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Identification of *Bacillus thuringiensis* Cry1AbMod binding-proteins from *Spodoptera frugiperda*

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ABSTRACT

Bacillus thuringiensis Cry toxins are currently used for pest control in transgenic crops but evolution of resistance by the insect pests threatens the use of this technology. The Cry1AbMod toxin was engineered to lack the alpha helix-1 of the parental Cry1Ab toxin and was shown to counter resistance to Cry1Ab and Cry1Ac toxins in different insect species including the fall armyworm *Spodoptera frugiperda*. In addition, Cry1AbMod showed enhanced toxicity to Cry1Ab-susceptible *S. frugiperda* populations. To gain insights into the mechanisms of this Cry1AbMod-enhanced toxicity, we isolated the Cry1AbMod toxin binding proteins from *S. frugiperda* brush border membrane vesicles (BBMV), which were identified by pull-down assay and liquid chromatography-tandem mass spectrometry (LC–MS/MS). The LC–MS/MS results indicated that Cry1AbMod toxin could bind to four classes of aminopeptidase (N1, N3, N4 y N5) and actin, with the highest amino acid sequence coverage acquired for APN 1 and APN4. In addition to these proteins, we found other proteins not previously described as Cry toxin binding proteins. This is the first report that suggests the interaction between Cry1AbMod and APN in *S. frugiperda*.

1. Introduction

Bacillus thuringiensis (Bt) is a Gram-positive bacterium that produces insecticidal Cry toxins that are currently used for insect pest control, since they are highly toxic to insects, safe to humans and environmental friendly [1,2]. The mode of action of the Cry1A proteins, that are active against lepidopteran species, involves several steps. After ingestion by the larvae the toxin is activated by midgut proteases, then the activated toxin binds to specific receptors (cadherin-like, APN, ALP), the toxin oligomerizes and forms pores in the apical membrane of the insect midgut cells causing cell lysis and insect death [3–5]. In particular, binding of the Cry1Ab toxin to cadherin facilitates further proteolytic activation of the toxin in which the N-terminal alpha helix-1 is cleaved facilitating toxin oligomerization [3]. The main threat to the use of Bt as a pest control agent is the evolution of resistance [6]. Cry1AbMod and Cry1AcMod toxins were engineered to lack the amino-terminal alpha helix-1 of the native toxins. They were shown to form oligomers *in vitro* without cadherin binding and were effective against Cry1A resistant insects [6,7].

The fall armyworm, *Spodoptera frugiperda* (J.E. Smith), is a lepidopteran pest that affects several important crops, such as corn, cotton, soybean, peanut, sorghum, sugarcane, bermudagrass, rice and cowpea, causing severe economic losses [8]. In recent years, there have been several reports describing the evolution of resistant *S. frugiperda* populations to corn expressing Cry1Fa in Puerto Rico [9], Brazil [10] and Florida [11]. Interestingly the Cry1AbMod toxin was shown to be effective against Cry1Fa-resistant *S. frugiperda* from Brazil while showing an enhanced toxicity against the susceptible *S. frugiperda* populations, as compared to Cry1Ab toxin [12].

Cry1A binding-proteins in the midgut of the susceptible larvae include cadherin, glycoproteins and glycosylphosphatidyl-inositol (GPI) membrane anchored receptors such as aminopeptidase N (APN) and alkaline phosphatase (ALP) [13]. Recently, the ABCC2 transporter was shown to be a functional receptor for Cry1A proteins in different lepidopteran species [14–17]. At present, there are no reports of the identification of Cry1A receptors in *S. frugiperda*, though the ribosomal protein S2 has been recognized as a binding protein for Vip3A toxin in this insect but in spite of its cellular location it is unlikely to function as

Abbreviations: ABCC2, ATP binding cassette subfamily C member 2; ALP, alkaline phosphatase; APN, aminopeptidase-N; BBMV, brush border membrane vesicles; Bt, *Bacillus thuringiensis*; CNBr, Cyanogen bromide; GPI, glycophosphatidylinositol; kDa, Kilodalton; LC–MS/MS, liquid chromatography-tandem mass spectrometry; M, molecular weight marker; RNAi, RNA interference

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a receptor of Vip3A [18]. Vip3A is also an insecticidal, pore forming toxin, produced by certain Bt strains during the vegetative phase of growth but it is not related to Cry1A toxins. On the other hand, for other *Spodoptera* species there is some information about the identification of proteins that are functional Cry1 toxin receptors. It was shown by RNAi assays that *S. exigua* with lower cadherin expression become tolerant to Cry1Ca, suggesting that cadherin could participate as receptor for Cry1Ca toxin [19]. Also, the silencing of cadherin expression by RNAi in *S. exigua* showed that cadherin is involved in Cry1Ac and Cry2Aa toxicity [20]. APN was proposed as receptor for Cry1Ca in *S. litura* by RNAi silencing experiments [21]. The APN1 of *S. exigua* was suggested as a receptor for Cry1Ca since the resistant insects lacked the expression of *apn1* gene [22]. Furthermore, silencing the expression of different APN isoforms in *S. exigua* larvae suggested that APN1, APN3 and APN6 are involved in Cry1Ca toxicity [23]. Finally, the participation of ABCC2 and ABCC3 in *S. exigua* were analyzed by RNAi showing that these ABC transporters are functional receptor for Cry1Ac and Cry1Ca in *S. exigua* [24].

Here we present the isolation and identification of different binding proteins for Cry1AbMod in *S. frugiperda* by pull-down assays and liquid chromatography-tandem mass spectrometry (LC–MS/MS). This is a powerful methodology that allows the identification of several proteins that interact with the toxin. The generated information of these proteins will help to understand the mode of action of Cry1AMod toxins, and provide tools for countering the development of resistant insects to Bt crops.

2. Materials and methods

2.1. Production of the recombinant Cry1Ab and Cry1AbMod toxins

The Bt 407- strain [25], producing Cry1Ab or Cry1AbMod toxin was grown at 30 °C until complete sporulation (3 days) in nutrient broth sporulation medium supplemented with erythromycin at 10 μg ml^{-1}. Spores/crystals were washed several times in 300 mM NaCl, 10 mM EDTA. Crystal inclusions were solubilized in an alkaline buffer (100 mM Na_2CO_3, 0.2% β-mercaptoethanol, pH 10.5) for 1 h at 37 °C. The activated toxins were obtained by treatment of the soluble protoxin with 1% of midgut juice from *S. frugiperda*. The activated toxins were purified by chromatography using a Mono Q ion-exchange column (Pharmacia Biotech, Montreal, Qc, Canada). Bound toxin was eluted with a 50–500 mM NaCl gradient in a 20 mM sodium carbonate buffer (pH 10.8). The Cry1AbMod protoxin and the purified toxin were analyzed by SDS-PAGE (10% acrylamide), with staining by Coomassie blue.

2.2. Preparation of brush border membrane vesicles (BBMV) and soluble BBMV proteins from S. frugiperda

An *S. frugiperda* colony has been maintained on an artificial diet [26] under laboratory conditions at 28 ± 2 °C and 65% ± 5% relative humidity, under a 12:12 (light-dark) photoperiod at the Institute of Biotechnology (IBT-UNAM) facilities since the year 2000. *S. frugiperda* midgut tissues from third instar larvae were dissected and stored immediately at −70 °C. BBMV were prepared by the magnesium precipitation method as described by [27] and stored at −70 °C until used. The concentration of BBMV protein was determined by the Lowry DC protein assay (BioRad, Hercules, CA) using bovine serum albumin as the standard. For BBMV solubilization, we optimized the solubilization procedure by comparing protein yields and solubilization of ALP and APN enzymatic activities after solubilization BBMV with two detergents (0.1% Triton X-100 or CHAPS 0.1%) in BBMV-solubilization buffer (50 mM Na_2HPO_4/NaH_2PO_4, 50 mM NaCl, 5 mM EGTA, 1 mM PMSF, pH 7,4). The data showed that higher yields of total soluble protein and of soluble ALP and APN were achieved with Triton X-100 (data not shown). Thus, one mg of BBMV protein was incubated with Triton-solubilization buffer (Triton X-100 1%, 50 mM Na_2HPO_4/NaH_2PO_4, 50 mM NaCl, 5 mM EGTA, 5 mM EDTA, 1 mM PMSF, pH 7.5) at 4 °C for 1 h and centrifuged at 50,000 rpm for 30 min. The supernatant was recovered and the protein was quantified by Bradford assay using bovine serum albumin as the standard.

2.3. Binding of Cry1Ab and Cry1AbMod to BBMV from S. frugiperda

Cry1Ab or Cry1AbMod toxins were biotinylated using Biotinamidocaproate N-hydroxysuccinamide ester (Amersham Biosciences) according to the manufacturer's instructions. Ten micrograms of BBMV protein were incubated in binding buffer (PBS, 0.1% BSA, 0.1% Tween 20) with different concentrations of Cry1Ab or Cry1AbMod. Samples were centrifuged (10 min at 14000 rpm) to remove unbound toxin. BBMV were washed three times with binding buffer and suspended in 10 μl PBS plus 3 μl of Laemmli buffer. Samples were boiled and separated by 10% SDS-PAGE and electrotransferred to a PVDF membrane. Proteins on the membrane were revealed using streptavidin coupled to peroxidase (1:5000). The membrane-bound complex was visualized with Super Signal chemiluminescence substrate (Pierce).

2.4. Ligand blot

Ten micrograms of BBMV protein were separated by 10% SDS-PAGE and transferred to PVDF membrane. The PVDF membrane was blocked with PBS supplemented with 5% skimmed milk and blots were incubated for 2 h with 5 nM of biotinylated Cry1Ab or Cry1AbMod toxins in washing buffer (0.1% Tween 20 in PBS) at room temperature. Unbound protein was removed by washing three times in washing buffer for 15 min. Bound toxin was identified by incubating the blots in PBS containing streptavidin–peroxidase conjugate (1:80000) for 1 h. The membrane-bound complex was visualized with Super Signal chemiluminescence substrate (Pierce).

2.5. Pull-down assay

Purified Cry1AbMod toxin was coupled with CNBr agarose (GE Healthcare), according to the manufacturer's instructions. Seven hundred μg of purified Cry1AbMod toxin were incubated with 150 μl CNBr agarose in 1 M $NaHCO_3$, 0.5 M NaCl buffer (pH 8.3) at 4 °C overnight. The noncoupled toxin was removed by centrifugation. The unreacted CNBr groups were blocked by incubation with 0.1 M Tris-HCl pH 8 at room temperature for 2 h. The resin was then washed 10 times with 500 μl PBS as described by the manufacturer. Finally, the coupled CNBr-Cry1AbMod agarose was stored in 20% ethanol (v/v) at 4 °C.

After incubation of 150 μl CNBr-Cry1AbMod agarose with 400 μg solubilized BBMV proteins in 500 μl of 50 mM Na_2HPO_4/NaH_2PO_4, 50 mM NaCl, 5 mM EGTA, 1 mM PMSF, pH 7,4 for 1 h at 4 °C, the unbound BBMV proteins were removed by centrifugation at 13,000 *rpm* for 10 min at 4 °C. The CNBr-Cry1AbMod agarose containing the bound proteins from BBMV was washed five times with 500 μl PBS supplemented with 1 M NaCl, followed by five washes with 500 μl PBS to remove unbound proteins. The proteins that remained bound to the CNBr-Cry1AbMod agarose were dissociated from the agarose by incubation at 100 °C for 5 min in 50 μl SDS-PAGE loading buffer (100 mmol/L Tris-Cl, 200 mmol/L DTT, 4% SDS w/v, 0.2% bromophenol blue w/v, 20% glycerol v/v, pH 6.8). The supernatant was loaded into SDS-PAGE. As negative control of this experiment, the activated CNBr agarose was incubated without Cry1AbMod protein, blocked as described earlier, and used for incubation with solubilized BBMV proteins.

2.6. Identification of the Cry1AbMod-binding proteins

The polyacrylamide gel stained by Coomassie blue was cut into four sections containing proteins with different molecular weights for