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Specific binding of activated Vip3Aa10 to *Helicoverpa armigera* brush border membrane vesicles results in pore formation

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ABSTRACT

Helicoverpa armigera is one of the most harmful pests in China. Although it had been successfully controlled by Cry1A toxins, some *H. armigera* populations are building up resistance to Cry1A toxins in the laboratory. Vip3A, secreted by *Bacillus thuringiensis*, is another potential toxin against *H. armigera*. Previous reports showed that activated Vip3A performs its function by inserting into the midgut brush border membrane vesicles (BBMV) of susceptible insects. To further investigate the binding of Vip3A to BBMV of *H. armigera*, the full-length Vip3Aa10 toxin expressed in *Escherichia coli* was digested by trypsin or midgut juice extract, respectively. Among the fragments of digested Vip3Aa10, only a 62 kDa fragment (Vip3Aa10-T) exhibited binding to BBMV of *H. armigera* and has insecticidal activity. Moreover, this interaction was specific and was not affected by the presence of Cry1Ab toxin. Binding of Vip3Aa10-T to BBMV resulted in the formation of an ion channel. Unlike Cry1A toxins, Vip3Aa10-T was just slightly associated with lipid rafts of BBMV. These data suggest that although activated Vip3Aa10 specifically interacts with BBMV of *H. armigera* and forms an ion channel, the mode of action of it may be different from that of Cry1A toxins.

1. Introduction

Helicoverpa armigera is one of the most serious pests of cotton in China. Although *Bacillus thuringiensis* (Bt) insecticide and Cry1A-expressing cotton had ever successfully controlled *H. armigera* (Wu et al., 2008; Wu and Guo, 2005), it is possible for susceptible pests to develop resistance to Bt due to the long-term use of it. In fact, several strains of *H. armigera* have been selected in the laboratory with high level of resistance to Cry1A toxins (Akhurst et al., 2003; Luo et al., 2006; Xu et al., 2005). Thus, searching for an alternative toxin to construct bivalent or trivalent insecticide with Cry1A toxins is of great significance. Vip3A is one such representative, which is secreted by Bt during the vegetative stage of growth. It shares no sequence homology with Cry toxins, and has insecticidal activity against a broad spectrum of lepidopteran insects (Estruch et al., 1996).

Many works have been done to investigate the core part that contributes to the insecticidal activity of Vip3A. Digested Vip3A by trypsin or susceptible insect midgut juice is still active (Yu et al., 1997). Deletion of 27 amino acids at the N-terminal end led to the loss of insecticidal activity of Vip3A-S184 against *Spodoptera exigua*, *H. armigera* and *Spodoptera litura* (Chen et al., 2003). However, mutant without N-terminal 39 amino acids of Vip3A is still active against *Chilo partellus*, while inactive against *S. litura*; deletion of 154 amino acids at the C-terminal end of Vip3A caused partial loss of toxicity against *C. partellus*, but complete loss of toxicity against *S. litura* (Selvapandiyan et al., 2001). These inconsistent data may result from the use of different target insects and Vip3A toxins in the bioassay.

The mode of action of Cry toxins, especially for Cry1A toxins, has been elucidated clearly (Bravo et al., 2007; Schnepf et al., 1998). After ingestion by susceptible insects, Cry1A protoxins is solublized in the alkaline and reducing environment of the insect midgut and then activated by the midgut enzymes. The activated Cry1A interacts with specific receptors (cadherin, aminopeptidase N (APN) and alkaline phosphatase) and forms an oligmer (Arenas et al., 2010; de Maagd et al., 1999; Gomez et al., 2002). With the assistance of APN, the oligomer of Cry1A toxins inserts into lipid rafts and forms stable ion channel, which leads to disruption of the ion balance between inside and outside of cells and finally results in insect death (Bravo et al., 2004; Zhuang et al., 2002). Unlike Cry1A toxins, the mode of action of Vip3A is still unclear. Histopathological results indicated that after ingestion by susceptible insects, Vip3A caused gut paralysis and complete lysis of gut epithelium cells, resulting in insect death (Yu et al., 1997). The 62 kDa fragment, produced by trypsin or lepidopteran midgut

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juice extract, can bind to BBMV and liposome and form an ion channel (Lee et al., 2003). To clearly elucidate the mode of action of Vip3A, it is essential to indentify the receptors of Vip3A on the BBMV. Previous reports showed that Vip3A does not share the same binding site with Cry1A toxins (Abdelkefi-Mesrati et al., 2011; Lee et al., 2003, 2006; Sena et al., 2009). Moreover, ligand blotting results indicated that Vip3A binds to two proteins on BBMV of *Manduca sexta*, whose molecular weights are approximately 80 kDa and 110 kDa, respectively (Lee et al., 2003), and two proteins on BBMV of *S. littoralis*, with molecular weights about 55 kDa and 100 kDa, respectively (Abdelkefi-Mesrati et al., 2011). However, it is still uncertain whether these proteins are the receptors of Vip3A.

In this paper, we demonstrated that although Vip3Aa10 is digested by trypsin or *H. armigera* midgut juice extract into several fragments, only the 62 kDa fragment (Vip3Aa10-T) specifically binds to BBMV of *H. armigera*. Density gradient centrifugation assay showed that Vip3Aa10-T is slightly associated with lipid rafts of BBMV. Ion channel activity was also observed when Vip3Aa10-T was incubated with BBMV of *H. armigera*.

2. Materials and methods

2.1. Preparation of Vip3Aa10 protein, cadherin fragment, APN fragment and their antibodies

The *vip3Aa10* gene was cloned from *B. thuringiensis* 8010 and inserted into pET-28a vector. The recombinant plasmid pET28a-*vip3Aa10* was transformed into *E. coli* BL21 (DE3) cells. Expression of *vip3Aa10* gene was induced by 0.3 mM IPTG. Vip3Aa10 with a His-tag at its N-terminal end was isolated from cell lysis solution by Ni^+ affinity chromatography, followed by Mono Q ion exchange chromatography. To obtain the 62 kDa fragment (Vip3Aa10-T), Vip3Aa10 was digested by 2% trypsin (wt/wt) at 37 °C for 2 h and then purified by Mono Q ion exchange chromatography. Vip3Aa10-T was dialyzed against buffer I (25 mM Tris–Cl (pH 7.8), 150 mM NaCl, 5 mM β-Mercaptoethanol) for BBMV binding assay, against phosphate-buffered saline (PBS, pH 8.0) for competitive binding assay or against buffer II (20 mM Tris–Cl (pH 8.6), 150 mM CsCl) for measurement of channel activity. Similarly, His-tagged cadherin fragment (1217-1580 amino acids) or His-tagged APN fragment (628-1014 amino acids) from *H. armigera* (Wang et al., 2003, 2005) was expressed in *E. coli* BL21 (DE3) cells containing pET28a-*cad* or pET28a-*apn* and purified by Ni^+ affinity chromatography and Mono Q ion exchange chromatography.

Purified Vip3Aa10, cadherin fragment and APN fragment were used as antigens for preparation of rabbit antibodies. Polyclonal antibodies were purified by ammonium sulfate precipitation from immunized rabbit sera.

The concentrations of proteins were determined by Bio-Rad protein assay kit with bovine serum albumin as a standard (Bradford, 1976).

2.2. Preparation of H. armigera midgut juice extract and Vip3Aa10 digestion

The midguts of third or fourth instar larvae of *H. armigera* were dissected longitudinally and centrifuged at 4 °C, 12,000*g* for 20 min. The supernatant was collected and stored at −80 °C. 40 μl Vip3Aa10 (0.48 mg/ml) was digested by 2% (wt/wt) *H. armigera* midgut juice extract at 37 °C for 0.5, 1, 2 and 8 h or by 1%, 2%, 250% and 500% (wt/wt) trypsin at 37 °C for 2 h. The reaction was then terminated by boiling with loading buffer. Samples were separated by 12% SDS-PAGE.

2.3. Preparation of BBMV

BBMV was prepared from third or fourth instar larvae of *H. armigera* by magnesium precipitation method (Wolfersberger et al., 1987) and finally resuspended in buffer III (25 mM Tris–Cl (pH 7.4), 150 mM NaCl, 5 mM EDTA). For channel activity assay, BBMV was prepared by calcium precipitation and resuspended by buffer IV (10 mM HEPES-Tris (pH 7.4), 300 mM Mannitol, 1 mM KCl) (Giordana et al., 1982).

2.4. Binding assay

To test the binding of digested Vip3Aa10 to BBMV, 50 μl Vip3Aa10 (approximately 25 μg) was digested by 2% (wt/wt) trypsin or 2% (wt/wt) *H. armigera* midgut juice extract at 37 °C for 2 h. The reaction was terminated by addition of 2 mM PMSF (final concentration). 2 μl digested Vip3Aa10 (about 1 μg) was then incubated with 50 μl BBMV (approximately 25 μg total proteins) in the final reaction volume of 100 μl at 25 °C for 1 h. After centrifugation at 4 °C, 30,000*g* for 20 min, the supernatant and pellet were separated by SDS-PAGE and subjected to Western blots. To examine the specific binding of Vip3Aa10-T to BBMV, Vip3Aa10-T was labeled by NHS-biotin according to the Pierce manual instruction and dialyzed against PBS. In the reaction volume of 100 μl, 1 μg biotin-Vip3Aa10-T was incubated with BBMV (25 μg total proteins) in the presence of PBS buffer or 1-fold or 10-fold excess of unlabeled Vip3Aa10-T or 10-fold excess of unlabeled trypsin activated Cry1Ab (from Dr. Liu CX, Institute of Plant Protection, Chinese Academy of Agricultural Sciences) at 25 °C for 1 h. The pellet was collected by centrifugation at 4 °C, 30,000*g* for 20 min and washed three times with PBS and analyzed by SDS-PAGE and Western blots.

2.5. Preparation of lipid rafts

For cholesterol extraction assay, BBMV (0.5 mg total proteins) was first incubated with 15 mM methyl-β-cyclodextrin (MβCD) at 37 °C for 40 min or 0.5% saponin at 4 °C for 40 min. Then BBMV was centrifuged at 4 °C, 30,000*g* for 20 min and the pellet was resuspended by buffer III. BBMV or BBMV pretreated by MβCD or saponin was incubated without or with approximately 50 μg Vip3Aa10-T at 25 °C for 1 h in buffer III containing 5% BSA and protein inhibitor cocktail (Roche). Samples were centrifuged at 4 °C, 30,000*g* for 20 min. Unbound Vip3Aa10-T was removed by washing the BBMV pellet three times. The BBMV pellet was resuspended by 400 μl pre-chilled buffer III containing 1% triton X-100 on ice for 30 min. Then 800 μl 60% Optiprep (Sigma–Aldrich) was added and the mixture was transferred into centrifuge tube, followed by successive overlaying of 2.5 ml 30% and 1.4 ml 5% Optiprep. After centrifugation at 2 °C, 260,000*g* for 3 h, fourteen fractions were collected from the top of the gradient (designated fraction 1 (top) to 14 (bottom)), with each fraction containing 360 μl sample.

2.6. Detection of biotin-Vip3Aa10-T, Vip3Aa10-T, cadherin and APN

Samples were separated by 10% SDS-PAGE, and blotted onto cellulose nitrate membranes. The membranes were blocked by phosphate-buffered saline containing 5% skim milk power and 0.05% Tween-20. After blocking, membranes were incubated with anti-Vip3Aa10, anti-cadherin, or anti-APN antibodies, respectively, followed by incubation with anti-rabbit horseradish peroxidase-conjugated secondary antibody. Blots were detected by enhanced chemiluminescence. For biotin-Vip3Aa10-T assay, the membrane blocked by bovine serum albumin was directly incubated with streptavidin-conjugated horseradish peroxidase and visualized by enhanced chemiluminescence.