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ARTICLE

Purification and characterization of trypsin produced by gut bacteria from *Anticarsia gemmatalis*

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Abstract

Purification of active trypsin in the digestive process of insects is essential for the development of potent protease inhibitors (PIs) as an emerging pest control technology and research into insect adaptations to dietary PIs. An important aspect is the presence of proteolytic microorganisms, which contribute to host nutrition. Here, we purified trypsins produced by bacteria *Bacillus cereus*, *Enterococcus mundtii*, *Enterococcus gallinarum*, and *Staphylococcus xylosus* isolated from the midgut of *Anticarsia gemmatalis*. The trypsins had a molecular mass of approximately 25 kDa. The enzymes showed increased activity at 40°C, and they were active at pH values 7.5–10. Aprotinin, bis-benzamidine, and soybean Kunitz inhibitor (SKTI) significantly inhibited trypsin activity. The L-1-tosylamido-2-phenylethylchloromethyl ketone (TPCK), pepstatin A, E-64, ethylenediamine tetraacetic acid, and calcium ions did not affect the enzyme activity at the concentrations tested. We infer the purified trypsins do not require calcium ions, by which they differ from the trypsins of other microorganisms and the soluble and insoluble trypsins characterized from *A. gemmatalis*. These data suggest the existence of different isoforms of trypsin in the velvetbean caterpillar midguts.

KEYWORDS
bacteria, pest control, protease inhibitor, trypsin

1 | INTRODUCTION

There is a growing interest in alternative pest control methods for agricultural use, and endogenous mechanisms of plant resistance against herbivory are a major focus for possible application in integrated pest management (El-Latif, 2015; Huffaker, 2015; Jayachandran, Hussain, & Asgari, 2013; Khandelwal et al., 2016; Lombardo, Coppola, & Zelasco, 2016; Scott, Thaler, & Scott, 2010). Protease inhibitors (PIs) are biopesticides widely recognized for their potential use in insect pest control, since the chronic ingestion of PIs affect the bioavailability of essential amino acids due to inhibition of digestive proteases (Lawrence & Koundal, 2002).

Many studies have shown that PIs negatively affect the development, behavior, biochemistry, and physiology of the velvet caterpillar *Anticarsia gemmatalis*, a key pest of soybean (Macedo, Freire, Kubo, & Parra, 2011; Moreira, Campos, Ribeiro, Guedes, & Oliveira, 2011; Paixão et al., 2013; Pilon, Oliveira, & Guedes, 2006). However, insects may adapt to PIs by an increase in protease production and synthesis of structurally different digestive enzymes or insensitive to PIs (Bown, Wilkinson, & Gatehouse, 1997; Oliveira et al., 2013; Oppert, Morgan, Hartzer, & Kramer, 2005; Souza et al., 2016; Tamaki & Terra, 2015). Furthermore, proteases secreted by bacterial from the midgut of insects may improve herbivore adaptation to PIs and other mechanisms of plant resistance (Sethi et al., 2011; Shinde et al., 2017; Visôtto, Oliveira, Guedes, Ribon, & Good-God, 2009a). In fact, there is strong evidence that trypsin activity in the midgut of *A. gemmatalis* is benefited by the insect gut microbiota (Visôtto et al., 2009a).

The successful use of PIs against insects depends on the target species and its ruling classes of proteases (Ahn & Zhu-Salzman, 2009; Moreira et al., 2011; Silva et al., 2015). Thus, the first steps toward understanding the adaptive mechanisms of *A. gemmatalis* to PIs must be supported by structural and functional studies of all isoforms of serine proteases, particularly trypsin, which is notoriously active in the insect gut. The trypsins (EC 3.4.21.4) cleave preferentially protein chains at the carboxyl side of basic amino acids, such as arginine and lysine (Mares-Guia & Shaw, 1965). The enzyme-kinetic characteristics and tertiary structure of these trypsins provide the understanding of the connection and affinity between enzyme inhibitor (Cuccioloni et al., 2016; Joshi, Mishra, Suresh, Gupta, & Giri, 2013; Liu, Zhang, Zeng, & Hu, 2017).

The characterization of unpurified enzymes of *A. gemmatalis*, such as trypsin linked to the peritrophic membrane (Xavier, Oliveira, Guedes, Santos, & Simone, 2005), soluble trypsin and cysteine proteases present in the insect gut (Mendonça, Oliveira, Visôtto, & Guedes, 2012; Oliveira, Simone, Xavier, & Guedes, 2005), and serine and cysteine proteases produced by gut bacterial (Pilon, Visôtto, Guedes, & Oliveira, 2013) has been performed. Thus, as a step forward for the development of pest control strategies, through the use of PIs, and for the elucidation of possible mechanisms of adaptation of the velvetbean caterpillar to the inhibitors, the objective of this study was to purify and characterize trypsins produced by bacteria isolated from the gut of *A. gemmatalis*.

2 | MATERIALS AND METHODS

2.1 | Microorganisms and culture condition

The bacteria *Bacillus cereus*, *Staphylococcus xylosus*, *Enterococcus mundtii*, and *Enterococcus gallinarum*, used in this study, were isolated from the midgut of *A. gemmatalis* by Visôtto, Oliveira, Ribon, Mares-Guia, and Guedes (2009b).

Preinoculums were prepared in 25 ml of brain heart infusion (BHI) culture medium with 0.1% bovine serum albumin (BSA) and maintained at 200 rpm and 37°C. An aliquot of 1 ml of the preinoculum of each bacterial culture was removed at an absorbance at 600 nm, equal to 0.2, transferred to 50 ml sterile BHI, with 0.1% BSA, and maintained at 200 rpm and 37°C for 24 h. At intervals of 2 at 2 h, 1 ml aliquots were taken for determination of absorbance, and the amount of protein and enzymatic activity in cultures.

2.2 | Preparation of the enzyme extract

One milliliter of each preinoculum was removed to an absorbance at 600 nm equal to 0.2, and individually inoculated in 200 ml of a BHI with 0.1% BSA, and maintained at 37°C, 200 rpm for 4 h to *E. mundtii* and *S. xylosus*, and 6 h for *B. cereus* and *E. gallinarum*. The bacteria were centrifuged at 10,000 rpm for 20 min at 4°C. The supernatant containing the enzyme extract was removed and concentrated by ultrafiltration in an Amicon Ultra-15 with a porous membrane molecular limit of 3 kDa (Millipore), and stored at −20°C for later use as the enzyme source for purification.

2.3 | Protein concentration and trypsin activity

The protein concentration of the extracts of bacterial cultures was determined as described by Bradford (1976), with BSA solutions of 0–0.2 mg/ml as standards.

Trypsin activity was determined as described by Erlanger, Kokowsky, and Cohen (1961), with a final concentration of a 0.5 mM *N*-α-benzoyl-L-Arg-*p*-nitroanilide (L-BApNA) in 0.1 M Tris-HCl buffer (pH 8.2). Initial rates were determined by the formation of ρ-nitroanilide product by measuring absorbance at 410 nm versus time (2.5 min). The calculations were performed considering the specific molar extinction coefficient of 8,800 M^{-1} cm^{-1} for the product.

2.4 | Purification of bacterial trypsin

Supernatants from enzyme extracts were subjected to precipitation with ammonium sulfate to 70% saturation. The amount of ammonium sulfate required to achieve this range of saturation was calculated based on the volume of supernatant of the culture that was subjected to precipitation (Scopes, 1994). The formed suspension was kept under agitation for 1 h, and 1 h to stand under cooling. Subsequently, the extracts were centrifuged at 12,000 rpm for 60 min at 4°C, and then the precipitates were collected. The precipitates were solubilized in Tris-HCl (10 mM, pH 7.5) and dialyzed overnight with the same buffer at 4°C, using molecular membranes with pore limit of 3 kDa (Sigma-Grade). After this period, centrifugation was performed 33,000 rpm for 60 min at 4°C to remove impurities. The supernatants were removed after being subjected to affinity chromatography on a column of ρ-aminobenzamidine agarose (2.5 ml) (Sigma®) equilibrated with Tris-HCl buffer (0.05 M, pH 7.5) and 0.5 M NaCl. Elution of the proteins was performed with glycine buffer (0.05 M, pH 3.0) with continuous flow of 1 ml/min and collected in 1.5 ml fractions. The eluted fractions were monitored by determining the Abs_{280}, and determining trypsin activity. The fractions corresponding to the elution peak were pooled and stored at −20°C for subsequent use in enzymatic characterization tests.

2.5 | Polyacrylamide gel electrophoresis

After each purification step, electrophoresis was performed with the samples following Laemmli (1970), with 12% polyacrylamide gel in the presence of 0.1% sodium dodecyl sulfate (SDS-PAGE). The experiment was conducted at a constant voltage of 100 V for 1 h and 20 min at room temperature. The staining of the gels was performed with silver nitrate, according to Blum, Beier, and Gross (1987). After the electrophoretic run, the gels were fixed in 100 ml of solution of methanol, glacial acetic acid, and water (50:12:38) for 2 h, followed by three washes of 10 min, with 50% ethanol solution. Subsequently, they were washed for 1 min in solution 0.02% sodium thiosulfate w/v, quickly washed with distilled water, and incubated for 15 min in a solution of 0.2% silver nitrate w/v containing 37 μl of 37% formaldehyde v/v. Gels were treated with the developing solution (4% sodium carbonate, 2 ml 0.02% sodium thiosulfate solution, and 50 μl of 37% formaldehyde) to visualize the protein bands. The reaction was stopped by adding acetic acid.

2.6 | Effect of pH and temperature

The pH profiles for trypsin activity from the bacterial isolates were determined with L-BApNA in the following buffer systems at 50 mM with 20 mM $CaCl_2$: citric acid/disodium phosphate (pH 3.0–6.5), Tris-HCl (pH 7.5–8.5), glycine/sodium hydroxide (pH 9.5–10.5), and disodium phosphate/sodium hydroxide (pH 11.0). The effects of

temperature on the enzyme activity were determined using the same substrate (L-BApNA) at temperatures ranging from 10°C to 50°C, using a spectrophotometer (Thermo Scientific UV-VIS, model Evolution 200) with temperature control system.

2.7 | Kinetic parameters

The determination of the kinetic parameters, K_M and V_{max}, were performed in the buffers, and in the best activities temperatures obtained using L-BApNA in a concentration range varying from 0.05 to 2.0 mM. Kinetics parameters were estimated by nonlinear regression (Michaelis-Menten equation) with the curve-fitting procedure of Sigma Plot (Systat, 2008).

2.8 | Effect of $CaCl_2$ and PIs

The effects of calcium ions on the enzyme activity were measured by the addition of different concentrations of $CaCl_2$ (5–30 mM) in the reaction mixture, using the temperature of 40°C and buffers with pH where best activity was obtained for each isolate (pH 7.0 to *B. cereus*, pH 9.5 to *E. mundtii*, pH 8.5 to *E. gallinarum* and *S. xylosus*).

Different PIs were selected and tested on enzyme activity in different concentration ranges according to their estimated K_i. Inhibition assays were performed with the following inhibitors: aprotinin (1.0–2.0 μM), bis-benzamidine (10–40 μM), soybean Kunitz trypsin inhibitor—SKTI (0.5–1.5 μM), *N*-α-tosyl-L-phenilalanine chloromethyl ketone—TPCK (5–20 μM), pepstatin A (1.0–2.0 μM), E-64 (5.0–50 μM), and ethylenediamine tetraacetic acid—EDTA (55–75 μM). The enzyme extracts were incubated for 25 min with each inhibitor prior to the addition of substrate to the reaction mixture, time required for the occurrence of possible enzyme–inhibitor interaction. After this incubation, the trypsin activity was subsequently determined as previously described.

The results were submitted to analysis of variance (ANOVA) and Tukey's test ($P < 0.05$) using the PROC GLM program; SAS Institute (2001).

3 | RESULTS

3.1 | Bacterial growth and trypsin activity

The profile of growth of *B. cereus*, *E. mundtii*, *E. gallinarum*, and *S. xylosus*, isolated from the midgut of *A. gemmatalis*, and the trypsin activity produced by these microorganisms were describe as a function of time (Figure 1). The bacteria growth and the enzyme activity began immediately after the microbial inoculation in culture medium. The exponential growth of the microorganisms occurred in 4 h of incubation for *B. cereus* and *E. mundtii*, and 6 h for *E. gallinarum* and *S. xylosus*. From these times, the growth speed was reduced and the cultures entered the stationary phase.

Enzyme production was increased in the exponential phase and early stationary growth stage. The greater activity of the trypsin was obtained after 4 h of incubation for *E. mundtii* and *S. xylosus*, and after 6 h for *B. cereus* and *E. gallinarum* (Figure 1). During the stationary phase, when the bacteria had reached peak enzyme, the trypsin activity was low, suggesting that the enzyme production is associated with growth and metabolism of the active microorganisms.

According to these results, the times of increased activity of trypsin were selected: 4 h for *E. mundtii* and *S. xylosus*, and 6 h for *B. cereus* and *E. gallinarum*, to obtain the enzyme extract necessary for the purification step.

3.2 | Purification of bacterial trypsin

The chromatographic profile for each purified enzyme (Figure 2) showed two predominant peaks after extensive washing with equilibration buffer. The first peak corresponds to proteins that do not have affinity for ρ-aminobenzamidine

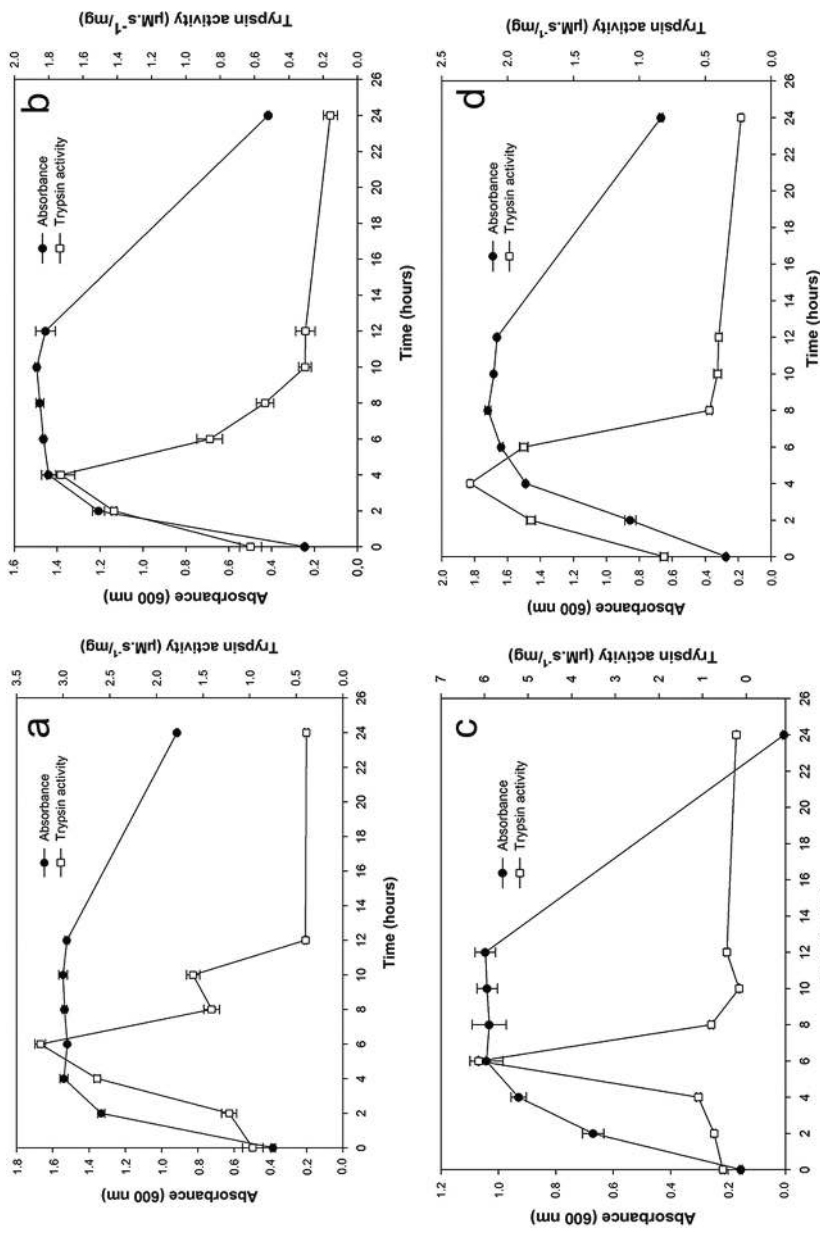

FIGURE 1 Growth curve and specific activity of serine protease of *Bacillus cereus* (a), *Enterococcus mundtii* (b), *Enterococcus gallinarum* (c), and *Staphylococcus xylosus* (d) isolated from the midgut of *Anticarsia gemmatalis*. Each symbol represents the mean and standard deviation of three replicates

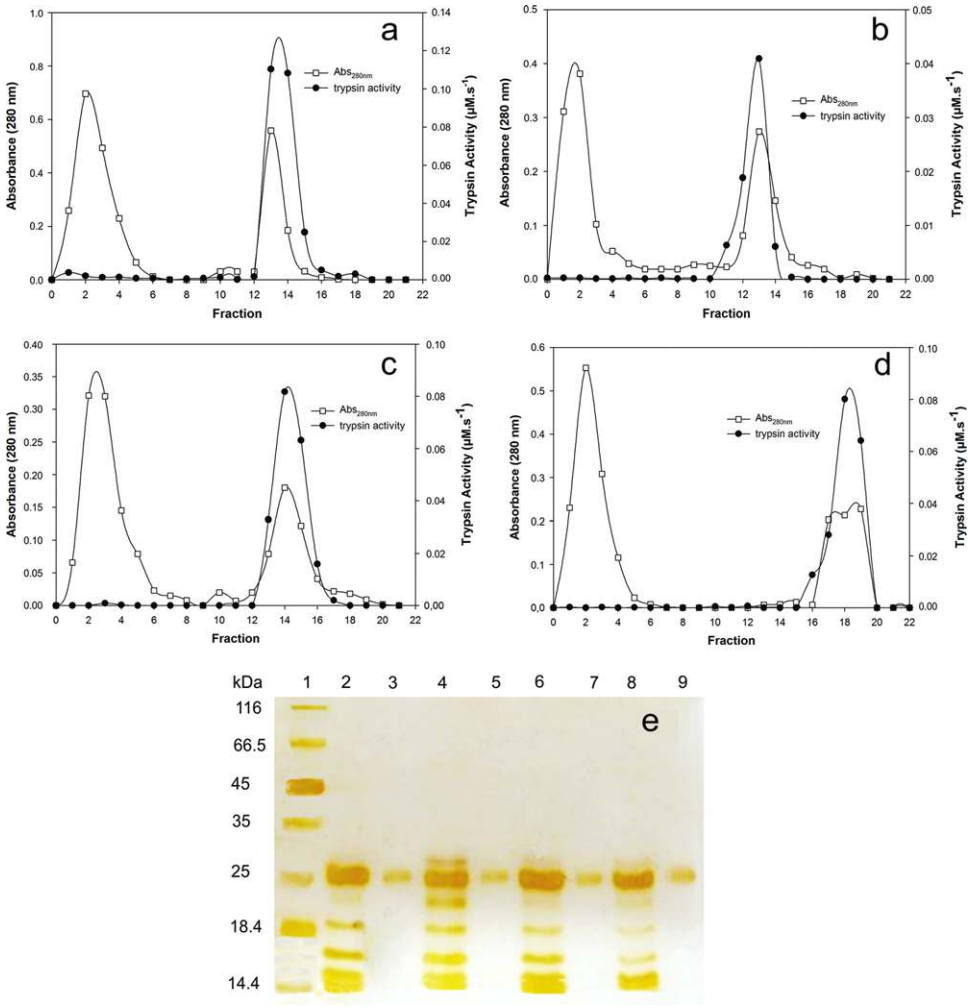

FIGURE 2 Elution profile of trypsin of *Bacillus cereus* (a), *Enterococcus mundtii* (b), *Enterococcus gallinarum* (c), and *Staphylococcus xylosus* (d) using the ρ-aminobenzamidine agarose column. Electrophoresis profile of the samples analyzed by SDS-PAGE (e); column 1: MW Standards; columns 2, 4, 6, and 8: crude enzyme extract of *B. cereus*, *E. mundtii*, *E. gallinarum*, and *S. xylosus*, respectively; columns 3, 5, 7, and 9: trypsin purified of *B. cereus*, *E. mundtii*, *E. gallinarum*, *and S. xylosus*, respectively

and do not possess the ability to efficiently hydrolyze the L-BApNA. The second minor protein peak corresponds to proteins that bind to ρ-aminobenzamidine, and has significant proteolytic ability against L-BApNA (Figure 2–d). These data are consistent with expectations, considering that trypsins are capable of hydrolyzing the L-BApNA substrate.

When subjected to electrophoresis, the samples eluted from the column migrate as a single band, while the crude extract showed several bands (Figure 2e), demonstrating the efficiency of the purification process. Comparing the distance of pattern migration (column 1) and the samples applied to columns 3, 5, 7, and 9, a molecular mass of approximately 25 kDa was obtained.

The steps and yields of purification (Table 1) show that the trypsin from *B. cereus* and *S. xylosus* has higher purification factor (155.65-, 152.63-fold, respectively), due to higher recovery of enzyme activity to the end of the process, followed by 143.62-fold of *E. gallinarum* and 140.36-fold of *E. mundtii*.

TABLE 1 Steps in purification of trypsin produced by bacteria isolated from the midgut of *Anticarsia gemmatalis*

Purification Steps	Bacteria Species	Total Protein (mg)	Total Activity ($\mu M \cdot s^{-1}$)	Specific Activity ($\mu M \cdot s^{-1}$/mg)	Purification Factor (X)	Yield (%)
Crude extract		2.03	0.16	0.08	1.00	100.00
Ammonium sulfate	*B. cereus*	0.16	0.14	0.87	10.87	87.50
ρ-Aminobenzamidine agarose		0.01	0.10	12.52	155.65	71.43
Crude extract		2.16	0.16	0.07	1.00	100.00
Ammonium sulfate	*E. mundtii*	0.22	0.15	0.69	9.50	93.75
ρ-Aminobenzamidine agarose		0.01	0.10	10.20	140.36	66.67
Crude extract		2.30	0.17	0.07	1.00	100.00
Ammonium sulfate	*E. gallinarum*	0.22	0.14	0.61	8.47	82.35
ρ-Aminobenzamidine agarose		0.01	0.09	10.37	143.62	64.29
Crude extract		1.99	0.16	0.08	1.00	100.00
Ammonium sulfate	*S. xylosus*	0.21	0.14	0.69	8.57	87.50
ρ-Aminobenzamidine agarose		0.01	0.10	12.26	152.63	71.43

3.3 | Effect of pH and temperature on trypsin activity

The trypsins were highly active in the pH range of 7–9.5, and showed different values (pH 7.0 for *B. cereus*, pH 9.5 for *E. mundtii*, and pH 8.5 for *E. gallinarum* and *S. xylosus*), but within the expected range of neutral to alkaline (Figure 3). As for the effect of temperature, all purified trypsin activity reached a plateau above 25°C (Figure 3).

3.4 | Kinetic characteristics

The K_M and V_{max} values of bacterial enzymes and soluble and insoluble extract of velvetbean caterpillar are shown in Table 2. The K_M values of trypsin of *B. cereus*, *E. mundtii*, *E. gallinarum*, and *S. xylosus* were 0.18, 0.22, 0.35, and 0.21 mM, respectively. The K_M obtained in this study are similar to those found for trypsins present in the soluble and insoluble extracts of the midgut of *A. gemmatalis*.

3.5 | Effect of $CaCl_2$ and proteinase inhibitors

The activity of the purified trypsin from *B. cereus*, *E. mundtii*, *E. gallinarum*, and *S. xylosus* was not affected by the presence of calcium ions in the concentrations tested. However, some inhibitors significantly reduced the enzymatic activity (Table 3). We found that aprotinin significantly reduced ($P < 0.05$) the activity of all enzymes due to increased concentration. The bis-benzamidine also affected significantly ($P < 0.05$) enzymatic activity in all concentrations; however, the concentration of 40 μM was more effective. The SKTI inhibitor was also tested on bacterial enzymes, and it showed to be very effective in the concentration of 1.5 μM for all enzymes. The TPCK, pepstatin A, E-64, and EDTA did not significantly affect ($P > 0.05$) the activity of the purified trypsins.

4 | DISCUSSION

Purification and characterization of insect trypsins have been performed in order to elucidate its structure and, thereby, to develop alternative insecticides molecules, such as PIs. Many active proteases in the intestine of insects are considered to be sensitive and others insensitive to PI, and little is known about the inhibitor and protease relationship of the associated microbiota. Recently, Shindle et al. (2017) reported that bacterial gut of *Helicoverpa armigera*

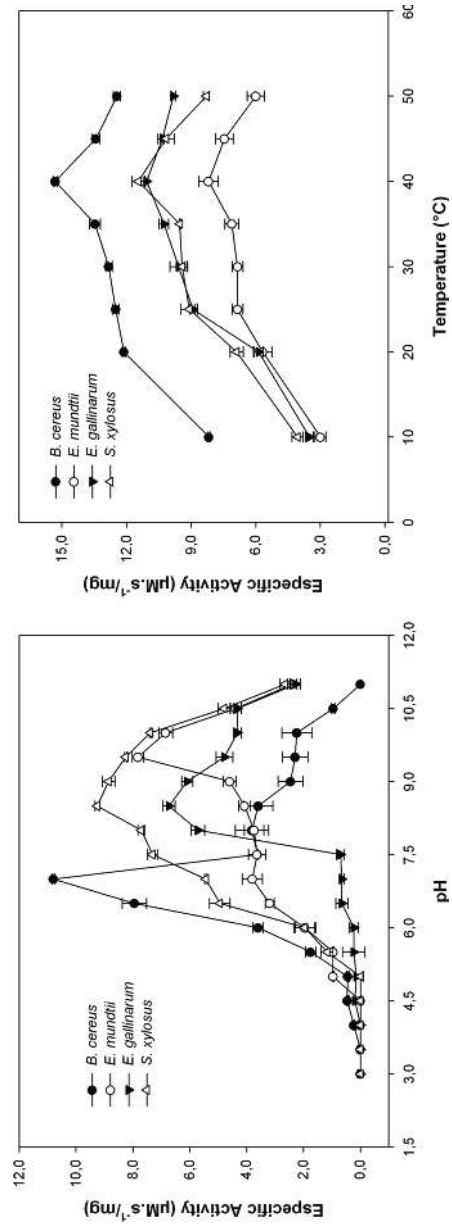

FIGURE 3 Effect of pH and temperature on the activity of trypsin purified from bacteria isolated from the midgut of *Anticarsia gemmatalis*. Each symbol represents the mean and standard error of three replicates

TABLE 2 Kinetic parameters of trypsin purified from bacteria isolated from the midgut *Anticarsia gemmatalis*

	Kinetics Parameters		
Species	**K_M (mM)**	**V_{max} (nM·s^{-1})**	**Reference**
Bacillus cereus (purified extract)	0.18	120.72	Present work
Enterococcus mundtii (purified extract)	0.22	117.89	Present work
Enterococcus gallinarum (purified extract)	0.35	116.79	Present work
Staphylococcus xylosus (purified extract)	0.21	105.43	Present work
Anticarsia gemmatalis (soluble extract)	0.32	480.00	Oliveira et al. (2005)
Anticarsia gemmatalis (insoluble extract)	0.23	21.80	Xavier et al. (2005)

TABLE 3 Effect of different inhibitors on the specific activity of trypsin purified from bacteria isolated from the midgut of *Anticarsia gemmatalis*

Inhibitor/Concentration (μM)	**Specific Activity of Trypsin (μM·s^{-1}·mg Protein)**			
Aprotinin	*B. cereus*	*E. mundtii*	*E. gallinarum*	*S. xylosus*
0.0	12.41 ± 0.23a	9.40 ± 0.38a	9.56 ± 0.19a	19.75 ± 0.09a
1.0	2.07 ± 0.29b	0.02 ± 0.01b	0.18 ± 0.06b	0.17 ± 0.08b
1.5	0.16 ± 0.10b	0.18 ± 0.09b	0.18 ± 0.02b	0.07 ± 0.02b
2.0	0.02 ± 0.02b	0.22 ± 0.11b	0.12 ± 0.01b	0.03 ± 0.03b
Bis-benzamidine				
0.0	12.41 ± 0.23a	9.32 ± 0.18a	10.75 ± 0.14a	14.05 ± 0.32a
10.0	7.55 ± 0.22b	2.21 ± 0.24b	6.15 ± 0.02b	6.75 ± 0.27b
20.0	5.22 ± 0.36c	1.00 ± 0.20c	3.96 ± 0.19c	4.09 ± 0.47c
40.0	2.89 ± 0.19d	0.31 ± 0.05d	2.13 ± 0.08d	1.28 ± 0.05d
SKTI				
0.0	12.41 ± 0.23a	7.02 ± 0.33a	10.68 ± 0.07a	14.00 ± 0.05a
0.5	7.30 ± 0.33b	4.10 ± 0.38b	0.46 ± 0.04b	0.70 ± 0.02b
1.0	3.95 ± 0.39c	0.03 ± 0.01c	0.37 ± 0.08b	0.13 ± 0.01c
1.5	1.51 ± 0.14d	0.04 ± 0.01c	0.14 ± 0.008c	0.03 ± 0.006c
TPCK				
0	13.07 ± 0.35a	7.70 ± 0.52a	9.63 ± 0.14a	11.50 ± 0.01a
5–20	nd	nd	nd	nd
Pepstatin A				
0	11.74 ± 0.06a	9.32 ± 0.18a	9.00 ± 0.32a	13.86 ± 0.33a
1–2	nd	nd	nd	nd
E-64				
0	12.25 ± 0.01a	9.04 ± 0.06a	8.21 ± 0.21a	9.51 ± 0.25a
5–50	nd	nd	nd	nd
EDTA				
0	9.67 ± 0.27a	7.80 ± 0.59a	10.40 ± 0.21a	14.44 ± 0.02a
55–75	nd	nd	nd	nd

Mean ± SEM. Means followed by the same letter vertically do not differ statistically by Tukey's HSD test ($P > 0.05$). nd: nondetectable levels of inhibition.

(Lepidoptera: Noctuidae) have an important impact on digestive physiology of this insect. Proteases synthesized by *Bacillus* sp. YP1 isolated from *H. armigera* significantly degraded the PIs present in seeds. Such a finding suggests that bacterial proteases have significant contribution in the degradation and detoxification of host seed protein rich in PIs (Shindle et al., 2017). In this sense, it is crucial to know the set and kinetic-enzymatic aspects of all proteases involved in the digestion process. In the present study, we purified and characterized the trypsin produced by the gut bacteria of *A. gemmatalis*.

Research related to the role of the gut microbiota of insects suggests that, microorganisms synthesize a variety of digestive enzymes that contribute to host digestion, and that the catalytic potential of these proteins is closely related to the characteristics of the physiological environment in which they are secreted (Anand et al., 2010; Liang, Fu, & Liu, 2015). We found that trypsin purified from bacteria showed bigger activity in neutral–alkaline medium. It is known that lepidopterous larvae have high luminal pH, which favors enzymatic activity at alkaline pH (Berenbaum, 1980; Terra & Ferreira, 1994). Our results corroborate those reported for chymotrypsin-like produced by *Bacillus subtilis* isolated from the gut of *H. armigera* (Shinde, Shaikh, Padul, & Kachole, 2012).

Proteases can be classified based on their sensitivity to various inhibitors (Elhoul et al., 2015; Rao, Aparna, Ghatge, & Deshpande, 1998). Enzymes isolated from *Streptomyces koyangensis* (Elhoul et al., 2015) and *Bacillus circulans* (Benkiar et al., 2013) were characterized and classified as serine proteases through biochemical evaluations, including determination of the effect of PIs. Here, purified enzymes from gut bacteria of *A. gemmatalis* were significantly inhibited by a natural peptide that acts as a competitive inhibitor of trypsin, aprotinin (Laskowski & Kato, 1980); by a synthetic molecule that acts as a parabolic partially competitive inhibitor of trypsin, called the bis-benzamidine (Junqueira, Silva, & Mares-Guia, 1992) and by a soybean trypsin inhibitor, SKTI. These results, together with the molecular mass obtained, and to the absence of enzymatic inhibition upon the treatment with inhibitors TPCK (irreversible inhibitor of chymotrypsin), pepstatin A (inhibitor of aspartyl protease), E-64 (irreversible inhibitor of cysteine proteases), and EDTA (chelating Ca^{2+} and other divalent metal and acts on metalloproteases and proteases activated by metals), suggest that the enzymes evaluated in this study are trypsins. The weak impact of EDTA on the activity of these enzymes also suggests that such proteases do not rely on any divalent cation, consistent with the fact that proteases are generally independent of cofactors (Uttatree & Charoenpanich, 2016).

The calcium ion regulates many biological systems by interacting with proteins with different affinities in different biological environments. The binding of Ca^{2+} to some proteins leads to an increase in stability and to changes in conformation of the calcium-binding proteins (Kotomán, Laczkó, Szabó, & Simon, 2003). Numerous studies confirm the positive effect of calcium ions (3–10 mM) on serine protease activity isolated from *B. circulans* (Benkiar et al., 2013; Rao, Sathish, Ravichandra, & Prakasham, 2009); *S. koyangensis* TN650 (Elhoul et al., 2015); *Bacillus* sp. strain B001 (Deng, Wu, Zhang, & Wen, 2011); *Bacillus laterosporus*-AK1 (Arulmani et al., 2007); and *Bacillus caseinilyticus* (Mothe & Sultanpuram, 2016). Many of these authors believe that the removal of Ca^{2+} from the strong binding site is associated with a significant decrease in stability, especially at high temperatures. Studies of DNA and sequence homology of bacterial proteases have demonstrated that the binding sites of calcium ions are variable in relation to the number and sequence of amino acids (Rao et al., 1998). Of the four calcium-binding sites in bacterial serine protease, two sites, that is, sites 3 and 4, are absent in the thermolabile neutral proteases of *Bacillus amyloliquefaciens* and *B. subtilis* (NprA), whereas in NprB, Asn187 in site 3 is replaced by Arg. Such changes are responsible for the loss of thermostability and can be detected by sequence homology studies (Tran, Wu, & Wong, 1991). In this study, the trypsin activities were not affected by calcium ions in the concentrations tested (5–30 mM). According to Rao et al. (1998), some proteases of bacterial origin do not require divalent ions to act efficiently.

Through the obtained results it can be inferred that bacteria present in the gut of *A. gemmatalis* synthesize trypsins. Previous studies have shown that these enzymes contribute significantly to protein digestion along with trypsins produced by the insect itself, demonstrating that there is a diversity of digestive trypsins active in *A. gemmatalis*. In addition, it provides basic information for analyses of molecular modeling and docking studies that will enable the development of potent organic PIs, peptides, or peptide mimetics, which could be used in plant protection and agricultural pest control.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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