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The overexpression and characterisation of Enoyl ACP Reductase from Brassica. napus: a component enzyme of fatty acid synthetase in plants

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The Overexpression and Characterisation

of Enoyl ACP Reductase from

Brassica napus

A Component Enzyme of Fatty Acid

Synthetase in Plants.

A thesis submitted to the

University of Durham

for the degree of

MASTER of SCIENCE

in
Plant Molecular Biology and Biochemistry

by
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1995

The Overexpression and Characterisation of Enoyl ACP Reductase from Brassica napus - A component Enzyme of Fatty Acid Synthetase in Plants.

J.W.Simon

Abstract

The main objecive of this research is to advance our understanding at the structural and expressional level of plant enoyl ACP reductase (ER), the enzyme which catalyses the last reductive step in the de-novo biosynthesis of fatty acids. Two different substrates (CoA and ACP) can be used for this enzyme. Large scale preparations of crotonyl CoA were prepared using crotonic anhydride and CoA and the reaction product was quantified and authenticated. ACP (*E.coli*) was prepared, using standard literature methods, and the homogeneity of the product was verified by SDS/PAGE and N-terminal amino acid sequence determination. Recombinant plant ER, under the control of the T7 promoter and the T7 polymerase, was produced in large quantities in *E.coli*, and purified to homogeneity using a procedure developed in this study. The recombinant protein was authenticated by both amino acid analysis and N-terminal amino acid analysis. Spectral determinations were performed which allowed for the first time an extinction coefficient (2.95×10^5) of the enzyme to be determined. Antibodies were raised in mice against denatured recombinant ER and two dimensional western blotting was performed to establish the complexity of isoform expression in rape seed and leaf material. CD spectroscopy was carried out on the protein, to make structural predictions of α-helix and β-sheet which were correlated with those determined directly from X-ray crystallographic studies. In collaboration with the University of Sheffield, plant ER was successfully crystallised, several heavy metal derivatives prepared and the complete 3-dimensional structure of the NADH enzyme complex at 1.9 Angstrom resolution was determined. Inhibitor studies using phenylglyoxal were performed and it was found that ACP (500µM) and CoA (10mM) would give total protection against loss of biological activity. Contrary to expectations no evidence could be obtained for the specific modification of a single arginine residue using phenylglyoxal, suggesting that this reagent may not be as specific as earlier reported.

Contents

Chapter 1

Introduction

Chapter 2

Materials and Methods.

Chapter 3

Results

**Purification, characterisation, enzyme kinetics
and crystallography of recombinant enoyl ACP
reductase.
Purification of acyl carrier protein from *E.coli*.
Isoforms of Enoyl ACP reductase in *Brassica napus*
plant extracts.**

Chapter 4

Results

**Enoyl ACP reductase active site investigation using
covalent modification studies.**

Chapter 5

General discussion and future work.

References

List of publications associated with this

research.

List of Figures

x

List of Tables

Abbreviations

A   Absorbance.

ACC   Acetyl CoA carboxylase.

ACP   Acyl carrier protein.

BSA   Bovine serum albumin.

CAPS   3-(Cyclohexylamino)-1-propane sulfonic acid.

CD   Circular dichroism.

CoASH   Coenzyme A.

DNA   Deoxyribonucleic acid.

DNAse   Deoxyribonuclease.

DTT   Dithiothreitol.

EDTA   Ethylene diamine tetra acetic acid.

ER   Enoyl ACP reductase.

FAS   Fatty acid synthetase.

FPLC   Fast performance liquid chromatography.

HPLC   High performance liquid chromatography.

IEF   Isoelectric focusing.

IPTG   Isopropyl-thiogalactosidase.

kDa   Kilodalton.

MWCO   Molecular weight cutoff.

NAD   Nicotinamide adenine dinucleotide.

NADH   Reduced nicotinamide adenine dinucleotide.

PAGE   Polyacrylamide gel electrophoresis.

PHB   Polyhydroxybuterate.

PVDF   Polyvinylidene difluoride.

rpm   Revolutions per minute.

SDS   Sodium dodecyl sulphate.

TAG   Tri-acylglycerol.

TEMED   N,N,N'N'-teramethylethylene diamine.

TFA   Trifluoroacetic acid.

TPCK   n-Tosyl-L-phenylalanine chloromethyl ketone.

TRIS   Tris (hydroxymethyl) aminomethane.

U   Units of enzyme activity.

Acknowledgements.

First in my list of acknowledgements must be Professor Toni Slabas for allowing me the opportunity to carry out this research whilst working in his laboratory. He was enthusiastic towards the idea of this research from the beginning and continued to give large amounts of his time to offer advice and encouragement throughout the research period. I would also like to thank Dr Brian Whitton who played no direct role in this research project but who was responsible for my initial schooling in science and has over the years continued to encourage and help with my career. I would also like to thank the Department of Biological Sciences at The University of Durham for allowing me the use of its facilities to carry out this research.

On a daily basis Dr Kieran Elborough and Dr Tony Fawcett offered valuable help and discussion and I thank them for this. John Gilroy as a friend showed a real interest in my research project, he was able to offer many useful suggestions and the benefits of his years of experience in protein chemistry and sequencing.

Finally to my wife Sarah, who has a firm belief that anything is possible, this kept me on the right track when things became difficult. Thank you for your tolerance and your help towards the completion of this thesis.

Declaration

The work presented within this thesis and submitted for the degree of Master of Science is the result of my own research. It has not been submitted previously for a degree in this or any other University.

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Chapter 1

Introduction

Biosynthesis of Lipids in Plants.

Lipids are a diverse group of chemicals which can be
broadly defined as insoluble in water and highly soluble
in non-polar organic solvents. Lipids are present in
cells as esterified fatty acids and the nature of the
fatty acid composition defines the lipids physical
properties.

In plants the major functions of lipids can be divided
into the following three categories:-

Structural - Lipids are an important component of
the bilayer of membranes in which they are present in
the form of phospholipids, galactolipids and sterol
esters. These plant membrane lipids are mainly long
chain, 16-18 carbon units in length with little
variation in their degree of saturation.

They provide the boundaries between subcellular
organelles, the external limits to cells, and also the
correct environment for a number of proteins and enzymes
to function.

Biosynthetic - Lipids act as intermediates in complex
lipid biosynthesis and as substrates for fatty acid

desaturation. Lipids also play a variety of less well defined roles such as defence chemicals, hormones and as attractants for insects.

Storage - The triacylglycerols (TAG) are a major form of carbon storage in the oil bodies of seeds and other lipid rich materials. The amount of this TAG in the seed from different species may vary from as little as 1-2% to as much as 60% of the total dry weight of the seed. Whilst there is little variation in the fatty acid composition of the structural lipids there is a wide variation in the composition of fatty acids found in the seed oils. The fatty acid properties of seed oils vary, and it is often the unusual fatty acid composition of a particular triglyceride which makes the oil of commercial value. The short chain (C12:0) (palm oil kernel and coconut) are used for detergents, the medium chain (C16-18) saturated and unsaturated (soybean, sunflower, olive and rapeseed) are generally edible and the longer chain (C22:1) (high erucic rapeseed) are used as lubricants.

Apart from these central roles within plants, lipid molecules have also been shown to play key roles in cell signalling (Truchet *et al.*, 1991), in determining host specificity in nodulating bacteria (Lerouge *et al.*, 1990) and in the structure of the cutin coat on the aerial surface of plants

1.1 The Economic Importance for the Study of the Pathways of Lipid Biosynthesis in Plants.

There are three main considerations which make the study of the biosynthesis of lipids in plants of economic importance.

a) Altering the lipid storage product in oil seeds.
Seed storage lipids from plants which include, sunflower, safflower, soybean, rapeseed, coconut and palm are a readily available source of lipids which represent a good feedstock for the chemical, detergent and food industry. The seeds when mature are generally easily harvested in a dry state and can be readily transported.

This source of natural oils accounted for almost 75% of the total world production of oils and fats in 1990 (Rattery, 1991) and this is expected to increase even further in the future. It represents a renewable resource which would help the demands for a sustainable agriculture and an environmentally friendly oil supply. Although a great deal of information exists on the composition of the various seed oils there is much less known on how the oils are synthesised and how this synthesis is regulated.

Elucidating the pathway of biosynthesis of lipids in plants is an essential component to understanding its regulation. This would allow the modification of naturally occurring products (which are not necessarily of optimal composition) and a potential increase in the yield of these products.

b) Engineering frost resistance in plants.

It has been shown that the degree of unsaturation of plant membrane lipids affects the fluidity of the membrane when subject to chilling temperatures (Murata 1983, Lynch and Thompson 1984, and Wada et al., 1990). The ability to be able to increase the degree of unsaturation in plant membrane lipids would allow them to have greater membrane-fluidity at lowered temperatures. Plants containing modified membranes would then be able to retain their biochemical functions during cold temperature acclimatisation.

c) Potential new Products.

There is a wide diversity of fatty acids currently synthesised by bacteria, plants and fungi, and many of these are considered commercially attractive. The genes for these products could be transferred to major oil seed crops which could then be grown in the conventional way to produce these new products. An example of this is

4

the ability to produce the polymer polyhydroxybutyrate
(PHB) by incorporating the genes encoding the three
enzymes (acetyl-CoA acetyl transferase, hydroxybutyryl
CoA dehydrogenase and PHB synthase) involved in PHB
synthesis from a bacteria (*Alcaligenes eutrophus*) into
Arabidopsis (Poirier *et al.*, 1992).

1.2 The Historical Background on Fatty acid Biosynthesis in Plants.

Early studies on the biosynthesis of plant fatty acids
involved the use of $[^{14}C]$ acetate in metabolic labelling
experiments. These experiments clearly demonstrated that
acetate can be incorporated into fatty acids and that
lipids are synthesised from acetyl CoA and malonyl CoA
(for reviews see Stumpf, 1980 and 1987).

The key biochemical steps involved are:-

i)   Provision of acetyl CoA from either the pyruvate
      dehydrogenase reaction inside the plastid, or from
      extraplastid production of acetate followed by its
      activation inside the plastid by acetyl CoA
      synthetase.

ii)   Conversion of acetyl CoA to malonyl CoA by the
      action of acetyl CoA carboxylase (ACC).

iii) Conversion of acetyl CoA and malonyl CoA to fatty
      acids by the fatty acid synthetase enzymes (FAS).

In plants the biosynthesis of fatty acids occurs in two
compartments:-

i)   Within the plastid, (chloroplasts in leaf and
     proplastids in seed) where the synthesis of C16
     (palmitoyl) and C18 (stearoyl) fatty acids takes
     place.

ii) Extra plastid, where acyl CoA thioesters can be
     further elongated by the action of a membrane bound
     FAS at the endoplasmic reticulum. The modified
     fatty acids are then either incorporated into
     storage lipids and phospholipids(by
     acyltransferases) or re-imported back into
     the plastid for incorporation into complex lipids.

An outline of this two compartment model is shown in
figure 1.2.1. below.

Acetate or other metabolites enter the plastid, and are
converted to acetyl CoA, which is then converted to
malonyl CoA by the action of acetyl CoA carboxylase.
These two substrates (acetyl and malonyl CoA) are then
used to synthesise fatty acids up to C18 in chain length
(as their ACP derivatives) by the action of fatty acid
synthetase (FAS). Desaturation can then occur by the
action of a soluble stearoyl- ACP desaturase, before the
lipids are either incorporated into complex plastid

lipids by acyl transferases or hydrolysed to free fatty
acids by acyl ACP thioesterase.
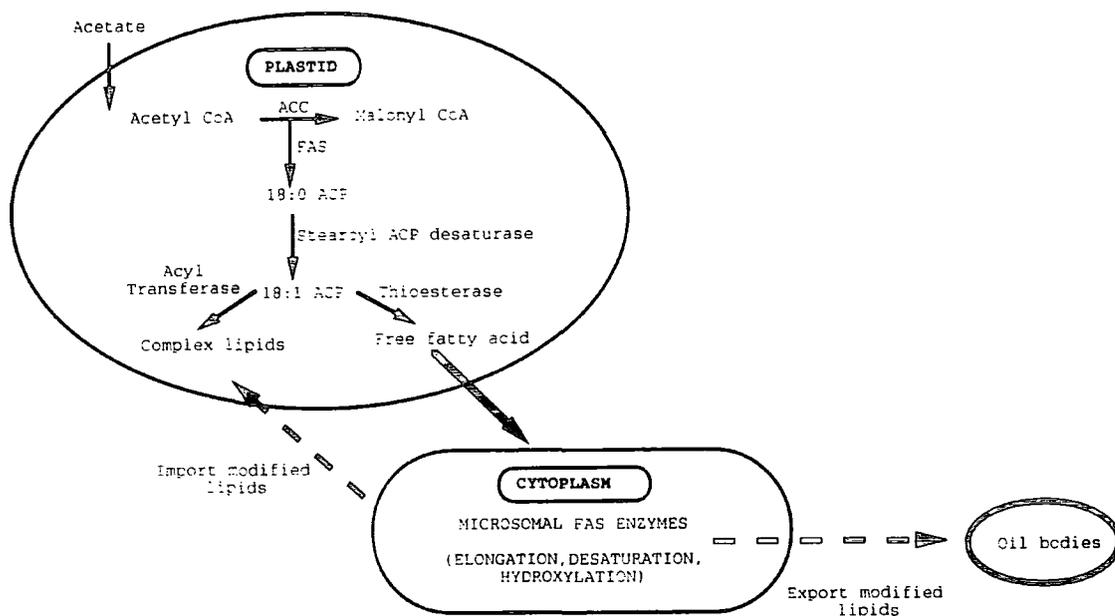
Figure 1.2.1
The two compartment model of plant fatty acid synthetase.

The free fatty acids are then exported from the plastid
to the cytoplasm, by a mechanism which has not been
strictly defined experimentally, for further elongation,
desaturation and hydroxylation before the lipid is
either reimported into the plastid or incorporated into
storage triglycerols.

Early pioneering work by Shimakata and Stumpf (1982)
amongst others, established the basic "core" facts for
fatty acid biosynthesis in plants. The model established
that at least six individual enzymes were involved in
the formation of C16 and C18 fatty acids in higher
plants. The formation of these fatty acids also involved

acyl carrier protein (ACP) as the acyl carrier between
these individual enzyme reactions.

1.3 The Plant Fatty Acid Synthetase.

There are two basic types of fatty acid synthetase (FAS)
which occur, classified according to their structural
organisation. A fully associated (Type I) which is
represented by the yeast (Lynen, 1969) and mammalian
systems (Bloch and Vance, 1977) where all the component
activities are present on one or two polypeptide chains.
For yeast the functional enzymes is $\alpha6\beta6$ and for animals
$\alpha2$.

The second type is a freely dissociable (type II)
represented by the plant and bacterial systems. In
contrast to type I the type II systems are characterised
by all of the individual component activities being
present as loosely associated polypeptides which can be
readily separated and purified.

The individual component reactions of plant fatty acid
synthetase are outlined in figure 1.3.1 below.

Figure 1.3.1 The individual component reactions of plant fatty acid synthetase.

ACETYL CoA -ACP TRANSACYLASE
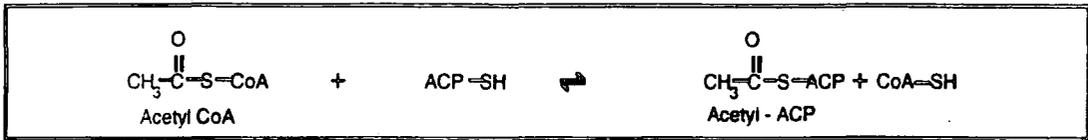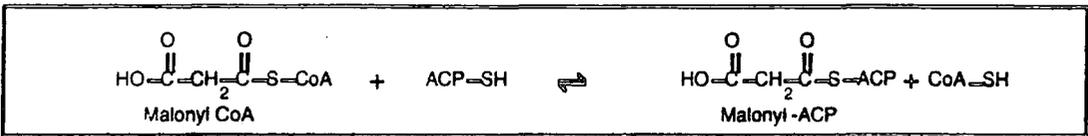
$$CH_3\!-\!\overset{\overset{\displaystyle O}{\|}}{C}\!-\!S\!-\!CoA \quad + \quad ACP\!-\!SH \quad \rightleftharpoons \quad CH_3\!-\!\overset{\overset{\displaystyle O}{\|}}{C}\!-\!S\!-\!ACP \; + \; CoA\!-\!SH$$

Acetyl CoA   Acetyl - ACP

MALONYL CoA-ACP TRANSACYLASE

$$HO\!-\!\overset{\overset{\displaystyle O}{\|}}{C}\!-\!CH_2\!-\!\overset{\overset{\displaystyle O}{\|}}{C}\!-\!S\!-\!CoA \quad + \quad ACP\!-\!SH \quad \rightleftharpoons \quad HO\!-\!\overset{\overset{\displaystyle O}{\|}}{C}\!-\!CH_2\!-\!\overset{\overset{\displaystyle O}{\|}}{C}\!-\!S\!-\!ACP \; + \; CoA\!-\!SH$$

Malonyl CoA   Malonyl -ACP

3-KETOACYL-ACP SYNTHASE III

$$CH_3\!-\!\overset{\overset{\displaystyle O}{\|}}{C}\!-\!S\!-\!CoA \; + \; HO\!-\!\overset{\overset{\displaystyle O}{\|}}{C}\!-\!CH_2\!-\!\overset{\overset{\displaystyle O}{\|}}{C}\!-\!S\!-\!ACP \quad \rightleftharpoons \quad CH_3\!-\!\overset{\overset{\displaystyle O}{\|}}{C}\!-\!CH_2\!-\!\overset{\overset{\displaystyle O}{\|}}{C}\!-\!S\!-\!ACP \; + \; CO_2 \; + \; CoA\!-\!SH$$

Acetyl CoA   Malonyl-ACP   Acetoacetyl-ACP

3-KETOACYL-ACP REDUCTASE

$$CH_3\!-\!\overset{\overset{\displaystyle O}{\|}}{C}\!-\!CH_2\!-\!\overset{\overset{\displaystyle O}{\|}}{C}\!-\!S\!-\!ACP \; + \; NADPH \; + \; H^+ \quad \rightleftharpoons \quad CH_3\!-\!\overset{\overset{\displaystyle OH}{|}}{C}\!-\!CH_2\!-\!\overset{\overset{\displaystyle O}{\|}}{C}\!-\!S\!-\!ACP \; + \; NADP^+$$

Acetoacetyl-ACP   D(-) hydroxybutyroyl-ACP

3-HYDROXYACYL-ACP DEHYDRASE

$$CH_3\!-\!\overset{\overset{\displaystyle OH}{|}}{C}\!-\!CH_2\!-\!\overset{\overset{\displaystyle O}{\|}}{C}\!-\!S\!-\!ACP \quad \rightleftharpoons \quad \text{Crotonyl-ACP} \; + \; H_2O$$

D(-) hydroxybutyryl-ACP

ENOYL-ACP REDUCTASE

$$\text{Crotonyl-ACP} \; + \; NADH \; + \; H^+ \quad \rightleftharpoons \quad CH_3\!-\!CH_2\!-\!CH_2\!-\!\overset{\overset{\displaystyle O}{\|}}{C}\!-\!S\!-\!ACP \; + \; NAD^+$$

                                       Butyroyl-ACP

3-KETOACYL-ACP SYNTHASE I

$$CH_3\!-\!CH_2\!-\!CH_2\!-\!\overset{\overset{\displaystyle O}{\|}}{C}\!-\!S\!-\!ACP \; + \; HO\!-\!\overset{\overset{\displaystyle O}{\|}}{C}\!-\!CH_2\!-\!\overset{\overset{\displaystyle O}{\|}}{C}\!-\!S\!-\!ACP \quad \rightleftharpoons \quad CH_3\!-\!CH_2\!-\!CH_2\!-\!\overset{\overset{\displaystyle O}{\|}}{C}\!-\!CH\!-\!\overset{\overset{\displaystyle O}{\|}}{C}\!-\!S\!-\!ACP$$

Butyroyl-ACP   Malonyl-ACP   3-ketohexanoyl-ACP

$$+ \; CO_2 \; + \; ACP\!-\!SH$$

9
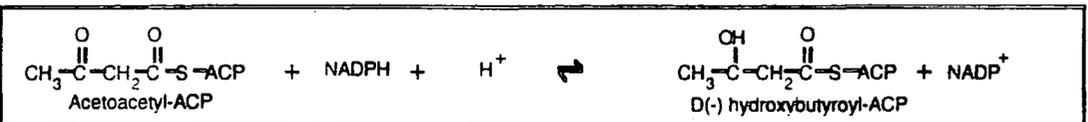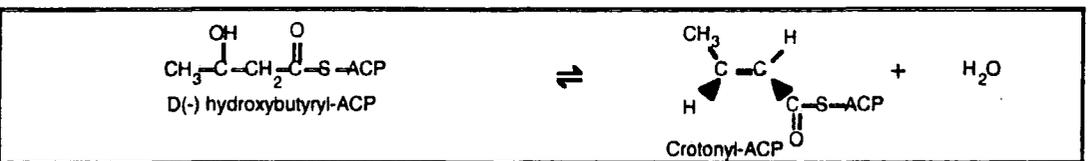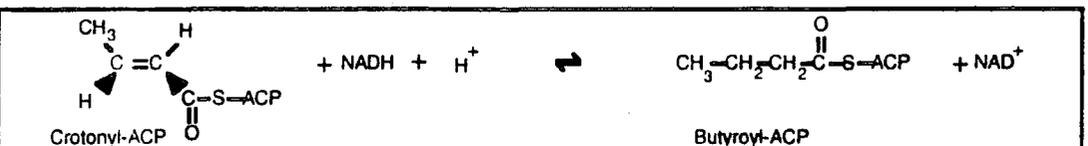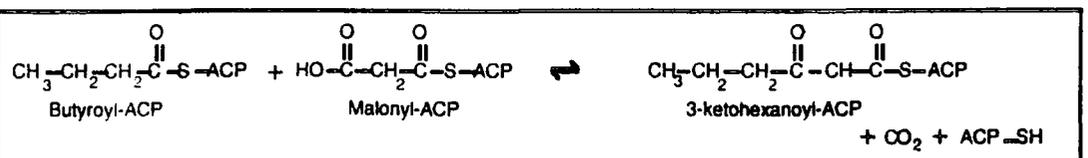
The initial reaction in plant FAS was believed until recently to be catalysed by acetyl ACP transacylase which adds an acetyl group onto ACP to form acetyl-ACP, which is then condensed with malonyl-ACP by the action of β-ketoacyl ACP synthetase (KAS I) to form acetoacetyl-ACP. However an alternative initial reaction involving the condensation of acetyl CoA with malonyl-ACP, (to form acetoacetyl-ACP) catalysed by β-ketoacyl-ACP synthetase III (KASIII), first discovered in E.coli (Jackowski and Rock, 1987) and more recently in plants (Jaworski et al., 1989 : Walsh et al., 1990) has been defined using cerulenin insensitivity. The subsequent condensations (up to C16) between the acyl-ACP intermediates and malonyl-ACP are catalysed by KAS I.

Following each condensation the β-ketoacyl-ACP intermediates are reduced (β-ketoacyl-ACP reductase), dehydrated (β-hydroxyacyl ACP dehydrase) and reduced again (enoyl ACP reductase) to yield a saturated acyl-ACP. The combined action of these four reactions is repeated adding two carbon units to the growing carbon chain until C16 is reached. The final elongation from C16 to C18, which yields the end product of plastid fatty acid synthetase (C18:0-ACP) is catalysed by a third condensing enzyme β-ketoacyl-ACP synthetase II (KAS II).

In 1982 rapid progress was made in isolating the soluble proteins of the plant FAS system with four independent laboratories reporting the separation of component activities from barley leaves (Hoj and Mikelsen, 1982), avacodo mesocarp (Caughey and Kegwick, 1982), safflower seeds (Shimakata and Stumpf, 1982), spinach leaves (Shimakata and Stumpf, 1982) and parsley suspension culture (Schulz *et al.*, 1982).

In particular Shimakata and Stumpf (1982) separated and characterised the substrate specificity's of β-ketoreductase, β-Hydroxyacyl (ACP) dehydrase and enoyl (ACP) reductase from spinach leaves. The native and denatured molecular weights were determined for all three enzymes using gel filtration and SDS PAGE.

Both of the reductive enzymes β-ketoreductase and enoyl (ACP) reductase could utilise NADH as reductant for activity, enoyl (ACP) reductase having an absolute requirement for NADH, whereas β-ketoreductase showed a preference for NADPH.

Since this early work a number of laboratories have been actively studying plant lipid biosynthesis and several of the soluble proteins have now been isolated from a number of different plant sources. The biochemistry of the subsequent membrane bound reactions of fatty acid desaturation and elongantion are however less understood

because of the difficulties associated with the
purification of membrane bound proteins.
For the purpose of this thesis the two most important
components for consideration are enoyl ACP reductase
(ER) and acyl carrier protein (ACP) and a more exacting
summary of available knowledge of these two FAS
components is given below.

1.4 Acyl Carrier Protein (ACP)

Early work on the characterisation of plant FAS
component enzymes concentrated on ACP. This was largely
because it was known, from work on *E.coli*, that it was
an abundant small molecule (approx. 10kDa) and that it
was relatively stable. Both acid precipitation and heat
denaturation were used as part of the purification
procedure. The protein is post translationally modified
and contains a 4'phosphopantotheine domain to which the
intermediates of fatty acid biosynthesis are attached
via the terminal sulphydryl.
The structure is shown schematically in figure 1.4.1.
below.

$$\text{O-P-O-CH}_2\text{-C-CH}_2\text{-C}_{\lessgtr}^O \quad \text{O}$$

~~G-A-D-S-L~~

ACP protein

Pantoic acid	β-alanine	β mercaptoethylene
4 phosphopantethenic acid		
4 phosphopantetheine		
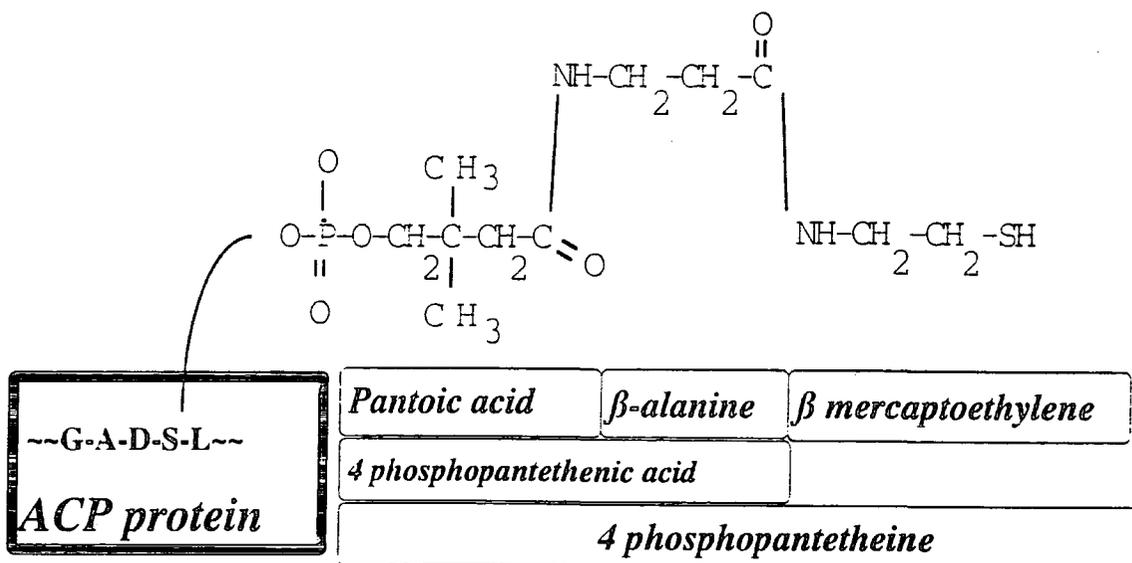
Figure 1.4.1 A schematic drawing showing the structure
of acyl carrier protein (ACP).

In comparison with other plant FAS component enzymes ACP
would be expected to be present in more than
stochiometeric amounts due to its central role in the
synthesis of fatty acids and as a substrate for complex
lipids biosynthesis. It was therefore considered as a
suitable target for the first isolation of a soluble
plant FAS component. ACP was purified from both spinach
(Kuo and Ohlrogge, 1984) and barely leaf (Hoj and

Svedson, 1983) and the amino acid sequence of both was determined.

The evidence from both the protein purification and from western blotting experiments indicated that there was a complexity of ACP types in plants, in barley at least three isoenzymes were identified two distinct molecular weight forms in leaf and one in seed. Two major forms were isolated from spinach leaves, which have been shown to have different amino acid sequences (Ohlrogge, 1987). Both forms are present in the chloroplast, but their relative expression was found to be different in light grown versus dark grown leaves.

Studies on the levels of ACP activity in both developing rape and soy bean seed showed ACP activity appeared just prior to the onset of storage lipid biosynthesis and therefore the gene could make a potential target for both temporal and specific regulation.

Purification of ACP from rape seed (Slabas *et al.*, 1987) proved more difficult than anticipated. It required detergent for solubilization and unlike the protein from *E.coli* proved to be neither heat nor acid stable and during chromatography rapidly lost biological activity. In order to circumvent this the ACP was acylated with [^{3}H] palmitate using *E.coli* ACP synthetase and the hydrophobic moiety introduced was used to assist in its purification.

Protein sequence comparisons of ACP from *E.coli* (Vanaman *et al.*,1986), spinach (Kuo and Ohlrogge, 1984), barley (Hoj and Svendson, 1983), rape (Safford *et al.*, 1988) and *Arabidopsis* (Post-Bettenmiller *et al.*, 1989) revealed several areas of high sequence homology especially at the region surrounding the phosphopantethylated serine residue. Ten independent cDNA clones for ACP from rape were cloned and sequence analyses of these clones showed a 51 amino acid N-terminus extension responsible for targeting it to the plastid (Safford *et al.*, 1992). The heterogeneity found within the mature portion of the sequences revealed at least three ACP species confirming what had previously been found during protein sequencing.

1.5 Enoyl ACP Reductase.

Enoyl ACP reductase (ER)the enzyme catalysing the second and final reductive step within FAS was first purified from plants by Shimakata and Stumpf (1982), who isolated the NADH specific form from spinach leaves.
Subsequently the enzyme was isolated from avocado mesocarp (Caughey and Kegwick 1982) and from *Brassica napus* (rape seed) (Slabas *et al.*,1986). The studies on

the rape seed enzyme showed that the levels of
biological activity and total protein increased
throughout the lipid deposition phase, therefore like
ACP, it would make a potential target for a seed
specific and temporal regulated gene.

Two forms of enoyl ACP reductase (type I and type II)
have been identified in *E.coli* (Weeks and Wakil,1968)
safflower (Shimakata and Stumpf,1982) and rape seed
(Slabas *et al.*, 1984). They differ in their specificity
for their hydrogen donor, type I requiring NADH and type
II requiring NADPH for activity.

The rape seed type I, NADH specific enzyme has been
purified to near homogeneity (Slabas *et al.*, 1986). It
can utilise either acyl CoA's, acyl ACP's or
acylcysteamines as substrate although the natural
substrate is ACP. The apparent kms for crotonyl CoA and
crotonyl ACP are 178μm and less than 1μm respectively,
the km for NADH is 7.6μm.

The rape enzyme is an α4 homotetramer of approximately
140 kDa molecular weight with a subunit mass of
approximately 35 kDa (Slabas *et al.*, 1986) and is not
inhibited by the herbicide diazoborine. This is in
contrast to the bacterial enzymes isolated from *E.coli*
and *Salmonella typhimurium* (EnvM protein, Bergler *et*
al., 1994) which have been found to be dimeric and are
inhibited by diazoborine compounds.

Extensive amino acid sequence information was obtained
for the mature rape ER protein and the N-terminus
(SESSESK) was identified. The cDNA from rape was cloned
and this showed that there is a leader sequence of 73
amino acids required to target the nuclear encoded
protein to the plastid (Kater *et al.*, 1991). Four genes
for enoyl ACP reductase from *Brassica napus* have been
identified, two of which were inherited from each
ancestor *B.oleracae* and *B.campestris* (Kater *et
al.*,1991). Four isoforms of the enzyme have been
identified using 2-dimensional western blotting (Fawcett
et al.,1994 part of this study). All four isoforms show
different patterns of temporal expression, the two most
abundant isoforms found in seed material are also the
two most abundant in leaf tissue.

The *B.napus* enoyl ACP reductase has been cloned and
overexpressed using the T7 promoter system in *E.coli*.
During the construction of the plasmid (pEAR2) for this
overexpression the DNA sequence encoding the N-terminal
amino acid was altered from serine to alanine to
facilitate cloning (Kater *et al.*,1991). This
substitution did not prevent a functional tetrameric
enzyme from being produced. However no kinetic
characterisation of this recombinant protein has been
reported.

Studies on the purification and characterisation of this recombinant plant protein form the basis of this thesis.

1.6 Objectives of this Study.

The main objective of this research work was to develop a stratergy which would allow purification of milligram quantities of recombinant *B.napus* enoyl ACP reductase from *E.coli*, for further studies on molecular structure and active site analyses. Previous attempts to investigate enzyme inhibition and active site amino acid residues (Cottingham *et al.*, 1989) were limited by the relatively small amount of protein available when purified directly from rape seed.

The relatively large amounts of purified material obtained during this work, would allow full structural characterisation of the plant enzyme which was not possible with the limited amount of material obtainable directly from rape seed. A poyclonal antibody could be raised and used in western blotting experiments to investigate the complexity of (ER) isoform expression using 2 dimensional electrophoresis.

Using the purified material spectral data could be collected which along with absolute amino acid analyses, could be used to establish a molar extinction coefficient for the plant enzyme.

A detailed molecular structural analysis of the plant enzyme could be initiated, starting with CD analyses (secondary structure) and extending into crystallography techniques and x ray diffraction analyses (full atomic structure).

Previous work using phenylglyoxal, a reagent which selectively modifies arginine residues (Cottingham *et al.*,1989) showed that one or possibly two arginine residues may be at or near the active site of enoyl ACP reductase from *B.napus*.

It is intended to repeat and extend the modification and substrate protection work to include peptide mapping and peptide sequencing experiments. The aim is to try to identify specific amino acid residues which are at or near the active site of the enzyme.

The aim of this research is to gain a firm structural understanding of the enzyme and its role in lipid biosynthesis.

Chapter 2.0

Materials, and Methods.

2.1 Materials and Reagents

Tryptone, yeast extract and agar were all obtained from
DIFCO Laboratories, PO box 14B, Central Avenue, West
Molesey, Surrey KT8 2SE.

CoASH, Crotonyl CoA, DTT (dithiothreitol), NAD, NADH,
and Phenylglyoxal were all obtained from Sigma Chemical
Co., Fancy Road, Poole, Dorset BH17 7NH.

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

HPLC grade Methanol, Isopropanol, Acetronitrile and TFA
(triflouroacetic acid) were from Rathburn Chemicals Ltd,
Walkerburn, Scotland EH43 6AU.

Acrylamide, Bis acrylamide, SDS, and TEMED were from
Biorad Laboratories Ltd. BioRad, Maylands Avenue, Hemel
Hempstead, Hertfordshire HP2 7TD.

Radioactive (14C labelled) Phenylglyoxal was obtained
from Amersham International Plc., Amersham PI, Little
Chalfont, Amersham, Buckinghamshire HP7 9NA.

All other chemicals and biochemicals were obtained from either Sigma Chemical Co. or BDH chemicals and were of the highest purity available.

2.1.2 Enzymes

Endoproteinase Lys-C and Endoproteinase Arg-C (Closripain) were both obtained from Promega, Delta House, Enterprise Road, Chilworth Research Centre, Southampton, Hants SO16 7NS.

DNase I, and lysozyme, were obtained from Sigma Chemical Co., Fancy Road, Poole, Dorset.

2.1.3 Chromatography media.

Blue Sepharose CL4B and Fast Flow Blue Sepharose were both obtained from Pharmacia, Milton Keynes, Buckinghamshire.

2.1.4 Prepacked Chromatography media.

Prepacked MonoQ and Highload MonoQ columns were obtained from Pharmacia and used on Pharmacia FPLC and Highload Chromatography systems respectively. For gel filtration experiments a superose 12 column was also obtained from Pharmacia and used on the FPLC system.

2.1.5 SMART Chromatography media.

MonoQ, MiniQ, MonoS, Phenyl superose, Superose 12, Fast Desalting, and C2/C18 reverse phase columns were all obtained from Pharmacia and used on the Pharmacia SMART chromatography system.

2.1.6 Microbore HPLC media.

Brownlee C8 and C18 reverse phase microbore columns (3cm x 2.1mm) were used on an Applied Biosystems (ABI) microbore HPLC system fitted with diode array detection to monitor absorbance and collect spectral data. Brownlee C8 and C18 reverse phase microbore columns (3cm x 2.1mm) were also used on the SMART system to separate peptide digests of covalently modified enoyl ACP Reductase.

A Vydac C18 high resolution microbore column 25cm x 2.1 mm ID was obtained from Technicol, Brook Street, Stockport Cheshire SK1 3HS. This column was used on the SMART system to isolate radioactively labelled peptides prior to protein sequencing.

2.1.7 Dialyses membranes.

Two types of dialyses membrane were used for buffer exchange throughout this research. For routine dialyses a general purpose membrane (obtained from Sigma) with a

molecular weight cut off (MWCO) approximately 12 kDa was used.

For the dialyses of ACP (molecular weight 10 kDa) a high specification Spectrapore membrane (obtained from Pierce & Warriner, UK Ltd, 44 Upper North Street, Chester CH1 4EF.) was used. This spectrapore membrane had a precise 6-8 kDa MWCO and was therefore more suitable for the dialyses of small molecules.

2.1.8 Bacterial Strain and Overexpression System for Enoyl ACP Reductase

The overexpressing *E.coli* strain BL21 (DE3), transformed with pEAR2 a plasmid containing a *Brassica napus* enoyl ACP reductase cDNA clone was used to overproduce biologically active enoyl ACP reductase (Kater *et al.*, 1991).

2.2. General Laboratory Methods.

2.2.1 High purity water.

Throughout all experiments freshly drawn Milli Q (Millipore UK.Ltd, The Boulevard, Blackmoor Lane, Watford, Hertfordshire WD1 8YW) water was used to make all solutions and buffers.

2.2.2 pH measurement.

pH measurements were made with a Jenway 3410 Electrochemistry analyser fitted with a general purpose combination electrode and calibrated with BDH standard buffer solutions. Temperature compensation was made using a PCT324 temperature electrode.

2.2.3 Conductivity measurement.

Conductivity measurements were made with the Jenway 3410 Electrochemistry analyser fitted with a PCM121 conductivity probe.

2.2.4 Protein estimation.

Protein concentration was determined using either the method of Bradford (1976), or the TCA precipitation method of Lowry (1951). BSA was used as the standard to produce calibration curves for both methods.

2.2.5 Concentration and buffer exchange of protein samples.

For protein samples up to 13 ml in volume Amicon Centriprep 30 concentrators (30000 MW cutoff) were used

in a Jouan MR18 22 centrifuge cooled to 4°C. These concentrators were centrifuged at a maximum 1500 x g according to the instructions supplied by Amicon. This allowed concentration down to a final volume of 500µl. For sample volumes less than 500µl and when rapid buffer exchange on small volumes was required Millipore Ultrafree MC microconcentrators (30000 MW cutoff) were used in a Hettich Mikrolitre benchtop centrifuge at 6500g according to the manufacturers instructions.

2.2.6 Spectrophotmetric measurement of protein samples.

Spectral data and the molar extinction coefficient of enoyl ACP reductase at 280nM was measured using a Pharmacia Ultraspec III spectrophotometer and quartz micro volume cuvettes. Spectral scans for purified enoyl ACP reductase were obtained between the wavelengths 240nm and 350nm using a Varian DMS90 UV / visible scanning spectrophotometer fitted with a Kipp and Zonen chart recorder.

2.3 Polyacrylamide gel electrophoresis and protein blotting.

2.3.1 Polyacrylamide gel electrophoresis.

Electrophoresis in the presence of SDS was performed by the method of Laemmli (1970) with a 3% stacking gel and

a 10% resolving gel. The ratio of acrylamide to bis-acrylamide was 37.5:1 for all SDS PAGE experiments except for experiments using Schagger gels (section 2.3.2) when a ratio of 49.5 : 3 was used.

In order to avoid possible N-terminal blocking all gels used for protein or peptide sequencing were prepared several hours before use and fresh running buffer was prepared from the highest purity reagents. In addition thioglycolic acid, at a concentration of 200 μM, was added to the cathodic buffer compartment and the gels pre run for 20 minutes at 50 volts. This was to eliminate free radicals from the system before the samples were applied.

BioRad mini ProteanII gel kits were used for all one dimension electrophoresis experiments and electrophoresis was carried out at 100 volts through the stacking gel and 200 volts through the resolving gel. Samples were loaded in a modified Laemmli sample buffer consisting of 2% SDS, 1% DTT, 0.01% Bromophenol blue, 10% Glycerol made up in 62.5mM Tris buffer pH 6.8 . This buffer was either used directly to solublize samples or added as a 5 times stock to liquid samples. In both cases samples were boiled for 3 minutes and then centrifuged in a Hettich Mikrolitre bench top centrifuge prior to loading gels. A continuous buffer system consisting of 0.1% SDS, 192 mM glycine,and 25mM tris at pH 8.3 was used for all protein electrophoresis experiments.

After electrophoresis protein bands were visualised in
the gel by staining with coomassie blue (2.3.3).

2.3.1.2 Molecular weight standards for electrophoresis.

Individual protein standards of Bovine serum albumin (MW
66 kDa), Ovalbumin (MW 45 kDa), and Trypsinogen (MW 24
kDa) were all made at 200 µg ml^{-1} in sample buffer,
boiled for 3.0 minutes, microfuged and stored at -20°C.
When needed 5 µl of each was loaded into a sample well
on a mini gel to give 1.0 µg protein bands.
From the position and intensity (after coomassie
staining) of these standard bands sample protein MW and
amounts was estimated.

2.3.2 Schagger peptide gels.

Peptide digests were separated using 16.5% resolving
gels with 5% stacking gels and the discontinuous buffer
system of Schagger(1987). The Acrylamide / Bis
acrylamide ratio was 49.5 : 3 for all Schagger gels.
The discontinuous buffer system used for all Schagger
gels consisted of 100mM tris, 100mM glycine, 0.1% SDS,
pH 8.25 as the cathodic buffer and 200 mM tris/HCl
pH 8.25 as the anodic buffer.
The modified Laemmli sample buffer described above was
used to solublise samples prior to loading gels (2.3.1).
Electrophoresis was carried out at 100 volts through

both the stacking and resolving gels as higher voltages resulted in the gels becoming warm.

After electrophoresis the peptide bands were either visualised in the gel by staining with coomassie blue or were blotted onto problot PVDF membrane (Applied Biosystems Ltd, Kelvin Close, Birchwood Science Park, Warrington Cheshire WA3 7PB) and used in protein sequencing experiments.

2.3.3 Coomassie Blue protein and peptide staining.

After electrophoresis protein and peptide bands were visualised in the acrylamide gel by first staining with coomassie I solution; (25% propan- 2-ol, 10% glacial acetic acid, 0.025% coomassie blue made up in MQ water). for 10 min at 60°C. The gel was then transferred to coomassie III; (10% glacial acetic acid, 0.003% coomassie blue made up in MQ water) for a further 10 min at 60°C. For both of these staining steps the gels and the staining solution were heated together in a microwave until they reached temperature and were then left at room temperature for the 10 minute staining period.

The second staining step resulted in protein bands not seen when stained only in Coomassie I becoming visible. This made it a more sensitive overall staining technique.

After staining the gels were destained by gently shaking
in a solution of 10% glacial acetic acid, 1% glycerol
made up in MQ water. In order to make the destaining
more rapid (<30 minutes) the gels in this destain
solution were heated to 60°C in a microwave.

2.3.4 Western Blotting and Transfer of Proteins and Peptides After Electrophoresis.

A KemEnTec semi dry blotter supplied by Whatman Labsales
was used to transfer proteins and peptides after
electrophoresis to either nitrocellulose (Hybond C,
Amersham) for antibody experiments or to PVDF (Problot,
Applied Biosystems) for protein sequencing experiments.
For transfer to nitrocellulose a continuous buffer
system containing 25mM Tris, 192mM glycine, 0.05% SDS
and 20% methanol at a constant current of 0.8mA cm^2 was
used for 1 hour.
Peptide digests were transferred to PVDF membrane using
a continuous buffer system of 10mM CAPS in 10% methanol
pH 11.0 with a constant current of 0.8 mA cm^2 for 30
minutes.

2.3.4.1 Ponceau Staining for Western Blots.

After electrophoretic transfer proteins were detected on
nitrocellulose by gently shaking the membrane in a
solution of 0.1% Ponceau S (Sigma) in 1% glacial acetic

acid for 1 min. The membranes were then destained with
several changes of 1% glacial acetic acid.
The stained protein bands appeared red on the white
background of the membrane after staining with
Ponceau S.

2.3.4.2 Rapid Coomassie Staining for PVDF membrane.

Peptides and proteins transferred to PVDF membrane for
protein sequencing were rapidly stained in a solution
containing 0.1% coomassie blue in 40% HPLC grade
methanol, 1% acetic acid. After staining the membranes
were destained with several changes of 50% HPLC grade
methanol. The destained membranes were then air dried
wrapped in SARANWRAP and stored at $-20^{O}C$ until the
protein sequencer was available.

2.3.5 Isoelectric Focusing

2.3.5. Isoelectric Focusing

Phast IEF gels pH 3-9 (Pharmacia) were used for
isoelectric focusing experiments on a Pharmacia Phast
electrophoresis system. Standard pi markers were used on
the same gels to produce a calibration from which the pi
of enoyl ACP reductase was estimated (fig 3.9.1).

2.3.6 Two Dimensional Electrophoresis and Western Blotting.

2.3.6.1 Two Dimensional Electrophoresis

Previous literature (Cottingham et al., 1988 and Slabas et al., 1992) had indicated heterogenity within the amino acid sequences of plant enoyl ACP reductase with at least two isoforms present in rape seed. To investigate the expression of these isoforms two dimensional electrophoresis was carried out on crude extracts of rape seed and rape leaf solubilised in lysis buffer (9M urea, 2%v/v triton X-100, 286mM β-mercaptoethanol, and 2% pharmalyte 3-10 (Pharmacia)) at room temperature. It was found that the solubilisation of proteins at room temperature was essential as higher temperatures (as low as $37^{\circ}C$) caused protein carbamylation, which resulted in a characteristic trail of spots towards the anode. This two dimensional electrophoresis was carried out using a Pharmacia multiphor II electrophoresis apparatus. Pre-cast immobilin dry-strips rehydrated and used according to the manufacturers instructions (Pharmacia) were used for IEF in the first dimension and pre-cast 8-18% SDS gels were used for the molecular weight separation in the second dimension. Carbamylated creatine kinase (CPK) was purchased from Pharmacia and used to calibrate the system. The IEF separation was carried out in three

phases according to the instructions supplied with the
immobiline strips. Phases 1 and 2 were pre-focusing and
sample application steps, both run at 300 volts, 1.0 mA,
for 900 and 5750 Vh respectively and phase 3, the sample
focusing step, run at 2000 volts, 1.0 mA for 16000 Vh.
This IEF was normally set-up to run overnight.
Following the IEF separation the strips were
equilibrated for 10 minutes in 50 mM tris/HCl pH 6.8
containing 30% glycerol, 1.0% SDS and 0.1% DTT, and then
for a further 10 minutes in the same solution without
DTT but containing 4% iodacetamide, and a few grains of
bromophenol blue. The strips were then carefully dried
and transferred to the SDS gel for the second dimension,
according to the instructions supplied with the
multiphor kit. The second dimension (SDS PAGE) was run
at 600 volts and 50mA for approximately 60 minutes.
Following electrophoresis the gel was carefully removed
from the backing film using the film remover (Pharmacia)
and the proteins were transferred to Hybond C nitro-
cellulose membrane using the semidry blotting protocol
outlined (2.3.4).

2.3.6.2 Antibody Labelling.

The denatured antibody raised against enoyl ACP
reductase(2.7) was not available at the time these
experiments were carried out, however an anti-rape
native enoyl ACP reductase antibody was available from

previous work (Slabas *et al.*,1990) and this was
therefore the antibody used.

After protein transfer to nitro-cellulose the membrane
was incubated in blocking solution (1.0% haemoglobin in
25mM tris / HCl pH 7.4 containing 0.8% NaCl, 0.2% KCl
and 0.1% tween 20 (TBST)) for 1 hour at room
temperature. The membrane was removed from the blocking
solution and washed three times with aliquots of TBST
before being incubated for 90 minutes in a 1/10000
dilution of primary antibody made up in 1% haemoglobin
TBS solution. The membrane was extensively washed with
TBST, to remove all unbound primary antibody and then
incubated for 90 minutes at room temperature in a 1/1000
dilution of [125I] secondary antibody made up in 1%
haemoglobin TBS solution.

The [125I] labelled membrane was again washed
extensively with TBST before it was carefully wrapped in
SARANWRAP and exposed to x-ray film at -80°C for four
days (figure 3.15). This analyses was carried out in
conjunction with Dr Tony Fawcett in this department.

2.4 Enoyl ACP reductase Enzyme Assays

Enoyl ACP reductase was assayed by following the
decrease in absorbance at 340 nm due to the crotonyl CoA
dependant oxidation of NADH (Slabas et al.,1986). The
standard assay mixture contained 10mM sodium phosphate
(pH6.2), 140µM NADH, 120µM Crotonyl CoA and a suitable
amount of enzyme in a total volume of 1.0 ml. A curve of

linearity versus amount of enzyme added to the assay reaction was constructed for each enoyl ACP reductase preparation.

Because of the difficulty and high expense of obtaining crotonyl CoA (prior to synthesising it directly (2.9.1) a micro assay was developed and used. This micro assay contained the same standard reaction mixture but the total volume was reduced to 100μl and the reaction was carried out in micro cuvettes. The reproducibility of this micro assay was tested for a range of enzyme concentrations and was shown to be linear and highly reproducible (figure 2.4.1).

The micro assay replaced the standard assay for all enoyl ACP reductase assays.

For both the standard assay and the micro assay the Ultraspec III spectrophotometer was used together with a Kipp and Zonen chart recorder to measure the decrease in absorbance against time.

Activity was expressed as enzyme units, defined as the amount of enzyme that converts 1 μmole of substrate to product in 1 minute under assay conditions. For this assay the absorbtion coefficient of NADH at 340nm (6.22 x $10^3 m^{-1} cm^{-1}$) was used.

Figure 2.4.1

Linearity of Micro Assay for Enoyl ACP Reductase Activity
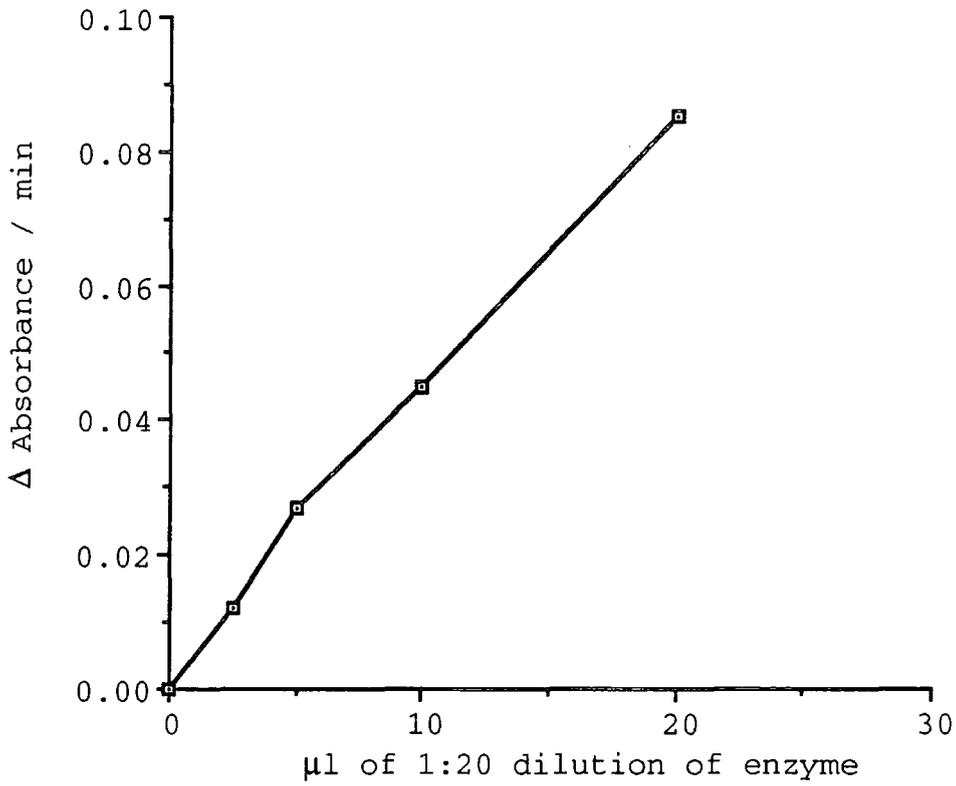
Purified enoyl ACP reductase was freshly diluted with assay

buffer to give a 1:20 stock. The decrease in absorbance at

340 nm due to the crotonyl CoA dependant oxidation of NADH was

measured in 100 µl quartz cuvettes.

2.5 Bacterial Growth, Recombinant Enoyl ACP reductase Induction and Extraction.

2.5.1 Bacterial Growth and Protein Induction.

2.5.1.1. Growth medium

The bacterial growth medium 2 x YT consisting of 1.0% yeast extract, 1.6% tryptone, 0.5% NaCl and 1% glucose was used to grow overexpressing *E.coli* BL21(pEAR2) throughout this project. Ampicillin at a concentration of $100\mu g\ ml^{-1}$ was added to all growth medium to maintain the plasmid selection.

The medium was sterilised by autoclaving without the glucose and ampicillin present. Stock glucose and ampicillin solutions were filter sterilised through a $0.22\mu m$ membrane filter and added to the medium after it had been sterilised.

2.5.1.2 Bacterial Growth and Protein Induction.

Agar plates of 2 x YT medium were inoculated from stock glycerol's of the bacterial strain which were stored at $-80^{O}C$. The plates were then grown overnight at $37^{O}C$.

In early experiments a 50 ml liquid culture was then inoculated from these seed plates and grown during the day shaken (120rpm) at $37^{O}C$. This liquid culture was used as the inoculum for 4 x 500ml liquid cultures which were grown overnight again shaken at $37^{O}C$.

The cells were harvested from these cultures by centrifugation (10000g for 10 min.) and then resuspended in fresh 1 x YT containing 0.2mM IPTG and grown for a further 3 hours. The cells were finally harvested again by centrifugation (10000g for 10 min.) and the cell pellets resuspended in a small volume of 50mM Tris/HCl extraction buffer pH8.0 containing 1mM EDTA and 5mM DTT. This paste of unbroken *E.coli* cells was then stored at -80°C until required for protein purification.

Later in this research project Dr Tim Hawkes at Zeneca Agrochemicals kindly arranged to grow a fermenter of this overexpressing strain. The cells were harvested and broken directly using a French press. This supplied large amounts of broken cell paste which were stored at -80°C ready for enoyl ACP reductase extraction and purification.

2.6 Extraction and Purification of Recombinant Enoyl ACP Reductase.

Throughout the purification of enoyl ACP reductase all procedures were carried out at 4°C. The purification detailed below is for a typical preparation on material grown in 2 litre batches and a typical purification table is given in chapter 3 (table 3.1.1). Later in the project it was possible to have the material grown in a large scale fermenter and have the cells broken and

extracted directly without the use of lysozyme. For
this material it was necessary to scale up the blue
sepharose step but all other aspects of the procedure
remained the same. A purification table for fermenter
grown material is given in chapter 3 (table 3.1.2).

The purification method was modified from that used to
isolate the native *Brassica napus* enzyme(Slabas
et al., 1986). It essentially involved the use of blue
sepharose affinity chromatography and high capacity Q
sepharose anion exchange chromatography. The blue
sepharose affinity matrix consists of Cibarcon Blue a
textile dye covalently attached to the cross linked
agarose gel sepharose CL6B. This matrix has a high
affinity for NADH and NAD requiring enzymes and has been
used extensively in their purification. Purification
tables for enoyl ACP reductase purified from batch grown
and fermenter grown material are shown (3.1.1 and
3.1.2).

2.6.1 Protein Extraction.

Unbroken cell paste (2.5.1.2) was thawed from $-80^{O}C$ and
lysozyme added to a concentration of 1.0 mg^{-1} ml^{-1}. The
slurry was stirred on ice for 1 hour after which $MgSO_4$
(1.0M stock) was added to give a final concentration of
5mM and DNase (10 mg^{-1} ml^{-1} stock) to give a final
concentration of 10 μg^{-1} ml^{-1}. The solution was further

stirred for 5.0 minutes to allow the DNase to cleave the DNA and the solution to loose its viscosity. The slurry was centrifuged in a Sorvall RC5B refrigerated superspeed centrifuge (cooled to $4^{\circ}C$) at 20000g for 10 minutes.

After centrifugation the supernatant was removed from the pellet containing cell debris and protamine sulphate (20 mg^{-1} ml^{-1} stock) was added to a final concentration of 1.0 mg^{-1} ml^{-1} and the solution stirred at room temperature for 5.0 minutes. This protamine sulphate step was included to precipitate fragments of DNA which would not be removed during normal centrifugation procedures.

After the protamine sulphate treatment the solution was centrifuged again at 20000g and the supernatant containing the enoyl ACP reductase activity removed from the precipitate.

A small aliquot was assayed immediately for enoyl ACP reductase activity (table 3.1.1) and a further aliquot kept for analysis by SDS PAGE (fig 3.1). The rest of the supernatant was adjusted to pH 6.2 with a small amount of 1.0M NaOH and batched with blue sepharose 2.6.2.

2.6.2 Blue Sepharose Chromatography

During this project blue sepharose fast flow replaced blue sepharose CL6B from Pharmacia and it was found that the binding capacity of fast flow blue sepharose was

much higher than that of CL6B. It was necessary to titrate amounts of blue sepharose against enoyl ACP reductase bound in order establish a suitable column size to use for standard preparations. The following method is for fast flow blue sepharose.

The supernatant (2.6.1) containing enoyl ACP reductase activity was batched with 25ml of blue sepharose (previously equilibrated in 10 mM sodium phosphate buffer pH 6.2, 1mM DTT (buffer A)) with gentle shaking at 4°C for 1 hour. The resin was then washed on a sinta glass funnel with several volumes of the phosphate buffer. After washing the resin was carefully poured into a Pharmacia column (C16/40) fitted with adjustable end fittings and packed at a flow rate of 5ml^{-1} min^{-1} on a Pharmacia Highload system. The bound enoyl ACP reductase was eluted from the column using a 250 ml^{-1} linear gradient from 0-2.5M NaCl in buffer A, at 5.0ml^{-1} min^{-1}. Fractions were collected during the elution and assayed for enoyl ACP reductase activity. A typical blue sepharose chromatography profile is shown in chapter 3 figure 3.2.1..

To remove the high salt before the next anion exchange chromatography step all fractions containing activity were pooled and dialysed over night against 3 X 2 litre changes of buffer A.

2.6.3 Highload Mono Q Chromatography

After the dialyses step the supernatant was checked for
pH and conductivity and adjusted if necessary to pH 6.2
and to a conductivity of less than 2.0mS. It was then
filtered through a 0.22μm millipore membrane filter to
remove any small particulates before it was loaded at a
flow rate of 5 ml^{-1} min^{-1} directly onto a Pharmacia
Highload Q sepharose 26/10 column.
The column was washed with several volumes of buffer A
to remove unbound material and the enoyl ACP reductase
was eluted with a 500ml linear gradient 0 - 1.0M NaCl in
buffer A.
Fractions were collected during the elution and assayed
for enoyl ACP reductase activity. The fractions
containing activity were analysed by SDS PAGE and
aliquoted before being stored at -80°C. A typical
highload chromatography profile is shown in chapter 3
figure 3.3.1. and a coomassie stained SDS PAGE gel is
shown in 3.3.1b.

2.7 Antibody production

An polyclonal antibody to denatured enoyl ACP reductase
was raised in mice using the following method.
Approximately 60μg of purified enoyl ACP reductase was
layered onto a preparative 10% SDS PAGE gel containing a
5% stacking gel but no sample wells. After
electrophoresis under normal running conditions (2.3.1)

the protein was transferred to nitrocellulose using the standard Western blotting protocol (2.3.4). The protein bands were located by Ponceau staining (2.3.4.1). The bands were cut from the nitrocellulose and ground in liquid nitrogen using a mortar and pestel. The ground nitrocellulose was transferred to a sterile falcon tube and 600μl of sterile 0.9% NaCl was added. The tube was vortexed carefully and 600 μl of complete Freunds adjuvant was added dropwise from a Hamilton syringe. Each of four white mice were immunised with 300 μl of the antigen mixture and boosts were given at two and four week intervals with the same amount of protein but without adjuvant. A final bleed was collected two weeks after the second boost and the antibody produced tested against purified protein and crude plant extracts (figures 3.5.1 and 3.5.2.).

2.8 Characterisation of Purified Recombinant Enoyl Reductase.

2.8.1 N-terminal Protein Sequence Analyses.

In order to confirm the N-terminal sequence of the purified recombinant *Brassica napus* enoyl ACP reductase, approximately 15μg (500pmole) was loaded across a preparative 10% SDS PAGE gel. After electrophoresis (2.3.1) the protein was semidry blotted from the gel (2.3.4) across to PVDF membrane. After rapid coomassie

staining the protein band was cut from the membrane and
applied directly to the blot cartridge of an Applied
Biosystems 477A pulse liquid phase protein sequencer
fitted with a 120 on-line PTH-amino acid analyser. The
blotted protein was analysed for the first ten amino
acid cycles.

The resulting sequence was 100% identical to the plant
sequence, except as expected the first residue was an
alanine in the recombinant protein instead of a serine
in the *Brassica napus* protein. This alanine for serine
substitution was introduced when the DNA sequence
encoding the N-terminal amino acid was altered from TCT
to GCT during the synthesis of the enoyl ACP reductase
construct ((Kater *et al.*,1991).

2.8.2 Stability of Purified Enoyl ACP reductase Under a Range of Storage Conditions.

Aliquots of the purified enzyme were stored under the
following conditions and assayed for enoyl ACP reductase
activity at time 0,4,7 and 14 days, in order to assess
the conditions under which the enzyme would retain
maximum biological activity.

1) $-20^{O}C$ + / - glycerol.

2) $-80^{O}C$ + / - glycerol.

3) $4^{O}C$ + sodium azide.

4) $4^{O}C$ + ammonium sulphate.

The results of this experiment are given in chapter 3 figure 3.6.1.. They show that after 14 days the enzyme had dropped to approximately 50% of its original activity under both -20°C and -80°C storage. There after it remained at a constant level. From this it was decided that for long term storage the purified material should be kept frozen at -80°C and aliquoted so that it would only undergo one freeze thaw cycle.

2.8.3. Kinetic Properties of Purified Enoyl ACP Reductase.

The kinetic properties of purified enoyl ACP reductase was measured under steady state conditions using the micro assay outlined (2.4). The initial rate of activity was measured for varying crotonyl CoA concentrations (100-1000μM) at a fixed NADH concentration (140μm) and for varying NADH concentrations (1-35μM) at a fixed crotonyl CoA concentration (300μM). A Lineweaver -Burk plot of both of the data sets collected was plotted (figure 3.7.1), and from this the apparent km's for both substrates was calculated (3.7).

2.8.4. Secondary Structure Analyses by Circular Dichroism.

The use of computer programmes to predicted secondary structural characteristics of a protein can provide useful information directly from protein sequence data. The two most popular of these programs (Chou and Fasman 1974. and Garnier-Robson 1987) rely on making statistical predictions for the unknown protein.

These predictions are made using data collected from the three dimensional structure of non-homologous proteins in a sample database.

Although the use of these programs can provide reasonable information the ability to make direct spectral measurements on a purified protein sample would provide more accurate information.

Most amino acids are optically active molecules which when in solution will cause left and right circularily polarised beams of light to be absorbed to different extents.

The secondary structures in a polypeptide chain have additional optical properties which may increase or decrease this overall absorbtion.

Circular dichroism is a spectrophotometric technique which measures the extent by which left and right circularly polarised beams of light are absorbed at different wavelengths. The use of CD spectroscopy on a pure protein in solution, and the use of computer programs to fit the observed spectrum to combinations of

the best fit reference spectra of α helices and β sheets in a database can provide reliable estimates of the secondary structural content of a protein, particularly α helices but also β strand.

CD analyses was carried out on a sample of purified enoyl ACP reductase (1.0mg) by Ms Sharon Kelly in the laboratory of Dr Nick Price at the University of Stirling. From this spectral data (shown figure 3.8) and an absolute estimate of the mass of the sample analysed (calculated from amino acid analyses) a secondary structure estimation using the "CONTIN" program of Provencher & Glockner (1981) was made (fig 3.8).

2.8.5 Amino Acid Analyses.

Several aliquots of purified enoyl ACP reductase were carefully weighed into pre-washed reaction vials and 5 μmole of α-amino butyric acid (AABA) was added to each vial to act as an internal standard. The aliquots were analysed after acid hydrolysis using a Waters pico-tag amino acid analyser. The results (chapter 3 table 3.12.1) from this analyses combined with absorbance data at 280nm for aliquots of the same material were used to calculate the molar extinction coefficient for enoyl reductase.

This amino acid analyses was kindly performed by Dr Artymuick at the University of Sheffield.

2.8.6 Native Molecular Weight Determination (Gel Filtration Chromatography).

The native molecular weight of purified enoyl ACP reductase was estimated using a superose 12 gel filtration column (Pharmacia) in 50mM sodium phosphate buffer pH 6.2, 200mM NaCl on a Pharmacia FPLC system. Later in the project a Pharmacia SMART chromatography system was available and as part of the evaluation procedure for this equipment the molecular weight of enoyl ACP reductase was re-measured using a superose 12 micro column (Pharmacia).

For both experiments suitable aliquots of the following standards were passed separately through the column and the retention time of each monitored against absorbance at 280nM. A calibration curve of MW against retention time was constructed (figure 3.11.1 and 3.11.2) and from this calibration the MW of enoyl ACP reductase was estimated.

MW Standards used for Gel filtration Chromatography, all made at 1.0 mg^{-1} ml^{-1} in 50mM sodium phosphate buffer, 1.0mM DTT.

Cytochrome C	MW 12.4 kDa
Bovine Serum Albumin	MW 66 kDa
Alcohol Dehydrogenase	MW 150 kDa
Appoferritin	MW 443 kDa

For the FPLC chromatography 100µg of each was used and for the SMART chromatography 10µg of each was used.

2.8.7 Spectral Properties of Purified Recombinant Enoyl Reductase.

The UV spectrum of purified enoyl ACP reductase was measured between 190 and 300 nM using the Ultraspec III spectrophotometer and quartz micro-volume cuvettes.
A sample of the same material which was sent for total amino acid analyses was used to determine the spectral properties of the enzyme. The results from both of these experiments was used to calculate a molar extinction coefficient for the purified enoyl ACP reductase.

2.8.8 Crystallisation and X ray Analysis of Purified Enoyl ACP Reductase.

Crystallisation trials were set up by the hanging drop vapour diffusion method, on solutions of purified enoyl ACP reductase mixed with an equal volume of a range of precipitants both in the presence and absence of NADH, NAD and crotonyl CoA. The solutions were suspended as droplets underneath siliconised glass coverslips over reservoirs of the various precipitants and slowly concentrated at 17°C for two weeks.
Resulting crystals were mounted in glass capillaries directly from the drop before positioning in the x ray beam for analyses. Initially X-ray data sets were collected from crystals grown in the presence of NAD or

crystals grown in the presence of crotonyl CoA. Later
crystals grown with NAD but then soaked with NADH were
used to resolve the nucleotide binding site.

Heavy atom derivatives of the crystals were produced
using gold, osmium and mercury, and diffraction patterns
were also collected for these heavy atoms crystals.

The additional diffraction data collected using these
heavy metal derivatives was used to refine and resolve
the diffraction data collected for the native crystals
of enoyl ACP reductase.

Typical crystals are shown in figure 3.13.1, a
diffraction pattern at 1.9 angstrom resolution is shown
in figure 3.13.2 and the resolved full x-ray structure
is shown in figure 3.13.3.

The crystallography and analysis was carried out by Dr
John Rafferty and Professor David Rice at the University
of Sheffield.

2.9 Synthesis and Purification of Crotonyl CoA

During this project Crotonyl CoA (the substrate used to
assay enoyl ACP reductase for biological activity)
became more and more difficult to obtain commercially.
It was therefore decided to attempt to synthesise it
directly using a modification of the crotonic anhydride
method (Stern 1955).

2.9.1 Synthesis of Crotonyl CoA using Crotonic
Anhydride and Coenzyme A.

A 10 mg[1] ml^{-1} solution of CoASH (13µM) made up in MQ
water and 400µl of 0.1M $KHCO_3$ pH 8.0 was stirred together
vigorously at 0^OC in a reacti-vial(Piearce). Crotonic
anhydride (4µl) was added dropwise from a hamilton
syringe while continuing to stir vigorously. After
allowing the reaction to incubate at 0^OC for 10 minutes
5µl was removed and checked for free SH using the DTNB
assay (2.9.2). When this assay resulted in no detectable
SH the reaction was stopped and adjusted to pH3.0 - 4.0
by the addition of 1M HCL and the pH was checked using
indicator paper.
The synthesised crotonyl CoA was separated from any free
acid by double extraction with diethyl ether. The
aqueous phase containing the crotonyl CoA was removed
from the ether phase and freeze dried. An aliquot of
freeze dried material was resuspended in 10mM sodium
phosphate buffer pH6.2 and assayed for its suitability
as a substrate for enoyl ACP reductase by comparing it
against commercially available crotonyl CoA in an enoyl
ACP reductase assay. The synthesised material was also
analysed for purity using reverse phase HPLC (2.9.3).

2.9.2 DTNB assay

In order to estimate the amount of free SH remaining and hence the efficiency of the reaction to synthesise crotonyl CoA (2.9.1) Ellmans reagent (5,5'-Dithiobis(2-nitrobenzoic acid)) was used in a colormetric assay which measures the yellow colour formed between free SH and DTNB by its absorbance at 412nm (Ellman 1959). Aliquots of the reaction mixture were incubated in the presence of buffer. DTNB was added and the assay was incubated for 25 minutes at room temperature after which the absorbance at 412nm was measured using a Pharmacia Ultraspec III spectrophotometer.

A calibration for this assay was produced using β-mercaptoethanol as the standard (figure 2.9.2).

2.9.3 Reverse Phase HPLC on Synthesised Crotonyl CoA.

In order to estimate the purity of the Crotonyl CoA synthesised in 2.9.1 it was analysed by reverse phase HPLC using a Brownlee C18 (30 x 2.1mm) reverse phase column fitted to the SMART chromatography system. A linear gradient from 6 to 20% Acetonitrile was used for elution and the crotonyl CoA peak was detected by monitoring its absorbance at 254nm. The chromatogram shown in figure 2.9.3 is from a typical batch of synthesised material and shows a <15% contaminant peak

Figure 2.9.2   DTNB Calibration Using β-mercaptoethanol

as Standard
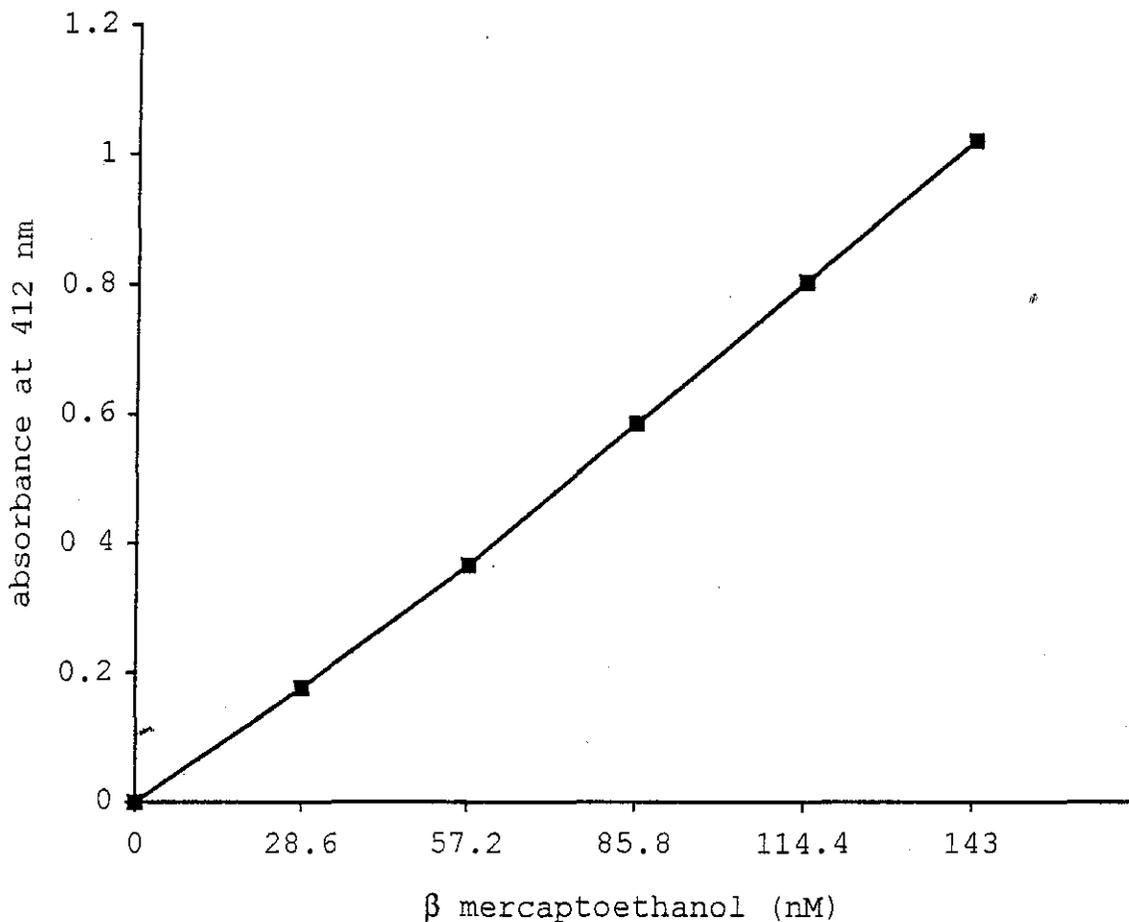
Dilution's of stock β-mercaptoethanol were made in 50mM sodium

phosphate buffer pH 7.0.   A 50μl aliquot of 10mM DTNB in 50mM

sodium phosphate buffer was added to a 1.0ml sample of each

standard. The solutions were mixed and allowed to incubate at room

temperature for 10 minutes. Detection was at 412 nm as described in

materials and methods (2.9.2).

Figure 2.9.3 Analyses of Crotonyl CoA Synthesised from Crotonic Anhydride and Co-enzyme A (2.9) by C18 Reverse Phase HPLC with a water / Acetonitrile Gradient.
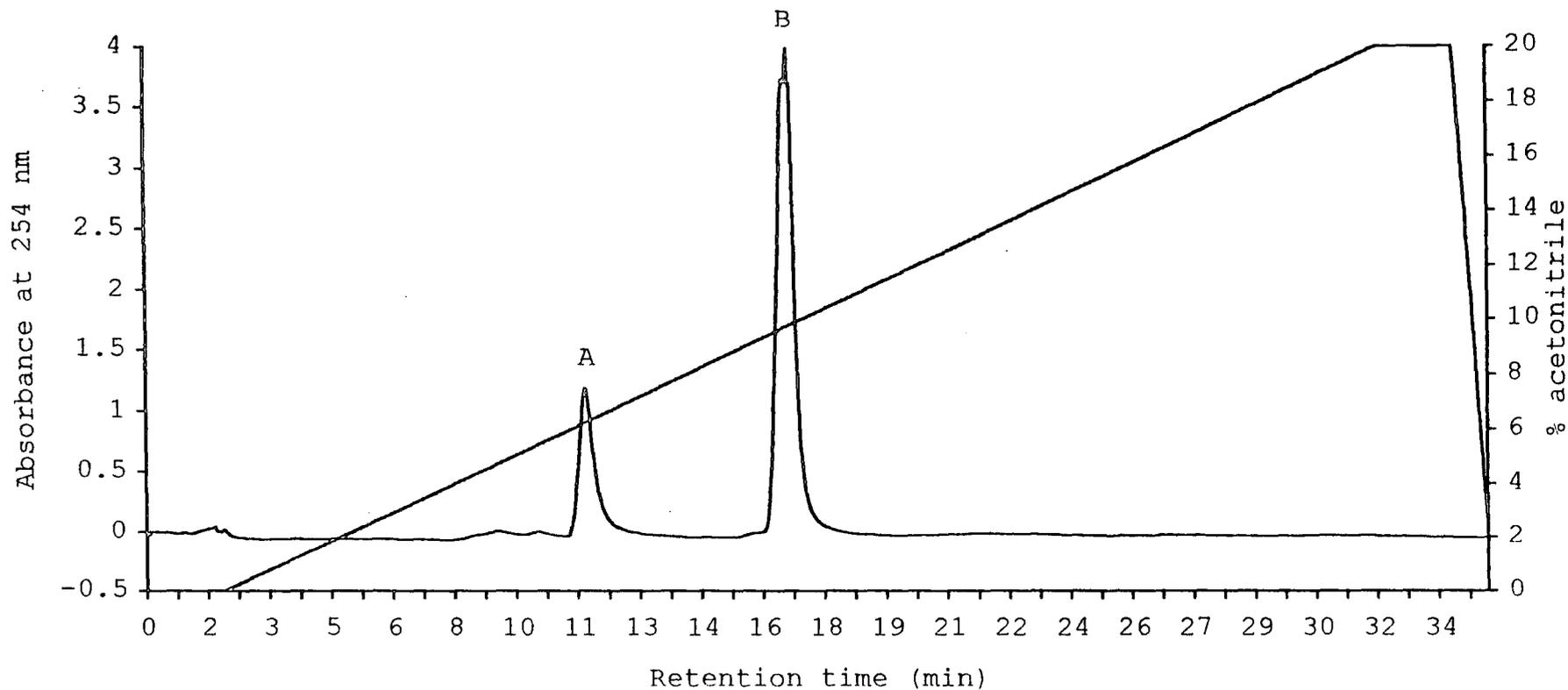
A 100 μl aliquot of crotonyl CoA synthesised from crotonic anhydride and co-enzyme A analysed on a Vydac C18 reverse phase column fitted to the SMART micro-chromatography system. A linear gradient from 0-20% acetonitrile was used to separate the crotonyl CoA (peak B) from the contaminating free CoASH (peak A).

at the same retention time as free CoASH. Attempts were made to remove this contaminant using the same reverse phase HPLC conditions, followed by freeze drying of the fraction containing the crotonyl CoA. this resulted in large losses of material and was not therefore used routinely.

The synthesised material (as analysed by reverse phase HPLC) was however of a high enough purity to be used in routine assays of column fractions or to monitor inactivation of the enzyme in phenylglyoxal labelling experiments. This synthesised crotonyl CoA was therefore the substrate used for these experiments during this project.

2.10 Acyl Carrier Protein (ACP) Purification from *E.coli*.

Since the natural substrates for enoyl ACP reductase are acyl esters of ACP it was decided that it would be necessary to have a suitable amount of purified ACP available during this research work.

This purified ACP would be used to either directly synthesise thioesters of ACP, to use as substrate for enzyme assays, or to use as a substrate protectant for active site residues in covalent modification experiments.

The method used for the purification of ACP was a modification of the method of Majerus *et al*, 1969.

2.10.1 ACP Extraction and Acid Precipitation.

Fermenter grown *E.coli* cells (strain K12) were harvested
and broken directly using a French press. The broken
cell slurry was diluted to a 50% paste in 10mM potassium
phosphate buffer and stored at -80°C.

Approximately 2.5L of cell paste was thawed from -80°C
and mixed with 1.0M potassium phosphate buffer pH 7.0 to
give a final concentration of 10mM. β mercaptoethanol was
added to give a final concentration of 1%. The paste was
stirred on ice and MgCl$_2$ (1.0M stock) to give 3mM and
DNase 50mg (Sigma crude) were added. The solution was
stirred on ice for a further 30 minutes before being
centrifuged at 60000g for 2 hours in a Beckman JA14
refrigerated centrifuge cooled to 4°C.

The supernatant was collected and re-adjusted to pH 7.0
with 4M NaOH. Finely ground ammonium sulphate was added
slowly to give 60% saturation (Dawson *et al.*, 1994), the
pH re-adjusted back to pH 7.0 and the solution stirred
slowly at 4°C.

After 30 minutes the solution was centrifuged at 30000g
for 30 minutes and the supernatant collected. The pH of
the supernatant was carefully adjusted to pH 1.0 with
concentrated hydrochloric acid. During this pH
adjustment the pH below 3.0 was monitored using pH paper
as the pH electrode could not be calibrated below this
pH. The acidified solution was left at 4°C for 3 hours
to allow the ACP to precipitate.

Because of the large volume the bulk of the supernatant was removed from the precipitate (containing the ACP) by siphoning and the remainder removed by centrifuging at 30000g for 30 minutes.

The pellets were carefully resuspended in a small volume of 1.0M Tris/HCl pH 8.0. The solution containing the resuspended pellets was dialysed in Spectrapore dialyses membrane (cutoff 6000-8000 kDa) against 3 x 2 litre changes of 10mM potassium phosphate buffer pH6.2, 0.1% β mercaptoethanol, overnight at 4°C.

After dialyses the supernatant was centrifuged at 15000g for 30 minutes poured off the resulting pellets and stored at -80°C.

2.10.2 Highload MonoQ Chromatography of ACP.

The supernatant (2.10.1) was thawed from -80°C filtered through a 0.2μm membrane filter to remove particulates, and loaded at 4.0 ml^{-1} min^{-1} onto a pre-equilibrated (buffer A) Highload MonoQ anion exchange column. The column was washed with 200 ml of 10mM potassium phosphate buffer pH6.2, 0.1% β mercaptoethanol (buffer A) and the bound ACP was eluted with a 500ml linear gradient from 0-0.5M lithium chloride in buffer A. Fractions were collected during the elution and assayed for ACP activity using a modification of the malonyl CoA -CO_2 exchange assay of Alberts et.al.(1963). The active fractions were pooled and dialysed in spectrapore dialyses membrane against 100mM imidazole/HCl pH 7.4,

1mM DTT, 1mM EDTA at 4°C and then re-dialysed against the same buffer containing 50% glycerol, aliquoted and stored at -80°C.

The purified ACP was analysed by SDS PAGE and quantified using the molar extinction coefficient for ACP at 280nm, (1.8 x10^3 (Rock & Cronan 1980)).

The ACP purified during this procedure was used for substrate protection during phenylglyoxal modification experiments of enoyl ACP reductase. It was also used for the synthesis of acyl ACP's and co-crystallography experiments with enoyl ACP reductase. The later two were not directly part of this research work.

2.11. Chemical Modification of Purified Enoyl ACP Reductase

Previous work on partially purified enoyl ACP reductase from *Brassica napus* (Cottingham *et al,*.1989) has shown inhibition of enzyme activity when the enzyme was incubated with the arginine side-chain modifying reagent phenylglyoxal.

Phenylglyoxal is a specific reagent for arginine residues when the reaction is carried out in bicarbonate buffers at pH 8.0 (Takahashi 1968 and 1977). Therefore for all modification experiments with phenylglyoxal the enoyl ACP reductase sample was first buffer exchanged into 85mM NaHCO$_3$ buffer pH 8.0 using Millipore Ultrafree

micro-concentrators (30000 MW cut-off) in a Hettich
mikrolitre bench centrifuge.

2.11.1 Inactivation of Enoyl ACP Reductase by
      Phenylglyoxal and Substrate Protection Studies.

Inactivation studies on enoyl ACP reductase were carried
out by incubating a known amount of purified buffer
exchanged enzyme with 0, 0.5, 1.0, 2.5 and 5.0 mM
phenylglyoxal at 30°C in a total reaction mixture of
50 µl. At 10 minute time points after the addition of
the phenylglyoxal 5µl aliquots were removed and assayed
for activity using the micro-assay method described
(2.4).

For substrate protection studies purified enoyl ACP
reductase was pre-incubated with concentrations of
purified ACP (2.10.2) or CoASH at 30°C for ten minutes.
Phenylglyoxal (2.5mM) was then added to the
enzyme/substrate mixture and the cocktail incubated at
30°C for 30 minutes. A 2.0 µl aliquot was then removed
and assayed for activity using the micro-assay method
described (2.4).
This 50 fold dilution into the assay prevented either
the ACP or CoASH from interfering with the assay,
although rates for both substrates without phenylglyoxal
present were also measured.

2.11.2 Radioactive Labelling of Enoyl ACP Reductase with [14C] Phenylglyoxal.

The loss of enoyl ACP reductase activity with the incorporation of radioactive phenylglyoxal was measured by incubating a known amount of purified buffer exchanged enzyme with 1.0mM [^{14}C] phenylglyoxal (specific activity 24mCi/mM) at 30°C.
A sample was taken at T= 0 and at 10 minute intervals for assay (micro assay 2.4). A further sample taken at each time point was diluted into bicine buffer, pH8.0 containing 10mM arginine. Ice cold 30% TCA was added to this sample and the solution kept on ice until all of the time points had been collected. The samples were left for a further 30 minutes incubation on ice before the resulting protein precipitate was pelleted by centrifugation at 13000g for 20 minutes. The pellet was washed with 200 µl ethanol repelleted, re-washed twice with ethanol and then re-dissolved in 100µl of formic acid. The solublised pellet was transferred to plastic scintillation vials, scintillation fluid (Ecoscinct, National Diagnostics) was added and the incorporated radioactivity measured using a Hewlett Packard 1600TR Tri-Carb scintillation counter.

2.11.3 Production of Enoyl ACP reductase Peptide Digests and Peptide Isolation.

In order to be able to identify arginine residues essential for biological activity of enoyl ACP reductase both a substrate protected sample and an unprotected sample was treated with radiolabelled phenylglyoxal and peptide digests produced on both lots of material. From these comparative digest a peptide with a radiolabelled arginine residue in the unprotected sample which was not radioactive in the substrate protected sample would indicate that the labelled residue was at or near the substrate binding site of the enzyme.

Several attempts were made to establish a protocol which would allow selective labelling of active site arginine residues, subsequent denaturing of the modified protein, and successful peptide mapping, isolation and protein sequencing.

2.11.3.1 Phenylglyoxal labelling and Peptide Digestion.

Two aliquots of purified enoyl ACP reductase (one protected by pre-incubation for 10 minutes with 10 mM CoASH) were incubated with [^{14}C] phenylglyoxal and the enzyme activity monitored against time (2.11.2). When the amount of enzyme activity remaining in the unprotected sample was less than 20% of the initial activity the protein was precipitated by adding 500µl of

ice cold 30% TCA and incubating on ice for 30 minutes. The TCA precipitate was pelleted by centrifugation at 13000g and the supernatant was removed.

The pellet was re-dissolved in 50 µl of 8M urea in 0.1M ammonium bicarbonate buffer pH8.0 and then the urea concentration was diluted to 2M with 0.1M ammonium bicarbonate buffer.

Peptide digestion of the denatured protein was attempted using Endo argC, Endo lysC, and TPCK treated trypsin.

For Endo LysC digestion 1:25 protease to protein ratio was used and the digest was incubated at $37^{O}c$ for 4 hours.

For Tryptic digestion TPCK treated trypsin was added at 1:25 protease to protein ratio and the digest incubated at $37^{O}C$ for 2 hours after which a second equal aliquot of trypsin was added and the digest re-incubated for a further 2 hours.

In Endo argC experiments 1:25 protease to protein ratio was used and the digest was incubated at $37^{O}c$ for 4 hours.

The efficiency of the various digestion methods was checked by analysing the digest on 15% Schagger gels and reverse phase HPLC.

Where necessary the peptide digests were stored at $-20^{O}C$ prior to analyses.

2.11.4 Reverse Phase HPLC on Radiolabelled Peptide Digests of Phenylglyoxal Modified Enoyl ACP Reductase.

Reverse phase HPLC using a C18 Vydac 25cm x 2.1mm I.D. high resolution peptide column on the SMART micro-chromatography system was used to both analyses the efficiency of the peptide digestion methods and to separate and purify peptide fractions produced. The reverse phase column was fitted to the SMART system using the column adaptor supplied with SMART and equilibrated in 0.1% TFA in MQ water at 100μl min-1. After the column equilibration the digests were applied via the internal micro sample loop and eluted from the column using a linear gradient 0 - 80% Acetonitrile containing 0.1% TFA.

During the separation the peptides were detected by monitoring the peptide bond at 214nM, and the peak fractionating facility of the SMART system was used to control the fraction collector and collect individual peptide peaks.

The chromatogram evaluation software (SMART manager) was used to compare peptide chromatograms and look for differences in the peptide maps produced.

An aliquot of each peptide fraction collected was transferred to plastic scintillation vials and Ecoscinct scintillation fluid (4.0ml) added before they were counted for radioactivity on a Hewlett Packard 1600TR Tri-Carb scintillation counter.

The remainder of the fraction was stored at -20°C prior
to sequencing on an Applied Biosystems 470A Gas-phase
sequencer.

2.11.5 Phenylglyoxal Modification and Sequencing of
Synthetic Peptides.

Two commercially available synthetic peptides Bradykinin
(R P P G F S P F) and αBag peptide (A P R L R F Y S)
were purchased from Sigma and labelled with [^{14}C]
phenylglyoxal under the same labelling conditions as
enoyl ACP reductase (2.11.2). The resulting radioactive
peptide was then separated from free phenylglyoxal using
the Vydac C18 column on the SMART chromatography system
and the same gradient conditions used for the separation
of enoyl ACP reductase peptide fragments (2.11.4).
Fractions were collected during the gradient and the
fraction containing the peptide was applied to the
Applied Biosystems 470A Gas-phase sequencer and
sequenced for the full length of the peptide.
During the sequencing run the sequencer was set up to
collect fractions from each amino acid cycle. The whole
fraction collected was transferred to scintillation
vials, Ecoscinct scintillation fluid was added and the
fractions collected were counted for radioactivity.
It was hoped to show by this experiment that only
arginine residues were labelled under these conditions
and that the radioactivity could still be detected after
both reverse phase HPLC and protein sequencing.

2.11.6   Protein Sequence Alignment and Conserved
        Sequence Analyses.

Any amino acids identified by phenylglyoxal modification
(2.11.4) would be expected to be conserved throughout
the two plant, four bacterial and one cyanobacterial
enoyl ACP reductase protein sequences available.
Conserved blocks of protein sequence within all seven
sequences were identified and the full sequences aligned
using the Multiple Alignment Construction Analysis
Program (MACAW) (Schuler *et al.*,1991).
This alignment data and the conserved blocks found are
shown (4.6)

Chapter 3

3.1 Overexpression and Purification of Enoyl ACP reductase.

Overexpressed *Brassica napus* enoyl ACP reductase was isolated and purified from the soluble fraction of transformed *E.coli* cell lysates. This was achieved using modifications of the published protocol for the purification of the native plant enzyme (Slabas *et al.*,1986).

Typically from 2 litre batch cultures of the transformed bacteria grown in the laboratory about 13 mgs of homogenous enzyme was produced. When material was grown in a large scale fermenter (at Zeneca) and the purification procedure scaled up then approximately 70 mgs of material was produced from a single purification run.

Fractions of material at each step during the purification were analysed on a 10% SDS PAGE gel stained with coomassie blue. The stained gel (figure 3.1) shows one major band at approximately 32 kDa (lane 4) in the fraction from the final purification step.

Purification tables for batch grown and fermenter grown material are shown in tables 3.1.a and 3.1.b As can be seen from the two tables enoyl ACP reductase was typically purified 25 fold with a final specific

Figure 3.1

A Coomasie Stained 10% SDS PAGE Gel Showing the Steps in a Typical Enoyl ACP Reductase Purification
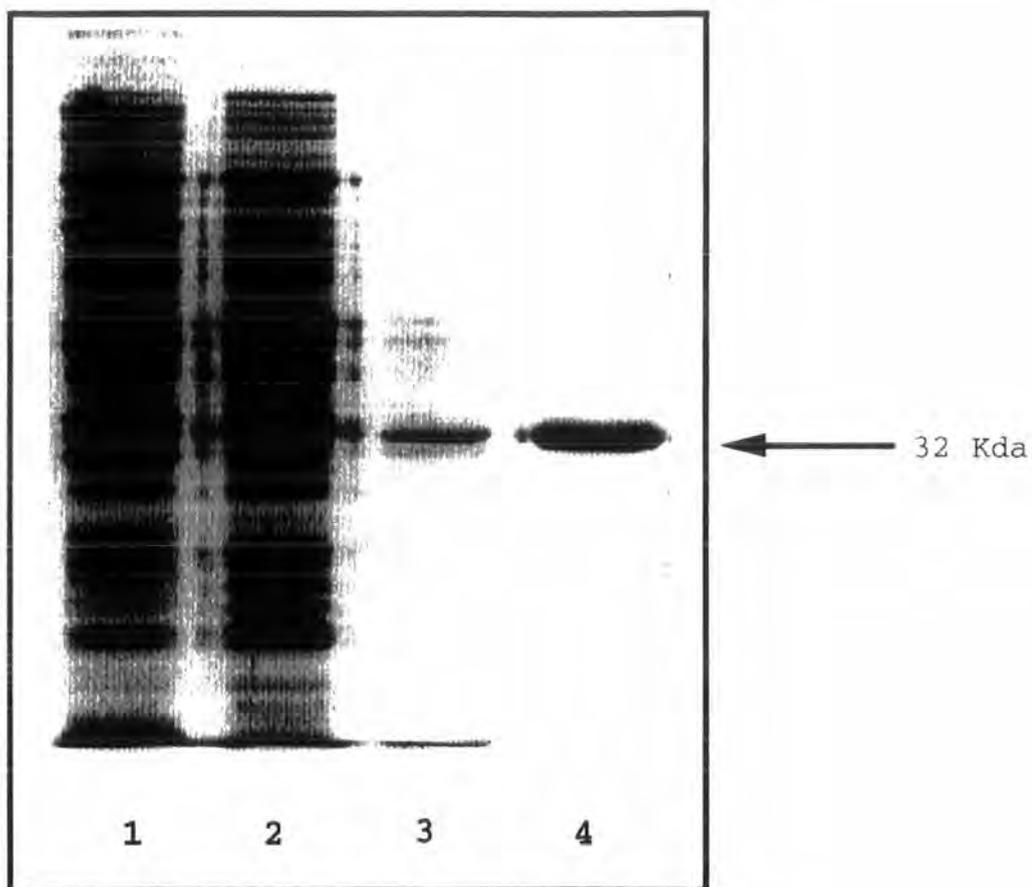
lane 1 shows the crude *E.coli* cell extract, lane 2 shows the supernatant after protamine sulphate treatment. Lane 3 is pooled material following blue sepharose chromatography, and lane 4 is the purified enoyl ACP reductase after the final highload Q chromatography.

Table 3.1a Purification From 2 Litres of Batch Grown *E.coli* BL21 (pEAR2)

Fraction	Total vol. (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Recovery (%)	Purification (fold)
Supernatant	37	744	80.3	0.109	100	1
Blue sepharose	94	280	52.3	0.186	65	1.7
Highload Q	26	13	35.5	2.73	44	25

Table 3.1b Purification from 30 ml of fermenter grown *E.coli* BL21 (pEAR2)

Fraction	Total vol. (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Recovery (%)	Purification (fold)
Supernatant	37	3600	570	0.158	100	1
Blue sepharose	200	1400	450	0.320	78	2
Highload Q	33	70	274	3.9	48	24

activity of between 2.7 and 3.9 U mg^{-1} min^{-1} after the final ion exchange chromatography step. This specific activity is somewhat lower than that previously found for enoyl ACP reductase purified directly from rape seed (Slabas _et al._, 1986 and Cottingham _et al._, 1988) where values of 200 and 155 U mg^{-1} were reported. This difference may be due either to the relatively small amounts of protein (63µg and 180µg) produced when enoyl ACP reductase was purified directly from rape seed, or that the specific activity of the recombinant protein is much lower, due to some processing artefact within the bacteria.

3.2 Blue Sepharose Affinity Chromatography.

Blue sepharose an affinity matrix (Pharmacia) consisting of sepharose CL6B covalently bound with a dye ligand of cibarcon blue was used as the first chromatography step in the enoyl ACP reductase purification. The dye ligand has a high affinity for NADH and NAD requiring enzymes. As enoyl ACP reductase is dependant on NADH for its biological activity it was therefore an ideal matrix to use as the first purification step.

After extraction, DNase treatment and centrifugation to remove cell debris the supernatant containing the enoyl ACP reductase activity was batched with blue sepharose at 4°c for 1 hour. The slurry was then packed into a column and washed with several volumes of buffer A.(2.6.2). The enzyme was eluted using a 0-2.5 M NaCl gradient.

Figure 3.2.1 Typical Blue Sepharose Elution Profile for Enoyl ACP Reductase
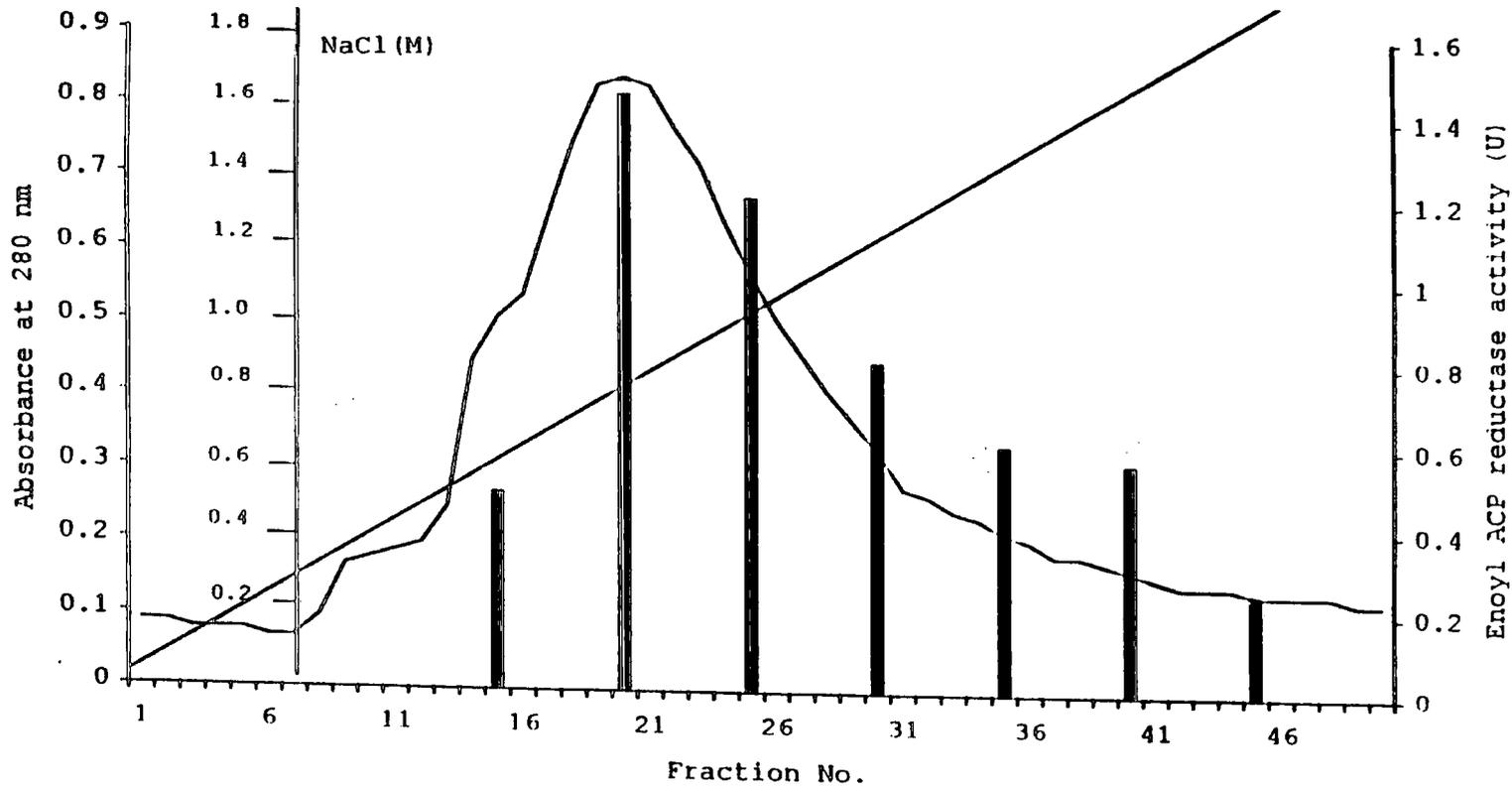
Enoyl ACP reductase eluted as a broad peak between approximately 600 and 1400mM NaCl (fractions 15 to 45). The enoyl ACP reductase activity expressed as U ml-1 (solid columns) was monitored by assaying a 5µl aliquot from every 5 fractions.

A typical blue sepharose profile is shown in figure
3.2.1. The enzyme activity eluted from the column over a
fairly broad peak between approximately 600 and 1400 mM
NaCl with a typical recovery of approximately 75% of the
starting activity.

3.3 Highload Mono Q Anion Exchange Chromatography

Following the blue sepharose chromatography step the
fractions containing the enoyl ACP reductase activity
were pooled and dialysed overnight at 4°C against
several changes of no salt buffer (10mM phosphate buffer
pH 6.2,1mM DTT). This dialyses was necessary to remove
the high salt used for the elution of enoyl ACP
reductase from the blue sepharose column. After the
dialyses the material was loaded directly onto a pre-
equilibrated Highload Q anion exchange column. The
enzyme was then eluted from this column using a 0-1.0M
NaCl gradient over 500 ml (2.6.3).
A profile from a typical highload run is shown in figure
3.3.1. and a coomassie stained SDS PAGE gel of the
fractions containing enoyl ACP reductase activity is
shown in figure 3.3.1b. The activity eluted as a
discreet peak at approximately 300 mM NaCl. The active
fractions collected from this column were pooled and
aliquoted for storage at -80°C. The coomassie stained
SDS PAGE gel shown in figure 3.1 shows a sample from

this pooled material lane 4. The enoyl ACP reductase was estimated to be >95% pure.

3.4 N-terminal Protein Sequence Analyses

In order to confirm that the single band visible on the coomassie gel in figure 3.1 lane 4 was purified *Brassica napus* enoyl ACP reductase it was decided to obtain N-terminal protein sequence data and compare it against that predicted for the overexpressed enzyme (Kater et al. 1991).

Approximately 500 pmole of the purified enzyme was loaded across a preparative 10% SDS PAGE gel and electrophoresis was carried out as described for protein sequencing gels (2.3.1). After electrophoresis the protein was blotted onto Problot PVDF membrane following the procedure outlined in (2.3.4) and stained using the rapid coomassie method (2.3.4.2). The single protein band detected was cut from the membrane and applied directly to the blot cartridge of an Applied Biosystems 477A pulse liquid phase protein sequencer fitted with a 120 on-line PTH amino acid analyser and analysed for the first five cycles. The comparison of the N-terminal sequence obtained against the sequence of the native plant enzyme for the first 5 amino acids is shown in figure 3.4.1.

Figure 3.3.1 Typical Highload monoQ Elution Profile for Enoyl ACP Reductase
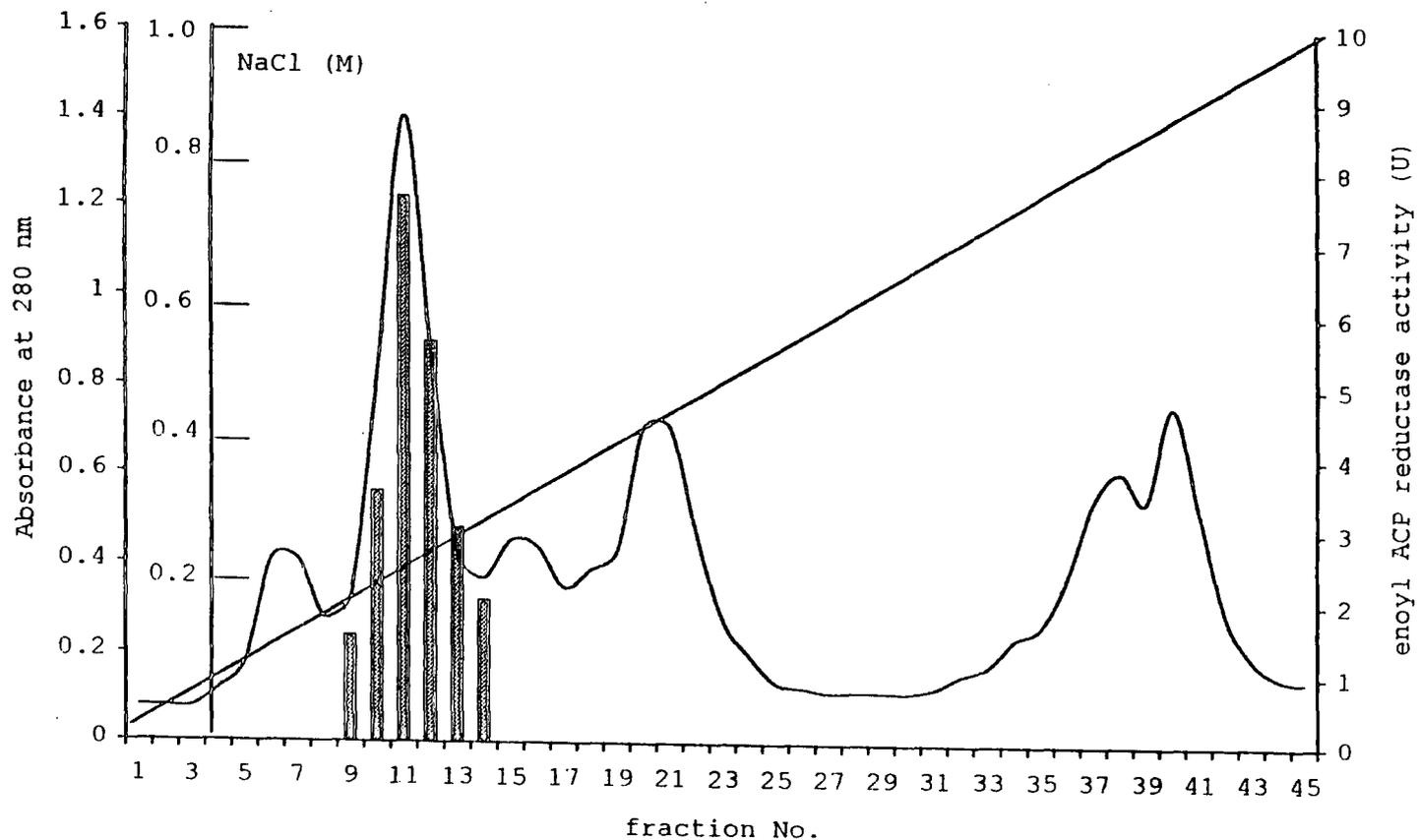
Enoyl ACP reductase activity eluted as a discreet peak betwen 200 and 300mM NaCl

(fractions 9 to 14). The activity expressed as U ml^{-1} (shaded columns) was monitored by

assaying a 5μl aliquot from every fraction.

Figure 3.3.1b

Coomassie stained 10% SDS PAGE gel of Active Fractions from a
Typical Highload Anion Exchange Chromatography Column.
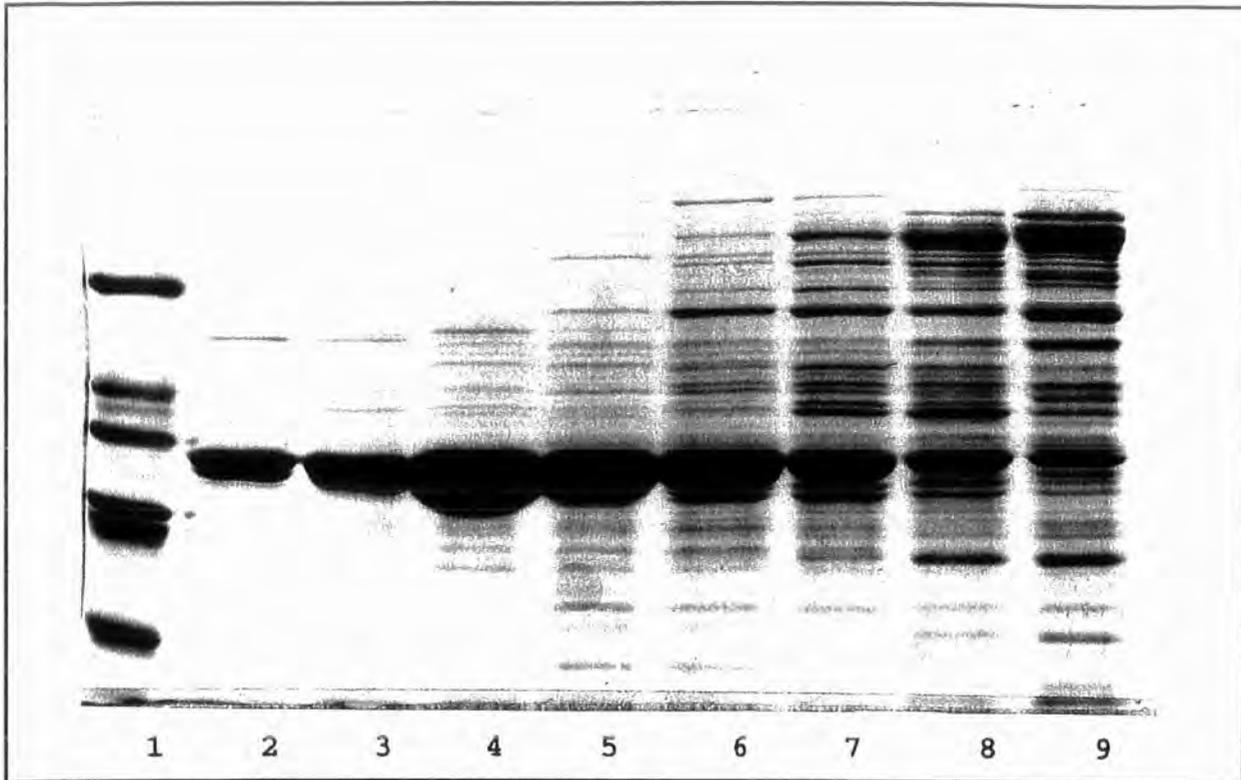
Lane 1 contains Sigma SDS VII molecular weight standards
(66,45,36,29,24,20 Kda).

Lanes 2 to 9 contain 5μl of fractions 9 - 16 from the highload
chromatogram shown in figure 3.3.1

An alanine residue was found at the N-terminus compared to a serine in the protein isolated from rape seed (Cottingham *et al.*, 1988).

This substitution was expected as it was introduced when the DNA encoding the N-terminal amino acid was altered from TCT to GCT during the synthesis of the enoyl ACP reductase construct (Kater *et al.*, 1991).

3.5 Evaluation of the Denatured Antibody Raised Against Purified Enoyl ACP Reductase.

The denatured polyclonal antibody raised against enoyl ACP reductase in mice (2.7) was tested for its specificity against crude rape seed extracts using the standard blotting protocol (2.3.4). An auto-radiogram of a typical blot obtained using ^{125}I labelled secondary antibody is shown in figure 3.5.1. The titre of the antibody was also measured by blotting a standard amount of purified enoyl ACP reductase onto nitro-cellulose and probing with serial dilution's of antibody solution. Figure 3.5.2 shows the ^{125}I labelled auto-radiogram of this nitro-cellulose blot.

Figure 3.4.1

N-Terminal Amino Acid Sequence Comparison

Between Purified Over-expressed Enoyl ACP

Reductase and the Protein Purifed from

Rape Seed.

	amino acid number				
	1	2	3	4	5
Over-expressed protein	Ser	Glu	Ser	Ser	Glu
Protein from rape seed	Ala	Glu	Ser	Ser	Glu
approx. pmole yield	330	100	50	50	100

Approximately 500 pmole of purified enoyl ACP
reductase was loaded across a preparative SDS
PAGE gel. Following electrophoresis the
protein was blotted to PVDF membrane and
detected using rapid coomassie staining. The
protein band was cut fom the membrane and
applied directly to the blot cartridge of the
sequencer. The sequence level obtained (pmole)
is given for each cycle. Serine usually
sequences low and two serines together give
even poorer yields.

Figure 3.5.1

Evalutaion of the Denatured Enoyl ACP Antibody Raised in Mice
Using Western Blot Analyses.
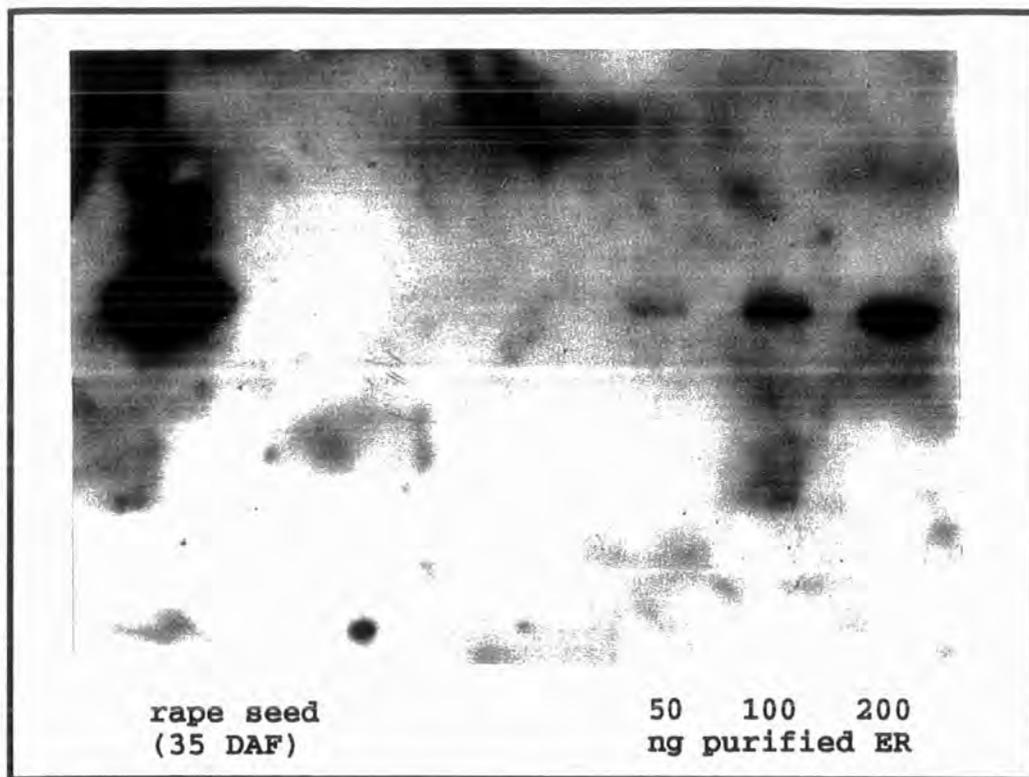
[125]I Labelled western blot on crude rape extract and varying
amounts of purified enoyl ACP reductase. The primary
antibody used to probe this blot was a 1/10000 dilution
of the polyclonal antibody against purified enoyl ACP
reductase raised in mice (2.7). Following labelling with
[125]I secondary antibody the blot was exposed to x-ray film
for 5 hours, before the film was developed.

Figure 3.5.2

Estimation of the Titre of the Denatured Enoyl ACP Antibody

Against Purified Enoyl ACP Reductase.
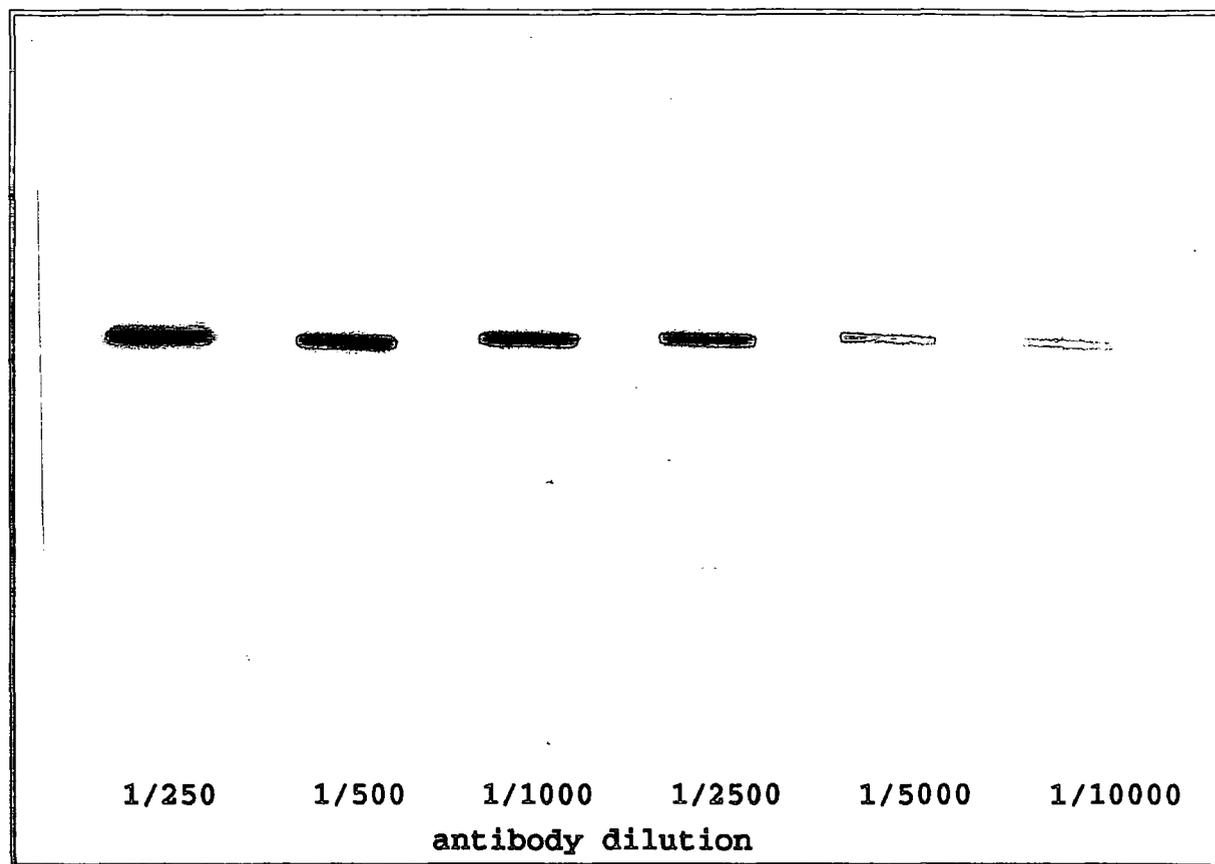
Purified enoyl ACP reductase (100 ng) was blotted onto
nitrocellulose membrane using a Biorad slot blot apparatus.
The individual slots were cut from the membrane and incubated
in diluted primary antibody solution. The membrane was then
washed and labelled with ^{125}I secondary antibody before exposure
to x-ray film.

3.6 Stability of Purified Enoyl ACP Reductase Under a Range of Storage Conditions.

In order to test the ability of purified enoyl ACP reductase to retain its biological activity under a range of possible storage conditions aliquots of the purified enzyme were stored under the following conditions.

-20°C + / - glycerol

-80°C + / - glycerol.

4°C + sodium azide.

4°C + ammonium sulphate

At time points 0,4,7, and 14 days a sample was removed from each of the aliquots and assayed for biological activity using the micro assay described in 2.4.

The results of these assays are shown in Figure 3.6.1. They showed that after 14 days the enzyme had dropped to approximately 50% of its initial activity under all of the storage conditions tested but thereafter remained at a constant level. It was also found that the purified enzyme rapidly lost biological activity when subject to repeated freeze thaw cycles. In view of this data the enzyme was aliquoted into small (500µl) aliquots and stored frozen at -80°C.

Figure 3.6.1

Stabillity of Purified Enoyl ACP Reductase Activity Under a Range of Storage Conditions
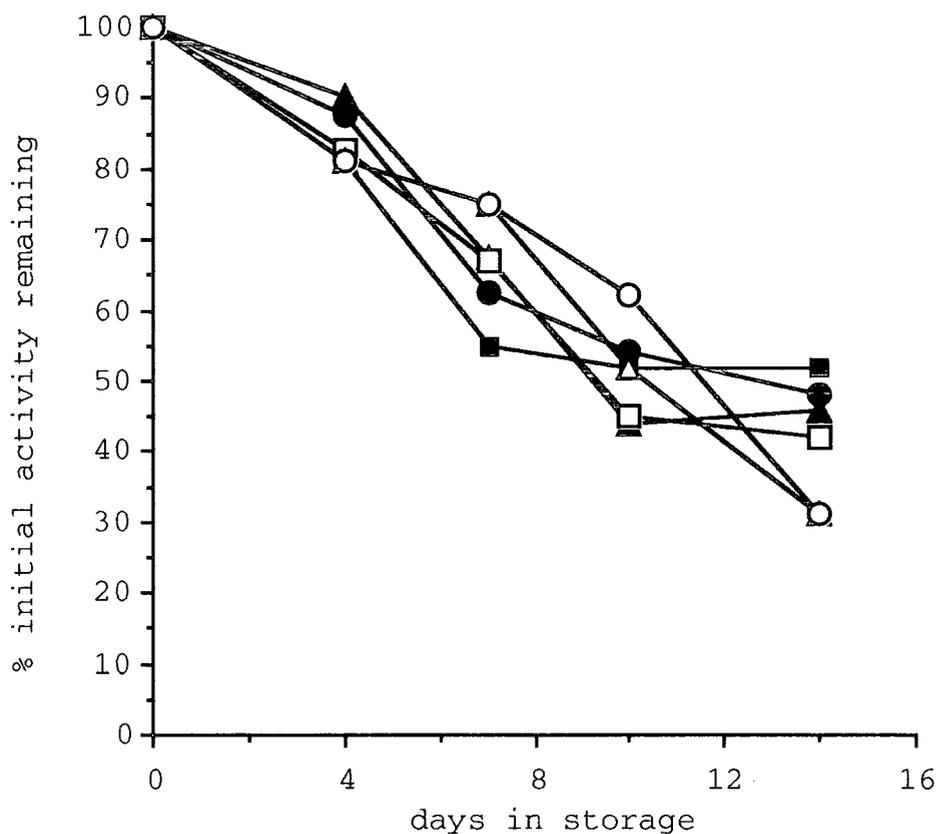
Remaining biological activity shown as a percentage of the initial activity for the following storage conditions; ■ -20°C, ▲-20°C + glycerol, ● -80°C, □ -80°C + glycerol, △ 4°C+ azide and O 4°C, + ammonium sulphate. Purified enoyl ACP reductase showed the same loss (approximately 50%) of its initial activity under all of the storage conditions tested.

3.7 Kinetic Properties of Recombinant Enoyl ACP Reductase.

The kinetic properties of recombinant enoyl ACP reductase were studied under steady state conditions. Initial rates of conversion of NADH to NAD were measured over a range of crotonyl CoA concentrations (100 to 1000 μM) using NADH at the standard assay concentration (140μM). The initial rate was also measured with varying NADH concentrations (1 to 35 μM) using a fixed concentration of crotonyl CoA (300μM).

Lineweaver - Burk plots for both sets of data are shown in figure 3.7.

The apparent Km for crotonyl CoA was found to be 385 μM and that for NADH 5.4 μM under these conditions.

The figure for NADH is close to the published (Slabas *et al.,* 1986) apparent Km value for enoyl ACP reductase from *Brassica napus* of 7.6 μM, and the figure for crotonyl CoA is twice that previously found for the plant enzyme.

3.8 Secondary Structure Analyses by Circular Dichroism.

From the spectral data (figure 3.8) obtained from the CD analyses carried out at the University of Stirling and an absolute estimate of the mass of the sample analysed, a secondary structure prediction for the purified enoyl ACP reductase was made using the "CONTIN" program of Provencher & Glockner (1981).

Figure 3.7 Lineweaver - Burke Plots of Initial Velocity Versus Substrate Concentration Using Purified Enoyl ACP Reductase.

saturating NADH [140μM]
varying crotonyl CoA
[100-1000μM]

apparent km
(crotonyl CoA) = 385μM

1/μM [crotonyl CoA]

fixed crotonyl CoA [300μM]
varying NADH [1-35μM]

apparent km
(NADH) = 5.4 μM

1/μM [NADH]
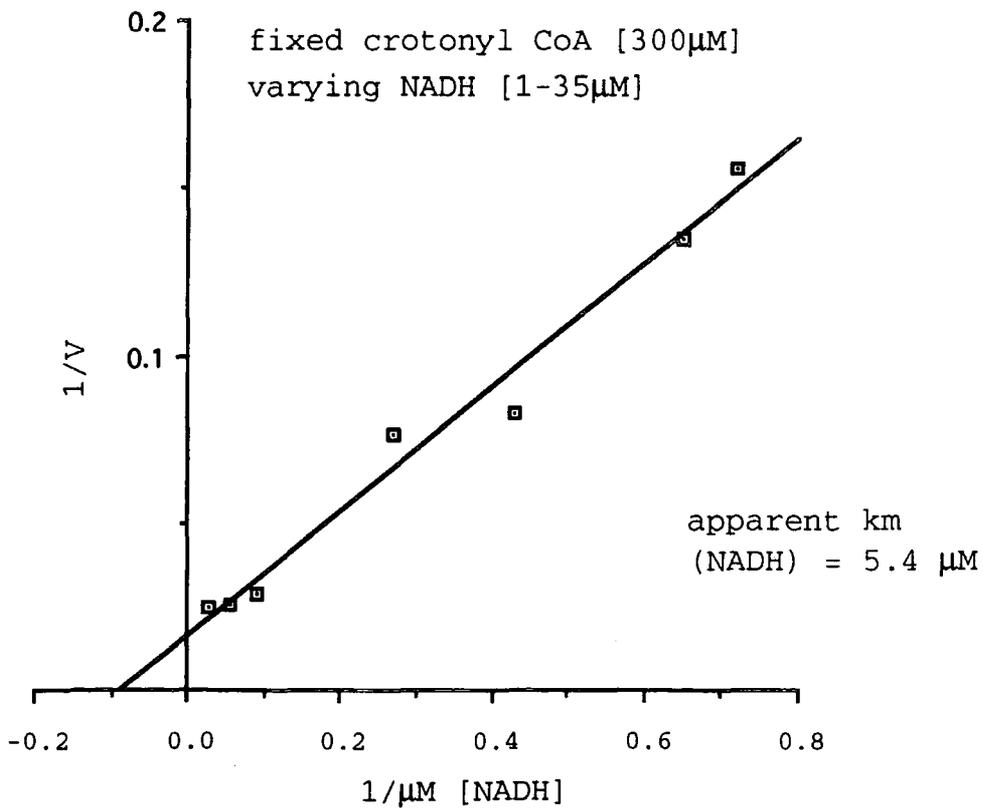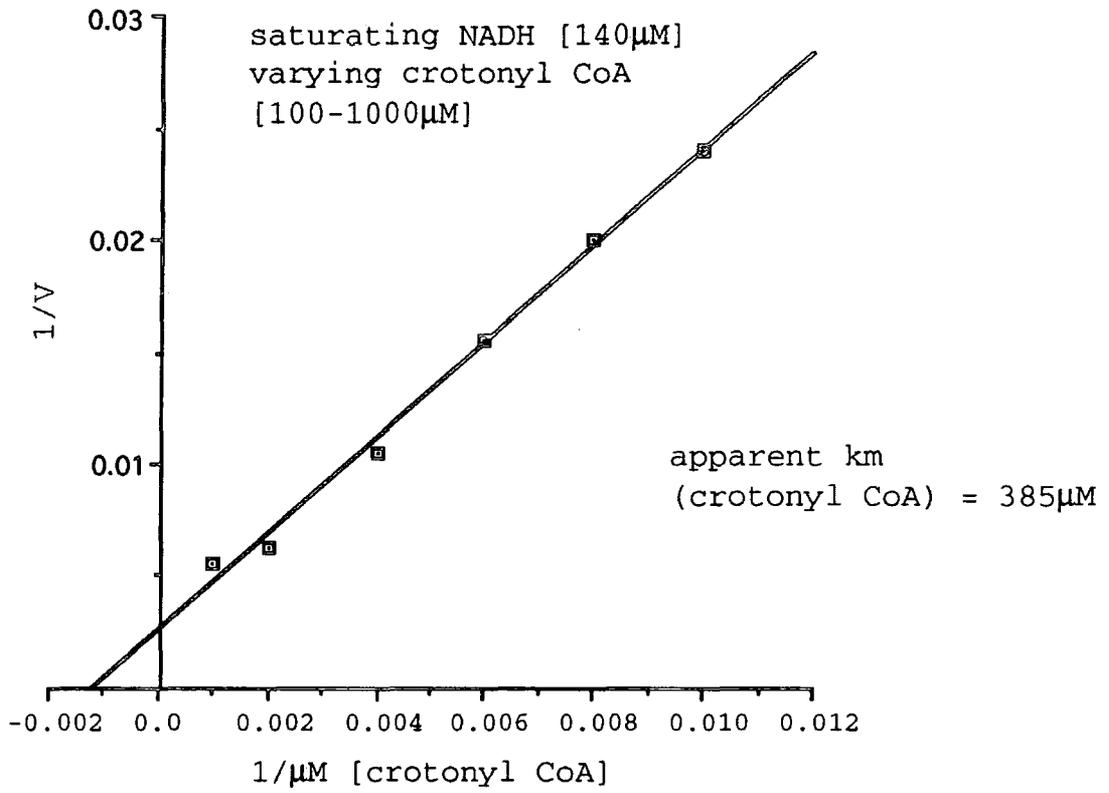
The data from this prediction is shown in figure 3.8 insert and suggests that almost half of the native enzyme is β-sheet and approximately one quarter α-helix. The remainder of the residues do not form any organised secondary structure. A secondary structure prediction for enoyl ACP reductase using the computer program of Chou and Fasman and the primary protein sequence data, was made, and gave the predicted structure as, 36% α-helices, 44% extended conformation and just over 9% each for turn and coil.

The information obtained from the resolution of the full X-ray structure of enoyl ACP reductase during this research work (section 3.13) revealed that each subunit domain within the tetrameric molecule is formed from 7 β-strands (creating a parallel β sheet), and 7 α-helices plus a number of loops of varying length. The results of this comparison show that the use of the CD spectra in the secondary structure prediction gave a more accurate prediction than the use of the computer program alone.

3.9 IEF Determination of Purified Enoyl ACP Reductase.

The Pi of recombinant enoyl ACP reductase was determined using Phast IEF gels (Pharmacia) calibrated with a range of Pi markers (2.3.5.1).

The resulting calibration and the position of enoyl ACP reductase on this gel is shown in figure 3.9.1. From

Figure 3.8   CD Spectra of Purified Enoyl ACP Reductase.

α-helix	β -sheet	remainder
24% +/-0.71%	47%+/-0.81%	28%+/- 1.4%
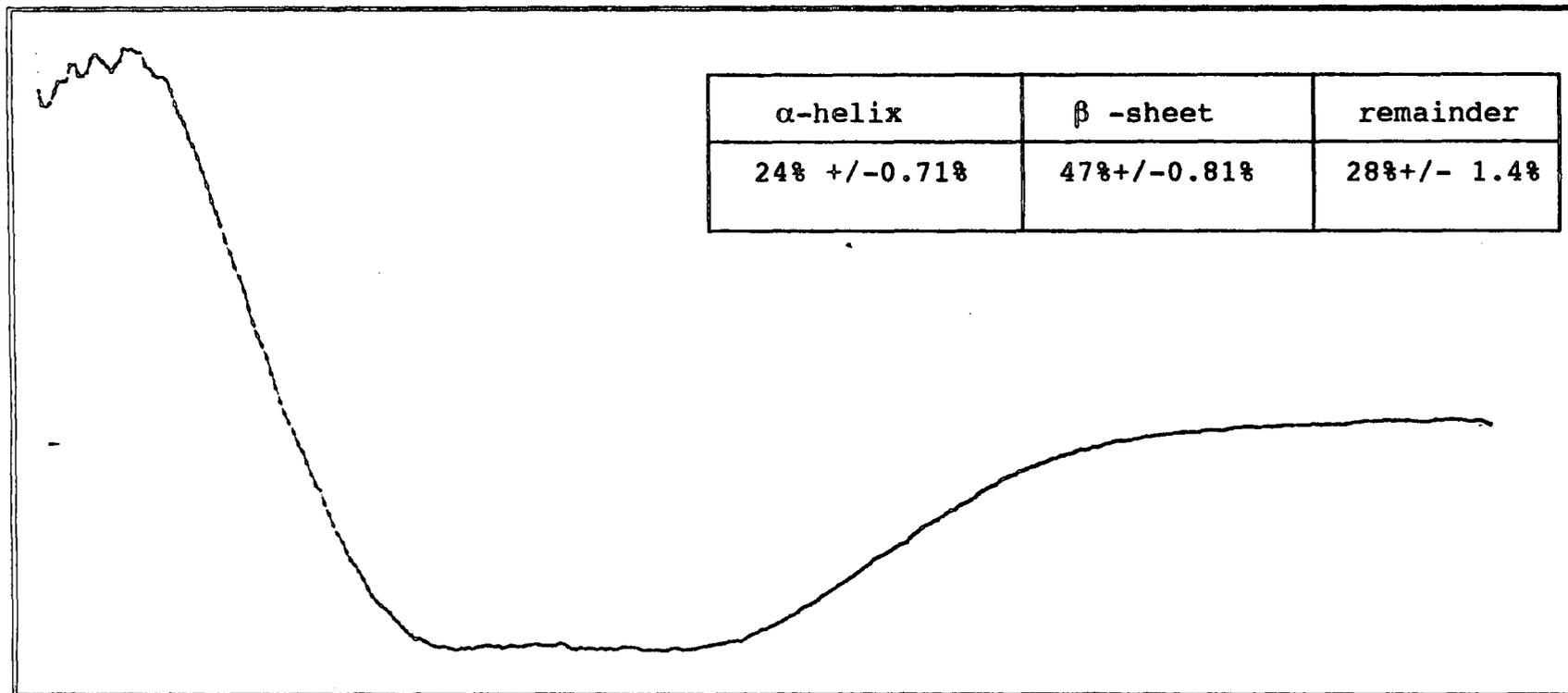
The CD spectra of purified enoyl ACP reductase obtained at the University of Sterling. The insert table shows the secondary structure calculation obtained using the spectra and the computer program CONTIN.

this the purified enzyme was estimated to have a Pi of 5.0.

This acidic Pi for purified recombinant enoyl ACP reductase is very similar to those observed for the four isoenzymes in extracts of *Brassica napus* seed which were found to have Pi points between 4.1 and 4.5 (Fawcett et al.,1994 part of this work).

3.10 Spectral Properties and Molar Extinction Coefficient for Recombinant Enoyl ACP Reductase.

The spectral properties of purified enoyl ACP reductase were analysed between 240 and 340nm using a scanning spectrophotometer and 100 μl quartz cuvettes(2.8.7). The enzyme solution (10mM phosphate buffer pH 6.2 + 1mM DTT) was measured against a reference cell containing buffer but no enzyme. The enzyme had a single absorbance maxima at about 278 nm (Figure 3.10.1).

Using this absorbance value at 278nm and the absolute concentration of the sample analysed (obtained from amino acid analyses carried out in (2.8.5) a molar extinction coefficient of 2.95×10^5 was calculated for the purified enzyme.

Figure 3.9.1

Phast Gel Calibration and Determination of the Native IEF of
Purified Enoyl ACP Reductase

distance (mm) from top of gel

rF for purified ER
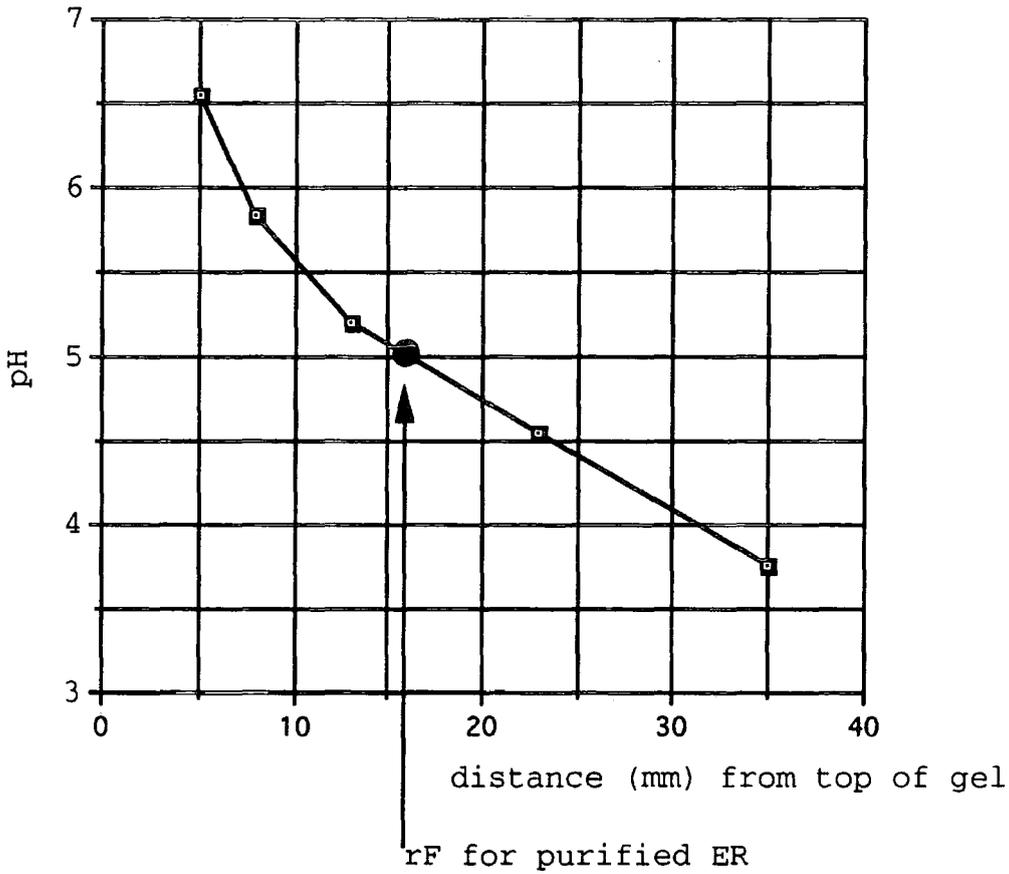
The pH 4.0 to 6.5 IEF gel was calibrated using a Pi
calibration kit (Pharmacia) and the Pi (pH 5.0) of purified
enoyl reductase was calculated from its running position
relative to these standards.

Figure 3.10.1

Spectral Characteristics of Purified Enoyl ACP Reductase
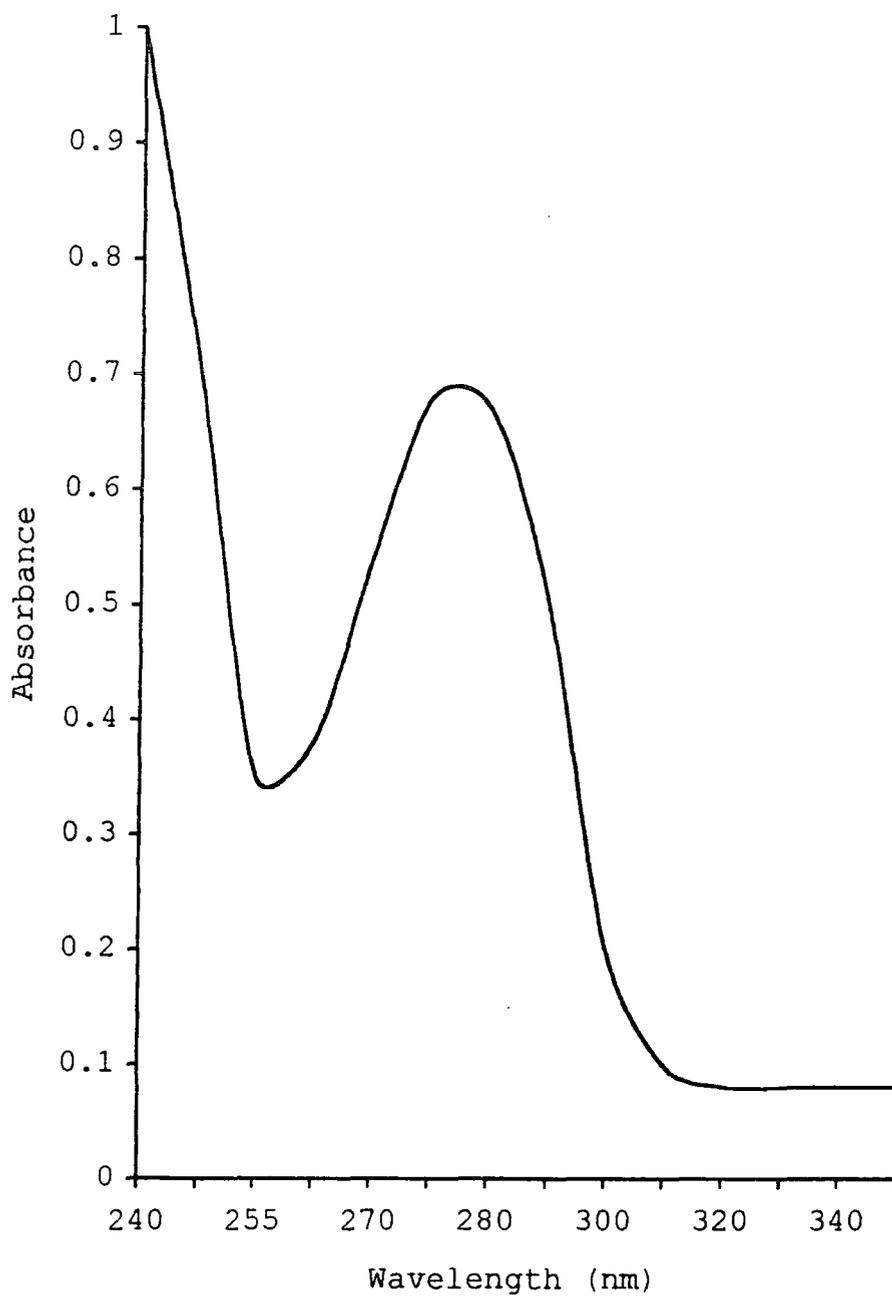
The purified enzyme had a single absorbance maxima at about 278 nm. This value was used to obtain the molar extinction coeficient for enoyl ACP reductase.

3.11 Native Molecular Weight of Recombinant Enoyl ACP Reductase.

The native molecular weight of recombinant enoyl ACP reductase was estimated using both a superose 12 gel filtration column on a FPLC system (Pharmacia) and a SMART superose 12 column on the SMART system (2.8.5). A calibration curve of elution time versus MW of a range of standards was used to calibrate both columns. From this calibration the elution position of a single peak of enoyl ACP reductase activity corresponded to an estimated molecular weight of 120kDa. A similar native molecular weight was found for the plant enzymes isolated from *Brassica napus* seed (Slabas et al., 1986) and spinach leaf (Shimakata & Stumpf, 1982). As the subunit molecular weight (analysed by SDS PAGE) was 32kDa this shows that the recombinant protein like the native plant enzyme exists as an $\alpha4$ tetramer. The calibration curves and the elution position of purified enoyl ACP reductase are shown in figures 3.11.1 and 3.11.2.

3.12 Amino Acid Analyses.

Total amino acid analyses using AABA as an internal standard was carried out at the University of Sheffield on several aliquots of purified enoyl ACP reductase. The error between the results for these samples was +/- 5% indicating good reproducibility of analyses.

Figure 3.11.1

Native Molecular Weight Determination of Purified Enoyl ACP

Reductase Using Gel Filtration Chromatography.
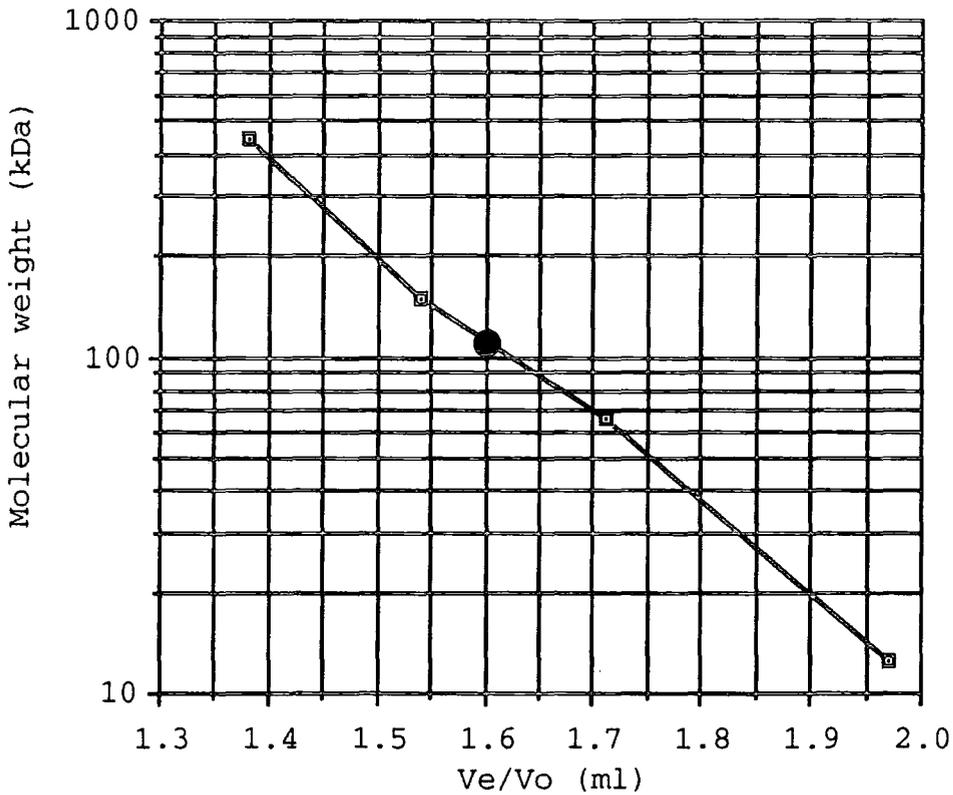
Superose 12 Gel Filtration column on a (Pharmacia) FPLC

system. The column was calibrated (2.8.6) using 1.0 mg

(100µl) of each of the following standards; cytochrome C

(12.4 kDa), ovalbumin (45 kDa), bovine serum albumin

(66 kDa) and β amylase (200 kDa). The elution position of

enoyl reductase is marked● and corresponds to an

approximate molecular weight of 120 kDa.

Figure 3.11.2

Confirmation of Native Molecular Weight of Purified Enoyl

ACP Reductase Using Gel Filtration Chromatography on the

SMART Micropurification System.
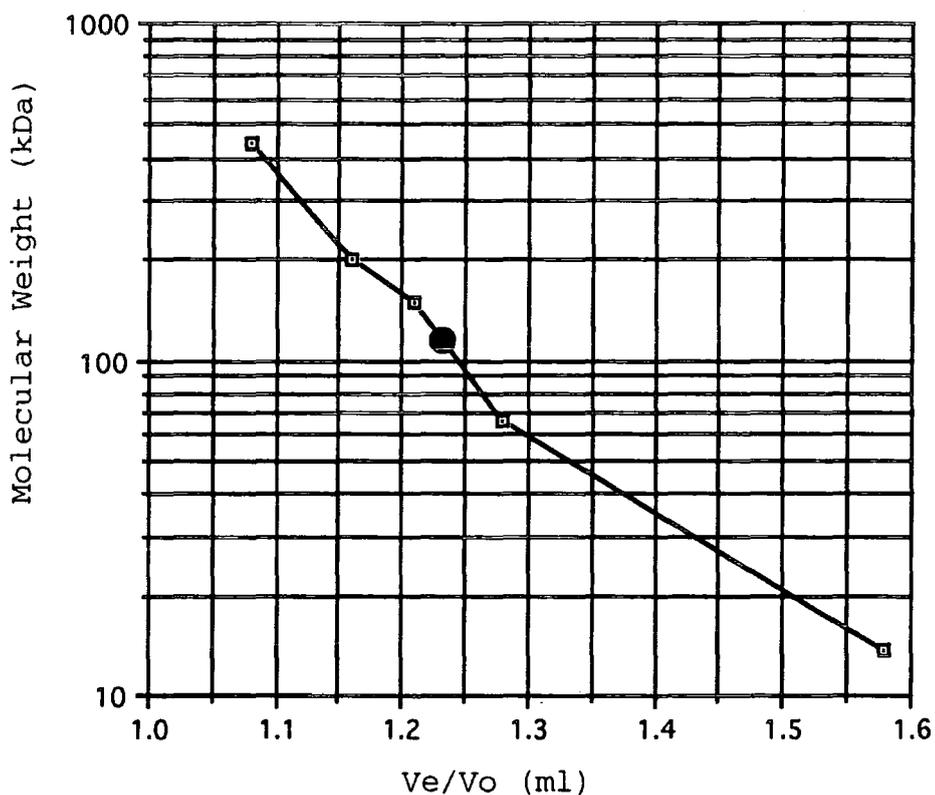
Superose 12 Gel Filtration micro-column on a (Pharmacia)

SMART system. The column was calibrated (2.8.6) using 20 μg

(20μl) of each of the following standards; cytochrome C

(12.4 kDa), ovalbumin (45 kDa), bovine serum albumin

(66 kDa) and β amylase (200 kDa).

The elution position of enoyl reductase is marked ●
and corresponds to an approximate molecular weight of

120 Kda.

Under the conditions of hydrolysis in 6N HCl which is
necessary to release amino acids quantitatively prior to
analyses, several amino acids undergo modifications.
Asparagine and glutamine will be hydrolysed
quantitatively to aspartic and glutamic acid. Yields for
threonine and serine are usually low, cysteine cannot be
determined and certain aliphatic amino acids are
released more slowly than other amino acids
(Ozols,1990).

Allowing for the above considerations when the results
obtained for the amino acids: glycine, arginine,
phenylalinine, leucine and lysine were compared with the
known amino acid sequence, deduced from the cDNA then
the analysis was shown to be accurate to within 6%.
The results of a typical set of analyses and a
comparison with amino acid values deduced from
cDNA data are shown in table 3.12.1.
This amino acid analyses, along with spectral analyses,
enabled, a molar extinction coefficient for purified
enoyl ACP reductase to be calculated. This accurate mass
determination was also necessary to translate the data
collected by CD analysis into a structural prediction.

3.13 Crystallisation and X-ray Analyses of Purified
   Enoyl ACP reductase.

Crystallography has proved to be one of the most
important tools for the elucidation of the detailed

Table 3.12.1

Amino Acid Analyses for Purified Enoyl ACP
Reductase and Comparison with the Known
Sequence Data Deduced from cDNA.

amino acid	MW	pmole	% of total	% of total deduced from cDNA
Asp	115	144.6	9.4	11.1
Glx	128.5	103.5	7.5	8.2
Ser	87	123.1	6.1	9.0
Gly	57	169.4	5.5	4.7
Arg	156.1	82.7	7.3	6.2
Thr	101	72.3	4.1	3.7
Ala	89.7	231.2	11.8	8.9
Pro	163.9	87.2	8.1	4.4
Tyr	163.1	39.0	3.6	5.0
Val	99.1	90.3	5.1	6.0
Met	131	32.2	2.4	2.0
Ileu	113.1	85.6	5.5	7.6
Leu	113.1	147.1	9.4	9.3
Phe	147.1	50.9	4.3	4.9
Lys	128.1	80.8	5.9	6.2

Several problems can occur during sample hydrolysis prior
to amino acid analyses and yeilds for some amino acids are
usually low (section 3.12) The results shown are for a
typical set of data and are close to that expected from the
translated cDNA (3.12).

structure of enzymes and proteins but one of the major
obstacles is the ability to produce suitable crystals
(Ollis & White, 1990).

The ability to produce large amounts of purified enoyl
ACP reductase enabled several lots of crystal trials to
be set up at the University of Sheffield (2.8.8). The
trials which included NAD (150µM) with
2.0 M ammonium sulphate in 0.1M HEPES (pH8.0) as
precipitant, gave octagonal crystals after two weeks
(Figure 3.13.1).

These crystals proved stable under X-ray analyses with
beam lifetimes of >24 hours, and an X-ray pattern with
1.9 angstrom resolution was obtained (figure 3.13.2)
(Rafferty et al., 1994).

Using more of the purified material a total of four
heavy atom derivatives (two gold, one osmium and one
mercury) of the crystals were produced. The x-ray
diffraction data collected for these derivatives was
used to resolve phase problems and refine the initial
crystal diffraction pattern.

The full x-ray structure of enoyl ACP reductase (figure
3.13.3) has now been resolved (Rafferty et al.,in press,
part of this work) and reveals a structure with a
classic dinucleotide binding fold. It consists of a
homotetramer, where each subunit forms a single domain
made up of 7 stranded parallel β-sheet flanked by 7 α-
helices (figure 3.13.3). The NADH binding site was
identified by soaking the crystals initially
crystallised in the presence of NAD, (where the bound

Figure 3.13.1
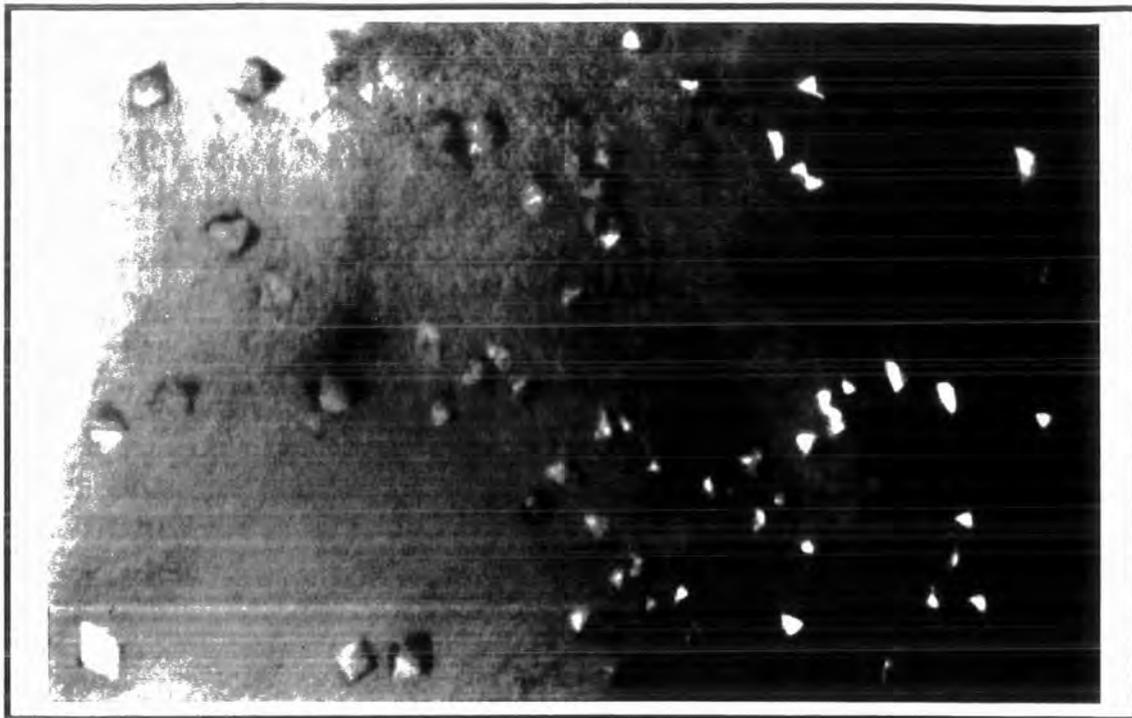
Isomorphous crystals of enoyl ACP reductase complexed with
NAD and grown by the hanging drop vapour diffusion method.
The enzyme crystallizes in the space group $P4_2 2_1 2$ with cell
dimensions a=b=70.5 Å, C=117.8 Å.
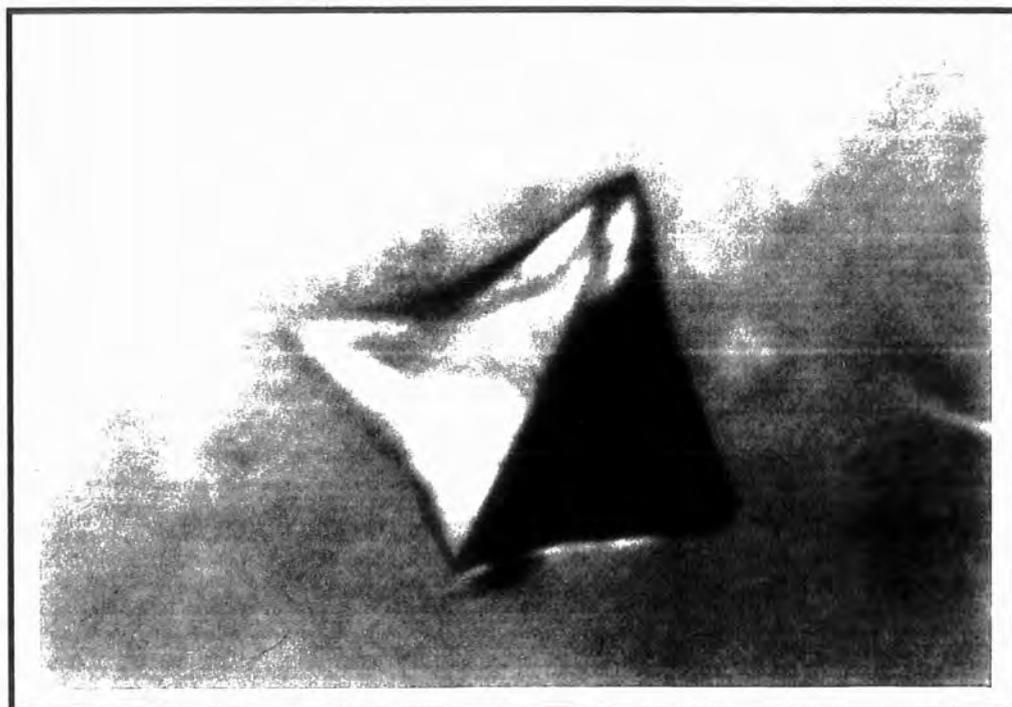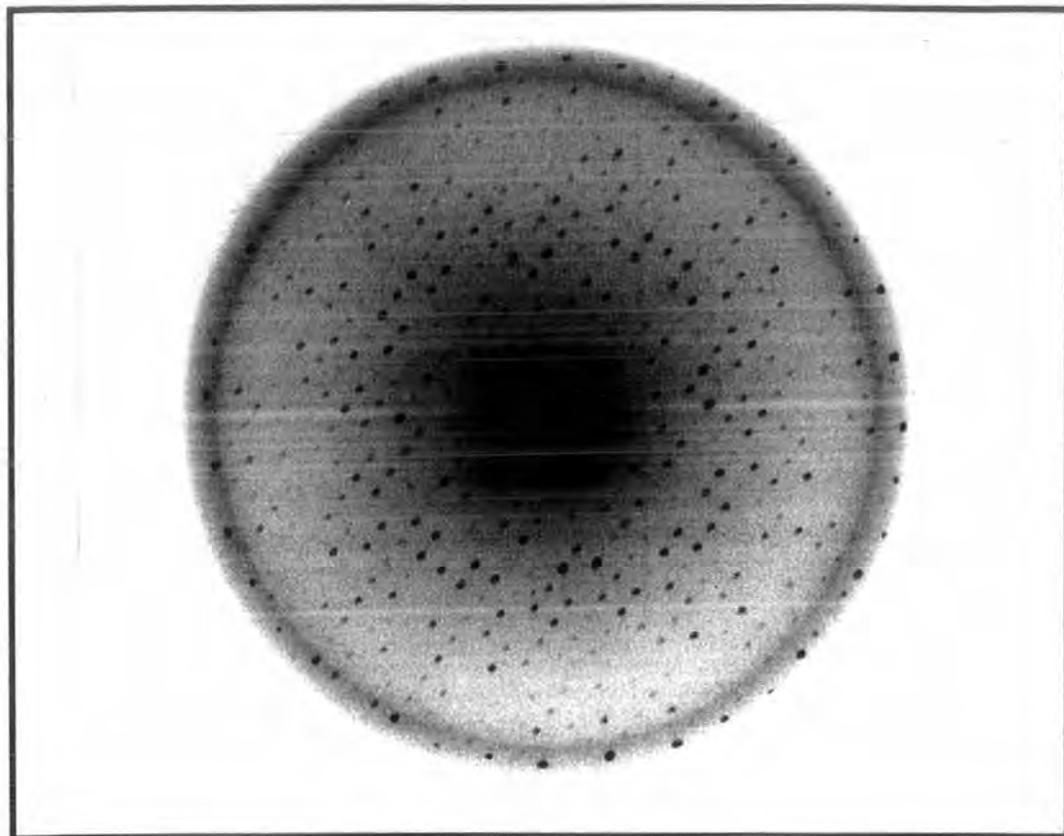
Figure 3.13.2

X-ray diffraction pattern obtained using the native enoyl ACP
reductase crystals shown in figure 3.13.1. The data collected
from this pattern for one crystal was 87% complete to 2.6 $\overset{\text{o}}{\text{A}}$
resolution and 83% complete to 1.9$\overset{\text{o}}{\text{A}}$ resolution.

The x-ray structure of enoyl ACP reductase was solved
using multiple isomorphous replacment techniques (MIR),with
this data set and further data collected using four heavy metal
derivatives of the enoyl ACP reductase crystals.

Figure 3.13.3

The X-ray Structure of Purified Enoyl ACP Reductase
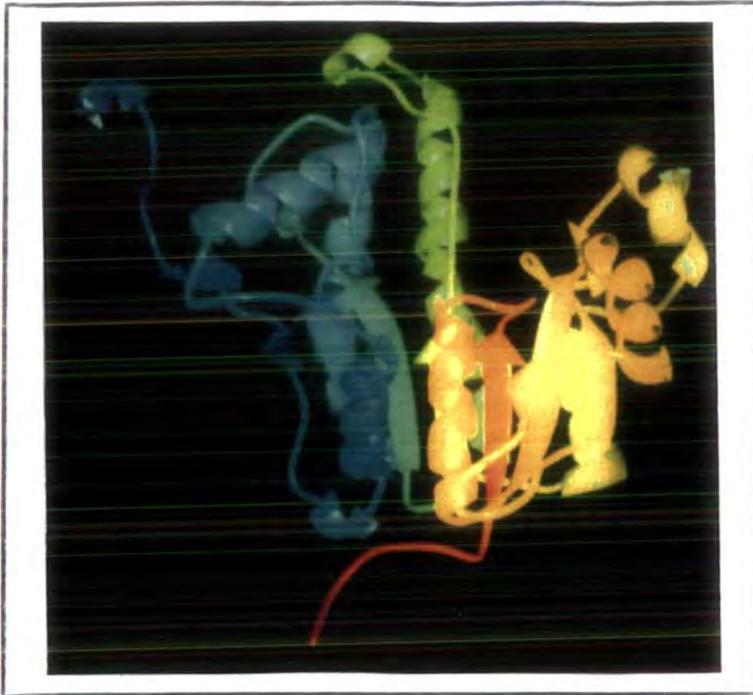
A ribbon diagram of a single subunit of enoyl ACP reductase
(dimensions 55x55x50 $\overset{O}{A}$). The subunit is formed from 7- β strands
flanked by 7 α helices plus a number of loops of varying length.
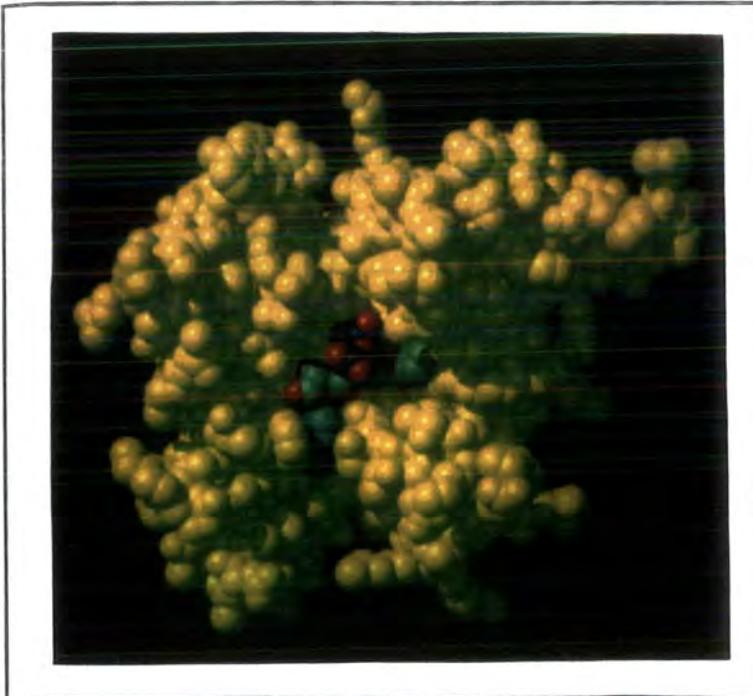
A space filled model of the same subunit were each amino acid is
represented by a sphere of the appropriate van de Walls space. The
nucleotide binding site is highlighted and lies in a cleft between
the COOH terminal end of a parallel β sheet and of flanking

Figure 3.13.3 (continued)

The X-ray Structure of Purified Enoyl ACP Reductase
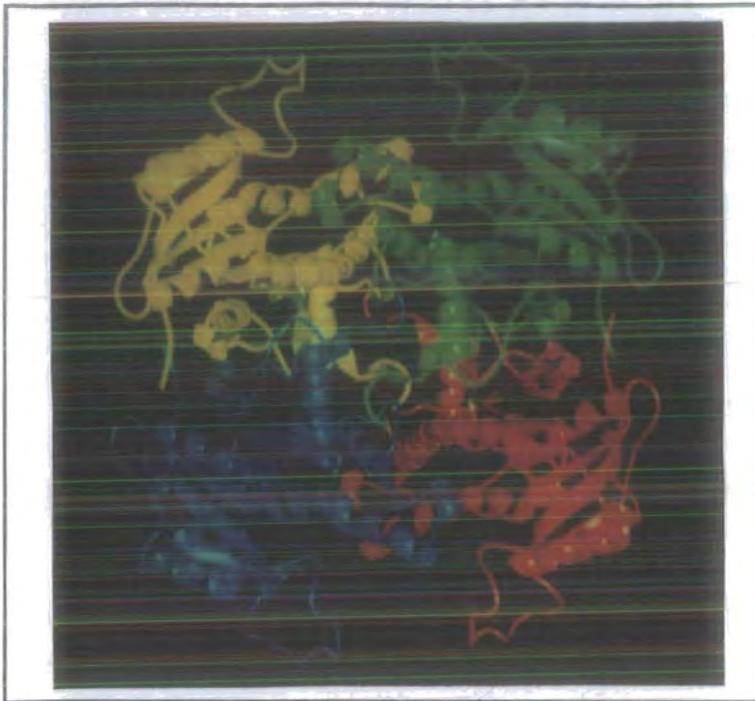
A ribbon diagram of a full tetrameric molecule of enoyl ACP reductase. Each monomer within the tetramer makes contact with all three symetery related partners.
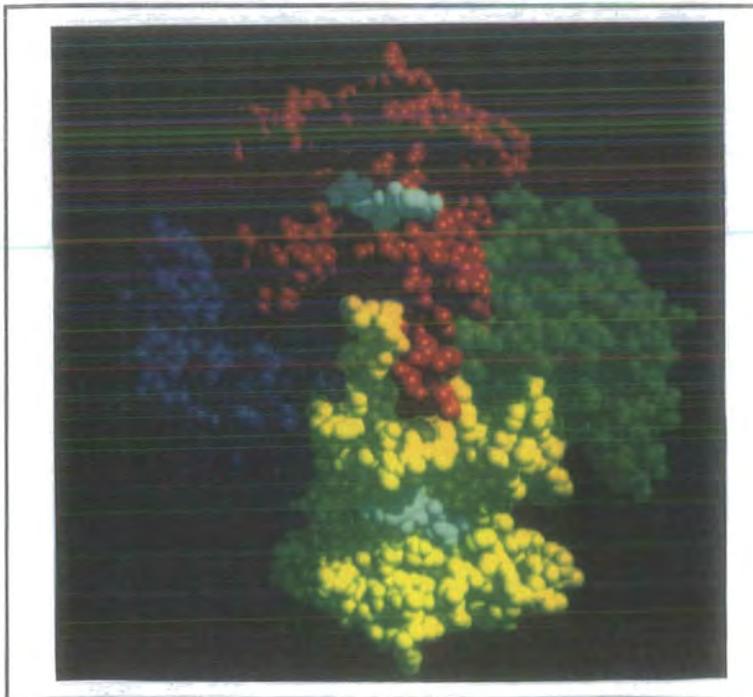
A space filled model of the same tetrameric moleule with the bound nucleotide highlighted in light blue.

NAD was disorded) with NADH. The nicotinamide ring of
the NADH became well ordered and using this data set and
difference fourier analysis the active site was located.
This NADH biding site lies in a cleft between the C
terminal end of a parallel β-sheet and flanking α-
helices (figure 3.13.3). Crystals were also grown in the
presence of the substrate analogue crotonyl CoA, and
diffraction data from these show that it may bind to the
same site as the NADH, and may therefore act as a
competitive inhibitor of the enzyme. The CoA appears to
be binding by its adenine and ribose moieties to the
same pocket as the adenine ring of the NADH. Attempts
are continuing to obtain crystals of enoyl ACP reductase
in the presence of n-acylcysteamine (another substrate
analogue which does not contain adenine and ribose
moieties) in order to identify the correct fatty acid
substrate binding site.

3.14 Purification of Acyl Carrier Protein (ACP) from E.coli.

As acyl esters of ACP are the natural substrates for enoyl ACP reductase a purification of ACP from *E.coli* was carried out during this research work.

ACP was purified from *E.coli* cell pastes (2.10) using a modification of the method of Majerus *et al.*, 1969. Purification by acid precipitation and anion exchange chromatography using a highload Q column (Pharmacia) resulted in approximately 200 mgs of >95% pure ACP from a 2.5 litre bacterial cell paste.

The elution profile of ACP from the highload chromatography step is shown in figure 3.14.1 and the active column fractions analysed on a 15% SDS PAGE gel stained with coomassie blue are shown in figure 3.14.2. The major band present shows the characteristic diffuse pattern typical of ACP on an SDS PAGE gel stained with coomassie blue. A spectral scan between 200 and 300 nm for the purified ACP is shown (figure 3.14.3) Using the molar extinction for ACP of 1.8×10^3 at 280nm (Rock & Cronan 1980 it was estimated that this single preparation yielded approximately 200mgs of pure ACP.
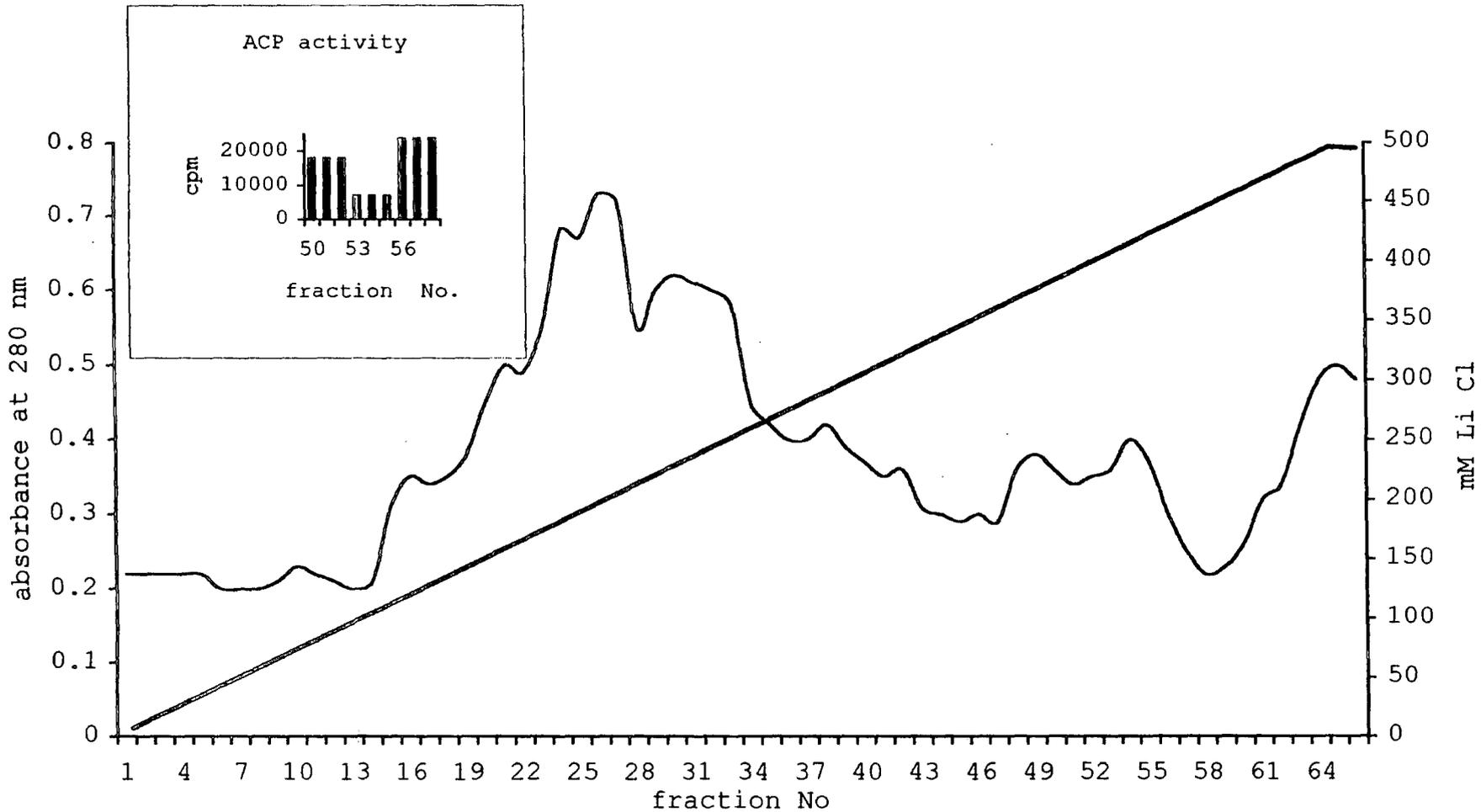
Figure 3.14.1 Typical Highload mono Q elution profile for *E.coli* ACP

Activity (insert) eluted between fractions 50 to 58, at approximately 400mM LiCl. A 5 µl aliquot of each of three fractions was pooled for assay. No ACP activity was found in any of the other fractions.

Figure 3.14.2

Active ACP Fractions From Anion Exchange

Chromatography Analysed on a 15% SDS PAGE gel

Stained with Coomassie Blue
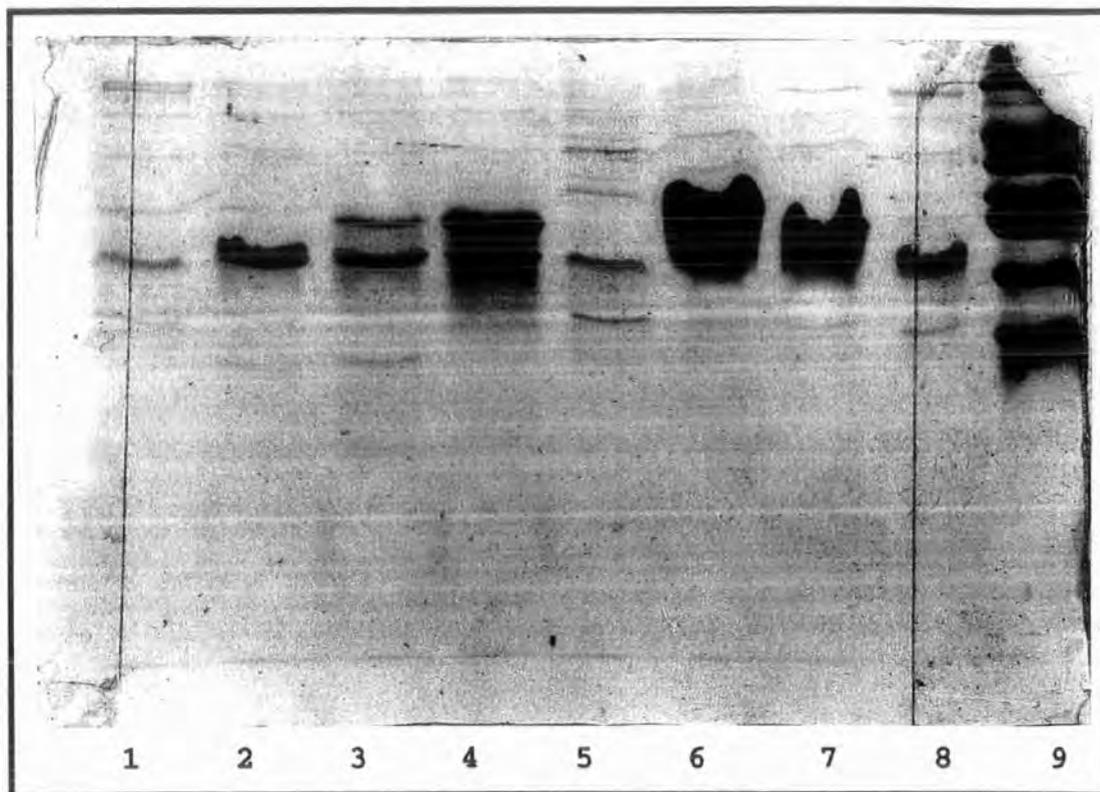
Lanes 1-8 contain 5µl of fractions 51 - 58 from the highload

chromatography shown in figure 3.14.1.

Lane 9 contains 5 µl of sigma SDS VII molecular weight

standards (66,45,36,29,24,20, and 14.2 kDa). The ACP can be

seen clearly in lanes 6 and 7 as a difuse band running at

approximately 20 kDa molecular weight. This is

characteristic of how ACP runs on SDS PAGE gels.

Figure 3.14.3

Spectral Characteristics of Purified *E.coli* ACP
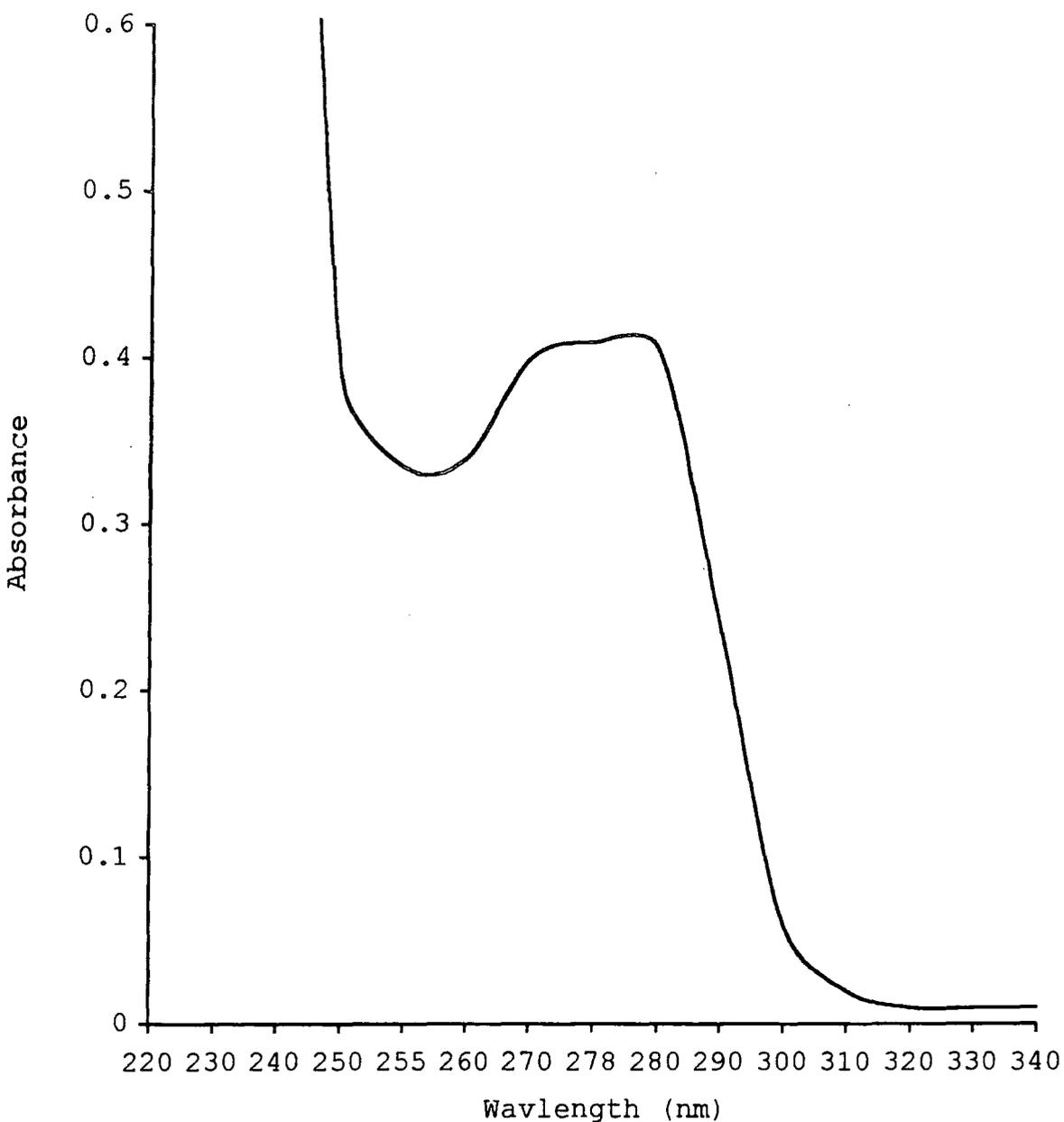
A spectral scan of purified *E.coli* ACP between 220 and 340 nm. The purified ACP has an absorbance maxima at 278 nm. The spectra shows the characteristic roughness between 250 and 260 nm caused by the two phenylalinine residues in the amino acid sequence.

3.15 Identification of Enoyl ACP Reductase Isoforms in Plant Material Using Two Dimensional Western Blot Analyses.

Evidence from previous literature had shown heterogeneity within the amino acid sequence of plant enoyl ACP reductase, this suggested the presence of at least two isoforms in rape seed (Cottingham et al., 1988 and Slabas et al., 1992). Southern blot analyses using a rape enoyl ACP reductase cDNA as a probe had also shown that *Brassica napus* contained four genes, two of which were inherited from each of its parents, B.*oleracea* and B.*campestris*.

To investigate the expression of these isoenzymes crude plant extracts of rape seed and rape leaf material were analysed by two dimensional western blotting (2.3.6). As the denatured antibody raised in mice against purified recombinant enoyl ACP reductase (2.7) was not ready at the time of this experiment an anti-rape native enoyl ACP reductase antibody (which was available from previous work) was used instead.

The results show clearly (figure 3.15) that the same four isoforms of enoyl ACP reductase (labelled I to IV) were detected in both seed and leaf material suggesting that each of the four genes are expressed in both of these tissues. The major two species (II and IV) in seed material also appeared as the most abundant in leaf. This analyses was carried out in conjunction with Dr Tony Fawcett in this department.

Figure 3.15.

Two Dimensional Western Blot Analyses of Isoforms of Enoyl ACP

Reductase in Crude Extracts of Rape Seed and Rape Leaf Material.
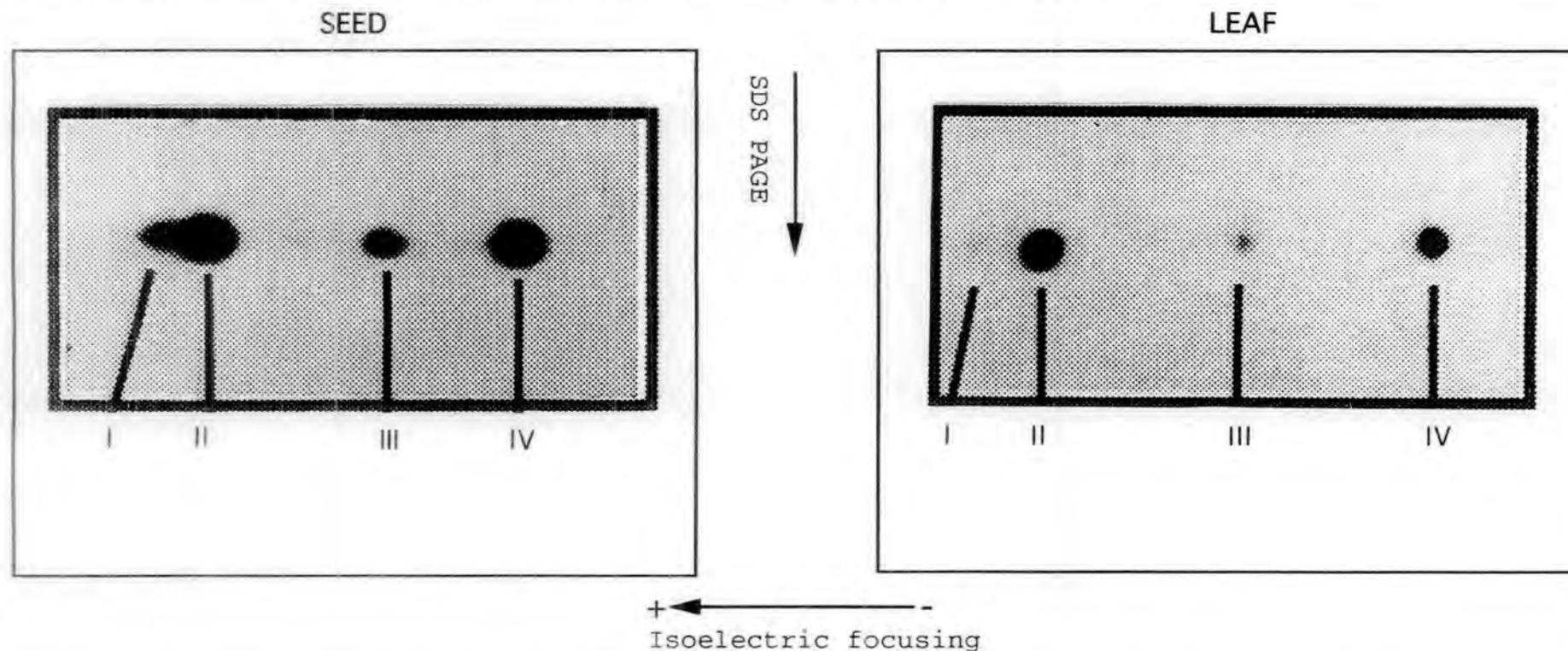
Isoform expression of enoyl ACP reductase in rape seed and leaf material analysed by

2 dimensional western blotting. The blots were labelled with a 1/10000 dilution of rabbit

anti-native rape seed primary antibody and a 1/1000 dilution of [^{125}I] labelled secondary

antibody. The same four isoforms are present in both tissues, with the two major spots present

in seed also present as the dominant spots in leaf.

Protein samples extracted from the equivalent of one seed from various stages of maturing rape were also analysed by 2D western blotting (not part of this study). This analyses showed that the four isoforms separated have different temporal expression.

Isoform II is the first protein detected and appears as the most abundant up to 42 days after flowering, isoform IV appears a little later than II and the other two isoforms I and III are always present in low concentrations (Fawcett et al., 1994).

Chapter 4

4.1 Covalent modification of Purified Recombinant Enoyl Reductase.

The chemical modification of proteins using group specific reagents, which covalently bind to the side chains of amino acids, has been widely used as a technique to identify specific amino acids which have a functional importance within a protein (Glazer *et al.*, 1985).

If the modification of an amino acid side chain with a group specific reagent results in the loss of biological activity, then that residue may have a functional role in substrate binding, enzyme catalysis, or result in an essential conformational change. However care must be used in the interpretation of covalent modification results, as the loss of activity may also be due to steric hindrance of the active site, or to a conformational change in the protein structure.

Several investigators have used covalent modification experiments to identify active site amino acids. In particular, lysine, (Poulose and Kolattukudy 1979 & 1983, Stole and Meister 1991), histidine (Vernon and Hsu 1985, Deka *et al.*, 1992) and arginine (Viale and Vallejos 1985, Mckee and Nimmo 1989, Mukherjee and Dekker 1992, Rider and Hue 1992) have all been successfully modified and identified as active site residues in a number of different enzymes.

Previous work on the inhibition and covalent modification of the native *Brassica napus* enoyl ACP reductase (Cottingham *et al*,.1989) by the arginine modifying reagent phenylglyoxal showed that the plant enzyme activity was inhibited after incubation with this reagent. Using substrate protection studies with CoASH and ACP this inactivation was shown to be related to the modification of two arginine residues in the Co-enzyme A. binding site of the plant enzyme. During this research the inactivation and substrate protection studies were repeated. Using the pure overexpressed enoyl ACP reductase and radioactive phenylglyoxal attempts were made to positively identify active site arginine residues by peptide mapping and sequencing.

4.2 Inactivation Kinetics of Enoyl ACP reductase Using Phenylglyoxal.

A known amount (approximately 10 μg) of purified enoyl ACP reductase was buffer exchanged into 85mM NaHCO$_3$ buffer pH 8.0 and incubated with 0, 0.5, 1.0, 2.5 and 5.0mM phenylglyoxal at a temperature of 30°C in a total reaction volume of 50μl (2.11.1).
At time = 0 and at 10 minute time points from the start of the incubation a 5μl aliquot was removed and assayed for biological activity (2.4).
Under these phenylglyoxal concentrations the purified enoyl ACP reductase rapidly lost biological activity.

Figure 4.2.1

Time Dependant Inactivation of Enoyl ACP Reductase Activity

with Increasing Phenylglyoxal Concentrations
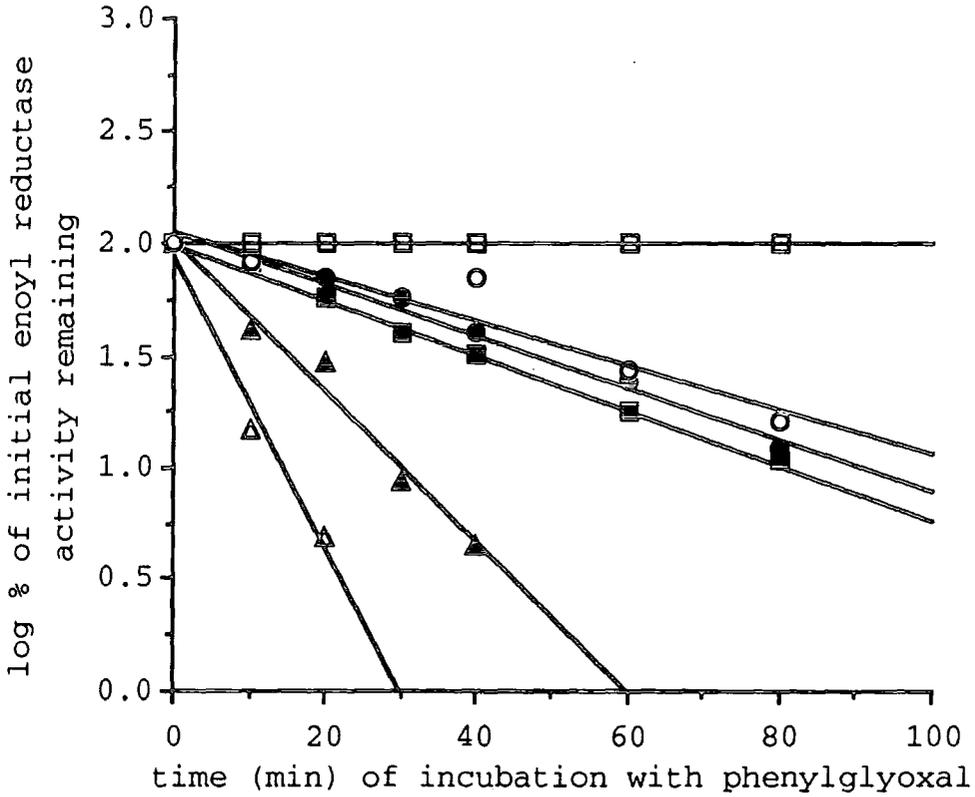
Purified enoyl ACP reductase was incubated with:□ 0,○ 0.25
● 0.5,■ 1.0▲2.5, and△ 5.0mM phenylglyoxal in bicarbonate
pH 8.0 at 30°C. At time intervals an aliquot was removed
from the incubation mixture and assayed for remaining
activity (2.11.1).

An inhibitor concentration of 1.0mM showed almost 50%
inhibition and a concentration of 5.0mM showed total
inhibition after 30 minutes incubation. This is shown in
figure 4.2.1. This data compares well with that shown by
Cottingham, who found that enoyl ACP reductase activity
decreased to less than 2% of initial activity, when
incubated with 4.8mM phenylglyoxal for 30 minutes. The
modification and inhibition was linear, very rapid and
appeared monophasic in nature. This suggested that a
single highly reactive arginine residue was involved.
The time dependant inactivation of enoyl ACP reductase
with increasing concentrations of phenylglyoxal is shown
in figure 4.2.1.

For each phenylglyoxal concentration the plot of the log
of the residual activity against time gave a linear line
showing that the inactivation was apparently first-
order.

4.3 Substrate Protection Studies.

The ability of *E.coli* ACP (purified in 2.10.1) and
co-enzyme A to protect against the inactivation of enoyl
ACP reductase by phenylglyoxal was examined using a
range of ACP and CoASH concentrations (2.11.1).

At a concentration of 500µM ACP gave total protection
against phenylglyoxal inactivation and CoASH at a
concentration of 20mM gave 70% protection. This data is
somewhat different to that found by Cottingham, who used

Figure 4.3.1

Substrate Protection Against Phenylglyoxal

Inactivation of Enoyl ACP Reductase Enzyme Activity
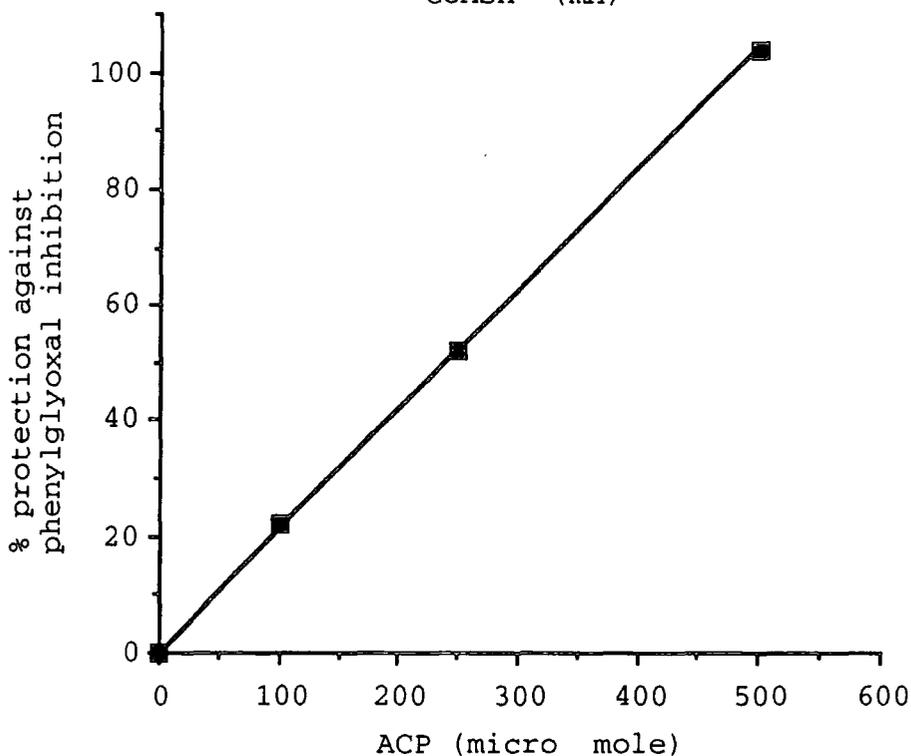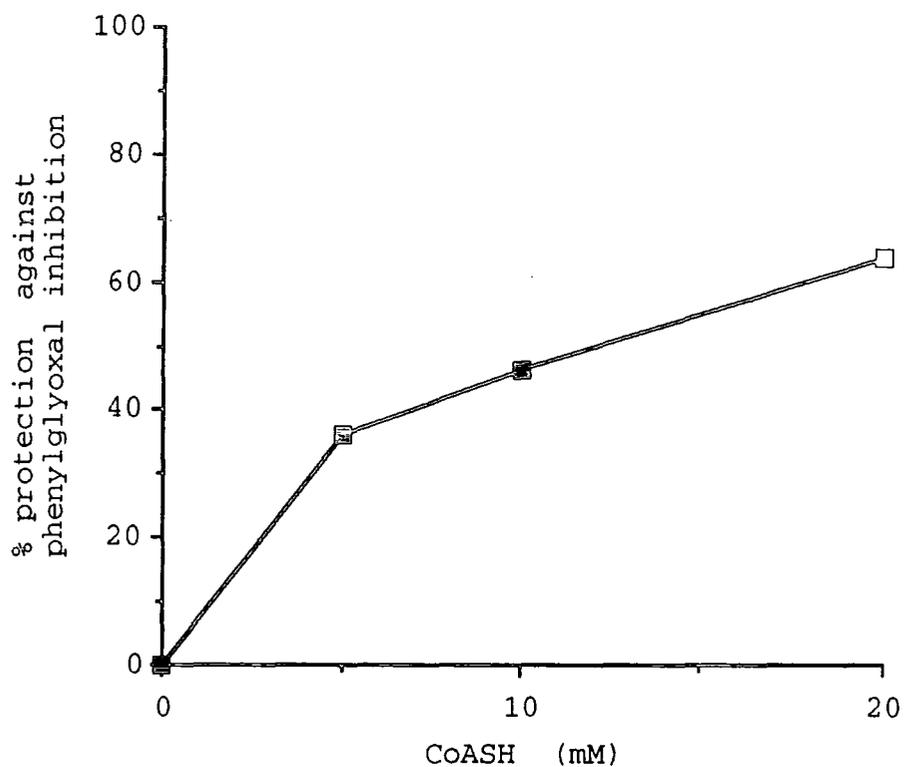
The top figure shows protection against phenylglyoxal inhibition when purified enoyl ACP reductase is pre-incubated with CoASH and the bottom figure shows protection against inhibition by preincubation with ACP.

partially purified (post blue sepharose) enoyl ACP reductase for his protection experiments and found that ACP at 9.0 μM and CoASH at 10 mM gave protection of enoyl ACP reductase activity of 35.4% and 98% respectively.

The data for these protection experiments is shown in figure 4.3.1.

Both of these reagents one a natural substrate and the other a substrate analogue gave almost total protection against the loss of enoyl ACP reductase activity after only a short pre-incubation with the protecting reagent. This substrate protection indicates that the modification and inhibitory effect of phenylglyoxal is at or near the substrate binding site of enoyl ACP reductase, or that the phenylglyoxal modification of a single arginine residue results in a conformational change in the tetrameric structure of the enzyme which inactivates it.

The results also show the suitability of CoASH to protect against covalent modification of active site arginine residues in later peptide mapping and sequencing experiments.

4.4 Radioactive Phenylglyoxal incorporation and loss of Enoyl ACP reductase Activity.

The loss of enoyl ACP reductase activity with the incorporation of radioactive phenylglyoxal was measured against time using [14C] labelled phenylglyoxal

Figure 4.4.1

Modification and Inhibition of Enoyl ACP Reductase
by [14]C Labelled Phenylglyoxal.
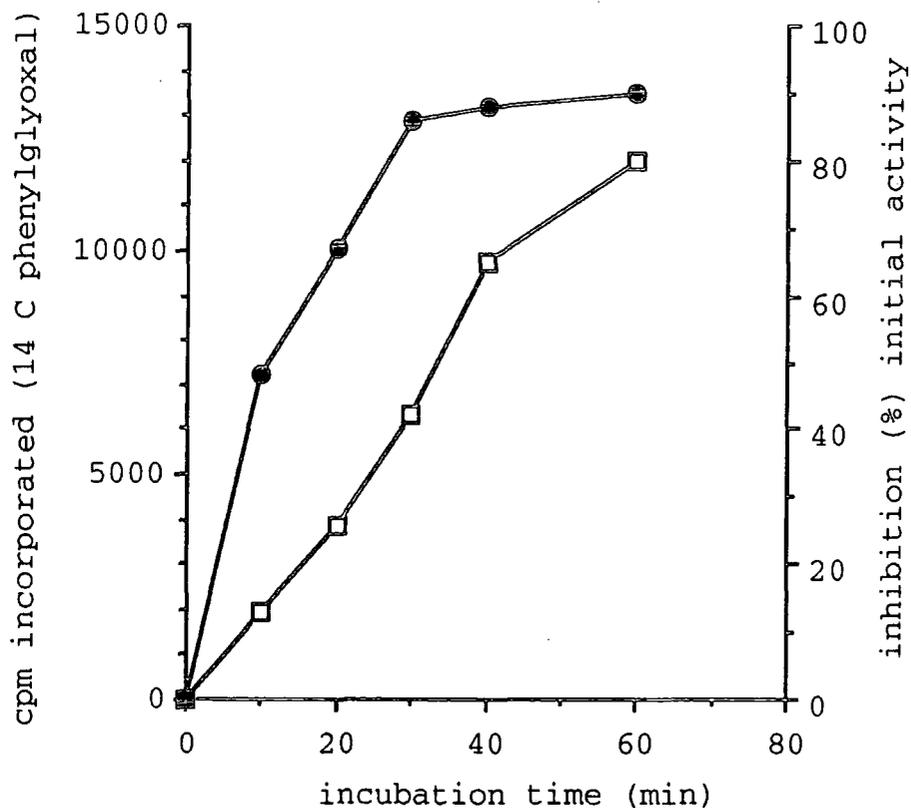
Figure 4.4.1 Shows the linear relationship between [14] C
phenylglyoxal incorporated (CPM) □ and the inhibition of
enoyl ACP reductase activity ● .   Approximately 1 nmole
of purified enoyl ACP reductase was incubated with radiolabeled
phenylglyoxal (2.11.2). At each time point samples were
removed and assayed for biological activity and radioactive
incorporation.

(2.11.2). The results show that a linear relationship exists between the loss of activity and the incorporation of radioactivity up to approximately 80% loss of activity.

The data is shown in 4.4.1. Using the specific activity of the isotope, the value for maximum inhibition, and the absolute amount of enzyme used in the experiment (calculated from amino acid data) it was estimated that approximately 2 moles of phenylglyoxal was bound per mole of native tetrameric enzyme when maximum inhibition occurred.

The stoichiometery of phenylglyoxal to arginine binding (two moles of phenylglyoxal / mole of arginine, (Takahashi 1968)) suggests that the activity of the tetrameric molecule is lost when only one arginine residue is modified. This is unlikely to be the result of a single active site within the tetramer being modified, but is more likely to suggest that the modification of a single residue results in a conformational change within the tetramer resulting in the loss of activity. The data collected during this work has not been able to answer this question conclusively.

This result was compared to the incorporation data given by Cottingham who found a linear relationship between phenylglyoxal binding and the inactivation of enoyl ACP reductase to at least 60% inhibition. The data was extrapolated to give a value for total inactivation when 4 moles of phenylglyoxal was bound per mole of enzyme.

Figure 4.4.2

Correlation of [14] C Phenylglyoxal Incorporation with
Inhibition of Enoyl ACP Reductase Activity.
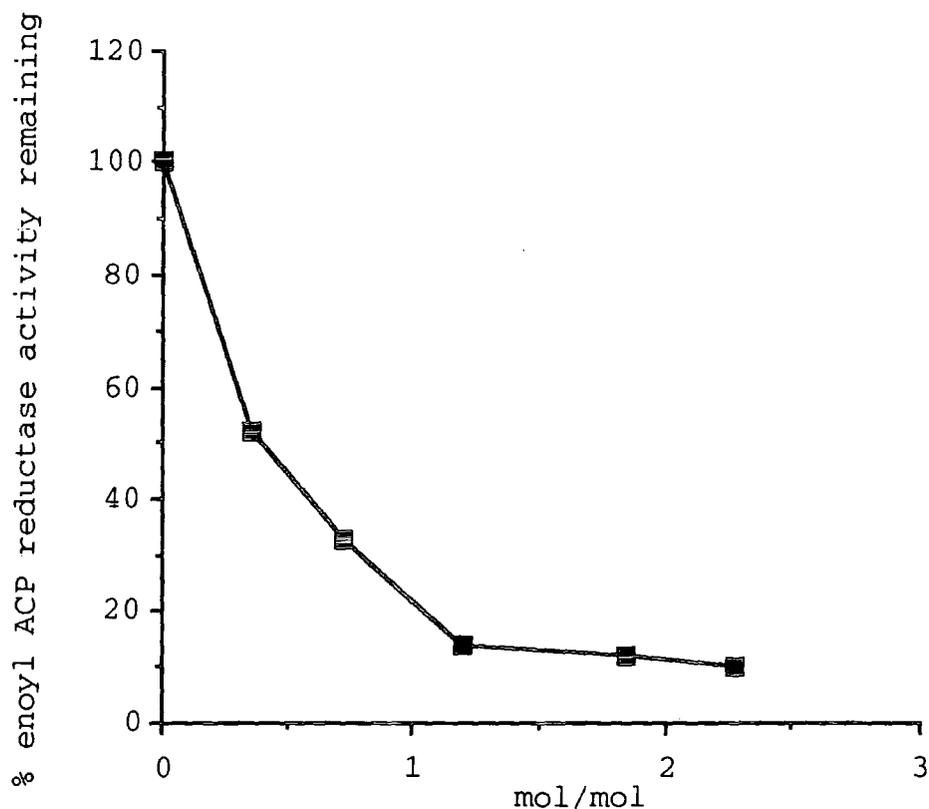
(purified enoyl ACP reductase/Phenylglyoxal)

The loss of enoyl ACP reductase activity plotted against the
incorporation of radiolabel described as mol/mol. This was
calculated from the specific activity of the phenylglyoxal
(supplied by the manufacturers Amersham) and the amount of
protein used in the experiment (calculated using the molar
extinction coeficient for purified enoyl reductase
(section 3.10) and the native molecular weight of
tetrameric enoyl ACP reductase (120 kDa).

The data could not be directly compared as Cottingaham used a partially purified enzyme in the experiment and the molecular mass of the monomer was used for the calculation.

4.5 Peptide Digestion and Peptide Mapping of 14C Phenylglyoxal Modified Enoyl ACP reductase.

Extensive attempts were made to inactivate purified enoyl ACP reductase with radiolabelled [^{14}C] phenylglyoxal both in the presence and absence of substrate protectants and then obtain comparative peptide maps by digestion with proteolytic enzymes (endo arg C, endo lysC, and TCK treated Trypsin) (2.11.3). Although it was possible to produce good reproducible digests with both the lys C and trypsin it was not possible to produce successful digest with arg C. Therefore for the experiments in which peptides were to be isolated for amino acid sequencing lys C and trypsin were the two enzymes used for the digestion.

For early experiments 15% Schagger gels were used to test the efficiency of the proteolytic digestion.

A typical coomassie stained schagger gel on peptide fragments is shown in figure 4.5.1.

Transfer of peptides from schagger gels to problot PVDF membrane followed by exposure to x-ray film was also tried in order to isolate peptides containing

Figure 4.5.1

Coomssie Stained 15% Schagger Gel on Lys C Peptide
Fragments of Purified Enoyl ACP Reductase.
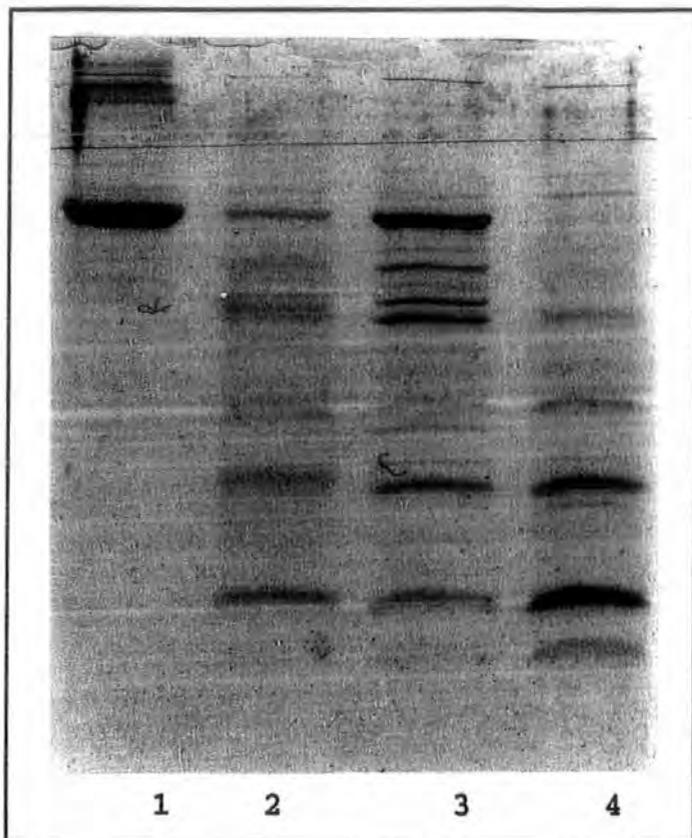
Lane 1 contains intact enoyl ACP reductase control

Lane 2 contains peptides produced after 2 hours
incubation with Lys C,

lane 3 peptides after 1 hours incubation.

lane 4 peptides after 3 hours incubation.

After 3 hours none of the intact enoyl ACP reductase
can be seen and a complete proteolytic digestion is
acheived.

Figure 4.5.1b

Fluorogram of Radioactive Peptide Fragments of

Purified Enoyl ACP Reductase
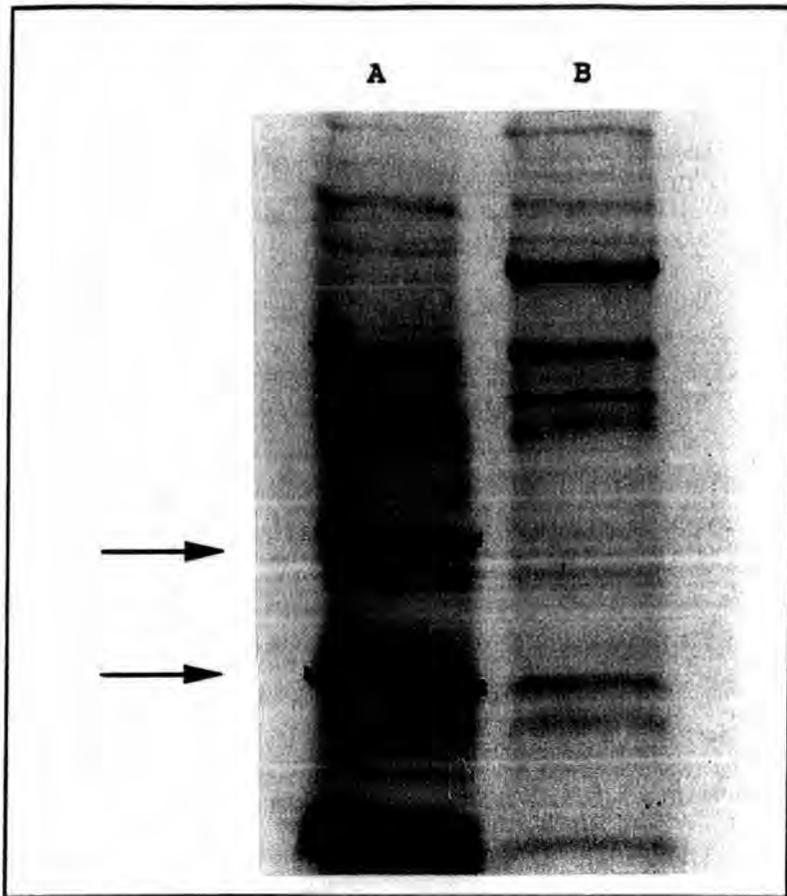
Purified enoyl ACP reductase was incubated with ^{14}C phenyglyoxal

with (B) and without COASH (A) as a substrate protectant.

Following the phenylglyoxal incubation the material was

incubated for 3 hours with lys C under standard digestion

conditions. The digests were separated on a 15% Schagger Gel and

then blotted over to PVDF membrane. The membrane was cut to give

lanes containing A and B on both halves, one half was coomassie

stained and one half exposed to x-ray film. The radioactive

bands present in A (marked) and not present in B were cut from

the coomassie stained membrane and applied to the protein

sequencer. No sequence data was obtained, either the peptides

were blocked during the procedure or they were present at too

low a level to sequence.

radioactive label for sequence analyses and
identification.

Although a successful fluorogram was obtained (figure
4.5.1b) no sequence data was obtained from the fragments
of blot applied to the protein sequencer. This could
have been because the peptides were N-terminal blocked
during the electrophoresis and blotting procedures or
that the blotting efficiency was low and the levels of
peptide applied to the sequencer were very low.
This proved to be an insensitive and (because of the
time required to obtain film results) time consuming
procedure, it was therefore decided to concentrate on
the use of reverse phase HPLC using the SMART micro
chromatography system (Pharmacia).

4.5.1 Enoyl ACP reductase Peptide Digest Analyses Using Reverse Phase HPLC on the SMART Chromatography System.

Two 500 pmole samples of purified enoyl ACP reductase
one protected by pre-incubation with 10mM CoASH were
incubated with [14C] labelled phenylglyoxal and the
biological activity remaining monitored against time.
After 60 minutes incubation when less than 25% of the
initial activity was detected in the unprotected sample
(figure 4.5.2.) further labelling was stopped by
precipitating the protein with 10% trichloro-acetic acid
(TCA). The pellets from the two samples were resuspended
and denatured in 8M urea before digestion with either

Figure 4.5.2

Inactivation of Enoyl ACP Reductase Activity by [14]C
Phenylglyoxal Prior to Digestion with Endo Lys C and Reverse
Phase HPLC of Peptides.
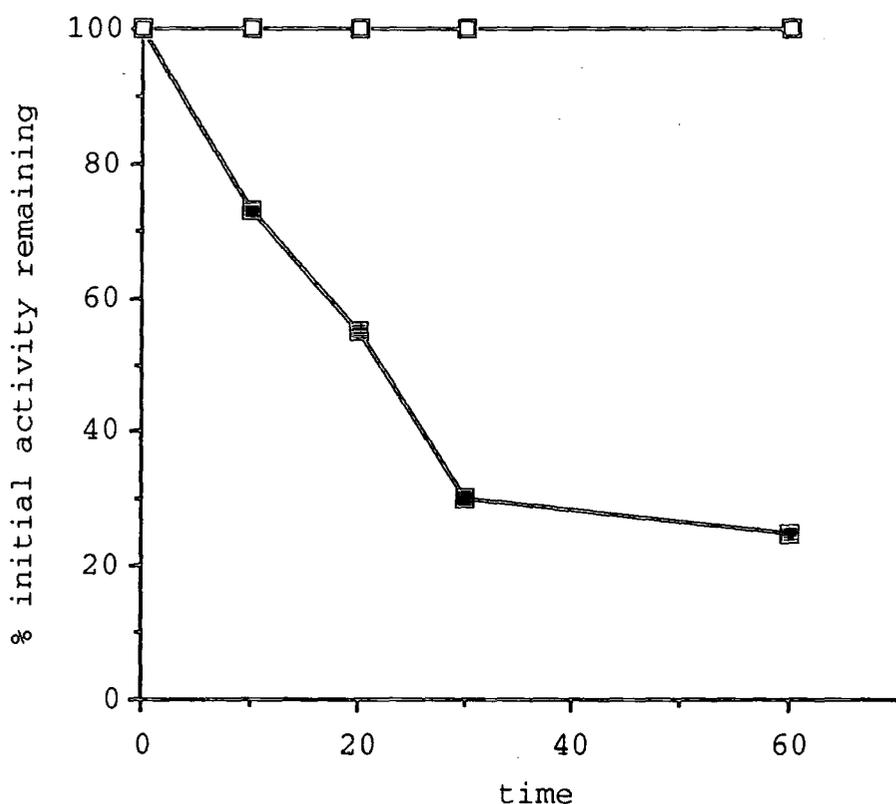
Enoyl ACP reductase activity in CoASH protected □ and
unprotected ■ (no substrate) samples after incubation with
[14]C labelled phenylglyoxal.

At t = 60 minutes the reaction was terminated and the
radiolabeled enoyl ACP reductase was precipitated by the
addition of 10% TCA (2.11.3) prior to peptide
digestion with endo LysC.

endo lysC or TCK treated trypsin. Figure 4.5.3 shows the comparative digest obtained from a lysC digestion and figure 4.5.4 shows comparative digests from a typical tryptic digest. Both sets of digests were analysed using a Vydac C18 microbore column with an 5-80% acetonitrile in 0.1% TFA gradient on the SMART chromatography system.

4.5.2 Sequencing of a Radioactive Peptide from [^{14}C] Phenylglyoxal Modified Enoyl ACP reductase.

After endo lysC digestion using the protocol above an enoyl ACP reductase peptide containing radioactive label was isolated (figure 4.5.5.) and successfully sequenced from the enzyme unprotected by substrate.

This peptide was shown to be the peptide after the first N-terminal lysine and continued to sequence correctly up to the next lysine residue. Although the sequencer was allowed to continue for a further five cycles (to confirm that this was a single lys C peptide) no further sequence data was obtained.

The sequence is given in the legend of figure 4.5.5. Although this appeared to be a successful experiment with a radioactive peptide containing an arginine residue isolated, sequenced and identified there were two observations which needed to be resolved.

i) The total amount of radioactivity associated with this peptide after reverse phase HPLC was 5.2 x 10^4 cpm.

Figure 4.5.3

C18 Reverse Phase HPLC on LysC Peptide Digests of Phenylglyoxal Modified Enoyl ACP Reductase.
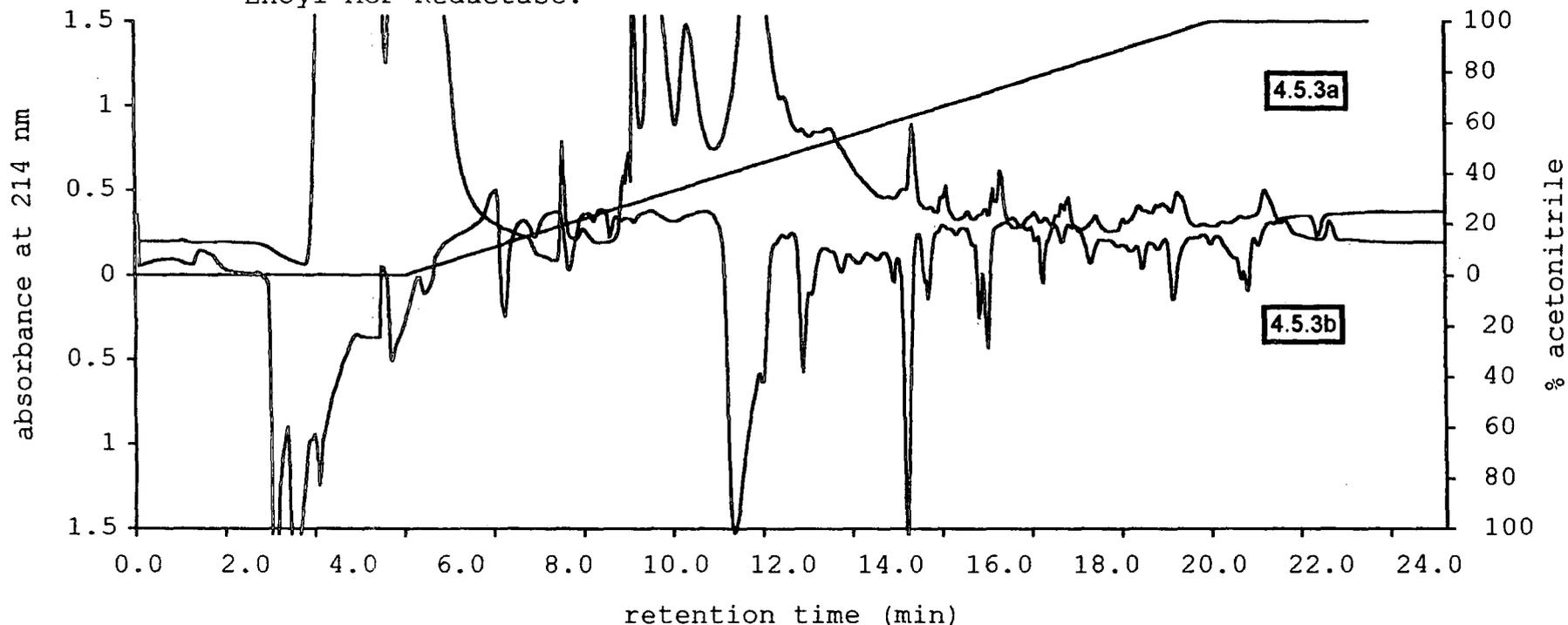
4.5.3a Enoyl ACP reductase without pre-incubation with substrate protectant was incubated directly with phenylglyoxal. Following LysC digestion the material was analysed on a Vydac C18 reverse phase column using a 0-80% acetonitrile gradient.

4.5.3b As (a) except the enoyl ACP reductase was pre-incubated with 10mM CoASH as substrate protectant prior to incubation with phenylglyoxal.

Figure 4.5.4

C18 Reverse Phase HPLC on Tryptic Peptide Digests of Phenylglyoxal Modified Enoyl ACP Reductase.
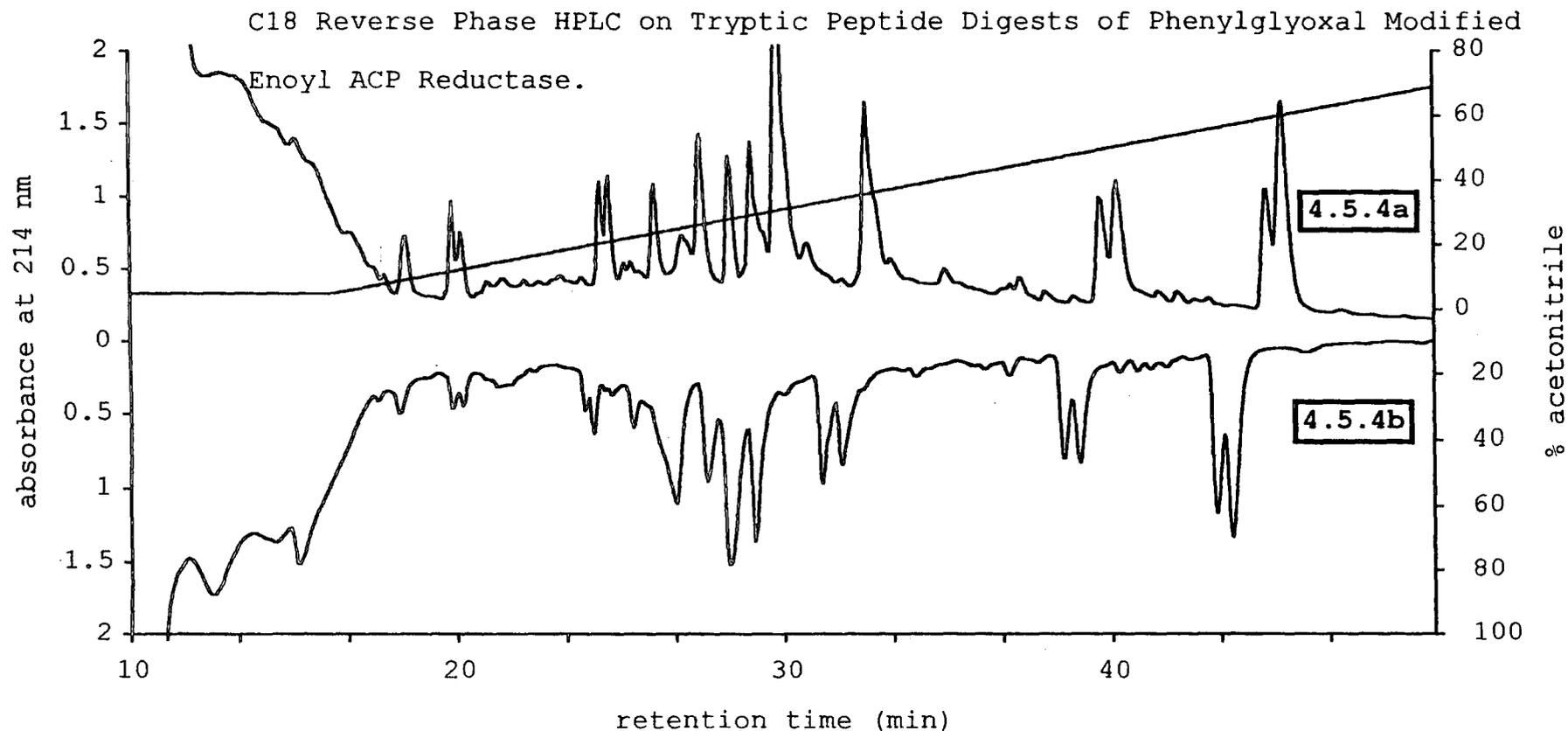
4.5.4a Enoyl ACP reductase without pre-incubation with substrate protectant was incubated directly with phenylglyoxal. Following tryptic digestion the material was analysed on a Vydac C18 reverse phase column using a 5-80% acetonitrile gradient.

4.5.4b As (a) except the enoyl ACP reductase was pre-incubated with 10mM CoASH as substrate protectant prior to incubation with phenylglyoxal.

From the specific activity of the [^{14}C] phenylglyoxal (24 mCi/mMol) this was calculated to be 4 nmole of phenylglyoxal incorporated. Although the modification was attempted on 500 pmole of enoyl ACP reductase the sequence data obtained was less than 50 pmole for each cycle.

A possible explanation for this large amount of radioactivity corresponding with low sequence yield could be that there was another peptide present containing a large amount of phenylglyoxal label and because it contained a blocked N-terminus it could not be sequenced.

ii) Following each Edman degradation reaction on the automatic protein sequencer only one third of the PTH-amino acid released is analysed by the HPLC. For this experiment the remaining two thirds of the sample from each cycle was collected in the sequencer fraction collector and counted for radioactivity.

In this way it was hoped that radioactive phenylglyoxal label could be conclusively associated with a specific amino acid residue.

No radioactivity was detected in any of the fractions collected possibly indicating that the label was lost during the early washing cycles of the sample disc on the sequencer.

These two observations indicated that having found a successful protocol to modify and isolate radioactive peptides from enoyl ACP reductase it was now necessary to show that the label remained during the peptide

Figure 4.5.5

Separation of [14C] Phenylglyoxal Labelled Enoyl ACP Reductase LysC

Peptides on C18 reverse Phase HPLC. (a) Enzyme pre-incubated with 10mM CoASH.

(b) Enzyme without substrate protection.

4.5.5a Enoyl ACP reductase was pre-incubated with 10mM CoASH as substrate protectant prior

to incubation with phenylglyoxal.

4.5.5b Enoyl ACP reductase without preincubation with substrate protectant was incubated

directly with phenylglyoxal.

Following digestion with LysC the digests were analysed on a Vydac C18 reverse phase HPLC

column using a 0-80% acetonitrile gradient. Three radioactive peaks were found (labelled

1,2,3) in the unprotected digest which were not present in the substrate protected digest.

The radioactive data for each peak is shown in the insert ([14C] cpm μl^{-1} of peptide

fraction). Peaks 2 and 3 were applied to the protein sequencer for analyses (chosen

because they were obviously different in the two digests). Sequence data was only obtained

for peak 3 (ASSGLPIDLRGK) and this was at a level of <50 pmole for each cycle (see 4.5.2)

absorbance at 214 nm

% acetonitrile

retention time (min)

4.5.5a

4.5.5b

1.2
1
0.8
0.6
0.4
0.2
0
0.2
0.4
0.6
0.8

100
80
60
40
20
0
20
40
60
80
100

5   10   15   20

Radioactivity

[14C] cpm

10000
5000
0

1   2   3

peptide peak

1

2

3

124
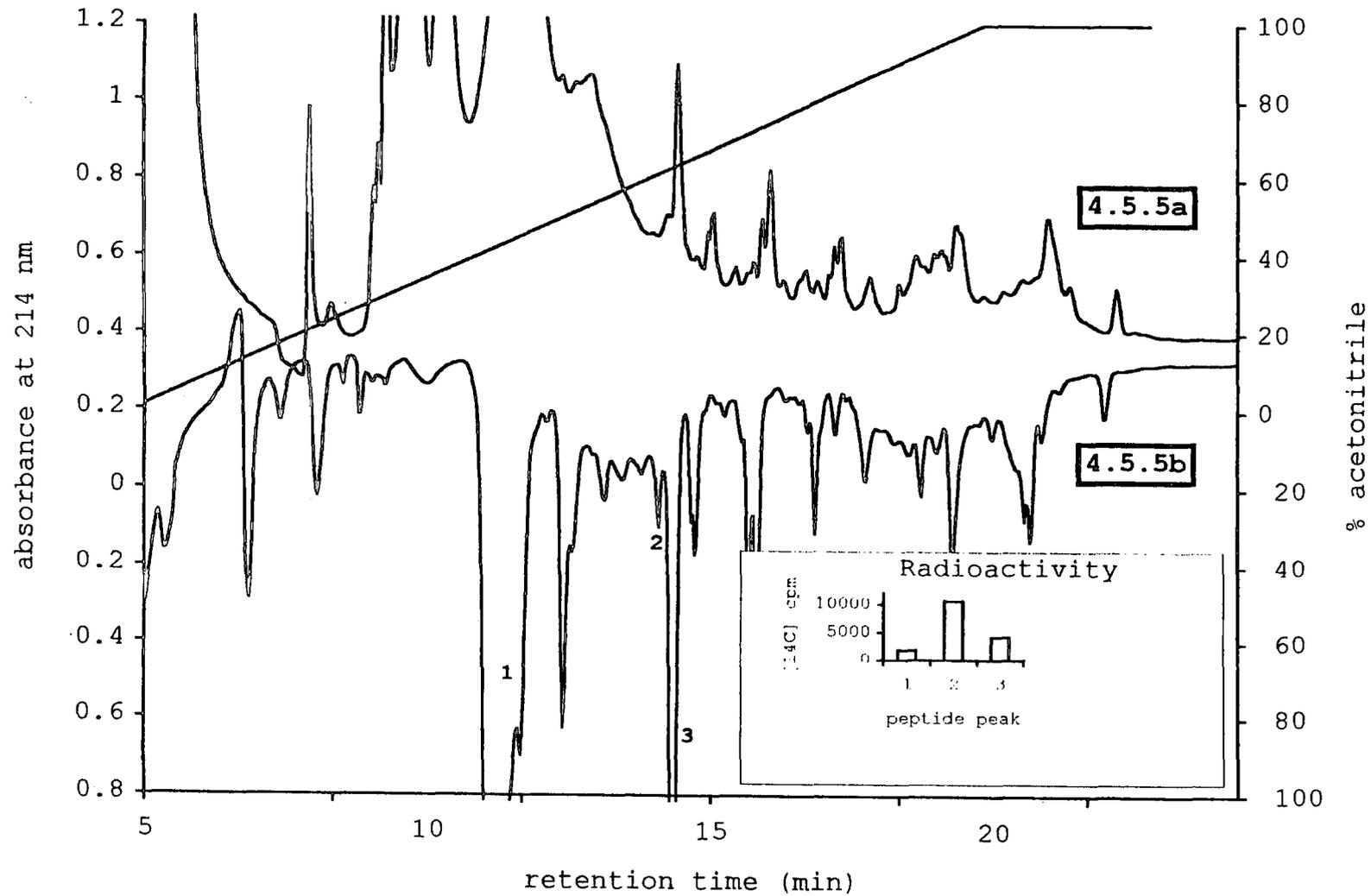
isolation and that it could be detected during protein

sequencing by collecting the fraction not analysed by

the HPLC.

Two commercially available synthetic peptides

(containing arginine residues) were purchased and used

to investigate whether it would be possible to follow

the radioactive label through reverse phase HPLC and

protein sequencing.

4.5.3 **[^{14}C] Phenylglyoxal Modification and Peptide Sequencing of Synthetic Peptides.**

In order to establish whether radiolabelled arginine

residues could be detected following reverse phase HPLC

and Edman degradation on the protein sequencer, two

synthetic peptides Bradykinin and α bag peptide (both

containing arginine residues) were purchased (Sigma).

These two peptides were first run directly on vydac C18

reverse phase HPLC and protein sequenced to show there

were no losses on these two instruments. They were then

both modified with [^{14}C] labelled phenylglyoxal under

the same reaction conditions as those used in the enoyl

ACP reductase experiments.

The modification was carried out on 1 nmole of each

peptide followed by reverse phase HPLC using the vydac

C18 column on the SMART chromatography system.

The resulting radioactive peptides were both N-terminal sequenced on the ABI protein sequencer and during each amino acid cycle the two thirds not analysed by the HPLC were collected and counted for radioactivity .

The expected amino acid sequence for each of the peptides and the sequence data obtained which confirmed this sequence in both cases is shown in table 4.5.3..

For both peptides the sequence level obtained was at approximately 400 pmole (the level expected when 1nmole is applied to the sequencer) for all amino acids except the arginine residues.

All of the arginine residues sequenced very low, for Bradykinin the N-terminal arginine sequenced at 50 pmole and the c-terminal arg sequenced at 17 pmole. The two arginines (positions 3 and 5) in the α bag peptide both sequenced at about the 50 pmole level.

All of the radioactivity associated with both peptides washed off either before or during the first cycle on the sequencer and could not be detected when the fractions from each sequencer cycle were counted.

These results confirmed that it would not be possible to detect radioactive label associated with arginine residues by collecting fractions during protein sequencing cycles.

Arginine residues consistently sequenced much lower than the other amino acids, an indication that phenylglyoxal modification was inhibiting arginine sequencing. This may be useful in identifying modified arginine residues when sequencing radioactive enoyl ACP reductase peptides

Table 4.5.3

Amino Acid Sequence of the Two Synthetic

Peptides Bradykinin and α Bag Peptide.

Bradykinin   Arg Pro Pro Gly Phe Ser Pro Phe Arg

α Bag Peptide   Ala Pro Arg Leu Arg Phe Tyr Ser

Following [14C] phenylglyoxal modification a 100µl

aliquot containing 1 nmole of each radiolabelled

peptide was separated on A vydac C18 reverse phase

column on the SMART micro-purification system (4.2.3).

The peak containing the peptide was collected and

applied directly to the protein sequencer for

analyses.

The sequence level (approximately 400 pmole) obtained

was correct when 1 nmole of sample is applied to the

sequencer, indicating that no loss had occured during

the peptide labelling and reverse phase HPLC

procedures.

as any sudden drop in sequencing yield for arginine may indicate that it has been modified.

4.5.4. Further [14] Phenylglyoxal Modified Peptide
Analyses on Enoyl ACP reductase.

Having shown using the two synthetic peptides outlined above (4.5.3) that it would not be possible to monitor radioactivity and associate it with specific residues using fractions from the protein sequencer.
Two further attempts were made to isolate and sequence radiolabelled peptide fragments from enoyl ACP reductase using trypsin for the digestion and the protocol outlined in 4.5.2.
In both of these further experiments successful inactivation was achieved on the enoyl ACP reductase sample not pre-incubated with substrate protectant and good comparative peptide maps were obtained by reverse phase chromatography on the SMART figure 4.5.6
During the chromatography peaks were collected and counted for radioactivity and the peaks which were present in the unprotected digests (not present in the substrate protected digest) which contained radioactivity were sequenced (2.11.4).
The sequence data obtained for these radioactive peptides is shown in figure 4.5.7 along with their position in the full enoyl ACP reductase protein sequence.

Figure 4.5.6

Separation of [14C] Phenylglyoxal Labelled Enoyl ACP Reductase Tryptic

Peptides on C18 reverse Phase HPLC. (a) Enzyme without substrate protection.

(b) Enzyme pre-incubated with 10mM CoASH.

4.5.6a Enoyl ACP reductase without pre-incubation with substrate protectant was incubated

directly with phenylglyoxal. Following tryptic digestion the material was analysed on a

Vydac C18 reverse phase column using a 0-80% acetonitrile gradient.

4.5.6b As (a) except the enoyl ACP reductase was pre-incubated with 10mM CoASH as

substrate protectant prior to incubation with phenylglyoxal.

The inserts show the radioactivity (cpm μl^{-1} of peptide fraction) associated with the

radioactive peptides found in digest(a) but not in digest (b) and the amino acid sequence

data obtained. The level of sequence data obtained was approximately 1 nmole which was the

level expected for 2.5 nmoles of starting material for each digest. The total

radioactivity associated with peaks 1 and 2, was equivalent to 107, and 70 pmoles

of phenylglyoxal respectively.

peak 1   66cpm μl-1[14C]   IIPGYGGGMSSAK
                               VNTISAGPLGSR
peak 2   47cpm μl-1[14C]   VLPDGSLMEIK

4.5.6a

4.5.6b

absorbance at 214 nm

% acetonitrile

retention time (min)

130

Figure 4.5.6

Separation of [14C] Phenylglyoxal Labelled Enoyl ACP Reductase Tryptic

Peptides on C18 reverse Phase HPLC. (c) Enzyme without substrate protection.

(d) Enzyme preincubated with 10mM CoASH.

4.5.6c Enoyl ACP reductase without pre-incubation with substrate protectant was incubated

directly with phenylglyoxal. Following tryptic digestion the material was analysed on a

Vydac C18 reverse phase column using a 0-80% acetonitrile gradient.

4.5.6d As (c) except the enoyl ACP reductase was pre-incubated with 10mM CoASH as

substrate protectant prior to incubation with phenylglyoxal.

The inserts show the radioactivity (cpm μl^{-1} of peptide fraction) associated with the

radioactive peptides found in digest(c) but not in digest (d) and the amino acid sequence

data obtained. The level of sequence data obtained was approximately 1 nmole which was the

level expected for 2.5 nmoles of starting material for each digest. The total

radioactivity associated with peaks 5, 6, and 7 was equivalent to 343, 392, and 465 pmoles
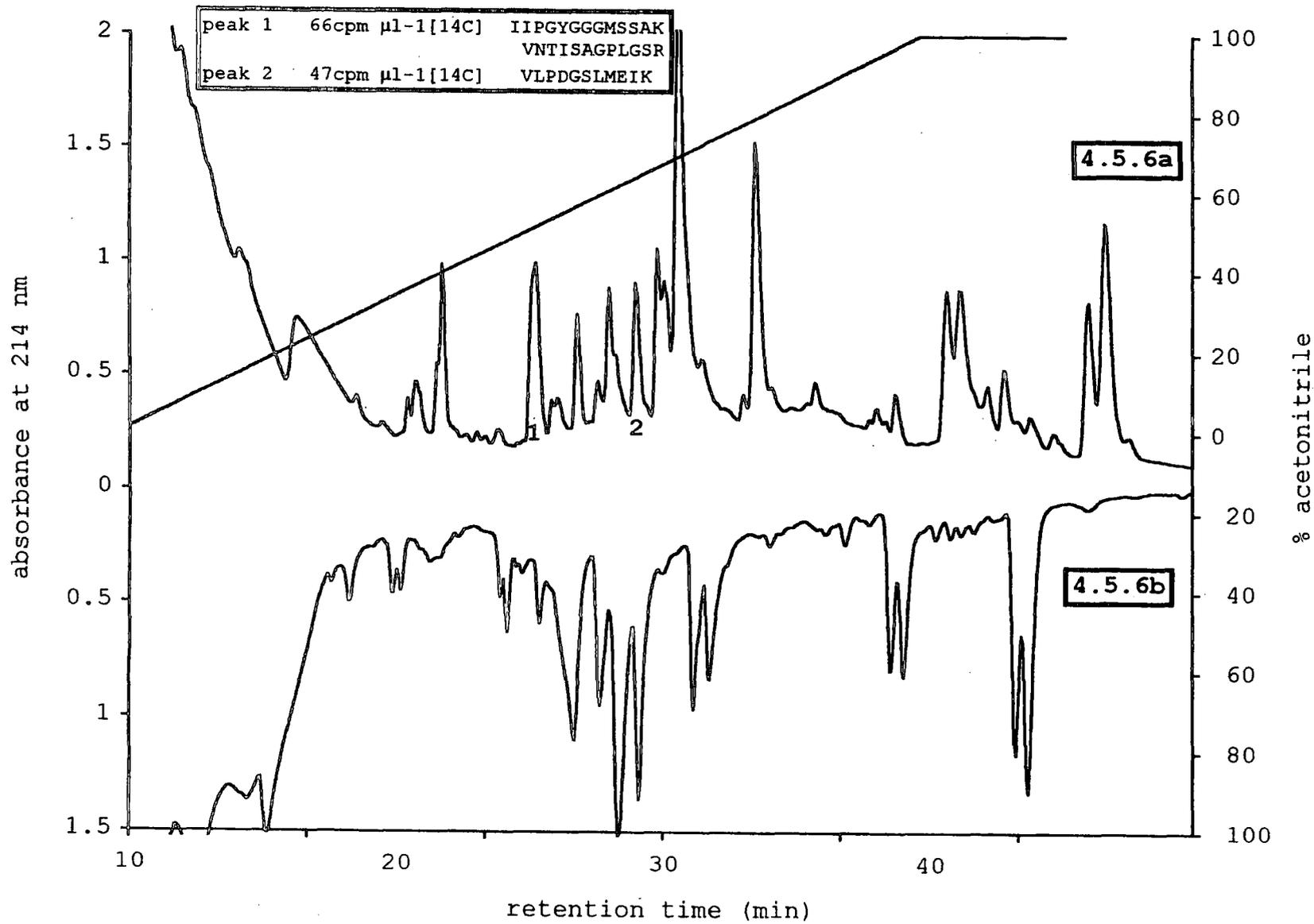
of phenylglyoxal respectively.

Radioactivity

Peptide sequence data obtained.
peak 5 = ASSGLPIDLR + VLPDGSLMEIK
peak 6 = ASSGLPIDLR
peak 7 = VLPDGSLMEIK

4.5.6.c

4.5.6.d

absorbance at 214 nm

% acetonitrile

retention time (min)

132
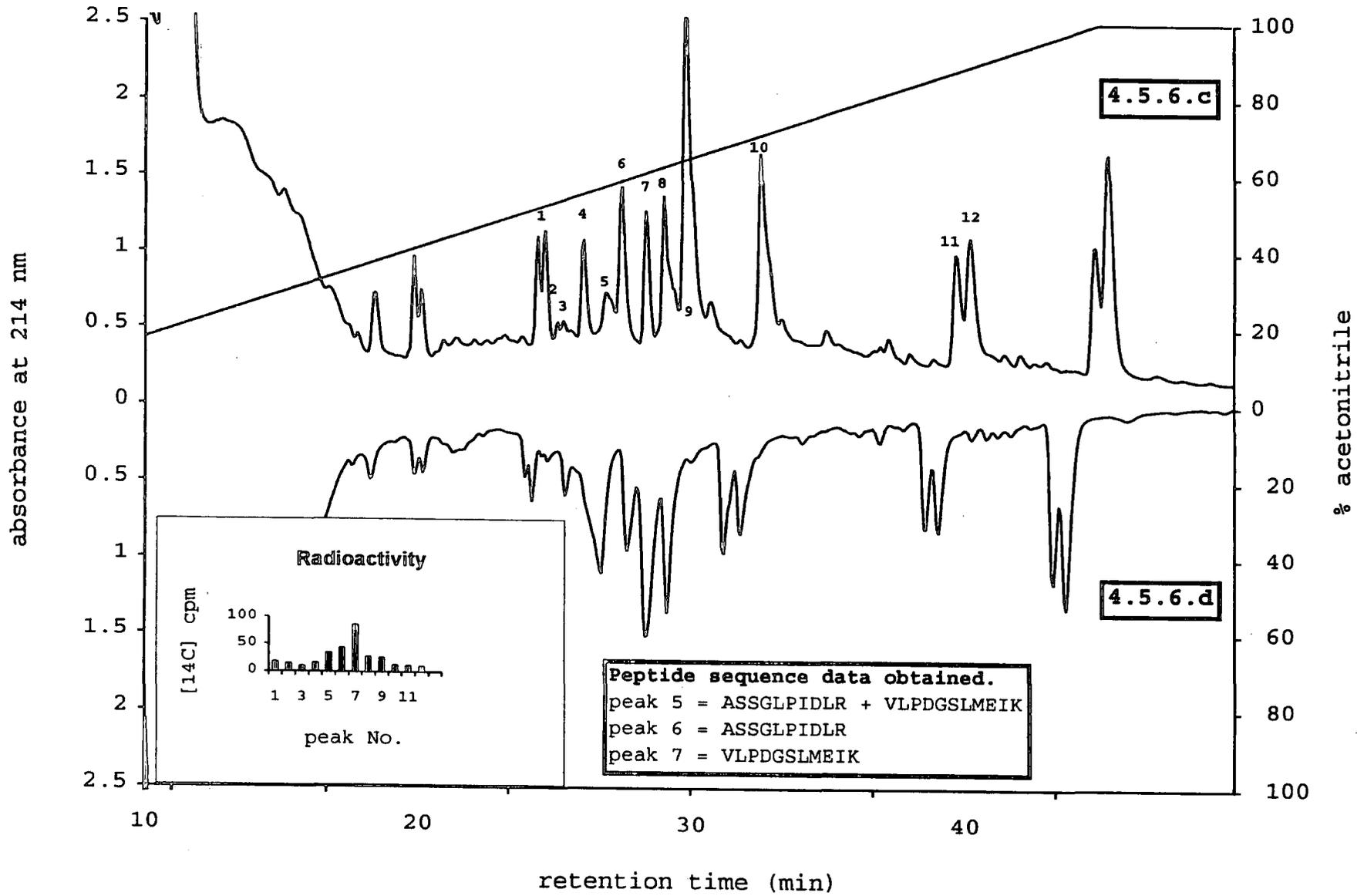
It has not been possible with the sequence data obtained during this project to conclusively identify the active site arginine residue which the phenylglyoxal inactivation data suggest is at the active site of the enzyme. It became apparent during this work that selectively labelling arginine residues within a protein using phenylglyoxal, producing peptide digests and obtaining peptide sequence data had unexpected complications associated with each step. The isolation of radioactive peptides that do not contain arginine residues suggested that phenylglyoxal was not entirely specific for arginine under the reaction conditions used.

The amount of radioactivity associated with all of the peptides isolated was much lower than expected for the stochiometery of phenylglyoxal to arginine of 2:1. This could indicate that the complex was breaking down and that the phenylglyoxal was being removed during the digestion and reverse phase HPLC steps. The arginine residues within the isolated peptides were at or near the C-terminus in all three cases and this naturally sequences low. Therefore the drop in sequencer yield observed for arginine with phenylglyoxal modified synthetic peptides was not clearly seen when sequencing the isolated peptides from modified enoyl ACP reductase. However in total four radioactive peptides were isolated and identified. Their respective positions in the full sequence is shown (figure 4.5.7). Two of the radiolabelled peptides isolated from the tryptic digests

Figure 4.5.7

Summary of Protein Sequence Data Obtained from the Radioctive Peptides

Isolated After [^{14}C] Phenylglyoxal Modification of Purified Enoyl ACP

Reductase.

Digestion	Thesis Section	Peptide Sequence	Position in Full Sequence
Endo LysC	(4.2.2)	ASSGLPIDLRGK	Ala 8 to Lys 19
Trypsin	(4.5.4 first attempt)	IIPGYGGGMSSAK VNTISAGPLGSR VLPDGSLMEIK	Ileu 194 to Lys 206 Val 228 to Arg 239 Val 73 to Lys 83
Trypsin	(4.5.4 second attempt)	VLPDGSLMEIK ASSGLPIDLR	Val 73 to Lys 83 Ala 8 to Arg 17

contained arginine residues at their c-terminus (arg 17
and 239) and the other two were identified as beginning
immediately after an arginine residue (arg 72 and 193).
Trypsin would not be expected to cleave arginine
residues after modification and the fact that all of the
arginine residues identified were shown to have been
cleaved at their c-terminus indicates that they probably
have not been modified.

A comparison of all of the known enoyl ACP reductase
sequences available in the Swissprot database(4.6)
showed three conserved arginine residues. Two of these
(arg 192 and 214) were identified from the molecular
structure data, obtained during the crystallography as
being involved with binding of the individual subunits
at the interfaces of the tetramer. The third (arg 227)
has not so far been assigned a functional role, but in
view of the phenylglyoxal inactivation data obtained
during this work could be an important residue in either
substrate binding or in stabilising the substrate
binding site of the molecule.

4.6 Multiple Sequence Alignment of Known Enoyl ACP
   Reductase Protein Sequences.

Seven available published enoyl ACP reductase protein
sequences (*Brassica napus, Arabidopsis, E.coli,
Salmonella typhimiurium, Anabaena and Mycobacterium
tuberculosis* (2530 and 2492))where aligned using the
Multiple Alignment Construction and Analysis program

Figure 4.6.1

Multiple Sequence Alignment (MACAW) of the Two Plant,

Four Bacterial and one Cyanobacterial Enoyl ACP

Reductase Sequences available in the Swissprot Database.

```
Rape         sesseskassglpicLRGKRAFIAGIADDNGYGWAVAKSLAAAGAEILVgtwvpalnife   60
Ecoli        mgf-----------LSGKRILVTGVASKLSIAYGIAQAMHREGAELAFtyqndklkgrv     48

S.typhimur.. mgf-----------LSGKRILVTGVASKLSIAYGIAQAMHREGAELAFtyqndklkgrv     48
Arabidopsis  sessenkapsglpicLRGKRAFIAGIADDNGYGWAIAKSLAAAGAEILVgtwvpalnife    60
anabaena     mttkismln------LTGKNALVTGIANNRSIAWGIAQQLHAAGANLGItylpdergkfe    54
2530         mtgl-----------LEGKRILVTGIITDSSIAFHIAKVAQEAGAELVLtgfdrlklvkr    49
mtu2492      mtgl-----------DDGKRILVSGIITDSSIAFHIARVAQEQGAQLVLtgfdrlrliqr    49


Rape         tslrrgkfdqsFvlpdgslmeikkvypldavfdnpedvpedvkankryagssnwtvqeaa   120
Ecoli        eefaaqlgssivlpcdvaedasidamfaelgnvwpkfdgfvhsigfapgdqldgdyvn--   106

S.typhimur.. eefaaqlgssivlpcdvaedasidamfaelgnvwpkfdgfvhsigfapgdqldgdyvn--   106
Arabidopsis  tslrrgkfdqsrvlpdgslmeikkvyaldavfdnpedvpedvktnkryagssnwtvqeaa   120
anabaena     kkvselveplnpsiflpcnvqndeqiqstfdtirdkw-----------------------   91
2530         iadrlpkpaplleldvqneehlstladritaeigeg-----------------------   85
mtu2492      itdrlpakaplleldvqneehlaslagrvteaigag-----------------------   85


Rape         ecvrqdfgsiGilvMslangpevskpll---ETSRKGYLAAISASSYSFVSLLSHFLPIM   177
Ecoli        --------------------------------AVTREGFKVAHDISSYSFVAMAKACRTML   135

S.typhimur.. --------------------------------AVTREGFKVAHDISSYSFVAMAKACRTML   135
Arabidopsis  ecvkkdfgsiGilvMslaigpevskpll---ETSRKDYLAVISASSYSFVSLLRHFLPIM   177
anabaena     -------grlGiliGclafanrddltgdfs-QTSRAGFATALDISTFSLVQLSGAAKPLM   143
2530         -------nkiGgvvGsigfmpqsgmginpffDAPYEDVSKGIHISAYSYASLAKAVLPIM   138
mtu2492      -------nklGgvvGsigfmpqtgmginpffDAPYADVSKGIHISAYSYASMAKALLPIM   138


Rape         NPGGASISLTYIASE-RIIPGYGGGMSSAKAALESDTRVLAFEAGRKQNIRVMTISAGFL   236
Ecoli        NPGSALLTLSYLGA-ERAIPNY-NVMGLAKASLEANVRYMANAMGPEG-VRVMAISAGFI   192

S.typhimur.. NPGSALLTLSYLGA-ERAIPNY-NVMGLAKASLEANVRYMANAMGPEG-VRVMAISAGFI   192
Arabidopsis  NPGASISLTYIASE-RIIPGYGGGMSSAKAALESDTRVLAYEASRKSNIRVMTISAGFL   236
anabaena     TEGGSIITLSYLGG-VFAVPNY-NVMGVAKAGLEASVRYLASELGSQN-IRVMAISAGFI   200
2530         NPGGGIVGMDFDP--TRAMFAM-NWMTVAKSALESVNRFVAREAGKVG-VRSNLVAAGFI   194
mtu2492      NPGGSIVGMDFDP--SRAMFAM-NWMTVAKSALESVNRFVAREAGKYG-VRSNLVAAGFI   194


Rape         GSRFAKAIGFIDTMIEYSYNNAPIQKTLT-----------ADEVGNAAAFLVSPLASAIT   285
Ecoli        RTLAASGIKDFRKMLAHCEAVTPIRRTVT-----------IEDVGNSAAFLCSDLSAGIS   241

S.typhimur.. RTLAASGIKDFRKMLAHCEAVTPIRRTVT-----------IEDVGNSAAFLCSDLSAGIS   241
Arabidopsis  GSRFAKAIGFIDTMIEYSYNNGPIQKTLT-----------ADEVGNAAAFLASPLASAIT   285
anabaena     RTLASSAVGGILDMIHHVEQVAPLRRTVT-----------QLEVGNTAAFLASDLASGIT   249
2530         RTLAMSAIVGgalgdeagqqmqlleegwdqraplgwnmkdPTPVAKTVCALLSDWLPATT   254
mtu2492      RTLAMSAIVGgalgeeagaqiqlleegwdqrapigwnmkdATPVAKTVCALLSDWLPATT   254


Rape         GATIYVDNDLNSMGValdspvfkdlnk--   312
Ecoli        GEVVHVLGDFSIAAMnelelk--------   262

S.typhimur.. GEVVHVDGDFSIAAMnelelk--------   262
Arabidopsis  GATIYVDNDLNAMGValdspvfkdlnskn   314
anabaena     GQVLYVDARYEIMGM--------------   264
2530         GTVIYADGDASTqll--------------   269
mtu2492      GDIIYADGDAHTqll--------------   269
```

The consereved blocks within the sequences are indicated
by upercase letters and the identical residues across all
seven sequences are highlighted.

(MACAW) (Schuler *etal.* 1991) in order to locate blocks of local similarity and identify conserved regions. The significant blocks found in all seven sequences are shown in figure 4.6 as uppercase letters and the full alignment for all seven sequences is shown. The alignment shows greater than 70% homology across all seven sequences. All of the conserved amino acid residues are highlighted and the arginines identified during peptide sequencing are marked (+).

Chapter 5

General Discussion and Future Work

5.1 The Aims of this Research.

The main objectives of the work presented in this thesis
was to produce large quantities of the enzyme enoyl ACP
reductase from *Brassica napus* using an overexpression
system in *E.coli*. A strategy had to be established which
would allow purification of milligram quantities of the
homogeneous enzyme.

Once purified this large amount of material would allow
characterisation studies, amino acid analyses, secondary
protein structure determination and crystallography
studies to be undertaken. These studies would not be
possible with the relatively small amount of material
which can be purified directly from *Brassica napus* seed.
Using covalent modification reagents, peptide mapping
and peptide sequencing the amino acid residues at the
active site of the enzyme would be investigated.

This data along with structural data obtained from x-ray
diffraction analyses of the enoyl ACP reductase crystals
would help target future site directed mutagenesis
experiments to specific amino acid residues which may be
important in binding and catalysis. All of the above
information should provide a greater understanding of
the reaction mechanism and molecular structure of the
first plant fatty acid synthetase component (enoyl ACP

reductase) studied in depth. Antibody studies performed on plant material would also allow the identification and expression patterns of specific genes encoding isoenzymes of enoyl ACP reductase, both in respect to temporal and specific expression.

5.2 Achievements of this Research.

E.coli strain BL21 (DE3) transformed with pEAR2 (Kater *et al.* ,1991) a plasmid containing an enoyl ACP reductase cDNA clone was grown in large quantities during this project. A successful purification strategy using blue sepharose affinity chromatography and anion exchange chromatography was developed. A typical preparation gave a 25 fold purification with one major 32kDa band visible when vastly over-loaded on an SDS PAGE gel stained with coomassie blue (figure 3.1). Using this procedure greater than 100 mgs of pure enoyl reductase was purified, which allowed full characterisation studies to be carried out.
The purified protein was N-terminal amino acid sequenced and the predicted (Kater *et al.*, 1991) alanine was found at the N-terminus in place of a serine in the protein isolated from rape seed (figure 3.4.1). A polyclonal antibody to the denatured protein was raised in mice and was shown to be highly specific when tested against crude rape seed extracts using western blot analyses (3.5)

Protein secondary structure predictions were made using data collected from CD spectra (figure 3.8) and also using the primary protein sequence and the Chou and Fasman computer program. When the CD prediction was compared with the data obtained from the full x-ray structure, it was shown to be more accurate than the prediction obtained using the computer program alone.

Spectral properties were measured and used along with total amino acid analyses to calculate a molar extinction coefficient for the recombinant enzyme (3.10). This absolute standard will allow future quantification of enoyl ACP reductase to be made directly. This has direct implications on studies currently being undertaken in this laboratory aimed at down regulating the expression of this enzyme in seed.

The native molecular weight (120kDa) of the purified enzyme was estimated by gel filtration chromatography (3.11) and found to be similar to that of the enzyme isolated from *B.napus* seed (Slabas *et.al.*, 1986) and spinach leaf (Shimikata and Stumpf, 1982). Previous literature (Cottingham *et al.*, 1988 and Slabas *et al.*, 1992) suggested the presence of at least two isoforms of enoyl ACP reductase in rape seed. The use of two dimensional western blot analyses was used as part of this study to try to investigate the presence of isoforms of the enzyme in both rape seed and rape leaf material. The results showed the same four isoforms present in both sets of material and the two most

abundant in seed are also the two most abundant in leaf
(3.15).

Acyl carrier protein (ACP) was purified from *E.coli*
(3.14) and used in substrate protection studies during
phenylglyoxal inhibition experiments. It was also used
in enzyme substrate / complex experiments and in ACP
crystallisation trials (not part of this work).

Crotonyl CoA was synthesised using crotonic anhydride
and CoASH (2.9) and used as the substrate to assay enoyl
ACP activity throughout this research work. Special
attention was made to the development of a micro-assay
which resulted in a reproducible analyses being
available using low amounts of substrate. This overcame
the economic and availability restrictions associated
with commercially available crotonyl CoA and therefore
extended the studies possible.

Work on the covalent modification and active site
identification of enoyl ACP reductase was re-
investigated as part of this research (4.5). Successful
modification and inhibition of the enzyme using the
apparent arginine specific modifying reagent
phenylglyoxal was achieved. The natural substrate ACP
and a substrate analogue CoASH were both found to
protect against this inhibition, 100% protection was
achieved using 500 µM ACP and 70% using 20mM CoASH.
The incorporation of radioactive phenylglyoxal was
correlated with the loss of enoyl ACP reductase
activity, 2 moles of phenylglyoxal were found to be
incorporated per mole of tetrameric enoyl ACP reductase

resulting in complete enzyme inhibition. This observation suggests that enoyl ACP reductase activity is lost when one arginine residue within the tetramer is modified. This loss of activity could either be caused by an active site modification or by a conformational change within the tetramer caused by the modification of a single arginine residue. This is contrary to earlier observations and has direct implications on the nature of the molecular structure essential for catalyses. Following [14C] phenylglyoxal labelling, proteolytic digestion, and peptide mapping using reverse phase HPLC, a number of radioactive peptides were isolated and N-terminal amino acid sequenced (4.5). The data collected proved inconclusive, although two potential arginine residues (arg 17 and 239) were identified (4.5). Although the selectivity of phenylglyoxal in causing inhibition of enoyl ACP reductase by specifically modifying a unique arginine residue is questioned, it was not possible within the time frame of this project to pursue this analyses further. However all of the techniques for radiolabelling, peptide digestion, peptide mapping using reverse phase HPLC and peptide sequencing at the picomole level are now well established because of the work carried out during this research.

The large quantities of purified recombinant enoyl reductase purified during this project allowed crystallisation trials to be set up at the University of Sheffield.

From these trials, crystals (Rafferty *et al.*, 1994) and heavy atom derivatives were obtained which produced good x-ray diffraction data. Using this data resolution of the full structure of enoyl ACP reductase from *Brassica napus* down to 1.9 angstrom resolution has been achieved. The NADH binding site of the enzyme has been identified. A proposed catalytic mechanism has now been worked out using data collected from this structural model and information obtained using the high 3 dimensional structural homology between enoyl ACP reductase and 3α,20β-hydroxysteroid dehydrogenase (HSD)(Rafferty *et al.*, in press).

Diffraction data was also collected from crystals formed in the presence of crotonyl CoA, and this data shows that the adenine and ribose moieties of the crotonyl CoA molecule appear to bind to the same pocket as the adenine of the NADH. This suggests that crotonyl CoA may therefore act as a competitive inhibitor of enoyl ACP reductase and also rationalises for the first time the inferior nature of CoA derivatives as substrates for enoyl ACP reductase activity.

A new substrate analogue (n-acylcysteamine) has recently been synthesised, which does not contain adenine and ribose moieties. Attempts are now continuing to obtain crystals in the presence of this substrate in order to try to identify the true fatty acid substrate binding site.

Recently the atomic structure of Inha the enoyl reductase from *Mycobacterium tuberculosis* was reported

(Dessen *et al.*, 1995), and when the two structures were compared they showed a very similar overall fold, not unexpected as the two protein sequences are similar with approximately 50% identity (figure 4.6.1).

This work represents a considerable advancement in our understanding of the structure and isoforms of enoyl ACP reductase.

5.3 Future Work.

Fatty acid biosynthesis in plants is catalysed by a freely dissociable type II fatty acid synthetase (FAS). This type II plant (FAS) is made up of nine freely separable catalytic components of which enoyl ACP reductase is one.

Several of the other components are being isolated and their cDNA's and genes cloned. Using the bacterial expression systems and purification strategies established during this project large quantities of several other FAS enzymes will become available.

The ability to purify large amounts of the component enzymes of plant FAS will enable rapid investigation into enzyme interactions, co-crystallography and detailed three dimensional enzyme structure to be carried out.

The investigation into the amino acid residues at the active site of enoyl reductase concentrated on the use of an arginine modifying reagent phenylglyoxal. This did

not result in conclusive identification of specific residues during this project. Reagents to specifically modify histidine, lysine, cysteine and arginine (other than phenylglyoxal) are available and could all be tried using similar methodology to that described in this thesis in order to further investigate amino acid residues at the catalytic site of the enzyme. However the greatest advancements are likely to come about as a consequence of information being available on the structure of enoyl ACP reductase at near atomic resolution.

The use of site directed mutagenesis experiments to make proteins with specific amino acid substitutions will provide a greater understanding of the catalytic site of the enzyme.

Although enoyl ACP reductase can use acyl ACP's, acyl CoA's, and acyl cysteamines as substrate, it's natural substrate is ACP (apparent km being less than 1 µM). The successful determination of the full x-ray structure and the resolution of the catalytic mechanism of this enzyme will eventually allow evaluation of substrate specificity and why acyl ACP's are better substrates than acyl CoA's. Further studies could be envisaged aimed at the rational design of specific plant enoyl ACP reductase inhibitors which could be of value to the agrochemical industry, especially due to considerations of the large structural differences between the animal and plant fatty acid synthetase.

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