



Bacillus thuringiensis pore-forming toxins trigger massive shedding of GPI-anchored aminopeptidase N from gypsy moth midgut epithelial cells

Algimantas P. Valaitis *

USDA Forest Service, 359 Main Road, Delaware, OH 43015, USA

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ABSTRACT

The insecticidal Cry proteins produced by *Bacillus thuringiensis* strains are pore-forming toxins (PFTs) that bind to the midgut brush border membrane and cause extensive damage to the midgut epithelial cells of susceptible insect larvae. Force-feeding *B. thuringiensis* PFTs to *Lymantria dispar* larvae elicited rapid and massive shedding of a glycosylphosphatidylinositol (GPI)-anchored aminopeptidase N (APN) from midgut epithelial cells into the luminal fluid, and depletion of the membrane-anchored enzyme on the midgut epithelial cells. The amount of APN released into the luminal fluid of intoxicated larvae was dose- and time-dependent, and directly related to insecticidal potency of the PFTs. The induction of toxin-induced shedding of APN was inhibited by cyclic AMP and MAPK kinase (MEK) inhibitors PD98059 and U0126, indicating that signal transduction in the MEK/ERK pathway is involved in the regulation of the shedding process. APN released from epithelial cells appears to be generated by the action of a phosphatidylinositol-specific phospholipase C (PI-PLC) cleavage of the GPI anchor based upon detection of a cross-reacting determinant (CRD) on the protein shed into the luminal fluid. Alkaline phosphatase was also released from the gut epithelial cells, supporting the conclusion that other GPI-anchored proteins are released as a consequence of the activation PI-PLC. These observations are the basis of a novel and highly sensitive tool for evaluating the insecticidal activity of new Cry proteins obtained through discovery or protein engineering.

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1. Introduction

Bacillus thuringiensis (Bt) is an aerobic, gram-positive bacterium that is used as a biopesticide for the control of insect pests. Most Bt strains produce parasporal proteinaceous crystals containing one or more pore-forming insecticidal proteins referred to as Cry toxins (Schnepf et al., 1998). The insecticidal activity of Cry proteins has been attributed to their ability to form ionic pores in the brush border membrane of midgut epithelial cells after interaction with specific receptors (Li et al., 1991). Several unique receptors for Bt toxins that may mediate the restricted host specificity of Cry toxins towards target insect pests have been identified in different insect species. A GPI-anchored aminopeptidase (APN-1) and a 270 kDa glycoconjugate (BTR-270) have been identified as high-affinity binding proteins for Cry toxins in *Lymantria dispar* larvae. APN-1 was found to exhibit unique specificity in its interaction with only Cry1Ac. In contrast, BTR-270 displayed high affinity interaction with a diverse number of highly toxic Cry toxins, suggesting that it is a common receptor for the insecticidal proteins (Valaitis et al., 1997, 2000; Valaitis et al.,

2001). In other insects, APNs, cadherins, a GPI-anchored alkaline phosphatase, and lipids have been characterized as toxin-binding receptors for various Cry proteins (Vadlamudi et al., 1995; Jurat-Fuentes and Adang, 2004; Griffiths et al., 2005).

Until recently, it was generally accepted that the toxic effect of the Cry proteins was due to osmotic lysis of the cells as a result of the permeabilization of the epithelial membranes. However, bacterial pore-forming toxins can promote cell death that is not directly related to membrane permeabilization. For example, the activity of *Pasteurella multocida* toxin (PMT) appears to be directly related to activation of a phosphatidylinositol-specific phospholipase, initiating a cascade of signaling events and a wide spectrum of cellular responses (Seo et al., 2000). Cry toxins have been reported to induce activation of adenylate cyclase and the elevation of intracellular cyclic AMP in intoxicated insect cell cultures. However, whether cyclic AMP plays a pivotal role in the pathogenic process in the activity of Bt remains unclear (Knowles and Farndale, 1998; Zhang et al., 2006).

A large number of membrane-associated proteins, including cytokines, growth factors and their receptors, cell adhesion molecules, and syndecans are secreted as biologically active molecules into the extracellular environment by a process referred to as shedding. Most shedding processes have been found to be mediated by a proteolytic cleavage (Almqvist and

* Tel.: +1 740 368 0020; fax: +1 740 369 2601.

E-mail address: avalaitis@fs.fed.us

Carlsson, 1988). The specific proteases involved in the shedding processes have been called sheddases or secretases and have been identified as members of the hydroxamate-sensitive ADAM family of membrane-associated proteases (Blobel, 2004). Although release of soluble forms of GPI-anchored proteins can be generated by endogenous membrane-associated phospholipases, ADAMs have also been implicated in the shedding of some GPI-anchored molecules (Wilhelm et al., 1999; Cavallone et al., 2001; Elwood et al., 1991). The bacterial PFTs, streptolysin O (SLO) and hemolysin (HlyA), induce the shedding of receptors for interleukin 6 and lipopolysaccharide, respectively (Walev et al., 1996), and *Staphylococcus aureus* α -toxin and the anthrax cholesterol binding pore-forming factor (AnLO) activate shedding of syndecans (Park et al., 2004; Popova et al., 2006). The kinetics of the shedding processes induced by bacterial PFTs is rapid, paralleling the kinetics of pore formation. One common feature of these shedding processes is that the activation is dependent on intracellular signaling pathways which can be blocked with specific inhibitors of the mitogen-activated protein kinase (MAPK) extracellular regulated kinase (ERK) pathway (Park et al., 2004). In addition, activation of the p38 MAPK signaling pathway has been demonstrated to play a role in cellular defense and recovery after attack by PFTs (Husmann et al., 2006; Aroian and van der Goot, 2007).

A specific aminopeptidase N isozyme (APN-1) has been identified as toxin-binding receptor in *L. dispar*, based on ligand blotting and surface plasmon resonance binding studies (Jenkins et al., 2000). However, subsequent efforts to demonstrate a functional role for APN-1 in *L. dispar*, as well as other APNs in insects, in the mode of action of Bt, have been only partially successful (Garner et al., 1999; Gill and Ellar, 2002; Sivakumar et al., 2007; Pigott and Ellar, 2007). The major finding of this study is that Bt Cry proteins are shown to induce massive shedding of APN-1 into the luminal fluid mediated by activation of GPI-specific phospholipase C via an intracellular signaling pathway. Since shedding of various cell surface proteins induced by bacterial pathogens has been implicated in promoting their virulence, shedding of APN may promote the cytotoxic action of Bt Cry toxins.

2. Materials and methods

2.1. Reagents

Suramin (8,8'-[carbonylbis[imino-3,1-phenylenecarbonylimino-4-methyl-3,1-phenylene]carbonyl-imino]]bis-1,3,4-naphthalenetrisulfonic acid), Piceatannol (3,4,3',5'-tetrahydroxy-trans-stilbene), and MRS 2179 (2'-deoxy- N^6 -methyl adenosine 3',5'-diphosphate) were purchased from Sigma (St. Louis, MO); PD98059 (2'-amino-3'-methoxyflavone) and U10126 (1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene) were from EMD Chemicals, Inc. (San Diego, CA); TAPI-1 was from Peptides International, Inc. (Louisville, KY); rabbit anti-CRD GP1-01 antibody was from Oxford Glycosystems (UK); Macro-Prep High Q and PVDF (polyvinylidene difluoride) membrane were from Bio-Rad Laboratories (Hercules, CA).

2.2. Insects, purification of APN and PI-PLC treatment

Gypsy moth larvae were obtained from Otis Methods Development Center, USDA, Otis ANGB, MA, and reared on artificial wheat germ diet (ICN Biomedicals) at 25 °C under 14 h light:10 h dark. Brush border membrane vesicles (BBMV) were prepared by the procedure of Wolfersberger et al. (1987), with the exception that the homogenization buffer was supplemented with 1 mM

PMSF. Purification of the Cry1Ac toxin-binding APN-1 from gypsy moth BBMV and generation of a polyclonal antibody specific towards Ld APN-1 was described previously (Valaitis et al., 1995). BBMV from control and Cry1Ac-intoxicated insect larvae were incubated with 0.2 U/ml of phosphatidylinositol-specific phospholipase (PI-PLC) purchased from Sigma for 1 h at 37 °C. The PI-PLC digests were centrifuged at 14,000g for 20 min and the amount of APN released was measured spectrophotometrically using leucine *p*-nitroanilide as a substrate as described below. The soluble aminopeptidase N (sAPN) in the luminal fluid from Cry1A toxin-treated insect larvae was purified by chromatography using a 5 ml MacroPrep High Q ion-exchange column and concentrated by ultra filtration using YM-30 ultra filtration membrane for immunochemical analysis.

2.3. Bacterial strains and purification of toxins

Bacillus thuringiensis subsp. *kurstaki* (HD-1 and HD-73), subsp. *tolworthi* (HD125), subsp. *sotto*, subsp. *tenebrionis*, a crystal minus derivative of subsp. *sotto* (4E3 Cry-) and *Escherichia coli* strains expressing the cloned Cry1Aa, Cry1Ab, Cry1Ac, Cry2A and Cry3A toxins were obtained from the *Bacillus* Genetic Stock Center at The Ohio State University. Sporulated cultures were produced by growing the Bt strains on nutrient agar plates for 6–7 days and from 0.5 L cultures of Bt strains grown at 30 °C for 5–6 days in a medium containing 1% glucose, 0.2% peptone, 0.5% NZ amine-A casein hydrolysate, 0.2% yeast extract, 15 mM (NH₄)₂SO₄, 23 mM KH₂PO₄, 27 mM K₂HPO₄, 1 mM MgSO₄, 0.6 mM CaCl₂, 17 μ M ZnSO₄, 17 μ M CuSO₄ and 2 μ M FeSO₄. The Cry1A proteins in HD-1, HD-73 and Bt *sotto* preparations were extracted from washed spore/crystal pellets obtained from sporulated Bt cultures by solubilization of the Cry proteins using 0.1 M carbonate, 10 mM EDTA and 10 mM DTT at pH 10.4 for 30 min at room temperature. The solubilized Cry protoxins were activated by digestion with affinity-purified gypsy moth trypsin (Valaitis, 1995) for 30 min at room temperature, and the activated toxins were purified by gel filtration using a 3.5 $\times$ 46 cm column of Sephacryl S-200 equilibrated in pH 9.6 50 mM carbonate–bicarbonate buffer containing 0.2 M NaCl and 10 mM EDTA. Cry2A was obtained from carbonate-extracted HD-1 spore/crystal pellets by solubilization of the crystals in 0.1 M NaOH and subsequently purified using Sephacryl S-200 gel filtration. Recombinant Cry1Aa, Cry1Ab and Cry1Ac, Cry2A and Cry3A toxins expressed in *E. coli* were purified by a procedure described previously (Lee et al., 1996). All the purified toxins used in this study were filter sterilized using 0.2 μ m syringe filters and stored at 7 °C in 20 mM Tris-buffer containing 0.2 M NaCl at pH 8.6. Vegetative insecticidal protein (Vip3A) was purified from a culture supernatant of *B. thuringiensis* HD-125 grown for 24 h at 30 °C in terrific broth using a 70% (w/v) ammonium sulfate precipitation step followed by anion exchange chromatography and gel filtration. The purity of the toxins was checked by 7.5% SDS–PAGE, and protein concentrations were determined spectrophotometrically using the Bio-Rad protein assay dye reagent with bovine serum albumin as a standard.

2.4. Sample preparations and enzyme assays

Serial dilutions of purified toxins were prepared in 0.2 μ m filter-sterilized 50 mM KCl–10% sucrose solution. A 5–8 μ l aliquot of each toxin dose was delivered into the larval midgut region with a 0.05 ml Hamilton 700 series syringe fitted with a 22-gauge needle using a model PG600 Hamilton repeating dispenser. For each assay data point a group of 5–10 larvae were force-fed the same dose and kept in the same container. Controls were force-fed an equal volume of 50 mM KCl–10% sucrose solution only. Crude

Bt samples of sporulated strains were obtained by growing of Bt strains on nutrient agar plates at room temperature for 7–10 days. A stock solution of 0.2 M cyclic AMP was prepared in 20 mM Tris-buffered saline and the pH was adjusted to 8.0. Stock solutions 50 mM of suramin and fluorescent brightener were prepared in deionized water; MRS 2179 and TAPI-1 were prepared in methanol; and piceatannol was prepared in ethanol. All of the agents were diluted 1:10 with the KCL-sucrose solution containing the toxin dose. Methanol and ethanol derived from the inhibitor stock solutions did not induce APN shedding. Luminal fluid samples were obtained by decapitating the larvae on absorbent paper, extruding the gut and extracting the luminal fluid in the gut by puncturing the gut and collecting the fluid in a microfuge tube. An equal volume of 25 mM Bis-Tris buffer containing 1 mM PMSF at pH 6.8 was added to the samples to inactivate the digestive serine proteases. After mixing the samples were centrifuged for 5 min in a microfuge at top speed and the pale-yellow supernatants were transferred to new microfuge tubes and stored at -20°C . The final concentration of the test agents in the gut fluid of the insects was determined by using a value of 83 μl obtained from the average volume of luminal fluid obtained from the insect larvae at this stage. APN activity was assayed using 2 mM L-leucine *p*-nitroanilide in 50 mM Tris-HCl pH 8.6 buffer at room temperature by continuous monitoring of *p*-nitroanilide released by the hydrolysis of LpNA at 410 nm using a Beckman DU 7400 spectrophotometer. An extinction coefficient of $8800\text{M}^{-1}\text{cm}^{-1}$ for *p*-nitroanilide at this wavelength was used. Alkaline phosphatase activity was measured using 2 mM *p*-nitrophenyl phosphate in 50 mM Tris-HCl, 10 mM MgCl_2 at pH 9.4 at 405 nm using an extinction coefficient of $18,500\text{M}^{-1}\text{cm}^{-1}$ for *p*-nitrophenol. One unit of enzyme activity was defined as the amount of enzyme catalyzing the hydrolysis of 1 μmol of substrate per min under these conditions.

2.5. Analytical gel electrophoresis and western immunoblot analysis

Proteins separated by 10% SDS-PAGE were electrophoretically transferred onto polyvinylidene difluoride (PVDF) membranes using a Bio-Rad mini trans-blot cell. The membranes were blocked for 1 h by incubation with 2% BSA in 20 mM Tris-buffered saline (TBS) at pH 8.0. The membranes were washed and then incubated with a specific APN antiserum and an anti-cross-reacting determinant (CRD) antibody. The membranes were washed three times with TBS containing 0.05% Tween-20 for 5 min each, incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG for 1 h. Detection was performed by staining with 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT).

3. Results

3.1. Cry1AC toxin induces APN shedding

Previous studies have demonstrated that APN-1 from *L. dispar* binds Cry1AC toxin, suggesting that it may serve as a receptor promoting the insecticidal activity of Bt (Jenkins et al., 2000; Lee et al., 1996). To determine if Cry1AC induced any changes in the GPI-anchored APN in the intestinal gut epithelial cells, APN activities in the BBMVs and luminal fluid of control and intoxicated insect larvae were examined. Insect larvae were force fed a 1:5 serial dilution of Cry1AC toxin. After 17 h, APN activity in the gut fractions was measured spectrophotometrically using LpNA as a substrate. The results showed that APN activity in the luminal fluid increased approx. 37-fold from 0.06 U/ml in the control

larvae to 2.2 U/ml in larvae force-fed 5 μg of Cry1AC in a dose-dependent manner (Fig. 1). The relationship between the Cry1AC dose and the increase in APN activity was linear at low toxin concentrations, and approached a plateau with Cry1AC doses in the microgram range. No further increase in APN activity in the luminal fluid was obtained when the 5 μg intoxicated insects were force fed an additional dose of toxin 17 h later, indicating that a maximal release of APN was reached with the initial dose. These results are consistent with the saturation kinetics observed with the irreversible insertion of the pore-forming Cry1A toxins using BBMVs in toxin-binding studies (Rajamohan et al., 1996).

To confirm that the increase of soluble APN in the luminal fluid was due to shedding of the membrane-bound GPI-anchored APN associated with the midgut epithelial cell membranes, the relative levels of APN activity in BBMVs prepared from control and Cry1AC-intoxicated larvae were determined. The specific activity of APN in the BBMVs prepared from the Cry1AC intoxicated larvae (1.25 U/mg protein) was 50% lower in comparison to the specific activity of APN (2.52 U/mg protein) in BBMVs prepared from an equal number of control larvae, indicating that approximately one half of the total membrane-anchored APN was released from the epithelial cell membranes in response to Cry1AC intoxication. PI-PLC treatments of BBMVs prepared from control and Cry1AC-intoxicated larvae revealed a 2.4-fold reduction in the amount of APN susceptible to solubilization by PI-PLC in the intoxicated larvae. The depletion of APN in BBMVs prepared from intoxicated larvae supports the conclusion that the soluble APN in the luminal fluid is generated from shedding of APN ectodomains from the cell surface and not from the release of cytosolic soluble aminopeptidase from damaged cells.

3.2. A diverse group of Bt pore-forming toxins induce APN shedding

Binding studies have shown that the purified APN from *L. dispar* bound Cry1AC but displayed no interaction with other Bt toxins such as Cry1Aa and Cry1Ab that have potent insecticidal activity to the insect larvae. The hypothesis that the shedding of APN from gut cell membranes may be specifically induced by Cry1AC was not supported by the observation that a diverse group of Cry toxins induce APN shedding (Fig. 2). Six doses (ranging from 60 pg to 200 ng/larva) of a 1:5 serial dilution of Cry1Aa, Cry1Ab, Cry1AC, Cry2A, DF1 and Cry3A toxins were administered to groups

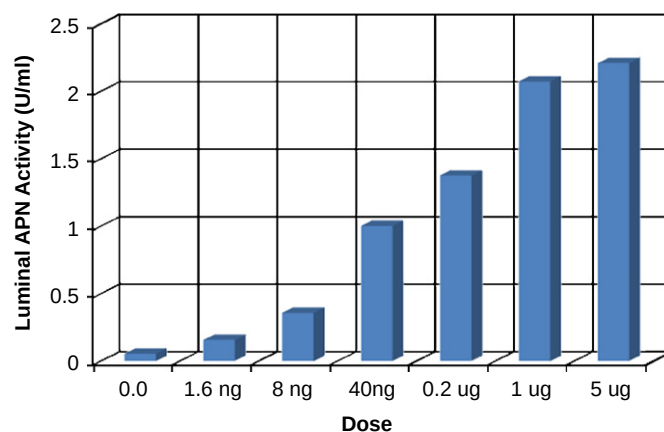


Fig. 1. APN shedding from gut epithelial cells into the luminal fluid in response to Cry1AC intoxication. Insect larvae (10 per dose) were force-fed a 1:5 serial dilution of Cry1AC toxin. After 17 h, APN activity in the pooled luminal fluid collected from each dose was measured spectrophotometrically using L-leucine *p*-nitroanilide (LpNA) as a substrate. APN activity in the luminal fluid increased 37-fold from 0.06 U/ml in control larvae to 2.2 U/ml in larvae fed 5 μg of Cry1AC in a dose-dependent manner.

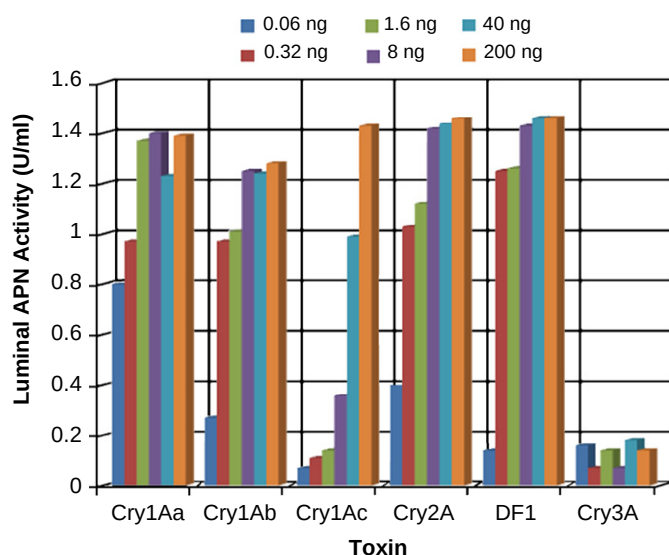


Fig. 2. A diverse group of Bt pore-forming toxins induce APN shedding. Six doses of a 1:5 serial dilution of Cry1Aa, Cry1Ab, Cry1Ac, Cry2A, DF-1 (a triple mutant of Cry1Ab) and Cry3A Bt toxins were administered to groups of 5–10 larvae per dose using the force-feeding technique. After 17 h, APN activity was measured in the pooled luminal fluid samples for each toxin dose using LpNA.

of 5–10 larvae per dose using the force-feeding technique. After 17 h, increase in APN activity was found in the luminal fluid with all of these toxins with the exception of Cry3A, which has no toxicity to gypsy moth larvae. APN increase was dose-dependent, and was not induced by heat-denatured Cry1A toxins (data not shown). Although a similar amount of luminal APN activity was obtained in larvae with all 5 insecticidal Cry toxins at high doses, significant differences were observed in their dose-response profiles at lower doses, correlating to their insecticidal potency. In response to a dose of 1.6 ng, an APN activity of 1.37, 1.01 and 0.14 U/ml was measured in the luminal fluid of Cry1Aa-, Cry1Ab- and Cry1Ac-intoxicated larvae, respectively. These results are consistent with the relative toxicity of Cry1Aa > Cry1Ab > Cry1Ac reported for *L. dispar* (Rajamohan et al., 1996). The increase in the luminal APN activity reached a plateau at approx. 1.4 U/ml for the all the Cry proteins that are toxic to *L. dispar*, indicating that permeabilization of the cells with the PFTs reaches a shared limit in the amount of APN that can be released from the epithelial cells.

The response values at both low and high doses with the Cry1Ab mutant DF1 were higher in comparison to Cry1Ab, consistent with the increased toxicity of DF1 compared to the wild-type Cry1Ab toxin (Rajamohan et al., 1996). Cry2A also was a potent inducer of APN shedding, displaying a similar profile observed with the Cry1Aa and Cry1Ab toxins. Since Cry2A has a distinct mode of action and may not share binding sites with Cry1A toxins, these results suggest that the APN shedding induced by the Cry toxins is not dependent on interaction with a specific toxin receptor but is most likely a consequence of osmotic stress caused by the PFTs. Overall, these results show that induction of APN shedding is restricted to Cry proteins which are toxic to *L. dispar*, and there is a correlation between APN shedding and potency of the Cry toxins.

The results of a time-course study using a mixture of Cry1Aa, Cry1Ab and Cry1Ac toxins purified from *B. thuringiensis* subsp. *kurstaki* HD-1 showed that the mixture was able to induce a maximum of approx. 3 U/ml in the luminal fluid of intoxicated insects. In response to a dose of 50 ng, a time-dependent increase of APN activity was measured at 5, 10, 30 and 60 min, reaching plateau at approx. 60 min and corresponding to a 60-fold increase

in comparison to that of untreated larvae. APN activity in the luminal fluid remained unchanged for more than 48 h, consistent with the irreversible nature of the shedding process and indicating that there is no significant loss of the enzyme activity in the luminal fluid of intoxicated larvae (data not shown).

3.3. A sensitive and reliable assay for evaluating the insecticidal activity of crude and purified Bt pore-forming toxins

Bt strains grown on nutrient agar plates were tested to determine if the APN assay can be applied for the evaluation of crude Bt samples. Bacterial spore/crystal mixtures from *B. thuringiensis* subsp. *kurstaki* (HD-1), subsp. *tolworthi* (HD-125), subsp. *sotto* (Bt sotto), a Cry minus derivative of subsp. *sotto* (4E3 Cry-) and subsp. *tenebrionis* (Bt *tenebrionis*) were administered to the insect larvae using the force-feeding technique. APN shedding was induced only with Bt strains that produce Cry proteins that are active towards *L. dispar* (Fig. 3). HD-1, which produces Cry1A and Cry2A toxins, and Bt sotto and HD-73, which produce Cry1Aa and Cry1Ac toxins, respectively, were potent inducers of APN shedding. HD-125, which produces the lepidopteran-active Cry9C toxin that is weakly active towards *L. dispar*, induced approx. 26% of the amount of APN shedding that was observed with HD-1. There was essentially no induction of APN shedding observed with Bt *tenebrionis*, which produces crystals containing the coleopteran-specific Cry3A toxin or the Cry minus Bt sotto derivative 4E3Cry-. The HD-125 strain which produces the vegetative insecticidal protein vip3A with insecticidal activity towards a broad spectrum of lepidopteran larvae was also a potent inducer of APN shedding. The magnitude of the responses with the crude spore/crystal samples was similar to that observed with purified Cry1A or Cry2A proteins, suggesting that the spores themselves do not play a role in inducing APN shedding. This is consistent with the negative results obtained with the Cry- Bt sotto strain. The results of this study indicate that the APN assay of the luminal fluid of Bt treated insect larvae could be a valuable tool in discriminating between inactive and active Bt strains for target insect pests.

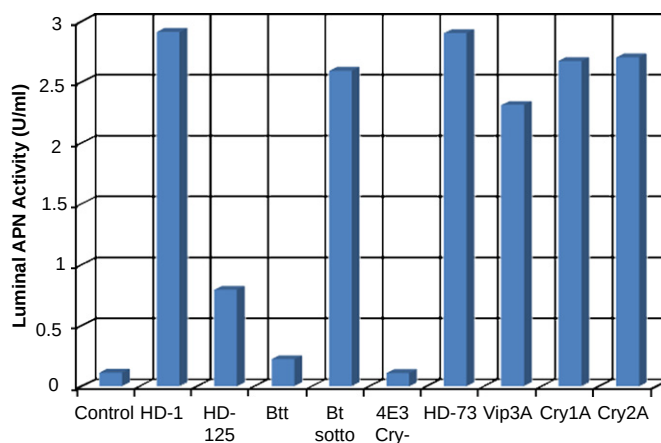


Fig. 3. A rapid and sensitive assay for evaluating the insecticidal activity of purified Bt toxins and crude Bt strains. Six µg of spore/crystal samples from sporulation cultures of *B. thuringiensis* subsp. *kurstaki* (HD-1), subsp. *tolworthi* (HD-125), subsp. *sotto* (Bt sotto), a Cry minus derivative of subsp. *sotto* (4E3 Cry-) and subsp. *tenebrionis* (Bt *tenebrionis*) grown on nutrient agar plates; 2 µg of purified Cry1A and Cry2A toxins from HD-1 and 2 µg of partially purified vip3A from HD-125 culture supernatant were administered to insect larvae (5 per group) using the force-feeding technique. APN released into the luminal fluid was measured after 3 h using LpNA as substrate.

3.4. Using inhibitors as probes to investigate the mechanism of APN shedding

A series of pharmacological drugs and inhibitors of proteins involved in cell signaling were used to examine the mechanism of APN shedding induced by Bt PFTs (Fig. 4). The effects of these agents were tested with a 50 ng dose of HD-1Cry1A toxins. TAPI-1 was used as a probe to determine whether APN release is mediated by a hydroxamate-sensitive ADAM metalloproteases. There was no inhibition of Cry1A toxin-induced shedding of APN with TAPI-1, suggesting that the process is not mediated by a proteolytic event. However, TAPI-1 may have been degraded by the milieu of digestive enzymes present in the gut fluid. Inhibition of shedding by cyclic AMP suggested that second messengers may be involved in some signal pathway controlling the induction of APN shedding. The inhibition was observed for several hours, after which the effect decreased, until no effect was seen after 17 h. Cyclic AMP has been reported to block light-induced photoreceptor shedding (Besharse et al., 1982), and more recently has been shown to activate the extracellular signal-regulated kinase (ERK) pathway in epithelial cells, promoting transient inhibition of apoptosis (Rudolph et al., 2004; Yamaguchi et al., 2000). Incorporation of 1 mM cyclic AMP in the diet did not inhibit Cry toxin activity towards neonate gypsy moth larvae (data not shown).

Since cyclic AMP is involved in carrying signals from receptors at the cell surface to proteins within the cell, suramin was used to determine if blocking the activation of receptors could inhibit shedding. Suramin is a broad-spectrum antagonist of cell surface receptors, inhibiting various signal transduction proteins. Suramin inhibited APN shedding (Fig. 4). The disulfonic acid stilbene fluorescent brightener shares structural similarities with suramin and also inhibited toxin-induced shedding of APN. Fluorescent brightener has been demonstrated to enhance the efficacy of baculoviruses towards target insect pests and has been shown to be an inhibitor of apoptosis in the larval midgut cells in *L. dispar* (Dougherty et al., 2006). Since fluorescent brightener is not a cell-permeable reagent, the site of action is most likely at the luminal surface of the epithelial cell membrane interfering with toxin binding or pore formation.

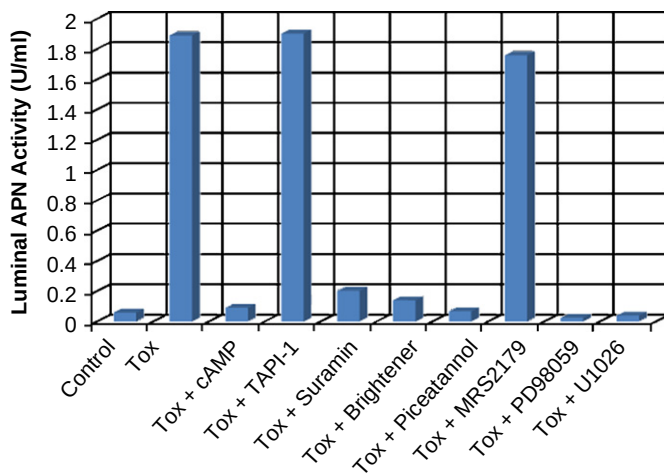


Fig. 4. Probing the mechanism of APN shedding induced by Bt pore-forming toxins using inhibitors. A 50 ng dose of Cry1A toxins purified from HD-1 was administered along with each inhibitor (0.3 mM of cyclic AMP, suramin, fluorescent brightener, MRS2179, and piceatannol; and 60 μ M of TAP-1, PD98059, and U1026) to groups of 5 larvae per experimental treatment. The final concentration of inhibitor was determined from an average value of soluble fluid recovered per larva. Luminal APN activity in the intoxicated larvae was determined 3 h after administering the sample to the larvae.

APN shedding also was inhibited by piceatannol, a potent inhibitor of the Syk tyrosine kinase which plays a crucial role in intracellular signal transduction. This observation is consistent with the hypothesis that activation of protein tyrosine kinases is an early event in the induction of shedding. MRS 2179, a P2Y₁ receptor antagonist, did not inhibit APN shedding, indicating that the stimulation or activity of this P2Y receptor is not involved in the shedding process.

The cell-permeable specific MAP kinase kinase (MEK) inhibitors, PD098059 and U1026, were highly effective in blocking the induction of the Cry1A toxin-induced APN release. However, the inhibition of APN shedding by a single dose of the MEK inhibitors like that observed with cyclic AMP was also transient. These observations imply that the extracellular signal-regulated mitogen-activated protein kinase (ERK MAPK) pathway plays an integral role in the toxin-induced APN shedding. PD 098059, a MEK1 inhibitor, and U1026, a dual MEK1 and MEK 2 inhibitor, are highly potent and specific inhibitors of the two ERK isozymes (ERK1/2) that are the terminal kinases of the MEK ERK pathway. How ERKs are involved in the cellular signaling pathway that controls induction of APN shedding remains unclear.

3.5. Bt toxins induce shedding of GPI-anchored alkaline phosphatase

To determine if shedding of other GPI-anchored proteins is induced by Bt toxins, analysis of alkaline phosphatase (ALP) activity in the luminal fluid of HD-1 Cry1A-intoxicated larvae was conducted. Dose studies analyzing the release of APN and ALP at 17 h clearly showed that the GPI-anchored ALP is released at concentrations similar to that observed for APN shedding (Fig. 5).

3.6. Shedding of APN is mediated by PI-PLC

The release of vast majority of cell-surface proteins has been attributed to the action of intrinsic membrane-bound proteinases which are sensitive to hydroxamic acid-based inhibitors such as TAPI-1. However, APN shedding was not inhibited with TAPI-1, suggesting that the toxin-induced activity was not mediated by

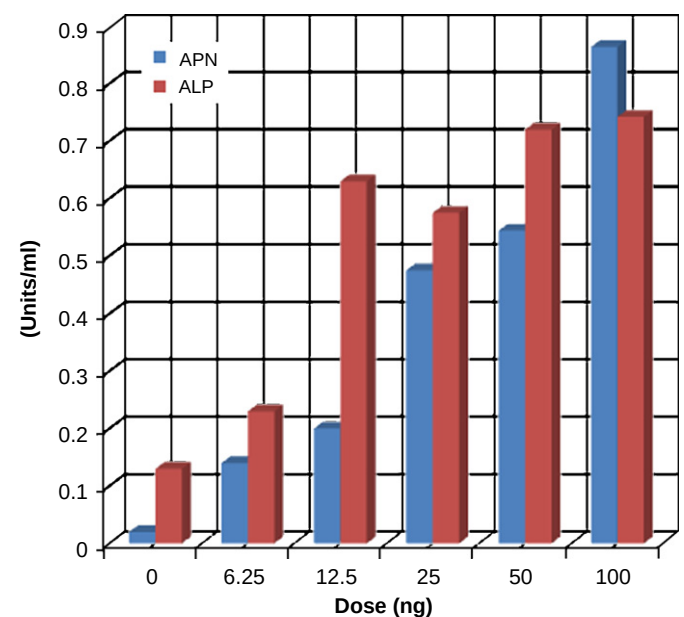


Fig. 5. Cry toxins induce the shedding of APN and ALP. Five doses of purified HD-1 Cry1A toxin were administered to insect larvae (5 per group) using the force-feeding technique. APN and ALP released into the luminal fluid were measured after 17 h.

proteolytic mechanism. Since gypsy moth APN is a brush border enzyme attached to the luminal side of the brush border membrane by a glycosylphosphatidylinositol (GPI) anchor, the release of APN in response to Bt PFTs may be due to the cleavage of the anchor by an endogenous glycosylphosphatidylinositol-specific phospholipase C (PI-PLC). To examine this possibility, western blots of soluble APN (sAPN) found in the luminal fluid of intoxicated larvae, and APN-1 purified from BBMV from control insects, were probed with rabbit polyclonal antibodies specific for APN-1 and an anti-cross-reacting determinant (CRD) antibody (Fig. 6).

SDS-PAGE followed by Coomassie staining showed the presence of a protein band with an apparent size of 100 kDa in the partially purified sAPN, along with several smaller protein bands. Probing with anti-APN antibodies revealed strong reaction with the upper protein band in the sAPN and with APN-1 purified from BBMV, supporting the conclusion that the sAPN is the hydrophilic form of membrane-bound APN-1 that is released in response to Bt PFTs. An antibody against the cross-reacting determinant (CRD) epitope generated after PIP2C digestion (anti-CRD) was used to test for the presence of a cleaved GPI anchor in the sAPN. The results obtained with the immunoblot analysis of sAPN revealed that the upper band clearly cross-reacts with the anti-CRD antibody, providing strong evidence that it is generated by PI-PLC mediated action. In addition, a smaller anti-CRD reacting band with an apparent size of 32 kDa was observed in the partially purified sAPN sample. The 32 kDa band did not cross-react with the anti-APN antibody, suggesting that another GPI-anchored protein is released in response to the bacterial PFTs. However, it is possible that the 32 kDa protein band may be a proteolytically generated fragment of the sAPN which does not cross-react with the anti-APN antibodies. As expected, the APN-1 purified from BBMV, without the use of exogenous PI-PLC, was not detected with the anti-CRD antibody. Additionally, alkaline phosphatase, which is also a GPI-anchored protein, was found to be shed into

the luminal fluid (Fig. 5), supporting the conclusion that *B. thuringiensis* PFTs trigger release of GPI-anchored proteins from epithelial cell membranes by a mechanism that is mediated by a PI-specific phospholipase C.

4. Discussion

The binding of Bt pore-forming toxins (PFTs) to midgut epithelial cells initiates a sequence of two irreversible events: formation of transmembrane pores and shedding of APN ectodomains. APNs have been identified as putative receptors or co receptors for Cry1A toxins in *L. dispar* and many other lepidopteran insects, but their precise role in the insecticidal mechanism of Bt toxins has remained controversial (Pigott and Ellar, 2007). The observations in this study clearly demonstrate that Bt insecticidal PFTs provoke shedding of APN into the luminal fluid, with concomitant depletion of the membrane-bound APN in the intoxicated epithelial cells. Previous investigations of microbial pathogens which secrete other PFTs have shown that membrane permeabilization by PFTs can trigger shedding of specific cell surface proteins, which promote bacterial virulence (Walev et al., 1996; Popova et al., 2006). Although shedding of surface proteins is a normal cellular function playing an important role in regulating activities of membrane proteins, bacterial pathogens can accelerate shedding, damaging target cells in a mechanism to enhance their virulence (Ratner et al., 2006). APN shedding in response to sublethal doses of Bt toxins may be interpreted as a potential defense mechanism, since soluble toxin-binding ectodomains may function as competitive inhibitors, preventing toxin interaction with the cell surface receptors, but the consequences of the massive loss of APN and other GPI-anchored proteins such as alkaline phosphatase from the affected cells are likely to impact the integrity of the epithelial cells and affect their viability. Furthermore, shedding is provoked by a diverse group of Bt toxins

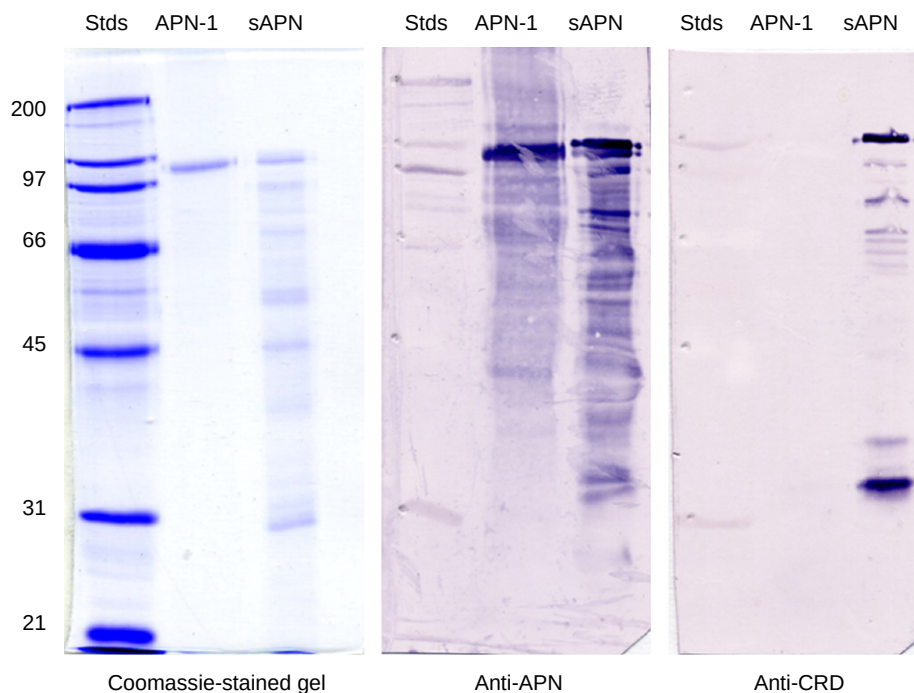


Fig. 6. Analysis of the soluble APN shed into the luminal fluid in Cry1A intoxicated insects by Western blots using polyclonal anti-APN and anti-CRD antibodies. The soluble APN (sAPN) found in the luminal fluid of Bt intoxicated insect larvae was partially purified by ion-exchange chromatography, concentrated by ultra filtration and analyzed by 10% SDS-PAGE and immunoblotting. APN-1 was purified from BBMV of control larvae by anion-exchange and size-exclusion chromatography using FPLC. High molecular weight standards were applied in lanes designated as Stds. Western blots were probed with antibodies specific for the toxin-binding APN-1 in *L. dispar* (Anti-APN) and antibodies specific for the cross-reacting determinant (CRD) epitope exposed by PI-PLC cleavage.

including proteins which do not interact with APN-1 in *L. dispar*, and therefore would not be expected to play a role in reducing toxicity. The initial trigger for initiation of the shedding process appears to be distinct from receptor binding, since Cry2A and vip3A, which do not share binding sites with Cry1A toxins (English et al., 1994; Alcantara et al., 2004; Estruch et al., 1996), were also strong inducers of APN shedding. This conclusion is consistent with the finding that shedding is not induced by pore-forming mutants which retain cell binding but are impaired in pore formation (Walev et al., 1996).

Investigation of the mechanism involved in the Bt toxin-induced shedding of APN in intoxicated larvae showed that the selective hydroxamic acid compound inhibitor of ADAM proteases, TAPI-1, did not inhibit the APN shedding, suggesting that the release of APN was not mediated by a proteolytic process. Detection of a cross-reacting determinant (CRD) epitope in the shed APN on western blots provided strong evidence that the APN found in the luminal fluid was generated by a GPI-specific PLC activity. The presence of a smaller antigenically distinct GPI-anchored protein in immunoblots of the luminal fluid APN from intoxicated insects and the release of GPI-anchored alkaline phosphatase (data not shown) suggest that shedding may not be restricted to APN. Further investigation is needed to determine if toxin-induced shedding is selective for a subset of membrane-anchored proteins.

Sublytic concentrations of bacterial PFTs can induce activation of survival signaling pathways in epithelial cells as a consequence of osmotic stress (Ratner et al., 2006). Osmotic stress in eukaryotic cells triggers a wide variety of responses, including activation of phospholipases. However, the mechanism involved in activation of phospholipases as a result of osmotic stress provoked by pore formation has not been biochemically defined. Identifying the components on the target epithelial cell membrane utilized by Bt toxins to initiate activation of endogenous PI-PLC will provide a mechanistic understanding of the role of cell signaling events in the cytotoxicity induced by Bt toxins. In all shedding systems investigated to date, activation of the MEK pathway appears to be critical. Inhibition of the MAP kinase ERK1/2 signaling pathway mediated by MEK using the highly specific MEK inhibitors PD908059 and U1026 inhibits Bt-induced APN shedding. These results support the conclusion that the MEK ERK1/2 cascade is crucial for induction of the PFT-triggered shedding process in *L. dispar* midgut epithelial cells. Activation of the P38 kinase, another member of the MAP kinase family, appears to be a conserved response in cells permeabilized by various bacterial PFTs and may play an important role in cellular recovery from sublytic concentrations of PFTs (Aroian and van der Goot, 2007). However, several of the receptor-activated and stress-induced shedding processes analyzed are refractive to the inhibition of p38 MAP kinase, suggesting that the ERK1/2 signaling cascade is the principal control for the activated shedding processes (Chung et al., 2006). The transient inhibition of APN shedding with exogenous cyclic AMP supports that an intracellular signaling pathway is involved in the shedding process. In addition, inhibition of APN shedding by cAMP may be due to the perturbation of the MEK ERK1/2 cascade based on reports that exogenous cyclic AMP can stimulate ERK1/2 activation in epithelial cells and provokes transient inhibition of apoptosis in intestinal cells (Rudolph et al., 2004; Yamaguchi et al., 2000). In agreement with results observed in a variety of other shedding systems (Park et al., 2000, 2004), APN shedding was impaired when protein tyrosine kinase was inhibited with piceatannol, a potent inhibitor of the Syk family of protein tyrosine kinases.

The general pathway for PI-PLC activation is through stimulation of receptors. Stimulation of G protein-coupled receptors (GPCRs) and protein tyrosine kinase receptors (RPTKs) activates β

and γ isoforms of PI-PLC. The inhibition of receptor activation by the broad spectrum receptor antagonist suramin and by the suramin analogue, fluorescent brightener, is in agreement with the conclusion that receptors are involved in the shedding process. It is possible that these agents interfere with the formation of toxin pores, inhibiting the permeabilization of the epithelial cell membrane. Fluorescent brightener was reported to inhibit apoptosis in *L. dispar* midgut cells, which suggests a relationship between toxin-induced APN shedding process and activation of an apoptotic signaling pathway (Dougherty et al., 2006).

Although a relationship between APN shedding and cell viability has not been directly established, these findings indicate that a massive loss of APN occurs concomitantly with intoxication, and suggest that APN shedding may promote the cytotoxic activity of Bt toxins. PFT-induced APN shedding may provide a rapid method to evaluate the insecticidal activity of new Bt strains towards target insect pests and facilitate elucidation of the biochemical processes governing the entomocidal activity of Bt toxins.

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