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

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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 major hydrolyzed substrates by the *P. nigrispinus* digestive tract, suggesting trypsin-like, chymotrypsin-like, and elastase activity in the stinkbug gut content. However, cysteine-like proteases that act in mildly acidic to neutral solution were most likely the enzymes that hydrolyzed the BApNA substrate because they were strongly inhibited by E-64 (a cysteine proteases inhibitor) (100% at 10^{-4} mM). Furthermore, TLCK besides TPCK also inhibited the BApNA hydrolysis (100% at 10^{-3} mM), which suggest they non-selectively inhibited cysteine-like proteases since TPCK does not inhibit trypsin-like proteases. Departing from 0.1 mM, TPCK, EDTA and PMSF showed increased inhibition towards SAAPLpNA hydrolysis causing 90, 70, 60% of inhibition at 1mM, respectively. The *in vitro* proteolytic activity of *P. nigrispinus* digestive contents against Cry1Ac suggests that this toxin cross the stinkbug digestive system virtually unprocessed.

Key words: predatory stinkbug, digestive proteinase, 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 (Hofte 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; Zanúncio *et al*., 2002). In North America, *Podisus maculiventris* (Say) (Heteroptera Pentatomidae) is probably the most important species in that context while *Podisus nigrispinus* (Dallas) (Heteroptera Pentatomidae) is the main stinkbug predator in the Central and South America (Zanúncio *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 proteinase 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 proteinases 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 perimicrivillar 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 raises 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* L. (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}) and centrifuged at 10,000 × g at 4 °C for five minutes. Thereafter, the supernatant containing the proteinases was carefully harvested and stored at −20 °C until use. Protein samples concentrations were determined by the bicinchoninic method (Smith *et al*., 1985), using a protein assay kit (Pierce Co., Rockford, IL, USA). The standard curve was established using bovine serum albumin (BSA). The protein concentration was standardized to 2.5 mg mL^{-1} total protein.

Determination of optimum pH for enzyme activity

To evaluate the activity of serine proteases, specific synthetic substrates were used: N-α-Benzoyl-L-arginine p-nitroanilide (BApNA) for trypsin, N-succinyl-ala-ala-pro-phe p-nitroanilide-(SAAPFpNA) for chymotrypsin, and N-succinyl-ala-ala-pro-leu p-nitroanilide (SAAPLpNA) for elastase. All substrates were obtained from Sigma (Sigma Co., St. Louis, MO, USA) and used at a final concentration of 1mM. The samples were diluted 1:10 in four different buffers: 0.1 M citric acid-NaOH (pH 3.0), 0.1 M sodium citrate (pH 4.0-6.0), 0.1 M Tris-HCl (pH 7.0-9.0), 0.1M glycine (pH 10); all buffers had 0.15 M NaCl and 20 mM $CaCl_2$. 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; N_{α}-Tosyl-L-lysine chloromethyl ketone hydrochloride (TLCK) for trypsin; and N-Tosyl-L-phenylalanine chloromethyl ketone (TPCK) for chymotrypsin-like proteins. 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 10^{0} 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 (Scientronix, 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 Coomassie Brilliant Blue R-250 solution, followed by a distaining 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.

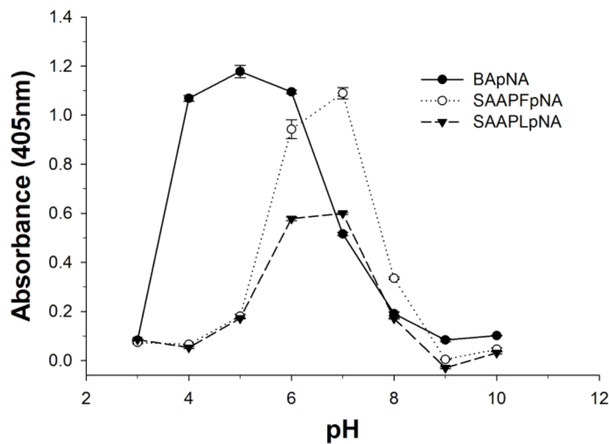

Figure 1. Proteolytic activity of adult *P. nigrispinus* gut contents proteases at different pH conditions. The points represent the mean absorbance (± SE) at 405 nm of three replicates.

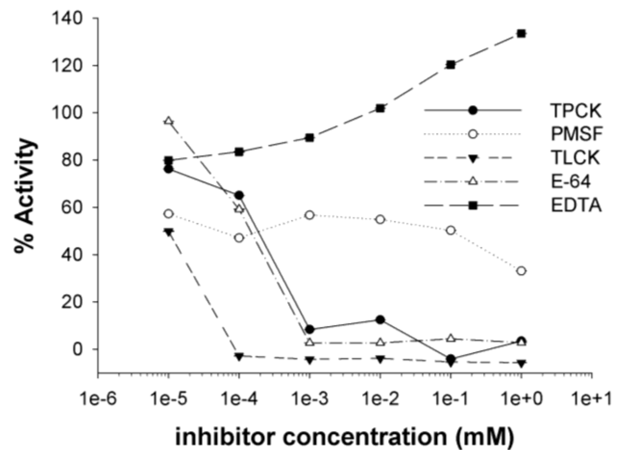

Figure 2. Inhibition of BApNA hydrolysis by *P. nigrispinus* digestive enzymes. The points represent the mean absorbance (± SE) at 405 nm of three replicates.

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 proteases, reduced about 40% BApNA hydrolysis at a concentration of 10^{-5} 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

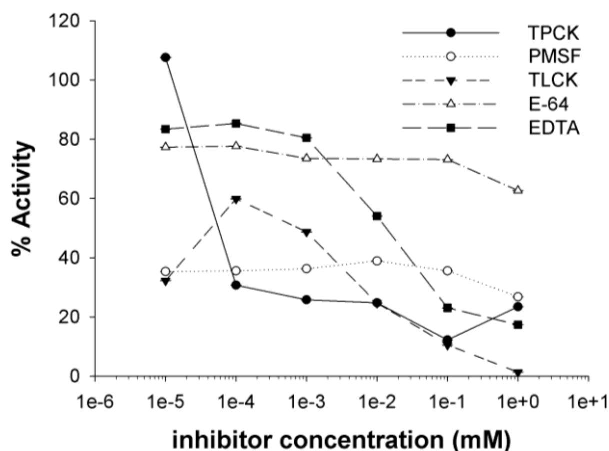

Figure 3. Inhibition of SAAPFpNA hydrolysis by *P. nigrispinus* digestive enzymes. The points represent the mean absorbance (± SE) at 405nm of three replicates.

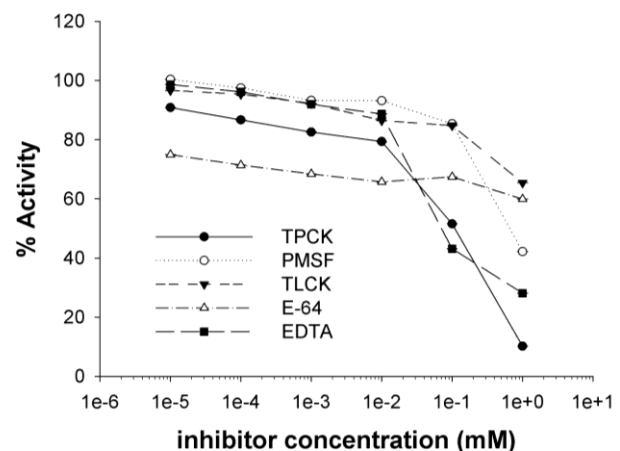

Figure 4. Inhibition of SAAPLpNA hydrolysis by *P. nigrispinus* digestive enzymes. The points represent the mean absorbance (± SE) at 405 nm of three replicates.

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.

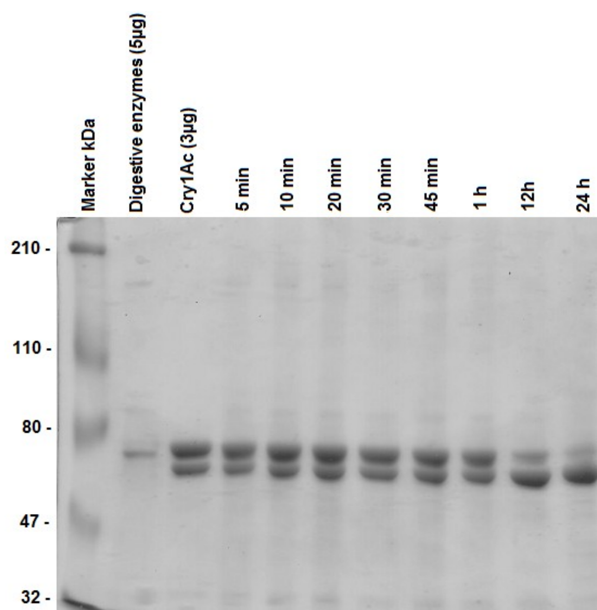

Figure 5. Cry1Ac-degradation electrophoretic profile after different incubation times with *P. nigrispinus* digestive enzymes.

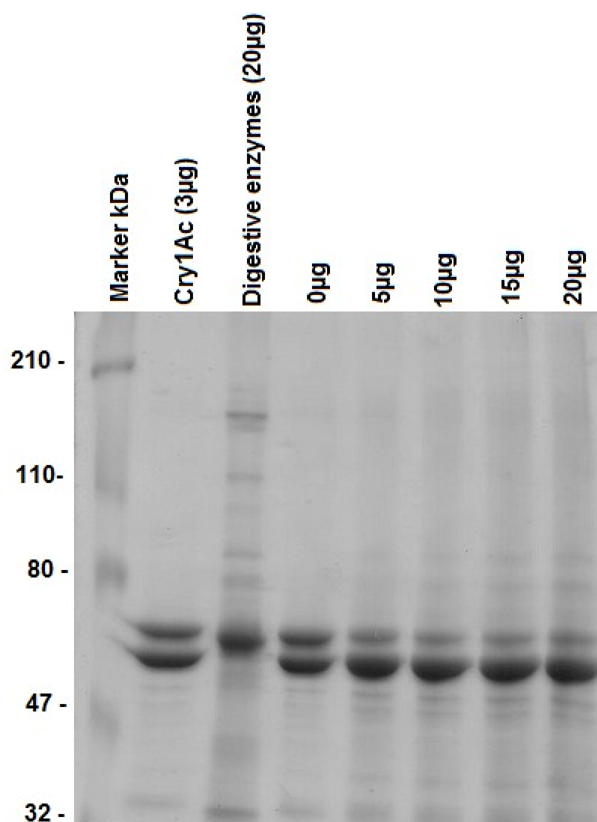

Figure 6. Cry1Ac-degradation electrophoretic profile after 12h of incubation times with different concentrations of *P. nigrispinus* digestive enzymes.

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* (Boisduval) (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* proteinases are 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 Hemiptera could result in the passage of the toxin unscathed through the predator gut. Digestion process begins before ingestion 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.

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