Proteolytic activity characterization of Podisus nigrispinus gut contents and apparent lack of Cry1Ac toxin hydrolysis

Andresa Cristina Batista de OLIVEIRA¹, Herbert Álvaro Abreu de SIQUEIRA¹, Valéria WANDERLEY-TEIXEIRA², Álvaro Aguiar Coelho TEIXEIRA²
¹Departamento de Agronomia (Entomologia), Universidade Federal Rural de Pernambuco, Brazil
²Departamento de Morfologia e Fisiologia Animal, Universidade Federal Rural de Pernambuco, Brazil

Abstract

The use of plants genetically modified with Bacillus thuringiensis Berliner (Bt) genes provides an unparalleled area wide protection to insect pests. However, the distribution and interaction of Cry toxins produced by Bt plants may impose harmful effects on non-target insects, including both phytophagous and predators exposed by direct feeding on plants or transfer via prey food chain. The stinkbugs, Podisus spp., serve as important beneficial agents against Lepidoptera larvae, which can feed on Bt plants. Therefore, this work aimed to determine the in vitro proteolytic activity of digestive enzymes of Podisus nigrispinus (Dallas) that plays a role (by hydrolysis) in these natural enemies' refractoriness to Bt Cry1Ac toxin. Firstly, a general characterization of proteases was performed to provide insights on this stinkbug hydrolysis. BApNA, SAAPFpNA, and SAAPLPNA were in this order the most active digestive enzymes of Podisus nigrispinus (Dallas) that plays a role (by hydrolysis) in these natural enemies' refractoriness to Bt Cry1Ac toxin. Secondly, this work aimed to determine the in vitro proteolytic activity of digestive enzymes of Podisus nigrispinus (Dallas) that plays a role (by hydrolysis) in these natural enemies' refractoriness to Bt Cry1Ac toxin. Lastly, a general characterization of proteases was performed to provide insights on this stinkbug hydrolysis. BApNA, SAAPFpNA, and SAAPLPNA were in this order the most active digestive enzymes of Podisus nigrispinus (Dallas) that plays a role (by hydrolysis) in these natural enemies' refractoriness to Bt Cry1Ac toxin. It is important to note that the stinkbug digestive system virtually unprocessed.

Key words: predatory stinkbug, digestive protease, Bt crops, refractoriness.

Introduction

Plant breeders have been able to create transgenic plants that express novel compounds with the advent of molecular genetic-based technologies. Perhaps the best known are the Bt plants, which express genes from the bacterium Bacillus thuringiensis Berliner (Andow and Hilbeck, 2004). This gram-positive bacterium occurs in soils (Hoffe and Whiteley, 1989), and during its sporulation phase produces crystalline inclusions containing delta-endotoxins or Cry proteins. When these crystals are ingested by insects, they are solubilized in the insect’s gut and activated by proteolytic enzymes. After activation, the toxins bind to specific receptors located on the brush border membrane of midgut cells, causing imbalance in ionic concentration, destroying cells, and ultimately leading to insect’s death (Knowles, 1994; Bravo et al., 2007; Gómez et al., 2007).

Bt plants expressing cry genes are in expansion, therefore it is increasingly important to study the effects of these toxins on non-target organisms (Andow and Hilbeck, 2004; Sanvido et al., 2007). The worldwide use of genetically modified plants has raised questions about their potential impact on biodiversity, particularly on phytophagous insects and natural enemies (Fontes et al., 2002). Insects and natural enemies can be exposed to toxins in many ways, either through direct exposure by feeding on the plant or its products or through indirect routes such as feeding on contaminated preys (O’Callaghan et al., 2005).

Stinkbug species of the genus Podisus are important predators of Lepidoptera larvae in the integrated pest management (De Clercq, 2000; Zanúnico et al., 2002). In North America, Podisus maculiventris (Say) (Heteroptera Pentatomidae) is probably the most important species in that context while Podisus nigrispinus (Dall) (Heteroptera Pentatomidae) is the main stinkbug predator in the Central and South America (Zanúnico et al., 2002). In addition to feeding on prey, these stinkbugs also feed directly on the host plants of their preys at early instars (Stoner et al., 1974; Valicente and O’neil, 1995; Coll, 1998). Such behaviour increases the stinkbugs risk of entering in contact with Cry toxins when exposed to Bt plants.

In Brazil, Bt cotton expressing the Cry1Ac toxin was commercially released in 2006 (CTNBIo, 2016). The P. nigrispinus feeds on the main cotton defoliators such as Alabama argillacea (Hubner) and Spodoptera spp., which are invariably exposed to Cry1Ac toxin. According to Torres et al. (2010), P. nigrispinus is unable to acquire the Cry1Ac toxin when feeding directly on transgenic cotton, but studies with P. maculiventris (Torres and Ruberson, 2008) showed that this predator acquires the toxin after ingesting larvae that have fed on transgenic cotton. Despite this, P. nigrispinus apparently suffers no adverse effects from the toxin (Torres et al., 2006). However, there is no information about the digestive physiology of P. nigrispinus and the underlying mechanisms of such Cry1Ac refractory response, which may allow the predator to tolerate or degrade Cry1Ac toxin.

Studies have assessed the effect of Bt plants expressing protease inhibitors on predators of the genus Podisus (Bell et al., 2003; 2005). Bell et al. (2003) reported re-
duction in fecundity of the generalist predator *P. maculiventris* using prey insects that had ingested transgenic potato leaves. Also, Bell *et al.* (2005) characterized the proteases in the gut and salivary glands of *P. maculiventris* by using inhibitors expressed in Bt plants and suggested alterations in the predator’s proteolytic activity. Second instar *P. nigrispinus* fed with *Spodoptera frugiperda* (Smith) reared on Bt cotton, expressing the Cry1Ac toxin, showed ultrastructural and histochemical changes in digestive cells of the middle region of the midgut resulting from the alteration of the perimicrovillar matrix of the predator gut (Cunha *et al.*, 2012).

Considering the agricultural importance of the *P. nigrispinus* in Brazil and that Cry1Ac toxin has been detected in excrements of non-target insects (Torres *et al.*, 2006), the current study tests the hypothesis that this predatory stinkbug may partially process the Cry1Ac toxin in its digestive system. Therefore, the current study evaluated whether the active toxin undergoes in vitro proteolytic processing after interaction with the digestive tract of *P. nigrispinus*. Also, gut protease activity was partially characterized, aiming to understand the basis of *P. nigrispinus* protease activity against Cry1Ac toxin.

### Materials and methods

**Insect rearing**

Adults of *P. nigrispinus* used in the experiments were obtained from an existing rearing colony at the Biological Control Laboratory, Universidade Federal Rural de Pernambuco (UFRPE). They were fed on larvae and pupae of *Tenebrio molitor* (Coleoptera Tenebrionidae), according to Zanuncio *et al.* (1994), at 26 ± 1.5 °C, 60-75% RH, and L12:D12 photoperiod.

**Extraction of gut contents**

Adults of *P. nigrispinus* were chilled to −20 °C for about 5 minutes and then dissected under a stereomicroscope (Olympus SZ61, Olympus®, Center Valley, PA, USA). The guts were immediately transferred to chilled 1.5-mL microfuge tubes containing 0.15 M NaCl solution (1 gut 40 µL 1.5-mL microfuge tubes containing 0.15 M NaCl solution (1 gut 40 µL 1.5-mL microfuge tubes containing 0.15 M NaCl and 20 mM CaCl2). Fifty microliters of each substrate diluted in the same buffers were added on top of 50 µl of diluted samples within microtiter plate wells to initiate the reaction. All tests were performed in triplicate and blanks were run in all appropriate cases. After 12 hours of incubation at 30 °C, the absorbance was monitored at 405 nm using a microplate reader (Biotek®, Winooski, VT, USA) linked to the Gen5® software. The experiments were repeated three times and means of activity were obtained from three replicates.

**Gut proteases inhibition**

The effect of proteinase inhibitors on the proteolytic activity of the digestive tract was evaluated toward the trypsin, chymotrypsin, and elastase activities. Five inhibitors were used: trans-epoxysuccinyl leucylamide-L-(4-guanidine) butane (E-64) for cysteine-like proteases; phenylmethylsulfonyl fluoride (PMSF) for serine-like proteases; ethylenediamine tetra-acetic acid (EDTA) for metallo-proteases; Nα-Tosyl-L-lysine chloromethyl ketone hydrochloride (TLCK) for trypsin; and N-Tosyl-L-phenylalanine chloromethyl ketone (TPCK) for chymotrypsin-like proteases. They were prepared in aqueous (E-64, TLCK, and EDTA), ethanol (TPCK), or methanol (PMSF) solutions at concentrations of 10−5, 10−4, 10−3, 10−2, 10−1, and 100 mM and stored at −20 °C. The incubations were performed at pH 6, with the gut contents pre-incubated with inhibitors at 30 °C for 15 minutes before adding the substrate. Absorbance was monitored at 405 nm, and the inhibitory activity was shown as a residual percentage when compared with the control test. All tests were performed in triplicate and blanks were run in all appropriate cases. The experiments were repeated three times and means of activity were obtained from three replicates.

**In vitro Cry1Ac toxin proteolysis**

The proteolytic processing of the Cry1Ac toxin was assessed by incubating 3 µg of active toxin with *P. nigrispinus* gut juice (8 µg of total protein) in a final volume of 13 µL of sodium citrate buffer (pH 6). The samples were incubated at 30 °C for eight different time intervals: 5, 10, 20, 30, 45, 60 minutes, 12 and 24 hours. The reactions were stopped by heating samples to 95 °C for 5 minutes. Two parts (26 µL) of Laemmli buffer (Laemmli, 1970) were added to samples and heated to 95 °C for 5 minutes before loading to a 7.5%-SDS polyacrylamide gel electrophoresis (SDS-PAGE) using an OmniPhor mini-electrophoresis apparatus (Scientonix, Inc., San Jose, CA, USA). Likewise, we evaluated the effect of four different concentrations of gut content proteins (5, 10, 15, and 20 µg) on the proteolytic processing of the Cry1Ac toxin (3 µg) along 12 h of incubation. After running, the gels were stained with Coomasie Brilliant Blue R-250 solution, followed by a destaining step. The Pierce® 3-Color Prestained Protein Molecular Weight Marker Mix (Thermo Scientific, Rockford, IL, USA) was used to follow the electrophoretic migration of the Cry1Ac protein.
Figure 1. Proteolytic activity of adult *P. nigrispinus* gut contents proteases at different pH conditions. The points represent the mean absorbance (± SE) at 405 nm of three replicates.

**Results**

The highest proteolytic activity towards the BApNA substrate took place at pH 5 while the highest activity towards SAAPFpNA and SAAPLpNA substrates occurred at pH 7, which suggest trypsin-like and chymotrypsin-like as well as elastase proteinases to have an optimum activity at different conditions. The activity of elastases was virtually half of the activities of trypsin- and chymotrypsin-like proteinases (figure 1).

Inhibitors of proteolytic enzymes were used to partially characterize the *P. nigrispinus* gut lumen proteinases. The most effective inhibitors against BApNA was TLCK inhibiting 100% of the activity at $10^{-4}$ mM followed by E-64 and TPCK that caused almost 100% of inhibition at $10^{-3}$ mM. PMSF, which specifically inhibits serine proteinases, reduced about 40% BApNA hydrolysis at a concentration of $10^{-2}$ mM but reduction did not reach 100% even at 1mM. Inhibition of BApNA-hydrolyzing enzymes from *P. nigrispinus* gut contents was not observed with increasing of the EDTA concentrations, conversely the activity was increased (figure 2).

The most effective inhibitor against the hydrolysis of SAAPFpNA was the TLCK with 100% of inhibition at the highest concentration of 1 mM. The EDTA, TPCK and PMSF also inhibited the hydrolysis of SAAPFpNA at 1 mM, causing at least 70% of activity inhibition. The PMSF indeed inhibited 65% of the activity even at its lowest concentration ($10^{-5}$ mM) evaluated when compared with EDTA and TPCK. The least effective inhibitor against SAAPFpNA was E-64, which caused only 30% of activity inhibition at the highest concentration assessed (figure 3).

The most effective inhibitor against SAAPLpNA was the TPCK, a specific inhibitor of chymotrypsin-like proteinases, which inhibited 90% of the substrate hydrolysis. EDTA and PMSF caused at 1 mM respectively 70% and 60% of SAAPLpNA-hydrolysis inhibition. The least effective inhibitors of SAAPLpNA hydrolysis were TLCK and E-64, which caused 30% and 40% of inhibition, respectively (figure 4).

Regarding the processing of the Cry1Ac toxin when
incubated with digestive enzymes for different intervals of time, no apparent toxin alteration was observed during shorter time intervals (5-60 minutes) (figure 5), but began to degrade by 12 h, with increased degradation being observed in the range of 24 hours. The Cry1Ac toxin showed little alteration when subjected to different concentrations of digestive enzymes (figure 6). However, slight increasing degradation of Cry1Ac occurred, that was proportional to the increase in the concentration of digestive enzymes of P. nigrispinus. All incubation times and concentrations of enzymes used showed a pattern of double bands of approximately 65 kDa, similar to that found in the activated toxin.

Discussion

Slightly acidic pH values (4.0-7.0) were found as optima for activity of P. nigrispinus gut proteases in agreement with a previous study in P. maculiventris where optimal enzymatic gut activity spanned between pH 5.5-6.5 (Bell et al., 2005) as well as in the P. nigrispinus midgut contents (pH ranging between 5.6 to 5.8) (Fialho et al., 2012). Hemipterans show in general acidic midgut contents (Terra and Ferreira, 1994). This condition is usually favourable to activity of cysteine-like proteases, which predominate in P. maculiventris (Bell et al., 2005) and P. nigrispinus gut (Fialho et al., 2012), respectively and inhibition of BAPNA hydrolysis by E-64 suggests this predominance, contradicting results found by Oliveira et al. (2006). BAPNA can be either hydrolyzed by cysteine-like proteases as observed by Novillo et al. (1997) in Leptinotarsa decemlineata (Say) (Coleoptera Chrysomelidae) or by trypsin-like proteases. However, the presence of cysteine-like proteases in the digestive tract of P. nigrispinus is likely because of the proteolytic optimum observed at an acidic pH (pH 5) (Nation, 2001), and the gut pH is a major determining factor of the activity of digestive enzymes (Terra and Ferreira, 1994), which can rule out the massive presence of trypsin-like proteases. According to these authors, cysteine-like proteinases are also inhibited by ketones such as TLCK and TPCK, which was also observed in this study. Despite that, presence of either trypsin-like or cysteine-like proteases appears to related to prey regime. Pascual-Ruiz et al. (2009) showed that the relative proteolytic activity in the P. maculiventris midgut depended on the prey consumed. They found trypsin- and chymotrypsin-like activities in P. maculiventris midgut when nymphs were fed on Spodoptera littoralis (Boisdvual) (Lepidoptera Noctuidae). The stinkbugs used in our experiments were fed with T. molitor, which expresses a diversity of proteases in its gut (Vinokurov et al., 2006), and that may have interfered in the results observed here.

The high hydrolysis of SAAPFpNA suggested the presence of chymotrypsin-like protease. The existing peak of activity at neutral pH (pH 7) and the effective inhibition of the SAAPFpNA hydrolysis by PMSF and TPCK support this hypothesis. Therefore, chymotrypsin-like proteases appear to be important enzymes for digestion in P. nigrispinus, although Fialho et al. (2012) did not observed the presence of chymotrypsin-like proteases in this insect. The great SAAPLPNA hydrolysis between pH 6 and 7 suggests the presence of elastase-
like proteases in the gut of *P. nigrispinus*. However, elastase activity in *P. nigrispinus* gut showed lower titration compared with cysteine-like and chymotrypsin-like proteases, being less common in this insect gut. Boyd et al. (2002) also found low levels of elastase in the anterior midgut of *Deraeocoris nebulosus* (Uhler) (Hemiptera: Miridae). The high inhibition of SAAPLpNA hydrolyses by TPCK and PMSF (Oppert et al., 1994) reinforce that *Podisus* protease is more likely to be chymotrypsin-like than elastase-like enzyme.

This paper represents the first study about the digestive physiology of *P. nigrispinus* associated with Bt toxins *in vitro*. Regarding the interaction of the enzymes of *P. nigrispinus* with activated Cry1Ac toxin, a little degradation of the toxin was observed in any of the treatments after a long period of incubation. Torres et al. (2006) showed that *Geocoris punctipes* (Say) (Hemiptera: Lygaeidae) was able to acquire the toxin diluted in water. They also showed that the toxin is excreted in the faeces of the insect, with peak of excretion occurring between 12 and 24 h, and no detection of toxin in the pirate bug body after 72 h. In 12 hours of incubation with digestive enzymes of *P. nigrispinus* there was little degradation of the toxin in our study. Thus, our findings suggest that the enzymes of the digestive tract of *P. nigrispinus* do not rapidly degrade the Cry1Ac toxin, and the fast excretion in hemipteran predators by the action of salivary enzymes (Cohen, 1995) and remains in the gut by the action of gut enzymes (Terra and Ferreira, 1994), so there may be differences in the proteolytic processing toxin *in vitro* and *in vivo*. However, Fialho et al. (2012) showed that extra-oral digestion is basically done by activity of collagenases to breakdown tissues, liquefying the prey for easy ingestion, and other enzymes would not be present or at very low titration. Although the effects of salivary enzymes on the Cry1Ac toxin was not evaluated in this study, the possibility of proteolytic activity from salivary glands towards this toxin is potentially ruled out. Although further evaluation of Bt toxin exposure to heteropteran gut lumen may be necessary, these results provide evidence to reject the hypothesis that the predator *P. nigrispinus* is able to completely degrade the Cry1Ac toxin. This may agree with Brandt et al. (2004), that studying Cry1Ac ingested by *Lygus hesperus* Knight (Heteroptera: Miridae) suggested that the toxin is mostly excreted because the active 65 kDa protein and degradation products were found in the faecal material. Nevertheless, whether the toxin passes through the digestive tract of *P. nigrispinus* and is eliminated intact in the excrements may account for its tolerance.

One challenge in integrated pest management is the conservation of natural enemies to aid in the control of pests. The use of Bt plants has drawn attention of researchers regarding its possible adverse effect on non-target organisms, including predators and parasitoids. There appears to have a good compatibility between the use of Bt plants and the predator stinkbug *P. nigrispinus*. It does not suffer adverse effects by toxins (Leite et al., 2014), which may be linked to apparent absence of enzyme complexes to degrade the Cry1Ac toxin, its rapid excretion, and plausible absence of specific receptors in the midgut.

**Acknowledgements**

We thank the FACEPE by granting scholarships to the first author, allowing this work. We also thank the National Research Council (CNPq) for financially supporting this work.

**References**


CTNIBIO, 2016.- *Aprovações comerciais*. Comissão Técnica Nacional de Biossegurança, Ministério da Ciência e Tecnologia, Brasília, Brazil.


Authors' addresses: Herbert Álvaro Abreu de Siqueira (corresponding author, herbert.siqueira@ufpr.br), Andreas Cristina Batista de Oliveira, Departamento de Agronomia (Entomologia), Universidade Federal Rural de Pernambuco, Rua Dom Manoel de Medeiros S/N, Bairro Dois Irmãos, Recife, PE - CEP 52171-900, Brazil; Valéria WANDERLEY-TEIXEIRA, Álvaro Aguilar Coelho Teixeira, Departamento de Morfologia e Fisiologia Animal (Histologia), Universidade Federal Rural de Pernambuco, Rua Dom Manoel de Medeiros S/N, Bairro Dois Irmãos, Recife, PE - CEP 52171-900, Brazil.

Received July 28, 2015. Accepted March 18, 2016.