RESEARCH

Identification of putative receptor proteins in Cylas formicarius *ssp.* elegantulus *midgut BBMVs for the Cry3Aa toxin of* Bacillus thuringiensis *ssp.* tenebrionis

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ABSTRACT

The use of *Bacillus thuringiensis* in biological pest control and to obtain insect resistant transgenic plants commonly rely on basic studies of the action mechanism of Cry toxins in the target insects. These studies are combined with toxin insecticide bioassays in Brush Border Membrane Vesicles (BBMVs) obtained from the insects' epithelial gut tissues. One of the toxins, Cry3Aa from *B. thuringiensis* ssp. *tenebrionis*, has shown activity against sweet potato weevil (*Cylas formicarius* ssp. *elegantulus* Fabricius), the main pest for this crop. Here is described for the first time the binding capacity of Cry3Aa to BBMVs vesicles obtained from the midgut of second-third instar larvae of this insect. BBMVs were purified and insecticide protein labelling and binding assays performed according to standard procedures. Four proteins (approximately 20, 30, 50 and 85 kDa, respectively) were detected as mediating binding of the CryAa toxin to the insect gut, as putative receptors. Peptides from these proteins were sequenced and aligned for homology detection against database sequences, leading to their identification as previously unreported proteins with this function. Additionally, storage roots from transgenic sweet potato plants expressing high levels of the Cry3Aa toxin were used in an in vivo bioassay to test insect control under lab conditions. The results suggested that the toxic activity of Cry3Aa from *B. thuringiensis* ssp. *tenebrionis* against sweet potato weevil could involve the toxin binding to some of the detected proteins in the insect gut.

Keywords: sweet potato, Bacillus thuringiensis ssp. tenebrionis, Sweet potato weevil, Cry3Aa toxin, binding assays

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RESUMEN

Identificación de proteínas de membrana intestinal de *Cylas formicarius* **ssp.** *elegantulus* **con función receptora para la toxina Cry3Aa de** *Bacillus thuringiensis* **ssp.** *tenebrionis***.** El uso de *Bacillus thuringiensis* como control biológico y para la obtención de plantas transgénicas resistentes a insectos se basa en estudios del mecanismo de acción de las toxinas Cry sobre insectos blanco. En este trabajo se describe por primera vez la unión de la toxina Cry3Aa a vesículas de membrana de borde en cepillo del epitelio intestinal del tetuán del boniato (*C. formicarius* sp. *elegantulus* Fabricius), la principal plaga de ese cultivo. Para esto se desarrollaron bioensayos con la toxina previamente marcada. Se confirmó la presencia de cuatro proteínas (alrededor de 20, 30, 50 y 85 kDa) en el intestino del insecto mediante Western Blot, presuntamente con función efectora. Péptidos de estas proteínas se secuenciaron y sus secuencias se compararon con las reportadas en bases de datos, lo que permitió su confirmación como proteínas no reportadas con esa función. Además, se evaluó la actividad insecticida de la toxina Cry3Aa en tubérculos de plantas transgénicas de boniato con la expresión a altos niveles de la toxina, en un bioensayo in vivo bajo condiciones de laboratorio. Los resultados sugieren que la toxicidad de la toxina Cry3Aa de *B. thuringiensis* ssp. *tenebrionis* contra el tetuán del boniato pudiera estar mediada por su unión a algunas de las proteínas identificadas en el intestino del insecto.

Palabras clave: boniato, *Bacillus thuringiensis* ssp. tenebrionis, tetuán del boniato, toxina Cry3Aa, ensayos de unión

*I*ntroduction

Insect infestations cause approximately 15 % of the global economic losses in relevant crops [1], with more than 9000 species affecting commercial crops [2]. Chemical insecticides have certainly helped, but they are very expensive [3], pose serious treats to the environment and induce insecticide resistance [4]. Among the alternatives, biological control agents have become a priority for agriculture, particularly the use of *Bacillus thuringiensis* toxins (Bt) [5-8]. Their use has dramatically reduced the application of conventional chemical insecticides [8].

More than 350 Bt toxins have been identified so far, and classified into 72 groups of Cry proteins [9]. Some reported toxins with activity against coleopteran

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are Cry7Aa obtained from BTS137J which belongs to the serovar H5 *galleriae* [10, 11] and Cry8Ca from *B. thuringiensis japonensis* [12, 13]. Particularly, Cry3A, Cry3B and Cry3C groups, produced by *B. thuringiensis tenebrionis* (Btt) [14], *san diego*, *morrisoni*, *tolworthi* and *galleriae*, [15-17] show specific activity against this insects' order.

In fact, Cry3Aa was the first δ-endotoxin discovered with coleopterocide activity [14], its gene being cloned and characterized [17]. Besides, the Cry3Aa crystal structure was the first ever elucidated for a Bt toxin [18]. Its simpler structure, as compared with those of other Cry toxins, makes it a useful model to explore the structure-function relationship of the toxin ligand-insect membrane receptor molecular complex [19]. That interaction in involved in the triggering events mediating the insect intoxication process [20]. Hence, the Cry3Aa action mechanism (crystal solubilisation, toxin activation and receptor binding) was investigated in coleopteran species, being described particularly in *Leptinotarsa decemliniata* [21].

But basic research is required on Bt toxins mechanisms of action for its potential use against the given target insects [22] as well as to identify their species' specific target receptors. Some mechanistic models have been developed using various methodologies including *in vitro* testing in Brush Border Membrane Vesicles (BBMVs). These are vesicles derived from insects' intestinal epithelial cells, which correspond to the plasma microvilli membranes of the lumen. They have been used to study the electric and chemical ion transport and binding of toxins to the receptor proteins [23], and are among the most widely used techniques for studying the toxin-receptor binding [24] and to determine its specificity [25].

Notoriously, the interactions Cry toxins-receptors and their downstream effects have been more extensively studied in lepidopteran [26-28]. Many Cry toxin binding models have been proposed [27]. Most of them involve three types of binding proteins as receptors: a cadherin-like protein, a glycosylphosphatidylinositol anchored aminopeptidase-N (APN) and an alkaline phosphatase (AP) [29]. In the case of Cry3Aa toxin, it has been found binding to the midgut receptors of several coleopteran insects such as *Leptinotarsa decemlineata* [21, 30-35], *Diabrotica undecempunctata* [35], *Tenebrio molitor* [32, 36], *Premnotrypes vorax* [22], *Diabrotica virgifera virgifera* [37], *Hypothenemus hampei* [2], *Cylas puncticolis* [38] and *Anthonomus grandis* [39].

A modular transmembrane protein (ADAM metalloprotease) was also identified in *Leptinotarsa decemlineata* as a Cry3Aa binding receptor [29].

However, in the case of the major sweet potato pest, the sweet potato weevil (SPW; *Cylas formicarius* ssp. *elegantulus*), no studies have been conducted resulting in an effective pest control by using Cry toxins. In fact, increasing the production of sweet potato is highly limited by the serious, difficult to avoid damages caused by this phytophagous insect due to its cryptic feeding behavior, with more than 40 % crop losses [40]. Moreover, no Bt-based coleopteran control products have been used because around 90 % of emerging pest larvae' life cycle occurs inside the tubers, limiting the efficacy of the biological control. For these reasons, the expression of Cry proteins specific against SPW in transgenic plants could provide an alternative strategy to control this pest [38].

Noteworthy, a correspondence between toxin expression and insecticide activity against SPW was observed [41], in spite of the low Cry3Aa toxin expression levels in sweet potato plants transgenic for the bacterial gene [42] or an optimized synthetic plant-like version [43]. In vitro binding assays using BBMVs from SPW and a highly purified Cry3Aa were standardized [44]. In vivo biological activity assays with transgenic tubers against SPW adults were performed after useless attempts with artificial diets in larval stages [41].

Since the elucidation of the Cry3Aa mechanism of action is paramount for establishing a successful pest control strategy, this work was aimed to identify the putative Cry3Aa receptor proteins at SPW gut level. The correspondence of the *in vitro* binding assays with the toxicity to insects in transgenic Cry3Aa sweet potato plants was also addressed. As far as we know, these binding assays were not previously tested for this particular weevil. Potential advantages includes assessing Bt proteins with different receptor binding properties, Bt toxins combinations by gene stacking or pyramiding in transgenic plants for increasing their toxicity or to delay the development of insect resistance to toxins.

Materials and methods

Insects and sweet potato tubers

C. formicarius ssp. *elegantulus* adults and larvae were collected from infected sweet potato tubers, harvested at the Experimental Station of the National Research Institute of Tropical Crops (INIVIT), Camagüey, Cuba, as well as all the vegetable material used in bioassays. Both adults and larvae were directly taken from the tubers at the time of experimentation.

Cry3Aa protoxin purification

Cry3Aa was purified from the sporulated culture of a Bt-EG2158 strain (kindly donated from the Bt stock of the Center for Genetic Engineering and Biotechnology, Havana, Cuba [45]) following previously described methods [46, 47]. The procedures included strain growing in SP medium (8 g/L Nutrient Broth, 1 mM $MgSO_4/H_2O$, 13.4 mM KCl, 0.01 mM $MnCl_2$, 0.2 mM $FeSO_4 \cdot 7\ H_2O$, 0.5 mM $CaCl_2$, pH 7.0) at 30 ºC for 72 h to complete sporulation and autolysis. Cells and crystals were harvested by centrifugation and washed with TET buffer (10 mM Tris-HCl, 1 mM EDTA, 0.05 % Triton X-100, pH 7.5). Spores and crystals were suspended in TTN buffer (20 mM Tris-HCl, 300 mM NaCl, 0.1 % Triton X-100, pH 7.2), disrupted by sonication and separated in discontinuous sucrose gradients. Crystals were solubilized in carbonate buffer (50 mM Na_2CO_3, pH 10.2) plus 0.1 % β-mercaptoethanol at 37 ºC for 12 h. Purified protoxin was quantified using the Bradford protein assay (Bio-Rad) with bovine serum albumin (BSA) as standard. The protein purity was evaluated by densitometric analysis of the Coomassie stained

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protoxin band with BSA reference resolved by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) at 12.5 % [48].

Toxin activation

The toxin was activated by using the protocol described by Bollag and Edelstein [49]. The optimal condition for soluble protoxin activation was trypsin (Sigma): protoxin molecular ratio 1:1 at 37 °C for 60 min. This process was followed by centrifugation at 13 000 *g* for 10 min. The supernatant was used as the active toxin preparation, quantified as abovementioned and checked by SDS-PAGE at 12.5 %.

Toxin labelling

Toxins were biotinylated using a biotin labeling kit (Roche Applied Science) according to the manufacturer's instructions, except for reactions being carried out in a $NaCO_3$ (pH 10) buffer, due to the limited solubility of the Cry3Aa toxin trypsinized form below pH 10 [33]. The non-reacted ester reagent was removed by gel filtration on a Sephadex Q25 column, the sample eluted with phosphate buffer solution (PBS; pH 7.4) and collected in 500 µL aliquots. Protein integrity was checked by SDS-PAGE 12.5 %.

BBMVs purification

BBMVs were isolated starting from 20 g of second-third instar SPW larvae using the differential magnesium precipitation method, as developed by Wolfersberg *et al*. [50]. Total proteins were quantified as previously described for protoxin quantification.

The presence, integrity and purity of purified vesicles were confirmed by SDS-PAGE at 12.5 %, scanning transmission electron microscopy and enzymatic assays, respectively.

The main bands detected in SDS-PAGE reacting with the Cry3Aa toxin were dissected from the gel and stored at –70 °C for mass spectrometry analysis.

Samples of 20 µL of BBMVs preparations were fixed in cooper grills, processed with uranyl acetate at 2 % and observed under a transmission electron microscope (MET-JEOL JEM 1400).

The aminopeptidase (AP) activity was determined by the Leucine AP Sigma Assay in the BBMVs final preparations and in the initial homogenates according to Wu *et al*. [19]. The comparison between both AP values supported the evaluation of BBMVs preparations purity.

In-gel protein digestion

The Coomassie blue stained bands were excised from SDS-PAGE gels, washed with milli-Q water (5 min) and incubated at 37 ºC with 50 % acetonitrile in 1 % ammonium bicarbonate (pH 8.3) until they become colorless. Gels were additionally cut in small cubes, dried in a SpeedVac concentrator (Savant) and further rehydrated in 25 mM ammonium bicarbonate buffer containing sequencing-grade trypsin (Promega, USA) at 12.5 ng/mL. The in-gel digestion proceeded overnight at 37 ºC. The resultant proteolytic peptides were eluted in 30 mL of ammonium bicarbonate at room temperature (30 min) and absorbed onto C18 ZipTip (Millipore, USA), previously equilibrated following the manufacturer instructions. The ZipTips were washed with formic acid solution (5 %, v/v), and eluted in 3.0 µL of 60 % acetonitrile, 0.1 % formic acid. The eluate was loaded into gold-coated borosilicate nanotips (Micromass, UK) for protein identification by mass spectrometry.

Mass spectrometry

The low energy ESI-MS and MS/MS spectra were acquired using a hybrid quadrupole orthogonal acceleration tandem mass spectrometer Q-Tof 2 (Micromass; Manchester, UK) fitted with a Z-spray nanoflow electrospray ion source. Other measuring conditions and data processing were the same as reported previously [51].

Cross-species protein identification

The most intense signals observed in the ESI-MS spectra were further analyzed by ESI-MSMS and these spectra were manually interpreted in order to obtain partial or complete sequence information of the analyzed peptides. The resultant sequences were directly loaded onto the MS BLAST program [52, 53] to perform the cross-species protein identification in the sequence database. For each analyzed band the sequences of all peptides were introduced into the MS BLAST program as a text file separated by hyphens, as suggested by Shevchenko *et al*. [52, 53]. Cross-species protein identifications were considered as correct when the alignment scores provided by the MS BLAST program were statistically significantly considered as positive hits. Sequence alignments were performed at http://dove.embl-heidelberg.de/Blast2/ using the default parameters [52, 53].

Binding experiments on intact BBMVs from SPW

For binding experiments, seven BBMVs concentrations ranging from 5 to 35 µg were mixed each with 5, 10, 15 and 20 ng of biotin-labeled toxin in 100 µL of PBS/Tween buffer (0.1 % Tween-20). After incubation for 1 h at room temperature, samples were centrifuged at 13 000 *g* for 30 min. The pellet was washed with 50 mM of $NaCO_3$ buffer (pH 10), centrifuged again and suspended in 30 µL of same buffer. BBMVs proteins bound to labeled toxin were plotted on nitrocellulose membrane slides (Hybond-C extra from Amersham Biosciences), slides further incubated overnight in blocking buffer solution (maleic acid 100 mM, 150 mM NaCl, 1 % skim milk as blocking reagent, pH 7.5). The blocked membrane was washed, incubated for 1 h with streptavidin-peroxidase conjugate 1:1000 in PBS and developed with ECL reagent (ECL Western blotting analysis system from Amersham Life Science). As control, sample vesicles without biotin-labeled toxin incubation were used at the same concentration and processed in the same way as the test samples.

Separation of BBMVs proteins of SPW and Western blotting analysis

BBMVs were suspended at 5 mg/mL in a buffer containing 20 mM Tris-HCl, 100 mM NaCl, 5 mM EDTA, pH 8.5 and stirred at 4 ºC overnight for solubilization. Insoluble material was removed by centrifugation at

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13 000 *g* for 1 h at 4 ºC. The supernatant was five-fold diluted with 20 mM Tris-HCl, pH 8.5 and syringe-filtered through a 0.2 µm membrane. Filtered BBMVs (50 mg) were loaded on a Sephadex G-75 ultrafine column and eluted in a phosphate buffer at a flow rate of 2 mL/min for 2 h. Seven fractions were collected, Amicon concentrated and quantified as described in above sections. Then, 20 µg of BBMVs proteins from each fraction were mixed with SDS-PAGE sample buffer, heated for 5 min at 100 ºC and loaded onto a 12.5 % polyacrylamide gel. After electrophoresis, separated proteins were transferred to nitrocellulose membrane according to Towbin *et al*. [54] (30 min at 20 V) and incubated overnight in a blocking buffer solution. The blocked membrane was washed, incubated for 1 h with 50 ng of biotin-labelled Cry3Aa, washed again and incubated with streptavidin-peroxidase conjugate 1:1000 in PBS. The signal was developed with ECL reagent (ECL Western blotting analysis system from Amersham Life Science). Proteins in fractions reactive to Cry3Aa toxin were processed by mass spectrometry. Sequences of some peptides of these proteins were subjected to analysis by homology comparison in databases according to Altschul *et al*. [55].

Bioassays

Bioassays were carried out using storage roots of two lines of transgenic Cry3Aa sweet potato tubers, regarded as high expression lines (with toxin levels ranging from 0.1-0.25 µg/g of fresh storage roots) and storage roots of the wild type as a control. Cry3Aa protein in storage roots was quantified using the commercial Agdia Cry3Aa DAS-ELISA test System (Agdia Inc., Elkhart, USA).

Four tubers were placed in plastic cages according to their sizes in a completely randomized design at 25 ºC and 70 % of relative humidity, where they were infested with two adult couples of SPW per tuber, accounting for eight females per cage. Sexing of weevils was conducted using the size of the antennal segment as reported by Cisneros and Alcazar [40]. After 48 h, adult couples were removed, expecting oviposition rates of 4.3 eggs per female [40]. Cages were then bunged and tubers were incubated until adult weevils emerged.

The toxic effect of transgenic storage roots on *C. formicarius* insects was evaluated by the number of emerged adults after 30 to 35 days of incubation. The bioassay was replicated three times. The average values of adults for each treatment and replicates were analyzed using a One way ANOVA assay and the differences were statistically compared using the F test. Data were processed by using Statgraphics Plus 5.1 for Windows.

Results and discussion

Cry3Aa protoxin purification

In this study, BBMVs from *C. formicarius* ssp. *elegantulus* were tested for the ability to bind the Cry3Aa from *B. thuringiensis* ssp. *tenebrionis*. Some BBMVs proteins were identified as putative receptors to this Cry toxin.

Cry3Aa crystals prepared from *B. thuringiensis* ssp. *tenebrionis* were composed of the expected 67 and 73 kDa protoxin-sized proteins, this last predominant in the preparation. This result was in agreement with previous reports on the predominance of the 73 kDa form in inclusions when *B. thuringiensis*. ssp. *tenebrionis* was grown in nutrient-rich broths, presumably due to a lower production of bacterial proteases under those conditions [56]. The trypsinized Cry3Aa toxin used in the binding assays migrated at 55 kDa (Figure 1), as expected [45]. The Cry3Aa was obtained at 1.3 mg/mL with a purity of 96.7 %, its trypsinized form rendering 0.9 mg/mL.

The presence of the 67 kDa form can be explained by the proteolytic process of the highest protein to the fully toxic Cry3Aa N-terminus product, losing the 49-57 residues segment during or after crystal formation. This modification has been extensively described [57-59], as well as the crystal protein being only soluble at pH values above 10 or below 4 [56]. The rich growing media and pH conditions tested could have also favored the preponderance of the 73 kDa form in the preparation. Moreover, the 55 kDa obtained after trypsin digestion can be explained by a mechanism as mentioned in similar assays performed at pH 10.5 [57].

The labeled toxin integrity was confirmed by SDS-PAGE 12.5 %. No degradation pattern was observed (data not shown).

Isolation and characterization of BBMVs from SPW

BBMVs yields, approximately 0.5 mg/mL, were very low considering the starting source of 20 g of entire larvae. This situation seems to be common for all *Coleoptera* spp., due to the small larval size [60]. The second-third instar SPW larvae used in the preparation reported in this work were between 4-6 mm long and 0.5-1 mm in diameter in the cephalic region (Figure 2). However, higher yields around 1.52-1.83 mg/mL have been obtained from the same starting material in small coleopteran larvae [61].

The vesicle preparation was considered as pure according to its aminopeptidase activity, which is a specific quality indicator to establish the purity of

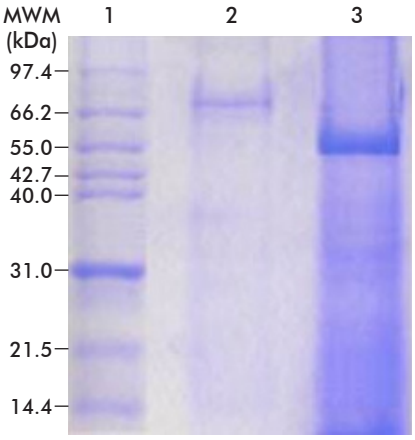

Figure 1. Purification of Cry3Aa protoxin in SDS-PAGE 12.5 %. Lanes: 1, Molecular weight marker (MWM, Mid-range; Promega); 2, Cry3Aa protoxin; 3, Cry3Aa solubilized and trypsinated.

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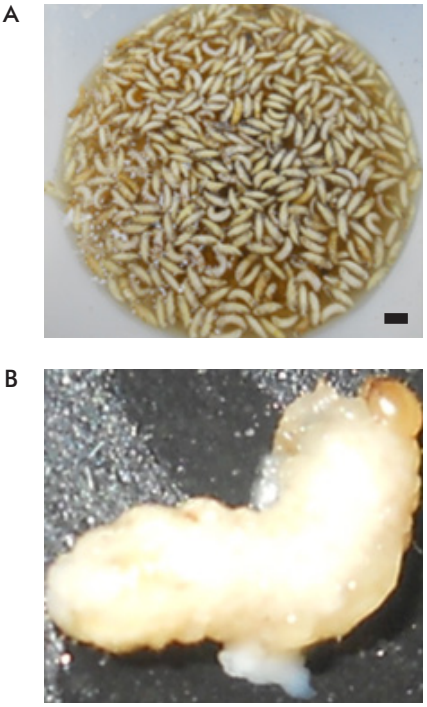

Figure 2. Sweet potato weevil (*Cylas formicarius* ssp. *elegantulus* Fabricius) larvae. A) Second-third instar larvae (20 g, approximately 350 larvae; Bar = 5 mm). B) Dissected single larvae.

BBMVs. Final AP values appear enriched almost 20 times as compared with the initial homogenate determination. Similar values of aminopeptidase activity have been described for BBMVs isolated from whole third instar of *C. scripta* larvae and midguts of *L. decemliniata* [19].

In BBMVs SDS-PAGE, seven major bands were observed at approximate sizes of 15, 20, 30, 50, 85, 100 and 150 kDa, respectively, which were sliced from the gel for further characterization (Figure 3).

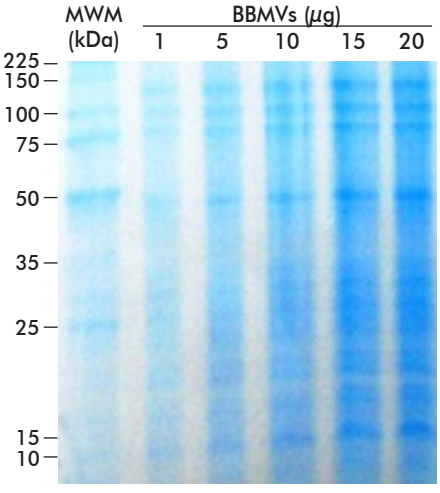

Figure 3. Purification of brush border membrane vesicles (BBMVs) from the sweet potato weevil (*Cylas formicarius* ssp. *elegantulus* Fabricius) larvae. MWM: Molecular Weight Marker (Mid-range; Promega).

Coinciding with this wide size range, Martínez and Cerón [61] reported on proteins between 24 to 116 kDa in a similar preparation of BBMVs from *Premnotrypes vorax*, the most intense bands with sizes 45, 60, 75 and 97 kDa. Only an equivalent size could be established for the 100 kDa band, while the rest are not perceptible in the preparation described in this work. Similar patterns were originally described in *P. brassicae* [50] and later in *A. aegypti* [62].

There are other coincidences in the SDS-PAGE of the SPW BBMVs with previous reports on protein bands molecular sizes above 100 kDa. Proteins with molecular sizes of approximately 120 kDa were described in *Manduca sexta* [63], 144 kDa in *T. molitor* [36], 148 kDa in *Spodoptera fhigiperda* and 103, 120 and 155 kDa in *Heliothis virescens* and *Heliothis zea*, all of them identified as Cry toxins receptors [22]. Other proteins of 120-170 kDa have been identified in some *Lepidoptera* spp. [64-68].

The fact that some midgut membrane proteins obtained, mainly those below 100 kDa, show no coincidences with the previously informed sizes in literature for other insects could be conditioned by varied factors. A plausible explanation is the proteolytic degradation of higher size proteins, rendering small signals. This situation could be present when higher-size putative receptors were proteolytically-degraded without affecting the toxin binding domain [69]. Though, the small bands could be binding proteins of such sizes. BBMVs presence and integrity was corroborated by transmission electron microscopy (Figure 4). Vesicles showed round shapes with sizes around 100 nm in diameter, coinciding with previous images of BBMVs obtained from *P. vorax* larvae midgut [22].

Binding experiments of the Cry3Aa toxin to intact SPW BBMVs

The binding assays with trypsinated-biotinylated Cry3Aa toxin confirmed its capacity to recognize BBMVs from *C. formicarius* ssp. *elegantulus*. Chemoluminescent signal detected in the immune-dot blot assay proportionally increased as the protein amount attached to the nitrocellulose membrane, indicating the affinity between the Cry3Aa toxin and certain proteins present in the purified vesicles from

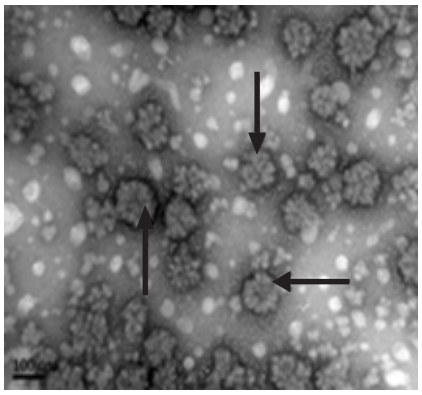

Figure 4. Trasmission electron microscopy micrographs of brush border membrane vesicles (BBMVs) from the sweet potato weevil (*Cylas formicarius* ssp. *elegantulus* Fabricius) larvae midgut. Arrows indicate the proper round-shaped vesicles.

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C. formicarius ssp. *elegantulus* (Figure 5). This fact suggests a possible molecular interaction between the intestinal proteins and the toxin structure.

Even when Cry3Aa was the first δ-endotoxin discovered with coleopterocide activity and its affinity to coleopteran midgut proteins has been widely described, this work is the first report of the binding of the Cry3Aa toxin to *C. formicarius* BBMVs.

Remarking the relevance of BBMVs binding assays, several studies have shown the direct correspondence between toxicity and toxin binding to a receptor for lepidopterans control [70]. Moreover, taking into account binding assays results, some products have been developed based on Bt and satisfactorily used in coleopteran pest control, such as: Trident®, M-One®, M-Trak® and Novodor®. Also, transgenic plants carrying Bt genes against coleopterans, like the transgenic Cry3Aa potato against Colorado Potato Beetle, commercialized since 1995 until 2000 were successfully introduced [71].

Western blot analysis

The binding of *C. formicarius* BBMVs to Cry3Aa was analyzed by Western blotting. From the seven major protein bands detected on SDS-PAGE gels, the four most intense corresponding to approximate molecular sizes of 20, 30, 50 and 85 kDa showed a recognition signal as indication of immunoaffinity reaction (Figure 6). Except for the 30 kDa protein band, which was almost twice as intense, signal intensity was similar for the other three.

These results could indicate that all proteins are Cry3Aa putative receptors, but perhaps the 30 kDa protein could exhibit higher affinity or is present in amounts higher than the others. In *Coleoptera* ssp. there are some reports of receptor proteins at insect gut level. Belfiore *et al*. [36] informed on a 144-kDa protein, present in the Yellow Meal Worm (*T. molitor*); Ochoa-Campuzano *et al*. [29] described for the first time Cry3Aa receptors of 30, 53, and 70 kDa in Colorado Potato Beetle (*Leptinotarsa decemliniata*); Martinez and Cerón [22] informed about a

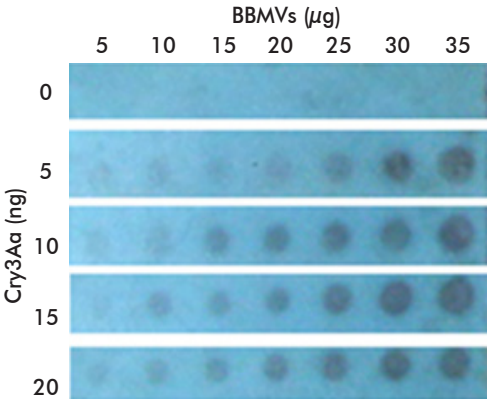

Figure 5. Recognition of *Bacillus thuringiensis* ssp *tenebrionis* Cry3Aa toxin by brush border membrane vesicles (BBMVs) proteins from the sweet potato weevil (*Cylas formicarius* ssp. *elegantulus* Fabricius) larvae midgut. Standard dotblot experiments were run onto nitrocellulose membranes with mixtures of BBMVs and biotin-labelled Cry3Aa toxin.

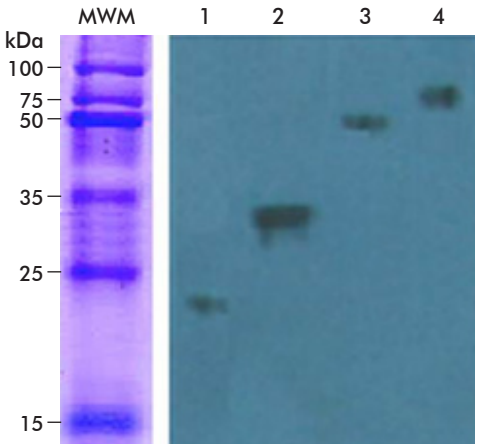

Figure 6. Cry3Aa recognition of electrophoresis-separated proteins from brush border membrane vesicles (BBMVs) of *Cylas formicarius* by Western blot. Lanes 1 to 4 correspond to 20, 30, 50 and 85 kDa proteins, respectively. MWM: Molecular weight marker (Mid-range; Promega).

70-kDa Cry3Aa-binding protein obtained from a BBMVs preparation of *Premnotrypes vorax*.

Also, in a finding regarding the Cry toxin-aphid gut interactions, Li *et al*. [72] confirmed the specific binding of Cry3Aa to 25 and 37 kDa proteins in the pea aphid, *Acyrthosiphon pisum*, by ligand blot analysis and competition assays.

According to the mass spectrometry results of the four proteins detected as recognizing Cry3Aa in BBMVs of *C. formicarius* (Table 1), sequence homology derived from peptide analysis indicated that the 30 kDa protein was identified as an annexin IX isoform like protein, which has a conserved motive in the annexin proteins family from the coleopteran *Dendroctonus ponderosae*. In line with these results, there is a report regarding a 30.32 kDa peptide identified as a Cry3Aa receptor in BBMVs of *Leptinotarsa decemliniata*, which corresponds to a Prohibitin-1 like protein from the coleopteran *Tribolium castaneum* [21].

The 20 kDa protein showed homology with a heat shock protein (HSP) Letal-2, comprising a motif conserved in the HSP family, which is also from *T. castaneum*.

Proteins of 60 and 85 kDa did not show similarity with any other reported proteins. The fact that *C. formicarius* genome is not sequenced could partially explain this situation. Nevertheless, it is interesting that in the case of the 85 kDa, Cry3Aa receptor proteins of 85.92 and 86.41 kDa found in BBMVs of *Leptinotarsa decemliniata* have been described as aconitase similar proteins from *Tribolium castaneum* [21].

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Table 1. Peptide sequence of proteins of BBMVs from *Cylas formicarius* that bind Cry3Aa toxin of *Bacillus thuringiensis* ssp *tenebrionis*

Protein molecular mass	Sequenced peptide	Score mascot	Description	Species
20 kDa	L/ISSDGVL/ISLTAPL/IATL/ISL/IWAHK	55	Annexin IX	*Dendroctonus ponderosae*
30 kDa	SAWEESL/IFNSL/IL/IL/ITR	58	Heat Shock Protein Lethal-2	*Tribolium castaneum*

It is noticeable that our finding did not coincide with the majority of Cry reported receptors, which have been identified as cadherins and APN [31, 73]. A partial *Diabrotica virgifera virgifera* cadherin fragment corresponding to cadherin repeat domains 8-10 was reported to bind activated Cry3Aa and to enhance toxicity in several beetles, suggesting that cadherin plays a functional role in Bt intoxication [46]. The synergizing mode of action is described for the coleopteran lesser mealworm (*Alphitobious diaperinus*). It is generally reported as Cry receptor in the midgut of several lepidopteran, coleopteran and dipteran insects [74-76]. In the particular case of *Aedes aegypti*, the role of cadherin mediating the toxicity is described, however is assured that it is not the main receptor [77]. A peptide containing the predicted toxin binding region from *T. molitor* cadherin (TmCad) bound Cry3Aa specifically and promoted toxin oligomerization in solution. Reduced levels of TmCad transcript in actively feeding larvae correlate with a reduction in toxicity [20]. Studies have demonstrated the involvement of Cry3Aa1 domain II loop 1 as the binding site of CR12 cadherin repeat in this insect species [47]. Cadherin receptors for Cry3Aa in *Leptinotarsa decemlineata* were also identified [46].

On the other hand, APN are considered one of the most important Bt receptors, over 140 cDNAs have been cloned from more than 20 lepidopteran species [78, 79]. They are the most abundant enzymes in the intestinal microvilli membranes, constituting the 55 % of brush membrane intestinal epithelium proteins in coleopterans [80]. Nevertheless, in this order of insects there is no description of any APN as receptor of Bt Cry toxins [21]. Only two proteins of 62 and 65 kDa in BBMVs of *Anthonomus grandis* have shown activity in binding experiments with Cry1Ba6 [39].

Nevertheless, in spite of the abundance of APN as Cry toxins receptor, it has not been described as a coleopteran receptor, while cadherins are the most widely distributed and well-studied as Cry receptors in coleopterans. The variability in the kind and abundance of the Cry receptors for different insects has been reported. The work of Oppert *et al*. using transcriptome profiling of the response of *T. molitor* to Cry3Aa revealed that only transcripts related to alkaline phosphatase were induced, while the ones related to APN and cadherin had similar numbers of reads in control and intoxicated larvae [81].

Bioassays

Test for in vivo Cry3Aa-insects assays using either fresh tuber discs or artificial diets supplemented with toxin were useless. Weevil attracting terpenes could be lost during exposure of tuber slices and fungal contamination interfered data analysis. Natural conditions in tuber slides are very difficult to achieve due to the cryptic feeding behaviour of this insect during larval stages to adults. However, bioassays were reported as successfully conducted with sweet potato weevils *C. puncticollis* and *C. brunneus* in Africa [11, 38, 82].

As an alternative, transgenic sweet potato tubers carrying a cry3Aa plant-like gene and expressing Cry3Aa toxin already obtained for plant-to-insect resistance purposes [41] were used for *in vivo* insecticide activity evaluation against SPW under lab conditions, higher expressing clones being selected (Table 2).

New adult weevils began to emerge at 31-35 days post-infestation. Adult emergence was recorded at 40 days, the observations in agreement with previous reports as evidencing that adult weevils fed in the same way either on transgenic or untransformed storage roots [11].

A smaller number of insects obtained from transgenic storage roots, with differences statistically significant, probably indicates loses in the larvae and pupae stages due to the toxic effect of the Cry3Aa expressed in plants. Nevertheless, there was not a total control of the SPW because there were insects emerging from transgenic tubers.

It should be taken into account that transgenic lines used of highest expression of the Cry toxin do not produce more than 0.25 µg/g of tuber tissue, which could be considered low according to similar reports [11].

Another plausible explanation, coinciding with this supposed insect resistance, could be that the chronology of Cry intoxication would be highly variable depending on the target insect. Some larvae can demonstrate clear symptoms within hours after intoxication, but in the case of Cry3Aa and the coleopteran *T. molitor*, larvae can survive for weeks without obvious signs of paralysis, which ultimately occurs [81].

Nevertheless, similar amounts of Cry3Aa in transgenic sweet potato plants have been toxic for *C. formicarius* under field conditions [42]. Even when an inverse correspondence between tuber damages by insect feeding and toxin expression was detected, no totally resistant clones were obtained.

Conclusions

In summary, the direct relation observed in this work between toxicity and binding experiments indicates the presence of proteins at *C. formicarius* gut, acting as putative receptors for Cry3Aa toxin and probably mediating a toxic mechanism. This is the first report on the specific binding of a Cry toxin in *C. formicarius*, as well as the detection of new proteins involved in such interaction. These results could help to characterize the mechanism of action of the Cry3Aa used in this work. Additionally, the same procedure could be used for some other Bt toxins with potential SPW control activity. The combination

Table 2. Sweet potato weevil (*Cylas formicarius*) adult insects emerged in bioassays with wild type or transgenic sweet potato plants expressing the *Bacillus thuringiensis* ssp *tenebrionis* Cry3Aa toxin.

Sweet potato tubers	Cry3Aa toxin content (µg/g of fresh storage roots)	Adults emerged
Wild type	Without Cry3Aa toxin	69 [a]
Transgenic clone 19	0.1	29 [b]
Transgenic clone 24	0.25	31 [b]

* Data with different letters have statistically significant differences (one-way Anova, Duncan test, p > 0.95)

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of binding assays with the identification by mass spectrometry of the proteins taking part rendered both, the detection of the supposed receptors and the finding of new proteins apparently related with the toxicity of the Cry3Aa on the major sweet potato pest worldwide.

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