Midgut proteases from larval spodoptera littoralis (lepidoptera: noctutoae)

Lee, Michael James

MIDGUT PROTEASES FROM LARVAL *SPODOPTERA LITTORALIS* (LEPIDOPTERA: NOCTUIDAE)

By

Michael James Lee

B.Sc. (Dunelm)

Being a thesis submitted for the degree of Doctor of Philosophy of the University of Durham.

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Hatfield College
University of Durham

DECLARATION

I hereby declare that the work presented in this document is based on research carried out by me, and that no part has been previously submitted for a degree in this or any other university.

ABSTRACT

The presence and properties of proteases present in larval midgut extracts from *Spodoptera littoralis* was investigated. Trypsin and chymotrypsin activities were found mainly in the midgut lumen while leucine aminopeptidase and dipeptidyl aminopeptidase IV activities were found solely in the midgut tissue. Hydrolysis of carboxypeptidase substrates indicated carboxyesterase A activity only. The characteristics of trypsin, chymotrypsin and leucine aminopeptidase were determined with respect to a variety of factors including pH, temperature, substrate concentration, P_1 amino acid specificity and molecular weight. Inhibition of the endopeptidases by a wide variety of protease inhibitors derived from chemical, microbial, plant and animal sources was also carried out. Endopeptidase activities were compared with proteases from *Helicoverpa armigera* and mammalian sources and the endopeptidase specificity discussed.

In addition, a haemolymph phenoloxidase (PO) was characterised (pH, substrate concentration), purified and the effects of various zymogen activating and PO inhibitory substances determined.

Finally, two parasites were studied. Firstly, the ultrastructure of stages in the life-cycle of a microsporidian parasite (*Nosema* sp.) from *H. armigera* and secondly, the morphological changes associated with a nuclear polyhedrosis virus (NPV) disease in *S. littoralis*, together with ultrastructural characterisation and comparison of restriction endonuclease patterns of the nucleic acids between NPVs from different sources.

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GLOSSARY

AApNA	N-acetyl-L-aspartic acid α-(pNA)
Abs	absorbance
AcA_3pNA	N-acetyl-alanyl-alanyl-alanine pNA
AlapNA	L-Alanine pNA
ALpNA	N-acetyl-L-leucine pNA
AMP	2-amino-2-methyl-1-propanol
AP	aminopeptidase
APpNA	arginyl proline pNA
ArgpNA	L-Arginine pNA
ATEE	N-acetyl-L-tyrosine ethyl ester
BAEE	N-α-benzoyl-L-arginine ethyl ester
BApNA	N-α-benzoyl-DL-arginine pNA
BBTI	Bowman-Birk trypsin inhibitor
BLpNA	N-α-benzoyl-L-lysine pNA
BM	Bratton-Marshall reaction
BSA	bovine serum albumin
BTEE	N-benzoyl-L-tyrosine ethyl ester
BTP	bis tris propane
BTpNA	N-benzoyl-L-tyrosine pNA
CAPS	(3-[cyclohexylamino]-1-propane-sulfonic acid
CEOI	Chicken egg ovoinhibitor
CEOM	Chicken egg ovomucoid
CK	chloromethyl ketone
CPA	carboxypeptidase A
CPB	carboxypeptidase B
CPTI	cowpea trypsin inhibitor
Da	Daltons
DDW	distilled deionized water
DFP	diisopropylphosphofluoridate
DMF	dimethyl formamide
DSS	2,2-dimethyl-2-silapentane-5-sulphonate
DTT	dithiothreitol
ε	coefficient of absorption
E-64	trans-epoxysuccinyl-L-leucylamido(4-guanidino)butane
E_a	activation energy
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(ß-aminoethyl ether) N,N,N',N'-tetraacetic acid
GlupNA	L-γ-glutamyl-pNA
GlypNA	L-Glycine pNA

H.a.	*Helicoverpa armigera*
HA	hippuryl arginine
hippuryl	N-benzoyl-glcyl-phenylalanyl
HPA	hippuryl phenylalanine
HPLA	hippuryl phenyllactic acid
i.d.	internal diameter
I_{50}	50% inhibition
IAAcid	iodoacetic acid
IAAmide	iodoacetamide
IVEM	Institute of Virology and Environmental Microbiology
K	Kelvin
K_i	inhibition constant
K_{ia}	K_i calculated using method of Dixon (1972)
K_{ib}	K_i calculated using method of Dixon (1953)
L-B	Lineweaver-Burk plot
LBTI	Lima bean trypsin inhibitor
leußNA	L-leucine-ß-naphthylamide
LeupNA	L-Leucine pNA
LyspNA	L-Lysine pNA
M	molar
MetpNA	L-Methionine pNA
M_r	molecular weight
N	no. of replicates
n	no. of substrate binding sites
N.D.	not determined
NEDD	N-(1-naphthyl)-ethylenediamine dihydrochloride
NEM	N-ethylmaleimide
NPV	nuclear polyhedrosis virus
p	pico- (10^{-12}) or probability
PAGE	polyacrylamide gel electrophoresis
pCMB	p-chloromercuribenzoic acid
pCMPS	p-chloromercuriphenyl-sulfonic acid
PhepNA	L-Phenylalanine pNA
PIB	Polyhedral inclusion body
PIPES	piperazine-N,N'-bis(2-ethane-sulphonic acid)
PMSF	Phenylmethylsulphonyl fluoride
pNA	p-nitroanilide or p-nitroaniline
PO	Phenoloxidase
PPO	Prophenoloxidase
ppt	precipitate
PropNA	L-Proline pNA

PVP	polyvinylpyrrolidine
Rel.Act.	relative activity
REN	restriction endonuclease
SA_2PLpNA	N-Succinyl-alanyl-alanyl-prolyl-leucyl pNA
SA_2PPpNA	N-Succinyl-alanyl-alanyl-prolyl-phenylalanyl pNA
SA_2VpNA	N-Succinyl-alanyl-alanyl-valinyl-pNA
SA_3pNA	N-Succinyl-alanyl-alanyl-alanyl pNA
SBTI	soybean trypsin inhibitor
SDS	sodium dodecyl sulphate
sem	standard error of the mean
S.l.	*Spodoptera littoralis*
spnt	supernatant
succinyl	3-carboxypropionyl
Suphepa	succinyl phenylalanine pNA
TAME	N-α-p-tosyl-L-arginine methyl ester
TCA	trichloroacetic acid
TEE	L-tyrosine ethyl ester
TEMED	N,N,N',N'-Tetramethylethylenediamine
TEW	Turkey egg white
TGPApNA	N-p-tosyl-glycyl-prolyl-arginine pNA
TGPLpNA	N-p-Tosyl-glycyl-prolyl-lysine-pNA
TLCK	N-α-p-tosyl-L-lysine chloromethyl ketone
TPCK	N-tosyl-L-phenylalanine chloromethyl ketone
Tris	tris(hydroxymethy)aminomethane
v/v	volume per volume
ValpNA	L-Valine pNA
V_e	eluted volume
V_o	void volume
w/v	weight per volume
x	mean
Z	N-carboxybenzoxy
ZAGPCK	Z-alanyl-glycyl-phenylalanine CK
ZGGLpNA	Z-glycyl-glycyl-leucine pNA
ZGLPCK	Z-glycyl-leucyl-phenylalanine CK
ZGPCpNA	Z-glycyl-prolyl-citrulline pNA

GENERAL INTRODUCTION

It has been estimated that 37% of the total potential crop production is lost to pest damage; insects accounting for 13% (Gatehouse and Hilder, 1988). Expenditure to control pests of the world's three major crops: cotton, maize and rice, amounted to 3.8 billion dollars in 1987 (Gatehouse and Hilder, 1988). Chemical control is expensive and has an effect on the environment by affecting non-target organisms.

Helicoverpa armigera, the corn earworm, is a major pest, particularly on cotton and legumes, throughout Africa, Asia, India, Australasia, the Pacific and Europe. (Hill, 1983; Fitt, 1989). *S. littoralis*, the Egyptian cotton worm, is a major pest of many crops in the Mediterranean area (Abul-Nasr, 1959; Hafez, 1958; Ben-Shaked, 1960; Ishaaya *et al*, 1971). Lepidoptera, along with other insects, tend to develop resistance to a range of pesticides and recent studies have suggested resistance to the synthetic pyrethroids (Sawicki and Denholm, 1987). Other means of control are necessary to complement the present chemical control and reduce damage by these pests.

A number of other control measures have been considered for lepidopteran insects, such as the use of nuclear polyhedrosis viruses (Benz, 1981), microsporidia (David and Novotny, 1990), fungi (Gillespie, 1988) and bacteria (Dulmage, 1981). Such parasites occur naturally; indeed, microsporidian and viral infections caused complete loss of insect stock at various times throughout this project. These potential control agents are detailed in chapters 4 and 5.

It has been shown that wounding of leaf tissues leads to the accumulation of proteinaceous proteinase inhibitors in plants (Green and Ryan, 1972; Brown *et al*, 1985; Wolfson and Murdock, 1990b; Broadway and Missurelli, 1990) which has led to speculation that these inhibitors are involved in plant defense mechanisms (Ryan and Green, 1974; Broadway *et al*, 1986). It has been proposed that these inhibitors be used to inhibit insect midgut proteinases and thereby act as a method for controlling insects (Boulter *et al*, 1989; Boulter *et al*, 1990a,b; Broadway and Duffey, 1986; Gatehouse and Hilder, 1983; Hilder *et al*, 1987; Murdock *et al*, 1988 and Shukle and Murdock, 1983). Using this strategy for insect pest control, resistant crop plants are produced by gene transfer of single genes encoding proteins which are toxic or unpalatable to the insect. Recent studies have suggested that plant proteinaceous inhibitors reduce insect growth and survival (Gatehouse and Boulter, 1983; Wolfson and Murdock, 1987). The Kunitz inhibitor from soybean has been shown to inhibit larval growth of *Manduca sexta* (Shukle and Murdock, 1983). *Spodoptera exigua* and *Heliothis zea* are lepidopteran pests which have been shown to be susceptible to protease inhibitors from soybean and potato in *in vivo* feeding trials (Broadway and Duffey, 1986). The corn borer (*Ostrinia nubilialis*) has also proved susceptible to SBTI (Steffens *et al*, 1978). Christeller *et al* (1990) demonstrated the presence of two endopeptidases in the black field cricket (*Teleogryllus commodus*) and then showed that the inclusion of protein inhibitors of these enzymes in diets reduced insect growth; proteins inhibitory to both

enzymes had the greatest effect (Burgess *et al*, 1991). In addition, Hilder *et al* (1987) showed that the introduction of the CPTI gene into tobacco plants resulted in a significant reduction of damage caused by the lepidopteran insect *Heliothis virescens*. Additive protective effects have been observed when two control measures have been tried simultaneously: Boulter *et al* (1990a) showed that transgenic plants encoding both CPTI and pea lectin genes exhibited enhanced resistance to *Heliothis virescens* while the insecticidal activity of *Bacillus thuringiensis* is potentiated by serine protease inhibitors (MacIntosh *et al*, 1990). Other methods for increasing the level of protease inhibitors in plants could be derived from interplant communication: certain signalling molecules, when applied to surfaces of plants, induce an increase in the inhibitor concentration in nearby plants (Farmer and Ryan, 1990).

It has been shown that a peptide is involved in the modulation of trypsin enzyme biosynthesis in mosquitoes (Borovsky *et al*, 1990). This peptide exhibits sequence similarities with mammalian, plant and several viral proteins. If such peptides are common throughout different insect orders, then this too may present possibilities for insect pest control.

It appears from the literature that midgut proteolytic activity varies from species to species with all four classes of proteinases found in insects (Wolfson and Murdock, 1990a; Terra, 1988). Therefore, knowledge of the proteases from the insects of interest would be of value if an insect control strategy is to be employed using protease inhibitors.

GENERAL MATERIALS AND METHODS

Insectary

Insects were reared in an insectary which was maintained at a temperature of 26 ± 2°C by an air conditioning unit (Prestair) and thermostatically controlled electric fan heater and at a humidity of 40 ± 10%. Air circulation was effected by electric fans and a constant photoperiodic regime of 14 hours light and 10 hours dark was in operation.

Stock animals

A population of *Helicoverpa* (*Heliothis*) *armigera* (Sudan strain) was obtained from the ODA Heliothis Unit, University College, Cleppa Park Field Research Station, Newport, Wales as first and second instar larvae (Authorized for retention by the Ministry of Agriculture, Fisheries and Food under licence number PHF346/832(88)). Insect larvae were reared as a population until the end of the second instar. Thereafter, the insect larvae were reared singly in 3oz plastic pots (supplied by DRG Products Ltd.) on an artificial diet which was in abundant supply. It was assumed that if phase changes did occur during the remaining three instars then these did not affect the experiments adversely. Phase changes are known to occur in lepidoptera (Mathee, 1945, 1947); in a crowd they are active, dark and velvety (gregaria); reared in isolation they vary in colour from green to pinkish grey. However, it is not clear either how long these phase changes take (at least one generation?) or what affect these have on the digestive physiology of the moth.

Populations of *Spodoptera littoralis* were obtained from Shell International Ltd. (Sittingbourne, Kent) as eggs laid on blotting paper. Dark, opaque eggs (an indication that hatching is imminent) were put into 3oz plastic pots and maintained on an artificial diet (composition given below) until the larvae had developed to the second instar. *Spodoptera littoralis* are not cannibalistic and can be maintained in the laboratory at relatively high densities. However, they are very susceptible to viral infection at high humidity (caused by water evaporation from food) and were transferred to larger boxes (27cm x 15cm x 9cm) containing large holes sealed with a fine mesh to help minimize this effect. Prepupal insects were transferred to boxes of the same dimensions containing peat to a depth of 1.5-2" in which the insects burrowed prior to pupation. Pupae were collected in 12oz open margarine containers (supplied by Joni Ltd., Randers, Denmark) and transferred to cylindrical cages made from aluminium and acetate sheet (29cm i.d. x 40cm^2) lined with tissue paper on which the female adult moths lay their eggs. The adult moths were supplied with cotton wool soaked with a liquid diet in an open margarine container.

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Composition of the diets

The diet was prepared as described below using ingredients in the following proportions (Singh, 1977):

i. Diet for larval *Spodoptera littoralis*

Casein	54.00g
Sucrose	54.00g
Bemax	70.00g
Cellulose	10.00g
Sorbic Acid	1.25g
p-Hydroxybenzoic Acid Methyl Ester	1.75g
Linseed Oil	1.13g
Agar	38.00g
Vanderzant Vitamin Mix*	14.40g
Salt Mix#	14.40g
Aureomycin	1.00g
Ascorbic Acid	0.50g
Ampicillin	0.15g
Formalin	0.50ml
Water	2000.00ml

The agar was dissolved by heating in 1300ml DDW. 700ml of DDW was then added to cool the temperature down to 60°C before adding all the other dietary components except Vitamin C, chlorotetracyclin and formaldehyde. The remaining ingredients were mixed in thoroughly once the temperature had fallen below 50°C. After mixing, the diet was put into plastic petri dishes, allowed to set and stored at 4°C until required.

ii. Diet for larval *Helicoverpa armigera*

Haricot bean seed finely ground	300.00g
Ascorbic acid	4.70g
p-Hydroxybenzoic Acid Methyl Ester	3.00g
Sorbic acid	1.50g
Aureomycin	7.50g
Linseed oil	12.00ml
Vitamin stock solution*	10.00ml
Water(a)	500.00ml
Yeast	48.00g
Agar °	17.25g
Water(b)	810.00ml

The agar was heated in water(b), allowed to cool to below 50°C and added to the

other ingredients premixed with water(a). The diet was then poured into trays and kept at 4°C until use.

iii. Diet for adult insects (made up to 1 litre with DDW)

Sucrose	50.04g
Honey	60.00ml
Yeast	1.01g
p-hydroxybenzoic acid methyl ester	2.00mg
Vitamin solution*	20.00ml

*2.01g Vanderzant Vitamin supplement for insects (United States Biochemical Corporation, Cleveland, Ohio) dissolved in 200ml DDW.

Vanderzant Vitamin supplement contains (composition per 1000g) (Singh, 1977) :

α-tocopherol (Vitamin E)	8g
ascorbic acid (Vitamin C)	270g
biotin (Vitamin H)	20mg
calcium pantothenate (Vitamin B_5)	1g
choline chloride	50g
folic acid	250mg
inositol	20g
niacinamide	1g
pyridoxine hydrochloride (Vitamin B_6)	250mg
riboflavin (Vitamin B_2)	500mg
thiamine hydrochloride (Vitamin B_1)	250mg
Vitamin B_{12} trituration in mannitol	2g

Wessons salts (ICN Biochemicals, Cleveland, Ohio) contain salts in the following amounts:

$CaCO_3$	120.00g
K_2HPO_4	129.00g
$CaPHO_4.2H_2O$	30.00g
$MgSO_4.7H_2O$	40.80g
NaCl	67.00g
$FeC_6H_5O_7$	11.00g
KI	0.32g
$MnSO_4.H_2O$	2.00g
$ZnCl_2$	0.10g
$CuSO_4.5H_2O$	0.12g

Treatment of glassware

All glassware was soaked overnight in a 2% solution of Teepol laboratory detergent, rinsed several times in cold tap water and twice in DDW.

Reagents

All mammalian enzymes and enzyme substrates were supplied by Sigma Chemical Co. Ltd., U.K. except Suphepa, which was obtained from Boehringer Mannheim Gmbh, Germany. All enzyme inhibitors, except CPTI, were obtained from Sigma. CPTI was a gift from Dr. A.M.R. Gatehouse and Dr. V. Hilder, University of Durham, U.K.. Standard proteins for gel filtration and SDS-PAGE were obtained from Sigma. Other chemical reagents were from Sigma and BDH Merck Ltd., U.K.. All reagents were of AnalaR grade or the purest commercially available. All solutions were prepared in DDW unless otherwise stated. Chromatography media (Sephadex G-25, G-75, Sephacryl S-200 and Q-Sepharose) were obtained from Pharmacia Limited, Uppsalla, Sweden.

Electron Microscopy

Karnovsky's fixative (Karnovsky, 1965) was prepared from two stock solutions of the following composition:

Solution A: 2g paraformaldehyde
40ml distilled water
2-6 drops 1M NaOH.

The paraformaldehyde and distilled water were warmed with continued shaking and sodium hydroxide slowly added dropwise until any precipitate had dissolved.

Solution B: 10ml 25% gluteraldehyde
50ml 0.2M sodium cacodylate-NaOH, pH 7.3.

Solutions A and B were kept separate at 4°C until required, when they were mixed in a 1:1 ratio. Animals were killed by decapitation, the alimentary tract removed by dissection and segments of midgut fixed for 1-1.5 hours at 0-4°C followed by post-fixation in 1% osmium tetroxide in sodium cacodylate buffer (pH 7.3) for 1 hour at 0-4°C.

The tissue was then dehydrated, at room temperature, through a graded series of ethanol solutions; fifteen minutes in each of 70% and 95% alcohol and 30 minutes in absolute alcohol. After 30 minutes in a 1:1 mixture of propylene oxide and absolute alcohol followed by 30 minutes in propylene oxide alone, the larval guts were infiltrated with a 1:1 mixture of propylene oxide and Araldite epoxy resin for 30 minutes at 45°C. They were then placed in absolute Araldite in specimen tubes at 45°C for thirty minutes with the lids off. Following infiltration with Araldite, the larval guts were embedded in Araldite and polymerization was effected for 12 hours at 45°C followed by 48 hours at 60°C. The Araldite mixture consisted of: 10ml Araldite (CY212), 10ml DDSA (dodecenyl succinic anhydride), 1ml dibutyl phthalate and 0.5ml DMP 2,4,6-tri(dimethylaminomethyl) phenol.

Silver/silver-gold sections of the anterior and posterior regions of the midgut of fifth instar larvae and the anterior region of the midgut of sixth instar larvae were cut on a

Reichert NK ultratome Model Om U2 or Om U3 using glass knives made with an LKB 7800 Knifemaker, expanded with chloroform vapour and mounted on uncoated copper grids. Sections were stained with uranyl acetate and lead citrate (Reynolds, 1963) before examination under a Philips EM 400T electron microscope.

Sections for light microscopy were also prepared from Araldite-embedded tissue. 'Thick' sections (1μm) were cut as described above, placed on glass slides, stained with toluidine blue and mounted in DePeX.

Estimation of Protein concentration

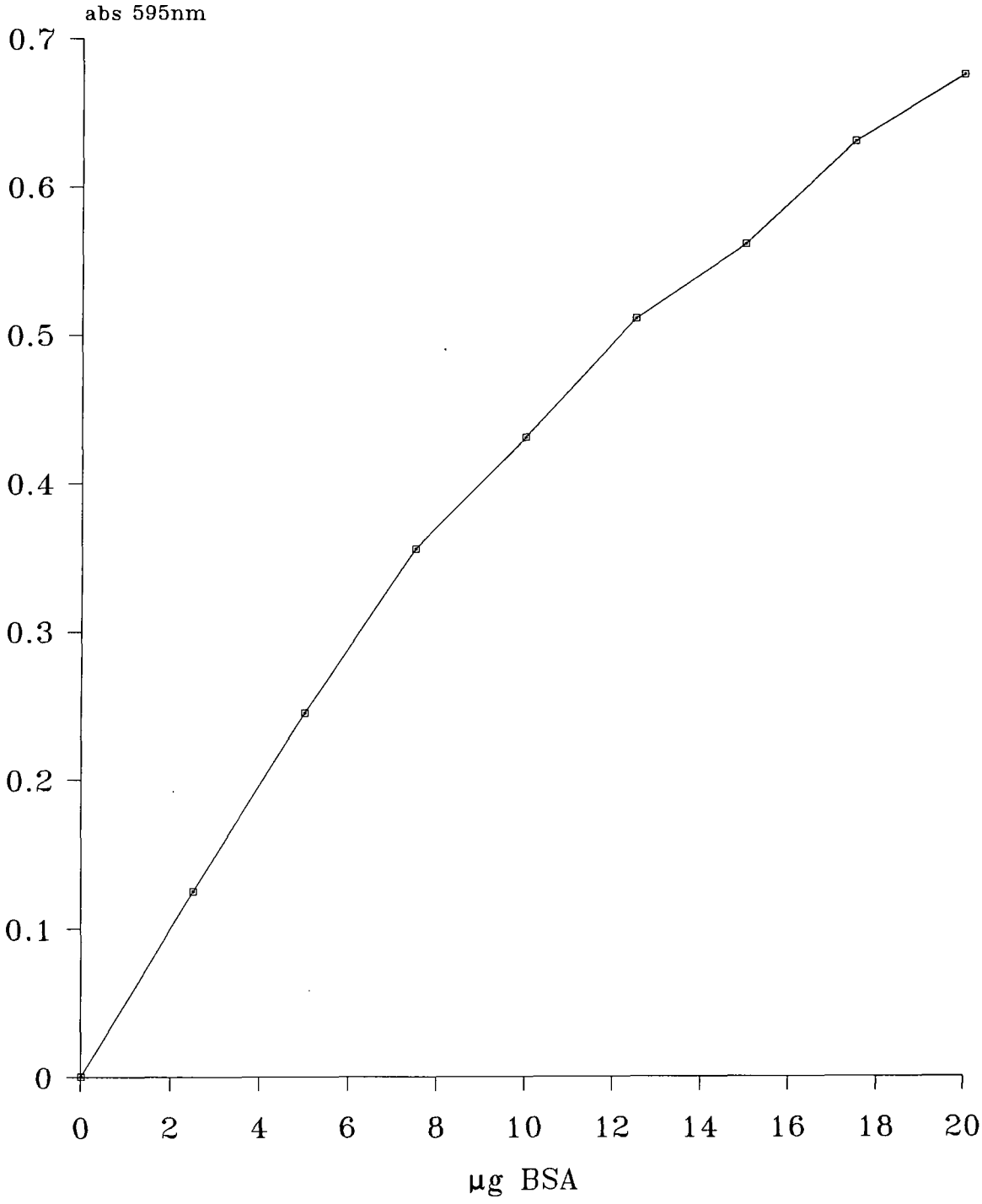
All protein determinations were made using a dye-binding assay with bovine serum albumin Fraction V as standard (Bradford, 1976). The protein reagent was made up as follows: 100mg Coomassie Blue was dissolved in 50ml of 95% ethanol. 100ml orthophosphoric acid (85%) was then added and the volume made up to 1 litre. Protein determinations were carried out by pipetting 0.1ml test sample into a test tube to which was added 10μl 1.1M NaOH and 1ml protein reagent. Tubes were left for ten minutes at room temperature, when the absorbance of each solution was read at 595nm on a Unicam SP1800 Ultraviolet Spectrophotometer. Bradford (1976) found that protein-dye complex formation was complete at 2 minutes and remained stable to within 4% for a period of 1 hour. However, all samples were read within 20 minutes as this is the flattest portion of the colour stability curve. Protein content was determined by reference to a freshly prepared standard curve relating protein concentration to absorbance (0-20μg BSA/100μl sample) (Fig. 0.1). Standard samples were treated in the same way as test samples.

Figure 0.1.

Standard curve of protein estimation using micromethod of Bradford (1976).

Legend: ordinate - absorbance at 595nm.
abscissa - amount protein (μg BSA).

Figure 0.1



CHAPTER 1

Characterization of midgut endopeptidases
from *Spodoptera littoralis* and *Helicoverpa armigera*

Introduction

Much less is known about invertebrate proteases than their mammalian and bacterial counterparts, at least partly as a consequence of the smaller size of the animals. The invertebrates comprise 9 of the 10 phyla and their evolutionary pathways diverged about 10^9 years ago (Zwilling and Neurath, 1981). For these two reasons, one might anticipate a more diverse structure and function in invertebrate proteases which may have no equivalent among the vertebrates.

The biochemistry of digestion in insects is varied; organic material of almost every kind in nature is eaten by insects (Gilmour, 1961; Wigglesworth, 1972), but the majority of insect species are phytophagous, while others are carnivorous, omnivorous or parasitic (Gillott, 1980). In accordance with their diversity of feeding habits, the structure, physiology and biochemistry of their digestive systems are highly varied. It is thought that the digestive enzymes of the alimentary tract reflect the type of food consumed (Dadd, 1970; Dow, 1986). Early work on insect proteases using natural proteins only as substrates, provided useful information regarding the presence and pH characteristics of endopeptidases, but did not allow the amino acid specificity of the protease to be determined (Powning *et al*, 1951). With the availability of artificial substrates, such as BAEE and ATEE, (Schwert *et al*, 1948; Hummel, 1959) and then the colorimetric substrates using pNA as the coloured product e.g. BApNA (Erlanger *et al*, 1961) the specificity of proteases, including those from insects, could be determined more easily. More recently, the introduction of tetrapeptide colorimetric substrates (Nakajima *et al*, 1979; Kasafirek *et al*, 1976) have facilitated the detection of endopeptidases. Cleavage of substrate by peptidases is more rapid with long peptide substrates (Bieth, 1989) due to an extended binding site in many proteases. For example, bovine chymotrypsin requires a minimum substrate size of tetrapeptides for optimal activity (Morihara *et al*, 1969). Microplate technology, as a method for assay volume reduction, has enabled the characterisation of the insect proteases to proceed at an increased pace and reduced the problems associated with the smaller size of insect bodies (e.g. Christeller *et al*, 1991).

The alimentary canal of insects is divided into three main regions: the foregut, midgut and hindgut. The foregut and hindgut are ectodermal in origin and therefore lined with cuticle, while the midgut is endodermal and therefore lacks a cuticular lining. The digestive enzymes which hydrolyze the food into products suitable for uptake and absorption are secreted mainly by the midgut, which is frequently where the majority of general digestion occurs. The enzymes secreted into the lumen function optimally within a limited range of pH and temperature and are adapted to the conditions within the midgut and to the diet on which the insects feed (Dow, 1986; Terra, 1990).

The pH of insect midgut contents is usually at pH 6-7.5 (House, 1974). Major exceptions are the very alkaline midgut contents (pH 8-12) of Lepidoptera (Dow, 1984; Chamberlain, 1990; Chao *et al*, 1991), scarab beetles (Bayon, 1980), detritus-feeding dipteran larvae (Dadd, 1975; Terra *et al*, 1979; Undeen, 1979; Sharma *et al*, 1984) and higher termites (Bignell and Anderson, 1980), the very acid middle region (pH 2.8-3.5) of the midgut of cyclorraphous Diptera (Greenberg, 1968) and the acid (pH 4.5-5) posterior region of the midgut of Hemiptera and Heteroptera (House, 1974). Thus, insect proteases function under a wide variety of conditions and the properties of the digestive enzymes must vary from species to species to enable activity at various different pH values.

The highly alkaline midgut lumen of Lepidoptera is thought to allow these insects to feed on plant material rich in tannins (Terra, 1988), which bind to proteins at lower pH, reducing the efficiency of digestion (Berenbaum, 1980) or to allow extraction of hemicelluloses from ingested plant cell walls (Terra, 1990). Surfactants, such as lysolecithin occur widely in insect digestive fluids (Martin and Martin, 1984) and may also be involved in the resistance to dietary tannins displayed by some insects (Bernays *et al*, 1981).

The site of high luminal pH generation in Lepidoptera is believed to be the goblet cells of the midgut (Dow, 1984), while the secretion of digestive enzymes (Wolfersberger, 1984) and absorption of nutrient products (Dow, 1986) are thought to be limited to the columnar cells. The lepidopteran larval midgut transports equal amounts of potassium and alkali from blood to midgut lumen. On this basis, Dow (1984) suggested that the goblet cell cavities in lepidopteran midgut allow coupling of electrogenic transport of potassium with the electrostatic removal of protons from transported bicarbonate inside the goblet cavity, producing a carbonate secretion system. Net transport of K_2CO_3 can thus generate the high pH characteristic of larval lepidopteran midgut.

The digestion of proteins may be divided into three phases: initial, intermediate and final digestion (Terra, 1988; 1990). The initial digestion consists of the dispersion and/or decrease in the M_r of the ingested proteins through the action of endopeptidases (e.g. trypsin). In the present work, the terms *protease* and *proteinase* have been used synonymously with the term *peptidase*, as it is apparent that many *proteases* are capable of acting as *peptidases* by cleaving small colorimetric substrates. Some workers (e.g. Barrett, 1986) differentiate between the term *proteinase* (meaning *endopeptidase*) and *protease* (meaning *exopeptidase* or *endopeptidase*). Intermediate digestion is the hydrolysis by aminopeptidases of peptides to dimers and/or small peptides, which are split into monomers by dipeptidases during the final digestion. Santos *et al* (1983) proposed that in lepidopteran larvae digestion occurs almost exclusively in the midgut and that initial digestion occurs in the endoperitrophic space, whereas intermediate and final digestion occurs at the level of the midgut cells. The main reason for the initial digestion to be limited to the endoperitrophic space while intermediate and final

digestion is confined to the midgut cells appears to be the size of the enzymes. Digestive enzymes enter the endoperitrophic space in the anterior midgut and are removed across the peritrophic membrane together with small hydrolysed proteins from the posterior midgut. The enzymes and nutrients are then displaced towards the anterior end of the midgut where final digestion and absorption of food takes place (Santos *et al*, 1984). Finally, the uptake of the products of protein digestion (i.e. amino acids) by the midgut from the lumen is carried out by a K^+/amino acid cotransporting mechanism localized on the apical surface of columnar cells in lepidopteran larvae (Sacchi *et al*, 1990; Wolfersberger, 1991; Hanozet *et al*, 1992). The main secretory role of the midgut is the active extrusion of potassium from haemolymph to lumen and this generates a large potassium and pH gradient together with a high transepithelial electrical potential difference. This in turn allows a potassium-dependent amino acid uptake.

The peritrophic membrane is usually a cylindrical sheet that envelops the food in the midgut. The roles ascribed to this membrane are: mechanical protection for midgut cells (Wigglesworth, 1972), a physical barrier for microorganisms (Richards and Richards, 1977), a barrier to prevent enzyme excretion (Terra and Ferriera, 1981; Terra *et al*, 1979) and a barrier to organize digestion (Terra, 1988, 1990). The majority of these functions depend on its permeability. Proteases (and other hydrolases such as α-D-glucosidase) restricted to the midgut cells are much larger than those penetrating the endoperitrophic space (Santos and Terra, 1986). The diameter of the pores of insect peritrophic membrane may be as high as 120nm according to Chapman (1985) corresponding with the diameters of the microvilli around which they are formed. However, the imposition of successive membranes may considerably reduce this, which may be a reason why the passage of dextrans and colloidal gold is more limited. Terra and Ferreira (1983) compared the size of the enzymes restricted to the ectoperitrophic space with those present in the endoperitrophic space and concluded that the pore size allowed soluble proteins less than 100kDa to permeate the membrane (corresponding to a pore size of 7-8nm). In addition, Wolfersberger *et al* (1986), using purified proteins in isolated peritrophic membranes from the lepidopteran, *Manduca sexta*, found a pore size of about 100kDa. This size of 7-8nm compares well with that determined by Zimmerman and Mehlan (1976) of 3nm and a size of 4nm determined using colloidal gold particles and labelled dextrans by Zhuzhikov (1970) and Peters and Weise (1986) respectively.

Variation in the endopeptidase characteristics that may exist between the two sexes has been investigated. Baumann (1990) reported that the endopeptidase activity was higher in males compared to females in *Periplaneta americana*, but offered no explanation for the difference. Gooding and Huang (1969) showed differences between the sexes in the beetle, *Pterostichus melanarius*, in their affinity for different protein substrates and total activity per midgut. However, they suggested the latter difference may be an artifact and it is noticeable that there are large standard errors associated with their affinity data. Yaginuma and Ushizima (1991) have shown that a sexual

dimorphism in proteolytic activities (in terms of size and total activity) was distinguishable by column chromatography in the fat body of *Bombyx mori*, but these differences were attributed to developmental changes occurring in the fat body. At present, there is no evidence to suggest that there are different endopeptidases in the male and female midgut involved with digestion.

In the present study, bond specificity of the endopeptidases under investigation are described using the nomenclature of Schecter and Berger (1967). In this system, the binding site, made up of a series of subsites called S, interact with a polypeptide substrate with amino acid residues called P. The amino acid residues on the amino-terminal side of the bond that is cleaved by the substrate are numbered P_1, P_2, P_3 etc. counting outwards. The residues on the carboxy-terminal side of the scissile bond are numbered P_1', P_2', P_3'. The subsites on the protease are termed to complement the substrate residues that interact with the enzyme.

The P_1 site of the substrate is usually the primary determinant of binding for endopeptidases and exopeptidases, but the presence of further sites in colorimetric peptide substrates usually increases the affinity of endopeptidases for the substrate (Dunn, 1989). Therefore, the specific activity of an endopeptidase will often be lower at any given substrate concentration while using smaller peptide substrates.

Investigations into the presence and properties of insect proteases are facilitated by using a number of artificial peptide and natural protein substrates. Those used in the present work are described below; hydrolysis of a substrate may indicate the nature of the hydrolysing enzyme.

A. Artificial pNA substrates

1. Trypsin substrates

a. BApNA (Erlanger *et al*, 1961) is used as a typical trypsin substrate to demonstrate the presence of a protease cleaving peptide bonds adjacent to arginine residues in the P_1 position. BApNA is also cleaved by bromelain and papain (cysteine proteases). For convenience, the BApNA-ase enzyme from *S. littoralis* and *H. armigera* is referred to as trypsin rather than trypsin-like or 'trypsin', while trypsins from other sources are named (e.g. bovine; mammalian). Other types of insect endopeptidase are specified in this way according to the hydrolysis of specific substrates and compared with named examples of proteases.

b. TGPLpNA is used as an additional substrate which is hydrolysed by proteases which cleave lysine in the P_1 position of the amino-terminal side of the scissile bond. It is also cleaved by plasmin (Lottenberg *et al*, 1981).

2. Mammalian chymotrypsin substrates

In general, chymotrypsin enzymes are characterised by their broad specificity with preference for aromatic amino acids (tryptophan, tyrosine, phenylalanine) and also leucine, methionine and alanine in the P_1 position (Keesey, 1987).

a. SA_2PLpNA is hydrolysed by bovine chymotrypsin and some elastases (Del Mar *et al*, 1980).

b. SA_2PPpNA is cleaved by bovine chymotrypsin (Del Mar *et al*, 1979; Nakajima *et al*, 1979), but also hydrolysed by some elastases, e.g. human pancreatic elastase 2 (Del Mar *et al*, 1980), but not others e.g. porcine elastase (Kasafirek *et al*, 1976). Additionally, hydrolysis occurs in the presence of human leukocyte cathepsin G at an optimum of pH 7.5 (Barrett, 1981), but is slow in the presence of trypsin, at a rate of 0.03% of cleavage compared with chymotrypsin (Del Mar *et al*, 1979).

c. BTpNA is hydrolysed by bovine chymotrypsin (Bundy, 1962; Bundy and Moore, 1966) and other enzymes with specificity for tyrosine in the P_1 position, such as carboxypeptidase Y from yeast (Hayashi *et al*, 1973).

d. Pancreatic chymotrypsins hydrolyse ALpNA, but more slowly than SA_2PLpNA, because of the smaller number of residues in the substrate (Dunn, 1989).

e. Suphepa (Nagel *et al*, 1965; Geiger, 1984) is hydrolysed by bovine chymotrypsin and other enzymes with specificity for phenylalanine in the P_1 position. However, porcine pancreatic chymotrypsin has a low affinity for this substrate; K_m is 5mM at pH 7.5 and 25°C in 0.02M phosphate buffer (Keesey, 1987) or 0.72mM in 50mM tris, 30mM $CaCl_2$ and 1% DMF (Boudier *et al*, 1981).

3. Elastase substrates

a. SA_3pNA is a pancreatic elastase substrate (Bieth *et al*, 1974), which is not cleaved by bovine trypsin or chymotrypsin. However, human pancreatic elastase 2 has a low affinity for this substrate and prefers the P_1 position to be occupied by aromatic residues.

b. SA_2VpNA is another elastase substrate; it is cleaved more rapidly by human leucocyte elastase than SA_3pNA (Kasafirek *et al*, 1976; Stein, 1983; Wenzel *et al*, 1980; Wenzel and Tschesche, 1981).

4. Subtilisin substrate

ZGGLpNA is digested by subtilisins and neutral endopeptidases (Lyublinskaya *et al*, 1974; Wilk and Orlowski, 1979).

5. Other pNA substrates

The presence of endopeptidase activity towards substrates with citrulline and aspartic acid in the P_1 positions can be investigated using ZGPCpNA and AApNA. The former is a substrate for the cysteine proteases papain, ficin and bromelain (Gray *et al*, 1984). AApNA is digested by certain bacterial proteases with specificity for aspartic acid as the N-terminal amino acid adjacent to the peptide bond, such as that secreted from *Pseudomonas fragi* (Drapeau, 1980; Noreau and Drapeau, 1979).

B. Artificial esterase substrates

Esterase substrates can also be used to assay for the activity of endopeptidases, but hydrolysis can also be due to the presence of esterases in a crude homogenate. ATEE, BTEE and TEE can be used for determining the activity of chymotryptic enzymes, while BAEE and TAME are more normally used to indicate the presence of trypsin enzymes.

1. ATEE can be used to determine chymotrypsin activity. However, it is also hydrolysed, albeit slowly, by trypsin (Hummel, 1959) and has a relatively low solubility in water. ATEE is also a substrate for subtilisin.

2. BTEE is a substrate for chymotryptic activity determination which is completely resistant to hydrolysis by trypsin (Hummel, 1959). It is soluble in methanol and ethanol.

3. TEE is similar to BTEE: it is not hydrolysed by trypsin and is soluble in methanol. However, it hydrolyses slowly in solution: 4% in 24 hours at 4°C and pH 7.0 (Dawson *et al*, 1986).

4. BAEE is soluble in water and is hydrolysed by trypsin, papain, bromelain, subtilisin and ficin. It is also a substrate chymotrypsin (Schwert *et al*, 1948) and hydrolyses slowly in solution (4% in 24 hours at 4°C and pH 8.0).

5. TAME is similar to BAEE, but is completely resistant to hydrolysis by chymotrypsin (Hummel, 1959). Autolysis is slow above pH 9.0.

C. Naturally-based protein substrates

1. Casein

Casein can be used to determine the activity of a wide variety of enzymes such as porcine pancreatic elastase, proteinase K, subtilisin, thermolysin and other endopeptidases.

2. Elastin-orcein

Hydrolysis of elastin-orcein indicates that elastolytic activity is present. However, the release of colour from this substrate is not linear with time (Shotton, 1970) and it is not soluble. Elastases have been defined as proteinases capable of solubilising fibrous elastin near neutral pH (Bieth, 1989) which excludes proteases which cleave elastin at more acidic pH such as pepsin (Wallach and Hornebeck, 1989). The classification of elastases as a separate family of serine proteases is no longer acceptable (Gertler *et al*, 1977) and the definition of an elastase as a serine protease that preferentially hydrolyses at alanine residues is too restrictive (Del Mar *et al*, 1980). It is possible for an enzyme to be classified as an elastase and be a serine, a metallo- or cysteine protease (Bieth, 1989).

Classification of proteolytic enzymes

Proteolytic enzymes can be classified according to their mechanism of action

(Hartley, 1960) and belong to four groups: (a) serine proteases, the active centre contains serine and histidine; (b) cysteine proteases, having a cysteine at the active centre; (c) aspartic proteases (also called carboxyl or acid) which have an acidic residue involved in the catalytic process; (d) metal proteases. The metalloproteases are frequently carboxypeptidases or aminopeptidases (i.e. exopeptidases) (see chapter 2).

Serine proteases comprise two distinct families: the mammalian serine proteases (e.g. trypsin, chymotrypsin, elastase) and the bacterial serine proteases (e.g. subtilisin) which have a differing amino acid sequence and three dimensional structure, but share a common active site geometry and catalytic mechanism. The active site has the catalytic triad of Asp 102, His 57, Ser 195 (chymotrypsin numbering). Catalysis proceeds via a tetrahedral transition state intermediate during both the acylation and deacylation steps of catalysis (Kraut, 1977). Serine proteases are usually active under alkaline conditions (pH 7-12) and their activity can be detected using a wide variety of substrates (e.g. BApNA and BTpNA for trypsin and chymotrypsin respectively). Calcium ions are occasionally required in the activation of some of the proenzymes and to stabilize some of the enzymes (Keesey, 1987). The specificity of the serine proteases are very diverse. Trypsin requires P_1 to be occupied by arginine or lysine. Other serine proteases show specificity for large hydrophobic sidechains (chymotrypsin, cathepsin G, chymase, subtilisin) or small aliphatic sidechains (elastases) at P_1. Many examples of insect endopeptidases which have been investigated so far belong to the serine group e.g. serine protease activity of *H. armigera* (Johnston *et al*, 1991).

The cysteine proteases include mammalian cathepsins, cytosolic calcium activated proteases (calpains) and the plant protease papain (Drenth *et al*, 1971). For papain, the major catalytic amino acid is cysteine 25, acting like serine 195 in chymotrypsin. Catalysis proceeds via a thiol ester intermediate and is facilitated by the side chains of adjacent histidine 159 and aspartic acid 158 (the two remaining amino acids involved in the catalytic triad of serine proteases). Cysteine proteases from plants (such as papain) show maximal activity at neutral or slightly alkaline pH (Zucker *et al*, 1985). Lysosomal cysteine proteases tend to be unstable at alkaline pH and have acidic pH optima, but if assays are carried out under favourable conditions, assays can reveal pH optima above neutral pH, suggesting that the catalytic mechanism may not be very different from plant enzymes (Willenbrock and Brocklehurst, 1984; Mason *et al*, 1985). Cysteine proteases contrast with serine proteases regarding the dominant subsite. Amongst the extended specificity site containing five to seven subsites (Berger and Schecter, 1970), the cysteine protease dominant subsite is S_2, which is a hydrophobic pocket which binds phenylalanine. Substrates such as BAEE and BApNA can also be used as substrates for cysteine proteases (Brocklehurst *et al*, 1987). Other peptide substrates with citrulline at the P_1 position allow detection of cysteine proteases at lower concentrations (Gray *et al*, 1984). Cysteine proteases are inhibited by cystatins, peptide diazomethanes, peptide epoxides (e.g. E-64), sulphydryl alkylating agents such as iodoacetate and iodoacetic acid, NEM and pCMPS. The diazomethanes were not tested

for their inhibitory activity of the lepidopteran proteases; at least some examples of the peptide diazomethanes are not entirely class specific, as they have been shown to react with plasma kallikrein, a serine protease (Zumbrunn *et al*, 1988). Cysteine proteases occur in haematophagous insects such as *Rhodnius prolixus* (Heteroptera) (Houseman, 1978; Garcia *et al*, 1978) and have been reported in a variety of Hemipteran insects: *Triatoma phyllosoma pallidipennis* (Houseman and Downe, 1981), *Cimex hemipterus* and *C. lectularis* (Houseman and Downe, 1982), *Eudchistus euschistoides* (Houseman *et al*, 1984) and *Phymata wolffii* (Houseman *et al*, 1985). Other cysteine proteases have been reported in the tick, *Ornithodoros tholozani* (Akov *et al*, 1976) and *Argas persicus* (Tatchell, 1964). They are also present in Coleopteran insects, such as *Tribolium castaneum* and *Callosobruchus maculatus* (Murdock *et al*, 1987); *Acanthoscelides obtectus* (Wieman and Nielsen, 1988) and *Leptinotarsa decemlineata* (Thie and Houseman, 1990). Cathepsin-like lysosomal enzymes have been reported in the lepidopteran larval fat body and midgut of *Mamestra brassicae* and *Pieris brassicae* (Sass *et al*, 1989), but their function in the fat body and midgut is thought to be associated with remodelling of cells rather than digestion.

The aspartic proteases include bacterial and mammalian pepsins and renin. These proteases have also been referred to as acid proteases as the majority of the enzymes are inactive above pH 6.0 (Rich, 1986b), but some enzymes now known to belong to the class act optimally on the substrate near pH 7.0 (Fruton, 1987). The characteristically active site residues are aspartic acids 33 and 213 (bacterial pepsin numbering) which are closely positioned (Kostka, 1985). In the enzymatically active pH range of 1.5-5, one is ionized, the other unionized. A potent inhibitor of aspartic proteases is pepstatin, a hexapeptide which resembles the normal substrates when in the transition state. As a generalization, aspartic proteases act best on peptide bonds between bulky hydrophobic amino acid residues. Thus Leu-Tyr, Tyr-Leu, Phe-Phe and Phe-Tyr bonds are the most susceptible in the insulin B chain (North, 1982). Aspartic proteases have functionally important specificity subsites on both sides of the catalytic site, so they will not cleave nonpeptide bonds, such as those between colorimetric leaving groups and peptides in synthetic substrates. Assays depend on the detection of cleavage of actual peptide bonds; substrates used may contain p-nitrophenylalanine which is environmentally sensitive (Pohl *et al*, 1983). Some aspartic proteases also catalyse the hydrolysis of most general protein substrates to peptides (e.g. pepsin) while others such as chymosin (rennin), cleave only a few bonds in casein. Renin, the kidney enzyme is highly specific, and cleaves angiotensinogen to produce angiotensin I in the physiological control of blood pressure. Aspartic proteases have been reported in the carrion-feeding Dipteran larvae of *Calliphora vicina* (Pendola and Greenberg, 1975), *C. vomitoria* (Fraser *et al*, 1961), *Musca domestica* (Greenberg and Paretsky, 1955), *Stomoxys calcitrans* (Lambremont *et al*, 1959) and *Sarcophaga ruficornis* (Sinha, 1975).

The presence, in biological material, of proteases and the class to which they belong can be determined by using selective substrates and inhibitors. Inhibitors may

react with more than one class of protease, specifically with one class or show a high degree of selectivity for a single protease. Similarly, substrates can be cleaved by a wide variety of proteases from different classes, or may be relatively specific for certain protease types. There are four types of inhibitor: artificially-derived chemicals (1) and inhibitors with microbial (2), plant (3) or animal (4) origins.

1. Artificially-derived inhibitors have been produced for all classes of enzymes (Powers and Harper, 1986a,b; Rich 1986a,b).

a. Peptide chloromethyl ketones

These irreversible inhibitors are useful active-site-directed protease-modifying reagents. Serine protease inhibition results from binding the inhibitor in a substrate-like manner followed by alkylation of the active-site histidine by the chloromethyl moiety. However, chloromethyl ketones are also known to inactivate cysteine proteinases and some have a poor specificity (Kettner and Shaw, 1981). In addition, they are susceptible to high pH (Shaw *et al*, 1965; Shaw, 1975) and some (e.g. TPCK) are only sparingly soluble in water (Keesey, 1987). TLCK inhibits bovine trypsin (Shaw *et al*, 1965), while TPCK inhibits bovine chymotrypsin (Schoellmann and Shaw, 1963).

b. Organophosphorus and sulphonyl fluoride inhibitors

Serine proteases are inhibited by DFP (diisopropyl-phosphofluoridate), which irreversibly inactivates serine proteases. DFP was not used in the present study due to its toxicity; it is an acetylcholine esterase inactivator. PMSF is safer to use as it is less volatile (Dunn, 1989) and inhibits by fluorinating the serine residue in the catalytic triad of serine proteases. PMSF does not react outside the reactive site under normal conditions of pH and temperature (Gold and Fahrney, 1964) and therefore other proteins in the enzyme preparation should not affect PMSF inhibition of the reactive enzymes. PMSF reacts more slowly with trypsin than chymotrypsin, while APMSF reacts much more rapidly with trypsin (Laura *et al*, 1980). PMSF is dissolved in methanol (Gold and Fahrney, 1964; Turini *et al*, 1969) as it decays relatively rapidly in solution, especially at high pH (James, 1978). PMSF reacts reversibly therefore with some cysteine proteases (Salvesen and Nagase, 1989) and is not entirely class specific. There is a wide variety of other classes of chemical inhibitor which inhibit serine proteases (Powers and Harper 1986a), including the coumarins, e.g. 3,4-dichloroisocoumarin, and peptide boronic acids (Harper *et al*, 1985; Kettner and Shevni, 1984).

c. Other artificial inhibitors

Benzamidine is a good reversible inhibitor of trypsin-like enzymes (Jeffcoate and White, 1974; Powers and Harper, 1986a). Other valuable inhibitors which may be used to characterise the protease include: iodoacetic acid (Jocelyn, 1972); iodoacetamide (Webb, 1966d; Benesch and Benesch, 1967); N-ethylmaleimide (NEM), dissolved in

methanol (Webb, 1966c); dithiothreitol (DTT), (Cleland, 1964); p-chloromercuri-benzoic acid (pCMB), (Boyer, 1954; Webb, 1966a,b); p-chloromercuriphenylsulfonic acid (pCMPS) (Velick, 1953; Jocelyn, 1972). Inactivation by pCMB and pCMPS can sometimes be reversed by the addition of cysteine (Webb, 1966a).

2. Microbial synthetic aldehyde and ketone peptides

The peptide aldehyde and ketone class of inhibitor was originally found by screening microorganisms for protease inhibitors (Umezawa and Aoyagi, 1975). They are all small peptides (M_r <1000) and numerous synthetic and naturally occurring peptides have been reported.

Chymostatin inhibits a wide variety of chymotrypsin-like enzymes including human cathepsin G (K_i = 0.28μM, Powers and Harper, 1985) and bovine chymotrypsin (K_i = 0.25μM, Umezawa and Aoyagi, 1977). Chymostatin does not inhibit trypsin-like enzymes, e.g. thrombin, plasmin and kallikrein (Umezawa *et al*, 1970). It is not very soluble in water and stock solutions are made up in DMF (Umezawa, 1976).

Elastatinal does not inhibit chymotrypsin or trypsin (Umezawa *et al*, 1973), but is a good inhibitor of elastases: K_i values for the inhibition of porcine pancreatic elastase and human leukocyte elastase are 0.24μM and 50-80μM, respectively (Umezawa and Aoyagi, 1977; Feinstein *et al*, 1976; Zimmerman and Ashe, 1977).

Leupeptin and antipain are reversible inhibitors referred to as 'transition-state analogues' as they mimic the tetrahedral intermediate formed during peptide bond hydrolysis. Leupeptin reacts equally well with serine and cysteine proteases; e.g. trypsin and cathepsin B (Umezawa, 1982; Sasaki *et al*, 1984; Rich, 1986) while antipain inhibits trypsin and plasmin as well as the cysteine proteinases, papain and cathepsin B (Umezawa, 1976; Suda *et al*, 1972). Chymotrypsin and thrombin are relatively poorly inhibited by leupeptin (Aoyagi *et al*, 1969a,b).

Pepstatin A was the first potent inhibitor of aspartic proteases to be found (Umezawa *et al*, 1970b; Umezawa, 1976a; Rich *et al*, 1985). Stock solutions are soluble in DMF. It is a remarkably potent inhibitor of pepsin (K_i = 4.5 x 10^{-11}M, Kunimoto *et al*, 1974).

Trans-epoxysuccinyl-L-leucylamido-(4-guanidino)-butane (E-64) is dissolved in a DMF-water mixture, (Hanada *et al*, 1978; Barrett *et al*, 1982). This inhibitor rapidly inactivates cysteine proteases: papain, cathepsin B and ficin.

Protein inhibitors of proteases

The nomenclature of protein protease inhibitors is confused, partly because the list of families is still preliminary, but the description here follows that of Laskowski and Kato (1980).

3. Legume seed proteinase inhibitors

The legume seed proteinase inhibitors fall into two groups: i. Kunitz family,

with a molecular weight around 22,000, relatively few disulphide bonds and a specificity primarily towards trypsin; ii. Bowman-Birk family, with a molecular weight around 8,000, a high concentration of cysteine (about 20%) and the capability to inhibit trypsin and chymotrypsin at independent sites.

i. Kunitz soybean trypsin inhibitor family

The soybean Kunitz inhibitor (Birk, 1976) can form a ternary complex between two molecules of either trypsin or chymotrypsin and the inhibitor (Bösterling and Quast, 1981), but trypsin is the most readily inhibited. The inhibition of trypsin by SBTI with BAEE as substrate is competitive, but with protein substrates the type of inhibition is unclear (Birk, 1986).

ii. Bowman-Birk protease inhibitor family

Inhibitors of this type are present in all leguminous plants (Birk, 1985; 1986). They consist of a tandem of homologous regions on the same polypeptide chain, each with a reactive inhibitory site. BBTI can form a 1:1 complex with either trypsin or chymotrypsin or a ternary complex with both enzymes. It has been reported as a potent inhibitor of many vertebrate trypsins and chymotrypsins. BBTI does not inhibit pancreatic elastase (Gertler and Birk, 1970), but does inhibit elastase from granulocytes (Scheissler *et al*, 1977). Other inhibitors of this type are similar to BBTI e.g. LBTI (Tauber *et al*, 1949; Fraenkel-Conrat *et al*, 1952; Jones *et al*, 1963) and CPTI (Gatehouse *et al*, 1980).

4. Animal protease inhibitors

Two main sources of protease inhibitor have been identified in animals, those of the pancreas and those identified in egg white. Inhibitors from the pancreas fall into two distinct families: (i) the bovine pancreatic trypsin inhibitor (Kunitz) family (BPTI) and (ii) the pancreatic secretory trypsin inhibitor family (Kazal). The inhibitors identified in egg white were found to have domains homologous to the Kazal family.

i. Kunitz inhibitor

The inhibitor aprotinin was used as an example of the Kunitz BPTI family (Trautschold *et al*, 1967; Gebhard *et al*, 1986). Aprotinin is resistant to high temperature, acid, alkali and proteolytic enzymes. Many trypsins from different sources are inhibited (e.g. cow, pig, human, turkey) and a few chymotrypsins (e.g. bovine), but it does not react with elastase.

ii. Kazal inhibitor

Pancreatic secretory trypsin inhibitor was not used in the study of the proteases from *S. littoralis*, but Kazal inhibitors from egg white were tried. Chicken ovomucoid is a single-headed inhibitor of trypsin (Fredericq and Deutsch, 1949; Lineweaver and

Murray, 1947), while the turkey ovomucoid is a double-headed one with one inhibitory site for trypsin and another site for chymotrypsin, subtilisin and elastase (Birk, 1986). Ovoinhibitor inhibits trypsins, chymotrypsins, elastase and microbial serine proteases (Kassell, 1970; Tomimatsu *et al*, 1966; Feeney *et al*, 1963; Davis *et al*, 1969).

α-macroglobulin is a non-specific inhibitor which inhibits most proteases. The molecule works by entrapping the protease (Barrett and Starkey, 1973) and is mechanistically different from most other inhibitors which are active-site-directed. As a result of steric hindrance, the complexed enzyme retains full activity against small substrates such as peptides, but is completely inactive against large ones (proteins > 30kDa). The effect of this inhibitor on the proteases was not investigated, because it is not class specific.

Serpins (*ser*ine *p*rotease *in*hibitor)

α_1-proteinase inhibitor, a serpin, belongs to a family of human plasma proteins (Carrell and Travis, 1985; Travis and Johnson, 1981; Brodrick *et al*, 1980). Serpins are small glycoproteins (M_r 40,000 to 60,000) and form 1:1 complexes with their target proteinase. α_1-proteinase inhibitor inhibits trypsin, chymotrypsin and elastase (Birk, 1986).

A number of other protein inhibitor families exist (Laskowski and Kato, 1980) including the *Streptomyces* subtilisin, potato I and II and *Ascaris* trypsin inhibitor families which were not tested against the insect proteases.

Insect endopeptidases usually belong to the serine or cysteine groups (Applebaum, 1985). Characterisation of proteases is most conveniently carried out by the effect of known inhibitors on specific substrates as described above. Comprehensive characterizations of insect proteases have been carried out (e.g. Christeller and Shaw, 1989).

Lepidopteran larval endopeptidases are extracellular serine proteases appearing free in the lumen. These exhibit high pH optima, which are well suited to the alkaline conditions of the midgut and are responsible for protein degradation from ingested food (Applebaum, 1985; Santos *et al*, 1986). There have been previous reports on endopeptidase activity from *H. armigera* (Akimenko *et al*, 1988; Rubinstein and Polson, 1983) and from *S. littoralis* (Ishaaya *et al*, 1971). However, the latter reports were confined to the pH optima for the digestion of gelatin and casein, and an M_r estimate. Akimenko *et al* (1988) investigated the trypsin protease from *H. armigera* with regard to pH optima, temperature and specificity of action on various peptides, but did not determine the action of inhibitors. Reports of chymotrypsin enzymes from insects are not widespread (Baumann, 1990), but this may be because the presence in insects of some enzymes digesting proteins (such as BSA, casein and haemoglobin) have not been further characterised with respect to their peptide bond specificity (e.g. Sasaki and Suzuki, 1982; Christeller *et al*, 1989). Consequently, as specific peptide substrates were not used, certain classes of protease appear absent, such as those with specificity

for peptide bonds adjacent to large residues such as leucine, phenylalanine and tyrosine.

The present study, therefore, has been undertaken to identify, partially purify and characterise the endopeptidases from the midgut of two insect species, *Helicoverpa armigera* and *Spodoptera littoralis*, together with an assessment of their interactions with a variety of inhibitors from artificial, microbial, animal and plant origins. Knowledge of the properties and interactions of these enzymes are essential in developing and optimising novel methods for pest control based on interference with gut function and to our understanding of the nature of protein digestion in these economically important insects. The characterisation of the proteases responsible for the initial digestion of proteins (i.e. the endopeptidases) are presented in this chapter, while those proteases confined to the tissue epithelia and responsible for the intermediate and final digestion of proteins (the exopeptidases) are detailed in the following chapter.

CHAPTER 1
Materials and Methods

Preparation of midgut homogenates

Feeding, final instar larvae of *Spodoptera littoralis* and *Helicoverpa armigera* of both sexes were killed by decapitation followed by severance of the posterior end of the abdomen. The carcass was then cut open ventrally and the midgut dissected free and placed in a homogenization tube containing 2mM DTT and sufficient PVP to bind dietary tannins (about 1g/10ml) at 0-4°C. Homogenization was carried out using a Camlab Tri-R, Stir-R homogenizer in a glass homogenization tube with a teflon pestle (clearance 0.1-0.15mm) with twenty passes of the pestle at approximately 1000 rpm. The resulting crude homogenate was then centrifuged at 10,000g at 0-4°C for 15 minutes in an MSE Prepspin 50 Ultracentrifuge to remove larger fragments of tissue/gut contents. The pellet was discarded and the whole-gut supernatant used as a crude enzyme preparation for estimating the relative activities of the various gut enzymes. Homogenates were kept on ice until used.

For all other studies on the midgut digestive enzymes, the animals were killed as described above, and their midgut contents separated from the gut wall while still retained within the layers of the peritrophic membrane. Any spilled midgut contents were washed from the tissue using DDW. Contents and tissue were collected and homogenized separately as described previously. Lipid was removed from this homogenate by the addition of an equal volume of chloroform to the homogenate followed by thorough mixing using a glass homogenizer and teflon pestle. The resulting mixture was then centrifuged at 2000rpm (400g) in an MSE Centaur 2 bench centrifuge or an MSE MicroCentaur at 13,000rpm or 10,000g, depending on the volume of homogenate, to separate the aqueous and chloroform fractions. The lipid-chloroform layer was discarded. The aqueous layer was then 95% saturated with ammonium sulphate to precipitate protein. This was then pelleted at 10,000g for 30 minutes, at 4°C, in an MSE Prepspin 50 Ultracentrifuge (but occasionally an MSE Europa 24M or an MSE High Speed 18) using an 8 x 50ml aluminium angle rotor. The pellets were collected, and stored frozen at -20°C until required when they were then suspended in an appropriate volume of DDW.

Gel filtration chromatography

Various Sephadex gel filtration media were used. These dry powders were swollen by slow addition to a large volume (about twice the final column volume) of elution medium, and then degassed under vacuum. Columns (Pharmacia, Sweden or Amicon Wright, England) were filled by pouring the degassed suspension down the inside wall until the column was full. Columns packed with Sephadex G-25 (56.0cm x i.d. 2.2cm) (using Amicon Wright columns), Sephadex G-75 (62.0cm x i.d. 1.6cm) or

Sephacryl S-200 (62.5cm x i.d. 1.6cm) (using Pharmacia C16/70 columns) were used. Each of these columns was equilibrated with several column volumes of elution medium before the addition of standards or samples. Initially, the elution medium was 0.2M glycine/NaOH (pH 7.0). In subsequent studies, 0.05M Tris/HCl buffer containing 1M NaCl at pH 7.2 was used. Salt was added to increase the ionic strength in order to prevent ionic interactions between proteins and the gel and help prevent sample/standard elution at an unexpected position (Pharmacia LKB, 1991) except when desalting (Sephadex G-25), when 1M NaCl was omitted. A neutral buffer pH was chosen to prevent more rapid autolysis at higher pH and to prevent breakdown of the enzymes at low pH.

Initially, samples were applied onto a drained bed surface. However, while this method gave adequate results, it occasionally disturbed the bed surface making it uneven; this can lead to reduced resolution (Pharmacia LKB, 1991). It was also time-consuming, as the sample must be allowed to drain from the bed surface into the column before the addition of more eluent. Therefore, it proved simpler and quicker to apply the sample below the eluent. The sample was made denser than the eluent by adding sufficient sucrose to ensure the sample did not float away from the bed surface. An aliquot of sample (usually 2ml) was then withdrawn into a syringe with a needle tip bent through 90°. The bent needle reduced the disturbance to the gel bed surface. The sample was then dispensed slowly as a layer above the bed surface and eluent flow could be started immediately. Each column (except Sephadex G-25) was then calibrated using a number of the following molecular weight standards: horse heart cytochrome c (M_r 12,400), horse skeletal muscle myoglobin (M_r 16,950), SBTI (M_r 20,100), bovine trypsinogen (M_r 24,000), bovine erythrocyte carbonic anhydrase (M_r 29,000), chicken egg ovalbumin (M_r 45,000), BSA (M_r 66,000), hexokinase from Baker's yeast (M_r 100,000), alcohol dehydrogenase from Baker's yeast (M_r 150,000), barley ß-amylase (M_r 200,000) and bovine liver catalase (M_r 240,000) (Andrews, 1964). Catalase has a molecular weight of 240,000 and may behave as if its molecular weight was 195,000 (Fisher, 1980). The void volume was determined with Blue Dextran. Eluant protein content was continuously monitored by measuring absorbance at 280nm using an LKB Uvicord II Ultraviolet Absorptiometer Detector Unit type 8303A together with an LKB Uvicord II Control Unit and plotted with an LKB Biocal.

A quantity of ammonium sulphate precipitate of crude midgut contents homogenate was resuspended in DDW. A 2ml aliquot of this enzyme preparation was applied to the gel column as described above and eluted using an LKB 12000 Varioperpex Peristaltic Pump. The eluant from the columns was collected in fractions of various sizes (2-10ml) using a flow rate of about 0.625 ml/min using a Frac-100 fraction collector (Pharmacia Ltd) or an LKB Ultrorac Fraction Collector 7000. Eluant protein content was measured as before and the enzyme activity determined in each fraction. Molecular weights of peaks were estimated by calculating the ratio between the eluted volume (V_e) and void volume (V_o) and reading from a plot of the ratio (V_e/V_o)

Figure 1.1

Calibration curve for determining the molecular weights of proteins in the range 3 to 80 kDa using a Sephadex G-75 column.

Legend: ordinate - V_e/V_o.
abscissa - Log_{10} Molecular weight (Da)

Proteins used to calibrate Sephadex G-75 column:

Protein	M_r (kDa)
Bovine serum albumin (BSA)	66
Egg albumin	45
Carbonic anhydrase	29
Trypsinogen	24
Soybean trypsin inhibitor (SBTI)	20.1
Myoglobin	16.95
Cytochrome c	12.4

Figure 1.2

Calibration curve for determining the molecular weights of proteins in the range 5 to 250 kDa using a Sephacryl S-200 column.

Legend: ordinate - V_e/V_o.
abscissa - Log_{10} Molecular weight (Da)

V_e is the volume required to elute the protein and V_o is the void volume of the column i.e. the volume required to elute a large molecule (Blue Dextran).

Proteins used to calibrate Sephacryl S-200 column:

Protein	M_r (kDa)
catalase	240
ß-amylase	200
Alcohol dehydrogenase	150
Bovine serum albumin (BSA) (dimer)	132
Hexokinase	100
Bovine serum albumin (BSA) (monomer)	66
Egg albumin	45
Carbonic anhydrase	29
Trypsinogen	24
Soybean trypsin inhibitor (SBTI)	20.1
Myoglobin	16.95
Cytochrome c	12.4

Figure 1.1

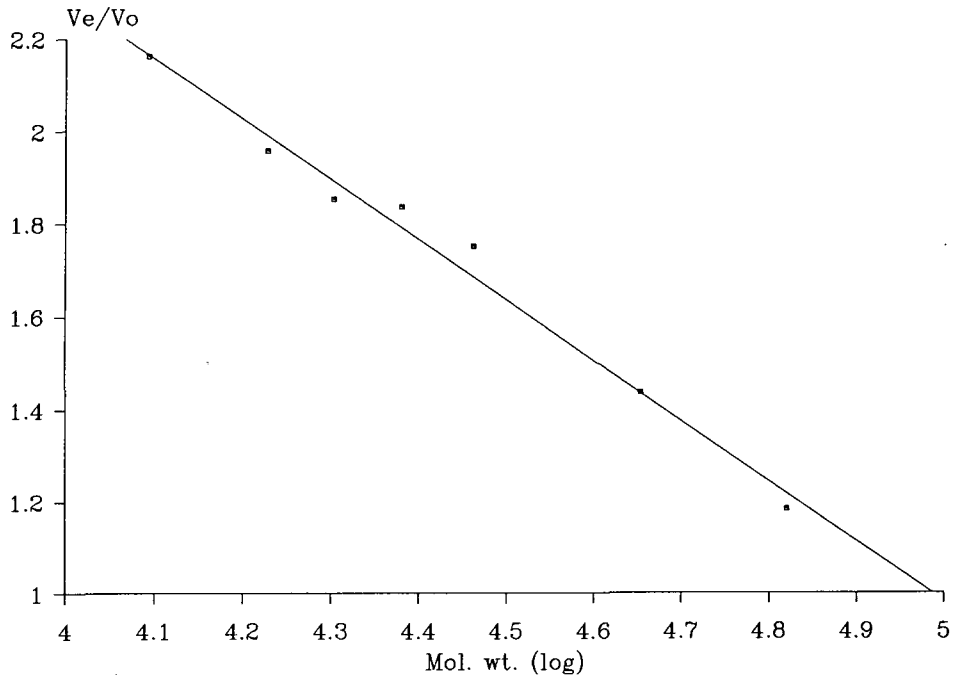
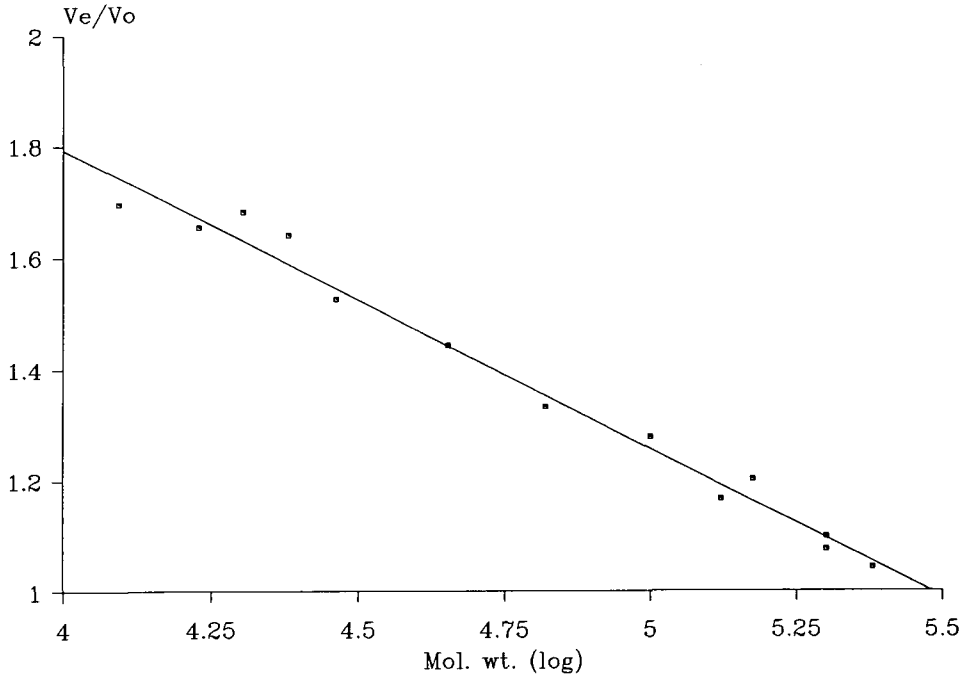


Figure 1.2



against $\log_{10}$ molecular weight for the standard proteins. Representative plots are shown in figures 1.1 and 1.2 for Sephadex G-75 and Sephacryl S-200 columns.

Anion-exchange chromatography

Crude gut contents or eluted fractions from a gel filtration column were subjected to anion-exchange chromatography. Initially such samples were dialysed overnight in DDW (with three changes of DDW) or desalted by application to a G-25 column. Dialysates were then added to an equal volume of double strength elution buffer (0.1M Tris pH 7.2) to give a final concentration of 50mM Tris buffer and loaded onto a column containing Q-Sepharose media. The gel was packed in a Pharmacia XK 16/20 to provide a column measuring (6cm x i.d. 1.6cm). The method of column packing was similar to that described for gel filtration. Columns were washed with several column volumes of buffer A (50mM Tris pH 7.2) at an elution rate of 1ml/min by a P-1 peristaltic pump (Pharmacia LKB). Samples were applied using a Pharmacia Gradient Programmer GP-250 Plus followed by elution of the protein with an increasing linear gradient of buffer B (50mM Tris and 1M NaCl pH 7.2) using the Gradient Programmer in conjunction with a gradient mixer. Fractions were collected using a Pharmacia Frac-100 fraction collector. Eluant protein content was continuously monitored by measuring absorbance at 280nm using an LKB 2158 Uvicord spectral detector and plotted on a Servoscribe RE 541.20 potentiometric recorder.

SDS-PAGE

The method employed was essentially that of Laemmli (1970) and was used as a method for the estimation of the M_r of various proteins (Weber and Osborn, 1969). An acrylamide/bis-acrylamide mix (29:1) was obtained from Sigma. This was dissolved in DDW to give a 30% solution which was stored in the dark at 4°C until required. Electrophoresis running buffer (x10 stock) consisted of 141g glycine, 30g Tris base and 10g SDS dissolved in 1 litre DDW. This stock was diluted x10 for use. The main gel was made up to the required percentage of acrylamide by mixing with buffer as follows:

% acrylamide in main gel	7.5%	10%	12.5%	15%
Acrylamide/bis-acrylamide (ml)	15	20	25	30
1.5M Tris/HCl pH 8.8 (ml)	15	15	15	15
DDW (ml)	29	24	19	14

This solution was then degassed using a vacuum pump and the following reagents added in this order:

i. 600μl 10% SDS

ii. 20μl TEMED

iii. 300μl 10% ammonium persulphate (made fresh daily)

The complete solution was mixed gently and immediately poured into the gel moulds to set. Gels (90mm x 80mm x 1mm) were usually run on an ATTO vertical

PAGE Dual Slab Kit AE.6450 (Genetic Research Instrumentation Ltd, England) but occasionally on larger gels (170mm x 160mm x 2mm) and consisted of a main gel (pH 8.8) and a loading or stacker gel (pH 6.8) with a lower concentration of polyacrylamide.

The stacker gel was prepared in the same way using the following reagents and volumes:

% acrylamide in stacker gel	5%	7.5%
Acrylamide/bis-acrylamide (ml)	2.0	3.75
0.5M Tris/HCl pH 6.8 (ml)	3.0	3.75
DDW (ml)	6.76	7.27
10% SDS (μl)	120	120
TEMED (μl)	10	10
10% ammonium persulphate (μl)	200	200

The stacker gel was prepared after the main gel had set, immediately poured on top of the main gel and the well-forming comb positioned. After the stacker gel had set, the comb was removed.

Samples for electrophoresis were mixed with an equal volume of a sample buffer. This was composed of the following ingredients made up to 100ml:

0.4M Tris/HCl pH 6.8	4.884g
10% SDS	10.0g
glycerol	10.0ml
bromophenol blue	0.01%

Samples were then boiled for 2 minutes and various volumes (5-30μl) applied to the gels. Molecular weight markers, obtained from Sigma as mixtures (SDS-7 for molecular weight determinations of 10,000-70,000 Daltons in 12.5% gels and SDS-200 for determinations of 30,000-200,000 in 7.5% gels), were also applied to the gel. After sample loading, 2-3μl of ß-mercaptoethanol was added to each well. A constant voltage of 75mV (and a current of 25-50mA) was provided during the running of the gel (running time of mini-gels at room temperature (10-20°C) was about 3 hours) by a Shandon Southern Vokam power pack. Electrophoresis was carried out until the tracker dye reached the gel base. The gels were removed and placed overnight in a Coomassie Blue fixative/stain (0.05% Coomassie Blue, 50% methanol, 7% ethanoic acid). Destaining took place in 7% ethanoic acid and 5% methanol. Gels were stored in destain solution at 4°C, until photographed.

Blotting

Proteins were blotted from acrylamide gels to nitrocellulose using the semi-dry blotting method of Kyhse-Andersen (1984). Samples loaded to gels to be used for blotting were not boiled, and the addition of ß-mercaptoethanol omitted. Transfer was carried out on an ATTO semi-dry Horizblot AE6670 electroblotter. Each transfer unit (containing one gel) comprised a sandwich arrangement of one sheet of 3MM paper

soaked in anode buffer 2 (25mM Tris, 20% (v/v) methanol, 0.1% SDS, pH 10.4); a sheet of nitrocellulose; the gel to be blotted; one sheet of 3MM paper soaked in cathode buffer (25mM Tris, 40mM 6-amino-n-hexanoic acid, 20% (v/v) methanol, 0.1% SDS, pH 9.4) and a sheet of dialysis membrane soaked in DDW. The anode plate was covered with two layers of 3MM paper soaked in anode buffer 1 (0.3M Tris, 20% (v/v) methanol, 0.1% SDS, pH 10.4) and the cathode plate with two layers of 3MM paper soaked in cathode buffer. The transfer units (up to six) were placed between the two electrode plates at a constant current of 0.15amps (about 50 Volts) for 2 hours using a Kikusui Electronics Corp. model PAB power pack.

After blotting, the nitrocellulose paper was removed and transferred to a p-nitroanilide substrate solution buffered to the enzyme optimum pH (the choice of both buffer and substrate was obviously dependent on the fractionated enzymes) and allowed to incubate at 30 ± 0.5°C for 1 hour. After incubation the paper was placed into a solution of 0.1% sodium nitrite in 1.0M HCl for 5 minutes. This solution was replaced with a freshly prepared solution of 0.5% ammonium sulphamate in 1.0M HCl for a further 5 minutes. Finally, the paper was transferred to a solution of 0.05% NEDD in 47.5% ethanol. The reaction was stopped by removing the paper from the NEDD solution when cleavage of the substrate was indicated by red zones. The nitrocellulose paper was then stored in air-tight plastic bags at -20°C until photographed.

Methods of enzyme assays

The activities of various enzyme preparations were investigated with a variety of peptidase substrates using one or more of the following methods:

Assay of a trypsin-like protease

i. discontinuous rate measurement using test-tubes.

The method employed essentially follows that of Erlanger *et al*, (1961). 2ml aliquots of 0.2M glycine-NaOH buffer pH 10.0 were equilibrated to 30 ± 0.5°C with 0.2ml of appropriately diluted enzyme homogenate and 0.2ml DDW for ten minutes. Enzyme activities were assayed in glass test-tubes incubated in a water bath controlled by a Grant SU5 temperature control unit. The reaction was started by the addition of 0.2ml 13mM BApNA and the change in absorbance at 405nm determined over a known incubation period of 15 to 30 minutes.

Routinely, trypsin activity was determined using BApNA (N-benzoyl-DL-arginine p-nitroanilide) as substrate, usually at a final concentration of 1.0mM. To overcome this substrate's low solubility in water, (about 0.7mM: Erlanger *et al*, 1961), it was necessary to dissolve it in an organic solvent. The substrate (13mM) was dissolved in 100% DMF with the minimum of heating and stored in a dark bottle or kept at -20°C to reduce the amount of autolysis. All other substrates dissolved in DMF were stored in this way.

Nagel *et al* (1965) used 100% DMF to dissolve 56mM BApNA by warming at

50°C to give a final concentration of 1.786% DMF and 1mM BApNA. Boehringer, quoted in Charnley (1975), found a final DMF concentration of 8% was necessary to solubilize high substrate concentrations. However, this caused 10% inhibition of bovine trypsin activity. Therefore, in the present investigation, preliminary studies were carried out in which the DMF concentration was altered in order to establish its suitability as a neutral organic solvent regarding enzyme activity.

The final absorbances were either read immediately, or the reaction stopped using 1ml of 30% acetic acid and the absorbance recorded later. Appropriate blanks were run and these contained boiled enzyme or DDW in place of the enzyme preparation. The rate (v) of product release, expressed as p-nitroaniline produced per minute, was either calculated by reference to a calibration curve (Fig. 1.3) or calculated using the following equation:

$$v = \mu\text{mol pna produced/min}$$
$$= (\text{abs. change/change in t}).(1/\varepsilon)$$

where ε is the difference between molar absorptivity for p-nitroaniline and benzoyl-arginine (ε = 10500 M^{-1} cm^{-1} at 405nm; Sarath *et al*, 1989).

Endopeptidase activity of midgut enzymes was investigated using another trypsin substrate, TGPLpNA (N-p-Tosyl-gly-pro-lys-pNA) (Lottenberg *et al*, 1981) in order to investigate digestion by the enzyme of peptides with lysine residues.

At low levels of product release, the sensitivity of the reaction was increased using a modification of the method of Preiser *et al* (1975). The proportions of the reactants described above was maintained, using volumes of 1.0ml glycine-NaOH buffer pH 10.0, 0.1ml enzyme solution, 0.1ml DDW/inhibitor solution and the reaction started with 0.1ml BApNA substrate. After incubation, the reaction was stopped using 160μl 2.5M HCl, followed by successive additions of 160μl 0.11% $NaNO_2$, 160μl 0.55% ammonium sulphamate and finally 160μl 0.055% NEDD. The absorbance value was read at 550nm using a Pye Unicam SP1800 Ultraviolet Spectrophotometer. The rate of product release was determined by reference to a standard curve (Fig. 1.4). It can be seen from the graph that there is a large increase in the sensitivity of the reaction (about 4.5 times). The concentration of acid required for full colour development is shown in fig. 1.5. 160 μl of various concentrations of acid were added to pNA solution followed by diazotization as described above.

The basis of the above reaction is to produce a diazotized product from p-nitroaniline. The hydrochloric acid is added to stop the reaction and move the pH to between 1 and 2, where the speed of the diazotization is most rapid. The concentration of acid used here (2.5M) was higher than that used by Preiser *et al* (1975) in order to reduce the volume of acid to be added to the solution and thereby reduce the dilution of the assay. In addition, the assay pH of lepidopteran midgut endopeptidases described here is higher (pH 10.0) than Preiser's assay (pH 8.0), which necessitates a higher

Figure 1.3

Standard calibration curve for the determination of p-nitroaniline released from pNA substrates when measured in cuvettes.

Legend: ordinate - absorbance at 405nm
abscissa - nmoles p-nitroaniline

Figure 1.4

Standard calibration curve comparing the absorbance of p-nitroaniline released from pNA substrates stopped (1) with acetic acid or (2) with HCl followed by diazotization with NEDD (N-(1-naphthyl) ethylene diamine dihydrochloride) in a Bratton-Marshall reaction. Absorbance measured in cuvettes.

Legend: ordinate: (1) - absorbance at 405nm
(2) - absorbance at 550nm
abscissa - nmoles p-nitroaniline

Figure 1.3

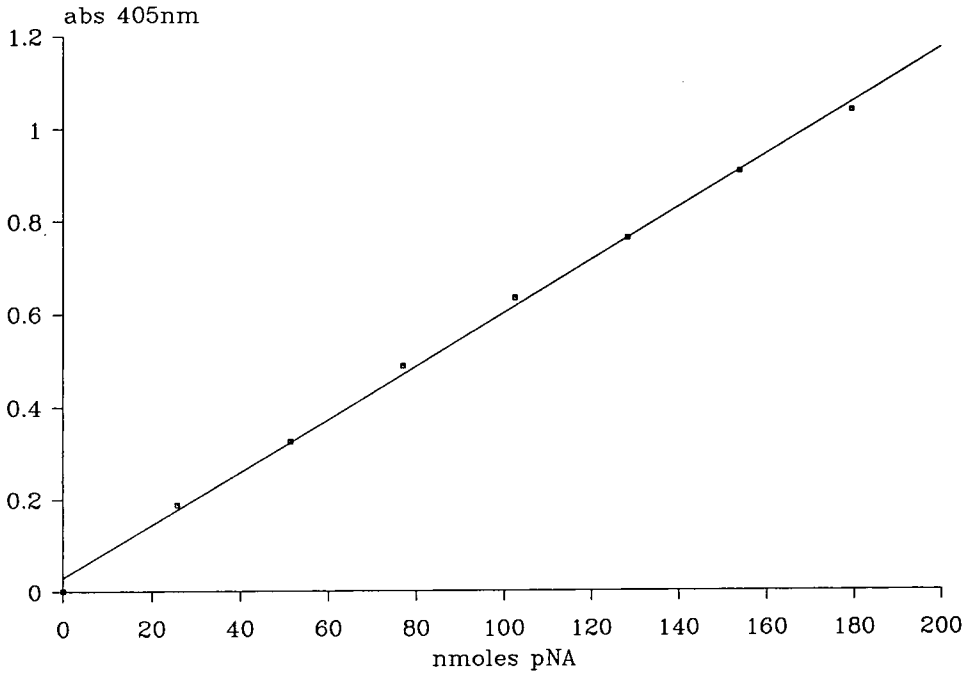


Figure 1.4

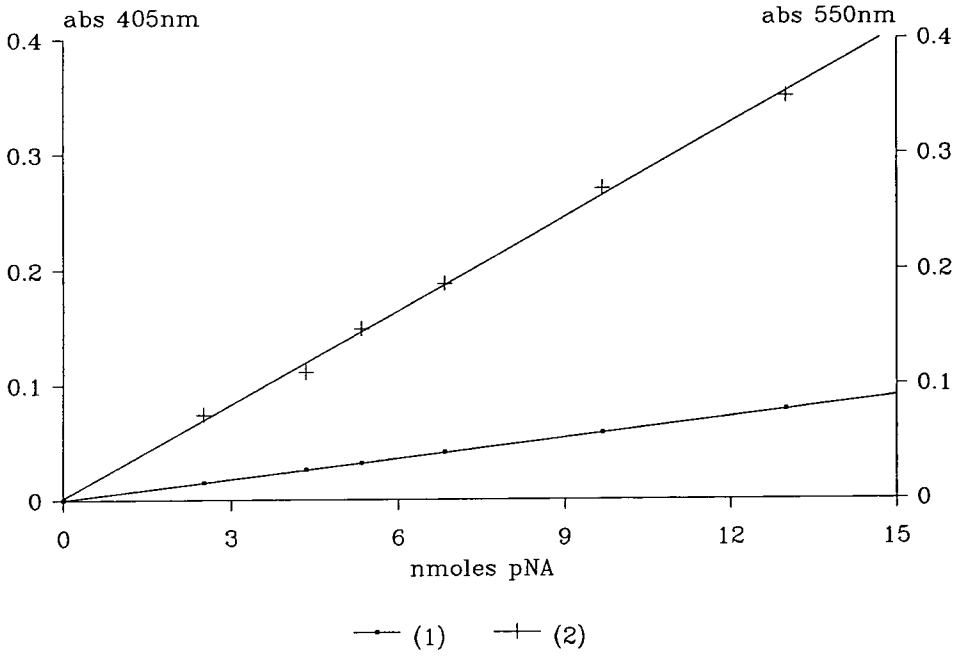


Figure 1.5

Full colour development of p-nitroaniline and NEDD requires the diazotization to occur at an acid pH (between pH 1-2). The minimum strength of acid which gave optimum absorption was determined by adding a fixed volume (160μl) of HCl to the 'reaction' mixture in test tube (containing 15nmoles pNA in 200μl DMF added to 2.4ml glycine buffer pH 10.0). This was then diazotized as described in the text.

Legend: ordinate - absorbance at 550nm
abscissa - Concentration of HCl added (M)

Figure 1.6

Standard calibration curve for the determination of p-nitroaniline released from pNA substrates when measured in microplates.

Legend: ordinate - absorbance at 405nm
abscissa - nmoles p-nitroaniline

Figure 1.5

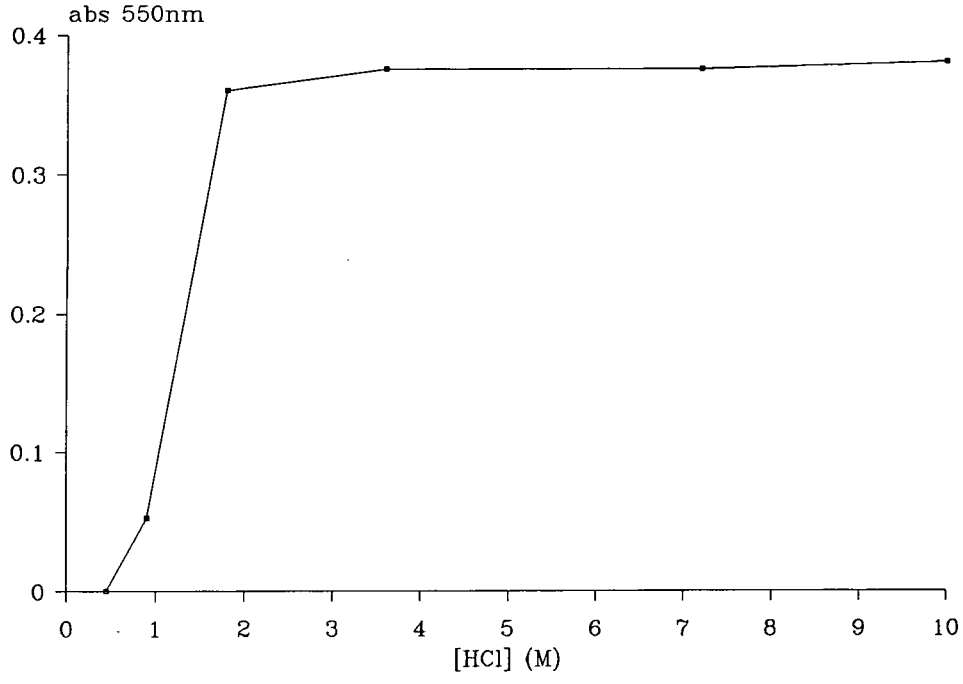
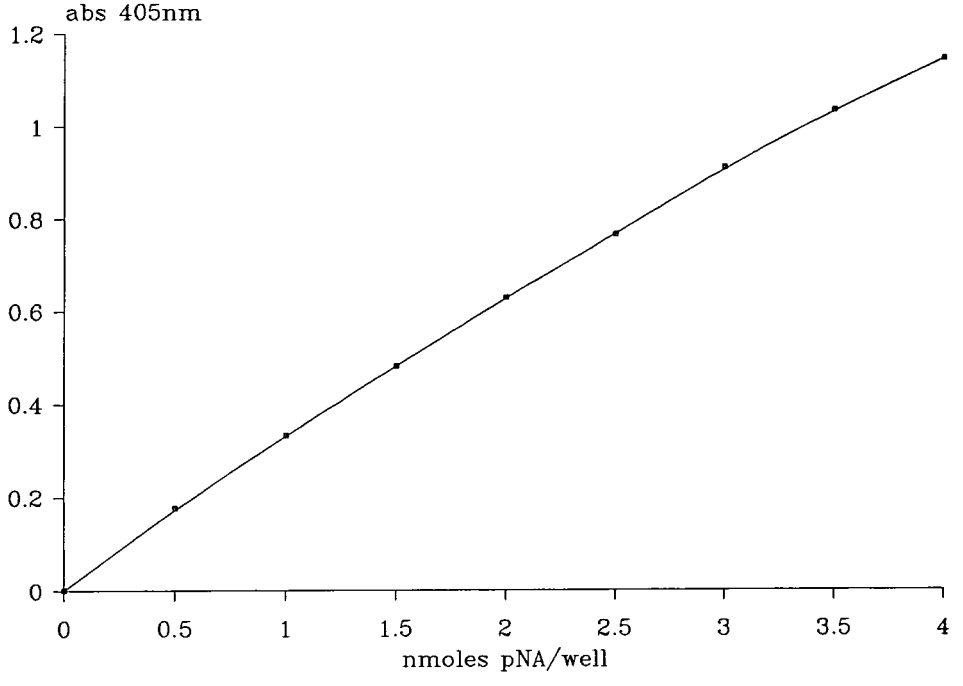


Figure 1.6



[HCl]. The HCl forms nitrous acid with the sodium nitrite which reacts with the pNA to produce a diazotised salt. Excess nitrite is removed by the addition of excess ammonium sulphamate. NEDD reacts with the diazo salt to form an azo dye (Norwitz and Keliher, 1981), which is coloured red.

ii. discontinuous measurement using microplates.

The use of microplates has many advantages over the use of test tubes; for example, the method allows the use of less enzyme preparation and other reaction media while allowing for a greater number of replicates. This is complimented by the speed in which the solutions can be dispensed using multichannel pipettes and by automatic reading and recording of the absorbance values of nearly 100 wells in less than one minute. This method was used extensively for the determination of active enzyme fractions from gel filtration and ion-exchange chromatography columns.

The relative composition of media was as described in (i) above. Thus, 150μl glycine-NaOH buffer pH 10.0 together with 15μl of enzyme preparation and 15μl DDW were pipetted into wells in the microplates (Flat bottomed microtitre plates, IS-FB-96 from Flow Labs, Rickmansworth, England). The plate was brought to temperature on the surface of a water-bath (Will *et al*, 1990) at 30 $\pm$ 0.5°C prior to the start of the reaction with the addition of 15μl of BApNA substrate to the wells at timed intervals. Well temperature was measured by inserting the tip of an RS Components Digital Thermometer fitted with a Type K thermocouple into a well to ensure thermoequilibration. The plate was then sealed with a Titertek plate sealer and submerged in the water bath. A minute before the end of the incubation, a known time which varied between 15 and 30 minutes, the plate was removed from the bath, dried, unsealed and the absorbances were either read immediately, or the reaction stopped using 0.1ml of 30% acetic acid and the absorbance recorded later using at 405nm using a Titertek Multiskan MCC/340 spectrophotometer. Appropriate controls were run as described in method (i). The rate of p-nitroaniline produced was calculated by reference to a calibration curve (Fig. 1.6).

Under conditions in which it was necessary to increase the sensitivity of the above, the Bratton-Marshall reaction (1939) as described by Preiser *et al*, (1975) in method (i) was miniaturised to detect low levels of p-nitroaniline product. The incubation proceeded as described above, but the reaction was stopped with 25μl of 2.5M HCl in place of 30% acetic acid. This was followed by successive additions of 25μl $NaNO_2$, 25μl ammonium sulphamate and finally 25μl NEDD. The absorbance was then measured on a Titertek Multiskan MCC/340 at 540nm. Peak absorbance of the reaction with NEDD occurs at 546nm (Norwitz and Keliher, 1982). The rate of product release was determined by reference to a standard curve (Fig. 1.7). From the calibration curve it can be seen that there is an increase in the absorption (and therefore sensitivity) by about 5.5 times, compared with undiazotized *p*-nitroaniline.

Figure 1.7

Standard calibration curve for the determination comparing the absorbance of p-nitroaniline before (1) and after (2) diazotization with NEDD (N-(1-naphthyl) ethylene diamine dihydrochloride). Absorbance measured in microplates.

Legend: ordinate: (1) - absorbance at 405nm
(2) - absorbance at 540nm
abscissa - pmoles p-nitroaniline

Figure 1.8

Standard calibration graph for the determination of orcein released from elastin-orcein by enzyme action. 40mg elastin-orcein was digested in 10ml of HCl in order to release the orcein. This was then serially diluted from the stock solution and made up to 6ml final volume in Sorensen phosphate buffer.

Legend: ordinate - absorbance at 590nm
abscissa - mg elastin-orcein

Figure 1.7

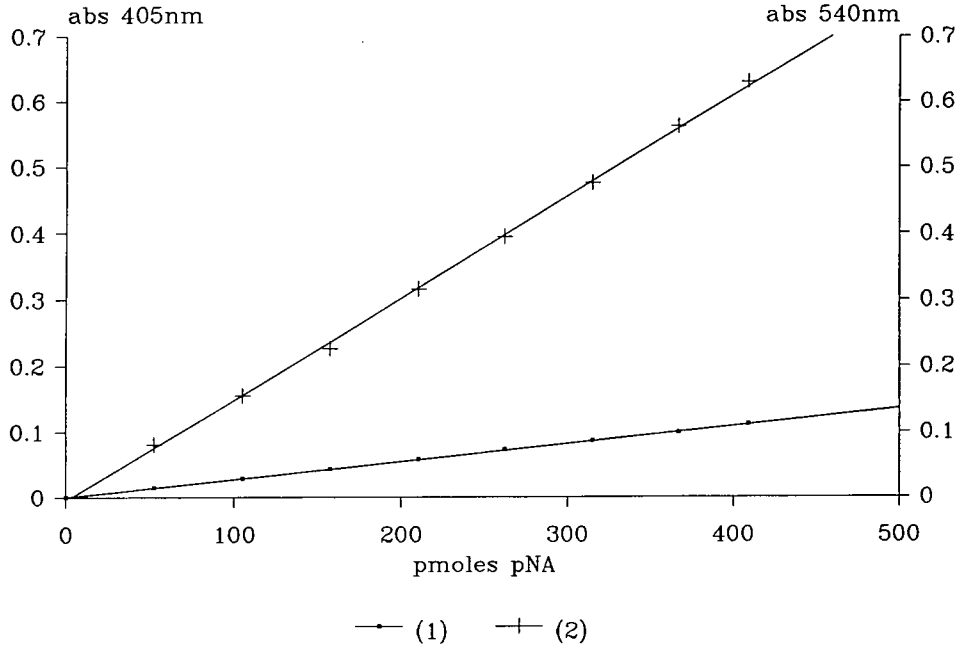
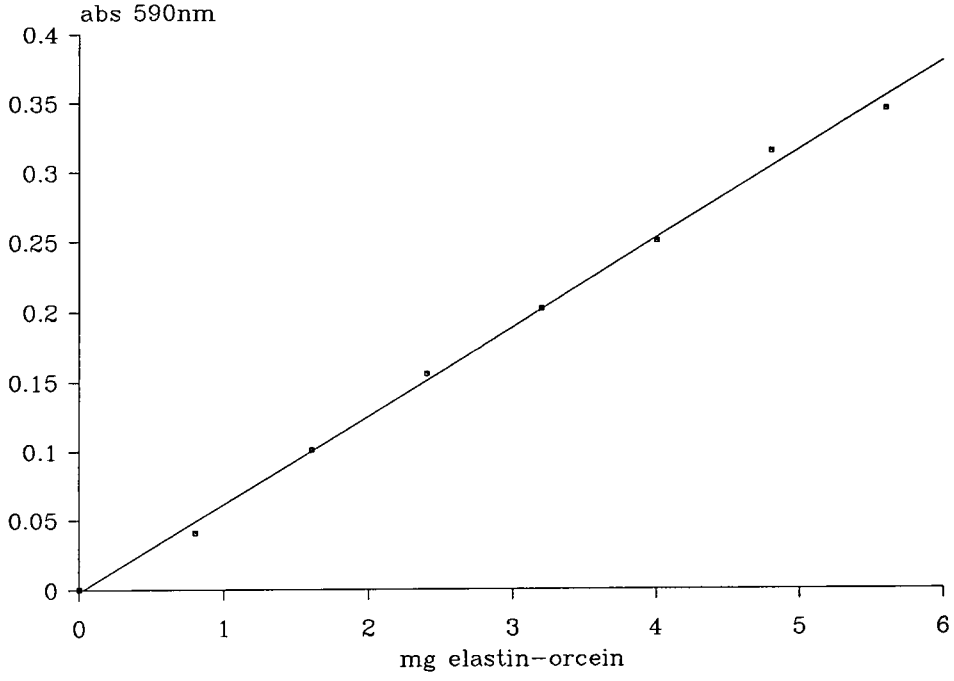


Figure 1.8



iii. continuous rate measurement

This method follows that of (Sarath *et al*, 1989). Reaction media were pipetted directly into cuvettes which were incubated to 30 ± 0.5°C inside the spectrophotometer's temperature control block. Quartz cuvettes were used when the spectrophotometer was set below 340nm, otherwise plastic cuvettes were used. As before, the reaction was started with BApNA and the contents mixed by inverting the cuvette when covered with a small piece of Nescofilm. During the measurement of p-nitroaniline production, the change in absorbance of the reaction was monitored continuously at 405nm for up to 3 hours using a Pye Unicam AR25 linear recorder. (When using the integrated rate equation, the reaction continued until all substrate had been depleted, see below). Appropriate blanks were assayed simultaneously to account for non-enzyme related substrate hydrolysis.

Other endopeptidase assays using p-nitroanilide:

The assay methods described above for BApNA hydrolysis were used to investigate the presence of the following classes of midgut endopeptidase activities with various other p-nitroanilide substrates:

a. Chymotrypsin activity was determined using Suphepa as substrate (N-succinyl-L-phenylalanine p-nitroaniline), (Geiger, 1984); BTpNA (N-Benzoyl-L-Tyrosine p-nitroanilide), (Bundy, 1962; 1966) or SA_2PPpNA, (N-Succinyl-Alanyl-Alanyl-Prolyl-Phenylalanine p-nitroanilide: Del Mar *et al*, (1979); Nakajima *et al*, 1979).

b. Substrates used normally to determine the elastase-like activity of mammalian enzymes were SA_2PLpNA (N-Succinyl-Alanyl-Alanyl-Prolyl-Leucine p-nitroanilide: Del Mar *et al*, 1980); SA_3pNA, (N-Succinyl-Alanyl-Alanyl-Alanine-p-nitroanilide: Bieth *et al*, 1974) and SA_2VpNA, (N-Succinyl-Alanyl-Alanyl-Valine-p-nitroanilide (Kasafirek *et al*, 1976; Stein, 1983; Wenzel *et al*, 1980; Wenzel and Tschesche, 1981).

c. Other p-nitroanilide substrates used in order to investigate the digestive enzymes present in whole gut homogenate were: acetyl-leucine-pNA, a chymotrypsin substrate; N-acetyl-L-aspartic acid α-(pNA), a bacterial protease substrate and Z-gly-pro-citrulline-pNA, a cysteine protease substrate. In addition, a sensitive substrate for subtilisins and neutral endopeptidases, Z-gly-gly-leu-pNA, was used (Lyublinskaya *et al*, 1974; Wilk and Orlowski, 1979).

Activity against protein substrates

Casein

Determination of endopeptidase activity by casein digestion followed a method modified from Kunitz (1947). Sodium-caseinate (5g/100ml) was dissolved by heating with an appropriate buffer solution; the latter varied depending on the pH conditions required. The pH of the solution was adjusted to the required value at 30 ± 0.5°C after the protein had dissolved and the pH was maintained above pH 7.0 at all times to avoid

protein precipitation. When using Tris buffer at near neutral pH, HNO_3 was used in place of HCl for adjusting the pH as the chloride salts of the protein precipitate readily. Assays were carried out in 1.5ml Eppendorf tubes. 0.8ml buffered-substrate solution was preincubated with 0.2ml DDW or 0.2ml inhibitor solution at 30 $\pm$ 0.5°C for 10 minutes, when the reaction was initiated by 0.2ml enzyme solution. The reaction was stopped after 30-60 minutes by the addition of 0.3ml of 30% trichloroacetic acid. Blanks were run in which TCA was added to the buffered substrate before the addition of enzyme. After the reaction had been terminated, the tubes were sealed and spun on an MSE MicroCentaur bench centrifuge at 13,000 rpm for 2 minutes to spin down undigested protein. The supernatant was removed to a quartz cuvette with a Pasteur pipette and its absorbance measured at 280nm. Enzyme activity was expressed as the change in absorbance/minute.

Elastin-orcein

Elastase activity was determined using a method modified from Mandl (1962). 20mg elastin-orcein substrate was added to 2ml buffer with 1ml enzyme solution and incubated for 14 hours at 30 $\pm$ 0.5°C with occasional mixing using a Fisons whirli-mixer. After the incubation period, 3ml of 0.7M Sorenson phosphate buffer (Dawson *et al*, 1986) pH 6 was added. The undigested substrate centrifuged off, and the absorbance of the supernatant read at 590nm. The rate of elastin-orcein digestion was calculated by reference to a calibration curve (Fig. 1.8) of acid-digested elastin-orcein serially diluted from a stock solution containing 40mg elastin-orcein in 10mls 10M HCl.

Activity against esterase substrates

Continuous rate measurement (method iii) was used to determine the activity of enzymes hydrolysing esterase substrates. N-Acetyl-L-tyrosine ethyl ester: ATEE, ε = 400, (Patat and Hirsch, 1966); L-Tyrosine ethyl ester: TEE, (Rovery *et al*, 1953; Schwert and Takenaka, 1955) and N-Benzoyl tyrosine ethyl ester: BTEE, ε = 964, (Hummel, 1959) were used to investigate chymotryptic esterase activity. ATEE is hydrolysed slowly by bovine trypsin (Inagami and Sturtevant, 1960). N-benzoyl-L-arginine ethyl ester: BAEE, ε = 808, (Kézdy *et al*, 1965; Baines *et al*, 1964) and p-Tosyl-L-arginine methyl ester: TAME, ε = 540, (Hummel, 1959; Trowbridge *et al*, 1963) were used for the assay of tryptic esterase activity. BAEE is hydrolysed slowly by bovine chymotrypsin (Schwert *et al*, 1948). ATEE and TEE hydrolysis were followed by a decrease in absorbance at 237nm and 233.5nm respectively (Rovery *et al*, 1953), whereas BTEE, BAEE and TAME hydrolysis resulted in an increase in absorbance at 256nm, 253nm and 247nm respectively.

Calculations of the velocity (amount of product produced by enzyme hydrolysis per minute) were made by using the difference between molar absorptivity for the

substrates and products in the following equation:

$v = \mu$mol pna produced/min

= (abs. change/change in t).$(1/\varepsilon)$

Enzyme activity and stability with pH

A variety of different buffers was used to provide an uninterrupted pH range for the investigation of the various enzyme activities and stabilities from 1.0-13.0 as required. The buffers used were: 0.2M KCl-HCl, pH 1.0 and 2.0 (Bower and Bates, 1955); 0.1M citric acid - 0.2M Na_2HPO_4, pH 3.0-8.0, (McIlvaine, 1921); 0.2M Na_2HPO_4 and 0.2M NaH_2PO_4, pH 5.8-8.0, (Gomori, 1955); 0.05M sodium barbitone-HCl, pH 6.8-9.6, (Britton and Robinson, 1931); 0.05M Bis tris propane-HCl, pH 6.5-9.5, (Good *et al*, 1966; Good and Izawa, 1972; Ferguson *et al*, 1980); 0.05-1M Tris-HCl, pH 7.0-9.0, (Bates and Bower, 1956); 0.2M glycine-NaOH, pH 8.6-11.0 (Gomori, 1955); 0.1M Na_2CO_3-0.1M $NaHCO_3$, pH 9.2-10.8, (DeLory and King, 1945); 0.025M borate-HCl/NaOH, pH 8.1-10.7, 0.025M phosphate-NaOH, pH 11.0-12.0 and 0.2M KCl-NaOH, pH 12.0-13.0 (Bates and Bower, 1956).

Treatment of enzyme-substrate affinity data

The Michaelis-Menten equation describes the effect of substrate concentration on the velocity of reaction. However, since the curve is asymptotic the Lineweaver-Burk (1934) equation has been used which enables K_m and V_{max} to be more accurately calculated by graphical representation (Dixon and Webb, 1964). V_{max} was calculated from the reciprocal of the intercept on the y axis and K_m from the negative reciprocal of the intercept on the x axis. To determine the intercepts on graphs, lines were generally fitted to the data by eye. However, Dowd and Riggs (1965) found that if used without adequate weighting the double reciprocal plot can result in larger errors in the kinetic constants than if Eadie/Hofstee's (1952) method of calculation is used. Therefore, weighted regression analyses were carried out on some reciprocal plots using Wilkinson's method, (1961).

Number of substrate binding sites

The number of sites was calculated using a computer program (devised by Dr. D. Hyde, University of Durham), which employs the Fibonacci search method (Atkins, 1973; Bannister *et al*, 1976) to fit enzyme-substrate affinity data to the following equation:

$$V = V_{max}[S]^n/[S]^n + K_m$$

Use of the integrated rate equation

An alternative method to initial velocities for estimating Michaelis-Menten parameters is the use of progress curves. For this method the reaction of the enzyme with substrate must continue until all substrate has been depleted. Analysis of the

progress curve uses the integrated form of the Michaelis-Menten equation:

$$[S]_o-[S]/t= -K_m.1/t.\ln[S]_o/[S] + V_{max}$$

A plot of $[S]_o-[S]/t$ versus $1/t.\ln[S]_o/[S]$ gives a straight line, where the slope is $-K_m$ and the intercept on the y axis is V_{max}. The laborious calculation of data points from a progress curve was later facilitated by the use of a simple program on a Casio fx-7000G calculator:

```
Lbl 1:?⇒B:?⇒C:-((A-B)/A-1)/C.
C^-1(ln(A/(A-B))).
Goto 1
```

Inhibitor studies

The effect of plant protein proteinase inhibitors on the hydrolysis of pNA substrates by insect protease activity was studied. Bovine trypsin was assayed for comparison at the same pH, but required the presence of 5mM calcium (see Fig. 1.11) for full activity. The effects of inhibitors were assayed using substrates against which there was established endopeptidase activity. When the action of inhibitors was determined, DDW was substituted with an equal volume of inhibitor solution (0.2ml in assay method i. & iii. and 15μl in assay method ii.). When the inhibitor was not dissolved in water, the control (i.e. without inhibitor) contained the alternative solvent. The enzyme was incubated with the inhibitor for ten minutes before substrate addition. Sufficient preincubation of the inhibitor is considered necessary for obtaining equilibrium data (DiPetro and Liener, 1989). However, Liu and Markakis (1989; 1990) have demonstrated that preincubation of the inhibitor with either bovine trypsin or chymotrypsin followed by the addition of substrate tended to underestimate the level of inhibition in comparison with preincubation of the inhibitor with the substrate followed by enzyme addition. In spite of this, it was decided to preincubate the inhibitor with the enzyme as a precaution against slow interaction between inhibitor and enzyme.

The effects of plant proteinaceous protease inhibitors on endopeptidases hydrolysing p-nitroanilide substrates were most easily assayed using the microplate method. A stock inhibitor solution was made to 13x the highest final concentration in the appropriate solvent. This stock was then serially diluted. The K_i of effective inhibitors could then be calculated using the method of Dixon (1972). This inhibition constant is called K_{ia} to distinguish it from the constant (K_{ib}) measured at pH 10.0 using the method of Dixon (1953).

CHAPTER 1
Results

Determination of midgut tissue and luminal pH

An estimate of the pH of the midgut contents and tissue of larval *H. armigera* was determined by placing a pH electrode (Beckman Model 3550) in pooled homogenates of midgut contents or midgut tissue dissected from feeding 6th instar larvae. A pH value of 9.6 $\pm$ 0.1 (N = 3) at 4°C for larval midgut contents and pH 7.2 $\pm$ 0.2 (N = 3) for larval midgut tissue was recorded by this means. At room temperature, the pH of regurgitated fluid from *S. littoralis* was measured at about pH 9.5 (BDH Universal Indicator) and at pH 9.5-10.0 using pH paper. However, the pH of midgut contents from *S. littoralis* was highly dependent on temperature (Fig. 1.9) and was only about pH 8.7 at 30°C. The pH change was non-linear with temperature.

Distribution of endopeptidase activities

Preliminary investigations on the tryptic and chymotryptic activities in midgut luminal contents and tissue indicated it was significantly higher in the contents from feeding larvae than in the lumen. Consequently, the presence and properties of the various midgut enzyme activities were investigated in midgut contents of larval *Spodoptera littoralis* and *Helicoverpa armigera* in subsequent studies.

Proteases characterised included chymotrypsin and trypsin from *S. littoralis*. A trypsin enzyme from *H. armigera* and bovine trypsin were included for comparison where stated.

The effect of DMF on BApNA and SA_2PLpNA activity

Initial experiments were carried out to investigate the effect of DMF on the activity of the endopeptidases from *S. littoralis*. Tryptic and chymotryptic endopeptidase activities were assayed in glycine-NaOH buffer at pH 10.0 using BApNA and SA_2PLpNA, respectively, as substrates and a range of concentrations of DMF (7.7% to 53.6% v/v). Examination of Figure 1.10 shows that both tryptic and chymotryptic activity were progressively inhibited with increasing concentrations of DMF in the incubation medium. Tryptic activity was less affected by DMF than chymotryptic activity. The concentration of DMF used in routine assays was a compromise between the need to ensure that the substrate remained in solution without affecting excessive enzyme inhibition. Consequently, all subsequent reactions requiring DMF-soluble substrates contained 7.7% DMF v/v unless otherwise stated.

The effect of Ca^{2+} and chelators on trypsin activity

In contrast to bovine trypsin, which was stimulated by over 50% in the presence of 0.75mM Ca^{2+} (Fig. 1.11), the activities of the tryptic proteases from *S. littoralis* and *H. armigera* were not significantly affected by Ca^{2+} concentration. Indeed, no

Figure 1.9

The effect of temperature on the pH of gut contents dissected from *S. littoralis*. Gut contents from 50 insects were pooled, cooled in ice and then warmed slowly in a water bath. The temperature and pH of the gut contents was continuously monitored.

Legend: ordinate - pH
abscissa - temperature (°C)

Figure 1.10

The effect of DMF concentration on the activity of the endopeptidases (trypsin and chymotrypsin) from *S. littoralis*.

Legend: ordinate - % activity
abscissa - % DMF (v/v)

Figure 1.9

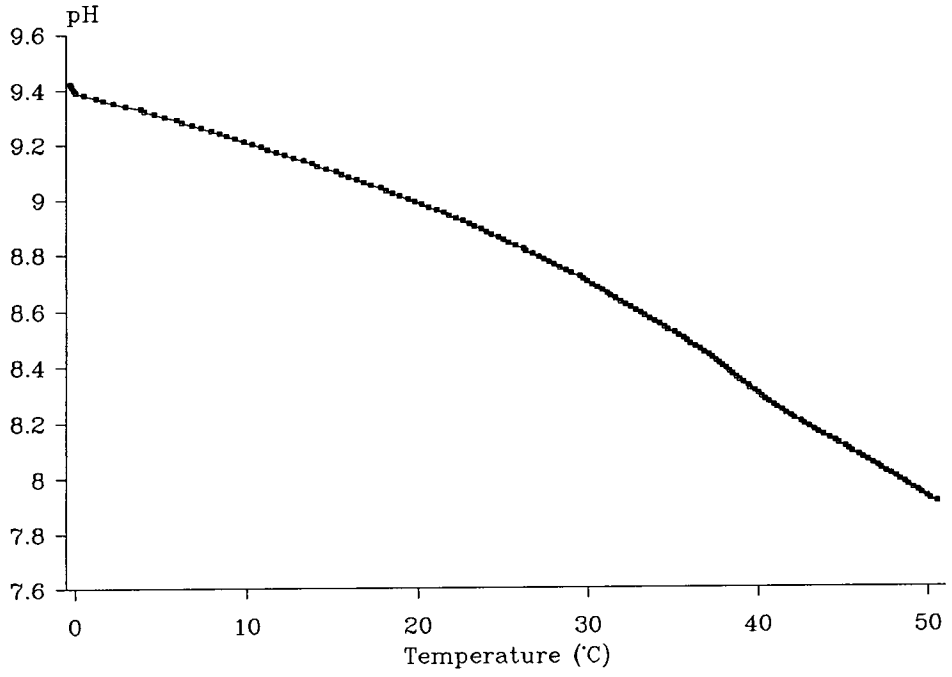


Figure 1.10

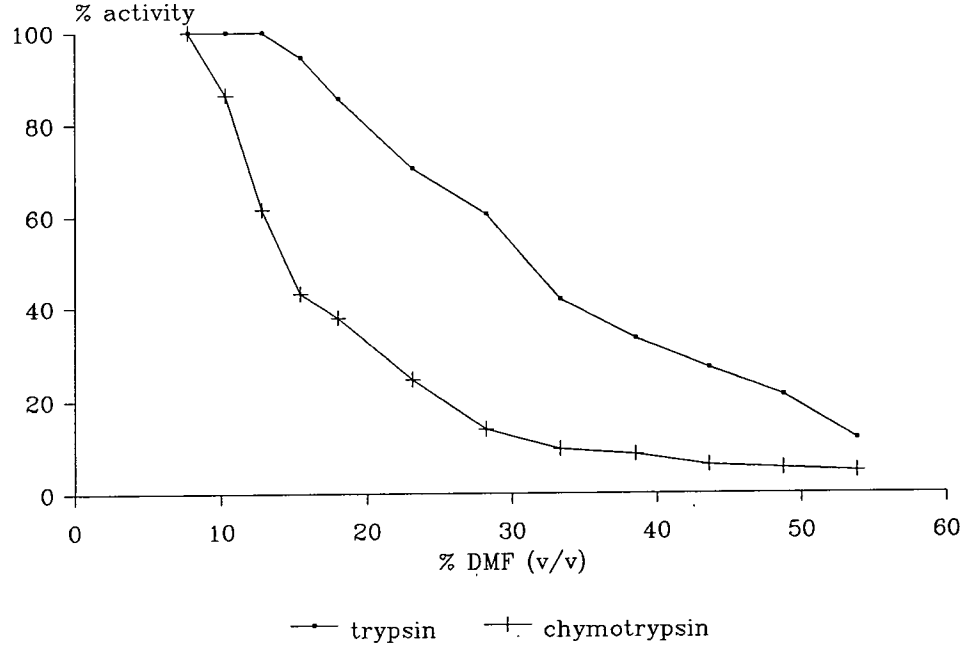


Figure 1.11

The effect of Ca^{2+} on the activity of bovine trypsin and trypsin enzymes from *H. armigera* and *S. littoralis*.

Legend: ordinate - % activity
abscissa - Ca^{2+} concentration (mM)

Figure 1.12

The effect of pH on the activity of trypsin towards BApNA from the midgut of larval *H. armigera*.

Legend: ordinate - % activity
abscissa - pH

Figure 1.11

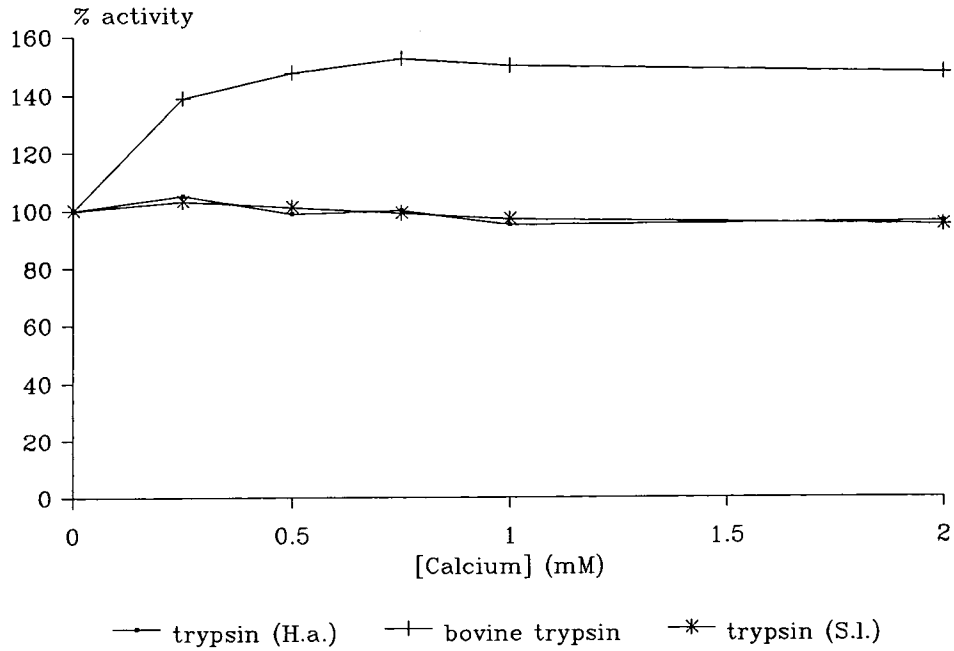
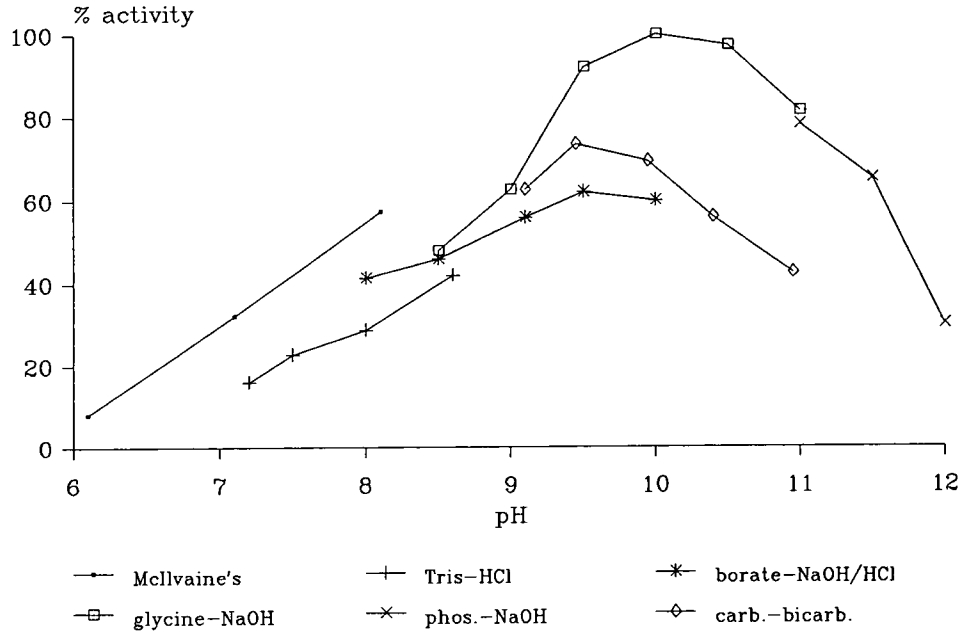


Figure 1.12



significant change in insect trypsin activities were observed over a range of Ca^{2+} concentration from 0-20mM. Similarly, the divalent cation chelators, EDTA (0-20mM) and EGTA (0-0.5mM), had no effect on the activities of trypsin-like enzymes from either species of insect. However, bovine trypsin activity assayed in the absence of added calcium ions was reduced by 30% in the presence of 5 mM EDTA and 25% by 5 mM EGTA. All subsequent assays of insect proteases took place in the absence of added calcium ions.

Characterisation of activity using BApNA as substrate

The effect of pH on activity

Figure 1.12 shows the effect of pH on enzyme activity from *H. armigera* against BApNA and how this is affected by different buffer systems. Tryptic activity was significantly greater when assayed in the presence of glycine-NaOH buffer compared to that observed with borate-NaOH (pH 8-10) and carbonate (pH 9-11) buffers at equivalent pH. Maximal activity was observed in the glycine-NaOH buffer at pH 10.0. Similarly at lower pH values, trypsin activity was influenced by the buffer system used. Thus, McIlvaine's and phosphate buffer appeared to stimulate the activity of the trypsin at low pH (pH 6-8) compared with activity of the enzyme in Tris-HCl buffer.

BApNA-ase activity from *S. littoralis* is shown in Figure 1.13. Maximal activity was again observed in glycine-NaOH buffer range (pH 8-10). All other buffers (McIlvaine's, phosphate, BTP, Tris and barbitone) appeared to stimulate the activity of the enzyme at low pH with respect to activity observed with glycine buffer at equivalent pH.

The apparent pH optima of pH 10.0 may in fact underestimate the true value due to substrate availability becoming limiting above pH 10.0, as a consequence of autolysis of pNA substrates with increasing pH (Sarath *et al*, 1989). Hence, all subsequent routine assays were carried out at or below pH 10.0 using glycine-NaOH buffer and care was taken to ensure that substrate availability did not become rate-limiting.

The effect of pH on stability of enzyme activity

1 ml aliquots of the enzyme preparation from *S. littoralis* were incubated with 2ml buffer at different values of pH (over the range 1-13) for 30 minutes at 30°C. At the end of this time, 3ml glycine-NaOH buffer pH 10.0 was added and the pH brought to pH 10.0 by the addition of HCl or NaOH as appropriate. The final volume was then made up to 10ml with DDW. Subsequently, trypsin activity was measured as described in the Materials and Methods. The results obtained are shown in Figure 1.14. It can be seen that the BApNA-ase enzyme is stable between pH 4.0 and 12.0 for 30 min and retains approx. 80% of its activity even at pH 3.0. Above or below these pH values enzyme activity was almost totally lost.

Figure 1.13

The effect of different buffers and pH on the activity of trypsin towards BApNA from the midgut of larval *S. littoralis*.

Legend: ordinate - % activity
abscissa - pH

Figure 1.14

The effect of pH on the stability of trypsin from the midgut of larval *S. littoralis*. Aliquots (1ml) of midgut enzyme preparation were incubated with buffer (2ml) at different values of pH, over the range 1-13 for 30 minutes at 30°C. Subsequently, 3ml glycine-NaOH pH 10.0 was added and the pH adjusted to pH 10.0 by the addition of HCl or NaOH as appropriate. The final volume was then made up to 10ml with DDW and the trypsin activity measured.

Legend: ordinate - % activity
abscissa - incubation pH

Figure 1.13

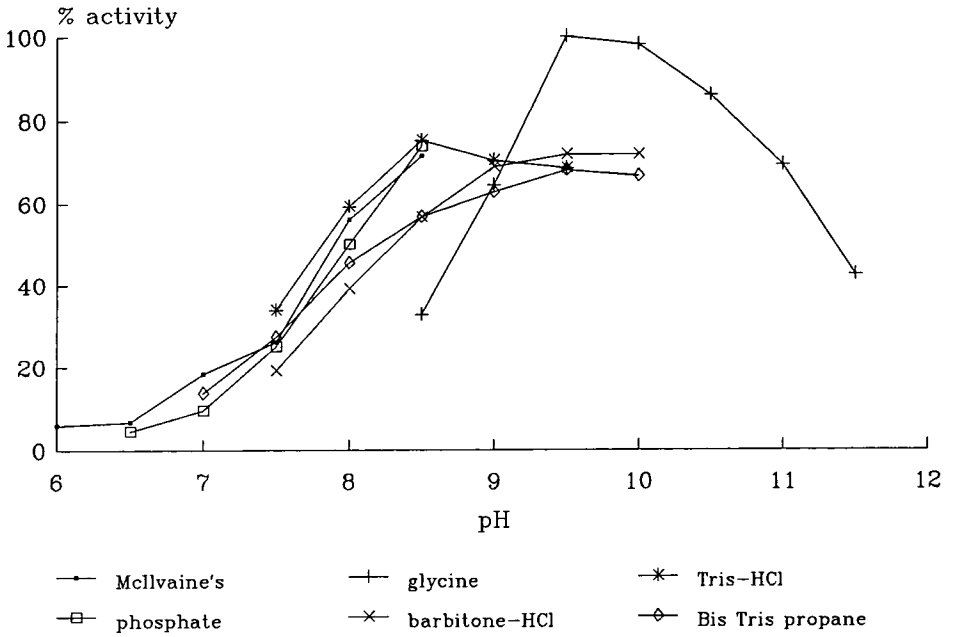


Figure 1.14

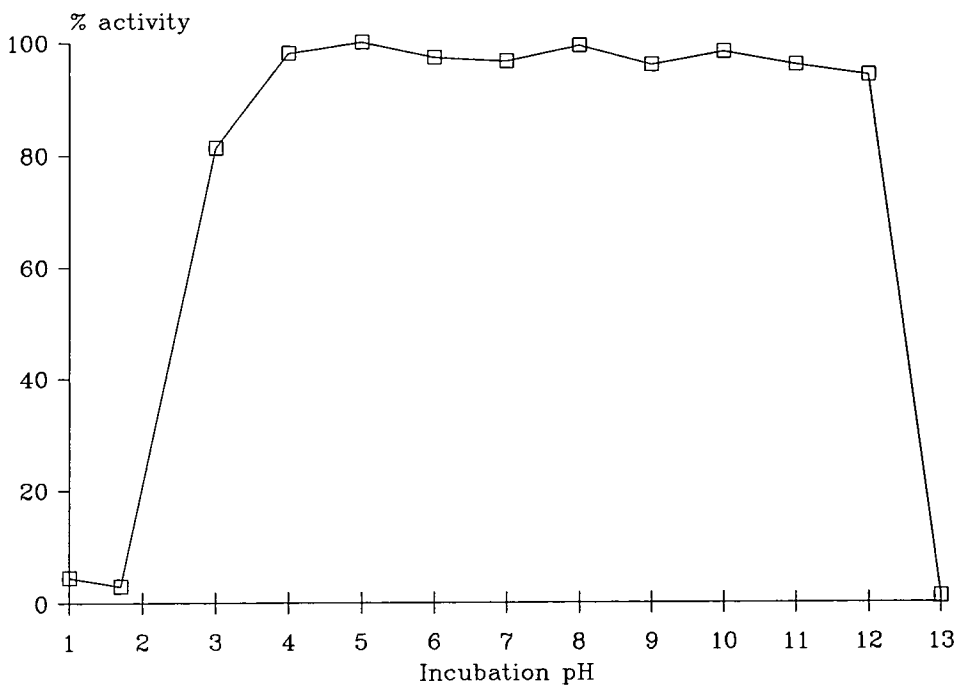


Figure 1.15

The determination of trypsin stability during a continuous assay in a whole time course study. 0.2ml enzyme, 0.2ml substrate and 2.6ml buffer (glycine pH 10.0) were incubated at 30°C. The production of pNA was continuously monitored until the reaction was complete. The reaction completion was considered complete after seven half-lives. For this test (Selwyn, 1965), all the parameters, except enzyme concentration, are kept constant and the results plotted against time multiplied by enzyme concentration. If the enzyme becomes denatured during the course of the reaction then the observations for different enzyme concentrations will fall on different curves.

Legend: ordinate - relative amount product formed
abscissa - relative [enzyme] x time

1, 2 and 4 indicate the relative amount of enzyme present for each curve.

Figure 1.16

As above, but activity determined at pH 8.0 (in Tris buffer) in order to check whether the denaturation of the enzyme is pH dependent.

Legend: ordinate - relative amount product formed
abscissa - relative [enzyme] x time

Figure 1.15

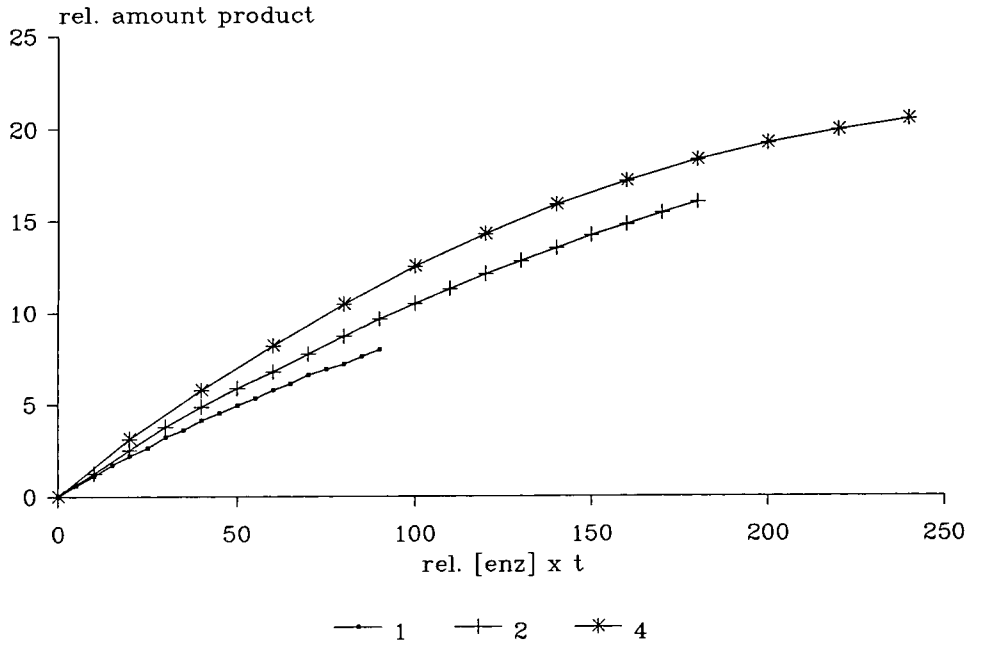


Figure 1.16

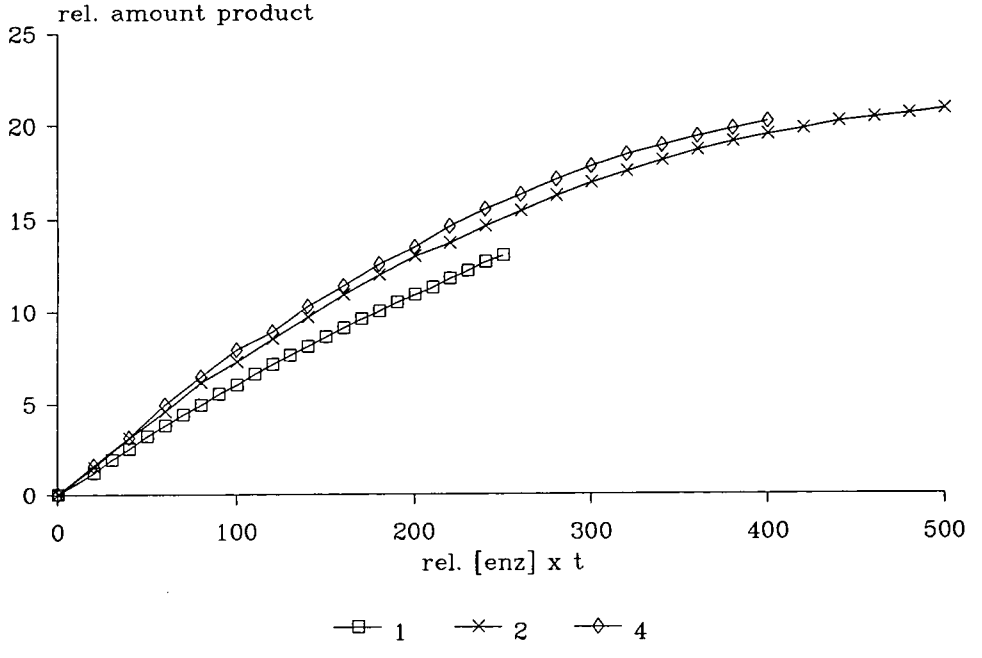


Figure 1.17

The effect of preincubation at various temperatures on the trypsin activity measured at 30°C. Enzyme solutions were buffered in glycine pH 10.0, incubated at the indicated temperature for 30 minutes and activity subsequently measured at 30°C.

Legend: ordinate - % activity
abscissa - temperature (°C)

Figure 1.18

The effect of temperature on the activity of the trypsin enzyme from the midgut of *S. littoralis* (Arrhenius plot). 2.2ml buffer (glycine pH 10.0) was thermoequilibrated at the appropriate temperature in testtubes. The reaction was started by the addition of 0.2ml substrate and 0.2ml enzyme at timed intervals. After 30 minutes incubation, the reaction was stopped by the addition of 1.3ml 30% acetic acid.

Legend: ordinate - ln Vel. (μmoles pNA/ minute/ mg protein)
abscissa - 1/temperature x 1000 (K)

Figure 1.17

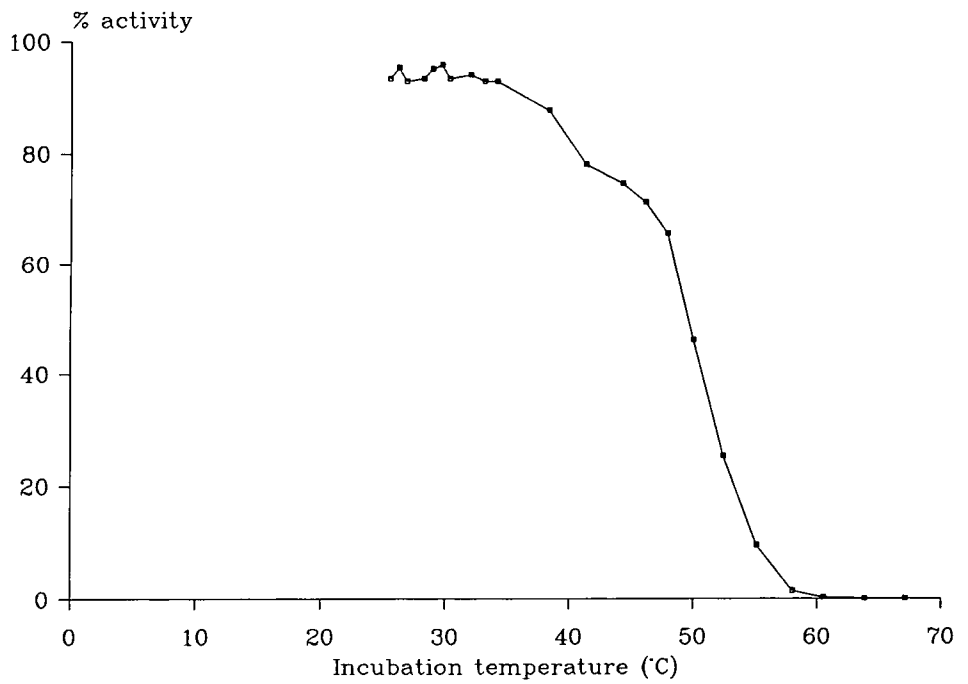
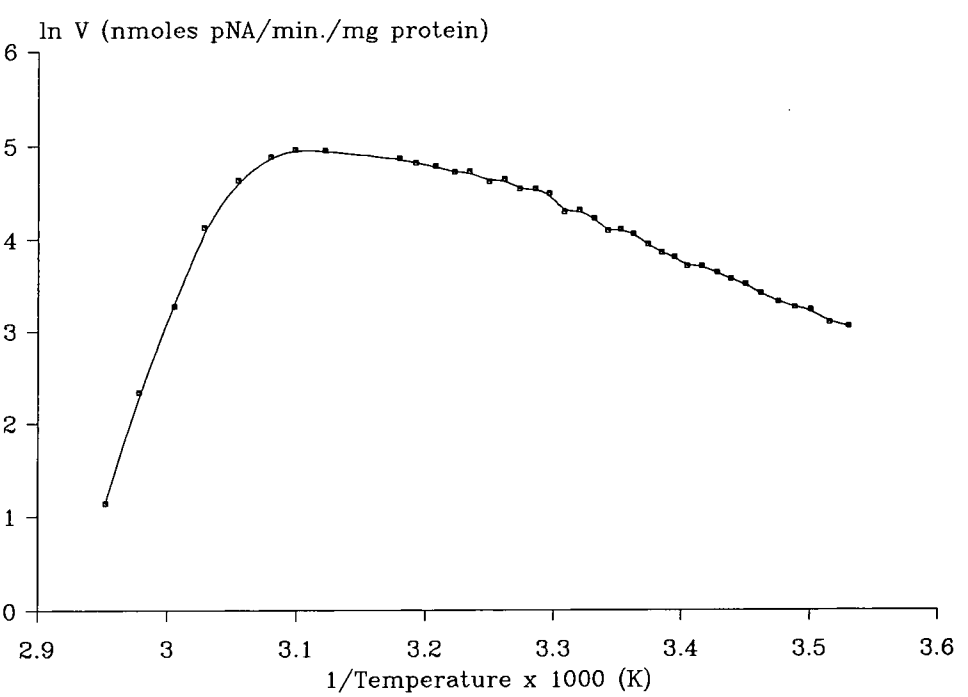


Figure 1.18



read at 405nm on a spectrophotometer. A temperature optimum of approximately 50°C was found over a 30 minute period (Fig. 1.18) for trypsin from *S. littoralis*. Arrhenius plots exhibited a 'break-point' at approximately 30°C (Fig. 1.18); the mean activation energy between 10 and 30°C was 57.4 ± 7.4 kJ/mole, while between 30 and 50°C it was 25.1 ± 1.6 kJ/mole.

Characterisation of activity with TGPLpNA as substrate

The endopeptidase activity of midgut enzymes was investigated using another trypsin substrate, TGPLpNA. Hydrolysis of this substrate was extremely rapid at pH 10.0. The mean K_m was 0.0564 ± 4.5E-3 mM (N = 3) and mean V_{max} was 3473 ± 278 nmoles pNA/mg protein/minute (N = 3), and this was the most rapidly hydrolysed peptide substrate tested.

Characterisation using SA_2PLpNA as substrate

The effect of pH on activity

Chymotryptic activity, measured using SA_2PLpNA, was affected by pH in a similar fashion to BApNA-ase activity (Fig. 1.19). The activity of the enzyme was highest at alkaline pH, which is characteristic of lepidopteran larval midgut endopeptidases. Maximal activity was observed in glycine buffer whilst McIlvaine's buffer appeared to stimulate activity at low pH compared with barbitone buffer. In addition, activity of the enzyme was higher at pH 8.0 in McIlvaine's buffer compared with that in glycine buffer at pH 8.6.

The effect of substrate concentration

The effect of SA_2PLpNA concentration on the rate of enzyme activity was determined in glycine buffer at pH 10.0 and 30°C over a concentration range of 0.1 to 1mM using a double reciprocal plot. The mean K_m was 0.49 ± 0.15 mM (N = 3) and mean V_{max} was 503 ± 25.0 (N = 3) nmoles pNA/mg protein/minute for *S. littoralis*. The value of n was 1.3 calculated as described previously for trypsin. This value indicates that there is a certain amount of cooperativity between aggregated binding sites or substrate activation. Gel filtration results presented below suggest that dimerisation of this enzyme can occur, as the molecular weight is twice that of the molecular weight found by SDS-PAGE.

Assays using other chymotryptic substrates

Assays involving the use of other substrates were used to investigate the presence of other enzymes which may be present and the specificity of those already found. Chymotryptic activity was not detected in homogenates of gut tissue or gut luminal contents using either Suphepa or BTpNA as substrates in the pH range 4.0 to 11.0. However, high levels of activity using SA_2PPpNA were present in the midgut contents: the mean K_m was 0.260 ± 0.015mM (N = 3) and mean V_{max} was 281 ± 28 nmoles

Figure 1.19

The effect of pH on the activity of chymotrypsin from the midgut of larval *S. littoralis*. Activity was measured using SA_2PLpNA as substrate.

Legend: ordinate - % activity
abscissa - pH

Figure 1.20

The effect of pH on the hydrolysis of TAME by midgut enzymes from *S. littoralis*. The change in absorbance was measured at 247nm.

Legend: ordinate - % activity
abscissa - pH

Figure 1.19

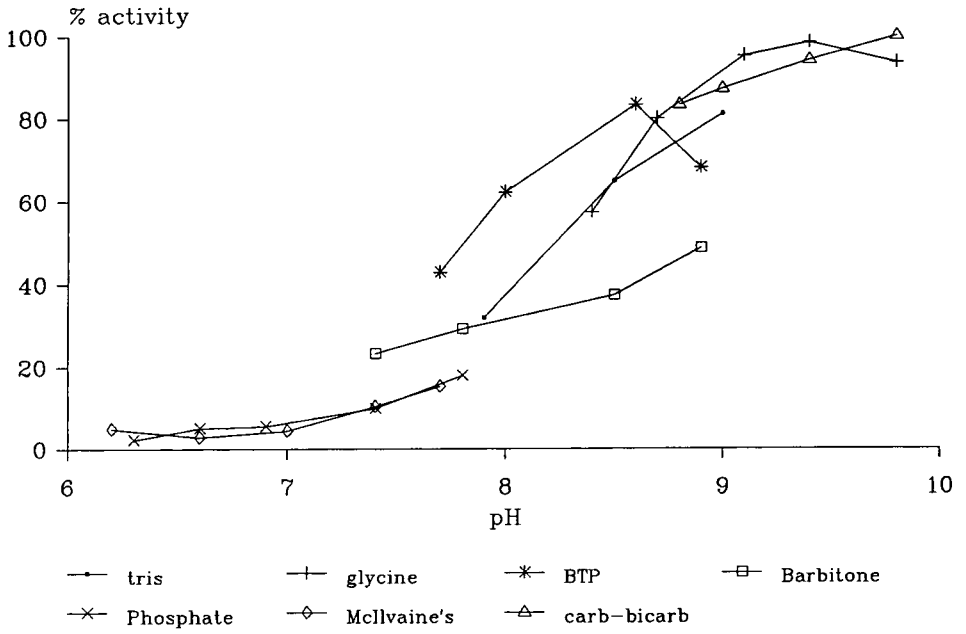
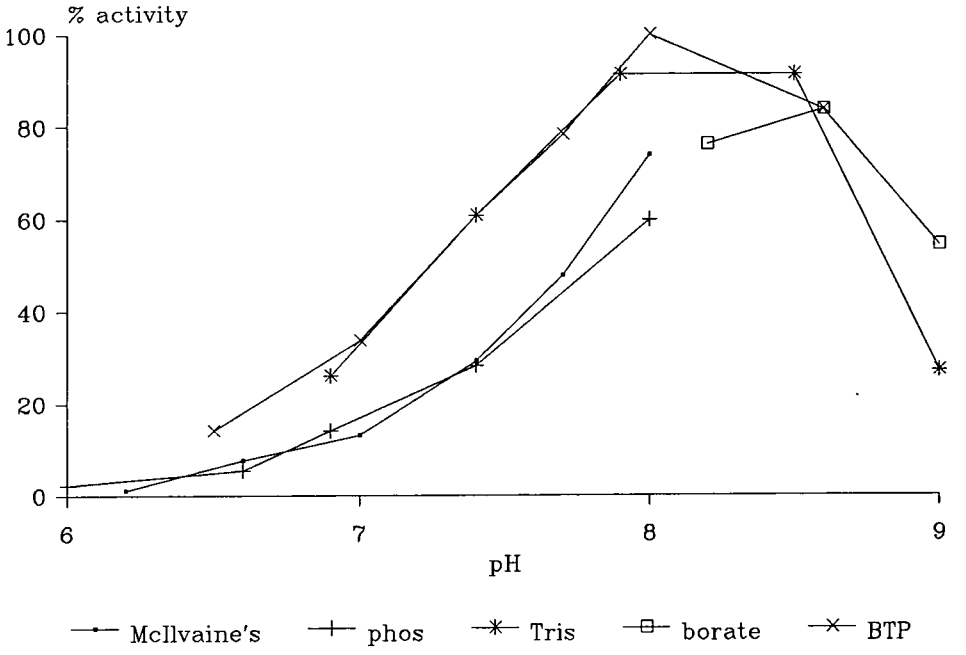


Figure 1.20



pNA/mg protein/minute (N = 3).

The specificity of peptide bond cleavage next to a leucine residue was investigated further using a variety of substrates with different numbers of N-terminal residues. The substrates used were LeupNA, ALpNA and ZGGLpNA at a concentration of 1mM. Activity was not detected with these substrates at pH 8.0 and 10.0.

Hydrolysis of elastase substrates

Hydrolysis of SA_3pNA was relatively slow compared with hydrolysis of SA_2PLpNA; the mean K_m was 3.35 $\pm$ 0.23 mM (N = 3) and mean V_{max} was 188 $\pm$ 13 nmoles pNA/mg protein/minute (N = 3) for *S. littoralis*.

No hydrolysis of the elastase substrate, 1mM SA_2VpNA, (Kasafirek *et al*, 1976), was detected at pH 8.0 or pH 10.0, although maximum hydrolysis of esterase and endopeptidase substrates occurred at these pH values, respectively.

Other pNA substrates

No measureable enzyme activity was detectable at pH 8.0 and 10.0 using 1mM AApNA and ZGPCpNA as substrates.

Esterase substrates

The presence of various esterase activities was investigated over a range of pH (6-11) using TAME, BAEE, BTEE, ATEE and TEE as substrates by using continuous rate assay as described in the materials and methods. Barbitone-HCl buffer absorbs strongly below 260nm and could not be used to assay esterase substrates.

The effect of pH on TAME, BAEE, BTEE and ATEE hydrolysis

The hydrolysis of TAME and BAEE (both cleaved by bovine trypsin) by midgut enzymes from *S. littoralis* was maximal at pH 8.0 and 9.5 respectively (Figs. 1.20 and 1.21). TAME is not suitable for high pH studies due to significant hydrolysis above pH 9.0 (Dawson *et al*, 1986). This may explain the lower pH optimum for TAME hydrolysis. The pH profile of TAME and BAEE breakdown differed from the profile for BApNA-ase activity; McIlvaine's buffer and phosphate-phosphate buffer tended to decrease activity compared with Tris and BTP buffers when using TAME and BAEE as substrates, whereas increased activity was observed using BApNA as substrate.

The hydrolysis of BTEE and ATEE (both cleaved by bovine chymotrypsin) by midgut enzymes was maximal at about pH 8.0 (Figs. 1.22 and 1.23). An optimum pH was not determined for TEE because the rate of hydrolysis was very low. Changes in the activity of the enzyme comparable with those obtained using TAME and BAEE with different buffers were not so apparent when using ATEE and BTEE as substrates. However, hydrolysis of BTEE was reduced by glycine buffer compared with BTP and Tris buffers.

Figure 1.21

The effect of pH on the hydrolysis of BAEE by midgut enzymes from *S. littoralis*. The change in absorbance was measured at 253nm.

Legend: ordinate - % activity
abscissa - pH

Figure 1.22

The effect of pH on the hydrolysis of BTEE by midgut enzymes. The change in absorbance was monitored at 256nm.

Legend: ordinate - % activity
abscissa - pH

Figure 1.21

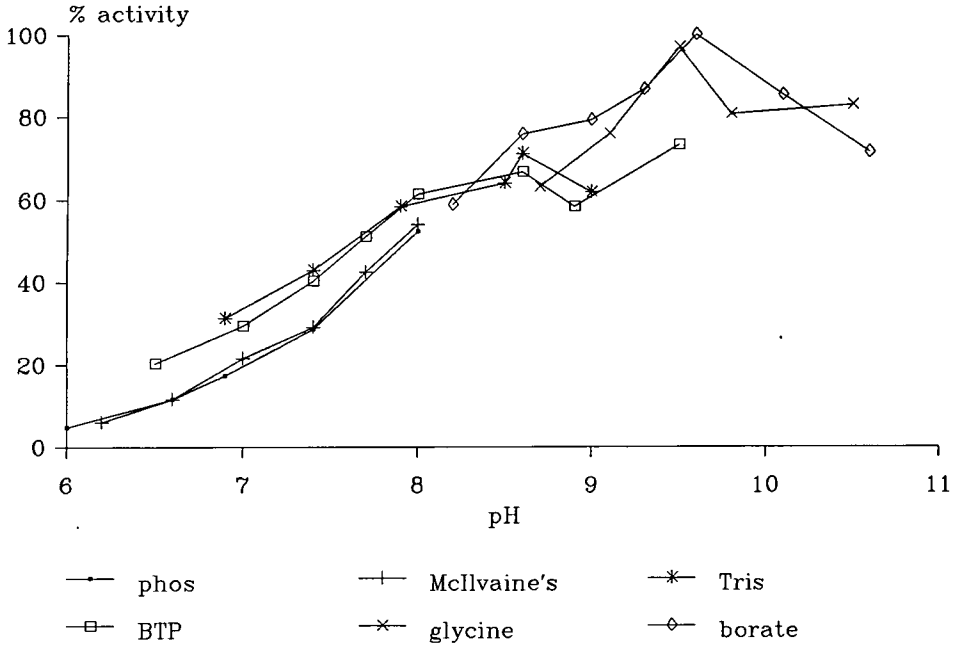


Figure 1.22

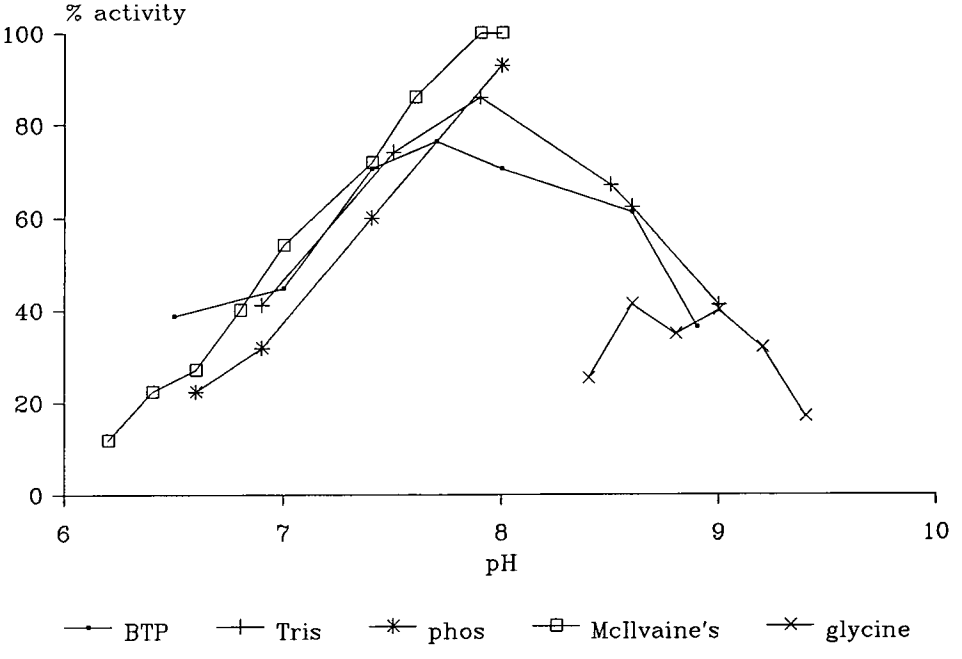


Figure 1.23

The effect of pH on the activity of ATEE by midgut enzymes. The change in absorbance was monitored at 237nm.

Legend: ordinate - % activity
abscissa - pH

Figure 1.24

The effect of pH on the stability of the BAEE hydrolysis by enzyme from the midgut of larval *S. littoralis*. Aliquots (1ml) of midgut enzyme preparation were incubated with buffer (2ml) at different values of pH, over the range 1-13 for 30 minutes at 30°C. Subsequently, 3ml glycine-NaOH pH 10.0 was added and the pH adjusted to pH 10.0 by the addition of HCl or NaOH as appropriate. The final volume was then made up to 10ml with DDW and the trypsin activity measured.

Legend: ordinate - % activity
abscissa - incubation pH

Figure 1.23

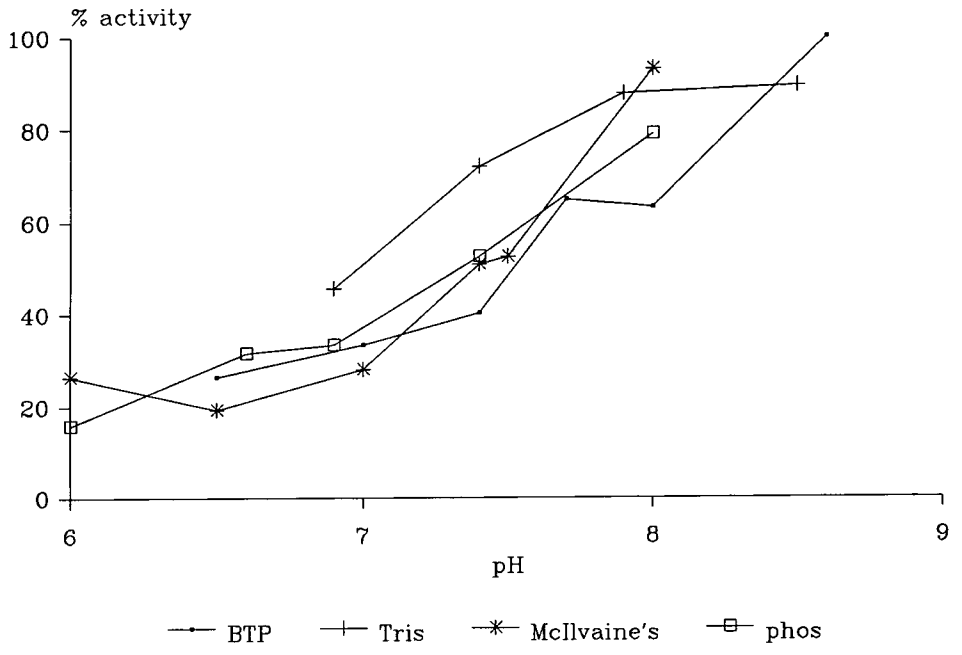
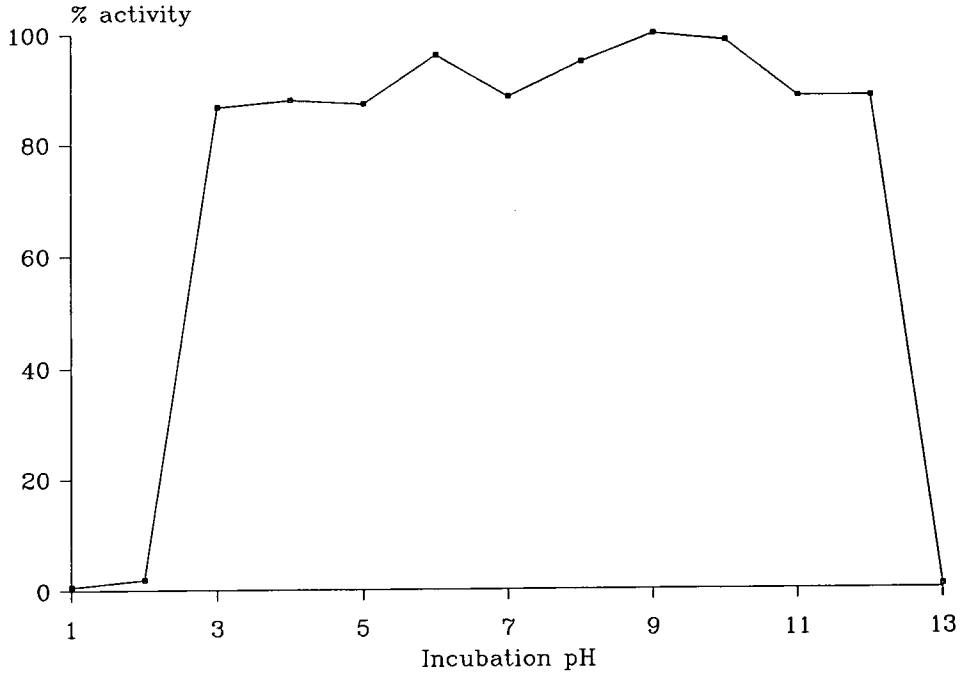


Figure 1.24



Enzyme stability with pH using BAEE and TAME as substrates

Aliquots of enzyme were incubated with buffer as described when using BApNA as substrate. However, at the end of the incubation time, 3ml of an appropriate buffer (glycine-NaOH buffer pH 9.5 when using BAEE and BTP buffer pH 8.0 when using TAME) was added and the final pH of the mixture brought to the required pH by the addition of HCl or NaOH as appropriate. The final volume was then made up to 10ml with DDW. Subsequently, activity was measured by continuous rate measurement as described in the Materials and Methods. From the results shown in Figure 1.24 it can be seen that the enzyme hydrolysing BAEE is characterised by a broad stability between pH 3.0 and 12.0 whereas hydrolysis of TAME occurred between pH 3.0 and 12.0, but its stability was highest at alkaline pH only (pH 9-11) (Fig. 1.25).

The effect of esterase substrate concentration

The change of enzyme activity with substrate concentration (0.025-1mM) was determined using a double reciprocal plot. Inhibition of activity was apparent when using BAEE concentrations above 0.4mM, but not with a similar concentration of TAME (Figs. 1.26 and 1.27). The kinetic parameters for BAEE substrate hydrolysis were determined using the linear part of the curve only. K_m for TAME and BAEE was about 59μM and 43μM respectively. V_{max} for TAME and BAEE was 4.8 and 6.9 μmoles product/mg protein/minute respectively.

Naturally-based protein substrates: casein

Linearity of the reaction with casein concentration

The effect of four different substrate concentrations (0.001, 0.1, 1 and 5g sodium caseinate/100ml) on caseinase activity was determined at pH 11.0. Over a period of 60 minutes a linear relationship was found between enzyme activity and time at a substrate concentration of 5g sodium caseinate/100ml (Fig. 1.28) and close to linearity for the same period for a concentration of 1g/100ml. Linearity using the lowest concentration of casein was limited to a few minutes only. The former casein concentration was used for all routine assays.

Caseinase activity: the effect of pH on activity

The activity of digestive enzymes from the midgut lumen of *Spodoptera littoralis* was assayed using casein as a substrate in the following buffers: Tris-HNO_3, pH 7-9; barbitone-HNO_3, pH 7-8.6; glycine-NaOH, pH 8.5-10.5; and phosphate-NaOH, pH 11-12. McIlvaine's buffer could not be used because the presence of phosphate ions in this buffer caused precipitation of the protein. Therefore, the apparent increase in activity observed with this buffer when using the substrates BApNA and SA_2PLpNA could not be investigated using casein. Maximal activity of the enzyme was observed between pH 10.5 and 11.5 as shown in Figure 1.29.

Figure 1.25

The effect of pH on the stability of TAME hydrolysis by midgut enzyme preparation.

Legend: ordinate - % activity
abscissa - incubation pH

Figure 1.26

The effect of BAEE concentration on the rate of hydrolysis by midgut preparation.

Legend: ordinate - 1/velocity (μmoles BA/minute/mg protein)
abscissa - 1/[BAEE] (mM)

Figure 1.25

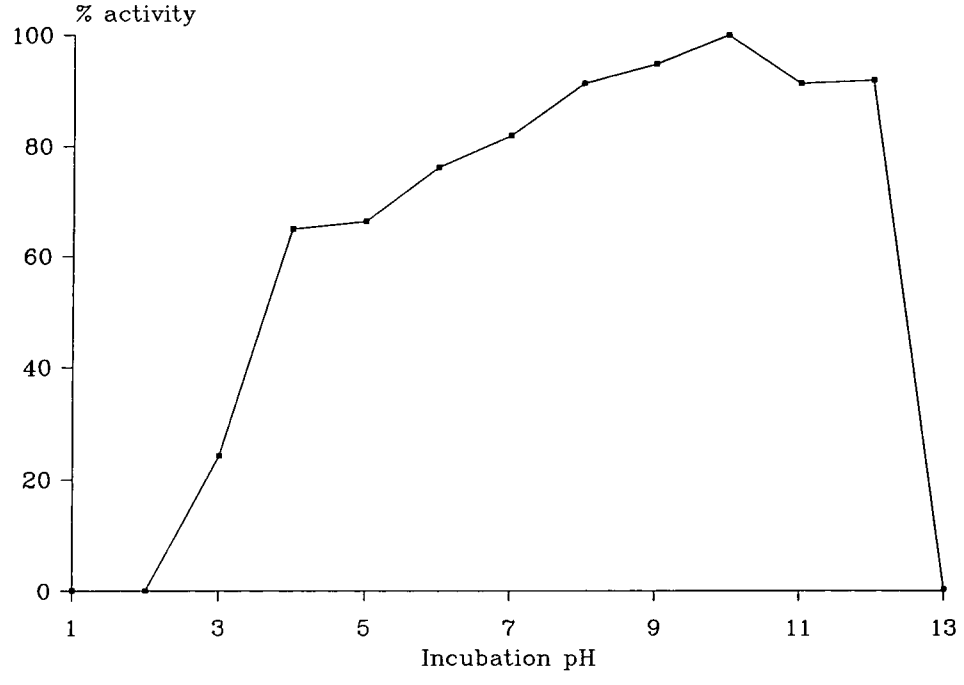


Figure 1.26

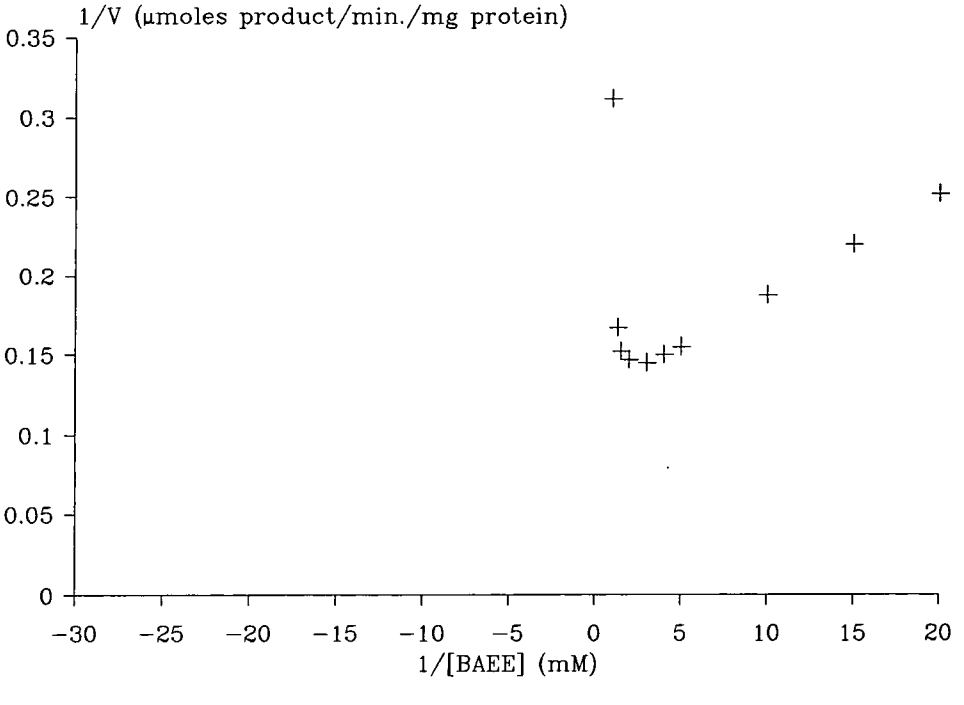


Figure 1.27

The effect of TAME concentration on the rate of hydrolysis by midgut preparation.

Legend: ordinate - 1/velocity (TA/minute/mg protein)
abscissa - 1/[TAME] (mM)

Figure 1.28

The linearity of casein hydrolysis with time. Crude enzyme preparation was incubated with casein-buffer at pH 11.0 for up to 1 hour. The casein was present at concentrations between 0.001 and 5g per 100ml. The reaction was stopped by the addition of 30% TCA and the undigested protein was removed by centrifugation. The relative amount of protein hydrolysed was measured at 280nm.

Legend: ordinate - Absorbance 280nm.
abscissa - time (hours)

Figure 1.27

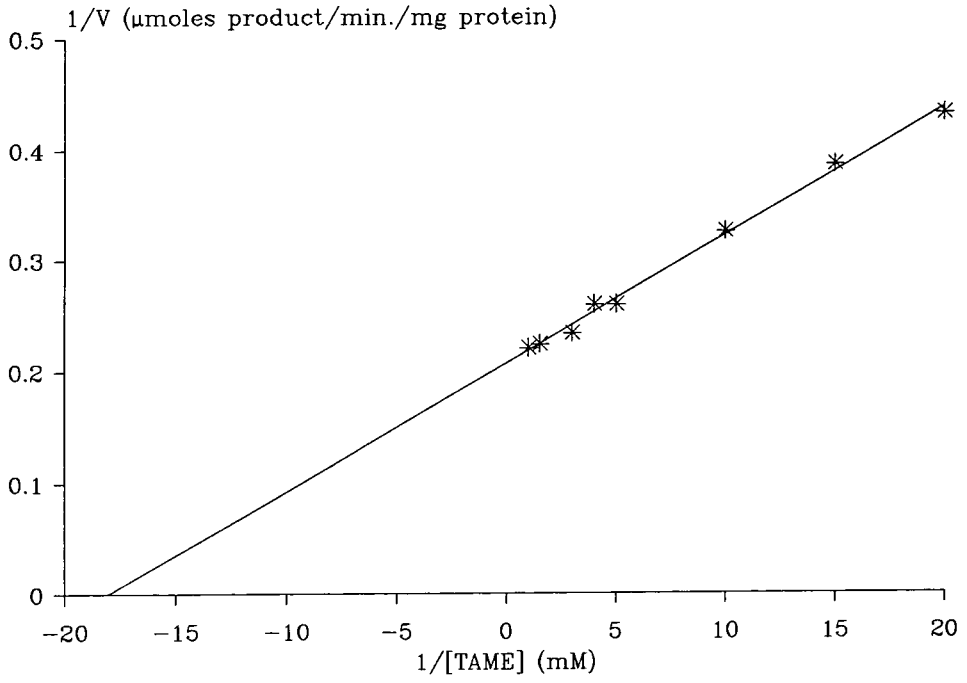


Figure 1.28

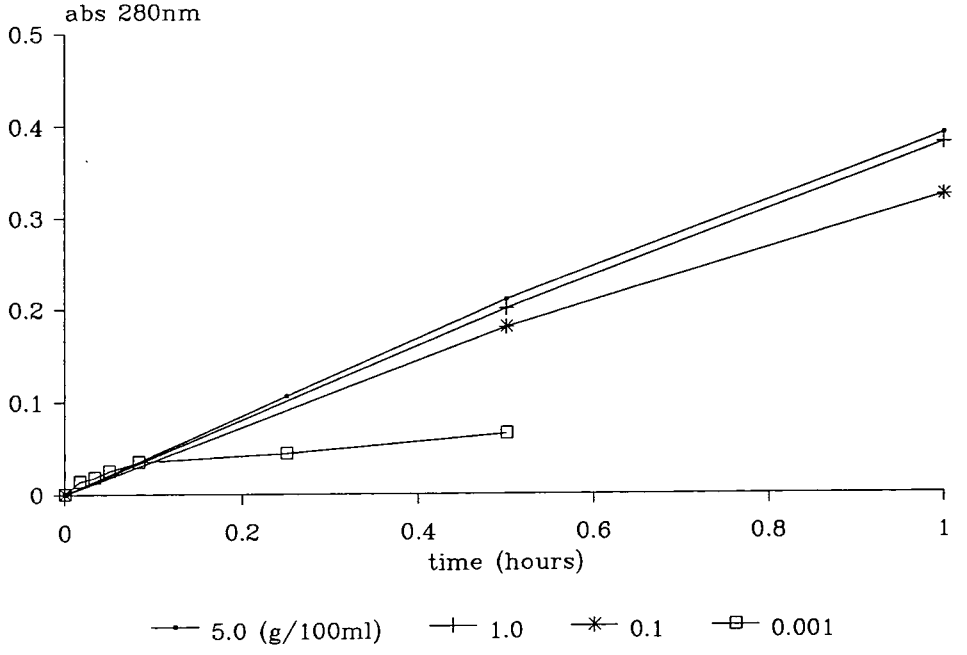


Figure 1.29

The effect of pH on casein hydrolysis by gut contents preparation. The amount of digestion was measured by the increase in absorbance at 280nm after precipitation of the undigested protein with 30% TCA.

Legend: ordinate - % activity
abscissa - pH

Figure 1.30

The effect of pH on the stability of caseinase from the midgut of larval *S. littoralis*. Aliquots (1ml) of midgut enzyme preparation were incubated with buffer (2ml) at different values of pH, over the range 1-13 for 30 minutes at 30°C. Subsequently, 3ml phosphate-NaOH pH 11.0 was added and the pH adjusted to pH 11.0 by the addition of HCl or NaOH as appropriate. The final volume was then made up to 10ml with DDW and the caseinase activity measured.

Legend: ordinate - % activity
abscissa - incubation pH

Figure 1.29

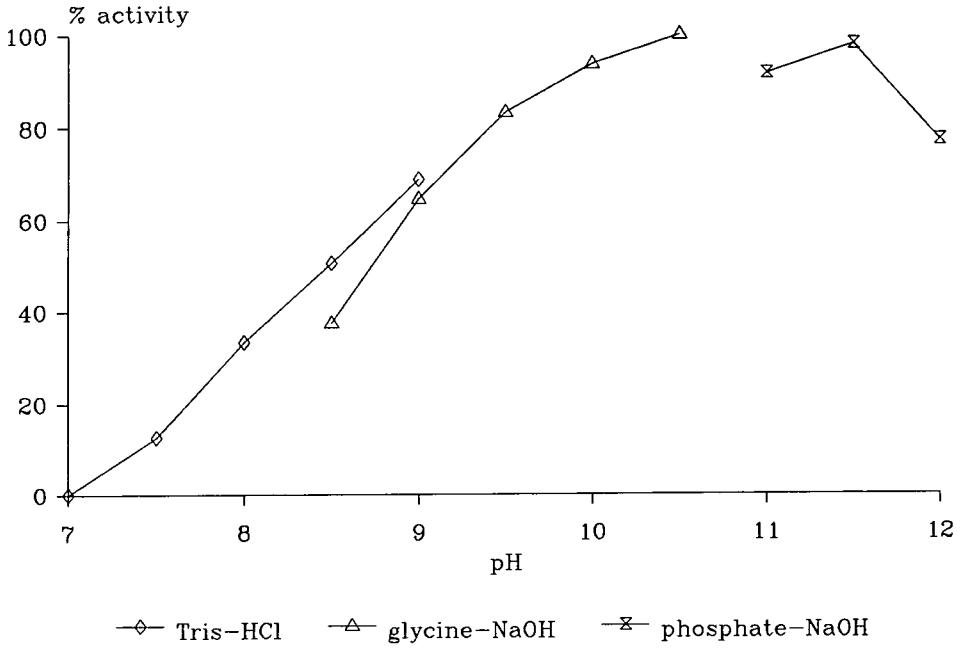
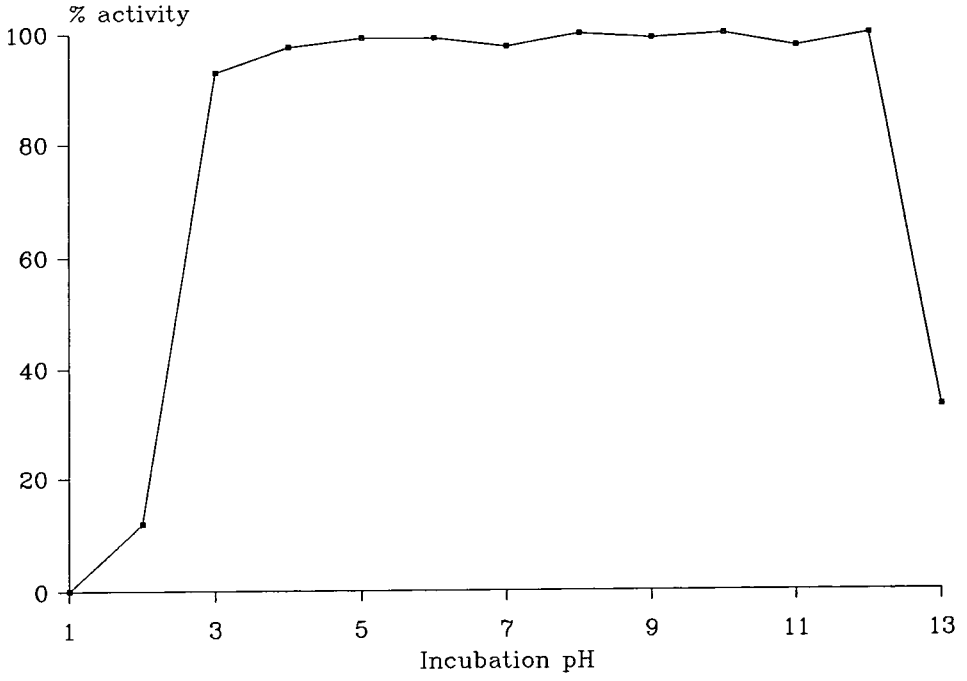


Figure 1.30



The effect of pH on stability

The enzyme preparation was incubated as described previously for trypsin stability, but 3 ml 0.05M phosphate-NaOH buffer pH 11.0 was added after 30 minutes at the incubation pH, and then the final pH was adjusted to pH 11.0 with NaOH or HCl as appropriate. The final volume was made up to 10 ml with DDW. 0.5ml enzyme-buffer mixture was then added to 0.5ml casein solution buffered to pH 11.0 with 0.05M phosphate-NaOH buffer and incubated at 30°C for 30 minutes. The assay was stopped by the addition of 0.5ml 30% trichloroacetic acid and the absorbance at 280nm read after centrifuging the precipitated protein. It can be seen that enzyme stability against casein was similar to that against BApNA (Fig. 1.30). A proportion of the activity was present even after incubation at pH 2.0, 3.0 and 13.0, but the enzyme is largely unaffected by the incubation pH between 4.0 and 12.0.

The effect of temperature on the activity of the enzyme

The effect of temperature on caseinase activity was determined under optimal conditions of pH (pH 11.0) using a 50mM phosphate-NaOH buffer over the temperature range 10-75°C and carried out in a similar manner to that described previously when using BApNA as substrate. All incubations were run for 30 minutes at the appropriate temperature. The results obtained are presented as an Arrhenius plot and are shown in Figure 1.31. Maximal activity was observed at approximately 50°C. It can be seen that the Arrhenius plot is non-linear and exhibited a break point at approximately 30°C. The mean activation energy between 10 and 30°C was calculated to be 88.6 kJ/mole, while between 30 and 40°C it was 26 kJ/mole. Activity decreased rapidly above 60°C.

Activity using elastin-orcein as substrate

Elastin-orcein was used as a substrate to determine the presence or absence of elastolytic activity in the midgut lumen of *Spodoptera littoralis*. To this end, homogenates were incubated with elastin-orcein substrate over a range of pH (6-11.5). The results obtained are shown in Figure 1.32. It can be seen that substrate hydrolysis occurred mainly between pH 8.5 and 11.5. However, the levels of activity were not high in the crude preparation used. In consequence, long incubation times were required in this study (14 hours). The rate of elastin hydrolysis was low, being approximately 20μg orcein/minute/mg protein and no further studies were undertaken with this substrate.

Enzyme activities of midgut homogenate

The relative activities of the midgut proteolytic enzymes are shown in Table 1.1. Enzyme activities are compared for data where the final substrate concentration was 0.1mM at the optimum pH (i.e. pH 10.0), except for TAME and BTEE where the pH optimum used was pH 8.0. A concentration of 0.1mM was used for all substrates in order to avoid the inhibition at higher levels by esterase substrates. Activity in the crude

Figure 1.31

The effect of temperature on the activity of the caseinase enzyme from the midgut of *S. littoralis* (Arrhenius plot). 2.2ml substrate-buffer (0.05M phosphate-NaOH and 5% sodium caseinate pH 11.0) was thermoequilibrated at the appropriate temperature in testtubes. The reaction was started by the addition of 0.2ml enzyme at timed intervals. After 30 minutes incubation, the reaction was stopped by the addition of 1.3ml TCA.

Legend: ordinate - ln Vel. (O.D./minute)
abscissa - 1/temperature x 1000 (K)

Figure 1.32

The effect of pH on the breakdown of elastin-orcein. The rate of reaction was slow and this necessitated a very long incubation time (12 hours) at 30°C.

Legend: ordinate - % activity
abscissa - pH

Figure 1.31

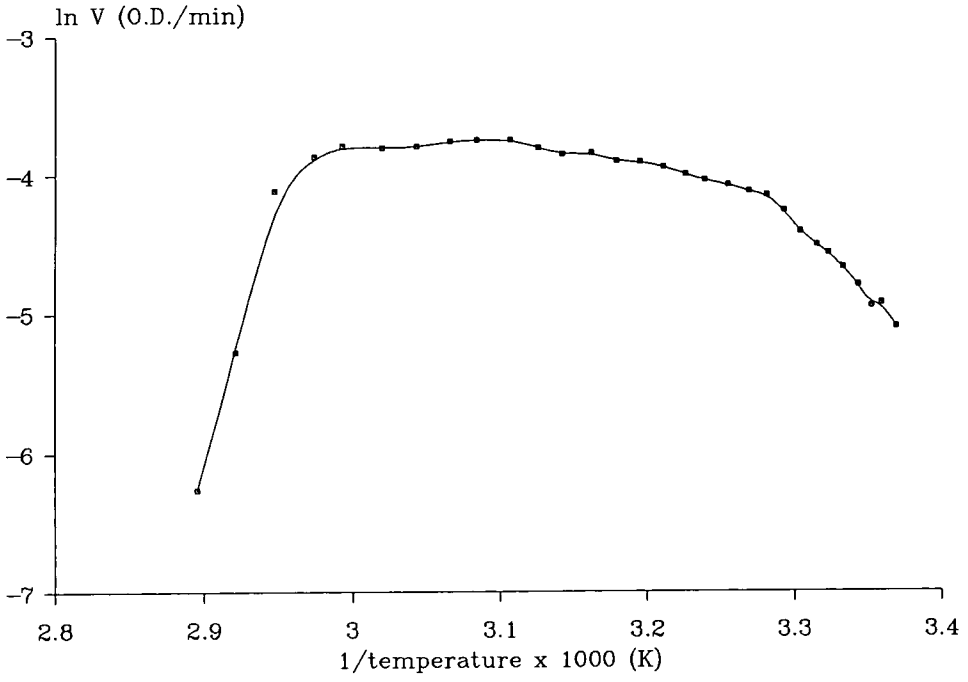
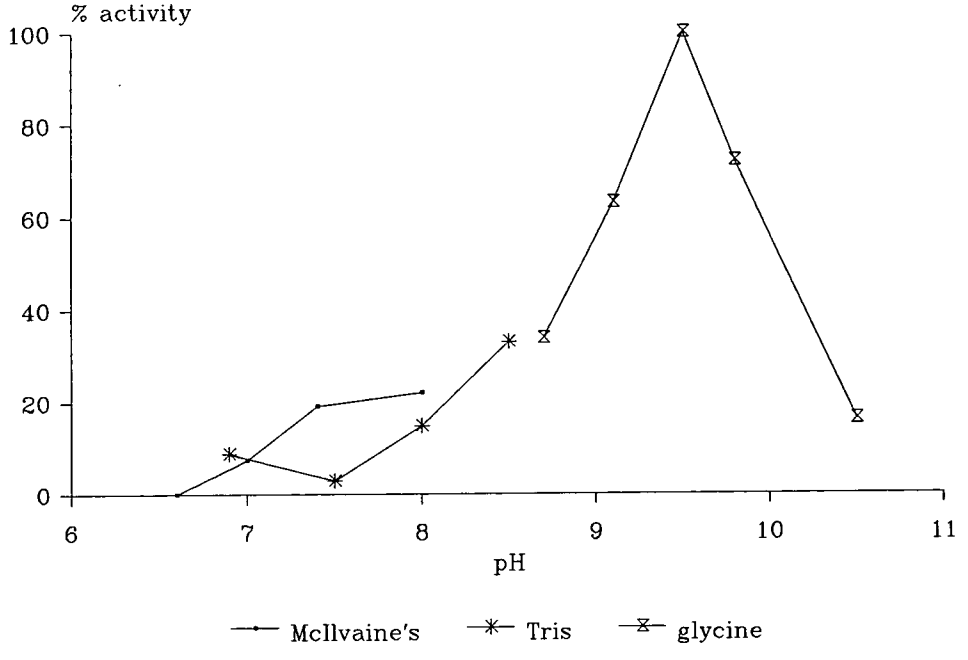


Figure 1.32



wholegut homogenates expressed as nmol product/min/midgut or nmol product/min/mg protein indicate that activity using esterase substrates is higher than the peptidase substrates and also that TGPLpNA was the most easily cleaved peptide substrate of those tested and SA_3pNA hydrolysed the slowest.

Table 1.1. Relative enzyme activities in wholegut homogenates of *S. littoralis*

Substrate	nmol/min /midgut	nmol/min/ mg protein	Rel.Act.
Trypsin-like			
BApNA	23.9 ± 1.6	15.6 ± 1.1	1.0
TGPLpNA	689.3 ± 55.2	448.5 ± 35.9	28.8
Chymotrypsin-like			
SA_2PLpNA	31.2 ± 1.5	20.3 ± 1.0	1.3
SA_2PPpNA	24.6 ± 2.4	16.0 ± 1.6	1.03
SA_3pNA	1.7 ± 0.1	1.1 ± 0.1	0.07
Esterase			
BAEE	1543 ± 82	1004 ± 53	64
TAME	939 ± 66	611 ± 32	39
BTEE	259 ± 23	169 ± 15	11

Activity of the proteases was determined at a substrate concentration of 0.1mM. In addition to the above list of substrates, other substrates were used, but the presence of activity could not be established using BTpNA, Suphepa, AApNA, ALpNA, SA_2VpNA, ZGPCpNA and ZGGLpNA. In all cases data represent mean (x) ± SEM for eight independent determinations (N = 8).

Table 1.2. Kinetic constants for midgut protease activities

Substrate	K_m	V_{max}	n*
Trypsin-like			
BApNA	0.257	306.5	0.74
TGPLpNA	0.056	3475	0.89
Chymotrypsin-like			
SA_2PLpNA	0.49	503	1.3
SA_2PPpNA	0.26	281	N.D.
SA_3pNA	3.35	189	1.17
Esterase			
BAEE	0.04	6920	0.93
TAME	0.06	4805	1.05

* this value refers to the amount of cooperativity or to the degree of substrate inhibition.

Partial purification of enzymes

Midgut contents homogenates were prepared and applied to chromatography columns as described in the methods.

Gel filtration

Column fractions from a Sephadex G-75 column were collected and assayed for trypsin activity, which generally resolved into two peaks (Fig. 1.33). The first emerged in the void volume indicating an M_r greater than about 80,000 and the second indicating an M_r of 24kDa. It has been reported previously that when void fractions were reapplied to gel filtration columns, the activity separated into void and eluted fractions again (Hamed and Attias, 1987). However, this was not the case with the present preparation. In an attempt to break-up the larger proteins, V_o protein was homogenized in 10% Triton X-114 and then the protein precipitated by saturation with ammonium sulphate and then centrifuged. This pellet was resuspended in a small volume of DDW and reapplied to the column. Trypsin activity was now present in the 24,000 M_r eluted fractions only and no detectable activity was observed in the void volume. On this basis it is assumed that the active V_o component is a consequence of polymerisation or binding to other components of the homogenate (such as dietary tannins) without total loss of activity.

BApNA and TAME were hydrolysed by those fractions eluting from the column which indicated a molecular weight of about 24 $\pm$ 0.36 kDa (N = 5). SA_2PLpNA, SA_3pNA, casein and BTEE were hydrolysed by proteins with a molecular weight of 19 $\pm$ 0.57 kDa (N = 3), provided the sample of midgut contents homogenate was applied from freshly dissected insects. Sample preparations which had been stored at -20°C eluted at a position corresponding to a molecular weight of 38,000 which suggests that the chymotrypsin dimerised under the storage conditions.

Active fractions eluted from the gel filtration column were subjected to SDS-PAGE in the presence of mercaptoethanol. The molecular weights of the main protein bands determined by this method were 22,750 and 23,500 for the chymotrypsin and trypsin enzymes respectively (Fig. 1.34). SDS-PAGE of unboiled samples from G-75 columns showing trypsin or chymotrypsin activity was also carried out in the absence of mercaptoethanol. Nitrocellulose blots were made and the distribution pattern of hydrolysed BApNA and SA_2PLpNA examined. The blots indicated that digestion of the two substrates was differently distributed and whilst the banding pattern was not comparable with that observed following sample boiling and in the presence of ß-mercaptoethanol, it was nevertheless possible to conclude that the chymotryptic and tryptic activities were distinct. Figures 1.35 and 1.36 show the different patterns obtained from hydrolysis of BApNA and SA_2PLpNA. Hydrolysis of the former is characterised by the presence of smearing and includes the formation of product in the stacker gel. Hydrolysis of SA_2PLpNA contrasts with BApNA hydrolysis by the formation of relatively tight product bands, indicating that the formation of complexes which are stable in the presence of SDS is more frequent with trypsin from *S. littoralis*

Figure 1.33

Resolution of trypsin and chymotrypsin midgut enzyme activities into two peaks (V_e and V_o). A comparison between trypsin and chymotrypsin shows that the ratio between V_o: V_e for trypsin is higher than chymotrypsin, where relatively little elutes in V_o. This indicates the trypsin enzyme aggregated more easily. The graph also shows the protein distribution after filtration and the digestion of casein.

Figure 1.33

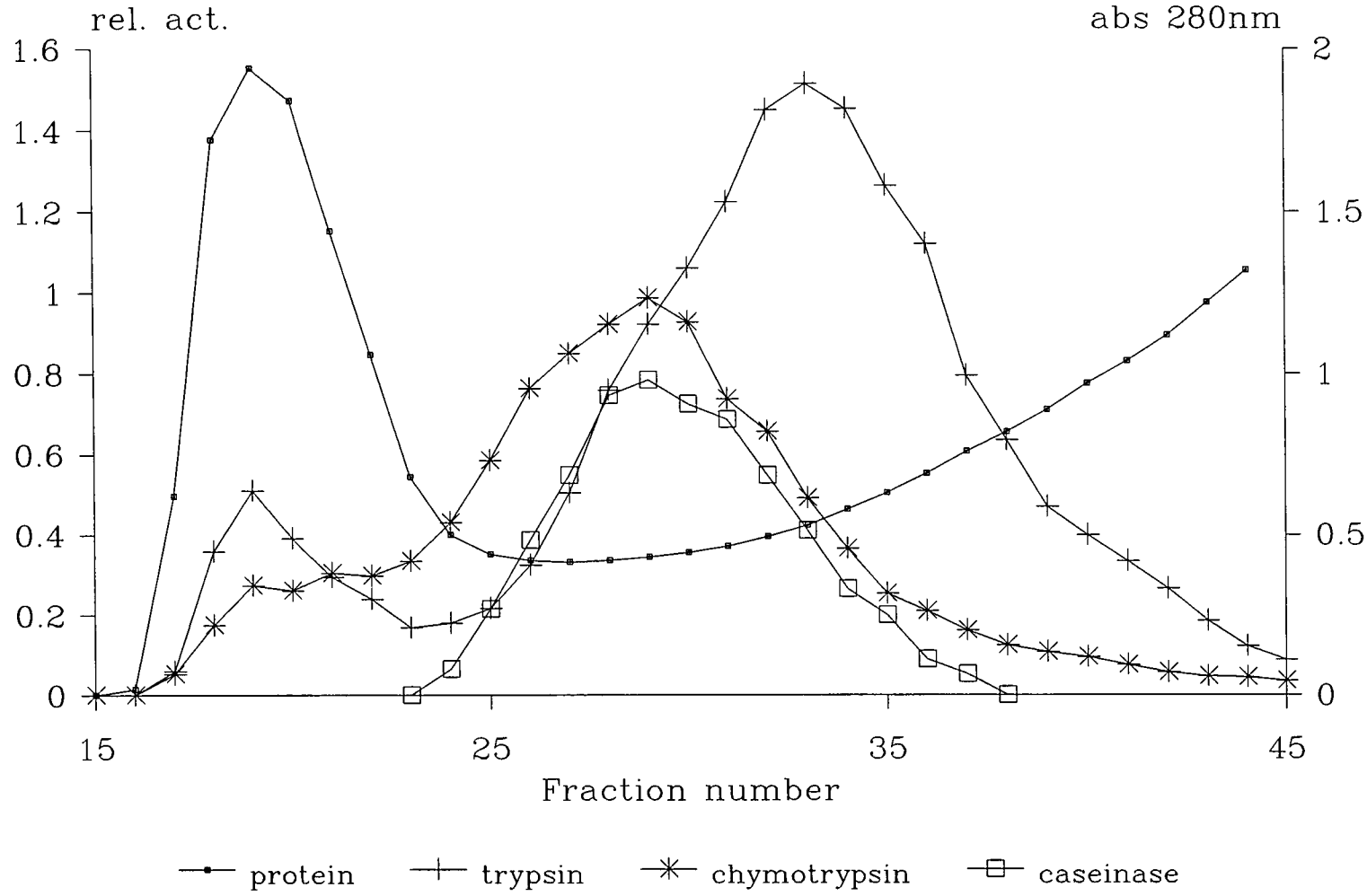
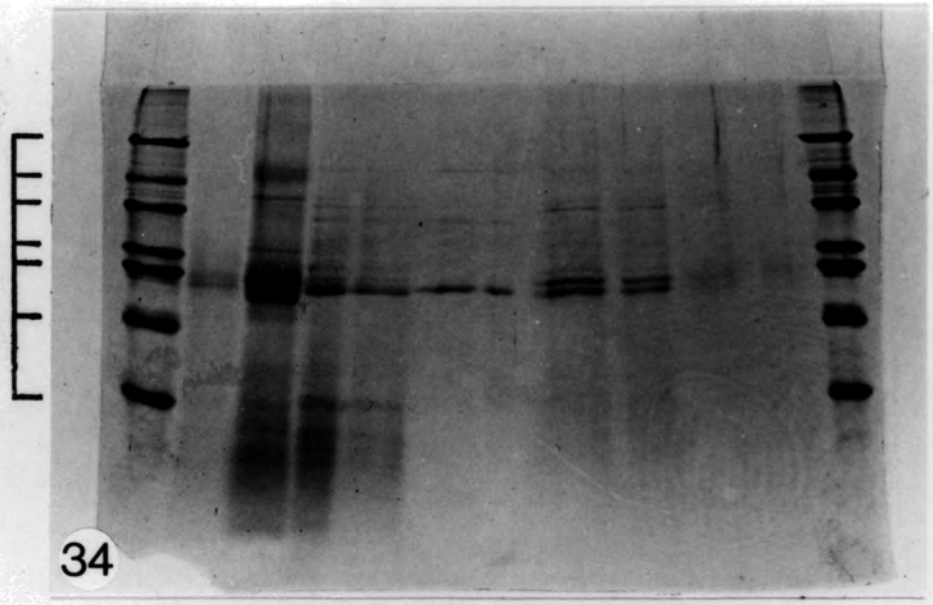

rel. act.
abs 280nm
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
2
1.5
1
0.5
0
15
25
35
45
Fraction number
protein
trypsin
chymotrypsin
caseinase

Figure 1.34

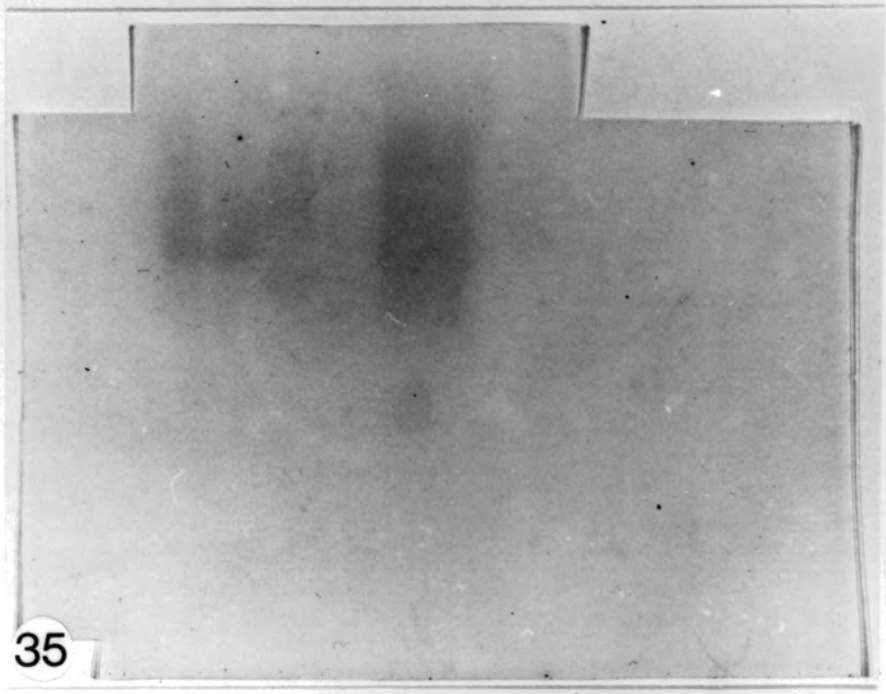
SDS-PAGE analysis of fractions from Sephadex G-75 gel filtration columns. Lane 2, crude midgut contents; lane 3, G-75, V_o (fraction 18); lane 4, fraction 20; lane 5, fraction 22; lane 6, fraction 25; lane 7, fraction 27; lane 8, fraction 30; lane 9, fraction 33; lane 10, fraction 40; lane 11, fraction 45.
Molecular weight markers (Lanes 1 and 12) were BSA (M_r 66 kDa), egg albumin (M_r 45 kDa), glyceraldehyde 3-phosphate dehydrogenase (M_r 36 kDa), carbonic anhydrase (M_r 29 kDa), trypsinogen (M_r 24 kDa), soybean trypsin inhibitor (M_r 20.1 kDa) and α-lactalbumin (M_r 14.2 kDa), as supplied by Sigma as SDS-70L.

Figure 1.35 and 1.36

The different patterns obtained after blotting the proteins from SDS-PAGE to nitrocellulose. Samples were not boiled and the gels were run in the absence of ß-mercaptoethanol, which would denature the proteases. However, in the absence of ß-mercaptoethanol, the proteases, especially trypsin (Figure 1.35), adheres to the polyacrylamide causing smearing.



34



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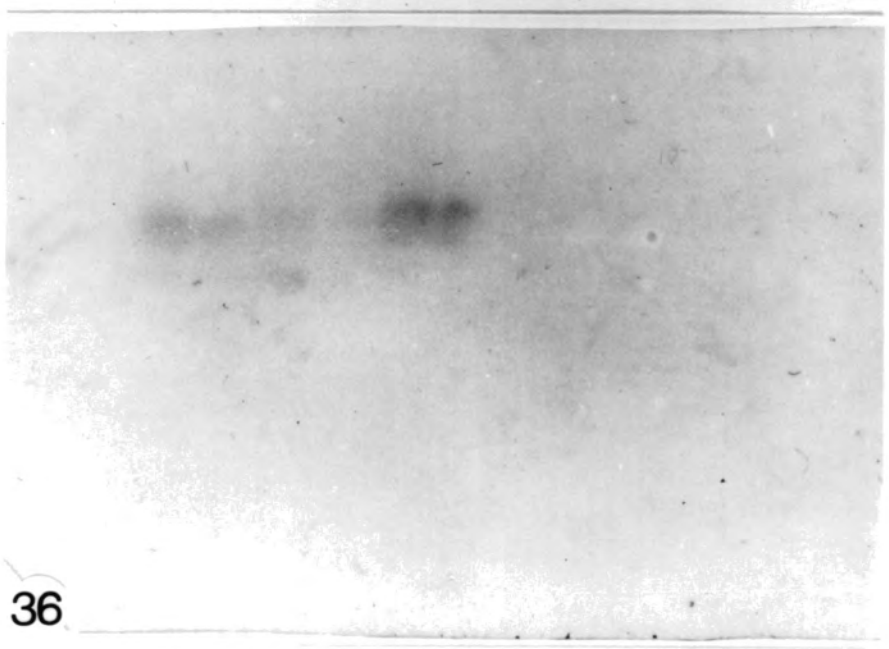


36

than chymotrypsin. Indeed, the formation of large protein complexes ($M_r > 80,000$) by trypsin rather than chymotrypsin was more apparent from gel filtration, where a larger proportion of activity eluted in the void volume.

Anion-exchange chromatography

Following gel filtration chromatography, anion-exchange chromatography (Q-Sepharose) was used in an attempt to further purify the endopeptidases. However, this procedure resulted in only a small increase in specific activity (x 1.6) and this was accompanied by a large drop in yield (x 0.4).

Table 1.3. Partial purification of trypsin from *S. littoralis*.

Step	Total activity	Specific activity*	Yield (%)
1. Crude	83.8	0.0569	100
2. $CHCl_3$ extract	58.6	0.0625	69.9
3. $(NH_4)_2SO_4$	53.6	0.0655	64.0
4. G-75	16.1	0.1648	19.2
5. Q-Sepharose	6.3	0.264	7.5

* measured as μM pNA/min/mg protein. Chymotryptic activity was not monitored during partial purification (steps 2 and 3 in Table 1.3), but the specific activity showed a similar increase between steps 1 and 4 (about 2.5x).

Effects of different inhibitors on enzyme activities.

In order to further characterise endopeptidase activities from *S. littoralis*, *H. armigera* and bovine trypsin, their responses to different concentrations of various protease inhibitors were investigated. Studies to determine I_{50} were carried out using partially purified endopeptidases produced from Sephadex G-75 gel filtration columns which were separated into aliquots, frozen and used as required. I_{50} values for the different inhibitors were determined using a BTP buffer system at neutral pH. This was necessary as several of the chemical inhibitors are unstable above pH 7.0 (e.g. half-lives are 35min at pH 8.0 for PMSF, and 5 minutes at pH 9.0 for TLCK and TPCK) whereas many animal, microbial and plant protease-inhibitor interactions are unstable at alkaline pH (Keesey, 1987; and Dawson *et al*, 1986). Furthermore, the use of a neutral assay pH allowed the effects of the different inhibitors to be compared. Figures 1.39 and 1.40 compare the effect of different inhibitors over a range of concentrations on the activity of trypsin while figures 1.47 and 1.48 show changes in chymotrypsin activity with different inhibitors.

Chemical Inhibitors

a. PMSF

Figure 1.37 shows the effect of PMSF on endopeptidases from *S. littoralis*, *H. armigera* and bovine trypsin. I_{50} for chymotrypsin from *S. littoralis* was 0.03mM for SA_2PLpNA hydrolysis and 0.039mM for casein hydrolysis. The apparent I_{50} obtained with casein is probably greater as a consequence of an increased incubation time (1 hour) leading to more inhibitor hydrolysis (half-life of 0.1mM PMSF at 25°C is 110min at pH 7.0 (James, 1978), but may be because the inhibitor could not be preincubated with the enzyme in buffer before the addition of substrate; substrate is dissolved in the buffer. An I_{50} value of 6-9mM for trypsin enzymes from both species of insect were obtained only after extrapolation of the logarithmic plot, because higher concentrations of PMSF crystallised out of solution. I_{50} for bovine trypsin was about 0.75mM under the conditions described.

b.TLCK

TLCK decomposes rapidly in aqueous solution at pH >7.5 (Shaw *et al*, 1965). The pattern of TLCK inhibition of all three trypsins (bovine trypsin and both insect trypsins) is very similar (Fig. 1.38). Chymotrypsin from *S. littoralis* was not inhibited by TLCK up to 0.25mM; TLCK inhibits endopeptidases which cleave specifically next to the lysine residue (Keesey, 1987). The I_{50} values determined for the trypsins from *S. littoralis* and *H. armigera* and for bovine trypsin were 0.021, 0.027 and 0.036mM respectively.

c. TPCK

TPCK is unstable in alkaline solution (Shaw, 1975), but 0.25mM TPCK did not inhibit the trypsins from either species of insect nor the chymotrypsin enzyme from *Spodoptera littoralis* at neutral pH. Although the chymotrypsin from *S. littoralis* cleaves the chymotryptic substrate, SA_2PPpNA, it was not inhibited by TPCK despite the fact that this inhibits other endopeptidases which cleave next to a phenylalanine residue (Keesey, 1987).

d. iodoacetic acid and iodoacetamide

The rate of reaction of these two compounds increases with increasing pH (Webb, 1966d). Therefore, inhibition of chymotrypsin and trypsin from *S. littoralis* by these compounds was investigated at pH 7.0 and 10.0. There was no inhibition at either pH of chymotryptic or tryptic activity from *S. littoralis*.

e. N-ethyl maleimide

The effectiveness of inhibition by this compound increases with increasing pH, if the inhibition is due to reaction with enzyme SH groups (Webb, 1966c). Therefore, assays were carried out at pH 7.0, 8.0, 9.0 and 10.0 using 5mM NEM. Enzyme activity

Figure 1.37

The effect of the serine protease inhibitor, PMSF, on the activity of bovine trypsin and the trypsin from *H. armigera* and on the activity of the endopeptidases, trypsin and chymotrypsin, from *S. littoralis*. The remaining activity was determined at pH 7.0 due to the susceptibility of the inhibitor to hydrolysis in alkaline conditions. All inhibitor studies involving chymotrypsin and trypsin activities were determined with SA_2PLpNA and BApNA respectively as substrates unless otherwise stated.

Legend: ordinate - % activity
abscissa - PMSF (mM)

Figure 1.38

The inhibition by TLCK of the activity of bovine trypsin and midgut trypsin enzymes from *S. littoralis* and *H. armigera*. TLCK did not inhibit the chymotrypsin enzyme from *S. littoralis*.

Legend: ordinate - % activity
abscissa - TLCK (mM)

Figure 1.37

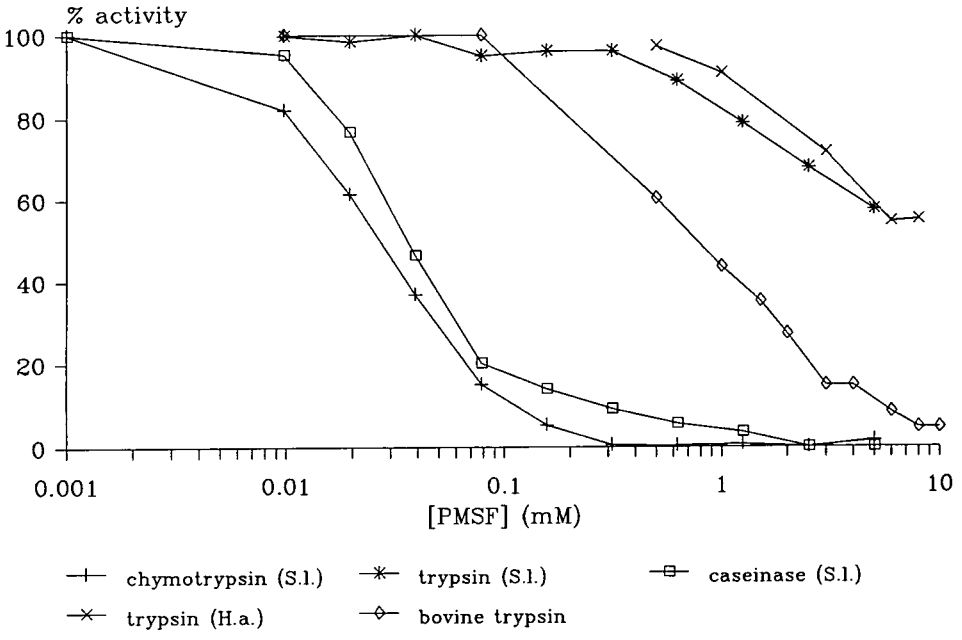
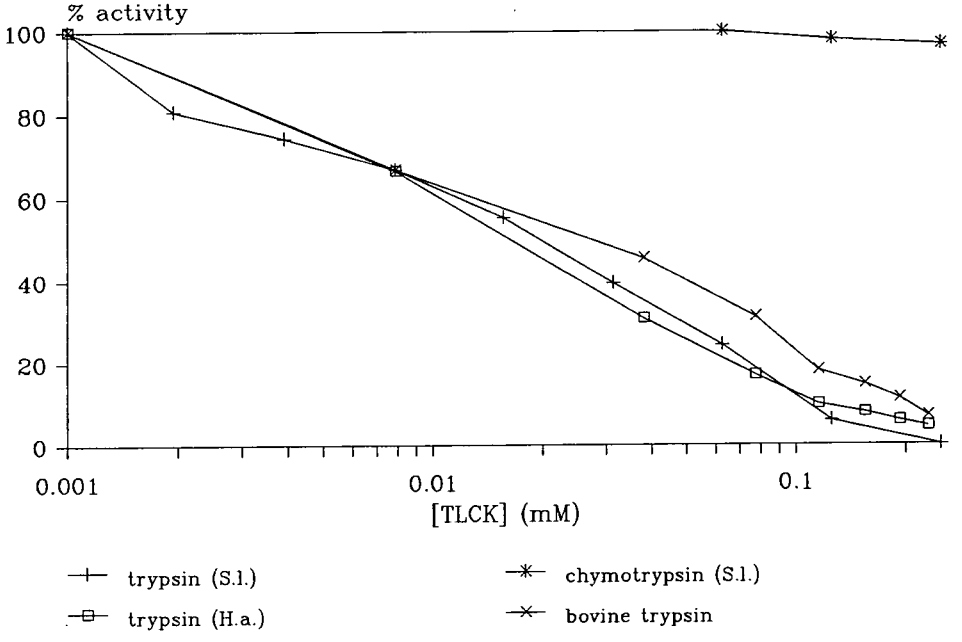


Figure 1.38



was not reduced at any pH.

f. p-chloromercuriphenyl-sulfonic acid

pCMPS was used as a more convenient alternative to pCMB due to its higher solubility. Figure 1.40 shows that 100% inhibition of trypsin was apparent at about 0.1mM pCMPS (I_{50} = 8μM). Following inactivation of trypsin using pCMPS, attempts were made to reactivate the enzyme using cysteine. It was found that 100% reactivation occurred in the presence of 10mM cysteine (pH adjusted prior to its addition in order to prevent a large pH change in the buffer) at pH 7.0 to 10.0. However, the reactivation time decreased with increasing pH from 55min at pH 7.0 to 24min at pH 10.0. In addition, the level of inhibition by a given concentration of inhibitor (0.373mM) was reduced with increasing pH from 100% at pH 7.0 and 8.0 to 77% at pH 10.0.

g. benzamidine

Benzamidine is an effective inhibitor of trypsin-like enzymes (Jeffcoate and White, 1974) and also inhibited trypsin from *S. littoralis* (Figs. 1.40 and 1.41). Benzamidine inhibition of insect endopeptidases was confined to trypsin activity (I_{50} = 11.9μM; K_{ib} = 11.6μM) and appeared not to affect chymotrypsin activity at 100μg/ml (about 0.64mM). Benzamidine has a similar affinity for bovine trypsin; K_{ib} = 18μM (Powers and Harper, 1986a); K_i = 16.7 μM (Mares-Guia, 1968).

h. 1,10-phenanthroline

The effect of 1,10-phenanthroline, a zinc chelator and metalloprotease inhibitor, on the activity of the two endopeptidases from *S. littoralis* was determined at concentrations of up to 100mM. Chymotrypsin activity was largely unaffected (< 15%) by 100mM inhibitor, but trypsin was only about 10% active at this concentration (Fig. 1.42). The I_{50} was 5.4mM for the latter enzyme at pH 7.0. The trypsin cannot, however, be described as a metalloprotease, because inhibition was noted with this inhibitor and EDTA only at high concentrations. There was no inhibition at the normal working concentrations (0.1mM) for inhibition of metalloproteases (Keesey, 1987).

i. DTT

DTT is a reducing agent and often stimulates activity of proteases following partial loss of activity with oxidizing agents e.g. clostripain (Mitchell and Harrington, 1971). Therefore, DTT stimulation of protease activity is an indicator that thiol protease activity is present. No inhibition or stimulation of trypsin activity was apparent up to 66mM DTT. The effect of DTT on chymotrypsin from *S. littoralis* was not determined.

Figure 1.39

The effect of the microbial inhibitors, leupeptin, E-64, chymostatin and antipain on trypsin activity from *S. littoralis*. The concentration of chymostatin is approximate, as it occurs naturally as a mixture of three peptides A (80%), B and C (leu, val and ile respectively). Substituent A is predominant, and the molarity has been calculated using an M_r = 606 from the structure given in Umezawa and Aoyagi (1983). The molarity of leupeptin is also approximate as this inhibitor is present as a mixture; one contains an N-terminal acetyl group, and the other a propionyl group.

Legend: ordinate - % activity
abscissa - [inhibitor] (μM)

Figure 1.40

The effect of a variety of inhibitors on the activity of trypsin from *S. littoralis* at pH 7.0.

Legend: ordinate - % activity
abscissa - [inhibitor] (μM)

Figure 1.39

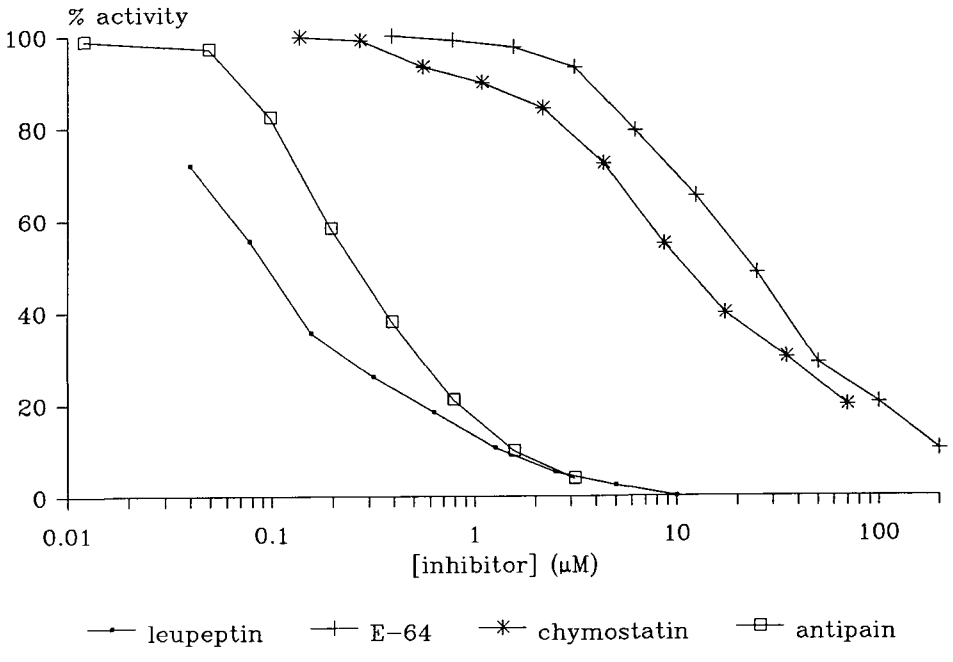


Figure 1.40

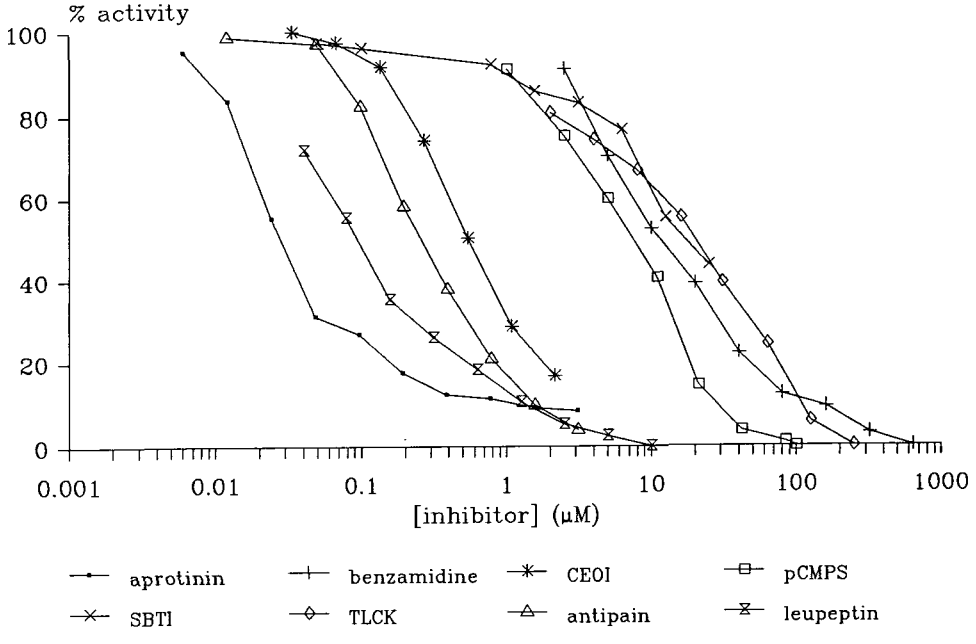


Figure 1.41

The effect of benzamidine on the activity of the trypsin enzyme at pH 10.0. Determination of K_{ib} using the Bratton-Marshall reaction to increase the sensitivity and plotted according to Dixon (1953).

Legend: ordinate - 1/relative velocity
abscissa - benzamidine (μM)

Figure 1.42

The effect of 1,10-phenanthroline on the activity of trypsin from *S. littoralis*.

Legend: ordinate - % activity
abscissa - 1,10-phenanthroline (mM)

Figure 1.41

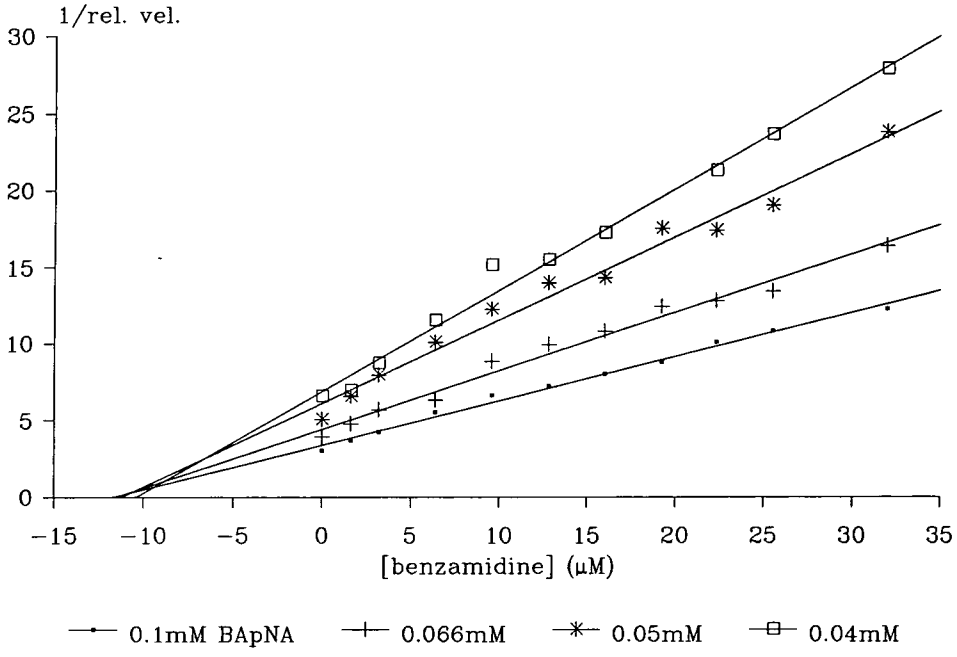
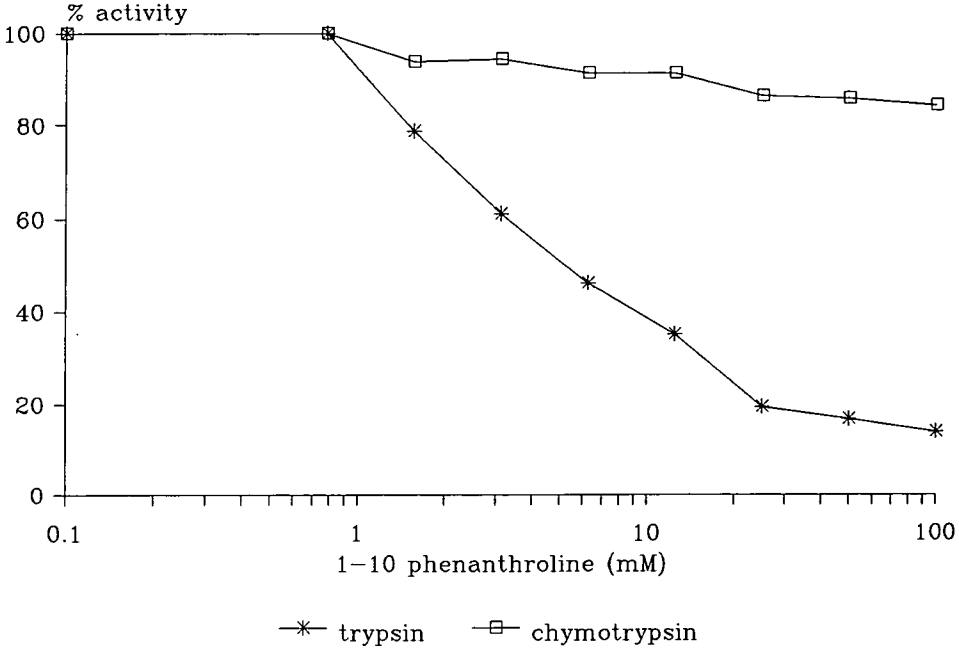


Figure 1.42



Microbial inhibitors

a. chymostatin

It can be seen from the summary of results in Table 1.4 that inhibition by chymostatin of both insect trypsins is very similar, but 50% inhibition occurs at lower chymostatin concentrations than for bovine trypsin; I_{50} for bovine trypsin is $>250\mu g/ml$ (Aoyagi and Umezawa, 1975). Chymotrypsin from *S. littoralis* was significantly more susceptible to inhibition than trypsin from the same insect (Fig. 1.43), by a factor of 610 for I_{50} and 4.85 for K_{ib}. For comparison, the I_{50} for bovine chymotrypsin was $1.5\mu g/ml$ under the assay conditions used (Johnston *et al*, 1991); however, other values have been reported: $0.15\mu g/ml$ (Dawson *et al*, 1986). Figures 1.44 and 1.45 show Dixon (1953) plots for the inhibition of chymotrypsin and trypsin from *S. littoralis* and confirm the higher susceptibility of chymotrypsin compared with trypsin.

Table 1.4. Effect of chymostatin on various peptidases

	I_{50}	K_{ib}
Trypsin (*H. armigera*)	$5.5\mu g/ml$	N.D.
Trypsin (*S. littoralis*)	$6.9\mu g/ml$	$14.6\ \mu g/ml$
Chymotrypsin (*S. littoralis*)	11.2ng/ml	$3.01\ \mu g/ml$
Chymotrypsin (bovine)	$1.5\mu g/ml$	N.D.
Trypsin (bovine)	$>100\mu g/ml^*$	N.D.

K_{ib} calculated using method of Dixon (1953) at pH 10.0. Results are presented as w/v rather than molar concentrations because chymostatin is a mixture of three components, with different amino acids: Leu, Val, and Ile. (Aoyagi and Umezawa, 1975). * by extrapolation of $\log_{10}$ plot (Fig. 1.43).

b. antipain

Antipain inhibits trypsin-like serine and some cysteine proteases (Suda *et al*, 1972; Umezawa, 1976). As before, preincubations were carried out for a period of ten minutes. This is necessary because a few minutes are required before steady-state non-competitive inhibition is established between bovine trypsin and antipain (Aoyagi and Umezawa, 1975). In contrast with endopeptidase inhibition by chymostatin, trypsin from *S. littoralis* was more strongly inhibited than chymotrypsin (Fig. 1.46) by a factor of 22.5 for I_{50}. Antipain does not inhibit either bovine elastase or bovine chymotrypsin (Aoyagi and Umezawa, 1975).

Figure 1.43

The effect of chymostatin on the activity of trypsin and chymotrypsin from *S. littoralis*, trypsin from *H. armigera* and bovine chymotrypsin and bovine trypsin.

Legend: ordinate - % activity
abscissa - ng/ml chymostatin

Figure 1.44

Dixon plot to determine K_i for the inhibition by chymostatin of chymotrypsin activity from *S. littoralis*.

Legend: ordinate - 1/rel. activity
abscissa - μg/ml chymostatin

Figure 1.43

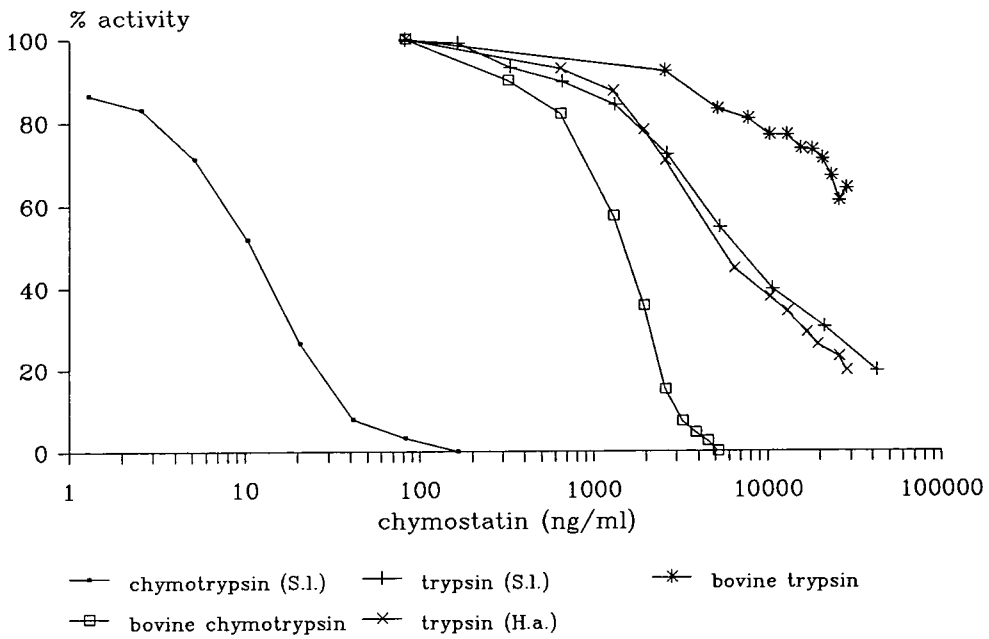


Figure 1.44

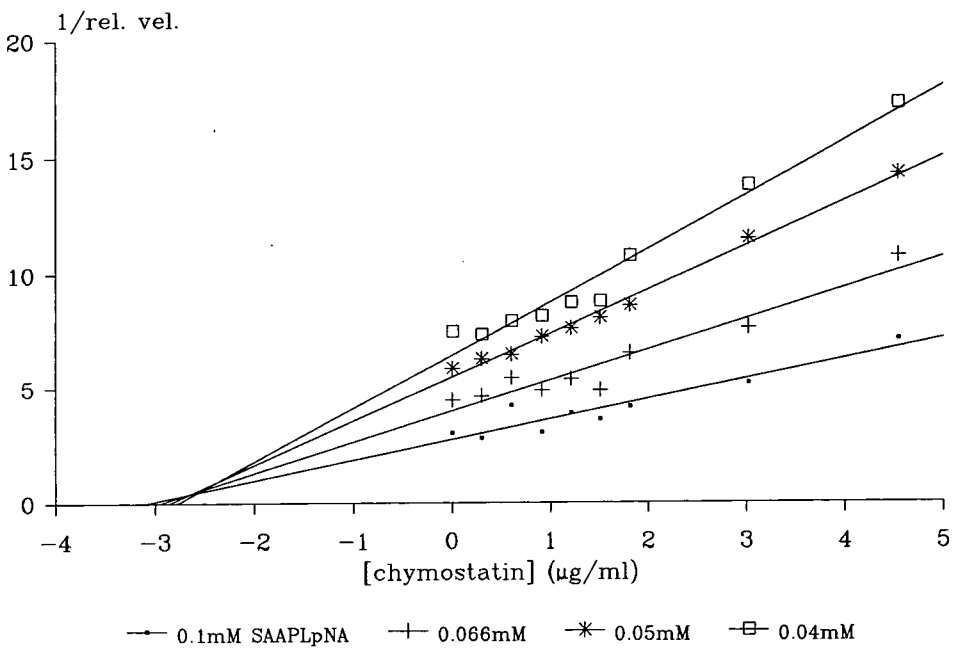


Figure 1.45

Dixon plot to determine K_i for the inhibition of trypsin from *S. littoralis* by chymostatin.

Legend: ordinate - 1/rel. activity
abscissa - μg/ml chymostatin

Figure 1.46
The inhibition of trypsin and chymotrypsin from *S. littoralis* by antipain.

Legend: ordinate - % activity
abscissa - μM antipain

Figure 1.45

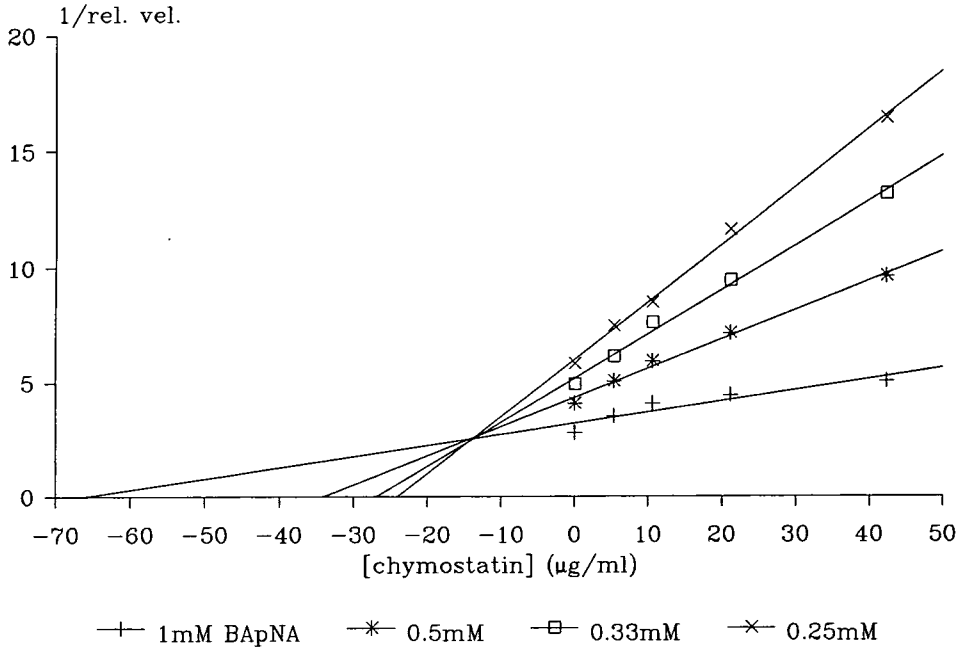


Figure 1.46

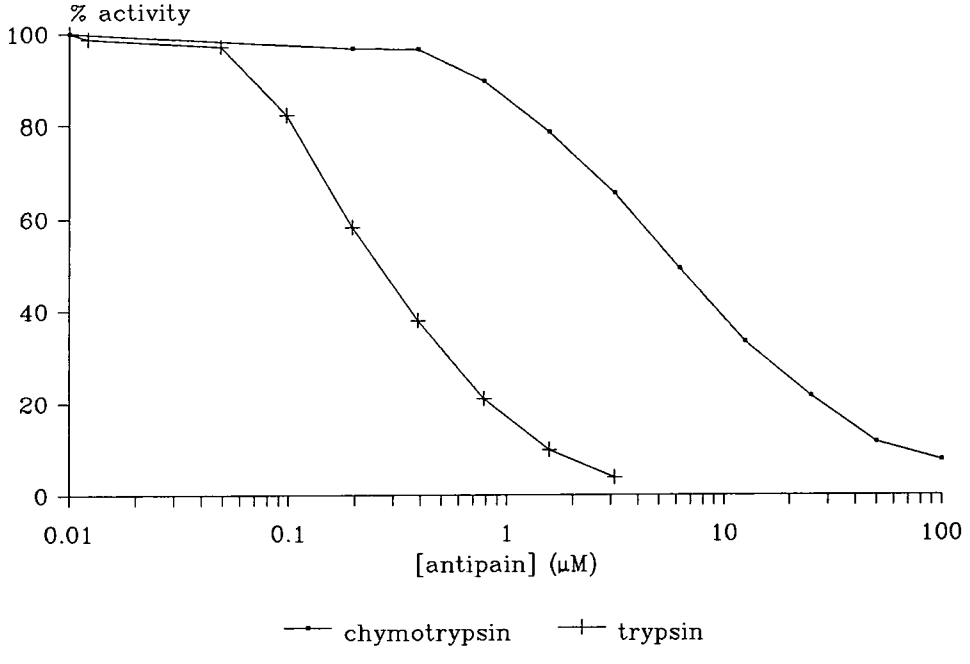


Table 1.5. Effect of antipain on various peptidases

	I_{50}
Trypsin (*S. littoralis*)	0.28μM
Chymotrypsin (*S. littoralis*)	6.20μM
Bovine trypsin*	0.43μM
Bovine chymotrypsin*	>400.00μM

K_{ib} were not determined. * Values quoted from Aoyagi and Umezawa (1975).

c. elastatinal

Elastatinal is a competitive inhibitor of elastase (Aoyagi and Umezawa, 1975). It inhibited neither trypsin nor chymotrypsin from *S. littoralis* sufficiently to determine an I_{50} value (see Fig. 1.48); this figure also shows that elastatinal is a far less potent inhibitor of chymotrypsin than chymostatin. The I_{50} for chymotrypsin and trypsin will be >100μM or 50μg/ml.

d. pepstatin A

Pepstatin A, an acid protease inhibitor (Rich *et al*, 1985), did not inhibit trypsin or chymotrypsin from *S. littoralis* at concentrations up to 100μM.

e. leupeptin

Leupeptin binds tightly to the active site of various serine and thiol proteases (Hanada *et al*, 1983). Leupeptin inhibits trypsin from *S. littoralis* (Fig. 1.39), but is not an effective inhibitor of chymotrypsin. The Lineweaver-Burk plot shown in Figure 1.49 demonstrates that inhibition of trypsin from *S. littoralis* by leupeptin is noncompetitive using BApNA as substrate; a Dixon plot to determine the K_i is shown in Figure 1.50.

Table 1.6. Effect of leupeptin on various peptidases

	I_{50}	K_{ia}	K_{ib}
Trypsin (*S. littoralis*)	48.5ng/ml	90.4ng/ml	35.4ng/ml
Chymotrypsin (*S. littoralis*)	>10μg/ml	N.D.	N.D.
Trypsin (bovine)*	2μg/ml	-	0.2μM
Chymotrypsin (bovine)*	>500μg/ml	-	-

K_{ia} calculated using method of Dixon (1972) at pH 7.0; K_{ib} calculated using method of Dixon (1953) at pH 10.0. Results are presented as w/v rather than molar concentrations because natural leupeptin (obtained from a microbial source) is not homogenous, but a mixture of propionyl and acetyl derivatives (Kondo *et al*, 1969). * Values quoted from Aoyagi and Umezawa (1975).

Figure 1.47

The effect of various plant protease inhibitors on the activity of chymotrypsin from *S. littoralis* at pH 7.0.

Legend: ordinate - % activity
abscissa - [inhibitor] (μM)

Figure 1.48

The effect of several inhibitors from a variety of sources on the activity of chymotrypsin from *S. littoralis* at pH 7.0.

Legend: ordinate - % activity
abscissa - [inhibitor] (μM)

Figure 1.47

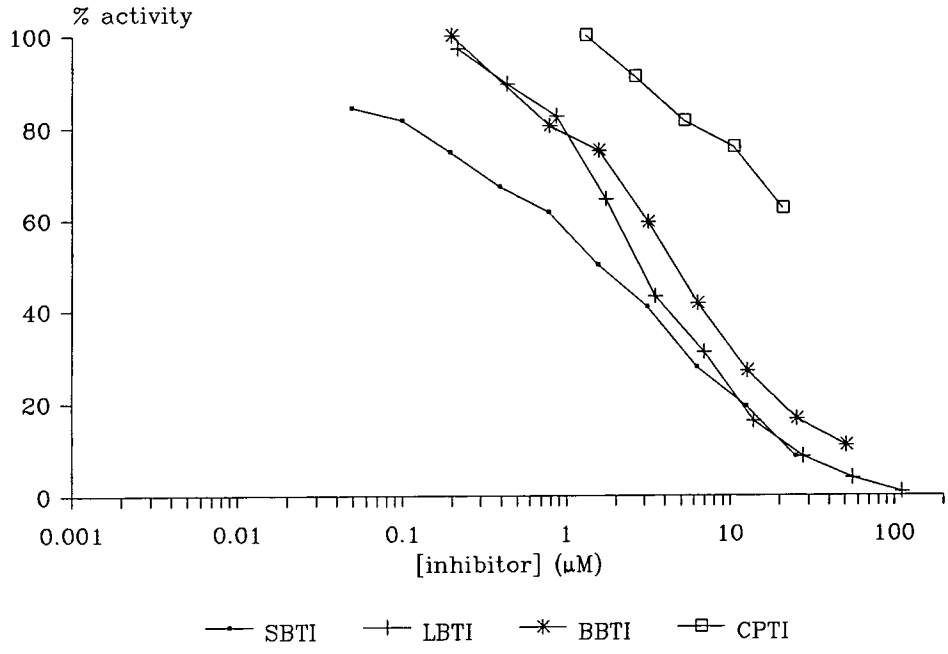


Figure 1.48

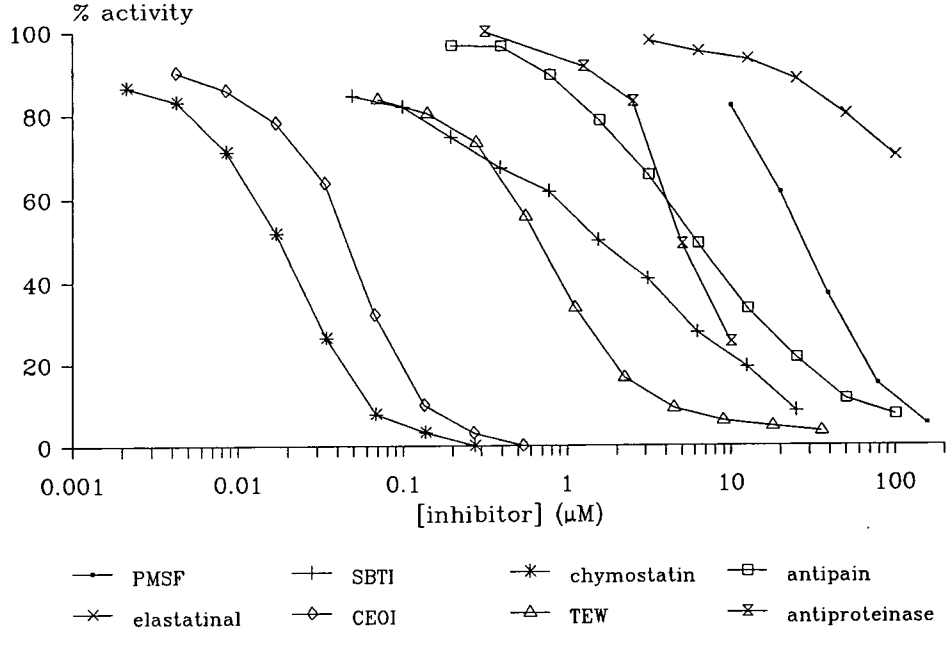


Figure 1.49

Lineweaver-Burk plot for the inhibition of trypsin from *S. littoralis* by leupeptin.

Legend: ordinate - 1/vel. (nmoles pNA/min./mg protein)
abscissa - 1/[BApNA] (mM)

Figure 1.50

Dixon plot for the inhibition of trypsin from *S. littoralis* by leupeptin.

Legend: ordinate - 1/rel. act.
abscissa - ng/ml leupeptin

Figure 1.49

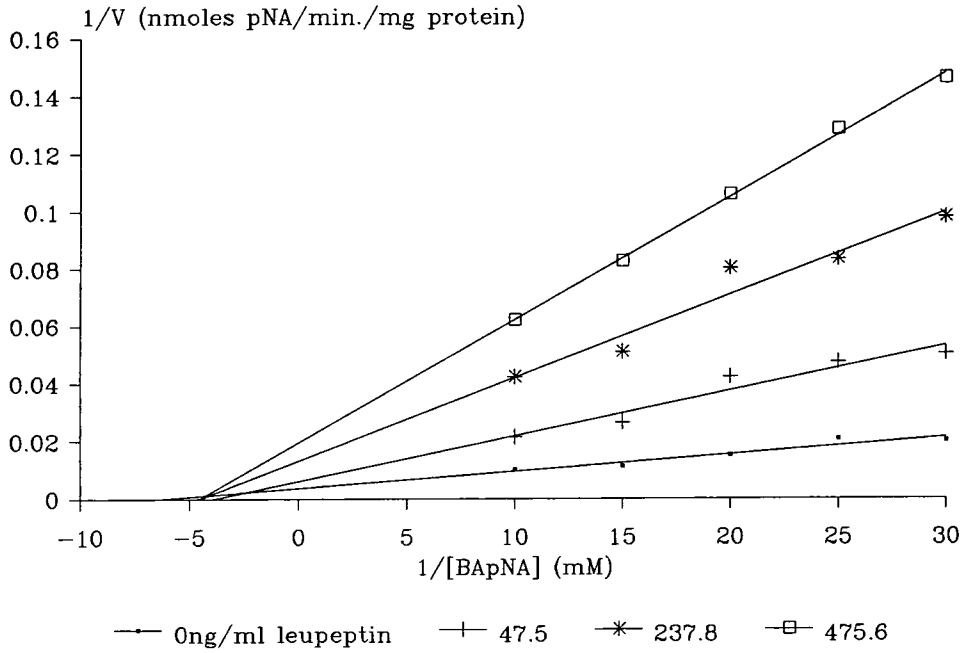
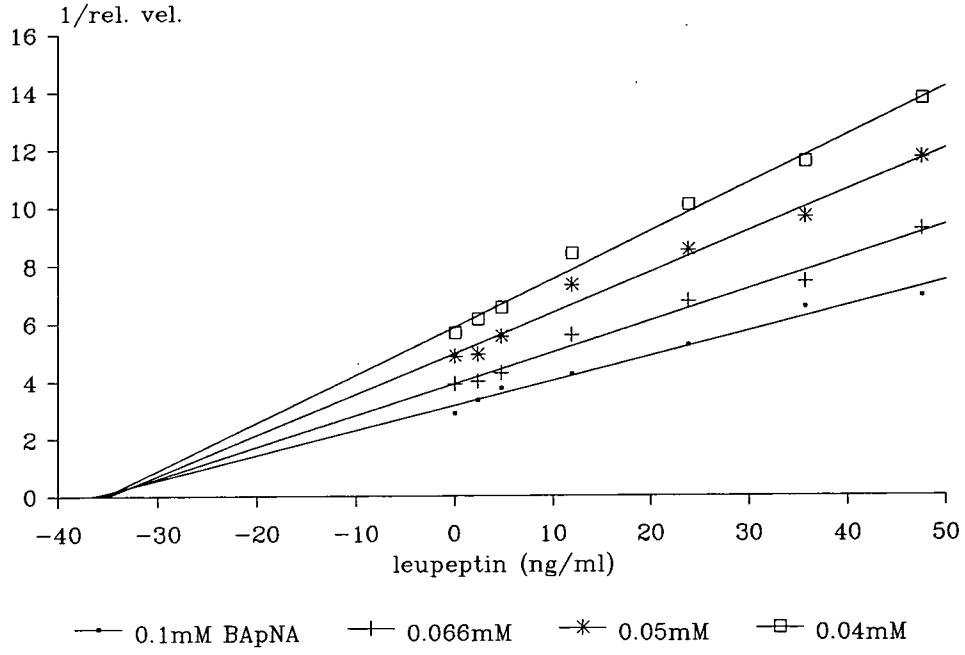


Figure 1.50



f. E-64

E-64 is a noncompetitive, irreversible inhibitor of thiol proteases originally isolated from culture medium of soil mold, *Aspergillus japonicus* TPR-4 (Hanada *et al*, 1978). Inhibition by E-64 is insensitive to pH between pH 5 and 9 and does not inhibit (I_{50} >250μg/ml = 700μM) serine proteases (Keesey, 1987). However, while E-64 did not inhibit chymotrypsin at 200μM at pH 7.0, it inhibited the trypsin enzyme from *S. littoralis* by almost 100% at this concentration (Fig. 1.39). However, the K_{ib} is over 6 times higher than for papain (Table 1.7 and Fig. 1.51). It is the least effective of the microbial inhibitors (Fig. 1.39) other than elastatinal, which did not affect trypsin activity.

Table 1.7. Effect of E-64 on various peptidases

	I_{50}	K_{ia}	K_{ib}
Trypsin (*S. littoralis*)	23.8μM	30μM	11.7μM
Chymotrypsin (*S. littoralis*)	>200.0μM	-	-
papain*	0.3μM	-	1.8μM

K_{ia} calculated using method of Dixon (1972) at pH 7.0; K_{ib} calculated using method of Dixon (1953) at pH 10.0. * quoted from data by Keesey (1987).

Plant protease inhibitors: a. Soybean trypsin inhibitor (SBTI)

The four endopeptidases from different sources were inhibited to varying extents by SBTI (Fig. 1.52 and Table 1.8 below). SBTI inhibited *H. armigera* trypsin and bovine trypsin noncompetitively (Johnston *et al*, 1991). The endopeptidases from *S. littoralis* also appear to be inhibited in a similar fashion, i.e. noncompetitively (Figs. 1.53 and 1.54). Both midgut endopeptidases had a higher K_{ib} than either the bovine trypsin or trypsin from *H. armigera*. Trypsin from *H. armigera* is more sensitive to SBTI inhibition than either trypsin and chymotrypsin from *S. littoralis* (ratio of K_{ib} of *H.a.* trypsin to proteases from *S.l.* 400 fold and 30 fold respectively) or bovine trypsin (ratio about 10 fold).

Table 1.8. Effect of SBTI on various peptidases

	I_{50}	K_{ia}	K_{ib}
Trypsin (*H. armigera*)	3.8nM	2.04nM	9.6nM
Trypsin (*S. littoralis*)	15.4μM	-	3.8μM
Chymotrypsin (*S. littoralis*)	1.6μM	4.00μM	286.0nM
Trypsin (bovine)	42.1nM	8.21nM	93.0nM

K_{ia} calculated using method of Dixon (1972) at pH 7.0; K_{ib} calculated using method of Dixon (1953) at pH 10.0. M_r of SBTI is 20,100 (Yamamoto and Ikenaka, 1967; Kim *et al*, 1985).

Figure 1.51

Dixon plot for the inhibition of trypsin from *S. littoralis* by E-64.

Legend: ordinate - 1/rel. act.
abscissa - μM E-64

Figure 1.52

The inhibition by SBTI of bovine trypsin and trypsin from *H. armigera*, together with trypsin and chymotrypsin from *S. littoralis*.

Legend: ordinate - % activity
abscissa - μM SBTI

Figure 1.51

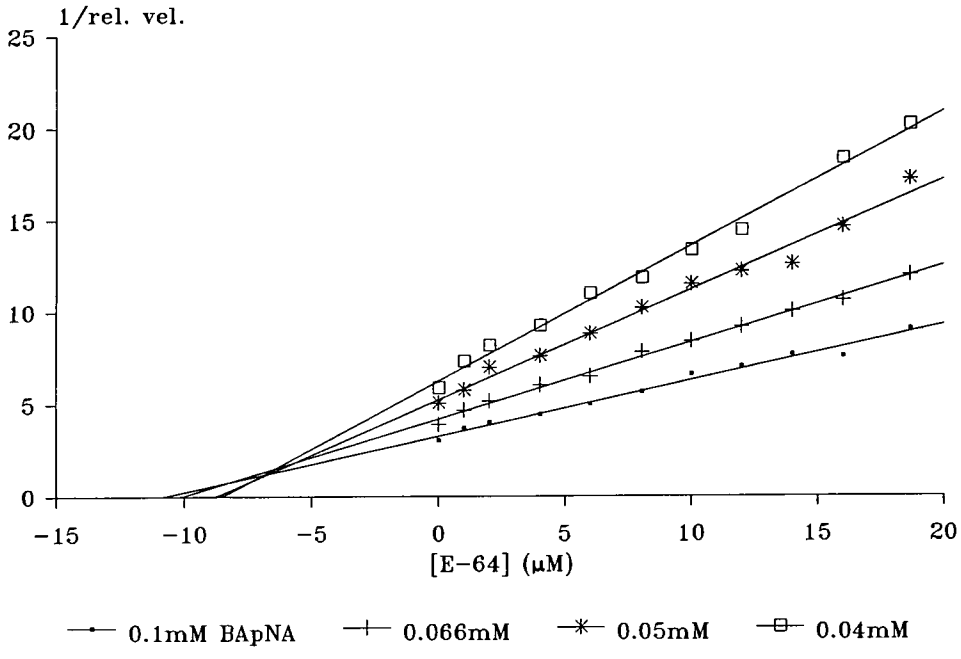


Figure 1.52

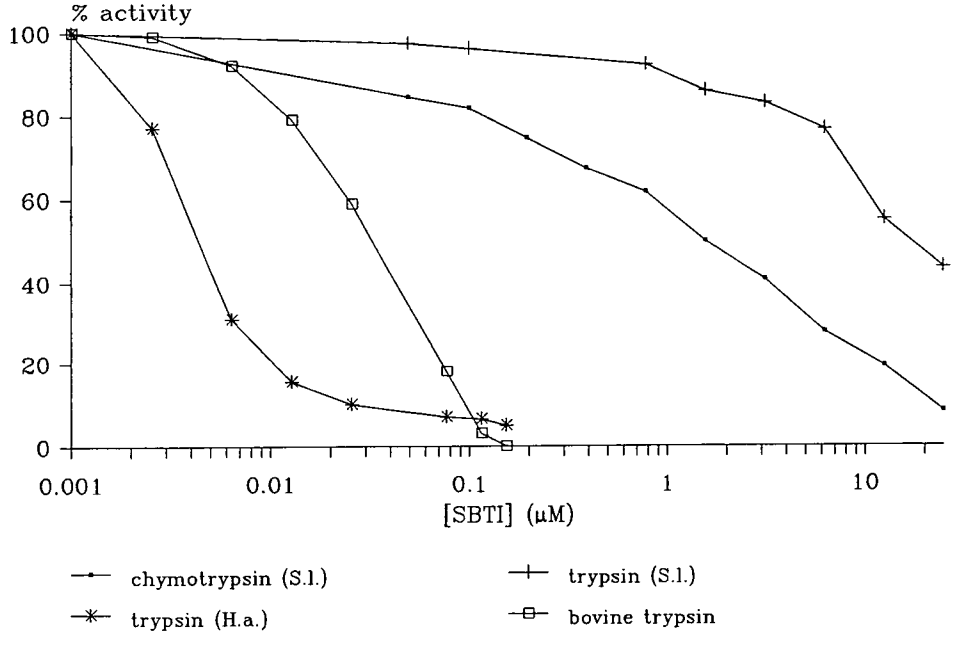


Figure 1.53

Dixon plot for the determination of K_i for SBTI inhibition of trypsin from *S. littoralis*.

Legend: ordinate - 1/rel. act.
abscissa - μM SBTI

Figure 1.54

Dixon plot for the determination of K_i for SBTI inhibition of chymotrypsin from *S. littoralis*.

Legend: ordinate - 1/rel. act.
abscissa - μM SBTI

Figure 1.53

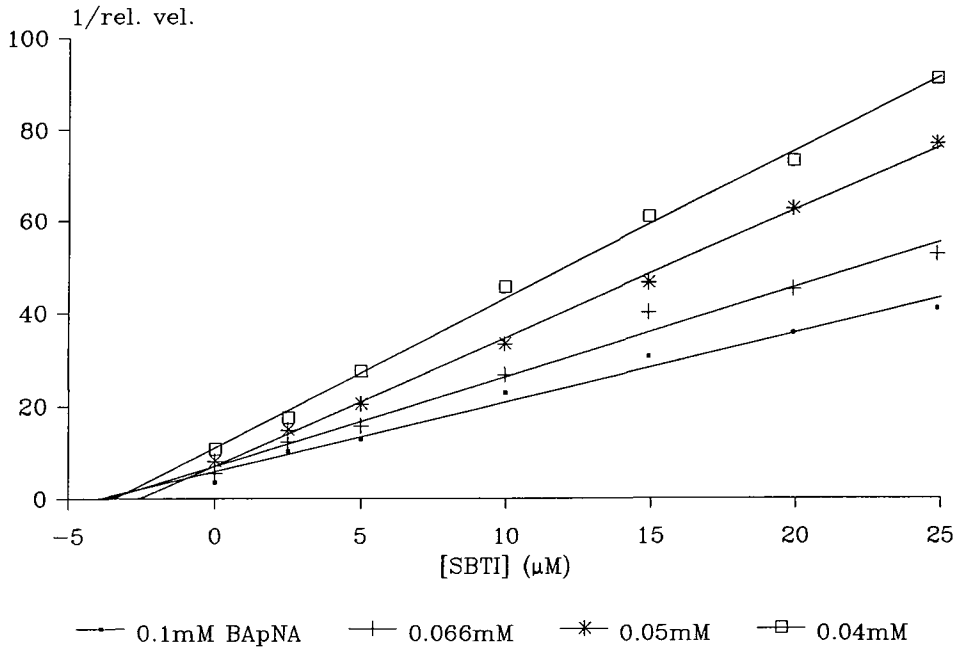
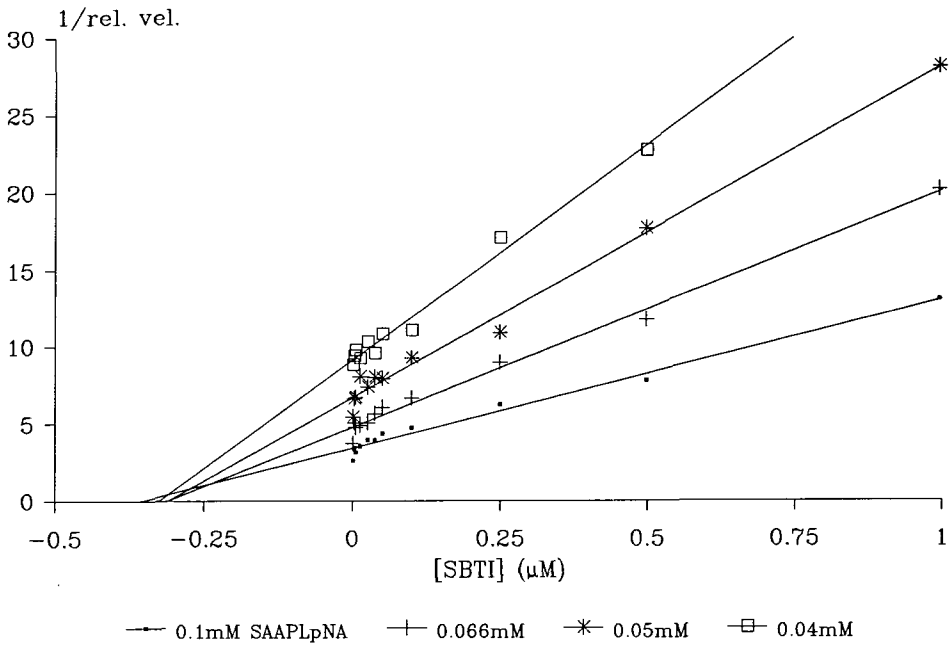


Figure 1.54



b. Bowman-Birk trypsin inhibitor (BBTI)

This is derived from soybean and is an inhibitor of vertebrate trypsin and chymotrypsin (Birk, 1985). Bovine trypsin was inhibited completely at a final concentration of 30nM, whereas the trypsin from *H. armigera* was over 40% active at this concentration (Johnston *et al*, 1991). Chymotrypsin from *S. littoralis* was inhibited at relatively high concentrations of BBTI, as shown in Figures 1.55 and 1.56. The I_{50} for BBTI inhibition of *S. littoralis* chymotrypsin was 20 or 100 times higher than for *H. armigera* and bovine trypsin respectively, but there was no measurable inhibition of trypsin from *S. littoralis* at 50μM BBTI.

Table 1.9. Effect of BBTI on various peptidases

	I_{50}	K_{ia}	K_{ib}
Trypsin (*H. armigera*)	0.25μM	0.64μM	0.25μM
Trypsin (*S. littoralis*)	>50.00μM	-	-
Chymotrypsin (*S. littoralis*)	4.95μM	5.80μM	1.86μM
Trypsin (bovine)	50.00nM	24.20nM	0.20μM

K_{ia} calculated using method of Dixon (1972) at pH 7.0; K_{ib} calculated using method of Dixon (1953) at pH 10.0. M_r of BBTI is about 7900 (Tan-Wilson *et al*, 1987; Hwang *et al*, 1977).

c. Cowpea trypsin inhibitor (CPTI)

CPTI is a competitive inhibitor of bovine trypsin (Johnston *et al*, 1991) of the Bowman-Birk family of serine protease inhibitors. CPTI was not an effective inhibitor of enzymes from *S. littoralis*; there was no inhibition of trypsin at concentrations up to 21μM and only about 40% inhibition of chymotrypsin at this concentration (Fig. 1.47). Extrapolation of the $\log_{10}$ plot would give an I_{50} of about 45-55μM for inhibition of chymotrypsin. CPTI was the least effective of the plant protein protease inhibitors tested (Fig. 1.47); being about 10-fold less potent than BBTI, SBTI and LBTI.

Table 1.10. Effect of CPTI on various peptidases

	I_{50}	K_{ib}
Trypsin (*H. armigera*)	-	88nM
Trypsin (*S. littoralis*)	>21μM	-
Chymotrypsin (*S. littoralis*)	50μM*	-
Trypsin (bovine)	-	176nM

K_{ib} calculated using method of Dixon (1953) at pH 10.0. M_r of CPTI is about 9000 (Gatehouse *et al*, 1980; Morhy and Ventura, 1987). * Estimated by extrapolation of $\log_{10}$ curve. Refer to text above.

Figure 1.55

The inhibition of various endopeptidases from by BBTI.

Legend: ordinate - % activity
abscissa - μM BBTI

Figure 1.56

Dixon plot for the determination of K_i for BBTI inhibition of chymotrypsin from *S. littoralis*.

Legend: ordinate - 1/rel. act.
abscissa - μM BBTI

Figure 1.55

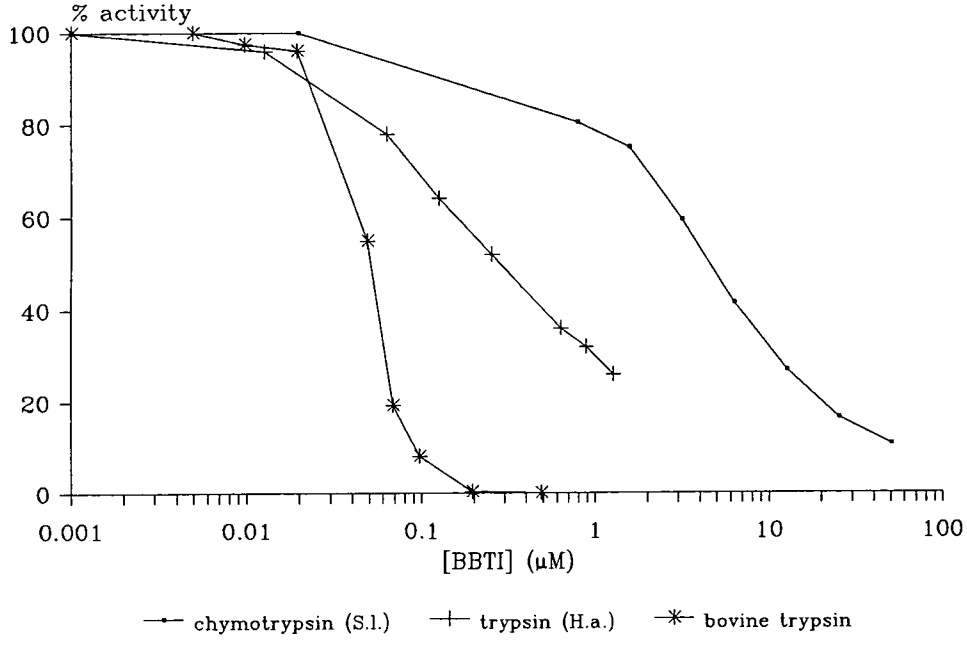
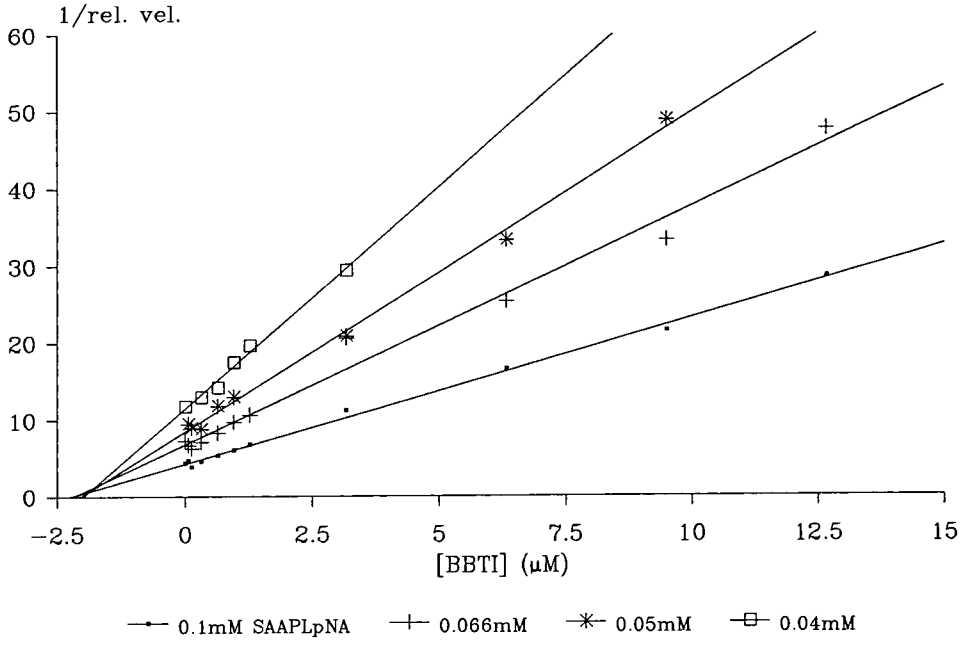


Figure 1.56



d. Trypsin inhibitor from lima bean, (LBTI)

Bovine trypsin was completely inhibited at an LBTI concentration of nearly 0.44μM, whereas nearly 20% of trypsin activity from larval *H. armigera* remained after incubation with 1.67 μM (Fig. 1.57). Inhibition of trypsin from *H. armigera* is competitive (Johnston *et al*, 1991). Trypsin from *S. littoralis* was not inhibited up to the highest concentration of inhibitor (110μM), but chymotrypsin was inhibited much more effectively (Figs. 1.57 and 1.58). However, this enzyme-inhibitor affinity was not as high as with bovine trypsin or trypsin from *H. armigera* (see Table 1.11).

Table 1.11. Effect of LBTI on various peptidases

	I_{50}	K_{ib}
Trypsin (*H. armigera*)	0.15μM	0.19μM
Trypsin (*S. littoralis*)	>110.00μM	-
Chymotrypsin (*S. littoralis*)	3.00μM	0.66μM
Trypsin (bovine)	60.00nM	0.19μM

K_{ib} calculated using method of Dixon (1953) at pH 10.0. M_r of LBTI is about 9000 (Jones *et al*, 1963; Tan and Stevens, 1971a,b; Haynes and Feeney, 1967)

Animal protease inhibitors: a. Aprotinin

Bovine lung aprotinin is a competitive inhibitor which forms a tight complex with serine proteases, blocking its active site. The protease inhibitor complex is unstable in aqueous solution above pH 10.0 (Keesey, 1987) and aprotinin is inactivated at pH > 12.8. Bovine trypsin was completely inhibited by aprotinin at a concentration of 120 nM. In contrast, about 20-30% of trypsin activity from *H. armigera* and *S. littoralis* remained at this concentration (Fig. 1.59). Chymotrypsin from *S. littoralis* was unaffected by this inhibitor at the highest concentration, 3.07μM. Aprotinin showed a high affinity for trypsin from *S. littoralis* comparable with bovine chymotrypsin, but several orders of magnitude lower than the affinity of aprotinin for bovine trypsin. Figure 1.60 shows a Dixon plot for the inhibition of *S. littoralis* trypsin by aprotinin.

Table 1.12. Effect of aprotinin on various peptidases

	I_{50}	K_{ib}
Trypsin (*H. armigera*)	20.9nM	N.D.
Trypsin (*S. littoralis*)	30.7nM	60nM
Chymotrypsin (*S. littoralis*)	>3.1μM	-
Trypsin (bovine)	43.0nM	60fM*
Chymotrypsin (bovine)	-	9nM*

K_{ib} calculated using method of Dixon (1953) at pH 10.0. M_r of aprotinin is 6511 (Laskowski and Kato, 1980). * data from Keesey, 1987.

Figure 1.57

The inhibition of bovine trypsin and trypsin from *H. armigera* together with chymotrypsin from *S. littoralis* by LBTI.

Legend: ordinate - % activity
abscissa - μM LBTI

Figure 1.58

Dixon plot for the determination of K_i for LBTI inhibition of chymotrypsin from *S. littoralis*.

Legend: ordinate - 1/rel. act.
abscissa - μM LBTI

Figure 1.57

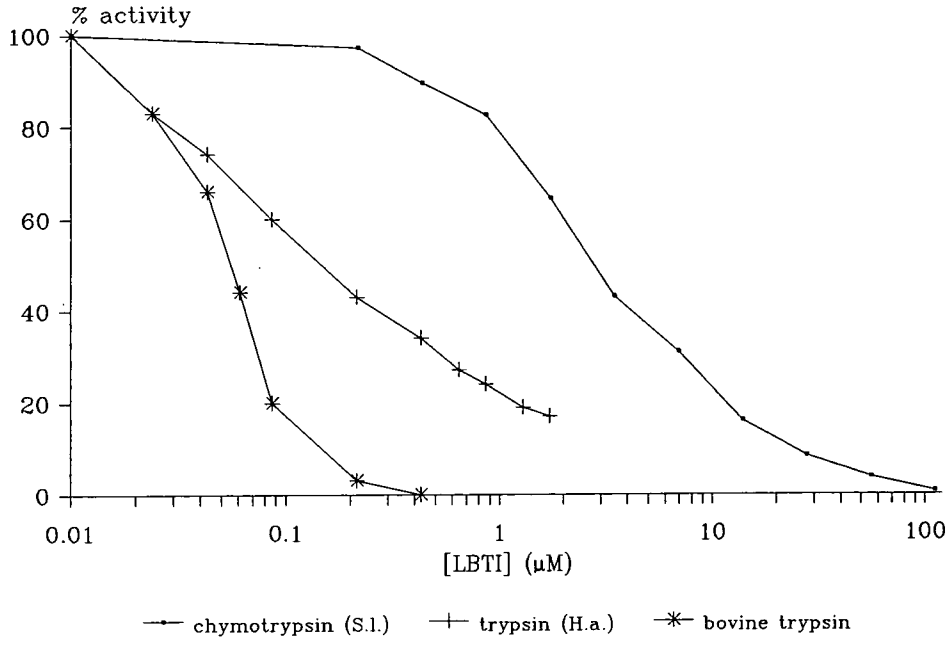


Figure 1.58

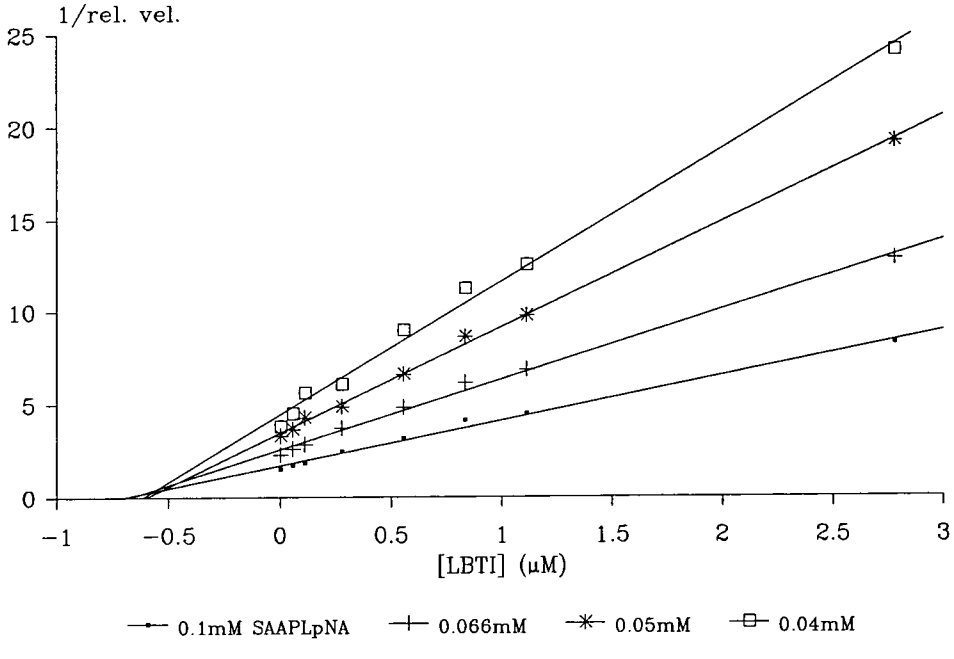


Figure 1.59

Inhibition of trypsin from *S. littoralis* and *H. armigera* and bovine trypsin by aprotinin.

Legend: ordinate - % activity
abscissa - μM aprotinin

Figure 1.60

Dixon plot for the determination of K_i for aprotinin inhibition of trypsin from *S. littoralis*.

Legend: ordinate - 1/rel. act.
abscissa - nM aprotinin

Figure 1.59

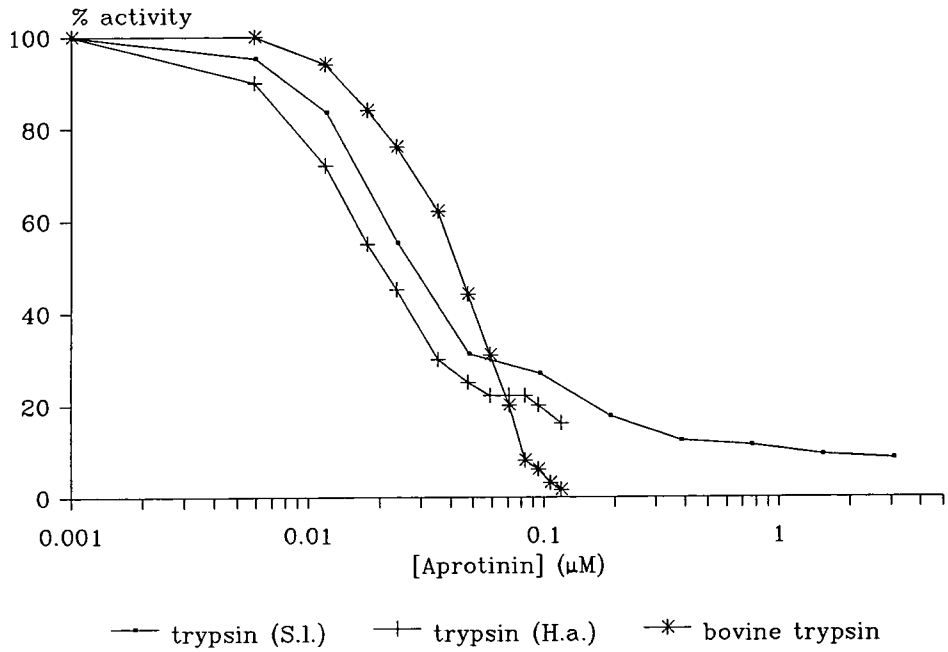
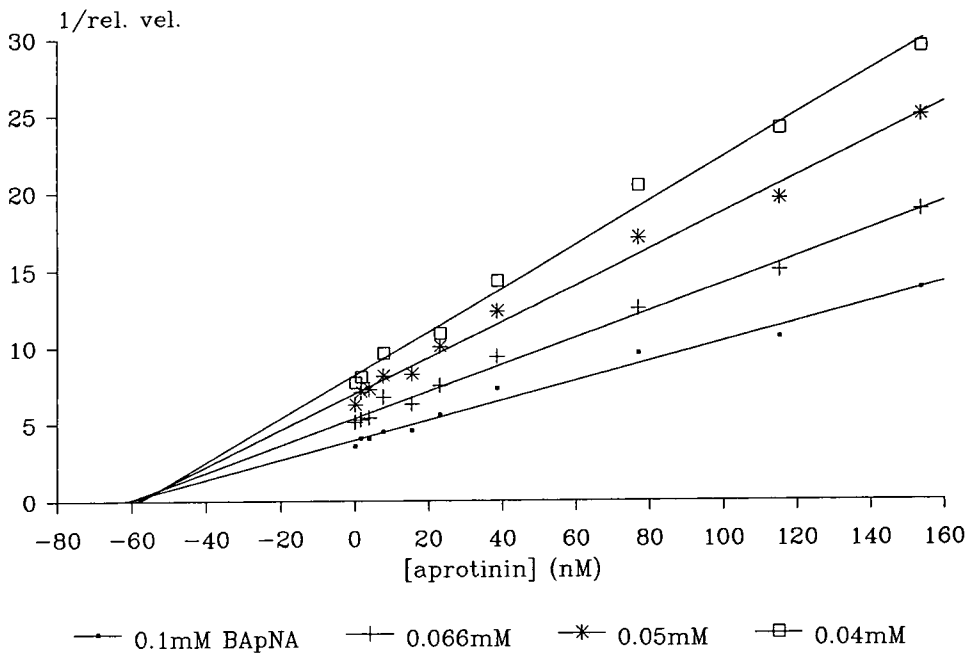


Figure 1.60



b. Chicken egg-white purified ovomucoid (CEOM)

CEOM is a Kazal type serine proteinase inhibitor, with a molecular weight of 28,000 (Kato *et al*, 1987). The effects of a range of ovomucoid concentrations (0-3.6μM) on insect and bovine trypsin activities was determined at pH 7.0 and 10.0. The endopeptidases from *H. armigera* and *S. littoralis* were unaffected over the concentration range of ovomucoid tested, whereas bovine trypsin was almost completely inhibited at nearly 28.6nM (Fig. 1.61). 50% inhibition of bovine trypsin was obtained by 4.93nM under the conditions described.

c. Chicken egg ovoinhibitor (CEOI)

CEOI inhibited both trypsin and chymotrypsin from *S. littoralis*. Chymotrypsin was more susceptible to inhibition by CEOI than the trypsin (Fig. 1.62). 50% inhibition of the caseinase occurred midway between I_{50} values for the two other enzymes. The greater affinity of CEOI for the chymotrypsin compared with trypsin is also shown in Figures 1.63 and 1.64.

Table 1.13. Effect of CEOI on various peptidases

	I_{50}	K_{ia}	K_{ib}
Trypsin (*S. littoralis*)	0.55μM	0.43μM	0.62μM
Chymotrypsin (*S. littoralis*)	50.00nM	13.10nM	4.30nM
caseinase (*S. littoralis*)	0.33μM	0.30μM	0.30μM

K_{ia} calculated using method of Dixon (1972) at pH 7.0; K_{ib} calculated using method of Dixon (1953) at pH 10.0; M_r of CEOI is 46,500 (Gertler and Ben-Valid, 1980).

d. Turkey egg white

Turkey egg ovomucoid is a Kazal-type serine protease inhibitor with a molecular weight of 28,000 (Gertler and Feinstein, 1971). Turkey egg white was used as a further alternative to chicken egg white derivatives. Ovomucoids demonstrate hypervariability of their amino acid residues which are in contact with proteases (Laskowski *et al*, 1987). Therefore, the absence of inhibition by ovomucoid from one avian species does not rule out the possibility of inhibition by ovomucoid from another. The egg white from turkey did not inhibit trypsin from *S. littoralis*, but did inhibit chymotrypsin: I_{50} = 0.68μM; K_{ia} = 0.43μM; K_{ib} = 68.9nM (see Figs. 1.65 and 1.66). It can also be seen that casein activity was reduced in the presence of this inhibitor, whereas trypsin activity was not, indicating that casein digestion is caused primarily by chymotrypsin.

e.α_1-proteinase inhibitor

α_1-proteinase inhibitor (α_1-antitrypsin) is a serpin with a molecular weight of about 52,000 (Carrell *et al*, 1982; Travis and Salvesen, 1983) which is active against mammalian thrombin, plasmin and trypsin (Rimon *et al*, 1966). However, it did not

Figure 1.61

The effect of CEOM on the activity of bovine trypsin

Legend: ordinate - % activity
abscissa - nM CEOM

Figure 1.62

The effect of CEOI on the activity of trypsin and chymotrypsin from *S. littoralis*.

Legend: ordinate - % activity
abscissa - nM CEOI

Figure 1.61

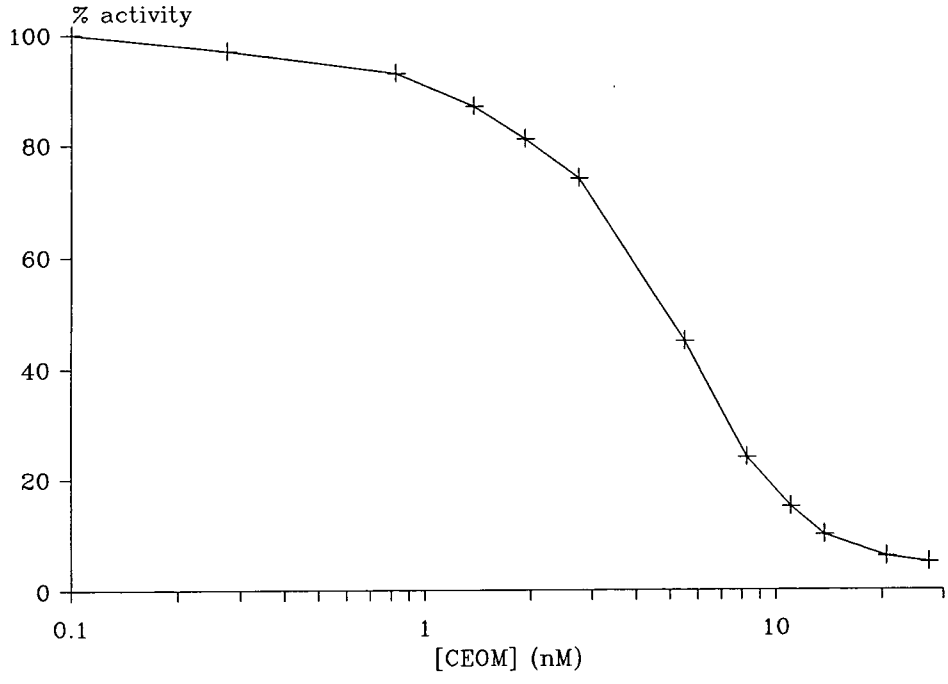


Figure 1.62

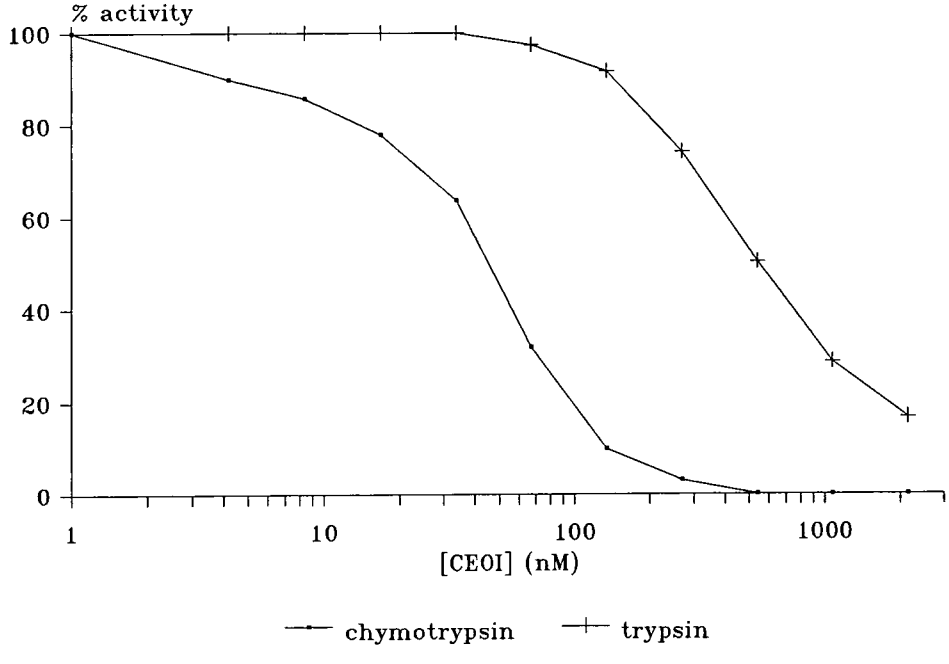


Figure 1.63

Dixon plot for the determination of K_i for CEOI inhibition of trypsin from *S. littoralis*.

Legend: ordinate - 1/rel. act.
abscissa - μM CEOI

Figure 1.64

Dixon plot for the determination of K_i for CEOI inhibition of chymotrypsin from *S. littoralis*.

Legend: ordinate - 1/rel. act.
abscissa - nM CEOI

Figure 1.63

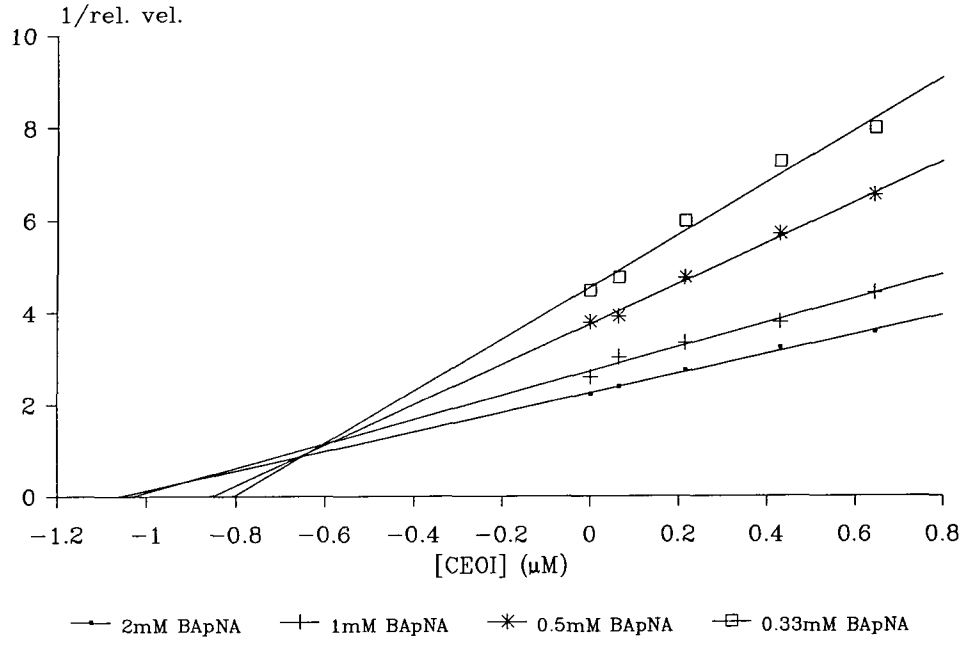


Figure 1.64

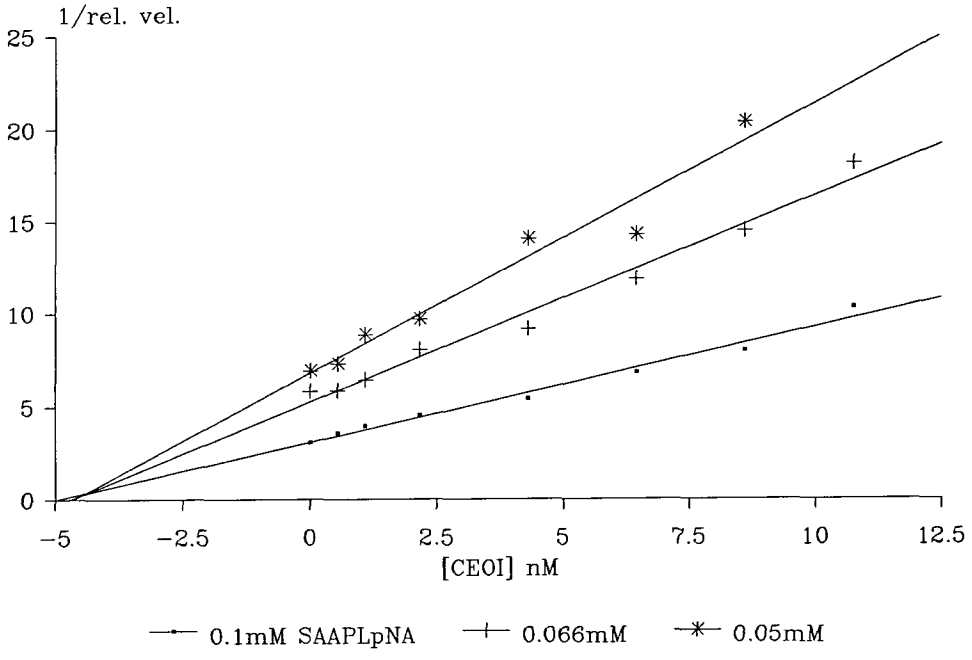


Figure 1.65

The effect of TEW on the activity of chymotrypsin activity (Sa_2PLpNA and casein hydrolysis) from *S. littoralis*.

Legend: ordinate - % activity
abscissa - μM TEW

Figure 1.66

Dixon plot for the determination of K_i for TEW inhibition of chymotrypsin from *S. littoralis*.

Legend: ordinate - 1/rel. act.
abscissa - nM TEW

Figure 1.65

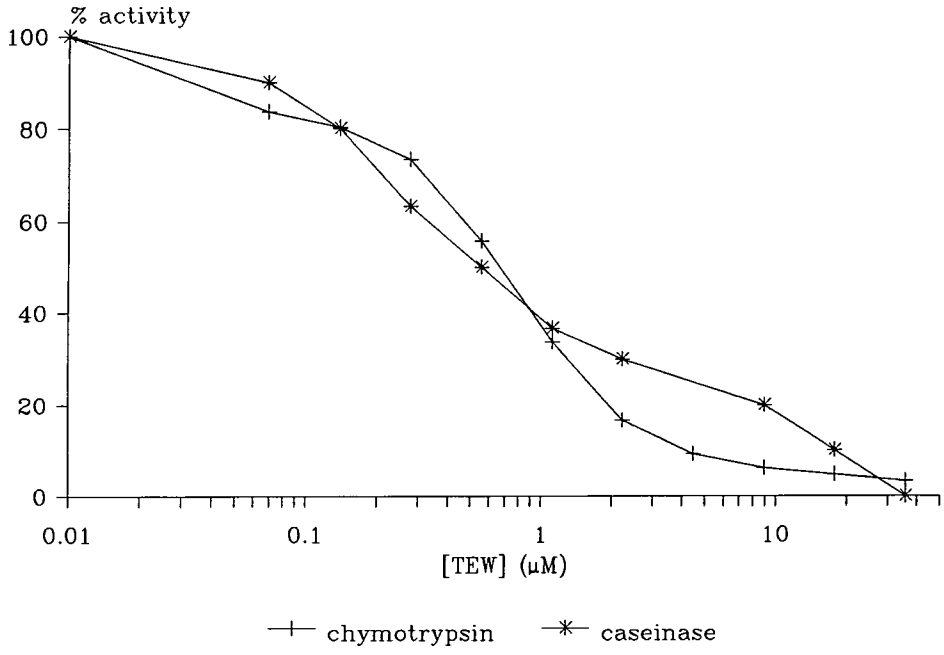
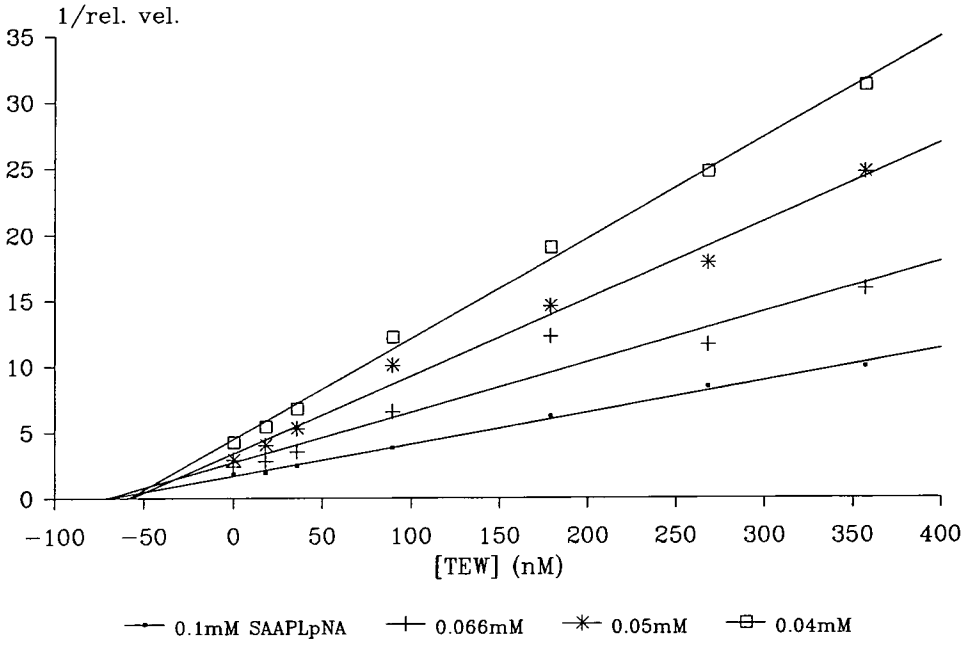


Figure 1.66



alter trypsin activity from *S. littoralis*, but chymotrypsin activity from the same insect was reduced at high concentrations; 5.0μM caused 50% inhibition at pH 7.0 (Fig. 1.48). Further investigations with this inhibitor were not carried out.

Characterization of enzymes hydrolysing other substrates.

After characterising the two endopeptidases with inhibitors using BApNA and SA_2PLpNA as substrates, the endopeptidases responsible for the hydrolysis of SA_2PPpNA, SA_3pNA and TGPLpNA could be established by the use of selective inhibitors. Enzyme activity was determined at pH 7.0 with and without benzamidine (0.64mM) and PMSF (0.3mM). In this way, hydrolysis of TGPLpNA could be attributed to the BApNA enzyme while SA_2PPpNA and SA_2PLpNA were hydrolysed by the same enzyme. SA_3pNA hydrolysis was not inhibited by benzamidine (0.64mM), leupeptin (20μM) or aprotinin (3.1μM), but was inhibited by PMSF (0.3mM), TEW (3.6μM), CEOI (0.65μM), SBTI (25μM), chymostatin (70μM), LBTI (11.1μM) and BBTI (12.7μM). Therefore, it is probable that hydrolysis of SA_3pNA is by the insect chymotrypsin.

CHAPTER 1
Discussion

The pH maxima of the endopeptidases from *H. armigera* and *S. littoralis* were alkaline. Maximal casein hydrolysis in *Heliothis zea* (Klocke and Chan, 1982) and *Spodoptera litura* (Ahmad *et al*, 1976) occurs at pH 11.0 and similarly high pH maxima have been reported in the wax moth, *Galleria mellonella* (Hamed and Attias, 1987), in *Bombyx mori* (Eguchi and Iwamoto, 1976) and in *Trichoplusia ni* digestive fluids (Pritchett *et al*, 1981). The pH optima of trypsin-like proteases found in the gut luminal contents of *H. armigera* and *S. littoralis* are consistent with these and other values published for proteases of other insects (Table 1.15). Furthermore, the pH values of the midgut are similar to those reported for the gut lumina of other larval lepidopterans e.g. Eguchi and Arai, (1983) measured the pH of *Bombyx mori* midgut at 10.84, the midgut pH of *Lichnoptera felina* measured pH 10.0 (Dow, 1984) while pooled digestive fluid from larvae of *Trichoplusia ni* measured pH 9.85 (Pritchett *et al*, 1981). In the present study, the midgut pH of *S. littoralis* was shown to be highly dependent on temperature (pH of 8.95 at 20°C and about 8.4 at 37°C). For the same species, Applebaum *et al*, (1964) found a pH of 8.5, while Ishaaya *et al*, (1971) reported it to be about 9.5. Unfortunately, the temperatures at which the pH was measured by these workers is not stated. However, given the temperature dependence referred to above, the discrepancies in pH reported may be explained in part on this basis. The pH gradient across the middle midgut is reversibly collapsed by anoxia, implying that it is sustained by a process requiring oxidative phosphorylation (Dow and O'Donnell, 1990). Therefore, the large temperature-dependent effect on pH of the midgut contents may be related to the absence of the midgut tissue and oxygen to sustain the high pH, rather than the entire change being a consequence of temperature effects on buffering ions. Another reason for the change in pH was suggested by Purcell *et al* (1992). They have measured the midgut pH of *Agrotis ipsilon*, *Heliothis virescens* and *H. zea* at pH 8.3, 9.7 and 8.9 respectively (temperature at which these pH measurements were made was not indicated). They suggest that in addition to the disruption of the energy requiring process by the dissection procedure, the presence of hydrolytic enzymes would lead to continued acid production and further lowering of the midgut fluid pH. This acidification will also have occurred during the pH measurement of gut contents from *S. littoralis*, but the degree of change this had on the measured pH is not known.

Hydrolysis of casein by endopeptidases from *S. littoralis* was maximal at a higher pH than that observed with BApNA or SA_2PLpNA. In the present study, it has been shown that hydrolysis of casein is a consequence of chymotryptic activity, as indicated by inhibition by PMSF and TEW and the absence of inhibition by TLCK, rather than tryptic activity responsible for BApNA hydrolysis. However, chymotryptic activity shows an extended binding site requirement (see below). Proteases from a single species of insect have different pH maxima for different substrates (e.g. Hamano and

TABLE 1.15. pH OPTIMA, SUBSTRATE AFFINITIES AND Mr OF VARIOUS ALKALINE DIGESTIVE ENDOPROTEASES

Species	Source	Optimum pH	K_m (app)	Substrate	M_r(kDa)	Reference
Acheta aegypti					21.5	Huang, 1971
A. domesticus		7.6		Hide powder		Teo & Woodring, 1988
A. mylitta					25.0	Kramer *et al*, 1973
A. pernyi					25.0	Kramer *et al*, 1973
A. polyphemus					25.0	Kramer *et al*, 1973
	cocoonase	8.3		BAEE	24.5	Berger *et al*, 1971
Attagenus megatoma		8.0	1.50mg/ml	casein		Baker, 1976
			7.60mg/ml	haemoglobin		
	CT-1	10.0	3.3 mM	BApNA	22.6	Baker, 1981
	CT-2	10.0	1.7 mM	BApNA	22.6	
	AT	8.5	0.21mM	BApNA	20.9	
Bombyx mori	adult	9.0		casein	40.0	Eguchi *et al*, 1972
	larval	11.2		casein		Eguchi & Iwamoto, 1976
			0.057 mM	BAEE		Kafatos *et al*, 1967a,b
	P-IIa		0.026 mM	BApNA		Sasaki and Susuki, 1982
	P-IIb		0.0281mM		22.0	
	P-IIIb		0.0298mM		23.0	
			0.697 mM	BApNA		Eguchi and Kuriyama, 1983
					24.0	Walsh, 1970
Calliphora erythrocephala		7.6-7.8	7.19mg/ml	azocasein		Evans, 1958
Costelytra zealandica	try-1	9.0-10.0	0.37mM	DL-BApNA	22.9	Christeller *et al*, 1989
	try-2		0.27mM		23.56	
Dermasterias imbricata		8.0-8.5		DL-BApNA	25.0	Camacho *et al*, 1970
Galleria mellonella	P1	10.5		Azocoll	12.5	Hamed & Attias, 1987
	P2	11.2			10.5	
Glossina morsitans		9.0-10.0	3.43mg/ml	haemoglobin		Gooding & Rolseth, 1976
			1.22mg/ml	casein		
		8.0-9.0		BApNA	19.1	Cheeseman & Gooding, 1985
		7.0		casein		Gooding & Rolseth, 1976
Gryllullus taiwanemma		8.3		casein		Nakashima *et al*, 1965
Helicoverpa armigera		10.0	0.257mM	DL-BApNA	24.0	Johnston *et al*, 1991

		10.0		gelatin	25.0	Akimenko *et al*, 1988
		8.0-9.0		gelatin	24.0	Rubinstein & Polson, 1983
Leucophaea maderae		9.0-9.5		casein-urea		Engelmann & Geraerts, 1980
Locusta migratoria		9.0		BAEE	18.2	Knecht *et al*, 1974
		9.0-9.3		BApNA		Charnley, 1975
		8.5		gelatin		Powning *et al*, 1951
Manduca sexta		8.1	0.0174mM	BAEE	24.0	Miller *et al*, 1974
Musca autumnalis		8.0		azocasein		Campbell *et al*, 1987
		8.0		BApNA		
		8.5		wool		Powning *et al*, 1951
Ostrinia nubilialis	P1	>10.0		BApNA		Houseman *et al*, 1989
Periplaneta americana		8.5		wool		Powning *et al*, 1951
	chymotrypsin			azocasein	25.0	Baumann, 1990
Pheropsophus aequinoctialis		8.5	0.13mM	BApNA	34.0	Ferreira & Terra, 1989
Phormia regina		7.9-8.3		casein		Brookes, 1961
Pieris rapae		8.0		TAME		Broadway, 1989
		9.0		BTEE		
Pterostichus melanarius	male	8.0	2.35mg/ml	haemoglobin	16.8	Gooding & Huang, 1969
	female		0.51mg/ml			
	male		9.40mg/ml	BSA V		
	female		16.7 mg/ml			
Simulium rugglesi		8.4	3.1mM	TAME		Yang and Davies, 1968
S. venustum		8.4	2.4mM	TAME		
Spodoptera littoralis		11.0		casein		Ishaaya *et al*, 1971
		11.0		casein	22.5	Present work
		10.0		BApNA	24	
		10.0		SA_2PLpNA	22.5	
		8.0		TAME		
		9.5		BAEE		
		8.0		ATEE		
		8.0		BTEE		
S. litura	P1	11.0	0.0057mM	casein	17.0	Ahmad *et al*, 1980
	P2	10.5	0.0029mM		21.0	
	P3	9.0	0.0002mM		53.0	
Stomoxys calcitrans		8.0		BApNA	19.5	Houseman *et al*, 1987
Tineola bisselliella		9.8		BApNA		Ward, 1975a,b
		9.4		casein		Ward, 1975a,b

Trichoplusia ni	9.8		casein		Pritchett *et al*, 1981
	8.5		TAME		Broadway, 1989
	8.0		BTEE		
Vespa orientalis	7.5-8.5		BAEE	27.0	Hagenmaier, 1971
				13.8	Jany *et al*, 1974
V. crabro				14.3	Jany *et al*, 1978a,b
Vertebrate proteases:					
Bovine trypsin	8.5		wool		Powning *et al*, 1951
		0.0109mM	BAEE		Hruska *et al*, 1973
				23.8	Kafatos *et al*, 1967a,b
	8.0	0.042mM	casein	24.0	Buck *et al*. 1962b
Human trypsin				22.9	Travis & Roberts, 1969
Porcine trypsin	8.0	0.029mM	casein	24.0	Buck *et al*, 1962b
cod chymotrypsin	7.8	0.14mM	BTEE	26.0	Asgeirsson & Bjarnason, 1991
Cyprinius carpio			TAME	25.0	Cohen *et al*, 1981
Mallotus villosus	8.0-9.0		BApNA	28.0	Hjelmeland & Raa, 1982

Mukaiyama, 1970; Houseman *et al*, 1989). This indicates either that (a) different enzymes are responsible for the substrate breakdown or (b) the observed pH maxima differences are a property of the substrate-enzyme-buffer interaction or (c) high pH causes substrate limitation of the observed enzyme activity. If (b) occurs *in vivo*, then the pH maxima reported for trypsin enzymes from both species of insect using BApNA as substrate may differ from the pH maxima operating *in vivo*. Large variations in pH maxima have been obtained for different substrates e.g. Gooding and Rolseth (1976) found breakdown of casein by a protease isolated from *Glossina morsitans* occurred maximally at pH 9.0-10.0, whereas BApNA hydrolysis was maximal at pH 8.0-9.0. Ward (1975a,b) found that using casein as substrate again resulted in a slightly higher pH maximum than with BApNA (pH 9.8 and 9.4 respectively) in *Tineola bisselliella*. Maximal breakdown of protein substrates does not, however, always occur at a higher pH than colorimetric peptide substrates: Baker (1981) reported a higher maximal activity from partially purified protease fractions using BApNA as substrate (pH 10.0) when compared with the activity of crude homogenate against casein/haemoglobin at pH 8.0 in *Attagenus megatoma* (Baker, 1976). However, much smaller differences in pH maxima have also been reported; proteases from various insects including *Musca domestica*, *Tineola bisselliella* and *Tenebrio molitor* hydrolysed the substrates azocasein, BApNA and wool maximally between pH 8.0 and 8.5 (Campbell *et al*, 1987; Powning *et al*, 1951). Similar maximal activities using different substrates have been described for proteases from *Locusta migratoria* using BAEE, pH 9.0 (Knecht *et al*, 1974); BApNA, pH 9.0-9.3 (Charnley, 1975) and gelatin, pH 8.5 (Powning *et al*, 1951). It is possible that in some of the instances mentioned above protein substrate hydrolysis was due to the presence of another endopeptidase with a different bond specificity to the trypsin enzyme, perhaps with an extended site specificity which was not therefore detected using more conventional substrates.

An additional reason for a lower apparent maximal pH of hydrolysis with pNA substrates may be a consequence of non-enzyme related hydrolysis, especially when a prolonged assay was used. This would cause substrate limitation of the rate of enzyme activity, whereas this does not occur at high pH (pH 10-12) using 5% casein as substrate. pNA substrates hydrolyse at a high pH in the absence of enzyme and consequently are not ideal substrates for studying enzyme activity at pH > 10.5; after 30 min at pH 11.5 less than 50% of a 1mM substrate remained. Similarly, Houseman *et al* (1989) reported that values of maximal hydrolysis were not obtained at pH > 10.0 due to rapid breakdown of BApNA. Nagel *et al* (1965) report that 50% of 0.06mM BApNA had hydrolyzed non-enzymatically at pH 10.8 when incubated for fifteen minutes at 25°C. On this basis, it is difficult to understand reports of higher maximal protease activity, e.g. maximal protease activity from *Bombyx mori* has been reported at pH 11.4 with 0.33mM BApNA as substrate (Eguchi and Kuriyama, 1983); one might have expected considerable substrate-limitation at this pH.

It has been shown that the various buffer systems used affected the relative

activity of endopeptidases from *H. armigera* and *S. littoralis* midgut. Similarly, Engelmann and Geraerts (1980) reported that proteolytic activity observed using casein-urea as substrate was markedly affected by the buffer system chosen; using glycine-NaOH, protease activity from *Leucophaea maderae* was still increasing at pH 10.6, but an optimum between 9.0 and 10.0 was obtained using AMP buffer. Buffer dependent shifts in pH optimum may also be of widespread occurrence in proteases not derived from insects; e.g. Frosco *et al* (1992) have shown that an elastase from *Aspergillus fumigatus* acts optimally at pH 7.4 in sodium borate buffer, but in Tris buffer, this optimum shifts to pH 8.8. This shift has also been observed with porcine pancreatic elastase (Shotton, 1970). Thus, whilst *in vitro* measurements of maximal activity of endopeptidases agree well with direct measurements of gut pH in this and other Noctuid insects (Ishaaya *et al*, 1971; Ahmad *et al*, 1980), various dietary factors which influence buffering capacity may influence optimal enzyme conditions *in vivo*.

Vertebrate trypsin is stabilized by Ca^{2+} (Nord and Bier, 1953; Green, 1953; Buck *et al*, 1962b) which also serve to activate substrate hydrolysis (Sipos and Merkel, 1968; Barrett and McDonald, 1980). In contrast, the activity of endopeptidases towards BApNA and SA_2PLpNA from *H. armigera* and *S. littoralis* was not stimulated by Ca^{2+}, but was, in fact, inhibited slightly at high concentrations of Ca^{2+}. A similar inhibition of trypsin activity from *Vespa orientalis* has been reported in the presence of $CaCl_2$ (Jany *et al*, 1978a). The absence of stimulation of protease activity by Ca^{2+} has been reported for the esterolytic activity of trypsin from the mosquitoes: *Aedes aegypti*, *Anopheles quadrimaculatus* and *Culex pipiens quinquefasciatus* (Briegel and Lea, 1975). No Ca^{2+} stimulation of an alkaline protease was found in another larval Noctuid, *Spodoptera litura* (Ahmad *et al*, 1980). Broadway and Duffey (1986) state that Ca^{2+} ions were a necessary component of the enzyme buffer to stabilize endopeptidase activity from the Noctuid insects: *Heliothis zea* and *Spodoptera exigua*, but did not describe the experimental work further. In *Ostrinia nubilialis*, two tryptic activities have been found (Houseman *et al*, 1989): a highly alkaline activity which increased 30% in the presence of calcium and low alkaline trypsin which was unaffected. Thus, the effect of Ca^{2+} on proteases from insects closely related to *Helicoverpa armigera* and *S. littoralis* is somewhat unpredictable. In the present study, it was possible that there was sufficient Ca^{2+} in the crude homogenate of luminal contents for full activition of a calcium requiring trypsin. Therefore, EDTA was added to establish whether activity could be significantly reduced through Ca^{2+} chelation. The activity of trypsins from *S. littoralis* and *H. armigera* was not altered by EDTA up to a concentration of 20mM or EGTA at a concentration of 1mM. Tryptic from *S. littoralis* was inhibited, nevertheless, by EDTA at higher concentrations (260mM). The effects of EDTA on digestive enzyme preparations in insects are well documented. Substantial inhibition with EDTA occurred in the P-2 protease from *Galleria mellonella* (Hamed and Attias, 1987) whilst Baker (1981) found that EDTA caused activation of two BApNA-hydrolyzing fractions and inhibition of the third from *Attagenus megatoma*. Ward (1975 a,b) has demonstrated two

EDTA-inhibited (but EDTA-Ca complex insensitive) metalloproteases in larvae of *Tineola bisselliella*. Digestive fluid and tissue protease activity from *Bombyx mori* was inhibited by EDTA (Eguchi and Iwamoto, 1976); the extent of the inhibition being similar to that observed in *H. armigera* (only about 10% inhibition at 50mM EDTA). Tryptic protease activity towards azocasein of *Ostrinia nubilialis* was inhibited by EDTA, although not activated by Ca^{2+} (Houseman et al, 1989), whereas BApNA hydrolysis was stimulated. Calcium chelating agents i.e. EDTA and EGTA had no effect on either BAEE or BTEE hydrolysis, but activated the BApNA-ase hydrolysis. Interpretation of this activation is difficult as they found that BApNA-ase activity was also increased in the presence of calcium ions.

The specificity of the endopeptidases from *S. littoralis* was investigated using pNA substrates. Trypsin hydrolysed TGPLpNA in addition to BApNA. Arginine and lysine specificity in the P_1 position is characteristic of trypsin-like enzymes (Keesey, 1987). Trypsin activity was much greater towards TGPLpNA than to BApNA which might suggest that the affinity of this trypsin is greater for P_1 = lysine. Mammalian trypsins have the reverse affinity specification (i.e. activity is higher for P_1 = arginine) unless the sequence of the enzyme is modified by site directed mutagenesis (Craik *et al*, 1985). This affinity specification (K_m higher for BLpNA) is duplicated in the hornet trypsin (Jany *et al*, 1978a). However, a comparison of activity and affinity towards different amino acids at P_1 is only appropriate when the remainder of the substrate is identical (as for SA_2PLpNA and SA_2PPpNA) because the cleavage of the scissile bond is often facilitated by increasing the peptide bond length (Dunn, 1989). Cleavage rates may be significantly altered by different residues in P_2-P_5: SA_2PLpNA was hydrolysed by endopeptidases from *S. littoralis* whereas ZGGLpNA was not. A similar effect of different P_2-P_5 on insect proteases has been reported by Amarant *et al*, (1991); the proteases from *Lomonia achelous* did not hydrolyse Ile-Pro-Arg-pNA, but activity was present against Pro-Phe-Arg-pNA. Therefore further studies will be necessary to characterise the tryspin specificity of this enzyme using the BApNA analogue, BLpNA, or the TGPLpNA analogue, TGPApNA (Abildgaard *et al*, 1977; Lottenberg *et al*, 1981).

Chymotrypsin activity from *S. littoralis* was investigated using a number of substrates to assess the effect of peptide chain length on its activity and primary substrate specificity. Peptide cleavage by chymotrypsin from *S. littoralis* was preferential when bonds were adjacent to bulky residues (P_1 = leucine, phenylalanine) which indicates the enzyme is a member of the chymotrypsin family (Bieth, 1989). In view of the more recent definition of an elastase: maximal hydrolysis of elastin occurs near neutral pH, the midgut endopeptidase from *S. littoralis* is described here using the term 'chymotrypsin' rather than 'elastase'. The enzyme responsible for the low rate of elastin hydrolysis could not be established as the midgut chymotrypsin due to the inherently low elastinolytic activity. Additionally, cleavage of SA_2PLpNA was relatively insensitive to elastatinal (<50% inhibition at 100μM), but was readily inhibited by

chymostatin (100% inhibition at 0.3μM), indicating a chymotryptic and not elastolytic nature. Aoyagi and Umezawa (1975) found that mammalian elastase was inhibited by 50% at a concentration of 0.29μg/ml (0.56μM), and K_i was 0.24μM (Umezawa and Aoyagi, 1983), whereas bovine trypsin and bovine chymotrypsin were unaffected at higher concentrations: >250μg/ml (490μM). Elastatinal has an alaninal at the terminal carbon and in view of the low activity of chymotrypsin with SA_3pNA (a comparison of relative activities for SA_3pNA : SA_2PPpNA is 0.054 : 1) only weak inhibition by elastatinal was expected.

Midgut chymotrypsin from *S. littoralis* was able to hydrolyse SA_2PLpNA and SA_2PPpNA rapidly, while SA_3pNA hydrolysis took place more slowly. 1mM SA_2VpNA was not hydrolysed, nor were BTpNA, Suphepa and ALpNA. The specificity of the chymotryptic endopeptidase from *H. armigera* and *H. zea* appear similar; preliminary studies showed that SA_2PLpNA was cleaved rapidly (K. Johnston, personal communication) while Suphepa and BTpNA were not hydrolysed over the pH range 4 to 11.8. The absence of activity against Suphepa and ALpNA compared with more rapid hydrolysis of SA_2PPpNA and SA_2PLpNA indicates that an extended peptide chain is necessary for efficient catalysis of peptide bonds. Tsai *et al* (1986) found that the specific activity of shrimp extract (*P. monodon*) for BTEE was 10 to 100-fold lower than that for SA_2PPpNA. They too concluded that the substrate size was important for the development of the full chymotrypsin activity. It has also been demonstrated that the K_m of bovine chymotrypsin is almost 10-fold lower for SA_2PPpNA than Suphepa (Del Mar *et al*, 1979) which reflects the importance of secondary binding sites in the kinetics of chymotrypsins. The absence of inhibition of the chymotrypsin enzyme by TPCK is possibly also a consequence of the need for effective binding. It would be interesting to study the effect of chloromethyl ketones with an extended peptide chain such as ZGLPCK, which has proved very effective against chymotrypsin from locusts (Sakal *et al*, 1988) or ZAGPCK which has been shown to be an effective inhibitor of shrimp chymotrypsin (Chen *et al*, 1991b).

There are several similarities such as size and to a certain extent their substrate specificity between this chymotrypsin enzyme and elastase enzymes from other sources. The inhibition of 'elastases' from a variety of lepidopteran larvae (*Helicoverpa punctigera* and *Cydia pomonella*) by eglin c (Christeller *et al*, 1992) proved to be relatively ineffective compared with the inhibition of human elastases (affinity for insect enzymes about 100-fold less), but similar in affinity to the inhibition by eglin c of bovine chymotrypsin (Seemüller *et al*, 1977). Christeller *et al* (1990) describe an 'elastase' enzyme from *Teleogryllus commodus* with apparently similar specificity to human and porcine pancreatic elastase II and to human leukocyte elastase. The enzyme from *S. littoralis* differs from these mammalian enzymes. Elastases such as human and porcine pancreatic elastase II exhibit characteristic chymotrypsin specificity (Del Mar 1980; Bieth, 1989), but solubilise elastin readily (Gertler *et al*, 1977) unlike the enzyme described here. Human leukocyte elastase prefers valine to alanine residues at P_1

(Wenzel *et al*, 1980), whilst in the present study, valine at this site rendered the enzyme from *S. littoralis* inactive; SA_3pNA, but not SA_2VpNA, was cleaved by midgut homogenate. Most other elastase enzymes differ from the endopeptidase described here e.g. porcine pancreatic elastase I does not cleave SA_2PPpNA (Kasafirek *et al*, 1976). However, while porcine elastase is readily inhibited by DMF (Bieth and Wermuth, 1973) like chymotrypsin from *S. littoralis*, bovine chymotrypsin is also inhibited by this solvent (Bundy and Moore, 1966; Maurel, 1978). It would be interesting to determine the effect of a variety of organic solvents and detergents on endopeptidase enzymes from *S. littoralis* in order to carry out a further comparison with other enzymes. Some organic solvents are reported to increase the rate of human leukocyte elastase hydrolysis of SA_npNA (N = 2 to 5) (Lestienne and Bieth, 1980). Wenzel *et al* (1990) has speculated that the activity of certain endopeptidases is controlled by the presence of detergents *in vivo*. It has been shown that the activity of certain endopeptidases can be increased *in vitro* in the presence of certain detergents. Triton X-100 increased the activity of human leukocyte elastase and Cathepsin G by 80% and 18% respectively while benzalkonium chloride led to a five fold increase in activity of human leukocyte elastase. Indeed, the proteolytic activity of caseinase activity from *Bombyx mori* was enhanced by the addition of Lubrol WX or Triton X-100 (Eguchi and Arai, 1983). Thus, surfactants may also aid digestion by preventing protein precipitation of tannins (see introduction) and by stimulating endopeptidase activity.

Chymotrypsin from *S. littoralis* appeared similar with respect to specificity and optimum pH to elastase from human aortic media (Hornebeck and Robert, 1989) and to proteinase K (Bond, 1989). It has been suggested that the existence of endopeptidases with a similar specificity to chymotrypsin described here may be of widespread occurrence in insects (Christeller *et al*, 1990). For example, Sasaki and Suzuki (1982), Eguchi and Iwamoto (1982) and Eguchi and Kuriyama (1985) have each reported two different endopeptidases with different substrate specificities in the midgut of the silkworm, *Bombyx mori*. On the basis of inhibitor and pH studies, one of these enzymes is comparable to trypsin from *S. littoralis* and the other to chymotrypsin. There have been other reports (Knecht *et al*, 1974; Giebel *et al*, 1971) of insect digestive proteinases which hydrolysed protein substrates such as casein and oxidised insulin B chain which were unable to degrade specific synthetic substrates for chymotrypsin such as glutaryl-L-phenylalanine-ß-naphthylamide. The choice of substrate is therefore very important. These observations are not inconsistent with the suggestion of the widespread nature of chymotrypsins in insects and arthropods in general; Chen *et al* (1991b) and Van Wormhoudt *et al* (1992) reported the presence of a SA_2PPpNA-hydrolysing enzyme from *Penaeus monodon* and *P. vannamei* respectively with similar specificity to the one described here.

The hydrolysis of the esters ATEE and BTEE may be a consequence of chymotryptic enzyme activity because the elution from gel filtration columns of activity towards these substrates coincided with that for SA_2PLpNA. However, the effect of

protease inhibitors on the esterase activity from *S. littoralis* were not determined. If chymotrypsin is responsible for both chymotryptic peptide and ester substrate hydrolysis then this endopeptidase must not require an elongated chain for ester substrate hydrolysis. The hydrolysis of esters by proteases normally occurs at a higher rate than the equivalent peptide (Hess, 1971). It has been shown that the BTEE activity in other lepidopteran insects, *Pieris rapae* and *Trichoplusia ni*, is totally inhibited by PMSF (Broadway, 1989). It was found that PMSF, as in this study, was also relatively poor at inhibiting trypsin activity, as determined by TAME hydrolysis. Baumann (1990) characterised the activity towards Z-tyrosine-p-nitrophenyl ester from cockroach intestine. The inhibition characteristics of this chymotrypsin were similar to the one present in *S. littoralis*: pCMB, TLCK, TPCK, EDTA and Ca^{2+} were ineffective inhibitors, whereas PMSF was a potent inhibitor of this protease. This is consistent with the suggestion that the hydrolysis of ester substrates by insect chymotrypsins can be inhibited by peptidase inhibitors. The presence of BTEE hydrolysing enzymes have been demonstrated in a number of insects, including species from families other than Lepidoptera. For example, Shukle *et al* (1985) have reported the presence of a chymotrypsin from the gut of *Mayetiola destructor*, a Dipteran, hydrolysing BTEE and casein with similar pH maxima characteristics for the two substrates and sensitivity to BBTI, SBTI, LBTI and duck ovomucoid. This inhibitory data together with the similar size of the proteases might suggest that the hydrolysis of ATEE and BTEE is due to chymotryptic activity with similar specificity for pNA peptides. Evidence of hydrolysis of BTEE by chymotrypsin has been identified in *Trichoplusia ni* (Pritchett *et al*, 1981), *Ostrinia nubilialis* (Houseman *et al*, 1989) and *Locusta migratoria* (Sakal *et al*, 1988) by TPCK and PMSF inhibition. It is possible that at least some of the BTEE-ases reported previously have similar synthetic peptide specificity.

Lepidoptera are frequently infected with microbes such as protozoa (Lee and Anstee, 1992) and viruses which produce associated proteases (Rubinstein and Polson, 1983). The possibility that endopeptidase production in insects are synthesized by such microbes has been considered in previous reports (Christeller *et al*, 1989; Lenz *et al*, 1991). The former report suggested that protease activity from *Teleogryllus commodus* was produced by the midgut tissue because only the hindgut contained bacteria and protozoa (Bauchop and Clarke, 1975). The low level of activity in midgut epithelium of many insects (Dadd, 1956) including *S. littoralis* and *H. armigera* (present study) might indicate microbial production by a midgut flora as the origin of the proteases. However, Lenz *et al* (1991) found that protease activity from the lepidopteran larvae of *H. zea* was not decreased when insects were reared axenically over those raised xenically. Evidence which suggests that the origin of endopeptidases from *S. littoralis* and *H. armigera* is not microbial is indicated by the absence of activity of subtilisins, the activity of which could not be demonstrated using ZGGLpNA, a specific subtilisin and neutral endopeptidase substrate (Lyublinskaya *et al*, 1974; Wilk and Orlowski, 1979).

Mammalian proteases such as trypsin are secreted in an inactive form

(zymogens) and are then converted to the active form by enteropeptidase. Therefore, another interpretation of the low midgut epithelial activity might be that protease secretion in insects is also in an inactive form. The existence of proenzymes (zymogens) was established in insects with reports on the cocoonase of silkmoths (Kafatos *et al*, 1967a,b; Berger, 1971) which exhibits certain sequence similarities with bovine trypsin (Kramer *et al*, 1973). The presence of an inactive zymogen involved in midgut digestion has been suggested for the Dipteran *Stomoxys calcitrans* (Moffatt and Lehane, 1990). It has been reported that the trypsin of *Bombyx mori* is secreted as an inactive molecule that is later activated by the high pH of the gut lumen (Eguchi and Arai, 1983; Kuriyama and Eguchi, 1985). Such alkaline conversion of the protease of *H. armigera* might explain why only low activity was detectable in gut tissue preparations. However, if this were so, then incubation of gut tissue homogenate might be expected to give significantly increased protease activity at high pH due to activation. A further report in favour of the secretion of an inactive molecule was made by Schlottke (1937) who found a positive effect of mammalian enterokinase on proteases from *Periplaneta americana*. However, further attempts to increase the levels of endopeptidase activity in *Locusta migratoria* by incubation with an extract of gut tissue (Khan, 1963) or in a variety of other insects (Powning *et al*, 1951) by incubation with enterokinase were not successful. It is concluded that the probable cause of low activity in the midgut tissue of *S. littoralis* is that the endopeptidases are secreted into the lumen rapidly following synthesis, where they accumulate. It is also possible that the midgut contains a protease inhibitor(s) which, in addition to a suboptimal pH, prevents proteolysis of the midgut tissue. Indeed, the presence of inhibitors in insect midgut has been demonstrated in the midgut of *Leucophaea maderae* (Engelmann and Geraerts, 1980). More recently, cDNA sequences of trypsin from the moth of *Manduca sexta* (Law *et al*, 1992) and the mosquito, *Aedes aegypti* (Barillas-Mury *et al*, 1991), have shown that the primary translation products are most likely to be proenzymes, which contain a signal sequence for secretion and an activation peptide, which must be cleaved to produce active enzyme. It has been suggested that the cleavage of the activation peptide is tryptic, as there is an arginine preceding the amino acid terminus of the active enzyme (Law *et al*, 1992), but at present, the nature of the enzymes involved in proenzyme (zymogen) activation are unknown.

A feature common to the effect of temperature on casein digestion and to BApNA hydrolysis with proteases from *S. littoralis* was the apparent discontinuity in the Arrhenius plot, which occurred at about 30°C. Discontinuities have been reported by Charnley (1975) from trypsin from *Locusta migratoria*. A number of explanations have been offered for the cause of this discontinuity, such as two different activation energies for two or more interconvertible enzymes catalysing the same reaction (Dixon and Webb, 1979). If the enzymes have a different activation energy, and the conversion from one to another is affected by temperature, then a sharp discontinuity will be observed (Dawes, 1964). A shift from one rate limiting step to another with a change in

temperature, as described by Crozier (1925) and discussed theoretically by Stearn (1949), would yield a smooth curvilinear transition, however, rather than a sharp inflection (Dixon and Webb, 1979). Whatever the cause of the discontinuity, the break coincides with that observed in other enzymes from insects e.g. trypsin from *Locusta migratoria*, at around 30°C (Charnley, 1975) and ß-glucosidase in *Trinervitermes trinervoides*, about 30°C (Hewitt *et al*, 1974).

Reports on the affinity of endopeptidases from insects for SA_2PLpNA and SA_3pNA appear to be limited to a characterisation of proteases from the black field cricket, *Teleogryllus commodus* (Christeller *et al*, 1990). The K_m reported for SA_2PLpNA digestion was 0.49 $\pm$ 0.15mM for *S. littoralis* which is not significantly different to that found for the cricket (1.14 $\pm$ 0.874mM), while that for SA_3pNA was 3.35mM $\pm$ 0.23mM for the *S. littoralis* enzyme, which is a lower affinity than that observed for the hydrolysis of this substrate by proteases from the cricket (0.24 $\pm$ 0.05mM). To my knowledge, previous reports of SA_2PPpNA hydrolysis by insect enzymes have been confined to a study on the proteinases in moulting fluid of the tobacco hornworm, *Manduca sexta* (Brookhart and Kramer, 1990). However, in contrast to the rate of hydrolysis of SA_2PLpNA by the chymotrypsin from *S. littoralis*, they found that this substrate was cleaved at a very low rate.

The substrate affinity of trypsin from *S. littoralis* and *H. armigera* for BApNA compare well with values obtained for other insect trypsins (Table 1.15). The presence of cooperative behaviour from substrate-velocity data analysis reveals n values of less than 1.0 for trypsins from both insects. This indicates that there is either negative cooperativity in a molecule with multiple active sites or that there is substrate inhibition. Using SDS-PAGE, a method which separates aggregated enzymes, the M_r of trypsin from *S. littoralis* was similar (about 24 kDa) to that obtained when measured by gel filtration. Multiple active sites are often the product of aggregates which are stable under normal conditions, but unstable in the presence of SDS; for example, leucine aminopeptidase (Barrett and McDonald, 1980). Therefore, the low value of n is probably due to substrate inhibition at high substrate concentrations, rather than negative cooperativity. Inhibition of trypsin occurred more noticeably using BAEE as substrate.

The molecular weights of trypsins from both species of insect and chymotrypsin from *S. littoralis* are similar to those reported for a number of other insect species (see Table 1.15). In the present study, the M_r of trypsin from *S. littoralis* was comparable with bovine trypsin (24 kDa). The M_r of trypsin from *H. armigera* (24 kDa) is similar to that reported by Akimenko *et al* (1988) (22-25.5 kDa) and by Rubinstein and Polson (1983) (22-24 kDa) for the same species. Other reports on M_r of trypsins from different species of insect indicate a large variation compared with vertebrate trypsins (Table 1.15). Most insect endopeptidases appear to have M_r in the range of 10,500 to 31,000 Da (Houseman *et al*, 1987), but there have been a number of reports of larger proteases. Ahmad *et al* (1980) used gel filtration and SDS-PAGE to purify three

alkaline proteases from *Spodoptera litura*, one of 50-53 kDa. Three proteases are reported to be present in the digestive juice of the silkworm, *Bombyx mori* (Akiko and Masaharu, 1978). Brookhart and Kramer (1990) reported a 100 kDa protease from *Manduca sexta* haemolymph, while the largest endopeptidase in insects was reported from the fat body by Yaginuma and Ushizima (1991) with an M_r of 232 kDa. These proteases appear not to be involved with digestion, but with intracellular metabolism. In view of the similar sizes of a large number of proteases from various sources (Table 1.15), many endopeptidases from insects and mammalian sources may show a high degree of sequence homology (e.g. Jany and Haug, 1983; Jany *et al*, 1983; Zwilling and Neurath, 1981). Sequences of proteases from insects include a report by Amarant *et al* (1991) of two proteases from the haemolymph of *Lonomia achelous* which had a degree of homology with thrombin. The inhibition characteristics of these two insect proteases were similar to those of the trypsin enzyme from *S. littoralis*. They suggest that inhibition by mercurials could derive from having a free cysteine in the active site. Mercurial inhibitors have also been shown to be effective inhibitors of trypsin described here from *S. littoralis*. This might indicate that an uneven number of cysteines also occurs in trypsin from *S. littoralis*. The presence of sequence similarities between the achelases and trypsin proteases from *H. armigera* and *S. littoralis* would explain the reaction with thiol proteinase-directed reagents such as E-64, pCMPS and leupeptin. Indeed, the trypsin from *H. armigera* may also be activated by thiol reagents such as ß-mercaptoethanol (Akimenko *et al*, 1988).

Trypsin from *S. littoralis* eluting in the void volume from a G-75 gel filtration column re-eluted in a position indicating an M_r of about 24 kDa following solubilisation with Triton X-114. However, it did not separate into smaller components following column reapplication without this detergent treatment. Hamed and Attias (1987) separated three active fractions from *Galleria mellonella*. The apparent M_r of these fractions was 11kDa, 20kDa and 29kDa, but rechromatography of the intermediate fraction, without the addition of detergent again yielded three peaks. This indicates that the three original protease fractions observed were molecular aggregates. Protease aggregation after detergent solubilisation has also been reported in *Bombyx mori* (Eguchi and Kuriyama, 1985).

The M_r of chymotrypsin from *Spodoptera littoralis* determined by gel filtration was 19 kDa. Voordouw *et al* (1974) have shown that an anomalously low M_r can be derived from gel filtration chromatographic methods and therefore the M_r was also determined with SDS-PAGE (22,500 Da). These values are comparable with the M_r value obtained for an endopeptidase from the black field cricket, *Teleogryllus commodus*, hydrolysing the same substrates: 19,500 and 23,600 Da for SA_3pNA/SA_2PLpNA and casein digesting enzymes respectively (Christeller *et al*, 1990). From the present work, it appeared that the chymotrypsin was able to cleave casein, whereas trypsin was not. This phenomenon may be common in insects; Christeller *et al* (1990) suggested that the caseinase and SA_2PLpNA enzymes may be

the same. In addition, data from Jany *et al* (1978a) showed that hydrolysis of Glutaryl-Phe-pNA (a chymotryptic substrate) by fractions from an extract of *Vespa crabro* eluting from an ion-exchange column was coincident with casein hydrolysis. Trypsin hydrolysis of BApNA occurred at a rate of seven times that of the nearest casein peak. In addition, while the affinity for BApNA by *Vespa crabro* and *Vespa orientalis* was ten fold higher, these insect trypsins hydrolysed azocasein, casein and haemoglobin at 10% the rate by bovine trypsin. Further comparison with chymotrypsin enzymes from insect sources hydrolysing SA_2PLpNA and SA_2PPpNA is not yet possible as they have only recently been discovered as suitable substrates for insect midgut endopeptidases (Christeller *et al*, 1990 and present work respectively). The higher activity of chymotrypsin for casein than trypsin reported here may be only a consequence of the amino acid content of the casein. Casein has various molecular species, some of which are particularly rich in aromatic amino acids (Eigel *et al*, 1984). The percent amino acid content of K-casein component A-1 (data for crude casein is unavailable, perhaps due to variability) of arginine and lysine is only 8.69%. The proportion which is made up by aromatic or hydrophobic amino acids i.e. those preferentially cleaved by chymotrypsin (leu, trp, phe, tyr and met) is over 14% (Woychik *et al*, 1966).

The inhibition of trypsin from *S. littoralis* is similar in many ways to other BApNA hydrolysing enzymes. It is characterised as a serine protease by inhibition with PMSF, but was less susceptible to PMSF than chymotrypsin. This is paralleled by the high relative efficacy of PMSF inhibition of bovine chymotrypsin compared with bovine trypsin (half-life time is 85 times greater for trypsin inhibition; Salvesen and Nagase, 1989). Trypsin endopeptidases from *S. littoralis* and *H. armigera* responded to TLCK inhibition in a similar way to bovine trypsin. Insects' alkaline proteases have been characterized as trypsin-like or chymotrypsin-like enzymes on the basis of inhibition by TPCK and TLCK (Law et al, 1977). However, two proteases from the greater wax moth, *Galleria mellonella* (L.) were not specifically inhibited by these chloromethylketone derivatives (Hamed & Attias, 1987). TLCK inactivates papain, a cysteine protease (Arnon, 1970) and also a protease from the bruchid beetle, *Callosobruchus maculatus* reported as a thiol protease (Campos et al, 1989). Neither TLCK nor TPCK were effective inhibitors of the chymotrypsin enzyme from *S. littoralis*.

A number of compounds proved to be unexpected inhibitors of one or both of the endopeptidases. pCMPS proved to be a potent inhibitor of trypsin from *S. littoralis*, but not chymotrypsin, which may be useful for purification work of the enzyme by affinity chromatography. Inhibition by pCMPS indicates sulfhydryl groups present in the enzyme are important for activity (Webb, 1966b; Jocelyn, 1972). Trypsin inactivated by pCMPS could be reactivated by the addition of cysteine. Reactivation by cysteine of protease activity inhibited by mercurial compounds has been observed in other Lepidoptera, such as *Trichoplusia ni* and *Plathypena scabra* (Wolfson and Murdock, 1990a). Iodoacetic acid and NEM did not affect activity of trypsin from *S.*

littoralis. Reports of protease inhibition by these two inhibitors is limited e.g. reduced streptococcal protease (Liu and Elliott, 1971). E-64, a thiol protease inhibitor, also reduced the activity of trypsin, but not as effectively as E-64 inhibition of thiol proteases such as bromelain (Hanada *et al*, 1978). Therefore, although trypsin from *S. littoralis* is over 30 times more sensitive to E-64 than mammalian serine proteases (I_{50} >0.72mM; Keesey, 1987), inhibition may be by another mechanism than formation of a thioether bond with the active thiol of thiol proteases; the I_{50} of thiol proteases such as stem bromelain is over 300 times lower than the I_{50} for trypsin from *S. littoralis*. Chymostatin inhibited trypsin almost completely at the highest concentration of inhibitor, whereas the I_{50} for bovine trypsin is >250μg/ml (Aoyagi and Umezawa, 1975). Inhibition of trypsin by leupeptin was non-competitive using BApNA as substrate. Aoyagi and Umezawa (1975) obtained the unusual observation that leupeptin shows competitive inhibition of the hydrolysis of TAME and BAEE (K_i is 0.34 and 0.13 μM respectively, Umezawa and Aoyagi, 1983), but also noncompetitive for BApNA. The effectiveness of the microbial inhibitors on trypsin was (in increasing effectiveness): E-64 < chymostatin < antipain ≤ leupeptin as shown in Figure 1.39. Except for aprotinin, leupeptin was the most effective inhibitor of those tried. α_1-proteinase inhibitor, sometimes referred to as α_1-antitrypsin, did not affect activity of trypsin, but caused inhibition of chymotrypsin at μM levels. Inhibition of trypsin from *H. armigera* also occurred with the serine inhibitors BBTI, SBTI, LBTI and aprotinin, although responses of bovine trypsin and insect trypsin at the same inhibitor concentration produced different relative amounts of inhibition. BBTI is known to inhibit both vertebrate chymotrypsin and trypsin (Birk, 1976) and can be split into two small fragments having either trypsin or chymotrypsin activity (Odani and Ikenaka, 1973). BBTI is a potent inhibitor of insect trypsins such as *Tenebrio molitor, Locusta migratoria* and *Bombyx mori* and forms a 1:1 inhibitor complex with *Tenebrio* trypsins (Levinsky *et al*, 1977). Inhibition of *S. littoralis* trypsin activity by SBTI, aprotinin and TLCK provides evidence for homology with vertebrate trypsins. While the insect trypsins from the two insects were found to be largely similar in their response to a variety of different compounds, differences between the two insects were apparent. For example, BBTI was not an effective inhibitor of the trypsin from *S. littoralis*, but did reduce chymotryptic activity. Indeed, trypsin from *H. armigera* appeared more sensitive to inhibition by the four plant protease inhibitors tested (BBTI, LBTI, SBTI and CPTI) than trypsin from *S. littoralis*.

Absence of inhibition by CEOM of trypsin and chymotrypsin from *S. littoralis* and *H. armigera* is also a property of human trypsin (Buck *et al*, 1962a). Similarly, CEOM did not inhibit trypsin from *Spodoptera litura* (Ahmad et al, 1980) or a protease from *Ostrinia nubilialis* (Houseman et al, 1989). TEW, the ovomucoid from turkey eggs, inhibited chymotrypsin from *S. littoralis*. A possible explanation for the different inhibition patterns of chymotrypsin by TEW and CEOM may be related to the fact that CEOM has an arginine in place of the lysine at the essential site of interaction with the

protease (Liu *et al*, 1968; Ozawa and Laskowski, 1966). If this was the reason, then it is probable that a variety of other ovomucoids would also be inhibitory towards chymotrypsin, as they have been shown to have the essential lysine residue (Feeney, 1971). Nonetheless, the ovomucoids probably vary in their effectiveness against insect chymotrypsin enzymes due to their hypervariability (Kato et al, 1987; Laskowski et al, 1987).

Chymotrypsin from *S. littoralis* is inhibited by α_1-proteinase inhibitor, although only at relatively high concentrations compared with its normal working concentration range (Witt and Lill, 1984). CEOI was a highly effective inhibitor, but cannot be used to characterise the enzyme as ovoinhibitors can act as elastase, trypsin and chymotrypsin enzyme inhibitors. Indeed, CEOI has two trypsin, two chymotrypsin binding sites, and one elastase binding site (Gertler and Ben-Valid, 1980). Chymotrypsin was also inhibited effectively by chymostatin, but not by elastatinal. The I_{50} for elastatinal inhibition of porcine pancreatic elastase is only 0.29μg/ml (Umezawa *et al*, 1973), which is additional evidence that the enzyme is chymotryptic in nature.

The presence of acid proteases operating in the midgut lumen of lepidoptera is unlikely. The lumen of most lepidopteran larvae, including the cotton earworm and the Egyptian cotton caterpillar, is highly alkaline and measured pH 9.5-10.0 (Johnston *et al*, 1991) at room temperature immediately after dissection and pH 8.7 at 30°C after warming from 0°C. Acid proteases might be active in the foregut and hindgut where pH may be as low as 4.0 (Dow, 1984). Pepstatin A specifically inhibits acid proteases except for proctase A, cathepsin D and renin (Umezawa *et al*, 1970b), but is not inhibitory against other proteases (Aoyagi *et al*, 1971, 1972). The two endopeptidase activities are not measurably active much below pH 6.0 in wholegut preparations. However, the absence of inhibition by pepstatin A, up to a concentration of 100μM, confirms the similarity of these endopeptidases with other non-acid proteases from other sources and also the absence of aspartic and cysteine protease activities. Table 1.16 and 1.17 show the effect of various chemical and proteinaceous inhibitors on endopeptidases from *S. littoralis*. In general terms, where an inhibitor effects activity of both endopeptidases, trypsin is less susceptible to inhibition with the exception of antipain and leupeptin. A number of inhibitors affect only trypsin activity: aprotinin, TLCK, pCMPS, E-64 and 1,10-phenanthroline, while others affect only chymotryptic activity: BBTI, CPTI, LBTI, and TEW. Amongst the four plant protease inhibitors, CPTI was the least effective inhibitor of chymotrypsin (Figure 1.47) while chymostatin and CEOI were the most effective inhibitors of those tested (Figure 1.48). NEM, IAAmide and IAAcid were ineffective, indicating the serine nature of the two endopeptidases. 1,10-phenanthroline and EDTA were only effective at high concentrations, which suggests that metal ions are not necessary for the activity of these enzymes. Chymostatin, antipain and CEOI were the only inhibitors which could significantly inhibit the activity of both enzymes.

A comparison of the relative susceptibility of the endopeptidases from *S.*

Table 1.16. Effect of inhibitors on endopeptidase activities (*S. littoralis*) of partially purified extracts at pH 7.0.

Inhibitor	% inhibition		concentration
	trypsin	chymotrypsin	(μM)
A. Serine endopeptidase inhibitors			
i. Chemical			
PMSF#	42	100	5000
TLCK#	100	0	250
TPCK#	0	0	250
benzamidine	100	0	6400
ii. Microbial			
leupeptin#@	100	20	5
chymostatin	80	100	40
antipain#@	100	95	6.25
elastatinal	0	30	100
iii. Plant			
SBTI	58	90	25
BBTI	0	90	50
CPTI	0	39	21
LBTI	0	100	110
iv. Animal			
aprotinin	90	0	0.76
CEOM	0	0	3.6
CEOI	83	100	2.15
TEW	0	100	357
α_1-antiproteinase	0	74	10
B. Cysteine/thiol protease			
IAAcid	0	0	1000
IAAmide	0	0	1000
NEM	0	0	5000
DTT	0	N.D.	66000
pCMPS	100	0	100
E-64	100	0	200
C. Acid protease			
pepstatin A	0	0	100
D. Metalloprotease			
1,10-phenanthroline	88	0	100000

also a thiol protease inhibitor
@ measured in μg/ml
N.D.: not determined
Inhibitor concentrations quoted are those yielding the maximum inhibition observed over the inhibition concentration range studied.

Table 1.17. Inhibition of endopeptidase activities from *S. littoralis*.

Inhibitor	I_{50} (μM)		K_{ia} (μM)		K_{ib} (μM)	
	trypsin	**chymotrypsin**	**trypsin**	**chymotrypsin**	**trypsin**	**chymotrypsin**
Serine endopeptidase						
i. Chemical						
PMSF	6-9x10³*	30.0	Irreversible		Unstable inhibitor	
TLCK	21.4	-	Irreversible		Unstable inhibitor	
benzamidine	11.9	-	Competitive		11.6	>6400
ii. Microbial						
leupeptin@	0.05	>10.0	0.09	>10.0	0.043	>5
chymostatin@	6.9	0.011	Competitive		14.6	1.72
antipain	0.3	6.2	Competitive		N.D.	N.D.
iii. Plant						
SBTI	15.4	1.6	>25.0	4.0	3.75	0.286
BBTI	-	5.0	>50.0	5.8	>50	1.86
LBTI	-	3.0	Competitive		>110	0.66
iv. Animal						
aprotinin	0.03	-	Competitive		0.06	>0.76
CEOI	0.55	0.05	Competitive		0.62	0.043
TEW	-	0.66	Competitive		>350	0.069
α_1-antiproteinase	-	5.0	Unknown (Keesey, 1987)		>10	N.D.
Thiol protease						
pCMPS	8.0	-	Irreversible			
E-64	23.8	>200	30	>200	11.7	>200
Metalloprotease						
1,10-phenanthroline	5.4x10³	-	-	-	N.D.	N.D.

* calculated by extrapolation of the $\log_{10}$ plot
@ measured in μg/ml
N.D. not determined
K_{ia} and K_{ib} determined by the methods of Dixon (1972), pH 7.0, noncompetitive inhibition and Dixon (1953), pH 10.0, respectively.
Also tested, but found ineffective (inhibition less than 50%) at the maximum concentration given in Table 1.16 were TPCK, elastatinal, CPTI, CEOM, IAAcid, IAAmide, NEM, DTT and pepstatin A.

littoralis and those from the black field cricket, *Teleogryllus commodus* (Christeller *et al*, 1991) to inhibition reveals that, the trypsins are more susceptible to inhibition by aprotinin than the respective chymotrypsins whereas the chymotrypsins are more easily inhibited by CEOI, LBTI, TEW, SBTI and BBTI than the trypsins. Further qualitative comparison is not possible; Christeller's study included a wider variety of plant protease inhibitors, while the present work included inhibitors from a wider variety of sources (most notably the microbial inhibitors) for which a comparison was not possible. These studies indicate a basic similarity among the proteases from *S. littoralis* and *Teleogryllus commodus* which may extend to other lepidopteran and orthopteran insect digestive proteases. Indeed, inhibition of general protease activity appeared quantitatively similar for a number of inhibitors in the two lepidopteran larvae *Trichoplusia ni* and *Plathypena scabra* (Wolfson and Murdock, 1990a). It would be of great interest to compare the primary sequences of a large variety of insect proteases in an attempt to establish the sequence changes which affect optimum pH, inhibition and substrate affinity and also to deduce the mechanism for the release of the endopeptidases into the midgut lumen. Furthermore, the midgut endopeptidases could be quantified using either tritiated DFP (Borovsky and Schlein, 1988), biotinylated chloromethyl ketones (Breton-Maintier *et al*, 1992) or radioimmunoassay (Sakal *et al*, 1992) for a variety of insects in an effort to understand if their concentrations are related to diet or other factors.

CHAPTER 2

Characterization and partial purification of midgut exopeptidases from *Spodoptera littoralis*

Introduction

Knowledge of protein digestion in Lepidoptera is largely confined to studies on endopeptidases present in the midgut lumen. Analyses of proteases of *Spodoptera littoralis* by other workers have been confined to the endopeptidases (Ishaaya *et al*, 1971; Rubinstein and Polson, 1983) and the presence or properties of the exopeptidases were not investigated. Endopeptidases, the primary proteolytic enzymes, are insufficient for complete digestion of proteins in the diet in insects (Houseman and Downe, 1983; Billingsley and Downe, 1985; 1988; Billingsley, 1990), as outlined in the introduction to Chapter 1. The endopeptidases are, however, necessary in order to decrease the molecular weight of the food molecules (Ferreira and Terra, 1989) in order that the products can cross the peritrophic membrane. The pores in this membrane allow proteins of a molecular weight < 50-100kDa to pass. Intermediate digestion is the hydrolysis by oligomer hydrolases (i.e. aminopeptidases and carboxypeptidases) of the partially digested food molecules to dimers. Final digestion concerns the breakdown of the dimers into monomers by dipeptidases. Therefore, the latter stages of digestion (intermediate and final) occur in the ectoperitrophic space and/or at the midgut microvillar surface by exopeptidase action.

Exopeptidases from mammals are metalloproteases. The metalloproteases CPA and CPB are homologous exopeptidases of similar structure and active sites. CPA, like chymotrypsin, prefers C-terminal aromatic and aliphatic side chains of hydrophobic nature, whereas CPB, like trypsin, prefers arginine and lysine residues. The mechanism of CPA towards ester and peptide substrates is still being determined, but the active site includes zinc, with three ligands of two glutamic acids and one histidine. Another glutamic acid side chain, 270, acts as the nucleophile, directly or with the participation of a water molecule. Metalloproteases show maximal activity at about pH 7.0. Like the aspartic proteases, the metalloproteases include some nonspecific enzymes, such as thermolysin and others which have a more rigid substrate requirement e.g. mammalian collagenase. The activity of aminopeptidases, such as leucine aminopeptidase can be determined with various amino acids linked to colorimetric leaving groups (e.g. leupNA, metpNA). Dipeptidyl aminopeptidase I-IV are characterised by a broad substrate specificity for attack at the unsubstituted NH_2 terminus, to catalyse the removal of amino acids as dipeptides. Dipeptidyl aminopeptidases I and II have acid pH optima, while III and IV have optima at about pH 8.0 (McDonald *et al*, 1971). The range of specificity displayed by dipeptidyl aminopeptidases is broad: dipeptidyl aminopeptidase I (cathepsin C) attacks peptides generally, but prefers aromatic groups in the P_1 position. Dipeptidyl aminopeptidase II and III prefer alanine and arginine at P_1, respectively, while dipeptidyl aminopeptidase IV prefers a proline in this position (McDonald *et al*,

1971). Amongst this group of enzymes, only the presence of dipeptidyl aminopeptidase IV activity was determined, using APpNA. Cystenyl aminopeptidase hydrolyses when the penultimate amino acid is cysteine and BCpNA was used as substrate.

A metalloprotease is one where a metal ion (usually zinc) participates in catalysis. It does not include proteases such a calpains and some serine proteases (e.g. bovine trypsin) whose activities are dependent on the presence of calcium. Both zinc-dependent and calcium-stabilized proteases can be inactivated by chelating agents (Powers and Harper, 1986b) such as EDTA and EGTA. 1,10-phenanthroline is preferred to EDTA as a zinc metalloprotease inhibitor, as its stability constant is about 1000 times higher for zinc than for calcium. Bestatin, an inhibitor of microbial origin, inhibits leucine aminopeptidase (K_i for mammalian aminopeptidase is about 60nM) (Umezawa *et al*, 1976; Umezawa, 1982) and is also an effective inhibitor of aminopeptidase B (K_i = 20nM) (Suda *et al*, 1976). Other microbial inhibitors of exopeptidases include diprotin A and B, which have been isolated from *Bacillus cereus* (Umezawa *et al*, 1984). Both inhibit dipeptidyl aminopeptidase IV from rat kidney.

Gut exopeptidase activity has been studied in insects from other orders: Coleoptera (Christeller *et al*, 1989; Ferreira and Terra, 1989); Diptera, (Gooding and Rolseth, 1976; Billingsley, 1990); Hemiptera, (Houseman and Downe, 1981; 1983) and Orthoptera (Christeller *et al*, 1990), but reports from Lepidoptera are few (Lenz *et al*, 1991). Therefore, as part of the study on the enzymes of the midgut involved in protein digestion from the larvae of *Spodoptera littoralis*, the properties of the exopeptidases were investigated. The main study in this chapter characterises the enzymes involved in intermediate digestion, but the presence of enzymes involved in final digestion was also established.

CHAPTER 2
Materials and Methods

Preparation and assay of midgut homogenates

Midgut tissue was dissected, homogenized and lipid extracted from final instar larvae of *S. littoralis* as described in chapter 1. Ammonium sulphate precipitates were collected from lipid extracted homogenates following centrifugation at 10,000g and stored frozen at -18°C until required for further studies.

Other methods carried out in a similar manner to those described in full in the preceding chapter included gel filtration, anion-exchange chromatography, SDS-PAGE and subsequent blotting.

Enzyme assays

N-terminal exopeptidases.

Aminopeptidase, cystenyl peptidase and dipeptidyl aminopeptidase IV activities were investigated using a variety of substrates. Apart from the substrates and buffers, the methods of assay were as described for the determination of trypsin activity in chapter 1.

Aminopeptidase activity was routinely determined using 1mM L-leucine pNA (Tuppy, 1962) as a substrate in 50mM barbitone buffer at pH 8.0. The rate of hydrolysis was measured at 405nm and compared to a standard curve as described previously. This substrate was readily soluble in water; a 13mM stock solution was stored at 4°C to reduce the rate of autolysis which occurs at higher temperatures. The N-terminal preference of the aminopeptidase of *S. littoralis* was investigated by using several additional amino acid pNA substrates dissolved in water: AlapNA, ValpNA, ArgpNA and LyspNA. PropNA was dissolved directly in buffer. The remaining substrates were dissolved in DMF: PhepNA, GlypNA, MetpNA and GlupNA. The ten substrates included examples of aliphatic (gly, ala, val, leu), basic (arg and lys), acidic (glu), sulphur-containing (met), aromatic (phe) and heterocyclic (pro) amino acid residues.

Dipeptidyl aminopeptidase IV was investigated using APpNA (Nagatsu *et al*, 1976) at a final concentration of 1mM while the presence of cystenyl aminopeptidase was determined using BCpNA dissolved in DMF (Tovey *et al*, 1973). However, the solubility of BCpNA was limited to an assay concentration of 0.25mM in barbitone buffer.

C-terminal exopeptidases

The presence of CPA and CPB activities were determined using the peptidase substrates HPA (CPA substrate) and HA (CPB substrate). HPLA, an ester substrate hydrolysed by CPA, was also used in order to compare relative peptidase and esterase activities of CPA. Reaction progress of HPA, HPLA and HA hydrolysis was

determined continuously at 254nm and the rate of change of absorbance together with the molar extinction coefficient used to calculate the amount of substrate hydrolysis according to Sarath *et al* (1989). All carboxypeptidase substrates were dissolved in water at final assay concentrations of 1mM. The extinction coefficients (ε) used were: HPLA, 592 (McClure *et al*, 1964; Whitaker *et al*, 1966); HA, 360 (Wolf *et al*, 1962), HPA, 280 (Davies *et al*, 1968).

Inhibitor and activator studies

The effect of a variety of inhibitors was determined on the activity of the aminopeptidase including bestatin (Umezawa *et al*, 1976; Umezawa, 1982), Tris buffer (Pfleiderer, 1970), EDTA (Keesey, 1987) and 1,10-phenanthroline (Powers and Harper, 1986b). Earlier studies have shown that metal ions stabilize, activate or inhibit certain aminopeptidases (Smith and Hill, 1960). Therefore, the effect of certain metal ions on the activity of AP was also investigated using the following ions: Ag^{+}, Ca^{2+}, Cd^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Fe^{3+}, Hg^{2+}, Mn^{2+}, Pb^{2+} and Zn^{2+}.

An activity effector of mammalian esterase and peptidase CPA activity, 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) (Epstein and Navon, 1969; Frye and Sebastian, 1990) was used in conjunction with the CPA substrates, HA and HPLA.

CHAPTER 2
Results

1. Aminopeptidases

Distribution of LAP activity

Investigations into the distribution of LAP activity in midgut lumen and tissue, using LeupNA as substrate, showed that it was present in the midgut tissue only. Consequently, further studies on the N-terminal exopeptidases were confined to midgut tissue homogenates.

The effect of pH on activity of LAP

LAP activity was studied routinely with LeupNA as substrate (Wachsmuth *et al*, 1966a,b). Figure 2.1 shows the effect of pH on LAP enzyme activity from *S. littoralis* using 1mM LeupNA as substrate. The graph shows that the activity of the LAP is increased in the presence of barbitone buffer compared with other buffers, particularly at pH above the maximum (pH 8.0-9.0). In addition, activity at more alkaline pH was increased in the presence of BTP buffer, but to a lesser extent. pH maxima could not be obtained with certain buffers; they showed either continuously rising or falling activity with pH. These included borate, glycine, McIlvaine's and phosphate buffers. The maxima observed with Tris buffer (pH 7.5) is lower than that observed with BTP and barbitone buffers (pH 8.0-8.5). A similar pH profile for the hydrolysis of GlypNA is shown in Figure 2.2, however with this substrate, increased activity in the presence of barbitone buffer is not apparent. This may be a consequence of the presence of DMF in the reaction mixture.

The effect of pH on LAP stability

Stability of the enzyme with respect to pH was determined in a similar manner to that previously described (Chapter 1). However, at the end of the 30 minute pH incubation at 30°C, 3ml 50mM barbitone buffer was added and the pH brought to pH 8.0 by the addition of HCl or NaOH as appropriate. The final volume was then made up to 10ml with DDW. The results are shown in Figure 2.3. It can be seen that the activity of the LAP is stable over a smaller range of pH than the endopeptidases, and is mostly inactive outside the pH range 5.0-12.0. The observation of sub-maximal activity at pH 10.0-12.0 may be due to the presence of suboptimal buffers. e.g. comparison with figure 2.1 shows that borate buffer decreases the activity by about half compared to barbitone buffer at pH 8.0. Borate buffer was still present in the reaction mixture when activity was measured.

The effect of substrate concentration

Enzyme assays were as described in the chapter 1, but LeupNA was dissolved in DDW. Care was taken to ensure that substrate availability did not become rate-limiting.

Figure 2.1

The effect of pH on the activity of leucine aminopeptidase from the midgut of larval *S. littoralis* towards LeupNA.

Legend: ordinate - % activity
abscissa - pH

Figure 2.2

The effect of pH on the activity of midgut leucine aminopeptidase towards GlypNA.

Legend: ordinate - % activity
abscissa - pH

Figure 2.1

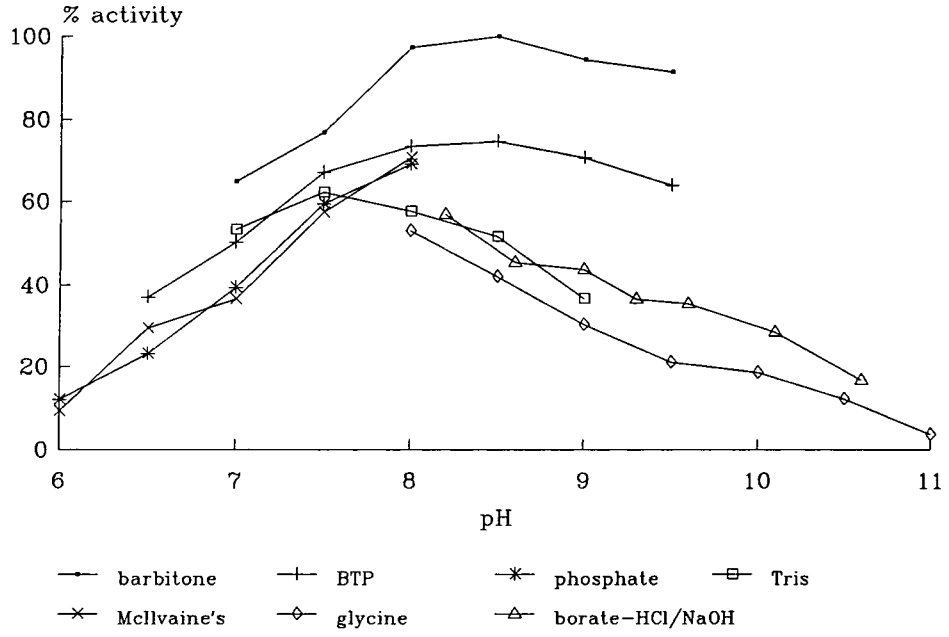


Figure 2.2

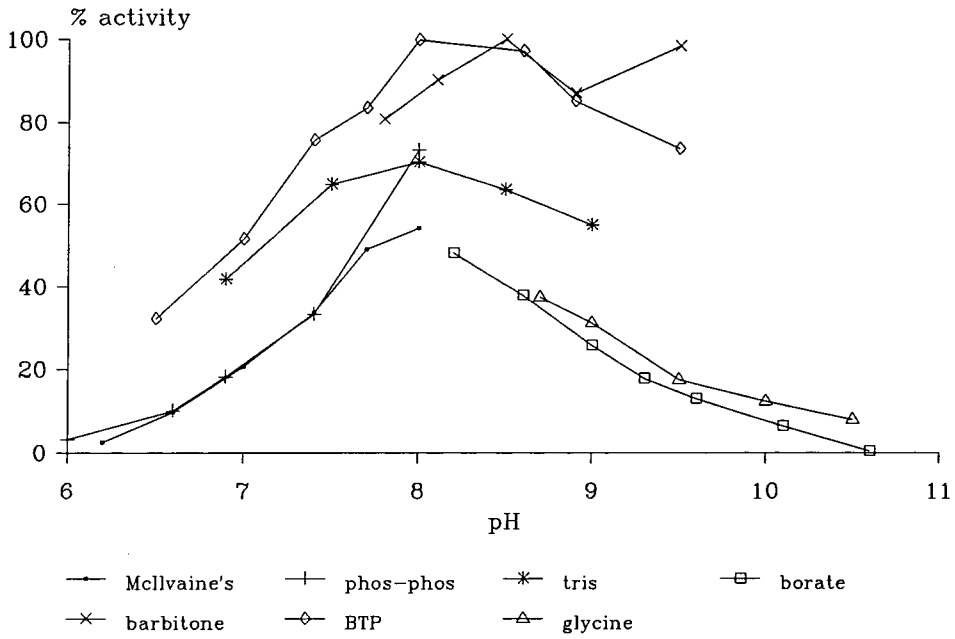


Figure 2.3

The effect of pH on the stability of leucine aminopeptidase. Enzyme preparation was incubated with buffer at different values of pH, over the range 1-13 for 30 minutes at 30°C. Subsequently, the pH was adjusted to pH 8.0 by the addition of an equal volume of barbitone buffer and HCl or NaOH as appropriate. The final volume was then made up to 10 ml with DDW and the activity measured.

Legend: ordinate - % activity
abscissa - incubation pH

Figure 2.4

The determination of leucine aminopeptidase stability during a continuous assay in a whole time course study. The release of product (pNA) was monitored continuously until the reaction was complete.

Legend: ordinate - relative amount product formed
abscissa - relative [enzyme] x time

Figure 2.3

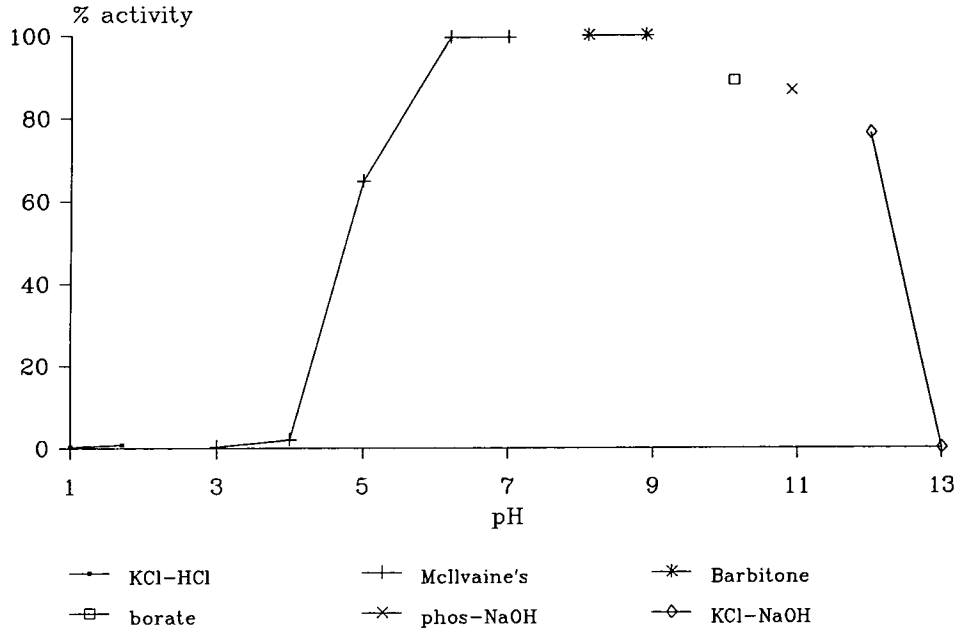
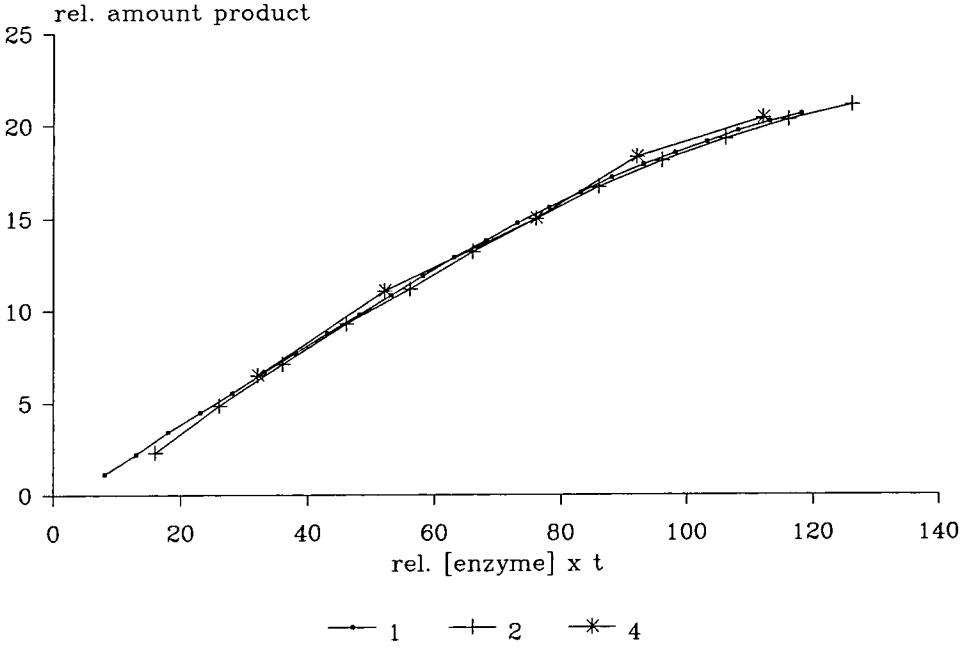


Figure 2.4



The mean K_m was 0.0756 $\pm$ 0.0021 mM and mean V_{max} was 17.81 $\pm$ 0.26 μmoles/min/mg protein using partially purified LAP from ion-exchange and S-200 chromatography columns.

The use of the integrated form of the equation in a whole time course study was valid according to Selwyn's test (1965) (Fig. 2.4). These progress curves were used to determine the change in V_{max} of LAP in crude homogenate with pH assayed in barbitone buffer. A plot of $\log_{10} V_{max}$ versus pH is of the form shown in Figure 2.5. Extrapolation of the linear portions of the plot gives the value of the pK_a at the point of intersection (Price and Stevens, 1989). Estimation of V_{max} using barbitone (pH 7.0-9.5) and McIlvaine's (pH 6.0-7.0) buffers and the pH range 6.0-9.5 indicates that only one ionizing group is active, presumably the unprotonated form and gives a pK_a value of 7.85.

The effect of temperature on the activity of LAP

Initially, the effect of temperature on enzyme stability was investigated. To this end, enzyme preparation was pre-incubated in barbitone buffer at pH 8.0 for 30 minutes over a range of temperatures (15-75°C). After this incubation, the preparation was returned to 30°C. The stability of the LAP from *S. littoralis* remained little altered by temperatures up to 30°C, but decline rapidly at higher temperatures (Fig. 2.6). No activity was present above 70°C.

The effect of temperature on the activity of LAP without preincubation was investigated as previously described. A temperature optimum of approximately 50°C was found over a 30 minute incubation period (Fig. 2.7) for LeupNA hydrolysis and the mean activation energy (E_a) was 38 $\pm$ 5 kJ/mole between 10°C and 30°C.

Partial purification of LAP

Midgut tissue homogenates were prepared and applied to gel filtration (S-200) and ion-exchange columns (Q-Sepharose) as described in Chapter 1. S-200 gel filtration of gut tissue homogenate revealed an activity eluting in the void volume (M_r > 250 kDa), but activity also eluted in a position indicating a smaller molecular weight (Fig. 2.8). Estimates of the molecular weight were 116 $\pm$ 11 kDa, obtained by comparison with a standard curve (see representative Fig. 1.2 shown in the methods, Chapter 1). It was not possible to carry out activity staining of blots from SDS-PAGE as, unlike the endopeptidases, the enzyme was totally inhibited by 0.1% SDS.

Anion exchange of crude gut tissue was also carried out; a representative example is shown in Figure 2.9. Ammonium sulphate precipitates of pooled active fractions were then applied to a S-200 column (Fig. 2.10). Active preparations were pooled and frozen for use in determining the kinetics of the partially purified enzyme against a variety of substrates.

A purification scheme for the enzyme is given below:

Figure 2.5

The effect of pH on the maximum velocity of the reaction of AP with LeupNA. The assays were performed at 30°C in 50mM barbitone buffer or McIlvaine's buffer and V_{max} determined from progress curves.

Legend: ordinate - V_{max} (μmoles product/minute/mg protein)
abscissa - pH

Figure 2.6

The effect of preincubation at various temperatures on the AP activity measured at 30°C. Enzyme solutions were buffered in 50mM barbitone buffer pH 8.0, incubated at the indicated temperature for 30 minutes and activity subsequently measured at 30°C.

Legend: ordinate - % activity
abscissa - temperature (°C)

Figure 2.5

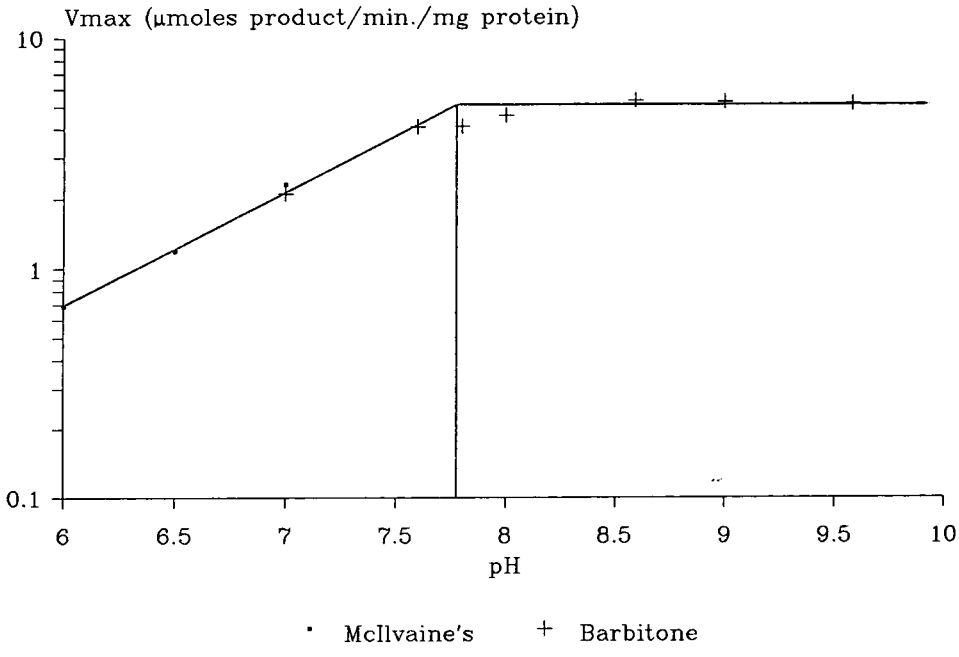


Figure 2.6

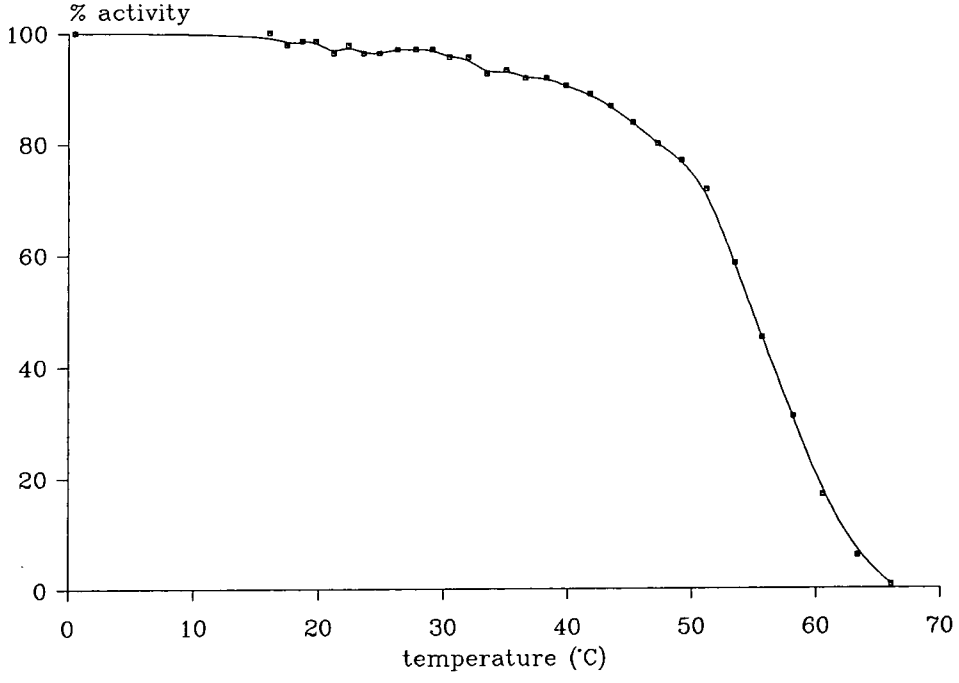


Figure 2.7

The effect of temperature on the activity of AP (Arrhenius plot). Aliquots of buffer were thermoequilibrated at the appropriate temperature in testtubes. The reaction was started by the addition of 0.2ml substrate and enzyme at timed intervals. After 30 minutes incubation, the reaction was stopped by the addition of 1.3ml 30% acetic acid.

Legend: ordinate - ln vel. (μM pNA/ minute/ mg protein)
abscissa - 1/temperature x 1000 (K)

Figure 2.8

The determination of AP molecular weight by gel filtration on Sephacryl S-200. Eluted fractions were tested for their activity towards LeupNA

Legend: ordinate (1) - protein (absorbance at 280nm)
(2) - activity
abscissa - fraction number

Figure 2.7

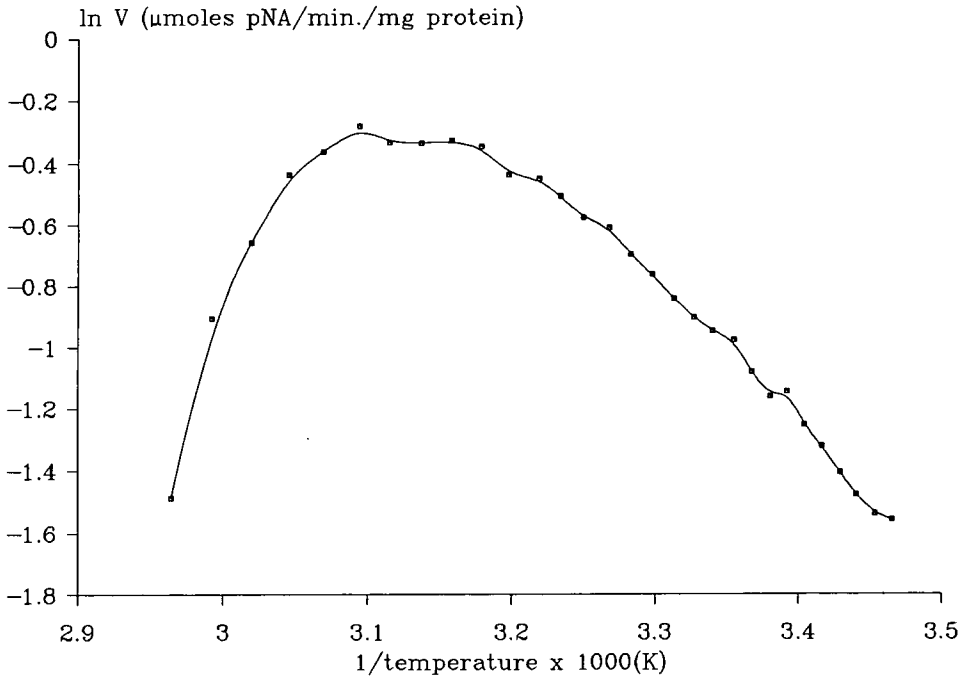


Figure 2.8

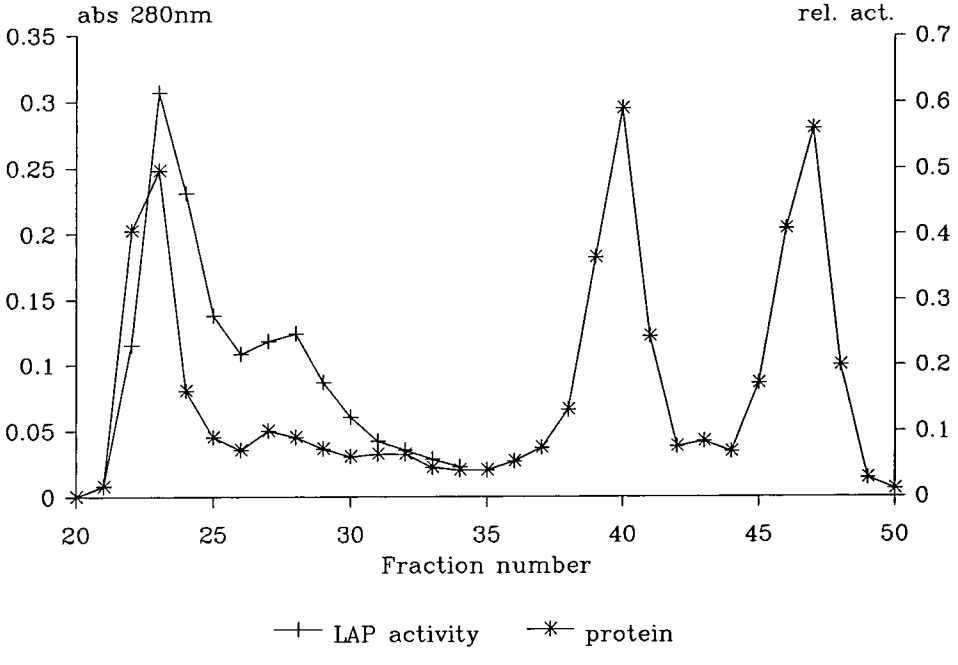


Figure 2.9

Anion exchange of dialysed ammonium sulphate precipitated proteins from *S. littoralis* larval midgut tissue on Q-Sepharose. Protein eluted by a NaCl gradient was measured by absorbance at 280nm and enzyme activity by LeupNA assays. The buffer used was 50mM barbitone pH 8.0.

Legend: ordinate (1) - protein or NaCl concentration (M)
(2) - rel. activity
abscissa - Fraction number

Figure 2.10

Further purification of LAP by gel filtration on Sephacryl S-200 following anion exchange chromatography. Eluted fractions were tested for their activity towards LeupNA

Legend: ordinate (1) - protein (absorbance at 280nm)
(2) - LAP activity, by LAP hydrolysis
abscissa - fraction number

Figure 2.9

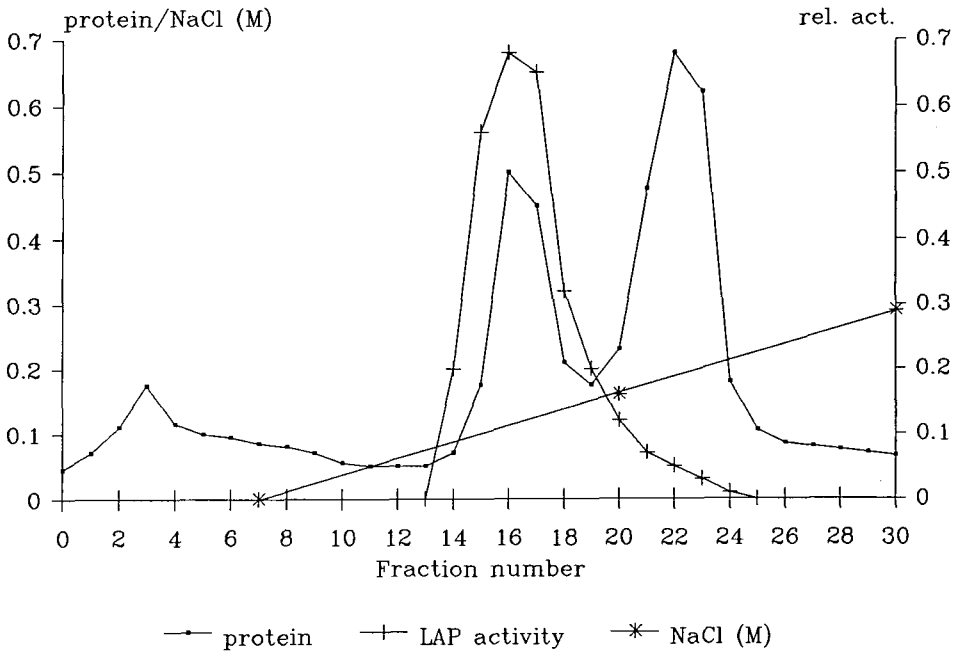


Figure 2.10

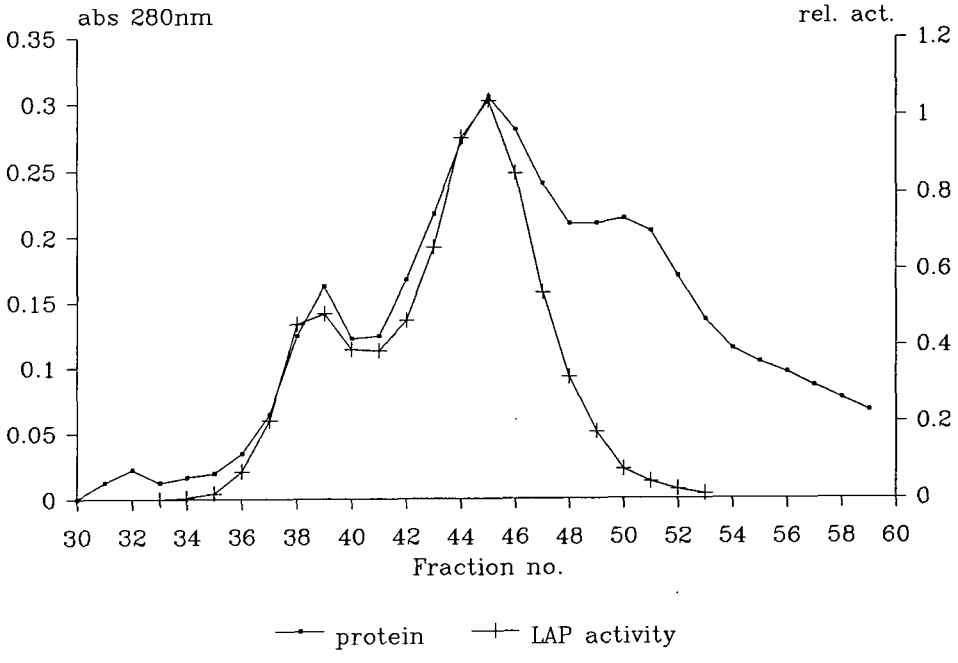


Table 2.1. Partial purification for LAP from *S. littoralis*

Step	Total activity μmoles/min	Total protein (mg)	Specific activity μmoles/min/ mg protein	Yield %
1. spnt after 10kg spin and $CHCl_3$ wash	1658	300	5.53	100
2. $(NH_4)_2SO_4$ spnt	21.88	21.14	1.0	1.3
3. $(NH_4)_2SO_4$ ppt.	1319	275	4.4	78.5
4. Q-Sepharose	410.2	37.1	11.1	24.4
5. S-200	160.9	18.3	19.1	10.0

Characterisation of LAP with other pNA substrates.

The N-terminal preference of the LAP was determined by using several amino acid-pNA substrates and making use of the partially purified leucine aminopeptidase preparation from anion exchange and gel filtration columns as described above. The kinetic parameters of *Spodoptera littoralis* midgut aminopeptidase is given in the table below determined from Lineweaver-Burk plots. All plots were linear; no examples of biphasic plots were demonstrated. Studies to determine the kinetics of the LAP were carried out using a single partially purified LAP preparation which was then diluted and aliquots stored frozen until required. This allowed the kinetics of the hydrolysis of the different substrates, particularly V_{max} and the relative specificity value $k(V_{max}/K_m)$, to be compared. Of all the kinetic parameters, k_{cat}/K_m ($= k \times V_{max}/K_m$) most accurately reflects the substrate specificity of the enzyme (Schellenberger *et al*, 1991).

It can be seen (Table 2.2) that the specificity of LAP was highest for LeupNA (rel. spec. = 235) followed by MetpNA (rel. spec. 150). V_{max} was highest using the substrates AlapNA and MetpNA (29.6 and 31.4 μmoles/min/mg protein respectively). Relatively high affinities were observed for all the substrates tested (K_m <0.7mM) except for propNA; LAP had the highest affinity for the substrate LeupNA (75.6μM) and the lowest for the heterocyclic amino acid propNA (K_m = 9.90 ± 1.33mM). Hydrolytic activity against glupNA was extremely low (< 1/750 of V_{max} with MetpNA) and could only be detected by long assay periods. Some substrates were dissolved in DMF while others were dissolved in DDW. Some enzymes are sensitive to the concentration of DMF (e.g. the chymotrypsin enzyme from *S. littoralis*). However, 7.7% DMF (the final [DMF] for assays involving DMF-solubilised substrates) did not affect LAP hydrolysis of LeupNA. Therefore, it is assumed that the observed differences in activities of LAP against substrates dissolved in DDW or DMF are a consequence of the change in the N-terminal amino acid only.

Table 2.2. Kinetic parameters of LAP from *S. littoralis*

Amino acid characteristics of the substrate			
Aliphatic	K_m (mM)	V_{max}	Rel. specificity
GlypNA*[2]	2.51 ± 0.45	0.84 ± 0.25	0.3
AlapNA#[1]	0.62 ± 0.02	29.6 ± 0.3	47.9
ValpNA#[1]	0.49 ± 0.03	2.1 ± 0.1	4.3
LeupNA#[1]	0.076± 0.002	17.8 ± 0.3	235.6
Basic			
ArgpNA[1]	0.35 ± 0.05	8.0 ± 0.1	23.0
LyspNA[1]	0.38 ± 0.03	6.6 ± 0.2	17.4
Acidic			
GlupNA[2]	0.21 ± 0.01	0.04 ± 0.01	0.2
S-containing			
MetpNA#[2]	0.21 ± 0.02	31.4 ± 0.4	150.2
Aromatic			
PhepNA#[2]	0.43 ± 0.07	11.9 ± 1.1	27.4
Heterocyclic			
PropNA#[1]	9.90 ± 1.33	2.8 ± 0.17	0.3

nonpolar

* polar, uncharged

V_{max} determined as (μmoles/min/mg protein).

Rel. spec. (relative specificity) was determined by dividing V_{max} by K_m. V_{max}/K_m is equal to $[E]_0 \times k_{cat}/K_m$, where $[E]_0$ is the molar concentration of the active sites in the solution. This latter ratio is perhaps the best indication of the specificity of an enzyme for a particular substrate (Schellenberger *et al*, 1991).

[1] substrate dissolved in DDW

[2] substrate dissolved in DMF (final concentration = 7.7%)

Effects of different substances on LAP activity

The effect of a variety of different substances on LAP activity was determined. These included a wide variety of serine, thiol and metalloprotease inhibitors and a number of metal ions.

The following protease inhibitors had no effect on the activity of the LAP at concentrations sufficient to inhibit endopeptidase enzymes (given in brackets): (a) serine protease inhibitors: PMSF (5 mM), TPCK (0.25 mM), TLCK (1 mM), TEW (3.6μM), SBTI (25μM), BBTI (12.7μM), LBTI (11.1μM), aprotinin (3.1μM), benzamidine (0.64mM), chymostatin (70μM), α_1-antiproteinase (10μM) and CEOI (0.65μM); (b) thiol/cysteine protease inhibitors: E-64 (200μM), leupeptin (20μM), NEM (5mM), iodoacetic acid (1mM) and pCMPS (0.675mM). Activity was measured at pH 7.0 in 50mM BTP buffer. DTT inhibited the LAP, but at high concentrations only (see Fig. 2.11), while the metalloprotease inhibitor EDTA proved relatively ineffective (< 10%) at 10mM (Fig. 2.12). The inhibition of LAP by 1,10-phenanthroline, a zinc chelator, was nearly 100%, but at high concentrations only (100mM). This is also reflected in the high I_{50} = 7mM.

The effects of different Tris buffer concentrations on the hydrolysis of LeupNA were investigated. Tris had little effect on LAP activity at concentrations below 0.25 M although marked inhibition was seen above this concentration (see Fig. 2.13).

The effect of a variety of metal ions on the activity of the LAP in 50mM Tris buffer at pH 7.5 (the optimum pH for this buffer) was studied. The following metal ions did not change the activity up to the maximum concentration shown: Mo (VI) (2mM), Co^{2+} (2mM), Mg^{2+} (10mM), Fe^{2+} (1mM) and Fe^{3+} (2mM).

The more effective inhibitory ions on LAP activity are shown in Figs. 2.14 and 2.15 and the table below.

Table 2.3. I_{50} for LAP inhibitory metal ions

Metal ion	I_{50} (mM)	Maximum inhibition(%)	Inhibitor conc. (mM)
Ag^{+}	-	31	1
Cd^{2+}	0.4	70	1
Cu^{2+}	0.58	100	10
Hg^{2+}	1.3	55	2
Mn^{2+}	20	52	20
Pb^{2+}	0.05	100	0.5
Zn^{2+}	0.17	100	2

The effect of bestatin on the LAP activity, an aminopeptidase inhibitor of microbial origin (Umezawa, 1982), is shown in Fig. 2.16. This was the most effective

Figure 2.11

The effect of dithiothreitol (DTT) on AP activity. Activity was determined in barbitone buffer pH 8.0 at 30°C. The pH was corrected for changes which occurred due to the presence of DTT.

Legend: ordinate - % activity
abscissa - mM DTT

Figure 2.12

The effect of EDTA and 1,10-phenanthroline on the activity of AP.

Legend: ordinate - % activity
abscissa - mM inhibitor

Figure 2.11

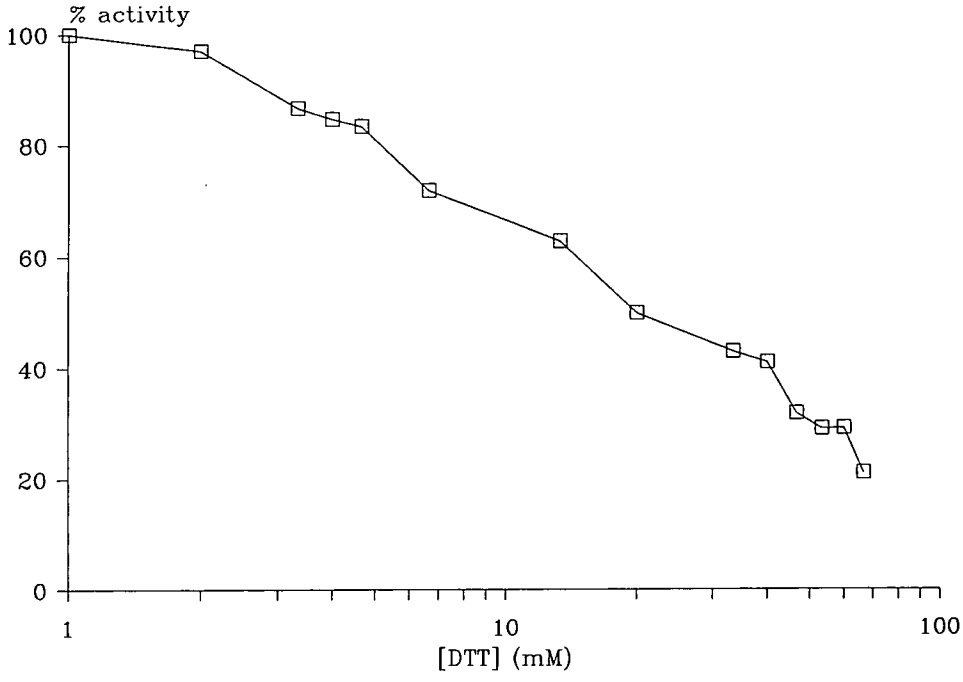


Figure 2.12

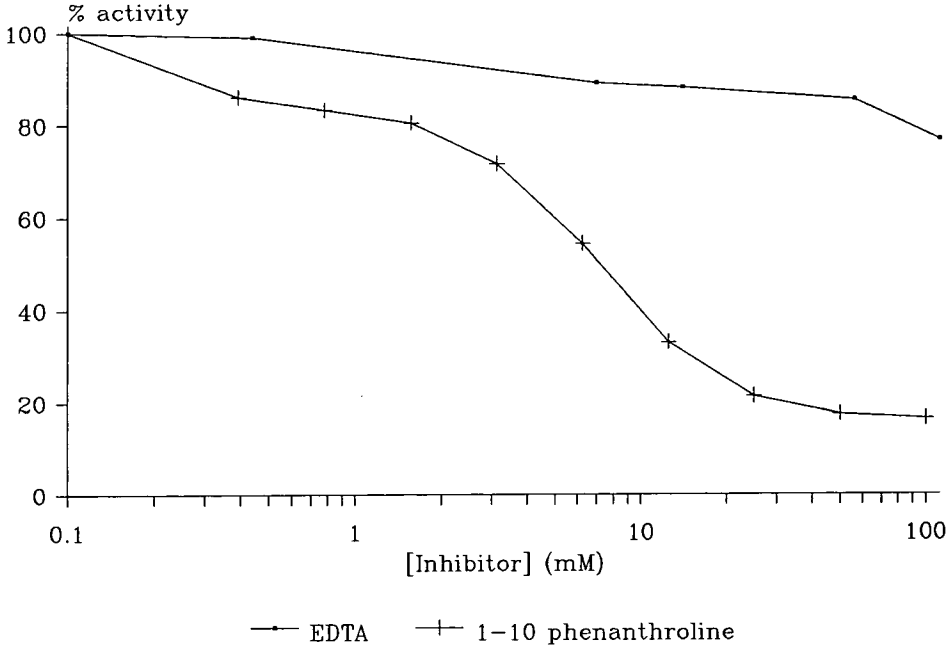


Figure 2.13

The effect of different Tris buffer concentrations on the activity of AP at pH 8.0.

Legend: ordinate - % activity
abscissa - mM Tris.

Figure 2.14

The effect of silver (I), mercury (II), zinc (II), lead (II) and cadmium (II) ions on the activity of AP. Ag^{+}, Zn^{2+} and Pb^{2+} were dissolved in Tris-HNO_3 rather than Tris-HCl, in order to maintain their solubility. Unlike some enzymes, such as α-amylases (Hori, 1969), the aminopeptidase activity did not appear to be affected by this change in the buffer.

Legend: ordinate - % activity
abscissa - mM metal ion

Figure 2.13

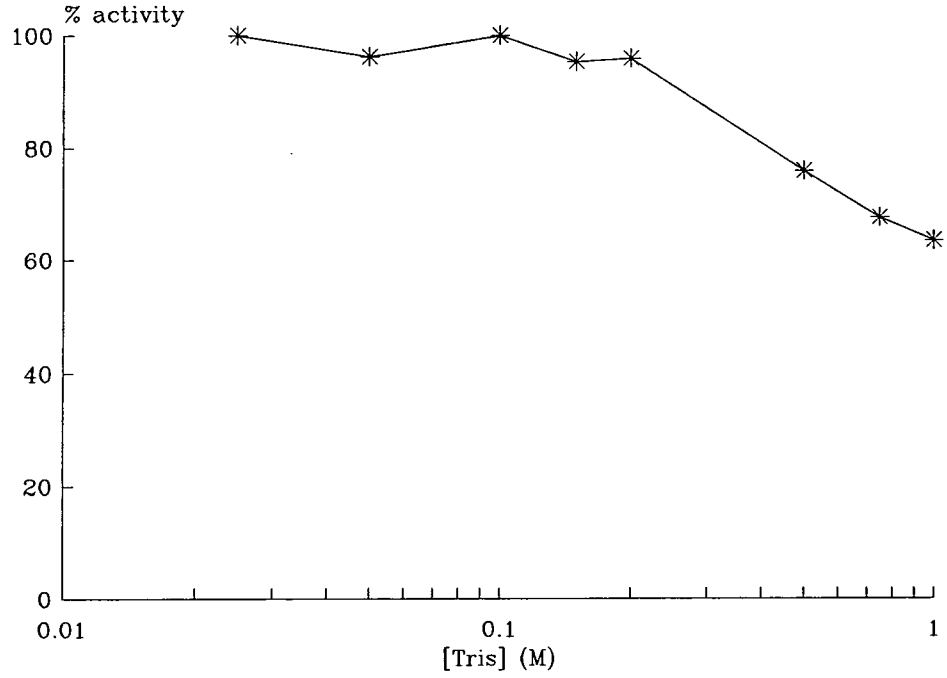


Figure 2.14

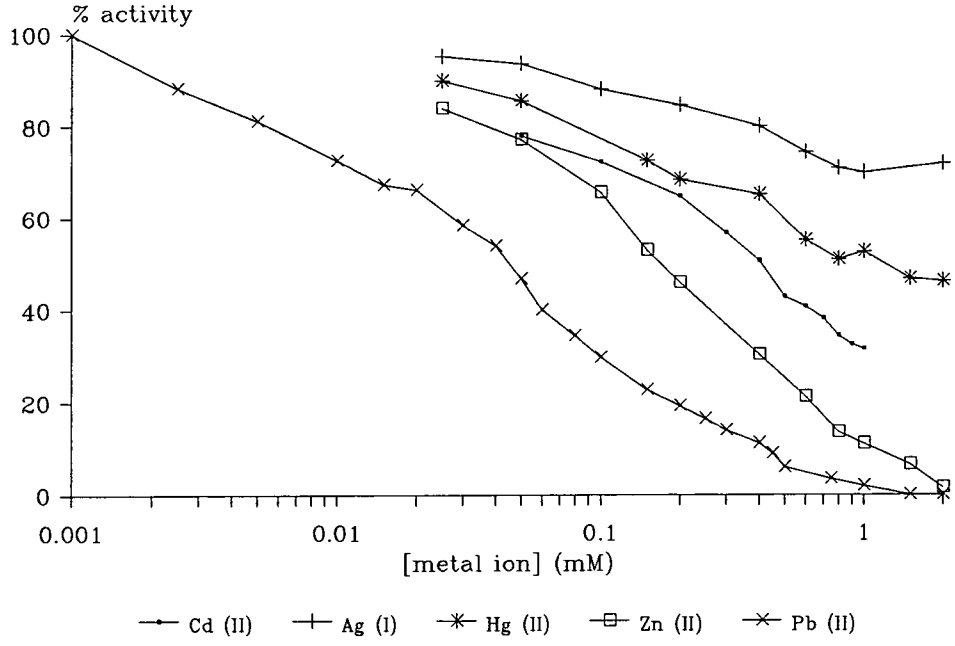


Figure 2.15

The effect of copper (II) and manganese (II) on the activity of AP.

Legend: ordinate - % activity
abscissa - mM metal ion

Figure 2.16

The effect of bestatin on the activity of AP.

Legend: ordinate - % activity
abscissa - μM bestatin

Figure 2.15

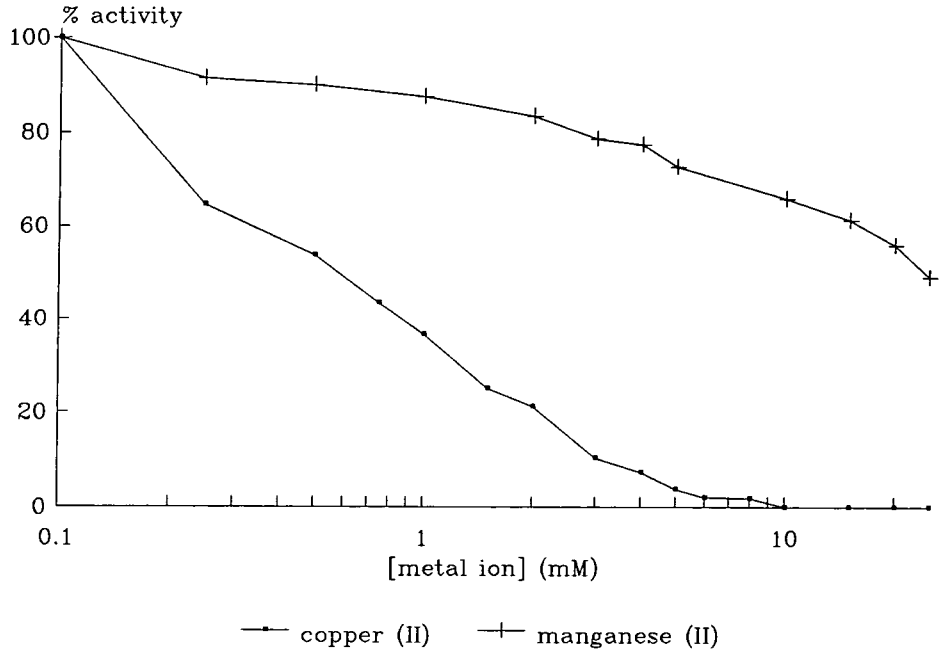
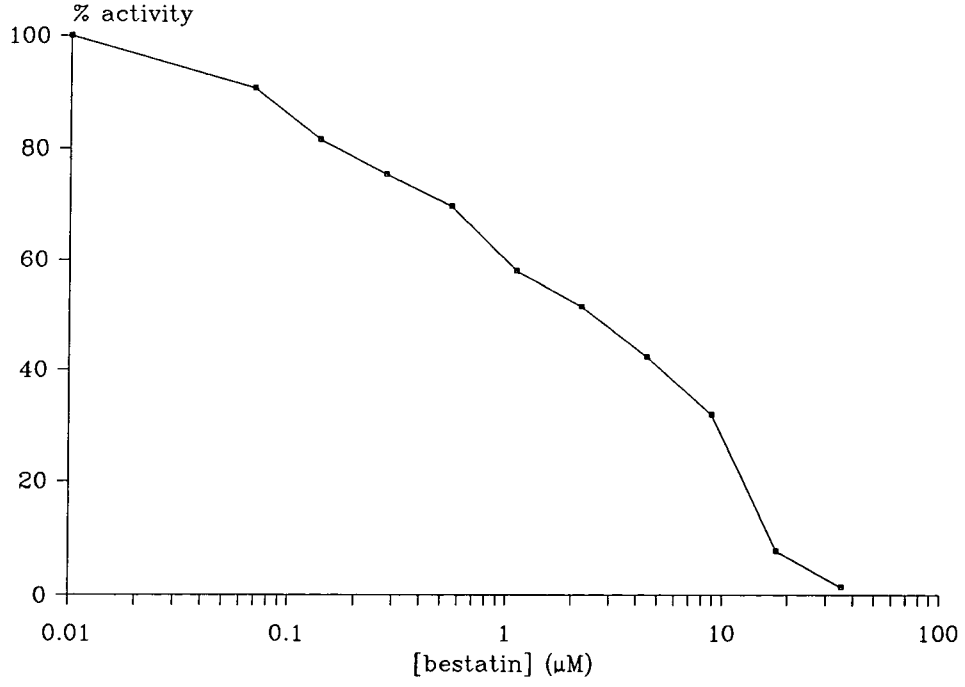


Figure 2.16



inhibitor of this enzyme of those tested, with an I_{50} of 2.3μM.

Other aminopeptidases of the midgut tissue

The presence of activity for the substrates BCpNA and APpNA was demonstrated which may indicate the presence of a cysteine aminopeptidase (Tovey *et al*, 1973) and a dipeptidyl aminopeptidase IV (Nagatsu *et al*, 1976). Both activities were present in the midgut tissue only.

Effect of pH and substrate concentration

The maximal activity of both the BCpNA-ase and the APpNA-ase occurred at a similar pH to that of LAP (about pH 8.0) (figs. 2.17 and 2.18). At this pH their activity was lower relative to that of the LAP in crude gut homogenates using 0.1mM substrate (relative activity of LAP: cystenyl LAP: dipeptidyl aminopeptidase IV = 4.8: 1: 1.2). The exopeptidase activity towards APpNA was lower in glycine buffer compared with BTP buffer (Fig. 2.17). The affinities of the enzymes for APpNA hydrolysis (K_m = 32.0μM) and BCpNA hydrolysis (K_m = 62.5μM) (see Fig. 2.19) were similar to the LAP affinity for LeupNA (K_m = 75.6μM). S-200 chromatography of gut tissue eluted the proteins capable of hydrolysing LeupNA and BCpNA coincidentally as two peaks, one in the void. This would arise if the two proteases were of similar M_r or if the hydrolysis of both substrates was due to just one enzyme.

2. Carboxypeptidases

Carboxypeptidase activity was investigated with substrates for CPA and CPB activity. The substrate HPLA was used to study CPA esterolytic activity. The effect of pH and different buffers on the activity of HPLA hydrolysis is shown in Fig. 2.20. At overlap points of the different buffers it can be seen that the activity of the enzyme was reduced using glycine buffer compared with the other buffers. Other buffers, such as barbitone, were unsuitable due to light absorption at 254nm. In addition, HPLA activity was not observed in the presence of McIlvaine's and phosphate buffers. An attempt was made to inhibit activity using DSS, a CPA esterase activity inhibitor (Frye and Sebastian, 1990). However, no decrease in the rate of HPLA hydrolysis was observed in the presence of 1-15mM DSS. The presence of CPA and CPB amidolytic activity could not be detected with the peptide substrates, HPA and HA, either in the presence or absence of DSS (DSS is a CPA peptidase stimulator, Epstein and Navon, 1967). This indicates that the HPLA activity could be a result of midgut esterases, rather than a peptidase, as peptidase activity could not be demonstrated at pH 7.0-10.0. Gel filtration indicated that the M_r of the HPLA hydrolysing enzyme was about 40,000Da and eluted in a position close to the chymotrypsin enzyme (Fig. 2.21) or slightly earlier (i.e. indicating a higher molecular weight). However, further studies on this enzyme were not continued, in view of the lack of evidence for any carboxypeptidase activity.

Figure 2.17

The effect of pH on the activity of dipeptidyl aminopeptidase IV using APpNA as substrate.

Legend: ordinate - % activity
abscissa - pH

Figure 2.18

The effect of pH on the hydrolysis of BCpNA.

Legend: ordinate - % activity
abscissa - pH

Figure 2.17

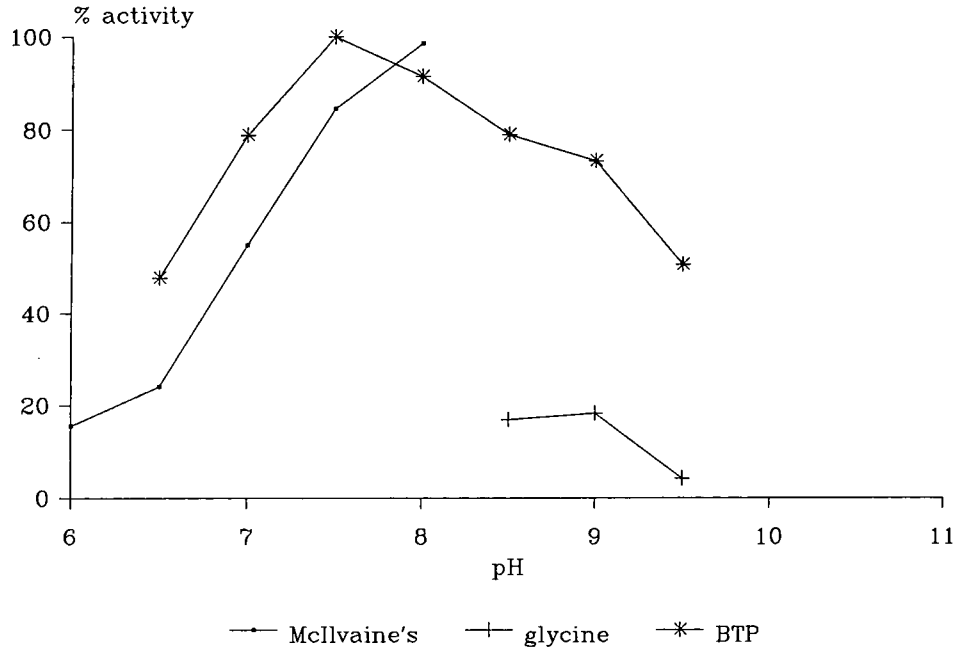


Figure 2.18

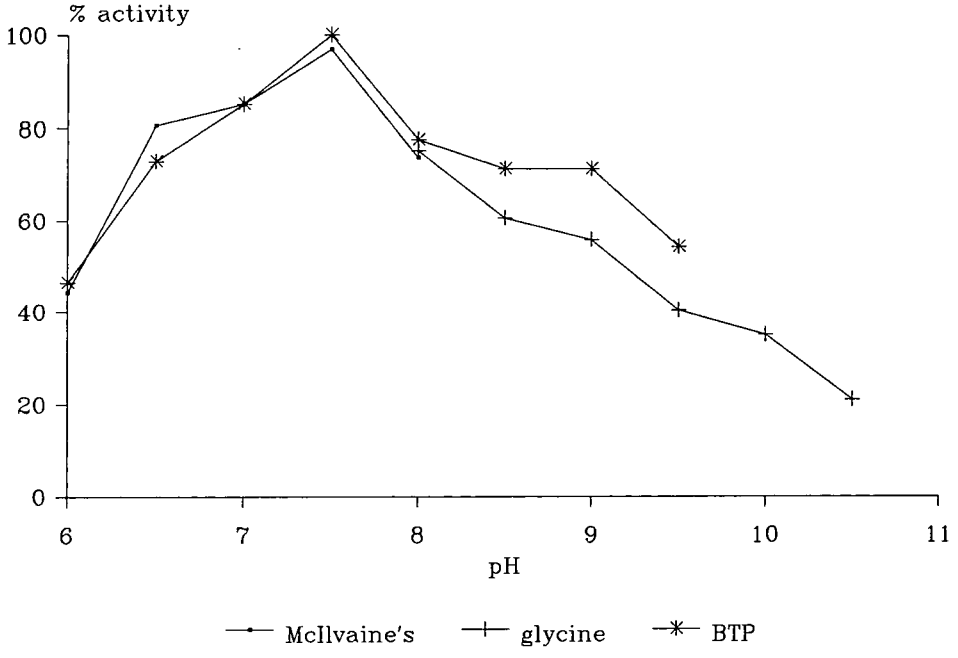


Figure 2.19

The effect of substrate on the hydrolysis of BCpNA and APpNA.

Legend: ordinate - 1/rel. velocity
abscissa - 1/[Substrate]

Figure 2.20

The effect of pH on the hydrolysis of HPLA. The rate of substrate hydrolysis was determined by the change in absorbance at 254nm.

Legend: ordinate - % activity
abscissa - pH

Figure 2.19

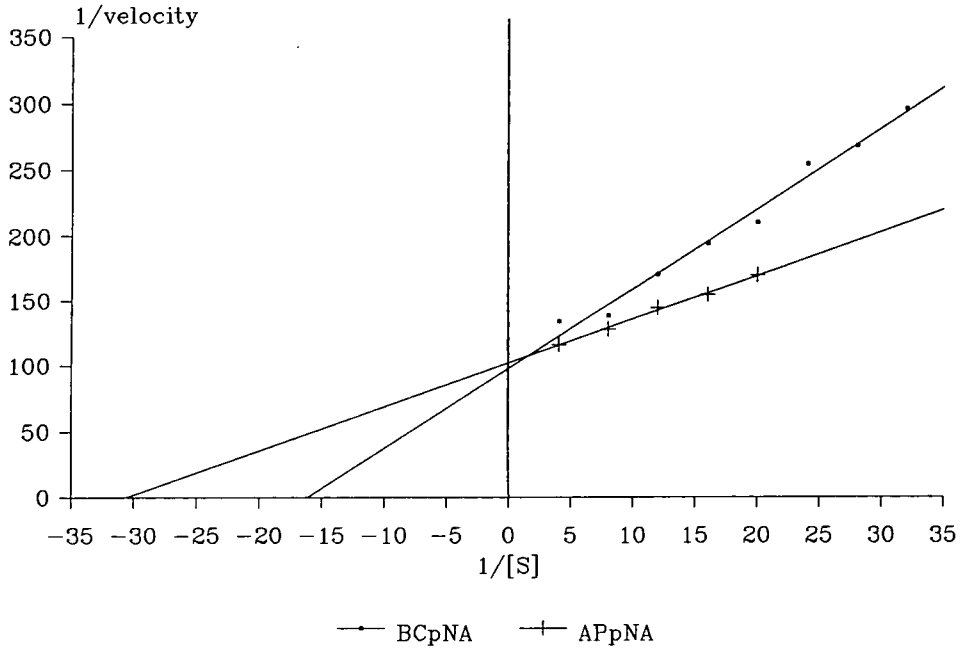


Figure 2.20

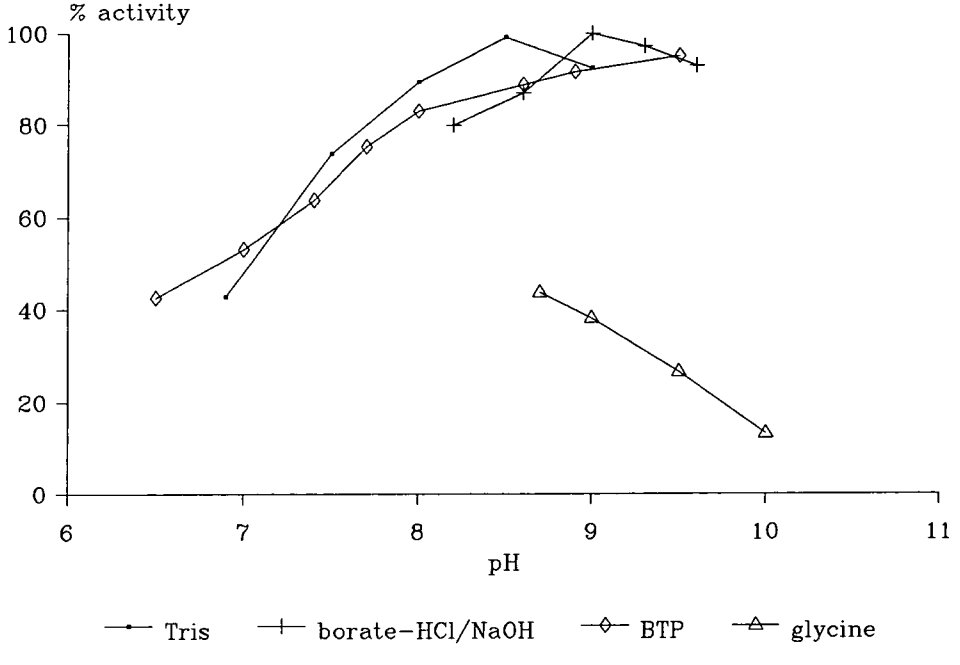


Figure 2.21

The determination of the molecular weight of the hydrolysing enzyme of HPLA on Sephadex G-75.

Legend: ordinate (1) - rel. act.
(2) - protein
abscissa - fraction number

Figure 2.22

The effect of small deliberate errors ($\pm$ 1-3%) in the estimation of the endpoint of progress curves.

Legend: ordinate - $[S]_o$-[S]/t
abscissa - $1/t.\ln[S]_o/[S]$

Figure 2.21

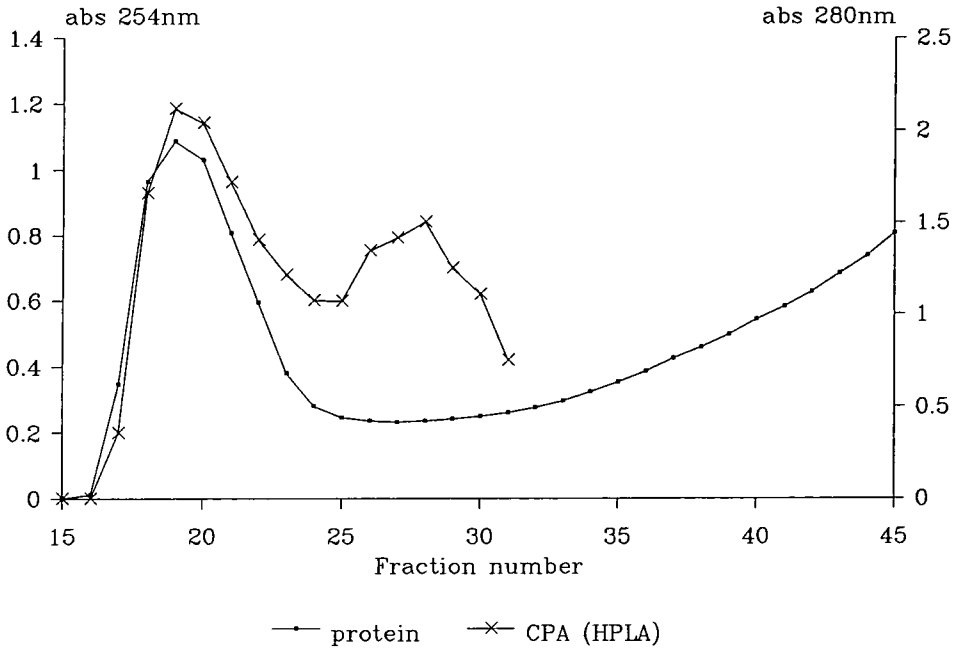
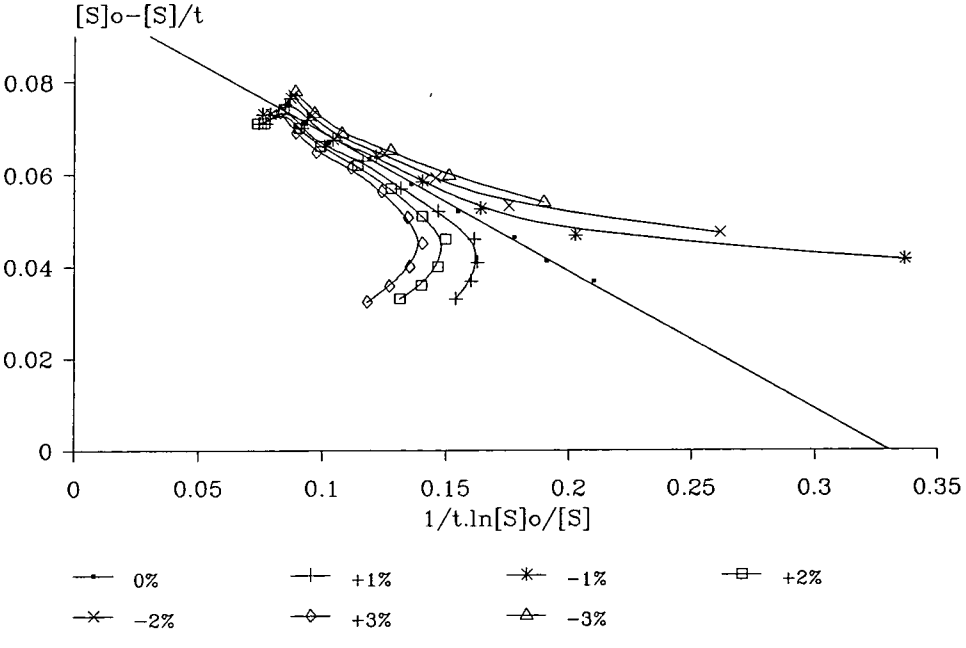


Figure 2.22



CHAPTER 2
Discussion

Aminopeptidase activities towards LeupNA, BCpNA and APpNA are maximally active at an alkaline pH (about pH 8.0) and together are able to hydrolyse a broad range of substrates. Figure 2.1 shows that the activity was markedly different in different buffers. Barbitone buffer, a protector of aminopeptidase activity from inhibitors (Moseley and Melius, 1967), caused stimulation of activity compared with the other buffers, particularly alkaline to the maximal pH. This could be compared with the activity of insect carbohydrases which is broader in the presence of Cl^- and NO_3^- (Hori, 1969). The dependence of mammalian aminopeptidase activity on metal ions has been demonstrated (Marks and Lajtha, 1970), but insect LAP activity was not increased by the presence of added metal ions. The effect of metal ions on LAP activity was determined in Tris buffer rather than barbitone buffer as this is known to reduce the sensitivity of aminopeptidases to certain compounds (Moseley and Melius, 1967). It was most affected by Pb^{2+} and Zn^{2+} (I_{50} values were 50μM and 170μM respectively), but activity was most notably unaffected by Mg^{2+} and inhibited by Mn^{2+}; both ions are stimulators of LAP enzymes from other sources (Delange and Smith, 1971). A further distinguishing feature of this enzyme is inhibition by Zn^{2+} (50% inhibition at 0.17mM); this ion has little or no effect on activity of swine LAP at 1mM concentration (Delange and Smith, 1971).

As already mentioned, aminopeptidases have been reported previously from different insect sources, although not as frequently as endopeptidase enzymes. The activity of the leucine aminopeptidase may be responsible for BCpNA hydrolysis in gut tissue homogenates from *S. littoralis* as activities eluted from an S-200 column coincidentally, but comparison with other reports is not possible; cystenyl aminopeptidases and dipeptidyl aminopeptidase IV have been reported infrequently in insects (e.g. Ferreira and Terra, 1984; 1989). The hydrolysis of BCpNA by LAP is paralleled in mammals; indeed, Tovey *et al* (1973) found that BCpNA could be attacked by LAP from serum. Recently, Tsujimoto *et al* (1992) have shown that the human placental LAP is identical with cystine aminopeptidase (also called oxytocinase). Therefore, hydrolysis of BCpNA and LeupNA is probably caused by only one insect enzyme. However, it can be seen from Figures 2.17 and 2.18 that the hydrolysis of APpNA is significantly reduced in the presence of glycine buffer and it is suggested that the aminopeptidase responsible for APpNA hydrolysis is different to that of the LeupNA and BCpNA hydrolysing enzyme.

Billingsley (1990) showed that the activity of the midgut aminopeptidase from the mosquito *Anopheles stephensi*, unlike the LAP from *S. littoralis*, was stimulated by EDTA and Mg^{2+}, but its inhibition by Tris and Mn^{2+} is similar. LAP from *A. stephensi* (Billingsley, 1990) and *Manduca sexta* (Brookhart and Kramer, 1990) were much more sensitive to 1,10-phenanthroline. I_{50} values were $<$ 0.02mM and 0.4mM respectively

compared with LAP from *S. littoralis* (I_{50} = 7mM). However, it has been shown that barbital buffer protects mammalian leucine aminopeptidase from inactivation by EDTA (Moseley and Melius, 1967) and it is conceivable that this buffer may be useful in stabilisation of the enzyme under other conditions as well (Delange and Smith, 1971) or that protection is also served by BTP buffer, which leads to this difference between the apparent susceptibility of these insect enzymes to metal chelators. Tris buffer, a known competitive inhibitor of LAP (Ferreira and Terra, 1984), also inhibited this enzyme with a susceptibility similar to the LAP from *Anopheles stephensi* (Billingsley, 1990).

The M_r of the aminopeptidase from *S. littoralis* (116 $\pm$ 11 kDa) is generally smaller than those from mammalian sources which are about 326 kDa (Keesey, 1987), but compare more favourably with those from other insect sources. Christeller *et al* (1989, 1990) reported the presence of LAP from the grass grub, *Costelytra zealandica* and from the black field cricket, *Teleogryllus commodus* with an optimum pH of 8.0 and M_r of 90-97 kDa and 94 kDa respectively. An M_r estimate of 114 kDa for the AP from *Teleogryllus commodus* as determined by Sephadex G-75 gel filtration, while nearly coinciding with the M_r of the LAP reported here for *S. littoralis*, must be treated with caution as determination by this gel is limited to proteins with an M_r <80 kDa (Pharmacia LKB, 1991). The size of AP from *Anopheles stephensi* was similar at 123kDa (Billingsley, 1990). However, it had a relatively low affinity for the substrate LeupNA (K_m = 1.55mM), compared with other insect AP enzymes e.g. *Attagenus megatoma,* (K_m = 0.32mM) (Baker, 1981) or LeußNA, e.g. *Bombyx mori*, (K_m = 0.34mM)(Sumida and Eguchi, 1983) which in turn were lower than the affinity of the LAP from *S. littoralis* for LeupNA (K_m = 76 μM). Other aminopeptidases of various sizes have been reported from *A. megatoma* and *Pheropsophus aequinoctialis* at 126kDa and 110kDa respectively (Baker, 1981; Ferreira and Terra, 1989) and in tsetse flies at 142-171kDa depending on the species of the insect (Cheeseman and Gooding, 1985).

The optimal pH (pH 8.0) of LAP from S. littoralis is similar to the optima found for other aminopeptidases. Most insect LAP enzymes appear to have optimal pH in the alkaline region 7.5-8.0 (e.g. Billingsley, 1990; Ferreira and Terra, 1989; Houseman and Downe, 1981; Gooding and Rolseth, 1976), which is similar to mammalian aminopeptidases (Wachsmuth *et al*, 1966a,b; Himmelhoch, 1970; Serafin *et al*, 1991). The pK_a of LAP was 7.85 as determined using progress curves (see materials and methods). The use of progress curves as a means of determining Michaelis-Menten kinetics has come under much scrutiny. The chief problem associated with this method is the estimate of the endpoint. An example of the effect of small errors in the estimation of the endpoint are given in Figure 2.22. This shows that such errors lead to serious deflections in the linearity of the line. Wharton (1983) and Moreno (1985) suggest that provided this can be resolved then this method is appropriate. Wharton (1983) recommends seven half-lives to elapse in order to reduce the end-point error. In addition, Atkins and Nimmo (1973) concluded that K_m and V_{max} could be determined from progress curves in the absence of systematic error (large deviations in the line

result from only small errors in the rate equation). The use of progress curves for kinetic analysis was deemed appropriate for LAP, which was stable according to Selwyn's test (1965) (Figure 2.4). Reports of pK_a of LAP from insects for comparison with the LAP of *S. littoralis* are few; Ferreira and Terra (1984) reported the pK_a of the aminopeptidase from *Rhynchosciara americana* to be 6.5. The pK_a value described for *S. littoralis* may be higher as a consequence of the buffers used; Ferreira and Terra (1984) used phosphate and borate buffers. Aminopeptidase from S. littoralis has an apparent optimum pH in borate buffer lower than pH 8.0 (Fig. 2.1) whereas barbitone buffer resulted in a more alkaline pH optimum which may be reflected in the higher pK_a.

Activity of the LAP in *S. littoralis* shows several similarities to its cleavage pattern compared with other insects: *Aedes aegypti* (Graf and Briegel, 1982) and *A. stephensi* (Billingsley, 1990). In all three, the enzymes show a preference for nonpolar amino acids (alanine, leucine and methionine). As described here, other insect aminopeptidases do not cleave acid amino acid substrates easily (Billingsley, 1990; Ferreira and Terra, 1984). In addition, the Michaelis constants of pNA substrate hydrolysis by LAP from *S. littoralis* are similar to those of the pig kidney AP (Wachsmuth *et al*, 1966b). It was shown that the rate of 1mM LeupNA hydrolysis by AP from *S. littoralis* was not altered significantly by the maximum [DMF] used to solubilize certain substrates (7.7%), whereas organic solvents are known to affect activity of AP enzymes from other sources (Wachsmuth *et al*, 1966b) e.g. DMF effects activity of LeupNA hydrolysis in a competitive manner. Inhibitors of other classes of hydrolase, such as serine protease inhibitors, did not have any effect on the activity of the LAP from *S. littoralis*, demonstrating that substrate hydrolysis was not due to the presence of contaminating endopeptidases. Neither pCMPS ~~and~~ nor iodoacetate changed the activity of AP, which contrasts with pig kidney aminopeptidase (Wachsmuth *et al*, 1966a) and may indicate the absence of functional SH groups (Pfleiderer *et al*, 1964).

Aminopeptidase activity was located in the midgut tissue in *S. littoralis*. Similarly, AP activity is reported in midgut tissue of various other species of insects; it has been located in the midgut cells of *Glossina morsitans* (Gooding and Rolseth, 1976; Gooding, 1977). The localization of aminopeptidases has been studied in a number of lepidopteran larvae and has been shown to be bound to the microvillar membranes of columnar cells in *Erinnyis ello* (Santos *et al*, 1986), in *Manduca sexta* (Wolfersberger, 1984) and in *Bombyx mori* (Sumida and Eguchi, 1983). Aminopeptidase activity is present on the midgut microvillar membranes of the hemipteran, *Rhodnius prolixus* (Billingsley and Downe, 1985). In *Drosophila melanogaster* aminopeptidase activity was associated with the cell membranes of the midgut and Malpighian tubules (Walker and Williamson, 1980). AP activity may be widely distributed within the insect body and have a variety of other functions other than intermediate and final digestion. It has been shown that the activity of AP from the Malpighian tubules and hindgut may be involved in the breakdown of adipokinetic hormones (Siegert and Mordue, 1992) and

neuropeptides (Puiroux and Loughton, 1992). In addition, AP activity has been reported in the intestinal lumen of *Haemopis medicinalis* and *H. sanguisuga* (Van der Lande, 1972) and in the haemolymph and midgut lumen of *D. melanogaster* (Walker *et al*, 1980) and from the haemolymph of *Calliphora erythrocephala* (Collett, 1989). However, hydrolysis of LeußNA and subsequent histochemical localization by the method of Seligman *et al* (1970) as described by Walker *et al* (1980) and Billingsley and Downe (1985) does not necessarily indicate aminopeptidase activity, as several enzymes are able to hydrolyse this substrate (Patterson *et al*, 1963). In *Rhynchosciara americana*, soluble aminopeptidases are involved in the terminal digestion of proteins in the ectoperitrophic space (Ferreira and Terra, 1982; 1984). This suggests that the midgut aminopeptidase is not only a membrane-bound enzyme (Gooding, 1977), but may also be secreted. However, as already mentioned, the presence of secreted aminopeptidases was not noted in the midgut of *S. littoralis*. Certain AP activities however, involved in digestion in fluid-filled spaces, have been due to membrane-bound aminopeptidases e.g. in the ectoperitrophic space of *Erinnyis ello* (Santos *et al*, 1983; 1984).

Activity of carboxyesterase activity was determined using HPLA. It was found that activity was not present in some buffers, namely phosphate, McIlvaine's, citrate and barbitone. Barbitone buffer is unsuitable due to its absorption at wavelengths below 260nm. Christeller *et al* (1990) also found that the HPLA hydrolysing enzyme from the black field cricket was inhibited by certain buffers (tricine and CAPS). Attempts at decreasing the carboxyesterase activity from *S. littoralis* with DSS (Frye and Sebastian, 1990) were not successful and this sulphonate did not increase the activity of the enzyme towards peptidase substrates as reported by Epstein and Navon, (1969). Lenz *et al* (1991) described the presence of carboxypeptidase B activity from *Heliothis zea* using the substrate HA, but activity was very low, and they describe the activity as 'only moderately above background'. Therefore, it seems that the presence of CPA and CPB peptidase activity in Lepidoptera has yet to be clearly demonstrated, Carboxyesterase (A) activity has been reported for a number of insects using the esterase substrate HPLA (e.g. Houseman *et al*, 1987), but frequently peptidase activity is not established. Baker (1981) established the presence of HPLA activity in the black carpet beetle, *Attagenus megatoma*, but could demonstrate only low activity towards the substrate HPA, a CPA substrate. No CPB activity could be demonstrated towards HA in either midgut tissue or luminal homogenates. Gooding and Rolseth (1976) have shown that the Dipteran, *Glossina morsitans,* has both HPA, HPLA and HA hydrolysing activity. Carboxypeptidase activity is present at high levels in other insect classes e.g. Orthoptera (Christeller *et al*, 1990), Coleoptera (Christeller *et al*, 1989) and Diptera (Cheeseman and Gooding, 1985). In conclusion, it appears that HPLA activity in *S. littoralis* is indicative of esterase activity rather than carboxypeptidase activity.

The broad pH optimum between pH 7.0-9.5 for HPLA hydrolysing activity was similar to other reports (Gooding and Rolseth, 1976; Houseman *et al*, 1987; Christeller

et al, 1989; 1990), but the rate of hydrolysis of HPLA by bovine CPA are independent of pH between pH 7.5 and 9.7 (McClure *et al*, 1964). The M_r estimate of the enzyme (40 kDa) by gel filtration appeared at least as large as the dimerised chymotrypsin enzyme from gut contents. The M_r of the carboxyesterase activity is in the range of molecular weights given for various insects in the above reports (range 26-43kDa), but the CPA enzyme from *Tineolla bisselliella* was estimated at 72kDa (Ward, 1975a).

CHAPTER 3

Characterization and partial purification of phenoloxidase from the haemolymph of *Spodoptera littoralis*

Introduction

The presence of phenolic compounds frequently upsets the conventional techniques by which enzymes are isolated from crude material (Van Sumere *et al*, 1975). When such material is ground in a buffer the hydrolases and phenolases released can thus catalyse reactions with these phenolics and quinones. Brown pigments, formed from them by polymerisation, may precipitate and/or inhibit many enzymes and subcellular organelles (Hulme and Rhodes, 1971). The presence of phenolic compounds together with the haemolymph PO from *S. littoralis* hindered purification of midgut endopeptidases. Gustavson (1954, 1963) established that both soluble and insoluble PVP form stable insoluble complexes with tannin material, while Loomis and Battaile (1966) have shown that phenols are effectively removed from hydrogen bonded complexes with protein by adding large amounts of PVP. Thus, PVP binds strongly to tannins and polymerised oxidation products of phenolases. The most satisfactory agents for tannin polymerisation seem to be insoluble PVP for enzymes (Loomis and Battaile, 1966). Pierpont (1970) suggests, amongst others, the use of insoluble PVP (polyclar AT) to remove polyphenols and the use of thiols to inhibit the polyphenoloxidase. It has been noted that inhibition of trypsin, amongst other enzymes, by condensed tannins from field peas and beans was much less marked in the presence of PVP (Tamir and Alumot, 1969; Griffiths, 1981). PVP has also been shown to restore trypsin activity of rats to levels comparable with those not fed tannins by reversing a tannin-trypsin complex (Griffiths and Moseley, 1980).

The midgut pH of Lepidopteran larvae is typically highly alkaline (Chapter 1). This helps to prevent the binding of tannins to midgut proteins. Indeed, this high midgut pH produces a substantial increase in digestibility of protein-tannin complexes in comparison with their digestibility at lower pHs. For example, increasing from pH 7.6 to pH 9.2 improves protein digestibility by the trypsin from the winter moth, *Operophtera brumata* (Feeny, 1969). Condensed tannins are bound to proteins independently of pH below 7-8, the binding decreasing rapidly above pH 8 because an ionized phenol (i.e. tannin, see below) cannot serve as proton donor for hydrogen bonding (Van Sumere *et al*, 1975). Phenols combine with proteins reversibly by hydrogen bonding, and irreversibly by oxidation followed by covalent condensations (Loomis and Battaile, 1966). The term 'phenols' also includes tannins; a polyphenol is regarded as a tannin when its molecular weight is between 500 and 3000.

The phenoloxidase of insects catalyses the hydroxylation of monophenols and the oxidation of diphenols to quinones (Lerch, 1988). Quinones are compounds which are able to react rapidly and non-enzymatically with many components, including digestive enzymes (Mason, 1955, 1959; Mason and Peterson, 1965; Brown, 1967). Polyphenolic

compounds form insoluble complexes with other macro-molecules such as proteins and this ability has long been associated with the observed reduction in nutritive value resulting from their inclusion in animal diets (Griffiths, 1986). Naturally occurring polyphenols, and in particular tannins, have been shown to inhibit a number of digestive enzymes including trypsin. Many polyphenolic compounds have been postulated as both retardants and stimulants to insect feeding (Harborne, 1979), but with increasing complexity of the structure enzyme inhibition appears to dominate.

Extraction of the midgut contents at a reduced pH near neutral levels would increase the activity of phenoloxidases which have pH optima at around 7.0 and also promote the binding of tannins to proteins. Conversely, extraction of the midgut contents at high pH (but 4°C) reduces binding of tannins to proteins of the midgut contents and the activity of the PO, but increases the self-digestion of the proteases. Therefore, midguts were pooled prior to chromatography at the pH of the midgut contents and methods for the inhibition of the PO and for tannin binding were investigated.

Phenoloxidase from arthropods is produced by the conversion of zymogenic prophenoloxidase to active phenoloxidase by serine proteases which also require activation (Leonard *et al*, 1985; Hergehahn *et al*, 1987; Söderhäll *et al*, 1988; Ashida and Yoshida, 1988; Andersson *et al*, 1989; Brehélin *et al*, 1989; Saul and Sugumaran, 1988). The activation of the prophenoloxidase (PPO) has been studied in a number of systems. In the cuticle of crayfish (Söderhäll, 1983), *Bombyx mori* (Dohke, 1973; Ashida *et al*, 1983), *Sarcophaga bullata* (Saul and Sugumaran, 1988) and *Manduca sexta* (Aso *et al*, 1985) PPO is activated by a serine protease which has a requirement for calcium ions (Ashida and Söderhäll, 1984). Activation of PPO also occurs in the haemolymph, and in *B. mori* and the crayfish *Pacifastacus leniusculus* two serine proteases are present as zymogens (Yoshida and Ashida, 1986; Aspán and Söderhäll, 1991). Activation of both plasma and cuticular phenoloxidase results in the release of a peptide and the subsequent polymerisation of the active enzyme (Ashida *et al*, 1974; Ashida and Dohke, 1980; Ashida and Yoshida, 1988).

Active PO enzyme hindered the purification of the endopeptidases of the midgut of *S. littoralis*. Therefore, a study was undertaken to characterise and purify the zymogen of PO in order that activity of the PO may be reduced and conversion of the PPO to the active enzyme minimized. This would then reduce phenol and quinone binding to enzyme proteins and facilitate endopeptidase purification.

CHAPTER 3
Materials and Methods

Preparation of haemolymph phenoloxidase

Haemolymph was collected from sixth instar larvae from *S. littoralis* by bleeding the carcass through the neck region. Collected haemolymph was pooled in 1.5ml Eppendorfs and frozen at -20°C until required. When required, samples were thawed and centrifuged at 13,000 rpm for 2 minutes using an MSE minifuge in order to precipitate insoluble material. The latter was discarded and the supernatant retained for enzyme assays.

Enzyme assays

Phenoloxidase activity was characterised with respect to pH, different metal ions and various other agents. The reaction medium consisted of 25μl of haemolymph in 150μl of McIlvaine's buffer (pH 7.5) and the reaction started by the addition of 50μl of the substrate, 4.5mM L-DOPA, (final substrate concentration of 1mM). Assays were usually carried out in microplates although in some cases 3ml cuvettes were used; in the latter, reaction volumes were increased proportionately (final reaction volume of 2.25ml). Absorbances were read at 492nm in both cases (Horowitz and Shen, 1952). Metal ions and potential agonists/inhibitors when used were dissolved directly in buffer and the pH corrected if necessary.

Purification of the prophenoloxidase

Supernatant obtained as above was subjected to gel filtration on a G-75 or S-200 column. The conditions of the column were as described in chapter 1. Fractions were tested for phenoloxidase (PO) activity before and after the addition of an equal volume of methanol in order to activate the prophenoloxidase (PPO). Organic solvents such as methanol are frequently used to activate prophenoloxidase (Preston and Taylor, 1970; Fisher and Brady, 1983). Aliquots of each putative PPO fraction were taken, treated with methanol and assayed for PO activity. Those fractions shown to contain PPO were pooled and desalted by dialysis. This was then subjected to anion-exchange chromatography (Q-Sepharose) and the proteins eluted by a gradient of sodium chloride. Fractions showing PO activity following methanol activation were pooled, and then reapplied to the ion-exchange column. Finally, these emergent fractions were assayed for activity.

CHAPTER 3
Results

The effect of pH on PO activity

The phenoloxidase present in the haemolymph of *S. littoralis* was assayed in appropriate buffers in the range 3.0 to 10.0. The optimum activity was found to occur at pH 7.5 in McIlvaine's buffer, or at pH 7.0 in 50 mM BTP (Fig. 3.1). However, PO activity was little affected by the buffer system used.

The effect of substrate concentration

The method used was similar to that described characterising endopeptidases. Lineweaver-Burk plots were again used to determine the mean apparent K_m (1.82 $\pm$ 0.24mM) and the mean V_{max} (4570 units $\pm$ 680) using the purified PPO activated with methanol. The specific activity of this purified enzyme is expressed in number of units, where one enzyme unit corresponded to an increase in optical density of 0.001 per minute per mg protein (Brehélin *et al*, 1989) at 30°C. The specific activity was determined to be about 2835 units with 1.92 mM DOPA as substrate.

Purification of PPO

Purification of the zymogenic form of the PO (PPO) from *S. littoralis* was effected to enable studies on PPO activation to be carried out. It was found that methanol activated the zymogenic form of phenoloxidase from *S. littoralis*. PPO eluted through Sephadex G-75 was not separated from active phenoloxidase and only void volume fractions hydrolysed the substrate, L-DOPA (Fig. 3.2). Elution through S-200 chromatographic media yielded a large PO peak, which after methanol treatment, was followed by a smaller peak of PPO (Fig. 3.3). The latter fractions were pooled and dialysed. Further purification was carried out by ion-exchange chromatography on Q-Sepharose and proteins eluted by an increasing gradient of sodium chloride. Three peaks eluted from this column (Fig. 3.4), of which only the third contained PO activity. The fractions from this final peak were again pooled, the salt content reduced by half by mixing the pooled fractions with an equal volume of buffer and then the ion-exchange step was repeated. The resulting elution pattern is shown in figure 3.5. Only the final peak contained PO activity and was stable for several weeks at 4°C.

Effects of different substances on PO activity

The effects of different substances on PO activity were carried out on cell-free haemolymph, except for (i), where purified PPO was used.

i. PPO activating substances

A variety of substances which activate other invertebrate PPO were incubated with this zymogen from *S. littoralis*. Methanol activation, as described earlier was an

Figure 3.1

The effect of pH on the activity of phenol oxidase from the haemolymph of *S. littoralis*. Activity was measured as the increase in absorbance at 492nm using 1mM L-DOPA as substrate.

Legend: ordinate - % activity
abscissa - pH

Figure 3.2

Gel filtration of PO activity through Sephadex G-75.

Legend: ordinate (1) - PO activity
(2) - protein absorption at 280nm
abscissa - fraction number

Figure 3.1

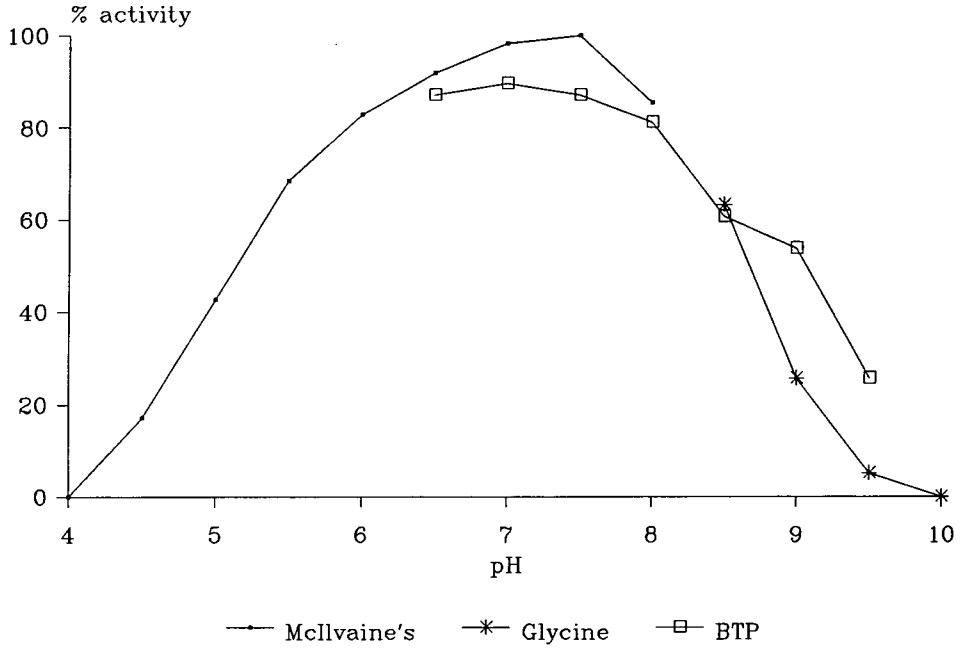


Figure 3.2

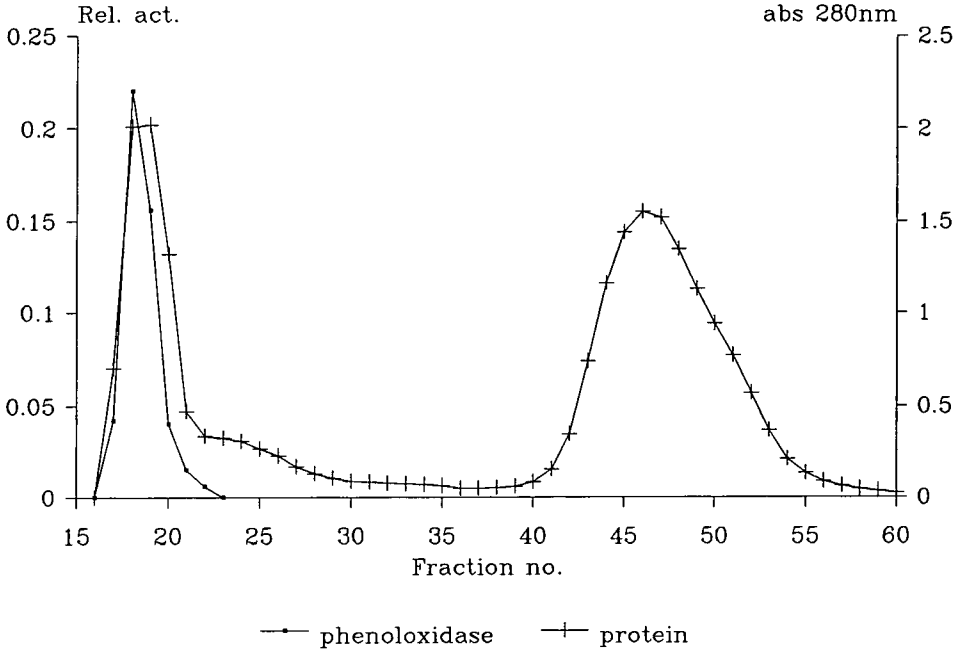


Figure 3.3

Gel filtration of PO activity through Sephacryl S-200. PO activity of the fractions was determined both before and after the addition of methanol. The second smaller peak is due to PPO only.

Legend: ordinate (1) - PO/PPO activity
(2) - protein absorption at 280nm
abscissa - fraction number

Figure 3.4

Anion exchange (1) following S-200 gel filtration and dialysis.

Legend: ordinate (1) - PO activity
(2) - % NaCl
abscissa - protein absorption 280nm

Figure 3.3

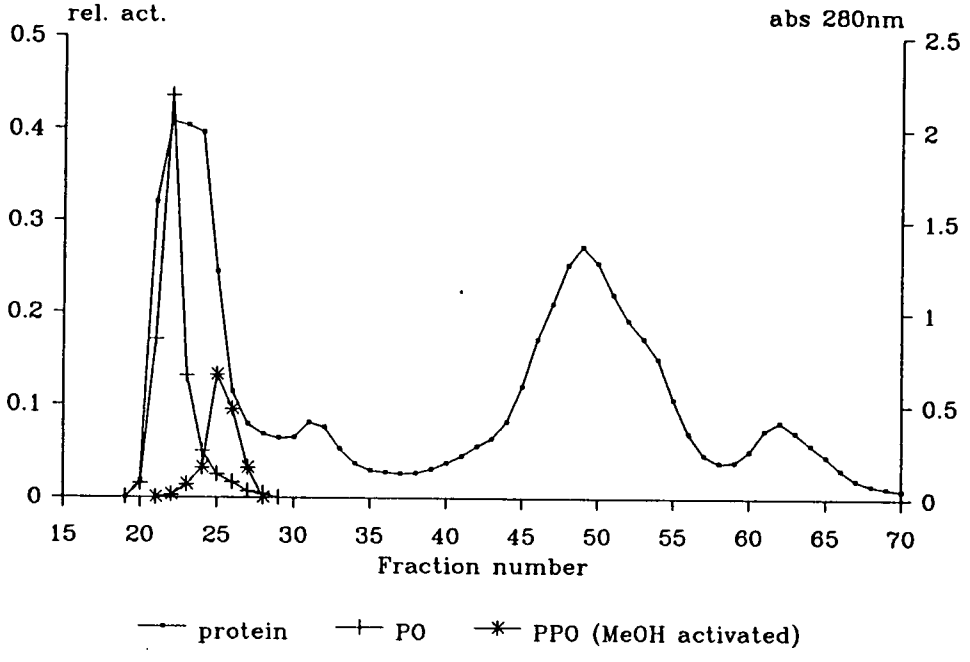


Figure 3.4

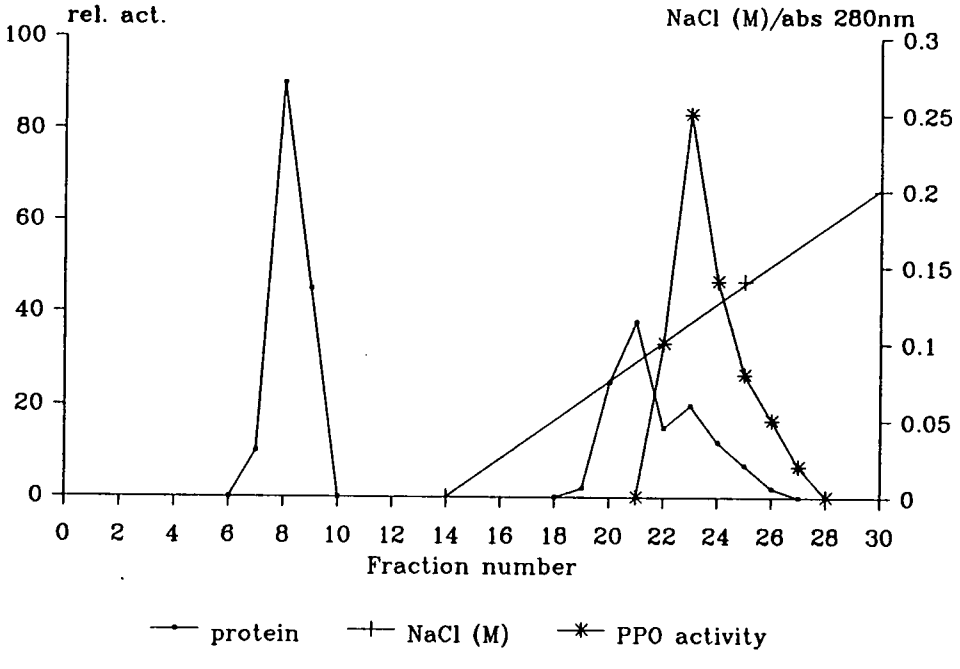


Figure 3.5

Anion exchange (2) chromatography of PO active fractions from the first ion exchange of S-200 fractions.

Legend: ordinate (1) - PO activity
(2) - % NaCl
abscissa - protein absorption

Figure 3.6

The effect of different salts (Na_2HPO_4, NaCl, Na_2SO_4 and KCl) on the activity of PO from haemolymph.

Legend: ordinate - % activity
abscissa - mM metal salt

Figure 3.5

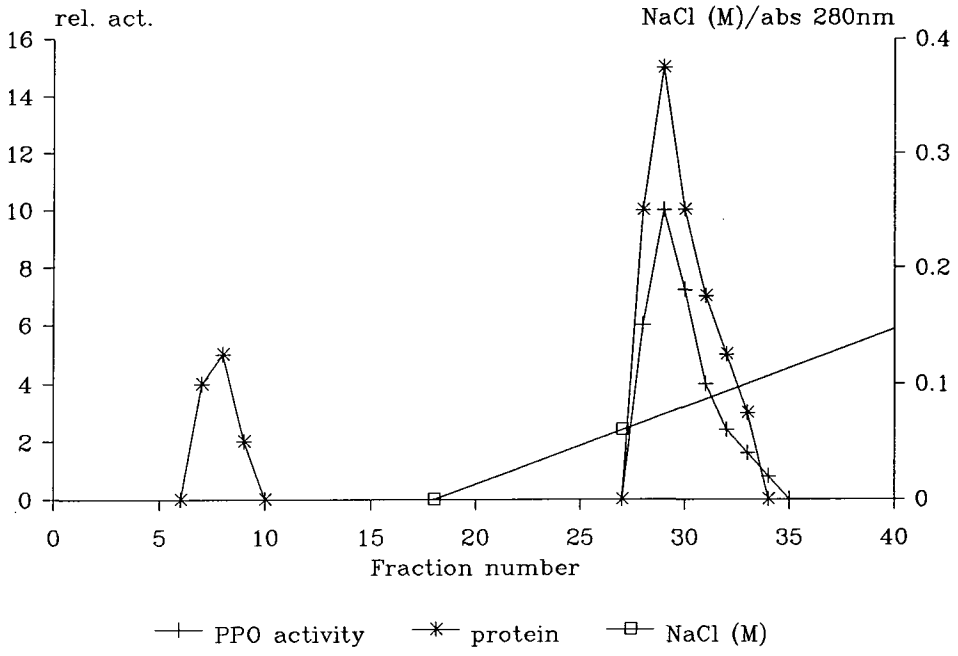
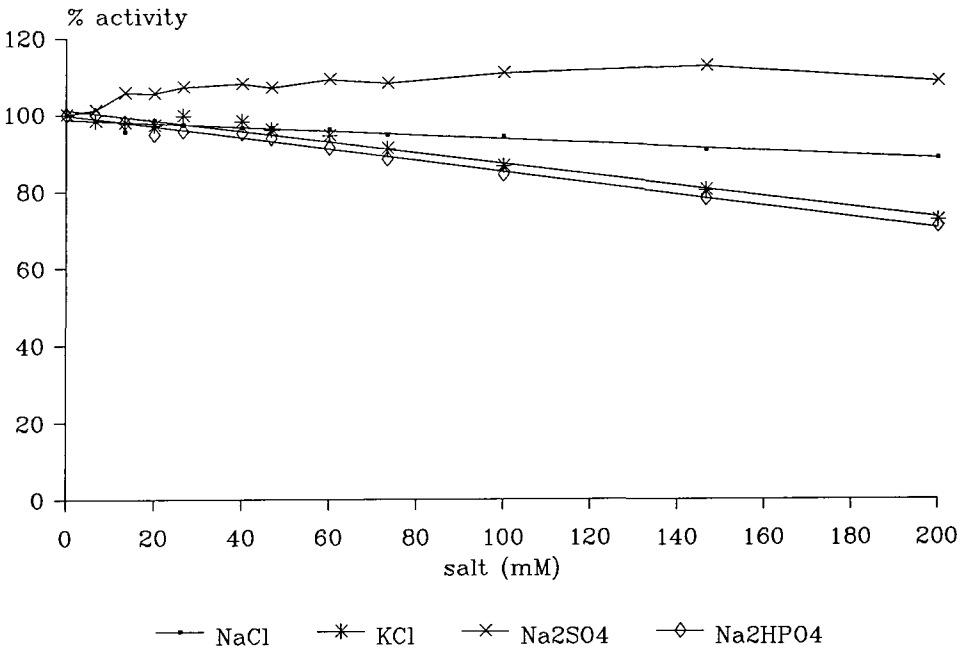


Figure 3.6



effective PPO activator. In addition, purified PPO was incubated at 30°C with lipopolysaccharide (LPS) (0.1 mg/ml), laminarin (1 mg/ml), trypsin (1 mg/ml) and chymotrypsin (1 mg/ml) and aliquots were removed at intervals for assay of PPO activity. It was found that, except for methanol, none of the above substances activated the zymogen within a two hour incubation period.

ii. metal ions

The effect of different metal ions on PO activity from cell-free haemolymph was determined. Sodium hydrogen-phosphate, sodium chloride and potassium chloride decreased PO activity only slightly (up to 30% at high concentrations) in a linear fashion with increasing salt concentration (Fig. 3.6). The divalent ions, Mg^{2+} and Ca^{2+} increased PO activity compared with the control (no added ions) at concentrations up to 100mM and 150mM respectively. At higher concentrations of these ions PO activity was lower than the control. PO activity peaked at an Mg^{2+} concentration of 26.7mM and a Ca^{2+} concentration of 20mM (Fig. 3.7). The effect of the divalent metal ion chelator, EDTA, on the PO activity was determined. The addition of even high concentrations of EDTA (160mM) did not inhibit PO activity significantly (< 3%).

iii. DTT

In vitro studies to test the effectiveness of the reducing agent, DTT, against PO of *S. littoralis* showed that it is sensitive at low concentrations. 50% inhibition occurs at about 0.02mM, while at 2mM, the concentration used for the preparation of midgut homogenates, inhibition is greater than 95% (Fig. 3.8). Therefore, DTT was used during the preparation of gut homogenates to inhibit PO and reduce its activity.

iv. kojic acid

In vitro studies indicated kojic acid is inhibitory towards *S. littoralis* PO activity. 50% inhibition occurred at 20μg/ml (140μM) (fig. 3.9). Lineweaver-Burk and Dixon plots of PO inhibition by kojic acid show that inhibition is competitive (Fig 3.10 and 3.11). K_{ib} determined from Dixon plots (Dixon, 1953) and K_{ia} (Dixon, 1972) were similar; K_{ia} was 17.4 $\pm$ 2.0 μg/ml (120μM) (fig. 3.12) while K_{ib} was 15.5 $\pm$ 2.5 μg/ml (110μM).

Figure 3.7

The effect of Ca^{2+} and Mg^{2+} on the activity of the PO from haemolymph.

Legend: ordinate - % activity
abscissa - mM metal salt

Figure 3.8

The effect of dithiothreitol (DTT) on the activity of phenol oxidase from haemolymph.

Legend: ordinate - % activity
abscissa - mM DTT

Figure 3.7

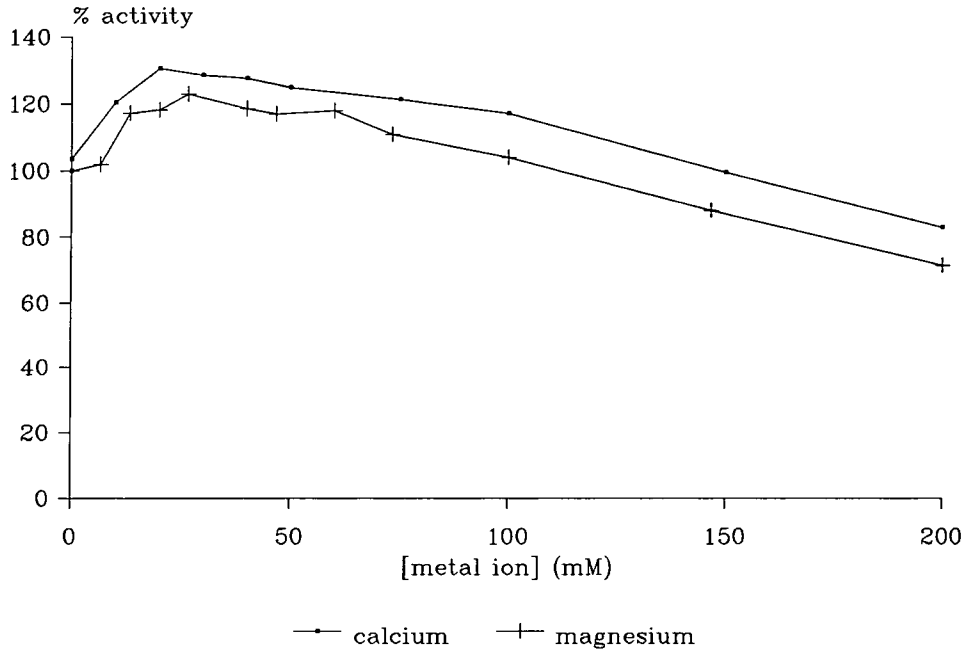


Figure 3.8

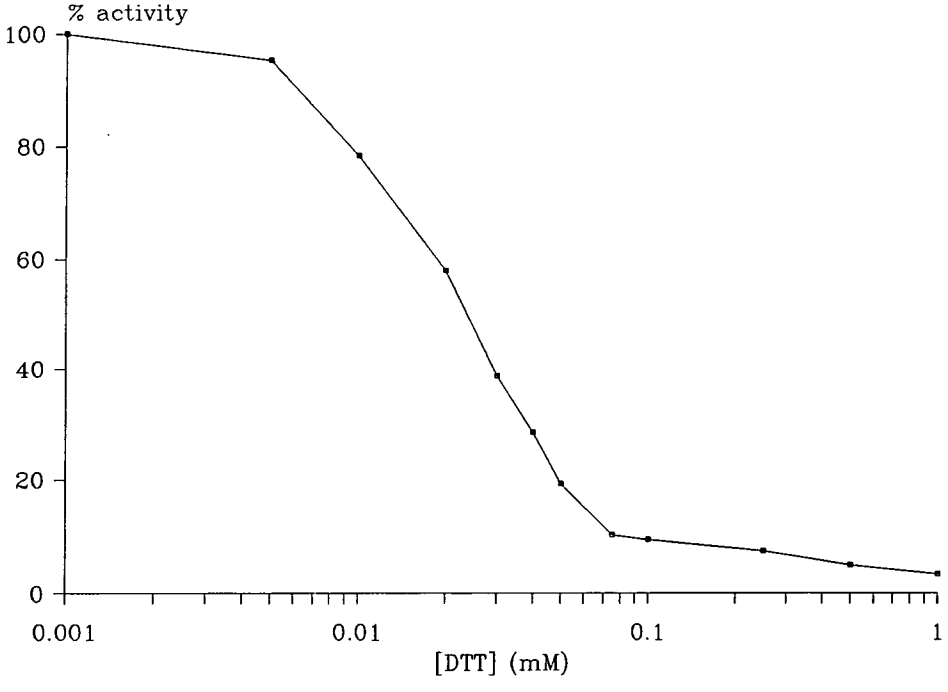


Figure 3.9

The effect of kojic acid on the activity of PO from the haemolymph.

Legend: ordinate - % activity
abscissa - mM kojic acid

Figure 3.10

Lineweaver-Burk plot of the inhibition of PO by kojic acid.

Legend: ordinate - 1/PO units
abscissa - 1/[L-DOPA] (mM)

Figure 3.9

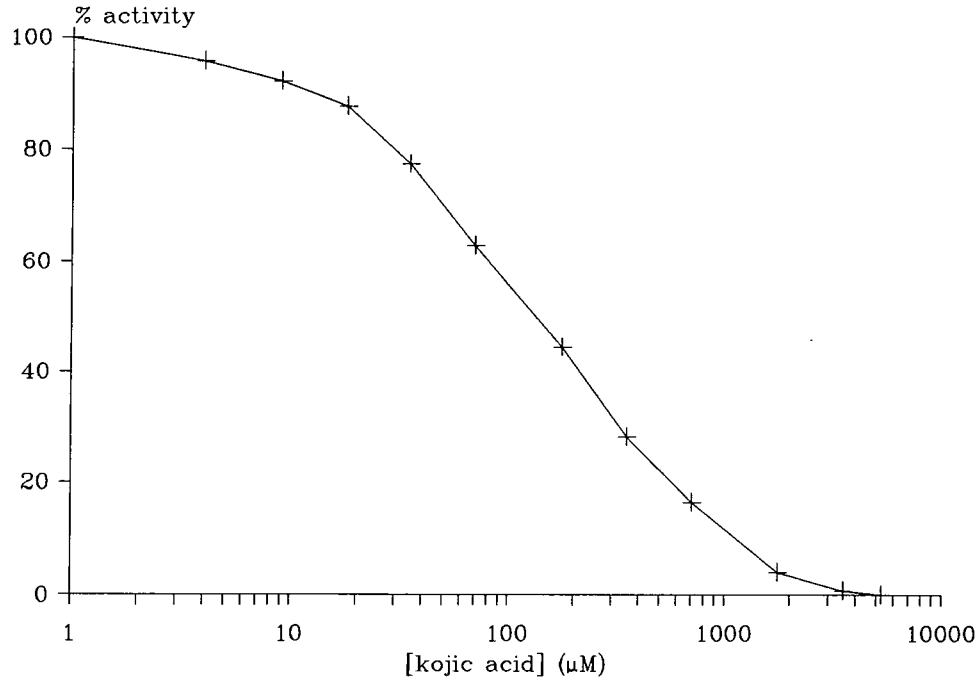


Figure 3.10

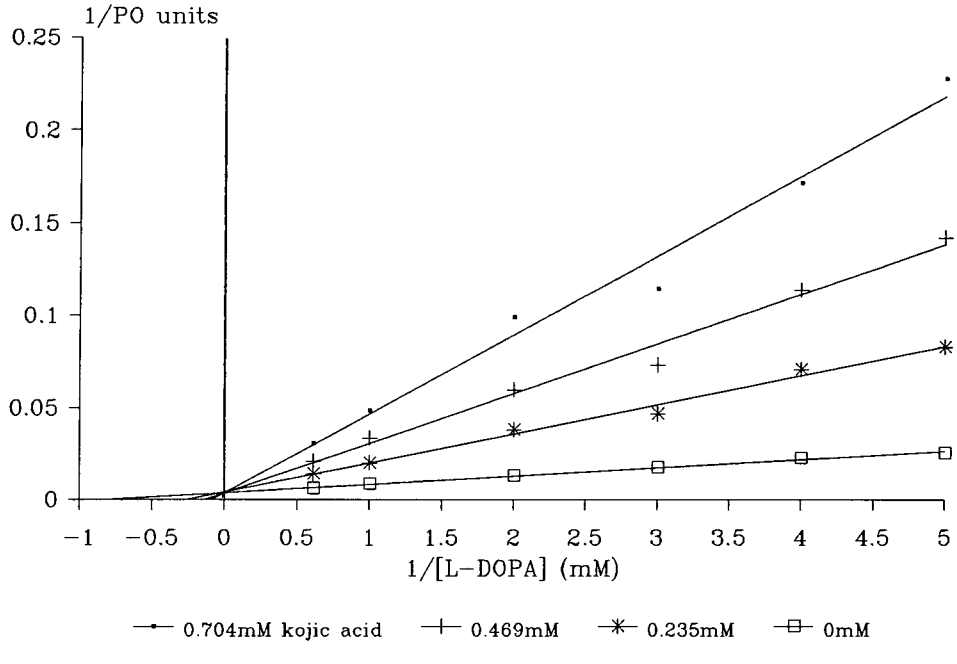


Figure 3.11

Dixon plot to determine the inhibition of PO by kojic acid.

Legend: ordinate - 1/rel. velocity
abscissa - μM kojic acid

Figure 3.12

Dixon plot (1972) to determine the inhibition of PO by kojic acid (K_{ia}). Diagonal lines intercept the curve at maximum activity/n, where n is an integer. The value of K_{ia} is the difference between any two intercepts on the x axis, or an average of several.

Legend: ordinate - % activity
abscissa - mM kojic acid

Figure 3.11

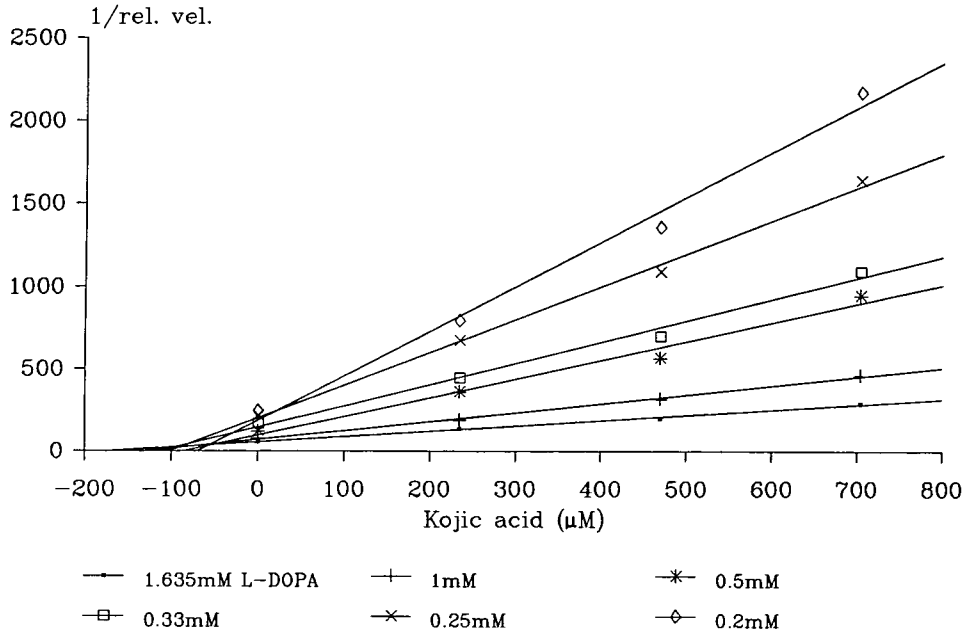
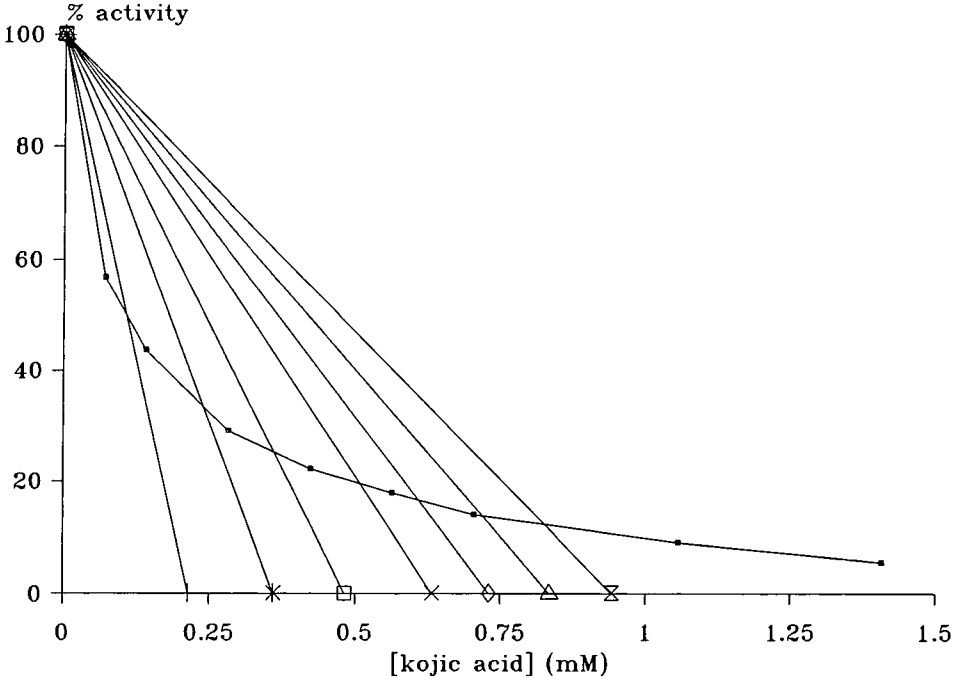


Figure 3.12



CHAPTER 3
Discussion

The phenol oxidase activity from *S. littoralis* was maximally active towards L-DOPA at near neutral pH (pH 7.5) and this was similar to the haemolymph pH (pH 7.2 $\pm$ 0.1 at 30°C, N = 3). The pH optimum is not too different from that of phenoloxidase activity from other insects e.g. the gypsy moth, *Lymantria dispar* (optimum at pH 8.0), the greater wax moth *Galleria mellonella* (optimum at pH 7.5) (Dunphy, 1991), *M. sanguinipes* (optimum at pH 7.5) (Bidochka *et al*, 1989), the red-humped oakworm, *Symmerista cannicosta* (optimum between pH 6.0 and 7.2) (Barrett, 1984) and the sheep blowfly, *Lucilia cuprina* (optimum between 6.5 and 7.0) (Barrett, 1987). However, the ability of the phenoloxidase from *Hyalophora cecropia* to oxidise L-DOPA was reported to be constant between pH 5.0 to 9.5 (Andersson *et al*, 1989). However, PO activity appeared less dependent on the different incubation buffers than other enzymes investigated from *S. littoralis* e.g. LAP activity was markedly different in glycine and BTP buffers.

The affinity of the phenoloxidase from *S. littoralis* for L-DOPA (Apparent K_m was 1.82 $\pm$ 0.24mM) was similar to that reported for the white shrimp (*Penaeus setiferus*), spiny lobster (*Panulirus argus*) and grass prawn (*Penaeus monodon*) (2.8 to 3.6mM) (Chen *et al*, 1991a; Simpson *et al*, 1988; Rolle *et al*, 1991). The specific activity of the purified prophenoloxidase from *S. littoralis* was determined to be about 2835 units with 1.92 mM DOPA as substrate. This activity can be compared with that reported for purified PPO from other arthropods at different substrate concentrations by estimating the specific activity from the Lineweaver-Burk plot. On this basis, activity would be about 4450 units at 10mM substrate concentration and 30°C. This level of activity is higher than the activity of grass prawn (900 units at 25°C) and similar to PPO activity for white shrimp (5400 units at 40°C) and lobster (7000 units at 25°C) reported by Chen *et al* (1991a), but lower than the specific activity of purified PPO from *Hyalophora cecropia* at about 20,000 units at 30°C (Andersson *et al*, 1989). However, quantitative comparison is difficult as specific activities for these enzymes were determined at different temperatures.

The activity of PO from crude cell-free haemolymph was determined in the presence of mono and divalent cations. PO activity from *S. littoralis* was increased in the presence of the divalent ions Ca^{2+} and Mg^{2+}. An increase in PO activity in the presence of metal ions has been observed in *Hyalophora cecropia* (Andersson *et al*, 1989). However, the PO from this latter insect was increased in the presence of both mono- and divalent ions. Calcium has been shown to enhance phenoloxidase activity from *Lymantria dispar* and *Galleria mellonella* (Dunphy, 1991; Brookman *et al*, 1989). Similar calcium-mediated PO activity enhancement has been reported for the Lepidopteran *Bombyx mori* (Ashida *et al*, 1983), for the Orthopteran *Schistocerca gregaria* (Dularay and Lackie, 1985) and *Locusta migratoria* (Brehélin *et al*, 1989) and

the dictyopteran *Blaberus craniifer* (Leonard *et al*, 1985). PO activity of cell-free haemolymph from *S. littoralis* peaked at concentrations of 20-50mM Ca^{2+} (Fig. 3.7); the concentration of this ion in the haemolymph, 36.1 $\pm$ 1.5mM (N = 30), determined by flame photometry (S. Al-Ahmedi, Durham University, personal communication) falls in this concentration range. High concentrations of Ca^{2+} reduced the level of phenoloxidase from *S. littoralis* cell-free haemolymph. The optimum concentration of calcium ions (20mM) for peak phenoloxidase activity in *S. littoralis* is higher than that required for orthopteran insects which is between 5 and 10mM (Dularay and Lackie, 1985; Brehélin *et al*, 1989; Brookman *et al*, 1989).

Kojic acid is an inhibitor of phenol oxidases which cause unfavourable darkening of food products through the oxidation of phenols to *o*-quinones. Kojic acid is a fungal metabolite produced by many species of *Aspergillus* and *Penicillium* (Kinosita and Shikata, 1964; Parrish *et al*, 1966) and has been shown to inhibit melanosis in the pink shrimp (Applewhite *et al*, 1990). Therefore, in view of the problems caused by traces of phenoloxidase from the haemolymph on the activity and purification of the digestive endopeptidases, the effect of kojic acid on the inhibition of PO activity was investigated. Kojic acid inhibition of the PO was effective: I_{50} was 20μg/ml (140μM). This is more effective than inhibition of PO enzymes from white shrimp and grass prawn: only 20% inhibited at this concentration; whereas the inhibition of spiny lobster PO is 80% inhibited at this concentration. However, the inhibitor constants (K_{ib}) for kojic acid inhibition of *S. littoralis* PO was considerably lower (as determined by Dixon plots) at 15.5μg/ml (110μM). K_i values for inhibition of PO from white shrimp, spiny lobster and grass prawn were 90, 70 and 50 μM respectively (Chen *et al*, 1991a) when DL-DOPA was used as substrate.

DTT, a reducing agent, was shown to be a highly effective inhibitor of the PO enzyme. 50% inhibition occurred at about 20μM DTT (3μg/ml) (Fig. 3.8), while at a concentration of 2mM the inhibition was greater than 95%. DTT was a more effective inhibitor than kojic acid and was therefore used exclusively in the preparation of midgut homogenates with PVP. In addition, kojic acid has been shown to induce mutations in various cell lines (e.g. Chinese hamster ovarian cells and rat liver cells) (Wei *et al*, 1991; Stark, 1980). It is also toxic to mice intraperitoneally (Morton *et al*, 1945), dogs intravenously (Friedemann, 1934) and to chick embryos (Lee *et al*, 1950).

Further preparations of midgut enzymes were carried out in the presence of 0.1g/ml of polyvinylpyrrolidone (tannin/phenol binding agent) and 2mM DTT (reducing agent; inhibited phenoloxidase effectively). Other compounds have been shown to be effective inhibitors of phenol oxidases or of the PPO activating system from various sources. For example: L-cysteine (Valero *et al*, 1991a); tropolone (Valero *et al*, 1991b) and melittin (Söderhäll, 1985). These compounds may also provide protection from the action of phenol oxidase activity during the preparation of midgut homogenates.

It has been shown in the results that prophenoloxidase can be activated by the

addition of methanol, which is similar to other PPO from other insects (Brehélin *et al*, 1989; Andersson *et al*, 1989). However, the action of some proteolytic enzymes such as trypsin or chymotrypsin (Preston and Taylor, 1970) or triggering of the endogenous activating system present in the haemolymph (Hughes and Price, 1976; Ashida and Yoshida, 1988) can also activate the PPO in arthropods. However, as described in the results section, purified PPO was not activated to the active enzyme by laminarin and lipopolysaccharide or by bovine trypsin and chymotrypsin. This is consistent with the report by Brehélin *et al* (1989) of the PO in *Locusta migratoria* that PO can be observed only in serum (i.e. with cellular material or cellular lysate) and never in plasma (cell free material). However, chymotrypsin has been shown to be an effective activator of purified PPO from *Bombyx mori* (Ohnishi, 1970). Serine proteases present in the haemolymph of insects convert PPO to PO, but require activation themselves (Aspán *et al*, 1990a,b; Aspán and Söderhäll, 1991; Andersson *et al*, 1989). Serine protease activity of arthropods is regulated by inhibitors in the haemolymph (Saul and Sugumaran, 1986; Hergenhahn *et al*, 1987; Brehélin *et al*, 1991). It may be possible to select an inhibitor of these proteases which could be included during the preparation and purification of midgut homogenates provided that this did not interfere with the activity of the midgut endopeptidases. To date, few studies have been carried out on the inhibition of the serine proteases of the haemolymph by serine protease inhibitors (e.g. Aspán *et al*, 1990a,b). The prophenoloxidase and its activation by serine proteases is not fully understood, but clearly merits further investigation.

CHAPTER 4

An ultrastructural study on various stages in the life-cycle of a microsporidian parasite (Microspora: Nosematidae) in *Helicoverpa (Heliothis) armigera* Hubner

Introduction

Many noctuid moths are important economic pests of cereals and other crops worldwide. *Helicoverpa (Heliothis) armigera* and *Heliothis* spp. remain as significant pests in Asia, Africa, the Americas and Europe and crop production would benefit from effective pest control. Certain parasitic organisms may offer a useful alternative approach to insect pest control compared with conventional chemical methods due to insect tolerance to an increasing number of pesticides (Burges, 1981). Insects act as hosts for numerous parasitic organisms, including representatives of fungi, bacteria, protozoa and certain metazoan phyla (Weiser & Briggs, 1971). Such agents may be valuable in insect pest control. For example microsporidia have been used against *Locusta migratoria* (Brooks, 1980; Burges, 1981) rangeland grasshoppers (Lockwood & Debrey, 1990) and to control the lepidopteran, *Lymantria dispar* (David & Novotny, 1990) and various coleopteran pests (Brooks, 1980; Khan & Selman, 1989). Such parasitic organisms have the advantage of being less indiscriminate in terms of host 'target' and infections occur relatively infrequently in vertebrates other than fish (Kudo, 1924; Sprague, 1977); microsporidian diseases in man are usually confined to those with immunodeficiency (typically AIDS) or stress related illness (Margileth *et al*, 1973; Desportes *et al*, 1985; Cali and Owen, 1988; Lowder *et al*, 1990; Cali, 1991; Weber *et al*, 1992).

During the course of rearing laboratory cultures of *H. armigera*, major population 'crashes' were noted from time to time. Examination of light microscopical sections of larval midgut and associated tissues (e.g. gut muscle tissue) revealed that insects were dying due to the presence of substantial microsporidian infection.

Since the early studies of Huger (1960a,b,c) on the ultrastructure of microsporidia, many species have been described on the basis of their ultrastructural features (Larsson, 1986). In 1982, Toguebaye and Bouix reported the presence of the microsporidian, *Nosema manierae*, in midgut tissue epithelium and other tissues of *Helicoverpa armigera* and later produced an ultrastructural description (Toguebaye and Bouix, 1983), the only one published for microsporidians occurring in this insect. The present study reports the presence of a nosematid parasite in larval *H. armigera*, describes the fine structure of various stages in its life-cycle and compares the morphology with that of similar microsporidian parasites, such as *N. manierae*.

Materials and Methods

Midguts and associated Malpighian tubules were dissected from recently moulted, actively feeding and non-feeding fifth and sixth instar larvae. Fixation and preparation of tissue was carried out as described in the general materials and methods.

CHAPTER 4
Results

The effects of microsporidian infection on insect viability went largely unnoticed in the stock population of *H. armigera* for about two generations following their discovery in sectioned tissues. Later, larvae were more heavily infected and showed acute paralysis. Such insects frequently died in the larval or pupal stages. These symptoms of the disease have already been described in *Spodoptera litura* diseased with *N. mesnili* (Abe, 1989); limited movement and paralysis of infected animals being a characteristic of microsporidian infection in insects (Youssef & Hammond, 1971). Mass infestation of muscle tissue did occur in *H. armigera* which would be consistent with these effects.

Light microscopy

Examination of the midgut sections revealed the presence of massive infestations of the midgut with large numbers of oval spores in both columnar and goblet cell cytoplasm (Fig. 4.1). In addition these were also visible in mucous droplets in the gut lumen (cf. *N. fumiferanae* infection of *Choristoneura fumiferana*, Nolan & Clovis, 1985). The musculature around the gut wall was also parasitized as were cells of the Malpighian tubules. The presence of the parasite in many different host tissues is encountered frequently in species infected with *Nosema* spp. e.g. *Nosema galerucellae* in *Galerucella luteola* (Toguebaye & Bouix, 1989).

Electron microscopy

Micrographs have been arranged and subsequently explained to show a developmental sequence; the fine structure and life cycle stages observed being consistent with the literature describing development in *Nosema* spp. (Vávra, 1976b; Larsson, 1986).

Cytology of the presporal stages

The meront stage (Fig. 4.2) of the microsporidian is rounded (2.5-3μm in diameter) with a large central diplokaryon which is approximately the same size as a mature spore (2.1 x 1 μm and 2.4 x 1.2 μm, respectively). The cytoplasm contained a poorly developed endoplasmic reticulum with numerous free ribosomes, and the unit plasma membrane adjacent to the host cell was surrounded by numerous host cell ribosomes. Thus, the ultrastructure of the meront cytoplasm is similar to that reported by Canning & Sinden (1973) for *Nosema algerae* in *Anopheles stephensi*. Characteristically for microsporidia, no mitochondria were present at any stage.

In contrast to the meront stage, the larger sporonts (measuring 4.5-6.5 x 2.2 μm) possessed a better developed R.E.R. which was arranged concentrically around the diplokaryon. Each sporont was separated from the host cell by an electron dense layer

Figure 4.1. Transverse section of the midgut showing numerous spores (S) in columnar (C) and goblet cells (G) and in mucus droplets (M) in the lumen (L). MV = microvilli. Light microscopy (x 550).

Figures 4.2-4.5. Meront and sporont stages of *Nosema* sp. found in the columnar cells of larval midgut of *H. armigera* (electron microscope).

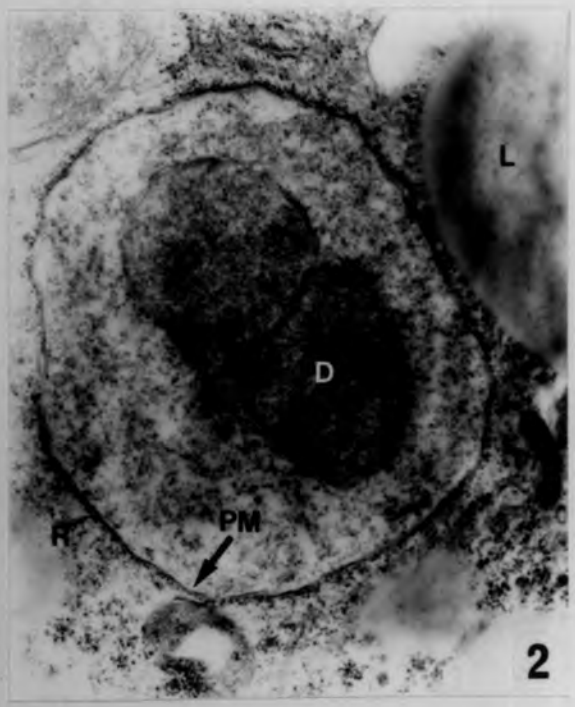
Figure 4.2. Meront with diplokaryon. D = diplokaryon; PM = plasma membrane; R = ribosomes of host cell surrounding parasite PM; L = lipid droplet (x 23,800).

Figure 4.3. Sporont showing formation of the wall (W) and the presence of a well-developed rough endoplasmic reticulum (RER) (x13,100).

Figure 4.4. Sporont showing late anaphase stage of mitosis. K = kinetochores; SP = spindle plaques; spindle microtubules (arrow).

Figure 4.5. Sporont showing prophase stage of mitosis. PV = polar vesicles; K = kinetochores; SP = spindle plaques; L = lipid droplet (x17,500).


S
L
M
MV
G
1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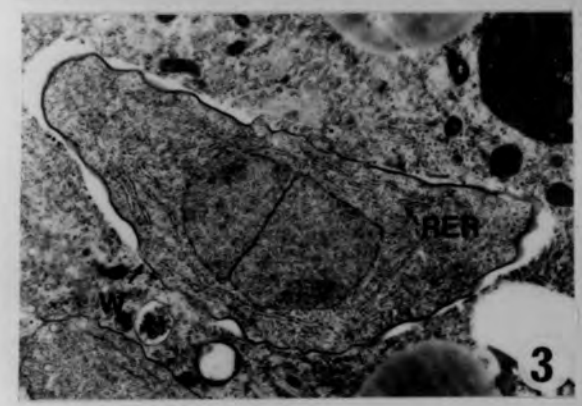
D
PM
2


RER
3


SP
4


SP
PV
L
5

Figures 4.6-4.11. Electron micrographs showing sporont and sporoblast stages of *Nosema* sp.

Figure 4.6. Immature sporont with developing wall (arrow) and indistinct nuclear envelope (x28,000).

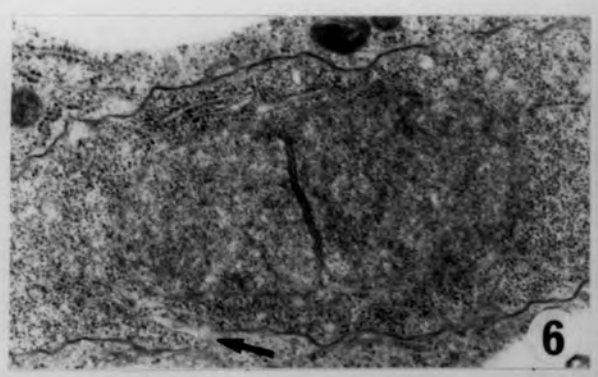
Figure 4.7. Two sporonts with interdigitating microvillar projections (MV) found in the basal (serosal) region of a host columnar cell. (x12,300).

Figure 4.8. Early sporoblast. Golgi (G); coalescences (C); anterior polar filament (PF); rough endoplasmic reticulum (RER); diplokaryon (D) (x24,000).

Figure 4.9. Sporoblast showing further development of the polar filament (PF); Golgi body (G); coalescences (C) (x26,000).

Figure 4.10. Sporoblast showing sections through the irregularly coiled polar filament (PF); Golgi (G) (x26,300).

Figure 4.11. Sporont with two diplokarya (D); host lipid droplet (L) (x20,400).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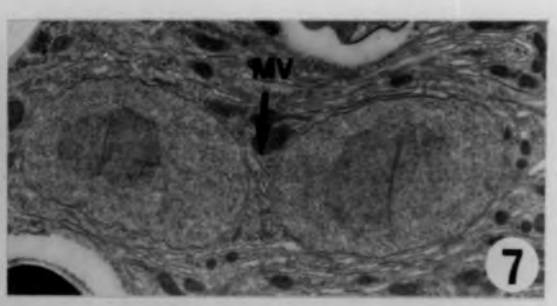
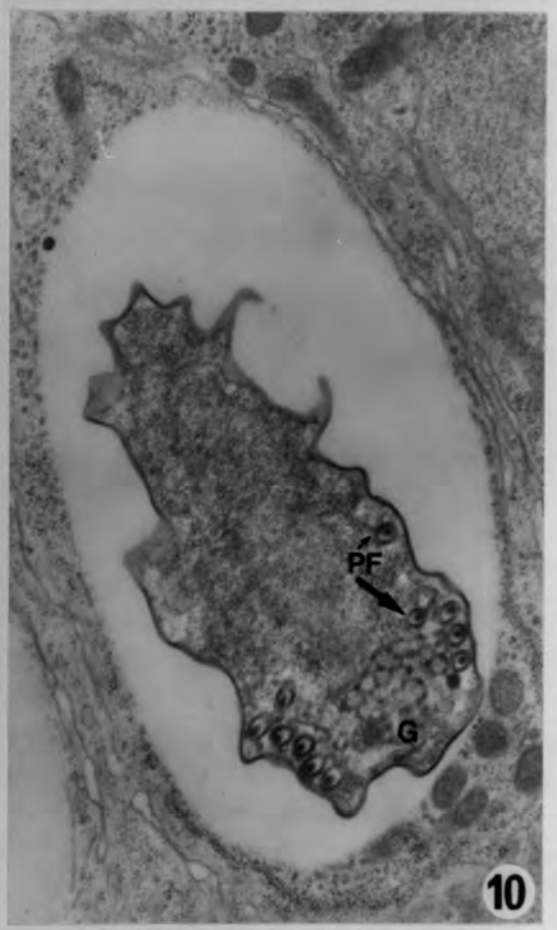
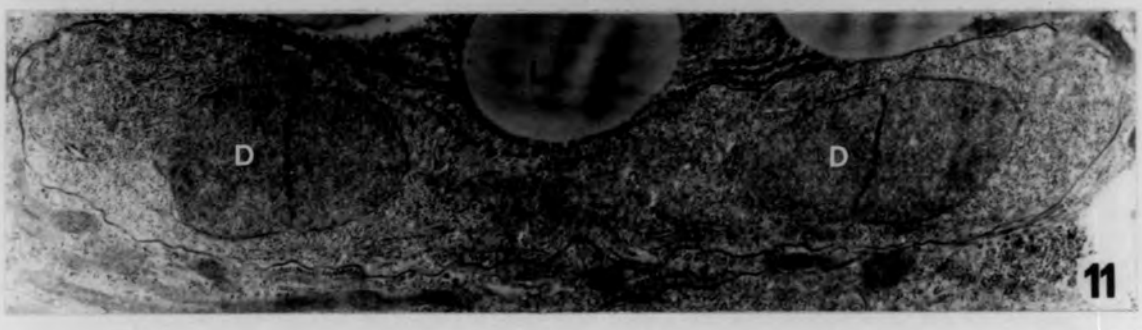

MV
7


D
RER
PF
G
8


PF
G
C
9


PF
G
10


D
D
11

surrounding the cell membrane. The host's ribosomes were noticeably less ordered in relation to the sporont being no longer attached to the parasites outer surface.

Throughout development of the parasite, each of the two closely opposed nuclei making up the diplokaryon had a structure typical of nuclei from eukaryotic cells and was surrounded by a double membrane (Figs. 4.3, 4.8 & 4.16). Consequently, the area of contact between the two nuclei appeared as a thickened electron-dense plate and occasionally the only part that was distinct (Fig. 4.6). Evidence of prophase during closed intranuclear pleuromitosis (Raikov, 1982) was found more frequently, but only in diplokarya (Fig. 4.5). The spindle plaque was seen as a thickened, electron-dense region of membrane and served as an attachment point for the microtubules which constitute the intranuclear spindle apparatus. The other ends of the microtubules are attached to the kinetochores of the chromosomes (Fig. 4.4 and 4.5). The plaque was about 230nm wide (Fig. 4.5) and a number of cytoplasmic vesicles were seen around the spindle plaques where these were depressed into the nucleus (Fig. 4.5). The stage shown in Figure 4.4 is probably a late anaphase in mitosis. This was seen more infrequently than the prophase stage of mitosis. Microtubules are shown extending between plaques on opposite sides of what appears to be a dividing nucleus. It is suggested that the chromosomes (seen as kinetochores) are subsequently pulled to the poles and the nuclear envelope pinches in two (Raikov, 1982).

Sporogony was disporoblastic; two separated diplokarya are shown in Figure 4.11, whereas Figure 4.7 indicates a similar structure apparently undergoing transverse fission into two similar sporonts with cytoplasmic filaments linking them. Elsewhere individual sporonts containing diplokarya were common (Fig. 4.3). Disporoblastic sporonts measured about 8.5 x 1.5-2.5 μm.

Sporoblasts were recognizable due to the presence of finger-like projections extending from their external surfaces into the spaces which typically surrounded each cell (Fig. 4.8). However, the possibility that such spaces represent fixation artifacts cannot be discounted. The Golgi body was found in the posterior half of the sporoblast (Fig. 4.8). In the younger stages this appeared as a collection of membrane-bound vesicles which subsequently become filled with electron-dense material. These coalescences were most obvious initially in the region of the Golgi body nearest the nuclei.

Figures 4.8-4.10 and 4.13-4.15 show the likely developmental sequence of the coiled polar filament as it extends from its site of production in the posterior region (Fig. 4.13) toward the anterior end where it terminated in the anchoring disc (Vávra, 1971). The latter was closely moulded to the inner contour of the spore apex in the mature spore (Fig. 4.15).

The spore

The average dimensions of mature spores were 2.43 $\pm$ 0.12 μm x 1.22 $\pm$ 0.06 μm (N = 13) (mean $\pm$ standard error) in the material prepared for electron

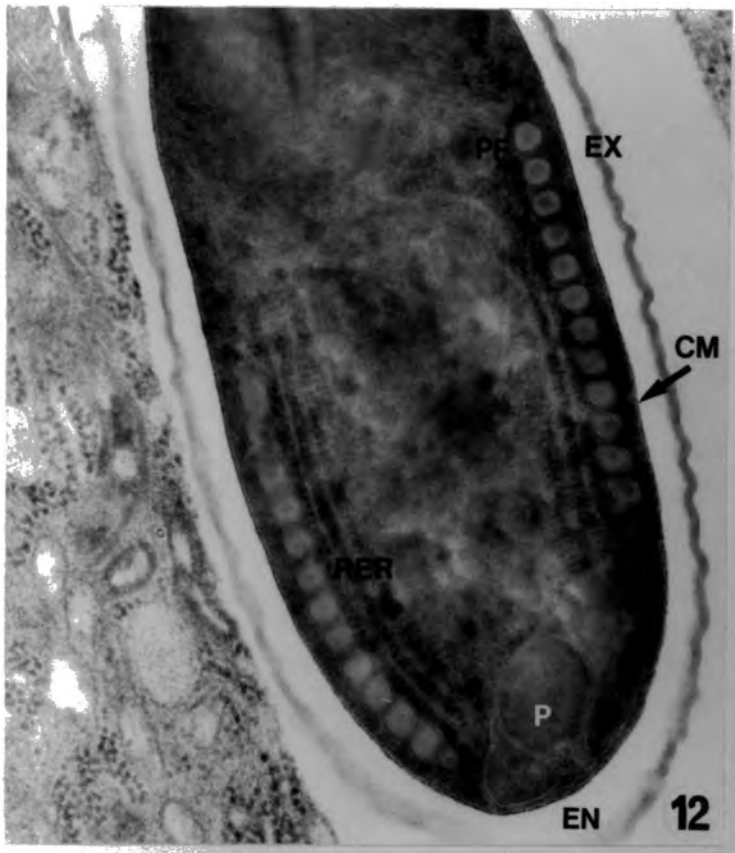
Figures 4.12-4.15. Electron micrographs of sporoblasts and spores of *Nosema* sp.

Figure 4.12. Posterior region of a spore showing 12-13 coils of the polar filament (PF); exospore (EX); endospore (EN) and cytoplasmic membrane (CM); rough endoplasmic reticulum (RER) and posterior body (P) (x58,000).

Figure 4.13. Sporoblast with anterior anchoring disc (AD); polar filament (PF); diplokaryon (D); Golgi body (G) (x27,200).

Figure 4.14. Immature spore. Anchoring disc (AD); lamellar polaroplast (LP); exospore (EX); diplokaryon (D) (x33,700).

Figure 4.15. Anterior region of mature spore showing exospore (EX); endospore (EN); anchoring disc (AD); lamellar polaroplast (LP); and the vesicular polaroplast (VP) (x100,000).


PE
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CM
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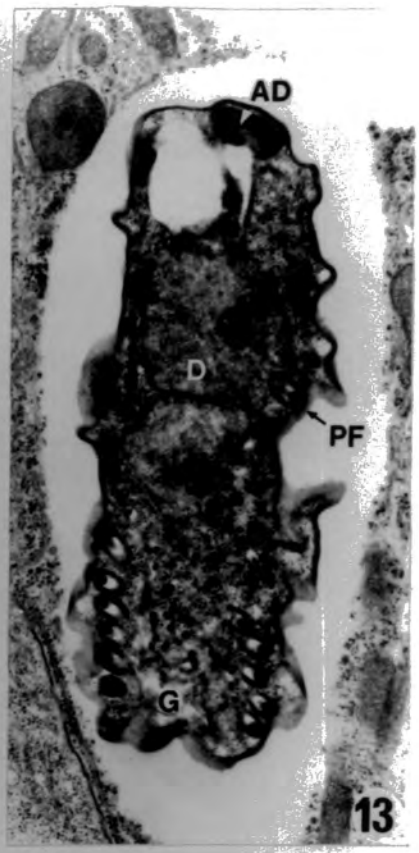
12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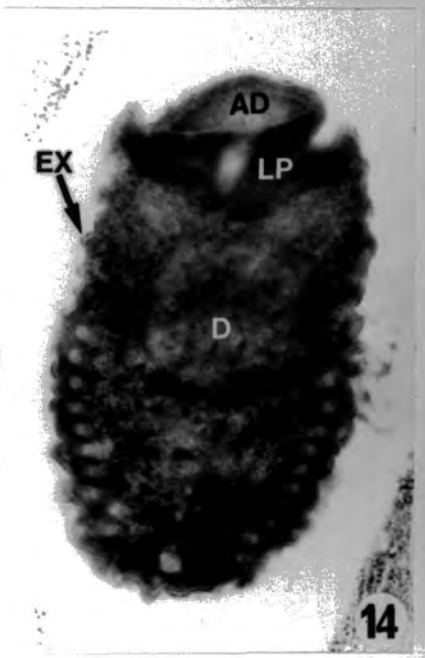
AD
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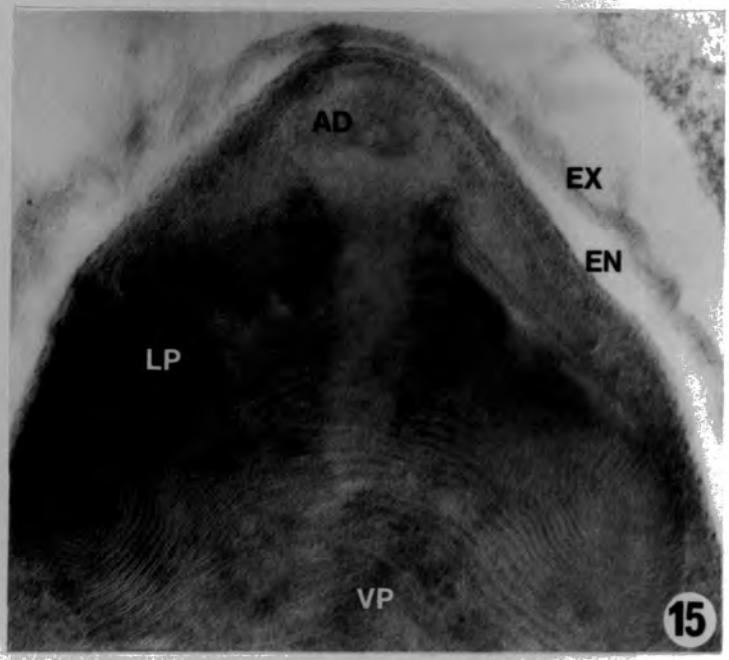
14


AD
EX
EN
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15

microscopy. Longitudinal sections through the spore revealed the presence of a peripherally situated coiled polar filament which was restricted to the posterior two-thirds of the cell.

The cytoplasm had numerous free ribosomes and areas of well-developed R.E.R., the latter being situated within helically coiled polar filament (Fig. 4.12). The diplokaryon was seen in the middle of the spore surrounded by the endoplasmic reticulum and the coiled polar filament (Fig. 4.16).

The polar filament develops from a simple tube filled with electron-dense material (Fig. 4.10) into a layered structure (Figs. 4.17 and 4.18) and up to nine layers can be seen in some sections through the polar filament (Fig. 4.18). The majority of the coiled filament is isofilar. In the mature spore the coils of the filament are arranged in a single layer, but irregular arrangements of the polar filament may be seen in the immature spores (Fig. 4.17). The polar filament was approximately 70nm in cross-sectional diameter and formed a 12-13 coiled helix in the posterior two-thirds of the spore (Fig. 4.12); although the uncoiled portion was wider, being about 90nm in diameter. The filament appeared elliptical or circular, depending on the plane of longitudinal sectioning (compare Figs. 4.12 and 4.16) (Burges *et al*, 1974). The angle of tilt of the polar filament coil with respect to the long axis of the spore was 65° (anterior coil) and 54° (posterior coil). In some sections, the diameter of the filament closest to the posterior body was smaller (50nm) (Fig. 4.12).

The posterior body (posterosome) in *Nosema* sp. infecting *H. armigera* was approximately oval and tilted like the polar filament; the angle being approximately 48° with respect to the longitudinal axis of the spore (Figs. 4.12 and 4.16). The posterior body is about one-fifth of the total spore length (excluding the spore wall, Fig. 4.12), and was limited by numerous layers of membranes (Fig. 4.16).

The exospore of the mature spore was 35nm thick and followed the corrugated surface of the underlying electron-lucid endospore. No tubuli were seen projecting from the exospore as reported in *Pleistophora debaisieuxi* (Vávra, 1976a). The corrugations of the spore wall may be real or a consequence of fixation and subsequent processing. The endospore was present only in the mature spore and measured about 50-60nm except at the anterior cap where it was considerably thinner (< 10nm) or perhaps absent (Fig. 4.15).

Production of the polaroplast starts after the polar filament has formed a cap in the sporoblast or immature spore (Fig. 4.13). Subsequently, the anterior region of the polaroplast is seen surrounding the uncoiled part of the filament as stacks of numerous flattened lamellae (Fig. 4.14) and the more posterior vesicular region developed later (Fig. 4.15 and 4.19).

Figures. 4.16-4.20. Electron micrographs showing spore structure.

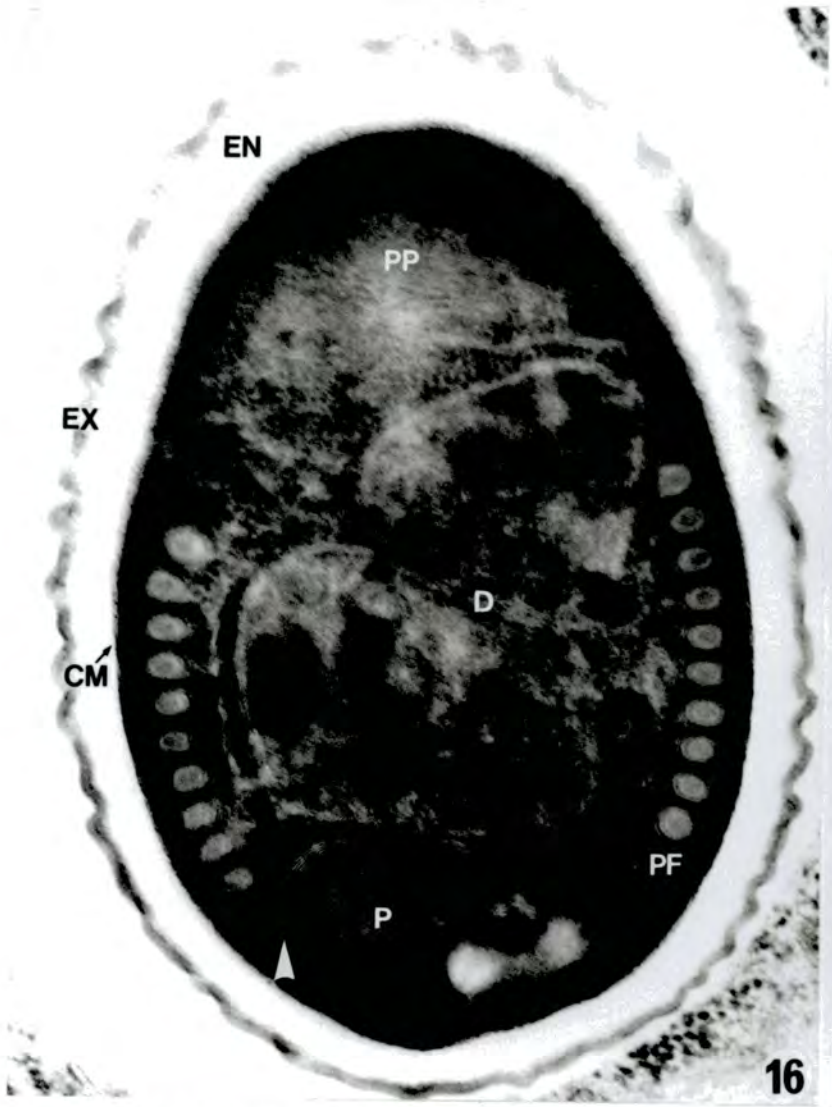
Figure 4.16. Posterior body (P); Diplokaryon (D); polar filament (PF); polaroplast (PP); cytoplasmic membrane (CM); endospore (EN); exospore (EX). continuation of polar filament membrane around posterior body (arrowhead) (x64,300).

Figure 4.17. Immature spore filament structure. Irregular coil (arrowhead); exospore (EX) (x160,000).

Figure 4.18. Mature spore filament structure showing lamellae of posterior-most filament. (x270,000).

Figure 4.19. Polaroplast of mature spore showing lamellar (LP) and vesicular portions (VP) (X100,000).

Figure 4.20. Fine structure of posterior body showing three subdivisions (arrows) and vesicular region (VR) (X100,000).


EN
PP
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CM
PF
P
16


EX
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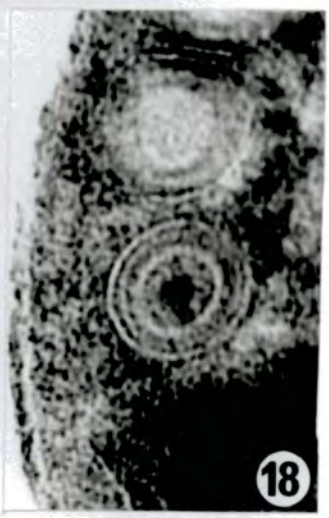
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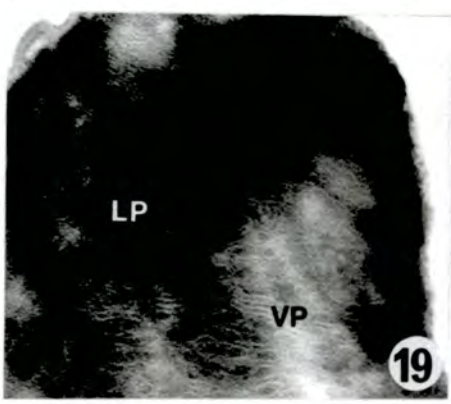
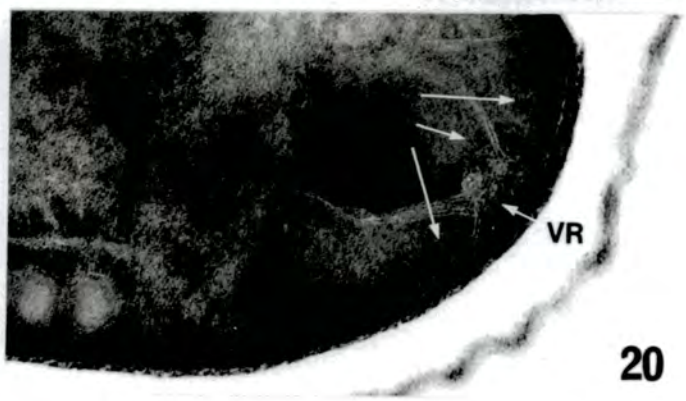

LP
VP
19


VR
20

CHAPTER 4
Discussion

The microsporidian parasite from *H. armigera* has a number of ultrastructural features that are characteristic of the genus *Nosema* (Weiser & Briggs, 1971; Sprague, 1977; Larsson, 1988; Toguebaye *et al*, 1988). Table 4.1 shows a comparison between the ultrastructural features of various species of the genus *Nosema* from lepidoptera and the microsporidium found in *H. armigera*. It can be seen that the species of *Nosema* sp. infecting *H. armigera* has ultrastructural features and morphology similar to several other species including *N. carpocapsae* (Malone & Wigley, 1981); *N. manierae* (Toguebaye and Bouix, 1983) and *N. transitellae* (Kellen *et al*, 1977). However, there are differences between these species and the *Nosema* species infecting *H. armigera*, e.g. no evidence of stages similar to the quadrinucleate meronts found in *N. manierae* were found and fixed spores from *H. armigera* were smaller than other *Nosema* spp. described (see Table 4.1). A complete identification of the current species must await further information concerning distribution, host specificity and the extent to which environmental conditions effect variability.

In the *Nosema* sp. infecting *H. armigera*, the polar filament consisted of several concentric layers. This agrees with other studies (Lom, 1972; Larsson, 1986) which report that the polar filament consists of several concentric layers. Indeed, layering is thought to be similar in all microsporidia polar filaments (Vávra *et al*, 1966). Eleven separate layers have been variously described by Vávra (1976a) and Larsson (1986). Using the nomenclature devised by the latter author, no subdivision of the central layer (Layer I) was distinguishable in the current study.

In some species of *Nosema* two distinct spore sizes have been reported, although no evidence of such dimorphism was found in the present study. Whilst there have been some suggestions that spore dimorphism does occur in certain spp. of *Nosema* (Larsson, 1983), Larsson (1986) has stated that this is a feature of the genus *Vairimorpha*.

The polar filament may form a specialized part of the posterior region of the spore called the posterior body. The location of the structure was identical to that occupied by the Golgi body earlier in the life-cycle (Fig. 4.8), suggesting that the posterior body is also elaborated by the Golgi body (Maurand and Vey, 1973). This view is supported by the apparent continuity between the membrane surrounding the posterior region of the polar filament, as seen in lateral longitudinal sections of the spore, and those associated with the posterior body (Fig. 4.16). Furthermore, it is reported that the posterior body spins vigorously during polar filament extrusion and is absent from the spore following filament discharge (Vávra, 1976a). Examination of Figure 4.20 shows the posterior body as being may made up of a number of membrane-bound sub-structures which may play a part in water uptake, thus providing the pressure needed for polar filament extrusion and the germination of the sporoplasm (Undeen, 1990).

The exospore of this insect microsporidian is formed from homogeneous electron-dense material and therefore conforms to that described as Type IB. The corrugations of the spore wall may be real or a consequence of fixation and subsequent processing. The polaroplast conformed to that described as Type I (nomenclature by Larsson, 1986) with closely packed lamellae in the anterior region and wider lamellae or more irregularly spaced vesicles in the posterior region. These lamellae extend to the periphery of the spore.

Many species of *Nosema* have been identified on the basis of the host in which they were first described. However, there is no evidence that *Nosema* spp. are in fact restricted to one host. For example, *Nosema eurytremae* has numerous different host species (Lie & Nasemary, 1973; Canning, 1975). Higby *et al*, (1979) have shown *N. eurytremae* may parasitise a variety of insect hosts and cell lines of *Xenopus* and *Aedes*, whilst cross infectivity and replication of *Nosema disstriae* and *Nosema algerae* has been demonstrated in different insect cell lines by Kurtti *et al* (1983) and Streett *et al* (1980), respectively. Similarly, *N. algerae* has been shown to be infective in a variety of different insect orders, in pig kidney cells (Undeen, 1975) and shrimps (Fournie *et al*, 1990) whilst *N. bombycis* infects some 20 species of lepidoptera and other insects (Machay, 1957; Kashkarova, 1981). These studies show microsporidians have a wide range of hosts.

Environmental conditions of the host are known to affect protozoan spore development. *H. armigera* has a high midgut luminal pH like other Lepidopteran larvae (Dow, 1984) and has been measured at pH 9.6 (unpublished result). This would suggest that the current microsporidian spores germinate effectively under alkaline conditions. Alkaline pH is a stimulus for spore germination in *N. locustae* (Whitlock and Johnson, 1990), e.g. *N. bombycis* (Ohshima, 1964); *N. fumiferanae* and *N. algerae* (Undeen, 1978). However, Undeen (1978) found *N. heliothidis* germinated best at about pH 7.0. Thus it would seem unlikely that the parasite investigated here is *N. heliothidis*.

Temperature is known to effect spore germination, viability and infectivity; peak evagination of spores occurred at 25°C in *N. bombycis* (Ohshima, 1964) with propagation and germination being inhibited at slightly higher temperatures (Whitlock and Johnson, 1990). From an analysis of data from Undeen and Maddox (1973), it can be shown that when host ranges are investigated, the hosts should be held at the same temperatures otherwise temperature dependent effects can be significant ($p < 0.01$).

Protozoan spore germination may be initiated by protease action, particularly chymotrypsin e.g. in *Eimeria tenella* (Wang and Stotish, 1975). It is possible that the use of chymotryptic inhibitors in transgenic plants could have an adverse effect on the sporulation of naturally occurring microsporidia infecting lepidopteran moths, or on those applied as a control measure.

A number of researchers have attempted to use small differences in spore size as an indication of genus e.g. Gassouma, (1972) compared *Plistophora leasi* and *P. simulii* (Debaisieux and Gastaldi, 1919), stating the former was larger by as little as 0.1μm.

However, the use of small differences in spore size should not be used alone as a taxonomic feature (Thomson, 1960) as spore size may vary between host species: e.g. *N. heliothidis* in *Campoletis sonorensis* (4.3 x 2.0μm) is smaller when infecting *Heliothis zea* (3.78 x 1.95μm) (Brooks & Cranford, 1972). Furthermore, spore size changes with geographic location of the same host species, *Heliothis zea*; 3.92 x 2.03μm in North Carolina and 4.40 x 1.79μm in Georgia (Brooks, 1968) and is affected by temperature (Maddox & Luckmann, 1966), age of the host and the host tissue infected (Blunck, 1954) and also the medium in which they are measured (Weiser, 1965). Fixation and staining may affect the size and shape of spores (Kudo, 1924; Larsson, 1989). Furthermore, significantly different spore sizes have been reported for the same parasitic species in different individual members of the same species of host (Blunck, 1954; Mercer & Wigley, 1987) and normal variations in spore size are to be expected as in any other population study (Walters, 1958; Lom *et al*, 1989). Therefore, small differences in dimensions from spores analyzed separately (e.g. in fixation, temperature etc.) should be treated with caution.

The factors discussed above make accurate identification to species level extremely difficult and therefore on this basis the *Nosema* sp. infecting *H. armigera* is not readily distinguishable from some species infecting other Lepidoptera. Nevertheless, given the potent insecticidal action noted in laboratory cultures of the lepidopteran pest, *H. armigera*, further studies on this nosematid are clearly warranted to determine host specificity and assess its potential as a control agent under field conditions.

CHAPTER 5

Nuclear Polyhedrosis Virus from the midgut of *Spodoptera littoralis*: ultrastructural characterisation and restiction endonuclease comparison with other NPVs.

Introduction

The genus *Spodoptera* contains nine species that occur throughout the subtropical and tropical areas of the world (Hostetter *et al*, 1990). Commonly called armyworms or climbing cutworms, they are polyphagous and frequently cause damage to ornamental plants and agricultural crops (Levy and Habeck, 1976; Hill, 1983; 1987). The species, *Spodoptera littoralis* is, together with *Helicoverpa armigera*, one of only seven lepidoptera which have IRAC (Insecticide Resistance Action Committee) Category I status (1990) as insecticide resistant crop pests (A. Devonshire, Rothamsted; pers. comm.). It is becoming increasingly difficult to control certain lepidopteran pests with insecticides; for example, *Plutella xylostella*, the diamondback moth, shows resistance to more than 46 insecticides (Miyata *et al*, 1986). The genus *Spodoptera* includes some of the world's major agricultural pests; their baculoviruses may be useful for biological control of these and other lepidopteran insect pests (Kelly, 1977; Granados and Federici, 1986; Padmavathamma and Veeresh, 1991). Baculoviruses possess unique features of specificity and efficacy against major insect pests that make them attractive as safe biological control agents (McIntosh and Ignoffo, 1986). Other than an increased mortality in the larval population of *S. littoralis*, NPVs cause reduced fecundity and/or egg hatchability of adult survivors (Santiago-Alvarez and Vargaz-Osuna, 1988) and additionally causes a proportionately higher reduction in the number of female pupae surviving compared with males (Vargaz-Osuna and Santiago-Alvarez, 1988).

NPVs nucleic acid is composed of double stranded DNA which exists as a covalently closed genome, contained within the rod-shaped, enveloped virions; they are thus in the genus *Baculovirus* (Vago *et al*, 1974; Matthews, 1979). In turn, the virions are frequently collected into 'bundles' and embedded in proteinaceous viral inclusions called polyhedra. NPVs have been isolated from a large number of insect species worldwide (Martignomi and Iwai, 1981), including *S. littoralis* (Cherry and Summers, 1985; Kislev and Edelman, 1982; Burgess, 1983). REN analysis has proved extremely useful in identifying insect viruses (Smith and Summers, 1978; Rohrman *et al*, 1978; Miller and Dawes, 1978a,b; Kelly *et al*, 1980; Tweeten *et al*, 1980; McIntosh and Ignoffo, 1983), detecting differences between genomic variants of the same virus species (Smith and Summers, 1978; 1979; Miller and Dawes, 1978a,b; Lee and Miller, 1978) and estimating the size of the viral genome (Rohrman and Beaudreau, 1977; Smith and Summers, 1978; Tweeten *et al*, 1980).

This chapter describes the morphological changes associated with the disease in *Spodoptera littoralis*, ultrastructural characterisation of the virus and comparison of restriction endonuclease patterns of the nucleic acids between NPVs from different sources.

CHAPTER 5
Materials and Methods

Electron microscopy

The preparation of midgut tissue from larval *Spodoptera littoralis* was prepared and sectioned as described in the General Materials and Methods.

Virus extraction and purification

The sample was homogenised in a 3ml glass homogeniser with 0.1% SDS on ice. The homogenate was then centrifuged on an MSE Chillspin for 5 minutes at 1000 rpm to remove insect debris. The supernatant was removed and subsequently centrifuged for 20mins at 4000 rpm. The resultant pellet was washed twice in distilled water to remove SDS. The final pellet was resuspended in 2 mls of distilled water and layered on a two step sucrose gradient; 50% sucrose (w/w) layered on top of a 3 ml cushion of 60% sucrose (w/w). This was then spun at 25,000 rpm for 1.5 hours in an MSE Highspeed 25 centrifuge. The virus band was removed, diluted 1:5 with distilled water and pelleted on the MSE Chillspin at 4000 rpm for 20mins. The virus pellet was again washed twice in distilled water in order to remove any sucrose. This final pellet was taken up in a small amount of distilled water and stored at 4°C.

DNA extraction

500μl of virus suspension at approximately 10^9 PIBs (polyhedral inclusion bodies)/ml was used. Firstly, 25μl of 1M Na_2CO_3 was added to the sample and incubated in a 37°C waterbath for 15 minutes until clear in order to break up the polyhedra. Secondly, 3μl protease K and 50μl of 10% SDS were added to the sample followed by incubation at 37°C for 30 minutes. The protease K breaks up any protein and SDS releases DNA from the virions. Finally, this was phenol/chloroform extracted to remove any protein.

Solutions used

a. TBE was made up as a 10x solution and diluted before use. The ingredients (Tris, 4.32g; boric acid, 270g; EDTA, 37.2g) were dissolved in 1 litre DDW and the pH adjusted to pH 8.3.

b. TE was also made up as a 10x solution. 12.1g Tris and 3.72g EDTA were dissolved in 1 litre DDW and the pH adjusted to pH 8.0.

An equal volume of TE-saturated phenol was added. The 2 phases were mixed together carefully by inverting the tube for about one minute, followed by a 2 minute high speed spin on a microfuge to separate the two phases. The upper phase containing the DNA was removed. This was repeated with more phenol and then with an equal volume of chloroform/isoamyl alcohol mix (24:1). The DNA solution was then dialysed against TE buffer at 4°C with 2 changes.

Endonuclease digest

50μl samples of the DNA were digested with various enzymes (*Eco*RI, *Hin*dIII, *Xho*I and *Pst*I) overnight at 37°C in a water bath. The digested DNA was run on a 0.6% agarose (Sigma, Type II) gel overnight at 50V using a large BRL Horizontal gel tank. The buffer used was TBE (see below), with ethydium bromide as a dye. The agarose was also made up in TBE buffer. Ethydium bromide was added at a concentration of 50μl/litre buffer. The DNA profiles were visualised on a UV light box and photographed. Molecular weight standards in Figure 5.6 (track 9) and Figure 5.7 (track 10) were lambda size marker bands produced after double digest with *Hin*dIII/*Eco*RI with sizes of 13.48, 3.27, 3.16, 2.71, 2.26, 1.29, 1.21, 1.00, 0.87, 0.62, 0.51 x 10^6 Da. Lambda DNA digests by *Hin*dIII alone, as for Figure 5.7 (track 1) gave 7 bands with molecular weights of 15.0, 6.12, 4.26, 2.84, 1.51, 1.32 and 0.32 x 10^6 Da. The molecular weight determination of the DNA fragments was determined using the corrected-reciprocal method of Southern (1979) shown in Fig. 5.1 and the markers in Figure 5.7, track 1. Mobilities of the bands were measured from an arbitrary point as the origin of the gel was not known. However, while this may have necessitated the correction, it affects neither the shape of the semilogarithmic or corrected reciprocal plots (Southern, 1979). The line on the corrected figure does not go through the origin, probably as a result of the estimate of the origin. The semi-logarithmic plot (Fisher and Dingman, 1971) gave large curvature in the high molecular weight range and was not used to calculate M_r (Fig. 5.2).

Figure 5.1

Lambda size markers produced after digest with *Hin*dIII were plotted according to the method of Southern (1979) to determine the molecular weight of NPV DNA fragments in Figure 5.7. The value of M_0 was calculated from the mobilities of bands 1, 6 and 7.

Legend: ordinate - molecular weight (MDa)
abscissa - $1/M\text{-}M_0$

Figure 5.2

Semi-logarithmic plot of Lambda size markers produced after digest with *Hin*dIII.

Legend: ordinate - distance from the estimated origin
abscissa - molecular weight (MDa)

Figure 5.1

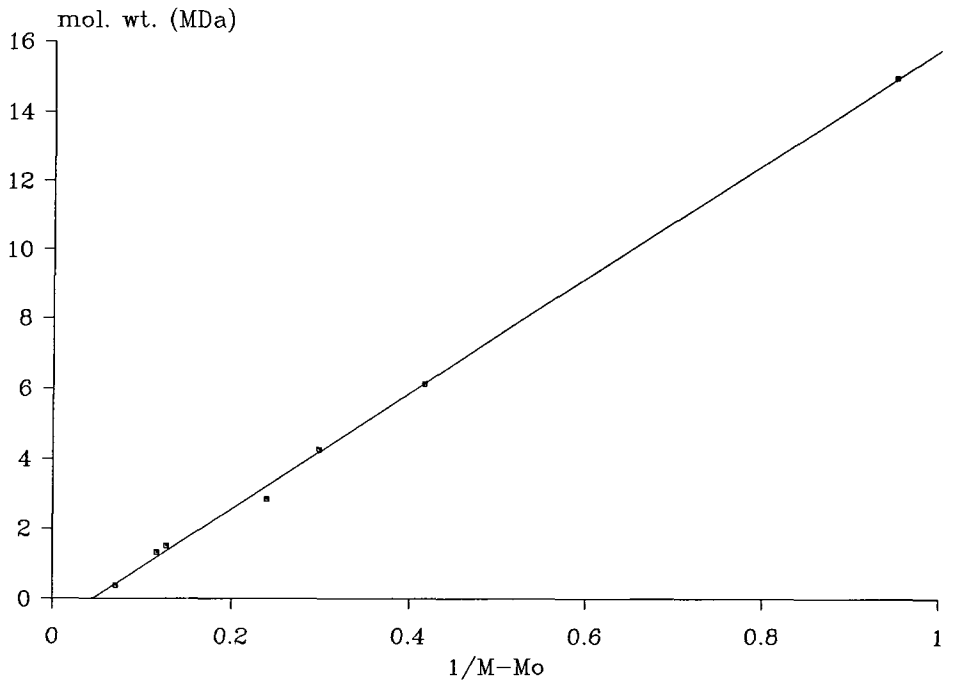
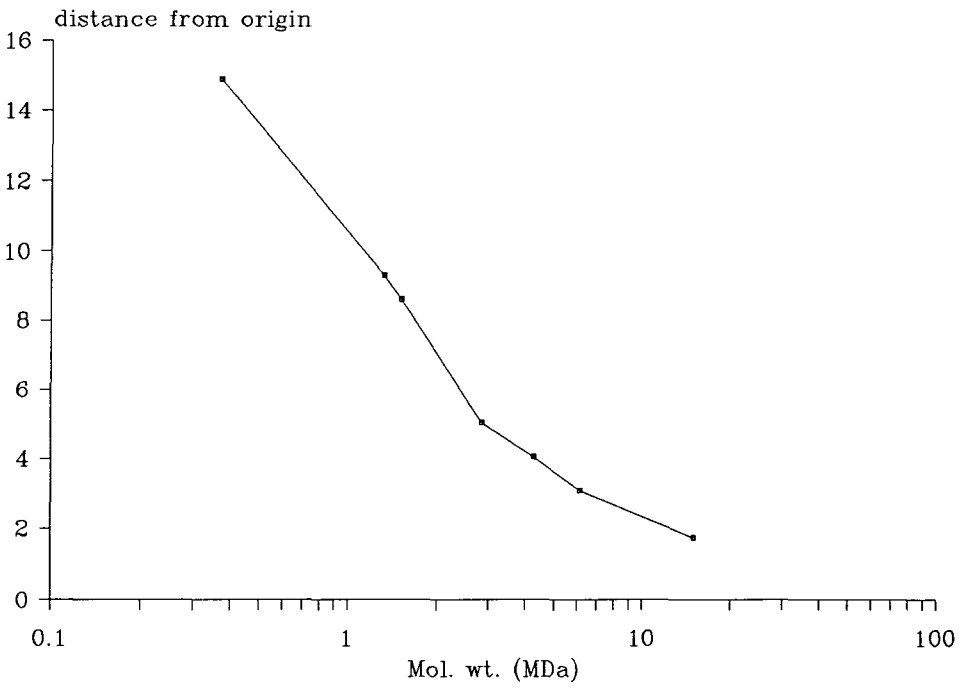


Figure 5.2



CHAPTER 5
Results

Animals infected with the NPV were noticeable among the stock colony due to the marked lack of dark pigmentation in their cuticle. Examples of such animals are shown in Figure 5.3. Infected late stage larvae frequently appeared glossy due to 'sweating'. In addition, animals with severe infection were very fragile and ruptured when handled. This was due to liquefaction of internal tissues, effectively leaving a thin cuticular 'bag' containing the destroyed tissue. This effect of polyhedrosis virus on lepidoptera is a characteristic of the infection and has been reported frequently (Steinhaus, 1960).

Electron microscopy

Electron microscopy of larval midgut indicated infection with an NPV (Fig. 5.4). The mean diameter of 17 randomly selected occlusion bodies observed within nuclei was 1.72 μm $\pm$ 0.06 (SEM) (range 1.25 to 2.05 μm). Each occlusion body contained an average of 17.3 $\pm$ 1.4 (SEM; N = 11) (range 11-27) bundles of nucleocapsids with 5.3 $\pm$ 0.35 (SEM; N = 26) (range 3-9) nucleocapsids (virions) per envelope (Fig. 5.5). Each virion measured about 35nm in diameter and 360nm in length.

Endonuclease digests

As shown in Figure 5.7, endonuclease digestion of NPV from IVEM and Durham isolates gave slightly different banding patterns between the two viruses. The fragmentation patterns of the eight NPVs digested with *Eco*RI are shown in Figure 5.6. It can be seen from this figure that the banding patterns for NPV IVEM and Durham isolates are similar. Figure 5.7 shows a comparison between these two NPVs when digested with a number of RENs. The fragmentation pattern by digestion with *Eco*R1 produces twenty-four fragments. Summation of these fragments gives an approximate molecular weight for the baculovirus DNA of 85.1 x 10^6 daltons and similar total weights with other endonucleases: *Hin*dIII digestion gives ten fragments with total weight of 80.0 x 10^6 daltons, *Xho*I digestion produces 23 fragments with a total weight of 80.6 x 10^6 daltons and *Pst*I digestion gives total of 81.2 x 10^6 daltons amongst eleven fragments. The average weight of the virus from the four digests is 81.7 x 10^6 $\pm$ 1.2 x 10^6 daltons.

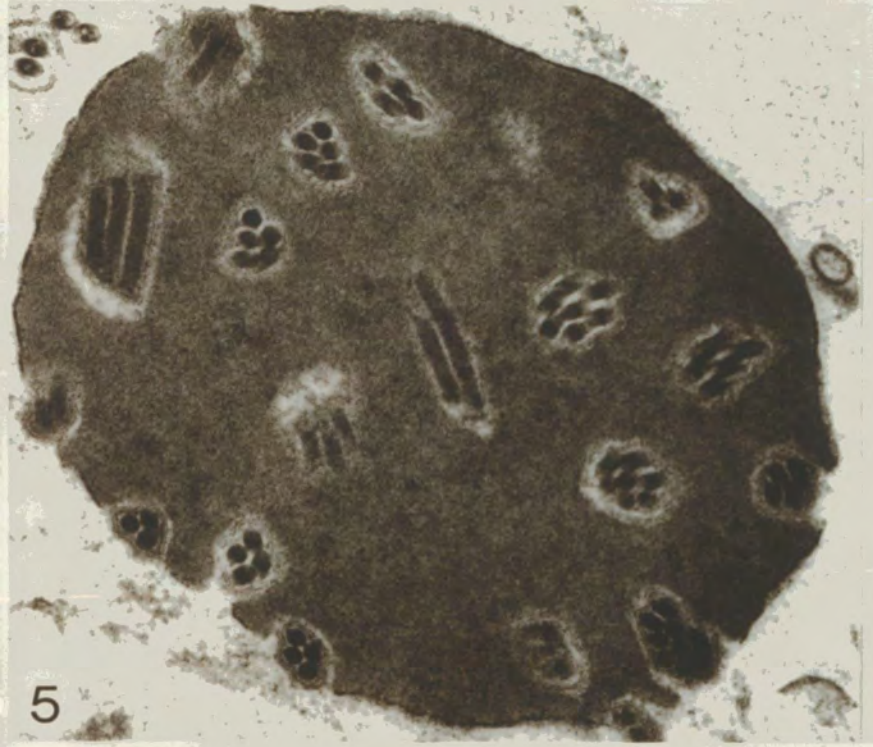
Figure 5.3

Colour change of *Spodoptera littoralis* larvae with NPV infection. the two animals on the left are heavily infected with the virus and appear glossy. The caterpillars on the right were feeding normally and did not display any symptoms of the disease.

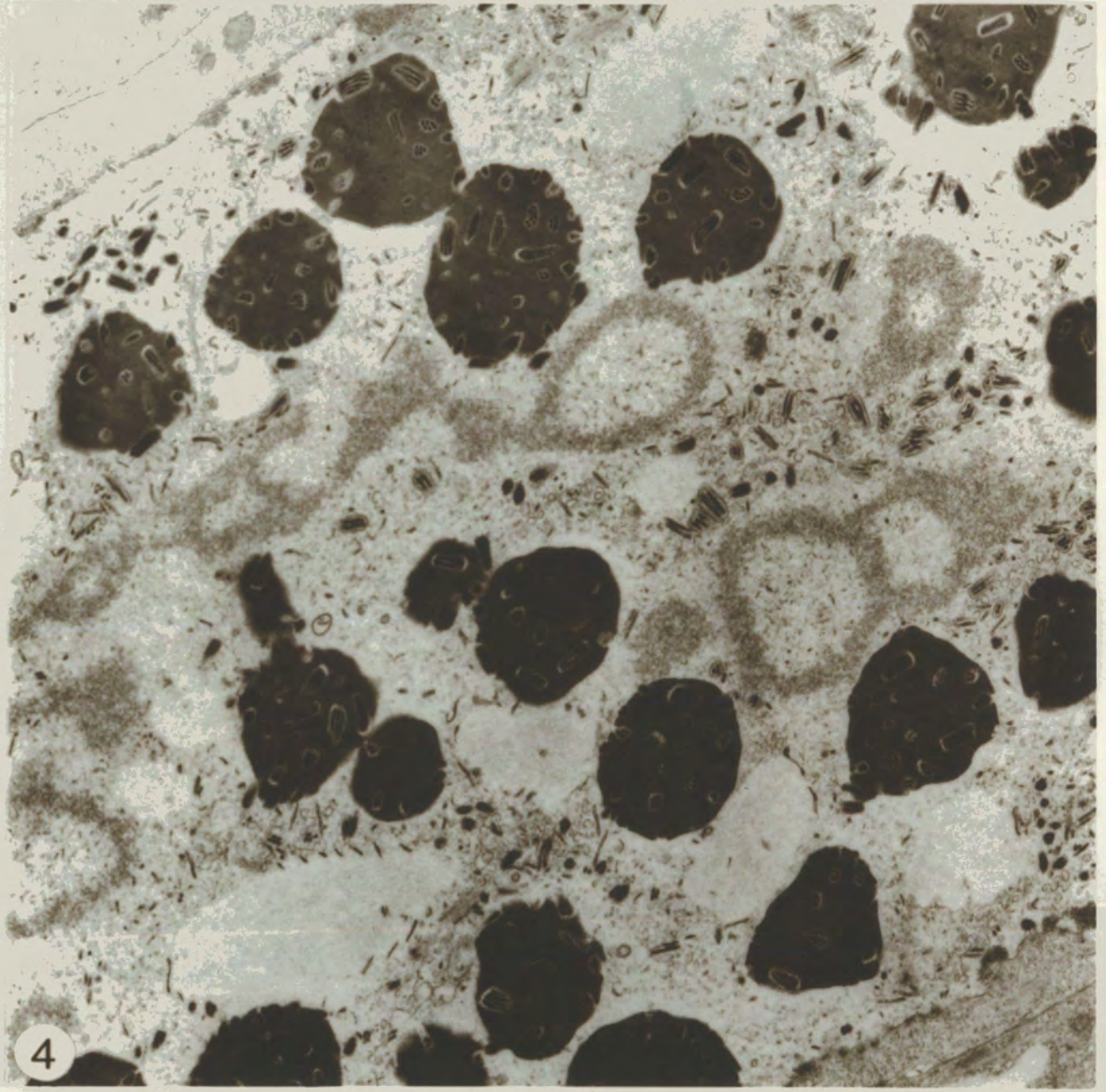
Figure 5.4 and 5.5

Electron micrographs of larval midgut. Figure 5.4 shows the nucleus of a columnar cell containing many polyhedra (occlusion bodies) containing a large number of nucleocapsid bundles (x16,000). The structure of the nucleocapsid bundles can be seen more clearly in Figure 5.5 (x56,000). Each bundle is surrounded by a double membrane and contains a variable number of virions.

3



5



4

CHAPTER 5
Discussion

The size of the polyhedra of the NPV infecting *Spodoptera littoralis* is comparable with those reported in other insects. Schwetzowa (1950) noted polyhedra of 1.5 to 2.0 microns in size in the greater wax moth *Galleria mellonella*. Aizawa (1962) found a size range of between 0.5 to 6 microns (mean 2.8 microns) also in *G. mellonella*, while a size of between 0.6 and 4.5 microns was found for the NPV infecting *Malacosoma alpicola* (Benz, 1963). In this case the number of virions per bundle varied up to nine; indeed, the number of nucleocapsids (virions) per bundle is often under ten (e.g. Hostetter *et al*, 1990), but in other species, such as *Bombyx mori*, the number of virions per bundle can be up to 19 (Berghold, 1963). Virion particle size found here in *S. littoralis* (35 x 360nm) is comparable with other measurements of NPVs from different sources: in virus particles of *Bombyx mori*, their dimensions are 75 x 370nm shrinking to 40 x 280 nm after purification (Berghold, 1953), whilst Benz (1963) found virions 35-41 nm x 270-370 nm, usually present as a pair in *Malacosoma alpicola*.

NPVs from *S. littoralis* and *S. litura* have been shown to be very variable and variability is quite common among wild-type baculoviruses (Kislev and Edelman, 1982). *Eco*R1 restriction digest patterns of the NPV isolate (lane 4 in Fig. 5.7) indicate that the Durham isolate falls into the SlNPV-B type (Cherry and Summers, 1985); this corresponds to the D isolate reported by Kislev and Edelman (1982) and to group II NPV described by Maeda *et al* (1990). The fragmentation pattern by digestion with *Eco*R1 produced twenty-four fragments. Summation of these fragments gives an approximate molecular weight for the baculovirus DNA of 85.1 x 10^6 daltons measured by the method of Southern (1979). The average molecular size as determined by the total fragment sizes of different RENs was 81.7 $\pm$ 1.2 x 10^6 daltons. This molecular weight estimate for the DNA is in the range reported for baculoviruses of between 50 to 94 x 10^6 daltons (Burgess, 1977), 50-150 x 10^6 daltons (Kislev and Edelman, 1982) or about 80 x 10^6 daltons (Entwhistle and Evans, 1985). This compares well with the average molecular weight of the D isolate (77.9 $\pm$ 3.0 x 10^6 daltons) calculated from the REN (restriction endonuclease) patterns using *Eco*RI fragments as standards (Kislev and Edelman, 1982) and the NPV isolate from *S. littoralis* reported by Burgess (1983) of 76.7 $\pm$ 3.6 x 10^6 daltons. However, it is significantly larger than the genome size of 62.4 $\pm$ 4.3 x 10^6 daltons reported for an NPV from *S. littoralis* by Kelly (1977) calculated by reassociation kinetics. A comparison of the REN analysis with published reports revealed that the REN digest patterns were unique. However, the REN digests were similar to those reported by Croizier *et al* (1989) and to the IVEM B-type NPV isolate shown on the same gel (Figure 5.7).

A comparison of Figures 5.6 and 5.7 shows that the Reading and IVEM isolate are very similar. However, the Durham isolate has a slightly different banding pattern,

but is still clearly a B-type NPV. Genotypic variances have been associated with differences in virulence (Gettig and McCarthy, 1982) and viral polypeptide profiles (Monroe and McCarthy, 1984). Sources for this genomic variation may include recombination between viruses and sequence deletion or reiteration within the viral genome (Cherry and Summers, 1985). Comparison of the REN digest with other published reports suggest that this virus has not been reported previously.

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