The mechanisms of action of insecticidal lectins from snowdrop (GNA) and jackbean (concanavalin a) ontomato moth larvae

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THE MECHANISMS OF ACTION OF INSECTICIDAL LECTINS FROM SNOWDROP (GNA) AND JACKBEAN (CONCANAVALIN A) ON TOMATO MOTH LARVAE.

A thesis submitted by Elaine Charlotte Fitches, B.Sc. in accordance with the requirements of the University of Durham for the degree of Doctor of Philosophy.

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ABSTRACT.

Artificial diet bioassays were carried out to investigate the impact of GNA and Con A upon the development of *L. oleracea* larvae. GNA, at 2 % of total dietary protein, exerted a significantly detrimental effect upon larval development, growth and consumption, with little effect upon survival. Con A was shown to be the more toxic of the 2 lectins. When tested at concentrations of 2.0 %, 0.2 % and 0.02 %, Con A caused a significant decrease in survival and larval development, and caused greater reductions in larval growth and consumption compared to GNA.

The potential for GNA and Con A to exert insecticidal effects via binding to the brush border membrane (BBM) and peritrophic membrane (PM) of *L. oleracea* larvae was investigated. Con A, which specifically binds α-D-mannopyranoside and α-D-glucopyranoside residues, was shown to bind *in vitro* to the majority of BBM and PM proteins. In contrast GNA, which exhibits strict specificity for α(1,3) and α(1,6)-linked D-mannose residues, bound to only 5 BBMV and 2 PM proteins. In agreement, higher levels of Con A, compared to GNA, were shown to accumulate in larval gut tissue after feeding the proteins *in vivo*. Despite this both lectins were shown to have a similar ability to disrupt the digestive capacity of the larval midgut. GNA and Con A stimulated similar short term elevations in BBM enzyme and soluble trypsin activities and a long term reduction in α-glucosidase activity. Increases in levels of trypsin activity in faecal material collected from lectin-fed larvae suggested that the proteins may act by disrupting mechanisms of enzyme recycling.

Aminopeptidase, an abundant and avidly binding BBM protein (120 kDa), was identified as a major Con A binding species in *L. oleracea*. A 98 kDa GNA-binding BBM protein was purified and amino acid sequence data was obtained from digest polypeptides allowing oligonucleotide primers to be designed. Subsequent attempts to amplify (by PCR and RT-PCR) fragments containing coding sequence corresponding to the 98 kDa protein were unsuccessful. This was attributed to oligonucleotide degeneracy together with the low abundance and relatively large size of the protein.

The potential for GNA and Con A to exert systemic effects upon *L. oleracea* was demonstrated by the detection of both lectins in the haemolymph of larvae exposed to experimental diets. GNA was detected in haemolymph of larvae exposed to experimental diet for just 2 hours. In contrast, no Con A was detectable in haemolymph extracted from larvae fed for 24 hours, although it was present in the haemolymph after 5 days of exposure to the diet. That GNA and Con A may act directly upon organs other than the insect gut was indicated by the detection of lectins *in vivo* in malpighian tubule and fat body tissue extracts. A significant reduction in haemocyte abundance in haemolymph samples extracted from lectin-fed larvae suggested that both GNA and Con A may also act by disrupting the immune system of *L. oleracea*.

DECLARATION.

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

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ABBREVIATIONS.

AD	artificial diet
AMV	Avian myeloblastosis
Bis-acrylamide	Bis (N,N'-methylene-bis-acrylamide)
bp	base pair
BB	brush border
BBMV	brush border membrane vesicle
BSA	bovine serum albumin
°C	degrees centigrade
CAPS	3-(cyclohexlamino)-1-propanesulphonic acid
Ci	curie
Con A	Concanavalin A
cpm	counts per minute
DMSO	dimethylsulfonide
DNA	dioxyribonucleic acid
DTT	ditheothreitol
EDTA	ethylene diamine tetraacetic acid
EMBL	European Bioinformatics Institute Nucleotide Sequence Database
EtBr	ethidium bromide
FITC	fluorosein
g	gram
gal	galactose
GalNAc	N-acetylgalactosamine
GlcNAc	N-acetylglucosamine
GNA	*Galanthus nivalis* agglutinin
hr	hours
IgG	immunoglobulin
Kb	kilobase pair
kDa	kilodalton
L	litre
m	metre
M	molar (concentration)
min	minute
MMLV	Moleney Murine Leukemia Virus
M_r	molecular weight
mRNA	messenger RNA
NBT	4-Nitroblue tetrazolium chloride
OD	optical density
O/N	overnight
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PM	peritrophic membrane
PVDF	polyvinylidene diflouride
r.h.	relative humidity
RNA	ribonucleic acid
rpm	revolutions per minute
RT	room temperature
SDS	sodium dodecyl sulphate
secs	seconds
SSC	saline sodium citrate
Taq	*Thermus aquaticus*
TBS	Tris buffered saline
v/v	volume for volume
w/v	weight for volume
X-phosphate	5-bromo-4-chloro-3-indoyl-phosphate

NUCLEIC AND AMINO ACID ABBREVIATIONS.

Code	Nucleic acid
A	adenosine
C	cytosine
G	guanine
I	inositol
N	unknown
T	thymidine
U	uracil

Codes	Protein
A ala	alanine
C cys	cysteine
D asp	aspartic acid
E glu	glutamic acid
F phe	phenylalanine
G gly	glycine
H his	histidine
I ile	isoleucine
K lys	lysine
L leu	leucine
M met	methionine
N asn	asparagine
P pro	proline
Q gln	glutamine
R arg	arginine
S ser	serine
T thr	threonine
V val	valine
W trp	tryptophan
Y try	tyrosine

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1. INTRODUCTION.

1.1. General introduction.

Whilst food production is increasing by approximately 1 % per year, the worlds population is growing annually at an estimated 2 % (Schmidt, 1995). Thus agriculture remains as one of the most important activities carried out by mankind. One of the first steps in agriculture was the domestication of specific plants as crops, involving selection for greater yield, ease of cultivation, and nutritional value. Over the centuries this selection process, together with the intensification of farming practises utilising high nutrient inputs to improve crop quality, has altered the coevolutionary balance between plants and their insect predators. As a result few cultivated species have retained the degree of insect resistance exhibited by their wild relatives (Feeny, 1976). Monocultures can favour drastic increases in the population of insects that feed upon these crops, with the numbers of herbivores exploiting a given host species increasing with the area occupied by that host (Strong, 1979). This situation occurs both in the field and during crop storage (Gatehouse & Gatehouse, 1998). Whilst the build up of pests to a specific host is typically reduced in diverse plant communities (Cromartie, 1981) agricultural practises that involve combining crops can have the opposite effect if each crop is susceptible to a particular pest. One such example is cotton and maize, both of which are hosts for the corn earworm *Helicoverpa zea* (Boddie) (Pimental *et al.*, 1977).

Total yield losses from all causes to all crops are roughly estimated to be $500 billion (US) per annum (Oerke *et al.*, 1994). The losses to pests (weeds, pathogens, and insects) has been estimated by Pimental (1991) to be 45 % worldwide. Most plant species experience insect predation although the severity and economic importance of that predation varies significantly among crop species. In addition to their direct action as pests insects also act as major vectors for plant diseases. Worldwide 15.6 % of the total production, valued at $90.5 billion (US) of the 8 principal food and cash crops (coffee, potato, soybean, maize, barley, cotton, rice and wheat) was lost to animal predators, primarily to insects in 1988-90 (Duck & Evola, 1997). Thus insect crop protection plays a vital and integral role in modern-day agricultural production.

Present day pest control depends essentially on the use of agrochemicals and, to a lesser extent, inherent varietal resistance. It is now generally accepted that the increasing reliance of agriculture upon chemical pesticides this century has further exacerbated problems of pest control. Whilst reforms in agricultural policy have facilitated a reduction in agrochemical expenditure within Europe elsewhere expenditure is rising. This in many cases has resulted in the rapid build up of resistance by insect pests to particular compounds, as exemplified by rapidly developed resistance by the cotton bollworm (*Heliothis virescens*) to organochlorine insecticides (Metcalfe, 1986). It is not unusual for resistance in a major pest to develop within one year of field use and examples of resistance developing after long term usage are common (Metcalf, 1986). In certain cases the indiscriminant application of pesticides has eliminated predatory species

along with primary pests, facilitating the emergence of new primary pests (Heinrichs & Mochida, 1983). The transformation of a secondary pest, the rice brown planthopper (RBP) (*Nilaparvata lugens*) to a primary pest with a greater impact on rice yield than the original pests is one such example (Heinrichs & Mochida, 1983). With increasing environmental awareness has come increasing concern over the environmental impacts of pesticide usage. As only around 1% of applied insecticide actually comes into direct contact with the target crop it is not surprising that toxic residues in ground water, soil and within the food chains of birds and animals, have been detected. A lack of correct safety practises and equipment for the 'safe' application of pesticides has resulted in serious consequences for the health of agricultural workers, particularly in developing countries. World Health Organisation estimates (1990) put the number of unintentional deaths attributable to pesticide poisons at about 20,000 per year (Dirham, 1993). Thus financial, environmental and health issues have together placed increasing pressure upon governmental bodies to invest in the development of alternative, safer, and more effective, strategies to combat the problem of pest control. The complete abolition of pesticide usage is not practical. However, it is hoped that the use of genetically engineered insect resistant crops, together with discriminant pesticide use, crop rotation, field sanitation and the use of pest free seeds, will go some way towards alleviating the existing shortfall in agricultural production. To date more than 60 plant species have been genetically engineered and nearly 3000 field tests of transgenic crops have been conducted worldwide (Schmidt, 1995).

There are a number of benefits associated with the use of genetic engineering to control crop pests. It affords season long crop protection (independant of weather), thereby abolishing the need to predict the often short time when toxicity is required. Thus insects are treated at their most sensitive developmental stage. Protection is provided where it is required and this includes plant tissue difficult to treat with chemical insecticides, such as the underside of leaves, roots, or new growth emerging between sprays. Ecological impact is limited as insecticidal activity is restricted to species that attack the crop and thus damage to harmless or beneficial insects is avoided. Problems of environmental pollution are also avoided as the defensive agent is biodegradeable and confined within the plant. Another potential benefit are financial savings in the avoidance of costs and problems associated with insecticidal application.

One of the main advantages of molecular plant breeding over conventional breeding is that it offers the possibility of introducing a new trait to an existing valuable variety without the co-transfer of possibly undesirable linked characteristics. Molecular plant breeding also allows the transfer of insect resistant genes between species, genera, and even Kingdoms. Recent advances in molecular techniques have led to the development of methods for the genetic transformation of a wide range of plants. Whilst problems of incompatability can arise (eg. poor *Bacillus thuringiensis* (Bt) expression in plants) these can usually be overcome through gene sequence manipulation. In a similiar fashion to the approach of plant breeders, biotechnologists hope to increase levels of crop protection

by 'pyramiding' genes. It is envisaged that the introduction of 'packages' of genes acting on different targets within insects will mimic the multimechanistic resistance that occurs in nature and thereby reduce the rate of resistance development.

The genetic engineering of crops for resistance does however bring its own problems and considerable technical challenges. The identification and isolation of suitable genes and subsequent testing of gene products to ensure effective control of a particular pest is a time consuming process. Transformation systems that permit the stable heritable insertion of foreign DNA must be further developed. Equally the encoded product must be expressed at levels adequate for significant insecticidal effects without compromising the performance of the crop in question. If biological control agents are to be used in conjunction with genetically modified crops possible implications for tri-trophic interactions must also be examined. Field trial methods need to be developed to demonstrate convincingly the enhanced resistance of the transgenic plants. To be economically viable the transgenic insect resistant crop has to meet with both grower and public approval and acceptance. At present the general public shows considerable distrust of biotechnology. This is based largely on failures in the past by the 'powers that be' to recognise and to promptly inform the public regarding damaging effects of certain insecticides such as dichlorodiphenyltrichloroethane (DDT). Bad publicity (eg. World in Action, 10 August 1998) and a lack of readily available consumer information has exacerbated this problem. There is concern over the possibility that recombinant genes may escape and enter the DNA of uncultivated species through hybridisation, thereby creating new, or exaggerating existing, weed problems. Genetically engineered species may themselves escape and invade communities and so cause ecological disruption. (Kareiva & Stark, 1994). Actually evaluating the risks involved for specific crops is a formidable task. Theoretically the use of transgenics appears as the safest and increasingly more practical approach to the issue of crop protection.

At present 2 main strategies for producing genetically engineered plants with increased resistance to pests have been successfully adopted. One approach is to use the entomicidal bacterium Bt (Berlinger) as a source of resistant genes and the second is to identify and utilise plant genes.

1.2. The use of Bacillus thuringiensis (Bt) in the genetic manipulation of crops for insect resistance.

The potential use of the pathogenic gram positive bacterium *Bacillus thuringiensis* (Bt) to control insect populations was recognised by Berlinger as early as 1909 (Peferoen, 1997). The use of Bt sprays over the past 30 years has demonstrated both their safety and specificity. In 1970, the isolation of HD-1, a strain far more potent that any other isolated to date, provided a major step forward for the commercial success of Bt (Dulmage, 1970). Most strains are active against Lepidoptera, with some specific to Diptera (Yamamoto & McLaughlin, 1981) and Coleoptera (Kreig *et al.*, 1983; Herrnstadt *et al.*, 1986). The main disadvantage of the use of Bt as a bioinsecticide is the relatively

short survival time of the toxin in the field, necessitating repetitively costly applications of the bacterium (Brunke & Meeusen, 1991). Thus the genetic engineering of plants to produce the Bt toxin as a cheaper alternative to Bt sprays has been actively pursued.

Bt produces a crystalline protein upon sporulation which, whilst exposed to the environment, acts to protect the bacterium from degradation. When injested by susceptible insects crystal proteins are dissolved from the crystals and protoxins are proteolytically activated to a trypsin resistant core fragment. The protein passes through pores in the peritrophic membrane (PM), binds to an epithelial brush border membrane (BBM) receptor and inserts into the membrane. Insertion induces pore formation and cell lysis. According to Knowles (1994) the sequence of events is: 1-5 mins - increased glucose uptake by gut cells and first histopathological signs in columnar cells; 5-10 mins - midgut paralysis, feeding cessation, increased blood pH and decreased lumen pH, apical membrane becomes permeable to dyes, columnar cell swelling, microvilli blebbing and first histopathological signs in goblet cells; 10-30 mins - increased potassium turnover in gut cells, increased blood potassium concentration, decrease in glucose and leucine transport to blood and general metabolic breakdown of gut cells; 30-60mins - gut cells lyse and slough from basement membrane and insect paralysis, followed by death within 1 to 3 days by starvation, and/or septicaemia if the insect had eaten Bt spores.

Cloning of the first crystal protein gene was achieved in 1981 (Schnepf & Whiteley, 1981) and the complete sequence was obtained in 1985 (Adang *et al.*, 1985, Schnepf *et al.*, 1985). The same crystal protein gene was found in many different strains and strains often carry multiple crystal protein genes. The mixing of spores and crystals from different strains in the environment, together with the wide range of crystal proteins providing access to new niches, is thought to account for the ecological success of the ubiquitous Bt bacterium (Lambert & Pefereon, 1992). To date only a fraction of the several 1000 unique Bt strains have been analysed in detail. In 1989, Höfte and Whiteley proposed a nomencalture and classification scheme for crystal protein gene sequences which divided genes into 4 groups based on their insecticidal activity (Lepidoptera specific type I; Lepidoptera and Diptera specific type II; Coleoptera specific type III; Diptera specific type IV), with further subdivisions determined by sequence homology. Due to inconsistencies and the ongoing discovery of crystal proteins with very different amino acid sequences and biocidal activities the original scheme was superceded by a scheme which ranks crystal proteins on the basis of their amino acid sequence alone. Crickmore *et al.* (1998) thus classified 96 crystal proteins reported by mid-1995 into 17 groups.

Crystal proteins commonly encode proteins of 130-140 kDa with a trypsin resistant core of 60 kDa (Pefereon, 1997). Insecticidal activity resides in the 60 kDa core derived from the N-terminal half of the crystal protein. The C-terminal portion is thought to be important for crystallisation of the protoxin. Sequence alignments demonstrate the presence of up to 5 conserved regions within the toxic moieties of crystal proteins and 3

in the C-terminal half (Pefereon, 1997). Structural information has been provided by elucidation of the 3-dimensional structure of Cry 3A by Li *et al.* (1991) and the toxic moiety of Cry 1Aa by Grochulski *et al.* (1995). The 3 domain structure is thought to be representative of the majority of toxic moieties of crystal proteins since the 5 conserved amino acid regions form the core of the structure (Pefereon, 1997). Domain I comprised of 250 N-terminal amino acids is thought to be responsible for pore formation since it, on its own, is able to insert itself into artificial lipid bilayers (Walters *et al.*, 1993; Von Tersch *et al.*, 1994). A central 200 amino acid domain II is thought to be responsible for interaction with target molecules in insect midgut epithelial cells. Binding studies with hybrid proteins have shown that binding is determined, at least in part, by domain II (Lee *et al.*, 1992). A sequence of 6 amino acids within domain II of Cry 1Aa has been shown to be essential for its binding to midgut epithelial cells. A 150 amino acid C-terminal domain II may be involved in structural stabalisation (Li *et al.*, 1991), specificity (Honée *et al.*, 1991; Bosch *et al.*, 1994), ion channel formation (Chen *et al.*, 1993) or binding (Aronson *et al.*, 1995).

Whilst studies have revealed an overall general scheme for the major steps between ingestion of the crystal and disruption of the insect gut the insecticidal activity of a particular crystal protein differs from species to species. Bt sequence homology is a poor indicator for insecticidal activity and processes responsible for the extreme specificity of Bt toxins are the focus of much attention in mechanisms of action studies.

The role of solubilisation and proteolytic activation for insecticidal activity in some cases has been demonstrated. Cry 7Aa (formerly Cry III C) had no insecticidal activity when tested as a crystal preparation against Lepidopteran and Coleopteran larvae. However, toxicity to the Colorado potato beetle was observed when Cry 7Aa was dissolved and trypsinised prior to oral administration (Pefereon, 1997). Non toxicity of Cry 7Aa to the tobacco hornworm (*Manduca sexta*) has been shown to be attributable to degradation of the dissolved crystal protein by gut proteases (Pefereon, 1997).

Detailed studies investigating the interaction of δ-endotoxins with the BBM of the insect midgut indicate that variability in the dynamics of interaction between toxins and receptors is largely responsible for determining the insecticidal specificity of a particular crystal protein. The specific binding of δ-endotoxins to the gut epithelium of a wide range of Lepidoptera (Van Rie *et al.*, 1989, 1990 a,b; Ferré *et al.*, 1991; Bravo *et al.*, 1992; Denolf *et al.*, 1993; Eschriche *et al.*, 1994) some Diptera (Ravoahangimalala *et al.*, 1993; Ravoahangimalala & Charles, 1995) and some Coleoptera larvae (Slaney *et al.*, 1992; Belfiore *et al.*, 1994) have been described. Whilst there are no crystal proteins which are toxic but do not bind to the BBM there are insects in which increased binding does not correlate with increased toxicity (Wolfersberger, 1990) and instances where crystal proteins bind without being toxic (Garczynski *et al.*, 1991). Lepidopteran larvae are often susceptible to more than one crystal protein and the presence of different binding sites for different crystal proteins in the same insect has been shown (Van

Frankenhuyzen *et al.*, 1993; Masson *et al.*, 1995; Luo *et al.*, 1996). It is thought that Cry 1A type proteins may compete for the same binding site whilst other crystal proteins appear to have a unique receptor (Pefereon, 1997). The interaction between an δ-endotoxin and its receptor appears to be a 2-step process (Pefereon, 1997). Initial binding is reversible and determined by the affinity and number of accessible binding sites. The subsequent insertion of at least part of the δ-endotoxin into the cell membrane is thought to render the interaction irreversible. Pore formation facilitates cell lysis and thus, as insertion appears essential for this process, a correlation between the irreversible binding and toxicity of δ-endotoxins is established (Chen *et al.*, 1995; Liang *et al.*, 1995; Ragamohan *et al.*, 1995).

To date all Cry IAc-binding proteins have been characterised as glycoproteins by virtue of their sensitivity to toxin-binding inhibition by amino-sugars or by the reduction of toxin binding by periodate treatment (Valiatus *et al.*, 1997). All of the purified toxin binding proteins, with one exception (a cadherin-like protein (Vadlamudi *et al.*, 1995)), have been identified as different isoforms of aminopeptidases. However the lack of binding of a large number of δ-endotoxins to an A-P isoform (APN-2) observed by Valiatus *et al.* (1997) suggests that not all APNs act as toxin binding proteins. The precise role of the receptor has not as yet been established. Receptors may act by concentrating δ-endotoxins at the cell membrane or by modifying δ-endotoxins in a way that facilitates membrane insertion or by becoming part of the pore itself (Pefereon, 1997). In addition the requirement for δ-endotoxin oligomerisation for pore formation remains to be established (Walters *et al.*, 1994). What is apparent however is that the specific action of δ-endotoxins is related to their pore-forming capacity which in turn is dependent upon receptor binding (Pefereon, 1997).

Initially the production of transgenic plants with significant insecticidal activity was hindered by low levels of Bt crystal protein expression. Over the past 10 years considerable modifications to crystal protein genes have largely overcome this problem. In the 1980s a truncated gene encoding the N-terminal portion of the crystal protein (known to be responsible for insecticidal activity) was introduced into a number of plant species. Whilst insecticidal effects were observed in some cases expression levels of crystal proteins were, at best, 0.01 % of total soluble proteins (Pfereon, 1997). Perlak *et al.* (1991) produced transgenes expressing levels of crystal protein of up to 0.3 % of plant proteins following modifications designed to improve the compatibility of the bacterial transgene with gene processing in plants. Modifications included the elimination of potential polyadenylation signals and ATTTA motifs; an increase in overall G + C levels and the replacement of bacterial codons with plant-preferred codons. It was concluded that the modifications improved the translational efficiency of the crystal toxin protein in plants. Modifications to a specific area in the Cry 1Ab coding region have been shown to drastically improve levels of expression in plants (van Aarssen *et al.*, 1995). More recently a chimeric gene with an unmodified coding sequence for the Cry 1Ac protoxin has been targeted to the chloroplasts of tobacco and expression levels of up to 3-

5 % of total soluble proteins have been achieved (McBride *et al.*, 1995).

To date Bt endotoxin genes with insecticidal activity against important crop pests have been introduced successfully into a number of crop species. Corn lines have been transformed with the cry 9C gene, which encodes a crystal protein exhibiting toxicity to an unusually wide range of Lepidoptera including important crop pests such as the European corn borer, black cutworm (*Agrotis ipsilon*) and South-western corn borer (*Diatraea grandiosella*) (Lambert *et al.*, 1996). Several crops expressing Bt endotoxins have recently been introduced as commercial products.

Whilst problems encountered in the production of transgenic crops expressing adequate levels of Bt crystal protein have been largely overcome there is increasing concern over the possibility that insects will develop widespread resistance to δ-endotoxins. With more than 500 insect species reported to be resistant to many different insecticides (Georghiou & Lagune-Tejeda, 1991) the capacity for resistance development to crops expressing Bt crystal proteins has obvious economic implications. So far the diamond back moth is the only insect that has been reported to have developed resistance to Bt in the field (Tabashnik *et al.*, 1990). Resistance was shown to be attributable to a change in the target binding site of Cry 1Ac with a dramatic reduction in the binding of Cry 1Ac proteins to the midgut observed (Tabashnik *et al.*, 1994 a,b). This phenomena has also been observed in diamond back moths in Florida (Tang *et al.*, 1996). Since Cry 1A proteins share binding sites it is not surprising that some levels of cross-resistance have been observed in the laboratory (Gould *et al.*, 1992; Müller-Cohn *et al.*, 1994; Tabashnik *et al.*, 1994b; Moar *et al.*, 1995). The contribution of factors such as exposure frequency, the genetics of resistance and the number of insect generations, to resistance development are currently being investigated. A number of strategies (such as targeting the pest with multiple Bt crystal proteins) based on theoretical models are being adopted in an attempt to manage the development of insect resistance to transgenic Bt crops (Pefereon, 1997).

1.3. Natural plant defence mechanisms - Inherent resistance.

Given the prodigous number of pest species it is not surprising that plants have evolved an equally impressive array of mechanisms to combat insect attack. Plant-based defence mechanisms depend primarily on 3 factors, namely, temporal avoidance, physical and chemical defences.

The life cycle of certain plants facilitates temporal avoidance, whereby plants avoid producing sensitive tissues during the period of maximal insect attack. This strategy is similarly adopted in agriculture where the use of 'early' or 'late' varieties may allow the crop to escape serious predation. Physical defences include the protection of vegetative tissues via the production of hairs, spines, or thickened cuticles and the protection of seeds with hard lignified coats. In chemical defense plants utilise their capacity for secondary metabolism to produce an extensive number of compounds that are either

antimetabolic, toxic, deterrant, or developmentally detrimental to predators. Examples of secondary metabolite classes thought to be involved in plant defense include terpenoids, steroids, glucosinolates, cyanogenic glycosides, pyrethrins, phenolics, alkaloids, flavanoids, rotenoids, saponins, and non protein amino acids. The efficacy of many of these natural products, such as nicotine and pyrethrum, has been proven by their widespread use as chemical pesticides in the first half of this century and the recent introduction of new products such as ryania and neem tree extracts (Margosan.O.) (Chilton, 1977). Secondary metabolites are generally produced by multigene pathways. Whilst the potential use of these compounds in plant genetic engineering is clearly vast at present understanding of biosynthetic pathways, enzymes, sites of synthesis, translocation and storage is limited. In addition plant genetic engineering is largely limited to the introduction and expression of single genes (with a second gene providing a selectable trait or marker). Vectors are capable of transferring as many as 4 genes but there has been no case yet where even 2 genes of a pathway have been transferred and co-ordinately expressed (Chilton, 1977). Research to increase understanding of complex biosynthetic pathways and towards the introduction of co-ordinately expressed genes is in progress. Thus it is highly likely that many such products will eventually become economically attractive targets for genetic engineering.

1.4. The potential use of plant-derived genes in the genetic engineering of crops for insect resistance.

A few plant defence mechanisms are based on a primary protein product. To have an effect these non-volatile proteins must be ingested and the target site is often the insect digestive system. Such proteins which can be utilised by present day biotechnological methods include enzyme inhibitors and lectins. To this end a wide range of single gene products have been investigated as potential insect control agents. Whilst both plant and bacterial proteins are suitable for current gene transfer methods those of plant origin are more likely to be compatible with the metabolic system of the transgenic host plant. Unlike Bt toxins these proteins have antimetabolic activity against a wide range of insects, although relatively high levels of expression in transgenic crops (around 2 % of total soluble proteins) are generally required for insecticidal effects.

1.4.1. The use of plant protease inhibitors.

Plant protease inhibitors, specific for animal and microbial enzymes (Laskowski & Sealock, 1971), are widely distributed throughout the plant kingdom. They are particularly abundant in seeds and storage organs where they account for up to 19 % of total protein (Ryan, 1981). The potential use of genes encoding plant protein protease inhibitors to produce transgenic crops with enhanced insect resistance has been explored for a number of years. Interest was initially fuelled by evidence to suggest that these proteins played a role in natural plant defence. The induction of systemic protease inhibitor synthesis by plants following insect feeding or mechanical wounding was first demonstrated by Green and Ryan in 1972. Evidence for a protective role in the 'field'

was first provided by Gatehouse *et al.* (1979) who found that elevated levels of inhibitors in a variety of cowpea (*Vigna sinensis* (L) Savi.) correlated with seed resistance to the bruchid *Callosobruchus maculatus* (F.), a major storage pest of this crop.

Plant enzyme inhibitors, functionally divided into protease inhibitors and inhibitors of other gut enzymes such as α-amylase, act primarily by binding to and inhibiting digestive enzymes of the insect gut. To date the molecular biology of insect digestive proteases has received little attention. Apart from Dipteran species, cDNA clones encoding digestive proteases have been isolated from only 3 phytophagous lepidopteran species, namely *M. sexta* (tobacco hornworm) (Peterson *et al.*, 1994,1995), *Choristoneura fumigerana* (spruce budworm) (Wang *et al.*, 1993,1995) and *Helicoverpa armigera* (Bown *et al.*, 1997, 1998 in press; Gatehouse *et al.*, 1997b). Insect serine protease sequences contain typical hydrophobic signalling (secretion) sequences, followed by sequences of 7-40 amino acid residues prior to the sequence of the N-terminus of the mature enzyme. The sequences contain an arginine residue at the putative activation cleavage site. Like mammalian serine proteases it is thought that at least some insect digestive enzymes are produced as zymogens, and are activated by proteolytic (perhaps tryptic) removal of an N-terminal peptide (Reeck *et al.*, 1997).

There are many reports in the literature about proteinase inhibitors and their effects on insect growth and development. Four classes,namely, serine; thiol; metallo- and aspartyl protease inhibitors have been identified. Most studies have focused on inhibitors of serine proteases, identified as major components of digestive fluids in numerous insects, and particularly in Lepidoptera (Applebaum, 1985). A common mechanism of serine protease action is the direct inhibition of digestive proteolytic enzymes whereby the inhibitor directly mimics the normal substrate for the enzyme and prevents the completion of normal peptide bond cleavage (Felton & Gatehouse, 1996). Many of these inhibitors are capable of simultaneously inhibiting two molecules of enzyme per inhibitor molecule (Gatehouse & Hilder, 1994). It is possible that a situation analogous to protease inhibitor effects in mammals exists in insects. In mammals major effects are the loss of nutrients through pancreatic hypertrophy and the overproduction of digestive enzymes (Liener, 1980). Comparitively little is known as yet about hormonal control and the synthesis/secretion of digestive proteases in insects. Enzyme secretion is thought to be largely constitutive in continuous feeders. However, virtually all insects studied show considerable changes in digestive enzyme levels during the digestion process and this is indicative of regulated secretion (Lehane *et al.*, 1996). Paracrine and/or prandial (i.e. the direct interaction of an element of the meal with digestive-enzyme producing cells) mechanisms are thought to be the main factors controlling enzyme synthesis and secretion (Lehane, 1995). However, distinguishing between the 2 probably interdependant mechanisms and the role of possible direct and indirect hormonal influences presents considerable difficulties. Thus details of the mechanisms involved remain to be established. Various studies have demonstrated that the insects physiological response to a diet containing enzyme inhibitors is complex and involves feedback

mechanisms that determine and adjust enzyme levels in the gut (Lehane *et al.*, 1996). The chronic ingestion of proteinase inhibitors results in the hyperproduction of proteolytic enzymes which, by limiting essential amino acids for protein synthesis, manifests as a reduction in growth and development (Broadway & Duffey, 1986). Accordingly the supplementation of methionine in the diet of *C. maculatus* has been shown to overcome the antinutritional effects of the cowpea trypsin inhibitor (CpTI) (Gatehouse & Boulter, 1983).

CpTI, a Bowman-Birk serine protease inhibitor, shows no toxicity to mammals at levels of 10 % total protein (Pusztai *et al.*, 1992) and has been shown to be effective against a wide range of economically important field and storage pests. The CpTI gene was the first of plant origin to be transferred successfully to another plant species resulting in enhanced insect resistance (Hilder *et al.*, 1987). Tobacco plants expressing CpTI at levels of up to 1 % of total soluble proteins were shown to have enhanced resistance to *H. virescens*, a serious pest of tobacco, cotton and maize. Significant protection afforded by CpTI was subsequently demonstrated for other Lepidopteran pests including *H. zea*, *Spodoptera littoralis* (Biosd) and *M. sexta* (Joh.). Significant protection of CpTI expressing tobacco plants against *H. zea* has also been shown in field trials (Hoffman *et al.*, 1991). Different CpTI expressing crops including potato, oil seed rape, rice and soft fruit have now been produced. CpTI transgenic rice has exhibited enhanced levels of protection against 2 major rice insect pests (*Sesamia inferens* and *Chilo suppressalis*) (Xu *et al.*, 1996). Significant resistance to the vine weevil (*Otiorhynchus sulcatus*) has recently been observed in CpTI transgenic strawberry plants (Graham *et al.*, 1995).

Transgenic plants with genes for other plant protease inhibitors have also been tested for their insecticidal activity. The tomato inhibitor II gene (PI-II), which encodes a trypsin inhibitor with some anti-chymotrypsin activity, when expressed in tobacco was shown to provide protection against *M. sexta*. Levels of expression were correlated with larval weight reduction (Johnson *et al.*, 1989). However, tobacco plants expressing tomato inhibitor I at similiar levels had no detrimental effects upon larval development (Johnson *et al.*, 1989). Carbonera *et al.* (1992) reported increased mortalities of the black cutworm *A. ipsilon* and armyworm *S.littoralis* exposed to plants transformed by a barley trypsin inhibitor. The gene encoding the potato inhibitor II (which inhibits chymotrypsin) has recently been engineered into tobacco. Larvae of the green looper (*Chrysodeixis enosmia*) exhibited significantly reduced growth on transgenic leaf tissue (Mcmanus *et al.*, 1994). Klopfenstein *et al.* (1993) has transformed trees (*Populus* hybrids) with the gene for PI-II and studies to evaluate insecticidal activity are planned. That proteinase inhibitors can synergise the insecticidal activity of Bt toxins has been indicated by recent work carried out by Zhao *et al.* (1996) who expressed 2 insecticidal genes in the same tobacco plant. One encoded CpTI and the other a toxin gene from Bt. Plants that contained both genes were more resistant to *H. armigera* larvae compared with plants containing either of the individual genes.

The potential for genes encoding cysteine protease inhibitors to produce transgenic crops with enhanced resistance, particularly to Coleopteran insects, is now being explored. Several studies have demonstrated *in vitro* inhibition of insect digestive proteases by cysteine protease inhibitors (Liang *et al.*, 1991; Hines *et al.*, 1991; Gillikin *et al.*, 1992; Michaud *et al.*, 1993). In some cases deleterious effects upon insects have been shown using artificial diets containing cysteine protease inhibitors (Chen *et al.*, 1992; Orr *et al.*, 1994; Edmonds *et al.*, 1996). Few studies have been reported of their insecticidal effects *in planta* although the oryzacystatin gene has been engineered into poplar trees for resistance towards *Chrysomela tremulae* F. (Leple *et al.*, 1995). The effects of inhibitors of metallo and aspartyl proteases on insects and their gut enzymes have been little studied although an inhibitor of aspartyl proteases has been reported in potato (Ritonja *et al.*, 1990) and inhibitors of carboxypeptidase-A (metallo protease) have been isolated from potato and tomato (Rancour & Ryan, 1968; Hass & Ryan, 1980).

The potential use of proteinaceous enzyme inhibitors in crop protection has been shown to hold great promise. However progress towards understanding the precise nature of interactions between inhibitors and insect digestive enzymes in different species is necessary. Additional problems due to insect adaptation or resistance are likely to develop in the field. Several insect species have been reported to adapt to proteinase inhibitors by altering their complement of secreted proteinases such that insensitive enzymes are predominant (Broadway, 1995; Jongsma *et al.*, 1995; Boulter & Jongsma, 1995). Adaptation has been shown to be influenced by host range with polyphagous insects better adapted to tolerate non-host proteinase inhibitors than more specialised feeders (Broadway & Villani, 1995). That adaptation through the induced production of inhibitor insensitive protease activity may be due to variants of existing enzymes rather than due to the induced activity of enzymes of different mechanistic classes has also been shown (Bown *et al.*, 1997). Bown *et al.* (1997) have suggested that multiple, varying protease encoding genes may represent an adaptive mechanism whereby insects can reduce the deleterious effects of plant protease inhibitors. Further studies in this area are required if adaptation and resistance to protease inhibitors in the field is to be managed successfully.

1.4.2. The use of plant α-amylase inhibitors.

A number of studies of inhibitors of enzymes other than proteases, mainly inhibitors of α-amylase, have been carried out. Whilst discussed separately it is recognised that α-amylase inhibitors from *Phaseolus* species are now considered as lectins (referred to as lectin-related proteins) following the recently revised definition of the term (section 1.4.4.1.). α-amylase inhibitors have been purified from many plants and are especially abundant in cereal grains (Garcia-Olmedo *et al.*, 1987). As wound induced synthesis of α-amylase inhibitors by insect attack has not been observed their role as defensive proteins remains speculative. Indirect evidence for a protective role is provided by the common bean (*Phaseolus vulgaris*) α-amylase inhibitor αAI which inhibits the activity of certain mammalian and insect α-amylases but not that of plant enzymes (Chrispeels,

1997). The common bean has long been known to be toxic to mammals and to contain factors that protect it against certain species of bruchid. The genes for 3 common bean defense proteins, namely phytohaemagglutinin (PHA), arcelin and αAI are encoded at a single locus in the *P. vulgaris* genome (Nodari *et al.*, 1993) and it is thought that the homologous genes have arisen by duplication of an ancestral gene. αAI has been found in most domesticated bean varieties and in wild accessions (Chrispeels, 1997). Bean αAI and wheat α-amylase inhibitors have been shown to have a variable effect on the activity of α-amylases of different origin (Gatehouse *et al.*, 1986; Ishimoto & Kitamura, 1988). The α-amylase of the cowpea weevil is strongly inhibited by bean αAI and this bruchid does not attack beans whereas least inhibited is the α-amylase of the Mexican bean weevil which is a major pest of beans (Ishimoto & Kitamura, 1989). Subsequent studies have established that this lack of inhibition is caused by digestion of the inhibitor by gut proteases (Ishimoto & Kitamura 1992; Ishimoto & Chrispeels, 1996). αAI is thus thought to be a major factor in the resistance of beans to the cowpea weevil.

αAI is synthesised as an initial translation product that is co-translationally and post-translationally modified to yield a holoprotein comprised of 2 glycoproteins α and β which form a complex (Chrispeels, 1997) of 20 - 60 kDa (Ho *et al.*, 1994). The αAI protein has no inhibitory activity until it is proteolytically processed at Asn ^{77} (Puego *et al.*, 1993). It is thought that proteolytic processing may bring about a conformational change that creates an active site allowing αAI to bind to its target enzyme (Chrispeels, 1997). The residues -Try-Gln-Trp-Ser-Try- have been shown by mutational analysis to be involved in the active site of the inhibitor (Chrispeels, 1997). Complex formation has a pH optimum of around 5.5 and therefore αAI inhibits amylases in the acid midgut of Coleoptera, but not in the alkaline midgut of Lepidoptera. For this reason αAI is well suited for defence against the starch eating bruchids.

The first αAI sequence was identified (Moreno & Chrispeels, 1989) on the basis of amino acid sequence identity of a purified heat stable αAI and a previously cloned cDNA referred to as 'bean lectin' based on its similarity to PHA (Hoffman *et al.*, 1982). This inhibitor is referred to as αAI-1. Expression of the αAI-1 cDNA in tobacco seeds confirmed that the sequence encoded an active inhibitor (Attabella & Chrispeels, 1990). Transgenic seeds exhibited antigenicity to bean α-amylase inhibitors and the expression of polypeptides of molecular weight corresponding to these particular inhibitors was shown. Seed extracts were found to be active against porcine pancreatic α-amylase and α-amylase present in the midguts of the meal worm *Tenebrio molitor* L. The potential for use of this gene to protect leguminous seeds from Coleopteran pests is being investigated. Recently Shade *et al.* (1994) produced αAI bean expressing pea plants. Transgenic αAI had a dramatic impact upon the azuki bean weevil, with larval death was closely correlated with the biochemical detection of αAI-1 in individual seeds. The response of the cowpea weevil was more variable although complete insect mortality was recorded for αAI levels of 0.8 - 1.0 %. Schroeder *et al.* (1995) have since shown that the αAI expressing pea seeds significantly inhibit the development of the pea weevil

(*Bruchus pisorum*), an insect that damages peas in the field. It is likely that protection to other starchy grain legumes that suffer bruchid attack could be similarly afforded by the αAI gene. At present the major constraint upon progress is the lack of suitable transformation methods for species such as cowpeas, mungbeans and chickpeas. Because αAI inactivates intraduodenal amylase in humans (Layer *et al.*, 1985) it is likely to be an antinutritional factor that must be heat denatured prior to consumption.

Many varieties of bean have been shown to contain 2 αAI inhibitors (Iguti & Lajolo, 1991), indicating that αAI, like the homologous protein PHA, may be encoded by 2 different genes (Chrispeels, 1997). αAI-2 showing 75 % amino acid sequence identity with αAI-1 has been purified from a wild accession of the common bean resistant to Mexican bean weevils (Mirkov *et al.*, 1994; Suzuki *et al.*, 1994). Unlike αAI-1, it is an effective inhibitor of Mexican bean weevil α-amylase (Suzuki *et al.*, 1993). The inhibition of Mexican bean weevil α-amylase αAI-2 expressed *in planta* has not yet been successfully demonstrated (Mirkov *et al.*, 1994). Recently a gene encoding an α-amylase inhibitor from wheat in tobacco has been shown to give increased protection against the army worm (*Spodoptera* spp.) and greasy cut worm (*Agrotic* spp.) (Carbonera unpublished).

1.4.3. The use of plant chitinases.

Chitinases are defined as enzymes with a specific hydrolytic activity directed against the homopolymer chitin, although some chitinases hydrolyse other related polymers. Chitin is a polymer of N-acetylglucosamine (GlcNAc) residues with β-1,4 linkages. Enzymatic cleavage occurs randomly at internal locations over the length of the chitin microfibril producing mainly chitbiose and other soluble low molecular mass multimers (e.g. chitotetraose and chitotriose) of GlcNAc. As one of the most abundant biopolymers it occurs as a structural component in the cuticles and shells of arthropods, in the cell walls of fungi and some algae, in nematodes, mollusks and in many other types of organisms (Kramer & Koga, 1986; Cabib, 1987; Gooday, 1990). The importance of chitin and chitinolytic enzymes in insect growth and development has fuelled recent investigations into the potential use of chitinases as insect defence proteins in transgenic plants and as microbial biological control agents.

Plant chitinases can be induced by viral attack (Métraux & Boller, 1986; Pan *et al.*, 1992; Margis-Pinheiro *et al.*, 1993) attack from bacteria (Métraux & Boller, 1986; Anuratha *et al.*, 1996) and in response to environmental stress (Boller *et al.*, 1983; Grosset *et al.*, 1990; Mauch, 1984 & 1998). This together with the absence of chitin or any other endogenous substrates in plants for purified plant chitinases (Sahai & Manocha, 1993) is indicative of a protective role in defence against fungi and perhaps insects, both of which contain chitinous exoskeletons.

Limited research into the role of plant chitinases in protection against insect attack has been carried out. Transgenic potato plants expressing a gene encoding bean chitinase

(BCH) under control of the constitutive CaMV 35S promoter were found to reduce the fecundity of the glasshouse potato aphid *Aulocorthum solani*, although this reduction was not statistically significant (Down, 1998). A synergistic effect was observed with transgenic potatoes expressing a double construct encoding GNA and BCH. A significant reduction in cumulative nymph production was observed on plants expressing both GNA and BCH compared to nymph production by aphids feeding on control and GNA only expressing plants (Down, 1998). Transgenic rice plants expressing relatively high levels of a rice chitinase (0.05 % total soluble proteins) were found to have no detrimental effects on the growth of the fall armyworm, *Spodoptera frugiperda* (Kramer *et al.*, 1997).

1.4.4. The use of plant lectins.

1.4.4.1. Plant lectins- definition, classification and distribution.

Plant lectins are a very large and heterogenous group of proteins most recently defined as proteins which irreversibly bind specific mono- or oligosaccharides and possess at least one catalytic domain (Peumans & Van Damme, 1995). Most lectins contain multiple sugar binding sites and can therefore, by cross-linking carbohydrate side chains of cell-surface glycoproteins, give rise to the formation of cell aggregates and visual agglutination of erythrocytes. The term haemagglutinin, still used as a synonym for lectin, dates back to 1888 when Stillmark observed that preparations from castor bean extracts agglutinated red blood cells. Haemagglutinin was replaced in 1948 by the term lectin (from the latin verb 'legere', meaning to select) following the discovery of the blood group specificity of certain agglutinins (Renkonnen, 1948; Boyd & Regrera, 1949). Accordingly an earlier definition of lectins as carbohydrate-binding proteins of non-immune origin which agglutinate cells or precipitate glycoconjugates was based primarily on the sugar specificity and inhibition of agglutination reaction (Goldstein, 1980). However, this definition excluded poorly agglutinating toxins known to contain lectin subunits (e.g. ricin) and those lectins that contain a second type of binding site interactive with non-carbohydrate ligands. Lectins were classically sub-divided into glucose/mannose-specific; galactose/ N-acetylgalactosamine (Gal/GalNAc)-specific; N-acetylglucosamine (GlcNAc)-specific; fucose-specific and sialic acid-specific (Goldstein *et al.*, 1986). This however, excludes many lectins such as the strictly mannose-specific snowdrop (*Galanthus nivalis* agglutinin) (GNA) lectin; those that bind only to oligosaccharides and it does not account for the higher affinity of many lectins for oligosaccharides containing 2 or more monosaccharide units.

The most recent definition of lectins is based upon the presence of functionally active carbohydrate binding domains and includes proteins and plant enzymes such as Class I chitinases, and Type-2 ribosome inactivating proteins (RIP e.g. ricin and abrin) which contain, respectively, an N-terminal chitin binding and catalytic domain (Collinge *et al.*, 1993) and an N-terminal toxic A chain and a C-terminal carbohydrate binding B-chain (Barbiera *et al.*, 1993). Also included are carbohydrate-binding proteins that possess

only one binding site and are therefore unable to precipitate glycoconjugates or agglutinate cells such as the mannose binding protein from orchids (Van Damme *et al.*, 1994 a,b). Lectin-related proteins, evolutionarily and structurally related to lectins but devoid of carbohydrate-binding activity (e.g. arcelins and α-amylase inhibitor from *Phaseolus* spp.) are also included (Mirkov *et al.*, 1994).

Thus 4 major types of lectins, namely, merolectins, hololectins, chimerolectins, and superlectins, are distinguished on the basis of the overall structure of the lectin subunits (Van Damme *et al.*, 1998).

Consisting exclusively of a single carbohydrate domain merolectins, due to their monovalent nature, cannot precipitate glycoconjugates or agglutinate cells (e.g. the monomeric mannose-binding protein from orchids (Van Damme *et al.*, 1994 a,b). Hololectins, also comprised solely of carbohydrate-binding domains, contain 2 or more identical or very homologous domains which bind the same, or structurally similar, sugars. With multiple binding sites this group, which includes most lectins, are capable of agglutinating cells and/or precipitating glyconjugates. Chimerolectins are fusion proteins composed of a carbohydrate-binding domain tandemly arrayed with an unrelated and independantly acting domain which may have a well defined catalytic (or other biological) activity. Chimerolectins with a single carbohydrate binding site behave as merolectins (e.g. Class I plant chitinases with only 1 chitin binding domain per molecule, cannot precipitate glycoconjugates or agglutinate cells). Chimerolectins with multiple carbohydrate sites behave as hololectins (e.g. Type 2 RIP, possess 2 carbohydrate-binding sites on each B chain and agglutinate cells). Superlectins are a particular type of chimerolectin. They are fusion proteins comprised of 2 tandemly arrayed carbohydrate-binding domains that are structurally distinct and recognise structurally unrelated sugars. To date only one super lectin, namely, TxLC-I from tulip bulbs has been described (Cammue *et al.*, 1986).

Lectins have been isolated from a wide variety of species encompassing almost every major taxonomical classification of flowering plants, and are also present in a variety of non-flowering and lower plants (Etzler, 1985). Chimerolectins belonging to Class I chitinases appear to be present in almost all plant species (Collinge *et al.*, 1993) whereas the presence of agglutinating lectins appears to be relatively scarce (Van Damme *et al.*, 1998).

Whilst lectins are found in virtually all vegetative tissues many plants accumulate lectins in storage tissue such as seeds, bulbs and bark. Lectins typically account for 0.1-5 % of total seed protein but can reach levels of up to 50 % as exemplified by *Phaseolus* species. Non seed lectins are also found at levels of 0.1 - 5 % of total proteins although values of >50 % have been reported for lectins in vegetative storage tissues of some species such as garlic cloves (Smeets *et al.*, 1997).

Although structurally diverse many lectins belong to a homologous family of proteins based on an amino acid chain of approx 220 residues, thought to have evolved through gene duplication and divergence (Chrispeels & Raikel, 1991). This is indicated by lectin and lectin-like genes in *Phaseolus vulgaris* which include 2 lectin isoforms phytohaemagglutinin L and E, the α–amylase inhibitor and arcelin storage proteins. The terms isolectin or isoform describe lectins encoded by the same gene but different due to variations in post translational modifications (Van Damme *et al.,* 1998). Four main evolutionary groups, namely legume lectins (Sharon & Lis, 1990); chitin-binding lectins (Raikhel *et al.,* 1993); Type 2 RIP (Barbiera *et al.,* 1993) and the monocot mannose-binding lectins (Van Damme *et al.,* 1998) have been identified.

Legume lectins, most commonly found in seeds of the Leguminoseae family, differ considerably in their carbohydrate-binding specificity. Distinguished by their preferential binding to monosaccharides most are specific for mannose/glucose (e.g. *Canavalia ensiformis* lectin (Con A)) or gal/GalNAc (e.g. *Arachis hypogaea* lectin). Lectins that interact exclusively with oligosaccharides (e.g. *Phaseolus vulgaris* (PHA) lectin) are also found. All legume lectins contain divalent cations (at specific metal binding sites) essential for their carbohydrate binding activity (Van Damme *et al.,* 1998). Synthesised as preproteins or preproteins on the endoplasmic reticulum (ER) legume lectins are converted into mature lectins after post-translational modifications both in the ER and during transport via the Golgi system to their final destination, typically vacuoles or vacuole derived organelles. Several legume lectins have been studied by X-ray crystallography (e.g. Con A) (Rongé *et al.,* 1991). All, irrespective of their sugar binding specificity, appear to be composed of monomers with a similiar 3-dimensional structure, typified by the occurrence of 2 anti-parallel pleated sheets (β structures). The majority of residues not involved in the β structures are located in loops and turns interconnecting the strands of the pleated sheets. Mn^{2+} and Ca^{2+} binding sites are highly conserved. Less conserved are amino acids in the carbohydrate binding sites, substitutions are thought to have facilitated evolution towards proteins with different sugar-binding specificity (Van Damme *et al.,* 1998).

Chitin-binding lectins, composed of one or more hevein domains (Raikhel *et al.,* 1993), have been isolated from several taxanomically unrelated families, including Gramineae, Urticaeae, Solanaceae and Euphorbiaceae. In addition to binding chitin, most of these lectins also react with GlcNAc, GlcNAc-oligomers and N-acetyl-D-neuraminic acid. Hololectins (true agglutinins), merolectins (monovalent and therefore non-agglutinating) and an extended group of chimerolectins, the Class I chitinases (hevein domain tandemly arrayed with a catalytic domain with chitinase activity) are found within this group.

Since the isolation of GNA by Van Damme *et al.* (1987) structurally and evolutionary related lectins have been isolated from 5 different families, namely, Amaryllidaceae, Alliceae, Araceae, Orchidaceae, and Liliaceae. Due to their unique specificity towards mannose and exclusive occurrence in monocotyledonous species they are classed as

monocot mannose-binding lectins. This single superfamily exhibits marked sequence homology (Van Damme *et al.,* 1991 a,b; 1992; 1994 a,b; 1995 a,b; 1996; Koike *et al.,* 1995). All monocot mannose-binding lectins are composed of lectin polypeptides of approx. 12 kDa. Important differences in processing and post-translational modifications have been identified (Van Damme *et al.,* 1998). Most of the lectins are composed of 2 or 4 identical subunits of approx. 12 kDa, synthesised as separate polypeptides. These homomeric lectins are synthesised as preproproteins and converted by co-translational cleavage of a signal peptide and post-translational removal of a C-terminal peptide, to a typically non-glycosylated mature lectin polypeptide (Van Damme *et al.,* 1991b; 1992; 1994 a,b). There are also several heteromeric forms. Heterodimers, built up of 2 different (but highly homologous) subunits (approx. 12 kDa), may be derived from separate preproproteins and processed similarly to homomeric precursors (e.g. *Allium ursinum* lectin (Smeets *et al.,* 1994). A second type are heterodimers or heterotetramers composed of 2 different types of subunit derived from a single precursor with 2 distinct lectin domains such as the heterodimeric garlic (*A. sativum*) lectin ASA-I (Van Damme *et al.,* 1992).

Unlike other plant lectin groups the monocot mannose-binding lectins are encoded by large families of closely related genes (Van Damme *et al.,* 1991 a,b; 1992 a,b; 1994 a,b; 1995 a,b; 1996). Sequence analysis of various cDNA clones has demonstrated that the expression of different lectin genes in a species can explain the occurrence of multiple lectin isoforms at the protein level (Van Damme *et al.,* 1992 a). Molecular modelling, based on X-ray crystallography of GNA (described in section 1.4.4.2.) and the homology and sequence identity of this group of lectins has suggested that the subunits of all monocot mannose-binding share a common 3-dimensional structure.

Type-2 ribosome-inactivating proteins (RIP) possess a highly specific rRNA N-glycosidase activity and are capable of catalytically inactivating eukaryotic (and in some cases prokaryotic) ribosomes. Type-1 and Type-2 RIPs differ in their subunit composition. Type-1 RIPs are monomeric proteins consisting of a single catalytically active subunit (approx. 30 kDa) and as such are not members of the lectin family. Type-2 RIPs are composed of 1,2, or 4 identical subunits. Each subunit consists of 2 structurally and functionally different polypeptides called A and B chains that are covalently linked by a disulphide bond. The A chain has N-glycosidase activity and sequence homology to Type-1 RIPs. The B chain is catalytically active and exhibits carbohydrate-binding activity. Both A and B chains are synthesised as large precursors containing 2 functional domains. Precursors are processed via the excision of a peptide linking the C-terminus of the first domain and the N-terminus of the second domain. This group, like the legume lectins, contains lectins with differing sugar-binding properties although most Type-2 RIPs are specific for gal or GlcNAc. Type-2 RIPs, found in members of taxonomically unrelated plant families, are considered as a superfamily of structurally related lectins (Van Damme *et al.,* 1998).

1.4.4.2. Snowdrop lectin Galanthis nivalis agglutinin (GNA).

Found at high concentrations in the bulbs of snowdrops, GNA is a hololectin which selectively agglutinates rabbit but not human erythrocytes. GNA belongs to the previously discussed group of monocot mannose-binding lectins. The 3-dimensional structure of GNA has recently been resolved by X-ray crystallography (Hester *et al.*, 1995). GNA, a tetrameric protein composed of 4 identical subunits of approx 12.5 kDa, is rich in leucine, asparagine and glycine and contains 3 cysteine residues. The structure corresponds to a β barrel composed of 3 antiparallel four-stranded β sheets interconnected by loops. Recognising exclusively mannose each lectin monomer possesses 3 identical mannose binding sites (composed of 4 amino acid residues) which can potentially accommodate 3 mannose residues (12 per tetramer). It is not glycosylated and occurs as a natural mixture of at least 6 isolectins that show variability in the C-terminus region (Van Damme, 1991). Mannose binding does not require the presence of metal ions or interactions with aromatic side chains in the binding site (Hester *et al.*, 1995). The presence of a mannose binding pocket in GNA is thought to explain the strict specificity requirements of this lectin family (Hester *et al.*, 1995).

1.4.4.3. Jack bean lectin Canavalia ensiformis (Con A).

Con A, first isolated from seeds of Jackbean and crystallised by Sumner *et al.* in 1936, is a hololectin belonging to the previously discussed group of legume lectins. This lectin exhibits greatest affinity for D-mannose and its glycosides in α-anomeric form. In oligo- and polysaccharides Con A reactivity is strongest with those containing non-reducing terminal α-linked mannose residues (Goldstein & Poretz, 1986). It also reacts with terminal α-linked glucose and GlcNAc but with less affinity (Goldstein & Poretz, 1986). At neutral pH Con A is composed of 4 non-glycosylated subunits of 26.5 kDa, some of which are broken and held together with non-covalent forces (Wang *et al.*, 1971). Below pH 5.6 the lectin is a 52 kDa dimer and above this pH it associates as a tetramer (Goldstein & Poretz, 1986).

Molecular cloning and biosynthesis studies have shown that Con A is not processed like other legume lectins (Van Damme *et al.*, 1998). The lectin is first synthesised in the developing seed as an inactive glycoprotein. Subsequent proteolytic splitting in the midchain position and removal of the leader peptide occurs. The broken peptide chain then undergoes a ligation reaction and the glycan side-chain is removed. Removal reveals the carbohydrate binding site and thus facilitates lectin activity (Carrington *et al.*, 1992). Activity can occur without proteolytic modification but deglycosylation is necessary for full activity (Min *et al.*, 1992). Con A, like other legume lectins is a metalloprotein with 1 Ca^{2+} and 1 Mn^{2+} per protomer which are essential for activity (Agrawal & Goldstein, 1967).

1.4.4.4. Insecticidal effects of plant lectins.

Showing marked stability under unfavourable conditions, stability over a wide range in

pH, heat resistance, and resistance to animal and insect proteases, lectins strongly resemble other defense related proteins (Peumans & Van Damme, 1995). Most plant lectins exhibit a much higher affinity for complex chains of animal glycoconjugates than for typical plant carbohydrates and some bind solely to carbohydrates not found (or rare) in plants (Peumans, 1995). In addition, accumulation in tissue susceptible to insect attack provides further indirect evidence for their role in plant defense. Work carried out to date which has identified lectins with insecticidal activity will be discussed by insect order.

Lectins active against Coleoptera.

An involvement of lectins in insect resistance was first proposed by Janzen *et al.* (1976) who showed that the PHA was toxic to developing larvae of the bruchid beetle *C. maculatus*. This was subsequently confirmed by Gatehouse *et al.* (1984) although a highly purified lectin preparation was found to be less effective than a crude preparation in retarding insect development. The contaminating protein with insecticidal activity was shown to be αAI, the α-amylase inhibitor (Huessing *et al.*, 1991 a). Other lectins toxic to *C. maculatus*, a major storage pest of cowpeas in many parts of the world, have since been identified.

Of 17 commercially available plant lectins screened by Murdoch *et al.* (1990), 5 were found to cause a significant delay in the development of *C. maculatus* larvae at dietary levels of 0.2 % and 1.0 % (w/w). Toxic lectins were specific for GalNAc/gal or GlcNAc. Wheat germ agglutinin (WGA) was the most toxic. Similar levels of toxicity to *C. maculatus* were later observed for rice (*Oryza sativa*) and nettle (*Urtica dioica*) lectins (UDA) which, like WGA, are specific for GlcNAc (Huessing *et al.*, 1991b). Gatehouse *et al.* (1991) have also shown that the winged bean (*Psophocarpus tetragonolobus* DC) lectin which is GlcNAc-specific, is toxic to *C. maculatus*. The use of lectins specific for N-acetylglucosamine was based on the fact that the insect midgut PM contains chitin, a polymer of GlcNAc (Richards & Richards, 1977) and was thus a target for lectins specific for this aminosugar. However, sugar specificity was shown to be a poor indicator of insecticidal activity. The lectin SNA-II from elderberry, specific for GalNAc, was ineffective against *C.maculatus*, whereas SNA-I, with specificity for 2,60-neuraminyl-gal/GalNAc was highly potent. Similarly the lectin from garden pea has little or no toxic effect, whereas lectins from *Dioclea* spp. are significantly toxic, as is GNA from snowdrop (Gatehouse & Gatehouse, 1998).

A similar approach was adopted by Czapla and Lang (1990) who screened 26 lectins for activity against the southern corn rootworm (SCR) (*Diabrotica undecimpunctata howardi* Barb.). This species is the easiest of the 3 corn rootworm species to rear on artificial diet although the western corn rootworm (*D. vergifera vergifera*) and northern (*D. barberi*) cause the greatest economic damage to maize (Czapla, 1997). Of the lectins tested, 3, from castor bean, pokeweed and green marine algae at 2 % in artificial diet were found to be toxic to neonate larvae. An additional 5 lectins, including WGA, inhibited larval growth by at least 40 % compared to larvae fed on control diet. All lectins exhibiting

insecticidal activity were specific for either GalNAc or GlcNAc. The mannose specific snowdrop lectin GNA has also been shown to be insecticidal towards the SCR (Edmonds, 1994). Both GNA and WGA were found by Allsopp and McGhie (1996) to be insecticidal for larvae of the sugarcane whitegrub (*Antitrogus parvulus*) a representative of a complex of 19 melolonthine whitegrub species which are major pests of sugarcane in Eastern Australia. GNA in semi-artificial diet at 0.05 % (v/v) caused significant mortality and growth inhibition after 28 and 22 days, respectively. WGA was active at similar concentrations although expression of the effects was slower. Recently an extensive study by Cavaliera *et al.* (1995) identified over 60 plant lectins, with different sugar specificities, that inhibited weight gain of neonate SCR by at least 40 % when tested at the 2 % level.

Lectins active against Homoptera.

In addition to severe host damage these insects act as major vectors of plant viruses. Two major pests of rice are the rice brown planthopper (RBP) (*Nilaparvata lugens*) and the rice green leafhopper (RGL) (*Nephotettix cinciteps*). Using an artificial diet system Powell *et al.* (1993) screened a series of lectins against the RBP. Whilst some had no effect on survival (e.g. garden pea and potato lectins), others exhibited significant effects. Of these GNA and WGA both gave 80 % mortality at a dietary concentration of 0.1 % (w/w). GNA was also found to be toxic to the RGL, causing 87 % mortality at a dietary concentration of 0.1 % (w/w).

Lectins have also been screened for insecticidal activity against the potato leafhopper (*Empoasca fabae*), a serious pest of alfalfa. Of 14 plant lectins screened by Habibi *et al.* (1993), 6 (PHA, WGA, jacalin, and lectins from *Lens culinaris* (lentil) pea and horsegram) were found to cause a significant reduction in insect survival at dietary levels of 0.2 - 1.5 % (w/w).

The susceptibility of 3 species of aphid to lectins has been reported. Rabhé and Febvay (1993) demonstrated that Con A had a significantly detrimental effect upon the survival and growth of the pea aphid *Acyrthosiphon* (Harr.). In contrast WGA was relatively ineffective. Both Con A and GNA at 0.1 % in artificial diet have been shown to cause a significant reduction in the growth and fecundity of the peach potato aphid (*M.persicae* (Sulz)) (Sauvion *et al.*, 1996). In this study the daffodil lectin (*Narcissus pseudonarcissus*) NPA, at a concentration of 40 µM and garlic (*Allium sativum*) lectin ASA, at a concentration of 63 µM, were seen to inhibit nymphal growth by 59 % and 26 %, respectively. The effect of 3 chitin binding lectins WGA, stinging nettle lectin (UDL), and lectin purified from *Brassica fruticulosa* and *B. brassicae* (BL), on the cabbage aphid (*Brevicoryne brassicae*) has been examined using artificial diet bioassays (Cole, 1994). UDL and BL caused 100 % mortality within 4 days at 0.25 % (w/v), and WGA (at the same dietary concentration) caused 100 % mortality within 8 days.

Lectins active against Lepidoptera.

To date comparitively few lectins have been tested in artificial diet studies and found to be toxic to Lepidoptera. Lectin insectidial effects have been examined against the European corn borer (ECB) (*O. nubilalis*) which is a major pest of maize in the USA, causing annual damage and control costs estimated at 800 million $ (US) (Czapla, 1997). Of 26 plant lectins screened by Czapla and Lang (1990), only 3 were shown to be insecticidal at 2 % levels in artificial diet. The lectins from castor bean (*Ricinus communis* L.), camel's foot tree (*Bauhinia purpurea*) and wheatgerm (WGA), specific respectively for GalNAc, GalNAc, and GlcNAc, were found to cause 100 % mortality when fed to neonates for 7 days. At 0.1 % topical applications WGA and the castor bean lectin inhibited larval weight gain by > 50 %. The lectin from soybean at a 1 % level was shown to inhibit the growth of the tomato hornworm (*M. sexta*) (Shukle & Murdock, 1983). In contrast, Czapla and Lang (1990) found that this lectin facilitated an increase in larval weights (by > 25 % compared to control larvae) of the ECB. Law & Kafir (1997) found that a mannose binding lectin from peanut (*Arachis hypogaea*) at 1.0 % and 0.5 % (m/m) in artificial diet caused a significant increase in mortality and a significant decrease in the length and mass of stem borer (*Chilo partellus*) larvae. In contrast, the pea (*Pisum sativum* L.) lectin had no insecticidal effect despite similar mannose/glucose binding properties.

Lectins active against Diptera.

The effects of plant lectins on blowfly (*Lucilia cuprina* (Weid.)) larvae have recently been examined in an attempt to identify possible control strategies for this pest which feeds on tissues and tissue fluids of susceptible sheep. Eisemann *et al.* (1994) observed a concentration dependant inhibition of larval growth rate and (at higher lectin concentrations) larval death following the ingestion of WGA, lentil lectin, and Con A. Deleterious lectin effects were not observed in the presence of inhibitory sugars indicating that activity was a consequence of specific lectin-ligand interactions.

1.4.4.5. Insect-resistant transgenic plants expressing lectins.

Artificial studies have identified several lectins with potent insecticidal activity against a number of important pest species. Unlike artificial diets the production of lectin-expressing transgenic plants has provided direct evidence that lectins can play a protective role against insects in plants.

A lectin isolated from pea (*Pisum sativum*) is readily broken down in the mammalian gut (Begbie & King, 1985) rendering it non toxic. A gene encoding the pea lectin (P-Lec) has been expressed in transgenic tobacco plants using the constitutive CaMV 35S promoter (Edwards, 1988). Plants expressing the lectin at up to 1.0 % of total protein were found to provide significant protection against attack by the tobacco hornworm (*H. virescens*). Survival was not affected but larval biomass and leaf damage were both found to be significantly reduced on the transgenic plants compared to controls (Boulter *et al.*, 1990).

Although resistant to gut proteolysis GNA exhibits no toxicity towards mammals (Pusztai, 1991). A gene encoding GNA has been constitutively expressed in tobacco and potato plants. Initially the GNA coding sequence under control of the CaMV 35S promoter was engineered into tobacco plants. Constructs containing a complete coding sequence for the preproprotein gave rise to GNA levels of up to 1 % of total protein in the leaf tissue of primary transformants. Selfed primary transformants produced progeny plants expressing GNA at up to 1.5 % of total protein. The functional integrity of GNA expressed in the transgenic tobacco was demonstrated by haemagglutination assay. Tobacco plants transformed with GNA were bioassayed against the tobacco budworm (*H. virescens*) (Gatehouse *et al.,* 1992) and the peach potato aphid (*M. persicae*) (Hilder *et al.,* 1995). Survival was not affected by feeding *H. virescens* larvae on GNA leaf discs. However, larvae reared on GNA discs weighed approx 60 % less than controls and a reduction of approx 50 % in GNA leaf disc area consumed, compared with controls, was recorded. Both whole plant and leaf disc assays demonstrated that GNA caused a significant reduction in the survival of *M. persicae* nymphs (Hilder *et al.,* 1995). A similar construct was used to produce transgenic potato plants which were vegetatively propagated to produce clonal replicates for bioassay. Comparable GNA expression levels to those found in tobacco were observed. GNA expressing potato plants were tested against the glasshouse potato aphid (*A. solani*). In laboratory trials a significant reduction (80 %) in fecundity was observed and a significant reduction in population build up was observed in glasshouse trials (Down *et al.,* 1996). Similar effects, in growth room trials, have been observed with the peach potato aphid (*M. persicae*) (Gatehouse *et al.,* 1996). GNA has now been expressed in transgenic rice (*Oryza sativa* L.) under control of the phloem specific promoter (RSs1) (Rao *et al.,* 1998). Plants expressing GNA, at up to 2.0 % of total soluble proteins, have shown resistance to attack by the RBP, evidenced as a decrease in survival, fecundity and developmental rate of the insects. GNA was also, as previously observed in artificial diet studies (Powell *et al.,* 1995), shown to have a deterrant effect on BPH feeding. GNA expressing potato plants have recently been shown to be significantly protected against attack by tomato moth larvae (*Lacanobia oleracea* L.) (Gatehouse *et al.,* 1997, Fitches *et al.,* 1997). The insecticidal effects of GNA upon *L. oleracea* are discussed in full detail in Chapter 4.

1.4.4.6. Mechanisms of lectin action.

Despite the potential of lectins for use as insect control agents little is known of the mechanisms by which their insecticidal effects are produced. As most edible plants contain lectins their toxicity to mammals has been studied in considerable detail over the past 20 years. Until recently most studies have been confined to a few selective lectins, namely, Con A, PHA and WGA. These lectins have generally detrimental effects on mammals. It has long been known that the inclusion of raw kidney beans in the diets of monogastric animals leads to rapid weight loss, and at high enough concentrations, to death within a few days (Pusztai, 1991). Much less is known about the effects of

potentially beneficial lectins such as the tomato lectin (Kilpatrick *et al.,* 1985). When administered orally at low doses many lectins have been shown to have beneficial effects on the digestive/absorptive efficiency of the gut, its immune system and bacterial ecology. Some lectins, via modulating gut hormone secretion, can influence the body's endocrine system with beneficial consequences for general metabolism (Pusztai, 1993). Accordingly the potential use of lectins as blockers of pathogens, immune stimulants, hormone modulators and metabolic agents in clinical-medical applications is now under investigation.

The vast range of biological responses to different lectins stems essentially from their degree of resistance to gut proteolysis. More than 90 % of fully reactive Con A, GNA and PHA has been recovered in the stomach and small intestine of lectin-fed rats (Pusztai, 1991).

The primary mode of lectin action in mammals is thought to depend upon the initial binding of lectins to specific oligosaccharides of microvilli of cells lining the gut wall (Pusztai, 1991). Lectins may also combine with appropriate secreted dietary and bacterial glycoconjugates throughout the digestive tract. Pusztai *et al.* (1990) demonstrated a firm correlation between the strength of binding of 6 lectins with differing sugar specificities to the rat BBM and their effectiveness as antinutrients.

The majority of BBM proteins including hormone and growth factor receptors, transport proteins and enzymes are glycosylated. Membrane lipids and gangliosides are also glycosylated, and all secreted mucins are carbohydrate-rich glycoproteins (Pusztai & Bardocz, 1996). Thus the scope of potential lectin-gut interactions is correspondingly vast. As a consequence of their sugar specificity not all lectins react with the epithelium. The binding of Con A to the rat BBM has been shown to induce disruptive changes in cell morphology, metabolism, and the proper functioning of the entire digestive system (Pusztai, 1991). In contrast, GNA, due to the relative scarcity of mannose containing BB glycans, does not initially exhibit binding to the rat BBM and this is thought to largely account for its lack of toxicity to mammals. Although recognition between lectins and receptors is instantaneous the strength of binding is dependant on the association constant between the lectin and the glycosyl group and the number of unoccupied sites (Pusztai & Bardocz, 1996). Lectin-ligand interactions are additionally influenced by variability in the structure of glycosyl side chains which depends on factors such as animal age, blood group specificity, genetics, mucosal cell types and their state of differentiation/maturation and position on the crypt/villus axis and along the gastrointestinal tract (Pusztai & Bardocz, 1996). To date the characterisation of specific mammalian gut surface lectin receptors has not been reported. Quantitative information on the precise carbohydrate structures of receptors, their location along the crypt/villus axis and changes occurring during normal turnover or under different conditions of age and dietary status is also limited. Consequently specific interactions *in vivo* between lectins and receptors remain largely hypothetical and proposed mechanisms of lectin action are based, in the main, on

immunohistochemical observations and on studies examining lectin-induced biochemical changes at the cellular and tissue level.

Receptor proteins are usually composed of more that one subunit. Signal molecules bind to glycosylated subunits exposed on the external side of the membrane. Subunits spanning the membrane are responsible for signal transmission and for the activation of second messenger system(s). Sugar structures may be present in or close to the active site of the receptor. They may be on the same subunit as the binding site for the natural ligand, or on another subunit. Whilst the lectin binding site is not the normal functioning binding site of the receptor, the resulting conformational change in receptor subunits embedded in the membrane and the ensuing signal transduction may be similiar irrespective of whether activation was by the physiological ligand or by the lectin. Thus lectins can mimic the effects of the natural ligand (e.g. growth factor, hormone). Lectin induced conformational changes in the receptor can send messages via second messengers to the cell. Thus, gene expression in the cell or tissue may be altered facilitating changes in the level and composition of enzymes produced in the cytoplasm or on the surface of the cell. In turn the cells ability to interact with growth factors and hormones may be altered. Therefore lectin binding may effect the entire metabolism of the cell. Alternatively bound lectin may physically block the active site of the receptor and thereby inhibit or destroy the physiological effect of the natural ligand. It is thought that so-called non- or anti-mitogenic lectins may act in this way (Pusztai & Bardocz, 1996).

The ability of PHA to mimic the effects of insulin is the most studied example of a lectin acting as an exogenous metabolic signal. This effect, observed both *in vivo* and *in vitro,* arises from the ability of PHA to recognise and bind to complex glycosyl moieties of insulin and insulin-like growth factor receptors (Pusztai, 1991). Similarities in the effects of insulin and PHA include activation of receptor tyrosine kinase; receptor autophosphorylation; stimulation of ion, glucose, amino acid and nucleic acid synthesis and cell growth/proliferation (Pusztai, 1991).

The growth factor activity of lectins is determined primarily by the strength and intensity of their binding to the gut wall and subsequent endocytosis by epithelial cells (Pusztai *et al,* 1990). Even when relatively weak, as is the case for Con A, the organisation of the epithelial membrane is sufficiently disturbed to allow a slight growth of the gut (King *et al.,* 1982; Pusztai *et al.,* 1990). Lectin induced damage to the microvillus membrane of epithelial cells causes the gut to become leaky and thereby compromises its protective and digestive/absorptive functions. To counteract these effects and prevent harmful compounds or bacteria entering the body the crypt cell proliferation rate (CCPR) is stimulated (Pusztai, 1991). The size of the crypts and number of cells they contain is also increased and with continuous exposure to PHA, the cell turnover time can decrease from 72 to 12 hours (Pusztai & Bardocz, 1996). The exact mechanism by which this occurs is still unknown (Pusztai *et al.,* 1988 a,b). Whilst during hyperplastic growth cell migration on the villi speeds up, the time taken for cellular differentiation remains stable.

Consequently there is an increase in the proportion of immature cells on the villi whose enzyme protein and enzyme patterns are typical of the immature cell type (Pusztai & Bardocz, 1996). This has been shown by immunofluorescence staining using antibodies raised against crypt cells (Pusztai, 1991) and is also evidenced as a reduction in the activities of maturation marker enzymes such as diamine oxidase (Sessa *et al.*, 1995); sucrase-isomaltase and alkaline phophatase (Pusztai *et al.*, 1996). As a result the capacity of cells to digest/absorb nutrients is reduced. Although GNA also binds to the crypt (following long term exposure to the lectin) its effect is the opposite of PHA as it appears to decrease the CCPR resulting in a decrease in the length and cell numbers of the crypts (Pusztai *et al.*, 1990).

Intracellular delivery of lectins results in the stimulation of cellular protein synthesis and increased levels of DNA and RNA (Pusztai *et al.*, 1988 a,b; 1989 a). At the tissue level cellular metabolism leads to growth both by aforementioned hyperplasia (increased rates of mucosal cell proliferation) and hypertrophy (increase in cell size) attributed to elevated levels of secretion of acidic mucinous glycoproteins (Pusztai *et al.*, 1988 a,b). Co-incident increases in levels of polyamines indicates that they play a role in the biochemical mechanism of gut growth (Pusztai *et al.*, 1988 a,b; 1989 a). Given the energy cost of maintaining high rates of protein, DNA and RNA synthesis the physical and nutritional consequences of hyperplasia are potentially serious. As there is a need for lectin-fed animals to maintain the integrity of the intestinal wall the utilisation of food for the direct benefit of systemic metabolism and the body is restricted. Indirect effects caused by lectin binding to gut neuroendocrine cells and subsequent stimulation of gut peptide hormone secretion into the systemic circulation are also observed (Pusztai, 1991). It is thought likely that toxic lectins such as PHA act to modulate the overall hormone balance of the body although precise mechanisms are not yet known. Thus lectin toxicity is not restricted to damage to the small intestine or to the nutritional and physiological problems associated with the stimulation of gut growth. However, dietary lectin intakes are typically low and thus the contribution of the growth stimulating activity of lectins to nutritional toxicity is thought to be relatively slight (Pusztai, 1991).

Recent studies suggest that most of the harmful consequences of so-called toxic lectins are due to their interaction with gut bacteria (Jayne-Williams & Burgess, 1974; Rattray *et al.*, 1974; Wilson *et al.*, 1980; Ceri *et al.*, 1988; Banwell *et al.*, 1988; Pusztai *et al.*, 1991). Despite extensive binding of PHA and subsequent growth stimulation of the small intestine, PHA is not toxic for germ free rats (Pusztai, 1991). Thus PHA toxicity observed at high dietary levels in conventional animals with a normal microbial microflora is not attributable to PHA alone. Similar studies with other animal species support the existence of a causative relationship between the presence of PHA in the diet and *E. coli* overgrowth and toxicity (Jayne-Williams & Burgess, 1974; Banwell *et al.*, 1984). Pusztai (1991) concluded that the induction of selective bacterial overgrowth in the small intestine compounds the erosive effects of some lectins on the BBM. Subsequent endocytosis of bacterial toxins and uptake into the blood circulation by

transcytosis is thought to result in deleterious effects by stopping vital cellular functions. The precise mechanisms of selective overgrowth and how this effects nutritional efficiency has not been established. It is known that lectins can directly interact with and agglutinate resident bacteria of the digestive tract (Pusztai & Bardocz, 1996). By increasing the CCPR and changing receptor glycosylation lectins can also effectively remove potential attachment sites for particular bacteria (Pusztai & Bardocz, 1996). Bacterial lectins are known to be involved in the adhesion of bacteria to the small intestinal epithelium (Pusztai & Bardocz, 1996). In normal healthy rats the villus epithelium is comprised largely of highly differentiated, mature enterocytes expressing complex saccharide groups and only a few terminal α-linked mannose residues on membrane and cytoplasmic glycoconjugates. The absence of GNA binding and high reactivity of PHA confirms that the residual population of Type-1, mannose-senstitive fimbriated *E.coli* is low in normal rats (Pusztai,1995; Pusztai & Bardocz, 1996). However, α-linked terminal mannosyl groups become numerous following PHA induced stimulation of the CCPR providing a significant increase in the availability of receptor sites for the bacteria and selective proliferation ensues (Pusztai & Bardocz, 1996).

Pusztai *et al.* (1993) demonstrated that an appreciable portion of endocytosed WGA was transported across the rat gut wall into the systemic circulation. This has subsequently been observed for other lectins (Pusztai & Bardocz, 1996). Endocytosis in the small intestine is increased in the presence of large numbers of commensal bacteria (Pusztai & Bardocz, 1996). Thus, in addition to their action upon the BBM and subsequent indirect systemic effects, lectins, via their uptake into the blood circulation, may directly influence peripheral tissues and body metabolism by mimicking the effects of endocrine hormones (Pusztai & Bardocz, 1996). Systemic effects of toxic lectins such as PHA include pancreatic enlargement; increases in liver weight and body fat catabolism; glycogen loss; thymus atrophy and a significant reduction in skeletal muscle weight (Pusztai, 1991). Due to the complexity of the mammalian endocrine system and the wide range in affected metabolic processes it is not yet known whether systemic effects are produced by direct or indirect mechanisms of lectin action (Pusztai, 1991).

Lectins, whether of plant or microbial origin act as mammalian gut immunogens (de Aizpurra & Russel-Jones, 1988). As lectins (particularly PHA) are either partially resistant to lysosomal breakdown or may be routed through non-lysosomal intracellular pathways after endocytosis (Pusztai, 1989 a,b,c) an appreciable portion of intracellularly absorbed PHA by columnar cells may be exocytosed in a fully functional form through the basolateral membranes into the systemic circulation. Lectins are also thought to be transported into sub-epithelial tissues in the M cell regions of the small intestine, where the lectin is taken up by subepithelial macrophages and lymphocytes and carried into the systemic circulation (Pusztai, 1991). That this is a minor route for transport is indicated by the fact that most of the sytemically absorbed PHA is bound to circulating glycoproteins and not to cells. The identity of blood cells which bind systemically absorbed PHA has not yet been established. However, that these cells include fully

immunocompetent lymphocytes is demonstrated by the fact that all animal species tested develop a powerful and selective humoral antibody response of the IgG type to the dietary lectin (Pusztai *et al.*, 1979; 1981b,; King *et al.*, 1983; Williams *et al.*, 1984; Begbie & King, 1985; Grant *et al.*, 1985). Amounts of systemically absorbed PHA may reach 5-10 % of that initially administered intragastrically (Pusztai 1989 a,b,c). In comparison the absorbed proportion of the less toxic tomato lectin is <1 % of that given initially (Kilpatrick *et al.*, 1985; Pusztai *et al.*, 1989 c). The local IgE based allergenic immune reaction contributes to the overall poor nutritional state of the body and acts to increase the nutritional toxicity of PHA. As feeding rats PHA is typically accompanied by a dramatic proliferation of bacteria in the small intestine it is thought that PHA may also have a generally detrimental effect upon the functions of the IgA based local immune system (Pusztai & Bardocz, 1996).

In insects it is similarly thought that lectin antimetabolic effects are primarly determined by lectins binding to suitably glycosylated targets in the insect gut. Ultrastructural studies have shown insecticidal lectins to be bound to the midgut epithelial cells of *L. oleracea* (Woodhouse S. pers comm); *A.solani* (Down, 1998); *A.pisum* (Sauvion *et al.*, 1995); *N.lugens* (Powell *et al.*, 1998); *L.cuprina* (Eisemann *et al.*, 1994) and *C.maculatus* (Gatehouse *et al.*, 1984, 1989). Subsequent disruption to microvilli and the appearance of epithelial cell damage has also been observed (Sauvion *et al.*, 1995; Powell *et al.*, 1998). However, an extensive study by Harper *et al.* (1995) found there to be no correlation between lectin binding and toxicity since many lectins which bound to the midgut epitheium of the Lepidopteran *O.nubilalis* and Coleopteran *D. vergifera* were not toxic to the insect.

In an investigation into the mechanisms of lectin action on blowfly (*L. cuprina*) larvae Eisemann concluded that the reduced growth (and death at higher lectin concentrations) of WGA, lentil lectin and Con A fed larvae was probably caused by at least 3 mechanisms of action. These were a reduced intake of diet, partial blockage of the PM pores, and the direct binding of lectins to midgut epithelial cells. Although no obvious disruption of epithelial cells was observed it was thought that binding could affect various functions of the cell membrane. Blockage of PM pores was evidenced as an inhibition of the free movement of gold particles across the PM and the subsequent formation of an impervious gel-like layer of undefined composition in the gut lumen side of the PM. There is indirect evidence that peritrophins may be involved in determining the porosity of the PM of *L. cuprina* (Willadsen *et al.*, 1993) and interestingly Con A and lentil lectin exhibit binding to all 3 characterised peritrophins, whereas WGA binds only to peritrophin-95. The potential for WGA (which exerted the strongest growth inhibitory effect) to additionally bind to chitin in the PM due to its specificty for GlcNAc was recognised. It was concluded that the first 2 mechanisms would restrict nutrient availability resulting in a general starvation effect. Antifeedant effects of lectins have also been observed in the Homopteran RBP (*N. lugens*). Powell *et al.* (1995) found that GNA (0.1 w/v) reduced honeydew droplet production by 96 % although some recovery

in excretion levels was observed after 24 hours. As the RBP has a well developed labium with a complex array of mechano- and chemoreceptors it was thought that GNA antifeedant effects may result from interference (possibly via lectin binding) with the normal functioning of chemoreceptor sites involved in diet recognition (Powell *et al.*, 1995).

That lectins have the potential to exert direct and/or indirect systemic effects in insects as they do in mammals has recently been demonstrated. Immunohistochemical studies carried out by Powell *et al.* (1998) have shown GNA to be present in the haemolymph of GNA-fed RBP (*N. lugens*) and have also shown it to be bound to fat bodies and ovarioles.

It has been demonstrated that the insecticidal effects of lectins are extremely variable. The same lectin can exert highly variable effects on different species and lectins with the same sugar specificity can also exert very variable effects on the same and different species. Four principle factors are thought to be responsible for this variability. Firstly, sugar specificity is based on binding to single carbohydrate residues and therefore does not account for binding affinities to oligomers or complex carbohydrates which may be dramatically different. Secondly, the characteristics of BBM glycosylation (and glycosylation of other sites of lectin action) will vary from species to species of insect as will the availability of carbohydrate side chains for lectin binding. Thirdly, as observed for the binding of lectins to the mammalian BBM and Bt toxins to the insect BBM, not every lectin-ligand will necessarily result in a toxic effect. Finally, possible multiple mechanisms of lectin action, as occurs in mammals, may be a contributory factor.

1.5. The insect midgut.

The alimentary tract of Lepidopteran larvae (and many other insects) is a continuous tube running from the mouth to the anus. The 3 major regions, namely, foregut, midgut and hindgut of the alimentary canal of *L. oleracea* larvae are depicted in Fig 3.1. The foregut is concerned primarily with ingestion, storage, grinding and transport of food to the midgut. The hindgut recieves remaining material from the midgut, together with urine from the malpighian tubules and is concerned with the absorption of water, salts and other valuable molecules prior to elimination of the faeces through the anus (Gullen, 1992). The midgut constitutes the major portion of the alimentary tract in *L. oleracea* (and many other Lepidopteran larvae) and is the major site of digestion. As such it is considered as the primary target of lectin action and therefore warrants some discussion.

The midgut is composed of a single layered epithelium resting on a continuous basement membrane. A discontinous latticework of muscles lie on the haemolymph side of the epithelium, the organisation of which varies according to species and diet (Billingsley & Lehane, 1996). The midgut epithelium is composed principally of digestive and absorptive cells. Endocrine cells of variable shape are also distributed throughout the epithelium, as are relatively undifferentiated regenerative cells (or nidi). In continuous

digesters the epithelium is typically morphologically and functionally differentiated along its length. The apical membrane of digestive cells is highly folded into microvilli and on the luminal surface of microvilli is a glycocalyx typically composed of a carbohydrate-rich electron dense layer (Houk *et al.*, 1986; Rudin & Hecker, 1989). The basolateral plasma membrane is usually highly and irregularly convoluted. The apical third of this membrane contains junctional complexes (often leaky) and is typically involved in cell to cell adhesion (Billingsley & Lehane, 1996). The secretion of digestive enzymes occurs via exocytosis of secretory vesicles near the base of the microvilli. Absorptive epithelial cells are difficult to distinguish from digestive cells but are typified by greater elaborations of the basolateral membrane and a lack of storage products (e.g. lipids and glycogens) and secretory activity (Billingsley, 1990). Lepidopteran larvae have a range of endo- and exopeptidases. Proteolytic activities in the guts of *L. oleracea* have been investigated and shown to be due largely to extracellular serine proteases with a high pH optima (Gatehouse *et al.*, in press). Of total proteolysis (measured using azoalbumin as a substrate and synthetic inhibitors) almost 40 % was due to trypsin-like activity, 30 % to chymotryptic activity and 15-20 % due to elastase activity. These results are similar to proteolytic activitites investigated in the guts of larval *H. armigera* and the closely related *H. virescens* (Johnson *et al.*, 1991,1995; Christeller *et al.*, 1992).

In most insects the midgut is separated from abrasive food particles by a semi-permeable peritrophic membrane or matrix (PM). PMs are classified as Type I or Type II based on their mode of formation (Wigglesworth, 1930). Most common, particularly in Lepidopterans, are Type I PMs (Tellam, 1996) which are synthesised by the entire midgut digestive epithelium. Type II PMs present in all Dipteran larvae are synthesised by specialised cells just posterior to the proventriculus (Tellam, 1996). Multiple functions are carried out by the PM by virtue of its ability to compartmentalise the gut and thereby partition molecules between the endo- and ecto-PM spaces. In addition to allowing the selective movement of small molecules from the gut lumen to digestive epithelial cells the PM retains larger molecules, as well as bacteria, viruses, and parasites. The porosity of the PM is thought to be a major factor regulating the movement of newly synthesised digestive enzymes from the midgut surface into the gut lumen (Tellam, 1996). Digestive enzymes, such as endoproteinases and endoglycosidases, degrade ingested polymeric macromolecules in the gut lumen. Degraded fragments then move into the ecto-PM space where they are further degraded by digestive enzymes, particularly exoproteinases and exoglycosides (Tellam, 1996). Digestive enzyme recycling is thought to occur in the endo-PM space. Terra and Ferreira (1981) hypothesised that such enzymes are recovered in the posterior midgut region by their movement across the PM into the ecto-PM space. A counter current water flow is thought to move these enzymes back towards the anterior midgut region where they again pass across the PM into the endo-PM space. PMs are composed principally of chitin, proteins and proteoglycans.The majority of PM proteins, the major component of PMs, appear to be glycosylated typically with oligosaccharides with terminal mannose or N-acetyl-D-galactosamine residues (Stamm *et al.*, 1978; Adang & Spence 1982; Rupp & Spence 1985; Dörner & Peters, 1988; East *et al.*, 1993). Thus

as both the BBM and PM are extensively glycosylated there is potential for both to act as primary targets for lectin binding which will obviously vary according to the nature of glycosylation and sugar specificity/affinity of a particular lectin.

1.6.Tomato moth Lacanobia oleracea (L.).

The tomato moth, *Lacanobia oleracea* (L.) is a member of the economically important noctuid group of lepidopterous pests. This polyphagous larvae is widely distributed throughout the greater part of Europe and Asia minor. In the UK this species was first noted as a pest of glasshouse tomatoes in the 1890s. Lloyd (1920) reported that the destruction of tomato foliage by larvae was less important than their habit of feeding on fruits and biting into plant stems. Whilst the use of synthetic organic insecticides in the 1950s largely controlled infestations there has been a recent re-emergence in their pest status. *L. oleracea*, next to whitefly, is now one of the most important pests in the Channel Islands (Trainer.N pers comm). This re-emergence has been attributed to 2 primary factors. Firstly to the stoppage of soil sterilisation techniques allowing pupae to overwinter in the soil, and secondly to the increasing use of biological control agents for other tomato pests and the consequent reduction in use of conventional (neurotoxic) insecticides that are incompatible with the survival of biocontrol agents (Foster, 1981; Jarett & Burges, 1982).

L.oleracea reared on an optimal artificial diet and kept under laboratory conditions (20°C and 65 % r.h.) progress through 6 larval instars over a period of around 35 - 38 days. Neonates hatch within 2-3 days of egg production. Larvae consume most of their intake in the dark although more or less continuous feeding occurs during daylight hours. Feeding ceases just prior to ecdysis and prior to pupal formation. The pupal period of larvae is variable but typically lasts for approximately 25-30 days.

1.7. Research objectives.

The overall aim of this project was to investigate possible modes of lectin insecticidal action by comparing the effects of GNA and Con A upon a model Lepidopteran pest *L. oleracea*. The main aims of this research are as follows:

1. To evaluate and compare the effects of GNA and Con A upon the development of *L. oleracea* larvae in artificial diet bioassays.
2. To compare the *in vitro* binding characteristics of GNA and Con A to the BBM and PM of *L. oleracea*.
3. To characterise putative GNA and Con A receptors in the midguts of *L. oleracea* larvae.
4. To establish possible mechanisms of action for the observed antimetabolic effects of the 2 lectins.

2. MATERIALS.

2.1. Chemical Reagents and Equipment Suppliers.

All chemical reagents, except those listed below, were supplied by B.D.H. Chemicals Ltd, Poole, Dorset, UK, and Sigma Chemicals Co, St Louis, U.S.A. Reagents were of analytical or best available grade.

Agarose - Gibco BKL Life Technologies Ltd, Paisley, Scotland.

Bacto Agar - Difco Laboratories, Detroit, Michigan, U.S.A.

Concanavalin A (Con A) - Vector Laboratories Inc, 30 Ingold Rd, Burlingame, CA 94010, U.S.A.

Developer - Ilford Phenisol - Ilford Ltd, Mobberly, Cheshire, UK.

DIG Glycan Differentiation kit - Boehringer Mannheim UK Ltd, Lewes, East Sussex, UK.

Disposable pippette tips and Eppendorf tubes - Greiner Labortechnik Ltd, Dorsley, Glostershire, UK.

DNA size markers and restriction enzymes - Northumbria Biochemicals Ltd, Cramlington, Northumberland, UK, or Boehringer Mannheim UK Ltd, Lewes, East Sussex, or Promega, Southampton, UK.

Fixer, Kodak Unifix - Phase Separation Ltd, Deeside, Clwd, UK.

GNA - Drs. W. Peumans and E.Van Damme, Catholic University of Leuven, Belgium.

Lambda ZAP II kit - Stratagene Ltd, 140 Cambridge Innovation Centre,Cambridge Science Park, Milton Rd, Cambridge, CB4 4GF, UK.

3MM paper and glass fibre - Whatmann Ltd, Maidstone, Kent, U.K.

Microcapillaries - Drummond Sci. Co. U.S.

Microsep™ centifugal concentrators - Flowgen Instruments Ltd, Broad Oak Enterprise Village, Broad Oak Rd, Sittingbourne, Kent, UK.

Microtitre plates and petri dishes - Bibby Sterilin Ltd, Stone, Staffordshire, UK.

National Diagnostics "Ecoscint" Scintillation fluid - B.S. & S. (Scotland Ltd), Edinburgh, Scotland.

Nitrocellulose filters, Schleider and Schwell grade BA-85 - Andermann & Co Ltd, Kingston-upon Thames, Surrey, UK.

Non-fat dried milk, and nappies - Boots Ltd, Nottingham, UK.

PCR cloning vector pGEMT, polyATract mRNA isolation kit, Wizard Minipreps DNA purification system,*Taq* polymerase, PCR buffers, and reverse transcriptases MMLV and AMV, - Promega Corporation, 2800 Woods Hollow Rd, Madison, WI 53711-5399, U.S.A.

Radiochemicals and the ECL Detection kit - Amersham International plc, Amersham, Bucks, UK.

Sephadex G-50, Ficoll-400, Hitrap-Q 1 ml ion exchange columns, hexadeoxyribonucleotides, and dNTPs - Pharmacia Fine Chemicals, Uppsaka, Sweden.

TOPO TA cloning kit - Invitrogen BV, De Schelp 12, 9351 NV, Leek, Netherlands.

Tripticase peptone - Becton Dickinson, Cowley, Oxon, UK.

X-ray cassettes - Genetic Research Instrumentation Ltd, Dunmov, Essex, UK.

X-ray films (Fuji-RX) - Fuji Photo Film (UK) Ltd.

Yeast extract - Umpath Ltd, Basingstoke, UK.

2.2. Treatment of glassware, plastic ware, general apparatus and reagents.

All apparatus coming directly or indirectly in contact with nucleic acids, enzymes, bacteria, and sensitive or sterile reagents, were sterilised by autoclaving. Items which could not be autoclaved were sterilsed by prolonged immersion or washing in 70 % (v/v) EtOH and if possible, flaming. All solutions for DNA work, except gels, were autoclaved, or made up with sterile water and sterile stock solutions in sterilised containers, or put through a sterilising filter. All water was distilled. As RNA is extremely sensitive to nucleases, great care was taken to ensure that all apparatus used with RNA was nuclease free. All glassware was baked O/N at 170 °C. Corex glass tubes for RNA extraction were siliconised using Repeltone prior to overnight baking. Sterile plastic disposables were either used directly from the pack, or DEPC (diethylpyrocarbonate) treated to inactivate nucleases by O/N soaking in 0.1 % (v/v) DEPC water prior to autoclaving. All solutions were similarly DEPC treated and

autoclaved. Remaining apparatus was freed from contamination by the chaotropic action of the guanidinium thiocyanate solution.

2.3. Antibodies.

Anti-Con A antibodies were prepared and supplied by Sigma Chemicals (St Louis, U.S.A.). Anti-GNA antibodies were prepared and supplied by Dr L.N. Gatehouse and Dr R. Croy, Department of Biological Sciences, University of Durham, U.K. HRP-conjugated secondary antibodies, reagents for Bradford assays, and silver staining of SDS-PAGE gels were supplied by Biorad Laboratories Ltd, Biorad House, Mayland Avenue, Hemel Hempstead, Herts HP2 7TD, UK.

2.4. Frequently used media, buffers and other solutions.

Protein extraction buffers.

Extract buffer	20 mM TrisHCl pH 7.5, 1 mM DTT
Buffer A	17 mM Tris/HCl pH 7.5, 300 mM mannitol,5 mM EGTA
Solubilisation buffer	20 mM Tris/HCl pH 7.5, 1 mM EDTA
Lysis sample buffer 1	50 mM Tris/HCl pH 6.8, 10 % (v/v) glycerol, 2 % SDS (w/v)
Lysis sample buffer 2	150 mM Tris/HCl pH 6.8, 10 % (v/v) glycerol, 3 % SDS (w/v), 0.002 % (w/v) bromophenol blue, 5 % β-mercaptoethanol (v/v)

SDS- PAGE buffers, solutions, and size markers.

Acrylamides	30 g acrylamide, 0.8 g bisacrylamide/ 100 ml
Resolving buffer	3.0 M Tris/HCl pH 8.8
Stacking buffer	0.5 M Tris/HCl pH 6.8
SDS sample buffer (2x)	0.2 M Tris/HCl pH 6.8, 20 % glycerol (v/v), 2 % (w/v) SDS, 0.002 % (w/v) bromophenol blue
Reservoir buffer (10x)	0.25 M Tris/HCl pH 8.3, 1.92 M glycine, 1 % (w/v) SDS
Stain	40 % (v/v) MeOH, 7 % (v/v) glacial acetic acid, 0.05 % (w/v) Kenacid blue
Destain	40 % (v/v) MeOH, 7 % (v/v) glacial acetic acid

Protein size markers (Sigma)

SDS 7	66 kDa Bovine albumin
	45 kDa Egg albumin
	36 kDa Glyceraldehyde-3-phosphate
	29 kDa Carbonic anhydrase bovine erythrocytes
	24 kDa PMSF- treated trypsinogen
	20 kDa Soybean trypsin inhibitor
	14 kDa Alpha-lactalbumin

SDS 6H	205 kDa Myosin 116 kDa β-galactosidase 97.4 kDa Phosphorylase b 66 kDa Albumin bovine 45 kDa Albumin egg 29 kDa Carbonic anhydrase bovine erythrocytes

Western blotting.

Anode buffer 1	0.3 M Tris/HCl pH 10.4, 20 % (v/v) MeOH
Anode buffer 2	0.025 M Tris/HCl pH 10.4, 20 % (v/v) MeOH
Cathode buffer	0.025 M Tris/HCl pH 9.4, 40 mM 6-aminohexanoic acid, 20 % (v/v) MeOH

Immunoblot assays.

Blocking solution	5 % (w/v) marvel, 1x PBS, 1 % (v/v) Tween 20
Antisera-buffer	5 % (w/v) marvel, 1x PBS, 0.1 % (v/v) Tween 20
PBS (10x)	KH_2PO_4 (0.015 M), Na_2HPO_4 (0.080 M), NaCl (1.37 M)

RNA/DNA electrophoresis.

TAE (50x)	2 M Tris/ acetic acid pH 7.7, 50 mM EDTA
Gel loading dye mix (DNA gels)	10 mM Tris/HCl pH 8.0, 10 mM EDTA, 30 % (w/v) glycerol, 0.1 % (v/v) fast orange G, prepared with distilled water
formaldehyde gel loading buffer (5x)	0.5 M MOPS pH 7.0, 40 mM sodium acetate, 5 mM EDTA pH 8.0 (NaOH)
formaldehyde gel running buffer	50 % (v/v) glycerol, 1 mM EDTA, pH 8.0 (NaOH), 0.25 % (w/v) bromophenol blue, 0.25 % (w/v) xylene cyanol FF

cDNA library screening and northern blotting.

chromatography column buffer (10x)	0.5 M Tris/HCl pH 7.5, 1.5 M NaCl, 0.1 M EDTA, 1 % (w/v) SDS
SSC (20x)	3 M NaCl, 0.3 M tri-sodium citrate pH 7.0 (HCl)
	Denhardts solution (50x), 1 % (w/v) Ficoll 400, 1 % polyvinylpyrrolidine (w/v), 1 % (w/v) BSA
Prehybridisation solution	5x SSC, 5x denhardts, 0.1 % (w/v) SDS
Hybridisation solution	5x SSC, 2x denhardts, 0.1 % (w/v) SDS
LB broth	1 % (w/v) NaCl, 1 % (w/v) tripticase peptone, 0.5 % (w/v) yeast extract, with distilled water
LB agar	LB broth plus 2 % (w/v) bacto-agar
NZY broth	0.5 % (w/v) NaCl, 0.2 % $MgSO_4$ $7H_2O$, 0.5 % (w/v) yeast extract, 1 % (w/v) NZ amine
NZY agar	NZY broth plus 1.5 % (w/v) bacto-agar
NZY top agar	NZY broth plus 0.7 % (w/v) agarose

3. METHODS.

3.1. Standard biochemical and molecular techniques.

All standard techniques are standard practice in the Department of Biological Sciences, Durham University, or based on protocols in Molecular Cloning: A Laboratory Manual (Sambrook, Fritsch, & Maniatis, 1989), or Protein Purification Methods: A Practical approach (eds Hames B.D. & Rickwood D, 1981) unless referenced otherwise.

3.2. Freeze-drying samples.

Leaf based artificial diet (AD), and larval faecal samples in 1.5 ml microcentrifuge tubes were frozen in liquid air and then lyophilised for 24 hr using a Flexi-Dry μP freeze drier (FTS Systems, New York, USA). Tubes had pierced lids to prevent sample loss on release of the vacuum.

3.3. Insect cultures.

Lacanobia oleracea eggs were obtained from Central Science Laboratories, MAFF, Slough, in October 1995. In the laboratory eggs, larvae, and pupae, were maintained at 20 °C and 65 % r.h., and adults at 24 ° C and 30 % r.h. All developmental stages were reared under a 16 hr light; 8 hr dark regime, with the exception of pupae which were maintained under constant darkness. Larvae were reared on AD of the composition shown in Table 1. Insect cultures were maintained by Gillian Davison.

Table 3.1. Larval artificial diet composition. All ingredients, except agar, were mixed well in 1.8 L of distilled water. Agar, dissolved in 0.92 L distilled water and cooled to below 50 °C was then added to the above mixture, poured into shallow trays and allowed to set.

weight/volume	Ingredients
60 g	Agar
2.72 L	Distilled water
332 g	Finely ground haricot bean seed
266 g	Wheatgerm
134 g	Finely ground soybean seed
98 g	Cascein
170 g	Finely ground brewers yeast
16 g	Ascorbic acid
8 g	Sorbic acid
13.2 g	Methyl-p-benzoate
26.6 g	Vanderzants vitamin mix
0.32 g	Tetracycline
10 ml	10 % (v/v) Formaldehyde solution

3.4. Insect Bioassays using artificial diets (AD).

The purity of GNA was estimated by spectrophotometry and SDS-polyacrylamide gel electrophoresis (PAGE) to be 70 - 90 %, depending on the batch. The protein was shown to be fully functionally active by haemagglutination assays (titre equivalent to published values for GNA). 100 % pure Concanavalin A (Con A) was purchased from Vector Laboratories. A diet based on freeze-dried potato leaf powder was used for all bioassays. This diet was formulated so as to eliminate additional lectins or sugars, components of many optimal lepidopteran diets, that might mask the effects of GNA or Con A. Diet composition is given in Table 2. Ratios of water to dry weight of components were adjusted to provide an optimal diet consistency. Leaf material was prepared by immediate freeze drying, following removal from the plant, to prevent any accumulation of compounds induced by wounding. Freeze-dried leaves were then ground to a fine powder in a mortar and pestle. Fresh diet was prepared every 4/5 days and stored at 4 °C in airtight containers. This diet when fed to neonate larvae supported normal growth and development up to the 5th or 6th instar, but was not optimized for the full life cycle of the insect. For short term assays larvae were initially reared on the optimal AD described in Table 3.1. Pre 5th instar larvae were then fed for 24 hr on control leaf based AD prior to exposure to lectin containing diets. To prevent AD dessication a small piece of wetted filter paper was placed alongside the diet. Fresh diet was given daily, and filter paper replaced regularly to prevent bacterial contamination. All assays were carried out under a 16 hr light : 8 hr dark regime. Unless otherwise stated lectins were incorporated into this diet at a single concentration estimated at 2.0 % (w/w) of the total dry weight of dietary proteins (7.3 mg pure lectin per g dry weight of diet). The control diet was supplemented with an equivalent weight of casein to account for the extra protein (i.e. GNA/ Con A) added to the experimental diet.

Table 3.2. Leaf based artificial diet composition. Components (A) were mixed thoroughly and added to boiled agar and water (C). Vitamins, antibiotics and GNA (B) were added once this mixture had cooled to below 45 °C

Weight/volume	Ingredient	Category
0.2525 g	Potato leaf powder	A
0.101 g	Caseine	A
2 ml	Distilled water	A
0.0101 g	Vitamin C	B
0.005 g	Methyl-p-hydroxybenzoate	B
0.0088 g	Aureomycin	B
0.00363 g	GNA or caseine	B
0.125 g	Agar	C
3 ml	Distilled water	C

3.4.1. Bioassay 1: Long term bioassay using AD containing GNA.

For each treatment (i.e.control and experimental (GNA 2 % total protein)) 30 neonate were placed individually into 250 ml clear plastic airtight pots. Small airholes were made in the lids 6 days after transfer. Survival and larval instar were recorded daily from 0-24 hr post hatch for each replicate pot. Larvae of each instar were identified by head capsule widths. In addition, the appearance of a newly formed clear head capsule (resulting from head capsule slippage) approximately 24-36 hr prior to ecydysis provided a useful indicator of the end of each instar. Once larvae were large enough to handle without causing damage (7 days post hatch) individual wet weights (+/- 0.1 mg) were recorded. Larvae were starved for approximately one hr prior to weighing which was carried out at the same time each day. Measurements of food consumption and faecal production were made on a dry, rather than wet weight basis, because determination of remaining diet and faecal fresh weight is prone to significant error due to variable water content after exposure. To this end a dry/wet AD weight calibration was carried out, by recording the initial wet weight of 10 variably sized diet pieces and respective dry weights following freeze-drying of the samples. The r^2 value obtained was 0.995. A known fresh weight of food was introduced daily, or more frequently if necessary, and the dry weight of introduced food was then calculated from the calibration. Leftover food and faeces were carefully separated, freeze-dried, and later weighed (+/- 0.1 mg). The dry weight of food consumed was thus calculated by subtracting the dry weight of food left over from the initial estimated dry weight of introduced food. Given that the error involved in the estimation of dry weight of introduced food is carried over when calculating dry weight consumption, care was taken to introduce as little excess food as possible. As larvae in this bioassay were kept as individuals, analysis of consumption could be made on an individual basis.

3.4.2. Data analysis 1.

All data analysis was carried out using the Statview (v.4.5; Abacus Concepts, Berkely, Ca, USA) software package on Apple Macintosh computers. The acceptance level of statistical significance was $P<0.05$ in all instances. The time taken (days) for each larvae to reach each successive instar was tabulated to facilitate statistical analysis of instar duration. A Mann-Whitney U-test was employed to detect significant differences in the median time taken to reach successive instars between the control and experimental samples. To detect significant differences in daily mean larval wet weights, mean larval consumption and faecal production, between treatments, analysis of variance (ANOVA) and a subsequent Fishers PLSD (probability least significant difference) test was employed.

3.4.3. Bioassay 2: Long term bioassay using AD containing Con A.

For each treatment (i.e.control and experimental (Con A)) 25 neonate larvae were placed individually into plastic containers. Con A was incorporated in AD at concentrations of 0.02 %, 0.2 %, and 2.0 % (w/w) of total dietary protein. Survival, instar, larval wet weights, consumption and faecal production were assessed as described for bioassay 1,

except that larval wet weights, and consumption and faecal production were recorded every 48 hr rather than daily.

3.4.4. Bioassay 3: Short term bioassay using ADs containing GNA and Con A, effects on gut enzyme activity.

For each treatment 30 5th instar larvae were placed individually into clear plastic pots and exposed to control diet, or diets containing GNA or Con A (2 % total protein) for 24 hr. Larval wet weights were recorded (+/- 0.1 mg) before and after exposure to the different diets. Intact guts (plus contents) were dissected over ice, blotted briefly to remove haemolymph and flash frozen in liquid air. Individual crude gut extracts were prepared by homogenisation in 400 µl distilled water, 0.125 mM DTT, using a TRI-R (Model S63C) homogeniser. Samples were again flash frozen in liquid air and stored at -20 ℃ for later analysis. Larval consumption and faecal output were analysed on a dry weight basis after freeze-drying diet and faeces as previously described (section 3.4.1.). The protein content, and tryptic activity, of individual faecal samples were also determined. Lectins in faecal samples were detected by SDS-PAGE, western blotting and ECL detection (as described in sections 3.10., 3.12 and 3.13, respectively). The intensity of bands was assessed, unless otherwise referenced using a Biorad GS 690 imaging densitometer. The samples were homogenised in 1 ml of 1x PBS , 0.125 mM DTT, shaken at 4 °C O/N, and spun for 15 min at 4 °C in a benchtop microcentrifuge. The resultant supernatant was analysed for protein concentration (section 3.9). Faecal samples were assayed for differences in trypsin activity between treatments (section 3.19.) .

3.4.5. Data analysis 2.

ANOVA analysis was carried out as described for bioassay 1 (section 3.4.2.) to determine any significant differences between treatments in the nutritional and physiological parameters measured, and between pmols product per min per gut, and pmols product per min per µg gut protein. P-nitrophenol standards were used to obtain a calibration of pmols product vs absorbance for alkaline phosphatase and α-glucosidase assays. P-nitroaniline standards were similarly used to allow determination of pmols product for aminopeptidase and trypsin assays. The r^2 values obtained for the linear calibration graphs were 0.999 and 0.998, respectively.

3.4.6. Bioassay 4: Short term bioassay using ADs containing GNA and Con A, comparing trypsin mRNA levels.

To determine if the differences in gut trypsin activity observed between treatments in bioassay 3 were attributed to differences in the levels of trypsin mRNA an identical short term assay (using 20 larvae per treatment) was carried out . Dissected guts were flushed clean with distilled water following dissection, blotted briefly and weighed (+/- 0.1 mg) prior to being flash frozen and stored at -80 °C. For each treatment 5 RNA extractions (4 larvae per replicate) were prepared using Tri Reagent (Sigma) according to the manufacturers instructions. Northern analysis was carried out as described in section

3.38. Filters were separately probed with two *Helicoverpa armigera* trypsin cDNA clones, HaTC16 (890 bp), and SR36 (849 bp) kindly provided by Dr D.Bown (Department of Biological Sciences, Durham University).

3.4.7. Bioassay 5: Long term bioassay using ADs containing GNA and Con A, effects on gut enzyme activity.

Neonate larvae (35 per treatment) were reared on either control diet, or diet containing GNA or Con A (2.0 % total protein) for 16 days, with daily changes of diet. Surviving larvae were used to assess the long term effects of lectins on gut enzyme activity. Larval wet weights were recorded prior to dissection (+/- 0.1 mg). Intact guts were removed, flushed with distilled water to remove peritrophic membranes (PM's) and gut contents, blotted briefly to remove excess haemolymph and individual gut wet weights were recorded (+/- 0.1 mg) to allow analysis of gut to larval weight ratio's. Crude gut extracts were prepared as described in bioassay 4 (section 3.4.6.). Samples were analysed for gut protein concentrations (section 3.9.), and for differences in aminopeptidase, alkaline phosphatase, and α-glucosidase activities between treatments (section 3.22.). Data analysis was carried out as described for bioassay 3 in section 3.4.5.

3.4.8. Bioassay 6: Detection of lectins in haemolymph and various tissues of larvae fed ADs containing GNA and Con A. Effects of lectin treatments on haemocyte abundance.

Newly eclosed 4th instar larvae (20 per treatment) were reared on control diet for 24 hr prior to placing on diets containing GNA or Con A (2.0 % total protein) for 5 days, with daily changes of diet. Larval wet weights were recorded prior to the onset of the feeding trial and prior to dissection (+/- 0.1 mg). Larval consumption was analysed as described for bioassay 1 in section 3.4.1., except that consumption was estimated per replicate (i.e.per 4 larvae) rather than on an individual basis. Various tissue (midgut, hindgut, malpighian tubule, fat body) and haemolymph samples were extracted as described in sections 3.5. and 3.4.8., respectively. Dissected tissues were pooled (4 larvae per sample) to give 5 replicates per treatment. Levels of lectin in each tissue sample replicate were analysed as described in section 3.13. Individual haemolymph samples extracted as described in section 3.4.8. were similarly analysed for the presence of GNA or Con A. Cell counts of individual haemolymph samples were recorded to investigate whether either lectin had a long term impact on non tissue associated haemocyte abundance. To this end 10 µl of haemolymph from each larvae was placed in 40 µl of prechilled isoosmotic TBS pH 7.4 buffer. Haemocyte counts (2 per sample) were recorded and the subsequent cell densities estimated using a Neubauer haemocytometer and Nikon (104) microscope. Haemocyte density was analysed for differences between treatments by ANOVA and subsequent Fishers PLSD (probability least significant difference) test.

3.4.9. Short term bioassays: investigating the uptake of lectins into the haemolymph of larvae fed on ADs containing GNA and Con A.

A number of short term assays were carried out to investigate the uptake of GNA and

Con A into the haemolymph of lectin fed larvae. Typically 5th instar larvae (20 per treatment) were fed for 24 hr on diets prior to sampling. Two time trials where haemolymph was extracted after 2, 4, 6 and 12 hr after exposure to GNA containing diet were also carried out. Larvae were starved for 2 hr prior to the onset of time trials to encourage immediate consumption of the diet. Pre-chilled larvae were blotted with EtOH and dried prior to haemolymph extraction which was carried out by piercing the cuticle using a fine needle. Droplets of extruded haemolymph were then taken up using 10 or 25 µl sterile microcapillaries and placed into pre-chilled 0.5 ml eppendorfs dusted with PPO (phenylthiocarbamide-phenol oxidase inhibitor). Using this technique 15 to 60 µl of haemolymph per 5th instar larvae was extractable. The presence of lectins was determined by running individual crude extracts on SDS-PAGE, western blotting, and ECL detection (sections 3.10, 3.12. and 3.13, respectively).

3.4.10. GNA levels in haemocyte and plasma fractions of haemolymph samples extracted from short term GNA-fed larvae.

The relative abundance of GNA in haematocyte and plasma fractions of haemolymph samples extracted from GNA-fed larvae was investigated. Larvae (20 per treatment) were treated as described in section 3.4.9. To obtain sufficient haemolymph for the preparation of a cell extract, equal volumes (typically 8-10 µl per sample) of crude haemolymph extracted from GNA-fed or control-fed larvae were pooled. Remaining haemolymph was analysed for the presence of GNA in individual samples by SDS-PAGE, western blotting and ECL detection (sections 3.10, 3.12.and 3.13, respectively). Pooled haemolymph samples were spun at 2,200 x g for 20 min at 4 ° C in a Beckmann J4202 rotor. The resultant supernatant, comprising the plasma, was removed and placed into fresh pre-chilled eppendorfs. The pellet, containing haemocytes, was washed 3 x with 50 µl isotonic PBS (305 - 310 mosmol/kg) and resuspended in 50 µl lysis buffer 1 by vortexing and sonication. Buffer osmolarity was measured and adjusted to the osmolarity of *L. oleracea* haemolymph (305 - 310 mosmol/kg Dr E. Richards pers comm) using a Vapro Vapour Pressure Osmometer 5520. Both plasma and cell preparations were then boiled for 3-5 mins and aliquots analysed for protein concentration . The relative abundance of GNA in the 2 fractions was analysed by running equal aliquots (to which around 10 µl lysis buffer 2 was added) on SDS-PAGE, western blotting, and ECL detection.

3.5. Dissection and protein extraction from various larval tissues.

Unless otherwise stated prechilled 5th instar larvae were dissected over ice Intact guts were extracted following decapitation and removal of a small rear portion of the larvae. Gut contents and PM's were removed by flushing with ice-cold extract buffer (20 mM Tris/HCl pH 7.5, 1 mM DTT, 5 µg/ml leupeptin) using a fine needle attached to a 2.5 ml syringe. Foregut, midgut and hindgut tissue, as designated in Fig 3.1, were removed under a dissecting microscope (model Nikon SM 2-1). Similarly malpighian tubules were teased from the hindgut and washed with extract buffer to remove surrounding fat bodies and haemolymph. PM's, carefully removed from intact guts and flushed, were

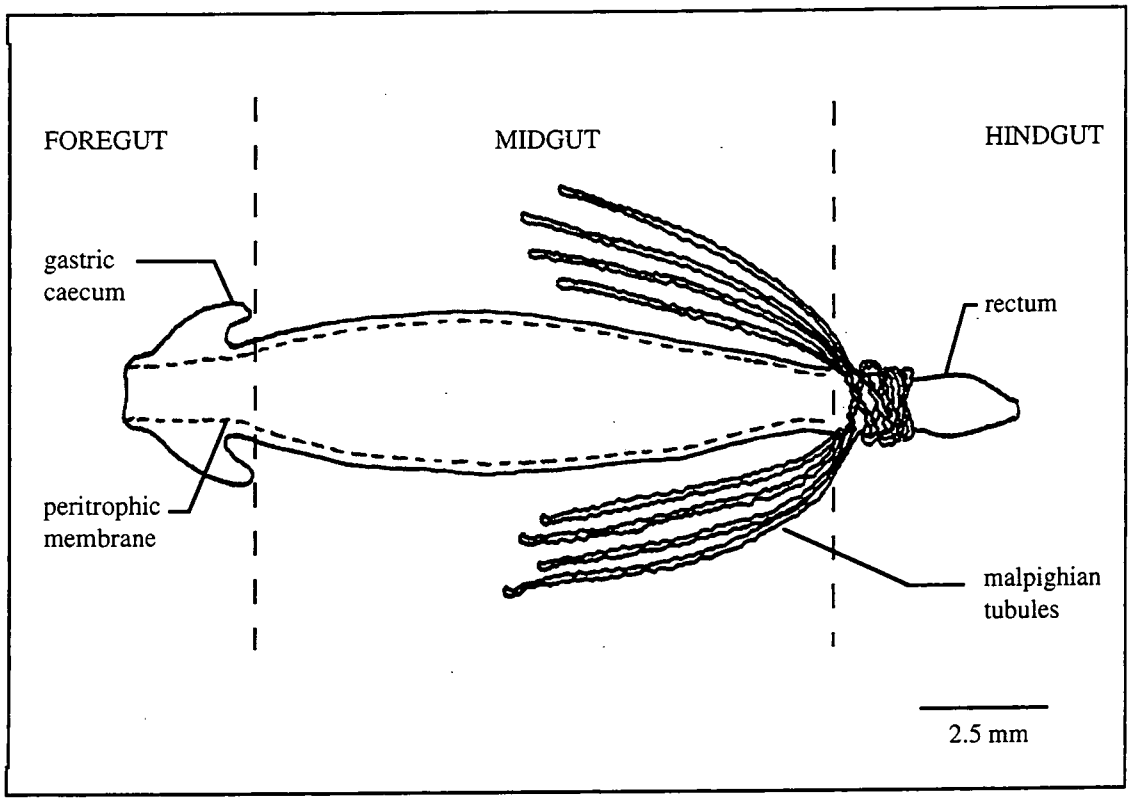


Fig 3.1. Diagrammatic representation of alimentary tract dissected from 5th instar *L. oleracea* larvae showing the 3 major divisions of the digestive tract.

placed in extract buffer (1 ml per 20 PM's) and washed 3 x to further remove contaminating gut contents. To obtain fat bodies a longitudinal cut through the cuticle was made from decapitated larvae. Larval guts were removed and the layer of fat bodies lying just beneath the cuticle were gently teased from the cuticle using a scalpel. Fat bodies were flushed with extract buffer to remove contaminating tissue and haemolymph. All samples were placed in extract buffer, frozen by immersion in liquid air, and stored at -20 °C. Crude extracts were prepared by homogenisation using a TRI-R (Model S63C).

3.6. Chloroform extraction of proteins from crude extracts.

Proteins were separated from lipids in crude extracts by chloroform extraction. 5 volumes of chloroform were added to the crude extract, the mix was vortexed and then spun for 5 min in a benchtop centrifuge. The resultant supernatant was removed, spun for an additional 2 min to remove chloroform traces, and the final supernatant stored at -20 °C.

3.7. Preparation of larval midgut brush border membrane vesicles (BBMV).

The $MgCl_2$ precipitation method of Wolfersberger *et al.* (1987), with slight modifications, was used for the preparation of BBMV's. The protease inhibitor leupeptin (5 µg/ml) was included in all solutions. A crude midgut homogenate was prepared as described in section 3.5, although a dissecting microscope was not used and guts were flushed clean with buffer A, as opposed to extract buffer. An equal volume of ice-cold 24 mM $MgCl_2$ solution was added to the homogenate and the resultant mix blended, using a polytron, and left to stand on ice for 15 min. This mixture was centrifuged at 4,500 rpm (2,500 xg) for 15 min at 4 °C in a Beckmann JA-20 rotor. The supernatant was decanted and centrifuged at 16,000 rpm (30,000 xg) for 30 min at 4 °C in the same rotor. The supernatant was decanted and stored on ice. The pellet was resuspended in 1/2 homogenate volume of 60 % ice cold buffer A, 40 % 24 mM $MgCl_2$, and centrifuged as for the original homogenate. The second 16,000 rpm pellet constituted the BBMV preparation. The combined 4,500 rpm pellets constituted cell debris, and the combined 16,000 rpm supernatants the cytoplasmic soluble protein fraction. The BBMV pellet was resuspended in approximately 1 ml ice cold half-strength buffer A per 100 midguts. For a typical BBMV preparation the midguts from 200 larvae (2.5-3 g fresh tissue) were used with a yield of around 2 mg of brush border (BB) proteins per g of fresh tissue.

3.8. Detergent solubilisation of BBMV and PM proteins.

BBMV and PM proteins were extracted by incubating an equal volume of BBMV preparation (5-10 mg/ml protein) or crude PM homogenate with solubilisation buffer and detergent, at 4 °C for 1 hr with gentle shaking. This mixture was spun at 50,000 rpm (100,000 xg) for 15 min in a TLA 100-4 rotor. The supernatant constituted solubilised proteins. To aid analysis of solubilisation efficiency the pellet was resuspended in an equal volume of solubilisation buffer prior to running on SDS- PAGE. Samples were stored at -20 °C. Of the various detergents, and detergent concentrations tested (CHAPS,

SDS, Triton X-100), Triton X-100, at a final concentration of 2 %, was found to be the most effective solubilising agent. Solubilisation efficiency was variable but typically estimated to be around 60 % of BBMV proteins.

3.9. Estimation of protein concentration.

A modified Bradford (1976) Assay, using the Biorad protein assay kit, allowed protein concentrations for various larval extracts to be estimated. Bovine serum albumin (BSA) was used as a protein standard, with dilutions prepared in the same buffer as that used for sample preparation. Protein samples were diluted to a volume of 160 µl with distilledwater. Blanks were prepared with distilled water in the first 3 wells of a microtitre plate (A1-A3). 40 µl of Bradford stock solution was added to each sample containing well. The plate was shaken briefly (900 rpm) on a microtitre plate shaker for 10 sec and the samples measured at 570 nm using a Dynatech MT 5000 microtitre plate reader (Dynatech Laboratories Ltd, West Sussex, U.K.). The protein content of solubilised BBMV and PM samples was estimated via a microtitre Bradford Detergent Compatible assay kit, according to the manufacturers instructions.

3.10. SDS-PAGE analysis.

Proteins were electrophoresed on polyacrylamide gels, run in a dissociating SDS-PAGE discontinuous buffer system (Laemmli 1970). Minigels (9 x 10 cm) were prepared as described by Hames (1981) and run in an ATTO AE-6450 apparatus. Samples were prepared by adding 2x SDS or 4x SDS sample buffer and boiled for 3-5 min prior to loading. 10 % ß-mercaptoethanol was added, if required for reducing, prior to boiling. Protein standards were also treated with ß-mercaptoethanol and boiled prior to loading. Gels were run at 70 v until samples had migrated through the stacker buffer, and then at 80-140 v.

3.11. Protein detection in polyacrylamide gels.

Proteins in the µg range were visualised in polyacrylamide gels by staining with 0.05 % (w/v) Coomassie blue, in 7 % (v/v) glacial acetic acid, 40 % (v/v) MeOH followed by destaining with 10 % (v/v) glacial acetic acid, 40 % (v/v) MeOH (Sambrook *et al.*, 1989). Silver staining allowed visualisation of proteins in the ng range. A Silver stain kit was used according to the manufacturers instructions (Biorad). Gels were stored in 10 % glycerol (v/v), 20 % MeOH (v/v), within heat sealed plastic bags prior to being photographed using a digital Mavica MVC-FD7 camera.

Table 3.3. Acrylamide gel composition.

This recipe is sufficient for two mingels. Acrylamide and distilled H_20 volumes were adjusted for different % Acrylamide gels.

Components	**12.5% Main Gel**
Acrylamides	6.25 ml
Tris/HCl, pH 8.8	1.875 ml
Tris/HCl, pH 6.8	-
Distilled water	6.165 ml
De-gas briefly	
10 % (w/v) SDS	150 µl
2 % (w/v) Ammonium persulphate	560 µl
TEMED	7.5 µl

3.12. Western Blotting.

The semi-dry blotting technique of Kyhse-Anderson (1974) was employed for the transfer of SDS-PAGE separated proteins to nitrocellulose. The stack comprised the following:

Anode Plate;
2 sheets of 3MM paper soaked in anode buffer 1;
1 sheet of 3MM paper soaked in anode buffer 2;
1 sheet of nitrocellulose soaked in distilled water;
1 acrylamide gel to be blotted;
1 sheet of 3MM paper soaked in cathode buffer;
1 sheet of dialysis membrane soaked in dH_20;
2 sheets of 3MM paper soaked in cathode buffer;
Cathode plate.

Proteins were electrotransferred at 1.25 mA for 1hr and the blotted gel stained with Coomassie Blue stain to check for successful protein transfer. The nitrocellulose was either immediately analysed, or stored between 3MM paper at 4 °C.

3.13. Detecting GNA and ConA in larval tissue and haemolymph extracts.

Western blotted larval proteins were analysed for the presence of GNA and Con A using the enhanced chemiluminesence (ECL) method. Nitrocellulose filters were incubated in blocking solution for at least 1 hr and probed with primary antibody polyclonal rabbit-anti-GNA or Con A antiserum (1 in 10 000 dilution) in blotto for 2 hr, with rotation, at 37 °C. Filters were then washed 3x 10 min in blotto and incubated with secondary

antibody affinity purified goat anti-rabbit IgG horseradish peroxidase conjugate (Biorad) for 1 - 2 hr at 37 °C. A further 3x 10 min washes in 1x PBS, 0.1 % (v/v) Tween 20, were carried out. Filters were then exposed to ECL detection reagents according to the manufacturers instructions, placed between two sheets of acetate in a film cassette and exposed to X-ray film. Autoradiographs were developed either manually or with an Automatic developer (X-ograph Imaging systems Compact X4, Malmesbury, Wiltshire U.K). The intensity of bands was assessed, unless otherwise referenced (typically using 2 different exposures for each filter) using a Biorad GS 690 imaging densitometer.

3.14. Identification of "in vitro" lectin binding proteins.

Identification of lectin binding proteins from various larval extracts was carried out using the DIG Glycan Differentiation Kit, according to the manufacturers instructions (Boehringer Mannheim UK Ltd). With this method carbohydrate moieties of glycoproteins bound to nitrocellulose can be characterised. Proteins were initially separated by SDS-PAGE and transferred to nitrocellulose by western blotting. The lectins in which filters were incubated are conjugated with steroid hapten digoxigenin and this enables immunological detection of the bound lectins. Bound lectin was detected using alkaline phosphatase labelled anti-digoxigenin antibodies followed by staining with 5-bromo-4-chloro-3-indoyl-phosphate (X-Phosphate) and 4-Nitro blue tetrazolium chloride (NBT). The kit contains control glycoproteins (e.g. carboxypeptidase Y) for demonstrating lectin specificity and for verifying the function of the kit.

3.15. GNA binding to haemocyte monolayers.

In vitro binding of GNA to haemocytes was investigated by overlaying cell monolayers with fluorosein (FITC)-labelled GNA purchased from Sigma (Poole, Dorset). Haemocyte monolayers were prepared by the following procedure: 6th instar larvae were bled as described in section 3.4.9. 50 µl of haemolymph was added to 400 µl prechilled Tris buffered saline (TBS) (0.05 M Tris/HCl to pH 7.4,with NaCl added to adjust osmolarity to 309 mosmol/kg). Cell density was estimated as described in section 3.4.8., and adjusted by dilution with TBS to a concentration of 8×10^5 - 1.0×10^6 cells/ml. For each treatment 50 µl of the cell suspension was added to each of 2 wells in an 8 welled microscope slide (CA Hendley, Essex UK) and incubated in a moist chamber for 20 mins at RT. After washing 6 x with TBS, using a glass pipette, monolayers were incubated for a further 10 min to "round up" cells. Monolayers were overlaid with 50µl of TBS, or FITC labelled GNA (5 µg/ml), or FITC (5 µg/ml), or FITC labelled GNA with 250 mM mannose. The osmolarity of all solutions was adjusted to 305 - 310 mosmol/kg (i.e. isoosmotic to larval haemolymph 309 mosmol/kg) to minimise cell damage. Overlays were left for 1 hr at RT, washed 6 x with TBS and viewed by phase contrast and under UV using a Leica DMLB microscope.

3.16. Haemagglutination assays.

The ability of GNA to agglutinate rabbit erythroctes (rbc) was analysed using a microtitre plate assay. A stock blood suspension of 50 % (v/v) venous rabbit blood and 50 % (v/v)

Alsevier's solution containing 1/30 th volume of anticoagulant was washed 3 x with 1 x PBS and resuspended to a final concentration of 2 % (v/v). 50 µl of GNA (0.1 mg/ml) was added to 50 µl each of 1 x PBS and rbc's in a microtitre well. Serial dilutions of GNA were made in subsequent wells containing 50 µl of 1x PBS and 50 µl rbc suspension. After incubation at RT for a minimum of 2 hr end points were recorded, by eye, as the reciprocal of the last dilution causing complete agglutination.

3.17. Ion exchange chromatography.

Partial purification of both the major 120 kDa Con A binding and 98 kDa GNA binding BBM glycopolypeptides was carried out by ion exchange chromatography. Solubilised BBMV proteins were fractionated on a GradiFrac Pharmacia system using a 1 ml Hitrap Q Sepharose Pharmacia Biotech ion exchange column equilibrated in 20 mM Tris/HCl pH 7.5 buffer containing 0.2 % (v/v) Triton X-100. After injection of an aliquot (2-10 mg protein, 2-4 ml) of solubilised BBMV, a gradient of 0 to 0.25 M NaCl over 50 ml was developed at a flow rate of 0.5 ml per min. Fractions (0.5 ml) were collected and analysed for purification and GNA binding properties using SDS-PAGE, and by probing blotted fractions with labelled lectins, according to the manufacturers instructions. Chromatography was carried out several times to process all of the samples.

3.18. Microsep ™ Ultrafiltration.

Fractions obtained from ion exchange chromatography containing either the 120 kDa Con A binding glycopolypeptide or the 98 kDa GNA binding glycopolypeptide were pooled. Further purification and concentration of the proteins was achieved using 30K Microsep™ ultrafiltration chambers. Pooled aliquots in ultrafiltration chambers were spun in a Mistral 2000 centrifuge at maximum speed (3660 rpm) until the desired concentration of protein was obtained. Filtrates were checked for the loss of protein by SDS-PAGE.

3.19. Deglycosylation.

The 98 kDa GNA binding protein present in solubilised BBMV preparations was deglycosylated using N-Glycosidase F (PGNase F, Boehringer). PGNase F hydrolyses all types of asparagine bound N-glycan chains from glycoproteins provided that the amino and carboxyl group are present in a peptide linkage. Preliminary trials were carried out to evaluate the concentration of enzyme required for full deglycosylation of the protein. The extent of deglycosylation was assessed by SDS-PAGE and subsequently by probing blotted polypeptides with labelled GNA according to the manufacturers instructions. Full deglycosylation was achieved by mixing approximately 25 µg of pure protein (preboiled for 3-5 min in the presence of 2 x SDS sample buffer and 10 % (v/v) ß-mercaptoethanol) with 0.2 units PGNase F in 0.25 M $NaPO_4$ pH 8.6 buffer. The mix was incubated O/N at 37 °C. Aliquots were then mixed with 2x SDS sample buffer and boiled (2-3 min) prior to analysis by SDS-PAGE and lectin blots. An absence of staining of the protein on blots probed with labelled lectins indicated that complete deglycosylation had been attained.

3.20.1. Enzymatic digestion of the 98 kDa GNA binding BBM protein.

Various proteases including trypsin, chymotrypsin, papain, and *S. aureus* (V8), were analysed for their ability to digest the purified 98 kDa GNA binding BBM protein. To this end preliminary trials were carried out using various ratios of protease:protein and 37 °C incubation periods. In all trials the protein was denatured by boiling in the presence of 2x SDS sample buffer, and 10 % (v/v) β–mercaptoethanol prior to incubation with protease dissolved in the appropriate buffer. Digest reactions were terminated by boiling and subsequently analysed by silver staining of SDS-PAGE gels. Of the proteases tested trypsin and V8 under optimised digestion conditions gave distinct polypeptide products of a sufficient concentration for amino acid sequencing.

3.20.2.Trypsin and Staphyloccocus aureus (V8) digests of the 98 kDa GNA binding BBM protein.

Aliquots of purified protein were denatured by boiling in the presence of 2x SDS sample buffer, and 10 % (v/v) β–mercaptoethanol, prior to incubation at 37 °C with trypsin (50:1 substrate:enzyme concentration ratio) in 1 mM HCl, 20 mM $CaCl_2$ pH 8.6 (O/N incubation), or V8 (5:1 substrate:enzyme concentration ratio) in protease buffer 0.125 M Tris/HCl pH 6.8, 0.1 % (w/v) SDS, 1 mM EDTA, 10 % (v/v) glycerol (3 hr incubation). Digest reactions were terminated by boiling.

3.21. Amino acid sequencing.

Tryptic and V8 digests of the 98 kDa GNA binding gut protein were subject to amino acid sequencing. Peptides were separated by SDS-PAGE, pre-run with 200 μM thioglycolic acid. Gels were soaked in electroblotting buffer (10 mM CAPS, 10 % (v/v) MeOH) prior to electrotransfer to a MeOH saturated Problott membrane (0.45 μm, Applied Biosystems, Warrington, Cheshire). The membrane was then washed, stained with Ponceau S, or 0.05 % (w/v) Coomassie Blue, 40 % (v/v) MeOH, and destained with 50 % (v/v) MeOH. Bands were excised and subject to gas phase amino acid sequencing carried out by John Gilroy at Durham University or by Cambridge protein sequencing, Cambridge University, Tennis Court Road, Cambridge.

3.22. Enzyme assays.

Enzyme assays were carried out using a microtitre plate-based system at 37 °C. Enzyme activities were determined with synthetic substrates as follows: aminopeptidase, L-leucine-p-nitroanilide; alkaline phosphatase, p-nitrophenyl phosphate; α-glucosidase, p-nitrophenyl-α-D-glucopyranoside; and trypsin, Nα-benzoyl-DL-arginine p-nitroanilide (BAPNA). In the standard assay procedure, buffer and enzyme were preincubated for 5 min at 37 °C, and reaction was initiated by addition of substrate to a final concentration of 1.0-1.5 mM in a total volume of 220 μl. Buffers used were as follows: aminopeptidase, 50 mM CAPS pH 9.5 ; alkaline phosphatase, 50 mM CAPS pH 10.0; α-glucosidase, 50 mM HEPES pH 7.5 ; and trypsin, 50 mM CAPS pH 11.0 . Following addition of substrate to the preincubated buffer and enzyme mix, the microtitre plate was shaken for 10 sec, incubated for 15 min at 37 °C, and the absorbance (405 nm) was measured at 0,

10, and 20 min using a Dynatech MT 5000 microtitre plate reader. Optimum pH and enzyme concentrations were determined prior to each assay using buffers ranging from pH 7 to 11.5, and 3 enzyme concentrations, respectively. Each sample was assayed in triplicate. The linearity of the relationship between change in absorbance with time was checked to ensure substrate concentrations were not limiting. Substrate and enzyme controls were run to ensure the validity of sample absorbance readings. P-nitrophenol standards were used to obtain a calibration of pmols product vs absorbance for alkaline phosphatase and α-glucosidase assays. P-nitroaniline standards were similarly used to allow determination of pmols product for aminopeptidase and trypsin assays. The r^2 values obtained for the linear calibration graphs were 0.999 and 0.998, respectively.

3.23. Estimation of nucleic acid concentration.

The integrity and concentration of nucleic acids samples were analysed on a Beckmann DU 7500 Spectrophotometer. A 1mg/ml DNA solution was assumed to give an OD 260 of 20, and a 1 mg/ml RNA solution an OD 260 of 25 (Sambrook *et al.,* 1989).

3.24. Preparation of total RNA.

RNA was prepared either using Tri Reagent (Sigma), according to the manufacturers instructions, or by the guanidinium thiocyanate method (described by Promega, D.E. Titus, 1991). Samples were stored at -80 °C in ribonuclease free deionised water or in 3 volumes of 75% (v/v) EtOH within 1 ml cryotubes (Nalge, Nunc Int, Denmark).

3.25. Isolation of messenger RNA.

Messenger RNA was isolated from total RNA using the PolyATract (Promega) kit according to the suppliers instructions.

3.26. Electrophoresis of RNA through formaldehyde agarose gels.

Formaldehyde agarose gel electrophoresis was used to assess RNA size distribution, prior to mRNA isolation, and to check concentrations of RNA extracted from *L. oleracea* gut tissue, prior to northern blotting, under denaturing conditions described by Sambrook *et al.* (1989). Electrophoresis was carried out in a horizontal slab gel using a Pharmacia GNA-100 minigel apparatus. Agarose gels (1.5 % (w/v)) were prepared by dissolving 0.84 g HGT agarose in 35 ml DEPC-treated water. To the cooled agarose 11 ml of 5x formaldehyde gel loading buffer, and 10 ml of formaldehyde (37 % (v/v) solution) were added. Samples of RNA (approx 5 µg) in 4.5 µl in RNAse free water were prepared by adding recommended ratios of DEPC treated formaldehyde gel running buffer, formaldehyde, and formamide, of total RNA. A similarly prepared standard pea ribosomal RNA sample was run alongside *L. oleracea* total RNA when assessing size distribution. Ethidium bromide (EtBr) staining was used to visualise RNA under UV illumination allowing a comparison of the size distribution of extracted RNA with the standard pea ribosomal RNA. Bands were detected by the orange fluorescence of the EtBr DNA complex under UV light illumination (254 nm) and recorded by photography through a red-orange filter (Kodak 23A Wratten) using transmitted UV light with

Polaroid Film type 667 3000.

3.27. Electrophoresis of DNA through agarose gels.

Electrophoresis of DNA was carried out in horizontal slab gels submerged in buffer as described by Sambrook *et al.* (1989). DNA was size fractionated in 1 - 1.5 % (w/v) agarose gels cast in 1x TAE buffer using Pharmacia GNA-100 minigel apparatus. Loading buffer (typically 10 µl) was added to DNA samples diluted in sterile water prior to loading. The gels were run in 1x TAE buffer at 90 v (constant voltage) and the separated DNA fragments were visualised as described for RNA electrophoresis. Markers lambda pst1, and Eco 471, were loaded alongside DNA samples to enable size estimation of dsDNA bands. Results were recorded by photography as described previously for formaldehyde agarose RNA gels.

3.28. Plasmid DNA preparation.

Plasmid DNA was isolated using a Wizard™ miniprep plasmid isolation kit (Promega) according to the manufacturers instructions. This method typically gave DNA concentrations of 100-250 ng/µl. The isolated DNA was stored frozen at - 20°C.

3.29. Restriction endonuclease digestion of DNA.

Restriction endonuclease reactions were carried out using the buffers and temperatures recommended by the manufacturers. Restriction digests of plasmid DNA were incubated at 37 °C for a minimum of 3 hr.

3.30. Recovery of DNA fragments from low melting point agarose.

DNA fragments were isolated using silica fines (supplied by Dr T.Fordham-Skelton pers comms). DNA fragments in 1 ml NaI (1M) were incubated at 70 °C for 10 - 15 min. 10 - 15 µl of silica fines was added and the mix left to stand for 15-20 min at RT. The DNA was pelleted by spinning for 2 min in a benchtop microcentrifuge. Following removal of the supernatant the pellet was washed with 70 % (v/v) EtOH and air dried. DNA was eluted by incubating the pellet for 5 - 10 min at RT in sterile distilled water and separated from silica fines by spinning for 2 min in a benchtop microcentrifuge. The isolated DNA was stored frozen at - 20 °C.

3.31. Radiolabelling DNA by random priming and G50 Sephadex column chromatography.

Trypsin cDNA restriction fragments were labelled with [α^{32}P] dCTP using the following method: 31 µl DNA (100-250 ng) in sterile water was boiled for 5 min and chilled immediately on ice. To this 10 µl OLB (see below); 2µl BSA (10 mg/ml); 5 µl (50 µCi) [α^{32}P] dCTP; and 2 µl Klenow polymerase (10 mg/ml) were added orderwise with mixing and the whole incubated at RT O/N. Labelled DNA was separated from unincorporated nucleotides by G50 Sephadex column chromatography as described by

Sambrook *et al.* (1989). This method is suitable for DNA greater than approximately 250 bp. For 400 µCi/mmol [α^{32}P] dCTP about 150 ng of probe will be synthesised for complete incorporation of the radionucleotide. Counts per minute of the labelled fraction (collected in approx 1 ml TE buffer) were detected by liquid scintillation in a Packard Tri-carb liquid scintillation analyser, model 1600 TR, using Ecoscint A as a scintillation fliud. Levels of specific activity achieved were 2.0 - 5.0 x 10^7 cpm.

OLB preparation:
1.Solution O: 1.25 M Tris/HCl pH 8.0, 0.125 M $MgCl_2$.
2.Solution A: 1 ml solution O plus 18 µl ß-mercaptoethanol, plus 5 µl each of 0.1M dATP,dGTP, dTTP made up in 3 mmM Tris/HCl pH 7.0, 0.2 mM EDTA.
3.Solution B: 2 M HEPES/NaOH pH 6.6
4.Solution C: Hexadeoxyribonucleotides in TE buffer at 9000_{260} units per ml.
5.OLB: Mix solutions A,B, and C in the ratio of 100: 250: 150, store 100 µl aliquots at -20 °C.

3.32. Preparation of λ ZAPII cDNA library.

A cDNA library of *L.oleracea* gut tissue was made using the ZAP-cDNA Stratagene kit according to the suppliers instructions. An estimated 6.84 µg of mRNA was used to produce this library. First and second strand synthesis controls and test ligation into the Uni-ZAP vector arms were not carried out but cDNA was quantified prior to ligation via the Ethidium Bromide Plate assay method recommended by Stratagene and carried out according to their instructions. Host MRF$^-$ cells are maintained as advised by Stratagene, by restreaking onto fresh LB plus tetracycline (12.5 µg/ml) 100 mm diameter plates weekly. The cDNA library is stored by two methods; in 15 ml Falcon tubes in the presence of 0.3 % (v/v) chloroform at 4 °C, and at -80 °C with the addition of 7 % (v/v) DMSO.

3.33. Titration and screening of cDNA library.

The phage library was titrated to enable plating of an appropriate number of plaques during primary screening. Serial dilutions of phage suspension in 100 µl SM buffer were mixed with 100 µl of host MRF$^-$ bacteria (grown O/N in LB + 0.2 % (w/v) maltose and 10 mM $MgSO_4$,tetracycline 12.5 µg/ml). The mix was adsorbed at 37 °C for 20 min, mixed briefly with approx 3 ml NZY top agar (maintained at 48 °C) and poured over 100 mm NZY agar plates. Plaques were counted following O/N incubation at 37 °C.

Phage were plated out, at a high density in an agarose (NZY) top layer, on large (22 x 22 cm) plates using host MRF$^-$ cells grown as described above. Plaques were allowed to grow to pin prick size (approx 0.5 mm) at which time further growth was prevented by cooling to 4 °C. Phage were lifted from agar plates to nitrocellulose filters and subsequently denatured and neutralised as described by Sambrook *et al.* (1989).

Replicate lifts were taken to minimise the chances of false positives appearing in secondary screening. Positively hybridising clones were purified to homogeniety by sequential rounds of plaque lifting at low phage density.

3.34. Hybridisation of filter-immobilised DNA/RNA to random labelled DNA probes.

All hybridisation reactions were carried out in heat sealed polythene bags contained in plastic boxes, or in Techne hybridisation tubes . Boxes were incubated in water baths and hybridisation tubes in Techne hybridisation ovens (S.H. Scientific, Northumberland). Hybridisation solutions containing dilutions of SSC, Denhardts solution, and herring sperm DNA, were equilibrated to the required temperature before use. Herring sperm DNA was prepared as described by Sambrook *et al.* (1989) before use. Filters were incubated for a minimum of 4 hr at 65 °C in prehybridisation solution containing 200 µg/ml herring sperm DNA. DNA probes were denatured by boiling for 5 min followed by rapid cooling on ice. The probe was then added to hybridisation solution containing 100 µg/ml herring sperm DNA ,and filters were probed by O/N incubation at 65 °C. Unless otherwise indicated filters were washed at 65 °C as follows, 2x for 15 min and 1 x for 30 min both in 2x SSC, 0.1 % (w/v) SDS, followed by 2 x for 5 min and 1x for 15 min both in 1x SSC, 0.1 % (w/v) SDS. A final high stringency wash for northern blots was 2x for 15 min in 0.1 x SSC, 0.1 % (w/v) SDS. Filters were air dried and autoradiographed as described in section 3.40.

3.35.1. Polymerase chain reaction.

PCR was used to amplify gene sequences from the *L. oleracea* gut cDNA library, and from DNA synthesised by reverse transcription (RT) of isolated *L. oleracea* gut mRNA. Oligonucleotide primers were synthesised by MWG Biotech (Ebersberg, Germany). A *M. sexta* full length trypsin cDNA clone (pMTPCR) kindly provided by Dr D. Bown was used for screening the cDNA library. Amino acid sequence data obtained for digest polypeptides of the 98 kDa GNA binding gut protein was used to design primers for amplification of clones from cDNA and RT reactions. These primers were used in conjunction with T7 and T3 primers specific for sequences either side of the multiple cloning site of the pBluescript SK phagemid (Stratagene Ltd, UK). Poly T-A, T-G and T-C primers consisting of 18 T bases and the specified terminal base were also used. The concentration of each primer and the amount of template DNA added was dependant upon the type of PRC being carried out. All the reaction components were placed into 0.5 ml thin walled microcentrifuge tubes, *Taq* polymerase was added last, and the whole gently mixed.

For a typical 100 µl amplification reaction the following were combined:

cDNA (preboiled and chilled on ice)	10.0 µl
10x PCR buffer (200 mM Tris pH 8.4, 500 mM KCl)	10.0 µl
$MgCl_2$ (25 mM)	3.2 µl
dNTP (1.25 mM)	16.0 µl
oligonucleotide primer 1 (20 µM)	5.0 µl
oligonucleotide primer 2 (20 µM)	5.0 µl
sterile distilled water	50.0 µl
Taq polymerase (5 U/ µl)	1.0 µl

For large numbers of reactions oligonucleotide primers were added to a master mix containing *Taq* buffer, dNTP's, $MgCl_2$, and sterile distilled water. Variable cDNA and $MgCl_2$ volumes were used for PCR optimisation. The amplification reaction was carried out using a Perkin Elmer (Gene Amp PCR system 2400) thermal cycler with the following reaction conditions:

Table 3.4. Polymerase chain reaction conditions.

	Temp (°C)	Time (sec)
denaturing	94	300
30 - 40 cycles of PCR amplification		
denaturing	94	30
annealing	42	30
extending	72	90
final extension	72	360
storage	4	∞

Aliquots (10 - 50 µl) of the completed PCR reactions were run on low melting point agarose gels as described in section 3.25. Remaining reaction mixtures were stored at -20 °C.

3.35.2. Reverse Transcriptase (RT) PCR.

DNA was synthesised from fresh mRNA (isolated from *L. oleracea* midguts using methods described in section 3.24 and 3.25) using Moloney Murine Leukemia Virus

(MMLV) and Avian myeloblastosis (AMV) reverse transcriptases. Typically 20 µl mRNA (approx 20 ng) was incubated with 1 µl poly TX (20 µM each of poly TA,TG, and TC), and 1 µl RNAse inhibitor at 70 °C, for 10 min. After cooling on ice a preheated mix of 8 µl RT 5 x buffer, 2 µl dNTP (10 mM each), 1 µl MMLV, and 7 µl DEPC treated water was added and the whole incubated at 37 °C for 1 hr, along with a control (no RT) mix. Of this reaction 2 µl was used for subsequent amplification by PCR as described in section 3.35.1. When AMV- RT was used the RT reaction was carried out for 1 hr at 50 °C.

3.36. Subcloning of PCR products.

PCR products separated by electrophoresis (section 3.27.), and purified using silica fines (section 3.30) were subcloned into the pCR 2.1 TOPO VECTOR using the TOPO Cloning Kit (Invitrogen) according to the manufacturers instructions. Single white transformed colonies were removed with sterile tooth picks and grown O/N at 37 °C in 10 ml LB broth containing 20 µg/ml ampicillin. Positive clones were then identified for sequencing by miniprep isolation (section 3.28), and restriction (section 3.29).

3.37. Glycerol stocks.

Single colonies were plated onto fresh LB/ ampicillin (50 µg/ml) plates and grown O/N at 37 °C. Two loop fulls of bacteria were added to 1 ml of sterile 80 % (v/v) glycerol in 1 ml glass tubes (BDH), and resuspended by vortexing. Glycerol stocks were stored at -80 °C.

3.38. Analysis of RNA by northern blotting.

Formaldehyde gels containing RNA were blotted onto nitrocellulose filters by capillary blotting as described by Sambrook *et al.* (1989). Gels were blotted in the conventional manner except that nappy liners were used in place of paper towels. After blotting, the position of the wells was marked on the filter with ink. To ensure transfer was complete the blotted gel was stained with EtBr and checked for fluorescence under UV light. Membranes were hybridised O/N in hybridisation solution containing random labelled $\alpha^{32}P$ labelled probes. Filters were washed to a stringency of 1x SSC, 0.1 % (w/v) SDS at 65 °C, and exposed to X-ray film.

3.39. DNA Sequencing.

Sequencing was performed by Julia Bartley, Gillian Storey and Vicky Kelly, (Department of Biological Sciences, University of Durham, U.K.) using the dideoxy terminator chemistry method (Sanger *et al.*, 1977) and fluorescent dye-linked M13 forward and reverse primers. The reaction was performed using a Perkin Elmer thermocycler 1 (Warrington, U.K.) using AmpliTaq®DNA polymerase, FSv (Perkin Elmer, Warrington, U.K.). Sequences obtained were analysed for homology to known gene sequences in the EMBL nucleotide sequence database via blast searches (http://www.ncbi.nlm.nih.gov/BLAST/).

3.40. Autoradiography and densitometry.

Autoradiography was used to detect ^{32}P labelled nucleic acids on filters. Filters were fixed on 3MM paper and for the detection of labelled DNA filters were oriented using radioactive ink. After placing in a film cassette the filters were covered with a polyethene sheet. For labelled DNA, detection X-ray film (Fuji RX) was sensitized by exposure to a low intensity flash light prior to placing between the filter and an intensifying screen within the cassette. The filter was then autoradiographed at -80 °C and films were developed as described in section 3.12. After autoradiography the intensity of bands was assessed (typically using 2 different exposures for each filter) using a Biorad GS 690 imaging densitometer.

4. RESULTS AND DISCUSSION.

The effects of GNA and Con A delivered via AD on the development of *L. oleracea* larvae.

AD bioassays were carried out to investigate the impact of GNA and Con A upon the development of *L. oleracea* larvae. Analysis of effects following acute oral exposure to either lectin, upon survival, development and consumption are presented. Differences and similarities in the effects of GNA and Con A are discussed. Such apparently basic information is required to assess the potential value of lectins in combating insect-related crop damage. In addition these bioassays provided a fundamental basis for subsequent investigations into the mechanisms of lectin action upon this model Lepidopteran pest.

4.1. Long term bioassay using GNA-containing AD.

An AD bioassay of GNA at 2.0 % total protein was conducted as described in section 3.4.1., with 30 neonate larvae placed individually on control and experimental diets. Fig 4.1.(A) shows that survival was not affected by feeding larvae AD containing GNA. Both control and experimentally reared larvae exhibited a gradual decline in survival during the first 5 instars, with 20 % mortality recorded for both treatments from day 0 to day 25. A subsequent steeper decline in survival was observed with 33 % of control, and 36 % of experimental larvae lost from day 27 to 30, and day 28 to 31, respectively. Although the suboptimal nature of the diet prevented larvae from attaining pupation, 66 % of control, and 56 % of experimentally reared larvae reached 6th instar.

In contrast to the lack of effect on survival, the presence of GNA in AD significantly inhibited larval growth, as shown in Fig 4.1. (B). Larvae fed on GNA-containing diet had a consistently lower mean wet weight (by approx. 20-30 %) compared to the control group. This difference was significant (ANOVA) from the onset of measurement (day 7 post hatch) until day 27, with no obvious trend in the ratio of control mean weight:experimental mean weight. The sharp increase in mortality after day 27, combined with a gradual increase in variance, largely accounted for the non-significance of statistical analysis for the later stages of development. A peak in mean larval weight was observed for both treatments at 30 days post hatch, at which point 80 % of the surviving control group but only 46 % of the surviving experimental group had reached their final instar. The maximum mean weight of larvae fed control AD was seen to be significantly greater (1.36 x), than that recorded for larvae reared on GNA-containing diet (ANOVA). In addition, reduced fitness of the experimental group was indicated by the ability of control larvae to withstand a significantly greater mean loss in weight prior to death (ANOVA) compared to GNA fed larvae (data not presented).

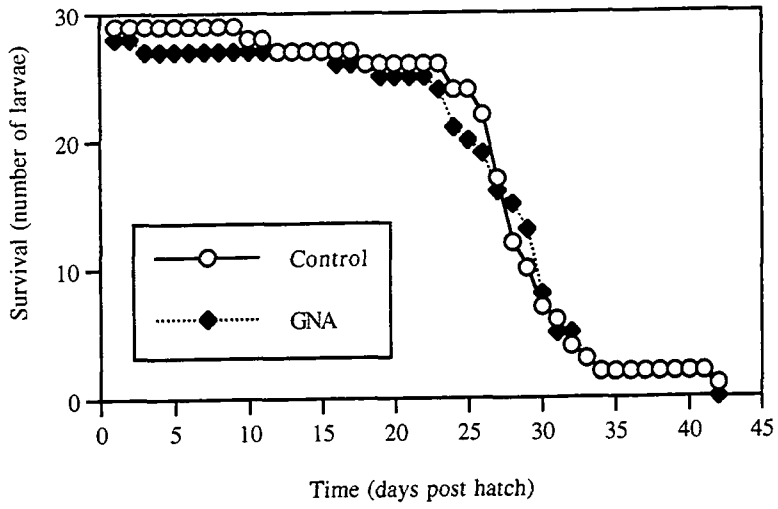
The presence of GNA in AD also significantly inhibited larval development. GNA-fed larvae consistently lagged behind control larvae in developmental rate. For all stages after 1st instar this translated into a delay of approximately 2 days in reaching instars (Fig.4.2.). This delay was largely attributed to the particularly slow development of around 10 % of the experimental group. The median time taken to reach all 6 instars

was significantly greater for GNA-fed larvae relative to the control group (Mann Whitney U-test).

Larvae reared on AD containing GNA exhibited a significant decrease in consumption compared with larvae fed control AD. This was presumably caused by the presence of the lectin. As shown in Fig. 4.3. (A), on a daily mean basis, larvae fed diet containing GNA consumed less than the control group throughout the bioassay. This difference was seen to be significant (ANOVA), for 13 out of the 37 days, for which consumption was assessed. Total daily consumption by the experimental group was also significantly less (ANOVA) than that recorded for the control group (results not presented). Cumulative values plotted in Fig 4.3. (B) show that overall, larvae raised on GNA-containing diet consumed 40 % less diet than that consumed by larvae fed control diet. Detailed analysis of diet consumption and faecal production on a per instar basis from 3rd to 6th instars was also carried out. Although moulting was not simultaneous, values were collated (e.g. values for day 14 to 19 correspond to 4th instar) to enable comparative analysis. Fig 4.4. shows that larvae reared on GNA-containing diet consumed less and produced less frass than the control group during each of the instars for which parameters were assessed. Values were highest for the 5th instar during which control and experimentally fed larvae consumed 31 % and 34 % of total consumption, and excreted 31 % and 34 % of total frass production, respectively. This correlated with results given in Fig 4.1. (B) and Fig 4.3. (A), which show that the greatest gain in mean weight was achieved by both groups during this instar (day 19 to 24). The greatest difference in consumption and faecal production between the 2 treatments occurred in the final instar, during which control larvae consumed 2 x, and produced 1.7 x that of the experimental group.

Fig 4.5. shows consumption of diet assessed as a ratio of body weight. Both control and experimental groups of insects exhibited a steady decline in this ratio from hatch until around day 30. A subsequent rise in this ratio reflected the voracious feeding of larvae in their penultimate and final instars (i.e. prior to the onset of pupation). The data was fitted to linear regressions of consumption/weight against time ($r > 0.85$) for both control and experimental groups (data not presented). The groups did not differ significantly when a non-parametric Wilcoxon signed rank test was used. These results suggested that although GNA depressed growth, and thus decreased diet consumption, it did not cause any long-term inhibition of feeding, since consumption as a fraction of body weight did not differ between control and experimental groups.

(A)



(B)

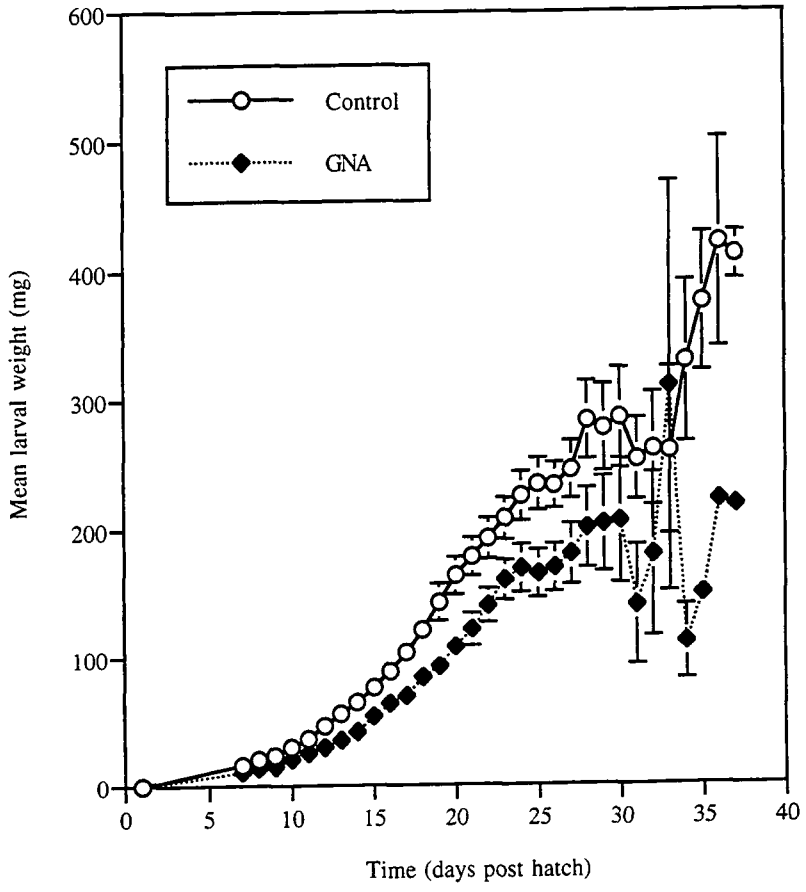


Fig 4.1. (A) Survival of *L. oleracea* larvae fed from hatch on control and GNA containing (2 % total protein) artificial diet. (B) Mean larval weight (wet) for *L. oleracea* larvae in the diet bioassay shown in (A). Error bars show mean +/- SE.

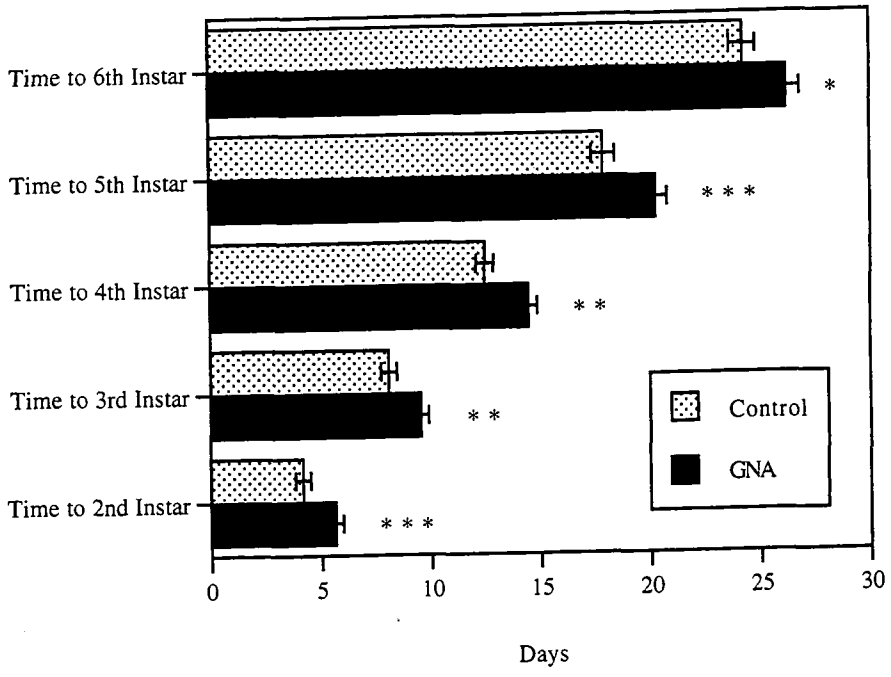


Fig 4.2. Mean time (days) taken to attain successive instars by *L. oleracea* larvae fed from hatch on control and GNA containing (2 % total protein) artificial diet. Error bars show means +/- SE; differences between control and GNA-fed groups are significant at * P<0.05, ** P<0.01, *** P<0.001 (Mann-Whitney U-test).

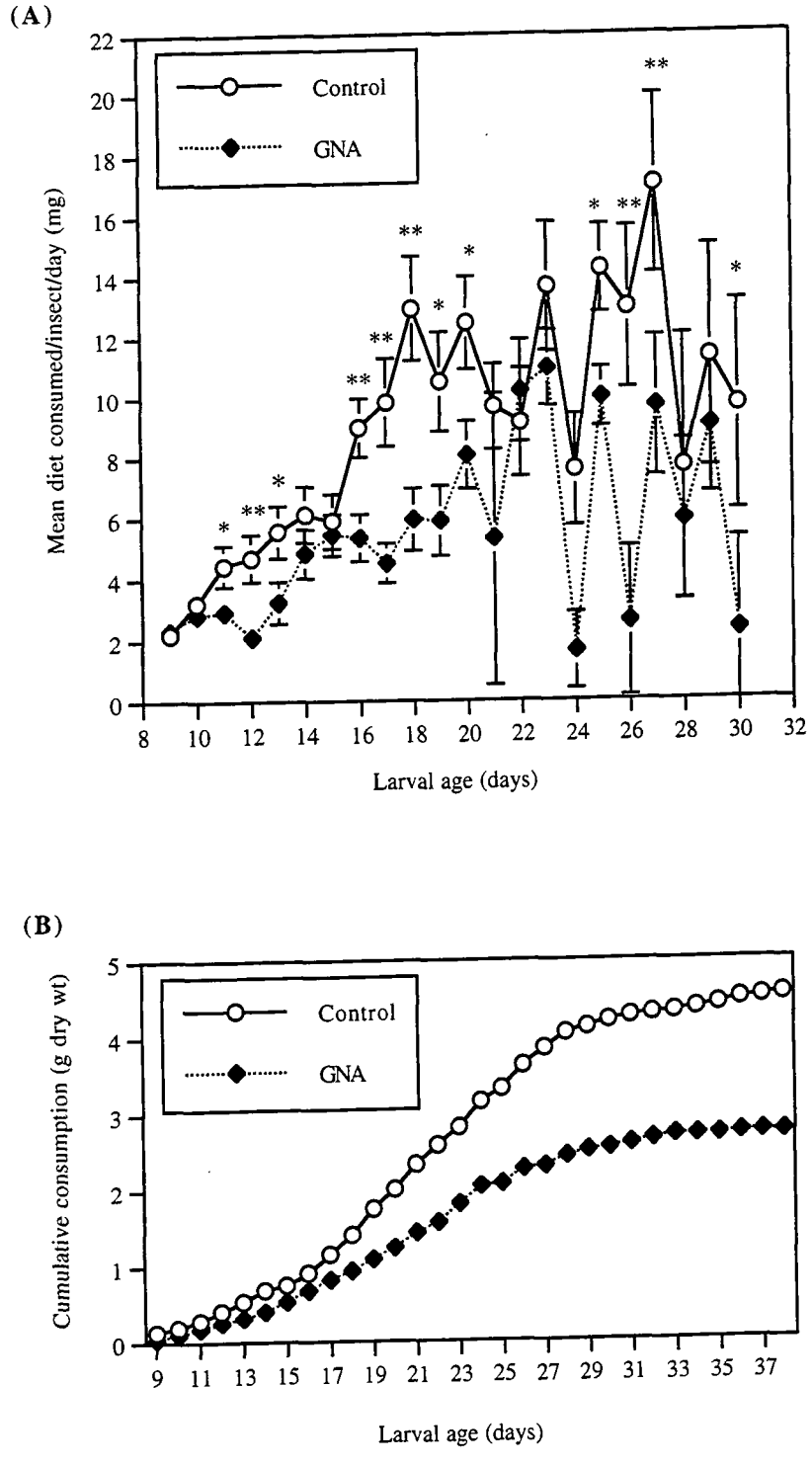


Fig 4.3. (A) Mean daily diet consumption (dry wt) for *L. oleracea* larvae fed control and GNA containing (2 % total protein) artificial diet. Error bars show means +/- SE; differences between control and GNA-fed groups are significant at * P<0.05, **P<0.01 (ANOVA). (B) Cumulative consumption of diet (total dry wt) by *L. oleracea* larvae shown in Fig 4.3 (A)

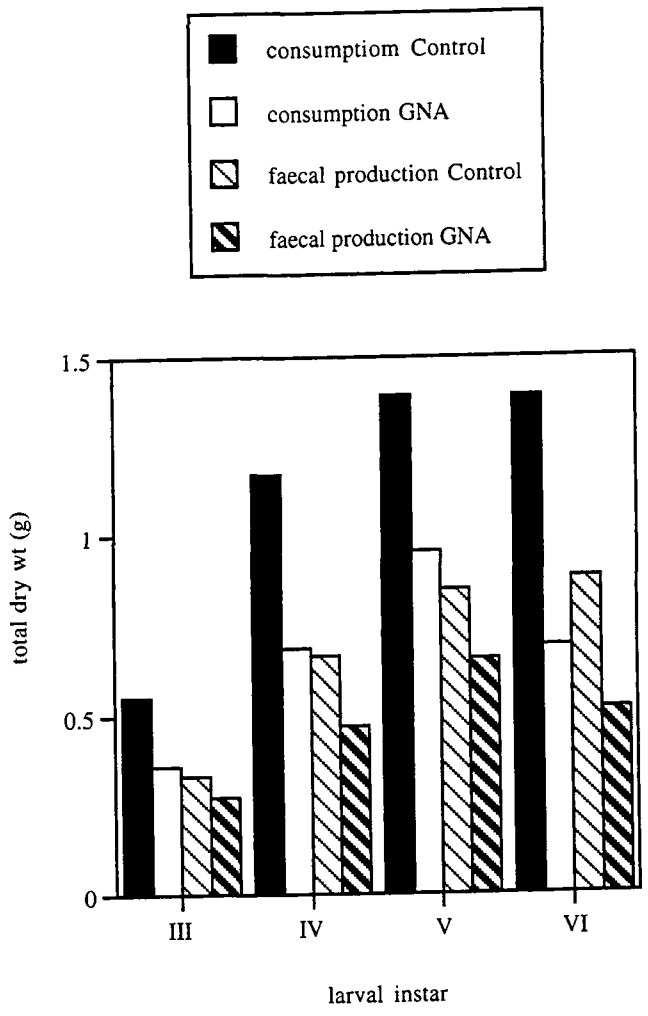


Fig 4.4. Total consumption and faecal production (g dry wt) on an instar basis by *L. oleracea* larvae fed from hatch on control and GNA containing (2 % total protein) artificial diet. Although moulting was not strictly simultaneous values were collated to allow comparitive analysis (days 11-14 correspond to III instar; days 15-19 correspond to IV instar; days 20-25 correspond to V instar; days 26-36 correspond to VI instar).

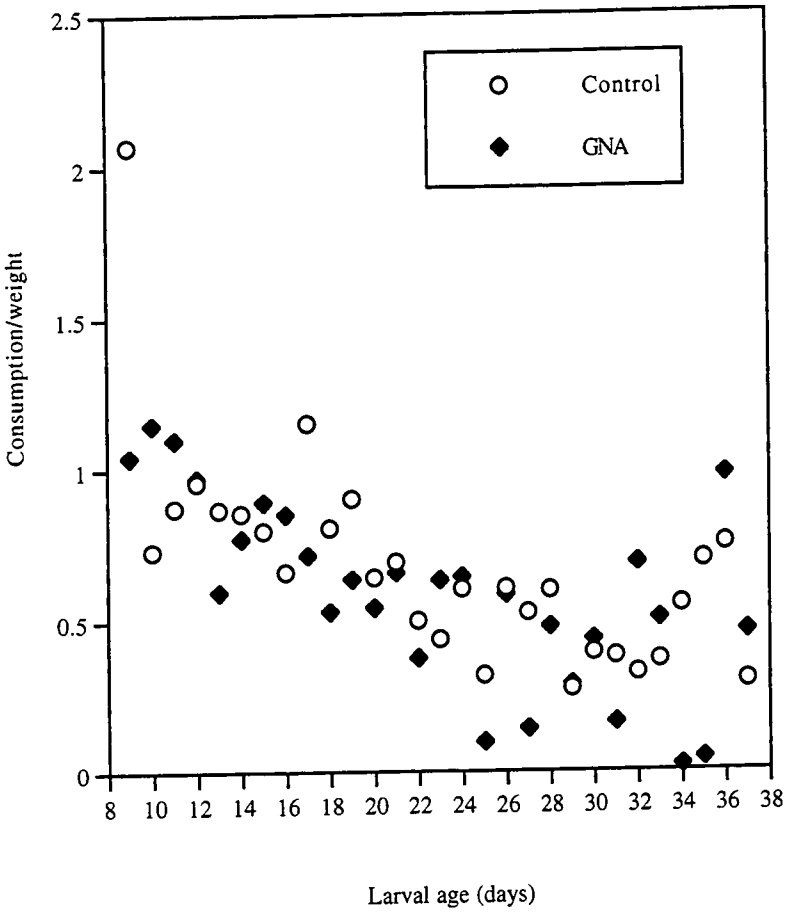


Fig 4.5. Mean daily diet consumption as a fraction of mean larval body weight, plotted against time (larval age) for *L. oleracea* larvae fed control and GNA containing (2 % total protein) artificial diet.

4.2. Long term bioassay using Con A-containing ADs.

An AD bioassay of Con A at 2.0 %, 0.2 %, and 0.02 % of total protein was conducted as described in section 3.4.3., with 25 neonate larvae per treatment placed individually on control and experimental diets. This bioassay was carried out with Dr. A.M.R. Gatehouse and G.M. Davison allowing direct comparisons with the effects of GNA to be made.

Fig 4.6. (A) shows that all 3 Con A treatments had a significant effect on larval survival and that this effect was apparent during the early stages of larval development. Survival on 2.0 % Con A was reduced by 92 % by day 12, compared with a reduction of only 12 % on control diet. Survival on diets containing 0.02 %, and 0.2 % Con A by this stage was reduced by 48 % and 40 %, respectively. However, the differences in survival between these two treatments were not significantly different from one another, but were significantly different from either the control or 2.0 % Con A groups.

The presence of Con A in AD also inhibited larval growth. Fig 4.6. (B) shows that larvae fed on Con A containing diets exhibited a lower mean weight than control fed larvae throughout the assay period. Whilst growth on 2.0 % Con A showed the greatest reduction of the three treatments (30 - 82 %), mean larval weight was not reduced in a strictly dose dependant manner. Larvae fed 0.02 % Con A exhibited a 20-47 % reduction, and larvae fed 0.2 % Con A exhibited a 0-25 % reduction, in mean weights relative to controls. The observed lower mean weights of Con A fed larvae were significant (ANOVA), only for larvae fed the highest concentration of lectin. This was largely attributed to small sample numbers resulting from decreased survival of the experimental groups.

The presence of Con A in AD significantly inhibited larval development. Whilst variable survival (and hence small sample numbers) in the assay made rigourous comparison difficult, instar distributions at all points exhibited significant differences between control and experimental groups. Fig 4.7. shows larval instar distribution for days 10, 16, and 22. At day 10, 95 % of control fed larvae were in 2nd or 3rd instars whereas all of surviving 2.0 % Con A fed larvae were still in their 1st instar. Similarly, by day 16 control larvae were evenly split between 3rd and 4th instars whereas all surviving larvae fed 2.0 % Con A diets were still in the 3rd instar. The trial was terminated on day 22 at which stage all surviving larvae reared on 2.0 % Con A diet were still in the 3rd instar whilst the majority of control fed larvae had reached the 5th instar with only approximately 5 % still in the 3rd instar. Compared with the parameters of survival and growth the effects of Con A on instar distributions appeared to be more closely related to lectin dose. A clear trend of the effects of concentration on instar distribution for larvae fed 0.2 %, and 0.02 % Con A diets is evident in Fig 4.7.

As a large reduction was observed in the number of larvae surviving on Con A containing diets the analysis of consumption is presented, for clarity, as mean cumulative consumption per larvae. Fig 4.8. shows that larvae fed on any of the Con A

diets consumed significantly less diet compared with those fed control diet. As for survival and growth, the effects of Con A were not strictly dose dependant. Although larvae fed 2.0 % Con A showed the greatest reduction in consumption relative to the control group, there was little difference in the amounts consumed between the 0.2 % and 0.02 % Con A treatments. Frass produced over the time coarse of the trial was directly related to diet consumed (data not presented).

In Fig 4.9. the consumption of diet is assessed as a ratio of body weight over time. Both control and experimental groups exhibited a decline in this ratio throughout the assay period. This suggested that although Con A depressed growth, and thus decreased diet consumption, it did not cause any long term inhibition of feeding, since consumption as a fraction of body weight was not reduced for Con A fed larvae relative to controls. The data in Fig 4.9. was fitted to linear regressions of consumption/ weight against time ($r > 0.65$) for control and experimental groups. A significant difference between experimental and control groups in this ratio was observed for the 2.0 % Con A and 0.02 % Con A treatments (non parametric Wilcoxon signed rank test). Higher ratios of consumption/weight observed for 2.0 % and 0.02 % Con A fed larvae for days 8, 10, 12, and 14, indicated that larvae may be attempting to compensate for reduced growth by consuming more diet in relation to their body weight.

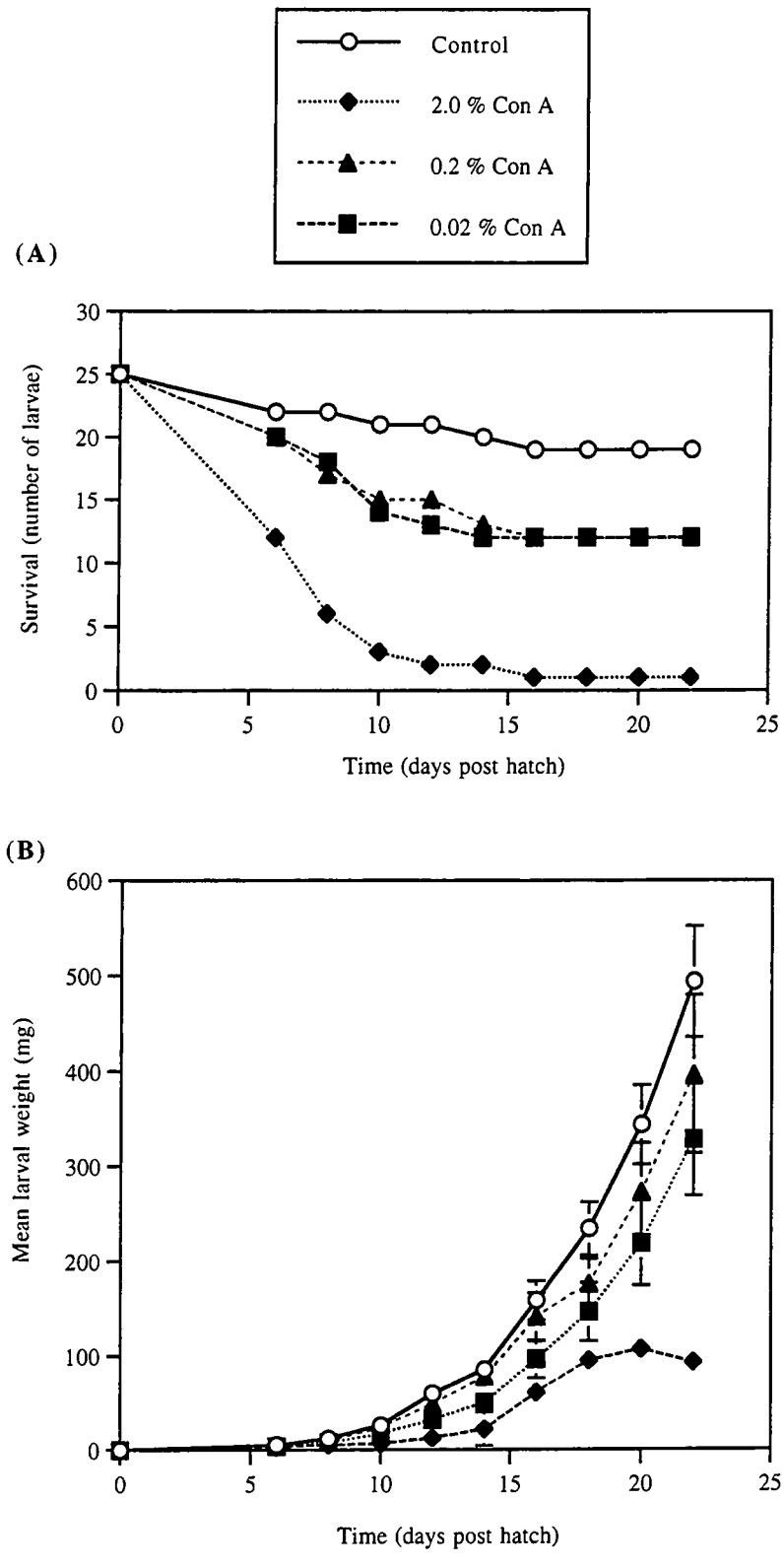


Fig 4.6. (A) Survival of *L. oleracea* larvae fed from hatch on control and Con A containing (2.0 %, 0.2 %, 0.02 % total protein) artificial diets. (B) Mean larval weight (wet) for *L. oleracea* in the diet bioassay shown in (A). Error bars show mean +/- SE.

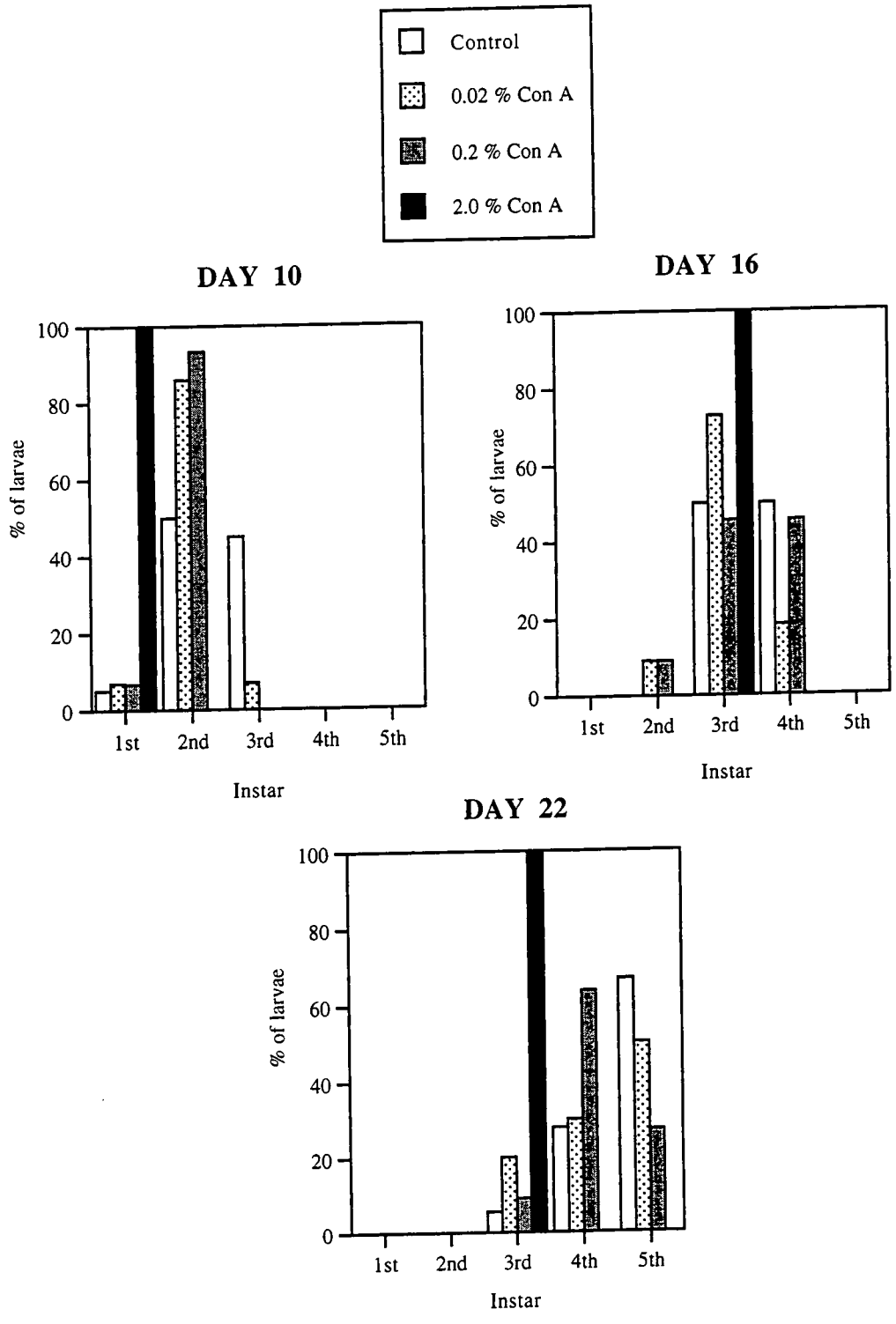


Fig 4.7. Effects of Con A, delivered in artificial diet at concentrations of 0 %, 0.02 %, 0.2 %, and 2.0 % total dietary protein, on larval instar distribution of *L. oleracea*. Panels show the proportion of surviving larvae in different instars at three time points during bioassays.

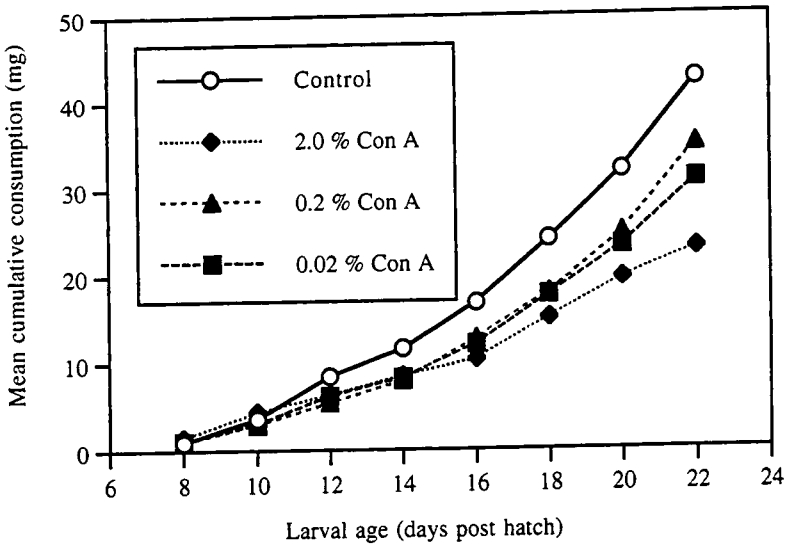


Fig 4.8. Cumulative consumption of diet (mean dry wt per larvae) by *L. oleracea* larvae fed from hatch on control and Con A containing artificial diets.

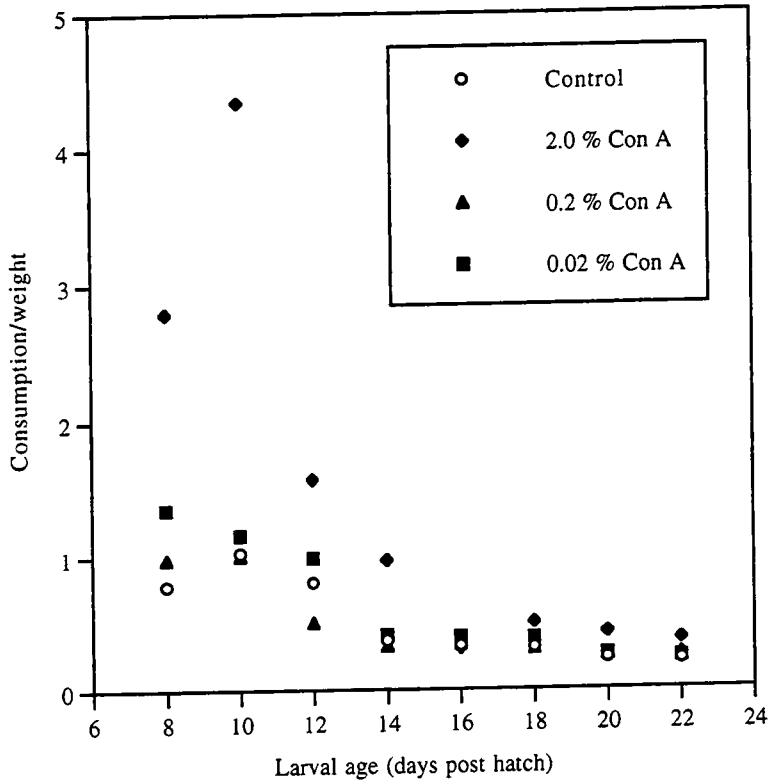


Fig 4.9. Mean daily diet consumption as a fraction of mean larval body weight, plotted against time (larval age) for *L. oleracea* fed on control and Con A containing artificial diets.

4.3. DISCUSSION.

This study provided a detailed analysis of the effects of two lectins, GNA and Con A, upon the survival, development, growth of, and consumption by, *L. oleracea* larvae.

When tested in AD at 2.0 % of total protein, GNA exerted a significantly detrimental effect upon larval development, growth, and consumption, with little effect upon survival. Larvae reared from hatch on GNA containing diet exhibited a significant and consistent delay of approximately 2 days in reaching successive instars throughout development (Fig 4.2.), and a consistently lower mean weight (approx. 20-30 %) compared with the control group (Fig 4.1. (B)). The reduction in weight was significant from the onset of measurement (7 days post hatch) until day 27. Thereafter smaller sample numbers, resulting from a sharp increase in mortality, together with an increase in variance, largely accounted for the non-significance of differences between control and experimental groups. Diet consumption was also significantly reduced by GNA. Experimental larvae consumed significantly less diet than control larvae for 13 of the 37 days for which consumption was assessed (Fig 4.3.(A)). Overall consumption by GNA-fed larvae was reduced by 40 % (Fig 4.3 (B)) compared to the control group.

Similiar effects upon *L. oleracea* have been observed in detached leaf bioassays the results of which have been published and are enclosed, see: Fitches E., Gatehouse A.M.R., and Gatehouse J.A. (1997) Effects of Snowdrop Lectin (GNA) Delivered Via Artificial diet and Transgenic plants on the Development of the Tomato Moth (*Lacanobia oleracea*) Larvae in Laboratory and Glasshouse Trials.(Journal of Insect Physiology Vol 43, No 8, 727-739). Feeding neonate larvae on potato leaves expressing relatively low levels of GNA (mean 0.068 % total soluble proteins; range <0.01-0.19 %) significantly inhibited larval development up until the 4th instar. A subsequent reduction in instar periods of transgenic fed insects resulted in a similar total developmental period for both control and experimental groups. GNA exerted a negative influence upon growth, evidenced as lower mean larval weights (3-28 % reduction for days 13-31 post hatch), compared with controls. However, differences were only significant for 3 of the 40 days for which measurements were taken. In the latter stages of development, prolonged compensatory feeding by larvae fed transgenic leaves actually resulted in their consuming 15 % more than the control group. These results indicated that the effects of GNA are at least to some extent dose dependant given that similiar but less dramatic effects were observed in the detached leaf assay, where the lectin was present at at least a 30 fold lower concentration than that used in the AD assay.

Glasshouse trials (Gatehouse *et al.*, 1997; Fitches *et al.*, 1997) have also shown GNA to confer resistance to attack by *L. oleracea.* in the short term. Following exposure of transgenic potato plants (expressing GNA at levels of up to 2.0 % total soluble proteins) to neonate larvae for 17-21 days Gatehouse *et al.* (1997) reported leaf damage to be reduced by >50 % relative to controls. Total insect biomass was reduced by 45-65 %, whilst survival was only slightly reduced (20 %). Fitches *et al.* (1997) also observed a

significant reduction in leaf damage, and a 48 % reduction in insect biomass per plant after 35 days of exposure to *L. oleracea* in glasshouse trials using transgenic plants expressing GNA at approximately 0.6 % of total soluble proteins.

GNA has been shown to be insecticidal towards a number of important insect pest species; including Homoptera such as aphids (Rahbé *et al.*, 1995; Down *et al.*, 1996; Sauvion *et al.*, 1996) and the rice brown planthopper (Powell *et al.*, 1993, 1995, 1998), and Coleoptera such as bruchid beetles and the sugar cane whitegrub (Gatehouse *et al.*, 1984; Allsopp & McGhie 1996). However, the effects of GNA ingestion vary from species to species of insect.

Con A when tested in AD at concentrations of 2.0 %, 0.2 %, and 0.02 %, of total protein, had a significant effect upon *L. oleracea* larval survival, growth, development, and consumption. Whilst no clear relationship between dose and subsequent toxicity was observed for the parameters analysed, the highest lectin concentration exhibited the greatest effects. Larvae reared on Con A diets from hatch for 22 days exhibited a variable (9-80 %) lower mean weight than control fed larvae throughout the assay period (Fig 4.6. (B)). Significant differences to controls were only observed for larvae fed the highest concentration of lectin. However, rather than a lack of effect, this was largely attributable to small sample numbers due to the high mortality of experimentally fed larvae (Fig 4.6. (A)). Rigourous analysis of instar duration was also restricted by small sample numbers although a significant delay in development was observed in the presence of Con A (Fig 4.7.). Total consumption was reduced by 92 %, 43 %, and 54.5 % by 2.0 %, 0.2 %, and 0.02 %, Con A treatments, respectively. Mean cumulative consumption was also significantly depressed by Con A. By day 22 mean cumulative consumption per larvae was reduced, relative to the control group by 46 %, 18 %, and 27 %, for 2.0 %, 0.2 %, and 0.02 % treatments, respectively.

Similiar effects upon *L. oleracea* have been observed using transgenic potato plants expressing Con A (Gatehouse *et al.*, in press). Feeding *L. oleracea* from hatch on detached leaves expressing relatively low levels of Con A (> 0.005 % total soluble proteins) caused a significant reduction in mean larval weight. By day 22, when many of the control insects were prepupal, the mean weight of larvae fed transgenic leaves was only approximately 52 % of the weight of control larvae. However, as observed in the GNA detached leaf assay, prolonged compensatory feeding by transgenic fed larvae in the latter stages of development resulted in their consuming 6.7 % more leaf material (dry weight) overall than the control group. The rate of development on transgenic leaves expressing Con A was also significantly delayed. In contrast, differences in survival were small and non significant. Whilst a clear relationship between dose and effect was not evident in the AD assay, a comparison of the 2.0 % Con A AD results with those of the detached leaf assay (where Con A was present at 400 fold lower levels) suggests that, like GNA, the effects of Con A were at least to some extent dose dependant. At present no glasshouse trials have been carried out for *L. oleracea* using Con A expressing plants.

A direct comparison of the results obtained for GNA and Con A assays suggested that Con A is the more toxic of the two lectins to *L. oleracea*. GNA has little or no effect upon larval survival even at concentrations of 2.0 % of total protein. In contrast Con A, at the same level in AD, caused a 92 % reduction in larval survival by day 12 (post hatch). Whilst GNA at 2.0 % total protein in AD caused a 20-30 % reduction in mean larval weight Con A at the same concentration caused a greater 30-82 % reduction. Con A was also seen to cause a greater reduction in larval consumption. Total consumption was reduced by 92 % by 2.0 % Con A compared to a 40 % reduction by 2.0 % GNA. This difference largely reflected the reduced survival of Con A treatments. Mean cumulative consumption per larvae relative to the control group also exhibited a greater reduction of 46 % for 2.0 % Con A fed larvae at day 22 (Fig 4.8.) compared with a reduction of 35 % for GNA fed larvae (results not presented). Both lectins induced a significant retardation in development although direct comparisons were hindered by small Con A sample numbers.

5. RESULTS AND DISCUSSION.

Identification of GNA and Con A binding glycopolypeptides in *L. oleracea* larvae; *in vitro* analysis.

This chapter describes *in vitro* experiments carried out in order to assess the potential for GNA and Con A to bind *in vivo* to various larval tissues. To this end labelled lectins were used to probe western blotted proteins extracted from larval guts, malpighian tubules, fat bodies, and heads. In mammals the strength of lectin binding to gut BBM proteins is an important determinant of lectin toxicity (Pusztai & Bardocz, 1996). Thus, the binding avidity of GNA and Con A to larval proteins was compared by *in vitro* sugar inhibition studies. These experiments were also carried out to identify which proteins bound lectins in the presence of high concentrations of inhibitory sugars as these proteins were considered to be potentially important targets for lectin action *in vivo*. As lectin toxicity in mammals is somewhat dependant upon age (Pusztai & Bardocz, 1996) the *in vitro* binding of GNA and Con A to *L. oleracea* guts throughout larval development was also investigated.

5.1. "In vitro" binding of lectins to larval BBM and PM proteins.

BBMV and PM extracts were prepared from 5th instar larvae and proteins were subsequently solubilised in the presence of 2.0 % Triton X-100. Proteins were separated by SDS-PAGE, blotted and glycopolypeptides which interact with GNA and Con A were subsequently detected by probing with digoxigenin labelled lectins. Results are presented in Fig 5.1. (A), (B) and (C). When compared with a gel stained for total proteins with Coomassie blue, the blot probed with Con A shows that this lectin bound to the majority of BBM and PM proteins. In contrast, GNA exhibited binding to only a limited subset of BBM proteins and very weak binding to only 2 PM proteins. The strongest GNA binding band represents an uncharacterised 98 kDa glycopolypeptide present in crude gut, BBMV and solubilised BBMV fractions. GNA also showed binding to 4 higher molecular weight uncharacterised glycopolypeptides in the crude gut extract. The molecular weights of these proteins were estimated, by running on 5 % acrylamide gels, to be approximately 180 kDa, 205 kDa, 210 kDa and >220 kDa (results not shown). These glycopolypeptides were not present in Triton X-100 solubilised fractions, and were not solubilised using CHAPS or SDS, at various concentrations in preliminary trials. This was thought to be indicative of a more intrinsic association with the gut membrane as compared to the 98 kDa binding protein. Both lectins exhibited binding to the most abundant glycopolypeptide in BBMV preparations (Fig 5.1.(A)) of approximately 120 kDa. This glycopolypeptide was characterised as aminopeptidase by ion-exchange chromatography and enzyme activity assays (see chapter 7). Fig 5.1. (C) shows that this abundant BBM and PM protein is a major Con A binding species but only binds GNA relatively weakly, as shown by faint staining of this protein in Fig 5.1.(B).

5.2. "In vitro" binding of lectins to proteins extracted from larval guts, malpighian tubules, fat bodies and head tissue proteins.

To establish if lectin binding, and hence membrane glycosylation, differed along the digestive tract of larvae, fore-, mid-, and hind-gut peptides were run on SDS-PAGE, blotted and probed with labelled GNA and Con A. Gut sections, as designated in Fig 3.1, were dissected from 20 5th instar larvae. Total protein concentrations of both crude and subsequent chloroform precipitated extracts were recorded to obtain a crude estimate of the relative abundance of proteins in different sections of the larval gut, and to allow equal aliquots to be run on SDS-PAGE gels. To identify potential targets, other than the gut, for lectin action malpighian tubules, fat bodies, and head tissue proteins, from 5th instar larvae were similarly prepared and analysed for lectin binding properties. Results are presented in Fig 5.2. (A), (B) and (C). A comparison of lanes 1, 2 and 3, in Fig 5.2. (A), (B) and (C), suggested that there are no major differences in the composition (Fig 5.2. (A)) and glycosylation status (Fig 5.2. (B) and (C)) of proteins, along the digestive tract. Poor photographic quality of Fig 5.2. (B) and (C) was due to weak staining of the blots. As observed in Fig 5.1.(B), at least 4 high molecular weight GNA binding proteins were present in the fore-, and mid-gut extracts, but only the smaller three were present in the hind-gut extract. The absence of glucose moieties in these higher molecular weight glycopolypeptides was indicated by an absence of staining in the Con A blot (Fig 5.2.(C)). GNA binding to the 98 kDa protein was most apparent in the midgut extract (visible as a distinct band on the blot but only just visible in Fig 5.2. (B)) , suggesting that the abundance and/or glycosylation status of this protein may be slightly different in fore- and hind-gut sections. Approximately 350 μg of total protein was extracted per larval gut, with 22 %, 64 %, and 14 % extracted from fore-, mid-, and hind-gut samples, respectively. No significant loss in protein concentration was observed following chloroform extraction. Thus whilst on a per μg basis the composition of proteins is similar, the majority of polypeptides were located in the midgut.

GNA and Con A binding to glycopolypeptides extracted from malpighian tubules, fat bodies, and larval heads, shown in lanes 4, 5 and 6, in Fig 5.2. (B) and (C) demonstrated that these tissues are all potential targets for GNA and Con A binding *in vivo*. An uncharacterised GNA binding glycopolypeptide, identical in size (98 kDa), to that observed in gut samples was present in these 3 samples. At least 2 higher molecular weight GNA binding proteins (180 kDa and 205 kDa) were present in the malpighian tubule extract (Fig 5.2. (B), lane 4). Similarly a 205 kDa glycopolypeptide, and 2 glycopolypeptides > 205 k Da, which bind GNA, were present in fat body and head tissue extracts (Fig 5.2. (B), lanes 5 and 6). Two major GNA binding proteins were present in both fat body, and head tissue extracts, the larger of which is identical in size (120 kDa) to that identified in gut samples as aminopeptidase. Similarly a 120 kDa Con A binding glycopolypeptide is present in malpighian tubule, fat body and larval head tissue extracts (Fig 5.2. (C), lanes 4, 5 and 6). In addition 2 major Con A binding species of approximately 75 kDa and 80 kDa, were present in fat body and head tissue extracts (Fig 5.2.(C), lanes 5 and 6). Both Con A, and to a lesser extent GNA,

exhibited binding to a number of lower molecular weight glycopolypeptides in malpighian tubule, fat body, and head tissue extracts.

5.3. "In vitro" inhibition of lectin binding to larval glycopolypeptides.

The inhibition of GNA and Con A binding *in vitro* to larval gut, malpighian tubule, fat body, and head tissue proteins was investigated by probing western blotted proteins with labelled lectins in the presence of various concentrations of mannose and glucose. Extracts were prepared as described in section 5.2. Gels for blots shown in Figs 5.2 (B), and (C), were run simultaneously with competition blots and hence represent controls for GNA and Con A inhibition blots, respectively. GNA inhibition was assessed by probing blots with labelled GNA in the presence of 0.2 M, 0.4 M and 0.6 M mannose. Results are presented in Fig 5.3.1. Con A inhibition was similarly assessed by probing blots with labelled Con A in the presence of either mannose or glucose at concentrations of 0.2 M, 0.4 M and 0.6 M, shown in Fig 5.3.2. and Fig 5.3.3., respectively. The extent of lectin binding is seen to be increasingly reduced in the presence of increasing concentrations of inhibitory sugars. A comparison of Fig 5.3.2. (A) and Fig 5.3.3. (A), with control Fig 5.2. (C), shows that Con A binding was more inhibited in the presence of 0.2 M mannose than in the presence of 0.2 M glucose. A comparison of Fig 5.2. (C), with Fig 5.3.2. (C) and Fig 5.3.3. (C), shows that Con A binding was almost completely inhibited in the presence of 0.6 M mannose and glucose. However aminopeptidase in the midgut sample still exhibited binding in the presence of 0.6 M mannose and glucose, evidenced as a faint band in Fig 5.3.2. (C) and Fig 5.3.3. (C), indicating that this may be the strongest lectin-glycoprotein interaction *in vivo* in the guts of larvae fed Con A containing diets.

When comparing, relative to control blots, GNA inhibition at 0.6 M mannose (Fig 5.2.(B). and Fig 5.3.1. (C)) with Con A inhibition at 0.6 M mannose and 0.6 M glucose (Fig 5.2.(C) with Fig 5.3.2. (C) and Fig 5.3.3. (C)) it appeared that overall GNA binding was less inhibited as compared to Con A. This suggested that whilst GNA binds to fewer proteins it may bind with more avidity than Con A. GNA binding to the 98 kDa protein in solubilised BBMV fractions (Fig 5.2.B) was inhibited in the presence of 0.2 M mannose (Fig 5.3.1. (A)). However, an uncharacterised 180 kDa protein in gut extracts showed binding in the presence of 0.6 M mannose, evidenced as a faint band in Fig 5.3.1. (C) indicating that this was potentially the strongest lectin-glycoprotein interaction *in vivo* in the guts of larvae fed on GNA containing diets. Similarly a 205 kDa glycopolypeptide present in fat body and larval head tissue extracts exhibited binding to GNA in the presence of 0.6 M mannose.

5.4. "In vitro" lectin binding to larval guts throughout development.

To establish if, during larval development, any major changes occur in the glycosylation of gut membrane proteins, crude gut extracts from each instar were run on SDS-PAGE, blotted and probed with labelled GNA and Con A. For instars 2-6 guts were dissected and flushed clean with buffer prior to homogenisation. For 1st instar larvae crude

extracts were prepared by homogenising whole bodies (minus head and distal portion) in buffer. Results are presented in Fig 5.4. (A), (B) and (C). Con A and GNA blots (5.4.(B) and (C)) show that there are no major changes in the glycosylation, at least with respect to glucose and mannose moeities, of gut proteins throughout larval development. Hence the potential for lectin binding in the guts of larvae fed GNA or Con A containing diets is similar at all stages of larval development. Reduced staining in both lectin blots for the 1st and 2nd instar extracts compared to other extracts was attributed to unequal protein loading which is apparent in the gel stained for total proteins with Coomassie blue (Fig 5.4. (A)), as opposed to differences in glycosylation. As observed in Fig 5.1. (B), GNA bound primarily to one 98 kDa protein and four higher molecular weight proteins. In addition GNA bound to a number of lower molecular weight proteins in crude extracts. Con A, as observed in Fig 5.1. (C) bound to the majority of gut proteins. This binding was observed at all stages in larval development (Fig 5.1.4. (A), (B), and (C)). Strong GNA binding to aminopeptidase and lower molecular weight proteins observed in this blot was thought to be a consequence of much more of the gut proteins being extracted by SDS treatment.

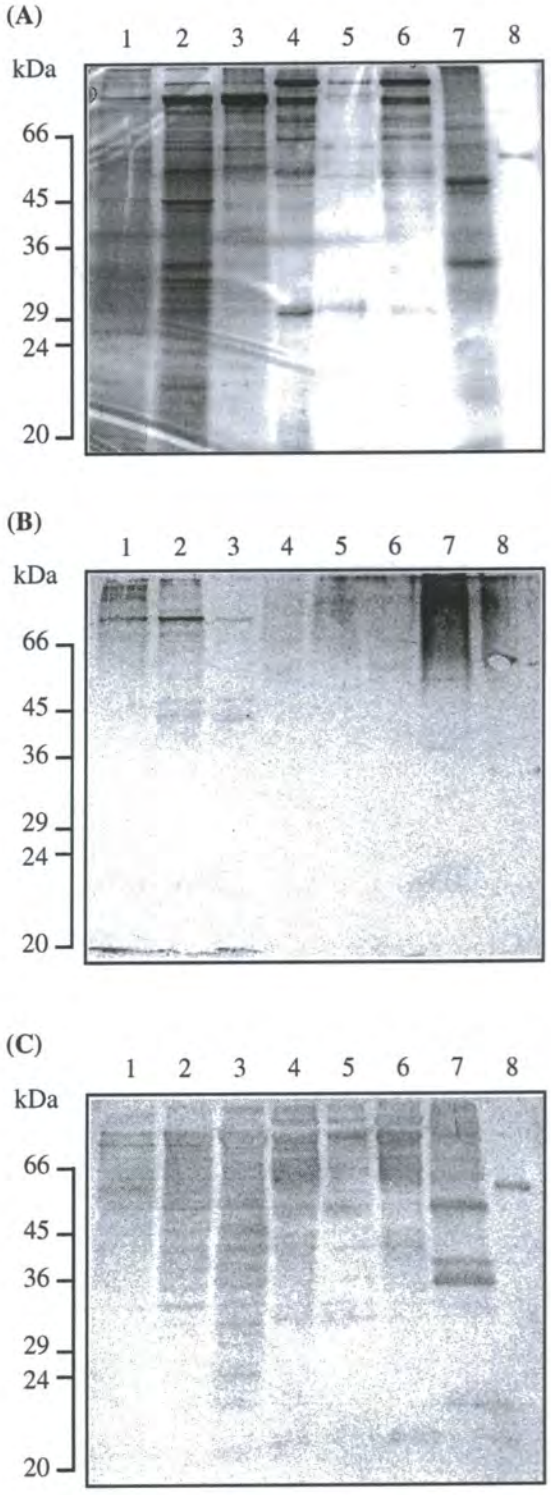


Fig 5.1. Binding of lectins to *L. oleracea* BBM and PM proteins *in vitro*. Proteins were extracted from larval guts and partially purified, prior to analysis by SDS-PAGE (12 % acrylamide gels). Samples were boiled in the presence of 2x SDS sample buffer and 10 % β-mercaptoehanol prior to loading. Proteins in (A) were detected by Coomassie blue staining. Replicate gels (B) and (C) were blotted onto nitrocellulose and probed with digoxigenin-labelled GNA (B) or Con A (C). Bound lectin was detected using alkaline-phosphatase-labelled anti-digoxigenin labelled antibodies, followed by staining with NBT/ X-phosphate. Loading is as follows, lane 1: crude gut homogenate (2.5 μg); lane 2: BBMV extract (2.5 μg); lane 3: Triton X-100 solubilised BBMV extract (2.5 μg); lane 4: PM homogenate (2.5 μg); lane 5: Triton X-100 solubilised PM extract (1.5 μg); lane 6: PM pellet (2.5 μg); lane 7: control diet (2.5 μg); for (B) and (C) lane 8: control glycoprotein carboxypeptidase Y (0.25 μg).

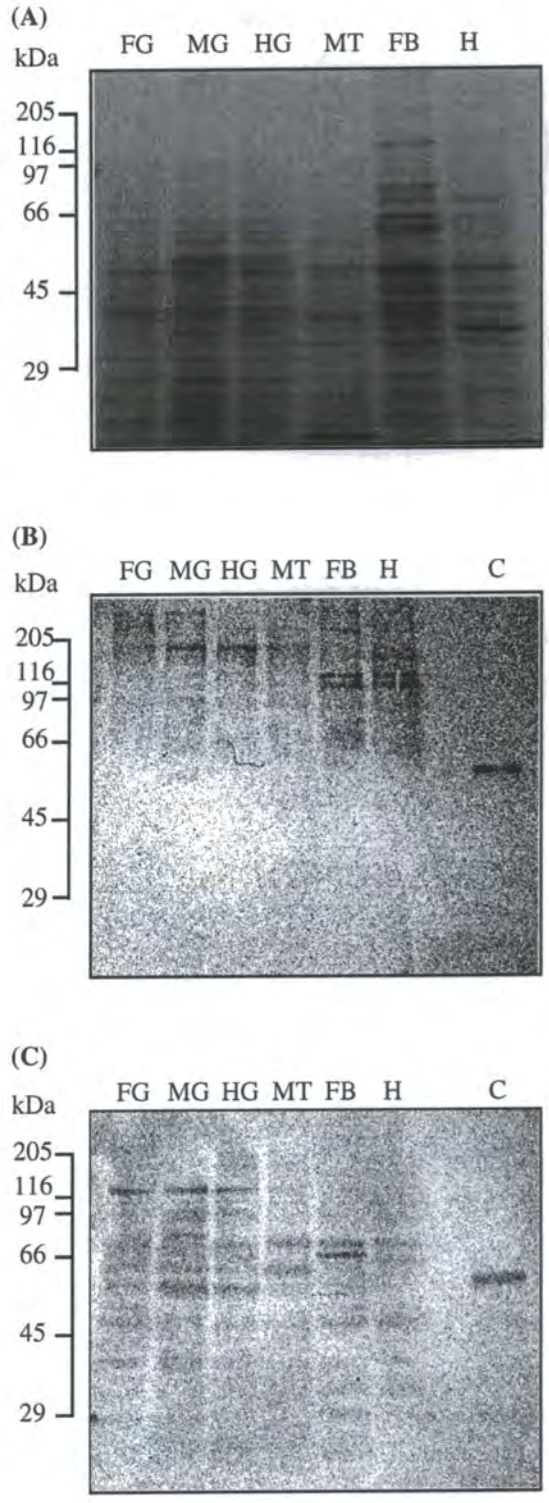


Fig 5.2. Binding of lectins to proteins extracted from *L. oleracea* guts, malpighian tubules, fat bodies and heads *in vitro*. Proteins were extracted from crude homogenates by chloroform precipitation prior to analysis by SDS-PAGE (10 % acrylamide gels). Samples (10 μg aliquots) were boiled in the presence of 2x SDS sample buffer and 10 % β-mercaptoehanol prior to loading. Proteins in (A) were detected by Coomassie blue staining. Replicate gels (B) and (C) were blotted onto nitrocellulose and probed with digoxigenin-labelled GNA (B) or Con A (C). Bound lectin was detected using alkaline-phosphatase-labelled anti-digoxigenin labelled antibodies, followed by staining with NBT/ X-phosphate. Loading is as follows, lane 1: foregut (FG); lane 2: midgut (MG); lane 3: hindgut (HG); lane 4: malpighian tubules (MT); lane 5: fat bodies (FB); lane 6: heads (H); for (B) and (C) lane 7: control (C) glycoprotein carboxypeptidase Y (0.25 μg).

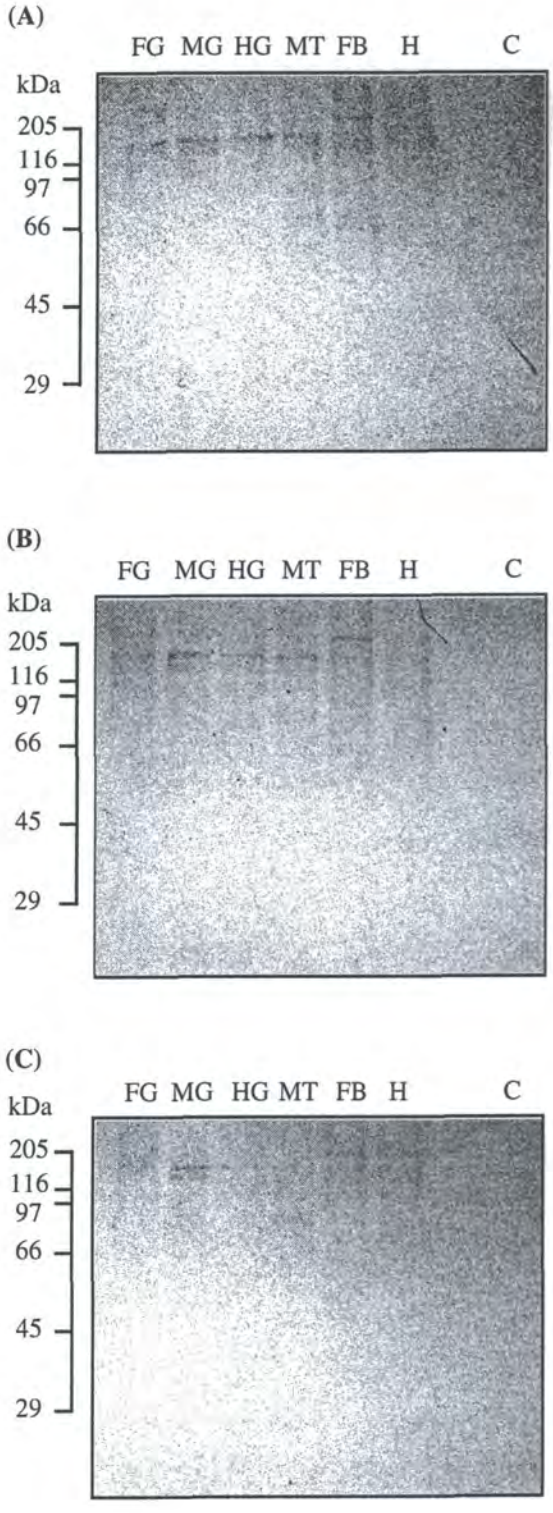


Fig 5.3.1. Inhibition of GNA binding to proteins extracted from *L. oleracea* guts, malpighian tubules, fat bodies and heads *in vitro*. Samples were prepared as described for fig 5.2, prior to analysis by SDS-PAGE (10 % acrylamide gels). Samples (10 μg aliquots) were boiled in the presence of 2x SDS sample buffer and 10 % β-mercaptoethanol prior to loading. Replicate gels were blotted onto nitrocellulose and probed with digoxigenin-labelled GNA in the presence of (A) 0.2 M mannose, (B) 0.4 M mannose and (C) 0.6 M mannose. Bound lectin was detected using alkaline-phosphatase-labelled anti-digoxigenin labelled antibodies, followed by staining with NBT/ X-phosphate. Loading is identical for (A), (B) and (C) as follows, lane 1: foregut (FG); lane 2: midgut (MG); lane 3: hindgut (HG); lane 4: malpighian tubules (MT); lane 5: fat bodies (FB); lane 6: heads (H); lane 7: control glycoprotein carboxypeptidase Y (0.25 μg).

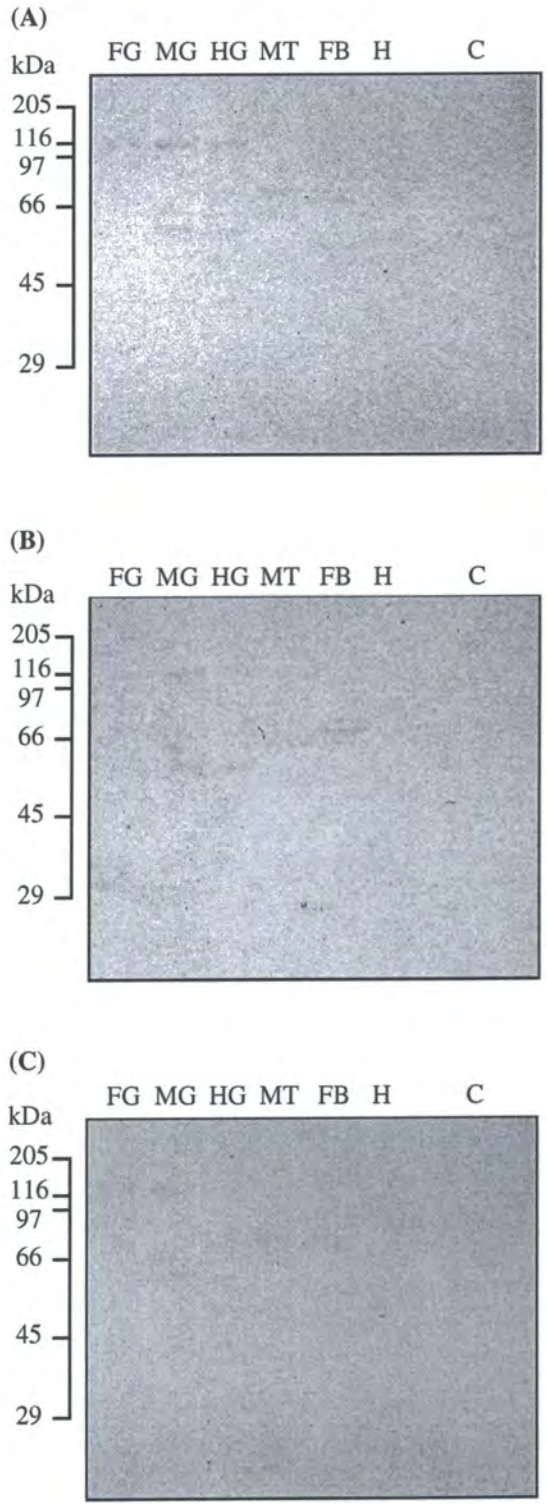


Fig 5.3.2. Inhibition of Con A binding to proteins extracted from *L. oleracea* guts, malpighian tubules, fat bodies and heads *in vitro*. Samples were prepared as described for fig 5.2, prior to analysis by SDS-PAGE (10 % acrylamide gels). Samples (10 μg aliquots) were boiled in the presence of 2x SDS sample buffer and 10 % β-mercaptoethanol prior to loading. Replicate gels were blotted onto nitrocellulose and probed with digoxigenin-labelled Con A in the presence of (A) 0.2 M mannose, (B) 0.4 M mannose and (C) 0.6 M mannose. Bound lectin was detected using alkaline-phosphatase-labelled anti-digoxigenin labelled antibodies, followed by staining with NBT/ X-phosphate. Loading is identical for (A), (B) and (C) as follows, lane 1: foregut (FG); lane 2: midgut (MG); lane 3: hindgut (HG); lane 4: malpighian tubules (MT); lane 5: fat bodies (FB); lane 6: heads (H); lane 7: control glycoprotein carboxypeptidase Y (0.25 μg).

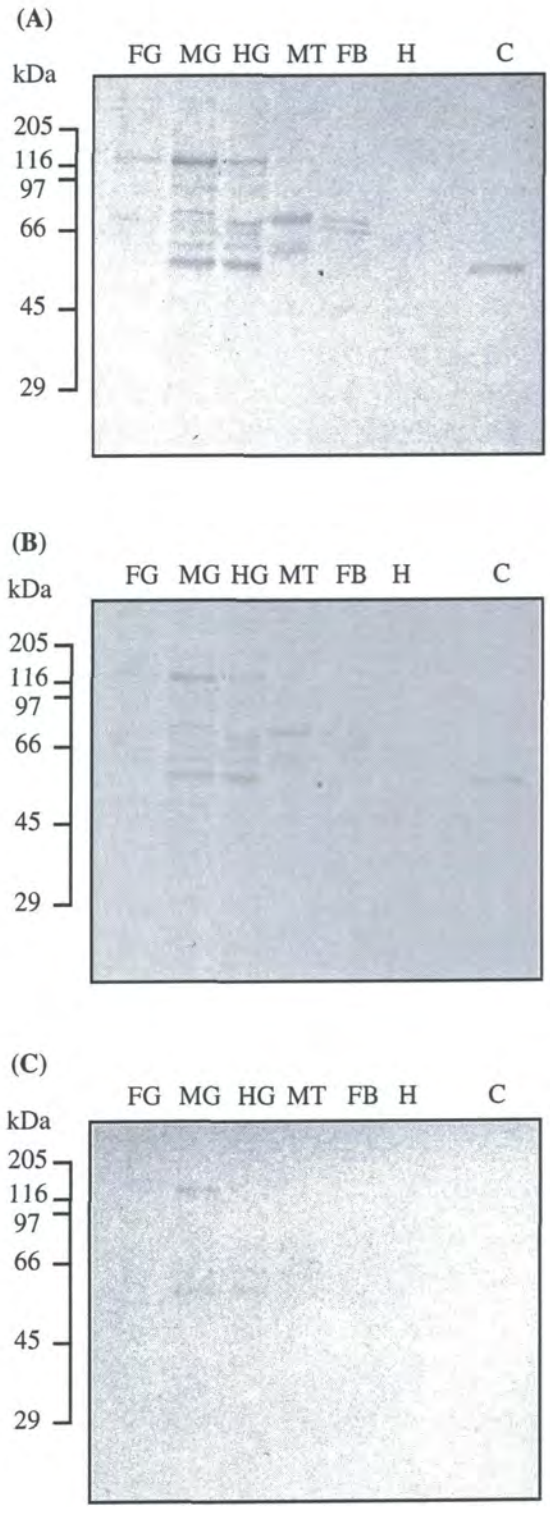


Fig 5.3.3. Inhibition of Con A binding to proteins extracted from *L. oleracea* guts, malpighian tubules, fat bodies and heads *in vitro*. Samples were prepared as described for fig 5.2, prior to analysis by SDS-PAGE (10 % acrylamide gels). Samples (10 μg aliquots) were boiled in the presence of 2x SDS sample buffer and 10 % β-mercaptoethanol prior to loading. Replicate gels were blotted onto nitrocellulose and probed with digoxigenin-labelled Con A in the presence of (A) 0.2 M glucose, (B) 0.4 M glucose and (C) 0.6 M glucose. Bound lectin was detected using alkaline-phosphatase-labelled anti-digoxigenin labelled antibodies, followed by staining with NBT/ X-phosphate. Loading is identical for (A), (B) and (C) as follows, lane 1: foregut (FG); lane 2: midgut (MG); lane 3: hindgut (HG); lane 4: malpighian tubules (MT); lane 5: fat bodies (FB); lane 6: heads (H); lane 7: control glycoprotein carboxypeptidase Y (0.25 μg).

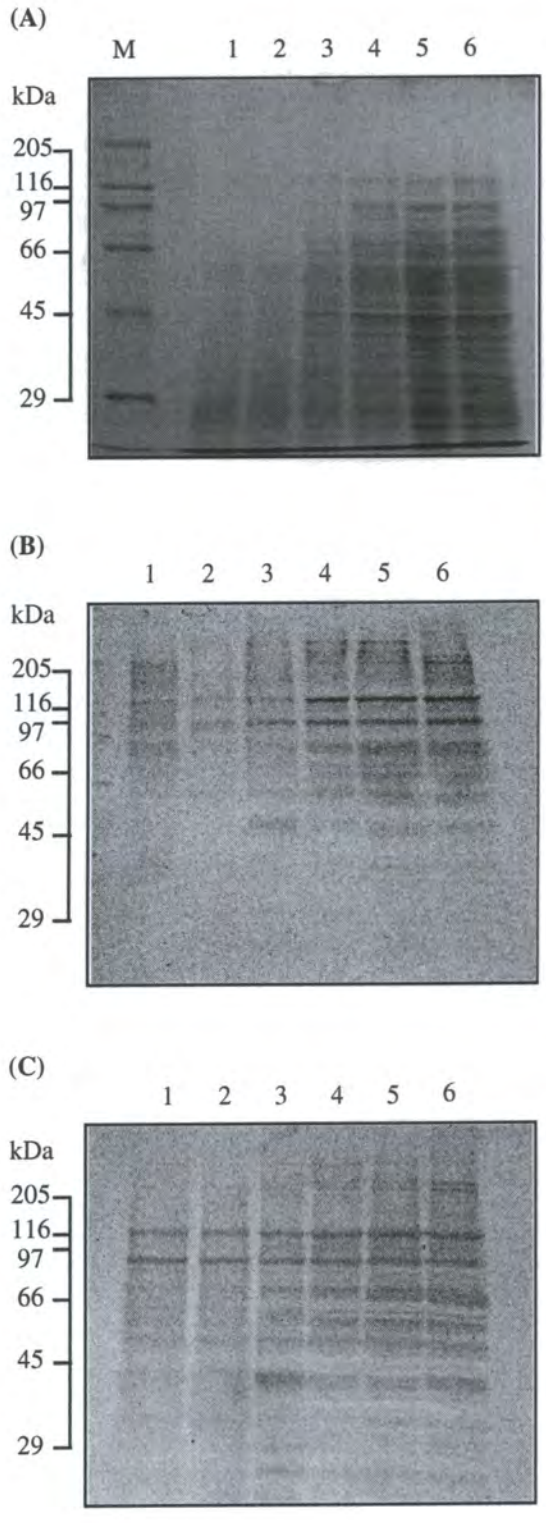


Fig 5.4. Binding of lectins to *L. oleracea* gut proteins throughout larval development. Crude extracts were prepared for each successive instar. Samples (10 μg aliquots) were boiled in the presence of 2x SDS sample buffer and 10 % β-mercaptoethanol prior to loading and separation by SDS-PAGE (10 % acrylamide gels). Proteins in (A) were detected by Coomassie blue staining. Replicate gels (B) and (C) were blotted onto nitrocellulose and probed with digoxigenin-labelled GNA (B) and Con A (C) Bound lectin was detected using alkaline-phosphatase-labelled anti-digoxigenin labelled antibodies, followed by staining with NBT/ X-phosphate. Loading is identical for (A), (B) and (C), except that (M) in (A) is molecular weight marker, as follows, lane 1: 1st instar; lane 2: 2nd instar; lane 3: 3rd instar; lane 4: 4th instar; lane 5: 5th instar; lane 6: 6th instar.

5.5. DISCUSSION.

The potential of GNA and Con A to bind *in vivo* to various larval tissues has been investigated by *in vitro* analysis of lectin binding properties, using labelled lectins to probe western blotted larval proteins. *In vitro* binding characteristics may not truly reflect the pattern of lectin binding *in vivo* since the availabilty of carbohydrate side chains cannot be determined by these methods, and thus the implications for possible effects *in vivo* are stated with caution.

Con A, which specifically binds α-D-mannopyranoside and α-D-glucopyranoside residues, was shown to bind *in vitro* to the majority of larval BBMV and PM proteins (Fig 5.1. (C)). In contrast GNA, which exhibits strict specificity for α(1,3) and α(1,6)-linked D-mannose residues, bound to only 5 BBMV glycopolypeptides and to 2 PM proteins (Fig 5.1.(B)). Differences between lectins in their *in vitro* binding capacity, which reflects the nature of gut membrane protein glycosylation, suggested that Con A has a greater potential, than GNA, to bind *in vivo*. Taken together with the results presented in Chapter 4, which demonstrated that Con A was the more antimetabolic of the two lectins, this suggested that, for *L. oleracea*, the degree of lectin toxicity was correlated with the degree of lectin binding in the insect gut. These results are analogous to the situation in mammals where the growth factor activity of lectins is determined primarily by the strength and intensity of their binding to gut BBM glycoproteins (Pusztai & Burdocz, 1996). Con A has been shown to bind, albeit patchily, to rat BBM glycoproteins causing slight growth of the small intestine following acute exposure to the lectin (Pusztai *et al.*, 1995). GNA, due to the relative scarcity of free terminal mannose residues on BBMs, does not bind initially, and consequently does not induce gut growth in rats following oral exposure to the lectin for 10 days (Pusztai *et al.*, 1990).

Con A, exhibited binding to a number of *L. oleracea* PM proteins (Fig 5.1. (C)), whereas GNA bound weakly to only 2 PM proteins in lectin blots (Fig 5.1. (B)). Eisemann *et al.* (1994), in a study of the effects of lectins upon the Dipteran *L.cuprina*, found that Con A bound to, and reduced the permeability of the PM of larvae. It was concluded that the observed reduction in growth of larvae fed Con A may at least in part be attributable to a blockage of the PM causing a subsequent restriction in the nutrients available to digestive cells. It was therefore concluded that in *L. oleracea* Con A similarly has the potential to disrupt the bidirectional movement of nutrient and digestive enzymes *in vivo*.

The ability of GNA and Con A to bind *in vitro* to various larval glycopolypeptides in the presence of increasing concentrations of inhibitory sugars has been compared. Whilst blots shown in Figs 5.3.1., 5.3.2. and 5.3.3. are not strictly comparable, greater overall inhibition of Con A binding by 0.6 M mannose and 0.6 M glucose, as compared to the inhibition of GNA binding by 0.6 M mannose, suggested that GNA may bind more avidily than Con A to gut glycopolypeptides. Since GNA does not bind to rat

BBM proteins but binds relatively strongly to *L. oleracea* BBM proteins it was concluded that this difference may account for the non toxicity of GNA to rats and antimetabolic effects on *L. oleracea.* larvae.

Inhibition studies also indicated that Con A binding to midgut aminopeptidase may be the strongest gut lectin-glycoprotein interaction *in vivo*, since binding was apparent in the presence of 0.6 M mannose and 0.6 M glucose (Fig 5.3.2. (C) and Fig 5.3.3. (C), respectively). Similarly an uncharacterised 180 kDa gut glycopolypeptide was bound by GNA in the presence of 0.6 M mannose (Fig 5.3.1. (C)), indicating that this may be the strongest *in vivo* gut lectin-glycoprotein interaction.

No major differences in the composition and glycosylation status (with respect to glucose and mannose moieties) of polypeptides were apparent in different sections of the digestive tract of *L. oleracea* (Fig 5.2. (A), (B) and (C)). In mammals surface glycosylation varies in different functional parts of the gut and therefore lectin binding is not uniform in the digestive tract (Pusztai & Bardocz, 1996). Results obtained for *L. oleracea* were thought to reflect the relatively poor differentiation of insect gut cells. Like most Lepidoptera, the midgut, as the major digestive region, dominates the body cavity of *L. oleracea* ; 66 % of total gut proteins were extracted from this region, in agreement with its being the major site for the production and secretion of digestive enzymes, and absorption of digestive products (Lehane & Billingsley, 1996).

No major differences in protein composition and lectin binding characteristics of gut extracts prepared from successive larval instars were observed (Fig 5.4.). This suggested that the composition and glycosylation of gut membrane polypeptides of newly hatched larvae is maintained throughout larval development. Hence the potential for lectin binding to the gut epithelium of larvae fed GNA or Con A containing diets is similar at all stages of larval development. Again this differs to the situation in mammals where age is known to be a determining factor in the structure of glycosyl side chains in the BBM, although quantative information on precise carbohydrate structure of gut surface receptors under different conditions of age is limited (Pusztai & Bardocz, 1996).

In vitro analysis demonstrated that malpighian tubules and fat bodies are both potential targets for lectin action *in vivo* since both extracts contained glycopolypeptides that bound GNA and Con A (Fig 5.2. (B) and (C)). The fat body is an organ of multiple metabolic functions, including detoxification of foreign compounds; metabolism of carbohydrate, lipid, and nitrogenous compounds; glycogen storage; fat and protein synthesis; blood sugar regulation; and the synthesis of major haemolymph proteins (Gullan & Cranston, 1992). Thus both lectins through their binding to fat body glycopolypeptides have the potential to disrupt correspondingly multiple metabolic processes. Malpighian tubules, which insert into the alimentary canal at the junction of the midgut and hindgut, together with the hindgut portion of the digestive tract, are responsible for excretory and osmoregulatory functions. Both GNA and Con A through binding to malpighian tubule and hindgut glycopolypeptides have the potential to disrupt

these processes *in vivo*. Both lectins also exhibited binding to glycopolypeptides extracted from larval heads (Fig 5.2.) The lack of antifeedant effects of either lectin in AD bioassays (see Chapter 4) suggests that if any interaction occurs *in vivo* between lectins and mouthpart glycoproteins it has no disruptive effect upon feeding behaviour. The possibilty for lectin interaction with glycopolypeptides in the brain and possible systemic effects cannot be discounted.

6. RESULTS AND DISCUSSION.

Characterisation of the major Con A and GNA binding glycopolypeptides in solubilised BBMV preparations from *L. oleracea.*

This chapter describes experiments that were carried out in an attempt to characterise major Con A and GNA binding glycopolypeptides from *L. oleracea* BBMV preparations. Preliminary *in vitro* studies (Chapter 5) indicated that whilst Con A bound to the majority of BBM glycopolypeptides, the major Con A binding species was an abundant 120 kDa glycopolypeptide (Fig 5.1.(C)). GNA bound to 5 higher molecular weight glycopolypeptides with a species of approximately 98 kDa evident in solubilised BBMV preparations (Fig 5.1.(B)). Failure to solubilise the remaining 4 glycopolypeptides was attributed to their being more intrinsically associated with the gut membrane. Preliminary experiments failed to obtain sufficient amounts of these glycopolypeptides (present in very low abundance in gut extracts) for N-terminal sequencing. The experiments described here are concerned with purification of the 98 kDa GNA binding BBM glycopolypeptide and attempts to characterise this protein. Partial purification of the 98 kDa GNA binding and 120 kDa Con A binding BBM glycopolypeptides was achieved simultaneously by ion exchange chromatography.

6.1. Partial purification of a major Con A binding BBM glycopolypeptide.

A major Con A binding gut glycopolypeptide was partially purified from Triton X-100 solubilised BBMV's prepared from 5th instar larval midguts. Solubilised proteins were fractionated by Hitrap Q ion exchange chromatography. A typical trace is presented in Fig 6.1. Fractions were subsequently analysed for purity and lectin binding properties by SDS-PAGE, and by probing blotted fractions with labelled Con A, respectively. Silver stained gels showing fractions which correspond to the ion exchange trace shown in Fig 6.1. are presented in Fig 6.2.(blots probed with Con A not presented). The major Con A binding glycopolypeptide of 120 kDa eluted as a broad peak between 0.1 M and 0.19 M NaCl (corresponding to fractions 26-48 in Fig 6.2. (B), (C) and (D)), with maximal elution at 0.155 M NaCl (corresponding to fractions 31 and 32 in Fig 6.2.(C)).

Similarly sized BBM polypeptides have previously been identified as aminopeptidases which bound Bt toxins in several Lepidoptera. These included 115 kDa and 106 kDa aminopeptidases from *M. sexta* (Garczynski & Adang 1995; Luo *et al.*, 1996); a 120 kDa aminopeptidase from *H. virescens* (Gill *et al.*, 1995); and a 100 kDa aminopeptidase from *L. dispar* (Valiatus *et al.*, 1995). In the light of this evidence fractions obtained from ion exchange chromatography of *L. oleracea* BBMV's were analysed for aminopeptidase activity using the synthetic substrate L-leucine-p-nitroanilide. The results are presented in Fig 6.3. A peak in aminopeptidase activity corresponding to the ion exchange elution peak of the major Con A binding glycopolypeptide (Fig 6.2.(C), fractions 31 and 32) was observed, indicating that the 120 k Da protein was aminopeptidase.

6.2. N-terminal sequencing of aminopeptidase.

Fractions that exhibited the highest degree of aminopeptidase purity by ion exchange chromatography (typically fractions 25-32 Fig 6.2 (C)) were concentrated by 30K Microsep™ ultrafiltration. Peptides were subsequently separated by SDS-PAGE (using 8 % acrylamide gels to ensure that sufficient separation of aminopeptidase from contaminating peptides was achieved), and blotted onto Problott membrane. N-terminal blockage was observed on two separate occasions using different samples and therefore confirmation of the identity of this protein as aminopeptidase was not obtained.

6.3. Purification of the major GNA binding glycopolypeptide in solubilised BBMV preparations.

The major GNA binding glycopolypeptide identified in solubilised BBMV preparations was purified simultaneously with the partial purification of aminopeptidase by ion exchange chromatography. The 98 kDa protein, analysed for purity by SDS-PAGE and by probing blots with labelled GNA, eluted between 0.075 M and 0.1 M NaCl (Fig 6.1.). This corresponds to fractions 15-22 shown in Fig 6.2. (A) and (E), and roughly to a small broad peak at 0.075 M in the ion exchange trace shown in Fig 6.1. The absence of a strictly corresponding peak in the trace was attributed to the relatively low abundance of this protein and the presence of contaminating peptides. The small peak observed at 0.105 M NaCl (corresponding to fraction 23 in Fig 6.2.) is the first fraction seen to contain aminopeptidase. Although ion exchange conditions were varied in preliminary trials the results presented represent optimal conditions achieved for the separation of the 98 kDa GNA binding BBM polypeptide. Chromatography was carried out several times and pure fractions were concentrated by 30K Microsep™ ultrafiltration to obtain sufficient protein for subsequent analysis. Fig 6.4. shows that the level of purification achieved by these methods was >90 %.

6.4. Deglycosylation of the major GNA binding glycopolypeptide in solubilised BBMV preparations.

Aliquots of pure protein were deglycosylated using N-glycosidase F as described in section 3.19. Complete deglycosylation shown in Fig 6.4.(A) lane 5, and confirmed by the absence of staining in the corresponding lectin blot in Fig 6.4.(B) lane 5, produced a polypeptide of approximately 75 kDa. Thus glycosylation was responsible for 23 % of the total molecular weight of this protein.

6.5. N-terminal sequencing of the purified GNA binding BBM glycopolypeptide.

Aliquots of the purified and concentrated protein were run on SDS-PAGE gels and blotted onto Problott membrane. However, N-terminal blockage of the protein was observed on two separate occasions using different samples.

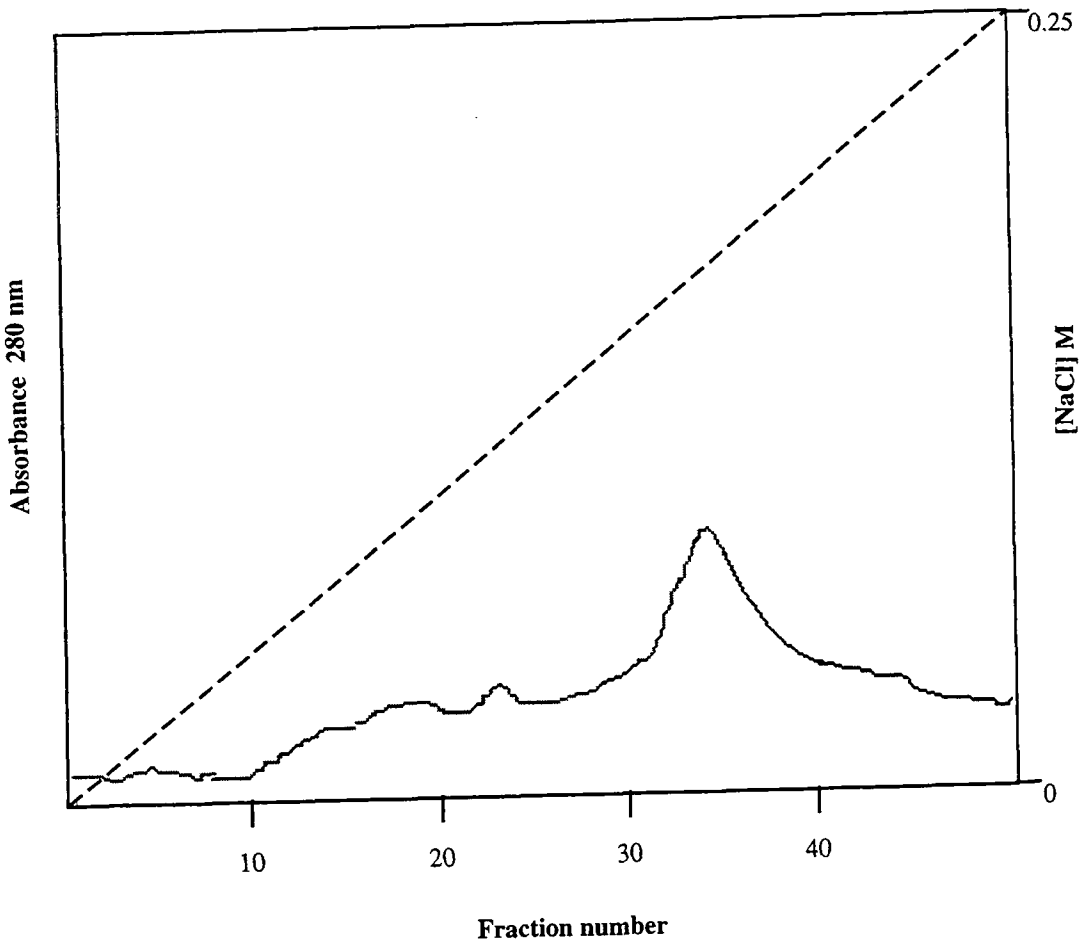


Fig 6.1. Purification of BBM Con A (120 kDa) and GNA (98 kDA) binding proteins. BBM proteins were solubilised with Triton X-100, and fractionated by ion exchange chromatography in 20 mM Tris / HCl buffer (pH 7.5) containing 0.2 % Trition X-100. A linear gradient of 0 - 0.25 M NaCl was controlled by a Pharmacia Biotech Gradifrac system at a flow rate of 0.5 ml / min.

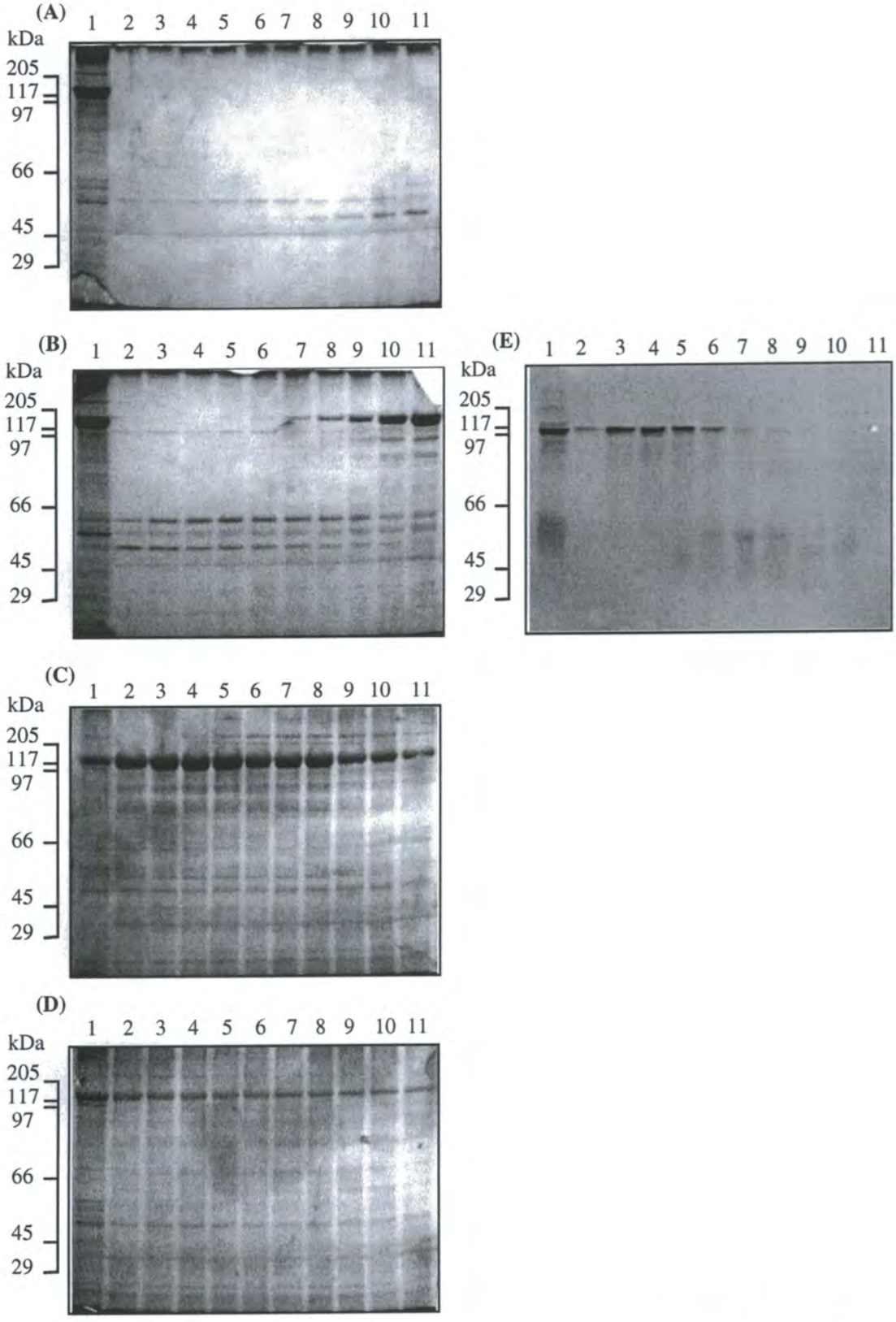


Fig 6.2. SDS-PAGE and lectin blot analysis of protein separation by ion exchange chromatography. Aliquots (20 μl) of fractions were boiled in the presence of 2x SDS sample buffer and 10 % β-mercaptoethanol prior to loading on 10 % acrylamide separating gels. Gels were silver stained or blotted (E) and probed with digoxigenin-labelled GNA. Bound lectin was detected using alkaline-phosphatase-labelled anti-digoxigenin antibodies followed by staining with NBT/ X-phosphate. Loading is as follows: lane 1 in all cases is solubilised BBMV (2.5 μg); (A) lanes 2-11: fractions 9-18, respectively; (B) lanes 2-11: fractions 19-28: respectively; (C) lanes 2-11: fractions 29-38, respectively; (D) lanes 2-11: fractions 39-48, respectively; (E) blot probed with GNA, lanes 2-11: fractions 19-28, respectively.

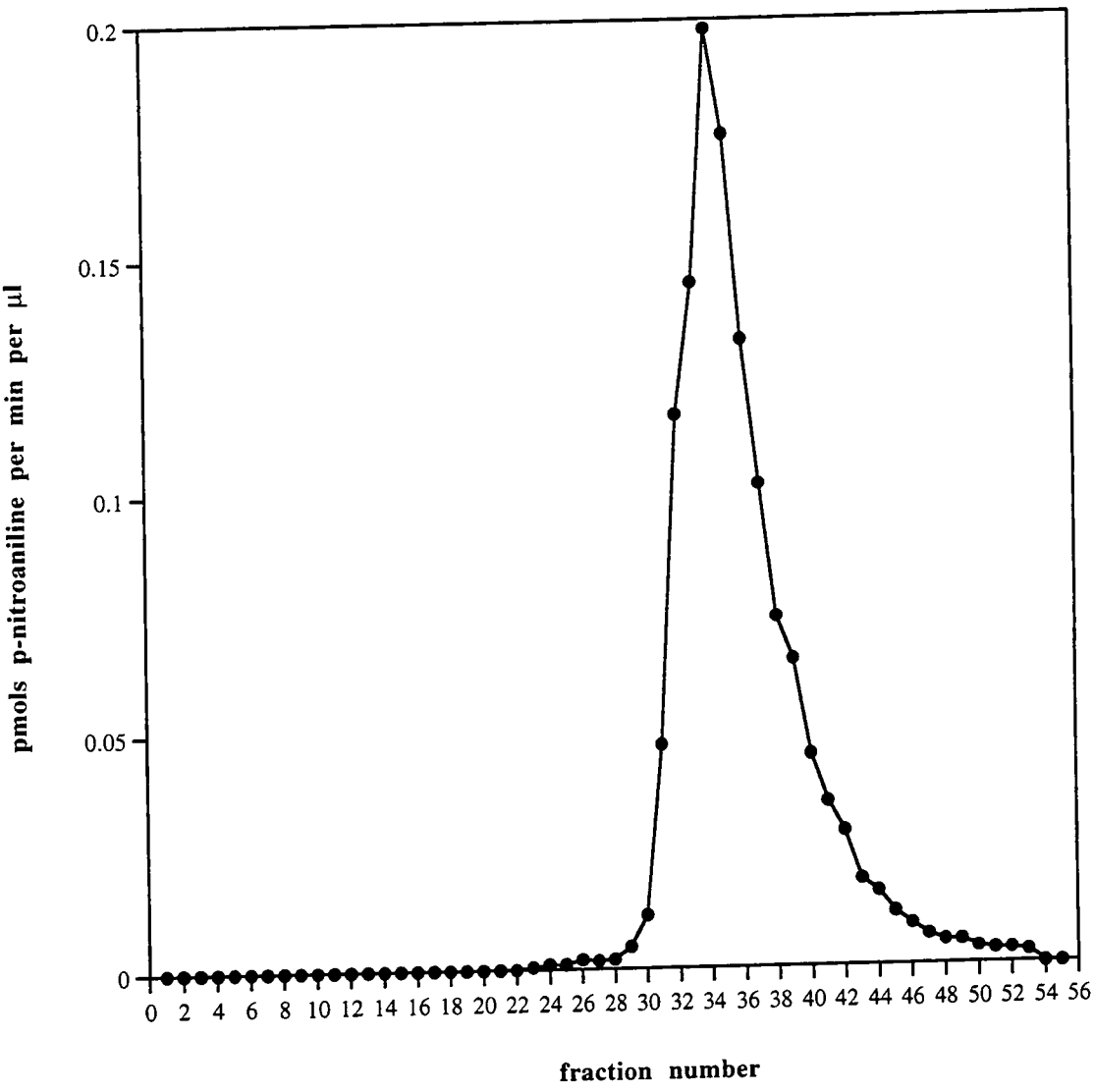


Fig 6.3. Aminopeptidase activity in ion exchange chromatography fractions of solubilised BBMV proteins. Activity was analysed using the synthetic substrate L-leucine-p-nitroaniline. Activity is expressed as pmols p-nitroaniline per min per µl of sample, derived from a calibration of product vs absorbance using p-nitroaniline standards.

6.6. Protease digestion and amino acid sequencing of the purified GNA binding BBM glycopolypeptide.

The protease V8 gave polypeptide products of approximately 45 kDa and 30 kDa when incubated with the purified GNA binding protein at a protease:protein ratio of 5:1 for 3hr at 37 °C. The 45 kDa V8 digest polypeptide subjected to amino acid sequencing is shown in Fig 6.4 (A) lane 6. Both V8 digestion polypetides are evident in Fig 6.4.(B), lane 6. The protease trypsin gave a distinct polypeptide product of approximately 36 kDa when incubated with the purified GNA binding protein at protease:protein ratio of 50:1, O/N at 37 °C (Fig 6.5). Digest products were subsequently blotted onto Problott membranes and subjected to amino acid sequencing. To ensure that bands excised for sequencing were genuine digestion products aliquots of pure protein and protease (incubated under the same conditions as the digest reactions) were run as controls on SDS-PAGE and blotted alongside digestion reactions. The trypsin digest polypeptide of approximately 36 kDa, subjected to amino acid sequencing on 2 occasions using different samples at Cambridge University and at Durham University, is shown in Fig 6.5. Amino acid sequence data obtained is presented in Fig 6.6.

6.7. Preparation of midgut cDNA library.

A cDNA library was prepared from *L. oleracea* midgut tissue polyA$^+$ mRNA (6.8 µg) using the lambda ZAP II cDNA Stratagene kit, according to the suppliers instructions. The integrity of the cDNA library was confirmed by screening the library with a cDNA probe encoding a *M. sexta* trypsin (600 bp), kindly provided by Dr D. Bown. Four positive clones were isolated. One of these clones was sequenced and shown to contain a coding sequence homologous to the *M. sexta* trypsin clone (results not shown). The nucleotide sequence of the full length *L. oleracea* trypsin clone was submitted to EMBL, accession no AJ007706.

6.8. Cloning of PCR and RT-PCR products.

PCR (using the cDNA library as a template), and RT-PCR (using gut tissue mRNA), methods were used, under various conditions, to obtain putative clones of the 98 kDa GNA binding glycopolypeptide. Degenerate oligonucleotide primers designed against amino acid sequence information obtained for the 98 kDa protein, are shown in Fig 6.7. As differences and ambiguities were observed in the amino acid sequence data obtained for the trypsin digest polypeptide (Fig 6.6.) 3 different primers, designated as TRY FOR 1, 2 and 3, were designed. In particular, 3 non-specific inosines, corresponding to amino acid number 7 in Fig 6.6., were placed at bases 13-15 of the TRY FOR 2 primer (Fig 6.7. (iv)) in an attempt to account for differences at this position in the amino acid sequences obtained. For PCR reactions these primers were used in conjunction with non-degenerate primers T7, and T3, specific for sequences either side of the multiple cloning site of the pBluescript ®SK phagemid. Oligo poly TA, poly TG, and poly TC primers were also used in PCR reactions. Figs 6.8. and 6.9. show examples of results obtained by PCR, and RT-PCR, respectively. Of the 10 amplification products obtained, 7 were successfully subcloned into the pCR 2.1 TOPO vector and sequenced.

None of these clones exhibited characteristics to indicate that they encoded predicted peptides corresponding to the protein in question. The cDNA and predicted amino acid sequences obtained from clones that exhibited homology to known genes via BLAST searches are given in Appendices 1-7. The sequence identification numbers of homologous sequences (as designated in BLAST searches) are given in brackets.

A PCR product of approximately 300bp, designated as clone 1, was obtained using V8 FOR and oligo dT primers. This clone contained a nucleotide sequence that encoded for the predicted amino acid sequence of the V8 primer in translation frame 1. However, the the amino acids following the primer sequence did not match those obtained for the original V8 digest product (Fig 6.6.). A 156 bp region of the clone exhibited 30 % identity (in frame 1) with a gene encoding a fat body protein isolated from *D. simulans* (GB AF045786). A PCR product of approximately 300 bp obtained using V8 REV and T3 primers, designated as clone 2, contained nucleotides encoding predicted amino acid sequences for both primers in translation frame 2. Homology to various adaptor proteins and protein tyrosine kinases in this frame was observed. An 84 bp region of the clone exhibited 38 % identity with an SH2/SH3 adaptor protein isolated from *D. melanogaster* (GI 1373390 (U57816)), and 50 % identity with protein-kinase receptors isolated from mice (SP Q02858 (X71426)). The sequence of this clone was only approximately 200 bp. As the predicted clone size (using the V8 FOR primer derived from a 45 kDa digest polypeptide) was around 1000 bp it was concluded that this clone did not encode any part of the 98 kDa protein.

Two PCR products of approximately 1300bp, and 890 bp, designated as clones 3 and 4, respectively, were obtained using the primers TRY FOR 1 and T7. Clone 3 contained neither primer but the predicted amino acid sequence in translation frame 3 exhibited homology to various alpha-amylase genes. This clone exhibited more than 60 % identity with alpha-amylase genes isolated from *O. nubilalis* (GI 436943(U04224); GI 435557 (U04226); GI 438387 (U04223); GI 435555 (U04225)). Clone 4 contained the T7 primer sequence but not the TRY FOR 1 predicted amino acid sequence and exhibited homology to various 26S protease regulatory subunit genes. A region of 185 bp of the clone exhibited 96 % identity (in protein translation frame 3) with a 26S protease regulatory subunit 4 gene isolated from *D. melanogaster* (SP P48601 (U39303)).

Two PCR products of approximately 600bp and 500bp, designated as clones 5 and 6, were obtained using TRY FOR 2 and T7 primers. Clone 5 did not contain the predicted amino acid sequence for either primer but exhibited homology (in protein translation frame 1) to various smooth muscle and calponin proteins. A 400 bp region of the 690 bp clone exhibited 46 % identity with two muscle specific proteins isolated from *D. melanogaster* (PIR A30128; SP P14318 (Y00795)), and 43 % identity to a calponin smooth muscle protein from pig (SP Q08092 (219538)). Clone 6 contained the T7 primer and nucleotide bases encoding 8 of the 9 predicted amino acids specified by the TRY FOR 2 primer in frame 3. Homology in protein translation frame 3 to various chymotrypsin genes was observed. A 282 bp region of the clone exhibited more than 50

% identity with chymotrypsins from *H. armigera* (GNL PID E1191405 (Y12279); GNL PID E1191359 (Y12273)) and *M. sexta* (GI 609526 (L34168)). The non match of the 5th predicted amino acid in the clone sequence to the sequence specified by the primer TRY FOR 3 and the small size of homologous chymotrypsin genes (approximately 100 amino acids) confirmed that this clone did not encode for the 98 kDa protein.

An RT-PCR product of approximately 980bp, designated as clone 7, was obtained using TRY FOR 3 and oligo dT primers. This clone contained both primer sequences. However, the predicted amino acid sequence following the primer sequence did not match the data obtained for the 98 kDa protein. Similarity of the clone to a small coding region and large 3' untranslated region of ADP/ATP translocase genes was observed. The 102 bp translated region exhibited 87 % identity with the coding region at the 3' end of ADP/ATP translocase genes isolated from *D. melanogaster* (GNL PID E293083 (Y10618); BBS 151320 (S71762); BBS 112436 (S43651)). Alignment of the majority of the sequence to untranslated regions of homologous genes indicated that this was not a clone encoding for any part of the 98 kDa protein.

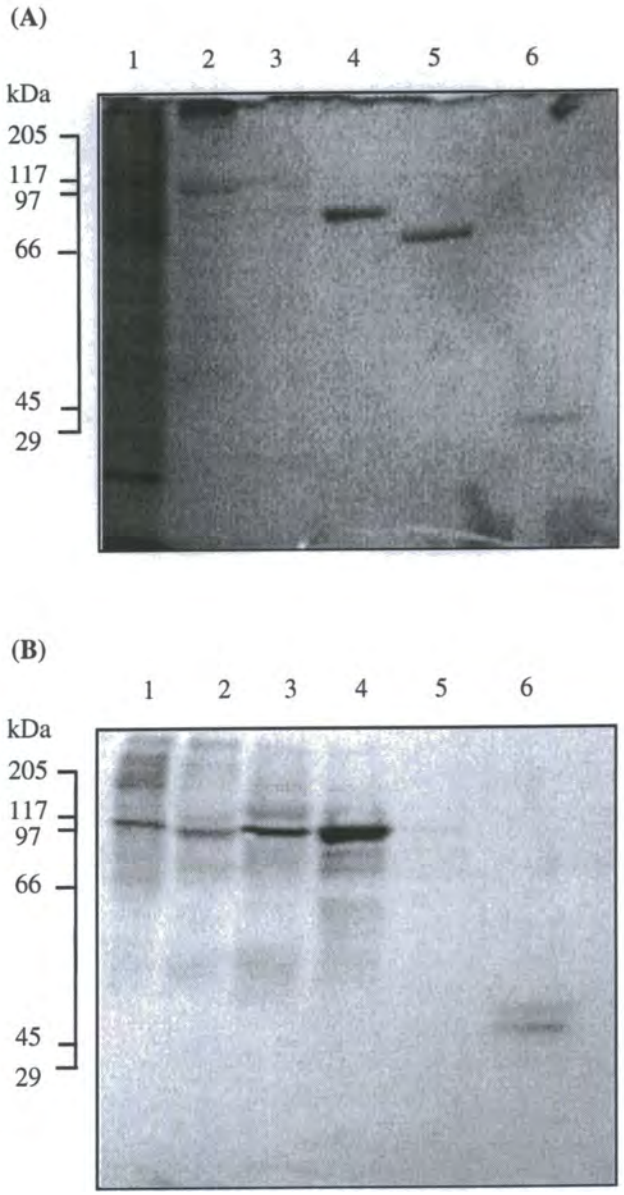


Fig 6.4. SDS-PAGE and lectin blot analysis of purification, deglycosylation and *S. aureus* (V8) digestion of the 98 kDa GNA binding BBM protein. The protein was purified from Triton X-100 solubilised BBMV preparations by ion exchange chromatography and concentrated by Microsep ™ ultrafiltration. Deglycosylation was achieved by O/N incubation at 37 °C in the presence of N-glycosidae F (0.008 U per μg pure protein). The protein was digested for 3 hrs at 37 °C using V8 at a substrate : enzyme ratio of 5 : 1. Loading is identical for (A) silver stained gel and (B) western blotted polypeptides probed with digoxigenin-labelled GNA. Bound lectin was detected using alkaline-phosphatase-labelled anti-digoxigenin antibodies, followed by staining with NBT/ X-phosphate. Lane 1: crude gut extract (2.5 μg); lane 2: BBMV (1.5 μg); lane 3: solubilised BBMV (1 μg); lane 4: purified protein (10 μg); lane 5: deglycosylated purified protein (20 μg); lane 6: V8 digest polypeptides (20 μg).

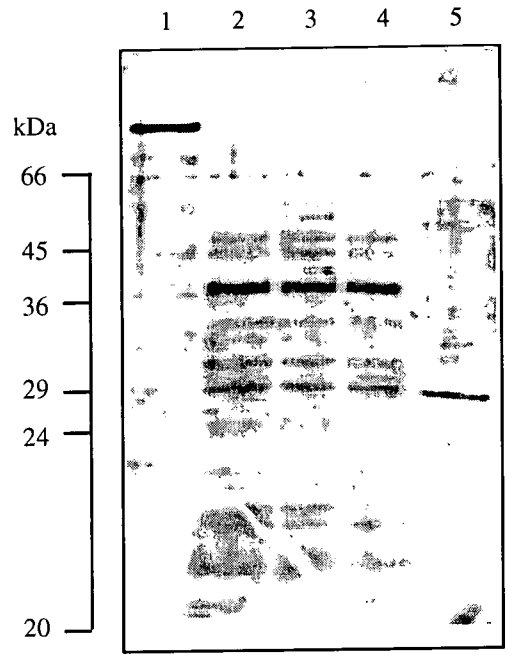


Fig 6.5. Blot showing the 36 kDa cleavage product obtained following digestion of the purified 98 kDa GNA binding BBM glycopolypeptide. The protein was digested by O/N incubation at 37 °C in the presence of trypsin (protease:protein ratio 50 : 1). Peptides were separated by SDS-PAGE (12 % acrylamide gel) and blotted onto Problott. The 36 kDa bands were excised and subject to amino acid sequencing. Loading is as follows: lane 1: control undigested 98 kDa protein (4 μg); lanes 2, 3, & 4 are trypsin digested peptides (10 μg) ; lane 5: control trypsin (4 μg).

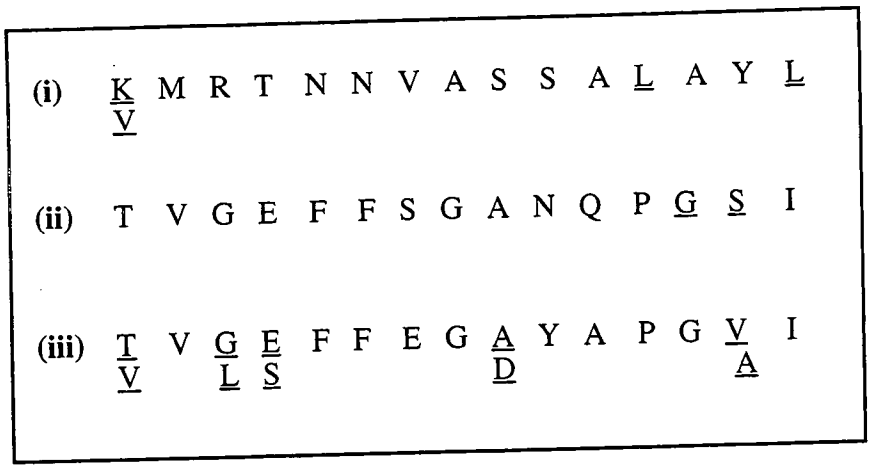


Fig 6.6. Amino acid sequence data obtained from digest polypeptides of the purified 98 kDa GNA binding BBM protein.
(i) amino acid sequence obtained from a 36 kDa polypeptide produced by V8 digestion of the 98 kDa protein.
(ii) & (iii) amino acid sequence data obtained from a 36 kDa polypeptide produced by trypsin digestion of the 98 kDa protein.
Digest products (approx 5 pmols) (i) and (ii), were sequenced at Durham University, and (iii), at the Department of Biochemistry, Cambridge University. Underlined letters denote uncertain or secondary sequence.

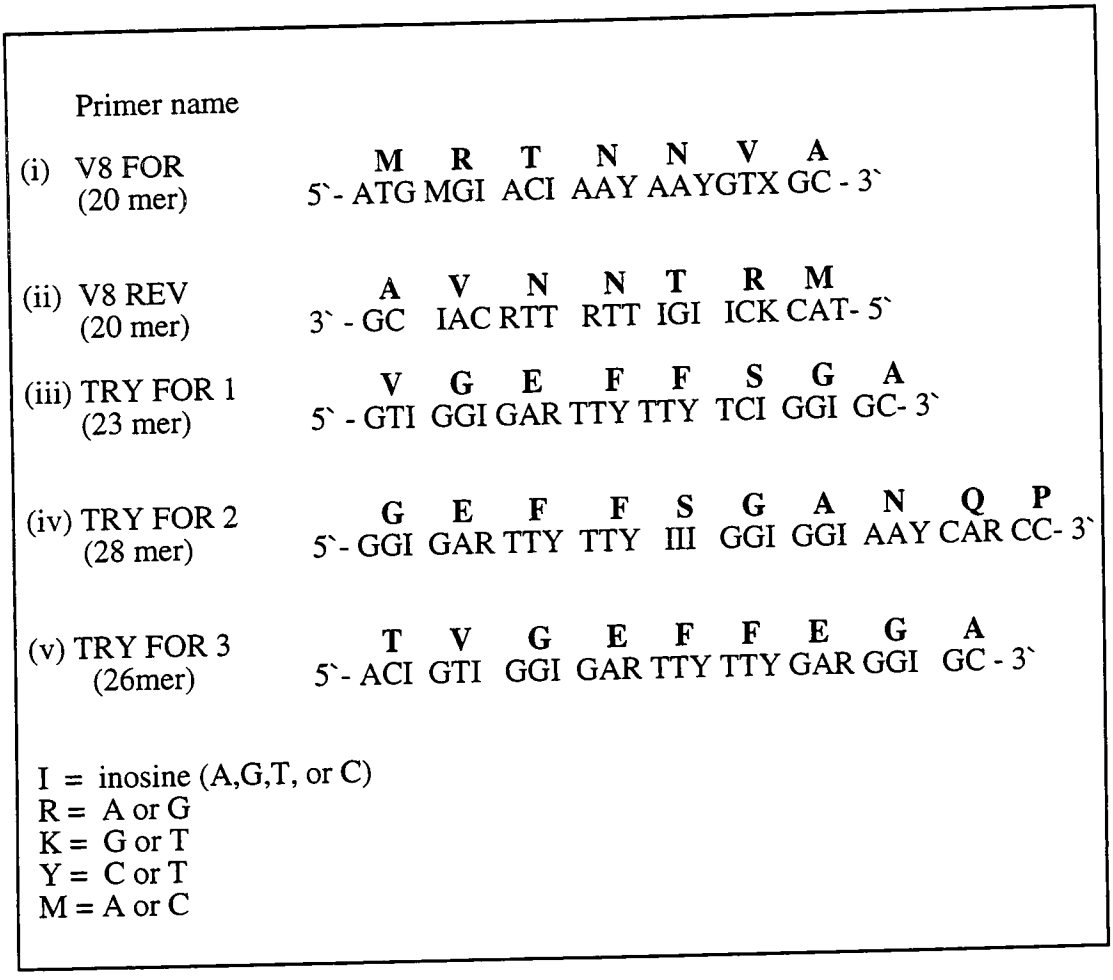


Fig 6.7. Oligonucleotide primers designed, according to the universal genetic code, against amino acid sequence information derived from digest polypeptides of the 98 kDa GNA binding BBM protein. Lypholised primers were synthesised by MWG Biotech, Germany. Letters in bold denote amino acids chosen, from sequence data shown in Fig 6.6., for primer design. (i) and (ii) are primers designed against amino acid sequence derived from the 45 kDa V8 digest polypeptide; (iii), (iv), and (v) are primers designed against amino acid sequence data derived from the 36 kDa trypsin digest polypeptide. Letters for degenerate bases are explained in the Fig.

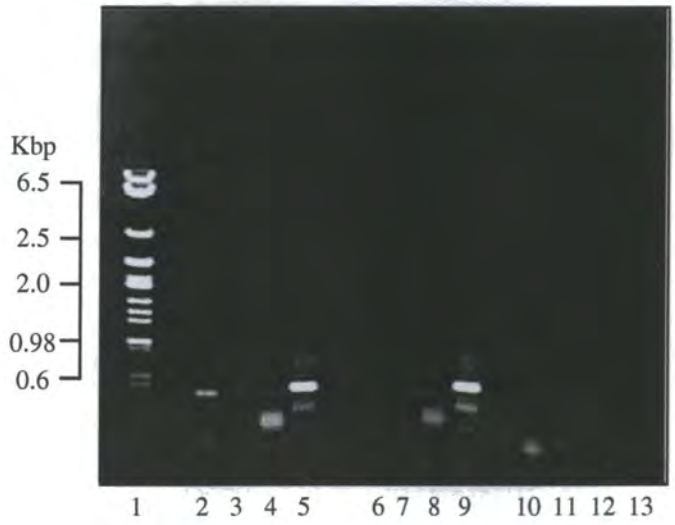


Fig 6.8. EtBr stained agarose gel (0.7 %) showing PCR products obtained using degenerate primers V8 FOR and V8 REV (designed against amino acid sequence data obtained from the V8 digest polypeptide of the 98 kDa GNA binding protein) and non degenerate primers T7 and T3 (specific for sequences either side of the multiple cloning site of the pBluescript phagemid). Lane 1: Eco 471 λ size marker; lane 2: primers V8 FOR & T7, 5 μl cDNA template; lane 3: primers V8 REV & T7, 5 μl cDNA template; lane 4 primers V8 FOR & T3, 5 μl cDNA template; lane 5: V8 REV & T3; lanes 6-9 are PCR reactions described for lanes 2-5, respectively, using 10 μl cDNA template; lanes 10-13 are controls containing primer combinations described for lanes 2-5, respectively, with no cDNA template.

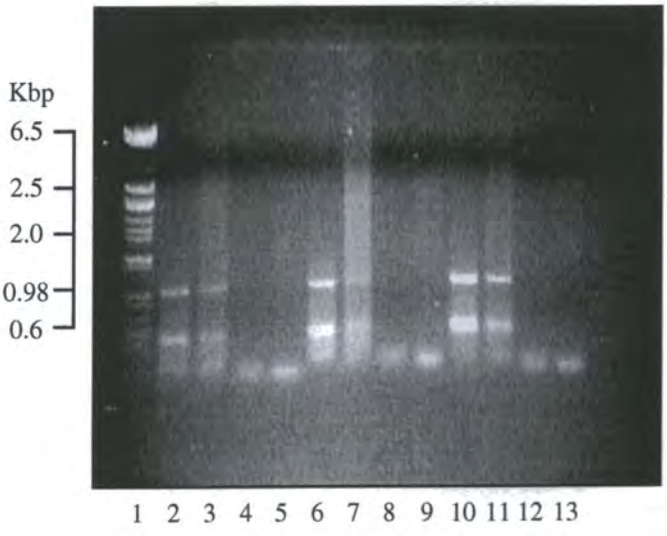


Fig 6.9. EtBr stained agarose gel (1.0 %) showing RT-PCR products obtained using the degenerate primer TRY FOR 3 (designed against amino acid sequence data obtained from the trypsin digest polypeptide of the 98 kDa GNA binding protein) and poly T-A, poly T-C and poly T-G primers. Approximately 20 ng of mRNA was isolated for 1st srand cDNA synthesis, carried out at 37 °C for 1 hr, using MMLV (200 U) reverse transcriptase. 2 μl aliquots of the RT reaction were used for subsequent amplification by PCR (annealing 42 °C). All pairs of reactions were carried out at 2 mM $MgCl_2$ & 5 mM $MgCl_2$ concentrations, respectively. Lane 1: Eco 471 λ size marker; lanes 2 & 3: RT reaction, primers TRY FOR 3 & poly T-A; lanes 4 & 5: control, no RT reaction, primers TRY FOR 3 & poly T-A; lanes 6 & 7: RT reaction, primers TRY FOR 3 & poly T-C; lanes 8 & 9: control, no RT reaction, primers TRY FOR 3 & poly T-C ; lanes 10 & 11: RT reaction, primers TRY FOR 3 & poly T-G; lanes 12 & 13: control, no RT reaction, primers TRY FOR 3 & poly T-C.

6.9. DISCUSSION.

The insecticidal effects of lectins are thought to be primarily determined by their binding to suitably glycosylated BBM proteins. Thus characterisation of target glycopolypeptides is fundamental to our understanding of the basis of lectin toxicity since it provides a means of identifying which physiological processes the lectin may disrupt.

A BBM glycopolypeptide of approximately 120 kDa was identified (Chapter 5) as a major Con A binding species on the grounds that it was the most abundant and avidly binding BBM protein. Partial purification of this glycopolypeptide was achieved by ion exchange chromatography (Figs 6.1. and 6.2.). Subsequent enzymatic analysis of ion exchange fractions provided strong evidence to suggest that the major Con A binding species was aminopeptidase. Aminopeptidase activity was observed only in those fractions containing the protein , and a peak in activity corresponded to the ion exchange elution peak of the protein (Figs 6.1. and 6.3.). Similarly sized aminopeptidases have previously been isolated from the BBM of several Lepidoptera; including 115 kDa and 106 kDa aminopeptidases from *M. sexta* (Garczynski & Adang 1995; Luo *et al.*, 1996) and a 120 kDa aminopeptidase from *H. virescens* (Gill *et al.*, 1995). Unfortunately N-terminal blockage prevented confirmation of this result. Attempts to deblock the N-terminus are required to confirm the identity of this protein. Failing this, further purification and digestion of the protein to yield smaller polypeptides for sequencing would provide amino acid sequence data. This information alone may be sufficient for characterisation and, if not, oligonucleotide primers designed against amino acid sequence data could be used to amplify the gene from the *L. oleracea* cDNA gut tissue library.

Attempts to characterise one of the 5 GNA binding BBM glycopolypeptides in *L. oleracea* were unsuccessful. Purification of the 98 kDa GNA binding protein in solubilised BBMV preparations was achieved by ion exchange chromatography (Figs 6.1. and 6.2.). Amino acid sequence data was obtained from V8 and trypsin digest polypeptides (Figs 6.4. and 6.5., respectively), allowing oligonucleotide primers to be designed. Subsequent attempts to amplify (by PCR and RT-PCR) fragments containing coding sequence corresponding to the 98 kDa protein sequence data obtained by amino acid sequencing were unsuccessful. That the clones were genuine was indicated by the significant homology of all 7 clones to known insect genes. Failure to obtain positive clones was largely attributed to the considerable degeneracy of the oligonucleotide primers which resulted both from degeneracy of the genetic code and ambiguities in the amino acid sequence data from which the primers were designed (Fig 6.6 and 6.7.). The low abundance and relatively large size of the protein further reduced the chances of the primers annealing to the correct gene using PCR and RT-PCR techniques. Additional amino acid sequence information would improve chances of success. Screening of the cDNA library with labelled oligonucleotide primers is another method that could be employed to obtain putative positive clones.

7. RESULTS AND DISCUSSION.

A comparison of the short and long term effects of GNA and Con A on the activities of soluble and BB enzymes of *L. oleracea* .

Experiments were carried out to establish whether the observed insecticidal effects of GNA and Con A were, at least in part, attributable to lectin induced disruption of the digestive capacity of the larval midgut of *L. oleracea*. To this end the effects of GNA and Con A at 2.0 % total protein, upon soluble endoprotease trypsin and membrane-bound exoprotease aminopeptidase activities were examined. This work has been published and is enclosed, see: Fitches E. & Gatehouse J.A. (1998) A Comparison of the Short and Long term Effects of Insecticidal Lectins on the Activities of Soluble and Brush Border Enzymes of Tomato Moth larvae (*Lacanobia oleracea*). (Journal of Insect Physiology Vol 44, No 12, 1213-1224). Trypsin-like proteases are major digestive enzymes in lepidopteran larvae (Bown *et al.*, 1997). Aminopeptidase is known to be a major component of BBM's and was previously identified as a major Con A binding species in *L. oleracea* (Chapters 5 and 6). Alkaline phosphatase, a midgut microvillar membrane marker in Lepidoptera, was also assayed to compare GNA-induced changes in *L. oleracea* with those in rats (Pusztai *et al.*, 1996). Finally, α-glucosidase was assayed to give an indication of possible effects of the lectins upon larval sugar metabolism. To examine short term effects on nutritional performance and gut enzyme activity 5th instar larvae were fed for 24 hr on lectin containing diets and sampled as described in section 3.4.4. Chronic effects were examined, as described in section 3.4.7., after feeding larvae from hatch for 16 days. To investigate whether metabolic disruption (as assessed by changes in enzyme activity) was related to the degree of lectin binding in the larval gut *in vivo* levels of lectins in the guts of chronically exposed larvae were analysed by western blotting.

7.1.Short term assay 1 - Nutritional data.

Larvae in the second day of the 5th instar were transferred to experimental diets, and exposed to the treatments for 24 hr. Under these conditions, the inclusion of lectins at 2.0 % total protein in AD had no effect on larval growth. After 24 hr, increases of 58 %, 58 % and 55 %, in mean wet weight were recorded for control larvae, and larvae fed on diets containing ConA and GNA, respectively. Fig 7.1. (A) shows that neither lectin exerted a short term depression of larval feeding. On the contrary, lectin-fed larvae consumed a greater amount of diet than control larvae. Differences for larvae fed on Con A-containing diet were not significant, but both mean consumption and faecal production over the 24 hr assay (Fig 7.1. (A)) were significantly greater (approximately 10 %) for larvae fed GNA compared to larvae fed control diet. From consumption values, estimates for mean lectin ingestion per larvae were 147 μg and 135 μg for GNA and Con A, respectively.

The mean total protein content of faecal extracts was similiar for control, GNA and Con A-fed larvae (0.296+/-0.081 mg, 0.285+/-0.093 mg and 0.290+/-0.087 mg,

respectively). However, Fig 7.1. (B) shows that control larvae produced faecal matter that contained a higher concentration of protein than that produced by lectin fed larvae. This difference was greatest for GNA samples which exhibited significantly lower mean protein levels per mg dry weight of frass (by approx. 15 %) compared to controls. The lower faecal protein concentrations for lectin-fed insects was reflected in higher gut protein levels. Fig 7.1. (C) shows that GNA and Con A both induced significant increases (approx. 15 % and 10 %, respectively) in the mean total protein content of crude gut extracts. The effect was again greatest for GNA-fed larvae, which exhibited significantly greater mean gut protein levels than both control and Con A-fed larvae.

7.2. Short term effects of lectins on digestive enzyme activity.

Differences in aminopeptidase, trypsin, α-glucosidase and alkaline phosphatase activities per gut, and per μg gut protein, between the control and lectin treatments are shown in Fig 7.2. Treatment with GNA and Con A significantly elevated total aminopeptidase activity, both in terms of total activity per larval gut (by 40 % and 45 % for GNA and Con A, respectively) and activity per μg gut protein (by 20 % and 33 % for GNA and Con A, respectively). Similarly, both lectin treatments resulted in elevated levels of trypsin activity per gut (13 %; GNA; not significant and 38 %; Con A; significantly different to GNA and control). The Con A-fed group also had significantly higher trypsin content on a per μg gut protein basis. Fig 7.3. shows that elevated levels of trypsin activity (total pmols per min) in faecal material were also observed. Lectin-fed insects had approximately 20 % more activity than controls. If expressed as mean activity per μg of faecal protein, this difference was significant for larvae fed GNA, but not for those fed Con A.

Whereas both protease activities that were assayed showed up-regulation by lectin treatments, results for the other digestive enzymes assayed were not as clear cut. GNA, but not Con A, induced a significant increase in α-glucosidase activity per gut (Fig 7.2.), but this difference was negligible when expressed as activity per μg gut protein due to the higher gut protein content in GNA-fed larvae. Although slightly elevated alkaline phosphatase activities (Fig 7.2.) were observed for both lectin groups, differences with the control group were not significant.

To ensure that the presence of lectins did not directly interfere with enzyme activity, 10 control samples were incubated for 15 mins with GNA and Con A (at a concentration equal to that incorporated in the experimental diet, i.e 0.6 mg/ml) and assayed for enzyme activity. No difference in enzyme activity between control and control plus lectin samples was recorded (results not presented, typical ANOVA $p = 0.9346$, $n = 10$).

7.3. Lectin recovery in the faeces of short term fed larvae.

The presence of GNA and Con A in proteins extracted from the faeces of a subset of short term fed larvae was shown by western blotting. Results are presented in Fig 7.4.. Levels of GNA and Con A in the faeces of lectin-fed larvae (estimated visually) were

similar. Both lectins accounted for approximately 5 % of total faecal proteins. Since not all of the ingested lectin had been fully processed by the larvae at the time of sampling it was not possible to obtain estimates for the total percentage of lectin recovered in faecal samples.

7.4. Northern blot assay for trypsin mRNA levels.

An identical short term assay, as described in section 3.4.6., was carried out to investigate if the observed increases in gut trypsin activity of lectin fed larvae were attributable to the upregulation of trypsin expression at the level of transcription. Northern blot hybridisation was used to compare the levels of mRNA species encoding trypsin in total RNA extracted from guts of larvae fed for 24 hr on control and lectin-containing diets. Five total RNA samples per treatment were transferred to nitrocellulose and probed with two different *H. armigera* trypsin cDNA clones. Clone SR36 (849 bp) has been shown to be upregulated in response to feeding *H. armigera* larvae on diet containing soybean Kunitz trypsin inhibitor (SKTI) whereas clone HaTC16 (890 bp) is downregulated (Bown *et al.*, 1997). As shown in Fig 7.5., both clones detected mRNA species of approx. 1.6 kb in gut RNA, but no difference was observed in the intensity of hybridisation (confirmed by densitometry) to RNA extracted from control and lectin-fed larvae. These results show that the increase in trypsin activity observed as a result of feeding lectins was not a result of specific increases in the levels of expression of trypsin genes.

7.5. Long term assay - Nutritional data.

Fig 7.6. shows that when lectins (at 2.0 % of total protein) were fed to *L. oleracea* larvae from hatch for 16 days both GNA and Con A caused a significant reduction in mean larval, and larval gut wet weights. Larvae fed on diets containing GNA and Con A exhibited a 66 % and 50 % reduction in larval weights, respectively, when compared to controls. These results were comparable to those obtained in preliminary bioassays presented in Chapter 4. However, no effect of Con A upon larval survival was observed in this assay. The reason for which remains unexplained. The only differences in assay procedures were the maintenance of larvae in individual pots (as opposed to 5 larvae per pot in this assay) and daily handling of larvae in the preliminary assay. Analysis of mean gut to larval wet weight ratios gave values of 0.157 (+/-0.028) for control, and 0.161 (+/-0.042), and 0.179 (+/-0.044) for GNA and Con A treatments, respectively. The ratio for the Con A treatment was significantly greater than the control group suggesting that this lectin caused enhanced growth of the gut relative to total larval size. In agreement with the decrease in larval size, the total gut protein content of lectin-fed larvae was significantly decreased when compared with control-fed insects (data not presented). When expressed as a fraction of gut weight, no significant differences in protein content between treatments were observed. Estimated values for mean mg protein per mg gut wet weight were 0.0523 +/-0.0186, 0.0589 +/-0.0174 and 0.0538 +/-0.0160 for control, GNA and Con A treatments, respectively.

7.6. Long term effects of lectins on digestive enzyme activity.

Differences in aminopeptidase, alkaline phosphatase and α-glucosidase activities per gut, and per μg protein, between the control and lectin-fed larvae in the long-term feeding experiment are shown in Fig 7.7. Since gut contents were removed from these insects, trypsin activity could not be assayed. The retarded growth of lectin-fed larvae is reflected by the significantly higher activity per gut observed for the control group with all three enzymes. However, when expressed as activity per μg protein, both lectin treatments caused increases in aminopeptidase and alkaline phosphatase activities of approximately 10-15 % relative to control extracts, although the increases were not statistically significant. In contrast to results in the short-term feeding experiments, treatment with GNA and Con A induced a significant reduction (approximately 20 %) in α-glucosidase activity per μg gut protein.

7.7. "In vivo" levels of lectin in chronically exposed larvae.

The presence of GNA and Con A, bound to polypeptides extracted from gut tissues of a subset of larvae chronically exposed to the lectins was shown by western blotting. Results are presented in Fig 7.8. Since the PM and gut contents were removed from these insects, the detection of lectins in these blots represents protein bound to gut walls. Densitometry of the blots was used to estimate approximate amounts of lectins present in 5 μg gut protein (n=5). Total gut protein estimates were then used to give mean bound lectin amounts per gut of 0.81 +/-0.39 μg and 6.51 +/-3.86 μg for GNA and Con A, respectively.

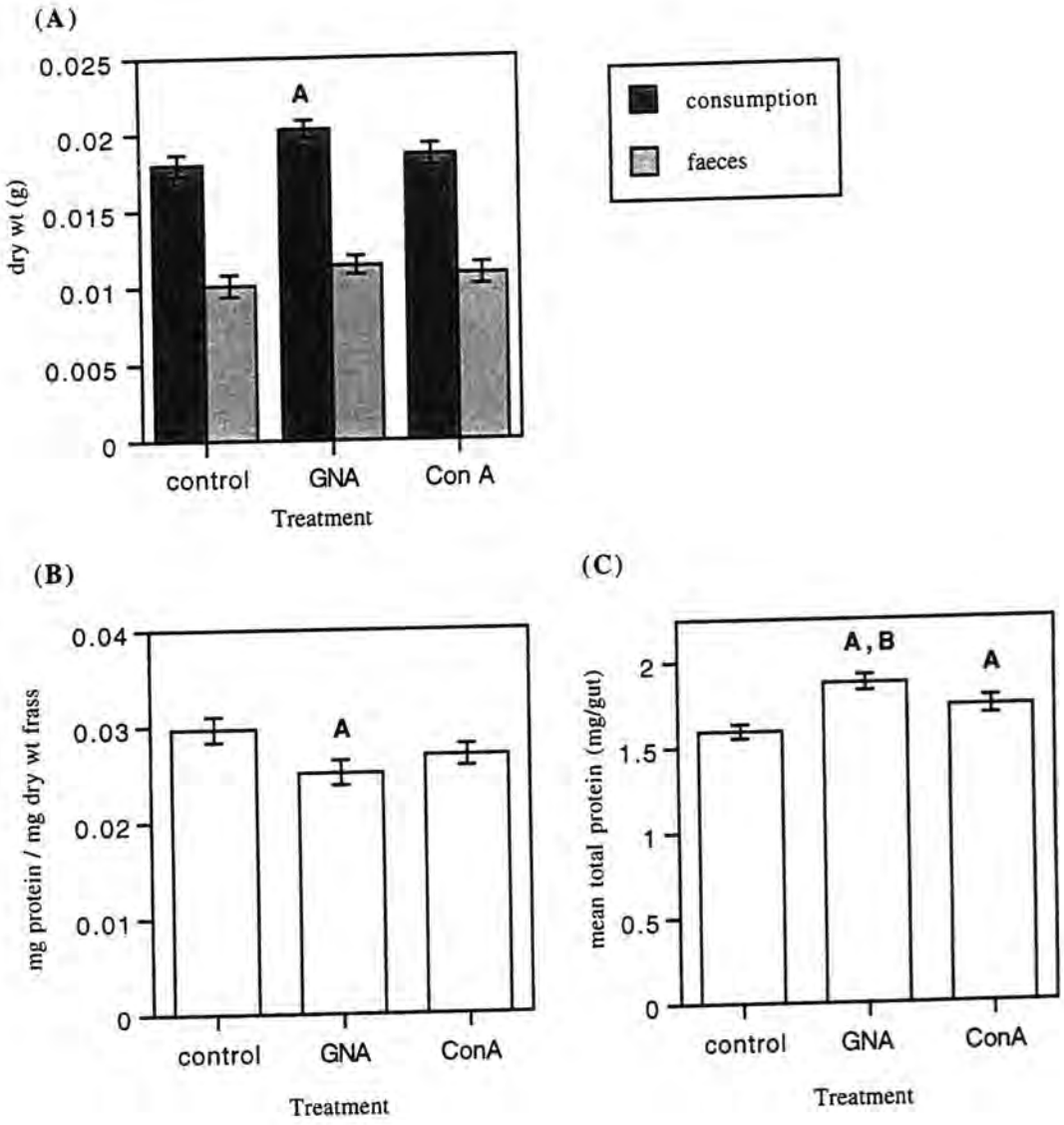


Fig 7.1. Physiological parameters measured for *L. oleracea* larvae in a short term lectin feeding trial. Larvae were fed for 24 hr on control diets, or diets containing GNA or Con A (2 % total protein). Bars show means +/- s.e.; A denotes a significant difference between the control and GNA fed treatments; B denotes a significant difference between the GNA and Con A treatments (ANOVA, n=30).

(A) Diet consumption and faecal production by larvae (mg dry wt).

(B) Protein content of faeces collected from larvae.

(C) Total protein in guts plus contents (mg/gut).

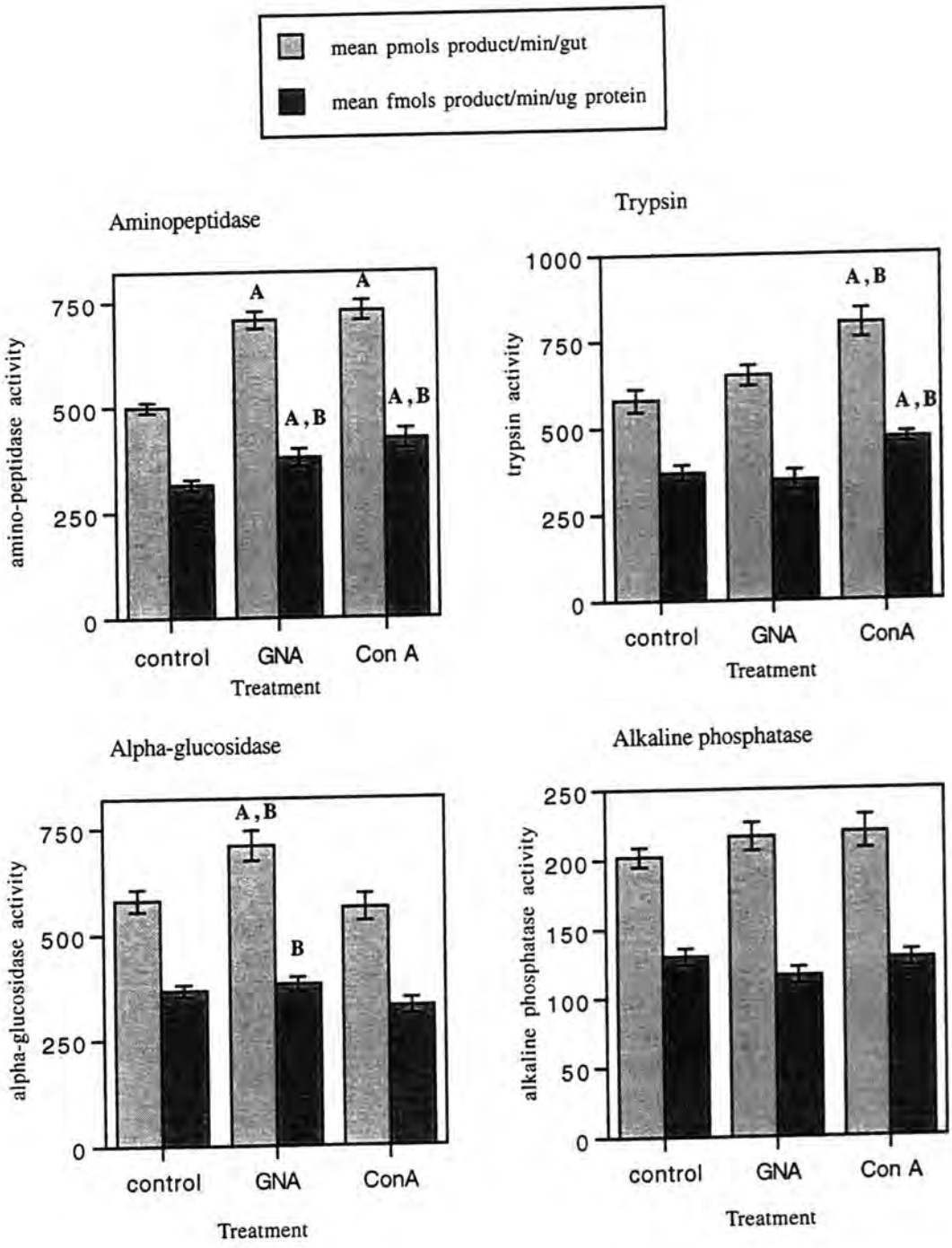


Fig 7.2. Digestive enzyme activities in *L. oleracea* larvae after exposure to lectins in the short term feeding trial. Larvae were fed for 24 hr on control diet or diets containing GNA or Con A (2.0 % total protein). Enzyme activities are expressed as mean of total pmols of product per min per gut, and mean fmols of product per min per μg protein. The products were p-nitroanilide for aminopeptidase and trypsin assays, and p-nitrophenol for alpha-glucosidase and alkaline phosphatase assays. Bars indicate means +/- s.e.; A denotes a significant difference between control and lectin treatments, and B a significant difference between the lectin treatments (ANOVA, n=30).

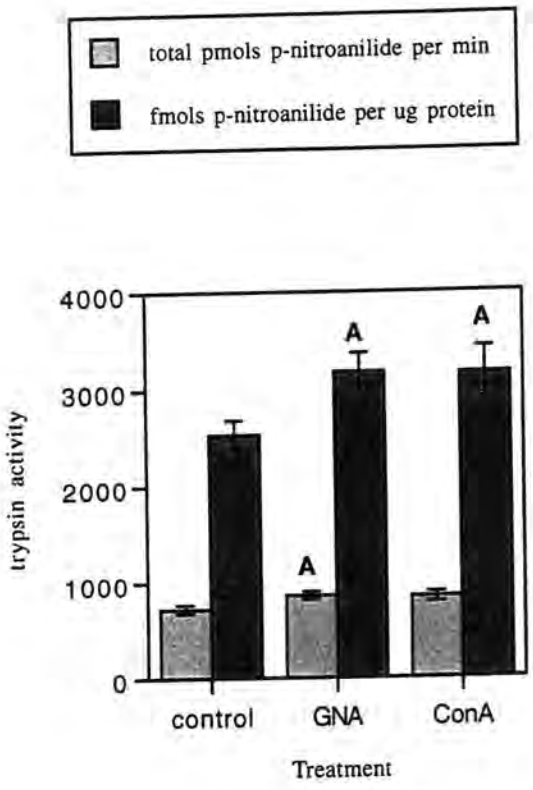


Fig 7.3. Trypsin activities in faeces collected from *L. oleracea* larvae after exposure to lectins in the short term feeding trial. Larvae were fed for 24 hr on control diet or diets containing GNA or Con A (2.0 % total protein). Trypsin activity is expressed as mean total pmols p-nitroanilide produced per min, and mean fmols p-nitroanilide produced per min per μg of faecal protein. Bars indicate means +/- s.e.; A denotes a significant difference between control and lectin treatments (ANOVA n=30).

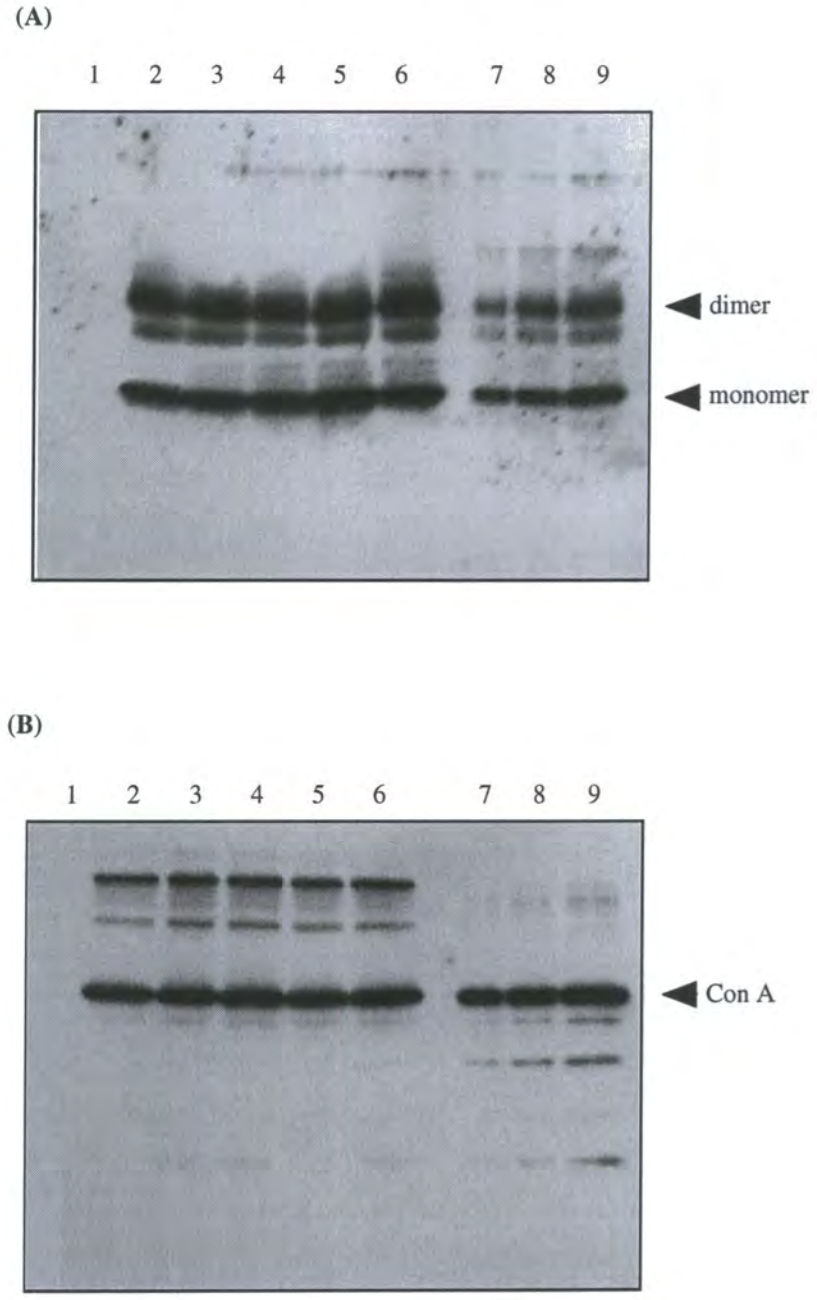


Fig 7.4. Levels of GNA (A) and Con A (B) in the faeces of lectin-fed *L. oleracea* larvae. Proteins were extracted from frass collected from larvae fed for 24 hr on lectin containing diets. Polypeptides were boiled in the presence of 2x SDS sample buffer and 10 % β-mercaptoehanol prior to separation by SDS-PAGE (12 % acrylamide gels). Gels were blotted onto nitrocellulose and probed with anti-GNA or anti-Con A antibodies. Bound antibodies were detected using peroxidase-labelled secondary antibodies followed by treatment with ECL reagents and exposure to X-ray film. 2 μg aliquots of proteins were loaded in all lanes, unless otherwise specified. Loading is as follows (A) lane 1: faecal proteins from insects fed on control diet; lanes 2-6; faecal proteins from insects fed on GNA containing diet; lanes 7, 8,& 9: GNA standards (25, 50 & 100 ng , respectively), (B) lane 1: faecal proteins from insects fed on control diet; lanes 2-6; faecal proteins from insects fed on Con A containing diet; lanes 7, 8 & 9: Con A standards (25, 50 & 100 ng , respectively). Arrows in (A) indicate monomeric and dimeric forms of GNA and in (B) intact Con A polypeptide.

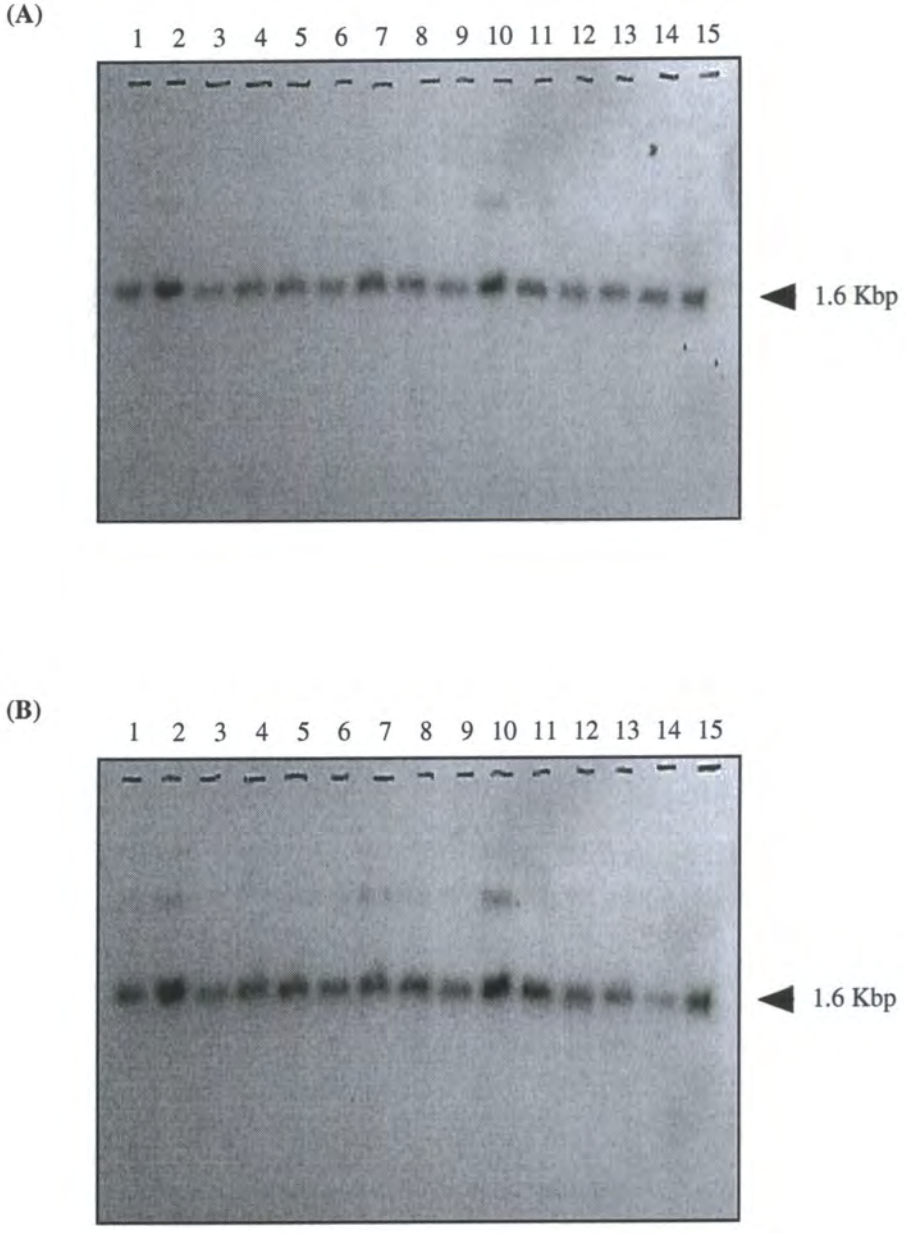


Fig 7.5. Northern blots of total RNA extracted from *L. oleracea* larvae after exposure to lectins in the short term feeding trial. Larvae were fed for 24 hr on control diet, or diets containing GNA or Con A at 2 % of total protein. Blots were probed with *H. armigera* trypsin cDNA clones (A) SB36 and (B) HaTC16 labelled with ^{32} P. Total RNA samples (5 μg per lane) were loaded as follows, lanes 1-5: control-fed larvae; lanes 6-10: Con A-fed larvae; lanes 11-15: GNA-fed larvae.

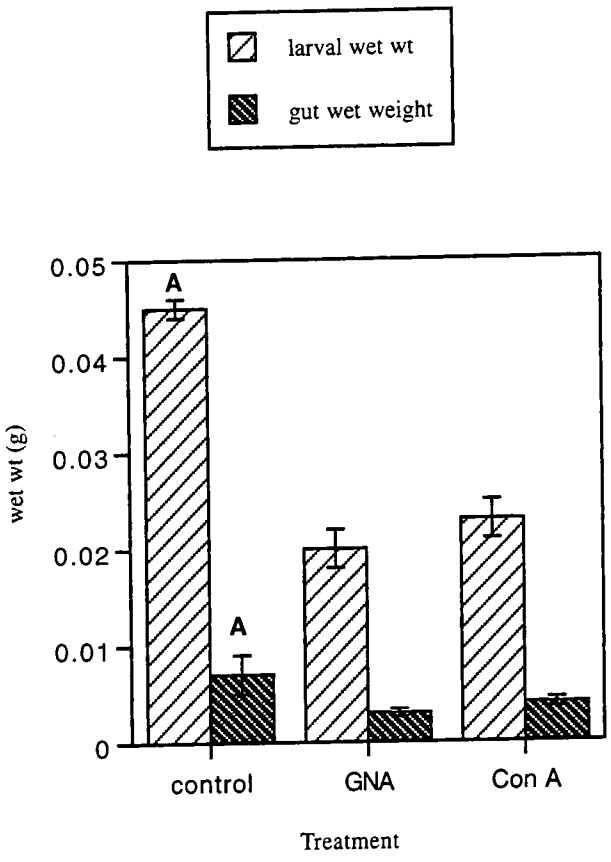


Fig 7.6. Larval weight, and weight of larval gut (after removal of contents) for *L. oleracea* larvae after exposure to lectins in the long term feeding trial. Larvae were fed from hatch for 16 days on control diet, or diets containing GNA or Con A (2.0 % of total protein). Bars indicate means +/- s.e. (control n=27; GNA n=32; Con A n=31). A denotes a significant difference between the control and lectin-fed treatments (ANOVA).

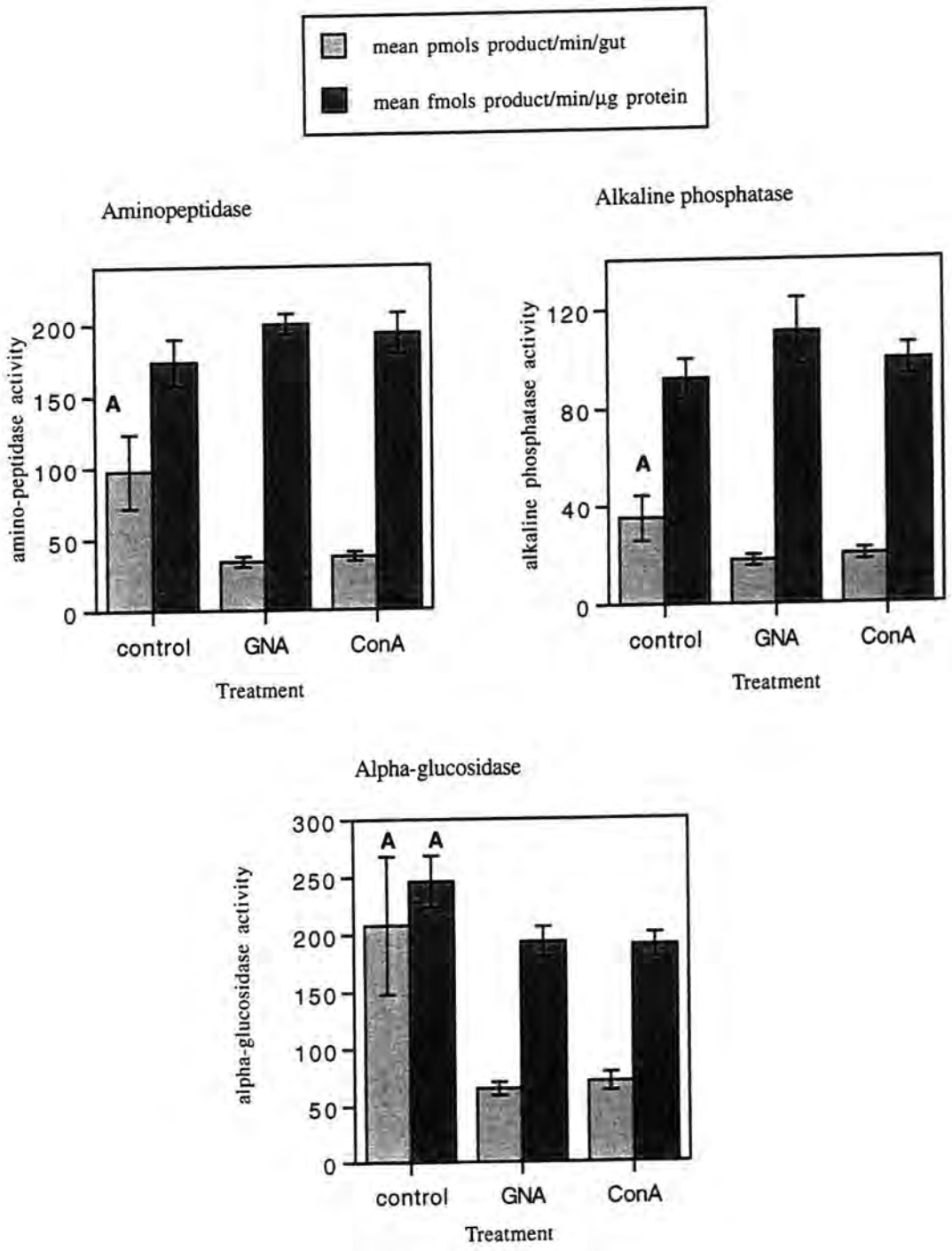


Fig 7.7. Digestive enzyme activities in *L. oleracea* larvae after exposure to lectins in the long term feeding trial. Larvae were fed from hatch for 16 days on control diet, or diets containing GNA or Con A (2.0 % total) protein. Enzyme activities are expressed as mean of total pmols product per min per gut, and mean fmols of product per min per µg gut protein. The products were p-nitroanilide for aminopeptidase, and p-nitrophenol for alkaline phosphatase, and α-glucosidase assays. Values for α-glucosidase activity per gut are divided by 10 to allow presentation on the same graph as values per µg gut protein. Bars indicate mean +/- s.e. (control n=27; GNA n=32; Con A n=31). A denotes a significant difference between the control and lectin treatments (ANOVA)

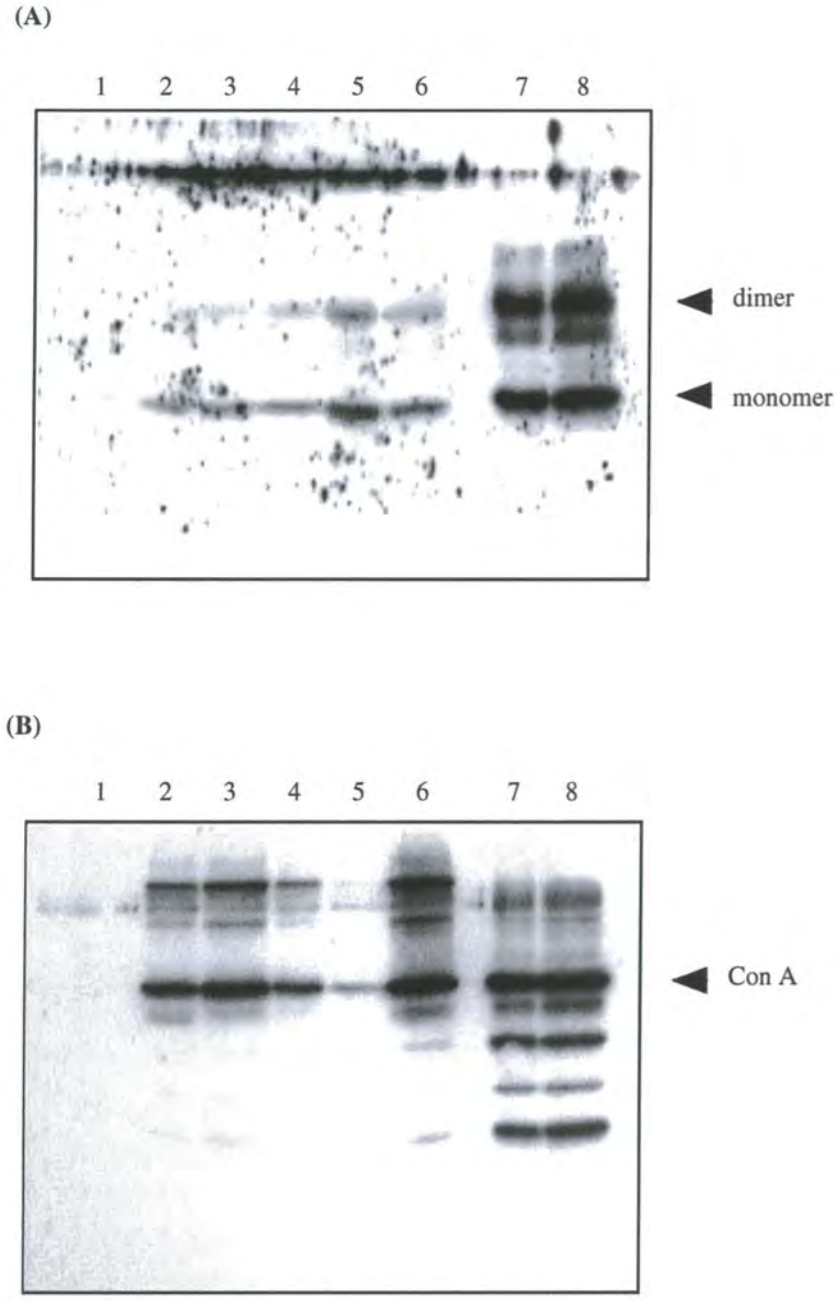


Fig 7.8. Binding of lectins to *L. oleracea* BBM proteins *in vivo*. Total gut proteins (after removal of contents) from insects used in the long term feeding trial were extracted, and boiled in the presence of 2x SDS and 10 % β-mercaptoethanol prior to separation by SDS-PAGE (12 % acrylamide gels). Gels were blotted onto nitrocellulose and probed with anti-GNA or anti-Con A antibodies. Bound antibodies were detected using peroxidase-labelled secondary antibodies followed by treatment with ECL reagents and exposure to X-ray film. 5 μg aliquots of proteins were loaded in all lanes, unless otherwise specified. Loading is as follows (A) lane 1: gut extract from insects fed on control diet; lanes 2-6; gut extracts from insects fed on GNA containing diet; lanes 7 & 8: GNA standards (50 & 100 ng , respectively), (B) lane 1: gut extract from insects fed on control diet; lanes 2-6; gut extracts from insects fed on Con A containing diet; lanes 7 & 8: Con A standards (50 & 100 ng , respectively). Arrows in (A) indicate monomeric and dimeric forms of GNA and in (B) intact Con A polypeptide.

7.8. DISCUSSION.

The effects of lectins on insect metabolism observed in the present study can be compared to effects observed in higher animals. Con A (but not GNA) is a known mitogen for many mammalian cell types, and both Con A, and to a lesser extent GNA, are known to act as growth factors in mammals (Pusztai, 1991). Similar effects are suggested by the increases in total protein per gut (Fig 7.1.(C)) observed in the short-term feeding assay. In addition, the significantly greater mean gut to larval weight ratio in larvae fed on Con A in the long-term feeding assay shows that this lectin stimulates larval gut growth, as it does in mammals. In the long-term feeding assay, GNA-fed larvae exhibited very little difference in relative gut size as compared to control larvae, in agreement with the absence of mitogenic activity observed for this lectin in higher animals. Pusztai *et al.* (1996) found that GNA, at 7% (w/w) total dietary protein, acted as a poor growth factor in mammals, inducing only slight hypertrophy of the small intestine when fed to rats for 10 days.

Con A, which specifically binds α-D-mannopyranoside and α-D-glucopyranoside residues, was previously shown to bind *in vitro* to the majority of larval BBMV and PM proteins (Chapter 5). GNA, which exhibits strict specificity for α(1,3)-and α(1,6)-linked D-mannose residues, bound to only five BBM and 2 PM proteins (Chapter 5). These differences in binding specificity observed for Con A and GNA *in vitro*, which reflect the nature of gut membrane protein glycosylation, were in agreement with the levels of accumulation of lectins in larval gut tissues after feeding the proteins *in vivo* (Fig 7.8.); i.e. higher levels of Con A *in vivo* correlated with the greater number of gut proteins to which Con A binds *in vitro* .

Whilst all larvae in the short term assay exhibited a similiar gain in wet weight, GNA and Con A induced an increase in consumption, and faecal production (Fig 7.1 (A)), and a reduction in mean faecal protein concentration (Fig 7.1. (B)). The lack of correlation between consumption and growth (weight gain of larvae) indicated that both lectins have an almost immediate negative impact upon larval nutrition. The long term feeding assay confirmed this conclusion, and shows that the negative effects are cumulative over the early stages of larval development (up to 4th instar). Larvae fed for 16 days on GNA and Con A-containing diets exhibited a 66 % and 50 % reduction in wet weights, respectively.

Although the lectins had a negative effect on insect growth, in the short term both lectins stimulated an increase in BBM enzyme and soluble trypsin activities. However, assays carried out on gut extracts from larvae in the long-term feeding experiment suggested that the lectin-induced stimulation of BBM enzyme activities observed in the short term is not maintained upon longer exposure to either lectin. Although gut extracts from GNA and Con A-fed larvae in this assay exhibited higher aminopeptidase and alkaline phosphatase activities (per μg gut protein) than controls, differences (<20 %) were not

significant. Pusztai *et al.* (1996) found activities of BBM aminopeptidase and alkaline phosphatase to be significantly increased in rats fed for 10 days on GNA-containing diet (7 % (w/w) protein). Larvae exposed to lectins showed reductions of α-glucosidase activity in the long term. Pusztai (1996) similarly observed a reduction of almost 50 % in BBM sucrase-isomaltase activity in rats exposed to GNA for 10 days. The causes, or physiological consequences, of these changes in BBM enzyme activities caused by the lectins are not clear. The increase in aminopeptidase activity may be a result of lectin binding to this enzyme. Both lectins have previously been shown to bind to BBM aminopeptidase (Chapter 5), with Con A binding very strongly, and GNA binding comparatively weakly.

The effects of lectins on the soluble trypsin activity in the short term assays suggest that both lectins induce a significant increase in levels of trypsin in larval guts. GNA increased trypsin activity in the faeces more than in the gut. This suggested that GNA may affect the recycling mechanisms for this enzyme more than Con A. The presence of GNA and Con A in the faeces of short term fed larvae (Fig 7.4.) confirmed that both lectins were resistant to proteolysis. Thus the elevated gut protein levels observed in the short-term feeding assay may partially reflect an accumulation of lectin bound to larval gut tissues, which in turn could lead to induction of trypsin activity. Numerous studies have shown a strong correlation between gut protein content and luminal proteinase levels in insects (Lehane, 1977; Houseman *et al* ., 1985; Billingsley and Hecker, 1991), leading to suggestions that secretagogue/prandial mechanisms may be operating (Lehane *et al* ., 1995). Interestingly, Blakemore *et al.* (1995) showed trypsin secretion in *Stomoxys calcitrans* to be stimulated by a range of soluble proteins that included the lectin WGA (wheat germ agglutinin). However, neither Con A nor GNA caused an increase in trypsin activity by stimulating up-regulation at the transcriptional level (Fig7.5.). Similarly, Gatehouse *et al.* (1997) found that changing the protein level of diet did not affect trypsin mRNA levels of polyphagous *H. armigera* larvae. These results are not necessarily inconsistent with the prandial mechanism of enzyme regulation, as feeding has been shown to effect control of digestive enzyme synthesis at either the transcriptional or translational level (Lehane *et al.*, 1995). The absence of correlation between gut protein content and the activities of epithelium bound aminopeptidase (Houseman and Downe, 1983; Schneider *et al* ., 1987; Billingsley and Hecker, 1991), or α-glucosidase (Billingsley & Hecker, 1991), has led authors to conclude that these enzymes are not controlled by the same secretagogue mechanism as extracellular proteases.

The disruption of rat gut epithelial cell morphology following exposure to various lectins has been well documented (Pusztai, 1991). Similarly, in this study, lectin binding may have indirectly affected enzyme regulatory mechanisms as a consequence of perturbation of the PM and/or BBM environment. If the lectins were acting solely as growth factors, it might be expected that all assayed enzymes would show a significant increase in activity, which was not the case. A change in the membrane environment and consequent disruption of enzyme recycling mechanisms may provide an alternative

explanation for the observed increases in the tryptic activity of faecal extracts collected from both GNA and Con A fed larvae.

8. RESULTS AND DISCUSSION.

Identification of GNA and Con A in the haemolymph of larvae exposed to lectin containing diets. Investigating the potential for systemic effects upon *L. oleracea.*

Studies in rats orally exposed to the toxic lectin PHA have shown that the lectin not only binds to gut epithelial cells but is endocytosed and subsequently exocytosed through basolateral membranes into the systemic circulation (Pusztai, 1991). As a result a powerful and selective humoral response of the IgG type to the dietary lectin occurs. In the light of this evidence several experiments were carried out to establish whether a similiar mode of transport might occur in *L. oleracea* . Fourth instar larvae exposed to control AD, and GNA and Con A containing AD (2.0 % total protein) for 5 days were sampled as described in section 3.4.8. Individual haemolymph samples were analysed for the presence of lectins by western blotting. Haemocyte counts were recorded to establish if either lectin under these conditions had an effect on the abundance of non-tissue associated haemocytes. The potential for further indirect mechanisms of lectin action was previously indicated by *in vitro* analysis which demonstrated both GNA and Con A were able to bind to glycopolypeptides extracted from malpighian tubules and fat bodies (Chapter 5). Thus proteins extracted from malpighian tubules, fat bodies, midgut and hindgut tissues dissected from lectin-fed larvae were analysed for the presence of GNA and Con A *in vivo* . Time trials to investigate the dynamics of lectin uptake into the haemolymph were carried out. The relative abundance of GNA in plasma and haemocyte fractions of haemolymph extracted from GNA fed larvae was examined. Finally the ability of haemocytes to bind GNA *in vitro* was investigated using fluorescent microscopy.

8.1. Short term assay - Nutritional data.

Larvae in the 2nd day of the 4th instar were transferred to experimental diets and exposed (as described in section 3.4.8.) to the treatments for 5 days. Under these conditions, the inclusion of lectins at 2.0 % total protein had no effect on larval survival, growth and consumption. Control larvae, and larvae fed on GNA and Con A containing diets exhibited increases of 0.0911 g, 0.0978 g and 0.0989 g, in mean larval wet weight (n=20) , respectively. Control larvae consumed a total 1.106 g (dry wt) of AD. GNA and Con A fed larvae consumed a similiar total 1.166 g and 1.180 g (dry wt) of AD, respectively.

8.2. "In vivo" levels of lectins in larval guts, malphighian tubules, and fat bodies.

The presence of GNA and Con A, bound to polypeptides extracted from midgut, hindgut, malpighian tubules and fat bodies, of larvae exposed to lectins (2.0 % total protein) for 5 days was shown by western blotting. For each treatment 5 replicates (4 larvae per replicate) of each of the aforementioned larval components were analysed for the presence of GNA and Con A by probing western blots with anti-GNA and anti-Con

QA antisera. No major differences, between replicate samples, in the levels of lectin (estimated visually) were observed for each treatment (results not presented). Thus, for ease of presentation, 1 replicate sample for each of the larval components were run on SDS-PAGE and western blotted. The summarised results are presented in Figs 8.1.and 8.2 which demonstrate that both GNA and Con A delivered via AD bind *in vivo* to midgut, hindgut, malpighian tubule, fat body and haemolymph glycopolypeptides. Since the PM and gut contents were removed from the insects, the detection of lectins in the midgut and hindgut samples represented protein bound to the gut BBM. Similarly malpighian tubules and fat bodies were thoroughly washed with buffer to remove contaminating tissues and haemolymph, and thus the detection of lectins in these samples represented protein bound to malpighian tubule and fat body glycopolypeptides. Approximate amounts of lectin present in each larval component were visually estimated. Both GNA and Con A accounted for 0.05 % - 0.1 % of total proteins in midgut and hindgut samples. Similar levels were observed in GNA-fed malpighian tubule extracts (0.05 - 0.1 % total proteins). Con A was less abundant (0.02-0.05 % total proteins) than GNA in malpighian tubule extracts. Con A and GNA accounted for approximately 0.02 - 0.05 %, and 0.01 - 0.02 % of total proteins extracted from fat bodies, respectively. Both lectins were present at similar levels in haemolymph samples where they accounted for 0.02 - 0.05 % of total proteins. These results indicated that both lectins (via binding to glycopolypetides) have the potential to disrupt osmoregulatory functions in *L. oleracea* carried out by malpighian tubules and the hindgut. Similarly both lectins have the potential to disrupt multiple metabolic functions carried out by fat bodies.

8.3. "In vivo" levels of lectins in the haemolymph.

The presence of GNA and Con A in individual haemolymph samples extracted from larvae exposed to lectins (2.0 % total protein) for 5 days was shown by western blotting. GNA was detected in 84 % (n=19), and Con A in 90 % (n=20) of the samples collected. Representative blots are shown in Fig 8.3. Levels of lectins in haemolymph samples were visually estimated. Both GNA and Con A accounted for 0.02 % - 0.05 % of total haemolymph proteins.

8.4. Haemocyte abundance and protein concentration in haemolymph samples.

The abundance of haemocytes in individual haemolymph samples (20 per treatment) extracted from larvae exposed to lectins (2.0 % total protein) for 5 days was estimated using a Neubauer haemocytometer. The protein content of each sample was also determined. Results are presented in Table 8.1. No significant differences were observed in the mean protein concentration of haemolymph samples. However, the density of haemocytes was significantly lower in haemolymph samples extracted from both GNA and Con A fed larvae (ANOVA). This indicated that the presence of lectins, via facilitating a reduction in haemocyte abundance, may have a detrimental effect upon the immune system of lectin-fed larvae.

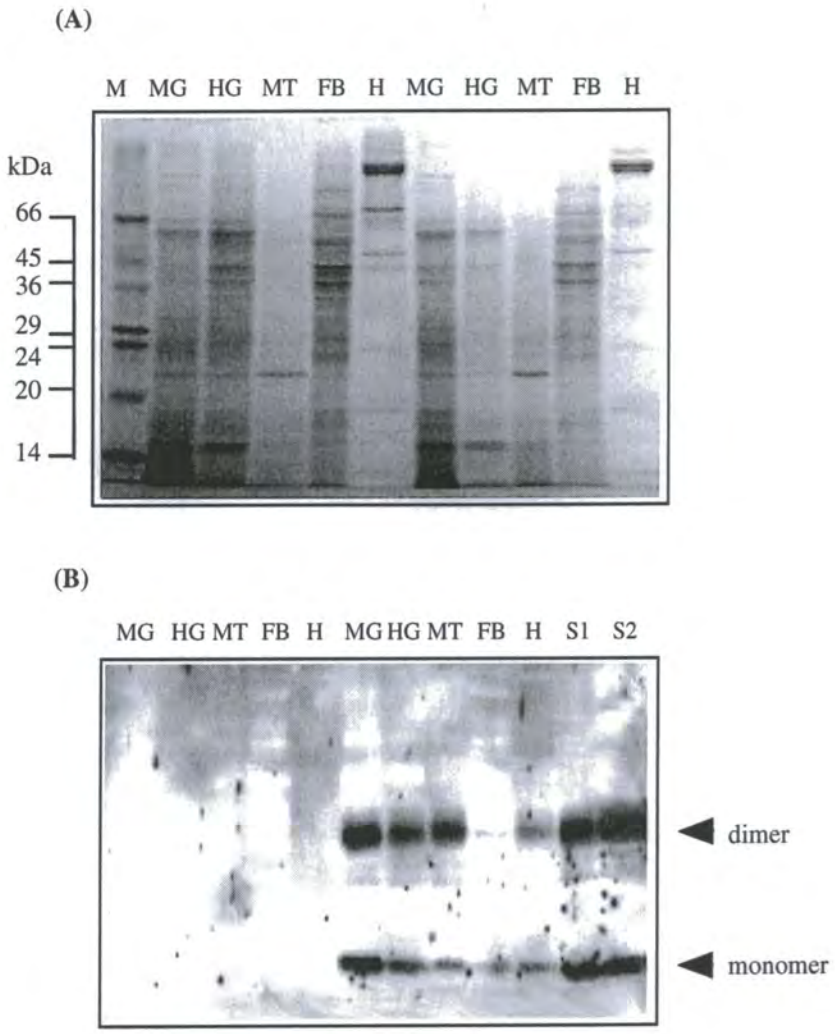


Fig 8.1. Binding of GNA to *L. oleracea* midgut, hindgut, malpighian tubule, fat body and haemolymph glycopolypeptides in vivo. Proteins were extracted from tissues dissected from larvae fed for 5 days on control diet, or diet containing GNA (2 % total protein). Polypeptides were boiled in the presence of 2x SDS sample buffer and 10 % β-mercaptoethanol prior to separation by SDS-PAGE (12 % acrylamide gels). Proteins (20 μg aliquots) in (A) were detected by Coomassie blue staining. Replicate gel (B) (10 μg aliquots) was blotted onto nitrocellulose and probed with anti-GNA antibodies. Bound antibodies were detected using peroxidase-labelled secondary antibodies followed by treatment with ECL reagents and exposure to X-ray film. Loading is as follows: (A) lane 1: (M) molecular weight marker; lanes 2-6 are extracts from control-fed larvae, lane 2: (MG) midgut; lane 3: (HG) hindgut; lane 4: (MT) malpighian tubule; lane 5: (FB) fat body; lane 6: (H) haemolymph; lanes 7-11 are extracts from GNA-fed larvae loaded as described for lanes 2-6. (B) Lanes 1-5: extracts from control-fed larvae, loaded as described for lanes 2-6 in (A); lanes 6-10: extracts from GNA-fed larvae, loaded as described for lanes 2-6 in (A); lanes 11 and 12 are GNA standards (10 & 20 ng, respectively). Arrows indicate monomeric and dimeric forms of GNA .

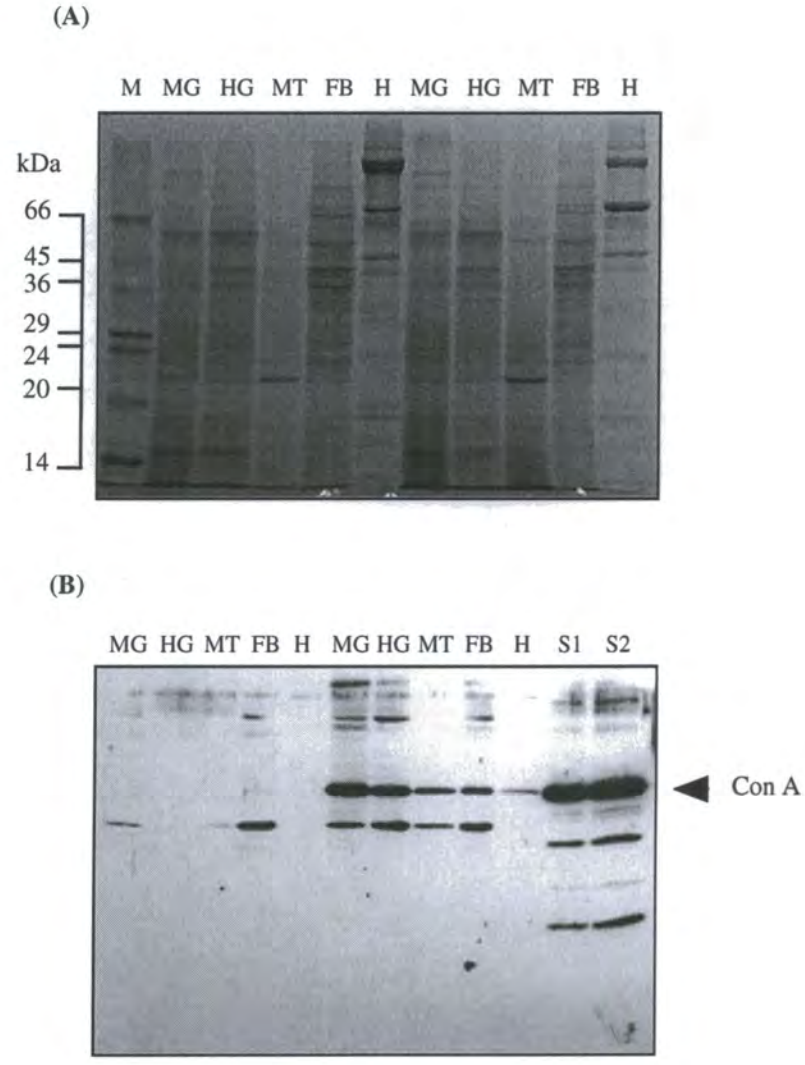


Fig 8.2. Binding of Con A to *L. oleracea* midgut, hindgut, malpighian tubule, fat body and haemolymph glycopolypeptides *in vivo*. Proteins were extracted from tissues dissected from larvae fed for 5 days on control diet, or diet containing Con A (2 % total protein). Polypeptides were boiled in the presence of 2x SDS sample buffer and 10 % β-mercaptoethanol prior to separation by SDS-PAGE (12 % acrylamide gels). Proteins (20 μg aliquots) in (A) were detected by Coomassie blue staining. Replicate gel (B) (10 μg aliquots) was blotted onto nitrocellulose and probed with anti-GNA antibodies. Bound antibodies were detected using peroxidase-labelled secondary antibodies followed by treatment with ECL reagents and exposure to X-ray film. Loading is as follows: (A) lane 1: (M) molecular weight marker; lanes 2-6 are extracts from control-fed larvae, lane 2: (MG) midgut; lane 3: (HG) hindgut; lane 4: (MT) malpighian tubule; lane 5: (FB) fat body; lane 6: (H) haemolymph; lanes 7-11 are extracts from Con A-fed larvae, loaded as described for lanes 2-6. (B) Lanes 1-5: extracts from control-fed larvae, loaded as described for lanes 2-6 in (A); lanes 6-10: extracts from Con A-fed larvae, loaded as described for lanes 2-6 in (A); lanes 11 and 12 are Con A standards (10 & 20 ng, respectively). Arrow indicates intact Con A polypeptide.

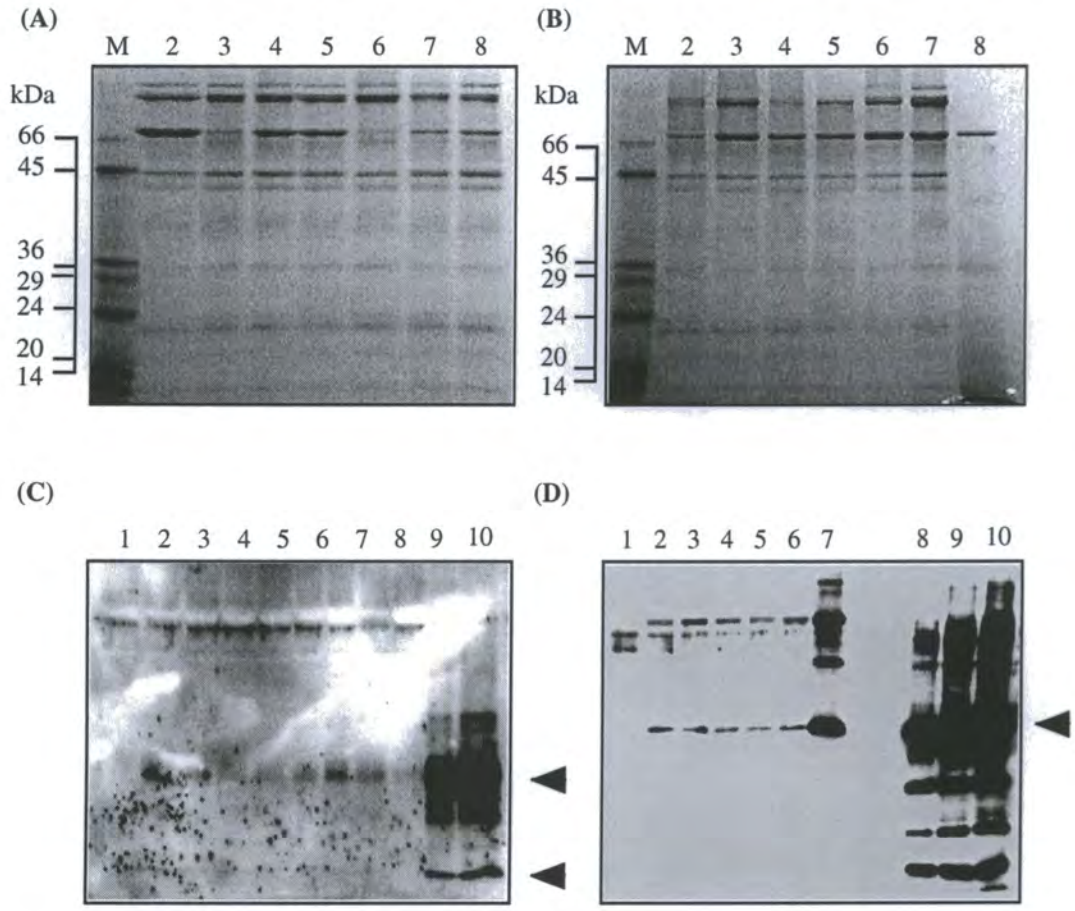


Fig 8.3. Detection of lectins in haemolymph samples extracted from *L. oleracea* larvae fed for 5 days on control diet, or diets containing GNA or Con A (2 % total protein). Aliquots (5 μg protein) of haemolymph were boiled in the presence of 2x SDS sample buffer and 10 % β-mercaptoethanol prior to separation by SDS-PAGE (12 % acrylamide gels). Proteins in (A) and (B) were detected by Coomassie blue staining. Replicate gels (C) and (D) were blotted onto nitrocellulose and probed with anti-GNA and anti-Con A antibodies, respectively. Bound antibodies were detected using peroxidase-labelled secondary antibodies followed by treatment with ECL reagents and exposure to X-ray film. Loading is as follows: (A) and (B) lane 1: (M) molecular weight marker; lane 2: haemolymph extracted from control-fed larvae; lanes 3-8: haemolymph extracted from GNA-fed (A) and Con A-fed (B) larvae; (C) and (D) lane 1: haemolymph extracted from control-fed larvae; lanes 2-7: haemolymph extracted from GNA-fed (C) and Con A -fed (D) larvae; lanes 8, 9 and 10: standards 25, 50 and 100 ng, respectively, for GNA (C) and Con A (D). Arrows in (C) indicate monomeric and dimeric forms of GNA and in (D) intact Con A polypeptide.

8.5. Investigating the dynamics of lectin transport to the haemolymph.

A number of time trials (as described in section 3.4.9.) were carried out to investigate the dynamics of GNA and Con A transport into the haemolymph of lectin-fed larvae. In all cases the presence of lectins in crude haemolymph collected from 5th instar larvae exposed to lectins at 2.0 % total protein in AD was detected by western blotting. Overall GNA was detected in the haemolymph of 50 % of larvae exposed to the lectin for 24 hr (n=84). However, the percentage of positive samples between different assays varied from 21 - 100 %. In one 24 hr assay the inhibitory sugar mannose was incorporated at a concentration of 10 mM in GNA containing AD. No GNA was detected in haemolymph extracted from larvae fed on this diet (n=10). In this assay GNA was detected in 80 % (n=10) of larvae fed GNA (no mannose) containing diet. Similarly no GNA was detected in 20 individual haemolymph samples extracted from larvae fed diet containing GNA that had been boiled for 30 min. Prior to diet preparation the boiled GNA was shown to be non-functional by a microtitre haemagglutination assay (results not shown). These results indicated that GNA uptake was dependant upon GNA binding to gut epithelial cells.

In two time trials haemolymph was extracted from larvae exposed to GNA containing diets for 2, 4, 6 and 12 hr. Larvae were starved for 2 hr prior to the onset of these trials to encourage immediate consumption of the diet. GNA was detected in 71 % (n=14) of larvae exposed to the diet for only 2 hr. However, no positive correlation between uptake and time of exposure to the diet was observed. GNA was detected in 71 %, 21 %, 57 %, and 21 % (n=14 for each sample point) of larvae exposed to diets for 2, 4, 6 and 12 hr, respectively. As time trials were carried out, as much as possible during daylight hours when feeding is sporadic, this variability was partly attributed to variability in the rate of feeding. Alternatively the lectin may be present at levels too low to be detected by western blotting methods.

Although Con A was present in the haemolymph of 90 % of larvae exposed to lectin containing diet for 5 days (section 8.4.) it was was not detectable in any haemolymph samples extracted from larvae exposed to the lectin at the same concentration, for 24 hr. This suggested that, compared to GNA, the uptake of Con A by *L. oleracea* was a significantly slower process. Alternatively the levels of lectin in the haemolymph were too low to be detected by western blotting.

8.6. The relative abundance of GNA in haemocyte and plasma fractions of haemolymph.

The potential for GNA to bind *in vivo* to glycopolypeptides present in plasma and haemocyte components of larval haemolymph was first examined by *in vitro* analysis. Plasma and haemocyte fractions were prepared from haemolymph extracted from 5th instar larvae fed on control diet as described in section 3.4.10. 1 µg aliquots were run on SDS-PAGE (10 % acrylamide gels), western blotted and probed for the presence of GNA binding glycopolypeptides using digoxigenin labelled GNA. Results presented in Fig 8.4. show that both fractions contained GNA binding glycopolypeptides. Of the 5

major GNA binding proteins evident in the plasma fraction 4 were >100 kDa and 1 was approximately 50 kDa. Two high molecular weight GNA binding proteins, of identical size to 2 of the 4 binding proteins in the plasma fraction, were evident in the haemocyte fraction. This indicated that GNA has the potential to bind *in vivo* to both plasma and haematocyte glycopolypeptides in the haemolymph of larvae exposed to lectin containing diet.

The relative abundance of GNA in plasma and haemocyte fractions of haemolymph extracted from 5th instar larvae fed for 24 hr on lectin containing diet was analysed as described in section 3.4.10. Results presented in Fig 8.5. show that both fractions contained GNA. On a per μg protein basis GNA was concentrated in the haemocyte fraction of haemolymph. However, the protein content of the plasma was approximately 20 fold greater than the protein content of the haemocyte fraction. Similiar results were observed on 3 separate occasions. This suggested that most of the GNA transported to the haemolymph of lectin fed larvae would be present in the plasma, with only a small proportion associated with haemocytes.

8.7. GNA binding to haemocytes "in vitro".

In vitro GNA binding to haemocytes was investigated by overlaying cell monolayers with FITC-labelled GNA, as described in section 3.15. These experiments were carried out at CSL, York, under the supervision of Dr E. Richards. Plates 1, 2, and 3, show low power (x 20 objective) fluorescence under UV light of GNA-FITC (5 μg/ml), control FITC (5 μg/ml), and control TBS (no FITC no GNA), overlaid monolayers, respectively. A comparison of the relatively intense staining observed in Plate 1 with that observed in control plates 2, and 3, suggested that GNA binds to haemocytes under these conditions. Relatively weak flourescence in the control Plates 2 and 3 indicated that fluorescence was not a consequence of non-specific interactions of FITC with haemocytes or endogenous haemocyte fluorescence. Plate 4 shows a representative view of a monolayer overlaid with FITC-labelled GNA in the presence of 250 mM mannose under UV at low power (x 20 magnification). The slightly reduced intensity of staining in Plate 4 was indicative of binding inhibition and suggested that GNA may bind to mannose moieties present on the external surface of the cell membrane. Further evidence for GNA binding is provided in Plate 5 which shows a representative high power (x 40 objective) view of the fluorescent staining of haemocytes overlaid with FITC-labelled GNA (5 μg/ml). Binding to the cell membrane and possible endocytosis of the lectin was indicated by the pattern of fluorescence observed in Plate 5 in relation to the same view under phase contrast in Plate 6. A comparison of Plates 5, and 6, with control plates 7 and 8, (which show monolayers overlaid with control FITC), under high power (x 40 magnification) with U.V. and phase contrast, provided further evidence that fluoresence is a consequence of GNA binding rather than non specific interactions of cells with FITC or autoflourescence. Although monolayers overlaid with control FITC exhibited some fluorescence this was associated generally with damaged haemocytes. Plates 9, and 10 show respectively a representative high power (x40 magnification) view under UV and under phase contrast light of monolayers overlain

with FITC-labelled GNA in the presence of 250 mM mannose. The reduced intensity of fluorescence under these conditions, as observed in Plate 4, provided further evidence for binding inhibition and suggested that GNA may bind to mannose moieties present on the external surface of the cell membrane.

Table 8.1. Mean protein concentration and haemocyte density estimates recorded for larvae fed for 5 days on control diet, or diet containing GNA and Con A (2.0 % total protein). * denotes a significant difference between the control and lectin treatments (ANOVA).

treatment	mean protein concentration(µg/µl)	s.e.(+/-)	mean haemocyte density (cells/µl)	s.e. (+/-)	sample no
control	6.27	0.23	6.59×10^4	4.91×10^3	20
GNA	5.84	0.42	5.37×10^4 *	3.60×10^3	19
Con A	6.27	0.27	5.11×10^4 *	4.18×10^3	20

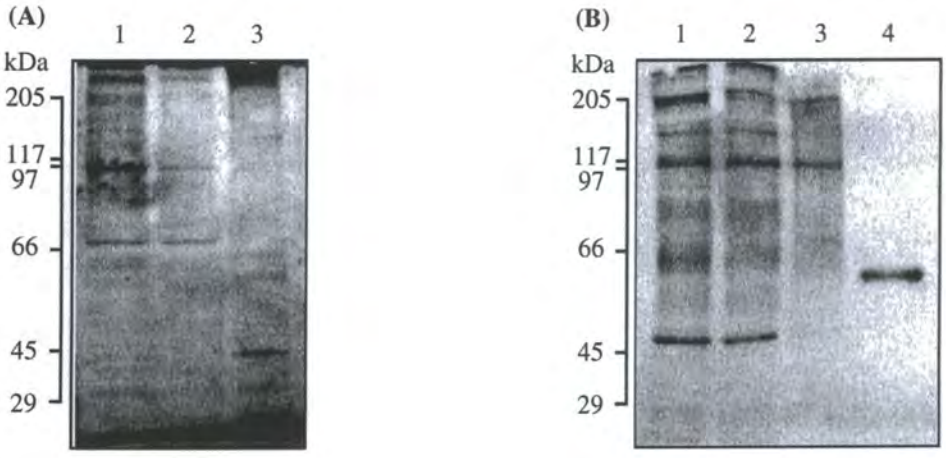


Fig 8.4. Binding of GNA to glycopolypeptides extracted from plasma and haematocyte components of *L. oleracea* larval haemolymph *in vitro*. Crude haemolymph extracts were centrifuged (2,200 x g, for 20 mins at 4 °C). The supernatant constituted the plasma fraction and the pelleted haemocytes were washed 3 x with isotonic PBS buffer prior to resuspension in lysis buffer. Polypeptides were boiled in the presence of 2x SDS sample buffer and 10 % β-mercaptoethanol prior to separation by SDS-PAGE (10 % acrylamide gels). Proteins (1 μg aliquots) in (A) were detected by silver staining. Replicate gel (B) was blotted onto nitrocellulose and probed with digoxigenin-labelled GNA. Bound lectin was detected using alkaline-phosphatase-labelled anti-digoxigenin labelled antibodies, followed by staining with NBT/ X-phosphate. Loading is as follows, (A) lane 1: crude haemolymph extract; lane 2: plasma extract ; lane 3: haemocyte extract; loading for (B) is as described for (A) except that lane 4 is control carboxypeptidase Y (0.5 μg).

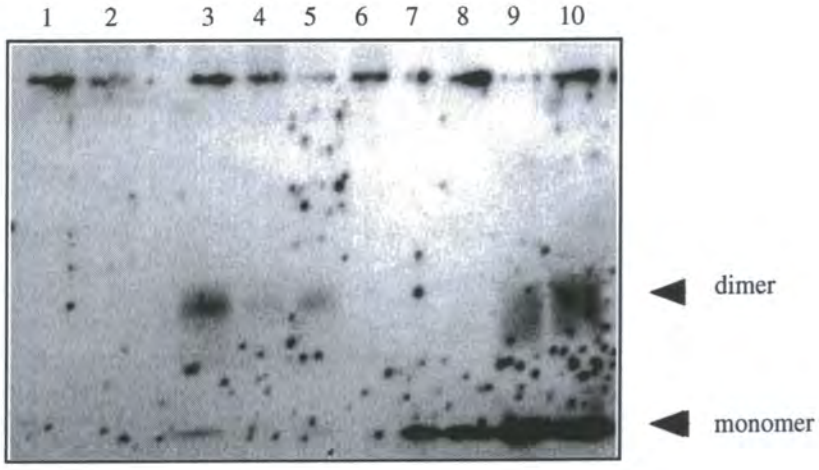


Fig 8.5. Binding of GNA to glycopolypeptides extracted from plasma and haematocyte components of *L. oleracea* larval haemolymph *in vivo*. Samples from 5th instar larvae fed GNA-containing diet (2 % total protein) for 24 hr were prepared as described in Fig 8.4. Polypeptides were boiled in the presence of 2x SDS sample buffer and 10 % β-mercaptoethanol prior to separation by SDS-PAGE (10 % acrylamide gel). The gel was blotted onto nitrocellulose and probed with anti-GNA antibodies. Bound antibodies were detected using peroxidase-labelled secondary antibodies followed by treatment with ECL reagents and exposure to X-ray film. 1 μg aliquots were loaded as follows, lane 1: haemocyte extract from control-fed larvae; lane 2: plasma extract from control-fed larvae; lanes 3 & 5: haemocyte extracts from GNA-fed larvae; lanes 4 & 6: plasma extracts from GNA-fed larvae; lanes 7, 8, 9 & 10 are GNA standards (10, 20, 50 & 100 ng, repectively). Arrows indicate monomeric and dimeric forms of GNA.

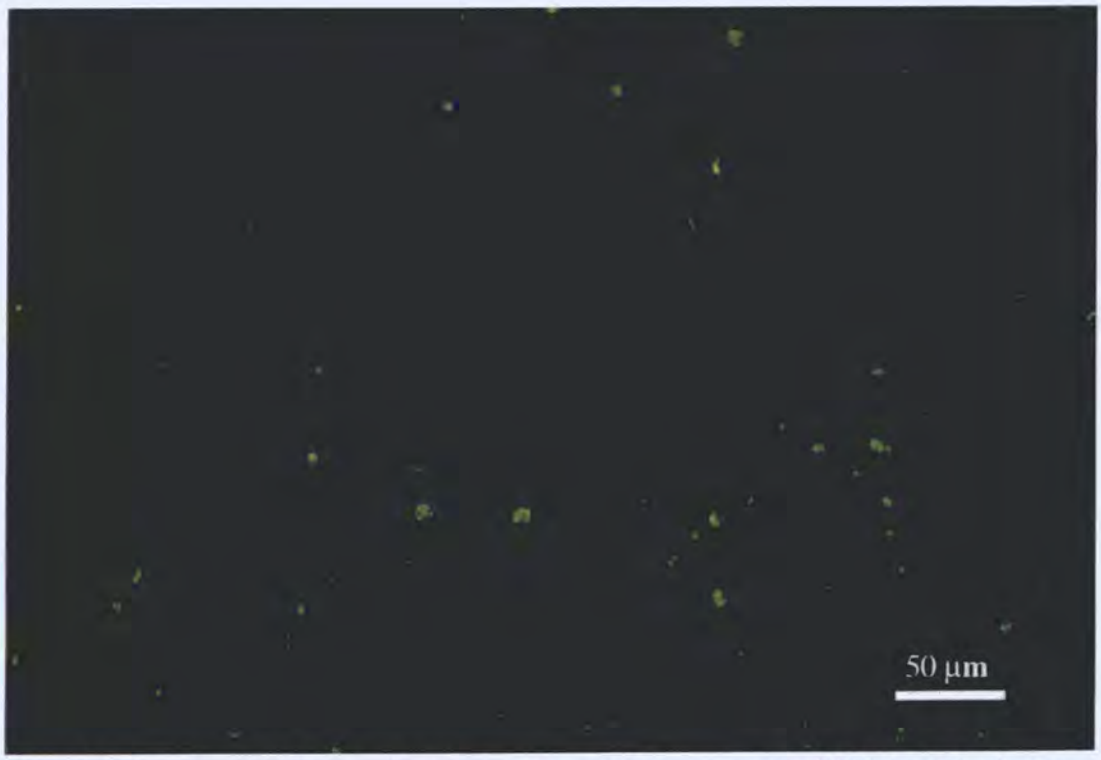


Plate 1: Low power (x 20 magnification) representative view under UV light of *L. oleracea* haemocyte monolayer overlain with FITC-labelled GNA (5 μg/ml).

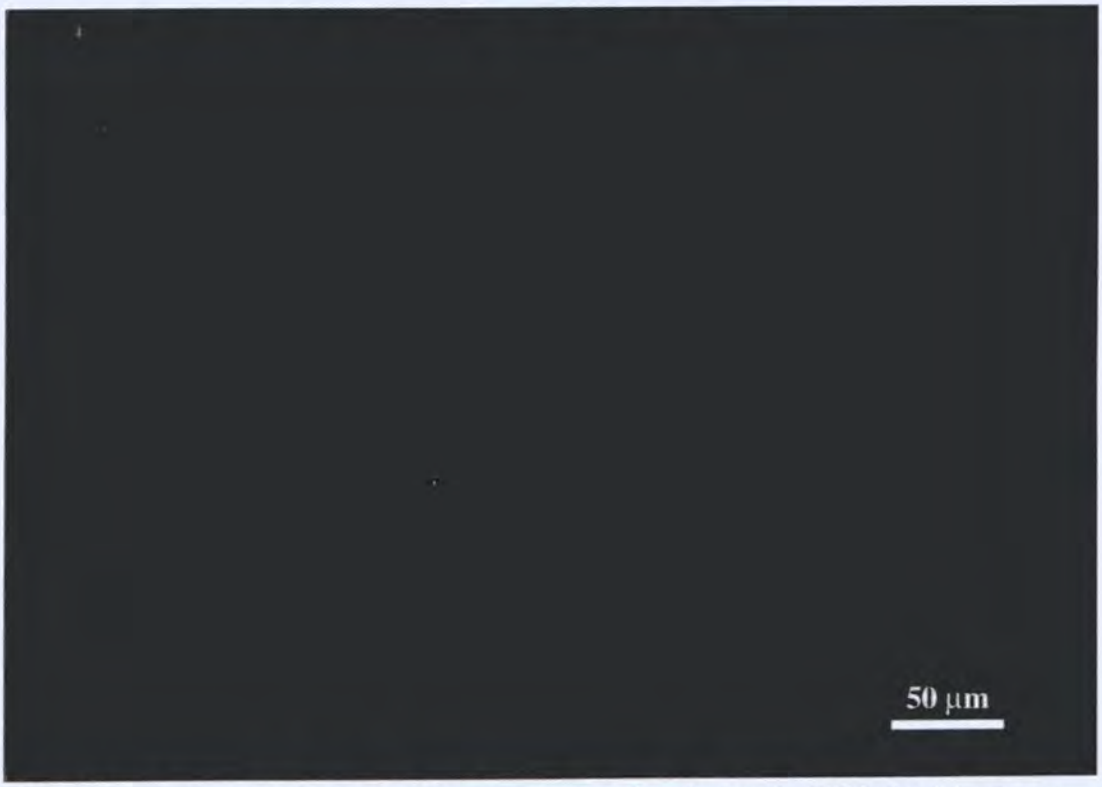


Plate 2: Low power (x 20 magnification) representative view under UV light of *L. olerace* haemocyte monolayer overlain with FITC (5 μg/ml).

Plate 3: Low power (x 20 magnification) representative view under UV light of *L. oleracea* haemocyte monolayer overlain with control isotonic TBS buffer.

Plate 4: Low power (x 20 magnification) representative view under UV light of *L. oleracea* haemocyte monlayer overlain with FITC-labelled GNA (5 μg/ml) in the presence of 250 mM mannose.

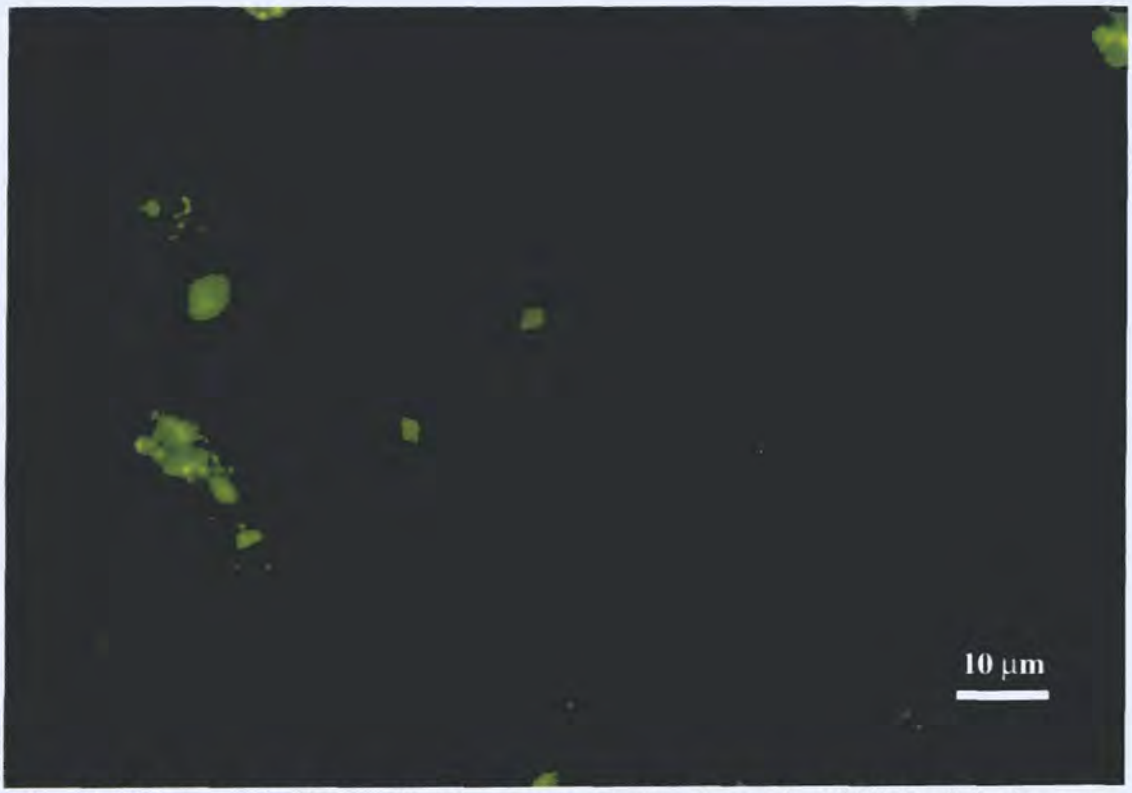


Plate 5: High power (x 40 magnification) representative view under UV light of *L. oleracea* haemocyte monolayer overlain with FITC-labelled GNA (5 μg/ml).

Plate 6: Replicate high power (x 40 magnification) view of plate 5 under phase contrast light.

Plate 7: High power (x 40 magnification) representative view under UV light of *L. oleracea* haemocyte monolayer overlain with FITC (5 μg/ml).

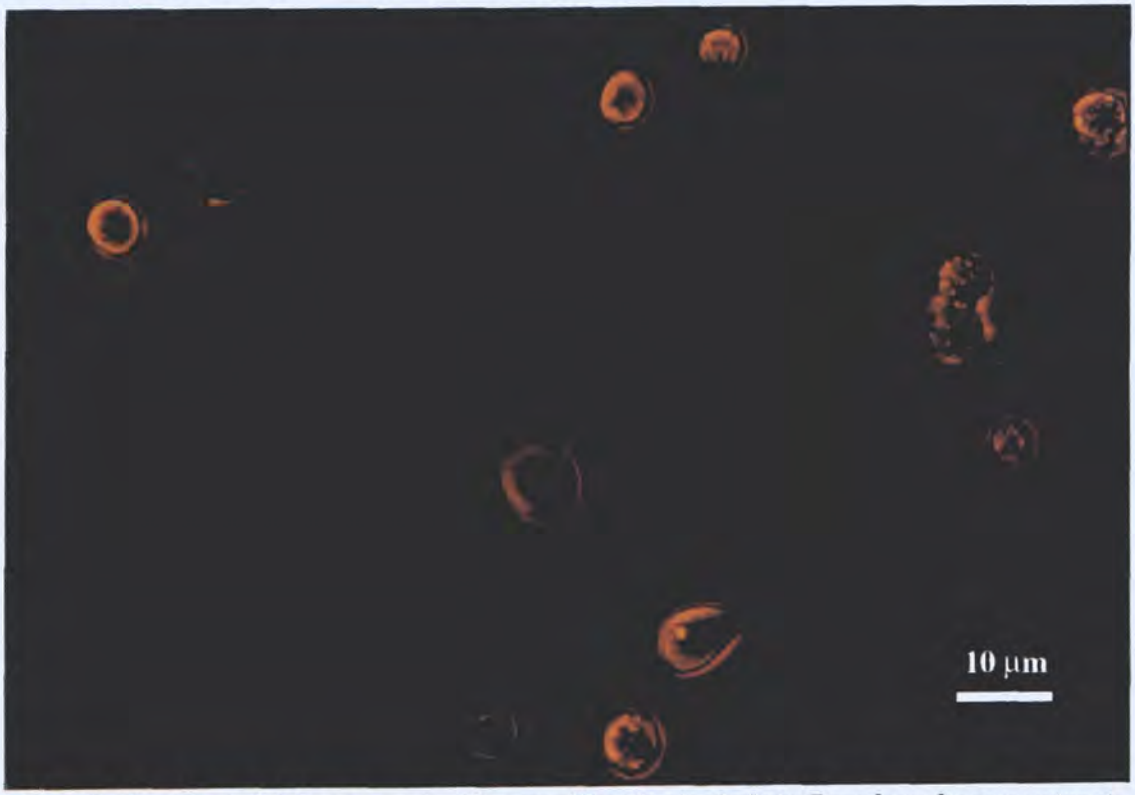


Plate 8: Replicate high power (x 40 magnification) of plate 7 under phase contrast light.

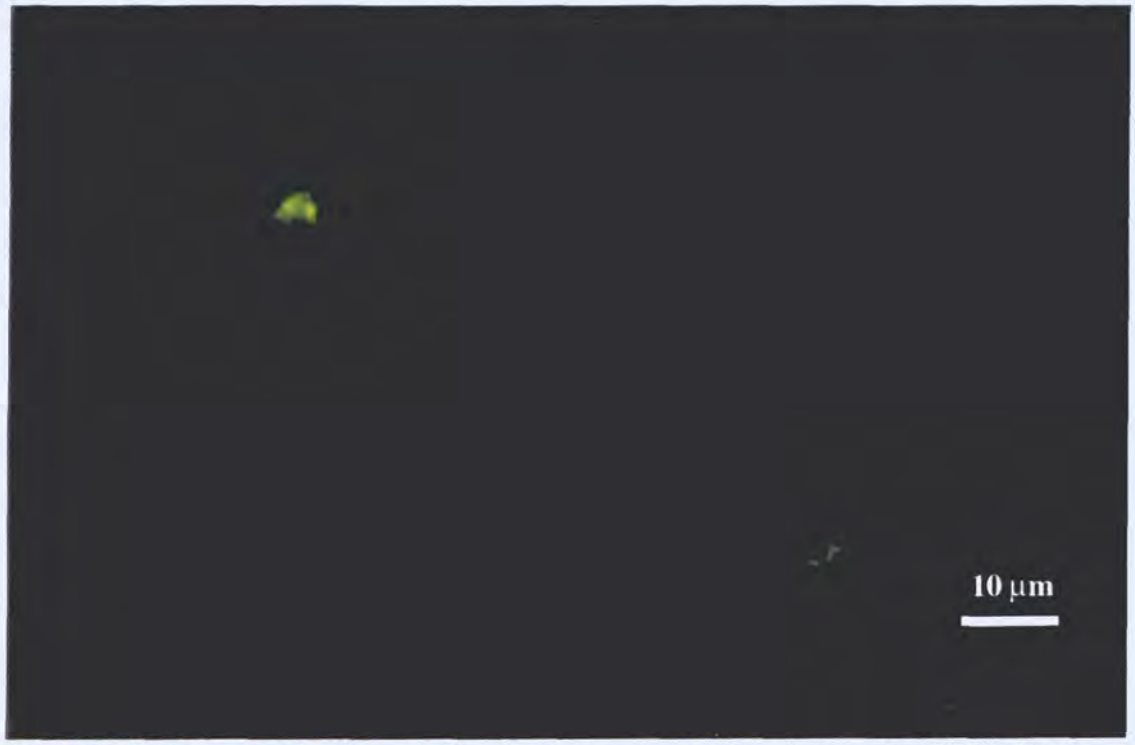


Plate 9: High power (x 40 magnification) representative view under UV light of *L. oleracea* haemocyte monolayer overlain with FITC-labelled GNA in the presence of 250 mM mannose.

Plate 10: Replicate high power (x 40 magnification) view of plate 9 under phase contrast light.

8.8. DISCUSSION.

The potential for GNA and Con A to exert systemic effects upon *L. oleracea* was demonstrated by the detection of both lectins in the haemolymph of larvae exposed to experimental diets for 5 days (Fig 8.3.). That GNA and Con A may act directly upon organs other than the insect gut was similarly indicated by the detection of lectins *in vivo* in malpighian tubule and fat body tissue extracts (Figs 8.1 and 8.2.). Both lectins were most abundant in midgut and hindgut samples where they accounted for approximately 0.05 - 0.1 % of total proteins. Lower levels of GNA and Con A were observed in malpighian tubule, fat body and haemolymph samples. Relatively high levels of lectin in the guts of lectin-fed larvae were in agreement with the gut as the primary target of lectin action.

Lectins, whether of plant or microbial origin, act as mammalian gut immunogens (de Aizpurva & Russel-Jones, 1988). Uptake into the blood circulation facilitates a humoral antibody response. That both GNA and Con A have an effect upon the immune system of *L. oleracea* was indicated by a significant reduction in the abundance of haemocytes in samples extracted from lectin-fed larvae relative to controls (Table 8.1.). Whilst this reduction may reflect a detrimental effect of the lectins upon nutrition no reduction in the growth of lectin-fed larvae was observed relative to controls. Given the ability of GNA and Con A to agglutinate rabbit erythrocytes it was thought that the observed reduction in haemocyte counts may be attributed to lectin mediated crosslinking and clumping of haemocytes in lectin-fed larvae. *In vitro* haemagglutination assays to investigate the ability of GNA to agglutinate haemocytes would indicate whether agglutination could occur *in vivo*. Alternatively the reduction in haemocyte abundance may be attributable to lectin-induced cell death. This could be evaluated, in a similarly run assay, by staining haemocyte samples with toludeine blue as staining is indicative of cell death. The ability of GNA to bind *in vitro* to haemocyte and plasma glycopolypeptides was demonstrated by probing western blotted fractions with dioxigenin labelled GNA (Fig 8.4.). GNA was also identified *in vivo* in both plasma and haemocyte fractions of the haemolymph (Fig 8.5.). That GNA binds to mannose moieties projecting from haemocyte membranes was indicated by *in vitro* studies using FITC-labelled GNA where a degree of binding inhibition was observed in the presence of 250 mM mannose (Plates 4, 9 and 10). Given the less stringent but overlapping sugar specificity of Con A it was predicted that this lectin was likely to exhibit a similiar ability to bind to glycopolypeptides of both plasma and haematocyte components of the haemolymph.

In time trials GNA was detected in the haemolymph of larvae exposed to experimental diet for just 2 hr. In contrast, no Con A was detectable in the haemolymph of larvae fed experimental diet for 24 hr although it was present in the haemolymph of larvae exposed to lectin-containing diet for 5 days. The reason for this significant difference was unclear. The greater binding avidity of GNA binding to BBM glycopolypeptides compared to Con A previously indicated by *in vitro* studies (Chapter 5) may facilitate more rapid endocytosis of the GNA. In mammals lectin endocytosis is known to be significantly increased in the presence of lectin induced bacterial proliferation (Pusztai

1991). The impact of lectins upon bacterial proliferation in the insect gut has not been studied. Thus the possibility that differing effects of GNA and Con A upon bacterial proliferation in the insect gut may facilitate variable rates of lectin endocytosis cannot be discounted. Strictly comparable studies in mammals on the dynamics of lectin uptake were not available. However, it is known that the initial rate of uptake into the blood circulation of rats of the homotetramer PHA-L_4 is almost 3x more rapid than that observed for PHA-E_4, although the amounts of both types of isolectins absorbed become the same after 3 hr (Pusztai, 1991). In rats amounts of systemically absorbed PHA have been shown to reach 5-10 % of that initially administered intragastrically (Pusztai, 1991).

On a per µg protein basis GNA was more concentrated in haemocyte fractions of larval haemolymph (Fig 8.5.). However, as plasma fractions exhibited a 20 fold greater abundance of proteins it was concluded that most of the transported lectin was in the plasma component of the haemolymph of lectin-fed larvae . Similarly, most of the internalised PHA in rats fed PHA containing diets is initially bound to serum glycoproteins, although the proportion associated with blood cells increases with time (Pusztai, 1991). The ability of haemocytes to endocytose GNA *in vitro* was indicated by fluorescent staining within haemocyte monolayers overlaid with FITC-labelled GNA (Plates 1, 4 and 5). In rats the irreversible absorption of PHA by blood cells similarly suggests that the lectin is taken up into blood cells by endocytosis (Pusztai, 1991).

9.1. GENERAL DISCUSSION.

The results obtained from preliminary artificial diet bioassays demonstrated that both GNA and Con A were insecticidal towards *L. oleracea* larvae. Detailed analysis of the effects upon larval development, following acute oral exposure to either lectin, provided a fundamental basis for subsequent work which focussed on establishing possible modes of lectin action. To this end the ability of GNA and Con A to bind *in vitro* to the larval BBM was examined and attempts were made to characterise major lectin binding BBM glycoproteins. Effects upon soluble trypsin and BBM enzymes were examined to establish if lectins disrupted the digestive capacity of the larval midgut. The ability of GNA and Con A to be taken up into the haemolymph of lectin-fed larvae and thereby act upon the immune system and tissues other than the gut was investigated. The following reports major findings from this work and their relevance to the overall objective of producing genetically engineered plants with improved resistance to insect pests. Much of this work was carried out on the basis of extensive studies of the mode of lectin action in mammals. The toxicity of lectins to mammals is of obvious importance in the field of crop protection. Thus results are compared with those obtained from mammalian studies and similarities and differences in modes of lectin action are discussed. The effects of GNA and Con A upon *L. oleracea* are also compared with their effects upon other insect species. Finally similarities and differences in the effects of GNA and Con A upon *L. oleracea* are discussed in relation to corresponding similarities and differences in their mode of action.

When tested in artificial diet at 2.0 % of total dietary protein GNA exerted a significantly detrimental effect upon larval development, growth and consumption, with little effect upon survival (section 4.1.). Similar effects have been observed in detached leaf bioassays (Fitches *et al.*, 1997) and glasshouse trials (Gatehouse *et al.*, 1997; Fitches *et al.*, 1997) where larvae were exposed to transgenic potato plants expressing GNA at levels of up to 2.0 % of total soluble proteins. Con A when tested in artificial diet at concentrations of 2.0 %, 0.2 % and 0.02 % of total dietary proteins had a significant effect upon larval survival, development, growth and consumption (section 4.2.). As for GNA, the effects were similiar to those observed in detached leaf bioassays using transgenic Con A expressing potato plants (Gatehouse *et al.*, in press). A direct comparison of GNA and Con A artificial diet bioassay results indicated that Con A was the more toxic of the two lectins to *L. oleracea*. GNA at 2.0 % of total dietary proteins had little effect upon survival whereas Con A significantly reduced survival even when present at a relatively low 0.02 % of total dietary proteins. Con A at 2.0 % of total dietary proteins also caused greater reductions in mean larval weights and diet consumption compared with GNA at the same dietary concentration (section 4.2.).

GNA and Con A have both been shown to be insecticidal towards a number of important insect pests although the effects of ingestion are highly variable from species to species of insect.

Down *et al.* (1996) found that GNA in artificial diet at 0.1 % w/v (approx. 21 μM) fed to the glasshouse potato aphid (*Aulacorthum solani*) had little effect upon survival but caused a significant (30 %) reduction in nymphal growth rate and a delay in development to adulthood. GNA also reduced the fecundity of *A. solani* by up to 65 % by delaying the onset, and by reducing the rate, of nymph production. Similar results were obtained for *A. solani* in growth cabinet assays using transgenic potato plants expressing GNA (0.3-0.4 % of total soluble proteins), and large scale glasshouse trials where the rate of population build up on GNA expressing plants was approximately 4 fold lower than on control plants (Down *et al.*, 1997). GNA has been shown to be toxic to the peach potato aphid (*Myzus persicae*). Sauvion *et al.* (1996) observed a 42 % reduction in survival and a significant reduction in growth rate when the lectin was administered in artificial diet at 1.5 % w/v (1500 μg/ml, approx. 30 μM) to *M. persicae* neonate nymphs for 8 days. As for *A. solani* GNA had a significantly detrimental effect upon aphid fecundity. Hilder *et al.* (1995) observed similar effects upon *M. persicae* in artificial diet (GNA 0.1 % w/v) and transgenic leaf assays where GNA was expressed at up to 2.5 % of total soluble proteins. Transgenic potato plants expressing GNA have also been shown to significantly reduce both the onset of nymph production, and cumulative production of nymphs (Gatehouse *et al.*, 1996). Significant effects have also been observed for the pea aphid (*Acyrhosiphon pisum*). Rahbé *et al.* (1995) observed a significant reduction in survival when GNA was administered in artificial diet at 0.25 % (250 μg/ml, approx. 5 μM) to *A. pisum* neonate nymphs for 7 days. 50 % growth inhibition occurred at 149 μg/ml (approx. 3 μM) a value 4 fold lower than that observed for *M. persicae* indicating that the pea aphid was the more sensitive of the 2 species to GNA.

Toxic effects of GNA have been observed for 2 important rice pest species *Nilaparvata lugens* (rice brown plant hopper) and *Nephotettix cinciteps* (leafhopper). Powell *et al.* (1993) found that GNA incorporated at 0.1 % w/v (approx. 21 μM) in artificial diet caused a significant reduction in the survival period of *N. lugens* and *N. cinciteps* . GNA was later shown to be toxic to *N. lugens* at concentrations as low as 0.02 % w/v (approx. 4.2 μM) (Powell *et al.*, 1995) and to cause a 50 % reduction in growth rate at 0.025 % (approx. 5.4 μM) (Powell *et al.*, 1998). Data from the *L. oleracea* artificial diet assay demonstrated that reduced feeding was a consequence of smaller larval size (Fig 4.5.) suggesting that GNA does not exert any long term antifeedant effect. This contrasts with the antifeedant effects of GNA observed for the *N. lugens.* Powell *et al.* (1995) found that GNA (0.1 % w/v) reduced honeydew droplet production by 96 % although some recovery in excretion levels was observed after 24 hr.

Toxic effects have also been recorded for the Coleopteran sugar cane white grub (*Antitrogus parvulus*). Allsopp & McGhie (1996) found that GNA administered in semi artificial diet at 0.05 % (v/v) caused significant mortality and growth inhibition after 28 days and 22 days, respectively.

Significant reductions in the survival of *L. oleracea* observed in artificial diet bioassays

where Con A was tested at concentrations of 2.0 %, 0.2 %, and 0.02 % (total dietary proteins) (section 4.2.) are analogous to highly significant reductions observed by Down (1998) in the survival of *A. solani* exposed to Con A at 0.1 %, 0.05 %, and 0.01 %, (w/v) levels in artificial diet. Con A induced retardation of *L. oleracea* growth is also paralleled by the reduced size of *A. solani* aphids exposed to 0.1 %, 0.05 %, and 0.01 % (w/v) Con A. However, only the highest lectin treatment in this case showed significant differences to control aphids (Down, 1998). Whilst Sauvion *et al.* (1996) found that GNA at 1.5 % (w/v) in artificial diet caused a significant reduction in the survival of *M. persicae*, Con A at the same concentration only reduced survival by 5 %. Similarly 50 % growth inhibition was observed at a Con A dose of 774 µg/ml, approximately 20 % greater than that recorded for GNA. Like GNA, Con A also adversely affected total fecundity and the reproduction dynamics of *M. persicae*. Both lectins, when expressed in potato plants at relatively low levels, caused a significant reduction in the fecundity of *M. persicae* aphids (Gatehouse *et al.*, 1996; Gatehouse *et al.*, in press). Interestingly unlike GNA, Con A caused no significant reduction in the onset of nymph production and a reduction in nymph size was only evident for 1 of the 3 transgenic lines tested. In fact insects were significantly larger (by approx. 30 %) on 2 of the 3 Con A lines tested. Rahbé *et al.* (1995) found Con A and GNA to be similarly toxic to *A. pisum*; significant mortality was caused by both lectins when administered in artificial diet at 0.25 % (w/v) (250 µg/ml) to neonate nymphs for 7 days. In this study Con A was tested on 5 other species of aphids at concentrations ranging from 0.005 - 1.5 % (w/v) (5-1500 µg/ml). Although dose-response curves differed substantially all species exhibited growth inhibition at higher doses. Whilst Powell *et al.* (1993) found GNA to be toxic to the *N. lugens* at levels as low as 0.02 % (w/v), Con A at 0.1 % (w/v) in artificial diet showed only marginal effects on mortality. Con A toxicity to the Dipteran *Lucilia cuprina* has been demonstrated by Eisemann *et al.* (1994) who observed a 50 % inhibition of larval growth at 4µM, and 100 % mortality at higher concentrations (25 µM).

Similar but less dramatic effects of lectins upon *L. oleracea* were observed in detached leaf assays where GNA and Con A were present at 30 and 400 fold lower levels, respectively, than those tested in artificial diet bioassays (Fitches *et al.*, 1997, Gatehouse *et al.*, in press). Thus it was concluded that the effects of both lectins were at least to some extent dose dependant. Dose dependancy has also been observed for GNA in aphid studies. Down (1998) found that GNA at 0.05 % (w/v) (500 ug/ml) in artificial diet caused no significant toxicity towards *A. solani* and actually resulted in a stimulation of nymph production. Sauvion *et al.* (1996) also reported that GNA at 0.001 % (w/v) (10ug/ml) caused a slight but significant growth stimulation (11 % increase in aphid weight compared to controls) of *M. persicae*. Analogous compensatory feeding by *L. oleracea* was observed in the GNA and Con A detached leaf assays which used plants expressing relatively low levels of lectin (Fitches *et al.*, 1997, Gatehouse *et al.*, in press). These results are somewhat analogous to the situation in mammals where some lectins, when administered orally at low doses, have been shown to exert beneficial effects upon general metabolism (Pusztai & Bardocz, 1996). With respect to

crop protection these results highlight the need to establish the level of a particular lectin required for significant insecticidal activity on a species-lectin basis. Furthermore adequate levels of expression must be achieved in the field in order to ensure that a viable degree of protection is conferred.

Whilst Con A appeared to be more toxic than GNA to *L. oleracea* this is not the case for other insect species. What is apparent in the literature is extreme variability in the sensitivity of different species to the 2 lectins. The effects of a particular compound incorporated into different artificial diets or transgenic materials are not strictly comparable, so only tentative comparisons can be made. *A. solani* appears to be more sensitive to Con A, showing significant reductions in survival at 0.1 % (w/v) Con A (Down 1998) but no reduction for 0.1 % (w/v) GNA (Down *et al.,* 1997). In contrast *M. persicae* and *N. lugens* appear to be more sensitive to GNA, both species exhibited significantly reduced survival for GNA treatments but little effect with equivalent Con A treatments (Powell *et al.,* 1993; Sauvion *et al.,* 1996). Both lectins show similiar toxicity to the *A. pisum* (Rahbé *et al.,* 1995). Differences in the sensitivity of different species to the 2 lectins were thought to be be attributable to 3 variable factors; assay procedures, the feeding habits of different species and the mechanisms of lectin action. Variability in the mechanisms of insecticidal lectin action are discussed in relation to known mechanisms of lectin action in mammals.

Feeding habits may contribute to variability observed in the effects of lectins upon different insect species. General detoxification mechanisms, or resistance to antimetabolic effects, are likely to be more developed in polyphagous species than in a monophagous insect. Monophagous insects are adapted to a specific host and thus sensitivity to introduced antimetabolic compounds may be greater compared with more general feeders. When testing the effects of Con A in artificial diet on 5 aphid species Rahbé *et al.* (1995) found that aphids could be grouped according to their host range characteristics. Oligophagous aphids, restricted to the Fabaceae were the most sensitive, while polyphagous aphids displayed the greater resistance to high levels of Con A. Although comparisons across insect orders are less valid it is noteworthy that the polyphagous species *L. oleracea*, *A. solani*, and *M. persicae* , show less sensitivity to GNA than the monophagous *N. lugens*.

Lectin toxicity in mammals is largely dependant on the binding of lectins to suitably glycosylated targets in the intestinal BBM (Pusztai, 1991). Pusztai *et al.* (1990) have demonstrated a correlation between the strength of binding of lectins to the BBM and their effectiveness as antinutrients. The binding of Con A to the rat BBM has been shown to induce disruptive changes in cell morphology, metabolism, and the proper functioning of the entire digestive system (Pusztai, 1991). In contrast GNA, due to the relative scarcity of mannose containing brush border glycans, does not initially exhibit binding to the rat BBM and this is thought to largely account for the lack of its toxicity to mammals (Pusztai *et al.,* 1990). By analogy to the situation in mammals, insecticidal effects of lectins may also be primarily determined by binding to suitably glycosylated

targets in the insect gut causing inhibition of nutrient absorption and/or midgut cell disruption (Pusztai, 1991). If the extent of lectin binding is the primary determinant factor of toxicity in insects then it is not surprising that Con A (which binds glucose and mannose residues), with a broader sugar specificity and hence greater capacity for binding to gut glycoproteins is more toxic to *L. oleracea* than the strictly mannose specific lectin GNA. Con A was shown to bind *in vitro* to the majority of larval BBMV and PM proteins (section 5.1.). In contrast GNA bound to only 5 BBMV and 2 PM glycopolypeptides and did not exhibit binding to PM proteins (section 5.1.). Correspondingly higher levels of Con A were detected *in vivo* in the guts of larvae fed on Con A containing diets as compared with detected levels of GNA (section 7.7.). Immunohistochemical studies in insects have demonstrated that Con A and GNA bind to, and cause disruption of, midgut epithelial cells. Examples where lectin binding has been observed include GNA and Con A in the midguts of *L. oleracea* (Woodhouse S., pers comm), GNA in the aphid *A. solani* (Down, 1998), GNA in the rice brown planthopper *N. lugens* (Powell *et al.,* 1998), and Con A in the blowfly *L.cuprina* (Eisemann *et al.,* 1994). Powell *et al.* (1998) demonstrated that GNA binding to the midgut epithelia of *N. lugens* facilitated disruption of microvilli and the appearance of epithelial cell abnormalities. Similarly Sauvion *et al.* (1995) observed marked Con A induced morphological changes in the digestive tract of *A. pisum.* Abnormalities were similiar to those observed in PHA fed rats and included epithelial cell distension, enlargement, and shedding in the midgut region (Pusztai, 1991). There is a need for detailed immunohistochemical studies to investigate differences in the extent of Con A and GNA binding to the BBM of *L. oleracea* to establish if the degree of binding is indeed correlated with toxicity. The possibilty that Con A, and to a lesser extent GNA, may additionally disrupt larval metabolism via binding to, and causing blockage of the PM, as has been observed in blowfly (*L. cuprina*) larvae (Eisemann *et al.,* 1994) also requires further investigation.

Thus there is growing evidence to support the view that the primary mechanism of lectin action is similiar in both mammals and insects. That is to say that lectin induced reductions in survival, development, and growth are, at least in part, facilitated by lectin binding and lectin induced damage to the insect gut epithelium. It is tempting to conclude therefore that GNA is toxic to insects but not to mammals, largely as a result of differences in gut glycosylation and that levels of toxicity are directly related to differences in the degree of lectin binding in the guts of different species. However, if the degree of binding was the sole determinant of toxicity then Con A, with a broader carbohydrate specificity, would be expected to be more generally toxic than GNA to insects which is clearly not the case. Variability in the nature of BBM glycosylation in different species together with variability in the binding affinity of lectins to single carbohydrate residues and to oligomers and complex carbohydrates are thought to be critical factors in determining subsequent toxicity. In addition an extensive study by Harper *et al.* (1995) found there to be no correlation between lectin binding and toxicity, since many lectins which bound to midgut epithelia in the Lepidopteran *Ostrina nubilalis* (European corn borer) and Coleopteran *Diabrotica vergifera* (Western corn

borer) were not toxic to the insect. This suggests, not surprisingly, that not every lectin-ligand interaction on the surface of the gut epithelium leads to a toxic effect. Similarly not all Bt crystal proteins that bind to the BBM are toxic to the insect (Garczynski *et al.*, 1991) and increased binding does not necessarily correlate with increased toxicity (Wolfersberger, 1990). Thus it seems likely that variability in the insecticidal effects of lectins may be largely attributable to variability in the strength and specificity of lectin-ligand interactions in the insect gut.

The inhibition of larval growth and development observed in both GNA and Con A bioassays suggested that both lectins may have acted by inhibiting nutrient uptake in *L. oleracea*. This is particularly evident for GNA in the detached leaf assays where consumption was almost identical for control and transgenic fed larvae (Fitches *et al.*, 1997). That GNA and Con A act, at least in part, by disruption of larval digestive metabolism was indicated by observed effects of lectin treatments upon soluble trypsin and BBM enzyme activities. Despite the greater potential for Con A, compared with GNA, to bind to the BBM and PM of *L. oleracea* both lectins were seen to have a similar ability to disrupt the digestive capacity of the larval midgut. In the short term a lack of correlation between consumption and growth (section 7.1.) indicated that both lectins have an almost immediate negative impact upon larval nutrition. An increase in levels of trypsin activity in faecal material (section 7.2.) collected from lectin-fed larvae suggested that the disruption of enzyme recycling processes may be a mechanism by which lectins exert their insecticidal effects. Significantly elevated protein levels observed in the guts of short term GNA and Con A fed larvae (section 7.1.) were thought to reflect an accumulation of proteolytically resistant lectin bound to the BBM. Numerous other studies have shown a strong correlation between gut protein content and luminal proteinase levels in several insects (Lehane 1977; Houseman *et al.*, 1985; Billingsley & Hecker, 1991) leading to suggestions that secretagogue/prandial mechanisms may be the main factors controlling digestive enzyme synthesis and secretion in insects (Lehane *et al.*, 1995). Thus it was concluded that the interaction of lectins with digestive enzyme producing cells may have facilitated the observed short term induction of trypsin activity in the guts of lectin-fed *L. oleracea*. A subsequent increase in demand for amino acids required for trypsin synthesis may in turn account for the observed short term increased consumption of diet by lectin-fed larvae, relative to controls (section 7.1.). Both GNA and Con A stimulated similar short term elevations in BBM enzyme and soluble trypsin activities (section 7.2.) and a long term reduction in α-glucosidase activity (section 7.6.). It is noteworthy that although differences were observed in the ability of GNA and Con A to bind to the abundant BBM aminopeptidase *in vitro* (section 5.2.) both lectins induced similar changes in the activity of this enzyme when fed to larvae in short and long term assays. Aminopeptidase was identified as a major Con A binding species (section 6.1.) whereas GNA bound relatively weakly (section 5.1.). Although binding to the BBM may be a causative factor in the toxicity of GNA and Con A to *L. oleracea* these results indicated that non-specific interactions between lectins and gut glycoproteins may account for some of the observed effects of lectins upon insects. In fact the presence of lectin in the gut may alone be sufficient to

disrupt metabolic processes. This mode of action has been indicated by a study carried out in rats. Although GNA does not initially bind to the rat BBM Pusztai *et al.* (1996) found activities of BBM aminopeptidase and alkaline phosphatase to be significantly increased, and α-glucosidase decreased, in rats fed for 10 days on GNA containing diet (7 % (w/w) protein).

As most membrane proteins including hormone and growth factor receptors, transport proteins, and brush border enzymes, are glycosylated the scope of lectin-gut interactions in both insects and mammals is vast. Therefore physiological responses to lectin treatments are correspondingly diverse and complex. Notable differences in the effects of GNA and Con A upon larval metabolism were thought to be indicative of differing modes of action faciltated perhaps by more specific lectin-ligand interactions. A significantly greater mean gut to larval weight ratio was observed for larvae chronically exposed to Con A whereas GNA fed larvae exhibited very little difference in relative gut size as compared with control larvae (section 7.5.) This suggested that Con A but not GNA stimulated larval gut growth and is analogous to the situation in mammals where orally administered Con A acts as a gut growth factor whereas GNA exhibits no significant mitogenic activity (Pusztai, 1991; Pusztai *et al.*, 1996). However, in mammals the growth factor activity of lectins is though to be determined mainly by the strength and intensity of their binding (Pusztai *et al.*, 1990). As yet specific mammalian gut lectin receptors have not been reported. *In vitro* studies of GNA and Con A binding to the BBM of *L. oleracea* in the presence of inhibitory sugars suggested that GNA binds with more avidity than Con A (section 5.3.). If these results reflect the situation *in vivo*, then the growth factor activity of Con A and GNA may not be dependant solely upon binding avidity as it appears to be in rats. Alternatively the growth factor activity of Con A may arise as a result of a specific lectin-ligand interaction i.e. Con A, but not GNA (which binds far fewer BBM glycoproteins), may bind to a specific receptor and thereby mimic the effect of endogenous growth factors in *L. oleracea*. The complexity of lectin-ligand interactions was further demonstrated by considerable differences in the dynamics of GNA and Con A transport to the haemolymph of lectin-fed larvae. In time trials GNA was detected in the haemolymph of larvae exposed to experimental diet for just 2 hr (section 8.5.). In contrast, Con A was not detectable after exposure to experimental diet for 24 hr (section 8.5) although it was present in the haemolymph of larvae exposed to lectin-containing diet for 5 days (section 8.3.). In rats lectins which bind avidly to epithelial cells are readily endocytosed (Pusztai & Bardocz, 1996). Hence it was concluded that the greater binding avidity of GNA, compared with Con A, to the BBM (section 5.3.) may facilitate more rapid endocytosis of the lectin and subsequent uptake into the haemolymph of *L. oleracea*. The observed difference in the dynamics of lectin uptake may occur as a result of differences in the dynamics of an interaction between GNA or Con A and a specific BBM receptor. This highlights the need for further work to identify lectin targets in the insect gut to increase our understanding of mechanisms by which lectins exert their insecticidal effects. Lectin endocytosis in mammals has been shown to be significantly increased in the presence of lectin induced bacterial proliferation (Pusztai, 1991). Thus differing effects of GNA and Con A upon

bacterial proliferation in the insect gut may provide an additional or alternative explanation for the observed differences in the time scale of lectin transport to the haemolymph of *L. oleracea* larvae.

That variability in the effects of lectins arises as a result of interactions of lectins with components other than that of the insect gut was previously indicated by studies which demonstrated antifeedant effects of GNA upon *N. lugens* (Powell *et al.*, 1993). As *N. lugens* has a well developed labium with a complex array of mechanoreceptors and chemoreceptors, Powell *et al.* (1993) concluded that GNA antifeedant effects may result from interference (possibly through binding of lectins to glycoproteins) with the normal functioning of chemoreceptor sites involved in diet recognition. Whilst neither GNA or Con A exerted antifeedant effects upon *L. oleracea* (section 4.1. and 4.2.) their potential to exert systemic effects was demonstrated by the detection of both lectins in the haemolymph of larvae exposed to experimental diets for 5 days (section 8.3.). That GNA and Con A may act directly upon organs other than the insect gut was also indicated by the detection of lectins *in vivo* in malpighian tubule and fat body tissue extracts (section 8.2.). Immunohistochemical studies have shown GNA to be present in the haemolymph and fat bodies of both larvae and adult *N. lugens* exposed to GNA containing artificial diets (Powell *et al.*, 1998). In mammals lectins have the ability to influence systemic metabolism by two different mechanisms. Indirect effects may be caused by lectin binding to gut neuroendocrine cells causing a stimulation of gut peptide hormone secretion into the systemic circulation. Alternatively, lectins transmitted through the gut wall into the blood circulation may directly influence peripheral tissues and body metabolism by mimicking the effects of endcocrine hormones (Pusztai & Bardocz, 1996). Systemic effects of toxic lectins such as PHA include pancreatic enlargement; increases in liver weight and body fat catabolism; glycogen loss; thymus atrophy; and a significant reduction in skeletal muscle weight. Due to the wide range in affected metabolic processes and complexity of the mammalian endocrine system it is not yet known whether systemic effects are produced by direct or indirect mechanisms of lectin action (Pusztai, 1991).

In analogy to the situation in mammals it is argued that lectins have the ability to influence the systemic metabolism of insects by two different mechanisms. Indirect effects may be caused by lectin binding to endocrine cells distributed throughout the epithelium of the insect gut. The transport of lectins into the haemolymph may directly influence peripheral tissues such as the malpighian tubules and fat bodies. The lack of effect of both GNA and Con A upon the survival and growth of 4th instar *L. oleracea* larvae fed on experimental diets for 5 days suggested that neither lectin had a toxic effect per se upon functions carried out by malpighian tubules and fat bodies (section 8.1.). However, detailed studies are required to establish if either lectin influences osmoregulatory functions and multiple metabolic processes carried out respectively, by malpighian tubules and fat bodies. Whilst not all lectin-ligand interactions may have damaging effects, the absorption or adherence of lectins to tissues other than the gut would presumably afford some overall nutritional cost to the larvae. In this way indirect

effects upon systemic metabolism may be cumulative.

An additional mode of lectin action in mammals is their ability to act as gut immunogens (de Aizpurva & Russel-Jones, 1988). Uptake into the blood circulation facilitates a humoral response antibody response. This local IgE based allergenic immune reaction contributes to the overall poor nutritional state of the body and acts to increase the nutritional toxicity of PHA. As feeding rats PHA containing diets is typically accompanied by a dramatic proliferation of bacteria in the small intestine it is thought that PHA may also have a generally detrimental effect upon the functions of the IgA based local immune system (Pusztai & Bardocz, 1996).

The fact that both GNA and Con A have an effect upon the immune system of *L. oleracea* was indicated by a significant reduction in the abundance of haemocytes, relative to controls, observed in samples extracted from larvae fed for 5 days on experimental diets (section 8.4.). GNA was identified in both plasma and haemocyte fractions of the haemolymph (section 8.6.) and its ability to bind *in vitro* to both components was demonstrated (sections 8.6 and 8.7.). It was predicted that Con A, which also binds mannose moieties, would demonstrate a similar, if not greater, ability to bind to plasma and haemocyte glycoproteins. Insect immunity (like other invertebrates) apparently lacks the specificity and memory exhibited by the vertebrate adaptive immune system. Instead insect immunity is thought to more closely resemble the innate vertebrate immune system (Gillespie *et al.,* 1997). Thus as insects cannot produce antibodies the effects of dietary lectins upon the immune system must differ to their effects upon the mammalian immune system. Insects do posssess a complex and efficient system of biological defence which involves rapid, relatively nonspecific responses, both cellular and humoral, to invading organisms (Gillespie *et al.,* 1997). The potential for lectin induced modulation of immune function is correspondingly complex and diverse.

The presence of the lectin itself may induce an immune response. In *D. melanogaster* the binding of a lectin from the snail *Helix pomatia* has been shown to induce an immune response evidenced as a stimulation in the synthesis of cecropins (Theopold *et al.,* 1996). Interestingly this lectin, like Con A, is an activator of mammalian lymphocytes. In addition to direct effects lectins may also act indirectly upon the insect immune system. Pathogenic recognition in insects relies on the specific binding of plasma or haemocyte proteins to bacterial or fungal polysaccharides (Gillespie *et al.,* 1997). Lectins in the haemolymph may compromise these mechanisms of recognition by competing for, and possibly blocking, sites on haemocyte and plasma peptides for pathogenic polysaccharides. Interference with functions carried out by endogenous lectins may also be facilitated by the presence of dietary lectins. Binding of endogenous lectins to bacterial surfaces is thought to act as a signal for recognition and subsequent immune responses by haemocytes (Gillespie *et al.,* 1997). Interestingly an insect lectin hemocytin, which stimulates haemocyte aggregation, cloned in *Bombyx mori* has been shown to contain a lectin domain homologous to the mammalian mannose binding

protein involved in immune responses (Kotani *et al.*, 1995). A variety of proteins and peptides that attack bacteria by several mechanisms are synthesised and secreted by fat bodies and haemocytes (Gillespie *et al.*, 1997). As both GNA and Con A were detected *in vivo* in fat body tissue of *L. oleracea* (section 8.2.) it was concluded that this is a further component of the insect immune system that could be adversely affected by lectins. Clearly introduced lectins have the potential to directly effect the synthesis of antibacterial proteins (via binding to fat body, plasma and haemocyte glycoproteins). In addition indirect effects resulting from the nutritional cost of storing a non degradable protein in the systemic circulation are also possible. The possible effects of lectins upon the insect immune system are particularly important with regards to the use of biological control in conjunction with genetically engineered crops. The insect immune system acts as the key defence against attack by various parasitoids. That GNA does to some extent compromise the immune system of *L. oleracea* has been indicated by studies which examined the the ability of *Eulophus pennicornis* to parasitise control and GNA-fed larvae. The ability of *E. pennicornis* to parasitise larvae was not compromised by the treatment of larvae with GNA. In fact for some of the parameters measured the incorporation of GNA in the larval diet was seen to significantly increase the performance of *E. pennicornis* (Bell H. pers comm). GNA has similarly been shown to have no effect upon the performance of the aphid parasitoid *Aphidius evvi*.. As for *E. pennicornis* some improvements in wasp performance on GNA-fed aphids were observed (Couty *et al.*, 1998). This suggests that the expression of GNA in crop plants is compatible with the use of biological control agents and may in fact improve the ability of introduced parasitoids to reduce pest populations. However, tri-trophic interactions must be studied in the field before any conclusive statements can be made.

In mammals the differential ability of lectins to bind to particular glycans and the dynamic nature of the structure of glycosyl side chains is known to depend upon several factors. These include animal age, blood group specificity, position along the gastrointestinal tract, mucosal cell types and their state of differentiation/ maturation (Pusztai & Burdocz, 1996). Accordingly the reactivity of the small intestine to lectins differs significantly in newborn and adult animals (Pusztai, 1990) For example the damage caused by intrapharyngeally infused Con A in neonatal guinea pigs is more serious in the gastric than in the small intestinal mucosa and results indicate that the neonatal gastrointestinal tract is more susceptible to the damaging effects of food lectins than the mature one (Weaver & Bailey, 1987). Whilst both GNA and Con A had significantly detrimental effects upon development when fed to neonate larvae (sections 4.1. and 4.2.) no effects were observed when the lectins were fed to 4th instar larvae for 5 days (section 8.1.). A lack of effect was also observed when GNA (at 2.0 % total dietary proteins) was fed to 3rd instar larvae for 2 weeks (results not presented). Insect gut cells are relatively poorly differentiated and no major changes in the nature of BBM glycosylation (with respect to glucose and mannose residues) were observed throughout the development of *L. oleracea* (section 5.4.). Thus, unlike mammals, the potential for lectin binding to the insect gut epithelium is likely to be similar at all stages of development. The observed lack of short term effects of either lectin may reflect

differences in the availability of carbohydrate side chains for lectin binding in the guts of neonate and more developed larave. Alternatively it may reflect a greater ability of older larvae to bear the nutritional costs afforded by the lectin treatment.

9.2. FUTURE WORK.

In the light of possible beneficial effects to insects of lectins at low concentrations, together with studies demonstrating resistance development to Bt toxins in genetically engineered crops, there is a need for longer term studies to assess the impact of Con A and GNA at various dietary concentrations on 2nd and subsequent generations. Down (1998) found that *A. solani* nymphs produced by GNA fed adults which were continued on GNA diet survived significantly longer than those produced by control aphids which were transferred to GNA diet. This indicated that acclimatisation to GNA could develop within 1 generation in aphids. At present 2nd generation studies of the effects of GNA and Con A are hindered by the need for very large sample numbers and the absence of an optimal artificial diet able to support larvae through to pupation. In order to realistically assess the potential of either lectin in the field further more long term studies are required.

An important aspect of mammalian lectin toxicity which has not been addressed by this work is the interaction of lectins with gut bacteria. Recent studies have demonstrated that most of the detrimental effects of toxic lectins upon mammals are due to their interaction with, and stimulation of, selective bacterial overgrowth (Pusztai & Bardocz, 1996). The damaging contribution of bacteria is shown by dramatic differences in the effects of PHA-containing diets on conventional and germ free rats which show essentially no toxic effects following exposure to raw kidney bean diets (Pusztai, 1991). Overgrowth may increase competition for food between the small intestine and proliferating bacteria. In addition the presence of adherent bacteria may disrupt the morphology and functions of the intestine. In order to establish if a similar mode of action occurs in insects comparable tests on conventional and germ-free larvae exposed to lectin-containing diets must be carried out.

It has been established that GNA and Con A may act by disrupting *L.oleracea* nutrition. However, the physiological consequences of the observed effects upon trypsin and BBM enzymes have not been elucidated. There is a need to characterise so called lectin receptors in order to identify which physiological processes might be affected. Receptor characterisation may also provide explanations for observed differences in the insecticidal effects of different lectins upon the same species and upon different species. In relation to crop protection characterisation of major lectin binding glycoproteins may be critical when considering resistance development in the field. In analogy to mechanisms of lectin action, a critical factor in determining Bt toxicity in various Lepidopteran pests is the binding of the Bt crystal proteins to high affinity receptors on the BBM. Binding is believed to be a critical preliminary step in catalysing the insert of a helical domain I of the Bt toxins in the lipid bilayer (Li *et al.*, 1991; Grochulski *et al.*, 1995), leading to the formation or disruption of ion channels (Slatin *et al.*, 1990;

Schwartz *et al.*, 1991,1993), and resulting in eventual larval death. Almost all receptors for Cry toxins have been characterised as aminopeptidases (Knight *et al.*, 1994; Sangadala *et al.*, 1994; Gill *et al.*, 1995; Valiatis *et al.*, 1995; Luo *et al.*, 1996) which, like lectins, exhibit binding inhibition in the presence of inhibitory sugars (Luo *et al.*, 1997; Valiatis *et al.*, 1997). That the development of resistance to Bt toxins is mediated via alterations in receptor protein structure has recently been investigated. Lee *et al.* (1995) reported an absence of Cry1Aa binding sites, but no decrease in Cry1Ab, and Cry1Ac binding sites in Cry1Ac resistant *H. virescens*. This suggested that the receptor A (AP-N) is modified in Bt resistant strains. Given the analogous reliance of lectin toxicity upon binding to BBM proteins modifications in major lectin binding 'receptors' following acute exposure may correlate with the almost inevitable development of resistance in the field.

The scope for future studies to investigate potential mechanisms of lectin action upon the insect immune response is clearly vast. The development of an ELISA method enabling quantitative analysis of lectin levels in different areas within an insect would be highly beneficial. Tissues, other than the gut, acting as lectin 'sinks' could be identified and differences in the levels of lectin in the same tissue from different insects may identify possible differences in modes of lectin action and thereby account for some of the variability in effects. Such information would also be pertinent to the proposed 'gene packaging' strategy whereby different genes acting on different targets within an insect are to be engineered into plants to provide more effective control of insect pests. Possible mechanisms of action previously discussed may be evidenced as a reduction in the ability of lectin-fed larvae to combat bacterial or fungal infection. Experiments designed to investigate the effects of lectins upon processes such as phagocytosis and encapsulation would provide indications of modes of lectin action upon the immune system. Preliminary studies carried out at CSL, York, have indicated that the ability of *L. oleracea* haemocytes to encapsulate FITC-labelled *E. coli* is compromised in GNA fed larvae relative to controls (Bell H., pers comm).

9.3. CONCLUDING REMARKS.

Insecticidal activity of the mannose specific lectin GNA to *L. oleracea* larvae has been demonstrated. As GNA is non toxic to mammals and likely to be compatible with the use of biological control agents it has considerable potential as a candidate for use in the field of crop protection. However, as its effects upon different pests are variable adequate levels of expression in the field must be established on a species basis and maintained throughout the growing season if a viable degree of protection is to be achieved.

Con A is known to be toxic to mammals (Pusztai, 1991). In this study Con A has been shown to be more toxic to *L. oleracea* than GNA. Con A, with a broader sugar specificity, has a greater potential than GNA to bind to the BBM of *L. oleracea* and this may account for its greater insecticidal activity. Con A, and to a lesser degree GNA, binds to the PM s and thus has potential to disrupt the protective function of the PM and

to disrupt the bidirectional movement of molecules across this membrane. Despite differences in the binding abilities of GNA and Con A to the PM and BBM both lectins in short and long term assays exhibited a similar capacity to disrupt larval digestive metabolism. In mammals so called toxic lectins exert systemic effects as a consequence of their uptake into the systemic circulation (Pusztai, 1991). Results suggest that a similar mode of action occurs in insects. Both GNA and Con A, as a result of their uptake into the haemolymph, have the potential to act upon organs other than the gut. Additional potential direct and indirect effects upon the insect immune system have been discussed.

Whilst both lectins had significant effects upon development when fed to neonates no effects upon 3rd or 4th instar larvae were observed. It is concluded that the primary mode of GNA and Con A action in *L.oleracea* is likely to be the disruption of the relatively fragile neonate BBM environment and ongoing nutritional costs of storing the proteolytically resistant lectins both in the gut and the systemic circulation. With regards to Con A disruption of both the PM and BBM environment is thought to afford a nutritional cost that is often too high for larvae to bear. GNA induced disruption acts to inhibit development from the onset. Differences in the effects of the 2 lectins were thought to be indicative of alternative modes of action which may be facilitated by more specific lectin-ligand interactions. Similar, albeit variable, effects of GNA and Con A upon other insect species are thought to be indicative of similar primary modes of lectin action.

APPENDIX 1.

DNA and predicted amino acid sequence of clone 1 (Chapter 6, section 6.8.) obtained by amplification of template *L.oleracea* gut tissue cDNA by PCR using V8 FOR (Fig 6.7.) and T7 primers. Amino acids are given in italics and * denotes those that correspond to the predicted amino acid sequence of the V8 FOR primer. Amino acids in bold show a region that exhibited 30 % identity with a fat body protein isolated from *D. simulans* (GB AF 045786, E=4.8) and underlined regions show homology to this protein. N in the nucleotide sequence (upper case) denotes uncertain bases and ? denotes corresponding uncertainty in the amino acid sequence.

5' - ATG CGG ACG AAC AAC GTG GCA GCT GCA GCC TTT ATC ATT CAC GAT CAA GAT

M R* T* N* N* V* A* A A A F I I H D Q D*

GTA ACA GTC CGT GGC GCT CAT GGA ACC AAC ATA ACT TCA GTC CAG GTA GCT

V T V R G A H G T N I T S V Q V A

CGT GTT GGC AAC ACC ATG CAT GCT ATC CCG ACT ATC AAA TCT GGA GGA CTC

R V G N T M H A I P T I K S G G L

GGG CAG AGC TTT GTC GTC ATC AAT ATT CTT GGA GTT AGA GGA AGT GCC TTC

G Q S F V V I N I L G V R G S A F

TCT TAC ACG ATC AGT GTT TAT GCA TCA ATT GGC TGC GCA AAT AGT TNA GGA

S Y T I S V Y A S I G C A N S ? G

NAT GGC TTT TTC TTC GTC TTG GAA AGT GGT TAC CAT TGT TGT GGT AGC CTT

? G F F F V L E S G Y H C C G S L

AAA - 3'

K

APPENDIX 2.

DNA and predicted amino acid sequence of clone 2 (Chapter 6, section 6.8.) obtained by amplification of template *L. oleracea* gut tissue cDNA by PCR using V8 REV (Fig 6.7.) and T3 primers. Amino acids are given in italics and * denotes those that correspond to the predicted amino acid sequence of the V8 REV primer are underlined. Amino acids in bold depict a region exhibiting 38 % identity with an SH2/SH3 adaptor protein isolated from *D.melanogaster* (GI 1373390 (U04224),E=2.0) and underlined regions show homology to this protein.

5' - GCA CGA GGA AAC GAT ACC AGA ATG ATG AAG CAA GCA TTA CTC GTT CTC GCT

A R G N D T R M M K Q A L L V L A

CTG GTG GGG CTC GCC TTC TCG GCT CCT CAG CCC AGG AAG CTG GTT CGG GAA

L V G L A F S A P Q P R K L F R E

CAC GTG GAG GAG TTC CTG GAC ATC ATC ATG GAG GAA TCT GGT CAT GAG ATG

H V E E F L D I I M E E S G H E M

GAG CAC CTG ATG GAA CAC TAC ATC GAG TAC GAC AAG TTC GTG CAA GCT CTG

E H L M E H Y I E Y D K F V Q A L

GAC TAC ATG CGC ACC AAC AAC GTC GCG - 3'

D Y M R* T* N* N* V* A**

APPENDIX 3.

DNA and predicted amino acid sequence of clone 3 (Chapter 6, section 6.8.) obtained by amplification of template *L. oleracea* gut tissue cDNA by PCR using TRY FOR 1 (Fig 6.7.) and T7 primers. Amino acids are given in italics and those in bold depict a region that exhibited more than 60 % identity to alpha-amylase genes isolated from *O. nubilalis*. Homologous amino acids to alpha-amylase (GI 436943 (U04224), E = 4 x 10^{-66}) are underlined. N in the nucleotide sequence (upper case) denotes uncertain bases and ? denotes corresponding uncertainty in the amino acid sequence.

5'- ATG CTG CAN GTC GCG GGA CAT GGT ACT GGT GGC AGC ACT GCT AAC TTC GGA

M L ? V A G H G T G G S T A N F G

AAC TGG CAC TAC CCT TCA GTT CCC TAC GGC AGG AAT GAC TTC AAC TTC CCT

N W H Y P S V P Y G R N D F N F P

CAA TGT GTC ATC TCT AGC AGT GAC TAC GGA TGC TGC CCT GAT AGG GTA CGC

Q C V I S S S D Y G C C P D R V R

AAC TGC GAG CTC TCC GGT CTT AAG GAC TTG AAC CAG GGT ACA GAA TAC GTG

N C E L S G L K D L N Q G T E Y V

CGT CAA ATG ATT GTG AAC TAC ATG AAC CAT CTC ATT AGC TTG GGT GTC GCT

R Q M I V N Y M N H L I S L G V A

GGT TTC AGA ATT GAC GCC GCC AAG CAC ATG TGG CCC GGA GAC TTG CGC GTC

G F R I D A A K H M W P G D L R V

ATC TAC GAC AGG CTA CAC AAC CTC AAC ACC GCC CAC GGT TTC CCC TCC GGT

I Y D R L H N L N T A H G F P S G

GCT CGT CCT TAC ATC TAC CAA GAA GTC ATT GAC CTC GGA GGC GAN GCC ATC

A R P Y I Y Q E V I D L G G ? A I

ACC CCG TGA CGA ATA TAC TCC TCT GGC TGC AGT CAC CCG AAT TCA AAT TCC

T P Z R I Y S S G C S H P N S N S

GGA TTG GAA CTT AGC CGA CCT TCA ACC CGC GGC AAC CAA CTT TAA ATG GCT

G L E L S R P S T R G N Q L Z M A

CTC CCA ACT TGG GGT ACT TGC CTG GGG TCT TCC TTG CTC CAC AAT GAT GCT

L P T W G T C L G S S L L H N D A

CCT TAC CTT CCA TTT GAT AAC CAC GAA CAA CCC AAA AAG TCC CNG GTG CTG

P Y L P F D N H E Q P K K S ? V L

GTT GGA AAC ATC CTG ACT TTA CAA ACA TCT TAA TTT ATA CAA GGT TGC TAT

V G N I L T L Q T S Z F I Q G C Y

CGC NTT CAT NTT TGG CCC ATC CTC CAT GGC TGG CCT CCA CTT GAA TAA - 3'

R ? H ? W P I L H G W P P L E Z

APPENDIX 4.

DNA and predicted amino acid sequence of clone 4 (Chapter 6, section 6.8.) obtained by amplification of template *L. oleracea* gut tissue cDNA by PCR, using TRY FOR 1 (Fig 6.7.) and T7 primers. Amino acids (in italics) in bold depict a region that exhibited 96 % identity with a 26S Protease regulatory subunit 4 isolated from *D.melanogaster* (SP P48601 (U39303), E = 4.0 x 10^{-38}) and underlined regions show homology tothis protein. N in the nucleotide sequence (upper case) denotes uncertain bases and ? denotes corresponding uncertainty in the amino acid sequence.

5' - TAC CGG GCC CCC CTC GAN GTC GAC GGT ATC GAT AAG CTT GAT ATC GAA TTC

Y R A P L ? V D G I D K L D I E F

CTG CAG CCC GGG GGG ATC CGC CCG CGG GAC ACT GGT ACT GGC AAG ACA TTG

L Q P G G I R P R D T G T G K T L

TTA GCT AAA GCT GTT GCC AAC CAG ACC TCT GCT ACT TTT CTG CGT GTT GTA

L A K A V A N Q T S A T F L R V V

GGC TCA GAG CTT ATC CAA AAG TAC TTG GGA GAT GGA CCC AAG CTA GTT CGT

G S E L I Q K Y L G D G P K L V R

GAG CTA TTC AGA GTT GCT GAA GAA CAT GCA CCA TCA ATT GTT TTC ATT GAC

E L F R V A E E H A P S I V F I D

GAA ATC GAT GCT GTA GGC ACA AAA CGT TAT GAT TCC AAC TCT GGT GGT GAG

E I D A V G T K R Y D S N S G G E

AGA GAA ATC CAA AGA ACT ATG TTA GAG TTG CTC AAC CAA CTT GAT GGA TTT

R E I Q R T M L E L L N Q L D G F

GAT TCA CGA GGC GAT GTT AAA GTT ATC ATG GCC ACA AAT CGC ATA NAA ACT

D S R G D V K V I M A T N R I ? T

TTA GAT CCG GCT TTG ATT CGC CCT GGT CGT ATT GAC AGA AAA ATC NAA TTT

L D P A L I R P G R I D R K I ? F

CCA TTG CCT TGA TGA GAA GAC CAA GAA AAA GAT CTT CCA CCA TTC CCA CAT

P L P Z Z E D Q E K D L P P F P H

CAA GAA ATG ACT CTG GCT TAG TAG TTC NAC TTG GTG TAG - 3'

Q E M T L A Z Z F ? L V Z

APPENDIX 5.

DNA and predicted amino acid sequence of clone 5 (Chapter 6, section 6.8.) obtained by amplification of template *L.oleracea* gut tissue cDNA by PCR, using TRY FOR 1 (Fig 6.7.) and T7 primers . Amino acids (given in italics) in bold depict a region exhibiting 46 % identity with a muscle specific protein isolated from *D. melanogaster* (PIR A30128, E = 8 x 10^{-29}) and underlined regions show homology to this sequence. N in the nucleotide sequence (upper case) denotes uncertain bases and ? denotes corresponding uncertainty in the amino acid sequence.

5' - ATG NGA GTT AGT TCA CTC ATT AGG CAC CCC AGG CTT TAC ANT TTA NGT TTC

M ? V S S L I R R H P R L ? L ? F

CGG TTG TAT GTT GNG GGA ATT GTG AGG GTA ACA ATT TCA CAC AGG AAA CAG

R L Y V ? G I V R V T I S H R K Q

TAT GAC CAT GAT TAC GCC AAG CTT GTA CNG AGN TCG GAT CCA TTA GTA ACG

Y D H D Y A K L V ? ? S D P L V T

GCC GCC AGT GTG TTG GAA TTC GCC CTT GGG AGT TTT TGG GGG GGN GAA TCA

A G S V L E F A L G S F W G G E S

GCC GAC ACC GTT GGA TCA GCC GAA AAC TTG TAT GAA GTC CTA AAA GAC GGT

A D T V G S A E N L Y E V L K D G

ANT CTG CTA TGC AAG CTC GTG AAC TCC TCT GCA GGA GGG CTC CGT GAA GAA

? L L C K L V N L L Q E G S V K K

GAT CAA CCA GTC CAC CAT GGC GTT TAA TGC ATG GAA AAC ATC AAC GCG TTC

I N Q S T M A F K C M E N I N A F

TTG GAT GCC GTG AAG AAG CTG GGA GTG CCA CCA CAG GAG ACG TTC CAG ACT

L D A V K K L G V P P Q E T F Q T

ATT GAC TTG TGG GAG AAG CAG AAC CTG TAC TCT GTC GTC GTG TGC CTG CAG

I D L W E K Q N L Y S V V V C L Q

TCT TTG GGA CGC AAA GCT GGG AAC TTC GGT AAG CCT TCC ATT GGG CCT AAG

S L G R K A G N F G K P S I G P K

GAG GCC GAC AAG AAC GTC CGT GAG TTC AGG AGG GAA CAA CTG AAG GCC GGG

E A D K N V R E F S E E Q L K A G

CAG AAC GTC ATC TCT CTC CAA TAC GGA ACC AAC AAG GGA CAG CAA TCA GGC

Q W V I S L Q Y G T N K G Q Q S G

ATC TCA TTC GGA GGC AGG CGC CAA ATG TAA - 3'

I S F G G R R Q M Z

APPENDIX 6.

DNA and predicted amino acid sequence of clone 6 (Chapter 6, section 6.8.). The 297 bp clone was obtained by amplification of template *L.oleracea* gut tissue cDNA by PCR, using TRY FOR 3 (Fig 6.7.) and T7 primers. Amino acids are given in italics and * denotes those that correspond to the predicted amino acid sequence of the TRY FOR 3 primer. Amino acids in bold depict a region exhibiting more than 50 % identity with chymotrypsin genes isolated from *H. armigera* and *M. sexta*. Homologous amino acids to a chymotrypsin isolated from *H. armigera* (GNL PID E1191405 (Y12279), E = 4 x 10^{-23}) are underlined.

5' - GGG GAG TTT TTT GGG GGG GCG AAC CAG CCG TTG AGC TAC GTG GAC TTG CCT

G E* F* F* G G* A* N* Q* P* L S Y V D L P*

GTG ATC TCG AAC GAG GTG TGT GCC CAG ACA TTC GGC GCT TTC GTC CTA CCC

V I S N E V C A Q T F G A F V L P

TCT AAC GTG TGC ACC AGC GGC GAA GGC GGC AAG AAC ATC TGC ACT GGT GAC

S N V C T S G E G G K N I C T G D

TCT GGT GGT CCT CTT GCC GTC ACC AGA GAT GGC AAA CCA CTT TTG ATT GGA

S G G P L A V T R D G K P L L I G

ATC ACT TCA TTT GGG ACA GTT ACG TGT GAA GGT GGA CAA CCC TCA GCC TAT

I T S F G T V T C E G G Q P S A Y

GCA AGA GTC ACC TTC TAC ATG GAC TGG GTC AAC GGC CAC CTT - 3'

A R V T F Y M D W V N G H L

APPENDIX 7.

DNA and predicted amino acid sequence of clone 7 (Chapter 6, section 6.8.) obtained by amplification of cDNA derived from gut tissue mRNA by RT-PCR using TRY FOR 3 (Fig 6.7.) and poly T-G primers. Amino acids are given in italics and * denotes those that correspond to the predicted amino acid sequence of the TRY FOR 3 primer. Amino acids in bold depict a region exhibiting 87 % identity with ADP/ATP translocases isolated from *D. melanogaster*. Homologous amino acids to an ADP/ATP translocase isolated from *D. melanogaster* (GNL PID E293083 (Y10618), E = 2.8 x 10^{-11}) are underlined.

5'- ACG GTG GGG GAA TTT TTC GAG GGG GCC TTC TCC AAC GTC CTC AGA GGT ACC

T V* G E* F* F* E* G* A* F S N V L R G T*

GGA GGT GCC TTC GTG CTT GTG TTA TAT GAT GAA ATT AAA AAG CTT CTC TAA - 3'

G G A F V L V L Y D E I K K L L Z

APPENDIX 8 PUBLICATIONS.

Fitches E., Gatehouse A.M.R. & Gatehouse J.A. (1997) Effects of Snowdrop Lectin (GNA) Delivered Via Artificial diet and Transgenic plants on the Development of the Tomato Moth (*Lacanobia oleracea*) Larvae in Laboratory and Glasshouse Trials. *J. Insect Physiol.* **Vol 43,** No 8, 727-739.

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Bell H.A. Fitches E.C., Down R.E., Marris G.C., Edwards J.A., Gatehouse A.M.R. (submitted) The Effects of Snowdrop Lectin (GNA) delivered via Artificial Diet and Transgenic Plants on *Eulophus pennicornis* (Hymenoptera: Eulophidae), a Parasitoid of the Tomato Moth *Lacanobia oleracea* (Lepidoptera: Noctuidae) *J. Insect Physiol.*

Fitches E., Woodhouse S., Leitch B.L., Gatehouse J.A. (in preparation) *In vitro* and *in vivo* binding of the Snowdrop lectin GNA and Jackbean lectin Concanavalin A to the alimentary tract of tomato moth larvae (*Lacanobia oleracea*) and uptake into the systemic circulation.

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