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IN VITRO CROSS RESISTANCE STUDIES WITH THE VEGETATIVE INSECTICIDAL PROTEIN VIP3A SUPPORT THE INSECT RESISTANCE MANAGEMENT STRATEGY FOR VIPCOTTM

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Abstract

The introduction into the US of cotton varieties expressing Vip3A will bring a novel toxin into a landscape that is dominated by Bollgard varieties expressing Cry1Ac. The recent introduction of Bollgard II has added Cry2Ab to this landscape. As part of the development of a resistance management strategy for Vip3A, we have undertaken a series of *in vitro* binding studies using brush border membrane vesicles prepared from the major cotton pests, *Heliothis virescens* and *Helicoverpa zea.* Competition binding studies and ligand blotting assays using biotinylated Vip3A, Cry1Ac, and Cry2Ab2 have demonstrated that these toxins have distinct binding sites and that cross-resistance between them is unlikely. The implications of these findings for resistance management for Vip3A are outlined.

Introduction

Bacillus thuringiensis δ–endotoxins (Cry toxins) have been successfully used to control many crop pests by either traditional spray application or transgenic plant approaches. However, a number of cases of insect resistance to the *B. thuringiensis* δ-endotoxins have been reported as a result of laboratory and more rarely, field selections (Ferre *et al*. 2002). Therefore, searching for a new family of insecticidal toxins, with a mode of action different from the δ-endotoxins, is one facet of current strategies designed to delay resistance development. Recently, a number of insecticidal proteins expressed during the vegetative growth phase of *Bacillus thuringiensis* have been identified (Estruch *et al*. 1996, 2001, Yu *et al*. 1997). These secreted vegetative insecticidal proteins (Vip) have a broad insecticidal spectrum including activity toward a wide variety of lepidopteran and also coleopteran pests (Estruch *et al*. 1996, 2001, Yu *et al*. 1997). The Vip3A toxin, an 88 kDa protein, is secreted into the culture media by *B. thuringiensis* and displays high toxicity against a range of Lepidopteran insects. The Vip3A mode of action has been examined and shown to be different from Cry1Ab δ-endotoxin in receptor binding and pore forming activity (Lee *et al*. 2003). These molecular events are also manifested behaviorally, as increasing amounts of Vip3A in an artificial diet correlate with interruption of feeding and gut clearance for susceptible insects (Yu *et al.* 1997). Currently, transgenic insect resistant cotton varieties expressing Cry1Ac, Cry2Ab2, and Cry1F *B. thuringiensis* δ–endotoxins are commercially available. In order to investigate the cross-resistance potential, we have examined the key steps involved in the mode of action and resistance development including proteolytic activation, receptor binding, and pore forming ability of Vip3A toxin and compared to the Cry1Ac and Cry2Ab toxins. Our findings indicate that Vip3A utilizes a different molecular target and forms ion channels that are distinct from those formed by δ-endotoxins.

Materials and Methods

Toxin preparation and proteolysis

Vip3A was overexpressed in *E.coli* as described in Yu *et al*. (1997) then purified with 18% ammonium sulfate precipitation followed by phenyl hydrophobic interaction column chromatography and DEAE ion exchange chromatography. In order to obtain the truncated Vip3A toxin (*ca.* 62 kDa), the 88 kDa full length toxin was incubated with 1% trypsin (w/w) in PBS buffer, pH 7.4 at 37°C for 1 h. Alternatively, lepidopteran gut juice was used to obtain a truncated Vip3A toxin. Gut juice was collected from lepidopteran larvae (*Manduca sexta, Heliothis virescens, Helicoverpa zea*) by gently inducing regurgitation; the gut juice was then centrifuged at 13,500 x *g* for 10 min at 4°C before use. Gut juice extracts (supernatants) were diluted 50-fold with PBS, added to the full length Vip3A toxin and incubated for 1 h at 29°C. Complete Protease Inhibitor cocktail (1 µl, resuspended per manufacturer's instructions, Roche Molecular Biochemicals, Indianapolis, IN) was added to stop the reaction. Toxin stability (all forms) was assessed by 8-12% gradient SDS-PAGE. Mark12™ molecular weight markers (Novex) were used. Cry1Ac toxin was expressed in *E. coli.* Inclusion body proteins were solubilized and activated with 2% trypsin at 37°C for 2h as described previously (Lee *et al*. 1993). Cry2Ab2 toxin was expressed in *E.coli* and purified using a His tag column as described in the manufacturer's instructions (Invitrogen Inc.)

Biotinylation of toxins

Full length Vip3A, trypsin activated Vip3A, trypsin activated Cry1Ac, full length Cry2Ab2, and trypsin activated Cry2Ab2 toxins were dialyzed against 0.1 M Borate buffer, pH 8.0, and then biotinylated using a Biotin labeling kit (Roche Molecular Biochemicals) per the manufacturer's instructions. Non-reacted biotin-7-NHS reagent was removed by gel filtration on a prepared Sephadex G-25 column per the manual instructions (Roche Molecular Biochemicals).

BBMV binding assays

Brush border membrane vesicles (BBMV) were prepared from fourth instar *M. sexta*, *H. virescens* and *H. zea* larval midguts by the differential magnesium precipitation method as described in Wolfersberger *et al*. (1987). The final pellet was resuspended in the binding buffer, consisting of 8 mM $NaHPO_4$, 2 mM KH_2PO_4, and 150 mM NaCl (pH 7.4) and protein concentration was measured using the Coomassie Protein Assay Reagent (Pierce Biotechnology, Inc., Rockford, IL). For qualitative estimation of competitive binding, 2.5 to 5 nM biotinylated toxin was incubated with 5-10 μg BBMV in the presence or absence of increasing amounts of the unlabeled toxin (2.5 to 50 fold excess). After 1h incubation at RT, reaction mixtures were centrifuged at 13,500 X *g* for 10 min. The pellets were washed three times with binding buffer containing 0.1% BSA. Final pellets were resuspended in the binding buffer without BSA and mixed with Laemmli sample buffer prior to SDS-PAGE (8%-16% gradient, Tris-Glycine® Novex gels, Invitrogen, Carlsbad, CA). For visualization, proteins were transferred to Novex polyvinylidene difluoride (PVDF) membranes (Invitrogen) and probed with streptavidin-conjugated peroxidase (Roche Molecular Biochemicals) and the SuperSignal® West Pico Chemiluminescence kit (Pierce).

BBMV ligand competition binding assays

BBMV proteins (2.5 μg) prepared from *H. virescens and H. zea* were separated via SDS-PAGE (4%-12% gradient) and transferred to PVDF membrane. 5nM of the biotinylated toxin was incubated with the membrane for 2 h at room temperature. The toxin-binding proteins were visualized with streptavidin-conjugated peroxidase (Roche Molecular Biochemicals) and the SuperSignal® West Pico Chemiluminescence kit (Pierce). SeeBlue® Plus2 (Novex) pre-stained standards were used.

Results and Discussion

Sequence homology and structural comparison of Vip3A with Cry1Ac and Cry2Ab2 toxins

One of the most important features of the Vip3A protein is that it shares no sequence homology with the known Bt delta endotoxin genes and other toxin genes. X-ray crystal structures of several Bt δ-endotoxins including Cry1Aa, Cry3A, and Cry2Aa have been solved (Grochulski *et al*.1995). These toxins are composed of three domains. Domain I consists of 7 α-helices and is responsible for pore formation. Domain II consists of three antiparrallel β-sheets and is involved in receptor binding. Domain III consists of two twisted, antiparrallel β -sheets and is important in receptor binding and pore formation. Cry2Aa, which has about 87% sequence homology with Cry2Ab2 in Bollgard II™, maintains the three domain structure similar to that of Cry1Aa toxin. While direct structural information is lacking for Vip3A, primary sequence divergence and an examination of predicted secondary structure give no indication of a similar domain organization or a putative α -helical bundle region within the polypeptide sequence, as exists for the Cry toxin channel. Protein folding blasts revealed that Vip3A might be a pore-forming protein that has a structure of β -barrels (unpublished data). The total lack of sequence homology between Vip3A and the Cry toxins as well as the marked structural differences between them strongly supports the probability that there will be no cross-resistance between Vip3A and Cry1Ac and Cry2Ab2 toxins in the target pests.

Proteolytic activation of Vip3A

In order to further understand the mode of action of Vip3A, we have examined selected steps critical to the mode of action of δ-endotoxins using Cry1Ab, Cry1Ac, and Cry2Ab2. Proteolytic activation, binding to isolated insect midgut brush border proteins, and an ability to form pores have been investigated. Full length Vip3A toxin (88kDa) is further processed to a 62kDa fragment (N terminus at 199) by *in vivo* digestion in the lepidopteran midgut or *in vitro* digestion with either trypsin or gut juice extracts (Figure 1). We have found that proteolysis occurs in both non-susceptible and susceptible insect midguts, suggesting that proteolysis alone is not a determining factor for insect specificity. Cry1Ac protoxin (130kDa) can be easily activated with either trypsin or lepidopteran gut juice extracts to a 65kDa core protein. Cry2Ab2 also gives rise to a core 50kDa trypsin- or chymotrypsin resistant fragment following treatment with either trypsin or *H. zea* midgut extracts or BBMVs.

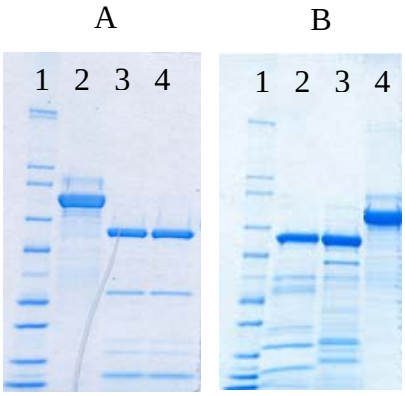

Figure 1. Trypsin and gut juice digestion of full length Vip3A toxin. (A) lane1, full length Vip3A; lane 2, Vip3A- trypsin for 1h; lane3, Vip3A-trypsin for overnight (B) lane 1, Vip3A-gut juice for 2 days ; lane2, Vip3A-gut juice for 1h; lane3, Vip3A full length.

Binding properties to the known Cry1A receptors

One of the key elements in the mode of action of Bt toxin is its binding to specific receptors on the surface of the midgut membrane. In general, biological activity and binding to the receptors are positively correlated. In binding studies with susceptible and resistant strains, a significant reduction of receptor binding has been observed in the resistant strains (Van Rie *et al*. 1990), indicating receptor binding is a critical element in resistance development. The recent publication on the mode of action of Vip3A toxin clearly demonstrated that Vip3A has unique receptor binding properties, which are different from those of the Cry1A toxin (Lee *et al*. 2003). BBMV competition assays with Vip3A and Cry1Ab toxin showed that Vip3A and Cry1Ab did not inhibit each others binding, indicating they recognize different receptors. More direct evidence came from the binding assays to the known Cry1A receptors. Vip3A toxin did not bind to the known Cry1A receptors, APN (120kDa) and Cadherin (>210kDa). In addition, unique Vip3A binding proteins (80kDa and 100kDa), which are distinct from Cry1A binding proteins, were identified in *Manduca sexta* BBMV. These findings strongly support the unique binding properties of Vip3A, and therefore the unique Vip3A mode of action.

BBMV competition binding assays

In this study, the possibility of cross resistance of Vip3A toxin with Cry1Ac and Cry2Ab2 toxin in two cotton pests, *H .virescens* and *H. zea*, has been examined by performing a series of *in vitro* binding assays (BBMV competition assays and BBMV ligand blotting assays). BBMV competition binding assays with *H .virescens* and *H. zea* BBMV were performed to demonstrate the binding site relationships among Vip3A, Cry1Ac, and Cry2Ab2 toxins (Figure 2). Biotin labelled Vip3A toxin bound to both BBMV in a competitive fashion, as an excess of unlabeled Vip3A significantly reduced the signal (Fig 2, A). No competitive binding was observed between Vip3A and Cry2Ab2 toxin. An excess amount of cold Cry2Ab2 toxin did not inhibit the binding of labelled Vip3A toxin, indicating that Cry2Ab2 does not bind to the Vip3A toxin receptor (B). Also, no competitive binding was observed between Vip3A and Cry1Ac. Excess cold Cry1Ac toxin did not inhibit the binding of labelled Vip3A toxin, indicating that Cry1Ac toxin does not bind to the Vip3A toxin binding sites (C).

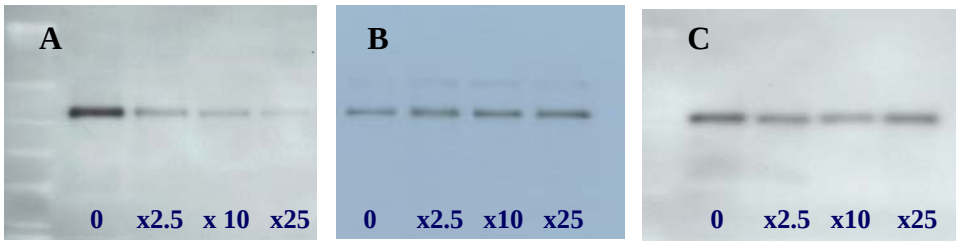

Figure 2. Competition binding assays with biotin labelled Vip3A toxins to *H. virescens* BBMV in the presence of increasing amount of unlabeled Vip3A (A), Cry2Ab2 (B), and Cry1Ac (C).

Biotin labelled Cry1Ac toxin bound to *H. virescens* BBMV competitively. Excess unlabeled Cry1Ac (50 times) significantly reduced binding (Fig3, lane 2). No competition between Cry1Ac and Vip3A was observed. Excess cold Vip3A toxin (50 times) did not inhibit the binding of labelled Cry1Ac toxin, indicating that Vip3A toxin does not bind to the Cry1Ac binding sites (lane 3). Binding assays with biotin labelled Cry2Ab2 toxin to

BBMVs of both species were also performed. Compared to Vip3A and Cry1Ac binding to BBMV, labeled Cry2Ab2 toxin showed much less binding to *H. virescens* and *H. zea* BBMV and the binding is not specific (labeled Cry2Ab2 toxin cannot be chased by increasing concentration of unlabeled Cry2Ab2 toxin) (data not shown). This non-specific binding phenomenon has been already demonstrated by English *et al.* (1994). From trypsin or chymotrypsin digestion, a resistant core of about 50kDa was observed. We also observed that incubation of Cry2Ab2 with *H. zea* BBMV can further process the 62kDa Cry2Ab2 toxin to a stable toxin of about 50 kDa. Biotinylated 50kDa Cry2Ab2 truncated toxin can bind to the *H. zea* BBMV but did not show specific binding either. This nonspecific binding of the Cry2Ab2 toxin was not inhibited by addition of cold Vip3A toxin, indicating Vip3A does not bind to the Cry2Ab2 binding sites. In summary, BBMV competition binding assays demonstrated that Vip3A toxin does not share the same binding sites with either Cry1Ac or Cry2Ab2 toxins in the two major cotton pests.

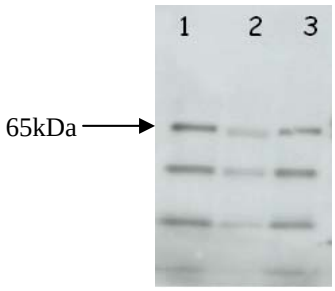

Figure 3. Competition binding assays with biotin labelled Cry1Ac to *H. virescens* BBMV in the absence (lane 1) or presence of unlabeled Cry1Ac (lane 2) and Vip3A (lane3) toxin. Two other bands represent the degradation products.

BBMV ligand blotting assays

BBMV ligand blotting assays were performed to identify putative toxin binding proteins (receptors) on BBMV proteins. Previously, several putative Cry1Ac toxin binding proteins have been reported (80kDa, 120kDa, 150kDa, 170kDa) (Lee *et al.* 1995, Luo *et al.* 1997). In this study, a similar pattern has been observed. For *H. virescens*, two major peptides were identified as Cry1Ac binding proteins (120kDa and 150-170kDa). Biotin labelled Vip3A toxin does not show any binding to these peptides. While an excess amount of cold Cry1Ac toxin competed for the binding of labelled Cry1Ac toxin to these peptides (competitive and specific binding), an excess amount of cold Vip3A toxin did not inhibit Cry1Ac binding, confirming Vip3A did not bind to the Cry1Ac binding proteins. For *H. zea*, more complicated binding patterns have been observed. Three major peptides were identified as Cry1Ac binding proteins (120kDa, 150kDa, and >210kDa). As in *H. virescens*, Vip3A does not show binding to any of these peptides. While excess cold Cry1Ac toxin competed off the binding of labelled Cry1Ac to these peptides (competitive and specific binding), excess cold Vip3A toxin does not inhibit the binding of Cry1Ac toxin, confirming that Vip3A does not recognize any of these Cry1Ac receptors. These *in vitro* binding studies have demonstrated that Vip3A toxin has distinct binding sites from Cry1Ac and Cry2Ab2 toxins. The lack of binding of Vip3A to the known Cry1Ac receptors strongly supports the use of Vip3A as a novel insecticidal agent, with little potential for cross-resistance.

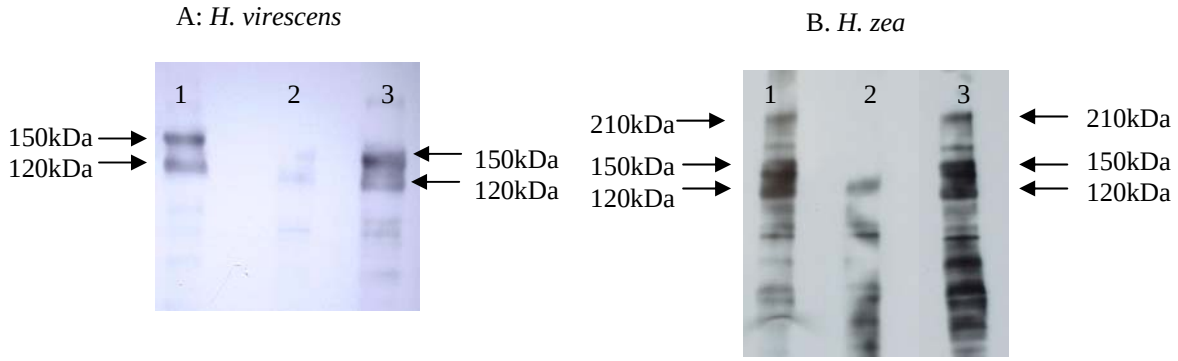

Figure 4. Binding of biotin labelled Cry1Ac toxins to protein blots of *H. virescens* (A) and *H. zea* BBMV (B) in the absence (lane 1) or presence of excess amount of unlabeled Cry1Ac (lane 2) and Vip3A toxin (lane 3).

Pore forming properties

In recent literature, the unique pore forming property of Vip3A has been demonstrated (Lee *et al*. 2003). In a voltage clamping assay with isolated midguts from the susceptible insect, *M. sexta*, activated Vip3A toxin showed pore forming activity, while full length Vip3A toxin did not form pores. As Vip3A pores are capable of destroying the transepithelial membrane potential, this suggests that pore formation may play a vital role in bioactivity. We found that the kinetics of Vip3A pore formation were at least eightfold slower than with an equimolar Cry1Ab toxin input. A lower saturation of functional Vip3A binding sites could be involved, as well as the possibility that assembly of and/or flux through the Vip3A pores differ from that with Cry1Ab. In planar lipid bilayers, Vip3A forms stable channels, which are voltage independent and highly cation selective; however, they differed considerably in their principal conductance state and cation specificity from those formed by Cry1A toxin. Therefore, we can conclude that these channels are structurally and functionally distinct from those of Cry1A type of toxins. A recent review of channel-forming proteins and peptides described 36 known families of pore-forming toxins (Saier *et al*., 2000). Excellent structural information exists for a few of these families with transmembrane segments formed by either a barrel of amphipathic β-sheets or a bundle of amphipathic alpha helices (Bt delta endotoxin Cry proteins). 7 alpha helices in domain I , of the Cry toxins have been identified as a pore forming domain. Bioinformatic tools predicted the Vip3 family does not have significant helices for channel formation like the Cry proteins and protein folding blasts revealed that Vip3 might be a pore-forming protein that has a structure of β -barrels.

Conclusion

The unique protein sequence, distinct receptor binding and ion channel properties of Vip3A indicate a marked lack of cross-resistance potential with Cry1Ac and Cry2Ab2, which are commercially available. This study supports the use of Vip3A as a novel insecticidal agent and also supports the insect resistance management strategy using Syngenta VipCot™.

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