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UNIVERSITÉ D'OTTAWA
UNIVERSITY OF OTTAWA

To my parents
and friends,

ABSTRACT

Based on the hydrolysis of synthetic substrates, pH optima for substrate hydrolysis and effects of potential activators and inhibitors, the identity and occurrence of cysteine proteinases in several major coleopteran families were investigated.

The larval midgut of the Colorado potato beetle, *Leptinotarsa decemlineata* contains the cysteine proteinases cathepsin B and H, the aspartate proteinase cathepsin D and leucyl aminopeptidase. This study which shows that cathepsin B and H occur in *L. decemlineata*, is the first investigation to adequately identify cysteine proteinases in the order Coleoptera and indicate that not all beetles use the commonly reported serine proteinases for digestion.

Cathepsin B and the serine proteinase trypsin were identified in the larval midgut of the yellow mealworm, *Tenebrio molitor*, and is the first time that cysteine and serine proteinases have been reported in the same insect midgut. Cysteine and serine proteinases have very different requirements for optimal activity, however, these differences appear to be accommodated by the midgut of *T. molitor* since cysteine proteolytic activity was greater in anterior compared to posterior portions of the midgut and serine proteolytic activity was greater in posterior compared to anterior portions of the midgut. The activity of both proteinases were greater in gut contents than gut wall, consistent with extracellular digestion in insects.

Cathepsin B and the serine proteinase chymotrypsin were identified in the maize

weevil, *Sitophilus zeamais*. The larger grain borer, *Prostephanus truncatus*, feeds on the same food source as *S. zeamais* but uses trypsin, chymotrypsin and leucyl and arginyl aminopeptidase and not cysteine proteinases for digestion. Results have not only indicated that beetles feeding on similar food sources may not necessarily use similar proteases for digestion, but also indicates that beetles have not evolved cysteine proteinases as a consequence to feeding on food sources that contain trypsin inhibitors.

Trypsin, leucyl aminopeptidase and arginyl aminopeptidase and not cysteine proteinases were identified in a predacious carabid beetle, *Pterostichus corvinus*, a predacious tiger beetle, *Cicindela sp.* and a saprophagous hide beetle, *Dermestes maculatus*, the first investigation to address whether cysteine proteinases occur in predacious and saprophagous beetles.

A survey of cysteine and serine proteinases from beetles representing 16 different families, 9 different superfamilies and two of the four recognized beetle suborders, indicated that cysteine and serine proteinases are both common in beetles and that cysteine proteinases can occur in beetles with a variety of different feeding habits including leaf, seed and pollen feeding beetles as well as predacious and saprophagous beetles. The presence of cysteine proteinases in beetles does not appear to be related to a cellulose digesting microflora but may be related to the evolutionary history of the different taxonomic groupings of beetles.

RÉSUMÉ

Nous avons identifié la nature et détécte la présence de protéinases de la cystéine chez plusieurs familles importantes de Coléoptères en nous basant sur l'hydrolyse de substrats synthétiques, les pH optima pour l'hydrolyse de ces substrats et les effets d'activateurs et inhibiteurs.

L'intestin moyen du doryphore de la pomme de terre, *Leptinotarsa decemlineata*, contient les protéinases de la cystéine cathepsine B et H, la protéinase de l'aspartate cathepsine D, et l'aminopeptidase de leucyl. Cette étude, mettant en relief la présence de la cathepsine B et H chez *L. decemlineata*, est la première à caractéuser précisément des protéinases de la cystéine chez les Coléoptères et elle montre que ces insectes n'utilisent pas tous des protéinases de la sérine.

Nous avons identifié la cathepsine B et la protéinase de la sérine trypsine dans l'intestin moyen de la larve du ténébrion meunier, *Tenebrio molitor*, et nous rapporons pour la première fois les protéinases de la cystéine et de la serine ont été trouvées dans l'intestin moyen d'un même insecte. Les conditions requises pour l'activité optimale des protéinases de la cystéine et de la sérine sont très différentes. Ces conditions se trouvent apparemment dans l'intestin moyen de *T. molitor* puisque l'activité protéolytique de la cystéine est plus grande dans la partie antérieure de l'intestin moyen (si on la compare avec la partie postérieure), et l'activité protéolytique de la sérine plus grande dans la partie postérieure de l'intestin moyen (si on la compare à la partie antérieure). L'activité de ces deux protéinases est plus importante dans le contenu de l'intestin que dans sa

paroi, indiquant une digestion extracellulaire chez les insectes.

Nous avons trouvé la cathepsine B et la protéinase de la sérine chymotrypsine chez le charançon du maïs, *Sitophilus zeamais*. Le grand capuçin du maïs, *Prostephanus truncatus*, a un régime alimentaire similaire au *S. zeamais* mais utilise pour sa digestion les protéinases de la trypsine et de la chymotrypsine et les aminopeptidases arginyl et leucyl, mais pas de protéinases de la cystéine. Ces résultats indiquent non seulement que des Coléoptères utilisant les mêmes sources de nourriture n'utilisent pas nécessairement les mêmes protéinases pour leur digestion, mais aussi que les protéinases de la cystéine n'ont pas évolué en réponse à une nourriture contenant des inhibiteurs de la trypsine.

Nous avons aussi identifié la trypsine et les aminopeptidases leucyl et arginyl, mais pas de protéinases de la cystéine, chez les prédateurs *Pterostichus corvinus* et *Cicindela sp.*, et le saprophage *Dermestes maculatus*. Cette étude est la première à définir si les protéinases de la cystéine se trouvent chez les Coléoptères prédateurs et saprophages.

Une étude d'ensemble des protéinases de la cystéine et de la sérine a été entreprise sur 16 familles différentes, 9 super-familles et 2 des 4 sous-ordres de Coléoptères. Il ressort que les protéinases de la cystéine et de la sérine sont toutes deux fréquentes chez les Coléoptères et que les protéinases de la cystéine se retrouvent chez des Coléoptères présentant des habitudes alimentaires variées, tels ceux se nourrissant de feuilles, graines ou pollen, ainsi que chez les prédateurs et les saprophages. La présence des protéinases n'apparaît donc pas associée à la présence d'une microflore digérant la cellulose, mais plutôt à l'histoire évolutive des différents groupes taxonomiques de Coléoptères.

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Sincere thanks are extented to all the people that I have met and become friends with as a graduate student here at the University of Ottawa.

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Finally, I would like to express my gratitude to my parents who have always worked hard in order to give me the best possible education. This thesis is not just a personal achievement but is also a reflection of the never ending support from my parents. This thesis is dedicated to my parents, Walter and Irmgard Thie.

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Ac-Tyr-OEt	*N*-acetyl-L-tyrosine ethyl ester
Arg-NHNa	*L*-arginine-β-naphthylamide
bis-Tris	bis(2-hydroxyethyl)-imino-Tris(hydroxymethyl)-methane
bis-Tris-propane	1,3-bis[Tris-(hydroxymethyl)methyl-amino]propane
Bt	*Bacillus thuringiensis*
Bz-Arg-NHNa	α-*N*-benzoyl-DL-arginine-β-naphthylamide
Bz-Arg-Np	α-*N*-benzoyl-DL-arginine-*p*-nitroanilide
Bz-Arg-OEt	α-*N*-benzoyl-L-arginine ethyl ester
Bz-Try-OEt	α-*N*-benzoyl-L-tyrosine ethyl ester
Bz-Tyr-Np	α-*N*-benzoyl-L-tyrosine-*p*-nitroanilide
$CaCl_2$	Calcium chloride
CPI	Potato carboxypeptidase inhibitor
DFP	Di-isopropyl flourophosphate
DIMBOA	2,4 dihydroxy-7-methoxy-1,4-benzoxazin-3-one
DTT	Dithiothreitol
E-64	*trans*-epoxysuccinyl-L-leucyl-amido(4guanidino)butane
E.U.	Enzyme unit
EDTA	Ethylenediamine tetraacetic acid
glutaryl-Phe-NHNa	L-glutaryl-L-phenylalanine-β-napthylamide

glutaryl-Phe-Np	L-glutaryl-L-phenylalanine-*p*-nitroanilide
HA	hippuryl-L-arginine
Hb	Haemoglobin
HPA	Hide powder azure
HPLA	Hippuryl-DL-phenyllactic acid
IAA	Iodoacetamide
Leu-NHNa	L-leucine-β-napthylamide
Leu-Np	Leucine-*p*-nitroanilide
LTI	Limabean trypsin inhibitor
MBOA	6-methoxybenzoxazolin-3-one
$MgCl_2$	Magnesium chloride
N-succinyl-ala-ala-ala-Np	*N*-succinyl-alanyl-alanyl-alanine-*p*-nitroanilide
NaCl	Sodium chloride
PKI	Pancreas kallicrein inhibitor
PMSF	Phenylmethyl sulfonyl floride
PTI	Potato trypsin inhibitor
STI	Soybean trypsin inhibitor
TLCK	Tosyl-L-lysine chloromethyl ketone
Tos-Arg-OMe	*p*-tosyl-L-arginine methyl ester
TPCK	Tosyl-L-phenylalanine chloromethyl ketone
Tris	Tris-(hydroxymethyl)-aminomethane

Z-Ala-Arg-Arg-MeONHNa	α-*N*-benzyloxycarbonyl-L-Ala-L-Arg-L-Arg-4-methoxy-β-napthylamide
Z-glycyl-L-leucine	*N*-carbobenzoxy-glycyl-L-leucine
Z-valyl-leucine	*N*-carbobenzoxy-L-valyl-L-leucine
Z-Phe-Val-Arg-Np	α-*N*-benzyloxycarbonyl-L-Phe-L-Val-L-Arg-*p*-nitroanilide

CHAPTER 1

GENERAL INTRODUCTION

1.1 Vertebrate protease characteristics

1.1.1 Classification

Our current knowledge of insect proteolytic enzymes is based on comparisons with proteases identified and classified from vertebrates. The nomenclature system used here to describe proteolytic enzymes is consistent with that recommended by the nomenclature committee of the International Union of Biochemistry (1984).

Proteases are enzymes that hydrolyse the peptide bonds of proteins. They include the proteinases, that cleave internal peptide bonds and peptidases that remove terminal amino acids from either the carboxyl end or from the amino end of proteins. The former can be classified into four classes based on their catalytic mechanism (Barrett and McDonald, 1980; Storey and Wagner, 1986): serine proteinases, such as trypsin and chymotrypsin, with a serine or histidine in the active site; cysteine proteinases such as cathepsin B and cathepsin H, with a cysteine in the active centre; aspartic proteinases, such as pepsin and cathepsin D, with a aspartic acid in the active site; and metallo-proteinases, such as collagenase and acrolysin, that require an essential metal for activity. Peptidases are grouped into eight different classes (McDonald and Barrett, 1980)

according to their substrate specificity, the site of attack and the size of the liberated fragment and include: aminopeptidases, carboxypeptidases, dipeptidyl peptidases, tripeptidases, tripeptidyl peptidases, dipeptidases, peptidyl dipeptidases and omega peptidases. When present in the alimentary tract the action of both proteinases and peptidases completely digests proteins. Protein hydrolysis is generally initiated by proteinases that cleave internal peptide bonds and proteolysis is completed by peptidases that hydrolyse peptides from the ends of fragments produced by proteinases.

Each protease class includes individual proteinases or peptidases, their recognition based on characteristics unique to each enzyme. These include for example substrate specificity, pH for optimal activity, molecular weight and physiological function. There are at least 59 different mammalian serine proteinases, 12 different cysteine proteinases, 15 aspartic proteinases, 11 metalloproteinases as well as numerous unclassified proteinases (Barrett and McDonald, 1980). It is beyond the scope of this thesis to list characteristics pertinent for the identification of each of these individual enzymes and I will concentrate on cysteine proteinases such as cathepsin B and H, serine proteinases such as trypsin and chymotrypsin, the aspartate proteinase cathepsin D and aminopeptidase enzymes. For this reason, characteristics used to identify these proteases are emphasized.

1.2.1 Identification

Both whole protein and synthetic substrates can be used to detect proteolytic activity. A model introduced by Schecter and Berger (1967) (Fig. 1) describes the binding of a polypeptide substrate to a corresponding series of domains on a protease, with each domain on the protease interacting with one amino acid on the substrate. Based on this model, proteases can be distinguished by substrate specificity, with the amino acid at the reactive site of the substrate (designated the P1 site) generally determining the specificity of the interaction with the active or catalytic site on the protease (designated the S_1 binding site). For example, trypsin has a preference for binding basic amino acids such as arginine and lysine, chymotrypsin for aromatic amino acids such as tryptophan, tyrosine and phenylalanine. The subsites adjacent to the P1 site are in the catalytic site of the protease and are often critical for substrate binding by increasing the specificity of the enzyme for bonds in polypeptide substrates.

Identifying individual proteases in crude homogenates on the basis of substrate specificity requires that suitable substrates be chosen. Whole protein substrates such as haemoglobin (Hb), azocasein and hide powder azure (HPA) are useful in detecting general proteolysis, but cannot be used to identify individual enzymes in crude gut preparations based on their hydrolysis alone, since they are themselves a collection of reactive sites susceptible to hydrolysis by all enzymes present. Moreover, some whole protein substrates such as azocasein are insoluble in buffers having pH values below 6.0 (Sarath *et al.*, 1989) and therefore are not useful for detecting proteases in acidic conditions. Synthetic

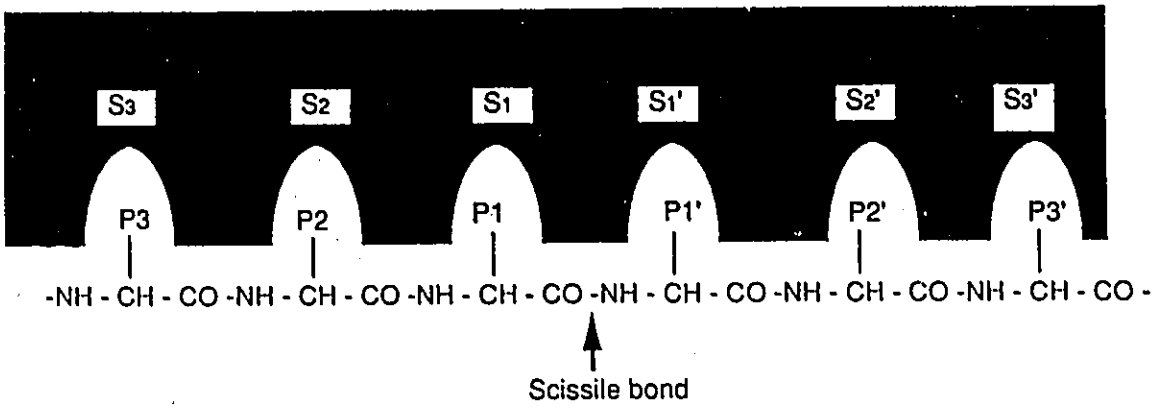

Fig. 1. The Schecter and Berger model for the binding of a polypeptide substrate to a protease (from Beynon and Bond (1989)). The C-terminus of the substrate is toward the right of the scissile bond, and the N-terminus toward the left of the scissile bond. The arrow indicates the catalytic site of the enzyme and corresponds to the point of cleavage of the substrate.

substrates have unique chemical structures, constructed around the chemistry of individual amino acids (or peptides) and are usually designed for hydrolysis by specific enzymes. Here substituents are added to either the α-NH_2 and/or α-COOH groups of amino acids, with the amino acid substituted in the reactive site of the substrate generally determining specificity. So, for example, many synthetic substrates for trypsin have the general formula of R-Arg-X, with the NH_2 terminus (R group) blocked by a number of reagents including benzoyl, benzoyloxycarbonyl, acetyl, tosyl and succinyl and the COOH-terminal or leaving group (X) by napthylamide, nitroanilide or methylcoumarin. Therefore, typical substrates often used to detect trypsin are Tos-Arg-OMe (*p*-tosyl-L-arginine methyl ester, Bz-Arg-OEt (α-*N*-benzoyl-L-arginine ethyl ester), Bz-Arg-Np (α-*N*-benzoyl-DL-arginine-*p*-nitroanilide) and Bz-Arg-NHNa (α-*N*-benzoyl-DL-arginine-β-napthylamide). The additional advantage of many synthetic substrates is that they are coupled through their α-COOH terminus to a chromophore such as nitroanilide that is released when hydrolysed, thus allowing for colourimetric quantification. More complex synthetic substrates are often designed with amino acids that bind with sites adjacent to the P1 site of the protease, and therefore increase the specificity of the interaction. For example, a more specific substrate used to detect trypsin is Z-Phe-Val-Arg-Np (α-*N*-benzyloxycarbonyl-L-Phe-L-Val-L-Arg-*p*-nitroanilide) (Barrett and McDonald, 1980).

A potential problem with synthetic substrates is that they can often be hydrolysed by other proteinases with similar substrate specificities. This is particularly true of the cysteine proteinase cathepsin B which has a specificity for arginine in the P1 position of small synthetic substrates (Barrett *et al.*, 1988), and thus hydrolyses synthetic substrates

used to detect trypsin. This problem can in some cases be alleviated when the pH for maximal substrate hydrolysis is determined. Maximum hydrolysis of Bz-Arg-NHNa and Bz-Arg-Np by cathepsin B occurs at pH 6.0 and by trypsin at pH 8.0 (Barrett and McDonald, 1980). But other enzymes in the same proteinase class may have similar pH optima and may potentially hydrolyse similar synthetic substrates such as the cysteine proteinase cathepsin H which can also hydrolyse Bz-Arg-NHNa in the mildly acidic pH range (Barrett and McDonald, 1980). Cathepsin H, however, is designated as an endoaminopeptidase because of its combination of both aminopeptidase as well as proteinase activity and can be distinguished from cathepsin B based on the hydrolysis of the aminopeptidase substrate Arg-NHNa (*L*-arginine-β-napthylamide) (Barrett and McDonald, 1980). Since this substrate is also a potential substrate of the peptidase arginyl aminopeptidase, identity again, can only remain tentative. Using the pH for maximal substrate hydrolysis to identify individual proteinases can also be complicated by using buffers that have variable ionic strengths over their buffering range, that are known to influence the catalytic activity of the enzyme. This problem can be overcome by using buffers developed by Ellis and Morrison (1982) that maintain constant ionic strengths.

Identification of individual proteinases can be made further when the pH and synthetic substrate hydrolysis are used in combination with the effects of potentially diagnostic activators and inhibitors on substrate hydrolysis. Potential activators, inhibitors and substrates commonly used to identify cathepsin B, cathepsin H, cathepsin D, trypsin chymotrypsin, leucyl aminopeptidase and arginyl aminopeptidase are given in

Table 1. Cysteine proteinases such as cathepsin B and H are characteristically activated by thiol compounds such as DTT (dithiothreitol) and often by chelating agents such as EDTA (ethylenediamine tetraacetic acid) and inhibited by thiol blocking reagents such as IAA (iodoacetamide) as well as inhibitors of microbial origin such as E-64 (*trans*-epoxysuccinyl-L-leucyl-amido(4guanidino)butane), isolated by Hanada *et al.* (1978) from an extract of *Aspergillus japonicus*. Cathepsin B can be distinguished from cathepsin H based on the hydrolysis of synthetic substrates such as Bz-Arg-NHNa in combination with inhibition by leupeptin, a microbial inhibitor isolated from various species of *Streptomyces* (Umezawa, 1976), which does not inhibit cathepsin H. Serine proteinases such as trypsin and chymotrypsin are often stabilized by divalent cations such as Ca^{2+} which have an apparent activation effect on activity and are inhibited by the general serine protease inhibitors DFP (di-isopropyl fluorophosphate) PMSF (phenylmethyl sulphonyl fluoride), aprotinin (from bovine lung) and STI (soybean trypsin inhibitor). Trypsin and chymotrypsin can be distinquished from each other based on maximal hydrolysis of synthetic substrates such as Bz-Arg-Np and Bz-Tyr-OEt (α-*N*-benzoyl-L-tyrosine ethyl ester) respectively, and effects of the synthetic inhibitors TLCK (tosyl-L-lysine chloromethyl ketone) and TPCK (tosyl-L-phenylalanine chloromethyl ketone), inhibitors specific for trypsin and chymotrypsin activities respectively. Table 1 reveals that arginyl aminopeptidase hydrolyses the same substrate as cathepsin H but can be distinguished from the latter enzyme based on maximal Arg-NHNa hydrolysis at a neutral pH and inhibition by the chelating agents EDTA and phenanthroline (indicating a Zn^{2+} metal ion involved in the catalytic mechanism), and no effects by cysteine proteinase inhibitors.

TABLE 1

Substrates, pH optima and potential activators and inhibitors used to identify the cysteine proteinases cathepsin B and H, the serine proteinases trypsin and chymotrypsin, the aminopeptidase enzymes, leucyl aminopeptidase and arginyl aminopeptidase and the aspartate proteinase, cathepsin D. Compiled from Barrett and McDonald (1980) and McDonald and Barrett (1980).

	Cysteine proteinases		Serine proteinases		Aminopeptidases		Aspartate proteinase
	Cathepsin B	Cathepsin H	Trypsin	Chymotrypsin	Leucyl aminopeptidase	Arginyl aminopeptidase	Cathepsin D
Potential substrate	Bz-Arg-NHNa	Arg-NHNa	Bz-Arg-Np	Bz-Tyr-OEt	Leu-Np	Arg-NHNa	Hb
pH optimum	6.0	6.0	8.0	7.5	9.0	7.0	4.0
DTT	+	+	-	-	-	-	N.E.
EDTA	+	+	N.E.	N.E.	N.E.	-	N.E.
Phenanthroline	N.E.	N.E.	N.E.	N.E.	-	-	N.E.
$CaCl_2$	N.E.	N.E.	+	+	+	N.E.	N.E.
$MgCl_2$	N.E.	N.E.	+	+	+	N.E.	N.E.
IAA	-	-	N.E.	N.E.	N.E.	N.E.	N.E.
Leupeptin	-	N.E.	-	N.E.	N.E.	N.E.	N.E.
E-64	-	-	N.E.	N.E.	N.E.	N.E.	N.E.
TLCK	-	-	-	N.E.	N.E.	N.E.	N.E.
TPCK	N.E.	N.E.	N.E.	-	N.E.	N.E.	N.E.
PMSF	N.E.	N.E.	-	-	N.E.	N.E.	N.E.
DFP	N.E.	N.E.	-	-	N.E.	N.E.	N.E.
STI	N.E.	N.E.	-	-	N.E.	N.E.	N.E.
Aprotinin	N.E.	N.E.	-	-	N.E.	N.E.	N.E.
Pepstatin	N.E.	N.E.	N.E.	N.E.	N.E.	N.E.	-

+ activation; - inhibition; N.E. No effect

Native leucyl aminopeptidase on the other hand is distinquished from arginyl aminopeptidase based on the hydrolysis of Leu-Np under alkaline pH conditions, inhibition by 1,10 phenanthroline (indicating a Zn^{2+} metal ion involved in the catalytic mechanism) but lack of inhibition by EDTA. Leucyl aminopeptidase can also be activated by $CaCl_2$ or $MgCl_2$ indicating the presence of either a Ca^{2+} or a Mg^{2+} activated enzyme, but unlike the native enzyme is reversibly inhibited by EDTA. The last protease mentioned on Table 1 is cathepsin D that can be identified based on the hydrolysis of haemoglobin in combination with inhibition by the aspartate specific inhibitor pepstatin.

Table 1 also reveals that inhibitors may also inhibit proteinases from more than one class. This includes leupeptin that inhibits trypsin as well as cathepsin B and TLCK that is known to inhibit various cysteine proteinases as well as being a specific inhibitor of tryptic activity.

In summary, reasonably accurate identification of enzymes should be based on the substrate hydrolysed, the pH at which maximal activity occurs, and the effects of biochemically diagnostic activators and inhibitors on activity. Final confirmation is made by purifying the enzyme and retesting with activators and inhibitors to confirm the identity of the individual enzymes. This last step is often difficult with insects because of their small size and problems in obtaining sufficient quantities of gut material for purification. Caution should also be used when analysing insect proteases, because even though they often share characteristics similar to their vertebrate counterparts they can differ in substrate specificity, effects with potentially diagnostic activators and inhibitors and molecular weight for example. These differences between vertebrate and insect

proteases will be evident on several occasions in the forthcoming sections.

For the purpose of this thesis, herbivorous insects are defined as those feeding on living plant tissue and include leaf feeders, leaf miners, stem and root borers, root feeders, juice feeders, fruit feeders, seed feeders and pollen feeders. Saprophagous insects are defined as insects that feed on dead organic matter either of plant or animal origin, and predacious insects are defined as those that feed on the tissue of living animals.

1.2 Insect proteases

Both proteinases and peptidases are generally required for complete digestion of a protein meal. Although peptidases have been reported in insects, their identity and occurrence has only been noted incidentally in studies on insect digestive enzymes, and most investigations have focused on proteinase activity. For a general review on peptidase activity in insects, see Applebaum (1985).

Of the four classes of proteinases, only serine, cysteine and aspartic activities have been reported in insects (Applebaum, 1985), with investigations on the serine proteinases trypsin and chymotrypsin being most common. This is probably due to the fact that trypsin and chymotrypsin were among the first enzymes isolated and characterized in vertebrates. Certainly vertebrate serine proteinases are considered to be the most thoroughly studied class of enzymes in the protease field and perhaps in all of enzymology (Dunn, 1989). As research on vertebrate proteases progressed, it became evident that there were many enzymes other than trypsin and chymotrypsin involved in

protein digestion. Until recently, trypsin and chymotrypsin were assumed to be the dominant digestive enzymes in all insects (see reviews by House, 1974; Law *et al.*, 1977; McFarlane, 1985), however, other proteinases particularly cysteine proteinases may also be important for digestion in insects (Applebaum, 1985), although the occurrence of these enzymes is not well understood.

Due mainly to their medical and veterinary importance, proteases from haematophagous insects, particularly the Order Diptera, have been thoroughly studied and characterized. The last two decades have seen increasing interest in the proteolytic enzymes of economically important herbivorous insects, including representative species from Coleoptera. These investigations are dominated with reports of serine proteinases although cysteine proteinases have more recently been detected in beetles and haematophagous and seed feeding Hemiptera. The intention of the forthcoming sections is to review the information gathered on serine proteinases in Coleoptera and selected herbivorous orders, as well as review the occurrence of cysteine proteinases in Hemiptera and Coleoptera. Peptidase activity identified in these orders is also addressed, but only to a minor extent.

1.2.1 Origin and production of insect proteases

The intestinal contents of most insects can support a variety of bacteria, yeasts or protozoa. These symbionts are thought to supply vitamins and other essential nutrients and may represent a possible source of luminal enzymes in insects (Applebaum, 1985). For

example, it has been suggested that proteolytic activity in haematophagous Hemiptera such as *Rhodnius prolixus* may be attributable to a proliferation of gut symbionts (Applebaum, 1985), since a peak of proteolytic activity in *R. prolixus* 6-7 days after blood ingestion (Houseman and Downe, 1983a) coincides with an increase in population size of the bacterium, *Nocardi rhodnii*, that *R. prolixus* harbours in its gut (Hill *et al.*, 1976). However, several electron microscopy studies have shown significant ultrastructural modifications of midgut cells as a result of feeding (Billingsley and Downe, 1983; Billingsley and Downe, 1985; Billingsley and Downe, 1988), indicating that the luminal proteases of *R. prolixus* originate from midgut cells and not the gut microflora. These ultrastructural modifications include changes in lysosomal structure as well as whorls of rough endoplasmic reticulum dispersing through the cytoplasm within 2 hrs of feeding (Billingsley and Downe, 1983). Both these changes have been found to correlate with the localization of aminopeptidase (Billingsley and Downe, 1985) and cathepsin B (Billingsley and Downe, 1988) in these organelles within the midgut cells of *R. prolixus*. Furthermore, studies by Billingsley and Downe (1988) suggest that the proteolytic enzymes such as cathepsin B are processed by the Golgi complex before secretion into the midgut lumen.

The investigations on *R. prolixus* indicate that the proteolytic enzymes required for digestion are endogenously produced. An endogenous origin for proteolytic enzymes has also been demonstrated for other haematophagous insects, including representatives from the Order Diptera (Richards, 1975; Rudin and Hecker, 1979) as well as representative species from other orders including Lepidoptera (Eguchi *et al.*, 1982;

Santos *et al.*, 1984) and Coleoptera (Baker, 1981a) suggesting that it may be relatively common in insects.

1.2.2. Serine proteolytic activity in beetles and other herbivorous insect orders.

1.2.2.1 Predacious beetles

Initial investigations on the proteolytic enzymes of the carabid beetle, *Pterostichus melanarius* reported maximal haemoglobin hydrolysis at pH 8.0 (Gooding and Huang, 1969). This study also reported trypsin activity based on the hydrolysis of Bz-Arg-OEt at pH 8.0 and inhibition by PMSF, while chymotrypsin was identified based on the hydrolysis of Bz-Tyr-OEt at pH 8.3 and inhibition by PMSF and TPCK, although the pH optima for these substrates was not reported. Trypsin and chymotrypsin have also been identified in the carabid beetles, *Calosoma calcidum* and *Carabus taedatus* (Cheung and Gooding, 1970). Trypsin in both beetles was identified based on maximal hydrolysis of Tos-Arg-OMe between pH 8.2-8.3 and inhibition by PMSF and TLCK and chymotrypsin based on the hydrolysis of Bz-Tyr-OEt, but the pH for maximal hydrolysis of Bz-Tyr-OEt was not reported. Vaje *et al.* (1984) also reported on trypsin and chymotrypsin activity in *Carabus auronitens*, *C. punctatoauratus*, *C. lineatus* and *C. splendens* based on the hydrolysis of Tos-Arg-OMe and Bz-Tyr-OEt respectively, both at pH 8.0, and inhibition by serine proteinase inhibitors, STI, limabean trypsin inhibitor (LTI), pancreas kallicrein

inhibitor (PKI) and ovomucoid, but again the pH for maximal substrate hydrolysis was not reported. Although these studies have reported on the identity of serine proteinases, many have not reported the pH for maximal synthetic substrate hydrolysis. The hydrolysis of a synthetic substrate at a fixed pH cannot be used to accurately identify proteolytic activity, since activity can be attributed to other proteinases such as cathepsin B that hydrolyse synthetic substrates with arginine in the P1 position of small synthetic substrates (Barrett *et al.*, 1988). This problem is evident again with recent investigations on predacious elaterid beetles, *Pyrophorus divergens* and *Pyrearinus termitilluminans* by Colepicolo-Neto *et al.* (1987), in which trypsin was identified based solely on the hydrolysis of Bz-Arg-Np at pH 8.5 and aminopeptidase based on the hydrolysis of Leu-Np (leucine-*p*-nitroanilide) at pH 8.0. By not reporting the pH for optimal hydrolysis and confirming the identity by using diagnostic inhibitors cannot preclude the presence of other proteinases, particularly cysteine proteinases, that also hydrolyse Bz-Arg-Np.

1.2.2.2 Saprophagous beetles.

There have been few reports on the proteolytic enzymes of saprophagous beetles, with one of the most thorough on the black carpet beetle, *Attagenus megatoma.* Initial studies reported maximal hydrolysis by midgut homogenates from *A. megatoma* of the whole protein substrate casein at pH 8.0 and reported on trypsin and chymotrypsin activity at pH 8.0 based on the hydrolysis of Tos-Arg-OMe and Bz-Tyr-OEt, respectively (Baker, 1976). Proteolytic activity in *A. megatoma* was later resolved by a combination

of ion exchange chromatography and gel electrophoresis into three trypsin-like enzymes (AT, CT-1 and CT-2) and four chymotrypsin-like enzymes (AC, CC-1, CC-2 and CC-3) (Baker, 1981b). All three trypsins maximally hydrolysed Bz-Arg-Np from pH 8.0-10.0, hydrolysed Tos-Arg-OMe and Bz-Arg-OEt and were inhibited by PMSF, TLCK and STI. All four chymotrypsins hydrolysed Ac-Tyr-OEt (*N*-acetyl-L-tyrosine ethyl ester), Bz-Tyr-OEt, Bz-Tyr-Np (α-*N*-benzoyl-L-tyrosine-*p*-nitroanilide) and glutaryl-Phe-Np (L-glutaryl-L-phenylalanine-*p*-nitroanilide) at pH 8.0, and were completely inhibited by PMSF and TPCK; AC and CC-1 also maximally hydrolysed Bz-Tyr-OEt at pH 7.5-10.0. Based on similar purification procedures two carboxypeptidases (Baker, 1981c) and an aminopeptidase (Baker and Woo, 1981) have also been identified from midgut homogenates of *A. megatoma*, and characterized based on maximal hydrolysis of synthetic substrates and inhibition by the metal chelating agents EDTA and 1,10 phenanthroline.

The only other saprophagous beetle that has received any significant amount of investigation has been the hide beetle, *Dermestes maculatus*, in which six trypsins, two chymotrypsins, one aminopeptidase and carboxypeptidase activities were demonstrated by polyacrylamide gel isoelectric focusing in combination with synthetic substrate hydrolysis and the effects of various inhibitors (Baker, 1982a). All 6 trypsins hydrolysed Bz-Arg-NHNa at pH 8.0 and were inhibited by PMSF and TLCK, both chymotrypsins hydrolysed glutaryl-Phe-Np at pH 8.0 and were inhibited by TPCK, although the pH for maximal substrate hydrolysis was not reported. Carboxypeptidase and aminopeptidase activities hydrolysed Z-glycyl-leucine (*N*-carbobenzoxy-glycyl-L-leucine) and Leu-NHNa (L-leucine-β-napthylamide) respectively at pH 7.5, but again the pH for maximal substrate

hydrolysis was not reported.

Although serine proteinases have been demonstrated in these two beetles, assay conditions used were designed to detect proteinases, particularly serine proteinases that have optimal activity in alkaline pH environments. These results cannot preclude the presence of cysteine proteinases that have maximal activity at acidic rather than the alkaline environments. Additional studies will be required in order to determine whether cysteine proteinases are also involved in digestion within this group.

1.2.2.3 Herbivorous beetles.

Some of the earliest research on digestive proteolysis in Coleoptera were on the stored grain product pests *Tenebrio molitor* (the yellow mealworm) and *Tribolium confusum* (the confused flour beetle) in which maximal casein hydrolysis of larval gut preparations occurred at pH 6.2 to 6.4 and 6.5 to 6.9, respectively (Birk *et al.*, 1962). Based on differential sensitivities to soybean trypsin inhibitor, two proteolytic fractions were identified in larval *T. molitor*; a soybean trypsin inhibitor fraction that contained trypsin-like activity detected based on the hydrolysis of casein and the synthetic substrate carbobenzoxy-tyrosine nitrophenyl ester at pH 6.5 and inhibition by STI, LTI, ovomucoid and DFP; and a soybean trypsin inhibitor resistant fraction that contained both carboxypeptidase B and aminotripeptidase based on the hydrolysis of the synthetic substrates Hippuryl-lysine and leucylglycylglysine, respectively (Applebaum *et al.*, 1964). This latter fraction was also reported to contain an unidentified activity that was thiol

activated and EDTA stabilized and activity attributed to either carboxypeptidase B or the cysteine proteinase cathepsin B (Applebaum *et al.*, 1964), however, this activity was not further investigated. Proteolytic activity in adult *T. molitor* was also resolved into an α- and β-protease (Zwilling, 1968; Zwilling *et al.*, 1972). The α-protease hydrolysed casein at pH 8.0 but differed markedly from bovine trypsin and chymotrypsin since it hydrolysed neither Bz-Arg-OEt nor Ac-Tyr-OEt nor was it effected by either TLCK or TPCK. On the other hand, the β-protease shared many vertebrate trypsin characteristics such as hydrolysis of Bz-Arg-OEt and inhibition by STI and LTI as well as PMSF, but its cleavage specificity towards the β-chain of oxidised insulin and a dodeca-peptide, derived from the active center of pig muscle lactate dehydrogenase, differed from vertebrate trypsin. Trypsin in larval *T. molitor* was later purified and resembled bovine trypsin with respect to its size, kinetic parameters, specificity towards the trypsin substrates Bz-Arg-Np and Tos-Arg-OMe and inhibition by trypsin inhibitors such as TLCK and STI, respectively, but differences in conformation from bovine trypsin were reported (Levinsky *et al.*, 1977).

Baker (1982b) reported that larval midgut homogenates from the Rice weevil, *Sitophilus oryzea*, the maize weevil, *S. zeamais* and the granary weevil, *S. granarius* hydrolysed the general proteinase substrate HPA over a broad pH range with a peak of activity from pH 3.0 to pH 4.0 and after a decline in activity to pH 5.0, there was an increase in activity to an optimum of 10.0. This investigation also reports on trypsin, chymotrypsin, aminopeptidase and carboxypeptidase in all 3 beetles, although the pH for maximal substrate hydrolysis was not reported. Trypsin was detected based on the

hydrolysis of Bz-Arg-OEt at pH 9.0, chymotrypsin based on the hydrolysis of Bz-Tyr-OEt at pH 9.0, aminopeptidase based on the hydrolysis of Leu-Np at 7.0 and carboxypeptidase based on the hydrolysis of Z-valyl-leucine (*N*-carbobenzoxy-L-valyl-L-leucine) and *N*-carbobenzoxy-glycyl-L-tyrosine at pH 9.0. The hydrolysis of Bz-Arg-Np was also reported as being fivefold higher by *S. granarius* midgut homogenates and Leu-Np hydrolysis was also reported as being greater in *S. granarius* compared to the other two species (Baker, 1982b) and both these activities were subsequently further identified in *S. granarius*. Here trypsin was identified based on the hydrolysis of Bz-Arg-Np at pH 9.0 and inhibition by TLCK and STI, and aminopeptidase based on the hydrolysis of Leu-Np at pH 7.0 and inhibition by 1,10 phenanthroline and the heavy metals Cd^{2+} and Hg^{2+}. Although this investigation by Baker (1982b) has detected activity based on the hydrolysis of synthetic substrates, neither the pH optima for hydrolysis nor the effects of diagnostic inhibitors on activity were reported for either *S. zeamais* or *S. oryzae* and cannot preclude the presence of other proteases with similar substrate specificities. Furthermore, the peak of activity between pH 3.0 and 4.0 detected by HPA hydrolysis in *S. zeamais* and *S. oryzae* and attributed to an acidic proteinase (Baker, 1982b) may also be attributable to a cysteine proteinase, but this was not examined.

Baker *et al.* (1984) reports that larval midgut homogenates from the sweet potato weevil, *Cylas formicarius elegantulus*, maximally hydrolyses the general proteinase substrate HPA over a broad pH range with a peak of activity from pH 9.0 to 11.5 and maximal casein hydrolysis occurred at pH 11.0. Aminopeptidase was also detected in this study by maximal hydrolysis of Leu-Np at pH 8.0, carboxypeptidase by maximal

hydrolysis of Z-valyl-leucine from pH 8.0 to 9.0 and trypsin by the hydrolysis of Bz-Arg-Np, but the pH for maximal hydrolysis of this latter substrate was not reported. Confirmation of activity using diagnostic inhibitors was not reported and cannot preclude the presence of other proteinases such as cysteine proteinases that hydrolyse the synthetic substrate Bz-Arg-Np.

Trypsin, aminopeptidase and carboxypeptidase A have been partially purified and characterized in grass grub larvae, *Costelytra zealandica* (Christellar *et al.*, 1989). Trypsin maximally hydrolysed Bz-Arg-Np at pH 9.0-10.0 and was inhibited by PMSF, TLCK, STI, LTI and aprotinin, aminopeptidase maximally hydrolysed Leu-Np at pH 7.5-8.5 and was inhibited by the heavy metal ions Cd^{2+}, Cu^{2+}, Hg^{2+} and Pb^{2+}, and carboxypeptidase A hydrolysed HPLA (hippuryl-DL-phenyllactic acid) with a maximum at pH 8.5 and was inhibited by potato carboxypeptidase inhibitor (CPI). This study also reports chymotrypsin and elastase activities based on the hydrolysis of Bz-Tyr-Np and *N*-succinyl-ala-ala-ala-Np (*N*-succinyl-alanyl-alanyl-alanine-*p*-nitroanilide) respectively, and inhibition by PMSF, as well as the lack of cysteine proteinase activity. This latter activity was tested for based on the hydrolysis of Bz-Arg-OEt at pH 6.0, in the presence of DTT and EDTA, and the lack of inhibition by either IAA or E-64 on substrate hydrolysis. Although Bz-Arg-OEt is a substrate hydrolysed by cysteine proteinases, it is generally regarded as a substrate for trypsin rather than cysteine proteinases (Barrett and McDonald, 1980) and assays for the detection of cysteine proteinases using Bz-Arg-NHNa as a substrate have been previously reported (Houseman, 1978).

1.2.2.4 Other herbivorous insect orders

The only other herbivorous insects that have received any significant amount of study are representatives from Orthoptera, Hymenoptera and Lepidoptera, with this latter order receiving the greatest number of investigations.

1.2.2.4.1 Orthoptera

In some of the earliest investigations on the African migratory locust, *Locusta migratoria*, protease activity was demonstrated in various regions of the digestive tract of adult insects based on the hydrolysis of the whole protein substrate azoalbumin at pH 8.0 (Khan, 1963) and dipeptidase activity reported based on the hydrolysis of DL-alanylglycine at pH 8.0 (Khan, 1962), but the pH for maximal substrate hydrolysis was not reported. Using ion-exchange chromatography Knecht *et al.* (1974) isolated four proteinase fractions (P_I-P_{IV}) in *L. migratoria*. All four fractions resembled serine proteinases based on hydrolysis of synthetic substrates and inhibition by PMSF. P_I and P_{IV} resembled vertebrate chymotrypsin and trypsin respectively, based on activity against casein, β-chain of oxidised insulin, hydrolysis of synthetic substrates and effects of various protease inhibitors. Trypsin (P_{IV}) maximally hydrolysed Bz-Arg-OEt at pH 9.0, hydrolysed Bz-Arg-NHNa and was inhibited by PMSF and TLCK. Chymotrypsin (P_I) hydrolysed casein with a pH maximum at pH 9.0, hydrolysed Bz-Tyr-OEt and *N*-acetyl-L-phenylalanine-napthylester at pH 8.0 and was inhibited by PMSF and TPCK, although the

pH for maximal hydrolysis of these latter two substrates was not reported. More recent investigations have purified two trypsins and chymotrypsin in *L. migratoria* (Sakal *et al.*, 1988; Sakal *et al.*, 1989) by DEAE-cellulose and affinity chromatography. Trypsin and chymotrypsin maximally hydrolysed Tos-Arg-OMe and Bz-Tyr-OEt respectively at pH 8.5-9.0 and were inhibited by PMSF, serine proteinase inhibitors from soybeans, chickpeas and chicken ovomucoid as well as TLCK and TPCK, that are specific inhibitors of trypsin and chymotrypsin respectively.

Trypsin, elastase, aminopeptidase and carboxypeptidase A and B have been partially purified and characterized in nymphs of the black field cricket, *Teleogryllus commodus* (Christellar *et al.*, 1990). Trypsin was identified based on maximal hydrolysis of Bz-Arg-Np at pH 9.0 and inhibition by PMSF, STI, aprotinin, wheat germ trypsin inhibitor and human inter-alpha trypsin inhibitor and elastase was identified based on maximal hydrolysis of *N*-succinyl-ala-ala-ala-Np at pH 9.0 and inhibition by STI, PMSF, alpha-1 antiproteinase inhibitor, secretory leukocyte trypsin inhibitor and chicken ovoinhibitor. Aminopeptidase was identified based on maximal hydrolysis of Leu-Np at pH 7.5 and inhibition by Cd^{2+}, Cu^{2+}, carboxypeptidase A based on maximal hydrolysis of HPLA between pH 8.0-9.0 and inhibition by CPI, and carboxypeptidase B based on hydrolysis of HA (hippuryl-L-arginine) at pH 8.0 and inhibition by CPI. This study also reports on chymotrypsin based on the hydrolysis of Bz-Tyr-Np at pH 8.0 as well as the absence of cysteine proteinase activity based on the hydrolysis of Bz-Arg-OEt at pH 6.0, in the presence of DTT and EDTA, and lack of inhibition by IAA, cystatin and E-64 on substrate hydrolysis.

The investigation on *T. commodus* has been the only attempt to address the presence of cysteine proteinases in an orthopteran insect. Not until more species are investigated, will the diversity of proteases that may exist within this order be fully understood.

1.2.2.4.2 Hymenoptera

The most extensive investigation on the proteolytic enzymes in this order have been on the honey bee, *Apis mellifera*. Initial investigations by Giebel *et al.* (1971) isolated four protease fractions (A-D) from the adult worker honey bee, *A. mellifera* midgut based on differential cleavage specificities of the β-chain of oxidized insulin, hydrolysis of synthetic substrates at pH 8.0 and effects of diagnostic inhibitors, but the pH for maximal substrate hydrolysis was not reported. Fraction A can be classified as trypsin based on the hydrolysis of Bz-Arg-OEt and Bz-Arg-NHNa and inhibition by TLCK and fraction D as chymotrypsin based on the hydrolysis of Ac-Tyr-OEt and glutaryl-Phe-NHNa (L-glutaryl-L-phenylalanine-β-napthylamide) and inhibition by TPCK. Fraction B was also reported to be similar to chymotrypsin, based on its hydrolysis of glutaryl-Phe-NHNa and inhibition by TPCK, but differed from bovine chymotrypsin in its cleavage specificity towards bovine insulin B-chain. Fraction C was reported to hydrolyse casein at pH 8.0 but was only slightly inhibited by PMSF, TLCK and TPCK and had no activity towards any of the synthetic substrates tested other than Bz-Arg-NHNa. Proteases of larval and adult honey bees were further purified (Dahlman *et al.*,

1978), but this investigation revealed only the presence of three proteinases, one of them with cleavage properties similar to trypsin (named protease A) and two similar to chymotrypsin (B_{III} and B_{IV}). This investigation revealed that protease A was absent in the larval midgut, although reported that they were able to purify a Bz-Arg-Np hydrolysing enzyme in larval *A. mellifera* that may be a catheptic protease based on a immunological experiment that demonstrated that this Bz-Arg-Np hydrolysing activity did not cross-react with protease A identified in adult honey bees.

Although serine proteinases have been detected in *A. mellifera*, assay procedures and substrates used, would only detect proteinases that are active in the alkaline pH range. The detection of an unknown Bz-Arg-NHNa hydrolysing activity reported by Giebel *et al.* (1971) and a Bz-Arg-Np hydrolysing catheptic protease reported by Dahlman *et al.* (1978), indicate that cysteine proteinases may be present within this insect, although the identity of this activity has remained unresolved.

1.2.2.4.3 Lepidoptera

Due to the large number of economically important herbivorous Lepidoptera, this order has received extensive investigation. Unlike previous investigations of serine proteinases in herbivorous insects, reports on Lepidoptera reveal enzymes with maximal activity at alkaline pH values of 9.0 or higher (Applebaum, 1985). This differs from vertebrates, in which maximal activity is typically around the pH range of 6.5-7.5 (Barrett and McDonald, 1980), indicating that even the commonly reported serine proteinases in

insects are unlike their vertebrate counterparts.

Initial investigations on larval army worm *Spodoptera litura* (Ahmad *et al.*, 1976), reported maximal casein hydrolysis at pH 11.0. Activity was resolved into three trypsin enzymes by gel filtration and ion exchange chromatography that maximally hydrolysed casein at three different pH values, 9.0, 10.5 and 11.0 and all three hydrolysed Bz-Arg-Np and Bz-Arg-OEt and were inhibited by PMSF and TLCK (Ahmad *et al.*, 1980). Larvae of the corn Earworm, *Heliothis zea* (Klocke and Chan, 1982) and the cabbage looper, *Trichoplusia ni* (Pritchett *et al.*, 1981), have optimal activities against casein at pH 9.8 and 11.0 respectively and tryptic and chymotryptic activities have been reported in *T. ni* (Pritchett *et al.*, 1981) and *H. zea* (Broadway and Duffey, 1986) based on the hydrolysis of Tos-Arg-OMe and Bz-Tyr-OEt respectively, however, the pH for maximal substrate hydrolysis was not determined in either study. Recent investigations by Lenz *et al.* (1991) confirm the presence of trypsin and chymotrypsin in *H. zea* as well as revealing the identity of carboxypeptidase A and B and aminopeptidase activities, based on the pH for maximal synthetic substrate hydrolysis. Maximal casein hydrolysis by larval silkworm, *Bombyx mori,* was reported at pH 11.2 by Eguchi and Iwamoto (1976) and using azocoll as a general substrate two alkaline proteinases with maximal activity at 10.5 and 11.0 in the wax moth, *Galleria mellonella* have been reported (Hamed and Attias, 1987). *Bombyx mori* activity was later attributed to 3 trypsin enzymes separated by gel filtration chromatography, distinguished by their specificity towards Bz-Arg-Np and Tos-Arg-OMe and inhibition towards DFP and PMSF (Eguchi and Iwamoto, 1982). Trypsin activity has also been partially purified in the larval midgut of the tobacco hornworm, *Manduca sexta*

(Miller *et al.*, 1974) by affinity chromatography and gel electrophoresis. Trypsin was identified based on the hydrolysis of Bz-Arg-OEt at pH 8.3 and inhibition by DFP, PMSF and TLCK, although the pH for maximal substrate hydrolysis was not determined. Recent investigations have identified trypsin and chymotrypsin in *M. sexta* using maximal synthetic substrate hydrolysis in combination with the effects of diagnostic inhibitors and reports on optimal activity greater than 10.0 (Houseman *et al.*, unpublished).

Investigations on the European corn borer, *Ostrinia nubilalis* (Houseman *et al.*, 1989), reported optimal azocasein hydrolysis at pH 10.0 or higher and reports on the identity of a high alkaline trypsin and a low alkaline trypsin and chymotrypsin in midgut homogenates. The high alkaline trypsin hydrolyses Bz-Arg-Np at pH values > 10.0 and is activated by the divalent cations Ca^{2+} and Mg^{2+}. The low alkaline trypsin maximally hydrolyses Bz-Arg-OEt at pH 9.0 but is not effected by either Ca^{2+} or Mg^{2+} and chymotrypsin maximally hydrolyses Bz-Tyr-OEt at pH 7.5-8.0. Both trypsin activities and chymotrypsin were further identified based on their inhibition by PMSF, STI and ovomucoid as well as specific inhibition of tryptic activity by TLCK and chymotryptic activity by TPCK. Recent investigations have partially purified and identified the serine proteinase elastase in *O. nubilalis* (Houseman and Moore, unpublished). Activity was identified based on optimal hydrolysis of *N*-succinyl-ala-ala-ala-Np and inhibition by serine proteinase inhibitors including the elastase specific inhibitor elastatinal.

1.2.3 Cysteine and aspartate proteinases in herbivorous Coleoptera and Hemiptera

Both cysteine and aspartic proteinases have been reported in herbivorous beetles and in haematophagous and seed feeding Hemiptera. The most comprehensive and thoroughly investigated occurrence of these proteinases to date have been in Hemiptera.

1.2.3.1 Hemiptera

Unlike the insects reviewed in the previous sections, Hemiptera are unique, in that most of the investigations have revealed the presence of enzymes other than the commonly reported serine proteinases. Here investigations have detected enzymes that have pH optima in the acidic pH range and hydrolyse synthetic substrates such as Bz-Arg-NHNa and Bz-Arg-Np, substrates that have historically been used to detect the serine proteinase trypsin.

Initial investigations on the blood sucking Hemiptera, *Rhodnius prolixus*, detected optimal proteolytic activity at pH 5.0 using haemoglobin (Gooding 1968, 1969) and at pH 5.5 with azocasein (Persaud and Davey, 1971) as substrates and Garcia *et al.* (1978) also reported on proteolytic activity in *R. prolixus* that showed maximal casein hydrolysis between pH 5.0 and 5.5 and was activated by thiol compounds. Proteolytic activity in *R. prolixus* was later attributed to the cysteine proteinase cathepsin B based on maximal hydrolysis of Bz-Arg-NHNa at pH 5.5, hydrolysis of Bz-Arg-Np at pH 5.5, activation by

DTT, cysteine, mercaptoethanol, glutathione and the chelating agent EDTA and inhibition by TLCK and IAA (Houseman, 1978; Houseman and Downe, 1980). Using similar assay conditions a similar proteolytic activity has also been detected in other haematophagous Hemiptera, including *Triatoma phyllosoma pallidipennis* (Houseman and Downe, 1981a), *Cimex hemipterus* and *C. lectularius* (Houseman and Downe, 1982a), the predacious Hemiptera, *Phymata wolffii* (Houseman *et al.*, 1985) and the seed feeding Hemiptera, *Euschistus euschistoides* (Houseman *et al.*, 1984). Both aminopeptidase and lysosomal carboxypeptidase B have also been identified in *R. prolixus* (Houseman and Downe, 1981b) and *Triatoma phyllosoma pallidipennis* (Houseman and Downe, 1981a). Aminopeptidase in both studies maximally hydrolysed Leu-Np at pH 8.0, was activated in the presence of $MgCl_2$ and inhibited by thiol compounds and EDTA. Lysosomal carboxypeptidase B maximally hydrolysed HPLA and *N*-carbobenzoxy-L-glutaryl-L-tyrosine in acidic conditions and inhibited by IAA and TLCK. The ability of the alkaline aminopeptidase to function optimally under the acidic conditions of the gut is reflected by the fact that aminopeptidase is located intracellularly in the gut wall whereas lysosomal carboxypeptidase B is located extracellularly in the gut lumen (Houseman and Downe, 1981a; 1981b). Aminopeptidase has also been identified in *C. hemipterus* and *C. lectularius* (Houseman and Downe, 1982a) and *E. euschistoides* (Houseman *et al.*, 1984). The aspartate proteinase cathepsin D has also been partially purified in *R. prolixus* by DEAE-cellulose chromatography, identified by maximal haemoglobin hydrolysis at pH 2.8 and inhibition by the aspartate specific proteinase inhibitor pepstatin (Houseman and Downe, 1982b). Based on similar characteristics, cathepsin D has also been identified in

6 species of Hemiptera representing 6 different families in the order (Houseman and Downe, 1983b).

1.2.3.2 Herbivorous beetles

Recent investigations indicate that cysteine proteinases are also involved in beetle digestion, but identity and occurrence of these enzymes in the order is not well understood.

Early investigations on bruchid beetles, *Callosobruchus chinensis*, the cowpea weevil, and *Acanthosceides obtectus*, the common bean beetle, reported weak proteolytic activity based on vertebrate trypsin and chymotrypsin assay techniques with casein and azocasein hydrolysis at pH 7.6 and the effects of serine proteinase inhibitors such as soybean trypsin inhibitor (Applebaum, 1964). Work by Xavier-Filho and Coelho (1980) on *Callosobruchus maculatus* also reported very weak activity in the alkaline pH range, but reported the presence of two acidic proteinases revealed by haemoglobin hydrolysis at pH 3.5 and azocasein hydrolysis at pH 5.5, although the pH for maximal substrate hydrolysis was not determined. Later it was found based on a pH optimum at 5.4 using sodium dodecylsulphate-polyacrylamide gel electrophoresis proteolysis assay, activation by DTT and inhibition by leupeptin and the thiol blocking reagent p-chloromercuribenzoic acid, that *C. maculatus* does not use serine proteinases, but cysteine like proteinases resembling cathepsin B (Gatehouse *et al.*, 1985). Cysteine proteolytic activity in *C. maculatus* was further confirmed by Kitch and Murdock (1986) based on

[^{3}H]methaemaoglobin maximal hydrolysis at pH 5.5, activation by thiol compounds and inhibition by IAA and E-64. Recently, three proteinases have been partially purified in *C. maculatus* (P1,P2 and P3) and resemble cysteine proteinases, based on optimal azocasein hydrolysis between pH 5.5 and 6, activation by DTT and inhibition by IAA, E-64 and a cysteine proteinase inhibitor isolated from cowpea seeds of *Vigna unguiculata* (Campos *et al.*, 1989). Proteolytic activity in *A. obtectus* has also been resolved as a cysteine-like proteinase (Wieman and Nielson, 1988) based on maximal hydrolysis of [^{3}H]methaemaoglobin by gut homogenates between pH 5.0-7.8 and purified preparations of Bz-Arg-NHNa between pH 4.5 and 7.0. Both crude and purified preparations were also reported to be activated by thiol compounds and inhibited by E-64 (Wieman and Nielson, 1988). Recently, Lemos *et al.* (1990) have also detected cysteine as well as aspartic proteinases in the bruchid beetle *Zabrotes subfasciatus*. Cysteine proteolytic activity maximally hydrolysed azocasein at pH 5.5, was activated by DTT and inhibited by E-64, IAA and TLCK, and the aspartic proteinase activity maximally hydrolysed haemoglobin at pH 3.5 and was inhibited by pepstatin. Using similar assay procedures, cysteine and aspartic proteinases have also been reported in the Colorado potato beetle, *Leptinotarsa decemlineata* (Wolfson and Murdock, 1987).

Cysteine proteolytic activity has been detected in other beetles, including a survey of Coleoptera from 11 different beetle species, using haemoglobin hydrolysis at pH 5.5 and inhibition by E-64 (Murdock *et al.*, 1987). Based on similar criteria Wolfson and Murdock (1990) have detected cysteine proteolytic activity in various other herbivorous Coleoptera. Based on his survey Murdock *et al.* (1987) suggests that cysteine proteinases

are common in Coleoptera.

It has only been since these recent studies on Coleoptera that it has become apparent that cysteine proteinases and not just serine proteinases are involved in digestion, but investigations have been limited primarily to seed feeding bruchid beetles. Furthermore, identification of these enzymes has been based principally on the hydrolysis of either whole protein substrates such as haemoglobin or the synthetic substrate Bz-Arg-NHNa in combination with thiol activation and inhibition by E-64. These are not adequate criteria to identify the individual proteinases used for digestion since both substrates are hydrolysed by more than one enzyme. Further investigations on these enzymes is warranted to determine the identity and occurrence of these proteases in the order.

1.2.3.3 Possible evolutionary origin for cysteine proteinases

While most studies on insect digestive proteases have focused on serine proteinases such as trypsin and chymotrypsin, other proteinase classes are important for digestion. A hypothesis of why present day Hemiptera feeding on protein food sources use catheptic cysteine and aspartate proteinases has been proposed by Houseman and Downe (1983b). The ancestor of present day Hemiptera is believed to have fed on plant sap, and as a consequence of feeding on this low protein food source lost its ability to produce alkaline proteinases. With a return to a high protein food source associated with a predatory life style, intracellular lysosomal cysteine and aspartic enzymes became the dominant digestive enzymes.

Preliminary research on Coleoptera indicates that cysteine proteinases are principally associated with seed-feeding bruchid beetles. These investigations and those on haematophagous and seed feeding Hemiptera has prompted Terra *et al.* (1988) to suggest that cysteine proteinases are an evolutionary adaptation by these insects to avoid feeding on food sources, such as seeds, that are high in serine proteinase inhibitors. Ryan (1990), includes other examples, such as the Colorado potato beetle, *L. decemlineata*, that feeds on potato and tomato tissues that contain serine proteinase inhibitors yet appear to use cysteine and aspartic proteinases for digestion.

The ancestor of modern beetles is thought to have been a detritovore feeding on decomposing plant material (Lawrence and Newton, 1982). Today, there are four recognized suborders: Archostemata, Myxophaga, Adephaga and Polyphaga. The Polyphagean suborder comprises over 90% of all described species and is considered to be the most recently evolved (Gillot, 1980), although the number of phyletic groups this suborder should be divided into is not clear. One major trend divides this suborder into the Scarabaeiformia, Bostrichiformia, Cucujiformia, Elateriformia and Stapyliniformia lineages (Crowson, 1960), the other trend into the latter three lineages corresponding to Crowson's series (Lawrence and Newton, 1982). But in either case, the ancestral beetle is thought to have been a mycophagous feeder, that is feeds on plant material altered by the action of fungal enzymes (Crowson, 1984). It is thought that mycophagy may have permitted beetles to exploit otherwise undigestible wood and leaf matter (Eastop, 1981) and the distribution of feeding habits in the presently recognized coleopteran suborders is thought to support the concept that general saprophagy or mycophagy was ancestral

within the order (Lawrence, 1987).

Phytophagous Coleoptera such as *L. decemlineata* feed on substrates that are high in cellulose and modern insects digest cellulose by means of enzymes similar to those produced by fungi, including the endoglucanases (Cx-cellulase), cellobiohydralases (C1-cellulase) and β-glucosidases (cellobiases) (Ljungdahl and Eriksson, 1985). Digestion of this polymer of glucose units is initiated by Cx-cellulase that disrupts the overall structure, with the chains then further broken down by C1-cellulase. The concerted action of Cx- and C1-cellulase results in the complete degradation of the original cellulose and the production of cellobiase units, that are then hydrolysed to glucose units by cellobiase. While many insects are able to produce Cx-cellulase and cellobiase (Martin, 1983, 1987), most insects (with the exception of the silverfish *Ctenolepisma lineata* (Lasker and Giese, 1956) and the Mexican bean beetle, *Epilachna varivestis* (Taylor, 1985)), cannot produce the full complement of cellulases because they cannot synthesize C1-cellulase (Martin, 1983). Insects compensate for their inability to synthesize C1-cellulase in one of four ways (Martin 1983); the presence of gut protozoa, as in termites and woodroaches; the presence of gut bacteria, as in Scarabid beetles; the use of cellulases from ingested fungi, as in fungus growing termites and siricid woodwasps; or by allowing microorganisms to feed on cellulose in one area of the gut and then digest them posteriorly. This latter, however, has not been demonstrated in insects.

Cellulose digestion has been reported in a number of beetles (Martin, 1983; Kukor *et al.*, 1988; Taylor, 1985) with reports of cellulolytic activity in the slightly acidic pH range. Taylor (1985) reports that all three cellulolytic enzymes in the midgut of *E.*

varivestis have optimal activities ranging from pH 3.5-5.5 consistent with the acidic pH detected along the digestive tract of *E. varivestis* and proposes that "the ability to digest cellulose would be a function, not only of the presence of cellulolytic enzymes, but also of a gut that allows these enzymes to function at their maximum rate". The significance of these results is that cysteine proteinases have similar acidic requirements (Barrett and McDonald, 1980), have been previously detected in larvae of *E. varivestis* (Murdock *et al.*, 1987) and are known to be produced by microorganisms (Ward, 1983). However, their have been no previous attempts to determine whether the presence of cysteine proteinases in beetles may be related to the presence of a gut microflora.

1.3 Why Investigate Insect Proteases ?

Investigations on insect proteases have not been centered around the idea of merely increasing our knowledge of insect biochemistry, but more with the goal that concerted efforts may ultimately lead to novel approaches to control pest species via their digestive enzymes. It is not surprising then, that the majority of investigations on insect proteases have been on insects that are either of medical, veterinary or agricultural importance.

Studying insect proteases has become that much more important with increasing demand for reduced insecticidal applications to control insects and demand for biological alternatives. One alternative is to increase the level of proteinase inhibitors found in plants in an effort to disrupt insect digestion. Green and Ryan (1972) reported that feeding by

the Colorado potato beetle, *L. decemlineata*, increased the natural level of trypsin inhibitors found in potato and tomato plant tissues. This response was viewed as a phytoprotective strategy for plants against insect attack, and by 1980 over 90 species of food plants were demonstrated as containing trypsin inhibitors (Liener and Kakade, 1980) and certainly many more have been found since then. This strategy was proven to be particularly effective at increasing resistance against insect pests when cowpea trypsin inhibitor was transferred into tobacco plants (Hilder *et al.*, 1987).

One of the most widespread biological control agents is *Bacillus thuringiensis* (*Bt*), a gram-positive soil bacterium that produces a toxic crystalline inclusion during sporulation that exhibits a highly specific insecticidal activity (Höfte and Whiteley, 1989). *Bacillus thuringiensis* has proven particularly effective in agriculture and forestry applications, with strains isolated so far found active against larvae of certain members of Lepidoptera and some showing toxicity against dipteran and coleopteran pests (Höfte and Whiteley, 1989). The potential for transgenic pest control has recently been demonstrated by Vaeck *et al.*, (1987) who introduced modified crystal protein genes into tobacco plants that were subsequently shown to be toxic to the tobacco hornworm, *Manduca sexta*. The importance for investigating insect proteases for this control strategy is realized when one considers that the mode of action of *Bt* is based on the ability of the crystal proteins to dissolve in the alkaline conditions of the insect midgut, which are then proteolytically processed by midgut proteases to yield smaller toxic fragments.

Digestive processes of insects are also potential targets for novel control strategies. For example, insect reproduction is often limited by the availability of protein

(McCaffery, 1975; Barton Browne *et al.*, 1980), and to make protein available for egg production requires that proteases be present to digest the meal. Thus control mechanisms aimed at protease inhibition may decrease protein available for egg production and therefore decrease the reproductive potential of pest insects.

To achieve desirable effects from control strategies aimed at insect proteases requires investigations to determine the mode of action of digestive inhibitors. Recent studies by Houseman *et al.* (in press) for example, investigated the mode of action of the maize derived growth inhibitor DIMBOA (2,4 dihydroxy-7-methoxy-1,4-benzoxazin-3-one) and one of its degradation products MBOA (6-methoxybenzoxazolin-3-one) on the European Corn borer, *Ostrinia nubilalis*. This investigation found that DIMBOA acts primarily as a digestive toxin by inhibiting the digestive enzymes of *O. nubilalis*, showing non-competitive inhibition of chymotrypsin activity, and MBOA acts as a postdigestive inhibitor through an unexplained mechanism.

Insect proteases as targets of biological control strategies may become an important alternative to chemical control. However, the effectiveness of these strategies will depend on studies to accurately identify the proteases involved in digestion, since in the past strategies such as plant proteinase inhibitors have assumed that insects use serine proteinases such as trypsin and chymotrypsin for digestion. Plant proteinase inhibitors and *Bt* may be effective against insects that use serine proteinases for digestion under alkaline pH conditions of the gut, but as indicated earlier, their is ample evidence that many insect pest species, particularly hemipterans and various beetles do not use serine proteinases for digestion. Clearly plant proteinase inhibitors, *Bt* toxin and other strategies to control

insects will be improved by accurate identification of the functional enzymes used for digestion, since strategies aimed at digestive proteinases may not be successful if the insect does not use serine proteinases.

1.4 Statement of problem and objectives of the research

Investigations on insect proteolytic enzymes have been dominated with reports on serine proteinases, yet recent investigations have revealed that cysteine proteinases are also important components for beetle digestive processes. However, identification of these enzymes in beetles has been made using poor criteria and it is not known whether cysteine proteinases are either the dominant digestive enzymes in this order or if they are used in conjunction with the more commonly reported serine proteinases. Based on only a limited number of investigations some researchers have also speculated on an origin for cysteine proteinases, however, a greater number of studies are required before a possible origin can be determined. Furthermore, cysteine proteinases function optimally in acidic environments and are known to be produced by microorganisms, but there have been no previous attempts to determine whether cysteine proteolytic activity in beetles may be related to the presence of a gut microflora.

With the limited information on cysteine proteinases and their potential importance in beetle digestion, the objectives of this research are:

1. To identify cysteine proteinases in several major Coleopteran families using synthetic substrates, pH optima for substrate hydrolysis, and the effects of potential activators and inhibitors.

2. To determine if cysteine proteinases are common as digestive enzymes, and if their occurrence is unique or if they are used in combination with the more commonly reported serine proteinases.

3. To determine whether cysteine proteinases are related to either the presence of a gut microflora and/or the evolutionary history of the different taxonomic groupings of beetles.

CHAPTER 2

MATERIALS AND METHODS

2.1 Insects

2.1.1 Maintained in colony

Adult Colorado potato beetles, *Leptinotarsa decemlineata* (Say) (Chrysomelidae), were kept in a glass covered tank (62 x 30 x 30 cm), with a soil depth of 10 cm, in a walk-in incubator at 25°C, 50 % relative humidity with a 16:8 (light:dark) photoperiod and maintained on fresh potato plant cuttings. Eggs deposited on potato leaves were collected and placed in covered petri dishes (10 x 1.5 cm) lined with dry Whatman No. 1 filter paper, and after hatching, 1st instar larvae were transferred to larger containers and maintained on potato leaves.

Adult and larval yellow mealworms, *Tenebrio molitor* Linnaeus (Tenebrionidae) were reared together in a covered plastic container (25 x 10 x 15 cm) in a walk-in incubator with the same humidity and photoperiod regime as above. Insects were maintained on wheat bran (obtained from a local health food store) and every two weeks a Granny Smith apple core was added as a moisture source.

Adult maize weevils, *Sitophilus zeamais* Motschulsky (Curculionidae) and adult larger grain borers, *Prostephanus truncatus* (Horn) (Bostrichidae), were maintained at

ambient lab temperature and light conditions. Insects were reared separately in 1 litre Mason jars containing re-cleaned corn kernels obtained from Richies Feed and Seed Co. in Ottawa. The bottom of *P. truncatus* colony jars also contained a 5 cm bed of a 20:1 corn flour:brewers yeast mixture.

Adult carabid beetles, *Pterostichus corvinus* (Dejean) (Carabidae), were collected along the shore of Ottawa river near Britannia Bay (45°25'N 75°47'W) and identified by Dr. Yves Bousquet from the Biosystematics Research Center at Agriculture Canada in Ottawa. Adults were reared in a desiccator chamber (20 x 15 cm), with 5 cm of water saturated soil. Collected larvae, were reared separately in 24 well tissue culture plates with each well 1.5 cm x 1.5 cm in size. Both larvae and adults were maintained on *T. molitor* larvae and kept in a walk-in incubator at 25°C, 50 % relative humidity with a 16:8 (light:dark) photoperiod.

Adult and larval hide beetles, *Dermestes maculatus* De Geer (Dermestidae) were obtained from Dr. C. Blomme at Laurentian University in Sudbury, Canada, and reared in a sealed plastic container (25 x 7 x 10 cm) with a 5 ml vial containing water as a moisture source. Insects were maintained on Purina rat chow and dried beef liver and kept at ambient lab temperature and light conditions.

2.1.2 Collected

Adult humpback beetles, *Gibbium psyloides* (Czenpinski) (Ptinidae) were obtained from Mr. Gerry Hilchie, Entomology Department at the University of Alberta, Canada.

Tiger beetle larvae, *Cicindela sp.* (Cincindelidae) were collected from the sandpits on Slack road in Nepean, Ontario (45°19′N 75°44′W). These larvae were captured and identified with the help of Dr. Bousquet and Dr. Henri Goulet from Agriculture Canada. The adult tiger beetle, *Cicindela scutellaris* Say (Cincindelidae) was also collected in this location by sweep netting and identified by Dr. Goulet.

Adult red milkweed beetles, *Tetraopes tetrophthalamus* (Forster) (Cerambycidae), adult soldier beetles, *Chauliognathus pennsylvanicus* De Geer (Cantharidae) and adult sevenspotted lady bird beetles, *Coccinella septempunctata* Linnaeus (Coccinellidae) were obtained by sweep netting in the open fields of the Britannia Bay area in Ottawa (45°23′N 75°47′W). Both adult *Alobates pennsylvanicus* De Geer (Tenebrionidae) and adult carabid beetle *Agorum melanarium* Dejean (Carabidae) were also obtained from this location, the former from inside old tree stumps and the latter by overturning rocks along the shore of the Ottawa River. Adult whirligig beetles, *Dineutus sp.* (Gyrinidae) were collected from the Rouge river in Quebec and adult carrion beetles, *Nicrophorus tomentosus* Weber (Silphidae) by pitfall trapping near Dunrobin, Ontario (45°29′N 76°02′W). Adult sap beetles, *Glischrochilus sp.* (Nitidulidae) were obtained from a compost heap at a home in Nepean Ontario (45°18′N 75°50′W) and an adult diving beetle (Dytiscidae) as well as adult grapevine beetles, *Pelidnota punctata* (Linnaeus) (Scarabaeidae) were obtained by using a fluorescent black light at dusk in west end Ottawa (45°22′N 75°47′W). All these beetles were identified to the family level using a dichotomous key from Elzinga (1987) and confirmation of the beetles to the species level, whenever possible, was made with the help of Dr. Bob Anderson from the Zoology

Division of the Canadian Museum of Nature in Ottawa.

2.2 Preparation of gut homogenates used for enzyme assays

Larval midguts and their contents used for proteolytic assays, were obtained by dissecting out the region that lies between the foregut and the start of the hindgut, marked by the insertion of the Malphighan tubules. The entire gut and its contents from adult beetles were used for proteolytic assays. To determine whether proteolytic activity was distributed differently in the larval midgut of *T. molitor*, anterior midgut versus posterior midgut, and midgut contents versus separated gut wall were assayed for their proteolytic activities. Anterior and posterior samples were collected by dividing the alimentary tract into two equal portions by cutting midway between the most anterior region and the insertion of the Malpighian tubules. Midgut contents were separated from gut wall by placing an intact gut in 0.15 M NaCl (sodium chloride) and splitting, using fine forceps, the wall longitudinally to free the intact peritrophic membrane and its contents. All guts were freed of adhering fat body and Malphighan tubules and samples collected were stored frozen at -15^{o}C in groups containing 5 to 15 guts depending on beetle species. When required, samples were thawed, homogenized in 0.15 M NaCl and supernatants after centrifugation at 13,500 g for 10 minutes, 4^{o}C, were adjusted to a volume (between 2 to 3 ml, depending on the species) and used for both protein and protease activity determinations.

2.3 Enzyme Assays

All substrates, potential activators and inhibitors were purchased from either Sigma Chemical Co. (St. Louis, Missouri), Calbiochem (San Diego, California) or Aminotech (Ottawa, Ontario). All assays were carried out in duplicate at 30°C using either a Beckman DU 65 or a Cary 2200 thermoregulated spectrophotometer.

2.3.1 Assays for protease activity

During this investigation the cysteine proteinases, cathepsin B and H, the aspartic proteinase cathepsin D, the serine proteinases trypsin and chymotrypsin and aminopeptidase activities were identified. The synthetic substrates used to detect cathepsin B proteolysis included Bz-Arg-NHNa, Bz-Arg-Np and a specific substrate Z-Ala-Arg-Arg-MeONHNa (Smith and Van Frank, 1975). Bz-Arg-NHNa, Bz-Arg-Np, Tos-Arg-OMe and Bz-Arg-OEt were used for trypsin activity, Arg-NHNa for cathepsin H and arginyl aminopeptidase activity, Leu-Np for leucyl aminopeptidase activity, Bz-Tyr-OEt for chymotrypsin activity and the whole protein substrate haemoglobin, for cathepsin D activity.

Bz-Arg-NHNa, Z-Ala-Arg-Arg-MeONHNa and Arg-NHNa hydrolysis were measured using the methods of Barrett (1972, 1976) as modified by Houseman (1978) and Bz-Arg-Np and Leu-Np hydrolysis were measured based on methods described by Houseman *et al.* (1985). A typical assay, consisted of adding midgut homogenate (10 -

25 µl) to 1.5 ml of 0.1 M buffer (containing 1 mM DTT and 3 mM EDTA for cathepsin B and H assays) and allowed to incubate at 30°C for 5 minutes before the reaction was started by adding 40 µl of substrate, 40 mg Bz-Arg-NHNa, Arg-NHNa, Bz-Arg-Np or Leu-Np/ml in dimethyl sulfoxide or 0.5 mg/ml Z-Ala-Arg-Arg-MeONHNa in dimethyl sulfoxide. Bz-Arg-NHNa, Z-Ala-Arg-Arg-MeONHNa and Arg-NHNa reactions were stopped by adding 2 ml of a colour reagent containing fast garnet GBC salt (Sigma Chemical Co.), 0.25 mg/ml, in mersayl-Brij reagent prepared by the method of Barrett (1976). Colour development was allowed to proceed for 10 minutes, before the absorbance at 520 nm was determined. For Bz-Arg-Np and Leu-Np hydrolysis, the reactions were terminated by addition of 0.5 ml of 30 % (v/v) acetic acid and absorbance at 410 nm determined. In order to account for any increased absorbance by homogenate or any spontaneous breakdown of substrate contributed to assay conditions, controls were run by adding gut homogenate after the stop reagent.

Bz-Arg-OEt, Tos-Arg-OMe and Bz-Tyr-OEt hydrolysis were measured based on similar methods as described by Houseman *et al.* (1989). Hydrolysis of these substrates, 1 mM in 0.15 M NaCl (containing 10% v/v methanol for Bz-Tyr-OEt) in the final reaction mixture, were measured for their change of absorbance from beginning to end of the reaction at 256 nm (Bz-Arg-OEt), 247 nm (Tos-Arg-OMe) or 254 nm (Bz-Tyr-OEt). Reactions were started by the addition of 0.5 ml of substrate to 0.5 ml of 0.2 M reaction buffer containing 5-10 µl midgut homogenate.

Haemoglobin hydrolysis was measured following the procedure of Houseman and Downe (1982b). To prepare this substrate, haemoglobin was dissolved in 0.15 M NaCl

and after centrifugation at 10,000 g for 15 minutes the supernant was dialysed, at 4°C, against several changes of 0.15 M NaCl. The dialysed protein was then adjusted to the desired concentration of 20 mg/ml. Reactions were started by adding 0.3 ml haemoglobin, in 0.15 M NaCl, to 0.3 ml of 0.2 M buffer containing 15-20 µl gut homogenate. The reaction was terminated by the addition of 0.6 ml of 20 % (v/v) trichloroacetic acid (TCA) and samples after approximately 8 hr at 4°C, were centrifuged at 13,500 g, at 4°C, for 10 minutes. The absorbance at 280 nm of the supernatant was then used for activity determinations. Controls were run to account for both spontaneous breakdown of substrate and any endogenous substrate contained in gut homogenates.

In order to determine the nanomoles of substrate hydrolysed the molar extinction coefficients of 27,000 for napthylamide Fast garnet GBC colour development (Barrett, 1977a), 8,800 for Bz-Arg-Np and Leu-Np (Erlanger *et al.*, 1961), 808 for Bz-Arg-OET (Kezdy *et al.*, 1965) and 540 for Tos-Arg-OMe (Hummel, 1959) were used. One enzyme unit (E.U.) was defined as the hydrolysis of 1 nmol of substrate per minute per milligram protein and one haemoglobin enzyme unit was defined as the increase of one absorbance unit at 280 nm, per minute per ml at 30°C.

2.3.1.1 The pH for maximal substrate hydrolysis

The pH at which maximal substrate hydrolysis occurs was determined, at 30°C, using buffers, 0.1 M in the final reaction mixture, that included; succinate, pH 4.0-6.0, sodium acetate, pH 4.0-6.0, potassium phosphate, pH 5.5-8.0, maleate, pH 5.5-7.0, citrate-

potassium phosphate, pH 2.5-7.0, Tris [Tris-(hydroxymethyl)-aminomethane], pH 7.0-9.0, bis-Tris [bis(2-hydroxyethyl)-imino-Tris(hydroxymethyl)-methane], pH 5.5-7.0, bis-Tris propane, (1,3bis[Tris-(hydroxymethyl)methyl-amino]propane), pH 6.5-9.0, and succinate-imadazole-diethanolamine, pH 4.0-10.0. Unlike the others, this latter buffer maintains a constant ionic strength over its complete buffering range (Ellis and Morrison, 1982).

2.3.1.2 The effect of diagnostic activators and inhibitors

Once the pH for maximal substrate hydrolysis was determined proteolytic activities were further identified by the effects of potentially diagnostic activators, incubated with gut homogenate at 30°C for 5 minutes before addition of substrate. Potential activators tested included the thiol compounds, DTT, cysteine, mercaptoethanol and glutathione, at 5 mM thiol equivalents and the chelating agent EDTA, tested at 1 and 3 mM. When significant thiol activation was detected, the pH for maximal substrate hydrolysis was redetermined and the effect of EDTA subsequently tested. Once thiol and EDTA conditions were optimized, then the effect of other compounds on substrate hydrolysis were tested under these conditions. Other chelating agents tested included EGTA and 1,10 phenanthroline, both at 1 and 3 mM. The effects of divalent cations were also tested using $CaCl_2$ and $MgCl_2$, both at 1 and 3 mM.

Using the pH for maximal activity the identity of the individual enzymes responsible for substrate hydrolysis were further determined using diagnostic inhibitors, of various proteases classes and tested at the indicated concentrations. Serine protease

inhibitors included, PMSF, added in 50 µl 1-propanol, TPCK, added in 25 µl methanol, aprotinin, STI, and PTI (potato trypsin inhibitor) 1 and PTI 2. Cysteine proteinase inhibitors tested included IAA and E-64 and the aspartate proteinase inhibitor pepstatin, added in 25 µl of methanol was also tested for its effect on substrate hydrolysis. The effects of TLCK and leupeptin both of which are inhibitors of cysteine and serine proteases, were also tested. The effects of TLCK or other chloromethylketones could not be tested on Bz-Arg-NHNa or any other napthylamide substrate due to chemical interference with assay conditions (Barrett, 1972) and the effect of STI could not be tested on napthylamide substrates that contained thiol compounds, due to formation of a precipitate when the colour reagent was added.

2.3.2 Assays for the survey of cysteine and serine proteinases

For the survey of cysteine and serine proteinases (Chapter 7) a set of assay procedures similar to those described in section 2.3.1 were designed to detect cathepsin B and trypsin. Cathepsin B activity was detected by Bz-Arg-NHNa hydrolysis at pH 5.0 in succinate-imadazole-diethanolamine buffer, activation by 2.5 mM DTT and inhibition by 1.0 µg E-64. Trypsin activity was detected by Bz-Arg-Np hydrolysis at pH 8.0 in 0.1 M succinate-imadazole-diethanolamine buffer and inhibition by 10 µg aprotinin.

2.3.3 Assay for C1-cellulase activity

C1-cellulase activity using microcrystalline cellulose as a substrate with assay conditions adapted from Taylor (1985) was used in order to determine the presence of a gut microflora (Chapter 7). A typical assay consisted of adding gut homogenate (50-100 µl) to 0.3 ml of 0.1 M succinate-imadazole-diethanolamine buffer, pH 5.0, that was allowed to incubate at 30°C for 5 minutes, before the reaction was started by adding 0.3 ml microcrystalline cellulose (50 mg/ml, suspended in 0.1 M buffer, pH 5.0) to the reaction mixture. The reaction was terminated 24 hrs later by adding 0.6 ml of 3,5-dinitrosalicyclic acid (Bernfeld reagent) and heating in a boiling water bath for 5 minutes. A 0.9 ml aliquot of distilled water was then added and the absorbance at 540 nm determined (Bernfeld, 1955). Controls were run with boiled gut homogenates. By the use of a standard curve generated with a 5 mg/ml D-glucose solution, increase in absorbance was converted into mg glucose equivalents liberated, and one enzyme unit was defined as the nanomoles of glucose liberated per hour per gut equivalent.

2.4 Protein determination

Protein was determined using bovine serum albumin standard (fraction V, Sigma Chemical Co.) by the method of Bramhall *et al.* (1969).

CHAPTER 3

IDENTIFICATION OF CYSTEINE PROTEOLYTIC ACTIVITY IN THE LARVAL MIDGUT OF THE COLORADO POTATO BEETLE, *LEPTINOTARSA DECEMLINEATA*

3.1 Introduction

The Colorado potato beetle is a pest of major econonomic importance throughout most parts of the world including Canada and the United States, being a major defoliator of the potato plant *Solanum tuberosum tuberosum* (L.) (Hare, 1980). It has also become a serious pest on other Solanaceous plants including tomato, *Lycopersicon esculentum* (Mill.), and eggplant *S. melongena* (L.) (Jansson *et al.*, 1988). Classic work by Green and Ryan (1972) found that feeding by *L. decemlineata* induced increased foliar levels of trypsin inhibitors in tomato and potato plants. The production of these inhibitors was proposed as a phytoprotective strategy for the plant against insect feeding and their effectiveness presumed to be based on the ability of these inhibitors to inhibit the digestive proteinases of *L. decemlineata* (Green and Ryan, 1972). Studies by Benz *et al.* (1985), however, found that when *L. decemlineata* larvae are fed on wounded potato plants with elevated trypsin inhibitor levels, little effect on growth and development of these beetles is observed.

Recent investigations have reported cysteine proteolytic activity in larval midgut homogenates from *L. decemlineata*, based on maximal haemoglobin hydrolysis between pH 5.0 and 6.0, activation by the thiol compound cysteine and inhibition by the cysteine specific proteinase inhibitor E-64 (Wolfson and Murdock, 1987). These investigators also reported an aspartate proteinase activity based on pepstatin inhibition of haemoglobin hydrolysis. Using similar assay techniques Murdock *et al.* (1987) has also reported on cysteine proteolytic activity within *L. decemlineata* as well as in 9 other beetle species and suggests that cysteine proteinases may be more common throughout the order.

Although cysteine proteinases appear to be involved with digestion in *L. decemlineata*, haemoglobin hydrolysis and inhibition by E-64 are not adequate criteria to identify individual proteinases since activity can be attributed to a variety of enzymes that can hydrolyse this substrate. The objective of this investigation is to identify the digestive proteinases in the alimentary tract of the Colorado potato beetle, *Leptinotarsa decemlineata*, using synthetic substrates, pH optima for substrate hydrolysis and the effects of potential activators and inhibitors.

3.2 Results

Maximal haemoglobin hydrolysis by potato beetle gut homogenates occurred at pH 4.5 in citrate potassium phosphate buffer (Fig.2). In the presence of 1 mM DTT and 3 mM EDTA, the synthetic substrates Bz-Arg-NHNa (Fig.3) in bis-Tris buffer, Bz-Arg-Np (Fig.3) in either bis-Tris or citrate potassium phosphate buffer, were maximally

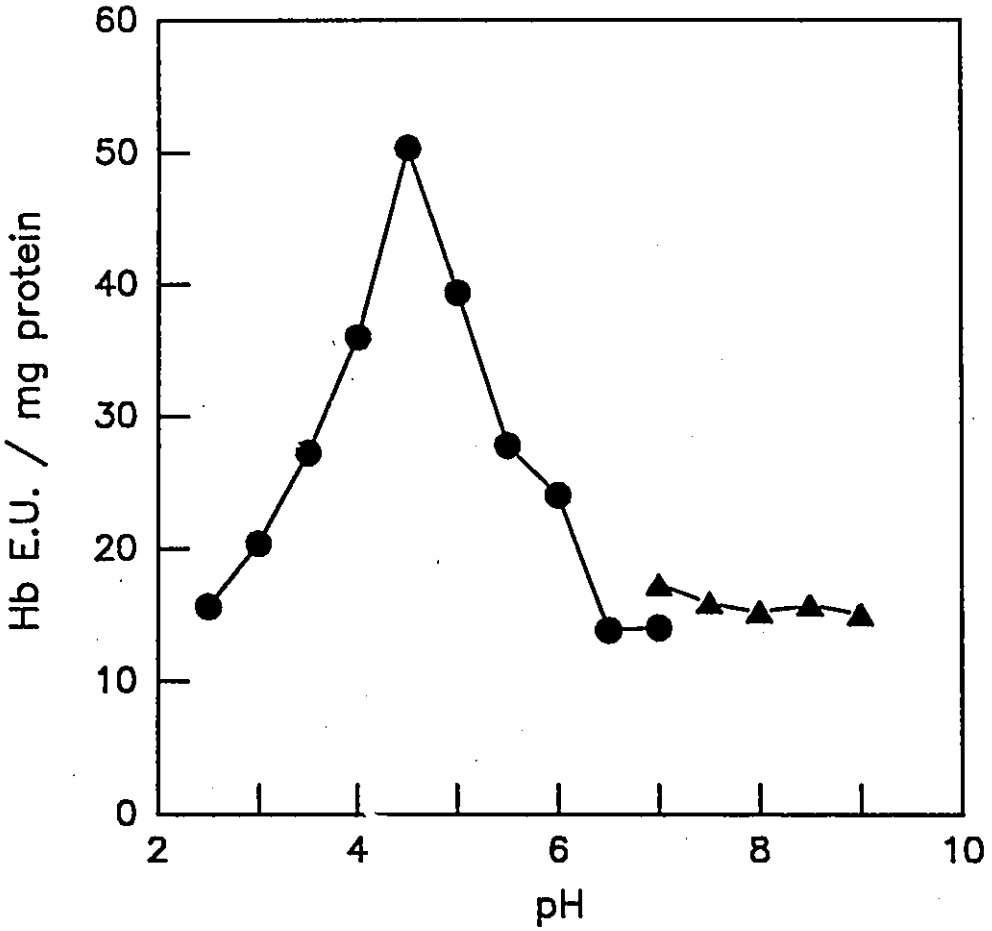

Fig. 2 The effect of pH on the hydrolysis of haemoglobin by *L. decemlineata* larval midgut homogenates. Buffers were citrate-potassium phosphate, pH 2.5-7.0 (●) and Tris, pH 7.0-9.0 (▲).

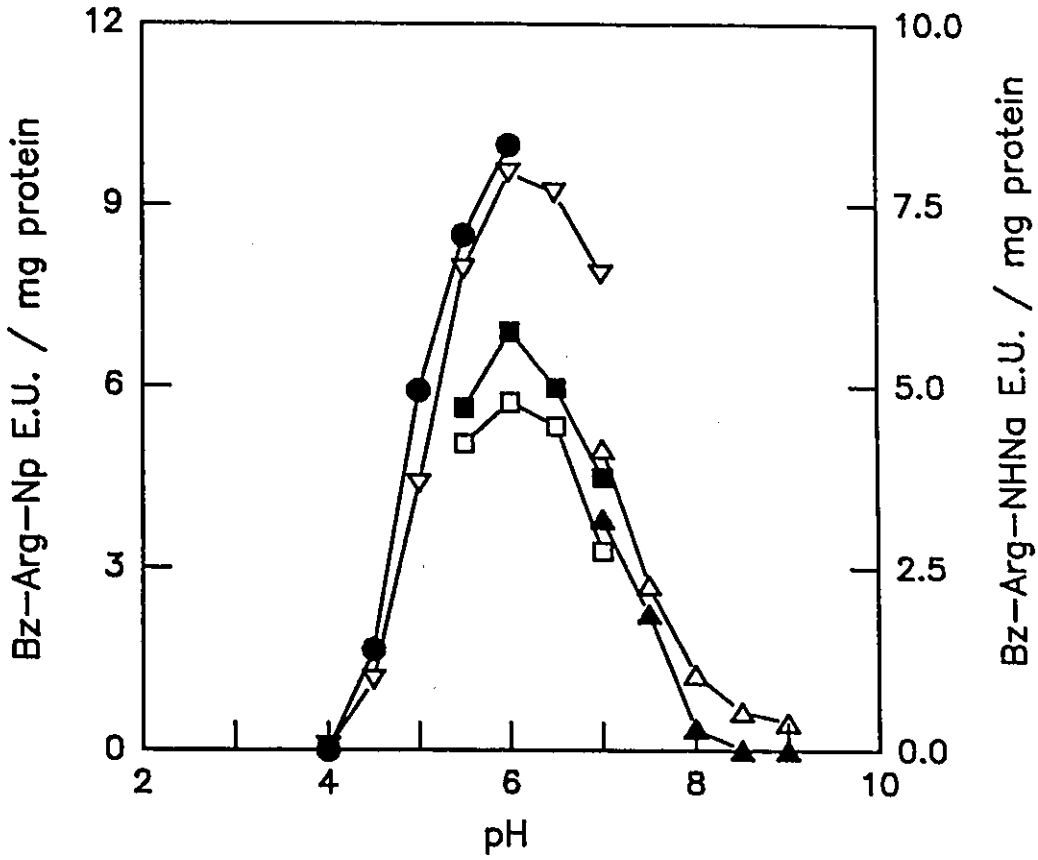

Fig. 3 The effect of pH on the hydrolysis of Bz-Arg-NHNa (solid symbols) and Bz-Arg-Np (open symbols) by *L. decemlineata* larval midgut homogenates. Buffers, containing 1 mM DTT and 3 mM EDTA, were succinate, pH 4.0-6.0 (●), citrate-potassium phosphate, pH 4.0-7.0 (▽), bis-tris, pH 5.5-7.0 (■, □) and Tris, pH 7.0-9.0 (▲ , △).

hydrolyzed at pH 6.0 and Arg-NHNa optimal hydrolysis occurred at pH 6.5 and 7.0 with bis-Tris and potassium phosphate buffers, respectively (Fig.4). Maximal Z-Ala-Arg-Arg-MeONHNa hydrolysis, in the presence of 1 mM DTT and 1.5 mM EDTA, occurred at pH 6.5 in maleate, and 7.5 with potassium phosphate and bis-Tris propane buffers (Fig. 5). Maximal Leu-Np hydrolysis, occurred at pH 8.0 in bis-Tris propane as buffer (Fig. 4).

The effect of thiol compounds and chelating agents are given in Table 2. Thiol compounds in the reaction mixture increased hydrolysis of Bz-Arg-NHNa, Bz-Arg-Np and Arg-NHNa with DTT being the best activator. Thiols had no effect on Z-Ala-Arg-Arg-MeONHNa hydrolysis. Cysteine inhibited Leu-Np hydrolysis but DTT, mercaptoethanol and glutathione had no effect. Mercaptoethanol inhibited Hb hydrolysis, cysteine showed slight activation and DTT and glutathione had no effect on hydrolysis of the natural substrate. EDTA had no effect on either the natural substrate or synthetic substrates and only 3 mM 1,10 phenanthroline inhibited Leu-Np hydrolysis.

The effects of TLCK, IAA, leupeptin, E-64 and pepstatin are given in Table 3. Iodoacetamide inhibited hydrolysis of Bz-Arg-NHNa, Bz-Arg-Np and Arg-NHNa, but only inhibited Z-Ala-Arg-Arg-MeONHNa when tested at 10.0 mM. Iodoacetamide had no effect on either Leu-Np or Hb hydrolysis. E-64 inhibited hydrolysis of all substrates other than Leu-Np and leupeptin inhibited all substrates other than Arg-NHNa and Leu-Np hydrolysis. TLCK inhibited Bz-Arg-Np, Z-Ala-Arg-Arg-MeONHNa and Hb hydrolysis, had no effect on Leu-Np, but could not be tested on either Bz-Arg-NHNa or Arg-NHNa hydrolysis due to chemical interference in the assay mixture (Barrett, 1972). The aspartic proteinase inhibitor pepstatin inhibited only Hb hydrolysis.

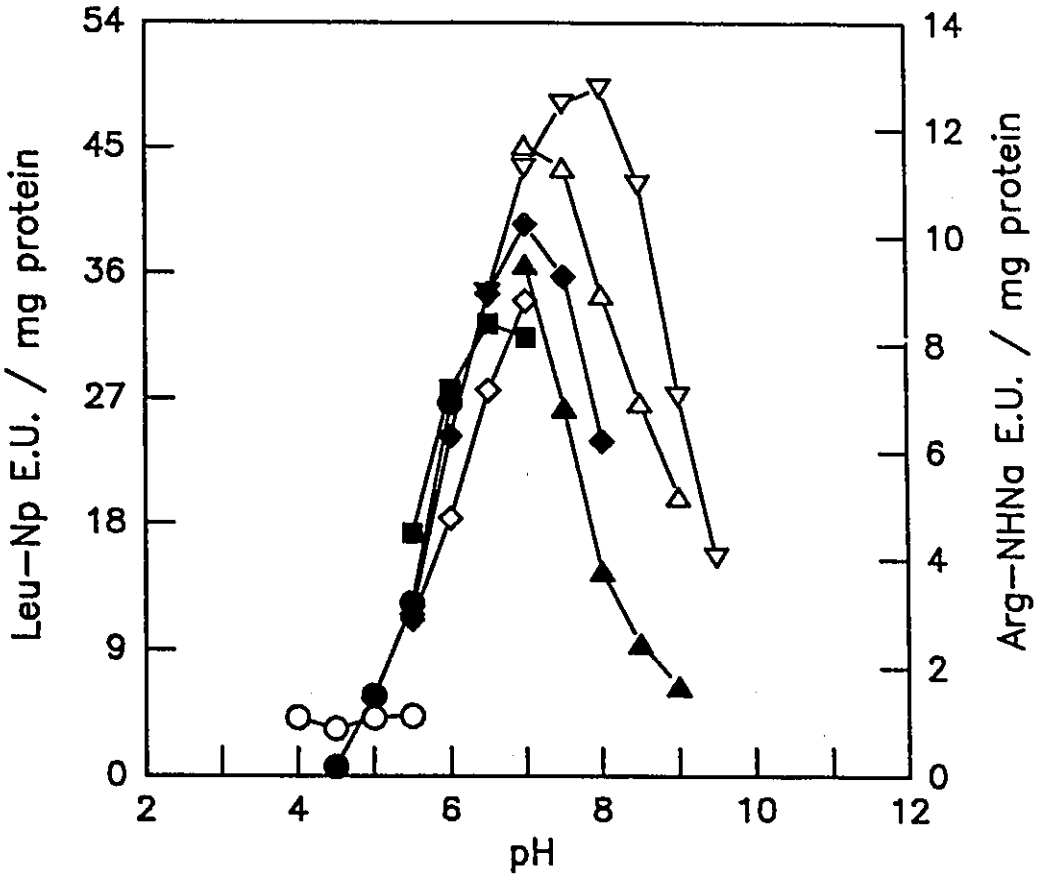

Fig. 4 The effect of pH on the hydrolysis of Arg-NHNa (solid symbols) and Leu-Np (open symbols) by *L. decemlineata* larval midgut homogenates. Buffers were succinate, pH 4.0-6.0 (● , ○), potassium phosphate, pH 5.5-8.0 (♦ , ◇), bis-Tris, pH 5.5-7.0 (■), Tris, pH 7.0-9.0 (▲ , △) and bis-Tris propane, pH 6.5-9.5 (▽). For Arg-NHNa hydrolysis, buffers contained 1 mM DTT and 3 mM EDTA.

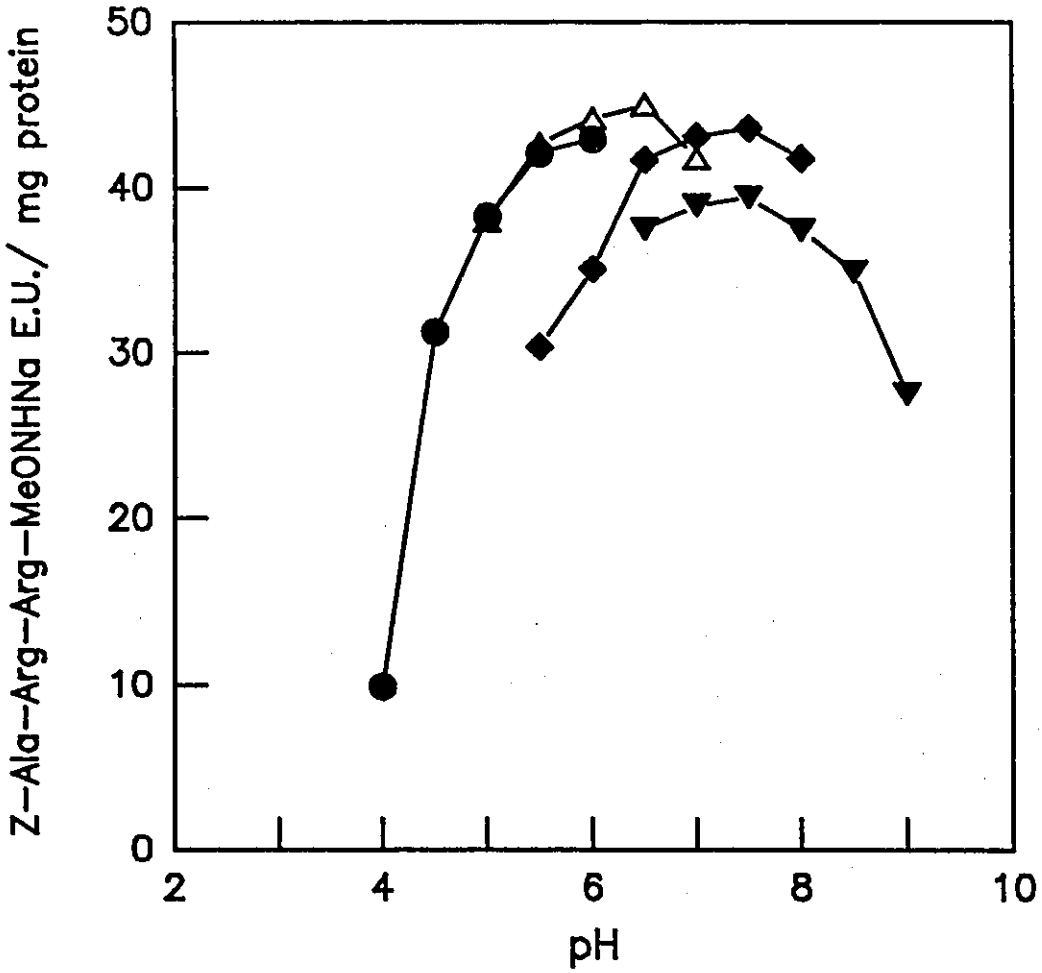

Fig. 5 The effect of pH on the hydrolysis of Z-Ala-Arg-Arg-MeONHNa by *L. decemlineata* larval midgut homogenates. Buffers, containing 1 mM DTT and 1.5 mM EDTA, were succinate, pH 4.0-6.0 (●), maleate, pH 5.0-7.0 (△), potassium phosphate, pH 5.5-8.0 (◆) and bis-Tris propane, pH 6.5-9.5 (▼).

TABLE 2

The effect of thiol compounds and chelating agents on substrate hydrolysis by larval midgut homogenates from *L. decemlineata*. Buffers used were: 0.1 M bis-Tris for Bz-Arg-NHNa and Bz-Arg-Np, pH 6.0, 0.1 M bis-Tris for Arg-NHNa, pH 6.5, 50 mM maleate for Z-Ala-Arg-Arg-MeONHNa, pH 6.5, 0.1 M bis-Tris propane for Leu-Np, pH 8.0 and 0.1 M citrate-potassium phosphate, pH 4.5 for Hb hydrolysis.

	% Relative activity					
	Bz-Arg-NHNa	Bz-Arg-Np	Z-Ala-Arg-Arg-MeONHNa	Leu-Np	Arg-NHNa	Hb
Buffer only	100	100	100	100	100	100
2.5 mM DTT	521[a]	483[c]	106[e]	88[f]	170[h]	88[j]
5 mM Mercaptoethanol	341[a]	288[c]	99[e]	86[f]	166[h]	59[j]
5 mM Glutathione	234[a]	271[c]	90[e]	85[f]	146[h]	100[j]
5 mM Cysteine	448[a]	103[c]	94[e]	60[f]	158[h]	118[j]
1 mM EDTA	102[b*]	103[d*]	102[e*]	84[f]	91[i*]	104[j]
3 mM EDTA	104[b*]	101[d*]	101[e*]	84[f]	92[i*]	94[j]
1 mM Phenanthroline	--	--	--	94[g]	--	--
3 mM Phenanthroline	--	--	--	73[g]	--	--

The following values (mean ± range/2 in E.U. per mg protein, n=2) represent 100% activity: a, 2.37 ± 0.11; b, 7.40 ± 0.10; c, 2.28 ± 0.12; d, 4.02 ± 0.07; e, 21.81 ± 0.08; f, 39.54 ± 1.11; g, 24.89 ± 0.78; h, 5.71 ± 0.10; i, 15.30 ± 0.01; j, 39.38 ± 1.61.

* Buffer contained 1 mM DTT

TABLE 3

The effect of TLCK, IAA, leupeptin, E-64 and pepstatin on substrate hydrolysis by larval midgut homogenates from *L. decemlineata*. Buffers used were: 0.1 M bis-Tris for Bz-Arg-NHNa and Bz-Arg-Np, pH 6.0, 0.1 M bis-Tris for Arg-NHNa, pH 6.5, all three containing 1 mM DTT and 3 mM EDTA, 50 mM maleate for Z-Ala-Arg-Arg-MeONHNa, pH 6.5 containing 1.0 DTT and 1.5 mM EDTA, 0.1 M bis-Tris propane for Leu-Np, pH 8.0 and 0.1 M citrate-potassium phosphate, pH 4.5 for Hb hydrolysis.

	% Relative activity					
	Bz-Arg-NHNa	Bz-Arg-Np	Z-Ala-Arg-Arg-MeONHNa	Leu-Np	Arg-NHNa	Hb
Buffer only	100	100	100	100	100	100
0.5 mM TLCK	--	33[c]	31[f]	97[g]	--	43[j]
0.5 mM IAA	8[a]	41[d]	98[f]	95[g]	51[h]	101[j]
1.0 mM IAA	10[a]	40[d]	86[f]	95[g]	52[h]	97[j]
10.0 mM IAA	--	--	60[f]	--	--	--
0.75 µg Leupeptin	21[a]	40[d]	37[f]	96[g]	94[i]	62[j]
0.5 µg E-64	9[b]	49[e]	61[f]	95[g]	43[i]	48[k]
1.0 µg E-64	11[b]	48[e]	61[f]	94[g]	41[i]	49[k]
5 µg Pepstatin	104[a]	103[d]	101[f]	100[g]	106[i]	27[l]
10 µg Pepstatin	100[a]	97[d]	100[f]	100[g]	106[i]	36[l]

The following values (mean ± range/2 in E.U. per mg protein, n=2) represent 100% activity: a, 7.40 ± 0.10; b, 5.08 ± 0.01; c, 5.17 ± 0.07; d, 1.94 ± 0.05; e, 5.17 ± 0.07; f, 5.21 ± 0.08; g, 40.93 ± 0.13; h, 7.42 ± 0.04; i, 17.90 ± 0.40; j, 62.12 ± 1.92; k, 23.65 ± 1.45; l, 23.79 ± 2.18.

STI and PTI 1 and 2 had no detectable effect on midgut homogenate hydrolysis of substrates tested (Table 4), however, the effects of STI could not be tested on either Bz-Arg-NHNa or Arg-NHNa hydrolysis due to precipitation in the reaction mixture when the colour reagent was added.

3.3 Discussion

Results from this investigation have found that at least four proteinases are involved in digestive proteolysis in the larval midgut of larval Colorado potato beetle and include the cysteine proteinases cathepsin B and cathepsin H, the aspartate proteinase cathepsin D and leucyl aminopeptidase.

The cysteine proteinase, cathepsin B, hydrolyzes substrates Bz-Arg-Np, Bz-Arg-NHNa and Z-Ala-Arg-Arg-MeONHNa and a thiol activator is required although activity may or may not require a chelator (Barrett and McDonald 1980). Z-Ala-Arg-Arg-MeONHNa is more specific for B, while Bz-Arg-Np and Bz-Arg-NHNa are hydrolyzed by both cathepsin B and H and as a consequence cannot be used to distinguish the two enzymes in whole midgut preparations. Cathepsin H is an aminoendopeptidase, which is also activated by thiol compounds, and can be distinguished by hydrolysis of specific substrate Arg-NHNa and by the absence of leupeptin inhibition which does inhibit cathepsin B. (Barrett and McDonald, 1980). Potato beetle midgut hydrolysis of all synthetic substrates other than Leu-Np, were optimal between pH 6.0-7.5, were activated

TABLE 4

The effect of STI and PTI 1 and 2 on substrate hydrolysis by larval midgut homogenates from *L. decemlineata*. Buffers used were: 0.1 M bis-Tris for Bz-Arg-NHNa and Bz-Arg-Np, pH 6.0, 0.1 M bis-Tris for Arg-NHNa, pH 6.5, all three containing 1 mM DTT and 3 mM EDTA, 50 mM maleate for Z-Ala-Arg-Arg-MeONHNa, pH 6.5 containing 1.0 mM DTT and 1.5 mM EDTA, 0.1 M bis-Tris propane for Leu-Np, pH 8.0 and 0.1 M citrate-potassium phosphate, pH 4.5 for Hb hydrolysis.

	% Relative activity					
	Bz-Arg-NHNa	Bz-Arg-Np	Z-Ala-Arg-Arg-MeONHNa	Leu-Np	Arg-NHNa	Hb
Buffer only	100	100	100	100	100	100
10 µg STI	--	93[b]	101[d]	98[f]	--	109[h]
50 µg STI	--	93[b]	102[d]	89[f]	--	107[h]
10 µg PTI 1	100[a]	104[c]	98[e]	--	102[g]	--
50 µg PTI 1	99[a]	106[c]	100[e]	--	104[g]	--
10 µg PTI 2	101[a]	106[c]	99[e]	--	103[g]	--
50 µg PTI 2	103[a]	113[c]	100[e]	--	112[g]	--

The following values (mean ± range/2 in E.U. per mg protein, n=2) represent 100% activity: a, 32.86 ± 0.22; b, 5.17 ± 0.07; c, 8.86 ± 0.09; d, 5.21 ± 0.08, e, 16.16 ± 0.26; f, 40.93 ± 0.13; g, 17.98 ± 0.03; h, 23.65 ± 1.45.

by thiol compounds, except for Z-Ala-Arg-Arg-MeONNHNa, inhibited by the thiol blocking agents TLCK and IAA and E-64 and not affected by the protease inhibitors STI, PTI 1 and 2 indicating that cysteine proteinases and not trypsin or chymotrypsin are present in the larval midgut. Arg-NHNa hydrolysis and the absence of leupeptin inhibition and Z-Ala-Arg-Arg-MeONHNa hydrolysis which was inhibited by leupeptin indicates that both cathepsin B and H activities are present in these preparations.

This investigation has also tentatively identified leucyl aminopeptidase based on maximal hydrolysis of Leu-Np at pH 8.0, inhibition by thiol compounds, the zinc chelating agent 1,10 phenanthroline (indicating an essential metal ion involved in the catalytic mechanism) and lack of an EDTA effect, characteristics that are similar to vertebrate leucyl aminopeptidase (McDonald and Barrett, 1980).

The aspartic proteinases cathepsin D and pepsin both hydrolyze protein under acid conditions and are inhibited by the aspartate specific proteinase inhibitor pepstatin (Barrett, 1977b). Pepsin has maximal activity at pH 2.0 while cathepsin D is active under less acid conditions ranging from 2.8-5.0 depending on the substrate used. The pH optima for Hb hydrolysis, inhibition by pepstatin and absence of STI inhibition are all consistent with the designation of cathepsin D. Inhibition of aspartate proteinases, such as cathepsin D, by thiol compounds reducing one or more strained disulfide bonds in the enzyme has been reported previously (Barrett, 1977b) and in a similar manner TLCK inhibition may result from alkylation of an essential group. During characterizations using crude midgut preparations and general proteinase substrate Hb other proteinases contained in the assay mixture may remain active. Decreased Hb hydrolysis detected in the presence of E-64 and

leupeptin may result from the residual activity of thiol activated enzymes in the assay mixture.

Previous reports of cysteine proteinases in insects, in particular cathepsin B, have been based either on Hb or Bz-Arg-NHNa hydrolysis, thiol activation and inhibition by E-64. These are not adequate characteristics to identify which cysteine proteinases are present since both substrates are hydrolyzed by more than one enzyme. By using specific substrates for cathepsin B and H, and the differential effect of leupeptin (Barrett and Kirschke, 1981) both cysteine proteases cathepsin B and H were detected. Cathepsin H was originally designated as cathepsin B1a or cathepsin B3 and was first described as cathepsin H by Kirsche *et al.* (1976). As a consequence previous reports of cathepsin B activity, based on Bz-Arg-NHNa hydrolysis, in Hemiptera (Houseman, 1978; Houseman and Downe, 1980; 1981a; 1982a; Houseman *et al.*, 1984; 1985) must remain tentative, and indicate only the presence of cysteine proteinases.

Green and Ryan (1972) found that increased foliar levels of trypsin inhibitors in potato and tomato plants was the result of feeding by the Colorado potato beetle. Based on the ability of these inhibitors to inhibit vertebrate proteinases, it was assumed that these inhibitors deter *L. decemlineata* feeding by inhibiting its digestive proteinases (Green and Ryan, 1972). Using two inhibitors isolated from potato plants, one that is specific for chymotrypsin (PTI 1) and the other for chymotrypsin and trypsin (PTI 2) (Ryan, 1984), this present investigation found no effects of these inhibitors on substrate hydrolysis. The ineffectiveness of these inhibitors is attributable to the fact that the digestive proteinases utilized by *L. decemlineata* are cathepsins B, D and H and leucyl

aminopeptidase and not trypsin or chymotrypsin that these inhibitors are targetted against. These results cannot over stress the importance of adequately identifying the functional proteinases involved in digestion if the role of digestive inhibitors of plant origin and their effects on insect digestion are to be fully understood.

CHAPTER 4

CYSTEINE AND SERINE PROTEOLYTIC ACTIVITY IN THE MIDGUT OF LARVAL YELLOW MEALWORM, *TENEBRIO MOLITOR*

4.1 Introduction

The yellow mealworm, *Tenebrio molitor* is an important cosmopolitan pest of stored grain products (Richards and Davies, 1977) and has been the subject of some of the most classic investigations on insect serine proteinases (reviewed in Applebaum, 1985) and insect digestive physiology in general (Dadd, 1970; House, 1974; Chapman, 1982).

Investigations on the proteolytic enzymes in *T. molitor* have focussed primarily on the identity and characteristics of the serine proteinase trypsin (Applebaum *et al.*, 1964; Zwilling, 1968; Zwilling *et al.*, 1972; Levinsky *et al.*, 1977 - for a complete review see section 1.2.1.3). An initial investigation on *T. molitor* reported that hydrolysis of the whole protein substrate casein, measured at pH 6.5, increased in the presence of the thiol compounds cysteine and glutathione and stabilized in the presence of the chelating agent EDTA (Applebaum *et al.*, 1964). Furthermore, this investigation reported that proteolytic activity in this beetle could be divided into two major components based on the susceptibility or resistance of proteolytic activity towards the serine proteinase inhibitors STI, LTI, ovomucoid and DFP; a susceptible fraction that contained tryptic activity; and

a resistant fraction that contained carboxypeptidase B and aminotripeptidase activity. The resistant fraction was also reported to be thiol activated, EDTA stabilized and contained esterolytic activity towards carbobenzoxy-tyrosine nitrophenyl ester. This latter activity was attributed to either carboxypeptidase B or cathepsin B (Applebaum *et al.*, 1964), a thiol activated EDTA stabilized cysteine proteinase, although the identity of this activity has remained unresolved.

The objective of this investigation is to determine, using synthetic substrates, pH for maximal substrate hydrolysis and the effects of potentially diagnostic activators and inhibitors, whether both cysteine and serine proteases are present in the alimentary tract of *T. molitor*.

4.2 Results

Maximum hydrolysis of Bz-Arg-NHNa in the presence of 1 mM DTT and 3.0 mM EDTA occurred at pH 5.0 with succinate and acetate as buffers (Fig. 6). In the presence of 1.0 mM DTT and 3 mM EDTA, optimal Bz-Arg-Np hydrolysis occurred at pH 8.0 and 8.5 with Tris and bis-Tris propane as buffers respectively (Fig. 6).

All four thiol compounds tested (Table 5) activated Bz-Arg-NHNa hydrolysis with DTT and cysteine being better activators than glutathione or mercaptoethanol. Bz-Arg-Np hydrolysis increased in the presence of DTT but substrate hydrolysis was not affected by

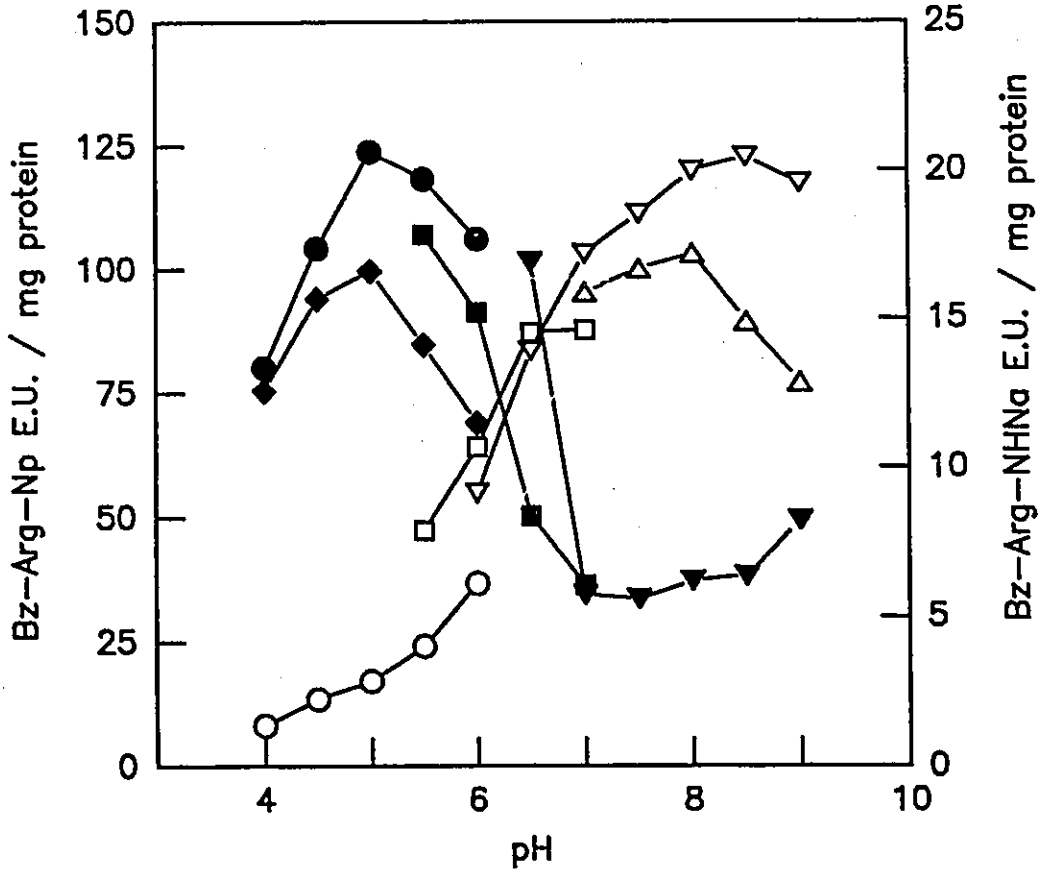

Fig. 6 The effect of pH on the hydrolysis of Bz-Arg-NHNa (solid symbols) and Bz-Arg-Np (open symbols) by *T. molitor* larval midgut homogenates. Buffers, containing 1 mM DTT and 3 mM EDTA, were succinate, pH 4.0-6.0 (● , ○), sodium acetate, pH 4.0-6.0 (◆), bis-Tris, pH 5.5-7.0 (■ , □), bis-Tris propane, pH 6.5-9.5 (▼ , ▽) and Tris, pH 7.0-9.0 (△).

TABLE 5

The effect of thiol compounds on substrate hydrolysis by larval midgut homogenates from *T. molitor*. Buffers used were; 0.1 M succinate, pH 5.0, for Bz-Arg-NHNa and 0.1 M Tris, pH 8.0, for Bz-Arg-Np hydrolysis.

	% Relative activity	
	Bz-Arg-NHNa	Bz-Arg-Np
Buffer only	100	100
2.5 mM DTT	689[a]	171[b]
5 mM Mercaptoethanol	294[a]	109[b]
5 mM Glutathione	347[a]	110[b]
5 mM Cysteine	610[a]	112[b]

The following values (mean ± range/2 in E.U. per mg protein, n=2) represent 100% activity. a, 5.01 ± 0.32; b, 21.51 ± 0.22.

the other thiols tested. Chelating agents EDTA, EGTA and 1,10 phenanthroline and the divalent cations Ca^{2+} and Mg^{2+} had no effects on the hydrolysis of either substrate (Table 6).

The effect of various diagnostic inhibitors are given in Table 7. Pepstatin had no effect whereas leupeptin inhibited hydrolysis of both substrates. Aprotinin and PMSF only inhibited midgut hydrolysis of the Bz-Arg-Np substrate and both IAA and E-64 inhibited only Bz-Arg-NHNa hydrolysis. STI was an inhibitor of Bz-Arg-Np hydrolysis, however, its effects on Bz-Arg-NHNa could not be determined due to formation of a precipitate in the reaction mixture when the colour reagent was added. Bz-Arg-Np hydrolysis was inhibited by TLCK, however, its effects on Bz-Arg-NHNa hydrolysis could not be observed because it chemically interferes with assay conditions (Barrett, 1972).

The anterior gut portion contained 71.3% of cysteine proteinase activity but only 33.1% of the serine proteolytic activity which was predominant in the posterior gut portion. Both proteinases were found at higher levels, 93.6% and 90.2% for cysteine and serine proteinases respectively, in the gut contents compared to the gut wall (Table 8).

4.3 Discussion

Both the serine proteinase trypsin as well as the cysteine proteinase cathepsin B were identified in larval midgut homogenates of *T. molitor*. Trypsin was identified based

TABLE 6

The effect of chelating agents and divalent cations on substrate hydrolysis by larval midgut homogenates from *T. molitor*. Buffers used were; 0.1 M succinate, pH 5.0, for Bz-Arg-NHNa and 0.1 M Tris, pH 8.0, for Bz-Arg-Np hydrolysis, both containing 1 mM DTT.

	% Relative activity	
	Bz-Arg-NHNa	Bz-Arg-Np
Buffer only	100	100
1 mM EDTA	108[a]	109[b]
3 mM EDTA	104[a]	110[b]
1 mM EGTA	110[a]	103[b]
3 mM EGTA	105[a]	108[b]
1 mM Phenanthroline	99[a]	103[b]
3 mM Phenanthroline	108[a]	99[b]
1 mM $CaCl_2$	98[a]	105[b]
3 mM $CaCl_2$	99[a]	108[b]
1 mM $MgCl_2$	98[a]	104[b]
3 mM $MgCl_2$	99[a]	105[b]

The following values (mean ± range/2 in E.U. per mg protein, n=2) represent 100% activity. a, 11.21 ± 0.04; b, 20.42 ± 0.01.

TABLE 7

The effect of TLCK, IAA, leupeptin, E-64, pepstatin, PMSF, aprotinin and STI on substrate hydrolysis by larval midgut homogenates from *T. molitor*. Buffers used were; 0.1 M succinate, pH 5.0, for Bz-Arg-NHNa and 0.1 M Tris, pH 8.0, for Bz-Arg-Np hydrolysis, both containing 1 mM DTT and 3 mM EDTA.

	% Relative activity	
	Bz-Arg-NHNa	Bz-Arg-Np
Buffer only	100	100
0.5 mM TLCK	--	71[c]
0.5 mM IAA	5[a]	95[c]
1 mM IAA	3[a]	88[c]
0.75 µg Leupeptin	23[a]	46[c]
0.5 µg E-64	6[a]	100[c]
1.0 µg E-64	1[a]	95[c]
5 µg Pepstatin	97[a]	103[c]
10 µg Pepstatin	103[a]	99[c]
5.0 mM PMSF	108[b]	68[d]
100 µg aprotinin	87[a]	10[a]
200 µg aprotinin	95[a]	13[a]
5 µg STI	--	12[c]
10 µg STI	--	14[c]

The following values (mean ± range/2 in E.U. per mg protein, n=2) represent 100% activity. a, 12.71 ± 0.11; b, 1.12 ± 0.01; c, 48.71 ± 2.31; d, 39.13 ± 1.32.

TABLE 8

Distribution of Bz-Arg-NHNa and Bz-Arg-Np hydrolytic activities in the larval midgut of *T. molitor*.

	Bz-Arg-NHNa (Cysteine proteinase)	Bz-Arg-Np (Serine proteinase)
	E.U. / gut portion	
Anterior portion	14.4 ± 1.3	14.5 ± 1.3
Posterior portion	5.8 ± 0.6*	29.3 ± 3.6*
Gut wall	1.1 ± 0.1	1.3 ± 0.3
Gut contents	16.0 ± 0.7*	12.0 ± 1.9*

* significantly different, $p \leq 0.01$, from corresponding gut portion.

on maximal hydrolysis of Bz-Arg-Np in the alkaline pH range and inhibition by PMSF, aprotinin, leupeptin, TLCK and STI, characteristics that are consistent with the identification of this proteinase (Barrett and McDonald, 1980) and with previous identifications of trypsin in *T. molitor* (Applebaum *et al.*, 1964; Zwilling, 1968; Zwilling *et al.*, 1972; Levinsky *et al.*, 1977). Cathepsin B was identified based on maximum hydrolysis of Bz-Arg-NHNa at pH 5.0, thiol activation and inhibition by IAA, E-64 and leupeptin (which does not inhibit cathepsin H). These characteristics are consistent with identification of cathepsin B (Barrett and McDonald, 1980) and confirm the identity of the unknown protease detected in the soybean resistant fraction reported by Applebaum *et al.* (1964).

Cysteine and serine class proteases require different pH conditions and activation requirements for maximal activity. Thiol compounds, or a reducing environment, required for optimal activity of cysteine proteases, are capable of inhibiting trypsin but in the mealworm tryptic activity is either activated or not affected by thiol compounds. These catalytic differences may be accomodated by the presence of the cysteine proteinase in the anterior midgut while the serine proteinases are restricted to the posterior portion. The anterior midgut appears to provide the acidic, reducing environment required by cysteine proteases, whereas the posterior portion may provide more alkaline pH conditions favourable for serine proteinase activity. Both activities were also found higher in gut contents compared to gut wall, consistent with extracellular digestion in insects. The differential distribution and requirements for activity of these two proteinases is also consistent with the pH values of 5.64 and 7.88 for the anterior and posterior regions

respectively of larval midgut of *T. molitor* reported by Terra *et al.* (1985). Terra *et al.* (1985) also reported tryptic activity in the posterior midgut of *T. molitor* and suggested that protein digestion is restricted to this region and that the acidic conditions in the anterior region of the midgut is more favourable for carbohydrate digestion. These results have overlooked the presence of proteolytic enzymes such as cysteine proteinases which can function in acidic environments in the midgut of *T. molitor*. The dominance of cathepsin B activity in the anterior region clearly indicates that protein digestion is not restricted to just the posterior midgut.

This is the first reported case in insects where both cysteine and serine proteinases have been detected in the same midgut and suggests that Coleoptera can be divided into at least three groups based on digestive proteolysis by either serine, cysteine or a combination of both activities.

CHAPTER 5

IDENTIFICATION OF DIFFERENT DIGESTIVE PROTEINASES UTILIZED BY THE MAIZE WEEVIL, *SITOPHILUS ZEAMAIS* AND THE LARGER GRAIN BORER, *PROSTEPHANUS TRUNCATUS*

5.1 Introduction

Both the maize weevil, *Sitophilus zeamais* and the larger grain borer, *Prostephanus truncatus* are serious stored product pests on corn as well as other cereal grains (Widstrom *et al.*, 1972; Subramanyam *et al.*, 1987). Digestion in *S. zeamais* and other *Sitophilus sp.* has been attributed to amylases (Baker, 1983; 1987) and proteinases (Baker, 1982b), however, to the best of my knowledge there have no such reports of either activity in *P. truncatus.*

Investigations on the proteinase activity in *S. zeamais* (Baker, 1982b) reported that gut homogenates hydrolysed the whole protein substrate HPA over a broad pH range, detecting a peak of activity from pH 3.0-4.0 and after a decline in activity to pH 5.0, there was an increase in activity to an optimum at pH 10.0. Baker (1982b) attributed activity to both acid and alkaline proteinases, but only the alkaline proteinases were further investigated. Trypsin and chymotrypsin were detected based on the hydrolysis of Bz-Arg-OEt and Bz-Try-OEt respectively at pH 9.0, aminopeptidase based on the

hydrolysis of Leu-Np at pH 7.0, and carboxypeptidase based on the hydrolysis of Z-L-valyl-L-leucine and Z-glycyl-L-tyrosine at pH 7.0, but the pH for maximal substrate hydrolysis was not reported for any of these substrates. Furthermore, proteolytic activity detected in the acidic pH range by HPA hydrolysis and attributed to an acid protease (Baker, 1982b), may be attributable to cysteine proteinases, but this was not examined.

Unlike investigations with *Sitophilus sp.* that belong to the Curculionidae family, reports on seed feeding beetles *C. maculatus* (Gatehouse *et al.*, 1985; Kitch and Murdock, 1986; Campos *et al.*, 1989), *Z. subfaceatus* (Lemos *et al.*, 1990) and *A. obtectus* (Wieman and Nielson, 1988) belonging to the Bruchidae family, have detected cysteine proteinases, that have optimal activity in the acidic rather than the alkaline pH range. Activity in these investigations was detected primarily based on the hydrolysis of haemoglobin and Bz-Arg-NHNa, activation by thiol compounds and inhibition by thiol blocking reagents such as IAA and TLCK.

Both *Sitophilus sp.* and Bruchid beetles belong to two different families, feed on similar food sources (i.e seeds), yet appear to use different proteolytic enzymes. Detection of cysteine proteolytic activity in bruchid beetles lead Terra *et al.* (1988) to suggest that Coleoptera use cathepsin-like cysteine proteinases, rather than serine proteinases such as trypsin, in order to avoid the harmful effects of feeding on food sources such as seeds that contain high levels of trypsin inhibitors. Thus serine proteinases will be inhibited by trypsin inhibitors found in seeds, but by using cysteine proteinases, beetles avoid the potential harmful effects of these ingested inhibitors. However, *S. zeamais* as well as other *Sitophilus sp.* use trypsin for digestion (Baker, 1982b) even though they feed on a food

source that is known to contain trypsin inhibitors and cysteine proteinase inhibitors are present in cowpea seeds and they are effective at inhibiting the proteinase reported in *C. maculatus* (Gatehouse *et al.*, 1985). Furthermore, corn trypsin inhibitor has no effect on either trypsin or chymotrypsin activity in the European corn borer, *O. nubilalis* (Laroque and Houseman, 1990).

Reports of cysteine proteolytic activity in seed feeding bruchid beetles indicate that these beetles appear to use similar proteinases for digestion, however, there have been no previous studies to determine whether two corn feeding beetles also use similar proteinases. The objective of this investigation is to further identify and compare digestive proteases in *S. zeamais* and *P. truncatus*, two beetles that both feed on corn a food source that is known to contain serine proteinase inhibitors (Laroque and Houseman, 1990), to determine whether they use similar proteinases for digestion.

5.2 Results

Maximal Bz-Tyr-OEt hydrolysis by *S. zeamais* midgut homogenates occurred at pH 7.5 with Tris and bis-Tris propane as buffers (Fig. 7). In the presence of 1 mM DTT and 3.0 mM EDTA, maximum Bz-Arg-NHNa hydrolysis occurred at pH 5.5 with acetate and succinate as buffers (Fig. 7). There was no detectable hydrolysis of Bz-Arg-Np by *S. zeamais* midgut homogenates.

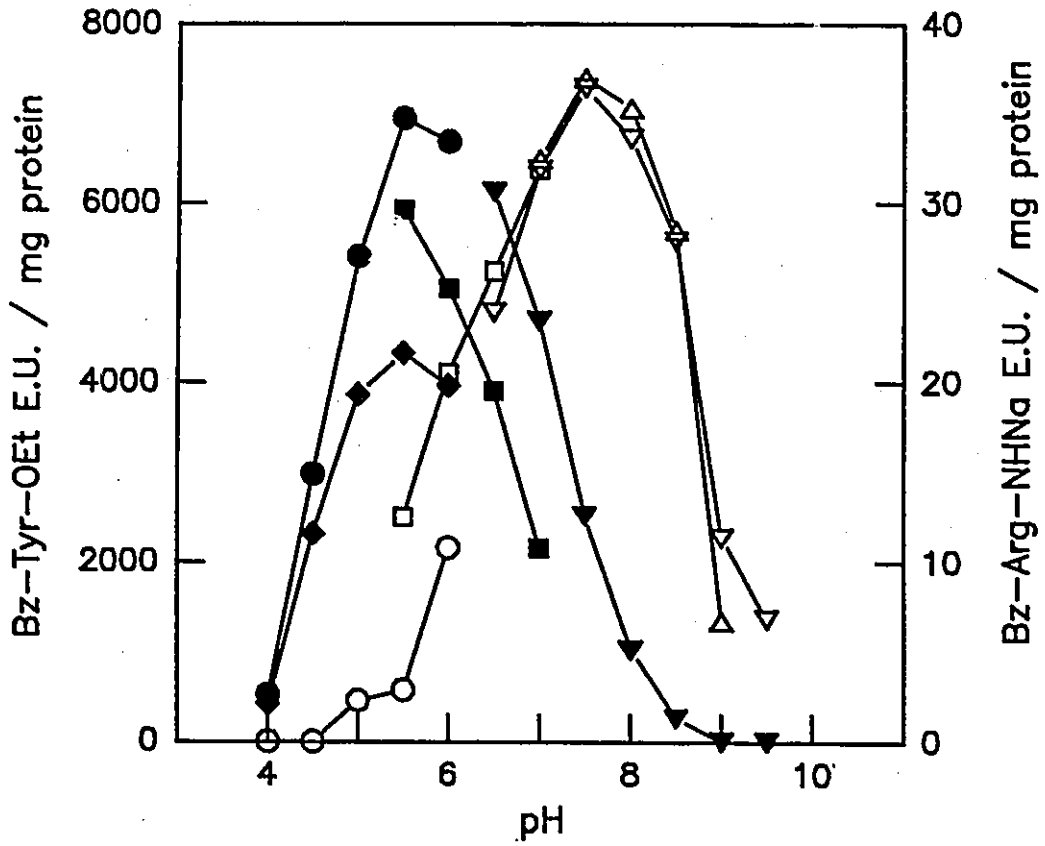

Fig. 7 The effect of pH on the hydrolysis of Bz-Arg-NHNa (solid symbols) and Bz-Tyr-OEt (open symbols) by *S. zeamais* larval midgut homogenates. Buffers were succinate, pH 4.0-6.0 (● , ○), acetate, pH 4.0-6.0 (◆), bis-Tris, pH 5.5-7.0 (■ , □), bis-Tris propane, pH 6.5-9.5 (▼ , ▽) and Tris, pH 7.0-9.0 (△). For Bz-Arg-NHNa hydrolysis, buffers contained 1 mM DTT and 3 mM EDTA.

The effect of thiol compounds and the chelating agent EDTA on substrate hydrolysis by midgut homogenates from *S. zeamais* are given in Table 9. Thiol compounds activated Bz-Arg-NHNa hydrolysis, with DTT and cysteine being the best activators. DTT and glutathione had no effect on Bz-Tyr-OEt hydrolysis and mercaptoethanol and cysteine both inhibited hydrolysis of the substrate. The chelating agent EDTA had no effect on the hydrolysis of either substrate.

The effect of potential inhibitors on substrate hydrolysis by midgut homogenates from *S. zeamais* are given in Table 10. IAA, leupeptin and E-64 inhibited Bz-Arg-NHNa hydrolysis, but had no effect on Bz-Tyr-OEt hydrolysis. Aprotinin and PMSF inhibited Bz-Tyr-OEt hydrolysis, but had no effect on Bz-Arg-NHNa hydrolysis. STI inhibited Bz-Tyr-OEt hydrolysis, however, its effects on Bz-Arg-NHNa could not be tested due to the formation of a precipitate when the colour reagent was added. Pepstatin had no effect on the hydrolysis of either substrate and only TPCK, of the two chloromethylketones tested, inhibited Bz-Tyr-OEt hydrolysis. The effects of TLCK and TPCK on Bz-Arg-NHNa could not be tested due to chemical interference with assay conditions (Barrett, 1972).

Maximal Bz-Arg-NHNa hydrolysis by *P. truncatus* midgut homogenates occurred at pH 8.5 in bis-Tris propane buffer (Fig. 8). Maximal Bz-Arg-Np and Bz-Tyr-OEt hydrolysis occurred at pH 9.0 and pH 8.5 respectively, in succinate-imadazole-diethanolamine buffer (Fig. 8) and maximal hydrolysis of Arg-NHNa and Leu-Np occurred at pH 7.0 and 7.5 respectively in bis-Tris propane buffer (Fig. 9).

The effect of thiol compounds on substrate hydrolysis by midgut homogenates

TABLE 9.

The effect of thiol compounds and EDTA on substrate hydrolysis by larval midgut homogenates from *S. zeamais*. Buffers, 0.1 M in the final reaction volume were: succinate, pH 5.5, for Bz-Arg-NHNa and Tris, pH 7.5 for Bz-Tyr-OEt hydrolysis.

	% Relative activity	
	Bz-Arg-NHNa	Bz-Tyr-OEt
Buffer only	100	100
2.5 mM DTT	675[a]	94[c]
5 mM Mercaptoethanol	138[a]	76[c]
5 mM Glutathione	150[a]	82[c]
5 mM Cysteine	525[a]	73[c]
1 mM EDTA	102[b*]	99[c]
3 mM EDTA	101[b*]	95[c]

The following values (mean ± range/2 in E.U. per mg protein, n=2) represent 100% activity. a, 1.96 ± 0.12; b, 25.46 ± 2.14; c, 12380.11 ± 1005.61.

* Tested in the presence of 1 mM DTT

TABLE 10.

The effect of TLCK, TPCK, IAA, aprotinin, pepstatin, leupeptin, E-64, STI and PMSF on substrate hydrolysis by larval midgut homogenates from *S. zeamais*. Buffers, 0.1 M in the final reaction volume were: succinate, pH 5.5, for Bz-Arg-NHNa containing 1 mM DTT and 3.0 mM EDTA and Tris, pH 7.5 for Bz-Tyr-OEt hydrolysis.

	% Relative activity	
	Bz-Arg-NHNa	Bz-Tyr-OEt
Buffer only	100	100
0.5 mM TLCK	--	81[b]
10 µM TPCK	--	78[b]
20 µM TPCK	--	66[b]
0.5 µM IAA	53[a]	101[b]
1.0 µM IAA	40[a]	102[b]
100 µg Aprotinin	98[a]	77[b]
200 µg Aprotinin	97[a]	62[b]
5 µg Pepstatin	100[a]	98[b]
10 µg Pepstatin	100[a]	107[b]
75 ng Leupeptin	12[a]	103[b]
5 ng E-64	17[a]	102[b]
10 ng E-64	8[a]	102[b]
5 µg STI	--	28[b]
10 µg STI	--	28[b]
125 µM PMSF	100[a]	22[b]

The following values (mean ± range/2 in E.U. per mg protein, n=2) represent 100% activity. a, 26.95 ± 2.25; b, 12380.11 ± 1005.61.

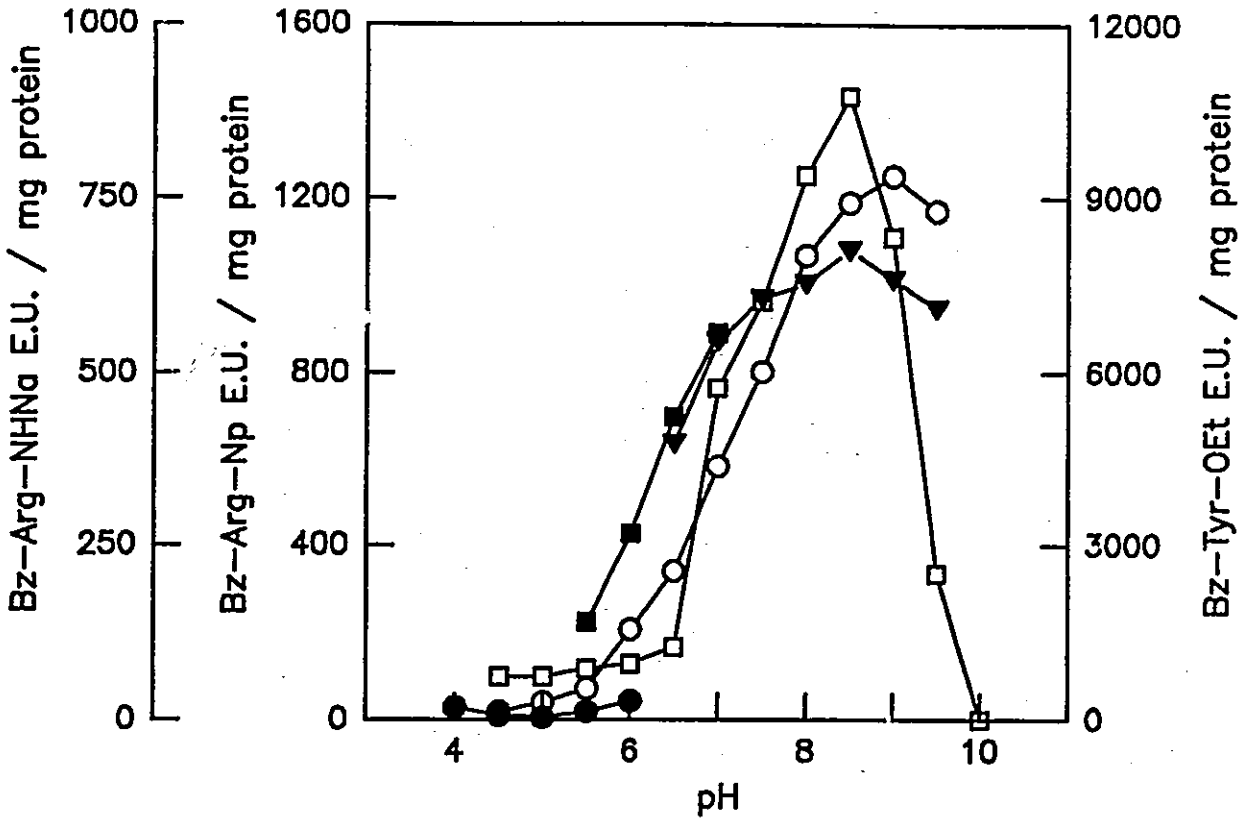

Fig. 8 The effect of pH on the hydrolysis of Bz-Arg-NHNa (solid symbols), Bz-Arg-Np (○) and Bz-Tyr-OEt (□) by *P. truncatus* larval midgut homogenates. Buffers were succinate, pH 4.0-6.0 (●), bis-Tris, pH 5.5-7.0, (■), bis-Tris propane, pH 6.5-9.5 (▼) and succinate-imadazole-diethanolamine, pH 4.5-10 (○, □).

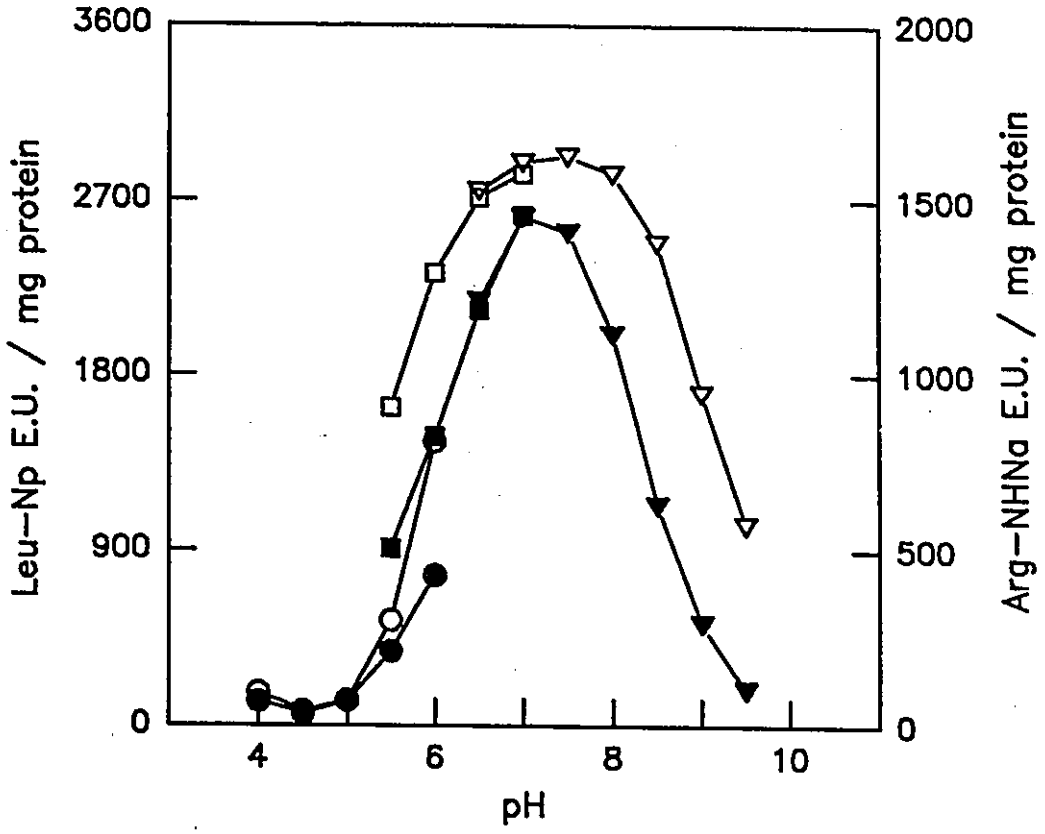

Fig. 9 The effect of pH on the hydrolysis of Arg-NHNa (solid symbols) and Leu-Np (open symbols) by *P. truncatus* larval midgut homogenates. Buffers were succinate, pH 4.0-6.0 (● , ○), bis-Tris, pH 5.5-7.0 (■ , □) and bis-Tris propane, pH 6.5-9.5 (▼ , ▽).

from *P. truncatus* are given in Table 11. DTT had no effect on the hydrolysis of any substrate, mercaptoethanol inhibited Bz-Arg-NHNa and Arg-NHNa but had no effect onthe other three substrates. Cysteine only inhibited Bz-Tyr-OEt and Arg-NHNa hydrolysis and glutathione only inhibited Arg-NHNa and Leu-Np hydrolysis. Divalent cations had no effect on substrate hydrolysis by *P. truncatus* midgut homogenates (Table 12) and only phenanthroline of the two chelating agents tested effected substrate hydrolysis, by inhibiting Arg-NHNa and Leu-Np hydrolysis (Table 12).

The effect of potential inhibitors on *P. truncatus* substrate hydrolysis are given in Table 13. Iodoacetamide, pepstatin and E-64 had no effect on the hydrolysis of any substrate tested and only TPCK of the two chloromethylketones inhibited Bz-Tyr-OEt hydrolysis. Both aprotinin and leupeptin inhibited Bz-Arg-NHNa and Bz-Arg-Np hydrolysis, had no effects on either Arg-NHNa or Leu-Np hydrolysis and only 100 ng aprotinin inhibited Bz-Tyr-OEt hydrolysis. STI inhibited Bz-Arg-NHNa, Bz-Arg-Np and Bz-Tyr-OEt, but had no effect on either Arg-NHNa or Leu-Np hydrolysis.

5.3 Discussion

Both the cysteine proteinase cathepsin B and the serine proteinase chymotrypsin were identified in larval midgut homogenates of *S. zeamais*. Cathepsin B was identified based on maximal Bz-Arg-NHNa hydrolysis at pH 5.5, thiol activation and inhibition by IAA, E-64 and leupeptin, characteristics consistent for the identification of this proteinase

TABLE 11.

The effect of thiol compounds on substrate hydrolysis by larval midgut homogenates from *P. truncatus*. Buffers, 0.1 M in the final reaction volume were: bis-Tris propane, pH 8.5, for Bz-Arg-NHNa, succinate-imadazole-diethanolamine, pH 9.0, for Bz-Arg-Np, succinate-imadazole-diethanolamine, pH 8.5, for Bz-Tyr-OEt and bis-Tris, pH 7.0 and 7.5, for Arg-NHNa and Leu-Np hydrolysis, respectively.

	% Relative activity				
	Bz-Arg-NHNa	Bz-Arg-Np	Bz-Tyr-OEt	Arg-NHNa	Leu-Np
Buffer only	100	100	100	100	100
2.5 mM DTT	103[a]	103[b]	96[c]	82[d]	100[e]
5 mM Mercaptoethanol	78[a]	98[b]	84[c]	59[d]	101[e]
5 mM Cysteine	97[a]	99[b]	79[c]	73[d]	99[e]
5 mM Glutathione	102[a]	100[b]	94[c]	55[d]	78[e]

The following values (mean ± range/2 in E.U. per mg protein, n=2) represent 100% activity: a, 621.82 ± 1.63; b, 3872.25 ± 58.52; c, 8415.89 ± 227.89; d, 1080.86 ± 13.11; e, 2944.14 ± 49.52.

TABLE 12

The effect of chelating agents and divalent cations on substrate hydrolysis by larval midgut homogenates from *P. truncatus*. Buffers, 0.1 M in the final reaction volume were: bis-Tris propane, pH 8.5, for Bz-Arg-NHNa, succinate-imadazole-diethanolamine, pH 9.0, for Bz-Arg-Np, succinate-imadazole-diethanolamine, pH 8.5, for Bz-Tyr-OEt and bis-Tris, pH 7.0 and 7.5 for Arg-NHNa and Leu-Np hydrolysis, respectively.

	% Relative activity				
	Bz-Arg-NHNa	Bz-Arg-Np	Bz-Tyr-OEt	Arg-NHNa	Leu-Np
Buffer only	100	100	100	100	100
1 mM EDTA	109[a]	101[b]	104[c]	98[d]	98[e]
3 mM EDTA	113[a]	104[b]	103[c]	99[d]	100[e]
1 mM Phenanthroline	102[a]	100b	--	18[d]	19[e]
3 mM Phenanthroline	99[a]	96[b]	--	5[d]	10[e]
1 mM $CaCl_2$	97[a]	113[b]	95[c]	100[d]	97[e]
3 mM $CaCl_2$	103[a]	110[b]	111[c]	101[d]	97[e]
1 mM $MgCl_2$	100[a]	112[b]	93[c]	100[d]	98[e]
3 mM $MgCl_2$	106[a]	112[b]	101[c]	101[d]	94[e]

The following values (mean ± range/2 in E.U. per mg protein, n=2) represent 100% activity: a, 494.44 ± 8.24; b, 3872.25 ± 58.52; c, 3697.75 ± 208.83; d, 1603.60 ± 14.25.

TABLE 13

The effect of TLCK, TPCK, IAA, pepstatin, aprotinin, leupeptin, E-64 and STI on substrate hydrolysis by larval midgut homogenates from *P. truncatus*. Buffers, 0.1 M in the final reaction volume were: bis-Tris propane, pH 8.5, for Bz-Arg-NHNa, succinate-imadazole-diethanolamine, pH 9.0, for Bz-Arg-Np, succinate-imadazole-diethanolamine, pH 8.5, for Bz-Tyr-OEt and bis-Tris, pH 7.0 and 7.5, for Arg-NHNa and Leu-Np hydrolysis, respectively.

	% Relative activity				
	Bz-Arg-NHNA	Bz-Arg-Np	Bz-Tyr-OEt	Arg-NHNa	Leu-Np
Buffer only	100	100	100	100	100
0.5 mM TLCK	--	93[c]	96[d]	--	100[f]
20 µM TPCK	--	--	15[d]	--	--
0.5 mM IAA	98[a]	95[c]	108[d]	98[e]	100[f]
1.0 mM IAA	100[a]	93[c]	93[d]	98[e]	100[f]
5 µg Pepstatin	105[a]	104[c]	106[d]	97[e]	102[f]
10 µg Pepstatin	100[a]	99[c]	106[d]	101[e]	101[f]
50 ng Aprotinin	34[b]	80[c]	91[d]	105[e]	103[f]
100 ng Aprotinin	35[b]	38[c]	73[d]	103[e]	100[f]
75 ng Leupeptin	15[b]	9[c]	87[d]	99[e]	101[f]
0.5 µg E-64	108[a]	93[c]	99[d]	99[e]	100[f]
1.0 µg E-64	92[a]	89[c]	98[d]	99[e]	100[f]
5 µg STI	6[a]	6[c]	6[d]	104[e]	103[f]
10 µg STI	5[a]	8[c]	3[d]	104[e]	103[f]

The following values (mean ± range/2 in E.U. per mg protein, n=2) represent 100% activity: a, 494.44 ± 8.24; b, 413.37 ± 5.35; c, 3872.25 ± 58.52; d, 3697.75 ± 208.83; e, 1603.60 ± 14.25; f, 2889.17 ± 18.48.

(Barrett and McDonald, 1980). Cathepsin B detected in this study may be responsible for the peak of HPA hydrolysis between pH 3.0 and 4.0 that had been previously reported by Baker (1982b). Chymotrypsin was identified based on maximal hydrolysis of Bz-Tyr-OEt at pH 7.5, inhibition by aprotinin, PMSF, STI, thiol compounds and TPCK. These characteristics are similar to vertebrate chymotrypsin (Barrett and McDonald, 1980), and confirm the identity of this proteinase previously reported by Baker (1982b). Bz-Arg-Np is hydrolysed by vertebrate trypsin (Barrett and McDonald, 1980), but under the assay conditions, *S. zeamais* gut homogenates did not hydrolyse this substrate. Baker (1982b) also reported no significant hydrolysis of this substrate by *S. zeamais* but detected trypsin activity based on the hydrolysis of Bz-Arg-OEt at pH 9.0. Further investigation will be required to identify this proteinase in *S. zeamais*, since the lack of Bz-Arg-Np hydrolysis is not a characteristic typical of trypsin activity (Barrett and McDonald, 1980).

Results from this study have identified trypsin, chymotrypsin, arginyl and leucyl aminopeptidase in the larval midgut of *P. truncatus*. Chymotrypsin was identified based on maximal hydrolysis of Bz-Tyr-OEt at pH 8.5, inhibition by aprotinin, STI, thiol compounds and TPCK. These characteristics are all similar to vertebrate chymotrypsin (Barrett and McDonald, 1980), although the lack of an effect by divalent cations that are generally recognized to stabilize and have an apparent activation effect on vertebrate chymotrypsin was not observed for *P. truncatus*. Bz-Arg-NHNa is hydrolysed by cathepsin B and H, two thiol activated cysteine proteinases (Barrett and McDonald, 1980). Hydrolysis of Bz-Arg-NHNa as well as Bz-Arg-Np by midgut homogenates from *P. truncatus* at pH 8.5 and 9.0 respectively, combined with no effects by either thiol

compounds or cysteine proteinase inhibitors, and the inhibition effects of aprotinin, STI and leupeptin, identify trypsin not cathepsin B in *P. truncatus*. Most of these effects are characteristic of vertebrate trypsin (Barrett and McDonald, 1980), but the lack of inhibition by TLCK, a trypsin specific inhibitor, indicates that *P. truncatus* trypsin may differ from its vertebrate counterpart.

Arg-NHNa is hydrolysed by both the cysteine proteinase cathepsin H and the peptidase, arginyl aminopeptidase (Barrett and McDonald, 1980; McDonald and Barrett, 1980). Maximum Arg-NHNa hydrolysis at pH 7.0, inhibition by 1,10 phenanthroline (indicating a Zn^{2+} ion involved in the catalytic mechanism) and thiol compounds and no effects by either serine or cysteine proteinase inhibitors, identify arginyl aminopeptidase and not cathepsin H activity in larval midgut homogenates from *P. truncatus*. Vertebrate arginyl aminopeptidase is inhibited by EDTA (McDonald and Barrett, 1980) but had no effect on *P. truncatus* arginyl aminopeptidase. Results from my investigation have also tentatively identified leucyl aminopeptidase in *P. truncatus* midgut homogenates based on the hydrolysis of Leu-Np at pH 7.5, inhibition by thiol compounds and 1,10 phenanthroline and lack of an EDTA effect. These characteristic are all similar to vertebrate leucyl aminopeptidase (McDonald and Barrett, 1980), although divalent cations that are known to activate this enzyme, had no effect.

Identification of only serine proteinases in *P. truncatus*, and a combination of cysteine and serine proteinases in *S. zeamais*, indicate that even though these two beetles feed on the same food source, they do not use the same proteinases for digestion. The use of trypsin and chymotrypsin rather than cysteine proteinases in *P. truncatus* is not

consistent with the hypothesis that cysteine proteases evolved in beetles feeding on food sources containing trypsin inhibitors to circumvent the harmful effects of these ingested inhibitors as suggested by Terra *et al.* (1988). Results from my investigation indicate that perhaps beetles such as *P. truncatus* may circumvent the harmful effects of ingested inhibitors by using proteases that are not sensitive to the inhibitory effects of serine proteinase inhibitors found in corn. Certainly this is a plausible strategy considering that the results from this present study have found that trypsin and chymotrypsin identified in *P. truncatus* do not show all of the characteristics typically associated with vertebrate trypsin and chymotrypsin. Previous studies have also found that corn trypsin inhibitor does not inhibit the trypsin and chymotrypsin activity of the European corn borer, *O. nubilalis* (Larocque and Houseman, 1990), although vertebrate trypsin and chymotrypsin are inhibited. Non the less the results obtained in my study clearly indicate that feeding on food sources containing trypsin inhibitors cannot explain the evolution of cysteine proteinases in beetles.

CHAPTER 6

IDENTIFICATION OF TRYPSIN AND AMINOPEPTIDASE IN LARVAE OF TWO PREDACIOUS BEETLES, *PTEROSTICHUS CORVINUS* AND *CICINDELA SP.* AND LARVAE OF A SAPROPHAGOUS BEETLE, *DERMESTES MACULATUS*

6.1 Introduction

Investigations on the proteolytic enzymes of predacious beetles have been dominated with reports on the serine proteinases trypsin and chymotrypsin (for a complete review see section 1.2.1.1). Identification of these proteinases has been based primarily on the hydrolysis of synthetic substrates and the effects of serine proteinase inhibitors. However, many of these investigations (Gooding and Huang, 1969; Vaje *et al.*, 1984; Colepicolo-Neto *et al.*, 1987) have not reported the pH for maximal substrate hydrolysis and synthetic substrates such as Bz-Arg-Np, Bz-Arg-OEt and Tos-Arg-OMe used to detect trypsin activity, are also hydrolysed by the cysteine proteinase cathepsin B that has a specificity for arginine in the P1 position of small synthetic substrates (Barrett *et al.*, 1988). Furthermore, these investigations have been limited to reports of proteolytic enzymes that have maximal activity in the alkaline pH range. These results can therefore not preclude the presence of cysteine proteinases that have maximal activity in the acidic

rather than the alkaline pH range (Barrett and McDonald, 1980) and have similar substrate specifities.

The black carpet beetle, *A. megatoma*, is a saprophagous beetle of economic importance in North America, with larvae that feed on a variety of products of animal origin, including wool, fur, skin and feathers (Bousquet, 1990). Based on maximal synthetic substrate hydrolysis in the alkaline pH range, in combination with the effects of diagnostic inhibitors digestion in this beetle has been attributed to a variety of proteases, including three trypsin-like and four chymotrypsin-like (Baker, 1981b) enzymes, two carboxypeptidases (Baker, 1981c) and aminopeptidase (Baker and Woo, 1981) (for a complete review see section 1.2.1.2). Trypsin, chymotrypsin, carboxypeptidase and aminopeptidase have also been detected in the hide beetle *D. maculatus* (Baker, 1982a) a saprophagous pest of economic importance that feeds on a variety of dead animal products (Bousquet, 1990). Trypsin in this study was identified based on the hydrolysis of Bz-Arg-NHNa at pH 8.0 and inhibition by PMSF and TLCK, chymotrypsin based on the hydrolysis of glutaryl-Phe-Np at pH 8.0 and inhibition by TPCK, carboxypeptidase based on the hydrolysis of Z-glycyl-L-leucine at pH 7.5 and aminopeptidase based on the hydrolysis of Leu-NHNa at pH 7.5. Although these characteristics can be tentatively used to identify these proteinases, the pH for maximal substrate hydrolysis were not reported for any of these substrates and cannot preclude the presence of cysteine proteinases that hydrolyse the synthetic substrate Bz-Arg-NHNa.

By the nature of the data presented, previous investigations on predacious and saprophagous beetles cannot preclude the presence of cysteine proteinases (in addition to

the already reported serine proteinases) that have optimal activity in acidic rather than an alkaline environment. The objective of this investigation is to determine whether the predacious beetles *P. corvinus* and *Cicindela sp.* and the saprophagous hide beetle, *D. maculatus*, utilize cysteine proteinases.

6.2 Results

Maximal Bz-Arg-NHNa and Bz-Arg-Np hydrolysis by midgut homogenates from *P. corvinus* occurred at pH 9.0 (Fig. 10). Maximal hydrolysis of both substrates occurred at pH 8.0 by midgut homogenates from *Cicindela sp.* (Fig. 11) and at pH 9.0 by midgut homogenates from *D. maculatus* (Fig. 12). Maximal Tos-Arg-OMe hydrolysis by midgut homogenates from *P. corvinus* (Fig. 10), *Cicindela sp.* (Fig. 11) and *D. maculatus* (Fig. 12) occurred at pH 8.5. Arg-NHNa and Leu-Np were maximally hydrolysed at pH 7.5 and 8.0 respectively, by midgut homogenates from *P. corvinus* (Fig. 13). Maximal hydrolysis of Arg-NHNa and Leu-Np occurred at pH 6.5 and 7.0 respectively, by midgut homogenates from *Cicindela sp.* (Fig. 14) and at pH 6.5 and 8.0 respectively, by midgut homogenates from *D. maculatus* (Fig. 15). The pH for maximal hydrolysis of all substrates were determined using succinate-imadazole-diethanolamine as buffer.

The effect of thiol compounds on substrate hydrolysis by midgut homogenates from *P. corvinus*, *Cicindela sp.* and *D. maculatus* are given in Tables 14, 15 and 16 respectively. Mercaptoethanol, glutathione and cysteine had no effect on either

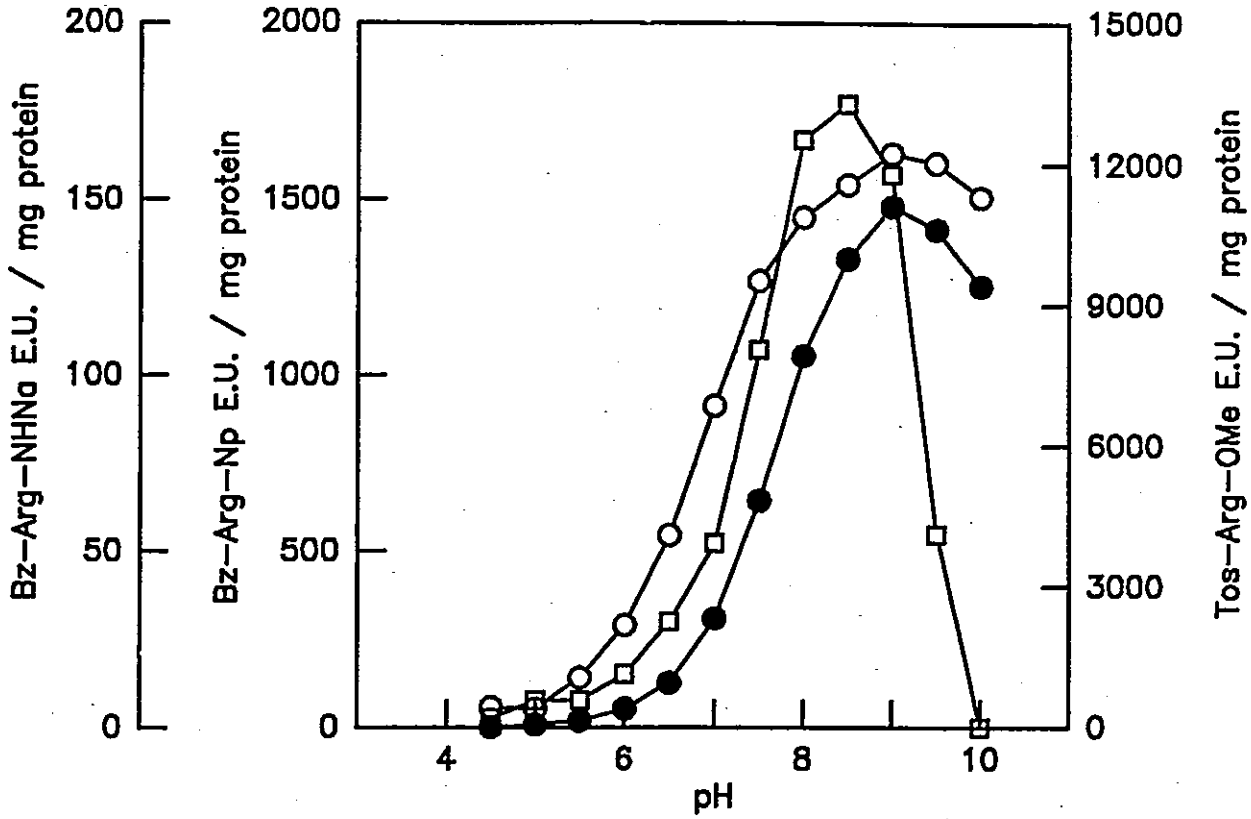

Fig. 10 The effect of pH on the hydrolysis of Bz-Arg-NHNa (●), Bz-Arg-Np (○) and Tos-Arg-OMe (□) by *P. corvinus* larval midgut homogenates. Buffer used was succinate-imadazole-diethanolamine, pH 4.5-10.0.

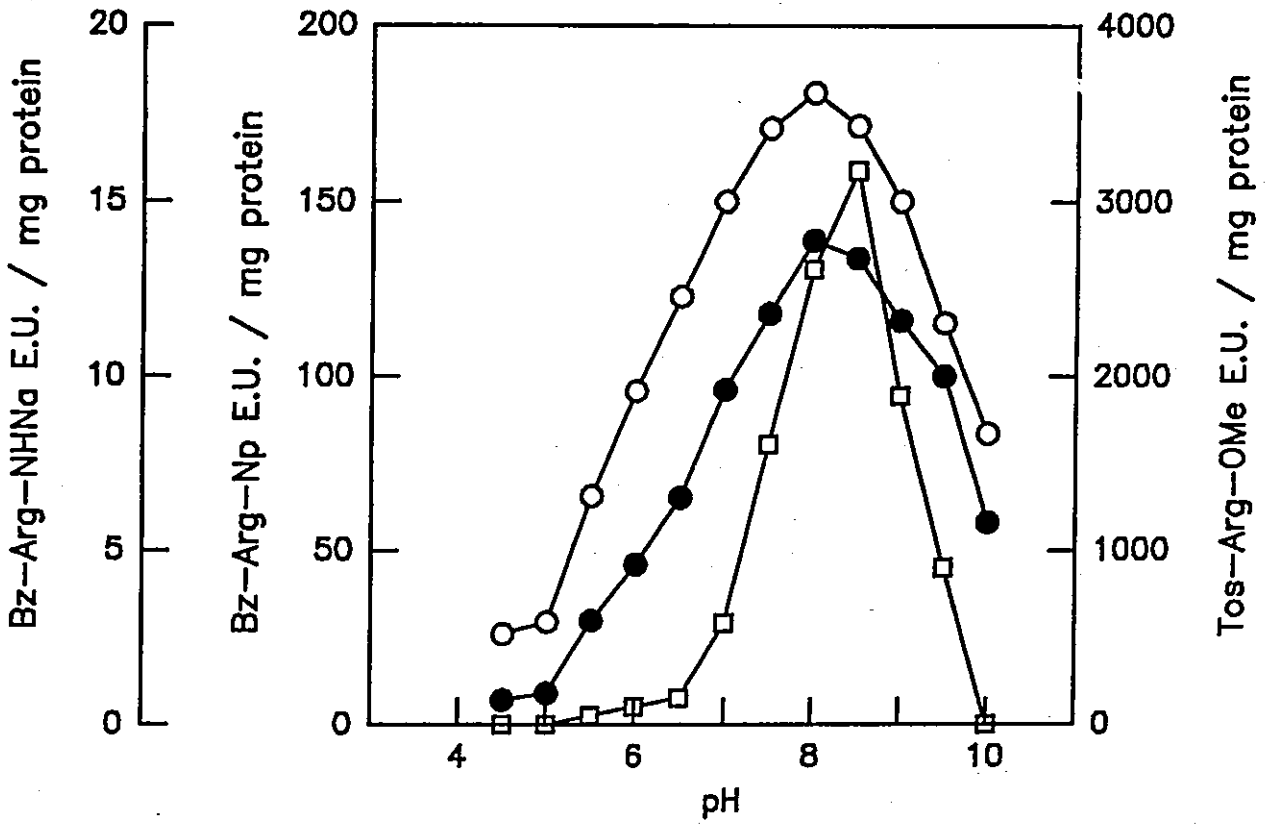

Fig. 11 The effect of pH on the hydrolysis of Bz-Arg-NHNa (●), Bz-Arg-Np (○) and Tos-Arg-OMe (□) by *Cicindela sp.* larval midgut homogenates. Buffer used was succinate-imadazole-diethanolamine pH 4.5-10.0.

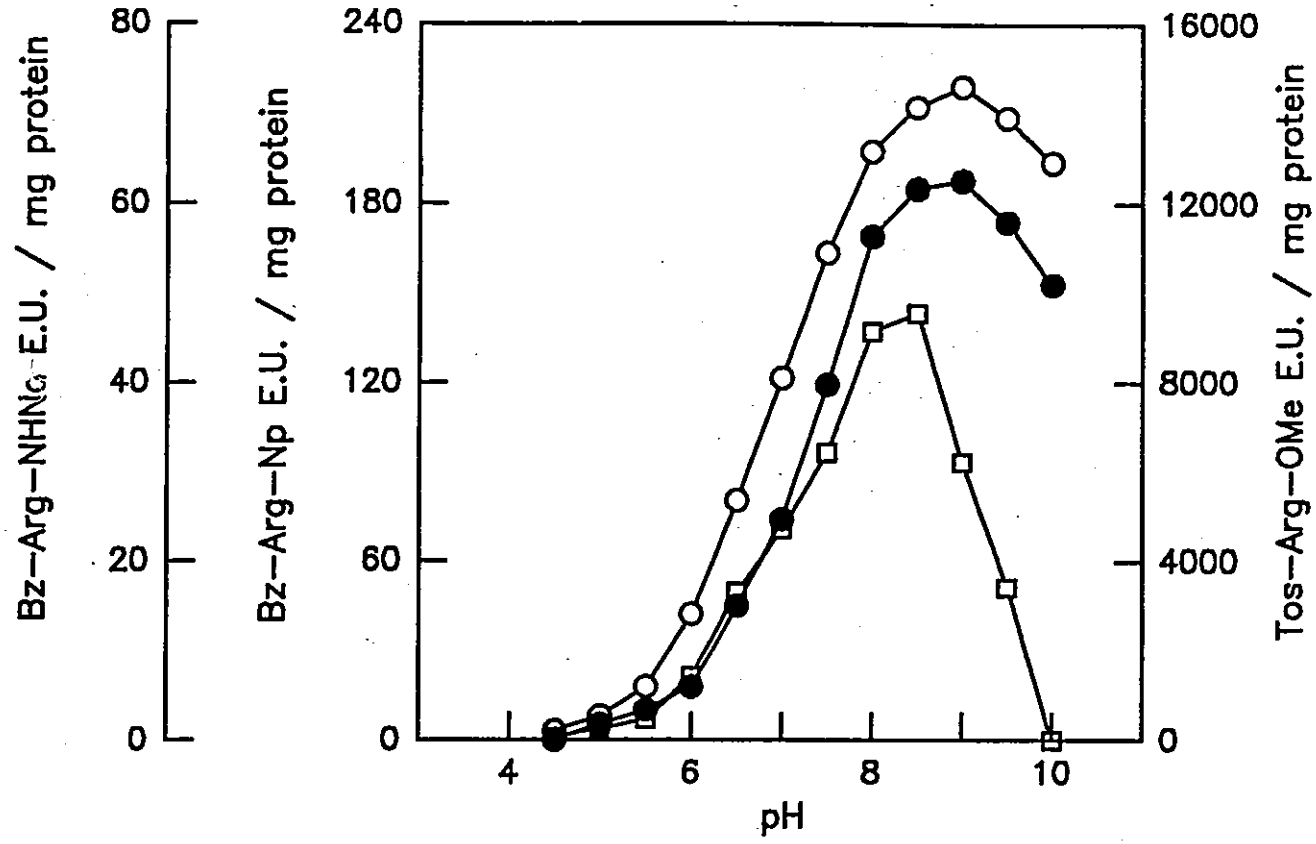

Fig. 12 The effect of pH on the hydrolysis of Bz-Arg-NHNa (●), Bz-Arg-Np (○) and Tos-Arg-OMe (□) by *D. maculatus* larval midgut homogenates. Buffer used was succinate-imadazole-diethanolamine, pH 4.5-10.0.

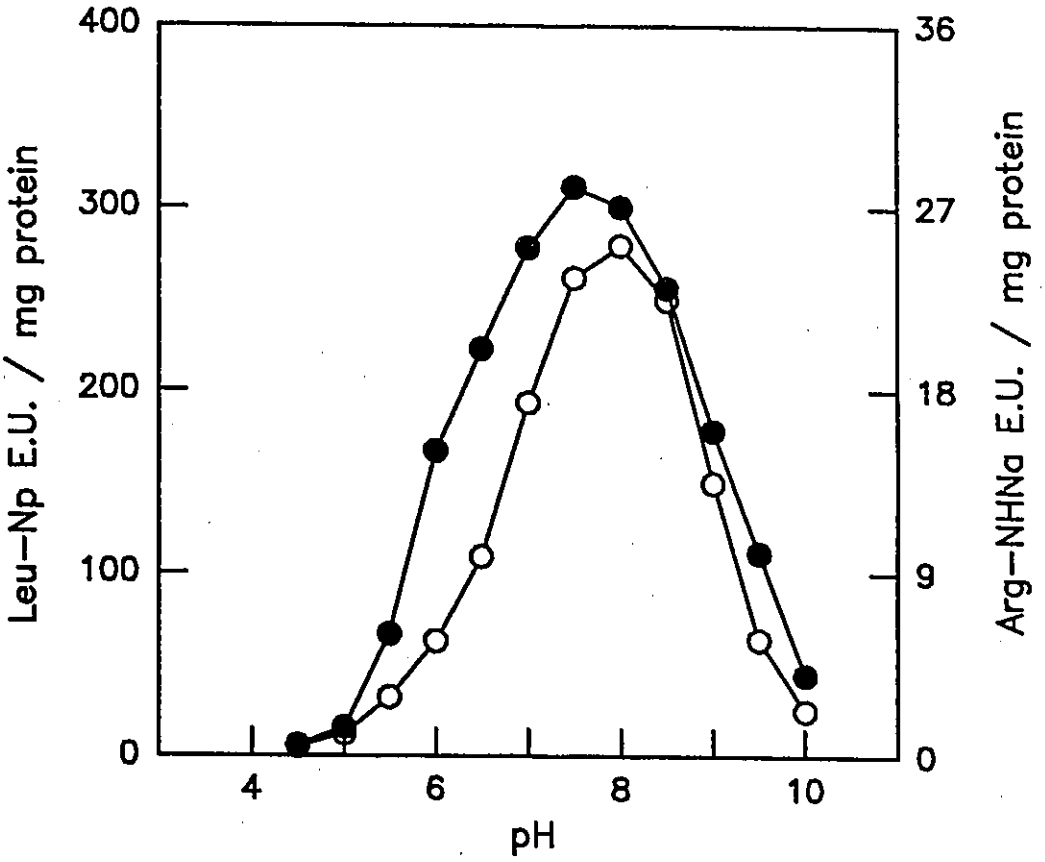

Fig. 13 The effect of pH on the hydrolysis of Arg-NHNa (●) and Leu-Np (○) by *P. corvinus* larval midgut homogenates. Buffer used was succinate-imadazole-diethanolamine, pH 4.5-10.0.

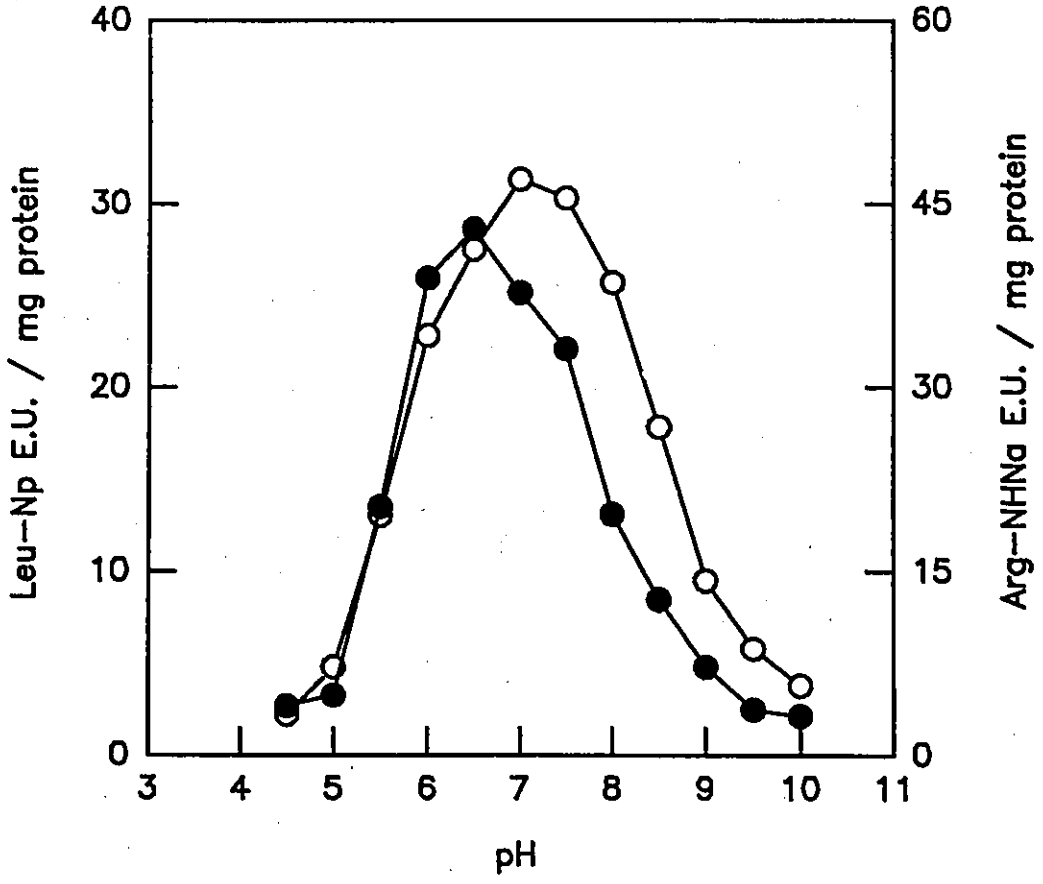

Fig. 14 The effect of pH on the hydrolysis of Arg-NHNa (●) and Leu-Np (○) by *Cicindela sp.* larval midgut homogenates. Buffer used was succinate-imadazole-diethanolamine, pH 4.5-10.0.

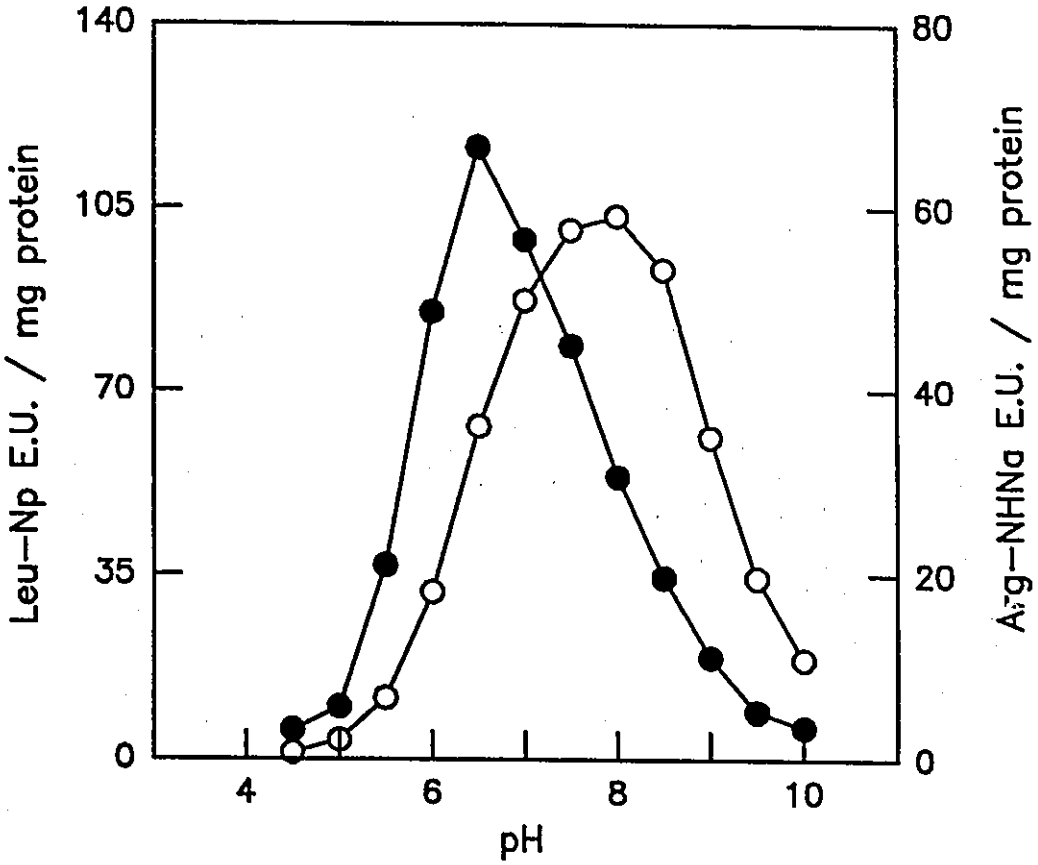

Fig. 15 The effect of pH on the hydrolysis of Arg-NHNa (●) and Leu-Np (○) by *D. maculatus* larval midgut homogenates. Buffer used was succinate-imadazole-diethanolamine, pH 4.5-10.0.

TABLE 14

The effect of thiol compounds on substrate hydrolysis by larval midgut homogenates from *P. corvinus*. Buffers, 0.1 M in the final reaction volume were: succinate-imadazole-diethanolamine, pH 9.0, for Bz-Arg-NHNa and Bz-Arg-Np and 0.1 M succinate-imadazole-diethanolamine, pH 8.0 and pH 7.5, for Leu-Np and Arg-NHNa hydrolysis, respectively.

	% Relative activity			
	Bz-Arg-NHNa	Bz-Arg-Np	Arg-NHNa	Leu-Np
Buffer only	100	100	100	100
2.5 mM DTT	73[a]	61[b]	112[c]	51[d]
5 mM Mercaptoethanol	84[a]	100[b]	64[c]	49[d]
5 mM Glutathione	110[a]	96[b]	72[c]	51[d]
5 mM Cysteine	102[a]	97[b]	57[c]	26[d]

The following values (mean ±/2 in E.U. per mg protein, n=2) represent 100% activity: a, 212.06 ± 1.10; b, 1892.23 ± 21.46; c, 27.47 ± 0.76; d, 197.86 ± 5.78.

TABLE 15

The effect of thiol compounds on substrate hydrolysis by larval midgut homogenates from *Cincindela sp.* Buffers, 0.1 M in the final reaction volume were: succinate-imadazole-diethanolamine, pH 8.0, for Bz-Arg-NHNa and Bz-Arg-Np and 0.1 M succinate-imadazole-diethanolamine, pH 7.0 and pH 6.5, for Leu-Np and Arg-NHNa hydrolysis, respectively.

	% Relative activity			
	Bz-Arg-NHNa	Bz-Arg-Np	Arg-NHNa	Leu-Np
Buffer only	100	100	100	100
2.5 mM DTT	78[a]	67[b]	88[c]	26[d]
5 mM Mercaptoethanol	95[a]	100[b]	104[c]	59[d]
5 mM Glutathione	99[a]	96[b]	87[c]	54[d]
5 mM Cysteine	94[a]	100[b]	67[c]	57[d]

The following values (mean ± range/2 in E.U. per mg protein, n=2) represent 100% activity: a, 26.76 ± 0.98; b, 148.33 ± 5.21; c, 12.07 ± 0.82; d, 58.60 ± 2.45;

TABLE 16

The effect of thiol compounds on substrate hydrolysis by larval midgut homogenates from *D. maculatus*. Buffers, 0.1 M in the final reaction volume were: succinate-imadazole-diethanolamine, pH 9.0, for Bz-Arg-NHNa and Bz-Arg-Np and 0.1 M succinate-imadazole-diethanolamine, pH 8.0 and pH 6.5, for Leu-Np and Arg-NHNa hydrolysis, respectively.

	% Relative activity			
	Bz-Arg-NHNa	Bz-Arg-Np	Arg-NHNa	Leu-Np
Buffer only	100	100	100	100
2.5 mM DTT	96[a]	88[b]	53[c]	12[d]
5 mM Mercaptoethanol	110[a]	105[b]	62[c]	25[d]
5 mM Glutathione	124[a]	102[b]	72[c]	33[d]
5 mM Cysteine	110[a]	104[b]	48[c]	17[d]

The following values (mean ± range/2 in E.U. per mg protein, n=2) represent 100% activity: a, 83.62 ± 0.30; b, 280.48 ± 2.85; c, 53.45 ± 1.72; d, 155.60 ± 1.22.

Bz-Arg-NHNa or Bz-Arg-Np hydrolysis of all three beetle species, whereas DTT inhibited Bz-Arg-NHNa and Bz-Arg-Np hydrolysis of *P. corvinus* and *Cicindela sp.* but had no effect on the hydrolysis of these two substrates by midgut homogenates from *D. maculatus*. All thiol compounds other than DTT were found to inhibit the hydrolysis of Arg-NHNa by midgut homogenates from *P. corvinus*, whereas only cysteine inhibited the hydrolysis of this substrate by *Cicindela sp.* midgut homogenates. All thiol compounds inhibited Arg-NHNa hydrolysis of *D. maculatus* midgut homogenates and all thiol compounds were found to inhibit Leu-Np hydrolysis of all three beetles. The effect of thiol compounds on Tos-Arg-OMe hydrolysis could not be tested due to a high U.V. absorbance encountered when this substrate was added to succinate-imadazole-diethanolamine buffer above pH 7.5 containing thiol compounds.

The effect of chelating agents and divalent cations on substrate hydrolysis by midgut homogenates from *P. corvinus*, *Cicindela sp.* and *D. maculatus* are given in Tables 17, 18 and 19 respectively. EDTA and phenanthroline were found to inhibit Arg-NHNa and Leu-Np hydrolysis of all three beetles, but had no effect on the other substrates tested. The divalent cations Ca^{2+} and Mg^{2+} at 3 mM inhibited the hydrolysis of Leu-Np by *D. maculatus* midgut homogenates but had no effects on the other substrates tested. The effect of phenanthroline could not be tested on Tos-Arg-OMe hydrolysis due to its high U.V. absorbance, previously reported as complicating certain assay procedures (Beynon and Salvenson, 1989).

The effect of potentially diagnostic inhibitors on substrate hydrolysis by larval midgut homogenates from *P. corvinus*, *Cicindela sp.* and *D. maculatus* are given in

TABLE 17

The effect of chelating agents and divalent cations on substrate hydrolysis by larval midgut homogenates from *P. corvinus*. Buffers, 0.1 M in the final reaction volume were: succinate-imadazole-diethanolamine, pH 9.0, for Bz-Arg-NHNa and Bz-Arg-Np, succinate-imadazole-diethanolamine, pH 8.0 and pH 7.5, for Leu-Np and Arg-NHNa respectively and succinate-imadazole-diethanolamine, pH 8.5, for Tos-Arg-OMe hydrolysis.

	% Relative activity				
	Bz-Arg-NHNa	Bz-Arg-Np	Tos-Arg-OMe	Arg-NHNa	Leu-Np
Buffer only	100	100	100	100	100
1 mM EDTA	99[a]	96[b]	103[c]	58[d]	49[e]
3 mM EDTA	102[a]	98[b]	99[c]	56[d]	50[e]
1 mM Phenanthroline	95[a]	93[b]	--	14[d]	15[e]
3 mM Phenanthroline	99[a]	94[b]	--	14[d]	13[e]
1 mM $CaCl_2$	98[a]	95[b]	105[c]	94[d]	101[e]
3 mM $CaCl_2$	98[a]	99[b]	111[c]	95[d]	96[e]
1 mM $MgCl_2$	99[a]	95[b]	104[c]	93[d]	95[e]
3 mM $MgCl_2$	100[a]	100[b]	103[c]	95[d]	97[e]

The following values (mean ±/2 in E.U. per mg protein, n=2) represent 100% activity: a, 212.06 ± 1.10; b, 1892.23 ± 21.46; c, 7755.56 ± 377.78; d, 27.47 ± 0.76; e, 197.86 ± 5.78.

TABLE 18

The effect of chelating agents and divalent cations on substrate hydrolysis by larval midgut homogenates from *Cincindela sp.* Buffers, 0.1 M in the final reaction volume were: succinate-imadazole-diethanolamine, pH 8.0, for Bz-Arg-NHNa and Bz-Arg-Np, succinate-imadazole-diethanolamine, pH 7.0 and pH 6.5, for Leu-Np and Arg-NHNa respectively and succinate-imadazole-diethanolamine, pH 8.5, for Tos-Arg-OMe hydrolysis.

	% Relative activity				
	Bz-Arg-NHNa	Bz-Arg-Np	Tos-Arg-OMe	Arg-NHNa	Leu-Np
Buffer only	100	100	100	100	100
1 mM EDTA	91[a]	102[b]	100[c]	17[d]	12[e]
3 mM EDTA	96[a]	99[b]	98[c]	17[d]	15[e]
1 mM Phenanthroline	88[a]	88[b]	--	0[d]	0[e]
3 mM Phenanthroline	83[a]	85[b]	--	0[d]	0[e]
0.3 mM Phenanthroline	--	--	--	35[d]	63[e]
1 mM $CaCl_2$	87[a]	109[b]	97[c]	94[d]	106[e]
3 mM $CaCl_2$	89[a]	105[b]	90[c]	94[d]	102[e]
1 mM $MgCl_2$	94[a]	109[b]	93[c]	98[d]	109[e]
3 mM $MgCl_2$	103[a]	109[b]	95[c]	104[d]	108[e]

The following values (mean ± range/2 in E.U. per mg protein, n=2) represent 100% activity: a, 26.76 ± 0.98; b, 148.33 ± 5.21; c, 12.07 ± 0.82; d, 58.60 ± 2.45.

TABLE 19

The effect of chelating agents and divalent cations on substrate hydrolysis by larval midgut homogenates from *D. maculatus*. Buffers, 0.1 M in the final reaction volume were: succinate-imadazole-diethanolamine, pH 9.0, for Bz-Arg-NHNa and Bz-Arg-Np, succinate-imadazole-diethanolamine, pH 8.0 and pH 6.5, for Leu-Np and Arg-NHNa respectively and succinate-imadazole-diethanolamine pH 8.5 for Tos-Arg-OMe hydrolysis.

	% Relative activity				
	Bz-Arg-NHNa	Bz-Arg-Np	Tos-Arg-OMe	Arg-NHNa	Leu-Np
Buffer only	100	100	100	100	100
1 mM EDTA	100[a]	104[b]	96[c]	8[d]	9[e]
3 mM EDTA	100[a]	107[b]	90[c]	8[d]	13[e]
1 mM Phenanthroline	97[a]	98[b]	--	5[d]	6[e]
3 mM Phenanthroline	97[a]	96[b]	--	5[d]	8[e]
1 mM $CaCl_2$	100[a]	101[b]	96[c]	102[d]	89[e]
3 mM $CaCl_2$	102[a]	109[b]	98[c]	97[d]	74[e]
1 mM $MgCl_2$	99[a]	100[b]	92[c]	98[d]	91[e]
3 mM $MgCl_2$	98[a]	105[b]	100[c]	98[d]	74[e]

The following values (mean ± range/2 in E.U. per mg protein, n=2) represent 100% activity: a, 196.90 ± 0.99; b, 772.50 ± 1.39; c, 6920.37 ± 441.67; d, 117.08 ± 1.76; e, 159.31 ± 1.42.

Tables 20, 21 and 22 respectively. Pepstatin, IAA and E-64 had no effect on substrate hydrolysis, whereas aprotinin, leupeptin and STI inhibited Bz-Arg-NHNa, Bz-Arg-Np and Tos-Arg-OMe of all three beetles, however, had no effects on either Arg-NHNa or Leu-Np hydrolysis. TLCK only inhibited the hydrolysis of Bz-Arg-Np by *Cicindela sp.* midgut homogenates, but could not be tested on Bz-Arg-NHNa or Arg-NHNa hydrolysis due to chemical interference with assay conditions (Barrett, 1972).

6.3 Discussion

Results from this study have tentatively identified trypsin, leucyl aminopeptidase and arginyl aminopeptidase in larval midgut homogenates of *P. corvinus*, *Cicindela sp.* and *D. maculatus* and reveal that the cysteine proteinases cathepsin B and H are absent in all three species.

The synthetic substrate Bz-Arg-NHNa is hydrolysed by both cathepsin B and trypsin. Hydrolysis of this substrate as well as Bz-Arg-Np and Tos-Arg-OMe in the alkaline pH range combined with STI, aprotinin, leupeptin, thiol compound inhibition and no effects of cysteine proteinase inhibitors confirm trypsin not cathepsin B in all three beetles. Although these results are characteristic of trypsin activity (Barrett and McDonald, 1980) a number of unusual results were obtained. In particular was the lack of inhibition by TLCK (a trypsin specific inhibitor (Barrett and McDonald, 1980)) on Bz-Arg-Np and Tos-Arg-OMe hydrolysis by either *P. corvinus* or *D. maculatus* gut homogenates. Although the lack of inhibition by TLCK is atypical of vertebrate trypsin,

TABLE 20

The effect of TLCK, IAA, pepstatin, aprotinin, leupeptin, E-64 and STI on substrate hydrolysis by larval midgut homogenates from *P. corvinus*. Buffers, 0.1 M in the final reaction volume were: succinate-imadazole-diethanolamine, pH 9.0 for Bz-Arg-NHNa and Bz-Arg-Np, succinate-imadazole-diethanolamine, pH 8.0 and pH 7.5, for Leu-Np and Arg-NHNa respectively and succinate-imadazole-diethanolamine, pH 8.5, for Tos-Arg-OMe hydrolysis.

	% Relative activity				
	Bz-Arg-NHNa	Bz-Arg-Np	Tos-Arg-OMe	Arg-NHNa	Leu-Np
Buffer only	100	100	100	100	100
0.5 mM TLCK	--	100[b]	100[c]	--	95[f]
0.5 mM IAA	98[a]	101[b]	98[d]	95[e]	99[f]
1.0 mM IAA	98[a]	101[b]	104[d]	96[e]	99[f]
5 µg Pepstatin	100[a]	102[b]	103[d]	97[e]	98[f]
10 µg Pepstatin	98[a]	102[b]	103[d]	97[e]	101[f]
50 µg Aprotinin	3[a]	5[b]	0[d]	106[e]	102[f]
100 µg Aprotinin	3[a]	4[b]	0[d]	107[e]	102[f]
5 ng Aprotinin	--	--	36[c]	--	--
75 ng Leupeptin	5[a]	4[b]	39[d]	95[e]	98[f]
0.5 µg E-64	105[a]	97[b]	100[d]	98[e]	101[f]
1.0 µg E-64	107[a]	99[b]	97[d]	97[e]	99[f]
5 µg STI	5[a]	6[b]	0[d]	104[e]	101[f]
10 µg STI	5[a]	6[b]	0[d]	104[e]	101[f]
10 ng STI	--	--	57[d]	--	--

The following values (mean ±/2 in E.U. per mg protein, n=2) represent 100% activity: a, 213.16 ± 3.30; b, 1782.99 ± 39.02; c, 3956.55 ± 71.94; d, 7755.56 ± 377.78; e, 27.19 ± 0.21; f, 189.50 ± 0.14.

TABLE 21

The effect of TLCK, IAA, pepstatin, aprotinin, leupeptin, E-64 and STI on substrate hydrolysis by larval midgut homogenates from *Cincindela sp.* Buffers, 0.1 M in the final reaction volume were: succinate-imadazole-diethanolamine, pH 8.0, for Bz-Arg-NHNa and Bz-Arg-Np, succinate-imadazole-diethanolamine, pH 7.0 and pH 6.5, for Leu-Np and Arg-NHNa respectively and succinate-imadazole-diethanolamine, pH 8.5, for Tos-Arg-OMe hydrolysis.

	% Relative activity				
	Bz-Arg-NHNa	Bz-Arg-Np	Tos-Arg-OMe	Arg-NHNa	Leu-Np
Buffer only	100	100	100	100	100
0.5 mM TLCK	--	66[b]	98[c]	--	106[e]
0.5 mM IAA	101[a]	107[b]	100[c]	92[d]	97[e]
1.0 mM IAA	101[a]	108[b]	97[c]	94[d]	100[e]
5 µg Pepstatin	106[a]	104[b]	98[c]	106[d]	104[e]
10 µg Pepstatin	107[a]	102[b]	100[c]	106[d]	101[e]
50 ng Aprotinin	16[a]	25[b]	0[c]	90[d]	100[e]
100 ng Aprotinin	7[a]	8[b]	0[c]	92[d]	101[e]
5 ng Aprotinin	--	--	40[c]	--	--
75 ng Leupeptin	71[a]	72[b]	75[c]	92[d]	100[e]
0.5 µg E-64	100[a]	100[b]	95[c]	94[d]	94[e]
1.0 µg E-64	107[a]	107[b]	95[c]	100[d]	100[e]
50 ng STI	59[a]	62[b]	30[c]	90[d]	103[e]
100 ng STI	36[a]	44[b]	25[c]	92[d]	102[e]

The following values (mean ± range/2 in E.U. per mg protein, n=2) represent 100% activity: a, 45.90 ± 0.24; b, 244.49 ± 8.02; c, 1285.88 ± 31.17; d, 5.09 ± 0.11; e, 61.47 ± 1.19.

TABLE 22

The effect of TLCK, IAA, pepstatin, aprotinin, leupeptin, E-64 and STI on substrate hydrolysis by larval midgut homogenates from *D. maculatus*. Buffers, 0.1 M in the final reaction volume were: succinate-imadazole-diethanolamine, pH 9.0, for Bz-Arg-NHNa and Bz-Arg-Np, succinate-imadazole-diethanolamine, pH 8.0 and pH 6.5, for Leu-Np and Arg-NHNa respectively and succinate-imadazole-diethanolamine, pH 8.5, for Tos-Arg-OMe hydrolysis.

	% Relative activity				
	Bz-Arg-NHNa	Bz-Arg-Np	Tos-Arg-OMe	Arg-NHNa	Leu-Np
Buffer only	100	100	100	100	100
0.5 mM TLCK	--	98[b]	93[c]	--	103[e]
0.5 mM IAA	90[a]	95[b]	90[c]	94[d]	101[e]
1.0 mM IAA	90[a]	96[b]	89[c]	98[d]	103[e]
5 µg Pepstatin	99[a]	90[b]	107[c]	99[d]	103[e]
10 µg Pepstatin	100[a]	90[b]	105[c]	98[d]	103[e]
50 µg Aprotinin	8[a]	3[b]	3[c]	102[d]	104[e]
100 µg Aprotinin	8[a]	3[b]	4[c]	100[d]	102[e]
75 ng Leupeptin	7[a]	7[b]	32[c]	99[d]	98[e]
0.5 µg E-64	89[a]	97[b]	96[c]	98[d]	101[e]
1.0 µg E-64	91[a]	95[b]	98[c]	101[d]	101[e]
5 µg STI	10[a]	4[b]	8[c]	103[d]	101[e]
10 µg STI	7[a]	6[b]	7[c]	105[d]	100[e]

The following values (mean ± range/2 in E.U. per mg protein, n=2) represent 100% activity: a, 95.23 ± 2.20; b, 622.68 ± 2.93; c, 6265.23 ± 245.38; d, 126.99 ± 2.47; e, 143.96 ± 0.68.

it has been previously reported in a study of trypsin activity in the face fly *Musca autumnalis* (Campbell *et al.*, 1987). TLCK also did not inhibit Tos-Arg-OMe hydrolysis by *Cicindela sp.* gut homogenates but did inhibit Bz-Arg-Np hydrolysis. The lack of an TLCK effect on Tos-Arg-OMe hydrolysis but inhibition on Bz-Arg-Np indicates that perhaps two different trypsins are active in the gut of *Cicindela sp.*

Cathepsin H is a thiol activated cysteine proteinase that hydrolyses the synthetic substrate Arg-NHNa (Barrett and McDonald, 1980). Arginyl aminopeptidase also hydrolyses this substrate but is inhibited by thiol compounds and the chelating agents EDTA, 1,10 phenanthroline, and not effected by the divalent cations Ca^{2+} and Mg^{2+} or cysteine proteinase inhibitors (McDonald and Barrett, 1980). Results from this study confirm the presence of arginyl aminopeptidase and not cathepsin H in all three beetles.

Vertebrate leucyl aminopeptidase hydrolyses the synthetic substrate Leu-Np in the alkaline pH range, is activated by divalent cations such as Mg^{2+}, inhibited by thiol compounds and the chelating agent 1,10 phenanthroline (indicating a Zn^{2+} ion involved in the catalytic mechanism) but not inhibited by the chelating agent EDTA (McDonald and Barrett, 1980). The results from this study have detected a similar enzyme in all three beetles based on the hydrolysis of Leu-Np and inhibition by 1,10 phenanthroline. This peptidase detected in all three beetles is not completely similar to its vertebrate counterpart because of the effects obtained with EDTA and divalent cations. EDTA does not inhibit native vertebrate leucyl aminopeptidase (McDonald and Barrett, 1980) but was found to inhibit the hydrolysis of Leu-Np by midgut homogenates from all beetles. EDTA inhibition is typical however of Ca^{2+} and Mg^{2+} activated vertebrate leucyl

aminopeptidase, but divalent cations had no effect on either *P. corvinus* or *Cicindela sp.* and inhibited *D. maculatus* substrate hydrolysis.

Investigations on the proteolytic enzymes in predacious and saprophagous beetles have reported primarily on the presence of the serine proteinases trypsin and chymotrypsin. These investigations would not have detected cysteine proteinases that have maximal activity in the acidic rather than the alkaline pH range and identified using characteristics much different to serine proteinases. The identification of trypsin and aminopeptidase in *P. corvinus* and *Cicindela sp.* in this present study are similar to previous reports of proteolytic activity in predacious beetles. However, it has been the first investigation to address the presence of cysteine proteinases in predacious beetles as well as being a thorough characterization of proteolytic enzymes in this feeding group, indicating that a consorta of enzymes and not just trypsin and chymotrypsin are responsible for digestion. In addition identification of both trypsin and aminopeptidase in *D. maculatus* confirms the identity of these enzymes previously reported by Baker (1982a) and is also the first investigation to address the presence of cysteine proteinases in a saprophagous beetle. Further investigations are required to determine whether the lack of cysteine proteinases is characteristic of these two feeding groups.

CHAPTER 7

A SURVEY OF CYSTEINE, SERINE AND C1-CELLULASE ACTIVITIES FROM REPRESENTATIVES OF 16 COLEOPTERAN FAMILIES

7.1 Introduction

Recent investigations on the proteolytic enzymes of Coleoptera have reported on the presence of cysteine proteinases principally associated with leaf and seed feeding beetles, and include investigations on bruchid beetles (Gatehouse *et al.*, 1985; Kitch and Murdock, 1986; Weiman and Nielson, 1988 Campos *et al.*, 1989; Lemos *et al.*, 1990) and surveys by Murdock *et al.* (1987) and Wolfson and Murdock (1990). Based on his survey, Murdock *et al.* (1987) has suggested that cysteine proteinases are common in Coleoptera. More recent studies have found, however, that beetles may use either serine (*P. truncatus*, *P. corvinus*, *Cicindela sp* and D. *maculatus* (Chapter 5, 6), cysteine (*L. decemlineata* (Chapter 3)) or even a combination of both enzymes (*T. molitor* and *S. zeamais* (Chapter 4, 5)) for digestion.

Previous investigations on the proteolytic enzymes in Coleoptera have reported on the presence of cysteine proteinases based principally on the hydrolysis of either haemoglobin or Bz-Arg-NHNa at pH 5.5 and inhibition by E-64 and includes Murdock *et al.* (1987) survey. These criteria cannot be used to either identify the proteinases responsible for digestion or assess the presence of serine proteinases that have optimal

activity at alkaline rather than acidic pH conditions. Furthermore, almost all previous reports of cysteine proteinases in Coleoptera have been either with seed or leaf feeding beetles. Only very recent investigations have identified cysteine proteinases in beetles based on maximal synthetic substrate hydrolysis in combination with the effects of diagnostic activators and inhibitors (Chapter 3,4 and 5) and addressed their presence in other beetle feeding habits (Chapter 6).

The ability of insects to digest cellulose has been the subject of numerous investigations (reviewed by Martin, 1983). Cellulose digestion is brought about by the action of three enzymes, Cx-cellulase, C1-cellulose and cellobiase (Ljungdahl and Eriksson, 1985). Insects that are not able to digest cellulose are related to their inability to synthesize C1-cellulase. This inability to synthesize C1-cellulase is overcome however by exploiting the cellulolytic potential of either gut bacteria or gut protozoa or the use of cellulases from ingested fungi (Martin, 1983). Cellulose digestion has been reported in many beetles that include representatives from the Scarabaeidae, that digest cellulose via gut bacteria (Martin, 1983) and representatives from Cerambycidae, that digest cellulose with the aid of ingested fungi (Martin, 1983; Kukor *et al.*, 1988). Taylor (1985) reports that the Mexican bean beetle, *Epilachna varivestis* (Coccinellidae) also digests cellulose but by its ability to synthesize the complete cellulolytic complex. Cellulose digestion has been reported to occur optimally in the acidic pH range (Taylor, 1985). Cysteine proteinases also work optimally in a similar acidic environment (Barrett and McDonald, 1980), are known to be produced by microorganisms (Ward, 1983), but previous investigations have not looked at whether cysteine proteolytic activity in Coleoptera may

be related to the presence of a gut microflora.

The synthetic substrates Bz-Arg-NHNa and Bz-Arg-Np are both substrates of the cysteine proteinase cathepsin B and the serine proteinase trypsin. Maximal hydrolysis of these substrates either in the slightly acidic pH range, thiol activation and inhibition by E-64 or maximal hydrolysis of these substrates in the alkaline pH range and inhibition by aprotinin are good indicators of cathepsin B and trypsin activity respectively. Since both substrates are hydrolysed by both enzymes, then a beetle that uses only cathepsin B can hydrolyse these substrates in the acidic pH range and hydrolysis will not be effected by aprotinin. Conversely, a beetle that uses only trypsin can hydrolyse both substrates in the alkaline pH range and hydrolysis will not be effected by either thiol compounds or E-64. A beetle that uses both enzymes, can hydrolyse both substrates and will show characteristics typical of cathepsin B and trypsin. Based on these criteria, this present investigation uses a set of assays to detect the cysteine proteinase cathepsin B, the serine proteinase trypsin and C1-cellulase activity in beetle species that are representative of 16 different beetle families and belong to one of four different feeding habits, in order to determine the occurrence of cysteine proteinases in Coleoptera, their relationship with serine proteinases, a gut microflora and coleopteran feeding habits.

7.2 Results

Results from the survey of leaf feeding beetles are given in Table 23. Bz-Arg-NHNa hydrolysis by adult and larval *L. decemlineata* increased when DTT was added to

TABLE 23.

Detection of cathepsin B and trypsin from gut homogenates of larval and adult herbivorous beetles. Cathepsin B was detected based on the ability of gut homogenates to hydrolyse Bz-Arg-NHNa, activation by DTT and inhibition by 1.0 µg E-64. Trypsin was detected based on the ability of gut homogenates to hydrolyse Bz-Arg-Np and inhibition by aprotinin. Buffers used were, 0.1 M succinate-imadazole-diethanolamine pH 5.0 and 8.0 for cathepsin B and trypsin, respectively.

		picomoles of substrate hydrolysed / minute / gut †				
		Cysteine proteinase (Detected by cathepsin B activity)			Serine proteinase (Detected by trypsin activity)	
	Family	Buffer only	2.5 mM DTT	1.0 µg E-64	Buffer only	10 µg Aprotinin
LEAF FEEDING BEETLES						
Leptinotarsa decemlineata adult	Chyrsomelidae	142.8 ± 6.4	230.2 ± 1.1	*12.5 ± 0.4	17.3 ± 0.2	17.5 ± 0.3
Leptinotarsa decemlineata larvae	Chyrsomelidae	62.3 ± 1.1	149.7 ± 1.0	*15.1 ± 0.4	14.9 ± 0.5	15.4 ± 0.2
Pelidnota punctata adult	Scarabaeidae	21.4 ± 0.3	36.7 ± 0.7	*35.3 ± 0.2	131.2 ± 1.2	49.1 ± 2.6
SEED FEEDING BEETLES						
Tenebrio molitor adult	Tenebrionidae	12.0 ± 0.6	29.6 ± 0.1	*4.4 ± 0.1	272.0 ± 6.8	31.5 ± 3.9
Tenebrio molitor larvae	Tenebrionidae	124.3 ± 0.2	281.6 ± 0.4	*37.7 ± 0.7	598.6 ± 10.0	165.5 ± 0.8
Sitophilus zeamais larvae	Curculionidae	1.0 ± 0.1	10.0 ± 0.1	*0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1
Prostephanus truncatus adult	Bostrichidae	0.1 ± 0.03	0.1 ± 0.02	0.1 ± 0.04	38.6 ± 0.2	2.4 ± 1.2
Prostephanus truncatus larvae	Bostrichidae	0.4 ± 0.1	0.7 ± 0.1	*0.7 ± 0.1	175.1 ± 10.9	11.1 ± 3.6
Gibbium psyloides adult	Ptinidae	--	--	--	14.8 ± 0.4	0.4 ± 0.1
POLLEN FEEDING BEETLES						
Chauliognathus pennsylvanicus adult	Cantharidae	0.4 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	220.7 ± 9.3	10.6 ± 5.1
Tetraopes tetrophthalamus adult	Cerambycidae	5.1 ± 0.8	110.3 ± 1.1	*3.9 ± 0.4	321.7 ± 7.4	25.3 ± 5.0

† Values represent mean ± range/2, n=2; * Tested in the presence of 2.5 mM DTT

the reaction buffer and decreased in the presence of E-64. The rate of hydrolysis of Bz-Arg-Np by adult and larval *L. decemlineata* gut homogenates was only 8% and 10% respectively of that observed for Bz-Arg-NHNa hydrolysis, and aprotinin did not inhibit. Bz-Arg-Np hydrolysis by gut homogenates from *P. punctata* decreased in the presence of aprotinin and Bz-Arg-NHNa hydrolysis increased in the presence of DTT but activity was not effected by E-64.

Results from the survey of seed feeding beetles are given in Table 23. Bz-Arg-NHNa hydrolysis by gut homogenates from adult and larval *T. molitor* and adult *S. zeamais* increased in the presence of DTT and decreased in the presence of E-64. Both *T. molitor* larval and adult gut homogenates hydrolysed Bz-Arg-Np and hydrolysis decreased when aprotinin was added to the reaction buffer. The rate of hydrolysis of Bz-Arg-Np by gut homogenates from *S. zeamais* was only 3% of that observed for Bz-Arg-NHNa hydrolysis and aprotinin had no effect. Adult and larval *P. truncatus* and adult *G. psyloides* gut homogenates hydrolysed Bz-Arg-Np and aprotinin decreased hydrolysis. The rate of hydrolysis of Bz-Arg-NHNa by gut homogenates from adult and larval *P. truncatus* was less than 1% of that obtained for Bz-Arg-Np and although hydrolysis increased in the presence of DTT with *P. truncatus* larvae, E-64 had no effect. Within detectable limits of the assay their was no hydrolysis of Bz-Arg-Np by gut homogenates from *G. psyloides*.

Both of the pollen feeding beetles surveyed (Table 23) hydrolysed Bz-Arg-Np and aprotinin decreased hydrolysis. Hydrolysis of Bz-Arg-NHNa by *T. tetrophthalamus* gut homogenates increased with DTT and inhibited by E-64. The rate of hydrolysis of Bz-

Arg-NHNa by *C. pennsylvanicus* gut homogenates was less than 1% of that observed for Bz-Arg-Np and hydrolysis decreased in the presence of DTT and E-64.

Results from the survey of predacious beetles are given in Table 24. Bz-Arg-Np was hydrolysed by gut homogenates of all beetles and substrate hydrolysis decreased when aprotinin was added to the reaction buffer. All beetles hydrolysed Bz-Arg-NHNa, however only gut homogenates from *C. septempunctata*, *Dineutus sp.* and an adult diving beetle, had a increase in substrate hydrolysis with DTT and decreased hydrolysis in the presence of E-64. Bz-Arg-NHNa hydrolysis by gut homogenates from all the other predacious beetles surveyed was not effected by either DTT or E-64, and the rate of hydrolysis of Bz-Arg-NHNa was 5% or less of that observed for the hydrolysis of Bz-Arg-Np.

Results from the survey of cathepsin B and trypsin for saprophagous beetles are given in Table 24. Both adult and larval gut homogenates from *D. maculatus* and gut homogenates from *Glischrochilus sp.* and *N. tomentosus* hydrolysed Bz-Arg-Np and hydrolysis decreased when aprotinin was added to the reaction buffer. Bz-Arg-NHNa was hydrolysed by gut homogenates from all beetles, but only gut homogenates from *Glischrochilus sp.* had increased hydrolysis with DTT and decreased hydrolysis in the presence of E-64. The rate of hydrolysis of Bz-Arg-NHNa by gut homogenates from adult and larval *D. maculatus* gut homogenates was 1.7% and 0.9% respectively of that observed for Bz-Arg-Np and hydrolysis decreased in the presence of DTT and was not effected by E-64. The rate of hydrolysis of Bz-Arg-NHNa was less than 1% of that observed for Bz-Arg-Np for gut homogenates from *N. tomentosus* and hydrolysis was not

TABLE 24

Detection of cathepsin B and trypsin from gut homogenates of larval and adult predacious and saprophagous beetles. Cathepsin B was detected based on the ability of gut homogenates to hydrolyse Bz-Arg-NHNa, activation by DTT and inhibition by 1.0 µg E-64. Trypsin was detected based on the ability of gut homogenates to hydrolyse Bz-Arg-Np and inhibition by aprotinin. Buffers used were 0.1 M succinate-imadazole-diethanolamine pH 5.0 and 8.0 for cathepsin B and trypsin, respectively.

		picomoles of substrate hydrolysed / minute / gut †				
		Cysteine proteinase (Detected by Cathepsin B activity)			Serine proteinase (Detected by trypsin activity)	
	Family	Buffer only	2.5 mM DTT	1.0 µg E-64	Buffer only	10 µg Aprotinin
PREDACIOUS BEETLES						
Alobates pennsylvanicus adult	Tenebrionidae	0.3 ± 0.1	--	--	131.2 ± 5.5	13.3 ± 0.9
Coccinella septempunctata adult	Coccinellidae	2.0 ± 0.1	5.3 ± 0.2	*1.3 ± 0.9	760.8 ± 25.1	60.0 ± 8.4
Dineutus sp. adult	Gyrinidae	29.6 ± 0.3	42.0 ± 0.1	*0.6 ± 0.2	10.5 ± 0.2	4.9 ± 0.8
Pterostichus corvinus adult	Carabidae	15.0 ± 0.1	16.5 ± 0.1	15.1 ± 0.9	4335.4 ± 1.1	412.0 ± 39.8
Pterostichus corvinus larvae	Carabidae	9.6 ± 0.2	11.0 ± 0.3	9.6 ± 0.2	3870.3 ± 19.5	366.7 ± 10.5
Agorum melanarium adult	Carabidae	3.8 ± 0.2	4.1 ± 0.2	3.8 ± 0.7	854.0 ± 12.4	30.9 ± 5.3
Cicindela scutellaris adult	Cicindelidae	18.0 ± 0.1	17.1 ± 0.7	17.1 ± 0.2	993.6 ± 2.2	60.7 ± 5.2
Cicindela sp. larvae	Cicindelidae	6.2 ± 0.1	6.8 ± 0.2	6.8 ± 0.1	136.1 ± 8.8	7.8 ± 0.6
Adult diving beetle	Dytiscidae	15.9 ± 0.1	47.0 ± 0.4	*18.1 ± 0.6	1313.1 ± 65.5	125.5 ± 13.4
SAPROPHAGOUS BEETLES						
Dermestes maculatus adult	Dermestidae	1.4 ± 0.1	0.7 ± 0.4	1.7 ± 0.2	84.25 ± 5.1	8.1 ± 1.1
Dermestes maculatus larvae	Dermestidae	1.6 ± 0.2	1.2 ± 0.5	1.3 ± 0.1	178.2 ± 2.3	23.4 ± 2.1
Glischrochilus sp. adult	Nitidulidae	1.1 ± 0.1	18.2 ± 0.1	*0.2 ± 0.1	13.9 ± 0.1	1.5 ± 0.1
Nicrophorus tomentosus adult	Silphidae	7.2 ± 0.8	8.1 ± 0.4	7.8 ± 1.8	5983.4 ± 28.1	299.6 ± 18.7

† Values represent mean ± range/2, n=2; * Tested in the presence of 2.5 mM DTT

activated by DTT or inhibited by E-64.

Gut homogenates from all beetles were tested for their ability to hydrolyse microcrystalline cellulose. Only larval and adult *L. decemlineata* and adult *T. tetrophthalamus* were found to hydrolyse this substrate (Table 25), measured within detectable limits of the assay.

7.3 Discussion

Based on the hydrolysis of Bz-Arg-NHNa at pH 5.0 in combination with activation by DTT and inhibition by E-64, the cysteine proteinase cathepsin B was detected in 8 different beetle species that included; *L. decemlineata*, *T. molitor*, *S. zeamais*, *C. septempunctata*, *T. tetrophalamus*, *Dineutus sp.*, *Glischrochilus sp.* and a adult diving beetle. Based on the hydrolysis of Bz-Arg-Np at pH 8.0 in combination with inhibition by aprotinin, trypsin was detected in all beetles other than *L. decemlineata* and *S. zeamais*.

A previous survey from representatives of 10 different families by Murdock *et al.* (1987) suggested that cysteine proteinases are common in beetles based on the ability of gut homogenates to hydrolyse haemoglobin at pH 5.5 combined with inhibition by E-64. This investigation did not detect the presence of serine proteinases that have optimal activity under alkaline conditions and are not inhibited by E-64. Furthermore the suggestion that cysteine proteinases are common in beetles was not evaluated based on the taxonomic groupings of this order. Based on a survey to detect both cathepsin B

TABLE 25

Detection of C1-cellulase from gut homogenates of larval and adult *L. decemlineata* and adult *T. tetrophthalamus*. Buffer used was 0.1 M succinate-imadazole-diethanolamine, pH 5.0.

	nanomoles of glucose liberated / hr / gut †
PHYTOPHAGOUS BEETLES	
Leptinotarsa decemlineata adult	448.16 ± 36.48
Leptinotarsa decemlineata larvae	553.29 ± 35.95
POLLEN FEEDING BEETLE	
Tetraopes tetrophthalamus adult	460.47 ± 33.32

† Values represent mean ± range/2, n=2

and trypsin, results from this present study have found that cysteine and serine proteinases are both common in Coleoptera and that they even can occur in combination. The conclusion that both activities are common in the order is based on the detection of these enzymes from a survey of beetles that are representatives of 16 different families that belong to 9 major beetle superfamilies (Chrysomeloidea, Cucujoidea, Curculionoidea, Bostrichoidea, Dermestoidea, Cantharoidea, Scaraboidea and Caraboidea) and belong to two of the four recognized beetle suborders (Adephaga and Polyphaga) (see Table 26 in chapter 8). Evaluating the results based on the taxonomic groupings is recognized when the results of this survey are interpreted. These results would initially indicate that serine proteinases are more common, since 16 of 18 beetle species had trypsin detected whereas only 8 of the 18 had cathepsin B detected. However, many of the beetles that had trypsin detected, in particular those that belong to the Caraboidea superfamily, have made up almost 30 % of the total number of beetles, 25 % of the total number of families, yet only represent one of the 8 superfamilies investigated. Results would only indicate that trypsin activity appears to be common in this superfamily, but it is not representative of the whole beetle order.

Almost all previous reports on cysteine proteolytic activity in Coleoptera have been limited to investigations on leaf and seed feeding beetles including surveys by Murdock *et al.* (1987) and Wolfson and Murdock (1990) and investigations on bruchid beetles. Results from this present investigation have detected cathepsin B like activity within all feeding habits, including predacious beetles (*C. septempunctata*, *Dineutus sp.* and an adult diving beetle) and a saprophagous beetle (*Glischrochilus sp.*). This indicates

that cysteine proteinases are not restricted to any one particular feeding habit but are found in leaf, seed and pollen feeding beetles as well as predacious and saprophagous beetles.

C1-cellulase and cathepsin B activity were detected together only in *L. decemlineata* and *T. tetrophthalamus*. It is not known if *L. decemlineata* or *T. tetrophthalamus* can synthesize the necessary enzymes for cellulose digestion, but a fungal association is probable for *T. tetrophthalamus* since Kukor *et al.* (1988) suggests that probably all cellulose digesting beetles that belong to the Cerambycidae family degrade cellulose with the aid of fungal enzymes. These results suggest that cysteine proteolytic activity in Coleoptera is not directly related to the presence of a gut microflora detected by C1-cellulase activity. However, confirmation of a gut microflora cannot be based solely on the detection of C1-cellulase activity, because many Coleoptera that feed on seeds and stored grain such as *Sitophilus* weevils are known to have a bacterial gut flora (Campbell, 1989) which could produce cysteine proteinases. In addition, the failure to detect cellulose digestion is also not considered evidence against the digestion of dietary cellulose especially in insects that are known to have bacteria in their gut (Martin, 1983). Furthermore, gut microorganisms are also known to ferment sugars, fix nitrogen, degrade uric acid, synthesize amino acids and many other processes other than cellulose digestion (Martin, 1983), including the ability to produce cysteine proteinases (Ward, 1983). Therefore it cannot be ruled out that cysteine proteolytic activity detected in this survey may be the result of the metabolic activity of a gut flora that was not detected by the C1-cellulase assay.

This survey has attempted to clarify the occurrence of cysteine proteinases in Coleoptera as well as their relationship with serine proteinases, a gut microflora and beetle feeding habits. Results have found that serine and cysteine proteinases are both common in Coleoptera and even can occur in combination and cysteine proteinases can be found in a variety of different beetle feeding habits, and are not restricted to leaf and seed feeding beetles. The presence of cysteine proteinases does not appear to be related to a cellulose digesting microflora detected by C1-cellulase activity, although the relationship between a gut flora and cysteine proteinases cannot be rule out since gut microorganisms perform functions other than digesting cellulose including the production of cysteine-like proteinases.

CHAPTER 8

GENERAL DISCUSSION

8.1 Cysteine proteinases in Coleoptera

The rational of this study was based on the knowledge that recent investigations on the proteolytic enzymes of beetles indicated that cysteine and not only the commonly reported serine proteinases may be important for digestion. However, all previous investigations on cysteine proteinases used poor criteria to identify these enzymes and furthermore, reports were limited primarily to seed and leaf feeding beetles. It seemed that if cysteine proteinases are important components for digestion in beetles and if biological control strategies aimed at digestive proteases are going to be effective alternatives to chemical control, that this warranted investigations to identify these enzymes as well as to try and clarify their occurrence in Coleoptera.

The objectives that were set out in this study were to identify cysteine proteinases in beetles that belong to several major coleopteran families and address the occurrence of these enzymes in relation to the more commonly reported serine proteinases. Furthermore, this study has attempted to determine whether cysteine proteinases are related to the presence of a gut microflora detected by C1-cellulase activity and/or the evolutionary history of the different taxonomic groupings of beetles. The following sections are organized in such a manner to discuss key results obtained in this study.

8.1.1 The occurrence of cysteine and serine proteinases in Coleoptera

Previous investigations on the Colorado potato beetle, *L. decemlineata* reported cysteine proteolytic activity based on maximal hydrolysis of Hb at pH 5.5 and inhibition by E-64 (Wolfson and Murdock, 1987). These criteria could not have identified the individual proteinases responsible for digestion since Hb is hydrolysed by more than one enzyme in crude gut preparations. Using synthetic substrates and the effects of diagnostic activators and inhibitors, this present study has identified the cysteine proteinases cathepsin B and H, the aspartate proteinase cathepsin D and leucyl aminopeptidase in *L. decemlineata*. Identification of cathepsin B and H in this beetle has been the first investigation to accurately identify cysteine proteinases in Coleoptera and reveal that cysteine proteinases do not always occur in combination with the more commonly reported serine proteinases.

One of the earliest and most classic investigations on the proteolytic enzymes of beetles were with the yellow mealworm *T. molitor* (Applebaum *et al.*, 1964). In this study trypsin as well as a thiol activated, EDTA stabilized enzyme were reported, although the latter activity was not further investigated. Results have confirmed the presence of trypsin in *T. molitor* and cathepsin B (Chapter 4), the first time that both cysteine and serine proteinases have been reported in the same insect midgut. Similar results were obtained with *S. zeamais* (Chapter 5) and with *T. tetrophthalamus*, *Glischrochilus sp.*, *C. septempunctata*, *Dineutus sp.* and an adult diving beetle (Chapter 7) and have revealed that the combination of cysteine and serine proteinases detected in *T. molitor* is not a

unique occurrence in the order but can be found in many other beetle species. Cysteine and serine proteinases have very different requirements for optimal activity (Barrett and McDonald, 1980) but this difference appears to be accommodated by the midgut of beetles exemplified with the results with *T. molitor* that found cathepsin B activity in anterior portions of the midgut and trypsin in the posterior portions. The ability of these two enzymes to exist in the same gut is also reflected by the fact that thiol compounds, such as DTT, that are known to inhibit trypsin (Barrett and McDonald, 1980), were in fact found to activate tryptic activity in *T. molitor*.

Previous investigations by Murdock *et al.* (1987) had suggested that cysteine proteinases are common in Coleoptera based on the ability of gut homogenates from beetles representing 10 different families to hydrolyse haemoglobin at pH 5.5 in combination with inhibition by E-64. Using assay conditions designed to detect the cysteine proteinase cathepsin B and the serine proteinase trypsin, in addition to investigating beetles that belong to 16 different families, 9 different superfamilies and two of the four recognized beetle orders (Chapter 7; summarized in table 26, pg 130), this present study has found that both cysteine and serine proteinases are common in beetles.

8.1.2 The occurrence of cysteine proteinases with beetle feeding habits

Previous investigations on the proteolytic enzymes of predacious beetles and saprophagous beetles reported primarily on the presence of the serine proteinases trypsin

and chymotrypsin. Trypsin in these studies was detected primarily based on the hydrolysis of synthetic substrates such as Tos-Arg-OMe, Bz-Arg-OEt and Bz-Arg-Np, but many did not report the pH for maximal substrate hydrolysis and included investigations on the predacious carabid beetles, *Pterostichus melanarius* (Gooding and Huang, 1969), *Carabus taedatus*, *C. auronitens*, *C. punctatoauratus*, *C. lineatus* and *C. splendens* (Vaje *et al.*, 1984), predacious elaterid beetles, *Pyrophorus divergens* and *Pyrearinus termitilluminans* (Colepicolo-Neto *et al.*, 1987) and the saprophagous hide beetle, *D. maculatus* (Baker, 1982a). These investigations could not preclude the presence of cysteine proteinases that have similar substrate specifities (Barrett *et al.*, 1988) and function under acidic rather than alkaline environments. In an attempt to address the presence of cysteine proteinases in these two feeding groups using synthetic substrates, the pH for maximal substrate hydrolysis and the effects of diagnostic activitors and inhibitors this study identified trypsin, leucyl and arginyl aminopeptidase in the predacious beetles, *P. corvinus* and *Cicindela sp.* and the saprophagous beetle *D. maculatus* and not cysteine proteinases (Chapter 6). Although these results are consistent with previous reports of proteolytic activity in the alkaline pH range for these two feeding groups, the only other report of aminopeptidase in predacious beetles was by Colepicolo-Neto *et al.* (1987) in which activity was reported based solely on the ability of midgut homogenates from *Pyrophorus divergens* and *Pyrearinus termitilluminans* to hydrolyse Leu-Np at pH 8.0. The identification leucyl and arginyl aminopeptidase in *P. corvinus* and *Cicindela sp.* has been one of the first adequate characterizations of these enzymes in predacious beetles.

Serine proteinases have been detected in beetles with a variety of feeding habits

and included investigations on predacious, saprophagous and herbivorous beetles. On the other hand investigations on cysteine proteinases were limited primarily to herbivorous beetles such as the leaf feeding beetle, *L. decemlineata* (Wolfson and Murdock 1987), and the seed feeding bruchid beetles, *C. maculatus* (Gatehouse *et al.*, 1985; Kitch and Murdock, 1986; Campos *et al.*, 1989), *A. obtectus* (Wieman and Neilson, 1988) and *Z. subfaciatus* (Lemos *et al.*, 1990). Results from my investigation have found that cysteine proteinases occur in leaf feeding, seed feeding, pollen feeding, saprophagous and predacious beetles (Chapter 7) and have indicated that cysteine proteinases are not restricted to just leaf and seed feeding beetles.

Results from this study have not only indicated that cysteine proteinases can occur in beetles with a variety of feeding habits, but have also found that beetles feeding on similar food sources may not necessarily use the same proteinases for digestion. The most striking example was found with *S. zeamais* and *P. truncatus* (Chapter 5), two beetles that feed on corn, yet *P. truncatus* uses serine proteinases but *S. zeamais* uses a combination of cysteine and serine proteinases for digestion. The ability of these two beetles to feed on the same food source yet utilize different proteinases for digestion may be attributable to a difference in the evolutionary histories of these two beetles (see section 8.1.4).

8.1.3 The relationship of cysteine proteinases with a gut microflora

The reason that most insects cannot digest cellulose is because they cannot synthesize C1-cellulase. This deficiency is overcome by the cellulolytic potential of either bacteria or protozoa or cellulases from ingested fungi (Martin, 1983). It seemed that since cysteine proteinases and cellulolytic enzymes in beetles have both been reported to function optimally in acidic conditions, that perhaps cysteine proteinases in beetles may be related to the presence of a gut microflora. Results from the survey of beetles (Chapter 7), however, detected C1-cellulase activity in only two beetles, *L. decemlineata* and *T. tetrophthalamus*. Although these results indicate that cysteine proteinases are not related to a cellulose digesting microflora, a relationship between a gut microflora and cysteine proteinases cannot be ruled out. This is because *Sitophilus* weevils such as *S. zeamais* are known to have a bacterial gut flora (Musgrave, 1964; Campbell, 1989) and gut microorganisms are known to perform many functions other than cellulose digestion, including the production of cysteine-like proteinases (Ward, 1983). Therefore, it is still possible that cysteine proteinases may be the product of a gut microflora, that was not detected by C1-cellulase activity.

Even though a direct relationship between a gut microflora and cysteine proteinases was not obtained in this study, it is still interesting to speculate on a possible association between cysteine proteinases and a gut microflora in the gut of a leaf feeding beetle such as *L. decemlineata*. Although plant foliage is low in total protein nitrogen (Strong *et al.*, 1984), *L. decemlineata* is still able to obtain proper nutrition for normal

growth and development. Perhaps the ability of *L. decemlineata* to survive on this protein poor food source is related to a symbiotic relationship with a gut microflora. Two possible associations are proposed: The first senario involves the beetle synthesizing both the cellulolytic enzymes to breakdown ingested plant foliage that maintains the gut microflora, and cysteine proteinases to turn over the gut microflora; the second senario involves the gut microflora digesting the ingested plant foliage and *L. decemlineata* synthesizing cysteine proteinases to turn over the gut microflora. In either of these senarios the digestion of the gut microflora may be providing the beetle with its protein requirement.

8.1.4 The relationship of cysteine proteinases with the evolutionary history of the different taxonomic groupings of beetles.

Reports of cysteine proteinases in seed feeding bruchid beetles and haematophagous and seed feeding Hemiptera lead Terra *et al.* (1988) to suggest that cysteine proteinases are a consequence of feeding on food sources such as seeds that are high in trypsin inhibitors. Results from this present study are not consistent with this hypothesis based on the finding that seed feeding beetles such as *P. truncatus* do not even use cysteine proteinases for digestion (Chapter 5) even though it feeds on corn, a food known to contain trypsin inhibitors.

The results from this present investigation indicate that cysteine proteinases in Coleoptera may be related to the evolutionary history of the different taxonomic

groupings of beetles. A summary of the results obtained from the survey of beetles (Chapter 7) are given in Table 26 and presented on an evolutionary tree for Coleoptera in figure 16 (from Gillot, 1980 - adapted from Crowson, 1960). Other than the detection of cysteine proteolytic activity in two beetles of the Caraboidea superfamily of the Adephagaen suborder (*Dineutus sp.* and a adult diving beetle), only those beetles that belong to the Cucujiformia lineage of the Polyphagaen suborder (Chrysomeloidea, Cucujoidea and Curculionoidea) had cysteine and C1-cellulase activities detected. All other beetles that belong to one of the other lineages within the polyphagean suborder (Bostrychiformia, Elateriformia, Scarabaeiformia and Staphyliniformia), had serine but not cysteine proteinases detected. The evolutionary history of Coleoptera and the occurrence of cysteine proteinases in the Cucujiformia lineage of the polyphagean suborder are also supported by previous research on the order (summarized in Table 27), which again finds cysteine proteolytic activity in either the Chrysomeloidea or Cucujoidea or Curculionoidea superfamilies. Although it is interesting to find that cysteine proteinases are found principally in beetles that belong to Cucujiformia lineage, further investigations on other coleopteran superfamilies are necessary before concluding that cysteine proteinases are predominately restricted to this evolutionary lineage.

TABLE 26
Detection of cysteine and serine proteinases
from representives of 9 coleopteran superfamilies (Chapter 7)

SUBORDER Superfamily: *Genus species*	Feeding Habit	Cysteine proteinase	Serine proteinase
POLYPHAGA			
Chrysomeloidea:			
Leptinotarsa decemlineata adult	Leaf feeding	+	-
Leptinotarsa decemlineata larvae	Leaf feeding	+	-
Tetraopes tetrophthalamus adult	Pollen feeding	+	+
Cucujoidea:			
Glischrochilus sp. adult	Saprophagous	+	+
Tenebrio molitor adult	Seed feeding	+	+
Tenebrio molitor larvae	Seed feeding	+	+
Alobates pennsylvanicus adult	Predacious	-	+
Coccinella septempunctata adult	Predacious	+	+
Curculionoidea:			
Sitophilus zeamais larvae	Seed feeding	+	+
Bostrichoidea:			
Prostephanus truncatus adult	Seed feeding	-	+
Prostephanus truncatus larvae	Seed feeding	-	+
Gibbium psyloides adult	Seed feeding	-	+
Dermestoidea:			
Dermestes maculatus adult	Saprophagous	-	+
Dermestes maculatus larvae	Saprophagous	-	+
Cantharoidea:			
Chauliognathus pennsylvanicus adult	Pollen feeding	-	+
Scarabaeoidea:			
Pelidnota punctata adult	Leaf feeding	-	+
Staphylinoidea:			
Nicrophorus tomentosus adult	Saprophagous	-	+
ADEPHAGA			
Caraboidea:			
Dineutus sp. adult	Predacious	+	+
Pterostichus corvinus adult	Predacious	-	+
Pterostichus corvinus larvae	Predacious	-	+
Agorum melanarium adult	Predacious	-	+
Cicindela scutellaris adult	Predacious	-	+
Cicindela sp. larvae	Predacious	-	+
Adult diving beetle	Predacious	+	+

(+) - Detection of enzymatic activity

(-) - No activity detected

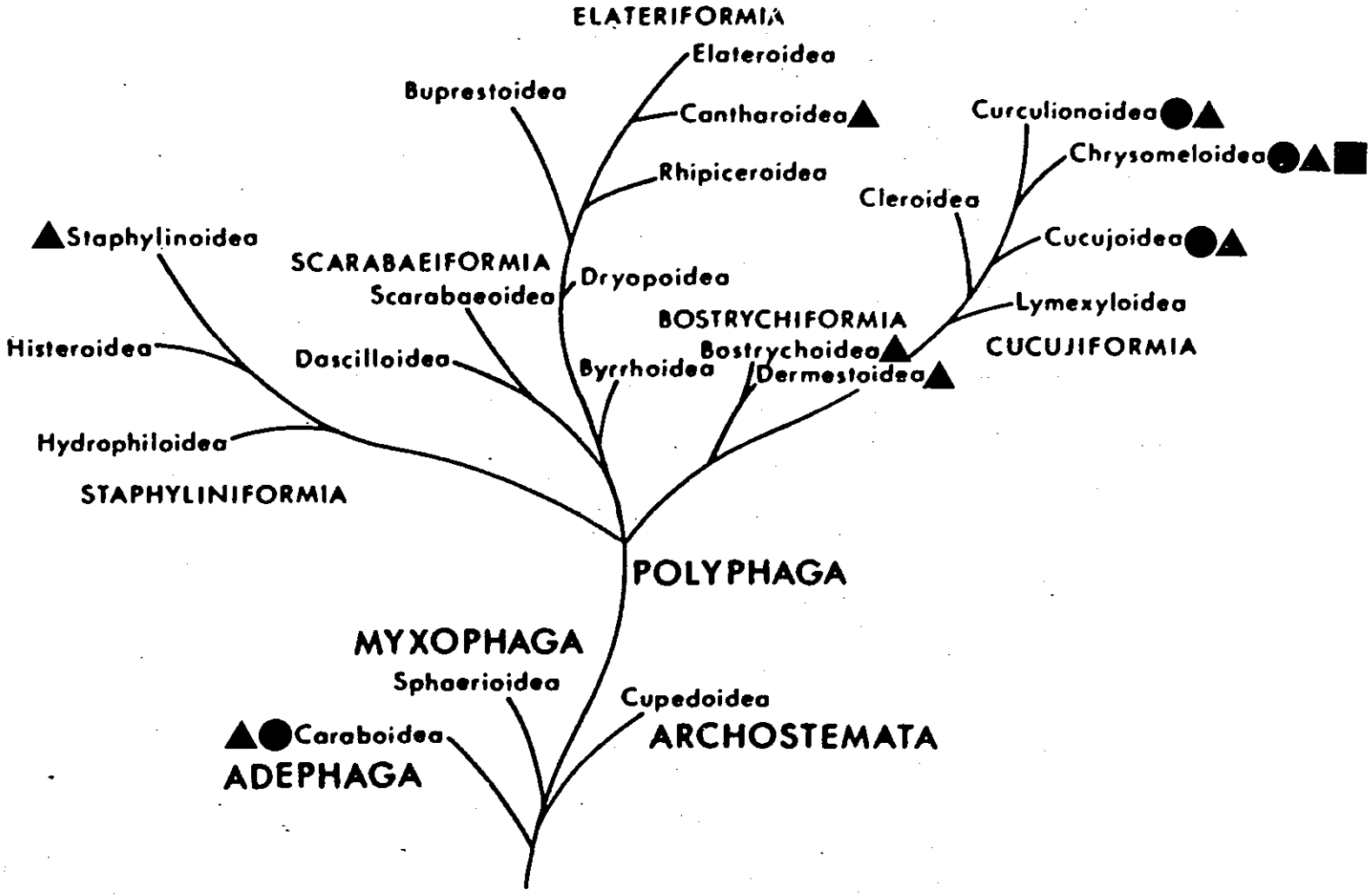

Fig. 16 Diagrammatic scheme of the possible evolutionary lineages of coleopteran superfamilies (figure from Gillot, 1980; p. 279). Detection of serine proteolytic activity is indicated by (▲), cysteine proteolytic activity by (●) and C1-cellulase activity by (■).

TABLE 27
Detection of cysteine and serine proteinases from previous research on the proteolytic enzymes of beetles

SUBORDER Superfamily: Family *Genus species*	Cysteine proteinase[a]	Serine proteinase[a]	Author(s)
POLYPHAGA			
Chrysomeloidea			
Bruchidae			
Callosobruchus maculatus	+		Gatehouse *et al.* (1985)
	+		Kitch and Murdock (1986)
	+		Murdock *et al.* (1987)
	+		Campos *et al.* (1989)
Acanthoscelides obtectus	+		Wieman and Neilson (1988)
Zabrotes subfaciatus	+		Lemos *et al.* (1990)
Chrysomelidae			
Leptinotarsa decemlineata	+		Murdock *et al.* (1987)
	+		Wolfson and Murdock (1987)
Crioceris asparagi	+	+	Wolfson and Murdock (1990)
Diabrotica sp.	+		Murdock *et al.* (1987)
Lema trilineata	+		Murdock *et al.* (1987)
	+	+	Wolfson and Murdock (1990)
Odonata horni	+		Wolfson and Murdock (1990)
Cerambycidae			
Tetraopes tetrophthalamus	+		Murdock *et al.* (1987)
Cucujoidea			
Tenebrionidae			
Tribolium castaneum	+		Murdock *et al.* (1987)
Nitidulidae			
Glischrochilus quadrisignatus	+		Murdock *et al.* (1987)
Meloidea			
Epicauta pestifera	+		Wolfson and Murdock (1990)
Coccinellidae			
Epilachna varivestis	+		Murdock *et al.* (1987)
	+		Wolfson and Murdock (1990)
Curculionoidea			
Curculionidae			
Anthonomus grandis	+		Murdock *et al.* (1987)
	+		Wolfson and Murdock (1990)
Sitophilus granarius		+	Baker (1982b)
Sitophilus oryzea		+	Baker (1982b)

...Continued

Table 27 continued.

Scarabaeoidea		
Scarabaeidae		
Costelytra zealandica	+	Christellar *et al.* (1989)
Elateroidea		
Elateridea		
Pyrophorus divergens	+	Colepicolo *et al.* (1987)
Pyrearinus termitilluminans	+	Colepicolo *et al.* (1987)
Dermestoidea		
Dermestidae		
Attagenus megatoma	+	Baker (1976; 1981b)
Dermestes maculatus	+	Baker (1982a)
ADEPHAGA		
Caraboidea		
Carabidae		
Pterostichus melanarius	+	Gooding and Huang (1969)
Calosoma calcidum	+	Cheung and Gooding (1970)
Carabus taedatus	+	Vaje *et al.* (1984)
Carabus auronitens	+	Vaje *et al.* (1984)
Carabus punctatoauratus	+	Vaje *et al.* (1984)
Carabus lineatus	+	Vaje *et al.* (1984)
Carabus splendens	+	Vaje *et al.* (1984)

[a](+) - Detection of enzymatic activity
- Blanks indicate that enzymatic activity was not reported.

The evolutionary history of beetles presented in Figure 16 also appears to explain the results obtained in this study that found beetles feeding on similar food sources (see Table 26) did not always have similar enzymes detected. The first example includes results on the seed feeding beetles, *T. molitor* and *S. zeamais*, that both had cysteine and serine proteinases and belong to the Cucujoidea and Curculionoidea superfamilies respectively of the Cucujiformia lineage. *Prostephanus truncatus* and *G. psyloides* are seed feeding beetles as well, yet had only serine proteinases detected but belong to the Bostrychiodea superfamily of the Bostrychiformia lineage. This difference in evolutionary histories therefore appears to be a plausible explanation of why two beetles such as *P. truncatus* and *S. zeamais* were found to utilize different proteolytic enzymes for digestion (Chapter 5), even though they feed on the same food source. Other examples include; *L. decemlineata*, a leaf feeding beetle that had cysteine and C1-cellulase activities, belongs to the Chrysomeloidea superfamily of the Cucujiformia lineage, yet the leaf feeding beetle *P. punctata* had only serine proteolytic activity, but belongs to the Scarabaeoidea superfamily of the Scarabaeiformia lineage; The saprophagous beetles *D. maculatus* (superfamily Dermestoidea) and *N. tomentosus* (superfamily Staphylinoidea) belong to the Bostrychiformia and Staphyliniformia lineages, respectively and had serine proteinase activity, whereas the saprophagous beetle *Glishrochilus sp.* (superfamily Cucujoidea) had both cysteine and serine proteinases, but belongs to the Cucujiformia lineage; The pollen feeding beetle, *C. pennsylvanicus* belongs to the Cantharoidea superfamily of the Elateriformia lineage had serine proteolytic activity detected, whereas *T. tetrophthalamus* had cysteine, serine as well as C1-cellulase activities but again belongs to the

Cucujiformia lineage.

The ancestor of modern beetles is thought to have been a mycophagous feeder, that is, feeds on decomposing wood and leaf matter altered by the action of fungal cellulases (Crowson, 1984) and it is thought that general saprophagy or mycophagy was ancestral in the order (Lawrence, 1987). Moreover, this food source is low in total nitrogen (Strong *et al.*, 1984), and perhaps the occurrence of cysteine proteinases in modern beetles that belong to the Cucujiformia lineage is a consequence of some beetles returning to feed on a high protein food source associated with seed and pollen feeding as well as predacious and saprophagous feeding habits in this group. Certainly the detection of C1-cellulase in *L. decemlineata* and *T. tetrophthalamus* that belong to the Cucujiformia lineage may be an ancestral characteristic maintained by these beetles, since detection of C1-cellulase is generally regarded as evidence of the entire cellulase complex in addition to enzymes comparable to fungal cellulases (Martin, 1983).

8.2 Proteolytic activity in herbivorous beetles in relation to other herbivorous insect orders.

Unlike previous investigations on herbivorous Hemiptera, Lepidoptera, Orthoptera and Hymenoptera, beetles investigated in this study that included investigations on leaf, seed and pollen feeding beetles, indicate that Coleoptera may be unique in their ability to use either cysteine, serine or a combination of both proteolytic activities for digestion. Cysteine proteinases in addition to serine proteinases, however, cannot be precluded in

other insect orders. This includes Hymenoptera, since previous studies on the proteolytic enzymes of the honey bee, *Apis mellifera* by Giebel *et al.* (1971) detected an unknown Bz-Arg-NHNa hydrolysing activity and Dahlman *et al.* (1978) reported a catheptic protease based on the hydrolysis of Bz-Arg-Np, that may prove with future research to be a cysteine proteinase.

8.3 Implications of the research

The results of this research have shed new light on the proteolytic enzymes in beetles and increased our understanding of digestive physiology in this order. This study has identified cysteine, serine and aminopeptidase enzymes in beetles that are representatives of several major coleopteran families and has found that both cysteine and serine proteinases are common in this order and that they can occur either together or separately. Furthermore, the results have found that the occurrence of cysteine proteinases in beetles is not related to either food source or a gut microflora but rather to the evolutionary history of the different taxonomic groupings of beetles. In addition, this investigation has found that both proteinases and peptidases are both important components for digestion in beetles such as *L. decemlineata* (Chapter 3) *P. truncatus* (Chapter 5), *P. corvinus*, *Cicindela sp.* and *D. maculatus* (Chapter 6), indicating that digestion in beetles is a coordinated event with a consorta of enzymes necessary for digestion.

Identifing the functional enzymes involved in digestion is important when assessing

the role of biological control strategies such as proteinase inhibitors aimed at disrupting insect digestion via digestive enzymes. For example, genetic manipulations to increase the natural level of trypsin inhibitors in potato plants or any other plant may prove ineffective against pests such as the Colorado potato beetle that use cathepsin B and H and not trypsin for digestion (Chapter 3). In addition, identification of the functional enzymes will improve other biological strategies such as *Bt* whose effectiveness has been based on the conversion of the crystal proteins into toxic fragments by alkaline proteases in the insect gut (Höfte and Whiteley, 1989). However, results from this study have found that beetles may use either acidic and/or alkaline proteases for digestion and this finding may direct attention to finding *Bt* strains that are effective at acidic as well as alkaline gut environments. The emphasis on the identification of the functional proteinases is also revealed by the fact that beetles may not use proteases that always share characteristics similar to their vertebrate counterparts. For example is the lack of inhibition by TLCK on Bz-Arg-Np hydrolysis by midgut homogenates from *P. truncatus* (Chapter 5), *P. corvinus* and *D. maculatus* (Chapter 6). This lack of inhibition by TLCK, a trypsin specific inhibitor (Barrett and McDonald, 1980) indicates that insect proteases cannot be assumed to be analagous or always share characteristics typical of vertebrate proteases. These results indicate if adequate identification of the functional proteinases involved in digestion are not determined that this could undermine the potential of digestive inhibitors as a useful biological control mechanism.

8.4 Future research

Suggested areas of future research are as follows:

1. To examine beetles that are representative of major superfamilies from all four suborders in order to further determine the presence of cysteine proteinases in relation to the evolutionary history of the different taxonomic groupings of beetles.

2. To further investigate the role of gut microorganisms in relation to the proteolytic enzymes of beetles.

3. To examine the effect of naturally occurring plant inhibitors on the growth and development of beetles that use cysteine proteinases for digestion.

CHAPTER 9

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