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Biochemical basis of insect resistance in winged bean (Psophocarpus tetragonolbus): characterisation of insecticidal proteins and their encoding genes

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Biochemical basis of insect resistance in winged bean (*Psophocarpus tetragonolobus*); characterisation of insecticidal proteins and their encoding genes.

A thesis submitted by David S. Howe BSc (UMIST) in accordance with the reqiurements for the degree of Doctor of Philosophy in the University of Durham.

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ABSTRACT

Biochemical basis of insect resistance in winged bean (*Psophocarpus tetragonolobus*); characterisation of insecticidal proteins and their encoding genes.

David S. Howe

Many pulses and beans grown for human comsumption are susceptible to insect attack. Winged bean, a high protein crop of the tropics, yield seeds which appear to be immune to infestation by the storage bruchid *Callosobruchus maculatus*.In this thesis the biochemical basis of this resisitance was investigated.

Insect bio-assays were carried out in which protein fractions from seeds of winged bean were incorporated at a range of concentrations into artificial seeds, and their effects upon the development of *C.maculatus* determined. Both albumin and globulin fractions were toxic to the developing larvae and their toxicity correlated with their haemagglutination activity. Assay of psophocarpin fractions A, B and C found the fraction psophocarpin B to be most insecticidal. On further purification this fraction yielded two lectin fractions and a protease inhibitor fraction. Purified basic lectin was highly insecticidal to *C. maculatus* larvae with an LC_{50} value of 0.35%. The physiological level of this protein in winged bean seeds is sufficient to account for their resistance to attack by *C maculatus*. Winged bean trypsin inhibitor was also purified and tested in artificial seeds against *C maculatus*. However, even at concentrations in excess of twice the physiological concentration it had no deleterious effects upon development. Winged bean protein fractions, incorporated in artificial diets, proved toxic to the Lepidopteran pests *Heliothis virescens* and *Spodoptera littoralis* in bio-assays, but it appeared that the basic lectin was not responsible for toxicity towards these insects.

Attempts to clone the gene encoding the winged bean basic lectin were made by constructing cDNA and genomic libraries, and heterologous lectin genes from soybean and *Phaseolus* were investigated as possible probes for the basic lectin gene. Purification of the basic lectin B3 and sequencing of 44% of its primary protein structure, along with comparisons with other legume lectin sequences allowed the synthesis of oligonucleotide primers for use in polymerase chain reaction experiments. However, all the PCR products obtained were shown to be the result of non-specific amplification. Further work needed to obtain the basic lectin gene is discussed.

Contents

FIGURES

TABLES

MEMORANDUM

Parts of the work presented in this thesis have been presented
previously in the following publication (see appendix) :

Gatehouse A.M.R., Howe D.S., Flemming J.E., Hilder V.A. and Gatehouse J.A.
(1991) Biochemical basis of insect resistance in winged bean (*psophocarpus
tetragonolobus*) seeds. *J. Sci. Food Agric.* **55** 63-74

STATEMENT

No part of this thesis has been previously submitted for a degree in this or
any other university. I declare that, unless otherwise indicated, the work
presented here is entirely my own.

ABBREVIATIONS

Abbreviations, where used, are those according to the Biochemical Society's instructions to authors, Biochemical Journal 1982 volume 209, pages 1-27. One letter notation for amino acids is given in the Biochemical Journal 1969 volume 113, pages 1-4. Any additional abbreviations are given below.

BAPNA	Nα-benzoyl-DL-arginine-p-nitroanilide
BTEE	N-benzoyl-L-tyrosine ethyl ester
bp	base pair
Ci	Curies
CPTI	Cowpea trypsin inhibitor
DABITC	4,4,-dimethylaminoazobenzene-4'-isothiocyanate
DABTH	4,4,-dimethylaminoazobenzene-4'-thiohydantoin
dATP	deoxyadenosine 5'triphosphate
dCTP	deoxycytosine 5'triphosphate
EtBr	ethidium bromide
IEF	isoelectric focusing
ITPG	isopropyl-β-D-thiogalactoside
Kbp	Kilobasepair
mRNA	messenger RNA
μCi	microcuries
μg	microgramme
μl	microlitre
O.D.	optical density
Oligo dT	cellulose immobilised thymidylic acid polymer
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PITC	phenylisothiocyanate
PMSF	phenylmethylsulphonyl floride
Poly A$^+$	polyadenylated RNA
RIP	ribosome inactivating protein
rRNA	ribosomal RNA
SDS	sodium dodecyl sulphate
SSC	saline sodium citrate buffer
Taq	*Thermus aquaticus*
TFA	trifluoroacetic acid
TPCK	N-tosyl-L-phenylalanine chloromethyl ketone
tRNA	transfer RNA
XGAL	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

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CHAPTER ONE

INTRODUCTION

1.1 General Introduction.

The best sources of protein for the human diet i.e. those yielding the highest protein efficiency scores, are those derived from animals. Individually, no plant can provide balanced amounts of all the amino acids required in a healthy diet. Cereals tend to be deficient in lysine and tryptophan and legumes are deficient in the sulphur containing amino acids, methionine and cysteine. However, with a mixed diet, plant protein becomes a valuable nutritional resource (Croy and Gatehouse 1985). Animal protein production per acre falls far below that of plant protein and it is clear that man will have to rely more and more on plant proteins as the world's population increases. A large increase in plant protein production could be achieved if the present crop losses due to disease, nematodes, insects and weeds could be reduced.

It is estimated that 37% of the total potential crop production is lost to pest damage, insects alone accounting for 13% (Gatehouse and Hilder 1989). A significant proportion of these losses are the consequence of agricultural techniques and breeding practices. Crops have been selected on a basis of yield, often under ideal conditions, and little account has been taken of inherent resistance mechanisms. Crops raised in foreign climes, removed from their natural habitat, fall prey to new local phytopathogens. Monoculture (which does not reflect the balance between plant and predator found in natural ecosystems) allows very little variation between plants in the same field, and they are all, therefore, susceptible to the same pest. Similarly the large scale storage of the same crop under one roof leads to pest population explosions.

Present methods of pest management rely heavily upon chemical control,

with the result that pesticide costs are an important economic factor in agriculture. Expenditure to control pests of the world's three major crops, cotton, maize and rice, amounted to 3,812 million dollars in 1987 (Gatehouse and Hilder 1989). This type of chemical control, besides being expensive, also has a devastating effect on the environment, either in being non-biodegradable or by affecting organisms higher in the food chain. It has become increasingly important to find alternative methods of control which are safe, efficient and cost effective. Changes in agricultural practice i.e. a turn away from monoculture, and the introduction of breeding programmes including natural resistance factors will be the long term aim of food producers worldwide, whilst in the short term environmentally-friendly pesticides and controls are sought.

1.2 Biological control and genetic engineering for resistance

Most plant breeders now recognise that selection for inherent pest resistance in crop varieties is just as important as selection on the basis of yields. However, the constraints of conventional breeding techniques mean that the transfer of a given trait without the concomitant transfer of undesirable ones is a long and laborious task, and that the transfer of any given trait across species is not possible. It is only with the advent of genetic engineering and gene transfer that it is now feasible to tackle these problems. The panoply of genetic engineering techniques that have been developed in recent years allow researchers to isolate genes with relative ease and fuse protein coding sequences to promoters and enhancers which will control their expression in the target organism. In plants, the target organisms can be any of those that can be transformed. To date these include many herbaceous dicotyledonous plants, woody dicotyledonous plants

and a few monocotyledonous plants, which means that a given gene may be transferred across large evolutionary barriers. Isolation of specific genes can be used to avoid the concomitant transfer of undesirable genes.

Commercial and ecological constraints on genetic engineers require that transformed plants be unaffected in terms of yield and quality, and when dealing with insect-resistant crops that the factors conferring resistance are either non-toxic to humans or not expressed in the organ to be eaten. In the latter case, potent toxins not previously introduced may still serve a role in insect-resistant crops of the future, as their expression can be controlled. Some problems arise with the expression and functional integrity of genes transferred across wide species barriers due to differing control signals affecting mRNA stability, protein translation, protein folding, and post-translational processing and targetting. Knowledge of the processing systems of both host and donor organisms can be employed to alleviate such problems for example, in the Bt system described later, the expression of the bacterial protein in the transformed plant was increased by the alteration of some of the bacterial codons to those favoured by the plant to be transformed (Fuchs *et al* 1989). The advantages afforded by genetic engineering techniques in the modification and improvement of crop plants make this a method that can supplement those of traditional plant breeding, hybrid seed production, farm mechanisation and the use of agrochemicals (in both provision of nutrients and pest control) in the search for sustainable crop yields under environmentally favourable agricultural practices.

The identification of useful genes to be transferred is perhaps the limiting factor in a genetic engineering approach to crop improvement. In the following section the defensive strategies produced by plants through their co-evolutionary struggle with phytopathogens will be discussed, with

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special attention being paid to stategies which can be duplicated by current genetic engineering techniques, i.e. the production of polypeptides and proteins. Other antimetabolites require a number of enzymes for their production; these are also discussed although the technology required to duplicate their synthesis in transgenic plants is not yet developed.

1.3 Plant defence mechanisms

1.3.1 Plant physical defences

Defensive methods used by plants against insect attack vary from the production of physical barriers to the synthesis of complex secondary metabolites and antimetabolic proteins. Among the array of physical barriers are thick cuticles, specialised trichomes and thick seed teguments. In some bean varieties certain leaf hoppers and aphids are known to become impaled on the hooked trichomes. Aphids coming into contact with the trichomes on wild potato cause them to rupture and release an exudate, which, upon oxidation, sets as an insoluble black substance upon the aphids legs , thus immobilising it. This form of resistance has been exploited and transferred to some modern cultivars by traditional breeding methods (Simmons 1981). In general, such physical factors are not amenable to genetic engineering, as they are controlled by a series of genes under complex regulation. However, the genes encoding a number of structural proteins (extensins and glycine-rich proteins), which accumulate in the extracellular matrix upon plant wounding or pathogen invasion, have been cloned and may prove useful in phytopathogen control.

Hydroxyproline-rich glycoproteins (extensins) are secreted as monomers into the wall matrix where they become insolubilised by a cross-linking process catalysed by a coordinately-expressed peroxidase enzyme. Both local and systemic induction of the bean extensins has been

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observed (Corbin *et al* 1987). The expression of glycine-rich wall proteins has also been reported (Keller *et al* 1989); when expressed these proteins are localised at the wound site. These responses are thought to be directly related to providing a physical barrier against invading fungal hyphae, and repairing damaged apoplast. They may also serve a role in insect defence or protection of the plant after insect attack (Bowles 1990).

A number of enzymes involved in the synthesis of cell wall polymers are known, and appear to be activated by wounding or pathogen invasion. Callose synthase, a plasma membrane-bound enzyme, catalyses the formation of β-1,3 glucan (callose) which is deposited around the cell membrane. Activation of this enzyme can be caused by treatment with chitosans (constituents of fungal cell walls and insect exoskeletons) (Kohle *et al* 1985), providing evidence for a possible role in insect defence. Elicitor treatment also activates the gene cinnamyl alcohol dehydrogenase (Walter *et al* 1988) which catalyses the production of all the cinnamyl alcohol precursors of lignin, a structural component of many plants. Genes for these enzymes may prove useful in providing physical barriers to insect pests and protecting insect-damaged plants against fungal attack.

1.3.2 Plant secondary metabolite defenses

The arsenal of plant secondary metabolites, many examples of which have been implicated in insect resistance, can be grouped into 3 classes in accordance with their pattern of synthesis;

1: Those derived from acetate and mevelonate (the acetogenins and isoprenoids).

2: Those derived from shikimic acid and amino acids (e.g. polyphenols and alkaloids)

3: Those derived from a mixture of 1 and 2 (e.g the flavanoids).

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Acetate and mevalonate derived secondary metabolites

While the low molecular weight acetogenins and isoprenoids (terpenoids) tend to be volatile, and are responsible for many plant scents and aromatic oils, the larger molecules (C_{15} and above) tend to be non-volatile and commonly give plants their astringent tastes or are synthesised into more complex units, e.g steroids. These secondary metabolites may therefore defend against insects at three levels; olfactory, feeding and growth.

Plants containing β-ocimene (a monoterpene) and the sesquiterpene (C_{15}) caryophyllene epoxide have dramatic repellent effects on the leaf cutting ant *Atta cephalotes*. The repellent effect of caryophyllene epoxide is thought to be due to its antifungal effect. The ants reject leaves with this terpenoid as it harms the fungal symbiont on which they feed (Hubbell 1983).

A number of lactone sesquiterpenoids are feeding deterrents; Glaucolide-A present in *Vernonica* plants has been shown to deter yellow striped armyworm at concentrations of 0.1%-1% in artificial diets (Burnett *et al* 1974). 8-deoxylactucin and lactupicrin, two sesquiterpene lactones from *Chichorium intybus*, have both proved to be insect antifeedants (Harborne 1985), while the bitter tasting triterpenoids found in members of *Cucurbitaceae* prove repellent to many insects tested, except those of the host-specific pest cucumber beetles.

Two insect hormones, juvenile hormone and ecdysone play a pivotal role in the normal metamorphosis and moulting in insect larvae. Both of these hormones have mimics within the plant kingdom, for example *Abies balsamea* (Balsam fir), used in the paper industry, produces the juvenile hormone mimic, juvabione, which is active against Pyrrhocoridae insects arresting

their normal metamorphosis. Other active juvenile hormone mimics have been found in the leaves of sweet basil (*Ocimum basilicum*) and roots of *Macropiper excelsum*. Evidence that these hormones are produced in response to insect attack has been provided by Purich and Nijholt (1974) who reported the production of the free acid juvabione in the costal fir *Abies grandis* when infested with balsam woolly aphid. Mimics of the ecdysones (moulting hormones) have also been found; these are triterpene derived, mainly of steroidal form. Saponins, glycoside-bound triterpenoids/steroids, have also proved toxic to insects in a number of cases; their mode of action may be to act on insect hormonal pathways following hydrolysis in the insect gut, or it may be directly related to their ability to lyse cells (Applebaum and Birk 1972).

Secondary metabolites derived from amino acids and shikimate

These include alkaloids and non-protein amino acids. The non-protein amino acids, are analogues of one of the twenty naturally occurring protein amino acids and as such they interfere with the normal metabolic processes involving amino acids. In this respect they are toxic to a great many organisms including man. The co-evolution of insects and plants has, however, meant that some insects have become resistant to non-protein amino acids, for example the Bruchid *Caryedes brasiliensis* is able to feed on the seeds of *Dioclea megacarpa* which contain the arginine analogue canavanine, as this insect's arginyl tRNA synthase discriminates between the protein and non-protein amino acid. *C. Brasiliensis* also has high levels of urease activity, which facilitate the conversion of canavanine to ammonia via urea (Rosenthal 1991).

The alkaloids (nitrogen-containing compounds derived from amino acid secondary metabolism) form the most familiar class of plant toxins as they

are toxic to an extremely wide range of animals., They include such familiar compounds as nicotine, strychnine and atropine (Gatehouse 1991). Few insects will feed on plant tissues containing nicotine, and, those that do, e.g. tobacco hornworm and *Musca domestica* (house fly), are able to cope with this toxic compound by excreting nicotine without metabolising it, or oxidising it to the inert nor-nicotine.

The mechanism of action of most alkaloids is poorly understood , except for those of the poly-hydroxy alkaloids. The toxicity of these metabolites is related to their structural similarity with sugars. Castanospermine, which occurs in the seeds of *Castanospermum australe* at concentrations of 0.1% , is an analogue of α-D-glucose and as such inhibits α-glucosidase. In feeding trials using castanospermine in artificial diets at concentrations of 0.03% , *Callosobruchus* larval growth is completely inhibited, and levels as low as 0.005% significantly inhibit larval development (Nash *et al* 1986). Swainsonine, a mannose analogue found in the pasture legume *Swainsona* is toxic to cattle, causing neurological symptoms, due to accumulation of mannose-based oligosaccharides. As yet no bioassays on insects have been performed.

Secondary metabolites derived from mixed metabolic pathways

This represents a very diverse class of secondary metabolites, which include the flavanoids, rotenoids and tannins. The flavanoids account for many of the plant colourations and some flavours found in food and drinks. Whether bitter tasting compounds and others like them are effective as insect feeding deterrent is a matter of conjecture but certainly oligomers of flavanoids, the condensed tannins, have proved insecticidal and their astringency is repellent to higher animals. Feeny (1970) reported the importance of tannins in the control of feeding of winter moth larvae

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(Operophtrea brumata) on oak trees. Their larvae cease to feed on oak in mid-June in response to a build up of tannins at that time. Tannins may also act by lowering the nutritional value of proteins in plants as they have the ability to complex protein rendering them impervious to proteolysis. The toxicity of tannins to seed eating beetles has also been recorded; Boughdad and co-workers (1986) have demonstrated that tannins present in the seed coats of *Vicia faba* seeds inhibit the development of the bruchid *Callosobruchus maculatus*.

The rotenoids are a class of isoflavanoids which have much potential as natural insecticides. Their major effect is to cause a dramatic decrease in oxygen uptake. Birk and co-workers (1989) have tested a number of rotenoids from *Lonchocarpus salvadorensis* and found them to be lethal to *C. maculatus* at physiological concentrations.

The use of chemical resistance mechanisms inherent in plants by commercial plant breeders has to date been limited, mainly because of the poor state of knowledge of both the mechanisms of resistance and their genetic linkage patterns. However, an increasing number of genes encoding enzymes involved in the production of these secondary metabolites have now been cloned :- Phenylalanine ammonia lyase, the first of the core enzymes of phenyl-propanoid biosynthesis (Lois *et al* 1989, Crammer *et al* 1989); coumarate CoA ligase (Douglas *et al* 1987), the isoforms of which, in soybean, are differentially involved in flavanoid or lignin biosynthetic pathways; chalcone synthase, the enzyme involved in the first committed step in flavanoid biosynthesis (Wingender *et al* 1989); casbene synthase, which catalyses the production of this cyclic diterpene from geranyl-geranyl-pyrophosphate (Moesta *et al* 1985). Many of these enzymes and their reactions are fundamental to processes in normal growth and development as well as in defence responses, and accordingly their

regulation is complex (Bowles 1990). It may,therefore, be some time before these genes may be used in a coordinated system of expression in transgenic plants to produce the desired secondary metabolites for insect defence purposes.

1.3.3. Plant protein antimetabolites

One strategy used by plants against insect predation is to protect seed and storage organs at the expense of other parts of the plant e.g. leaves. A corollary to this is the accumulation of many antimetabolic storage proteins. These include enzyme inhibitors, hydrolases, lectins and other toxins. Many of these proteins are encoded by small gene families and their patterns of expression are well understood. Their expression is normally spatially and temporally regulated in seeds and other storage organs, but they may also be expressed in other tissues in response to phytopathogens. Many of these antimetabolites are small, single polypeptide proteins, another quality which makes them ideal candidates for genetic manipulation for crop resistance improvement.

Alpha amylase inhibitors

The presence of proteinaceous α-amylase inhibitors in plants was first demonstrated by Kneen and Sandstedt (1943), who discovered an α-amylase inhibitor in aqueous extracts of wheat. Proteinaceous α-amylase inhibitors have since been discovered in many other cereals including oats (Elliot and Leopold 1953), rye (Kneen and Stanstedt 1946), sorghum (Miller and Kneen 1947), Barley (Mundy *et al* 1983; Weseleke *et al* 1983) and millet (Chandresekhar *et al* 1981). α-Amylase inhibitors are not confined to monocotyledonous plants; a survey by Jaffé and co-workers (1973), using porcine pancreatic α-amylase, detected the presence of α-amylase inhibitors in 79 of 95 legume cultivars tested. Prominent inhibitory activity was

found mainly in *Phaseolus* species and *Dolichos* species; lentils , cowpea and chickpea displayed little activity. Other major sources of α-amylase inhibitors have been tuberous plants e.g. *Solanum tuberosum* (Kamaryt *et al* 1982), local yam (*Disscorea alat*) and *Colocasia* species (Shivaraj *et al* 1979).

These proteins belong to a number of sequence families and often consist of subunits of relatively low molecular weights (5-15,000). In cereals they are normally present as monomers or dimers, in legumes, however they are more often found as the higher molecular weight proteins, e.g. the Phaseolus α-amylase inhibitor, which has a M.W. of 49,000 (Marshall and Lauda 1975). The mechanism of action of these proteins is poorly defined. In wheat each subunit of the inhibitor is thought to have a binding site for the amylase enzyme (which is thought to have two sites for binding). The binding is considered to be ionic in nature as binding is pH dependent and optimum inhibition always occurs at either the pI of the inhibitor or when opposite electrical charges are present on the the enzyme and inhibitor.

Inhibitors of exogenous and endogenous plant amylases have both been isolated and as yet no relationship between the two forms has been found. Indeed, Konarev (1982) reports that the inhibitor of the endogenous wheat amylase has a higher molecular weight (19,500) than its exogenous inhibiting counterparts (13-15,000). The role of the endogenous inhibitors is uncertain, although they are believed to be involved with starch metabolism within the seed kernel as their activity has been correlated to amylolytic activity in both malting barley and steeped wheat kernels (Silano 1987).

The role of the inhibitors of exogenous proteins is considered to be two fold. First, they appear to have a role as storage proteins. The

expression of inhibitors of exogenous α-amylase in wheat kernels starts 8 days after fertilisation and increases during maturation with a final content in the mature kernel of 1% ; then decreases rapidly on germination. This pattern, matched with the heterogeneity of the inhibitors found within each species, is typical of storage proteins whose biological role is essentially that of being broken down to supply a nitrogen source to the developing embryo (Silano 1987). Secondly, they function as antimetabolites toward predators, and are involved especially in insect resistance.

A number of workers have demonstrated either *in vivo* and/or *in vitro* effects of α-amylase inhibitors on insect amylases. Yetter and co-workers (1979) were able to show that an α-amylase inhibitor from winter wheat was deleterious to the rice weevil (*Sitophilus oryzae*), and yellow mealworm (*Tenebrio molitor*) using both *in vitro and in vivo* assays. An α-amylase inhibitor from maize inhibits the gut amylases of *Sitophilus zeamais, Rhyzopertha dominica* and *Tribolium casteneum* (Blanco-Labra 1981), whilst that of the red kidney bean inhibits the amylases of the red flour moth larvae (*Anagasta kühniella*), the confused flour beetle and yellow mealworm (Powers and Culbertson 1982).

A number of α-amylase inhibitors have been shown to be bifunctional in their activity as they are also able to inhibit proteases. These include WASI, a subtilisin/α-amylase inhibitor from wheat (Mundy *et al* 1984), BASI, a similar protein from barley (Weseleke 1983) and I-1(TAI), a trypsin/α-amylase inhibitor from ragi (Campos and Richardson 1983). A number of other α-amylases have proved resistant to trypsin degradation and may prove to be bifunctional inhibitors (Silano 1987). An α-amylase inhibitor isolated from Jobs tears (*Croix lacrimajobi*) is an unusual bifunctional protein in that it has chitinase catalytic activity (Ary *et al* 1989). These bifunctional inhibitors may prove to be an exciting prospect

for biotechnologists; however no work has been done to show that these inhibitors are any more insecticidal than α-amylase inhibitors or trypsin inhibitors on their own. None of the genes encoding these bifunctional proteins have been cloned, and clearly more work in this particular area of insecticidal proteins/genes is needed.

The protease inhibitors

The presence of protease inhibitors in plant tissues was first demonstrated as long ago as 1938, when Read and Haas reported an aqueous extract from soybean which inhibited the ability of trypsin to liquefy gelatin. Two protein factors responsible have since been purified by Kunitz (1945) and Birk (1961), and both are representatives of two different types of protease inhibitor families that have since been found to be distributed throught out the plant kingdom, and to be particularly abundant amongst the leguminosae. (Richardson 1977).

The Kunitz inhibitor family of proteins are generally characterized by a relatively high polypeptide molecular weight (20-25KDa), relatively few disulphide bonds and a specificity toward one protease; whilst those of the Bowman-Birk type are characterized by low polypeptide molecular weights (6-8KDa), are rich in cysteine and have two binding sites (for this reason they are often referred to as double headed inhibitors). The Bowman-Birk inhibitors have marked stability toward heat, acid and alkali, which is attributed to the stabilizing effect of disulphide bonds on the protein structure (Liener and Kakade 1980).

Although inhibitors of all the major classes of proteases have been documented, i.e. inhibitors of metallo-, sulphydryl-, aspartyl- and serine protease inhibitors, it is the serine proteases that appear to be the most abundant and best documented.

All inhibitors of serine proteinases so far isolated employ the same

competitive mechanism of inhibition (Laskowski and Kato 1980). The mechanism of action proposed for the Kunitz inhibitors is thought to involve the formation of a tetrahedral adduct between the inhibitor and the enzyme, which is stabilised by a very close complementary fit between the two proteins. The recognition site for the proteases can normally be found in a sequence region which is highly conserved, especially amongst legume inhibitors . It is small variations within this region that generally give rise to the change of inhibitor specificity. e.g. an (arginine / lysine)-serine dipeptide changed to a (leucine / aromatic amino acid)-serine dipeptide at the 'active site' changes the specificity from trypsin to chymotrypsin inhibition.

The effects of plant protease inhibitors on insect development have been studied by a number of groups. Lipke and co workers (1954) reported the inhibition of growth and protease activity of the flour beetle *Tribolium confusum* when reared on soybeans. Birk and Applebaum (1960) reported a similar effect on another flour beetle *T.casteneum* ; the toxic factor in these two cases was later purified and proved to be a larval protease inhibitor (Birk *et al* 1963a).

Both the Kunitz and Bowman inhibitors from soybean have proved to be active against *Tenebrio molitor* gut proteases (Applebaum 1964) and more recently the Kunitz inhibitor has been shown to inhibit larval growth of *Manduca sexta* (Shukle and Murdock 1983), a Lepidopteran pest. Two other Lepidopteran pests susceptible to protease inhibitors in *in vitro* gut protease assays and in *in vivo* feeding assays are *Heliothis zea* and *Spodoptera exiqua*. In these cases, inhibitors from soybean and potato proved effective (Broadway and Duffey 1986). The corn borer (*Ostrinia nubilalis*) has also proved susceptible to a soybean trypsin inhibitor (Steffens *et al* 1978).

The fact that protease inhibitors very rarely inhibit endogenous plant proteases would suggest that they serve a role other than that of preventing proteolysis and aiding seed storage protein accumulation during seed maturation.

Support for the concept that plant protease inhibitors have evolved as part of a defensive mechanism against invading pests has been presented by Ryan and co-workers (1972). Trypsin inhibitors are found most abundantly in seeds and tubers but the mechanical damage of potato leaves by the colorado beetle leads to an accumulation of protease inhibitors I and II (Green and Ryan 1972). Within 2-3 days of first damage inhibitors account for 1% of the total soluble protein of leaves and remain for long periods stored in vacuoles. Trypsin inhibitors can also be found at the wound site in crown galls of tobacco (Wong *et al* 1976), whilst tomato produces trypsin inhibitors in response to attack from pathogenic fungi (Peng and Black 1976). This latter response mechanism appears to be mediated by an oligosaccharide factor, which, when isolated, stimulates the production of protease inhibitors in many plant species (McFarland and Ryan 1974).

A comprehensive study of the cowpea trypsin inhibitors by Gatehouse, Hilder and co-workers has also led to compelling evidence of the role of trypsin inhibitors in insect resistance. A single variety of cowpea (TVu 2027) was selected from 5,000 accessions, in being the only cultivar which possessed seed resistance to *C. maculatus* (the major storage pest of cowpea). This resistance appeared to correlate with significantly higher levels of protease inhibitors in the resistant line (Gatehouse *et al* 1979). The albumin fraction obtained from this variety was shown to be toxic to *C. maculatus* when incorporated in artificial diets at the 10% level; this toxicity could be removed by selective absorption of the trypsin inhibitors in this fraction by the use of affinity chromatography columns.

Further-more, addition of the affinity-purified inhibitor fraction at 0.8% levels in artificial diets was found to be strongly antimetabolic (Gatehouse *et al* 1980). Other workers in this field have suggested that the inherent resistance in these seeds does not simply correlate with trypsin inhibitor content and that a more complex multi-mechanistic system is at work (Redden *et al* 1983). Further studies by Hilder and co-workers have, however, shown that CPTI can be used to confer insect resistance. Chemical studies showed the cowpea trypsin inhibitors to be small polypeptides of around 80 amino acids, belonging to the Bowman-Birk type of double-headed serine protease inhibitors and to be products of a multi-gene family (Hilder *et al* 1989). The purified protein was tested in artificial diets against a broad range of both field and storage insect pests; those insects that proved susceptible to physiological levels of this inhibitor included the Lepidopteran pests, *Heliothis virescens, H. zea, Spodoptera littoralis, Chilo partellus* and *Manduca sexta*, and the Coleoptera pests *Anthonomus grandis, Diabrotica undecimpunctata, Tribolium confusum* and *Callosobruchus maculatus*. It has been shown that the effect of CPTI is mediated by interaction with insect digestive enzymes and that supplemention of diets with sulphur amino acids overcame its toxicity. (Gatehouse and Boulter 1983).

Cowpea seeds can be eaten raw by humans without any obvious toxic effect (Peterson 1984) and recent feeding trials show that the inhibitor in untreated seed meal does not adversely effect the growth of rats (Boulter *et al* 1990). This apparent lack of toxicity toward mammals in conjunction with the small size of the protein has made it an ideal candidate for genetic engineering.

A pUC19 cDNA library was constructed from poly A^+ RNA, prepared from the cotyledons of cowpea. This library was screened with a mixed synthetic

oligonucleotide probe specific to a region of amino acid sequence of the major CpTI protein isolated, and a partial cDNA CpTI clone previously isolated. Direct nucleotide sequencing of a cross hybridising clone (pUSSRc3/2) revealed a long leader sequence containing a number of in frame start codons, the entire coding sequence of CpTI and a short 3' non-translated region. A 550bp Alu1-Sca1 restriction fragment containing the cowpea sequences from pUSSRc3/2 was transferred to the Sma1 site of the *Agrobacterium tumefaciens* Ti plasmid binary vector pROK2. The resulting construct contained a nos-neo gene (to allow transformants to be selected on the basis of kanamycin resistance), the cowpea sequence under the control of the constitutive CamV 35S promoter, and the nopaline synthase gene transcription termination sequence bound by the T-DNA right and left borders. At this stage both constructs containing the the CpTI gene in the correct orientation (PROK-CpTI+5) and those in the reverse orientation (pROK-CpTI-2) were mobilised into the *Agrobacterium tumefaciens* and used to transform tobacco (*Nicotiana tabaccum)* leaf discs. Those plants transformed with the construct in the reverse orientation acted as controls. Transformants were selected on the basis of their resistance to kanamycin and transformed plants were regenerated from shootlets by transfer to root inducing, kanamycin containing agar medium. Cuttings were taken from the original transformants to produce sufficient clonal plants for insect bioassays.

Transformants contained 3-7 unrearranged copies of the construct which were simply and stably inherited. Expression of CpTI in the pROK-CpTI+5 plants and not in the pROK-CpTI-2 plants was confirmed by dot -immunobinding assays using polyclonal antibodies raised in rabbits against total CpTI. Expression in the pROK-CpTI+5 plants ranged from below the limit of detection to ~1% total soluble protein.

Correct processing and functional integrity of CpTI in tobacco was confirmed by western blot analysis of leaf tissue samples run on SDS-PAGE and by direct *in vitro* trypsin inhibitor assays using bovine trypsin.

The critical test on these CpTI expressing plants was a bioassay to test their respective levels of insect resistance or tolerance. The results obtained when newly emerged larvae of the Lepidopteran *Heliothis virescens* (tobacco budworm) were used to infest plants sealed in individual planteria and kept under controlled light and temperature regimes, showed that plants expressing higher levels of CpTI were more tolerant to insect attack. Those transformants showing high levels of CpTI expression were used in further feeding trials with *H.zea* (corn earworm), *Spodoptera littoralis* (armyworm) and *Manduca sexta* (Tobacco hornworm). Approximately 75% of the larvae died within 2-3 days, whilst the plants were only partially damaged. Control plants were reduced to stalks within 7 days by these pests.

These experiments point to the role in insect resistance for plant protease inhibitors. Confirmation of this role has been shown in similar transgenic plants expressing potato protease inhibitors, which have also proved resistant towards insect attack (Ryan et al 1989)

The lectins

Lectins are a ubiquitous class of proteins found throughout the plant and animal kingdoms; they are currently defined as sugar binding proteins, or glycoproteins, of non-immune origin which agglutinate cells and/or glycoconjugates (Goldstein *et al* 1980). Their presence in plants has been known now for more than 100 years, since the discovery by Stillmark of a factor from castor bean capable of agglutinating erythrocytes. Because of their wide-spread use in isolation and analysis of complex carbohydrates, cell separation and studies of cell surface architecture, a great deal of

information is available on lectin structure and specificity. In general, lectins from plants within particular taxonomical groups have distinctive properties that distinguish them from lectins of less closely related plants, i.e. lectins found within the same tribe tend to have the same sugar binding specificities, molecular weights and subunit compositions and lectins within the same families have close sequence homologies (Etzler 1985). Lectins found within the leguminosae can be divided into two major classes; those that have identical (or nearly identical) subunits Mr ~26,000 (the one chain lectins) and those of the Vicieae tribe that are made up of two different subunits (the two chain lectins), tetramers of 2α light chains (Mr ~6000) and two β heavy chains (Mr~20,000). The leguminosae lectins all show extensive homologies, those of the Vicieae tribe appear to have arisen as a result of a proteolytic processing of a single chain precursor (Mr~26,000) at an Asn-X peptide bond (Richardson 1981). As a further variant, two lectins from the Dioclea tribe are circular permutations of the standard single chain form.

A number of invariant regions have been found within the lectin sequences. These have been related via chemical modification studies to the lectin binding sites for carbohydrates, Mn^{2+} and Ca^{2+}, and a hydrophobic binding site.

Many lectins are glycoproteins. Two forms of glycosylation occur, the common N-linked glycosylation found in animals and a rarer arabinose form, confined to the solanaceae tribe. Agglutination activity of lectins does not appear to be affected by the removal of their carbohydrate moieties. Evidence from the study of two chain lectins and concanavalin A suggests that the carbohydrate side chain may be involved in processing of the preprolectin (Richardson 1981).

The biological role of lectins in plants remains unclear and a number

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of hypotheses have been proposed. Recent discoveries of different lectins in different tissues of the same plant (Van Damme *et al* 1990) may indicate that during evolution lectins have become adapted for different functions in different tissues and that no clear role can be assigned. The evolution of the lectin genes in *Phaseolus vulgaris* may be an example of this; the two lectins PHA-L and PHA-E are closely homologous to a non-lectin protein (arcelin) and an α-amylase inhibitor. The genes for these proteins all lie close together on the same chromosome and are thought to have arisen from gene duplication events followed by divergence (Chrispeels and Raikhel 1991).

The developmentally regulated pattern of expression of most legume lectins and their abundance in seeds has suggested that they either function as storage proteins or are involved in seed maturation/germination. The mitogenic activity of lectins towards lymphocytes may parallel similar activities found in germinating seeds on embryonic cells (Howard *et al* 1972); however as yet only weak or no activation of cell division has been observed when lectins are used on host cell protoplast suspensions (Howard *et al* 1977, Nagl *et al* 1972). Lectins may be responsible for the organisation of glycoproteins in multienzyme systems held within the seed protein bodies. The two proposed functions of lectins that have received most attention are their role as mediators of symbiosis between plants and microorganisms and their protection of plants against phytopathogens (Lis and Sharon 1986).

There is now strong evidence that lectins are involved in recognition and attachment of *Rhizobium* as a first step in nodulation. The lectins of both soybean and clover have been shown to specifically agglutinate the corresponding infective strains of *Rhizobia japonicum* and *R.trifolii* (Bohlhool and Schmidt 1974). Furthermore, roots expressing pea lectin in transgenic clover bind *R.leguminosarum* bv. viciae (Diaz 1989), a rhizobium

strain usually specific for the pea cross inoculation group.

The protective role of lectins against phytopathogens has been suggested by a number of workers. Mirelman *et al* (1978) reported the inhibition of growth of the fungal pathogens *Fusarium* and *Trichoderma* by wheat lectins, and inhibition of growth of *Penicillium and Aspergillus* by lectin has been recorded by Barkai-Golan and co-workers (1978). The galling of tomato caused by the root knot nematode *Meloidogyne incognita* can be controlled by watering of the plants with dilute solutions of concanavalin A at a concentration of $3\mu g/250ml$ soil (Marban-Mendza *et al* 1987). Janzen *et al* (1976) were able to show that the *Phaseolus vulgaris* lectin is toxic to the larvae of the bruchid beetle *Callosobruchus maculatus*; an LD_{50} of 0.6% dietary protein was determined in this study. Other insecticidal lectins that have been reported include those of *Pisum sativum* (Gatehouse *et al* 1990), rice lectin, nettle lectin (Huesing *et al* 1991) and wheat germ agglutinin (Murdock *et al* 1990) all of which have been shown to be toxic to weevils. The wheatgerm agglutinin (WGA) has also been shown to be toxic to european corn borer and southern corn rootworm (Czapla *et al* 1991).

The gene for the *Pisum sativum* lectin has been cloned and fully characterised (Gatehouse *et al* 1987). This gene has been used to create transgenic tobacco plants. The gene under the control of the CamV 35S promoter is correctly expressed and the transgenic plants have been shown to contain active lectin protein in their leaves at levels of 7.99 $\pm$ $0.2\mu g/mg$ of protein. This level of expression was enough to confer resistance to the lepidopteran pest *Heliothis virescens* (tobacco budworm). A statistically significant ($p<0.001$) drop in insect biomass was observed when insects reared on control plants were compared to those reared on lectin positive transgenic plants. The leaf area eaten by the insects was also significantly different ($p<0.05$) with $5.5 \pm 0.9 \text{ cm}^2$ eaten in control

plants in comparison to 3.2 ± 0.6 cm^2 in the lectin plants. First selfed

generation tobacco plants expressing this lectin have been crossed with a

CPTI expressing tobacco plant, homozygous for the CPTI gene (Boulter *et al*

1990). The resulting lectin plus CPTI expressing plants had a directly

additive toxic effect on the test organism *Heliothis virescens,* when

compared with hererozygous lectin only and CPTI only expressing plant. The

lack of interference between these two genes shows the efficacy of using

standard plant breeding methods to combine new insecticidal genes together.

A degree of synergism or interference may have been expected in this

system as both gene products (lectin and CPTI) are thought to exert their

toxic effects within the insect gut. The fact that this was not observed

suggests that the toxic effects of these two proteins are mediated through

different mechanisms.

1.4 Plant proteins which may be exploitable as insect antimetabolites

(i) Chitinases

Chitinases are a class of endohydrolases that catalyse the hydrolysis

of chitin (the cell wall polymer of microbes and insect exoskeletons). The

chitinases isolated to date contain polypeptides in the range 25-35Kd and a

number of isoenzymes are usually present in any one plant species. The

chitinase genes cloned are members of multigene families. Chitinases are

known to accumulate under developmental regulation as well as in response

to defence related stimuli (Mauch *et al* 1980). In studies with bean,

activation of chitinase genes was found to be rapid, 10 fold stimulation

being observed within 5 minutes of elicitor addition (Hedrick *et al* 1988).

Using Chitinase-GUS transcriptional fusion constructs the temporal and

spatial expression of the chitinase 5B gene from bean was seen to occur at

the site of infection (Roby *et al* 1990). The activity of chitinases against

fungal pathogens has been demonstrated both *in vitro* and *in vivo*. As yet no reports have been published involving insect bioassays, but it is possible that the chitinases could act on the insect exoskeleton or gut peritrophic membrane.

(ii) Ribosome inactivating proteins

Ribosome inactivating proteins (RIPs) are particularly potent toxins, capable of inactivating ribosomes by hydrolysing a sugar base linkage in ribosomal RNA. Since this RNA sequence is invariant amongst all eukaryotes it is likely that these proteins would be toxic to insects as well. Although RIPs are known to be very toxic to mammalians, very little work has been done on their activity toward insects. In one study Gatehouse and co-workers (1990) were able to show that both Type II (two subunit) and Type I (single subunit) type RIPs were toxic toward Coleoptera such as bruchid beetles and Orthoptera e.g. locust, but ineffectual towards Lepidopteran pests tested. The lack of activity towards lepidoptera was considered to be due to digestion of the toxin in the insect guts.

(iii) Lipoxygenases

Lipoxygenases are dioxygenase enzymes widely distributed in plants that catalyse the hydroperoxidation of cis-cis-pentadiene moieties in unsaturated fatty acids. Lipoxygenase activity can lead to the formation of other compounds e.g. carbonyls, epoxides. No convincing role for lipoxygenases in the physiology of plants has been put forward, despite their widespread distribution in higher plants (for example they represent 1% of total protein in soybean seeds). Shukle and Mudock (1983) have reported an antimetabolic effect of lipoxygenase from soybean on *Manduca sexta* (a non pest species) by artificial diet bioassay.

(iv) Thionins

The thionins are a family of small (MW ~5000) basic proteins rich in

cysteine that can be found in many monocotyledonous and dicotyledonous plants. They appear to have two subcellular locations, either in protein bodies in the seed endosperm, or in the cell wall in leaf. Thionins are induced to accumulate in the leaf in response to pathogen challenge (Bohlmann *et al* 1988) and their toxicity towards a range of organisms has been demonstrated (Bowles 1990), but not, as yet, toward insects. In barley the thionins are members of a large (50-100), heterogeneous, multigene family which appear to have arisen from gene duplication events, as they all lie on the same chromosome.The thionin from *Sorghum* has been shown to have α-amylase inhibitory properties (Bloch and Richardson 1991); this α-amylase inhibitory property is thought to be due to the ion chelating nature of the protein.

1.5 Insecticidal proteins of non-plant origin

Bacterial toxins

Spore preparations of the gram-positive bacteria *Bacillus thuringiensis* (Bt) have been used for the last 20 years as biological insecticides. The insecticidal properties of these spores is attributable to the presence of polypeptides (delta endotoxins) found in crystal inclusion bodies. These delta endotoxins vary in structure and the number of forms present from strain to strain vary. These differences between strains of Bt are known to lead to a difference in their spectra of insecticidal activity. Many are specific to Lepidopteran pests although a few have been shown to be toxic toward Diptera and Coleoptera (Vaeck *et al* 1987).

Isolation of the bacterial toxins was done on the basis of the unusually low solubility of the crystal proteins. These isolates have been used to produce polyclonal and monoclonal antibodies (Höfte 1986).

The identification and cloning of the toxin genes has been made easier by the observation that, in most cases, the gene resides on a plasmid. Each endotoxin is the product of a single gene. Restriction fragments from isolated plasmids containing Bt genes have been detected by screening for expression in *E.coli* with the appropriate anti-toxin antibodies.

This method was used in the isolation of the bt2 gene from *Bacillus thuringiensis* strain berliner 1715, which encodes an endotoxin shown to be insecticidal to both *M. sexta* and *Pieris brassicae*. Characterisation of the recombinant polypeptide expressed in *Escherichia coli* revealed a protoxin 1,155 amino acids long. This protoxin Mr 130,000 remains innocuous to the insect until it is cleaved in the alkaline conditions of guts of Lepidopteran larvae to generate a smaller polypeptide (Mr 60,000) which possesses full toxic activity. Mapping of the protein revealed that the toxic region of this protein lay in an amino terminal region between amino acids 29 and 607 (Höfte *et al* 1986).

Chimeric bt genes were constructed in the plant co-integration expression vectors pGSH160 and pGSH150. When transformed into *Agrobacterium tumefaciens* containing the disarmed mobilising plasmid pGV2260, recombination between pGV2260 and the expression vector mediated through homologous pBR322 sequences produced Ti plasmids with T-DNA constructs containing the intact Bt2 gene fused to the promoter of the TR DNA, a truncated Bt2 gene (encoding 610 amino acids) and two Bt2 genes (encoding 683 and 724 amino acids respectively) fused with the neo gene at the 3' end.

Each strain was used to transform a series of leaf discs of *Nicotiana tabaccum* var. petit havana SR1 and transformants were selected for on a basis of neomycin phosphotransferase expression (i.e. kanamycin

resistance). Quantitative detection of *B.thuringiensis* toxin in leaves of transformed plants was performed using ELISA and and amino terminal specific monoclonal antibodies.

Plants transformed with truncated bt2 genes or the bt2-neo fusion constructs were found to express the toxin at levels of 0.02% of the total soluble protein. This level is ten fold lower than for the neomycin phosphotransferase gene under the control of the same promoter. The lower level of expression of transformants containing the bacterial genes reflects the incompatibility (in terms of differential RNA stability and translational efficiency) of plant and bacterial gene expression. Southern analysis of transformed plants containing the bt:neo fusion gene revealed the presence of 1 - 5 copies per genome. These copies were stably incorporated as determined by analysis of the F_1 progeny.

Correlation between insecticidal activity and the Bt protein content of transformed plants was detected when transformed plants were used in insect bioassays with the Lepidopteran pest *Manduca sexta* (tobacco hornworm). Plants expressing as little as 0.004% total soluble protein were capable of producing 100% mortality of these insects within six days, whilst those expressing higher levels (0.03%) from the Bt2-neo fusions were capable of producing 100% mortality in 3 days. This level of expression may be too low to protect against members of the *Noctuidae* such as *Heliothis* and *Spodoptera* organisms, which are less sensitive to the berliner toxin. The use of more powerful promoters and isolation of other specific Bt genes for the production of transgenic plants effective against these and other pests is being actively pursued. The origin of these protein antimetabolites from organisms other than plants does present problems in expression.

Transgenic tomato plants with the truncated Bt gene have been tested

against *Heliothis zea* and *Heliothis virescens* ; despite low levels of expression, resistance to these insect larvae was enhanced over controls although resistance levels were low and probably insufficient for adequate control (Fischhoff *et al* 1989).

Enhanced promoters and modifications of codon usage have been used in expressing elevated levels of Bt toxin (Fuchs *et al* 1989) Bt plants have now been used in field trials and initial results have shown that they are capable of coping with heavy infestations as was the case in Florida, where substantial protection against the tomato pinworm *Keiferia lycopersicella*, a major pest of tomatoes of Florida , California and Mexico, was afforded (Fischhoff 1989).

1.6 The Winged Bean.

The winged bean, *Psophocarpus tetragonolobus* (L.) DC. (from the greek *psophos*, 'noise' and *karpos*, 'fruit'.) is a tropical legume native to Southeast Asia and Western Africa. Although initially discovered in 1690, it has only relatively recently become the subject of scientific attention. The plant itself is a climbing herbaceous perennial which can grow to heights of up to 4 metres when supported. Axillary racemes, which may bear three to twelve flowers , white, purple or blue in colour, and normally produce one to two fruits (Eagleton 1985). The fruit, which gives the winged bean its name, are pods of 6cm to 36cm in length with four longitudinal jagged 'wings'. Each fruit may contain up to 20 globular seeds, white, brown, black or yellow, with variable markings, of average dry weight 250mg. Seeds sown in the rainy season grow slowly during the first three to five weeks but the plants once established grow rapidly and pods can be harvested as early as six to ten weeks (Anon 1974). *Psophocarpus sp.* can be highly nodulated and have a high nitrogen fixation

capacity (Maesfield 1961) which may equip them well for growth in poor soils. A study in 1974 by the National Academy of Science recognised the winged bean's potential as a major food crop. It has a high seed protein and oil content, on average 34% and 17% respectively, which is directly comparable to that of soybean (*Glycine max*). The tuberous roots also contain high amounts of protein, up to 20% dry weight, as compared with the 1%-2% found in the other main root crops of the tropics, i.e. cassava, sweet potato and yams.

Insect resistance of winged bean

The winged bean has been reported to have relatively few natural insect pests, however, this may be due to the lack of scientific investigation in this area. It is noteworthy that none of the insect pests of winged bean, thus far reported, are known to attack the seed or maturing cotyledons of this plant. *Ophiomyia phaseoli*, the most important pest of winged beans, attacks the stalks of young plants, its larvae boring holes in them causing leaf mining (Valíek 1989). The black aphid (*Aphis craccivora*) is a phloem feeder, while the larvae of *Maruca testularlis*, a Pyralide, infest the flowers. *Polyphagotarsonemus latus* is a cosmopolitan species that has been reported to destroy as much as 60% of the crop harvest, it however, does not attack the seeds. Dobie and co-workers (1979), having surveyed the literature, were unable to find reports that the mature winged bean seed was susceptible to attack by storage bruchidae. Studies by these workers have shown that the winged bean seeds are toxic towards a number of *Callosobruchus* species and the New World bruchids *Acanthoscelides obtectus* and *Zabrotes subfaciatus*, and that the major toxic or inhibitory component of winged beans does not reside in the testa but in the cotyledons.

Winged bean seed proteins

Analysis of winged bean seed protein by Cory *et al* (1971) indicated a

better amino acid composition than for most legumes, particularly with respect to lysine and sulphur containing amino acids. However the levels of cysteine and methionine limit the nutritional value of the seed.

Early studies by Gillespie and Blagrove (1978) on the isolation and composition of winged bean seed proteins has revealed the presence of two major proteins with sedimentation coefficients of 2S and 6S and a minor 8S component. They appear to be devoid of an 11S legumin-like storage protein which is the seed protein common throughout the leguminosae. Gillespie and Blagrove (1978) were able to isolate three protein fractions on the basis of solubility and isoelectric precipitation; these were designated Psophocarpin A, B, and C .

Psophocarpin B accounts for over 30% of the extractable winged bean protein. It is a heterogeneous fraction containing protease inhibitors, lectins and the major storage albumin WBA-1, the major 2S component, which constitutes 15% of the total extractable winged bean protein (Kortt *et al* 1989). Recent sequence analysis of this storage protein have lead to suggestions that the Kunitz inhibitors have evolved from the 2S albumins to fulfill a protective role against various predators, as the 2S albumin in winged bean shares sequence homologies with the Kunitz family trypsin inhibitors, particularly in the C-terminal third of the sequence where identities of 47% and 37% have been found with soybean and winged bean trypsin inhibitors respectively.

Psophocarpin A, a tetramer of subunits MW 40,000 (each consisting of a light (MW 16,000) and heavy chain (MW 24,000)), is the minor 8S component of the seed protein which cross reacts with antibodies raised to the soybean 7S globulin light and heavy chains. N-terminal sequencing of the two subunits have confirmed their homologies with other 7S seed storage proteins e.g. γconglutin (Hirano 1989).

Psophocarpin C consists of a number of polypeptides, MW 17,000-80,000; in a recent study many had blocked N-terminals and therefore their homologies with other seed storage proteins are unknown (Hirano 1989).
Antimetabolic proteins of winged bean.

Winged bean seeds and tubers contain significant amounts of protease inhibitors and haemagglutinins, around 5% and 4% respectively in both tissues (Shibita et al 1986, Kortt and Caldwell 1985)

It is suggested that there are as many as 10 different types of protease inhibitor present in the seeds of winged bean. (Shibita et al 1986). Initial analysis of the trypsin inhibitors has revealed that there are two main forms, WBTI-1 and WBTI-II (Kortt 1979); both consist of single polypeptide chains of molecular weights of 20,000 and each contains four cysteine residues per subunit. WBTI-1 has been fully sequenced, and this has confirmed its relationship to other members of the Kunitz inhibitor family. WBTI-1 is known to be present in two isoinhibitor forms of pI 8.5 and 9.4; this difference has been explained by a difference in their amino acid composition at positions 73 and 152 (Yamamoto et al 1983) and they are thus likely to be the products of two separate genes. A Bowman-Birk type trypsin inhibitor has been reported (Hirano 1989, Shibita et al 1986) which has a molecular weight around 8,000 and is in low abundance.

A trypsin inhibitor related to WBTI-1 can be found in the mature winged bean tubers, as determined by serological cross reactivity (Madiah et al 1989). It has a subunit molecular weight 21,000, and has been designated WBT-TI-II and makes up 2% of the inhibitory component . A second trypsin inhibitor present in the tubers WBT-TI-1, Mr 45,000 is a dimer composed of two subunits Mr 22,500. It does not cross react immunologically with either WBT-TI-II or a winged bean seed extract .

Four chymotrypsin inhibitors have been identified in winged bean

seeds, WCI-1, 2, 3 and 4 (Shibata *et al* 1986). WCI-2 and 3 inhibit

α-chymotrypsin in a stoichiometric ratio of 1:2, whilst WCI-1 and 4 inhibit

in the ratio 1:1 and also inhibit trypsin (but not simultaneously). All

four chymotrypsin inhibitors cross react with antibodies raised against

WCI-3, but these antibodies do not cross react with trypsin inhibitors. The

gene encoding WCI-3 has recently been cloned and sequenced, and has

homology to other Kunitz-like inhibitors. Expression of WCI-3 is controlled

developmentally, transcripts appearing in the maturing cotyledons 35 d.a.f.

and increasing to a maximum up until 50 d.a.f. (Peyackoknagul 1989).

The haemagglutinins

The presence of lectin in winged bean seeds has been known for some

time, with several workers showing that extracts from seeds agglutinate

human erythrocytes of types A, B and O (Renkonen 1948, Lee *et al* 1977, Boyd

1961).

Pueppke (1979), using a Bio-Gel lactobionate adsorbent was the first

to isolate a phytohaemagglutinin from winged bean seeds. A MW of 46,000 was

determined using ultracentrifugation techniques whilst SDS-PAGE

electrophoresis revealed subunits of 29,000 +/- 3000, suggesting that the

lectin was present as a non-covalently linked dimer. The lectin

agglutinated all three types of human erythrocyte upon desialyation and

treatment with trypsin, but remained inactive against natural cells.

D-galactose was found to be the most potent inhibitor of the

haemagglutination reaction., Little difference was observed between α and β

D-galactopyranosides although lactose was four times more potent an

inhibitor than was melibiose. (Pueppke 1979).

Appukuttan and Basu (1980) described the preparation of a winged bean

seed lectin specific for N-acetyl galactosamine, for use in the study of

glycoconjugates. A Sepharose-N-Caproyl-D-galactosamine affinity column was

employed to purify a MW 41,000 protein (determined by gel filtration on Bio Gel P100). SDS-PAGE gel electrophoresis revealed a single polypeptide of MW 35,000. This lectin was capable of agglutinating human A, B and O type erythrocytes in their native forms.

In a study of the lectins of winged bean seed by Higuchi and Iwai (1984) two forms of lectin were isolated on the basis of haemagglutination activity and pI. One of these lectins, the acidic, resembled the protein isolated by Pueppke, but no lectin similar to that isolated by Appukkan and Basu was observed.

Other workers have since confirmed the existence of two distinct forms of lectin present in the winged bean seed. Using gel filtration and ion exchange chromatography. Kortt *et al* (1985) were able to isolate two lectin fractions, pI=5.5 and pI=8.5 . These fractions, acidic and basic lectin, have distinct haemagglutination and sugar binding specificities. The basic fraction agglutinated rabbit erythrocytes much more strongly than the acidic lectin and was unable to agglutinate human O^+ erythrocytes. Lactose was four times more potent as an inhibitor of haemagglutination against acidic lectin than basic. The basic lectin binds α-galactosides e.g. melibiose, raffinose, stachyose whereas the acidic lectin binds β-galactosides e.g. lactose.

Physiochemical analysis of the acidic and basic lectins has led to a better understanding of their structures. The basic lectin fraction is composed of three isolectins B1,B2 and B3 (as determined by differences in pI) Mr 58,000. Each is a dimer composed of 2 non-covalently linked subunits MW 29,000 each. Isoelectric focusing in 8M urea revealed that there are three different subunits α, β and γ. B1 is composed of two α subunits, B2 an α and a β and B3 a β and a γ. The amino acid compositions of the three isolectins are very similar. N-terminal sequencing of the B3 isolectin to

40 residues revealed no microheterogeneity and therefore the subunits β and γ have identical N-terminal sequences and differences in pI are related to other amino acid substitutions, postranslational modifications or deamidation on extraction . The 11 N-terminal residues of B2 are identical with those of B3, further evidence for the identity of α, β and γ subunits. The lectins isolated by both Higuchi (1985) and Kortt (1984) are glycoproteins, in contrast to those isolated previously. The lectins have approximately 7% sugar and analysis assuming N-linked Asn glycosylation of a similar nature to that seen in mammalian systems, suggests two carbohydrate side chains per subunit.

The acidic lectin fraction was also composed of three isolectins, Mr 54,000. SDS-PAGE was used to estimate subunit molecular weights of 31,000. The lectins are non-covalently bound dimers. The charge heterogeneity of these subunits and of the isolectins was very small. N-terminal sequencing and amino acid composition analysis of A1, A2 and A3 revealed that A2 and A3 appeared to be identical whereas A1 had a number of amino acid differences. Glycosylation differences may explain the differences between A2 and A3 but, as is the case with the basic lectin. Small differences in amino acid sequence are more likely to explain the differences between A1 and A2, A3.

Carbohydrate analysis was carried out on a mixture of acidic lectins prepared on a lactose-Sepharose column. Mannose, N-acetyl glucosamine, fucose and xylose were present in the M ratio 9: 4.4: 1.6: 7.0 and the total carbohydrate content was estimated as 7.0% (Kortt 1985).

Expression and distribution of lectin

Although the acidic lectins and the basic lectins are closely homologous, as shown by similar N-terminal sequences, clearly a number of genes are involved in the expression of lectins in the winged bean plant.

Studies by Kortt (1988) with affinity purified acidic and basic lectins from a variety of cultivars from different regions of S.E. Asia showed very few varietal differences in either the basic or acidic lectins present (Kortt and Caldwell 1986). It would appear, therefore, that the divergence of these two lectins, from a common ancestor, happened early on in this plant's evolution.

The specific activity of winged bean lectin (measured in haemagglutination units/mg) throughout the plant during its life cycle has been measured by Shet and co-workers (1988). Mature seeds are the main source of lectin in winged bean, with the bulk of the activity residing in the cotyledons, and smaller amounts in the seed coat and embryo. During germination and early development of the plant no lectin activity is detectable in either the epicotyl, hypocotyl or cotyledonary leaves. However, as the plants mature, lectin can be detected in the stems, shoots and roots of the winged bean plant. The amount of lectin activity in the young shoots begins to increase gradually around the 6th week after germination whilst the lectin activity in the stems increases gradually after 15 weeks.

The presence of lectin activity in roots can be detected 4 weeks after germination and continues to increase steadily during the plants growth. Young flower buds and opened flowers both have lectin activity as do young pods and immature seeds and tubers. The precise build up and expression of lectin in developing winged bean pods and seeds has been investigated by Higuchi and co-workers (1989). Lectin activity in both pod and seed could first be detected 30 days after flowering and increased to a maximum around 65 d.a.f. . The lectin appearing at 30 days was found to be an acidic lectin (as determined by haemagglutination and immunogenic properties), whilst the basic lectin could not be detected until 40 d.a.f. .

Both the mature pods and seeds possessed similar haemagglutination specificities in this study and the authors suggest that this reflects similar contents of lectin isoforms.

1.7 Aims and objectives

A number of groups have now produced insect resistant plants using modern genetic engineering techniques (Fischhoff *et al* 1987, Hilder *et al* 1987, Attabella *et al* 1990). In each case this resistance relies on a different type of antimetabolic protein, be it bacterial toxin, trypsin inhibitor, lectin or α-amylase inhibitor. That these investigations have proved successful means that a greater emphasis now has to be placed on the discovery and investigation of other plant defence factors that can be exploited in a similar manner. The aims of this thesis were to investigate the biochemical factor(s) involved in the insect resistant properties of the mature winged bean seed. Any information gained was to be used in an attempt to isolate new/novel genes that could possibly be used in transgenic plants to confer insect resistance.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Materials

2.1.1 Glassware and plasticware.

All glassware and plasticware used for DNA and RNA manipulations was siliconised with dimethyldichlorosilane (2% v/v in 1,1,1-trichloroethane), washed in distilled water, dried and autoclaved. In addition, glassware used in RNA work was baked overnight at $170^{\circ}C$ after siliconisation.

2.1.2 Plant Material.

Garden pea seeds (*Pisum sativum* var.Feltham First) were provided by Dr. J.A. Gatehouse, Department of Biological Sciences, University of Durham, U.K. . Cowpea seeds (*vigna unguiculata*, Californian black-eyed beans) were obtained from Maggie's Farm, New Elvet, Durham, England. All seeds were stored at $4^{\circ}C$.

2.1.3 Insects.

Callosobruchus maculatus, *Heleothis virescens* and *Spodoptera littoralis* were generously provided by Dr. A.M. Gatehouse, Department of Biological Sciences, University of Durham, U.K. . These insects were kept in controlled temperature and humidity chambers ($27^{\circ}C$, 70% relative humidity)

2.1.4 Bacterial strains.

Esherichia coli strains K803, LE392 and P2392 were generously provided by Dr. A.P. Fordham-Skelton, Department of Biological Sciences, University of Durham, U.K. . *E.coli* frozen competent cells strain DH5α were supplied by GIBCO-BRL Ltd., Paisley, Scotland. Strain genotypes and source references are listed in Sambrook *et al* (1989) and Fedoroff (1983).

2.1.5 Nucleic acids.

Plasmids pUC18 and pUC19 were obtained from Boehringer Mannheim U.K., Lewes England. λ EMBL3 DNA was from Stratagene, California, USA. λ DNA size markers were purchased from Northumbrian Biologicals, Cramlington, England. pDUB128 was provided by Dr. G.A. Edwards, Shell Biologicals, Sittingborne, England. pPVL134 was provided by Dr. R.R.D. Croy, Department of Biological Sciences, University of Durham, U.K. . Phage λL9-4 was generously donated by Professor R. Goldberg, UCLA, California USA. Poly A$^+$ RNA from cotyledons of garden pea (*Pisum sativum* var. Feltham First) was generously supplied by Dr. I.M. Evans, Department of biological Sciences, University of Durham, U.K. . Oligonucleotides were synthesised in this department by Mr J. Gilroy, using an ABI 381A DNA synthesizer.

2.1.6 Biological and Chemical Reagents.

All reagents, with the exception of those noted below, were obtained from BDH chemicals Ltd., Poole, Dorset, U.K. and were either of analytical grade or the best avaliable.

Acridine orange, adenosine 5'triphosphate, ampicillin, bovine serum albumen, dithiothreitol, ethidium bromide, glyoxal, herring sperm DNA, IPTG, N-lauryl sarcosine, lysozyme, N,N-methylene-bis-acrylamide, ε-aminocaproylgalactosamine-agarose, polyvinylpyrolidine, pronase-P, RNAase, spermidine and tRNA were from Sigma Chemical Co., Poole, Dorset, U.K. .

Boric acid, caesium chloride and sodium dihydrogen orthophosphate were from Koch-Light Ltd., Harverhill, Suffolk, U.K. .

Ficoll 400, Klenow fragment, Sephadex G-50 and T4 DNA ligase were from Pharmacia Fine Chemicals, Uppsala, Sweden.

Nitrocellulose filters (BA85, 0.45μm) were from Schleicher and Schull,

Anderman and Co. Ltd., Kingston-upon-Thames, Surrey, U.K. .

Whatman 3MM paper and 2.5cm GFC discs were from Whatman Ltd., Maidstone, Kent, U.K..

Bacto-agar, Bactotryptone and Bacto-yeast extract were from Difco Laboratories, Detroit, Michigan, USA.

Restriction enzymes and buffers were from Northumbria biologicals Ltd., Cramlington, Northumberland ; Bethesda reasearch laboratories, Bethesda, Maryland. ; Pharmacia Fine Chemicals ; Boeringer Corporation (London) Ltd., Lewes, East Sussex.

Alkaline phosphatase, Polynucleotide kinase, tris(hydroxymethyl)aminomethane and XGAL were from Boeringer Corporation (London), Lewes, East Sussex, U.K. .

Taq DNA polymerase was obtained from Promega, Epsilon House, Enterprise Rd., Southampton, England.

Nick translation kits and radionucleotides were from Amersham International plc., Amersham, Bucks, U.K. .

Kodak X-ray film cassettes were kindly donated by the Freeman Hospital, Newcastle upon Tyne, England.

Goat anti-rabbit IgG (H+L) Horseradish peroxidase linked antibodies were supplied by Bio-Rad Laboratories, 1414 Harbour Way South, Richmond, California, USA.

Immobilised melibiose and Immobilised lactose were obtained from Pierce and Warriner (UK) Ltd., 44 Northgate Street, Chester, England.

2.2 METHODS

2.2.1 Haemagglutination assay for lectin activity.

The method used was that of Lis and Sharon (1972).

Rabbit blood (New Zealand White) was used for basic lectin assays whilst blood of human group O^+ was used for acidic lectin assays. The appropriate blood was collected in an equal volume of Alsever's solution (2% Glucose (w/v), 0.8% Sodium citrate (w/v), 0.42M Sodium chloride, pH 6.1) in a heparinised tube (Sarsdet U.K.) and stored at 4^OC. 1.0ml of cells were washed in PBS (0.15M sodium chloride, 0.05M Phosphate buffer pH 7.2) four times and then trypsinised by incubation in 6ml PBS, 200μl Bovine trypsin (0.2mg/ml in 10mM HCl) at 37^OC for 15 minutes. The cells were then washed and resuspended in PBS to give a 2% working strength solution. The material to be tested was either lyophilised and dissolved directly in PBS or if already in solution an equal volume of 2×PBS was added to prevent haemolysis. Assays were performed in microtitration trays with U-shaped wells (Sterillin U.K). 50μl of extract was serially diluted with 50μl PBS followed by addition of 50μl of 2% erythrocyte suspension. Titres were recorded after 30 minutes-1 hour.

2.2.2 Assay of protein fractions for trypsin inhibitory activity.

The method is essentially that of Erlanger et al (1961).

The assays were performed in plastic cuvettes. The standard assay components were :-

   2890-Xμl Buffer, 0.046M Tris-HCl pH 8.1, 0.015M calcium chloride

   Xμl Test fraction

   10μl Bovine trypsin (2mg/ml in 0.01M HCl)

   100μl BAPNA (16mg/ml in DMSO)

The bovine trypsin was assayed and titrated to a suitable concentration in order that a linear initial rate response could be observed over 3 minutes.

The trypsin and sample to be tested were preincubated for 5 minutes at room temperature, before the addition of substrate and the rate of reaction monitored for 3 minutes by recording the change in O.D.$_{410nm}$ spectrophotometrically.

2.2.3 Assay of protein fractions for chymotrypsin inhibitory activity.

The method used was that of Hummel (1959).

The assays were performed in 3ml plastic cuvettes. The standard assay components were :-

2680-Xμl Buffer, 0.05M Tris-HCl, 0.02M CaCl$_2$ pH 8.2

20μl Bovine chymotrypsin 100μg/ml in 0.01M HCL

300μl BTEE 3.13mg/ml in 50%(v/v) methanol/water

Xμl Test fraction

The chymotrypsin was assayed and titrated to a suitable concentration in order that a linear initial rate response could be observed over 3 minutes.

The fraction to be tested was preincubated with the chymotrypsin before addition of the enzyme substrate (BTEE). The rate of reaction was monitored for 3 minutes by recording the change in O.D.at 256nm.

2.2.4 Assay of protein fractions for α-amylase inhibitory activity.

The inhibition of starch hydrolysis by α-Amylase was determined by following the release of reducing sugars, using the dinitrosalicylate reaction as described by Bernfeld (1955).

Assay buffers differed depending on the source of amylase in the test reaction :-

Spodoptera littoralis   50mM glycine/NaOH 0.1M NaCl pH9.0

Bacillus licheniformis 50mM sodium acetate/ acetic acid pH 6.9

Porcine   50mM sodium acetate/ acetic acid pH 7.0

Locust   20mM Sodium phosphate buffer, 6mM NaCl pH 7.8

Each amylase source was assayed and titrated to a suitable concentration in order that a linear colormetric response was observed. The assay mixture consisted of :-

250µl α-amylase (varying concentrations in assay buffer)

250µl inhibitor (1,5,10,50 and 100 × α-amylase concentration)

250µl Assay buffer

250µl 1% Starch solution (w/v)

1000µl Total volume

The enzyme and the inhibitor were preincubated at 30°C for 25 minutes, before the addition of the starch substrate and a further incubation at 30°C for 10 minutes. The reaction was stopped by the addition of 1.0ml of dinitrosalicylate solution (1g dinitrosalicylate in 20ml 2N NaOH, 50ml distilled water, 30g potassium tartrate) and boiling in a water bath for 10 minutes. The reaction was allowed to cool and then diluted five fold before the O.D.$_{530nm}$ was determined.

2.2.5 Isolation of winged bean seed protein albumin and globulin fraction.

The method used was that of Croy (1977).

Winged bean seeds were ground in a Janke and Kunkel water cooled mill and defatted with hexane. Meal was extracted overnight at 4°C in 50mM sodium borate buffer pH 8.2 (10ml/g). After centrifugation of the suspension (10,000×g for 30 minutes at 4°C) the supernatant was dialysed exhaustively against 25mM sodium acetate pH 4.8 at 4°C. The globulin fraction

precipitated at this stage was recovered by centrifugation (10,000×g for 30 minutes at 4°C). The supernatant was pooled as the albumin fraction. Both fractions were dialysed against distilled water, to remove salts, before lyophilisation.

2.2.6 Ammonium sulphate fractionation of winged bean seed proteins.

Defatted meal was extracted in 50mM sodium borate buffer pH 8.2 (1g/10ml) at 4°C with stirring overnight. The suspension was centrifuged at (9,000×g for 25 minutes at 4°C) and the pellet discarded. Ammonium sulphate was added to the supernatant gradually to 40% saturation at 4°C. After 2 hours stirring the suspension was centrifuged (9,000×g for 30 minutes at 4°C). The pellet formed the 40% cut. The remaining supernatant was subsequently taken through 60%, 80% and finally 100% cuts. All cuts were then redissolved in 0.1M ammonium hydrogen carbonate and desalted on a column of sephadex G25 (60×2.2cm) equilibrated with 100mM ammonium hydrogen carbonate before freeze drying.

2.2.7 Isolation of winged bean seed psophocarpin fractions.

The method used was that of Gillespie and Blagrove (1976) and the procedure is detailed in the following flow chart.

10g defatted seed meal

Stir for 4h at 4°C with
500 ml acetate-chloride buffer, pH4.5;
centrifuge

Precipitate

(Discard)

Supernatant

Add ammonium sulphate
to 90% saturation;
stir for 2h; centrifuge.

Precipitate

stir with 0.15M phosphate, pH 7;
dialyse against 0.05M phosphate pH7;
centrifuge.

Supernatant

(Discard)

Precipitate

Crude psophocarpin A. Dissolve
in acetate-chloride buffer, titrate
to pH 4.8 and centrifuge off the
precipitate containing impurities.
Supernatant contains pure A.

Supernatant

Dialyse against deionised
water in cold, titrate to
pH 5.4 with imidazole;
stir; centrifuge

Precipitate

pH 5.4-insoluble fraction;
essentially psophocarpin C

Supernatant

pH 5.4-soluble fraction;
essentially psophocarpin B

2.2.8 Isolation of winged bean trypsin inhibitor.

The method is essentially that of Gatehouse *et al* (1980).

20g of defatted meal was extracted overnight with stirring at 4°C in 200ml of extraction buffer (0.1M sodium acetate, 0.3M sodium chloride, 0.01M calcium chloride pH 4.0). The suspension was clarified by centrifugation at 10,000×g for 30 minutes followed by filtering through glass wool and loaded onto a trypsin/sepharose 4B affinity column equilibrated with extraction buffer. The column was washed extensively with extraction buffer to remove unbound substances, and then washed with one column volume of unbuffered wash (0.3M NaCl, 0.01M $CaCl_2$). Trypsin inhibitor was eluted from the column by washing the column in elution buffer (0.01M HCl, 0.3M NaCl, 0.01M $CaCl_2$ pH 2.0). Fractions corresponding to a single peak of trypsin inhibitory activity were pooled and precipitated by addition of ammonium sulphate to 95% saturation. The precipitated fractions were resuspended in 0.1M NH_4HCO_3 and desalted on a sephadex G25 column equilibrated with the same buffer before lyophilisation and storage at 4°C.

2.2.9 Isolation of the acidic and basic lectins from winged bean seed.

The method used was essentially that of Kortt (1984).

300mg of Psophocarpin B (see Methods 2.2.7) was dissolved in 15ml 0.05M Tris-HCl, 0.1M NaCl, pH8, clarified by centrifugation at 3000×g for ten minutes and applied to a Sephacryl S-200 column (190×2.6cm) equilibrated with the same buffer. Flow rate was 15ml/hr and 5ml fractions were collected and assayed for haemagglutination activity. Fractions agglutinating rabbit erythrocytes were pooled, dialysed against distilled water and lyophilised (crude basic lectin); fractions agglutinating human O^+ erythrocytes were pooled separately, dialysed and lyophilised (crude acidic lectin) .

2.2.10 Isolation of winged bean acidic and basic lectin using affinity chromatography

The method is that of Kortt and Caldwell (1985).

1g of hexane defatted seed meal was extracted overnight with stirring at $4^\circ C$ in 20ml 5mM phosphate buffer, 0.1M sodium chloride pH7.1 (PBS). The extract was clarified by filtration through muslin and by centrifugation (10,000×g for 30 minutes at $4^\circ C$) before it was applied to a immobilised melibiose affinity column (3.0 × 0.5cm) the eluate from this column was then applied to a column containing immobilised lactose. Both columns were washed extensively with PBS until the absorbance at 280nm was reduced and remained constant. Lectins were eluted from the columns using 0.3M galactose in PBS. 1ml fractions were collected, and their $O.D._{280nm}$ recorded. Fractions were also assayed by SDS-PAGE after precipitation using TCA.

2.2.11 Ion exchange chromatography of winged bean acidic lectin

The method is that of Kortt (1985).

20mg of an acidic lectin fraction purified from psophocarpin B by gel filtration (2.2.9) was resuspended in 0.01M citrate buffer pH5.1 and applied to a SP-Sephadex C-25 (Pharmacia) ion exchange column (25cm × 1cm) equilibrated with with 0.01M citrate buffer pH5.1. The column was then washed with this buffer before a 1000ml linear gradient of 0.01M - 0.1M citrate buffer pH 5.1 was used to elute bound proteins at a flow rate of 20ml/hr. 5ml fractions were collected and assayed by SDS-PAGE following TCA precipitation.

2.2.12 Isolation of pea lectin.

The method was essentially that of Trowbridge (1974).

200g of mature pea seeds were ground into a fine meal in a Janke and Kunkel water cooled mill and extracted with stirring at $4^{O}C$ with 800ml 25mM Sodium acetate pH4.8, for at least 3 hours. After centrifugation at 1500×g for 30 minutes, the supernatant was retained and was brought to 60% saturation with ammonium sulphate by addition of solid, followed by stirring at $4^{O}C$ for at least 2hr. Precipitated protein was recovered by centrifugation at 3000×g for 30 minutes, dissolved in 80ml of distilled water, and dialysed at $4^{O}C$ against two 1l volumes of 0.15M Sodium chloride, 0.01M Phosphate buffer pH 7.2 for 3 hours each.The crude lectin extract was loaded onto a Sephadex G-100 column (500ml). The Column was washed with 0.15M Sodium chloride, 0.01M Phosphate buffer pH 7.2 until the O.D.$_{280}$ was less than 0.05 and the pea lectin was then eluted with the same buffer containing 0.2M Glucose. 10ml fractions were collected using a FRAC-100 fraction collector (Pharmacia) and their O.D. 280nm monitored and plotted on a chart recorder. Fractions correlating to the peak of released lectin were pooled and dialysed exhaustively against distilled water followed by freeze drying, weighing and storage at $4^{O}C$.

2.2.13 Reduction and carboxymethylation of proteins.

4mg of affinity purified basic lectin was dissolved in 1ml of 6M guanidine HCl, 0.6M Tris-HCl pH 8.6 . 10µl of β-mercaptoethanol was then added and the solution kept under nitrogen for 3 hours. The reduced protein was then carboxymethylated by the addition of 20mg of iodoacetic acid in 100µl of 0.1M sodium hydroxide, and was kept in the dark for 20 minutes. The solution was then dialysed against 5l of 5mM ammonium bicarbonate in the dark for 26hrs before freeze drying.

2.2.14 Digestion of winged bean lectin with trypsin.

3mg of reduced and carboxymethylated, affinity purified winged bean basic lectin protein was digested with 0.1mg of bovine trypsin (TPCK-treated) in a minimal volume of 0.2M N-ethylmorpholine digestion buffer pH 8.5 for approximately five hours at 37°C.

2.2.15 Liquid-phase manual sequencing of peptides.

(1) Coupling

The sample to be sequenced (5-10nmol) was resuspended in 80µl of 50% aqueous pyridine ; to this 40µl of DABITC (2.8mg/ml in pyridine) was added. The tubes were purged with nitrogen, sealed and incubated at 52°C for 50 minutes. 10µl of PITC was then added and the tubes again purged with nitrogen before incubation at 52°C for a further 20 minutes.

(2) Washing

The coupling reaction was vortexed with 1ml of heptane : ethylacetate (2:1), and the phases separated by brief centrifugation in a bench centrifuge. The upper phase was removed by aspiration and discarded. The lower aqueous phase was subjected to two further washes, as above, before drying down under vacuum.

(3) Cleavage

The dried down samples were cleaved by the addition of 50µl of anhydrous TFA, followed by purging with nitrogen and incubating at 52°C for 15 minutes . Before extraction the TFA was removed by drying under vacuum.

(4) Extraction

200µl of n-butyl acetate and 50µl of water were added and the mixture vortexed well before brief centrifugation in a bench centrifuge to separate the phases. The upper organic phase was removed into a conversion tube and

dried down under vacuum. The remaining aqueous phase was also dried down under vacuum and was then subjected to a further cycle.

(5) Conversion

50μl of 50% TFA was added to the conversion tube and the tube was incubated at 80°C for 10 minutes before drying down again under vacuum.

(6) Analysis of DABTH-derivatives using thin-layer chromatography

Dried down converted samples were re-dissolved in 5μl of ethanol and spotted onto 30mm square polyamide layer sheets 4mm from either edge in the bottom left hand corner. A standard (DABTH-diethylamine) was also spotted on the sheet at this position. The chromatogram was developed in two dimensions in covered beakers. The solvent for the first dimension was glacial acetic acid : water, 1:2 (v/v) and the solvent front was allowed to run to the top of the sheets. The sheets were dried in a stream of warm air and then developed in the second dimension in toluene : n-hexane : acetic acid, 2:1:1 (by vol) until the solvent front was 2-3mm from the top of the sheet. The DABTH-derivative was visualised and identified by exposing the chromatograms to a stream of hydrochloric acid fumes produced by directing a gentle stream of air into a winchester containing 100-200ml of concentrated hydrochloric acid.

2.2.16 General extraction of proteins from plant tissue.

Tissue was ground under liquid nitrogen using a pestle and mortar and lyophilised . Proteins were extracted, from 20mg of lyophilised tissue, in 1ml protein extraction buffer (Phosphate buffered saline pH7.5, PMSF 100μg/ml, Leupeptin 0.5μg/ml) by mixing overnight at 4°C. The supernatant was recovered following centrifugation (15,000×g for 10 minutes at 4°C) and the protein precipitated using trichloroacetic acid (method 2.2.17).

2.2.17 Precipitation of proteins using trichloroacetic acid.

Proteins were precipitated from solution, where necessary, by the addition of one fifth volume of 70% Trichloroacetic acid and cooling on ice for 20 minutes. Protein was pelleted by centrifugation (13,000×g for 10 minutes at 4°C) and washed three times with ethanol before drying on a vacuum desiccator for 5 minutes.

2.2.18 Preparation of dialysis tubing.

Dialysis tubing for use with both proteins and DNA was prepared by boiling for 10 minutes in a large volume of 2% Sodium bicarbonate, 1M EDTA followed by rinsing and boiling for a further 10 minutes in distilled water. Tubing was stored in 70% ethanol and washed exhaustively before use.

2.2.19 Polyacrylamide gel electrophoresis of proteins

The method employed was essentially that of Laemmli (1970), gels were run in a Studier type apparatus as described by Hames (1981). 180 × 150 × 10mm gels of the appropriate percentage acrylamide were prepared according to the following recipes :-

Resolving Gel   34.0ml 30% Acrylamide stock[*]

                     22.5ml 1.0M Tris-HCl pH8.8

                     1.4ml Distilled water

            Mix and deaerate under vacuum

                     1.5ml Ammonium persulphate (15mg/ml)

                     0.6ml SDS (10%w/v)

                     20µl TEMED

                     60.02ml TOTAL VOLUME

              Mix and pour gel immediately

Stacking Gel   2.0ml 30% Acrylamide stock[*]

                      14.8ml Distilled water

                      2.5ml Tris pH6.8

                      Mix and deaerate under vacuum

                      0.5ml Ammonium persulphate (15mg/ml)

                      0.2ml SDS (10%w/v)

                      20µl TEMED

                      20.02ml TOTAL VOLUME

                      Mix and pour immediately

      [*](30% acrylamide, 1% bisacrylamide)

The resulting gels were placed in the electrophoresis apparatus, and the reservoirs filled with running buffer (192mM Glycine, 25mM Tris base). Samples were dissolved in 10% sucrose, 2% SDS (w/v) and heated (100°C) for 5 minutes before loading. The gel was run at 25mA for ~5 hours at constant current. After electrophoresis, proteins were visualised by staining the gel for ~4hrs in 200ml Kenacid Blue Stain (0.05% (w/v) coomasie brilliant blue R250 in 50% (v/v) methanol, 7% (v/v) acetic acid). Excess stain was removed by soaking the gel overnight in Destain solution (50% (v/v) methanol, 7% (v/v) acetic acid). Gels were preserved by vacuum drying between two layers of cellophane, using a flat bed gel drying apparatus.

2.2.20 Transfer of proteins from acrylamide gels to nitrocellulose.

The semi dry blotting method of Kyse-Andersen (1974) was used. Transfer was carried out on a Sartorius electroblotter according to the instructions supplied. Each transfer unit comprised one sheet of 3MM paper soaked in anode buffer 2 (25mM Tris, 20% (v/v) methanol, pH 10.4); a sheet of nitrocellulose ; the gel to be blotted ; one sheet of 3MM paper soaked

in cathode buffer (25mM Tris, 40mM 6-amino hexanoic acid, 20% methanol, pH 9.4) and a sheet of dialysis membrane soaked in distilled water. The anodic plate was covered with two layers of 3MM soaked in Anode buffer 1 (0.3M Tris, 20% (v/v) methanol, pH 10.4), and the cathodic plate with two layers of 3MM paper soaked in cathode buffer. The transfer units were placed between the two plates and transfer took place for 1.5 hours at $0.8mA/cm^2$.

2.2.21 Immunological detection of immobilised protein on nitrocellulose filters.

The method is modified from that of Towbin *et al* (1979), using the blocking agent described by Johnson *et al* (1984).

All incubations took place at $40^{o}C$ with shaking, and all washing steps were conducted in the same conditions for 10 minutes each. Filters were incubated and washed once in BLOTTO (5% (w/v) non-fat dried milk powder in 20mM Tris-HCl pH 7.2, 0.9% (w/v) sodium chloride). Filters were then incubated with rabbit anti-(winged bean basic lectin) antiserum (50µl/50ml BLOTTO) for 2hrs, washed three times in BLOTTO and incubated with (horse radish peroxidase)-linked goat anti-(rabbit IgG) immunoglobulins (20µl/50ml BLOTTO). Filters were washed three times in BLOTTO and once with 20mM Tris-HCl pH 7.2, 0.9% sodium chloride. 50mg Diaminobenzoic acid was dissolved in 100ml 20 mM Tris-HCl pH7.2, 0.9% sodium chloride and 100µl of 30% (v/v) hydrogen peroxide and 3ml 1% CoCl added. Filters were incubated in this staining solution until bands developed, and were then transferred to water.

2.2.22 Standard diet and rearing for lepidopteran bio-assay.

The standard diet used in the the assay of lepidopterans was that published by Singh (1980).

Haricot bean flour 237g, alfalfa meal 237g, wheatgerm 237g, brewer's yeast 106g, ascorbic acid 3.3g, and methyl-p-hydroxybenzoate 6.6g were mixed together along with 1300ml of molten agar solution which had been allowed to cool to $50^{o}C$ (42.63g agar in 1300ml of water). To this mixture was added vitamin C 10.6g, Aureomycin 300mg, formaldehyde 6.6ml and 700ml of distilled water. The mixture was plated out into sterile petri dishes and allowed to cool. The diet was stored at $-20^{o}C$. All Lepidoptera (other than neonates) used in bio-assay were reared on this diet prior to bio-assay. Bioassays were conducted at $26^{o}C$ and 70% relative humidity.

2.2.23 Standard diet and bioassay using bruchid beetle

Feeding trials were performed as described by Gatehouse and Boulter (1983).

Known amounts of test protein/protein mixes were dissolved in 15ml of distilled water.This solution was mixed thoroughly with 2.5g of milled and sieved chick peas and lyophilised . The diet was then powdered and sufficient distilled water (approx. 1ml) was added to the dried slurry in order to mould five replicate pellets . Pellets were dried over silica gel and allowed to equilibrate for seven days in the insectary, 70% r.h., $26^{o}C$, prior to sealing in clingfilm. The artificial seeds were inoculated by placing in an active culture of *C.maculatus* for 1-2 days. The inoculated pellets were then placed in individual vials and adult emergence monitored each day for 42 days. On termination of the feeding trial each pellet was dissected and live adults, pupae, larvae scored.

2.2.24 Storage of bacterial strains.

Bacteria which required storage for long periods of time were preserved in storage solution, 60% L Broth and 40% glycerol. Bacterial lawns grown from

single colonies on selective agar plates were transfered to 2ml aliquots of storage solution, mixed and kept at $-80^\circ C$.

2.2.25 Preparation of competent cells.

5µl of a glycerol of *Escherichia coli* JM101 were incubated overnight at $37^\circ C$ in 10ml YT broth (8g/l bacto-tryptone, 5g/l bacto-yeast extract and 2.5g/l NaCl pH7.0). 500µl aliquot of the the overnight culture was added to 50ml YT broth on a rotary shaker at $37^\circ C$ and the growth rate monitored. At $O.D_{660nm}$ ~ 0.6 the cells were harvested by centrifugation at 4,000×g for 10 minutes at $4^\circ C$ and resuspended in 1ml of cold 0.1M calcium chloride. After one hour the cells were harvested again by centrifugation and resuspended in 1ml of cold 0.1M calcium chloride. Cells were used within three days of preparation and kept at $4^\circ C$. Transformation frequencies of the order 10^5/µg pUC18 were normally obtainable.

2.2.26 Transformation of DH5α competent cells.

The method is that according to the suppliers protocol.

Ligation reactions were diluted 5 fold and 1µl of the dilution was added to 100µl of DH5α competent cells that had been allowed to thaw on ice. The cells were incubated at $0^\circ C$ for 30 minutes, heat shocked for 45 seconds at $42^\circ C$ and then placed on ice for a further 2 minutes. 0.9ml of S.O.C. broth (2% bactotryptone, 0.5% yeast extract, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$ and 20mM glucose) was added and the cells incubated for 1 hour at $37^\circ C$ with shaking. 100µl and 10µl of these cells were plated out on YT AMP XGAL agar plates (100µg/ml ampicillin, 50µg/ml X-GAL).

2.2.27 *In Vitro* packaging of concatamerised λ DNA.

Freeze/thaw and sonicated extracts were optimised for packaging using

concatamerised λ 1897 sam7. The optimised extracts were allowed to thaw on ice, the freeze/thaw extract was added to the sonicated extract and mixed gently. 1μg of DNA dissolved in 5μl 10mM Tris-HCl pH 7.9, 10mM $MgCl_2$ was added before the combined extracts had totally thawed and incubated for 1 hour. 1ml of SM buffer (5.8g/l NaCl, 2g/l $MgSO_4$, 0.05M Tris-HCl pH 7.5 and 2% gelatin) was added to the mixture and the phage titre measured. A control, with no DNA, was used in order to determine the background titre due to the extract.

2.2.28 Spectrophotometric analysis of nucleic acid solutions.

Absorbance spectra for nucleic acid solutions were obtained by using a Pye Unicam SP8-150 UV/vis spectrophotometer operated in scanning mode between 320nm and 230nm . The purity of the solution was determined by the $O.D_{260/280}$ ratio (1.8 for pure DNA and 2.0 for pure RNA) and the $O.D_{260/235}$ ratio being higher than the $O.D_{260/280}$ ratio. A 1μg/ml solution of DNA has an $O.D_{260}$ of 0.020 whilst that of RNA is 0.024.

2.2.29 Alcohol precipitation of DNA .

DNA was precipitated from solution by addition of 0.1 volumes of 3M Sodium acetate pH 5.2 and 2.0 volumes of cold ethanol, followed by cooling at $-80^{O}C$ for 30 minutes or at $-20^{O}C$ overnight. The DNA was then pelleted (12000×g, 15 minutes at room temperature), washed twice with 70% (v/v) ethanol, dried briefly under vacuum before resuspending in T.E. buffer.

2.2.30 Extraction of proteins from DNA using phenol.

Solutions of DNA were deproteinised by 'Phenol/chloroform extraction'. An equal volume of redistilled phenol under T.E buffer was added to the DNA solution and mixed by vortexing for 15 seconds. The aqueous layer after

centrifugation (12000×g, 5 minutes at room temperature) was then transfered to another tube and re-extracted with phenol. The aqueous layer was separated again by centrifugation and transfered to another tube. At this stage the phenol was back extracted with a small volume of T.E. buffer (to recover as much DNA as possible) and the aqueous layers combined. Phenol extraction was then followed by chloroform extraction. Equal volumes of chloroform/isoamyl alcohol (24:1 v/v) under T.E. buffer were used order to remove any remaining phenol. After phenol/chloroform extractions DNA was recovered by alcohol precipitation. In some cases when the DNA solution was very viscous the solution was extracted three time with diethyl ether and remaining diethyl ether removed by aspirating the sample with nitrogen for 5 minutes. High molecular weight DNA was extracted using a phenol/cresol mix, 500µl phenol: 70µl cresol : 1.5mg 8-Hydroxyquinoline (Kirby 1965).

2.2.31 Extraction of high molecular weight DNA from plant tissue

The method was that of Ellis *et al* (1984).

1g of fresh young leaves were frozen in liquid nitrogen and ground to a fine powder in a pre-cooled pestle and mortar. 5ml of filter sterilised extraction buffer (0.45M sodium chloride, 0.045M trisodium citrate, 0.1M sodium diethyldithiocarbamate, 0.1M EDTA, pH 8.9) was added and the material mixed thoroughly after thawing on a warm water bath and the addition of 100µl 20% (w/v) SDS. 10ml chloroform:isoamylalcohol (24:1) was mixed with the resulting paste and the phases separated by low speed centrifugation in an MSE bench centrifuge (4000rpm). The aqueous phase was removed with a wide bore pipette and transferred to sterile 100ml glass beakers, the solution was gently overlaid with 20ml absolute ethanol and the nucleic acid removed from the interface by spooling onto a disposable plastic pipette tip. The nucleic acid was air dried and redissolved

overnight in 500μl TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0). This solution was extracted once with phenol/cresol (see 2.2.30) before the nucleic acid was reprecipitated with two volumes of absolute ethanol and resuspended in 500μl TE buffer. The yield was approximately 800μg DNA with a molecular weight >50kb.

2.2.32 Agarose gel electrophoresis of DNA

Preparation and electrophoresis of DNA was as described by Maniatis *et al* (1982).

1.6g of agarose (0.8% gel) was boiled with 200ml of Alec's gel buffer (40mM Tris-acetate pH 7.7, 2mM EDTA) until the agarose had fully dissolved. The solution was cooled to 50°C before addition of ethidium bromide to 0.5 μg/ml, and the gel was poured and allowed to set in a perspex mould (190×150×6mm) with a well forming comb, cemented onto a glass plate using silicone grease. Gels were submerged (1-2mm) in a electrophoresis tank containing Alec's gel buffer and EtBr at 0.5μg/ml. DNA samples containing 10% loading buffer (10% glycerol, 10mM Tris-HCl pH7.4, 1mM EDTA pH8.0) were loaded into the wells and subsequently subjected to electrophoresis at 120V for 2 hours or overnight at 30V. The DNA was visualised on a UV Transilluminator (300nm) and photographed through a Kodak 23A Wrattan filter, using a Polaroid MP-4 Land Camera with Polaroid type 667 film (exposure 4s at f16).

2.2.33 Capillary Blotting of DNA.

The method is that of Southern (1975).

Following electrophoresis DNA fragments in the gel were denatured by washing twice with 1.5M Sodium chloride, 0.5M Sodium hydroxide for 15 minutes. The gel was then neutralised with successive 30 minute washes with

1.5M Sodium chloride, 0.5M Tris-HCL pH 7.2, 1mM EDTA and placed on a bridge of Whatman 3MM paper set in a reservoir of 20×SSC. A carefully cut nitrocellulose or nylon membrane was placed on top of the gel followed by three layers of Whatman 3MM paper, a stack of absorbent pads, a glass plate and a small weight. Transfer by capillary action was allowed to proceed for 16 hours. Nitrocellulose filters were placed between two sheets of blotting paper and baked in a vacuum oven at 80°C for 1-2 hours. Nylon membranes were air dried, wrapped in cling-film and placed DNA side down on a Transilluminator (300nm) for 2 minutes.

2.2.34 Preparation of M13 replicative form DNA from *E.Coli* cells.

The method is that of Maniatis et al (1982).

Cells were harvested by centrifugation (10,000×g, 10min at 4°C) from 10ml overnight cultures in YT-AMP broth. The cell pellet was resuspended in 200μl solution 1 (Lysozyme 2mg/ml, 50mM glucose, 10mM EDTA, 25mM Tris-HCl pH 8) and transfered to a sterile 1.5ml plastic tube. 200μl solution 2 (0.2M Sodium hydroxide, 1% SDS) was added and the samples placed on ice for 5 minutes before addition of 200μl of solution 3 (3M sodium acetate pH 4.8) Following a further 5 minute incubation on ice the samples were centrifuged (14,000×g, 10min at 4°C) and the supernatant Phenol/chloroform extracted. DNA was recovered by ethanol precipitation and resuspended in 50μl T.E buffer. (approx. concentration 500ng/μl).

2.2.35 Isolation of small amounts of plasmid DNA from *E.coli* using silica fines

1.5ml of an overnight culture was centrifuged for 1min. The medium was removed by aspiration, leaving the bacterial pellet as dry as possible.

The pellet was resuspended by vortexing in 0.35ml of a solution containing 8% (w/v) sucrose, 0.5% (v/v) Triton X-100, 50mM EDTA (pH8.0) and 10mM Tris-HCl (pH8.0). The tube was incubated on a boiling water bath for 90seconds and then centrifuged immediately for 10min at room temperature in a minicentrifuge at 12,000xg. The pellet was removed from the tube with a sterile toothpick and discarded. 0.7ml of sodium iodide solution (90.8g sodium iodide, 1.5g disodium sulphite and 100ml distilled water, filter sterilised and saturated by addition of 0.5g disodium sulphite) was added and mixed well before addition of 10μl of a silica fines slurry. DNA bound to the glass fines during incubation at room temperature for 10mins. The fines were then pelleted by centrifugation for 15 secs in a microcentrifuge at 12,000xg and the supernatant was removed by aspiration. Fines were resuspended in 1ml 70% ethanol, pelleted once more and the ethanol removed by aspiration. The pellet was briefly dried under vacuum and the DNA eluted by incubation of the fines at 37°C for 10 minutes with 50μl T.E. buffer. 5μl of the resulting sample contained approximately 1μg of plasmid DNA.

2.2.36 Digestion of DNA with restriction endonucleases.

Enzymes were used at a concentration of 2-5 units/μg DNA, for two hours, at the temperature specified by the supplier. High molecular weight genomic DNA was restricted with 5-10 units/μg DNA for five hours.

2.2.37 Electroelution of DNA from agarose gel.

The method is that of McDonnell et al (1971).

The segment of gel containing the desired DNA fragment was excised using a sterile scalpel and placed in dialysis tubing with approximately 200μl of 1x TBE buffer (0.045M Tris-borate, 1mM EDTA) and sealed using mediclips. The dialysis tubing was then submerged in a mini gel tank containing 1x TBE

buffer and electrophoresed for 20 minutes at 60mA, or until the DNA had left the gel slice (observed under U.V. light). The polarity was reversed for 20 seconds and the buffer removed from the dialysis sac using a micropipette.This buffer containing the electroeluted fragment was phenol extracted and the DNA ethanol precipitated and washed in 70% ethanol.

2.2.38 Ligation of DNA.

DNA molecules with cohesive termini were ligated at 4°C for 12-16 hours using 1 Weiss unit of T_4 DNA ligase/μg DNA in a minimal volume of ligase buffer (20mM Tris-HCl pH7.6, 10mM $MgCl_2$, 10mM DTT and 0.6mM ATP). Blunt ended PCR products were ligated to vector DNA in a five fold excess, under the same conditions followed by a further 2 hour incubation at 20°C after the addition of 1 Weiss unit of DNA ligase.

2.2.39 Dephosphorylation of DNA molecules using alkaline phosphatase.

The 5' phosphate groups of DNA molecules were removed by treatment with calf intestine alkaline phosphatase in 50mM Tris-HCl pH9.0, 1mM $MgCl_2$, 0.1mM $ZnCl_2$ and 1mM spermidine, for 1 hour at 37°C using 0.2U/μg DNA.

2.2.40 Sau3A partial restriction of winged bean DNA.

The method is that of Kaiser and Murray (1985).

10μg of winged bean DNA was restricted with 0.05 units of Sau3A in 100μl Sau3A buffer (6mM Tris-HCl pH7.5, 6MM $MgCl_2$, 50mM NaCl, 1mM DTT) for 100 minutes at 37°C. 10μl aliquots were removed from the main reaction at 10 minute intervals from the start of the incubation. The reaction in these aliquots was stopped by adding EDTA to 20mM and heating at 70°C for 10 minutes and assayed for DNA molecules in the range 18-22 kbp by electrophoresis in agarose gel (0.3% w/v).

2.2.41 ^{32}P Labelling of synthetic oligonucleotides.

The method used can be found in Sambrook *et al* (1989).

The reaction was performed in a 1.5ml plastic tube and the standard

reaction mix was :-

> 1.0µl Oligonucleotide solution (15pMoles)
>
> 3.0µl 10× Polynucleotide Kinase buffer (0.5M Tris-HCl, 0.1M
>
>   MgCl$_2$, 50mM dithiothreitol, 1mM spermidine, 1mMEDTA)
>
> 5.0µl ^{32}P γATP 50µCi
>
> 20.5µl Distilled deionised water
>
> 0.5µl Polynucleotide kinase (2.5 units)
>
> 30.0µl Total volume

The reaction was incubated at 37°C for 30 minutes and the reaction mix was

then filtered directly into the hybridisation solution using a polysulphone

filter (Acrodisc).

2.2.42 ^{32}P Labelling of DNA using random oligonucleotide primers

DNA was labelled by using random oligonucleotides as primers according to

the method of Feinberg and Vogelstein (1983), using an Amersham Random

Prime labelling kit. 0.5µg DNA denatured by heating for 10 minutes at 95°C

was mixed with 10µl nucleotide/buffer solution (containing dATP, dTTP and

dGTP), 5µl of [α^{32}P]-dCTP, (50µCi ; 125pmole, 1µl random hexanucleotides

), water to 45µl and 5µl of enzyme solution (Containing 2.5 units Klenow

enzyme) followed by incubation at 37°C for 2 hours. The reaction was

stopped by addition of 2µl EDTA, 0.2mol/l pH8.0 and unincorporated

nucleotides removed by chromatography on a sephadex G50 (superfine grade)

column equilibrated and run in column buffer (50mM Tris-HCl pH 7.5, 0.1%

SDS, 150mM NaCl, 10mM EDTA). The excluded peak of radioactivity was

collected and the specific activity of the labelled probe was determined by scintillation counting of 1μl aliquots.

2.2.43 Hybridisation of radiolabelled DNA to membrane bound DNA and RNA

Membranes were placed in sealed polythene bags and prehybridised with 1ml/cm^2 prehybridisation solution (7.5ml 20×SSC, 1.25ml 100× Denhardts solution (2% ficoll, 2% bovine serum albumin, 2% polyvinylpyrollidine), 10% SDS and 0.5mg denatured herring sperm DNA). After 1 hours incubation at 65°C the prehybridisation solution was replaced with fresh prehybridisation solution containing 1μg probe (1×10^{8}dpm). The bag was heat sealed and the filter hybridised 25°C below the Tm of the duplex for 16 hours (Bonner, 1973). This was usually around 65°C. Washing of filters varied according to the probe used and is described in the text.

2.2.44 Autoradiography.

Autoradiography of ^{32}P decay was carried out using Fuji-RX film (Fujimex, Swindon, Wiltshire, U.K.). The film was preflashed once and left to expose at -80°C with an intensifying screen (Dupont , Wilingham, Delaware, USA.) in a Kodak film cassette.

2.2.45 Direct extraction of RNA in hot SDS.

The method is that of Buchbinder and Hall (1978).

10g of frozen cotyledons were warmed to -20°C before addition of solid DTT to give 5mM DTT after addition of extraction buffer. 26ml of extraction buffer(0.2M Sodium borate, 1% SDS, 30mM EGTA, pH9.0), warmed to 100°C, was then added. The mixture was immediately homogenised for 20 seconds using a Polytron (speed setting 10). Isoamyl alcohol was added to reduce foaming and the mixture was allowed to cool below 40°C before the addition of

Proteinase K (0.3mg/ml). After incubation at 37°C for 1 hour, 2ml of 2M

potassium chloride was then added and the mixture cooled to 4°C to

precipitate the insoluble potassium dodecyl sulphate, which was removed by

centrifugation (10,000 ×g, 10 minutes). Solid lithium chloride (0.0848g/ml)

was added and the RNA allowed to precipitate overnight at 0°C. RNA was

recovered by centrifugation (10,000×g, 10minutes) and washed twice with

cold 2M Lithium chloride before resuspension in 5ml 0.2M Potassium acetate

pH 5.5. The solution was clarified by centrifugation (10,000×g, 10 minutes)

and the nucleic acid reprecipitated overnight at -20°C by the addition of

2.5 volumes of ethanol.

2.2.46 Formaldehyde gel electrophoresis of RNA.

The method is that of Miller (1988).

A 1.5% high gelling temperature horizontal submarine agarose gel was

prepared by dissolving 1.4g of HGT agarose in 67ml H_2O and 9.3ml

10×MOPS/EDTA (0.5M MOPS, 10mM disodium EDTA pH7.0) then cooling to 70°C

before addition of 17ml Formaldehyde (37%). The gel was cast in a 11×14cm

gel former and allowed to set for at least 1 hour at room temperature. The

gel was subjected to pre-electrophoresis for 3 minutes at 60 Volts in 1×

MOPS/EDTA before loading samples. RNA samples (in 1μl) were prepared for

electrophoresis by addition of 4.4μl of Buffer A (294μl 10×MOPS/EDTA, 706μl

H_2O) and 11.6μl of formaldehyde/formamide (89μl 37% formaldehyde, 250μl

formamide), heating at 70° C for 10 minutes and chilling on ice. 1.5μl of

gel loading buffer was added to each sample before loading. The samples

were electrophoresed for 2 hours at 100 volts with constant circulation of

buffer using a peristaltic pump. Following electrophoresis the gels were

stained in the dark for a maximum of 5 minutes in 5μg/ml EtBr in H_2O. Gels

were then destained in the dark for a maximum of 1 hour in H_2O and

visualised on a UV transilluminator (300nm).

2.2.47 Isolation of winged bean RNA from root tissue

RNA was extracted by homogenisation of tissue in guanidinium thiocyanate (Chirgwin *et al* 1977).

Roots expressing lectin, as determined by haemagglutination assays, were collected from plants grown hydroponically, washed in sterile distilled water, 70% ethanol and sterile distilled water again . 10g of root material was homogenised with 25ml of extraction buffer (4M Guanidine thiocyanate, 25mM sodium citrate pH 7.0, 0.5M sodium N-lauryl sarcosine, 0.1M β-mercapto-ethanol) using a Polytron . The supernatant was clarified by centrifugation at 15,000×g, 4°C for 30 minutes and layered onto 12ml cushions of 5.7M CsCl, 0.1M EDTA pH 7.0 and centrifuged for 24 hours at 30,000×g, 4°C. The supernatant was carefully removed by aspiration and the pellet resuspended in 1ml of 7.5M Guanidine HCl. The suspension was clarified by centrifugation, 6,000×g for 15 minutes at 4°C and the supernatant precipitated at -20°C by addition of 0.025 volumes acetic acid and 0.5 volumes ethanol. The pellet was collected by centrifugation at 9,000×g for 15 minutes at 0°C , washed with 70% ethanol and resuspended in 500μl sterile distilled water. RNA was stored for short periods at -80°C or for longer periods under liquid nitrogen.

2.2.48 Transfer of RNA to nitrocellulose membranes.

The immobilisation of RNA on nitrocellulose was performed in the same manner as per southern blotting (except that no depurination or subsequent neutralisation was required before blotting).

2.2.49 Selection of polyadenylated RNA.

Poly A$^+$ was isolated using oligo (dT)-cellulose in either a batch or column chromatography procedure, according to Maniatis *et al* 1982.

A 1ml column of oligo(dT)-cellulose was washed with DEPC treated water, 0.1M NaOH, 5mM EDTA and then washed again with DEPC treated water until the pH of the column had returned to neutral. The column was washed with five column volumes of sterile loading buffer (20mM Tris-HCl pH 7.6, 0.5M NaCl, 1mM EDTA and 0.1% SDS) and equilibrated in this buffer. Total RNA was dissolved in sterile distilled water, heated to 65°C for 5 minutes, an equal volume of 2× loading buffer was added and the sample allowed to cool to room temperature. The sample was loaded onto the column, the flow through collected reheated to 65°C cooled and reapplied to the column. The column was washed with ten column volumes of loading buffer, followed by four column volumes of loading buffer containing 0.1M NaCl to elute rRNA.

Poly A$^+$ was selected with two to three column volumes of 10mM Tris-HCl pH7.5, 1mM EDTA and 0.05% SDS. The poly A$^+$ was selected again by repeating the procedure after adjusting the sodium chloride concentration of the eluted mRNA to 0.5M. Purified poly A$^+$ was precipitated with 0.3M sodium acetate pH 5.2 and 2.2 volumes of ethanol at -20°C.

2.2.50 cDNA synthesis and cloning.

cDNA was synthesised according to the method of Gubler and Hoffmann (1983) using a Pharmacia cDNA synthesis kit.

Poly A$^+$ RNA was resuspended in DEPC treated water (20μl) and heated to 65°C for 10 minutes and then chilled on ice. This solution was added to a first strand reaction mix containing Murine reverse transcriptase, RNA-guard, RNase/DNase free BSA, Oligo(dT)$_{12-18}$ primer, dATP, dTTP, dGTP and dCTP. The reaction was incubated at 37°C for one hour. The first strand reaction mix

was then added to 100μl of a second strand reaction mix containing RNase-H, *E.coli* DNA polymerase I and dNTPs. The second strand reaction was incubated at 12°C for one hour and 22°C for one hour. 1μl of Klenow fragment was added and the mixture incubated at 37°C for a further 30 minutes. cDNA produced was phenol/chloroform extracted and ethanol precipitated twice.

cDNA was resuspended in 6μl of sterile deionised distilled water and to this solution was added 1μl EcoRI adaptor solution, 1μl T$_4$ ligase buffer (NBL), 1μl dATP solution and 1μl T$_4$ DNA ligase. Following ligation of the adaptors overnight with incubation at 12°C the T$_4$ DNA ligase was denature by heating at 65°C for ten minutes and the DNA phosphorylated (method 2.2.41). Unincorporated adaptors and dATP were separated from the cDNA by gel filtration on Sepharose CL4B (method 2.2.42) the cDNA was phenol/chloroform extracted and ethanol precipitated.

A number of trial ligations of the adaptor linked cDNA with dephosphorylated pUC18 plasmid vector (method 2.2.38, 2.239) were performed at insert to vector ratios of 6:1, 3:1 and 1:1 and recombinants selected for on YT XGAL AMP agar plates.

2.2.51 Standard PCR conditions.

The method is essentially that of Saiki *et al* 1989.

Unless otherwise stated in the text all PCR experiments were performed in the following standard reaction conditions :- 200μM dATP, dTTP, dGTP and dCTP, 50mM KCl, 10mM Tris-HCl pH 8.3, 1.5mM MgCl$_2$, 0.01% (w/v) gelatin, 33μM of each primer, 1μg of template DNA and 2.5U of *Taq* DNA polymerase. Reaction volumes were either 50μl or 100μl. The reaction mixture was overlaid with mineral oil to prevent evaporation. PCR amplifications were performed using a Hybaid 'Intelligent heating block' to control the temperature cycling. The standard temperature/time conditions used in each

amplification reaction were 30 cycles of 92°C/90sec, 45°C/90sec and 72°C/90sec with an initial denauration step of 94°C for 2 minutes, however in attempts to improve specificity these conditions varied and are, therefore, given in the text where appropriate.

RESULTS

CHAPTER THREE

BIOCHEMICAL INVESTIGATION OF WINGED BEAN SEED ANTIMETABOLITES

an indication of the increase in the development period of insects on the

diets; the number of days after oviposition to the first adult emergence

increased from 29 days for the control to 35 and 37 days respectively for

Figure 3.2

The effect of different ammonium sulphate precipitated protein fractions from winged bean seed on the development of *C.maculatus*.

The numbers of adults surviving on artificial diets containing different ammonium sulphate fractions from a total winged bean seed extract at 0%, 2.5% 5% and 7.5% (w/w) incorporation were recorded 42 days after oviposition. Error bars indicate the standard error of the estimate for the number of adults surviving (n=5).
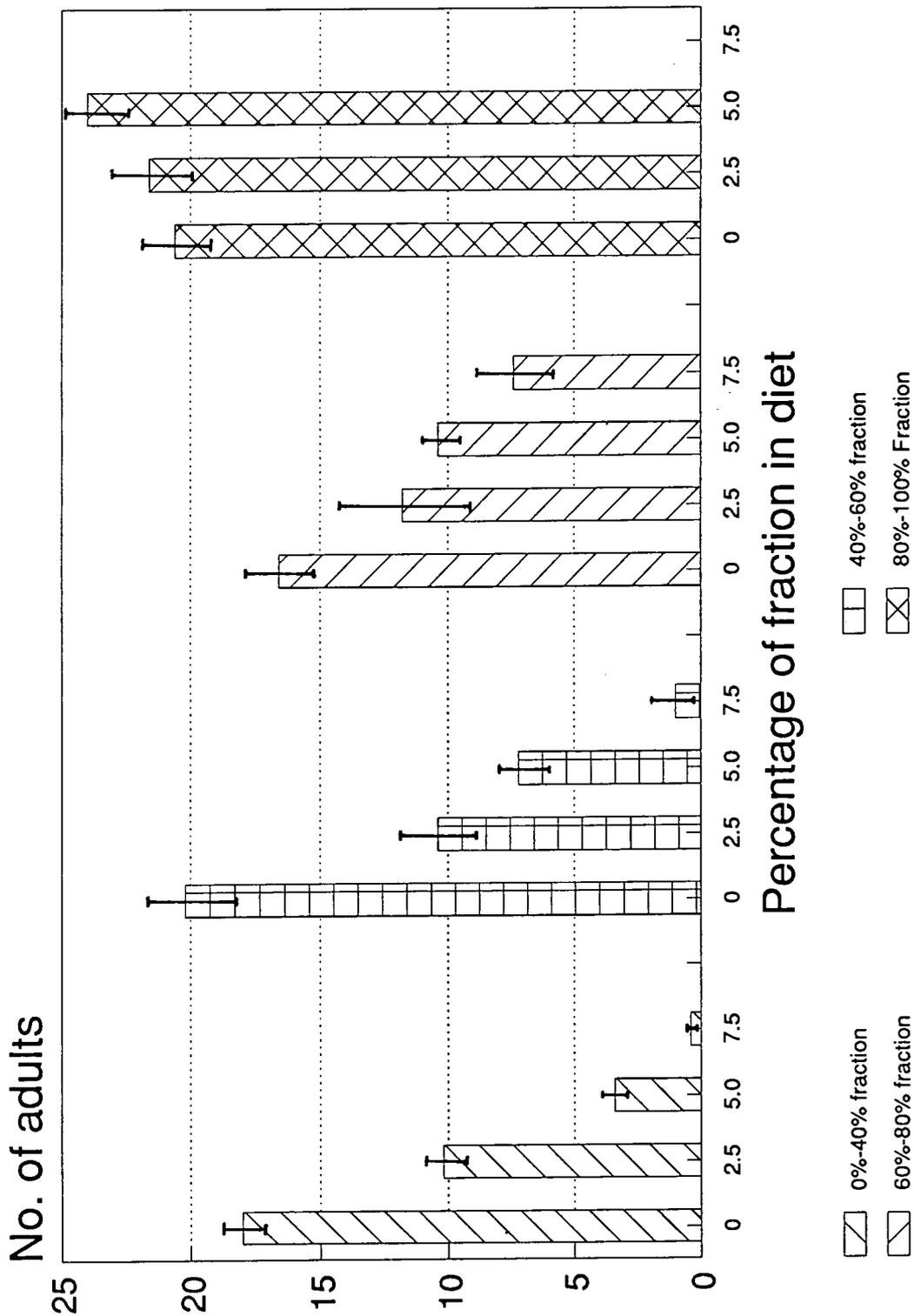
the 2.5% and 5.0% diet. The adults present in the 7.5% diet did not emerge before the termination of the assay on the 42nd day.

The winged bean seed globulin also proved toxic to *C.maculatus* when fed in artificial diets. Figure 3.1b shows the numbers of adults and larvae surviving on diets containing different concentrations of this fraction. The percentage survival of adults compared to that of the control varied from 90% at 2.5% incorporation to 6% at 10% incorporation, again the statistical significance, of a difference in the results for each treatment was confirmed, using the Kruskal-Wallis test ($p>0.01$). The median period of development increased with increased incorporation of the fraction in the artificial diet, the median period of development for each diet i.e. 0% , 2.5% , 5% and 10% increased from 31 to 34, 36 40 and 42 days respectively. The LC_{50} of the albumin and globulin fractions prepared from mature winged bean seeds were estimated at 2.5% and 5% respectively.

In an attempt to further elucidate the protein components causing insect toxicity, total proteins extracted from mature winged bean seeds were fractionated using ammonium sulphate precipitation. Proteins precipitating in the range 0-40%, 40-60%, 60-80%, and 80-100% saturation by ammonium sulphate were used in bio-assays with *C. maculatus*. Results of these bio-assays are presented in figure 3.2.

The 0-40% fraction and the 40-60% fraction were found to be the most insecticidal. Both had similar LC_{50} values of approximately 2.5% (w/w) incorporation although the 40% fraction was slightly more potent. The 80%-100% ammonium sulphate fraction appeared to have little toxic activity and the numbers of larvae surviving to adulthood increased with increasing incorporation of this fraction in the diet. An increased median development period was observed for all diets, at 2.5%(w/w) incorporation

Figure 3.3

Separation of polypeptides present in crude winged bean protein fractions.

Polypeptides present in 20μg samples of four ammonium sulphate fractions and the albumin and globulin fractions used in the insect bio-assays were separated by SDS-PAGE in 17.5% gels. 20μl samples of a 10mg/ml solution of protein extracts in gel loading buffer were boiled ($100^{o}C$, 5 minutes) and loaded onto the gel. Proteins were visualised following electrophoresis (~5 hours at 20mA) by staining with coomassie brilliant blue (R250).

1. 0%-40% Ammonium sulphate fraction
2. 40%-60% Ammonium sulphate fraction
3. 60%-80% Ammonium sulphate fraction
4. 80%-100% Ammonium sulphate fraction
5. Albumin fraction
6. Globulin fraction
7. Total winged bean seed extract.
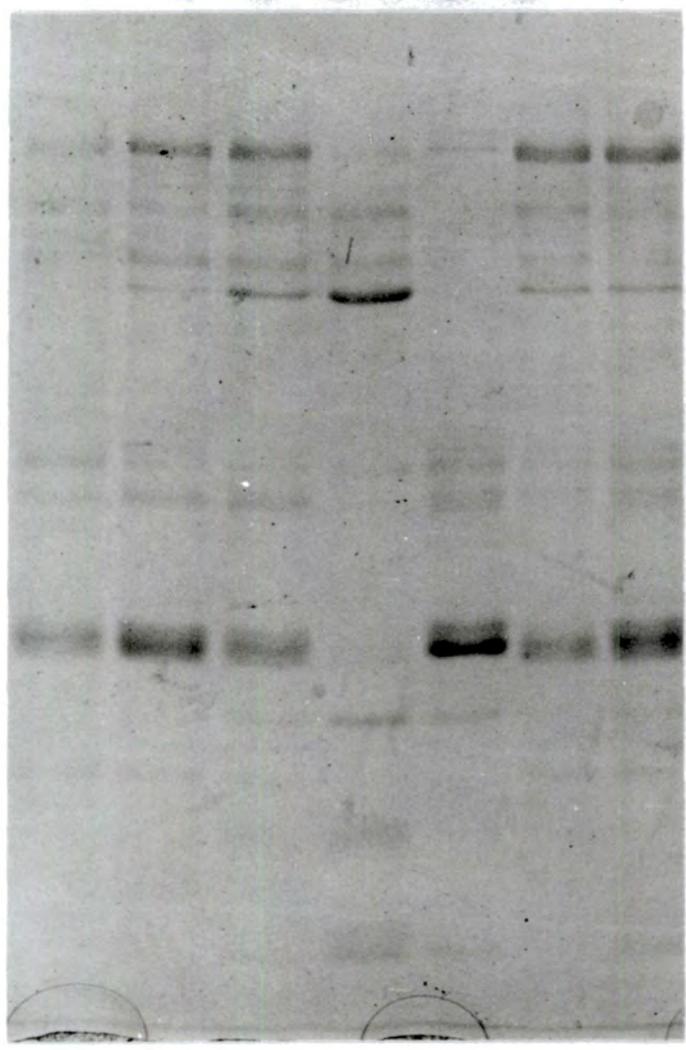
the increase in the median development period was 8% , 8% , 3% , and 5% for the 40% , 60% , 80% and 100% fractions respectively. A Kruskal-Wallis test comparing the the numbers of surviving insects for each diet was statistically significant (p >0.01).

The protein fractions used in bio-assay were analysed by SDS-PAGE, results are shown in figure 3.3. Although differences in the polypeptide compositions of these fractions could be observed , it was apparent that the ammonium sulphate fractionation process was not efficient in separating distinct groups of proteins. The 60% and 80% fractions were very similar, consisting of polypeptides in the range MW 10,000-80,000 the most abundant polypeptides being those around MW 20,000. The 40% fraction had a greater proportion of lower molecular weight polypeptides (MW 40,000- MW 10,000) but the higher molecular weight polypeptides found in the 60% and 80% fractions were still visible. The 100% fraction was deficient in polypeptides in the MW 20,000-40,000 range and the predominant proteins in this fraction were those of MW 40,000 and above, and MW 18,000 and below. This fraction was the least toxic of those tested and therefore the proteins in the range MW 20,000-40,000 were implicated in insect toxicity. The albumin and globulin fractions assayed on SDS-PAGE showed differences in the presence of high molecular weight proteins, these predominating in the globulin fraction. A slightly higher concentration of polypeptides in the MW 20,000-40,000 range was observed in the albumin fraction.

The complexity of the ammonium sulphate and albumin and globulin fractions made it hard to implicate any one protein in the insecticidal effects of the protein fractions. As a consequence, although these assays show that the winged bean seed proteins are insecticidal, they do not

provide more than circumstantial evidence in identifying which proteins are responsible for toxicity.

3.2 Preparation and Bio-Assay of protein fractions Psophocarpin A, B and C

Gillespie and Blagrove (1978) were able to resolve three protein fractions from winged bean seed extracts by electrophoresis on cellulose acetate paper, which were termed psophocarpin A , B and C according to their mobility. The fractions could be partially purified on a basis of differential solubilities; Psophocarpin A was insoluble at pH 7.0 in low ionic strength buffers, Psophocarpin C was insoluble at pH 5.4, whilst Psophocarpin B was essentially soluble at this pH.

Psophocarpins A , B and C were prepared from 10g of hexane defatted winged bean meal according to method 2.2.7. The yields were 70mg, 490mg and 650mg respectively, representing 0.7%, 4.9% and 6.5% of the dry weight of the seeds. Assuming winged bean seeds are on average 25% protein and that 70% of the total protein can be recovered in the initial extraction, one would expect a yield of approximately 1.75g; therefore the fractions account for 1.21g/1.75g $\times$ 100% = 69% of the total seed protein.

The three psophocarpin fractions were incorporated into artificial diets at concentrations of 0% , 1% , 2% and 5% (w/w) and their antimetabolic effects on *C. maculatus* determined by bio-assay.

The results, Table 3.1, show that all three fractions have a deleterious effect on larval development to adulthood and on the median development period of the insect. No adult or larval development was observed on diets containing any of the fractions at concentrations of 5%. No larvae survived to adulthood in diets where psophocarpin A , B and C had been added at concentrations of 2% and above. At the 1% level

Protein fraction	Median development period (days)[a]	No. of larvae g^{-1} diet ±SD	No. of pupae g^{-1} diet ±SD	No. of adults g^{-1} diet ±SD	Percentage of adult surviving relative to control
Control	32.0	0	1.6±1.49	39.2±8.63	100
+1%(w/w) psophocarpin A	37.5	16.0±4.0	1.0±1.0	4.8±2.79	25
+2%(w/w) psophocarpin A	>42.0	5.0±1.0	0	0	0
+5%(w/w) psophocarpin A	>42.0	0	0	0	0
+1%(w/w) psophocarpin B	>42.0	9.6±2.3	0	0	0
+2%(w/w) psophocarpin B	>42.0	0	0	0	0
+5%(w/w) psophocarpin B	>42.0	0	0	0	0
+1%(w/w) psophocarpin C	37.0	16.4±6.98	0.4±0.8	3.2±1.46	13
+2%(w/w) psophocarpin C	>42.0	9.0±4.12	0	0	0
+5%(w/w) psophocarpin C	>42.0	0	0	0	0

Table 3.1

The effect of psophocarpin fractions A,B and C on the development of *Callosobruchus maculatus*.

The median development period, number of adults, number of larvae and number of pupae was recorded 42 days after ovipostion on artificial diets containing 1%, 2% and 5% added psophocarpin fractions.

Figure 3.4

Separation of polypeptides present in 20µg samples of Psophocarpin A,B and C by electrophoresis in 17.5% polyacrylamide gels in the presence of SDS.

1. Psophocarpin A

2. Psophocarpin B

3. Psophocarpin C

4. Total protein extract from winged bean seed.
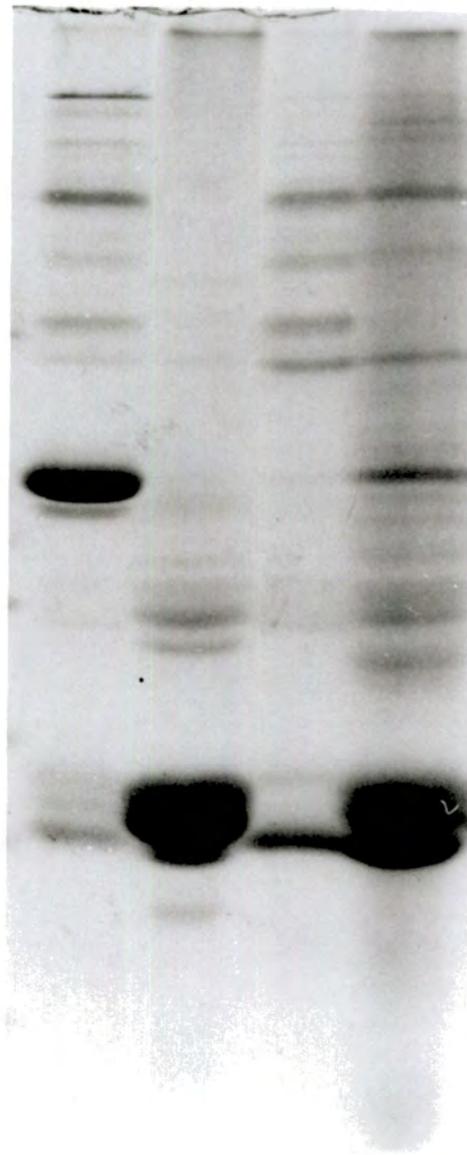
psophocarpins A and C caused a decrease in adult survival of 75% and 87%
respectively and in both cases the median development period for the
insect was increased by 15% i.e. 32 days to 37 days.

Psophocarpin B had the most marked effect on insect development of the
fractions, there was no adult emergence observed after 42 days at any of
the test concentrations. Only the 1% diet had any developing larvae
present. These assays confirmed the antimetabolic character of winged bean
seed proteins and identified Psophocarpin B as the most toxic fraction.

3.3 Analysis of Psophocarpins A, B and C

When assayed on SDS-PAGE differences in polypeptide compositions of
these samples were observed (Figure 3.4). Psophocarpin A consisted
predominantly of a 40,000 M.W. polypeptide, whilst other polypeptides in
this fraction include those of M.W.s 66,000 , 50,000 and 20,000. The most
abundant polypeptides present in psophocarpin B ranged between 20,000 and
22,000 M.W. whilst approximately 10% of the Psophocarpin B fraction
consisted of polypeptides in the range 27,000 to 34,000 M.W. Few
polypeptides were observed with M.Ws >35,000 in this fraction. Psophocarpin
C contained approximately equal proportions of polypeptides of approximate
M.Ws 20,000, 45,000, 50,000, 57,000 and 60,000.

3.4 Assays for toxic proteins

Psophocarpins A, B and C were further assayed for known protein
antimetabolites. Haemagglutination assays carried out according to method
2.2.1 confirmed an abundance of both the winged bean basic and acidic
lectin in the psophocarpin B fraction in comparison to either psophocarpin
A or C (Table 3.2).

	Haemagglutination titre*	
Fraction	Human O$^+$	Rabbit
Psophocarpin A	8	0
Psophocarpin B	128	8
Psophocarpin C	16	2

Table 3.2

Haemagglutination titres for protein fractions psophocarpin A, B and C against untrypsinised human O$^+$ and rabbit erythrocytes as determined by serial dilution in microtitration plates with a 2% suspension of the appropriate erythrocytes. (Method 2.2.1)

* Maximum dilution of fraction before loss of activity / 100µg protein

Figure 3.5

The mean percentage of trypsin inhibition activity present in psophocarpin fractions A, B and C, as determined by trypsin inhibition assays. Error bars indicate standard error , n = 5 .

Percentage inhibition

Microgramme of fraction
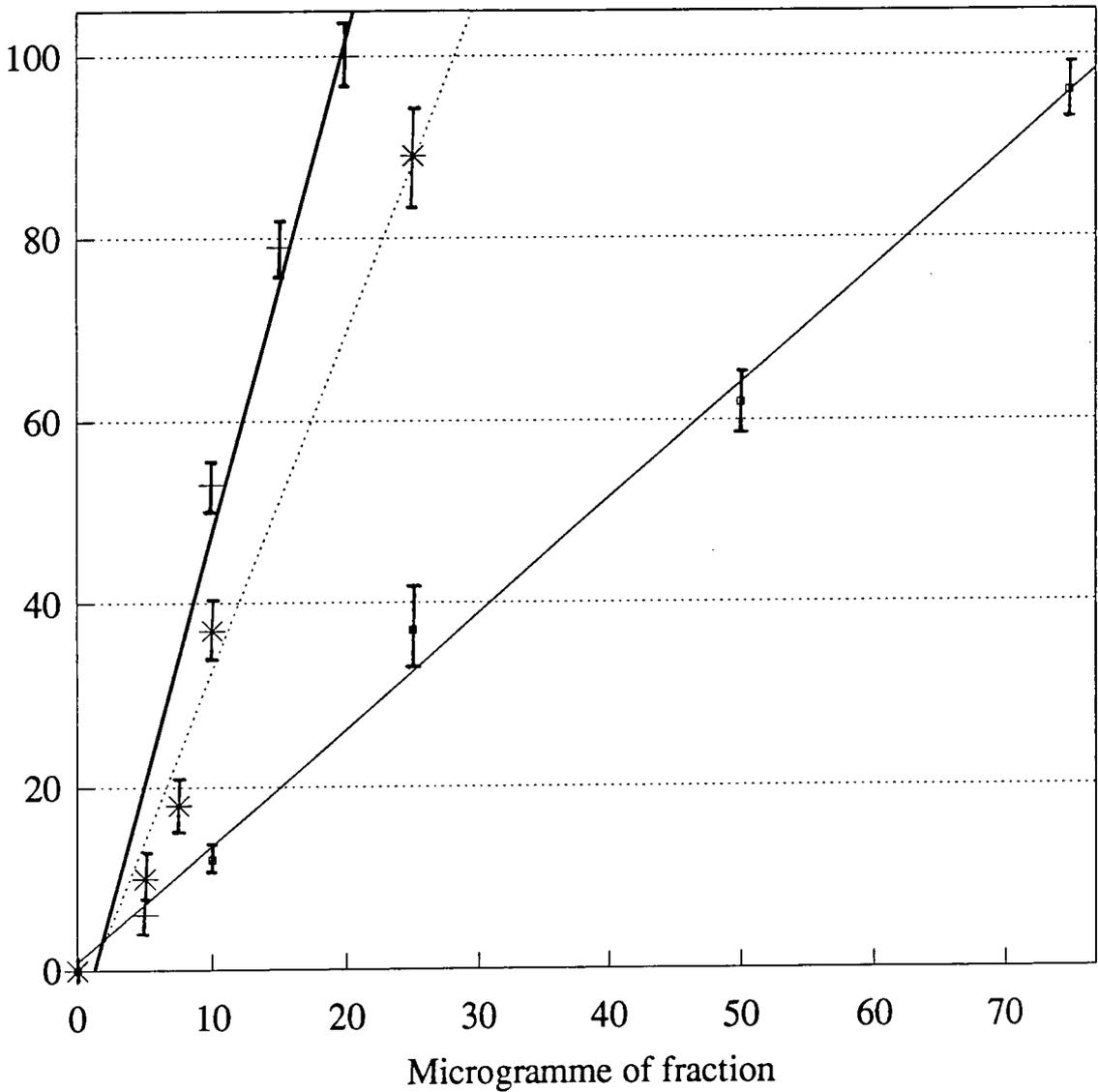
—■— Psophocarpin A   ＋ Psophocarpin B

··✳·· Psophocarpin C

Fractions psophocarpin A,B,C and a total winged bean extract were assayed for α-amylase inhibitory activity against porcine and *Bacillus* α-amylases and against gut extracts from locust and *Spodoptera* using the appropriate pH assay buffer (Method 2.2.4). Neither the total winged bean seed extract nor the psophocarpin fractions had any detectable inhibitory activity on any of the α-amylase activities tested, even at α-amylase to fraction ratios of 1 :100. The lack of polypeptides in these fractions in the range 10-15,000, as observed by SDS-PAGE, is further evidence for the lack of α-amylase inhibitors within the seeds of winged bean.

The psophocarpin fractions were assayed for protease inhibitory activity. Trypsin inhibitor assays carried out according to method 2.2.2 confirmed a predominance of trypsin inhibitor in the psophocarpin B fraction with a $I_{50} = 12\mu g$ of protein fraction, compared to $15\mu g$ and $41\mu g$ of protein fraction for psophocarpin C and A respectively (figure 3.5).

Chymotrypsin inhibitor activity in these fractions paralleled that of the trypsin inhibitors with psophocarpin B having the greatest activity $(I_{50}=10\mu g)$, followed by psophocarpin C $(I_{50}=17\mu g)$ and A $(I_{50}=38\mu g)$.

3.5 Purification and bio-assay of winged bean trypsin inhibitor

The trypsin inhibitors of the mature winged bean seed were purified from a protein extract by affinity chromatography on a column of bovine trypsin coupled to Sepharose 4B . Binding and washing of the extract was done at pH 4; subsequent elution of the inhibitors took place at pH 2. The fraction were desalted on a column of Sephadex G-25 and freeze dried. The resultant protein fraction was tested for haemagglutination activity, and chymotrypsin inhibitory activity. No haemagglutination activity was

detected toward either human or rabbit erythrocytes using the microtitration plate method. Chymotryptic inhibitory activity was detected at very low levels (200 fold excess of WBTI : chymotrypsin for inhibitory activity). Analysis of the fraction revealed two major polypeptide components, one of MW 20,000 and at MW 9,000 ± 1000 (This latter component is not present in the winged bean seed protein extract and is likely to be an artifact of the purification process, (see discussion).The yield of trypsin inhibitor using this method was 1.25mg/g seed meal.

The trypsin inhibitors purified in this way were offered to *C.maculatus* at concentrations ranging from 0% to 5% in artificial diets . The results, Table 3.3 , indicate that far from being toxic the addition of the protein to the diet has caused a decrease in the developmental period and an increase in the number of larvae surviving to adulthood at 42 days. 134% survival on diets containing 5% added winged bean trypsin inhibitor was observed compared to the control diet (taken as 100% survival) and there was a decrease in the median development period of 10%.

3.6 Purification of winged bean lectins

One of the roles suggested for lectins is in insect defense, and both *Phaseolus* and black bean lectins have proved insecticidal (Janzen *et al* 1978, Gatehouse *et al* 1984). Winged bean lectins themselves have been studied in relation to mammalian toxicity.

The role of lectin in the resistance mechanisms of the winged bean seed has been implicated from the results of feeding trials using crude extracts and the lack of toxicity of purified winged bean trypsin inhibitors. The toxicity of the albumin, globulin and psophocarpin

Protein fraction	No. of days to first adult emergence	No. of larvae g^{-1} diet ±SD	No. of pupae g^{-1} diet ±SD	No. of adults g^{-1} diet ±SD	Percentage of adults surviving relative to control
Control	40	28.0±11.84	12.40±4.38	14.2±0.1	100
+0.5%(w/w) WBTI	38	12.4±5.95	11.55±3.36	18.7±1.89	111
+1.0%(w/w) WBTI	37	39.3±8.4	10.50±2.0	20.1±3.05	102
+1.5%(w/w) WBTI	37	27.0±0.75	6.65±2.3	15.5±7.5	63.3
+2.0%(w/w) WBTI	38	28.0+2.75	10.35±2.3	19.0±1.3	100.7
+2.5%(w/w) WBTI	37	24.8±2.75	8.40±0.96	15.1±8.9	108.6
+3.0%(w/w) WBTI	37	22.2±9.8	11.75±2.92	20.9±4.6	133.5
+5.0%(w/w) WBTI	36	20.6±3.2	8.40±0.03	23.1±4.7	134.2

Table 3.3

The effect of winged bean trypsin inhibitor on the growth and development of *Callosobruchus maculatus*.

The median development period, number of adults, number of larvae and number of pupae was recorded 42 days after ovipostion on artificial diets containing 0.5%-5.0% added winged bean trypsi inhibitor.

fractions mirrors their lectin content. Affinity chromatography methods were used in an attempt to purify both acidic and basic lectin separately in large enough quantities, in order that they could be used in insect feeding trials. A number of methods using affinity chromatography media have been developed for the isolation of winged bean acidic and basic lectins. The method employed was that of Kortt and Caldwell (1985). Analysis of results was done using a combination of SDS-PAGE and spectrophotometry at 280nm.

Sieved defatted seed meal (1g) was extracted in PBS and the extract was sequentially applied to columns containing, the affinity resins immobilised melibiose and immobilised lactose, designed to the bind basic and acidic lectin respectively (method 2.2.10). 1ml samples were collected during elution of the affinity columns with 0.3M galactose buffer and those with positive O.D. readings in comparison to the control were pooled and TCA precipitated according to method 2.2.17, before analysis by SDS-PAGE. No bands representing lectin could be observed using this method. Yields of approximately 2.5mg g^{-1} acidic and basic lectin are expected from winged bean seeds and thus a total O.D. increase in the eluted buffer from each column of approximately 2.5 units was expected, but this was not observed. A total O.D. increase of 0.088 units was observed which represents 88μg of protein, however since there were no bands observed on SDS-PAGE it appears that this increase in O.D. may be due to other factors .

Haemagglutination assays of initial washes of both affinity columns in the absence of galactose confirmed that a great majority of the lectins were in fact passing straight through the columns and that binding was not taking place efficiently.

It was possible that a direct protein extract was high in factors

Figure 3.6

SDS-PAGE analysis of protein extracts eluted from immobilised melibiose. 100µl samples of extracts, taken before and after elution from the affinity resin immobilised melibiose, were precipitated using trichloroacetic acid, washed and resuspended in SDS-loading buffer and subsequently analysed by SDS-PAGE.

1. Extract passed through column

2. Initial wash of column

3. Winged bean seed extract applied to column

4. Fraction 1 eluted with 0.3M galactose

5. Fraction 2 eluted with 0.3M galactose

6. Fraction 3 eluted with 0.3M galactose

7. Fraction 4 eluted with 0.3M galactose

8. Fraction 5 eluted with 0.3M galactose
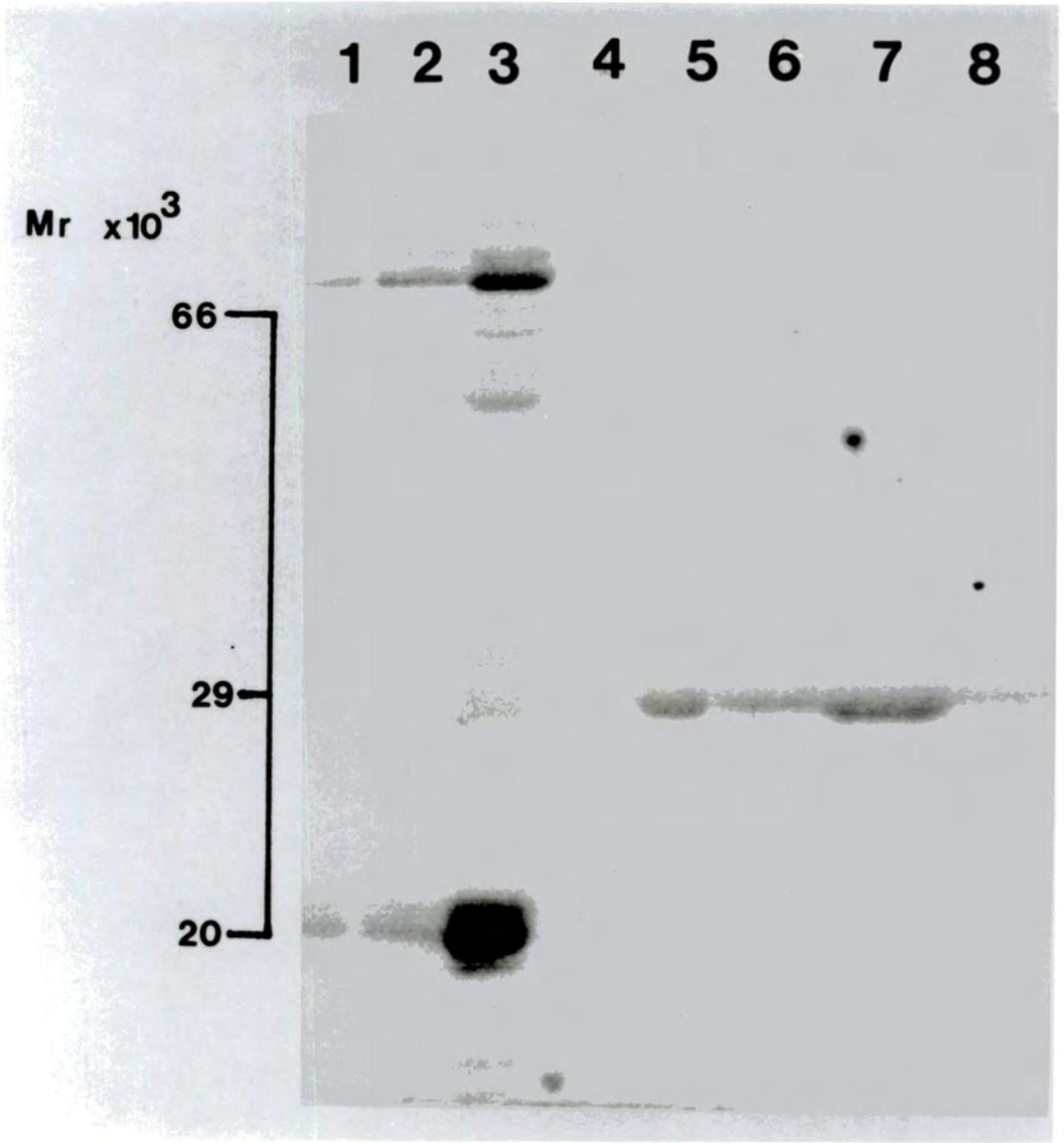
inhibiting binding , for example sugars or polysaccharide . It was decided to use an ammonium sulphate precipitate, collected at 95% saturation after resuspension and dialysis in the column loading buffer used (in this case PBS).

Binding, of the basic lectin, was much improved on the immobilised melibiose column (as determined by haemagglutination assays with rabbit blood, of extract applied and eluted from the column) and lectin eluted with 0.3M galactose in PBS could be observed on SDS-PAGE gels following TCA precipitation of samples (see figure 3.6). The yields were, however, poor (approx. 600μg/g seed meal) in comparison to previously published results (Kortt 1985, Higuchi *et al* 1983 , Puepkke 1979). As basic Lectin obtained from this affinity purified system was homogeneous, as determined by SDS-PAGE and N-terminal sequencing, it was used later used in protein sequencing experiments, (see chapter 7) and in the production of antibodies used in western blotting experiments (see chapter 5). No binding of the acidic lectin was observed using the immobilised lactose column under these conditions. A further attempt to purify the acidic lectin using a column of immobilised N-caproyl-galactoseamine using the same extraction, binding and elution conditions also proved unsuccessful. The seemingly anomalous binding of both lectins from this winged bean cultivar made it difficult to purify large amounts of them, by affinity chromatography, for use in insect bio-assays.

The lectin fractions (acidic and basic) used in the insect bioassays were separated from other seed proteins on a basis of size and charge using gel filtration and ion exchange chromatography.

Crude samples of acidic and basic winged bean lectins were purified from 300mg of Psophocarpin B by gel filtration using Sephacryl S-200 resin

Figure 3.7

Chromatography of psophocarpin B on Sephacryl S-200 (190×2.6cm) in 0.05M Tris-HCl, 0.1M NaCl pH8.0 . 150mg of lyophilised protein was dissolved in the same buffer and applied to the column, the flow rate was adjusted to 15ml/hr and 5ml fractions were collected. Haemagglutination titres of each fraction were obtained by serial dilution of each fraction in microtitration plates with a 2% suspension of rabbit (basic lectin) and human O^+ (acidic lectin) erythrocytes.
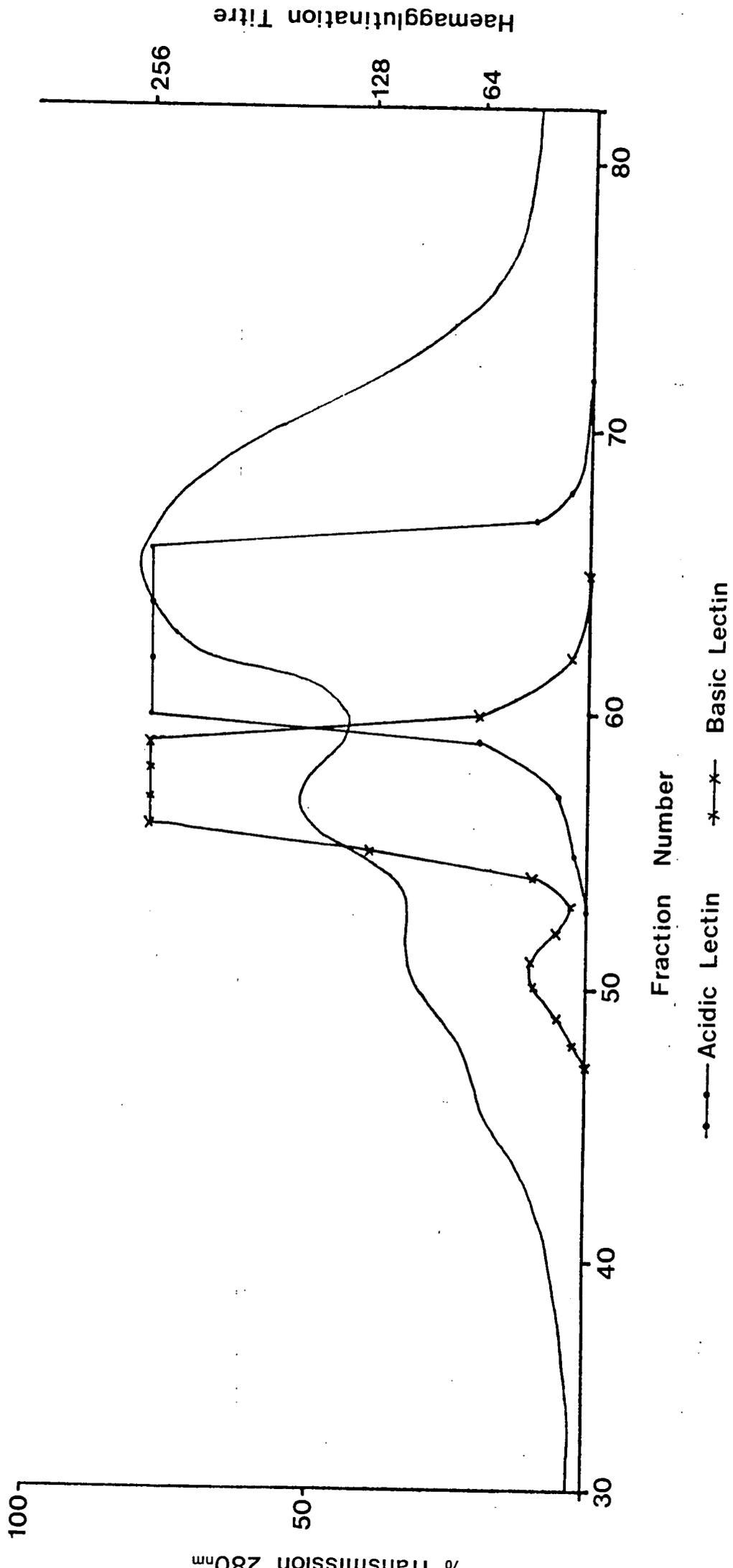
(see method 2.2.9). The resulting chromatogram and the haemagglutination activity of each fraction towards both rabbit and human O^+erythrocytes (figure 3.7) shows the four major peaks that were eluted and that the acidic and basic lectin activity occurred in fractions between the third and fourth peaks. Fractions were pooled with as little overlapping activity as possible. The results obtained from this gel filtration experiment were reproducible and very similar to those of Kortt (1985) who used a similar procedure to separate psophocarpin proteins by molecular weight. This procedure was repeated a number of times to isolate sufficient acidic and basic lectin fractions for insect bioassay. 300mg of psophocarpin B typically yielded 15mg and 25mg of basic and acidic lectin fractions respectively.

SDS-PAGE analysis and amino-terminal sequencing of the two fractions revealed that they were both contaminated by the winged bean trypsin inhibitors (trypsin inhibitor assays also confirm this) but the majority of the contaminating component as determined by N-terminal sequencing was from WBA-1, the winged bean seed albumin. The acidic lectin fraction may contain as high as 80-90% contaminating polypeptides. The basic lectin is only slightly contaminated, with approximately 5% trypsin inhibitor/WBA-1, as determined by relative intensities of N-terminal DABTH amino acid derivatives.

An attempt was made to further purify the acidic lectin fraction obtained from Psophocarpin B using ion exchange chromatography.The method was essentially that of Kortt and Caldwell (1985) (see method 2.2.11). 30mg of an acidic lectin fraction obtained after gel filtration of psophocarpin B on Sephacryl S-200 was applied to a cation exchange column and a 1000ml gradient of 0.01M-0.1M citrate buffer was used to elute bound protein.

Figure 3.8

Ion-exchange chromatography of an acidic lectin fraction on SP-Sephadex.
20mg of an acidic lectin fraction (see figure 3.7) was applied to a
SP-Sephadex C-25 column equilibrated in 0.01M citrate buffer pH5.1,
the column was washed with this buffer and bound proteins eluted with
a gradient of citrate buffer 0.01M-0.1M pH5.1. Flow rate was 20ml/hr
and 5ml fractions were collected.

Fraction Number
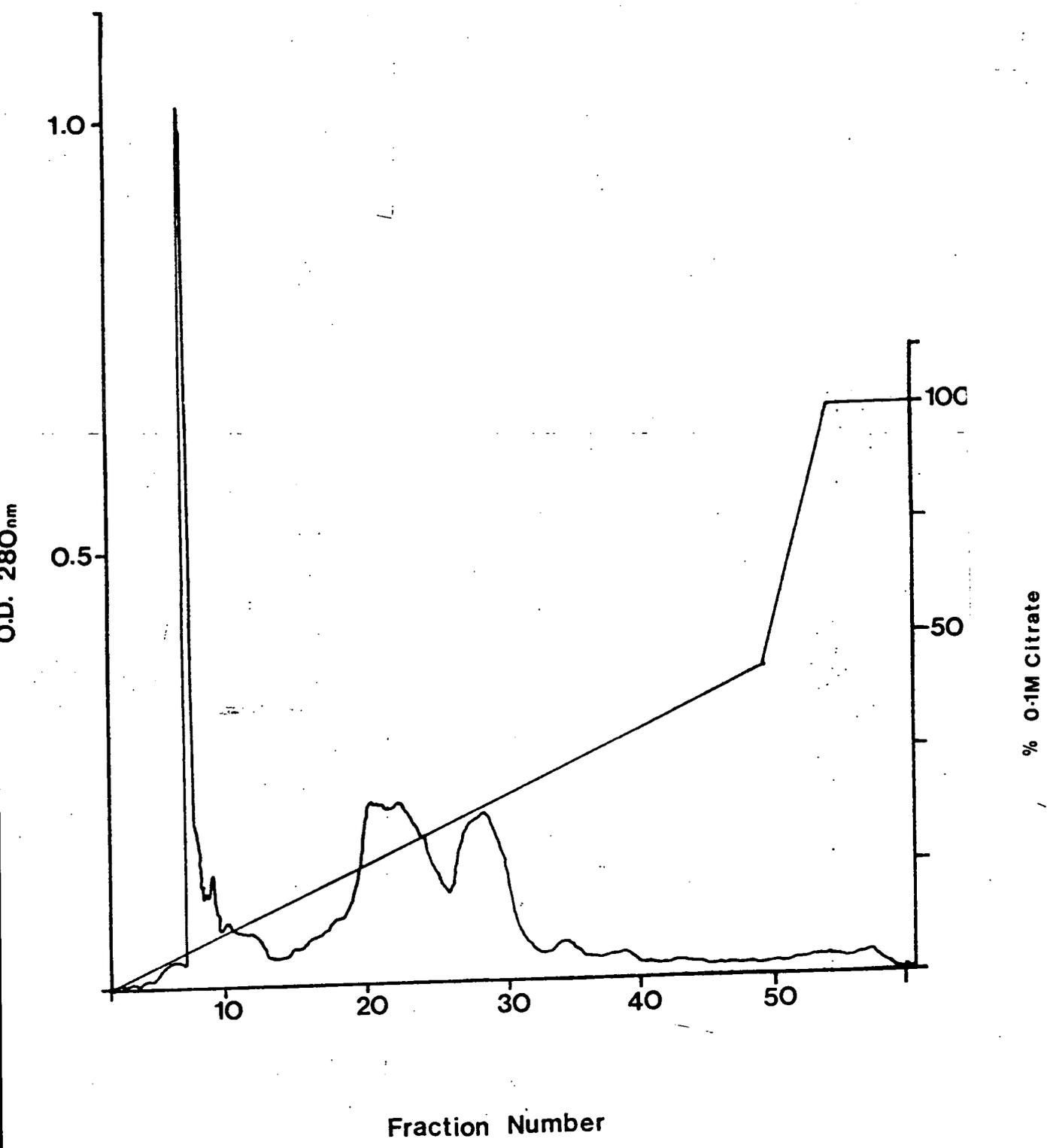
Figure 3.8 shows a typical elution profile obtained. The acidic lectin is eluted very early on in the gradient (at approx. 0.02M citrate) as determined by haemagglutination assay of the fractions using human O^+ erythrocytes. A similar effect was noticed by Kortt (1985); however he found the bulk of lectin activity eluted much later on the gradient as two peaks. No such result was observed in this or repeated experiments, which suggests a weak affinity of the acidic lectin due to an altered pI value for this protein, or an abundance of the acidic lectin isoform I and the absence of other isoforms in this variety of winged bean. With the low yield of the acidic lectin and the impurities still present in the fraction obtained by ion exchange it was decided to use the acidic lectin without further purification.

3.7 The effect of Winged Bean lectin on Larval development of *C.maculatus*

The physiological concentrations for the acidic and basic lectins within mature winged bean seed have been estimated at 0.27% and 0.34% respectively (Kortt 1985, Higuchi *et al* 1984); with this in mind both types of lectin, purified from psophocarpin B by gel filtration on S-200, were offered to *C.maculatus* larvae at 0%, 0.1%, 0.2% and 0.5% (w/w)concentrations in the artificial beans. Insect emergence and total numbers of adults surviving were monitored up to 42 days after oviposition.

The basic lectin fraction had a very detrimental effect upon adult emergence, with adult emergence decreasing with increased lectin in the diet, (figure 3.9a). An LC_{50} value of approximately 0.35% was estimated for the basic lectin. This lectin fraction also had an effect on the

Figure 3.9a

The effect of winged bean basic lectin on the development of

Callosobruchus maculatus.

The number of surviving adults was recorded 42 days after oviposition on an artificial diet containing 0%, 0.1%, 0.2% and 0.5% lectin. The number of adults surviving per gram of diet is indicated as is the standard error of estimate (n=5).

Figure 3.9b

The effect of winged bean basic lectin on the emergence of

Callosobruchus maculatus.

The total number of adults emerging from artificial diets containing basic lectin at 0%, 0.1%, 0.2% and 0.5% was recorded each day after oviposition. The average number of insects per gram of diet is indicated.

A

No. of insects

Percentage of Basic lectin in diet

⊠ Adults

B

No. of insects

Days after oviposition

- Control
+ 0.2% Basic lectin
* 0.1% Basic lectin
⊟ 0.5% Basic lectin
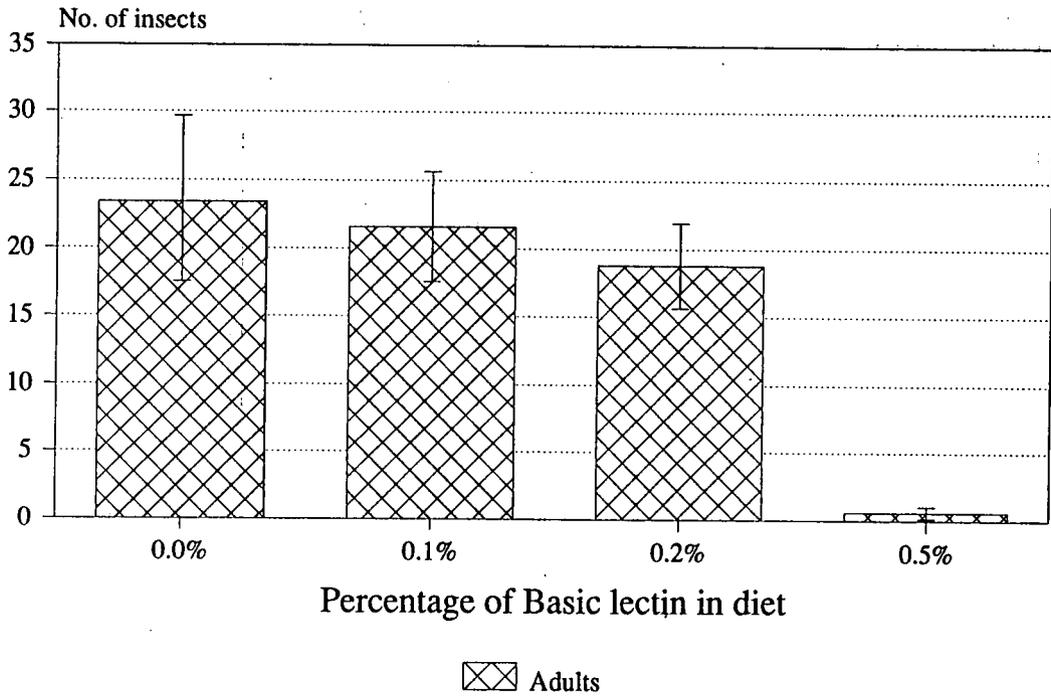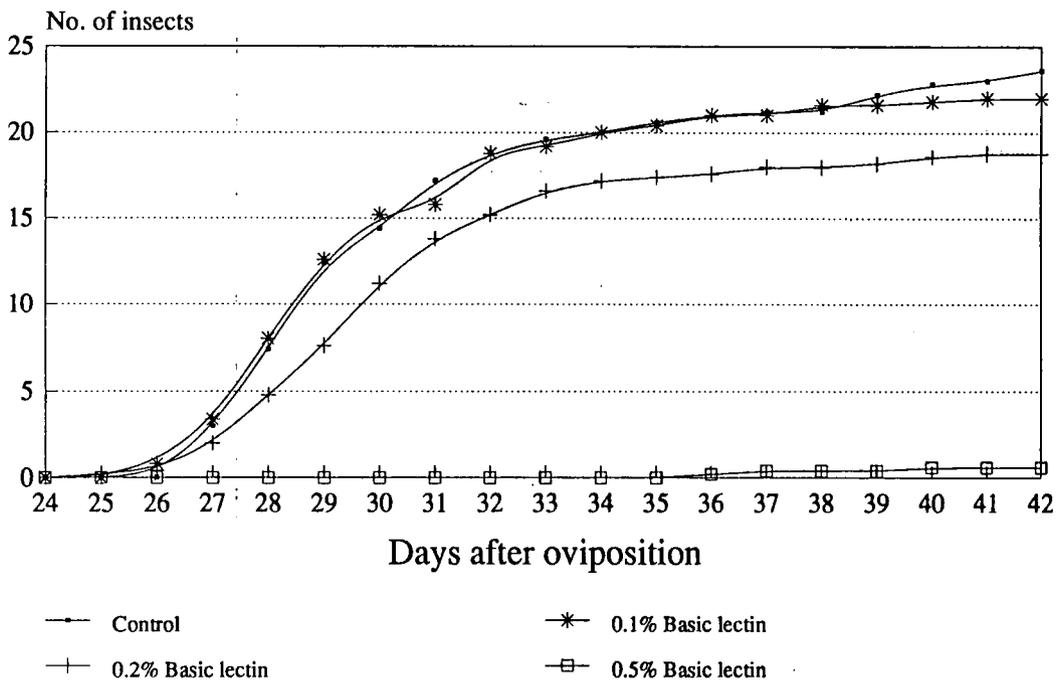
developmental period of *C.maculatus*, with a 26% increase in the median development period at 0.5% incorporation in the artificial diet figure 3.9b. There was a significant difference between the emergence of adults from different diets as determined by a Kruskal-Wallis test ($p > 0.01$).

The acidic lectin fraction had little effect upon both survival and developmental period of *C.maculatus*, at the highest concentrations of incorporation (0.5%). No significant difference could be observed when the test diets were compared to the control diets, however the relative concentration of acidic lectin in this fraction was ten fold lower than that of the basic lectin.

CHAPTER FOUR

LEPIDOPTERAN BIOASSAY AND DEVELOPMENT OF DIETS

4.1 Bioassay of Lepidoptera

The winged bean has proved resistant to a number of Coleopteran pests i.e.
Callosobruchus maculatus, C.chiensis, C.analis, C.phaseoli, C.rhodesians,
Zabrotes subfasciatus and *Acanthoscelides obtectus* (Dobie *et al* 1979). The
winged bean seed lectins are likely to be the antimetabolites involved in
this resistance, as has been shown to be the case with *C.maculatus.*

Bioassays were conducted with two economically important Lepidopteran
pests i.e. tobacco budworm (*Heliothis virescens*) and the armyworm
(*Spodoptera littoralis*) with a view to determining the possible use of
winged bean lectins as biological controls of other insect pests,
particularly those of the order Lepidoptera.

Initially bioassay using total winged bean seed meal was performed
using *H.virescens* as the test organism. The performance of the larvae
reared on a standard artificial diet containing haricot bean meal (2.2.22)
was compared to that of an experimental diet in which the haricot bean meal
constituent had been replaced by winged bean seed meal. Five first instar
larvae were inoculated onto eight 10g replicate diets of the standard
artificial diet (control diet) and the diet containing winged bean meal
(test diet). Diets were placed in plastic pots in a controlled
temperature/humidity room (70% relative humidity, 23°C). Insect survival
was recorded after 24 hours. 30%±15% of the total number of larvae reared
on the control diet survived compared to 7.5%±9.6% on the test diet. The
statistical significance of this result can not be analysed using
standard parametric techniques as the survival of Lepidopteran larvae on
artificial diets does not follow a normal distribution. The non-parametric
test used in this case was the two sample Mann-Whitney U test, the null
hypothesis being that there is no difference in the survivorship of the
insects feeding on the two different diets . The value obtained, U=41, did

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not fall within the critical regions at the 2%, 5% or 10% levels and hence the null hypothesis was accepted the difference in the mean survival of the larvae on these two diets was not statistically significant. However, the low survival of control larvae may mask any effect of the winged bean seed proteins.

A similar experiment comparing the survivability of Spodoptera larvae, on the control and test diets used above, was performed. In this case ten first instar larvae were inoculated onto eight replicates of the two diets. The diets were placed in a controlled humidity/temperature chamber as before and the number of larvae surviving after 24 hours was determined. 65%±20% of the larvae on the control diet survived compared with 20%±16% on the test diet incorporating the winged bean seed meal. A two sample Mann-Whitney U test confirmed that in this instance there was a statistical difference (p<0.02) between the survivorship of S.littoralis feeding on the two different diets, suggesting that the winged bean seed meal was detrimental to this insect's development.

To determine whether the toxic factor in the winged bean meal was heat labile, a bioassay was conducted in which winged bean seed meal was compared to winged bean seed meal that had been autoclaved ($130^{o}C$ for 60 minutes) and subsequently dried in an oven ($103^{o}C$, 12 hours) . These two components were used in artificial diets, as before, in place of the haricot bean meal constituent. Third instar larvae were used in order that differences in larval biomass between diets could be monitored. Survival of the larvae on these two diets were very similar, after ten days the mean survival rates were 54%±23% and 57%±26% for the winged bean diet and the autoclaved winged bean diet respectively. The development of the larvae on the two diets was, however, significantly different. The average biomass of larvae reared on the diet containing autoclaved winged bean meal was

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approximately four fold larger than than that of the larvae reared on the diet containing untreated winged bean seed meal (1.80g±1.36g compared to 0.475g±0.35g). A Mann-Whitney U test confirmed the statistical significance of this result (p<0.02). This suggested that a heat labile factor may be responsible for the seed meal's antimetabolic effect. The winged bean seed meal and the autoclaved seed meal were assayed for lectin activity with trypsinised rabbit erythrocytes; titres of 2^1 and 2^8 were recorded for 20mg/ml extracts.

It was noted that there was an increased viability of the older Lepidopteran larvae reared on the winged bean diets in comparison to the first instar larvae. This was investigated further. Five fourth instar larvae were allowed to develop for 3 days on eight replicates of control, winged bean meal and autoclaved meal diets. The original biomass placed on each of these diets had similar means and variance (1.2g±0.293g, 1.2g±0.44g ,1.23g±0.252g) and a Kruskal-Wallis analysis confirmed that the initial distribution of biomass between these diets contained no significant differences. Insect survival over this period was 100% for all diets. The development of these larvae as determined by mean biomass of the larvae differed slightly from diet to diet, those on the control diet being more successful (1.94g±0.52g) than those reared on the autoclaved diet (1.88g±0.308g) and the untreated winged bean diet (1.78g±0.395g). Statistical analysis of these results using the Kruskal-Wallis test for different treatments suggested that the differences in means were not significant and that in fact the biomass was evenly distributed throughout the diets. The late instar larvae were therefore able to develop more successfully on the winged bean diet than the early instar larvae.

Development of Insect Bioassays.

Large quantities of material were required for the use of *in vivo* assays using the standard, rearing, diet and therefore these tests were suitable, mainly, for bean meals and direct protein extracts. These assays are based on optimal diets and may mask the effect of deleterious proteins which exert chronic rather than acute effects. A number of other systems, which may be used to detect and study the toxic effects of insecticidal proteins were investigated. Lepidopteran larvae will feed on Glass fibre filter discs (Whatman GFC) and these have been used by some workers to determine a number of insect feeding deterrents in 24 hour bioassays. Communion hosts have also been used as minimal diets in these assays(Gatehouse A.M.R personal communication). An initial experiment was performed to determine the survival of 3rd instar larvae on these two diets . One larva was placed on each of ten replicates of GFC, Host and no diet (control) media. The larvae on the the GFC discs and no diet control had 100% mortality after 48 hours whilst 90% of the larvae on the host diet survived. After 4 days the survival on hosts was 80% , and at 6 days this had dropped to 20%.

A host based system was used to assay the winged bean fractions psophocarpin A, B and C. A positive control of PHA and a negative control of host only were used to monitor the assay. Protein fractions were incorporated at 5% by absorption of a solution of the respective protein in sterile distilled water. The discs were then dried under vacuum and allowed to equilibrate for 7 days at 70% R.H. and 23°C. Second instar larvae of *Heliothis virescens* were placed on each of 10 hosts and their survival recorded after 3 days. At this stage 9/10 control larvae survived compared to 10/10 for PHA, 3/10 psophocarpin A, 5/10 psophocarpin B and 7/10 psophocarpin C. Psophocarpin A therefore appeared to be the most toxic

fraction followed by psophocarpin B and C. This suggested that there may be a winged bean seed protein that could be exploited as an antimetabolite against Lepidoptera, and that winged bean lectins may not be the most active antimetabolites against these insects.

4.2 The development of natural bioassays for Lepidoptera

In assays using communion hosts there was a constant drop in insect biomass despite their relative longevity on this type of diet. This drop in weight is not desirable as it shows that the larval mortality may be affected by starvation or water loss when using this type of diet, and the toxic effect of the proteins added may be masked by these factors. A diet that reflected the natural diet of the Lepidopteran more closely and on which the larvae were able to develop and increase in weight was considered to be a more sensible means of assaying the potential of any compound as an antimetabolite of insects in a natural environment.

The natural diet of both *Heliothis* and *Spodoptera* includes the leaves of the tobacco plant. A diet of tobacco leaves suspended in agar base was tested. Tobacco leaves taken at random from a Petite Havana cultivar were ground under liquid nitrogen and lyophilised. These leaves were then incorporated into a 2% (w/w) agar medium at 10%(w/w) and allowed to set in sterile 90ml petri dishes. Because of the potential for bacterial infection/ fungal growth, chlorotetracycline was added at a concentration of 150μg/ml to a control set of diets to access its potential as an inhibitor of fungal growth. The toxicity of this compound could also be tested. Survival of five second instar *Spodoptera* larvae on eight replicates of the the tobacco, tobacco and tetracycline, and a 2% agar diet were monitored over a 10 day period. After 24 hours the survival on each diet was 48%, 60% and 32 % respectively. This decreased to 35% , 47.5% and

12% after 5 days, and to 10%, 27.5% and 2% after 10 days. The biomass of insects on each diet containing tobacco increased slightly over the first 2 days then remained constant for 7 days before dropping. A number of diets without added antibiotic became infected by molds and survival of insects on these was severely limited. Some diets containing antibiotic also became infected but larvae appeared to survive better on these. The high water content of these diets led to problems of condensation in the storage containers, and larval growth was reduced in these diets compared to larvae on plants. Lack of leaf material prevented the development of these diets further but this type of system would clearly be useful in directly assaying transgenic plants expressing antimetabolites in leaf tissue.

CHAPTER FIVE

CONSTRUCTION OF cDNA AND GENOMIC LIBRARIES

5.1 Construction of a winged bean cDNA library

An efficient means of isolating a given gene is via the screening of a cDNA library. Although this does not give the gene itself, if successful it yields a cDNA probe which can be used to isolate clones from a genomic library with high efficiency. The library is prepared from total mRNA extracted from tissue which most abundantly expresses the desired gene's protein, since the number of clones in the library containing the required cDNA sequence should be directly proportional to the percentage of total mRNA represented by the specific sequence sought, and by inference, the proportion of total protein expressed in that tissue. In the case of the winged bean lectins, the developing seed cotyledons express lectin at the highest percentage compared to any other tissues of the plant. Estimates of seed lectin expression vary from 1.7% to >2.5% total protein (Higuchi and Iwai 1985, Kortt and Caldwell 1985), and it therefore follows that libraries of the order of 1000 clones would be sufficient to ensure inclusion of at least one lectin cDNA clone in the library produced from total developing seed mRNA.

Maximum lectin expression in developing winged bean seeds occurs around 60 days after flowering (Higuchi *et al* 1988); seeds were harvested at this point, and the testas removed, and the cotyledons frozen under liquid nitrogen and stored until further use at -80°C. A haemagglutination assay was performed using a single cotyledon, to ascertain that the seeds collected were expressing lectin. A haemagglutination titre of 2^{4} was recorded using untrypsinised rabbit erythrocytes, using a 20mg/ml extract.

An attempt to purify total RNA from frozen cotyledon tissue was made using the hot SDS method (2.2.45). The resulting RNA showed severe degradation when run on a formaldehyde gel. It was noticed that during the homogenisation step of this procedure that there was very little frothing

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as is normally observed at this stage, with pea cotyledons for example, suggesting that insufficient detergent to denature endogenous RNAses had been present. An alternative method (2.2.47), using guanidinium thiocyanate to prevent ribonuclease activity, was used with greater success. 5g of cotyledon tissue yielded approximately 500μg of intact RNA as determined by formaldehyde gel electrophoresis. This total RNA was used to isolate polyA$^+$ mRNA via oligo dT affinity chromatography. The yield of polyA$^+$ after two rounds of chromatography was approximately 2μg of RNA.

In a control experiment 1μg of pea cotyledon polyA$^+$ was used to prepare cDNA using a commercial cDNA synthesis kit (Pharmacia). 50ng of the resulting cDNA was blunt end ligated into HincII restricted pUC18, and transformed into competant *E.coli*. Recombinant clones were selected for on a blue/white basis on YT XGAL AMP agar plates. 115 white colonies and 425 blue colonies were observed. Minipreparations of plasmid DNA from 12 white colonies followed by restriction analysis revealed 9 plasmids with inserts between 200bp and 540bp. The number of clones obtained per μg of polyA$^+$ RNA was approximately 500. This experiment was repeated in an attempt to increase the yield , using EcoRI adaptors to clone the blunt ended cDNA fragments into the EcoRI site of pUC18. 30ng of phosphorylated adaptor linked cDNA was used in a ligation reaction with 150ng of dephosphorylated EcoRI restricted pUC18. 50 white colonies were observed after selection on YT XGAL AMP agar plates. The size of the inserts of the resulting recombinants was not determined, but the number of recombinants per μg cDNA using this system was approximately 1700.

1μg of winged bean polyA$^+$ RNA was used to prepare cDNA using the commercial cDNA synthesis kit as before. The second strand synthesis was monitored by measuring the incorporation of ^{3}H deoxythymidine in TCA precipitable material. It was estimated that approximately 400ng of cDNA

was produced. EcoRI adaptors were ligated to the winged bean cDNA and phosphorylated using T_4 polynucleotide kinase. Excess adaptors were removed from the cDNA by column chromatography on DNA grade Sephadex G50. 50ng of adaptor linked cDNA was ligated to 200ng of dephosphorylated EcoRI restricted pUC18 vector overnight at $15^{\circ}C$. The ligation mixture was diluted five fold with T.E. buffer and a proportion was used to transform competant *E.coli* DH5α. A number of white colonies (recombinants) were detected after selection on YT XGAL AMP agar plates. None of those tested had plasmids containing inserts. The large number of white colonies with no inserts suggested the incorporation of adaptor dimers. The separation of excess adaptors in a repeat experiment was done using Sepharose CL4B. The background of white colonies due to adaptors was reduced dramatically. The number of clones containing cDNA inserts in this experiment was low i.e. 3, this represented a total library size of 30 clones; this was not enough to obtain a lectin cDNA.

Lack of developing cotyledon material prevented the isolation of more of mRNA from this source. The level of expression of lectin in the mature roots of winged bean is comparable to that of seeds (72 haemagglutination units/mg cotyledon protein in comparison to 82 haemagglutination units/mg root protein). Total RNA was prepared from mature (6 months) roots of winged beans grown hydroponically (0.44g Phostrogen/litre). 10g of roots were washed in sterile water and RNA purified using the guanidinium thiocyanate method as before. 250μg of total RNA was prepared, and appeared to be undegraded as determined by visualisation on ethidium bromide stained formaldehyde agarose gel (Figure 5.1). PolyA$^+$ RNA was prepared via a batch method using oligo dT cellulose (the yield was not determined). The polyA$^+$ was used for cDNA synthesis; ^3H labelled dTTP was added to the first strand synthesis and the presence of cDNA was confirmed by determining TCA

Figure 5.1

Formaldehyde gel electrophoresis of winged bean root RNA. 1μg and 10μg
of total RNA, extracted from mature winged bean root tissue using a
guanidinium isothiocyanate buffer (2.2.47), was analysed by
electrophoresis through formaldehyde agarose gel (100volt, 2 hours)
and visualised with staining in ethidium bromide (5μg/ml for 5
minutes).

1. 10μg winged bean root total RNA
2. 1μg winged bean root total RNA
3. 10μg pea leaf total RNA
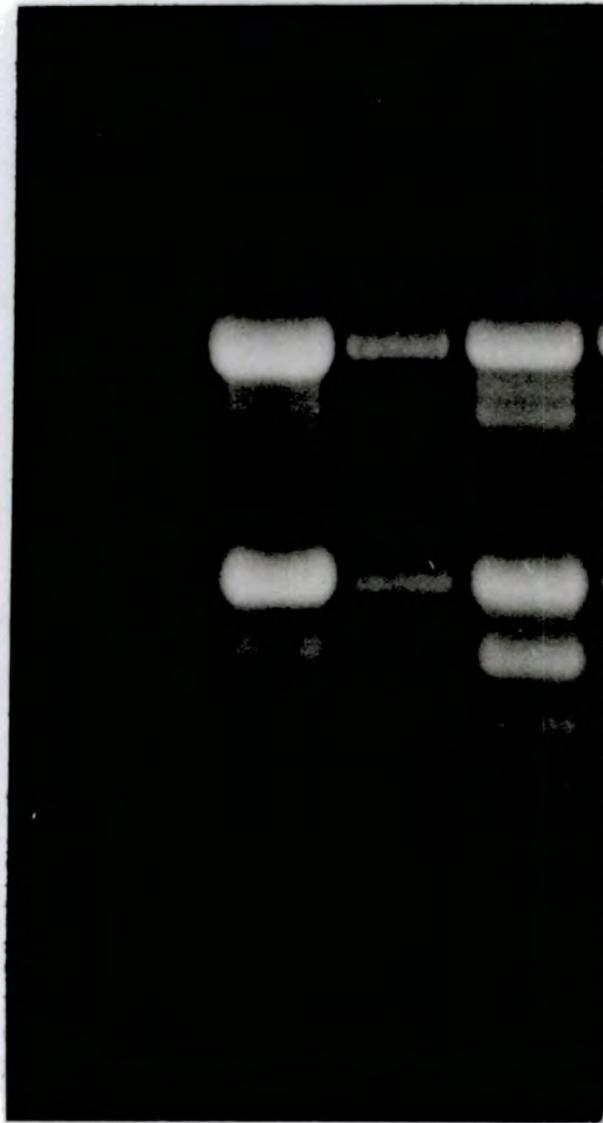
Figure 5.2

Detection of lectin idiotypes present in tissues of winged bean plants using rabbit anti-basic lectin antibodies. 20mg/ml extracts of tissue were were electrophoresed on on SDS polyacrylamide gels and blotted onto nitrocellulose filters. The filters were washed with rabbit anti-lectin antibodies and subsequent binding was monitored using horse radish peroxidase goat anti-rabbit antibodies and diamino-benzoic acid.

A) Stained polyacrylamide gel

1. Autoclaved seed meal extract

2. Seed meal extract, post harvest

3. Pod tissue extract, 50 d.a.f.

4. Tuber tissue extract, 6 month old plant

5. Stem tissue extract, 30 week

6. Leaf tissue extract, 30 week

7. Protein molecular weight markers (SDS-7 Sigma Co.)

B) Above gel after blotting and probing with anti-basic lectin antibodies

1. Autoclaved seed meal extract

2. Seed meal extract, post harvest

3. Pod tissue extract, 50 d.a.f.

4. Tuber tissue extract, 6 month old plant

5. Stem tissue extract, 30 week

6. Leaf tissue extract, 30 week
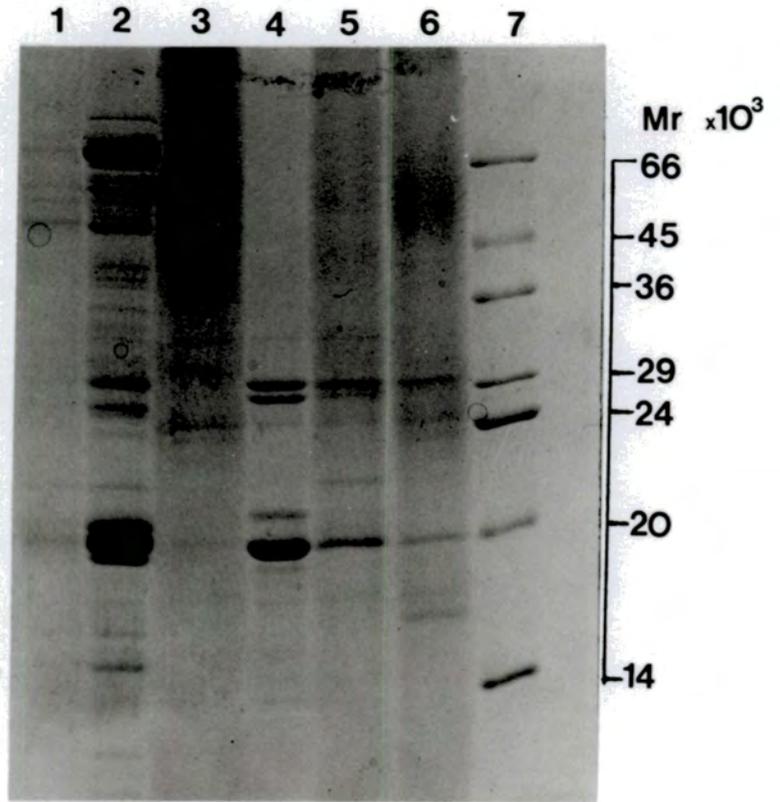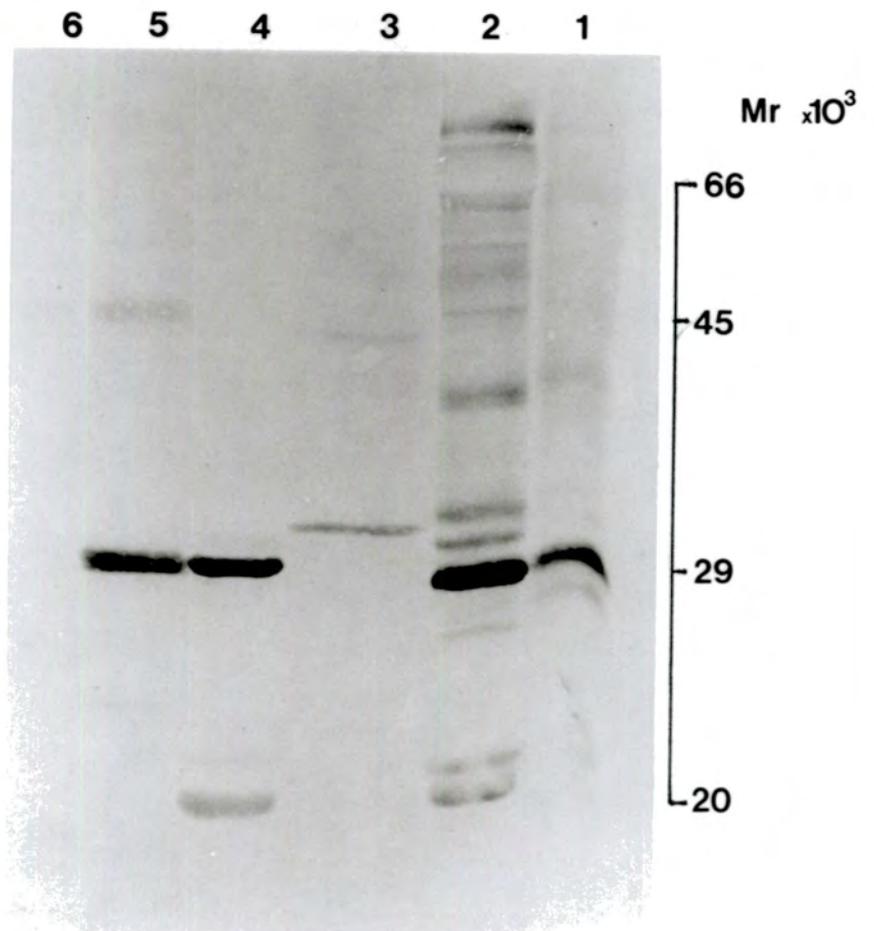
precipitable β emissions. It was estimated that approximately 100ng of cDNA was prepared. Adaptor linked cDNA was ligated with pUC18 vector as before and recombinants screened for on YT XGAL AMP plates. No recombinant clones were obtained in experiments using cDNA prepared from winged bean root material.

5.2 Expression of winged bean lectin

Shet and Madaiah (1987) have assayed the expression of lectin within different tissues of the winged bean plant at different stages of development using haemagglutination techniques, whilst Higuchi and co-workers (1988) have used both haemagglutination and immunoprecipitation techniques to assay the expression of lectin in developing seeds and pods of the winged bean. These studies suggested that the level of lectin expression is highest in seed, mature root and pod. Little or no expression of lectin was determined in other tissues. Western blotting studies were performed using antibodies raised against affinity purified basic lectin (see chapter 3) in an attempt to determine whether other tissues (particularly leaf tissue) were expressing lectin at lower levels than than those that could be determined using haemagglutination assays. Protein extracts from mature leaf, stem, pod were assayed alongside an extract from winged bean cotyledon. No lectin could be detected in leaf tissue using this method, whilst the antibodies bound to proteins (MW~30,000) from extracts of pod, seed and stem (figure 5.2). No lectin expression was detected in leaves taken at different stages of plant development (10 days to 6 months). Experiments on the expression of lectin during the germination of winged bean seeds demonstrated that the anti-basic lectin antibodies did not bind to proteins in extracts from young shoot root or stem tissues from which RNA extraction is made easier by the lack of lignified tissue.

Figure 5.3

Detection of basic lectin in the germinating seeds of winged bean
using rabbit anti-lectin antibodies. Cotyledon extracts were taken
from germinating winged bean seeds at two day intervals from 0-20 days
after imbibition. 20μl samples were analysed by SDS-PAGE and blotted
onto nitrocellulose before detection of lectin using rabbit anti-basic
lectin antibodies and Horse raddish linked goat anti-rabbit
antibodies.

A) Stained polyacrylamide gel

1. Seed extract four days after imbibition

2. Seed extract eight days after imbibition

3. Seed extract six days after imbibition

4. Seed extract two days after imbibition

5. Seed extract zero days after imbibition

6. Protein molecular weight markers (SDS-7, Sigma Co.)

B) Above gel after transferring to nitrocellulose and probing with
anti-basic lectin antibodies

1-5. As above
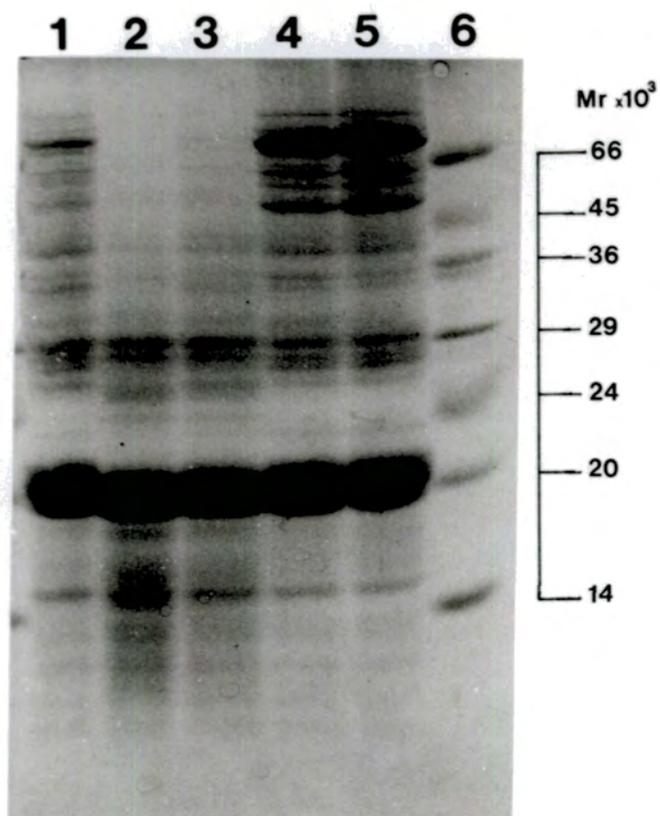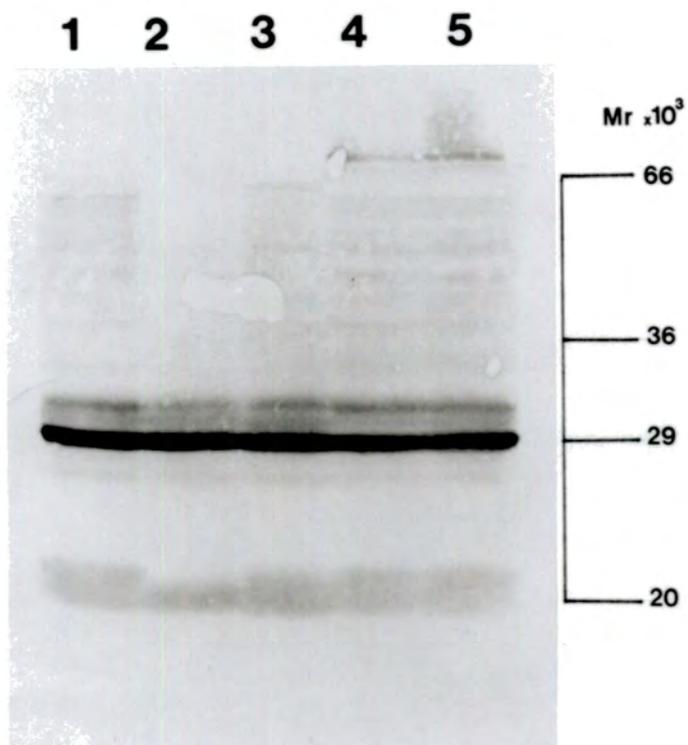
While using anti-basic lectin antibodies, to detect basic lectin during the germination of seeds, it was interesting to observe that the lectin present in the mature seeds before imbibition appeared to remain intact in the cotyledons throughout the germination of the seed over the twenty day time period for which they were assayed (see figure 5.3a, 5.3b). This is evidence that the lectin may serve another role rather than that of a storage protein.

5.3 Construction of a winged bean genomic library.

The lack of suitable tissue from which to isolate lectin mRNA, made it necessary to consider the isolation of the lectin gene from a genomic library.

The size of the winged bean genome is unknown. The winged bean is however considered to be related to soybean, and they both have similar chromosome counts (i.e. 22). By extrapolation they may both have similar genome sizes at around 4×10^9 bp per haploid genome.

The method of construction of the library was based on the method of Frischauf and co-workers (1983) where size selection of insert DNA is negated by the use of a phosphatase step. The vector EMBL3 was used, which accepts DNA inserts between 9kb and 23kb and therefore a library of approximately 2×10^6 clones is required in order to be within 90% confidence limits that a single copy gene from this genome will be represented.

Winged bean DNA was prepared from young leaf tissue from the same plant, using diethyldithiocarbamate as a DNA nuclease inhibitor, (method 2.2.31). 4g of leaf tissue yielded 800μg of high molecular weight DNA which could be restricted by a number of common restriction endonucleases. Further preparation of the winged bean DNA involved partial digestion with Sau3A followed by phosphatasing of the resultant DNA fragments. 10μg of high molecular weight DNA was used in a pilot scale digestion with Sau3A

over a 100 minute period (2.2.40) , aliquots were taken from the reaction at 10 minute intervals for analysis on 0.4% agarose gel, see figure 5.4. A digestion time of 60 minutes appeared to yield the highest mass of DNA in the 23kb-9kb size range, as determined by relative fluorescence. The modal average length DNA of the 23kb-9kb size range was therefore present in the 30 minute digest as predicted by Seed and co-workers (1982). Partial digestions (10μg) were carried out for 20, 30 and 40 minutes, under the same conditions of the pilot scale reaction, the DNA was phenol/chloroform extracted, ethanol precipitated and resuspended in distilled water before dephosphorylation with calf intestine alkaline phosphatase. These DNA fractions were assayed for fragments of the correct length by electrophoresis on 0.4% agarose gel as before. The 20, 30 and 40 minute fractions appeared similar to those observed in the pilot scale reaction.

Ligation assays were performed on the insert DNA before and after dephosphorylation. Before dephosphorylation insert DNA was capable of ligating to its self , as observed by visualisation of ligation products on 0.8% agarose gel. The integrity of the DNA termini was demonstrated by cloning low molecular weight de-phosphorylated winged bean DNA into pUC18 plasmid vector restricted with BamHI, a number of white, recombinant clones were observed against a background of non recombinant blue colonies.

Vector DNA (EMBL3) was restricted with BamH1 and EcoRI and the stuffer fragment was removed by differential precipitation of the vector arms using Isopropanol. Trial ligations of vector and insert DNA from the 30 minute partial digest were performed in 10μl reaction volumes at 12°C overnight in the ratios 0.25:1, 0.5:1 , 1:1 , 2:1, and 4:1 insert DNA to vector, 1μg of vector DNA was used in each case. Ligated DNA was packaged using a freeze/thaw packaging extract (2.2.27). 10ng of control wild type concatenated λ DNA was also packaged to monitor packaging efficiency. 10μl

Figure 5.4

Partial restriction of winged bean genomic DNA with the enzyme Sau3A. 10 aliquots were taken from a 100μl restriction digest reaction at ten minute intervals, the reaction stopped by addition of EDTA and heating at 68°C for 10 minutes and the products electrophoresed on a 0.4% agarose gel.

1. 10 minutes after reaction initiation
2. 20   "   "   "   "
3. 30   "   "   "   "
4. 40   "   "   "   "
5. 50   "   "   "   "
6. 60   "   "   "   "
7. 70   "   "   "   "
8. 80   "   "   "   "
9. 90   "   "   "   "
10. 100   "   "   "   "
11. DNA size markers (λDNA restricted with EcoR1)
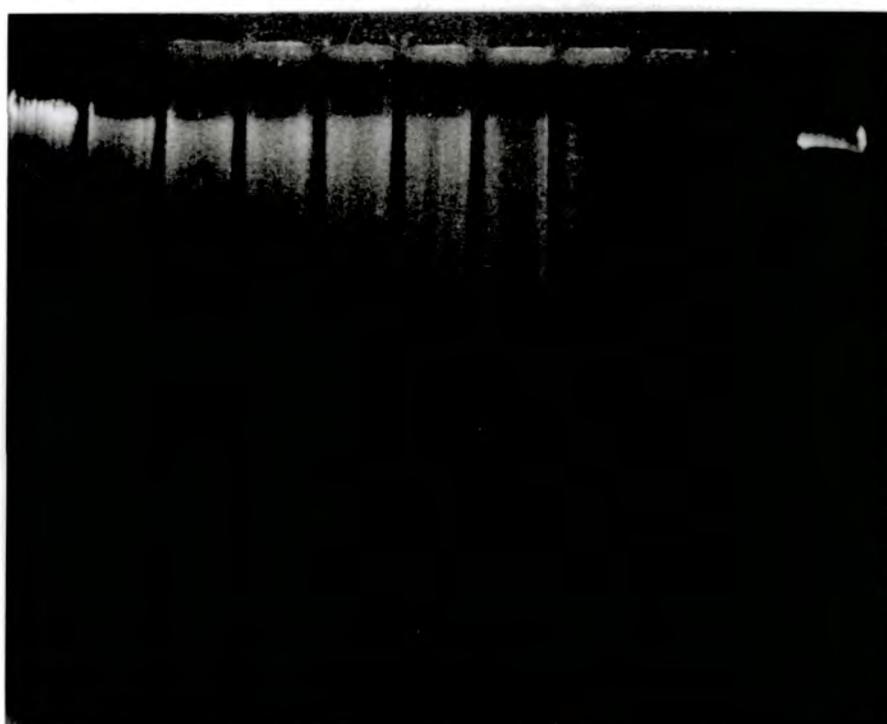
Ligation Ratio insert:vector	Sau3A Digest Time (min)	Permissive Host LE392	Restrictive Host P2392
control	N/A	2356	0
0.25:1	30	0	0
0.5:1	30	0	0
1.0:1	30	0	1
2.0:1	30	10	3
4.0:1	30	16	19
control		2016	0
4.0:1	30	42	19
6.0:1	30	22	23
control		2155	0
4.0:1	40	9	2
2.0:1	40	77	46
control		2289	0
2.0:1	20	0	0
3.0:1	20	0	0
4.0:1	20	0	0

Table 5.1

Numbers of plaque forming units obtained from 10 μl aliquots of *in vitro* packaged ligation reactions on restrictive and permissive *E.coli* host strains.

aliquots of packaged phage were used to transfect *E.coli* strains LE392 and P2392. The results are presented in table 5.1. In each packaging experiment the control λDNA was packaged successfully, and the efficiency was in the order 2×10^8/μg DNA. At ligation ratios below 2:1 insert to vector only one recombinant plaque was observed. At ratios of 2:1 and 4:1, 3 and 19 recombinant plaques were observed with a background of 7 and 3 non-recombinant plaques respectively. 2.4×10^3 pfu / μg insert DNA were therefore obtained in this trial ligation experiment. A 100-1000 fold increase in yield was required to produce a library large enough to screen for a single copy gene. A series of trial ligations were carried out using DNA from the 30 minute and 40 minute digests, at ligation ratios of 4:1 and 6:1 . The ligated DNA was packaged as before and plated out on permissive and restrictive *E.coli* host strains LE392 and P2392. The results are shown in table 5.1 . At a ratio of 4:1 insert:vector DNA digested for 30 minutes produced a similar number of plaques when compared to the previous trial ligation i.e. 19 plaques, but the number of non-recombinant plaques was greater, at 23. At a ratio of 6:1 insert to vector slightly more recombinant clones were observed, 23 whilst the number of non-recombinant clones was negligible. A ligation using the 40 minute DNA digest fraction produced the highest number of recombinant plaques. Forty six plaques were observed, however this was against a background of thirty one non-recombinant plaques. Approximately 5.7×10^3 pfu / μg of insert DNA were obtained in this experiment. The total number of recombinant clones obtained in these experiments was 1.13×10^4 which represents approximately 5-10% of the total winged bean genome. A final attempt to increase the size of the library used DNA from the 20 minute digest. This was ligated at ratios of 2:1, 3:1 and 4:1 insert to vector DNA, 1μg of vector DNA was used. No plaques were observed on either the permissive or restrictive host strains.

CHAPTER SIX

ANALYSIS OF HETEROLOGUS PROBES

6.1 Heterologus DNA probes

Any winged bean library that was produced was to be screened with an oligonucleotide probe that was designed using the N-Terminal amino acid sequence data obtained by Kortt (1984) and confirmed by manual sequencing of the isolated lectin protein. The screening of libraries with such probes is difficult at the cDNA level and even more so when attempting to screen genomic libraries due to high background hybridisation signals (Maniatis *et al* 1982). Ideally a library should be screened with a second probe to confirm cross hybridising colonies/plaques. Three lectin clones from *Pisum sativum*, *Phaseolus vulgaris* and *Glycine max* were obtained for use as heterologous probes to detect the winged bean lectin genes.

6.2 Pea Lectin

Samples of high molecular weight winged bean genomic DNA (20μg) were restricted with the enzymes EcoRI and BglII, and the products electrophoresed on 0.8% agarose gels along side 10μg of pea genomic DNA restricted with EcoRI (Figure 6.1). The DNA was transferred to nitrocellulose by Southern blotting. The nitrocellulose filters were prehybridised for approximately 2hrs at $42^{o}C$ in prehybridisation buffer (method 2.2.43).

The pea lectin probe used was a 1.2 Kb HindIII/EcoRI fragment isolated from the plasmid pDUB128. This fragment contains the entire coding sequence for the pea lectin. 50ng of this DNA was random prime labelled using the BRL random prime kit and 50μCi of dCTP (specific activity >4000Ci/mmol). The probes produced had a specific activity $>5 \times 10^{8}/\mu g$. The nitrocellulose filters were allowed to hybridise with freshly boiled probe in

Figure 6.1a

Agarose gel electrophoresis of restricted winged bean and pea genomic DNA. 20μg samples of winged bean DNA were restricted with EcoRI and BglII and electrophoresed alongside 10μg of pea genomic DNA digested with EcoRI. The gel was stained, photographed and the DNA was transfered to nitrocellulose by Southern blotting.

1. DNA size markers, λDNA restricted with HindIII

2. 20μg Winged bean DNA restricted with EcoRI

3. 20μg Winged bean DNA restricted with BglII

4. 10μg Pea genomic DNA restricted with EcoRI

5. DNA size markers, λDNA restricted with HindIII

Figure 6.1b

Hybridisation of pea *LecA* to winged bean and pea genomic DNA.

A 1.2Kb fragment containing the pea lectin coding sequence was labelled with ^{32}P-dCTP and hybridised to winged bean genomic DNA and pea genomic DNA immobilised on nitrocellulose. Hybridisation was at 42°C , subsequent washing was at 50°C in 5×SSC. Filters were autoradiographed for one week at -80°C.

1. DNA size markers, λDNA restricted with HindIII

2. 20μg Winged bean DNA restricted with EcoRI

3. 20μg Winged bean DNA restricted with BglII

4. 10μg Pea genomic DNA restricted with EcoRI

5. DNA size markers, λDNA restricted with HindIII

A

1 2 3 4 5

Kbp
— 14.1
— 6.4
— 4.3
— 2.3
— 1.3

B

1 2 3 4 5

Kbp
— 16·9
— 8·3
— 4·0
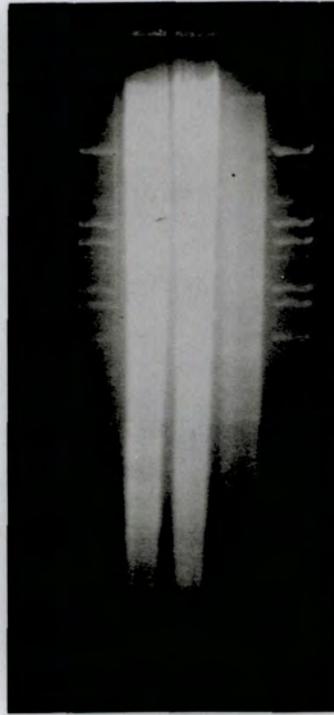
hybridisation buffer for 20hrs with shaking at 42^oC . After hybridisation the filters were washed in twice in 5×SSC at 42^oC for 25 minutes and then autoradiographed. Subsequent washes and autoradiography were done at 50^oC and 60^oC in 5×SSC. Non-specific binding of the probe to both winged bean and pea DNA occurred under the low stringency washing conditions of 42^oC and 5×SSC. At a wash temperature of 50^oC non-specific binding was still observed, but this was only, faint hybridisation to a band >16Kbp in the winged bean genomic DNA that had been digested with BamH1 (see figure 6.1b). At 65^oC and under washing conditions of 5×SSC the pea lectin probe bound specifically to EcoR1 fragments (4.0Kb, 8.3Kb and >16Kb) in the pea genomic DNA, but there was no hybridisation to DNA from winged bean . The 16kB and The 4.0kb bands observed at higher stringency washes in pea genomic DNA represent the pea lectin gene and an unknown related gene (Gatehouse *et al* 1987)

6.3 Phaseolus lectin.

20µg samples of winged bean genomic DNA were restricted with the enzymes BglII, EcoR1 and Xba1 respectively, the products were electrophoresed on a 0.8% agarose gel as before and Southern blotted. Positive controls of 10pg, 25pg and 50pg of the 900bp Pst1 restriction fragment from the plasmid pPVL134 (which contains the PHA lectin-like sequence) were also present on this gel and blot. The 900bp lectin coding fragment was used as a probe and 200ng was labelled to a specific activity of $2.85×10^9$ dpm/µg using the random prime nick translation procedure as before. Filters were prehybridised at 42^oC for 14 hours with shaking in prehybridisation buffer. Freshly denatured probe was added and this was allowed to hybridise for 14 hours with shaking at 42^oC. After hybridisation

Figure 6.2a

Agarose gel electrophoresis of restricted winged bean genomic DNA. 20μg samples of winged bean DNA were restricted with EcoRI and BglII and XbaI and electrophoresed on 0.8% agarose gel along side 10pg, 25pg and 50pg of a 900bp *phaseolus* lectin like sequence. The gel was stained, photographed and the DNA was transfered to nitrocellulose by Southern blotting.

1. DNA size markers, λDNA restricted with PstI

2. 20μg Winged bean DNA restricted with EcoRI

3. 20μg Winged bean DNA restricted with BglII

4. 20μg Winged bean DNA restricted with XbaI

5. DNA size markers, λDNA restricted with PstI

Figure 6.2b

Hybridisation of *Phaseolus vulgaris* lectin-like gene with winged bean DNA A PstI fragment from the plasmid pPVL134 was labelled with ^{32}P dCTP and hybridised to winged bean genomic DNA restricted with EcoRI, XbaI and BglII immobilised on nitrocellulose filters. Hybridisation was at 42°C and subsequent washing was at 50°C in 1×SSC. Filters were autoradiographed overnight.

1. DNA size markers, λDNA restricted with PstI

2. 20μg Winged bean DNA restricted with EcoRI

3. 20μg Winged bean DNA restricted with BglII

4. 20μg Winged bean DNA restricted with XbaI

5. DNA size markers, λDNA restricted with PstI

6. 10pg pPVL134 900bp restriction fragment

7. 25pg pPVL134 900bp restriction fragment

8. 50pg pPVL134 900bp restriction fragment

A
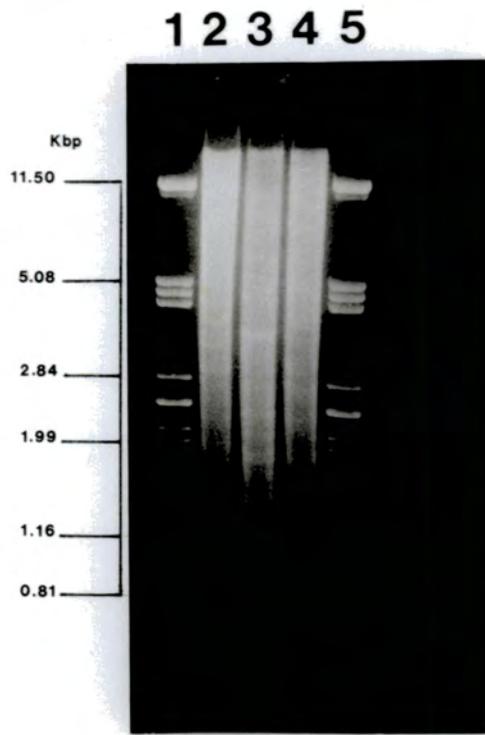
B
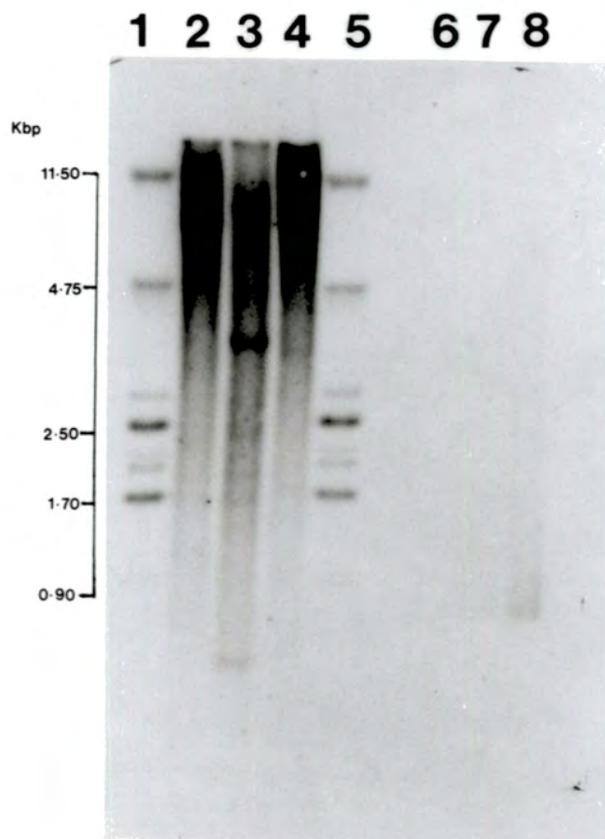
filters were washed three times in 1×SSC at 42° and autoradiographed; subsequent washes and autoradiography took place at 50°C and 65°C with 1×SSC. Under conditions of washing of 1×SSC and 50°C an overnight autoradiograph revealed three distinct bands above a background of non specific binding (figure 6.2). The probe had bound to two EcoR1 fragments (3.7Kb and ~800bp) and one BglII fragment (8.1Kb) in the winged bean DNA digests. However, these three bands corresponded with prominent bands in the ethidium bromide stained gel and suggested that the probe had bound to repeat sequence DNA. On further washing of the filter to 65°C and 1×SSC and with a one week exposure the no hybridisation to winged bean DNA could be observed on the resulting autoradiograph. The single copy equivalent of the *Phaseolus vulgaris* lectin clone was just visible.

6.4 Soybean lectin.

Restriction digests of winged bean DNA with the enzymes BglII, EcoR1 and Xba1 were performed as before, the products were run on agarose gel along side a 10pg and 100pg of a 1.5Kb DNA fragment containing the coding sequence of the soybean lectin gene. The 1.5 Kb Kpn1 fragment from the plasmid pL9-2.7(pUC18 containing a 2.7Kb Xba1 fragment from the genomic clone λL9-4 (Vodkin *et al* 1983) was used to probe the southern blot of this gel. This probe contains the 857bp coding sequence for the soybean lectin along with 370bp of 5' flanking sequence and 300bp of 3' flanking sequence. Prehybridisation and hybridisation was done at 42°C in 6×SSC. Initial washes and autoradiography were at 42°C, 2×SSC and 50°C, 1×SSC. Under the low stringency conditions (42°C) the probe was found to bind to a number of high molecular weight fragments of winged bean DNA and no clear pattern could be discerned. At 50°C the probe bound to a number of distinct

Figure 6.3

Hybridisation of soybean lectin coding sequence to winged bean DNA.
A 1.5Kb Kpn fragment from the plasmid pλL9-2.7 was labelled with ^{32}P dCTP
and hybridised to winged bean genomic DNA restricted with EcoRI, XbaI and
BglII immobilised on nitrocellulose filters. Hybridisation was at 42°C and
subsequent washing was at 50°C in 1×SSC. Filters were autoradiography for
five days at -80°C.

1. 100pg 1.5Kb pL9-2.7 Kpn1 restriction fragment

2. 10pg 1.5Kb pL9-2.7 Kpn1 restriction fragment

3. DNA size markers, λDNA restricted with PstI

4. 20μg Winged bean DNA restricted with EcoRI

5. 20μg Winged bean DNA restricted with BglII

6. 20μg Winged bean DNA restricted with XbaI

7. DNA size markers, λDNA restricted with PstI
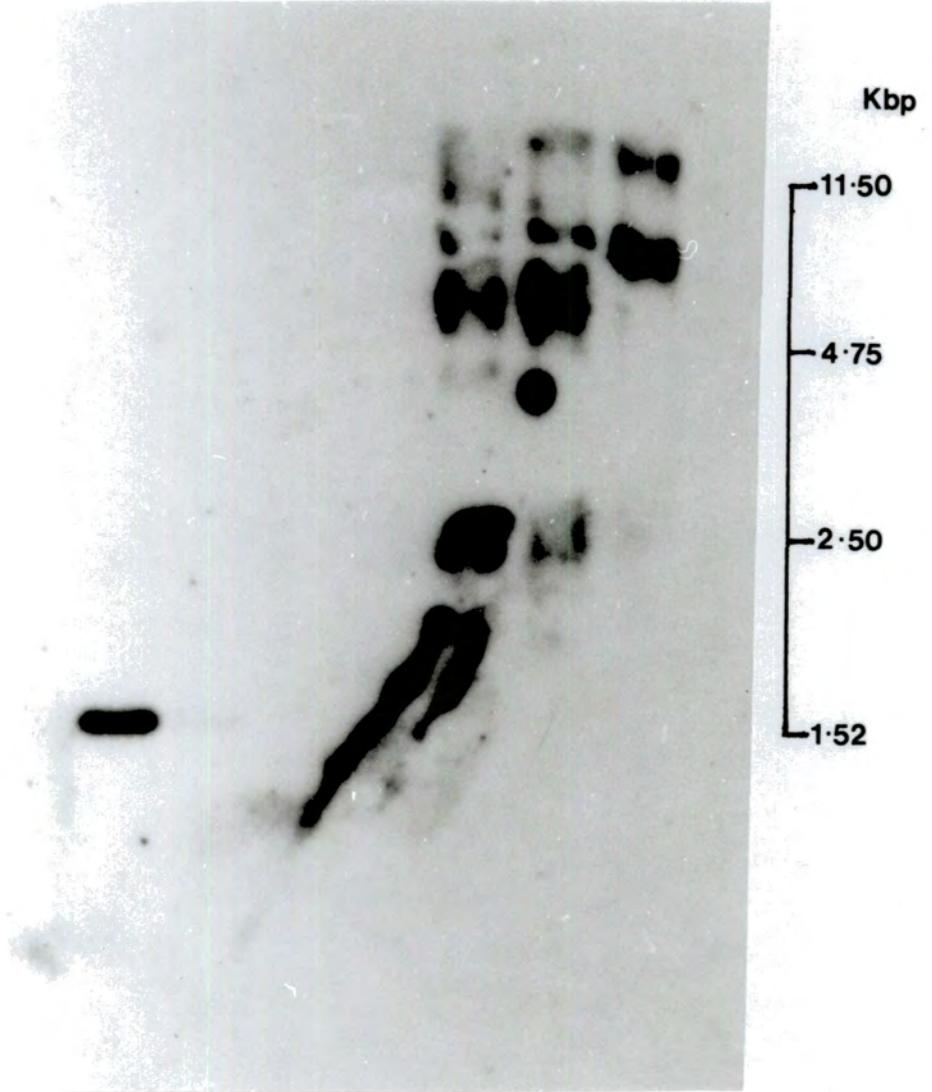
fragments from the EcoR1 digest (>11.5Kb, 8.3Kb), the BglII digest (>11.5Kb, 8.9Kb, 6.0Kb, 2.4Kb) and the Xba1 digest (10.7Kb, 8.3Kb, 5.6Kb, 4.2Kb) (see figure 6.3), however, at 65°C, 1×SSC, no hybridisation to these bands was observed; the 10 copy equivalent soybean lectin control could be observed under these conditions.

6.5 Northern blotting.

No information was gained from the southern blotting experiments with heterologous probes as to the size of the restriction fragment containing the lectin gene. A northern blot of RNA isolated from maturing winged bean cotyledons was probed with two oligonucleotide probes, in an attempt to ascertain the winged bean lectin message size and relative abundance in the RNA extract which was to be used for cDNA synthesis.

The two probes used were the C-terminal primer used in the PCR experiments and the internal oligo used as a probe for PCR products; both of these probes are complementary to the RNA. 15pM of each oligonucleotide were end labelled with γ ^{32}P dATP (>5000 Ci/mmol) using polynucleotide kinase (method 2.2.41). The nitrocellulose filter was hybridised at 22°C for 2 hours in prehybridisation solution (6×SSC, 5×Denhardt's, 0.5% SDS, 100μg/ml Herring sperm DNA and 0.05% Sodium pyrophosphate) and then hybridised with the oligonucleotide probe for 48hrs in the same solution. The filter was washed at 22°C in 3×SSC, 0.1% SDS wrapped in Saran wrap and autoradiographed. No hybridisation was seen using the internal oligonucleotide probe. The C-terminal probe hybridised to two bands; these were the ribosomal 18S and 25S RNAs.

6.6 Use of lectin specific antibodies as probes.

Anti-pea lectin antibodies

The possible use of pea lectin antibodies as a probe for the winged bean basic or acidic lectin was investigated. A total seed extract containing both acidic and basic lectin (as determined by haemagglutination activity of this extract toward rabbit and human erythrocytes) was separated on SDS-polyacrylamide gel, transferred to nitrocellulose and probed with anti-pea lectin antibodies. Purified pea lectin (Method 2.2.12) was used as a positive control. No binding to winged bean polypeptides was observed whilst the two bands of pea lectin preprolectin and β subunit hybridised successfully (The α subunit does not react with these anti-pea lectin antibodies (Edwards 1988))

Anti-basic lectin antibodies

The production of lectin specific antibodies that could be used in the detection of winged bean lectin from expression libraries has already been mentioned (chapter 5). The antibodies were specific for winged bean proteins (as they do not bind to the protein molecular weight markers) and hybridisation to the lectin protein was very much stronger than to any of the other winged bean seed proteins (figure 5.2), some non specific binding of the antibodies was observed but this background is unlikely to prevent this polyclonal antibodies use as a probe for the winged bean lectin.

CHAPTER 7

PROTEIN SEQUENCING AND THE POLYMERASE CHAIN REACTION

7.1 Primer design and Peptide sequencing.

A number of examples have now been reported where specific amplification of genes has been achieved using degenerate primers deduced from protein sequence data. (Lee 1989, Giris 1989). Primers containing inosine, which has the ability to base pair with all bases and therefore reduce the degeneracy, have also been used effectively (Knoth 1988, Fordham-Skelton *et al* 1990). This latter approach was used in an attempt to isolate the winged bean basic lectin gene(s).

As the N-terminal sequence for both the basic and acidic lectins had already been determined (Kortt *et al* 1986) and a number of lectin protein sequences were available, only a small amount of manual sequencing of peptides was needed to obtain sequences whose position in the protein's primary structure could be predicted.

It was necessary to obtain sequence data from peptides as close to the C-terminal as possible and to check that the N-terminal sequence was in accordance with that previously published and that the protein being sequenced was homogeneous.

Basic winged bean lectin (4mg) was prepared from mature winged bean seeds by affinity chromatography, (see method 2.2.10) and the lectin's purity was determined by SDS-PAGE. The lectin was reduced and carboxy-methylated and subsequently cleaved with trypsin . Separation of the peptides was achieved by gel filtration through a column of Biogel P-6 (Bio-Rad) (200 × 1cm) in 0.1M ammonium bicarbonate. The results are presented in figure 7.1. Each of the nine peaks obtained were pooled separately and freeze dried. Peptides were separated further from these freeze dried samples using reverse phase high pressure liquid chromatography on a Vydac C_{18} column (0.5x25cm) with a linear

Figure 7.1

Gel filtration of winged bean basic lectin, after enzymatic digestion with trypsin, on a Bio-Gel P6 (1×200cm) column in 0.1M NH_4HCO_3 (flow rate 4ml/hour, fraction size 1ml). Fractions pooled are labelled.

O.D. 230nm

Fraction No.

WBT1
WBT2
WBT3
WBT4
WBT5
WBT6
WBT7
WBT8
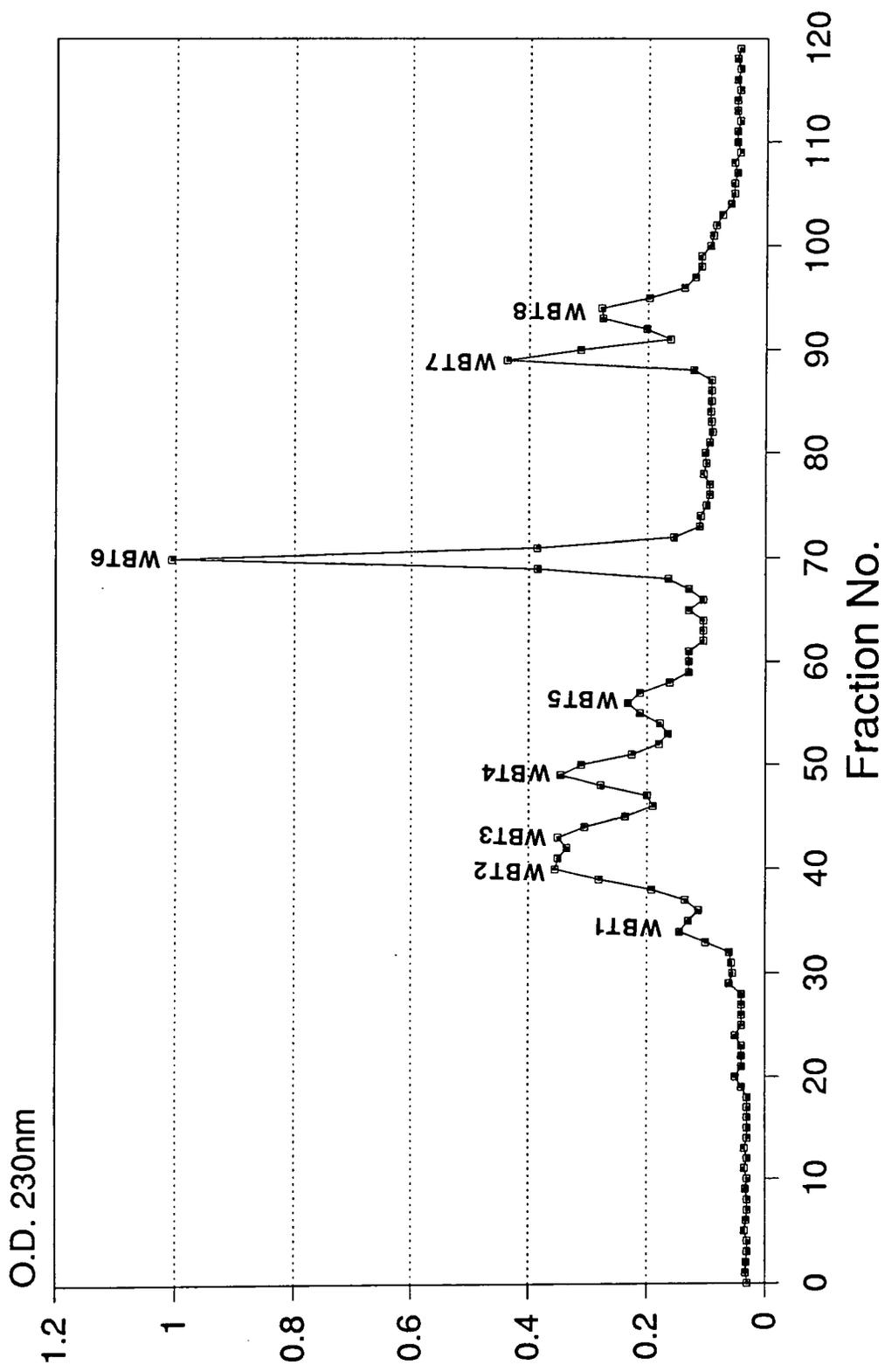
Figure 7.2

Reverse-phase HPLC separation of the peptides of pooled fractions WBT2, WBT3 and WBT4 obtained by enzymatic digestion of basic lectin with trypsin. The peptides were dissolved in 400µl 6M guanidine hydrochloride, 0.1% TFA, applied to a Vydac C_{18} analytical column (4.6mm × 25cm) equilibrated in 0.1% aqueous TFA and eluted with a gradient of acetonitrile, 0.1% TFA at a flow rate of 1ml/min. Peptides were detected by monitoring the absorbance at 216nm. Peaks indicated were pooled lyophilised and used for sequencing.

A) Peptides separated from fraction WBT2 (figure 7.1)

B) Peptides separated from fraction WBT4 (figure 7.1)

C) Peptides separated from fraction WBT3 (figure 7.1)

A

% Acetonitrile

O.D. 216nm

Time (Minutes)

B

% Acetonitrile

O.D. 216nm

Time (Minutes)

C

% Acetonitrile

O.D. 216nm

Time (Minutes)
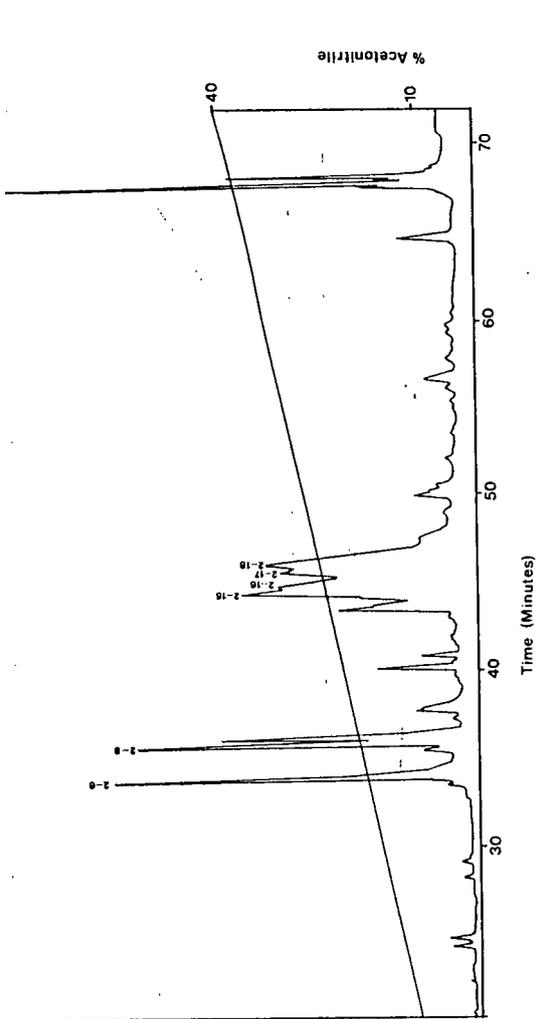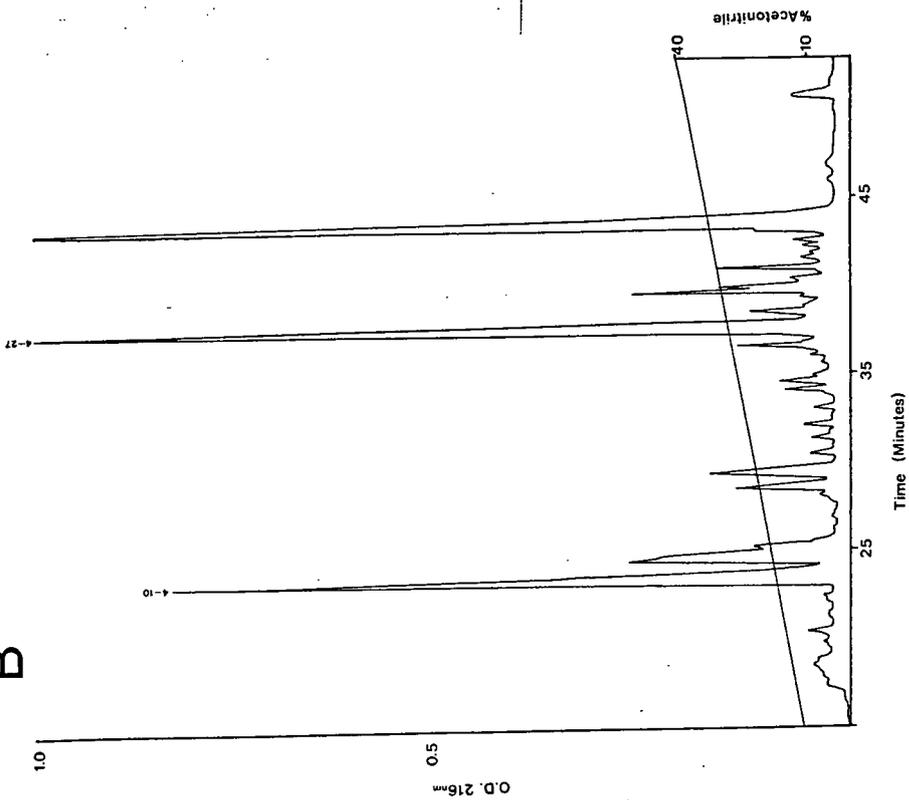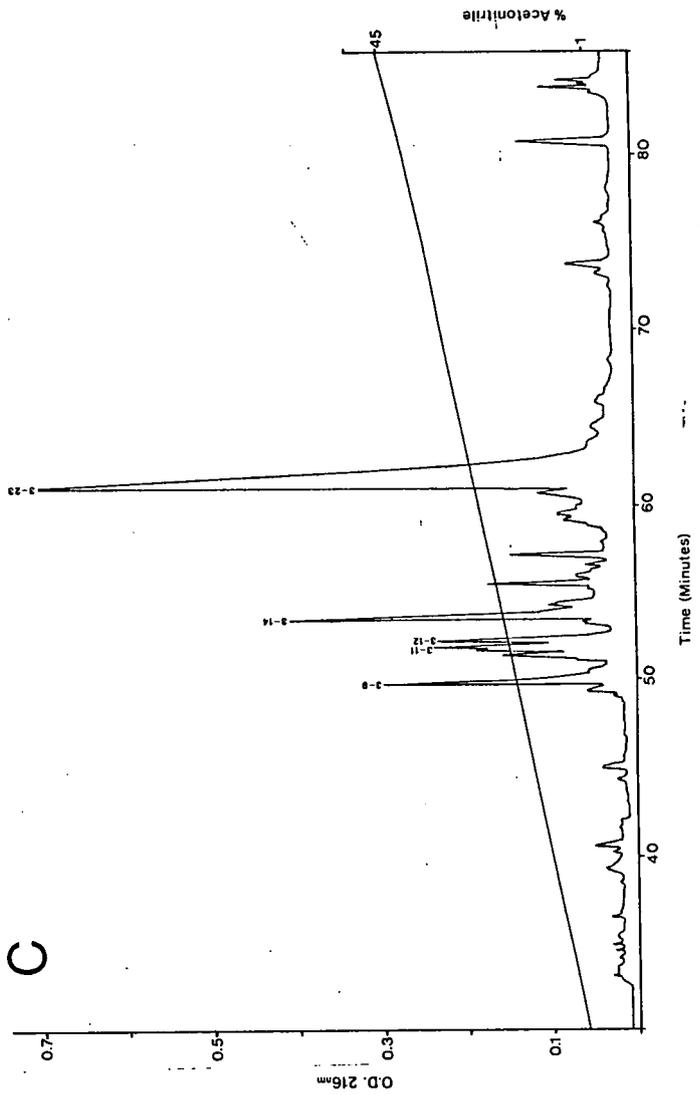
PEPTIDE	SEQUENCE
WBT2-6	QVLPESVNVGFSAATGD
WBT2-8	QATETHDLLS?
WBT2-15	QATETHDLLS?SFAS?
WBT2-16	QATETHDLL??SFA?SL
WBT2-17	QATETHDLLS?W?SFF?S?L?
WBT2-18	---NO SEQUENCE---
WBT3-9	QATETHDL
WBT3-11	XRLA
WBT3-12	QATETHDL?
WBT3-14	PVQL?DT
WBT3-4	QVLPESVNVGFSAATGDPSG
WBT4-10	VVNGPTV?
WBT4-27	KTLSFNFNQFHQNEE
WBT5-15	TGP,AAH,TNS three peptide mix
WBT5-26	VLSGVVT?, SFDASVPGTN two peptides
WBT5-32	LDSDGL
WBT6-10	AV
WBT6-13	AVLY?

Table 7.1   Sequences of tryptic peptides of winged bean basic lectin derived using the manual DABITC/PITC microsequencing method. The one letter amino acid code is used, ? after an amino acid denotes a very weak signal. X indicates that the amino acid at this position could not be determined. An L in the sequence indicates either a Lecuine or Isoleucine as these two amino acid derivatives could not be distinguished from each other with the method used.

gradient of 0% to 70% acetonitrile in 0.1% trifluoroacetic acid controlled by a Varian 5000 HPLC system. Peaks with high O.D.s and/or which appeared well separated were used for manual sequencing (see fig 7.2) which was carried out as described in the methods section (2.2.15). The results of the manual sequencing of 18 peptides are given in table 7.1.

It is not unusual to find that the most abundant peptides arise from the amino and carboxy terminals, as these may be least protected by the proteins folding . In this case of the 18 peptides sequenced 8 peptides could be assigned to the C-terminal positions and 2 to NH_2 positions by homology. Peptides 4-27, 4-10, 3-4 and 2-17 provided the most useful data and their predicted position in the basic lectins primary structure was determined, see figure 7.3.

7.2 Designing PCR primers

When working from protein data, the degeneracy of the genetic code makes the construction of non-degenerate oligonucleotide primers extremely difficult as few amino acids have specific codons. Although the design of an efficient set of specific PCR primers is to a certain extent empirical, there is little doubt that the primers are the single most important factor in determining the success of any PCR experiment (Anderson 1990). It is widely accepted that primers should have close to a 50:50 G+C : A+T ratio, avoid sequences that have significant secondary structure and that the primers should not have regions of complementarity. In general primers greater than 15 base pairs are required so that annealing temperatures can be kept as high as possible. Mixed oligonucleotide primers derived from amino acid sequences are useful when only a limited amount of sequence data is available. It is however the degeneracy of the genetic code that places the biggest constraint on the redundancy of primers. Selection of amino

Figure 7.3

Alignment of winged bean tryptic peptide sequences with homologous legume lectin sequences. Peptide sequences of the winged bean basic lectin (in bold) are placed above their homologous regions in the other lectin sequences. Conserved regions between the lectins are indicated by □, 7/9 matches between sequences and ■, ≥8/9 matches between sequences.

```
BASIC     KT-LS-FNFNQFH--QNEE                      VVNG-
          -□-□--□-■----■---□-□□□□■■■---------■-■■□---□□-
EcorL     VE-T-IS-FSFSEFEPGNDNLTLQGDS*P-ETSGVLQLTKINQNGM
DBL       AN-I-QS-FSFKNFNS--PSFILQGDATV--SSGKLQLTKVKENGF
Favin     TDE-I-TS-FSIPKFRPDQPNLIFQGGGYT--TKEKLTLTKAV----
LCL       TE-T-TS-FSTTKFSPDQQNLIFQGDGYT--GKEGLTLTKVS----
PSL       TE-T-TS-FLITKFSPDQQNLIFQGDGYT--TKEKLTLTKAV----
PHA-L     SNDIYFN-FQRFN--ET--NLILQRDASVS-SSGQLRLTNLNGNGE
SBA       AE-T-VS-FSWNKFVPKQPNMILQGDAIV-TSSGKLQLNKVDENGT
SL        AENT-VS FDFSKFLSGQENLILQGDTVTDDSNRCLVLTR-ENNGR
conA      -Q-TDALHFMFNQFSKDQKDLILQGDATT-GTNGNLELTRVSSNGS


BASIC     PTV                      SFDAS-VP-GTN
          □-----■■□■■□-■□□■■■--□□-■■-■-□-■-□-□---□-----■
EcorL     PAWDSTGRTLYAKPVHIWDMTTGTVASFETRFSFS*EQPYTRP*PA
DBL       PLRFPSGRAFYSSPIQIYDKFTGAVASWATSFTVKIS-APSKASFA
Favin     -K-NTVGRALYSLPIHIWDSETGNVADFTTTFIFVID-APNGYNVA
LCL       -K-ETGGRALYSTPIHIWDRDTVNVANFVTNGSQVFRESPNGYNVA
PSL       -K-NTVGRALYSSPIHIWDRETGNVANFVTSFTFVIN-APNSYNVA
PHA-L     PRVGSLGRAFYSAPIQIWDNTTGTVASFATSFTFWIQ-VPNNAGPA
SBA       PKPSSLGRALYSTPIHIWDKETGSVASFAASFNFTFY-APDTKRLA
SL        PVQDS GRVLYQTPIHLWDKQIDKEASFETSFTFFIY-RENINRGG
ConA      PEGSSVGRALFYAPVHIWES-SATVSAFEATFAFLIK-SPDS-HPA


BASIC              PVQLDT
          ■■--■□--■-□--■--□□■□--■■-■----------□□■■■■■■■□
ECorL     DGLVFFMGPTKSKPAQGYGY--LGIFFNSKQDNSY-QTLGVEFDTF
DBL       DGIAFALVPVGSEPRRNGGY--LGVF-DSDVYNNSAQTVAVEFDTL
Favin     DGFTFFIAPVDTKPQTGGGY--LGVF-NGKDYDKTAQTVAVEFDTF
LCL       DGFTFFIAPVDTKPQTGGGY--LGVF-NSAEYDKTTQTVAVEFDTF
PSL       DGFTFFIAPVDTKPQTGGGY--LGVF-NSAEYDKTTQTVAVEFDTF
PHA-L     DGLAFALVPVGSQPKDKGGF--LGLFDGS---NSNFHTVAVEFDTL
SBA       DGLAFFLAPIDTKPQTHAGY--LGLF-N--ENESGDQVVAVEFDTF
SL        DGITFFLAPTDTQPKSGGGY--LGIFKDAE-SNET--VVAVEFDTF
ConA      DGIAFFISNIDSSIPSGSTGRLLGLFPDAN AD-T--IVAVELDTY


BASIC
          --■---■■■-----■■■■□■■■□□■--■--■-□-■□--□-□-■-□-
ECorL     -SNP--WDPPQ-VPHIGIDVNSIRSIKTQPFQLDNGQVANVVIKYD
DBL       -SNS-GWDPSM--KHIGIDVNSIKSIATVSWDLANGENAEILITYN
Favin     -YNAA-WDPSNGKRHIGIDVNTIKSISTKSWNLQNGEEAHVAISFN
LCL       -YNAA-WDPSNKERHIGIDVNSIKSVNTKSWNLQNG----------
PSL       -YNAA-WDPSNRDRHIGIDVNSIKSVNTKSWKLQNGEEANVVIAFM
PHA-L     -YNKD-WDP--TERHIGIDVNSIRSIKTTRWDFVNGENAEVLITYD
SBA       -RNS--WDPPN--PHIGINVNSIRSIKTTSWDLANNKVAKVLITYD
SL        -SNR--WDPAN  SHIGINVNSVKSKITTPWGLKNDYF-TVTITYD
ConA      -PNTDIGDPSYP--HIGIDIKSVRSKKTAKWNMQDGKVGTANIIYN




BASIC                                        QV I PESVNVGFSA
                                               L
          □----■---□-□■--------□----□--■-□□-□□■■■■□-■□■■
EcorL     ASSKILHAVLVYPSSG-----AIYTIAEIVDVKQVLPEWVDVGFSA
DBL       AATSLLVVSLVHPSRR-----TSYILSERVDITNELPEYVGVGSFA
Favin     ATTNVLSVTLLYPN       LTGYRLSEVVPLKDVVPEWVRIGFSA
LCL                          VTSYTLNEVVPLKDVVPEWVRIGFSA
PSL       AATNVLTVSLTYPNSLEEENVTSYTLSDVVSLKDVVPEWVRIGFSA
PHA-L     SSTKLLVASLVYPSQK-----TSFIVSDTVDLKSVLPEWVSVGFSA
SBA       ASTSLLVASLVYPSQR-----TSNILSDVVDLKTSLPEWVRIGFSA
SL        ATRS-LSVSSFYRNKPD----DIFTVKASVHLRDALPQWVRIGLSA
ConA      S▼DKRLSAVVSYPN------ADATSVSYDVDLNDVLPEWVRVGLSA


BASIC     ATGDPSGQATETHD II SWSF F S I
                        LL      A L
          ■□-------□---■-■■□■-■-■---□
EcorL     ATG-AQRDAAETHDVYSWSFQAS*PETND-AV
DBL       TTG-LSEGYIETHDVLSWSFASRLPDDST-AEPLDLARYLVRNVL
Favin     TTG-AEYATHE---VLSWTFLSELTGPSN
LCL       TTG-AEFAAQE---VHSWSFNSQLGHTSKS
PSL       TTG-AEYAAHE---VLSWSFHSELSGTSSSKQAADA
PHA-L     TTGINKGNV-ETHDVLSWSFASKLSDGTTSEGLNLANLVLNKIL
SBA       ATG-LDIPG-ESHDVLSWSFASNLPHASSNIDPLDLTSFVLHEAI
SL        ATG-DLVEQHRL---YSWSFKSVLPLDSST
ConA      STG-LYKETNTI---LSWSFTSKLKSNSTH
```

acids with the minimum number of codons is preferred to obtain the least redundant primers possible. Information in Table 7.2. was used to decide on regions with the least redundancy. With the manual sequencing method used no distinction could be made between isoleucine or leucine, as these could not be separated by thin layer chromatography, therefore nine possible codons could code for this amino acid option.

The region QATETHD provided the longest C-terminal sequenced region in which the amino acids had low numbers of redundant codons. The oligonucleotide primer designed for this region had a redundancy of 1024 fold, this was further reduced to 4 fold by the use of inosine residues at some positions of redundancy. The C-terminal reverse primer was therefore 5'-TC ITG IGT T/C TC IGT IGC T/C TG-3' (see figure 7.4a). The region QATETHD was chosen from the C-terminal region because it was well conserved amongst other legume lectin sequences and of low redundancy.

The NH$_2$terminal region chosen for designing the forward primer again represents a region which is relatively well conserved and allows for a primer with low redundancy. The oligonucleotide primer synthesised for this NH$_2$ terminal region, FNFNQFH, has a redundancy of 128 fold . The sequence of the forward primer was 5'-TT(T/C) AA(T/C) TT(T/C) AA(T/C) CA(A/G) TT(T/C) CA(T/C) CA-3', (see figure 7.4b).

CODON DEGENERACY

No. of Codons	Amino Acid
1	M, W
2	C,D,E,F,H,K,N,Q,Y
3	I
4	A,G,P,T,V
6	L,R,S
9	L/I

Table 7.2

A table of the number of codons each amino acid can be coded by genetically. The single letter amino acid code is used, L/I represents the Leucine/Isoleucine amino acid ambiguity inherent in the manual sequencing method used.

A)   **N-TERMINAL OLIGONUCLEOTIDE FOR PCR**

Amino acid sequence :- NH_2--F---N---F---N---Q---F---H---Q--COOH

Sense strand :-   5′ --TT$^{T}_{C}$ AA$^{T}_{C}$ TT$^{T}_{C}$ AA$^{T}_{C}$ CA$^{C}_{A}$ TT$^{T}_{C}$ CA$^{T}_{C}$ CA-- 3′

OLIGONUCLEOTIDE PRIMER 116

5′ --TT$^{T}_{C}$ AA$^{T}_{C}$ TT$^{T}_{C}$ AA$^{T}_{C}$ CA$^{C}_{A}$ TT$^{T}_{C}$ CA$^{T}_{C}$ CA-- 3′

B)   **C-TERMINAL OLIGONUCLEOTIDE FOR PCR**

Amino acid sequence :- NH_2--Q---A---T---E---T---H---D---COOH

Sense strand :-   5′--CA$^{A}_{G}$ GCN ACN GA$^{A}_{G}$ ACN CA$^{T}_{C}$ GA$^{T}_{C}$--3′

Complementary strand :- 3′--GT$^{T}_{C}$ CGN TGN CT$^{T}_{C}$ TGN GT$^{A}_{G}$ CT$^{A}_{G}$--5′

5′--$^{A}_{G}$TC $^{A}_{G}$TG NGT $^{T}_{C}$TC NGT NGC $^{T}_{C}$TG--3′

OLIIGONUCLEOTIDE PRIMER 222

Inosines in place of A/G and N third base options.

5′--TC ITG IGT $^{T}_{C}$TC IGT IGC $^{T}_{C}$TG--3′

Figure 7.4 A) Design of the N-terminal oligonucleotide primer for
the amplification of the winged bean basic lectin gene.
B) Design of the C-terminal oligonucleotide primer
for the amplification of the winged bean basic lectin gene
I=Inosine , N=A,G,C or T

7.3 Polymerase chain reaction using degenerate primers.

An initial amplification reaction was carried out under high stringent annealing conditions (i.e. $55^{\circ}C$). The thermal cycling regime involved a denaturing step of $94^{\circ}C$ / 4 mins followed by automatic cycling through the following temperature/time cycles in mode 4 on a Hybaid Intelligent heating block (No control of ramping is available on this device, heating and cooling cycles are approximately $2^{\circ}C$/min and $1^{\circ}C$/min respectively.) $92^{\circ}C$ / 2 min denaturing, $55^{\circ}C$ / 2 min annealing, $72^{\circ}C$ / 2 min extension for 30 cycles. A further extension at $72^{\circ}C$ / 2 min followed the last cycle. The reaction mixture was as stated in the methods section (2.2.51).

Two amplification products could be detected following agarose gel electrophoresis. They had approximate sizes of 400bp and 300bp; these two products were observed in approximately equimolar proportions as determined visually.

The two amplification products detected were considered to be too small to be lectin genes and a further amplification reaction was attempted with a lower annealing temperature of $50^{\circ}C$, in an attempt to encourage primer-template binding. The time cycle was reduced to 90sec for all steps i.e. denaturing, annealing and extension. Analysis of the PCR reaction mixture by agarose gel electrophoresis, after the amplification procedure, yielded no obvious amplified products, only a sub 100bp 'primer smear' could be detected.

Figure 7.5

The effect of magnesium ion concentration on the amplification of PCR products from winged bean genomic DNA. PCR primers designed to amplify winged bean lectin coding sequences (figure 7.4a,b) were used in PCR amplification reactions using winged bean genomic DNA. Six 100μl reactions contained 1μg of genomic DNA, 0.33μM of each of the two primers, 200μM dNTPs and either 1.5, 3.5, 5.5, 7.5, 9.5 or 11.5mM magnesium ions. The reactions were subjected to 30 temperature/time cycles of 92°C/90sec denaturing, 45°C/90sec annealing, and 72°C/90sec extension. Products were electrophoresed on a 0.8% agarose gel and visualised under UV.

1. DNA size markers, Pst1 restricted λDNA
2. 1.5mM magnesium PCR reaction
3. 3.5mM magnesium PCR reaction
4. 5.5mM magnesium PCR reaction
5. 7.5mM magnesium PCR reaction
6. 9.5mM magnesium PCR reaction
7. 11.5mM magnesium PCR reaction
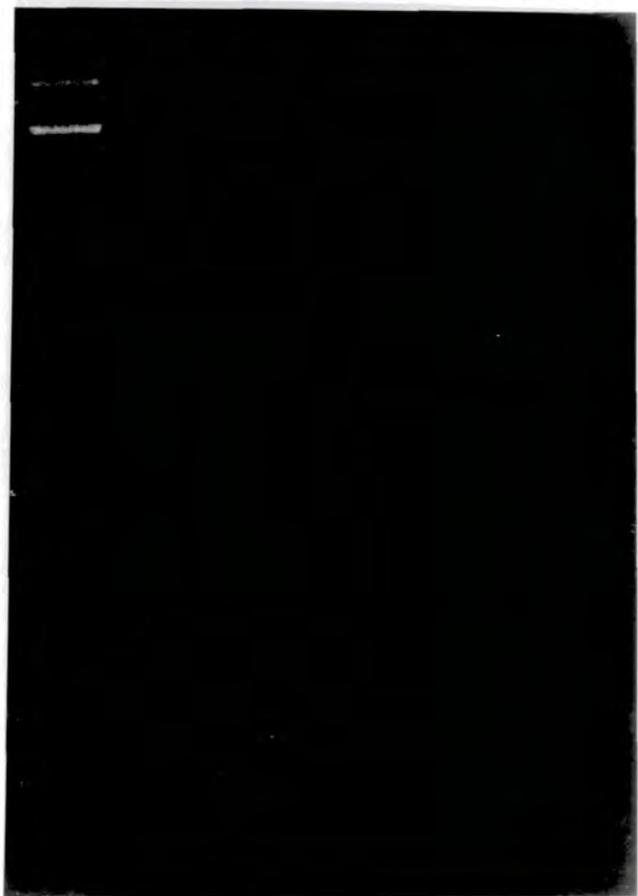
7.4 Magnesium ion titration experiments.

In another amplification reaction the annealing temperature was reduced further to 45°C, and at the same time the magnesium ion concentration within the reaction mix was varied. The reaction mixes were prepared as before with addition of 0,1,2,3 and 4µl of 100mM $MgCl_2$ to provide final Mg^{2+} ion concentrations of 1.5, 3.5, 5.5, 7.5 ,9.5 and 11.5mM. These reaction mixes were subjected to 30 cycles of 92°C / 90sec, 45°C / 90sec and 72°C / 90sec were controlled automatically as before on a Hybaid intelligent heating block and the products visualised on a 0.7% (w/v) agarose gel following electrophoresis. The results are presented in figure 7.5. A single amplified band could be detected in the reaction mixes containing 3.5, 5.5 and 7.5 mM Mg^{2+}; the most prominent band could be detected in the reaction mix containing 5.5mM Mg^{2+} . This band appeared was approximately 550bp in size, i.e.close to the 600bp-700bp expected.

The band containing the 550bp PCR product was excised from the gel and electroeluted according to method (2.2.37). Approximately 50ng of the resulting fragment was used in a ligation reaction with 10ng of plasmid vector pUC19 restricted with HincII. The reaction mix was used to transform competant *E.coli* DH5α cells (2.2.26). Recombinant clones were selected for on XGAL/AMP agar plates. A number of white colonies were observed and 12 were chosen at random for plasmid minipreping according to method 2.2.34.

Plasmids containing inserts were detected by restriction with endonuclease BamHI and visualisation on 0.7% (w/v) arose gel following electrophoresis. Five clones appeared to contain inserts of approximately 550bp. One clone pDH1.PCR was purified further using silica fines (2.2.28) and sequenced.

Figure 7.6

Nucleotide sequence and deduced amino acid sequences of the pDH1.PCR insert. The insert was sequenced in both directions using forward and reverse m13 dye labelled primers and the dideoxy chain termination method (Sanger *et al* 1977) and data collected on an ABI model 373A DNA sequencer. The three rows below the nucleic acid sequence correspond to the amino acid reading frames 1,2 and 3 respectively. These were determined using the programme DNAsys, starting from the first sequenced base. The single letter amino acid code is used, Stp indicates the position of a stop codon.

B:DH1.PCR.SEQ is 606 base pairs long.
Translation from 1 to 605.

```
     Stp S  R  T  L  T  S  S  T  K  Stp V  H  N  Q  K  I  Q  Q  L
        R  V  E  L  Stp P  V  P  P  N  E  Y  I  I  R  K  F  S  S  C
        E  S  N  F  N  Q  F  H  Q  M  S  T  Stp S  E  N  S  A  A  A
  1     TAGAGTCGAACTTTAACCAGTTCCACCAAATGAGTACATAATCAGAAAATTCAGCAGCTG


        Q  V  S  P  F  A  P  H  Y  N  C  C  K  R  T  H  A  Q  F  L
        K  S  H  L  L  L  L  T  I  I  V  V  R  E  H  M  L  N  F  Stp
        S  L  T  F  C  S  S  L  Stp L  L  Stp E  N  T  C  S  I  F  N
 61     CAAGTCTCACCTTTTGCTCCTCACTATAATTGTTGTAAGAGAACACATGCTCAATTTTTA


        T  P  T  P  S  M  H  Q  D  H  S  K  P  Stp K  E  A  H  S  A
        L  P  L  Q  V  C  I  R  I  T  P  S  L  E  R  R  H  I  Q  L
        S  H  S  K  Y  A  S  G  S  L  Q  A  L  K  G  G  T  F  S  F
121     ACTCCCACTCCAAGTATGCATCAGGATCACTCCAAGCCTTGAAAGGAGGCACATTCAGCT


        S  L  L  L  F  W  R  D  F  Y  L  G  W  L  G  P  L  M  V  G.
        H  S  F  Y  S  G  G  I  S  I  W  A  G  W  G  R  Stp W  W  G
        T  P  S  I  L  A  G  F  L  F  G  L  A  G  A  A  D  G  G  V
181     TCACTCCTTCTATTCTGGCGGGATTTCTATTTGGGCTGGCTGGGGCCGCTGATGGTGGGG


        S  Y  P  P  N  W  S  N  L  S  W  N  S  S  C  Stp C  F  I  N
        P  I  L  Q  I  G  P  T  S  H  G  I  H  L  V  D  A  S  S  I
        L  S  S  K  L  V  Q  P  L  M  E  F  I  L  L  M  L  H  Q  S
241     TCCTATCCTCCAAATTGGTCCAACCTCTCATGGAATTCATCTTGTTGATGCTTCATCAAT


        L  S  K  C  W  D  N  A  A  I  Stp S  E  D  H  C  Y  Y  F  F
        S  L  N  A  G  I  M  Q  Q  F  E  V  K  I  I  V  I  I  S  F
        L  Stp M  L  G  Stp C  S  N  L  K  Stp R  S  L  L  L  F  L  S
301     CTCTCTAAATGCTGGGATAATGCAGCAATTTGAAGTGAAGATCATTGTTATTATTTCTTT


        L  P  StpStp T  N  R  S  N .V  T  R  K  S  M  Y  V  V  F  E
        C  H  D  K  Q  T  G  Q  M  S  Q  E  K  V  C  M  W  C  L  K
        A  M  I  N  K  Q  V  K  C  H  K  K  K  Y  V  C  G  V  Stp K
361     CTGCCATGATAAACAAACAGGTCAAATGTCACAAGAAAAAGTATGTATGTGGTGTTTGAA


        N  V  L  T  H  S  S  V  R  A  K  K  K  I  G  F  Y  Y  N  E
        M  F  S  P  T  Q  V  F  A  L  K  K  K  L  A  F  T  I  M  K
        C  S  H  P  L  K  C  S  R  Stp K  K  N  W  L  L  L  StpStp N
421     AATGTTCTCACCCACTCAAGTGTTCGCGCTAAAAAAAAAATTGGCTTTTACTATAATGAA


        T  L  L  P  F  S  S  L  F  L  L  N  F  L  T  N  Q  K  R  T
        L  C  C  L  F  P  L  Y  S  S  L  T  S  Stp Q  T  K  R  E  Q
        S  V  A  F  F  L  F  I  P  P  StpL  L  N  K  P  K  E  N  N
481     ACTCTGTTGCCTTTTTCCTCTTTATTCCTCCTTAACTTCTTAACAAACCAAAAGAGAACA


        T  Q  R  Q  T  P  T  C  V  Q  A  T  E  T  H  E  T  C  R  H
        L  N  D  K  H  Q  P .V  C  R  P  P  R  P  T  R  P  A  G  M
        S  T  T  N  T  N  L  C  A  G  H  R  D  P  R  D  L  Q  A  C
541     ACTCAACGACAAACACCAACCTGTGTGCAGGCCACCGAGACCCACGAGACCTGCAGGCAT


        A
         Q
          K
601     GCAAG
```

7.5 Sequencing of PCR products.

Sequencing was performed according to the ABI protocol using

ABI-21mer M13 dye labelled primers and the dideoxy sequencing method

(Sanger *et al* 1977). The sequence data obtained using an ABI model 373A DNA

sequencer is presented in figure 7.6 along with an amino acid translation

in all three reading frames. A C-terminal primer site could be detected at

the 3' end of the clone; this had 100% homology to the C-terminal primer.

All the inosine residues in the primer were replaced by guanosine residues

in the sequenced DNA . The 5' sequence of the insert had 100% homology

with the last 20bp of the N-terminal primer, the first 2bp partly coding

for phenylalanine were not present. The insert size was 579bp.

An amino acid translation of the DNA sequence in all three reading

frames was obtained using a computer sequence analysis programme DNAsys.

Both the N-terminal and the C-terminal amino acid sequences could be

detected. The first amino acid of the N-terminal was absent, the final

amino acid of the C-terminal became glutamic acid due to insertion of the

PCR fragment into the Hinc II site of the vector. No lectin like sequence

could be detected amongst the open reading frames and no significant

homologies were observed to proteins in the NIH protein sequence data base.

A further PCR experiment was performed in which the annealing

temperature was reduced to 43°C. Figure 7.7 shows the specific

amplification product obtained in this experiment. The DNA fragment was

approximately 600bp in size. This fragment was of a similar size to that

previously amplified but the two did not cross hybridise. The DNA fragment

was electroeluted from an agarose gel, ethanol precipitated and

subsequently resuspended in sterile distilled deionised water before

"polishing" the ends with T_4 polymerase and blunt end ligation

Figure 7.7

The effect of annealing temperature on the amplification of PCR
products from winged bean genomic DNA. A 100μl reaction containing 1μg
of genomic DNA, 0.33μM of the two primers, 200μM dNTPs and 5.5mM Mg^{2+}
ions was subjected to 30 temperature/time cycles of $92^{O}C$/90sec
denaturing, $43^{O}C$/90sec annealing and $72^{O}C$/90sec extension. Products
were visualised following electrophoresis in 0.8% agarose gel.

1. DNA size markers, Pst1 restricted λDNA.

2. $43^{O}C$ annealing temperature PCR reaction.
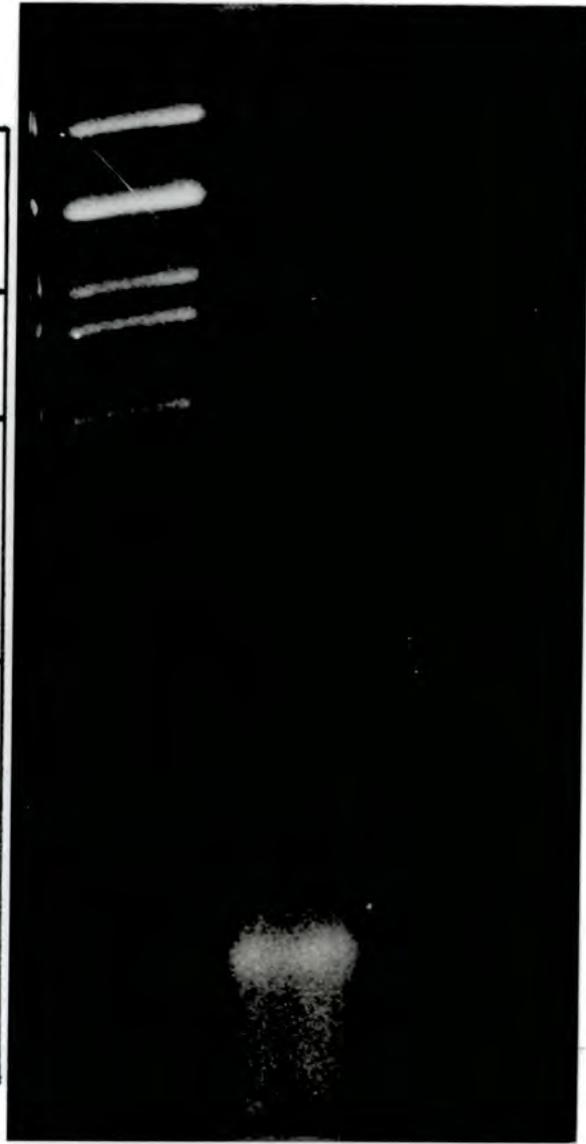
Figure 7.8

Nucleotide sequence and deduced amino acid sequences of the pDH20.PCR
insert. The insert was sequenced in both directions using forward and
reverse m13 dye labelled primers and the dideoxy chain termination
method (Sanger *et al* 1977) and data collected on an ABI model 373A DNA
sequencer. The three rows below the nucleic acid sequence correspond
to the amino acid reading frames 1,2 and 3 respectively. These were
determined using the programme DNAsys, starting from the first
sequenced base. The single letter amino acid code is used, Stp
indicates the position of a stop codon.

B:DH20.PCR.SEQ is 665 base pairs long.
Translation from 1 to 664.

```
      K   L   A   C   L   Q   V   F   N   F   N   Q   L   H   H   T   I  Stp  D   F
        S   L   H   A   C   R   S   L   T   L   I   S   C   I   T   Q   Y   K   T   L
          A   C   M   P   A   G   L  Stp  L  Stp  S   V   A   S   H   N   I   R   L  Stp
1     AAGCTTGCATGCCTGCAGGTCTTTAACTTTAATCAGTTGCATCACACAATATAAGACTTT


      N   L   M   A   F   R   K   M   V   V   S  StpStpStp  F   S   C   K   S   L
        T   L   W   H   S   G   K   W   L   S   R   N   N   D   S   R   A   R   A   S
          P   Y   G   I   P   E   N   G   C   L   V   I   M   I   L   V   Q   E   P   Q
61    AACCTTATGGCATTCCGGAAAATGGTTGTCTCGTAATAATGATTCTCGTGCAAGAGCCTC


      K   L   L   Y   S   G   N   I   I   K   N   Q   K   N   L   F   H   T   Q   P
        S   C   S   I   P   A   T   S   S   R   T   K   K   T   F   F   I   L   S   L
          A   A   L   F   R   Q   H   H   Q   E   P   K   K   P   F   S   Y   S   A   C
121   AAGCTGCTCTATTCCGGCAACATCATCAAGAACCAAAAAAACCTTTTTCATACTCAGCCT


      V   I   L   H   C   L   D   S   M   T   R   R   F   D   V   N   I   D   R   G
        L   F   F   I   V   L   I   P  Stp  H   V   D   L   M   L   I  Stp  I   E   V
          Y   S   S   L   S  Stp  F   H   D   T  Stp  I  Stp  C  Stp  Y   R  Stp  R   L
181   GTTATTCTTCATTGTCTTGATTCCATGACACGTAGATTTGATGTTAATATAGATAGAGGT


    Stp  L   V   Y   N   V   C   I   N   S   F   A   L   I   F   I   T   I   L   H
        N   W   Y   T   M   S   V  Stp  I   V   L   H  Stp  Y   S   S   Q   F   C   I
          I   G   I   Q   C   L   Y   K  Stp  F   C   I   N   I   H   H   N   F   A   F
241   TAATTGGTATACAATGTCTGTATAAATAGTTTTGCATTAATATTCATCACAATTTTGCAT


      L  StpStp  K   S   Y   K   W   K   E   V   I   I   K   F   Q   C   I   N   S
        Y   N   K   S   H   I   S   G   K   K  StpStp  L   N   F   N   V   L   T   V
          I   I   K   V   I  Stp  V   E   R   S   N   N  Stp  I   S   M   Y  Stp  Q  Stp
301   TTATAATAAAAGTCATATAAGTGGAAAGAAGTAATAATTAAATTTCAATGTATTAACAGT


      D   S   L   Y   Q   L   S   W   Y   K   Y   L   Q   W   L   I   N   G   H   L
        T   V   Y   T   N  Stp  A   G   I   S   I   F   S   G   S  Stp  M   A   I   S
          Q   F   I   P   I   K   L   V  Stp  V   S   S   V   A   H   K   W   P   S   L
361   GACAGTTTATACCAATTAAGCTGGTATAAGTATCTTCAGTGGCTCATAAATGGCCATCTC


      W   T   M   Y  Stp  A   L   L   S   R   T   L   T   P   L   K   Q   T   R   G
        G   P   C   T   K   P   Y   S   A   A   L   S   H   L   L   S   K   P   E   A
          D   H   V   L   S   P   T   Q   P   H   S   H   T   S  Stp  A   N   Q   R   P
421   TGGACCATGTACTAAGCCCTACTCAGCCGCACTCTCACACCTCTTAAGCAAACCAGAGGC


      P   H   W  Stp  L   T   K   L   Y   T   A   R   A   R   V   V   L   K   I   P
        R   I   G   D  Stp  Q   N   Y   I   Q   P   E   Q   G  Stp  F  Stp  K   F   L
          A   L   V   T   N   K   T   I   Y   S   P   S   K   G   S   F   E   N   S   S
481   CCGCATTGGTGACTAACAAAACTATATACAGCCCGAGCAAGGGTAGTTTTGAAAATTCCT


      P   Y   R   R   S   L   P   Y   G   L   H   H   Q   C   Q   H   S   A   V   T
        H   T   A   D   P   Y   H   T   D   S   I   I   S   A   N   I   Q   Q  Stp  R
          I   P   Q   I   P   T   I   R   T   P   S   S   V   P   T   F   S   S   D   V
541   CCATACCGCAGATCCCTACCATACGGACTCCATCATCAGTGCCAACATTCAGCAGTGACG


      L   L   Q   D   T   E   T   H   E   T   L   E   D   P   R   V   P   S   S   N
        Y   F   R   T   P   R   P   T   R   L  Stp  R   I   P   G   Y   R   A   R   I
          T   S   G   H   R   D   P   R   D   S   R   G   S   P   G   T   E   L   E   F
601   TTACTTCAGGACACCGAGACCCACGAGACTCTAGAGGATCCCCGGGTACCGAGCTCGAAT


      L
      Stp
661   TTAg
```

into the vector pUC18 restricted with HincII. A resulting clone
(pDH20.PCR) was sequenced using both the forward and reverse dye labelled
primers as before.

The insert was 605bp in length, and both primer sites could be
detected. Each primer site had a one base pair mismatch in comparison
to their parent primers, a guanosine for the thymidine/cytosine option
at position 18 of the N-terminal primer and a thymidine for a
guanosine in position 16 of the C-terminal primer. The inosine
residues of the C-terminal primer were replaced by guanosine residues
in the sequenced DNA.

The amino acid translation in all 3 reading frames yielded no
open reading frames encoding lectin (see fig 7.8). Both primer amino acid
sequences could be detected but no further homology with winged bean lectin
or any other lectin sequence could be detected. A search of a protein
sequence data base with the largest open reading frames yielded no
significant homologies with any other proteins.

The annealing temperature was lowered further to $41^{o}C$ and the
reactions carried out at four different magnesium ion concentrations.
Figure 7.9 shows the PCR products amplified under these conditions. At
magnesium ion concentrations of 4,5 and 6mM six bands could be visualised
on agarose gel (1.5kb, 1.15kb, 0.75kb, 0.60kb , 0.40kb and 0.30Kb). At the
lower magnesium ion concentration of 2mM the 1.5kb and 1.15Kb bands could
not be seen. Bands corresponding to the 1.5kb, 1.15Kb, 0.75Kb and 0.6Kb
products were excised and electroeluted from the gel as before. The ends of
the resulting DNA fragments were polished with T_4polymerase and ligated to
pUC19 restricted with Hinc II before transformation into *E.coli* DH5α
competent cells. Four clones, pCR1.8, pCR2.6, pCR3.4 and pCR4.7,
representing the four different size PCR products were sequenced using the

Figure 7.9

The effect of annealing temperature on the amplification of PCR product from winged bean genomic DNA. Four 100μl reactions containing 0.33μM primers , 200μM dNTPs and 2, 4, 5 and 6mM Mg^{2+} ions respectively were taken through 30 time/temperature cycles as in figure 7.6 except that the temperature of the annealing step was $41^{O}C$. PCR products were visualised on 0.8% agarose gel.

1. DNA size markers, Pst1 restricted λDNA.

2. 2mM Mg^{2+} reaction.

3. 4mM Mg^{2+} reaction.

4. 5mM Mg^{2+} reaction.

5. 6mM Mg^{2+} reaction.

Arrows indicate PCR products electroeluted for subsequent cloning.
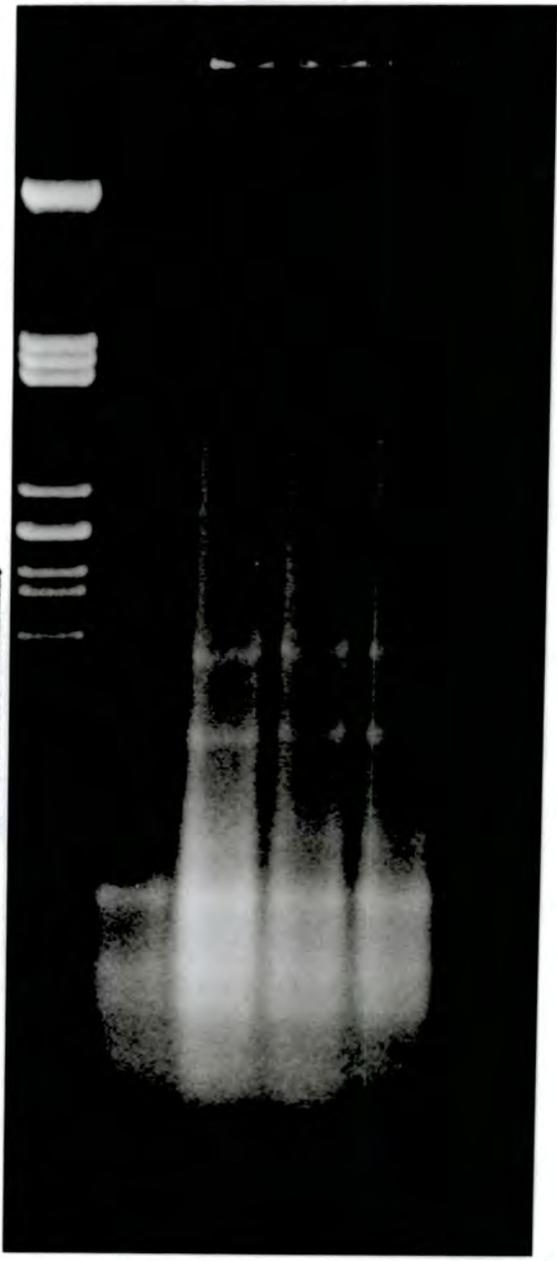
M13 dye labelled forward primer as before. The sequences obtained and their derived amino acid s equences are presented in figures 7.10 and 7.11. Two of the four sequences contain a region homologous to one of the primers used, however none of these clones contained large open reading frames, or part of an open reading frame similar to any of the legume lectins.

7.6 Design and use of an oligo nucleotide probe for winged bean basic lectin genes.

The number of variable parameters in PCR reactions make the sequencing of clones to determine the nature of the PCR products produced each time a long and laborious task. An oligonucleotide probe was designed which could be used in hybridisation experiments involving southern blots from agarose gels with the PCR products from different reactions. The oligonucleotide, shown in figure 7.12, was designed using the protein sequence data of a peptide known to reside near the C-terminal end of the mature protein, 10 amino acids upstream of the C-terminal peptide sequence used to design the C-terminal PCR primer. All subsequent PCR reactions were assayed for the lectin gene fragment using this internal oligonucleotide probe.

7.7 Design of a alternative N-terminal primer.

Although products from the previous PCR experiments appeared to be specific in that they amplified the same size fragments consistently in repeated experiments, sequencing had established that they were products of non-specific amplification events. A second N-terminal oligonucleotide primer was made (figure 7.13) in order that the N-terminal and C-terminal primers had similar properties i.e length, incorporation of inosine nucleotides and calculated melting temperatures (both minimum and maximum), this was to ensure that the two primers were likely to behave similarly

Figure 7.10

Nucleotide sequences and derived amino acid sequences of PCR clones containing a 1.5Kb and a 1.15Kb insert. Both inserts were sequenced using the forward m13 primer, data was collected on an ABI 373A DNA sequencer and amino acid reading frames were determined using the software package DNAsys.

B:PCR1.8.SEQ is 359 base pairs long.
Translation from 1 to 358.

```
         S  K  L  C  L  T Stp S  C  Q  S  T  L  N  L  W  I StpN  S
         Q  N  S  A  L  R  K  V  A  R  V  R  L  T  S  G  F  E  I  P
         K  T  L  P  Y  V  K  L  P  E  Y  A Stp P  L  D  L  K  F  L
1        TCAAAACTCTGCCTTACGTAAAGTTGCCAGAGTACGCTTAACCTCTGGATTTGAAATTCC


         C  L  Y  T  R  Y  W  P Stp F  T  R  T  L  C  S  L  S  N  R
         A  Y  I  P  G  I  G  H  N  L  Q  E  H  S  V  V  L  V  T  G
         L  I  Y  P  V  L  A  I  I  Y  K  N  T  L Stp S StpStp Q  G
61       TGCTTATATACCCGGTATTGGCCATAATTTACAAGAACACTCTGTAGTCTTAGTAACAGG


         G  K  G Stp G  F  T  R  C  E  I  S  H  C  S  R  N  P  R  C
         G  R  V  K  D  L  P  G  V  R  Y  H  I  V  R  G  T  L  D  A
         E  G  L  R  I  Y  P  V Stp D  I  T  L  F  E  E  P Stp M  L
121      GGGAAGGGTTAAGGATTTACCCGGTGTGAGATATCACATTGTTCGAGGAACCCTAGATGC


         C  R  S  K  G  S  S  T  R  P  F Stp C  V  V  D  S  Y  P  R
         V  G  V  K  D  R  Q  Q  G  R  S  S  A  L Stp I  L  I  Q  D
         S  E Stp R  I  V  N  K  A  V  L  V  R  C  R  F  L  S  K  T
181      TGTCGGAGTAAAGGATCGTCAACAAGGCCGTTCTAGTGCGTTGTAGATTCTTATCCAAGA


         L  V  S  F  E  N  A  M Stp I  A  R  K  M Stp S  V  W  L  T
         L  Y  H  L  K  M  P  C  E  S  L  E  R  C  K  V  Y  G Stp P
         C  I  I Stp K  C  H  V  N  R Stp K  D  V  K  C  M  A  N  P
241      CTTGTATCATTTGAAAATGCCATGTGAATCGCTAGAAAGATGTAAAGTGTATGGCTAACC


         H Stp R  E  F  R  K  G  I  G  A  G  H  R  D  P  R  D  L
         T  N  A  S  F. V  R  E  L  E  Q  A  T  E  T  H  E  T  C
         L  T  R  V  S Stp G  N  W  S  R  P  P  R  P  T  R  P
301      CACTAACGCGAGTTTCGTAAGGGAATTGGAGCAGGCCACCGAGACCCACGAGACCTGC=
                                   ‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾
```


B:PCR2.6.SEQ is 295 base pairs long.
Translation from 1 to 294.

```
         L  V  A  G  V  T  N  L  L  L  K  I Stp S  K  E  I  I  E  K
         W  L  Q  V Stp P  T  C  Y Stp R  Y  S  R  K  K StpStp K  R
         G  C  R  C  N  Q  P  A  T  K  D  I  V  E  R  N  N  R  K  E
1        TTGGTTGCAGGTGTAACCAACCTGCTACTAAAGATATAGTCGAAAGAAATAATAGAAAAG


         S  S  S  I  K  I  P  I  S  T Stp T  D  C  I  P  P  L  I  N
         A  P  V Stp R  S  P  L  V  R  K  P  I  V  Y  H  H StpStp T
         L  Q  Y  K  D  P  H Stp Y  V  N  R  L  Y  T  T  T  D  K  H
61       AGCTCCAGTATAAAGATCCCCATTAGTACGTAAACCGATTGTATACCACCACTGATAAAC


         T  R  I  G  Y  I  N  W  A Stp S  T  P  S  S  K  S  L  Y  R
         P  E Stp A  I  L  T  G  P  R  A  P  P  R  V  K  A  S  T  A
         Q  N  R  L  Y Stp L  G  L  E  H  P  L  E Stp K  P  L  P  Q
121      ACCAGAATAGGCTATATTAACTGGGCCTAGAGCACCCCCTCGAGTAAAAGCCTCTACCGC


         R  L  T  N  L  R  I  P  N  C  V  S  N  R  S  Y  M Stp R  I
         G Stp P  T Stp G  S  Q  I  A Stp A  I  G  L  T  C  K  G  S
         V  D  Q  P  E  D  P  K  L  R  E  Q Stp V  L  H  V  K  D  P
181      AGGTTGACCAACCTGAGGATCCCAAATTGCGTGAGCAATAGGTCTTACATGTAAAGGATC


         L  Y  P  C  L  K  S  P  L  P  S  H  R  N  P  R  V  P
         C  T  H  A  S  K  V  P  C  Q  A  T  E  T  H  E  Y
         V  P  M  P  Q  K  S  L  A  K  P  P  K  P  T  S  T
241      CTGTACCCATGCCTCAAAAGTCCCTTGCCAAGCCACCGAAACCCACGAGTACCT=
                                  ‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾
```

Figure 7.11

Nucleotide sequences and derived amino acid sequences of PCR clones containing a 0.75Kb and a 0.60Kb insert. Both inserts were sequenced using the forward m13 primer, data was collected on an ABI 373A DNA sequencer and amino acid reading frames were determined using the software package DNAsys.

B:PCR3.4.SEQ is 194 base pairs long.
Translation from 1 to 193.

```
      R  G  V  T  N  I  G  L  K  S  R  Q  M  L  V  G  R  K  L  E
       G  A  S  Q  I  L  V Stp K  A  A  K  C  W  L  E  E  S Stp N
        G  R  H  K  Y  W  F  E  K  P  P  N  V  G  W  K  K  V  R  T
1     CGGGGCGTCACAAATATTGGTTTGAAAAGCCGCCAAATGTTGGTTGGAAGAAAGTTAGAA


      L  S  L  F  V  M  N  F  L  R  L  M  D  V  L  V  I  W  Q  C
       F  L  C  L  S Stp T  F  L  G Stp W  M  F  W  L  S  G  S  V
        F  F  V  C  H  E  L  S Stp A  D  G  C  S  G  Y  L  A  V  L
61    CTTTCTTTGTTTGTCATGAACTTTCTTAGGCTGATGGATGTTCTGGTTATCTGGCAGTGT


      C  L  S  S  S  I  L  R  L  H  Q  H  S Stp V  G  L  P  N  I
       V  C  Q  A  V  F  C  D  F  T  N  I  P  R  L  G  C  Q  I  L
        F  V  K  Q  Y  S  A  T  S  P  T  F  L  G  W  V  A  K  Y  C
121   TGTTTGTCAAGCAGTATTCTGCGACTTCACCAACATTCCTAGGTTGGGTTGCCAAATATT


      V  Y  G  A
       F  M  V  R
        L  W  C
181   GTTTATGGTGCGG=
```


B:PCR4.7 .SEQ is 234 base pairs long.
Translation from 1 to 233.

```
      N  G  H  L  N  D  V  S  T  L  M  R  V  R  A  L  Q  R  L  D
       M  G  I  S  M  T  Y  P  L StpStp G  F  E  L  C  N  D  L  T
        W  A  S  Q Stp R  I  H  F  D  E  G  S  S  F  A  T  T Stp R
1     AATGGGCATCTCAATGACGTATCCACTTTGATGAGGGGTTCGAGCTTTGCAACGACTTGAC


      V  A  F  N  Q  M  K  D  N  I  N  A  L  I  A  S  K  K  Q  L
       S  H  L  T  R Stp R  T  I  S  T  P Stp L  P  A  K  N  S  L
        R  I Stp P  D  E  G  Q  Y  Q  R  L  N  C  Q  Q  K  T  A  Y
61    GTCGCATTTAACCAGATGAAGGACAATATCAACGCCTTAATTGCCAGCAAAAAACAGCTT


      I  E  G  I  A  H  E  L  R  N  T  V  S  A  P  A  L  S  K Stp
       L  K  V  S  L  T  N  C  E  T  P  L  V  R  L  R  Y  R  N  E
        Stp R  Y  R  S  R  T  A  K  H  R Stp C  A  C  V  I  E  M  K
121   ATTGAAGGTATCGCTCACGAACTGCGAAACACCGTTAGTGCGCCTGCGTTATCGAAATGA


      R Stp A  I  N  L  S  D  D  D Stp P  V  R Stp N  R
       D  E  R Stp T Stp A  T  T  T  N  L  Y  V  E  I  V
        M  S  D  K  P  E  R  R  R  L  T  C  T  L  K  S Stp
181   AGATGAGCGATAAACCTGAGCGACGACGACTAACCTGTACGTTGAAATCGTGA=
```

N-TERMINAL OLIGONUCLEOTIDE PRIMER FOR PCR

Amino acid sequence:- NH$_2$-**F**---**H**---**Q**---**N**---**E**---**E**---**Q**--COOH

Sense strand :   5' TT$_C^T$ CA$_C^T$ CA$_G^A$ AA$_T^C$ GA$_G^A$ GA$_G^A$ CA$_G^A$ 3'

With Inosines in place of A/G options.

PRIMER 267 :- 5' TT$_C^T$ CA$_C^T$ CAI AA$_T^C$ GAI GAI CA 3'

Figure 7.12 Design of oligo 267, an N-terminal primer for the amplification of the winged bean basic lectin.

Internal oligonucleotide probe for winged bean lectin sequences

Protein sequence data :-

$$\text{NH}_2\text{-V-----N------V----G-----F---COOH}$$

Sense strand :- $5'\text{G T N A A } {}^{T}_{C}\text{ G T N G G N T T } {}^{T}_{C}\ 3'$

Antisense strand:- $3'\text{C A N T T } {}^{A}_{G}\text{ C A N C C N A A } {}^{A}_{G}\ 5'$
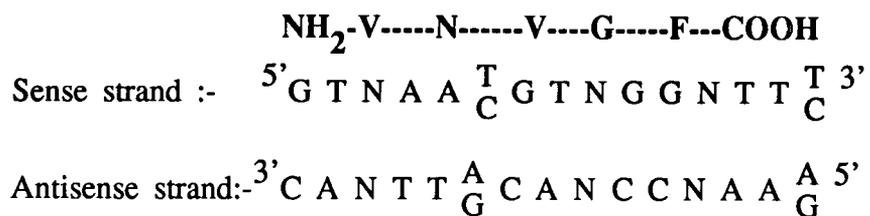
Internal oligonucleotide probe 257

$$5'\text{A A N C C N A C } {}^{A}_{G}\text{ T T N A C }\ 3'$$

Figure 7.13 Design of the oligonucleotide probe used in probing PCR products for lectin sequences.

under the same annealing conditions. The oligonucleotide primer was used in conjunction with the previous C-terminal oligonucleotide primer in PCR reactions with annealing temperatures of 50°C, 45°C and 41°C.

At 50°C PCR products observed appeared to be similar to those obtained using the previous N-terminal primer, i.e. a prominent band around 600bp in size accompanied by two less prominent bands of 300bp and 400bp. Additional faint bands of 900bp and 750bp could also be seen. These PCR products, immobilised on nitrocellulose, failed to hybridise with ^{32}P end labelled internal oligonucleotide when washed with 2×SSC at 25°C, whilst the 4507bp restriction fragment, from the λ DNA size markers used, did hybridise under these conditions. These products also failed to hybridise to the coding sequence of soybean lectin when washed at 50°C in 2×SSC.

At 45°C products of 600bp and 300bp were observed, however, when the PCR products were run on agarose gel no hybridisation to the internal oligonucleotide probe was observed. At 41°C two strong bands were observed when the PCR products were visualised on agarose gel, these were approximately 600bp and 400bp. Faint bands of 500bp and 300 bp could also be detected. The 600bp and 400bp products appeared to correspond to those seen using the original N-terminal oligo at this temperature, (see figure 7.14a), however none of these products hybridised with the ^{32}P labelled lectin specific oligonucleotide, number 257, under non stringent washing conditions of 25°C and 3×SSC (figure 7.14b).

Figure 7.14a

PCR products amplified from genomic winged bean DNA using oligonucleotide primer numbers 267 and 222. The PCR products amplified using the primers 267 and 222 with an annealing temperature of $41^{O}C$ were visualised on gel.

1. PCR reaction with no genomic DNA, 5mM Mg^{2+} ions, primers 116, 222.

2. 4mM Mg^{2+} ion amplification reaction using primer numbers 116, 222.

3. 5mM Mg^{2+} ion amplification reaction using primer numbers 116, 222.

4. DNA size markers, Pst1 restricted λDNA.

5. 5mM Mg^{2+} ion amplification reaction using primer numbers 267, 222.

6. 4mM Mg^{2+} ion amplification reaction using primer numbers 267, 222.

7. PCR reaction with no genomic DNA, 5mM Mg^{2+} ions, primers 267, 222.

Figure 7.14b

Hybridisation of a diagnostic probe for the winged bean lectin gene to DNA on a southern blot of the gel in figure 7.15a. An internal oligonucleotide was labelled with using polynucleotide kinase and $\gamma^{32}P$ dATP, this probe was allowed to hybridise with the southern blot for 48 hours at $25^{O}C$ in the presence of 6×SSC, then washed three times in 2×SSC at $25^{O}C$. The Filter was autoradiographed overnight at $-80^{O}C$. Lanes 1-7 are as described in figure 7.14a.

A

1 2 3 4 5 6 7

Kbp
0.80
0.51
0.34

B

1 2 3 4 5 6 7

Kbp
0·80
0·51
0·34
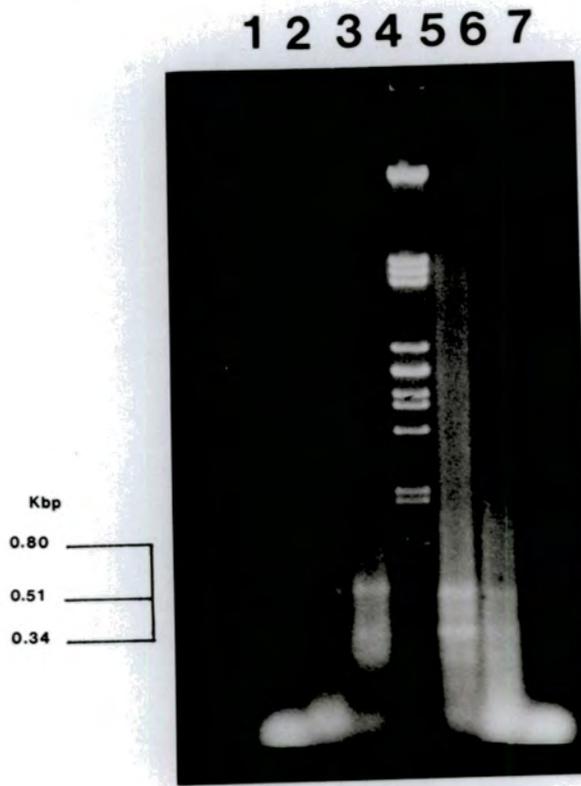
7.8 Effect of primer concentration on PCR specificity.

The effect of primer concentration on the specificity of the PCR reaction was studied in an attempt to isolate the winged bean lectin gene. The concentration of oligonucleotide primers was reduced 10, 100 and 1000 fold for both primer pairs i.e. 267, 222 and 222, 116. At $41^{o}C$ and 4.0mM Mg^{2+} there was no significant change in the size of PCR products when compared to those previously obtained using either set of primers i.e. 116 and 222 or 267 and 222. In general, a lowering of the primer concentration reduced the yield of products from a level similar to those obtained when using primers at a concentration of $30\mu M$ to a level at which no products at all were obtained (one thousand fold dilution). A lack of hybridisation of the diagnostic oligonucleotide to any products produced in these experiments, after washing with $2\times SSC$ at $22^{o}C$, confirmed that there had been no change in the specificity in any of these experiments.

In a further experiment, altering the primer concentrations, to take account of the redundancy differences between the primer pairs, yielded novel PCR products. Using the oligonucleotide primers numbers 116 and 222 at a ratio of 16:1, with a $45^{o}C$ annealing temperature and 3.5mM MG^{2+} an 850bp and a 700bp product was obtained after 33 amplification cycles. The use of primer numbers 267 and 222 at a ratio of 2:1 produced a 500bp product not normally amplified under the same annealing conditions of $45^{o}C$ and 3.5mM. In both experiments however, these different molecular weight products did not hybridise to the diagnostic probe under low stringency conditions.

7.9 Attempts to improve PCR specificity

The product Perfect Match (Stratagene) was used to destabilise any mis-matched primer-template complexes that would otherwise result in non-specifically amplified products. Amplification reactions

were repeated using primers 267 and 222 at annealing temperatures of 50°C and 45°C, in each case there were no high molecular weight primer products produced and only a 'primer smear' was observed. This product was not tested at lower annealing temperatures. This product was also tested on the primer pair 116 and 222. At 55°C and 50°C under Mg^{2+} concentrations of 1.5mM and 3.5mM no products were observed. This product may not be suitable for use with inosine containing primers it was, therefore, decided to use formamide to destabilise mismatched primers. Formamide was used at a concentration of 1% and 3% in standard PCR reactions with an annealing temperature of 45°C and Mg^{2+} concentration of 3.5mM. When using primer numbers 267 and 222 at ratios of 1:1 and 2:1 there was no PCR product observed. Primer numbers 116 and 222, when used at a ratio of 1:1 or 16:1, produced a 600bp product; when tested however, this product did not hybridise to the internal oligonucleotide probe.

A number of methods were used to amplify the winged bean basic lectin gene t able 7.3 summarises the products observed using oligonucleotide pairs 222 and 116, and 222 and 267 at different annealing temperatures under a variety of conditions. None of these experiments were successful in isolating a winged bean lectin gene.

Table 7.3 Summary of the reactions/ methods used in an attempt to clone the winged bean lectin gene. Standard PCR reactions were performed according to method 2.2.51 with alterations to either primer concentrations, magnesium ion concentration or annealing temperature as indicated in the table. The products obtained in each reaction and the means by which the product was tested for the presence of lectin like sequences is also indicated.

Primer Ratio	Mg^{2+} Conc. μM	Annealing Temperature ^{o}C	Amplified Products	Identification Method
Primers 116 and 222				
1:1	1.5	55	~400bp and 300bp	Too small
1:1	1.5	50	No products	N/A
1:1	3.5	50	No products	N/A
1:1	3.5	45	~550bp	Cloned
1:1	5.5	45	~550bp	Cloned
1:1	7.5	45	~550bp	Cloned
1:1	5.5	43	~600bp	Cloned
1:1	2.0	41	~750bp,600bp,400bp,300bp	Cloned
1:1	4.0	41	1.5Kb,1.15Kb,750bp,600bp,400bp,300bp	Cloned
1:1	5.0	41	1.5Kb,1.15Kb,750bp,600bp,400bp,300bp	Cloned
1:1	6.0	41	1.5Kb,1.15Kb,750bp,600bp,400bp,300bp	Cloned
1:1	4.0	37	No products	N/A
0.1:0.1	4.0	41	~600bp	Hybridisation
0.01:0.01	4.0	41	~600bp and ~400bp	Hybridisation
0.001:0.001	4.0	41	No products	Hybridisation
16:1	1.5	45	~850bp,~700bp,~600bp	Hybridisation
16:1	3.5	45	~850bp,~700bp,~600bp	Hybridisation
1:1	1.5	55+Perfect Match	No products	N/A
1:1	3.5	55+Perfect Match	No products	N/A
1:1	1.5	50+Perfect Match	No products	N/A
1:1	3.5	50+Perfect Match	No products	N/A
1:1	3.5	45+Formamide at 1%	~600bp	Hybridisation
1:1	3.5	45+Formamide at 3%	~600bp	Hybridisation
16:1	3.5	45+Formamide at 1%	~600bp	Hybridisation
16:1	3.5	45+Formamide at 3%	~600bp	Hybridisation
Primer 267 and 222				
1:1	1.5	55	No products	N/A
1:1	1.5	50	~700bp	Hybridisation
1:1	3.5	50	~750bp,~600bp,~400bp,~300bp	Hybridisation
1:1	5.5	50	900bp,750bp,600bp,400bp,300bp	Hybridisation
1:1	1.5	45	~600bp and ~400bp	Hybridisation
1:1	3.5	45	~600bp and ~400bp	Hybridisation
1:1	5.5	45	~600bp and ~400bp	Hybridisation
1:1	1.5	41	~600bp,~500bp,~400bp,~300bp	Hybridisation
1:1	3.5	41	~600bp,~500bp,~400bp,~300bp	Hybridisation
1:1	5.5	41	~600bp,~500bp,~400bp,~300bp	Hybridisation
0.1:0.1	4.0	41	~500bp and ~400bp	Hybridisation
0.01:0.01	4.0	41	~400bp	Hybrisiation
0.001:0.001	4.0	41	No products	Hybridisation
2:1	1.5	45	~600bp,~500bp,~300bp	Hybridisation
2:1	3.5	45	~600bp,~500bp,~300bp	Hybridisation
1:1	1.5	55+Perfect Match	No products	N/A
1:1	3.5	55+Perfect Match	No products	N/A
1:1	1.5	50+Perfect Match	No products	N/A
1:1	3.5	50+Perfect Match	No products	N/A
1:1	3.5	45+Formamide at 1%	No products	Hybridisation
1:1	3.5	45+Formamide at 3%	No products	Hybridisation
2:1	3.5	45+Formamide at 1%	No products	Hybridisation
2:1	3.5	45+Formamide at 3%	No products	Hybridisation

CHAPTER EIGHT

DISCUSSION

8.1 Initial analysis of winged bean seeds for toxic components.

A number of workers have studied the development of storage bruchidae on legume seeds, with a view to determining insect resistance factors present within the seeds (for reviews see Janzen (1969), Applebaum and Birk (1972), Southgate (1978), Birch *et al* (1985), Birch *et al* (1989).). In one such study by Dobie *et al* (1979), a number of cosmopolitan storage bruchidae were allowed to infest mature winged bean seeds. Both old and new world bruchids were used including *Callosobruchus maculatus, C. chinensis, C. analis, C. phaseoli, C. rhodesians, Zabrotes subfasciatus and Acanthoscelides obtectus*. All of these species used were able to lay eggs and their newly hatched larvae penetrated the testa of the beans. The larvae died subsequently during the first or second instars. The possible presence of toxins in the seed testa was investigated by allowing *C. maculatus* to develop in diets of cowpea flour meal mixed with winged bean seed testa. There was no statistically significant difference in survivability of larvae reared on this diet when compared to those reared on a cowpea control diet. The cotyledons of winged beans were, therefore, deemed to be either nutritionally deficient for , or toxic to, developing bruchid larvae (Dobie *et al* 1979).

Simmonds *et al* (1989), whilst assaying 182 accessions of *Phaseolus sp.* for attack by old and new world bruchids, described six different stages in the progression of the bruchid-legume interaction at which resistance may occur. These were, 1) oviposition, 2) embryo development, 3) penetration of the testa, 4) growth of larvae within the cotyledon, 5) pupation and 6) emergence. It was noted that the most effective and consistent resistance in the *Phaseolus sp.* operates against new world bruchids at early stages of development i.e. stages 1-3 described above. Resistance to the old world bruchids occurred in the later stages of development i.e. stages 4-6.

It appears then that the old world bruchids e.g. *C. maculatus* are affected at a metabolic level and are relatively catholic in their choice of seeds on which they lay their eggs, whilst the new world bruchids e.g. *Zabrotes subfasciatus* tend to be more species specific in their egg laying and more tolerant of metabolic resistance factors, this being explained by the close evolutionary relationship between the new world bruchids and *Phaseolus*.

The bioassays used in these studies used *C. maculatus*, an old world bruchid, whose catholic egg laying and susceptibility to toxic seed components make it useful in determining the toxic factors present in the seeds.

Lipid antimetabolites have been reported in the the winged bean; these include fatty acids such as eicosenoic acid, which has been implicated in causing myocardial lesions, and behenic acid which is antinutritional at levels of 0.58mg/g. The winged bean is also known to contain a number of potential protein antimetabolites, namely trypsin inhibitors, chymotrypsin inhibitors and lectins. Initially crude protein extracts were used to determine the toxicity of any of these proteins present. The seeds were defatted with hexane and all protein fractions were either dialysed against distilled water/ 0.1M ammonium bicarbonate or desalted on a gel filtration column to ensure the removal of lipid and low molecular weight compounds e.g. sugars and non-protein amino acids.

The protein content of winged bean seeds varies between 20% and 30% (w/w), with the major albumin component WBA-1 accounting for 15% of total protein (Kortt *et al* 1989). The other albumins make up an estimated 30% of the extractable protein, and globulin fraction proteins account for the remaining 55%. The distribution of antimetabolites between these two fractions commonly finds the protease inhibitors in the albumin fraction whilst the lectins are often found in the globulin fraction. The results of

the bioassays using these two groups of proteins showed that they both conferred resistance to the artificial seeds and that the toxicity was statistically significant (p>0.01) at levels of incorporation which paralleled the physiological concentrations found in the mature winged bean seeds. On termination of the bioassays it was apparent that the decrease in adult emergence and the increased median development period was due to the poor growth of the larvae as dissection of the bioassay pellets revealed that those larvae left were small and poorly developed. The increased median development period and poor larval development reflect a chronic rather than acute toxic effect of the added protein fractions. In a few cases adults were fully formed but found dead just beneath the seed surface, unable to make the normal window in the testa by which they would escape. This effect is generally considered to be due to malnutrition (Simmonds *et al* 1981), an effect consistent with the presence of antimetabolic proteins within these fractions.

The toxic effects of both the albumin and globulin fractions confirmed the existence of a proteinaceous defense factor in the mature winged bean seed capable of preventing the normal development of *C. maculatus* larvae. The albumin fraction appeared to be more toxic to the developing larvae than that of the globulin fraction which suggested a role for the winged bean trypsin inhibitor in the defense mechanism. Analysis of these two fractions using SDS-PAGE confirmed the presence of the 20,000 MW Trypsin inhibitor in both fractions.

Two fractionations (ammonium sulphate fractionation and isoelectric precipitation) were performed to try and further isolate the toxic components of the winged bean seed proteins. SDS-PAGE analysis of the ammonium sulphate fractions of the seed protein extract demonstrated that the majority of the proteins precipitated over a fairly narrow range. This

phenomenon has also been observed by others workers (Gillespie and Blagrove 1978). The most toxic ammonium sulphate fractions (in terms of LC_{50}) were precipitated over the range 40%-60% and analysis of these fractions revealed a complex mixture of polypeptides and, therefore, no correlation could be made to toxicity and the causal polypeptides. The bioassays did, however, confirm the presence of proteinaceous defense factors. The fractionation of winged bean seed proteins using differential solubility/ isoelectric precipitation proved more effective in the separation of groups of polypeptides and bioassay of these fractions helped to rule out the toxicity of some proteins, particularly fraction psophocarpin A as this consisted, essentially, of the 40,000MW protein psophocarpin A (Gillespie and Blagrove 1978). Analysis of this fraction for both lectin activity and serine protease inhibitory activity demonstrated that there was a very low level of lectin activity and a reasonably low level of trypsin and chymotrypsin inhibitory activity in comparison to the the other psophocarpin fractions tested. Fraction psophocarpin B had by far the highest haemagglutination titres of the three fractions and marginally higher trypsin and chymotrypsin inhibitory activity than the psophocarpin C fraction. The bioassays using the these fractions identified psophocarpin B as the most toxic fraction and in general the toxicity of each fraction paralleled the the activity of the trypsin inhibitor, and lectin.

8.2 α-Amylase inhibitory activity of winged bean seeds.

Experiments on a total protein extract and on psophocarpin A, B and C show that there was no α-amylase inhibitor present active against Porcine, locust, *Bacillus*, *Aspergillus*, and *Heliothis* α-amylases. Ideally, as there are a number of reports of the differing specificities of α-amylase inhibitors, these protein samples should have been tested with gut extracts from *C. maculatus* to ascertain that an α-amylase inhibitor specific to this

organism was not present, but the small size of the larvae and their

maturation within hard seed pellets make isolation of the gut extracts for

the assay difficult; in addition α-amylase inhibitors specific to *C.*

maculatus amylase have not been observed in other legume species. There

have been no reports in the literature of the presence of α-amylase

inhibitory activity in winged bean, and the seeds have few low molecular

weight polypeptides (<20,000 MW) typical of α-amylase inhibitors in other

legumes.

8.3 Protease inhibitors of winged bean

The trypsin inhibitors indigenous to winged beans are predominantly of

the Kunitz type i.e. MW 20,000 and low in sulphur. A low molecular weight

Bowman-birk type inhibitor has been identified (Shibita *et al* 1986, Hirano

1989) but this is present in very low amounts and has not been purified.

The total trypsin inhibitor preparation purified by affinity chromatography

gave a diffuse band at approximately 20,000 MW on SDS-PAGE (suggesting

slight heterogeneity in polypeptide molecular weight), in agreement with

the data presented by Shibita and co-workers who estimated the trypsin

inhibitors to be in the range 20,000 to 20,300 MW. A chymotrypsin inhibitor

present in winged bean seeds at 0.1-0.7% (Shibita *et al* 1986) which also

has trypsin inhibitory activity is likely to be isolated by this system.

Chymotrypsin inhibition assays confirmed the presence of a chymotrypsin

inhibitor, but the low activity in comparison to the inhibitory activity

present in a total winged bean seed extract suggests that the inhibition is

due to the trypsin/chymotrypsin inhibitor and not contaminating

chymotrypsin inhibitors. In addition to the bands of approximately 20,000

MW observed on SDS-PAGE analysis of the purified trypsin inhibitor fraction

used in the insect bioassays,polypeptides of 9,000 ± 1,000 MW were also

observed as a minor component. This lower molecular weight polypeptide is

not seen in direct protein extracts from winged bean seeds and is likely to
be an artifact generated by cleavage of the native trypsin inhibitor when
using the trypsin-Sepharose affinity column; such cleavage of inhibitors
during purification on affinity columns has been noted by a number of
workers . That the low molecular weight product was a by- product of
purification could have been determined by N-terminal sequencing of the the
polypeptide as the entire protein sequence of the Kunitz type winged bean
trypsin inhibitor has been determined (Yamamoto *et al* 1983). The
physiological concentration of trypsin inhibitors in the winged bean seed
has been estimated at 2-3% (Shibita *et al* 1986, Yamamoto *et al* 1983, Kortt
et al 1979) and the bioassays were performed with this in mind. The results
showed a statistically significant increase in the numbers of larvae
surviving to adulthood on diets containing >2.5% added trypsin inhibitor,
therefore a role for the trypsin inhibitor in defense against bruchid
attack seems unlikely , certainly in terms of being the primary
antimetabolite. The increase in the number of larvae surviving to adulthood
and the increased growth rate is likely to be due to the increased protein
content of the diet, particularly the essential amino acids, arginine,
leucine, threonine and valine which along with the sulphur amino acid
cysteine are abundant in the winged bean trypsin inhibitors (Yamamoto *et al*
1983, Kortt 1979) and make up for deficiencies of these in cowpea seeds.

The trypsin inhibitors that have been studied with respect to insect
defense include inhibitors from the Bowman-Birk (Gatehouse *et al* 1980)
Kunitz and potato families (Green *et al* 1972). The Bowman-Birk inhibitors
appear to be toxic to a wider variety of insects than than those of the
other classes, which may be explained in terms of their stability within
the insect gut. Bowman-Birk inhibitors do show considerable stability
toward heat and pH extremes due to the high degree of disulphide bonding

whilst their compact tertiary structure make them more stable to insect proteases. In this respect the winged bean inhibitor may be susceptible to the pH conditions or proteases in the bruchid gut.

8.4 The Lectins of winged bean seeds.

The purification of lectins to homogeneity on a large scale presents problems, as most lectins appear in multiple forms that differ only slightly in their chemical and physical properties. The isolation of the winged bean basic and acidic lectin isomers on a large enough scale to use in insect bioassays was considered impractical and therefore the isomers that constituted the basic lectin fraction and the isomers present in the acidic lectin fraction were purified together. A number of affinity matrices have been used in the isolation of winged bean lectins (see table 8.1) and this method apparently offers a simple means of their purification. However, the most commonly employed matrices, namely Sepharose-6-amino-caproyl-D-galactose and p-amino-phenyl-β-D-galactoside Sepharose 4B have given variable results in the literature. Puepkke (1979) reported that neither of these resins bound lectin from winged bean, while both Appukattan and Basu (1981), and Higuchi and co-workers (1984) have isolated basic and acidic lectins respectively using these resins. Kortt (1984) reported that Sepharose N-caproyl-galactoseamine was a non-specific affinity matrix for winged bean lectins. It is unlikely that varietal differences between winged bean lines explains this phenomenon, since in the seeds of the related species *Psophocarpus scandens* contain lectins with similar physiological and chemical properties to those of *P.tetragonolobus*. Kortt (1988) used columns containing lactose and melibiose linked to a solid support successfully in the isolation of acidic and basic seed lectins from *Psophocarpus tetragonolobus* and *P.scandens*, and lectins from *P.tetragonolobus* tubers, and was also able to purify lectins from a number

Affinity Matrix	Elution buffer	Lectin isolated	Authors
Lactobionate AE Biogel P-150		Acidic	Puppeke (1979)
Sepharose-6-amino caproyl-D-galactoseamine	200mM lactose 200mM galactose	Acidic Basic	Appukutan +Basu (1980) Khan (1986)
p-aminophenyl-β-D-galacto-pyranoside sepharose 4B	100mM galactose	Basic	Higuchi and Iwai (1985)
Lactosyl AE Biogel P-200	200mM galactose	Acidic	Pantajali (1988)
Cross linked Guar gum	100mM galactose	Tuber lectin (basic)	Shet and Madaiah (1987)
Lactose-sepharose 6B	300mM galactose	Acidic	Kortt + Caldwell (1985)
Mellibiose Biogel P-150	300mM galactose	Basic	Kortt + Caldwell (1985)

Table 8.1

Affinity media used in the preparation of acidic and basic lectins

from winged bean. Elution conditions and lectins purified are indicated

of different winged bean varieties. Despite the apparent reproducibility of this system neither the acidic or basic lectin could be isolated, in the present study, in the quantities expected, i.e. 2-3mg /g seed meal. Haemagglutination assays showed that the lectins were not binding to the columns, and therefore a protein extract was isolated by ammonium sulphate fractionation in an attempt to remove inhibitors of binding. The haemagglutination activity per mg of protein was two fold higher in the 95% ammonium sulphate fraction when compared to the total extract, and an increase in the binding of the basic lectin was observed; however the yield was still much lower than anticipated, suggesting very weak binding to the column. Kortt reported the unexpected failure of *P.scandens* acidic lectins to bind to lactose-Sepharose under conditions that quantitatively adsorb the *P.tetragonolobus* acidic lectins, even though the affinity for lactose is very similar (Kortt 1988)

The acidic and basic lectins can be separated by gel filtration on Sephacryl S-200, even though their molecular weights are very similar (54,000 and 58,000). This is a result of differential retardation of the lectins on the column due to their differing sugar binding activities; Sephacryl, being a polymer of allyl dextran and acrylamide, interacts weakly with both the acidic and basic lectin. Kortt (1985) was able to demonstrate a shift in the mobility of these two proteins on gel filtration with a similar media (Ultrogel Ac44) with buffer containing 0.3M galactose. The acidic lectin apparent molecular weight changed from 32,000 to 45,000 whilst the basic lectin apparent molecular weight changed from 38,000 to 58,000. It was decided not to use galactose to aid in the separation of the acidic and basic lectin by gel filtration because of the risk of contaminating the columns with bacteria (especially as bacteriostatic compounds could not be used in fear of carrying them over into insect

bioassays). The basic lectin used in the bioassays was of approximately 95%

purity, as judged by SDS-PAGE. The statistically significant reduction

in the numbers of *C.maculatus* larvae surviving to adulthood, and the

increase in the median development period of these larvae, provide

convincing evidence for the this lectin's role in insect defense against

storage bruchidae. Because of the impurities present in the acidic lectin

fraction used in the insect bioassays the antimetabolic effects of this

protein have not been fully elucidated. The differing sugar binding

specificities of the two lectins present in the winged bean seeds may mean

that that they serve different roles within the same tissue.

On the basis of the present results, there appears to be a role for

winged bean lectin in the resistance of seeds to attack by storage

bruchidae; earlier results have suggested that other lectins may play a

similar protective roles against bruchid attack, e.g. PHA, wheat germ

agglutinin, rice lectin , datura lectin, tomato lectin, nettle lectin and

pea lectin have all be shown to be toxic toward the storage bruchidae

C.maculatus (Chrispeels *et al* 1990, Boulter *et al* 1990). More recently the

potential of lectins as insect resistance factors has been surveyed;

Murdock and co-workers (1990) carried out an extensive bio-assay of

commercially available lectins, although a number of the preparations used

in the bio-assays were not pure they contained significant lectin content

and a number of lectins were deemed toxic toward *C. maculatus* because of

the increase in development period which they caused. Five lectins, peanut

agglutinin, osage orange, potato, jimson weed and wheat germ agglutinin

caused statistically significant increase in development of 9.8, 8.5, 3.6,

4.1 and 6.5 days at 1% incorporation in an artificial seed diet. These

workers were also able to detect a dosage response of 0.49 day and 1.47 day

increase in development period for each 0.1% increase in incorporation of

peanut agglutinin and wheat germ agglutinin in the artificial diets. The authors suggest (based on calculations using these figures) that natural seeds containing 1% wheat germ agglutinin would significantly affect the population build up of storage bruchids in a large seed store when compared to a lectin deficient seed. The potato, jimson weed and wheat germ agglutinins are all N-acetyl glucosamine binding proteins, and it was proposed that their effect may be mediated by interaction with the insect peritrophic membrane. Murdock and co-workers (1990), in contrast to other workers, were unable to detect statistically significant decreases in the development of bruchid beetles when using pea lectin, PHA or datura lectin in artificial diets. Total numbers of surviving adults were not recorded in this survey, and it may be that these lectins reduce the total number of surviving insects without affecting their developmental period. It has been argued that, in the case of PHA, the lectin used in previous trials were not pure and that the toxic factor was an α-amylase inhibitor (Chrispeels 1990).

The activity of lectin proteins against other insects has hardly been explored; there have been few bioassays of lectins with other orders of insect, presumably because lectins are expressed in seeds and therefore not encountered by the majority of phytophagous insects. Two lectins, from pea and soybean, have been tested against lepidopterans. The pea lectin has been tested in *in vivo* assay on transgenic tobacco plants. Although reduction in insect survival was not statistically significant, the insect biomass (mean weight of survivors) and the leaf area eaten were all significantly reduced in this assay, with the insect biomass reduced four fold compared to control plants (Boulter *et al* 1990). Soybean lectin has been tested in artificial diets against *Manduca sexta* and was found to have a significant effect on larval growth at 1% incorporation in the diet. The

artificial diet used was similar to that used in the assays here, and neonate larvae were used in this assay. The effect of the lectin was monitored by recording biomass (Shuckle and Murdock 1983). No studies on the effects of these proteins on late instar lepidoptera have been performed.

8.5 Bio-assay of Lepidoptera

With the possibility of expressing lectins in the leaves and other tissues in transgenic plants it is worth while determining the toxicity of these proteins toward other pests, those of the order Lepidoptera were chosen here as this order accounts for a significant percentage of pest control expenditure.

Although *Spodoptera littoralis* is not regarded as a major pest of any one plant the genus *Spodoptera* has been recorded as feeding on 40 different plant families containing at least 87 species of economic importance. *Heliothis virescens* is also a polyphagous pest of many economically important crops. The ability of these two insects to grow on a number of different diets makes them easy to culture on artificial diets that can be used to detect deleterious compounds.

The artificial diets in this study were based on those of Singh (1980). This diet has been optimised for the growth of many phytophagous lepidopterans including *H.virescens and S.littoralis*. First instar larvae (neonates, 1-2 days old) were used in the early feeding trials. These larvae will not have fed on any diet prior to inoculation onto the test diets and are of equivalent biomass, two important factors in ensuring experimental reproducibility. *Heliothis* larvae have cannibalistic tendencies so these were not incorporated on replicates at levels higher than five larvae/replicate.

There was no statistical decrease in the survival of *Heliothis* grown

on an artificial diet including winged bean meal from ones reared on the control diet. However, there was a marked decrease in biomass, which, when analysed, was found to be statistically significant($p<0.02$). The winged bean meal was incorporated in the test diet at ~11% as a direct replacement to the normal haricot bean meal constituent, and it was not determined whether the change in insect biomass compared to those on a control diet correlated with the amount of meal in the diet. This would be an obvious experiment to pursue, although the variance in the data obtained may make it difficult to determine a statistically significant correlation. The number of insects that would have to be analysed in order that 95% of the sample data lay between 5% of the mean survival figure is estimated to be between 200 and 300 i.e. 20 to 30 replicates of each diet. (This was calculated on the assumption that the data was normally distributed).

In the assay of *S.littoralis* against winged bean seed meal the detrimental effects of this diet were more pronounced, and there was a statistically significant ($p<0.02$) difference in the survivability of insects reared on this diet compared to those on the control diet. These two experiments have therefore shown that the winged bean seed meal may be detrimental to the development of Lepidopteran larvae. That a protein may be involved in this effect was demonstrated by bioassay with autoclaved winged bean meal. Autoclaving of winged bean meal has been shown to be an effective means of denaturing the toxic constituents, namely lectin and trypsin inhibitors (anon 1974). This meal when tested for haemagglutination activity gave a much lower titre than the untreated meal. In this assay older (2nd-3rd instar) lepidopteran larvae were used in order that the change in insect biomass could be monitored (neonates being to small to weigh accurately). Although a statistically significant correlation between the three diets could be made i.e. that the larvae were

able to survive better on the autoclaved diet, it was apparent that the older instar larvae were able to survive better than the first instar larvae used in the other assays. A bioassay using 4th instar larvae was unable to show a deleterious effect using the winged bean diet. This has shown the importance of using young larvae in these experiments. Late instar larvae consume large amounts of food (up twice their own body weight in 24 hours) but a large percentage of this food is not used by the insect and passes through the gut, the percentage passed through depending on the quality of the food. On artificial diets that have been optimised for the insect concerned the food will be of good quality and passed through at a high rate, which may mean that chronic antimetabolites may not have time or be present in high enough local concentrations to exert an affect. In neonate larvae the food is not passed through the gut so quickly so the affects of antimetabolites in the diet become more pronounced. The feeding of older larvae under non-ideal conditions in which the the passage of food thorough the gut will be slower may favour the detection of chronic antimetabolites.

8.6 Developing Lepidopteran diets

Although the method of using glass fibre discs, as a medium to absorb compounds, to assay for feeding deterrent properties has been useful in detecting feeding deterrents of Lepidopterans (Gatehouse A.M.R. personal communication) the loss of weight and quick death of larvae on this diet was not considered appropriate for the study of proteins that may exert their effect through a digestive mechanism, i.e poor uptake through the gut wall or inhibition of digestive proteins. The communion hosts were used as larvae survived on these for longer and the presence of minimal amounts of carbohydrate and protein in the hosts ensured some gut enzyme activity. The toxic effects of psophocarpin A, B and C were assayed using this system,

and all three fractions proved toxic. Psophocarpin A was the most toxic fraction, followed by psophocarpin B and C . Winged bean trypsin inhibitor has been incorporated in artificial diets of Lepidoptera at levels up to 5% without any decrease in the survivability of neonate larvae (J. Flemming, personal communication), and it is therefore possible that the winged bean lectin is again the insect antimetabolite present. The lectin PHA used at the 5% level in this diet was not toxic, with larvae reared on this diet faring better than those on the control diet suggesting that they were able to utilise the protein. The role of winged bean lectin as an antimetabolite to Lepidopterans has not been assayed as yet, but this host bio-assay system may prove useful in the analysis of both chronic and acute antimetabolites, having the advantage of there being few complicating factors in the diet. It does unfortunately become a very time consuming and labour intensive assay method if sufficient numbers of larvae are to be screened to gain statistically significant results from the trial.

The idea of designing a more natural diet based on tobacco leaves (a natural diet for both *Spodoptera* and *Heliothis*) would clearly have significant advantages. Application of the protein directly to leaves was considered but this has the problem of spreading the protein in an even layer over the the leaf. If for example an area of leaf is left uncovered it would be difficult to determine whether this was the area eaten (or not eaten) by the larvae. The diet devised in which protein could be mixed in with the agar before it had set ensures an even distribution throughout the diet. Two disadvantages of this system became obvious, bacterial growth and water condensation and evaporation grossly affected insect development. The problem of bacterial growth was remedied with the inclusion of chlorotetracycline in the diet, which was shown to be non-toxic to lepidopterans at the concentration used. The problem of water evaporation

and condensation might be overcome by using a higher percentage agar. However, the diet was not developed further because of the problems with leaf material. This bio-assay system offers the best potential for studying the toxicity of antimetabolic proteins whilst using a 'natural' diet. However, expression of the protein in a transgenic plant would offer a superior alternative to any of the bio-assay systems used for Lepidopterans.

Insect survival for neonates appeared to be a fair method of studying the toxicity of antimetabolites as these organisms are more susceptible. Older larvae must have their development assayed by monitoring changes in biomass as they are more resistant to antimetabolites. Both Spodoptera and Heliothis are armyworms and as such will move on from one food source to the next as it is used up, and this ability to utilise a number of diets in the later stages of development may explain their resistance to antimetabolites in general when they are in a mature larval form. Whether plants can express protein in a high enough concentration to cope with attack by late instar larvae is unknown. Bioassays of transgenic plants, with older larvae have shown low levels of resistance compared to first instar larvae (Hilder V.A. personal communication), and this is an aspect of genetic engineering of plants for insect resistance that needs to be addressed.

8.7 Mechanisms of lectin toxicity.

Lüning and Bartels (1926) were probably the first workers to suggest a connection between bean toxicity and haemagglutinin content, and since this time much information has been gathered on the toxicity of lectins. Lectins from legume seeds have proved toxic to a number of organisms including rats (Honvar et al 1962), chicks (Jayne-Williams 1973) and insects (Janzen 1976). The winged bean lectins have been shown to be toxic to rats (Higuchi

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et al 1982) and chicks (Higuchi *et al* 1981).

The precise mechanism of toxicity of lectins remains unclear. Not all lectins produce toxic effects in experimental animals, and toxicity very often depends on the animal species being tested. One explanation of these variations may be related to the differences in the susceptibility to digestion of different lectins; for example , pepsin inactivates soybean lectin rapidly and this lectin is much less toxic to rats than is kidney bean lectin which is not completely inactivated by pepsin even after 6 days digestion (Jaffé 1980). It is clear from studies using immunofluorescence techniques that the lectins bind to the gut wall in both mammals (King *et al* 1979) and insects (Gatehouse *et al* 1984). In bruchid beetle larvae PHA lectin binds to the mid-gut epithelium. In a more detailed study using rat gut the major lectin-positive sites for the same lectin were found to be in the non-crypt regions of the villi in the proximal region of the small intestine. In both cases there was evidence of disruption of the cell surfaces, and lectin could be localised on the distal side of the gut epithelium. Binding to the gut wall and cell surface disruption leads to reduced villi, reduced absorptive surface and a concomitant decrease in food absorption. Binding or disruption by lectins of brush border enzymes e.g. carbohydrases and dipeptidases, is considered to interfere with the final stages of nutrient hydrolysis and transport (Kim *et al* 1976). *In vitro* experiments using intestinal loops (Pope *et al* 1975) and everted sac techniques (Jaffé and Canjeo 1961) were able to show decreased active and passive transport of nutrients through the gut wall following binding of bean lectin. In particular, nitrogen loss in lectin fed animals is very pronounced; this is not only due to the passage of food through the gut without absorption but to the loss of endogenous proteins of the gut wall. A number of other effects of lectin have been reported for mammalian

organisms, including modulation of endocrine hormonal control e.g. the decrease in insulin (Pusztai *et al* 1986), interaction with the immune system leading to hypersensitivity (Puzstai *et al* 1986) and impairment of body defense mechanisms leading to tissue invasion by intestinal microflora (Jayne-Williams and Burgess 1974), a theory supported by the fact that lectins toxic to conventional rats and chicks are not toxic to their germfree counterparts.

In contrast to the body of work on higher animals the primary, secondary and systemic effects of lectins in the diets of insects has not been investigated. It is likely that the lectin disrupts the transport of nutrients across the gut wall in an analogus fashion to that found in mammals and interaction of lectins with insect peritrophic membranes may also be a causative factor in lectin toxicity. The binding of lectin to the insect gut wall has been demonstated (Gatehose *et al* 1984). Lectins have been shown to cross the insect gut wall in *in vitro* experiments (Gatehouse J.A personal communication) there may, therefore, be systemic effects of lectins on insects.

8.8 The potential of lectins as insect resistance factors.

Although many lectins have the potential to be used as insect resistance factors in crop protection, it is debatable whether commercial organisations will encourage the introduction of genes that code for proteins that are toxic to either humans or livestock. There are few examples of lectins toxic toward insects being innocuous to other animals. It has been suggested that pea lectin is not noxious to humans (Boulter *et al* 1990), but it has however proved to be toxic to pigs, monogastric animals of similar gut physiology (Huisman *et al* 1983). When the precise mechanisms of lectin binding and toxicity are discovered it may be possible to search for lectins that are more insect specific. Chrispeels (1990) for

example has suggested that the chitin-binding lectins may be toxic to insects because they bind to the peritrophic membrane lining the insect gut (although the mechanism of toxicity was not explained), this structure is not present in the mammalian gut and thus the lectins might not be toxic. However, no experimental evidence to support this conclusion has been presented.

Among the main advantages of using lectin genes in conferring insect resistance is that they are plant derived genes, and hence the correct expression and processing of the subsequent protein should present few problems. Lectins are targeted to the protein bodies of cells of the cotyledon and tuber or vacuoles in other tissues i.e. root and stem. Such sequestering of the protein should not affect the physiology of the plant; indeed expression of pea lectin (Boulter *et al* 1990) and soybean lectin (Goldberg *et al* 1984) under the control of a constitutive promoter in tobacco have had negligible yield penalties, and no physiological differences of these plants in comparison to controls were observed. The use of tissue specific promoters to express the resistance gene enables the expression of the toxic gene to be restricted to defined plant tissues without affecting the commercial product. For example, the expression of a lectin gene solely in potato leaves would leave tubers free from the toxic gene product, but resistance to leaf eating predators would still be conferred. This approach is being pursued by at least one biotechnology company (Edwards G.A. personal communication).

Lectins are normally highly expressed, easily extracted, have a convenient assay for activity, produce antibodies of high avidity and belong to families for which there is a considerable amount of amino acid sequence data available. Given these criteria, the cloning and isolation of lectin genes should, in principle, be a relatively straightforward process.

The winged bean lectin whilst proving toxic to the bruchid beetle *Callosobruchus maculatus* and being the likely toxic factor in this beans resistance to *Chilo parlatus* (Singh and Sarup 1985) , other *Callosobruchus sp.*, *Acanthoscelides obtectus* and *Zabrotes subfasciatus* (Dobie *et al* 1979) is also toxic to rats (Higuchi *et al* 1984) and chicks (Higuchi *et al* 1981) Its toxicity toward lepidopterans has not been fully determined. The toxicity of this lectin toward mammals may not make it an immediate choice for the production of insect resistance crops.

8.9 Heterologous probing experiments.

A number of different methods have been used to isolate lectin genes. These include probing of cDNA libraries with oligonucleotides, probing of genomic libraries with cDNA clones, and the production of cDNA libraries highly enriched for lectin sequences using polysome isolation with lectin antibodies. The *Dolichos biflorus* lectin gene was isolated from a cDNA library using a degenerate oligonucleotide probe that had been designed to the N-terminal amino acid sequence that encompassed the conserved phenylalanine residues at positions six and ten (Schnell and Etzler 1987). The oligonucleotides used in the PCR experiments in this thesis were to be used in a similar manner to probe for the winged bean basic lectin gene. Although probing of a northern blot with these oligonucleotides did not prove successful it was hoped that they may still have been useful for that purpose, as the failure of the probes to hybridise to a lectin sequence could be due to the combined effects of probing with a degenerate oligonucleotide and poor RNA transfer (the transfer of RNA from ethidium bromide stained gels is less efficient than in unstained gels (Sambrook *et al* 1989). Haemagglutination assays on extracts of cotyledons used in the isolation of RNA confirmed expression of the basic lectin and therefore the presence of lectin messenger RNA.

A soybean lectin cDNA was originally isolated from a library which had been enriched for lectin messenger RNA by immunoprecipitating polysomes with a lectin specific antibody (Vodkin *et al* 1983). Although enrichment for winged bean DNA sequences was possible, as the appropriate antibodies were available, a library constructed from all RNA messages was preferred as it would also have contained a number of trypsin inhibitor clones (another gene that may have insect resistance potential).

The lectin from *Phaseolus lunatus* was isolated from a cDNA library by making use of a previously isolated *Phaseolus* agglutinin gene and the soybean lectin gene. These two genes were used as heterologous probes of a maturing cotyledon cDNA library (Imbrie-Milligam *et al* 1989). Clones hybridised to these two probes at washing stringencies of 0.1×SSC at 65°C, a stringency (~94%) much higher than the relative homologies of these genes 57.9% (soybean lectin) and 70.0% (PHA). This information suggests that although no conclusive hybridisation of these probes was detected in the the Southern blotting experiments described in this thesis they may well be useful in the detection of lectin cDNA clones from a winged bean cDNA library. Heterologous probing experiments (chapter six) were relatively unsuccessful in determining the presence of a lectin gene and the optimum hybridisation stringencies. It was apparent that the pea lectin gene could not be used as a probe for winged bean lectin, as it did not hybridise to winged bean sequences at low stringencies. More extensive tests on the hybridisation of the *Phaseolus* and Soya lectin cDNA clones pPVL134 and pL9-2.7 with winged bean genomic DNA would have to be done to determine appropriate washing conditions. In standard southern blotting assays 10μg of genomic DNA is sufficient to detect single copy genes when using high specific activity probes (>10^7) 20μg samples used in these experiments should have allowed detection of low copy number genes, it was possible

that if a lectin gene family is present in the winged bean genome that the high hybridisation signals detected were not background and may have been lectin specific, this should have been investigated further. In *Phaseolus vulgaris* there are at least four genes encoding lectin polypeptides, or polypeptides with homology to lectin (PHA-L, PHA-E, α-amylase inhibitor and arcelin) and winged bean may be similar in containing a multigene family encoding lectins; it might contain at least two genes, one for acidic and basic lectins respectively, and the protein sequence variants found in the basic isolectins suggest multiple encoding genes. In soybean and pea however, there is only one active lectin gene although both plants have one or more pseudogenes which will cross-hybridise with a lectin cDNA probe at low stringency.

8.10 Homology amongst legume lectins.

Eight legume lectin gene sequences were compared in order to determine homology at the gene level, and to assess the possible hybridisation strategies that may be needed when using these genes as heterologous probes. The sequences of eight lectin clones were extracted from the Micro-Genie data base and were edited such that the coding sequences (including the signal peptide leader sequence) could be compared using the microcomputer software programs ALIGN and RDF2 (Pearson and Lipman 1988). The first program aligns the sequences pairwise and gives a percentage identity result , the second gives an optimised statistical score (Z value). This value is derived from the mean number of matches divided by the standard deviation, as such the higher the Z value the more likely that the the sequences compared are closely related. Pearson and Lipman suggest that any Z value greater than 10 is statistically significant. The results of these two tests when applied to the lectin genes from *Phaseolus vulgaris, Phaseolus lunatus, Dolichos biflorus, Glycine max* and *Pisum*

	PHAL	PINTO	PHAE	DBIF	PVUL	PLUN	GMAX	PSAT
PHAL	------	92.6	90.6	72.9	67.4	70.0	62.5	53.7
PINTO	236.18	------	91.1	73.6	70.4	69.8	62.2	53.9
PHAE	461.87	285.35	------	72.0	67.8	69.0	61.7	54.4
DBIF	106.54	150.75	193.28	------	61.3	61.0	60.8	54.8
PVUL	79.29	69.36	68.12	32.61	------	66.4	53.1	50.5
PLUN	126.78	97.24	179.57	87.90	49.42	------	57.9	53.1
GMAX	112.10	141.02	131.97	110.62	23.51	99.48	------	57.4
PSAT	50.20	52.84	84.77	60.75	28.18	35.33	78.17	------

Table 8.2.

Comparison of the nucleotide coding sequences of eight leguminosae lectins.
The open reading frames of the eight lectins were aligned, pairwise, using the
software package ALIGN and their percentage identities were recorded (figures
to the right of the diagonal). These aligned sequences were then used with the
software package RDF2 and Z values were recorded (figures to the left of the
diagonal). PHAL *phaseolus vulgaris* agglutinin L subunit, PHAE
Phaseolus vulgaris agglutinin E subunit, PINTO *P.vulgaris* cv.pinto
agglutinin, DBIF *Dolichos biflorus* agglutinin, PVUL *P.vulgaris*
α-amylase inhibitor PLUN *P.lunatus* agglutinin, GMAX *Glycine max*
agglutinin, PSAT *Pisum sativum* agglutinin

sativum are recorded in table 8.2.

Clearly the *Phaseolus* lectins are closely related to one another, with homologies ranging from 66.4% to 92.6% and their use as heterologous probes of other *Phaseolus sp.* lectin genes should be very successful using high stringency washing conditions. The *Glycine max* and *Dolichos biflorus* genes are less homologous to the other members of the Phaseolae tribe (53.1% to 73.6%), but it should be possible to use these as lectin gene probes for the *Phaseolus* family using slightly lower stringency washing conditions (e.g. 3×SSC at 50°C). The genera *Psophocarpus* is a member of the Phaseolae tribe, and is considered to be closely related to the genera *Glycine*, it is therefore likely that the lectins from *Psophocarpus sp.* share large regions of homology with the other Phaseolae lectins. This conclusion is supported by the observation that peptides sequenced from the winged bean basic lectin showed greater homology with the sequences of lectins from Phaseolae tribe than those of the Viciae tribe (figure 7.4). Use of a *Phaseolus* lectin cDNA as a heterologous probe was therefore not unreasonable.

Although the pea lectin is a double chain lectin, the gene is arranged such that the β subunit is coded for first, and thus the sequence arrangement is directly homologous to that in the single chain lectins. The homology of this lectin to the other lectins compared here was, however, relatively poor (54%) and this accounts for the lack of hybridisation observed in the southern blotting experiments of winged bean DNA with this probe.

A method of isolating the lectin gene that does not rely on the use of DNA probes is that of characterising clones in the library into different expression classes. The *Pisum sativum* lectin coding sequence *LecA* was originally isolated by probing a cDNA library with mRNA from which the

library had been derived in order to detect clones of different expression classes. The pea lectin gene was expressed at approximately 2% in this library (Gatehouse *et al* 1987). A similar method could be used in conjunction with heterologous probing or probing with oligonucleotides to isolate a winged bean cDNA clone. With this method it may be possible to distinguish between the expression of the acidic and basic lectin genes (if two genes exist), as the acidic lectin protein is expressed earlier than than the basic lectin protein during seed development (Higuchi *et al* 1988).

8.11 cDNA library construction.

Maturing winged bean seeds were chosen as optimal material for the isolation of RNA containing lectin mRNA as they produce lectin in relatively large quantities. Seed cotyledons are also suitable tissue for RNA extraction, as they have little lignified tissue or phenolic compounds which can make the extraction of nucleic acid difficult. Isolation of RNA from root tissue was considered to be more difficult, because of the high level of ribonucleases present in this tissue. The expression of lectin in root tissue was, however, considered to be at a high enough level to make this tissue a reasonable second source of RNA.

In the initial RNA isolation experiments, using cotyledon material, it was found that the Hot-SDS method, was not capable of inhibiting all the ribonuclease activity present within the cotyledons. This may well have been due to the high lipid content of the winged bean cotyledons limiting the effectiveness of the SDS detergent in the extraction buffer. A more potent ribonuclease inhibitor, not susceptible to high lipid concentrations, is guanidinium hydrochloride, the major constituent of the buffer used in the method of Chirgwin and co-workers (1977). This buffer enabled the extraction of relatively undegraded RNA.

Messenger RNA was isolated using oligo dT affinity chromatography in

either column or batch fashion. Although the column chromatography method was probably more efficient in the separation of polyA$^+$ RNA from ribosomal RNA it is more likely to lead to the introduction of ribonucleases in the purification process. The quality and yield of the mRNA isolated is the limiting factor in the length and yield of cDNA products. In the experiments performed here, the production of cDNA was monitored by the incorporation of ^{3}H dTTP into the first strand of cDNA synthesised using reverse transcriptase; in each case the yield of cDNA was lower than expected suggesting a poor quality polyA$^+$. A new commercial product for the isolation of poly A$^+$uses biotinylated oligo dT which binds strongly to streptavidin linked to magnetic particles, a rare earth magnet is then used in order to physically separate the mRNA from the ribosomal RNA. This system appears to be particularly efficient in both separation and production of good quality mRNA. This would be the method of choice in any subsequent polyA$^+$ isolation.

A final factor that has limited the size of the winged bean cDNA library is the poor yield of recombinants from the cDNA produced. Blunt end cloning of large DNA molecules is particularly inefficient and the addition of either adaptors or linkers to blunt ended molecules to create cohesive termini greatly improves the cloning efficiency (Sambrook *et al* 1989). In this case EcoR1 adaptors were used as they avoided a number of DNA manipulation steps needed when using linkers which may have reduced the yield further. Initially a problem in the separation of unincorporated adaptors was thought to be affecting the efficiency of cloning; however, following chromatography using sepharose CL4B (which allows separation of the adaptor dimers from small cDNAs) no marked increase in the efficiency of cloning was observed.

The cDNA library was to be screened differentially or with the use of

heterologous probes (Chapter 6). However, the production of antibodies specific to the winged bean lectin would have made the production of an expression library a reasonable alternative strategy to isolate the lectin gene. The use of lambda phage insertion vectors such as λgt11 or Lambda Zap (Stratagene) in any future experiment would combine the advantages of being able to probe for the lectin gene using both nucleic acid and antibodies, with the increased efficiency in cloning gained by using *in vitro* packaging of Lambda vector.

8.12 Genomic library construction.

Because of the lack of tissue available for further attempts to construct a cDNA library, an attempt was made to construct a genomic library using the Lambda replacement vector EMBL3 (Frischauf *et al* 1983). DNA yields from large winged bean leaves ($\geq$ 2 weeks old) were poor in comparison to young leaflets, presumably due to a combination of effects, including larger cells, and thus less DNA per unit fresh weight, an increase in toughening secondary cell wall components and the presence of phenolic compounds. The method used was, however, successful in purifying winged bean DNA in a reasonable yield (approximately 800μg/g leaf) which was of a high enough molecular weight for subsequent use in library construction. Genomic DNA partially restricted by Sau3A could be religated to itself (as observed by differential mobility on agarose gels). The insert DNA was dephosphorylated using calf intestine alkaline phosphatase to avoid a size fractionation step. This dephosphorylating appeared to have worked as the DNA could not be religated to itself. Integrity of the cohesive ends of these molecules was confirmed by subcloning a low molecular weight fraction into the plasmid pUC18, which had been restricted with the enzyme BamH1. In the method of library construction used the vector was prepared using a double digestion technique followed by isopropanol

precipitation to prevent competition of the central stuffer fragment in the ligation reaction. Small aliquots of the restricted vector were assayed for digestion on agarose gel before use in the ligation reactions. 1.13×10^4 clones were obtained from subsequent ligation experiments. This low efficiency was not due to inefficiency of the *in vitro* packaging extract, as the control vector (concatenated $\lambda c1857$ sam7) yielded the expected number of clones. That some spi resistant clones were obtained suggested that the poor efficiency was due to the low amount of packageable DNA, either because of incorrect insert size or more probably because of DNA molecules with damaged ends. The theoretical concentrations of vector and insert DNA required in a ligation reaction in order to obtain concatenated DNA can be calculated. It is, however, more sensible to use an empirical approach ,as used here, to cope with the problems of DNA molecules with damaged ends. In this respect the maximum ligation efficiency occurred at a insert to vector ratio of 4:1, a ratio higher than the 0.5:1 expected in theory and again suggesting that the insert DNA was not fully ligatable due to damaged ends.

Lambda replacement vectors having a polylinker region containing a Xho1 site can be used to increase the concatermerisation efficiency by using a filling in reaction with both vector and insert, which leads to the production of compatible half sites preventing self-ligation of both insert and vector DNA. This method can also be used without the need to size fractionate the insert DNA, and would be the method of choice. in any future genomic cloning experiments. An increase in the cloning efficiency has been reported with the use of bacterial strains which have mutations in their restriction systems i.e. mcrA,B. This can increase the efficiency of cloning by ten fold for eukaryotic genomes, particularly of those which have highly methylated DNA (Raleigh 1987).

8.13 Protein sequencing

Sequence data obtained from tryptic digests of purified winged bean lectin amounted to 44% of the basic lectin sequence (assuming a subunit molecular weight of 29,000). 28% of the sequence could be assigned positions in the primary sequence by homology with other lectin sequences. The peptide WB4-27 was identical to the previously published N-terminal sequence of the basic lectin B3 (Kortt 1984) and was confirmed to be the N-terminal sequence of the affinity purified lectin by N-terminal sequencing of the native protein. No microheterogenity was observed in this peptide or in the N-terminal sequence of the native protein, although this was expected as both Hirano (1989) and Kortt(1984) have shown that there are a number of basic lectin isomers that differ slightly in their N-terminal sequences (See Figure 8.1). Kortt has estimated the relative proportion of the different basic lectin isomers (B1, B2 and B3) to 0.3:1.0:0.9 respectively. It could be that in the present work that the affinity column was more specific for the B3 lectin, or that in the winged bean variety used, this is the predominant isomer. The region SFNFN-F-QN in the N-terminal sequence is highly conserved between the lectins of *Psophocarpus sp* (figure 8.1), which was why it was subsequently used to design oligonucleotide primers. The peptide VVNGVP was useful in confirming that the lectin being sequenced was the B3 lectin, as this peptide overlaps with the last amino acids that Kortt (1984) obtained for the N-terminal sequence of the B3 lectin using automatic sequencing. The probability that this peptide has a C-terminal lysine residue is very high as trypsin cleaves at basic amino acids and a lysine is present in Kortt's sequence next to this peptide sequence. Other peptide sequences obtained were clearly of lectin origin as they shared homology to other legume lectin sequences (see figure 4.3).

ACIDIC LECTINS   WS-20 TETQSFNFDHFEENSNELNL

WS-21 TEGQSFNFDXFEEN

WS-24 TETQSFNFDXFEENSNELN

SA1   TEIQSFNFNGFVPEN

SA3   TETQSFNFNVFEPEN

A1   TETQSFNFDHFEENSNELNLQRDALI

A3   TTEQSFNFDNFEENDNELNLQTDAL

BASIC LECTINS   WS-2 KTISFNFNQFDQNEEQL

WS-17 DTISFNFNQFFQ

WS-19 KTISFNFNQFFQNEEQDLLLP

B3   KTISFNFNQFHQNEEQLKLQRDARISSNGVLNL(T)KVVNG

SB1   ETISFNFNQFQQND

SB2   SQTQSFNFNKFEQNK

GSL   TETISFSFNNFEQDSNELIL

Figure 8.1

N-terminal amino acid sequences determined for *Psophocarpus*

tetragonolobus and *P.scandens* acidic and basic lectins. WS-21, WS-24

and WS-20, *P. tetragonolobus* acidic lectins (Hirano 1989); A1 and A3

P.tetragonolobus acidic lectins I and III (Kortt 1984); SA1 and SA3

P.scandens acidic lectin I and III (Kortt 1988); WS-2, WS-17 and WS-19

P.tetragonolobus basic lectins (Hirano 1989) SB2 and SB3 *P.scandens*

basic lectins II and III (Kortt 1988); B3 *P.tetragonolobus* basic

lectin B3; GSL *P.tetragonolobus* green shell lectin (Yagi *et al* 1990).

An X in the sequence indicates an undetermined amino acid.

8.14 Attempts to clone the winged bean lectin gene using PCR.

The PCR reaction, cycle conditions.

Conceptually the PCR reaction can be likened to the natural process of DNA replication, in that the number of DNA molecules generated by the PCR reaction doubles after each cycle. The important factor controlling this process is the choice of oligonucleotides which will flank a specific stretch of DNA to be amplified. These can form an initiation duplex from which the DNA polymerase can prime.

As the amino acid sequence and corresponding gene sequences of a number of lectins has now been reported it was possible to determine the codon specificities for the Phaseolae tribe (table 8.3) and possibly select peptide sequences with amino acids that had strict codon specificities. It was noticed that there were preferences in codon usage for the amino acids phenylalanine, serine, alanine, aspartic acid, asparagine, threonine, and isoleucine , which were as high as 90% e.g. ATC, the isoleucine codon favoured by the *Phaseolus vulgaris* lectin like sequence PHVL134. A preference for codons ending in C was widespread, however, no unequivocal codon bias could be assigned and therefore no amino acid preference was made in this respect.

The two oligonucleotides designed initially for the amplification of the winged bean basic lectin gene were the N-terminal primer number 116 (see figure 7.4.a) and the C-terminal primer number 222 (see figure 7.4b). These oligonucleotides were synthesised using data from stretches of amino acid sequences which were unambiguous, large enough to be able to design reasonable size oligonucleotides, i.e. ≥ 20 bases (such that the annealing temperature could be kept as high as possible) and had amino acids which were least redundant . Inosine was used in the design of the C-terminal

		DBIF	GMAX	PHVL	PHAL			DBIF	GMAX	PHVL	PHAL
AAA	Lys	2	5	5	6	CAA	Gln	4	5	7	3
AAG	Lys	7	7	5	3	CAG	Gln	1	1	1	4
AAC	**Asn**	**13**	**10**	**16**	**16**	CAC	His	3	3	3	0
AAT	Asn	1	6	4	8	CAT	His	0	3	0	2
ACA	Thr	2	3	2	5	CCA	Pro	1	8	0	0
ACG	Thr	4	3	3	5	CCG	Pro	1	1	0	0
ACC	Thr	7	8	11	9	CCC	Pro	8	3	3	8
ACT	Thr	3	4	3	2	CCT	Pro	2	4	2	1
AGA	Arg	3	2	1	0	CGA	Arg	0	0	0	1
AGG	Arg	1	2	1	3	CGG	Arg	0	1	1	1
AGC	**Ser**	**7**	**7**	**9**	**7**	CGC	Arg	5	1	2	2
AGT	Ser	0	1	0	0	CGT	Arg	1	0	2	1
ATA	Ile	0	2	0	1	CTA	Leu	1	0	1	2
ATG	Met	1	1	2	1	CTG	Leu	3	0	2	4
ATC	**Ile**	**10**	**7**	**12**	**10**	CTC	Leu	8	8	5	9
ATT	Ile	5	6	1	2	CTT	Leu	4	8	4	5
GAA	Glu	7	5	5	5	TAA	Stop	0	1	0	0
GAG	Glu	4	4	4	4	TAG	Stop	1	0	1	1
GAC	**Asp**	**10**	**8**	**11**	**11**	TAC	Tyr	6	2	4	4
GAT	Asp	4	9	5	5	TAT	Tyr	3	3	4	1
GCA	Ala	3	3	1	1	TCA	Ser	2	1	4	1
GCG	Ala	0	1	1	0	TCG	Ser	1	3	1	0
GCC	**Ala**	**14**	**10**	**8**	**10**	**TCC**	**Ser**	**18**	**7**	**10**	**10**
GCT	Ala	5	7	1	4	**TCT**	**Ser**	**6**	**11**	**9**	**10**
GGA	Gly	0	4	2	4	TGA	Stop	0	0	0	0
GGG	Gly	5	3	2	4	TGG	Trp	4	6	4	5
GGC	Gly	8	1	5	9	TGC	Cys	0	0	0	0
GGT	Gly	4	5	0	3	TGT	Cys	0	0	0	0
GTA	Val	2	1	1	0	TTA	Leu	1	1	1	1
GTG	Val	7	6	6	10	TTG	Leu	4	7	0	3
GTC	Val	8	5	8	5	TTC	**Phe**	**12**	**10**	**10**	**12**
GTT	Val	4	6	6	6	TTT	Phe	3	4	2	4

Table 8.3

Codon usage amongst legume lectins. The coding sequences of four legume lectins have been compared against protein sequence data and codon usage recorded. The amino acids related to each codon are indicated by their three letter code. Figures in bold indicate preferred codons.

primer to reduce the redundancy at wobble base positions. Inosine has the ability to base pair with Adenine, Thymine or Cytosine (Ohsuka *et al* 1985). Due to the redundancy of primer 116 and the inosine content of primer 222 it was not possible to use the relevant computer software to determine whether either of these primers had any significant degree of secondary structure (particularly at their 3' ends) or whether they had any complementarity to each other which may lead to erroneous priming and primer dimer formation. In the case of primer 116 the 3' sequence was made as unambiguous as possible by not including the wobble base of the final codon in the synthesised oligonucleotides as a DNA duplex with the 3' termini of the primer is essential for synthesis by *Taq* (Erlich *et al* 1991). It was chosen not to design restriction sites onto the ends of the primers to avoid any miss-priming that these may have encouraged (although there is no evidence that the addition of restriction site sequences at the 5' end of PCR primers affects their specificity and a number of workers have obtained clones using this system).

The melting temperature (Tm) of the two primers 116 and 222 were calculated using the formula :- (Stubbs et al 1980) $Tm = 4 \times (G+C) + 2 \times (T+A)$. This equation is normally used when the oligonucleotide is present in high salt concentrations (0.9M NaCl) and does not take into account the conditions in which most PCR reactions take place, but it suffices as a rough approximation for use in the planning of annealing temperatures in PCR reactions. The minimum and maximum melting temperatures for the oligonucleotides that constitute primer 116 were $52^{o}C$ and $66^{o}C$ respectively, and for primer 222 were $48^{o}C$ and $68^{o}C$ respectively. The temperature ranges, therefore, over which the two primers could anneal were very similar. It is, however, possible that whilst one primer anneals at a high temperature, e.g $66^{o}C$, the opposite primer might not bind because it's

melting temperature is much lower, e.g. 48$^{\text{o}}$C.

8.15 PCR conditions

The PCR reaction is a cyclic reaction of three steps, denaturation, annealing and extension. The denaturation step is probably the least critical part of the PCR process; the temperature for this part of the cycle has to be high enough such that there is dissociation of the duplex DNA and low enough such that the DNA polymerase remains active up to the completion of the PCR reaction. The half life of *Taq* DNA polymerase at 95$^{\text{o}}$C is 130 minutes (Gelfand 1989) and as such the denaturation step in a typical 25 cycle PCR experiment of 2 minutes at 92$^{\text{o}}$C will not significantly deplete the number of active polymerase molecules, whilst at this temperature and time period most DNA duplexes will have dissociated (Fenton-Williams 1987). The extension phase of the PCR cycle is generally carried out at 72$^{\text{o}}$C, which is the optimum temperature for the enzyme. At this temperature the enzyme will incorporate approximately eighty nucleotides/sec and hence any extension time greater than twenty seconds was considered long enough for the amplification of a lectin gene (which would be less than 1500 bp since no lectin yet sequenced contains introns). The annealing temperature is considered to be the most important part of the amplification cycle, as the temperature and time period here control the annealing of the primers, Too high a temperature causes the primers to dissociate and there is no amplification, too low a temperature allows the primers to bind non-specifically. Because the *Taq* polymerase is active at the lower temperature at which annealing takes place the time between annealing and extension is also important. Primers that bind non-specifically at the lower temperature may be extended sufficiently that they are not dissociated during the extension cycle. Annealing times less than a 2 minutes followed by rapid ramping rates (1$^{\text{o}}$C/sec) between

annealing and extension phases of the cycle should reduce this possibility. Annealing temperatures should be theoretically calculable using the primer base composition; however it is usual to work empirically, and alter the annealing temperature within the range 55°C (the melting temperature of a 50% GC rich 20mer oligonucleotide) to 35°C. In most of the PCR experiments performed the denaturing step and the extension step were kept the same, i.e. 92°C, 90 sec denaturing and 72°C, 90 sec extension. The annealing step was varied in attempts to improve primer specificity.

8.16 PCR experiments.

The initial amplification reaction at 55°C yielded two low molecular weight products (300 and 400 bp) which could have coded for sequences of 133 and 100 amino acids. Neither of these were considered to be the product required, as the expected length of the amino acid sequence was of the order of 200 amino acids, i.e approximately 600 bp. The annealing temperature used was fairly high, and it was likely that at this temperature only one of the primers was able to bind, leading to non-specific amplification products. Lowering of the annealing temperature to 50°C prevented the production of the lower molecular weight products, but no specific amplification product was observed at this temperature. At 45°C a single 579bp DNA fragment was amplified, but cloning and subsequent analysis revealed no homology to the lectin or any other protein in the data base. It has been suggested by (Saikai 1989) that the magnesium ion concentration in the reaction buffer has an effect on the polymerase enzyme specificity and therefore a magnesium ion titration was done at this temperature. The change in concentration appeared to have an effect on the yield of the amplification products but no obvious alteration of specificity was observed. The optimum magnesium ion concentration for this reaction was approximately 5mM. Lowering of the annealing temperature a

further $2^{O}C$ to $43^{O}C$ produced a specific product of 605bp. Again, however, when cloned and sequenced no reasonable homology could be observed with other lectins. A further lowering of the annealing temperature by $2^{O}C$ yielded a number of amplification products. Those in the size range expected and those that may have included introns. i.e. 1.5kb were isolated, cloned and sequenced. Again none of these clones contained lectin inserts. It was assumed that in all cases non-specific binding of primers had occurred.

8.17 Sequence analysis of PCR products.

Analysis of the six DNA sequences obtained from cloned PCR products formed under different reaction conditions revealed four sequences that contained regions homologous to the C-terminal primer (PCR 1.8, PCR 2.6, DH1.PCR and DH20.PCR), two of which on further sequencing were found to have a regions homologous to the N-terminal primer, see figures(7.6 and 7.8). No homology could be found to the N-terminal or C-terminal primers in either of the two remaining PCR products cloned. These two clones, PCR 3.4 and PCR 4.7, were derived from the lower molecular weight products (750bp and 600bp). They may have primer binding sites at the ends 3' to those sequenced but as they contained no lectin like sequences they were not studied further. Other workers have reported the formation of PCR products with only one primer binding site distinguishable in the resulting cloned sequence. None of the sequences cloned bore any significant comparison to one another as determined by nucleotide alignment software (NNCALN). Apart from the expected terminal primer binding sites no other obvious primer derived sequences could be observed which may have been the result of non-specific primer interactions. The G+C content of the clones (from 38% to 47%) and the size of the PCR products (i.e. above those expected for a primer dimer product) confirms that the products are due to

non-specific binding of the primers to random DNA segments and subsequent amplification.

8.18 Use of new primers, and oligonucleotide probes.

Alteration of both the magnesium ion concentration and the the temperature seemed to have little effect on the specificity of the reaction. It was decided therefore to design a new oligonucleotide primer. The primer was based on at the N-terminal amino acid sequence of the basic lectin as this region had the longest continuous stretch of amino acids from which to design a new oligonucleotide primer. A primer was designed (see figure 7.14) which had similar properties to the C-terminal oligo 222. The redundancy of the two primers was of the same order, 4 : 8, the number of inosine bases was similar as were the length of the oligos (21mer and 20mer) and the G+C content. The minimum and maximum melting temperature of the primers 267 and 222 were also closely matched, at 48-68°C and 50-62°C respectively. An oligonucleotide was also made for use as a probe of the PCR products, to the region VNVGF sequenced and thought to lie in a position internal to the primers, as deduced by homology to other lectins (figure 7.3). A number of experiments carried out with these new primers (see table 7.3), followed by probing of the PCR products, has failed to show the presence of an amplified lectin gene.

8.18 Attempts to increase primer specificity

A number of approaches have been used in attempts to increase the primer specificity and hence obtain a lectin clone. These include both altering of primer concentrations, and the addition of compounds to the PCR reaction which destabilise mis-matched duplexes. Reducing the primer concentration serves to limit mispriming (Saiki 1989) and therefore to increase specificity. When the concentration of the PCR primers was reduced there was a decrease in the yield of non-specific products; however, there

was no concomitant increase in the yield of specific products. It has been considered that an excess of one primer over another, induced by their differing redundancies, may alter the specificities of the PCR reaction. Although workers using degenerate primers have not reported this to be a problem, reactions in which the primer concentrations had been altered, to allow for differences in redundancies, were undertaken. No lectin specific products were observed when probing with the lectin specific probe despite differences in PCR products being observed. These empirical methods of controlling specificity by altering primer concentrations in the PCR reaction were also complemented with the use of compounds that destabilised mismatched duplexes i.e formamide and the product Perfect Match (Strategene). Formamide has been used by other workers to improve the specificity of PCR (Sarkar *et al* 1990) its effect mediated by its ability to destabilise mismatched duplexes. The use of formamide with both primer pairs did not yield a specific lectin product that would hybridise to the internal oligonucleotide probe. DMSO has also been used by some workers to destabilise non-specific priming (Winship 1989). However, DMSO (at 5% v/v) is known to inhibit DNA synthesis by *Taq* polymerase by 50% and was therefore not used. No specific products were produced when the product Perfect Match was used in PCR with annealing temperatures of 55°C and 50°C. Use of this product at lower annealing temperatures may lead to more specific products, but it is also possible that the presence of inosine oligonucleotides in the the primers may affect the activity of Perfect Match. It is not known what the nature of this commercial product is.

The C-terminal primer was used to probe a northern blot of cotyledon RNA, to determine whether this primer was capable of binding to the lectin sequence. No hybridisation was observed at low stringency washes, although annealing to ribosomal RNA was observed at this stringency. Possibly this

primer is the cause of the failure to amplify lectin sequences.

A number of possible experiments remain to be explored which may yield a lectin gene. In a recent report Robbins and Morris (1991) have reported the amplification of the gene dihydroflavanol reductase using redundant oligos and cDNA but the gene could not be amplified from genomic DNA. It would appear that the abundance of the sequence in the poly A^+ RNA makes the PCR reaction much more feasible, possibly be because the lack of specificity of the primers is outweighed by the less complex mix of starting sequences. It should be feasible to attempt to PCR the lectin message from cotyledons, pod or mature root RNA, following reverse transcription of the messages. (Frohdam et al 1988). Trimethylammonium chloride has also been used as a specificity enhancer in PCR reactions (Hung et al 1990) this chemical has not been tested, but it may successfully enhance the specificity of the primers used here.

Ideally, the complete sequencing of the lectin protein would allow a much better design of primer i.e. optimal length and redundancy with a concomitant increase in specificity of binding.

8.19 Future experiments

Recently Sawhney and Shobhana (1990) reported the use of desialylated mucin immobilised in gluteraldehyde cross linked gelatin to purify winged bean acidic and basic lectins by elution with D-galactose and D-lactose respectively. This method may be useful in the purification of these lectins in large quantities in order to continue the areas of research considered in this thesis.

More studies need to be done with both the acidic and basic lectin to determine their range of insect toxicity. These would include a number of economically important pests such as Diabrotica sp. (rootworms), Colorado beetle, locust, etc. . Tests using minimal artificial diets may prove most

useful in these studies. Acidic lectin toxicity has not been confirmed or refuted in this thesis, and it would therefore be desirable to compare this lectin's toxicity to insects with the basic lectin.

The mechanism of action of lectins as toxins/antimetabolites to insects has not been studied extensively. The availability of the basic lectin antibodies would allow immunofluoresence studies of lectin binding to be carried out, which may help elucidate the mechanism.

An interesting aside may be the involvement of lectins in chitin binding or chitinase activity. A number of lectins toxic to insects have chitin binding properties, and more recently a number of enzyme activities have shown to be stimulated by lectins e.g. invertase (Vattuone *et al* 1991). It would be interesting to see A) whether winged bean lectin has any chitin binding activity B) whether it stimulates chitinases activity.

Lectin expression has been studied using haemagglutination activity, but no detailed study has been conducted as to to the expression of lectin as a pathogenesis related response. This could be investigated using a dot blot immuno-detection system using wounded leaves, salicylic acid or elicitors. These experiments may help to address the question of the role of plant lectins.

The protein signature data base Prosite (Bairoch 1991) contains two sequence signatures that can be used to recognise all the the legume lectins sequenced to date, these are:-

1)   [LIV]-X-[EDQ]-V-[FYW]-V-X-[LIV]-G-[LF]-[ST]

2)   [LIV]-[STAG]-V-[EQV]-[FL]-D-[ST]

The first signature contains the invariant Ca^{2+} binding domain while the second is a signature for an invariant region in the α subunit.

These signatures are capable of detecting all 36 legume lectin sequences on the EMBL data base. Oligonucleotides designed using these sequences may be

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Figure 8.2

The nucleotide alignment of legume lectin coding sequences using CLUSTAL 4. The open reading frames of the eight lectins were aligned, using the software package CLUSTAL 4 with gap fix and gap vary parameters of ten. PHAL *phaseolus vulgaris* agglutinin L subunit, PHAE *Phaseolus vulgaris* agglutinin E subunit, PINTO *P.vulgaris* cv.pinto agglutinin, DBIF *Dolichos biflorus* agglutinin, PVUL *P.vulgaris* α-amylase inhibitor PLUN *P.lunatus* agglutinin, GMAX *Glycine max* agglutinin, PSAT *Pisum sativum* agglutinin

```
PHAE  - PHASEOLU  ATGAATGCATACA---TGGCTTCCTCCAA----CTTA-----CTCTCCCTAGCCCT--CT
PHAL  - PHASEOLU  ATGAATGCATACA---TGGCTTCCTCCAA----GTT--------CTTCACTGTCCT--CT
PVUL  - PHASEOLU  ATG---------------GCTTCCTCCAA----GTTA------CTCTCCCTAGCCCT--CT
PINTO - PINTO B   ATGGCTTCCTCCA------ACTTACTC---------T---------CCCTA--GCCCT--CT
PLUN  - PHASEOLU  ATGGCTTCTTCCG---T--CCTACTC-------GTT------CTCTCACTGTCACT--GT
PSAT  - PISUM SA  ATGATCTC------GTT-------CT----ACGCG---ATATTTCTATCCATTCTCTTAACA
GMAX  - GLYCINE   ATGGCTACTTCAAAGTTGAAAACCCAGAATGTGGTTGTATCTCTCTCCCTAACCTTAACC
DBIF  - DOLICHOS  ATGGCTTCCTCCA---CAGTCTCT---------GTTG-----TCCTCT----CCCT--CT
                  ***                                                          *

PHAE  - PHASEOLU  TC-----CTTGTGCTTCTCACCCAC---GCAAACTCAGCCAGCCAAACCTCCTTCAGCTTC
PHAL  - PHASEOLU  TC-----CTTGTGCTTCTCAGCCAC---GCAAACTCAGCAACGATATCTACTTCAACTTC
PVUL  - PHASEOLU  TC-----CTTGCGCTTCTCAGCCAC---GCAAACTCAGCCACCGAAACCTCCTTCATCATC
PINTO - PINTO B   TC-----CTTGTGCTTCTCACCCAC---GCAAACTCAGCCAGCCAAACCTTCTTCAGCTTC
PLUN  - PHASEOLU  TC----CTAGTTCTACT-ACTTAC---GCAAGCCTCAGC--TGAACTCTTTTTCAACTTT
PSAT  - PISUM SA  ACAATCCTT-----TTCTT-----CAAGGTGAACTCAACTGAAACCACTTCCTTCTTGATC
GMAX  - GLYCINE   TTGGTACTGGTGCTACTGACCAGCAAGGCAAACTCAGCGGAAACTGTTTCTTTCAGCTGG
DBIF  - DOLICHOS  TC-----CTGCTGCTTCTCACCCAA---GCAAACTCAGCCAACATCCAATCCTTCTCCTTC
                         **     * **         *   * *               *   ***

PHAE  - PHASEOLU  CAAAGGTTCA------ACGAAACCAACCTT-ATCCTCCAACGCGATGCCACCGTCTCATCC
PHAL  - PHASEOLU  CAAAGGTTCA------ACGAAACCAACCTT-ATCCTCCAACGCGATGCCTCCGTCTCATCC
PVUL  - PHASEOLU  GATGGGTTCA------ACAAAACCAACCTT-ATCCTTCAAGGCGATGCCTCCGTCTCATCC
PINTO - PINTO B   GATAGGTTCA------ACGAAACCAACCTT-ATCCTCCAACGCGATGCCTCCGTCTCATCC
PLUN  - PHASEOLU  CAAACGTTCA------ACGCAGCCAACCTT-ATCCTCCAAGGCAATGCA---GTCTCATCC
PSAT  - PISUM SA  ACCAAGTTCAGCCCCGACCAACAAAACCTAATCTTCCAAGGAGATG--GCTATACCA-CA
GMAX  - GLYCINE   AACAAGTTCGTGCCCGAAGCAACCAAACATGATCCTCCAAGGAGACGCTATTGTGACCTCC
DBIF  - DOLICHOS  AAAAACTTCA------ACTCACCCAGCTTC-ATCCTCCAAGGTGACGCCACCGT---ATCC
                  ***           *   *      *  ***  ****   * * *        *     *

PHAE  - PHASEOLU  AAAGGCCAGTTACGACTAACCAATGTTAATGACAACGGAGAACCCACGTTGAGCTCTCTG
PHAL  - PHASEOLU  TCCGGCCAGTTACGACTAACCAATCTTAATGGCAACGGAGAACCCAGGGTGGGCTCTCTG
PVUL  - PHASEOLU  AACGGCCAACTACAACTATCCTAT---------------AATTCATAC--GACTCTATG
PINTO - PINTO B   TCCGGCCAGTTACGACTAACCAATGTTAACAGCAACGGAGAACCACGGTGGGCTCTCTG
PLUN  - PHASEOLU  AAAGGCCACTTACTACTAACCAATGTTACACACAACGGAGAACCCAGTGTGGCATCTA-G
PSAT  - PISUM SA  AAAGAGAAGCTGACACTGACCAA-------GGCAGTAAAGAAC-----------ACTGT-
GMAX  - GLYCINE   TCGGGAAAGTTACAACTCAATAAGGTTGACGAAAACGGCACCCCAAAACCCTCGTCTCT-
DBIF  - DOLICHOS  TCCGGCAAGTTACAACTCACCAAAGTCAAGGAAAACGGCTT-TCCACTCCGTTTTCCCTC
                  *   *  *  ***    *                                *

                           A  F  Y  S  A  P  I  Q  I  D
PHAE  - PHASEOLU  -GGCCGTGCCTTCTACTCCGCCCCCATCCAAATCTGGGACAACACCACCGGCGCCGTGGC
PHAL  - PHASEOLU  -GGCCGCGCCTTCTACTCCGCCCCCATCCAAATCTGGGACAACACCACCGGCACCGTGGC
PVUL  - PHASEOLU  -AGCAGAGCCTTCTACTCCGCCCCCATCCAAATCAGGGACAGCACCACCGGCAACGTCGC
PINTO - PINTO B   -GGCCGCGCCTTCTACTCCGCCCCCATCCAAATCTGGGACTACACCACCGGCAAC---GC
PLUN  - PHASEOLU  -GGCCGCGCCTTGTACTCCGCCCCCCATCCAAATCAGGGACAG----CACCGGCAAC---GC
PSAT  - PISUM SA  TGGCAGAGCCCTCTATTCCTCACCTATCCATATCTGGGATAGAGAAACAGGCAACGTTGC
GMAX  - GLYCINE   TGGTCGCGCCCTCTACTCCACCCCCATCCACATTTGGGACAAAGAAACCGGTAGCGTTGC
DBIF  - DOLICHOS  CGGTCGCGCCTTCTACTCCTCCCCCATCCAAATCTATGACAAGTTCACAGGCGCCGTCGC
                  *  *  ***  *  **  ***  *  **  *****  **      **      **  **    *    **

PHAE  - PHASEOLU  C-GCTTCGCCAACCTCCTTCACATTCAATATCGACGTTCCCAA-CAATTCAGGAC-CCGC
PHAL  - PHASEOLU  CAGCTTCGCCA-CCTCCTTCACATTCAATATACAGGTTCCCAA-CAATGCAGGAC-CCGC
PVUL  - PHASEOLU  CAGCTTCGACA-CCAACTTCACAATGAATATCCGCACTCACCG-CCAAGCAAATT-CCGC
PINTO - PINTO B   CAGCTTCGACA-CCAACTTCACATTCAATATCCTCGTTCCCAA-CAATGCAGGAC-CCGC
PLUN  - PHASEOLU  GAG-TTCGACA-CCAACTTCACA-TATCTTCA-TATACTCTCCAACAAATTTTCCAAAATGTCAC
PSAT  - PISUM SA  TAATTTTG-TAACTTCCTTCACTTTTGTCATAAATGCACCCA-ACAGT-TACAACGTTGC
GMAX  - GLYCINE   CAGCTTCGCCG-CTTCCTTCAACTTCACCTTCTATGCCCCT-GACACAAAAAGGC-TTGC
DBIF  - DOLICHOS  CAGCTGGGCCA-CCTCCTTCACAGTCAAGATTTCAGCTCCCAG-CAAGGCATCCT-TCGC
                  *  *      *        *****    *       *                   *   -  -    *

PHAE  - PHASEOLU  CGATGGCCTTGCCT-------TTGTTCTCCTCCCCGTGGGCTCTCAGCC-CAAAGACAAAG
PHAL  - PHASEOLU  CGATGGACTTGCCT-------TTGCTCTCGTCCCCGTGGGCTCTCAGCC-CAAAGACAAAG
PVUL  - PHASEOLU  CGTTGGCCTTGACT-------TTGTTCTCGTCCCCGT------CCAGCC-CGAATCCAAAG
PINTO - PINTO B   CGATGGCCTTGCCT------TTGCTCTCGTCCCCGTGGGCTCTCAGCC-CAAAGACAAAG
PLUN  - PHASEOLU  AGACGATCCTGCCTGGCTTTTTGCTCTCGTCCCGGTCGACTCTCAGCC-CAAAGAAAAAG
PSAT  - PISUM SA  CGACGGGTTTAC----GTTCTTCA---TCGCACCTGTAGATACTAAGCCGCAGAC-CGGCG
GMAX  - GLYCINE   AGATGGACTTGCCT-----TCTT--TCTCGCACCAATTGACACTAAGCCACAAACACA-TG
DBIF  - DOLICHOS  CGATGGGATTGCCT------TCGCTTGGTACCCGTCGGGTCTGAGCC-CAGACGCAACG
                  *  *      *          *     *     **  *      ****  *   *      *

                                                                 V  A
PHAE  - PHASEOLU  GCGGTCTTCTAGGTCTGTTCAACAACTACAAATACGACAGCAATGCCCATACTGTGGCTG
PHAL  - PHASEOLU  GGGGTTTTCTAGGTCTTTTCGACGGCAGCAA------CAGCAATTTCCATACTGTGGCTG
PVUL  - PHASEOLU  GCG-----------------------------------------ATACTGTGACTG
PINTO - PINTO B   GCGGTTTTCTAGGTCTTTTCGACGGCAGCAA------CAGCAATTTCCATACTGTGGCTG
PLUN  - PHASEOLU  GCCGTCTTCTAGGTCTTTTCAACAAATCCGAAAACGATATAAACGCCCTTACTGTGGCTG
PSAT  - PISUM SA  GTGGATATCTCGGAGTTTTCAATAGCGCAGAGTATGATAAAACCACTCAAACTGTTCGCTG
GMAX  - GLYCINE   CAGGTTATCTTGGTCTTTTCAACGAAAACGAGTCTGGTGA-------TCAAGTCGTCGCTG
DBIF  - DOLICHOS  GTGGCTATCTAGGTGTTTTCGACAGCGATGTCTACAACAACTCCGCCCAGACTGGTGGCAG
                                                                       **  * *  *

                  V  E  F  D  T                                    H  I  G
PHAE  - PHASEOLU  TGGAGTTCGACACCCTCTACAACGTTCACTGGGACCC------CAAACCGCGTCATATTG
PHAL  - PHASEOLU  TGGAGTTCGACACCCTCTACAACAAGGACTGGGACCC------CACAGAGCGTCATATTG
PVUL  - PHASEOLU  TGGAGTTCGACACCTTCCTCAGC---------------------------CGTATTA
PINTO - PINTO B   TGGAGTTCGACACCCTCTACAACAAGGACTGGGACCC-------CAGAGAGCGTCATATTG
PLUN  - PHASEOLU  TGGAGTTCGACACTTGCCACAACCTGGACTGGGAC----------AAAAAC-TC------
PSAT  - PISUM SA  TGGAGTTTGACACTTTCTATAATGCTGCATGGGATCCAAGCAACACAGAGATAGACATATTG
GMAX  - GLYCINE   TTGAGTTTGACACTTTCCGGAACTC----TTGGGATCC-------ACCAAATCCACACATCG
DBIF  - DOLICHOS  TGGAGTTCGACACGTTGTCCAACAGCGGCTGGGACCC------CAGCATGAAGCACATTG
                  * *****  *****          *                                *
```

```
                                  I   D  V  N
PHAE  -- PHASEOLU  GCATCGACGTGAACTCCATCAAGTCTATCAAAACGACGACGTGGGATTT-TGTCA------
PHAL  -- PHASEOLU  GCATCGACGTGAACTCCATCAGGTCTATCAAAACGACGCGGTGGGATTT-TGTGA-----
PVUL  -- PHASEOLU  GCATCGACGTGAACAACAACGA---TATCAAAAGCGTGCCTTGGGATGTACACGACTACG
PINTO -- PINTO B   GCATCGACGTGAACTCCATCAAGTCTATCAAAACGACGCCGTGGGATTT-TGTCA-----
PLUN  -- PHASEOLU  --CATCGCCGTGAAC------CTGGG---TATCGGAAGCGTGCCTTGGAATTTCCGCGACTACG
PSAT  -- PISUM SA   GAATCGATGTGAACAGTATCAAATCCGTAAACACTAAGTCGTGGAAGTTGC------AGA
GMAX  -- GLYCINE    GAATTAACGTCAATTCTATCAGATCCATCAAAACGACGTCTTGGGATTTG-GCCA--ACA
DBIF  -- DOLICHOS   GGATCGACGTGAACTCCATCAAGTCTATCGCAACGGTGTCGTGGGATTT-GGCCA-----
                       **    ** **            *     *    *   *** *  *

PHAE  -- PHASEOLU  AAGGAGAAAACGCGGAGGTTCTGATCACCTATGACTCCTCCACGAAGCTCTTGGTGGCTT
PHAL  -- PHASEOLU  ACGGAGAAAACGCCGAGGTTCTGATCACCTATGACTCCTCCACGAATCTCTTGGTGGCTT
PVUL  -- PHASEOLU  ACGGACAAAACGCCGAGGTTCGGATCACCTATAACTCCTCCACGAAGGTCTTCTCGGTTT
PINTO -- PINTO B   ACGGAGAAAACGCCGAGGTTCATATCACCTATGAGTCCTCCACGAAGCTCTTGGTGGCTT
PLUN  -- PHASEOLU  ACGGACAAAATGCCGATGTTCTGATCACCTATGACTCCTCCACGAAGTTCTTGGCGGTTT
PSAT  -- PISUM SA   ATGGTGAAGAGGCTAATGTTTGTGATAGCTTTTAATGCTGCTACTAATGTGTTAACTGTTA
GMAX  -- GLYCINE    ATAAAGTA---GCCAAGGTTCTCATTACCTATGATGCCTCCACCAGCCTCTTGGTTGCTT
DBIF  -- DOLICHOS   ACGGCGAAAACGCCGAGATTCTGATCACGTACAACGCCGCCACCTCCCTTTTGGTGGTTT
                     *       *   ** *   **    ** *  *   *   *  * **    * **    * *

PHAE  -- PHASEOLU  CTCTGGTTTACCCT----TCTCT--------GAAAACA-----AGCTTCATCGTCTCTGACA
PHAL  -- PHASEOLU  CTCTGGTTTACCCT----TCTCA--------GAAAACG-----AGCTTCATCGTCTCTGACA
PVUL  -- PHASEOLU  CTCTGTCAAACCCT---TCTAC--------GGGAAAG-----AGCAACAACGTCTCTACCA
PINTO -- PINTO B   CTCTGGTTTACCCT----TCTCT--------GAAAACA-----AGCTTCACCGTCTCTGACA
PLUN  -- PHASEOLU  CTCTGTTTTACCCT----ATTAC--------GGGAAAG-----AGAAACAACGTCTCTGCAA
PSAT  -- PISUM SA   GTTTGACCTATCCTAATTCACTTGAGGAAGAGAATGTAACTAGTTATACTCTTAGCGACG
GMAX  -- GLYCINE    CTTTGGTCTACCCT----TCACA--------GAGAACC-----AGCAATATCCTCTCCGATG
DBIF  -- DOLICHOS   CTCTGGTTCACCCT----TCTCG---------CAGAACG-----AGCTACATCCTCTCCGAAA
                      * **     * ***          **      ** *   *   *

PHAE  -- PHASEOLU  CAGTGGACCTGAAGAGCGTTCTTCCCGAGTGGGTGATCGTTGGGTTCACTGCCACCACTG
PHAL  -- PHASEOLU  CAGTGGACCTGAAGAGCGTTCTTCCCGAGTGGGTGAGCGTTGGGTTCTCTGCCACAACTG
PVUL  -- PHASEOLU  CAGTGGACCTGGAGAAAGAAGTTACGACTGGGTGAGCGTTGGGTTCTCTGCCACCACTCAG
PINTO -- PINTO B   CAGTGGACCTGAAGAGCGTTCTTCCCGAGTGGGTGAGCGTTGGGTTCTCTGCCACCACTG
PLUN  -- PHASEOLU  ATGTGGAGCTGGAGAAAGTTCTTGACGACTGGGTGAGCGTTGGGTTCTCTGCCACCTCAG
PSAT  -- PISUM SA   TTGTGTCTTTGAAGGATGTTGTTCCTGAGTGGGTAAGGATTGGTTTCTCAGCTACCACAG
GMAX  -- GLYCINE    TGGTCGATTTGAAGACTTCTCTTCCCGAGTGGGTGAGGATAGGGTTCTCTGCTGCCACGG
DBIF  -- DOLICHOS   GAGTGGACATCACTAATGAACTTCCCGAGTATGTGGGCGTTCTCCGCCACCACCG
                     **        *              **    ** *  **       * ** *** * **    *  *
                                                H  D  V  L  S  W  S  F  A  S
PHAE  -- PHASEOLU  GGATTACTAAAGGGAACGTTGAAACGAACGACATCCTCTCTTGGTCTTTTGCTTCCAAGC
PHAL  -- PHASEOLU  GGATTAATAAAGGGAACGTTGAAACGAACGACGTCCTCTCTTGGTCTTTTGCTTCCAAGC
PVUL  -- PHASEOLU  GGGCTTATCAATGGAGCTATGAAACGCACGACGTCCTCTCTTGGTCTTTTTCTTCCAAGT
PINTO -- PINTO B   GGATTACTAAAGGGAACGTTGAAACGAACGACATCCTCTCTTGGTCTTTTGCTTCCAAGC
PLUN  -- PHASEOLU  GGGC---ATA------------CGAGACGCACGACGTGCTCTCTTCGTCTTTTGCTTCCAAGC
PSAT  -- PISUM SA   GAGC-----------AGAATA-TGCAGCACATGAAGTTCTTTCATGGTCTTTTCATTCTGAGT
GMAX  -- GLYCINE    GACTCGACATA----CCTGGGGAATCGCATGACGTGCTTTCTTGGTCTTTTGCTTCCAATT
DBIF  -- DOLICHOS   GGCTTTCCGAAGGCTACATTGAAACTCACGACGTCCTCTCTTGGTCTTTTGCTTCCAGGC
                     *                 *     *   *  **   *  ** **  *  *******   ***

PHAE  -- PHASEOLU  TCTC----CGATGGCACCACATCTGAAGCTTTGAATCTTGCCAACTTCGCCCTCAACCAAA
PHAL  -- PHASEOLU  TCTC----CGATGGCACCACATCTGAAGGTTTGAATCTCGCCAACTTGGTCCTCAACAAAA
PVUL  -- PHASEOLU  TCAT----CAAT---CTTAAGGACCAAAAATCTGAACGTT-CCAACATCGTCCTCAACAAGA
PINTO -- PINTO B   TCTC----CGATGGCACCACATCTGAAGGTTTGAATCTCGCCAACTTGGTCCTCAACCAAA
PLUN  -- PHASEOLU  TCTC----C------TCCCTTGACGAATGCCCTACAGGT-GAAACC----TCCTCAACAAGA
PSAT  -- PISUM SA   TG--------AGTGGAACTTC------AAGTTCTAA-----GCAAGCTGC-----------AGA
GMAX  -- GLYCINE    TGCCACACGCTAGCAGTAACATTGATCCTTTGGATCTTACAAGCTTTGTGTTGCATGAGG
DBIF  -- DOLICHOS   TCCC----GGATGACTCCACCGCTGAACCTCTGGACCTTGCTCGCTACCTCGTCCGCAACG
                     *              *         *            *              *          *

PHAE  -- PHASEOLU  TCCTCTAG
PHAL  -- PHASEOLU  TCCTCTAG
PVUL  -- PHASEOLU  TCCTCTAG
PINTO -- PINTO B   TCCTCTAG
PLUN  -- PHASEOLU  TCCTCTAG
PSAT  -- PISUM SA   TGCA-TAG
GMAX  -- GLYCINE    CCATCTAA
DBIF  -- DOLICHOS   TCCTCTAG
                      **
```
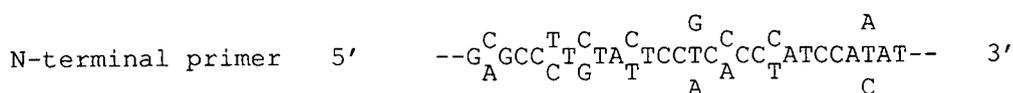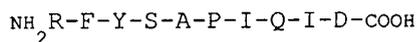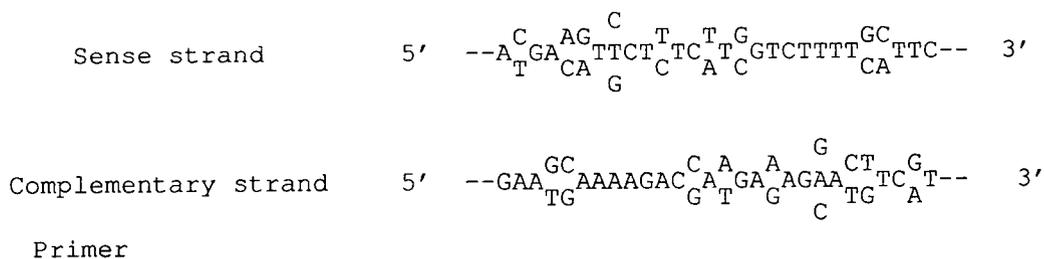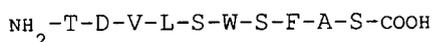
N-terminal oligonucleotide primer

Amino acid coding region associated with conserved sequence:-

NH₂R-F-Y-S-A-P-I-Q-I-D-COOH

(shown with LaTeX: NH_2R-F-Y-S-A-P-I-Q-I-D-COOH)

N-terminal primer   5′   --G C GCC T C TA C TCCTC G C C A ATCCATAT--   3′
                                  A   C T G T   A A T   C

(degenerate positions shown stacked:)

```
                                  C   T C   C   G C C   A
N-terminal primer   5′   --G GCC  TA  TCCTC CC ATCCATAT--   3′
                          A  C T G T     A A T      C
```

C-Terminal oligonucleotide primer

Amino acid coding region associated with conserved sequence:-

NH_2-T-D-V-L-S-W-S-F-A-S-COOH

Sense strand   5′   --A C GA AG C TTCT T TC T G GTCTTTT GC TTC--   3′
                            T   CA   C   C A C   CA
                                      G

```
                          C   AG  C      T   T G        GC
Sense strand   5′   --A GA   TTCT TC  TC   GTCTTTT  TTC--   3′
                     T   CA  G     C   A C         CA
```

Complementary strand   5′   --GAA GC AAAAGAC C A A G CT G T--   3′
Primer   TG   G A GA GA C TG A
                                        (with stacked: C A A / G T G / G, CT / TG, G / A, T)

```
                              GC        C A   A  G CT  G
Complementary   5′   --GAA   AAAAGAC   A  GA  AGAA  TC  T--   3′
strand                TG              G T   G     C TG   A
Primer
```

Figure 8.3

Design of two oligonucleotide primers for use in PCR.

An N-terminal and C-terminal oligonucleotide primer were designed

using using sequence information from conserved regions found

between legume lectin nucleotide sequences

useful, either as primers for PCR experiments or as a diagnostic probe for lectin genes. The redundancy that would have to be incorporated into these primers in order that they encode these two signatures is high and therefore there use as diagnostic probes is more likely considering the problems of nonspecific priming in PCR experiments undertaken so far.

Nucleotide sequence alignment of eight legume lectin genes isolated using clustal 4 (see figure 8.2) yielded two stretches of nucleotide homology near the N-terminal and the C-terminals that were relatively invariant between all the sequences. These regions have 20 and 19 identical bases between all sequences over a 28bp region in each case. Redundant primers designed to this region (see figure 8.3) should be able to amplify all lectin genes so far isolated and therefore hopefully the winged bean lectin genes.

Differential expression of acidic and basic lectin within the developing winged bean seed provides an interesting system for the study of the controlling factors of these genes. The expression of lectin in maturing stem and root tissue also leads to the question of whether the same gene is responsible for the lectin in these tissues as is the case in soybean (Golberg *et al* 1984).

The study of winged bean lectin genes is dependent on the isolation of genomic clones, and probably the most favoured strategy to obtain such clones would be to produce more cDNA for either library construction or PCR amplification experiments. Finally, obtained a nucleotide sequence encoding the basic lectin would allow the production of transgenic plants expressing this protein constitutively, by analogy with the experiments of Boulter and co-workers (1990). Such plants would allow the efficacy of the lectin as a protective agent against insect attack to be rigorously tested both in the laboratory and in the field.

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Biochemical Basis of Insect Resistance in Winged Bean (*Psophocarpus tetragonolobus*) Seeds

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ABSTRACT

Mature seeds of the winged bean (Psophocarpus tetragonolobus) *are toxic to developing larvae of a range of cosmopolitan storage Bruchidae of economic importance, including the cowpea seed weevil,* Callosobruchus maculatus. *Insect feeding trials were carried out in which protein fractions from seeds of winged bean were incorporated at a range of concentrations into artificial seeds, and their effects upon development of* C maculatus *determined. Both albumin and globulin fractions were toxic to the developing larvae and their toxicity correlated with their haemagglutinating activity. Assay of Psophocarpin A, B and C fractions demonstrated Psophocarpin B to be the most insecticidal and to contain the highest haemagglutinating activity. Purified basic seed lectin was highly insecticidal to* C maculatus *larvae, with an LC_{50} value of c. $3 \cdot 5 \, g \, kg^{-1}$. The physiological level of this protein in winged bean seeds is sufficient to account for their resistance to attack by* C maculatus. *Winged bean trypsin inhibitor was also purified and tested in artificial seeds against* C maculatus. *However, even at concentrations in excess of twice the physiological concentration it had no deleterious effects upon development.*

Key words: Psophocarpus tetragonolobus, Callosobruchus maculatus, bruchids, lectins, toxicity, resistance.

INTRODUCTION

The winged bean, *Psophocarpus tetragonolobus*, is a tropical leguminous crop which in recent years has received much attention, since it represents a crop with great

potential for the developing countries. All parts of the plant are edible and the mature seeds not only provide a valuable source of edible oils, but also have a high protein content (*c* 35%). Apart from its nutritive value, winged beans can be grown under a range of environmental conditions and although indigenous in the humid tropics, they can be grown in drier regions as the crop responds well to irrigation. Furthermore, it is able to grow at high altitudes and in poor soils deficient in nitrogen, being significantly better at nitrogen fixation than most other legumes (Anon 1975).

Most pulses grown for human consumption suffer severe damage and loss during storage due to a range of insect pests of the family Bruchidae. Post-harvest loss of *Phaseolus vulgaris* seed, as a result of infestation by two members of the Bruchidae, *Acanthoscelides obtectus* (Say) and *Zabrotes subfasciatus* (Boheman) has been estimated at approximately 25% (Gatehouse *et al* 1987). In the case of cowpea, *Vigna unguiculata*, a 100% loss over a 5 month period due to the bruchid beetle *Callosobruchus maculatus* has been reported (Singh 1978). Mature winged bean seeds, however, appear to be immune to infestation by bruchids. Dobie *et al* (1979), therefore investigated their susceptibility to infestation by a range of cosmopolitan storage Bruchidae to which the seed is liable to be exposed. All species laid eggs and the newly hatched larvae penetrated the testa, but in all cases the developing larvae died during their first or second instars.

Legume seeds are known to contain a range of secondary compounds, and direct or circumstantial evidence has been obtained for the role of some of these compounds in protection against insect attack (Janzen *et al* 1976; Gatehouse *et al* 1979, 1984, 1990; Evans *et al* 1985). The present paper demonstrates that resistance of mature winged bean seeds to insect attack has a biochemical basis and can be accounted for by the presence of the seed lectins.

EXPERIMENTAL

Biological materials

A culture of *Callosobruchus maculatus*, originally from Campinas, Brazil, was reared and maintained on cowpea seeds in controlled temperature and humidity chambers (70% RH, 27 ± 1°C). Cowpea seeds (Californian black-eyed beans) were obtained locally and winged bean seeds were a gift from TISTR, Bangkok, Thailand.

Chemicals

Sephadex G-25, Sepharose 4B and Sephacryl S-200 for column chromatography were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Trypsin (Type I from bovine pancreas), the enzyme substrates BAPNA and BTEE, Con A, PHA lectin and cyanogen bromide were obtained from Sigma London Chemical Company Ltd, Poole, Dorset. Other chemicals were of analytical grade as appropriate.

Preparation of proteins and protein fractions

Albumin and globulin fractions were prepared from mature hexane-defatted seeds

of winged bean as detailed by Croy (1977). Ammonium sulphate precipitated protein fractions were prepared as follows: defatted meal was extracted in 50 mM sodium borate buffer pH 8·2 (100 g litre^{-1}) at 4°C with stirring, overnight. The suspension was centrifuged at 9000 × g for 30 min at 4°C and the pellet discarded. Solid ammonium sulphate was then added to the clear supernate to 40% saturation (226 g litre^{-1}) at 4°C and after c 2h of continual stirring the suspension was centrifuged as above; the pellet obtained formed the 40% cut. The remaining supernate was subsequently taken through 60%, 80% and, finally, 100% cuts. All four ammonium sulphate cuts were desalted on a column of Sephadex G-25 (60 × 2·2 cm) equilibrated with 100 mM NH$_4$HCO$_3$ and freeze dried.

The protein fractions described as Psophocarpin A, B, C were prepared from hexane defatted seed meal according to Gillespie and Blagrove (1978), and were desalted by dialysis against distilled water. The seed lectins were purified from Psophocarpin B by dissolving it in 0·05 M Tris-HCl, 0·1 M NaCl, pH 8 (20 mg ml^{-1}) followed by centrifugation at 3000 × g for 10 min at 4°C to clarify and applied to a Sephacryl S-200 column (190 × 2·6 cm) equilibrated with the above buffer. The flow rate was adjusted to 15 ml h^{-1} and 5-ml fractions collected. Fractions agglutinating a suspension of rabbit erythrocytes were pooled and lyophilised to make the basic seed lectin; those agglutinating human O$^+$ erythrocytes were pooled separately and lyophilised to give the acidic seed lectin preparations.

Trypsin inhibitors were purified from the seeds by affinity chromatography on a column of trypsin linked to Sepharose 4B (Gatehouse *et al* 1980). Cyanogen bromide activation of the resin and coupling of the enzyme was carried out as detailed by March *et al* (1974).

Protein fractions were analysed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970) and tested for haemagglutination activity by serial dilution using microtitration plates with a suspension of 2% v/v rabbit or human group O erythrocytes (Lis and Sharon 1973); Con A and PHA lectin were used as standards. Trypsin and chymotrypsin inhibition assays were carried out using the synthetic substrates BAPNA (Erlanger *et al* 1961) and BTEE (Walsh and Wilcox 1970), respectively.

Feeding trials

The insecticidal effects of the protein fractions, purified lectins and trypsin inhibitors were investigated by allowing *C maculatus* larvae to develop in pellets of chickpea meal to which the components had been added at a range of concentrations. Pellets were prepared, and bioassays were carried out as described by Gatehouse and Boulter (1983). Where appropriate, the LC$_{50}$ value was determined.

RESULTS AND DISCUSSION

Effect of crude protein fractions on larval development

Since the mature seeds of several different species of edible legume have been shown to contain insecticidal proteins (Janzen *et al* 1976; Gatehouse *et al* 1979, 1984; Ishimoto and Kitamura 1988) various protein fractions were prepared in

order to identify potentially toxic components. Initially, albumin and globulin protein fractions were prepared and each incorporated into artificial seeds at a range of concentrations and offered to *Callosobruchus maculatus*. Results showed that both protein fractions were toxic to the developing larvae with an LC_{50} value of $25 \, g \, kg^{-1}$ for the albumins and $50 \, g \, kg^{-1}$ for the globulins (Fig 1); in the present study these proteins constitute approximately 40 and $110 \, g \, kg^{-1}$ of the total dry weight of the seed respectively. The toxicity of these fractions correlated with the haemagglutination titres.

Further fractionation studies based on ammonium sulphate precipitation also showed a strong correlation between toxicity and the haemagglutination titre. Each of four fractions 0–40% (40% cut), 40–60% (60% cut), 60–80% saturation (80% cut) and the proteins soluble at 80% saturation were incorporated into artificial pellets at 0, 25, 50 and $75 \, g \, kg^{-1}$ and their effects upon larval development and survival was monitored. Although the 80% cut did not have a marked effect, both the 40% and 60% cuts did, with the LC_{50} values being 30 and $36 \, g \, kg^{-1}$ respectively. Haemagglutination assays of the protein fractions using a 2% v/v suspension of rabbit erythrocytes showed that the 40% cut contained the highest level of haemagglutination activity, being marginally higher than the 60% cut, and considerably greater than the 80% cut.

Effect of Psophocarpin A, B, C on larval development

Not only have the winged bean seed proteins been separated into albumin and globulin type proteins, but they have also been separated, on the bases of their solubilities, into three fractions known as Psophocarpin A, B, and C (Gillespie and Blagrove 1978) (Fig 2). Their physiological concentrations within the mature seed are estimated to be 7, 49 and $65 \, g \, kg^{-1}$ respectively. Each of the three fractions were incorporated into the artificial beans at 0, 10, 20 and $50 \, g \, kg^{-1}$ and their effects, both on percentage of adult emergence and median development period were determined 42 days after initial oviposition. At all three concentrations tested the crude protein fractions had a marked deleterious effect with no larvae surviving to adult on beans containing any of the added proteins at levels of $20 \, g \, kg^{-1}$ and above (Table 1). At $10 \, g \, kg^{-1}$ incorporation adult emergence was reduced to 25% and 13% for Psophocarpin A and C respectively, relative to the control beans, and in both cases the median development period was increased by approximately 15%. Psophocarpin B, on the other hand, was very potently insecticidal at this level, allowing no adult emergence. Haemagglutination assays carried out on these fractions showed that the protein fraction having the most adverse effect upon bruchid development had the highest lectin content. Titre values of 2^4, 2^3 and 2^2 haemagglutination units mg^{-1} protein were obtained for psophocarpin B, C and A respectively.

Effect of lectins on larval development

Since psophocarpin B was the most insecticidal of the protein fractions tested it was further purified by gel filtration on Sephacryl S-200. Protein peaks corresponding to the basic lectin, based on agglutination of rabbit erythrocytes (Subunit M_r 29 000), and the acidic lectin, based on agglutination of human group O erythrocytes
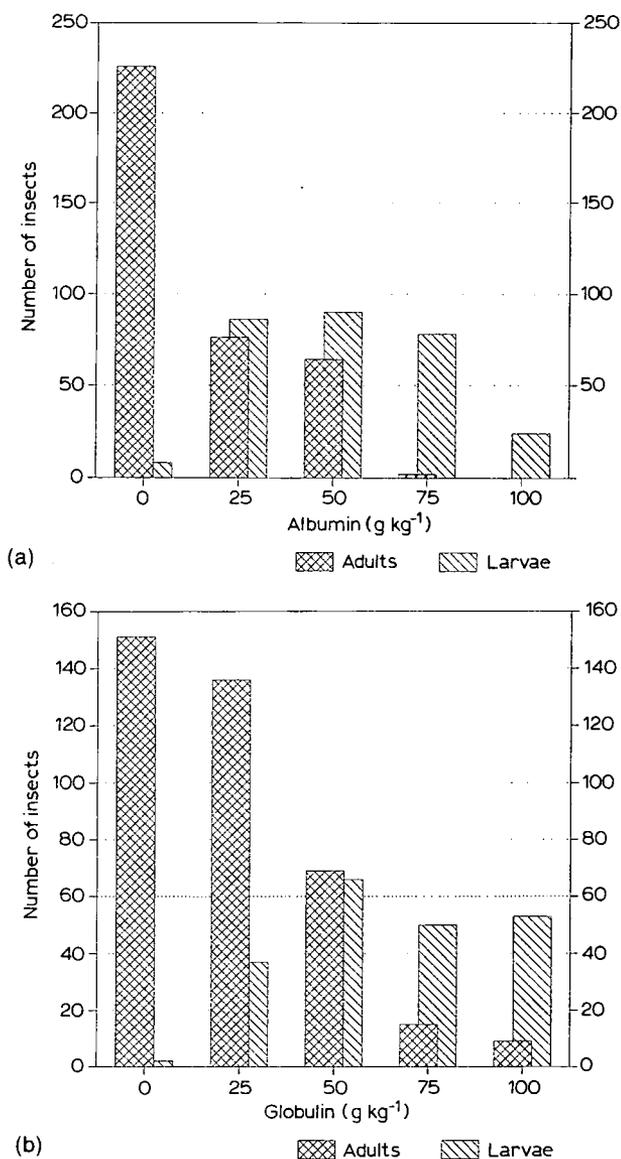
Fig 1. Dosage responses showing the effects of added winged bean (a) albumin and (b) globulin proteins on development of *Callosobruchus maculatus*. Bioassays were carried out over a developmental period of 42 days. Standard error of estimate for number of adults g^{-1} was <10% where $n = 5$.

(Subunit M$_r$ 30–32 000), were pooled. Analysis on SDS-PAGE showed that whilst the basic lectin had been purified to homogeneity (Fig 2), the acidic lectin was highly contaminated with the 2S albumin protein, WBA-1 (Kortt *et al* 1989). Not only do these two lectins have slightly different subunit molecular weights, but they also differ in their isoelectric points and, perhaps of most interest
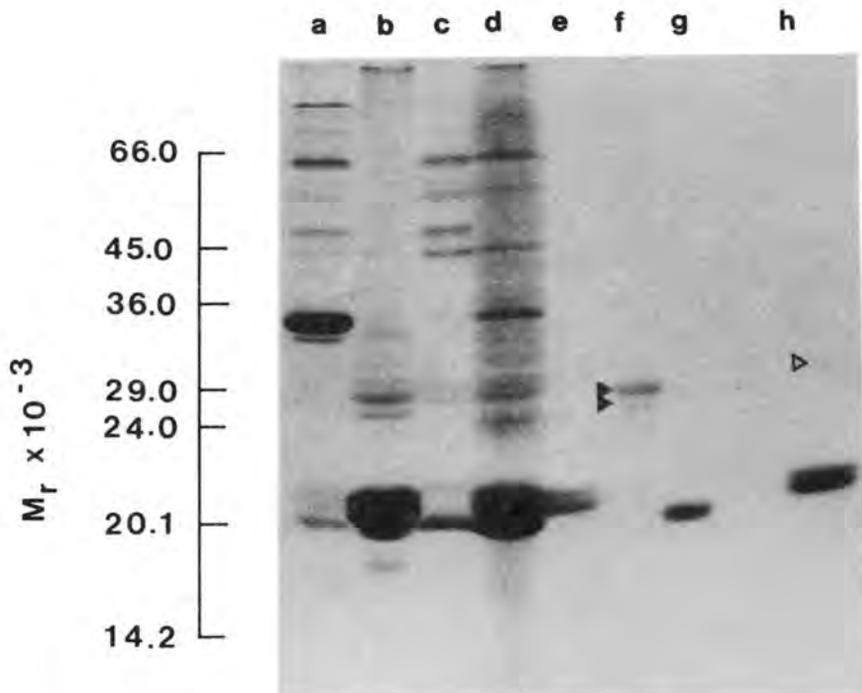
Fig 2. SDS-PAGE (on 170 g kg^{-1} gel slabs), under reducing conditions, of protein extracts from seeds of *Psophocarpus tetragonolobus*. Tracks (a) psophocarpin A; (b) psophocarpin B; (c) psophocarpin C; (d) total seed protein; (e, h) acidic lectin prep. (track (h) at higher loading to show lectin band); (f) basic lectin; (g) trypsin inhibitor. Basic lectin polypeptides are indicated by closed arrowheads; acidic lectin polypeptide by open arrowhead. Molecular weight calibration was determined using Sigma SDS 7 markers.

in this particular study, in their sugar specificities (Kortt and Caldwell 1985). The physiological concentration of these two different lectins within the mature winged bean seed have previously been estimated as 3·4 and 2·7 g kg^{-1} for the basic and acidic lectins respectively (Kortt and Caldwell 1985). Both types were offered to *C maculatus* larvae at 0, 1, 2 and 5 g kg^{-1} in artificial beans. The basic lectin had a very detrimental effect upon larval development with an LC$_{50}$ value of approximately 3·5 g kg^{-1} (Fig 3). Not only did this protein effect adult emergence (Fig 3(a)), but it also had a deleterious effect upon the developmental period and at the highest concentration tested, ie at 5 g kg^{-1}, the median development period was increased by *c* 26% (Fig 3(b)). At this concentration the acidic lectin, on the other hand, had a negligible effect upon both survival and development period. Since the physiological concentration of the basic lectin present in mature winged bean seeds is 3·4 g kg^{-1}, these results clearly demonstrate that it is present at sufficiently high levels to confer protection against the bruchid pest, *C maculatus*. The fraction containing acidic lectin, on the other hand, did not appear to be insecticidal towards *C maculatus* in the present study, even at the highest levels tested. However, a protective role for the acidic lectin can not be excluded as the amounts of acidic lectin used were below the physiological level in the seed.

TABLE 1

Effect of added winged bean psophocarpin fractions to artificial beans on development of *Callosobruchus maculatus*

| Protein fraction	Median development period (days)^a	No. of larvae g^{-1} diet $\pm$ SD	No. of pupae g^{-1} diet $\pm$ SD	No. of adults g^{-1} diet $\pm$ SD	Percentage of adults survival relative to control
Control	32·0	0	1·6±1·49	39·2±8·63	100
+10 g kg^{-1} Psophocarpin A	37·5	16·0±4·0	1·0±1·0	4·8±2·79	25
+20 g kg^{-1} Psophocarpin A	∞	5·0±1·0	0	0	0
+50 g kg^{-1} Psophocarpin A	∞	0	0	0	0
+10 g kg^{-1} Psophocarpin B	∞	9·6±2·32	0	0	0
+20 g kg^{-1} Psophocarpin B	∞	0	0	0	0
+50 g kg^{-1} Psophocarpin B	∞	0	0	0	0
+10 g kg^{-1} Psophocarpin C	37·0	16·4±6·98	0·4±0·8	3·2±1·46	13
+20 g kg^{-1} Psophocarpin C	∞	9·0±4·12	0	0	0
+50 g kg^{-1} Psophocarpin C	∞	0	0	0	0

^{a} ∞ = No development observed.
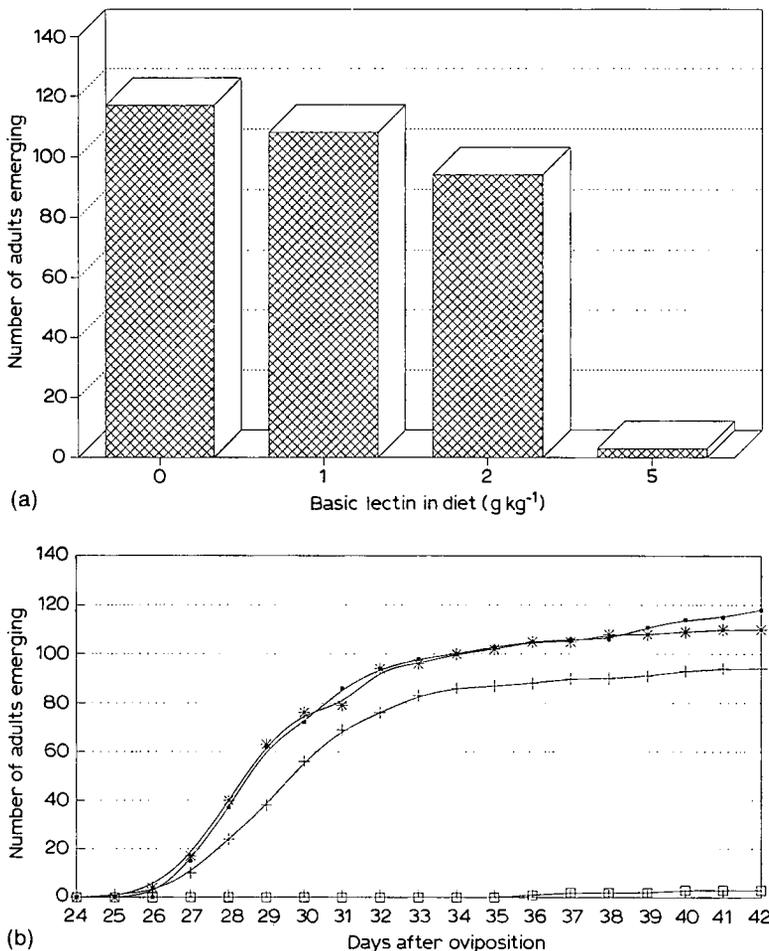
Fig 3. Response of development of *Callosobruchus maculatus* to different concentrations of basic lectin purified from winged bean seeds. (a) Effect on adult emergence, (b) effect on medium development period ($\bullet$, control; *, $10 \, g \, kg^{-1}$ basic lectin; +, $20 \, g \, kg^{-1}$ basic lectin; $\square$, $50 \, g \, kg^{-1}$ basic lectin).

Furthermore, lectins may have different effects upon different insect species, particularly in view of their differing sugar specificities.

The insecticidal properties of a lectin isolated from another legume species, *Phaseolus vulgaris*, have been demonstrated towards *C maculatus*, a non pest species (Janzen *et al* 1976; Gatehouse *et al* 1984). In this instance the LC_{50} value of the lectin purified from cv. Processor by high voltage electrophoresis was *c.* $13 \, g \, kg^{-1}$ (Gatehouse *et al* 1984). Recent studies have shown that the presence of the lectin gene from garden pea, *Pisum sativum*, when expressed in transgenic tobacco plants, confers resistance against the tobacco budworm, *Heliothis virescens* (Boulter *et al* 1990).

Although toxicity of the winged bean lectin towards insects has not previously been directly demonstrated, toxicological studies have indicated that the seed lectin

of winged beans was toxic towards mammals (Higuchi *et al* 1984). Singh and Sarup (1985) found that diets containing winged bean seed meal were not suitable to sustain larval growth of the Lepidopteran, *Chilo partellus*, but no further attempts were made in that study to identify the toxic component(s).

Effect of trypsin inhibitors on larval development

Preliminary studies to elucidate the insecticidal component(s) present in the mature winged bean seed demonstrated the toxicity of the albumin protein fraction. Although lectins are often present in both the albumin and globulin fractions, protease inhibitors are almost exclusively associated with the albumin fraction of legume seeds and often constitute a major component of that fraction. The mature seeds of winged bean contain approximately 20 g kg^{-1} trypsin inhibitor (unpublished data). Earlier studies carried out by Gatehouse *et al* (1979) demonstrated that the protease inhibitors of cowpea (*Vigna unguiculata*) were antimetabolic to *C maculatus*. Recently transgenic tobacco plants containing the cowpea trypsin inhibitor (CpTI) gene and expressing the protein at levels of *c* 10–20 g kg^{-1} were found to be resistant to attack by many economically important insect pests, including Lepidoptera (Hilder *et al* 1987; Gatehouse *et al* 1990). Protease inhibitors have clearly been shown to be involved in some instances of insect resistance in plants and, in some of these, to be induced by insect attack (Ryan 1985; Brown *et al* 1985).

The winged bean trypsin inhibitors (WBTI) therefore represent possible candidates for seed protection of winged beans. The affinity purified inhibitor was incorporated into artificial pellets at seven different concentrations from 0–50 g kg^{-1} and the effects upon survival and rate of development monitored. The results (Table 2) clearly demonstrate that WBTI, even at over twice the physiological concentration found in the mature seeds, had no deleterious effects upon survival of *C maculatus*. Indeed, at the highest levels of incorporation the presence of inhibitor resulted in increased survival, and a decrease in development period, relative to the controls. The deficiency of sulphur amino acids in legume seeds in general can limit the development of insect predators; thus Gatehouse and Boulter (1983) showed that addition of sulphur amino acids to resistant cowpea meal rendered it non-toxic to *C maculatus*. The serine protease inhibitors in winged bean belong predominantly to the Kunitz inhibitor family (Peyachoknagul *et al* 1989), and the sequence of WBTI shows that it contains significant amounts of cysteine (2·6 mol %) as well as potentially limiting amino acids such as tryrosine and tryptophan (Kortt *et al* 1989). One possible explanation for the observed enhanced development of insects is therefore that the larvae are able to partially digest the WBTI and utilise the amino acids it contains. The WBTI was also shown to be innocuous to the Lepidopteran *Spodoptera littoralis* (unpublished data), an insect pest sensitive to CpTI; the insecticidal properties of WBTI are clearly different from Bowman–Birk inhibitors such as CpTI.

CONCLUSION

The mature seeds of winged bean (*Psophocarpus tetragonolobus*), a potentially important food crop in developing countries, are resistant to insect attack. The

TABLE 2
Effect of added WBTI to artificial beans on development of *Callosobruchus maculatus*

Diet	No. of days to first adult emergence	No. of larvae g^{-1} diet ± SD	No. of pupae g^{-1} diet ± SD	No. of adults g^{-1} diet ± SD	Percentage of adults survival relative to control
Control	40	28·0 ± 11·84	12·4 ± 4·38	14·2 ± 0·1	100
+0·5% WBTI	38	12·4 ± 5·95	11·55 ± 3·36	18·65 ± 1·89	111
+1·0% WBTI	37	39·3 ± 8·4	10·5 ± 2·0	20·1 ± 3·05	102
+1·5% WBTI	37	27·0 ± 0·75	6·65 ± 2·3	15·5 ± 7·5	63·3
+2·0% WBTI	38	28·0 ± 2·75	10·35 ± 2·3	18·95 ± 1·3	100·7
+2·5% WBTI	37	24·75 ± 2·75	8·4 ± 0·96	15·05 ± 8·9	108·6
+3·5% WBTI	37	22·15 ± 9·8	11·75 ± 2·92	20·85 ± 4·6	133·5
+5·0% WBTI	36	20·6 ± 3·2	8·4 ± 0·034	23·1 ± 4·7	134·2

The standard error for each treatment is given ($n = 5$).

basic lectin purified from the mature seeds was shown to be toxic to the developing larvae of the bruchid beetle *Callosobruchus maculatus*, with an LC_{50} value of $35 \, g \, kg^{-1}$, ie similar to the levels of basic lectin present in winged bean seeds (Kortt and Caldwell 1985). The basic lectin could therefore play a major role in the protection of winged bean seeds to attack by this polyphagous pest. The trypsin inhibitors present in the seed and tested in the reported bioassays, do not appear to confer protection against *C maculatus* at physiological levels, although they may have effects against other insect species. In addition, the protective role against *C maculatus* attributed to the basic lectin may not apply to other insect species, since the lectin from *P vulgaris*, although toxic to *C maculatus*, has no effect on a bruchid species normally a pest of this legume, *Acanthoscelides obtectus* (Gatehouse *et al* 1989). The possibility that insecticidal compounds not identified in the present study are present in winged bean seeds cannot be excluded.

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