



Acta Scientiarum. Biological Sciences

ISSN: 1679-9283

eduem@uem.br

Universidade Estadual de Maringá

Brasil

Torres Cruz Reis, Denise; Rennó dos Mares-Guia, Thiago; Silvano de Oliveira, Jamil; Martins Costa Santos, Alexandre; Matos Santoro, Marcelo; Almeida Oliveira, Maria Goreti

Purification of a membrane-bound trypsin-like enzyme from the gut of the velvetbean caterpillar  
(*Anticarsia gemmatalis* Hübner)

Acta Scientiarum. Biological Sciences, vol. 34, núm. 3, julio-septiembre, 2012, pp. 263-270

Universidade Estadual de Maringá

.png, Brasil

Available in: <http://www.redalyc.org/articulo.oa?id=187123686003>

- How to cite
- Complete issue
- More information about this article
- Journal's homepage in redalyc.org

redalyc.org

Scientific Information System

Network of Scientific Journals from Latin America, the Caribbean, Spain and Portugal

Non-profit academic project, developed under the open access initiative



## Purification of a membrane-bound trypsin-like enzyme from the gut of the velvetbean caterpillar (*Anticarsia gemmatilis* Hübner)

Denise Torres Cruz Reis<sup>1\*</sup>, Thiago Rennó dos Mares-Guia<sup>2</sup>, Jamil Silvano de Oliveira<sup>3</sup>, Alexandre Martins Costa Santos<sup>3,4</sup>, Marcelo Matos Santoro<sup>4</sup> and Maria Goreti Almeida Oliveira<sup>1</sup>

<sup>1</sup>Departamento de Bioquímica e Biologia Molecular, Universidade Federal de Viçosa, Av. P. H. Rolfs, s/n, 36570-000, Viçosa, Minas Gerais, Brazil.

<sup>2</sup>Departamento de Bioquímica, Núcleo de Terapia Celular e Molecular, Instituto de Química, Universidade de São Paulo, São Paulo, São Paulo, Brazil.

<sup>3</sup>Departamento de Bioquímica e Imunologia, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil. <sup>4</sup>Departamento de Ciências Fisiológicas, Universidade Federal do Espírito Santo, Vitória, Espírito Santo, Brazil. \*Author for correspondence. E-mail: denisetcr@yahoo.com.br

**ABSTRACT.** Disruption of protein digestion in insects by specific endoprotease inhibitors is being regarded as an alternative to conventional insecticides for pest control. To optimize the effectiveness of this strategy, the understanding of the endoprotease diversity of the target insect is crucial. In this sense, a membrane-bound trypsin-like enzyme from the gut of *Anticarsia gemmatilis* fifth-instar larvae was purified. Non-soluble fraction of the gut extract was solubilized with 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) and subjected to a p-aminobenzamidine affinity chromatography followed by anion-exchange chromatography. The yield of the purified enzyme was 11% with a purification factor of 143 and a final specific activity of 18.6  $\mu\text{M min}^{-1} \text{mg}^{-1}$  protein using N- $\alpha$ -benzoyl-L-Arg-p-nitroanilide (L-BApNA) as substrate. The purified sample showed a single band with proteolytic activity active and apparent molecular mass of 25 kDa on SDS-PAGE. Molecular mass determined by MALDI-TOF mass spectrometry was 28,632  $\pm$  26 Da. Although the low recovery and the difficulties in purifying large enzyme amounts limited its further characterization, the results contribute for the understanding of the proteases present on *A. gemmatilis* gut, which are potential targets for natural or specifically designed protease inhibitors.

**Keywords:** insect-plant interaction, soybean pest control, trypsin-like enzyme purification.

## Purificação de uma enzima “tipo tripsina” não-solúvel do intestino da lagarta da soja (*Anticarsia gemmatilis* Hübner)

**RESUMO.** Comprometer a digestão de proteínas dos insetos pelo uso de inibidores específicos de endoproteases tem sido amplamente estudado como um método de controle de pragas alternativo ao uso dos inseticidas convencionais. No processo de otimização desta estratégia, o conhecimento da diversidade das endoproteases do inseto alvo torna-se crucial. Neste sentido, uma enzima “tipo-tripsina” ligada à membrana obtida do intestino de larvas do 5º instar de *A. gemmatilis* foi purificada. A fração insolúvel do extrato do intestino foi solubilizada com 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) e submetida à uma cromatografia de afinidade em uma coluna de p-aminobenzamidina, seguida por uma cromatografia de troca-aniónica. O rendimento da enzima purificada foi de 11% com fator de purificação de 143 e uma atividade específica final de 18.6  $\mu\text{M min}^{-1} \text{mg}^{-1}$  de proteína usando N- $\alpha$ -benzoyl-L-Arg-p-nitroanilide (L-BApNA) como substrato. Após a separação da amostra purificada por SDS-PAGE e incubação subsequente com caseína, uma única banda ativa com massa molecular aparente de 25 kDa foi observada. A massa molecular determinada por espectrometria de massa (MALDI-TOF) foi de 28,632  $\pm$  26 Da. O baixo rendimento e as dificuldades em purificar grandes quantidades da enzima limitaram caracterização complementar. A observação desta enzima, no entanto, é mais uma etapa no processo de conhecer as endoproteases presentes no intestino de *A. gemmatilis*, alvos potenciais de inibidores de proteases naturais ou especificamente projetados.

**Palavras-chave:** interação planta-inseto, controle da lagarta da soja, purificação de enzima “tipo-tripsina”.

### Introduction

The velvetbean caterpillar, *A. gemmatilis* Hübner (Lepidoptera: Noctuidae) is one of the main pests of soybean crop, causing important yield losses due to herbivorous attack, which can cause a 100% defoliation (PRAÇA et al., 2006). Soybean has a great social and economical value worldwide,

and the understanding of some aspects of *A. gemmatilis* behaviour, physiology and plant-insect interactions is crucial for the development of crop protection methods which does not rely exclusively on the use of agrochemicals. Implementation of an Integrated Pest Management program to control this insect using the nucleopolyhedrovirus of

*A. gemmatalis* (AgMNPV) has already led to great economic and environmental savings (MORALES et al., 2001). A promising approach to integrate management strategies is the development of insect-resistant transgenic plants expressing inhibitors that target digestive proteolytic enzymes (ZAVALA et al., 2004).

Interfering with pest protein digestion by expressing protease inhibitors is a strategy widely employed by plants to defend themselves against pest attack (FERRY et al., 2006). It has been demonstrated that soybean also employs inducible protease inhibitors as defense mechanism against *A. gemmatalis* (FORTUNATO et al., 2007). The protease inhibitors (PIs) increase mortality and retard growth and development of insects when added to artificial diets (BROADWAY, 1995) or expressed in transgenic plants (ZAVALA et al., 2004). However, insects can adapt to PIs presence by different mechanisms (BOWN et al., 1997; DE LEO et al., 2001; JONGSMA; BOLTER, 1997; JONGSMA et al., 1995; VOLPICELLA et al., 2003) and it turns out that an understanding of digestive enzymes of the target insect is essential to plan successful and sustainable strategies using PIs (CHRISTOU et al., 2006; DE LEO et al., 2001; FERRY et al., 2006; TELANG et al., 2005; VOLPICELLA et al., 2003).

The dominant mechanistic class of digestive protease in Lepidoptera is serine-protease, particularly trypsin (EC 3.4.21.4), which is involved in the initial phases of protein digestion and have been implicated in the adaptation of lepidopteran insects to plant proteases inhibitors (MAZUNDAR-LEIGHTON et al., 2000; TERRA; FERREIRA, 1994). The trypsins isolated from the midgut of various insects typically exhibit relative molecular mass (Mr) from 20,000 to 35,000 and alkaline pH optima (TERRA; FERREIRA, 1994). Although trypsins have high sequence similarity, the insect trypsins differ in responses to protein inhibitors and in their specificities to substrates (BOWN et al., 1997; BROADWAY, 1997; TERRA; FERREIRA, 1994; TERRA; FERREIRA, 2005). Additionally, the secretion route of trypsin, which is common among vertebrates, may occur in a different form in Lepidoptera (LEMOES; TERRA, 1992). Evidences that in these insects the soluble trypsin is derived from a trypsin form that is associated with vesicle membranes led to a model for trypsin secretion in larval midgut (JORDÃO et al., 1999). Membrane-bound trypsin-like activity has already been reported in gut extracts from *A. gemmatalis*, but no further purification was attempted (OLIVEIRA et al., 2005;

PEREIRA et al., 2005; XAVIER et al., 2005). Considering the economical relevance of soybean culture, the damages caused by *A. gemmatalis* attack and the fundamental role of trypsin-like enzymes in these insect digestion, analysis of biochemical aspects of its digestive processes involving enzyme characterization is important to go further on the development of pest control methods acting through the digestive system, especially based on disruption of protein metabolism using proteinase inhibitors.

In this context, the goal of this work was the purification and partial characterization of a membrane-bound trypsin-like enzyme from the gut of *A. gemmatalis* larvae.

## Material and methods

### Insects culture

A laboratory population of *A. gemmatalis* was reared in an artificial diet described by Hoffman-Campo et al. (1985), maintained at  $25 \pm 5^\circ\text{C}$ ,  $70 \pm 10\%$  of relative humidity and 14:10h light : dark photoperiod.

### Membrane-bound enzyme extract preparation

*A. gemmatalis* fifth-instar larvae were chilled on ice and their guts were dissected out in 1 mM HCl. The dissected guts were submitted to a series of nitrogen freezing and thawing at  $37^\circ\text{C}$  water bath, followed by centrifuging at 100,000 g for 45 minutes at  $4^\circ\text{C}$ . The resulting supernatant was discarded and the corresponding pellet was suspended in 1 mM HCl containing 0.5% (w v<sup>-1</sup>) CHAPS. After incubation for 16h at  $4^\circ\text{C}$  the resuspended pellet was centrifuged at 44,800 g for 60 min. at  $4^\circ\text{C}$ . The supernatant was collected, concentrated using a stirred ultrafiltration cell 8400 (Millipore) with a 10 kDa membrane cut-off and stored frozen at  $-20^\circ\text{C}$ . This membrane-bound enzyme extract (MBEE) was used in the enzyme assays and submitted to purification.

### Enzyme assays and protein determination

Trypsin-like activities were assayed in 1 mL reaction volumes using synthetic substrates L-BAPnA, at a final concentration of 0.25 mM in 0.1 M tris-HCl pH 9.0 buffer. The buffer (850  $\mu\text{L}$ ) and the substrate (50  $\mu\text{L}$ ) were pre-incubated for 5 minutes at  $35^\circ\text{C}$  and the reaction was started by the addition of MBEE sample (100  $\mu\text{L}$ ). After incubation for 15 minutes at  $35^\circ\text{C}$ , the reaction was stopped by the addition of 250  $\mu\text{L}$  of 60% (v v<sup>-1</sup>) acetic acid. Rates were adjusted to  $\mu\text{moles}$  of substrate hydrolyzed using the extinction coefficient for pNA at 410 nm, which is  $8800 \text{ M}^{-1} \text{ cm}^{-1}$ . Protein was determined according to Bradford (1976) using bovine serum albumin (BSA) as a standard.

### Enzyme purification

A MBEE sample obtained from 200 guts was loaded onto a p-aminobenzamidine-agarose (2.5 mL) column (Sigma) equilibrated with 0.05 M tris-HCl buffer pH 7.5, containing 0.5 M NaCl at 4°C. The column was washed with the same buffer and bound proteins were eluted with 0.05 M glycine-HCl buffer, pH 3.0. The elution flow rate was 1 mL min.<sup>-1</sup> and 1.5 mL fractions were collected. Eluted fractions were monitored at 280 nm and analyzed for proteolytic activity using BApNA as substrate (as described previously). Active fractions were pooled, adjusted to pH 8.6 by the addition of 0.1 M NaOH, and loaded onto a Resource Q (6.4 x 30 mm) column in a FPLC system (Pharmacia LKB Biotechnology), equilibrated with 0.05 M glycine-HCl buffer pH 8.6. Elution was carried out with a 30 min., gradient of 0-1 M NaCl, in the same buffer, at room temperature. The flow rate was 1 mL min.<sup>-1</sup> and 1 mL fractions were collected and adjusted to pH 3 with 1 M formic acid solution. Most active fractions were pooled, stored at -20°C and named partially purified sample (PPS).

### SDS-PAGE and casein zymogram

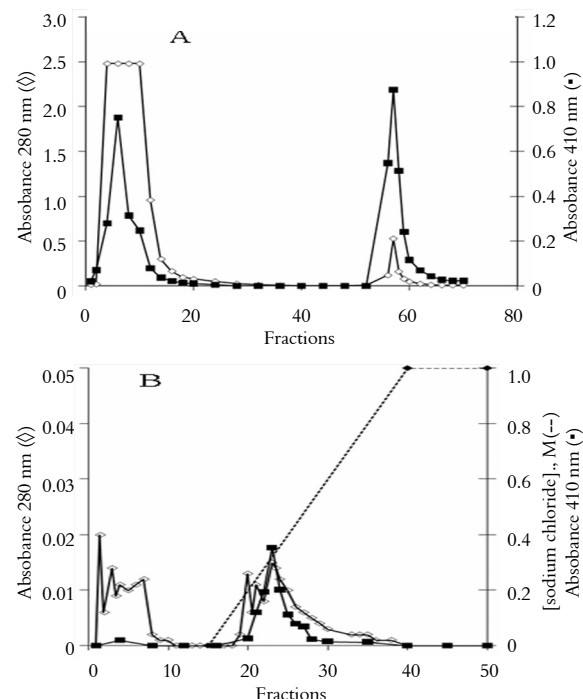
The MBEE and the PPS were subjected to SDS-PAGE on 12.5% (w v<sup>-1</sup>) gels. For *in gel* detection of proteolytic activity, after electrophoresis the gel was immersed in 50 mM Tris-HCl buffer pH 8 for 30 min. at 4°C, with gentle agitation, in order to decrease de SDS concentration. Subsequently, the gel was soaked into a 2% casein solution in 0.05 M Tris-HCl pH 7.5 and incubated for 30 - 90 minutes (GARCIA-CARREÑO et al., 1993). The gel was stained with Coomassie Brilliant Blue R-250 and destained with 40% (v v<sup>-1</sup>) ethanol in 10% (v v<sup>-1</sup>) acetic acid.

### Reverse phase chromatography and Mass spectrometry

PPS was loaded onto LiChrospher® 100 RP-18 (250 x 4 mm) (Merck™) column. The sample was eluted by a 60 min., gradient from 0.1% (v v<sup>-1</sup>) trifluoroacetic acid (TFA) in water to 0.1% (v v<sup>-1</sup>) TFA, in 80% acetonitrile, at a flow rate of 1 mL min.<sup>-1</sup>. Fractions were collected and dissolved in a saturated  $\alpha$ -cyano-4-hydroxycinnamic acid matrix solution (1:3, v v<sup>-1</sup>), spotted onto a MALDI target plate and dried at room temperature for 15 min. Average masses were obtained in an Autoflex III (Bruker™) in linear positive mode, with external calibration, using the Protein Calibration Standard II (Bruker™) for Mass Spectrometry calibration molecules. Software FlexAnalysis (Bruker Daltonics) was used to interpret mass spectra.

### Results

The non-soluble fraction of gut extract from *A. gemmatilis* fifth-instar larvae was solubilized with CHAPS and loaded on a p-aminobenzamidine-agarose column. Two active peaks, using L-BApNA as substrate, were observed (Figure 1A). The non-bound peak was related to non-specific hydrolysis of the substrate L-BApNA by enzymes other than trypsin-like, which are present in the insect gut extract (TERRA; FERREIRA, 1994). Active fractions from bound peak (56-60) were pooled and further purified by anion-exchange chromatography (Figure 1B).



**Figure 1.** Chromatographic purification of membrane-bound enzymatic extract (MBEE) obtained from the gut of *A. gemmatilis*. (A) Affinity chromatography of MBEE on a p-aminobenzamidine column equilibrated with 0.1 M tris-HCl buffer pH 7.5 at 4°C. Elution was performed with 0.05 M glycine buffer pH 3. (B) Anion-exchange chromatography of the active fractions (56-60) from the previous chromatography on Resource Q column equilibrated with 0.05 M glycine buffer pH 8.6. Elution was performed with a 30 min., gradient of 0-1 M NaCl in the same buffer. Substrate used: L-BApNA.

An anionic active peak eluted with approximately 0.3 M NaCl (Figure 1B). The corresponding active fractions (22-24) were pooled and, at the end of purification procedures, the enzyme yield was 11% with a specific activity of 18.6  $\mu$ M min.<sup>-1</sup> mg<sup>-1</sup> protein (Table 1). A 143-fold increase in the enzyme specific activity was achieved, but the protease recovery was low. Low recovery of the midgut protease from silkworm larvae, *Bombyx mori*, was also reported, although the purification factor was 594-fold (EGUCHI et al., 1982). Solubilization and recovery of

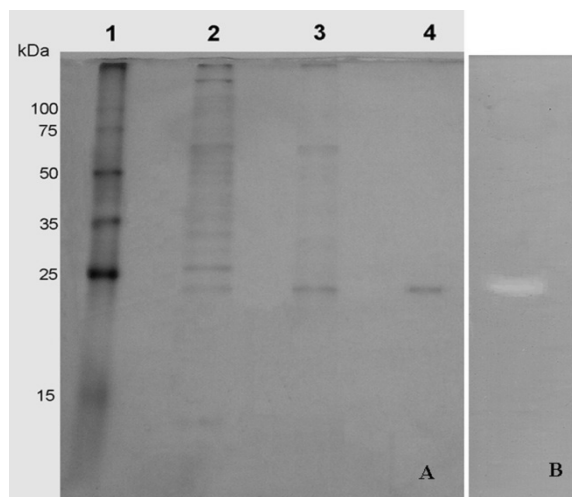
membrane-bound trypsin from *Musca domestica* and *Spodoptera frugiperda* were described to be lower than most other intrinsic proteins of midgut cell membranes (JORDÃO et al., 1996, 1999).

**Table 1.** Purification of membrane-bound trypsin-like from the gut of *A. gemmatilis*<sup>a</sup>.

Purification Step	Total prote (n <sup>o</sup> mg)	Total activity (μM min <sup>-1</sup> )	Specific activity (μM min <sup>-1</sup> mg <sup>-1</sup> )	Purification (fold)	Recovery (%)
Extract (MBEE)	47.68	6	0.13	1	100
Affinity chromatography	0.69	3.5	5.1	39	58
Anion-exchange chromatography	0.036	0.6	18.6	143	11

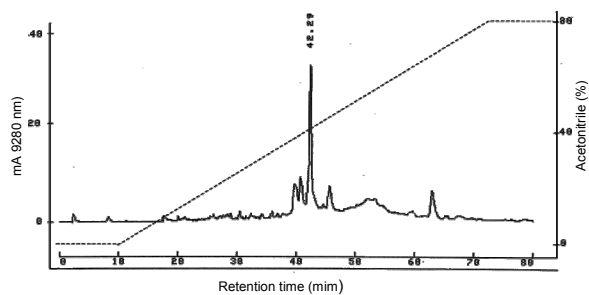
<sup>a</sup>Enzymatic activities were established at 410 nm, in a substrate of 0.25 mM BApNA.

Purity of the sample obtained after the anion-exchange chromatography was assessed by SDS-PAGE and a single protein band with an approximate molecular mass of 25 kDa was observed (Figure 2A). The casein zymogram analysis (Figure 2B) revealed the presence of a single active band of molecular mass corresponding to that observed in silver-stained SDS-PAGE.



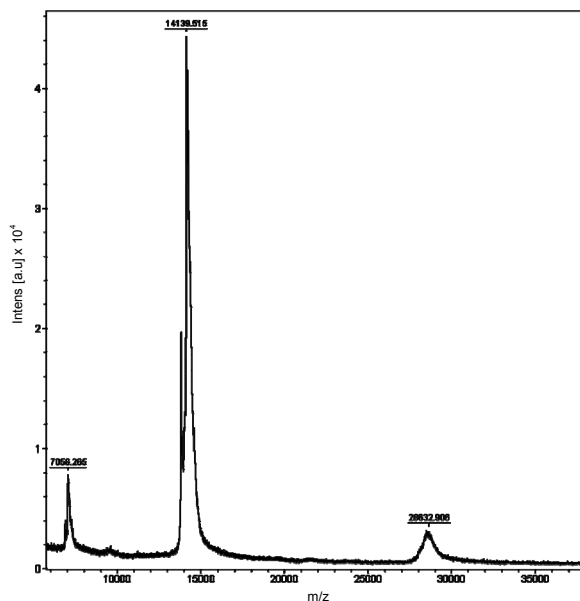
**Figure 2.** (A) SDS-PAGE and casein zymogram of the membrane-bound enzymatic extract (MBEE). Lane 1: molecular weight markers; lane 2: MBEE; lane 3: affinity chromatography fractions (56-60); lane 4: anion-exchange chromatography fractions (22-24). The gel was silver-stained. (B) Casein zymogram of anion-exchange chromatography fractions (22-24). The clear area corresponds to casein hydrolysis. The amount of protein loaded in all lanes was about 20 μg, except at lane 3 (15 μg).

The sample obtained after the anion-exchange chromatography was subjected to a reversed phase chromatography before mass spectrometry analysis. A predominant peak with a retention time of 42.29 min was observed (Figure 3). This peak was collected and submitted to MALDI-TOF analysis.



**Figure 3.** Reversed phase chromatography of the sample obtained after the anion-exchange chromatography. Elution was performed with a 60 min., gradient from 0.1% (v v<sup>-1</sup>) trifluoroacetic acid (TFA) in water to 0.1% (v v<sup>-1</sup>) TFA in 80% acetonitrile at a flow rate of 1 mL min<sup>-1</sup>.

The MALDI-TOF mass spectrometry analysis of the reversed phase eluted sample revealed an abundant ion of 14,149.7 daltons (Da), and two less abundant ions of 7,068.6 Da and 28,547.7 Da (Figure 4).



**Figure 4.** MALDI TOF mass spectrum of the purified trypsin-like from *A. gemmatilis*.

## Discussion

Lepidopteran insects primarily depend on proteases like trypsin for protein digestion. Impairment to digestion caused by specific protease inhibitors reflects not only on abnormal growth and development of the larvae but also on the fecundity and the fertility of the adult (ZAVALA et al., 2004). Taking in account the relevance to normal development of insects, trypsin-like enzymes have been considered key targets for potential insecticidal agents acting through the larvae gut, like the protease inhibitors. However, protease/inhibitor interactions in host pest systems are complex and the choice of a protease inhibitor that could act as an

insecticidal agent, showing high inhibitory activity against insect pest, should consider the diversity of proteases present on the pest assessed (LAWRENCE; KOUNDAL, 2002). Membrane-bound trypsin activity has been described in lepidopteran larvae gut cells and there are some evidences that it is the precursor of the trypsin soluble form (JORDÃO et al., 1999). In *A. gemmatalis*, the membrane-bound trypsin-like activity corresponded to 18% of total trypsin-like activity in the midgut (PEREIRA et al., 2005). Thus, in order to improve the knowledge about the *A. gemmatalis* digestive proteases, this study describes the purification of an anionic membrane-bound trypsin-like enzyme from the fifth instar larvae.

Purification of insect trypsin-like enzymes is usually achieved by affinity or ion-exchange chromatography (LEMOS; TERRA, 1992; LOPES; TERRA, 2003). However, a combination of both of them was applied in this work. Although the hydrolysis of the substrate used (BAPNA) is not a definitive proof of the trypsin-like character of the enzyme, as other enzymes are able to act on this substrate, there is now enough evidence that most insect midgut enzymes, hydrolyzing this substrate, are trypsin-like (TERRA; FERREIRA, 1994). A substantial increase in specific activity was observed after anion-exchange chromatography, when compared to the activity measured after the first affinity chromatographic step. Nevertheless, the enzyme yield and specific activity after this preparation were only 11.2% and  $18.6 \mu\text{M min}^{-1} \text{mg}^{-1}$  protein, respectively. Low recovery of trypsin from midgut tissue was already reported for *Spodoptera frugiperda* and *Musca domestica* (FERREIRA et al., 2005; JORDÃO et al., 1996), and, consequently, no kinetic characterization was addressed in this study. The anionic character of the purified enzyme is in agreement with the observation that most of the purified trypsins from insects are anionic (DIÁZ-MENDONZA et al., 2005) although some cationic enzymes have also been described (LAM et al., 2000).

The SDS-PAGE analysis of anion-exchange fractions revealed a single protein band of approximate 25 kDa as shown in Figure 2A, lane 4, that corresponds to the only active band seen on casein zymogram, presenting the same molecular weight (Figure 2B). More bands were visible in the specifically-eluted fractions from the affinity column (Figure 2A, lane 3) and some of them did not show proteolytic activity in casein zymogram analysis (data not shown). These bands could be related to trypsin-like proteins which possess characteristic signature motifs and retains the residues believed to

interact with inhibitors, but the serine 195 is replaced by other amino acid. As a consequence, these enzymes play physiological roles other than digesting proteins (MAZUNDAR-LEIGHTON et al., 2000).

The congruence between mass estimation by SDS-PAGE and mass determination by MALDI-TOF mass spectrometry indicates that the *A. gemmatalis* enzyme is composed by two polypeptide chains of 14,139 Da or by a single peptide chain of 28,632 Da, and in this case the 14,139 Da peak represents its double charged species. Although MALDI is considered a “soft ionization” method and predominantly generates singly charged ions (TRAUGER et al., 2002), multiple charged MALDI ions can be generated, depending on the experimental conditions, such as the matrix used, matrix solution, matrix analyte/ratios, sample deposition methods. The charge state, as well as the signal intensity is related to the way the sample is spotted (LIU; SCHEY, 2008). Therefore, the 14,139 Da peak observed in the mass spectra does not necessarily represents the singly charged ion or the most abundant species in the sample. The trypsin structure is highly conserved among invertebrates and vertebrates and consists of a single peptide chain (MUHLIA-ALMAZÁN et al., 2008). Trypsins isolated from insects’ midgut typically exhibit molecular mass from 20,000 to 35,000 Da (TERRA; FERREIRA, 1994), although oligomeric forms have been described, resulting in observations of higher molecular masses (BRITO et al., 2001). Even though it could be considered that the *A. gemmatalis* enzyme is formed by two polypeptide chains of 14,139 Da, the conserved structure described for insect trypsins, the SDS-PAGE analysis and the MALDI-TOF spectra analyzed strongly supports that this enzyme is a monomer of 28,632 Da. The possibility of enzyme autolysis could also be considered, supporting the difference in amount of the two species in MALDI-TOF, but in this case, 14,139 Da major component should be visible on the electrophoretogram. Nevertheless the discrepancy between molecular mass from SDS-PAGE and MALDI-TOF is relatively large; it is known that many factors can influence the electrophoretic mobility on SDS-PAGE, giving unreliable mass measurements for some proteins (HAMES, 1990). A significant difference between molecular mass determination on SDS-PAGE and MALDI-TOF for two trypsin-like from the midgut of *Locusta migratoria* was also described (LAM et al., 2000). The difficulties imposed by SDS-PAGE are avoided by MALDI-TOF mass spectrometry determination.

Studies of immunolocalization of this enzyme, as performed by Jordão et al. (1999), may provide the basis for understanding the secretory mechanism of trypsin-like enzyme in *A. gemmatalis*. There were observed similarities in the chromatographic and electrophoretic profiles between the membrane – bound enzyme and a soluble form of a trypsin-like enzyme purified from the *A. gemmatalis* gut extracts using the same procedures described above (data not shown). Besides that, further characterization may also lead to a successful exploration of these enzymes as targets of protease inhibitors in the strategies of insect pest control acting through the digestive tract.

### Conclusion

A membrane-bound trypsin-like enzyme from the gut of *Anticarsia gemmatalis* fifth-instar larvae was purified using a combination of p-aminobenzamidine affinity chromatography followed by anion-exchange chromatography. Molecular mass determined by MALDI-TOF mass spectrometry was  $28,632 \pm 26$  Da. Further characterization was limited by the low recovery and the difficulties in purifying large enzyme amounts. Studies of immunolocalization could provide the basis for understanding the secretory mechanism of trypsin-like enzyme in *A. gemmatalis* as there were observed similarities in the chromatographic and electrophoretic profiles between the membrane – bound enzyme and a soluble form of a trypsin-like enzyme purified from the *A. gemmatalis* gut extracts using the same purification process described above (data not shown).

### Acknowledgements

The hospitality and structure provided by the Marcos Luiz dos Mares Guia Laboratory at Federal University of Minas Gerais (UFMG) is appreciated. This work was supported by grants from the Minas Gerais State Foundation for Research Aid (Fapemig) and National Council of Scientific and Technological Development (CNPq).

### References

BOWN, D. P.; WILKINSON, H. S.; GATEHOUSE, J. A. Differentially regulated inhibitor-sensitive and insensitive protease genes from the phytophagous insect pest, *Helicoverpa armigera*, are members of complex multigene families. **Insect Biochemistry and Molecular Biology**, v. 27, n. 7, p. 625-638, 1997.

BRADFORD, M. M. A rapid and sensitive method for the quantification of microgram quantities of proteins

utilizing the principle of protein dye binding. **Analytical Biochemistry**, v. 72, n. 7, p. 248, 1976.

BRITO, L. O.; LOPES, A. R.; PARRA, J. R. P.; TERRA, W. R.; SILVA-FILHO, M. C. Adaptation of tobacco budworm *Heliothis virescens* to proteinase inhibitors may be mediated by the synthesis of new proteinases. **Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology**, v. 128, n. 2, p. 365-375, 2001.

BROADWAY, R. M. Are insects resistant to plant proteinase inhibitors? **Journal of Insect Physiology**, v. 41, n. 2, p. 107-116, 1995.

BROADWAY, R. M. Dietary regulation of serine proteinases that are resistant to serine proteinase inhibitors. **Journal of Insect Physiology**, v. 43, n. 9, p. 855-874, 1997.

CHRISTOU, P.; CAPELL, T.; KOHLI, A.; GATEHOUSE, J. A.; GATEHOUSE, A. M. R. Recent developments and future prospects in insect pest control in transgenic plants. **Trends in Plant Science**, v. 11, n. 6, p. 302-308, 2006.

DE LEO, F.; BOTTINO, M. B.; CECI, L. R.; GALLERANI, R.; JOUANIN, L. Effects of a mustard trypsin inhibitor expressed in different plants on three lepidopteran pests. **Insect Biochemistry and Molecular Biology**, v. 31, n. 6/7, p. 593-602, 2001.

DIÁZ-MENDONZA, M.; ORTEGO, F.; LACOPA, M. G.; MAGAÑA, C.; DE LA POZA, M.; FARINÓS, G. P.; CASTAÑERA, P.; HERNÁNDEZ-CRESPO, P. Diversity of trypsins in the Mediterranean corn borer *Sesamia nonagrioides* (Lepidoptera: Noctuidae), revealed by nucleic acid sequences and enzyme purification. **Insect Biochemistry and Molecular Biology**, v. 35, n. 9, p. 1005-1020, 2005.

EGUCHI, M.; IWAMOTO, A.; YAMAUCHI, K. Interratation of proteases from the midgut lumen, epithelia and peritrophic membrane of the silkworm, *Bombyx mori*. **Comparative Biochemistry and Physiology**, v. 72, n. 2, p. 359-363, 1982.

FERREIRA, C.; CAPELLA, A. N.; SITNIK, R.; TERRA, W. R. Digestive enzymes in midgut cells, endo- and ectoperitrophic contents and peritrophic membranes of *Spodoptera frugiperda* (Lepidoptera) larvae. **Archives of Insect Biochemistry and Physiology**, v. 26, n. 4, p. 299-313, 2005.

FERRY, N.; EDWARDS, M. G.; GATEHOUSE, J. A.; CAPELL, T.; CHRISTOU, P.; GATEHOUSE, A. M. R. Transgenic plants for insect pest control: a forward looking scientific perspective. **Transgenic Research**, v. 15, n. 1, p. 13-19, 2006.

FORTUNATO, F. S.; OLIVEIRA, M. G. A.; BRUMANO, M. H. N.; SILVA, C. H. O.; GUEDES, R. N. C.; MOREIRA, M. A. Lipoxigenase-induced defense of soybean varieties to the attack of the velvetbean caterpillar (*Anticarsia gemmatalis* Hubner). **Journal of Pest Science**, v. 80, n. 4, p. 241-247, 2007.

- GARCIA-CARREÑO, F. L.; DIMES, L. E.; HAARD, N. F. Substrate-gel electrophoresis for composition and molecular weight of proteinases or proteinaceous proteinase inhibitors. **Analitical Biochemistry**, v. 214, n. 1, p. 65-69, 1993.
- HAMES, B. D. One-dimensional polyacrylamide gel electrophoresis. In: HAMES, B. D.; RICKWOOD, D. (Ed.). **Gel electrophoresis of proteins: a practical approach**. New York: Oxford University Press, 1990. p. 1-147.
- HOFFMAN-CAMPO, C. B.; OLIVEIRA, E. B.; MOSCARDI, F. **Criação massal de lagarta da soja (*Anticarsia gemmatilis*)**. Londrina: Embrapa-CNPSo, 1985. p. 23.
- JONGSMA, M. A.; BAKKER, P. L.; PETERS, J.; BOSCH, D.; STIEKEMA, W. J. Adaptation of *Spodoptera exigua* larvae to plant proteinase inhibitors by induction of gut proteinase activity insensitive to inhibition. **Proceedings of the National Academy of Sciences USA**, v. 92, n. 17, p. 8041-8045, 1995.
- JONGSMA, M. A.; BOLTER, C. The adaptations of insects to plant proteinase inhibitors. **Journal of Insect Physiology**, v. 43, n. 10, p. 885-895, 1997.
- JORDÃO, B. P.; TERRA, W. R.; RIBEIRO, A. F.; LEHANE, M. J.; FERREIRA, C. Trypsin secretion in *Musca domestica* larval midguts: a biochemical and immunocytochemical study. **Insect Biochemistry and Molecular Biology**, v. 26, n. 4, p. 337-346, 1996.
- JORDÃO, B. P.; CAPELLA, A. N.; TERRA, W. R.; RIBEIRO, A. F.; FERREIRA, C. Nature of the anchors of membrane-bound aminopeptidase, amylase, and trypsin and secretory mechanisms in *Spodoptera frugiperda* (Lepdoptera) midgut cells. **Journal of Insect Physiology**, v. 45, n. 1, p. 29-37, 1999.
- LAM, W.; COAST, G. M.; RAYNE, R. C. Characterization of multiple trypsins from the midgut of *Locusta migratoria*. **Insect Biochemistry and Molecular Biology**, v. 30, n. 1, p. 85-94, 2000.
- LAWRENCE, P. K.; KOUNDAL, K. R. Plant protease inhibitors in control of phytophagous insects. **Electronic Journal of Biotechnology**, v. 5, n. 1, p. 93-109, 2002.
- LEMOES, F. A.; TERRA, W. R. Soluble and membrane-bound forms of trypsin-like enzymes in *Musca domestica* larval midguts. **Insect Biochemistry and Molecular Biology**, v. 22, n. 7, p. 613-619, 1992.
- LIU, Z.; SCHEY, K. L. Fragmentation of multiply-charged intact protein ions using MALDI TOF-TOF mass spectrometry. **Journal of the American Society for Mass Spectrometry**, v. 19, n. 2, p. 231-238, 2008.
- LOPES, A. R.; TERRA, W. R. Purification, properties and substrate specificity of a digestive trypsin from *Periplaneta americana* (Dictioptera) adults. **Insect Biochemistry and Molecular Biology**, v. 33, n. 4, p. 407-415, 2003.
- MAZUNDAR-LEIGHTON, S.; BABU, C. R.; BENNETT, J. Identification of novel serine proteinase gene transcripts in the midguts of two tropical insect pests, *Scirpophaga incertulas* (Wk.) and the *Helicoverpa armigera* (Hb.). **Insect Biochemistry and Molecular Biology**, v. 30, n. 1, p. 57-68, 2000.
- MORALES, L.; MOSCARDI, F.; SOSA GÓMES, D. R.; PARO, F. E.; SOLDORIO, I. L. Fluorescent brighteners improve *Anticarsia gemmatilis* (Lepidoptera: Noctuidae) nucleopolyhedrovirus (AgMNPV) activity on AgMNPV susceptible and resistant strains of the insect. **Biological Control**, v. 20, n. 3, p. 247-253, 2001.
- MUHLIA-ALMAZÁN, A.; SÁNCHEZ-PAZ, A.; GARCIA-CARREÑO, F. L. Invertebrate trypsins: a review. **Journal of Comparative Physiology B**, v. 178, n. 6, p. 655-672, 2008.
- OLIVEIRA, M. G. A.; SIMONE, S. G.; XAVIER, L. P.; GEUDES, R. N. C. Partial purification and characterization of digestive trypsin-like proteases from the velvet bean caterpillar, *Anticarsia gemmatilis*. **Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology**, v. 140, n. 3, p. 369-380, 2005.
- PEREIRA, M. E.; DÖRR, F. A.; PEIXOTO, N. C.; LIMA-GARCIA, J. F.; DÖRR, F.; BRITO, G. G. Perspectives of digestive pest control with proteinase inhibitors that mainly affect the trypsin-like activity of *Anticarsia gemmatilis* Hübner (Lepidoptera: Noctuidae). **Brazilian Journal of Medical and Biological Research**, v. 38, n. 11, p. 1633-1641, 2005.
- PRAÇA, L. B.; SILVA NETO, S. P.; MONNERAT, R. G. *Anticarsia gemmatilis*/Hübner, 1818 (Lepidoptera: Noctuidae). **Biologia, amostragem e métodos de controle**. Brasília: Embrapa Recursos Genéticos e Biotecnologia, 2006. (Série Doc., 196).
- TELANG, M. A.; GIRI, A. P.; SAINANI, M. N.; GUPTA, V. S. Characterization of two proteinases of *Helicoverpa armigera* and their inter action with proteinase inhibitors. **Journal of Insect Physiology**, v. 51, n. 5, p. 513-522, 2005.
- TERRA, W. R.; FERREIRA, C. Insect digestive enzymes: compartmentalization and function. **Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology**, v. 109, n. 1, p. 1-62, 1994.
- TERRA, W. R.; FERREIRA, C. Biochemistry of digestion. In: GILBERT, L.; IATROU, K.; GILL, S. S. (Ed.). **Comprehensive Molecular Insect Science, Biochemistry and Molecular Physiology**. Oxford: Elsevier, 2005. v. 4, p. 171-224.
- TRAUGER, S. A.; WEBB, W.; SIUZDAK, G. Peptide and protein analysis with mass spectrometry. **Spectroscopy**, v. 16, n. 1, p. 15-28, 2002.
- VOLPICELLA, M.; CECI, L. R.; CORDEWENER, J.; AMERICA, T.; GALLERANI, R.; BODE, W.; JONGSMA, M. A.; BEEKWILDER, J. Properties of purified gut trypsin from *Helicoverpa zea*, adapted to proteinase inhibitors. **European Journal of Biochemistry**, v. 270, n. 1, p. 10-19, 2003.



XAVIER, L. P.; OLIVEIRA, M. G. A.; GUEDES, R. N. C.; SANTOS, A. V.; DE SIMONE, S. G. Trypsin-like activity of membrane-bound midgut proteases from *Anticarsia gemmatalis* (Lepidoptera: Noctuidae). **European Journal of Entomology**, v. 102, n. 2, p. 147-153, 2005.

ZAVALA, J. A.; PATANKAR, A. G.; GASE, K.; HUI, D.; BALDWIN, I. T. Manipulation of endogenous trypsin proteinase inhibitor production in *Nicotiana attenuata*

demonstrates their function as antiherbivore defenses. **Plant Physiology**, v. 134, n. 3, p. 1181-1190, 2004.

*Received on January 21, 2010.*

*Accepted on September 15, 2010.*

License information: This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.