cDNA cloning and expression of Bacillus thuringiensis Cry1Aa toxin binding 120 kDa aminopeptidase N from Bombyx mori

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1 The sequence is present in GenBank under accession number AF084257.
toxic activity [5–8,14]. Recently, two putative Cry1 toxin receptor proteins were identified in lepidopteran insects. One is a cadherin-like glycoprotein receptor for Cry1Aa and Ab in *Bombyx mori* [15,16] and *Manduca sexta* [17], and the other is aminopeptidase N (APN), a receptor for Cry1Aa, Ac and 1C in *B. mori* [18], *M. sexta* [19–22], *Heliothis virescens* [23], *Lymantria dispar* [24] and *Plutella xylostella* [25]. APN cDNA from several insects has been cloned: 120 kDa APN, a Cry1Ac toxin receptor, from *M. sexta* (*M. sexta* APN1) [26] and *H. virescens* [23] and 120 kDa APN, a Cry1Ab toxin receptor, from *M. sexta* (*M. sexta* APN2) [22]. The Cry1A toxins enhance Rb⁺ leakage from phospholipid vesicles reconstituted with 120 and 170 kDa APN purified from *M. sexta* [19] and *H. virescens* [27], respectively. Moreover, it was also reported that Cry1Aa, Ac and 1C toxins form ion channels in a planar lipid bilayer in the presence of *M. sexta* APN [28]. Therefore, APN is likely to be a functional Cry1A toxin receptor in vivo.

We previously reported the purification of the GPI-anchor-linked 120 kDa Cry1Aa binding protein from the *B. mori* midgut, which was identified as APN, and determined its N-terminal and internal amino acid sequences [18]. In this study, we describe the cloning and expression of APN. The Cry1Aa toxin binding ability of expressed APN was analyzed subsequently.

Total RNA was obtained from the midgut of early fifth instar larvae of a hybrid race of *B. mori*, Kinshu×Showa, using a Quickprep Total RNA Extraction Kit (Pharmacia). mRNA was prepared with an oligo dT column using an mRNA Purification Kit (Pharmacia). cDNA was synthesized from the mRNA with an oligo dT primer using a cDNA Synthesis Module Kit (Amersham) and a cDNA library was constructed in *V* MOSElox using a cDNA rapid cloning module (Amersham).

First, the cDNA fragment was amplified by PCR using Taq DNA polymerase. Degenerate primers were designed from the N-terminal and internal amino acid sequences of *B. mori* APN [18]: F1, GA-(CT)CC(ACGT)GC(ACGT)TT(CT)(AC)GI(CT)TI-CC and R1, A(AG)(ACGT)CCCCA(AG)TT(CT)TCAT(ACGT)GC(ACGT)CC. Subsequently, nested PCR was performed with nested primers: F2, CC-(ACGT)(AC)GICA(CT)TA(CT)CA(AG)GT(ACGT)-AC and R2, (AG)AA(AG)TCIGG(AGT)AT(ACGT)GC(ACGT)GC(CT)TG. The amplified fragment was subcloned into the T-overhang vector, p123T (Mo Bi Tec). Dideoxy double-stranded sequencing of the cDNA insert was performed using an ALFred DNA sequencer (Pharmacia) according to the manufacturer’s instructions and confirmed that the amplified fragment was the expected one. This fragment was labeled with fluorescein using an ECL random prime labeling and detection system (Amersham) and used as a probe to screen a *V* MOSElox cDNA library. Hybridized probe was detected with anti-fluorescein antibody conjugated to horseradish peroxidase (HRP) by following the manufacturer’s instructions. Approximately 5×10⁵ clones were screened. The positive clone was plaque-purified and analyzed by PCR. The PCR-positive clone was automatically subcloned to pMOSElox by cre-mediated excision of plasmids in vivo in *Escherichia coli* BM25.8. The insert was mapped by restriction enzymes and the resulting fragments were subcloned into pBluescript II SK+ (SK++; Stratagene) and transfected into *E. coli* XL1-Blue. The double-stranded DNA insert in SK+ was completely sequenced in both directions.

The cDNA fragment encoding the *B. mori* APN was amplified by PCR. The amplified fragment was subcloned into GST fusion protein expression vector, pGEX-4T-3 (Pharmacia) and transfected into *E. coli* BL21. The transfected cells were cultured and expression was induced with 1 mM IPTG for 4 h at 37°C. The protein was produced as an inclusion body and partially purified by sonication and centrifugation. Immunoblotting and ligand blotting were performed using a previously described method [18]. The expressed proteins were separated by SDS-PAGE and transferred from the gel onto a PVDF membrane.

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Fig. 1. The sequence of *B. mori* APN cDNA and the deduced amino acid sequence. The polyadenylation signal is in capitals. The putative N-terminal cleavable signal peptide and the GPI signal peptide are underlined. The probable cleavage/anchor addition site is indicated by arrow. The consensus N-glycosylation site is double-underlined. The zinc-metallopeptidase signature is boxed. Partial amino acid sequences obtained from the purified protein are broken underlined, and the N-terminal residue of the mature protein is indicated by arrow.
filter (Bio Rad). The membrane filter was incubated in a 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 mouse anti-\textit{B. mori} APN antiserum or Cry1Aa toxin (1 nM) for 1.5 h. After being washed 3 times with TBST for 15 min each time, the filter was then incubated in blocking buffer containing goat anti-mouse IgG (H+L) HRP conjugate (Bio-Rad) or HRP-conjugated mouse anti-Cry1Aa monoclonal antibody (lane 3), respectively. Lane 1 was stained with Coomassie brilliant blue.

PCR using degenerate primers F1 and F2 gave products of several sizes. Using nested PCR with primers F2 and R2, only one DNA fragment of approximately 800 bp was amplified and this was used as a probe to screen the \textit{B. mori} midgut cDNA library. A single clone hybridized with this probe. It contains an open reading frame of 2958 bp, which corresponds to 986 amino acids (Fig. 1). The clone contains the criteria for a Kozak consensus translational initiation site, ATAAATG [29]. A polyadenylation signal, AATAAA [30], is observed in the 3' untranslated region preceding a poly(A) tail. The deduced amino acid sequence matches the partial amino acid sequences obtained from the purified APN perfectly. There is a potential N-glycosylation site at amino acid residues 717–719. The zinc-metallopeptidase signature [31] is observed at amino acid residues 359–363. The N-terminal amino acid sequence of the mature APN starts at the 40th amino acid residue, indicating that 39 amino acids are cleaved from the N-terminal of the mature APN. The first 20 amino acids seem to be a signal sequence. There is also a long hydrophobic region from amino acid residues 969 to 986 in the C-terminal. In several insects, it is reported that the midgut APN is linked to a GPI anchor [22,23,32,33]. Previously, we reported that \textit{B. mori} APN was released by PIPLC from BBMV [18], indicating that \textit{B. mori} APN is linked to a GPI anchor protein. In this study, the presence of a long hydrophobic C-terminus preceded by a domain of three small residues (Asp965–Ala967), which is a putative cleavage-anchor addition site [34], also indicates that \textit{B. mori} APN is a GPI anchored protein. N- and C-terminal proteolytic cleavage result in a 105 kDa protein containing 926 amino acids residues. Since the APN isolated from the \textit{B. mori} midgut is 120 kDa, the difference in the molecular masses of these two proteins may be derived from the presence of a carbohydrate or some other post-translational modification.

In order to demonstrate that the protein encoded by the cDNA has Cry1Aa toxin binding ability, the \textit{B. mori} APN cDNA was expressed as a GST fusion protein in \textit{E. coli} cells. The approximately 130 kDa protein expressed in the cells is likely the GST-APN fusion protein, since the molecular masses of GST and APN are 27 and 105 kDa, respectively (Fig. 2, lane 1). Moreover, anti-APN antiserum reacted with this protein (Fig. 2, lane 2). Next, the binding ability of the fusion protein was analyzed by ligand blotting using Cry1Aa toxin. The Cry1Aa toxin bound to the 130 kDa GST-APN fusion protein (Fig. 2, lane 3), but not to GST itself (data not shown), indicating that the recombinant \textit{B. mori} APN had Cry1Aa toxin binding ability. Several smaller bands were also observed. These might be degraded products of the fusion protein, since anti-APN antiserum also reacted with these proteins (Fig. 2, lane 2).

Recently, a partial cDNA encoding APN was isolated from \textit{B. mori} midgut by Hua et al. [35]. Its deduced amino acid sequence shows a very high similarity with our sequence (97.7% identity). The differences between the sequences of Hua et al. and our
Fig. 3. Comparison of the deduced *B. mori* APN amino acid sequence with the sequences of APNs from *M. sexta* and *H. virescens*.
sequence are found within Met$^{1}$−Ala$^{13}$, Gln$^{477}$−His$^{485}$, Asn$^{529}$−Val$^{537}$, Asn$^{608}$ and His$^{738}$−Glu$^{739}$. In this region, our sequence shows a similarity with the APNs of M. sexta and H. virescens, especially with M. sexta APN1, while similarity with the sequence reported by Hua et al. is not observed. Moreover, in our study, the recombinant APN expressed by E. coli cells bound to Cry1Aa toxin, while Hua et al. did not confirm Cry1Aa toxin binding ability. Consequently, we believe that the full-length cDNA obtained in this study encodes the Cry1Aa toxin binding APN reported previously by us [18]. It is possible that the difference between our sequence and that of Hua et al. is the result of cloning cDNA coding another isoform or an experimental error in the sequence analysis of Hua et al.

GalNAc is reported to inhibit the binding of Cry1Ac to M. sexta APN1 [36,37] and H. virescens 170 kDa APN [27], but not the binding of Cry1Aa and Ab [27,37]. Moreover, the Cry1Aa toxin bound to the recombinant fusion protein expressed in E. coli cells in this experiment (Fig. 2). This indicates that Cry1Aa toxin can bind to the B. mori APN by recognizing a protein structure without a natural sugar chain. The deduced amino acid shows 29.0−73.7% identity to APN from other insects that are reported to be Cry1A toxin receptors (Fig. 3). B. mori APN has a very high similarity to APN from M. sexta APN1 (73.7% identity), which binds to Cry1Aa [37]. On the other hand, 120 kDa H. virescens APN (41.1% identity) is not reported to bind to Cry1Aa. It is possible that the Cry1Aa toxin recognizes the portion of the structure of B. mori APN that is conserved in M. sexta APN1 and not in the 120 kDa H. virescens APN.

The recombinant protein produced by E. coli in this study may be useful to analyze the Cry1Aa toxin binding site of B. mori APN. Furthermore, a comparison with the structures of other insect APN may lead to an understanding of how the binding specificity and insecticidal specificity of B. thuringiensis toxins are determined.

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References