Antimetabolic proteins from plants and their potential use in conferring resistance against corn rootworms

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ANTIMETABOLIC PROTEINS FROM PLANTS AND THEIR POTENTIAL USE IN CONFERRING RESISTANCE AGAINST CORN ROOTWORMS (*DIABROTICA* SP.)

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ABSTRACT

The major digestive enzymes of larval *Diabrotica undecimpunctata howardi*, the southern corn rootworm (SCR), have been investigated. A number of proteases have been identified, the majority cysteine proteases, some aspartic acid proteases are also present. *In vitro* assays of cysteine proteases showed that almost all activity could be arrested by E64 or chicken egg-white cystatin. This activity was also affected by TLCK, CpTI and thaumatin.

Two inhibitory activities were demonstrated in protein extracts from rice seed. The first, oryzacystatin-I, caused marked inhibition of both the insect cysteine proteases and papain. The second produced strong inhibition of insect cysteine proteases but left papain virtually unaffected. Amino acid sequence data for this novel inhibitor was obtained, and significant homology demonstrated to the rice allergenic proteins (Izumi *et al.*, 1992; Adachi *et al.*, 1993). Oryzacystatin-I was expressed as a fully functional fusion protein (Rozc) in *Escherchia coli*, isolated, characterised and employed in feeding trials with larval SCR, a significant rise in mortality was demonstrated. Other protease inhibitors were also assayed *in vivo*, but none exhibited the efficacy of Rozc.

A single iso-form of α-amylase was identified and characterised. *In vitro* assays with amylase inhibitors demonstrated the effectiveness of WAAI and the weak effect of BAAI. WAAI, employed in bioassay, produced a significant decrease in survival.

Five lectins were tested by bioassay. WGA and GNA caused marked alterations in larval development, GNA was most effective. Saporin caused little effect when incorporated into artificial diet. WAAI, CpTI and GNA were employed in combination bioassays. An enhanced level of effectiveness was demonstrated with the double and triple combinations assayed.

While further work is necessary, especially assaying protein efficacy *in planta* and establishing mechanisms of action, this project has successfully demonstrated the clear potential of plant antimetabolic proteins for use in the enhancement of inherent resistance of crop plants to insects, and of employing a number of proteins in a multi-mechanistic defence.

CONTENTS

FIGURES

Abbreviations

AMC	-	7-amido-4-methylcoumarin
APS	-	ammonium persulphate
b(p)	-	base (pair)
BAAI	-	common bean α-amylase inhibitor
BApNA	-	α-*N*-benzoyl-DL-arginine-p-nitroanilide
BSA	-	Bovine Serum Albumin
Bz-	-	α-*N*-benzoyl
(c)DNA	-	(complementary) deoxyribonucleic acid
CP	-	cysteine protease
CPI	-	cysteine protease inhibitor
CpTI	-	Cowpea trypsin inhibitor
(k)Da	-	(kilo) Dalton
DFP	-	diisopropylphosphofluoridate
DMSO	-	dimethyl sulphoxide
DNase	-	deoxyribonuclease
DNSA	-	3,5-dinitrosalicylic acid
DTT	-	dithio threitol
E64	-	L-*trans*-Epoxysuccinyl-leucylamido(4-guanidino)butane
ECL	-	Enhanced Chemiluminescent
EDTA	-	Ethylenediaminatetra-acetic acid
GNA	-	*Galanthus nivalis* agglutinin (snowdrop lectin)
HPLC	-	high pressure liquid chromatography
IPTG	-	isopropyl-ß-D-thiogalactoside
L	-	litre
LGE	-	SCR larval gut extract
mRNA	-	messenger ribonucleic acid
NCR	-	Northern Corn Rootworm
O.D.	-	Optical Density
ozc	-	oryzacystatin

PAGE	-	polyacrylamide gel electrophoresis
PBS	-	phosphate-buffered saline
pCMB	-	p-Chloromercuribenzoic acid
pCMS	-	p-Chloromercuriphenylsulphonic acid
PMSF	-	phenylmethylsulphonyl fluoride
RIP	-	ribosome inactivating protein
RNase	-	ribonuclease
Rozc	-	recombinant oryzacystatin
SAGE	-	SCR adult gut extract
SCR	-	Southern Corn Rootworm
SDS	-	sodium dodecyl sulphate (or lauryl sulphate)
sp.	-	species
TBS	-	Tris-buffered saline
TCA	-	trichloroacetic acid
TEMED	-	NNN'N'tetramethylethylenediamine
TLCK	-	N-tosyl-L-lysine chloromethyl ketone
TPCK	-	N-tosyl-L-phenylalanine chloromethyl ketone
Tris	-	Tris(hydroxymethyl)aminomethane
Tween-20	-	Polyoxyethylenesorbitan monolaurate
U	-	Units
WAAI	-	wheat α-amylase inhibitor
WAGE	-	WCR adult gut extract
WCR	-	western corn rootworm
WGA	-	wheat germ agglutinin (wheat germ lectin)
Xgal	-	5-bromo-4-chloro-3-indolyl-ß-D-galactoside
Z-	-	α-*N*-benzyloxycarbonyl

Single letter abbreviations for amino acids and bases are as specified in : *Biochem. J.* (1984) 219, 345-373 and *Biochem. J.* 229, 281-286, respectively.

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CHAPTER 1 INTRODUCTION

In recent years we have become increasingly aware of the problems of pest control confronting today's farmers. A multitude of insecticides is widely available and used extensively, resulting in a total global expenditure on insecticides for 1990 of $7,655 million; but despite this, pest resistance and resurgence, pollution of the environment, and death and illness of humans and wildlife, are clear indications that we are far from winning the "war" against insect pests.

Probably the most expensive insect pests in North America are the corn rootworms, members of the *Diabrotica* genus, the larvae of which feed voraciously on the roots of field maize and a number of other crops. Rootworm control is complicated by the subterranean habit of the larvae, relying largely on soil insecticides, which, by 1976, were routinely applied to 50-60% of the total maize acreage. Despite this, by 1986, the toll of larval feeding on maize roots, in terms of treatment costs and crop losses, had reached around $1 billion per year, with an additional cost of $40-50 million for the aerial sprays applied during extensive outbreaks, and another $50-100 million of damage, per year, caused by *Diabrotica* species attacking other crops, such as peanut and sweet potato.

Rootworms have already developed resistance to a number of insecticides, such as heptachlor and cyclodiene, and, with the growing expanse and intensity of maize farming in the American corn-belt, they have managed to increase their distribution remarkably within the last 50 years, the resistant strains spreading further than those susceptible to insecticide treatment. Unquestionably, alternative strategies are needed to improve control and reduce the pressures for resistance development, this project aimed to investigate the potential of one such approach.

One way of decreasing the amount of insecticide necessary would be to enhance the inherent resistance of crop plants to pest attack. This

could be achieved with traditional breeding programs where resistant lines are available, or, as in the case of maize and the corn rootworms, where no effective resistance is displayed within a species, genetic engineering could be used to transfer resistance between species. There is a vast array of proteins produced by plants which exhibit toxic or antimetabolic properties towards insects, and which could be expressed in transgenic plants to confer insect resistance or to increase tolerance to insect attack.

The successful enhancement of resistance by the transfer of genes encoding such proteins has recently been demonstrated with the expression of CpTI, a trypsin inhibitor from cowpea (*Vigna unguiculata*) in transgenic tobacco (Hilder *et al.*, 1987), plec, a lectin from pea (*Pisum sativum*) in transgenic tobacco and potato (Boulter *et al.*, 1990; Barsby, 1991), and protease inhibitors from both tomato and potato in transgenic tobacco (Johnson *et al.*, 1989).

The aims of this project were to investigate the presence of such natural resistance factors in a variety of plants and to evaluate their potential for use in the protection of maize from corn rootworm attack.

DIABROTICA SPECIES - THE CORN ROOTWORMS

The genus *Diabrotica* Chevrolat is largely neotropical and includes some of North America's most destructive and expensive insect pests. The larval stage is most pernicious in the case of maize, causing reduction in yield both by actual damage to the root system and through fallen plants which cause harvesting problems. Adults also cause damage, feeding on the aerial parts of the plants, often causing poor kernel set, and can act as vectors for some plant diseases, such as maize chlorotic mottle virus and cowpea severe mosaic virus (Gergerich *et al.*, 1986).

The western corn rootworm (WCR), *Diabrotica virgifera virgifera* LeConte, and northern corn rootworm (NCR), *D. barberi* Smith and Lawrence, are widely distributed throughout the central and mid-western United States where their primary and most economically important host is field maize, *Zea mays* L. (Chiang, 1973). The NCR and WCR have a similar life-cycle, the eggs overwinter in the soil and hatch in late spring (Branson, 1987). The larvae feed on only a limited range of plants in the Gramineae family, the most important host plant being maize. In mid-summer adult emergence begins, the adults feeding on the pollen, silks and leaves of maize, along with other plant species. A few weeks later, egg laying starts and continues until the beetles are killed by frost.

The southern corn rootworm (SCR), *D. undecimpunctata howardii* Barber, has a broader host range and is an economic pest of peanuts, sweet potato and maize in the south-eastern United States. Unlike the northern and western corn rootworms, the SCR overwinter as hibernating adults, which, unable to survive the sub-zero conditions of the central and northern regions of North America, migrate to the southern United States for winter. During the following warm season these adults migrate again, extending their range into most of North America east of the Rocky Mountains, as indicated in Figure 1 (redrawn from Krysan, 1986).

Distribution of *Diabrotica undecimpunctata howardi*, the southern corn rootworm in North America, shown as shaded regions. (Redrawn from Krysan, 1986).

The eggs, laid in spring, soon hatch and the polyphagous SCR larvae feed on the roots of many species including members of the Cucurbitaceae, Leguminosae, Solanaceae, Compositae, Cyperaceae and Convolvulaceae, as well as plants of the Gramineae family (Krysan, 1986). Adult emergence begins in mid-summer.

In all species, the newly hatched larvae feed primarily on the root hairs and outer cortical tissue. As they become older and the food requirement increases, they burrow deeper into the cortical parenchyma, causing channelling to occur in the stele, thus causing greater damage to the plant. Feeding is not limited to the roots, in the insectary larvae will readily feed on the kernel and stem of newly germinated maize, but in the field situation, where maize plants are more established, the only susceptible point for attack may be the new roots and root hairs.

The tolerance of many maize cultivars to corn rootworm infestation has been evaluated on the basis of root volume, root dry weight and pulling resistance (see Smith, 1989), but the generally recognised economic threshold of only 10 rootworm larvae per maize plant indicates both the level of damage caused per larva and the susceptibility of most of the varieties commonly grown.

Control of *Diabrotica* species

Various tactics have been employed in the attempt to control corn rootworms and their damage. For many years, a 4-year rotation of maize, oats and clover, used by farmers in the mid-western United States, successfully kept corn rootworm populations below economically damaging levels (Flint & van den Bosch, 1981). The arrival of effective soil insecticides, which decreased larval numbers in the root zone when applied at the time of planting or first cultivation (Mayo & Peters, 1978; Mayo, 1980), however, made this 4-year rotation unnecessary, and, consequently, it was abandoned by almost all maize growers, since producing maize every year

was far more profitable. This discontinuation of the successful rotation programs led to the resurgence of rootworm populations, requiring an increase in pesticide usage, which in turn resulted in increased soil pesticide residues. Broadcast insecticide applications were also employed to suppress the adult population, reducing the number of eggs laid, and thus depressing the ensuing larval population and lowering subsequent damage (Pruess *et al.*, 1974).

Soon there were reports of ineffective control from the insecticides, and resistant biotypes were demonstrated in several areas, first in WCR populations (Ball & Weekman, 1962, 1963). Some of these strains, resistant to insecticides such as aldrin, heptachlor and cyclodiene, showed an increased fitness over the susceptible race, and spread rapidly throughout the corn-belt, causing competitive displacement of the susceptible strains (Krysan, 1986). Sutter (1982) found that SCR larvae were more tolerant of a number of insecticides than were larvae of the WCR. Similarly, Chio *et al.* (1978) showed that while substantial resistance to aldrin and heptachlor occurred in the northern, western and southern corn rootworms, SCR larvae also demonstrated marked resistance to the effects of a number of other insecticides, including DDT, fonofos, malathion and diazinon. The emergence of resistant biotypes, however, was not the only cause of the decreasing efficacy of insecticides, the adaptation of soil microbes, in areas of extensive usage, to utilise persistent insecticides, rapidly degrading them soon after application, dramatically reduced the active concentration of soil insecticides, the resultant lack of insecticide efficacy often appearing at first sight to be caused by resistance in the insects (Felsot *et al.*, 1982). Environmental factors have also been shown to greatly influence the efficacy of soil insecticides; some years an application might be successful, while under different environmental conditions, another year, the WCR population might actually be increased by the application of the same insecticide (Gray *et al.*, 1992).

Despite the decreasing returns from such extensive pesticide use, farmers were and still are, for economic reasons, unwilling to return to the 4-year rotation plan. Many insecticides were removed from the recommended list for use in rootworm control, either because of the emergence of resistant biotypes or the occurrence of adverse environmental effects, and the few products replacing them were used to such an extent that pressure for the development of resistance to these new chemicals was immense.

Clearly, novel insecticides or alternative strategies were urgently needed to overcome this mounting problem. Much of the present day control effort, however, still involves the widespread application of insecticides and the problem is now such that rootworms are probably the most expensive insect pests in North America. Increased public pressure for environmental protection (such as protecting non-target organisms from damage and ground water from contamination), and the future threat of tighter legal constraints in the United States on traditional pesticide use, has increased interest in the development of more biorational corn rootworm management technology.

Alternative control strategies

Methods concentrating on cultural and edaphic factors, such as fall-ploughing and no-tillage systems, have been investigated. With fall-ploughing, tilling the land during the autumn months, the soil was broken into clumps which created a larger surface exposed to the winter cold, exposing the eggs to lower temperatures and so reducing their viability. This, however, relies on the soil remaining in clumps after ploughing, if the soil crumbles, then ploughing tends to move the eggs to a greater depth, giving them greater protection from the cold, hence increasing, rather than decreasing, the population (Chiang, 1973).

Effective herbicides have minimised the need for cultivation, allowing a no-tillage system being employed. The dense ground cover, common to

this practice, attracts adults for oviposition, yet because the eggs remain undisturbed throughout their development, they are concentrated along the preceding year's rows. Planting between these rows in the following year has been shown to reduce the population as larvae have a limited host-search range (Strnad & Dunn, 1990; Brust, 1991).

Delayed planting of maize has also been used, with the aim of reducing the survival of larvae by the delayed chronological occurrence of lifestages, but the results have been greatly variable (Fisher *et al.*, 1990). Investigations into the potential use of feeding deterrents, instead of insecticides, have also produced some promising results (Landis & Gould, 1989).

Research into the possible use of semiochemicals (behaviour modifying chemicals) and baited traps has drawn much interest, with the idea of employing sex pheromones and kairomones, the chemicals used in host-location, to lure the adults away from crops and oviposition sites, to traps baited with insecticides. The potential of such attractants was demonstrated by Weissling *et al.* (1989) using semiochemicals encapsulated in a starch borate matrix (SBM), allowing a sustained release of the active chemical over a period of time. Baits employing these SBM-encapsulated semiochemicals, along with insecticides, were shown to both attract and kill corn rootworm adults. This strategy is particularly attractive because of the selectivity of the control (Weissling *et al.*, 1991), and the restriction of insecticides to the traps, allowing high doses of toxic insecticides to be used with little threat of harm to non-target species or damage to the environment. The effectiveness of these traps, however, was dramatically affected by the seasonal responses of rootworms to the semiochemicals used, possibly restricting their overall usefulness in a rootworm control program.

Increasing inherent resistance

Natural resistance has been found to occur in maize in the form of a recessive gene (Sifuentes & Painter, 1964). Xie *et al.* (1992) have suggested that a contributing factor might be the levels of hydroxamic acid in maize roots. However, despite the appreciable improvements in yield demonstrated in several professed rootworm-tolerant maize hybrids (Smith, 1989), the corn rootworms still caused serious amounts of damage. With recurrent selection and backcross breeding having had only limited success in increasing these levels of resistance (Smith, 1989), there appears to be no natural resistance factor, capable of providing effective protection against the rootworms, present within the genetic pool encompassed by the maize species available to conventional breeders. Recent advances in technology have opened up a further possibility, the transfer of resistance between species, by the use of genetic engineering to transfer the genes necessary for the production of the resistance factor.

Increasing inherent resistance by genetic engineering

Some plants exhibit few defence mechanisms, while in others, such as some crop plants, resistance factors have been actively bred out to increase their palatability. Many plants, however, over the course of evolution, have developed a sophisticated array of mechanisms to protect themselves from attacking insects. These range from the physical armaments of spines, sticky hairs or tough leathery surfaces, to the production of chemicals that deter, injure or kill attackers. Physical defences are not really suitable for transfer between species due to the large changes in phenotype necessary. Those secondary metabolites produced as a result of complex biosynthetic pathways, are also not generally suitable to date, due to the complicated transfer of the numerous genes involved in the compound's synthesis and the restricting state of current technology.

Primary Gene Products

Despite these constraints, a range of defence mechanisms remain available, and highly appropriate, for transfer between species. These are the protein antimetabolites, a number of different types of protein with toxic or antimetabolic properties. As primary gene products, their expression in transgenic plants would require the transfer of only a single gene. The beauty of using genetic engineering to confer the defence mechanism of one plant to another is that the protection is then present when required throughout the season, whether constantly or as a result of triggering, regardless of the weather conditions, and is actually contained within the tissues where protection is needed, and thus causes no threat to non-target organisms.

Experiments designed to test whether any penalty in terms of yield, vigour, or stress resistance would be paid by plants because of the expression of a foreign protein (in this case CpTI in transgenic tobacco plants) demonstrated no reduction in plant quality because of CpTI expression, other than the small penalty resulting from the transformation/regeneration process (Hilder & Gatehouse, 1991).

In nature, wild plants generally display several different defence mechanisms, any one of which alone is not completely effective in protecting the plant from predators, but which together provide an effective level of protection. Using this method of employing primary gene products to enhance resistance, it would be possible to mimic this natural situation by the introduction of more than one type of resistance gene into individual crop plants. The validity of this multi-genic, multi-mechanistic approach was demonstrated by Boulter *et al.* (1990) with the combined expression of CpTI and plec in transgenic tobacco, demonstrating that expression of the two proteins provided greater protection from *Heliothis virescens* (tobacco budworm) than did each protein separately.

Insect digestive enzymes

Unless a protein functions as a repellent or feeding deterrent, then its defence mechanism must act through the ingestion of the protein. One obvious mode of action, employed by a number of the plant antimetabolic proteins, is the inhibition of enzyme activity in the gut, leading to nutritional or metabolic problems. Clearly the effects of ingested proteins *in vivo* are most accurately examined by the use of feeding trials, incorporating proteins into artificial diet or, preferably, using transgenic plants expressing the protein at known levels. In the case of enzyme inhibitors, however, preliminary assays are advisable, in order to establish the types of enzyme activity demonstrated in the gut of the target insect, allowing a considered choice of the inhibitors to be tested. The digestive enzymes of insects are numerous and varied, the most extensively studied are the proteases and amylases, although others, such as maltases, glucosidases, galactosidases, mannosidases, trehalases and lysozymes have been identified (Colepicolo-Neto *et al.*, 1986, 1987; Gatehouse & Anstee 1983; Gatehouse *et al.*, 1985b; Santos *et al.*, 1983; Espinoza-Fuentes & Terra, 1987; also review Applebaum, 1985). Cellulases, presumably of bacterial origin, have also been identified in insect gut extracts (Colepicolo-Neto *et al.*, 1986, 1987). As the majority and the best studied of the plant enzyme inhibitors are those which affect the activities of various proteases and α-amylases, it was the study of these enzymes and inhibitors on which this project was concentrated. Accordingly, only these two classes of enzyme will be discussed here.

Proteolytic enzymes

Proteases, the enzymes which catalyse protein digestion, are classified into four groups according to their catalytic mechanism: serine proteases with a serine residue in the active centre; cysteine proteases with a cysteine residue in the active centre; aspartic acid proteases with an acidic

amino acid residue; and metalloproteases with an essential metal ion involved in the catalytic mechanism. (Storey & Wagner, 1986)

Serine proteases

The serine proteases form a large group of enzymes, widely distributed in nature, distinguished by the reactivity of a serine residue in the active site and by the inhibition of their activity by DFP (see Dixon & Webb, 1979). Serine proteases generally act as endopeptidases, cleaving within the peptide, cleavage of a terminal bond generally being inhibited by the charge on the carboxyl or amino group of the terminal residue.

In humans, and other vertebrates with a distinct pancreas, serine proteases are produced as inactive zymogens (Edwards & Hassall, 1971) and converted to the active enzyme by the action of trypsin, trypsin itself also being produced in this way. The most extensively studied of the serine proteases are chymotrypsin and trypsin, both of which are employed in the human digestive system.

Chymotrypsin shows a preference to cleave peptide chains at the carboxyl-side of aromatic amino acid residues (Laskowski, 1955), while trypsin shows a greater specificity, hydrolysing bonds on the carboxyl-side of the basic amino acids, lysine and arginine (Walsh & Wilcox, 1970). The active site of chymotrypsin has been shown to occur in a depression in the surface of the enzyme, next to a deep cleft which holds the aromatic side chain of the residue next to the sissile peptide bond (see review, Eisenberg, 1970). Active site-directed inhibition, pH assays and chemical substitutions have confirmed the involvement of an activated serine residue in the active centre, and also demonstrated the close involvement of a histidine residue, His^{57} (Ong *et al.*, 1965), and a buried aspartic acid residue, Asp^{102}, acting as a charge relay system, activating the serine residue (Blow *et al.*, 1969).

Most serine proteases can be inhibited by PMSF (phenylmethanesulphonyl fluoride), by the sulphonation of the active serine

residue in the active centre of the enzyme (Gold, 1967; James, 1978). Although no inhibition is demonstrated towards metalloproteases, aspartic acid proteases and most cysteine proteases, papain and other similar cysteine proteases are inhibited by PMSF, as are some non-protease enzymes, thus limiting the use of PMSF with crude enzyme preparations. Trypsin and chymotrypsin can be distinguished by the use of the inhibitors, TLCK and TPCK. TLCK (N-tosyl-L-lysine chloromethyl ketone) irreversibly inhibits proteases which cleave specifically next to a lysine residue, such as trypsin (but not chymotrypsin) by forming a covalent bond with the imidazole group of a histidine residue in the active centre of the enzyme (Mumford *et al.*, 1981). TPCK (N-tosyl-L-phenylalanine chloromethyl ketone) irreversibly inhibits chymotrypsin and other proteases which cleave specifically next to a phenylalanine residue, trypsin is not inhibited (Ong *et al.*, 1965). TPCK, like TLCK, inhibits by mimicking the substrate, forming a covalent bond with a histidine in or near the active centre of the protease. Both inhibitors also inhibit some cysteine proteases.

Cysteine proteases

Many proteases depend for their activity on the highly reactive thiol group of a cysteine residue at their catalytic site. These cysteine proteases are not DFP-sensitive. Mammalian cysteine proteases, located in the lysosomes of cells in the liver, kidney and spleen (Turk, 1986), are responsible for much of the bulk turnover of proteins in the cell. However, cysteine proteases are not employed in the digestive systems of mammals.

The best studied cysteine protease is papain, from the fruit of *Carica papaya*, first isolated in a crystalline form by Balls *et al.* (1937). The single polypeptide chain of papain is folded into two distinct sections, divided by a groove inside which lies the active centre of the enzyme (see review, Eisenberg, 1970). In the native crystalline form the thiol group is blocked, allowing little protease activity to occur. The addition of mild reducing

agents, such as cysteine, free the thiol group and activate the enzyme; optimum activation can be achieved by the simultaneous application of a thiol compound, such as cysteine, and a heavy metal-binding agent like EDTA (Arnon, 1970). Protease activity can be inhibited by heavy metal ions, such as Zn^{2+}, Cd^{2+}, Cu^{2+}, Hg^{2+}, Fe^{2+} and Pb^{2+}, in these cases reactivation is possible by the addition of cysteine and EDTA. Thiol-blocking reagents, such as iodoacetic acid and pCMB also cause inhibition of cysteine proteases, but their reactivity with low molecular weight thiol compounds causes problems when used under the usual conditions of assay of cysteine proteases, ie. in the presence of thiol activators, and they cannot be relied on to exhibit stoichiometric inhibition under these conditions (Barrett *et al.*, 1982). TLCK and TPCK, as mentioned earlier, cause loss of papain activity, acting specifically on the active thiol group of the enzyme, rather than the imidazole group of a histidine residue as in the case of trypsin or chymotrypsin, resulting in a stoichiometric inhibition. A non-competitive, irreversible inhibitor specific for cysteine proteases is E64 (L-*trans*-Epoxysuccinyl-leucylamido(4-guanidino)butane), isolated from cultures of *Aspergillus japonicus*, causing stoichiometric inhibition by the formation of an ether bond with the active thiol of the enzyme, E64 does not react with other thiol compounds (Barrett *et al.*, 1982).

The cysteine proteases form a group of homologous proteins, known as the "papain superfamily", and including the mammalian lysosomal enzymes, Cathepsins B, L and H, and the plant cysteine proteases, papain, chymopapain, ficin and actinidin. The amino acid sequences of these enzymes have been found to be closely related, and the enzymes demonstrated to have similar folding structures and employ identical catalytic groups (Takio *et al.*, 1983; Portnoy *et al.*, 1986; Ishidoh *et al.*, 1987a, 1987b). Their proteolytic specificities, however, are vastly divergent, for example, while all are believed to act as endoproteases, Cathepsin H may also act as an aminopeptidase, cleaving at the amino-terminus of the

substrate, and Cathepsin B as a carboxypeptidase, attacking bonds at the carboxy-terminus as well as those within the substrate molecule (Koga *et al.*, 1990; Barrett and Kirschke, 1981). Using the differences in specificity, and employing synthetic peptides as substrates, allows one to distinguish between the mammalian cysteine proteases: Cathepsin H will readily hydrolyse H-Arg-AMC and has a greater affinity for Bz-Arg-2-naphthylamide than does Cathepsin B, but is unable to digest either Z-Phe-Arg-AMC or Z-Arg-Arg-AMC; Cathepsin B hydrolyses Bz-Arg-2-naphthylamide, Z-Phe-Arg-AMC and Z-Arg-Arg-AMC; and Cathepsin L, while demonstrating far greater proteolytic activity than Cathepsin B (Turk, 1986), is more specific and consequently unable to hydrolyse many of the synthetic substrates of Cathepsin B, it does, however, show a strong affinity for Z-Phe-Arg-AMC (Barrett and Kirschke, 1981). Cathepsin L was found to be similar to papain, having analogous folding structures and substrate specificities, both showing a preference for hydrophobic amino acids at the P_2 sub-site, the residue next but one to the sissile peptide bond of the substrate, although Cathepsin L showed a greater preference for bulky, hydrophobic amino acids at the S_3 sub-site of the enzyme than did papain (Koga *et al.*, 1990).

Aspartic acid proteases

The best studied aspartic acid protease is pepsin, an endopeptidase which, unlike the serine and cysteine proteases, hydrolyses only peptide bonds, and not those of esters or amides. Pepsin, on a molecular basis, acts more slowly than other proteases on proteins, and even more slowly on peptides. Pepsin shows a preference for substrates with aromatic amino acids on either side of the sissile bond, hydrolysing bonds mainly on the amino-side of phenylalanine and tyrosine residues (Edwards & Hassall, 1971). pH and chemical substitution experiments have demonstrated that two carboxyl groups, on the active site residues Asp^{32} and Asp^{215}, are catalytically important to the activity of the enzyme.

Pepsin, in mammals, is produced as the zymogen pepsinogen, secreted by cells known as chief cells, located in the wall of the stomach. Pepsinogen is unstable below pH6 and is autocatalytically converted to the active pepsin. Maximal protease activity is demonstrated around pH1.8 - 2.0 (Herriott, 1955). Pepsin activity can inhibited by pepstatin, which forms a tight 1:1 complex with aspartic acid proteases, and which has no effect on the activities of cysteine or serine proteases (Umezawa, 1976; Marciniszyn *et al.*, 1976).

Metalloproteases

Metalloproteases possess an essential metal ion, involved in the catalytic mechanism of the enzyme. Carboxypeptidases are the best studied metalloproteases, these enzymes act only on the terminal amino acid residues at the carboxyl end of the protein. Carboxypeptidase requires a free carboxyl group in the substrate, and is unable to hydrolyse the amides and esters of simple alcohols that are readily digested by serine and cysteine proteases. The specificity of carboxypeptidase is determined primarily by the amino acid bearing the free carboxyl group and only affected to a small extent by the section of the molecule on the N-terminal side of this residue (Dixon & Webb, 1979). Cleavage is most rapid with residues with aromatic and branched aliphatic side chains (Eisenberg, 1970). A good substrate for metalloproteases is the synthetic peptide, Succinyl-Ala-Ala-Phe-AMC, the Ala-Phe bond being hydrolysed (Mundy & Strittmatter, 1985; Mumford *et al.*, 1980).

Carboxypeptidase contains one tightly bound zinc ion per molecule. Determination of the tertiary structure of carboxypeptidase revealed that this zinc ion was held in the active centre of the molecule, co-ordinated to three groups on His^{69}, Glu^{72} and His^{196}, with the fourth co-ordination position occupied by a water molecule in the free enzyme, this water being displaced by the binding of a substrate molecule (Hartsuck & Lipscomb,

1971; Reeke *et al.*, 1967). Removal of the zinc ion, by dialysis or the addition of a chelating agent such as EDTA, results in the complete loss of activity. Activity may be restored by the addition of zinc ions or other metal ions including Fe^{2+}, Mn^{2+}, Co^{2+} and Ni^{2+} (Coleman & Vallee, 1960). Carboxypeptidase activity can also be inhibited by structural analogues of specific substrates, notably aromatic D-amino acids and certain aromatic and heterocyclic carboxylic acids, the most effective inhibitor is ß-phenylpropionic acid (Neurath, 1955).

Insect proteases

Corn rootworms, like other insects, obtain essential amino acids from their diet by means of extracellular proteases acting in the lumen of the gut. Essential amino acids are those which either cannot be synthesized in the body, or cannot be produced at sufficient speed to satisfy the needs of the body, and such amino acids must be obtained from the diet, usually by the breakdown of dietary proteins. Free amino acids are necessary for the synthesis of enzymes and tissue proteins, the turnover rate of which varies in different tissues.

The digestive serine proteases, chymotrypsin and trypsin, and the aspartic acid protease, pepsin, were the first proteases to be studied in insects because of their principal roles in the digestive systems of vertebrates. Several groups reported that trypsin was the chief digestive protease in insects (see review, Applebaum, 1985). Indeed many insects have been found to exhibit serine protease activity, notably the Lepidopteran larvae, (Applebaum, 1985), but insects are now known to employ a variety of different proteases spanning all the classes of proteolytic enzymes.

Cysteine and aspartic acid proteases have been identified from members of the Hemiptera (Houseman & Downe, 1982a, 1982b) and in the Coleopteran species *Leptinotarsa decemlineata* (Colorado potato beetle) (Thie

& Houseman, 1990b), and *Callosobruchus maculatus* (cowpea weevil) (Gatehouse *et al.*, 1985a, Campos *et al.*, 1989; Silva & Xavier-Filho, 1991). Pepsin-like enzymes have also been characterised in some Dipteran species, such as *Musca domestica* (Espinoza-Fuentes & Terra, 1987). An alkaline gut has been found to be characteristic of Lepidopteran larvae, along with the presence of trypsin-like serine proteases whose pH optima correspond to that of the gut (Applebaum, 1985). Serine protease activity has also been confirmed in the guts of various Coleopteran species such as the carabid beetles *Pterostichus melanarius* (Gooding & Huang, 1969), *Carabus auronitens*, *C. punctatoauratus*, *C. lineatus*, and *C. splendens* (Vaje *et al.*, 1984), the sweet potato weevil *Cylas formicarius elegantulus* (Baker *et al.*, 1984), the elaterid beetle *Pyrophorus divergens* (Colepicolo-Neto *et al.*, 1986, 1987), the scarab beetle (grass grub larva) *Costelytra zealandica* (Christeller *et al.*, 1989), and the yellow mealworm *Tenebrio molitor* (Thie & Houseman, 1990a). It must be noted that while some of these enzymes resemble trypsin or chymotrypsin, others are distinct in their substrate specificities and their susceptibility to inhibition.

Cysteine protease activity, as mentioned above, has been identified in the cowpea weevil *Callosobruchus maculatus* and the Colorado potato beetle *Leptinotarsa decemlineata*. Thie & Houseman (1990a) demonstrated cysteine protease activity in extracts from *Tenebrio molitor*, and Murdock *et al.* (1987), who investigated the digestive protease activity of a variety of seed and leaf eating beetles, including *Diabrotica* sp., *Leptinotarsa decemlineata* and *Tribolium castaneum*, demonstrated cysteine protease activity *in vitro* for ten out of the eleven species tested, with some species found to employ cysteine proteases as their principal digestive proteolytic enzymes.

It appears, then, that cysteine proteases are commonly used throughout the Coleoptera, although not ubiquitously so, the balance presumably resulting from the composition of their normal diet or else reflecting phylogenetic traits (Colepicolo-Neto *et al.*, 1987). Direct

comparisons between these insect proteases and the plant and mammalian cysteine proteases discussed earlier, are complicated by the lack of highly specific inhibitors and substrates, although the use of synthetic peptides allows some useful comparisons to be made. Determination of amino acid sequences for insect proteases would be a great step forward allowing sequence, and possibly structural, comparison with both mammalian and plant enzymes.

All four classes of protease are represented in the digestive systems of insects as a group, with some insects known to possess a few specialised proteases and others exhibiting a broader range. One cannot, therefore, simply assume that certain proteases will be present in any one insect species, rather it is essential to individually characterise the digestive enzymes of each specific target insect, if an intended control strategy is to be based on the action of protease inhibitors.

Amylases

Starch is the principal reserve polysaccharide in higher plants, large amounts are accumulated in the storage organs, the roots, rhizomes, tubers, stem pith and seeds of various species, and also accumulated in the chloroplasts during photosynthesis. Starch is made up of two components, amylose and amylopectin. Amylose is a linear polymer of glucose residues linked by α-1,4-glycosidic bonds, the same type of polymer makes up amylopectin, but this molecule has a branched structure, the chains, at the branching points, being linked by α-1,6-glycosidic bonds.

Maltose, produced by the digestion of starch, is further hydrolysed by maltases, releasing glucose. This glucose, by way of the glycosidic pathway and the Kreb's cycle, is metabolised to produce energy. Glucose is generally the major source of energy for the metabolic processes carried out in the cell, such as the enzyme reactions, nutrient degradation, amino acid production, and protein degradation and synthesis, enabling an

organism to survive. Glucose can also be derived from sucrose and other oligo- and polysaccharides, other sources of energy include other carbohydrates, excess amino acids and lipids.

The enzymes which catalyse the hydrolysis of starch, the α-amylases and ß-amylases, both attack α-1,4-glycosidic bonds, but differ in the location of the particular bond selected. Neither enzyme cleaves α-1,6-glycosidic bonds. α-amylases act mainly as endoamylases, the location of the bond to be hydrolysed within the molecule, apparently selected at random, although cleavage of terminal bonds is slower that internal bonds. The result, in the initial stages of the reaction, when the molecules are first split into a few large sections, is a rapid decrease in the molecular weight of the substrate, while the final products are maltose, limit dextrins (the highly branched core of amylopectin), and small amounts of glucose. α-amylases from different sources vary in their electrophoretic mobility, solubility and pH tolerance, and in their specific activity. Human salivary α-amylase has an associated Ca^{2+}, the removal of which causes no marked loss of activity (Bernfeld, 1955). Full activity has, however, been demonstrated to require the presence of a chloride ion, the removal of this by dialysis reduces activity, reactivation can be achieved by the addition of Cl^-, or a weaker activation with Br^-, NO_3^-, and I^- (Bernfeld, 1955; Dixon & Webb, 1979).

ß-amylases act as exoamylases, selecting only the penultimate bond from the non-reducing end group of the substrate. A succession of maltose units is released, the enzyme producing an inversion at the bond split as each is released. This successive degradation is halted only when the enzyme nears a branching point in amylopectin, the final products of digestion being maltose and limit dextrin. ß-amylase is apparently unaffected by the presence or absence of chloride or calcium ions, but is inhibited strongly by reagents selective for thiol groups such as pCMB, $AgNO_3$, isobenzoate and $CuSO_4$ (Bernfeld, 1955).

Amylase activity can be monitored by assaying the increase in reducing power of a solution of amylopectin or soluble starch, or the decrease in the viscosity of a starch paste, these will detect both α-amylase and ß-amylase activity (Bernfeld, 1955). One method of distinguishing between these activities is by their ability to alter the iodine-staining properties of amylose. α-amylases, by producing a rapid decrease in the molecular weight of its substrate, cause a rapid change in iodine-staining properties of amylose, an effect produced by the cleavage of very few bonds, accompanied by only a small increase in reducing power and a slight decrease in the viscosity of a starch paste. ß-amylases produce a slow decrease in molecular weight as successive maltose units are removed from the substrate, hence only a slow change in iodine-staining properties is produced.

Insect amylases

With starch as the principal reserve polysaccharide in plants, it is not surprising that amylases are widely distributed amongst insects, those feeding on cereals or legume seeds often possessing higher proportions of amylase activity than insects with a low starch diet (Baker, 1988a).

Much of the research into insect amylase activities has been associated with investigations into the effects of plant derived amylase inhibitors. Early work looking at the effects of plant amylase inhibitors on insect enzymes were conducted with extracts from *Tenebrio molitor* (see reviews by Buonocore *et al.*, 1977; Warchalewski, 1983), but now a wide range of agriculturally important insects have been tested (Gatehouse *et al*, 1986; Baker, 1987; Campos *et al.*, 1989; Bloch & Richardson, 1991; Gutierrez *et al.*, 1990, 1993). Much of this work has been conducted with crude extracts, only in a few cases have the individual iso-α-amylases been identified and their respective susceptibilities been studied. Iso-amylases from *Sitophilus zeamais*, *S. granarius* and *S. oryzae* were identified by Baker (1983, 1987).

Chen *et al.* (1992a) confirmed the presence of two iso-amylases in *S. oryzae* adults, and identified two α-amylase isozymes from *Tribolium castaneum* adults and a single α-amylase from *Tenebrio molitor* adults. Four α-amylase isozymes were identified in *Callosobruchus maculatus* larvae (Campos *et al.*, 1989). Interestingly, not only are amylases from different sources differentially inhibited by various plant amylase inhibitors, but the separate iso-amylase from individual insects are also differentially affected (Baker, 1987; Campos *et al.*, 1989; Gutierrez *et al.*, 1990, 1993).

The absolute importance of *in vivo* testing with inhibitors, rather than reliance on *in vitro* assays, was demonstrated by Gatehouse *et al.* (1986). This group demonstrated that the strong inhibition of extracted *Tribolium confusum* α-amylases by the wheat 0.28 α-amylase inhibitor, was not at all reflected in its effects on larvae *in vivo*, their growth and development being very little affected. This emphasises the fact that not only must susceptibility of specific enzymes be investigated *in vitro*, but the result with an extracted enzyme may be quite different to the situation found with a live insect. The pH of the gut may differ from that used in the assay, possibly reducing or enhancing inhibition, other enzymes present in the gut may act upon the inhibitor denaturing it, or the enzyme may be shielded in some way, preventing inhibition. *In vivo* studies are absolutely essential in order to gain a genuine knowledge of the true effects of plant inhibitors.

Antimetabolic proteins from plants

Not all of the antimetabolic proteins produced by plants act by the inhibition of enzymes. The four categories into which the proteins fall are outlined below, along with some examples:

i) Protease inhibitors

The presence in plants of protease inhibitors active against mammalian digestive enzymes has been known for many years, and was confirmed when

Kunitz first isolated and characterised a trypsin inhibitor from soybean, *Glycine max* (Kunitz, 1946, 1947). Since then, it has become clear that protein protease inhibitors are widespread in plants (Richardson, 1977). Because of the importance of serine proteases in the mammalian digestive system, and the consequential medical and nutritional significance of their inhibitors, the focus of much of the early research was the characterisation of various plant-derived serine protease inhibitors. More recently, the effect of these inhibitors on insect proteases has been investigated, and the lack of apparent serine protease activity in some cases and the lack of inhibition in others, has lead to investigation of the other types of protease employed within the digestive systems of insects and to a search for inhibitory proteins from plants. While inhibitors of serine proteases have been well studied and numerous examples identified, fewer cysteine protease inhibitors have been discovered. To the author's knowledge, inhibitors of metalloproteases have only been isolated from potato and tomato (Rancour & Ryan, 1968; Hass & Ryan, 1980), while aspartic acid protease inhibitors have been reported only in potato (Ritonja *et al.*, 1990).

The mode of action of these protease inhibitors is not clear, they may work by direct inhibition of enzymes, reducing the breakdown of dietary protein and causing a lack of free essential amino acids, alternatively inhibition may induce hyperproduction of proteases to compensate for the inhibited enzymes, and this enzyme synthesis, itself, depleting the available supply of essential, sulphur-containing amino-acids and causing problems with metabolism. Either way, the supplementation of the diet with methionine alone or with other free amino acids in has been shown to reduce some of the detrimental effects in some cases (Gatehouse & Boulter, 1983; Broadway & Duffey, 1986; Hines *et al.*, 1990). Broadway and Duffey (1986) speculated that high endogenous levels of methionine in plant tissues or seeds may increase their susceptibility to predatory insects.

Serine Protease Inhibitors

The serine protease inhibitors are the best studied of the plant protein inhibitors and appear to be numerous and widespread. Because of their possible antinutritional role, most investigations have focused on members of the Leguminosae, Gramineae and Solanaceae, the families containing important staple food crops. These inhibitors fall into several families exhibiting distinct form and specificity of action.

The soybean inhibitor isolated by Kunitz was a double-headed inhibitor of trypsin (Bosterling & Quast, 1981). Similar inhibitors were identified in the seeds of other leguminous species, such as the winged bean (*Psophocarpus tetragonolobus*) (Yamamoto *et al.*, 1983) and *Erythrina latissima* (Joubert *et al.*, 1985) and also in the Mimosoideae and Gramineae. While the legume inhibitors do not appear to affect endogenous enzymes, suggesting a possible protective role, the Kunitz-type inhibitors isolated from cereals seem to have a regulatory role in endogenous, germination specific α-amylase activity (see review, Richardson, 1991).

Another inhibitor isolated from soybean, the soybean "Bowman-Birk inhibitor" (Birk *et al.*, 1963; Odani & Ikenaka, 1972), shares homology with a number of other inhibitors widely distributed throughout the Leguminosae and Gramineae. These are generally double-headed inhibitors, usually of trypsin and chymotrypsin. One prominent member of this group is CpTI, the trypsin inhibitor from cowpea. In feeding trials using purified CpTI incorporated into artificial diets, a number of economically important insect pests were found to be affected adversely. These included the Lepidopterans, *Heliothis virescens*, *H. zea*, and *Spodoptera littoralis*, the Coleopterans, *Callosobruchus maculatus*, *Anthomonas grandis*, and *Diabrotica undecimpunctata*, and also *Locusta migratoria* (Hilder *et al.*, 1989). These workers also screened a number of cowpea varieties for resistance to *Callosobruchus maculatus*, demonstrating that resistance was related to high levels of CpTI in the seed (Gatehouse *et al.*, 1979). Xavier-Filho *et al.*

(1991) have, however, found varieties where this correlation does not hold, and the resistance has been discovered to result from a multi-mechanistic defence strategy. The gene encoding CpTI was genetically engineered into tobacco (Hilder *et al.*, 1987), as mentioned earlier, where it was demonstrated to confer increased resistance to *Heliothis virescens*, the tobacco budworm, thus affirming a protective role for these protein inhibitors in plants.

Serine protease inhibitors identified in potato and tomato by Green and Ryan (1972) were demonstrated to rapidly accumulate as a response to mechanical wounding or insect damage to leaves. This accumulation was found to occur not only in the wounded tissue, but throughout the plant (Ryan, 1978). Broadway *et al.* (1986) confirmed that this response did act to increase protection against insects, in that the available food quality of the plant tissues was markedly decreased, demonstrated by a significant reduction in the growth of larval *Spodoptera exigua* fed on a diet of wounded leaf material compared to those on uninduced leaf diet.

These potato and tomato inhibitors have homologues in other species and are divided into two families. Members of the inhibitor I family are strong inhibitors of chymotrypsin and subtilisin, but only weak inhibitors of trypsin, while those from the inhibitor II family powerfully inhibit trypsin and chymotrypsin. Johnson *et al.* (1989) produced transgenic tobacco plants expressing tomato inhibitor I, tomato inhibitor II or potato inhibitor II in their leaves. Feeding trials with this transgenic material, conducted with larval *Manduca sexta* (tobacco hornworm) demonstrated a significant reduction in larval growth due to the inhibitor II proteins and little effect resulting from the presence of inhibitor I protein, indicating that the inhibition of trypsin was the cause of the reduced growth rate.

Recently another family of inhibitors was discovered, the low molecular weight trypsin inhibitors isolated from members of the Cucurbitaceae (Hojima *et al.*, 1982; Wieczorek *et al.*, 1985; Hara *et al.*, 1989). Despite their relatively small size, these inhibitors are incredibly potent, having

association constants among the highest ever recorded for trypsin inhibitors (Wieczorek *et al.*, 1985).

The cereals also possess a class of homologous proteins which includes numerous enzyme inhibitors, active against serine proteases or amylases, some active against two or more enzyme types, and some with unknown function. This family of proteins will be discussed in greater detail later, with regard to amylase inhibitors.

Despite the differences in specificity of the various serine protease inhibitors, they all seem to react with the enzymes according to a common, substrate-like standard mechanism. While the majority of the structures possessed by the different groups of inhibitors are unrelated, all have an exposed binding loop, compact in shape and containing a hydrophobic core. This exposed loop interacts with the enzyme active site in a similar manner to that of substrates, the exposed side-chains of the loop residues form mostly hydrophobic links with the protease subsites. Intra- and inter-molecular interactions help to stabilize the complex, with the result that the binding is so tight that decomposition seldom occurs. The carboxypeptidase inhibitor from potato acts in a similar manner, although the complex formed takes the form of a enzyme:product-like complex, the carboxy-terminal residue being split off from the inhibitor, but remaining bound within the active centre of the enzyme (see review, Bode & Huber, 1992).

Cysteine Protease Inhibitors

Although much is known about the proteinaceous cysteine protease inhibitors (CPIs) existing in animals, relatively little is known about plant-derived CPIs. Until recently only a few papers were available, dealing with the CPIs in pineapple (Reddy *et al.*, 1975), barley, wheat and rye (Fossum, 1970). In recent years, with the identification of cysteine protease activity in a number of insects, interest in the inhibitors of these enzymes has increased. Cysteine proteases should be extremely suitable targets for

insect control by inhibition, because of their absence from the digestive systems of mammals. Rele *et al.* (1980) assayed a number of seed extracts for protease inhibitory activity and found that while inhibitors of serine proteases were present in some but not all the extracts, inhibitors of papain were present in every tissue tested. These extracts were from *Phaseolus vulgaris, P. aconitifolius, Arachis hypogaea, Glycine max, Vigna unguiculata, Triticum vulgare, Sorghum vulgare, Zea mays, Oryza sativa* and *Brassica oleracea.* Actively growing plant callus and tissue culture were also screened and CPI activity confirmed in most species tested. These data indicate that CPIs are more widespread throughout the plant kingdom than was previously believed, and show that their presence is not restricted to storage tissue. This group (Rele *et al.*, 1980) also purified four iso-inhibitors of papain from cowpea (*Vigna unguiculata* subsp. *cylindrica*) seeds. More recently CPIs have also been identified in rice (Abe *et al.*, 1987b; Kondo *et al.*, 1990b), potato (Rowan *et al.*, 1990; Hildmann *et al.*, 1992; Krizaj *et al.*, 1993), maize (Abe & Whitaker, 1988; Abe *et al.*, 1992) and wisteria seeds (Hirashiki *et al.*, 1990) among others. Sequence data from the CPIs of cowpea (Fernandes *et al.*, 1993), rice, potato (Hildmann *et al.*, 1992), maize and wisteria have shown that the proteins are highly homologous and also show strong homology to members of the cystatin superfamily of CPIs of animal origin (Barrett, 1987; Turk & Bode, 1991). The CPI from potato, reported by Rowan *et al.* (1990), shows an inhibition specificity different to many of the cystatins and an apparent similarity to the Kunitz family of trypsin inhibitors. Krizaj *et al.* (1993) obtained the complete amino acid sequence of a similar CPI from potato, and demonstrated extensive sequence homology with the Kunitz soybean trypsin inhibitor.

The cowpea CPI is reported to occur at almost constant levels (around 0.001%) in the seed, far below the concentration of CpTI which varies from 0.1% to 0.2% (Rele *et al.*, 1980; Xavier-Filho, 1991). A cDNA encoding this CPI, employed in a northern blot analysis of a resistant and a susceptible

line of cowpea, confirmed the similar levels of CPI expression (Fernandes *et al.*, 1991, 1993). Xavier-Filho (1991) demonstrated that the cysteine protease activity of *Callosobruchus maculatus* could be completely inhibited by the cowpea CPI, but as the concentrations of this inhibitor showed no correlation with resistance levels, the group concluded that this inhibitor was, unlike CpTI, associated with physiological processes and endogenous control, rather than defence against insect attack. Recent studies indicate that resistance may be related to a variant form of vicilin storage protein which is resistant to digestion by the midgut proteases and consequently limits the food supply to the larva (Pereira de Sales *et al.*, 1992).

Two CPIs have been identified in the kernels of maize, *Zea mays*, a 9kDa protein with similar inhibitory activity and physical characteristics to the rice CPI, oryzacystatin-I (ozcI) was reported by Abe and Whitaker (1988), and a 12 or 15.5kDa protein (denoted corn cystatin-I) was described concurrent, with this work, by Abe *et al.* (1992). Abe *et al.* (1992) isolated a full-length cDNA clone for a cystatin-like protein. The amino acid sequence of the encoded protein, deduced from the nucleotide sequence, showed 71% homology with ozcI in the region between residues 36-135 (corn cystatin-I numbering), and maintained the Gln-Val-Val-Ala-Gly central region, highly conserved amongst the cystatins (Barrett, 1987). When expressed as a fusion protein in *E. coli*, high levels of papain inhibitory activity were demonstrated. No native protein was purified from maize, but corn cystatin-I mRNA was demonstrated in maize kernel, confirming the transcription of the DNA, if not the protein's expression *in vivo*.

Oryzacystatin

The most extensively studied of the plant CPIs are those from rice. Two distinct inhibitors have been isolated (Kondo *et al., 1990b*), the first, termed oryzacystatin (later oryzacystatin-I) (Abe *et al.*, 1987b) is a potent inhibitor of papain and of the cysteine protease responsible for the

hydrolysis of the reserve glutelin during seed germination. Despite marked similarities in the amino acid sequence of this oryzacystatin-I and the second, oryzacystatin-II (approximately 55% identity) and significant homology with several of the animal cystatins (Abe *et al.*, 1987a; Kondo *et al.*, 1991), Kondo *et al.* (1990b) showed they were remarkably distinct in their enzyme specificities and the developmental patterns of their mRNAs in the ripening stages of the seeds. Oryzacystatin-I was found to inhibit papain more effectively (K_i $3{\cdot}0x10^{-8}M$) than cathepsin H (K_i $0{\cdot}79x10^{-6}M$), while oryzacystatin-II was more effective against cathepsin H (K_i $1{\cdot}0x10^{-8}M$) than papain (K_i $0{\cdot}83x10^{-6}M$). Neither was effective against Cathepsin B or L (Michaud *et al.*, 1993). Also while the mRNA for oryzacystatin-I was expressed at a maximum level 2 weeks after flowering and could not be detected in mature seeds, the mRNA for oryzacystatin-II was expressed constantly throughout the maturation stages and was clearly detectable in the mature seeds.

The oryzacystatins are the first well-defined cystatins of plant origin. Cystatins are characteristically tight, reversibly binding inhibitors of papain-like cysteine proteases (Bode *et al.*, 1988), forming equimolar complexes with papain, leaving the active site of the enzyme inaccessible to substrates (Anastasi *et al.*, 1983; Lindahl *et al.*, 1988). The cystatin binds to the enzyme in a simple manner, different from the binding of the substrate, and in marked contrast to the more complicated interactions of serine protease inhibitors with their target enzymes (Björk & Ylinenjärvi, 1990; Lindahl *et al.*, 1991). Oryzacystatins are known to occur in a wide range of cultivars of the rice variety sub-species *Oryza sativa* L. *japonica* (Kondo *et al.*, 1989a), although little is known of their occurrence in *Oryza sativa* L. *indica*.

Liang *et al.* (1991) demonstrated that nearly all the proteolytic activity demonstrated against casein in larval midgut extracts of two stored grain pests, the rice weevil (*Sitophilus oryzae*), and the red flour beetle

(*Tribolium castaneum*), could be inhibited by oryzacystatin-I. If this inhibition were exerted *in vivo* as well as *in vitro*, this would then affirm the role of CPIs in protection, as well as the endogenous regulatory role previously demonstrated for both cowpea CPI and oryzacystatin.

Recently the X-ray crystal structures of two members of the cystatin superfamily, chicken egg-white cystatin (Bode *et al.*, 1988) and stefin B in complex with an *S*-carboxymethylated papain (Stubbs *et al.*, 1990) have been elucidated. Both structures composed a long central α-helix wrapped in a 5-stranded, antiparallel ß-pleated sheet, with a subsidiary trunk of helix or strand. At one end of the molecule is an exposed first ß-hairpin loop, comprising the QVVAG region, highly conserved in the cystatins, and flanked on either side by a second hairpin-loop and the projecting amino terminal "trunk". In the enzyme:inhibitor complex, both of these exposed loops interact with subsites adjacent to the catalytic residues of the enzyme, while the trunk segment loops over the catalytic cysteine residue of the enzyme and binds to subsites nearby, preventing the access of substrates to the enzyme's catalytic site through pure steric hindrance (Bode & Huber, 1992). Clearly, this mechanism is quite different to that employed by the serine protease inhibitors and the potato carboxypeptidase inhibitor.

ii) Amylase inhibitors

Inhibitors of α-amylases are particularly abundant in the seeds of various cereals and legumes, although they are also found in other species. Many of these inhibitors have been demonstrated to be active against mammalian α-amylases, with which the majority of their characterisation has been conducted. Where activity against insect enzymes has been investigated, it has often been found that inhibitors active against mammalian amylases are ineffective against the insect enzymes, and vice versa. The cereal inhibitors form a multi-gene family including monomeric, dimeric and tetrameric α-amylase inhibitors and also serine protease

inhibitors, as mentioned earlier. The amylase inhibitors from legumes appear to belong to a novel inhibitor family, some showing homology to the lectins. Others, such as the α-amylase inhibitor from ragi (*Eleusine coracana*), show no homology to other known inhibitors (Campos & Richardson, 1984).

Wheat α-amylase Inhibitors

Wheat grains contain a complex of albumin proteins, many of which belong to a group of proteins including the α-amylase inhibitors. These have been separated, largely on the basis of gel-filtration, into three families of iso-inhibitors with molecular weights of around 12kDa (0.28 family), 24kDa (0.19 family) and 60kDa (0.53 family). The inhibitors are accumulated in wheat endosperm during grain ripening, reaching a maximum at maturity, and decrease rapidly on germination, no inhibitory activity being detectable in either root or shoot of the seedling (Pace *et al.*, 1978). Inhibition by the wheat α-amylase inhibitors is typically reversible and non-competitive (see review, Warchalewski, 1983). Investigations into the effects of α-amylase inhibitors on humans, using feeding trials with a diet containing wheat α-amylase inhibitors at concentrations higher than those normally present in the diet, recorded no decrease in hydrolysis of dietary starch due to amylase inhibition, concluding that no adverse effects were incurred (Warchalewski, 1983). Members of the α-amylase/trypsin inhibitor family from wheat and barley have, however, been shown to be involved with baker's asthma, an allergy common amongst workers exposed to inhalation of flours, most of the allergenic proteins characterised so far belonging to this family (Gomez *et al.*, 1990; Mena *et al.*, 1992), although this is an inhalation problem and not associated with digestion of the proteins.

It is interesting to note that at no stage of development are wheat α-amylases inhibited by these wheat amylase inhibitors (see review, Buonocore *et al.*, 1977), instead, at germination α-amylases are synthesized *de novo* following signalling by giberellins. This suggests that the major role of

these inhibitors is one of protection against external attack. Endogenous α-amylase inhibitors have been isolated but these are different to the main group of inhibitors.

Silano *et al.* (1975) investigated the inhibitory activities of the three albumin fractions against amylases from various sources. In general, the 12kDa (0.28) fraction was found most effective against insect amylases, while being inactive towards amylases from avian and mammalian species. This group found that the amylases of those insects that normally attacked wheat and wheat products were susceptible to inhibition by all three groups of inhibitor, while amylases derived from non-pest species were largely resistant to inhibition. This result implies that those insects which could attack wheat and suffer no nutritional problems do not, while those whose enzymes are inhibited if they attack wheat, do so. It is entirely possible that enzymes which are inhibited *in vitro*, may not be affected *in vivo* due to some detoxification step or shielding of the enzyme, as was demonstrated to occur with *Tribolium confusum* by Gatehouse *et al.* (1986). It seems peculiar however, even if this were the case, that these insects are not out-competed by those insects that do not need to protect themselves.

The differential effects of wheat α-amylase inhibitors on the two iso-enzymes present in *Sitophilus oryzae* (rice weevil) and the single amylase of *S. granarius* (granary weevil) were demonstrated by assaying across the elutants from HPLC separation of wheat proteins (Baker, 1987). Further studies of the effects of a 0.28 inhibitor from wheat (designated 0.31) on these enzymes showed similar levels of inhibition, and inhibitor:enzyme complex formation for all three was in an apparent 2:1 molar ratio (Baker, 1988b). The activity of another member of the 0.28 family, denoted WRP-25, was investigated by Chen *et al.* (1992a), effective inhibition was demonstrated against all the amylases extracted from *Sitophilus oryzae*, *Tribolium castaneum* (red flour beetle) and *Tenebrio molitor* (yellow mealworm), with the exception that one of the *S. oryzae* iso-enzymes was 10-

fold less sensitive to WRP-25 inhibition than were the others. WRP-25 was reported to inhibit human salivary amylase and porcine pancreatic amylase by only 6% and 12%, respectively, under conditions where insect enzymes were inhibited by over 88%. These enzymes were also assayed with a 12kDa inhibitor from corn, and although no inhibition of mammalian amylases was reported, the inhibition α-amylases from *T. castaneum* and *T. molitor*, although not those of *S. oryzae*, is particularly interesting, not only for its differential specificity, but also because this protein is also known to inhibit bovine trypsin and human activated Hageman factor, demonstrating a bifunctional inhibitory property. This demonstrates the care that is necessary in characterising proteins for defensive roles. Some vital inhibitory property may not be tested for and therefore be overlooked possibly leading to problems with palatability for mammals, in other cases inhibition may be strongly affected by pH, thus assays must be conducted under physiological conditions where possible.

A correlation has been suggested between resistance of some wheat varieties and the inhibitory ability of their extracts against certain insect α-amylases (Yetter *et al.*, 1979). The *in vivo* effects of the 0.28 inhibitor of Kashlan and Richardson (1981) has been demonstrated with *Callosobruchus maculatus* (Gatehouse *et al.*, 1986). Effective inhibition was demonstrated *in vitro* against extracted α-amylases of both this insect and *Tribolium confusum*, but, as mentioned above, only *C. maculatus* was affected *in vivo*.

Common Bean α-amylase Inhibitors

The bean α-amylase inhibitors, isolated from several varieties of the common bean, appear to be very closely related. They are glycoproteins, generally around 15% carbohydrate, with a molecular weight of 43-50kDa estimated for the native protein by gel filtration. The subunits which make up these tri- or tetra-meric proteins are 15-18kDa in mass, and may or may not be identical (Moreno *et al.* 1990). Bean α-amylase inhibitors have been

shown to be strong, non-competitive inhibitors of mammalian amylases, inhibitor:enzyme complexes forming in a 1:1 molar ratio (Marshall & Lauda, 1975)

Marshall and Lauda (1975) extracted an inhibitor from white kidney beans (*Phaseolus vulgaris* variety Great Northern) which they named "Phaseolamin". Inhibition was demonstrated against porcine, human and snail amylases, but no activity was found against any of the plant, bacterial or fungal enzymes tested. No insect α-amylases were tested. Inhibitors isolated from red and black kidney beans, have been shown to demonstrate inhibitory activity against the α-amylases of *Tenebrio molitor* and *Callosobruchus maculatus* (Pueyo *et al.*, 1993; Huessing *et al.*, 1991c).

The inhibitor isolated by Moreno and Chrispeels (1989) from *Phaseolus vulgaris* variety Greensleeves was also characteristic of this group. Resolution by SDS-PAGE showed five bands, found to represent glycoforms of two different polypeptides. Analysis of the sequence of these polypeptides revealed their correspondence to the two halves of a lectin-like protein encoded by an already identified gene, or else one closely related to it. Subsequent studies with this novel inhibitor type indicated that the bean α-amylase inhibitor was a typical bean lectin-like protein, synthesized on the rough endoplasmic reticulum, modified in the Golgi, and transported to storage vacuoles (Moreno *et al.* 1990).

As can be seen, the structure and specificity of enzyme inhibitors in plants varies greatly. Many plant species exhibit multiple inhibitor types, some presumably involved in regulation of endogenous enzyme activity and physiological processes, while others clearly exhibit a protective role. This variety provides an immensely wide base on which to search for suitable insect enzyme inhibitors for use in control mechanisms.

iii) Lectins

Lectins are carbohydrate-binding proteins of non-immune origin that agglutinate cells or precipitate polysaccharides or glycoconjugates (Goldstein *et al.*, 1980). They are structurally diverse proteins, present in many different plant species and in many different organs and tissues, although the highest concentrations occur in seeds and other storage tissues. Most of the lectins so far characterised are secretory proteins, accumulated in vacuoles, cell walls and intercellular spaces. It has been suggested (Chrispeels & Raikhel, 1991) that lectins have evolved through gene duplication and divergence, as indicated with the family of lectins and lectin-like proteins from the common bean, or by incorporation of lectin-domains into other genes, as is suggested by the characteristic domain common to lectins such as hevein, WGA (wheat germ agglutinin), rice lectin, tomato lectin and UDA (nettle lectin).

A possible defence role for these proteins was first proposed by Janzen *et al.* (1976), after demonstrating the toxicity of phytohemaglobin (PHA), the lectin from *Phaseolus vulgaris*, to larval *Callosobruchus maculatus*. This was apparently confirmed by Gatehouse *et al.* (1984), although this group showed that the impure PHA was more effective than pure PHA in retarding larval development. The toxic, contaminating compound has now been identified as the *Phaseolus vulgaris* α-amylase inhibitor (Huessing *et al.*, 1991c) discussed previously, and the rigourously purified PHA shown to be non-toxic to cowpea weevil larvae (Murdock *et al.*, 1990).

The toxic effects of some lectins towards mammals has been shown to be a consequence of binding to glycoprotein receptors in the gut, causing disruption of nutrient absorption across the gut wall (Liener, 1986; Puztai, 1991). The mode of action of lectins in insects is unknown, but probably, as in mammalian toxicity, involves binding to midgut cells and consequential disruption of cell function (Gatehouse *et al.*, 1989). The midguts of larval *C.*

maculatus (sensitive) and *Acanthoscelides obtectus* (tolerant), fed on *P. vulgaris* lectin, were analysed by indirect immunofluorescence. Fluorescence on the surface of *C. maculatus* midgut cells indicated binding of the lectin, and the absence of this fluorescence on *A. obtectus* midgut cells confirmed a lack of lectin binding with this species, indicating that binding is directly involved with lectin activity. The actual effects of this binding are not known. If lectins were to bind to midgut epithelial cells, nutrient absorption may be inhibited, or endocytosis stimulated, allowing entry into the cell of the lectin and possibly other toxins present in the gut. Binding to the peritrophic membrane, the chitin-rich structure lining the midgut of most phytophagous insects, could prevent correct formation of the membrane, or might disrupt the membrane such that movement across the membrane is either prevented or enhanced.

Recently, more reports on the insecticidal properties of lectins have emerged. A range of lectins have now been tested in artificial diet against *Callosobruchus maculatus* (Murdock *et al.*, 1990, Huessing *et al.* 1991a, 1991b), *Ostrinia nubilalis* (European corn borer), *Diabrotica undecimpunctata howardi* (Southern corn rootworm) (Czapla and Lang, 1990), *Nilaparvata lugens* (rice brown planthopper) and *Nephotettix cinciteps* (rice green leafhopper) (Powell *et al.*, 1993). WGA (wheat germ lectin), UDA (nettle lectin) and rice lectin were all found to cause marked retardation of larval development in *C. maculatus* (Huessing *et al.* 1991a, 1991b), while thornapple lectin and tomato lectin had significantly less effect (Murdock *et al.*, 1990).

In bioassays where lectins were applied topically to diet, WGA, *Bauhinia purpurea* lectin and castor bean lectin all proved toxic to larval *O. nubilalis*, none of the other lectins tested having any marked effect on survival or development. When these three lectins were incorporated into artificial diets at 0.5% (w/w) mortality on each was 100%. 50% mortality was shown to be caused by 0.059% (w/w) WGA, 0.073% (w/w) *Bauhinia purpurea* lectin and 0.029% (w/w) castor bean lectin. Lectins from *Phaseolus vulgaris*,

Pisum sativum, *Glycine max* and *Viscum album* (mistletoe) had no toxic effect on *O. nubilalis* or SCR. Topical application bioassays with larval SCR produced 100% mortality only with the castor bean lectin, and 35% mortality with *Codium fragile* lectin and pokeweed (*Phytolacca americana*) lectin. Marked reduction in development was demonstrated with *Mandura pomifera* lectin, *Codium fragile* lectin, *Bandeiraea simplicifolia II* lectin, *Artocarpus integrifolia* (Jackfruit) lectin, *Vicia villosa* (hairy vetch) lectin, and WGA. WGA caused a 58% reduction in weight gain and a 10% increase in mortality. *Sambucus nigra* (elderberry) bark lectin caused no alteration in survival but reduced weight gain by 40%. When lectin was incorporated into artificial diet a 50% reduction in weight of SCR larvae was produced by WGA at 0.3% (w/w) (see Chrispeels & Raikhel, 1991).

The insecticidal properties of lectins was again demonstrated by their effects on the nymphal stages of two important rice pests, the rice brown planthopper (*Nilaparvata lugens*) and the rice green leafhopper (*Nephotettix cinciteps*) (Powell *et al.*, 1993). Of the eight lectins tested, only two, *Galanthus nivalis* agglutinin (GNA) and WGA, were shown to exhibit antimetabolic effects towards the rice brown planthopper when incorporated into artificial diet at 0.1% (w/v). Only GNA, the D-mannose specific lectin from snowdrop bulbs (Van Damme *et al.*, 1987, 1991), was found to be effective against the rice green leafhopper.

Although the binding specificities of most lectins have been deduced, classified by their binding to single residues, for example both WGA and pokeweed lectin are specific for *N*-acetylglucosamine, their binding affinities to oligomers may be much higher (Goldstein and Poretz, 1986). This more complex specificity may account for why one lectin is effective against an insect, while other lectins with the same single residue biding specificity have no effect. The binding to oligomers in nature allows far greater variation than the binding specificity to single residues. As a result of this

more complex situation, it is necessary to investigate the specific activity *in vivo* of any lectins chosen as putative components of a control program.

iv) Ribosome inactivating proteins

Many plants possess ribosome inactivating proteins (RIPs), enzymes capable of specifically removing a single adenine residue from the highly conserved stem-loop structure present in all 23S, 26S and 28S ribosomal RNA, resulting in the permanent inactivation of ribosomes, preventing their use during protein synthesis. Plant RIPs fall into two categories, type-I exist as single polypeptide chains representing the active enzyme, and type-II RIPs, where the enzyme is linked, via a disulphide bond, to a second polypeptide which in all cases studied to date is a galactose-specific lectin. These type-II RIPs are the more potent of the two, the lectin component binding the RIP to selective sites on cell surfaces, thus promoting the RIP's activity within the cell. The most well known examples of RIPs are saporin, a type-I RIP from the tissues of *Saponaria officinalis*, and ricin, the highly toxic type-II RIP from castor bean (*Ricinus communis*) seeds. Little evidence is available for the effectiveness of RIPs against insects. Gatehouse *et al.* (1990), however, recently reported the extreme toxicity of both ricin and saporin to two species of Coleoptera, *Callosobruchus maculatus* and *Anthomonas grandis*, both with LD_{50} values of less than 0.01% (dry weight). Interestingly the Lepidopteran species tested, *Spodoptera littoralis* and *Heliothis virescens*, proved resistant to the effects of both RIPs, suggesting that these species could hydrolyse the proteins, inactivating them before any detrimental effect could be exerted. Strangely, ricin and saporin were observed to have similar effects on the Coleopterans tested, despite saporin lacking the lectin polypeptide which is considered necessary for RIP binding and subsequent activity in intact cells.

Due to the extreme toxicity of these proteins to mammals, their involvement in an insect resistance program is largely theoretical at

present. The coupling of RIP-enzyme polypeptides to lectins or antibodies, capable of binding to specific cell surfaces, was seen as the pathway to controlled RIP utilisation in pest control, but the comparable levels of activity with and without the lectin polypeptide observed by Gatehouse *et al.* (1990) is indicative the vast gaps in our understanding of RIP action, many of which must be filled before any such control methods can be contemplated.

Specific aims of the project

The initial aim was the characterisation of the major digestive protease and amylase activities present in the larval gut of *Diabrotica undecimpunctata howardii*, the southern corn rootworm, with some comparison, where possible, with the enzymes of the adult gut of this species and those of the western corn rootworm, *D. virgifera virgifera*. To this end, a variety of *in vitro* assays would be used, and enzymes, extracted from the insect guts, assayed against various diagnostic inhibitors.

Having determined the principal enzyme types present in the gut, suitable plant protein inhibitors would be sought, by one of three methods: using plant proteins prepared previously within the department or elsewhere; preparing protein samples from plant material using published procedures, where possible; or isolating novel inhibitors from plant material. These proteins would then be assayed, using the same *in vitro* assays as used for the enzyme characterisation, to examine their inhibitory effects on the insect enzyme activity. Inhibitors shown to cause marked inhibition of activity *in vitro* would then be tested for *in vivo* effectiveness using bioassays, incorporating the protein into an artificial diet and determining its effect on larval survival and development, measuring development as the weight gained during a given period. Other antimetabolic proteins, such as

lectins, would also be tested in insect feeding trials, in order to evaluate the level of control provided. The possible additive or synergistic effects of employing a multi-protein treatment would also be investigated, with a view to possible multi-genic, multi-mechanistic control in transgenic crop plants. Using the results from these *in vitro* and *in vivo* studies, the potential of each of the different antimetabolic proteins, taken separately or in combination, for use in a genetically engineered control strategy would be evaluated.

CHAPTER 2 MATERIALS AND METHODS

Chemical Reagents and Equipment

Unless otherwise stated, chemicals were of analytical grade and supplied by BDH-Merck Ltd., Lutterworth, Leics., Medicell dialysis tubing was also obtained from BDH.

The following reagents were supplied by Sigma Chemical Co. Ltd., Poole, Dorset: acrylamides, antibiotics, azocasein, BApNA, Brij 35 solution, BSA, casein, α-cellulose, cholesterol, cyanogen bromide, D-sucrose, DTT, E64, ethidium bromide, ß-mercaptoethanol, methyl paraben, myoglobin, papain, pCMB, pCMS, pepstatin A, porcine pancreatic α-amylase, potato carboxypeptidase inhibitor, protein size markers (SDS7), RNase, soluble potato starch, sorbic acid, Soybean (kunitz) trypsin inhibitor, thaumatin, TLCK, Tween 20.

Z-Phe-Arg-AMC, Z-Arg-Arg-AMC and AMC were supplied by Novabiochem (UK) Ltd., Nottingham.

Agarose minigel apparatus, S-sephadex (S-200), S-sepharose FF and Q-sepharose FF were supplied by Pharmacia Biosystems Ltd., Milton Keynes.

ATTO protein gel apparatus and semi-dry blotting apparatus, and X-ray cassettes were supplied by Genetic Research Instrumentation Ltd., Dunmow, Essex.

Anti-oryzacystatin I and anti-oryzacystatin II antisera were kindly supplied by S.Arai, Department of Agricultural Chemistry, Tokyo University, Japan.

Immuno-Blot western blotting kit and secondary antibodies were supplied by Biorad Laboratories Ltd., Hemel Hempstead, Herts.

ECL western blotting detection reagents were supplied by Amersham, Life Science, Little Chalfont, Bucks.

Agarose, plasmid pUC8 and DH5α competent cells were supplied by Gibco BRL, Life Technologies Ltd., Paisley, Scotland.

DNA size markers, IPTG, restriction enzymes and Xgal were supplied by Northumbria Biologicals Ltd., Cramlington, Northumberland.

Restriction enzymes and glycogen were supplied by Boehringer Mannheim UK Ltd., Lewes, E. Sussex.

Nitrocellulose, Schleicher and Schuell, grade BA-85, was supplied by Anderman and Co. Ltd., Kingston-upon-Thames, Surrey.

3MM paper was supplied by Whatman Labsales Ltd., Maidstone, Kent.

X-ray film, Fuji-RX, was supplied by Fuji Photo Film (UK) Ltd., London. Developer, Ilford phenisol, was supplied by Ilford Ltd., Mobberly, Ches. Fixer, Kodak Unifix, was supplied by Phase Separations Ltd., Deeside Clwyd.

DNA and protein sequencers were supplied by Applied Biosystems Inc., Warrington, Ches.

Agar (Bacto) was supplied by Difco Ltd., W. Molesey, Surrey.

Yeast Extract (Oxoid) was supplied by Unipath Ltd., Basingstoke, Hants.

Bacto-tryptone was supplied by Becton Dickinson, Cowley, Oxon.

Wesson's salt mix and Vanderzant's vitamin mix were supplied by ICN Biochemicals, Cleveland, Ohio, USA.

Yeast was supplied by Phillip Harris Education, Shenstone, Lichfield, Staffs.

Wheat germ and honey were supplied by Naturally, Spennymoor.

Cabbage was supplied by the local supermarket.

Levington's M3 potting compost and vermiculite (medium grade) were supplied by Klondyke Garden Centre Ltd., Chester-le-Street, Co. Durham.

Biological Materials

Rice, *Oryza sativa* L. var. "Simular Roma" (long grained variety) and var. "Originaro" (short grained variety), were supplied by Maggie's Farm, Durham.

Isolated rice germ (variety unknown) was kindly supplied by Dr. Carmen Benedito de Barber, of the Instituto de Agroquimica y Tecnologia de Alimentos, Spain.

Rice plant material, *Oryza sativa* L. var. Taichung Native 1, was kindly supplied as seed by the International Rice Research Institute, Manilla, Philippines, and grown by Kevin S. Powell in the Department of Biological Sciences, Durham University.

Diabrotica undecimpunctata howardi and *D. virgifera virgifera* were supplied as eggs by French Agricutural Research Inc. (Lamberton, MN) and cultures reared and maintained in departmental insectaries.

Maize kernels, *Zea mays* var. Kodak F1, Dawn F1, or Earle F1 were supplied by Yates Seeds Ltd., Macclesfield, Ches.

Plant antimetabolic proteins: Cowpea trypsin inhibitor (CpTI), GNA, plec, and α-amylase inhibitors from bean and wheat (WAAI and BAAI) were purified at Durham University, using standard techniques (Gatehouse *et al.*,1980; Van Damme *et al.*, 1987; Trowbridge, 1974; Moreno & Chrispeels, 1989; Kashlan & Richardson, 1981). Elderberry lectins, SNA1 and SNA2, and GNA were kindly supplied by W.Peumans, Leuven, Belgium. Enterolobin was kindly supplied by M.V.Sousa, University of Brasilia. Saporin was kindly supplied by F.Stirpe, University of Bologna. WGA and cystatin were supplied by Sigma Chemical Co. Ltd., Poole, Dorset.

Commonly used Solutions and Buffers

Acrylamides: 30g acrylamide, 0.8g bisacrylamide $100ml^{-1}$

Resolving Buffer: 3.0M Tris HCl pH8.8 (36.3g Tris $100ml^{-1}$)

Stacker Buffer: 0.5M Tris HCl pH6.8 (6g Tris $100ml^{-1}$)

2xSDS Sample Buffer: 0.2M Tris pH6.8 (1.52g $100ml^{-1}$)
20% glycerol (20ml $100ml^{-1}$)
2% SDS (2g $100ml^{-1}$)
0.002% Bromophenol blue (2mg $100ml^{-1}$)

10x Reservoir Buffer:
0.25M Tris (30.3g L^{-1}) pH 8.3
1.92M glycine (144g L^{-1})
1% SDS (10g L^{-1})

Stain:
40% Methanol, 7% Glacial Acetic Acid
0.05% Kenacid Blue R or Coomassie Brilliant Blue

Destain:
40% Methanol, 7% Glacial Acetic Acid

Protein size markers:
(SDS7: Sigma)

66k	Bovine albumin
45k	Egg albumin
36k	Glyceradehyde-3-phosphate dehydrogenase
29k	Bovine erythrocyte carbonic acid
24k	PMSF-treated trypsinogen
20k	Soybean trypsin inhibitor
14k	Alpha-lactalbumin

10xPBS (L^{-1})
2g KH_2PO_4
11.5g Na_2HPO_4
2g KCl
80g NaCl

YT medium (L^{-1})
8g tripticase
5g yeast extract
5g NaCl (autoclaved before use)

Solution I (ml^{-1})
2mg lysozyme
9mg glucose (50mM)
50µl 0.2M EDTA (10mM)
25µl 1M Tris-HCl pH8.0 (25mM)
925µl $d_3.H_2O$

Solution II (ml^{-1})
200µl 1N NaOH (0.2N)
50µl 20% SDS (1%)
750µl $d_3.H_2O$

Solution III
3M Na-acetate pH 4.8
(adjusted with glacial acetic acid)

TAE buffer	40mM Tris-acetate pH7.7 10mM EDTA
TE buffer	10mM Tris-HCl pH7.5 1mM EDTA
SOC medium	2% Bacto-tryptone 0.5% Yeast Extract 10mM NaCl 2.5mM KCl 10mM $MgCl_2$ 10mM $MgSO_4$ 20mM Glucose

METHODS

(1) REARING AND HANDLING OF *DIABROTICA*

Eggs, obtained from French Agricutural Research Inc. (Lamberton, MN), were reared to adult and a culture maintained, using the techniques recommended by Branson *et al.* (1975), modified slightly to suit the available facilities. These methods were chosen for their closeness to the natural environment of the corn rootworm.

Adults

Adults were maintained at 25°C(±2°C), 10-15%RH and with a 16L:8D lighting regime. Beetles were held in perspex cages and provided with artificial diet (a 1:1:1 mixture of yeast, honey and wheat germ) and cabbage as food, the diet replaced fortnightly and cabbage replaced at least twice weekly. The cabbage was also used to provide cover for the oviposition site, a tray of damp, dark-coloured pebbles. Eggs were collected from the oviposition site twice weekly. Adults were transferred to clean cages fortnightly to reduce risk of disease.

Eggs

Eggs were washed from the pebbles onto a mesh sheet, retaining the stones in a metal sieve. The eggs were collected into one area of the sheet, this folded so the eggs were uppermost and placed in a closed container until hatching began. Eggs were maintained in total darkness at 25°C and kept damp.

Larvae

As larval emergence began, the egg sheets were transferred to mats of seedling maize (see below), and left for 1 week, allowing larvae to hatch and move onto the rearing medium, away from the egg mass.

Trays of seedling maize thus infested with 1st instar larvae were kept moist and at 25°C. After 1 week the original mat was split in half and fresh half mats used to provide more food for the developing larvae. Larvae migrated towards the fresh seedlings, allowing the old (dead) half to be replaced with another new half when necessary (usually after 1 week).

When larvae reached prepupal stage, the trays were caged using a wire framework and a mesh. Emerging adults were removed regularly and transferred to the adult cage. Once a prepupal stage was reached no further fresh maize was added.

Maize

Maize (*Zea mays* variety Kodak F1, Dawn F1 or Earle F1) mats were grown in trays 25x35cm^2, in a 1:1 mixture of Levington's M3 potting compost and vermiculite (medium grade). Kernels were washed in ethanol to remove the fungicide treatment, rinsed thoroughly, several times and soaked in water for 24hrs. After further washing the maize was sown. Maize mats used for larval rearing were usually planted 7 days prior to use.

(2) ENZYME PREPARATION FROM *DIABROTICA* SPECIES

Preparation of crude enzyme extracts

Midguts from larvae or adults were excised, placed immediately in cold d.H_2O or 1mM DTT, and homogenised for 30sec with a teflon-glass homogeniser. The homogenate was chloroform extracted in order to reduce the lipid content of the extract. 4 volumes of chilled chloroform was added to the extract, vortexed to ensure thorough mixing and centrifuged at 13,000rpm, 4°C for 20min. The aqueous phase was carefully removed, aliquoted, frozen in liquid nitrogen and stored at -20°C. Stock extract for protease assays contained 47 larval or 40 adult guts per 2ml 1mM DTT (unless otherwise stated), while extract for amylase assays only 17 guts per 1ml d.H_2O.

Extracts for use with azocasein as substrate were prepared by a slightly modified method, excised guts (50 larval guts per 1ml) were kept in chilled d.H_2O for 10min, vortexed and centrifuged, as above. The supernatant was chloroform extracted, as above, to remove lipids.

Differential extraction of enzymes

Digestive tracts of larvae were dissected (as above) and placed in d.H_2O or PBS (140mM NaCl, 6mM Na-phosphate pH 7.2), 1 gut per 20µl. These excised, intact guts were centrifuged at 13,000rpm (Hawksley centrifuge) and 4°C for 5min. The supernatant (lumen contents) was chloroform extracted (see above), aliquoted and stored at -20°C. The pellet (gut walls) was rinsed twice in 200µl of the extraction fluid and soluble proteins extracted by homogenisation in d.H_2O or PBS, 20µl per gut, and centrifugation. This supernatant (gut walls) was chloroform extracted, aliquoted and stored at 20°C.

Extraction of soluble protein from carcasses (remains after removal of digestive tracts) and intact larvae was achieved following the same method as for gut wall extract.

(3) ENZYME AND INHIBITOR ASSAYS

Assay of BApNA-ase activity

2.4µl gut extract or 1.4µg papain (1mg/ml in 0·02M reduced glutathione) was mixed with 890µl 0·1M K-phosphate buffer pH3.4 or 7·5, respectively (or buffer plus inhibitor). Following preincubation of this assay mixture at 25°C for 5 min, the reaction was initiated by the addition of 70µl of substrate (BApNA 16mg/ml in DMSO). The rate of BApNA hydrolysis, resulting in the production of p-nitroaniline, was monitored by measuring the rate of change of optical density at 410nm.

A thermoregulated dual-beam spectrophotometer (Pye Unicam SP8-150 UV/VIS Spectrophotometer) was used in order that a temperature of 25°C could be maintained, and rate of BApNA autolysis compensated for by using an assay mixture of 930µl buffer alone, run concurrently in the back beam, with the enzyme assay.

Assay of protease activity by Myoglobin digestion and electrophoresis

10µl McIlvaine's buffer (Dawson *et al.*, 1986), 4µl distilled H_2O (or inhibitor/activator) and 2µl enzyme were centrifuged briefly to mix and incubated at 30°C for 5 min to equilibrate. 4µl of 2mg/ml myoglobin stock was added, mixed and incubated at 30°C for a set time period (ranging from 0 to 24 hr). After this time 20µl of 2xSDS sample buffer was added, mixed briefly and heated to 100°C for 90 seconds.

15µl samples were then loaded onto 12·5% or 15% polyacrylamide minigels (see below), with approximately 1µl 2-mercaptoethanol per sample. Each gel was also loaded with SDS7-molecular weight marker and a 3µg myoglobin standard.

Control assays were conducted with no enzyme (or inhibitor), allowing correction for substrate autolysis. For inhibition assays controls were also conducted with (i) inhibitor and no enzyme, to check for proteolytic activity in the inhibitor sample; and (ii) enzyme and no inhibitor, allowing calculation

of inhibition from normal digestion levels. In controls the enzyme or inhibitor (or both) were replaced by distilled water.

Quantification of these results was obtained by scanning the stained gels using an LKB Bromma Ultroscan XL Enhanced Laser Densitometer. A standard curve produced for myoglobin concentration was approximately linear between 0·1µg and 4·0µg, allowing the amount of myoglobin on the gel to be calculated from the band's density. Comparison with a standard, undigested myoglobin band on the stained gel allowed % digestion to be calculated. Percent inhibition was calculated by the reduction in hydrolysis compared to the uninhibited control.

General protease assay using azocasein digestion

Using a method modified from that of Lemos *et al.* (1990), azocasein was used as a general protease substrate. 60µl of enzyme sample (1.5 larval gut equivalents) was incubated with 315µl prewarmed 0.3M acetate buffer pH5.5 at 30°C for 5min. The reaction was initiated by the addition of of 375µl prewarmed 1% azocasein solution (azocasein dissolved in buffer, heated to ensure complete solution), and terminated after 30min incubation at 30°C by the addition of 250µl chilled 20% TCA. After 15min standing at room temperature the samples were centrifuged for 2min (microfuge) and 0.6ml of supernatant removed and added to an equal volume of 2N NaOH. Optical density was measured at 440nm. Distilled water was used as a blank. 1 unit of protease activity was taken as that giving an O.D. of 0.100 under the conditions used.

In order to find the optimum pH for protease activity, assays were conducted replacing the acetate buffer used above with the following: 0.2M Na-acetate buffer for pH4.0 - 5.5; 0.2M Na-phosphate for pH6.0 - 8.0.

All assays were conducted in duplicate and no-enzyme controls carried out concurrently to allow correction for any free peptides present in the substrate stock solution and also for substrate autolysis.

Inhibitor (and enhancer) assays were carried out by replacing 5µl buffer with inhibitor solution (in buffer at 1mg ml^{-1}) and preincubating enzyme, inhibitor and buffer for 5min prior to starting the reaction. Assays were again conducted in duplicate, with no-enzyme controls, and with additonal controls: (i) containing no inhibitor, run as standards; and (ii) controls containing inhibitor but no enzyme, conducted to check for activity in each inhibitor assayed.

Analysis of protease activity by elecrophoresis and selective staining

SDS-polyacrylamide-gelatin gels were made using a method modified from that of Heussen and Dowdle (1980), resulting in 11% SDS-acrylamide gels containing 0·12% (or 0.25%) gelatin. Both minigel and full-sized gel electrophoresis was carried out in order to demonstrate the electrophoretic mobility of proteolytic activities. Resolving gels were made as follows:-

	Minigel	Full-gel
Acrylamides (30% acrylamide, 0·8% bisacrylamide)	5·482ml	21.93ml
3·0M Tris-HCl pH 8·8	1·869ml	7.48ml
Gelatin stock (1%)	1·760ml (3.75ml)	7.04ml (15.0ml)
d.H_2O	5·557ml (3.57ml)	22.23ml (14.27ml)
Degas for 5 min		
10% SDS	166·0µl	0.60ml
APS (20mg/ml)	149·5µl	1.125ml
TEMED	16·6µl	20.0µl

(numbers in parentheses refer to 0.25% gelatin gel)

The main gel was overlaid with 0·05% gelatin in 0·3M Tris HCl pH 8·8 and allowed to polymerise at room temperature. Stacking gels were made as normal (see below; also Hames, 1981), and also polymerised at room temperature. Samples were prepared by diluting with approximately 1/4 volumes 2xSDS Sample Buffer, and these used without any heat treatment. After equilibration at 4°C, samples were loaded (5-20µl for minigel, 20µl for

full gel), along with protein standards (SDS7, SIGMA) at 4x supplier's recommended loading. Reservoir buffer was made as normal (see Materials section).

Electrophoresis was carried out at 4°C, in the absence of ß-mercaptoethanol, at a constant current of 8mA (the full gel being electrophoresed overnight). Following electrophoresis, SDS was removed from gels by washing for 30min at room temperature in 2·5% Triton X-100 (in aqueous solution). Gels were then rinsed several times in distilled water and incubated in McIlvaine's buffer at pH 5.0 (Dawson *et al.*, 1986) for 3hr at 37°C.

Inhibition and stimulation assays were carried out with full sized gels by cutting the gel in half to form duplicates and incubating one section in buffer alone, and the other in buffer plus 5mM cysteine or buffer plus 20µM E64.

Gels were stained with an aqueous solution containing 40% methanol, 7% glacial acetic acid and 0·05% Coomassie Brilliant Blue R. at room temperature for 1hr, and destained briefly in the same solution without the stain. Proteolytic activity was observed as colourless zones against the dark blue background. Standard proteins (SDS7) were run on the gels in order to aid estimation of molecular weights.

Gelatin used directly as protease substrate

This assay followed the same procedure as that used with myoglobin, the substrate in this case being 1% gelatin. Samples were incubated for 0 or 17hr. The results were visualised on 10% acrylamide gel (Hames, 1981). An assay using myoglobin as substrate was run simultaneously as a control for enzyme activity.

Fluorimetric Assay: Z-Phe-Arg-AMC or Z-Arg-Arg-AMC as substrate

The reagents and method used were modified from those of Barrett and Kirschke (1981). The assay buffer generally employed was 340mM Na-acetate, 60mM acetic acid, 4mM Na_2EDTA pH 5◦5, made to 8mM DTT before use.

The enzyme sample (1-4µl) plus inhibitor (0-20µl), were diluted to 500µl with diluent (0◦1% Brij35 solution in d.H_2O), and 250µl of prewarmed assay buffer added. 2min preincubation at 30°C was allowed for activation of the enzyme and temperature equilibration, after which the reaction was initiated by the addition of 250µl of substrate (20µM Z-Phe-Arg-AMC or Z-Arg-Arg-AMC in DMSO, diluted with d.H_2O from 1mM stock). Assays were conducted at 30°C for 10min, unless otherwise stated, after which 1ml of chilled stopping reagent (100mM chloroacetic acid, 30mM Na-acetate, 70mM acetic acid pH 3.0) was added.

The fluorescence of the free aminomethylcoumarin was determined by excitation at around 358nm and emission at 450nm. A Baird-Atomic 'Fluoripoint' Spectrofluorimeter was used. Distilled water was used as a blank, and a standard of 2.5µM product (AMC in DMSO, diluted from 1mM stock in a 1:1 mixture of assay buffer and stopping reagent) was used as a standard, representing the total concentration of product possible from the assay. A standard curve of product concentration against fluorimeter reading was produced and used to calibrate the results. Activity was calculated in mUnits, taking 1mU as 1nmol AMC released per min at 30°C.

For pH range assays 0.2M Na-acetate pH 4.0 - 5.5 and 0.2M Na-phosphate pH 6.0 - 8.0 were used. These buffers contained neither Na_2EDTA or DTT, rather, a sample of diluent was prepared to 4mM Na_2EDTA and 8mM DTT, 250µl added to the enzyme sample and this diluted to 500µl with normal diluent.

All assays were conducted in duplicate with a corresponding pair of no-enzyme controls. For inhibition assays additional controls were used with (i) enzyme and no inhibitor, and (ii) inhibitor and no enzyme.

Assay of Amylase Activity

Amylase activity was measured according to the method of Bernfeld (1955), with a number of modifications. 10µl enzyme sample (0.17 gut equivalents extracted in $d.H_2O$) and 80µl Mc'Ilvaine's buffer of the appropriate pH (or 40µl buffer and 40µl inhibitor solution) were mixed and preincubated at 31°C for 30min. 50µl substrate (1% starch, brought to a gentle boil to obtain complete solution, and cooled) was then added, mixed thoroughly and this incubated for exactly 30min at 31°C. The reaction was terminated by the addition of 100µl of DNSA reagent (1g 3,5-dinitrosalicylic acid in 20ml 2M NaOH with 30g Rochelle Salt (sodium potassium tartrate), made up to 100ml with distilled water) and boiling for 5min. 1ml $d.H_2O$ was added and this allowed to stand at room temperature for 15min before measuring the optical density at 540nm. Activity was measured in mg maltose liberated per 30min at 31°C by 10µl enzyme sample, calibrating the results using a standard curve of maltose in the same solutions.

A pH curve for *Diabrotica* gut α-amylase was established by using no inhibitor and assaying activity with a range of buffers from pH3.0 to 7.0. No preincubation was necessary for these assays. The optimum pH thus determined, was used in all subsequent assays. Inhibitors used were dissolved in buffer of this pH. The controls employed during inhibition assays were: inhibitor with no enzyme; enzyme with no inhibitor; neither enzyme or inhibitor.

All assays were conducted in duplicate. No-enzyme controls were employed in all assays, to allow the correction of results for substrate autolysis. For inhibition assays, additional controls were used with (i) enzyme and no inhibitor, and (ii) inhibitor and no enzyme.

Assay of isoamylase activity by electrophoresis

Enzyme samples were electrophoresed on native polyacrylamide gels (see below) at 4°C and amylase activity analysed using a method modified from that of Brewbaker *et al.* (1968). Following electrophoresis gels were rinsed in 0.2M Na-acetate buffer pH5.0 for 5min at room temperature before being incubated for 2hrs in 1% (w/v) starch solution (in the same buffer), at 37°C. After this incubation, gels were rinsed in 0.1M acetic acid for 5min at room temperature and stained in acidified iodine/KI solution. Amylase activity was apparent as colourless zones in the dark stained gel. The effect of inhibitor was investigated by preincubating enzyme and inhibitor for 30min at 31°C prior to electrophoresis.

Native Acrylamide Gel Electrophoresis

Non-dentauring polyacrylamide gels, used for enzyme analysis were made according to Hames (1981), resulting in 10% acrylamide minigels. Solutions were prepared as for SDS-PAGE, with SDS omitted from sample and reservior buffers.

	Resolving Gel	Stacking Gel
Acrylamides	5.00ml	1.25ml
Tris-HCl pH8.8	1.875ml	-
Tris-HCl pH6.8	-	2.5ml
d.H_2O	7.375ml	5.75ml
De-gas for 5mins		
1.5% APS	0.75ml	0.5ml
TEMED	7.5µl	7.5µl

Gels were polymerised at room temperature and equilibrated to 4°C. Samples, prepared in 1xSample buffer, were neither boiled, nor was ß-mercaptoethanol added. Electrophoresis was carried out at 4°C, at 100V for 2-4hrs.

(4) ESTIMATION OF pH OF SCR LARVAL GUT

Dissection

Larval guts were excised and stained with universal indicator solution. The subsequent colouration allowed estimation of pH.

Regurgitation

Larvae were induced to regurgitate by gentle agitation. A small amount of universal indicator solution was added to this liquid, and the pH deduced.

Feeding with coloured diets

Indicators (bromocresol green (pH3.6 - 5.2; yellow to blue), methyl red (pH4.2 - 6.3; red to yellow) and litmus (pH5.0 - 8.0; red to blue) were incorporated into artificial diet in aqueous solution. The diet was allowed to cool and set. 2nd and 3rd instar larvae were then placed on the diet and the colour of food in the gut investigated after one and five days of feeding. Colouration of food in the gut was taken to result from the pH of the gut and its contents. Dissection of the gut allowed further investigation of gut pH from fore- to hind-gut.

(5) INHIBITOR PREPARATION FROM PLANTS

i) Analytical techniques

SDS-Polyacrylamide Gel Electrophoresis

SDS-polyacrylamide minigels were prepared according to Hames (1981). Unless otherwise stated, 12.5% acrylamide gels were used (see below). Solutions used are detailed in the Materials section. For alternative concentrations, adjustments were made to this basic recipe according to those suggested by Hames (1981). Samples, in 1xSDS Sample Buffer, were

boiled for 3min (unless otherwise stated), and 2-25µl loaded per well along with 1µl ß-mercaptoethanol.

	Resolving Gel	Stacking Gel
Acrylamides	6.25ml	1.25ml
Tris-HCl pH8.8	1.875ml	-
Tris-HCl pH6.8	-	2.50ml
d.H_2O	6.165ml	5.65ml
De-gas for 5mins		
10% SDS	0.15ml	0.10ml
2% APS	0.56ml	0.50ml
TEMED	7.5µl	7.5µl

Electrophoresis was carried out in an ATTO electrophoresis tank, following the supplier's instructions. After eletrophoresis, gels were stained with a solution of 0.05% Kenacid Blue R or Coommassie Brilliant Blue in 40% methanol, 7% acetic acid, and destained breifly in the same solution minus the dye. Where necessary gels were analysed using an LKB Bromma Ultroscan XL Enhanced Laser densitometer.

Transfer of proteins from Acrylamide gels to Nitrocellulose

The semi-dry blotting technique of Kyhse-Anderson (1974) was employed. 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 H_2O; 1 acrylamide gel to be blotted; 1 sheet of 3MM paper soaked in cathode buffer; 1 sheet of dialysis membrane (cellophane) soaked in d.H_2O; 2 sheets of 3MM paper soaked in cathode buffer; CATHODE PLATE.

Where anode buffer 1 is 0.3M Tris, 20% (v/v) methanol, pH10.4; anode buffer 2 is 25mM Tris, 20% (v/v) methanol, pH10.4; and the cathode buffer is 25mM Tris, 40mM 6-aminohexanoic acid, 20% (v/v) methanol, pH9.4. Electrotransfer of proteins was carried out at 2.5mA cm^{-2} for ½ - 1hr. The blotted gel was stained to check transfer had been successful, and the

nitrocellulose stored between 3MM paper or immediately analysed using an immunoblot technique (see below). In all cases of western blotting, duplicate gels were produced, one being blotted as above, then stained, the other being stained for reference only.

Tissue Immunoblot

Tissue immunoblots were conducted using a method modified from that of Pereira *et al.* (1992). A nitrocellulose filter was soaked in d.H_2O and placed on dry filter paper. Tissue sections were freshly cut and pressed lightly onto the filter. The filter was then rinsed in d.H_2O, soaked in 2% periodic acid for 10min to reduce endogenous peroxidase activity, and rinsed again in d.H_2O. Immunoblot analysis was continued as below.

Immunoblot Analysis

1: Colour development technique

A BIO-RAD Immuno-blot kit was used, employing colour development reagents which react with alkaline phosphatase-labelled secondary antibodies to produce a purple colouration. Detection was carried out according to the supplier's instructions, the conditions outlined in the following:

Wash in TBS (20mM Tris, 500mM NaCl, pH7.5) 5mins; block in TBS, 3% gelatin 1hr; rinse 2x in TBS, 0.05% Tween-20 10mins; primary antibody (anti-oryzacystatinI antiserum at 1 in 1000) in TBS, 0.05% Tween-20, 1% gelatin overnight; rinse 2x in TBS, 0.05% Tween-20 10mins; secondary antibody (alkaline phosphatase-labelled, at 1 in 3000) in TBS, 0.05% Tween-20, 1% gelatin 3hrs; rinse 2x in TBS, 0.05% Tween-20 10mins; rinse in TBS 10mins; colour development solution until sufficient colouration developed.

Developed blots were rinsed in d.H_2O; blotted dry, photographed and stored dark and dry. Throughout this method incubations were carried out at room temperature and with gentle shaking.

2: Enhanced Chemiluminescent (ECL) technique

Amersham ECL western blotting detection reagents were used for this technique, which works by the oxidation of luminol in the presence of hydrogen peroxidase, enabling the detection of immobilized antigens, conjugated with horseradish peroxidase-labelled antibodies, by the emission of light. The procedure was modified slightly from that suggested by the supplier's, the following conditions being used. Unless otherwise stated all steps were conducted at room temperature and with gentle shaking.

Wash in PBS 10mins; block (PBS, 5% Marvel, 1% Tween-20) 1 hour; rinse in antisera buffer (PBS, 5% Marvel, 0.1% Tween-20) 3x 5mins; primary antibody (anti-oryzacystatinI antiserum at 1 in 10,000) in antisera buffer 3-4hrs or at 4°C overnight; rinse in antisera buffer 3x 5mins; secondary antibody (Horseradish peroxidase-conjugated antibody at 1 in 5000) in antisera buffer 2-3hrs or at 4°C overnight; rinse 3x in PBS, 0.1% Tween-20 (2x5min and 1x15min); rinse in water with at least one change, 5min.

The remaining steps were carried out in the dark room. Equal volumes of Amersham detection reagents were mixed to a total volume of 0.125ml cm^{-2} filter. Using flat-ended forceps, the filter was drained over absorbent paper towel, placed in a clean container, protein-side uppermost, and the detection reagent mixture poured over. After exactly 1min the filter was drained, placed between two acetate sheets inside an autoradiograph cassette and immediately exposed to photographic film for 15sec and then to another film immediately afterwards for 1min. Subsequent re-exposures depended on these initial results.

Amino acid Sequencing

Automated protein sequencing was performed on an ABI model 477a protein sequencer, by Mr. J.S. Gilroy, according to the supplier's instructions, and using ABI reagents.

ii) Protein purification techniques

Initial protein extraction from seed material

Seed tissue was milled to size 2, unless stated otherwise, using a Disc mill (type 11-500, supplied by Glen Creston Ltd., Stanmore, Middx.), and extracted by stirring overnight at 4°C, in 25mM Na-phosphate buffer pH7.0 with 0.15M NaCl, using approximately 1L buffer per 500g milled seed. The seed debris was removed by centrifugation (Beckman, 13,800g, 4°C, 25min). This extraction method was employed to provide crude protein extracts for SDS-PAGE

Heat-treatment of Seed Extracts

Extracted material was, unless otherwise stated, heated in a 80°C water-bath for 10min, and immediately centrifuged to remove any precipitate formed (Beckman, 13800g, 4°C, 25min).

Ammonium Sulphate Precipitation

Unless otherwise stated, a 30-65% ammonium sulphate cut was taken. Ammonium sulphate was added, gradually, in the calculated proportions, with stirring at 4°C. Stirring at this temperature was continued for at least 1.5hr, and the precipitate collected by centrifugation (Beckman 13800g, 4°C, 25min). In order to obtain a 30-65% cut, ammonium sulphate was first added at 16.4g $100ml^{-1}$, and this precipitate (0-30% cut) removed. A second addition of ammonium sulphate was then made to the supernatant, at 21.4g $100ml^{-1}$, the resultant precipitate being those proteins precipitating out between 30 and 65% saturation of ammonium sulphate. Fractions required for analysis were resuspended in distilled water, dialysed exhaustively against this and freeze dried (using an Edwards Molulyo freeze drier), allowing samples to be assayed for inhibitory activity or analysed by SDS-PAGE.

Column Chromatography: 1. Gel Filtration

S-200 Sephadex was used for a separation of proteins based on molecular size. The column (135cm^3) was equilibrated with 0.1M ammonium bicarbonate, and the sample also dissolved in this and filtered or centrifuged, as necessary, to obtain a clear solution. A flow rate of 11.4ml hr^{-1} was produced using a peristaltic pump, and the elution monitored at 280nm and collected at a rate of 6 fractions per hr. Following analysis on SDS-PAGE, elutant was pooled by peak, freeze dried and assayed for inhibitory activity.

Column Chromatography: 2. Ion-exchange

S-sepharose Fast Flow and Q-sepharose Fast Flow (Pharmacia) were used in conjunction with a Pharmacia gradient programmer (LKB G250) and pump system. Unless otherwise stated, column volume was 28cm^3, and flow rate 1ml min^{-1}. Q-sepharose columns were equilibrated with 20mM Tris buffer at pH8.0 and run against a linear salt gradient over a period of 3-4hrs. S-sepharose columns were similarly used with a linear salt gradient, but buffer varied, Na-acetate or formate buffer as indicated in each particular step.

Samples for use in ion-exchange chromatography were dissolved in the appropriate buffer and clarified, by filtration or centrifugation as necessary, before being loaded onto the column. In all cases elution was monitored at 280nm and collected in 3min fractions. Resultant fractions were generally assayed for inhibitory activity, pooled, dialysed exhaustively against distilled water and freeze dried.

Column Chromatography: 3. HPLC

HPLC was carried out using a reversed phase Vydac (Cat. No. 218TP54) C_{18} peptides column and a Gilson International gradient manager 702 (v.3.0). Samples were dissolved in less than 1ml 0.1% TFA, and filtered

before loading. The column was equilibrated with 0.1% TFA and run at 1ml min^{-1} with a linear gradient from 0 to 50% acetronitile, 0.1% TFA, over a period of 100min. Major peaks, as detected at 214nm, were collected, neutralised with ammonium bicarbonate where stated, and freeze dried before undergoing further analysis by assay, SDS-PAGE or sequencing.

Cyanogen Bromide Cleavage

Cyanogen bromide cleavage was carried out according to the method of Allen (1981). Protein was dissolved at 5mg ml^{-1} in 70% formic acid and an equal weight (to protein) of CNBr (suspended at 2g ml^{-1} in acetronitrile). The solution was mixed and flushed thoroughly with N_2, before being incubated in the dark for 24hrs at room temperature. After this time the reaction was diluted at least 15x with distilled water and was lyophilised.

iii) Preparation of plant CP inhibitors

Rice: Location of CPI within the seed

Germ and endosperm were separated by simple dissection and ground with a pestle and mortar. Taking an equal mass of each, proteins were extracted overnight at 4°C in 25mM Na-phosphate buffer pH7.0, 0.15M NaCl. The supernatant resulting from a 25min centrifugation (12,000g, 4°C) was heat-treated (80°C for 10min) and the resultant precipitate removed by centrifugation, as above. The proteins retained in the supernatant were assayed for inhibitory activity against SCR cysteine proteases and papain, using the substrate Z-Phe-Arg-AMC. These fractions were also analysed by SDS-PAGE and Western blotting.

Tissue immunoblotting was conducted using almost mature rice seeds, used directly from the rice plant. The rice seeds were bisected longitudinally and pressed lightly onto a moist nitrocellulose filter. The filter, after incubation in 2% periodic acid for 10min, was analysed using the ECL immuno-blot technique.

Rice: Purification of CPIs from rice seed

Throughout the purification procedures, fractions were assayed for inhibitory activity against rootworm cysteine proteases, with 2µl (0.047 gut equivalents) per assay, using Z-Phe-Arg-AMC as a substrate. Some fractions were also tested against papain activity. Fractions were also analysed by SDS-PAGE and western blotting.

Initial extraction method

Seed material was milled and a protein extract prepared as usual, and heat treated (80°C for 10min). The fraction precipitated between 30 and 65% saturation with ammonium sulphate at 4°C was resuspended in distilled water, dialysed and freeze dried. This fraction was redissolved in 50mM Na-acetate buffer pH4.9, filtered to clear, and subjected to ion exchange chromatography (S-sepharose FF), using this buffer with a linear gradient from 0 to 0.4M NaCl over 2.5hrs. The resultant inhibitory fractions were pooled, dialysed against distilled water and freeze dried.

This inhibitory fraction was resuspended in 0.1M ammonium bicarbonate, filtered to clear, and purified further by gel filtration (S-200 Sephadex). The resultant peaks were pooled, dialysed, freeze dried and assayed for inhibitory activity. The inhibitory peak was redissolved in 25mM formate buffer pH4.1 and subjected to ion exchange chromatography (S-sepharose FF), using this buffer with a linear gradient from 0 to 0.4M NaCl over 3.5 - 4hrs. Resultant peaks were pooled, dialysed exhaustively against distilled water and freeze dried.

The inhibitory fraction was dissolved in 600µl distilled deionised water and further purified by HPLC, using 0.1% TFA and a linear gradient from 0 to 50% acetronitrile. Major peaks were collected, freeze dried and assayed for inhibitory activity. The fraction causing the greatest relative inhibition was analysed for N-terminal amino acid sequence data.

Modified extraction method

Initial extraction of proteins from seed material was conducted as above, this extract heat treated (80°C for 10min) and the precipitate formed between 30 and 65% saturation with ammonium sulphate (at 4°C) collected, dialysed and freeze dried, as above. This inhibitory extract was resuspended in 20mM Tris buffer pH8.0, clarified by centrifugation and fractionated by anion exchange chromatography (Q-sepharose FF), using this buffer with a linear NaCl gradient from 0 to 0.5M over 3-4hrs.

The inhibitory fractions were pooled, dialysed and freeze dried, prior to cation exchange chromatography (S-sepharose FF), using 25mM formate buffer pH4.1 or 50mM Na-acetate pH4.1, as stated, and a linear salt gradient from 0 to 0.4M NaCl over 3.5-4hrs. Fractions were pooled by peak, dialysed, freeze dried and assayed for inhibitory activity against SCR enzymes and papain.

The fraction which caused only inhibition of insect enzymes was purified further by HPLC, using the same conditions as before. Major peaks were collected and the 0.1% (approximately 0.01M) TFA neutralised, unless stated otherwise, by the addition of a tenth volume of 0.5M ammonium bicarbonate, prior to freeze drying. The fraction demonstrated to possess the greatest inhibitory activity was subjected to both SDS-PAGE and sequence analysis.

Large scale extractions of rice CPIs were conducted using this method, although centrifugations were altered to deal with the increase in volume, and chromatography volumes and flow rates altered because of the greater concentration of proteins. Anion exchange chromatography was conducted using a Q-sepharose FF column of 133cm^3 with a flow rate of 2.5ml min^{-1} and salt gradient applied over a period of 5hrs. S-sepharose chromatography was conducted as normal.

Extraction of CPIs from other plants

Crude extracts from rice, wheat and maize

0.4g of seed material was ground with pestle and mortar and extracted overnight at 4°C in 1.2ml PBS. Insoluble material was removed by centrifugation, as usual, and samples of these supernatants assayed for CPI activity in 20min assays using Z-Phe-Arg-AMC as a substrate, with 2µl (0.047gut equivalents) SCR larval gut extract. These samples were also analysed by SDS-PAGE and western blotting.

Wheat extraction for CPIs

2g milled wheat grain was extracted overnight in 50mM Na-acetate pH4.9 at 4°C. After removal of insoluble material by centrifugation, proteins in the supernatant were precipitated by the addition of ammonium sulphate to 100% saturation at 4°C. This precipitate was resuspended in distilled water, dialysed exhaustively and lyophilised. A sample of this fraction was heat treated (80°C for 10min), the precipitate removed by centrifugation, and the supernatant tested for CPI activity, as before.

Maize extraction for CPIs

Untreated maize kernels were extracted, as normal, in 25mM Na-phosphate buffer, pH7.0, 0.15M NaCl overnight at 4°C. Debris was removed by centrifugation, and proteins in the supernatant collected by precipitation between 0 and 65% saturation with ammonium sulphate. This fraction was resuspended in distilled water, dialysed exhaustively and lyophilised, prior to assaying for CPI activity and analysis by SDS-PAGE and western blotting. Inhibition assays were conducted with 10x diluted enzyme stocks and using an incubation period of 5min.

(6) PRODUCTION OF RECOMBINANT ORYZACYSTATIN

Plasmid DNA Minipreparation

Single colonies from agar plates, or loopfulls from glycerol preserved cells, were innoculated into 10ml aliquots of YTamp (YT medium with 50µg ml^{-1} Ampicilin) and incubated overnight at 37°C on a rotating wheel. Plasmid DNA was prepared from cultures using a scaled up version of the method of Birnboim and Doly (1979).

Cells were harvested by centrifugation, resuspended in 200µl Solution I and kept on ice for 30mins. 600µl Solution II were added, the tube inverted to mix and kept on ice for 5min. 450µl Solution III were added, the tube inverted several times to mix and incubated on ice for 1hr. Following a 5min, 12,000g centrifugation, 1100µl of the clear supernatant were removed, transferred to a second eppendorf, and 500µl cold (-20°C) isopropanol added, the contents mixed and placed at -20°C for 30min.

The resultant precipitate was collected by a 2min, 12,000g centrifugation at 4°C, resuspended in 400µl 0.1M Na-acetate, 0.05M Tris-HCl pH6.0 and reprecipitated with 1ml -20°C ethanol for 10min at -20°C, centrifuging as above.

The pellet was resuspended and reprecipitated as in the last steps and the DNA finally dried for 5min under vacuum, resuspended in 100µl d_3H_2O and stored at -20°C. 5µl aliquots were used for restriction analysis.

Plasmid DNA restrictions

Restriction of plasmid DNA was performed at 37°C for 2hrs using buffer supplied with the restriction enzymes. A restriction enzyme to DNA ratio of at least 1 unit μg^{-1} was used, concentration of DNA being estimated from agarose gel. Final dilution of restriction enzymes were at least 10-fold.

Agar Plates

Agar plates were made using YT medium, autoclaved with 15g l^{-1} agar. For YTampXgal plates, Ampicilin was added to 50µg ml^{-1} and Xgal to 40µg ml^{-1}. Plates were poured and allowed to set in a sterile flow cabinet, then being sealed and stored at 4°C. Prior to use plates were warmed to room temperature.

Agarose Gel Electrophoresis

Electrophoresis of DNA was carried out in a Pharmacia GNA-100 minigel apparatus or on 15.5 x 18.5 x 0.6cm gels in tanks holding 2.1l of buffer as described in Maniatis *et al.* (1982). TAE buffer was used. Running buffer and gel contained 1µg ml^{-1} ethidium bromide. 0.2-0.5 volumes of dye (10mM Tris-HCl, 10mM EDTA, pH8.0, 1mg ml^{-1} fast orange G., 30% glycerol) were added to samples before loading. A commercially prepared restriction digest of lambda DNA was run as a size marker. Electrophoresis was carried out at 2-10 volts cm^{-2} for approximately 2hrs and gels photographed using transmitted UV light.

Retrieval of DNA from agarose gels

Slices of agarose gel containing DNA were cut from the gel and placed in prepared dialysis tubing (Maniatis *et al.*, 1982) with the minimum volume of TAE buffer, all the air excluded and the ends clipped shut. The tubing was then placed in a Parmacia GNA-100 minigel apparatus, perpendicular to the field and covered with TAE buffer. Electrophoresis was carried out for 15min at 50V. After checking over UV that the DNA had migrated from the gel to the edge of the dialysis membrane, the polarity was reversed for 15sec. Using a micropipette the buffer, containing the DNA, was removed from around the gel slice and the tubing rinsed with a small (50-100µl) volume of TAE. In order to remove any contaminating enzymes, the DNA was then purified by phenol extraction and ethanol precipitation.

Phenol extraction and ethanol precipitation of DNA

Phenol extraction was carried out using the following method, vortexing for 15sec and centrifuging for 3min at 12,000g at each stage. The sample volume was adjusted up to 200µl with TE buffer if necessary, and extracted with 200µl phenol. After vortexing and centrifuging the aqueous phase was taken and retained. The phenolic phase was extracted with 100µl TE buffer, vortexed and centrifuged. This aqueous phase was added to the previous aqueous phase and extracted with 300µl chloroform/isoamyl alcohol (24:1) vortexing and centrifuging as above. The aqueous phase was removed, re-extracted as above and the final aqueous phase removed and ethanol precipitated.

DNA was ethanol precipitated using 1µl (20µg) glycogen carrier, adding 1/25 volumes of 5M ammonium acetate, 0.25M $MgCl_2$ pH 5.2 and two volumes of ethanol cooled to -20°C. This was mixed and placed at -20°C for at least 1hr followed by centrifugation at 4°C, 12,000g for 20 mins. The ethanol was carefully poured from the tube and replaced by 70% ethanol at -20°C. Tubes were inverted to mix, centrifuged as above for 5min and the liquid poured off. The DNA was dried under vacuum for 5min and resuspended in H2O.

Subcloning procedure

The insert to be subcloned was restricted with *PstI*, phenol and chloroform/isoamyl alcohol extracted, and ethanol precipitated. The vector, pUC8, was processed in the same way. Ligations containing equimolar amounts of insert and vector (using 0.1-0.2µg vector) were set up in 10µl volume and incubated overnight at 15°C. 1 unit of T4 DNA ligase was used in each restriction with the buffer supplied with the enzyme.

Transformation of competent cells

DH5α competent cells were transformed following the suppliers instructions: ligated plasmid was gently mixed with freshly thawed cells and incubated on ice for 20min. Cells were heat shocked for 2min at 42°C, four volumes of SOC medium added and the cells incubated at 37°C for 1hr to express ampicilin resistance. Aliquots were then spread over YTampXgal plates and incubated overnight at 37°C. White colony formation was indicative of recombinant transformants.

Minipreparation of plasmid DNA for sequencing

Bacteria containing plasmid DNA were grown overnight as above and harvested by centrifugation. DNA was purified for sequencing by a further modification of the Birnboim and Doly method (1979). Cells were resuspended in 200µl of solution I, kept on ice for 30min, 400µl of solution II added and the tube mixed by inversion and kept on ice for 5min. 300µl of acid potassium acetate (600µl 5M potassium acetate + 115µl glacial acetic acid + 285µl H_2O) were mixed in by inversion and the mixture placed on ice for 30 min.

The tube was then centrifuged for 30min at 12,000g and 700µl of the clear supernatant removed. To this was added 2µl of 10 mg ml^{-1} RNase (DNase free) and the tube incubated at 37°C for 20min. The solution was then extracted once with phenol/chloroform/isoamyl alcohol (25:24:1) and once with chloroform/isoamyl alcohol (24:1), vortexing and centrifuging for 3min at both stages. DNA was then ethanol precipitated as usual.

The DNA pellet was resuspended in 16.8µl H_2O, 3.2µl of 5M NaCl were then mixed in, followed by 20µl of 13% polyethylene glycol 8000 and the contents mixed and stood on ice for 20min. DNA was recovered by centrifugation for 10min at 12,000g, the supernatant removed by micropipette, 1ml of -20°C 70% ethanol added and the tube spun for a further 5min. The precipitate was resuspended in 15µl H_2O and 1µl aliquots

restricted and electrophoresed with known amounts of standard DNA to estimate the concentration prior to sequencing.

Automated DNA sequencing

Automated DNA sequencing was performed on an ABI model 373A DNA sequencer, by Ms J. Bartley using ABI reagents in a modification of the dideoxy chain termination method with Taq polymerase (see 373A user's manual).

Storage of cells in glycerol

Cells were grown overnight at 37°C on YTampXgal plates and scraped off into two 1ml aliquots of YT. These aliquots were vortexed to achieve a thorough distribution of cells, and 1ml 80% glycerol added to each, and vortexed to mix. Stocks of cells were stored in glycerol at -80°C.

Crude protein extraction from *E. coli* for SDS-PAGE and Western blot analysis

Transformed bacterial cells were grown overnight in 5ml YTamp as above, harvested by centrifugation and resuspended in 100µl 1x SDS Sample Buffer. Cells were disrupted by boiling for 5min, and cell debris removed by centrifugation for 5min (microfuge). Samples were then electrophoresed as normal, with 5-15µl sample loaded per well and ß-mercaptoethanol being added where necessary. For western blot analysis proteins were transferred to nitrocellulose from unstained gels as normal, and probed using anti-ozcI antiserum and the ECL detection technique.

Disruption of transformed cells by sonication

Harvested cells were resuspended in 10mM Tris pH8.0, 2mM EDTA and transferred to corex tubes, such that tubes were two thirds full of liquid. An MSE Soniprep 150 sonicator was employed, using 10sec bursts, with 10 or

20sec intervals. Where possible samples were kept on ice to prevent heat gain during sonication. Cell debris was removed by centrifugation (Beckman 15900g, 10min).

Purification of Rozc from *E.coli*

Cells containing the recombinant transformant were grown in 2x5ml YTamp for 8hrs at 37°C, then transferred to 2x1L YTamp plus 0.5mM IPTG, and allowed to grow for a further 15h at 37°C. Cells were harvested by centrifugation, resuspended in 10mM Tris pH8.0, 2mM EDTA and disrupted by sonication. Cell debris was removed by centrifugation (Beckman, 15900g, 10min). The supernatant was heated in a 82°C water bath for 15min, allowed to cool to 4°C, and the resultant precipitate removed by centrifugation, as before. The precipitate formed in the supernatant between 0 and 65% saturation with ammonium sulphate was collected by centrifugation, resuspended in 20mM Tris pH8.0 and dialysed exhaustively against this buffer. Final purification of Rozc was achieved by ion exchange chromatography (Q-sepharose FF), using 20mM Tris pH8.0 with a linear gradient from 0 to 1M NaCl. The fraction containing pure Rozc was dialysed exhaustively against d.H_2O and lyophilised.

Throughout the purification procedure, fractions were tested for papain-inhibitory activity, using Z-Phe-Arg-AMC as a substrate, and analysed with SDS-PAGE and western blotting, using anti-ozcI antiserum, for the presence and purity of Rozc.

(7) BIOASSAYS

Larvae for use in bioassay

Larvae for use in bioassay were reared in one of two ways: (1) reared as normal on mats of maize seedlings, being sorted from the compost and washed before use; or (2) reared in closed, vented, containers on

germinated maize (free from compost). For the latter technique, maize kernels were washed and soaked as usual, but were germinated on damp filter paper in large covered petri-dishes. The egg sheet was transferred to a larger container when hatching began and the germinating maize kernels placed around the eggs. The container was closed, but vents (sealed with fine mesh) ensured that, although humidity was maintained at a high level, condensation was kept to a minimum. These larvae were reared for up to 1 week in total darkness under normal insectary conditions. This second method allowed smaller larvae to be used in bioassay, without time consuming searches through rearing mats and with reduced damage to the larvae.

Artificial Diet

The artificial diets used for feeding trials with larval SCR were modified from that of Sutter *et al.* (1971), as recommended by Branson *et al.* (1975). Three versions of the diet were used: Diet 1, close to that suggested by Sutter *et al.* (1971), used for the majority of bioassays; Diet 2, similar to that suggested by Marrone *et al.* (1985); and Diet 3, modified for use in assays with α-amylase inhibitors, by the addition of starch and the reduction of sucrose levels. Diet 1 was shown by Sutter *et al.* (1971) to sustain larval development through to pupation and emergence of adults.

Diets were made according to the recipes in Figure 2. Agar was dissolved in water by heating in an autoclave for 20mins at 120°C, creating a sterile base for the diet. For Diet 3, starch was suspended in 10ml water and this added to the hot agar with stirring, dissolving the starch with the minimum amount of denaturation. The agar was then placed in an electric blender with group (c) ingredients (see table A), and mixed at high speed for 1min. After the diet had cooled slightly, to around 50°C, the components of group (d) were added, the diet mixed for a further 2mins and dispensed into 9ml aliquots in 20ml pots. These pots were placed in a water

bath at 50°C to retard gelling, allowing the addition of "inhibitors", in aqueous solution (1ml added 9ml aliquot), to the diet. After thorough mixing, the diets were divided, unless stated otherwise, into 2ml fractions in small 7-10ml pots. Once poured and slightly cooled, the diets were covered and allowed to set. Diet was stored at 4°C upside-down, to reduce condensation effects. The concentration of inhibitors is given in %(w/v), for example 13mg inhibitor per 10ml diet represents a concentration of 0.13% (w/v) or 1% dry weight.

Figure 2.

		Diet 1	Diet 2	Diet 3
a)	$d.H_2O$	148ml	148ml	138ml
	Agar (Difco)	2.90g	2.90g	2.90g
b)	Starch	-	-	6.45g
	$d.H_2O$	-	-	10ml
c)	Wheat germ	5.50g	5.50g	5.50g
	Casein	6.45g	6.45g	6.45g
	D-sucrose	6.45g	7.70g	0.5g
	Alpha-cellulose	2.75g	2.75g	2.75g
	Wessen's Salt mix	1.85g	1.85g	1.85g
	Methylparaben	0.20g	0.20g	0.20g
	Sorbic acid	0.125g	0.125g	0.125g
	Cholesterol	0.0125g	0.0125g	0.0125g
	Raw linseed oil	0.05ml	0.08ml	0.05ml
d)	Streptomycin	0.025g	0.025g	0.025g
	Aureomycin	0.025g	0.025g	0.025g
	Vitamin mixture	3.0ml	3.0ml	3.0ml
	10% KOH	2.0ml	2.0ml	2.0ml
	Formalin	0.20ml	0.20ml	0.20ml
e)	$d.H_2O$	20.0ml	20.0ml	20.0ml
	Total	200.0g	201.3g	200.5g

Bioassay method

Larvae for use in bioassay were selected so as to be of similar size. They were cleaned and weighed in groups of 3 or 5, prior to being

introduced onto the diet. The diet was first equilibrated to room temperature and its surface scored roughly with a sterile needle to facilitate larval burrowing. Each pot was then capped and incubated for 9 days at 25°C and total darkness, unless stated otherwise. After 9 days the surviving larvae were counted and weighed. Depending on the condition of diets and larvae at this stage, the assay was either terminated or continued for another 2 days or more. Where dead larvae were present these were removed from the diet pot. Dead larvae were not weighed.

"No diet" controls were conducted by placing 1 or 5 larvae in pots containing no diet. In order to maintain the high humidity necessary for larval survival and development, the centre of each lid was removed and replaced with a fine mesh, the pots were then stood in a small amount of water in a closed container. This step was unnecessary for the other pots as the diet maintained humidity at a high level.

Choice of diet

The suitability of diets 1 and 2 for bioassay use was assessed by comparison of larval survival and development on control diets (ie. with no inhibitor added). Successful modification of the diet, for use with α-amylase inhibitors, was assessed, again, by the survival and development of larvae on a range of control diets, made according to the recipe for diet 3, but containing differing amounts of sucrose (0% to 1.25% w/w).

Statistical Methods

Statistical analysis of results was conducted according to the methods described by Sokal & Rohlf (1973) and Parker (1979). Unless otherwise stated, the acceptance level employed was $p=0.05$.

The *G*-test of independence was used to analyse the significance of differences in survival between controls and treatments. Treatments were compared to controls one at a time, the data arranged in a matrix:

	Live	Dead	(Total)
Control	a	b	$(a+b)$
Treatment	c	d	$(c+d)$
(Total)	$(a+c)$	$(b+d)$	$(a+b+c+d) = n$

In cases where the total number of larvae under consideration, n, was less than 200, Yate's correction was applied to the figures. If $(ad) > (bc)$ then 0.5 was subtracted from each of a and d, and 0.5 added to each of b and c, or vice versa if $(ad) < (bc)$. Three quantities were then calculated, using the amended values of a, b, c and d:

(1) $a\ln a + b\ln b + c\ln c + d\ln d$

(2) $(a+b)\ln(a+b) + (c+d)\ln(c+d) + (a+c)\ln(a+c) + (b+d)\ln(b+d)$

(3) $n\ln n$

The statistic G was computed as $G_s = 2x[(1)-(2)+(3)]$ and compared to the tabulated values of chi-squared for one degree of freedom (df).

The *F*-test for the equality of two variances was used to determine whether two sets of WGSL or WGL data possessed equal variances. A basic assumption of the test was that data was normally distributed. The variance ratio, F, was computed:

$$F_s = s_1^2/s_2^2$$

where s represents the standard deviation of the sample set, and $s_1 > s_2$. The probability that these data deviate significantly from the null hypothesis that the variances are equal (H_o: $\sigma_1^2=\sigma_2^2$) was determined by examining the tabulated values of F with (n_1-1) and (n_2-1) degrees of freedom (df).

Student's *t*-**test** was employed to analyse the difference between two samples, for example, in order to establish whether a treatment caused a significant decrease in larval weight gain from that achieved on control diets. For this test it was assumed that distributions were normal and that the variances of the two samples were equal, as demonstrated using the *F*-test. Formulae for the computation of t_s depended on the size of the samples (Sokal & Rohlf, 1973):

(i) where sample size was unequal and one or both were less than 30:

$$t_s = \frac{\ddot{y}_1 - \ddot{y}_2}{\sqrt{[S_c \times N_c]}} \qquad df = n_1 + n_2 - 2$$

$$\text{where} \quad S_c = \frac{(n_1-1)s_1^2 + (n_2-1)s_2^2}{(n_1 + n_2 - 2)} \qquad \text{and} \quad N_c = \frac{(n_1 + n_2)}{(n_1 n_2)}$$

(ii) where $n_1 = n_2$:

$$t_s = \frac{\ddot{y}_1 - \ddot{y}_2}{\sqrt{[{}^1/_n(s_1^2 + s_2^2)]}} \qquad df = 2(n - 1)$$

(iii) where sample size was unequal and both were greater than 30:

$$t_s = \frac{\ddot{y}_1 - \ddot{y}_2}{\sqrt{[(s_1^2/n_1) + (s_2^2/n_2)]}} \qquad df = n_1 + n_2 - 2$$

A modified version of the *t*-test was employed in cases where the variances of two treatments differed significantly (Parker, 1979). Here, the formulae for t_s were used as usual, but with a reduced number of degrees of freedom (f), calculated as follows:

$$1/f = U^2/(n_1-1) + (1-U)^2/(n_2-1)$$

$$\text{where} \quad U = (s_1^2/n_1) \; / \; [(s_1^2/n_1)+(s_2^2/n_2)].$$

Analysis of variance (ANOVA) was employed for WGSL and WGL data where variances were shown to be equal (using the *F*-test), and where a number of treatments could be included, thus making the repeated use of the *t*-test inefficient and improper. A basic assumption of the ANOVA is that

distributions are normal and variances equal. For *a* groups or treatments, each composed of *n* individual measurements, the variance ratio, *F*, was computed and presented in the following way:

	Source of variation	*df*	*SS*	*MS*	F_s
1	Variation among groups	a-1	$n(a-1)s_y^2$	SS/df	MS_1/MS_2
2	Variation within groups	$a(n-1)$	$\Sigma(n-1)s^2$	SS/df	
3	Total	an-1	SS_1+SS_2		

where *SS* represents *sums-of-squares* and *MS*, the *mean-squares*. s^2 represents the variance of the various treatments, while s_y^2, the variance of the means of these treatments around a grand mean. Where group sizes differed, the average *n* was calculated as:

$$n_o = 1/(a-1) \times [\Sigma n - (\Sigma n^2/\Sigma n)]$$

A priori tests were conducted following ANOVA, allowing individual or group means to be compared with others, recalculating SS_1. For example, a comparison between the control (Y_c) and a group of three treatments (Y_1, Y_2 and Y_3) could be made:

$$SS_1 = \frac{(\Sigma Y_c)^2}{n_c} + \frac{(\Sigma Y_1+\Sigma Y_2+\Sigma Y_3)^2}{(n_1+n_2+n_3)} - \frac{(\Sigma Y_c+\Sigma Y_1+\Sigma Y_2+\Sigma Y_3)^2}{(n_c+n_1+n_2+n_3)}$$

as effectively two groups are being compared, degrees of freedom in this case would be taken as 1, and F_s calculated as usual.

Correlation analysis was employed to examine the relationships between initial weight and weight gain, and between treatment dosage and response, calculating the correlation coefficient, *r*, and, where appropriate, the coefficient of determination, r^2 (Sokal & Rohlf, 1973; Parker, 1979). The correlation coefficient, *r*, was computed:

$$r = \frac{\Sigma xy - (\Sigma x \Sigma y/n)}{\sqrt{[(\Sigma x^2-(\Sigma x)^2/n)(\Sigma y^2-(\Sigma y)^2/n)]}}$$

where x represents the independent variable (initial weight or treatment concentration), and y the dependent variable (weight gain or mortality). The coefficient of determination was computed as the square of r.

Regression analysis was employed, where the linear component was found to be significant, to obtain the regression line, the equation whereby the variation in y not accounted for in terms of the variation in x is at a minimum. The regression equation in the form $y = mx + c$, was deduced by calculating m, using the formula below, and substituting the mean values of x and y into the equation to obtain c.

$$m = \frac{\Sigma xy - (\Sigma x \Sigma y / n)}{\Sigma x^2 - (\Sigma x)^2 / n}$$

Correction of weight gain data

Dependence of weight gain on the initial weight of larvae was investigated using correlation and regression analysis. A correction term was formulated from the regression equation, and the success of this correction examined by correlation and regression analysis.

Determination of distribution type

The distributions of the corrected WGSL and corrected WGL data were examined. By grouping both the control results and, using standard normal deviates, the theoretical normal data, the significance of any deviation from the normal distribution was assessed using the G-test.

Data Analysis

Weight gain

The effects of treatments on larval development was investigated using the parameter of weight gain, calculated by subtracting the initial

weight from the final weight of each larva. Where larvae were weighed in groups, the mean individual mass was used. This weight gain was multiplied by a correction term in order to annul its variation due to initial weight. Weight gain was considered in two forms: the weight gain per surviving larva (WGSL) was analysed to investigate the possible mode of action of the plant proteins; and the weight gain per larva (WGL), including all larvae, both surviving and dead, was analysed to give an overall image of the effect on the population. Dead larvae were not weighed, but were considered to have a biomass of 0mg, thus weight gain was the negative of the initial weight.

The sum, mean and standard deviation of the corrected WGSL and corrected WGL data for each treatment were calculated and these values employed in analysis for variation between treatments and controls. The *F*-test was used to establish the equality of sample variances, analysing two samples at a time. Where variances were found to be equal, the variation between samples was analysed with either the *t*-test, or, where a number of treatments showed equal variances, analysis of variance (ANOVA) was employed. Where variances were shown to be significantly different, the differences between samples was evaluated using the *t*-test, with a reduced number of degrees of freedom. Correlation analysis was employed to examine dosage response, where relevant.

Survival

Larval survival was examined using the *G*-test of independence to determine whether survival was affected by a given treatment, and, where appropriate, correlation analysis was employed to evaluate the relationship of treatment dosage and survival.

CHAPTER 3 CHARACTERISATION OF INSECT DIGESTIVE ENZYMES

RESULTS

Part A: PROTEASES

(i) BApNA Hydrolysis

Some larval protease activity was demonstrated using BApNA as substrate. BApNA, α-N-benzoyl-DL-arginine-p-nitroanilide, is a chromogenic substrate. Digestion results in the release of p-nitroaniline, a coloured compound, which can be monitored by direct spectrophotometry at 410nm. Rate of substrate hydrolysis is inferred from the rate of p-nitroaniline production, measured as the increase in $O.D._{410nm}$ sec^{-1}. Steady and similarly low rates of hydrolysis were demonstrated with both the larval gut extract and papain (a plant-derived cysteine protease). The rate of p-nitroaniline production, ie. BApNA digestion, was established as 1.27 $x10^{-4}$ $O.D._{410nm}$ sec^{-1} for insect proteases (0.056 gut equivalents) and 1.25 $x10^{-4}$ $O.D._{410nm}$ sec^{-1} for papain (1.4µg), under the described conditions. These rates were close to the lower limits of the spectrophotometer's sensitivity.

(ii) Myoglobin

Using this method high levels of protease activity were demonstrated. Myoglobin is a general protease substrate which, when subjected to SDS-PAGE, appears as a single distinct band on stained gel at around 18kDa. The extent of myoglobin digestion resulting from incubation with protease was determined by calculating the reduction in myoglobin present in assay samples, compared to an undigested control, scanning the stained gel with a laser densitometer and calibrating results using a myoglobin standard curve.

Initial assays, conducted with gut extracts from SCR larvae at pH3.6 resulted in total digestion of myoglobin within 24hrs, while assays at pH6.0 resulted in the occurrence of little hydrolysis. Assaying protease activity over a range from pH2.7 to pH7.2, over a 30min period, demonstrated that

substantial protease activity occurred over a broad pH range. It was observed that 0.047 larval gut equivalents (2µl) of protease activity were ample to cause marked digestion of 8µg myoglobin over the 30min incubation period, throughout much of the pH range. Initial assays suggested that a peak of activity occurred at around pH4.6, although activity was high over the whole range tested (see Figure 3). Subsequent assays again showed the broad spectrum of activity, showing consistently high activity from pH3.0 towards pH6.0, but failed to exhibit a peak at pH4.6 (Figure 4). At very low pH (below 3.0), hydrolysis of myoglobin seemed slightly increased, whilst above pH6.5 protease activity was minimal.

Inhibition assays were conducted in order to establish the major type of protease activity present. Compounds were tested by replacing the 4µl distilled water in the assay mixture by an equal volume of inhibitor solution. Percent inhibition was calculated by the reduction in hydrolysis in the test assay compared to an uninhibited control. Levels of 22% to 43% inhibition were observed with the cysteine protease inhibitors E64, pCMB and pCMS (Figure 5). Assays with varying concentrations of inhibitor were not conducted, thus no estimation of maximal inhibition levels was possible. As can be seen from Figure 5, the levels of digestion achieved varied a little amongst the various assays collated here. 0.047gut equivalents of larval protease activity were demonstrated to hydrolyse between 4.24 and 5.6µg of the 8µg of myoglobin present in the assay medium in 30min at 30°C. It can be extrapolated that 1 gut equivalent of larval protease activity would be capable of digesting between 90 and 120µg of myoglobin in 30min, under these conditions. The proteolytic activity of extracts taken from adult insects was demonstrated to be higher than that of larval extracts, 0.04 gut equivalents of activity shown to hydrolyse 7.36µg of myoglobin in 30min at 30°C, ie. around 180µg myoglobin digested per adult gut equivalent in 30min, under these conditions.

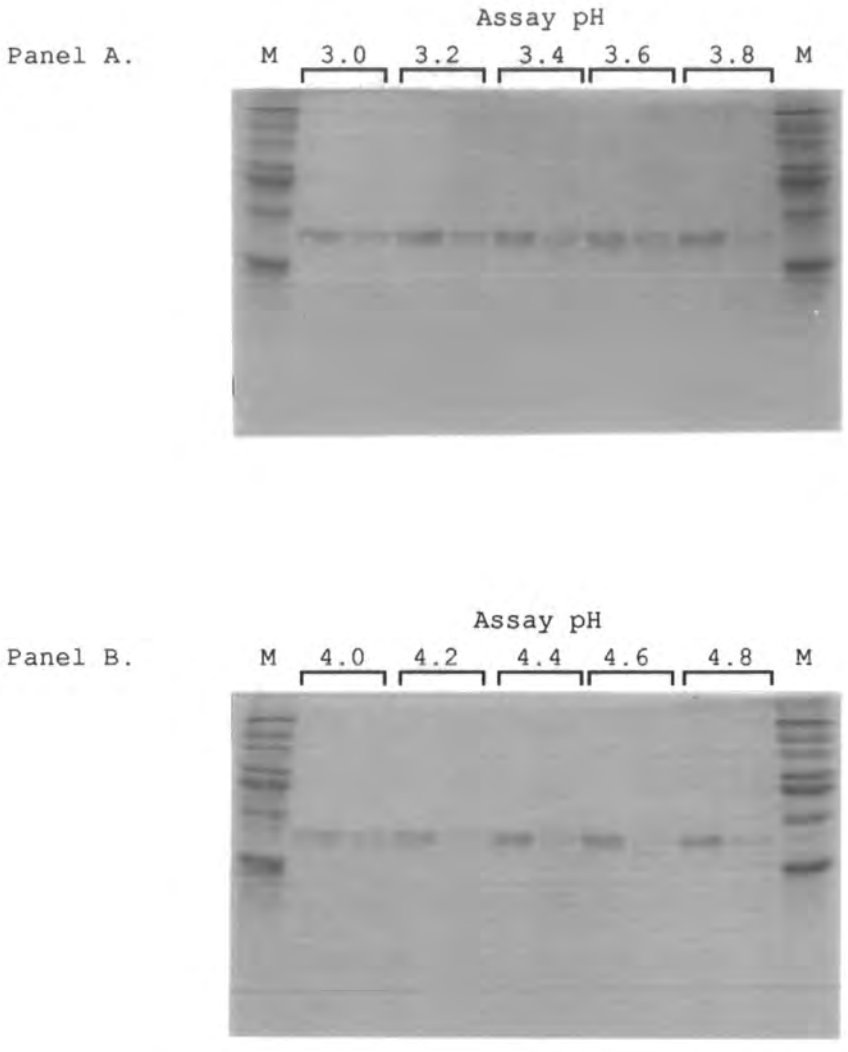


Figure 3. Polyacrylamide gels showing the influence of pH on larval gut protease activity, as determined by myoglobin digestion. Paired assay lanes show no-enzyme control (left) and enzyme assay (right). Lanes M, molecular weight markers.

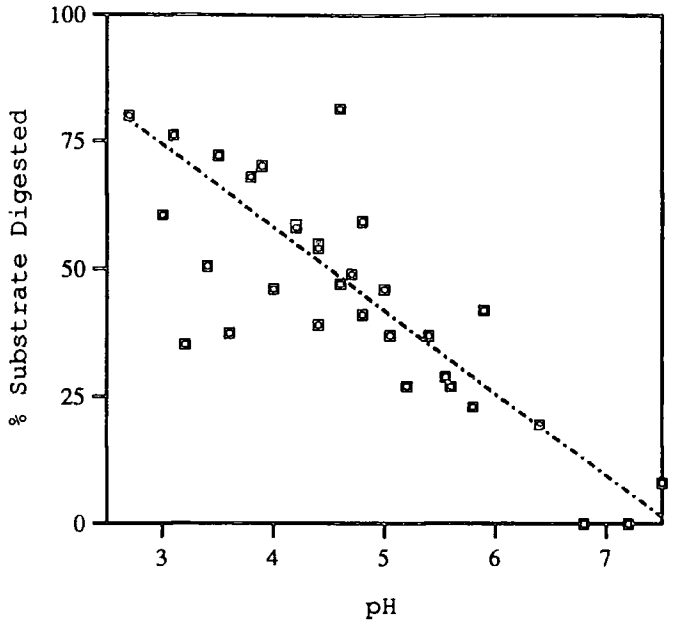


Figure 4. The influence of pH on insect protease activity as detected by myoglobin digestion. 2µl insect gut extract (0.047 larval gut equivalents) were used per 30min assay. Activity is given as a percentage decrease in myoglobin concentration, with 8µg myoglobin used per assay.

Enzyme	Control Dign	Inhibitor	% Inhibition
LGE	5.44	7.0mM pCMB	24.5
LGE	4.24	0.5mM pCMS	33.5
LGE	5.60	2.9mM E64	22.3
SAGE	7.36	2.9mM E64	43.5

Figure 5. Each assay mixture contained 2µl enzyme sample in 20µl. Levels of control digestion are given as µg of myoglobin digested (from the original 8µg) in 30min. Inhibition is given as the percent reduction in this activity. Results are calculated directly and taken from a number of assays.

(iii) Azocasein

Larval protease activity was also demonstrated using azocasein as substrate. Azocasein is another general protease substrate, produced from casein by the coupling of tyrosine and histidine side-chains with diazotized sulphanilic acid (or sulphanilamide in alkali). The azo-coupling confers an intense yellow colouration on the protein. Digestion of azocasein releases azo-coupled peptides which are soluble in a dilute solution of TCA. Such a TCA solution precipitates any remaining intact proteins, allowing quantification of the released peptides by their absorbance at 440nm. It was found to be necessary to use as much as 1-2 gut equivalents of activity per reaction to achieve sufficient activity for general use (ie. over 1unit). The use of no-enzyme controls allowed correction for any peptides present in the stock solution or produced by substrate autolysis.

Assays were conducted over a range of pHs using the buffers: 0.2M sodium acetate for pH4.0 - 5.5 and sodium phosphate for pH6.0 - 8.0 (See Figure 6.). Activity over a broad pH range was demonstrated. From pH4.0, the level of activity increased rapidly with pH to pH5.5.. A dip in activity was consistently recorded at pH6.0. From this pH up to pH7.5, activity levels were demonstrated again to rise with pH, with a peak of activity apparent around pH7.0 - 7-5. The level of activity demonstrated was remarkably high from pH4.5 to pH8.0, showing a distinct difference from the range demonstrated with myoglobin as substrate.

Inhibition assays were conducted using a range of potential inhibitors and enhancers. 1.5 gut equivalents were used per assay. 5µl buffer was replaced with inhibitor solution. Three sets of controls were employed, as for inhibitor assays with myoglobin. Inhibitors were all prepared and used at stock concentrations of 1mg ml^{-1}, and therefore do not represent the amount needed for maximal inhibition levels. The results, shown in Figure 7, show that 73% of protease activity was inhibited by E64 and 71% by cystatin. Activity was also demonstrated to be enhanced by 25% by the

addition of DTT. 34% inhibition of activity was also seen to be caused by the aspartic protease inhibitor, pepstatin A. Little (around 10%) inhibition of activity was caused by either of the trypsin inhibitors.

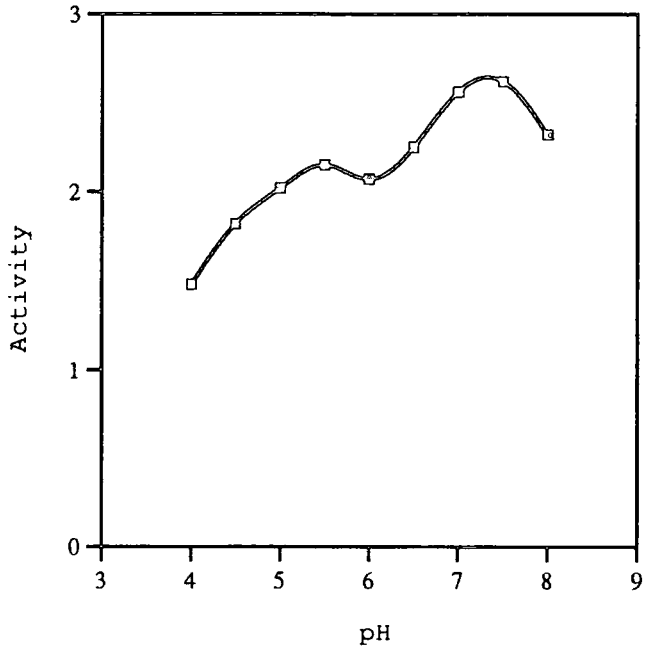


Figure 6. The influence of pH on SCR larval protease activity. 60µl gut extract (1.5 gut equivalents) was used per assay. Azocasein was employed as a substrate. Activity units are given as that giving an OD. of 0.100 under the conditions used.

Inhibitor/Enhancer	Concentration	% Res.Act.
Control		100
E64	18.66µM	27
Cystatin	0.51µM	29
Soybean trypsin Inhibitor	0.33µM	89
CpTI	6.7µg ml^{-1}	90
Pepstatin A	9.72µM	66
Potato carboxypeptidase inhibitor	6.7µg ml^{-1}	95
DTT	43.23µM	125

Figure 7. Concentrations are given for inhibitor or enhancer in reaction mixture (750µl). % Res.Act. represents enzyme activity, expressed as a percentage of the control activity in the absence of inhibitor or enhancer.

(iv) Gelatin

The medley of proteases present in the insect gut was analysed using gelatin as substrate, co-polymerised into polyacrylamide gel by a method modified from that of Heussen and Dowdle (1980). Samples of extracted gut or papain were electrophoresed on 11% minigels containing 0.12% gelatin at 4°C. Gels were then washed in 2.5% Triton X-100 to remove SDS, and incubated in McIlvaine's buffer at pH5.0 at 37°C. Subsequent staining with Coomassie Brilliant Blue revealed proteolytic activities as colourless zones against a dark background.

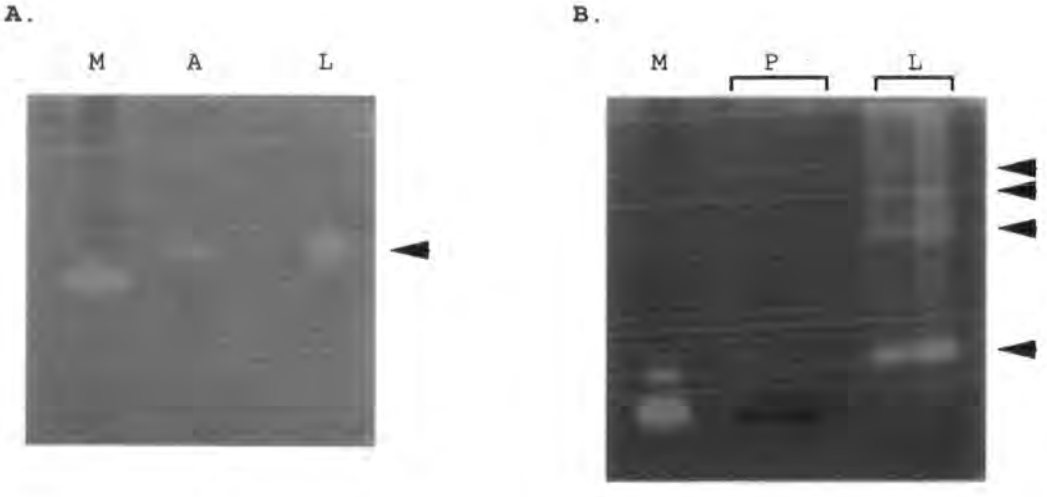


Figure 8. Protease digestion by SCR gut enzymes, detected following electrophoresis on polyacrylamide gel co-polymerised with gelatin. Panel A, 0.33 gut equivalents of adult protease activity (A) and 0.39 gut equivalents of larval protease activity (L). Panel B, 2.5µg (left) and 5.0µg (right) papain activity (P) and 0.059 (left) and 0.118 (right) gut equivalents of larval gut protease activity. Lanes M, molecular weight markers. Arrows indicate the zones of major activity in the insect extracts.

Figure 8 shows initial minigels, panel A demonstrating one main zone (possibly composed of two distinct zones) of protease activity in the midgut extracts of both larval and adult SCR. This activity appeared on gel at around 30kDa and some streaking was apparent towards the top. Two bands of activity were seen in the molecular weight marker track. An attempt to incorporate cystatin, as well as gelatin, into the gel resulted in no alteration in observed activity (results not shown).

Subsequent gels revealed the presence of further activities, more clear zones being visible on gel (Figure 8, panel B). In order to distinguish more clearly between these different zones of activity and investigate the type of activity observed, full-sized gels were employed. Samples from a differential extraction of SCR larvae were used, allowing the location of the proteases to be examined. The equivalent of extract from one insect was loaded per well. Following electrophoresis, gels were sectioned and the effects of cysteine and E64 on protease activity investigated.

The resultant gels (Figure 9) revealed more clearly the multiple zones of protease activity. At least 8 distinct proteolytic activities were observed, ranging in size from around 24kDa to over 60kDa. All these activities appear to be stimulated by incubation with 5mM cysteine, as shown on panels C and D. Incubation in buffer plus 20μM E64 resulted in no proteolytic activity being detected (results not shown). Preincubation of enzyme sample and 1mM $CuSO_4$ resulted in only slight inhibition of activity. The observed activities were grouped, rather arbitrarily, on the basis of mobility on SDS-gel (without ß-mercaptoethanol), into four groups: group I consisting of one activity with Mwt of approximately 24kDa; group II comprising at least two activities with Mwts between 30 and 35kDa; group III containing at least two activities at 45 to 50kDa, and group IV with at least three activities of over 55kDa.

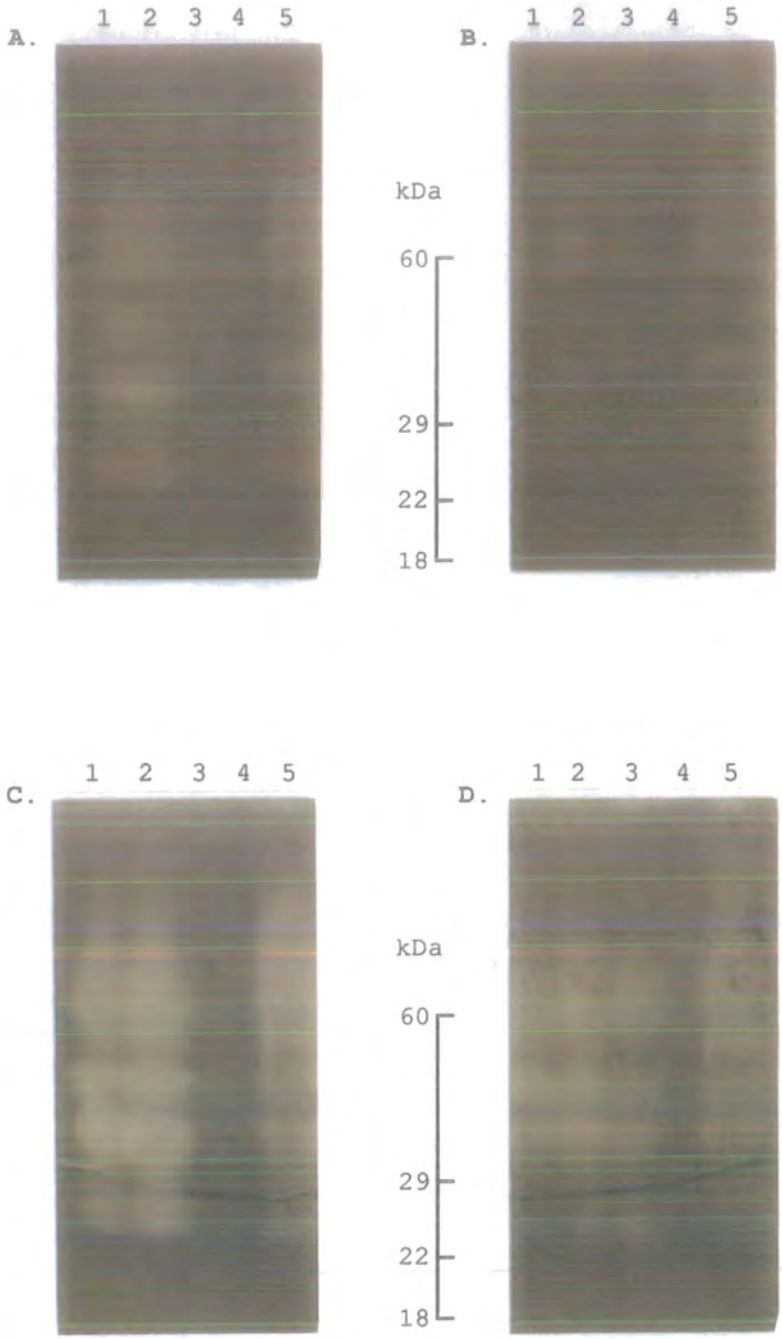


Figure 9. Protease digestion of gelatin co-polymerised into polyacrylamide gel. Lanes show digestion by luminal contents (2); gut wall fraction (3); carcass fraction (4); extract from total larva (5); and luminal contents preincubated with Cu2+ (1). The enzyme fractions used in panels A and C were extracted in distilled water, those in panels B and D were extracted in PBS. Incubation of gels was conducted in McIlvaine's buffer pH5.0 (panels A and B), or buffer plus 5mM cysteine (panels C and D). The equivalent of 0.8 larval extracts were used per lane.

Panel A (extraction in water) demonstrates that the activity observed during previous assays is virtually all limited to the contents of the gut lumen, only a small amount of activity being visible in the gut wall fraction and this only after stimulation of activity with cysteine (see panel C). Panel B (extraction in PBS) shows less overall activity, but demonstrates protease activity to be present in both gut lumen and wall, although less is visible in the wall extract. Almost all the bands observed in the water extracted fractions are also demonstrated in those extracted in PBS, although some only after cysteine stimulation. One band of activity, however, present in the water extracted luminal contents at around 31kDa, is present in the PBS extracted material only as a smudge of activity following stimulation with cysteine.

(v) Fluorimetric Assays

Z-Phe-Arg-AMC as substrate

High levels of cysteine protease activity in the midgut extracts of SCR and WCR were confirmed by their ability to digest Z-Phe-Arg-AMC, a synthetic substrate specific for cysteine proteases such as papain, Cathepsin B and Cathepsin L. Digestion of Z-Phe-Arg-AMC, a barely fluorescent compound, liberates AMC which, following termination of the reaction with monochloroacetate, can be quantified by its intense fluorescence. Assays using trypsin yielded no digestion of the substrate. Papain, the plant-derived cysteine protease, was used as a control and assays conducted to compare the activities of insect and plant enzymes. Activity was converted to mU, 1mU representing 1nmol of AMC released per min.

Activity levels in extracts from SCR larvae and adults and also WCR adults were shown, at pH5.5, to be extremely similar (figure 10). Extrapolation of these results allows activity levels for 1 gut equivalent to

be determined, ie. 3.62mU per gut equivalent for SCR larvae, 4.42mU per gut equivalent in adult SCR, and 4.05mU per gut equivalent with adult WCR.

Extract	Amount	Activity (mU)
SCR larvae	0.047gt	0.170
SCR adult	0.040gt	0.177
WCR adult	0.040gt	0.161
papain	1µg	0.154

Figure 10. Comparison between the digestive powers of rootworm enzymes and papain, using the substrate Z-Phe-Arg-AMC. Amount indicates the quantity of enzyme sample used, in gut equivalents (gt) or mass. Activity, given in mU, is the mean of duplicate assays, corrected for autolysis of the substrate.

Inhibition assays were conducted by the substitution of 1 to 20µl buffer with inhibitor solution. Three sets of controls were used, as usual for inhibition assays. Preliminary assays with E64 and cystatin confirmed that both inhibitors were capable, at low concentrations, of causing almost complete inhibition of the detectable activity of larval and adult cysteine proteases. 1µg cystatin or 0.5µg E64 both caused around 98% inhibition of the activity demonstrated by 2µl SCR larval gut extract. The activity of extracts from adult SCR and WCR (0.04 gut equivalents) were almost completely arrested by 1µg cystatin.

Assays conducted with the differentially extracted fractions from larval SCR confirmed the results of the gelatin assays, as shown in figure 11. With the fractions extracted in water, the proteolytic activity demonstrated in the extract from complete insect was shown to be limited to the gut luminal contents. Fractions extracted in PBS exhibited activity in all but the carcass fraction. Markedly less activity was demonstrated with the PBS extracted fractions, especially in the extract from total insect.

Fraction	Extraction Medium	
	$d.H_2O$	PBS
Lumen contents	0.080	0.036
Gut wall	0	0.034
Carcass	0	0
Total larva	0.108	0.008

Figure 11. Comparison of proteolytic activity in extracts from SCR larvae. Each assay contained extract from 0.1 insects. Activity is shown in mU.

Estimation of Enzyme Titre using E64

E64 is known to cause tight, irreversible inhibition of many cysteine proteases by the production of equimolar complexes, while having no effect on serine proteases (Barrett & Kischke, 1981; Barrett *et al.*, 1982). Chicken egg-white cystatin has also been shown to form equimolar complexes with papain-like cysteine proteinases (Anastasi *et al.*, 1983; Lindahl *et al.*, 1988). Because of this stoichiometric inhibition, it was expected that inhibition of insect cysteine proteases by E64 and cystatin would be directly proportional to the concentration of inhibitor used.

Initial E64 inhibition assays, using normal amounts of enzyme (2µl gut extract or 1µl papain), failed to produce the expected linear relationship, demonstrating instead a sigmoidal relationship (results not shown). Similar results were obtained with cystatin. When diluted enzyme stocks (10x diluted) and shortened incubation times (5min) were employed, inhibition of papain by E64 was roughly proportional to the inhibitor concentration, demonstrating that the sigmoidal relationship observed originally was due to assay conditions. All kinetics studies and pH assays using this substrate were conducted using these modified conditions. In these cases the volume of enzyme is given according to the equivalent amount of original stock solution used.

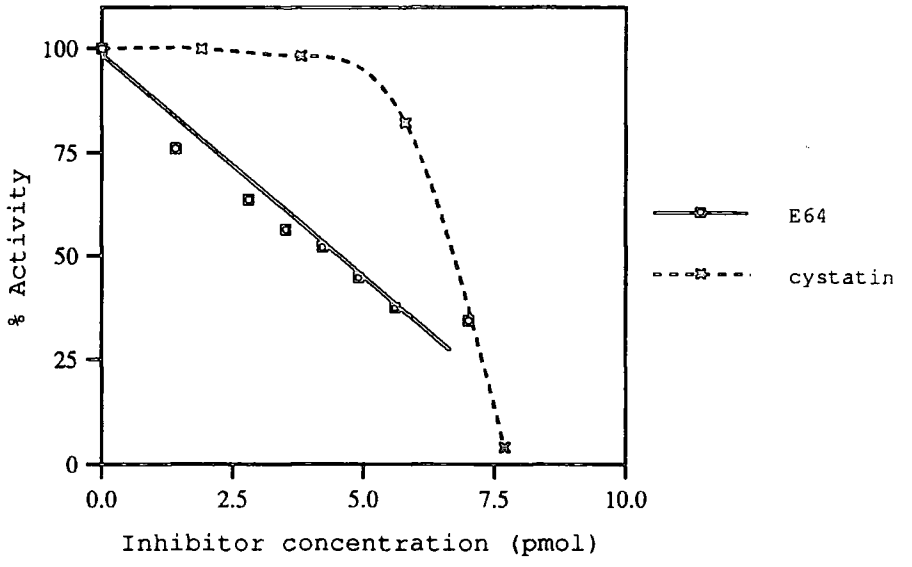


Figure 12. Inhibition of papain activity by E64 and cystatin. 0.1µg (4pmol) papain was used per 5min assay. Z-Phe-Arg-AMC was employed as a substrate. Remaining activity is given as a percentage of the uninhibited activity.

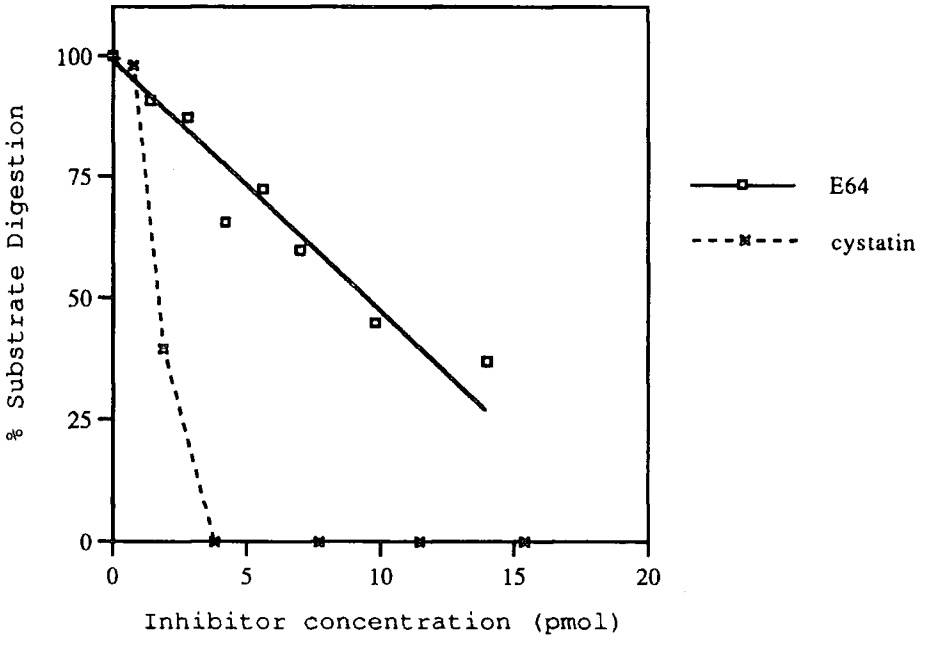


Figure 13. Inhibition of larval gut enzyme by E64 and chicken egg white cystatin. 0.2µl gut extract (0.0047 gut equivalents) were used per 5min assay. Digestion is given as a percentage of uninhibited enzyme activity, using the substrate Z-Phe-Arg-AMC.

E64 inhibition of 0.1µg papain was roughly proportional to inhibitor concentration, using a 5min assay. Figure 12 shows that 100% inhibition of papain was caused by around 9.5pmol E64, allowing a total enzyme stock concentration of 95µM to be calculated.

The action on 0.2µl larval gut extract by E64 also resulted in approximately linear inhibition, ie. inhibition proportional to the inhibitor concentration (Figure 13). 100% inhibition under these circumstances occurred when 19.4pmol E64 was present, giving a total stock cysteine protease concentration of 97µM, assuming molar inhibition by E64.

Figures 12 and 13 also show the effects of cystatin, the chicken egg-white cysteine protease inhibitor, on the activities of larval gut proteases and papain. As can be seen, under these conditions, inhibition by cystatin was not linear, but it is interesting to note the far greater effect of cystatin on the insect protease activity in comparison to its effect on papain.

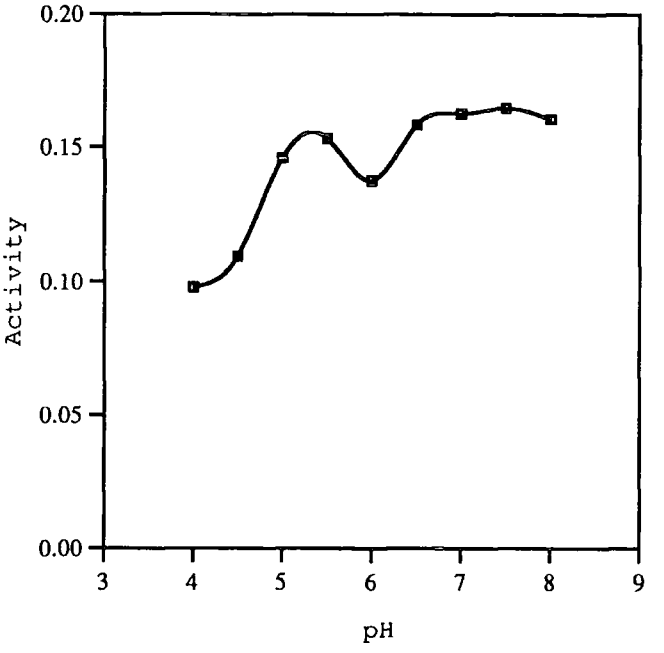


Figure 14. The influence of pH on protease activity in SCR larval gut extract, using the substrate Z-Phe-Arg-AMC. Activity is given in mU, with 0.2µl gut extract (0.0047 gut equivalents) used per assay.

Influence of pH on protease activity

Assays investigating the effect of pH on activity were conducted using 0.2M sodium acetate for pH4.0 - 5.5 and sodium phosphate for pH 6.0 - 8.0, as in the azocasein pH studies. 10x diluted enzyme samples were used in 5min assays. The resultant activity, as illustrated in Figure 14, is similar to that achieved with the azocasein assays (see Figure 6), activity being high over the whole range of pH tested, peaking at around pH7.0 - 7.5. Once more a dip in activity was demonstrated at pH6.0.

Enzyme	Z-Phe-Arg-AMC	Z-Arg-Arg-AMC
2µl LgE	0.701	0.430
1µg papain	0.295	0

Figure 15. Comparison of protease activity with two fluorimetric substrates. Activity given in mU, calculated from duplicate results and adjusted for substrate autolysis.

Z-Arg-Arg-AMC as substrate

Unlike Z-Phe-Arg-AMC, the synthetic substrate Z-Arg-Arg-AMC is known to be unaffected by Cathepsin L and H, being specific for Cathepsin B-like enzymes. The method and principle of this assay are precisely analogous to that using Z-Phe-Arg-AMC as substrate, allowing a direct comparison of results. Assays were conducted with 10x diluted enzyme stocks and an incubation period of 5min, unless otherwise stated. Initial assays comparing the maximal digestion of the two substrates demonstrated dramatic differences between papain and at least part of the activity present in the gut extracts (see Figure 15). These assays were conducted with normal stock concentrations of enzyme, using a 10min incubation period and using the substrate at an initial concentration of 40µM. With SCR larval extracts, only 60% of the activity level demonstrated with Z-Phe-Arg-AMC

was achieved with the substrate Z-Arg-Arg-AMC. Papain was found to have no effect on Z-Arg-Arg-AMC.

Digestion of the two fluorimetric substrates

Time course assays were conducted with the digestion of both substrates by the insect enzymes assayed over a period of 0 to 30min. 10x diluted enzyme stocks were used. The results, shown in Figure 16, demonstrate marked differences in the digestion rates of the two substrates. The rate of Z-Arg-Arg-AMC digestion is constant, giving a linear increase of product with time. This rate, however, is low, only 25% of the substrate being digested within 30min. In comparison the digestion of Z-Phe-Arg-AMC is rapid, but the rate of this digestion is not constant. The initial rate is very high, but within minutes this decreases and settles to a constant but lower rate. This lower rate is still greater than that achieved with the substrate Z-Arg-Arg-AMC, and after 30min of digestion only 14% of the substrate remains undigested.

The kinetics involved in this digestion were investigated by varying the substrate concentration in a 5min assay. Figure 17 shows the resultant plots. Using the formula:

$$\frac{1}{V} = \frac{Km}{Vmax} \times \frac{1}{[S]} + \frac{1}{Vmax}$$

approximate values of Km were calculated for each substrate, as were Vmax values (see Figure 17). Similar strength of binding was demonstrated with the two substrates, Km values determined as 4.4 $x10^{-6}$M and 6.7 $x10^{-6}$M for Z-Phe-Arg-AMC and Z-Arg-Arg-AMC, respectively. The distinct digestion rates demonstrated in Figure 16 were quantified in the calculation of Vmax, values of 0.50mU and 0.15mU obtained for Z-Phe-Arg-AMC and Z-Arg-Arg-AMC, respectively.

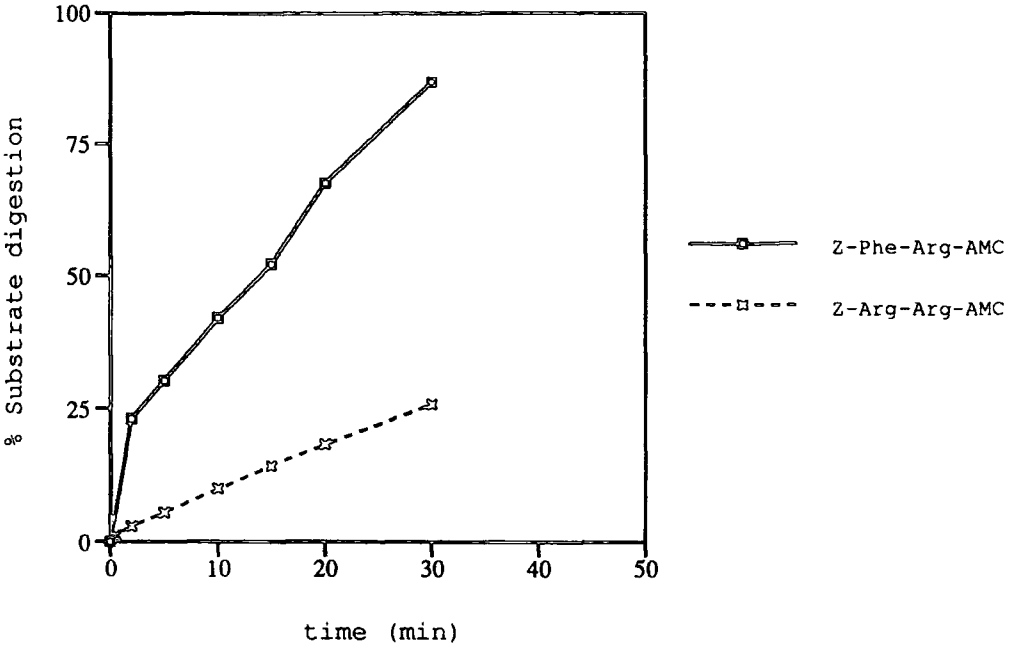
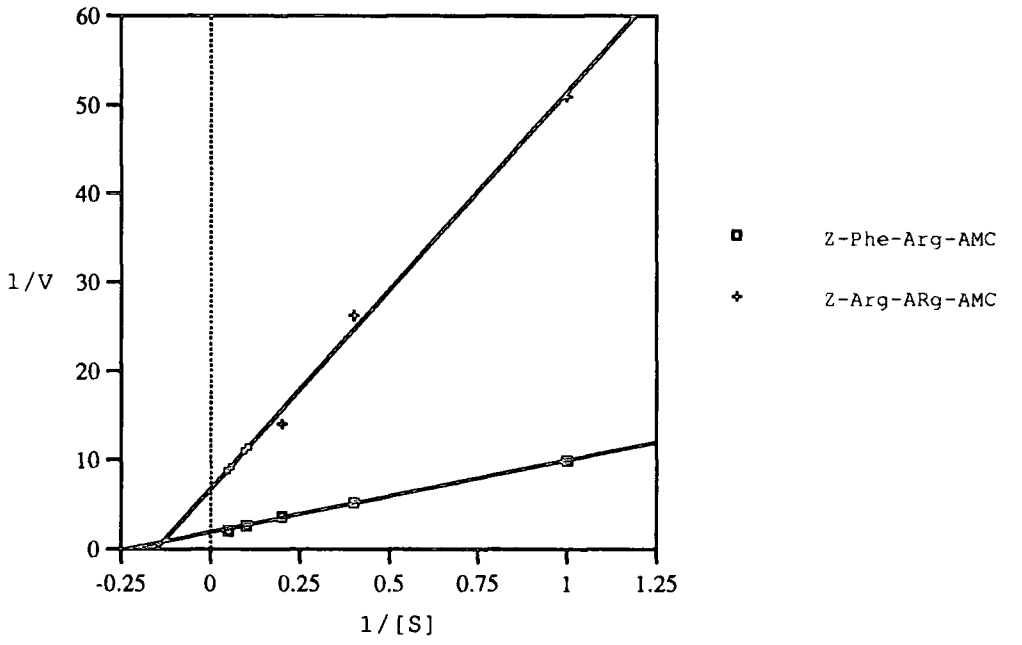


Figure 16. Comparative digestion of the two fluorimetric substrates by SCR larval digestive cysteine proteases. 0.0047 gut equivalents of enzyme extract were used per assay.



Substrate	Km	Vmax
Z-Phe-Arg-AMC	4.4×10^{-6}M	0.50mU
Z-Arg-Arg-AMC	6.7×10^{-6}M	0.15mU

Figure 17. Digestion of Z-Phe-Arg-AMC and Z-Arg-Arg-AMC, by larval gut proteases, with varying concentrations of substrate. Results are shown as the reciprocal of the substrate concentration (μM) and velocity (mU). Approximate values of Km and Vmax are shown for the two substrates.

Inhibition Assays with Z-Arg-Arg-AMC

Inhibition of gut protease activity, as detected by Z-Arg-Arg-AMC, was investigated using E64 and cystatin, employing the same methods as used with Z-Phe-Arg-AMC. The results, shown in Figure 18, demonstrate that E64 inhibition again approximates to a straight line, increasing in proportion to the concentration of inhibitor. Inhibition levels, however, are low and no estimation of enzyme concentration can be made. Cystatin inhibition is interesting in that initial inhibition is rapid but levels off at about 60% inhibition, the remaining 40% activity seemingly unaffected despite increasing amounts of inhibitor. This effect was found to be highly reproducible. It was also notable that all activities measured here were small in comparison with those found with the other fluorimetric substrate, in fact digestion levels were measured to be only 20% of those found with Z-Phe-Arg-AMC (results not shown).

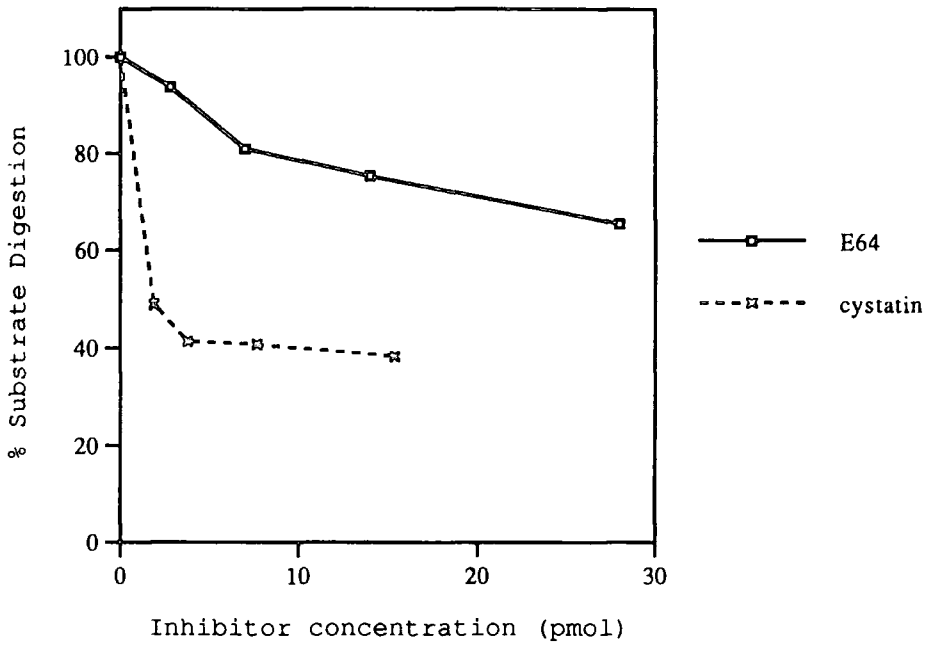


Figure 18. Inhibition assays showing the inhibitory activities of E64 and cystatin against larval proteases, as detected with the substrate Z-Arg-Arg-AMC. Activity is given as a percentage of uninhibited activity. 0.2µl gut extract (0.0047) gut equivalents) were used per 5min assay.

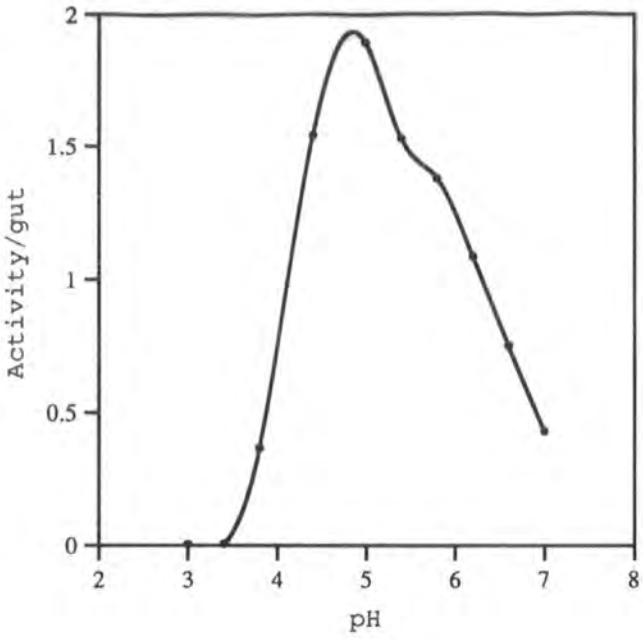


Figure 19. The effect of altered pH on the activity of larval SCR digestive amylases. Activity is given in mg maltose liberated per 30min at 30°C, per gut equivalent of enzyme activity.

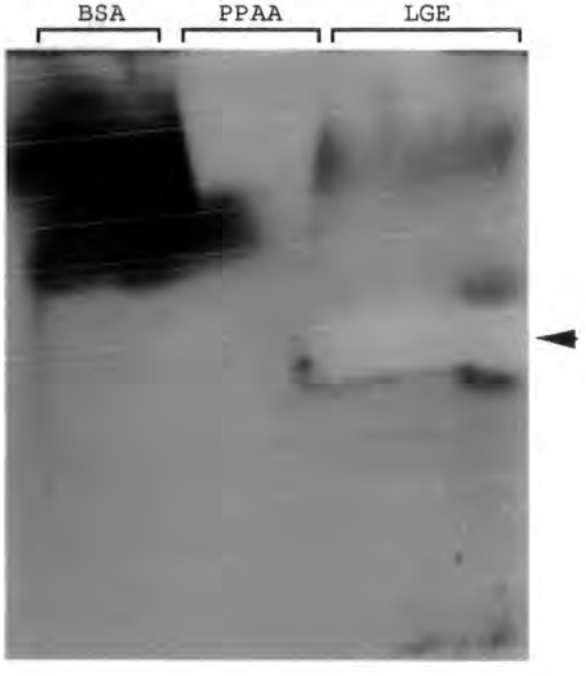


Figure 20. Analysis of larval amylase activity by native polyacrylamide gel electrophoresis, starch infiltration and iodine staining. Lanes, 20µg (left) and 10µg (right) BSA; 65µg porcine pancreatic α-amylase (PPAA); and 0.042, 0.085 and 0.170 gut equivalents (left to right) of SCR larval gut extract (LGE). Larval amylase activity is indicated by the arrow.

Part B: AMYLASES

Amylase activity was demonstrated using a starch hydrolysis method modified from that of Bernfeld (1955), assaying activity at 30°C for 30min. This method uses the increase in the reducing power of a solution of soluble starch, which results from starch digestion, to measure amylase activity. Units were calculated as mg maltose liberated per 30min at 30°C by 10µl enzyme sample, ie. 0.17 gut equivalents. High levels of α-amylase activity were detected in the larval gut extract. Assays over a range of pHs were conducted and indicated a peak of activity at around pH5.0, although activity was high over a broad range of pHs from pH4.0 to 6.5 (see Figure 19). Little activity was demonstrated at pH3.4 and below. The activity of 1gut equivalent was determined to be 1.89mg maltose liberated per 30min at pH5.0 and 31°C.

Analysis of the isoforms of α-amylase present in SCR larval gut was conducted by electrophoresis of enzyme samples on native polyacrylamide gels at 4°C, followed by infiltration with starch, incubation at 37°C to facilitate enzyme activity, and staining according to the method of Brewbaker *et al.* (1968). Amylase activity appears on the gel as a colourless zone on the dark stained background. Porcine pancreatic α-amylase was used on gel as a control, although, as can be seen from Figure 20, this activity remained close to the top of the gel. A single band of amylase activity was detected on gel in the gut extract tracks. Some activity was also visible at the top of the gel, although on subsequent gels this activity was minimal.

Part C: ESTIMATION OF INSECT GUT pH

Dissection and Staining

Larval guts were dissected out and Universal Indicator solution used to stain for pH. The excised gut appeared red (pH4.0 - 5) while the surrounding fluid was yellow (around pH6).

Regurgitation

Regurgitation was induced by gentle agitation of larvae, and the resulting liquid stained by the addition of a small amount of Universal Indicator solution. The regurgitant on mixing with indicator became red in colour, indicating a pH of 4 - 5.

Artificial Diets containing Indicator

Universal indicator solution was incorporated into artificial diet at a concentration of 0.2%. This showed that the diet was of pH5 - 6. The indicator solution, however, contained propan-2-ol, and this proved toxic to larvae, causing mortality within one or two hours and preventing any feeding occurring.

Subsequently diets were prepared incorporating various indicators: bromocresol green, methyl red and litmus, added in aqueous solution. Of these three, the litmus diet proved the most successful. On the methyl red diet little colour change could be observed in the gut as a result of the presence of indicator.

Larvae feeding on diet impregnated with bromocresol green were observed to have fed within 30min of being introduced onto the diet, as coloured diet was clearly visible within their guts. This colouration appeared more of a green-blue than the diet, indicating that the gut contents were more acidic than was the blue diet. It was difficult to establish any accurate estimations of pH of the gut contents using the bromocresol green indicator.

Litmus incorporated into artificial diet gave rise to the most interesting and useful results. The diet was coloured purple by the indicator, showing it, as expected, to be mildly acidic. After feeding for one day the insect gut contents appeared more red than the diet, ie. more acidic. On dissection of gut larvae it was discovered that while the majority of the gut contents (fore- and mid-gut) were coloured red (acidic),

the hind-gut contents were markedly blue in colour. After feeding was allowed to continue on the diet for five days, the excised gut appeared different to those feeding on the diet for only one day. The majority of the gut contents appeared a deeper red colour, slightly purple, and no distinct blue colouration was visible in the hind-gut.

CHARACTERISATION OF INSECT DIGESTIVE ENZYMES - DISCUSSION

For an insect control strategy, using plant-derived inhibitors, to be successful, it is essential that the inhibitors are active against enzymes employed by the target insect. Thus before any such strategy can be formulated, it is necessary to determine the major types of enzyme active in the particular insect in question. A wide range of enzymes are used by insects, but as the majority of known plant inhibitors are active against either proteases or amylases, it is these enzymes on which this project has concentrated. As was discussed in the introduction, all four classes of protease are found represented within the insects, at least three of the four being found within the order Coleoptera. Within each class, like with mammalian proteases, different types of insect proteases have been shown to exist, such as the two types of cysteine protease (Cathepsin B- and Cathepsin H-like) found in the larval Colorado potato beetle, *Leptinotarsa decemlineata* (Thie & Houseman, 1990b). Likewise the isoamylases identified in a number of Coleopteran species exhibit different susceptibilities to inhibition (Baker, 1987,1983; Campos *et al.*, 1989; Chen *et al.*, 1992a). Obviously, the individual characterisation of the enzyme activities for any specifically targeted species is essential.

To establish the foundation of this project, the digestive enzymes of *Diabrotica undecimpunctata howardi*, the SCR, were extracted and the proteases and amylases investigated. The major proteolytic activities were determined as cysteine proteases. The general location of protease activity

was also investigated. High levels of amylase activity were demonstrated using the DNSA detection method of Bernfeld (1955). The influence of pH on protease and amylase activity was examined, as was the actual pH of the larval gut contents.

Part A: PROTEASES

BApNA, a substrate commonly used for detection of trypsin-like enzymes, was employed in initial assays with insect gut extracts in order to establish whether digestive serine proteases were present in the SCR larvae. Extremely low rates of digestion were demonstrated (1.27×10^{-4} $O.D._{410nm}$ sec^{-1}), possibly indicating that the assay lacked sensitivity. Comparison of these activities with those of papain (1.25×10^{-4} $O.D._{410nm}$ sec^{-1}) and trypsin, implies that the low rates of activity observed were instead due to the type of protease present in the extract; BApNA proving sensitive to trypsin digestion but insensitive to digestion by papain. This indicates that the major digestive proteases of the SCR larva are not of the serine kind.

To investigate the effects of cysteine protease inhibitors on proteolytic activity myoglobin was used as substrate. Unlike BApNA, myoglobin acts as a general protease substrate, being susceptible to digestion by a variety of types of protease. When electrophoresed on SDS-polyacrylamide gel, myoglobin appears as a single sharp band, relative amounts of which may be quantified by scanning with a laser densitometer. This allows one to calculate the amount of hydrolysis, calibrating the results according to a myoglobin standard curve and a standard undigested band on the stained gel.

As expected, high levels of protease activity were demonstrated by the digestion of myoglobin. It was calculated that 1 gut equivalent of SCR larval protease activity would be sufficient to digest between 90 and 120µg myoglobin in 30min, under the assay conditions. A higher rate of digestion

was demonstrated with extracts from adult SCR, around 180µg myoglobin was digested per adult gut equivalent in 30min at 30°C.

Inhibitor assays indicate that at least a proportion of proteolytic activity is due to cysteine proteases, as partial inhibition is caused by the synthetic inhibitors, E64 and pCMB which are specific for this class of proteases. Inhibition by these inhibitors is far from complete, only up to 44%. Assays using different concentrations of inhibitor were not conducted using this substrate, hence it was not determined whether the inhibition demonstrated was at maximal levels, or whether the remaining activity was due to the presence of other classes of protease.

The use of cysteine proteases in the SCR digestive system agrees with the findings of Murdock *et al.* (1987). These workers, however, demonstrated a reduction in proteolytic activity of 93% as a result of pCMB inhibition (50µM pCMB with 0.05 gut equivalents), far greater than that shown here, despite the concentration of inhibitor used being much lower. Concurrent with this work, Purcell *et al.* (1992) also report inhibition of SCR larval protease activity by the cysteine protease inhibitor, E64. These workers reported inhibition levels of 69% by this inhibitor, much greater than that observed in this work, using myoglobin as substrate. The concentration of E64 was 28µM, lower than that used here. The amount of gut extract used in these assays, in terms of gut equivalents, is unclear, "aliquots of midgut fluid containing 25µg protein" were used, hence it is impossible to make any direct comparison between the two assays. It is evident however, that a large proportion of larval proteolytic activity is due to cysteine type proteases, a greater proportion, it appears, than has been demonstrated here .

Proteolysis of azocasein

In order to study the nature of the gut proteases further, azocasein, another general protease substrate, was used, following a method modified

from that of Lemos *et al.* (1990), similar to the casein assay of Kunitz (1947). This method was found to be less sensitive than the myoglobin assay, requiring 1-2 larval gut equivalents of activity per reaction, compared to the 0.047 gut equivalents used in the myoglobin assay. However, the results were shown to be highly reproducible and the method readily adaptable for pH range and inhibition assays.

Inhibition assays were carried out with a range of inhibitors using stock concentrations of 1mg ml^{-1}. Assays with varying inhibitor concentrations were not conducted, thus the resulting inhibitions cannot be assumed to represent maximal levels. Only 10% inhibition was caused by the Soybean and Cowpea trypsin inhibitors, as would be expected from the previously demonstrated low level of BApNA-ase activity. Although this inhibition may be due to the presence of low concentrations of trypsin-like enzymes, some serine inhibitors have also been found to inhibit cysteine proteases weakly (Gatehouse *et al.*, 1985a) and this may be the case here. Pepstatin reduced activity by 34%, demonstrating the presence of aspartic acid proteases, although not as major contributors to the digestive proteolytic activities of the larvae.

With azocasein as a substrate, the cysteine protease inhibitors, E64 and cystatin, inhibited gut protease activity by over 70%, confirming the dominating presence of cysteine proteases in the larval digestive system. The enhancement of activity by DTT demonstrated here also denotes cysteine protease activity, and thus corroborates the inhibitor assay results.

These results correlate well with the inhibition reported by Purcell *et al.* (1992): 4% inhibition from 0.5µM soybean trypsin inhibitor; 69% inhibition by 28µM E64; and 22% inhibition by 7µM pepstatin. It would appear conclusive then that the major proteolytic enzymes found in the SCR larval gut are cysteine proteases, with a minor component of aspartic acid proteases.

Inhibition of proteolysis of the two substrates, myoglobin and azocasein, by E64 differed substantially. With myoglobin as a substrate, the protease activity of 0.047 gut equivalents was reduced by only 22.3% by the addition of E64 at 2.9mM, whereas using azocasein as a substrate, the proteolytic activity of 1.5 gut equivalents was inhibited by over 70% by only 18.66µM E64. The high concentration of E64 employed to produce 22% inhibition of the 0.047 gut equivalents of activity demonstrated with myoglobin as substrate, suggests that an apparent maximal level of inhibition has been reached in this case. Inhibition of 70% of proteolytic activity by the same inhibitor using a different substrate demonstrates the dominant presence of cysteine proteases in the extract, and indicates that the specificity of the two substrates is different. The low pH range demonstrated for enzyme activity with myoglobin as a substrate may indicate that myoglobin is more susceptible to digestion by aspartic acid proteases, and this may explain why only low decreases in activity were demonstrated with inhibitors of cysteine protease. The presence of aspartic acid proteases in the insect extract was confirmed by inhibition with pepstatin, using the substrate azocasein.

Analysis of iso-proteases on polyacrylamide gel

To further characterise the different proteolytic enzymes and iso-enzymes present in the larval gut, gelatin, co-polymerised into polyacrylamide gel, was used as substrate. It must be recognised that this method of analysis is limited to those enzymes able to renature successfully following SDS treatment and electrophoresis, and with activities capable of hydrolysing gelatin. As a result it is likely that the number of enzymes detected by this technique is an underestimation of the actual activity. It is also possible that whole groups of enzymes may remain undetected, thus biasing the results. It must also be considered that the success of any inhibition assays conducted by incubating enzyme and inhibitor prior to

electrophoresis, is limited to those cases where the enzyme:inhibitor complex formed is of a covalent nature capable of withstanding dissociation during electrophoresis on SDS polyacrylamide gel.

Initial assays demonstrated the presence of a number of proteases in both larva and adult SCR extracts. Two main bands of protease activity were apparent, with some indistinct areas of activity higher on the gel. The activity in the two extracts appeared comparable, suggesting little difference between the major digestive proteases of larval and adult SCR. Similarities might be expected as both stages preferentially feed on maize, although the nutritional composition of the diet (root versus aerial tissues) would imply the need for different digestive capabilities.

No activity was demonstrated with any samples of papain. Assays were conducted with gelatin as substrate using the myoglobin assay method (data not shown). This confirmed the capability of papain to hydrolyse gelatin, thus indicating its inability to renature following SDS-PAGE.

Surprisingly, two bands of activity were observed on initial gels in the tracks containing the molecular weight markers, SDS7. The only protease supposed to be present in the SDS7 is PMSF-treated trypsinogen. Trypsinogen is the almost inactive form in which trypsin is secreted from the pancreas. Following PMSF treatment and the heat treatment (boiling for 3min, according to the supplier's instructions) employed during preparation of the marker stock solution, the trypsinogen should be completely inactivated. It seems most likely that the activity demonstrated was due to some contamination of the stock SDS7 solution, subsequent to heat treatment. Following a further heat treatment of this stock, no further activity was observed.

Using full-sized gels it was possible to achieve a better separation between the zones of activity, at least eight distinct bands of enzyme activity were apparent. Incubation with cysteine appeared to stimulate all the enzymes, indicating that all the proteolysis demonstrated here was due

to cysteine proteases. Inhibition of these enzymes by preincubation with 1mM $CuSO_4$ prior to electrophoresis was only slight, less than was expected considering that Cu^{2+} is a known inhibitor of many cysteine proteases (Arnon, 1970). Having demonstrated the presence of cysteine proteases in the extract, this result implies that either enzyme:inhibitor complex formation was slow in forming in this case, or else dissociation of the complex has occurred during electrophoresis. Incubation of gel sections in 20µM E64 led to the apparent inhibition of all activities, there being no proteolysis observable on the stained gel. This confirms the results from cysteine incubation, indicating that all activities demonstrated with this method were of the cysteine protease type.

Location of enzyme activity within larvae

The location of protease activity within the larvae was investigated by conducting a differential extraction. The luminal contents of the digestive tract were separated from the gut wall, and this from the rest of the carcass. Extract was also taken from whole insects, for comparison. These extracts were run on gelatin-polyacrylamide gels and also used in assays with Z-Phe-Arg-AMC as a substrate, the activities demonstrated with these two assays were in complete agreement.

Differences in protease distribution were demonstrated. The fractions extracted in distilled water showed that virtually all activity was limited to the lumimal contents of the gut, with just small amounts present in the gut wall fraction. With fractions extracted in PBS, while the overall level of protease activity was lower, proteolysis was demonstrated in both lumen and wall samples. It is possible that the activity found in the gut wall may be due to incomplete separation of the wall and luminal fractions, but as the water and the PBS extractions were prepared simultaneously, it would be expected that the fractionation of the two would have been the same. It is possible that PBS may act either to enhance the binding of enzymes to the

gut wall, or may impair release of enzymes from the epithelial cells, either of which would result in the reduced separation seen in the extracts.

The location of the protease activity was not fully determined because of this discrepancy of results. Activity was shown to be limited to the digestive tract, there being virtually no activity demonstrated in the carcass fractions assayed. The precise location of the enzymes, as regards the lumen or the cells of the gut wall, remains unclear as activity was observed in both fractions, despite being greater in luminal fractions. The proteases present in the lumen of the digestive tract must originate from one of three sources: the epithelial cells of the gut wall, as the majority would be expected to; from any gut flora present; or from the food itself. Extracts from maize were shown to exhibit little or no cysteine protease activity with Z-Phe-Arg-AMC as a substrate (see *Chapter 4*) discounting the possibility of the enzymes being of food origin. Although the contribution of gut flora cannot be totally ruled out, from the similarity of the activities observed on gel between luminal and wall samples, it is apparent that at least the majority of activity originates from the epithelial cells. It is possible that activities present only in the luminal contents may still originate from these epithelial cells, as they may have been produced as inactive zymogens, secreted into the gut lumen and there processed into an active form (Moffatt & Lehane, 1990). The general conclusions to be drawn from these assays are that the proteolytic activities observed originate from the digestive tract, not elsewhere in the body, and that the majority are of the cysteine protease class.

Comparison with WCR proteolytic activities

The recently published findings of Gillikin *et al.* (1992), concerning the characterisation of the digestive proteases of WCR larvae, agree well with those demonstrated here for SCR larvae. Gelatin co-polymerised gels were also used by these workers and gave similar patterns of activity to

those reported here. Proteolytic activity was classified into three groups as shown in Figure 21, demonstrating some obvious similarities between the species.

Group	WCR data		SCR data	
	Mwt range	Members	Mwt range	Members
I	20-25	≥4	24	1
II	33-36	≥2	31-34	2-3
III	>45	≥2	45-50	2-3
IV			>55	≥3

Figure 21. WCR data represents that of Gillikin *et al.* (1992), SCR data is as presented in this work. Members indicates the number of activities found in each group.

Inhibitor	WCR Data		SCR Data	
	Conc.	% Act.	Conc.	% Act.
Control		100		100
E64	20.0μM	70	18.6μM	27
Cystatin	10.0μM	31	0.5μM	29
Pepstatin	7.3μM	38	9.7μM	66
DTT	5.0mM	172	43.0μM	125

Figure 22. WCR data represents that of Gillikin *et al.* (1992), SCR data is as presented in this work. **Conc.** represents the concentration of inhibitor employed. **% Act.** represents residual activity expressed as a percentage of the uninhibited/unstimulated control activity.

A comparable differential extraction was also carried out by this group. No activity was found in carcass fractions. Activity from all groups occurred in the luminal contents, however, only activities of group II were detectable in the gut wall fraction. For SCR, as described above, most activities observed in the lumen fraction, were also apparent in the gut wall

fraction. This discrepancy is probably due more to the exact extraction method used than to real differences in the enzyme distribution between the two species.

These workers also demonstrated the stimulation of these activities by cysteine and confirmed the inhibition of activity by preincubating with Cu^{2+} and E64 prior to electrophoresis. Strong inhibition was apparent with Cu^{2+}, although E64 gave only weak inhibition.

Gillikin *et al.* (1992) also conducted a wide range of inhibitor assays using the substrate [^{14}C]methyl-BSA. The conclusions drawn from these corresponded well with those found with SCR proteases assayed with azocasein. Figure 22 presents the results with those inhibitors common to both works. While resultant inhibitions from cystatin are almost identical, and the enhancement of activity due to DTT is similar, results from E64 inhibition show a marked difference, with 73% inhibition demonstrated in SCR, compared to only 30% inhibition displayed with WCR proteases. This variation may be due to dissimilarity of the substrates' specificities or the effects of different assay conditions (pH, etc.). The other marked difference between the results is found with inhibition due to pepstatin. Pepstatin inhibits SCR protease activity by 34%, while apparently having almost twice this effect on WCR proteases. These discrepancies may be due to real differences in enzyme type between the two species, but this cannot be confirmed without identical assays being conducted on the two species, under the same conditions, preferably by the same group of workers. It must be noted, however, that in the assays conducted for this project where the proteolytic activities of adult WCR and SCR have been compared, no marked differences have been demonstrated. For example, using Z-Phe-Arg-AMC as a substrate, comparable levels of cysteine protease activity were established in extracts from adults of the two species, 4.42mU and 4.05mU per gut equivalent calculated for SCR and WCR extracts respectively, and almost complete inhibition of this activity was demonstrated by 1µg chicken

egg-white cystatin. No direct comparison of larval proteases was undertaken.

Fluorimetric assays

High levels of cysteine protease activity have been confirmed with assays using the fluorimetric substrates Z-Phe-Arg-AMC and Z-Arg-Arg-AMC, the amount of free AMC released during digestion measured by an increase in fluorescence. Digestion of Z-Phe-Arg-AMC is indicative of general, although not total, cysteine protease activity, as it is insensitive to Cathepsin H activity. Z-Arg-Arg-AMC is a more specific substrate, known to be sensitive to digestion by Cathepsin B, while remaining unaffected by Cathepsins L and H. Assays specific for Cathepsin H activity were not conducted.

Enzyme preparations from the digestive tracts of both larvae and adults (SCR and WCR) displayed strong proteolytic activity towards Z-Phe-Arg-AMC, between 3.6 and 4.4mU of activity per gut equivalent, almost all of which (around 98%) could be arrested by cystatin or E64. This high level of activity reconfirms the predominance of cysteine protease activity in the insect gut. Levels of protease activity in extracts from adult insects were found to be greater than those in larval extracts, 4.0 to 4.4mU per gut equivalent in adult extracts, compared to 3.6mU per gut equivalent in larval extracts. This is in agreement with the activities demonstrated using myoglobin as a substrate, with 180µg and 90-120µg myoglobin digested in 30min by adult and larval protease extracts, respectively. Interestingly the activity exhibited here, using Z-Phe-Arg-AMC as substrate, is far greater than any demonstrated by the differentially extracted fractions. Here maximal digestion was achieved with the complete larva extract (extracted in water), showing an activity of only 1.01mU per larva equivalent. The general use in extractions of 1mM DTT, an enhancer of cysteine protease

activity, and its absence from the differential extraction, at least partly explains the depressed activities demonstrated in these differential fractions.

Inhibition of cysteine protease activity

Due to its ease and reliability, and the detection of only cysteine protease activity, this assay was used for kinetics studies of the larval cysteine proteases and also for the majority of inhibition assays with plant derived putative cysteine protease inhibitors. Assaying trypsin activity using this substrate confirmed that no digestion occurred, affirming the specificity of the substrate towards cysteine proteases. Papain was also shown to readily hydrolyse this substrate, and strong inhibition of this activity by cystatin and E64 was also demonstrated. It must be recognised that this assay does not detect the activities of all types of cysteine protease, for example Cathepsin H activity would be undetected, thus some of the enzymes present in the gut extract may be unaccounted for by these assays. A comparison with the results obtained using other substrates will hopefully reduce this error margin.

Initial E64 inhibition studies using Z-Phe-Arg-AMC as substrate and 2µl larval gut extract or 1µl (1mg ml^{-1}) papain, demonstrated sigmoidal relationships between E64 concentration and the level of inhibition caused (results not shown). Cystatin was found to give similar results. Both E64 and cystatin are known to form equimolar complexes with papain-like cysteine proteases, although while E64 binds irreversibly to enzyme molecules, cystatin binding is reversible (Barrett *et al.*, 1982; Anastasi *et al.*, 1983; Björk & Ylinenjärvi, 1990). It would be expected, therefore, that inhibition by either inhibitor would be directly proportional to its concentration, unlike that obtained here. Further investigation proved that incubation time and enzyme concentration were too high, allowing the rate to become limited by lack of available substrate. Consequently the incubation

period was reduced to 5min and the concentration of enzyme samples decreased ten-fold.

Subsequent assays demonstrated approximately linear responses to E64 inhibition (see Figures 12 and 13). From these results, approximate concentrations were calculated, assuming the equimolar inhibition by E64, stock enzyme concentrations of 95μM and 97μM estimated for papain and larval gut extract, respectively. This apparent concentration of papain was over twice the maximum expected value (42.5μM for a 1mg ml^{-1} stock), implying that either E64 inhibition of papain has not occurred in a mole for mole fashion, although this is unlikely as papain is known to possess only one active site, or that the actual concentration of the E64 solution used was below that expected, possibly due to impurity of the solid employed. This disparity between E64 and papain concentration makes it impossible to produce any realistic estimate for the concentration of cysteine proteases in the larval gut extract. The results can, therefore, only be taken as relative values, but they do present an indication of the comparable levels of protease activity (as determined by E64 inhibition) exhibited by the two samples.

Inhibition assays conducted with chicken egg-white cystatin still failed to exhibit a linear relationship between inhibitor concentration and reduction in protease activity, despite the decrease in enzyme concentration and incubation time. At low concentrations almost no inhibition was demonstrated, but then a small increase in inhibitor concentration causes the activity to decrease rapidly. The differences observed between the activities may be an effect of the reversibility of the inhibition caused by cystatin (Bode *et al.*, 1988) and the irreversible inhibition of E64 (Barrett *et al.*, 1982).

The different concentrations of protease apparent from inhibition by E64 and cystatin, both expected to inhibit in a equimolar manner, is noteworthy, and indicates one of two possible explanations: one of the

inhibitors does not act in the expected stoichiometric manner; or some of the enzymes present are susceptible to inhibition by one inhibitor and not the other. Due to the mixture of proteases already demonstrated to be present in the larval extract, and the similar levels of inhibition caused to papain by the two inhibitors, the second explanation seems the more probable.

Without isolating the different proteases of the insect gut extract, and undertaking mechanistic assays to establish the exact interaction stoichiometry of the inhibitors with these enzymes, firm conclusions cannot be drawn.

These results are, however, in agreement with those obtained using azocasein as a substrate, as here E64 and cystatin demonstrated comparable inhibitory activities, causing 73% and 71% inhibition at 18.6μM and 0.5μM respectively. These figures support the evidence that cystatin inhibition of the insect cysteine proteases is greater than that produced by E64. Interestingly, these results also help to explain the apparent contradiction discussed earlier concerning the findings of Gillikin *et al.* (1992), where the protease activity of larval WCR extracts could be inhibited by only 30% with 20.0μM E64, while 10.0μM cystatin caused up to 69% inhibition. Clearly this concentration of E64 was insufficient to produce a maximal level of inhibition, while that demonstrated in this work using azocasein as substrate appears to be closer to this maximum.

The most intriguing revelation of these inhibition assays, is the impressive effectiveness of cystatin inhibitory activity on SCR larval cysteine proteases, especially relative to the effects of E64 and the impact of these inhibitors on papain. If this strong inhibitory activity was also demonstrated by cystatins of plant origin, they could prove to be appropriate candidates for use in rootworm control strategies. The lack of cysteine proteases in the digestive system of mammalian species reinforces the suitability of cysteine protease inhibitors as tools for pest control.

Differences between insect cysteine proteases and papain

Use of the second fluorimetric substrate, Z-Arg-Arg-AMC, confirmed suspected differences between the action of papain and that of at least some of the enzymes present in the larval gut preparation. Papain, as discussed earlier (see Chapter 1), has specificities analogous to Cathepsin L, different to those of Cathepsins B and H (Koga *et al.*, 1990). It was therefore expected that no activity would be detected for papain using this substrate. This was confirmed to be the case. Assays with larval gut extract showed some digestion of this substrate, although at lower rates than achieved using Z-Phe-Arg-AMC, maximal digestion with Z-Arg-Arg-AMC gave levels of released AMC equivalent to only 60% that obtained with Z-Phe-Arg-AMC. A comparison between the rates of digestion of the two fluorimetric substrates (Figures 16 and 17), demonstrated the marked difference between the activities detected. Z-Arg-Arg-AMC is affected only by Cathepsin B-like protease activity, while Z-Phe-Arg-AMC is also digested by Cathepsin L-like enzymes. Considering the complexity of proteases already shown to be present in the gut extract, it seems most likely that the digestive tract contains a mixture of different cysteine protease types, some capable of acting on Cathepsin B substrates, and others more similar to papain or Cathepsin L in their substrate specificities. It is essential to recognise that while one enzyme may digest the same substrate as another enzyme, the two enzymes are not necessarily identical, either in their substrate specificity, or in their susceptibility to inhibition by different compounds. Thus, while some activity was demonstrated with Z-Arg-Arg-AMC, the enzymes responsible are undoubtedly different to Cathepsin B and distinct in numerous ways.

Kinetics studies with the two substrates

It is interesting that in time course assays, while the rate of digestion of Z-Arg-Arg-AMC is constant, that of Z-Phe-Arg-AMC is initially

very high, but rapidly settles to a lower constant rate. This effect is indicative of tight binding between enzyme and product, allowing rapid initial reaction and release of free AMC, but, due to the slow dissociation of the enzyme:product complex, the rate of subsequent digestion is limited by the lack of free enzyme. This phenomenon does not occur with Z-Arg-Arg-AMC, implying that either the binding of the enzyme:product complex is less tight in this case, or else different enzymes are responsible for the two activities. Recognising the complex mixture of proteases present in the gut, the latter explanation is most likely. This could be determined by using both substrates together, an additive effect confirming the presence of enzymes with different substrate specificities. Such an assay was not conducted here.

Approximate values were calculated for the binding of larval gut cysteine proteases with the two substrates. Rates of hydrolysis, at various concentrations of substrate, were measured, a Michaelis-Menton plot produced, and approximate values of Km and Vmax calculated. The complexity of the enzyme preparation used allows these values to be, at best, approximate, but some indication of relative activities can be derived. Km values of 4.4μM (for Z-Phe-Arg-AMC) and 6.7μM (Z-Arg-Arg-AMC) were obtained, indicating that binding to both substrates is strong. Approximate values of Vmax of 0.5mU and 0.15mU (for Z-Phe-Arg-AMC and Z-Arg-Arg-AMC, respectively), emphasise the difference in rate achieved in the two assays.

Inhibition of Z-Arg-Arg-AMC proteolysis

Inhibition assays using Z-Arg-Arg-AMC as substrate, showed the inhibition of activity caused by E64 and cystatin to follow similar trends to that observed with Z-Phe-Arg-AMC, cystatin producing greater levels of inhibition than E64. Inhibition, per mole of inhibitor added, was, however, lower for Z-Arg-Arg-AMC than Z-Phe-Arg-AMC and an apparent maximum

level of inhibition was achieved with cystatin, with around 40% of activity still unaffected. The use of higher concentrations of cystatin caused virtually no further decrease in activity. Inhibition by E64 approximated to a straight line, proportional to inhibitor concentration. As E64 is working on the same concentration of enzymes regardless of substrate, identical inhibition levels would be expected with both substrates, however, E64 may not compete effectively with substrate for the binding site of those enzymes hydrolysing Z-Arg-Arg-AMC; since E64 contains a leucine side chain as its specificity determinant, it would be expected to mimic Z-Phe-Arg-AMC better than Z-Arg-Arg-AMC.

It must be noted that of the medley of cysteine proteases represented in the gut extract, not all appear to be capable of digesting Z-Phe-Arg-AMC. Evidence for this lies in the inhibition demonstrated by cystatin. While all the activity demonstrated with Z-Phe-Arg-AMC can be inhibited by cystatin, only 60% of activity seen with Z-Arg-Arg-AMC is susceptible to cystatin inhibition. This implies that the remaining "immune" 40% was not detected by the first substrate. Again the results obtained using azocasein as a substrate, demonstrating that comparable levels of protease inhibition are achieved with E64 and cystatin, indicates that only a small proportion of cysteine proteases, if any, are susceptible to E64 and not cystatin.

This situation emphasises the difficulties involved in enzyme characterisation, from the choice of suitable assays and substrates, to the precise specificities of enzymes and inhibitors. Much of the classification of insect enzymes published to date refers to enzymes such as "trypsin-like enzymes" or "Cathepsin B-like enzymes", but while all the activity responsible for the digestion of Z-Arg-Arg-AMC here could be termed Cathepsin B-like, the differential effects of cystatin indicate that at least two enzymes are present, neither of which may really resemble Cathepsin B. Clearly great care needs to be taken in the terminology employed in enzyme classification if over-simplification is to be avoided.

It is also important to note that if 40% of the activity demonstrated with Z-Arg-Arg-AMC is unaffected by cystatin, then this small proportion of enzymes may provide a source of resistance were control by cystatin inhibition attempted. In order to assess the danger of such an event, purification and complete characterisation of the individual enzymes would be necessary. Unfortunately time did not permit for such purification and individual characterisation to be conducted.

The influence of pH on protease activity

Various assays were employed in order to establish an optimum pH for the activity observed in the SCR digestive tract. Variable results with myoglobin assays over a range of pHs made it difficult to establish any optimum pH for this proteolytic activity. Activity was high over the whole range from pH3.0 to pH6.0, although little activity could be detected above pH6.5. Initial assays indicated that optimal activity occurred at pH4.6, although later assays showed great variability and did not confirm this optimum. This broad spectrum of activity is indicative of multiple enzyme activities, as would be expected from a crude enzyme preparation.

The apparent increase in activity observed at very low pHs is most probably due to instability of the substrate in more extreme acid conditions. This degradation was unfortunately not assayed for, the control used in these assays being a zero time control, not a no-enzyme control for each pH, which would have removed any such autolysis from subsequent calculations. Another feasible explanation for increased activity could be possible alterations in substrate conformation under strongly acid conditions, rendering it more sensitive to digestion by certain insect proteases.

Assaying relative activity levels over a range from pH4.0 to pH8.0 using the substrate azocasein, showed high levels of proteolysis from pH5.0 to pH8.0, reaching a peak around pH7.0 - 7.5, a profile markedly different

from that demonstrated with myoglobin digestion.. The apparent dip in activity recorded at pH6.0 is most likely due to the change in buffer at this point, rather than being a real decrease. The same pattern of response to pH was observed with the digestion of the fluorimetric CP substrate Z-Phe-Arg-AMC. As the peaks of activity in azocasein digestion are mirrored with the cysteine protease substrate, the dominating presence of the cysteine proteases in the gut extracts are once more indicated. The broad range of pH over which high levels of activity are exhibited is indicative of a mixture of activities, but such a range of activity might be well expected considering that the vast volume of food ingested in comparison with the total size of the insect. It would therefore be expected that whatever food is taken in would have a great effect on conditions within the gut, quite possibly affecting the pH of the lumen. In these conditions, the digestive enzymes would still need to be fully active, hence possibly explaining the broad range of activity demonstrated.

This broad range of activity is similar to the profiles reported by Purcell *et al.* (1992) and Wolfson and Murdock (1990) for SCR larval gut protease activity. Purcell and workers demonstrated a somewhat narrower range, using the general protease substrates azocoll and azocasein. They showed that below pH4.0 activity was minimal. Above this, activity was demonstrated to rise rapidly with pH, peaking around pH6.0 and falling slowly, considerable activity still being exhibited at pH10.0. This profile, while showing activity over a range similar to that reported here, does differ markedly in the peak of activity observed. In this work activity was found to reach a broad peak ranging from pH5.0 to 8.0, whereas Purcell *et al.* demonstrated a narrow peak from pH6.0 to 7.0.

Wolfson and Murdock assayed enzyme activity, using tritiated methaemoglobin as substrate, over a range from pH2.0 to 12.0, using seven buffers, unfortunately none of which overlapped in pH range. The pH profile reported corresponds well with the results presented here. The dip

in activity observed with both azocasein and Z-Phe-Arg-AMC is also seen in this group's work. Unfortunately this too coincides with a change in buffer, preventing firm conclusions being drawn about the reality of this activity dip.

It is widely recognised that the buffer used can markedly affect the resultant activity of an enzyme. A good example of this was presented by Thie and Houseman (1990b) in their report on Cathepsin-like enzymes in the midgut of *Leptinotarsa decemlineata*, the Colorado potato beetle. Three buffers were employed with overlapping pH ranges. The resultant activity, while still indicative of the pH profile, was significantly affected by the buffer used. For example, at pH5.5, hydrolysis (of the substrate *N*-benzoyl-DL-arginine-p-*β*-naphthylamide) with a succinate buffer was 1.5-fold that achieved with a bis-tris buffer. This then demonstrates the danger of directly comparing assays when different buffers have been used. As previously discussed, the reality of the dip in activity observed at pH6.0 is dubious due to the change of buffer at this point, although the fact that is has been observed with two different pairs of buffers somewhat substantiates its validity. Unfortunately time did not permit for these assays to be repeated using a single range or overlapping buffers. Interestingly, Purcell's group, who used two buffer types (100mM glycyl-glycine/100mM acetate for pH3-5 and 100mM ACES/50mM Tris/50mM ethanolamine for pH6-10), report a peak in activity at pH6.0 (Purcell *et al.*, 1992). It is difficult, because of the extensive changes in buffer used, to draw any real conclusions from the pH profile presented, other than the fact that activity is high over a broad range of pHs.

The effects of employing different substrates

The activities demonstrated using azocasein and Z-Phe-Arg-AMC, and those observed using myoglobin as substrate, are immensely different. This effect may be due to differences in substrate susceptibility to digestion by

the different protease types, or else to some structural differences between the substrates. Both myoglobin and azocasein are general protease substrates, so their susceptibility should be comparable. Gillikin *et al.* (1992) reported a similar phenomenon with their assays on the crude extracts from WCR larval guts. Using the general protease substrates, [^{14}C]methyl-casein and azocoll, significant activity was detected over the range of pH4.0 to 9, with an optimal pH between pH5 and 6. With the substrate [^{14}C]methyl-BSA, a different response was observed, with maximal activity apparent at pH4.0 and activity limited to the range pH3 to 6. The digestion follows a pattern closely resembling that found here with myoglobin as substrate. These workers hypothesized that the difference in activity was due to differences in the tertiary structure of the substrates. To substantiate this, digestion of heat denatured [^{14}C]methyl-BSA was assayed over the pH range from pH3 to 10. This showed a pH profile similar to that demonstrated with [^{14}C]methyl-casein, with an optimum at around pH6 and a range of activity far broader than that of the native BSA. These results validate the hypothesis that the discrepancies observed here are due to structural differences in the substrates, rather than distinct specificities.

Comparison of pH influence on SCR and WCR digestive proteases

Comparison of this SCR data with data available on WCR enzymes demonstrates both similarities and differences between the species. Gillikin *et al.* (1992) reported a broad pH profile, activity being high from pH4.0 to 9.0, peaking around pH5-6, a more acidic optimum than that exhibited by SCR enzymes. This is similar to the WCR larval profile published by Wolfson and Murdock (1990), which shows a sharp peak at pH6.0, the activity demonstrated over the remaining pH range reaching only 50% that of the peak, but remaining fairly high from pH4.0 to 10. As previously the

excessive changing of buffer types brings into question the clarity of this profile.

Wolfson and Murdock also reported pH profiles for adult SCR and WCR. They found that while adult WCR proteases maintained activity over a broad range of pHs (2.5-11), SCR adult extracts exhibited little activity outside a range from pH2.0 to 6.0. These apparent differences between adults and larvae and between the two species are surprising considering the similarity of CP activity levels demonstrated in this work.

Further comparison between the species, particularly comparison between the larvae, was impossible due to the loss of the WCR culture from the departmental insectaries. Comparison of the larval SCR results with the characterisation of WCR larval digestive proteases by Gillikin and workers (1992) reveals great similarities between the two, both in terms of broad pH profile and in the general characteristics of the proteases present, as has been discussed earlier.

Some difference in enzyme activity between the two species would be anticipated due to the difference in host ranges between them, the SCR displaying a far greater host range. The host ranges obviously overlap (with maize, the main host of both species) and in concordance with this, there appears great similarity in at least some of the enzymes present.

Wolfson and Murdock (1990) also report the results of inhibition assays with SCR adult proteases. They found that 79% of activity, when assayed at pH4.5, could be inhibited by pepstatin, while a decrease of only 7% was produced by E64, indicating the dominance of aspartic proteases in the digestive extracts. The results presented in this work demonstrate the maintenance of high levels of cysteine protease activity in adult SCR. The presence of aspartic proteases in adult rootworms was not investigated.

Conclusion: Proteases

SCR larvae have been shown to possess a number of proteolytic activities, the majority of which are of the cysteine protease type, and active over a wide range of pH conditions. This enzyme activity can be drastically inhibited by the cysteine protease inhibitors, cystatin and E64, and partially inhibited by pepstatin, an inhibitor of aspartic proteases. The mixture of proteases contains enzymes with similar substrate requirements to Cathepsin B and Cathepsin L, but displaying unique inhibitory susceptibilities and individual substrate specificities. The majority of these activities can be completely inhibited by cystatin, but a small proportion, detectable with Z-Arg-Arg-AMC and not digesting Z-Phe-Arg-AMC, are unaffected by cystatin inhibition. The relative effectiveness of cystatin and E64 towards the activities of these insect proteases and papain is remarkable, cystatin having a far greater effect on the insect enzymes. This strength of inhibition indicates the potential use of this type of inhibitor in a control program.

These results are in general agreement with other findings regarding SCR larval proteases and show apparent high similarity with WCR larval proteases (Gillikin *et al.*, 1992). Assays with Z-Phe-Arg-AMC as substrate have demonstrated similar high levels of cysteine protease activity to be also present in the adults of both SCR and WCR.

Digestive cysteine proteases in insects seem an eminently suitable target for control by inhibition, because, as mentioned earlier, these enzymes are not employed in the digestive system of mammals. Protease inhibitors, present in the diet of insects, have been shown to result in increased mortality, decreased weight gain, or reduced fecundity (Broadway & Duffey, 1986; Wolfson & Murdock, 1987). Along with these direct effects of ingested inhibitors, it may also be possible to combine more highly insecticidal compounds, within the diet, these being protected from

degradation and detoxification in the insect gut by the action of the protease inhibitors.

Part B: AMYLASES

α-amylase activity was readily demonstrated in SCR larval gut extracts, using the DNSA-reagent detection method of Bernfeld (1955). Assays were conducted using McIlvaine's buffers over a range of pH from 3.0 to 7.0. Activity was high within the range from pH3.8 to 6.6, reaching a maximum at pH5.0.

Amylase activity was also analysed by electrophoresis on native polyacrylamide gels at 4°C, followed by infiltration with starch, incubation and staining. Only one band of amylase activity was detected. Some weak activity was also apparent at the top of the gel, indicative of proteins retarded either by contaminating carbohydrates, or by their high molecular weight or high isoelectric point. Porcine pancreatic α-amylase, used as a positive controls on the gels, also remained high on the gel.

Conclusion: Amylases

The α-amylase activity demonstrated in digestive extracts from larval SCR appears to be the result of a single isoform of the enzyme. It is possible that two isoforms may run to identical positions on gel, but this occurrence is unlikely. Many other insects are known to possess multiple forms of amylase, each form having slightly different specificities (Baker, 1983, 1987; Campos *et al.*, 1989; Chen *et al.*, 1992a), often where one amylase is inhibited the others are used to a greater extent to compensate for the loss of activity. Due to the lack of isoforms apparent in the SCR larval digestive system, this enzyme appears to be highly suitable as a target enzyme for control by inhibition. Although α-amylases are employed in mammalian digestive systems, inhibitors have been discovered which possess specificity towards certain amylases, some having no effect on mammalian

amylase, while strongly inhibiting those from other sources. If such an inhibitor, causing no inhibition of mammalian amylases, was found to be highly effective against the insect enzymes, then the larvae, would be unlikely to develop resistance to the inhibitor, there being no other form of the enzyme in reserve to allow survival.

Part C: ESTIMATION OF INSECT GUT pH

Estimation of the pH of the larval gut, *in vivo*, was conducted using three methods. Staining of excised guts with Universal indicator solution, caused the tissues of the gut to become red, indicating a pH of 4 to 5, while the surrounding body fluids became yellow, showing a pH of around 6. It was unclear from the results whether this method merely stained the external wall of the gut rather than its contents, thus while it does indicate that the gut is acidic, it fails to confirm the pH of the luminal contents.

Regurgitation was induced in some larvae and the resultant liquid, the gut contents, determined, by staining, to have a pH of 4 to 5, confirming the findings of the dissection assay above. It is possible, however, that this regurgitant may represent only the contents of the foregut. When a similar regurgitation technique was employed by Slaney *et al.* (1992), these workers, however, demonstrated the gut fluid of SCR larvae to be pH7.0, far less acidic than that observed here.

It is well documented (Wigglesworth, 1972) that some insects have changes of pH during the course of their digestive tract. In order to study the possibility of this phenomenon in SCR, the larvae were fed an artificial diet into which was incorporated various indicator dyes. The most successful indicator diet was that containing litmus. In this case the mildly acid diet was coloured purple. The insects were observed to have fed on the diet within 30min of being introduced onto it. After one day of feeding the coloured gut of the larvae was visibly red (acid), even through the bodies of the live larvae. Upon dissection the gut revealed an interesting

result. The fore and midgut contents were a deep red colour, indicative of acid conditions, whereas the contents of the hind gut were markedly blue in colour. This is strong evidence for the existence of an active mechanism for the control of pH within the main area of the gut. The midgut appears to be maintained at low pH, possibly by the recycling of acid or the expulsion of alkaline compounds into the hindgut. Such a mechanism could serve to maintain near optimal conditions for enzyme activity within the gut regardless of the pH of ingested food.

After five days of feeding on the artificial diet, the larvae appeared to have become acclimatised to the diet. Dissection of the gut after this length of feeding revealed a deeper red (slightly purple) colouration, with no marked change of colour into the hindgut. This alteration of pH pattern must be due to some habituation to the conditions of the diet, the acid pH of the diet requiring no special mechanism to maintain the gut pH, showing possibly even greater control of the gut conditions. It might be that the maize seedling diet, on which the larvae were feeding prior to introduction to the coloured artificial diet, was less acidic, thus requiring the use of an active control mechanism to maintain a suitable gut pH for digestive processes.

These feeding trials also demonstrated that food was ingested within 30min of the larvae being placed on the diet, having just recently been removed from the rearing medium, and that residence time of diet within the gut was apparently less than 24hrs, the larvae observed to be producing coloured frass after this time.

Conclusion: pH

The contents of the larval gut of SCR were demonstrated to be acidic, possibly with an active mechanism present to maintain this pH. Staining of gut contents demonstrated a pH of between 4 and 5. This correlates well with the amylase optimum of pH5.0 established in this work, and is within

the range of high activity demonstrated for protease activity, although an optimum for at least part of this activity would appear to lie at a higher pH. In nature, enzymes are sometimes found under conditions suboptimal for their activity, so although activity of SCR proteases has been determined to be highest at around pH7.0, activity *in vivo* may be somewhat lower than this due to the more acidic conditions encountered. Obviously, it is important in enzyme assays to reproduce the physiological conditions as far as possible, thus, in this case, assays should be conducted around pH5.0, otherwise both proteolytic behaviour and the effects caused by inhibitors may give misleading results. It must always be remembered that the behaviour and susceptibilities of an enzyme *in vitro* may be greatly different from its activity *in vivo*, also the strong effects of inhibitors *in vitro* may not be reflected in results *in vivo* (Gatehouse *et al.*, 1986). The presence of other enzymes, the protection of membranes, and the exact composition of the gut contents may all act to shield an enzyme or to detoxify an inhibitor. Thus, while characterisation of enzyme activity *in vitro* is invaluable in the choice of inhibitors for potential use in a SCR control strategy, the effects of such compounds *in vivo* must also be carefully assessed.

CHAPTER 4 CYSTEINE PROTEASE INHIBITORS FROM PLANTS

EXTRACTION OF CYSTEINE PROTEASE INHIBITORS FROM RICE - RESULTS

Preliminary Investigation of CPI activity in Rice Seed

Initial assays, using crude extracts of rice seeds, confirmed the presence of inhibitors, capable of inhibiting corn rootworm digestive cysteine proteases. The 30-65% fraction from ammonium sulphate precipitation of a crude extract was found to almost completely inhibit activity of the larval proteases, causing up to 94% inhibition.

Location of CPI in the seed

Weighing separated germ and endosperm established that germ makes up approximately 2% of the intact seed by mass. Equal masses of germ and endosperm were ground, protein extracted and subjected to heat treatment (80°C, 10min). The precipitate resulting from heat treatment of the germ extract was far greater than the wispy precipitate formed in the endosperm extract, possibly indicating the relative proportions of protein present in the two extracts. Analysis by SDS-PAGE showed that a large number of proteins were present in the crude germ extract, while little protein of any sort was found in the endosperm extract (see Figure 23). Heat-precipitated material was removed and the supernatant assayed for inhibitory activity using the substrate Z-Phe-Arg-AMC, and analysed using SDS-PAGE and Western blotting. Short and long grain cultivars of indica rice were compared for their relative content of CPI (see figure 24). In both varieties tested, the germ fraction caused 70% inhibition of larval gut cysteine proteases, while the endosperm fractions gave only 14% and 3% inhibition for extracts from Simular Roma and Originaro, respectively. Both varieties exhibited far greater levels of inhibitory activity towards SCR gut enzymes than towards papain, papain activity being inhibited by only 5% or 16.5% with germ

fractions (Simular Roma and Originaro, respectively), and up to 2% by endosperm fractions.

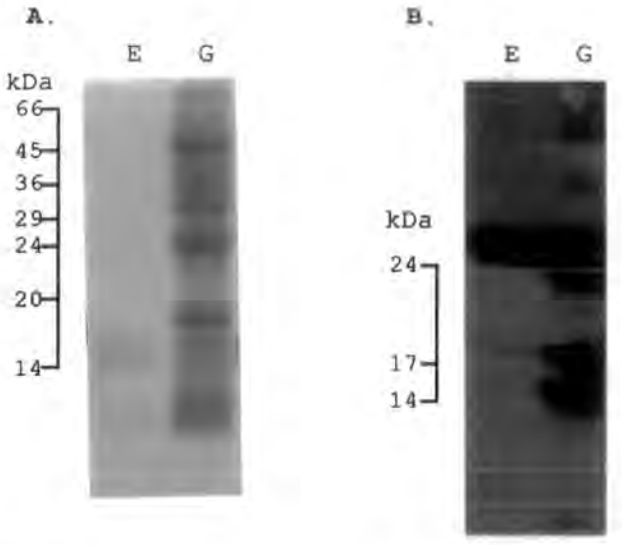


Figure 23. SDS-PAGE (panel A) and western blot (panel B) analysis of crude protein extracts from rice, using anti-ozcI antiserum for antigen detection on western blot. Lane E, endosperm fraction; and lane G, germ fraction.

Variety	Grain	Extract	Larval CP	Papain
Originaro	short	endosperm	3%	0%
"	"	germ	70%	16.5
Simular Roma	long	endosperm	14%	2%
"	"	germ	70%	5

Figure 24. Inhibitory activity demonstrated by heat soluble extracts from separated endosperm and germ rice. Inhibitory values are given as % decrease from activity in the absence of inhibitor. All results are corrected according to no-enzyme controls. No CP activity was demonstrated by any of the rice extracts.

Western blot analysis using anti-ozcI antibodies demonstrated that ozcI was present mainly in the germ fraction of the seed (see Figure 23). This

is in agreement with the inhibition assays, which demonstrated that most CPI activity was restricted to the germ. Some cross reaction was observed with the endosperm material although this was of higher molecular weight (around 25kDa) and was assumed to be due to contamination of the antisera with antibodies to other common rice proteins, as discussed later.

Tissue immuno-blot of rice seeds

Slightly immature seed was taken freshly from rice plants, bisected longitudinally and used for immuno-blotting with anti-ozcI antisera. This demonstrated the location of ozc in the seed section extremely clearly (see Figure 25). In these almost mature seeds the germ can be distinguished clearly as the area of highest concentration of ozc in the seed, with some protein also apparent in the aleurone layer surrounding the endosperm. In more immature seed, where no distinct germ can be observed on the actual seed, this clear zonation could not be seen, rather, some general reaction was visible throughout the seed.

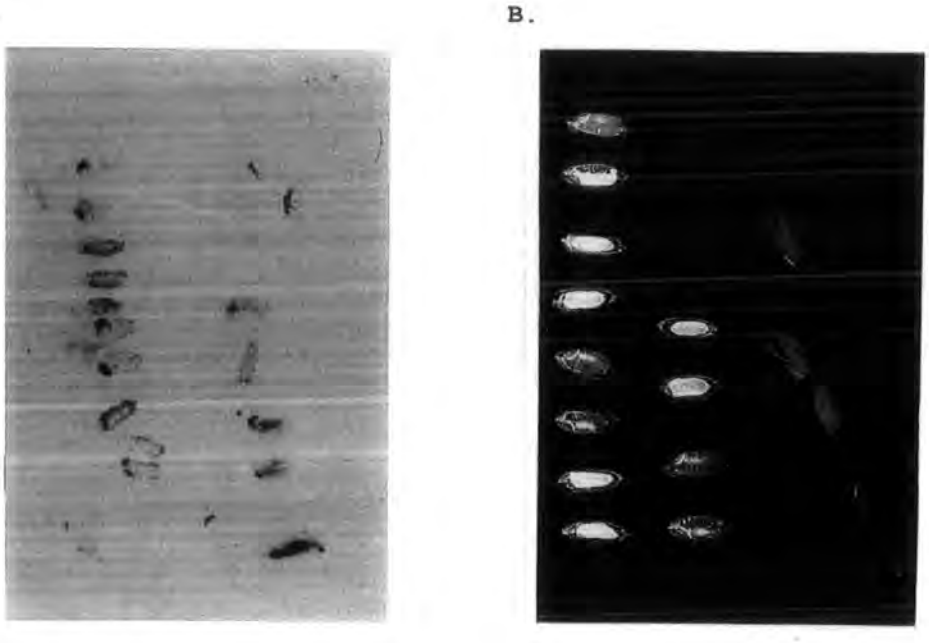


Figure 25. Tissue immuno-blot of rice seeds (panel A) and the seeds used (panel B). Anti-ozcI antiserum was used to detect ozcI within the seed.

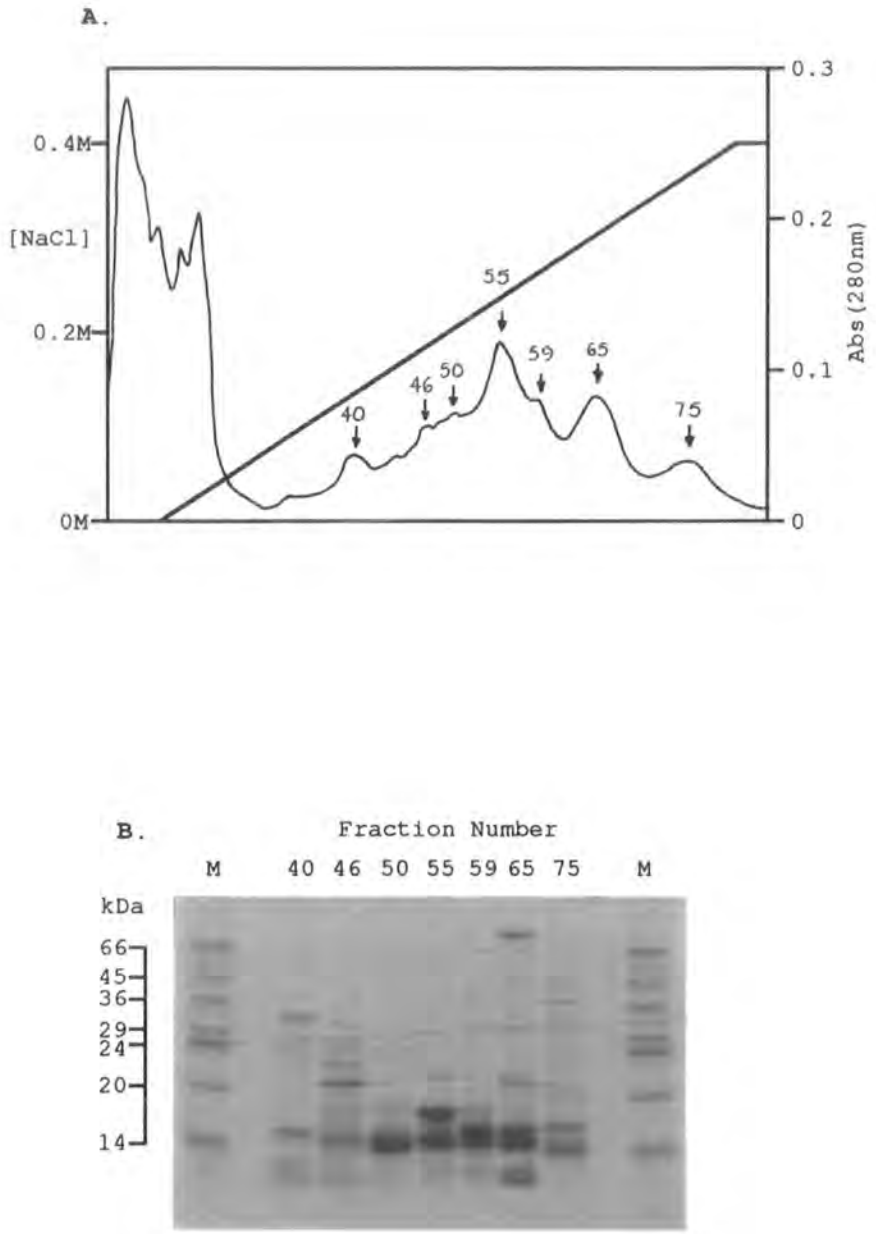


Figure 26. Panel A: Column profile from S-sepharose chromatography of ammonium sulphate precipitated rice fraction, using 50mM Na-acetate buffer pH4.9, with a linear salt gradient from 0-0.4M NaCl. The peaks tested are indicated. Panel B: SDS-PAGE analysis of peak fractions from column elution (40-75). Lane M, molecular weight markers.

Purification of a CPI from rice seed

Protein extracts were prepared as normal, heat treated, fractionated by ammonium sulphate precipitation and subjected to cation exchange chromatography. Of the seven resulting peaks (see Figure 26), only one (#40) appeared to cause inhibition of larval gut proteases, this eluting off the column at around 0.14M NaCl (data not shown). This fraction was shown, on stained gel, to contain two major components, proteins with apparent Mwts of 15kDa and 30-33kDa, along with a number of other less abundant proteins.

This inhibitory peak was further purified by gel filtration, separating the fraction into two peaks. It was established that peak B possessed inhibitory activity towards larval gut protease activity, while peak A exhibited virtually no such activity. On SDS-polyacrylamide gel, the impure nature of this inhibiting sample was illustrated (Figure 27). The lyophilised peak B was then subjected to cation exchange chromatography using a formate buffer (Figure 28).

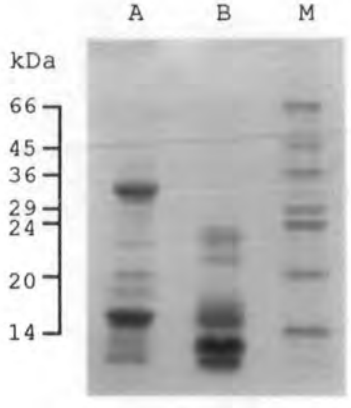


Figure 27. SDS-PAGE analysis of peaks A and B resulting from gel filtration of rice preparation, in the presence of ß-mercaptoethanol. Lane M, molecular weight markers.

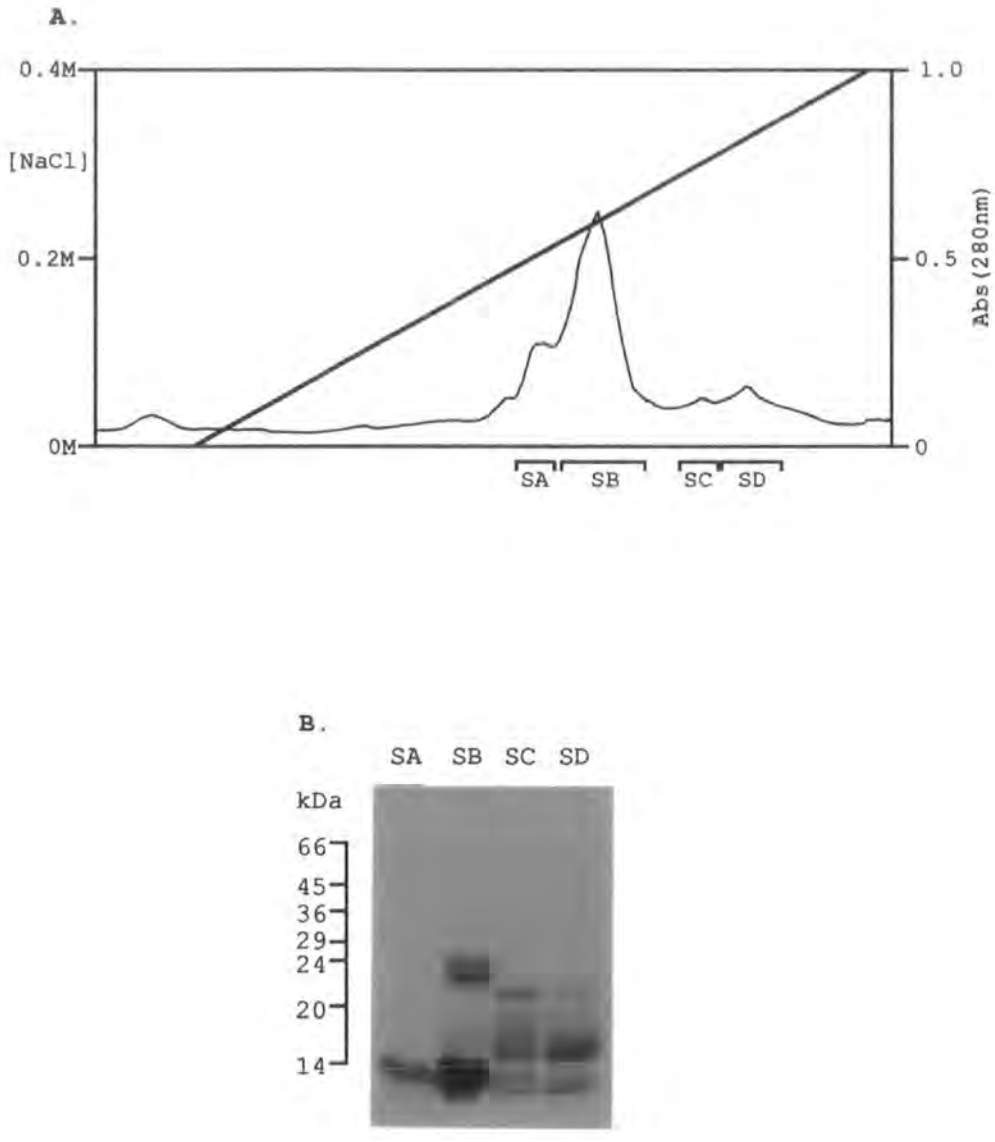


Figure 28. Panel A. Profile of elution from cation exchange chromatography of rice fraction B, using 25mM formate buffer pH4.1 with a 0-0.4M NaCl gradient, peaks SA-SD indicated. Panel B, SDS-PAGE analysis of samples from SA-SD.

Attempts to assay the four resultant peaks gave no consistent inhibition of papain or larval proteases (results not shown). It was found that after exhaustive dialysis, inhibitory activity could be detected from one peak (SB), eluting off the column at 0.24M NaCl (Figure 29). This inhibitory fraction was demonstrated to cause strong inhibition of larval gut cysteine proteases, up to 86% inhibition demonstrated here with a 16µl sample of SB, whereas no activity was exhibited against papain, even with 16µl SB no inhibition of papain activity was observed.

A.

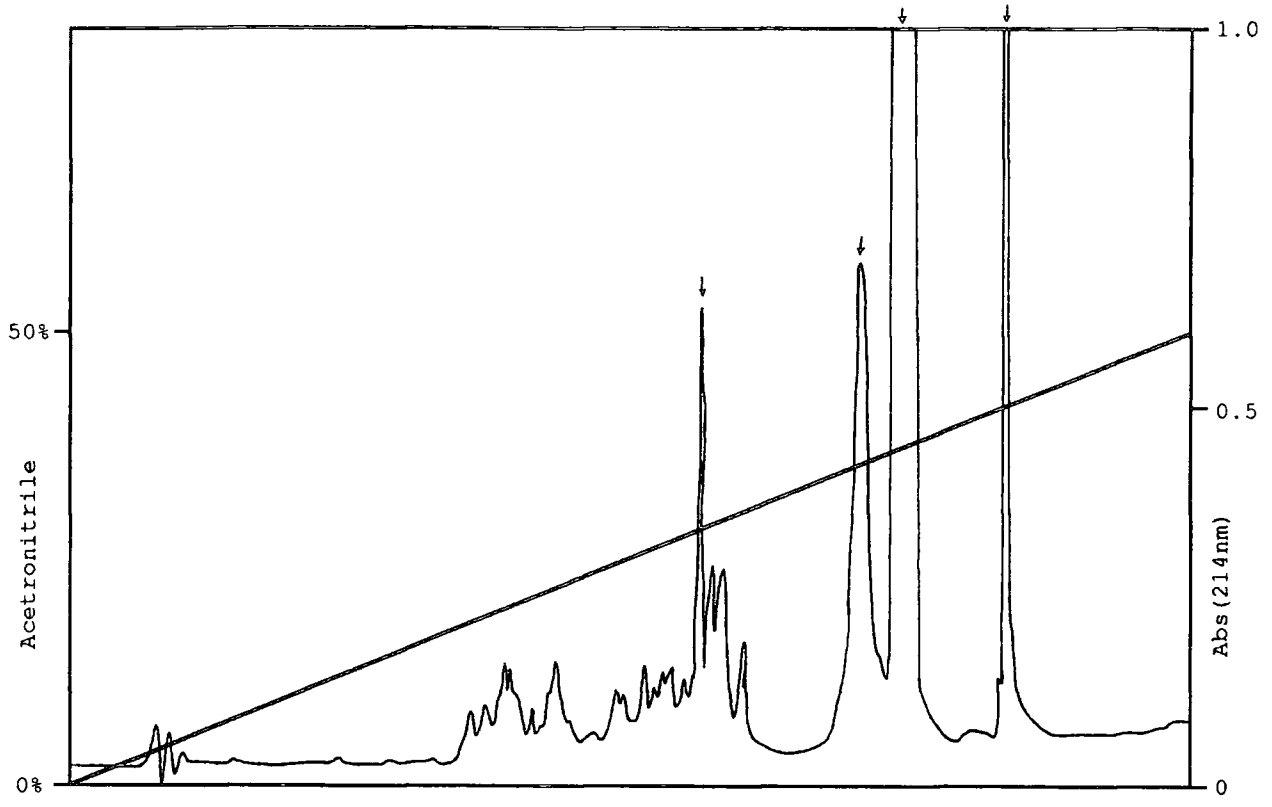
Assay	% Inhibition
2µl cystatin	95.1
8µl 30-65% ext.	30.6
8µl SA	6.4
8µl SB	29.0
8µl SC	9.6
8µl SD	9.6

B.

Assay	LgE	Papain
2µl cystatin	100	100
2µl SB	23	N/D
4µl SB	37	0
8µl SB	55	0
16µl SB	86	0

Figure 29. Table A shows the results from 20min assays, with 2min preincubation, using Z-Phe-Arg-AMC as substrate and 2µl gut extract per assay. % Inhibition was calculated as the percent reduction from uninhibited control activity. Table B shows the effects of varying the concentration of SB on the activity of 2µl LgE or 1µl papain, using Z-Phe-Arg-AMC as a substrate. Figures shown indicate % inhibition of activity. All assays were conducted in duplicate with zero time and no-enzyme controls.

A.



B.

Peak	% of peak	% Inhn
#22	50	18
#23	25	42
#25	40	45

Figure 30. Panel A, Profile of elution from HLPC treatment of rice fraction SB. Acetronitrile gradient is shown. Major peaks, indicated by arrows, are #18, #22, #23 and #25, from left to right. Panel B, Inhibition of larval gut protease activity by samples from peaks #22, #23 and #25. The amount of sample used in each assay is given as a percentage of the total peak.

Fraction SB was purified further by reversed phase HPLC, the three main peaks obtained, #22, #23 and #25, eluting at 35, 37 and 41.9% acetronitrile respectively (Figure 30). These peaks were assayed for inhibitory activity (also shown in figure 30). Considering the relative size of each peak and the amount used in assay, #25 was determined to exhibit the greatest inhibitory activity of the three.

Sequence data from the first 20 N-terminal amino acids of SB#25 was obtained, this compared to known sequences using an NBRF protein database, and shown to be highly homologous to proteins of the thaumatin-like family, as shown in Figure 31.

```
                    10        20
SB#25         VTFTIVNKCGYTVWPAALPS
               ::.:::.:.:::::.::  .
ThaumatinI    ATFEIVNRCSYTVWAAASKGDAALDAGGRQLNSGESWTINVEPGTNGGKIWA
                    10        20        30        40        50
```

Alignment	% Identity	Overlap
Tobacco PR-protein R (precursor)	73.7	19aa
Thaumatin I	75.0	16aa
Thaumatin II precursor	75.0	16aa
Tobacco TMV-induced protein	68.4	19aa
Maize α-amylase/trypsin inhibitor	58.8	17aa
Tobacco curled leaf protein P10	45.0	20aa

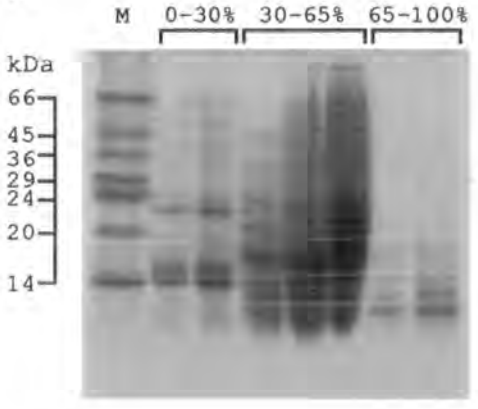
Figure 31. N-terminal amino acid sequence obtained for SB#25 and homology with Thaumatin-I and other thaumatin-like proteins. ":" indicates identity of residues, "." indicates conservative change.

CPI Extraction from Excised Rice Germ

A slightly modified procedure, similar to that of Liang *et al.* (1991), was used in subsequent extractions from both whole rice seed and from rice germ. Dialysed ammonium sulphate precipitates (0-30%, 30-65% and 65-100%) were assayed for inhibitory activity, and this activity demonstrated to be

limited to the 30-65% fraction, as shown in Figure 32. This fraction caused around 97% inhibition of CP activity in all the insect extracts assayed (SCR larval and adult, and WCR adult). Virtually no inhibition was demonstrated with the other fractions. Papain activity was found to be inhibited by only around 43%, a result in accordance to that found previously.

A.



B.

Enzyme	0-30%	30-65%	65-100%
SCR larval gut CPs	9%I	97%I	4%I
SCR adult gut CPs	11%I	96%I	8%I
WCR adult gut CPs	10%I	97%I	6%I
Papain	4%I	43%I	4%I

Figure 32. Panel A, SDS-polyacrylamide gel of ammonium sulphate precipitated fractions from rice extract. Figures represent ammonium sulphate saturation of solution. 5µl and 10µl (left to right) of each sample were used, with an additional lane of 2µl 30-65% fraction (left-most lane). Panel B, inhibition of protease activity by ammonium sulphate precipitated fractions. Each assay was conducted with 2µl insect enzyme sample or 1µg papain, in duplicate, with control assays to correct for substrate autolysis. %I represents the percent inhibition relative to assays composed of enzyme alone.

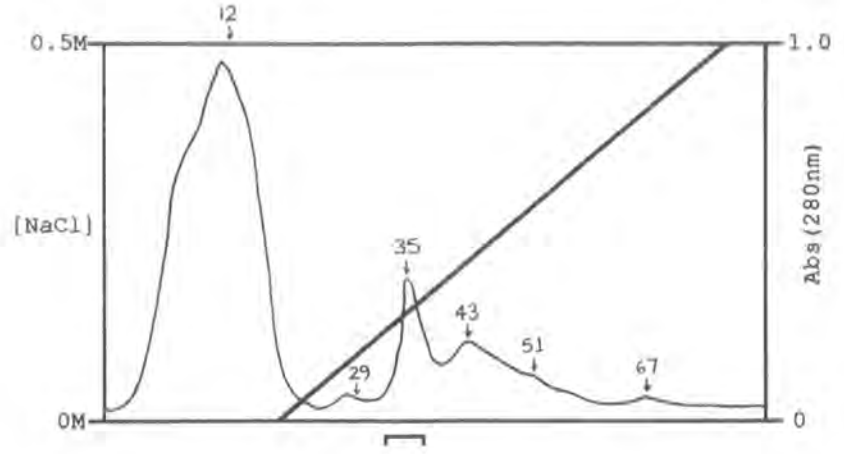


Figure 33. Profile of ammonium sulphate precipitated rice germ fraction on chromatography with Q-sepharose, 20mM Tris pH8.0, 0-0.5M NaCl.

A.

Fraction	[NaCl]	%Inhn
1µg cystatin	-	97
#12*	0M	9
#29	0.10M	0
#35*	0.16M	94
#43*	0.22M	21
#51	0.28M	3
#67	0.40M	13

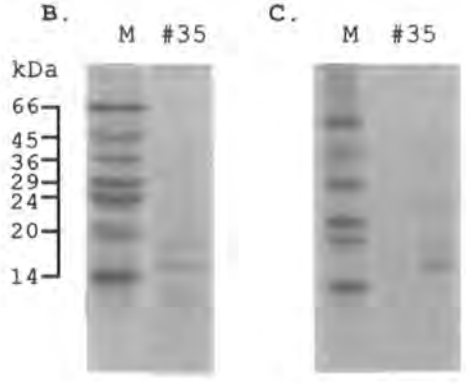


Figure 34. Panel A, Inhibitory activity of elution fractions using the substrate the substrate Z-Phe-Arg-AMC and 2µl SCR adult gut extract. % inhibition (%Inhn) is given relative to assays composed of uninhibited enzyme. [NaCl] indicates the point of elution of each fraction. Major peaks are indicated by *. Panels B and C, SDS-PAGE analysis of #35 (denoted "QRG"), with (B) and without (C) ß-mercaptoethanol. 15µl (75µg) QRG was loaded in both cases.

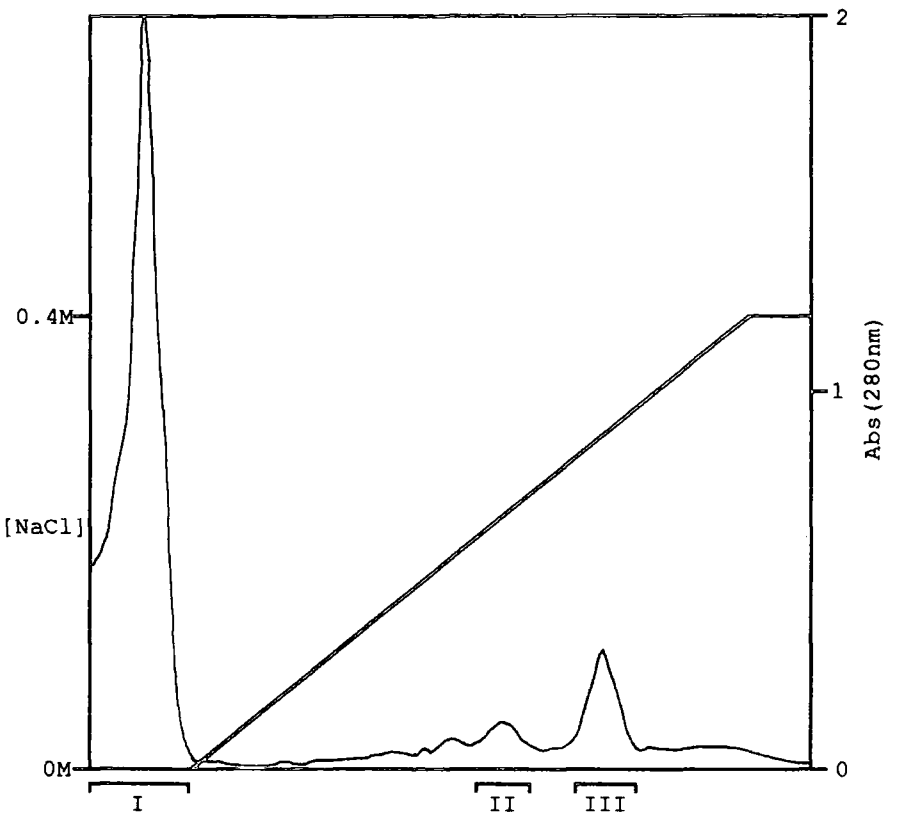


Figure 35. Column profile from S-sepharose chromatography of QRG, using 25mM formate buffer pH4.1, 0-0.4M NaCl. Peaks I, II and III were pooled to give SQRG-I, SQRG-II and SQRG-III, respectively.

Fraction	[NaCl]	Papain	WAgE
1µg cystatin	-	96%I	97%I
SQRG-I	0M	59%I	94%I
SQRG-II	0.22M	0%I	71%I
SQRG-III	0.29M	0%I	15%I

Figure 36. Inhibition of papain and WCR adult CP activity by dialysed rice germ fractions following S-sepharose chromatography. 20min assays comprised 2µl WAgE or 1µl papain plus 4µl rice sample, and were run in duplicate. [NaCl] indicates the point of elution from column. %I indicates the percent inhibition of enzyme activity. Concentration of rice fractions, although known to be comparable, were not measured.

When this inhibitory fraction was subjected to anion exchange chromatography, three main peaks were obtained (Figure 33), of which one (denoted "QRG"), eluting at approximately 0.16M NaCl, exhibited strong inhibitory activity towards insect gut enzymes, as shown in figure 34, causing 94% inhibition. Other fractions gave between 0 and 21% inhibition of SCR cysteine protease activity.

QRG was then subjected to cation exchange chromatography, (figure 35), the three resultant peaks, denoted SQRG-I, SQRG-II and SQRG-III, were pooled, dialysed and freeze dried before any consistent inhibitory activity was demonstrated. Figure 36 illustrates the differential inhibition of papain and insect gut protease caused by these fractions. Fraction SQRG-II caused no reduction in papain activity, while producing a decrease of 71% in insect enzyme activity, whereas SQRG-I inhibited both enzyme activities. SQRG-III has no effect on papain activity and little on that of WAgE, only 15% decrease.

The inhibitory fractions were subjected to SDS-PAGE and analysed by western blotting, using anti-ozcI and anti-ozcII antisera (figure 37). It was confirmed that ozcI was present in the fraction SQRG-I and that ozcII was lacking. The blots also affirmed that neither ozcI or ozcII were present in the fraction SQRG-II. Crude extracts of long and short grained varieties of indica rice were also tested on this western blot, and both shown to contain the two forms of ozc.

The results presented are taken from a single extraction, although other CPI preparations, using this method and either whole seed or rice germ, produced similar results (results not shown). Inhibition of SCR larval and adult digestive CPs was found to be analogous to the WCR adult extract results shown here.

SQRG-II was further purified by reversed phase HPLC. Three major peaks were obtained (Figure 38), eluting from the column at 27.5%, 40.5% and 65.7% acetronitrile. The fractions were neutralised prior to freeze drying,

and assayed against larval gut extract and papain for inhibitory activity (Figure 38).

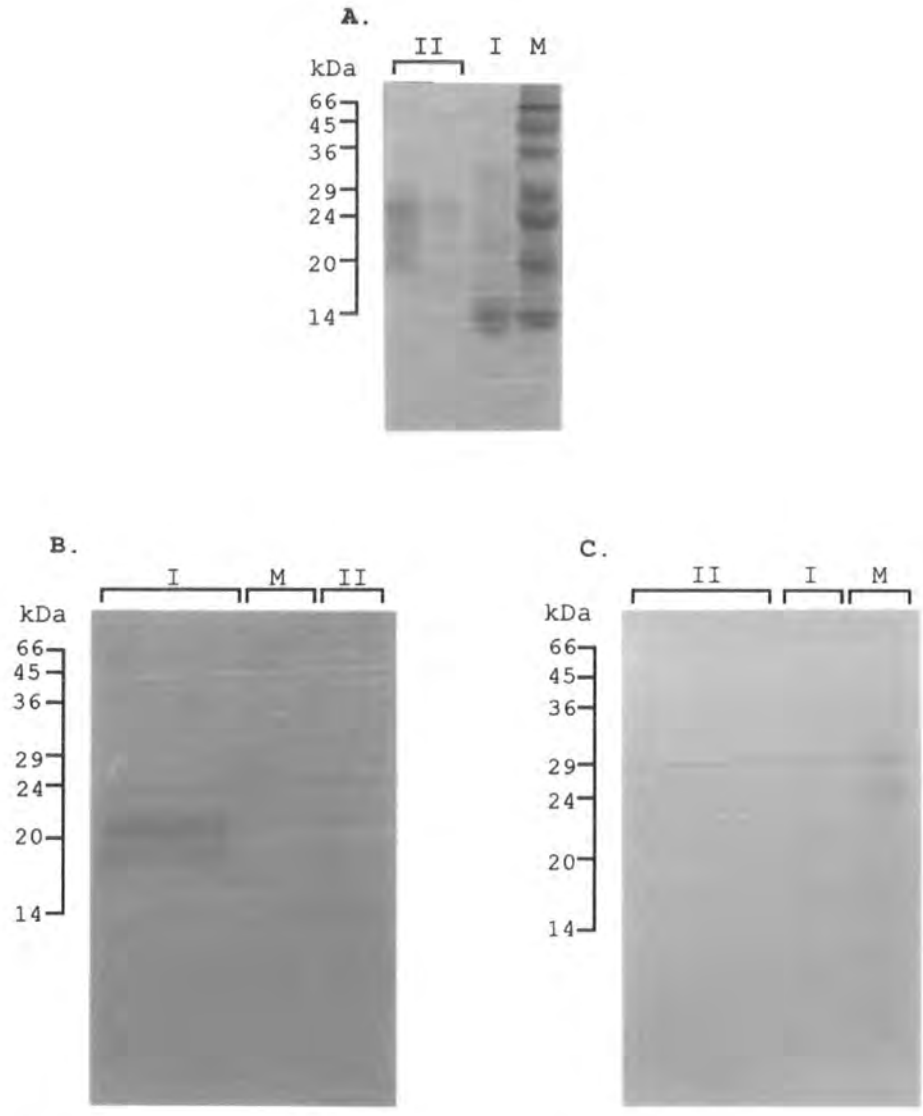
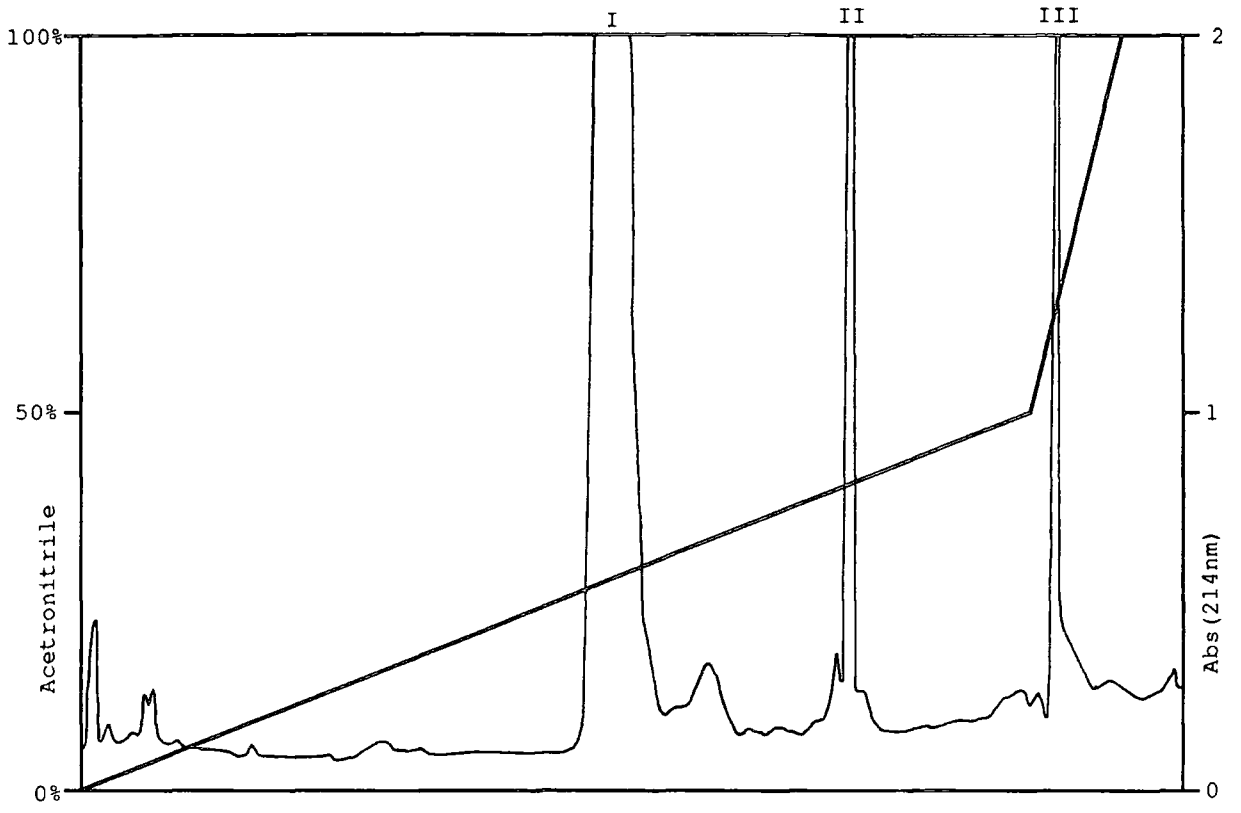


Figure 37. Analysis of SQRG-I (I) and SQRG-II (II) by SDS-PAGE (panel A) and western blot analysis, using antisera raised against ozcI (panel B) and ozcII (panel C). The colour detection method was employed for the visualisation of blots.

A.



B.

Peak	%Acn	Papain	LGE
I	27.5	1%I	1%I
II	40.5	24%I	90%I
III	65.7	1%I	2%I

Figure 38. Panel A, HPLC profile from chromatography of SQRG-II. Acetronitrile gradient is shown. The three major peaks, denoted I, II and III, are #5, #10 and #14, respectively. Panel B, Inhibition of cysteine protease activity by rice germ fractions I, II and III (above), following neutralisation. %Acn (% Acetronitrile) indicates the point of elution from HPLC. Assays were conducted in duplicate for 10mins, with 2µl larval gut extract (LGE) or 1µl papain and 10µl approximately 2mg/ml rice fraction.

Peak II (#10) was the only factor in which inhibitory activity could be demonstrated. This peak caused 90% inhibition of larval gut cysteine protease activity, whereas papain activity was decreased by only 24%. The other fractions caused only 1 or 2% inhibition of either enzyme activity. When peak II was analysed on SDS-PAGE, it migrated as a single band with an apparent molecular weight of 15-16kDa (Figure 39).

This inhibitory peak (#10) was twice resuspended in distilled water and freeze dried, in order to remove contaminating volatile salts which would interfere with sequencing. When sequence analysis was attempted on an aliquot of this sample, no sequence was obtained either because of blocking of the N-terminus or failure to resuspend the protein. The remaining sample was resuspended in 0.1% TFA with 6M guanadine, passed once more through the HPLC and the protein peak (eluting in the same position as before) freeze dried. Sequencing was again attempted, once more without success, the peptide appearing to be N-terminally blocked.

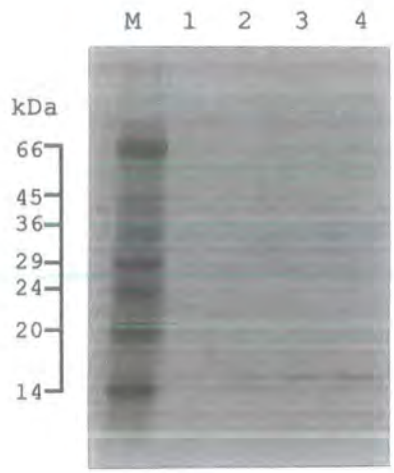


Figure 39. SDS-polyacrylamide gel of SQRG-II#10, electrophoresed in the presence of ß-mercaptoethanol. Lanes 1-4, 5µl, 10µl, 15µl and 20µl SQRG-II fraction #10 (around 90% of the mass due to salts present in the sample); lane M, molecular weight markers.

Separation of rice endosperm and germ

Attempts made to separate the germ by coarse milling of the whole seed, followed by air or water separation, produced no simple and satisfactory method of separation (results not shown).

Large scale rice CPI extraction from whole seed

A scaled-up procedure for extraction from whole rice seed was employed to purify larger quantities of rice CPI, mainly following the second method of extraction. Alterations were made where necessary to cope with the bulk of the samples used.

A protein extraction was prepared from 2kg rice seed, heat treated (80°C for 10min) and the protein remaining in solution precipitated between 0-30% and 30-65% saturation with ammonium sulphate. After exhaustive dialysis against distilled water and freeze drying, these two fractions were analysed by SDS-PAGE and western blotting, using anti-ozcI antisera for detection (Figure 40). Although cross-reaction at around 25kDa can be seen in both fractions, clearly the majority of ozcI is limited to the 30-65% cut. This is in agreement with the previous findings that CPI activity is limited to this fraction (see figure 32).

This ozc containing fraction was subjected to Q-sepharose chromatography, a larger column employed to accommodate the large amount of protein. Inhibition assays were conducted and two zones of inhibitory activity demonstrated (Figure 41). The inhibiting fractions were pooled (as BB3-1 and BB3-2), dialysed and freeze dried. BB3-1 was subjected to S-sepharose chromatography, using a Na-acetate buffer at pH4.1 (Figure 42 panel A). Inhibition assays with larval gut extract demonstrated one peak of activity, which caused up to 93% inhibition, but which spanned a number of the peaks apparent from the column profile. These inhibitory fractions were pooled, dialysed against the starting buffer, and passed through the S-sepharose column twice more (Figure 42 panels B & C).

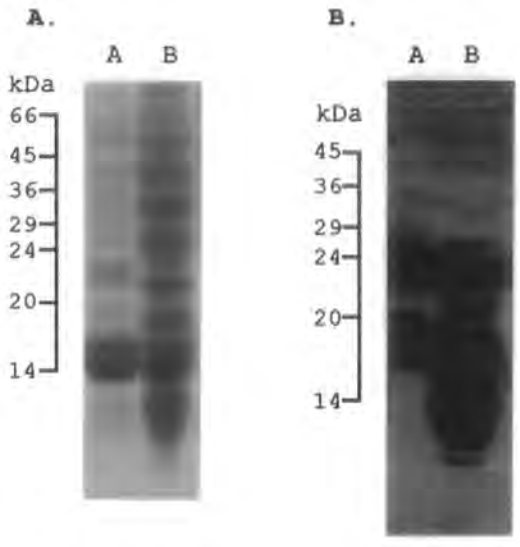


Figure 40. SDS-PAGE (panel A) and western blot (panel B) analysis of fractions precipitated between 0-30% (lane A) and 30-65% (lane B) saturation with ammonium sulphate.

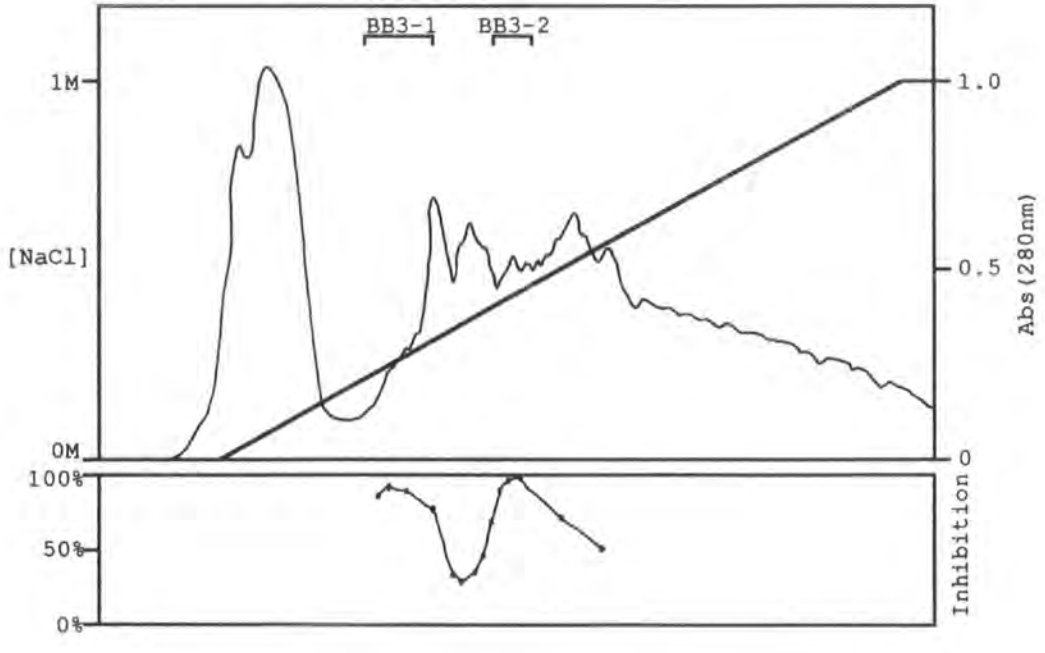


Figure 41. Q-sepharose chromatograhy profile of the ammonium sulphate precipitated fraction of BB3, rice extract. 0-1M NaCl gradient is shown. The inhibition caused to larval gut extract (LGE) enzymes is presented below the profile.

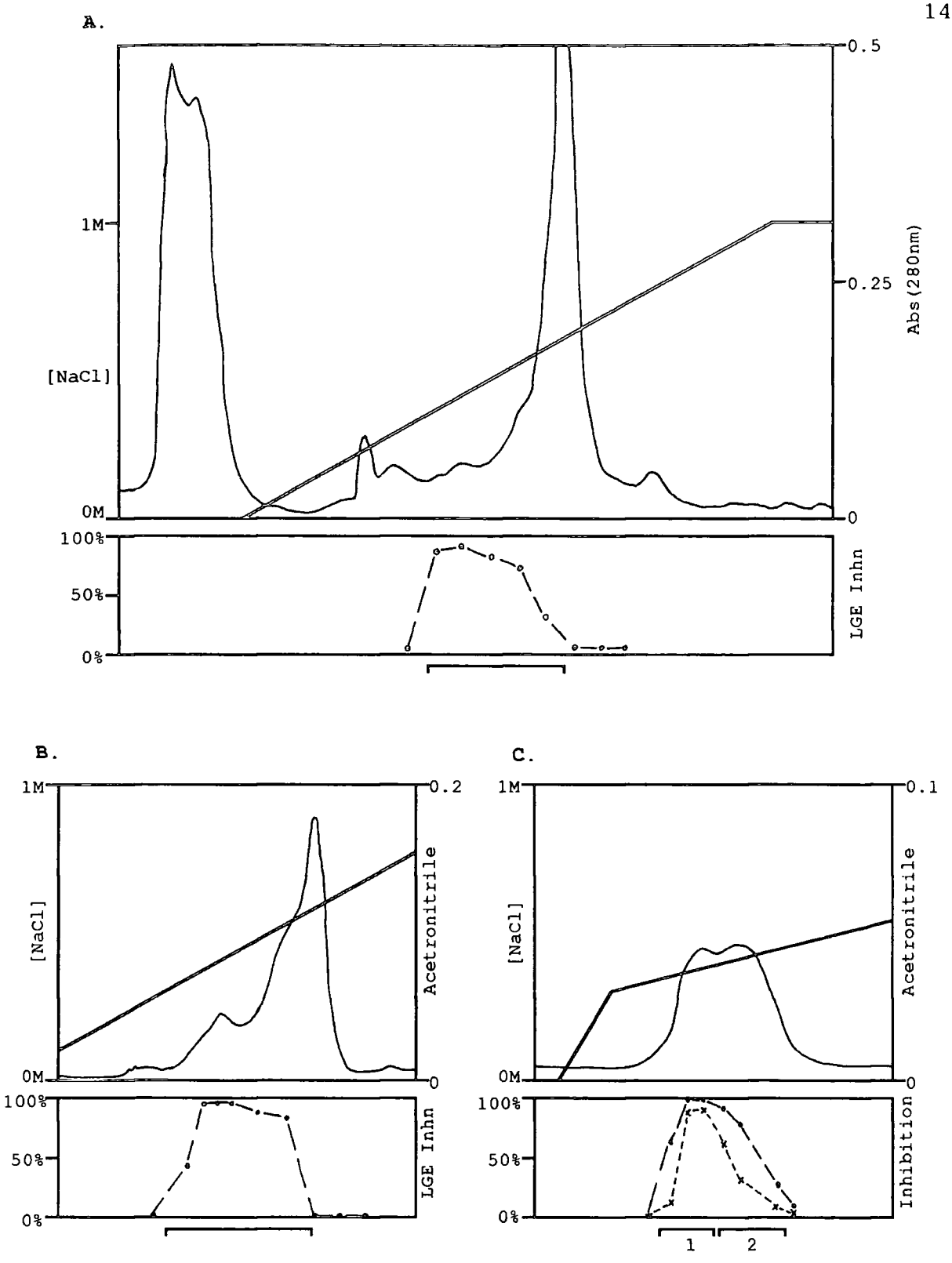


Figure 42. S-sepharose treatment of BB3-1, using 50mM Na-acetate buffer pH 4.1 with a linear gradient from 0-1M NaCl, and the inhibition caused by the resultant fractions. Panels A and B represent the first and second applications of BB3-1 to S-sepharose, in each case the fractions indicated were pooled and reapplied to the column. Panel C represents the third application, inhibition assays were conducted on LGE (∘) and papain (×). Fractions were pooled as indicated, 1 and 2 denoted BB3-11 and BB3-12, respectively.

Although inhibitory activity remained spread between the two protein peaks obtained, a marked difference was demonstrated in assays with papain and larval proteases, papain being inhibited strongly (90%) by fractions from the first peak of protein (BB3-11), whereas inhibition by fractions from the second peak (BB3-12) was much weaker (around 30%). Fractions from both peaks caused substantial inhibition of larval gut CP activity (96% and 80% for BB3-11 and BB3-12, respectively). The two peaks were pooled, largely on the basis of protein peak on the trace, but partially on the grounds of inhibitory characteristics, dialysed exhaustively and freeze dried.

BB3-2, the other inhibitory fraction from Q-sepharose, was also subjected to S-sepharose chromatography. Inhibition assays showed a similar inhibition pattern to that demonstrated with BB3-1 (Figure 43). A peak of inhibitory activity was demonstrated against both enzyme samples in the elution at 0.37M NaCl, causing over 90% inhibition of both enzyme activities. In the fractions following this peak, papain inhibitory activity dropped off rapidly, whereas inhibitory activity towards larval gut CPs decreased only gradually.

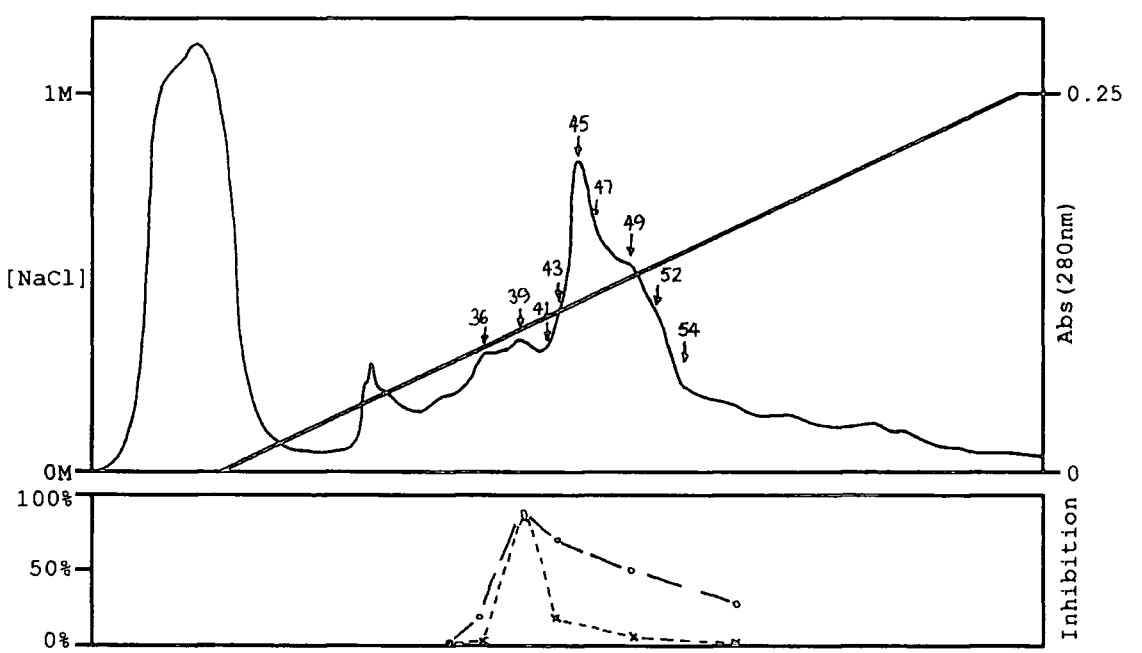


Figure 43. Profile obtained from S-sepharose chromatography of BB3-2. Chromatography was conducted using the same conditions as described in Figure 40. Inhibition assays were conducted, using the substrate Z-Phe-Arg-AMC, against LGE (-o-) and papain (-x-).

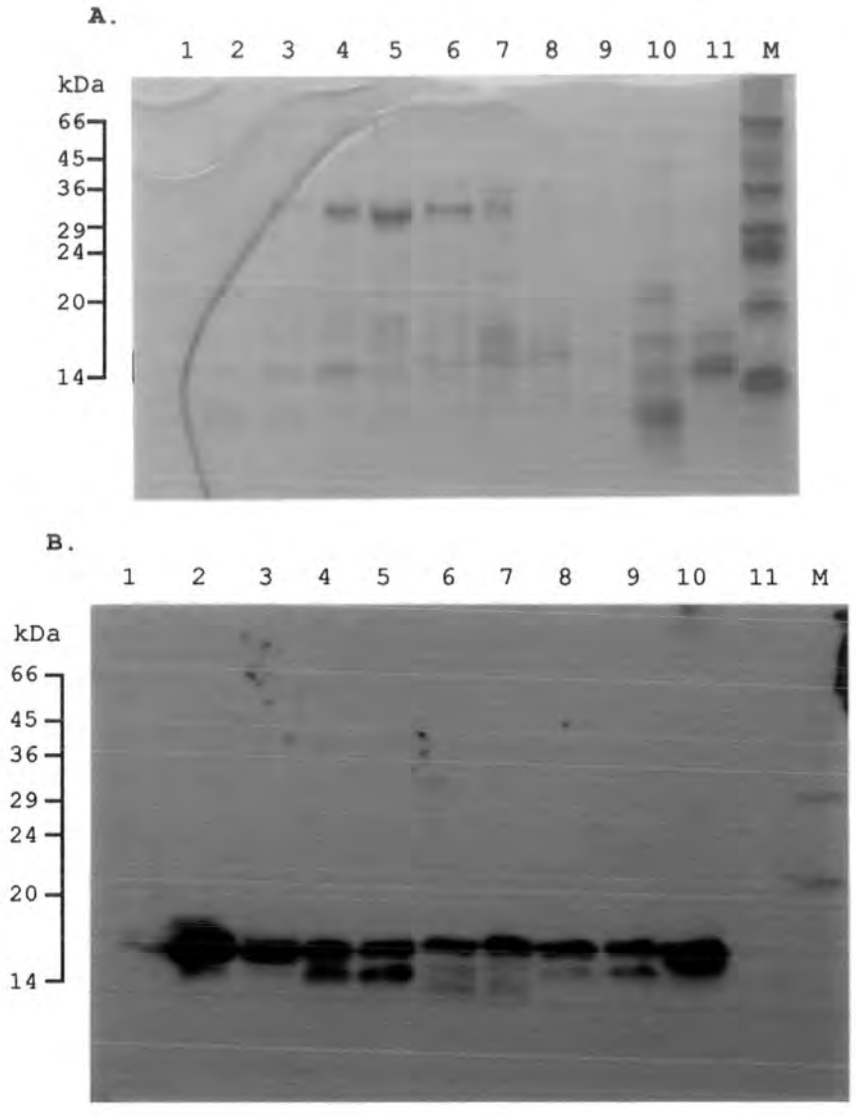


Figure 44. SDS-polyacrylamide gel (panel A) and western blot (panel B) of rice fractions following cation-exchange chromatography. Lanes 1-9, fractions 36, 39, 41, 43, 45, 47, 49, 52 and 54, respectively, from the chromatography of BB3-2; lane 10, BB3-11; lane 11, BB3-12; and M, molecular weight markers.

These inhibitory fractions from BB3-2, and the pooled fractions, BB3-11 and BB3-12, were electrophoresed on SDS-polyacrylamide gel and analysed by western blotting for the presence of ozcI. The resultant blot (Figure 44) demonstrated that ozcI was present in all the inhibitory fractions from BB3-2, and also in BB3-11, although no representative band was visible on SDS-PAGE. No ozcI was detected in fraction BB3-12.

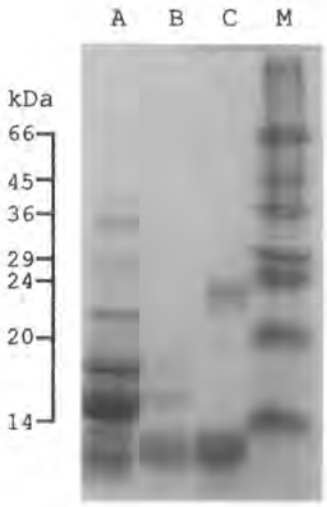
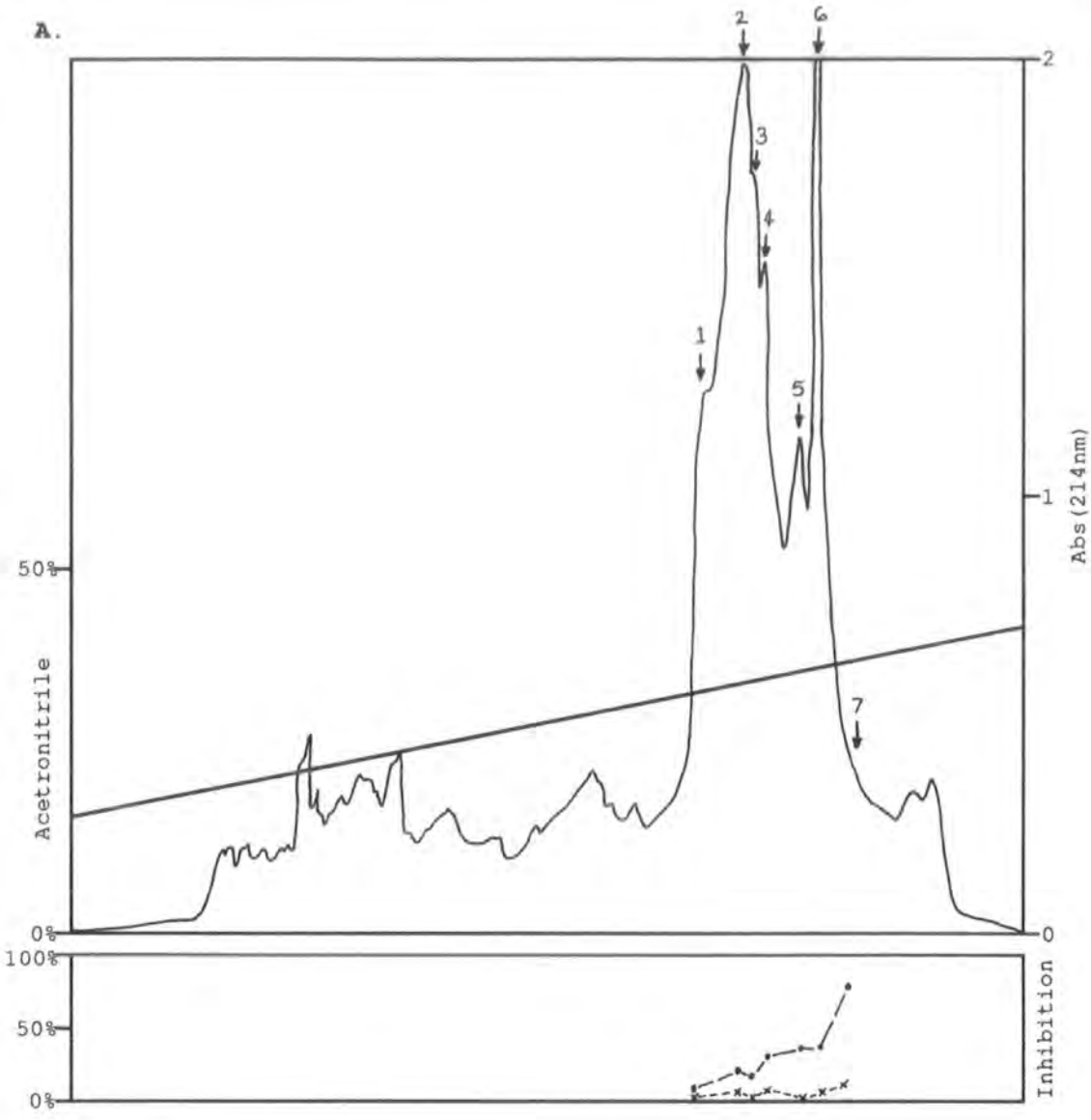


Figure 45. SDS-PAGE analysis of BB3-12 (lane A), SQRG-II (lane B) and SB (lane C), in the presence of ß-mercaptoethanol. Lane M, molecular weight markers.

The composition of BB3-12 was compared, by SDS-PAGE to both SQRG-II and SB prior to HPLC purification (Figure 45). Three main bands were visible in fraction BB3-12, with apparent Mwts of 21kDa, 18kDa and 15kDa. It was unclear from the gel whether the 15kDa band, the major component of BB3-12, represented a single protein, or two proteins with similar mobilities. Lower Mwt proteins were also present in this fraction, visible on stained gel as a sharp band at 13kDa and a less distinct band around 10-12kDa.



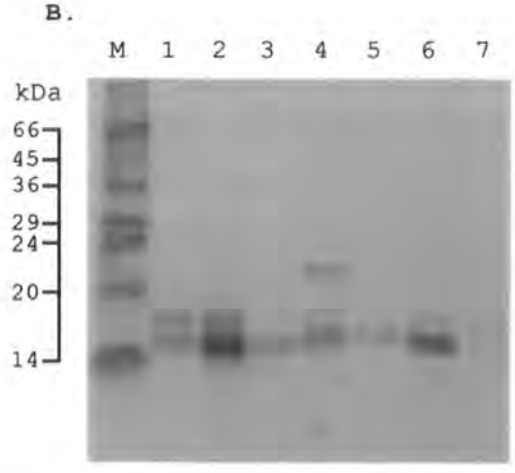


Figure 46. Panel A, profile of BB3-12 separation by HPLC. Inhibition assays using the eluted fractions are presented below the relevant peaks (1-7) of the profile, showing inhibition of LGE (-•-) and papain (-×-). Panel B, Samples from peaks 1-7 electrophoresed on SDS-polyacrylamide gel in the presence of ß-mercaptoethanol. 4% of each sample was loaded. Lane M, molecular weight markers.

The stained gel showed that fraction SQRG-II contained one or more proteins of 11-12.5kDa, a protein of 15kDa, and smaller amounts of proteins of around 14.5kDa and 18kDa. The 15kDa and 18kDa (and possibly the 14.5kDa) proteins corresponded to two of the major bands in BB3-12. This 15kDa protein in fraction SQRG-II corresponds to the protein (#10) previously purified by HPLC (see figure 39). The major components of fraction SB were visible as multiple bands around 11-13kDa and 22-23kDa. A band at 13kDa apparently corresponds to the 13kDa protein in BB3-12. Surprisingly, no 15kDa band was shown to be present in SB, although previous gels showed some indistinct bands at this point (see figure 28), and earlier in the purification a protein of this size was demonstrated as a major component (Figure 26).

BB3-12 was subjected to reversed phase HPLC, and the fraction separated into seven fractions, including two major peaks. These were not neutralised, but were freeze dried, assayed for inhibitory activity and analysed by SDS-PAGE (Figure 46). From the gel analysis, it was noted that none of the samples from HLPC were pure, all appeared to contain a contaminant of around 18kDa. Although little inhibitory activity was demonstrated in most of the fractions, around 40% inhibition of larval cysteine protease activity was caused by fractions #5 and #6, and 80% inhibition was caused by fraction #7. Inhibition of papain activity did not exceed 17%. Because #7 represented only the tail of peak #6, an aliquot of fraction #6 (denoted BB3-12#6) was analysed for sequence data. It was noted that the amount of protein apparent from the sequence analyser was far less than the concentration expected from gel analysis. The sequence obtained was found, by the use of a composite protein sequence database, to be highly homologous (>70% identity) to superoxide dismutase, a protein of around 17.4kDa (Figure 47).

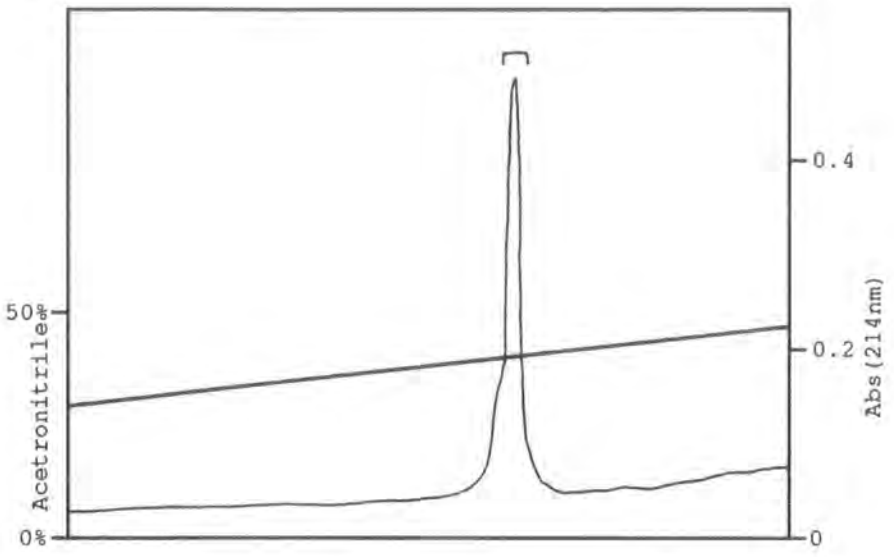
```
                                10        20
BB3-12#6                        VKAVAVLASSEGVKGTIFFS
                                :.:::::::::::::::::
Superoxide dismutase            AKGVAVLNSSEGVKGTIFFTHEGNGATTVTGTVSGLRPGLHGFHVHALGDNT
                                  10        20        30        40        50
```

Figure 47. N-terminal amino acid sequence derived from sample BB3-12#6 and alignment with the N-terminus of superoxide dismutase from white cabbage. ":" indicates identity of residues, "." indicates conservative change.

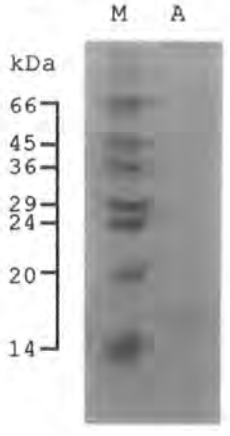
The remaining BB3-12#6 fraction was purified further by repeated application to the HPLC, maximising the purity of the sample, denoted BB3-6 (figure 48). An aliquot (termed BB3-neut6) of the resultant sample was neutralised prior to freeze drying. BB3-neut6 was assayed for inhibitory activity and analysed by SDS-PAGE (Figure 48). 33% inhibition of larval gut CP activity was demonstrated with 8µl of the sample, this amount of BB3-neut6 producing a slight increase in the activity of papain. SDS-PAGE analysis confirmed the size of the protein as 16kDa and also the purity of the sample.

The remaining BB3-6 was cleaved with cyanogen bromide, and the resultant peptides purified by reversed phase HPLC (figure 49), the individual peaks being chromatographed again to increase sample purity. Three main peaks were obtained, one, CB6, eluting at the position of the native protein, and the others, CB3 and CB4, at 33 and 34% acetronitrile, respectively. The purified peaks of CB3 and CB4 were analysed for amino acid sequence data, and the sequences obtained compared to others in a composite protein sequence database. Both sequences exhibited strong homology to the rice allergenic (RA) proteins, recently described by Izumi *et al.* (1992) and Adachi *et al.* (1993), as shown in figure 50.

A.



B.



C.

Assay	%Inhn
0.2µl LGE + 0.5µg cystatin	100
0.2µl LGE + 8µl BB3-neut6	33
0.1µg papain + 0.5µg cystatin	93
0.1µg papain + 8µl BB3-neut6	-7

Figure 48. Panel A, profile of BB3-12#6 separation by HPLC, collected peak indicated. Panel B, SDS-PAGE analysis of fraction BB3-neut6 in the presence of ß-mercaptoethanol. Lane M, molecular weight markers. Panel C, inhibition of protease activity of larval gut extract (LGE) and papain by cystatin and fraction BB3-neut6, using the substrate Z-Phe-Arg-AMC.

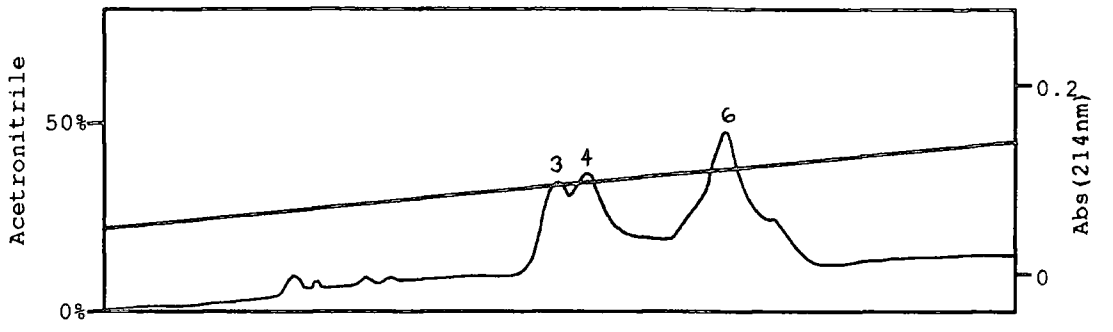


Figure 49. HPLC profile following cyanogen bromide cleavage of BB3-12#6. Major peptide peaks are indicated.

```
          60         70         80         90        100        110        120
BB3-CB-3                                 MTEVFPGERRGDLERAAASLP
                                          A    C
                                         :?:::::?:::::::::::::
RA17      GWCRCGALDHMLSGIYRELGATEAGHPMAEVFPGCRRGDLERAAASLPAFCN VDIPNGPGGV
                                         :::::::::::::::::::??::::
BB3-CB-4                 MLSGIYRELGATEAGHPMAEVFPGNWRGDL
                                              CR
```

Figure 50. Alignment of BB3-6 cyanogen bromide peptides with cDNA clone, RA17. Residues which were ambiguous in analysis are shown in italic type with the possible alternative printed below the main sequence. ":" indicates identity of residues, "?" indicates the possible identity of ambiguous residues.

EXTRACTION OF CYSTEINE PROTEASE INHIBITORS FROM RICE - DISCUSSION

As discussed in the introduction, CPIs have now been identified in a variety of plants, ranging from potato to cowpea, and from rice to pineapple. Rele *et al.* (1980) demonstrated the presence of papain inhibitors in extracts from a range of plant species, suggesting that their presence in plants may in fact be ubiquitous, a view shared by Ryan, in his review in 1990. The most highly characterised of the plant CPIs are the cystatins from rice, oryzacystatins I and II. These are known to display differential specificities towards papain and Cathepsin H (Kondo *et al.*, 1990b), ozcI having stronger affinity towards papain, while ozcII displays a preference towards Cathepsin H. Neither ozcI or ozcII exhibit inhibitory activity towards Cathepsins B or L (Michaud *et al.*, 1993).

With the extensive characterisation reported for the oryzacystatins (Abe *et al.*, 1987a, 1987b, 1988; Kondo *et al.*, 1989a&b, 1990a&b, 1991; Arai *et al.*, 1991), these seemed to be a sound starting point for investigations of plant CPIs and their effects on SCR enzymes. Because of the high levels of cysteine proteolytic activity demonstrated in SCR extracts in this work, and the strong inhibition of this activity caused by cystatin (another member of the "cystatin superfamily"), it was anticipated that ozcI may prove an effective tool for SCR control. Inhibition assays with cystatin, using Z-Phe-Arg-AMC as substrate, demonstrated the greater inhibitory activity of this inhibitor towards SCR cysteine proteases relative to papain, indicating that, should ozcI act in a manner similar to cystatin, then its activity towards the gut CPs should be even stronger than that towards papain. Cathepsin H activity was not specifically assayed for, but from the high levels of cysteine protease activity demonstrated using the substrates Z-Phe-Arg-AMC and Z-Arg-Arg-AMC, both of which are resistant to Cathepsin H digestion, it was assumed that Cathepsin H-like enzymes, if present, were not the dominant protease type.

Initial investigations, using Z-Phe-Arg-AMC as substrate, demonstrated that SCR cysteine protease activity could be inhibited strongly by crude protein extracts from rice. The fraction precipitated by 30-65% saturation with ammonium sulphate was capable of arresting almost all detectable CP activity, only 4% of the original activity remaining. These results confirm that the CPIs present in rice seed are actually capable of inhibiting the CPs of larval SCR, justifying further purification of these inhibitors, and investigation into their effects and their possible use in a plant defence mechanism.

Interestingly, inhibition of SCR larval gut CPs was far stronger than that of papain. This is in general agreement with the relative effectiveness of cystatin against the insect digestive CPs and papain. The difference observed here may be due solely to a greater affinity of ozcI for the insect CPs, otherwise, a proportion of the inhibition demonstrated may result from the presence of other CPIs in the seed, possibly ozcII, showing greater specificity towards the insect gut enzymes.

Location of CPIs in the rice seed

The general location of CPI activity within the rice seed was examined by separating the germ (embryonic tissue) from the endosperm. Extracts from these fractions were assayed for inhibitory activity and also analysed by SDS-PAGE and western blotting. The results of assays using Z-Phe-Arg-AMC as substrate (Figure 24), demonstrate a dramatic restriction of CPI activity to the germ tissue, contrary to the findings of Kondo *et al.* (1990b) who report that ozcI occurs in rice endosperm, but substantiated by further experiments in this work. Two indica rice (*Oryza sativa* L. *indica*) cultivars were tested, the long grained, Simular Roma, and a short grained cultivar, Originaro. Levels of inhibitory activity varied between the two, but in both cases by far the majority of inhibition was caused by the germ fraction. Workers in Japan established that the amount of ozcI present in japonica

rice (*Oryza sativa* L. *japonica*) varies greatly between cultivars (Kondo *et al.*, 1989a), substantiating the validity of the differences found between the cultivars tested here.

Western blot analysis of these extracts once more demonstrated the limitation of ozcI to the germ of rice seed, a fact reaffirmed by the lucid results from the tissue immuno-blotting experiment with longitudinally bisected rice seeds. OzcI was found to be present in highest amounts in mature seed, where it was limited mainly to the germ and, to a lesser degree, to the aleurone layer surrounding the endosperm. It was noted that in seeds where the germ was clearly discernible, ie. in mature seed, ozcI detection was strong and was concentrated in this germ region. In less mature seeds, where the position of the germ was barely distinct, no concentrated area of ozc was visible on the immuno-blot. Kondo *et al.* (1990b) found that ozc content within rice seeds increased rapidly during initial development, reaching a maximum five weeks after flowering, a pattern different to that of the main bulk of protein amassed during the maturation process. The developmental rate of rice seed varies markedly depending on the growth conditions provided, consequently no direct comparison was possible between the findings of Kondo *et al.* (1990b) and the observations made in this work. All the results presented here, despite their contradiction of the reports from the Japanese group, indicate clearly that the majority of CPIs present in rice, including ozcI, occur in the germ.

Extraction of CPIs from rice seed

Following a method modified from that Abe *et al.* (1987b), an attempt was made to purify ozcI from rice seed. Throughout the purification, fractions were assayed for inhibitory activity towards rootworm cysteine protease activity (2µl), using Z-Phe-Arg-AMC as substrate. It was established that following cation exchange chromatography using a formate buffer, no consistent inhibitory activity could be detected, prior to

exhaustive dialysis against distilled water, when activity was recovered, presumably by some conformational change. The inhibitory fraction obtained, SB, was intriguingly discovered to inhibit larval gut cysteine proteases strongly (up to 86% inhibition was recorded here), while possessing no apparent activity against papain. This behaviour indicates that the activity demonstrated belongs neither to ozcI or ozcII, both of which inhibit papain activity, but rather to another, unknown inhibitor.

Final purification of the inhibitor from the fraction SB was achieved by reversed phase HLPC. Two of the three main peaks obtained caused some inhibition of larval gut extract. The fraction which possessed the greater relative inhibitory activity (#25) was subjected to Liquid-phase protein sequencing, and the first twenty N-terminal amino acids derived:

VTFTIVNKCGYTVWPAALPS

This sequence was found to be highly homologous (75% identity) to proteins of the thaumatin-like family.

Thaumatin-like proteins

Thaumatin, the sweet tasting protein from the fruit of the West African shrub, *Thaumatococcus daniellii* Benth (van der Wel & Loeve, 1972; Edens *et al.* 1982), shares extensive sequence homology with a number of seed proteins (Richardson *et al.*, 1987; Roberts & Selitrennikoff, 1990; Hejgaard *et al.*, 1991), and a group of pathogenesis-related (PR) proteins. Thaumatin-like PR-proteins, induced by stress or pathogen infection, have been discovered in several plant species: tobacco (Cornelissen *et al.*, 1986; Linthorst *et al.*, 1989; Singh *et al.*, 1989; Pierpoint *et al.*, 1992), tomato (King *et al.*, 1988; Rodrigo *et al.*, 1991), potato (Pierpoint *et al.*, 1990), wheat (Rebmann *et al.*, 1991), barley (Bryngelsson & Green, 1989), maize (Frendo *et al.*, 1992) and rice (Reimmann & Dudler, 1993). These proteins, along with many of the thaumatin-like seed proteins, have been found to exhibit antifungal activities (see also Hejgaard *et al.*, 1991; Vigers *et al.*, 1991).

The 22kDa thaumatin-like protein from maize, reported by Richardson *et al.* (1987) to be a bifunctional inhibitor of mammalian trypsin and insect α-amylases, is now thought to be identical to zeamatin, an antifungal protein from maize (Roberts & Selitrennikoff, 1990; Vigers *et al.*, 1991). The complete amino acid sequence of zeamatin has been obtained and shown to be identical to the bifunctional inhibitor (Richardson,M., personal communication). No inhibitory activity towards either enzyme was demonstrated by zeamatin, but it has since been demonstrated that the apparent activity demonstrated by Richardson *et al.* (1987) was actually due to the action of a protease co-purified with the 22kDa protein (Richardson,M., personal communication). No inhibitory activity towards either serine proteases or α-amylases was demonstrated by the thaumatin-like seed proteins R and S, from barley grains, although these too possess antifungal activity similar to that of zeamatin (Hejgaard *et al.*, 1991). Vigers *et al.* (1991) reported that thaumatin also exhibits a zeamatin-like antifungal activity.

Interestingly, while a thaumatin-like PR-protein of around 15.7kDa has been reported in rice (Reimmann & Dudler, 1993), no group has yet reported a thaumatin-like rice seed protein. Vigers *et al.* (1991) reported zeamatin-like proteins in seed extracts from five other plant species; barley, flax, oats, sorghum and wheat. All these seed proteins had antifungal activity, were approximately 22kDa in mass, exhibited cross-reactivity to anti-zeamatin antiserum, and the three for which N-terminal amino acid sequence was obtained, showed high levels of homology to zeamatin. No such protein or antifungal activity was demonstrated by Vigers *et al.* with rice extracts.

None of the published data on thaumatin-like proteins, to the author's knowledge, report assays for cysteine protease inhibitory activity. It would appear that the general function of thaumatin-like proteins, whether natural seed proteins or PR-proteins, is one of protection against fungal attack. However, little or no antifungal activity was demonstrated in rice extracts by Vigers *et al.* (1991), suggesting that the rice thaumatin-like seed protein

isolated in this work may lack the normal antifungal capacity of this group of proteins. No assays for anti-fungal activity were conducted in this work. Subsequent extractions of CPIs from rice, along with assays for CPI activity in thaumatin (see below), indicate that the thaumatin-like protein sequenced was a contaminant in the CPI sample and probably possessed little or no CPI activity.

Interestingly, using SDS-PAGE, a protein with an apparent Mwt of 22kDa was demonstrated to occur in the fraction SB. The thaumatin-like seed proteins reported to date, show a characteristic Mwt of 22kDa. An obvious hypothesis, then, would be that the protein sequence obtained corresponded to the 22kDa protein observed on stained gel. Following HPLC treatment, no fraction from SB was analysed by SDS-PAGE, so the composition of SB#25, the sequenced fraction, cannot be confirmed. This is, however, the first report of a thaumatin-like seed protein from rice.

Purification of CPIs from rice germ

To purify the larger quantities of CPI necessary for use in extensive inhibition assays *in vitro* and insect bioassays (*in vivo*), a scaled up procedure for its extraction was needed. Due to the virtual restriction of CPIs to the germ of rice seed, and the small proportion of the seed made up by the germ (only 2% by mass), the most obvious method of increasing the yield without dealing with unmanageable quantities of rice tissue, was purification from detached rice germ.

The extraction procedure was slightly modified from that used initially, making it more simple and closer to the method by which ozcI was purified by Liang *et al.* (1991). This method was also used for extractions from whole rice seed, giving comparable results. Where rice germ was used, 50g of milled rice germ, the equivalent of 2500g of intact seed, were extracted as normal. Fractionation by ammonium sulphate precipitation resulted in the inhibitory activity being limited to the 30-65% fraction, virtually no

inhibition being caused by the other fractions. Inhibition by this fraction was demonstrated against the digestive CPs of larval and adult SCR and adult WCR (all inhibited by over 96%), and also against papain, although papain activity was reduced by only 43%. This differential inhibition correlates with the previous results and provides further firm evidence for the similarity of enzymes utilised in the digestive systems of SCR and WCR.

Successive chromatography of this fraction yielded two inhibitory peaks. The first, SQRG-I, the flowthrough material, possessed inhibitory activity towards both papain and insect CPs; the second, SQRG-II, was inhibitory towards the insect enzymes alone. The assay results presented (Figure 36) were conducted with WCR adult gut extracts, but subsequent germ and whole seed extracts with comparable characteristics were assayed against both larval and adult SCR CPs with analogous results. It would seem then that the CPs detected here, active in the digestive system of WCR and SCR, are much the same throughout the larval and adult stages. They also show immense similarities in specificity between the species, contrary to the reports of Wolfson and Murdock (1990), who suggest that great differences occur between the two species, and also that the major digestive enzymes of the SCR adults are aspartic acid proteases. While the pH profiles of protease activity may vary between the two stages and two species, the differences in actual, general protease activity and specificity seem less pronounced.

Confirmation of ozcI and novel CPI

As SQRG-II caused inhibition of insect CPs while leaving papain activity unaffected, this inhibitory activity was unlikely to be due to the presence of either of the oryzacystatins, both of which cause inhibition of papain. Western blot analysis of SQRG-I and SQRG-II confirmed this hypothesis, as no reaction was seen with antiserum raised against ozcI or ozcII. SQRG-I was demonstrated to contain ozcI, but not ozcII. As this

sample was not pure, as was demonstrated by SDS-PAGE, it is possible that some contaminants may also act as CPIs. SQRG-I was, however, employed as an impure positive for immuno-blotting for ozcI and also used in insect bioassay as an initial test as to the possible effectiveness of ozcI *in vivo* on insect survival and development. Crude extracts from the two rice cultivars, Simular Roma and Originaro, were also analysed on these western blots, both possessed marked quantities of both ozcI and ozcII, as detected by these antisera.

SQRG-II was further purified by reversed phase HPLC and the three main peaks obtained neutralised with ammonium bicarbonate solution prior to freeze drying. This step was taken in an attempt to regain some of the activity which seems lost following HPLC. It appears that certain buffers, such as the formate used for cation exchange and either the TFA or acetronitrile from HPLC, affect the inhibitory activity of the protein, possibly by promoting a conformational change. In the case of formate, this is corrected by dialysis against distilled water, the inhibitory activity being fully restored. The neutralisation of fractions from HPLC, and subsequent freeze drying, would appear to have a similar effect, one of the neutralised peaks obtained here from SQRG-II caused around 90% inhibition of larval CP activity. SDS-PAGE of this peak, SQRG-II#10 revealed a single band, with an apparent molecular weight of 15-16kDa (under reducing conditions), illustrating that the protein had been purified to homogeneity.

The elution point of SQRG-II#10, showed a strong similarity to that of the peak SB#25, from which the thaumatin-like N-terminal sequence was obtained. The behavioural characteristics of the two fractions, during the extraction procedure, were also analogous. The 15-16kDa protein revealed in SQRG-II#10 by SDS-PAGE does not, however, appear to correspond to the protein purified from fraction SB. On the gels shown in figures 26 and 28, a protein of 15-16kDa was shown to be a major component of SB during the early stages of its extraction, however, following the final ion-exchange

chromatography step, SB is shown to be composed largely of two proteins with apparent Mwts of around 14kDa and 22kDa, with only minor, indistinct bands visible around 14-16kDa. This difference was shown most clearly when the two fractions were electrophoresed on the same gel (Figure 45), bands below 14kDa occurred in both fractions, but the 15-16kDa protein isolated from SQRG-II was apparently absent from SB, while the 22kDa protein, thought to correspond to the thaumatin-like protein purified from SB, was absent in SQRG-II.

To confirm that the protein present in SQRG-II#10 was different to that sequenced from fraction SB#25, SQRG-II#10 was analysed for N-terminal amino acid sequence data, but without success, the N-terminus being blocked, preventing any sequence analysis. There are two possible explanations for this: either the protein was naturally blocked (in vivo), proving its dissimilarity to SB#25; or the protein may have become blocked, *in vitro*, during the purification, possibly due to the use of formate buffers or ammonium salts, both of which are known to occasionally cause problems during the preparation of proteins for sequencing (Harris & Angal, 1990). Further investigation was undertaken in order to establish the identity of the inhibitory protein.

Larger scale extraction of the novel CPI from whole rice seed

Because of the limited supply of excised rice germ, further bulk purifications were not possible using this material. Attempts were made to develop a simple system for germ separation, using coarse milling and air or water separation, but no satisfactory method was found. The simplest formula seemed to be dealing with the bulk of the whole rice seed, rather than developing complex methods to remove the germ.

Larger scale extraction from rice seed was conducted mainly following the normal procedure with some alterations necessary to cope with the greater volumes used. Western blot analysis of ammonium sulphate fractions

confirmed the separation of ozcI into the 30-65% fraction, although some cross-reaction was displayed at around 25kDa, present in both fractions. This phenomenon is likely to be due to contaminants in the ozcI preparation used for antibody production, thus raising contaminating antisera, specific to rice proteins common to both fractions. Had time permitted, this theory could have been tested by raising antiserum against the recombinant ozc purified from *E. coli* (see Chapter 5), unfortunately time did not allow.

Chromatography of the 30-65% ammonium sulphate fraction, using a large volume Q-sepharose FF column, resulted in the production of two inhibitory peaks, BB3-1 and BB3-2, which both arrested over 90% of insect cysteine protease activity. These fractions, BB3-1 and BB3-2, were chromatographed on S-sepharose using acetate (rather than formate) buffer. Assays of samples taken across the inhibitory peaks from both BB3-1 and BB3-2, showed a differential inhibition of papain and larval gut extract, a pattern possibly due either to the greater affinity of ozcI to the insect proteases, or else to a mixture of ozcI and another CPI with behavioural characteristics comparable to those found in SQRG-II and SB.

The inhibitory peak from BB3-1 was passed through the column twice more in an attempt to obtain a single protein peak corresponding to the inhibitory activity demonstrated, but still, the inhibitory peak spanned the two protein peaks apparent from the column profile. This resistance to purification, and the differential inhibitory activity observed across these two peaks, implied that two inhibitory factors were present. The first, restricted mainly to the first protein peak, caused strong inhibition of both papain and the larval CPs, whereas the second, presumably concentrated in the second protein peak, was highly effective against the insect enzymes, while leaving papain less affected.

The extent of ozcI activity in the fractions was demonstrated by western blot analysis. All the inhibitory fractions from BB3-2 were shown to contain ozcI, as was BB3-11, only BB3-12 demonstrated virtually no cross

reaction with the antiserum. No distinct band corresponding with this ozcI was visible on the stained gel, indicating that the concentration of this protein was sufficiently low to be easily detected by the ECL method, but not discernible by Kenacid blue R staining on gel. The division of BB3-11 and BB3-12 had been based largely on the trough between the peaks on the column trace, thus, considering the tail normally observed on peaks from column chromatography, it was surprising that such a strict limitation was made of ozcI into fraction BB3-11. This suggests that ozcI might not be solely responsible for the first protein peak observed, rather it may occur as a peak close to, or enclosed within, the leading end of this first peak.

Due to the lack of ozcI detected in BB3-12, and the similarity of inhibitory characteristics to that found previously (the high level of inhibition of larval enzymes along with only low inhibition of papain), this indicates the presence of a novel CPI, similar or identical to that identified previously in samples SQRG-II and SB. A comparison of BB3-12 with samples of SQRG-II and SB prior to their purification by HPLC, using SDS-PAGE, demonstrated some interesting similarities and differences (Figure 45). The 15-16kDa protein purified from SQRG-II, absent from fraction SB, as was previously discussed, appears to be a major component of BB3-12. A 18kDa protein present as a minor component of SQRG-II, was also demonstrated in BB3-12. No 22kDa protein, corresponding to that present in SB, was found in BB3-12, although lower Mwt proteins demonstrated on gel may have been common to all three fractions.

BB3-12, the inhibitory fraction shown by western blot analysis to contain no ozcI, was further purified by reversed-phase HPLC. Weak inhibitory activity (39%) was demonstrated by two peaks #5 and #6, greater inhibition strangely being caused by the tail of peak #6 than by the peak itself. As discussed previously, the inhibitory activity of this protein becomes diminished in the presence of formate buffer or that of TFA and acetronitrile. Activity can generally be restored by dialysis of formate

samples or neutralisation of HPLC fractions prior to freeze drying, suggesting that the loss of activity is due to some conformational change in the presence of these particular compounds. The lyophilised fractions obtained from SB by purification with HPLC showed reduced inhibitory activity, whereas those from SQRG-II, neutralised prior to freeze drying, possessed their normal high levels of inhibitory activity. Because this neutralisation step may have been the cause of N-terminal blocking, no neutralisation was employed with BB3-12 fractions, hence a reduced inhibitory activity was to expected. One possible explanation for this apparent anomaly could be a delay between the HPLC recorder and the emergence of fractions. However, this delay was estimated to be only seconds, not long enough to account for a shift of peak #6 elutant to fraction #7, this would require at least 1min. SDS-PAGE analysis of these HPLC fractions showed that all contained a minor contaminant with apparent molecular weight 17-18kDa. BB3-12#6, was found to contain large amounts of a 15-16kDa protein, the density of this band affirming that this fraction, and not #7, represented the peak demonstrated on the HPLC profile, and its position indicating the presence of the protein purified in SQRG-II#10.

A sample of fraction BB3-12#6 was analysed for sequence data and the first 20 amino acid residues of the N-terminus determined. This sequence showed high homology to superoxide dismutase, a protein of 17.4kDa. The amount of protein present in the sample according to the sequence output was far lower than that expected from the estimated amount on gel. This, along with the size of superoxide dismutase, 17.4kDa, corresponding to the minor contaminant observed on stained gel at 17-18kDa, indicates that the protein sequenced was this contaminant and that the CPI, the 15-16kda protein, was again N-terminally blocked. Since neither formate or ammonium bicarbonate had been employed during this preparation of the protein, it appears that the protein is blocked *in vivo*.

```
                    10         20         30         40         50
RA17       D-HH--Q-VYSPGEQCRPGISYPTYSLPQCRTLVRRQ-CVG-RGASAA--DEQVWQDCCRQLAAV
RAG2       D-HHKDQVVYSLGERCQPGMGYPMYSLPRCRAVVKRQ-CVG-HGAPGGAVDEQLRQDCCRQLAAV
RA14       D-HHKDQVVYSLGERCEPGMGYPMYSLPRCRAVVLRQ-CVGYR--SPGAVDEQLAQDCCRELAAV
RA5        EYHHQDQVVY-TRARCQPGMGYPMYSLPRCRALVKRQ-C---RG-SAAAA-EQVRRDCCRQLAAV
WAAI               SGPWSWCNPATGYKVSALTGCRAMVKLQ-CVGSQVP-----EAVLR-DCCQQLADI
Barley TI            FGDSCAPGDALPHNPLRACRTYVVSQICHQG--PRLLTSDMKRR--CCDELSAI

            60         70         80         90         100        110
BB3-CB-3                                  MTEVFPGERRGDLERAAASL--P
                                           A    C
BB3-CB-4                 MLSGIYRELGATEAGHPMAEVFPGNWRGDL
                                              CR
RA17       DDGWCRCGALDHMLSGIYRELGATEAGHPMAEVFPGCRRGDLERAAASL--PAFCNVDIPNGPG-
RAG2       DDSWCRCSALNHMVGGIYRELGATDVGHPMAEVFPGCRRGDLERAAASL--PAFCNVDIPNGTG-
RA14       DDSWCRCSALNHMVGGIYRELGATDVGHPMAEVFPGCRRGDLERAAASL--PAFCNVDIPNGTG-
RA5        DDSWCRCEAISHMLGGIYRELGAPDVGHPMSEVFRGCRRGDLERAAASL--PAFCNVDIPNGGG-
WAAI       NNEWCRCGDLSSMLRAVYQELGVRE-GK---EVLPGCRKEVMKLTAAS-V-PEVCKVPIPNPSGD
Barley TI  P-AYCRCEALRIMQ-GVVTWQGAFE-GAYF-KDSPNCPRERQTSYAANLVTPQECNLGTIH--G-

          120        130
RA17        --GVCY--WLGYPRTPRTGH
RAG2        --GVCY--WLGYPRTPRTGH
RA14        --GVCY--WLGYPRTPRTGH
RA5         --GVCY--WL-----ARSGY
WAAI        RAGVCYGDWAAYPDV
Barley TI   SA-YCPELQPGYG
```

Figure 51. Alignment of BB3-6 cyanogen bromide peptides with deduced amino acid sequences of the rice allergenic proteins, WAAI and Barley trypsin inhibitor. Dashes represent introduced gaps to maximise alignment. Residues which were ambiguous in analysis are shown in italic type with the possible alternative printed below the main sequence. Numbering is according to RA17.

The remainder of fraction BB3-12#6 was further purified by HPLC, until appearing homogeneous both by HPLC and SDS-PAGE, on which a single band, of 15-16kDa, was visible. Neutralisation of a sample of this fraction prior to freeze drying did not result in the recovery of full inhibitory activity, indicating that the conformation of the protein had been permanently altered by the initial HPLC treatment and subsequent freeze drying. This pure protein sample (BB3-6) was subjected to cyanogen bromide cleavage. Three resultant peptides were purified by HPLC and sequence data was obtained from two, BB3-CB-3 and BB3-CB-4. No attempt was made to sequence the third peptide. These peptides were compared to sequences in a composite protein sequence database (OWL 17.0; at SEQNET data facility, Daresbury, U.K.) using the SWEEP (v3.02) homology searching programme (written by A.J.Bleasby), and found to exhibit strong homology (over 80% identity) with the rice allergenic (RA) proteins described recently by Izumi *et al.* (1992) and Adachi *et al.* (1993). The greatest homology was to the cDNA clone RA17. A comparison of the amino acid sequences of these cDNAs is presented in Figure 51. The two peptides produced actually appear to overlap in the RA protein, due to cleavage at different methionine residues.

Little characterisation of these allergenic proteins has been conducted. Investigations have been conducted into their location and accumulation within the rice seed, along with their reaction with IgE from sera of allergic patients. Assays against bovine trypsin and bacterial α-amylase have been conducted but no inhibitory activity demonstrated (Matsuda,T., personal communication). Interestingly, the majority of these proteins were found to be located in the endosperm of the rice seed, as opposed to the bran, the outer layer of the seed (Matsuda *et al.*, 1988). These fractions were separated by polishing material off the grains, the bran fraction comprising the first 8% (by weight) of material removed, and endosperm, the following 10%. No distinction between endosperm and germ material was made.

Interestingly, the main bands seen in the endosperm fraction, following SDS-PAGE, correlate well with the bands observed with ammonium sulphate precipitated fractions from both whole grain and germ, suggesting the presence of germ material in the "endosperm" fraction of Matsuda *et al.* (1988).

Along with the isolation of cDNA and genomic clones for these proteins, this group have isolated the major allergenic protein, and successfully obtained N-terminal amino acid sequence data from it. As there is no mention of additional steps necessary to unblock the protein, this implies that the protein in japonica rice is not N-terminally blocked.

Interestingly the family of RA proteins also show marked homology with the cereal trypsin and amylase inhibitors, including barley trypsin inhibitor and WAAI, the major 0.28-family α-amylase inhibitor from wheat. When WAAI was assayed for activity against SCR cysteine proteases, in this work, no CPI activity was demonstrated.

In order to confirm that this allergenic protein was indeed responsible for the CPI activity demonstrated, the isolation of more of the purified protein would be necessary, allowing thorough characterisation of its inhibitory properties. This protein could be obtained from two sources: using larger scale purification from rice seed or isolated germ; or expressing it in bacteria as a recombinant protein. As has been demonstrated in the extractions conducted in this work, this inhibitory protein is present in only very small amounts in rice seed, thus extractions from kilograms of rice would be necessary to obtain sufficient protein for characterisation. The simpler method may be the production of an expression plasmid, allowing expression of a recombinant form of the protein in bacteria. Several options are available: If the cDNA clone (RA17) could be obtained from the Japanese group this could be engineered to allow expression in bacteria, resulting in the expression of the japonica rice RA protein, which may not be identical to

that purified here from indica rice, and which may or may not exhibit inhibitory activity. Otherwise, this RA17 cDNA could be used to screen an indica rice cDNA library. Alternatively, oligonucleotides could be produced using the published RA17 cDNA sequence and the corresponding RA encoding sequence amplified from indica rice seed cDNA. The production of a recombinant form of the indica rice RA protein, the native form of which has been isolated in this work, would be most useful as this would confirm the presence of an analogous family of proteins in indica rice, and allow characterisation of the indica rice novel CPI protein. Unfortunately time did not allow this work to be conducted.

INVESTIGATION OF CPI ACTIVITY IN MAIZE AND WHEAT - RESULTS

Crude protein extracts were obtained from rice seed, maize kernel and wheat grain, by extraction in PBS. Insoluble material was removed by centrifugation, although the maize extract remained notably cloudy. The supernatants, assayed for CPI activity against larval gut proteases, (Figure 52) caused different levels of inhibition. Maize and wheat extracts caused 70 to 80% inhibition, whereas, surprisingly, the crude rice extract gave only 16% inhibition. The wheat extract exhibited low levels of endogenous CP activity. The 0.28 family α-amylase inhibitor from wheat, WAAI, was assayed for both CP and CPI activity, as shown in figure 52, but neither protease or inhibitory activity was demonstrated.

Another wheat extract was prepared, extracting in acetate buffer and precipitating proteins by 100% saturation with ammonium sulphate. This extract, after resuspension and dialysis, was assayed for CPI activity, minimal amounts of inhibition (around 1%) caused to insect protease activity. Heat-treating the wheat extract (80°C for 10mins) had no affect on this activity (results not shown).

A similar preparation was made from milled untreated maize kernels, extracting in Na-phosphate buffer, collecting the proteins precipitated between 0 and 65% saturation with ammonium sulphate. This maize extract was assayed for inhibitory activity (see figure 53), no activity was demonstrated towards either insect gut cysteine proteases or towards papain.

Assay	µU	% Activity
LgE alone	37.0	100
10µl wheat ext.	11.7	32
10µl rice ext.	31.2	84
10µl maize ext.	8.7	24
10µg WAAI	47.4	124
5µg WAAI	48.6	126
Control wheat ext.	5.8	-
Control WAAI	0.0	-

Figure 52. Assay of the CPI activity exhibited by crude seed extracts. Assays contained 2µl LgE, except those denoted "Control". Activity is given in µU, where 1µU represents 1pmol free AMC liberated per min. % Activity refers to percent residual activity, corresponding to that occurring in the absence of any inhibitor.

Assay	µU	% Inhibition
LgE	49.2	-
LgE + 5µl maize	49.2	0
papain	39.4	-
papain + 5µl maize	39.0	1
Control maize	0	-

Figure 53. The inhibitory activity of maize extract, demonstrated using a 5min assay with the substrate Z-Phe-Arg-AMC, and 2µl diluted LgE or 1µl diluted papain. Activity is given in µU, as above, and % inhibition is the decrease in activity caused for each enzyme.

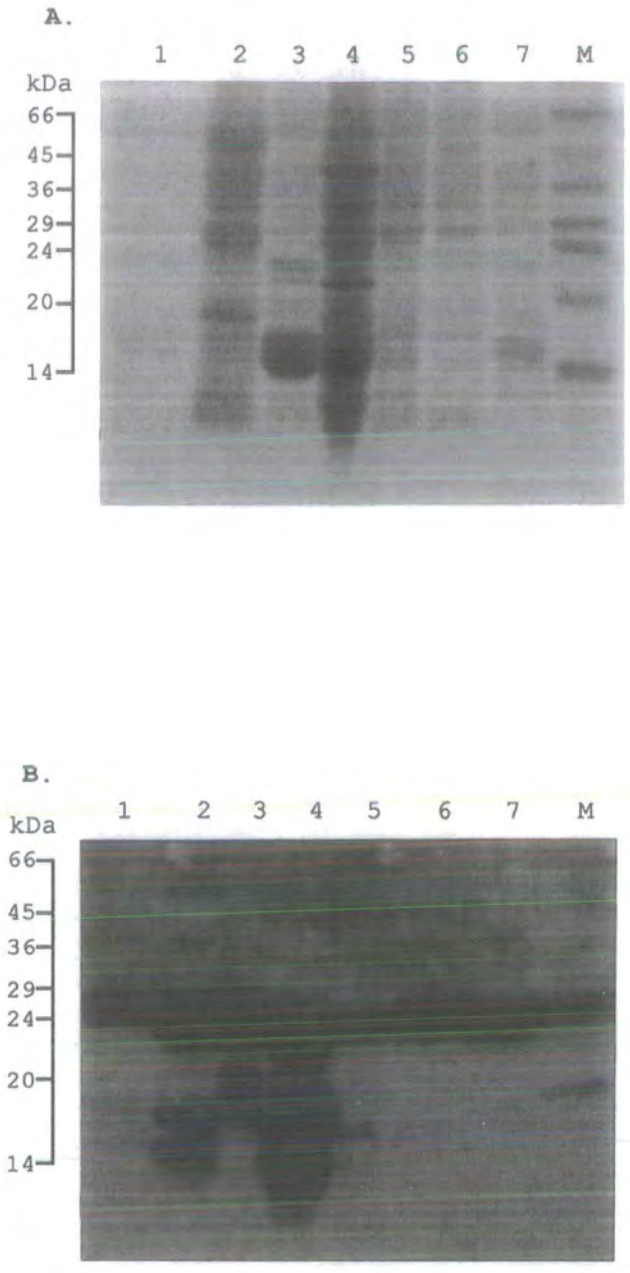


Figure 54. SDS-PAGE (panel A) and western blot (panel B) analysis of protein extracts from rice (lanes 1-4), maize (lanes 5 and 6) and wheat (lane 7). Lanes 1 and 2, crude rice endosperm and germ extracts, respectively; lanes 3 and 4, 0-30% and 30-65% ammonium sulphate fraction from rice, respectively; lane 5, 0-65% ammonium sulphate fraction from maize kernel; lanes 6 and 7, crude extracts from maize and wheat, respectively; and lane M, molecular weight markers. Western blot analysis was conducted using anti-ozcI antiserum.

The fraction was also analysed by western blotting, for reaction with anti-ozcI antiserum (Figure 54). The blot also shows crude extracts from wheat grain, maize kernel, and endosperm, germ and ammonium sulphate fractions from rice. The western blot shows cross reaction in all the samples around 25 to 26kDa. No other bands are visible in the crude wheat extract. In the maize extract, especially in the more pure fraction, a band was visible at around 16kDa, corresponding to a band present in both the crude rice germ fraction and in a 30-65% ammonium sulphate fraction from rice.

CPI ACTIVITY IN WHEAT AND MAIZE - DISCUSSION

CPIs are now known to occur in a wide range of plants (Rele *et al.*, 1980), including wheat (Fossum, 1970) and maize (Abe & Whitaker, 1988; Abe *et al.*, 1992). An investigation was conducted into the occurrence in wheat grains and maize kernels of CPIs capable of inhibiting the CPs of larval SCR. Crude protein extracts were taken from the ground seeds of wheat, maize and rice, extracting in PBS. Inhibition assays revealed that while the wheat and maize extracts inhibited larval gut CP activity by 70 to 80%, the rice extract inhibited activity by only 16%, a remarkable result considering the high levels of inhibition demonstrated previously by rice extracts. The low level of CPI activity demonstrated by this extract, knowing the amounts of inhibitor present in rice seed, suggests one of the following: that PBS was not a good extraction medium for use with rice seed, although no reason is obvious as to why this should be so unless inhibition is masked by endogenous CP activity; that the concentrations of inhibitor in the wheat and maize fractions were surprisingly high; or that the inhibition demonstrated was not merely due to CPI activity, but also to contaminating carbohydrates or the presence of proteins which compete with the substrate for enzyme. To investigate these possibilities, further purification of the wheat and maize proteins was undertaken.

Activity of wheat proteins towards SCR larval Cysteine Proteases

Because of the homology between the rice allergenic proteins and the wheat α-amylase inhibitor, WAAI, along with the high apparent levels of inhibitory activity present in wheat fractions, WAAI was tested for CPI activity. No inhibition of larval digestive CPs was demonstrated and no endogenous CP activity was exhibited.

Extraction of wheat proteins with acetate buffer and ammonium sulphate precipitation, achieved a purer sample than assayed initially. Inhibition assays demonstrated virtually no inhibitory activity with this sample. This apparent loss of inhibitory activity was most probably due to the removal of carbohydrate contaminants and some PBS-soluble proteins from the fraction. It is possible that the inhibitory activity was due to a protein soluble in PBS but insoluble in the acetate buffer, but as all inhibitors obtained from rice were soluble in this buffer, this was thought unlikely. Another possible reason for apparent loss of inhibitory activity could be the presence of endogenous proteases, insensitive to inhibition by the native CPI, but able to cause hydrolysis of the substrate, masking the inhibition of the insect enzymes. Since a low level of CP activity had been demonstrated by the initial crude wheat extract, the possibility of an active endogenous enzyme was investigated, heating the extract to 80°C for 10mins, a treatment sufficient to denature any endogenous proteases. Assaying this heat treated fraction demonstrated no altered inhibitory activity towards the larval cysteine proteases. It was concluded that no protein inhibitor of larval SCR digestive cysteine proteases, corresponding to the inhibitors found in rice extracts, was present in the soluble fraction of wheat grain, and that the apparent inhibition displayed with the crude extract was merely due to the competition of other proteins for the active site of the enzymes, or interference from other contaminants.

Maize CPIs and their activity towards SCR enzymes

As discussed in the introduction, two CPIs have previously been identified in the kernels of maize (Abe & Whitaker, 1988; Abe *et al.*, 1992), both of which show similarities to the cystatins. The 9.2kDa protein extracted from the endosperm of maize kernels by Abe & Whitaker (1988) demonstrated strong, heat stable, inhibitory activity towards both papain and ficin, and was reported to have very similar physical characteristics to oryzacystatin, and comparable specificities against proteases. The CPI reported by Abe *et al.* (1992) showed strong sequence homology to the cystatins, and a striking resemblance (71% homology between residues 36-135) to ozcI, as might be expected as the cDNA clone was isolated by screening a cDNA library from immature maize kernels with a mixture of cDNA inserts for ozcI and ozcII.

Corn cystatin-I, expressed in *E. coli* as a fusion protein consisting of 15 amino acid residues N-terminal, originating from pUC19, and 105 residues (Gln^{31}-Ala^{135}) from corn cystatin-I, was demonstrated, in a crude extract, to cause high levels of papain inhibition. This fusion protein appeared to exhibit the usual cystatin characteristics, but, no native protein was purified from maize to confirm this inhibitory activity (Abe *et al.*, 1992).

Considering the presence of these two proteins and their similarity to oryzacystatin, together with the apparent inhibitory activity demonstrated by the crude maize extract, it is amazing that corn rootworms should choose to, and indeed, be able to feed on maize at all. Yet SCR larvae not only feed on the roots of maize, but will readily feed on germinating maize kernels, such behaviour was often observed during this work. It would therefore appear that the insect enzymes are shielded from or unaffected by the maize CPIs, or else these inhibitors are degraded rapidly during the initial stages of germination.

A protein extraction was prepared from maize kernels, following a similar method of the rice CPIs, assayed for inhibitory activity and analysed

by Western blotting, probing the filter with anti-ozcI antiserum. This immuno-blot analysis was conducted to examine the possible homology between the reported maize CPIs and ozcI. Crude samples of wheat and rice extracts were also analysed.

One or two bands of cross-reaction are apparent throughout the samples, occurring at around 25 to 26kDa. As these are not present uniformly across the blot, but vary with samples, they are not likely to be due to gel buffer contamination, but instead due to cross reaction with storage proteins, common to all three species, probably because of the presence of contaminating antisera, as has been discussed earlier in this chapter.

In the maize extract (0-65% ammonium sulphate fraction) a band was clearly visible at around 16kDa, corresponding to a band present in the germ and 30-65% fractions from rice. A faint band at 16kDa was also visible in the crude extract from maize. No such band was discernible in the crude wheat extract. Cross reaction of rice fractions has already been discussed. The apparent molecular weight of this maize protein coincides with the 15.5kDa molecular mass of the complete corn cystatin-I, provided the putative signal region were not removed (processing of this would produce a protein of around 12kDa). This cross reaction with the anti-ozcI antiserum indicated that a high level of homology was maintained between the proteins of the two species, and suggested, from the homology of the corn cystatin-I sequence and ozcI, that this cross reacting protein was either identical to the corn cystatin-I or a closely related protein. Because of the immuno-cross-reactivity and high level of sequence homology, it seemed most probable that this protein would possess similar inhibitory capabilities to those of ozcI, and as such would probably be an effective inhibitor of the larval CPs, *in vitro* if not *in vivo*. Interestingly, despite this cross reaction with the antiserum, no inhibition was demonstrated by the maize extract towards either papain or the insect enzymes. Two possible conclusions can

be drawn from this: that the cystatin-like protein from maize is inactive, despite the fusion protein produced by Abe *et al.* exhibiting strong papain inhibitory activity; or that the protein present in this extract is an inactive form, possibly requiring processing, such as the removal of a signal region of polypeptide, for activation. The inhibition demonstrated by the crude maize extract arises from the presence of activated CPIs, removed from the purer extract, or, as suggested with the wheat extract, may have been due to contaminating carbohydrates or other proteins competing for the enzyme active sites. Because of the lack of inhibition demonstrated in the purer extracts from wheat and maize, and the limited time available, no further investigation into the CPIs from these species was conducted.

CPI ACTIVITY *IN VITRO* FROM OTHER SOURCES - RESULTS

Enterolobin

Assay	Rate	% Inhibition	I.Units
40µg papain	1.10	-	-
+ 40µg enterolobin	0.81	26.3	0.263
40µg papain	1.25	-	-
+ 40µg enterolobin	0.92	26.4	0.264
+ 80µg enterolobin	0.82	34.4	0.172
+ 120µg enterolobin	0.72	42.4	0.141

Figure 55. Inhibition of papain by enterolobin. Assay mixtures consisted 40µg (1mg ml^{-1}) papain and 890µl (buffer plus inhibitor). Rate is given as the change in OD_{410nm} sec^{-1} $x10^4$. % inhibition was calculated as the decrease from the control, and I.Units (in inhibitory units per µg enterolobin) calculated using the formula (B x C/100)/A, where Aµg enterolobin inhibited Bµg papain by C%. Results represent single assays.

Enterolobin was assayed for inhibitory activity against papain, using BApNA as substrate. A low level of inhibition was demonstrated (figure 55), between 26 and 42%, but activity towards SCR enzymes was never investigated due to the high levels of inhibitor necessary, 120µg enterolobin was necessary to create a 42% reduction in the activity of 40µg papain. The inhibition units per µg enterolobin demonstrate that relative inhibitory power decreased markedly with increased inhibitor concentration.

TLCK

Inhibition of SCR larval cysteine protease activity by TLCK was confirmed using the substrate Z-Phe-Arg-AMC. 2µl larval gut extract were used per 20min assay and the effect of TLCK investigated by adding 2 or 4µg of 1mg ml^{-1} TLCK. Inhibition was calculated from assays containing no inhibitor, the results being corrected for autolysis of the substrate. Inhibition levels of 83% and 93% were demonstrated for 2µg and 4µg TLCK, respectively. No assay was conducted to demonstrate the inhibitory activity of TLCK towards papain.

CpTI

CpTI was also demonstrated to possess inhibitory activity against the insect enzymes (WCR adult extract) using the fluorimetric substrate Z-Phe-Arg-AMC. The protease assay was conducted as described above for TLCK inhibition, with the effect of CpTI investigated by adding 2µg of 1mg ml^{-1} CpTI to the assay mixture. Inhibition was calculated in the usual manner, a decrease in control activity of 37% produced, this amount of CpTI having no effect on an equivalent amount of papain activity.

The inhibitory activity of CpTI had also been assayed using aszocasein as a substrate, as reported in Chapter 3. 1.5gut equivalents of larval protease activity were inhibited by 10% by the addition of 5µg CpTI (6.7µg ml^{-1} in assay solution), an equivalent of 3.3µg CpTI per SCR larval gut

equivalent shown to cause a 10% decrease in the total protease activity, compared to the 37% inhibition of cysteine protease activity demonstrated by the equivalent of 50µg per WCR adult gut equivalent.

Thaumatin

Thaumatin was assayed for inhibitory activity towards larval digestive cysteine proteases and also for cysteine protease activity. 20min assays, using Z-Phe-Arg-AMC as substrate, were conducted with 2µl larval gut extract and 20µg or 50µg thaumatin, producing 4 and 16% inhibition of protease activity, respectively. 20µg thaumatin, when assayed alone, in the absence of papain or insect gut extract, displayed no cysteine protease activity as determined by the digestion of the substrate Z-Phe-Arg-AMC.

IN VITRO PROTEASE INHIBITION BY OTHER COMPOUNDS - DISCUSSION

Compounds fom various sources were tested for CPI activity effective against the rootworm digestive cysteine proteases, including: enterolobin, a protein isolated from the tissues of *Enterolobium contortisiliquum* (Sousa, 1991); the cowpea trypsin inhibitor, CpTI, previously demonstrated to cause adverse effects to the SCR *in vivo* (Gatehouse *et al.*, 1984); thaumatin, the homologue of the protein SB#10 isolated from rice in this work; and, although not a plant protein, TLCK (N-tosyl-L-lysine chloromethyl ketone), the trypsin inhibitor was also assayed.

Enterolobin

Enterolobin, a cytolytic protein isolated from the seed of *Enterolobium contortisiliquum* (Sousa, 1991) has been reported to exert a toxic effect on the larvae of *Callosobruchus maculatus* when applied incorporated in artificial seed (Sousa *et al.*, 1993). The protein was demonstrated to act as a weak inhibitor of papain activity, displaying a maximum inhibitory activity of only 0.26 inhibitory units per µg of inhibitor. Because only weak inhibition

was demonstration, even with quite high concentations of inhibitor, and because of the limited supply of enterolobin available, the activity of this protein against larval SCR enzymes was not assayed. The scarcity of the protein also prevented assaying its effects *in vivo* on larval SCR development.

TLCK

TLCK, as discussed in the introduction, is a selective inhibitor of serine proteases, capable of inhibiting trypsin but not chymotrypsin, although it is known to inhibit some cysteine proteases, such as papain, ficin and bromelain. Because of the differences demonstrated between papain and at least some of the enzymes of the insect gut, the inhibitory effects of TLCK were investigated. Inhibition of protease activity was demonstrated, over 90% of cysteine protease activity, as determined by the substrate Z-Phe-Arg-AMC, was susceptible to inhibition, indicating a surprising level of similarity in the susceptibilities of papain and the insect cysteine proteases to inhibition. It would have been interesting to test whether the activity detected by the digestion of Z-Arg-Arg-AMC was also susceptible to TLCK inhibition. Unfortunately this assay was not conducted.

Cowpea trypsin inhibitor

CpTI, the trypsin inhibitor from Cowpea, was found to show a substantial level of inhibitory activity towards the insect gut enzymes. No inhibition of papain was demonstrated, as was expected. Inhibition assays were conducted, 3.3µg CpTI causing 10% inhibition of one gut equivalent of SCR larval total proteases and 50µg CpTI causing 37% inhibition of one gut equivalent of WCR adult cysteine proteases, using the substrates azocasein and Z-Phe-Arg-AMC respectively. This demonstrates that the relative effectiveness of the inhibitor is greater as demonstrated using using SCR enzymes, and azocasein as substrate, the 15-fold increase in inhibitor used

in the Z-Phe-Arg-AMC (with WCR adult enzymes) causing only a 4-fold increased in inhibition. While this difference may be due in part to variation between the SCR larval and WCR adult enzymes, although such differences have been insignificant in the assays conducted thus far, it may result from the limited detection capabilities of the substrate Z-Phe-Arg-AMC, or may reflect a maximal level of inhibition by this inhibitor. The results are interesting in that cysteine proteases are demonstrated to be inhibited by CpTI, serine proteases, such as trypsin, demonstrating no ability to hydrolyse Z-Phe-Arg-AMC. Considering the similarity of papain and insect cysteine proteases to TLCK inhibition, it is surprising that as much as 37% of activity could be inhibited by a compound which has no effect on the activity of papain.

The low levels of serine protease activity demonstrated using BApNA as substrate, indicated that the reduction produced by CpTI, in total protease activity, as determined by azocasein digestion, must have been largely due to the inhibition of non-serine proteases, the results from the Z-Phe-Arg-AMC assays indicating that the activity inhibited was that of cysteine proteases. This inhibition again confirmed differences in enzyme type between papain and the various cysteine proteases of the insect gut.

Thaumatin

Thaumatin, the sweet tasting protein from *Thaumatococcus daniellii*, homologous to the first protein isolated and sequenced from rice in this work (SB#10), was assayed for inhibitory activity towards SCR larval cysteine proteases. Only weak activity was demonstrated, the greatest inhibition demonstrated was 16% produced by 50µg thaumatin, little compared to the high levels of inhibition caused by the rice CPIs. To determine whether this low level of inhibition was real, or due to endogenous cysteine protease activity in thaumatin masking the inhibition, assays were conducted with thaumatin in the absence of any enzyme sample. Such cysteine protease

activity was demonstrated by Cusack *et al.* (1987) in preparations of thaumatin, but no cysteine protease activity was measured here, using the substrate Z-Phe-Arg-AMC. These results, along with the later results from rice extractions indicated that the thaumatin-like protein sequenced from SB#10 was a co-purified protein, not the protein responsible for the major inhibitory activity. It is interesting, however, considering that this is the case, that any CPI activity was demonstrated at all by thaumatin, suggesting that the rice thaumatin-like protein may have exhibited some CPI activity.

CHAPTER 5 EXPRESSION OF OZCI IN *ESCHERICHIA COLI*

RESULTS

A construct (p231F26) encoding the complete sequence of the mature ozcI protein, with the 5'-noncoding region and intron removed, had previously been prepared (Gatehouse,L. unpublished work). Plasmid DNA was prepared from cells containing this construct, by the method of Birnboim and Doly (1979). Using *Pst*I digestion, the ozcI encoding region was isolated, and ligated into the multi-purpose cloning site of pUC8. The expression plasmid, shown in Figure 57, which maintained ozcI in frame with the *lacZ* gene (figure 56), was transformed into *E. coli* (DH5α) competent cells, producing a fusion protein consisting of 14 amino acid residues from the multiple cloning site of pUC8 N-terminal and 102 residues of the full length encoding sequence for ozcI.

M T M I T N S R G S V D L Q *M S S D* ...
ATG ACC ATG ATT ACG AAT TCC CGG GGA TCC GTC GAC CTG CAG *ATG TCG AGC GAC* ...

Figure 56. DNA and deduced amino acid sequence of the ozc expression plasmid, pHV, at the region of fusion between pUC8 and the 5' end of the ozc encoding sequence. The sequence starts at the initiation codon of the *lacZ* gene from pUC8. Bases and deduced amino acid residues derived from pUC8 are given in normal type, those from the ozc encoding sequence are given in italics. "..." denotes the continuation of the sequence.

The initial transformants were streaked onto YTampXgal plates and grown overnight at 37°C. Eight white colonies (5 true white and 3 blue-white), denoted pHV1 to pHV8, and a single blue colony (negative), pHV9, were picked off the plate, a sample streaked onto a grid on another plate to check for reversion, and the remaining cells grown overnight in 10ml YTamp. All the eight putative recombinant transformants were found to grow white

at the start of the streak, becoming pale blue further along the streak where the density of colonies was lower.

DNA was prepared from the nine cultures grown overnight and was restriction mapped with *Sac*I and *Eco*RI (Figure 57). This confirmed the size and correct orientation of the insert in four out of the eight putative recombinant transformants, pHV1, pHV2, pHV4 and pHV5. High yields of DNA were recovered from pHV2 and pHV4.

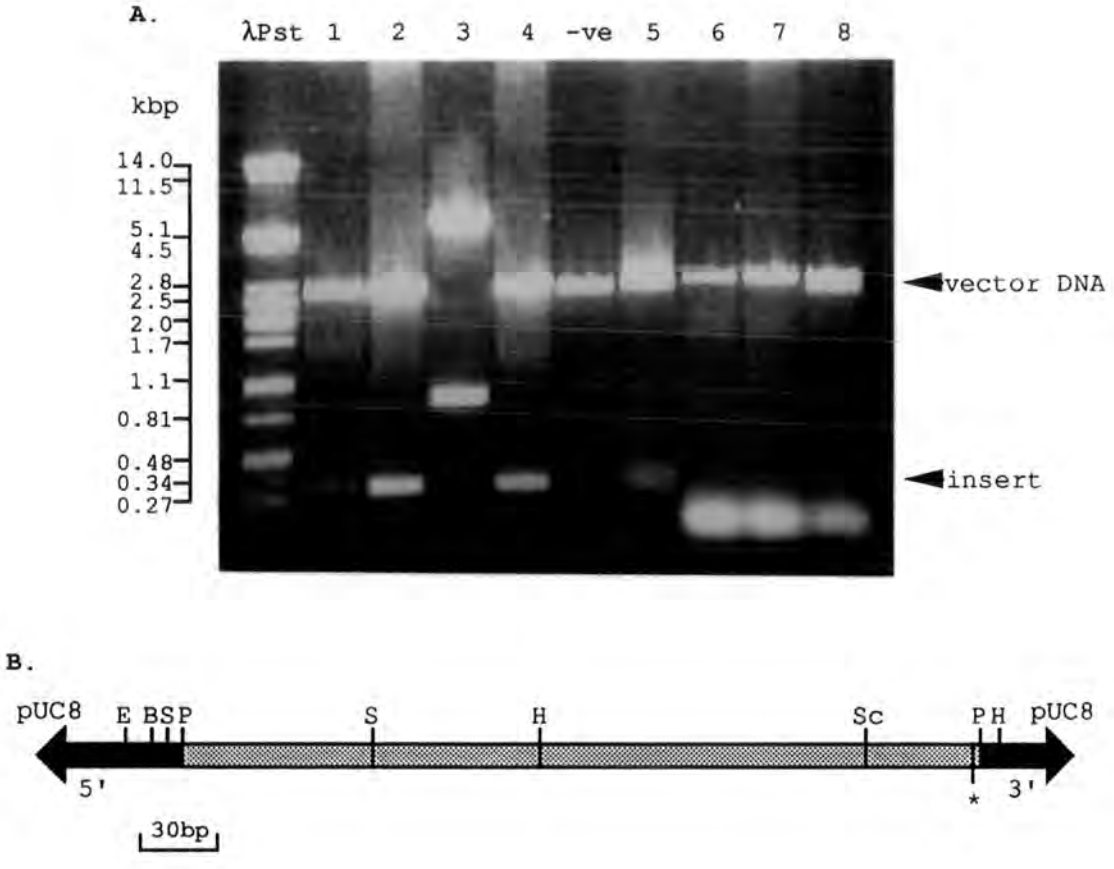


Figure 57. Panel A, Agarose gel of restriction digested pHV transformants using *Sac*I and *Eco*RI. Lanes 1-8, transformants pHV1-pHV8; lane "-ve", pHV9 (negative), and λ*Pst*, standard marker. Panel B, restriction map of pHV, showing the area of insert in pUC8. Restriction enzymes are abbreviated as: E, *Eco*RI; B, *Bam*HI; S, *Sal*I; P, *Pst*I; H, *Hind*III; Sc, *Sac*I. The ozcI encoding insert is shown patterned, and pUC8 in black. * indicates the stop codon.

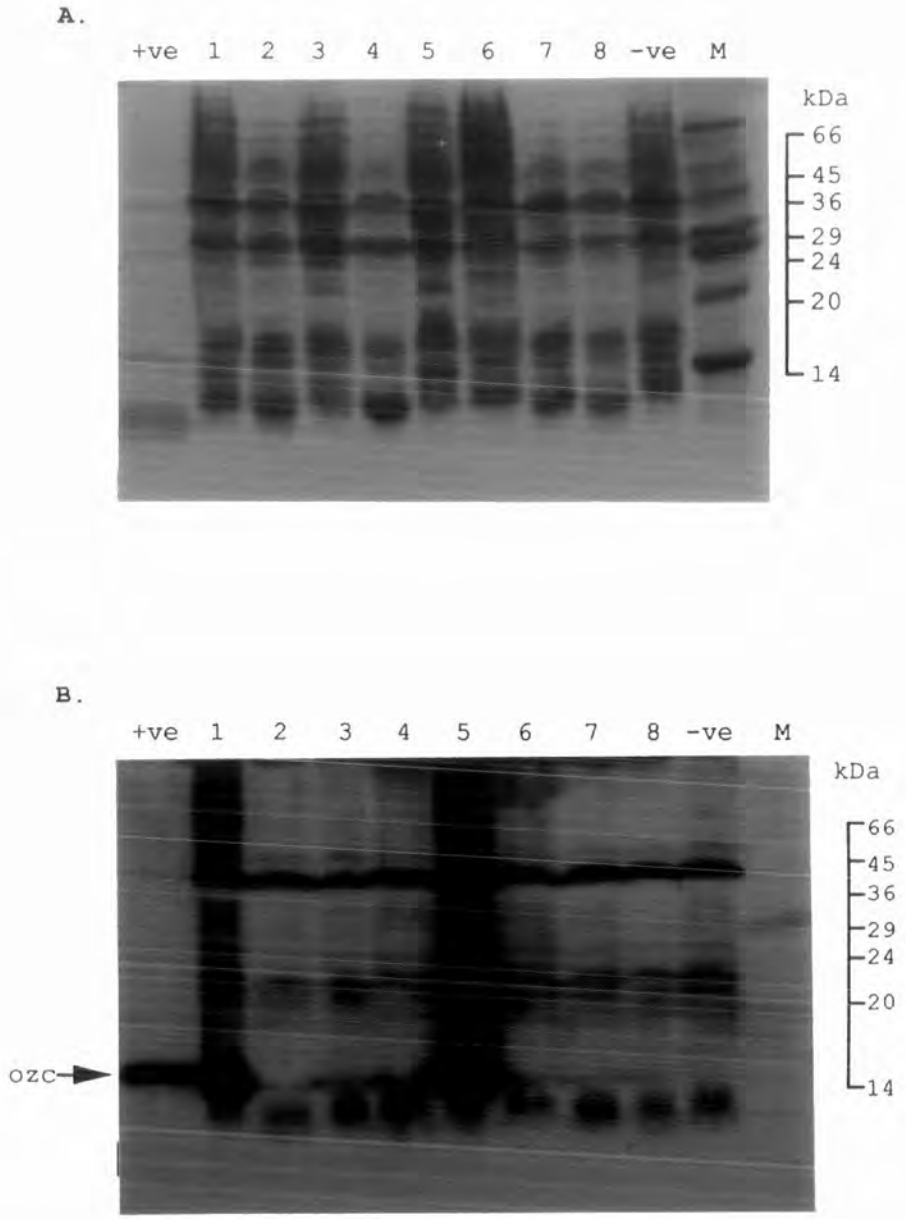


Figure 58. SDS-PAGE (panel A) and western blot (panel B) analysis of protein extracts from transformed cells. Lanes 1-8, pHV1-pHV8; lane "+ve", SQRG-I, the partially purified ozcI from rice seed; lane "-ve", pHV9, the negative control; and lane M, molecular weight markers. Anti-ozcI antiserum was employed for antigen detection of western blot.

Cells containing the nine transformants were grown overnight in YTamp, harvested and crude protein extracts prepared from the pelleted cells by resuspension and boiling in 1x SDS sample buffer. Cell debris was removed by centrifugation. Samples from the supernatants were subjected to analysis by SDS-PAGE and western blotting, using anti-ozcI antiserum for detection. SQRG-I (crude ozcI sample from rice, see Chapter 4) was used as a positive control and pHV9 as a negative control. The immuno-blot (Figure 58) showed high levels of cross reaction with bacterial proteins (or aggregated or processed ozc), but confirmed the presence of the recombinant ozcI (Rozc) in the tracks of pHV1, pHV4 and pHV5, the highest apparent expressor being pHV5. Rozc, from its mobility on gel, had an apparent Mr of around 15kDa.

pHV1, pHV2, pHV4 and pHV5 were again streaked out onto an YTampXgal agar plate and grown overnight. All grew white, but with some blue colouration also. White colonies from each transformant were picked from this plate, streaked onto a second, and again allowed to grow overnight. Every one of the streaks on this plate grew with a pale blue colouration. To test whether the plasmids in these cells were actually losing their inserts, blue colonies from each streak were grown overnight in YTamp medium, and the crude protein extracts prepared from the cultures analysed by SDS-PAGE and Western blotting. pHV9 was employed as a negative control and SQRG-I as positive control. Both gel and blot demonstrate clearly the presence of Rozc in all of the four samples (Figure 59). All samples appeared to contain similar amounts of Rozc, indicating that these transformants are expressing Rozc to approximately the same levels. The apparent inconsistency of DNA yields and protein levels obtained here is most probably due to variance in the cell concentration of cultures used for preparations.

pHV5 (now denoted pHEV1(ozc)) was picked as possibly the highest expressor. Plasmid DNA prepared from this transformant was sequenced in

both directions and the nucleotide sequence obtained compared to that published by Abe *et al.* (1987a). The sequences obtained confirmed the fusion and showed the insert sequence to be identical to that published for ozcI. pHEV1(ozc) was stored as glycerol at -80°C and this transformant used in all future preparations of Rozc.

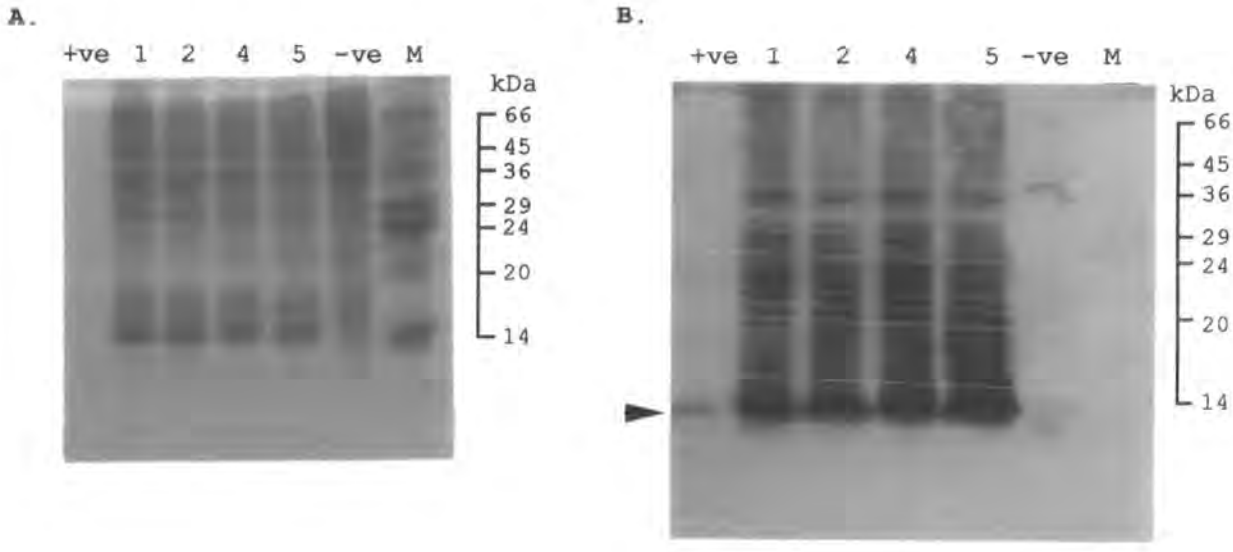


Figure 59. SDS-PAGE (panel A) and western blot (panel B) analysis of protein extracts from transformed cells. Lanes 1, 2, 4 and 5, extracts from pHV1, pHV2, pHV4 and pHV5, respectively; lane "+ve", SQRG-I, the impure ozcI sample from rice seed; lane "-ve", pHV9, the negative control; and lane M, molecular weight markers. Anti-ozcI antiserum was used in western blot analysis. The position of Rozc is indicated with an arrow.

Purification of Rozc from E. coli

Two 1L cultures of pHEV1(ozc) cells were prepared, cells harvested by centrifugation, resuspended in 10mM Tris pH8.0, 2mM EDTA and disrupted by sonication. After removal of cell debris, the supernatant was subjected to heat treatment (82°C for 15min). This heat treatment step was found necessary as, in initial extractions, proteins of 12-13kDa were found to

appear and increase in concentration throughout the purification procedure, indicating that proteolytic degradation of Rozc was occurring, reducing the purity of the sample substantially. Abe *et al.* (1988) employed a step of heating in a boiling water bath for 15min, this was tried but proved too fierce as the majority of Rozc was irretrievably lost into the precipitate. Extractions using the 82°C water bath heat treatment step produced samples of Rozc free from contaminating proteolytic fragments.

Following heat treatment, the supernatant was fractionated by ammonium sulphate precipitation. In an initial extraction the precipitates from 0-30% and 30-65% saturation with this salt were collected, according to the method used for the extraction of CPI from rice seed. Following resuspension and dialysis against 20mM Tris pH8.0, the presence of Rozc was demonstrated in both 0-30% and 30-65% precipitated fractions by SDS-PAGE (results not shown) and Z-Phe-Arg-AMC assays (Figure 60). These enzyme assays also confirmed the biological activity of the recombinant protein, the two samples causing marked inhibition of both papain and larval CP activity. Accordingly, in ensuing preparations, the precipitate formed between 0 and 65% saturation with ammonium sulphate was collected. Figure 61 shows the extent of Rozc separation from bacterial proteins at the various stages of the purification.

Assay	% inhn
4µl 0-30% fraction	95.5
8µl 0-30% fraction	96.3
4µl 30-65% fraction	91.8
8µl 30-65% fraction	95.5
0.5µg cystatin	100.0

Figure 60. Inhibition of larval SCR digestive cysteine proteases by ammonium sulphate precipitated fractions from *E. coli* extract. Inhibition is given as the percent decrease from uninhibited activity. 2µl larval gut extract (0.047 gut equivalents) was used per 10min assay.

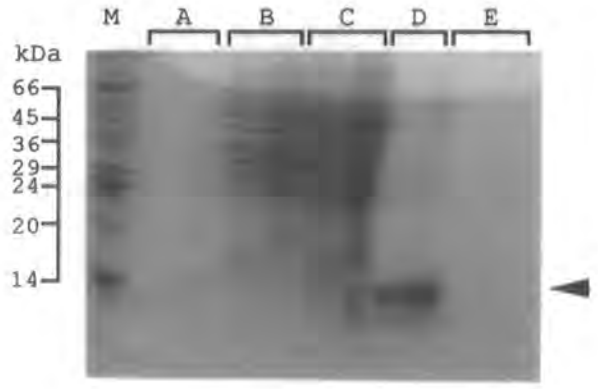


Figure 61. SDS-polyacrylamide gel of Rozc purification steps. Protein samples taken from the following fractions: A, culture supernatant after cell harvesting (concentrated by ammonium sulphate precipitation (0-65% saturation)); B, cell debris following sonication; C, heat-treatment precipitated proteins; D, 0-65% ammonium sulphate fraction; E, proteins remaining in solution at 65% saturation with ammonium sulphate; and M, molecular weight markers. The position of Rozc is indicated.

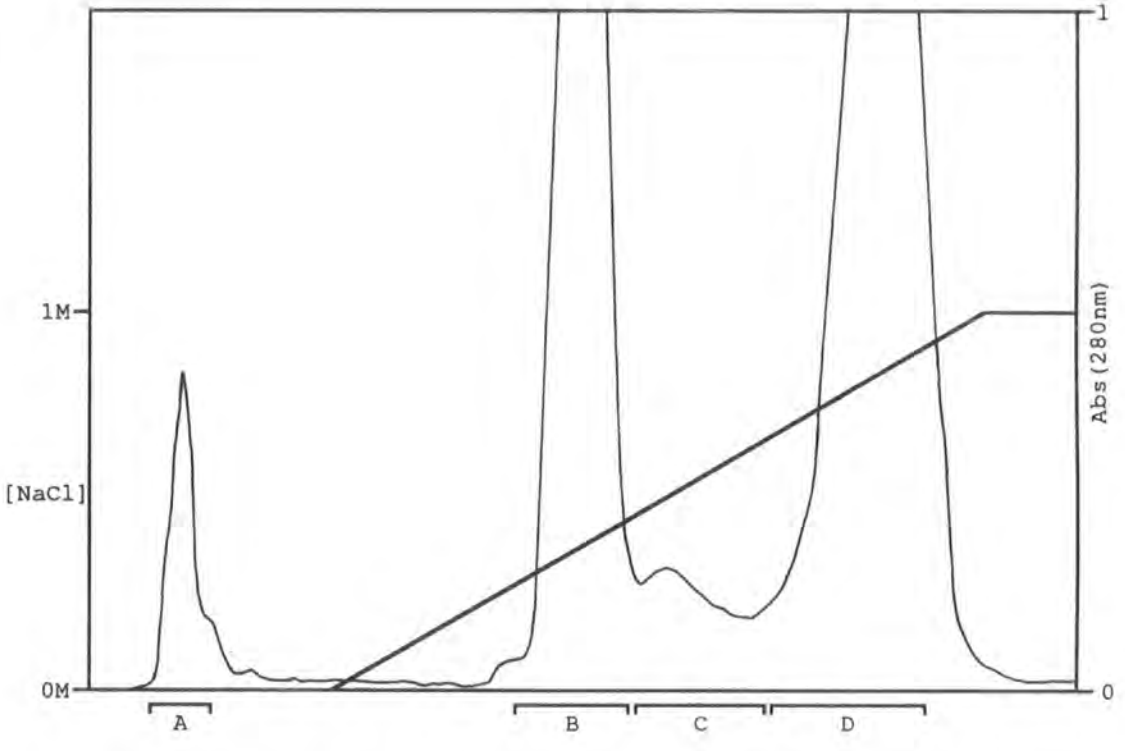


Figure 62. Profile of *E. coli* extract separation achieved by Q-sepharose chromatography. Pooled fractions are indicated.

After exhaustive dialysis against 20mM Tris pH8.0, the Rozc containing fraction was subjected to Q-sepharose chromatography, using this buffer with a linear gradient from 0 to 1M NaCl. The resultant elution profile showed 2 major (B and D) and two minor (A and C) peaks, as shown in Figure 62. Western blot analysis revealed that peak B contained high levels of Rozc, with lower levels being detected in the other peaks (Figure 63). Rozc in fraction B appears to have been purified to homogeneity, as demonstrated by the single band visible on stained SDS-polyacrylamide gel. From the western blot, however, it was obvious that some polymerisation of Rozc had occurred, dimers being apparent on the blot, although these were not visible on the stained gel, indicating the small proportion of Rozc polymerised.

These fractions were also assayed for inhibitory activity towards papain and larval CPs using the substrate Z-Phe-Arg-AMC. Almost complete inhibition of both enzyme samples was demonstrated by fraction B (see Figure 64). Inhibition of larval CPs was also caused by fraction C. Production and purification by this method yielded around 126mg of essentially pure, biologically active Rozc from 2litres of culture.

Fraction	LgE	Papain
A	15%	8%
B	100%	97.5%
C	83%	13%
D	0%	0%

Figure 64. Inhibitory activity of Rozc towards papain and larval gut CPs. Percentages of uninhibited digestion are given. A-D represent the peaks derived from Q-sepharose chromatography.

Effect of Rozc inhibitory activity *in vitro*

Characterisation of the inhibitory properties of Rozc was conducted employing identical methods as were used to examine enzyme:inhibitor

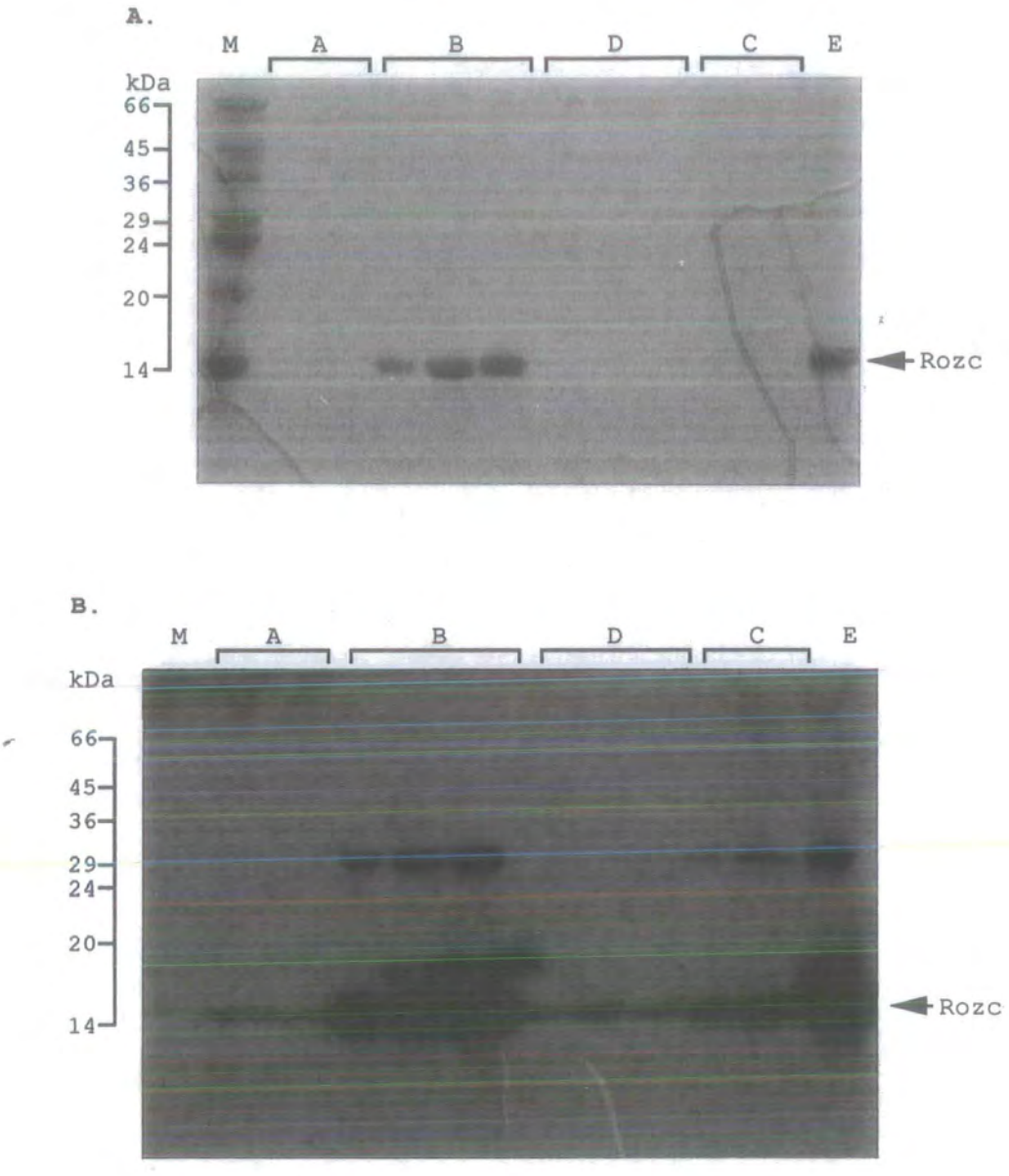


Figure 63. SDS-PAGE (panel A) and western blot (panel B) analysis, using anti-ozcI antiserum, showing the Rozc content of protein peaks produced by anion exchange chromatography. Lanes A-D, peaks A-D as shown in Figure 62; lane E, Rozc preparation prior to chromatography; lane M, molecular weight markers. Bands detected off-line in lanes B (panel B) result from gel movement prior to transfer of proteins onto nitrocellulose filter. Samples were dialysed after chromatography, diluted 1x in 2xSDS sample buffer, and loaded (left to right): A and C at 5µl and 15µl; B and D at 2µl, 5µl and 10µl; and E at 5µl.

After exhaustive dialysis against 20mM Tris pH8.0, the Rozc containing fraction was subjected to Q-sepharose chromatography, using this buffer with a linear gradient from 0 to 1M NaCl. The resultant elution profile showed 2 major (B and D) and two minor (A and C) peaks, as shown in Figure 62. Western blot analysis revealed that peak B contained high levels of Rozc, with lower levels being detected in the other peaks (Figure 63). Rozc in fraction B appears to have been purified to homogeneity, as demonstrated by the single band visible on stained SDS-polyacrylamide gel. From the western blot, however, it was obvious that some polymerisation of Rozc had occurred, dimers being apparent on the blot, although these were not visible on the stained gel, indicating the small proportion of Rozc polymerised.

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Effect of Rozc inhibitory activity *in vitro*

Characterisation of the inhibitory properties of Rozc was conducted employing identical methods as were used to examine enzyme:inhibitor

interactions with E64 and cystatin. The results obtained with Rozc are, therefore, directly comparable to these other inhibitors.

Inhibition of papain and larval cysteine protease hydrolysis of the substrate Z-Phe-Arg-AMC by Rozc, cystatin and E64 is shown in figures 65 and 66. The similarity of the inhibitory behaviour of Rozc and cystatin is striking. These inhibitors gave almost complete inhibition of papain at 15 and 9pmoles inhibitor per assay, respectively, and caused complete inhibition of the larval gut protease activity at 9 and 4pmoles inhibitor per assay, respectively. While 9pmoles cystatin caused almost complete inhibition of papain activity, 9pmoles Rozc produced only 70% inhibition. With the insect cysteine proteases, complete inhibition was caused by 4pmoles cystatin, and this amount of Rozc reduced proteolytic activity by 77%.

Inhibition assays employing Z-Arg-Arg-AMC as substrate, again demonstrated the comparable inhibitory activity of Rozc and cystatin (Figure 67). 4pmoles cystatin (per assay) caused 60% inhibition of larval cysteine protease activity, greater amounts caused little further decrease in proteolysis. 4pmoles Rozc gave around 50% inhibition of larval gut cysteine protease activity, greater amounts causing only slightly higher levels of inhibition.

The influence of pH on the inhibitory activity of Rozc was also investigated, employing the same methods as used to assess the effect of pH on enzyme activity, with the addition of 50ng Rozc (3.7nM) per assay. Controls with no inhibitor were used to calculate percent inhibition at each pH. The results, illustrated in figure 68, show that inhibitory activity responds to pH in a similar way to enzyme activity. Between pH4 and 6, where protease activity is lower, inhibition caused by Rozc is between 20 and 40%. From pH6 to 8, when the uninhibited enzyme activity is higher, the inhibitory power of Rozc ranges from 40 to 65%.

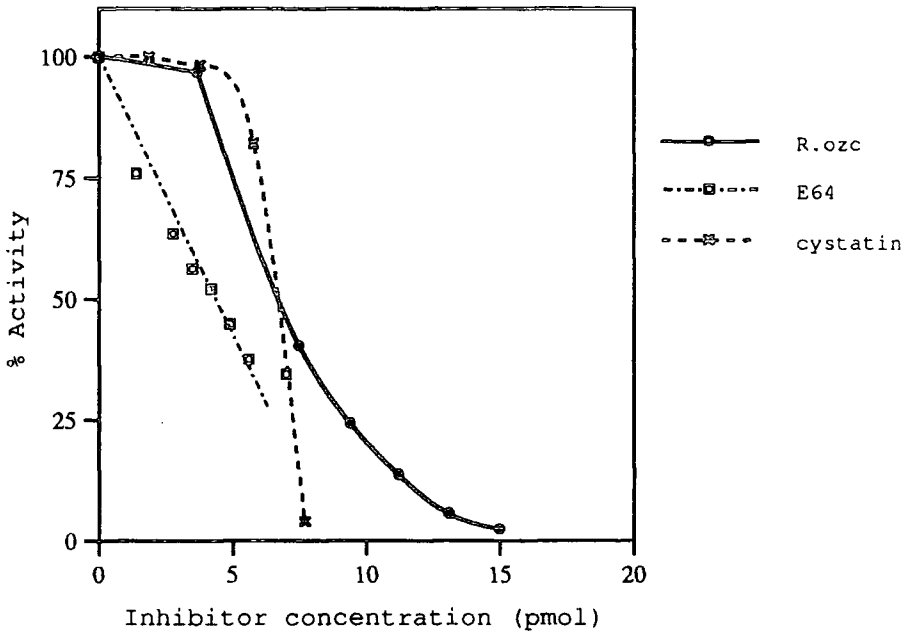


Figure 65. Inhibition of papain activity by Rozc, and comparison to inhibition by E64 and cystatin. 0.1µg papain was used per 5min assay. Z-Phe-Arg-AMC was employed as a substrate. Remaining activity is given as a percentage of the uninhibited activity.

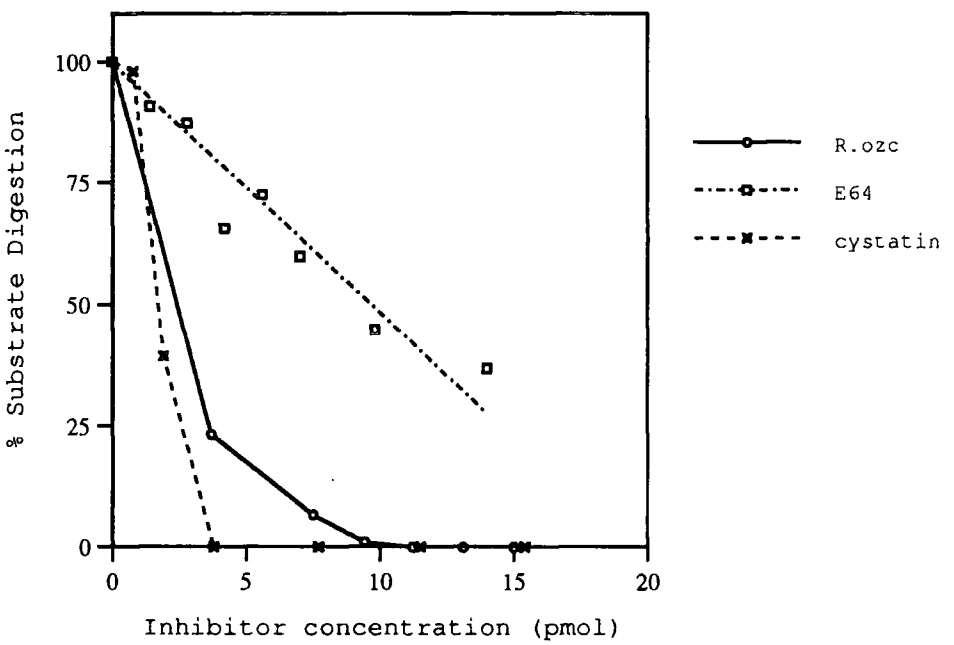


Figure 66. Inhibition of larval gut enzyme by Rozc, and comparison with E64 and cystatin inhibition. 0.2µl gut extract (0.0047 gut equivalents) were used per assay. Digestion is given as a percentage of uninhibited enzyme activity, using the substrate Z-Phe-Arg-AMC.

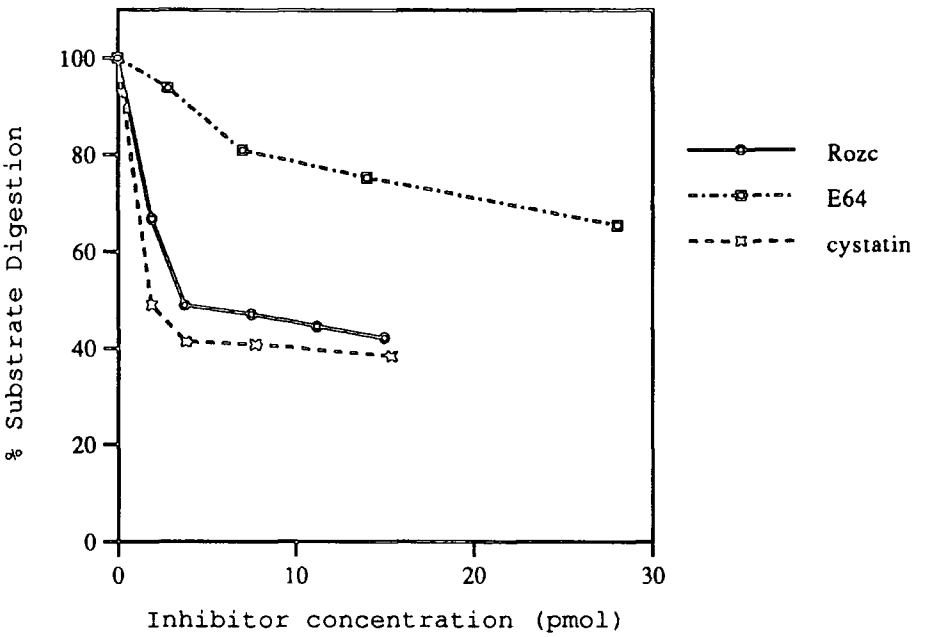


Figure 67. Inhibition assays showing the inhibitory activities of Rozc, E64 and cystatin against larval gut proteases, as detected with the substrate Z-Arg-Arg-AMC. Activity is given as a percentage of uninhibited activity. 0.2µl gut extract (0.0047gut equivalents) were used per 5min assay.

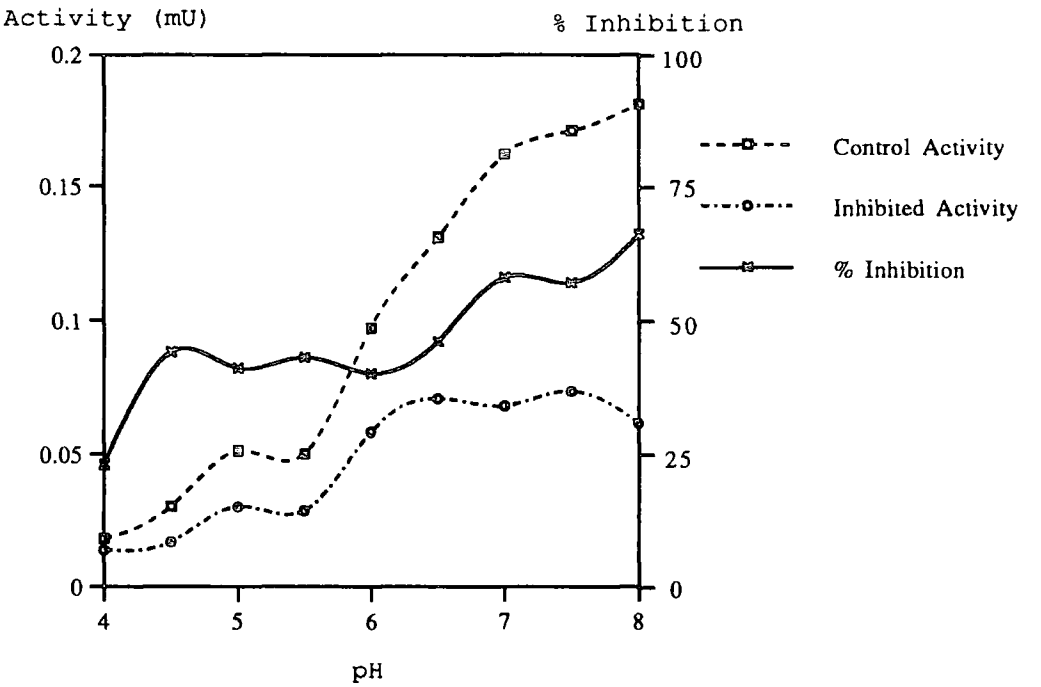


Figure 68. The influence of pH on Rozc inhibitory activity. Dashed lines represent uninhibited and inhibited enzyme activities, as detected by digestion of Z-Phe-Arg-AMC. 0.2µl larval gut extract (0.0047 gut equivalents) and 3.75pmol Rozc were used per 5min assay.

EXPRESSION OF OZCI IN *E. COLI* - DISCUSSION

OzcI is present at very low concentrations in the seeds of rice, kilograms of rice seed yielding only microgram amounts of ozcI. Due to the relatively large amounts of protein required for extensive bioassays and characterisation of ozcI's inhibitory specificities towards insect enzymes, coupled with the difficulty of its purification, isolation of sufficient material from rice seed was not completely viable. Recombinant ozcI was successfully expressed in *E. coli* using pUC8 as the expression vector. The construct encoded a simple fusion protein with 14 amino acid residues N-terminal, originating from the polylinker of pUC8, and 102 residues of the full length ozcI encoding sequence, the biological activity of the inhibitor apparently being fully retained.

Four of the original eight putative recombinant transformants were demonstrated to express Rozc. Of these four, picked originally as white (positive) colonies following transformation, all were subsequently found to grow pale blue. The simple blue/white screening method used to select recombinant transformants, utilises the fact that the plasmid's *lacZ* gene is disrupted by the presence of an insert, allowing easy identification of recombinant transformants, when grown on media containing Xgal, by their white (colourless) colonies (Old & Primrose, 1985). Cells containing the expression plasmid, in this case pUC8, and no insert, will form blue colonies on Xgal media. The reversion of colonies from white to blue, suggests the plasmids in these cells are losing their inserts. The confirmation of Rozc expression in these pale blue colonies, demonstrated that no such loss was occurring.

Restriction mapping, SDS-PAGE, Western blot analysis, and papain inhibition assays were used to confirm the success of the transformation and the expression of a biologically active protein. From the four recombinant transformants, pHV5, now denoted pHEV1(ozc), was picked as possibly the highest expressor of the recombinant protein. The construction and

successful manipulation was ultimately confirmed by sequencing the insert in this transformant in both directions and comparing this sequence data with the published nucleotide sequence of a cDNA clone for ozcI (Abe *et al.*, 1987a). pHEV1(ozc), stored in glycerol, was used subsequently in all preparations of Rozc.

Expression of ozcI in *E. coli* by other groups

Expression of ozcI in *E. coli* has been reported by Abe *et al.* (1988) and Chen *et al.* (1992b). The various fusion proteins produced are illustrated in Figure 69. Abe *et al.* (1988) expressed ozcI cDNA and various truncated fragments in *E. coli*, producing 6 different fusion proteins, all but two of which retained their normal papain inhibitory activities and specificities. Activity of the fusion proteins lacking the N-terminal 21 amino acid residues from ozcI was found to be unaffected, as were those lacking the C-terminal 11 residues, hence indicating these residues to be nonessential for normal papain inhibitory activity. However, when 38 amino acids of ozcI were missing from the C-terminus, inhibitory activity was dramatically reduced, and the lack of 35 N-terminal amino acid residues of ozcI rendered the resultant fusion protein completely inactive. Inhibitory activity of the four fully active fusion proteins was found to be comparable to that of wild-type ozcI.

The sequences of cystatins are characterised by the conservation of certain residues, thought to be involved in their inhibitory activity. The sequence Gln-X-Val-Y-Gly (Gln^{53}-Val^{54}-Val^{55}-Ala^{56}-Gly^{57} in ozc) is most highly conserved (Barrett, 1987; Turk & Bode, 1991). Experiments using cassette or site-directed mutagenesis to produce variants in the Gln-X-Val-Y-Gly region have demonstrated the importance of this region for the inhibitory activity of ozcI (Arai *et al.*, 1991) and chicken egg white cystatin (Auerswald *et al.*, 1992), the inhibition of some enzymes being markedly affected by only small alterations in this region.

```
                                                          10         20
OzcI                                                MSSDGGPVLGGVEPVGNENDLHLVD
Rozc                                          MTMITNSRGSVDLQMSSDGGPVLGGVEPVGNENDLHLVD
ppPOC26-5'-1                  MITNSSSVPGDQFRAE*AHRAGGEGEEK*MSSDGGPVLGGVEPVGNENDLHLVD
ppPOC26-5'-22                                       MITNSSSVPGDPLESHLVD
ppPOC26-5'-22G                                      MITNSSSVPDPLESHLVD
ppPOC26-5'-39
ppPOC26-3'-91                 MITNSSSVPGDQFRAE*AHRAGGEGEEK*MSSDGGPVLGGVEPVGNENDLHLVD
ppPOC26-3'-67                 MITNSSSVPGDQFRAE*AHRAGGEGEEK*MSSDGGPVLGGVEPVGNENDLHLVD
ppT7OC 9b (ROC)                                                   MARIRARGSSRVD
ppKKOC 9b                                           MSSDGGPVLGGVEPVGNENDLHLVD

                        30         40         50         60         70
OzcI                  LARFAVTEHNKKANSLLEFEKLVSVKQQVVAGTLYYFTIEVKEGDAKKLYEA
Rozc                  LARFAVTEHNKKANSLLEFEKLVSVKQQVVAGTLYYFTIEVKEGDAKKLYEA
ppPOC26-5'-1          LARFAVTEHNKKANSLLEFEKLVSVKQQVVAGTLYYFTIEVKEGDAKKLYEA
ppPOC26-5'-22         LARFAVTEHNKKANSLLEFEKLVSVKQQVVAGTLYYFTIEVKEGDAKKLYEA
ppPOC26-5'-22G        LARFAVTEHNKKANSLLEFEKLVSVKQQVVAGTLYYFTIEVKEGDAKKLYEA
ppPOC26-5'-39          MITNSSSVPGDPNSLLEFEKLVSVKQQVVAGTLYYFTIEVKEGDAKKLYEA
ppPOC26-3'-91         LARFAVTEHNKKANSLLEFEKLVSVKQQVVAGTLYYFTIEVKEGDAKKLYEA
ppPOC26-3'-67         LARFAVTEHNKKANSLLEFEKLVSVKQQVVAGTLYYFTIEVKGLAS
ppT7OC 9b (ROC)       LARFAVTEHNKKANSLLEFEKLVSVKQQVVAGTLYYFTIEVKEGDAKKLYEA
ppKKOC 9b             LARFAVTEHNKKANSLLEFEKLVSVKQQVVAGTLYYFTIEVKEGDAKKLYEA

                       80         90        100
OzcI                  KVWEKPWMDFKELQEFKPVDASANA
Rozc                  KVWEKPWMDFKELQEFKPVDASANA
ppPOC26-5'-1          KVWEKPWMDFKELQEFKPVDASANA
ppPOC26-5'-22         KVWEKPWMDFKELQEFKPVDASANA
ppPOC26-5'-22G        KVWEKPWMDFKELQEFKPVDASANA
ppPOC26-5'-39         KVWEKPWMDFKELQEFKPVDASANA
ppPOC26-3'-91         KVWEKPWMDFKELQVAS
ppPOC26-3'-67
ppT7OC 9b (ROC)       KVWEKPWMDFKELQEFKPVDASANA
ppKKOC 9b             KVWEKPWMDFKELQEFKPVDASANA
```

Figure 69. Amino acid sequences of ozcI (Abe *et al.*, 1987a), Rozc and the published fusion proteins of Abe *et al.* (1988), denoted pPOC26-5'-1 through pPOC26-3'-67, and Chen *et al.* (1992b), denoted ppT7OC 9b (ROC) and ppKKOC 9b. Amino acid residues noted in bold type were derived from the vector, those printed in italics originate from the 5'-noncoding region of ozcI. Numbering is according to the wild-type ozcI.

There is also evidence for a role in activity by the amino terminus of the molecule (Bode *et al.*, 1988). There is much variability in this region of the cystatins, but one particular glycine residue (Gly^9 of chicken egg white cystatin (Barrett, 1987); Gly^5 (Abe *et al.*, 1988) or Gly^{10} (Turk & Bode, 1991) in ozc) is conserved in all of the inhibitory cystatins (Barrett, 1987; Turk & Bode, 1991). It is surprising then, that the lack of the 21 N-terminal residues (containing Gly^5 and Gly^{10}) from fusion proteins ppPOC26-5'-22 and ppPOC26-5'-22G had no apparent effect on their inhibitory activities. The group concluded that despite this glycine residue being one of the most conserved residues in the cystatins, it was not essential for the papain inhibitory activity of ozc, rather the crucial residues were elsewhere on the polypeptide.

Loss of activity was demonstrated when then protein lacked 35 residues from the N-terminus or 38 residues C-terminal, indicating that the central region of the polypeptide containing the conserved Gln-Val-Val-Ala-Gly sequence was not sufficient to produce inhibition of papain. This suggests that other structural features of the protein are also of importance for activity. Recently, the structure of two cystatins has been revealed by X-ray analysis, and three contact regions demonstrated in complexes with papain (Bode *et al.*, 1988; Stubbs *et al.*, 1990; reviewed by Bode & Huber, 1992). These three contacts consist of the N-terminal region containing the conserved Gly^9 residue, an exposed ß-hairpin loop (comprising the conserved Gln-X-Val-Y-Gly sequence) and a second hairpin loop. If the inhibitory activity of cystatins is dependant on the formation of all three contacts, this would explain the loss of inhibition demonstrated with the ozc fusion proteins ppPOC26-5'-39 and ppPOC26-3'-67, as here either the N-terminal region or the second loop would be absent. The retention of activity by fusion proteins lacking the 21 N-terminal residues may result from these residues being replaced by the 14 or 15 residues originating from the vector. If this were the case then one of the contacts, that covering the

enzyme's active centre, is actually made by the region of fusion protein originating from the vector, demonstrating a remarkable resilience on the part of ozc.

Chen *et al.* (1992b), expressed cDNA clones of ozcI in *E. coli* using the vectors pKK233-3 and pT7-7. Two expression constructs were produced, pKKOC 9b encoding the entire amino acid sequence of ozcI with no residues originating from the vector, and pT7OC 9b, encoding a fusion protein containing 11 amino acid residues N-terminal originating from the vector and 79 amino acids of the C-terminus of ozcI, lacking 23 residues of the wild type ozcI. Expression of the non-fusion ozcI with the construct pKKOC 9b was extremely low, a small amount of inhibitory activity was demonstrated, but no protein was detectable by SDS-PAGE. pT7OC 9b was successfully used to express recombinant truncated ozcI (ROC), a protein of around 10kDa, at levels 10% of the total bacterial protein mass. The inhibitory activity of ROC was demonstrated to be comparable with that of ozcI and chicken cystatin against papain activity in assays using [^{3}H]casein as substrate. Inhibition of papain by E64 was demonstrated to be approximately 3-fold stronger than ROC or chicken cystatin.

<u>The inhibitory activity of Rozc</u>

Characterisation of the inhibitory activity of Rozc, the recombinant ozcI produced in this work, revealed a high level of activity towards papain, showing a pattern comparable to that of chicken egg-white cystatin. Inhibition by E64 was higher than that achieved with Rozc. No direct comparison could be made between Rozc activity and native ozcI, as no pure sample of this protein was available, however, the behavioural analogy between Rozc and cystatin, and the contrast with E64 inhibition, indicate that the fusion protein does exhibit the normal ozcI behavioural characteristics. These results are in general agreement with those of Chen

et al. (1992b), cystatin the recombinant ozcI causing similar inhibition levels with papain, while E64 proved a stronger inhibitor.

The effect of Rozc on insect enzymes *in vitro*

In vitro inhibition assays were carried out with enzyme extracts from larval SCR (0.0047 gut equivalents per 1ml assay). These assays again indicated a behavioural parallel with cystatin. Like cystatin, Rozc strongly inhibits enzyme activity, around 80% inhibition being achieved by Rozc at 3.7nM (approximately 0.79µM Rozc per larval gut equivalent). Total inhibition of activity was caused by this concentration of cystatin, whereas with this amount of E64 only around 20% inhibition occurred. The strength of the inhibition caused to these enzymes by cystatin and Rozc is remarkable, especially in comparison with E64 inhibition. Again because of the similarity of Rozc inhibition with that of cystatin, it must be assumed that this activity is essentially identical to that of wild-type ozcI.

Investigations into the influence of pH on the inhibitory power of Rozc demonstrated that inhibitory activity increased between pH4 and pH8. Interestingly this enhancement corresponded roughly to the increase observed in enzyme activity with larval gut cysteine proteases, demonstrating a surprising suitability of this inhibitor for use against these insect enzymes.

In vitro insect assays were also conducted by Chen *et al.* (1992b). Enzyme extracts (0.01-0.02 midgut equivalents per 100µl assay) were taken from 7 species of stored product pest. Gut protease activity from rice weevil, *Sitophilus oryzae*, was inhibited by 80% at around 5µM ROC in assay solution, at which point an apparent maximum level of inhibition was reached. Similar apparent maximum levels of inhibition were demonstrated by ROC with midgut proteases from the confused flour beetle, *Tribolium confusum*, the red flour beetle, *Tribolium castaneum* and the cadelle beetle, *Tenebriodes mauritanicus*. PMSF (0.1mM), the serine protease inhibitor, gave a maximum

inhibition level of around 20% with all four enzyme extracts. In each case almost complete inhibition could be obtained where both ROC and PMSF were used. ROC inhibition of protease activities from midgut extracts of the dark mealworm, *Tenebrio obscurus*, the yellow mealworm, *Tenebrio molitor* and the Indian meal moth, *Plodia interpunctella*, reached their apparent maximums at only 39%, 31% and 18%, respectively. This lower level of inhibition is due to a lower ratio of cysteine protease to serine protease activity found in these three species, as confirmed by the higher levels of PMSF inhibition demonstrated.

These results are in agreement with an earlier publication from this group (Liang *et al.*, 1991), which reported that 20μM wild-type ozcI would inhibit the enzymes of rice weevil and the red flour beetle by 88 and 90% respectively, employing the same assay as above. This substantiates the group's conclusions that ROC acted in an essentially identical manner to that of the wild-type protein.

Both the findings of these American workers and those reported in this work, indicate that for insect species where cysteine proteases are employed as the major digestive proteases, ozcI may present a useful tool for defence against their attack. It must be recognised that while all these results are from *in vitro* studies, investigating the effects of inhibitors on the activity of extracted enzymes, these effects, as discussed earlier (see Chapter 1), may be very different to those seen *in vivo*, where shielding of enzymes or denaturation of inhibitors may be occur.

It is interesting that all the attention thus far in the published reports of cysteine protease inhibitors as defence against insects has been concentrated on protection against stored product pests, such as the flour beetles, as is demonstrated by the above papers. A similar application to the improvement of resistance to insects, such as the rootworms, is perfectly

logical, given that cysteine proteases have been shown to play such a principal role in their digestion of dietary proteins (see Chapter 3).

Production of Rozc for bioassays

The successful production of large quantities of recombinant ozc, confirmed to be biologically active and shown to exhibit comparable behavioural characteristics to ozc, overcomes the problems of low ozc yields obtained from rice seed. The quantities of ozc required for bioassays were almost impossible to achieve from rice seed, but such amounts of Rozc can be readily produced.

CHAPTER 6 PLANT α-AMYLASE INHIBITORS *IN VITRO*

RESULTS

Wheat 0.28 α-amylase inhibitor, WAAI, was prepared by the method of Kashlan and Richardson (1981) from *Triticum aestivum* variety Slejpner. Figure 70 shows several preparations of WAAI on SDS-polyacrylamide gel, electrophoresed in the presence of ß-mercaptoethanol. Two main bands are clearly visible at 15-16kDa in the samples containing WAAI.

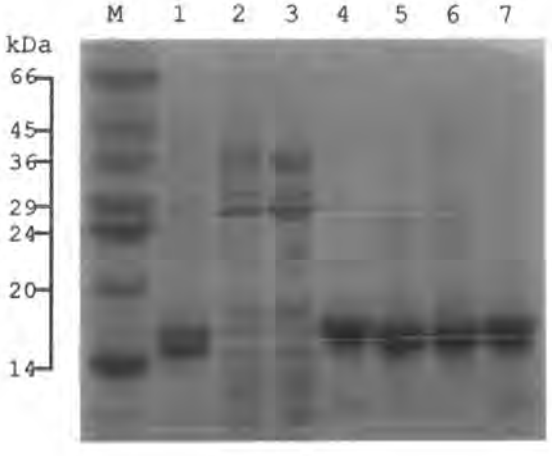


Figure 70. Analysis of seven preparations of WAAI using SDS-PAGE. Lanes 1 and 4-7, extracts containing WAAI as the major component; lanes 2 and 3, extracts not containing WAAI; lane M, molecular weight markers. All extracts were loaded at 10µl of 2mg/ml.

Bean α-amylase inhibitor, BAAI, was prepared from haricot beans (*Phaseolus vulgaris*), using a method modified from that of Moreno and Chrispeels (1989), the affinity chromatography replaced by cation exchange (S-sepharose with 30mM Na-acetate pH5.0 and a linear salt gradient).

Assays of SCR α-amylase activity at pH5.0, in the presence of various concentrations of WAAI or BAAI, were conducted. Inhibitors were used at a range of concentrations, from 5mg ml^{-1} to 9.5ng ml^{-1} (1.4mg ml^{-1} to 2.7ng ml^{-1} in assay mixture) for WAAI, or 5mg ml^{-1} to 0.5µg ml^{-1} (1.4mg ml^{-1} to

0.14µg ml^{-1} in assay mixture) for BAAI. 0.17 larval gut equivalents (10µl extract) and 40µl of inhibitor was used per assay (140µl).

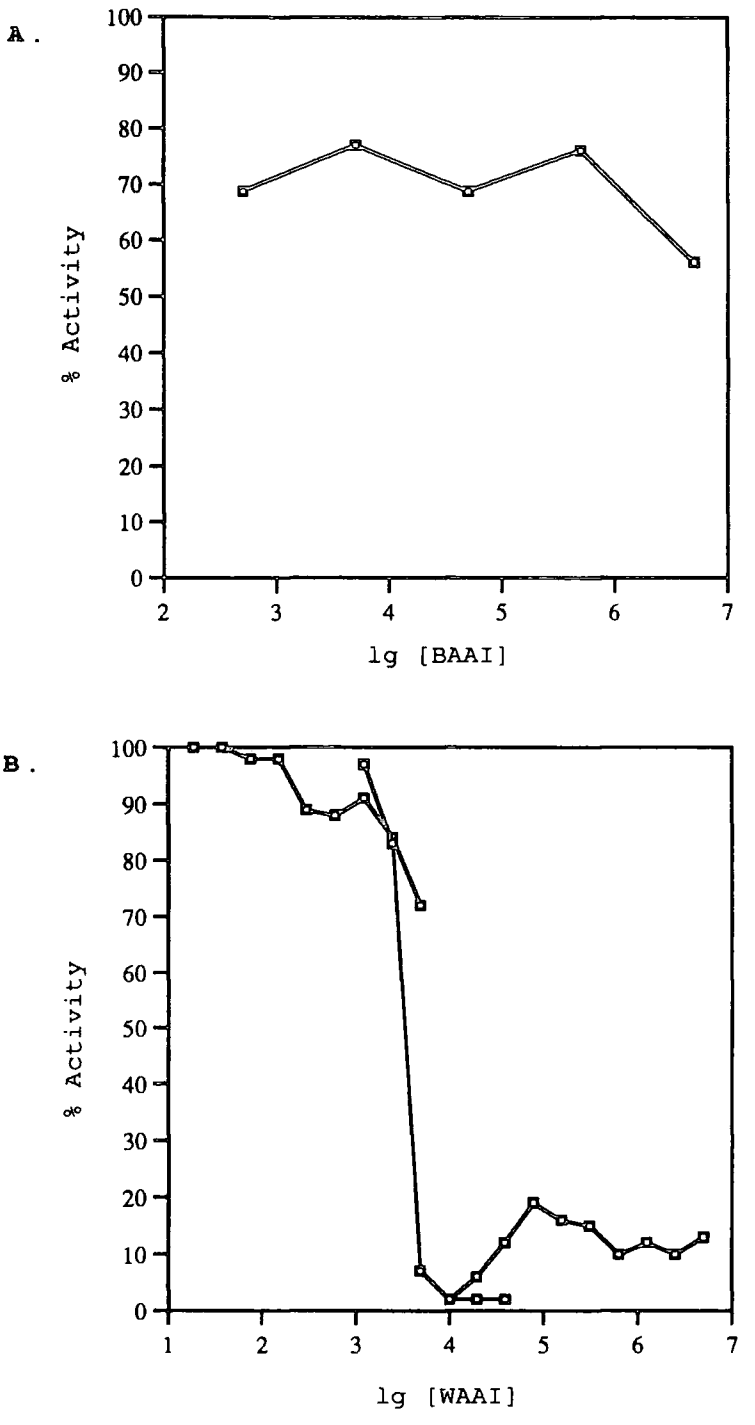


Figure 71. *In vitro* inhibition of SCR larval gut amylase activity by WAAI and BAAI. Activity is givenas a percent of the uninhibited enzyme activity. Inhibitor concentration is given as the log of the concentration in the assay solution, in µg/ml. The results with WAAI, from separate assays are presented as separate lines.

The results, shown in Figure 71, demonstrate the inhibition observed. Activity is presented as a percentage of the uninhibited activity. Inhibitor concentration is given as ng ml^{-1} in the assay solution. Inhibition by BAAI was variable, but gave consistantly low levels of inhibition, with only 44% inhibition caused by the highest concentration of BAAI assayed, 1.4mg ml^{-1} (in assay mixture). Assays were conducted in duplicate and corrected by the use of no-enzyme controls. The results presented represent values from a single assay, other assays demonstrated similar levels of inhibition.

WAAI inhibition was also somewhat varied, but less so than with BAAI. The WAAI plot presented (Figure 71) represents a number of assays, each conducted in duplicate. 50% inhibition of larval amylases occurred between 0.80 and 1.40µg ml^{-1} WAAI in the assay mixture, an ID_{50} was estimated such that the α-amylase activity of one larval gut equivalent was inhibited to 50% by 0.90µg WAAI. No comparison could be made with porcine pancreatic α-amylase, as no inhibition of this enzyme was caused by WAAI (data not shown).

The inhibitory effect of WAAI was also demonstrated by electrophoresis and selective staining. Enzyme extract and inhibitor were preincubated for 30min at 31°C, and cooled to 4°C, prior to electrophoresis on native polyacrylamide gel at 4°C. Samples of enzyme with no inhibitor, and inhibitor with no enzyme were also preincubated as above and used as controls. Porcine pancreatic α-amylase, alone or with WAAI, was also preincubated and run on gel.

Figure 72 represents one such gel following starch infiltration and iodine staining. Inhibition of α-amylase activity was visible as the decrease in band size demonstrated in lanes 6-8, inhibition increasing with increased WAAI concentration (lanes 8 to 6). Little or no inhibition of porcine pacreatic α-amylase activity was demonstrated (lane 3). No activity was observed with WAAI alone (lane 1). Some ill-defined activities were present

higher on the gel, and two distinct bands of activity of higher molecular weight were also visible in lanes 6 to 8.

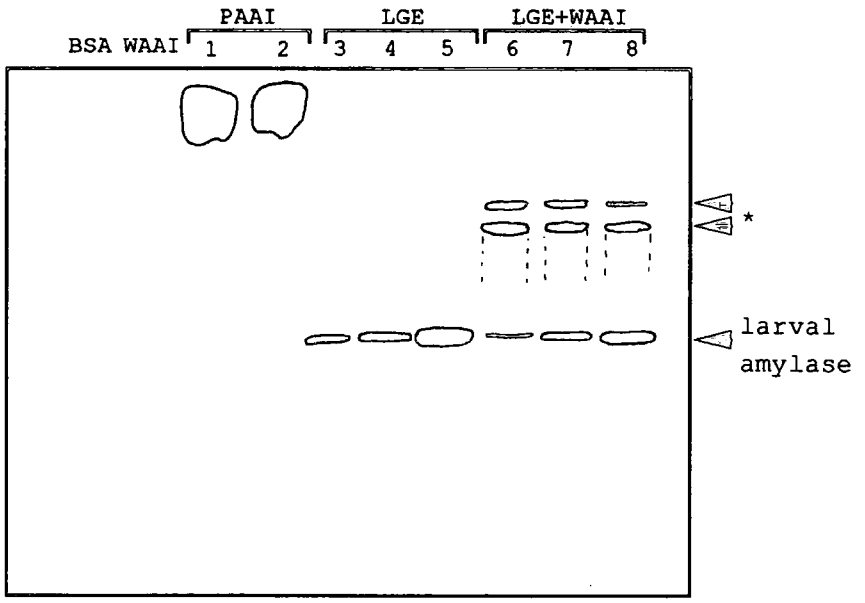


Figure 72. Diagram of polyacrylamide gel, showing analysis of WAAI inhibition of larval gut enzymes (LGE), using electrophoresis followed by starch digestion. Lane BSA, 10µl BSA; lane WAAI, 29.3ng WAAI; lanes PAAI, 19.5ng porcine pancreatic α-amylase, alone (1) and with 29.3ng WAAI (2); lanes LGE, 0.5µl (3), 1.0µl (4) and 1.5µl (5) larval gut extract (0.008, 0.017 and 0.025 gut equivalents, respectively); lanes LGE+WAAI, 1.5µl LGE preincubated with 29.3ng (6), 22.0ng (7) and 14.6ng (8) WAAI. Normal larval amylase activity is indicated, others are indicated by *.

α-AMYLASE INHIBITORS AND THEIR EFFECT *IN VITRO* - DISCUSSION

The α-amylase inhibitors from wheat and beans are the most highly characterised of the plant α-amylase inhibitors. These inhibitors have been shown *in vitro* to efficiently inhibit α-amylases from various sources, including insects and mammals, as discussed in the introduction. Members of the 0.28 family, monomeric inhibitors from wheat, are known to be highly effective against some insects, including species of *Tenebrio, Tribolium,*

Sitophilus, Oryzaephilus and *Callosobruchus.* while not affecting the enzymes of birds or mammals (Silano *et al.*, 1975). Bean αAIs have been demonstrated to exhibit inhibitory activity towards *Tenebrio molitor* and *Callosobruchus maculatus*, among others (Heusing *et al.*, 1991c, Pueyo *et al.*, 1993).

High levels of inhibitory activity against SCR larval α-amylases were demonstrated with WAAI, around 0.90μg WAAI inhibiting 1gut equivalent of activity by 50% (an equivalent of 1.09μg ml^{-1} WAAI in assay solution with 0.17 gut equivalents). Almost complete inhibition of α-amylase activity was demonstrated by higher concentrations of inhibitor. *In vivo* analysis of WAAI effects on development and survival of SCR larvae was also investigated using insect feeding trials (see Chapter 7).

The bean αAI, BAAI, was shown to only weakly inhibit SCR larval α-amylase activity, producing a decrease in activity of only 44% when used at a concentration of 1.4 mg ml^{-1} in the assay mixture, an equivalent amount of WAAI causing almost complete inhibition of the insect amylase activity. Due to the low level of inhibition demonstrated and the limited supply of the protein, no further investigation of BAAI effects was conducted.

Visualisation of WAAI inhibition of larval enzymes was achieved using starch infiltration and iodine staining of native polyacrylamide gels. Samples were preincubated for 30min prior to electrophoresis, allowing enzyme:inhibitor complex formation. Clear inhibition of activity was demonstrated, this inhibition increasing with increased WAAI concentration. No amylase activity was demonstrated with the WAAI sample. Tracks containing different loadings of the larval extract alone showed one a single band of α-amylase activity, as demonstrated previously. In lanes containing the enzyme/inhibitor mixture, two bands of activity were apparent higher on the gel, with weak activity forming a smudge between these bands and the main α-amylase band. This activity is most probably due to dissociation of the enzyme:inhibitor complex following electrophoresis, and to a lesser degree during electrophoresis (ie. forming the smudge), allowing starch

digestion by the freed enzyme. An increase in the activity in these bands occurred with increasing WAAI concentration, substantiating this dissociation theory. The presence of these bands also demonstrates that the inhibition caused to the insect enzymes by WAAI is reversible. It is possible that dissociation of the enzyme:inhibitor complex is promoted by the presence of the substrate, as has been suggested previously with αAIs (Marshall and Lauda, 1975), due to the predominant affinity of the enzyme for the substrate. This, along with the increased temperature following electrophoresis, would account for the extent of dissociation and consequential activity demonstrated in these upper bands.

The presence of the two discrete bands of higher molecular weight indicated that two distinct complexes are formed between the enzyme and WAAI, showing that the WAAI sample is impure, comprising at least two inhibitors of closely related affinities, probably both members of the 0.28 family. The analysis of WAAI samples by SDS-PAGE (Figure 70) showed that the preparation was obviously composed largely of two proteins, of slightly different molecular mass (14-16kDa), co-purifying, both seemingly are inhibitory to the larval α-amylases. The difference in molecular mass of the two components demonstrated on SDS-PAGE, bears remarkable similarity to the difference between the two bands of activity demonstrated in Figure 72.

Silano *et al.* (1975) found that of the insects tested, α-amylases were either susceptible to all the wheat inhibitors, or resistant to them all. Inhibition of insect enzymes was strongest with the 0.28 monomeric family, whereas no inhibitory activity against avian or mammalian α-amylases was exhibited by this group. Gutierrez *et al.* (1990, 1993) demonstrated a differential inhibition exhibited by the various wheat inhibitors towards insect enzymes. Testing several Lepidopteran pests, they showed that the tetramerics (0.53 family) caused the most inhibition, inhibition decreasing with the number of subunits present. In a study of agricultural pests (Gutierrez *et al.*, 1990) a differential inhibition was shown against several

Coleopteran species. The dimerics (0.19 family) were found to be more effective against *Leptinotarsa decemlineata* and *Oryzaephilus surinamensis* α-amylases than were the monomerics (0.28 family), while the reverse was true for inhibition of α-amylases from species such as *Tenebrio molitor* and *Sitophilus oryzae*.

CHAPTER 7 EFFECTS OF PLANT ANTIMETABOLIC PROTEINS ON DEVELOPMENT OF LARVAL *DIABROTICA* IN ARTIFICIAL DIET BIOASSAYS

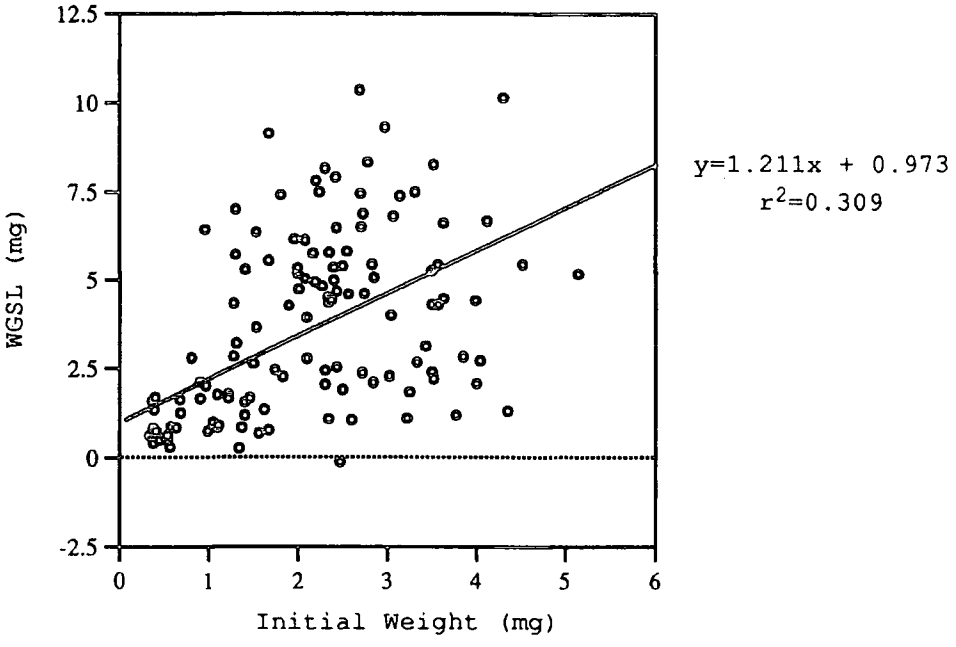
RESULTS

Dependence of weight gain on initial weight

Despite attempts within assays to use larvae of similar weights, the larvae employed for these bioassays were not of a uniform size. The influence of this initial weight on subsequent weight gain was investigated using control data for day 9 surviving larvae, collated from all bioassays conducted using the normal diet (diet 1). Correlation analysis was conducted with the paired data, taking the initial weight as the independent variable and weight gain as the dependent variable.

The correlation coefficient, r, was computed as 0.566, with 316 degrees of freedom (figure 73), showing a significant relationship between weight gain (weight gain per surviving larva, WGSL) and initial weight ($p<0.001$). Regression analysis was employed to deduce the equation, $y = 1.211x + 0.973$, the regression line, also shown in Figure 73. It must be noted that this line cannot be extrapolated to meet the y-axis, as no larva could possess an initial weight of 0mg.

The coefficient of determination, r^2, was computed as 0.309, indicating that 30.9% of the variation within the weight gain data could be explained by the initial weight of the larvae. This represents only a portion of the variation present in the weight gain data. Other factors, possibly peculiar to a single bioassay, such as a temporary drop in temperature in the insectary, may also increase the variation within the data, but the degree of influence of such factors cannot be calculated, thus the only factor for which a correction can be made, is the initial weight. As demonstrated by the coefficient of determination, r^2, a correction for initial weight would represent a decrease of over 30% of the total variation within the data.



IW		WGSL		Σ*xy*	*df*	*r*	
Σ*x*	644.040	Σ*y*	1089.240	2704.913	316	0.556	***
Σ*x*²	1716.523	Σ*y*²	5684.290				
n	318	*n*	318				

Figure 73. Correlation analysis of weight gain results, comparing initial weight and WGSL. *** indicates significance at $p<0.001$. Also regression plot of WGSL vs. initial weight, showing regression line and equation.

As the regression line represents the equation by which the amount of variation in the weight gain data accountable by the initial weight is at a maximum, a correction term for weight gain was formulated, based on this equation, applying the following theory:

$$Y_{observed} = k + jX_{observed}$$

where $Y_{observed}$ is the weight gain obtained in the bioassay, $X_{observed}$ is the actual initial weight, and k and j are constants. It also follows that:

$$Y_{expected} = k + jX_{expected}$$

where $X_{expected}$ can be taken as the mean initial weight, and $Y_{expected}$ is the independent weight gain value (independent of initial weight as this always approximates to the mean initial weight). Combining these equations gives:

$$\frac{Y_{expected}}{Y_{observed}} = \frac{k + jX_{expected}}{k + jX_{observed}}$$

and

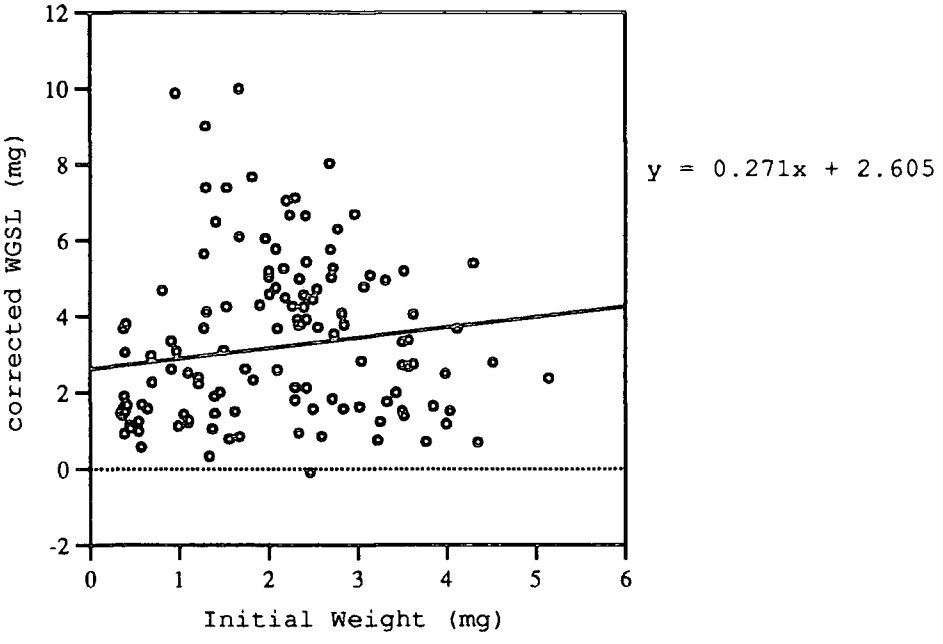
$$Y_{expected} = \frac{(Y_{observed})(k + jX_{expected})}{k + jX_{observed}}$$

As $X_{expected}$, the mean initial weight, is a constant, the term $(k + jX_{expected})$ is also constant. The values of k and j were obtained from the regression line as 0.973 and 1.211 respectively. As this correction term had been calculated using data from only the surviving larvae, the mean initial weight employed in subsequent corrections was taken as the mean of this data (1.9036), and not the mean from the entire pool of control larvae.

The success of this correction term in annulling the effect of initial weight was tested by further correlation analysis of this control data, using the initial weight and the corrected WGSL data (see Figure 74). A regression line of $y = 0.271x + 2.605$ was obtained, the correlation coefficient, r, being 0.159, which, with 316 degrees of freedom, shows no significant dependence on initial weight.

Control WGSL data from days 10, 11 and 12 were treated with this correction term and analysed, using t-tests, to establish any deviations from the day 9 data used above. No significant differences (at p=0.05) were demonstrated (data not shown). Control data from assays employing the starch diet (diet 3) were also corrected in this way, and found using t-

tests, to show no significant deviation from the data from normal diets (data not shown). Accordingly, all WGSL and WGL (weight gain per larva, dead or alive) data were treated with this correction term.



IW		c.WGSL		Σxy	*df*	*r*	
Σx	644.040	Σy	1002.869	2142.807	316	0.159	NS
Σx^2	1716.523	Σy^2	4351.225				
n	318	*n*	318				

Figure 74. Correlation analysis of weight gain results, comparing initial weight and corrected WGSL. NS indicates no significance. Also regression plot of WGSL vs. initial weight, showing regression line and equation.

Distribution of corrected WGSL and WGL data

The distributions of corrected WGSL and corrected WGL data were assessed by *G*-test for difference from the normal distribution. Differences

were found to be not significant ($p>0.05$), although the normal distribution of the corrected WGL was affected by the zero weight value assumed for dead larvae. Normal distributions were assumed for subsequent comparisons.

Choice of diet

A bioassay was conducted to investigate the suitability of diets 1 and 2 (see Figure 2) for use in bioassays. The assay was carried out as normal, but using five replicate pots each containing 10 larvae. Surviving larvae were counted and weighed after 8 days, 13 days and 16 days. The resultant survival, c.WGSL and c.WGL were analysed, the results are presented in Figure 75.

The survival of larvae on the two diets was identical on day 8 (54%), but by day 13, fewer larvae were surviving on diet 2 (40% compared to 48%), and by day 16 the difference had increased further, with only 26% of larvae surviving on diet 2 compared to 36% on diet 1. Using the *G*-test of independence, the difference between these rates was determined as not significant.

Corrected WGSL data revealed that on each occasion of weighing, larvae on diet 1 showed a greater weight gain than those on diet 2. Comparison of these data, however, using the *t*-test, demonstrated no significant differences between the rate of larval development on the two diets.

Examination of the corrected WGL data revealed once more a rate of development for larvae on diet 1, greater than that with diet 2. The mean corrected WGL with diet 2 was found to be negative on all the days of measuring, showing an overall WGL loss for the test population. The mean corrected WGL for larvae on diet 1 was low but positive on days 8 and 13, and dropped to only -0.07mg by day 16. *t*-test analysis demonstrated no significant difference between the data on any day of measurement.

While there was no significant difference between the survival or development of larvae on the two diets, diet 1 produced consistently higher levels of survival and greater increases in larval weight, thus this diet was chosen for routine use in bioassays.

Survival

Day	Diet 1 No.alive	Diet 1 Total	Diet 2 No.alive	Diet 2 Total	G_s	df	
8	27	50	27	50	-		
13	24	50	20	50	0.0365	1	NS
16	18	50	13	50	0.7496	1	NS

Corrected WGSL

Day	Diet 1 Mean	Diet 1 s^2	Diet 1 n	Diet 2 Mean	Diet 2 s^2	Diet 2 n	t_s	df	
8	2.186	2.570	27	1.599	0.766	27	1.670	40	NS
13	3.146	3.972	24	2.380	4.942	20	1.205	42	NS
16	3.490	8.077	18	3.157	7.762	13	0.324	29	NS

Corrected WGL

Day	Diet 1 Mean	Diet 1 s^2	Diet 1 n	Diet 2 Mean	Diet 2 s^2	Diet 2 n	t_s	df	
8	0.274	5.741	50	-0.026	3.576	50	0.695	98	NS
13	0.482	8.550	50	-0.199	6.457	50	1.243	98	NS
16	-0.007	9.822	50	-0.593	6.964	50	1.011	98	NS

Figure 75. Statistical analysis of results from diets 1 and 2. NS indicates no significance ($p>0.05$).

Artificial diet for α-amylase inhibitor bioassay

The successful modification of diet 1 for use in bioassays investigating the *in vivo* effects of α-amylase inhibitors, by the removal of the majority of the sucrose and the addition of starch, was examined using two bioassays (see Figure 76).

In the first bioassay, six diets (all versions of diet 3, see Figure 2) were used, with sucrose concentrations ranging from 0 to 1.25% (w/w). The survival of larvae varied from 93% on diets with 0% and 0.25% sucrose, to 67% and 60% on diets containing 1.25% and 0.5% sucrose respectively. Surprisingly, the rates of survival observed demonstrated no apparent relationship to the concentration of sucrose in the diet. *G*-tests revealed no significant difference in survival, even between the 0% and 0.5% sucrose diet results.

The corrected WGSL data were analysed by ANOVA and *t*-test. No significant differences were demonstrated between the weight gains, except for those larvae on the 1.0% sucrose diet, where the c.WGSL was significantly lower than for the 0% or 0.25% diets. *t*-tests were also employed to analyse the differences between corrected WGL data from the six diets. While the gains on the 0% and 0.25% diets were markedly greater than those on any of the other diets, only the difference between these and the 0.5% diet results were found to be significant.

A second bioassay was conducted as a direct comparison of the 0% and 0.25% sucrose diets, these demonstrated to be most successful, in terms of survival and development, in the first bioassay. Survival of the larvae in this bioassay was markedly lower than in the previous one, only 52% and 60% surviving by day 9 on the 0% and 0.25% diets respectively.

Corrected WGSL data exhibited equal variances and, while the mean corrected WGSL on the 0.25% diet was higher than that on the 0% diet, a *t*-test revealed that no significant difference existed between the two.

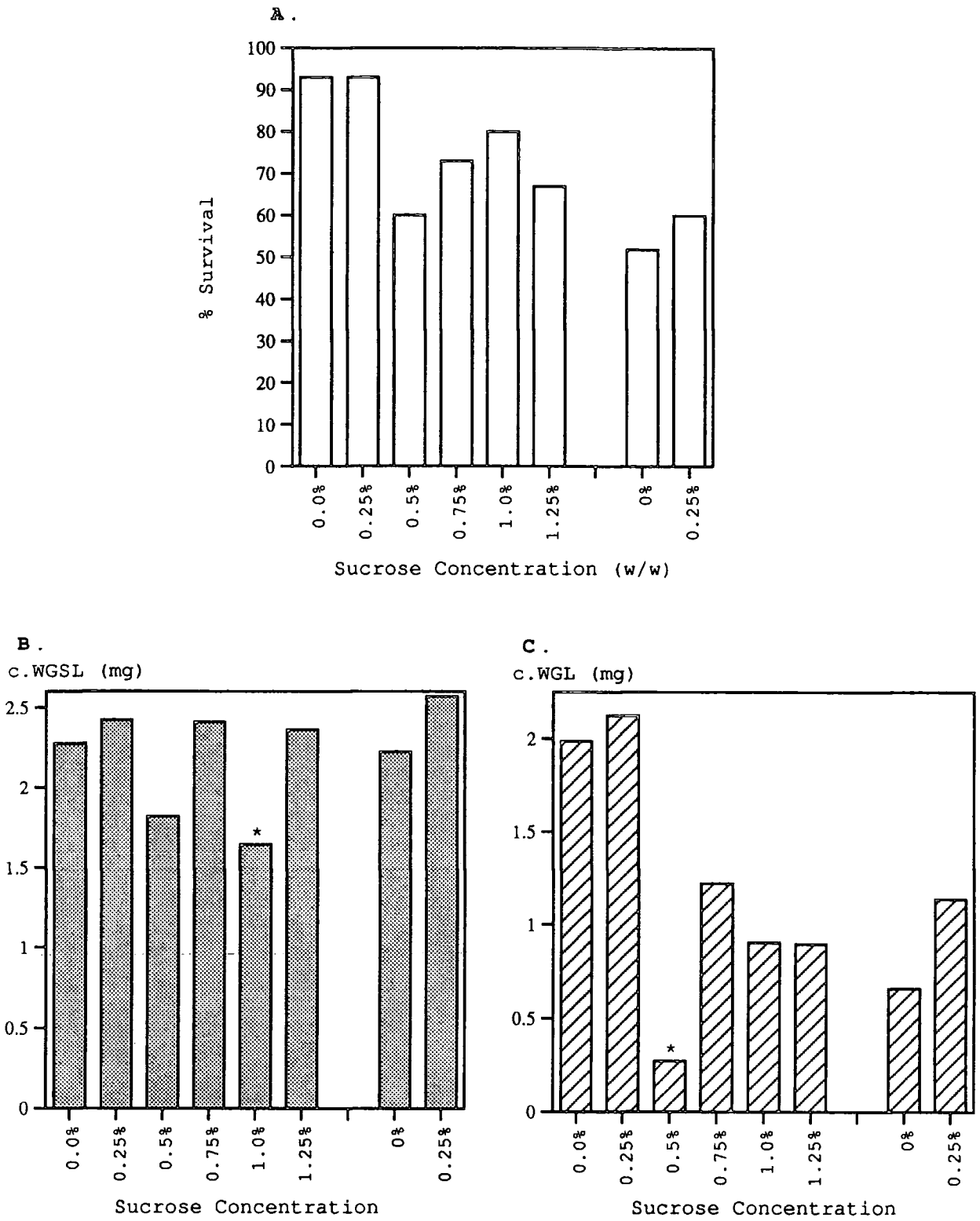


Figure 76. Survival (panel A), corrected WGSL (panel B), and corrected WGL (panel C) of SCR larvae on artificial diet modified for use in amylase inhibitor bioassays. Concentrations indicate sucrose content of diets. Significant differences from the 0% sucrose diet data are indicated by * ($p<0.05$).

Corrected WGL data revealed a marked difference between the two diets, the mean corrected WGL on the 0.25% diet being almost twice that on the sucrose free diet, however, no significant difference could be demonstrated. As the diet containing 0.25% sucrose, (diet 3, see Figure 2), was demonstrated to give consistently, although not significantly, higher survival and development rates than the other diets, this diet was chosen for use in bioassays involving α-amylase inhibitors only.

Survival of larvae with no diet

No-diet controls were conducted to discover the length of time that larvae survive in the absence of food. 30 larvae with a mean initial weight of 0.436mg were held singularly or in groups of 5, with no diet, but in conditions of high humidity. The size of the group had no effect on larval survival. Survival was recorded after 3, 6 and 7 days, as 67%, 10% and 7%. All larvae were dead by day 9. Unless otherwise stated, survival and weight gain of larvae in subsequent bioassays, was recorded on day 9.

BIOASSAY RESULTS

A number of proteins were tested in bioassay for their *in vivo* effects on larval SCR survival and development. The results discussed below consider each antimetabolite in turn, complete bioassay results are given in the Appendix.

LECTINS

(1) Elderberry Lectins

SNA1 and SNA2, lectins from *Sambucus nigra*, elderberry, were incorporarted into artificial diet at 0.26% (w/v) and tested against SCR larvae. The results, relative to controls, are given in Figure 77. Little difference was demonstrated between survival on the lectin treatments and controls in either bioassay. The corrected WGSL data from bioassay II

showed a greater increase in weight than was found in bioassay I, with a significant increase from the controls demonstrated for larvae on SNA1-treated diet in bioassay II. In both assays weight gain was greater with the SNA1 treatment than the SNA2 treatment. The c.WGL data mirrored that of c.WGSL.

(2) Pea Lectin

Plec, a lectin from pea, *Pisum sativum*, was tested against SCR larvae by incorporation into artificial diet at a concentration of 0.26% (w/v), as shown in Figure 78. In both assays where plec was tested, no notable alteration of larval survival was demonstrated. On both days of recording, survival was close to or equal to that on the control diet. The corrected WGSL data showed distinct differences between the two assays. In bioassay IV, the c.WGSL data showed a decrease from the control, while in bioassay V, an increase was demonstrated which, by day 12, was notable but not significant. This difference in weight gain was also shown in the corrected WGL data, a decrease in c.WGL demonstrated in bioassay IV and a marked, but not significant increase found with bioassay V.

(3) Wheat Germ Lectin

WGA, incorporated into artificial diet at 0.26% (w/v) (see Figure 79), caused a slight enhancement of larval survival. At day 9, there was no difference in survival, but by day 12, survival on the WGA-treated diet was greater than on the control. This enhancement, however, was definitely not significant, the G_s value obtained, 0.139, indicating no significance at a level of $p>0.50$. The corrected WGSL data demonstrated a significant reduction in weight gain caused by WGA treatment on both days of recording. This reduced weight gain in surviving larvae, is also reflected in the corrected WGL data, a significant decrease from the control data demonstrated on day 9, although not on day 12.

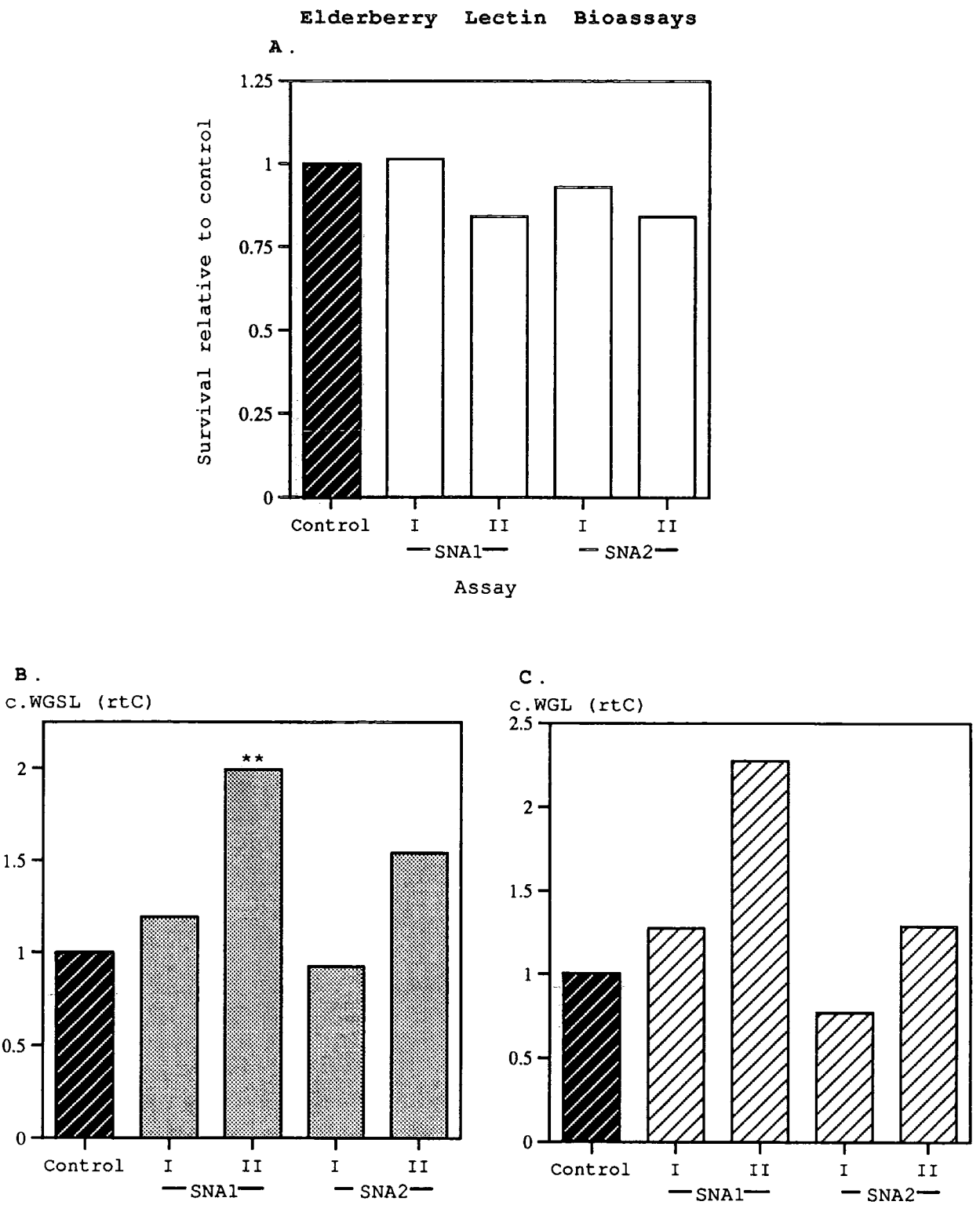


Figure 77. Survival (panel A), corrected weight gain per surviving larva (c.WGSL, panel B), and corrected weight gain per larva (c.WGL, panel C) of SCR larvae on artificial diet treated with elderbery lectin, SNA1 or SNA2, at 0.26% (w/v). All values are given relative to Controls (rtC). Both assays were conducted for 8 days. Significance of differences from the control are indicated by *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; no asterix indicates no significance, $p>0.05$.

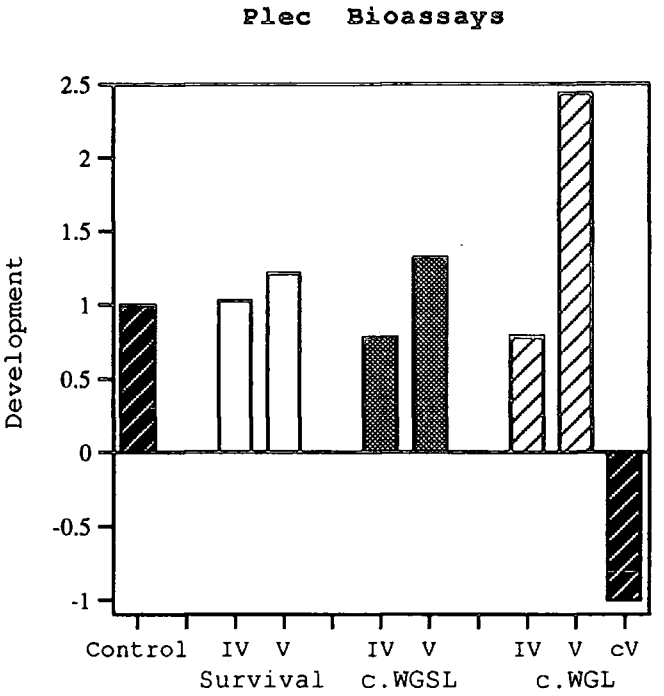


Figure 78. Survival, corrected weight gain per surviving larva (c.WGSL) and corrected weight gain per larva (c.WGL) of SCR larvae on artificial diet treated with plec at 0.26% (w/v). All values are given relative to Controls, the c.WGL control (cV) is -1, as shown. Assays IV and V were conducted for 11 and 12 days, respectively. Significance of differences from the control are indicated by *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; no asterix indicates no significance, $p>0.05$.

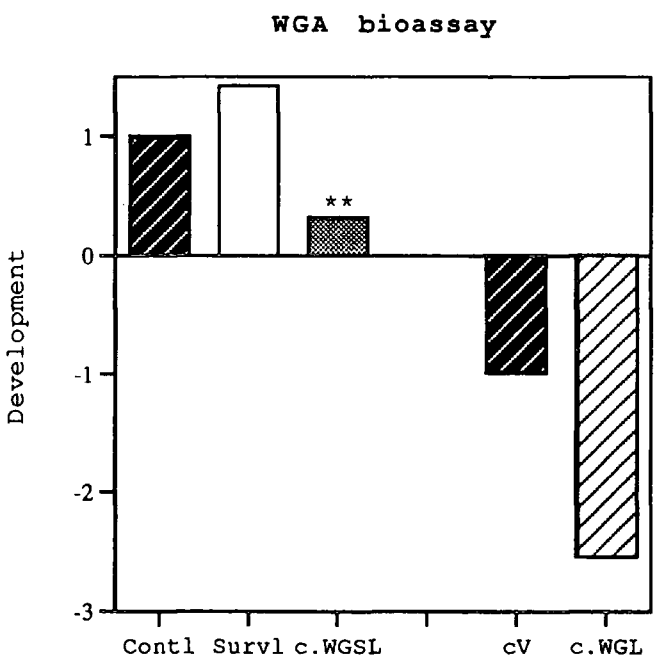


Figure 79. Survival, corrected weight gain per surviving larva (c.WGSL) and corrected weight gain per larva (c.WGL) for SCR larvae on artificial diet treated with WGA, incorporated at 0.26% (w/v). All values are given relative to Controls, control (cV) for c.WGL is -1, as shown. The assay was conducted for 12 days. Significance is indicated as described in Figure 78.

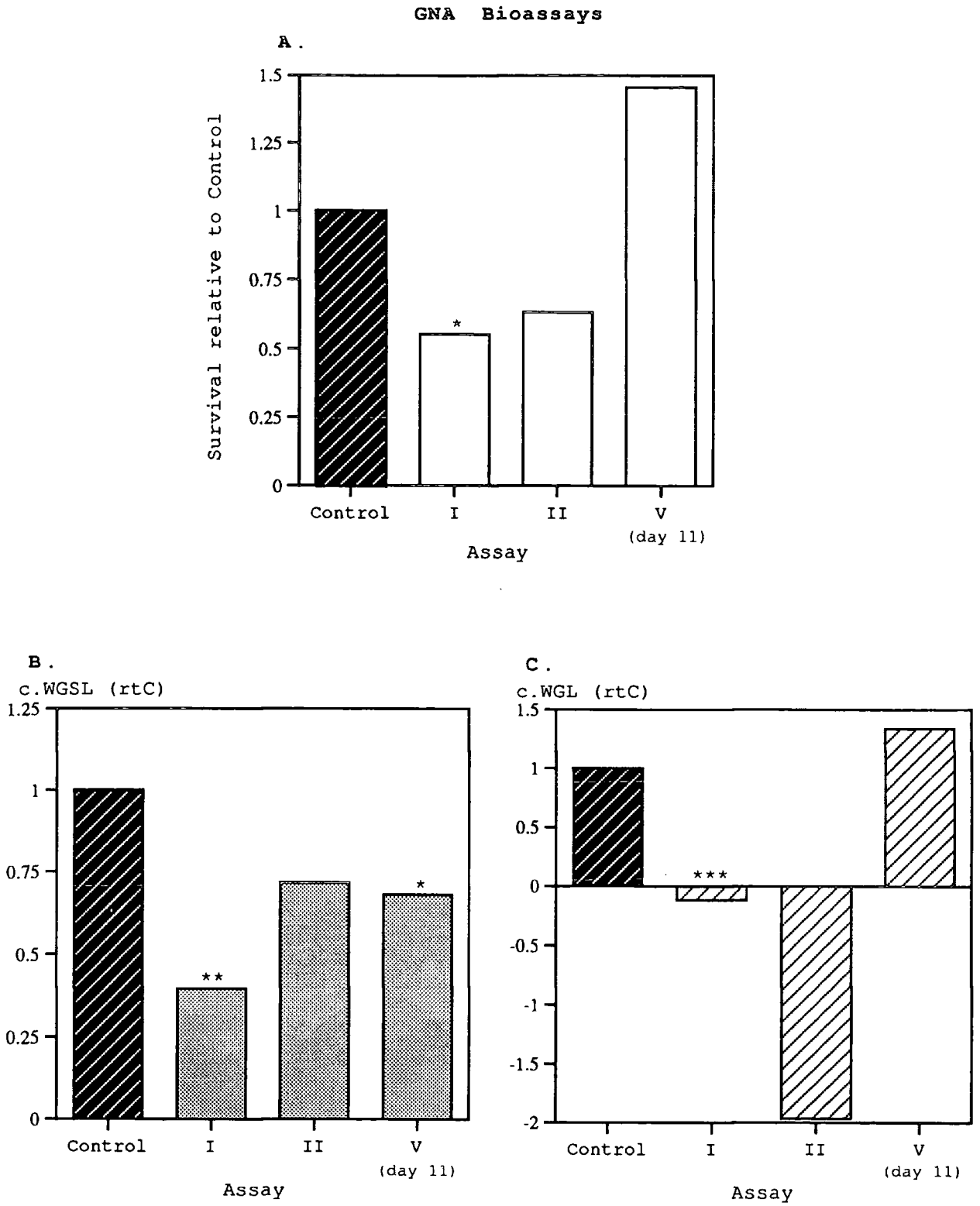


Figure 80. Survival (panel A), corrected weight gain per surviving larva (c.WGSL, panel B), and corrected weight gain per larva (c.WGL, panel C) of SCR larvae on GNA treated artificial diet, with GNA at 0.26% (w/v). All values are given relative to Controls (rtC). Assays were conducted for 8 days, unless otherwise stated. Significance of differences from the control are indicated by *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; no asterix indicates no significance, $p>0.05$.

(4) Snowdrop Lectin

GNA, a lectin from snowdrop, was incorporated into artificial diet at 0.26% (w/v). The effects of this treatment on survival and development in larval SCR are shown in Figure 80.

In bioassays I and II, GNA caused a marked increase in mortality, the survival of larvae in assay I was demonstrated to be significantly lower on the treated diet than on the control. In assay V, no increase in mortality was seen, rather, a substantial enhancement of survival was demonstrated, this increased survival was not significantly greater than the control.

A decrease in corrected WGSL was found, in all assays, to result from GNA treatment, the reduction in weight gain was significant in assays V ($p<0.05$) and I ($p<0.01$). The corrected WGL data demonstrates the overall effect of the treatment on the population. The decrease in both survival and weight gain caused by GNA in bioassays I and II, result in a dramatic reduction in c.WGL, highly significant in the case of assay I. The increased survival rate demonstrated in assay V, produced c.WGL data slightly greater than that of the controls, despite the significant decrease in c.WGSL.

RIPs - Saporin

Saporin was tested against larval SCR by incorporation into artificial diet at concentrations of 0.0013% and 0.0071% (w/v) in bioassays I and II, respectively. The results, relative to the control, are given in Figure 81.

At 0.0013% (assay I), the treatment was found to cause almost no alteration of survival or weight gain. At 0.0071% (assay II), saporin caused a significant increase in mortality of larvae, with a survival rate of less than 50% that of the controls. Those larvae surviving, however, showed an increased weight gain over the controls which, although not significant, gave a mean corrected WGSL almost twice that of the control. The corrected WGL data from the higher concentration treatment showed a dramatic decrease from the control, although this difference was not significant.

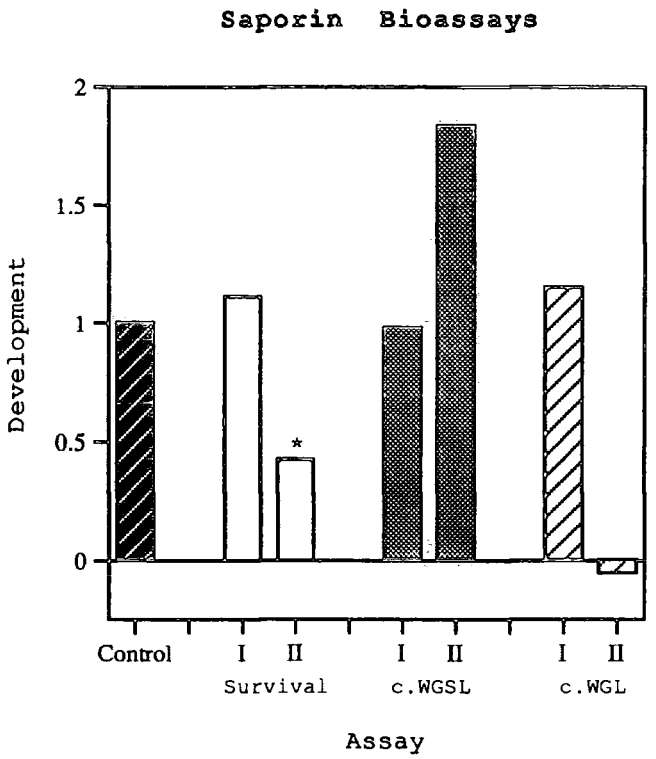


Figure 81. Survival, corrected weight gain per surviving larva (c.WGSL) and corrected weight gain per larva (c.WGL) of SCR larvae on artificial diet treated with saporin. All values are given relative to Controls. Saporin was incorporated into diet at 0.0013% (w/v) in assay I and at 0.0071% (w/v) in assay II. Assays were conducted for 8 days. Significance of differences from the control are indicated by *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; no asterix indicates no significance, $p>0.05$.

SERINE PROTEASE INHIBITORS - CpTI

CpTI was incorporated into artificial diet at 0.13%, 0.26% and 0.65% (w/v) and gave the results shown in Figure 82. Although the results were varied, the general trend demonstrated was a decrease in all three parameters.

In assay I, the 0.13% treatment had little effect on survival or development. The 0.26% treatment had no effect on corrected WGSL, but resulted in a decreased survival rate and reduced c.WGL. The 0.65% treatment, surprisingly, had no effect on larval survival, but caused a significant decrease in c.WGSL and a c.WGL reduced below that of the 0.26% treatment.

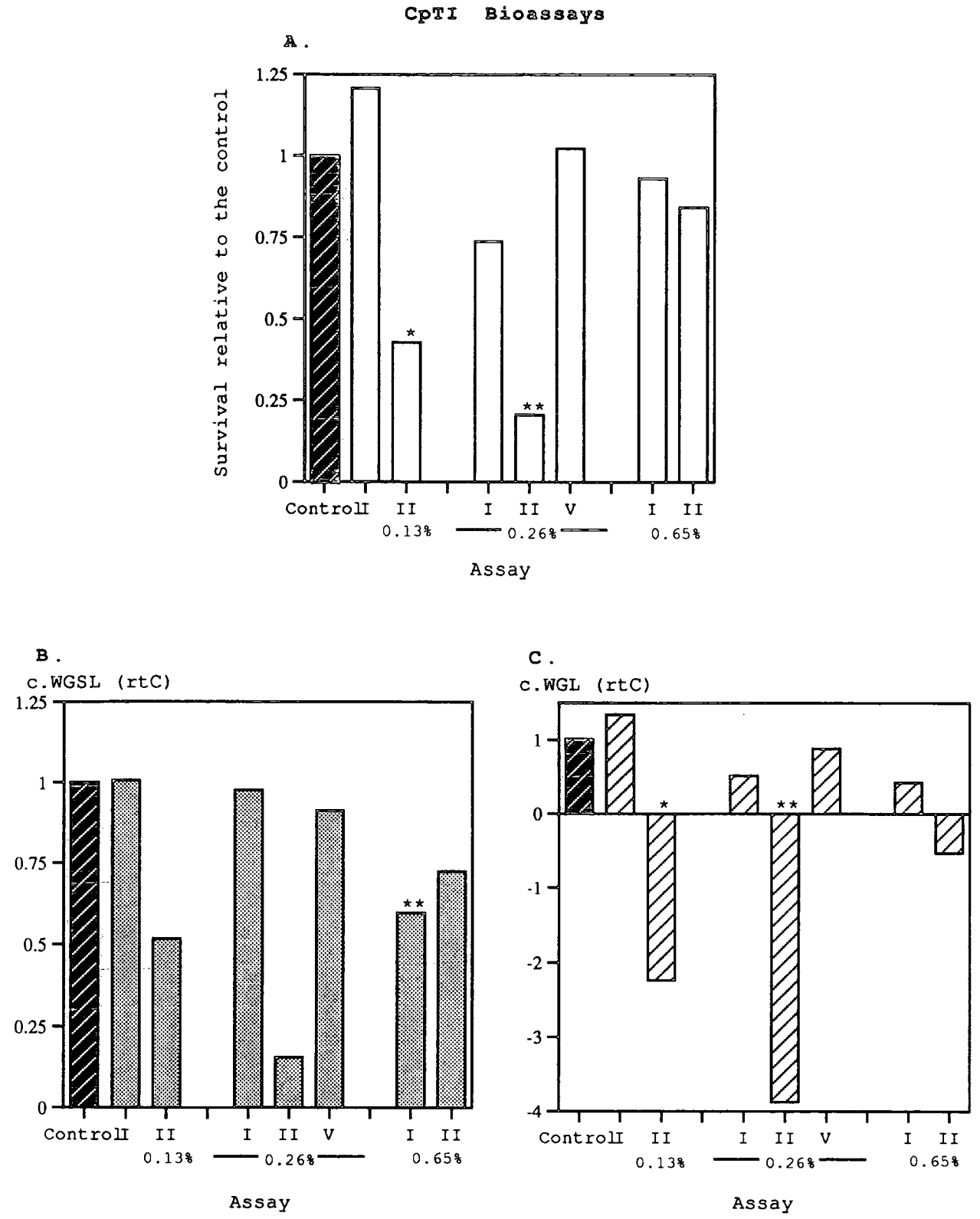


Figure 82. Survival (panel A), corrected weight gain per surviving larva (c.WGSL, panel B), and corrected weight gain per larva (c.WGL, panel C) of SCR larvae on artificial diet treated with CpTI incorporated at 0.13%, 0.26% and 0.52% (w/v), as indicated. All values are given relative to Controls (rtC). Assays I and II were conducted for 8 days, assay V results, for 11 days. Significance of differences from the control are indicated by *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; no asterix indicates no significance at $p>0.05$.

In assay II, the three treatments were again employed. In this case, the 0.13% and 0.26% treatments both resulted in decreased weight gain and significantly increased mortality, the adverse effects being greater in the more concentrated treatment. Once again, the 0.65% treatment differed in its effects, survival again remaining largely unaffected while c.WGSL and c.WGL were decreased only marginally.

Strangely, the 0.26% CpTI treatment employed in bioassay V, caused a small decrease in weight gain but had no impact on the survival of the larvae.

CYSTEINE PROTEASE INHIBITORS

(1) Cystatin

Chicken egg white cystatin was incorporated into artificial diet at a concentration of 0.0167% (w/v). Survival of larvae fed on this diet was decreased only marginally from those on a control diet, while corrected WGSL was increased slightly on the treated diet, as shown in Figure 83. Corrected WGL was largely unaffected by the treatment.

(2) SQRG-I

The ozcI sample partially purified from rice seed, SQRG-I, was assayed at 0.13% (w/v) in artificial diet, the effects on SCR larvae are shown in Figure 83. Small decreases were demonstrated in survival and weight gain for larvae on the treated diet, compared to those on control diet. Neither reduction was significant, but, as demonstrated in the corrected WGL data, the overall effect was quite notable.

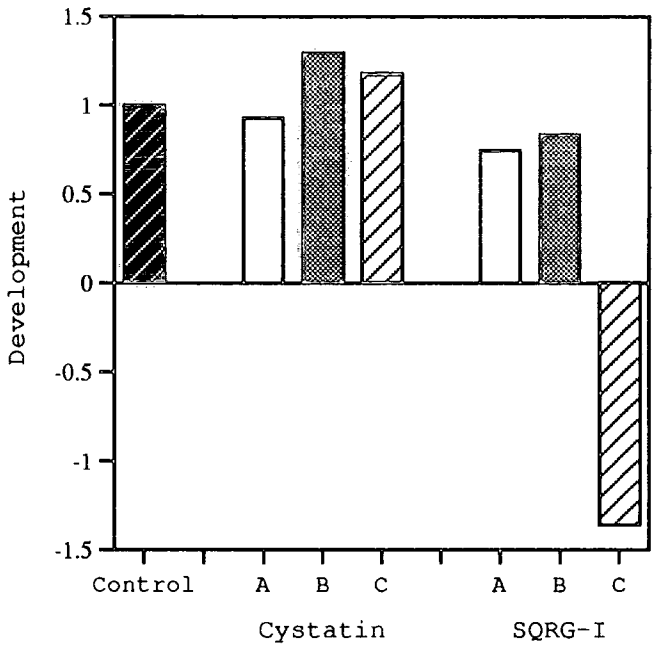


Figure 83. Data for the survival (A), corrected weight gain per surviving larva (c.WGSL, B) and corrected weight gain per larva (c.WGL, C) for SCR larvae on artificial diet treated with cystatin (assay I) and SQRG-I (assay II), incorporated at 0.0167% and 0.13% (w/v), respectively. All values are given relative to Controls. Assays were conducted for 8 days. None of the results were significantly different from the controls.

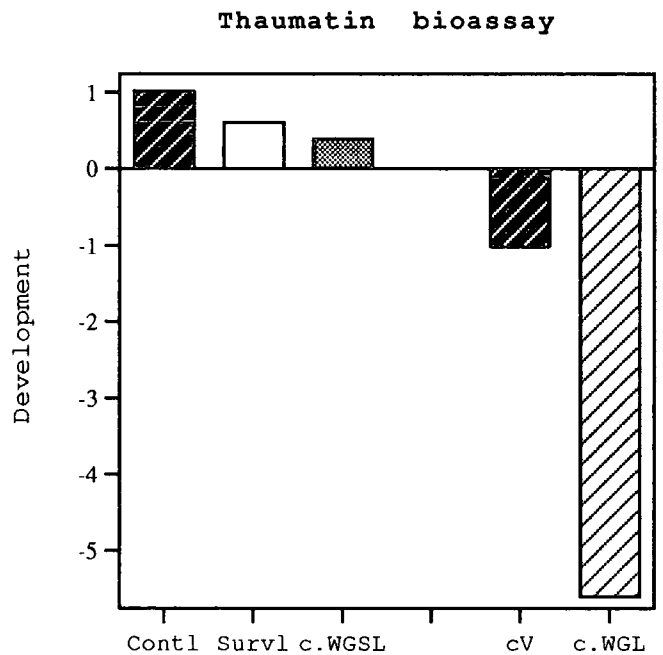


Figure 84. Survival (Survl), corrected weight gain per surviving larva (c.WGSL) and corrected weight gain per larva (c.WGL) of SCR larvae on artificial diet treated with thaumatin, incorporated at 0.26% (w/v). All values are given relative to Controls, for c.WGL, the control (cV) is -1. The assay was conducted for 12 days. None of the results were significantly different from the controls.

(3) Thaumatin

Survival of larvae on diet containing 0.26% (w/v) thaumatin was, by day 9, identical to that on the control diet, but decreased by day 12 to be 40% lower than the controls (see Figure 84).

Corrected WGSL was also reduced by the thaumatin treatment, although not significantly, the mean c.WGSL value for day 12 on the thaumatin diet being approximately half that achieved by the controls. Corrected WGL was dramatically reduced by the thaumatin treatment, although the reduction from the controls was not significant.

(4) E64

The efficacy a treatment of E64, a specific inhibitor of cysteine proteases, on larval SCR was assayed by incorporation into artificial diet at 0.013% (w/v) in bioassays I, II, III, VI and VII and 0.026% in bioassay III (Figure 85). All assays showed a marked increase in mortality due to the presence of E64 in the diet, except for bioassay I where a small increase in survival was demonstrated. This reduction in survival levels was significant in bioassay III and by day 10 in bioassay VII. Treatment with 0.026% (w/v) E64 resulted in a further increase in mortality, giving a survival equivalent to only 25% that of the controls.

Weight gain (c.WGSL) on E64 treated diets was dramatically reduced from that observed on the control diet. With the 0.013% treatment, the mean corrected WGSL values from the various bioassays were all below 0.5mg, significantly lower than those for the controls, which ranged from 1.7mg to 4.7mg. Interestingly, c.WGSL was less affected by the higher concentration of E64, the mean c.WGSL value being as high as 1.25mg. Corrected WGL was, in all cases, significantly reduced from the controls ($p<0.01$).

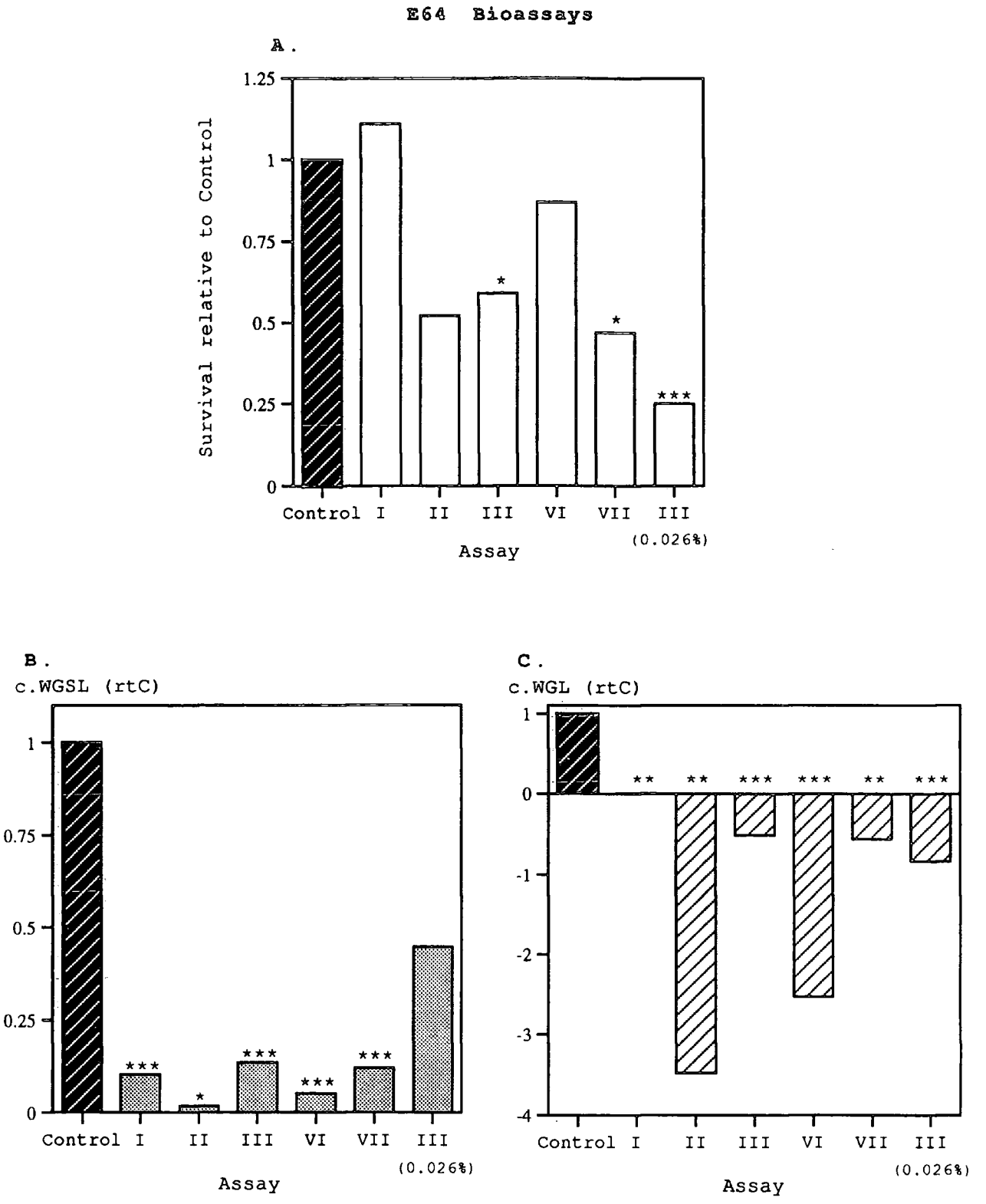


Figure 85. Survival (panel A), c.WGSL (panel B), and c.WGL (panel C) of SCR larvae on E64 treated artificial diet (at 0.013% (w/v), except where stated). All values are given relative to controls (rtC). Assays were conducted for 8 days (I and II), 9 days (III and VI), and 10 days (VII). Significance of differences from the control are indicated by *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; no asterix indicates no significance, $p>0.05$.

(5) Rozc

Rozc, the recombinant oryzacystatin produced in this work (see Chapter 8), was incorporated into artificial diet (diet 1) at concentrations of 0.065%, 0.130% and 0.260% (w/v) for bioassay VI, with an additional concentration of 0.0325% (w/v) employed in bioassay VII. The results, relative to the controls are presented in Figure 86.

Survival of larvae on the Rozc diets was lower than on the controls, survival, in most cases, decreased markedly with increased inhibitor concentration, although no significant correlation was demonstrated in either bioassay (Figure 87). Survival on the 0.26% treatment was significantly lower than that of the controls in both bioassays, and below that with E64 treatment (see Figure 85).

Marked variation was observed in the corrected WGSL data both between the two bioassays and between the treatments. In bioassay VI, all Rozc treatments produced a decrease in weight gain from the control. This decrease, however, was least in the 0.13% treatment, and greatest in the 0.065% treatment where a significant reduction from the controls was demonstrated. Conversely, in the day 10 data from bioassay VII, all treatments were found to cause increased weight gain, except the 0.13% treatment which produced a c.WGSL significantly lower than the control. The gains demonstrated with the 0.26% treatment were significantly greater than those achieved by the controls.

In bioassay VI, while a clear decrease in corrected WGL is demonstrated with the 0.065% treatment, dramatic and highly significant decreases from the controls were demonstrated with the 0.13% and 0.26% treatments. Although corrected WGL decreased with increasing inhibitor concentration, no significant correlation between the two was found to exist (see Figure 87). In bioassay VII, by day 10, the reduction in c.WGL was less marked, but a significant correlation ($p<0.02$) between Rozc concentration and corrected WGL could be demonstrated.

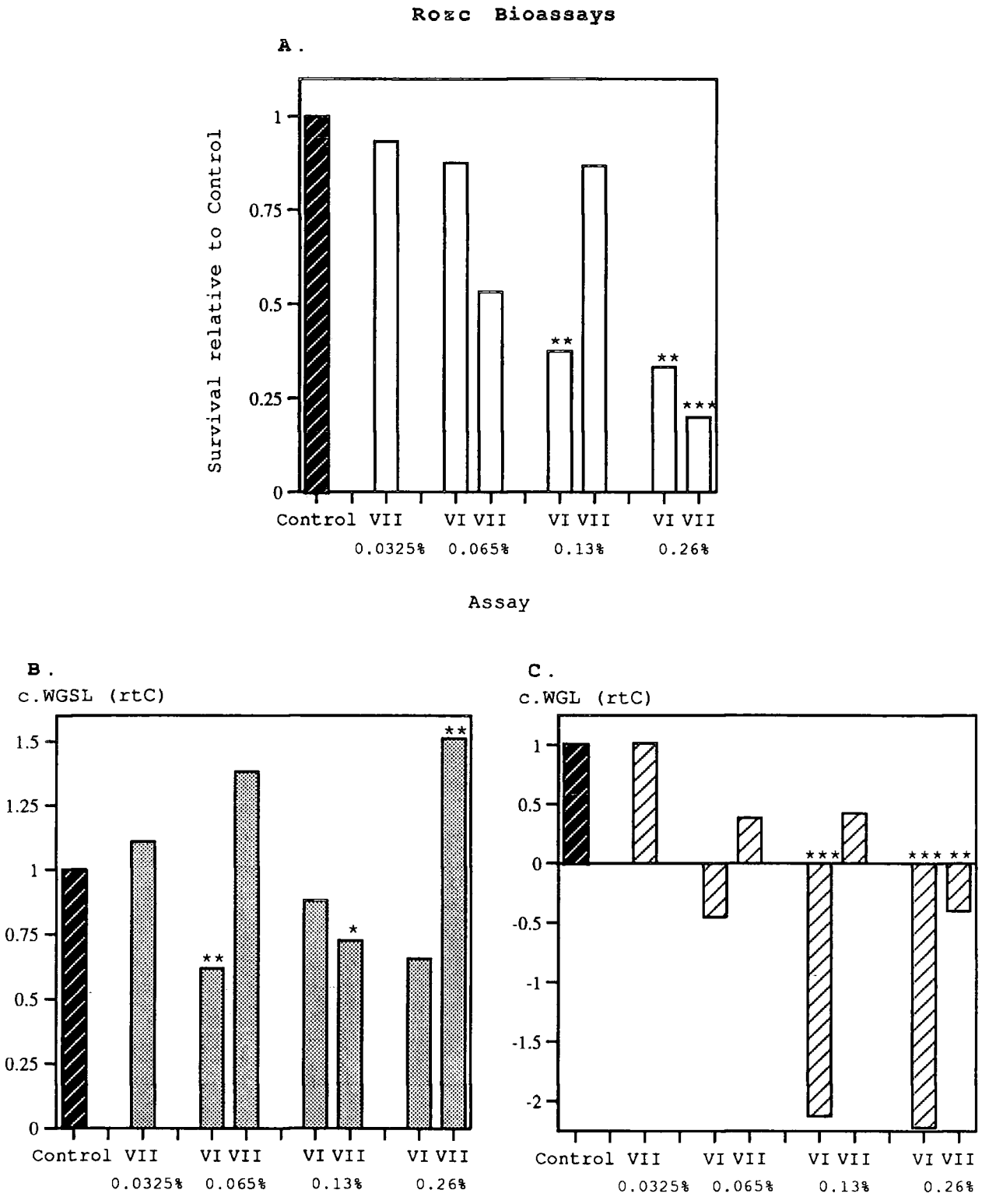


Figure 86. Survival (panel A), c.WGSL (panel B), and c.WGL (panel C) of SCR larvae on artificial diet 3 (starch diet) treated with Rozc, incorporated at 0.0325%, 0.065%, 0.13% and 0.26% as indicated. All values are given relative to Controls (rtC). Assay VI was conducted for 9 days, and assay VII for 10 days. Significance of differences from controls are indicated by *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; no asterix indicates no significance, at $p>0.05$.

Survival

[Rozc]	Mortality	Σxy	df r
Bioassay VI			
Σx 0.455	Σy 276	28.3817	2 -0.533 NS
Σx^2 0.0887	Σy^2 19848		(p»0.1)
n 4	n 4		
Bioassay VII			
Σx 0.4875	Σy 288	34.970	3 0.833 NS
Σx^2 0.08978	Σy^2 18208		(p>0.05)
n 5	n 5		

Corrected WGL

[Rozc]	c.WGL	Σxy	df r
Bioassay VI			
Σx 0.455	Σy -1.544	-0.3593	2 -0.884 NS
Σx^2 0.0887	Σy^2 1.7642		(p>0.1)
n 4	n 4		
Bioassay VII			
Σx 0.4875	Σy 2.512	0.01007	3 -0.954 *
Σx^2 0.08978	Σy^2 2.6962		(p<0.02)
n 5	n 5		

Figure 87. Correlation analysis of survival and corrected WGL data from bioassays VI and VII (Day 10), incorporating Rozc into artificial diet. Level of significance is indicated by NS, Not Significant; *, p<0.05; **, p<0.01; or ***, p<0.001.

α-AMYLASE INHIBITORS - WAAI

Bioassays were conducted to assay the effects of WAAI, wheat α-amylase inhibitor, on the survival and development of SCR larvae, comparing the effects when incorporated into the normal artificial diet (diet 1) and the starch diet (diet 3) at levels of 0.065%, 0.13%, 0.26% and 0.52% (w/v). The results, relative to the controls, are given in figures 88 (diet 3) and 90 (diet 1). The results from the incorporation into diet 3 will be discussed first.

Survival in both bioassays was substantially reduced with all WAAI concentrations, in comparison with the controls. In bioassay VIII, survival in all treatments was significantly lower than the control. Between the 0.13% and 0.52% treatments, mortality increased with inhibitor concentration, but no significant correlation, could be demonstrated between WAAI concentration and mortality (see Figure 89).

In bioassay IX, a slight reduction in survival was demonstrated with the treatments at day 9, by day 10 mortality on the treatments had increased and a significant correlation was demonstrated between inhibitor concentration and mortality ($p<0.05$).

No significant alteration of weight gain was demonstrated with the corrected WGSL data from bioassay VIII, the 0.065% treatment showed a slight increase and the 0.26% treatment a decrease in weight gain. In bioassay IX, the weight gains demonstrated by larvae fed on WAAI treated diets were either equivalent to or greater than the controls, the 0.26% treatment produced weight gains significantly greater than the controls on both days of measuring.

All the concentrations of WAAI employed in bioassay VIII produced corrected WGL significantly lower than the controls, the greatest reductions were demonstrated in the 0.26% and 0.52% treatments, but no significant linear relationship was demonstrated between WAAI concentration and corrected WGL (see Figure 89).

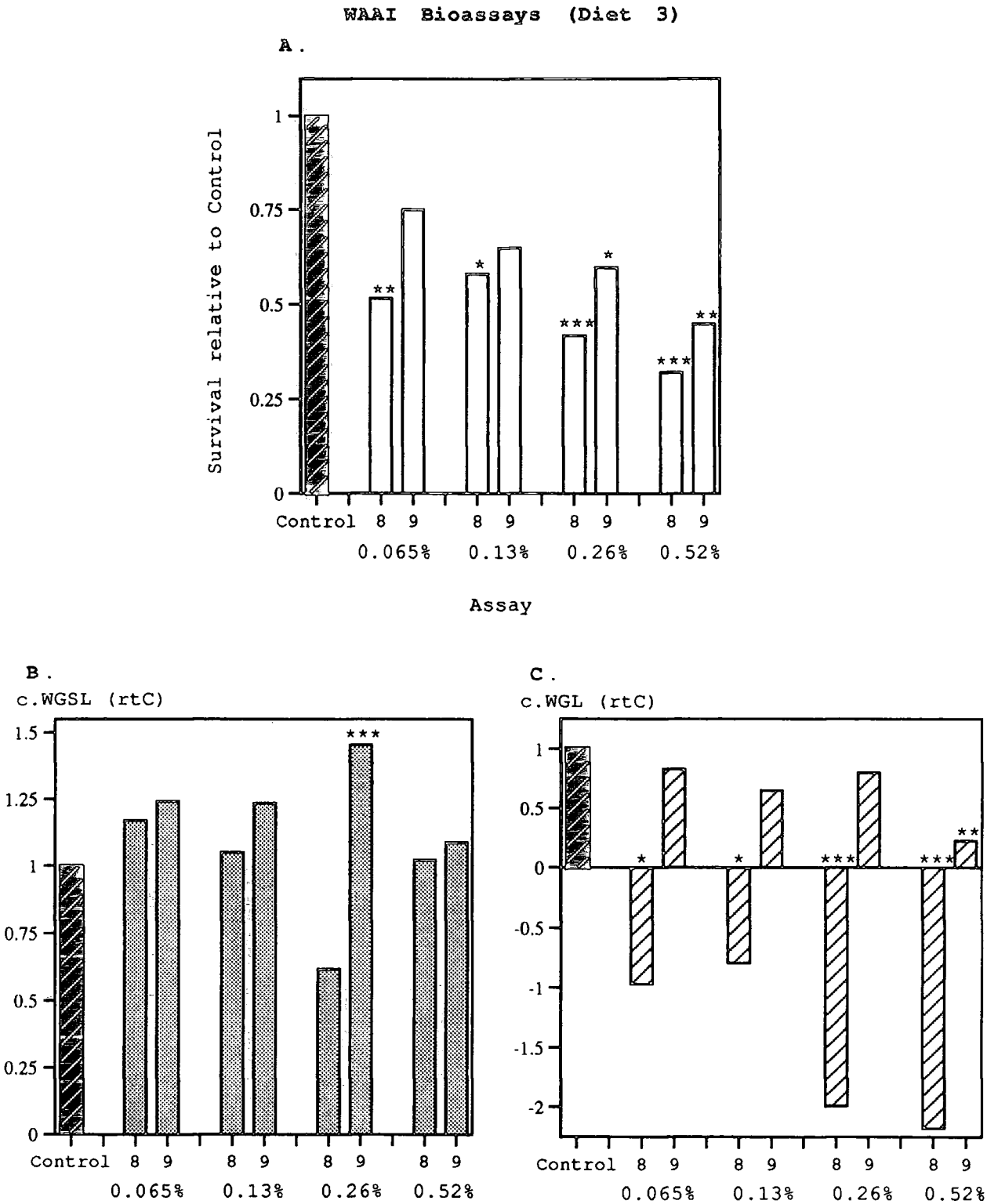


Figure 88. Survival (panel A), corrected WGSL (panel B), and corrected WGL (panel C) of SCR larvae on artificial diet 3 (starch diet) treated with WAAI, incorporated at 0.065%, 0.13%, 0.26% and 0.52%, as indicated. All values are given relative to Controls (rtC). Assay 8 was conducted for 9 days, and assay 9 for 10 days. Significant differences from controls are indicated by *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

In bioassay IX, little variation from the control was demonstrated on day 9, but, by day 10, although the reduction was less marked than in assay VIII, a significant correlation was seen between inhibitor concentration and corrected WGL (see Figure 89), and a significant decrease in weight gain produced in the 0.52% treatment.

Survival

[WAAI]	Mortality	Σxy	*df*	*r*	
Bioassay VIII					
Σx 0.975	Σy 324	73.580	3	0.783	NS
Σx^2 0.3591	Σy^2 22040				(p>0.1)
n 5	n 5				
Bioassay IX					
Σx 0.975	Σy 224	55.640	3	0.890	*
Σx^2 0.3591	Σy^2 11104				(p<0.05)
n 5	n 5				

Corrected WGL

[WAAI]	c.WGL	Σxy	*df*	*r*	
Bioassay VIII					
Σx 0.975	Σy -2.396	-0.8828	3	-0.821	NS
Σx^2 0.3591	Σy^2 2.6628				(p>0.05)
n 5	n 5				
Bioassay IX					
Σx 0.975	Σy 6.119	0.80788	3	-0.909	*
Σx^2 0.3591	Σy^2 8.5493				(p<0.05)
n 5	n 5				

Figure 89. Correlation analysis for bioassay VIII and IX (Day 10) results. Level of significance is indicated by NS, Not Significant; *, p<0.05; **, p<0.01; or ***, p<0.001.

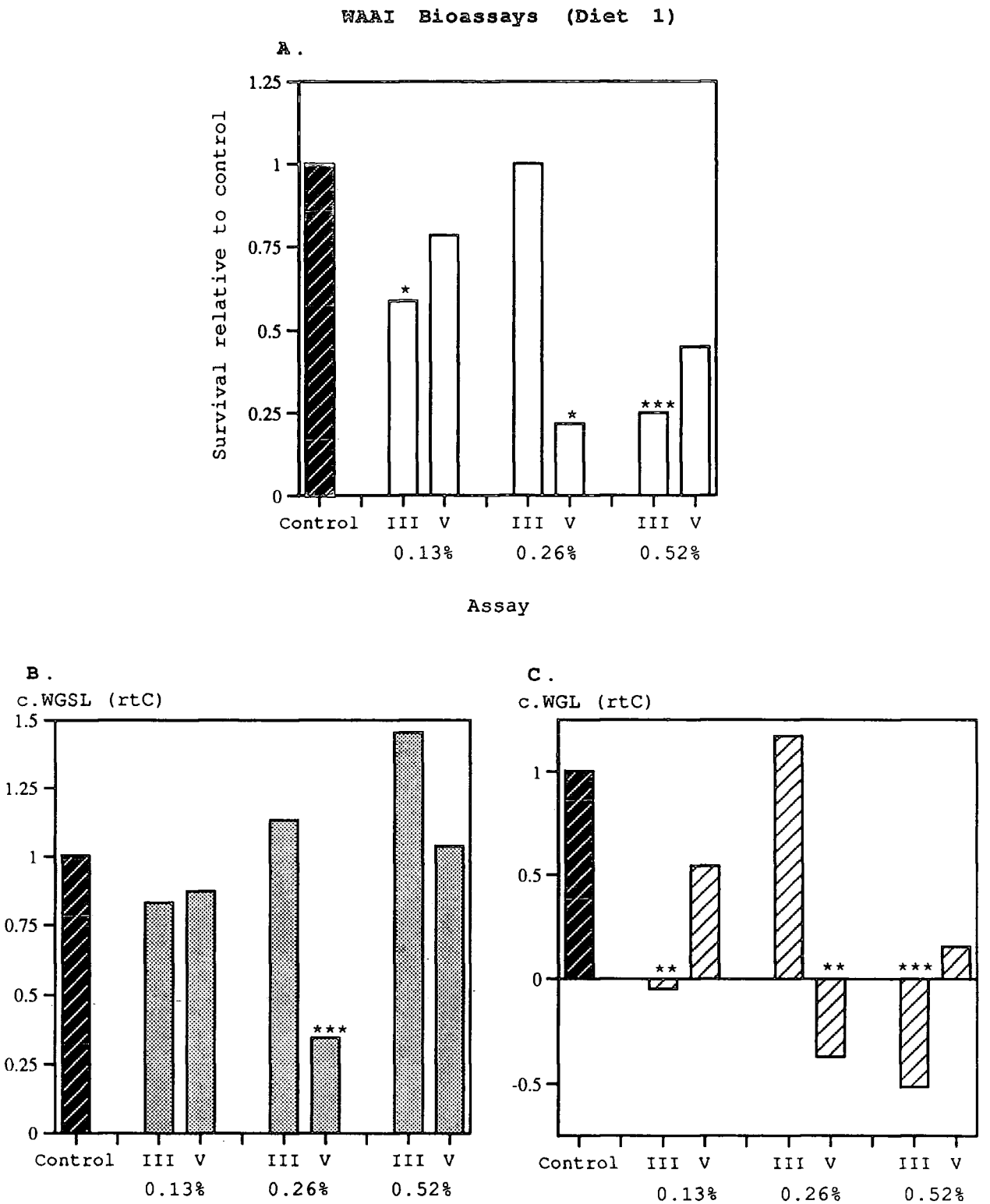


Figure 90. Survival (panel A), corrected WGSL (panel B), and corrected WGL (panel C) of SCR larvae on artificial diet 1 treated with WAAI, incorporated at 0.13%, 0.26% and 0.52% as indicated. All values are given relative to Controls (rtC). Assays were conducted for 11 days. Significant differences from controls are indicated by *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

The effect of WAAI using the normal artificial diet (diet 1) was investigated in bioassays III and V (see Figure 90). The results exhibited a greater variability that on diet 3, but interestingly, the same patterns were seen as with the starch diet, little alteration seen in the corrected c.WGSL data, except for a significant decrease recorded at day 11 on the 0.26% treatment in bioassay V. The mortality, except for the 0.26% treatment in bioassay III, showed a general increase with increasing inhibitor concentration.

TREATMENT COMBINATIONS - CpTI+WAAI & CpTI+WAAI+GNA

CpTI, WAAI and GNA were employed to assess the effect of combining treatments. Figure 91 shows the results obtained from combining CpTI and WAAI (panel A), and CpTI, WAAI and GNA (panel B), all at 0.26% (w/v). Both the combination treatments were found to result in an increased mortality rate. While the effect of the two treatments on larval survival was similar, their effect on weight gain, in bioassay V was most distinct, the CpTI+WAAI combination produced a c.WGSL significantly increased from the control, while the triple combination showed a significant reduction. The c.WGSL data from bioassay IV showed almost no deviation from that of the control. Corrected WGL data, in both combinations, showed a marked decrease from the controls, this difference being significant in both cases in bioassay V.

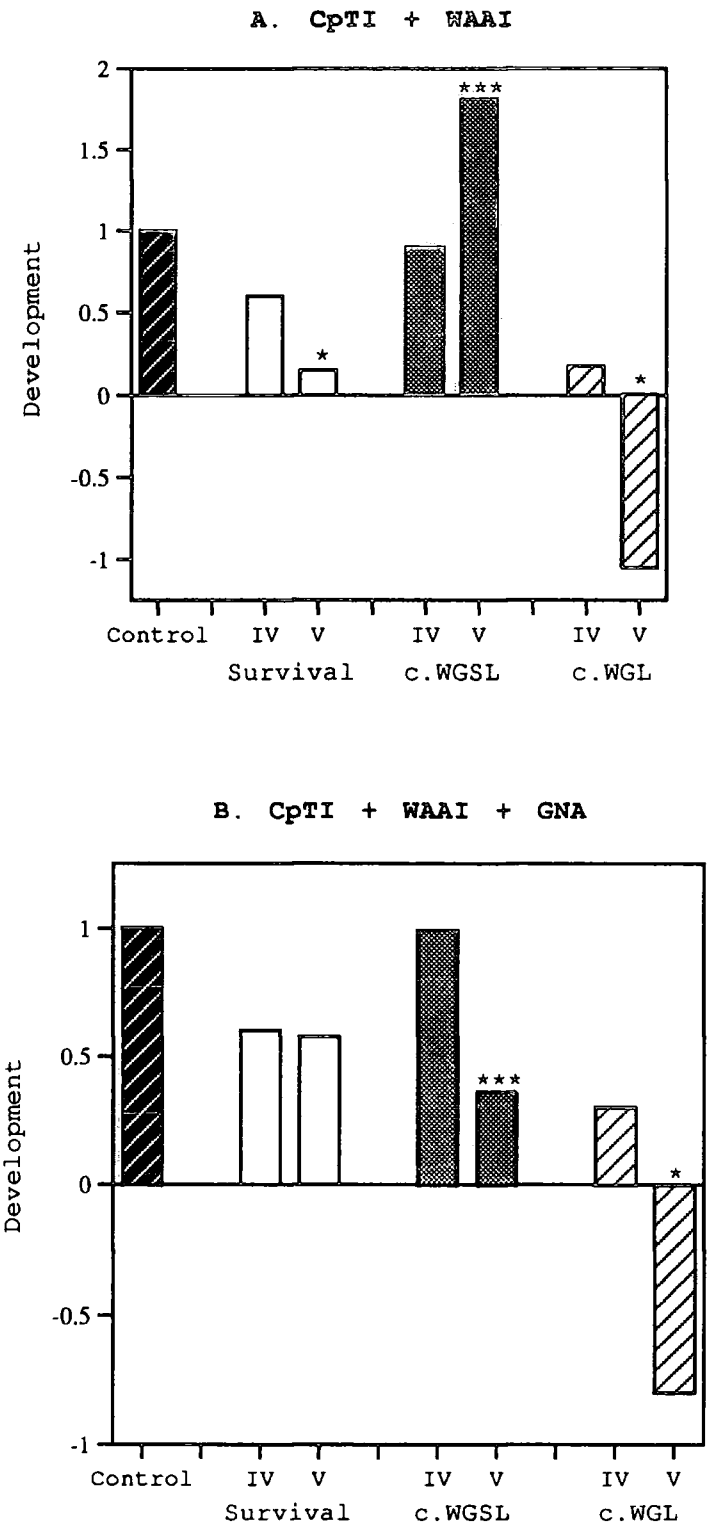


Figure 91. Data for the survival, corrected WGSL and corrected WGL for SCR larvae on artificial diet treated with CpTI and WAAI (Panel A), or CpTI, WAAI and GNA (Panel B), all incorporated at 0.26% (w/v). All values are given relative to Controls. Both assays were conducted for 11 days. Significant differences from the controls are indicated by *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

IN VIVO EFFECTS OF PLANT ANTIMETABOLIC PROTEINS - BIOASSAY DISCUSSION

The need for *in vivo* testing of inhibitor effects

The high levels of cysteine protease and α-amylase activity demonstrated in SCR larval gut extracts, indicates the potential for using CPIs and α-amylase inhibitors as tools for enhancing resistance to SCR. Yetter *et al.* (1979) demonstrated, in some wheat varieties, a correlation between *in vivo* resistance to *Tenebrio molitor* and the extent of *in vitro* inhibition of the larval α-amylase by the extracted inhibitors, indicating that α-amylase inhibitors in plant tissues may confer resistance to some insects. Similarly, Huessing *et al.* (1991c) demonstrated, both *in vitro* and *in vivo*, the effectiveness of bean α-amylase inhibitor (purified according to Moreno and Chrispeels, 1989) on α-amylase activity and development of *Callosobruchus maculatus*, again indicating a role for α-amylase inhibitors in protection. Evidence for the role of protease inhibitors in plant defence was provided both by the wound-induced accumulation of protease inhibitors in tomato (Green & Ryan, 1972), and by the enhanced resistance to insect attack demonstrated by transgenic plants expressing protease inhibitors (Hilder *et al.*, 1987; Johnson *et al.*, 1989). These examples, however, are all concerned with the inhibitors of serine proteases, less work having been conducted with the cysteine proteases. The cysteine protease inhibitor, ozcI, has been expressed in transgenic tobacco and lettuce (Masoud *et al.*, 1993; AGC, unpublished results), but no enhanced levels of resistance to insect attack have, as yet, been reported.

Gatehouse *et al.* (1986), as discussed previously, reported the major 0.28 protein to be inhibitory *in vitro* to the α-amylases of both *Callosobruchus maculatus*, the cowpea weevil, and *Tribolium confusum*, the confused flour beetle, a storage pest of wheat products. *In vivo*, however,

while the protein, incorporated into artificial seeds, proved toxic to *C. maculatus*, no detrimental effects were observed with *T. confusum*.

Clearly, high levels of inhibitory activity *in vitro*, even when demonstrated to occur against enzymes from a specific insect, are not reliable criteria for the selection of resistance factors, rather *in vivo* studies are absolutely necessary to identify any shielding or detoxifying mechanisms exhibited by the insect. Consequently, bioassays were conducted, incorporating various proteins into artificial diet, monitoring the effects on the survival and development of larvae feeding on these diets, comparing the results to those from larvae on control diets. Development was measured as weight gain.

Influence of initial weight on weight gain

Throughout the insect feeding trials in this work, attempts were made to employ larvae of a uniform size. However, the size of insects used in one bioassay often varied from those used in other bioassays, depending on the availability of larvae. When the weight gain (WGSL, weight gain per surviving larva) data was computed, a high level of variability was observed within the data, even within the control data. In order to establish whether the variability of the initial weight was influencing the subsequent WGSL, the control data was collated and the relationship between initial weight and WGSL (day 9) examined by correlation analysis. This analysis revealed a highly significant correlation between the two measurements, the coefficient of determination demonstrating that 30.9% of the variation occurring in the WGSL data was due to the influence of the initial weight of larvae.

Obviously, other factors must also act to cause variation in weight gain, but as all the bioassays were conducted in the same manner and, as far as is known, under the same conditions, no influencing factors other than that of the initial weight could be corrected for, and as this correction would, as shown by the coefficient of determination, decrease variability by

over 30%, it was judged essential. A correction term was formulated such that the initial weight of all larvae would be made approximately equal to the mean initial weight, thus annulling the variation caused by differences in the initial weight.

The success of this correction term was demonstrated by correlation analysis of the initial weight and the corrected WGSL data, with no significant dependence demonstrated. Analysis of WGSL data from days 10, 11 and 12, and from assays employing diet 3, corrected using this correction term, demonstrated no significant deviation from the corrected day 9 data. All data, both WGSL and WGL (weight gain per larva, dead or alive), were treated with this correction term.

Distribution of corrected WGSL and WGL data

To analyse the corrected WGSL and WGL data, it was first necessary to determine the type of distribution formed by the data. As most natural probability distributions tend towards the normal distribution, it was expected that the weight gained by larvae of approximately equal initial weight, over a given time period and under the same conditions, would exhibit a distribution close to the normal distribution. The corrected WGSL and WGL control data represent two such distributions, the initial weights corrected to be approximately equal to the mean initial weight, these were analysed and neither found to deviate significantly from the normal distribution.

As no significant deviation from the normal was demonstrated with the corrected control data, it was taken that all the corrected WGSL and corrected WGL data could be assumed to be normally distributed, thus allowing analysis of the data using Student's *t*-test and ANOVA, both of which take the normal distribution of data as a basic assumption.

Choice of diet

Two artificial diets were assayed for their suitability for use in bioassay: the first, diet 1, as recommended by Branson *et al.* (1975) and the second, diet 2, modified from that of Marrone *et al.* (1985), the only difference being the increased levels of sucrose and linseed oil in diet 2.

Survival on the diets was fairly low, only 54% of larvae surviving to day 8, but as no estimate for the natural mortality rate of larval SCR was available, it was impossible to infer whether this survival rate represented the maximum level of survival to be expected, or whether the low level was indicative of sub-optimal conditions presented by the artificial diet. From the vast number of eggs laid, the maximum number observed for a single SCR female recorded as 1198 eggs (see Krysan, 1986), one would realistically expect a large number to be inviable, and a considerable proportion of the larvae to die during development, otherwise the problems of SCR infestation of crops would be far greater than it actually is. Consequently, it was accepted that the expected survival rate of SCR larvae was quite low, and as no alternative diet was readily available for testing, diets 1 and 2 were assumed to be suitable for sustained larval growth.

No significant differences were demonstrated between the two diets, in terms of survival, corrected WGSL and corrected WGL, although all measurements were greater for larvae on diet 1. Accordingly, diet 1 was employed in the majority of subsequent bioassays.

Starch diet

The one flaw in the composition of diet 1, was the high concentration of sucrose and the lack of starch. This made the diet unsuitable for bioassays where inhibitors of α-amylase were to be tested. The diet was modified, replacing the sucrose with an equivalent mass of starch (3.225% w/w).

It has been suggested by Branson (1986) that sucrose, at a concentration of around 2%, was necessary in the diet of SCR larvae as a feeding stimulant. Sucrose was thus added to the modified diet at six different concentrations in the range 0 to 1.25% (w/w), and tested in bioassay. Surprisingly, no trend in larval survival and development was observed corresponding to the concentration of sucrose in the diet, and no feeding stimulus was derived from the higher sucrose concentrations, as would be indicated by increased weight gain. No significant differences in weight gain were demonstrated on the diets, except with the 1.0% sucrose diet, on which corrected WGSL was markedly reduced. As the survival on the 0% and 0.25% sucrose diets was notably higher than with the other diets, these two diets were compared in another bioassay. Again no significant differences were demonstrated between larvae on the two diets, although both survival and weight gain were consistently higher on the 0.25% sucrose diet. This being the only difference, the 0.25% sucrose diet was chosen for use with α-amylase inhibitors.

BIOASSAYS

A range of compounds were tested for their effectiveness against larval survival and development *in vivo*, using bioassays. The results obtained showed a high degree of variability, even between control data. A certain level of variability would be expected, that inherent in the insect population. The stability of the population has been noted to vary throughout the year, becoming least stable, and most prone to crashing, during the winter months, possibly due to an increase in temperature fluctuations. The bioassays reported in this work were conducted at the following times of year: bioassay II in January; bioassay III in March; bioassays VI and VIII in April; bioassay IX in May; bioassay VII in June; bioassay IV in August; bioassay V in August/September; and bioassay I in

early Dec. This difference in timing may, at least partially, explain the increased variability demonstrated by the results of some bioassays.

LECTINS

(1) Elderberry lectins

The lectins SNA1 and SNA2, from elderberry, *Sambucus nigra*, bark (Broekaert *et al.*, 1984; Kaku *et al.*, 1990) were tested against SCR larvae in bioassays I and II, both were incorporated into artificial diet at a concentration of 0.26% (w/v). These lectins exhibit different binding specificities, SNA1 showing high affinity for N-acetylneuraminic acid linked by α2,6-linkages to galactose or N-acetylgalactosamine (Shibuya *et al.*, 1987), while SNA2 is simply Galactose/N-acetylgalactosamine specific (Kaku *et al.*, 1990). The results showed that larval survival was little affected by the treatments, while weight gain (c.WGSL) was slightly increased. Larval weight gain was most enhanced by treatment with SNA1. Clearly, the desired results from these bioassays would be a significant decrease in survival or weight gain, or both, indicating that a compound was detrimental to the insect. No such adverse effects were demonstrated with either of the elderberry lectins.

Interestingly, Czapla and Lang (1990) tested elderberry bark lectin (SNA1) against larval SCR, by topical application of the lectin onto the surface of the diet. By day 7, the mortality rate was equal to the control, but the weight recorded for larvae on treated diet was significantly lower than the controls. This lack of affect on survival is in agreement with the results obtained in this work, but the weight gain decrease observed by Czapla and Lang (1990) is contrary to that demonstrated with either of the two elderberry lectins used in this work.

(2) Pea lectin

Plec, a D-mannose/D-glucose specific lectin isolated from pea, *Pisum sativum* (Trowbridge, 1974), was tested, at a concentration of 0.26% (w/v), against SCR larvae in bioassays IV and V. Survival of the larvae was unaffected by the treatment. Weight gain data gave variable results, the plec treatment in bioassay IV showed a slight decrease in corrected WGSL from the control, while in bioassay V it produced an increase. Neither the increase or decrease in weight gain from the controls was significant, indicating that plec has no effect on SCR larval development, beyond possibly increasing the variability of their weight gain.

Boulter *et al.* (1990) demonstrated the efficacy of plec against *Heliothis virescens* (tobacco budworm) when expressed in transgenic tobacco plants. Plec caused a reduction in survival of almost 20%, while insect biomass (total) was around 25% of that on control material. Clearly, no such dramatic effects were produced by the lectin when ingested by SCR larvae, once again demonstrating the necessity of testing individual compounds with individual pest species.

(3) Wheat germ lectin

WGA, the N-acetyl-ß-D-glucosamine binding lectin from wheat germ (Allen *et al.*, 1973), incorporated into diet at 0.26% (w/v), was demonstrated, in bioassay V, to slightly enhance larval survival. The corrected WGSL data demonstrated a significant reduction in weight gain caused by WGA treatment, an effect also seen in the corrected WGL data.

Clearly this lectin possesses a greater ability to adversely affect the survival and development of SCR larvae than was demonstrated by the other lectins tested. The differences observed in their efficacies is presumably due to the specificity of their carbohydrate binding properties.

WGA was also tested against SCR by Czapla and Lang (1990). A topical application of lectin to the diet surface resulted, by day 7, in a 10%

decrease in survival and 58% weight loss when compared to the controls. The difference in weight recorded for the WGA treatment and control was shown to be significant. These results are in agreement with that demonstrated in this work, with survival rates little affected, and the reduction in weight gain, the mean corrected WGSL for day 9 demonstrated here, being around 50% lower than that of the controls. Chrispeels and Raikhel (1991) report that Czapla also demonstrated that WGA, when incorporated into artificial diet at 0.3% (w/w), caused a 50% reduction in the weight of SCR larvae in comparison to those on control diets. This is in direct agreement with that reported here. Clearly, WGA is a more suitable for use in a SCR control program than the elderberry lectins or plec, WGA producing greater adverse effects in the larvae.

(4) Snowdrop lectin

GNA, the mannose-specific lectin isolated from snowdrop (*Galanthus nivalis*) bulbs by Van Damme *et al.* (1987), was tested against larval SCR, at a concentration of 0.26% (w/v) in artificial diet. In bioassays I and II the GNA employed was supplied by W. Peumans (Leuven, Belgium), while that used in bioassay V was a 25:40 mixture (w/w) of this and GNA prepared in Durham, using the same method (Van Damme *et al.*, 1987).

In both bioassays I and II, GNA was seen to cause an increase in mortality, while in bioassay V, the GNA mixture produced a small enhancement of survival. A decrease in weight gain (c.WGSL) was demonstrated in all cases. The marked difference between the effects of the Peumans GNA and the GNA mixture used in bioassay V, was most visible in the corrected WGL data, the former producing a marked reduction in corrected WGL, while the latter had little effect.

The results from bioassays I and II indicate that GNA acts to both increase mortality in SCR larvae, and decrease the weight gained by those surviving. The results from treatment with the GNA mixture show a reduced

efficacy, the adverse effects only being apparent in the reduction of weight gain, indicating that the Durham preparation of GNA was different to that supplied by Peumans, and was either less effective or acted in a different manner. A haemagglutination assay conducted with the two GNA samples demonstrated that the Peumans GNA was approximately 64-fold more effective in agglutination than that prepared in Durham (Gatehouse,AMR., personal communication). This shows that, although the two GNA samples were purified by the same method (Van Damme *et al.*, 1987), the lectins isolated possess different properties, both in terms of haemagglutination ability and insecticidal properties, although strong agglutination properties are not necessarily a direct indication of efficacy against insects. It has been suggested that GNA represents one member of a family of lectins from snowdrop bulbs (Gatehouse,L. and Bown,DP., personal communication), the difference in activity, demonstrated here, probably being due to the isolation of different forms, possibly because of varietal variation within the snowdrop bulbs used for the extraction.

GNA, as discussed in the introduction, has been shown to exhibit antimetabolic effects against the rice brown planthopper, *Nilaparvata lugens*, and the rice green leafhopper, *Nephotettix cinciteps* (Powell *et al.*, 1993). Adverse effects have also been observed with a number of other insects (Gatehouse,AMR., personal communication). From the results observed in this work, GNA would seem a suitable candidate for use in a transgenic protection strategy for crop plants. It would seem advantageous to assess the effects of the various forms of snowdrop lectin, chosing the most effective form for further testing, both in transgenic plants and for adverse effects on any non-target organisms, especially mammals.

RIPs - saporin

Saporin, a type-I ribosome-inactivating protein (RIP) from *Saponaria officinalis*, was tested for anti-insect activity against SCR larvae,

incorporating the protein into artificial diet at two concentrations, 0.0013% and 0.0071% (w/v), in bioassays I and II respectively. Clear differences were observed between the two bioassays, the lack of effect demonstrated in bioassay I possibly arose from the low concentration of saporin used. The results from the higher concentration treatment showed that saporin caused increased mortality in SCR larvae, but, interestingly, those surviving gained more weight than those on control diet, showing an enhancement of development.

As discussed in the introduction, saporin was demonstrated by Gatehouse *et al.* (1990) to be highly toxic to two species of Coleoptera, *Callosobruchus maculatus* and *Anthomonas grandis*, while having little effect on the two Lepidopteran species tested, *Spodoptera littoralis* and *Heliothis virescens*. It was anticipated that the effect of saporin on SCR larvae would be similar to that reported with the other Coleopteran species. Gatehouse *et al.* (1990) demonstrated LD_{50} values of less than 0.01% (dry weight), a level of saporin corresponding to that employed here in bioassay I. SCR larvae appear to be far less susceptible to saporin toxicity than these other species, possibly due to an ability to hydrolyse the protein, inactivating it and consequently providing additional amino acids from the breakdown of the protein, thus supplementing the diet and promoting weight gain. From the significantly reduced survival rate demonstrated in this work, it would appear that any such ability to hydrolyse and detoxify the protein may vary from larva to larva. This would explain the high level of survival on the diet containing only a low concentration of saporin, more larvae demonstrating sufficient hydrolysing power to inactivate this low level of RIP. However, the unaltered weight gain in these surviving larvae, suggests that the hydrolysed protein was not subsequently employed as a dietary supplement. Obviously, if the ability exists for SCR larvae to detoxify certain levels of saporin, then, should this protein be employed in a control strategy, high concentrations would be necessary to prevent the survival of

those larvae with high detoxification capabilities, preventing the population from becoming increasingly resistant to the protein. The low levels of efficacy demonstrated in this work, however, dispute the suitability of saporin for use in any rootworm control program.

SERINE PROTEASE INHIBITORS - CpTI

CpTI, the cowpea trypsin inhibitor, was shown by *in vitro* enzyme assays to cause a substantial decrease in rootworm proteolytic activity: a decrease in cysteine protease activity of 37% demonstrated with gut extracts from adult WCR, using Z-Phe-Arg-AMC as a substrate, an a 10% decrease in the total protease activity of larval SCR gut enzymes, using azocasein as a substrate. The *in vivo* effects of CpTI on SCR larvae were investigated by feeding trials, the inhibitor incorporated into artificial diet at concentrations of 0.13%, 0.26% and 0.65% (w/v).

The influence of CpTI treatment on survival and development showed great variability between bioassays, all three concentrations were represented in bioassays I and II, but no clear relationship between level of inhibitor and the strength of effect was demonstrated.

In assay I, an increase in mortality resulted from the 0.26% treatment, while the 0.65% CpTI diet caused no alteration of survival but reduced the developmental rate, causing a significant decrease in c.WGSL. In assay II, survival and weight gain were both adversely affected by the 0.13% and 0.26% treatments, although no such effect was demonstrated in the 0.65% treatment. The treatment used in assay V also failed to cause any adverse effect.

A number of preparations of CpTI were used to accumulate sufficient material for use in bioassay. The great variability demonstrated in the results, a variability too great to be explained by the natural variation within the insect population, suggests that the purity of these CpTI samples may have varied, resulting in less CpTI than expected being present in some

assays, and also in the presence of contaminants. The results can, therefore, be interpreted in one of two ways: that the significant decreases in survival and development demonstrated in these assays was due to the action of CpTI in a relatively pure form, the reduced activity in other treatments being due to the presence of contaminants in the preparation; or that the adverse effects demonstrated with some of the treatments were due to contaminants in the CpTI preparation, possibly such as the cowpea cysteine protease inhibitor, and that the smaller effects were demonstrated with the purer CpTI samples. Without careful investigation of the CpTI preparations, and bioassays using exhaustively purified protein, it is impossible to know whether the effects demonstrated here were due to CpTI or a contaminant.

CYSTEINE PROTEASE INHIBITORS

(1) cystatin

Chicken egg-white cystatin, the cysteine protease inhibitor shown in *in vitro* enzyme assays to be extremely effective against the cysteine proteases of the larval gut, was tested *in vivo* in bioassay I, added to the artificial diet at a concentration of 0.0167% (w/v). From the high levels of protease inhibition caused by low concentrations of cystatin *in vitro*, it was expected that cystatin, even at this low concentration would prove detrimental to larvae, in reality, however, the survival rate was decreased only marginally and the corrected WGSL data showed a significant increase in weight gain over that of the controls.

After demonstrating *in vitro* the prominence of cysteine proteases amongst the digestive enzymes of larval SCR, and the effectiveness of cystatin as an inhibitor of this enzyme activity, the results from this bioassay were most surprising, the only effect demonstrated being a stimulatory one. It is possible that the lack of effect was due to the low

concentration of cystatin employed in the bioassay, but the results cast grave doubts as to the potential of CPIs for use in SCR control strategies.

(2) SQRG-I

SQRG-I, the sample of oryzacystatin I partially purified from rice germ, was employed in bioassay with larval SCR, to investigate the kind of activity that might be expected with the pure protein. The preparation was incorporated into artificial diet at a concentration of 0.13% (w/v).

An increased rate of mortality was demonstrated, along with a small decrease in corrected WGSL, creating a quite notable effect overall, certainly far greater than that demonstrated with the low concentration of cystatin. These results indicated that oryzacystatin, in a pure form, could have significant adverse effects on larval survival and development, justifying the continuation of work concerning this protein and further attempts to isolate sufficient pure protein for use in bioassay.

(3) Thaumatin

Thaumatin, the intensely sweet-tasting protein from *Thaumatococcus danielli*, was tested in bioassay against larval SCR, because of its homology with the protein purified from rice in fraction SB (see Chapter 4). *In vitro* enzyme assays had demonstrated that thaumatin possessed weak inhibitory activity towards the cysteine proteases of the insect gut, an inhibition level insignificant when compared to the almost complete inhibition demonstrated by the rice CPI. While later work confirmed that the thaumatin-like protein was a co-purified protein and not the major source of the inhibitory activity, thaumatin was assayed *in vivo* against SCR larvae to establish whether the weak inhibition demonstrated *in vitro* would be reflected in its effects on larval development *in vivo*.

Both survival and weight gain of larvae on the thaumatin-treated diet were reduced in comparison with the controls, producing a c.WGL

dramatically lower than the control. This effectiveness of thaumatin was interesting, while not having any significant effect on larval survival and development, notable adverse effects were demonstrated, effects apparently greater than those of the wheat germ lectin, WGA, and at least comparable with those demonstrated with the impure oryzacystatin fraction.

(4) E64

E64, the specific inhibitor of cysteine proteases isolated from *Aspergillus japonicus* (Barrett *et al.*, 1982), which had previously been shown to be highly effective against the cysteine protease activities present in larval gut extracts (see Chapter 3), was assayed *in vivo* at 0.013% and 0.026% (w/v) in artificial diet feeding trials. The efficacy of E64 treatments was surprising. In almost all cases mortality was increased markedly, while surviving larvae showed virtually no weight gain above the initial weight.

This almost complete lack of weight gain, suggests that E64 was acting as an antifeedant. If the action of the compound were purely one of enzyme inhibition, some reduction in weight gain would be anticipated, resulting from the lack of free amino acids available from the diet, but other nutrients, such as sucrose, would be accessible in the diet, enabling some growth to occur. With E64, the situation seemed to be different, the almost complete lack of growth, as determined by corrected WGSL, suggested that feeding was at a minimum, showing a greater effect than would be expected with just a single protease inhibitor. Although an increase in mortality was demonstrated, a comparison with the no-diet control results showed that some feeding must have occurred in order to maintain such relatively high numbers of larvae, all the larvae in no-diet controls being dead by day 9, and the majority dead by day 7.

One explanation for the results may be that E64, a small molecule, might somehow be able to pass through the gut wall and affect enzyme activity elsewhere in the body, however, as virtually no protease activity

was detected in the rest of the larva (see Chapter 3), this possibility seems most unlikely..

Another conceivable explanation may be that E64, while acting as an enzyme inhibitor, also serves as an antifeedant, discouraging the larvae from feeding. Any diet consumed would provide some protein along with a small amount of E64. This E64 concentration may be insufficient to cause extensive inhibition of proteases, thus some amino acids might be derived from the diet. While their lack of nutrition may entice larvae to further feeding, the compulsion to eat may be largely overcome by their repulsion at the presence of an antifeedant. Following this course the larvae would eventually die of starvation or from nutritional problems caused by enzyme inhibition.

This theory, however, was not upheld by the results obtained for the 0.026% E64 treatment, the corrected WGSL in this case was far greater than that demonstrated with the lower E64 concentration, suggesting rather, that the sole action of E64 was that of a potent cysteine protease inhibitor. The action of E64 will be discussed again, later, in comparison with the Rozc bioassay results.

(5) Recombinant oryzacystatin

Rozc, the recombinant oryzacystatin produced in *E. coli* in this work, shown to be an effective inhibitor of papain, the plant derived cysteine protease, and of the larval gut cysteine proteases *in vitro*, was assayed for its effectiveness against larval SCR *in vivo*, by incorporation into artificial diet at concentrations of 0.0325%, 0.065%, 0.13% and 0.26% (w/v) in bioassays VI and VII. E64, at 0.013%, was assayed concurrently for comparison.

Survival of larvae decreased with an increased concentration of Rozc, in comparison with the controls, although no significant correlation could be demonstrated in either bioassay between Rozc concentration and mortality. Weight gain appeared to become more variable as a result from Rozc

treatment, assay VI showing consistent reductions in c.WGSL, while assay VII revealed both increases and decreases from the control.

The corrected WGL data show an overall view of the effect of Rozc on the population. Both assays demonstrated a marked reduction in c.WGL, generally increasing with inhibitor concentration, a significant correlation ($p<0.02$) being demonstrated in bioassay VII.

In a comparison of these results and those demonstrated with the SQRG-I treatment, the observations of decreased survival coupled with little effect on weight gain, correlate well with the results of the lower concentration treatments with Rozc. *In vitro* assays, comparing Rozc and cystatin, indicated that the recombinant protein possessed inhibitory properties equivalent to those of native oryzacystatin, thus the results demonstrated in bioassay are assumed to represent those that would occur with the native oryzacystatin.

Concurrent assays with E64 provided an interesting comparison (see Appendix), both inhibitors acting on the cysteine protease activity of the larval gut. While the survival of larvae on E64 treated diets was greater than that with the 0.26% Rozc treatment, the weight gain with E64 treatments was consistently negligible. Comparison of the surviving larvae showed that while larvae on E64 treatments appeared normal, ie. like the controls apart from their almost complete lack of weight gain, those on the Rozc treatments seemed bloated in appearance, and when compared with normal larvae, reared normally and not used in the bioassay, of roughly the same size, the larvae from the Rozc treatments appeared fatter, almost swollen.

The action of Rozc on larval development was expected to be one of protease inhibition, causing a reduction in the availability of free essential amino acids, by one or both of the following: inhibiting protein hydrolysis, and thus decreasing the release of essential amino acids from dietary proteins; or stimulating hyperproductivity of cysteine proteases to replace those inhibited, a process which would also deplete the levels of available

essential amino acids. From the above observations, it was speculated that this deprivation of free amino acids may, as discussed for E64, cause a compulsion to feed in order to ingest greater quantities of protein, and so increase the theoretical amount of available amino acids. Along with the ingested proteins, however, would be more Rozc, causing greater inhibition of enzymes of consequently a greater lack of amino acids. An effect of this kind would eventually lead to the situation demonstrated here with Rozc treatment in bioassay VII, the greater the Rozc concentration, the greater the tendency to feed, the larvae becoming bloated in their attempt to maintain an amino acid supply. Comparison with the E64 treatments, once again suggests that E64 also causes some antifeedant effect, stopping the larvae from the overfeeding activity apparent on the Rozc diets.

α-AMYLASE INHIBITORS - WAAI

WAAI, the α-amylase inhibitor from wheat, shown in this work to cause strong inhibition of SCR larval amylase activity, was assayed against larvae in bioassays using both the normal artificial diet (diet 1) and diet 3, the modified diet with only 0.25% (w/w) sucrose and 3.225% (w/w) starch. As diet 1 contains no starch, but rather, 3.225% (w/w) sucrose, there is no component in the diet on which the insect amylases can work, thus inhibiting these amylases should not result in any nutritional deficiency. Bioassays were, however, conducted on the normal artificial diet to investigate whether any adverse effects would be produced. The results from assays employing the modified diet will be discussed first.

WAAI was incorporated into diet 3 at concentrations of 0.065%, 0.13%, 0.26% and 0.52% (w/v) and the effect on larval survival and development recorded. Survival of larvae was notably reduced with all WAAI concentrations, mortality increasing with inhibitor concentration, a significant correlation demonstrated between inhibitor concentration and mortality

($p<0.05$) for bioassay IX (day 10). Corrected WGSL was largely unaffected by the WAAI treatment, although variability, as with the Rozc treatment, appeared to be increased. Corrected WGL was reduced from the control in all cases, a significant correlation demonstrated between WAAI concentration and c.WGL for bioassay IX (day 10).

The results show that while having little effect on weight gain, WAAI caused a significant increase in larval mortality. Larvae were not killed immediately after feeding, survival on day 9 of bioassay IX being close to that on control diets (see Appendix), demonstrating that the mechanism of WAAI was not merely one of toxicity, instead, larvae were found to grow apparently normally for several days, presumably during which the inhibitor arrested amylase enzymes, preventing the breakdown of dietary starch, thus reducing the availability of soluble sugars, resulting in a decreased energy supply and consequential metabolism problems, the weakened larvae eventually reaching the limit of their resources, and the population crashing.

The effects of WAAI on SCR larvae feeding on the normal artificial diet was investigated in bioassays III and V. The results exhibited a greater variability that on diet 3, but interestingly, the same patterns were seen as with the starch diet. Little alteration was seen in the corrected WGSL data, while mortality showed a general increase with increasing inhibitor concentration. Assuming that the effects demonstrated with WAAI treatment using the starch diet did result from inhibition of amylase activity, then for the same effect to be demonstrated in larvae fed on a starch-free diet, where amylase activity is not required for the steady supply of glucose necessary to sustain normal metabolic processes, this glucose supply must again become limited, thus leading to the same effects. Of course, the insect amylases will be inhibited by WAAI, irrespective of whether or not there is substrate present, but insects feeding on diet 1 should be largely unaffected by this inhibition as, in this case, glucose, along with fructose, should be available from the digestion of dietary sucrose. The fact that

larvae are affected in a similar manner, regardless of the composition of the diet, suggests that either sucrases are not present and the insect amylases are necessary in the absence of dietary starch or glucose, for the mobilisation of carbohydrates stored within the insect; or that the sucrases present in the digestive system of the insect are also inhibited by the wheat protein. No investigation of sucrase activity, or its inhibition by WAAI, was conducted *in vitro*, thus no confirmation of either theory is possible. The possibility of WAAI action through the inhibition of cysteine proteases was dismissed using *in vitro* studies (see Chapter 4), no CP or CPI activity was demonstrated.

COMBINATIONS - CpTI+WAAI & CpTI+WAAI+GNA

To investigate the possible additive effects of proteins on the survival and development of SCR larvae, two combinations were employed in bioassay, CpTI and WAAI, and a triple combination of these two plus GNA, all proteins were used at a concentration of 0.26% (w/v).

Both combinations resulted in an increased mortality rate, but their effects on weight gain were distinct. The CpTI+WAAI combination produced a weight gain equal to or increased from the control, while the triple combination showed either no alteration of the growth rate or a significant reduction. It was notable that the results of the CpTI assay conducted in bioassay V concurrent with the combination trial (see Appendix), differ little from the controls, suggesting that the majority of the activity demonstrated by the CpTI+WAAI combination was derived from the WAAI component. The WAAI treatments conducted in bioassay V, reveal the clear pattern of reduced survival and largely unaffected weight gain also demonstrated in the double combination treatment results.

The different effects on weight gain demonstrated in the two combination assays may have resulted from the GNA employed, that used in assay IV was the pure GNA supplied by Peumans, while for bioassay V the

mixture of GNA (Peumans and Durham) was employed. Comparing the effects of these samples of GNA alone, the Peumans GNA (used in assays I and II) decreased weight gain and increased mortality, while the GNA mixture caused reduced weight gain but enhanced survival. Surprisingly then, the results show a reduction of survival in both triple combination assays and differing weight gains, a significant reduction in weight gain caused in the combination with the GNA mixture, while the Peumans GNA in combination caused no alteration of weight gain from the control. By direct comparison with the CpTI and GNA data from bioassay V (see Appendix), a strong additive effect can be seen in the combination treatments, greater mortality occurring in the double combination than with either protein taken individually, and this coupled with a reduction in weight gain with the triple combination.

SUMMARY

Lectins

Two lectins were demonstrated to cause adverse effects to developing larvae, WGA, an N-acetyl-ß-D-glucosamine binding lectin from wheat germ, and GNA, a D-mannose specific lectin from snowdrop. The latter was found to be the most potent of the four lectins tested, but further work would be necessary to identify the form of GNA most effective against these insects, this form then being employed in the transformation of test plant material. Interestingly, while the D-mannose specific GNA causes dramatic reductions in weight gain of SCR larvae, plec, a lectin with similar specificities, binding both D-mannose and D-glucose, produces no notable adverse effects. The elderberry lectins, SNA1 and SNA2 were also found to produce no adverse effects in SCR larvae. From the various reports of the anti-insect properties of lectins, they appear to demonstrate a surprising selectivity in their action. This specificity could be usefully employed in an insect control

program to enable selectivity of effect. If lectins, as suggested by Gatehouse *et al.* (1989), do act by binding to midgut cells, then they may also be useful in combination with other proteins, to facilitate their entry into the epithelial cells of the midgut, promoting their action within the insect.

RIPs

Saporin, a type-1 RIP, caused no adverse effects in SCR larvae when provided in artificial diet at a concentration of 0.0013% (w/v). At the higher concentration of 0.0071% saporin (w/v), larval mortality was markedly increased. It was expected that RIPs would prove highly toxic to SCR larvae, even at low concentrations, the results, however, suggest the existence of some tolerance mechanism in the insects, possibly an ability to detoxify low levels of the protein by degradation. This casts doubt on the suitability of saporin, and possibly of other type-1 RIPs, for use in a control program for corn rootworms.

Enzyme inhibitors

Those proteins found to cause inhibition of larval cysteine protease activity were, where possible, tested for *in vivo* efficacy in bioassays, E64 was also tested for comparison. CpTI, although generally found to cause detrimental effects to the larvae, gave inconsistent results, treatment sometimes producing an increased mortality with weight gain unaffected, while another concentration may cause no alteration of survival but decrease weight gain markedly. Cystatin, surprisingly, caused virtually no adverse effects when present in the diet at 0.0167% (w/v), a result most remarkable because of its potency *in vitro*. Despite the disappointing lack of *in vivo* efficacy demonstrated by chicken egg white cystatin, the partially purified ozcI sample, SQRG-I, produced a marked increase in mortality of larvae in bioassay, suggesting that the lack of effect from the cystatin treatment was

due to the low concentration employed. The decreased survival demonstrated with the impure ozcI fraction was also observed with the recombinant form of the protein, Rozc. This protein demonstrated a definite potential for use in a control strategy, resulting in an overall reduction in development, determined as c.WGL, showing a significant negative correlation with the concentration of Rozc. The results from Rozc and E64 assays provided an interesting comparison, while both treatments affect the survival of larvae, Rozc having the greater effect, the effects on weight gain were completely distinct. Larvae feeding on Rozc treated diets showed variable weight gains, some being smaller than the controls, while others appeared large and bloated. Larvae fed on the E64 treated diet, however, all showed an almost complete lack of weight gain, suggesting that E64 may also possess antifeedant capabilities.

Thaumatin produced a surprisingly large effect on SCR survival, comparable to that observed with the partially purified ozcI sample. The strength of this effect was most surprising as the protein showed only weak inhibitory activity towards the insect cysteine proteases, suggesting that thaumatin may also act by a mechanism other than merely protease inhibition. It would be interesting to investigate the mode of action of thaumatin further, examining possible inhibitory effects to other enzymes employed by the insect.

The α-amylase inhibitor, WAAI, also produced a notable detrimental effect to SCR larvae when supplied in artificial diet, mortality increasing with the concentration of inhibitor. Interestingly, the same pattern was demonstrated whether the diet was one containing starch (diet 3) or one with sucrose (diet 1). These results are similar to those demonstrated by Gatehouse *et al.* (1986) with *Callosobruchus maculatus*, a dramatic increase in mortality produced as a result of feeding on WAAI treated diet. Further investigation of the enzymes present in the insect gut, along with the action

of WAAI on these, would be necessary to establish the mode(s) of action of this protein.

Comparing *in vivo* and *in vitro* responses

The results from the enzyme inhibitor assays and bioassays exposed differences between the responses demonstrated *in vitro* and those produced by a protein *in vivo*. Cystatin, which acts as potent inhibitors of enzymes *in vitro* had surprisingly little effect *in vivo*, while SQRG-I and Rozc, similar to cystatin in their *in vitro* effects, dramatically reduced the survival and developmental rates of larvae. Another difference demonstrated was that, whilst a compound might be known to possess one inhibitory property *in vitro*, its effects *in vivo* may reveal the existence of other properties, for example, the different effects observed *in vivo* with the two cysteine protease inhibitors, E64 and Rozc, E64 appearing to act also as an antifeedant. WAAI, the α-amylase inhibitor from wheat, also produced surprising results *in vivo*, causing similar effects even when amylase activity was expected to be unnecessary, ie. when larvae were fed on a diet containing high sucrose levels but no starch.

Conclusions

A number of proteins have been demonstrated to have the potential for use in conferring enhanced resistance against corn rootworm attack, although further work, with exhaustively purified proteins and with transgenic material would be required. These proteins include the lectins GNA and WGA, and the enzyme inhibitors ozcI and WAAI. Investigation into the mode of action of such proteins would also be necessary, along with evaluation of the risks of resistance build-up. Any compounds to be used in such a strategy would, of course, also need to be screened for toxicity or other risks towards mammals. Investigation of the mode of action of

thaumatin would also provide interesting and useful information, with a view to the use of novel types of antimetabolite for pest control.

The results from the combination assays confirm the potential of a multi-genic, multi-mechanistic approach to enhanced resistance, an additive effect being demonstrated in the double and triple combinations. Further combination assays would have provided useful information, assaying, for example, the effects of Rozc in combination with an α-amylase inhibitor and a lectin. Time and the scarcity of materials did not allow for further assays, or for the further purification of samples, such as the CpTI preparations.

CHAPTER 8 CONCLUDING DISCUSSION

Aims of the project

The aim of this project was to evaluate the potential of natural resistance factors from plants for use in the protection of maize from corn rootworm attack, this has been completed with some degree of success. Antimetabolic proteins of different types were investigated. Many of these act by the inhibition of enzymes, consequently, the initial aim was the characterisation of the principal digestive enzymes of larval SCR. These enzyme preparations were then assayed, *in vitro*, against various proteins of known and unknown inhibitory capability, and were employed to aid the isolation of novel inhibitors from plant material. Those inhibitors shown to be active against the insect enzymes were then used in bioassay, feeding larvae on artificial diets containing inhibitor and assessing the effect *in vivo*. *In vivo* assays were also conducted using a number of lectins and saporin, the ribosome inactivating protein. Assays were also conducted to assess the potential of a strategy employing a number of different antimetabolites.

Enzymes

The major digestive proteolytic enzymes from *Diabrotica undecimpunctata howardi* have been identified. A number of proteases are employed, cysteine proteases being the principal type, although aspartic acid proteases are also present. The protease activities show similarities to those of the western corn rootworm, *D. virgifera virgifera*, as reported by Gillikin *et al.* (1992) and confirmed in this work. High levels of cysteine protease activity were demonstrated in larval SCR extracts between pH5.0 and pH8.0, in agreement with the findings of Purcell *et al.* (1992) and similar to that reported by Wolfson and Murdock (1990) for SCR larvae. A similar range of activity was demonstrated by Gillikin *et al.* (1992) for larval WCR. The

addition of E64 or chicken egg white cystatin caused almost complete inhibition of this activity, demonstrated using Z-Phe-Arg-AMC as a substrate. High levels of amylase activity were also demonstrated in larval SCR gut extracts, although only one isoform of α-amylase was identified. Activity was demonstrated at high levels over a pH range from 4.0 to 6.5. Investigation of the physiological pH of the larval gut demonstrated that the gut contents were acidic, between pH4 and pH6, correlating with the acidic optima established for the amylase and protease activities.

Inhibitors *in vitro*

Having established the principal types of proteolytic and amylolytic activities present in the insect gut, suitable plant protein inhibitors were sought. Plant proteins previously isolated were assayed for inhibitory activity *in vitro*. CpTI, enterolobin and thaumatin were assayed for inhibitory activity against cysteine proteases, and α-amylase inhibitors from wheat (WAAI) and bean (BAAI) were tested against the insect amylases. The wheat α-amylase inhibitor (WAAI) was also tested for CPI activity. Of these proteins, CpTI and thaumatin possessed CPI activity which caused a low level of inhibition of insect cysteine proteases, and WAAI caused almost complete inhibition of larval amylase activity, but demonstrated no CPI activity. Enterolobin showed only weak CPI activity and BAAI caused only a small reduction in amylase activity.

Oryzacystatin I was partially purified from rice seed and demonstrated to cause strong inhibition of papain, the plant-derived cysteine protease, and the cysteine proteases in larval and adult gut extracts from SCR and WCR. A recombinant form of ozcI was produced in *E. coli*, isolated and shown to cause strong inhibition of insect cysteine proteases. This recombinant protein possessed an N-terminal extension of 14 amino acid residues originating from the multipurpose cloning site of pUC8, but comparison with the activity of the impure sample of the native protein, and

also that of chicken egg white cystatin, indicated that the fusion protein possessed the inhibitory activity of wild-type ozcI.

Assaying the comparative inhibitory activity of rice fractions against the plant enzyme, papain, and the insect cysteine proteases led to the purification of a novel inhibitor, a protein causing little inhibition of papain activity, but a potent inhibitor of the SCR cysteine proteases. Sequence analysis of this protein was hindered by the N-terminal blockage of the polypeptide chain, but cyanogen bromide cleavage produced peptides which, when sequenced, demonstrated a high level of homology (over 80% identity) with the rice allergenic proteins recently reported by Izumi *et al.* (1992) and Adachi *et al.* (1993). Unfortunately, due to the very low concentrations of this protein in rice, sufficient material was not accumulated to allow any analysis of its *in vivo* efficacy against the development of SCR larvae.

Bioassays:

Enzyme inhibitors *in vivo*

A number of proteins, successfully shown to inhibit larval digestive proteases *in vitro*, were incorporated into artificial diet and used in bioassay to assess their effects *in vivo*. Of those tested, the recombinant form of ozcI was the most potent, causing a significant rise in the mortality rate of the larvae. Native ozcI, tested using the partially purified sample from rice germ, also produced a marked reduction in survival of larvae, compared to those on a control diet, indicating that the action of the recombinant form was the same as the wild-type ozcI. Surprisingly, cystatin, which *in vitro* demonstrated inhibitory properties similar to ozcI, caused no notable effects on larval development, although the low concentration used in bioassay may have contributed to this.

The α-amylase inhibitor, WAAI, shown *in vitro* to effectively inhibit the insect α-amylase activity, *in vivo* produced a significant decrease in survival, apparently independent of the composition of the diet as regards

the starch/sucrose content. The mode of action and other inhibitory properties of WAAI would require investigation prior to this proteins employment to enhance inherent resistance.

Lectins and RIPs *in vivo*

Five lectins were assayed for *in vivo* activity against SCR larvae: two elderberry lectins, SNA1 and SNA2; wheat germ lectin, WGA; snowdrop lectin, GNA; and a pea lectin, plec. Only two of these, WGA and GNA, caused marked alterations in larval development. The results obtained with WGA are in agreement with the work of Czapla and Lang (1990; Chrispeels & Raikhel, 1991). Interestingly, plec, which binds both D-mannose and D-glucose, did not affect larval development, while GNA, which is specific for D-mannose, caused a dramatic reduction in weight gain and also a reduced level of survival. Both GNA and WGA have been successfully shown to possess marked potential for use in corn rootworm control strategies.

Saporin, a ribosome inactivating protein, produced surprisingly little effect on larval development when assayed *in vivo*. Considering the known toxicity of many RIPs to mammals, and the low level of efficacy demonstrated here against rootworms, the inclusion of saporin in a resistance enhancement program would seem unlikely.

Enhancing protection using a number of proteins

The use of a number of antimetabolic proteins to provide an elevated level of protection from insect pests was illustrated by Boulter *et al.* (1990), as described earlier. In nature, plants generally express a complex array of defence mechanisms, each individually unable to provide complete protection, but, which, when presented as a whole, generally supply a level of protection sufficient to enable the plant to survive and grow. To explore the possibilities for combining a number of proteins together to enhance resistance, two assays were conducted, one combining WAAI and CpTI,

resulting in an effect slightly enhanced from that of either component alone, and another combining WAAI, CpTI and the lectin, GNA. This triple combination assay showed a marked enhancement of the anti-insect properties demonstrated by any of the proteins individually or of the WAAI and CpTI taken together. Clearly the use of multi-genic and multi-mechanistic protection would provide a level of resistance superior to any individual factor employed alone, and it would also decrease the risk of resistance build-up within the insect population, the emergence of biotypes resistant to all the protection mechanisms expressed by a plant would be highly unlikely.

Conclusions

This project has successfully demonstrated the great potential for enhancing the inherent resistance of maize by the expression of certain introduced antimetabolic proteins. Unfortunately, no transgenic material was available during the time of this project, so no *in planta* bioassays could be conducted. Such bioassays are essential to establish the effects of proteins when ingested along with the insect's natural diet, and to establish the levels of protection provided. The lectins GNA and WGA, and the enzyme inhibitors WAAI and ozcI, appear, from the results presented here, to hold the greatest potential for effective control using this method.

Much research is, however, still necessary, as many questions remain to be answered. Further investigation of the modes of action of these proteins, using exhaustively purified proteins, would provide information useful in the evaluation of each protein and allow a reasoned choice of proteins for use in a multi-mechanistic approach. For example, the presence of a protease inhibitor may protect another protein from hydrolysis and thus allow it to remain active against the insect. Alternatively, proteins which bind to the surfaces of certain cells might aid the action of other proteins.

Further investigation would also be necessary to establish the level of protein(s) necessary to provide effective resistance in the plant. It must be recognised, however, that the level of antimetabolite necessary when incorporated into artificial diet may, because of the difference in composition of the diet, differ markedly from the concentration required *in planta* for effective control.

One important consideration in the design of such a control strategy is the effect of the protein(s) on the plant itself. Obviously, if a protein produces an antimetabolic effect within the plant, then such a plant, if it survives, may be weakened and thus more, not less, susceptible to insect attack. Clearly these problems will be faced during the initial production of the transgenic plants, and thus are likely to be of less concern during later work. Hilder and Gatehouse (1991), however, showed that the expression of CpTI in transgenic tobacco caused no reduction in plant quality. Similarly, transgenic plants expressing the cysteine protease inhibitor, ozcI, have been produced (Masoud *et al.*, 1993; AGC., unpublished results) without any obvious penalties. Others, expressing serine protease inhibitors, have been produced and demonstrated to possess enhanced insect resistance (Hilder *et al.*, 1987; Boulter *et al.*, 1990; Johnson *et al.*, 1989; Barsby, 1991), affirming the potential of this approach to pest control.

Another concern is the effect of these antimetabolites on non-target organisms, that is, both the mammals, including humans, for whom a food crop may be destined, and those predators who feed on the insect pests. An unexpected problem with many insecticides has been their adverse effects on the predators of pest species, a decrease in the predator population often resulting in a resurgence of pests. Examination of the adverse effects that any protein(s) would cause to these predatory species, or to humans and other mammals, would be most useful in providing a clearer picture of the protection to be expected in the field situation, and of any risks involved in the process.

One vital factor that must be considered and investigated as thoroughly as possible is the possibility of resistance build-up within the rootworm population. As discussed in the introduction, strains of corn rootworm have emerged which show increased tolerance to a wide range of insecticides. Such a pattern has also been observed in many other pest species, over 500 pest species are known to have developed resistance to at least one insecticide type. Part of the problem has been the extensive and indiscriminate application of large amounts of insecticide, a few individuals surviving by chance by being genetically better equipped, and these going on to produce the next generation which will consequently contain a higher proportion of individuals carrying the resistance genes. Recently, much hope has been placed on the use of bacterial toxins, crystalline compounds highly toxic to specific species of insect. Farmers have used sprays containing of a mixture of *Bacillus thuringiensis* toxins for more than two decades with no sign of resistance. Because of this history of maintained efficacy with no resistance build-up, along with the specificity of the different strains of toxin to different insect species, *B.t.* toxins were considered ideal for use in the protection of crops by their expression in transgenic plants. However, during the 1980's, with the decreasing efficacy of many chemical insecticides, sales of *B.t.* toxin sprays increased 4-fold world-wide to over $M100, and, in 1985, the first example of *B.t.* resistance was found in moths in grain storage bins in the American Midwest. In 1990, resistant diamondback moths were found in Hawaii, and since then in Florida, New York, Japan and parts of the Asian mainland. Laboratory tests have also demonstrated that a wide range of insects have the capacity to develop resistance to *B.t.* toxins (see review Holmes, 1993). Clearly the problem of resistance development is one which must be considered in great depth, otherwise, rather than easing the pest problem and reducing our reliance on insecticides, we could merely exacerbate the situation.

Taking all of these factors into account, this project has demonstrated that the use of antimetabolic proteins to enhance the resistance or tolerance of crop plants to rootworm attack holds great potential. Expressing a number of proteins in transgenic plants would increase the resistance and serve to reduce the possibility of resistant biotypes emerging. The correct choice of antimetabolites, with the aid of good cultural or edaphic practices, and the judicious application of certain specific insecticides when necessary, could provide a long awaited release from our mounting pest problems and our extensive reliance on insecticides.

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APPENDIX

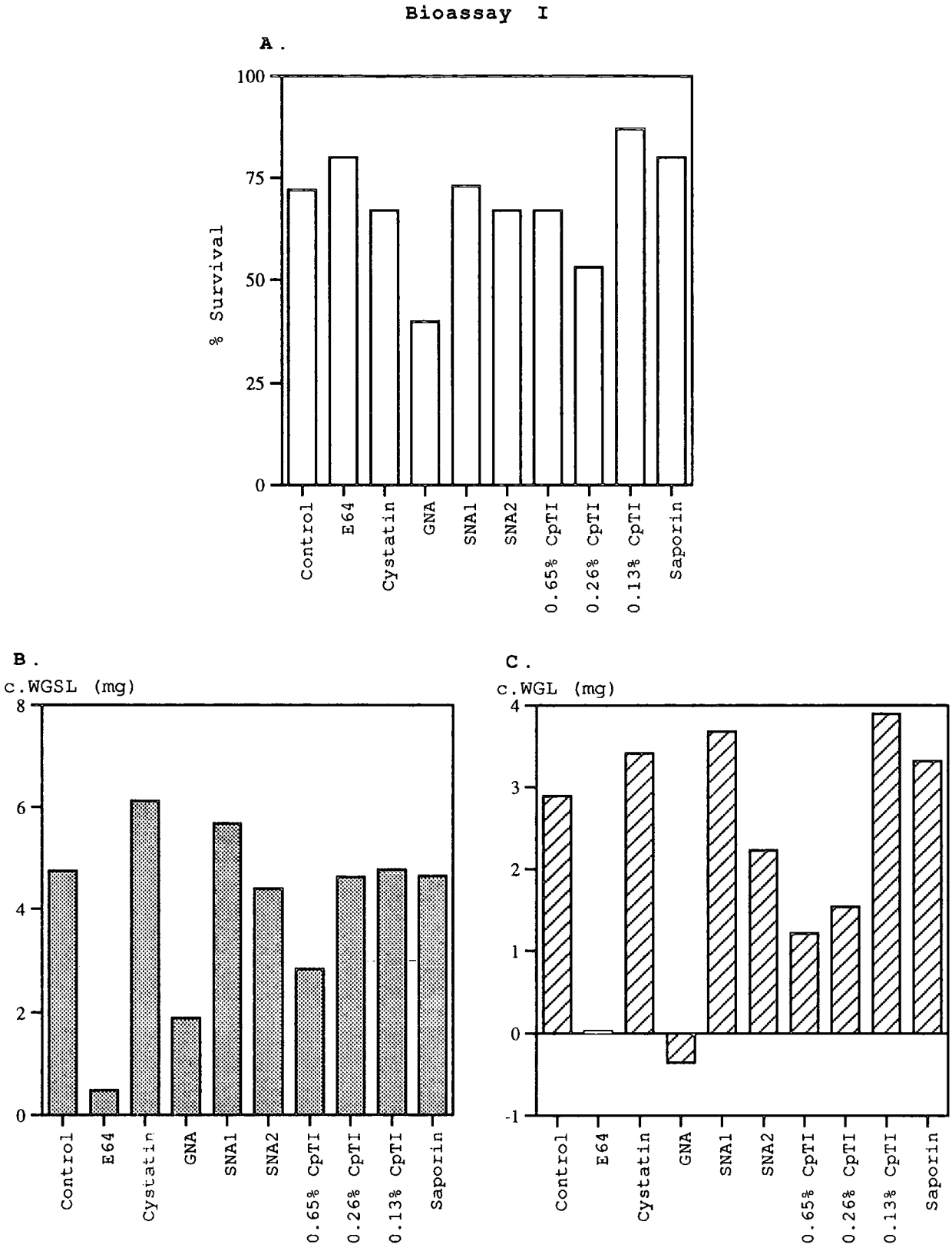

Figure 92. Bioassay I. Effect of various treatments on the survival (panel A) and development of SCR larvae. E64 was added at 0.013% (w/v), cystatin at 0.0167% (w/v), and saporin at 0.0013% (w/v). Other proteins were added at 0.26% (w/v) or as indicated. Development is given as corrected weight gain per surviving larva (c.WGSL, panel B), and corrected weight gain per larva (c.WGL, panel C). The assay was conducted for 8 days.

Survival

Assay	Control No.alive	Control Total	Treatment No.alive	Treatment Total	G_s	*df*	
GNA	54	75	6	15	4.175	1	*
0.26% CpTI	54	75	8	15	1.200	1	NS

Corrected WGSL

Assay	Control Mean	Control s^2	Control *n*	Treatment Mean	Treatment s^2	Treatment *n*	t_s	*df*	
E64	4.747	3.553	54	0.485	0.173	12	7.746	64	***
cystatin	4.747	3.553	54	6.117	4.343	6	1.673	58	NS
GNA	4.747	3.553	54	1.882	9.778	6	3.292	58	**
SNA1	4.747	3.553	54	5.675	3.382	11	1.494	63	NS
SNA2	4.747	3.553	54	4.396	1.423	10	0.566	62	NS
0.65% CpTI	4.747	3.553	54	2.836	3.679	10	2.937	62	**
0.26% CpTI	4.747	3.553	54	4.629	3.276	8	0.166	60	NS
0.13% CpTI	4.747	3.553	54	4.776	2.782	13	0.051	65	NS
saporin	4.747	3.553	54	4.652	0.891	12	0.169	34	NS

Corrected WGL

Assay	Control Mean	Control s^2	Control *n*	Treatment Mean	Treatment s^2	Treatment *n*	t_s	*df*	
E64	2.891	11.533	75	0.029	1.026	15	3.222	75	**
cystatin	2.891	11.533	75	3.413	19.149	9	0.422	82	NS
GNA	2.891	11.533	75	-0.351	7.070	15	3.4/84	88	***
SNA1	2.891	11.533	75	3.684	14.092	15	0.811	88	NS
SNA2	2.891	11.533	75	2.226	11.009	15	0.0695	88	NS
0.65% CpTI	2.891	11.533	75	1.213	8.003	15	1.791	88	NS
0.26% CpTI	2.891	11.533	75	1.537	13.337	15	1.392	88	NS
0.13% CpTI	2.891	11.533	75	3.898	7.745	15	1.077	88	NS
saporin	2.891	11.533	75	3.318	8.323	15	0.455	88	NS

Figure 93. Bioassay I. Statistical analysis for bioassay I results. Level of significance is indicated by NS, Not Significant; *, $p<0.05$; **, $p<0.01$; or ***, $p<0.001$.

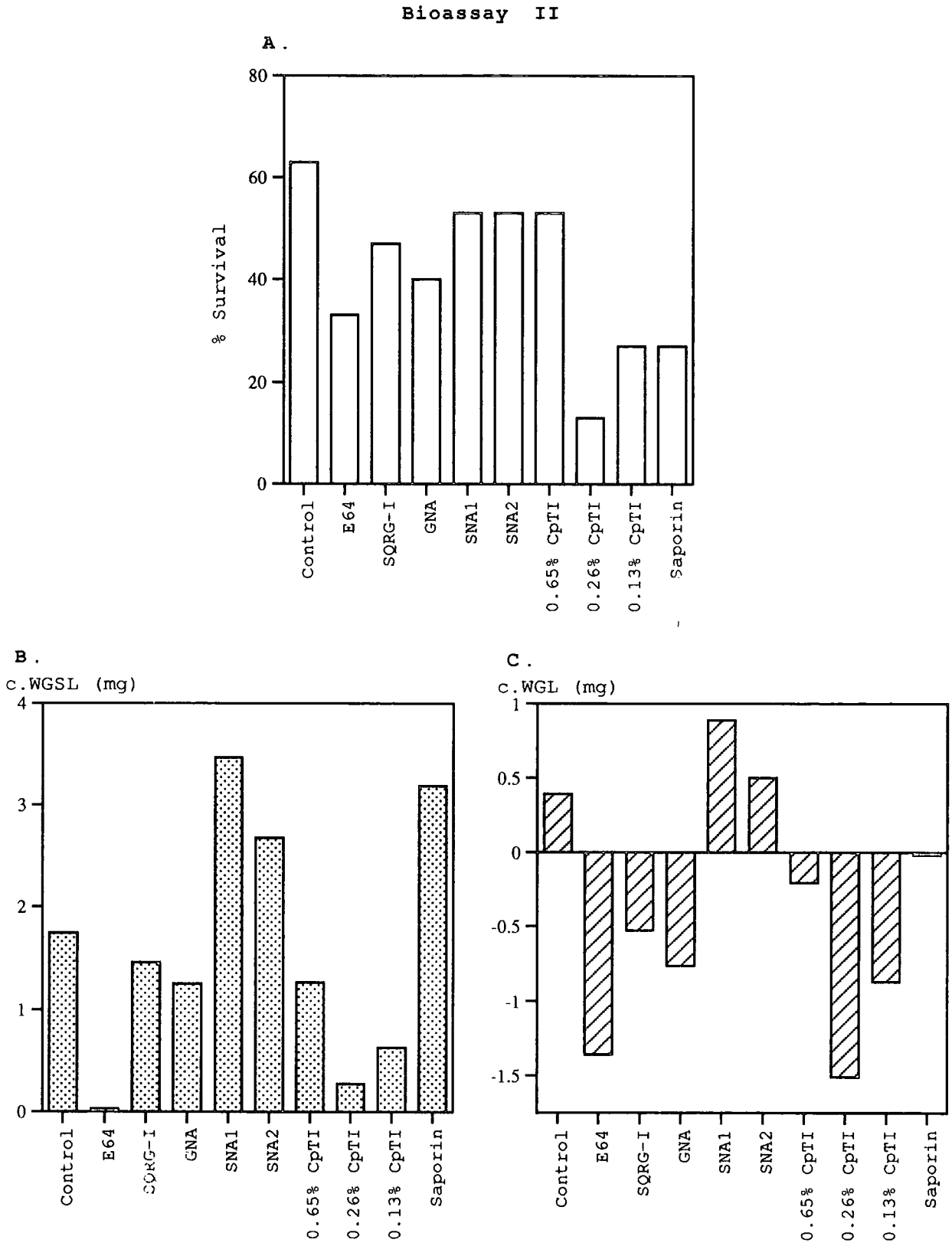

Figure 94. Bioassay II. Effect of various treatments on the survival (panel A) and development of SCR larvae. E64 was added at 0.013% (w/v), SQRG-I at 0.13% (w/v), and saporin at 0.0071% (w/v). Other proteins were added at 0.26% or as indicated. Development is given as corrected weight gain per surviving larva (c.WGSL, panel B), and corrected weight gain per larva (c.WGL, panel C). The assay was conducted for 8 days.

Survival

Assay	Control No.alive	Control Total	Treatment No.alive	Treatment Total	G_s	df	
0.26% CpTI	47	75	2	15	10.93	1	**
0.13% CpTI & saporin	47	75	4	15	5.226	1	*
E64 & SRA1	47	75	5	15	3.256	1	NS

Corrected WGSL

Assay	Control Mean	Control s^2	Control n	Treatment Mean	Treatment s^2	Treatment n	t_s	df	
E64	1.741	2.538	47	0.029	0.063	5	2.379	43	*
SQRG-I	1.741	2.538	47	1.455	1.841	7	0.464	52	NS
GNA	1.741	2.538	47	1.249	0.635	6	0.733	51	NS
SNA1	1.741	2.538	47	3.468	2.839	8	2.183	53	**
SNA2	1.741	2.538	47	2.861	1.868	8	1.871	53	NS
0.65% CpTI	1.741	2.538	47	1.262	0.694	8	0.827	17	NS
0.26% CpTI	1.741	2.538	47	0.272	0.142	2	1.290	3	NS
0.13% CpTI	1.741	2.538	47	0.629	0.709	4	1.371	4	NS
saporin	1.741	2.538	47	3.190	4.076	4	1.715	49	NS

Corrected WGL

Assay	Control Mean	Control s^2	Control n	Treatment Mean	Treatment s^2	Treatment n	t_s	df	
E64	0.390	4.765	75	-1.357	1.059	15	3.022	44	**
SQRG-I	0.390	4.765	75	-0.531	4.499	15	1.498	88	NS
GNA	0.390	4.765	75	-0.767	3.133	15	1.927	88	NS
SNA1	0.390	4.765	75	0.888	9.579	15	0.748	17	NS
SNA2	0.390	4.765	75	0.501	6.791	15	0.174	88	NS
0.65% CpTI	0.390	4.765	75	-0.206	3.041	15	0.994	88	NS
0.26% CpTI	0.390	4.765	75	-1.511	0.699	15	3.312	58	**
0.13% CpTI	0.390	4.765	75	-0.872	1.073	15	2.183	43	*
saporin	0.390	4.765	75	-0.020	4.910	15	0.662	88	NS

Figure 95. Bioassay II. Statistical analysis. Level of significance is indicated by NS, Not Significant; *, $p<0.05$; **, $p<0.01$; or ***, $p<0.001$.

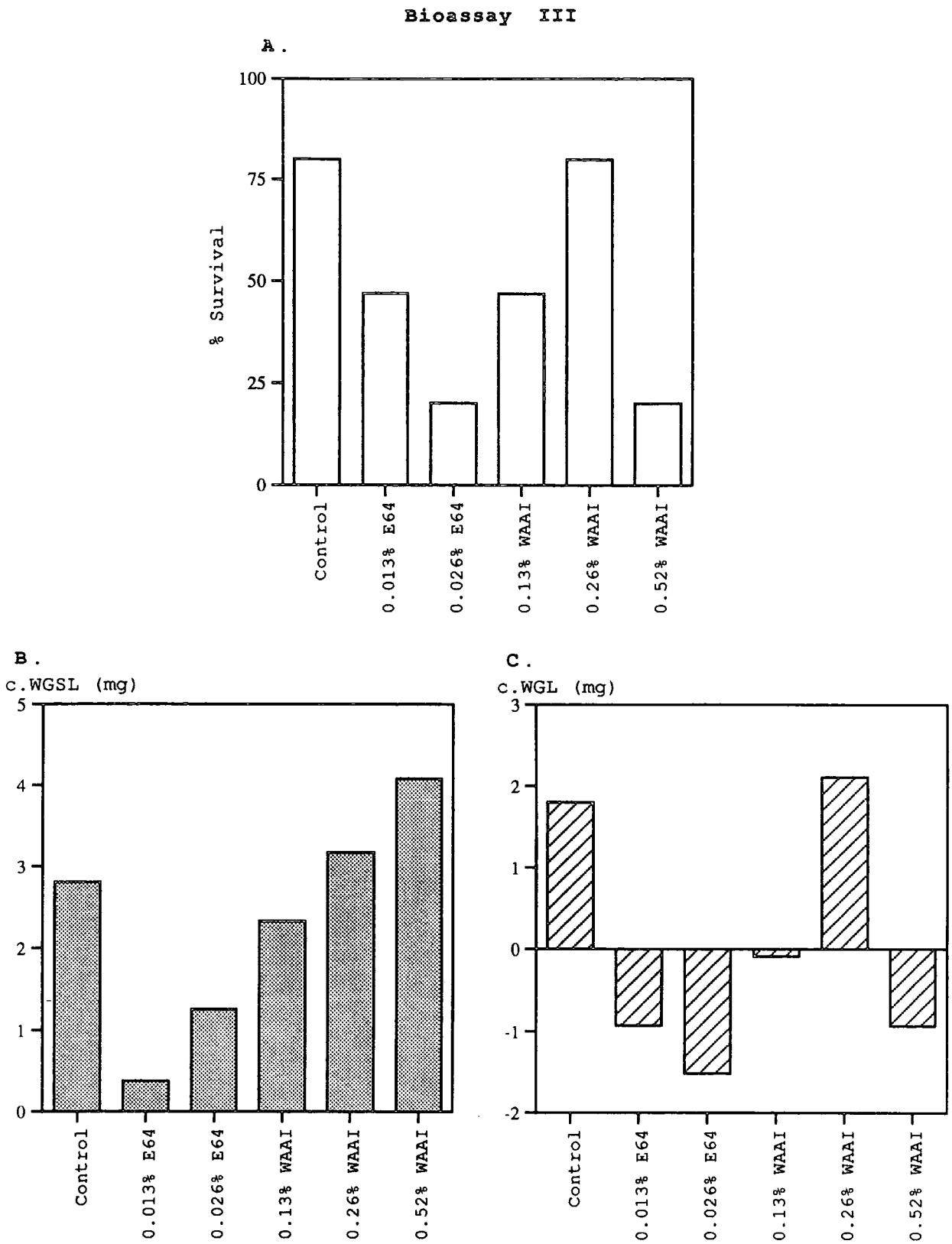

Figure 96. Bioassay III. Effect of E64 and WAAI on the survival (panel A) and development of SCR larvae. Development is given as corrected weight gain per surviving larva (c.WGSL, panel B), and corrected weight gain per larva (c.WGL, panel C).

Survival

Assay	Control No.alive	Control Total	Treatment No.alive	Treatment Total	G_s	*df*	
0.026%E64 & 0.52%WAAI	60	75	3	15	17.231	1	***
0.013%E64 & 0.13%WAAI	60	75	7	15	5.083	1	*

Corrected WGSL

Assay	Control Mean	Control s^2	Control *n*	Treatment Mean	Treatment s^2	Treatment *n*	t_s	*df*	
0.013%E64	2.803	1.860	60	0.377	0.271	7	4.640	65	***
0.026%E64	2.803	1.860	60	1.254	1.094	3	1.933	61	NS
0.13% WAAI	2.803	1.860	60	2.331	2.487	7	0.853	65	NS
0.26% WAAI	2.803	1.860	60	3.173	1.913	12	0.856	70	NS
0.52% WAAI	2.803	1.860	60	4.078	3.467	3	1.558	61	NS

Corrected WGL

Assay	Control Mean	Control s^2	Control *n*	Treatment Mean	Treatment s^2	Treatment *n*	t_s	*df*	
0.013%E64	1.803	5.541	75	-0.928	1.711	15	4.348	35	***
0.026%E64	1.803	5.541	75	-1.515	2.214	15	5.240	30	***
0.13% WAAI	1.803	5.541	75	-0.085	6.554	15	3.068	88	**
0.26% WAAI	1.803	5.541	75	2.111	6.320	15	0.457	88	NS
0.52% WAAI	1.803	5.541	75	-0.931	7.225	15	4.010	88	***

Figure 97. Bioassay III. Statistical analysis. Level of significance is indicated by NS, Not Significant; *, $p<0.05$; **, $p<0.01$; or ***, $p<0.001$.

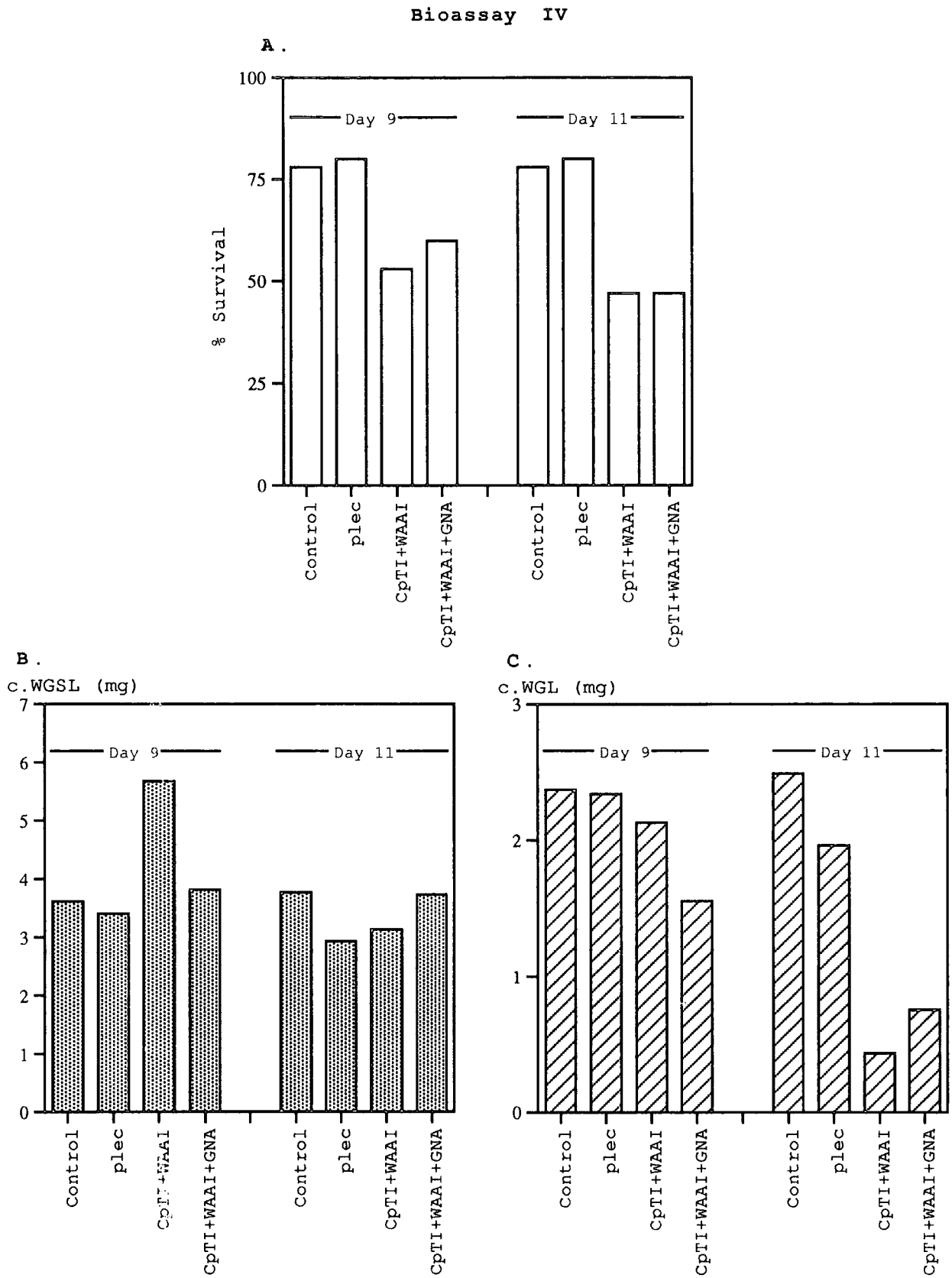

Figure 98. Bioassay IV. Effect of various treatments on the survival (panel A) and development of SCR larvae. All proteins were incorporated into diet at 0.26% (w/v). Development is given as corrected weight gain per surviving larva (c.WGSL, panel B), and corrected weight gain per larva (c.WGL, panel C). Results shown represent the mean values on days 9 and 11.

Survival (day 11)

Assay	Control No.alive	Total	Treatment No.alive	Total	G_s	*df*	
C/W & C/W/G	21	27	7	15	2.8684	1	NS

Corrected WGSL

Assay	Control Mean	s^2	*n*	Treatment Mean	s^2	*n*	t_s	*df*	
Day 9									
plec	3.609	3.087	21	3.406	4.880	12	0.273	19	NS
C/W	3.609	3.087	21	5.668	9.315	8	1.798	9	NS
C/W/G	3.609	3.087	21	3.813	5.949	9	0.227	12	NS
Day 11									
plec	3.760	3.972	21	2.931	2.253	12	1.350	28	NS
C/W	3.760	3.972	21	3.137	11.896	7	0.543	7	NS
C/W/G	3.760	3.972	21	3.731	2.322	7	0.040	26	NS

Corrected WGL

Assay	Control Mean	s^2	*n*	Treatment Mean	s^2	*n*	t_s	*df*	
Day 9									
plec	2.373	7.930	27	2.341	8.702	15	0.034	40	NS
C/W	2.373	7.930	27	2.131	19.963	15	0.197	20	NS
C/W/G	2.373	7.930	27	1.554	11.601	15	0.793	40	NS
Day 11									
plec	2.491	8.916	27	1.960	6.574	15	0.606	40	NS
C/W	2.491	8.916	27	0.436	11.958	15	1.935	40	NS
C/W/G	2.491	8.916	27	0.751	9.333	15	1.783	40	NS

Figure 99. Bioassay IV. Statistical analysis. Level of significance is indicated by NS, Not Significant; *, $p<0.05$; **, $p<0.01$; or ***, $p<0.001$. C/W and C/W/G represent CpTI+WAAI and CpTI+WAAI+GNA repectively.

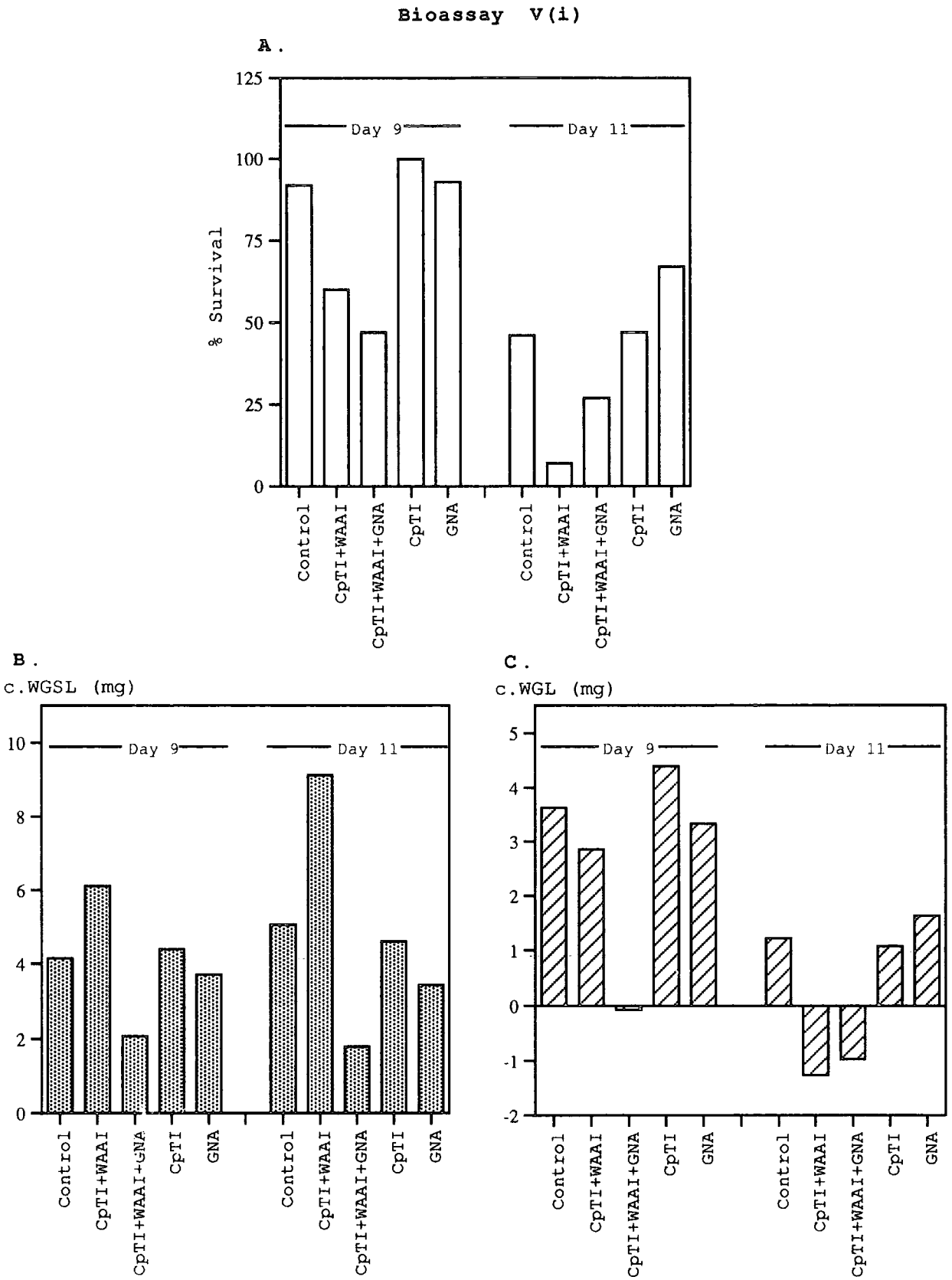

Figure 100. Bioassay V(i). Effect of various treatments on the survival (panel A) and development of SCR larvae. All proteins were added to diet at 0.26% (w/v). Development is given as corrected weight gain per surviving larva (c.WGSL, panel B), and corrected weight gain per larva (c.WGL, panel C). Results are presented for days 9 and 11.

Survival (day 12)

Assay	Control No.alive	Control Total	Treatment No.alive	Treatment Total	G_s	*df*	
thaumatin	5	15	3	15	0.171	1	NS
WGA	5	15	7	15	0.139	1	NS

Corrected WGSL

Assay	Control Mean	Control s^2	Control *n*	Treatment Mean	Treatment s^2	Treatment *n*	t_s	*df*	
Day 9									
thaumatin	4.629	1.798	14	3.885	0.949	14	1.679	26	NS
WGA	4.629	1.798	14	2.301	0.699	14	5.512	26	***
plec	4.629	1.798	14	5.091	2.719	13	0.801	25	NS
Day 12									
thaumatin	3.548	1.228	5	1.731	2.839	3	1.873	6	NS
WGA	3.548	1.228	5	1.136	1.496	7	3.496	10	**
plec	3.548	1.228	5	4.648	1.331	6	1.602	9	NS

Corrected WGL

Assay	Control Mean	Control s^2	Control *n*	Treatment Mean	Treatment s^2	Treatment *n*	t_s	*df*	
Day 9									
thaumatin	4.183	4.682	15	3.478	3.356	15	0.963	28	NS
WGA	4.183	4.682	15	2.008	1.940	15	3.273	28	**
plec	4.183	4.682	15	4.131	8.744	15	0.054	28	NS
Day 12									
thaumatin	-0.246	8.060	15	-1.377	3.017	15	1.316	28	NS
WGA	-0.246	8.060	15	-0.625	3.553	15	0.431	28	NS
plec	-0.246	8.060	15	0.597	12.187	15	0.725	28	NS

Figure 101. Bioassay V(i). Statistical analysis for bioassay V part (i). Level of significance is indicated by NS, Not Significant; *, $p<0.05$; **, $p<0.01$; or ***, $p<0.001$.

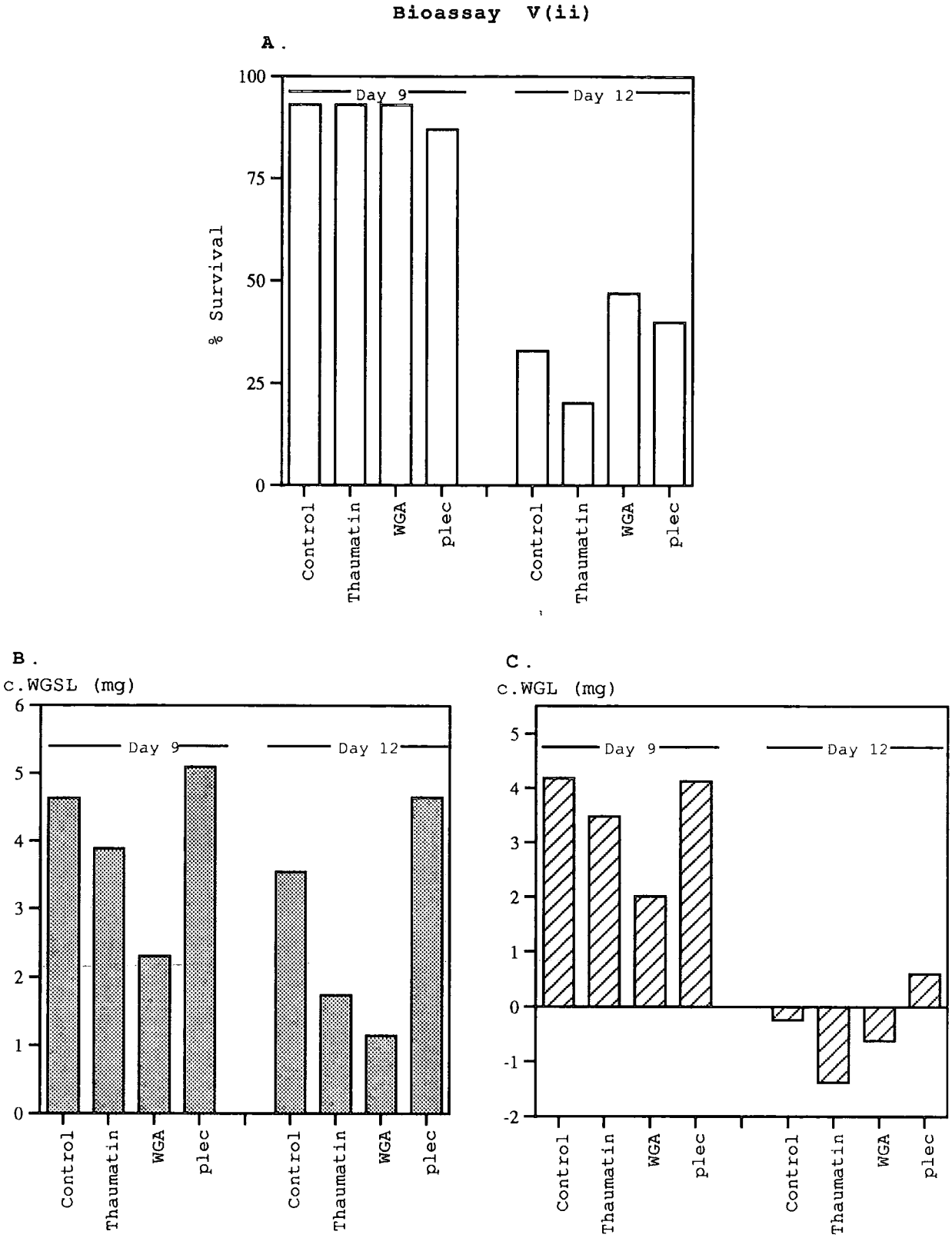

Figure 102. Bioassay V(ii). Effect of various treatments on the survival (panel A) and development of SCR larvae. All proteins were added to artificial diet at 0.26% (w/v). Development is given as corrected weight gain per surviving larva (c.WGSL, panel B), and corrected weight gain per larva (c.WGL, panel C). Results are presented for days 9 and 12.

Survival

Assay	Control No.alive	Total	Treatment No.alive	Total	G_s	*df*	
Day 9							
C/W	22	24	9	15	3.826	1	NS
C/W/G	22	24	7	15	7.569	1	**
Day 11							
C/W	11	24	1	15	5.497	1	*
C/W/G	11	24	4	15	0.749	1	NS
GNA	11	24	10	15	0.890	1	NS

Corrected WGSL

Assay	Control Mean	s^2	*n*	Treatment Mean	s^2	*n*	t_s	*df*	
Day 9									
C/W	4.147	2.253	22	6.108	4.036	9	2.642	29	*
C/W/G	4.147	2.253	22	2.068	0.396	7	5.215	24	***
CpTI	4.147	2.253	22	4.395	0.461	15	0.680	31	NS
GNA	4.147	2.253	22	3.713	1.633	14	0.927	34	NS
Day 11									
C/W	5.048	0.213	11	9.126	0	1	29.27	10	***
C/W/G	5.048	0.213	11	1.785	0.001	4	23.20	10	***
CpTI	5.048	0.213	11	4.606	6.457	7	0.455	6	NS
GNA	5.048	0.213	11	3.440	3.122	10	2.792	10	*

Corrected WGL

Assay	Control Mean	s^2	*n*	Treatment Mean	s^2	*n*	t_s	*df*	
Day 9									
C/W	3.635	5.058	24	2.861	19.237	15	0.633	15	NS
C/W/G	3.635	5.058	24	-0.079	4.486	15	5.128	37	***
CpTI	3.635	5.058	24	4.395	0.461	15	1.546	29	NS
GNA	3.635	5.058	24	3.336	3.640	15	0.427	37	NS
Day 11									
C/W	1.219	13.025	24	-1.272	8.283	15	2.258	37	*
C/W/G	1.219	13.025	24	-0.976	2.972	15	2.550	35	*
CpTI	1.219	13.025	24	1.078	14.432	15	0.116	37	NS
GNA	1.219	13.025	24	1.633	9.018	15	0.371	37	NS

Figure 103. Bioassay V(ii). Statistical analysis for bioassay V part (ii). Level of significance is indicated by NS, Not Significant; *, $p<0.05$; **, $p<0.01$; or ***, $p<0.001$. C/W and C/W/G represent CpTI/WAAI and CpTI/WAAI/GNA repectively.

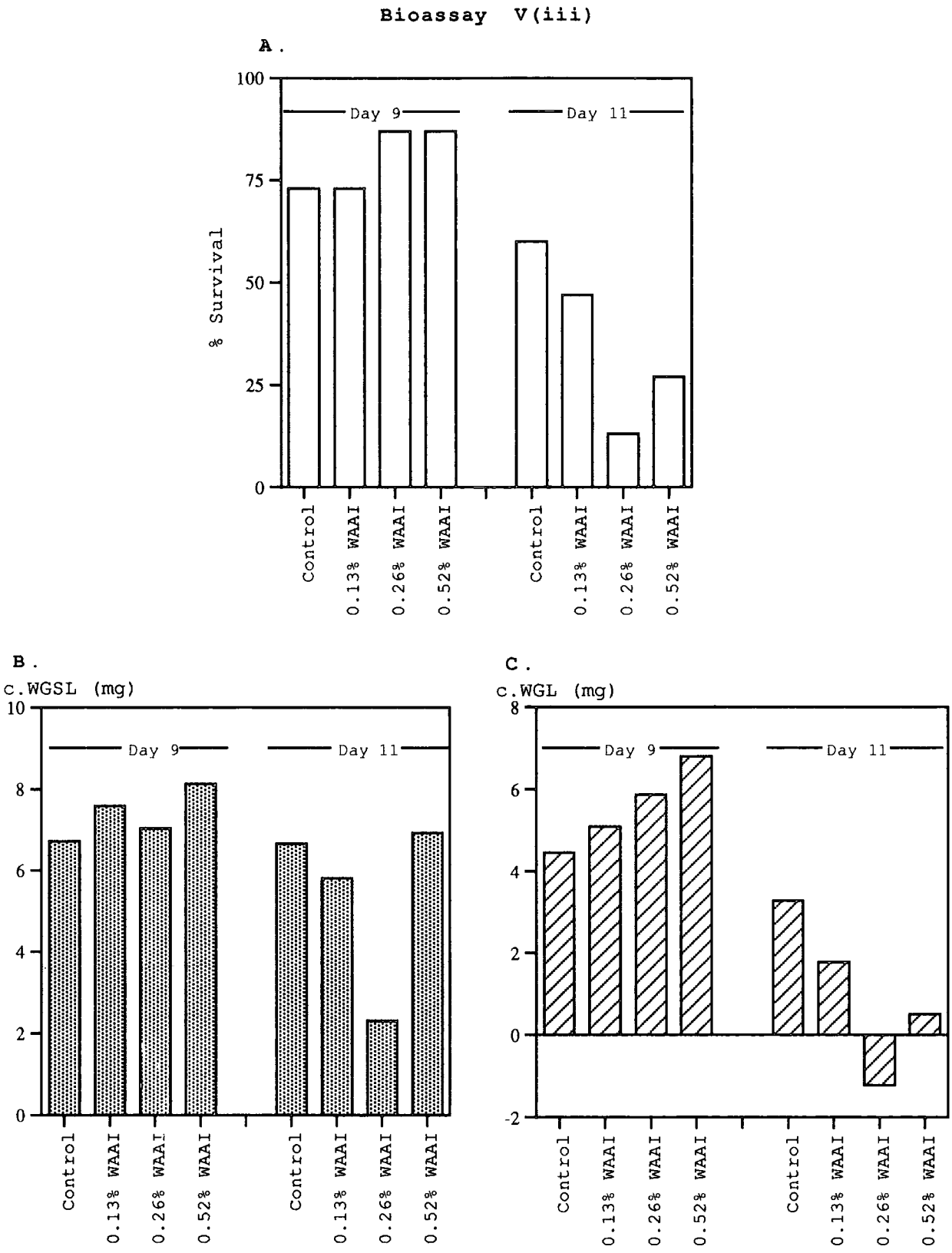

Figure 104. Bioassay V(iii). Effect of various treatments on the survival (panel A) and development of SCR larvae. Protein concentrations are given as % (w/v) in artificial diet. Development is given as corrected weight gain per surviving larva (c.WGSL, panel B), and corrected weight gain per larva (c.WGL, panel C). Results are presented for days 9 and 11.

Survival (day 11)

Assay	Control No.alive	Control Total	Treatment No.alive	Treatment Total	G_s	df	
0.26%WAAI	9	15	2	15	5.386	1	*
0.52%WAAI	9	15	4	15	2.201	1	NS

Corrected WGSL

Assay	Control Mean	Control s^2	Control n	Treatment Mean	Treatment s^2	Treatment n	t_s	df	
Day 9									
0.13%WAAI	6.712	4.117	11	7.582	1.685	11	1.198	20	NS
0.26%WAAI	6.712	4.117	11	7.036	15.476	13	0.246	18	NS
0.52%WAAI	6.712	4.117	11	8.126	17.364	13	1.033	18	NS
Day 11									
0.13%WAAI	6.660	2.560	9	5.814	7.562	7	0.774	14	NS
0.26%WAAI	6.660	2.560	9	2.308	0	2	8.160	8	***
0.52%WAAI	6.660	2.560	9	6.917	4.640	4	0.264	11	NS

Corrected WGL

Assay	Control Mean	Control s^2	Control n	Treatment Mean	Treatment s^2	Treatment n	t_s	df	
Day 9									
0.13%WAAI	4.455	17.935	15	5.094	19.413	15	0.405	28	NS
0.26%WAAI	4.455	17.935	15	5.870	22.724	15	0.859	28	NS
0.52%WAAI	4.455	17.935	15	6.800	27.165	15	1.352	28	NS
Day 11									
0.13%WAAI	3.276	19.874	15	1.780	18.499	15	0.935	28	NS
0.26%WAAI	3.276	19.874	15	-1.220	2.062	15	3.718	17	**
0.52%WAAI	3.276	19.874	15	0.507	17.007	15	1.766	28	NS

Figure 105. Bioassay V(iii). Statistical analysis for bioassay V part (iii). Level of significance is indicated by NS, Not Significant; *, $p<0.05$; **, $p<0.01$; or ***, $p<0.001$.

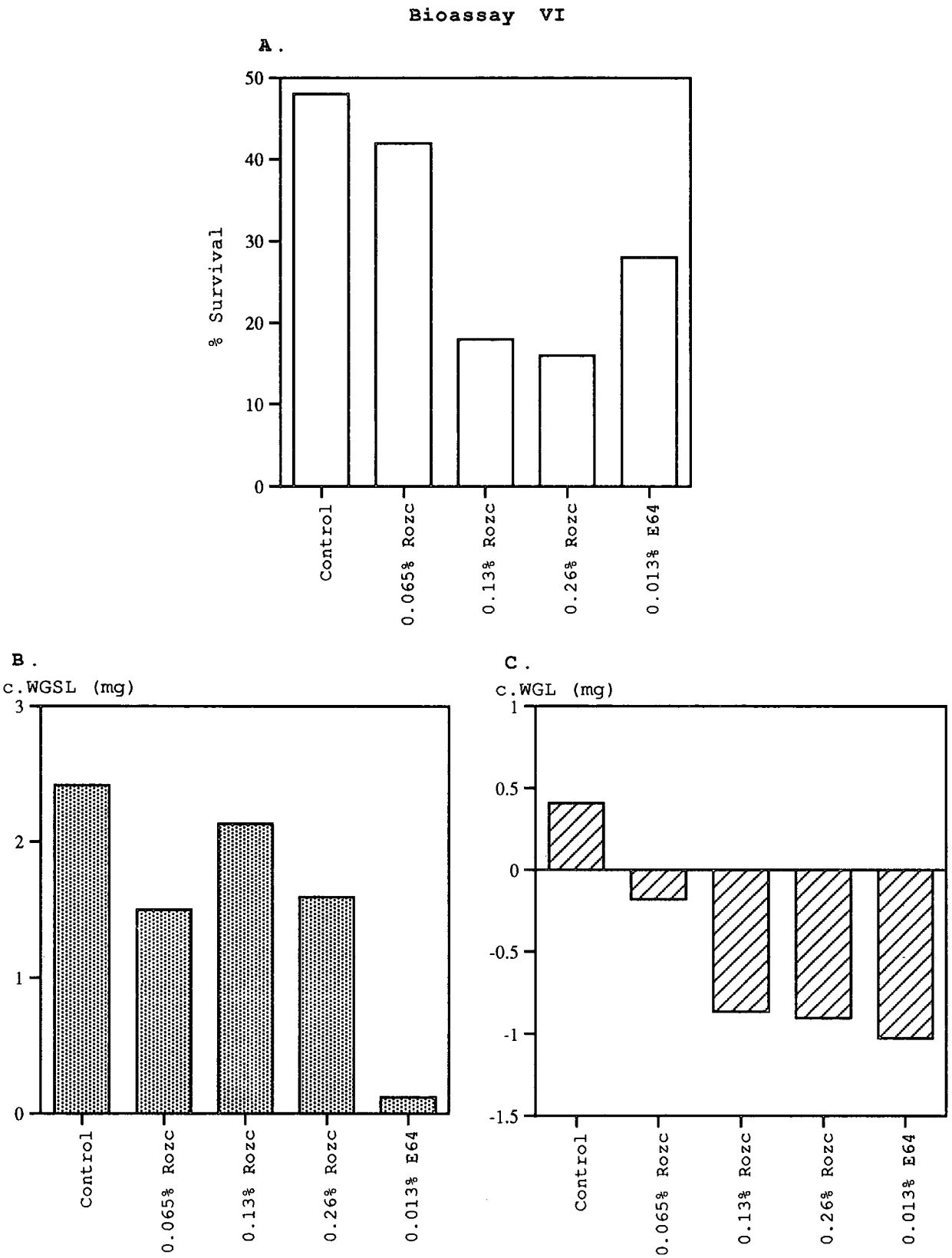

Figure 106. Bioassay VI. Effect of Rozc and E64 on the survival (panel A) and development of SCR larvae. Protein concentrations are given as % (w/v) in artificial diet. Development is given as corrected weight gain per surviving larva (c.WGSL, panel B), and corrected weight gain per larva (c.WGL, panel C).

Survival

Assay	Control No.alive	Control Total	Treatment No.alive	Treatment Total	G_s	*df*	
0.13%Rozc	24	50	9	50	9.079	1	**
0.26%Rozc	24	50	8	50	10.657	1	**
E64	24	50	14	50	3.463	1	NS

Corrected WGSL

Assay	Control Mean	Control s^2	Control *n*	Treatment Mean	Treatment s^2	Treatment *n*	t_s	*df*	
0.13%Rozc	2.417	1.332	24	2.131	0.929	9	0.660	31	NS
0.26%Rozc	2.417	1.332	24	1.591	4.318	8	1.071	8	NS
0.065%Rozc	2.417	1.332	24	1.497	1.162	21	2.750	43	**
E64	2.417	1.332	24	0.121	0.109	14	9.125	29	***

Corrected WGL

Assay	Control Mean	Control s^2	Control *n*	Treatment Mean	Treatment s^2	Treatment *n*	t_s	*df*	
0.13%Rozc	0.407	4.444	50	-0.865	2.167	50	3.498	88	***
0.26%Rozc	0.407	4.444	50	-0.904	1.833	50	3.700	83	***
0.065%Rozc	0.407	4.444	50	-0.182	2.560	50	1.574	91	NS
E64	0.407	4.444	50	-1.028	0.553	50	4.539	61	***

Figure 107. Bioassay VI. Statistical analysis. Level of significance is indicated by NS, Not Significant; *, $p<0.05$; **, $p<0.01$; or ***, $p<0.001$.

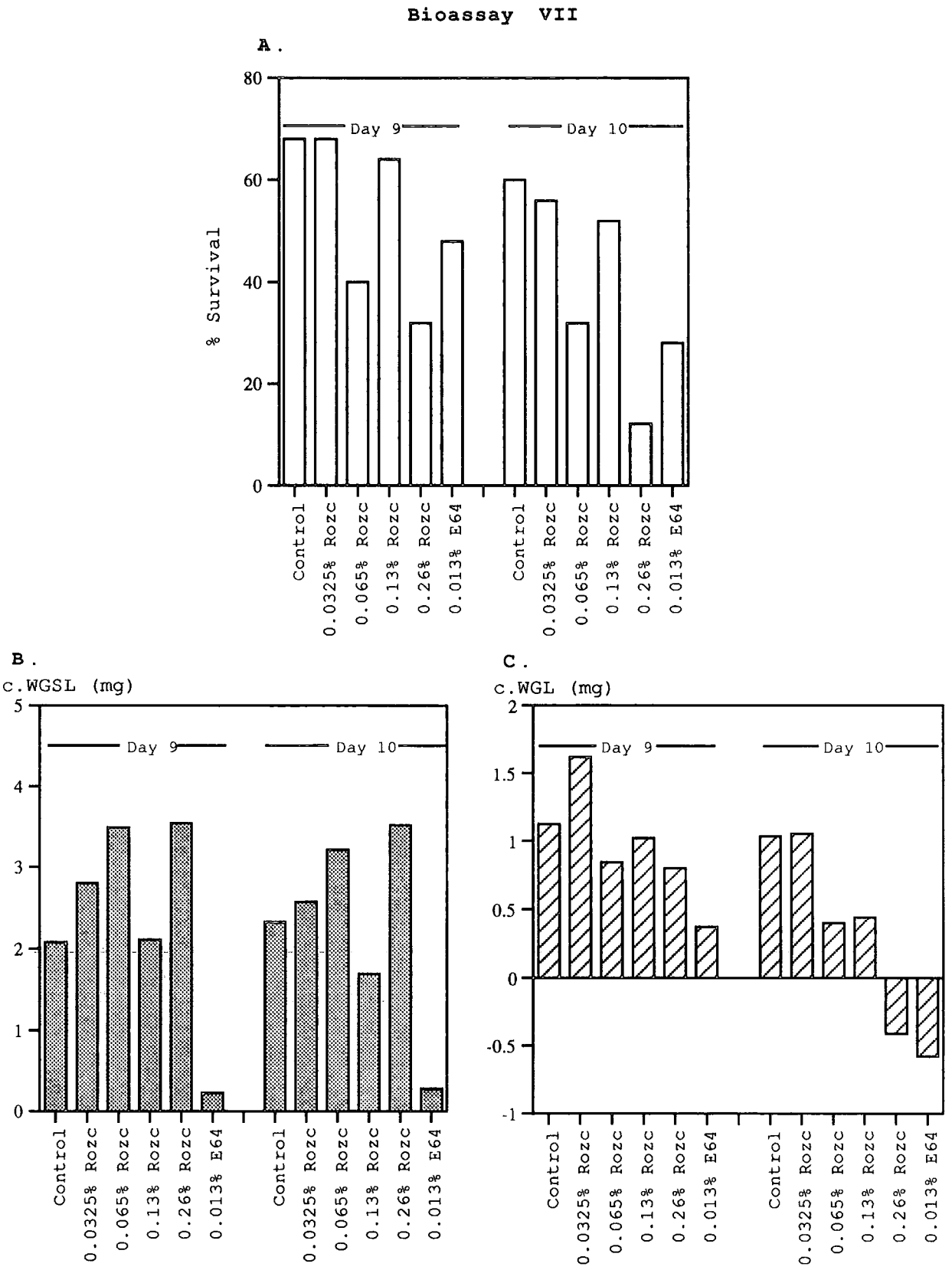

Figure 108. Bioassay VII. Effect of Rozc and E64 on the survival (panel A) and development of SCR larvae. Development is given as corrected weight gain per surviving larva (c.WGSL, panel B), and corrected weight gain per larva (c.WGL, panel C). Protein concentration is indicated as % (w/v). Results from days 9 and 10 are presented.

Survival

Assay	Control No.alive	Total	Treatment No.alive	Total	G_s	df	
Day 9							
0.26%Rozc	17	25	8	25	5.211	1	*
0.065%Rozc	17	25	10	25	2.928	1	NS
Day 10							
0.26%Rozc	15	25	3	25	11.079	1	***
0.065%Rozc	15	25	7	25	2.928	1	NS
E64	15	25	7	25	4.035	1	*

Corrected WGSL

Assay	Control Mean	s^2	n	Treatment Mean	s^2	n	t_s	df	
Day 9									
0.0325%Rozc	2.081	1.020	17	2.804	0.170	17	2.732	21	*
0.065%Rozc	2.081	1.020	17	3.485	2.849	10	2.390	13	*
0.13%Rozc	2.081	1.020	17	2.110	0.160	16	0.109	21	NS
0.26%Rozc	2.081	1.020	17	3.541	0.392	8	4.422	21	***
E64	2.081	1.020	17	0.222	0.047	12	7.354	18	***
Day 10									
0.0325%Rozc	2.326	1.293	15	2.575	0.160	14	0.797	18	NS
0.065%Rozc	2.326	1.293	15	3.213	2.292	8	1.453	11	NS
0.13%Rozc	2.326	1.293	15	1.692	0.044	13	2.118	15	*
0.26%Rozc	2.326	1.293	15	3.515	0.109	3	3.394	12	**
E64	2.326	1.293	15	0.278	0.026	7	6.831	15	***

Corrected WGL

Assay	Control Mean	s^2	n	Treatment Mean	s^2	n	t_s	df	
Day 9									
0.0325%Rozc	1.125	2.696	25	1.621	3.204	25	1.021	48	NS
0.065%Rozc	1.125	2.696	25	0.842	5.915	25	0.482	42	NS
0.13%Rozc	1.125	2.696	25	1.020	2.304	25	0.235	48	NS
0.26%Rozc	1.125	2.696	25	0.800	4.264	25	0.616	48	NS
E64	1.125	2.696	25	0.370	0.360	25	2.159	30	*
Day 10									
0.0325%Rozc	1.033	3.367	25	1.052	3.158	25	0.037	48	NS
0.065%Rozc	1.033	3.367	25	0.401	4.541	25	1.123	48	NS
0.13%Rozc	1.033	3.367	25	0.438	1.798	25	1.309	48	NS
0.26%Rozc	1.033	3.367	25	-0.412	2.196	25	3.063	48	**
E64	1.033	3.367	25	-0.581	0.307	25	4.210	28	**

Figure 109. Bioassay VII. Significance is indicated as for figure 107.

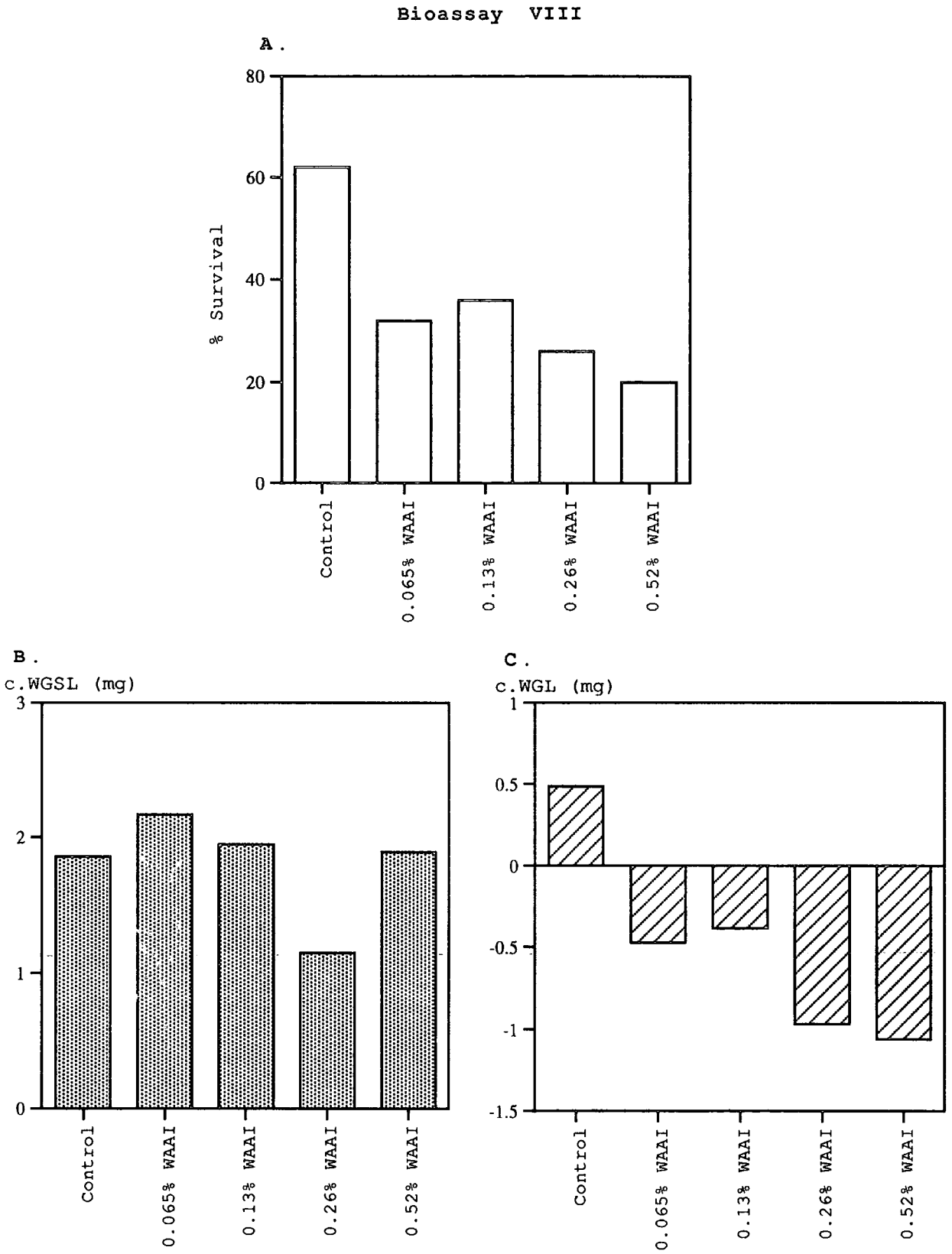

Figure 110. Bioassay VIII. Effect of WAAI on the survival (panel A) and development of SCR larvae, using artificial diet 3 (starch diet)I. Protein concentrations are given as % (w/v) in artificial diet. Development is given as corrected weight gain per surviving larva (c.WGSL, panel B), and corrected weight gain per larva (c.WGL, panel C).

Survival

Assay	Control No.alive	Control Total	Treatment No.alive	Treatment Total	G_s	df	
0.065%WAAI	31	50	16	50	7.976	1	**
0.13%WAAI	31	50	18	50	5.819	1	*
0.26%WAAI	31	50	13	50	11.985	1	***
0.52%WAAI	31	50	10	50	17.101	1	***

Corrected WGSL

Assay	Control Mean	Control s^2	Control n	Treatment Mean	Treatment s^2	Treatment n	t_s	df	
0.065%WAAI	1.856	1.409	31	2.168	0.861	16	0.990	45	NS
0.13%WAAI	1.856	1.409	31	1.948	1.369	18	0.263	47	NS
0.26%WAAI	1.856	1.409	31	1.146	1.100	13	1.968	42	NS
0.52%WAAI	1.856	1.409	31	1.893	7.913	10	0.040	10	NS

Corrected WGL

Assay	Control Mean	Control s^2	Control n	Treatment Mean	Treatment s^2	Treatment n	t_s	df	
0.065%WAAI	0.486	3.98	50	-0.471	3.614	50	2.455	98	*
0.13%WAAI	0.486	3.98	50	-0.385	3.614	50	2.230	98	*
0.26%WAAI	0.486	3.98	50	-0.967	1.877	50	4.244	87	***
0.52%WAAI	0.486	3.98	50	-1.059	3.679	50	3.946	98	***

Figure 111. Bioassay VIII. Statistical analysis. Level of significance is indicated by NS, Not Significant; *, $p<0.05$; **, $p<0.01$; or ***, $p<0.001$.

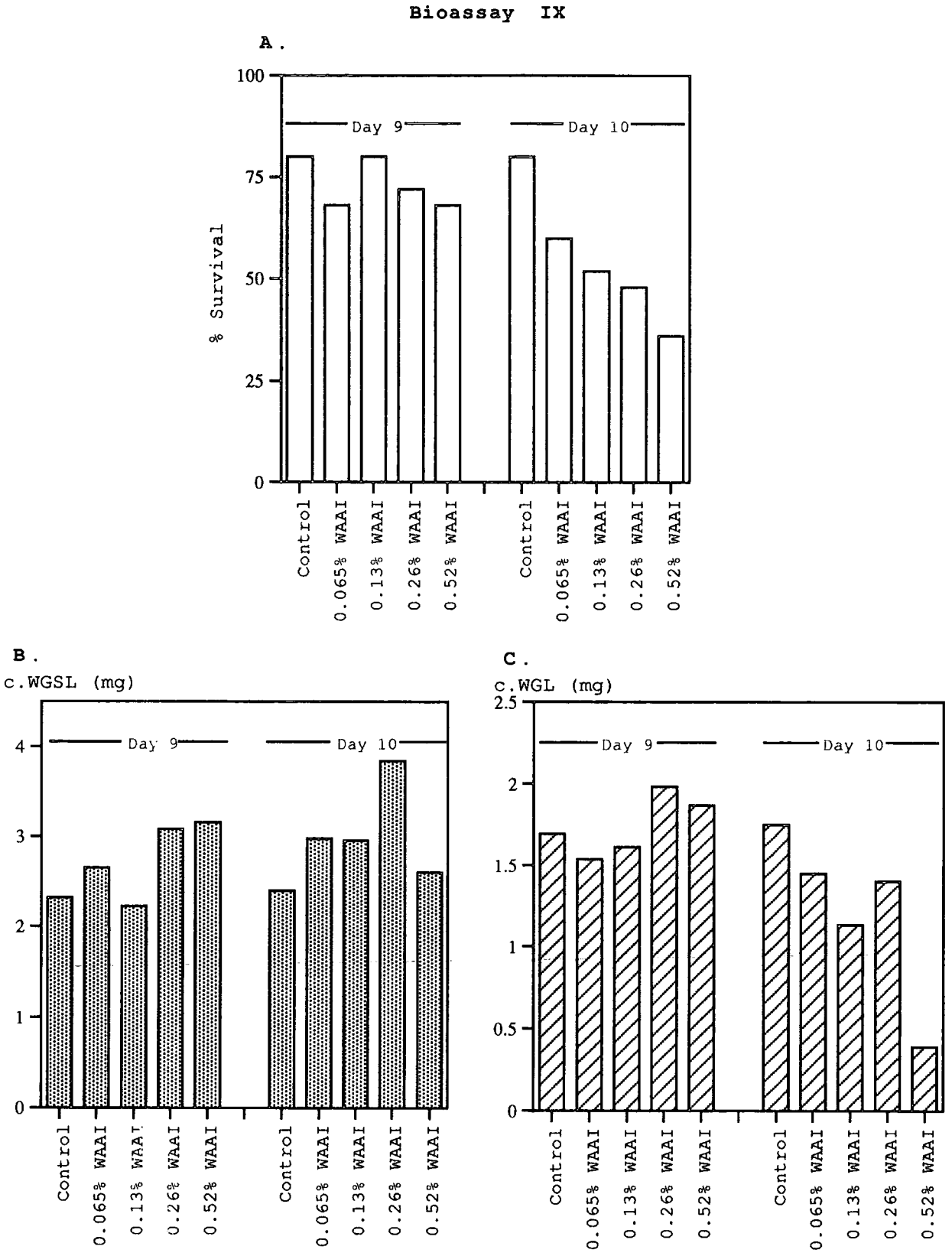

Figure 112. Bioassay IX. Effect of WAAI on the survival (panel A) and development of SCR larvae, using artificial diet 3. Concentration are given as % (w/v) in artificial diet. Development is given as corrected weight gain per surviving larva (c.WGSL, panel B), and corrected weight gain per larva (c.WGL, panel C). Results from days 9 and 10 are presented.

Survival (day 10)

Assay	Control No.alive	Total	Treatment No.alive	Total	G_s	*df*	
0.52%WAAI	20	25	9	25	8.481	1	**
0.26%WAAI	20	25	12	25	4.339	1	*
0.13%WAAI	20	25	13	25	3.261	1	NS

Corrected WGSL

Assay	Control Mean	s^2	*n*	Treatment Mean	s^2	*n*	t_s	*df*	
Day 9									
0.065%WAAI	2.324	1.020	20	2.655	1.486	17	0.890	35	NS
0.13%WAAI	2.324	1.020	20	2.224	0.226	20	0.401	27	NS
0.26%WAAI	2.324	1.020	20	3.085	1.073	18	2.288	35	*
0.52%WAAI	2.324	1.020	20	3.157	0.103	17	3.487	23	**
Day 10									
0.065%WAAI	2.398	0.801	20	2.979	1.107	15	1.763	33	NS
0.13%WAAI	2.398	0.801	20	2.960	0.645	13	1.833	31	NS
0.26%WAAI	2.398	0.801	20	3.841	1.719	12	3.705	30	***
0.52%WAAI	2.398	0.801	20	2.604	0.699	9	0.794	27	NS

Corrected WGL

Assay	Control Mean	s^2	*n*	Treatment Mean	s^2	*n*	t_s	*df*	
Day 9									
0.065%WAAI	1.690	2.487	25	1.535	3.767	25	0.310	48	NS
0.13%WAAI	1.690	2.487	25	1.611	1.745	25	0.192	48	NS
0.26%WAAI	1.690	2.487	25	1.983	4.012	25	0.575	48	NS
0.52%WAAI	1.690	2.487	25	1.869	3.740	25	0.358	48	NS
Day 10									
0.065%WAAI	1.749	2.384	25	1.449	4.305	25	0.580	48	NS
0.13%WAAI	1.749	2.384	25	1.132	4.092	25	1.212	48	NS
0.26%WAAI	1.749	2.384	25	1.399	6.502	25	0.587	39	NS
0.52%WAAI	1.749	2.384	25	0.390	3.097	25	2.902	48	**

Figure 113. Bioassay IX. Statistical analysis. Level of significance is indicated by NS, Not Significant; *, $p<0.05$; **, $p<0.01$; or ***, $p<0.001$.