2003 BELTWIDE COTTON CONFERENCES, NASHVILLE, TN – JANUARY 6-10

VIP: A NOVEL INSECTICIDAL PROTEIN WITH BROAD SPECTRUM LEPIDOPTERAN ACTIVITY

Frank Shotkoski and Eric Chen
Syngenta Biotechnology, Inc.
Research Triangle Park, NC
Victor J. Mascarenhas
Syngenta Crop Protection
Leland, MS
Roy Boykin
Syngenta Crop Protection
Greensboro, NC

Abstract

Vip3A is a member of the recently discovered family of vegetative insecticidal proteins isolated from *Bacillus thuringiensis*. The protein possesses insecticidal activity against the major lepidopteran pest insects including black cutworm (*Agrotis ipsilon*), fall armyworm (*Spodoptera frugiperda*), beet armyworm (*S. exigua*), tobacco budworm (*Heliothis virescens*), corn earworm (*Helicoverpa zea*), soybean looper (*Pseudoplusia includens*) and cotton leaf perforator (*Bucculatrix thurberiella*). Vip3-insecticidal proteins have been found in ~75% of *Bacillus* strains analyzed. Unlike the δ-endotoxins, Vip3 insecticidal proteins are expressed during the vegetative stage of development as well as during sporulation and are secreted proteins that do not undergo N-terminal processing. The Vip3A protein has no homology with any known proteins and work is being conducted to better understand this protein's mode of action. Vip3A requires an activation step by the insect gut enzymes for receptor-specific binding. Competition binding assays have shown Vip3A and Cry1Ab do not share the same receptor. Characterization of voltage-clamping of larval midguts and synthetic planar lipid bilayer membrane assays have demonstrated that activated Vip3A forms a unique ion channel that has properties very different from that of Cry1Ab protein. Histological studies have shown that the Vip3A protein targets midgut epithelium cells of susceptible insects initiating a series of cytological changes comprising profuse vacuolization and swelling prior to cell lysis and larval death. *Vip3A* has been transformed into several crop plants including maize, rice and cotton in an effort to provide protection against the major lepidopteran pests of these crops. Our transgenic maize events offer excellent protection against black cutworm, armyworm species and corn earworms. In cotton, Vip3A offers excellent control of the bollworm complex, beet armyworm, fall armyworm and loopers. Field trials have provided data suggesting that our Vip cotton is very competitive with bollgard cotton. We show here that Vip cotton has a novel mode of action, which is different to the Bt cotton on the market now, and therefore promises to offer growers a valuable alternative for use in resistance management strategies.

Introduction

Vip3A, a recently discovered insecticidal protein derived from *Bacillus thuringiensis* (Berliner) is highly insecticidal to numerous economically important lepidopteran insect pests (Estruch et al, 1996 & 2001, Warren et al., 1997 and Yu et al., 1997). Although Vip3A is derived from *Bacillus thuringiensis* (Berliner), several factors separate it from the many delta-endotoxins reported in the literature such as the CryIAc found in Bt cotton. Vip3A protein is secreted during the vegetative stages of bacterial development thus it is classified as an exotoxin. In contrast, CryIA proteins are only found during the sporulation phase and are classified as endotoxins. Vip3A has no sequence or structural homology between Vip3A and any of the delta-endotoxins. *Bacillus thuringiensis* δ-endotoxins have been successfully used to control many crop pests by either traditional spray application or transgenic plant approaches. 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 δ-endotoxins, is one facet of current strategies designed to delay resistance development.

Reported here are results of studies designed to assess the mode of action of Vip3 protein. These studies demonstrate that after ingestion, Vip3A protein is activated by gut fluid, the activated proteins bind to specific gut receptor proteins and eventually form channels across the gut cell membrane initiating apoptosis, cell lysis and eventual insect death. These studies establish that Vip3A and Cry1Ab do not compete binding sites in competition assays. It is also shown that Vip3A channels clearly differ from those of Cry1Ab based on the *in vitro* responses, conductance capacity and the underlying structures. Our findings indicate that while Vip3A shares some steps in the mode of action with Cry1Ab δ-endotoxin, it utilizes a different molecular target and forms distinct ion channels as compared to Cry1Ab.

Materials and Methods

Toxin Preparation, Proteolysis and Biotinylation

Cry1Ab toxin was expressed in *E. coli*, purified and trypsin-activated as described previously (Lee et. al., 1992). Vip3A was expressed in *E.coli* as described in Yu et al. 1997. To obtain the truncated Vip3A toxin, the full length toxin (Vip3A-F) was incubated with 1% trypsin or alternatively, lepidopteran gut juice was used to obtain a truncated Vip3A toxin (Vip3A-G). Gut juice was collected from *M. sexta* or *O. nubilalis* larvae by gently inducing regurgitation. Complete Protease Inhibitor cocktail was added to stop the reaction. Toxin stability (all forms) was assessed on 8-12% gradient SDS-PAGE. Vip3A-F, Vip3A-G, and Cry1Ab toxin were biotinylated using a Biotin labeling kit (Roche Molecular Biochemicals) per the manufacturer's instructions.

BBMV Binding Assays

Brush border membrane vesicles (BBMV) were prepared from last-instar *M. sexta and O. nubilalis* larval midguts by the differential magnesium precipitation method as described in Wolfersberger *et al*. 1987. For qualitative estimation of competitive binding, 5 nM biotinylated Cry1Ab or Vip3A-G toxin was incubated with 10 µg BBMV in the presence or absence of 250-fold excess unlabeled Cry1Ab or Vip3A-G, respectively.

Identification of Vip3A and Cry1Ab Toxin–Binding Proteins by BBMV Ligand Blotting

BBMV proteins (20 µg) from *M. sexta* were separated via SDS-PAGE (4%-12% gradient) and transferred to PVDF membrane. Biotinylated Cry1Ab and Vip3A-G toxins were incubated with the membrane for 4 h at room temperature. The toxin-binding proteins were visualized with streptavidin-conjugated peroxidase and the SuperSignal® West Pico Chemiluminescence kit. SeeBlue® Plus2 (Novex) pre-stained standards were used.

Binding of Toxins to the Purified *M. sexta* APN and Cadherin Ectodomain TBR

Purified APN protein (2.5 µg) was blotted onto PVDF membrane following SDS-PAGE. The membrane was blocked with 3% BSA for 1 h and incubated with 10nM biotinylated Vip3A-G or Cry1Ab toxin for 2 h at RT. After washing with TTBS buffer, the bound toxins were probed with streptavidin-conjugated HRP and the SuperSignal® West Pico Chemiluminescence kit (Pierce). Similarly, cadherin ectodomain TBR protein (6 µg) was blotted onto a nitrocellulose membrane following SDS-PAGE. After blocking, the membrane was incubated with 10 nM biotinylated Cry1Ab or biotinylated Vip3A-G proteins for 2 h at RT. After washing the membrane, bound toxins were probed with streptavidin-conjugated HRP and ECL Chemiluminescence kit (Amersham Pharmacia Biotech, Piscataway, NJ).

Voltage Clamping Assays

Voltage clamping assays were performed as described previously by Chen et al. 1993. Late 4th instar *M. sexta* or *D. plexippus* larvae were dissected, and midguts were mounted on a disk 3.9 mm in diameter. A standard chamber buffer (32 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 240 mM sucrose in 5 mM Tris, pH 8.3) was used to bathe the isolated midgut. After 20 to 30 min, allowing for stabilization of the midgut conditions, 15 nM Vip3A-F, Vip3A-G, or Cry1Ab toxin was added to the lumen side of the chamber and the change in short circuit current (I_{sc}) over time (min) was recorded. During the experiment, the chamber solution was bubbled continuously with oxygen. The I_{sc} was tracked with a Kipp and Zonen chart recorder (Bohemia, NY), and data were collected with the MacLab™ data acquisition system (ADInstruments, Grand Junction, CO) on a Macintosh computer.

Planar Lipid Bilayers

Planar lipid bilayers were formed in aqueous solution by painting lipids across a 0.20 to 0.25 mm aperture in a Delrin plastic bilayer chamber. Bilayer thinning was monitored visually with 30X zoom stereomicroscope and through membrane capacitance measurements. Cry1Ab or Vip3A toxins were typically added from 50 mM Tris, pH 8.5 solutions to the cis side of the bilayer to a concentration of 75 to 150 ng/µl. All additions were accompanied by 30 s to 1 min of stirring. Bilayer membranes were voltage-clamped in the outside-out submode using a Dagan 3900A integrating patch-clamp amplifier with Ag/AgCl electrodes connected to *cis* or *trans* sides via agar bridges. The *trans* side served as the ground with a positive membrane potential affording a positive current from *trans* to *cis*. Currents were typically low-pass Bessel-filtered at 500 Hz then sampled at 2.5 kHz using Axoscope 8 data acquisition software. Following observation of single-channel-type currents, current-voltage relationships were formed from mean current amplitudes of the predominant single channel type present over stepped voltages typically between ± 80 mV.

Results

Proteolysis of Vip3A Toxin by Lepidopteran Gut Proteases

Proteolytic activation of the 88 kDa Vip3A-F to an approximately 62 kDa occurred via the action of either trypsin or gut juice extracts from the susceptible, *M. sexta*, or non-susceptible, *O. nubilalis,* insects. These data indicate that the proteolytic activation process alone is not a key factor in insect toxicity and specificity. After *in vivo* feeding of biotinylated-Vip3A-F *to M. sexta*, Vip3A-F was also converted to the 62 kDa truncated form confirming this activation step.

BBMV Binding Assays

The biotinylated-Vip3A-G bound to *M. sexta* BBMV in a competitive fashion, as a 250-molar excess amount of unlabeled Vip3A-G significantly reduced the remaining signal. Biotinylated-Vip3A-G also bound to *O. nubilalis* BBMV, however, the total amount of bound Vip3A-G was significantly reduced for this non-susceptible insect as compared to *M. sexta*. Vip3A-G binding to *O. nubilalis* BBMV was also competitive.

***In vitro* Binding Assays to Known Cry1A Receptors**

APN blotting experiments demonstrated that biotinylated Cry1Ab bound strongly to APN as expected, while biotinylated Vip3A–G showed no binding. Furthermore, no binding was observed with Vip3A-G to APN where the protein was directly blotted onto PVDF membrane under non-denaturing conditions. In addition, *in vitro* binding to the ectodomain (EC) of the 210 kDa cadherin-like protein was examined. Biotinylated Cry1Ab showed strong binding to this ectodomain TBR, whereas Vip3A-G did not show any binding.

BBMV Ligand Blotting

Biotinylated Cry1Ab showed binding to 120 kDa APN-like and 210 kDa Cadherin-like molecules. Vip3A-G did not. Biotinylated-Vip3A-G toxin primarily showed binding to two proteins of *ca*. 80 kDa and 110 kDa. The latter protein migrated different than the 120 kDa APN-like protein which bound Cry1Ab.

Voltage Clamping Analysis

Voltage clamping experiments were performed with both *M. sexta* and *D. plexippus* larval midgets. With *M. sexta* midgut and 15nM Cry1Ab, the I_{sc} inhibition had a slope of - 8.1 µA/min. Vip3A-F did not show any pore forming activity even at 150 nM, however subsequent addition of Cry1Ab toxin to the Vip3A-F-treated midgut demonstrated responsiveness with an I_{sc} inhibition slope of - 7.6 µA/min. Vip3A-G differed from Vip3A-F in that it clearly possessed pore-forming activity at 15 nM with an I_{sc} inhibition slope of – 1.1 µA/min. With a 10-fold molar increase, Vip3A-G showed only a slightly steeper response, with an I_{sc} inhibition slope of -1.8 µA/min. To further examine the relationship between Vip3A biological activity and these *in vitro* assays, the voltage-clamp response of a nonsusceptible insect larval midgut, *D. plexippus*, was tested. While Cry1Ab (which can exhibit toxicity to *D. plexippus* larvae) showed a pore-forming response with an I_{sc} inhibition slope of - 8.8 µA/min, Vip3A-G (nontoxic to *D. plexippus*) failed to show any pore formation. Again, a subsequent addition of Cry1Ab toxin to this intact midgut demonstrated the typical Cry1Ab I_{sc} inhibition response (slope = - 8.3 µA/min).

Ion Channels Formed by Activated Vip3A and Activated Cry1Ab

Vip3A toxin which was activated *in vitro* by either incubation with lepidopteran gut fluid or trypsin demonstrated the ability to form stable ion channels in the planar lipid bilayer system. This is in contrast to full-length Vip3A which to date has not shown ion channel activity in this same assay system. The channels formed by activated Vip3A were characterized by long open times and a predominant open state of 312 ± 5 pS (n = 3) under conditions of symmetrical 300 mM KCl. Gating to other discrete open states was present on occasion suggesting that the main open state of activated Vip3A is represented by a complex structure that is capable of some degree of ion conduction prior to the fully formed main open state of the channel. Under symmetrical conditions, the channels were voltage-independent over the range of voltages tested. Activated Cry1Ab formed ion channels with an apparent variety of open states, however a main conductance state of approximately 730 pS was observed under conditions of symmetrical 300 mM KCl.

Discussion

In the present study, the mode of action of Vip3A, a member of a new family of *B.t.* toxins, has been investigated. As with δ-endotoxins, the mode of action of Vip3A appears to be complex, involving a number of discrete steps. Vip3A is processed in the lepidopteran gut. We have found that a dominant, stable, *ca* 62 kDa protein is formed by the action of susceptible or non-susceptible lepidopteran gut juice extract as well as by trypsin. After *in vivo* feeding assays with *M. sexta*, the 88 kDa Vip3A-F toxin is also processed into the stable 62 kDa form. Previously, Yu et al. 1997, observed that Vip3A-F processed by the action of gut juice from the non-susceptible insect, *O. nubilalis,* could be rendered toxic to susceptible insects. Taken together, these data suggest that while proteolysis of Vip3A occurs, it alone is not a determining factor for insect specificity. We propose, however, that this processing is minimally required for the toxin bioactivity, and can be considered as an activation step, as the full-length, unprocessed Vip3A-F is incapable of forming pores *in vitro*, which we propose to be critical in Vip3A mode of action.

Interaction with midgut epithelium is the next likely step in Vip3A mode of action. Yu et al., 1997 conducted *in vivo* immunolocalization studies that showed Vip3A binding is restricted to the midgut tissue of a susceptible (*Agrotis ipsilon*) indicating that midgut epithelial cells of susceptible insects are the primary target. Since the aminopeptidase N (APN) family and cadherin-like glycoprotein have been identified as putative Cry1A toxin receptors in many different insects, we also examined whether Vip3A-G could specifically recognize these proteins. First, Vip3A-G does not show binding to a 120 kDa *M. sexta* APN whereas Cry1Ab toxin shows strong binding. In addition, Vip3A-G does not show binding to a recombinant-

expressed cadherin ectobinding domain similar to that which was reported to interact strongly with Cry1A toxins. Again, we found that Cry1Ab toxin shows strong binding to this receptor.

Biotinylated Vip3A-G toxin predominantly binds to low abundance *ca* 80 kDa and 110 kDa bands, generating a pattern that clearly differs from that of Cry1Ab. Marginal binding is also observed toward a >200 kDa molecule which does not co-migrate with a cadherin-like protein. Therefore, our binding data strongly suggests that Cry1Ab and Vip3A do not share the same binding sites. The toxic events that follow binding of Vip3A to insect midgut epithelia could potentially be diverse. Based on two separate voltage-clamping techniques, our data support the existence of a pore-forming step in the mode of action of Vip3A following the binding to midgut epithelial receptors.

Upon investigation of Vip3A-F and Vip3A-G toxins with isolated *M. sexta* midgut, we discovered that Vip3A-G is able to form pores while Vip3A-F toxin is not, even after prolonged incubation or increased concentration. As Vip3A-G pores are capable of destroying the transmembrane potential, this suggests that pore formation may play a vital role in bioactivity. Planar lipid bilayer experiments validate and extend the conclusions from our isolated midgut voltage clamp data in that processed Vip3A demonstrates the ability to form distinct ion channels in the absence of any receptors. The Vip3A channels are quite stable over time, capable of long open times (>1 s), and of a large conductance status (>300 pS). Therefore, the cumulative effects of Vip3A channel formation provide the simplest explanation for the previously observed pore forming response. Furthermore, the channels observed *in vitro* could reasonably be expected to account for the documented histological changes in susceptible insect midgut tissue and the associated toxicity. As the biophysical properties of these channels also differ somewhat from those of Cry1Ab, we can conclude that these channels are structurally and functionally distinct from those of Cry1Ab and by extension, perhaps from other homologous Cry-type toxins.

Previously, it was proposed that Vip3A might induce an apoptotic pathway after binding to its respective midgut receptors in susceptible insects, Estruch et al., 2001. Toxic agents derived from sporulated *B.t.* cultures could induce apoptosis in cultured midgut cells from *H. virescens* (Loeb et al., 2000 and 2001). While we hypothesize that the Vip3A pore-forming properties described herein might alone account for observed toxicity, this does not preclude that unique binding events and/or pore formation will be found to mediate other aspects of Vip3A bioactivity.

Several important strategies have been put in place to address concerns of resistance to *B.t.* δ-endotoxins, particularly with their presentation in the form of transgenic crops. Our data suggest that the unique midgut interactions involved with Vip3A and its receptor(s) render it potentially useful as a second generation *B.t.* toxin for resistance management. Interestingly, in a laboratory culture of *S. exigua*, Moar *et al*., 1995 found that the presence of HD1 *B.t.* var. *kurstaki* spores reduced the development of resistance to Cry1C. As this strain is a source of the Vip3A gene, it is quite possible that expression of Vip3A may have contributed to these observations. Donovan et al., 2001 recently demonstrated that Vip3A production was able to account for a significant component of toxicity via the "spore effect" in an HD1 strain toward *A. ipsilon* or *S. exigua*. While the development of resistance to *B.t.* δ-endotoxin outside of the laboratory environment has only been documented in isolated cases (Ferre et al., 2002), it remains a concern for the future to better preserve and extend the usefulness of these important insect control agents. One strategy involves the presentation of several toxins together, especially if a differing mode of action involving different receptors is available. We have shown that the mode of action of Vip3A differs in several steps as compared to Cry1Ab, therefore, incorporation of Vip3A into insect control programs may serve to address δ-endotoxin resistance concerns in addition to its own value as a control agent.

References

Chen, X. J., M. K. Lee, and D. H. Dean. 1993. Site-directed mutations in a highly conserved region of *Bacillus thuringiensis* δ-endotoxin affect inhibition of short circuit current across *Bombyx mori* midguts. Proc. Natl. Acad. Sci. USA 90:9041-9045.

Donovan, W. P., J. C. Donovan, and J. T. Engleman. 2001. Gene knockout demonstrates that *vip3A* contributes to the pathogenesis of *Bacillus thuringiensis* toward *Agrotis ipsilon* and *Spodoptera frugiperda.* J. Invertebr. Pathol. 78:45-51.

Estruch, J. J., G. W. Warren, M. A. Mullins, G. J. Nye, J. A. Craig, and M. G. Koziel. 1996. Vip3A, a novel *Bacillus thuringiensis* vegetative insecticidal protein with a wide spectrum of activities against lepidopteran insects. Proc. Natl. Acad. Sci. USA 93:5389-5394.

Estruch, J. J., and C.-G. Yu. September, 2001. Plant pest control. U. S. patent 6,291,156 B1.

Ferre J., and J. Van Rie. 2002. Biochemistry and genetics of insect resistance to *Bacillus thuringiensis.* Annu. Rev. Entomol. 47:501-533.

Lee, M. K., R. E. Milne, A. Z. Ge, and D. H. Dean. 1992. Location of a Mobyx mori receptor binding region on a *Bacillus thuringiensis* •-endotoxin. J. Biol. Chem. 267:3115-3121.

Loeb M. J., R. S. Hakim, P. Martin, N. Narang, S. Goto, and M. Takeda. 2000. Apoptosis in cultured midgut cells from *Heliothis virescens* larvae exposed to various conditions. Arch. Insect Biochem. Physiol. 45:12-23.

Loeb M. J., P. A. W. Martin, N. Narang, R. S. Hakim, S. Goto, and M. Takeda. 2001. Control of life, death, and differentiation in cultured midgut cells of the lepidopteran, *Heliothis virescens*. In Vitro Cell. Dev. Biol. – Animal 37:348-352.

Moar, W. J., M. Pusztai-Carey, H. van Faassen, D. Bosch, R. Frutos, C. Rang, K. Luo, and M. J. Adang. 1995. Development of *Bacillus thuringiensis* Cry1C resistance by *Spodoptera exigua* (Hubner) (Lepidoptera:Noctuidae). Appl. Environ. Microbiol. 61:2086-2092.

Warren, G. W. 1997. Vegetative Insecticidal Proteins: Novel Proteins for Control of Corn Pests, p. 109-121. *In* N. Carozzi and M. Koziel (ed.), Advances in Insect Control, Taylor & Francis, Bristol, PA.

Wolfersberger, M., P. Luthy, A. Maurer, P. Parenti, P. V. Sacchi, B. Giordana. 1987. Preparation of brush border membrane vesicles (BBMV) from larval lepidopteran midgut. Comp. Biochem. Physiol. 86A: 301-308.

Yu, C.-G., M. A. Mullins, G. W. Warren, M. G. Koziel. and J. J. Estruch. 1997. The *Bacillus thuringiensis* vegetative insecticidal protein Vip3A lyses midgut epithelium cells of susceptible insects. Appl. Environ. Microbiol. 63:532-536.