Bacillus thuringiensis Cry1Aa toxin-binding region of *Bombyx mori* aminopeptidase N

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Abstract The *Bacillus thuringiensis* Cry1Aa toxin-binding region of *Bombyx mori* aminopeptidase N (APN) was analyzed, to better understand the molecular mechanism of susceptibility to the toxin and the development of resistance in insects. APN was digested with lysylendopeptidase and the ability of the resulting fragments to bind to Cry1Aa and 1Ac toxins was examined. The binding abilities of the two toxins to these fragments were different. The Cry1Aa toxin bound to the fragment containing 40-Asp to 313-Lys, suggesting that the Cry1Aa toxin-binding site is located in the region between 40-Asp and 313-Lys, while Cry1Ac toxin bound exclusively to mature APN. Next, recombinant APN of various lengths was expressed in *Escherichia coli* cells and its ability to bind to Cry1Aa toxin was examined. The results localized the Cry1Aa toxin binding to the region between 135-Ile and 198-Pro.

Key words: Aminopeptidase N; δ-Endotoxin; *Bacillus thuringiensis*; *Bombyx mori*

1. Introduction

Bacillus thuringiensis, a Gram-positive bacterium, produces various types of crystal inclusions during sporulation [1–3]. These crystal inclusions are composed of an insecticidal protein, δ-endotoxin. δ-Endotoxin has a high insecticidal activity, a high specificity and is not toxic to mammals, so it is used as a biological control agent. When susceptible insects ingest crystal inclusions, the crystal inclusions are solubilized in the alkaline environment of the insect midgut and processed proteolytically to yield smaller active toxins [3,4]. The toxins bind to specific receptor molecules on the midgut epithelial cells of host insects [5–8] and disrupt the permeability of the midgut cell membrane, resulting in a net influx of ions and an accompanying influx of water, so that the cell swells and lyses [9–11].

Each toxin has a specific insecticidal spectrum in vivo. In many cases, this spectrum is correlated with the presence of a specific receptor in the midgut membrane [5–8,12]. Binding of the toxin to the specific receptor is necessary for insecticidal activity in vivo, so the presence of a specific receptor is one of the most important factors for determining insecticidal specificity.

Recently, two types of putative Cry1 toxin receptor proteins were identified in lepidopteran insects. One is a cadherin-like glycoprotein receptor found in *Bombyx mori* [13–15] and *Manduca sexta* [16], and the other is aminopeptidase N (APN), which is found in *B. mori* [17], *M. sexta* [18–21], *Heliothis virescens* [22,23], *Lymantria dispar* [24] and *Plutella xylostella* [25]. Cry1A toxins enhance Rb+ leakage from phospholipid vesicles reconstituted with 120 kDa and 170 kDa APN purified from *M. sexta* [18] and *H. virescens* [23], respectively. The Cry1Aa, 1Ac and 1C toxins also form ion channels in a planar lipid bilayer in the presence of *M. sexta* APN [26]. Therefore, APN is likely a functional Cry1A toxin receptor in vivo.

N-Acetylgalactosamine (GalNAc) inhibits Cry1Ac toxin-binding to APN from *M. sexta* [27] and *H. virescens* [23], but not Cry1Aa toxin-binding. It is believed that Cry1Ac toxin binds to the sugar chain of APN, while no interaction between Cry1Aa and the sugar chain has been reported. The precise mechanism of Cry1Aa toxin recognition is unknown. Identifying and comparing the recognition mechanisms of Cry1Aa and 1Ac toxins is important to an understanding of the molecular mechanism that determines the susceptibility of insects to the toxin and how they acquire resistance.

Previously, we reported the cDNA cloning and expressing of *B. mori* APN [28]. Cry1Aa toxin bound to *B. mori* APN expressed on *Escherichia coli* cells [28]. In this study, we examined the Cry1Aa toxin-binding region on *B. mori* APN.

2. Materials and methods

2.1. Preparation of Cry1Aa and 1Ac toxins

Cry1Aa and 1Ac toxins were obtained from *B. thuringiensis* subsp. *sotto* T84A1 and *B. thuringiensis* subsp. *kurstaki* HD73, respectively. The preparation of activated toxin is described elsewhere [17].

2.2. Ligand blotting analysis

Ligand blotting analysis was carried out using a previously described method [17]. The proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto PVDF membrane (Bio-Rad). The membrane filter was incubated in blocking buffer, TBST (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.05% Tween 20) containing 2% bovine serum albumin, for 1 h at room temperature. The filter was then incubated in blocking buffer containing 10 nM Cry1Aa or 1Ac toxin for 1.5 h. After washing it with TBST, the filter was incubated for 1.5 h in blocking buffer containing mouse anti-Cry1A toxin monoclonal antibody that cross-reacted to Cry1Aa and Ac toxins. After washing, the filter was incubated in blocking buffer containing goat anti-mouse IgG(H+L)-HRP conjugate (Bio-Rad). Bound antibody was detected using the ECL Western blotting detection system (Amersham).

2.3. Protease digestion of APN and ligand blotting of digested fragments

Protease digestion of APN was carried out using a previously de-

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scribed method [17]. The purified APN was subjected to SDS-PAGE. The gels were stained with Coomassie brilliant blue and the stained APN band was excised. This APN was again subjected to SDS-PAGE and digested with lysylendopeptidase (Wako Pure Chemical) in a stacking gel during electrophoresis. The separated fragments of the digested protein were transferred onto PVDF membrane (Bio-Rad) and ligand blotting analysis was carried out.

2.4. Expression of APN and ligand blotting of recombinant APN

Recombinant APN was produced using a previously described method [28]. The cDNA fragments encoding the various parts of *B. mori* APN were amplified by PCR. The amplified fragments were subcloned into a GST fusion protein expression vector, pGEX-4T-3 (Pharmacia), and transfected into *E. coli* BL21. The transfected cells were cultured and gene expression was induced with 1 mM IPTG for 4 h at 37°C. The recombinant protein was produced as inclusion bodies and partially purified by sonication and centrifugation. Ligand blotting was used to determine the Cry1Aa toxin-binding ability of the recombinant protein.

3. Results and discussion

3.1. Ligand blotting analysis of purified B. mori APN

Purified *B. mori* APN was subjected to SDS-PAGE and tested for the ability to bind Cry1Aa and 1Ac toxins by a ligand blotting assay. Although both toxins bound to APN, the signal from Cry1Aa toxin-binding to APN was more intense than that of Cry1Ac toxin (Fig. 1, lanes 1 and 2).

3.2. Cry1Aa toxin-binding ability of digested APN fragments

In order to analyze the Cry1Aa and 1Ac toxin-binding regions, *B. mori* APN was digested with lysylendopeptidase and the Cry1Aa and 1Ac toxin-binding ability of the resulting fragments was tested. Protease digestion produced several fragments, observed with SDS-PAGE (Fig. 2A, lane 3). The binding abilities of Cry1Aa and 1Ac to these fragments were different (Fig. 2A, lanes 1 and 2), suggesting that the recognition mechanisms of the two toxins are not identical.

Cry1Aa toxin bound to the 80 and 30 kDa fragments, but not to the 50 kDa fragment (Fig. 2A, lane 1). The N-terminal amino acid sequences of these three fragments were analyzed. The sequences of the 80 and 30 kDa proteins were the same as the N-terminal sequence of mature 120 kDa APN [17], which starts at 40-Asp, while the N-terminal amino acid sequence of the 50 kDa protein was different (Fig. 2B). The latter sequence, QAAIPDFSAGA, is identical to the deduced amino acid sequence from 314-Gln to 324-Ala [28]. Moreover, the 313th amino acid residue is lysine, which is a lysylendopeptidase cleavage site. Therefore, the N-terminal of the 50 kDa fragment, which does not bind Cry1Aa toxin, was thought to start at 314-Gln. The C-terminal amino acid residues of each fragment were predicted from the deduced *B. mori* APN amino acid sequence, using the molecular weight and the lysine positions (Fig. 2C). These results suggest that the Cry1Aa toxin-binding site is located in the region between 40-Asp and 313-Lys.

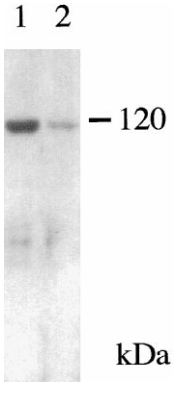

Fig. 1. Ligand blotting analysis of purified APN with Cry1Aa and 1Ac toxins. APN was subjected to SDS-PAGE and a ligand blotting analysis was performed with Cry1Aa (lane 1) and 1Ac (lane 2) toxins.

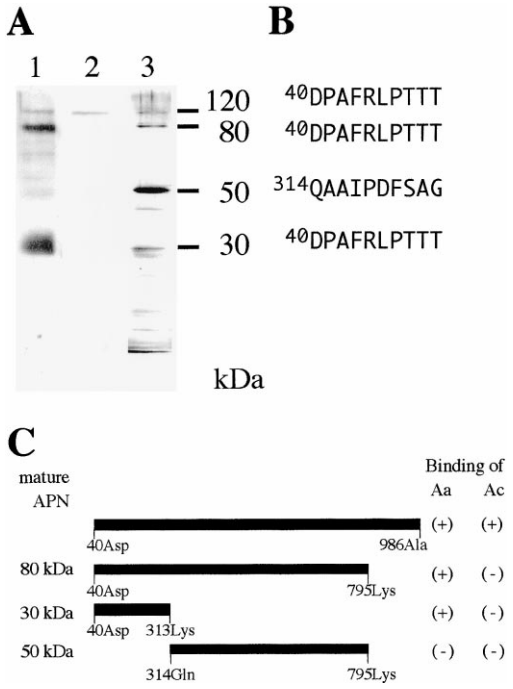

Fig. 2. Protease digestion of *B. mori* APN and the Cry1Aa and 1Ac toxin-binding abilities of the resulting fragments. *B. mori* APN was digested with lysylendopeptidase. The fragments were separated by SDS-PAGE (12.5% polyacrylamide) and ligand blotting analysis was performed. (A) Lane 1, ligand blotting with Cry1Aa toxin; lane 2, ligand blotting with Cry1Ac toxin; lane 3, Coomassie brilliant blue-stained gel. (B) The N-terminal amino acid sequences of the 80, 50 and 30 kDa fragments and mature 120 kDa APN. (C) The binding ability of each digested fragment.

On the other hand, Cry1Ac toxin bound exclusively to mature APN (Fig. 2A, lane 2). It is speculated that the lysylendopeptidase-digested fragments did not contain the complete Cry1Ac high-affinity binding region. It is possible that the Cry1Ac-binding region exists on a fragment that is too small to be detected by SDS-PAGE or is cleaved by the lysylendopeptidase. Using a different protease might reveal the Cry1Ac toxin-binding region.

The binding of Cry1Ac toxin to the 80 and 30 kDa fragments that bound to Cry1Aa toxin was very faint and was only detected after a long exposure (data not shown). It has been reported that Cry1Ac toxin has different affinities to the two *M. sexta* APN-binding sites and shares one of the two binding sites with Cry1Aa toxin [27]. It is possible that the 30 kDa fragment containing 40-Asp–313-Lys, the putative Cry1Aa toxin-binding region, contains the lower affinity Cry1Ac toxin-binding site shared with Cry1Aa toxin.

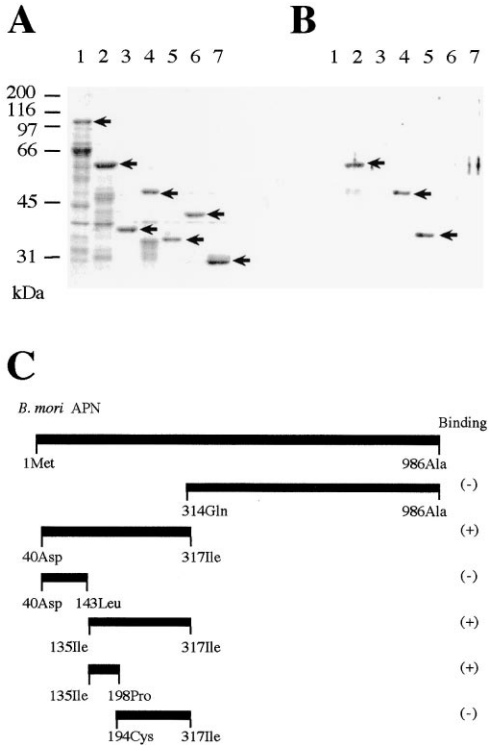

Fig. 3. Analysis of the Cry1Aa toxin-binding ability of various recombinant *B. mori* APN fragments. Recombinant APN fragments of various lengths were expressed as GST fusion proteins in *E. coli* cells. The recombinant APN fragments were subjected to SDS-PAGE and the Cry1Aa toxin-binding ability was analyzed using ligand blotting. (A) Coomassie brilliant blue-stained gel and (B) ligand blotting with Cry1Aa toxin. Lanes 1–7, GST fusion proteins with APN fragments, 314-Gln–986-Ala, 40-Asp–317-Ile, 40-Asp–143-Leu, 135-Ile–317-Ile, 135-Ile–198-Pro, 194-Cys–317-Ile, and GST alone, respectively. The arrows indicate recombinant proteins. (C) The binding ability of the recombinant APN fragments.

3.3. *Precise analysis of the Cry1Aa toxin-binding region using recombinant APN*

In order to pinpoint the putative Cry1Aa toxin-binding region within 40-Asp–313-Lys, we expressed recombinant APN fragments in *E. coli*. Previously, we reported that Cry1Aa toxin bound to the mature type of recombinant *B. mori* APN expressed in *E. coli* cells [28]. In this study, recombinant APN fragments of various lengths were expressed as GST fusion proteins in *E. coli* cells (Fig. 3A) and their Cry1Aa toxin-binding ability was analyzed (Fig. 3B). The smallest fragment that bound Cry1Aa toxin was 135-Ile–198-Pro (Fig. 3B,C), suggesting that the toxin binds to an amino acid structure without a sugar chain in the region from 135-Ile to 198-Pro. Although Cry1Ac toxin also bound to this fragment, the binding signal was too weak to conclude whether Cry1Ac also bound to the region without a sugar chain (data not shown).

3.4. *Cry1Aa toxin recognition structure*

Recently, it was reported that GalNAc inhibits the binding of Cry1Ac toxin to APN from *M. sexta* [27] and *H. virescens* [23], suggesting that the APN sugar chain is very important for binding to Cry1Ac toxin. On the other hand, inhibition of Cry1Aa toxin was not reported. Cry1Aa toxin bound to recombinant *B. mori* APN [28] and APN fragments expressed in *E. coli* cells. Therefore, it is believed that Cry1Aa toxin binds to an amino acid structure without a sugar modification. In this study, Cry1Aa toxin bound to the *B. mori* APN between 135-Ile and 198-Pro, indicating that an amino acid structure in this region plays an important role in the recognition of Cry1Aa toxin.

B. mori is more sensitive to Cry1Aa toxin than it is to Cry1Ac toxin and the binding affinity of Cry1Ac to *B. mori* APN is lower than that of Cry1Aa [29]. On the other hand, *M. sexta* is very sensitive to both Cry1Aa and Cry1Ac toxins, and both toxins bind to *M. sexta* APN with similar high affinity [27]. This study revealed that the recognition structures on APN for Cry1Aa and 1Ac toxins are not identical (Fig. 2A). The different insecticidal activities of Cry1Aa and Cry1Ac toxins in *B. mori* might result from the different APN recognition mechanisms of the two toxins.

This study revealed that Cry1Aa toxin binds directly to a structure consisting of 64 amino acid residues. Recently, Nakanishi et al. reported that the *P. xylostella* APN fragment containing this region was expressed in *E. coli* cells and confirmed its ability to bind Cry1Aa toxin [30]. In this region, 27 of the amino acid residues were conserved in *B. mori* and *P. xylostella* APN. It is possible that these amino acids play an important role in the recognition by Cry1Aa toxin. Comparing the amino acid sequences from many insect APNs that do and do not bind to Cry1Aa toxin may help to determine the mechanism of Cry toxin-binding specificity and the subsequent insecticidal specificity. In addition, comparing the amino acid sequences in susceptible and resistant insects may be an effective approach to learn about the molecular mechanism for the development of insect resistance.

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