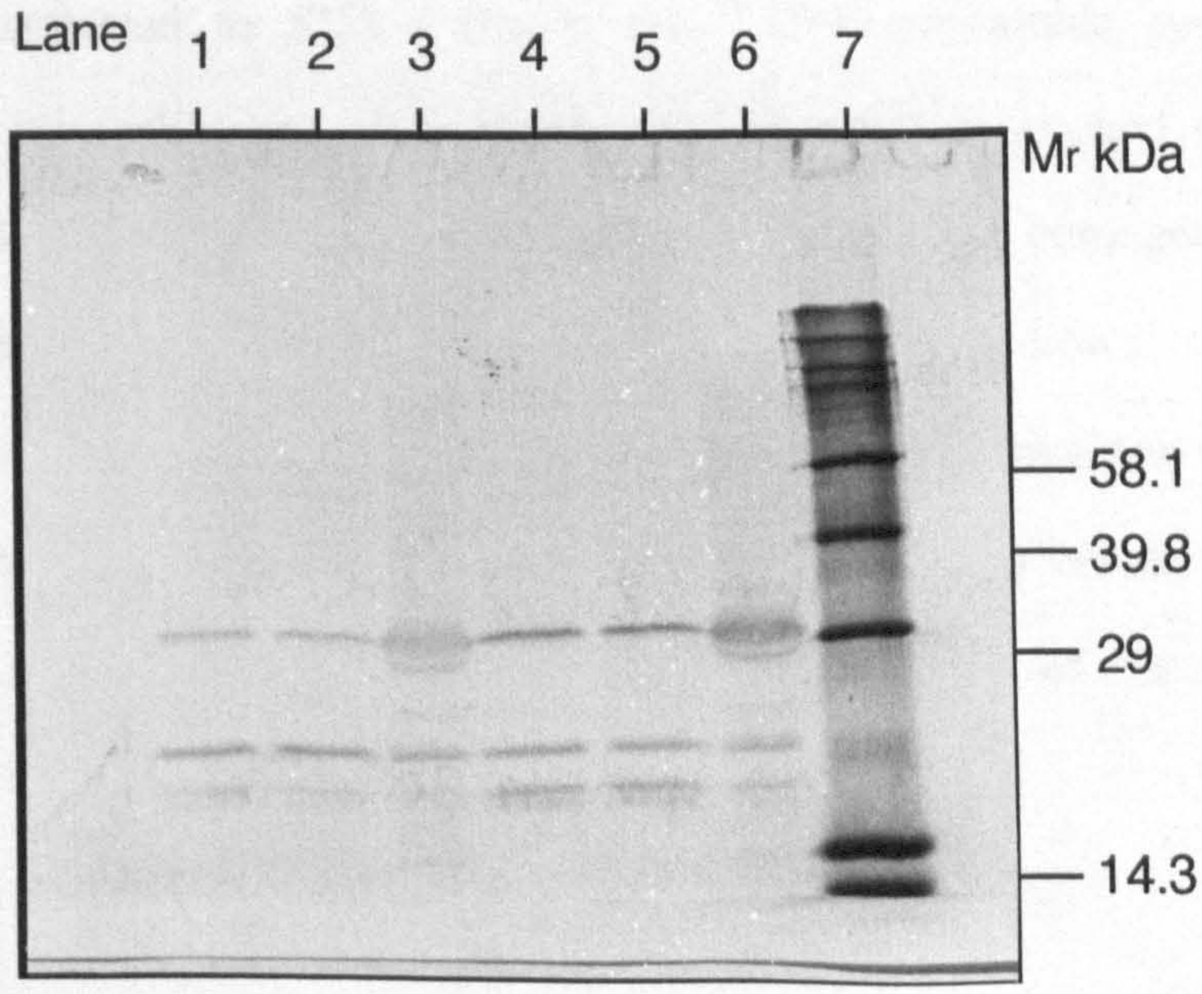
Figure 5.6 B Western blot showing the biotinylated proteins in *E.coli* BL21(λDE3) harbouring pETBP4.

A culture of *E.coli* BL21(λDE3) containing pETBP4 was grown up as described above except one 50ml culture was supplemented with 0.5mM D-biotin (Lanes 4-6). The culture was spun down and re-suspended in 5ml of 50mM Tris-HCl pH8.0, 2mM EDTA. Proteins were extracted by freezing in dry ice and thawing in ice-water three times, the cell debris was removed by centrifugation for 2 minutes in a microfuge and the supernatant run in lanes 1 and 4 (Johnson & Hecht, 1994). Proteins were extracted further by 1 minute of sonication the cell debris was removed by centrifugation for 2 minutes in a microfuge and the supernatant run in lanes 2 and 5. A total cell extract was made by boiling the freeze-thawed and sonicated cells in 1 × SDS sample buffer for 2 minutes, lanes 3 and 6. Lane 7 contains biotinylated protein standards.

A


Lane
1
2
3
4
5
Mr kDa
205
116
97.4
66
45
29

B


Lane
1
2
3
4
5
6
7
Mr kDa
58.1
39.8
29
14.3

To see whether the overexpressed protein was biotinylated and to see if supplemental biotin in the media affected the biotinylation status of the protein two batches of cells were grown up in LBamp as previously described, one batch containing 0.5mM D-biotin. After 3 hours post-induction growth the cells were harvested in a 50ml polypropylene centrifuge tube by centrifugation at 3,000 × g for 10 minutes and the media poured off into the growth flask for autoclaving. Each batch of cells was re-suspended in 5ml of 50mM Tris-HCl pH8.0, 2mM EDTA and subjected to three rounds of freeze-thawing (Johnson & Hecht, 1994). Cells were frozen in dry-ice and thawed by placing in ice-water. After three rounds 80µl was removed into an microfuge tube, spun at top speed in a microfuge for 2 minutes, the supernatant transferred to a fresh microfuge tube, and stored on ice. The remaining cells were sonicated with a sonicating probe for 3 minutes at maximum power and amplitude. Another 80µl sample was removed into an microfuge tube, spun at top speed in a microfuge for 2 minutes, the supernatant transferred to a fresh microfuge tube, and stored on ice. Another 80µl sample was removed, but this was not centrifuged. This was done for both batches of cells. Twenty microlitres of 5 × SDS sample buffer was added to each sample and they were boiled for 2 minutes. After cooling 10µl of each sample was subjected to SDS / PAGE on a 12% acrylamide gel before being transferred to nitrocellulose. The membrane was blocked, probed with anti-biotin antibodies and donkey anti-sheep IgG : alkaline phosphatase conjugate before being developed with BCIP / NBT. The resulting Western blot is shown in Figure 5.6 B. Lanes 4-6 shows the protein produced by cell grown in the presence of 1mM biotin. The signal is stronger from the cells grown in the presence of biotin, suggesting that biotin in the external media aids the complete biotinylation of the over expressed protein.

5.4.3 Purification of overexpressed BCCP protein

Two times 1L of LBamp were inoculated with a seed stock of *E.coli* BL21(λDE3) cells harbouring pETBP4. The cells were grown at 37°C with shaking at 220 rpm to an OD_{600} of 1.0. The cells were induced by addition of IPTG to 1mM from a 500mM stock. After 3 hours post-induction growth the cells were transferred equally to six 500ml Beckman centrifuge tubes and spun at 3,000 × g for 30 minutes to harvest the cells. After centrifugation the media was poured back into the flasks and the cells re-suspended in a total volume of 100ml of 50mM Tris-HCl pH7.2, 1mM EDTA and 5mM DTT. The cells were passed twice though a French press cell disrupter with an extra 150ml of 50mM Tris-HCl pH7.2, 1mM EDTA and 1mM PMSF. The 250ml of broken cells were spun in a Beckman L-70 Ultracentrifuge with a 45Ti rotor for 1hour at 105,000 × g to remove the cell debris and membranes.

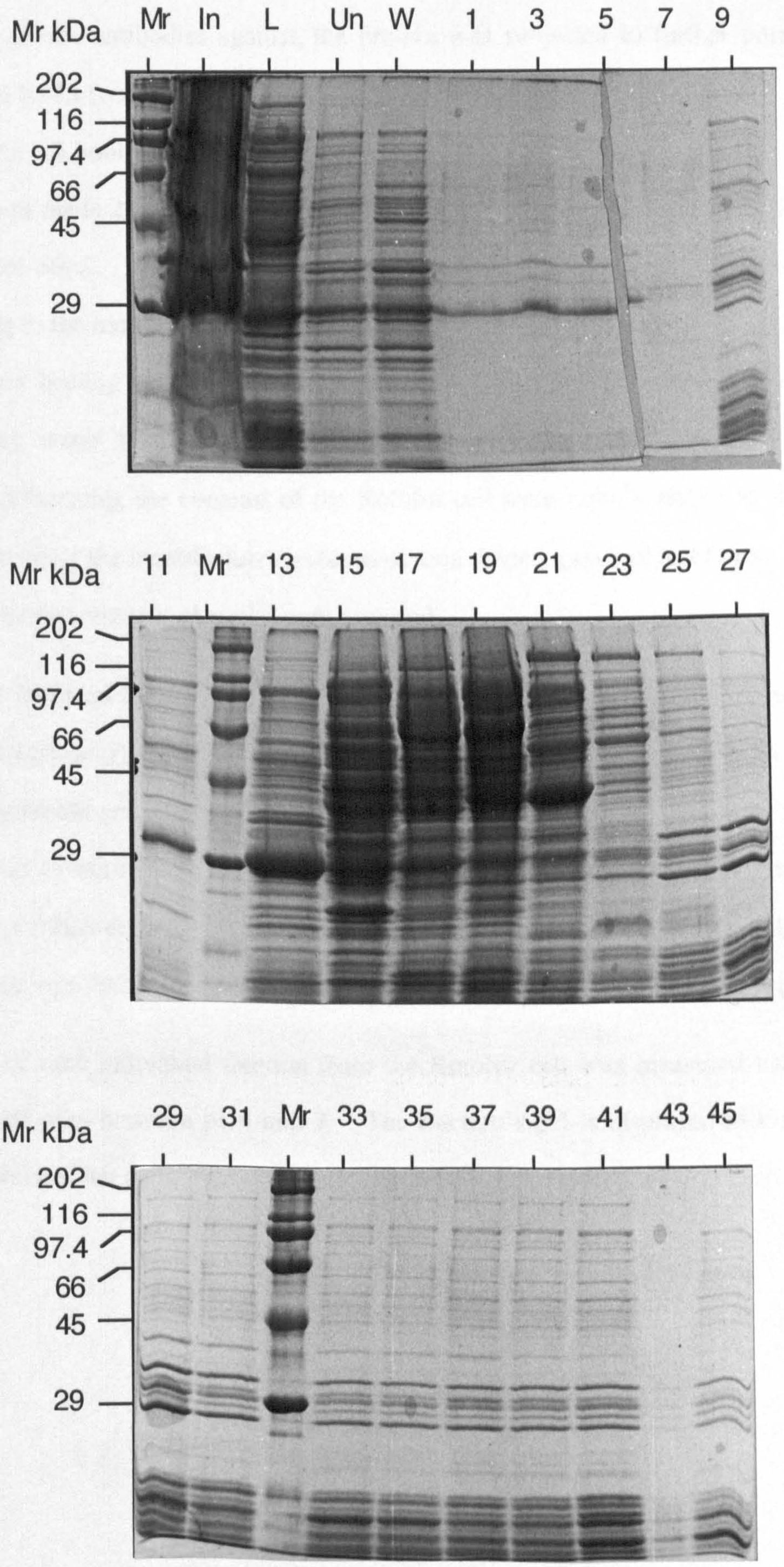
The supernatant from the ultracentrifugation was filtered through a 0.22μm filter and applied to a pre-equilibrated 50ml Hi-Load Mono-Q ion exchange column (PHARMACIA) at a rate of 2ml/min. The column had been previously equilibrated with 5 volumes of 50mM Tris-HCl pH 7.2, 1mM EDTA and the Abs_{280} on the in-line UV monitor set to zero. After the bacterial supernatant had been applied to the column, it was washed with 50mM Tris-HCl pH7.2, 1mM EDTA until the Abs_{280} was < 5%. Proteins were eluted from the Hi-Load Mono-Q with a linear gradient of NaCl from 0mM to 2M over 300ml at 2ml / min flow rate. Fractions of 5ml were collected.

Every other fractions was analysed by SDS / PAGE for protein content (Figure 5.7). As samples contained a high level of NaCl which interferes with the running of the gel, 100μl of each fraction to be analysed was methanol : chloroform. The resulting protein pellet was re-suspended in 100μl of 1 × SDS sample buffer and 20μl was run on a 12% acrylamide gel. Fraction 13 contained a large amount of a 30kDa protein which was presumed to be the overexpressed protein.

Figure 5.7 SDS/ PAGE analysis of protein fractions from *E.coli* BL21(λDE3) expressing pETBP4 after Hi-Load Mono-Q separation.

Protein from 2L of *E.coli* BL21(λDE3) cells harbouring pETBP4 were extracted into 250ml of 50mM Tris-HCl pH7.2, 1mM EDTA, 5mM DTT and 1mM PMSF using an automated French press . The 250ml of broken cells were spun in a Beckman Ultracentrifuge for 1hour at 105,000 × g to remove the cell debris and membranes. The supernatant was filtered through a 0.22μm filter and the proteins separated on a 50ml Hi-Load Mono-Q column by elution of bound proteins with a linear gradient of 0-2M NaCl. Fractions of 5ml were collected, of which 20μl was run on a 12% acrylamide gel

The lanes show; Mr, SDS-6H protein markers; In, insoluble protein from ultracentrifuge run; L, protein loaded onto column; Un, protein not bound to column; W, protein in wash; 1-45, Fractions from Mono-Q column.


Mr kDa
Mr
In
L
Un
W
1
3
5
7
9
202
116
97.4
66
45
29
Mr kDa
11
Mr
13
15
17
19
21
23
25
27
202
116
97.4
66
45
29
Mr kDa
29
31
Mr
33
35
37
39
41
43
45
202
116
97.4
66
45
29

As the fraction containing the majority of overexpressed protein was not pure enough to raise specific antibodies against, the protein was subjected to further purification. Fractions 10-13 (containing the purest 30kDa protein) were dialysed against 2 × 2L of ddH_2O for 12 hours with stirring to remove traces of salt and buffer. The dialysed protein was made 2% with respect to 3/10 BIO-RAD ampholytes and 3M urea in a final volume of 50ml. This solution was placed in a BIO-RAD Rotofor cell, assembled according to the manufacturers instructions, and electrophocused for 4 hours at 12W. To prevent heating the cooling core was kept at 0°C throughout by attachment to a circulating water bath containing refrigerated 20% anti-freeze. After 4 hours isoelectricfocusing, the contents of the Rotofor cell were rapidly eluted as individual fractions, using the manufactures vacuum-elution device, into 5ml test tubes. The test tube fractions were stored at 4°C until required.

Eighteen fractions from the Rotofor electrophocusing cell were analysed for protein content by SDS / PAGE. Ten microlitres of each fraction was loaded onto duplicate 12% acrylamide gels. One gel was stained with Coomassie R-250 and the duplicate transferred to nitrocellulose. The membrane was blocked, probed with anti-biotin antibodies followed by donkey anti-sheep IgG : alkaline phosphatase conjugate and developed with BCIP / NBT. The gels and Western blots are shown in Figure 5.8.

The pH of each individual fraction from the Rotofor cell was measured using a pH probe calibrated between pH4 and 7. The fraction's pH is displayed in Figure 5.8. This indicates that the pI of the overexpressed protein is between pH 5.3 and pH6.0.

Figure 5.8 A Coomassie stained 12% acrylamide gel of proteins separated by preparative isoelectric focusing in the BIO-RAD Rotofor cell.

Fractions 10-13 from the Mono-Q separation in Figure 5.7 were separated by isoelectricfocusing in the presence of 2% 3/10 ampholytes and 3M urea.

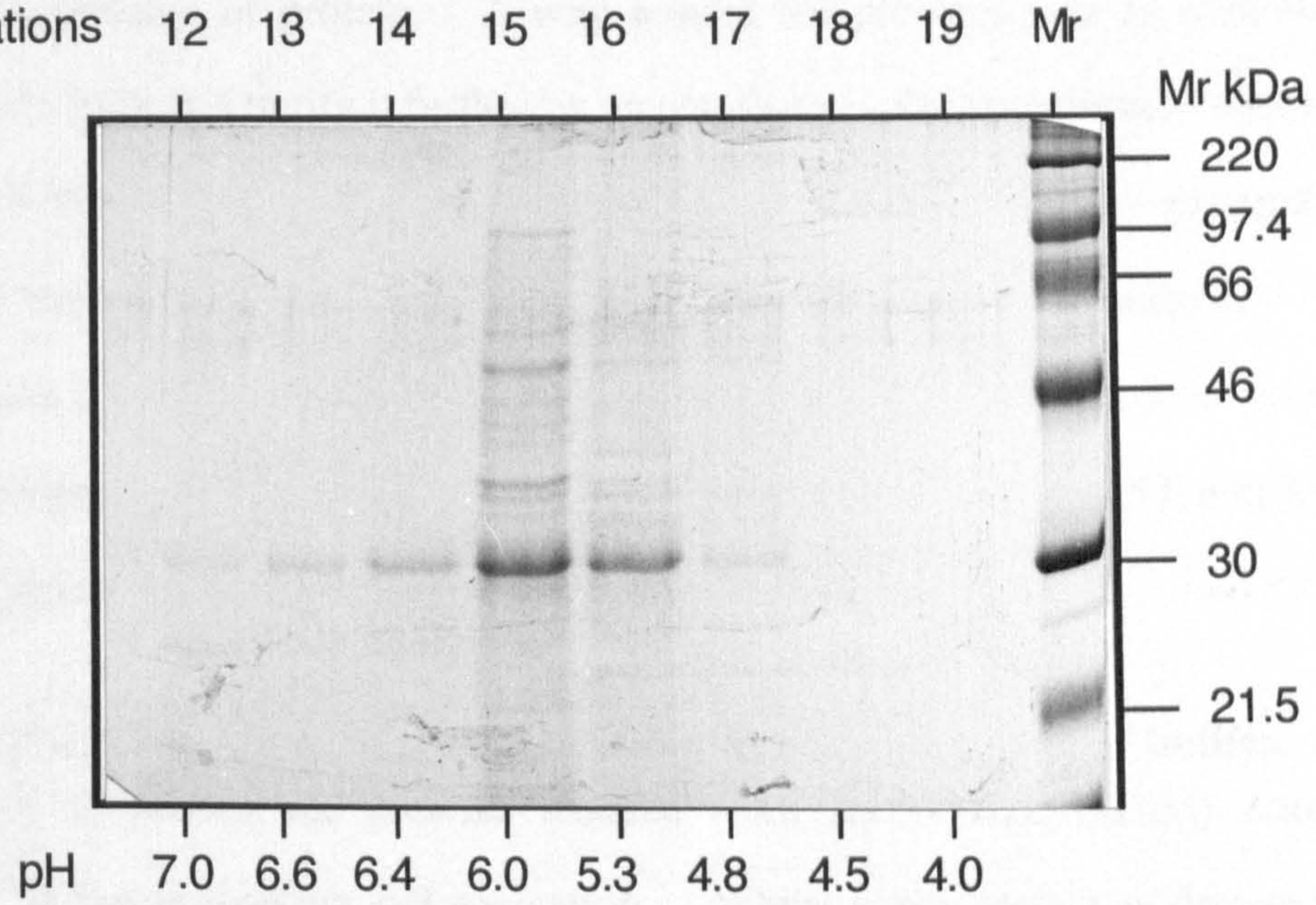
Lanes contain the following; M_r, protein standards, 2μg each protein; 12-19, fractions from the Rotofor cell.

Figure 5.8 B Western blot with anti-biotin antibodies of proteins separated by preparative isoelectric focusing.

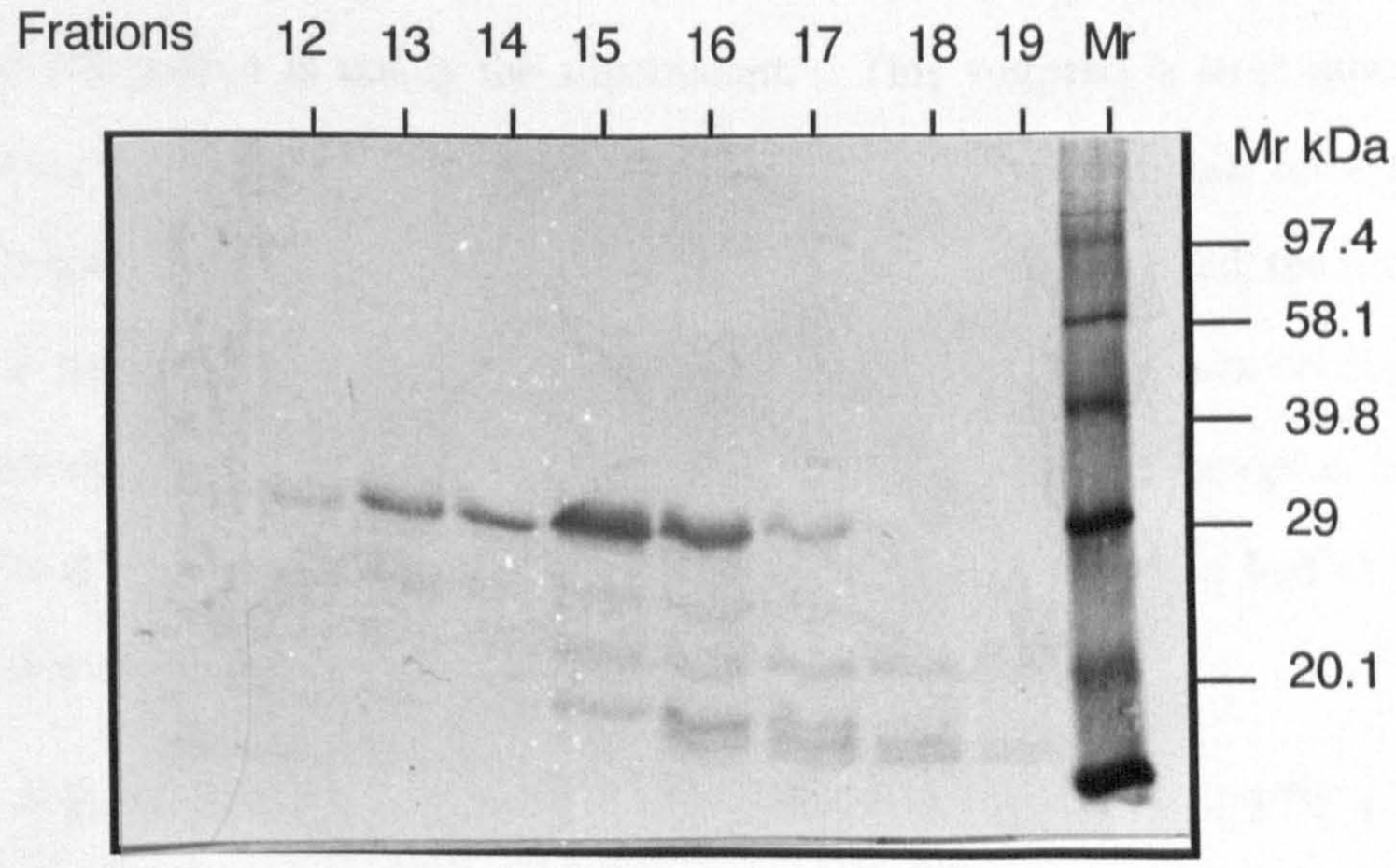
A duplicate gel to that shown in A was transferred to nitrocellulose and probed with anti-biotin antibodies, donkey anti-sheep IgG : alkaline phosphatase conjugate and developed with BCIP NBT.

Lanes contain the following; M_r, biotinylated protein standards; 12-19, fractions from the Rotofor cell.

A


Frations 12 13 14 15 16 17 18 19 Mr
Mr kDa
220
97.4
66
46
30
21.5
pH 7.0 6.6 6.4 6.0 5.3 4.8 4.5 4.0

B


Frations 12 13 14 15 16 17 18 19 Mr
Mr kDa
97.4
58.1
39.8
29
20.1

Fractions 15 and 16 contained the most biotinylated overexpressed BCCP. The protein in these fractions had precipitated out of solution, even in the presence of 3M urea. This is most likely to be because it was at or near it's pI at which point a protein carries no net charge and becomes less soluble as a result. Attempts to solubilise these fractions with higher concentrations of urea and buffers at pH7.2 failed to solubilise significant amounts of protein. A way around the problem was to solubilise the protein with SDS and purify it further by preparative gel electrophoresis. As the yield from the Rotofor cell was approximately 100μg this was not used for preparative gel electrophoresis, but protein was prepared from freshly grown bacteria. These experiments have determined that the pI of the over-expressed protein in solution is between pH6.0 and 5.3. This is close to the predicted value of 5.53 and provides valuable information for future analysis of 2-D Westerns.

5.4.4 Preparation and purification of pETBP4 inclusion bodies.

Figure 5.6 B shows the proteins isolated from *E.coli* BL21(λDE3) harbouring pETBP4 by freeze-thawing and sonication. Whilst some protein is detected in the supernatants after these processes, when compared the amount detected in the total protein of the same amount of whole cells it can be seen that the majority of the overexpressed protein is not in the supernatant. This suggests a large amount of protein is aggregating into inclusion bodies and remaining insoluble after freeze-thawing or sonication. Whilst the soluble fraction was partially purified, the low yield from 2L of bacterial culture made purification of the insoluble fraction more desirable. Purification of inclusion bodies can produce protein which is pure enough to be used directly for the raising of antibodies. If it is not, then the inclusion bodies can be solubilised with SDS and purified using preparative SDS /PAGE.

One litre of *E.coli* BL21(λDE3) harbouring pETBP4 was grown up at 37°C to OD_{600} of 1.0 in LBamp as before and induced with 1mM IPTG. After 3 hours post-induction growth the bacteria were harvested by centrifugation at 3,000 × g. Bacteria were re-suspended in 50mM Tris-HCl pH8.0, 2mM EDTA and kept on ice until required. Inclusion bodies were isolated by lysis with lysozyme and detergent and re-

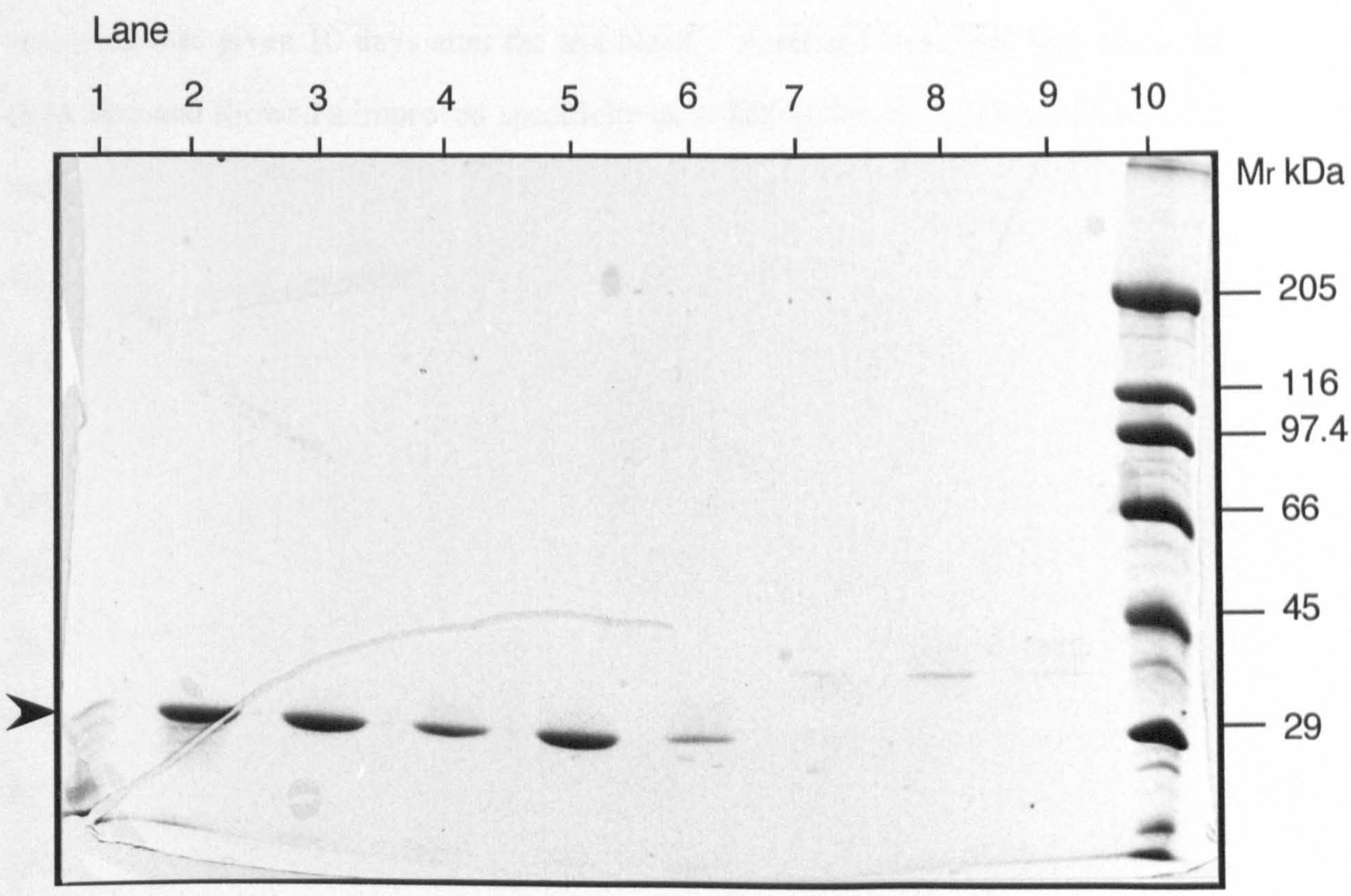
suspended in 10ml of 50mM Tris-HCl pH8.0, 2mM EDTA, 0.1% Triton-X100 and pelleted again by centrifugation at 12,000 × g for 15 minutes at 4°C. This washing step helped to remove excess soluble proteins. As the inclusion bodies not completely free from contaminating proteins, the protein was purified further by continuous elution preparative SDS / PAGE. A 6cm, 10% preparative gel was cast. An estimated 2mg of protein was solubilised in 1ml of 1 × SDS sample buffer by boiling for 2 minutes. The cooled solution was spun at full speed in a microfuge for 1 minute and the soluble protein applied to the top of the gel. The gel was run at 10W constant power and 20, 5ml fractions were collected from the bottom of the gel when the dye front reached the bottom at a rate of 1ml /min. Initial fractions contained the dye front (bromophenol blue) and were coloured accordingly. Fractions immediately after the dye front which did not contain blue dye were analysed. 100µl of each fraction was methanol chloroform precipitated, re-suspended in 20µl of 1 × SDS sample buffer and analysed on a 12% acrylamide gel. The analysis of fractions is shown in Figure 5.9. This shows that the BCCP protein to be purified to homogeneity in a simple purification procedure, suitable for the raising of antibodies.

The fractions containing pure BCCP were pooled and the protein precipitated by methanol : chloroform precipitation. Organic solvent extraction removed the SDS from the protein sample and concentrated it. The precipitated protein was pooled, re-suspended in 500µl of sterile water and stored at -20°C until required. This procedure yielded approximately 500µg of purified protein. In this state the protein was not soluble but remained in small particles as a milky white suspension. The purified protein was used for raising anti-BCCP antibodies.

Figure 5.9 Purification of inclusion bodies from *E.coli* BL21(λDE3) cells expressing pETBP4 by continuous elution gel electrophoresis.

Inclusion bodies were isolated from 1L of *E.coli* BL21(λDE3) harbouring pETBP4 after 3 hours growth from OD_{600} of 1.0 in the presence of 1mM IPTG. Inclusion bodies were purified on a 10% preparative gel and the proteins collected in 5ml fractions. The fractions immediately after the dye front which did not contain bromophenol blue were analysed on a 10% gel stained with Coomassie blue. The picture shows the proteins in the first 9 fractions after the dye front eluted.

The lanes contain the following; Lanes 1-9, 100μl of each of the first nine fractions from the preparative gel after the dye front; Lane 10, SDS-6H protein standards, 2μg each protein.


Lane
1
2
3
4
5
6
7
8
9
10
Mr kDa
205
116
97.4
66
45
29

5.5 Anti-BCCP sera

Antisera was produced to the purified, insoluble BCCP as described in section 2.4.15. For the initial injection into a New Zealand White Rabbit, 200μg of BCCP in 1.2 ml of sterile PBS was emulsified with 1.2 ml of FCA.. For booster injections, 100μg of BCCP in 1.2ml of sterile PBS was emulsified with 1.2ml of FIA. 1ml of the FIA emulsion was given forty days after the initial injection. A test bleed taken fourteen days after the first booster showed a good reaction to BCCP at 1:1000 dilution (data not shown) but the background considered too high. A second booster of 1ml of FIA emulsion was given 10 days after the test bleed. A second test bleed was taken 14 days later and showed a improved specificity at 1:2000 dilution. This test bleed was used to probe a developmental and tissue Western blot shown in Figure 5.10.

The Western blot shows that the antisera detects several bands very strongly at 35, 31 and 30kDa. A 35kDa band was previously identified as the biotinylated subunit of Type II acetyl-CoA carboxylase (Elborough *et al.*, 1996). Many other bands are also detected by the antisera which are probably background antibodies in the serum. These could be removed from the picture by developing for less time, using a higher dilution of the antisera or giving further boosters to the animal to produce a higher titre serum which could be used at even lower dilutions with improved specificity. The antisera also picks up a high M_r band at about 220kDa. This could be the large form ACCase but this was not determined. Due to the highly conserved nature of the BCCP protein, antigenic determinants on the BCCP may be similar to those on other biotin-containing or lipoic-acid binding proteins, which are known to have strong homology to BCCP (Toh *et al.*, 1993).

Figure 5.10 A Western blot with anti-BCCP antisera of total protein extracts from *Brassica napus* leaf, root and developing seed material

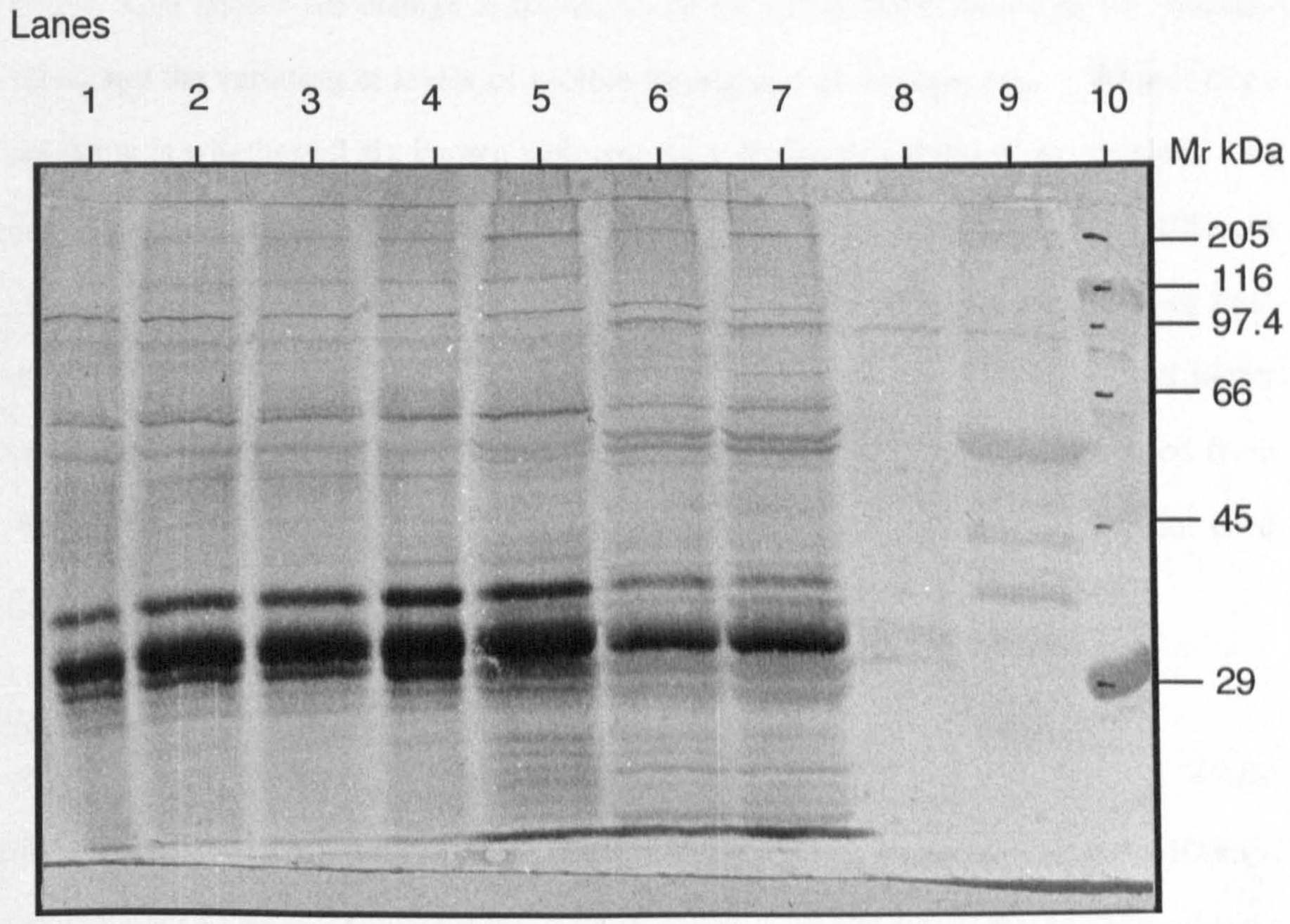
Extracts were prepared as described in section 2.4.2 and equal amounts (in weight of starting material) were applied to each lane of a 12% acrylamide gel. Proteins were transferred to nitrocellulose and probed with anti-BCCP sera, sheep anti-rabbit IgG : alkaline phosphatase conjugate and developed with BCIP / NBT.

The lanes contain total protein extracts from the following; Lanes 1-7, developing embryos; Lane 8, root; and Lane 9, leaf. Lane 10 contains protein standards, 2μg each protein. The developing embryo extracts were from embryos 38, 44, 47, 51, 56, 61 and 70 daf.

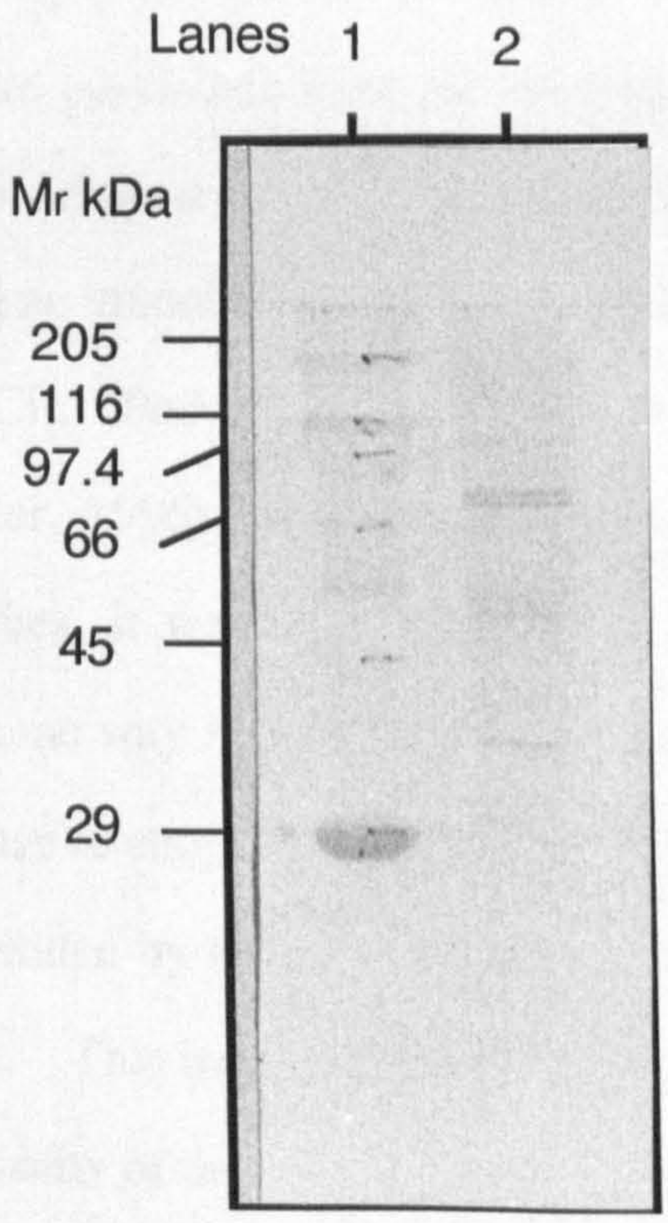
Figure 5.10 B Western blot with pre-immune serum from the rabbit used for anti-BCCP antisera.

A bleed was taken from the rabbit 14 days before the initial injection. A total protein extract prepared as described in section 2.4.2 from embryos 49daf was run on a 12% gel (Lane 2) with SDS-6H protein standards (Lane 1). Proteins were transferred to nitrocellulose and detected with a 1:500 dilution of the sera. The secondary antibody was sheep anti-rabbit : alkaline phosphatase conjugate and the image was developed with BCIP/NBT.

A


Lanes
1
2
3
4
5
6
7
8
9
10
Mr kDa
205
116
97.4
66
45
29

B


Lanes
1
2
Mr kDa
205
116
97.4
66
45
29

5.6 Analysis of BCCP isoforms in developing seeds of Brassica napus

Figure 5.10 shows the change in the levels of BCCP in different tissue of *Brassica napus* and the variation in levels of protein throughout embryogenesis. What it does not show is whether all six known isoforms have the same pattern of expression. In order to investigate the possibility that the isoforms of *Brassica napus* have different expression patterns and hence different functions, 2-dimensional Western blots of total protein extracts from developing embryo were probed with affinity purified anti-biotin antibodies. Anti-biotin antibodies were employed because they could be purified from the sera to create a very specific antibody. The anti-BCCP antibodies were not used because they were not available at the time these experiments were carried out.

5.6.1 Affinity purification and recovery of anti-biotin IgG

Anti-biotin antibodies were previously purified as described in section 3.6.2. Whilst these antibodies were better than crude sera, the harsh elution conditions (100mM glycine pH2.5) were of some concern in that they could denature the antibody during the purification. In addition, by eluting with acid it would be possible to elute proteins that were perhaps not specifically recognising the biotin residue, that is non-specific antibodies. Antibodies have previously been successfully purified using $MgCl_2$ to elute from the antigen-matrix (Harlow & Lane, 1988). Affinity purification of enoyl-ACP reductase antibodies on an immobilised enoyl-ACP reductase column and elution of antibodies with 4M $MgCl_2$, 20mM Tris-HCl base (no pH) produced improved specificity antibodies (Fowler, 1996). Whilst this purification has been shown to purify high affinity antibodies, it was demonstrated that the enoyl-ACP reductase antibodies were altered in some way in that when they were attached to a protein-A agarose matrix. When the native enoyl-ACP was bound to the antibodies the majority of the protein could not be eluted by 4M $MgCl_2$, 20mM Tris-HCl base, or indeed by any "gentle" elution method. This implies that affinity purification of antibodies can change the specificity and avidity of the antibodies, possibly detrimentally.

Previous purifications of biotin-binding proteins have used a chemical analogue, 2-iminobiotion. At pH11 the 2-iminobiotin has a similar structure to biotin and biotin-binding proteins are able to bind. When the pH is reduced to pH4 the conformation of 2-iminobiotin changes such that the affinity of biotin-binding proteins is reduced and they can be eluted. This has been used very successfully to purify avidin and streptavidin (Fudem-Goldin & Orr, 1990). This was rejected as an improved purification scheme on the basis that the extreme of pH could possibly affect the antibody in a similar way to 4M $MgCl_2$, 20mM Tris-HCl base or 100mM glycine pH2.5 tried previously.

Anti-biotin antibodies were purified with biotin-agarose utilising the fact that the affinity and probably the $t_{1/2}$ for the dissociation of polyclonal anti-biotin antibodies is most likely less than that for avidin. Avidin can be purified with biotin-agarose but the problem is that the $t_{1/2}$ for the release is >200days, so the protein has to be eluted at pH1.5 in 6M guanidine-HCl (Fudem-Goldin & Orr, 1990). In order to purify anti-biotin antibodies it was assumed that they $t_{1/2}$ for the release of hapten from antibody would be measurable in hours instead of days as a result of the lower affinity of some of the polyclonal antibodies.

All steps were carried out at 4°C in a cold room and all buffers and sera were filtered though a 0.22μm filter before use. Ten millilitres (packed bed volume) of biotin-agarose matrix (SIGMA) was poured into a 35ml separating funnel with a glass scinter above a tap at one end and a glass stopper at the other. This was fastened to a rotary wheel which enabled the separating funnel to be rotated end over end at approximately 2 rpm. The matrix was washed five times with 20ml of PBS. Twenty five millilitres of sheep anti-biotin sera was poured into the funnel with the tap in the closed position. The stopper was secured tightly and the funnel left to rotate at 2 rpm for >4 hours. This was judged sufficient time for all the available biotin residues on the matrix to be bound by an antibody. After the elapsed time the wheel was stopped with the stopper uppermost. The stopper was removed and the tap opened allowing the sera to drain

off into a vial, the matrix could not drain away due to the presence of the glass scinter. The matrix was allowed to drain under gravity until flow stopped, but the matrix remained wet at all times. Twenty five millilitres of PBS was poured gently into the vertical funnel with the tap open and allowed to drain through, washing the matrix, this wash was discarded. The tap was closed and 25ml of PBS +500mM NaCl was added to the funnel. The stopper was replaced and the wheel set rotating. The matrix was washed with one change of PBS +500mM NaCl for 1 hour. The washing with 500mM NaCl ensured that there were no proteins bound to the matrix or through ionic interactions to other proteins which were not specific for biotin. The final wash was allowed to drain through the tap until dripping stopped. The tap was closed and 10ml PBS + 1mM biotin +0.2% azide were added. The stopper was replaced securely and the wheel set rotating. The matrix was left like this for 24 hours after which the wheel was stopped, the liquid drained off through the tap into a 50ml polypropylene centrifuge tube and the matrix extensively washed with PBS to remove residual biotin (at least 10 changes of 25ml of PBS).

The procedure was repeated three times in all yielding 30ml of affinity purified antibodies. The 30ml was concentrated down to 3ml with an AMICON Centriprep 30 concentrator. This works by ultrafiltration in which only small molecules can pass through the filter and molecules with a native $M_r > 30,000$ are retained. Consequently the majority of the free biotin would have been removed. Remaining biotin, bound and unbound, was removed by exhaustive dialysis against PBS. The 3ml of antibody solution was placed in 1cm diameter dialysis tubing and sealed with clips. This was dialysed against 4 × 1L of PBS in a flask with stirring for a total time of 48 hours. A final dialysis for 24 hours against 1L of PBS + 2mg avidin ensured that all biotin was removed from the antibodies.

The purified anti-biotin antibodies were examined for purity by SDS / PAGE. Antibodies are made up of two chains, a heavy chain and a light chain. Figure 5.11 A shows the purified antibodies stained with Coomassie R-250 after running on a 10% acrylamide gel. Three major bands are present, two of which are the IgG heavy and light chains. These have a M_r of 55,000 and 25,000 (Harlow & Lane, 1988). The third higher M_r dominant band of near 60,000 may be the IgM heavy chain. Also present are some unknown contaminants at high M_r.

The specificity of the purified antibodies was tested by Western blot analysis of total protein extracts (Figure 5.11 B). The results are better than the previous Western blots (Figure 3.4). The main difference between this and previous affinity purification procedures is that the resulting titre is much higher, probably due to the concentration step. The result on Western blots is a stronger signal than from previous anti-biotin antibody purifications, and more specific detection of biotin-containing proteins with less background. Previous Westerns (Figure 3.4) were unable to clearly show the presence of a large subunit ACCase, but Figure 5.11 B demonstrates that it is detectable with the improved purification of anti-biotin antibodies.

These improved, affinity purified anti-biotin antibodies were used to probe 2-D Westerns blots of the same material used for Figure 5.11 B

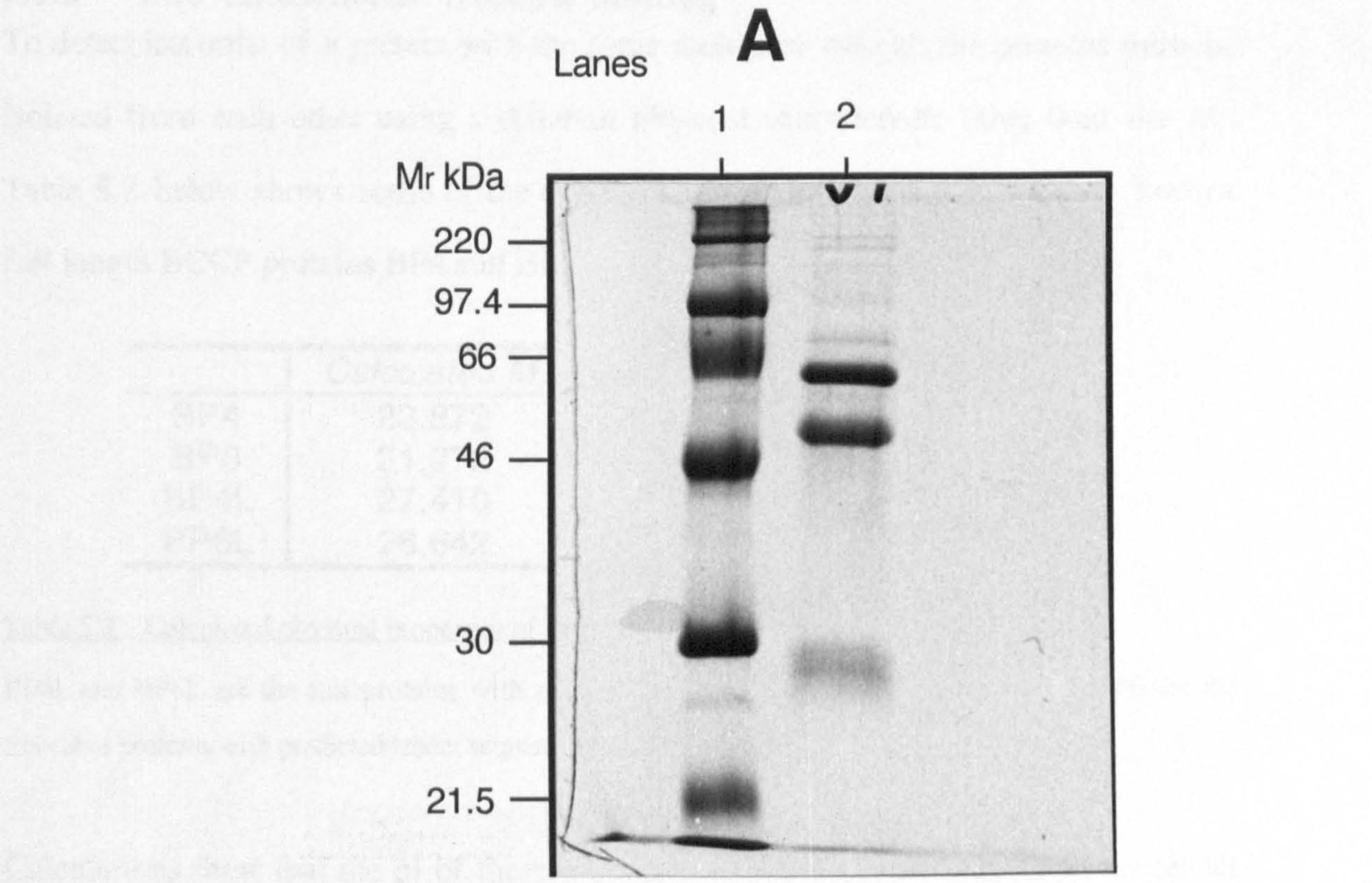
Figure 5.11 A Coomassie 10% stained gel of purified anti-biotin IgG.

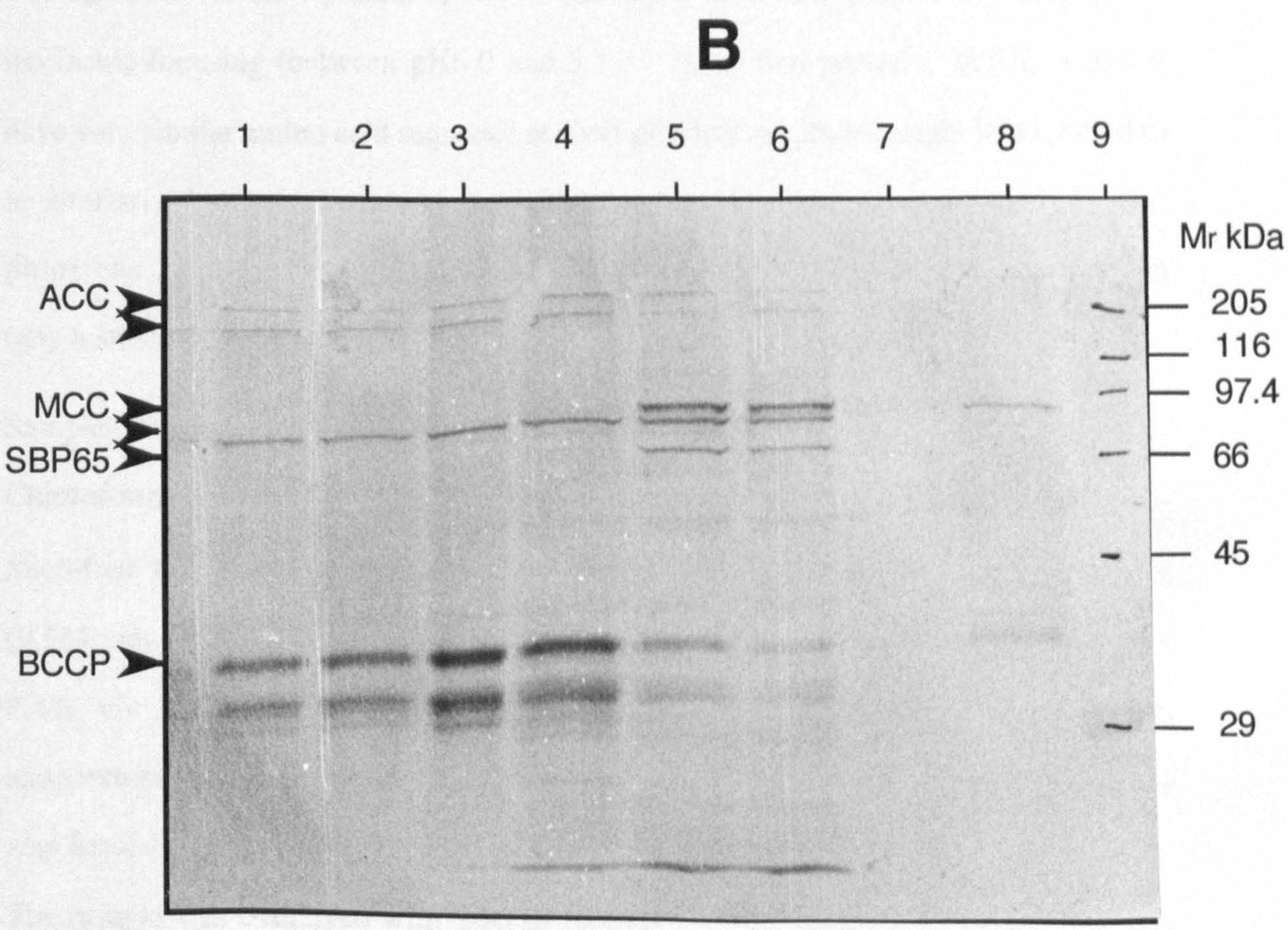
Biotin-binding proteins were purified by affinity chromatography on biotin-agarose from 75ml of sheep anti-biotin sera. Proteins were eluted with 1mM biotin in PBS and concentrated to 3ml. A microlitre of the purified antibody fraction was made 1 × with SDS sample buffer and run on a 10% acrylamide gel. The gel was stained with Coomassie R-250.

Figure 5.11 **B** Western blot using affinity purified anti-biotin IgG of total protein extracts from *Brassica napus* leaf, root and developing seeds.

Extracts were prepared as described in section 2.4.2 and equal amounts (in weight of starting material) were applied to each lane of a 12% acrylamide gel. Proteins were transferred to nitrocellulose and probed with affinity purified anti-biotin antibodies, sheep anti-rabbit IgG : alkaline phosphatase conjugate and developed with BCIP / NBT.

The lanes contain total protein extracts from the following; Lanes 1-6, developing embryos; Lane 7, root; and Lane 8, leaf. Lane 9 contains biotinylated protein standards. The developing embryo extracts were from embryos 44, 47, 51, 56, 61 and 70 daf.


A
Lanes
1
2
Mr kDa
220
97.4
66
46
30
21.5


B
1
2
3
4
5
6
7
8
9
Mr kDa
ACC
MCC
SBP65
BCCP
205
116
97.4
66
45
29

5.6.2 Two dimensional Western blotting

To detect isoforms of a protein with the same molecular weight, the proteins must be isolated from each other using a different physical characteristic other than the M_r. Table 5.2 below shows some of the calculated physical properties of the two known full length BCCP proteins BP4 and BP6

	Calculated M_r	*Net charge*	*Calculated pI*
BP4	22,872	-1.99	5.53
BP6	21,972	-1.83	5.85
BP4L	27,410	+2.01	8.24
BP6L	26,642	+2.51	8.26

Table 5.2 Calculated physical properties of known full length BCCP proteins.

BP4L and BP6L are the full proteins with predicted leader sequence intact, and BP4 and BP6 are the truncated proteins with predicted leader sequence cleaved.

Calculations show that the pI of these proteins vary by 0.02 pH units with the leader sequence intact but by 0.32 pH units when the proposed leader sequence is cleaved. The value for the BP4 protein (5.53) is consistent with that observed in preparative isoelectric focusing (between pH6.0 and 5.3) These two proteins, BCCP 4 and 6 have very similar amino acid sequence so their physical properties might be expected to be similar. Less similar proteins might be expected to have more variant physical properties. Nonetheless, theoretically it should be possible to separate proteins with only a small difference in pI if the correct pH gradient is used.

Samples for isoelectric focusing were prepared as described in section 2.4.2. Chloroform : methanol precipitated proteins were re-suspended with the aid of an microfuge tube disposable homogeniser, in 100µl of First Dimension sample buffer (9.5M urea, 2.0% Triton X-100, 5% 2-mercaptoethanol, 1.6% v/v 5/8 Pharmalyte, 0.4% v/v 3/10 Pharmalyte). Samples were vortexed for 10 minutes at room temperature to ensure complete solubilisation. Twenty five microlitres of each sample was loaded onto the top of a 1st dimension rod with the aid of a Hamilton syringe. The sample was overlayed with 20µl of Sample Overlay buffer (9M urea, 0.8% v/v 5/8 Pharmalyte, 0.2% v/v 3/10 Pharmalyte, 0.0025% Bromophenol blue) diluted 1:1

with ddH_2O. Samples were isoelectrofocused for 500V at 10 minutes followed by 750V for 3.5 hours.

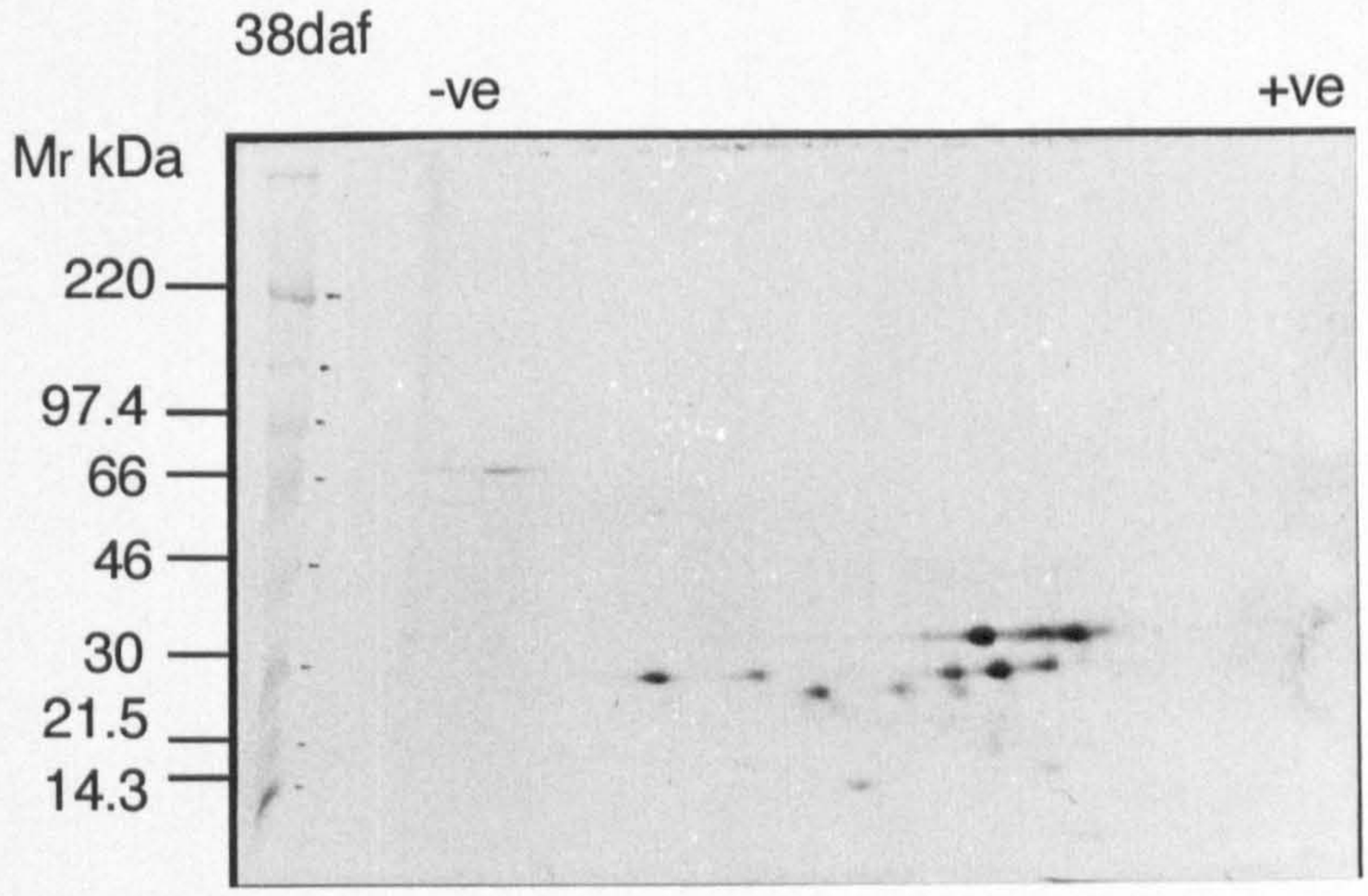
Isoelectricfocusing separates proteins by their pI, the second dimension separates proteins according to their M_r. Gradient gels (5-15%) were used for best resolution of proteins. When isoelectric focusing was complete, the gel rods were either used immediately or frozen at -20°C. There was no observed difference between using the gel rods immediately or storing them for several weeks at -20°C. To run the second dimension the gel rods were loaded onto the gel and equilibrated for 5 minutes by overlaying with 150µl of SDS sample equilibration buffer (0.0625M Tris-HCl pH6.8, 2.3% w/v SDS, 5% v/v 2-mercaptoethanol, 10% w/v glycerol, 0.0025% Bromophenol blue). The second dimension gels were run at 20mA per gel for 1 hour until the bromophenol blue reached the bottom of the gel.

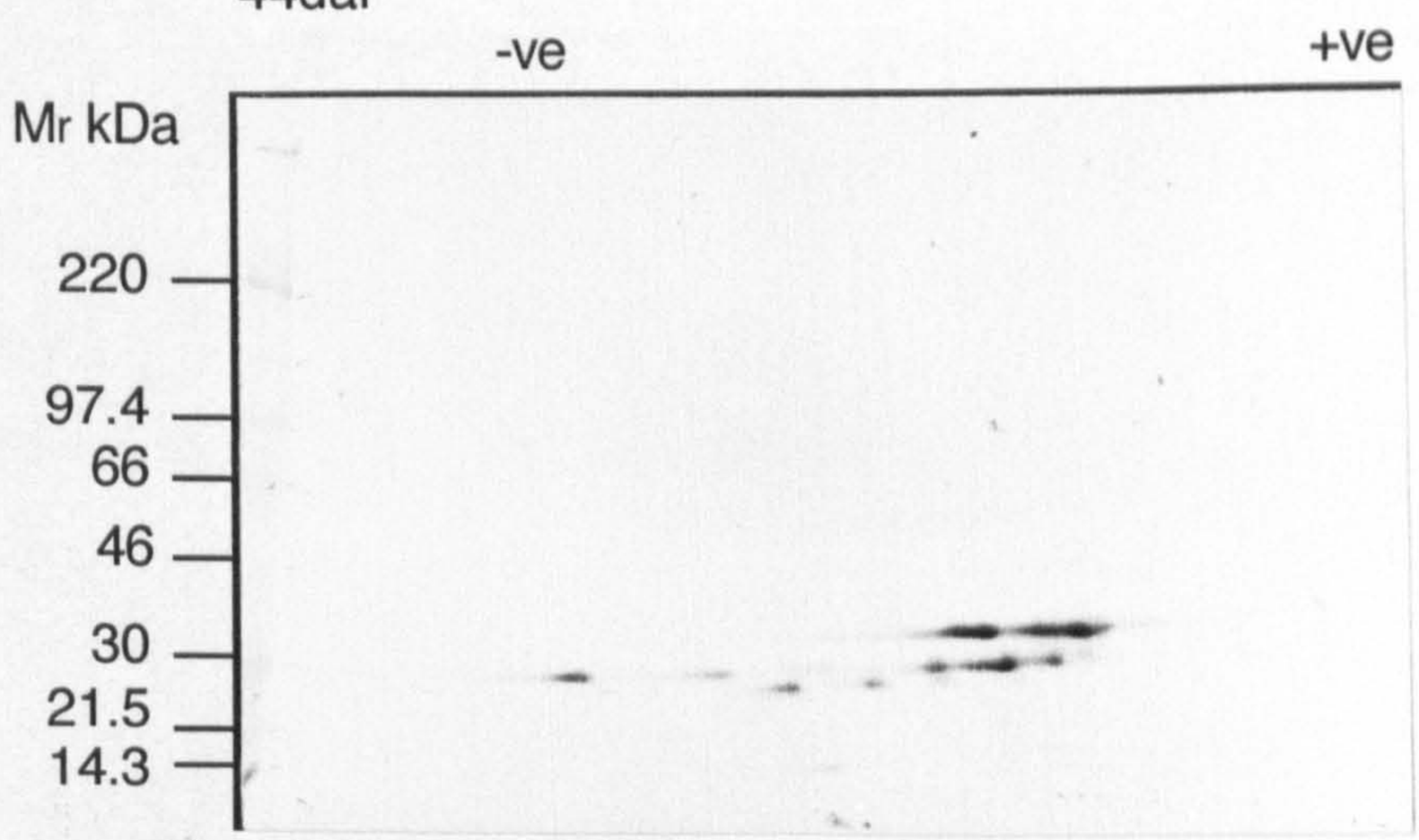
Gel proteins were either stained with Coomassie R-250 or transferred to nitrocellulose. 2-D Western blots were prepared in this manner with total protein extracts from leaf, root and embryos 38, 44, 47, 51, 56, 61 and 76 daf. The Western blots were probed with the affinity purified anti-biotin antibody (Figure 5.11). The antibody was detected with donkey anti-sheep IgG : alkaline phosphatase conjugate and developed with BCIP/NBT. The resulting Western blots are shown in Figure 5.12 A, B and C. No proteins were detected in the root sample so this has not been shown.

Figure 5.12 A 2-D anti-biotin Western blots of total protein extracts from seeds at various stages of embryogenesis.

Protein samples were isoelectrofocused in 6cm gel rods and separated on 5-15% acrylamide gels before transferring to nitrocellulose. The Western blot was probed with affinity purified anti-biotin antibodies, donkey anti-sheep IgG : alkaline phosphatase conjugate and developed with BCIP / NBT.

Pictures shows from top to bottom protein samples from 38daf, 44daf and 47daf.


38daf
-ve
+ve
Mr kDa
220
97.4
66
46
30
21.5
14.3


44daf
-ve
+ve
Mr kDa
220
97.4
66
46
30
21.5
14.3

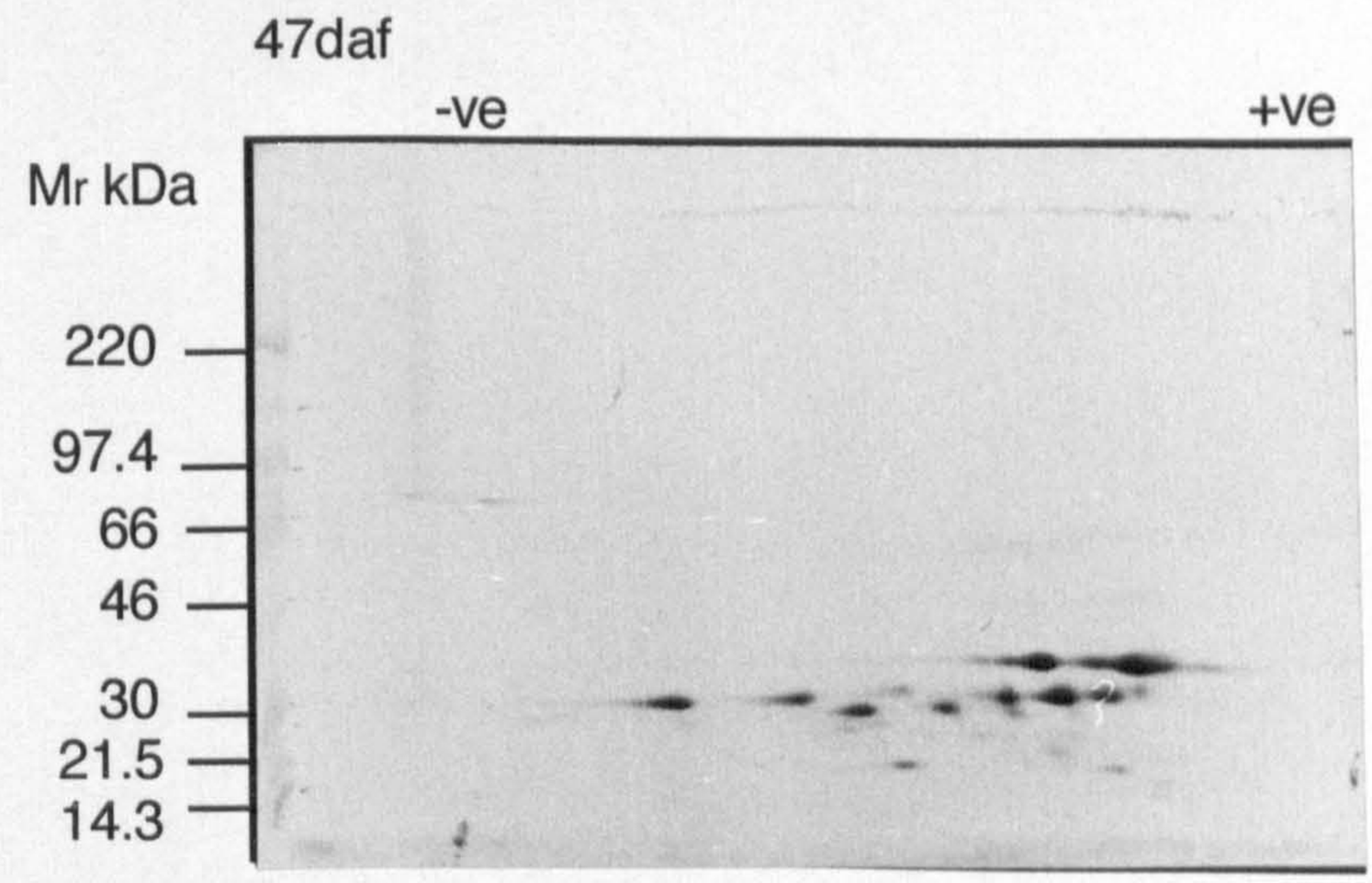

47daf
-ve
+ve
Mr kDa
220
97.4
66
46
30
21.5
14.3

Figure 5.12 B 2-D anti-biotin Western blots of total protein extracts from seeds at various stages of embryogenesis.

Protein samples were isoelectrofocused in 6cm gel rods and separated on 5-15% acrylamide gels before transferring to nitrocellulose. The Western blot was probed with affinity purified anti-biotin antibodies, donkey anti-sheep IgG : alkaline phosphatase conjugate and developed with BCIP / NBT.

Pictures shows from top to bottom protein samples from 51daf, 56daf and 76daf.

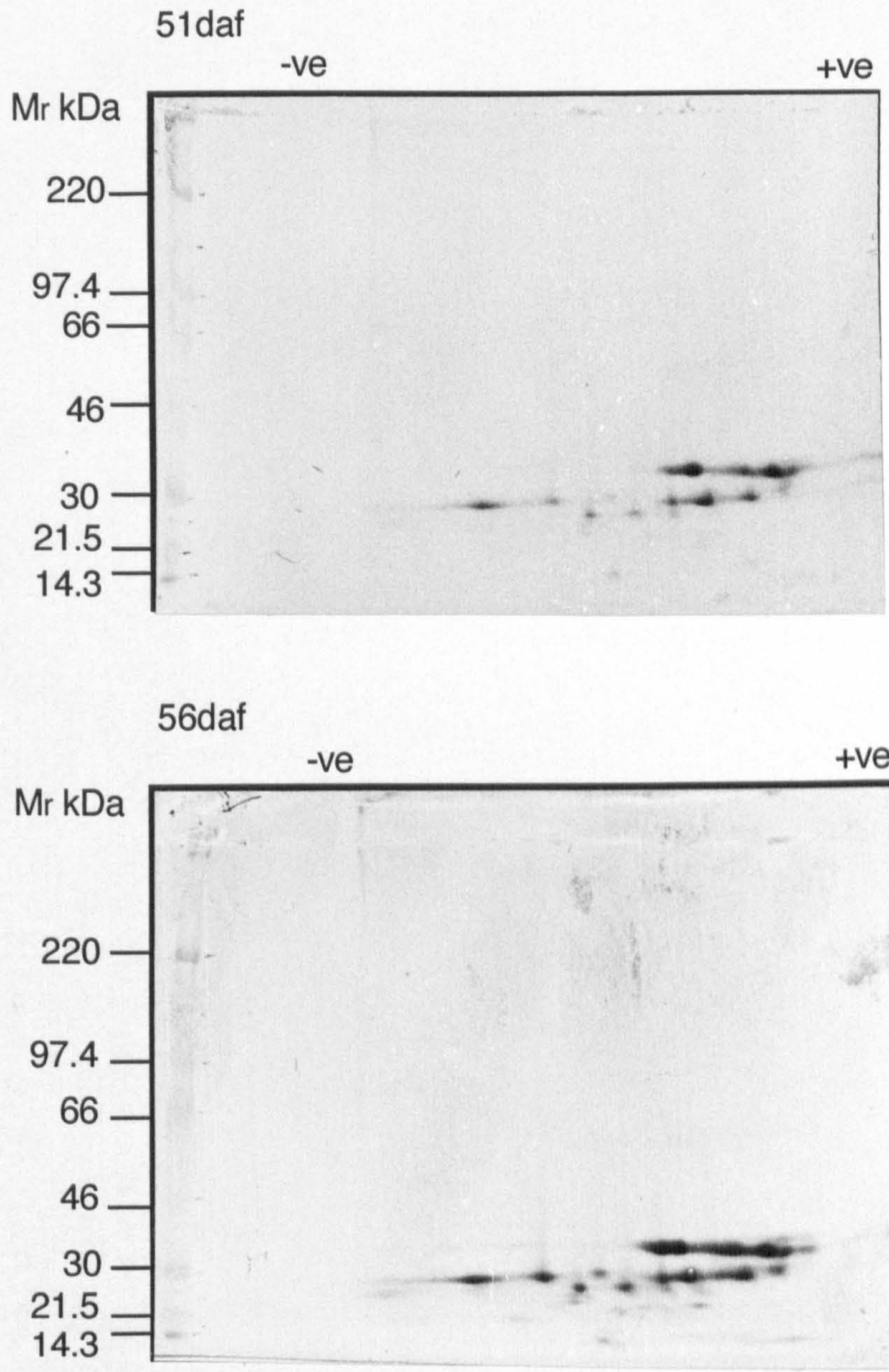
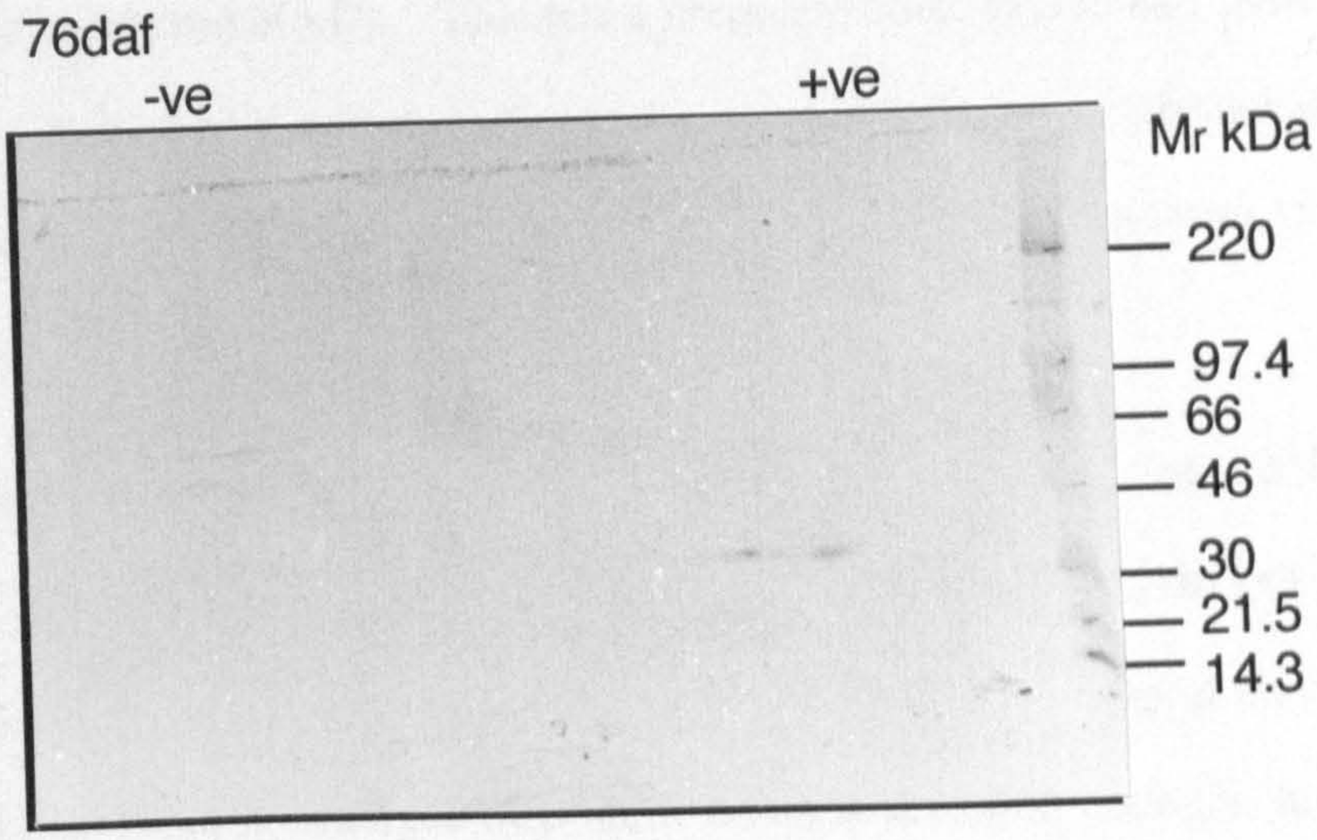

51daf
-ve
+ve
Mr kDa
220
97.4
66
46
30
21.5
14.3
56daf
-ve
+ve
Mr kDa
220
97.4
66
46
30
21.5
14.3
76daf
-ve
+ve
Mr kDa
220
97.4
66
46
30
21.5
14.3

Figure 5.12 C 2-D anti-biotin Western blot of leaf total protein extract.

Protein samples were isoelectrofocused in 6cm gel rods and separated on 5-15% acrylamide gels before transferring to nitrocellulose. The Western blot was probed with affinity purified anti-biotin antibodies, donkey anti-sheep IgG : alkaline phosphatase conjugate and developed with BCIP / NBT.

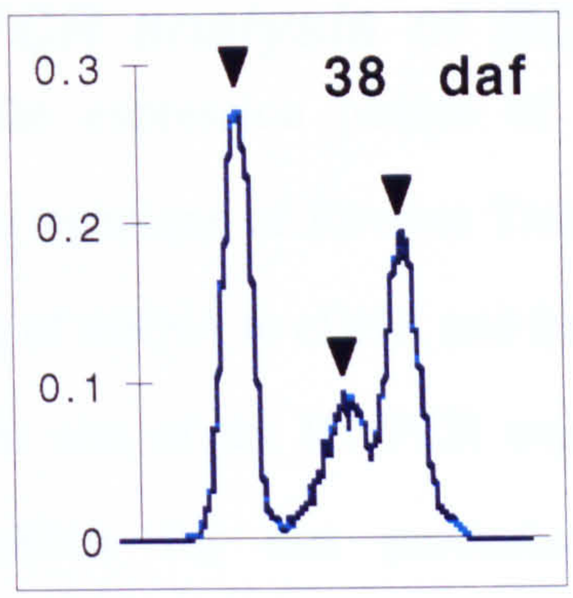

76daf
-ve
+ve
Mr kDa
220
97.4
66
46
30
21.5
14.3

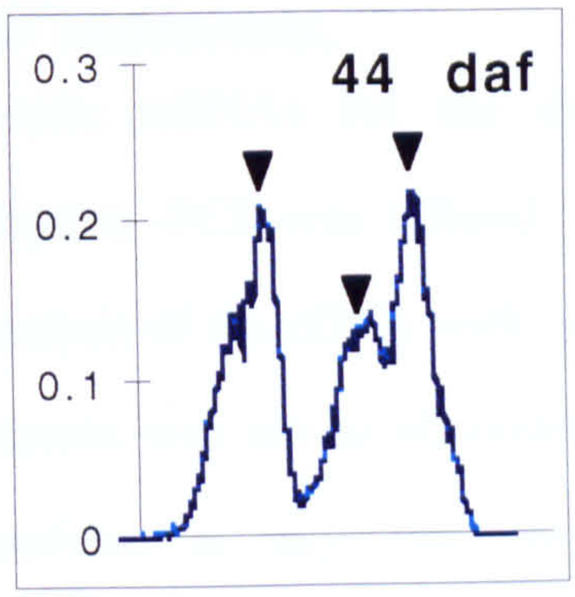
5.6.3 Analysis of 2-D anti-biotin Western blots

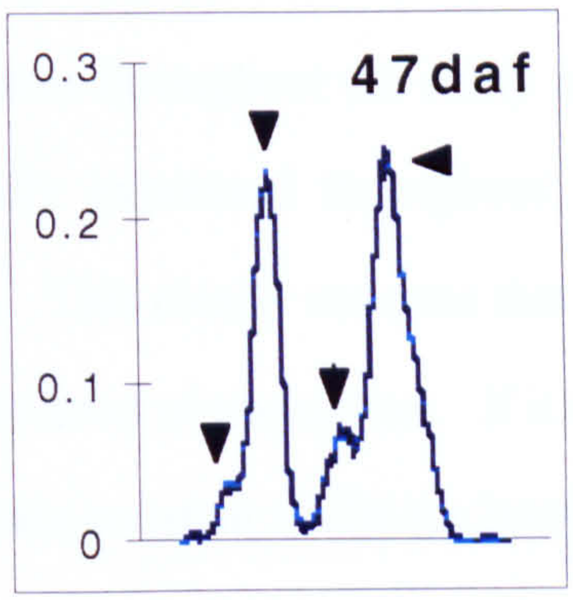
The two dimensional western blots in Figure 5.12 show that the pattern of expression of BCCP isoforms varies throughout embryogenesis. In particular, at 56 daf at least six spots can be seen with an estimated M_r of 35kDa whereas at 38 daf only three spots are visible. The leaf 2-D anti-biotin Western blot shows two very faint spots at 36kDa. To emphasise these changes in the pattern of observed spots, the Westerns were processed using a Bio-Rad GS-650 Densitometer. Images were scanned at 600dpi resolution and the optical density profile of the pattern of spots shown at 35kDa calculated. These profiles are shown in Figure 5.13.

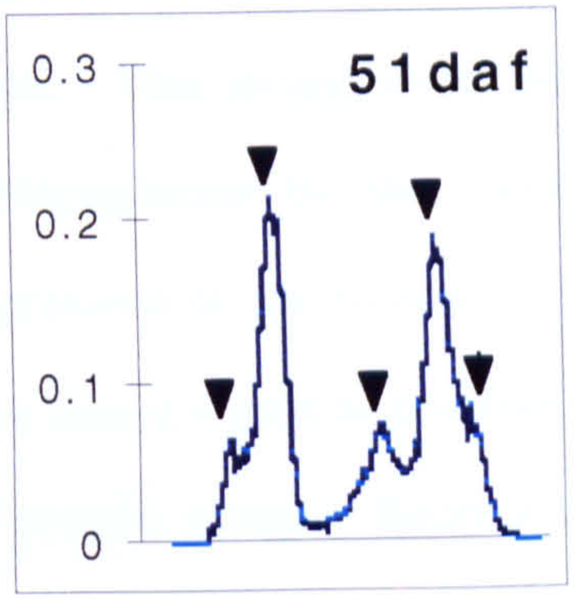
The only other biotinylated protein detected on 2-D blots was the β-methylcrotonyl-CoA protein at around 80kDa. This has a predicted basic pI and can therefore be seen as a smear near the negative end of the isoelectricfocusing dimension. Other biotinylated proteins cannot be seen due to constraints on the amount of protein that can be loaded onto the 1st dimension gel rods (5μg maximum).

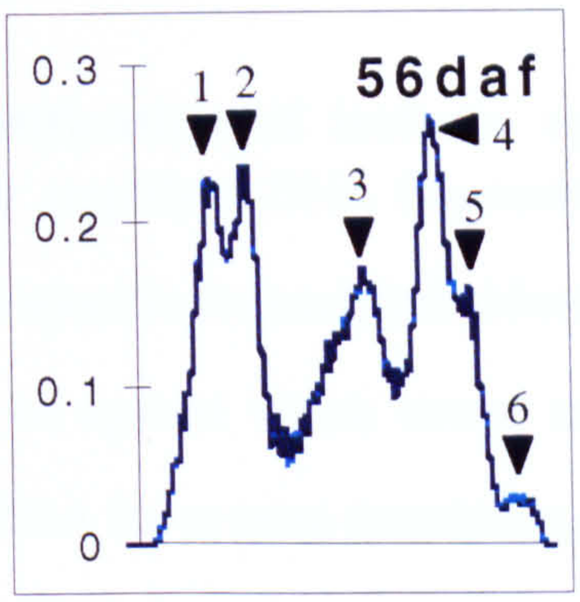
The profiles make clear the appearance and disappearance of BCCP isoforms throughout embryogenesis. What 2-D anti-biotin Western blots do not make clear is which isoform corresponds to which isolated cDNA. It was hoped that this could be achieved by correlating changes in protein isoform levels to changes in the levels of mRNA for each known cDNA.

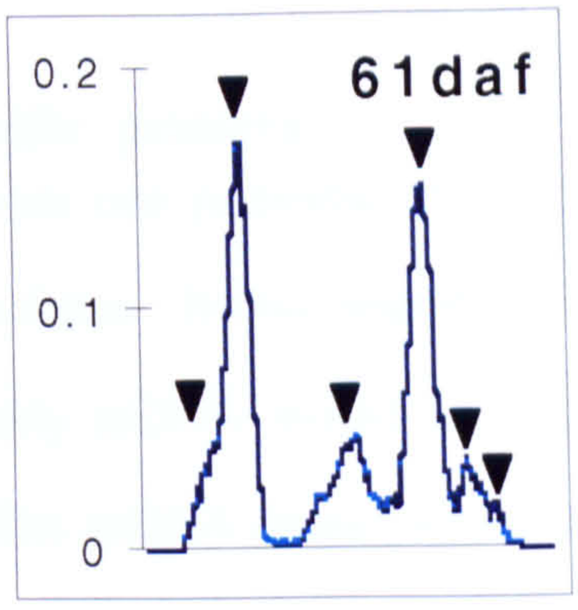


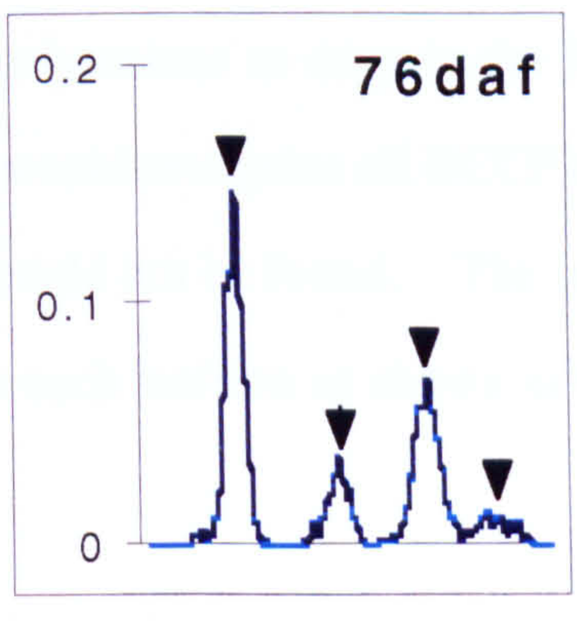












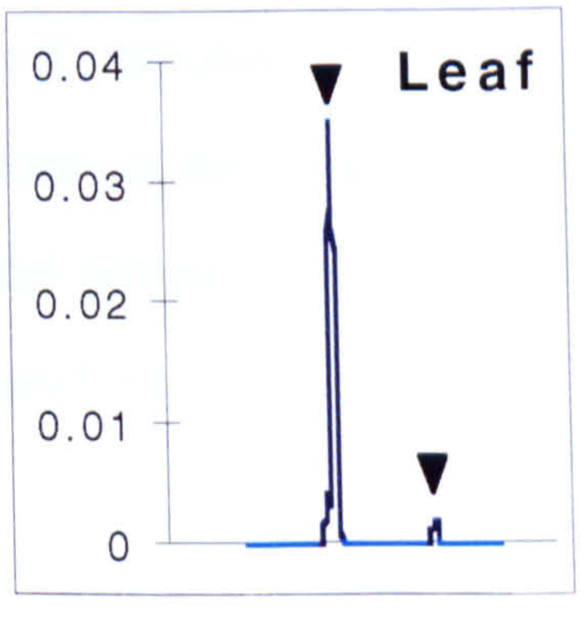


Figure 5.13 Pattern of expression of biotinylated 35kDa proteins thought to be isoforms of BCCP throughout embryogenesis.

The arrows mark the peaks. The numbering of peaks follows the order seen in 56daf where all six peaks are visible.

5.7 RT-PCR analysis of BCCP isoforms.

To identify the expression pattern of specific mRNAs for the different BCCP isoforms, the technique of Reverse Transcription -PCR was utilised. This enabled the converting of mRNA to cDNA and the analysis of the cDNA with isoform specific primers. The aim of the RT-PCR experiments was not to absolutely quantify the levels of mRNA for one particular isoform at any one stage throughout embryogenesis, but rather to obtain a pattern of expression of mRNA for each particular isoform throughout the many stages. This should reveal the isoforms that are differentially expressed throughout embryogenesis by the sudden increase in mRNA levels. This clearly assumes that expression of the protein is not significantly regulated at the level of translation. If it were then it would be possible for the level of mRNA to remain constant whilst the level of protein varied. Bearing this assumption in mind, the levels of mRNA were analysed for each isoform.

5.7.1 Identification of isoform specific primers.

To specifically amplify a DNA fragment from one particular isoform primers were designed to be specific for each individual isoform. It was important to decide which primer would be against which strand as only ssDNA would be made by oligo dT priming of mRNA for reverse translation. The ssDNA strand would be the antisense strand, therefore the first primer would have to be in the sense orientation at the 5' end for the DNA polymerase to copy in the 5' to 3' direction. It was decided to use a primer which would recognise all BCCP isoforms at the 5' end as a specific primer for each isoform could not be found. The second primer, at the 3' end was designed to be specific for each isoform as shown in Figure5.14.

Figure 5.14 Alignment of BCCP DNA sequences and the isoform specific primers.

Sequences were aligned using the PILEUP program from the Wisconsin GCG package with default settings. The area of strongest homology was used to design a primer (Universal BP) which would recognise all six known isoforms. Areas were identified in each cDNA of least homology between known sequences. These were used to design primers which would be specific for each known cDNA.

Universal BP

```
701                                                          750
BCCP1  CACCCTTTAT TAAGGTTGGA GACAAAGTGC AGAAGGGACA AGTTCTATGC
BCCP2  CACCCTTTAT CAAGGTTGGA GACAAAGTGC AGAAGGGACA AGTTCTATGC
BCCP4  CTCCTTTTGT CAAGGTTGGA GATAAGGTGC AGAAGGGTCA AGTTGTTTGC
BCCP6  CTCCTTTTGT CAAGGTCGGA GATAAAGTGC AGAAGGGTCA AGTTGTTTGC
BCCP7  CTCCTTTTGT CAAGGTTGGA GATAAGGTGC AGAAGGGTCA AGTAGTTTGC
BCCP3  CCCCTTTTGT CAAGGTTGGA GATAAGGTGC AGAAGGGTCA AGTTGTTTGC
       751                                                   800
BCCP1  ATCGTTGAAG CTATGAAGTT AATGAATGAA ATAGAGTCTG ...ACAACGG
BCCP2  ATCGTTGAAG CCATGAAGCT AATGAATGAA ATAGAGTCTG ATCAAACGGG
BCCP4  ATTATTGAAG CTATGAAACT CATGAATGAG ATTGAGGCTG AGAAATCAGG
BCCP6  ATTATCGAAG CTATGAAACT GATGAATGAG ATTGAGGCTG AGAAGTCAGG
BCCP7  ATCATTGAAG CTATGAAACT CATGAATGAG ATTGAGGCTG AGAAATCAGG
BCCP3  ATTATTGAAG CTATGAAACT GATGAACGAG ATTGAGGCTG AGAAGTCAGG
       801                                                   850
BCCP1  AACCGTAGTG GATATCGTTG C...AGAGAT GCAACCTGTT AGCCTCGACA
BCCP2  AACCGTAGTG GATATCGTTG CAGAAGATGG CAAGCCTGTT AGTCTCGACA
BCCP4  AACCATCACT GAACTACTAG CTGAAGATGG AAAACCGGTT AGCGTTGACA
BCCP6  AACCATCACT GAATTACTGG CTGAAGATGG AAAACCCGTC AGCGTTGATA
BCCP7  AACCATCACT GAACTACTGG CTGAAGATGG AAAACCGGTT AGCGTTGACA
BCCP3  AACCATCACC GAGTTACTGG CTGAAGATGG AAAACCGGTC AGCGTTGATA
       851                                                   900
BCCP1  CTCCTCTGTT TGTGGTTCAA CCGTAGAATC GGCACCATGA GAAGTGGAGA
BCCP2  CTCCTCTGTT TGTGGTTCAA CCGTAGAATC GACACCACGA GAGATGGAGT
BCCP4  CGCCTCTGTT TACCATCGTG CCTTGAAGCG .......... TCATTTTAGC
BCCP6  CGCCTCTGTT TACCATCGCT CCTTGAAATG AAGAAGCTGT CATTTTGAGC
BCCP7  CGCCTCTGTT TACCATCGTG CCTTGAAGCG .......... TCATTTTAGC
BCCP3  CGCCTCTGTT TACCATCGTT CCTTGAAATG AAGAAGCTG. TCATTTTAGC
       901                                                   950
BCCP1  AAAGAGG... .......... .......CTA CTTGTGTCCT GAGACTACTT
BCCP2  AAAGAGGGCT ACTTTAAATC GATCTATGTG CTTTGGTAAT ACTGTTTCTT   BP2
BCCP4  TGG*TCAG*A CA...TCTTC AAGTCTTGGT TTAGCTAATA CCGACGTGCT  BP4i
BCCP6  TGCTTCAGAA CA...TCATC AA.....ACT CCTGCTTATA CCGACGTGCT
BCCP7  TGGTTCAGAA CA...TCTTC AA....AGTC TTGGTTTATA TCGACGGGCT
BCCP3  TGGTTCAGAA CACCTACTTC AAACTCTTGG TTTAGCTATA CCAACGTGCT
       951                                                  1000
BCCP1  CTTGAGACT. ........CT TGTGTCCTTT TCGTTGTTAC CGTTAAAAGT
BCCP2  GAGTTTAAG. ........AA TGTGTCTTCT TCATTTGTTA CTGTCAAACT
BCCP4  G*AAATCA*T ..AAAGTAA* TGTTTCGGTC ATA*TGGTTT GG........
BCCP6  GACAATCGTT .AAAAGTAAT CATCTCGGTC AGGTTGGTTC GGGTTCGGCT
BCCP7  GAAAATCATT ..CAAGTAAT TGTTTCGGTT TTTTCTTTTT GCTTGAACTG  BP7ii
BCCP3  GACAATCGTT AAAAAGTAAT TATTTCTGTC AGGCTGGTTT GG........
       1001                                                 1050
BCCP1  CTGTTAGTTT TTTCTTTTGG TTCGATGAGG GAGGTGGTTA AAGAAGATGA
BCCP2  CTGTTAGTTT TTTTTTTTGG TCTGGTGAGG AAGAAAGAGT TTTTAGTTTC
BCCP4  ...TTTGTTC GTTTTTTTGT TTTTCCTCAC CTT....... ..........
BCCP6  AATTATGTTT TTTTTTTCTT TTTCTCTCAA CTTTCTGAAC TCACTT....
BCCP7  TCACATTTTG TGTGAACCAG TTAACGTATT AAAATTGCAT GTTTTTAGGA
BCCP3  ..TTATGGTT GTCTTTTTTT TTTATTAAAA AAAACTTTCT GAACTGTCAC
       1051                                                 1100
BCCP1  GAGCTCTGTT TTATTGACAC ACGAATCTTT GGTTCTGTTT TTTTGCAATT
BCCP2  TA...TTGGT TAAACAAAAA AAAAAGATGA GGCTATGTTT TATTTTTTTT
BCCP4  ....TTGTAT GAATCACGTG ACGTATTAAT ATTGCATG*T TTTAAGATAA
BCCP6  ..TCTTTTGA GAATCGCGTA ACATATCAGT TATGCATGTC TTTCGAACAA
BCCP7  TAATTTGGAC AAATCAATTC AGTTTACAGT GGGTTGTACT ATCTCCCCTG  BP7i
BCCP3  CTTCTGTTGA GAATCACGTA ACATATCAGA TTTACAAGGT GAAACGGCTT  BP3
       1101                                                 1150
BCCP1  TTCTATATCA AATTTTTTTA TCACCCTCTC CCAACCACAC GCTCTCGTCT  BP1
BCCP2  CA..... ..... ..... ..... .....
BCCP4  TTTCGACCAA ACAAT..... ..... ..... .TCAGTTTAC           BP4ii
BCCP6  ATCAGACAAA CTAATAGTAT GCTGTACTAT GAACTGCCCT ATTAGGGTGA  BP6
BCCP7  GTTTGATATG ATTTGTGTTG TTCGGATGAT ATAACTTTAT AGAAAGTGTT
BCCP3  GGAGGAGGCT GAACGATTGG AAGTTTCGAC ATGGTTACAT TTCCAGTAAT
```

The sequence of the individual primers designed is shown in Figure 5.15.

UniBP	GAA GAY AAR GTG CAG AAG GG	Tm 54.0°C
BP1	CGA GAG CGT GTG GTT GG	Tm 51.7°C
BP2	CAA GAA ACA GTA TTA CCA AAG CAC	Tm 52.1°C
BP3	TCA GCC TCC TCC AAG CC	Tm 51.7°C
BP4i	GTC GGT ATT AGC TAA ACC AAG AC	Tm 53.3°C
BP4ii	GTA AAC TGA ATT GTT TGG TCG A	Tm 49.1°C
BP6	GGG CAG TTC ATA GTA CAG CAT. AC	Tm 55.1°C
BP7i	GAA CAA CAC AAA TCA TAT CAA ACC	Tm 50.4°C
BP7ii	CAC AAA ATG TGA CAG TTC AAG C	Tm 50.9°C

Figure 5.15 Primers and their Tms designed for RT-PCR of specific isoforms of BCCP.

UniBP is the only primer in the sense orientation and has two redundant positions, Y (C/T) and R (A/G) so at least 25% of the primers will be 100% specific, the other 75% will have 90 or 95% specificity.

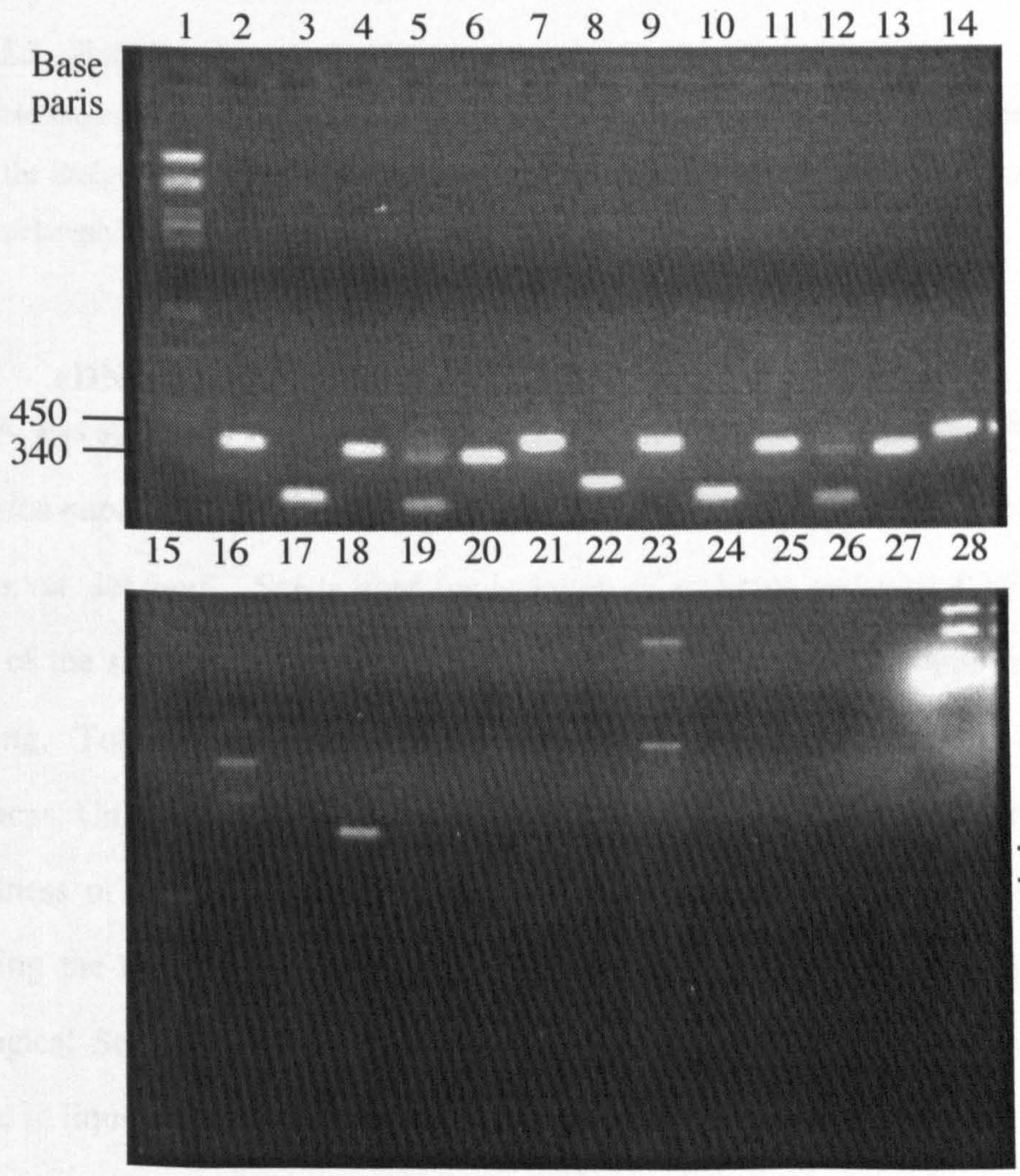
Primers were tested for suitability in PCR and their specificity in PCR reactions with Vent® DNA Polymerase using the isolated plasmids for each of the BCCP cDNAs. A mixture of the plasmids for all known six cDNAs was included for each primer as a control. PCR was carried out using AmpliWax™ PCR Gem 100 and Gene Amp Thin-Walled Reaction Tubes. The reaction was carried out over 30 cycles of 94°C 1 minute, 50°C 1 minute, 74°C 40 seconds. When finished, the reactions were cooled to room temperature and 10µl was run on a 1% agarose gel. The gel was recorded with a BIO-RAD Gel Documentation System, and is reproduced in Figure 5.16.

The results in Figure 5.16 show that the primers produce a single product from plasmid copies of the cDNA. The primers all appear to be specific apart from BP4i (Lane 5 and 12) which produces both the expected product size and another unexpected product. The predicted product sizes from the different primers are listed in Table 5.3.

Consequently, all the primers except BP4i could be used to asses the levels of mRNA in embryos of *Brassica napus*.

Figure 5.16 Demonstration of the specificity of the designed gene specific primers by PCR.

PCR reactions were set up to test the specificity of the primers for their targeted cDNAs. The PCR products were separated on a 1.5% agarose gel. PCR reactions contained the following templates and primers; Lane 2, pBP1with primers UniBP and BP1; Lane 3, pBP2 with primers UniBP and BP2; Lane 4, pBP3 with primers UniBP and BP2; Lane 5, pBP4 with primers UniBP and BP4i; Lane 6, pBP4 with primers UniBP and BP4ii; Lane 7, pBP6 with primers UniBP and BP6; Lane 8, pBP7 with primers UniBP and BP7i. Lanes 9-27 shows the products when using a mixture of plasmids pBP1-7 (1ng each plasmid) with the following primers; Lane 9, UniBP and BP1; Lane 10, UniBP and BP2; Lane 11, UniBP and BP3; Lane 12, UniBP and BP4i; Lane 13, UniBP and BP4ii; Lane 13, UniBP and BP6; Lane 15, UniBP and BP7i; Lane 16, BP1; Lane 17, BP2; Lane 18, BP3; Lane 19, BP4i; Lane 20, BP4ii; Lane 21, BP6; Lane 22, BP7i; Lane 23, UniBP.


1 2 3 4 5 6 7 8 9 10 11 12 13 14
Base paris
450
340
15 16 17 18 19 20 21 22 23 24 25 26 27 28
Base paris
450
340

Primer pair	*Expected product size base pairs (with intended cDNA)*
UniBP + BP1	394
UniBP + BP2	233
UniBP + BP3	383
UniBP + BP4i	214
UniBP + BP4ii	359
UniBP + BP6	406
UniBP + BP7	386
UniBP + BP7ii	275

Table 5.3 Size of product produced from intended target gene with specific primers.

The table shows the predicted length of RT-PCR products when the intended cDNA clone is amplified using the designed gene specific primers. If the primers were not gene specific, then a product of different length would be expected.

5.7.2 cDNA template for PCR

cDNA was synthesised from total RNA isolated from embryos dissected from seeds of *Brassica napus* var. Falcon as well as from total RNA isolated from leaves of *Brassica napus* var. Jet neuf. Seeds used for isolation of embryos and total RNA preparation were of the same time course as used for protein analysis by 2-D anti-biotin Western blotting. Total RNA was isolated by Mr Russell Swinhoe, Department of Biological Sciences, University of Durham, from embryos 38, 44, 49, 54, 58 and 63 daf. The intactness of the RNA was confirmed by electrophoresis on a formamide gel and viewing the ribosomal bands (carried out by Mr Russell Swinhoe, Department of Biological Sciences, University of Durham, results not shown). Total RNA was stored in liquid nitrogen from the time of preparation until required.

cDNA synthesis was performed with the SUPERTSCRIPT™ Preamplification System for First Strand cDNA Synthesis from LIFE TECHNOLOGIES, GIBCOBRL. New sterile disposable plastic tips and reaction tubes were used throughout along with DEPC treated water to eliminate RNase contamination. Five micrograms of total RNA and 0.5µg oligo dT primers were combined in a total volume of 12µl. The RNA was denatured by heating to 70°C for 10 minutes in a dry heating block, and placed on ice to cool for 1 minute allowing the oligo dT primers to anneal. 2µl each of PCR buffer

(200mM Tris-HCl pH8.4, 500mM KCl), 25mM $MgCl_2$ and 0.1M DTT were added plus 1µl of 10mM dNTP mix. The reaction components were mixed gently by flicking the reaction tube and centrifuged briefly to bring the liquid together at the bottom of the tube. The reaction was heated to 42°C for 5 minutes prior to addition of 1µl (200U) of SUPERSCRIPT II RT. The first strand synthesis was incubated at 42°C for 50 minute to allow the reverse transcriptase to copy the RNA. The reaction was terminated by heating to 70°C for 15 minutes. The reaction was cooled on ice before 1µl of DNase free RNase H was added, mixed and incubated at 37°C for 20 minutes. The synthesised cDNA was stored at -20°C until required.

A separate first strand synthesis reaction was performed for total RNA from leaf and the different stages of developing embryo.

5.7.3 Quantifiable amplification of DNA by PCR

PCR is an exponential process as the products produced by the reaction can serve as templates for the next round of PCR reactions. This means that during the initial cycles of the PCR reaction, the rate of accumulation of PCR products increases exponentially. Towards the end of the reaction the concentration of reagents such as primers and dNTPs required for the reaction decrease. This leads to a relative reduction in the rate of accumulation of product and the reaction is no longer exponential. This has implications for the quantification of PCR reactions. When comparing the levels of products generated from two PCR reactions containing exactly the same amount of primers and dNTPs but different amounts of starting template, it is important that samples are analysed within the exponential phase of PCR. If the reaction containing more starting template is allowed to progress through the exponential phase into the linear phase, then the amount of product in the other PCR reaction containing less starting template will quickly catch up with the levels in other reaction. This is because the first reaction containing more starting template would now be accumulating product linearly whereas the second PCR reaction containing less starting template is still accumulating product exponentially.

To ensure that the PCR reactions did not leave the exponential phase of the reaction, a PCR reaction was set up and samples were removed from the reaction every 5 cycles and analysed on 1.5% agarose gels to monitor the progress of the reaction. A rule of thumb is that 5 cycles should produce twice as much product during the exponential phase (Clontech, 1991).

One microlitre of the first strand cDNA synthesis reaction was used per PCR reaction. PCR was carried out using primers BP4ii and UniBP, AmpliWax™ PCR Gem 100 and Gene Amp Thin-Walled Reaction Tubes. The same conditions and concentration of components was used as in the test reaction (Figure 5.16). The thermal cycler was programmed to carry out cycles of 94°C 1 minute, 50°C 1 minute and 74°C 40 seconds for 40 cycles.

Samples of 10μl were taken from the reactions after 20, 25, 30, and 40cycles and run on 1% agarose gels. The products obtained are shown in Figure 5.17. Lane 9 contains the control reaction using pBP4 as a template. By comparing the intensity of the band produced in this lane to the 800 base pairs band in the λPst I markers it is possible to say that the amount of product is increasing exponentially for 30 cycles. There is little increase from between 30 and 40 cycles where this PCR reaction will have entered the linear phase. The PCR reactions from the synthesised cDNA have a lower starting concentration of DNA and therefore more cycles were required to produce a product. Product can just bee seen after 30 cycles in lane 5. The products are clearly visible after 40 reactions, suggesting that the PCR reactions are exponential up to 40 cycles from the small amount of starting material in this synthesised cDNA.

Figure 5.17 A Products produced by 20, 25, and 30 cycles of RT-PCR with primers BP4 and UniBP.

PCR was carried out using 1µl of first strand synthesis, primers UniBP and BP4 with AmpliWax™ PCR Gem 100 and Gene Amp Thin-Walled Reaction Tubes. The same conditions and concentration of components were used as in the test reaction (Figure 5.16). The thermal cycler was programmed to carry out cycles of 94°C 1 minute, 50°C 1 minute and 74°C 40 seconds for 40 cycles.

Samples of 10µl were removed after 20, 25 and 30 cycles and run on a 1% agarose gel. Lanes contained, Lane 1 and 11, 1µg of λPst I markers; Lanes 2-7, PCR from cDNA synthesised from embryos, Lane 2 38daf; Lane 3, 44daf; Lane 4, 49daf; Lane 5, 54daf; Lane 6, 58daf, Lane 7, 63daf. Lane 8, PCR from cDNA synthesised from leaf mRNA; Lane 9, PCR from pBP4 plasmid (positive control); Lane 10, PCR from pBC1/2/3/6/7 plasmids (negative control).

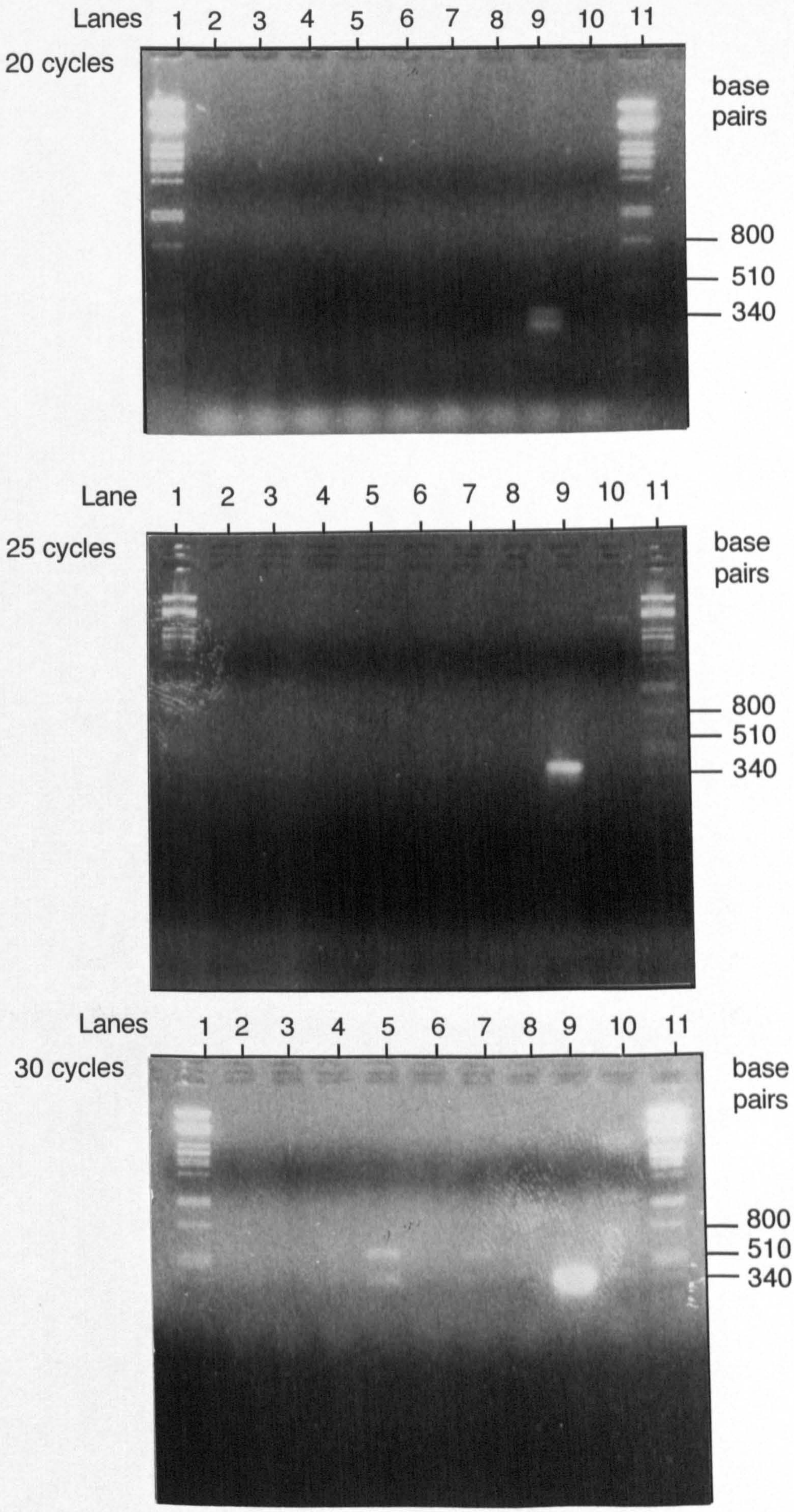
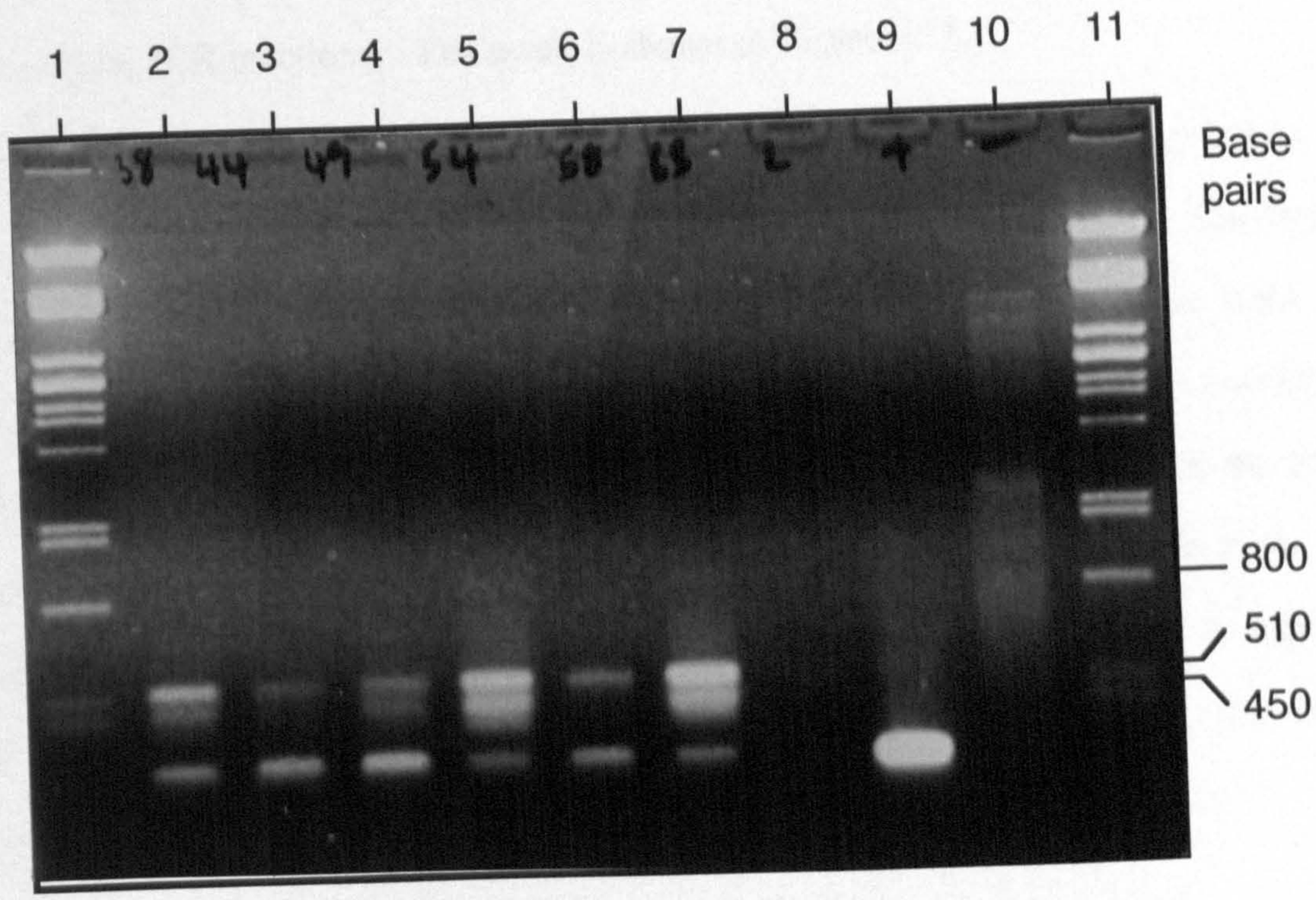

Lanes 1 2 3 4 5 6 7 8 9 10 11
20 cycles
base pairs
800
510
340
Lane 1 2 3 4 5 6 7 8 9 10 11
25 cycles
base pairs
800
510
340
Lanes 1 2 3 4 5 6 7 8 9 10 11
30 cycles
base pairs
800
510
340

Figure 5.17 B Products produced by 40 cycles of RT-PCR with primers BP4 and UniBP.

PCR was carried out using 1µl of first strand synthesis, primers UniBP and BP4 with AmpliWax™ PCR Gem 100 and Gene Amp Thin-Walled Reaction Tubes. The same conditions and concentration of components were used as in the test reaction (Figure 5.16). The thermal cycler was programmed to carry out cycles of 94°C 1 minute, 50°C 1 minute and 74°C 40 seconds for 40 cycles.

Samples of 10µl were removed after 40 cycles and run on a 1% agarose gel. Lanes contained, Lane 1 and 11, 1µg of λPst I markers; Lanes 2-7, PCR from cDNA synthesised from embryos, Lane 2 38daf; Lane 3, 44daf; Lane 4, 49daf; Lane 5, 54daf; Lane 6, 58daf, Lane 7, 63daf. Lane 8, PCR from cDNA synthesised from leaf mRNA; Lane 9, PCR from pBP4 plasmid (positive control); Lane 10, PCR from pBC1/2/3/6/7 plasmids (negative control).


1
2
3
4
5
6
7
8
9
10
11
Base pairs
800
510
450

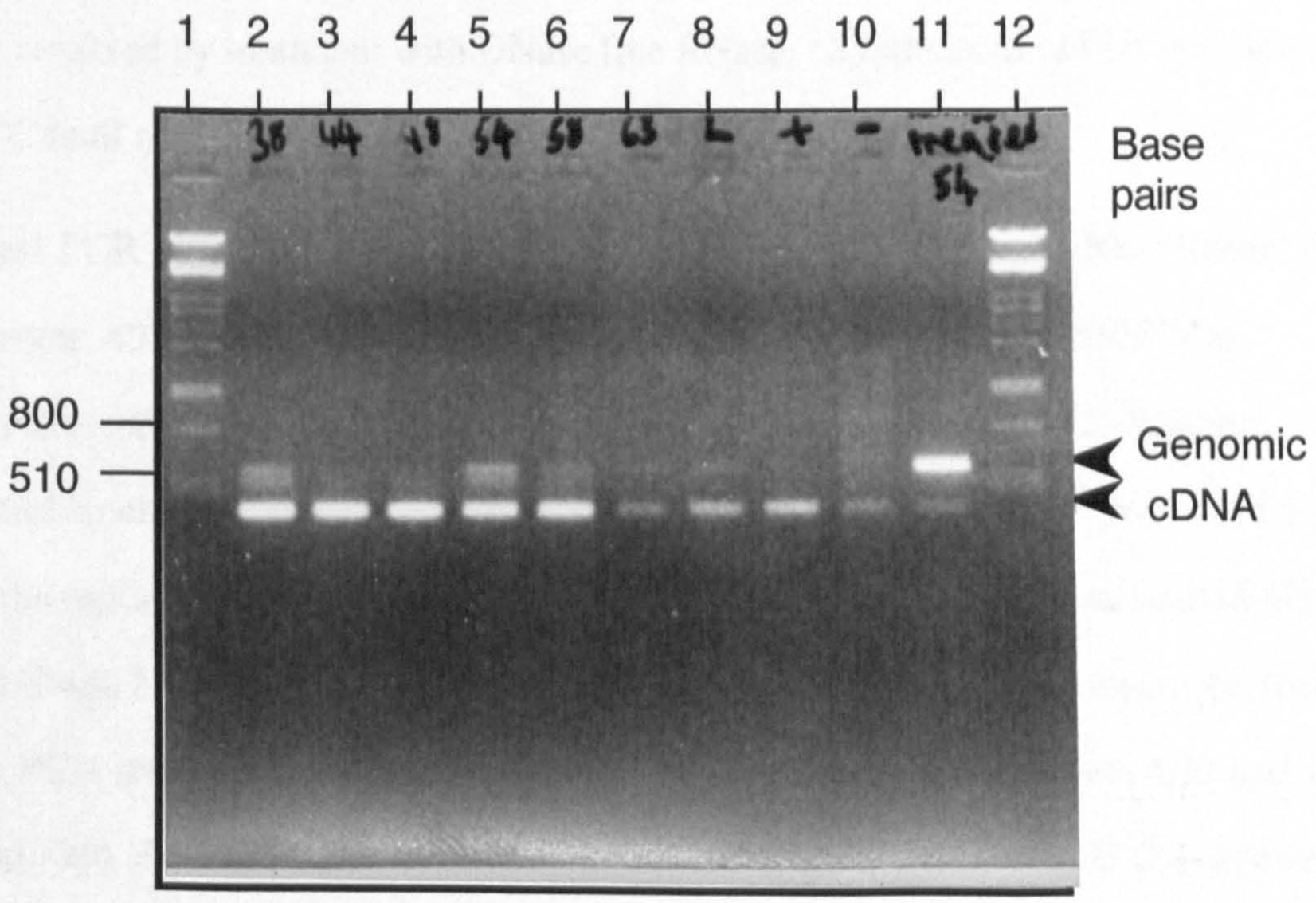
The products produced by the RT-PCR reactions are more complex than the product from the control reaction. This could be because the primers are not specific for the BP4 gene, but previous results have demonstrated that they are (Figure 5.16). The more likely reason is that the total RNA is contaminated with trace amounts of genomic DNA. To test this hypothesis the PCR reaction was repeated with an additional sample as a control. 1µg of total RNA was treated with DNase free RNase in 5µl of 20mM Tris-HCl pH8.4, 2.5mM $MgCl_2$, 20mM KCl for 30 minutes at 37°C. This sample was used as a control for DNA contamination. RNA was removed by the RNase but any DNA contamination would remain intact. The sample was used directly in PCR reactions. The result is shown in Figure 5.18.

These results show that the larger band produced is a result of DNA contamination of the original total RNA preparations (marked Genomic in Figure 5.18). The larger band was produced in Lane 11 which contained only RNase treated total RNA as template. The only possible template in the PCR reaction shown in Lane 11 was DNA contamination in the original total RNA preparation. This interferes with the PCR reaction by providing a template for amplification which is not derived from the mRNA. This can adversely affect the quantification of the reaction as it may be amplified preferentially to the cDNA. It was necessary to remove the DNA contamination for further analysis.

Figure 5.18 RT-PCR using DNase free RNase treated sample as a control (Lane11) with primers BP4 and UniBP.

PCR was carried out using 1µl of first strand cDNA synthesis reaction, primers UniBP and BP4 with AmpliWax™ PCR Gem 100 and Gene Amp Thin-Walled Reaction Tubes. The same conditions and concentration of components were used as in the test reaction (Figure 5.16). The thermal cycler was programmed to carry out cycles of 94°C 1 minute, 50°C 1 minute and 74°C 40 seconds for 30 cycles.

Samples were analysed on a 1.5% agarose gel. Lanes contained the following; Lane 1 and 12, Pst I markers; Lanes 2-7, PCR from cDNA synthesised from embryos, Lane 2 38daf; Lane 3, 44daf; Lane 4, 49daf; Lane 5, 54daf; Lane 6, 58daf, Lane 7, 63daf. Lane 8, PCR from cDNA synthesised from leaf mRNA; Lane 9, PCR from pBP4 plasmid (positive control); Lane 10, PCR from pBC1/2/3/6/7 plasmids (negative control); Lane 11, DNase free RNase treated 54daf total RNA.


1 2 3 4 5 6 7 8 9 10 11 12
38 44 43 54 58 63 L + - treated 54
Base pairs
800
510
Genomic
cDNA

5.7.4 Removal of genomic DNA

Genomic DNA was removed from preparations of total RNA by treating with RNase free DNase. A known amount of total RNA (final concentration close to 0.2µg / µl) was treated with 1U of RNase free DNase in 20mM Tris-HCl pH8.4, 2.0mM $MgCl_2$, 50mM KCl, at 25°C for 15 minutes. The DNase was inactivated by heating to 65°C for 15 minutes before cooling on ice.

One microgram of DNase treated total RNA was used for cDNA synthesis as before with 0.5µg of oligo dT in 20mM Tris-HCl pH8.4, 2.5mM $MgCl_2$, 50mM KCl, 10mM DTT, 0.5mM dNTPs and 200U of SUPERSCRIPT II RT at 42°C for 50 minutes. RNA was removed by treatment with DNase free RNase. Synthesised cDNA was stored at -20°C until required.

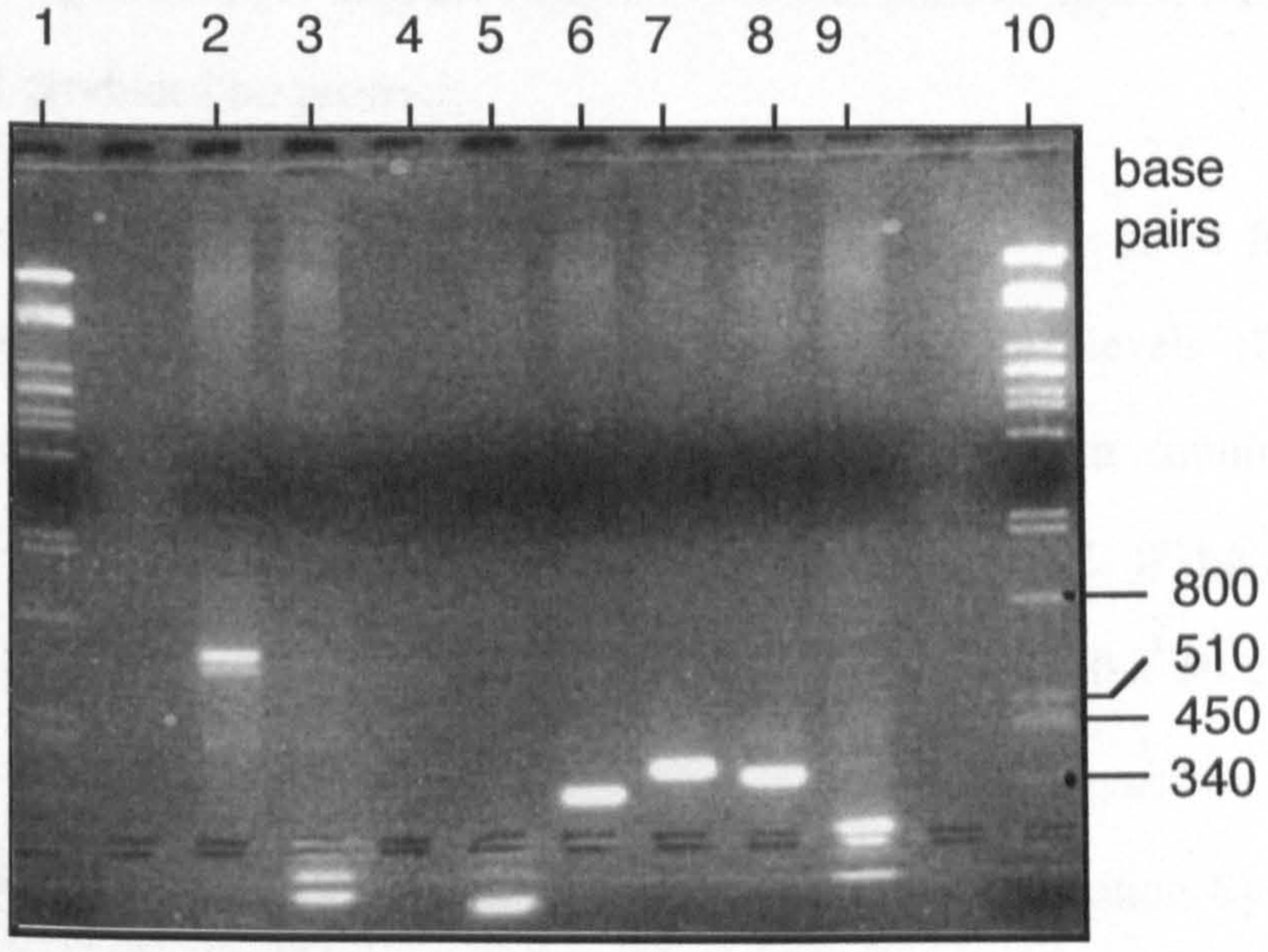
A test PCR was carried out with cDNA synthesised from total RNA isolated from embryos 49 daf. Reactions contained UniBP + BP1/2/3/4i/4ii/6/7i/7ii. One microlitre of synthesised genomic DNA free cDNA was used per PCR reaction. As a control to check specificity, the plasmid DNA of all known cDNAs was used except for the one against which the specific primer was targeted (i.e. BP2 contained plasmid BP1/3/4/6/7 but not BP2), so if the primer was specific, no product would be formed. The PCR reactions were done as before with AmpliWax™ PCR Gem 100 and Gene Amp Thin-Walled Reaction Tubes. 30 cycles of 94°C 1 minute, 50°C 1 minute and 74°C 40 seconds were done before 10µl was combined with 2µl of DNA loading dye and run on a 1% agarose gel. The gel is shown in Figure 5.19.

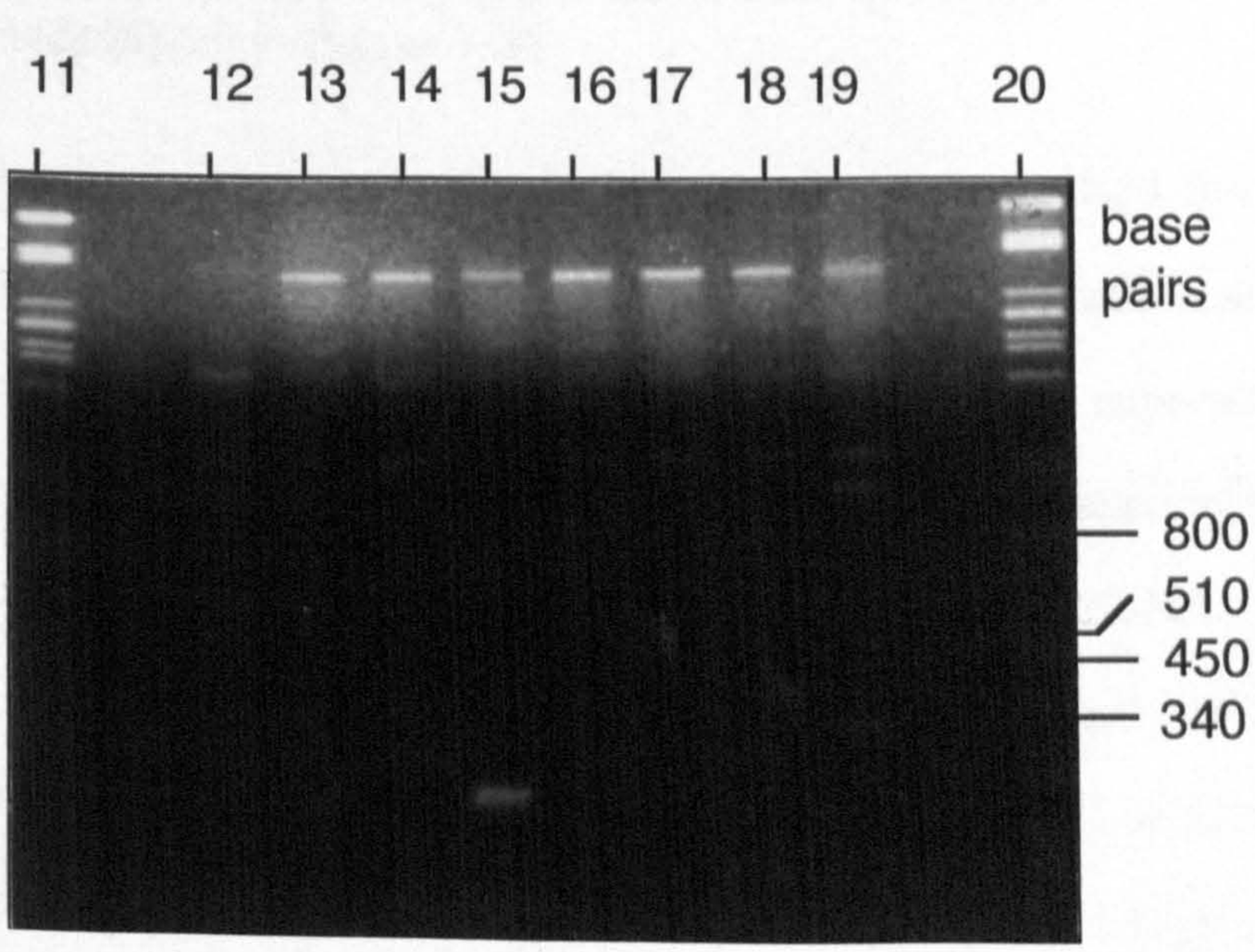
This shows that single products are produced from the cDNA synthesised from genomic DNA free total RNA isolated from embryos 49daf. This demonstrates that the previous larger bands were from genomic DNA contamination which has now been removed. A much larger product than expected was obtained using primer UniBP and BP1 (approximately 700 base pairs instead of the 394 base pairs expected). This could be from a hnRNA which still contains an intron or from the primers binding at a non-intended site.

Figure 5.19 Test PCR using synthesised cDNA from total RNA of 49daf embryos with UniBP + BP1/2/3/4i/4ii/6/7i/7ii.

One microgram of DNase treated total RNA was used for cDNA synthesis. One microlitre of first strand cDNA synthesis reaction was used per PCR reaction. PCR was carried out using AmpliWax™ PCR Gem 100 and Gene Amp Thin-Walled Reaction Tubes. The same conditions and concentration of components was used as in the test reaction (Figure 5.16). The thermal cycler was programmed to carry out cycles of 94°C 1 minute, 50°C 1 minute and 74°C 40 seconds for 30 cycles.

Samples of 10µl were analysed on a 1.5% agarose gel. Lanes contain products from PCR reactions with the following primers and templates. Lanes 2-9, PCR using cDNA synthesised from DNase treated total RNA from 49daf embryos. Lanes 11-19, PCR using a mixture of all pBP plasmids expect; Lane 12, no pBP1; Lane 13, no pBP2; Lane 14, no pBP 3; Lane 15 + 16, no pBP4; Lane 17, no pBP6; Lane 18+19, no pBP7. All PCR reaction contained the UniBP primer. The products shown are from PCR with UniBP and the following specific primers. Lanes 2+12, BP1; Lanes 3+13, BP2; Lanes 4+14, BP3; Lanes 5+15, BP4i; Lanes 6+16, BP4ii; Lanes 7+17, BP6; Lanes 8+18, BP7i; Lanes 9+19, BP7ii.. Lanes 1, 10, 11 and 20 each contain 1µg of λ Pst I markers


1 2 3 4 5 6 7 8 9 10
base pairs
800
510
450
340


11 12 13 14 15 16 17 18 19 20
base pairs
800
510
450
340

5.7.5 Analysis of BCCP mRNA levels throughout embryogenesis

The specificity of the primers for their intended cDNAs has been demonstrated in Figure 5.19. Primers BP4i and BP7ii were found to amplify a fragment from cDNAs other than their target one and were therefore not used for RT-PCR analysis. The expected amplified product was seen in each case except for BP1 and BP3. BP1 produced a fragment larger than the expected 394 base pairs of approximately 750 base pairs. BP3 produced no product.

One microlitre of cDNA synthesised from 1μg of total RNA prepared from leaf and embryos 38, 44, 49, 54, 58 and 63 daf, was analysed for the levels of each BCCP mRNA by PCR. DNA amplifications were prepared and run simultaneously for 30cycles of 94°C 1 minute, 50°C 1 minute and 74°C 40 seconds using AmpliWax™ PCR Gem 100 and Gene Amp Thin-Walled Reaction Tubes. After 30 cycles 10μl of the reaction was combined with 2μl of DNA loading dye and analysed on a 1% agarose gel. The gels were analysed using a BIO-RAD Gel Documentation System and the pictures are reproduced in Figure 5.20.

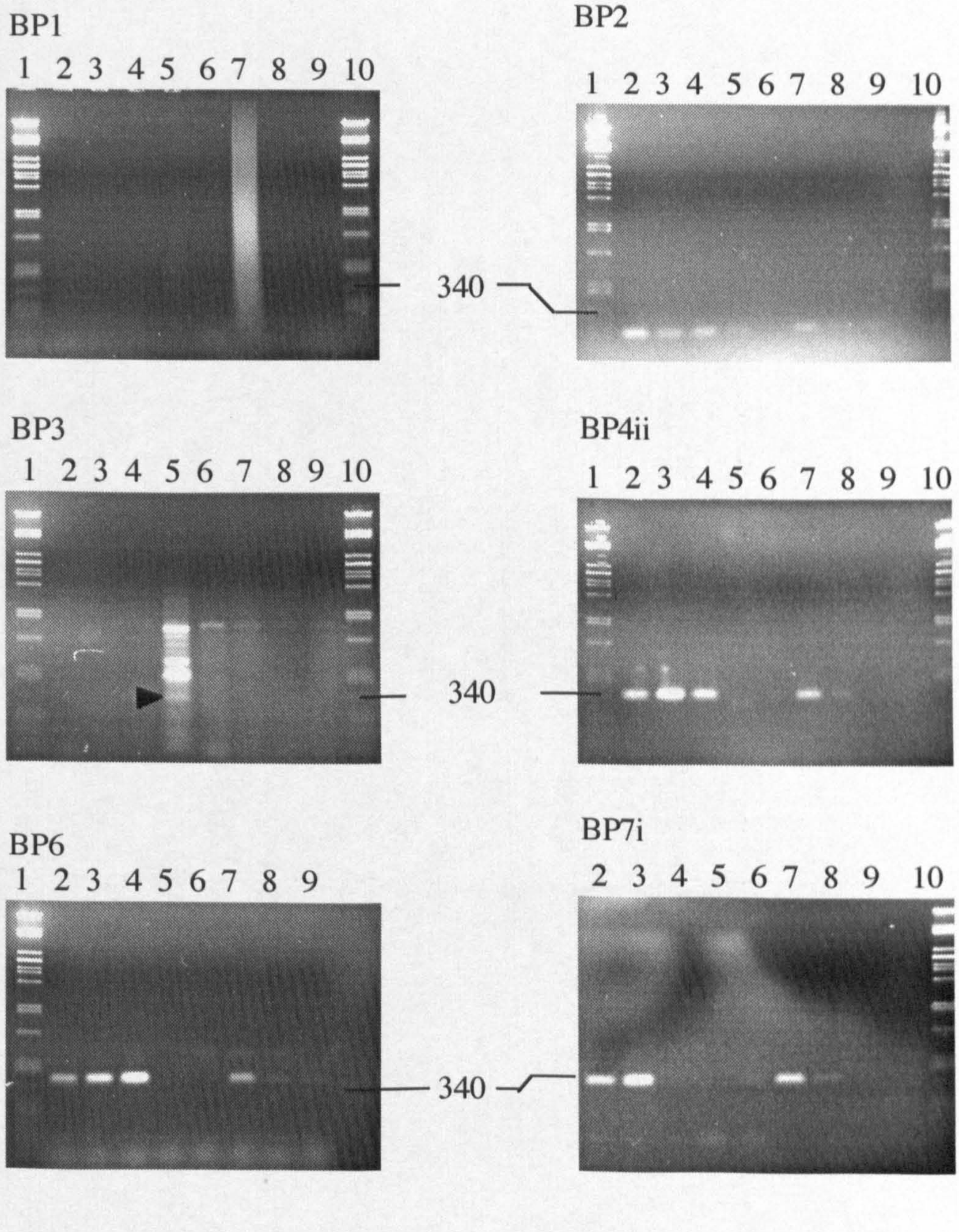
Only very small amounts of product were using cDNA synthesised from total RNA isolated from embryos 54daf (Lane6), possibly because the synthesis reaction failed to work efficiently for unknown reasons. No products of the expected sizes were obtained for BP1 or BP3 (394 base pairs and 383 base pairs respectively). Several products were obtained using primers UniBP and BP3 when the cDNA synthesis was primed with primer BP3. One product may have been the correct size (marked with arrow). As this product was not produced from cDNA primed with oligo dT, the possibility that the mRNA was not poly-adenylated was investigated further.

The PCR reactions for BP2, 4ii, 6 and 7i all worked well and were analysed using the Molecular Analyst® software from BIO-RAD. No product was seen in Lane 4 with primers BP7i and UniBP, probably due to an error in the set-up of the PCR reaction.

Figure 5.20 1% agarose gel showing the PCR products from RNA throughout embryogenesis and in leaf tissue for different BCCP isoforms.

One microlitre of cDNA synthesised from 1µg of total RNA prepared from leaf and embryos 38, 44, 49, 54, 58 and 63 daf, was analysed for the levels of each BCCP mRNA by PCR. DNA amplifications were prepared and run simultaneously for 30 cycles of 94°C 1 minute, 50°C 1 minute and 74°C 40 seconds using AmpliWax™ PCR Gem 100 and Gene Amp Thin-Walled Reaction Tubes. After 30 cycles 10µl of the reaction was analysed on a 1.5% agarose gel.

The lanes show the PCR products from cDNA generated from mRNA with an oligo dT primer unless stated otherwise, of; Lane 1, λ Pst I markers; Lane 2, 38daf embryo, Lane 3, 44daf embryo, Lane 4, 49daf embryo; Lane 5, 49daf embryo cDNA synthesised with the gene specific primer BP3; Lane 6, 54daf embryo; Lane 7, 58daf embryo; Lane 8, 63daf embryo; Lane 9, Leaf. The arrow on the gel with primers UniBP and BP3 shows a possible band or the correct number of base pairs in the lane using cDNA synthesised with the gene specific primer BP3 (Lane 5).


BP1
1 2 3 4 5 6 7 8 9 10
340
BP2
1 2 3 4 5 6 7 8 9 10
BP3
1 2 3 4 5 6 7 8 9 10
340
BP4ii
1 2 3 4 5 6 7 8 9 10
BP6
1 2 3 4 5 6 7 8 9
340
BP7i
2 3 4 5 6 7 8 9 10

As the gels has been digitally captured using the Bio-Rad Gel Documentation system it was possible to directly assess the levels of product in each PCR reaction by the intensity of band produced.

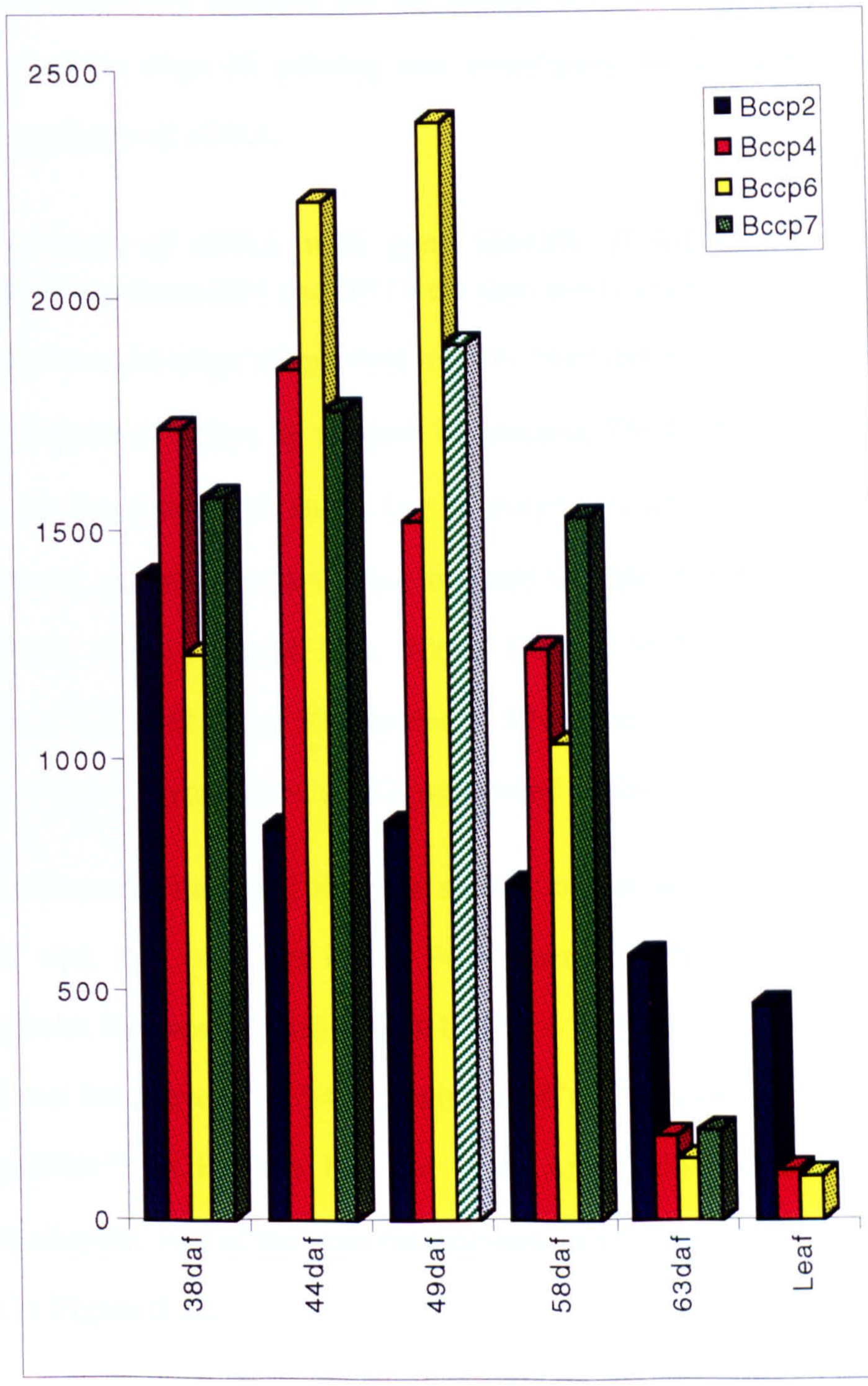


Figure 5.21 Relative levels of mRNA for BP2 (blue), BP4 (red), BP6 (yellow) and BP7 (green).

Bars represent the intensity of product produced by PCR from cDNA of each stage when run on a 1% agarose gel and stained with ethidium bromide. Imaging was done using a BIO-RAD Gel Documentation System and the Molecular Analyst Software. The hatched bar for BP7 represents an estimated level from Figure 5.19.

The levels of expression of mRNA for BP2, 4, 6 and 7 have been assessed in Figure 5.20 and 5.21 and they show the variation in levels of mRNA for each isoform during embryogenesis. The RT-PCR did not produce a product for BP1 and 3. The possibility that these two isoforms did not contain polyA$^+$ tails and hence no cDNA was synthesised by oligo dT priming was investigated by using the gene specific primers for synthesis of cDNA.

5.7.6 Synthesis of cDNA with gene specific primers

The gene specific primers BP1 and BP3 were each used to synthesise cDNA from total RNA preparations and oligo dT purified mRNA from leaf and embryo 49daf. Total RNA was prepared as before by removal of genomic DNA contamination. 1μg of genomic DNA free total RNA and 0.1μg of polyA+ isolated mRNA were used for cDNA synthesis as before with 0.05μg of gene specific primer in 20mM Tris-HCl pH8.4, 2.5mM $MgCl_2$, 50mM KCl, 10mM DTT, 0.5mM dNTPs and 200U of SUPERSCRIPT II RT at 42°C for 50 minutes. RNA was removed by treatment with DNase free RNase. Synthesised cDNA was stored at -20°C until required.

PCR was performed with 1μl of the gene specific primer synthesised cDNA. cDNA synthesised with BP1 was put into a PCR reaction with UniBP + BP1, cDNA synthesised with BP3 was amplified with UniBP + BP3 primers. PCR was set up as before and run for 30cycles of 94°C 1 minute, 50°C 1 minute and 74°C 40 seconds using AmpliWax™ PCR Gem 100 and Gene Amp Thin-Walled Reaction Tubes. After the final cycle, 10μl of the reaction analysed on a 1% agarose gel. The products are shown in Figure 5.22.

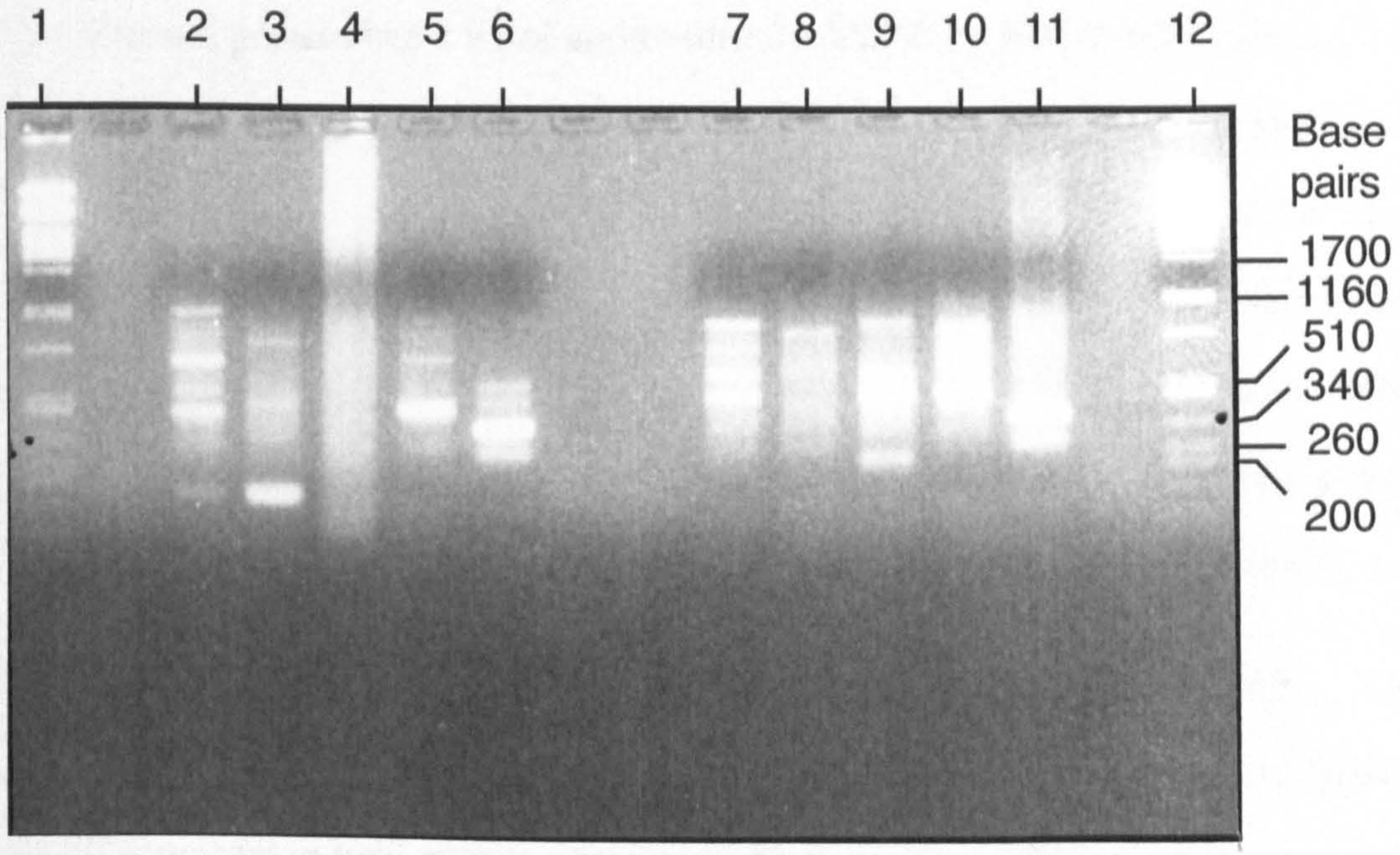
The control reactions in Lanes 6 and 11 respectively shows the expected products from the BP1 and BP3 plasmids respectively (394 base pairs and 383 base pairs respectively). No products were synthesised of this length from the mRNA primers with the gene specific primers for BP1 and BP3. Consequently, it was not possible to asses the level of expression of these two mRNAs. This could possibly be due to a very low level of expression for these two mRNAs which were not detected.

Figure 5.22 Analysis of total RNA and polyA+ mRNA by RT-PCR for BP1 and BP3 from leaf and embryo.

The gene specific primers BP1 and BP3 were each used to synthesise cDNA from total RNA preparations and oligo dT purified mRNA from leaf and embryo 49daf.

PCR was performed with 1µl of the gene specific primer synthesised cDNA. cDNA synthesised with BP1 was put into a PCR reaction with UniBP + BP1, cDNA synthesised with BP3 was amplified with UniBP + BP3 primers. PCR was set up as before and run for 30cycles of 94°C 1 minute, 50°C 1 minute and 74°C 40 seconds after which 10µl was analysed on a 1.5% agarose gel.

The lanes show the following; Lane 1 + 12, 1µg of λ Pst I markers. Lanes 2-5, PCR reactions with UniBP + BP1 with cDNA synthesised with the primer BP1 from the following RNA; Lane 2, total RNA 49daf embryo; Lane 3 poly A+ mRNA, 49daf; Lane 4, total RNA leaf; Lane 5, polyA+ mRNA leaf. Lane 6, PCR from pBP1 with primers UniBP+BP1. Lanes 7-10, PCR reactions with UniBP + BP3 with cDNA synthesised with the primer BP3 from the following RNA; Lane 7, total RNA 49daf embryo; Lane 8 poly A+ mRNA, 49daf; Lane 9, total RNA leaf; Lane 10, polyA+ mRNA leaf. . Lane 11, PCR from pBP3 with primers UniBP+BP3.


1
2
3
4
5
6
7
8
9
10
11
12
Base pairs
1700
1160
510
340
260
200

5.8 Discussion

Biotin Carboxyl Carrier Protein in *Brassica napus* is transcribed as at least six different isoforms, the cDNAs of which have been isolated (Elborough *et al.*, 1996). The expression of these isoforms has been studied in this chapter, in an attempt to reach conclusions as to how these six isoforms may be involved with changes in detected acetyl-CoA carboxylase activity throughout embryogenesis.

The cDNA for one of the isoforms of BCCP, BP4, has been overexpressed using a pET vector, in *E.coli*. The overexpressed protein was expressed as the holoprotein in that the predicted leader peptide was not expressed at the start of the protein. The overexpressed protein had a M_r of approximately 30,000 by SDS/PAGE compared to a predicted M_r of 23,000. This discrepancy in molecular weight can be attributed to the fact that the protein structure is very similar to the *E.coli* BCCP in that the central region is rich in proline and alanine residues. This has been shown to cause peculiar mobility during electrophoresis (see Section 1.3.3.). The overexpressed protein is also biotinylated by the biotin holoenzyme synthetase of *E.coli*. These two physical properties show that the protein has qualities in common with the BCCP from *E.coli*.

The overexpressed 30kDa protein was used to raise antibodies in a rabbit. These antibodies prominently distinguish a 35kDa protein and several other protein bands of 29-31kDa present in total protein extracts of oilseed rape embryos. A protein pattern like this was also observed when total protein extracts were probed with anti-biotin antibodies suggesting that the proteins are one and the same. This demonstrates that our cloned genes encode a protein which is biotinylated and expressed at a high level in the tissues of developing oilseeds.

There exists a discrepancy between the size of the over-expressed protein and the size of the protein observed on Western blots of protein extracts from all tissues. Western blots have shown that BCCP exists within chloroplasts as a biotinylated protein with M_r of 35kDa (this study, Elborough *et al.*, 1996). Import of proteins into chloroplasts involves the targeting of the protein to the chloroplast by means of a transit peptide.

The transit peptide facilitates the import of the apoprotein into chloroplasts but once the protein has been imported, the transit peptide is cleaved off by a specific protease to create the holoprotein. The protein targeting sequence of BCCP was identified by a computer program, comparing the BCCP sequence to known targeting sequences and predicting from this where the transit peptide would be cleaved (Figure 5.1).

The predicted size of the transit peptide is 4.5kDa. The overexposed holoprotein has a M_r of approximately 30,000. The BCCP protein in purified *Brassica napus* chloroplasts is 35kDa. This evidence suggests that the BCCP apoprotein is imported into chloroplasts of oilseed rape without cleavage of the targeting peptide. How this is important to the function of BCCP is not clear, nor has it been proven beyond doubt. Other post-translational modifications could be responsible for the decreased mobility on SDS / PAGE of the holoprotein extracted from seeds of *Brassica napus*, such as glycosylation. There are no reports of BCCP protein being glycosylated and the possibility has not been investigated in this study although conserved glycosylation sites are to be found in both BP1 and BP4. Complete cDNAs were isolated for only two BCCP isoforms, pBP4 and pBP6 (Elborough *et al.*, 1996). The cDNA sequences are 1008 and 1133 base pairs respectively which corresponds to the size of the mRNA on northern blots (1100 base pairs). A similar size has also been reported for the mRNA from *Arabidopsis thaliana* (Choi *et al.*, 1995). This excludes the possibility that there could be a start codon further towards the 5' end of the cDNA as our DNA is nearly the same length as the mRNA on northern blots.

Final proof of the nature of the transit peptide could come from studies with the overexpressed apoprotein or by purifying and sequencing the BCCP protein itself. Labelled apoprotein could be used for chloroplast import assays, if the size of the imported apoprotein is not diminished upon import then clearly the transit peptide is not cleaved. Sequence from a purified BCCP protein would reveal the N-terminal sequence, and hence whether the transit peptide has been cleaved. It could also indicate whether any other post-translational modifications have occurred such as phosphorylation or glycosylation and at which residues.

The expression of isoforms of the 35kDa biotinylated protein has also been investigated. A novel purification procedure for anti-biotin antibodies has been utilised to produce a fraction which detects biotinylated proteins at a high titre and with minimal background. Purification of proteins from our anti-biotin sera on biotin-agarose produced a fraction containing two proteins with high M_rs of approximately 50,000 and 25,000. These are thought to represent the heavy and light chains associated with IgG subtype antibodies (Harlow & Lane, 1988). A larger band in this gel, of approximately 60,000 daltons may represent the heavy chain from a different antibody subtype, possibly IgM which is thought to have a substantially larger constant region than IgG (Harlow & Lane, 1988).

The highly pure anti-biotin antibodies detected up to six proteins with a M_r of 35,000 in two dimensional SDS/PAGE. These all had an acidic pI. Overexpressed BP4 protein also had an acidic pI of 5.3-6 compared with a calculated pI of 5.5. Previously it was suggested BCCP may be imported into the chloroplast without cleavage of the transit peptide. The predicted pI of the protein with leader peptide is around pH8 due to the very basic nature of the leader peptide. There are several post-translational modifications which could affect the pI of the protein, notably biotinylation and glycosylation which both attach to a basic residue, and phosphorylation. Phosphorylation of the BCCP protein has not been investigated, but it would explain the difference between observed pI and calculated pI. Phosphorylation has been well documented for animal ACC where in response to hormonal control (insulin) its activity is decreased (Hardie, 1989), and could represent a mechanism in plants for regulation of ACCase activity. All the BCCP isoforms contain several known conserved protein kinase sites.

The levels of the six isoforms has been assessed by the use of a densitometer which allows quantification of the fraction of BCCP expressed by the individual isoforms at any one time. The two-dimensional Western blots were not all processed at the same time so this means comparison of levels of protein between the blots cannot be accurately assessed. Instead, the data were combined with the expression data

obtained for the 35kDa biotinylated protein in Chapter 3 (see Figure 3.4 B and 3.5 B). The values obtained from that work for the expression of all 35kDa biotinylated proteins by 1-D Western blots have been divided into the relative levels observed for the individual isoforms in 2-D analysis.

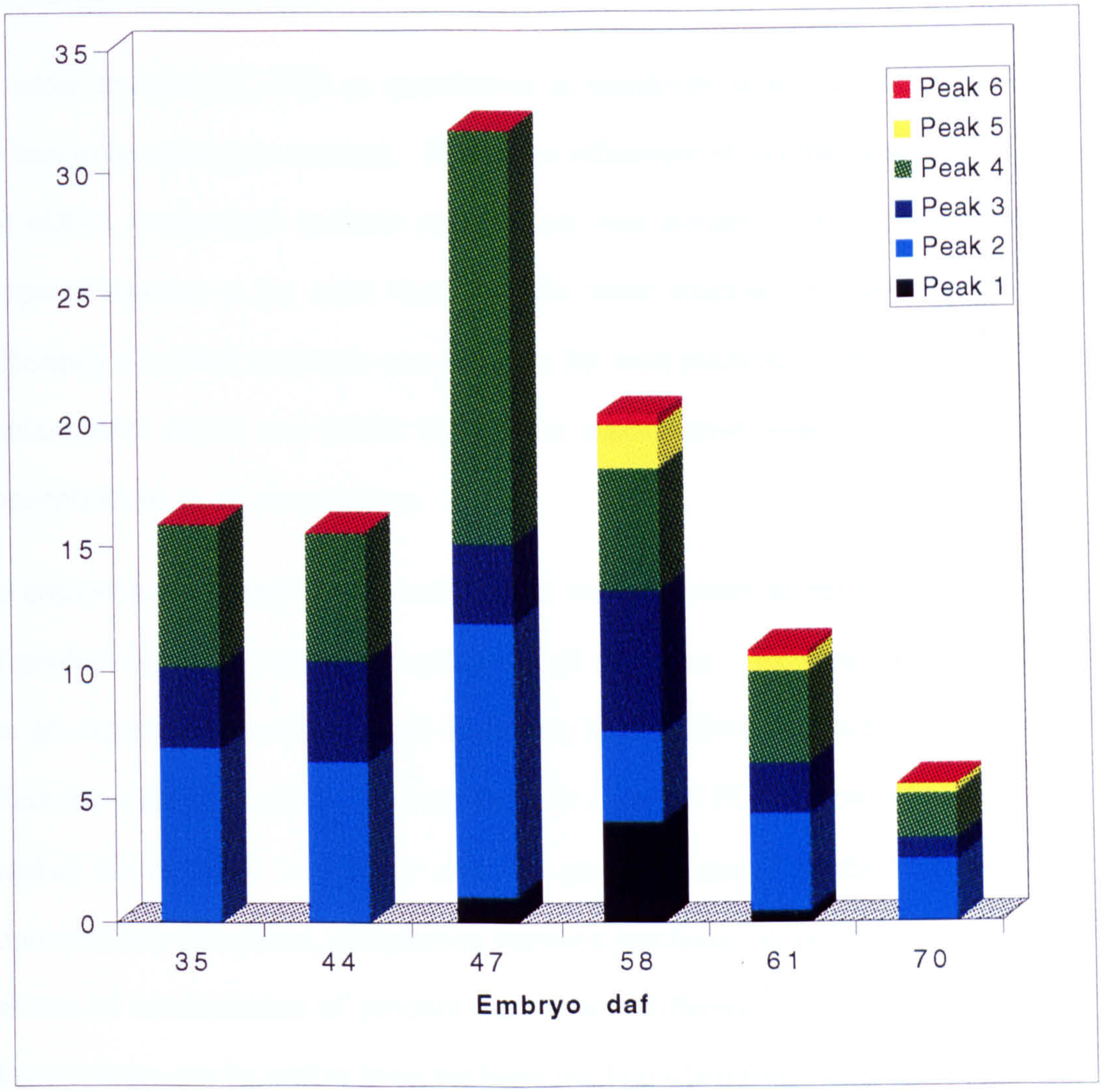


Figure 5.23 The expression of BCCP isoforms throughout embryogenesis.

Peaks are numbered in the order they occur in Figure 5.13, 56 daf from left to right. The data obtained by analysis of extracts in 1-D Westerns with anti-biotin antibodies was used for total protein levels. Each peak of total protein level was fractionated into isoforms according to the relative levels observed on 2-D blots.

This analysis of the data reveals that peak 4 makes the biggest contribution to the increase in BCCP during embryogenesis followed by peak 2. The later expression of peaks 1, 5 and 6 suggests they have a special function later in embryogenesis.

To obtain information on the specific genes responsible for this differential expression of BCCP isoforms RT-PCR has been used to identify changes in mRNA levels which may correspond to the observed changes in protein isoforms. To obtain meaningful results from the RT-PCR experiments it was necessary to ensure that the process was as quantitative as necessary.

In order to make RT-PCR as quantitative as sensitivity would allow, there were two factors to be taken into account. Firstly the efficiency of synthesis of cDNA. As all the cDNA synthesised in these experiments was isolated from similar sources and prepared together at the same time with the same reagents, it was assumed that the efficiency of cDNA synthesis was the same for each reaction. This assumes levels of contaminants which may inhibit the reverse transcriptase were minimal or of similar concentrations in all preparations.

To ensure the PCR step was quantitative, it was necessary to ensure that accumulation of product occurred only exponentially in all reactions. Because PCR amplifications are an exponential process, small variations in amplification efficiency can drastically affect the yield of products. During the later stages of PCR the rate of accumulation of product slows down to a linear rate, the plateau phase. If the PCR reactions are continued into this phase, comparison between reactions can produce invalid results as the rate of accumulation of product can become different in separate reactions. The plateau phase can be said to have not been reached when five additional cycles does not yield twice as much product (Clontech, 1991). By keeping within the exponential phase of PCR, it appears that RT-PCR can detect changes in mRNA levels of greater than two-fold without the using of internal standards. Internal standards are used to provide a mimic for the PCR reaction. Internal standards are normally synthesised ssDNA templates of an absolute known concentration which contain the primer sites of interest. These can be used to assess precisely the amount of product generated from a known quantity of starting material. This is important when comparing products from different primers. PCR primers may act as substrates for DNA synthesis with differing efficiencies so precise quantification requires that the

efficiency is known. In these experiments, one primer is the same for all PCR reactions so the efficiency is likely to be the same for that primer at least. The efficiency of the other primer is unknown, but as PCR reactions are to be compared with other PCR reactions using the same primers, the efficiency of DNA synthesis should be the same for all reactions.

The levels of expression of the different identified genes for BCCP have been shown in Figure 5.21. To try and establish if any of our identified genes co-ordinated their expression with the expression of protein isoforms the data was compared.

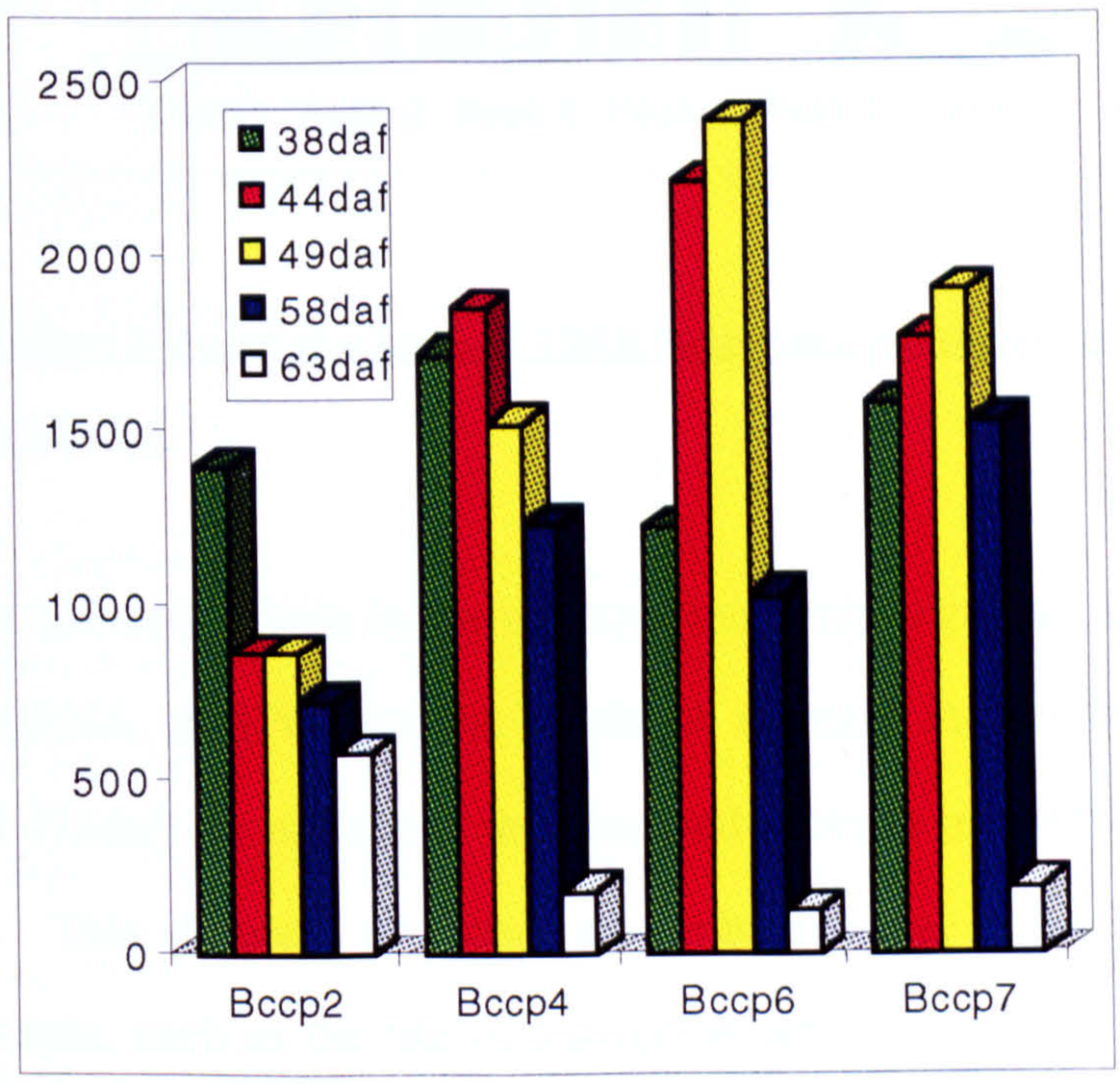


Figure 5.24 A Changes in the relative levels of BCCP mRNAs detected by RT-PCR.

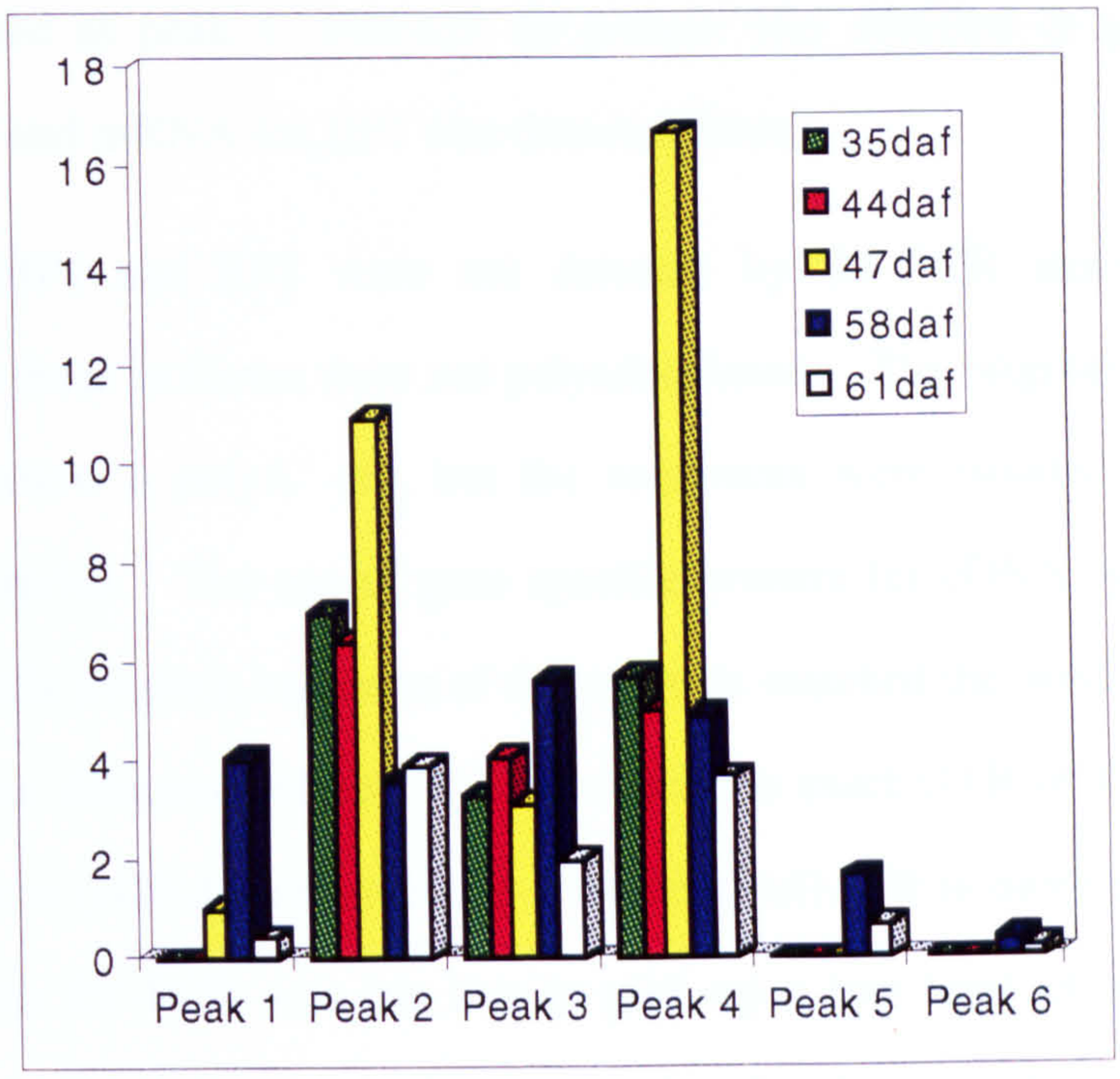


Figure 5.24 **B** Changes in the relative levels of 35kDa biotinylated protein isoforms detected by 2-D anti-biotin Western blotting.

The single most induced protein isoform throughout embryogenesis is peak 4. The most induced mRNA is BP6, but the levels of increase in protein (nearly 3-fold between 38 and 478daf) do not match the observed increase in mRNA levels through PCR (2-fold). This suggests that there are probably other factors influencing the synthesis of protein, such as the rate of translation which, if it were higher at 47daf than at 38daf would produce more protein. With this in mind, the single most highly expressed BCCP isoform at 38daf is peak 2. The level of mRNA for BP2 was highest at the start of embryogenesis so the isoform observed at peak 2 may be encoded for by BP2. Although the levels of peak 2 rise at 47daf and the level of detected mRNA is lower at this stage that at the start of embryogenesis, if the rate of translation was increased at this stage of embryogenesis, an expected increase of protein product would be seen. Peak 3 remains fairly constant throughout embryogenesis as do the levels of BP4 and BP7 mRNA. As the detected level of BP4 mRNA drops from 44daf and this mRNA is detected in leaf, it is tentatively suggested that this mRNA could encode for peak 3. That would infer that BP7 encodes the

protein visualised as peak 1, although no protein was detected in peak 1 early in embryogenesis and mRNA for BP7 was detected there.

The isoforms BP1 and BP3 were not detected by RT-PCR analysis. It was considered that these isoforms were not polyadenylated. The original DNA sequence data does not show a polyA$^+$ tail, but the sequences were isolated from oligo dT synthesised libraries. The use of gene specific primers for cDNA synthesis did not produce a specific product, and none of the products matched the predicted size. It is possible that our designed primers did not match the exact UTR of these isoforms in the variety Falcon (cDNAs were isolated from Jet neuf). It is more probable that the sensitivity of the RT-PCR was too low to pick up a low level of mRNA for these isoforms. Peaks 5 and 6 appear only at a very low level around 56daf. If translation of the mRNA was high it could be possible that very few copies of the mRNA were present at that time and therefore not detected in these experiments.

Throughout this chapter, most of the detection of BCCP isoforms was through the use of affinity purified anti-biotin antibodies. These were used as they have very low background and the BCCP antibodies show many other bands in Western blots of *Brassica napus* total protein extracts. They also have another advantage, they do not detect BCCP protein which is not biotinylated and therefore not active. Previous reports on methylcrotonyl-CoA carboxylase have demonstrated that this enzyme can be controlled by biotinylated of the apoenzyme (Wang *et al.*, 1995). Western blots have been presented in this chapter using affinity purified anti-biotin antibodies and anti-BCCP antibodies. Both Western blots used the same protein extracts with the same amount loaded onto each gel and were processed identically except for the primary antibody incubations. The observed levels were compared in each case.

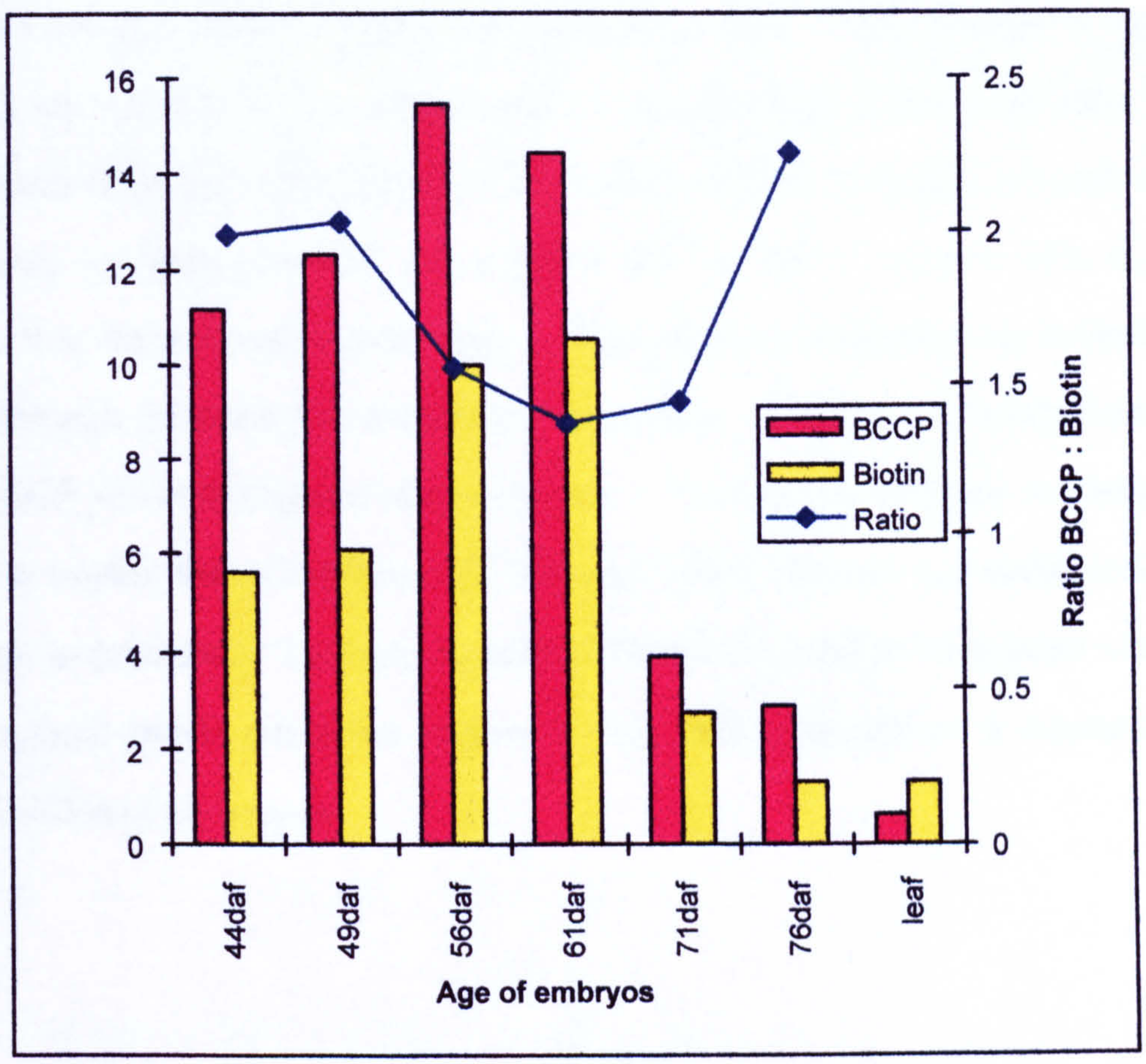


Figure 5.25 Biotinylation status of BCCP in embryos.

Comparison of the levels of BCCP (35kDa) detected in total protein extracts of *Brassica napus* embryo and leaf (from Figures. 5.10 A & 5.11 B). The ratio protein detected with anti-BCCP : anti-biotin antibodies is shown.

Figure 5.25 shows that the levels of BCCP protein increases to a maximum around 56daf. This coincides with the maximal rate of lipid biosynthesis. Also at this time, the ratio of biotinylated protein to BCCP protein is at its highest. As BCCP protein is quickly degraded after 56daf, the ratio of detected protein with anti-biotin : anti-BCCP nearly doubles. This implies that the biotin moiety is removed before protein is degraded. Clearly, the enzyme cannot be active without the biotin moiety attached, so it is possible that this is a mechanism for deactivating the enzyme.

In conclusion, the data presented in this chapter shows that BCCP is a highly regulated protein, the expression of which is complex and varied. The existence of isoforms which are specific to mid embryogenesis and the huge increase in total BCCP detectable at this time reflects its role as a subunit of Type II acetyl-CoA carboxylase. Evidence has been presented which shows that the BCCP isoform BP6 is highly induced in the mid-embryogenic stage. This implies a mechanism for its induction, both through increased transcription and translation. Finally, the biotinylation status of BCCP varies throughout embryogenesis. The sudden decrease in biotinylated protein implies that a biotinidase is activated which removes the biotin before the protein is degraded. This may be because biotin is needed to be recycled into other biotinylated protein which are induced in late embryogenesis, or a mechanism for deactivation of the enzyme.

Chapter 6: Investigations into the relationship between the Biotin Carboxylase and BCCP subunits of the Type II acetyl-CoA carboxylase from *Brassica napus*.

6.1 Introduction

Type II acetyl-CoA carboxylase in higher plants is a dissociable multisubunit complex consisting of several protein subunits (Kannangara & Stumpf, 1972; Alban *et al.*, 1995). This is in contrast to the Type I ACCase which is made up of a single polypeptide (Elborough *et al.*, 1994b). Like the *Escherichia coli* protein, Type II acetyl-CoA carboxylase can be divided into three components, a biotin carboxylase, a biotin carboxyl carrier protein and a carboxyltransferase (Alberts & Vagelos, 1972; Guchhait *et al.*, 1974). These components catalyse the two-step reaction sequence common to all biotin-dependent carboxylases.

$$\text{BCCP-biotin} + \text{ATP} + HCO_3^- \xrightarrow[\text{Biotin carboxylase}]{Mg^{2+}} \text{BCCP-biotin-}CO_2^- + \text{ADP} + P_i \quad (1)$$

$$\text{BCCP-biotin-}CO_2^- + \text{acetyl-CoA} \xrightarrow[\text{Carboxyl transferase}]{} \text{BCCP-biotin} + \text{malonyl-CoA} \quad (2)$$

Reaction 1, catalysed by the biotin carboxylase subunit, was found to be ATP and Mg^{2+} dependent and catalysed the carboxylation of biotin (Alberts & Vagelos, 1972). The carboxyltransferase subunit catalysed reaction 2 which transfers the CO_2 from carboxybiotin to the substrate acetyl-CoA. In *Escherichia coli* the enzyme was found to be dissociable into a biotin-containing E_a fraction and a non-biotin-containing fraction E_b. Neither of these fractions was able to catalyse the complete reaction alone, but by recombining the protein fractions activity could be reconstituted (Alberts & Vagelos, 1972). That the biotin carboxylase subunit functions independently of the carboxyltransferase subunit was demonstrated by the incorporation of $H^{14}CO_3^-$ into E_a-^{14}C. This was then isolated from unincorporated $H^{14}CO_3^-$ by sephadex chromatography and incubated with E_b in the presence of acetyl-CoA. The product

[-^{14}C] malonyl-CoA was formed demonstrating the two independent activities (Alberts & Vagelos, 1972).

The plant enzyme has been shown to be composed of a similar system by isolating chloroplasts and measuring ACCase activity. Enzyme activity was lost in chloroplasts when ruptured, possibly due to the presence of an inhibitor (Kannangara & Stumpf, 1972), but regained when the E_b fraction of ACCase from *E.coli* was added. This suggested two things, 1) the acetyl-CoA carboxylase from higher plant chloroplasts was readily dissociable into protein components and 2) the carboxyltransferase protein from *E.coli* could easily interact with the plant biotin carboxyl carrier protein.

The primary structure of the subunits of the Type II acetyl-CoA carboxylase in higher plants has been derived by the cloning and sequencing of cDNAs for these proteins (Sasaki *et al.*, 1993; Shorrosh *et al.*, 1995; Choi *et al.*, 1995; Elborough *et al.*, 1996; Shorrosh *et al.*, 1996). The genes have all been identified by sequence homology with algal and bacterial proteins. The biotin carboxylase and biotin carboxyl carrier protein have been purified from pea chloroplasts (Alban *et al.*, 1995) but no amino acid sequence data was reported for comparison with the proposed sequences from tobacco (Shorrosh *et al.*, 1995), *Arabidopsis* (Choi *et al.*, 1995) or oilseed rape (Elborough *et al.*, 1996). The function of these isolated genes has therefore been inferred only by homology to known genes.

Little information is available on the characteristics of the complete Type II acetyl-CoA carboxylase. This is inevitably due to the readiness of the complex to dissociate into separate subunits, making purification of the entire protein complex in its native state problematic (Alban *et al.*, 1995). It is thought that the enzyme consists of four subunits in a similar fashion to the *E.coli* protein. The biotin carboxylase protein has an observed M_r of 32,000 (pea chloroplasts, purified enzyme, Alban *et al.*, 1995) or 47,000 (pea chloroplasts, Western blot, Shorrosh *et al.*, 1995) and 50,000 in castor seeds (plastids, Western blot Shorrosh *et al.*, 1995). Biotin carboxyl carrier protein has been observed as a 38kDa protein in *Arabidopsis* (Choi *et al.*, 1995) and pea

(Alban *et al.*, 1995), and a 35kDa protein in *Brassica napus* (this study and Elborough *et al.*, 1996). These two subunits which make up the E_a fraction of *E.coli* ACCase have been shown to strongly interact in pea (Alban *et al.*, 1995; Roesler *et al.*, 1996) to the extent that they copurify.

The carboxyltransferase component of the enzyme, or E_b fraction of the *E.coli* enzyme, is made up of two proteins, both in higher plants and *E.coli* (Li & Cronan, 1992b; Sasaki *et al.*, 1993; Shorrosh *et al.*, 1996). The CT-β subunit is encoded by a single gene on the chloroplast genome and has a M_r of 87,000 on SDS/PAGE (Sasaki *et al.*, 1993). Immunoprecipitation of proteins from pea chloroplasts with antibodies against the CT-β subunit revealed the 87kDa CT-β subunit was associated with other subunits of M_r 91,000, a diffuse band of polypeptides <50,000 and a biotinylated protein of 35,000. The 91kDa protein has subsequently been identified as the α-subunit of Type II acetyl-CoA carboxylase previously known as IEP96 (Shorrosh *et al.*, 1996). The α and β subunits of CT in pea have been shown to be tightly associated with each other, as have the biotin carboxylase and biotin carboxyl carrier protein subunits. These two protein complexes are thought to interact with each other in a weaker association, probably by ionic interactions as they are dissociable on DEAE sepharose (Shorrosh *et al.*, 1996).

6.2 Aims

The aims of the research presented in this chapter were to characterise the biotin carboxylase protein from *Brassica napus*, by isolation of a *Brassica napus* cDNA sequence for biotin carboxylase, expression of the protein in *E.coli* using the pET system and the raising of anti biotin-carboxylase antibodies. These would enable an analysis of the protein by Western blotting. As the Type II acetyl-CoA carboxylase is made up from multiple subunits, it is of interest to ascertain whether the levels of all the subunits are equally regulated. It might be expected that the expression of all four subunits would be co-ordinated, but equally it could be the case that the levels of one subunit alone change in response to biochemical requirements, making that subunit the

rate limiting factor in enzyme activity. This could be a function of the rate of turnover of that protein. Western blots with antisera against different subunits of Type II ACCase would provide initial evidence for the pattern of expression of the different subunits.

The large amounts of protein produced by overexpression would also be used to investigate the interaction of the biotin carboxylase and BCCP subunits. By combining the BC and BCCP proteins it may be possible to demonstrate the enzyme activity and hence biological function of the cloned genes, as biotin carboxylase should be able to label BCCP when provided with $H^{14}CO_3^-$ and ATP. Currently, the function of these genes has only been inferred from their homology to other biotin-dependent carboxylases. Biochemical activity would provide substantial evidence that the cloned genes were part of a Type II acetyl-CoA carboxylase enzyme present in *Brassica napus*.

6.3 Isolation of a cDNA encoding biotin carboxylase

Several projects world-wide are focusing on the sequencing of random cDNA sequences from important plant species, called expressed sequence tags (ESTs) (Newman *et al.*, 1994; van de Loo *et al.*, 1995). This is a result of the automation of DNA sequencing making it a simple and less time consuming task. Sequences are compared against known cDNA / protein sequences for identification. Clones which show no significant similarity to known sequences remain unassigned.

By using one such clone identified as having similarity to *Anabena* ACCase biotin carboxylase, the sequence for the biotin carboxylase subunit of Type II ACCase was deciphered in *Nicotina tabacum* (tobacco, Shorrosh *et al.*, 1995). This reported cDNA sequence was compared by Dr. Kieran Elborough, Department of Biological Sciences, University of Durham, to the known *Arabidopsis* EST database. One *Arabidopsis* clone (VBVXB09, accession number F14110) was identified as having homology to the *N.tabacum* sequence. The most conserved regions between the *Arabidopsis* EST clone and the known *N.tabacum* sequence were used to design primers for PCR. This was to ensure that there would be maximum possibility of the primer's sequence being very similar in *Brassica napus*. These would be used to either amplify a probe from *Arabidopsis* DNA for library screening or to directly PCR the complete clone from a suitable *B.napus* library in conjunction with T_3 and T_7 primers. The PCR strategy is explained diagramatically in Figure 6.1.

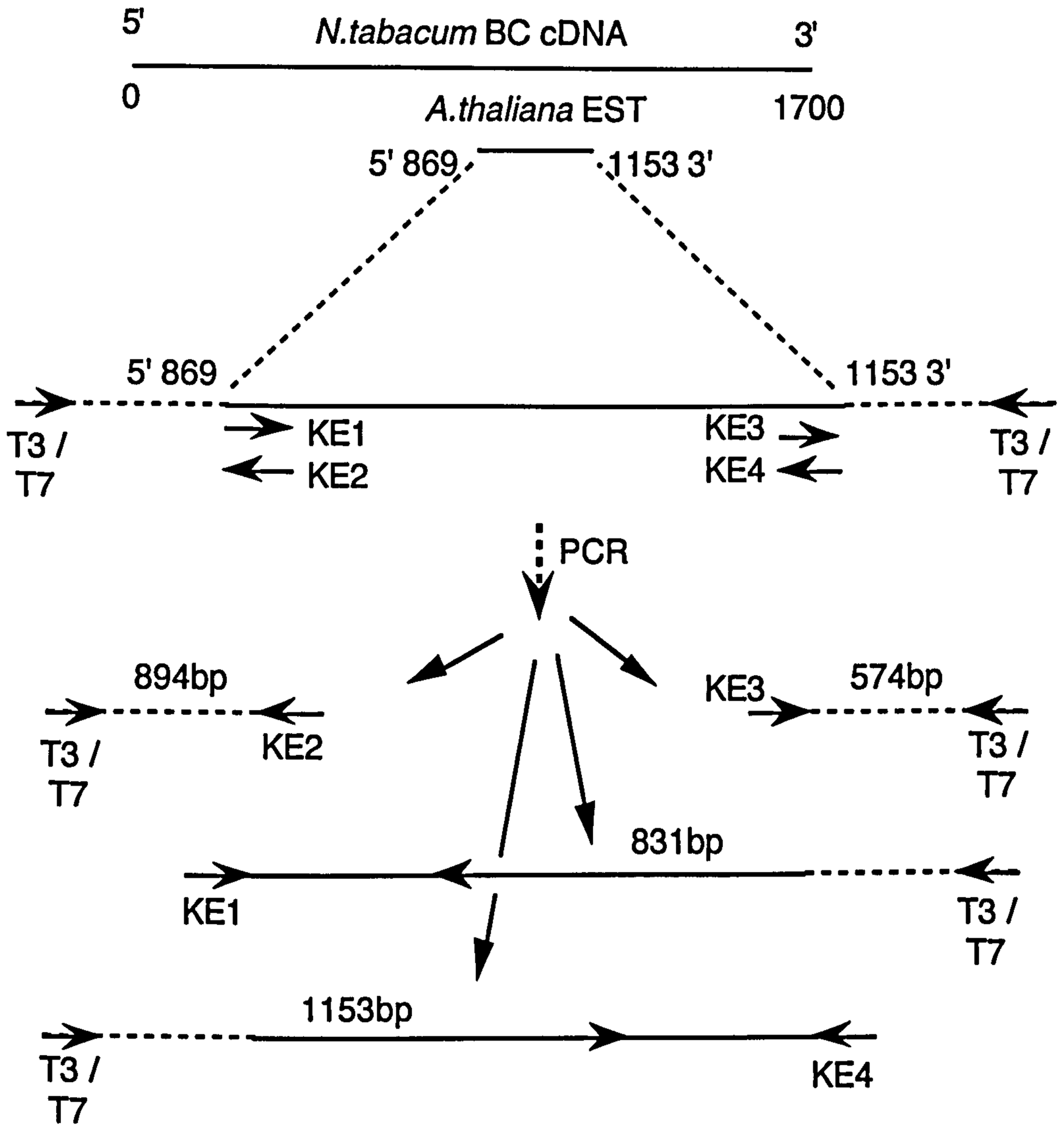


Figure 6.1 PCR strategy for cloning *B.napus* biotin carboxylase.

Primers were designed to PCR fragments 5' and 3' from the *Arabidopsis* EST utilising T3 or T7 as the second primer present in the cloning site of λZAP II. A 284 base pair fragment of the *Arabidopsis* EST would serve as a control to check the identity of products. The predicted length of the products from the *N.tabacum* sequence is shown above each PCR product in number of base pairs.

The sequence of the primers KE1-4 is shown below;

KE1 $^{5'\ 869}$ GGG AAT GAT GGA TGT TAT CTG GAG AAG $^{894\ 3'}$

KE2 $^{5'\ 894}$ CTT CTC CAG ATA ACA TCC ATC ATT CCC $^{869\ 5'}$

KE3 $^{5'\ 1126}$ GAT GAG AGA GGT TCC TTC TAC TTC ATG G $^{1153\ 3'}$

KE4 $^{5'\ 1153}$ CCA TGA AGT AGA AGG AAC CTC TCT CAT C $^{1126\ 3'}$

6.3.1 PCR of biotin carboxylase cDNA

The library used was constructed by Johan T. M. Kroon, Department of Biological Sciences, University of Durham, and had previously been used for cloning partial cDNAs for the Type I ACCase from *B.napus* (Elborough *et al.*, 1994b). The library had been constructed from polyA$^+$ isolated mRNA from mid-development embryos of *Brassica napus* var. Jet neuf. cDNA had been synthesised with random N_6 oligomers and ligated into the vector with adapters which contained a Not I restriction site. The Not I restriction site contained within the adapter sequence would facilitate cloning of PCR products into Not I / Eco RV cut pSK-.

The desired DNA was amplified from the library by PCR. Each reaction contained 5µl of library with a tire of 1×10^8p.f.u./ml as template, one of the BC primers KE1-4 and either the T3 or T7 primer. The DNA was denatured in the PCR reaction for 5 minutes at 94°C before amplification of the desired fragments over 30 cycles of 94°C 1 minute, 50°C 1 minute and 74°C 2 minutes.

Of each PCR reaction, 10µl was run on a 1% LMP agarose gel and viewed under long wave UV illumination (302nm) to minimise DNA damage. The PCR products obtained are shown in Figure 6.2 A. The results show that the primers do not amplify a single product, but a dominant product can usually be seen in each case. As two PCR reactions were done with each BC primer (KE1-KE4) but with either the T3 or T7 primer, a major product of similar size in the pairs of reactions could be identified as the expected product. For example, in Figure 6.2 A Lane A① a single band of approximately the predicted number of base pairs (831) was visible. In Lane A②, which shows products amplified with the same BC primer KE1 but using T7 instead of T3 as the second primer, the pattern of products is more complex. The dominant product was identified as having a similar size to that in Lane A① and was taken to be the expected product.

Figure 6.2 A 1% LMP agarose gel showing the bands obtained by PCR with biotin carboxylase primers KE1 - KE4 from a random primer cDNA library from *Brassica napus* embryo.

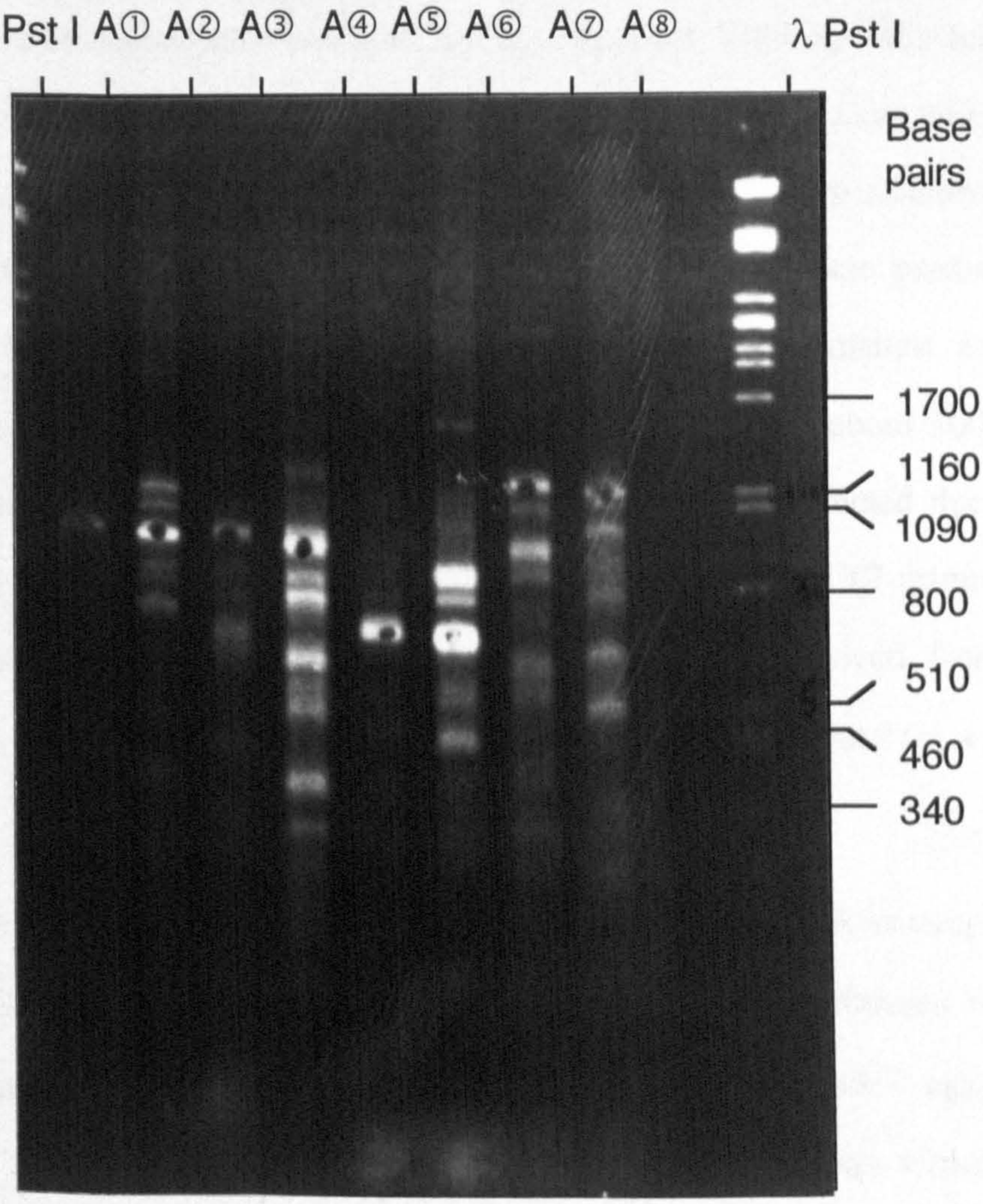
Lanes marked λPst contained 1μg each of λ Pst I markers. Other lanes contain PCR products using the following primer combinations; Lane A①, KE1+T3; Lane A②, KE1+T7; Lane A③, KE2+T3; Lane A④, KE2+T7; Lane A⑤, KE3+T3; Lane A⑥, KE3+T7; Lane A⑦, KE4+T3; Lane A⑧, KE4+T7.

Figure 6.2 B 0.8% agarose gel showing the bands obtained by the second round of PCR with the same primers.

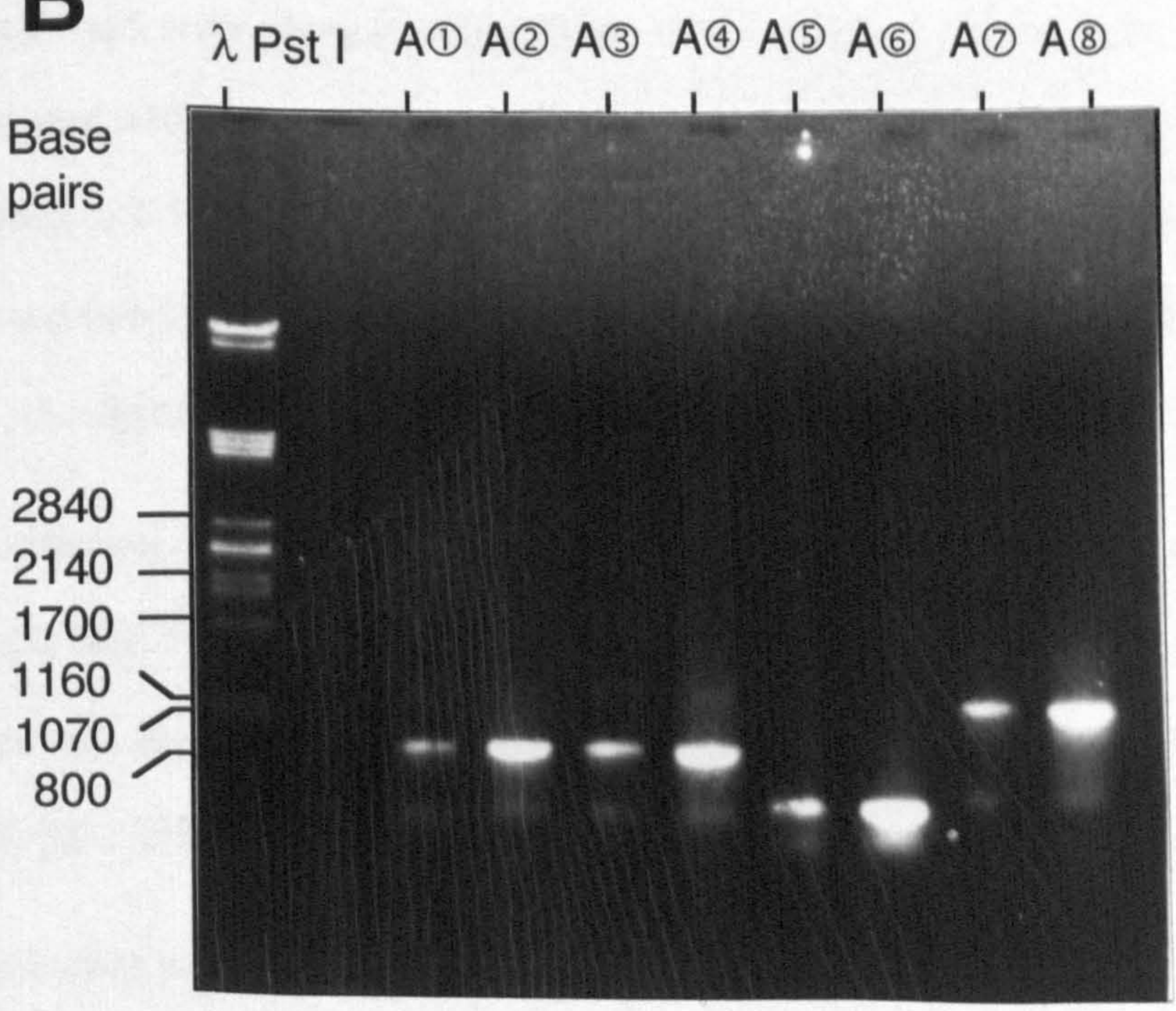
PCR was repeated with the same primers as before using the agarose plug from Figure 6.2A as a template, the holes were the plugs were removed are visible.

The lane marked λPst contained 1μg of λ Pst I markers. Other lanes contain PCR products using the following primer combinations; Lane A①, KE1+T3; Lane A②, KE1+T7; Lane A③, KE2+T3; Lane A④, KE2+T7; Lane A⑤, KE3+T3; Lane A⑥, KE3+T7; Lane A⑦, KE4+T3; Lane A⑧, KE4+T7.

A


λ Pst I
A① A② A③ A④ A⑤ A⑥ A⑦ A⑧
λ Pst I
Base pairs
1700
1160
1090
800
510
460
340

B


λ Pst I
A① A② A③ A④ A⑤ A⑥ A⑦ A⑧
Base pairs
2840
2140
1700
1160
1070
800

6.3.2 Subcloning PCR fragments

Previous PCR reactions had shown that the primers were not 100% specific but that a dominant band was evident of approximately the predicted number of base pairs (KE1 831; KE2, 894; KE3, 574; KE4, 1153). These dominant bands were matched to the predicted size of the bands from Figure 6.1. The largest bands were produced by KE4 with T3 or T7 (predicted 1153bp, actual 1200) and the smallest by KE3 (predicted 574bp, actual 750bp). KE1 and KE2 produced bands about 1000bp in length (predicted 831bp, 894bp). The bands were bigger than predicted due to the vector DNA on one end between the Eco RI cloning site and the T3 or T7 primer sites. Using a sterile Pasteur pipette, a 1.0mm diameter plug was removed from each identified band, placed in a new, sterile thin walled reaction tube and used for a second round of PCR.

Reactions were set up as before using the DNA plug from the first PCR as template for the corresponding primers. A second round of 30 cycles was performed with the same conditions and 10µl was analysed by electrophoresis through a 0.8% agarose gel (Fig. 6.2 B). This shows that the fragments have been amplified, are virtually free from contaminating bands and at a relatively high concentration. The remaining 90µl was extracted with phenol : chloroform (1:1) and then chloroform. The DNA was precipitated with ethanol and the DNA was collected by centrifuging at top speed in a microfuge for 10 minutes. The DNA was dried in a Jouan SpeedVac for 10 minutes and re-suspended in 20µl of ddH_2O. The DNA was digested with Not I overnight at 37°C. Additionally 2µg of pSK- was digested with Not I and Sma I.

The restriction digests were run on a 1% gel and the fragments isolated, 200ng of each fragment and 50ng of vector were ligated together with T4 DNA. Half of each ligation was transformed into *E.coli* DH5α and plated onto LB + 50µg/ml ampicillin with X-gal / IPTG. Over 30 white colonies were obtained per each transformation.

Two colonies were taken from each plate and used to inoculate 10ml of LB + 50µg/ml ampicillin. Cultures were grown overnight at 37°C and used to prepare plasmid DNA

by the Wizard Miniprep method. One microlitre of the resultant DNA was digested with Eco RI and Not I for 2 hours at 37°C to excise the insert and run on a 1% agarose gel (Figure 6.4).

By digesting the subcloned PCR fragments with Eco RI and Not I it was possible to check that the cloned fragments were of the correct number of base pairs in each case. The results showed that all the white colonies analysed contained the expected insert size except A⑦2 which contained a smaller insert than expected.

Control PCRs using primers KE1 and KE4 with the plasmid DNA from A①, A②, A⑦ and A⑧ all produced a product which matched the predicted size 284bp (data not shown) except for A⑦2 which doesn't appear to contain the correct insert (Figure 6.4). DNA from the same preparations shown in Lane A①1, A②1, A⑤1, A⑥1, A⑦1 and A⑧1 were used for DNA sequencing using forward and reverse primers. Sequence was compiled to make a complete sequence.

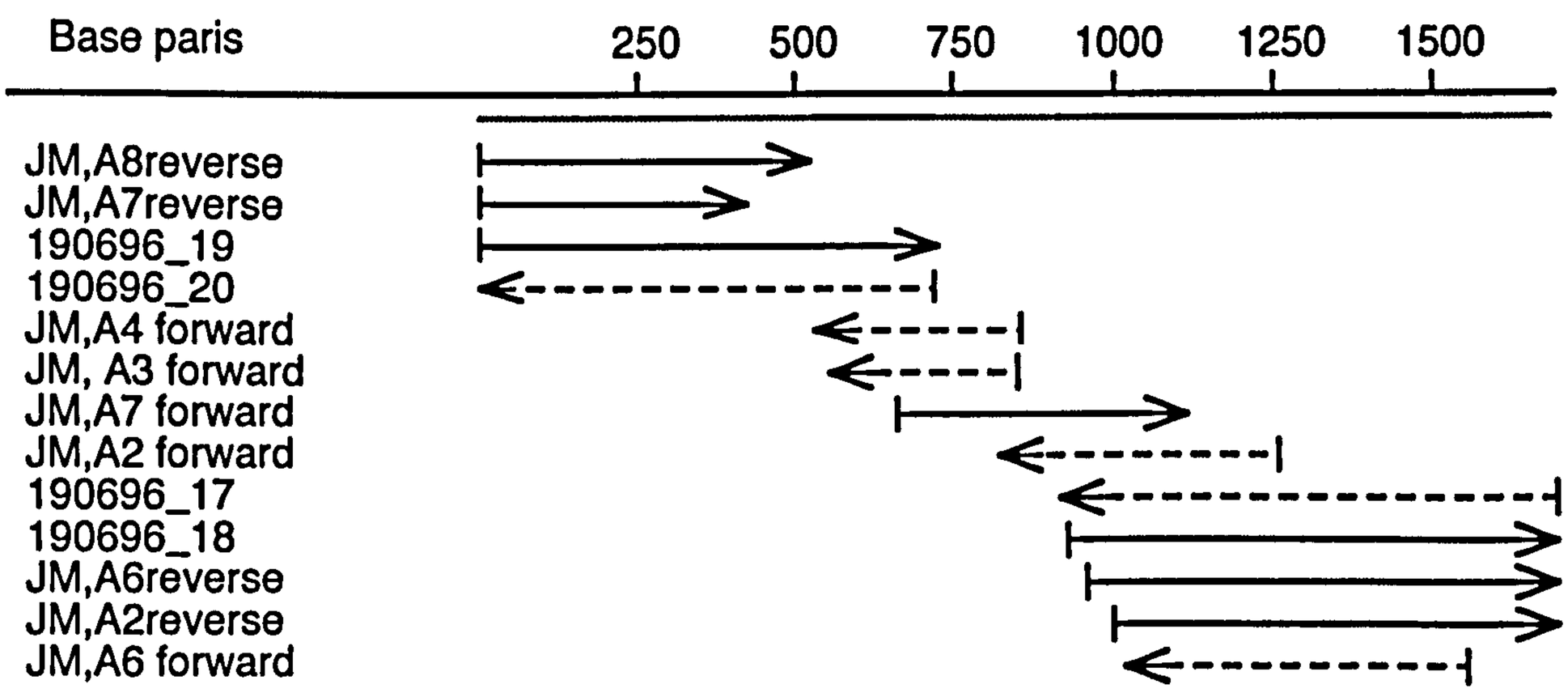
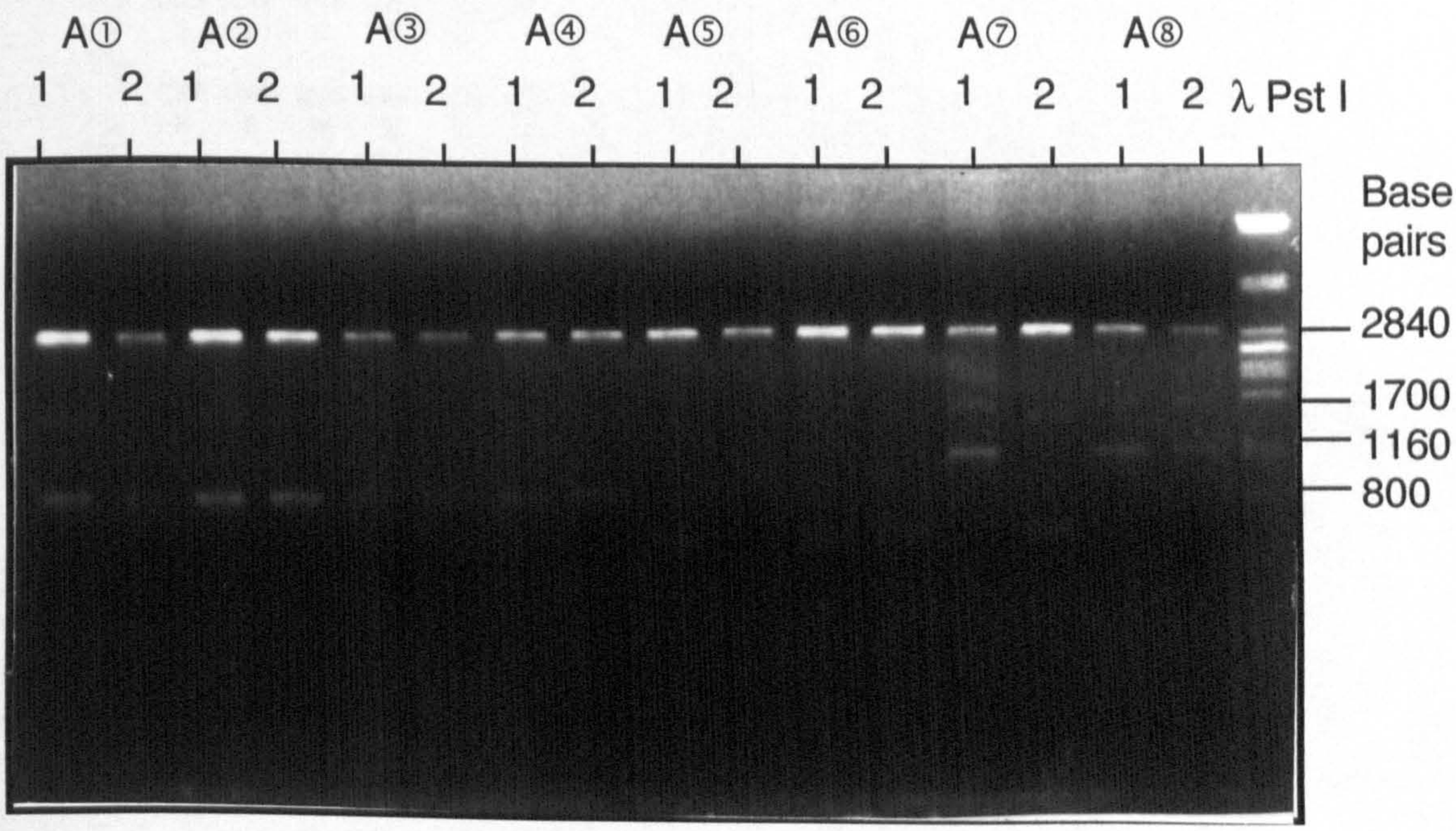


Figure 6.3 Compilation of BC sequences using the SEQMAN program from DNASTAR.

Figure 6.4 Restriction digests of plasmids containing the PCR fragments of biotin carboxylase.

PCR fragments generated in Figure 6.2 B were cut with Not I and ligated into pBluescript™ SK- cut with Not I and Sma I. Ligations were transformed into *E.coli* DH5α and two colonies from each plate were grown for preparation of plasmid DNA. Plasmid DNA was analysed for insert by digestion with Not I and Eco RI.

Pairs of lanes contain plasmids from the same ligation and transformation but isolated from different colonies. Lanes contain plasmids resulting from ligation of pSK- Not / Sma I ligated to Not I cut PCR products. The PCR products in each case were amplified with the following primers; Lane A① 1+2, KE1+T3; Lane A② 1+2, KE1+T7; Lane A③ 1+2, KE2+T3; Lane A④ 1+2, KE2+T7; Lane A⑤ 1+2, KE3+T3; Lane A⑥ 1+2, KE3+T7; Lane A⑦ 1+2, KE4+T3; Lane A⑧ 1+2, KE4+T7. The lane marked λPst contained 1μg of λ Pst I markers


A①
A②
A③
A④
A⑤
A⑥
A⑦
A⑧
1 2 1 2 1 2 1 2 1 2 1 2 1 2 1 2 λ Pst I
Base pairs
2840
1700
1160
800

6.3.3 Sequence of *Brassica napus* biotin carboxylase

The complete sequence was obtained by sequencing the various subclones of BC generated by PCR and aligning the sequences using the SEQMAN program from DNASTAR. The sequence and translation is shown in Figure 6.5.

```
1/1                                                 31/11
*GA CAA GAA GGA TCT CAG TCT CTT ATC CAG CAA ATG GAC GCC TCA ATG ATT ACT AAT TGC
     Q   E   G   S   Q   S   L   I   Q   Q   M   D   A   S   M   I   T   N   C
61/21                                               91/31
AAA TCC ACT GCC TCC CTT CCC TCT TTG TTC CTG GGG AGA TCG GGA GGT AGT CTT AGA AGC
 K   S   T   A   S   L   P   S   L   F   L   G   R   S   G   G   S   L   R   S
121/41                                              151/51
TCC CAG TGT AAT GTC ATG ATG GGG AAA ACC ATT ACC TTC CCG AGC CAA AAG ACT CAG ACT
 S   Q   C   N   V   M   M   G   K   T   I   T   F   P   S   Q   K   T   Q   T
181/61                                              211/71
TTG AAG GTT AGC CGG AAG AAC TTG AAT AGG AGG TGT GGT GGT GGT GCG CTT GGT GCT ACC
 L   K   V   S   R   K   N   L   N   R   R   C   G   G   G   A   L   G   A   T
241/81                                              271/91
TGC AGT GGG GAT AAG ATT CTT GTG GCT AAC AGA GGT GAA ATT GCT GTC CGT GTG ATC CGA
 C   S   G   D   K   I   L   V   A   N   R   G   E   I   A   V   R   V   I   R
301/101                                             331/111
ACT GCT CAT GAG ATG GGG ATT CCT TGT GTT GCT GTT TAC TCA ACC ATC GAC AAG GAT GCA
 T   A   H   E   M   G   I   P   C   V   A   V   Y   S   T   I   D   K   D   A
361/121                                             391/131
TTG CAT GTC AAA TTG GCT GAC GAG GCT GTT TGT ATT GGT GAA GCT CCT AGC AAC CAG TCG
 L   H   V   K   L   A   D   E   A   V   C   I   G   E   A   P   S   N   Q   S
421/141                                             451/151
TAT TTG GTG ATA CCG AAT GTT CTG TCA GCT GCA ATC AGC CGA AGA TGC ACA ATG CTT CAT
 Y   L   V   I   P   N   V   L   S   A   A   I   S   R   R   C   T   M   L   H
481/161                                             511/171
CCT GGA TAT GGA TTC CAT TCC CAA AAC GCT CTT TTT GTT GAG ATG TGC AGA GAG GAG AGA
 P   G   Y   G   F   H   S   Q   N   A   L   F   V   E   M   C   R   E   E   R
541/181                                             571/191
GTG AAT GTG TcA TTG GGA CCA AAT CCT GAT AGC ATC CGT GTC ATG GGT GAC AAA TCA ACT
 V   N   V   S   L   G   P   N   P   D   S   I   R   V   M   G   D   K   S   T
601/201                                             631/211
GCC AGG GAG ACG ATG AAG AAT GCA GGT GTC CCA aCT GTA CCA GGG AGT GAT GGA CTA TTa
 A   R   E   T   M   K   N   A   G   V   P   T   V   P   G   S   D   G   L   L
661/221                                             691/231
AAG AgC ACA GAA GAA GGA gTC AAG CTC gCC AAT GAG ATT GGG TTT CCT GTG ATG ATC AAG
 K   S   T   E   E   G   V   K   L   A   N   E   I   G   F   P   V   M   I   K
721/241                                             751/251
GCA ACA GCT GGT GGT GGT GGA CGT GGA ATG CGT CTT GCT AAT GAA CCG TCA GAG TTT GTG
 A   T   A   G   G   G   G   R   G   M   R   L   A   N   E   P   S   E   F   V
781/261                                             811/271
AAA CTG CTG CAG GCA GCA AAG AGT GAG GCC GCG GCT GCT TTT GGG AAT GAT GGA TGT TAT
 K   L   L   Q   A   A   K   S   E   A   A   A   A   F   G   N   D   G   C   Y
841/281                                             871/291
CTG GAG AAG TAC GTC CAA AAT CCC CGC CAT ATT GAG TTC CAG ATT CTT GCA GAT AAA TTT
 L   E   K   Y   V   Q   N   P   R   H   I   E   F   Q   I   L   A   D   K   F
901/301                                             931/311
GGA AAT GTT GTG CAC TTC GGC GAG CGT GAC TGC AGC ATC CAG AGA CGG AAC CAA AAG TTG
 G   N   V   V   H   F   G   E   R   D   C   S   I   Q   R   R   N   Q   K   L
961/321                                             991/331
CTG GAa GAA GCA CCT TCT CCT GCA CTG ACC CCT GAG TTG GGA AAA GCC ATG GGT GAT GCA
 L   E   E   A   P   S   P   A   L   T   P   E   L   G   K   A   M   G   D   A
1021/341                                            1051/351
GCT GTT GCA GCA GCA GcG TCG ATT GGC TAC GTT GGT GTT GGT ACC GTG GAG TTC CTT TTG
 A   V   A   A   A   A   S   I   G   Y   V   G   V   G   T   V   E   F   L   L
1081/361                                            1111/371
GAT GAG AGA GGT TCC TTc TTA TTT CAT GGA GAT GAA CAT AGA AAT CCA GGT GGA GCA TCC
 D   E   R   G   S   F   L   F   H   G   D   E   H   R   N   P   G   G   A   S
1141/381                                            1171/391
TGT GAC AGA GAT GAT TTA TCT GTT GAT TTG ATT GAA GAA CAG ATT CGT GTT GCA ATG GGA
 C   D   R   D   D   L   S   V   D   L   I   E   E   Q   I   R   V   A   M   G
1201/401                                            1231/411
GAG AAA CTG CGT TAT ACA CAG GAT GAG ATT GTG CTA AGA GGA CAC TCT ATT GAA TGC CGT
 E   K   L   R   Y   T   Q   D   E   I   V   L   R   G   H   S   I   E   C   R
```

```
1261/421                                                    1291/431
ATC AAT GCA GAG GAC CCA TTC AAA GGA TTC AGA CCT GGA CCT GGA AGA ATA ACA GCA TAT
 I   N   A   E   D   P   F   K   G   F   R   P   G   P   G   R   I   T   A   Y
1321/441                                                    1351/451
CTG CCA TCT GGA GGT CCT TTT GTT AGA ATG GAT AGT CAT GTG TAT CCC GAC TAT GTT GTT
 L   P   S   G   G   P   F   V   R   M   D   S   H   V   Y   P   D   Y   V   V
1381/461                                                    1411/471
CCT CCA AGC TAT GAC TCT CTT CTT GGA AAG CTT ATT GTA TGG GCT CCA ACG AGG GAA AGG
 P   P   S   Y   D   S   L   L   G   K   L   I   V   W   A   P   T   R   E   R
1441/481                                                    1471/491
GCA ATT GAG CGG ATG AAA CGT GCA CTT AAT GAC ACT ATT ATT ACA GGG GTT CCC ACC ACC
 A   I   E   R   M   K   R   A   L   N   D   T   I   I   T   G   V   P   T   T
1501/501                                                    1531/511
ATT GAG TAC CAC AAA CTT ATC CTT GAA GTT GAG GAT TTC AAG AAC GGA AAA GTT GAC ACT
 I   E   Y   H   K   L   I   L   E   V   E   D   F   K   N   G   K   V   D   T
1561/521                                                    1591/531
GCT TTC ATC CCC AAG CAT GAG GAG GAA TTA GCA GAG CCT CAT GAA ATT GTG CTA GTG AAA
 A   F   I   P   K   H   E   E   E   L   A   E   P   H   E   I   V   L   V   K
1621/541                                                    1651/551
GAT TTG ACA AAC GCA GCT GCT TAGAATCCATCAGCTCTTAAGATGAAACAGTAATTTTCAG
 D   L   T   N   A   A   A   *
```

Figure 6.5 Sequence and translation of biotin carboxylase from *Brassica napus.*

Sequence was arranged and translated by DNASTRIDER.

To confirm that the sequence encoded biotin carboxylase the predicted amino acid sequence was compared with the identified sequence from *Nicotina tabacum* as shown in Figure 6.6.

```
                1                                                    50
B.napus     mdasmitnck stasl...ps lflgrsggsl rssqcnvmmg ktitfpsqkt
N.tabacum   mdsaaltsvc gksalrftpg lflgrtng.i rssqcsfmag nrinfprqra
               51                                                   100
B.napus     qtlkvsrknl nrrcgggalg ATCSGDKILV ANRGEIAVRV IRTAHEMGIP
N.tabacum   qayrvstkss tr...ggala ATCRAEKILV ANRGEIAVRV IRTAHEMGIP
              101                                                   150
B.napus     CVAVYSTIDK DALHVKLADE AVCIGEAPSN QSYLVIPNVL SAAISRRCTM
N.tabacum   CVAVYSTIDK DALHVKLADE SVCIGEAPSN QSYLVIPNVL SAAISRGCTM
              151                                                   200
B.napus     LHPGYGFHSQ NALFVEMCRE ERVNVSLGPN PDSIRVMGDK STARETMKNA
N.tabacum   LHPGYGFLAE NAVFVEMCRE HGINF.IGPN PDSIRVMGDK STARDTMKNA
              201                                                   250
B.napus     GVPTVPGSDG LLKSTEEGVK LANEIGFPVM IKATAGGGGR GMRLANEPSE
N.tabacum   GVPTVPGSDG LLQSTEEGVR LAEEIGYPVM IKATAGGGGR GMRLAKEPDE
              251                                                   300
B.napus     FVKLLQAAKS EAAAAFGNDG CYLEKYVQNP RHIEFQILAD KFGNVVHFGE
N.tabacum   FVKLLQQAKS EAAAAFGNDG VYLEKYVQNP RHIEFQVLAD KYGNVVHFGE
              301                                                   350
B.napus     RDCSIQRRNQ KLLEEAPSPA LTPELGKAMG DAAVAAAASI GYVGVGTVEF
N.tabacum   RDCSIQRRNQ KLLEEAPSPA LTPELRNAMG DAAVAAAASI GYIGVGTVEF
              351                                                   400
B.napus     LLDERGSFLF HGDEHRNPGG ASCDRDDLSV DLIEEQIRVA MGEKLRYTQD
N.tabacum   LLDERGSFYF MEMNTRIQVE HPVTEMISSV DLIEEQIRVA MGEKLRYKQE
              401                                                   450
B.napus     EIVLRGHSIE CRINAEDPFK GFRPGPGRIT AYLPSGGPFV RMDSHVYPDY
N.batacum   DIVLRGHSIE CRINAEDAFK NFRPGPGRIT AYLPAGGPFV RMDNHVYPDY
              451                                                   500
B.napus     VVPPSYDSLL GKLIVWAPTR ERAIERMKRA LNDTIITGVP TTIEYHKLIL
N.tabacum   VVPPSDDSLL GKLIVWAPTR EGAIERMKRA LNDTIITGVP TTIEYHKLIL
              501                                                   541
B.napus     EVEDFKNGKV DTAFIPKHEE ELAEPHEIV. .LVKDLTNAA A
N.tabacum   DIEDFKNGKF DPSFIPKHGG ELAPPHKMVP AATKEMVNAS A
```

Figure 6.6 Alignment of the amino acid sequences of biotin carboxylase from *Brassica napus* and *Nicotina tabacum* (Shorrosh *et al.*, 1995).

The sequence in lower case is the proposed chloroplast transit peptide and the underlined sequence the putative ATP binding site.

The sequence comparison in Figure 6.6 clearly shows that the cloned cDNA represents the biotin carboxylase from *Brassica napus* due to its strong homology (76.9% similarity, 19.4% divergence) to the identified sequence in *N.tabacum*.

6.4 Overexpression of Biotin Carboxylase

The cDNA of biotin carboxylase from *Brassica napus* was used to overexpress the biotin carboxylase protein using a pET vector. The overexpressed protein was required to raise anti-BC antibodies and to study the biochemical properties of the protein. Previously, expression of the BCCP subunit had been successful using pET11a, and this vector was used again for biotin carboxylase.

6.4.1 Construction of a full length biotin carboxylase cDNA

The subclones sequenced to produce the entire sequence of BC were two overlapping clones, neither of which contained the entire deduced open reading frame alone. To overexpress the biotin carboxylase as an entire protein, the two fragments had to be joined together to make an complete open reading frame. The fragments amplified using primers KE1 with T7 and KE4 with T3 subcloned as A② and A⑦ contain an overlapping region (between primers KE1 and KE4). The sequence in this region was examined for unique restriction sites.

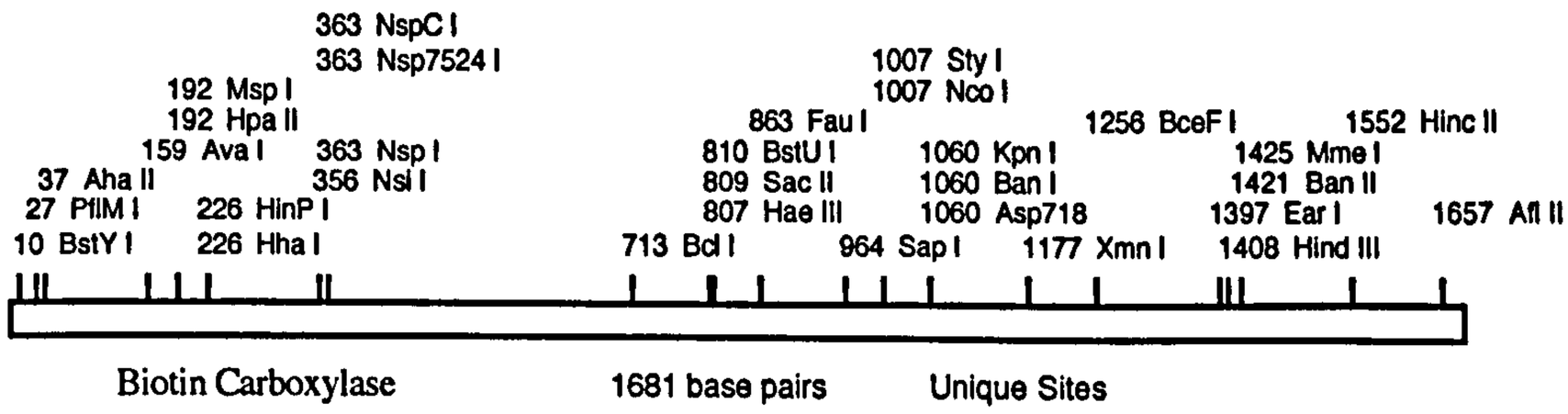


Figure 6.7 Unique restriction sites in the biotin carboxylase cDNA sequence from DNASTRIDER.

The primers KE1 and KE4 border the region between 823 and 1109. There are several unique sites in this region, but it was necessary to find one which would not cut the cloning vector pSK- so Nco I was chosen.

Of each subclone A②1 and A⑦1 3μg of DNA was digested with Not I / Nco I in Boehringer Mannheim SureCut Buffer H at 37°C overnight. Also digested simultaneously was 2μg of pSK- with Not I at 37°C overnight. The digests were run on a 0.8% agarose gel and viewed under long-wave UV light (302nm) to prevent DNA damage. The gel picture is shown in Figure 6.8 A and shows the size of the two fragments from the subclones A②1 and A⑦1 when digested with Nco I and Not I. The 850bp and 1000bp BC fragments in lanes 1 and 2 respectively, and the pSK- vector cut with Not I in lane 3 were isolated from the gel. That the isolated fragments had been recovered was checked by running 1μl on a 0.8% agarose gel (Figure 6.8 B).

The full length construct was made by ligating together in the same reaction, 1μl of Not I cut pSK-, 2μl of A② Not I / Nco I fragment and 1μl of A⑦ Not I / Nco I fragment with 3U of T4 DNA ligase in a total volume of 10μl overnight at 16°C. Half of the ligation was transformed into Subcloning Efficiency DH5α™ Competent Cells and plated onto LB + 50μg/ml ampicillin plus X-gal / IPTG. Transformed bacteria were grown at 37°C overnight. Four white colonies were obtained and these were used to inoculate 10ml cultures of LB + 50μg/ml ampicillin for preparation of plasmid DNA. The plasmid DNA obtained from these four cultures was analysed for insert by digestion with Not I / Nco I and by digestion with Not I alone. The fragments resulting from these digests were analysed by separation through a 0.8% agarose gel as shown in Figure 6.8 C.

These digests showed that the Not I / Nco I fragments had clearly been ligated together into the Not I cut pSK-. Although in the ligation reaction some of the biotin carboxylase fragments would have been ligated together in the wrong orientation (i.e. Not I - Not I) this would leave a fragment with Nco I ends which could not be ligated into the vector. Consequently, a full length biotin carboxylase gene had been constructed called pBC, and this could be used for PCR to generate a suitable fragment for ligation into pET11a.

Figure 6.8 A Digests of A②1 and A⑦1 with Nco I / Not I run on a 0.8% agarose gel.

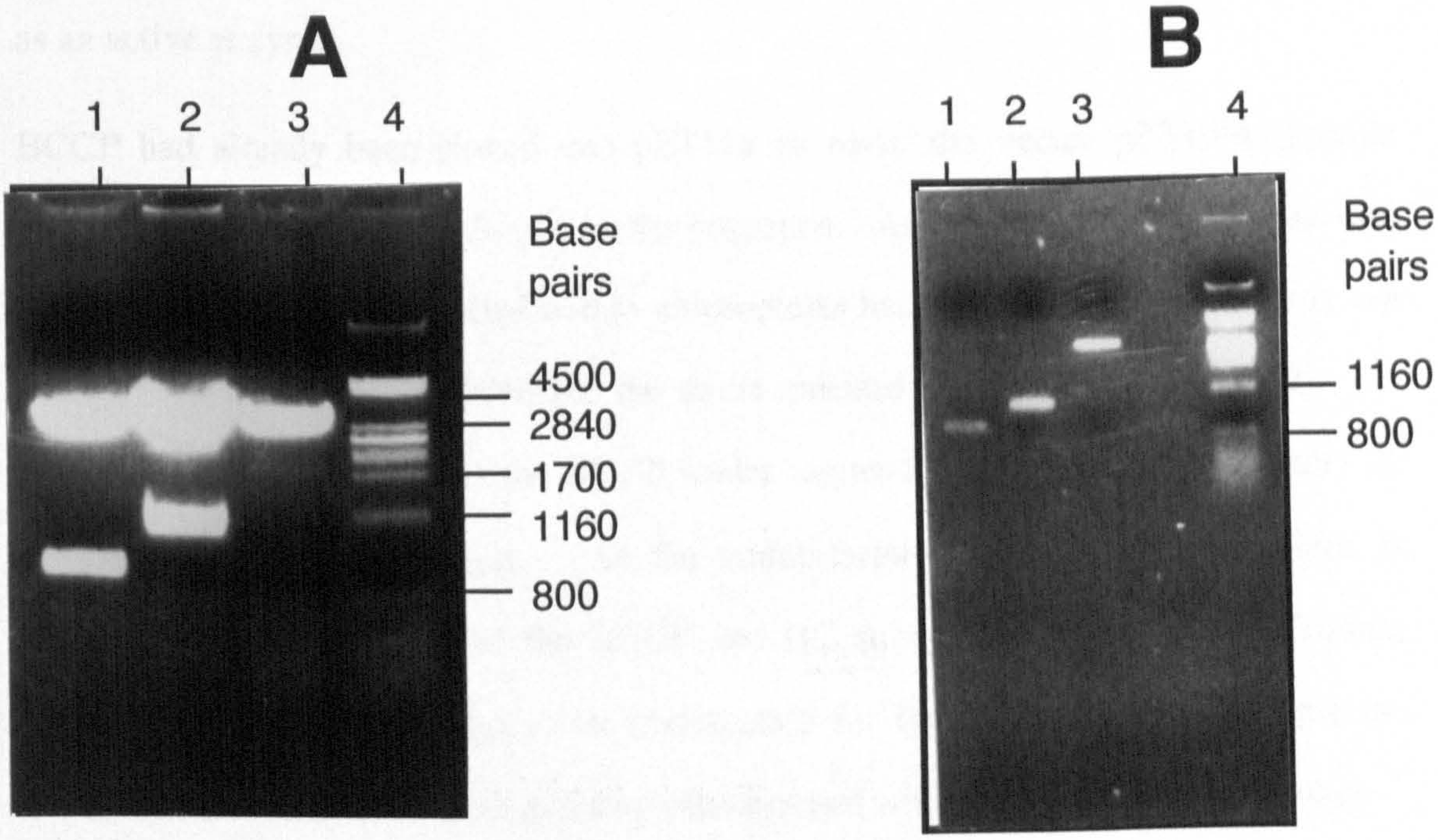
Digests of 3μg of each subclone A②1 and A⑦1 was with Not I / Nco and 2μg of pSK- with Not I were separated on a 0.8% agarose. Lanes contain the following; Lane 1, A②1 Nco I / Not I digest; Lane 2, A⑦1 Nco I / Not I digest; Lane 3, pSK- Not I digest; Lane 4, 1μg of λ Pst I markers.

Figure 6.8 B Isolated Not I - Nco I biotin carboxylase fragments and the Not I cut pSK- for ligation.

The relevant bands from Figure 6.8 A were isolated from the gel and 1μl run on a 0.8% agarose gel to check the purification. Lanes contain the following ; Lane 1, 850 base pair Nco I / Not I fragment of A②1; Lane 2, 1000 base pair Nco I / Not I fragment of A⑦1; Lane 3, Not I digested pSK- fragment; Lane 4, 1μg of λ Pst I markers.

Figure 6.8 C Analysis of the plasmids contained within white colonies obtained from ligation of Not I-Nco I BC fragments and Not I cut pSK-

Plasmid DNA was prepared from *E.coli* DH5α cells transformed with the ligation of Not I cut pSK- and the Not I / Nco I fragments from A②1 and A⑦1. Four colonies were isolated and named pBC. Lanes contained the following; Lanes 1+10, 1μg of λ Pst I markers; Lane 2-5, Not I digest of pBC1-4 respectively; Lanes 6-9, Not I / Nco I digest of pBC1-4 respectively.

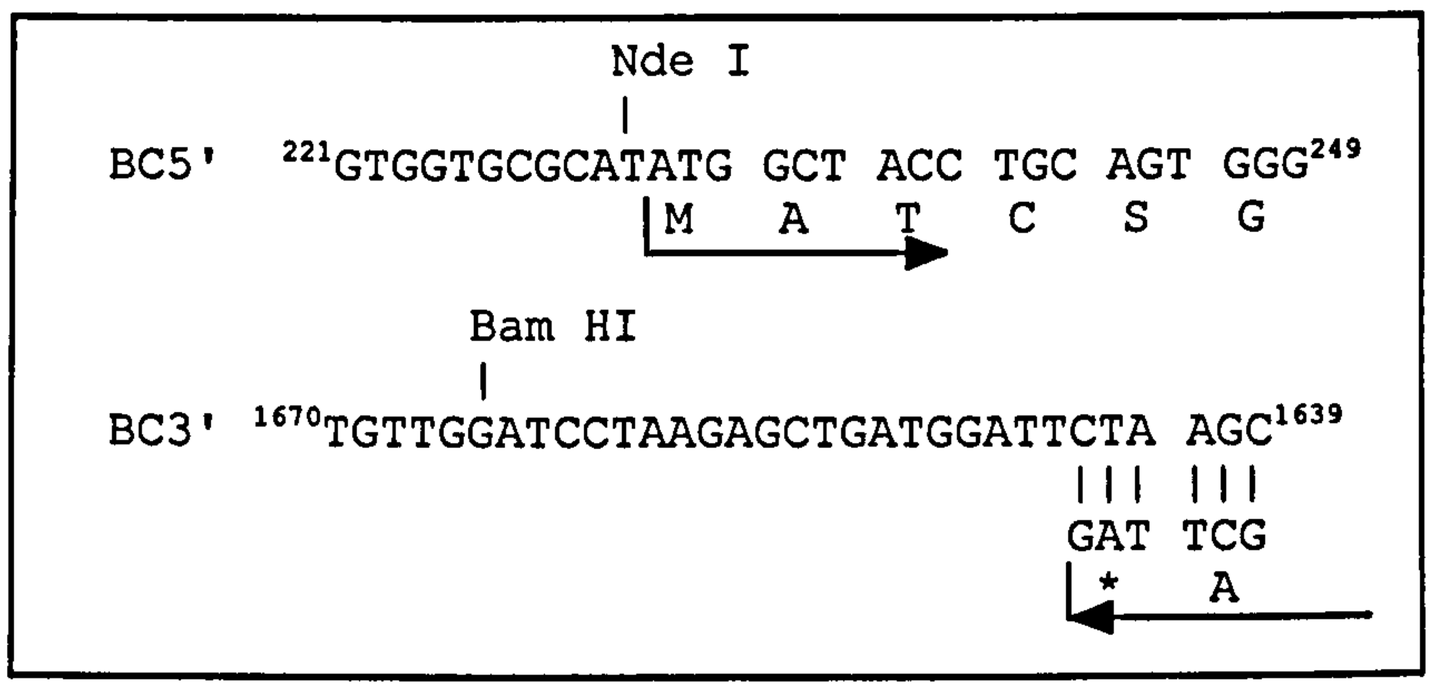

A
1 2 3 4
Base pairs
4500
2840
1700
1160
800
B
1 2 3 4
Base pairs
1160
800
C
1 2 3 4 5 6 7 8 9 10
Base pairs
1700
510
Base pairs
2840
1980
1160
800

6.4.2 Construction of the expression vectors pETBC and pETBP+L.

The cDNA sequence for biotin carboxylase was cloned into pET11a without the DNA encoding for the leader sequence proposed in Figure 6.6. This would overexpress holoprotein form of the subunit which would normally be found inside the chloroplast as an active enzyme.

BCCP had already been cloned into pET11a to make the vector pETBP4 (section 5.4.1) but this clone was without a leader sequence. As previously discussed (section 5.8), the BCCP protein detected within chloroplasts has a M_r of 35,000. This is not consistent with the M_r observed for the overexpressed BCCP protein (30,000). It seems possible therefore that the BCCP leader sequence is not cleaved upon entry of the protein to the chloroplast. As the amino-terminus of the BCCP protein is important in the interaction of the BCCP and BC subunits in *E.coli* (Li & Cronan, 1992a), if this interaction was to be investigated for the *Brassica napus* enzyme the BCCP subunit may have needed to be overexpressed with the leader sequence intact.

As previously described (section 5.4.1) to clone genes in frame into pET11a an Nde I site must be created as the start codon at the 5' end and a Bam HI site at the 3' end. With this in mind the primers shown in Figure 6.9 were designed.



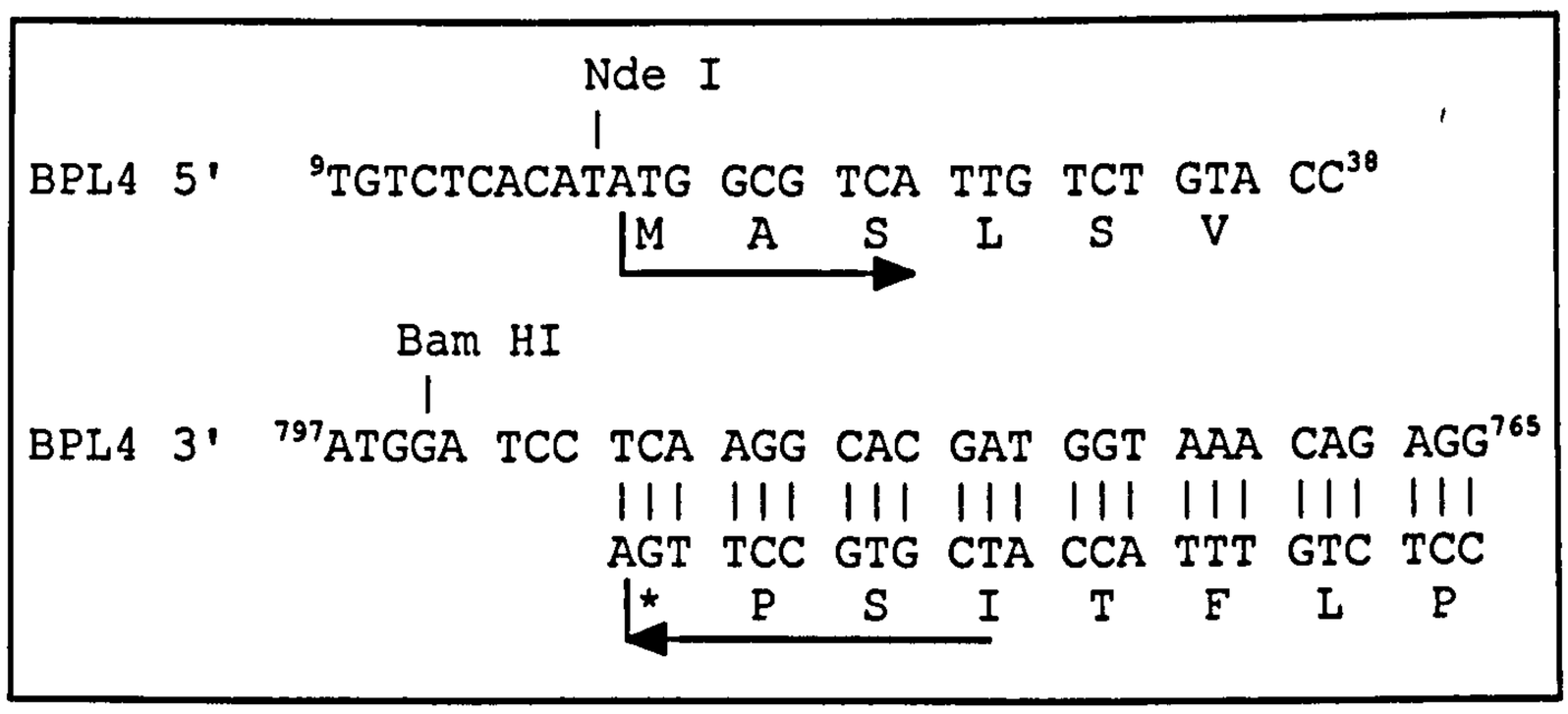


Figure 6.9 Primers designed for expression of biotin carboxylase and BCCP + leader sequence.

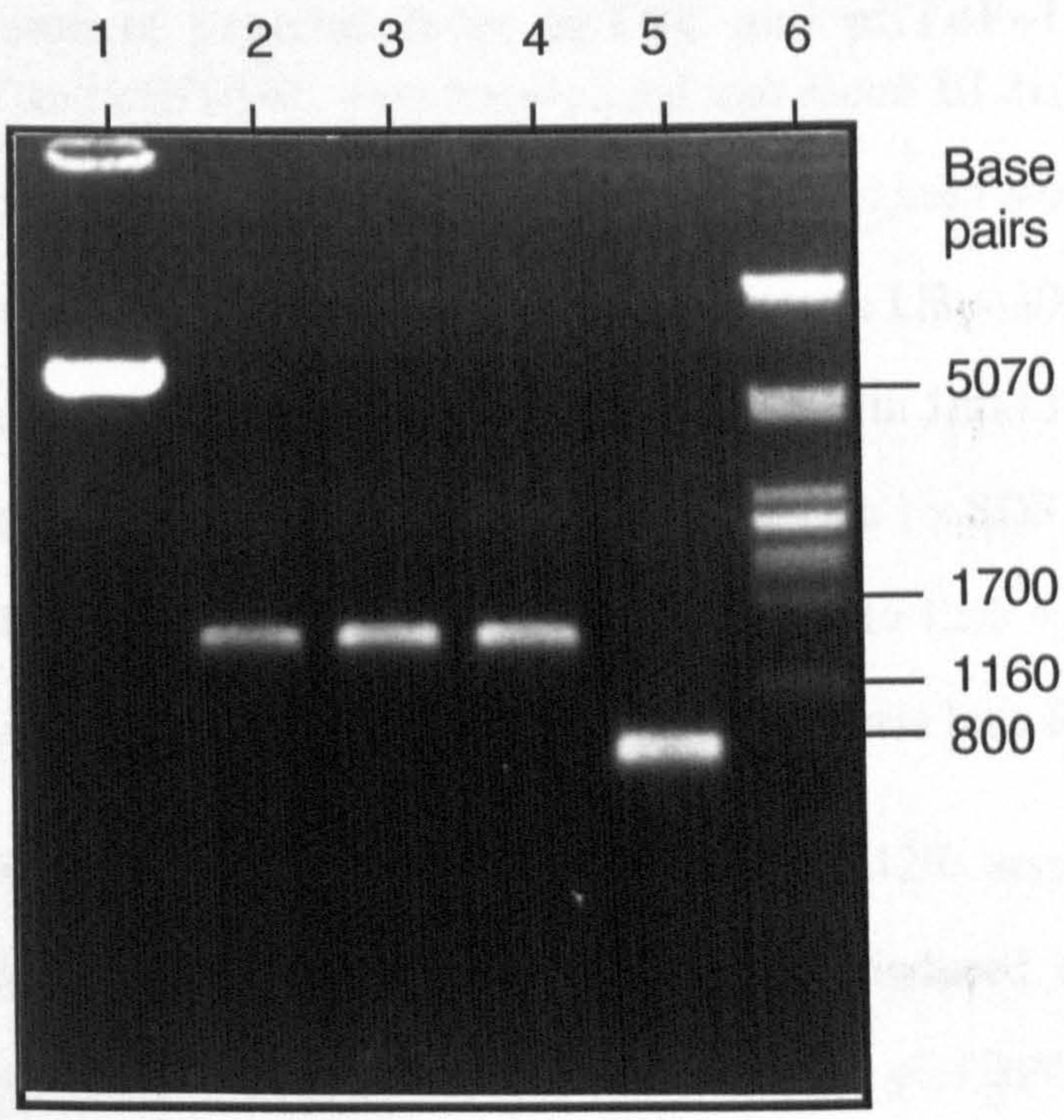
The primers for biotin carboxylase are BC5' and BC3' (top) and the primers for BCCP + leader sequence are BPL4 5' and BPL4 3' (bottom). Both sets of primers will allow ligation into pET11a.

PCR was carried out with the primers shown in Figure 6.9 and 2ng of the relevant plasmid template. Amplification was performed for 30 cycles of 94°C 1 minute, 55°C 1 minute and 74°C 2 minutes. The PCR reaction was run on a 0.8% agarose gel and the fragments isolated. The isolated fragments and 2μg of pET11a were digested with Nde I and Bam HI at 37°C overnight. The digests were run on a 0.8% agarose gel as shown in Figure 6.10 and the fragments isolated. These isolated fragments were used to create the required vectors by ligation. pET11a cut with Bam HI and Nde I was ligated with either the similarly digested BC or BCCP fragments generated by PCR. Approximately equimolar amounts of the DNA fragments were ligated in a total volume of 10μl with 3U of T4 DNA ligase.

Figure 6.10 0.8% agarose gel showing restriction digests of the isolated PCR fragments and pET11a for ligation to make the pETBC and pETBP+L expression vectors.

PCR was carried out with the primers shown in Figure 6.9. The PCR reaction was run on a 0.8% agarose gel and the fragments isolated. The isolated fragments and 2μg of pET11a were digested with Nde I and Bam HI at 37°C overnight. The restriction digests are shown opposite run on a 0.8% agarose gel. The fragments were isolated fragments ligated together to produce the pET vectors pETBC and pETBP+L.

The lanes contain the following; Lane 1, 2μg of pET11a digested with Nde I and Bam HI; Lanes 2-4, isolated PCR product of primers BC5' and BC3' from pBC digested with Nde I and Bam HI; Lane 5, isolated PCR product of primers BPL4-5' and BPL4-3' from pBP4 digested with Nde I and Bam HI; Lane 6, 1μg of λ Pst I markers.


1
2
3
4
5
6
Base pairs
5070
1700
1160
800

Half of the ligation was used to transform 200µl of *E.coli* DH5α and the cells were plated out on LB + 50µg/ml ampicillin (section 2.5.14). Bacteria which grew were used to inoculate 10ml of LB + 50µg/ml ampicillin and left to grow at 37°C with shaking at 220rpm overnight. Plasmid DNA was prepared from bacteria and analysed for the presence of the required insert by digesting 1µg of DNA with Nde I and Bam HI for 2 hours at 37°C and running the DNA on a 0.8% agarose gel. The results confirmed that all transformed bacteria which grew contained plasmids with the correct insert (not shown).

6.4.3 Expression of proteins from pETBC and pETBP+L

Plasmids pETBC and pETBP+L were transformed into *E.coli* BL21(λDE3) cells with $CaCl_2$. Colonies which grew after 16 hours were made into seed stocks and stored at -80°C. Seed stocks were used to inoculate 50ml cultures in LB + 100µg/ml ampicillin and these were grown up to an OD_{600} of 1.0 and induced with 1mM IPTG for 3 hours. Samples of uninduced cells and induced cells were boiled in 1 × SDS sample buffer for 2 minutes, spun for 1 minute in a microfuge and loaded onto 12% acrylamide gels for separation of proteins. Proteins were stained with Coomassie blue R-250.

Figure 6.11 shows Coomassie R-250 stained protein in 12% acrylamide gels. It shows the proteins in *E.coli* BL21(λDE3) cells when uninduced and induced with IPTG. The bacteria containing the plasmids pETBC and pETBP4 showed obvious expression of proteins with M_rs of 46,000 (Fig. 6.11 A, lanes 2, 4 and 6) and 30,000 (Figure 6.11 B, lane 4) respectively, in the presence of IPTG. These proteins were not expressed when pET11a alone was induced in the cells so the novel protein expressed in the bacteria containing the pETBC and pETBP4 constructs was considered to be the cloned gene of interest.

The bacteria harbouring the vector pETBP+L show very little or no expression of a novel protein when induced with IPTG (Figure 6.11 C, lane 3). To confirm that a novel protein was being produced, the proteins synthesised by the pETBP+L vector were labelled with ^{35}S-methionine.

Figure 6.11 **A-C** Coomassie stained over-expressed proteins separated on a 12% acrylamide gel.

E.coli BL21(λDE3) cells various overexpression vectors and were grown up to an OD_{600} of 1.0 in 50ml cultures of LB + 100μg/ml ampicillin and induced with 1mM IPTG for 3 hours. Samples of uninduced cells and induced cells were boiled in 1 × SDS sample buffer for 2 minutes, spun for 1 minute in a microfuge and loaded onto 12% acrylamide gels for separation of proteins. Proteins were stained with Coomassie blue R-250. Arrows mark the visible overexpressed proteins in each gel.

A Expression from pETBC in *E.coli* BL21(λDE3) cells.

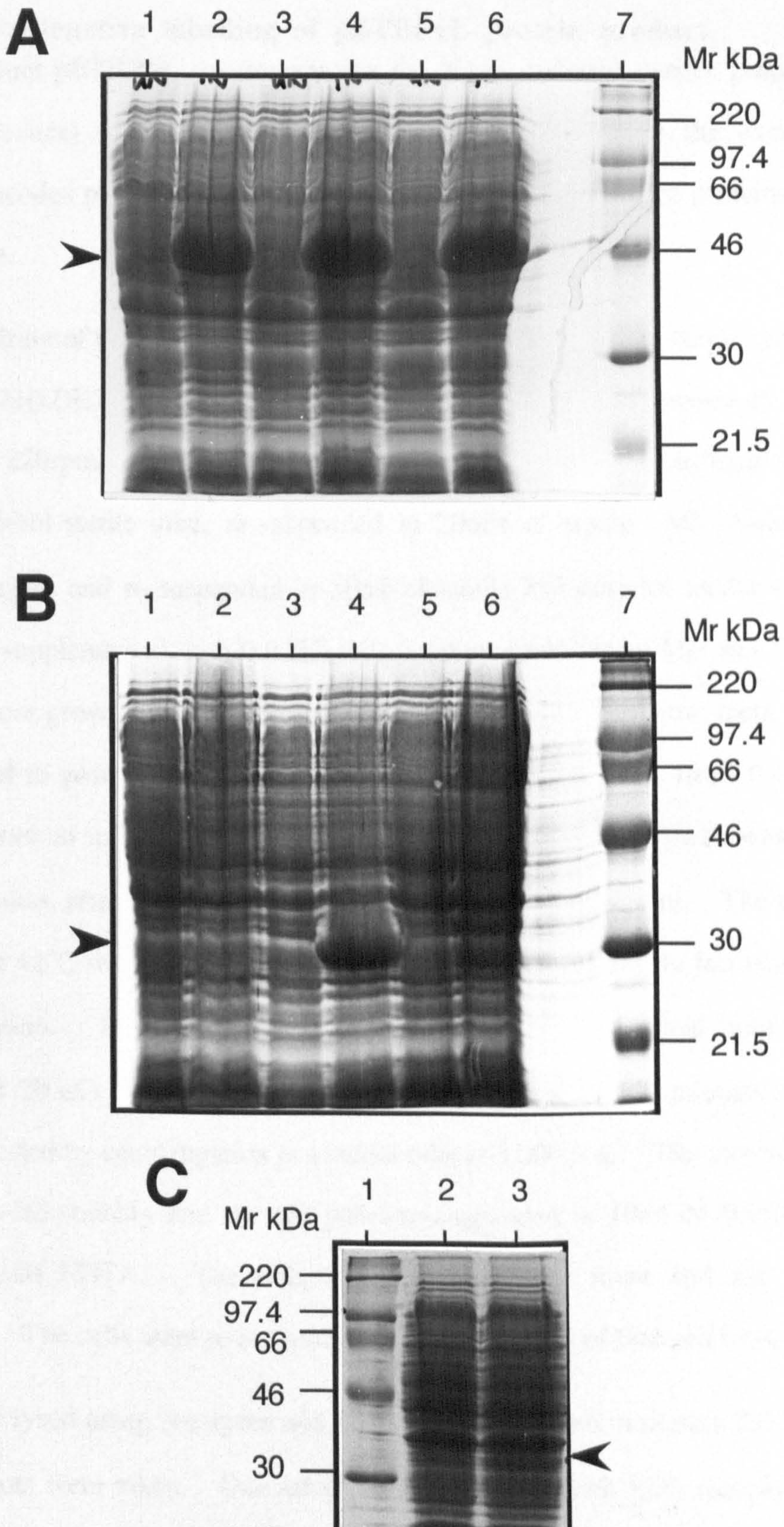
Lanes show the following; Lanes 1, 3 and 5 uninduced *E.coli* BL21(λDE3) cells containing pETBC; Lanes 2, 4 and 6 induced BL21(λDE3) cells containing pETBC; Lane 7 Rainbow markers, 2μg each protein.

B Expression of pET11a, pETBP4 and pETBP+L in *E.coli* BL21(λDE3) cells.

Lanes show the following; Lanes 1, 3 and 5, uninduced cells; Lanes 2, 4 and 6, induced cells; Lanes 1+2, cells containing pET11a; Lanes 3+4, cells containing pETBP4; Lanes 5+6, cells containing pETBP+L; Lane 7, Rainbow markers, 2μg each protein.

C Expression of pETBP+L in *E.coli* BL21(λDE3) cells.

Lanes show the following; Lane 1, Rainbow markers, 2μg each protein; Lane 2, uninduced cells; Lane 3 induced cells.


A
1 2 3 4 5 6 7
Mr kDa
220
97.4
66
46
30
21.5
B
1 2 3 4 5 6 7
Mr kDa
220
97.4
66
46
30
21.5
C
1 2 3
Mr kDa
220
97.4
66
46
30
21.5
14.3

6.4.4 Radioactive labelling of pETBP+L protein product.

The construct pETBP+L (overexpressing the biotin carboxyl carrier protein with a leader sequence) showed no obvious expression of protein so the expression of plasmid encoded protein was checked by labelling plasmid encoded proteins with ^{35}S-methionine.

A 50ml culture of LB + 100μg/ml ampicillin was inoculated with a single seed stock of *E.coli* BL21(λDE3) harbouring pETBP+L. The culture was grown at 37°C with shaking at 220rpm to an OD_{600} of 0.5. Cells were collected by centrifugation at 3,000 × g in a 50ml sterile tube, re-suspended in 20mls of sterile M9 minimal media, collected again and re-suspended in 50ml of sterile M9 minimal media + 100μg/ml ampicillin supplemented with 0.005% (w/v) amino acids except Met and Cys. The bacteria were grown for 90 minutes in the minimal media to allow them to become accustomed to growth in minimal media. IPTG was added to 1mM from a sterile 100mM stock to induce expression of the T7 polymerase and growth was continued for 30 minutes, after which time rifampicin was added to 200μg/ml. The culture was placed in a 42°C water bath with gentle shaking for 20 minutes to facilitate uptake of the rifampicin. The bacteria were then grown for an additional hour before the addition of 20 μCi of ^{35}S-Met. Growth was continued for 30 minutes before cells were harvested by centrifugation in a sterile tube at 3,000 × g. The radioactive media was discarded suitably and the cell pellet re-suspended in 10ml of 50mM Tris-HCl pH8.0, 2mM EDTA. The cells were collected once more and the supernatant discarded. The cells were re-suspended in a final volume of 5ml and frozen at -20°C.

Cells were lysed using lysozyme and detergent as described in section 2.4.14. Two 20μl aliquots were taken. One sample was made 1 × with SDS sample buffer and boiled for 2 minutes, this sample was total cell protein. The other was centrifuged for 5 minutes in a microfuge and the supernatant taken, made 1 × with SDS sample buffer and boiled for 2 minutes, this sample was the solubilised supernatant. The sample were run on a 12% acrylamide gel with ^{14}C labelled M_r markers (AMERSHAM), and the

proteins stained with Coomassie R-250. The gel was dried and exposed to a High Sensitivity phosphor imaging screen overnight. The stained gel is shown in Figure 6.12 A along with the processed phosphor imaging picture in Figure 6.12 B.

This shows that some protein is being expressed by these cells, but at a significantly lower level than observed with the other expression vectors pETBP4 and pETBC. Most of this protein was not present in the solubilised supernatant (lane 4). This suggests that it was expressed in inclusion bodies. The data does reveal the relative mobility of the protein on SDS / PAGE as 35,000 daltons. This coincides exactly with the observed value for the BCCP protein in chloroplasts.

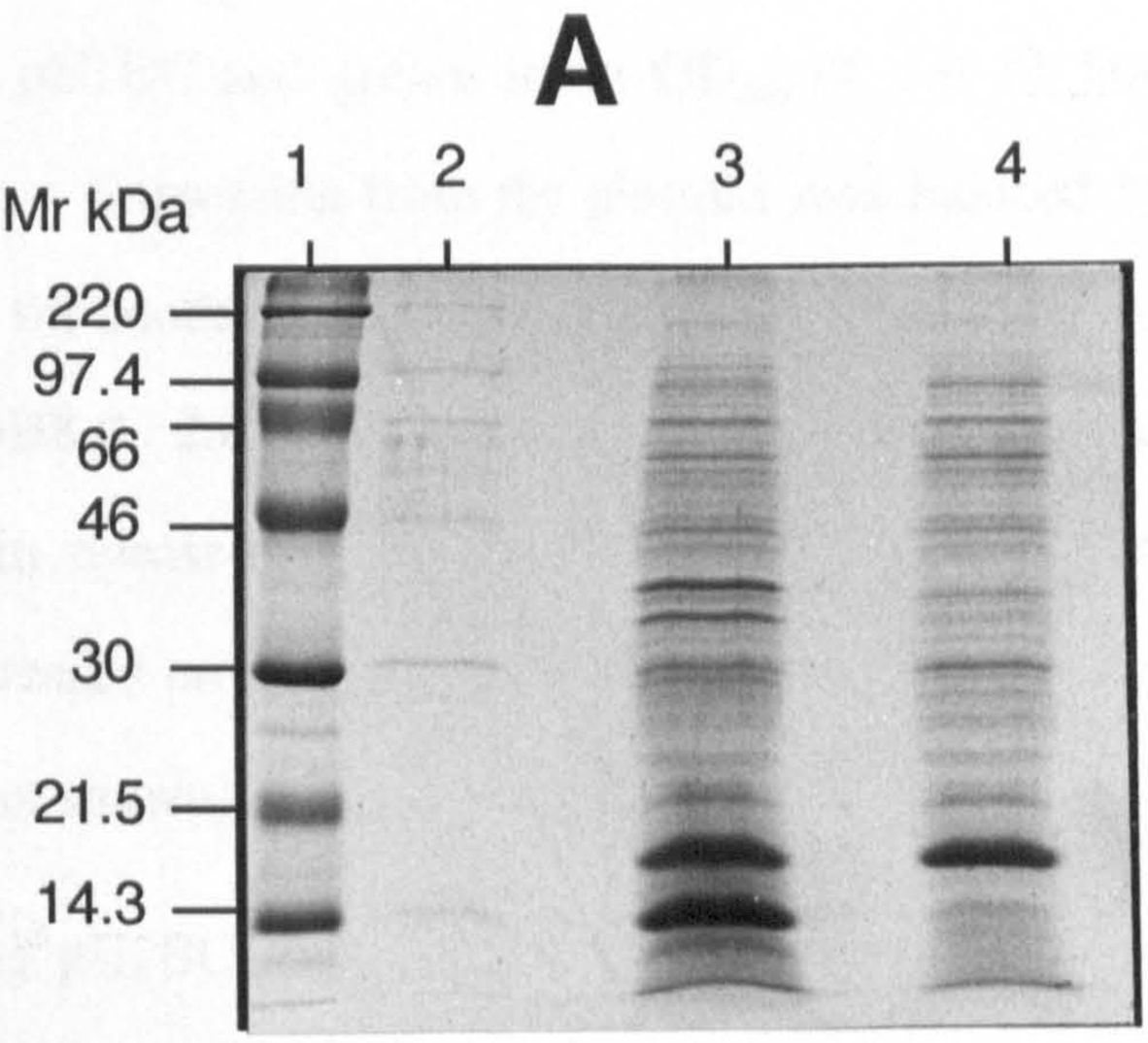
Figure 6.12 A Coomassie stained 12% acrylamide gel of proteins from *E.coli* BL21(λDE3) harbouring pETBP+L.

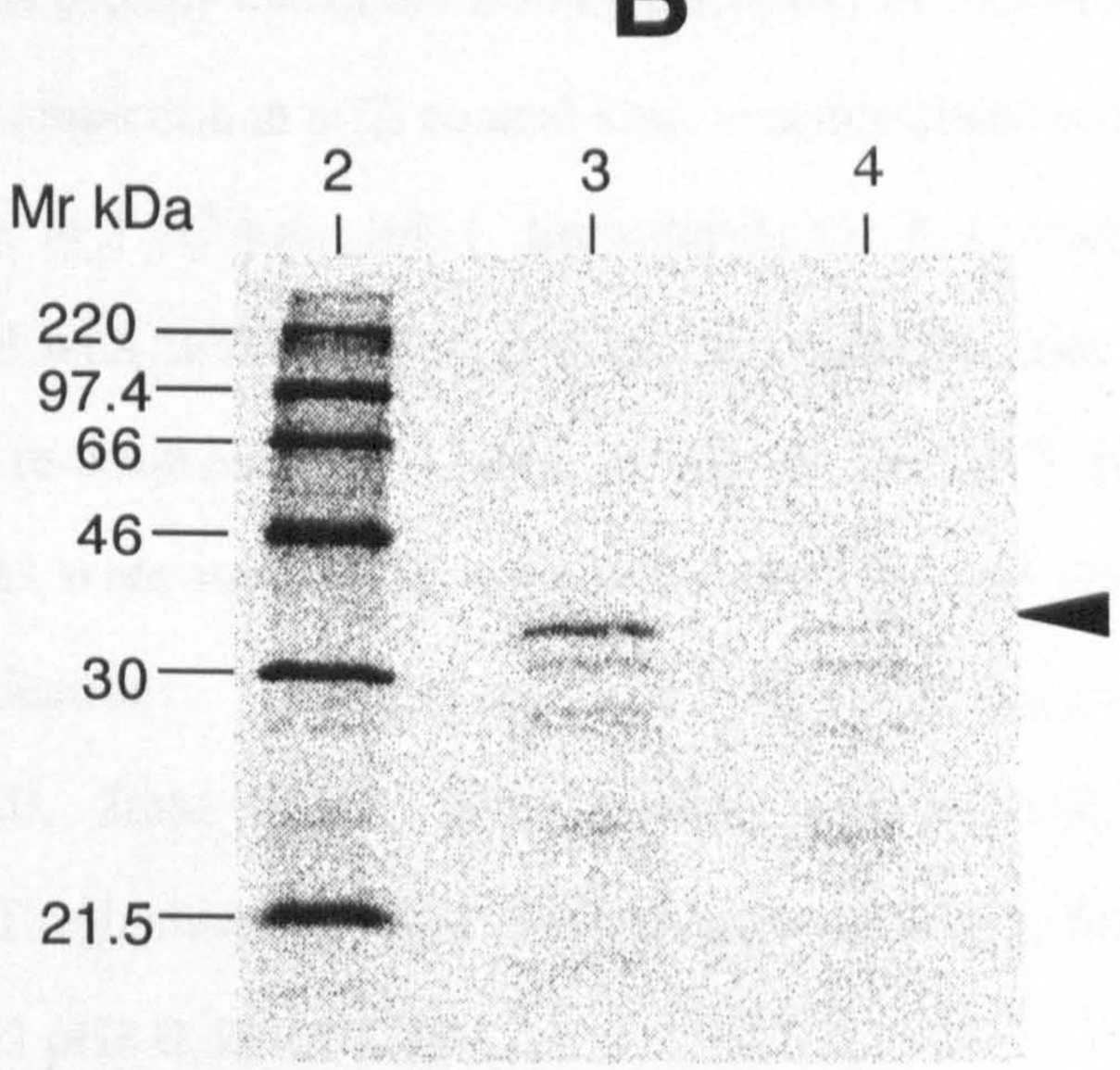
Expression was induced from pETBP+L in *E.coli* BL21(λDE3) cells at log phase with IPTG. Bacterial transcription was inhibited by the addition of rifampicin to 200μg/ml. The bacteria were then grown for an additional hour before the addition of 20 μCi of ^{35}S-Met. Growth was continued for 30 minutes before cells were harvested by centrifugation in a polypropylene centrifuge tube at 3,000 × g. Cells were lysed with lysozyme and sonication.

The lanes show the following; Lane 1, Rainbow markers, 2μg each protein; Lane 2, ^{14}C labelled protein standards; Lane 3, total protein from induced cells harbouring pETBP+L; Lane 4, soluble protein from induced cells harbouring pETBP+L.

Figure 6.12 B Image from the gel shown in A after exposure to a High Sensitivity phosphor imaging screen and scanning at 100μm resolution.

The gel shown in Figure 6.12 A was exposed to a high sensitivity phosphor imaging screen for 16 hours, after which the image was scanned at 100μm resolution and processed with BIO-RAD's Molecular Analyst® software.


A
1
2
3
4
Mr kDa
220
97.4
66
46
30
21.5
14.3


B
2
3
4
Mr kDa
220
97.4
66
46
30
21.5

6.4.5 Purification of overexpressed biotin carboxylase protein

The BCCP protein was initially partially purified by breaking cells through an automated French press and applying the protein to a HiLoad Mono-Q column, although with a low yield (section 5.4.3). A similar approach was tried with biotin carboxylase. Two 1L cultures of LB + 100μg/ml ampicillin were each inoculated with one seed stock of pETBC and grown to an OD_{600} of 1.0 (8 hours) at 37°C with shaking at 220rpm. Expression from the plasmid was induced by adding IPTG to 1mM and growing for another 3 hours. Cells were pelleted, re-suspended in 50mls of 50mM Tris-HCl pH8.0, 2mM EDTA and broken with an automated French press. The soluble protein released by breaking the cells by this method contained no detectable overexpressed protein when run on 12% acrylamide gels and stained with Coomassie (data not shown).

Bacteria harbouring pETBC overexpressed BC in large quantities (Figure 6.11 A, lanes 2, 4 and 6), but as no protein could be detectable in the soluble phase it was assumed that this protein was in the insoluble fraction as inclusion bodies. A litre of TB + 200μg/ml ampicillin in a 2L conical flask was inoculated with a single seed stock and left to grow at 37°C overnight. Immediately the following morning the culture was made 1mM with IPTG and left to grow for a further three hours. Cells were harvested and re-suspended in 100ml of 50mM Tris-HCl pH8.0, 2mM EDTA. Inclusion bodies were isolated by lysis with lysozyme and detergent and the DNA sheared by sonication. The crude inclusion body pellet was re-suspended in 50mM Tris-HCl pH8.0, 2mM EDTA, 500mM NaCl and pelleted by centrifugation at 12,000 × g. The inclusion bodies were re-suspended in a final volume of 5ml of 50mM Tris-HCl pH8.0, 2mM EDTA.

Ten microlitres of isolated inclusion bodies were placed in a microfuge tube and diluted to 50µl with 50mM Tris-HCl, 2mM EDTA. The inclusion bodies were treated with various detergents by adding detergent and buffer to a final volume of 100µl. Treatments included; 50mM Tris-HCl, 2mM EDTA; 0.5% Triton X-100; 100mM $CaCl_2$; 0.1% SDS; 0.25% CHAPS; 0.5% Nonidet P-40; 0.1% Tween-20 and 10µl of 10N NaOH and 1 × SDS sample buffer. After the addition of detergents the inclusion bodies were left vortexing at room temperature for 30 minutes. Protein not solubilised by this treatment was centrifuged to the bottom of the microfuge tube by centrifuging at top speed for 15 minutes in a microfuge. The supernatant was removed from each tube, made 1 × with SDS sample buffer, boiled for 2 minutes, and 5µl run on a 12% gel. The protein pellet was re-suspended in 1 × SDS sample buffer, boiled for 2 minutes and run on a 12% gel. Gels were stained with Coomassie R-250.

The results shown in Figure 6.13 B demonstrated that the inclusion bodies were solubilised by SDS (lane 4) and high pH (lane 8) and several contaminant proteins were removed by the use of the non-ionic detergent Nonidet P-40 (lane 6).

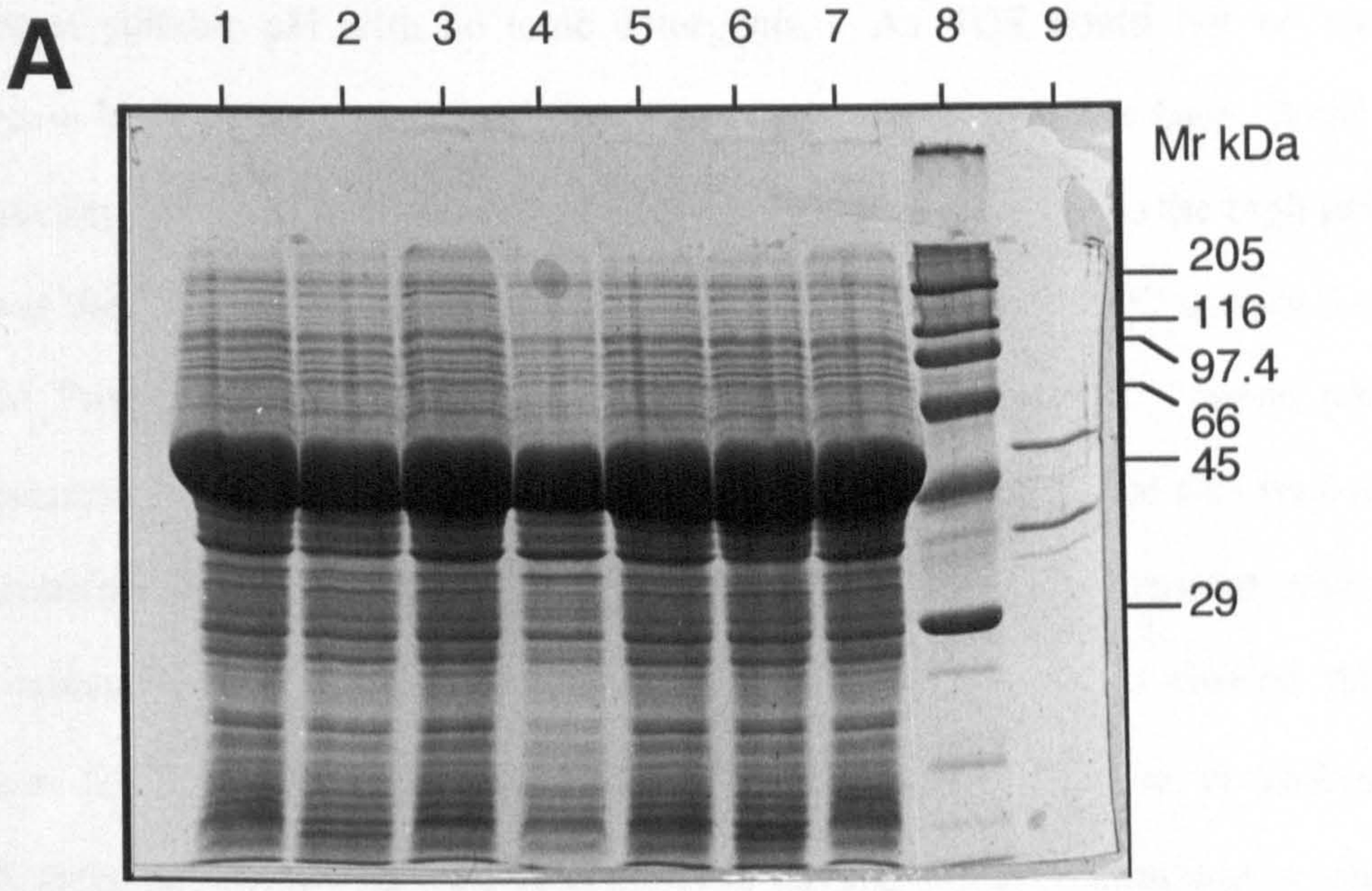
Figure 6.13 A Isolated inclusion bodies washed with various detergents and salt treatments.

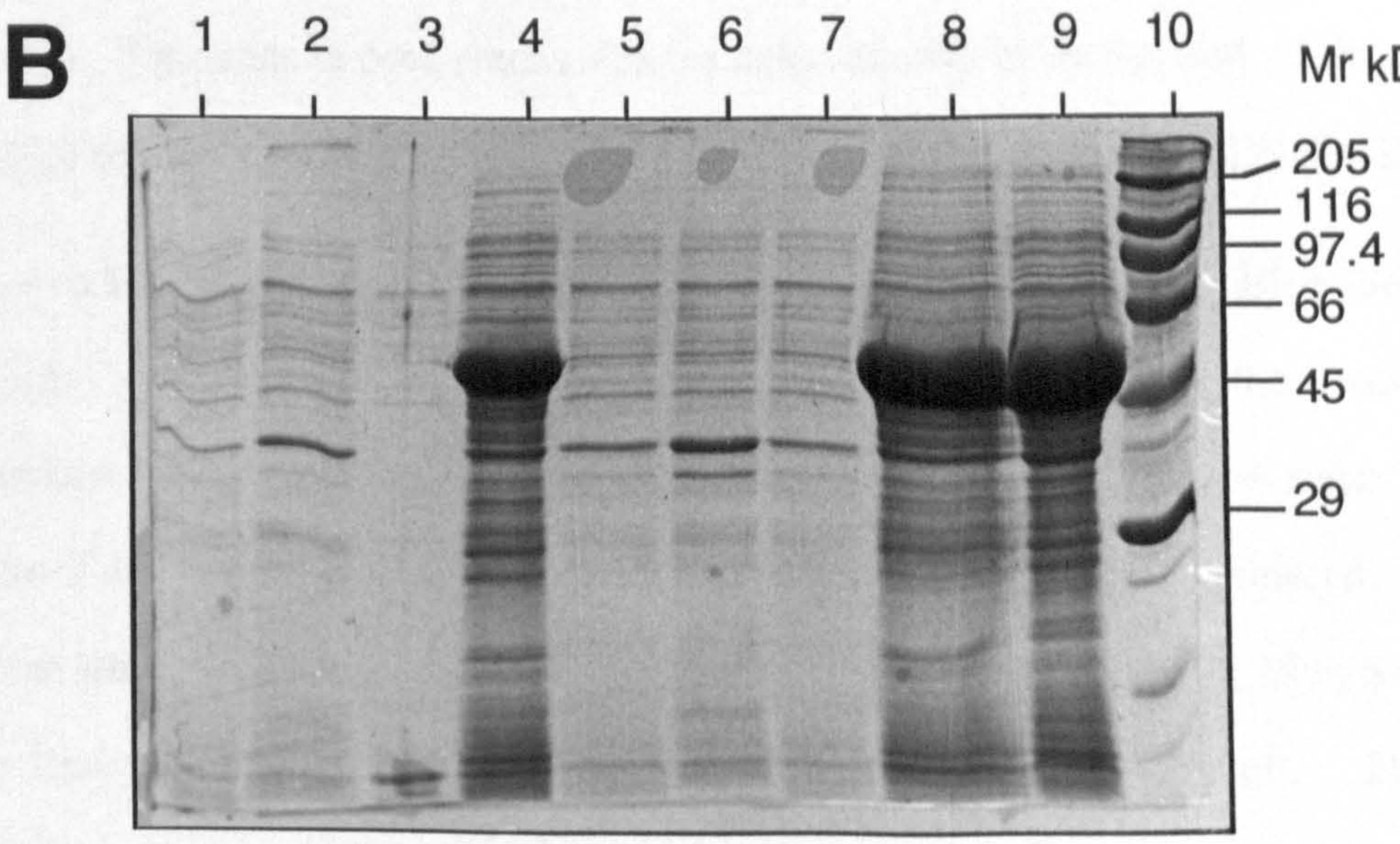
Inclusion bodies were isolated from *E.coli* BL21(λDE3) cells harbouring pETBC grown in 1L of terrific broth, by lysis with lysozyme and detergent and centrifuging at 12,000 × g for 15 minutes.

The protein pelleted by a 15 minute, 12,000 × g centrifugation after the following treatments; Lane 1, 50mM Tris-HCl pH8.0, 2mM EDTA; Lane 2, 0.5% Triton X100; Lane 3, 100mM $CaCl_2$; Lane 4, 0.1% SDS; Lane 5, 0.25% CHAPS; Lane 6, 0.5% Nonidet P-40; Lane 7, 0.1% Tween-20; Lane 9, 10μl of 10N NaOH. Lane 8 contained SDS-6H M_r markers, 2μg each protein.

Figure 6.13 B Soluble protein from above treatments not pelleted by a 15 minute, 12,000 × g centrifugation after treatment

The Lanes contain protein solubilised by the following treatments. Lane 1; 50mM Tris-HCl pH8.0, 2mM EDTA; Lane 2, 0.5% Triton X-100; Lane 3, 100mM $CaCl_2$; Lane 4, 0.1% SDS; Lane 5, 0.25% CHAPS; Lane 6, 0.5% Nonidet P-40; Lane 7, 0.1% Tween-20; Lane 8, 10μl of 10N NaOH; Lane 9, 1 × SDS sample buffer. Lane 10 contained SDS-6H M_r markers, 2μg each protein.


A
1 2 3 4 5 6 7 8 9
Mr kDa
205
116
97.4
66
45
29


B
1 2 3 4 5 6 7 8 9 10
Mr kDa
205
116
97.4
66
45
29

Although a high percentage of the solubilised inclusion bodies was BC protein, a significant amount was contaminant *E.coli* proteins and in order to use the expressed protein for antibodies or biochemical studies it would have to be purified. To purify the protein it would need to be in solution and, if chromatography was to be used, in a buffer of suitable pH with no ionic detergents. As SDS could not be used, the inclusion bodies were solubilised by re-suspension in 50mM Tris base, 2mM EDTA and stirring on ice for 10 minutes. This led to solubilisation due to the high pH. The pH was then brought down to pH8.0 by dialysis with stirring at 4°C against 3 × 2L of 50mM Tris-HCl pH8.0, 2mM EDTA over a period of 20 hours. Some precipitate was observed in the dialysis tubing when dialysis was complete but this was removed by centrifugation at 12,000 × g for 15 minutes. The clear supernatant was pH8.2. The supernatant containing solubilised BC inclusion bodies was filtered through a 0.22μm filter and applied to a 20ml Blue Sepharose Fast Flow at 2ml/min pre-equilibrated in 50mM Tris-HCl pH8.0, 2mM EDTA. The column was washed with 100ml of 50mM Tris-HCl pH8.0, 2mM EDTA until the Abs_{280} was near zero. Proteins were eluted from the column by a 200ml gradient of NaCl from 0M to 2M at 2ml/min. Fractions of 5ml were collected automatically from the start of the gradient and 20μl of every other fraction was analysed for protein content by SDS/PAGE.

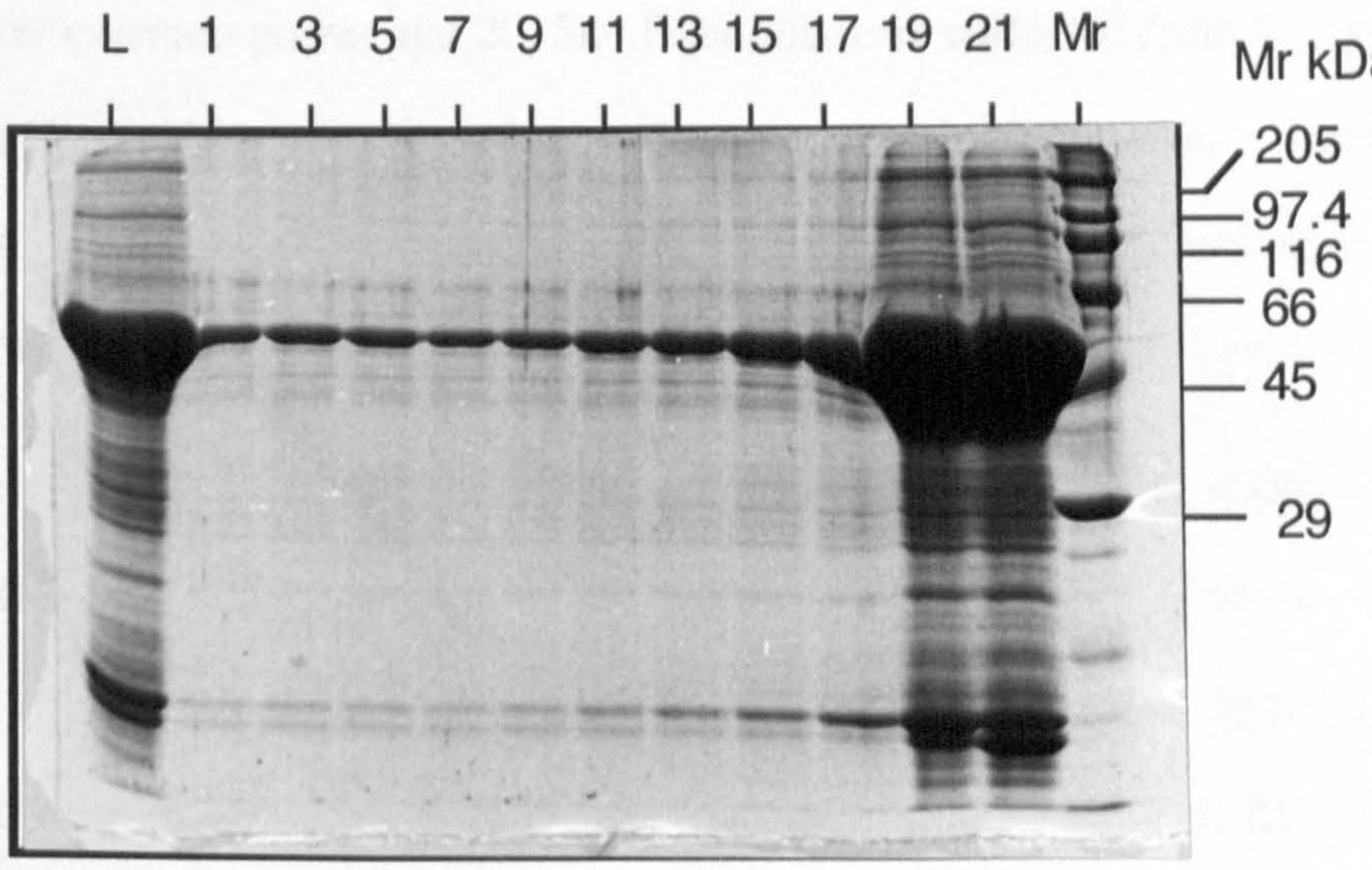
Figure 6.14 shows the protein content of the fractions from the Blue Sepharose column. There is no purification of the desired protein. Although the contaminants in the first fractions look minimal the original sample would give the same banding pattern if less protein was loaded. Several possibilities were considered. 1) the column was run incorrectly but repeated attempts to purify BC using Blue Sepharose were made under a variety of conditions but all gave the same result. 2) All the proteins in the sample bind to Blue Sepharose; if this were truly the case it would be expected that they would bind with different affinities and therefore be separated from each other when the binding was disrupted with NaCl. Also, HiLoad Mono-Q chromatography gave similar results, all proteins were eluted together by increasing concentrations of NaCl. 3) Under the buffering conditions, the proteins were not

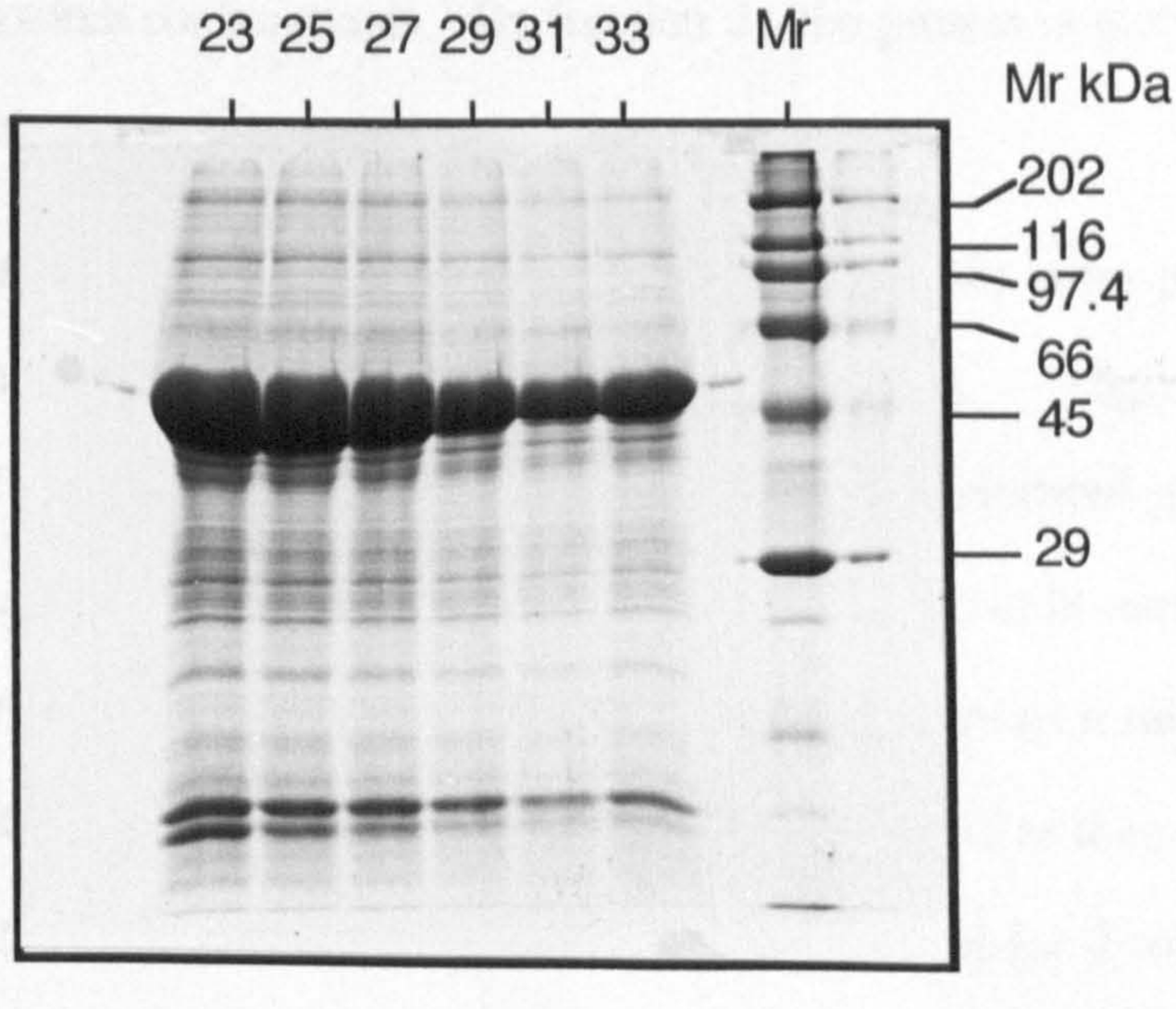
fully dissociated from each other and bound to the column and each other non-specifically.

Figure 6.14 Analysis of fractions from Blue Sepharose FF column eluted with a 0-2M NaCl gradient on 12% acrylamide gels.

Inclusion bodies isolated from *E.coli* BL21(λDE3) cells containing pETBC were solubilised by re-suspending in 50mM Tris base (no pH), 2mM EDTA. The pH was brought to pH 8 by dialysis with stirring at 4°C against 3 × 2L of 50mM Tris-HCl pH8.0, 2mM EDTA. Precipitated protein observed in the dialysis tubing was removed by centrifugation. The soluble protein was filtered through a 0.22μm filter and applied to a 20ml Blue Sepharose FF column. Proteins which did not bind were eluted in a 100ml wash with 50mM Tris-HCl pH8.0, 2mM EDTA. Protein which did bind was eluted by a gradient of NaCl from 0-2M.

20μl of every other fraction was run on a 12% acrylamide gel. The gels are illustrated opposite. Lanes contain; L, protein loaded onto the column; 1-33, Fractions eluted from the column; M_r SDS-6H M_r markers, 2μg each protein.


L 1 3 5 7 9 11 13 15 17 19 21 Mr
Mr kDa
205
97.4
116
66
45
29


23 25 27 29 31 33 Mr
Mr kDa
202
116
97.4
66
45
29

As a consequence of the difficulties in trying to purify the BC protein using conventional chromatography techniques BC inclusion bodies were solubilised in 1 × SDS sample buffer and the desired protein purified by continuous elution gel electrophoresis. An estimated 5mg of protein was boiled in 1 × SDS sample buffer, cooled, spun for 1 minute in a microfuge and loaded onto a 10cm 7% gel. The gel was run at 10W constant power and 20, 5ml fractions were collected from the bottom of the gel when the dye front reached the bottom at a rate of 0.5ml /min. Initial fractions contained the dye front (bromophenol blue) and were coloured accordingly. Fractions immediately after the dye front which did not contain blue dye were analysed.

Every third fraction was analysed for protein content by taking 100µl samples and precipitating the proteins with methanol / chloroform. Proteins were re-suspended in 20µl of 1 × SDS sample buffer and analysed on a 12% acrylamide gel. The analysis of fractions is shown in Figure 6.15 A. This shows the low M_r contaminating proteins in the initial fractions (1-9). The BC protein begins to elute in fraction 15 but still with some contaminants. By fraction 21 the protein is eluting as essentially pure BC protein.

The fractions containing purified BC (fractions 21-40) were pooled and the protein precipitated by methanol : chloroform precipitation. Organic solvent extraction removed the SDS from the protein sample. The precipitated protein was pooled, re-suspended in 500µl of sterile water and stored at -20°C until required. In this state the protein was not soluble but remained in small particles as a milky white suspension. To check the concentration of protein in this sample, 1µl of the purified isolated protein was diluted to 20µl with 1 × SDS sample buffer, boiled for 2 minutes and loaded onto a 12% gel for analysis of purity (Figure 6.15 B). This showed the BC protein to be essentially pure and of a high concentration (5-10µg/µl) by estimation with the protein standards. As the gel shows 1µl out of 500µl was purified this meant >2.5mg of pure BC protein had been obtained.

Figure 6.15 A Coomassie stained 12% gel of the fractions from preparative gel electrophoresis of BC inclusion bodies.

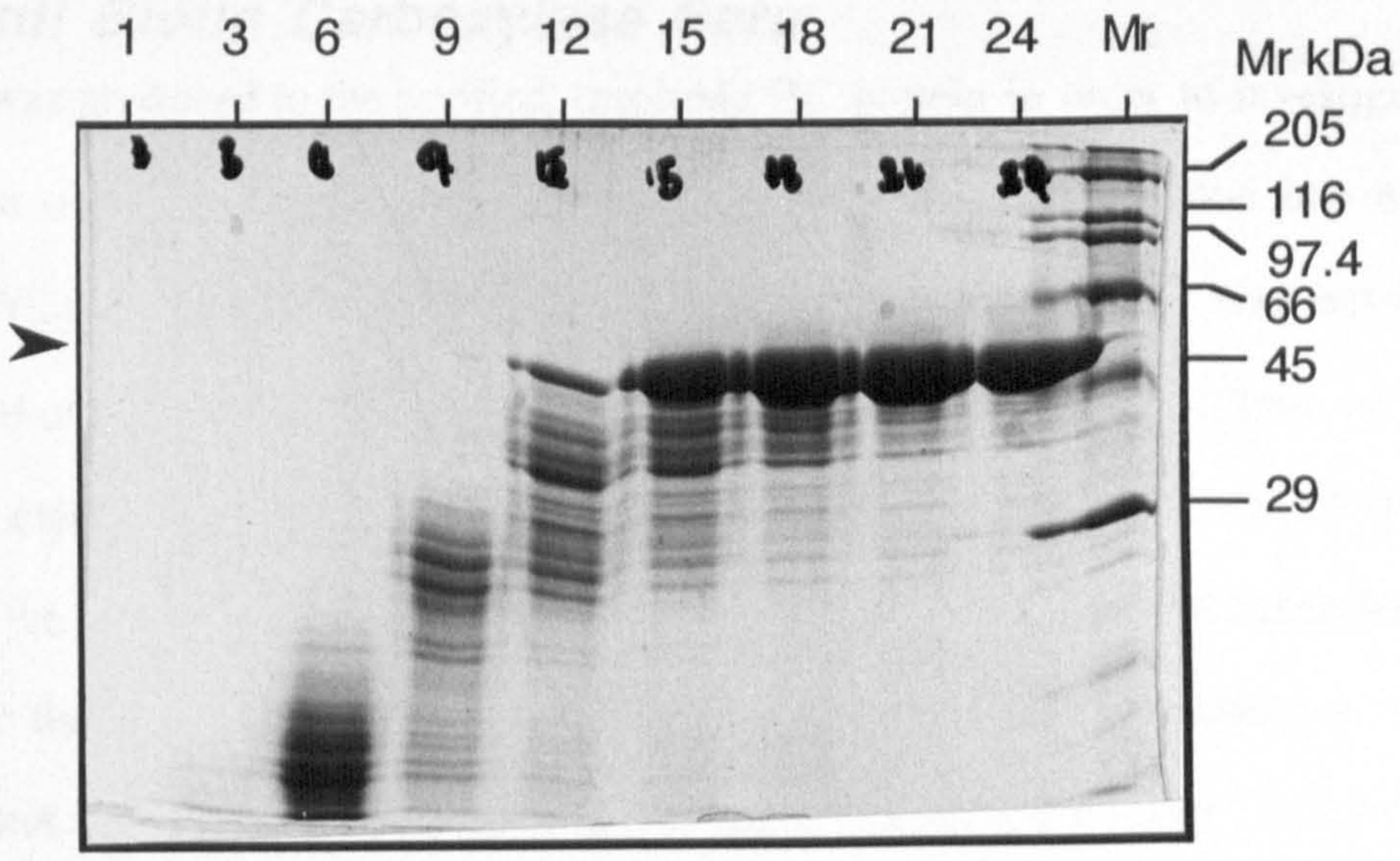
An estimated 5mg of BC protein was solubilised in 1 × SDS sample buffer and loaded onto a 10% preparative, 10cm, 7% acrylamide gel. Five millilitre fractions were collected in SDS running buffer at 1ml /min after the dye front reached the bottom of the gel.

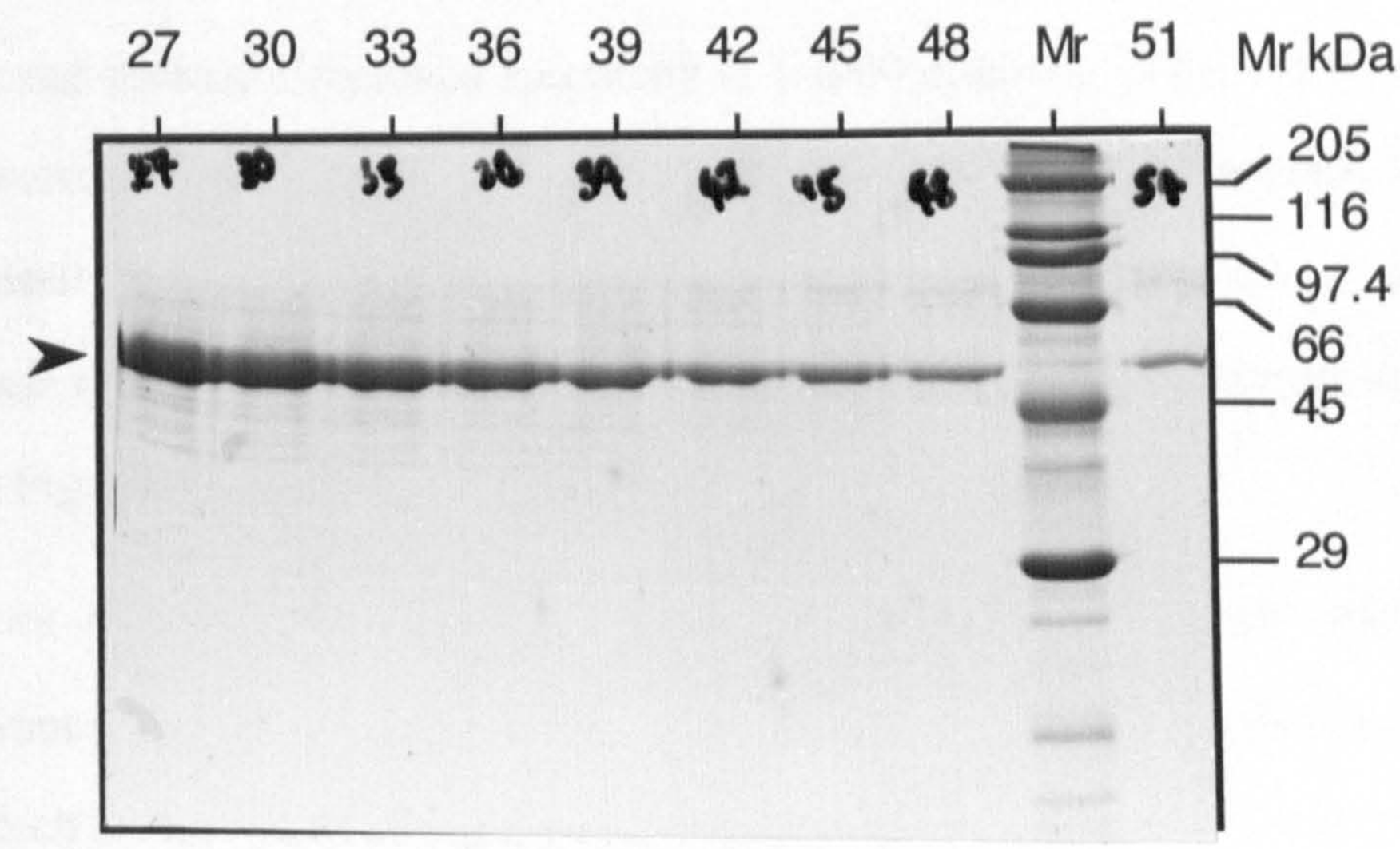
Every third fraction was analysed for protein content by SDS / PAGE on 12% gels. 100µl of each fraction was methanol / chloroform precipitated, re-suspended in 1 × SDS sample buffer and loaded onto a gel. The gels opposite show the fractions (1-51). Lanes marked M_r contain SDS-6H protein standards (SIGMA), 2µg each protein.

Figure 6.15 B Analysis of purified BC protein on 12% acrylamide gel.

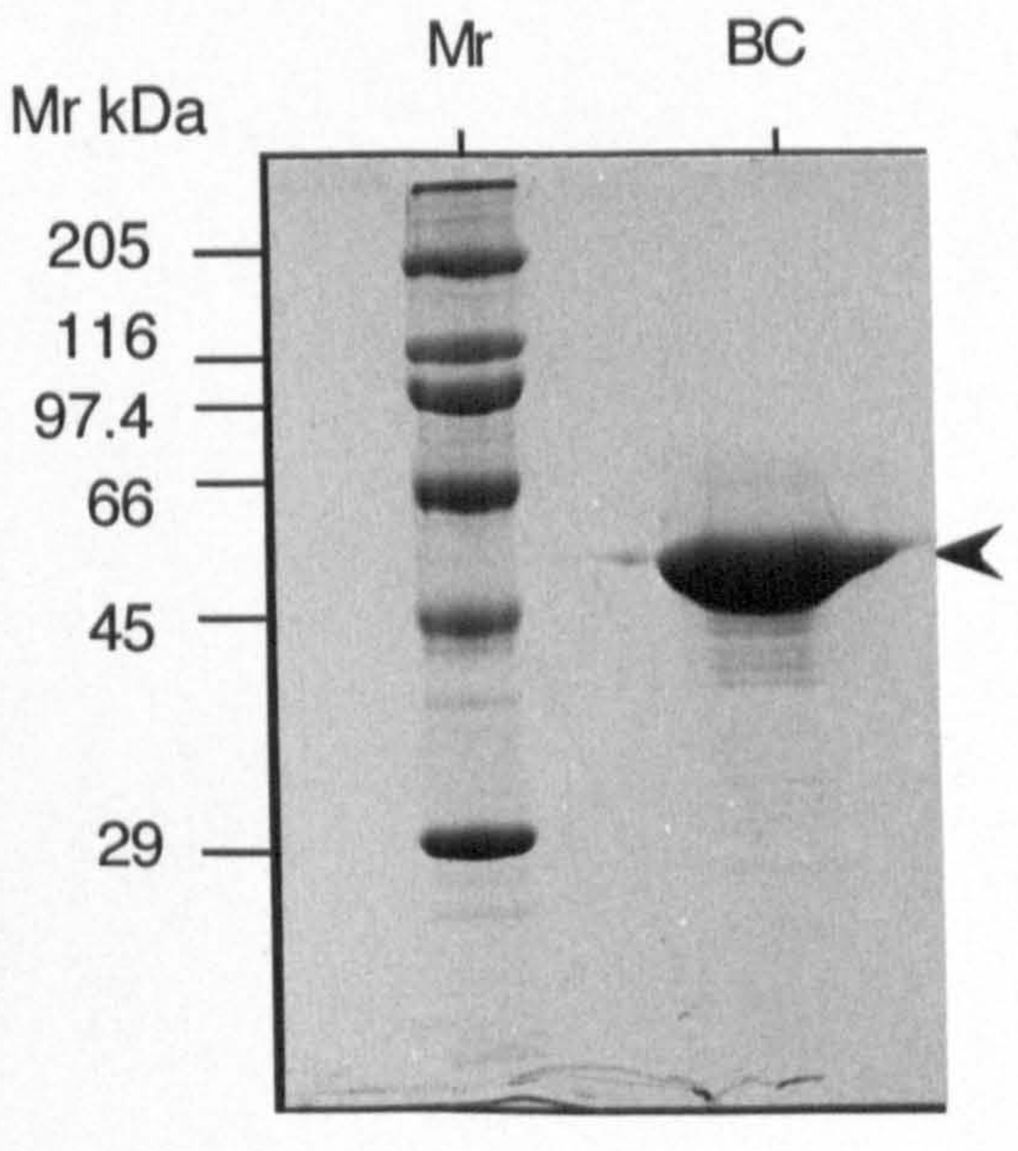
Fractions 21 - 30 were pooled and the protein precipitated by methanol / chloroform extraction. The precipitated protein was re-suspended in 500µl of ddH_2O, 1µl of which was solubilised in 1 × SDS sample buffer and run on a 12% gel (lane marked BC). The lane marked M_r contains SDS-6H protein standards, 1µg of each.

A


1 3 6 9 12 15 18 21 24 Mr
Mr kDa
205
116
97.4
66
45
29


27 30 33 36 39 42 45 48 Mr 51
Mr kDa
205
116
97.4
66
45
29

B


Mr
BC
Mr kDa
205
116
97.4
66
45
29

6.5 Anti Biotin Carboxylase Sera

Antisera was produced to the purified, insoluble BC protein in order to investigate the expression of this protein in *Brassica napus*. For the initial injection into a New Zealand White Rabbit, 500μg of BC protein in 1.2ml of sterile PBS was emulsified with 1.2ml of FCA. For booster injections, 200μg of BC protein in 1.2mls of sterile PBS was emulsified with 1.2mls of FIA. For the booster injection, 1ml of the FIA emulsion was given forty days after the initial injection. A test bleed taken fourteen days after the first booster showed a good reaction to pure BC protein at 1:1000 dilution (not shown) but the background was high. A second booster of 1ml of FIA emulsion was given 10 days after the test bleed. A second test bleed was taken 14 days later and showed a improved specificity at 1:2000 dilution. This test bleed was used to probe a Western blot of different tissues at different developmental stages. Immunoreactive proteins were detected with sheep anti-rabbit IgG : alkaline phosphatase conjugate and developed with BCIP / NBT. The developed image is shown in Figure 6.16.

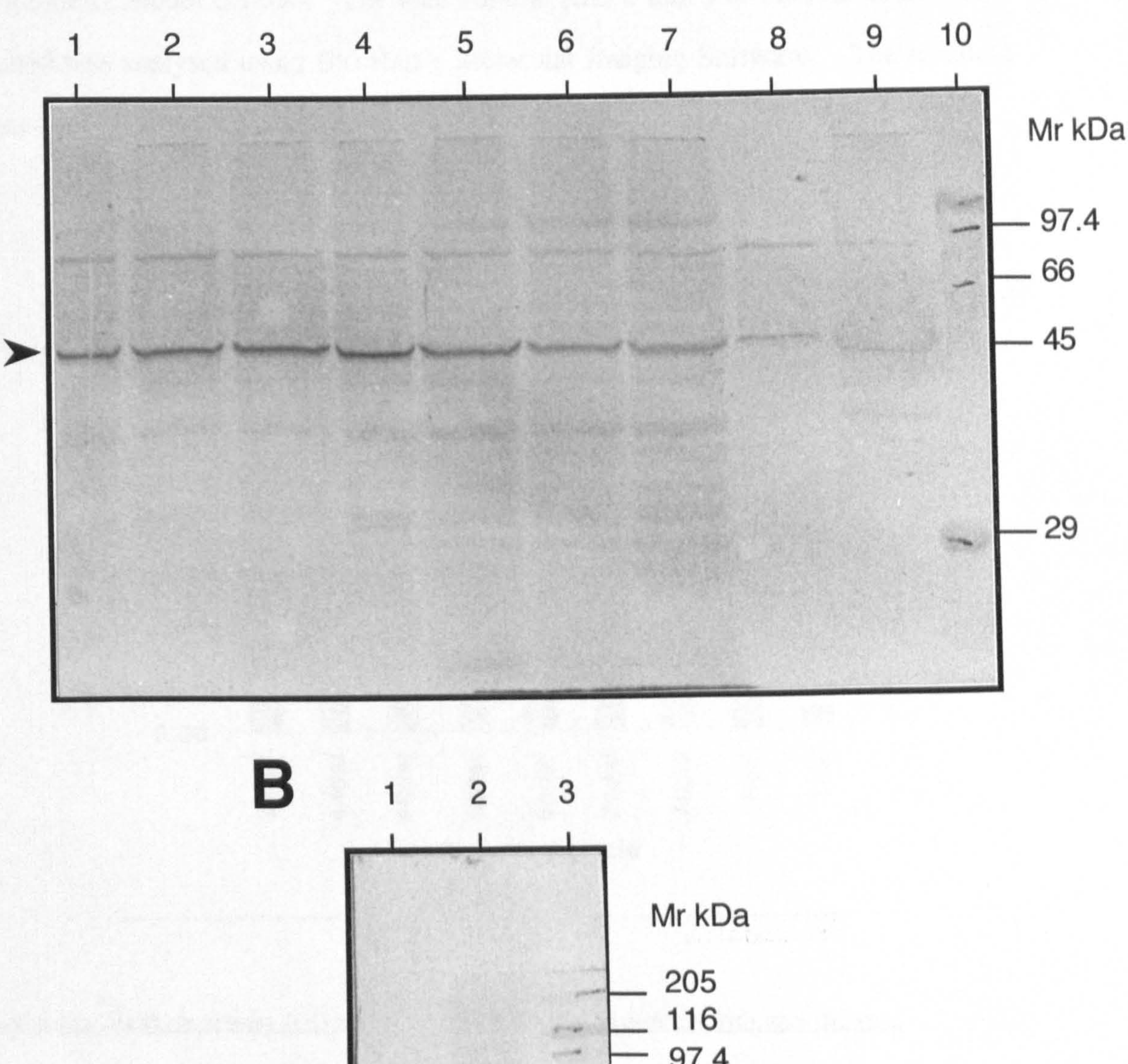
This shows the antisera detects a protein with a M_r of approximately 48,000 in embryo, root and leaf tissues. The signal is strongest in mid-stage embryos (lane 4, 49daf) which are known to be rapidly accumulating lipid.

Figure 6.16 A Western blot with anti-BC antisera of total protein extracts from *Brassica napus* leaf, root and developing seed material

Extracts were prepared as described in section 2.4.2. Samples were re-suspended in 1 × SDS sample buffer by boiling for 2 minutes and vortexing for 10 minutes. Equal loadings (25µl) were applied to each lane. The lanes contain total protein extracts from developing embryos, lanes 1-7; root, lane 8; leaf lane 9; and M_r markers lane 10. The developing embryo extracts were from embryos 38, 44, 47, 51, 56, 61 and 70daf. Samples were electrophoresed on a 12% acrylamide gel and transferred to nitrocellulose membrane. Proteins were detected with 1:1000 dilution of the rabbit anti-BC sera. The secondary antibody was sheep anti-rabbit : alkaline phosphatase conjugate and the image was developed with BCIP/NBT.

Figure 6.16 B Western blot with pre-immune serum from the rabbit used for BC antibodies.

A bleed was taken from the rabbit 14 days before the initial injection. A total protein extract prepared as described in section 2.4.2 from embryos 49daf (Lane 2) was run on a 12% gel with SDS-6H M_r markers (Lane 3). Lane 1 contains 0.1 µl of the purified BC protein shown in Figure 6.15 B. Proteins were transferred to nitrocellulose and detected with a 1:500 dilution of the sera. The secondary antibody was sheep anti-rabbit : alkaline phosphatase conjugate and the image was developed with BCIP/NBT.


A
1
2
3
4
5
6
7
8
9
10
Mr kDa
97.4
66
45
29
B
1
2
3
Mr kDa
205
116
97.4
66
45
29

6.5.1 Densitometric analysis of BC expression

To assess differential levels of expression of BC throughout embryogenesis and in leaf and root tissues, the Western is Figure 6.16 A was scanned with a BIO-RAD Imaging Densitometer Model GS-690. The total volume (OD × mm^2) of the individual bands obtained was analysed using BIO-RAD'S Molecular Imaging Software. The resulting values have been displayed graphically in Figure 6.17.

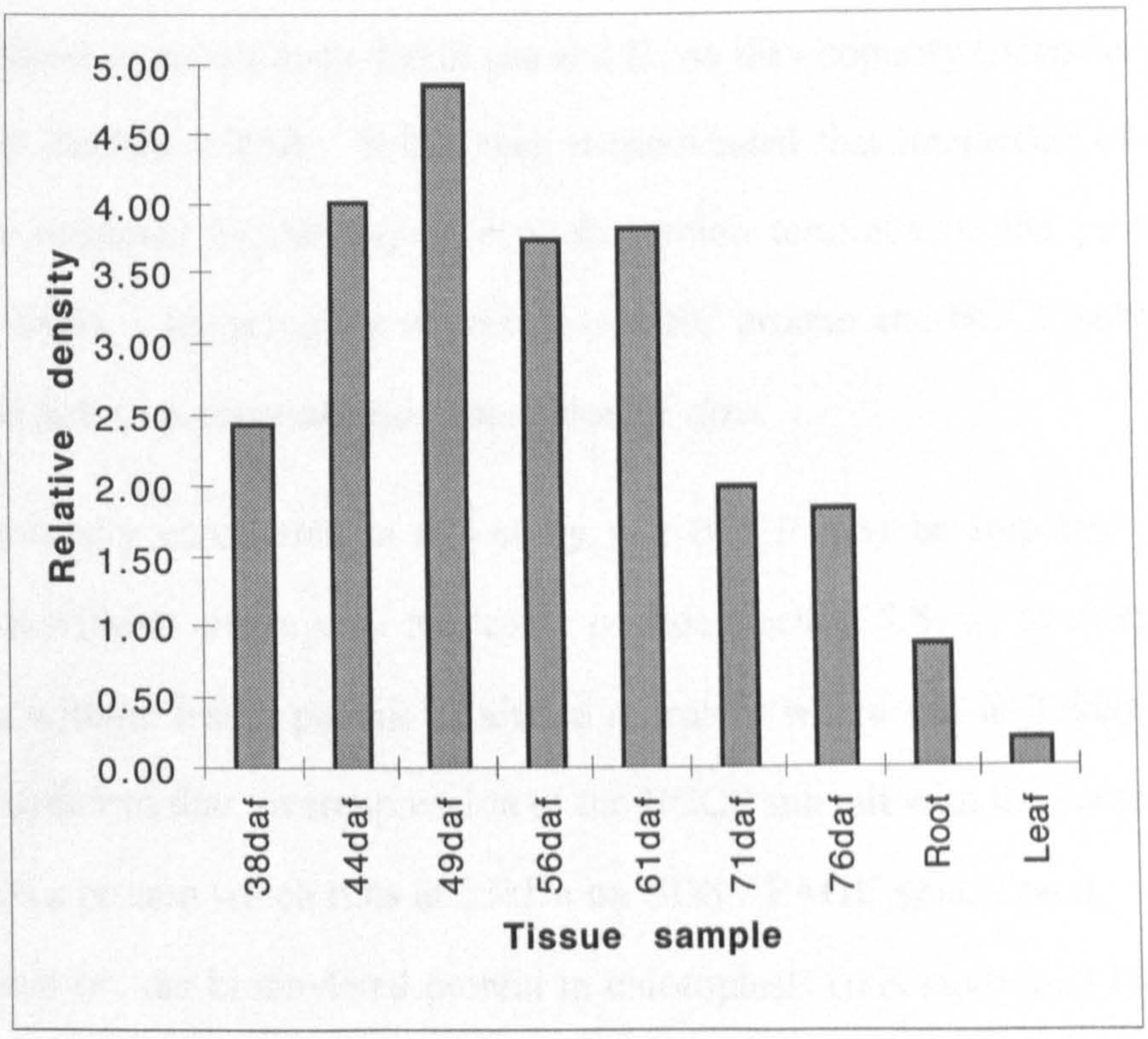


Figure 6.17 Relative density (OD × mm^2) of the 46kDa band detected with anti-BC sera.

The Western and the densitometric analysis show that BC protein levels are altered to a significant extent during embryogenesis. There was a 2.7 fold difference between the maximum and minimum levels observed in embryo (49 and 76daf respectively), a 5.5 fold difference between the highest levels observed in embryo and the level observed in root and a 22 fold difference between the levels observed in 49daf embryo and leaf. These figures are consistent with a role for the biotin carboxylase subunit of Type II acetyl-CoA carboxylase in embryogenesis, presumably as part of the enzyme complex to generate malonyl-CoA for lipid biosynthesis.

6.6 Biotin Carboxylase as a subunit of Acetyl-CoA carboxylase

The biotin carboxylase subunit of Type II acetyl-CoA carboxylase can be assayed independently of the other subunits as it is able to carboxylate free biotin (Guchhait *et al.*, 1974). It can also be assayed by its ability to carboxylate the biotin cofactor attached to biotin carboxyl carrier protein (Alberts & Vagelos, 1972). This indicates that the two subunits interact and the interaction between biotin carboxylase and biotin carboxyl carrier protein is such that in pea and *E.coli* they copurify (Alban *et al.*, 1995; Nenortas & Beckett, 1996). It has been demonstrated that interaction of these two subunits is mediated by the sequence at the amino terminus of the protein (Li & Cronan, 1992a). By using the overexpressed BC protein and BCCP subunit it was hoped to be able to reconstitute this interaction *in vitro*.

It was previously considered in this study that BCCP may be imported into intact chloroplasts without cleavage of the leader peptide (section 5.8) as overexpression of the protein without leader peptide produced a protein which ran at 30kDa on SDS / PAGE. Further to that, overexpression of the BCCP subunit with the leader sequence intact yields a protein which runs at 35kDa on SDS / PAGE gels, exactly the same as that observed for the biotinylated protein in chloroplasts (this study and Elborough *et al.*, 1996). This implied that if the amino terminus facilitates the interaction of BCCP and BC then the leader peptide may be of importance in this interaction.

BCCP and BC proteins have both been purified from *E.coli* by preparative gel electrophoresis. Whilst this gave protein which was suitable for the raising of antibodies, it would be no use for biochemical characterisation. To study the interaction of BC and BCCP two soluble proteins in their native conformations would be required.

6.6.1 *In vitro* transcription and translation of BC and BCCP.

By using *in vitro* transcription and translation, it was hoped to produce radiolabelled proteins which would be soluble and could be visualised by native gel electrophoresis and exposure to a high sensitivity phosphor imaging screen. Interacting proteins ought to produce a protein band with a higher Rf value than the single subunits alone.

Transcription and translation was done using the TNT® Coupled Reticulocyte Lysate System from Promega. This system is recommended by the manufacturers for expression of multiple proteins for the studying of protein-protein interactions. Proteins were labelled using L-[U-^{14}C]Leucine, >300mCi/mmol and 50μCi/ml at a final concentration in the reaction of 5μCi/ml. The reaction system contained, 1μg of total plasmid DNA (where two plasmids were used, 0.5μg of each plasmid DNA was used per reaction), 50% TNT® Rabbit Reticulocyte Lysate, 0.02mM all amino acids minus Leucine, 2μl of TNT® Reaction Buffer, 1μl of TNT® T7 RNA Polymerase, 40U pancreatic RNase inhibitor (PHARMACIA) and 5μl of L-[U-^{14}C]Leucine in a final volume of 50μl. The reaction was incubated for 90 minutes at 30°C. Eight reactions were carried out containing different DNA templates; 1) pETBP4, 2) pETBP+L, 3) pETBC, 4) pETBC + pETBP4, 5) pETBC + pETBP+L, 6) pp2A, 7) pET11a, 8) Luciferase control from PROMEGA. All the reactions contained 1μg of DNA in total. pp2A is a subunit of a protein phosphatase which Dr. Marta Evans, Department of Biological Sciences, University of Durham was attempting to express from pET11d. The luciferase positive control was provided by PROMEGA and encoded a protein 61kDa in size.

Proteins synthesised and labelled by the TNT® Coupled Reticulocyte Lysate System were analysed by native electrophoresis on 5-20% acrylamide gels. A 20μl sample from the labelling reaction was made 1 × with native PAGE sample buffer and loaded onto the stacker. The gel was run at 20mA per gel and the dye front was allowed to elute from the gel carrying with it the unincorporated label. The gel was stained with Coomassie R-250 and dried in cellophane to preserve it. The dried gel was exposed

to a high sensitivity phosphor imaging screen overnight and scanned at 200μm the following morning. A picture of the stained gel is shown in Figure 6.18 A and the developed image from the phosphor imaging screen in Figure 6.18 B.

These gels show that the BC protein is expressed and radiolabelled as a soluble protein by the *in vitro* expression kit (Figure 6.18 B, lane 3). The BCCP protein with leader sequence was also expressed by this system (Figure 6.18 B, lane 2). The BCCP protein without leader sequence was not expressed (Figure 6.18 B, lane 1). The experiment was repeated twice, and on both occasions the faintest of bands was observed in the reaction co-expressing the biotin carboxylase and biotin carboxyl carrier protein with leader sequence with a high Rf value. This high Rf band was not seen in lanes expressing the biotin carboxylase or the biotin carboxyl carrier protein with leader sequence alone. This suggested that possible there was an interaction between the two subunits that could be observed by native gel electrophoresis. Neither the anti-BC or anti-BCCP antibody were available at the time these experiments were carried out so the proteins could not be transferred to nitrocellulose and identified with antibodies. An anti-biotin antibody could not be used either because the system would not have biotinylated the BCCP protein.

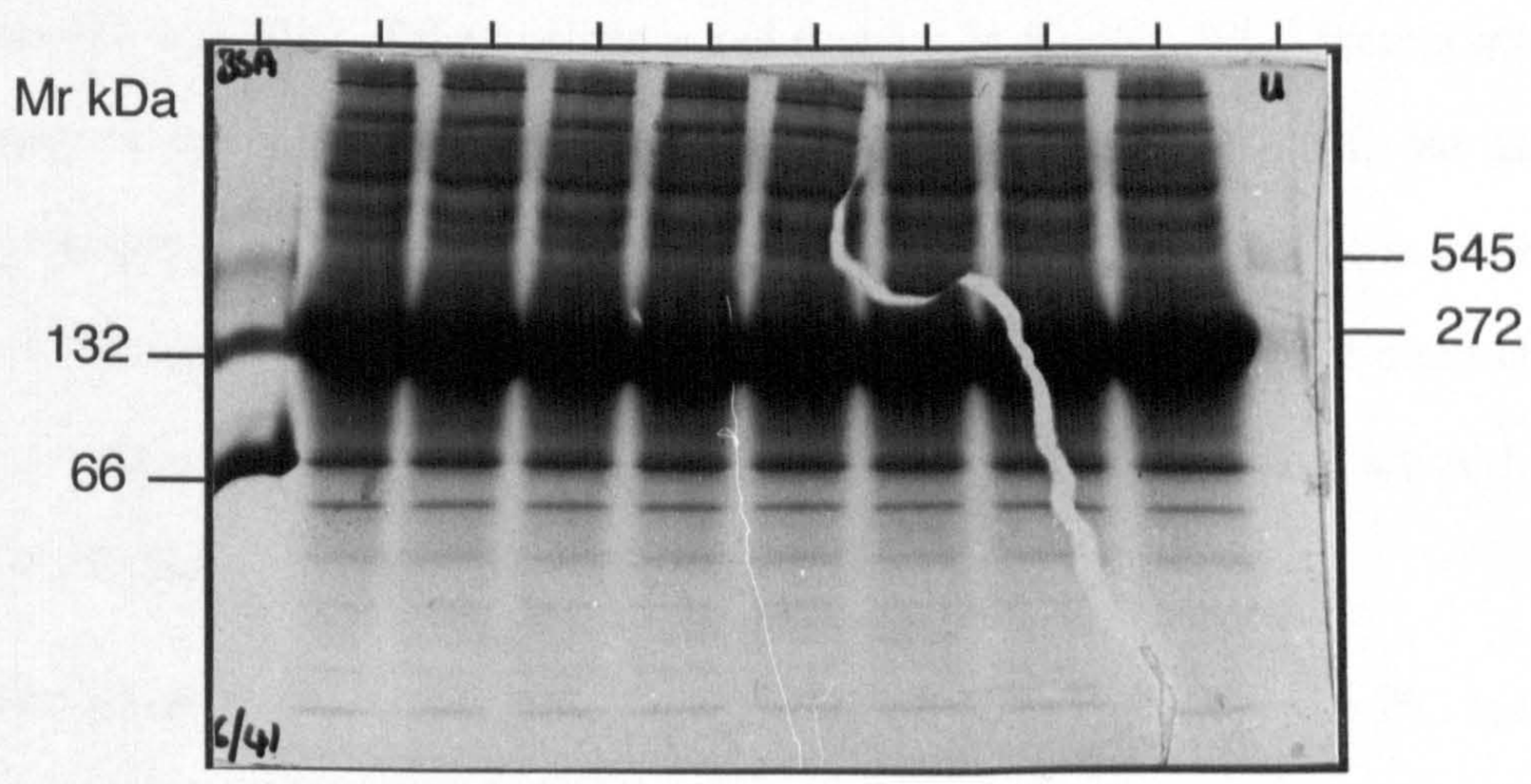
Figure 6.18 A Coomassie stained 5-20% native gel of the proteins produced by the TNT® Coupled Reticulocyte Lysate System.

Products from the following plasmid vectors labelled with [U-^{14}C]-leucine were run on a 5-20% native gel; Lane 1, pETBP4; Lane 2, pETBP+L; Lane 3, pETBC; Lane 4, pETBC and pETBP4; Lane 5, pETBC and pETBP+L; Lane 6, pp2A; Lane 7, pET11a; Lane 8, Luciferase control, 61kDa. Other lanes contain; BSA, bovine serum albumin, 5μg total protein; U, urease, 200ng total protein.

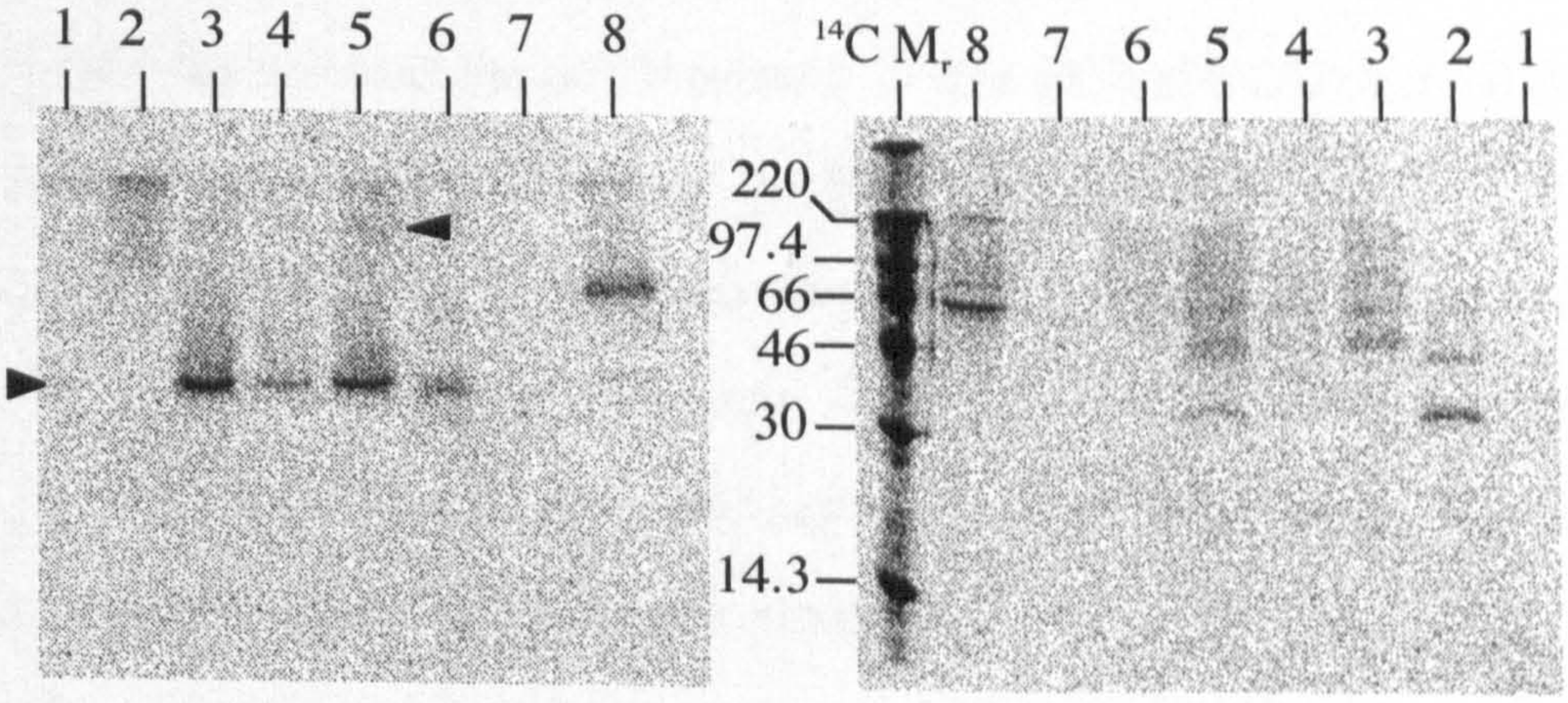
Figure 6.18 B Labelled proteins produced by the TNT® Coupled Reticulocyte Lysate System detected on a high sensitivity phosphor imaging screen.

The gel above was exposed to a High Sensitivity Phosphor Imaging screen and scanned at 100μm (left). A denaturing gel of the same samples was also run and exposed to the screen (right). Lanes contain the same as for A. Lane ^{14}C M_r, contains ^{14}C-methylated protein standards (AMERSHAM). The lower arrow marks the position of the BC subunit on the native gel. The upper arrow located on the picture marks the faint high Rf band.

A


BSA 1 2 3 4 5 6 7 8 U
Mr kDa
132
66
545
272
BSA
U
C/41

B


1 2 3 4 5 6 7 8
14C Mr 8 7 6 5 4 3 2 1
220
97.4
66
46
30
14.3

6.6.2 Improving solubility of protein during expression in *E.coli*

In vitro expression of the BC and BCCP subunits using the TNT® Coupled Reticulocyte Lysate System provided tentative evidence that the two subunits may interact, but due to the very low amounts of protein expressed in this system it was difficult to draw conclusive results

In order to try and obtain more protein for study, several attempts were made to improve the solubility of the overexpressed proteins in *E.coli*. What factors affect the solubility of protein when expressed in *E.coli* are currently unknown, but as every individual protein is unique, predicting whether a certain protein will be soluble under one set of conditions versus another is not possible. Therefore, several methods were tried to improve the solubility of the overexpressed *B.napus* biotin carboxyl carrier protein and biotin carboxylase proteins in *E.coli*.

Overexpression of the *E.coli* BCCP in *E.coli* required expression of the BC subunit at the same time for efficient translation (Li & Cronan, 1992a; Nenortas & Beckett 1996). This was probably because the two genes in *E.coli* were co-transcribed on the same mRNA and therefore presumably co-translated in stochiometric quantities (Li & Cronan, 1992a). This suggested that the biotin carboxylase subunit may in some way stabilise the expression of the BCCP subunit. When BC and BCCP from *E.coli* were overexpressed in *E.coli* purification of the BCCP subunit was made difficult by the strong binding of the BC subunit (Nenortas & Beckett, 1996). This association was disrupted by 2M urea. It seemed likely that efficient overexpression of the biotin carboxyl carrier protein and biotin carboxylase from *B.napus* in *E.coli* could also depend upon the two subunits being co-expressed.

There were two ways of creating a system for co-expression of the two subunits. One way would be to create a gene fusion between the BCCP and BC cDNAs in a pET vector. This would encode an mRNA similar to the native *E.coli* BCCP and BC mRNA with a 10bp gap between the end of BCCP and the start of BC with the sequence TGA CGAGGCGAAC ATG, the box representing the ribosome binding

site, the underlined TGA the stop of BCCP and the underlined ATG the start of BC. The 10 base pair insert is the sequence found between the *E.coli* BCCP and BC. Creation of such a gene fusion was considered but the cloning procedure would be lengthy and difficult with no guaranteed expression of the BC subunit at the end. The alternative method to overexpress both subunits was to use two plasmids with different antibiotic resistance.

pET24a(+) was used as this vector has exactly the same properties as pET11a(+) used previously except that it carrier the kanamycin resistance gene. The inserts from pETBP4 and pETBP+L were cut from plasmid preparations of these vectors using Nde I and Bam HI. pET24a was also digested with Nde I and Bam HI. The inserts and the vector were isolated and the new vectors pET24BP4 and pET24BPL4 were created by ligation of the inserts from pETBP4 and pETBP+L into the Nde I / Bam HI digested respectively. The ligations were transformed into *E.coli* DH5α and plated onto LB + 30μg/ml kanamycin. Transformants were grown at 37°C overnight and bacteria which grew were used to inoculate 10ml LB + 30μg/ml kanamycin cultures for preparation of plasmid DNA. The prepared plasmid DNA was analysed by restriction digestion with Nde I and Bam HI which showed that all transformants contained the vector plus relevant insert.

Approximately equal amounts of plasmid pETBC and pET24BP4 or pET24BPL4 were used for co-transformation of *E.coli* BL21(λDE3) cells which were plated on LB + 50μg/ml ampicillin, LB + 20μg/ml kanamycin and LB + ampicillin and kanamycin. The amount of plasmid used affected the ratio of colonies on LBamp : LBKAN. In extreme cases colonies would only grow on LBamp or LBKAN suggesting that too much of one plasmid was being used. By varying the amount of each plasmid used, it was possible to obtain an approximately equal number (within a factor of 2) of both LBamp and LBKAN and this gave the highest yield of transformants on LBamp/KAN. These were used to create seed stocks as they were assumed to have been equally transformed by the two plasmids.

Fifty millilitre cultures of LB containing the appropriate antibiotics were grown from a single seed stock and expression was induced with 1mM IPTG at mid log phase. Figure 6.19 A shows the inclusion bodies isolated from these cultures containing the different plasmids. No soluble proteins could be detected by Coomassie staining of the soluble protein when separated on a 12% gel (data not shown).

Figure 6.19 B shows the effect of growing the bacteria in 50ml LB (containing 10% of normal NaCl) culture containing the appropriate antibiotics + 2.5mM Betaine and 1000mM sorbitol. This growth media has been reported as able to produce highly expressed recombinant protein in an active form (Blackwell & Horgan, 1991). Betaine is a 'compatible solute' which is taken up in response to low water potential. The effect of betaine is to cause thermodynamically unfavourable 'preferential hydration' which supposedly minimises protein-solvent contact and aids in stabilisation of protein structure. Expression of biotin carboxylase and biotin carboxyl carrier protein under these conditions did not seem to affect the solubility of the protein product. Inclusion bodies were still formed as in Figure 6.19 A and the soluble fraction shown in Figure 6.19 B contained no detectable overexpressed protein by Coomassie staining and comparison with control lanes.

Figure 6.20 shows a Western blot of the soluble fractions from the cells grown in the presence of 2.5mM betaine and 1000mM sorbitol with anti-biotin antibodies. The 35kDa band and 30kDa band produced by the pETBP+L and pETBP4 vectors respectively were just visible but the levels of these proteins were too low to consider purification.

Figure 6.19 A SDS / PAGE of inclusion bodies from *E.coli* BL21(λDE3) cells expressing subunits of Type II acetyl-CoA carboxylase.

Cultures of *E.coli* BL21(λDE3) cells containing pET vectors overexpressing subunits of Type II ACCase, were grown to mid-log phase and induced with 1mM IPTG for 3 hours. Inclusion bodies were isolated from the induced cells and analysed on a 12% acrylamide gel.

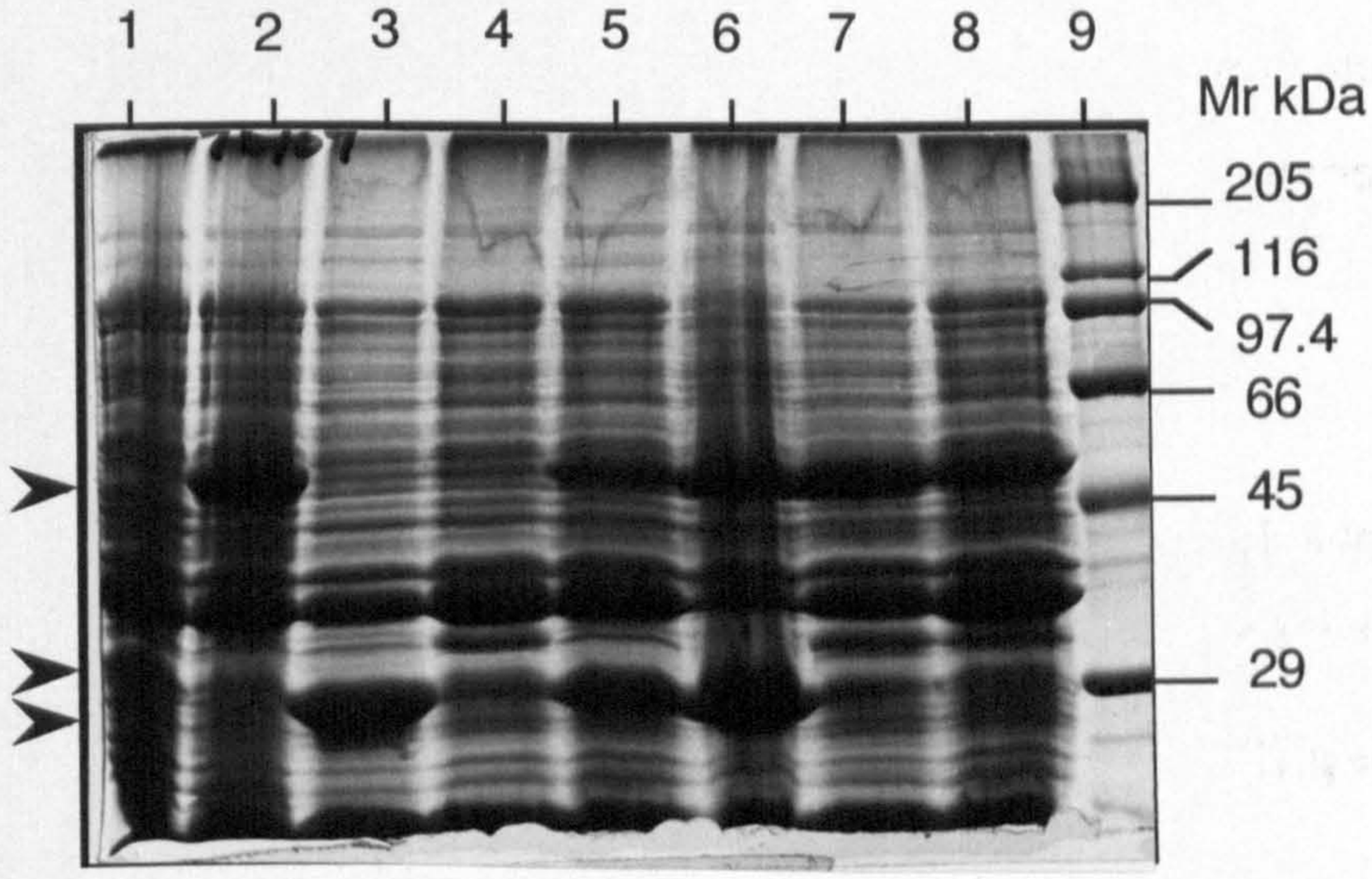
Lanes contain inclusion bodies isolated from *E.coli* BL21(DE3) cell containing the following plasmids; Lane 1, pET11a; Lane 2, pETBC; Lane 3, pETBP4; Lane 4, pETBP+L; Lanes 5; pETBC + pET24BP4; Lane 6 pETBC + pET24BP4 grown at 30°C; Lane 7, pETBC + pET24BPL4; Lane 8; pETBC + pET24BPL4 grown at 30°C. Lane 9 contains SDS-6h protein standards, 2μg each protein. Arrows mark the position of the overexpressed proteins.

Figure 6.19 B SDS / PAGE of purified soluble protein from *E.coli* BL21(λDE3) cells expressing subunit of Type II acetyl-CoA carboxylase grown with 2.5mM betaine.

Cultures were grown in the same way as for A except the LB contained 10% of the normal NaCl, 2.5mM betaine and 1000mM sorbitol

Lane 1 contains SDS-6h protein standards, 2μg each protein. Other lanes show soluble proteins from *E.coli* BL21(λDE3) cells containing the following plasmids; Lane 2, pETBC + pET24BPL4 grown at 30°C; Lane 3 pETBC + pET24BPL4; Lane 4, pETBC + pET24BP4 grown at 30°C; Lane 5, pETBC + pET24BP4, Lane 6, pETBP+L; Lane 7, pETBP4; Lane 8, pETBC; Lane 9 pET11a; Lane 10 no plasmid.

A


1 2 3 4 5 6 7 8 9
Mr kDa
205
116
97.4
66
45
29

B

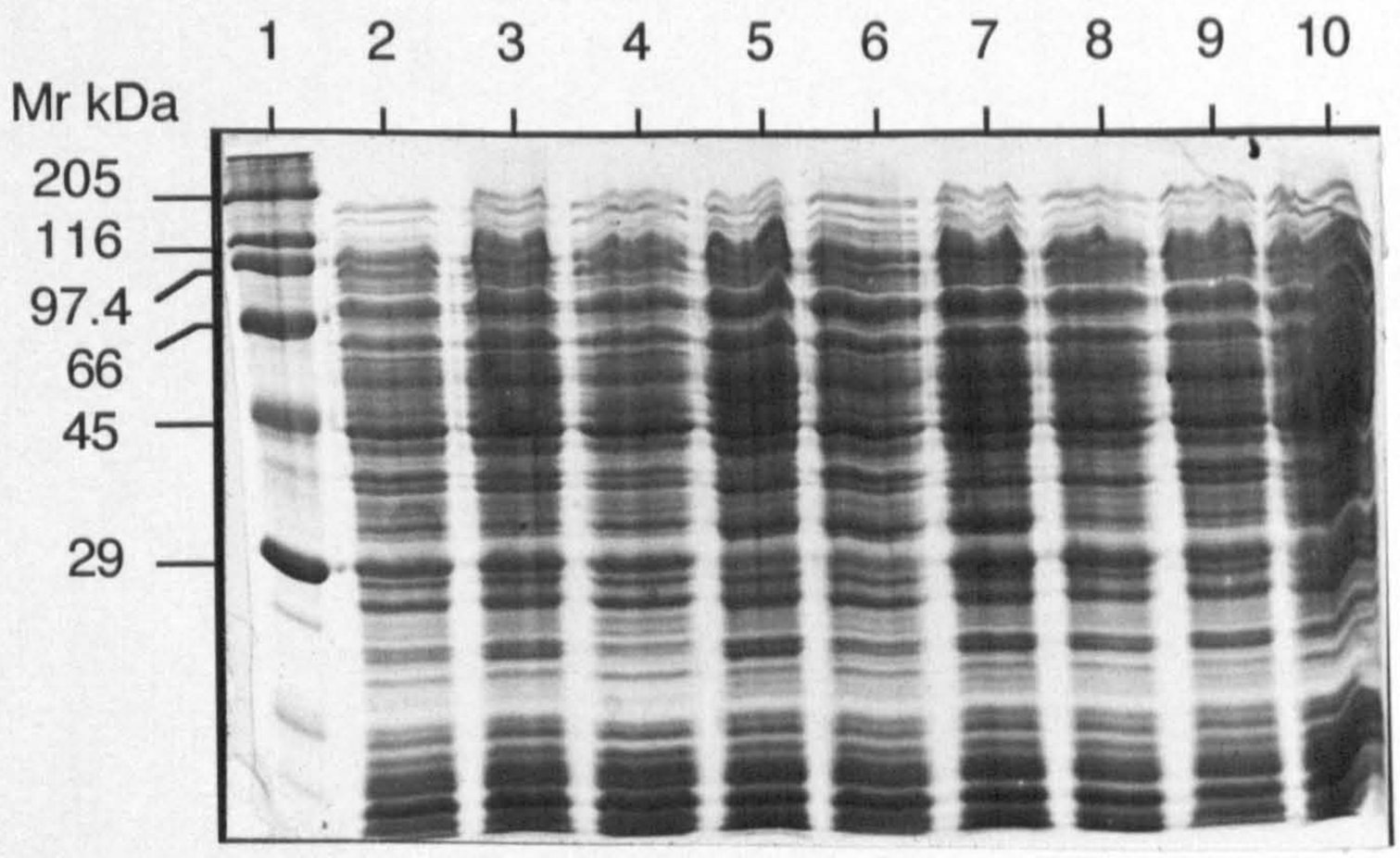
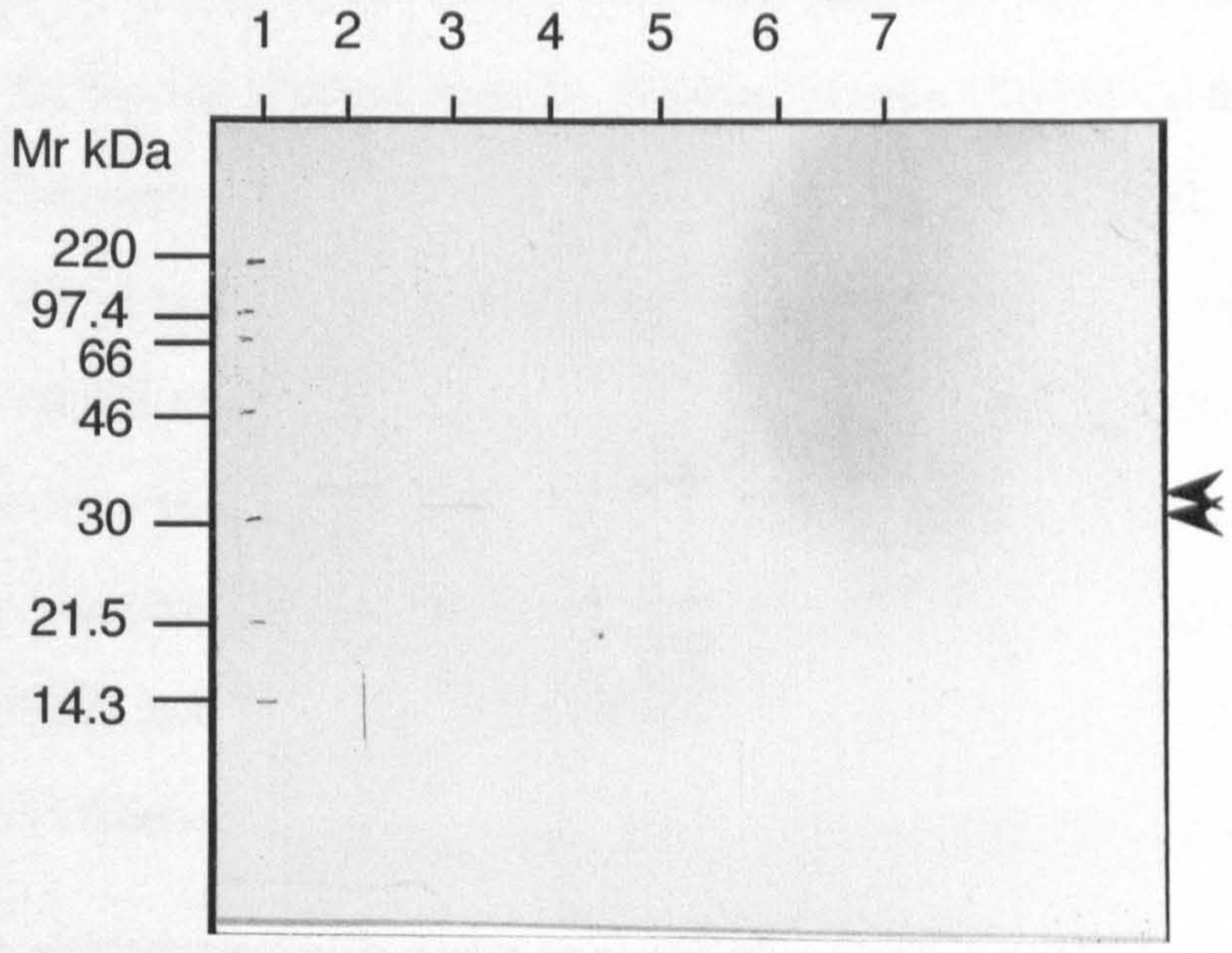

1 2 3 4 5 6 7 8 9 10
Mr kDa
205
116
97.4
66
45
29

Figure 6.20 Anti-biotin Western blot of the soluble proteins expressed by *E.coli* BL21(λDE3) cells harbouring plasmids overexpressing BCCP and BC protein in the presence of 2.5mM betaine and 1000mM sorbitol.

The soluble proteins analysed in Figure 6.19 B were subjected to Western blotting with anti-biotin antibodies. Proteins were separated on a 12% SDS / PAGE gel and transferred to nitrocellulose. The membrane was probed with affinity purified anti-biotin antibodies and donkey anti-sheep IgG : alkaline phosphatase conjugate. The image was developed with BCIP / NBT.

Lanes show the following soluble biotin-containing proteins from *E.coli* BL21(λDE3) cells containing the following plasmids; Lane 2, pET24BPL4 + pETBC; Lane 3, pET24BP4 + pETBC; Lane 4, pETBC; Lane 5, pETBP+L; Lane 6, pETBP4; Lane 7, pET11a. Lane 1 contains Rainbow markers, 2μg each protein. The arrows mark the barely detectable, overexpressed BCCP proteins.


1 2 3 4 5 6 7
Mr kDa
220
97.4
66
46
30
21.5
14.3

6.6.3 GroESL Molecular chaperones to increase soluble protein

As growth in the presence of betaine and growth at 30°C had failed to increase the amount of soluble protein, a different approach was required to try and produce soluble proteins. Chaperonins are abundant, indispensable proteins that facilitate protein folding in an ATP-dependent manner enhancing the yield of properly folded substrate protein under conditions where spontaneous folding does not occur (Todd *et al.*, 1994). As inclusion bodies consist essentially of denatured protein (Hartley & Kane, 1988) the use of the *E.coli* chaperonins GroES and GroEL may aid in the expression of proteins in their native, correctly folded and presumably soluble state.

An expression plasmid pT7groE, for the GroE molecular chaperones under the control of the T7 promoter was obtained from Dr. George Lorimer, DuPont Co Inc., Centre of Research & Development, Division of Molecular Biology, POB 80402, Wilmington, DE 19880. This plasmid was capable of expressing the groE operon, which contains the genes for groES and groEL, to a high level as soluble protein in cells containing a chromosomal copy of T7 polymerase (Kalbach & Gatenby, 1993). GroEL is made up of 57kDa subunits and binds to non-native proteins. GroES consists of 10kDa subunits and binds to GroEL when is has bound a non-native protein. Together, they facilitate the ATP dependent refolding of non-native proteins (Schmidt *et al.*, 1994).

pT7groE was a chloramphenicol resistant plasmid so could be used in conjunction with the BC and BCCP overexpressing plasmids pETBC, pET24BP4 and pET24BPL4. *E.coli* BL21(λDE3) cells were transformed with pT7groE and plated onto LB + 50μg/ml chloramphenicol (cm). Bacteria which grew on LBcm were made competent using $CaCl_2$. These bacteria were co-transformed with pETBC and pET24BP4 of pET24BPL4 (section 6.6.2). Bacteria from the co-transformation were plated onto LBamp/KAN/cm and those bacteria which grew at 37°C overnight were made into seed stocks.

A single seed stock was used to inoculate 50ml LB containing the appropriate antibiotics and the cultures were grown at 37°C until OD_{600} was 1.0 when the cultures

were made 1mM IPTG. Three hours later the cells were harvested and fractionated into soluble and insoluble parts.

The soluble and insoluble fractions were run on a 12% acrylamide gel as shown in Figure 6.21. The expression of groESL in the soluble phase could clearly be seen and it was also possible to see BC protein in the soluble phase (Figure 6.21 A), although the amount was tiny compared to the amount in the insoluble phase (Figure 6.21 B). It was difficult to tell from the rather overloaded gels of Figure 6.21 whether BCCP was present in the soluble phase or not, certainly large amounts were present in the insoluble fraction.

To check for the presence of BCCP in the soluble phase, less protein was run on another 12% acrylamide gel, next to diluted insoluble protein and soluble fractions were probed with anti-biotin antibodies on a Western blot (Figure 6.22 A and B).

These gels and Westerns clearly show that a small amount (approximately 100ng on the gel), but significantly more than in previous experiments, of biotin carboxylase is expressed in the soluble phase in the presence of groE chaperonins. Presumably this protein is in the native state. BCCP protein is also detectable in the soluble phase of these bacteria. This demonstrates that the presence of the groE chaperonins in the bacteria aids in the correct folding and expression of soluble protein. As there was detectable amounts of biotin carboxylase and biotin carboxyl carrier protein in the soluble phase, these protein extracts were analysed for interaction of proteins by gel filtration chromatography.

Figure 6.21 A Expression of biotin carboxylase and BCCP in the presence of GroE chaperonins, soluble phase.

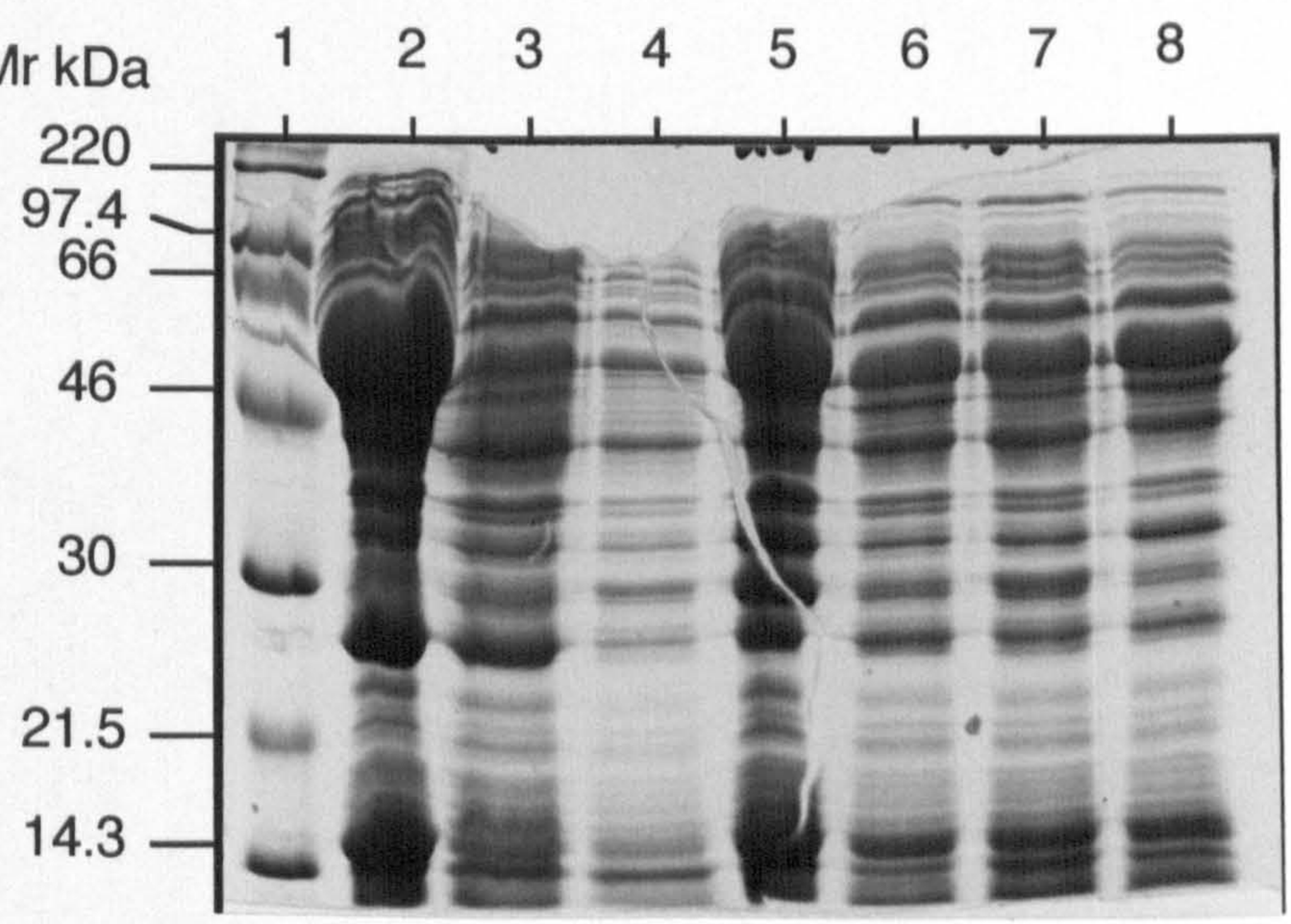
Lanes show the soluble proteins from *E.coli* BL21(λDE3) cells after 3 hours of growth in the presence of 1mM IPTG containing the following plasmids; Lane 2, pT7groE; Lanes 3-8 all contained proteins expressed from pT7groE and; Lane 3; pET11a; Lane 4, pETBP4; Lane 5, pETBP+L; Lane 6, pETBC; Lane 7, pETBC + pET24BP4; Lane 8, pETBC + pET24BPL4. Lane 1 contains Rainbow M_r markers, 2μg each protein.

Figure 6.21 B Expression of biotin carboxylase and BCCP in the presence of GroE chaperonins, insoluble phase.

The insoluble fraction from Figure 6.21 A was solubilised in 1 × SDS sample buffer and 5μl was loaded onto a 12% gel.

Lanes show the insoluble proteins from *E.coli* BL21(λDE3) cells after 3 hours of induction containing the following plasmids; Lane 2, pT7groE; Lanes 3-8 all contain pT7groE and; Lane 3; pET11a; Lane 4, pETBP4; Lane 5, pETBP+L; Lane 6, pETBC; Lane 7, pETBC + pET24BP4; Lane 8, pETBC + pET24BPL4. Lane 1 contains Rainbow M_r markers, 2μg each protein.

A


Mr kDa
1
2
3
4
5
6
7
8
220
97.4
66
46
30
21.5
14.3

B

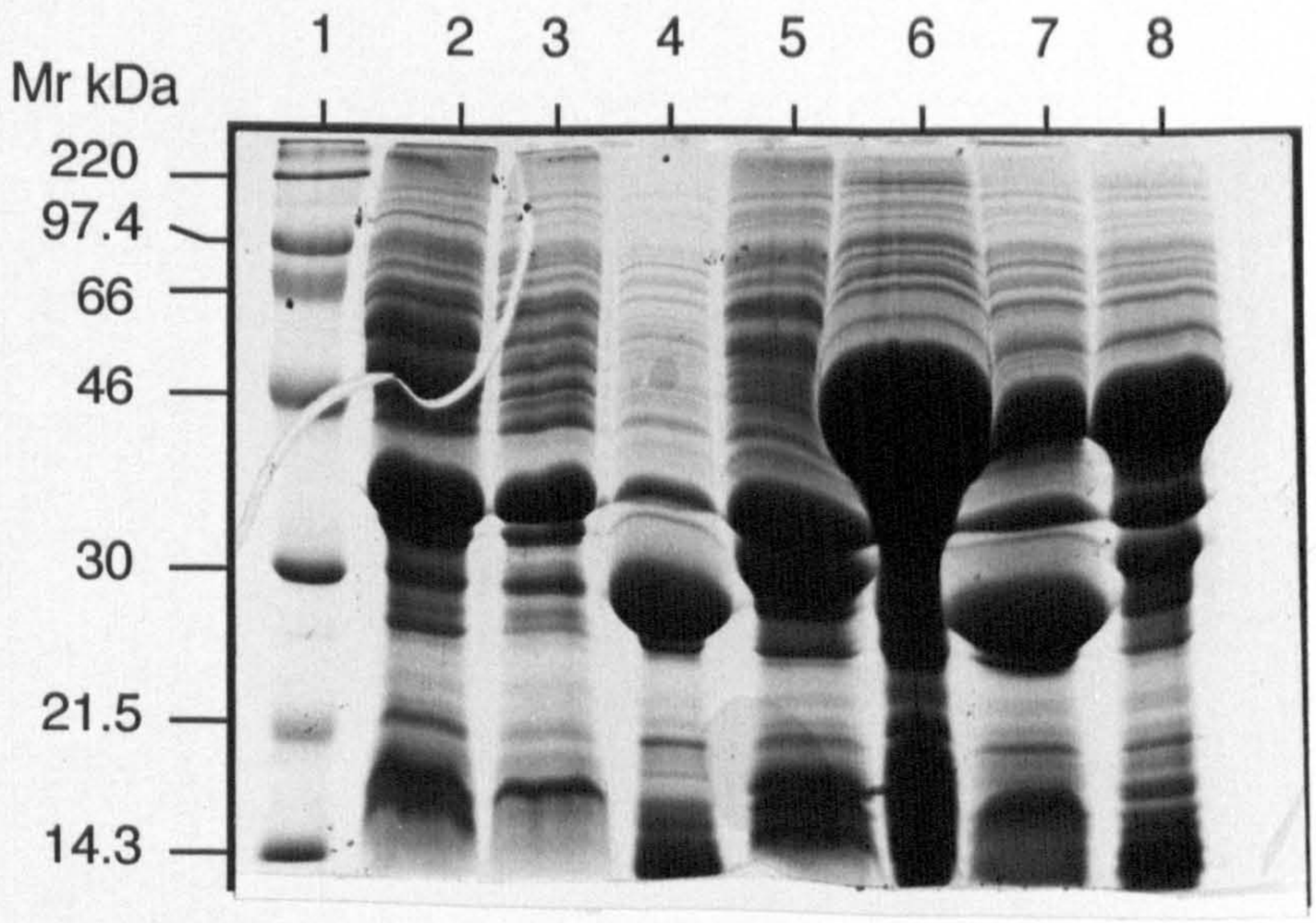

Mr kDa
1
2
3
4
5
6
7
8
220
97.4
66
46
30
21.5
14.3

Figure 6.22 A Comparison of soluble and insoluble fractions of *E.coli* BL21(λDE3) cells harbouring pT7groE and expressing pET24BP4 or pET24BPL4 with or without the presence of pETBC.

The soluble and insoluble phases shown separately in Figure 6.21 A and B were compared for soluble protein by running a smaller amount (1/10th less) on a 12% SDS gel, with soluble and insoluble fractions side by side.

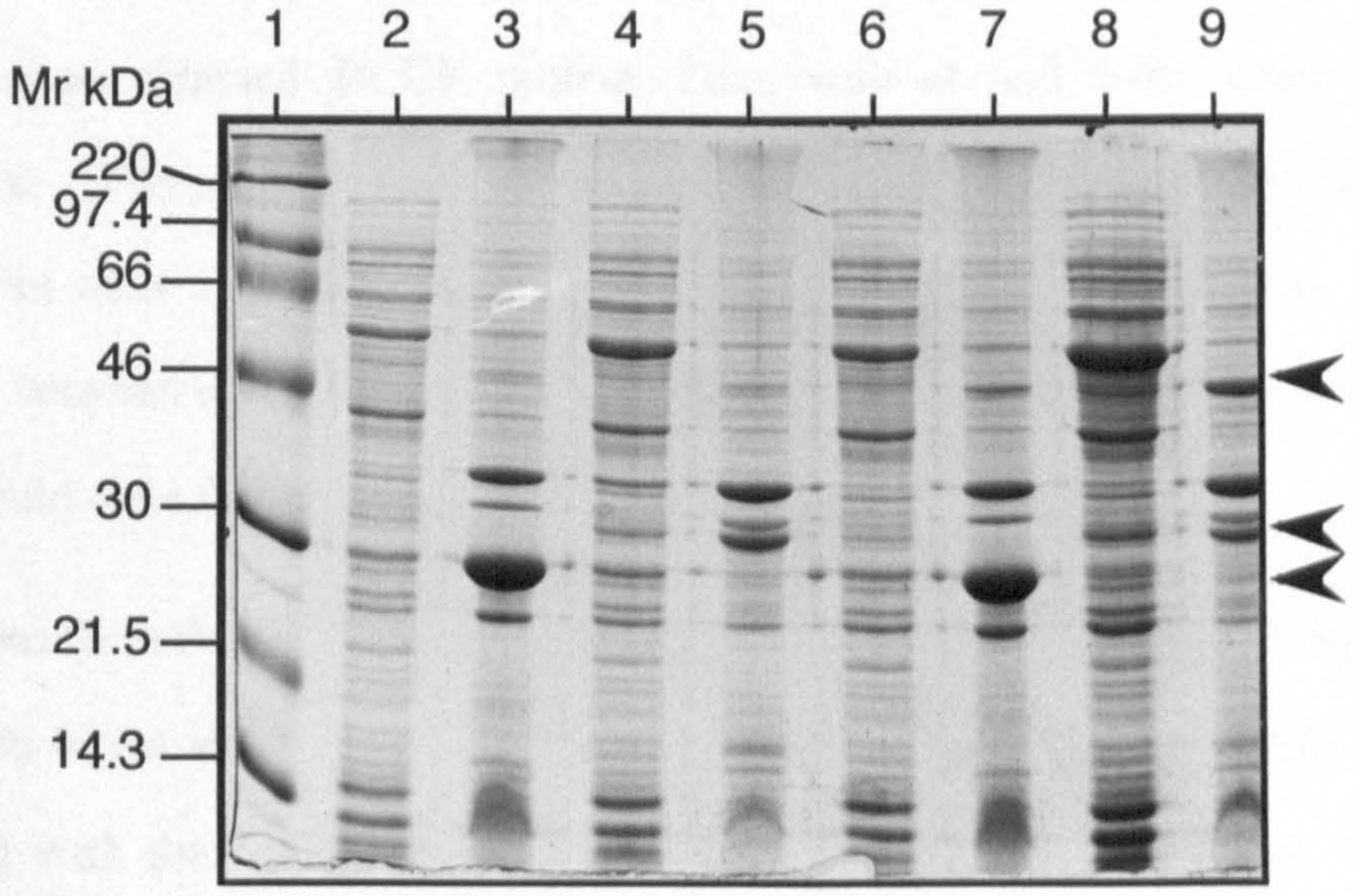
Lanes contained protein from *E.coli* BL21(λDE3) cells containing pT7groE and the following plasmids; Lanes 2+3, pETBP4; Lanes 4+5, pETBP+L; Lanes 6+7, pETBC + pET24BP4; Lanes 8+9, pETBC + pET24BPL4. Lanes 2, 4, 6 and 8 contain soluble proteins, Lanes 3, 5, 7 and 9 contain insoluble proteins. Lane 1 contains Rainbow markers, 1μg each protein. Arrows mark the position of recombinant proteins.

Figure 6.22 B Analysis of the soluble fractions from Figure 6.22 A by Western blotting with anti-biotin antibodies.

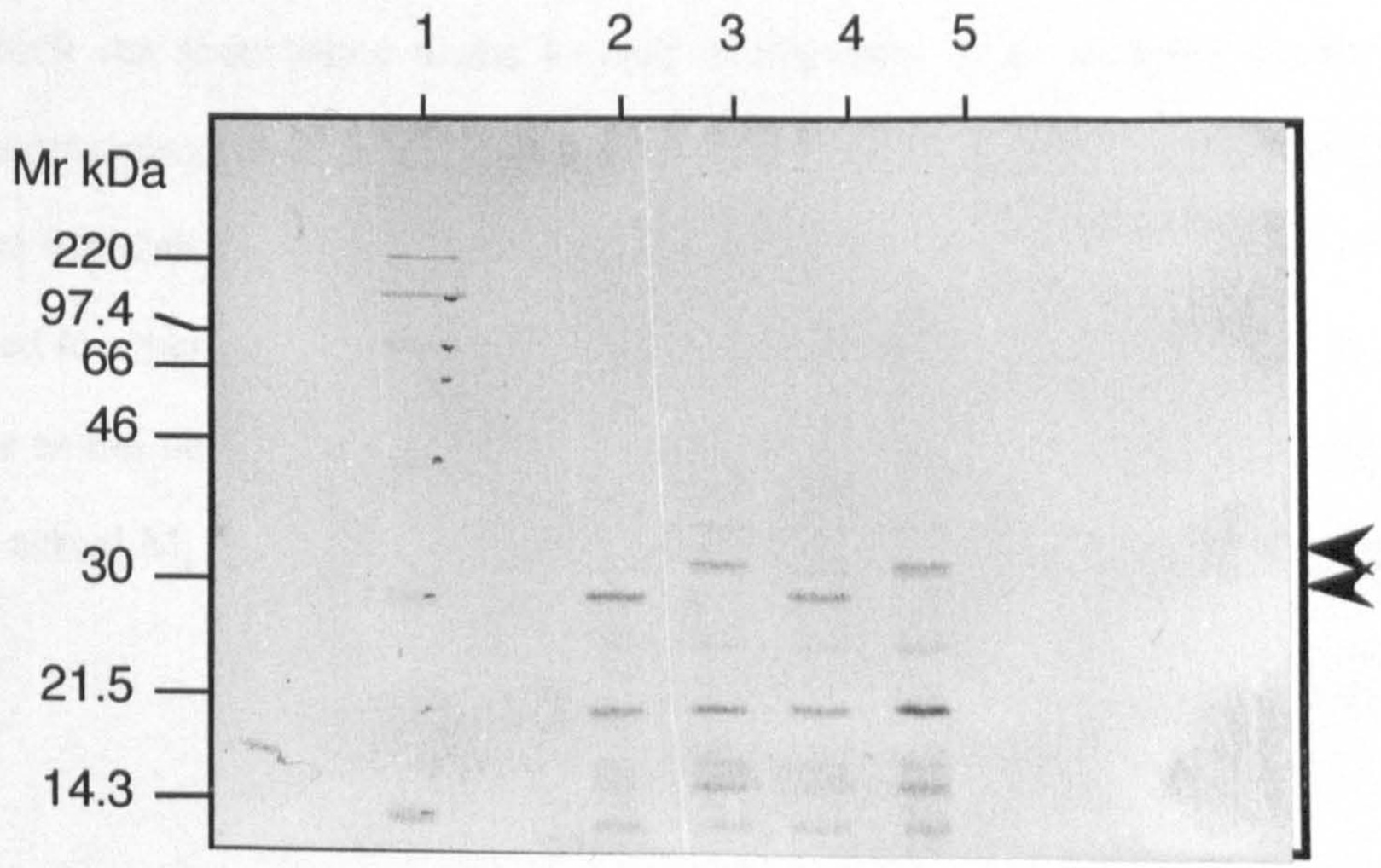
The insoluble fractions run in Lanes 2, 4, 6 and 8 in Figure 6.22 A were analysed by Western blotting with anti-biotin antibodies.

Lanes show the biotinylated soluble protein from *E.coli* BL21(λDE3) cells containing pT7groE and the following plasmids; Lane 2, pETBP4; Lane 3, pETBP+L; Lane 4, pET24BP4 + pETBC; Lane 5, pET24BPL4 + pETBC. Lane 1 contains Rainbow markers, 1μg each protein. The arrows mark the position of the recombinant BCCPs.

A


1 2 3 4 5 6 7 8 9
Mr kDa
220
97.4
66
46
30
21.5
14.3

B


1 2 3 4 5
Mr kDa
220
97.4
66
46
30
21.5
14.3

6.6.4 Gel filtration chromatography of BC and BCCP subunits

The soluble fractions shown in Figure 6.22 contained significant amounts (approximately 10μg/ml) of soluble BC protein and detectable amounts of BCCP protein on Western blots. To discover whether these two proteins were able to interact, each of the total protein samples shown in Figure 6.22 was separated by gel filtration chromatography using a superose-12 column. The first two samples contained overexpressed BCCP protein alone without and with leader sequence respectively. The second pair of samples contained the same overexpressed BCCP proteins, but also contained overexpressed BC protein. If there was a specific interaction between the BC and BCCPs in the second pair of samples, then the BCCP protein should elute from the gel filtration column at an earlier stage.

The fractions from the gel filtration run were initially analysed by ELISA with affinity purified anti-biotin antibodies. The ELISA results are shown graphically in Figure 6.23 along with the calibration of the column using proteins of known M_r. The ELISA shows that the majority of biotinylated proteins are eluted in the first fractions (fraction 7) as high M_r species. A second smaller peak was observed in fraction 9 after which the absorbance drops to near background in all samples except for the sample containing biotin carboxylase and biotin carboxyl carrier protein with leader sequence together. In this sample the absorbance remains high in fractions 10 and 11 compared to other samples. This correlates to a M_r of 45,000 to 60,000. This could correlate to the native M_r of the combined BC and BCCP+L subunits, remembering that the actual M_r of BCCP+L is not 35,000 as seen on SDS / PAGE gels but 27,000.

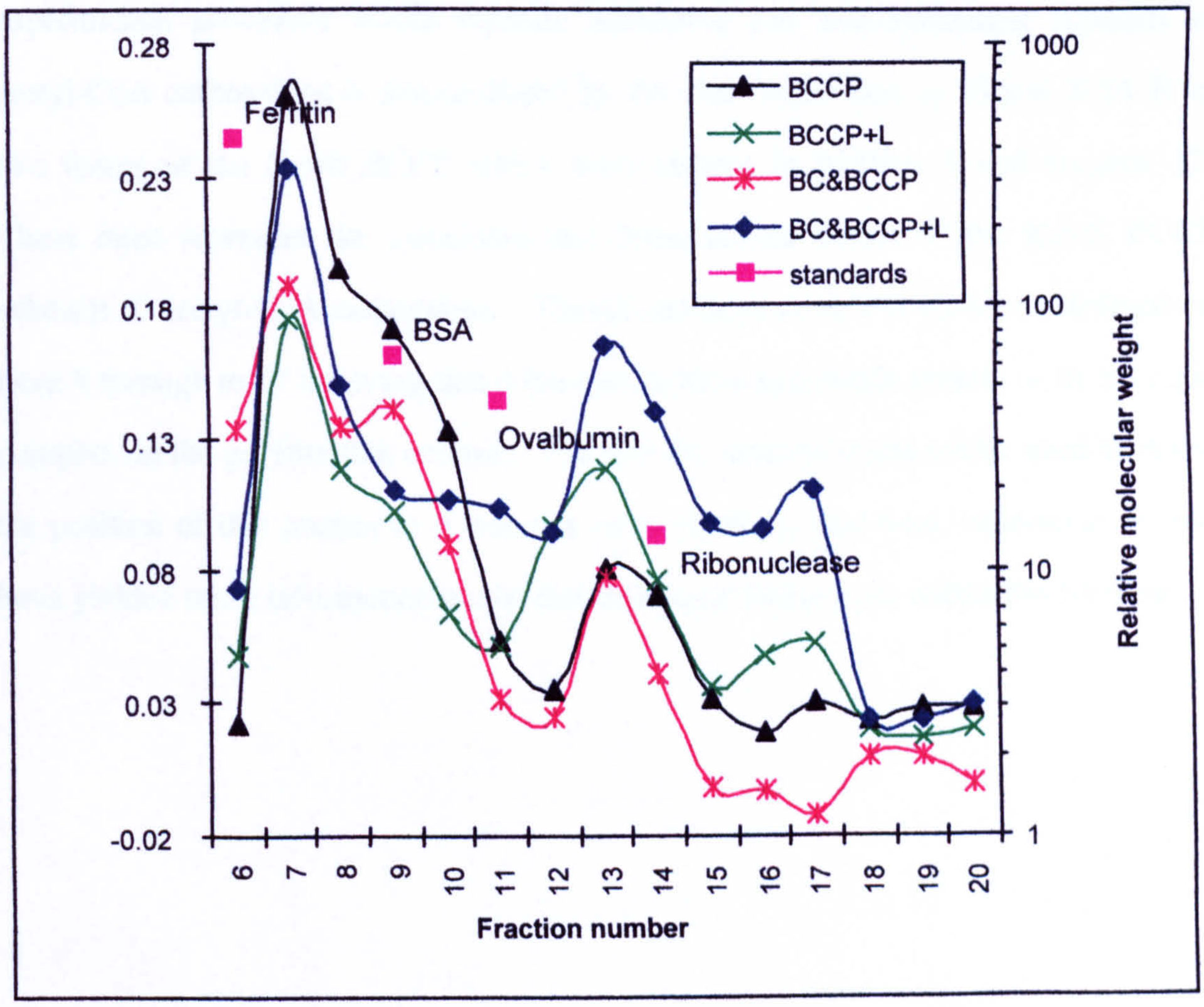


Figure 6.23 Analysis of fractions from superose-12 column by ELISA.

Biotinylated proteins from *E.coli* BL21(λDE3) cells overexpressing subunits of *B.napus* Type II acetyl-CoA carboxylase were separated by gel filtration and detected by ELISA with anti-biotin antibodies. Absorbance was corrected for background absorbance against BSA.

Fractions 6, 7, 8, 9, 12, 10, 13, 14, and 17 were analysed for protein content by Western blotting with anti-biotin antibodies and are shown in Figure 6.24. Of each fraction, 20μl was run on a 12% acrylamide gel and transferred onto nitrocellulose where they were detected with affinity purified anti-biotin antibodies, donkey anti-sheep : alkaline phosphatase conjugate and developed with BCIP / NBT.

The recombinant biotinylated protein is clearly visible in the Western blots as shown in Figure 6.24 A-D, but there seems to be no detectable difference between protein expressing *B.napus* BCCP alone or together with the BC subunit. That the

experimental procedure would separate associated and non-associated subunits of acetyl-CoA carboxylase is demonstrated by the clear separation in Figure 6.24 B of two forms of the *E.coli* BCCP which were present in fraction 8 and fraction 17. These must represent the associated and disassociated forms of the *E.coli* BCCP subunits of acetyl-CoA carboxylase. The recombinant protein is visible in all fractions from 8 through to 17 implying that it does not behave as a single protein or as a simple complex on the gel filtration column. The anti-BC antisera could not be used to detect the position of this protein as it was not yet available at that time, otherwise, it may have yielded more information on the distribution of that protein within the fractions.

Figure 6.24 Analysis of fractions from superose-12 column by Western blotting with affinity purified anti-biotin antibodies.

E.coli BL21(λDE3) containing pT7groE and a plasmid(s) expressing a subunit(s) of Type II ACCase that had been induced by 1mM IPTG were fractionated into soluble and insoluble protein. Proteins in the soluble phase were separated by gel filtration chromatography and the biotinylated proteins detected by ELISA. Those fractions containing significant amounts of biotinylated protein were analysed by Western blotting.

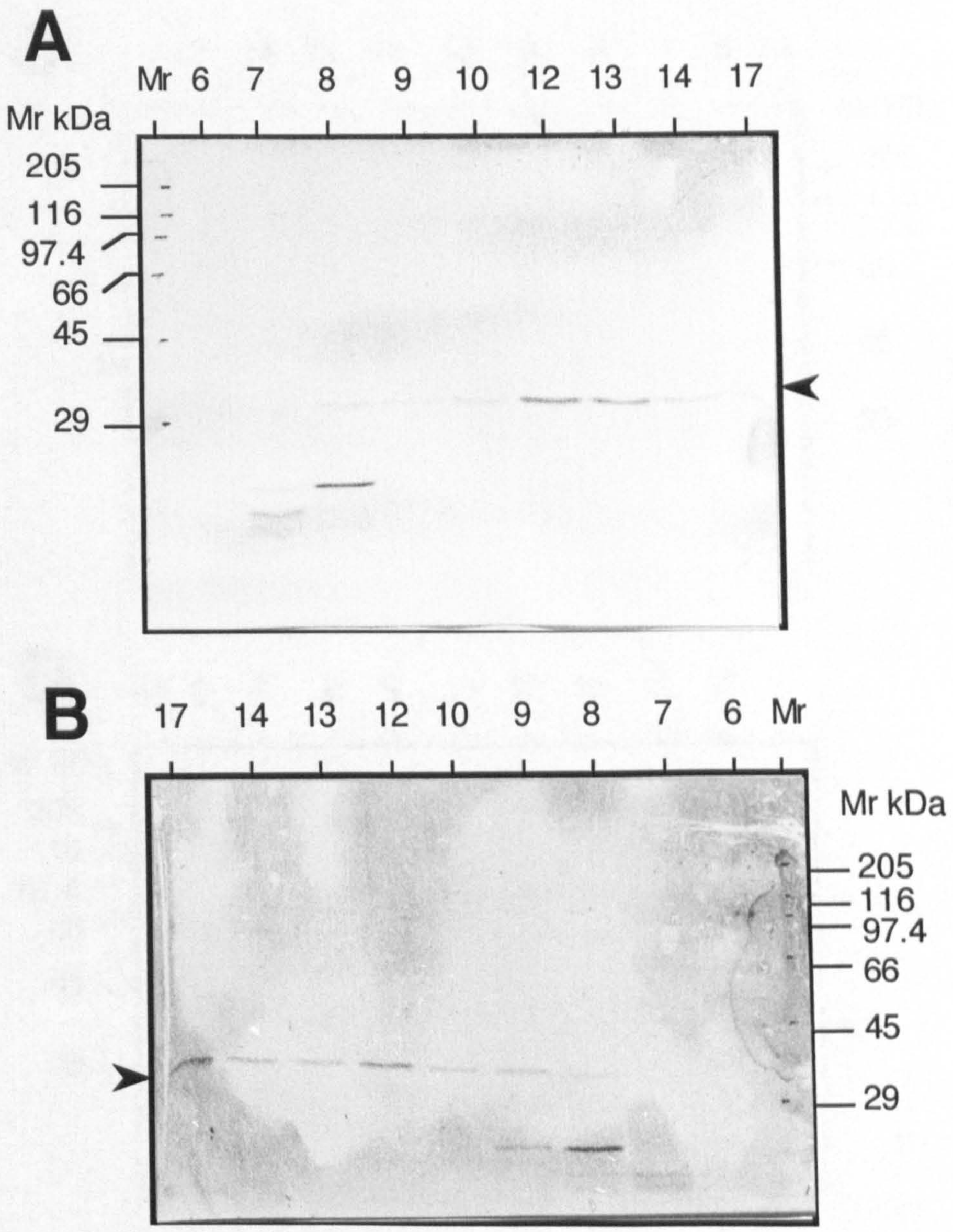
A Size fractionated soluble proteins from *E.coli* BL21(λDE3) cells expressing BCCP4.

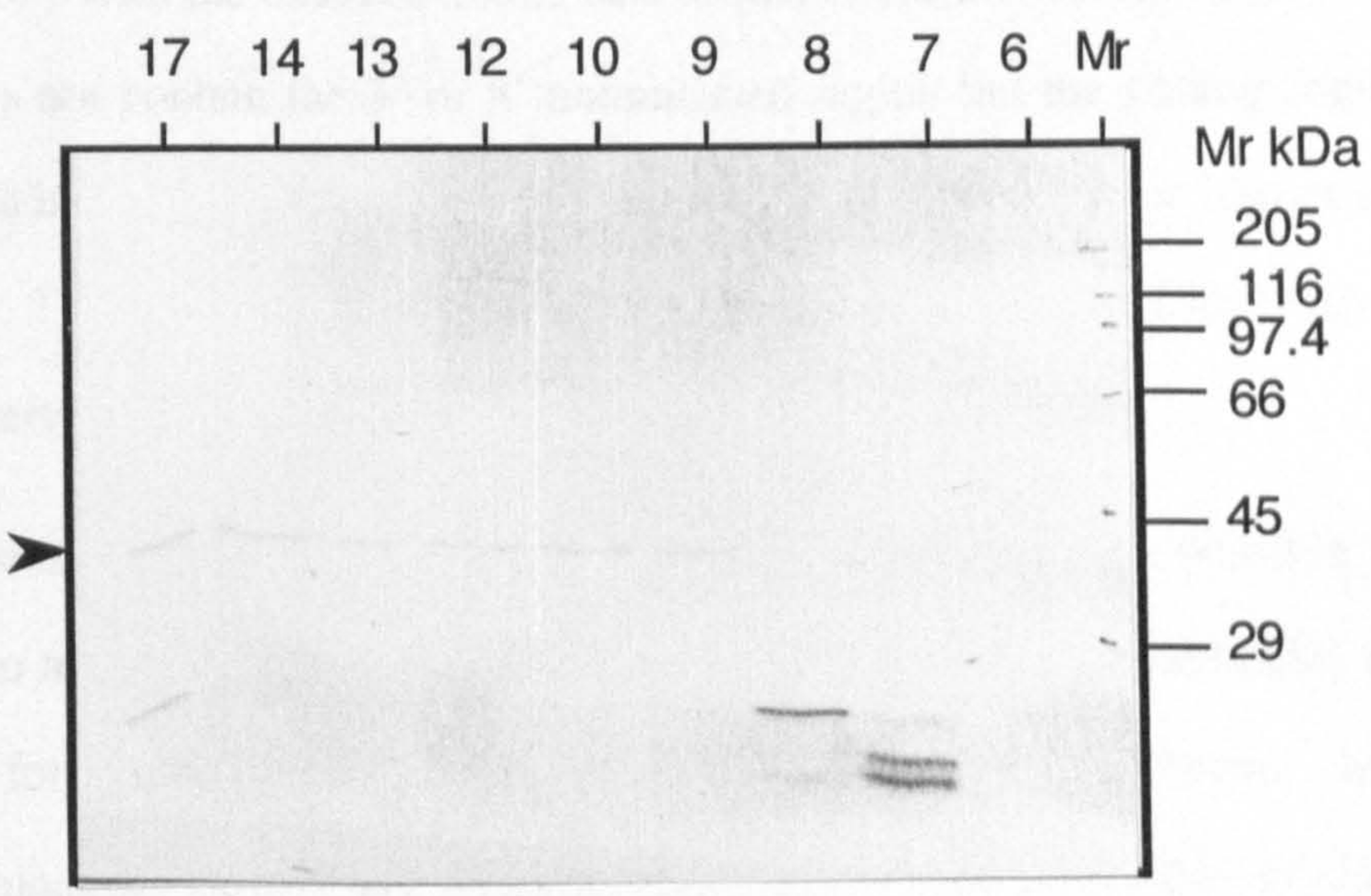
B Size fractionated soluble proteins from *E.coli* BL21(λDE3) cells expressing BCCP+ leader sequence.

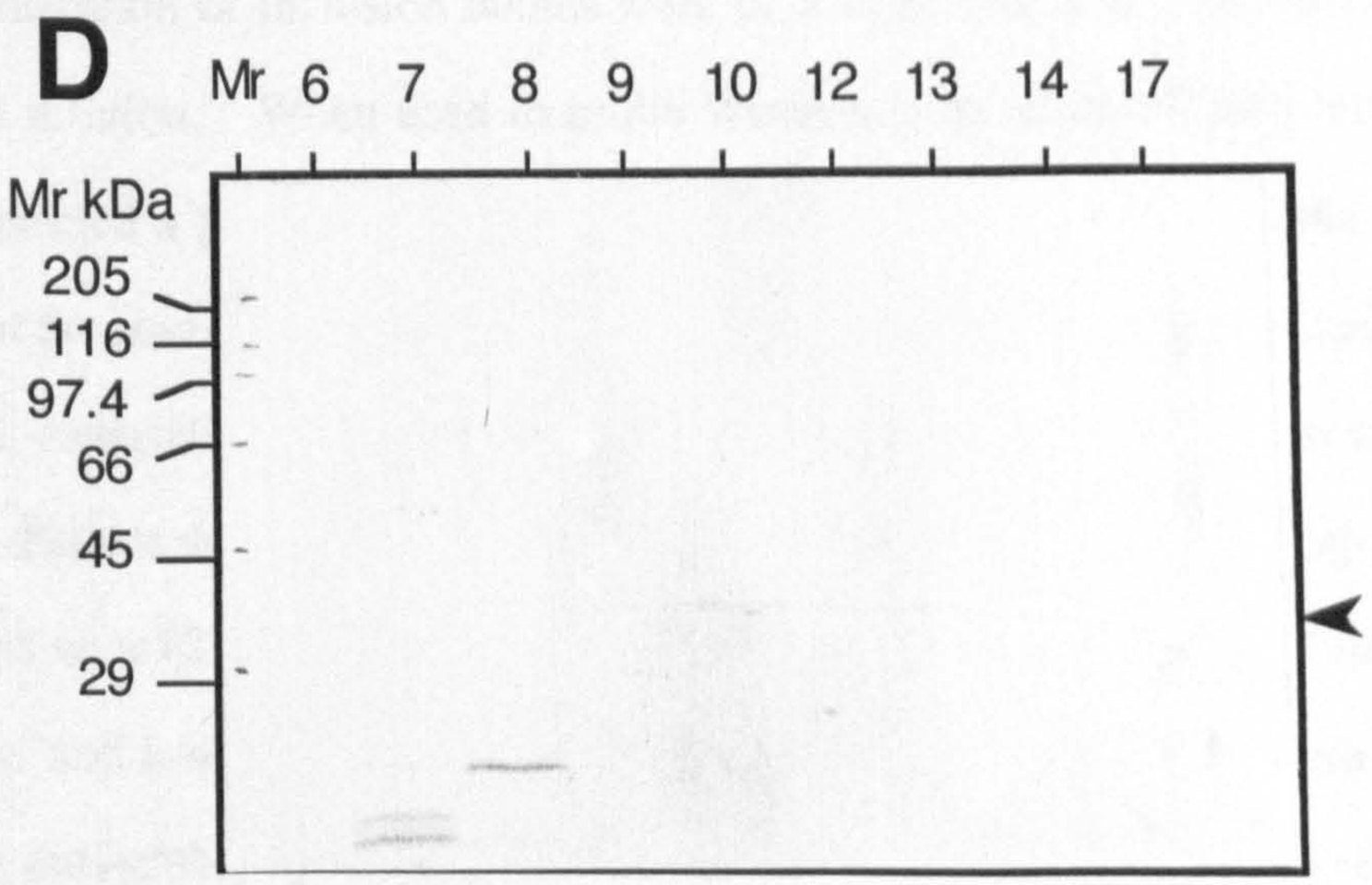
C Size fractionated soluble proteins from BL21(λDE3) cells expressing BC and BCCP4.

D Size fractionated soluble proteins from *E.coli* BL21(λDE3) expressing BC and BCCP + leader sequence.

In all pictures the lanes show 20μl of 1ml fractions run on a 12% acrylamide gel and transferred to nitrocellulose. The Western blot was probed with affinity purified anti-biotin antibodies and donkey anti-sheep IgG : alkaline phosphatase conjugate. The image was developed with BCIP / NBT. Lanes contain the following; M_r, SDS-6H protein standards; Lanes 6-17, fraction numbers from superose-12 column. The recombinant BCCP is marked with an arrow.


A
Mr 6 7 8 9 10 12 13 14 17
Mr kDa
205
116
97.4
66
45
29
B
17 14 13 12 10 9 8 7 6 Mr
Mr kDa
205
116
97.4
66
45
29


C
17 14 13 12 10 9 8 7 6 Mr
Mr kDa
205
116
97.4
66
45
29


D
Mr 6 7 8 9 10 12 13 14 17
Mr kDa
205
116
97.4
66
45
29

6.7 Discussion

The Biotin Carboxylase subunit of *Brassica napus* has been isolated by PCR from a random primed cDNA library of mid-embryogenesis mRNAs. The full sequence of the cDNA for this subunit of Type II acetyl-CoA carboxylase from *Brassica napus* has been discovered and is presented in this work. The deduced amino acid sequence has strong homology with the deduced amino acid sequence from *Nicotina tabacum.* The sequence does not contain the 3' or 5' untranslated region but the coding region has been identified in its entirety including the proposed leader peptide for import into the chloroplast. The ATP binding site identified for *N.tabacum* BC (Shorrosh *et al.*, 1995) is conserved in the *B.napus* protein sequence.

By cloning the cDNA for this protein from *Brassica napus* it has been possible to raise an antibody to another subunit Type II acetyl-CoA carboxylase. Antibodies provide key tools for the studying of protein *in vivo* by Western analysis, immunoprecipitation, ELISA and immunolocalisation. The antibodies produced by the purification of inclusion bodies were of a high titre and reasonable specificity at 1:2000 dilution. When used to probe Western blots of developing embryo and leaf they detected a protein with an M_r of ≈ 48,000 (predicted from sequence of 51,054 without proposed leader sequence). The exact M_r is difficult to estimate from SDS / PAGE, especially in leaf, as the protein migrates at a M_r close to the rubisco protein which distorts the gel (see Figure 3.3 B for a picture of Coomassie stained chloroplast proteins on a 12% gel) (Shorrosh *et al.*, 1995). The Western blot of developing seed material and leaf and root tissues reveals that levels of the BC protein are maximum during mid-embryogenesis, as are the levels of BCCP. The observed levels of BC protein by Western blotting vary up to 3 fold through embryogenesis, compared to a greater than 5 fold change in the observed levels of BCCP. The BC protein was detected in leaf and root samples, whereas BCCP is barely detectable in leaf and not at all in root. This suggests a higher basal level of BC protein than BCCP in tissues of *Brassica napus.* The reason for this is not clear but suggests that the BCCP subunit may have a more potent role to play in the regulation of the enzyme than the BC

subunit. The actual amount of BC protein detected also appears much lower than for BCCP. This could be because of the different polyclonal antibodies used in each case, less efficient transfer of higher M_r proteins from the gel onto nitrocellulose or because the protein is blocked from the antibodies by the rubisco protein. These problems could be overcome by using antibodies in ELISA experiments. These would be able to accurately determine the exact amounts of each subunit in moles of protein giving precise information on the variation in the levels of each protein.

In order to demonstrate the biological activity of the cloned protein, the biotin carboxylase was overexpressed in *Escherichia coli*. All of the overexpressed protein was found in inclusion bodies under normal conditions, as determined by SDS / PAGE. At the time, antibodies were not available against BC and consequently the soluble fractions could not be specifically probed via Western blots for the presence of this protein. Inclusion bodies could not be purified by normal chromatographic techniques. Although the protein appeared to be soluble in that they were not pelleted by a 15 minute 12,000 × g spin and were filtered through a 0.22μm filter, it was not possible to separate the protein from contaminant on Blue Sepharose or Mono-Q columns. The reasons for this are unclear but may be a result of the denatured state of the protein. In such a state, hydrophobic surfaces which would normally be located internally in the protein, would be exposed and these may cause non-specific binding to other proteins and the chromatography matrix. Alternatively, it was possible that the protein was not truly in solution but existed as a very fine suspension of particles.

Attempts to express the protein with increased solubility focused on the use of reagents to aid in the correct folding of the protein during expression. Betaine was reported to cause increased production of soluble protein with a subsequent increase in activity (Blackwell & Horgan, 1991). Under these growth conditions there was no obvious increase in the solubility of BC protein and the BCCP subunits also appeared to be largely insoluble. It was not possible to assay directly for activity as the native *E.coli* protein would also be present and incorporate $H^{14}CO_3^-$ under the assay conditions (Alban *et al.*, 1995).

The molecular chaperones encoded by the *groE* operon were used for try and improve the solubility of the overexpressed proteins by ensuring correct folding. The expression plasmid pT7*groE* was used to express the groES and groEL chaperonins in *E.coli* BL21(λDE3) cells. Whilst the vast majority of the protein was present in inclusion bodies a small amount was detectable in SDS / PAGE gels stained with Coomassie blue R-250. A Western blot of the soluble fractions also demonstrated the expression of the recombinant BCCP protein in a soluble biotinylated form.

As it appeared that both subunits BC and BCCP were found in the soluble fraction of cells expressing the *groE* chaperonins, these were used for gel filtration experiments. If the two subunits were interacting when expressed together then the BCCP protein should have less retention time than when it is expressed in isolation. The biotinylated BCCP would be detected with anti-biotin antibodies. These experiments already contained an internal control. The *E.coli* protein is readily dissociable into subunits (Alberts & Vagelos, 1972) and therefore a similar effect should be seen for the *E.coli* BCCP, it should elute with differing retention times. The *E.coli* BCCP did elute in two fractions as predicted (Figure 6.24 C), but the overexpressed *Brassica napus* BCCP did not. The protein eluted in all fractions from fraction 8 - 17. No difference in elution was seen between protein samples which contained the overexpressed biotin carboxylase and those that did not. This could be because, 1) the BCCP subunit was not truly soluble and was present as extremely minute particles of varying M_r which did not pellet at 12,000 × g, 2) the BCCP subunit was soluble but was interacting with many other *E.coli* proteins and therefore did not behave as a single protein, or 3) the BCCP subunit was interacting with the superose matrix through hydrophobic and therefore did not elute as a single peak .

These experiments showed that it was not possible to overexpress biotin carboxylase in its native state under the conditions used here. Although, soluble BCCP protein was produced in earlier experiments in this study (section 5.4.3), the amount of soluble protein produced could not be consistently repeated and expression in the presence of the plasmids pT7groE and pETBC reduced the amounts of soluble protein.

In vitro transcription and translation of the pETBC plasmid produced a soluble, labelled protein with an estimated M_r of 46kDa. Similar experiments with the vectors pETBP4 and pETBP+L failed to produce protein on a native gel. Whilst no labelled products were produced by the pETBP4 vector in this system, the pETBP+L vector did overexpress a protein but it did not run on the native electrophoresis system used. This is probably because the predicted pI of the protein is basic and the stacking gel is acidic. Under such conditions the protein would be expected to be neutral or positively charged which is why it did not run into the gel. This made it difficult to ascertain whether there was protein-protein interaction when the two subunits were *in vitro* transcribed and translated together. A very faint band was evident with a low mobility but its identity could not be deciphered. The experiment was repeated twice and the band found to be present both times. Now that anti-BC protein antisera is available, the experiment could be repeated and the proteins transferred to nitrocellulose and the identity of any products with a high Rf value discerned.

It has been possible for other workers to overexpress the biotin carboxylase in a soluble form (B. Shorrosh, personal communication) by creating a GST-fusion protein. This often employed method is known to increase the product solubility, but was not used in these experiments because it may have affected the subunit interaction. There were several options for which there was not the time to explore, to perhaps produce soluble proteins. The use of a vector expressing thioredoxin instead of groE, is thought to have a similar affect to expressing a protein as a GST-fusion, by changing the redox environment within the cell (Yasukawa *et al.*, 1995). Prokaryotic and eukaryotic cells are thought to have a quite different redox environment (Kondo *et al.*, 1993). This suggests another alternative would be to express the cells in a eukaryotic expression system. Finally, now that the genes for all the subunits of Type II acetyl-CoA carboxylase have been isolated(Sasaki *et al.*, 1993; Shorrosh *et al.*, 1995; Elborough *et al.*, 1996; Shorrosh *et al.*, 1996), it may be possible to co-overexpress all four subunits. Whilst this may not seem like a solution, studies with other multisubunit enzymes have indicated that even without the presence of just one

subunit, none of the subunits can be expressed in a soluble form (S. Rawsthorne, personal communication).

In conclusion, a partial cDNA for biotin carboxylase encoding the full open reading frame, has been cloned by PCR. This cDNA has been sequenced and has been shown to be homologous to the sequence from *N.tabacum* (tobacco, Shorrosh *et al.*, 1995). The cDNA has been used to overexpress the biotin carboxylase protein in *E.coli* using the vector pET11a but the protein product from this vector was entirely in inclusion bodies. Purified inclusion bodies were used to raise antibodies in a rabbit. The antisera detected a protein with an estimated M_r of about 48,000 which accumulates to a high level during mid-embryogenesis, this is consistent with the detected protein in castor which was shown to be biotin carboxylase (Shorrosh *et al.*, 1996). Compared to another subunit of Type II acetyl-CoA carboxylase, the biotin carboxylase subunit did not appear to be as highly regulated, possibly due to a higher basal level of expression. Co-expression of the biotin carboxylase and biotin carboxyl carrier protein did not increase the yield of soluble protein. The molecular chaperones encoded by the groE operon were able to increase the yield of soluble BC and BCCP protein, but the BCCP protein was not separated as a single peak by gel filtration so no definite conclusion about the interaction of the two subunits could be drawn. *In vitro* transcription and translation of the biotin carboxylase and biotin carboxyl carrier protein with leader sequence produced two labelled bands with M_rs of 46,000 and 35,000. Native PAGE with these two subunits together showed a faint high molecular weight band which could have been the result of the subunits interacting. This provides preliminary evidence that the protein products of the cloned genes for BCCP and BC could complex into a protein of large native M_r *in vitro*.

Chapter 7: Discussion and future work

The objectives of this research were to characterise biotinylated proteins from *Brassica napus* and *Arabidopsis thaliana.* Previous research on these organisms had demonstrated the existence of a 220kDa acetyl-CoA carboxylase (Hellyer *et al.*, 1986). Other biotinylated proteins had been identified in *Daucus carota* (carrot, Wurtele & Nikolau, 1990) and activities for the enzymes acetyl-CoA carboxylase, methylcrotonyl-CoA carboxylase, propionyl-CoA carboxylase and pyruvate carboxylase had been demonstrated in this species and in *Brassica napus*.

This study has demonstrated the existence of seven biotinylated protein in *Brassica napus* with M_rs of 220, 150, 76, 71, 65, 35 and 30kDa by detecting them with anti-biotin antibodies on Western blots. Previous work on *Daucus carota* (carrot) identified six biotinylated proteins of 210, 140, 73, 50, 39 and 34kDa, by detection with [^{125}I] labelled streptavidin (Wurtele & Nikolau, 1990). At the time, little was known about the identity of biotinylated protein in plants and these workers could only speculate on the identity of the detected proteins by comparison with the animal and yeast enzymes. Since 1990, many of these biotinylated proteins have been purified and their cDNAs isolated. Consequently, it is now possible to assign a function to most of the known biotinylated protein in *Brassica napus* with a degree of confidence. The 220kDa protein was purified from seeds of *Brassica napus* and identified as acetyl-CoA carboxylase (Hellyer *et al.*, 1986). A partial cDNA was also isolated for this enzyme showing it to be temporally expressed throughout embryogenesis (Elborough *et al.*, 1994b). As the identity of the 220kDa protein had been determined it was not investigated further to allow investigation into the identity of the other biotinylated proteins.

β-Methylcrotonyl-CoA carboxylase has been purified from a number of sources (Alban *et al.*, 1993) and found to be an enzyme of approximately 74-85kDa. In this study, a cDNA for β-methylcrotonyl-CoA carboxylase was characterised from *Arabidopsis*. This was used to probe a northern blot of mRNA from embryos of *Brassica napus*

throughout development. The results showed that the mRNA was nearly 2,800 base pairs in size and was expressed highly in root and leaf. The expression of the mRNA for β-methylcrotonyl-CoA carboxylase was also higher in late embryogenesis than in early. This pattern of expression is consistent with previously observed results (Clauss *et al.*, 1993), and with the potential role of MCCase in leucine catabolism (Aubert *et al.*, 1996). The observed pattern of expression, the size of the mRNA in *Brassica napus*, and the M_r of β-methylcrotonyl-CoA carboxylase from other species, indicated that the 76kDa biotinylated protein detected on Western blots of *Brassica napus* protein extracts, was probably β-methylcrotonyl-CoA carboxylase.

The 65kDa protein detected in very late developing seeds of *Brassica napus* was probably the SBP65 storage protein detected in large quantities in pea seed (Duval *et al.*, 1994). The existence of this protein has been demonstrated albeit at lower levels, in seeds of *Brassica napus* (A. R. Slabas, personal communication from Roland Douce).

The only detected biotinylated protein in isolated chloroplasts of *Brassica napus* was the 35kDa protein and this has since been identified as the biotin carboxyl carrier protein (BCCP) of a Type II acetyl-CoA carboxylase common to all plants except the *Gramineae* (work in this study, Elborough *et al.*, 1996, Konishi *et al.*, 1996). The major biotinylated protein(s) in *Brassica napus* seeds is (are) the 30kDa biotinylated band(s). This may consist of one or more proteins with similar M_rs. This protein has been demonstrated to be related to the BCCP protein as anti-BCCP antisera cross-reacts with this protein. The function and subcellular localisation of this protein is unknown, but it does not appear to be located within chloroplasts isolated from leaves of *Brassica napus*.

The remaining two proteins with M_rs of 150 and 71kDa have not been assigned a definite function in this study. There are reports of plants being able to catalyse the

ATP dependent, avidin sensitive carboxylation of geranyl-CoA in maize, and a 120kDa biotinylated protein has been partially purified from maize leaves (Caffrey *et al.*, 1995). This enzyme has not been investigated in this study. The characterisation of this enzyme will probably require the complete purification of the protein from plant tissues, as the only other known source of this enzyme is *Pseudomonas citronellolis*, and the cDNA or gene for this enzyme has not been cloned from this source (Fall & Hector, 1977).

The remaining biotinylated protein at 71kDa has also not been studied and its identity remains in doubt. It has been demonstrated in this study that the activity of pyruvate carboxylase in *Brassica napus* is not increased by acetyl-CoA, and as a result it was possible to conclude that this enzyme represents a Type II pyruvate carboxylase as characterised from some bacteria and fungi (Scrutton & Taylor, 1974; Cohen *et al.*, 1979; Tozo *et al.*, 1992). This enzyme is made up of two subunits, the biotinylated subunit of which has an M_r of about 65,000 (Cohen *et al.*, 1979; Tozo *et al.*, 1992). If the Type II enzyme was made up of two subunits in *Pseudomonas* and *Leptosphaeria*, it may well have a similar structure in higher plants. The function of pyruvate carboxylase in higher plants is not known, therefore speculation about the possible regulation of the protein is not possible. The pattern of expression of the 71kDa protein throughout developing seeds of *Brassica napus* indicates that the levels of the protein do not change significantly. There are two feasible possibilities as to the identity of this protein, it could represent an isoform of MCCase, which has two genes in *Arabidopsis thaliana* and four genes in *Brassica napus*, or it could represent a biotinylated subunit of pyruvate carboxylase. Previous work on biotin proteins did not detect the 71kDa protein as the signal from the 76kDa protein would have masked it (Wurtele & Nikolau, 1990). As anti-biotin antibodies give a sharp, precise band, this protein has been detected in seeds of *Brassica napus*. Identification of this protein will probably require the purification of the enzyme. Maturing seeds of *Brassica*

napus var. Jet neuf, would seem an obvious choice of tissue as the activity reported in this study was substantially greater than values previously reported.

The conserved nature of the biotinylation site enabled the mistaken isolation of several clones which expressed biotinylated proteins. The characterisation of these clones revealed the sequence for MCCase from *Arabidopsis* and the sequence of an unknown *Arabidopsis* gene. The unknown gene (pBC1.6) was demonstrated to encode for a biotinylated protein when expressed from λZAPII bacteriophage. The identity of the expressed protein was not discerned as expression of open reading frames from the cDNA as fusion proteins in the pMAL-c2 system failed to produce a biotinylated protein. The characterised cDNA contained an intron sequence for which splice site borders were identified by homology with known intron / exon boundaries in *Arabidopsis*. This was shown not to be an artefact by PCR amplification of the sequence from genomic DNA of *Arabidopsis*. Whilst the discovery of an intron in a cDNA sequence is not unheard of, it proved to be interesting in this case as when the cDNA sequence was used to probe a northern blot, two hybridising bands were revealed in late embryos. The second hybridising band was 1000 base pairs larger than the other band which was observed in embryos and root tissues. The origin of this band is unknown, but it could have been the result of differential splicing of the gene, in which an intron (or introns) was not removed, leading to a change in the protein product and consequently function. This was mostly speculation and to investigate this further a complete cDNA would have been required. The northern blot indicated that the full length gene was 2,800 base pairs. Several libraries were screened but without success. The most likely explanation for the lack of success in isolating a cDNA for this clone is that the clone was not represented in any of the libraries screened. This could be a result of the observed low level of expression for this mRNA in *Brassica napus*, and the fact that only amplified libraries were screened. A fresh un-amplified library made from isolated *Arabidopsis* mRNA would probably have to be screened in order to isolate a full length clone.

Recently through, stronger indications as to the identity of this gene have come from gene and protein homology searches of databases. The isolation and sequencing of the Bloom's syndrome gene has lead to the identification of a family of recQ DNA helicases with four known members (Ellis *et al.*, 1995; Watt & Hickson, 1996). As the cDNA or protein sequence for pBC1.6 reported in this study gives highest scores against all the protein in this group when used to search DNA or protein databases, it provides strong evidence that the pBC1.6 gene product may be a recQ DNA helicase. Especially significant is the very strong homology of the cDNA sequence to the conserved helicase domains, as these are essential for the gene products function (Gorbalenya *et al.*, 1989). Further work is required to demonstrate the activity of this gene's protein product. The isolation of a full length gene or cDNA is crucial as this will identify the remaining helicase domains and the important DExH box, characteristic to these helicases. The possibility that this gene product is involved in DNA repair may be investigated by the growth of plants in the presence of DNA mutagens and subsequent analysis of the mRNA. The fact that the original clone came from a library made with mRNA from inflorescence and that the expression in *Brassica napus* was found in growing tissues such as roots and embryos suggests that the protein product may be involved in DNA replication. The requirements and function of this mRNA's protein product could be investigated by transforming *Arabidopsis* with antisense expression constructs and observing the phenotype of recovered plant cells. It might be expected that the phenotype of these cells would be similar to the phenotype observed in Bloom's syndrome patients. This is typified by a high incidence of mutation due to DNA cross linking, and genome instability through hyper-recombination and chromosome non-disjunction as a result of failure to complete recombination (Ellis *et al.*, 1995;Watt & Hickson, 1996). It is feasible that strongly antisensed plants may not be able to grow as normal plants and revert to callus or die.

Much of the work in this study has focused on the important enzyme acetyl-CoA carboxylase, in particular the Type II form of this enzyme. This is in part due to the strong commercial interest in acetyl-CoA carboxylase as a means of altering the flux of carbon into lipid biosynthesis in order to increase the flux of carbon into other pathways, such as polyhydroxybutarate biosynthesis (Nawrath *et al.*, 1994). Apart from this there is strong academic interest in the discovery of a prokaryotic form of acetyl-CoA carboxylase in higher plants. This presents further evidence of an endo-symbiotic origin for chloroplasts as one of the genes for the Type II acetyl-CoA carboxylase is chloroplast encoded. Questions must arise as to how the levels of four different subunits are controlled and how they are assembled into an active enzyme within the chloroplast. How the Type I and Type II enzymes individually contribute towards lipid biosynthesis will be determined by antisense experiments of the individual enzymes.

The studies presented here have shown that acetyl-CoA carboxylase in the chloroplasts of *Brassica napus* is a multi-subunit enzyme, of which the biotinylated subunit is 35kDa and the biotin carboxylase subunit is 50kDa. The levels of both of these subunits were induced to mid-embryogenesis and decreased thereafter, consistent with the increase in total lipid throughout embryogenesis. Studies on the interaction of these subunits were significantly hampered by the difficulty of expressing these proteins in a soluble form in *E.coli.* Whilst 100μg of soluble BCCP protein was initially purified, the BC protein was completely insoluble. The solubility of the BC protein was improved by co-expression of the BC protein with the BCCP subunit and the groE chaperonins. Whilst this system produced a small amount of soluble protein, it was not able to show conclusively that the two proteins were complexed. *In vitro* transcription and translation did not produce conclusive results either due to the small amount of protein produced. A way around the problem would be to express the proteins in a baculovirus system which usually produces soluble proteins. There are vectors available which can co-express two proteins at a time. This system would also be well suited to these experiments as there is not a Type II dissociable acetyl-CoA

carboxylase in insect cells. As this system is very time-consuming to set up it was not possible to perform these experiments in the period of this study. Further work could also concentrate on the expression of all the Type II acetyl-CoA carboxylase subunits *E.coli*. This may produce soluble protein as it may be necessary for all the subunits of the enzyme to be present for the correct folding of the protein.

Characterisation of the biotin carboxyl carrier protein in *Brassica napus* included the demonstration that the protein is expressed as multiple isoforms. Six genes were initially isolated for this enzyme and the variation in expression of four of these at the mRNA level has been demonstrated. The other two mRNAs (pBP1 and 3) could not be detected in these experiments. That these isoforms exist in *Brassica napus* has been demonstrated by the isolation of clones from a *Brassica napus* genomic library which produce specific products when used as a template in a PCR reaction with primers BP1 or BP3 and UniBP (data not presented, genomic clone isolated by Dr. Martha Evans, Department of Biological Sciences, University of Durham). Multiple protein isoforms were detected by 2-D Western blotting with anti-biotin antibodies, three of these isoforms appear to be house-keeping genes in that they are present at all stages throughout seed development and in leaf tissue where the rate of lipid biosynthesis is minimal. The other three isoforms are expressed specifically during mid to late embryogenesis. This may reflect the changes in demand for malonyl-CoA during lipid biosynthesis in developing seeds.

The physical characteristics of the BCCP subunit contains some quirks which are difficult to explain. The initial observation was that the BCCP in isolated chloroplasts is a 35kDa protein, the same size as the overexpressed BP4 with proposed leader sequence. This observation depends on the isolated BCCP cDNAs being full length as further sequence could exist at the 5' end of the cDNA which could contain a different initiation of translation codon and therefore encode a different leader peptide. There are two pieces of evidence to support the claim that pBP4 and pBP6 contain full or almost full length cDNAs and that the initiation of translation codon is the correct one. 1) The cDNA on northern blots is 1100 base pairs, approximately the same size

as pBP6. 2) pBP6 contains several stop or nonsense codons upstream from the proposed start codon. These facts ensured that a full length cDNA was isolated and that the initiation of translation codon was the correct one.

There are two possibilities as to why the protein detected in chloroplasts runs at 35kDa on SDS / PAGE. The leader sequence may not be cleaved upon transport into the chloroplast or there may be a post-translational modification which causes the protein to have altered mobility. Such a post-translational modification may be glycosylation, but changes in mobility usually require a high degree of glycosylation and highly glycosylated proteins are usually structural in function. It is more likely therefore that the protein runs at 35kDa because the leader peptide is not cleaved. If the transit peptide is not cleaved, then a second physical quirk is noticeable, the protein has a very different pI from the predicted one. The overexpressed BCCP without leader sequence was shown to have a pI consistent with the calculated value. The calculated value for the BCCP with leader sequence was basic at approximately pH 8, but the protein was found at the acidic end of the isoelectricfocusing gel. This may be due to the structure of the leader peptide, which are known to by amphipathic helices (Theg & Geske, 1992), or a result of post-translational modifications such as phosphorylation or attachment of other acidic lipid groups.

The final characteristic of this protein which is hard to explain is the identification of BCCP as a series of proteins from 31-29kDa. These proteins are susceptible to proteolytic degradation as demonstrated by early extraction procedures which failed to take adequate precautions against proteolysis, leading to a variety of bands at between 14 and 30kDa. The 31-29kDa proteins appear to be BCCP because they are immunoreactive to the anti-BCCP antisera but they are not located in chloroplasts purified from leaves of *Brassica napus*. Whilst these proteins are also shown to be biotinylated proteins when detected with affinity purified anti-biotin antibodies, the ratio of protein detected with anti-BCCP antibodies to anti-biotin antibodies was a minimum of 5.5, whereas for the 35kDa protein it was a maximum of 2.5. Finally these proteins are the same size as the overexpressed BCCP protein without leader

sequence. These proteins could represent a cytoplasmic form of Type II acetyl-CoA carboxylase, but this is very unlikely as the β-CT subunit is encoded by the chloroplast genome, and their are no reports of chloroplast encoded proteins being exported to form a cytoplasmic enzyme.

This evidence points towards a possible model for the expression of BCCP in *Brassica napus*, a possible means of regulation of acetyl-CoA carboxylase and a way in which the protein turnover is regulated. Speculation about the data presented in this study has lead to the following model of how BCCP may be produced and its levels regulated in chloroplasts of *Brassica napus*. The BCCP protein is synthesised from nuclear encoded genes, biotinylated and transported to the chloroplast. The protein enters the chloroplast, but the transit peptide is not cleaved immediately. As the BCCP protein must combine with the biotin carboxylase and the two CT subunits, it is immediately bound by chaperonins which refold-the protein and complex it with the biotin carboxylase. These proteins are then transported to the thylakoid membrane where they combine though ionic interaction with the CT subunits to produce malonyl-CoA for lipid biosynthesis. The formation of this complex is controlled by the levels of BCCP being imported into the chloroplast. The complex is disassociated in response to an unknown signal and the BCCP leader sequence is removed. This disassociation of the complex could be facilitated by a specific (de)phosphorylation event and protease. Once the BCCP leader sequence has been removed, the protein is targeted for degradation and is exported out of the chloroplast. Once removed from the plastid, a cytoplasmic biotinidase rapidly removes the biotin residue, scavenging it for recycling and the remaining protein is degraded.

Such a model is only speculative but may provide a useful paradigm within which to carry out further studies. An indication of whether the leader peptide remains intact on the imported protein could come from chloroplast import studies. Labelled, soluble BCCP with leader sequence could be incubated with isolated chloroplasts to demonstrate the protein uptake. SDS / PAGE analysis of the imported proteins would show whether the leader sequence had been removed or not. These experiments

could not be carried out in this study due to the lack of soluble BCCP with leader sequence. Improved *in vitro* transcription and translation may produce soluble protein for these studies, the protein from which could now be detected with anti-BCCP antibodies. Purification of the BCCP protein itself from intact chloroplasts and sequencing of the N-terminus would be one way of demonstrating whether the leader sequence is intact on the active protein *in vivo*. Other experiments could use leader peptide specific antibodies to detect the protein. Provided they were specific, anti-leader peptide antibodies could be used to immunolocalise the BCCP leader sequence. Other components of the system, such as the specific complex dissociation factor, possibly a protein kinase / dephosphorylase or the leader peptide protease would need to be identified. These could possibly be identified by using labelled soluble BCCP with leader sequence protein in an assay system. Such an assay could perhaps, be used to screen an expression library for a specific protein kinase, by *in vitro* transcription / translation and incubation of the protein with the *in vitro* synthesised protein and [γ-^{32}P]dATP. By screening the library in aliquots and looking for the production of phosphorylated BCCP with leader sequence, it may be possible to isolate a specific protein kinase.

Biotinylated proteins have a key role to play in much of basic carbon biochemistry. A unique controlling mechanism, the biotinylation status of the enzyme, may exist for all of these enzymes. Whilst this study has tried to address some of these questions, there are still biotinylated proteins whose identity remain unproven and whose roles in carbon biochemistry are unknown. Acetyl-CoA carboxylase is an enzyme into which intensive research is ongoing, and the molecular mechanisms of its regulation are inevitably going to be discovered. Hopefully, much of the work in this study will aid in subsequent investigations and provide useful tools for further analysis for both acetyl-CoA carboxylase and all biotin-containing enzymes.

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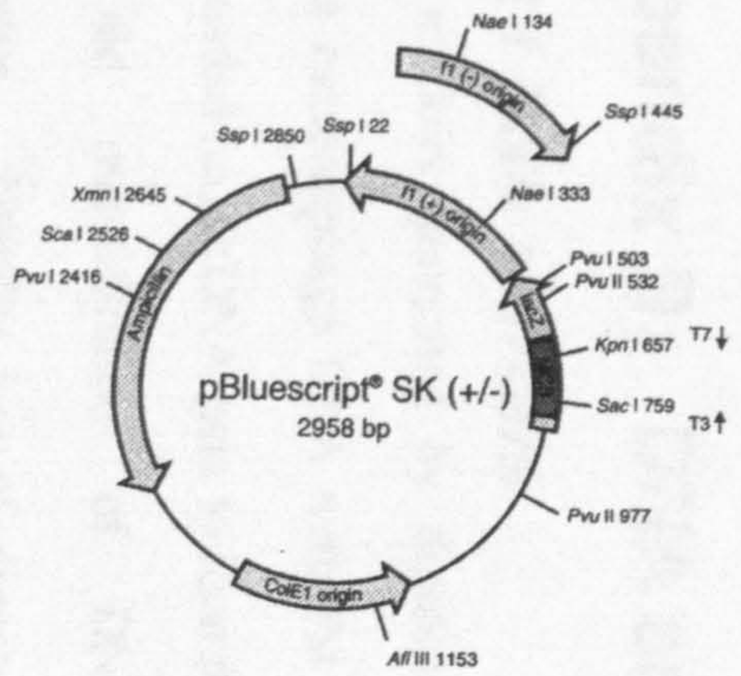
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Appendix A; pSK+/-

A map of the plasmid pBluescript® II SK (+/-) is provided on the right for the readers reference (Stratagene catalogue, 1996). The plasmid pSK- is derived from λZAP II. Sequencing was usually performed with the reverse and M13 -20 (forward) primers. The other primers used in this study were the T3 and T7 primers. The sequence and restriction site in the MCS are shown below to aid in identification of sites used to create nested deletions.




```
Reverse primer                                    T3 primer
5' GGAAACAGCTATGACCATG 3'                  5' AATTAACCCTCACTAAAGGG 3'

                                                                  BstXI      Eag I          SpeI        SmaI       Eco RI
          MET                  BssH II  T3 promoter  +1→          Sac I   SacII  Not I    Xba I      BamHI      PstI
5' GGAAACAGCTATGACCATGATTACGCCAAGCGCGCAATTAACCCTCACTAAAGGGAACAAAAGCTGGAGCTCCACCGCGGTGGCGGCCGCTCTAGAACTAGTGGATCCCCCGGGCTGCAGG
3' CCTTTGTCGATACTGGTACTAATGCGGTTCGCGCGTTAATTGGGAGTGATTTCCCTTGTTTTCGACCTCGAGGTGGCGCCACCGCCGGCGAGATCTTGATCACCTAGGGGGCCCGACGTCCTTAA
          |                    |                    |
         816                  792                  759
          β-Galactosidase →

                  Bsp106I HincII          Eco0109I
                  ClaI    AccI            DraII
     EcoRV HindIII        SalI  XhoI      ApaI   KpnI                              BssHI
AATTCGATATCAAGCTTATCGATACCGTCGACCTCGAGGGGGGGCCCGGTACCCAATTCGCCCTATAGTGAGTCGTATTACGCGCGCTCACTGGCCGTCGTTTTACAA
   GCTATAGTTCGAATAGCTATGGCAGCTGGAGCTCCCCCCCGGGCCATGGGTTAAGCGGGATATCACTCAGCATAATGCGCGCGAGTGACCGGCAGCAAAATGTT
                                                |          ←+1 T7 promoter          |
                                               657                                619
                                                    3' CGGGATATCACTCAGCATAATG 5'  3' TGACCGGCAGCAAAATG 5'
                                                          T7 primer                    M13 -20 primer (forward)
```

Appendix B; DNA and protein standards

λPst I DNA markers

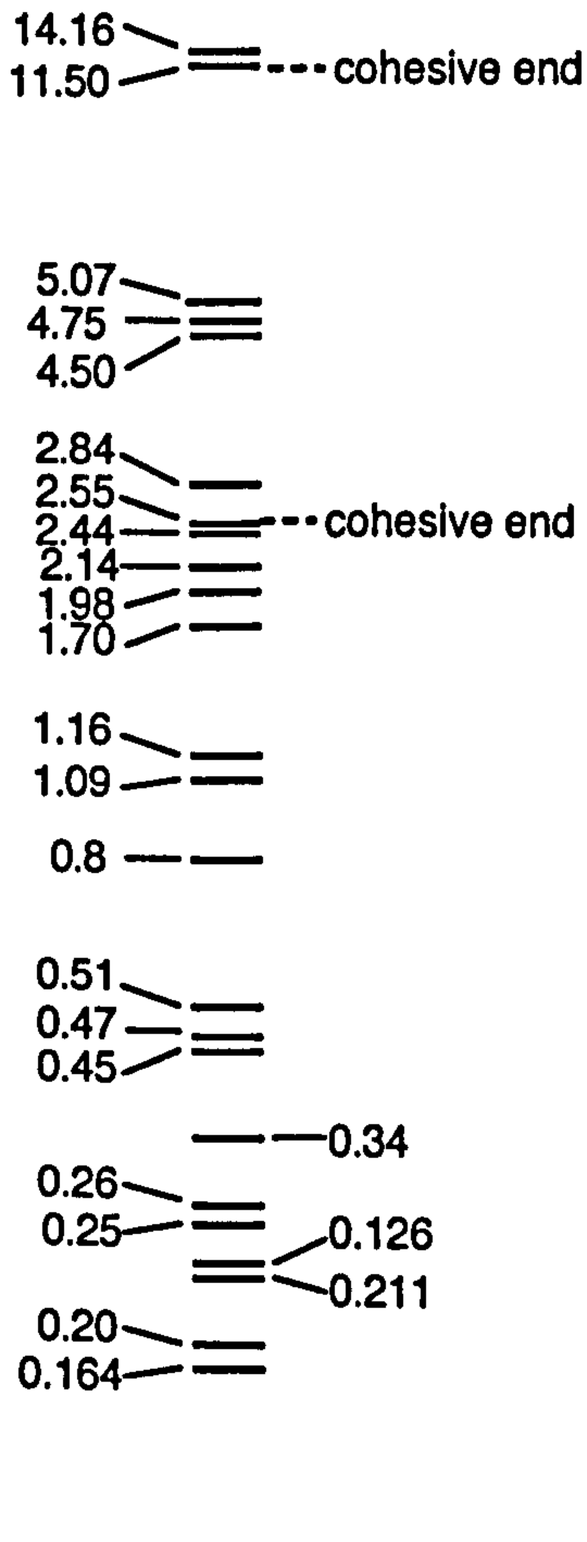
DNA markers generated by digesting lambda bacteriophage DNA with Pst I were loaded on all DNA gels to identify and aid quantification of DNA fragments. Usually 1µg of digested lambda DNA would be loaded.

Quantification of fragments was carried out by matching the intensity of the unknown band to the band of closet intensity in the lambda markers. The quantity of DNA in any one Pst I fragment of lambda DNA was worked out in nanograms as follows;

$$ng = \frac{\text{size of fragment}}{48.5} \times \text{ng loaded}$$

Where ng is the amount of DNA per fragment; size of fragment refers to the size in kilo base pairs of the lambda Pst I fragment being compared to and ng loaded is the total amount of lambda DNA loaded in that lane.

Fragments produced by digesting lambda DNA with Pst I; sizes are in thousands of base pairs.



Protein standards

The following protein standards were used throughout this study. The M_r of the standard proteins is indicated in kilo daltons.

Protein	Molecular weight	SDS-7	SDS-6H	SDS-6B	Rainbow	[^{14}C]-methylated
Myosin	220,000				X (blue)	X
α_2-Macroglobulin	205,000		X			
β-Galactosidase	116,000		X			
Phosphorylase B	97,400		X	X	X (brown)	X
BSA	66,000	X	X		X (red)	X
Catalase	58,100			X		
Ovalbumin	45,000	X	X		X (yellow)	X
Alcohol dehydrogenase	39,800			X		
GPDH	36,000	X				
Carbonic Anhydrase	29,000	X	X	X	X (orange)	X
Trypsinogen	24,000	X				
Trypsin Inhibitor	20,100	X		X	X (green)	X
Lysozyme	14,300			X	X (magenta)	X
Lactalbumin	14,200	X				

When using rainbow markers the dye alters some protein's M_r; ovalbumin, 46,000; carbonic anhydrase, 30,000; and trypsin inhibitor, 21,500.

Appendix C: Publications

Part of the work presented in this thesis has resulted in a publication and is bound here for the reader's reference and for completion.

Biochem. J. (1996) **315**, 103–112 (Printed in Great Britain)

Biotin carboxyl carrier protein and carboxyltransferase subunits of the multi-subunit form of acetyl-CoA carboxylase from *Brassica napus*: cloning and analysis of expression during oilseed rape embryogenesis

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In the oilseed rape *Brassica napus* there are two forms of acetyl-CoA carboxylase (ACCase). As in other dicotyledonous plants there is a type I ACCase, the single polypeptide 220 kDa form, and a type II multi-subunit complex analogous to that of *Escherichia coli* and *Anabaena*. This paper describes the cloning and characterization of a plant biotin carboxyl carrier protein (BCCP) from the type II ACCase complex that shows 61% identity/79% similarity with *Anabaena* BCCP at the amino acid level. Six classes of nuclear encoded oilseed rape BCCP cDNA were cloned, two of which contained the entire coding region. The BCCP sequences allowed the assignment of function to two previously unassigned *Arabidopsis* expressed sequence tag (EST) sequences. We also report the cloning of a second type II ACCase component from oilseed rape, the β-carboxyltransferase subunit (βCT), which is chloroplast-encoded. Northern analysis showed that although the relative levels of BCCP and βCT mRNA differed between different oilseed rape tissues, their temporal patterns of expression were identical during embryo development. At the protein level, expression of BCCP during embryo development was studied by Western blotting, using affinity-purified anti-biotin polyclonal sera. With this technique a 35 kDa protein thought to be BCCP was shown to reside within the chloroplast. This analysis also permitted us to view the differential expression of several unidentified biotinylated proteins during embryogenesis.

INTRODUCTION

Plant acetyl-CoA carboxylase (ACCase; EC 6.4.1.3) is one of the pivotal enzymes of fatty acid biosynthesis in both leaf and seed tissue [1]. It catalyses the ATP-dependent carboxylation of acetyl-CoA to form malonyl-CoA and is thought to be an important regulatory step in *de novo* fatty acid synthesis in chloroplasts [2]. Its central role reflects its importance as a target for the commercial aryloxyphenoxypropionate-type herbicides used presently [3]. The herbicides' action differentiates between the two forms of ACCase found in dicotyledonous plants [4].

All the ACCases so far studied have a biotin moiety, covalently bound to a lysine residue, that is thought to act as a molecular arm to pass the carboxy group between the subunit active sites [5]. However, the molecular organization of the enzyme differs according to the source of the enzyme. The mammalian form (type I) is a large multifunctional polypeptide [6–8] of at least 200 kDa made up of three fused domains [biotin carboxylase (BC), biotin carboxylase carrier protein (BCCP), and the carboxyltransferase (CT)]. The protein represents one of the four biotinylated enzymes in mammalian cells and one of three in yeast [9,10]. In contrast, the *Escherichia coli* form (ACCase type II) consists of a polypeptide complex [11–13] consisting of BC dimer, BCCP dimer and CT tetramer ($\alpha_2\beta_2$) subunits. These are encoded by four separate genes, which in *E. coli* are named *acc*C, *acc*B, *acc*A and *acc*D respectively. The BCCP subunit is biotinylated *in vivo* but, in contrast with BCCP from mammals and yeast, represents the only biotinylated protein in *E. coli* [10]. In higher plants at least six biotinylated proteins are present in crude extracts [14].

Initially, plant ACCase was thought to be of the type I form, as indicated by the isolation of both plant/photosynthetic algal ACCase protein and cDNA/genomic clones indicative of the 220 kDa form [15–25]. The early understanding of ACCase in plants as being solely of the mammalian-like type I form was in retrospect over-simplistic. As far back as 1972 Kannagara and Stumpf reported a smaller plant ACCase indicative of type II [26]. In addition, functional ACCases similar to the multi-subunit complexes in *E. coli* were observed in spinach chloroplasts, barley chloroplasts and avocado plastids [26–29]. However, until recently these observations were largely discounted because ACCase I was shown to be highly susceptible to protease degradation [30], and most reports studied a form of at least 220 kDa.

In 1992 the sequencing of the *acc*D gene, encoding the β-subunit of the carboxyltransferase (βCT) subunit of the *E. coli* ACCase [13], enabled a putative function to be assigned to an open reading frame (ORF) reported in the chloroplast genome of several plant species. The ORFs included pea [31,32], tobacco [33], the parasitic plants *Cuscuta reflexa* (G. Haberhausen;

Abbreviations used: ACCase, acetyl-CoA carboxylase; BC, biotin carboxylase; BCCP, biotin carboxyl carrier protein; βCT, β-carboxyltransferase subunit of type II ACCase; EST, expressed sequence tag; MCCase, methylcrotonyl-CoA carboxylase, ORF, open reading frame; d, days; UTR, untranslated region.

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The DNA sequences presented in this paper have been submitted to the EMBL/Genbank data libraries under accession numbers X90727–X90732 and Z50868.

EMBL accession number X69803) and *Epifagus virginiana* [34], *Pinus thunbergii* [35], *Marchantia polymorpha* [36], *Physcomitrella patens* [37] and *Angiopteris lygodiifolia* [38]. Despite the knowledge of these homologous ORFs, much less was known about their expression and the association between their predicted products and ACCase activity. Indeed, the pea chloroplast DNA ORF was first designated as a putative DNA binding protein containing a zinc-finger motif [39]. The transcriptional analysis of the *acc*D homologues in plants has so far been confined to pea, where it has been shown to be complex as a result of multiple transcription start sites and co-transcription with genes both 5′ and 3′ [31,39].

The realization that there were two forms of ACCase in dicotyledonous plants was brought about by Li and Cronan [40] initially, and more exhaustive studies followed soon afterwards [41,42]. Antibodies generated to an oligopeptide derived from the pea βCT sequence were used to immunoprecipitate an 87 kDa *acc*D-like protein from pea leaf chloroplast extract [42]. This led to the loss of ACCase activity from the extract. The protein antigen co-precipitated with two other proteins of 91 and 35 kDa, suggestive of a complex *in vivo* [42]. These polypeptide subunits were shown to be organized into a large complex similar to that of *E. coli* ACCase. The 35 kDa protein was biotinylated *in vivo* and was located in the chloroplast, highly indicative of its being BCCP, whereas the 91 kDa associated protein was thought to be the BC subunit. A recent study by Shorrosh et al. [25] described the cloning and characterization of the tobacco BC subunit from type II ACCase.

With two forms of ACCase present in dicotyledonous plants the possibility arose that only one or both types might play a part in *de novo* lipid synthesis. In pea leaves it has been shown that both types are present, but in different cellular and subcellular compartments [41]. The larger type I is found in the cytosol of epidermal cells whereas type II is in the chloroplasts of mesophyll cells. It has been suggested that because plant ACCase I is induced by UV irradiation and fungal elicitors, its role is mainly biosynthesis of flavonoids and waxes [43,44] through a fatty acid chain extension activity. The chloroplast is thought to be the site for *de novo* fatty acid synthesis in mesophyll cells [45] and BCCP resides within the chloroplast. It is therefore reasonable to suppose that a type II *Brassica napus* ACCase would be mainly associated with *de novo* lipid synthesis.

As part of the *Arabidopsis* genome project, random cDNA clones are continually being partly sequenced and compared with the available databases [46]. If no significant similarity is shown the clone remains unassigned. Although *Arabidopsis* provides an ideal model for plant genetic manipulation it is not suitable as an agricultural crop. Oilseed rape is the major oil-bearing crop in Europe and is amenable to genetic engineering. Several groups are working on the manipulation of its fatty acid content [47] and on the production of alternative products such as polyhydroxybutyrate [48].

Because both BC [25] and βCT [31–38,42] have been cloned from different plants, a full understanding of the type II ACCase requires a full-sized ORF BCCP subunit cDNA clone. We report here the successful use of an EST sequence to clone *B. napus* BCCP cDNAs. The BCCP sequence data obtained allowed us to assign an identity to two previously unassigned ESTs i.e. 5′ BCCP gene. Using degenerative primers we also cloned a second member of the ACCase II complex, the chloroplast-encoded βCT. Both the BCCP and βCT oilseed rape clones were used as a tool for the first comparative study of the RNA expression of two type II ACCase subunits during oilseed rape embryo development. Western analysis of biotinylated protein levels during oilseed rape embryogenesis allowed us to view the temporal expression of BCCP and other unidentified biotinylated proteins.

MATERIALS AND METHODS

cDNA library construction

The cDNA library used was generated by using poly(A)$^+$ mRNA isolated by the method of Logemann et al. [49] from mid-cotyledon-stage developing embryos of *B. napus* cv. Jet neuf. First-strand synthesis was performed with poly(dT) oligonucleotide primers in accordance with the manufacturers' instructions (Amersham International). The resulting cDNAs generated were cloned into the *Eco*RI/*Xho*I sites of λ-ZAP II as recommended by the manufacturers (Stratagene). The host bacterium used was XL-1 Blue (Stratagene).

Strategy for βCT cloning and sequencing

Degenerate oligonucleotide primers were designed against the conserved peptide motifs MDEDMV and FGMLGD (residues 293–298 and 432–437 respectively in the tobacco protein) found in the predicted amino acid sequences of all the published *acc*D homologues. These oligonucleotides were used with PCR to amplify a single product of approx. 440 bp from (i) *B. napus* chloroplast DNA, (ii) plasmid psbLa containing a 4.9 kb *Hin*dIII insert of *Arabidopsis thaliana* chloroplast DNA shown previously to encode an *acc*D homologue (as judged by partial sequence analysis; W. Schuster, unpublished work), and (iii) total DNA extracted from *B. napus* (results not shown). The PCR product from total *B. napus* DNA was cloned into the *Xba*I/*Bam*HI sites of pBluescript (pβCT1) and sequenced. Sequence analysis showed that pβCT1 encoded an *acc*D homologue. On the basis of amplification of a PCR product of identical size from both chloroplast DNA and total DNA, the insert from pβCT1 was used as a probe to screen a total genomic library of *B. napus* cv. Jet neuf. Several clones were isolated. A *Hin*dIII fragment of approx. 5 kb was present in several overlapping genomic clones and was recognized by pβCT1. The *Hin*dIII fragment from one clone was subcloned into pBS (pβCT2) and sequenced 5′ and 3′ from within the coding region identified in pβCT1 by using overlapping primers.

Probe preparation and labelling

The cDNA probes used for screening the rape libraries were generated by appropriate restriction endonuclease digestion of cDNA containing plasmids. The DNA fragment required was separated from vector DNA by TAE agarose electrophoresis and isolated by using the GeneClean II kit (Bio 101) or by freezing and ultrafiltration.

The probe (200–300 ng) was either radiolabelled with [α-^{32}P]dCTP with the Megaprime kit as recommended by the manufacturers (Amersham International) to a level of 5×10^9 c.p.m./μg or 3′ end labelled with Klenow polymerase. Unincorporated label was removed by using Biospin chromatography columns (Bio-Rad).

Library screening

For the rape poly(dT)-primed cDNA libraries 150000 plaque-forming units were screened as previously described [20]. The filters were hybridized at 65 °C and washed four times with 2 × SSC, 0.1 % SDS within 30 min at 65 °C. Plasmid rescue of cDNA clones was carried out as described in the Stratagene protocol for *in vivo* excision of pSK from λ-ZAP II.

Sequencing of DNA clones

Sequencing was performed with an Applied Biosystems Inc. 373A DNA sequencer (Ms. J. Bartley, Durham University Sequencing Service). Both forward and reverse primers (−21m13 and M13RP1) were used initially for all clones. Further sequencing was performed with a combination of forward and reverse primers and synthesized oligonucleotide primers. Primers were made by using a 381A DNA synthesizer (Applied Biosystems Inc.). Computer analysis of DNA sequence was performed with the SEQNET GCG package from the SERC facility at Daresbury, and DNA Strider shareware [50].

Northern blot analysis

Total RNA (for βCT blot) and poly(A)$^+$ mRNA (for BCCP blot) was prepared from 5 g of young leaf, 5 g of root and 5 g of embryos harvested at 15, 22, 29, 36, 42 and 49 d after anthesis, with the recommended procedure (Pharmacia mRNA purification kit). Poly(A)$^+$ mRNA (1–5 μg) and total RNA (10 μg) were loaded onto a 1% (w/v) formamide/formaldehyde agarose gel for electrophoresis. The Northern blot procedure was performed as described previously [20].

B. napus protein extracts

Embryos were removed from seeds of *B. napus* at defined days after flowering and stored in liquid nitrogen until required. Ten embryos from different days after flowering were used for protein extraction. Embryos were ground in liquid nitrogen with a pestle and mortar, weighed and kept frozen in 15 ml polypropylene tubes. Ten volumes of boiling extraction buffer [50 mM Tris, 1 mM EDTA, 0.5 mM DTT, 10% (v/v) glycerol, 1 mM PMSF, 0.5% Triton X-100 and 2% (w/v) SDS] were added, vortexed and placed in a vigorously boiling water bath for 20 min. The extracts were cooled and spun at 10000 *g*. The supernatant from between the fat layer and the pellet was removed and used for SDS/PAGE analysis. Root and leaf extracts were also prepared by this method.

Chloroplasts were isolated from 10-day-old rape seedlings by pelleting through a 40% Percoll layer (a gift from Dr. I. M. Evans).

Western blot analysis

SDS/PAGE was performed with 12% gels. Equal loading of all protein extracts were made. Biotinylated SDS molecular mass markers (Sigma) were also loaded. The protein was transferred to nitrocellulose (Hybond C extra, Amersham) by wet-blotting in 25 mM Tris, 192 mM glycine, 0.038% SDS, 20% (v/v) methanol at 40 mA for 16 h. The nitrocellulose was then exposed to 2% (w/v) SDS at 100 °C and allowed to cool. After extensive washing with Milli-Q water and PBS-T [137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.75 mM KH_2PO_4, 0.1% (v/v) Tween-20, pH 7.4] the nitrocellulose was blocked in 3% (w/v) BSA in PBS-T containing 0.02% azide for 2 h. This and all subsequent steps were performed at 4 °C. The nitrocellulose was incubated with a 1:250 dilution of affinity-purified sheep anti-biotin antibodies for 2 h in 1% BSA in PBS-T containing 0.02% thimerosal. After being washed three times for 10 min with PBS-T, the membrane was incubated for a further 2 h with donkey anti-sheep IgG conjugated with alkaline phosphatase (Serotec) in 1% BSA in PBS-T containing 0.02% thimerosal. The membrane was washed a further three times for 10 min with PBS-T. The blot was developed with BCIP/NBT for approx. 5 min at room temperature until the background began to develop, at which time the membrane was transferred to distilled water for extensive washing.

RESULTS

BCCP cDNA cloning

ACCase catalyses a pivotal step in fatty acid synthesis. Isolation of the ACCase II genes from oilseed rape is therefore desirable for the manipulation of fatty acid synthesis in the crop *B. napus*. To clone the BCCP subunit from the multi-subunit form of ACCase we required a probe. Previous data from this laboratory had shown that *Arabidopsis* DNA sequences are highly homologous with those of rape (results not shown). As a first step we analysed the *Arabidopsis* EST database for any *E. coli* BCCP-like sequences. A 410 bp sequence entry was identified that showed similarity to the *accB* gene (BCCP subunit) from *E. coli*. It contained a homologous biotin-binding domain sequence but its ORF was shorter than that of *E. coli* because a large part of the clone was 3′ untranslated region (UTR). The EST cDNA clone (EST-ID:ATTS1191, GenBank-ID:Z25714) was obtained from the Ohio State Genetic Resource Centre.

Having identified a heterologous probe we used it to screen 150000 plaque-forming units from both leaf- and embryo-derived poly(dT)-primed mRNA libraries from *B. napus* cv. Jet neuf. The primary round of leaf library screening yielded no hybridizing plaques. However, with the embryo library 24 positive plaques were identified, which were taken through three rounds of screening to plaque purity. Complete sequencing of several clones revealed six different classes (Figure 1) typified by pBP1 (842 bp), pBP2 (421 bp), pBP3 (925 bp), pBP4 (1008 bp), pBP6 (1133 bp) and pBP7 (720 bp), which all had significant similarity to other bacterial and algal BCCP sequences. The six classes fell into two groups based on their 3′ UTR and ORF similarity (group 1, pBP1 and pBP2; group 2, pBP3, pBP4, pBP6 and pBP7). The percentage derived amino acid sequence similarity/identity between pBP1 and pBP2 (subgroup 1) was 89.4%/83.3%, pBP4 and pBP6 (representatives of subgroup 2) 85.7%/80.1%, and pBP1 and pBP4 (representatives of subgroups 1 and 2) 65.9%/50.8%. Other inter- and intra-group comparisons showed very similar figures, further demonstrating the existence of the two subgroups. The *Arabidopsis* EST cDNA (EST-ID:ATTS1191) used to isolate the clones showed similarity to all the clones in the ORF region but only to the group 1 clones in the 3′ UTR region. The pBP2 insert was 79% identical at the DNA level, throughout the whole length, with the EST cDNA sequence. Analysis of the two longest clones pBP4/pBP6 identified a putative translational start methionine codon, which showed 100% homology with the consensus sequence for plants AACAAUGGC [51]. In addition both clones had classical poly(A) tails at their 3′ ends. Both features together suggest that pBP4 and pBP6 are full-length clones with respect to the coding region. Computer analysis, based on the method of von Heijne [52], of the sequence identified a putative transit peptide sequence cleavage site that is conserved between both full-length ORF clones, pBP4 and pBP6.

Comparison of the derived amino acid sequence from the longest clone (pBP6) with that of *Anabaena* and *E. coli* BCCPs shows that the similarity between the proteins is primarily at their N- and C-termini (Figure 2). A comparison of *Anabaena* and *E. coli* BCCPs is also presented pictorially to demonstrate the known divergences (Figure 2). The direct amino acid GAP comparison (BBSRC Computing Facility, Daresbury) with the *E. coli* BCCP shows similarity throughout the length of the *E. coli* protein with the longest continuous stretch occurring around the biotinylation site (Figure 2). It has been shown previously

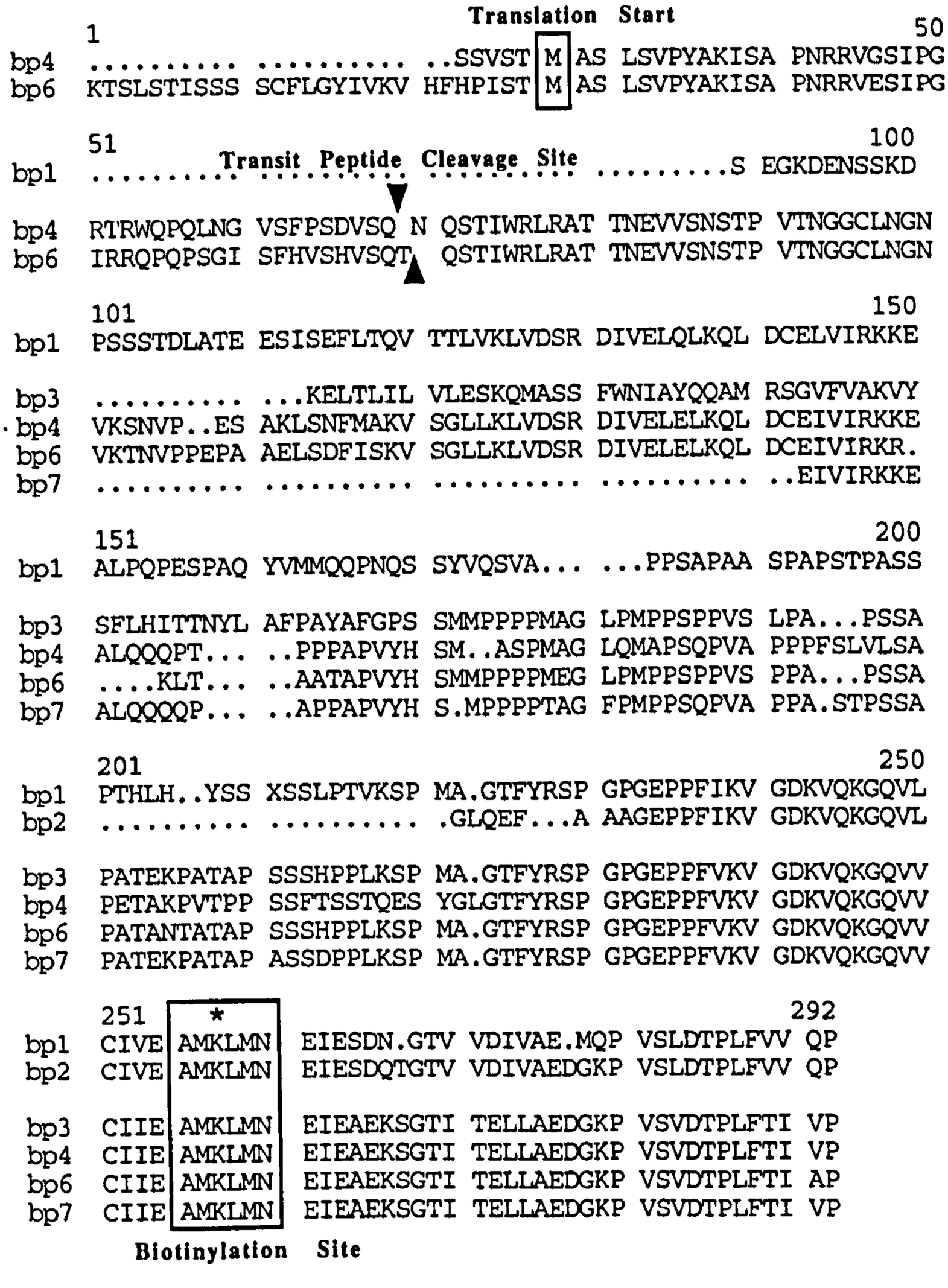


Figure 1 Derived amino acid sequences of BCCP

ORF-derived amino acid sequences of the *B. napus* BCCP cDNA clones pBP (shown as bp in the figure) 1/2/3/4/6/7 (EMBL accession numbers X90727–X90732 respectively). Sequences were aligned by using the CLUSTAL programme from Daresbury. The boxed regions show putative translational start and transit peptide cleavage sites as well as the conserved biotinylation site.

that approximately 30 amino acid residues on each side of the biotinylated site are important for biotinylation *in vivo* [53–55]. Although the rape BCCP biotinylation site identified in the derived amino acid sequences of all the clones differs from that of *E. coli* BCCP it is identical with that of *Anabaena* (Ala-Met-Lys-Leu-Met). A TBLASTN search [56] of all available databases with the pBP6-derived amino acid sequence showed *E. coli*, *Pseudomonas* and *Anabaena* BCCPs to have the highest scores of 237 (44% identical/59% similar over the last 104 residues), 255 (48% identical/64% similar over the last 102 residues) and 248 (61% identical/79% similar over the last 72 residues) respectively. Scores of over 100 are generally thought to be significant. The only other non-BCCP biotinylated protein that featured in the top 50 derived protein sequences was human propionyl-CoA carboxylase, with a score of 97. Most of the other proteins that showed similarity with the translated pBP6, with the TBLASTN method, had a high proline amino acid content, a key feature of the central region of the translated BCCP gene (Figure 1). The derived BCCP sequences showed an extraordinary number of proline/alanine residues, consisting of 35% of the amino acids

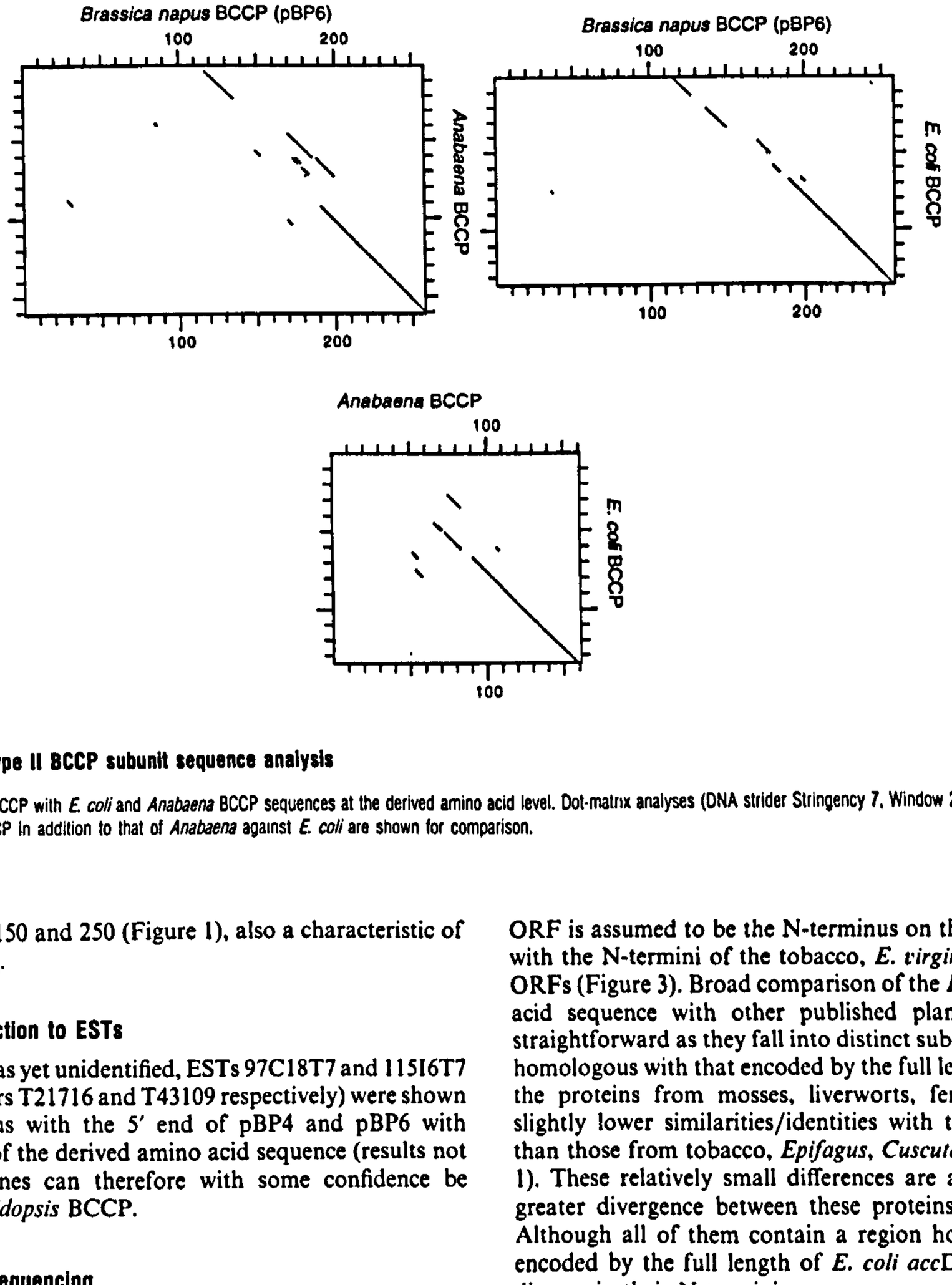


Figure 2 ACCase type II BCCP subunit sequence analysis

Comparison of *B. napus* BCCP with *E. coli* and *Anabaena* BCCP sequences at the derived amino acid level. Dot-matrix analyses (DNA strider Stringency 7, Window 23) of *B. napus* BCCP against *Anabaena* and *E. coli* BCCP in addition to that of *Anabaena* against *E. coli* are shown for comparison.

between residues 150 and 250 (Figure 1), also a characteristic of *E. coli* BCCP [13].

Assignment of function to ESTs

The overlapping, as yet unidentified, ESTs 97C18T7 and 115I6T7 (accession numbers T21716 and T43109 respectively) were shown to be homologous with the 5′ end of pBP4 and pBP6 with identity in 70% of the derived amino acid sequence (results not shown). The clones can therefore with some confidence be assigned as *Arabidopsis* BCCP.

βCT cloning and sequencing

Using all the available *acc*D homologue sequence data, degenerate oligonucleotides were used to amplify a single product of approx. 440 bp from *B. napus* total DNA. A PCR product of identical size was amplified from both rape chloroplast DNA and an *Arabidopsis* subclone containing the *acc*D homologue. The 440 bp PCR product was subcloned and fully sequenced (pβCT1), which showed it to be an *acc*D homologue. The 440 bp was used to screen a total genomic library of *B. napus* cv. Jet neuf and several clones were isolated. A *Hin*dIII fragment of approx. 5 kb was present in several overlapping genomic clones that hybridized to the pβCT1 insert. The *Hin*dIII fragment from one clone was subcloned (pβCT2) and sequenced (results not shown, accession number Z50868).

The ORF within pβCT2 encodes a protein of 489 amino acids that has 66% identity and 78% similarity over its full length with the *acc*D homologue encoded by the tobacco chloroplast DNA [33]. Three methionine residues are found very close to the first stop codon at the N-terminus. The residue giving the longest ORF is assumed to be the N-terminus on the basis of similarity with the N-termini of the tobacco, *E. virginiana* and *C. reflexa* ORFs (Figure 3). Broad comparison of the *B. napus acc*D amino acid sequence with other published plant sequences is not straightforward as they fall into distinct subclasses. In the region homologous with that encoded by the full length of *E. coli acc*D, the proteins from mosses, liverworts, ferns and *Pinus* have slightly lower similarities/identities with the *B. napus* protein than those from tobacco, *Epifagus*, *Cuscuta* and pea (see Table 1). These relatively small differences are a reflection of much greater divergence between these proteins in their N-termini. Although all of them contain a region homologous with that encoded by the full length of *E. coli acc*D (Table 1) [13] they diverge in their N-termini.

The mosses, liverworts and ferns [36–38] and *Pinus* [35] form a group in which the *acc*D proteins all possess a 25-residue extension, with a high degree of identity, beyond the N-terminus of the *E. coli* protein. Alignment of the five other plant protein sequences, which are much longer, revealed that the *B. napus*, tobacco and *E. virginiana* ORFs and amino acid sequences were most similar (Figure 3). The pea ORF contains two large inserts, one in each of the N- and C-terminal halves that result from repeated DNA sequences identified previously [31,32]. The alignment also showed the deletion of a region from the pea protein (from between Ile-72 and Thr-73) that is conserved in *B. napus*, tobacco, *E. virginiana* and *C. reflexa* (Figure 3). The ORF from *C. reflexa* also contains an insert (from Ser-69 to Asn-112) by comparison with the other amino acid sequences but is otherwise very similar. The functional significance of the N-terminal halves of the higher-plant polypeptides can, at present, only be speculated on. The presence of substantial regions of identity within this domain suggests conservation of structure

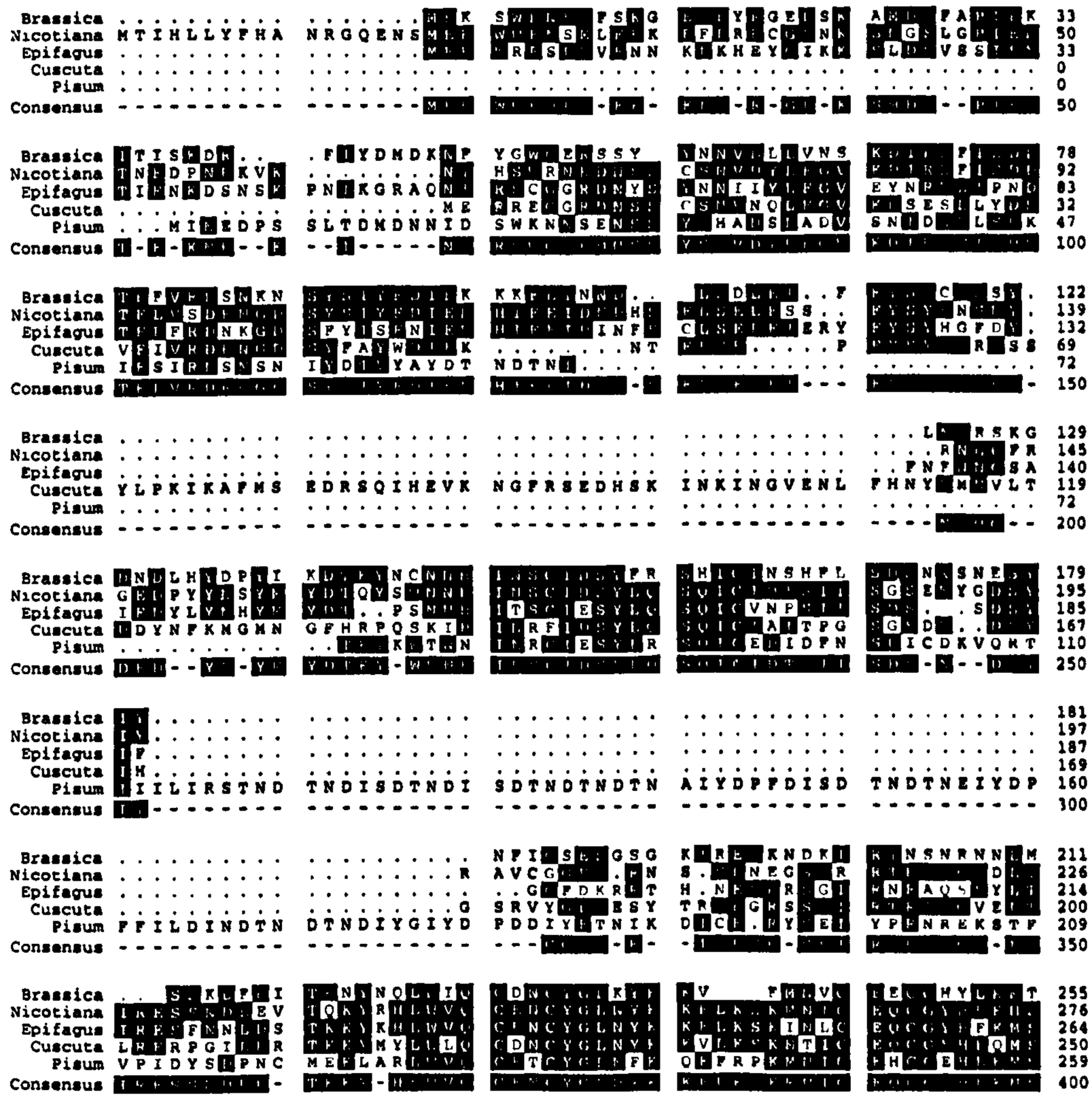


Figure 3 ACCase type II β-carboxyltransferase subunit sequence analysis

Amino acid alignment of the N-terminal halves of the predicted higher-plant polypeptides with similarity to the βCT subunit of ACCase [40]. Sequences were aligned by using the PILEUP and LINEUP programs and were taken from pea [31], tobacco [42], *Epifagus virginiana* [4] and *Cuscuta reflexa* (G. Haberhausen; EMBL accession number X69803). Identical residues are boxed.

and/or function. A search of the translation products from the EMBL Database with the conserved polypeptide motifs found within this region failed to identify any significant relationship with any other polypeptides. *B. napus* βCT is exceptional within the C-terminal region of the *acc*D homologues. The zinc-finger motif previously identified [31,32] is found as $CXXCX_{18}CXXC$ (where X is any amino acid) in all sequences, including that of *E. coli*, except for *B. napus* where the central domain contained only 12 residues (Figure 3). Apart from this difference and the insert present in pea, the C-terminal halves of all the *acc*D homologues are remarkably similar (Table 1).

Northern blot analysis of BCCP and βCT expression

Storage lipid synthesis occurs in *B. napus* seed with a maximal rate of production during the middle stage of embryogenesis. Turnham and Northcote [56] showed that ACCase enzymatic activity in rape embryos followed the rate of lipid deposition; more recently Kang et al. [57], using different extraction techniques, showed that ACCase activity increased before lipid deposition. In both cases the activity decreased before the maximal accumulation of lipid occurred. To study the mRNA expression of the BCCP genes, which are thought to be involved in *de novo* storage lipid synthesis, we screened a developmental Northern blot. Each lane on the blot had an equal amount of root, leaf or embryo poly(A)$^+$ mRNA from different stages of *B. napus* embryogenesis. The blot shown in Figure 4A was generated by screening with the pBP7 insert and was identical when all six cDNA types were used on separate occasions (results not shown). During embryogenesis, BCCP mRNA expression (1.2 kb full length) rose steeply, peaking during the middle portion of embryogenesis. Maximal mRNA expression occurred at 54 d, after which the level of BCCP mRNA dropped markedly. This profile mimicked that of the other *B. napus* fatty acid synthesis components enoyl reductase, β-keto reductase and ACCase I [20]. Although there was a notable amount of BCCP mRNA present in root there was a comparatively low level in leaf. The integrated phosphoimager data showed that the level of BCCP mRNA expression in 54-day embryos is 26 times that of leaves, which is itself only twice that of background.

Table 1 Comparison of the predicted amino acid sequence from *B. napus* with those of all other published sequences with similarity to the βCT subunit of ACCase [40]

The comparison was made by using the BESTFIT program and is restricted to residues 220–489 of the *B. napus* sequence. To enable sensible comparison the insert in the pea sequence (residues 234–394) has been deleted. Sequences are taken from the references described in Figure 3 and also: *Angiopteris lygodiifolia* [38], *Marchantia polymorpha* [36], *Physcomitrella patens* [37] and Black pine [35].

	B. napus	
Species	βCT identity (%)	βCT similarity (%)
Tobacco	79	86
E. virginiana	77	87
C. reflexa	72	83
Pea	68	81
M. polymorpha	69	79
Black pine	67	77
P. patens	66	76
A. lygodiifolia	66	76
E. coli	41	62

The *B. napus* chloroplastic genome-encoded βCT subunit was used in a Northern blot against total RNA from the same batch of whole seeds used for the BCCP Northern data (see Figure 4A). The cDNA insert hybridized to several bands of 2.4, 1.6, 1.3 and 1.1 kb (Figure 4B), which were expressed proportionally throughout embryogenesis, the strongest association being with a band of approx. 2.4 kb. Because all the bands are proportional we made the assumption that they all contain the βCT gene. Northern analysis of pea βCT was complicated in a similar way as a result of multiple transcription start sites and co-transcription with genes both 5′ and 3′ [31,39]. Nagano et al. [31] showed that three transcripts with sizes of 5.0, 2.8 and 2.4 kb contained the entire ORF of *acc*D and ORFs of both *trn*Q and *psa*I genes. The blot also shows (Figure 4B) that in leaf material there is one extra hybridizing RNA species not present in embryo tissue (2.7 kb) and a band visible in embryos (2.6 kb) not present in leaves.

It can be seen from a comparison of Figures 4A and 4B that the rape βCT mRNA expression was very similar to that of BCCP during embryogenesis. Because both proteins are thought to associate *in vivo* to form part of the type II ACCase complex, this was perhaps to be expected. The peak of expression occurred at 54 d after anthesis, followed by the characteristic decrease in expression. However, in contrast with BCCP the relative amount of βCT expression in leaf was higher with an embryo:leaf ratio of 7:2.

Anti-biotin Western blot of rape embryos

To gain a real insight into BCCP expression it was important to study protein levels as well as mRNA. In the absence of specific BCCP antibodies, and because BCCP is biotinylated *in vivo*, biotin-specific antiserum was used for this purpose. To generate biotin-specific antibodies, keyhole limpet haemocyanin was coated with biotin and used as an antigen in rabbits. To minimize false background signals in a Western blot the antibodies generated were affinity-purified. This was achieved by column chromatography on a biotin-agarose matrix. The generated antibodies were more specific and had a higher titre than those available commercially.

Previous work describing the prokaryotic BCCP studies showed that the proline/alanine-rich region of *E. coli* BCCP confers an SDS gel anomaly [13]. The protein runs at 35% less

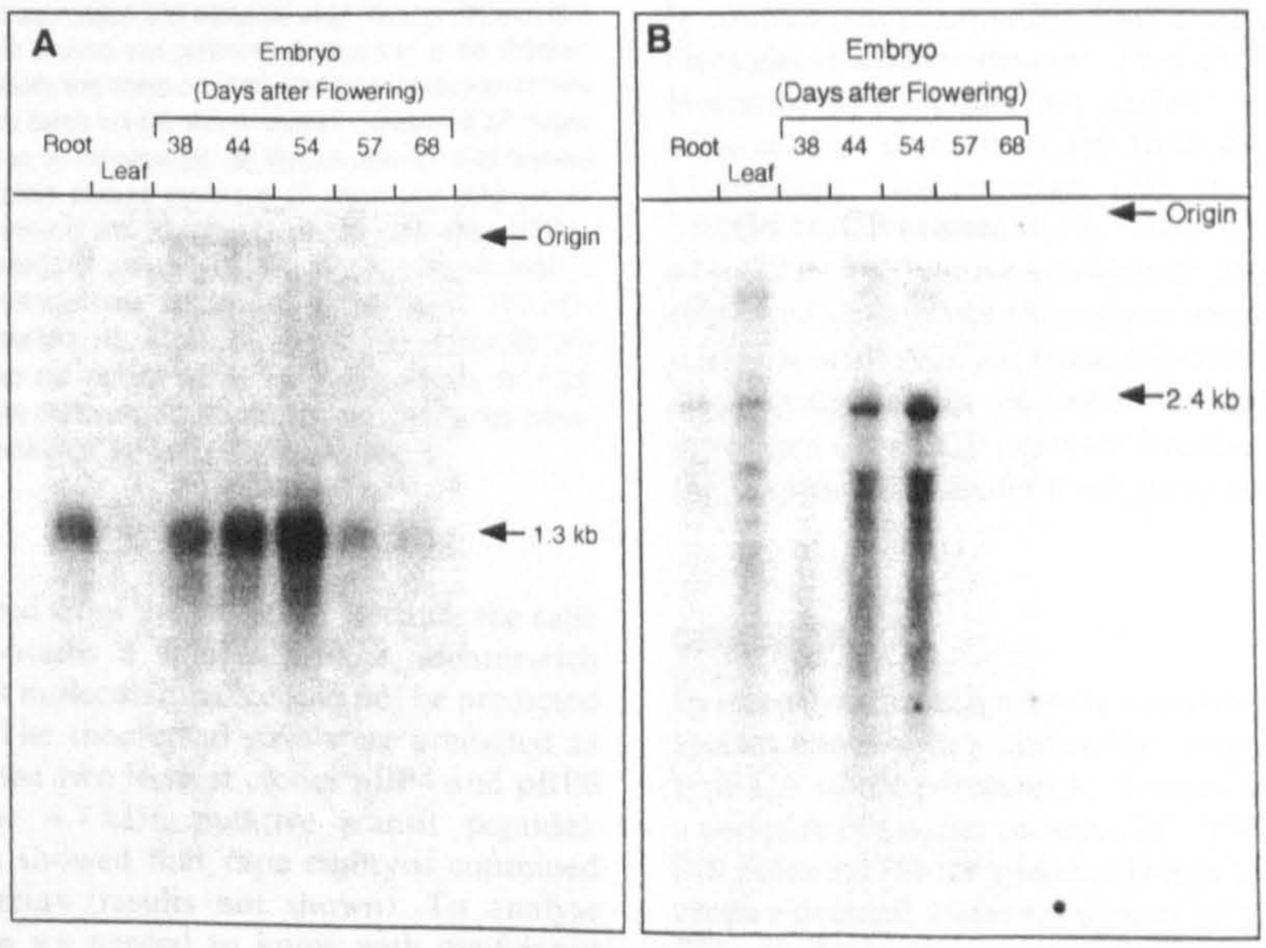


Figure 4 Northern blot analysis of ACCase type II BCCP and βCT subunit expression during rapeseed embryogenesis

(**A**) Northern analysis with the BCCP cDNA (pBP7 insert) as a probe: 1 μg of poly(A)$^+$ RNA from embryos 38, 44, 54, 57 and 68 d after anthesis and from root and young leaf was used for the blot. Hybridization and washing conditions were as described in the Materials and methods section. Phosphorimaging screens were exposed for 2 h. Molecular mass markers were revealed by ethidium bromide/UV. (**B**) Northern analysis with the rape β-carboxyltransferase domain DNA: 10 μg of total RNA from the same batch of embryos as in (**A**) was used for the blot. Hybridization and washing conditions were the same as in (**A**). The hybridizing bands were revealed by autoradiography with an exposure time of 7 d.

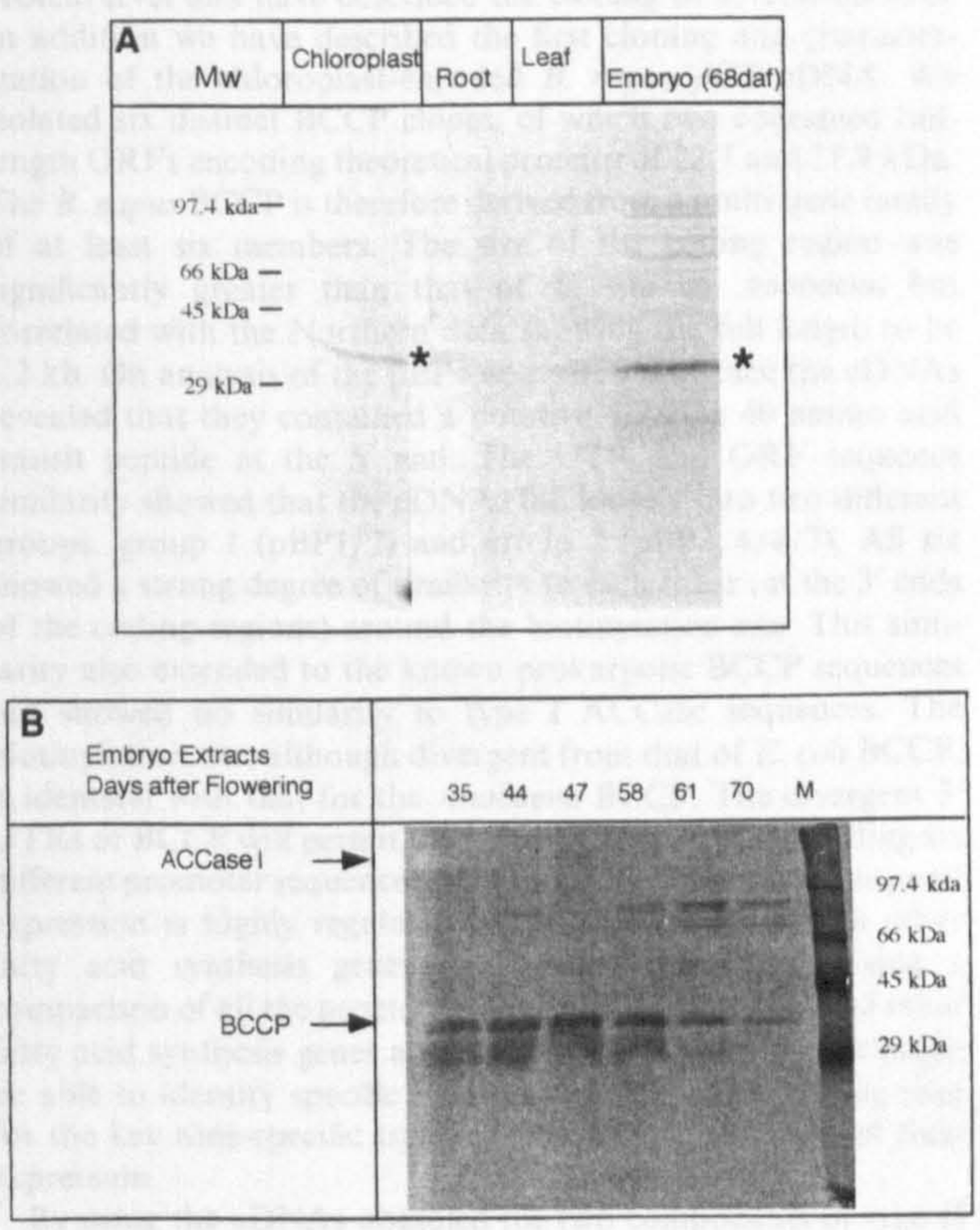


Figure 5 Western blot analysis of biotinylated proteins present in *B. napus* and identification of BCCP protein

(**A**) Extracts were derived from leaf chloroplasts, root, leaf and late embryogenesis embryos (as determined by morphology at 68 d after anthesis). SDS/PAGE was performed on 12% gels with an equal loading of root, leaf or embryo protein extract (5 μg total protein per lane). Intact chloroplasts (1 μg of total protein) and biotinylated SDS molecular mass markers (M) were also loaded in the respective lanes. Western analysis was performed as described in the Materials and methods section. The primary antibody was sheep anti-biotin and the secondary an alkaline phosphatase-conjugated antiserum. The bands marked with an asterisk represent BCCP protein at 35 kDa. All the lanes shown were run on the same gel. (**B**) Western analysis of biotinylated proteins. Western analysis of biotinylated proteins present in *B. napus* was performed on embryo extracts representative of the whole of embryogenesis (judged by morphology and days after anthesis). Volume-equalized loadings of extracts (adjusted for the respective mass of embryos used, i.e. 30–50 ng of embryos) were separated by SDS/PAGE on 12% gels. Biotinylated SDS molecular mass markers (M) were also loaded. Western analysis was performed as described in the Materials and methods section. The primary antibody was rabbit anti-biotin and the secondary an alkaline phosphatase-conjugated antiserum. The bands marked with the ACCase label indicate the position of the type I 220 kDa ACCase.

mobility than that predicted from the sequence. Because the rape BCCP sequence also contains a similar proline/adenine-rich region (Figure 1) the exact molecular mass could not be predicted from SDS gel mobility. The theoretical sizes were predicted as 22.7 and 21.9 kDa from the two longest clones pBP4 and pBP6 respectively (without the 4.7 kDa putative transit peptide). Earlier work in Durham showed that rape embryos contained several biotinylated proteins (results not shown). To analyse BCCP protein expression we needed to know with confidence which biotinylated protein band on SDS/PAGE was BCCP. The only other plant precedents [58,59] showed that the one biotinylated protein in pea chloroplasts was the 35 kDa BCCP subunit of ACCase. Similarly a Western blot of *B. napus* chloroplast protein showed that only one biotinylated protein resides within the chloroplast (Figure 5A). It is very probable that this represents the BCCP protein allowing identification of the 35 kDa biotinylated protein in root, leaf and embryo as BCCP (indicated by the asterisks in Figure 5A). Purification of plastids from rapeseed embryos also revealed that the 35 kDa biotinylated polypeptide is localized within the plastid [60]. Although the same levels of protein were loaded in each lane, BCCP levels were low in both root and leaf in comparison with that in embryo. In the rape embryo BCCP is the major biotinylated protein present (approx. 40%). There is also independent evidence that BCCP migrates more slowly on SDS/PAGE than expected from its predicted size. The pBP4 insert was cloned into an overexpression vector and overexpressed by inducement by isopropyl β-D-thiogalactoside (results not shown). The expressed protein (22.7 kDa theoretical size without the putative targeting sequence) was shown to be biotinylated *in vivo* and ran at 33 kDa on SDS gels (results not shown).

As in all organisms the presence of polypeptides, and their amounts, vary throughout embryogenesis. Previously the question of how many biotinylated proteins are in plants had been addressed, and it was shown to be about six. So far nobody had shown (i) how many were present in rapeseed and (ii) the differential expression in the embryo during embryogenesis. Figure 5B shows the Western blot of the embryos at different stages. During the first three stages there were three biotinylated proteins present at 220, 82 and 35 kDa, which most probably represented ACCase I, methylcrotonyl-CoA carboxylase (MCCase) and BCCP from the ACCase II complex. The smeary bands below the BCCP were most probably partial degradation products of BCCP because they were much more pronounced, and the 35 kDa BCCP levels lower, when samples were prepared by the relatively slow method of maceration/ammonium sulphate precipitation/dialysis (results not shown). At 58 d after anthesis three more biotinylated proteins appeared at 50, 70 and 88 kDa. Their levels rose to a peak at 61 d and dropped slightly at 70 d, just before the desiccation stage. Another protein band of 75 kDa appeared at 61 d and stayed present at the same level until 70 d. In contrast, the putative MCCase levels remained fairly consistent throughout embryogenesis. This profile was supported by a Northern blot (results not shown) with *Arabidopsis* MCCase sequenced at Durham (a gift from Dr. Reddy, Colorado State University). This contrasts with the 220 kDa ACCase I and 35 kDa BCCP protein levels, which decreased significantly from 47 to 70 d. The relative amounts of ACCase I and BCCP protein mimicked each other throughout embryogenesis. Although the overall level of ACCase I detected was much lower, a quantitative comparison cannot be made because the Western blot was optimized for BCCP analysis. Because ACCase I is much larger the efficiency of transfer from gel to membrane was much lower.

DISCUSSION

In recent years it has become apparent that dicotyledonous plant species contain two distinctive forms of the enzyme ACCase: type I, a single polypeptide of more than 220 kDa; and type II, a complex of smaller proteins BC, BCCP and CT. To achieve the full potential for the genetic manipulation of lipid products from crops a detailed understanding of fatty acid synthesis is required. This is dependent on a full characterization of the proteins involved, and more specifically on the pivotal enzyme ACCase. We have previously described the cloning of partial-length cDNAs from the *B. napus* type I ACCase [20].

In this paper we have studied two constituents of the multipeptide type II ACCase from *B. napus*. We have outlined the analysis of BCCP from a plant source at the mRNA, DNA and

protein level and have described the cloning of several cDNAs. In addition we have described the first cloning and characterization of the chloroplast-encoded *B. napus* βCT cDNA. We isolated six distinct BCCP clones, of which two contained full-length ORFs encoding theoretical proteins of 22.7 and 21.9 kDa. The *B. napus* BCCP is therefore derived from a multi-gene family of at least six members. The size of the coding region was significantly greater than that of *E. coli* or *Anabaena* but correlated with the Northern data showing the full length to be 1.2 kb. On analysis of the pBP4 and pBP6 sequence the cDNAs revealed that they contained a putative 4.7 kDa 40 amino acid transit peptide at the 5′ end. The UTR and ORF sequence similarity showed that the cDNAs fall loosely into two different groups, group 1 (pBP1/2) and group 2 (pBP3/4/6/7). All six showed a strong degree of similarity to each other (at the 3′ ends of the coding regions) around the biotinylation site. This similarity also extended to the known prokaryotic BCCP sequences but showed no similarity to type I ACCase sequences. The biotinylation site, although divergent from that of *E. coli* BCCP, is identical with that for the *Anabaena* BCCP. The divergent 3′ UTRs of BCCP will permit the isolation of the corresponding six different promoter sequences. This study has shown that temporal expression is highly regulated and almost identical with other fatty acid synthesis genes that we have isolated. Using a comparison of all the promoter sequences from BCCP, and other fatty acid synthesis genes available in our laboratory, we might be able to identify specific sequences representing binding sites for the key time-specific transcription factors that control their expression.

By using the cDNAs obtained for two components of type II ACCase we were able to compare their expression directly at the RNA level. The βCT and BCCP mRNA expression levels during embryogenesis were very similar. Previous work has shown that rape type I ACCase and several other lipid synthesis genes are expressed in a very similar manner [20]. However, the Northern analysis of the βCT was complicated by the presence of several hybridizing bands. This was also observed by Sasaki et al. in their analysis of pea leaf βCT [42]. Given that the βCT primary transcript size is 2.4 kb in *B. napus* and yet the ORF is less than 1.5 kb long, there may be another ORF on the transcript. In the chloroplast DNAs of several other species such as pea the *acc*D gene is followed closely (by less than 180 bp) by the *psa*I gene, which has a very small ORF (120 bp in pea [39]). As in pea there may be several transcription start signals at the 5′ end of the ORFs that may all be used, giving rise to several bands seen on the Northern analysis.

The Western blot data that we present here show that rape leaf chloroplasts contain the biotinylated 35 kDa BCCP protein, which in turn implies that the putative transit peptide sequence is chloroplastic. In addition because leaf-to-embryo BCCP protein ratios are higher than that for mRNA we can only assume that either protein turnover is much greater in embryos or the translational control is much less prevalent in the leaf. The protein was larger on Western analysis than the theoretical size expected but the cDNA sequence showed that, like *E. coli* BCCP, the central region was rich in alanine and proline. This particular sequence characteristic has been shown previously to reduce the mobility of proteins on SDS/PAGE, i.e. the *E. coli* BCCP runs as a 22 kDa band in contrast with its 17 kDa theoretical size. The rape BCCP similarity to *E. coli* BCCP also extends to the *E. coli* BCCP N-terminus. It has been shown before that the N-terminal region represents the site for protein–protein interaction [61], whereas the proline–alanine central region is thought to act as a flexible arm to allow the carboxy group to move between subunit active sites. The BCCP genes will allow the construction of antisense constructs to study the function of type II ACCases *in vivo*. We have already generated antisense type I ACCase transgenic rape plants, which in combination with BCCP antisense plants will allow us to differentiate between the roles of both types of ACCase within dicotyledonous species.

Using the anti-biotin antibodies generated for this study we analysed all the biotinylated proteins during rapeseed development. The major biotinylated protein in the embryo was the BCCP, which showed expression correlating to its mRNA expression. This is in marked contrast with that in pea where the BCCP has not been detected in the embryo [62]. This may reflect the different major storage products used by the two plants. Peas have only 2% of their mass as oil whereas rape has approx. 45%. With embryo morphology as a marker, the peak of BCCP expression occurred simultaneously to that of maximal lipid synthesis, after which expression was markedly reduced. We obtained enough affinity-purified antibody to generate an anti-biotin immunochromatography column. This could be used to eventually purify the BCCP from seed extracts for protein analysis.

Several other biotinylated proteins were identified, some of which can be tentatively attributed to type I ACCase and MCCase. Without functional analysis the identification of the other proteins was impossible but previous work detected pyruvate carboxylase activity in embryo extracts, a biotinylated enzyme (results not shown). Some of the bands might also represent the rape equivalent of the biotin-binding protein in pea embryos [62] and geranoyl-CoA carboxylase recently partly purified from maize [63].

Recent research has isolated the biotin carboxylase [49] and previous research the β-carboxyltransferase subunit clones [31–38,42] from different plant sources. In this report we have described the first isolation of plant full-sized BCCP and in addition a βCT clone from the same species. With the isolation of the α-carboxyltransferase subunit and the purification of expressed gene products the stage will be set for the full characterization of type II ACCase from plants at the protein level. Because the subunits are thought to form a complex *in vivo* by protein–protein interactions it may be important to study the subunits from the same plant source. In isolating two of the three or four subunit types we have gone some way to achieving this in the agriculturally relevant crop *B. napus*.

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