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Inhibition kinetics of digestive proteases for *Anticarsia gemmatalis*

ADRIANA M. PATARROYO-VARGAS, GLÁUCIA CORDEIRO, CAROLINA R. DA SILVA, CAMILA R. DA SILVA, EDUARDO G. MENDONÇA, LILIANE E. VISÔTTO, JOSÉ C. ZANUNCIO, WELLIGTON G. CAMPOS & MARIA GORETI A. OLIVEIRA

Abstract: *Anticarsia gemmatalis* Hübner, 1818 (Lepidoptera) is a major pest of soybean in the Brazil. It is known that the reduction of proteolytic activity by the ingestion of protease inhibitors reduces digestion and larval development of the insects. Control via inhibition of the digestive enzymes necessitates deeper knowledge of the enzyme kinetics and the characterization of the inhibition kinetics of these proteases, for better understanding of the active centers and action mechanisms of this enzyme. Trypsin-*like* proteases found in the gut of *Anticarsia gemmatalis* were purified in a p-aminobenzamidine agarose column. Kinetic characterization showed K_M 0.503 mM for the L-BApNA substrate; V_{max}= 46.650 nM s^{-1}; $V_{max}/[E]$= 9.256 nM s^{-1} mg L^{-1} and $V_{max}/[E]/K_M$= 18.402 nM s^{-1} mg L^{-1} mM. The K_i values for the inhibitors benzamidine, berenil, SKTI and SBBI were 11.2 µM, 32.4 µM, 0.25 nM and 1.4 nM, respectively, and all revealed linear competitive inhibition. The SKTI showed the greatest inhibition, which makes it a promising subject for future research to manufacture peptide mimetic inhibitors.

Key words: Enzyme kinetics, inhibitor competitive, inhibition kinetics, velvet bean caterpillar.

INTRODUCTION

Soybean plants can be damaged by insects throughout their whole cycle, with *Anticarsia gemmatalis* (Lepidoptera: Noctuidae) being one of this crop main pest (Bernardi et al. 2012). This insect reduces production quantities and requires rapid control to prevent economic losses (Vianna et al. 2011).

The study of the endogenous resistance mechanisms of plants against insect herbivores promises a safe strategy for pest control as an alternative to the use of chemical insecticides that affect other non-target organisms and cause environmental impact (Gatehouse 2002, Ferry et al. 2006, Scott et al. 2010, Mills & Kean 2010, Patarroyo-Vargas et al. 2017). The inhibition mechanism of the digestive enzymes is an alternative strategy for pest control, although the structure and function of these macromolecules must be studied.

Plants possess protease inhibitor (PI), which are important multi-mechanistic components of defense against pests (Silva-Filho & Falco 2000). PI are generally small and stable molecules, specific for the major digestive proteases (serine, cysteine, and aspartyl proteases) of pest herbivores (Jamal et al. 2012). The serine proteases, trypsin and chymotrypsin, are the major digestive enzymes occurring in the midgut of Lepidoptera (Terra & Ferreira 1994, Shi et al. 2013). PI ingestion reduces the proteolytic activity, and consequently, the larval digestion,

growth and development, as well as adult fertility and fecundity (Mahdavi et al. 2013, Shi et al. 2013). However, insects have developed defense mechanisms for protection against the deleterious effects of PI. These defenses include increasing the enzymatic synthesis of the class that is being inhibited, as well as of enzymes insensitive to the inhibitors, in response to the inhibitory effect (Oliveira et al. 2005, Pilon et al. 2009, Scott et al. 2010, Oliveira et al. 2013, Wielkopolan et al. 2015).

Deeper knowledge of the enzyme and the inhibition kinetics of the *A. gemmatalis* digestives proteases allows for a better understanding of the active centers, of the action mechanisms of these enzymes and the protein and/or synthetic inhibitors that need to be applied as inhibitors of the complex systems of the insect's digestive proteases.

The objective of this work was to study the inhibition kinetics of the intestinal serine proteases of *Anticarsia gemmatalis* Hübner, 1818 (Lepidoptera: Noctuidae) using synthetic and protein proteases inhibitors. This is a pioneer study on the characterization of the inhibition model of the trypsin-*like* enzyme in the midgut of *A. gemmatalis* and will enable mapping of the active centers of these enzymes. In addition, it will provide a basis for applied ecological studies of how insect resistance occurs to plants of interest. They may also demonstrate how plant-herbivore interactions should be considered in strategies for Integrated Pest Management.

MATERIALS AND METHODS

The experiments were performed in the Laboratory of Enzymology, Biochemistry of Proteins and Peptides of the Institute of Biotechnology Applied to Agriculture and Laboratory of Insect Rearing of the Department of Biochemistry and Molecular Biology at the Federal University of Viçosa in Viçosa, Minas Gerais State, Brazil.

Anticarsia gemmatalis

The fourth and fifth instar larvae of *A. gemmatalis* were reared in the Laboratory of Insect Rearing of Department of Biochemistry and Molecular Biology of Federal University of Viçosa on an artificial diet (Hoffman-Campo et al. 1985), and maintained in climatized chambers with 25 ± 2ºC, relative humidity 70 ± 10% and photoperiod 14 hours.

Crude midgut extract preparation

The larvae were immobilized on ice. Their guts were removed and placed in 10^{-3}M HCl solution, in the proportion of five guts/mL solution at -20° C. The crude enzyme extract was obtained by cell disruption after passing through nine cycles of freezing in liquid nitrogen followed by thawing in a water bath at 37° C. The suspension obtained was centrifuged at 100,000 rpm for 45 minutes at 4° C (Oliveira et al. 2005).

Enzyme purification

The crude enzyme extract was then subjected to affinity chromatography on a p-aminobenzamidine agarose column (2.5 mL) (Sigma®) balanced with Tris-HCl buffer 0.05 M at pH 7.5 containing NaCl 0.5 M. Proteins were eluted under continuous flow of 1 mL/min of glycine buffer 0.05 M at pH 3.0. The fractions corresponding to the activity peak were pooled and stored at -20° C for future use during enzyme kinetic assays.

Protein assays

The protein concentrations of the samples of crude and purified enzymatic extracts were measured using the ABS 0.2 mg mL^{-1} as the standard (Bradford 1976).

Enzymatic activity

The activity of trypsin-*like* serine proteases was determined in Tris-HCl buffer 0.1 M at pH 8.0 containing $CaCl_2$ 20 mM with the chromogenic substrate L-BApNA 1.2 mM (Erlanger et al. 1961). The initial rates of the product p-nitroanilide were determined at 410 nm in function of time (2.5 min) using the molar extinction coefficient of 8800 $M^{-1}cm^{-1}$ for the product. The experiment was conducted in a series of three repetitions (Oliveira et al. 2005).

Kinetic characterization

The K_M and V_{max} were determined using the Tris-HCl buffer 0.1 M at pH 8.0 containing $CaCl_2$ 20 mM and the purified enzyme at a final concentration of 5.04 µg mL^{-1} and increasing the concentrations of the L-BApNA chromogenic substrate (from 0.1 mM to 1.0 mM). The initial rates were determined as described above.

The K_i were determined with Tris-HCl buffer 0.1 M at pH 8.0 containing $CaCl_2$ 20 mM in the presence of the chromogenic substrate L-BApNA in a concentration range of 0.1 mM to 0.4 mM, used in all the analyses. Synthetic and protein inhibitors and their concentrations were as follows: synthetic inhibitors benzamidine (10 µM to 50 µM) and berenil (20 µM to 100 µM), protein inhibitors SKTI (0.1 nM to 1.0 nM) and SBBI (0.5 nM to 4.0 nM). The initial rates were determined as described earlier. The kinetic parameters were calculated by nonlinear regression using the software Sigma Plot 10.0, adopting the simple uni reaction kinetic model.

The general equation rate for this model is:

$$v = \frac{V_{max}.[S]}{K_M + [S]} \quad (1)$$

The linear method used was the double reciprocal graph of Lineweaver-Burk in which the linearization was represented by the equation:

$$\frac{1}{V_{max}} = \frac{K_M}{V_{max}} . \frac{1}{[S]} + \frac{1}{V_{max}} \quad (2)$$

The K_i of the inhibitors were obtained by the intersection of the lines corresponding to the substrate concentrations (Dixon et al. 1979). Equation (3) represents the Dixon plot, followed by the inhibition kinetics model:

$$\frac{1}{v} = \frac{K_M}{V_{max}.K_i.[S]} . [I] + \frac{1}{V_{max}} . \left(1 + \frac{K_M}{[S]}\right) \quad (3)$$

The competitive inhibition model was determined using the double reciprocal graph methods of Lineweaver-Burk (Equation 4) and the double reciprocal graph of the slopes (Equation 5):

$$\frac{1}{v} = \frac{K_M}{V_{max}} . \left(1 + \frac{[I]}{K_i}\right) . \frac{1}{[S]} + \frac{1}{V_{max}} \quad (4),$$

$$Slope_{\frac{1}{S}} = \frac{K_M}{V_{max}.K_i} . [I] + \frac{K_M}{V_{max}} \quad (5)$$

RESULTS

Partial purification of the serine proteases

The crude enzyme extract from the guts of the *A. gemmatalis* larvae was subjected to affinity chromatography on a p-aminobenzamidine agarose column with fractions containing the activity of the trypsin-*like* serine proteases, eluted with glycine buffer 0.05 M, corresponding to the second absorbance peak (Figure 1). Proteins that did not adhere to the p-aminobenzamidine agarose column were identified in the first absorbance peak. This procedure resulted in a purification factor of 6.0 and an original activity yield of trypsin-*like* serine proteases of approximately 124% (Table I). The partially purified sample showed two bands with an approximate molecular mass of 66 kDa and 35 kDa on 15% polyacrylamide gels (data not shown).

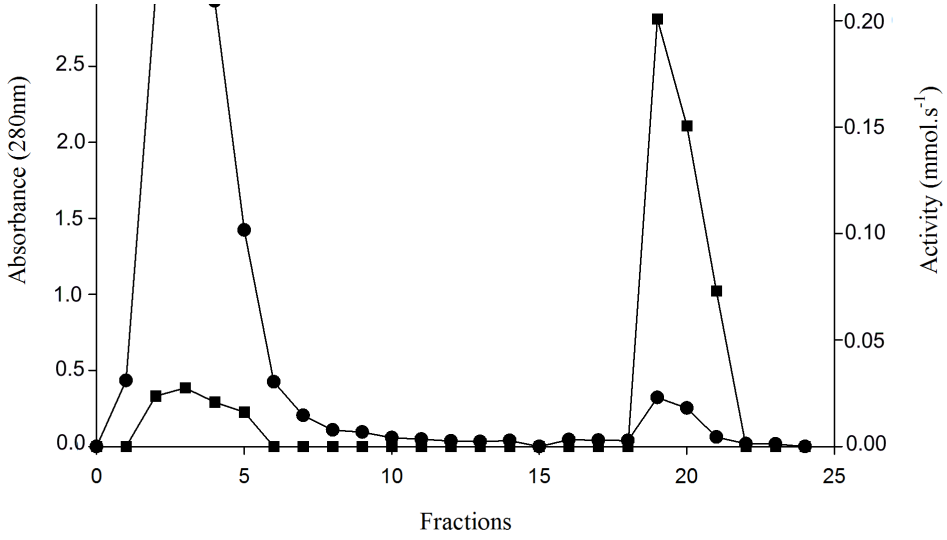

Figure 1. Chromatographic elution profile of trypsin-*like* serine proteases of *Anticarsia gemmatalis*is (Lepidoptera: Noctuidae) in p-aminobenzamidine agarose column. Absorbance of 280 nm (●), enzymatic activity of μM s^{-1} (■).

Table I. Partial purification of digestive serine proteases from *Anticarsia gemmatalis* (Lepidoptera: Noctuidae).

Fractions	Total protein (mg)	Total activity (μM s^{-1})	Specific activity (μM s^{-1} mg)	Purification factor	Yield (%)
Crude extract	1.58	0.144	0.091	1	100
p-aminobenzamidine agarose	0.328	0.179	0.546	6	124.3

Determination of the kinetic parameters

The kinetic parameters estimated for the serine proteases were apparent because the system used was partially purified (Table II). Data were compared against those of the bovine β-trypsin as standard.

The amidolytic activity generated a concentration-speed hyperbolic curve following the Michaelis-Menten kinetic model for the substrate concentration range used (Figure 2). The K_{Mapp} and V_{maxapp} were confirmed by linearization of the Michaelis-Menten curve generating the double reciprocal graph of Lineweaver-Burk with an R^2 value of 0.9860 (inset of Figure 2).

The partially purified serine proteases show a faster transformation rate of approximately 5.6 times more than that of β-trypsin (Table II).

Determination of the inhibition constant

The Lineweaver-Burk graphs (Figure 3) were constructed utilizing variable concentrations of the inhibitor and substrate to determine the inhibition of the serine proteases trypsin-*like* partially purified. The four Lineweaver-Burk graphs showed inhibition of the competitive type. The increase in the slopes of the lines with rising concentrations of the inhibitor indicates that the enzyme is distributed in larger quantities in the form of the EI complex. The presence of the inhibitor did not change the value of V_{max} where the intersection of the line in axis 1/V corresponds to $1/V_{max}$ in the Lineweaver-Burk graphs.

The slopes graph obtained from the reciprocal plots of the data (Figure 4) show a

significant linear curve. This confirms that the pattern of inhibition of the amidolytic activity of the partially purified enzyme against the inhibitors benzamidine, berenil, SKTI and SBBI is of the competitive type, where an inhibitor molecule binds to the enzyme forming a type of EI binary complex.

The data from the Dixon plot, fixing the concentration of the tested inhibitors showed that an increase in the substrate concentration triggered a decrease in the degree of inhibition; however, fixing the substrate concentration and increasing the inhibitor concentration increased the degree of inhibition (Figure 5).

The calculated inhibition constants by the Dixon et al. (1979) method of the inhibitors benzamidine, berenil, SKTI and SBBI were obtained (Table III) and the data compared with the β-trypsin.

The K_i of benzamidine, SKTI and SBBI for the partially purified enzyme were lower than the K_i for the β-trypsin. This shows the higher affinity of these inhibitors for the active center of the partially purified trypsin-*like* protease of *A. gemmatalis* compared with the β-trypsin. The berenil showed a higher K_i, suggesting a lower affinity of this inhibitor for the active center of the partially purified trypsin-*like* enzyme.

Table II. Kinetic parameters of the substrate hydrolysis L-BApNA by trypsin of the digestive serine proteases from *Anticarsia gemmatalis* (Lepidoptera: Noctuidae).

Parameters	β-trypsin [(a)]	Trypsin-*like*
K_M (mM)	0.580	0.503
V_{max} (nM s^{-1})	-	46.650
K_{cat} (1 s^{-1})	1.630	-
$V_{max}/[E]$ (nM s^{-1} mg L^{-1})	-	9.256
K_{cat}/K_M (1 s^{-1} mM)	2.810	-
$V_{max}/[E]/K_M$ (nM s^{-1} mg L^{-1} mM)	-	18.402

[(a)] Data from Nakata & Ishii (1972).

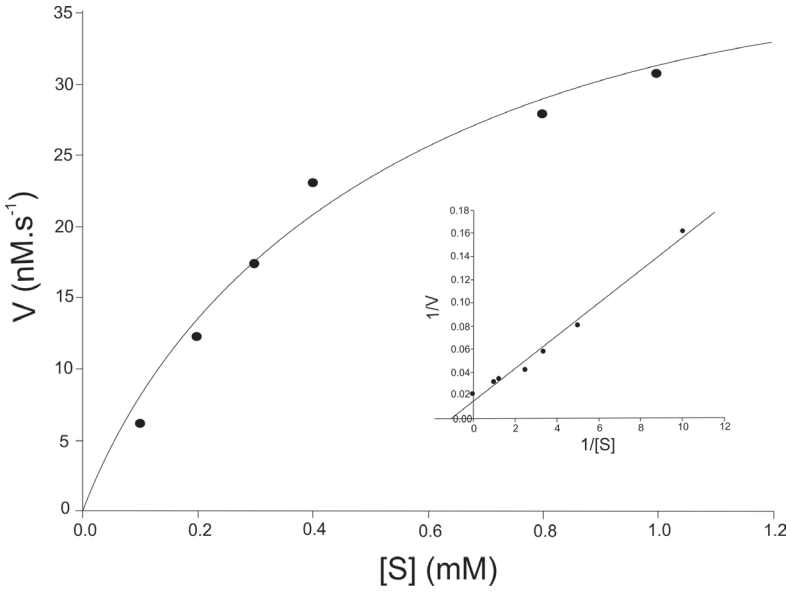

Figure 2. Michaelis-Menten graph for the hydrolysis of L-BApNA catalyzed by the enzyme. The substrate concentrations ranged from 0.1 mM to 1 mM. The points are experimental. Insertion: graph of the double reciprocal Lineweaver-Burk. The line drawn was calculated by linear regression with R^2 of 0.9860.

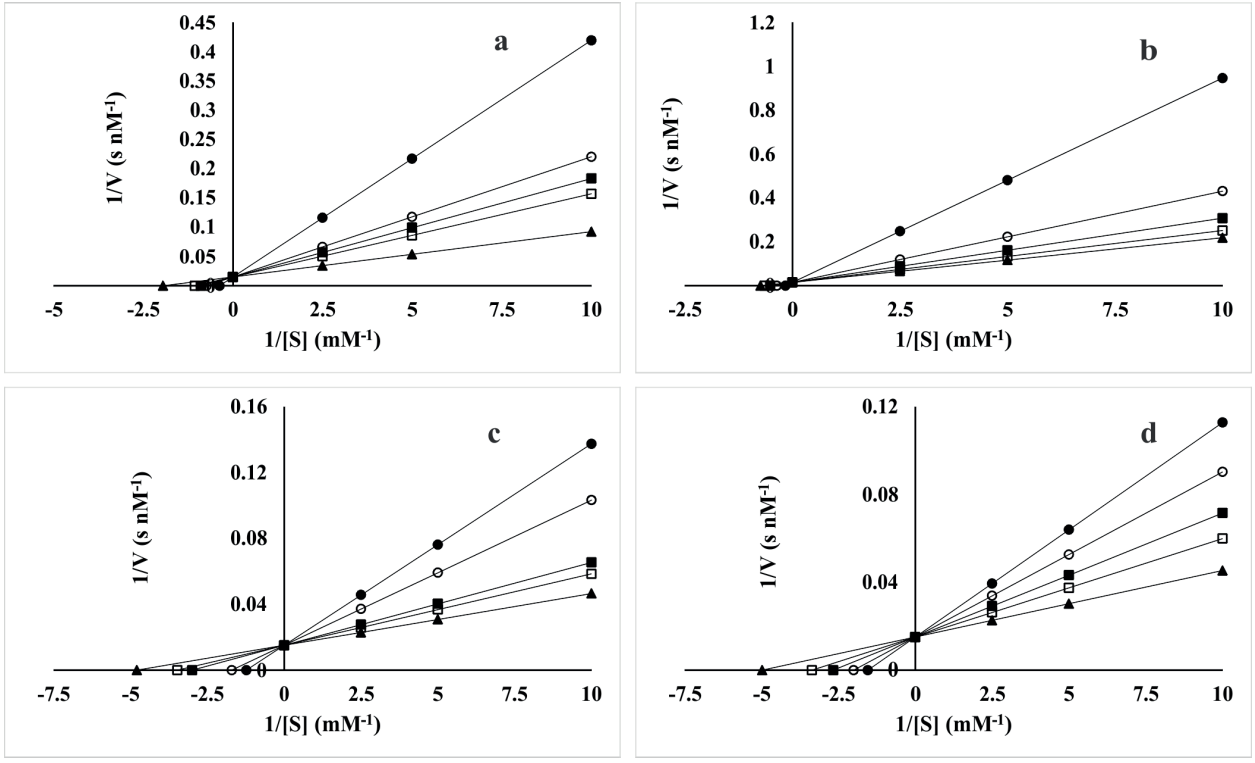

Figure 3. Lineweaver-Burk graphic of the inhibition (I) of the intestinal trypsin-*like* of *Anticarsia gemmatalis* (Lepidoptera: Noctuidae) by the inhibitors (**a**) benzamidine, (**b**) berenil, (**c**) SKTI and (**d**) SBBI in the presence of L-BApNA substrate. The lines were calculated by linear regression using the parameters of Table II. The points are experimental. I1 (▲), I2 (□), I3 (■), I4 (○), I5 (●).

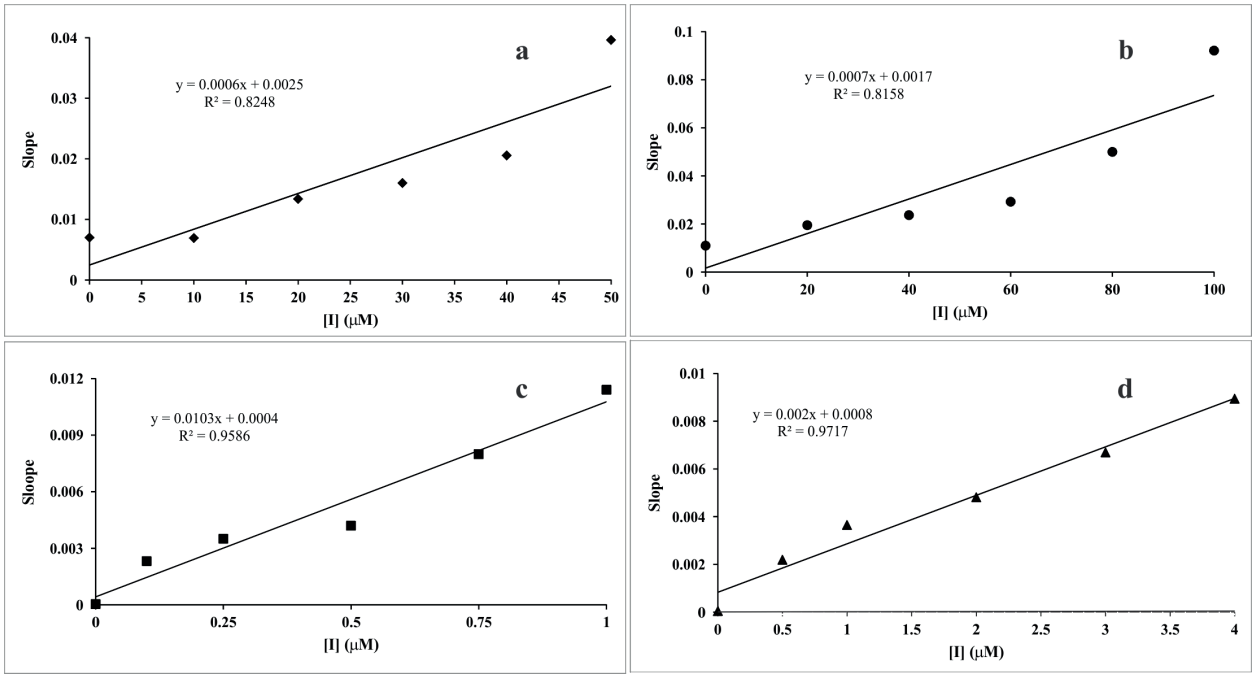

Figure 4. Graphic slopes of the Lineweaver-Burk graphic versus concentration of I for the hydrolysis of L-BApNA by partially purified trypsin-*like* in the presence of the inhibitors (**a**) benzamidine, (**b**) berenil, (**c**) SKTI and (**d**) SBBI.

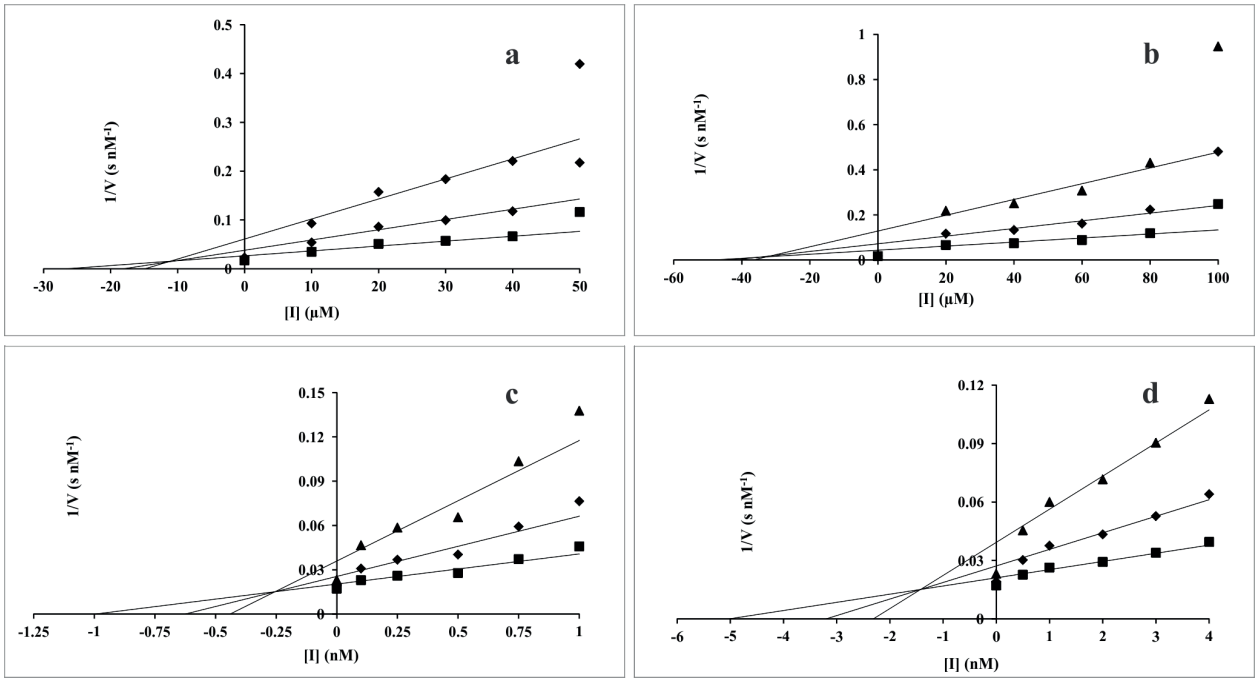

Figure 5. Dixon's plot of the inhibition of intestinal trypsin-*like* of *Anticarsia gemmatalis* (Lepidoptera: Noctuidae) by the inhibitors (a) benzamidine, (b) berenil, (c) SKTI and (d) SBBI in the presence of L-BApNA substrate (S). The lines were calculated by linear regression using the parameters of Table II. The points are experimental. S1 (▲), S2 (◆), S3 (■).

Table III. Inhibition constant of inhibitors of serine proteases of *Anticarsia gemmatalis* (Lepidoptera: Noctuidae) in the presence of L-BApNA substrate.

Inhibitor	K_i trypsin-*like*	K_i β-trypsin
Benzamidine	11.2 µM	18.4 µM [(a)]
Berenil	32.4 µM	1.79 µM [(b)]
SKTI	0.25 nM	0.6 nM[(c)]
SBBI	1.4 nM	1.6 nM[(c)]

[(a)] Data from Mares-Guia & Shaw 1965, [(b)] Data from Junqueira et al. 1992; [(c)] Data from Zollner 1999.

DISCUSSION

In order to promote a plant defense against pests by eliminating the use of pesticides, we have been working to obtain a protein molecule, which is own from physiological environment. Therefore, this protein molecule needs have an inhibitory action over the proteases in the intestines of insects.

To construct a peptide or a mimetic peptide with great potential to use in the protection of plants is necessary to know the kinetic model of inhibition of insect pest gut proteases.

In this sense, we performed the kinetic characterization of the trypsin-*like* inhibition of *A. gemmatalis* to understand the inhibition from the point of view of structure/physiological function. From the kinetic model it is possible

to propose an inhibitor molecule ecologically acceptable for the control of agricultural pests by lipoxygenase pathway.

The benzamidine, a potent competitive inhibitor of the trypsin-*like* serine proteases, occupies the S_1 subsite of this enzyme, which is the specificity site. The first eluting peak probably corresponds to the cysteine proteases present in the midgut enzyme extract of *A. gemmatalis,* the enzymes lacking a p-aminobenzamidine affinity but capable of hydrolyzing the substrate L-BApNA (Terra & Ferreira 1994). The increase in the total activity of the trypsin-*like* enzyme following affinity chromatography (Table I) is due to the separation of its probable inhibitors present in the crude extract during the purification process. This was also reported for the purification of the trypsins of *Bombyx mori* Lineu, 1758 (Eguchi & Kuriyama 1985), *Locusta migratoria* (Linnaeus, 1758) (Lam et al. 2000) and *Heliothis virescens* (Fabricius, 1781) (Brito et al. 2001). Purification of the midgut extract of *A. gemmatalis* in the p-aminobenzamidine agarose column showed a better yield (124.3%) than the aprotinin-agarose column (66.7%) (Oliveira et al. 2005).

The band with mass close to 35 kDa of the soluble trypsin-*like* enzyme from *A. gemmatalis* is found to be consistent with the masses of the trypsins of most insects, ranging between 20 kDa and 35 kDa (Terra & Ferreira 1994), similar to those isolated from other Lepidoptera: 27 kDa and 24 kDa of *Sesamia nonagrioides* (Lefèbvre, 1827) (Novillo et al. 1999), 26 kDa and 29 kDa of *Helicoverpa armigera* (Hübner, 1805) (Telang et al. 2005) and 28.7 kDa of *Diatrea saccharalis* (Fabricius, 1794) (Lopes et al. 2006). Multiple trypsins commonly occur in the intestine of Lepidoptera and are usually related to the adaptability of the insect to plant protease inhibitors (Brito et al. 2001, Volpicella et al. 2003, Budatha et al. 2008). The band with a mass of 66 kDa is consistent with the molecular mass of 67 kDa and 70 kDa of *H. virescens* (Brito et al. 2001). Proteins reported with a molecular mass of 66 kDa to 91 kDa represented either the clustering of the molecules of trypsin-*like* proteases or an evolutionary adaptation of *A. gemmatalis* due to the constant exposure to protease inhibitors (Oliveira et al. 2005). The molecular mass of a band, practically the double of the other, indicates the enzyme dimerization (Oliveira et al. 2005).

The similar K_M value of the partially purified trypsin-*like* serine proteases and that of the β-trypsin in the presence of L-BApNA (Table II) shows the similar interaction of this substrate with the active center of both enzymes. The kinetic parameters of the trypsin-*like* serine proteases from *A. gemmatalis* partially purified on a column of aprotinin showed a K_M of 0.32 mM for the substrate L-BApNA (Oliveira et al. 2005). The transformation rate in the semi-pure systems can be expressed as $V_{max}/[E]$ and specificity constant by $V_{max}/[E]/K_M$. The faster rate of product formation by the action of the trypsin-*like* serine proteases on the substrate L-BApNA does not correspond to a greater interaction by the substrate because the K_M values were similar for the enzymes compared. The partially purified trypsin-*like* enzyme showed a greater specificity constant than the β-trypsin, and therefore, was more efficient in the formation of the ES complex. The semi-purified enzyme showed the greater adaptation between the substrate and the active center in the transition stage, which indicates the better catalytic efficiency of the trypsin-*like* enzymes from *A. gemmatalis*.

Benzamidine, berenil, SKTI and SBBI acted as pure competitive inhibitors of the partially purified trypsin-*like* serine proteases in the analyzed concentration ranges of S and I. The competitive inhibitor increased the K_M of the substrate, requiring a higher concentration of the substrate for the enzyme to reach any

fraction of V_{max}. The factor $\left(1 + \frac{[I]}{K_i}\right)$ can be considered an important statistical dependent factor on [I] which describes the free form of enzyme distribution and in the EI form. The highest concentration of the inhibitor increases the K_M and the slope of the line by the factor $\left(1 + \frac{[I]}{K_i}\right)$. The inhibitor and the substrate are mutually exclusive, for competing for the same binding site in the region of the active center of the enzyme.

The characterization of three different types of graphic profiles shows that the inhibitors analyzed with the pure competitive inhibition of the trypsin-*like* enzyme in the concentration range of the inhibitors and substrates analyzed. This is the first time that an inhibition model of the trypsin-*like* enzyme of the midgut from *A. gemmatalis* is characterized and, therefore, deserves special attention given to the standardization of the model. The characterization of an inhibition kinetic model requires more than one type of plot adjusted to more than one kinetic equation, because different kinetic models can present the same profile of a given graph. The Lineweaver-Burk graph of the bovine β-azotrypsin with partially hyperbolic competitive inhibition showed the same profile of pure competitive inhibition (Oliveira et al. 1993). The characterization of the inhibition of the bovine trypsin that exhibits the partially parabolic competitive inhibition graph showed the Lineweaver-Burk graph with the same profile of pure competitive inhibition (Junqueira et al. 1992).

The values of the inhibition constants can be explained by the chemical and structural characteristics of the inhibitors tested. The benzamidine, with K_i 18.4 μM, is a synthetic aromatic amide that competitively inhibits the trypsin, electrostatically interacting with the Asp_{189} of the trypsin and hydrophobically with specificity site of the active center of this enzyme (Oliveira et al. 1993, Patarroyo-Vargas et al. 2017). The inhibition of the serine proteases partially purified by benzamidine indicates that they are trypsin-*like* proteases. The berenil interacts electrostatically with the Asp_{189} of the trypsin and hydrophobically with the specificity site of the enzyme active center and strongly with the secondary binding site in a way similar to the Asp_{17} of the bovine pancreatic trypsin inhibitor (BPTI) (Oliveira et al. 1993). The S_2' region is very close to the active site of the trypsin and presents the residues Tyr_{39}, His_{40} and Tyr_{151}. The residue, Tyr_{151} modification in the S_2' subsite of the bovine trypsin shows that the tyrosine residue participates in the activation of the enzyme making it permanently more active (Oliveira et al. 1993).

The lower K_i values for the inhibitors SKTI and SBBI compared with the K_i of synthetic inhibitors allows them to occupy all the binding sites available in the active center of the enzyme and thus an optimized adjustment occurs in the EI complex formation. The synthetic inhibitors, being smaller, do not occupy all the available sites of the enzyme, forming a little less stable EI complex. The protein inhibitors reveal K_i in the same order of magnitude; however, the highest affinity of the SKTI to the active center of the enzymes can be explained by the differences in the architecture of these inhibitors (Volpicella et al. 2003). The Kunitz-type inhibitor interacts with the enzyme through the amino acid residue at the position P_1, arginine or lysine, and this direct interaction between the reactive site residues of the inhibitor with the catalytic site of the enzyme characterizes a competitive mechanism of inhibition. The canonical conformation of the reactive site loop of SKTI interacts with the reactive site of the enzyme through the electrostatic links and hydrogen interactions (Bode & Huber 1992), forming a stable EI complex.

The Bowman-Birk inhibitors also present the canonical loop conformation that interacts with the active center of the enzyme. The presence of seven disulfide bonds stabilizes the structure of the inhibitor, making it less flexible to adapt to the partially purified trypsin-*like* enzyme when compared with SKTI. Another hypothesis is that through evolution, the Lepidopteran trypsins showed increased hydrophobicity of the subsites of the active site by ingesting plant protease inhibitors. These inhibitors possess, in the active region, a hydrophilic sequence that does not adapt to the hydrophobic trypsin sites. The change in the primary specificity of the trypsin in Lepidoptera is an evolutionary adaptation in resistance to the plant trypsin inhibitors (Lopes et al. 2006). This makes it difficult for the interaction between the inhibitor SBBI and the partially purified enzymes to occur.

Inhibition kinetic studies are tools for understanding the multi-mechanistic system of enzymes. Our study demonstrated, for the first time, the adaptation of trypsin-*like* enzymes in the gut of *A. gemmatalis* against different inhibitors. The SKTI soybean inhibitor showed the greatest inhibition of these enzymes. This is the reason underlying the aim of studying the production of peptide-mimetic inhibitors that may be produced by the plant or applied to it, to act as inhibitors of the complex system of the insect's digestive proteases. However, the adaptive mechanisms of the insect should be carefully considered. The possibility of producing resistant plants expressing PI makes the development of new studies necessary to increase the chances of success of this method (Chitgar et al. 2013). In future, these studies should include the expression levels of the PI, its inhibition constant, the stability of the PI in the gut and the adaptation ability of the insect to the inhibitors via alteration in gene expression. Besides the important economic benefits, these studies may also provide environmental and social benefits by enabling the possibility of developing cultivars resistant to the insect pests and thus significantly reduce the use of pesticides. They, also, provide the basis for applied ecological studies on how insect resistance occurs in plants of interest. This also demonstrates how the plant-herbivore interactions need to be considered in the strategies for Integrated Pest Management. This knowledge will be of great importance for plant breeding programs aiming producing cultivars resistant to insect pests.

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ADRIANA M. PATARROYO-VARGAS[1]
https://orcid.org/0000-0002-5831-1931

GLÁUCIA CORDEIRO[1]
https://orcid.org/0000-0002-9950-5409

CAROLINA R. DA SILVA[1]
https://orcid.org/0000-0002-2186-4129

CAMILA R. DA SILVA[2]
https://orcid.org/0000-0003-1393-5115

EDUARDO G. MENDONÇA[1]
https://orcid.org/0000-0003-2755-3898

LILIANE E. VISÔTTO[3]
https://orcid.org/0000-0002-6086-4076

JOSÉ C. ZANUNCIO[4]
https://orcid.org/0000-0003-2026-281X

WELLIGTON G. CAMPOS[5]
https://orcid.org/0000-0001-9836-0466

MARIA GORETI A. OLIVEIRA[1]
http://orcid.org/0000-0001-5603-9471

[1]Departamento de Bioquímica e Biologia Molecular, Instituto de Biotecnologia Aplicada a Agropecuária/ BIOAGRO, Universidade Federal de Viçosa, Avenida Peter Henry Rolfs, s/n, 36570-900 Viçosa, MG, Brazil

[2]Instituto de Ciências Agrárias, Universidade Federal de Viçosa, Campus Rio Paranaíba, Km 7, Zona Rural, MG-230 - Rodoviario, s/n, 38810-000 Rio Paranaíba, MG, Brazil

[3]Instituto de Ciências Biológicas, Universidade Federal de Viçosa, Campus Rio Paranaíba, Km 7, Zona Rural, MG-230 - Rodoviario, s/n, 38810-000 Rio Paranaíba, MG, Brazil

[4]Departamento de Entomologia, Instituto de Biotecnologia Aplicada a Agropecuária/ BIOAGRO, Universidade Federal de Viçosa, Avenida Peter Henry Rolfs, s/n, 36570-900 Viçosa, MG, Brazil

[5]Departamento de Engenharia de Biossistemas, Universidade Federal de São João Del-Rei, Praça Dom Helvécio, 74, 36301-160 São João Del-Rei, MG, Brazil

Correspondence to: **Maria Goreti A. Oliveira**
E-mail: malmeida@ufv.br

Author contributions

Adriana Maria Patarroyo-Vargas performed the experiments and scientific writing, Gláucia Cordeiro contributed to the literature review and scientific writing, Carolina Rocha da Silva contributed to scientific writing and grammar review, Camila Rocha da Silva, Eduardo Gomes Mendonça and Liliane Evangelista Visôtto contributed to the evaluation of work in the field of biochemistry and scientific writing, José Cola Zanuncio and Welligton Garcia Campos contributed to scientific writing and review of the manuscript, Maria Goreti de Almeida Oliveira guided the work and supervised the steps previously described.