Journal of Invertebrate Pathology 112 (2013) 152–158

Contents lists available at SciVerse ScienceDirect

Journal of Invertebrate Pathology

journal homepage: www.elsevier.com/locate/jip

Bacillus thuringiensis Cry1Ia10 and Vip3Aa protein interactions and their toxicity in *Spodoptera* spp. (Lepidoptera)

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ARTICLE INFO

Article history:
Received 17 April 2012
Accepted 17 November 2012
Available online 5 December 2012

Keywords:
In vitro competition assays
Receptor binding
Spodoptera frugiperda
Spodoptera albula
Spodoptera eridania
Spodoptera cosmioides

ABSTRACT

The polyphagous pests belonging to the genus *Spodoptera* are considered to be among the most important causes of damage and are widely distributed throughout the Americas'. Due to the extensive use of genetically modified plants containing *Bacillus thuringiensis* genes that code for insecticidal proteins, resistant insects may arise. To prevent the development of resistance, pyramided plants, which express multiple insecticidal proteins that act through distinct mode of actions, can be used. This study analyzed the mechanisms of action for the proteins Cry1Ia10 and Vip3Aa on neonatal *Spodoptera frugiperda*, *Spodoptera albula*, *Spodoptera eridania* and *Spodoptera cosmioides* larvae. The interactions of these toxins with receptors on the intestinal epithelial membrane were also analyzed by binding biotinylated toxins to brush border membrane vesicles (BBMVs) from the intestines of these insects. A putative receptor of approximately 65 kDa was found by ligand blotting in all of these species. *In vitro* competition assays using biotinylated proteins have indicated that Vip3Aa and Cry1Ia10 do not compete for the same receptor for *S. frugiperda*, *S. albula* and *S. cosmioides* and that Vip3Aa was more efficient than Cry1Ia10 when tested individually, by bioassays. A synergistic effect of the toxins in *S. frugiperda*, *S. albula* and *S. cosmioides* was observed when they were combined. However, in *S. eridania*, Cry1Ia10 and Vip3Aa might compete for the same receptor and through bioassays Cry1Ia10 was more efficient than Vip3Aa and showed an antagonistic effect when the proteins were combined. These results suggest that using these genes to develop pyramided plants may not prove effective in preventing the development of resistance in *S. eridiana*.

1. Introduction

Spodoptera spp. (Lepidoptera: Noctuidae) are important pests that cause significant damage to maize, cotton, bean and soybean crops (Santos, 2001). Several species, such as *Spodoptera frugiperda*, *Spodoptera albula*, *Spodoptera eridania* and *Spodoptera cosmioides*, are widely distributed in the American tropics and are currently being controlled by the Cry and Vip proteins from *Bacillus thuringiensis* via the adoption of *Bt* crops. *S. frugiperda* is sensitive to several Vip3 and Cry1 proteins, but there is already evidence that this species is less sensitive to some Cry1A proteins (http://www.glfc.cfs.nrcan.gc.ca/bacillus). Cry1I proteins act specifically on the Lepidoptera and Coleoptera orders and, unlike other Cry proteins, are soluble and do not accumulate as crystals (Tailor et al., 1992). Similarly, Vip3Aa toxins are produced in the vegetative phase and act specifically on Lepidoptera pests.

Cry toxin binding to the epithelial membrane of mid-intestinal cells is mediated by specific receptors or binding sites. Such binding is essential for insect toxicity, although different toxin receptors may be present (Hofmann et al., 1988). Different protein receptors for Cry toxins have been identified, such as cadherins, glycophosphotidylinositol (GPI)-anchored alkaline phosphatase (AP), GPI-anchored aminopeptidase-N (APN), a 270 kDa glycoconjugate receptor (GCR) and 250 kDa P252 (Bravo et al., 2011). According to the model proposed by Bravo et al. (2004), Cry toxin monomers bind to cadherins on the mid-intestinal epithelium of lepidopteran larvae, changing their conformation, which results in toxin oligomerization and increases their ligation affinity for APN (Pardo-López et al., 2006) and for AP (Jurat-Fuentes and Adang, 2004. Activated Vip3Aa toxin acts in the insect's intestinal epithelium, similar to the Cry proteins. Because Vip3Aa proteins are already soluble, they are able to bind to receptors more rapidly, once the Cry protein needs to be solubilized. This action results in progressive degeneration of the epithelial layer (Yu et al., 1997). Cry1A protein resistance in laboratory insect strains may or may not be linked to cadherin genes (Tabashnik et al., 2005), since there is already reports of changes in the genes coding for AP or APN (Tiewsiri and Wang, 2011; Jurat-Fuentes et al., 2011; Caccia

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0022-2011
http://dx.doi.org/10.1016/j.jip.2012.11.011

et al., 2012). Resistance to the proteins encoded by the *cry* transgenes in crops, would eliminate the benefit of reduced chemical insect application, which is toxic to the environment and to human beings (Maia, 2003).

Studies on Cry and Vip proteins and their interactions with receptors on the intestinal epithelium membrane of insect pests is thus very important, as there are no reports of competition for a single receptor by these proteins. Using toxins that do not compete for the same receptor may diminish the chances of resistance development. Moreover, they can act synergistically enhancing their toxicity to insect larvae. This strategy seeks to develop pyramided genetically modified crops that express Cry and Vip3A proteins to manage resistance and to control pests as effectively as possible. Therefore, this study aimed to analyze the toxicity of the proteins Cry1Ia10 and Vip3Aa, which were expressed in *Escherichia coli*, and their synergistic effects on neonatal *S. frugiperda*, *S. albula*, *S. eridania* and *S. cosmioides* larvae. Also, our results indicate a correlation between the mode of action of these toxins in bioassays and their biding to the receptors in the brush border membrane vesicles (BBMV) of these insects.

2. Materials and methods

2.1. Cry1ia10 and vip3Aa cloning

The *cry1Ia10* gene was isolated from the *B. thuringiensis* var. *thuringiensis* T01-328 strain, and the complete gene (2160 bp) was cloned into the pET28a(+) expression vector (Bergamasco et al., 2011). The *vip3Aa* gene was isolated from the *B. thuringiensis* HD-1 line, and the complete gene (2350 bp) was cloned into the pET SUMO expression vector (Mendes, 2011). Both of the expression vectors added a polyhistidine tag (6 His) to the end of the recombined genes for protein detection and purification. The vectors containing the genes were used to transform competent *E. coli* BL21(DE3) cells by thermal shock (Hanahan, 1983) to induce recombinant gene expression.

2.2. Induction of protein expression and purification

Cry1Ia10 and Vip3Aa expression was induced by inoculating a pre-culture containing 20 ml LB media and 50 μg/ml kanamycin with a single colony from one of the clones containing the expression vector with the specific gene. The culture was grown at 37 °C and agitated at 250 rpm for 16 h. The pre-culture was transferred to 200 ml of LB media and 50 μg/ml kanamycin and agitated until an OD_{600} of 0.6 was reached. IPTG was then added to a final concentration of 1 mM (Vip3Aa) or 5 mM (Cry1Ia10) to induce expression. The culture was maintained at 25 °C (Vip3Aa) or 30 °C (Cry1Ia10) for 24 h with agitation (190 rpm).

Cell lysis and solubilization of the proteins were performed as described by Bergamasco et al. (2011). Gene expression was confirmed by resolving the total protein on a 10% SDS–PAGE gel stained with Coomassie Blue and by Western Blot using an anti-histidine antibody (Sigma Aldrich). Lysate from *E. coli* BL21 (DE3) without the gene inserts was used as a negative control. Lysates containing Vip3Aa (approximately 100 kDa) (Mendes, 2011) and Cry1Ia10 (approximately 81 kDa) (Bergamasco et al., 2011) in the lysate were quantified by densitometry via the Bionumerics software (Applied-Maths) and a bovine serum albumin (BSA) standard curve before use in the bioassays and BBMV binding studies.

2.3. Bioassays

Neonatal *S. frugiperda* larvae were provided by the Applied Ecology Laboratory, FCAV/UNESP. Neonatal *S. eridania, S. albula* and *S. cosmioides* larvae were provided by the Insect Biology Laboratory, ESALQ/USP, Piracicaba, SP, Brazil.

Bioassays were performed using lysates from *E. coli* BL21 (DE3) clones containing the Cry1Ia10 and Vip3Aa proteins individually, to determine the insecticidal activity of the proteins, or together to evaluate their synergistic or antagonistic interaction. The bioassays were carried out using the methods described by Mendes (2011) for neonatal larvae from four *Spodoptera* species. Treatments were applied to the surface of an artificial diet contained in 128-well polystyrene plates (Bio-Assay Tray – Bio-Serv).

To determine the 50% and 90% lethal concentrations (LC_{50} and LC_{90}) of the individual toxins, seven different Cry1Ia10 and Vip3Aa protein concentrations (0.05 ng/cm^2, 0.5 ng/cm^2, 1 ng/cm^2, 5 ng/cm^2, 25 ng/cm^2, 50 ng/cm^2 and 500 ng/cm^2) were tested by applying 50 μl of each dilution to the artificial diet. For bioassays using both proteins, seven different concentrations (0.0025 ng/cm^2, 0.025 ng/cm^2, 0.25 ng/cm^2, 1 ng/cm^2, 2.5 ng/cm^2, 25 ng/cm^2 and 250 ng/cm^2) at a 1:1 ratio for each of the protein concentrations were tested by applying 25 μl of Vip3Aa + 25 μl of Cry1Ia10 from each dilution to the artificial diet. The same procedure was performed for the two controls, one with lysis buffer and the other with untransformed *E. coli* BL21 (DE3) lysate.

Each bioassay was repeated five times, with 16 neonatal larvae per replication, for each toxin concentration. The insects were incubated at 28 ± 2 °C, 65 ± 5% relative humidity, and 14:10 L:D photoperiod. The bioassays were evaluated after 7 days, and the LC_{50} and LC_{90} were calculated by Probit analysis. The equality and parallelism analyses of the regression lines were compared using the maximum likelihood ratio test using the Polo Plus program (Robertson et al., 2007) to test the hypotheses of parallelism and equality between the lines. The synergism and antagonism analysis were evaluated according to Tabashnik's method (Tabashnik, 1992) and Wu et al. (1994).

2.4. BBMV collection and preparation

Approximately 5 g of intestines were dissected (Escriche et al., 1995) from last-instar *S. frugiperda*, *S. eridania, S. albula* and *S. cosmioides* larvae. The intestines were washed in cold MET buffer (250 mM Mannitol, 17 mM Tris–HCl and 5 mM EGTA [pH 7.5]), frozen in liquid nitrogen and stored at −80 °C (Hernández et al., 2004). BBMVs were prepared by the differential magnesium precipitation method described by Wolfersberger et al. (1987) from the dissected intestines. The protein concentrations of the BBMV preparations were determined by the Bradford assay (Bradford, 1976) using BSA as a standard.

2.5. Purification, pro-toxin activation and biotin labeling

Lysates containing Vip3Aa and Cry1Ia10 proteins were purified on His-Trap HP columns (GE Healthcare) based on the affinity of the nickel contained in the column for the polyhistidine (6× His) tag in the recombined proteins. Twenty mM imidazole was added to the lysate and centrifuged for 10 min at 7400× g and 4 °C. The supernatants were put on the His-Trap Chelating HP – 1 ml (Amersham) purification column charged with 0.1 M $NiSO_4$ that was previously prepared according to the manufacturer's instructions. The protein was eluted with elution buffer (50 mM phosphate buffer [pH 8.0], 0.5 M NaCl and 500 mM imidazole). Fractions of 1 ml were collected in Eppendorf tubes and then analyzed by electrophoresis on a 10% SDS–PAGE gel.

Purified Vip3Aa and Cry1Ia10 pro-toxins were trypsinized using 1% bovine trypsin (Sigma Aldrich) at 37 °C and 100 rpm agitation for 1 h. Trypsin digest efficiency was confirmed by SDS–PAGE (Escudero et al., 2006). The activated Vip3Aa toxin (62 kDa) and activated Cry1Ia10 toxin (60 kDa) were labeled with biotin using

the ECL Kit Biotinylation Module (GE Healthcare), following the manufacturer's instructions. The labeled proteins were eluted using Sephadex G25 – P10 Desalting columns (GE Healthcare) and PBS buffer (phosphate buffered saline, pH 7.4). Ten microliter aliquots of the collected fractions were applied to a polyvinylidene fluoride (PVDF) membrane (GE Healthcare) for dot blot analysis to show the fraction of the proteins that were biotinylated. The membranes were incubated with peroxidase (HRP)-conjugated streptavidin (GE Healthcare), which specifically binds to biotin, and developed by the addition of the SIGMAFAST DAB with Metal Enhancer (Sigma Aldrich) chromogenic substrate, following the manufacturer's instructions.

2.6. Ligand blotting

Ligand blotting was performed in accordance with the procedures reported by Abdelkefi-Mesrati et al. (2011a). Quantified (20 μg) BBMVs from *S. frugiperda*, *S. eridania, S. albula* and *S. cosmioides* were separated on two 9% SDS–PAGE gels and electrotransferred to PVDF membranes. After a 2-h incubation in a solution containing 3 μg of trypsinized and biotinylated Vip3Aa and Cry1Ia10 protein, the membranes were incubated for 1 h with peroxidase (HRP)-conjugated streptavidin (GE Healthcare) at a 1:1500 dilution and visualized using SIGMAFAST DAB with Metal Enhancer (Sigma Aldrich), according to the manufacturer's instructions.

2.7. Biotinylated Cry1Ia10 and Vip3Aa competition assays

Competition assays were performed as suggested by Sena et al. (2009). Biotinylated Vip3Aa (50 ng) and Cry1Ia10 (50 ng) were incubated with BBMVs (20 ng) from *S. frugiperda*, *S. eridania, S. albula* and *S. cosmioides*, in 0.1 ml of binding buffer (PBS pH 7.4, 0.1% BSA) for 1 h, in the absence or the presence of unlabeled competitor proteins. Competitor proteins were mixed and incubated with the BBMVs after incubation with the biotinylated proteins. For the homologous competition assays, activated unlabeled protein (200× in excess) was used for BBMVs incubated with the same biotinylated proteins. For heterologous competition assays, the biotin-labeled proteins were incubated with the unlabeled competitor at 200-, 500- and 1000-fold excess in the presence of BBMVs from each of the insect species.

After incubation, the BBMVs were washed, centrifuged and resuspended in 10 μl of sterile Milli-Q water and 5 μl of electrophoresis sample buffer (Laemmli, 1970). Samples containing biotinylated proteins and BBMV but no competitor and another sample containing only biotinylated protein without BBMV were used as negative controls. The samples were electrophoresed on a 9% SDS–PAGE gel and, after electrophoresis, electrotransferred to a PVDF membrane (GE Healthcare). Toxins on the membrane were detected with peroxidase (HRP)-conjugated streptavidin (GE Healthcare) and developed with SIGMAFAST DAB with Metal Enhancer (Sigma Aldrich), following the manufacturer's instructions. The detected bands were quantified by densitometry using the Bionumerics software (Applied-Maths) and compared to the positive control containing biotinylated proteins and BBMV.

3. Results and discussion

3.1. Bioassays

Neonatal *S. frugiperda*, *S. eridania, S. albula* and *S. cosmioides* larvae were sensitive to Vip3Aa and Cry1Ia10 protein and had low LC_{50} levels for both the individual and the combined toxins (Table 1). Vip3Aa was more efficient than Cry1Ia10 when tested individually on *S. frugiperda*, *S. albula* and *S. cosmioides*. However, Cry1Ia10 is slightly but significantly more efficient than Vip3a for *S. eridania.* The highest LC_{50} levels for Vip3Aa and Cry1Ia10, when tested individually, were observed for *S. frugiperda.* Exposing the neonatal larvae from this species to the negative controls (lysis buffer and untransformed *E. coli* BL21) did not cause death. Unlike the control, the surviving larvae from the toxin treatments remained in the first instar until evaluation on the seventh day. This result demonstrates the sub lethal effect of these toxins, which is important for controlling population growth by slowing the life cycle of surviving pests.

Table 1
Dose–response bioassay for Cry1Ia10 and Vip3Aa, individually and combined (1:1 ratios), in four different *Spodoptera* spp. species, evaluated after 7 days.

Treatment/ species	LC_{50} (ng/cm^2) (CI min.–max.)[b]	LC_{90} (ng/cm^2) (CI min.–max.)[b]	$b \pm$ (SEM)[c]
S. frugiperda			
Cry1Ia10[a]	69.52 (30.235–140.130)	2161.02 (612.190–53096.123)	0.839 ± 0.167
Vip3Aa[a]	24.66 (9.731–44.510)	262.74 (153.178–560.339)	1.233 ± 0.207
Cry1Ia + Vip3Aa	5.67 (3.832–8.764)	217.12 (107.683–542.839)	0.809 ± 0.063
S. albula			
Cry1Ia10	15.03 (11.421–20.122)	301.26 (176.727–617.743)	0.984 ± 0.083
Vip3Aa	3.90 (2.787–5.464)	122.22 (71.559–241.467)	0.857 ± 0.063
Cry1Ia + Vip3Aa	1.47 (1.008–2.188)	64.47 (33.687–148.646)	0.780 ± 0.058
S. cosmioides			
Cry1Ia10	10.51 (7.919–14.197)	215.35 (128.124–424.794)	0.977 ± 0.076
Vip3Aa	2.78 (2.304–3.370)	15.24 (11.408–22.126)	1.736 ± 0.135
Cry1Ia + Vip3Aa	1.08 (0.790–1.505)	19.65 (11.874–37.332)	1.018 ± 0.075
S. eridania			
Cry1Ia10	1.93 (1.479–2.507)	16.38 (11.138–27.200)	1.380 ± 0.101
Vip3Aa	3.44 (2.641–4.529)	56.76 (35.744–103.451)	1.053 ± 0.081
Cry1Ia + Vip3Aa	7.23 (4.614–12.103)	503.90 (215.228–1557.833)	0.695 ± 0.055

[a] Results obtained by MENDES (2011).
[b] (CI min.–max.): confidence Interval (CI 95%).
[c] $b \pm$ (SEM): angular coefficient of the line and standard error.

Of the species analyzed in this study, *S. cosmioides* was the most sensitive to Vip3Aa and was approximately eight times more susceptible than *S. frugiperda*, the least sensitive. In our bioassays, Vip3Aa was highly toxic for the four *Spodoptera* species, with values lower than those found in the Specificity Database (http://www.glfc.forestry.ca/bacillus) for *S. frugiperda*, and has an LC_{50} value of 49.3 ng/cm^2 for neonatal *S. frugiperda* larvae when the toxin is placed on the food surface (Sena et al., 2009). Lee et al. (2003) reported a value of 55.9 ng/cm^2 for larvae from this species. The Vip3Aa toxicity for the various other Lepidoptera species has also been evaluated. In a study by Doss et al. (2002), Vip3Aa1 was more active against *Spodoptera litura* than against *Agrotis ipsilon* and *Plutella xyllostella*, with LC_{50} of 45 ng/cm^2, 80 ng/cm^2 and 220 ng/cm^2, respectively. Vip3Aa16 acted efficiently against *Spodoptera littoralis*, with LC_{50} of 305 ng/cm^2 (Abdelkefi-Mesrati et al., 2011a) and against *Ephestia kuehniella*, with LC_{50} of 36 ng/cm^2 (Abdelkefi-Mesrati et al., 2011b). In addition to Vip3Aa, other Vip3 proteins were toxic against *Prays oleae* (Abdelkefi-Mesrati et al., 2009), *Helicoverpa armigera*, *S. litura* and *Spodoptera exigua* (Chen et al., 2003).

Furthermore, in the species tested here, Cry1Ia10 was 36 times more toxic against *S. eridania* than against *S. frugiperda* (the least sensitive). The observed LC_{50}values for Cry1Ia10 show that it is highly toxic to all four *Spodoptera* species, with values lower than

those found in the Specificity Database (http://www.glfc.forestry.ca/bacillus), which shows a value of 289 ng/cm^2 for neonatal *S. frugiperda* larvae (Martins et al., 2008). The recombinant Cry1Ia12 protein was also toxic to first-instar *S. frugiperda* larvae, with LC_{50} of 5 μg/ml (Grossi-de-Sá et al., 2007). High toxicity was also shown by Bergamasco et al. (2011), who measured Cry1Ia10 toxicity against *S. frugiperda* neonates and found LC_{50} of 17 ng/ml. In studies on other pests, Cry1Ia was found to be active against *P. oleae* (Dammak et al., 2010), *Ostrinia fumacalis* and *Prays xylostella*. (Li-Ming et al., 2007). This difference in toxicity may be due to different larval phases, distinct populations in this species and different bioassay protocol.

The different Cry protein toxicities against Lepidoptera (Escudero et al., 2006) and between Cry and Vip3 (Sena et al., 2009) have also been previously studied. In a study by Escudero et al. (2006), bioassays using different species of Lepidoptera and Coleoptera showed that *Leptinotarsa decemlineata* was more sensitive to Cry1Ia7 than the others. Sena et al. (2009) measured the toxicity of Vip3Aa, Vip3Af, Cry1Ab and Cry1Fa in *S. frugiperda*, and demonstrating that these Vip3A proteins are more toxic than these Cry1 proteins in the *S. frugiperda* population studied. The same observation was made in the *S. frugiperda, S. albula* and *S. cosmioides* populations used in this study, in which the Vip3Aa toxin was more toxic than Cry1Ia10, but was not true for the *S. eridania* population.

However, analysis of the two toxins combined resulted in lower LC_{50} doses compared to the individual LC_{50} values for Vip3Aa and Cry1Ia10 (Table 1), supporting a synergistic activity between these proteins in *S. frugiperda, S. albula* and *S. cosmioides*. Synergistic activity was also shown by Lee et al. (1996), where combinations of Cry1Ab-Cry1Ac and Cry1Aa-Cry1Ac acted synergistically on larvae of *Lymantria dispar*. The mechanisms underlying their synergism have still not been completely elucidated (Sharma et al., 2010). It has been hypothesized that pore formation by different toxins causes osmotic breakdown in the cells, causing the majority of the mortality (Lee et al., 1996), and that the formation of hetero-oligomers, i.e., oligomerization among different toxins, also improves their ability to bind compared to homo-oligomers (Chakrabarti et al., 1998) and may prevent nonspecific binding (Schnepf et al., 1998).

Nevertheless, the two toxins acted antagonistically in the *S. eridania* population when tested together, as indicated by the significant increase in the LC_{50}, which was higher than the sum of the individual LC_{50} values for Cry1Ia10 and Vip3Aa. This type of antagonism has also been shown by Lee et al. (1996), where Cry1Aa and Cry1Ab combined were three times less effective than expected for *L. dispar* larvae. One possible explanation for the antagonism between Vip3Aa and Cry1Ia10 in *S. eridania* may be the properties of their interactions with the receptors described by Vanden Heuvel et al., 2002. These authors stated that this result could be due to antagonism by partial agonism or dualism, in which the two toxins may have similar affinities for the same receptors, but with intrinsically different activities, and thus initially act synergistically due to the high availability of receptors. However, as the receptors are depleted, the toxins begin to act antagonistically, i.e., the partial agonist occupies the receptors and acts less effectively, blocking the activity of the total agonist (Vanden Heuvel et al., 2002). To define the mechanism of this antagonism, the receptors involved in this activity should be better characterized. These results clearly demonstrate that the *B. thuringiensis* Vip3Aa and Cry1Ia10 toxins are very active against *Spodoptera* populations and that they act differently on *S. eridania*.

3.2. Purification, pro-toxin activation and biotin labeling

Recombinant protein expression was confirmed by SDS–PAGE (Fig. 1A) and Western blot by binding the polyhistidine tag with an anti-His antibody (Sigma Aldrich) (Fig. 1B). Bands were detected at approximately 100 kDa for Vip3Aa (approximately 89 kDa for Vip3Aa and an additional 11 kDa for the ubiquitin and polyhistidine tag in the pET-SUMO vector) and 81 kDa for Cry1Ia10 in IPTG-induced bacterial lysates. The lysates were purified and activated by trypsin. The data on Vip3Aa and Cry1Ia10 activation in this study corroborate data published elsewhere (Abdelkefi-Mesrati et al., 2009; Lee et al., 2003), resulting in 62 kDa activated toxins for both Vip3Aa and Cry1Ia10 (Fig. 1A). These toxins were biotinylated to test their binding to BBMVs from the studied species.

Cry1Ia10 and Vip3Aa activation by trypsin is an essential step for determining their toxic effects *in vitro*. This procedure is performed to reproduce the proteolysis of the pro-toxin that creates the active toxin in the midgut of the larvae *in vivo*. Proteolysis is essential for Vip and Cry activity due to the inability of the unprocessed proteins to bind to BBMVs in *in vitro* binding assays. This activation can be performed using intestinal juices from the studied insect larvae or with trypsin. The choice to use trypsin for activation in this study is based on Lee et al. (2003), who determined that both intestinal juice and trypsin predominantly create a 62 kDa Vip3A toxin. This fragment size was also verified by trypsinizingVip3Lb (Abdelkefi-Mesrati et al., 2009), and a 60 kDa fragment was confirmed by Escudero et al. (2006) for Cry1Ia7 activation using trypsin.

3.3. Ligand blotting

Ligand blotting analysis allowed us to show biotinylated Vip3Aa and Cry1Ia10 binding to putative receptors found on the BBMVs by detecting a band with a biotin-specific antibody. Both biotinylated Vip3Aa and Cry1Ia10 putatively bind to approximately 65 kDa receptors on BBMVs in *S. frugiperda, S. albula, S. cosmioides* and *S.*

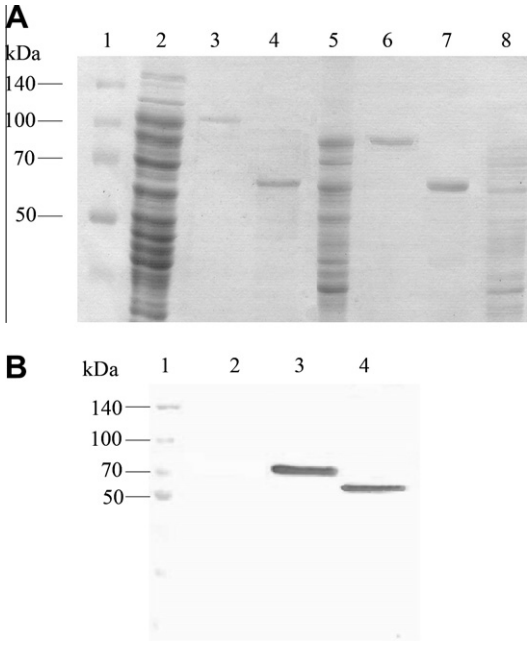

Fig. 1. Vip3Aa and Cry1Ia10 protein expression. (A) 10% SDS PAGE gel detection of purified and activated Cry1Ia10 and Vip3Aa. Lanes: 1 – Spectra™ Multicolor Broad Range Protein Ladder (fermentas), 2 – lysate containing Vip3Aa, 3 – purified Vip3Aa protein, 4 – trypsinized Vip3Aa protein, 5 – lysate containing Cry1Ia, 6 – purified Cry1Ia protein, 7 – trypsinized Cry1Ia protein, 8 – negative control: *E. coli* BL21 (DE3) lysate. (B) Protein band visualization by Western blot of the protein lysates incubated with a monoclonal anti-His antibody. Lanes: 1 – Spectra™ Multicolor Broad Range Protein Ladder (fermentas), 2 – negative control: *E. coli* BL21 (DE3) lysate, 3 – lysate containing Vip3Aa, 4 – lysate containing Cry1Ia10.

eridania (Fig. 2), suggesting that the Vip3Aa and Cry1Ia receptors have a similar size in the four studied species.

In similar studies, Bravo et al. (2007) identified several receptors for the Cry toxins in the midguts of susceptible insect larvae from the Lepidoptera order and showed the importance of glycophosphotidylinositol (GPI)-anchored proteins for Cry1 toxin interactions in this order. Among these proteins are alkaline phosphatases with molecular weights between 62 kDa and 68 kDa (Jurat-Fuentes and Adang, 2004, 2006; Fernández et al., 2006) and aminopeptidase-N with a molecular weight between 112 kDa and 120 kDa (Rajagopal et al., 2003; Hua et al., 2004; Pacheco et al., 2009), or a 130 kDa which may be a dimer of 65 kDa protein (Malik et al., 2006).

In addition to GPI anchored proteins, the best-characterized Cry targets are cadherin-type receptors with a molecular weight of approximately 210 kDa (Hua et al., 2004; Flanagan et al., 2005; Jurat-Fuentes and Adang, 2006).

Based on this information, the putative 65 kDa receptor for Cry1Ia10 and Vip3A may consist of an aminopeptidase-N or alkaline phosphatase monomer. In contrast to the Cry toxins, several putative receptors were detected for Vip3A toxins, but they have not yet been characterized (Singh et al., 2010). Studies on the Vip3Aa16 toxin have shown binding to putative receptors at 65 kDa in *E. kuehniella* (Abdelkefi-Mesrati et al., 2011b) and to two putative receptors at 55 kDa and 100 kDa in *S. littoralis* (Abdelkefi-Mesrati et al., 2011a). Lee et al. (2003) detected two putative Vip3A-G receptors at 80 kDa and 100 kDa in BBMVs from *Manduca sexta*. Vip3LB has also been shown to bind a putative 65 kDa receptor in the midgut of *P. oleae* (Abdelkefi-Mesrati et al., 2009).

Receptor models in some studies have suggested that an insect may have, at varying levels, several classes of receptors that can be recognized by different toxins (Rajagopal et al., 2003; Malik et al., 2006; Perera et al., 2009). This receptor diversity may explain the heterogeneity of the BBMVs used to determine Cry toxin affinity (Malik et al., 2006) and that receptor binding is essential for toxicity.

Thus, we can infer that the putative Vip3Aa and Cry1Ia10 receptors, of approximately 65 kDa, in *S. frugiperda, S. albula, S. cosmioides* and *S. eridania* have similar size, but may not be necessarily the same type of receptor. Their size is comparable to the size of GPI-anchored alkaline phosphatase receptors and monomeric GPI-anchored aminopeptidase. The characterization of these putative receptors by enzymatic activity or amino-acid sequencing would determine the similarities or dissimilarities between them and shed light on the binding mechanisms between them and Vip3Aa or Cry1Ia.

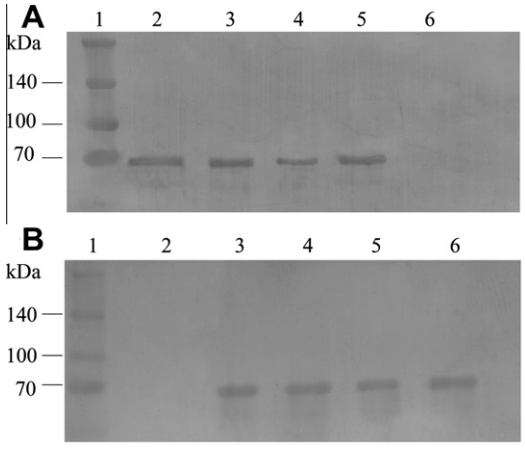

Fig. 2. Detection of the putative receptor (65 kDa) for biotinylatedVip3Aa (A) and Cry1Ia10 (B) toxins in the midgut of *Spodoptera* spp. by ligand blotting. (A) Lanes: 1 – Spectra™ Multicolor Broad Range Protein Ladder (Fermentas), 2 – *S. frugiperda* BBMV 3 – *S. albula* BBMV, 4 – *S. eridania* BBMV,5 – *S. cosmioides* BBMV, 6 – BSA negative control. (B) Lanes: 1 – Spectra™ Multicolor Broad Range Protein Ladder (Fermentas); 2 – BSA negative control, 3- *Spodoptera frugiperda* BBMV, 4 – *S. albula* BBMV, 5 – *S. eridania* BBMV, 6 – *S. cosmioides* BBMV.

3.4. Biotinylated Cry1Ia10 and Vip3Aa binding assays

Biotinylated Vip3Aa and Cry1Ia10 toxins specifically bind BBMVs from *S. frugiperda*, *S. eridania, S. albula* and *S. cosmioides* (Fig. 3, lanes 2 and 8). This binding was confirmed by homologous competition with an excess of an unlabeled version of the same toxin, substantially reducing the labeled toxin binding to the BBMVs (Fig. 3, lanes 3 and 7).

Heterologous competition with an excess of unlabeled Cry1Ia10 did not reduce Vip3Aa binding significantly, indicating that it does not compete with Vip3Aa for receptors in the midgut in *S. frugiperda*, *S. albula* and *S. cosmioides* (Fig. 3A–C, lanes 4–6). Therefore, the receptors for the analyzed proteins must be different in these three species.

In contrast, the heterologous competition in *S. eridania* (Fig. 3D, lanes 4–6) with an excess of unlabeled Cry1Ia10 competitor partially reduced the labeled Vip3Aa binding to BBMVs. However, even at 1000× excess, the Cry1Ia10 competitor did not completely ablate labeled Vip3Aa. This reduction was quantified by densitometry, and competition with an excess of 200, 500 and 1000× reduced binding by 33%, 55% and 57.33%, respectively. This fact confirms that there was competition but that this competition is not due to an exclusive affinity for the same receptor. The displacement of Vip3Aa may be due to partial competition, i.e., there may be one receptor site for each protein, and Vip3Aa receptor shared with Cry1Ia10. Although Cry1Ia10 has a low affinity for the Vip3Aa receptor, it is sufficient to partially displace Vip3Aa binding on the BBMV receptor in the midgut of *S. eridania*.

This same binding analysis for Cry and Vip protein receptor binding was also studied by Lee et al. (2006), who found that there was no competition between Vip3A and Cry1Ac in *Heliothis virescens* and *Helicoverpa zea* BBMVs. Gouffon et al. (2011) showed by testing the competition between radioactively labeled Cry2Ae and the competitors Cry1Ab, Cry1Ac, Cry1Fa, Vip3A, Cry2Ae and Cry2Ab in BBMVs from *H. virescens, H. zea* and *H. armigera* that Cry2Ae does not compete with the Cry1 and Vip3 proteins. In studies performed on *S. frugiperda*, Sena et al. (2009) showed that Cry1

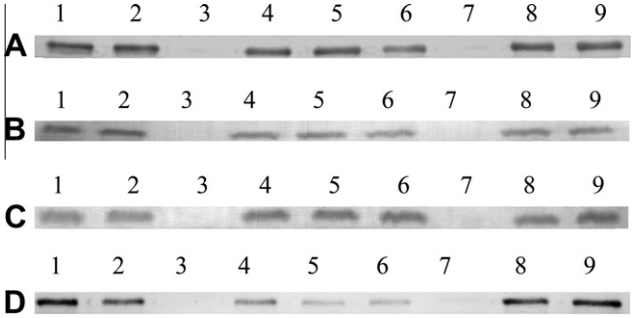

Fig. 3. Vip3Aa and Cry1Ia10 toxin binding to BBMVs in *S. frugiperda* (A), *S. albula* (B), *S. cosmioides* (C) and *S. eridania* (D). Lanes: 1 – Biotinylated Vip3Aa (control without BBMVs), 2 – biotinylated Vip3Aa bound to BBMV (no competitor), 3 – homologous competition: biotinylated Vip3Aa and unlabeled Vip3Aa (200× excess), 4 – heterologous competition: biotinylated Vip3Aa and unlabeled Cry1Ia10 (200× excess), 5 – heterologous competition: biotinylated Vip3Aa and unlabeled Cry1Ia10 (500× excess), 6 – heterologous competition: Biotinylated Vip3Aa and unlabeled Cry1Ia10 (1000× excess), 7 – homologous competition: biotinylated Cry1Ia10 and unlabeled Cry1Ia10 (200× excess), 8 – biotinylated Cry1Ia10 bound to BBMV (no competitor), 9 – Biotinylated Cry1Ia10 (control without BBMV).

and Vip3a occupy distinct binding sites and that Cry1Fa occupies the same binding site as Cry1Ab, while Vip3Af1 and Vip3Aa1 compete for the same receptor. The binding of various *B. thuringiensis* Cry1Ia proteins to the intestinal epithelium of Lepidoptera larvae was also characterized by BBMV binding. A study by Ibargutxi et al. (2006) showed that biotinylated Cry1Ia toxin and Cry1Ac did not compete in BBMVs from *Earias insulana*. In a study by Escudero et al. (2006), biotinylated Cry1Ia7 did not occupy the same binding site as Cry1Ab or Cry1Ac in the *E. insulana* and *Lobesia botrana* intestine. Since there are no reports of competition for bidding to receptors among the Vip3 and Cry toxins, at the same time there are no reports of this kind of competition between the Cry1Ia toxins and other Cry toxins. The Vip3Aa and Cry1Ia share some characteristics (Espinasse et al., 2003); both proteins do not accumulate as crystals and their similar mode of action when compared to the other Cry toxins seem to indicate this action relatedness would permit competition for binding to a particular receptor. Of the four studied *Spodoptera* species, these toxins were antagonistic, and there was receptor-binding inhibition due to competition between the toxins only in *S. eridania*. This instance is the first report of *in vivo* and *in vitro* receptor binding competition between the Cry and Vip proteins in a *Spodoptera* species.

Based on the results found in this study, we conclude that Cry1Ia10 does not compete with Vip3Aa for the same binding site in *S. frugiperda, S. albula* and *S. cosmioides* and that there is partial competition in *S. eridania*. Our binding competition analysis corroborates the bioassay results, in which these toxins were very effective against all four studied species. At the same time for *S. eridania* there was detected antagonism between both proteins and for the other species, a higher level of action between them, which can be seen as a certain degree of synergism. Although the amino acid sequence of Vip3Aa and Cry1Ia10 are different, the mechanism of synergism remains unknown. Due to the unequal mechanisms of action in the *Spodoptera* species, an indication regarding the use of Vip3Aa and Cry1Ia10 in combination to control these polyphagous pests will be possible after their receptors are characterized. A combination of the *vip3Aa* and*cry1Ia*genes can be used to obtain pyramided *Bt* plants to prevent resistance in *S. frugiperda, S. albula* and *S. cosmioides*, as these proteins do not compete for the same receptor site. However, this combination in pyramided plants may allow a certain level of resistance for *S. eridania*, resulting from a mutation in the receptor site common to *vip3Aa* and *cry1Ia*. Therefore, these results suggest that pyramiding plants with certain genes may not always be effective for preventing resistance in various insect–pest species of the same genus.

Acknowledgments

I thank Prof. Dr. José Roberto Postali Parra and the team in the Insect Biology Laboratory of the Entomology and Acarology Department at ESALQ – USP for kindly providing the *Spodoptera* spp. samples. This work was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico-CNPq (National Council of Scientific and Technological Advancement) and by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior-CAPES (Coordination for the Improvement of Higher Education Personnel) scholarship.

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