Brazilian Journal of Medical and Biological Research (2005) 38: 1633-1641
ISSN 0100-879X

Perspectives of digestive pest control with proteinase inhibitors that mainly affect the trypsin-like activity of *Anticarsia gemmatalis* Hübner (Lepidoptera: Noctuidae)

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Abstract

The present study describes the main characteristics of the proteolytic activities of the velvetbean caterpillar, *Anticarsia gemmatalis* Hübner, and their sensitivity to proteinase inhibitors and activators. Midguts of last instar larvae reared on an artificial diet were homogenized in 0.15 M NaCl and centrifuged at 14,000 *g* for 10 min at 4ºC and the supernatants were used in enzymatic assays at 30ºC, pH 10.0. Basal total proteolytic activity (azocasein hydrolysis) was 1.14 ± 0.15 absorbance variation min^{-1} mg $protein^{-1}$, at 420 nm; basal trypsin-like activity (N-benzoyl-L-arginine-p-nitroanilide, BApNA, hydrolysis) was 0.217 ± 0.02 mmol p-nitroaniline min^{-1} mg $protein^{-1}$. The maximum proteolytic activities were observed at pH 10.5 using azocasein and at pH 10.0 using BApNA, this pH being identical to the midgut pH of 10.0. The maximum trypsin-like activity occurred at 50ºC, a temperature that reduces enzyme stability to 80 and 60% of the original, when pre-incubated for 5 and 30 min, respectively. Phenylmethylsulfonyl fluoride inhibited the proteolytic activities with an IC_{50} of 0.39 mM for azocasein hydrolysis and of 1.35 mM for BApNA hydrolysis. Benzamidine inhibited the hydrolysis with an IC_{50} of 0.69 and 0.076 mM for azocasein and BApNA, respectively. The absence of cysteine-proteinases is indicated by the fact that 2-mercaptoethanol and L-cysteine did not increase the rate of azocasein hydrolysis. These results demonstrate the presence of serine-proteinases and the predominance of trypsin-like activity in the midgut of Lepidoptera insects, now also detected in *A. gemmatalis*, and suggest this enzyme as a major target for pest control based on disruption of protein metabolism using proteinase inhibitors.

Key words
- *Anticarsia gemmatalis* (Lepidoptera: Noctuidae)
- Azocasein hydrolysis
- BApNA hydrolysis
- Proteinase inhibitors
- Protein digestion
- Serine-proteinases

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Presented at the XVIII Annual Meeting of the Federação de Sociedades de Biologia Experimental, Curitiba, PR, Brazil, August 27-30, 2003.

Part of a Master's thesis presented by F.A. Dörr to the Post-graduation Program in Toxicologic Biochemistry, Universidade Federal de Santa Maria, RS, Brazil.

F.A. Dörr and J.F. Lima-Garcia were recipients of CAPES fellowships.

Received April 6, 2005
Accepted August 30, 2005

Introduction

As soybean becomes a major world food source, the expansion of its cultivation is likely to increase crop vulnerability to insect pests (1). Among these pests, the velvetbean caterpillar, *Anticarsia gemmatalis* Hübner (Lepidoptera: Noctuidae), is the most important defoliator (2). The larvae have caused economic losses in the South of the United States and in Latin America (1), with the applications of insecticides being required for their control. In Brazil, the successful implementation of an Integrated Pest Management program to control this insect involving the use of the nucleopolyhedrovirus of *A. gemmatalis* (3) has led to great economic and environmental savings (4).

The development of crops resistant to insect pests using *Bacillus thuringiensis* (5) is considered to be an essential component of this Integrated Pest Management program. Genetic engineering enables the transfer of novel genes to economically important plants in order to produce resistant cultures (6). Genes encoding inhibitors that target digestive proteolytic enzymes of herbivore insects are candidates for plant transformation (7). Proteinase inhibitors are found in a variety of plant species, where they are believed to have a role in defense against pests (8). The potential use of these inhibitors as resistance factors has been demonstrated (9-13). The stable binding of these inhibitors to digestive proteinases causes a delay in protein digestion. The induction of protein synthesis and consequently the pernicious hypersecretion of digestive enzymes occurring to compensate for this inhibition lead to the depletion of essential amino acids, and this negative amino acid balance may result in retarded development and eventually in death (14).

Proteinases are divided into four classes based on the amino acid or metal ion involved in the catalytic site which cleaves peptide bonds: serine, cysteine, aspartic, and metalloproteases. All these classes of proteases have been demonstrated in insects (15). Except for some hemipteran and coleopteran species, in most insect groups initial protein digestion relies on serine-proteinases, particularly trypsin and chymotrypsin (15). In Lepidoptera larvae, these enzymes have been shown to have a high pH optimum, which is consistent with the alkaline conditions in their midgut (16).

Digestive enzymes have been selected as targets for pest control methods and their participation in the activation of *Bacillus thuringiensis* endotoxins, as well as their efficacy against *A. gemmatalis* have also been demonstrated (17,18). Although there is a vast literature dealing with proteolytic enzymes in Lepidoptera larvae, few reports are available regarding *A. gemmatalis*. Since proteolytic enzymes are responsible for protein digestion and consequently for the supply of amino acids needed for development, knowledge about the activity of these enzymes and their sensitivity to inhibitors is fundamental for future programs of pest control. In the present paper, we report the partial characterization of proteolytic activities in the midgut of *A. gemmatalis* larvae.

Material and Methods

Material

Sulfanilamide-azocasein, N-benzoyl-L-arginine-p-nitroanilide (BApNA), N-benzoyl-L-tyrosine-p-nitroanilide (BTpNA), phenylmethylsulfonyl fluoride (PMSF), benzamidine (BZD), and iodoacetic acid were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Reagen (Rio de Janeiro, RJ, Brazil) supplied ethylenediaminetetraacetic acid (EDTA), L-cysteine, dimethyl sulfoxide and bovine serum albumin fraction V. All other reagents used were of analytical grade.

Insect rearing

The study was conducted in the Labora-

tory of Toxicologic Biochemistry, Department of Chemistry, Universidade Federal de Santa Maria, Santa Maria, RS, Brazil, during the period from March 2002 to March 2004.

Eggs of *A. gemmatalis* were obtained from the Soybean Research National Center (Londrina, PR, Brazil), a unit of the Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA). Larvae were reared on an artificial diet with a 14-h photoperiod at 25 ± 2ºC and 50 ± 10% relative humidity, as proposed by Hoffmann-Campo et al. (19).

Sample preparation

Actively feeding last instar larvae were chilled and their midguts were dissected free over ice. After homogenization in cold 0.15 M NaCl (1:10, w/v), the material was centrifuged at 14,000 *g* for 10 min at 4ºC. The resulting supernatants were pooled, divided into aliquots and frozen at -20ºC until required. To obtain gut wall and lumen content, after dissection, midguts were split lengthwise and the walls were washed with cold 0.15 M NaCl; the lumen content and gut wall were homogenized separately (1:10, w/v) and then centrifuged as described for total midgut. The samples were stored at -20ºC, since no alteration of both total proteolytic activity and trypsin-like activity was verified by freezing. A pool of different larval midguts was used for each enzymatic assay.

pH estimation

The pH of gut content was estimated using narrow range pH indicator paper (Merck, Darmstadt, Germany). Immediately after dissection, midguts were split lengthwise and the papers introduced in the middle region. Measurements were obtained from four midguts, and each determination was carried out in triplicate. The pH paper was compared to the values obtained using standard solutions of known pH (20).

Enzymatic assays

Total proteolytic activity was determined by the method of Marchetti et al. (21), with some modifications. Typically, the sample (15 µL containing 10-20 µg protein) and 0.1 M Tris (21-24) buffer, pH 10.0 (360 µL), were pre-incubated for 5 min at 30ºC before the addition of 20 µL 2% azocasein (w/v, in glass-distilled water). After 20 min of incubation, the reaction was stopped using 400 µL 10% trichloroacetic acid (w/v). Tubes were kept on ice for 10 min and then centrifuged at 5,000 *g* for 5 min; 500-µL aliquots of the supernatant were withdrawn and mixed in a cuvette with 500 µL 1 M NaOH and absorbance at 420 nm was determined. Blanks (test tubes without samples) were run in all cases. The basal activity obtained in the absence of inhibitor or activator at 30ºC and pH 10.0 was 1.14 ± 0.15 variation in absorbance at 420 nm (ΔAbs) min^{-1} mg $protein^{-1}$.

Trypsin-like activity was assayed using the chromogenic substrate BApNA (25). Briefly, 0.1 M Tris buffer, pH 10.0 (1.35 mL), and the sample (10 µL containing 5-15 µg protein) were pre-incubated for 5 min at 30ºC before the addition of 0.2 mL 7.8 mM BApNA (in 13% dimethyl sulfoxide; 1 mM final concentration) to start the reaction. After 15 min of incubation, the reaction was stopped with 0.75 mL 30% acetic acid and absorbance was measured at 410 nm (ε = 8800 M^{-1} cm^{-1}). Assays were carried out in triplicate and appropriate blanks were run in all cases. The basal specific activity determined in the absence of inhibitor or activator at 30ºC and pH 10.0 at 410 nm was 0.217 ± 0.02 mmol p-nitroaniline formed min^{-1} mg $protein^{-1}$.

Chymotrypsin-like activity was determined using BTpNA according to the method of Christeller et al. (26). Assays containing 1 mM BTpNA and 1.35 mL 0.1 M Tris buffer, pH 10.0 (21) at 410 nm, were carried out as previously described for trypsin-like activity.

Effect of pH on enzymatic activities

The effect of pH on azocasein hydrolysis was evaluated at pH 7.0, 8.0, 9.0, 9.5, 10.0, 10.5, and 11.0 for 0.1 M Tris buffer and at pH 8.0, 9.0, 9.5, 10.0, 10.5, and 11.0 for 0.1 M glycine buffer. BApNA hydrolysis was evaluated at pH 8.0, 8.5, 9.0, 9.5, 10.0, 10.5, and 11.0 for both buffers. Buffers were always prepared at the concentration of 0.1 M (21,23,24) and the pH was corrected by the addition of 1 M HCl or 1 M NaOH to reach the pH values desired for both Tris and glycine. Activities were assayed as described above.

Effect of temperature

Briefly, 0.1 M Tris buffer and samples were pre-incubated for 5 min at given temperatures before the addition of the substrate to start the reaction. Assays were carried out as previously detailed. To assess the stability of the enzyme, the buffer and the sample were pre-incubated at given temperatures for 5 or 30 min (21). Test tubes were then transferred to a water bath at 30ºC. After 2 min, time enough to reach thermoequilibration, the activity was assayed as previously described.

Inhibition studies

In order to identify the major proteinases present in the midgut of *A. gemmatalis*, inhibitors and activators of different mechanistic classes were tested. In all cases, samples (15 µL containing 10-20 µg protein), 0.1 M Tris buffer (360 µL) and inhibitor/activator were pre-incubated together for 10 min (20 min for PMSF) before the addition of substrate to start the reaction.

To investigate the effects of PMSF on proteinase activities the assays were carried out at 30ºC in 0.1 M Tris, pH 7.0, because this compound precipitates in alkaline medium (27,28). The inhibitor was tested at concentrations ranging from 0 to 0.5 mM for total proteolytic activity and at concentrations ranging from 0 to 2.5 mM for trypsin-like activity. The effects of BZD on both activities were tested using inhibitor concentrations ranging from 0 to 0.5 mM for total proteolytic activity and from 0 to 0.04 mM for trypsin-like activity, in 0.1 M Tris, pH 10.0, at 30ºC. IC_{50} values for both inhibitors were calculated by the method of Dixon and Webb (29).

The effects of EDTA, Ca^{2+} and Mg^{2+} (10 mM) and iodoacetic acid, L-cysteine and 2-mercaptoethanol (1 mM) were also investigated at appropriate pH as detailed in Table 1 and preincubated as described earlier. Data are reported as percent relative activity compared to the respective controls (samples without inhibitors/activators).

Table 1. Effect of inhibitors and activators on azocasein and BApNA hydrolysis.

Inhibitor/activator	Concentration (mM)	Azocasein (% control)	BApNA (% control)
EDTA	10	93	98
Iodoacetic acid	1	96	98
L-cysteine	1	72	96
2-Mercaptoethanol	1	94	100
Ca^{2+}	10	120	130
Mg^{2+}	10	136	141

Data are reported as means of three or four independent determinations. Standard errors were less than 10% of the means. Assays were carried out in 0.1 M Tris, pH 7.0, at 30ºC using 0.1% azocasein or 1 mM BApNA as substrates. Ca^{2+} and Mg^{2+} assays were carried out in 0.1 M Tris, pH 10.0. BApNA = N-benzoyl-L-arginine-p-nitroanilide; EDTA = ethylenediaminetetraacetic acid.

Protein estimations

Protein measurements in midgut extracts were done according to the dye-binding method of Bradford (30) using bovine serum albumin fraction V as standard.

Results

Proteinase activities

The pH of the midgut content of *A. gemmatalis* larvae ranged from 9.5 to 10.0. Using BApNA as substrate, 82% of trypsin-like activity was observed in midgut lumen

content and 18% in the midgut walls (Table 2). These results are consistent with the view that trypsin is a soluble enzyme secreted by midgut cells into the lumen during digestion (31).

Total proteolytic activity of midgut extracts in terms of azocasein hydrolysis increased with pH increase from 7.0 to 11.0, with a maximum rate at pH 10.5 for both Tris and glycine buffers. Only 16% of the maximum activity was observed in Tris, pH 7.0, and glycine, pH 8.0, buffers. Trypsin-like activity showed a maximum rate of BApNA hydrolysis at pH 10.0 when Tris buffer was used. However, the optimum pH shifted slightly to pH 10.5 with glycine buffer (Figure 1).

Attempts to detect chymotrypsin-like activity using BTpNA as substrate failed. It is known that some insect chymotrypsins only cleave synthetic substrates with more than one amino acid residue (13,27,32).

Effect of temperature

Proteolytic activity on the basis of BApNA hydrolysis was temperature dependent and maximum activity was obtained at 50ºC (Figure 2). The thermal stability of trypsin-like activity in the midgut samples pre-incubated for 5 min remained unchanged at temperatures up to 40ºC. However, pre-incubation for 30 min at temperatures of 40ºC or more sharply reduced the proteolytic activity, which was almost abolished at 60ºC (Figure 3).

Inhibition studies

PMSF inhibited the proteolytic activities with an IC_{50} of 0.39 mM for azocasein hydrolysis and of 1.35 mM for BApNA hydrolysis (Figure 4). BZD also inhibited the hydrolysis of both substrates (Figure 5), with an IC_{50} of 0.69 and 0.076 mM for azocasein and BApNA, respectively.

The cysteine-proteinase inhibitor iodoacetic acid had no significant effect on total proteolytic activity (only 4% inhibition; Table 1). The absence of cysteine-proteinases is confirmed by the fact that 2-mercaptoethanol and L-cysteine did not enhance azocasein hydrolysis. The inhibition of proteolytic activity by L-cysteine may be a consequence of interactions with the substrate, since higher concentrations of L-cysteine precipitated azocasein. EDTA, a metal-chelating agent, did not inhibit azocasein hydrolysis to a great extent, suggesting that metalloproteinase is not a major constituent of midgut extracts. Trypsin-like activity was

Table 2. Compartmentalization of trypsin-like enzymes in midgut of *Anticarsia gemmatalis* larvae.

Site	Specific activity (mmol p-nitroaniline min^{-1} mg^{-1})	Relative activity (%)
Gut walls	0.065 ± 0.002	18
Gut contents	0.300 ± 0.006	82

Data are reported as means ± SEM of three independent determinations.

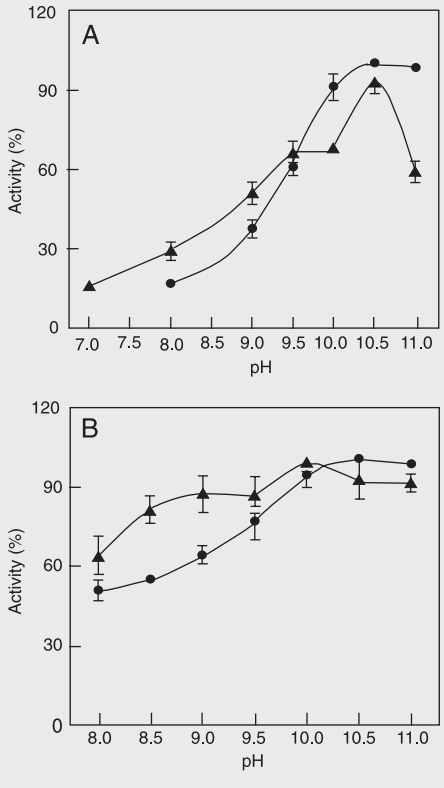

Figure 1. pH curves of proteinase activities from the larval midgut of *Anticarsia gemmatalis*. *A*, Effect of pH on the hydrolysis of 0.1% azocasein. *B*, Effect of pH on the hydrolysis of 1 mM N-benzoyl-L-arginine-p-nitroanilide. Buffers used were 0.1 M Tris (triangles) and 0.1 M glycine (circles). Data are reported as means ± SEM of four independent determinations.

not affected by any of the thiol reagents tested, i.e., iodoacetic acid, 2-mercaptoethanol and L-cysteine. EDTA also had no effect. Ca^{2+} and Mg^{2+} enhanced azocasein and BApNA hydrolysis around 30% (Table 1).

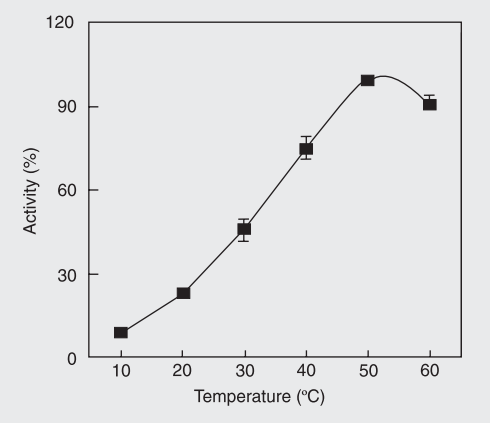

Figure 2. Effect of temperature on trypsin-like activity from the larval midgut of *Anticarsia gemmatalis*. N-benzoyl-L-arginine-p-nitroanilide (1 mM) hydrolysis was measured at given temperatures in 0.1 M Tris, pH 10.0, for 15 min. Data are reported as means ± SEM of three independent determinations.

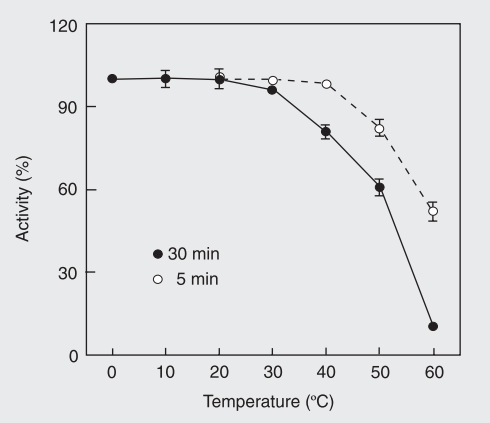

Figure 3. Thermal stability of trypsin-like enzymes from the larval midgut of *Anticarsia gemmatalis*. Samples were pre-incubated at given temperatures in 0.1 M Tris, pH 10.0, for 5 min (open circles) and 30 min (filled circles). After 2 min of thermoequilibration at 30°C, N-benzoyl-L-arginine-p-nitroanilide (1 mM) was added to start the reaction (15 min). Data are reported as means ± SEM of three independent determinations.

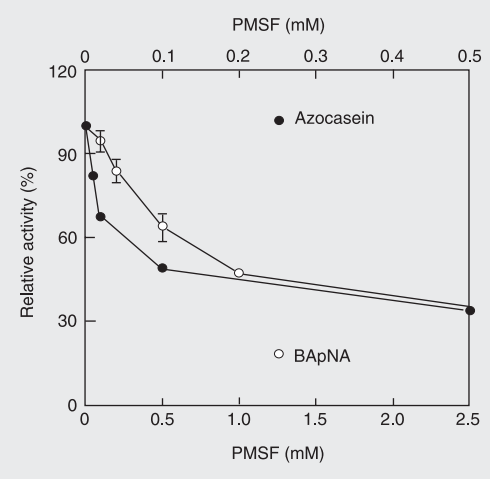

Figure 4. Inhibition of trypsin-like and total proteolytic activities from the larval midgut of *Anticarsia gemmatalis* by phenylmethylsulfonyl fluoride (PMSF). Assays were carried out in 0.1 M Tris, pH 7.0, at 30°C. Data are reported as means ± SEM of three independent determinations. BApNA = N-benzoyl-L-arginine-p-nitroanilide.

Discussion

The alkaline pH (9.5-10.0) of the midgut of *A. gemmatalis* is similar to that reported in other investigations of Lepidoptera larvae, whose midgut content had a pH of 8.0 to 12.0 (24,27,31,33). Teo et al. (34) have reported a pH of 7.6 for the midgut content of *A. gemmatalis* larvae. This discrepancy may be due to the different methods used by these investigators to estimate midgut pH that disrupted the regulatory process necessary to maintain it (35). The hydrolysis of the substrates, BApNA and azocasein, is influenced by the buffers used in pH studies. The effects of buffer systems on enzymatic assays have been reported previously for insect digestive enzymes (27,36).

The pH value of 10.5 for maximal proteolytic activity using both buffers corresponded to the second peak of pH 10.0 reported for this insect by Teo et al. (34), who also found a peak of activity at pH 7.6. The alkaline optimum pH for azocasein hydrolysis strongly suggests the presence of serine-proteinases in midgut extracts, confirming the occurrence of protein digestion in this insect (13).

The trypsin-like activity was strongly temperature dependent and was similar to that reported for other Lepidoptera larvae (21, 27,37). The effect of temperature on the metabolism of these insects and consequently on their life cycle is well known (38). Leppla et al. (39) have reported that, at 21.1ºC, larvae require almost twice the time to complete their development to pupae when compared to larvae reared at 32.2ºC under laboratory conditions.

The principal catalytic activities responsible for proteolysis were determined using inhibitors and activators of different mechanistic classes. The presence of serine-proteinases was confirmed by the fact that PMSF (Figure 4), an inhibitor considered to be diagnostic for this class of proteinase, caused 50% inhibition of azocasein hydrolysis at

0.39 mM and 50% inhibition of BApNA hydrolysis at 1.35 mM. This small difference in IC_{50} between the two activities suggests an important contribution of trypsin-like activity to the total proteolytic activity detected on azocasein hydrolysis.

To confirm this participation we also tested the effect of BZD, an inhibitor considered to be characteristic for trypsin, on total proteolytic and trypsin-like activities (Figure 5). The results demonstrated a pronounced difference in the sensitivities of enzymatic activities to the inhibitor, since an inhibitor concentration about 10 times higher was necessary to inhibit azocasein hydrolysis (IC_{50} = 0.69 mM) compared to BApNA hydrolysis (IC_{50} = 0.076 mM). These results agree with those reported by Oliveira et al. (22) who detected a higher sensitivity of the proteolytic activity of the partially purified fraction to BZD than to PMSF. The absence of important effects of other compounds tested rules out a strong participation of other proteinase activities. As a whole, these results demonstrate the presence of trypsin-like enzymes in midgut extracts due to their high sensitivity to the inhibitor, high rate of BApNA hydrolysis, and occurrence mainly in midgut content (Table 2). In addition, these results agree with data reported by other investigators who detected the presence of trypsin-like activity in several species of Lepidoptera (15,16), as well as in the *A. gemmatalis* species (22).

In contrast with other reports, which showed that trypsin-like enzymes from insect sources do not need divalent cations to reach their maximum activities (25, 35), we observed that calcium and magnesium activated BApNA and azocasein hydrolysis. Similar results were obtained by Oliveira et al. (22) using Ca^{2+}, and by Houseman et al. (40) and Bernardi et al. (37), who reported that Mg^{2+} was the best activator of trypsin-like activity when crude midgut extracts were assayed. Interestingly, the addition of EDTA to the reaction medium prevented the activation effects (data not shown).

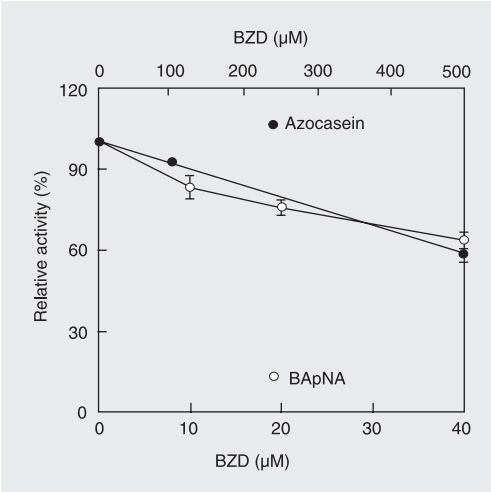

Figure 5. Inhibition of trypsin-like and total proteolytic activities from the larval midgut of *Anticarsia gemmatalis* by benzamidine (BZD). Assays were carried out in 0.1 M Tris, pH 10.0, at 30°C. Data are reported as means ± SEM of three independent determinations. BApNA = N-benzoyl-L-arginine-p-nitroanilide.

The present study was carried out using crude extracts of midgut obtained after homogenate centrifugation and after partial characterization. Thus, it was possible to demonstrate that we were working with a mixture of proteinases with features similar to those of trypsin. The present study shows the predominance of trypsin-like proteases in the midgut of *A. gemmatalis*, which could be targeted with specific inhibitors to achieve insect control.

Acknowledgments

The authors gratefully acknowledge EMBRAPA - Soja (Londrina, PR, Brazil), especially Dr. F. Moscardi, for supplying the eggs of *Anticarsia gemmatalis*.

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