Eur. J. Entomol. **102**: 147–153, 2005
ISSN 1210-5759

Trypsin-like activity of membrane-bound midgut proteases from *Anticarsia gemmatalis* (Lepidoptera: Noctuidae)

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Keywords. Trypsin, proteases, velvetbean caterpillar, Lepidoptera, Noctuidae, *Anticarsia*

Abstract. Membrane-bound proteases from preparations of the midgut of 5th instar velvetbean caterpillars, *Anticarsia gemmatalis* (Hübner) were obtained by resuspension of the pellet obtained after 100,000 *g* centrifugation. As expected of trypsin-like proteases, they hydrolyzed casein and the synthetic substrates *N*-α-benzoyl-L-Arg-p-nitroanilidine (L-BApNA) and *N*-α-p-tosyl-L-Arg methyl ester (L-TAME). Higher activities were observed at 50°C, and at pH 8.5 and 8.0 for both synthetic substrates L-BApNA and L-TAME. The membrane-bound proteases were inhibited by EDTA, phenylmethan sulphonyl fluoride (PMSF), tosyl-L-lysine chloromethyl ketone (TLCK), benzamidine and aprotinin. TLCK and benzamidine were particularly active inhibitors. The K_M-values obtained were 0.23 mM for L-BApNA and 92.5 μM for L-TAME. These results provide evidence for the presence of membrane-bound trypsin-like proteases in the midgut of the velvetbean caterpillar, a key soybean pest in warm climates. The interaction between *A. gemmatalis* digestive proteases and soybean protease inhibitors has potentially important consequences for soybean breeding programs.

INTRODUCTION

An understanding of insect-plant interactions is important for predicting host plant colonization by insect-pests and for developing alternative control methods (Pedigo, 1989; Boulter, 1993; Bernays & Chapman, 1994; Carlini & Grossi-de-Sá, 2002). Plants usually contain an array of proteins toxic to insects, particularly the legumes that are rich in nitrogen (Bernays & Chapman, 1994; Koiwa et al., 1997; Hilder & Boulter, 1999; Stotz et al., 1999; Carlini & Grossi-de-Sá, 2002). Arcelin, lectin, α-amylase and protease inhibitors make up some of the nitrogen-rich defensive compounds present in legumes (Ryan, 1990; Felton & Gatehouse, 1996; Jongsma & Bolter, 1997; Koiwa et al., 1997; Stotz et al., 1999; Carlini & Grossi-de-Sá, 2002).

Earlier reports on plant proteins active against insect proteases led to investigations of the interactions between insect digestive proteases and plant protease inhibitors, which have consequences for biotechnology and plant breeding (Hilder et al., 1987; Boulter, 1993; Hilder & Boulter, 1999; Carlini & Grossi-de-Sá, 2002). Serine proteases are the best-studied proteases and are present in viruses, prokaryotes and eukaryotes, suggesting they are vital for the survival of organisms. They are also frequently recorded in insects, particularly Lepidoptera, where they are the main family of proteases (Terra & Ferreira, 1994). The most frequently studied serine proteases, trypsin- and chymotrypsin-like, act in a wide range of physiological processes including digestion, protein activation in the melanization cascade, antibacterial activity and insect immune response (Nakajima et al., 1997; Gorman et al., 2000a,b; Ma & Kanost, 2000).

Trypsin-like enzymes preferentially cleave internal peptide bonds on the carboxyl side of basic L-amino acids, such as arginine and lysine, and are irreversibly inhibited by N-α-tosyl-L-lysine chloromethyl ketone (TLCK). The digestive activity of trypsin-like enzymes from insect midgut were studied and characterized in Auchenorrhyncha (Foissac et al., 2002), Blattodea (Elpidina et al., 2001; Marana et al., 2002; Lopes & Terra, 2003), Coleoptera (Zhu & Baker, 1999; Wagner et al., 2002), Diptera (Gorman & Paskewitz, 2001; Muharsini et al., 2001) and Lepidoptera (Lee & Anstee, 1995; Valaitis, 1995; Novillo et al., 1999).

In Lepidoptera, it is reported that the soluble trypsins found in secretory vesicles are derived from membrane-bound trypsins (Eguchi & Iwamoto, 1976; Eguchi et al., 1982; Santos & Terra, 1984; Santos et al., 1986). Jordão et al. (1999) suggest that the trypsin from *Spodoptera frugiperda* (Lepidoptera: Noctuidae) is probably processed

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by the Golgi complex and transported in secretory vesicles. These vesicles migrate through the cell microvilli merging with the apical membrane and are released in the larvae lumen. The present study provides evidence of the presence of membrane-bound trypsin-like proteases in midgut preparations of the velvetbean caterpillar, a key soybean pest in warm climates, and the likely occurrence of members of other protease families. The interaction between *A. gemmatalis* digestive proteases and protease inhibitors has potentially important consequences for soybean breeding programs.

MATERIAL AND METHODS

Insects and chemicals

Anticarsia gemmatalis was reared on the artificial diet described by Hoffman-Campo et al. (1985) and maintained under controlled conditions of 25 ± 5°C, 70 ± 10% r.h. and 14L : 10D photoperiod. Fourth and fifth instar larvae were used in the experiments. All reagents were purchased from Sigma-Aldrich Brasil Ltda (São Paulo, SP, Brazil).

Preparation of midgut extract

Larvae were rinsed with water, chilled on ice and dissected in 10^{-3} M HCl at 4°C. One hundred midguts (with contents) were subjected to nine cycles of nitrogen freezing and thawing at 37°C in a water bath. Aliquots of 1 ml of midgut extract were centrifuged at 100,000 *g* for 45 min at 4°C and the pellets that formed resuspended in 10^{-3} M HCl with 0.5% Triton X-100 for 2 h at 4°C. Another 100,000 *g* centrifugation for 30 min at 4°C followed. The resulting supernatant was stored at –18°C and later used as an enzyme source after dialysis against 100 volumes of 0.1 M Tris-HCl buffer (pH 8.5).

Protein assays

Protein concentration was measured following Bradford (1976). Solutions of 0–0.2 mg/ml of bovine serum albumin (BSA) were used as standards.

Enzyme assays

Proteolytic activity was determined using casein as a substrate and monitored at a wavelength of 280 nm as described by Kunitz (1947) and adapted by Oliveira et al. (1993). Amidolytic activity was determined using *N*-α-benzoyl-L-Arg-p-nitroanilidine (L-BApNA; 0.5 mM) as a substrate. For the determination of esterolytic activity *N*-α-p-tosyl-L-Arg methyl ester (L-TAME; 0.10 mM) was used (Hummel, 1959). Optimum pH conditions for enzyme activity were determined at 25°C using L-BApNA and L-TAME as substrates. These substrates were also used at temperatures varying from 15 to 65°C to assess the effect of temperature on enzyme activity. Three replicates were always used to quantify enzyme activity.

Effect of calcium and protease inhibitors

Calcium chloride ($CaCl_2$) was added to 0.5 mM L-BApNA (25°C) in 0.1 M Tris-HCl (pH 8.5) at final concentrations varying from 0 to 30 mM to assess the effect of Ca^{++} on enzyme activity. The same substrate and buffer system was used to test the effect of the following protease inhibitors on protease activity: ethylenediamine-tetracetic acid (EDTA; 0–100 mM), phenylmethan sulphonyl fluoride (PMSF; 0.04–3.20 mM), tosyl-L-lysine chloromethyl ketone (TLCK; 0.005–1.0 mM), benzamidine (0.001–1.0 mM) and aprotinin (0.05–4.0 mM). Enzyme samples were incubated for 25 min with each protease inhibitor prior to the addition of L-BApNA to the mixture, which was then incubated for 2.5 min and the amidolytic activity determined as previously described.

Kinetic assays

The determination of the kinetic parameters K_M and V_{max} was carried out in 0.1 M Tris-HCl (pH 8.5) with L-BApNA concentrations ranging from 0.019 to 1.2 mM. The kinetic parameters were determined for L-TAME at concentrations ranging from 0.013 to 0.8 mM in 0.1 M Tris-HCl (pH 8.0) by non-linear regression using the software Enzfitter (Elsevier, Biosoft).

RESULTS

Proteolytic activity and trypsin-like properties

The proteolytic activity, detected with casein, followed the hyperbolic model of Michaelis-Menten (Fig. 1). Trypsin substrates are proteins, peptides and synthetic amides as well as esters with amides or ester bonds in their structures formed by the carboxyl groups of lysyl and arginyl basic residues (Neurath & Schwert, 1950). Therefore two synthetic substrates, the amide L-BApNA and the ester L-TAME, were used to recognize the presence of trypsin-like proteases in the midgut epithelium of *A. gemmatalis.*

The temperature profile of protease activity, using L-BApNA as a substrate, showed three peaks, at temperatures 35, 50 and 60ºC, with the highest activity at 50ºC (Fig. 2A). The existence of more than one peak of activity suggests the presence of different forms of proteases in the microsomal fraction of the midgut homogenate of *A. gemmatalis*. When L-TAME was used as a substrate, only one peak of protease activity was observed at 50ºC (Fig. 3A). The different peaks of activity observed with these two substrates is not surprising since L-TAME is a specific substrate for serine proteases, unlike L-BApNA, which is a substrate for serine and cysteine proteases. Therefore, the activity associated with these substrates is probably affected by non trypsin-like proteases, as reported in other Lepidoptera (Terra & Ferreira, 1994; Valaitis, 1995; Jongsma & Bolter, 1997).

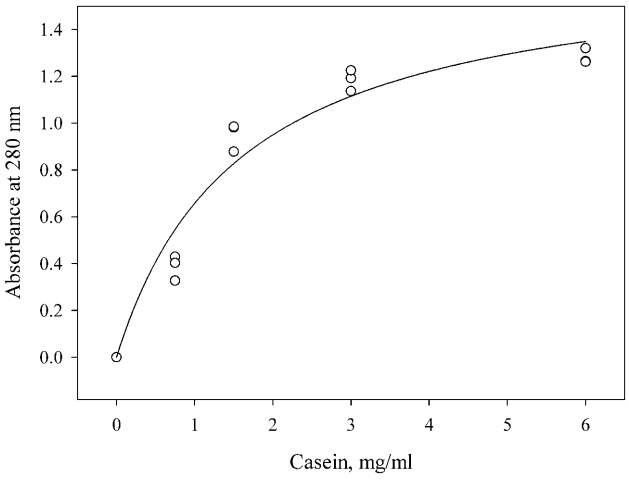

Fig. 1. Casein hydrolysis by membrane-bound midgut proteases of *A. gemmatalis*. Casein concentrations varied from 0.075 to 0.6% (w/v).

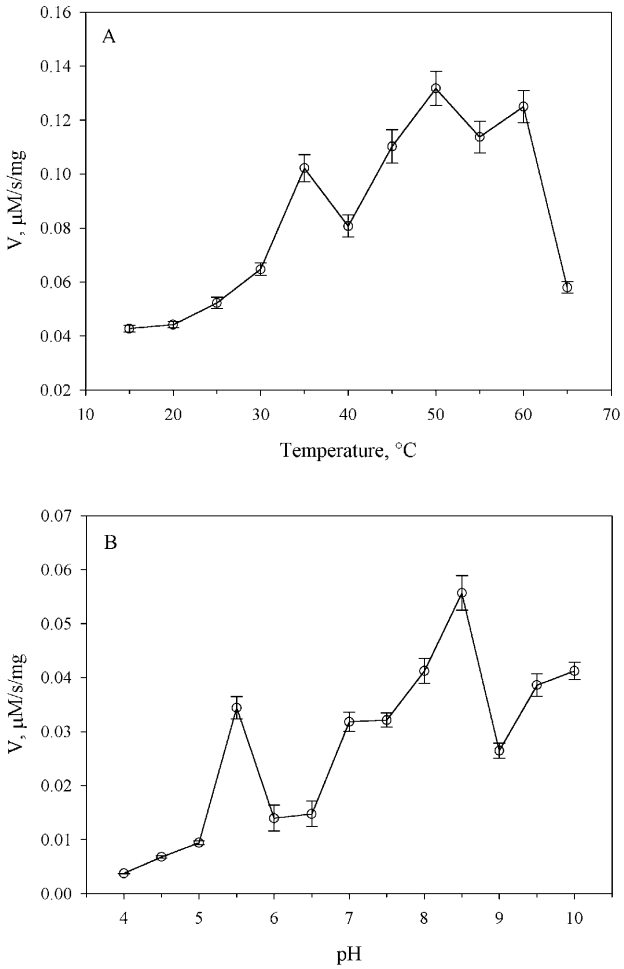

Fig. 2. Effect of temperature (A) and pH (B) on the membrane-bound protease activity of *A. gemmatalis* midgut using L-BApNA as a substrate. Each symbol represents the mean of three replicates (n = 3). Vertical bars indicate standard errors of the means.

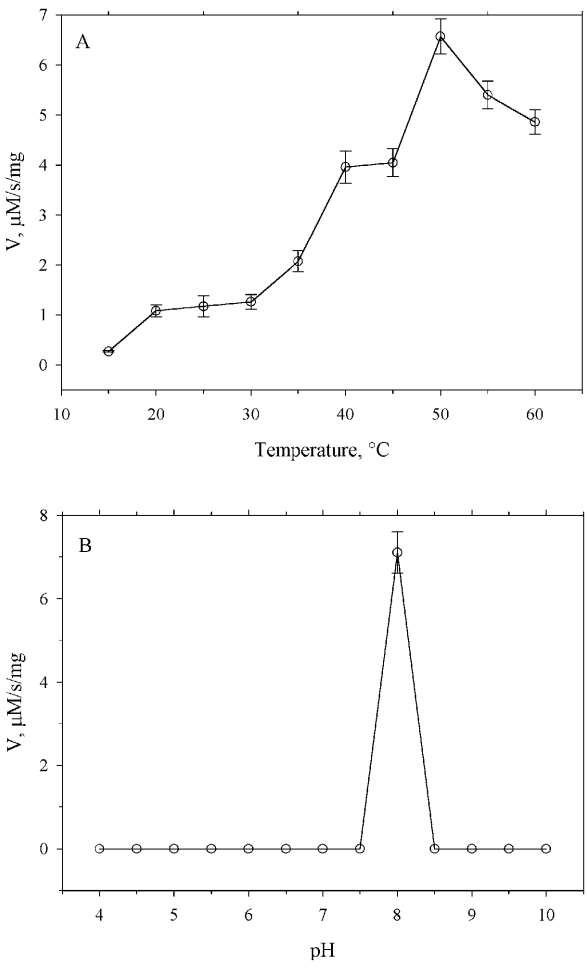

Fig. 3. Effect of temperature (A) and pH (B) on the membrane-bound protease activity of *A. gemmatalis* midgut using L-TAME as a substrate. Each symbol represents the mean of three replicates (n = 3). Vertical bars indicate standard errors of the means.

Highest activity of membrane-bound proteases from *A. gemmatalis* midgut were observed at pH 5.5 and 8.0 (highest) when L-BApNA was used as a substrate (Fig. 2B) providing support for the presence of two distinct trypsin-like enzymes. Only one peak at pH 8.0 was observed in the activity profile of the insect proteases using L-TAME as a substrate (Fig. 3B). Increased protease activity was observed when 20 mM Ca Cl_2 (Fig. 4) was used, which accords with an earlier report that Ca^{++} stabilize bovine trypsin against autolysis at this concentration (Dias & Rogana, 1986).

Inhibition studies

Trypsin-like activity of the membrane-bound proteases from *A. gemmatalis* was further characterized by the use of specific inhibitors in concentration-inhibition bioassays. The established curves followed the probit model (low χ^2 and $p > 0.05$), which allowed the I_{50} values to be estimated (i.e., inhibitor concentration required to achieve 50% inhibition of the protease activity) (Table 1). The metallo-protease inhibitor EDTA, which also inhibits proteases with metals as activity modulators, decreased the membrane-bound protease activity of *A. gemmatalis*

TABLE 1. Relative inhibition of membrane-bound proteases from *A. gemmatalis*, by five protease inhibitors.

Inhibitor	I_{50} (95% CL)	Degrees of freedom	χ^2	Probability (*p*)
EDTA	89.77 (71.43–136.30) mM	2	4.58	0.10
PMSF	0.63 (0.48–0.91) mM	2	2.89	0.23
TLCK	39.24 (23.59–60.63) µM	2	0.19	0.91
Aprotinin	11.27 (6.23–42.84) µM	2	0.88	0.64
Benzamidine	475.13 (245.15–1181.00) µM	2	4.37	0.11

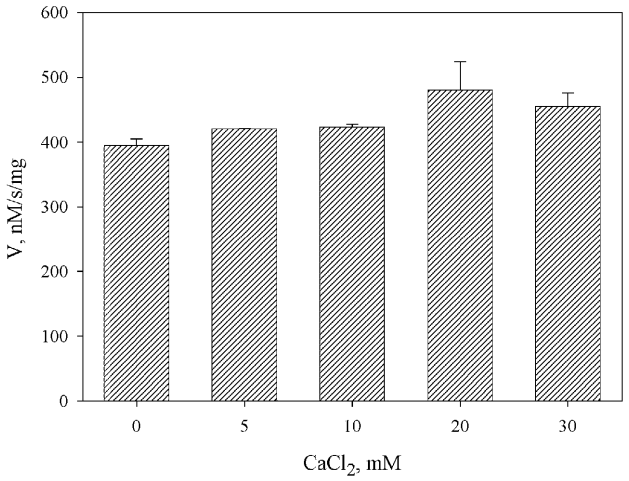

Fig. 4. Effect of Ca^{++} on the membrane-bound protease specific activity of *A. gemmatalis* midgut using L-BApNA as a substrate. Each bar represents the mean of three replicates (n = 3). Vertical bars indicate standard errors of the means.

(Table 1). Similar findings reported for trypsin-like proteases from *S. littoralis* and *Melolontha melolontha* (Coleoptera: Scarabaeidae) lead to the suggestion that Ca^{++} are involved in enzyme function (Lee & Anstee, 1995; Wagner et al., 2002).

PMSF is a serine protease inhibitor that reacts with a serine residue in the reactive site of the enzyme (Barret, 1994). It inhibits serine-proteases, such as trypsin and trypsin-like enzymes, and in the present study inhibited membrane-bound proteases from *A. gemmatalis* (Table 1). TLCK, an irreversible inhibitor of trypsin-like serine proteases, alkylates an histidine residue close to the reactive serine of trypsins (Shaw et al., 1965). The membrane-bound proteases of *A. gemmatalis* were inactivated not only by PMSF, but also by TLCK (Table 1) that inhibits trypsins (Bernardi et al., 1996; Gatehouse et al., 1999; Novillo et al., 1999; Zhu & Baker, 1999; Lam et al., 2000).

Aprotinin is a small peptide (6.5 KDa) that strongly inhibits trypsin-like enzymes by tightly binding its Arg_{17} residue close to the active site of the enzyme (Röhlmann et al., 1973; Geiger & Fritz, 1981; Oliveira et al., 1993). Aprotinin was preferred to soybean trypsin inhibitor (SBTI) as it is more frequently used for recognizing such enzymes (Röhlmann et al., 1973; Oliveira et al., 1993), what was the main objective of the present work. Membrane-bound midgut proteases of *A. gemmatalis* were significantly inhibited by aprotinin with an I_{50} of 11.22 µM (Table 1), as expected for trypsin-like proteases and observed in *Ostrinia nubilalis* and *S. littoralis* (Bernardi et al., 1996; Marchetti et al., 1998). The synthetic trypsin inhibitor benzamidine (Mares-Guia et al., 1981) also inhibited the activity of the membrane-bound proteases of *A. gemmatalis* (Table 1) further confirming their trypsin-like nature, as in other species (Bernardi et al., 1996; Novillo et al., 1999; Zhu & Baker, 1999; Lam et al., 2000).

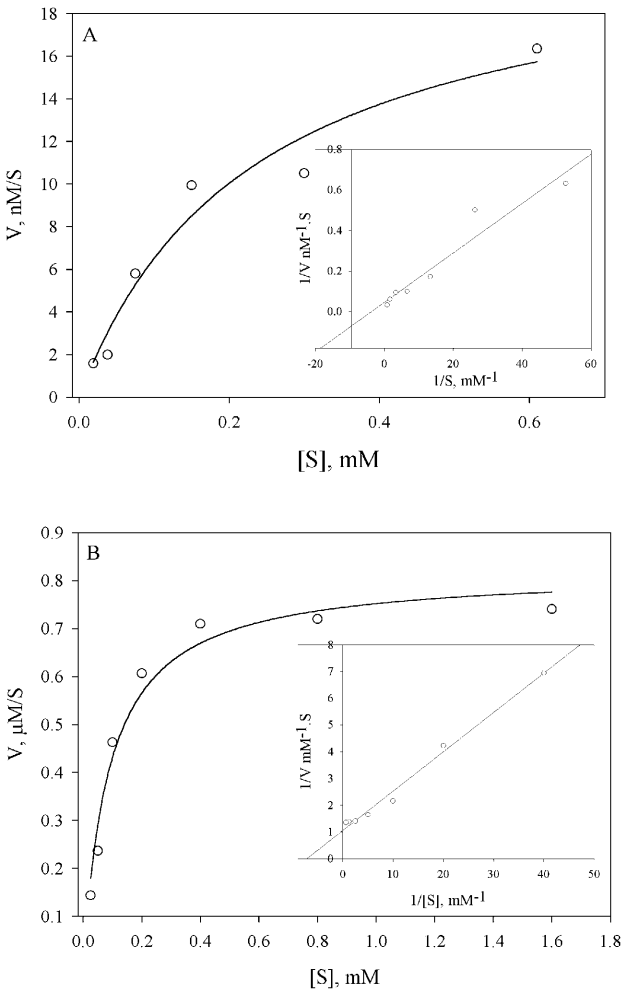

Fig. 5. Michaelis-Menten plot of the membrane-bound trypsin-like protease activity of *A. gemmatalis* midgut using L-BApNA (A) and L-TAME (B) as substrates. Insertion: Lineweaver-Burk plot (double reciprocal). Each symbol represents the mean of three replicates (n = 3).

Kinetic studies

The kinetic parameters of the membrane-bound midgut proteases of *A. gemmatalis* estimated using amide and ester synthetic substrates are given in Table 1. Over the range of substrate concentrations used both amidolytic and esterolytic activity showed the concentration-velocity curve with hyperbolic profile following the Michaelis-Menten kinetic model (Fig. 5). The membrane-bound midgut proteases of *A. gemmatalis* had K_M values of 0.23 mM in the L-BApNA and 95.4 µM in the L-TAME hydrolyses (Table 2).

TABLE 2. Kinetic parameters of the membrane-bound trypsin-like proteases of *A. gemmatalis.*

Substrate	K_M	V_{max}
L-BApNA	0.23 mM	21.8 nM/s
L-TAME	95.4 µM	0.82 µM/s

DISCUSSION

In insects, the processing of precursors and the secretory mechanism of digestive enzymes differ from that found in other animals (Terra & Ferreira, 1994). This study of the process is therefore an important contribution to cell biology and may provide new targets for alternative control methods. The first study of the mechanism of enzyme secretion in Lepidoptera was done using larvae of *Bombyx mori* and indicated that membrane-bound trypsin-like proteases are transported from the tissues to the lumen of the gut where they are solubilized and converted into an active form. Some of the trypsin-like proteases are incorporated in the peritrophic membrane, an extracellular sheath which protects the midgut epithelial cells (Eguchi et al., 1982; Kuriyama & Eguchi, 1985).

Enzymes are bound in cell membranes by means of a hydrophobic peptide or a GPI-anchor. Enzymes bound by hydrophobic peptides are usually solubilized by high (e.g., octylglucoside, CHAPS) or low (e.g., Triton X-100) CMC (critical micellar concentration) detergents and are at least partially released from membranes by the action of papain or trypsin (Hooper & Turner, 1988). Enzymes anchored via GPI are released into solution by high CMC detergents or by GPI-PLC treatment (Hooper & Turner, 1988). Trypsins were found in fractions of ectoperitrophic fluid obtained by differential centrifugation (Ferreira et al., 1994). The detection of proteolytic activity in the insoluble fraction from the midgut of *A. gemmatalis*, after treatment with Triton X-100 and centrifugation at 100,000 *g*, indicates the occurrence of membrane-bound proteases that may be at least partially transferred to the peritrophic membrane.

The membrane-bound digestive proteases of *A. gemmatalis* hydrolyzed both protein and synthetic substrates. The protein hydrolytic activity was assessed using casein as a substrate, which showed a similar kinetic profile to those obtained using the synthetic substrates L-BApNA and L-TAME. Activity towards these substrates was expected of trypsin-like proteases and was observed for the membrane-bound proteases from *A. gemmatalis*. The activity profiles obtained using these substrates differed, which suggests the presence of other proteases, probably cysteine proteases, since L-BApNA is also a substrate for this family of enzymes.

EDTA decreased the membrane-bound activity of *A. gemmatalis* midgut proteases, probably by sequestering Ca^{++} associated with the amino acid residues of the protein structure. At least some insect trypsin-like proteases are likely to have Ca^{++} as a co-factor (Houseman et al., 1989; Wagner et al., 2002), as may be the case for *A. gemmatalis*, where Ca^{++} positively modulate membrane-bound trypsin-like activity in the midgut. These results, however, contrast with those from other insect trypsins, which are unaffected by Ca^{++} (Lewinsky et al., 1977; Johnston et al., 1991; Lemos & Terra, 1992).

The serine protease competitive inhibitor PMSF also inhibited the membrane-bound protease activity of *A. gemmatalis*. TLCK, a trypsin inhibitor, decreased L-BApNA hydrolysis in the present study probably by alkylating the histidine residue of the catalytic triade of these enzymes (Shaw et al., 1965). These results indicate that serine proteases or, more particularly, trypsin-like serine proteases are present as membrane-bound proteases, since they are inhibited by PMSF and TLCK. The stronger inhibition provided by TLCK, a specific trypsin-like inhibitor, than PMSF, a serine protease inhibitor, indicates the occurrence of other serine proteases in the velvetbean caterpillar and protease enzymes, probably cysteine protease, as also previously indicated by the activity profiles of L-BApNA and L-TAME. In addition, Ca^{++} seem to be a co-factor positively modulating the trypsin-like activity, which is inhibited by EDTA. Aprotinin and benzamidine, two well-known trypsin inhibitors (Mares-Guia & Shaw, 1965; Mares-Guia, 1968; Mares-Guia et al., 1981; Bernardi et al., 1996), also inhibited the activity of the membrane bound proteases of *A. gemmatalis* and provide further support for their being trypsin-like serine proteases.

Inhibition of mammalian and insect proteases may differ indicating that the disruption of insect protein digestion when fed on transformed plants containing protease inhibitors requires the prior selection of the appropriate inhibitors for the digestive proteases of the insect pest species to be targeted (Reeck et al., 1997). Protease activity in *A. gemmatalis* was inhibited by aprotinin (the bovine pancreatic trypsin inhibitor, BPTI), which may be useful for transferring the soybean genome aimed at insect resistance. Nevertheless, validation by characterizing *A. gemmatalis* soluble midgut proteases and subsequent feeding trials are needed before attempting crop transformation. In addition, insect resistance to plant protease inhibitors has been reported in several species of insect (Broadway, 1995; Jongsma & Bolter, 1997) and therefore an understanding of the mechanism by which the velvetbean caterpillar enzyme couple with soybean protease inhibitors will aid the selection of inhibitors that will prevent or delay the development of resistance.

The K_M value for L-BApNA hydrolysis was 2.4× higher than that for L-TAME. Therefore the membrane-bound trypsin-like proteases of *A. gemmatalis* show a better binding to L-TAME than to L-BApNA, as reported for bovine trypsin (Nakata & Ishii, 1972; Magalhães-Rocha et al., 1980). This higher efficiency against the ester substrate may be due to the reaction mechanism catalyzed by serine proteases. These enzymes show a slow acylation during their amidolytic activity followed by fast deacylation for product formation; the opposite occurs during esterolytic activity (Inagami, 1972; Fastrez & Fersht, 1973). Therefore the rate-limiting step in amide hydrolysis by these trypsin-like enzymes is acylation, while in ester hydrolysis it is deacylation.

The induction of host plant protease inhibitors via the lipoxygenase (LOX) pathway is a defensive response to attack by insects and pathogens (Farmer & Ryan, 1992). This response to insect injury can inhibit the activity of insect midgut proteases leading to an hyperproduction of proteolytic enzymes and reduced availability of essential amino acids for insect growth and development

(Broadway & Duffey, 1986; Broadway, 1995). Plant-induced protease inhibitors can inhibit insect midgut proteases leading to death and such a control method may be exploited in one of two ways against insect-pests like *A. gemmatalis*. First by applying peptides or mimetic peptides, which are potent inhibitors of insect midgut proteases, like insecticides. Secondly, by transforming the soybean genome for increased expression of a potent protease inhibitor. Future characterization of purified midgut proteases of *A. gemmatalis* and the assessment of their relevance in soybean-*A. gemmatalis* interactions will direct future efforts to develop more effective control methods.

ACKNOWLEDGEMENTS. The financial support provided by the CAPES Foundation, National Council of Scientific and Technological Development (CNPq) and the Minas Gerais State Foundation for Research Aid (FAPEMIG) is greatly appreciated. The comments and suggestions provided by three anonymous referees are also greatly appreciated and acknowledged.

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Received July 1, 2004; revised and accepted November 29, 2004