Binding specificity of *Bacillus thuringiensis* Cry1Aa for purified, native *Bombyx mori* aminopeptidase N and cadherin-like receptors

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Abstract

**Background:** To better understand the molecular interactions of Bt toxins with non-target insects, we have examined the real-time binding specificity and affinity of Cry1 toxins to native silkworm (*Bombyx mori*) midgut receptors. Previous studies on *B. mori* receptors utilized brush border membrane vesicles or purified receptors in blot-type assays.

**Results:** The *Bombyx mori* (silkworm) aminopeptidase N (APN) and cadherin-like receptors for *Bacillus thuringiensis* insecticidal Cry1 toxins were purified and their real-time binding affinities for Cry1 toxins were examined by surface plasmon resonance. Cry1Ab and Cry1Ac toxins did not bind to the immobilized native receptors, correlating with their low toxicities. Cry1Aa displayed moderate affinity for *B. mori* APN (75 nM), and unusually tight binding to the cadherin-like receptor (2.6 nM), which results from slow dissociation rates. The binding of a hybrid toxin (Aa/Aa/Ac) was identical to Cry1Aa.

**Conclusions:** These results indicate domain II of Cry1Aa is essential for binding to native *B. mori* receptors and for toxicity. Moreover, the high-affinity binding of Cry1Aa to native cadherin-like receptor emphasizes the importance of this receptor class for Bt toxin research.

**Background**

*Bacillus thuringiensis* (Bt) has been sold commercially and used as a biopesticide worldwide for over half a century. However, growing public concern surrounding Bt use has sparked worldwide debate over current policies [1]. For example, in India, fear over a potential *Bombyx mori* (silkworm) epizootic, or microbial pathogen outbreak, inspired a governmental ban on the use of Bt, despite the nation’s continuing use of traditional chemical pesticides [2].

While pest control with Cry toxins that possess low *B. mori* activity (*i.e.* Cry1Ac) is a viable solution in affected countries, it is worthwhile to investigate the specific molecular mechanisms that make Cry1Aa highly active. Early work took advantage of the fact that Cry1Aa, but not Cry1Ac, is toxic to *B. mori*. For example, Ge *et al.*[3] exchanged hypervariable regions between genes encoding the two toxins and localized the toxicity specifying region of Cry1Aa to residues 332–450 in domain II. A follow-up study demonstrated the toxicity specifying residues were involved in binding *B. mori* brush border membrane vesicles. [4]
icicles [4]. More specifically, alanine substitution or deletion of residues 365 to 371 removed nearly all toxicity and binding to B. mori BBMV [5].

Recently, research on B. mori has focused on purifying and cloning the midgut epithelial receptors targeted by Cry1Aa toxin. The first toxin-binding receptor purified from B. mori was a 120-kDa aminopeptidase N (APN), which appears around 110-kDa on SDS-PAGE gels when preparative conditions are used that cleave its glycosyl-phosphatidylinositol (GPI) anchor. This receptor was shown to bind Cry1Aa with a 7.6 nM affinity, as determined by Scatchard analysis with ELISA binding assays [6]. The APN was cloned and expressed in E. coli and demonstrated to bind Cry1Aa toxin on ligand blots [7]. These results indicate the Cry1Aa-APN interaction was specific and that APN glycosylation was not required for Cry1Aa binding. This is not altogether surprising because Cry1Aa binding to Manduca sexta APN has not been found to be modulated by sugar binding [8] and the B. mori APN sequence is 73.7% identical to M. sexta APN-1. Sequence alignments with Plutella xylostella APN receptor indicate that a highly conserved region of APN likely functions as the toxin binding site [9]. By testing for toxin binding to lysylendopeptidase-digested B. mori APN fragments, the toxin binding site was suggested to be between Ile135 and Pro198. A later study by these authors identified 120-kDa and 115-kDa APNs coeluting from an anion-exchange column that together yielded a Cry1Aa affinity of 53 nM [10]. These APNs eluted just prior to a 120 kDa APN with 7.6 nM affinity. It is unclear whether the 120- and 115-kDa proteins represent uncleaved and cleaved GPI-anchor isozymes. Interestingly, this study also showed that Cry1Ac toxin binds to the 120/115 kDa APN fraction with equal affinity as Cry1Aa, and only 4-fold reduced affinity to the isolated 120-kDa APN. Nonetheless, Cry1Aa is 210 times more toxic than Cry1Ac to B. mori [4]. As a whole, B. mori APN research indicates the presence of at least three genetic isoforms [7,11,12], with toxin affinities ranging from nanomolar to none at all.

In addition to APN, a completely different toxin receptor class has been affinity precipitated by toxin from solubilized B. mori midgut proteins. In this manner, Nagamatsu et al. [13] purified a 175-kDa glycoprotein (Btr175) that bound Cry1Aa toxin. Interestingly, these authors did not observe binding of Cry1Aa to APN-sized bands in ligand blot studies with BBMV. Antibodies produced to Btr175 blocked toxin binding to the receptor in BBMV. The antibody serum also reduced Cry1Aa activity against B. mori when it was fed to larvae prior to toxin addition to the diet [14]. The same group cloned and introduced the Btr175 gene with a baculovirus vector into Spodoptera frugiperda Sf9 cells. Addition of Cry1Aa caused swelling and lysis of only the Sf9 cells expressing Btr175. Based on sequence analysis, the receptor was characterized as a cadherin-like glycoprotein containing nine cadherin repeats, a membrane proximal region, one transmembrane region, and a small cytoplasmic domain [15]. Ihara et al. [16] also purified and partially sequenced what was presumed to be the same cadherin-like receptor. Binding studies indicated that the affinity of the cadherin for Cry1Aa is equivalent to that of the brush border membrane vesicles from B. mori [16], an affinity that is substantially lower than the APN affinities reported. Recently, cDNA variants of Btr175 have been discovered, showing at least three alleles of the cadherin-like receptor are found in B. mori [17]. It is likely that glycosylation plays a major role in cadherin-like receptor isoforms as well, as glycosylation has been observed previously for the M. sexta cadherin-like receptor BT-R [18].

Progress in research on silkworm receptors for Bt toxins has provided a means for assaying mutant toxins with potentially altered binding and activity. In this study, we tested the hypothesis that Cry1Aa binds to both the 120-kDa B. mori APN and the 175-kDa B. mori cadherin-like protein. Based on the previous work of Ge, et al. [3] and Lee, et al. [4], we also postulated that domain II of Cry1Aa is the significant binding domain. These hypotheses were tested for the first time in studies with purified, native B. mori receptors (rather than BBMV) under real-time, non-labeled toxin binding conditions.

Results

Bombyx mori aminopeptidase N and cadherin-like receptor purification

To investigate the specificity of Cry toxins for B. mori receptors, the two known B. mori midgut receptors were purified from B. mori BBMV. Solubilized B. mori BBMV proteins were separated by Q Sepharose anion-exchange chromatography and all eluted fractions were tested for APN enzymatic activity. Additionally, Cry1Aa toxin binding capability was assayed by "slot blotting" all fractions and probing with biotin-Cry1Aa. The chromatogram in Fig. 1 displays the separation of cadherin and APN from BBMV proteins. APN isozymes of 100- and 110-kDa were detected that did not show Cry1Aa-binding in slot blot assays (Fig. 1; fractions 24–25 and 30–31). Such isoforms have been reported previously [11,12]. In addition, a 115-kDa APN was detected with Cry1Aa-binding capability (Fig. 1; fractions 33–36). As expected, fractions were also observed that exhibited no APN enzymatic activity but bound Cry1Aa on slot blots (Fig. 1; fractions 26–27). Initially these fractions were predicted to contain the cadherin-like Cry1Aa-binding protein [13,14,16]. The candidate receptor fractions for APN and cadherin were separately loaded on a size-exclusion column for
further purification (Fig. 2A and 2B). A protein with APN enzymatic activity eluted 75 minutes after injection (Fig. 2A; fractions 15–16), approximately 4 minutes after the 120-kDa L. dispar APN elutes on the same column [19]. The candidate cadherin-like receptor fraction eluted in fractions 9–11 at around 180 kDa (Fig. 2B).

**Analysis of receptor purity**

The pooled and concentrated candidate receptor fractions were examined by SDS-PAGE before and after size-exclusion purification to assess purity (Fig. 3). The putative cadherin-like receptor material appears at a molecular size around 180 kDa, both before and after secondary purification (Lanes 2 and 1, respectively). Several BBMV proteins appear present in the APN-containing fraction prior to size-exclusion purification (Fig. 3; Lane 4). The molecular weight of the final, purified APN was estimated to be 115–120 kDa (Fig. 3; Lane 3). It is not known whether the GPI anchor is still intact on the APN receptor; however, in the current study, phosphatidylinositol-specific phospholipase C (PIPLC) was not used during BBMV preparation. It was shown previously that APN may be purified with intact GPI-anchors if PIPLC is omitted from the preparation buffer [6]. It is likely that our APN has similarly retained the GPI anchor.

In view of the fact that B. mori BtR175 possesses sequence similarity to M. sexta BT-R1, the candidate fraction was probed on a slot blot with anti-BT-R1 polyclonal antiserum. A weak to moderate cross-reactivity with anti-BT-R1 was observed for B. mori BBMV as well as the putative cadherin-like receptor fraction, providing strong evidence that the material is a cadherin-like protein (Fig. 4). Anti-Bt-R1 antibody recognition was not observed for fractions eluting before and after the cadherin material, nor for the purified APN (Fig. 4). Similar antibody assays were not performed to substantiate the identity of the purified APN because it clearly displayed strong, characteristic APN enzyme activity.

The purity of both receptors was further examined by a Cry1Aa toxin ligand blot (Fig. 5). Both APN and the cadherin-like receptor fractions bound biotinylated Cry1Aa. No other toxin-binding bands were apparent, and neither purified receptor sample was visibly cross-contaminated with the other receptor (Fig. 5).
Affinity estimation by surface plasmon resonance

Cry toxin binding studies have been reported previously for *B. mori* that used BBMV assays or used purified receptors in ELISA assays or blots; however, no Cry toxin studies concerning *B. mori* have been published employing SPR analysis. The affinity of Cry1Aa binding to *B. mori* APN and *B. mori* cadherin receptors was evaluated by real-time kinetic analysis on a BIAcore 2000. Simple bimolecular binding of Cry1Aa was observed to both *B. mori* APN and cadherin (Fig. 6A and 6B). Toxin-receptor on-rates for association (kₐ), off-rates for dissociation (kₜ), and overall binding affinity (kₜ/kₐ, or Kᵤ) were calculated for toxin binding. The apparent rate constants for wild-type Cry1Aa and *B. mori* APN were kₐ = 2.0 × 10⁴ M⁻¹s⁻¹ (+/- 1.3 × 10²), kₜ = 1.5 × 10⁻³ s⁻¹ (+/- 1 × 10⁻⁵), and Kᵤ = 75 nM. To *B. mori* cadherin, significantly tighter affinities were obtained: kₐ = 1.3 × 10⁴ M⁻¹s⁻¹ (+/- 6.1), kₜ = 3.3 × 10⁻⁵ s⁻¹ (+/- 1 × 10⁻⁵), Kᵤ = 2.6 nM. This apparent off-rate clearly accounted for Cry1Aa’s higher affinity for cadherin than for APN. The cadherin off-rate observed in this study could have significant consequences in vivo: slow toxin dissociation may enable protracted lingering near the brush border membrane surface, greatly facilitating toxic (domain I) insertion and subsequent pore formation. The overall affinity determined in the present study for Cry1Aa to BtR175 (2.6 nM) agrees well with the findings of Ihara et al. [16] by a different assay (0.8 nM).

We also explored the specificity of Cry1Aa for the native *B. mori* receptors by comparing the binding response of Cry1Aa with the binding of Cry1Ab, Cry1Ac, and domain-switched toxin 4109 (Fig. 7A and 7B). Hybrid toxin 4109 is particular useful in this context, because it is comprised of domains I and II from Cry1Aa and domain III from Cry1Ac (Aa/Aa/Ac) [3]. Hybrid-toxin 4109 binding to both receptors was not noticeably different from Cry1Aa: for APN binding, kₐ = 1.9 × 10⁴ M⁻¹s⁻¹ (+/- 1.4 × 10²), kₜ = 1.5 × 10⁻³ s⁻¹ (+/- 1.6 × 10⁻⁵), and Kᵤ = 78 nM; for cadherin binding, kₐ = 1.3 × 10⁴ M⁻¹s⁻¹ (+/- 2 × 10²), Kᵤ = 78 nM.
**Discussion**

The dissociation constants presented are the first determined for *B. mori* Cry receptors by the use of SPR technology. Additionally, the apparent affinity of Cry1Aa for the cadherin-like receptor is the highest observed affinity to date for Cry toxin binding to purified receptors using SPR. This finding emphasizes the important biological role that this receptor class plays for Bt toxins. Recently, using phage display technology, a scFv molecule with short sequence homology to *M. sexta* and *B. mori* cadherin-like receptors was shown to bind domain II of Cry1Aa, Cry1Ab, and Cry1Ac toxins [20]. In the present study, only Cry1Aa shows measurable binding to the purified, native cadherin-like receptor from *B. mori*. This finding may be the result of purification of a particular receptor variant with Cry1Aa specificity (e.g., one of po-

$k_d = 3.34 \times 10^{-5} \text{s}^{-1} (+/- 2 \times 10^{-5})$, and $K_D = 2.6 \text{nM}$. In stark contrast, Cry1Ab and Cry1Ac showed no apparent binding to either receptor (Fig. 7A and 7B). These results are entirely consistent with the hypothesis that Cry1Aa domain II (alone) is essential for binding to both the APN and cadherin-like receptors as purified in the present study.

**Figure 5**

Ligand blot of purified *B. mori* APN and cadherin-like receptors (5 µg each lane, 6% SDS PAGE) probed with biotinylated Cry1Aa toxin (50 µg). M, pre-stained molecular weight standards; APN, purified *B. mori* APN; cad, purified cadherin-like receptor.

**Figure 6**

Real-time binding of Cry1Aa to *B. mori* APN and cadherin-like protein, BtR175. Representative BIAcore response curves for toxin injections at 200, 300, 500, and 1000 nM. Experimental curves (gray) are shown overlaid with fitted curves (black) obtained with the 1:1 Langmuir binding model. Response units (RU) are shown at left (1 RU = 1 pg/mm² of protein bound). (A) Cry1Aa wt binding to *B. mori* APN. (B) Cry1Aa wt binding to *B. mori* BtR175.
The apparent Cry1Aa affinity for purified APN measured in this study, 75 nM, is 10-fold higher than the value reported by Yaoi et al. [6] for purified 110-kDa APN using a separate technique (7.6 nM). In the aforementioned study, an ELISA assay was used to indirectly calculate affinity by incubating receptor-bound toxin with a peroxidase-conjugated anti-Cry1Aa antibody over 1.5 hours at 37°C. It is possible that the difference in binding constants reflects our condition of more direct receptor-binding measurement in "real-time", as well as the different binding buffers and temperature used.

Yaoi et al. [21] estimated the toxin-binding region of B. mori APN to be between Ile135 and Pro198 based on toxin blot overlays with protease-digested APN fragments. BLAST sequence alignments [22] yielded 81% identity and 96% similarity between this 63 residue stretch and the homologous region of M. sexta APN-1, which also binds Cry1Aa toxin (Jenkins, unpublished observation). Interestingly, in L. dispar APN-1, which does not bind Cry1Aa, the same stretch is only 37% identical and 56% similar (4% unaligned gaps). These results appear to support the findings of Yaoi et al. [21]. However, Heliothis virescens (tobacco budworm) APN is only 45% identical and 53% similar to B. mori APN, yet it binds Cry1Aa toxin with high affinity [23]. Moreover, sequence alignments with APN from Lactobacillus, Streptococcus, Saccharomyces, Arabidopsis, rat, pig, yeast, and human yielded more similarity than H. virescens APN to the putative Cry1Aa-binding region of B. mori APN. It is likely that as the X-ray crystal structures of APNs are solved, structural alignments of APNs will help resolve the specificity-determining regions more accurately. Additionally, structural information will aid in the rational construction of toxins with reduced binding for beneficial insects without losing activity to target pests. In this context a unique Cry1Aa binding epitope within domain II has been identified that, when mutated, results in specific reduction of toxicity to B. mori (You, et al., unpublished manuscript). The application of protein engineering to B. thuringiensis insecticidal proteins is entering a new era of tailoring pesticides with reduced activity to beneficial insects as well as increasing activity against pest insects [24].

**Conclusions**

Domain II of Cry1Aa is both necessary and essential for tight binding to two B. mori midgut receptors, the cadherin-like and aminopeptidase N receptors, a finding that correlates with biological activity data. The Cry1Aa binding affinity, as well as the dissociation rate for the cadherin-like receptor, are the lowest measured using the surface plasmon resonance technique. The SPR method presented here may be useful for screening other Cry toxins or Cry toxin variants specifically engineered to...
reduce or eliminate specificity for receptors from this non-target insect.

Materials and methods

Mutant toxin construction and preliminary analysis

Hybrid toxin 4109 consisting of domains I and II of Cry1Aa and domain III of Cry1Ac was constructed as previously described [3]. Force-feeding bioassays on B. mori and BBMV binding assays were conducted as described [4]. Crystal proteins were solubilized and trypsinized, and active toxins were column purified as carried out previously [19].

Receptor purification

B. mori midguts were dissected from 4th or 5th instar larvae and brush border membrane vesicles were prepared by the Wolbersberger method [25]. B. mori BBMV (10 mg in 10 ml) was solubilized in 5 mg/ml CHAPS zwitterionic detergent (Roche) overnight at 4°C with gentle rocking. Solubilized BBMV was centrifuged at 10,000 x g for 10 min and supernatant was concentrated to 2 ml by Amicon YM30 ultrafiltration. The sample was then loaded on a Q Sepharose HR 10/30 anion-exchange column. All column chromatography was carried out on an ÄKTA Explorer (Amersham Pharmacia Biotech). Low salt buffer (buffer A) consisted of 20 mM Tris, 5 mM MgCl2, 0.4 mg/ml CHAPS, pH 8.6, and the high salt buffer used was buffer A containing 1 M NaCl. A step gradient of salt was used to elute BBMV proteins. All fractions were tested for APN enzymatic activity by the LpNA assay. Briefly, 390 µl of sample are mixed with 10 µl of 2 mM leucine-p-nitroanilide (containing a leucine-phenylalanine dipeptide). A yellow chromophoric change indicates amidopetidase N activity, defined as the ability to cleave a neutral amino acid from the N-terminus of a polypeptide. Cry1Aa binding ability was also checked by slot blotting fractions to PVDF membrane and probing with biotinylated Cry1Aa [26]. Fractions with Cry1Aa-binding ability and APN enzymatic activity were concentrated to 2 ml volumes and loaded on a Superdex 200 size-exclusion column (120 ml bed volume) using Hepes-buffered saline (HBS; 10 mM Hepes, 150 mM NaCl, 3.4 mM EDTA, pH 7.4) as running buffer. Absorbance was monitored at 280 nm and 260 nm to judge protein purity of collected peaks relative to flow through. Fractions eluting around 115–120 kDa, the MW of APN, were collected and protease inhibitors were added after a final concentration. Anion-exchange fractions with Cry1Aa-binding ability but without APN enzymatic activity were also size-purified, and fractions eluting around 175–250 kDa, the MW of BtR175, were collected and concentrated. Approximately 0.10 mg (in 0.25 ml) of cadherin-like receptor and 0.30 mg of APN (in 1 ml) were obtained.

Analysis of receptor purity

Candidate receptor fractions were analyzed by 10% SDS-PAGE (40 µl/lane) and stained with Coomassie brilliant blue. For slot blot assays, 5 µg of M. sexta or B. mori BB-MVs and 40 µl of candidate receptor fractions were blotted onto PVDF membrane and assays was carried out as reported previously [27], except for using 1:1000 anti-Bt-R1 polyclonal antiserum. For ligand blot assays, samples separated by SDS-PAGE (6%) were transferred to PVDF overnight, blocked with 5% dried milk in TTBS (50 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20, pH 7.5). Samples were probed with 50 µg biotin-Cry1Aa and streptavidin-conjugated horseradish peroxidase for 1 hr each, with 45 min TTBS washes, and developed in DAB/Urea (BioRad).

Surface plasmon resonance with purified midgut receptors

B. mori APN and cadherin were immobilized on a CM5 sensor chip by the amine-coupling method (Biacore AB). Receptors were diluted into ammonium acetate, pH 4.2 prior to immobilization. An HBS (pH 7.4) buffer flow rate of 50 µl/min was used for all injections. Randomized toxin concentrations varying from 100 nM to 1000 nM were injected (110 µl) over the receptor surfaces. Surfaces were regenerated with 6 µl pulses of 10 mM NaOH, 250 µM ethylene glycol, pH 11.0 at 100 µl/min. Signal responses from a blank flowcell containing ethanolamine as a blocking agent were subtracted from all response curves and data were fitted using BIAevaluation 3.0. The curves were fit to a simple 1:1 Langmuir binding model to obtain apparent rate constants (A + B ↔ AB).

List of abbreviations

Bt, Bacillus thuringiensis; APN, aminopeptidase N; GPI, glycosyl-phosphatidylinositol; BBMV, brush border membrane vesicles; LpNA, leucine-p-nitroanilide; PIPLC, phosphatidylinositol-specific phospholipase C; HBS, Hepes-buffered saline; SPR, surface plasmon resonance

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References

1. Jayaraman K: A caveat: Bt must be allowed, but with caution and after carefully controlled trials. Curr. Sci 1991, 60:465
2. Van Driesche RG, Bellows JTS: Biological Control. New York: Chapman and Hall, 1996
3. Lee MK, Milne RE, Ge AZ, Dean DH: Location of the Bombyx mori specificity domain on a Bacillus thuringiensis δ-endotoxin protein. Proc. Natl. Acad. Sci. USA 1989, 86:4037-4041
4. Ge AZ, Shivarova NI, Dean DH: Location of a Bombyx mori receptor binding region on a Bacillus thuringiensis δ-endotoxin. J. Biol. Chem 1992, 267:3115-3121
Cry IA δ-endotoxin binding and pore formation. Insect Biochem. Mol. Biol. 1997, 27:735-743
24. Rajamohan F, Alzate O, Curtiss A, Dean DH: Protein engineering of Bacillus thuringiensis δ-endotoxin: mutations at domain II of Cry IA enhancer receptor affinity and toxicity towards gypsy moth larvae. Proc. Natl. Acad. Sci. USA 1996, 93:14338-14343

25. Woltersberger M, Lusty P, Maurer A, Parenti P, Sacchi FV, Giordana B, Hanoez GM: Preparation and partial characterization of amino acid transporting brush border membrane vesicles from the larval midgut of the cabbage butterfly (Pieris brassicae). Comp. Biochem. Physiol. 1987, 86A:301-308

26. Lee MK, Young BA, Dean DH: Domain III exchanges of Bacillus thuringiensis Cry IA toxins affect binding to different gypsy moth midgut receptors. Biochem. Biophys. Res. Commun 1995, 216:306-312

27. Lee MK, You TH, Young BA, Valaitis AP, Dean DH: Aminopeptidase N purified from gypsy moth BBMV is a specific receptor for Bacillus thuringiensis Cry IA toxin. Appl. Environ. Microbiol 1996, 62:2845-2849

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