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Aminopeptidase N isoforms from the midgut of *Bombyx mori* and *Plutella xylostella* – their classification and the factors that determine their binding specificity to *Bacillus thuringiensis* Cry1A toxin

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Abstract **Novel aminopeptidase N (APN) isoform cDNAs, *BmAPN3* and *PxAPN3*, from the midguts of *Bombyx mori* and *Plutella xylostella*, respectively, were cloned, and a total of eight APN isoforms cloned from *B. mori* and *P. xylostella* were classified into four classes. *Bacillus thuringiensis* Cry1Aa and Cry1Ab toxins were found to bind to specific APN isoforms from the midguts of *B. mori* and *P. xylostella*, and binding occurred with fragments that corresponded to the BmAPN1 Cry1Aa toxin-binding region of each APN isoform. The results suggest that APN isoforms have a common toxin-binding region, and that the apparent specificity of Cry1Aa toxin binding to each intact APN isoform seen in SDS–PAGE is determined by factors such as expression level in conjunction with differences in binding affinity.** © **2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Aminopeptidase N; Cry1A toxin; Receptor; *Bombyx mori*; *Plutella xylostella*; *Bacillus thuringiensis*

1. Introduction

Aminopeptidase N (APN) (EC 3.4.11.2) is an enzyme that preferentially cleaves neutral amino acids from the amino-terminus of proteins or oligopeptides. This enzyme is widely distributed within the plant and animal kingdoms. In animals, APN is most abundant in the brush border membrane of the intestine, and is an enzyme involved in the digestion of protein in food. In addition, APN is a major *Bacillus thuringiensis* Cry toxin receptor candidate in the midgut of insects.

The Gram-positive bacterium *B. thuringiensis* produces several insecticidal proteins called Cry toxins. Cry toxins possess insecticidal specificity, but do not affect mammals. Consequently, Cry toxins have been used as microbial insecticides. Recently, Cry toxin genes have been used in the genetic development of insect-resistant plants. However, it is not clear why each Cry toxin kills only specific insects.

Cry toxins produced as protoxins are solubilized and activated proteolytically in the midgut of a susceptible insect [1–3]. The activated toxin binds to specific receptors on epithelial cells in the midgut [4,5], subsequently creating a pore in the cell membrane that eventually leads to cell death [6]. Therefore, the toxin-specific binding to the receptor is one of the key factors determining insecticidal specificity. Several APNs [7–13] as well as cadherin-like molecules [14,15] have been identified as Cry toxin receptor candidates. Experiments involving membranes reconstituted with APN suggest that APN promotes the insertion of Cry toxins into the cell membrane, and is also involved in pore formation initiated by three toxins [12,16,17].

Eighteen APN isoform cDNAs from eight lepidopterans have been cloned and registered in databases: from *Bombyx mori*, *Heliothis virescens*, *Plutella xylostella*, *Helicoverpa punctigera*, *Manduca sexta*, *Limantria dispar*, *Plodia interpunctella*, and *Epiphyas postvittana*. Oltean et al. [18] found that APN isoforms from insects were grouped into at least four classes according to dendrogram analyses of APN sequences. However, no insect has been reported to have isoforms from all four classes. There are uncloned APN isoforms, such as *M. sexta* Cry1C toxin-binding 106-kDa APN [8] and *L. dispar* APN2 [19] and the fourth APN isoform in *B. mori* [20]. Not all APN isoforms in lepidopterans have been cloned, and it is not known how many classes of APN are present in lepidopterans or whether lepidopterans possess APN isoforms from all four classes.

Eight of 18 cloned APN isoforms, *B. mori* APN1 [21], *M. sexta* APN1 [22], APN2 [23], *H. virescens* 120-kDa APN [11], 170-kDa APN [18], 110-kDa APN [24], *L. dispar* APN1 [25] and *E. postvittana* APN [26] are reported to bind to Cry toxin. There are reports that indicate that different Cry toxins bind to distinct APN isoforms in the brush border membrane vesicle (BBMV). In *M. sexta*, both Cry1Aa and Cry1Ac toxins bind to APN1 [22], Cry1Ab toxin binds to APN2 [23], and Cry1C toxin binds to 106-kDa APN [8]. In *H. virescens*, Cry1Aa and Cry1Ab toxins bind to 170-kDa APN only, while Cry1Ac toxin binds to 120-kDa, 110-kDa, and 170-kDa APN [11,12,18,24]. In *L. dispar*, Cry1Ac toxin binds to APN1, but Cry1Aa, Cry1Ab, Cry1C, Cry2A, and Cry3A toxin do not bind to either APN1 or APN2 [9,10]. Therefore, it is not known whether each APN isoform binds to Cry toxin and whether each toxin binds to a specific class of APN isoform from various lepidopterans.

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The receptor domains involved in the binding of Cry toxin have been studied. In the APN isoform derived from *B. mori*, Cry1Aa toxin binds to a Cry1Aa toxin-binding region between Ile135 and Pro198 of BmAPN1 [27]. Moreover, the same region of PxAPN3 binds to the Cry1Aa toxin [28]. By contrast, in cadherin-like protein, only eight amino acid residues are identified as Cry1Aa and Cry1Ab toxin-binding epitopes [29].

This study sought to clarify the binding specificity of Cry toxin to the classes of APN, and investigated the molecular mechanisms involved in this process. We found a novel APN cDNA sequence, derived from the midgut of both *B. mori* and *P. xylostella*, which suggests that at least four classes of APN are present in the midgut of lepidopterans. Moreover, we showed that Cry1Aa and Cry1Ab toxins bind to specific APN isoforms when intact APN molecules are used, although every APN isoform from all four classes had a common Cry1Aa and Cry1Ab toxin-binding region. We predicted the factors determining the Cry1A toxin-binding specificity to each APN isoform. All APN isoforms seem to have a common partial structure that both Cry1Aa and Cry1Ab toxins can bind to, and this structure determines the binding ability of Cry1Aa and Cry1Ab toxins with APN. Other factors might also have an effect on the binding of Cry1A toxin.

2. Materials and methods

2.1. Preparation of Cry1Aa and Cry1Ab toxins

Cry1Aa and Cry1Ab crystals were purified from *B. thuringiensis* serovar *sotto* strain T84A1 and recombinant *Escherichia coli* expressing Cry1Ab toxin [30] (provided by Sumitomo Chemical), respectively. The purified crystals were solubilized and then activated as described elsewhere [13].

2.2. Cloning of APN isoform cDNAs from the B. mori and P. xylostella midgut

Total RNA, from the midgut of fifth instar *B. mori* (Kinshu× Showa) and fourth instar *P. xylostella*, was isolated and cDNAs were synthesized according to previously described methods [28]. Degenerate primers used for PCR amplification were designed from three conserved peptide sequences that are found in APNs from a number of organisms: GAMEWG (S1, 5′-GIGCNAYNGARAAYTGGG), YRVNYD (R1, 5′-RTCRTAVTTIACDCKGTA) and WLNEGFA (R2, 5′-GCRAANCCYTCRTTNARCCA). Another degenerate primer (S2, 5′-TITWYMGIYTICCNACNACNAC) was designed from the peptide sequence YRLPTTT, which is conserved in *B. mori* APN1, *H. virescens* 120-kDa APN, and *M. sexta* APN1. PCR was performed with template cDNA derived from the midgut of *B. mori* and *P. xylostella*. PCR products were cloned into the T-overhang vector p123T (MoBi Tec) and sequenced. Following sequence analysis, a partial cDNA of a novel APN-like protein derived from *B. mori*, named *BmAPN3*, was identified.

The APN cDNA fragment (*PXfrg1*) from *P. xylostella* has been cloned previously [28]. The *BmAPN3* and *PXfrg1* sequences were used to design oligonucleotide primers specific to the respective APNs. Using these primers, the complete nucleotide sequences of *BmAPN3* and *PXfrg1* were determined utilizing 5′- and 3′-RACE.

2.3. Preparation of specific antisera to four APN isoforms derived from B. mori

cDNA fragments encoding the non-conserved regions of four *B. mori* APN isoforms (Fig. 1), amino acid residues Thr57–Thr173 of BmAPN1 and the homologous parts of another BmAPN, were amplified by PCR and cloned into the GST fusion protein expression vector, pGEX-4T-3 (Amersham Pharmacia Biotech) and then transfected into *E. coli* BL21. Recombinant APN fragments were produced using a previously described method [28]. Recombinant APN fragments, purified by SDS–PAGE and electroelution, were used to raise class-specific antisera in mice.

2.4. Immunoblot and ligand blot analyses of B. mori and P. xylostella BBMV

BBMV from the midgut of *B. mori* and *P. xylostella* was prepared according to the method described by Worfersberger et al. [31]. BBMV proteins were separated by SDS–PAGE and transferred to a nitrocellulose membrane. The membrane was blocked with 2% bovine serum albumin (BSA) in TBST (10 mM Tris–HCl pH 8.0, 150 mM NaCl, 0.05% Tween 20). For the immunoblot, the membrane was incubated with mouse anti-APN antiserum, followed by goat anti-mouse IgG–HRP conjugate (Bio-Rad). The bound antibody was detected using ECL Western blotting detection systems (Amersham Pharmacia Biotech). For the ligand blot, the blocked membrane was incubated in 10 nM Cry1Aa or Cry1Ab toxin in TBST containing 2% BSA, followed by mouse anti-Cry1Aa toxin antiserum that cross-reacted with both Cry1Aa and Cry1Ab toxins. The membrane was incubated in goat anti-mouse IgG–HRP conjugate and the bound antibody was detected as described above.

2.5. Expression and toxin-binding ability of the Cry1Aa toxin-binding region in APN classes from B. mori and P. xylostella

The toxin-binding regions from each APN class of *B. mori* and *P. xylostella* were expressed for GST fusion proteins as described above. The binding ability of APNs to Cry1Aa and Cry1Ab toxins was assessed using ligand blot analysis.

3. Results

3.1. cDNA cloning of APN isoforms from the midgut of B. mori

RT-PCR strategy was used to clone APN isoform cDNAs from the midgut of *B. mori*. Primer pair S1/R1 amplified an approximately 900-bp fragment. This fragment was cloned into the p123T vector and sequenced. Three different sequences were identified. Sequence analysis showed that the fragments were identical to the sequences of *B. mori APN1* [21], *APN2* [20], and *APN4* (GenBank AB013400). Another primer pair, S2/R2, produced a 1000-bp product that was identified as a novel APN-like sequence, named *BmAPN3*, after sequencing and aligning. Full-length *BmAPN3* was obtained by 5′- and 3′-RACE and sequencing. The sequence data of *BmAPN3* has been deposited in GenBank (accession number AF352574).

BmAPN3 is 3240 bp long and contains a 3015-bp open reading frame encoding a putative 1005-amino acid protein with a theoretical molecular mass of 113.6 kDa. The predicted start codon is embedded in a consensus Kozak translation initiation sequence (AAGATGG) [32]. There is a putative polyadenylation signal sequence (AATAAA). Analysis of the N-terminal region with the program SignalP (http://www.cbs.dtu.dk/) predicted a signal peptide sequence (MANYKVIIFLAACVLAQA, residues 1–18) with a cleavage site between Ala18 and Phe19. The glycosylphosphatidylinositol (GPI) anchor signal sequence [33], consisting of three small amino acids (DAA, residues 982–984) and a stretch of hydrophobic residues (PVSTFLSVAVVALVAVVNLIM, residues 985–1005), is found at the C-terminus. Six potential *N*-glycosylation sites (NXS/T) [34] and 11 *O*-glycosylation sites, predicted using the program NetOGlyc 2.0 (http://www.cbs.dtu.dk/), were found to be present. BmAPN3 is characterized by a consensus zinc-binding/gluzincin motif (HEXXHX$_{18}$E, residues 371–394) [35] and a gluzincin aminopeptidase motif (GAMEN, residues 335–339) [36]. BLAST searches of the GenBank database using the protein sequence revealed that BmAPN3 was most similar to other lepidopteran APNs, including *H. virescens* 120-kDa APN [11] (64.6% identical), *L. dispar* APN1 [25] (65.9% identical), and *P. interpunc-*

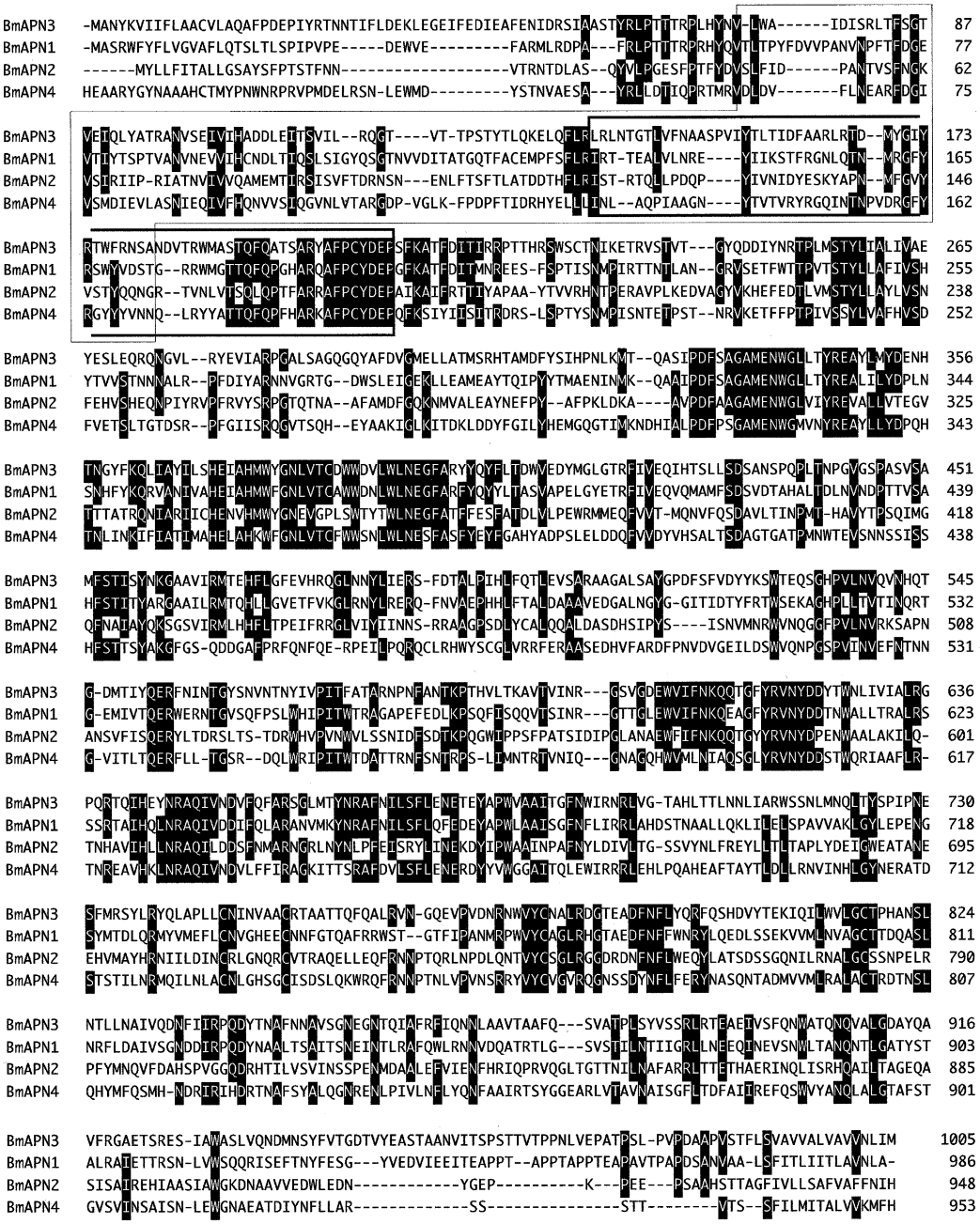

Fig. 1. Comparison of the deduced amino acid sequence of *BmAPN3* with those of *BmAPN1* [21], *BmAPN2* [20], and *BmAPN4* (GenBank AB013400). Multiple-sequence alignment was performed using the program ClustalX. Highly conserved residues have dark backgrounds. The large boxed region was used as the antigen to raise specific antiserum. The area surrounded by the bold line is the region corresponding to the Cry1Aa toxin-binding region of BmAPN1.

tella APN1 [37] (63.4% identical). The deduced amino acid sequence of *BmAPN3* was aligned with other *B. mori* APN isoforms using ClustalX (Fig. 1). BmAPN3 contained almost all the conserved residues found in three other *B. mori* APN isoforms.

3.2. cDNA cloning of APN isoforms from the midgut of *P. xylostella*

Various APN isoform cDNAs from the midgut of *P. xylostella* were cloned as above. Three different cDNA sequences, obtained using primers S1 and R1, were identical to the sequences of *P. xylostella APN1* [23], *APNA* [38], and *APN4* (GenBank AJ222699). The full length of previously cloned *PXfrg1* [28] was sequenced using 5′- and 3′-RACE. The 5′-end of *PXfrg1* was not determined, although a 5′-truncated cDNA was obtained and renamed *PxAPN3*. The sequence data of *PxAPN3* has been deposited in GenBank (accession number AF109692).

PxAPN3 contained a 2826-bp open reading frame encoding a putative 942-amino acid protein. There was a sequence (AATAA) similar to a polyadenylation signal sequence (AATAAA). The *PxAPN3* sequence also appeared to have a pos-

sible GPI anchor signal sequence. The sequence contained four putative *N*-glycosylation sites (NXS/T) and 22 *O*-glycosylation sites. PxAPN3 is characterized by a consensus zinc-binding/gluzincin motif ($HEXXHX_{18}E$) and a gluzincin aminopeptidase motif (GAMEN). BLAST searches of the GenBank database using the protein sequence revealed that *PxAPN3* was highly similar to lepidopteran APNs.

3.3. Classification of lepidopteran APNs

The deduced amino acid sequences encoded by *BmAPN3* and *PxAPN3* were aligned with 18 other lepidopteran APNs using ClustalX. The lepidopteran APNs were grouped into four classes on the phylogenetic tree derived from a ClustalX alignment (Fig. 2). The group composed of BmAPN1 [21], MsAPN1 [22], Hv170kDaAPN [18], HpAPN1 [39], and PxAPNA [38] was tentatively named class 1. The group composed of BmAPN2 [20], MsAPN2 [23], PxAPN1 [23], and Ld λ APN2 [25] was categorized as class 2. The group composed of Hv120kDaAPN [11], LdAPN1 [25], PiAPN1 [37], HpAPN3 [39], EpAPN [26], BmAPN3, and PxAPN3 was named class 3. The group composed of Hv110kDaAPN [24], BmAPN4 (AB013400), HpAPN2 [39], and PxAPN4 (AJ222699) was named class 4. In both *B. mori* and *P. xylostella*, class 1, 2, and 4 APNs were evident. In addition, class 3 APNs were evident in *B. mori* and *P. xylostella*.

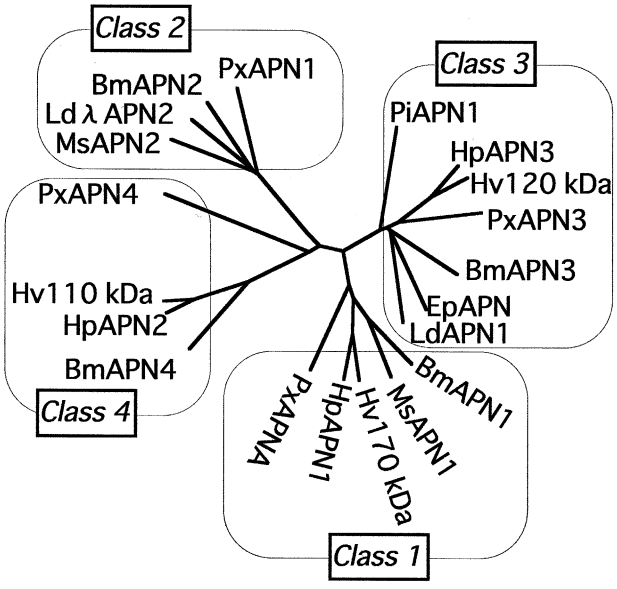

Fig. 2. Phylogenetic tree derived from a ClustalX alignment of lepidopteran midgut APNs. GenBank accession numbers are as follows: *B. mori* APN1 (BmAPN1), AF084257 [21]; *M. sexta* APN1 (MsAPN1), X89081 [22]; *H. virescens* 170-kDa APN (Hv170kDa), AF173552 [18]; *H. postvittana* APN1 (HpAPN1), AF217248 [39]; *P. xylostella* APNA (PxAPNA), AF020389 [38]; *B. mori* APN2 (BmAPN2), AB011497 [20]; *M. sexta* APN2 (MsAPN2), X97877 [23]; *P. xylostella* APN1 (PxAPN1), X97878 [23]; *L. dispar* λ APN2 (Ld λ APN2), AF126443 [25]; *B. mori* APN3 (BmAPN3), AF352574 (this study); *H. virescens* 120-kDa APN (Hv120kDa), U35096 [11]; *P. xylostella* APN3 (PxAPN3), AF109692 (this study); *L. dispar* APN1 (LdAPN1), AF126442 [25]; *P. interpunctella* APN1 (PiAPN1), AF034483 [37]; *H. postvittana* APN3 (HpAPN3), AF217250 [39]; *E. postvittana* APN (EpAPN), AF276241 [26]; *B. mori* APN (BmAPN4), AB013400; *H. virescens* 110-kDa APN (Hv110kDa), AF378666 [24]; *P. xylostella* APN (PxAPN4), AJ222699; *H. postvittana* APN2 (HpAPN2), AF217249 [39].

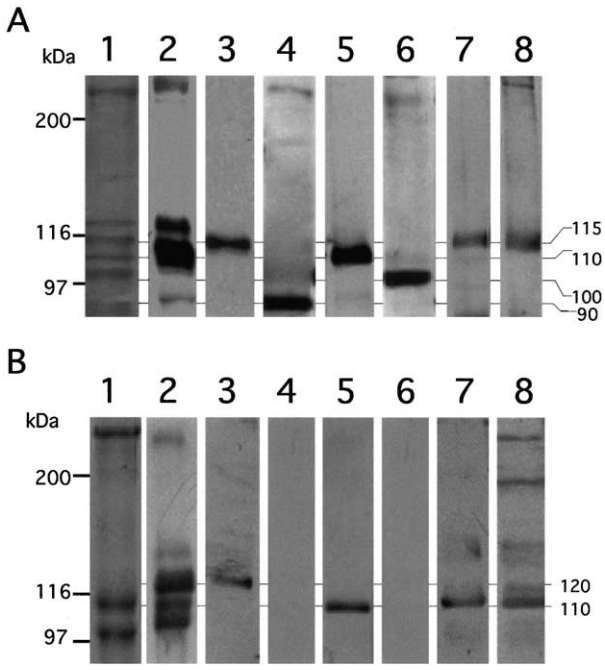

Fig. 3. SDS–PAGE, immunoblot, and ligand blot analyses of *B. mori* (A) and *P. xylostella* (B) BBMV proteins. *B. mori* and *P. xylostella* BBMV proteins were separated by 5% SDS–PAGE and then transferred to nitrocellulose membranes: Coomassie brilliant blue-stained gel (lanes 1). Immunoblot blot analyses were performed with anti-APN antiserum (lanes 2), anti-BmAPN1 antiserum (lanes 3), anti-BmAPN2 antiserum (lanes 4), anti-BmAPN3 antiserum (lanes 5), and anti-BmAPN4 antiserum (lanes 6). The secondary antibody was goat anti-mouse IgG–HRP conjugate and ECL was the method of detection. Ligand blot analyses were performed with 10 nM Cry1Aa (lanes 7) and Cry1Ab (lanes 8) toxin. Binding toxins were detected as described in Section 2. The numbers on the left are molecular masses. The molecular sizes on the right are discussed in the text.

3.4. Immunoblot and ligand blot analyses of B. mori and P. xylostella BBMV

To identify the four APN isoforms in *B. mori* BBMV, an immunoblot analysis was performed using class-specific antisera (Fig. 3A). The anti-BmAPN1, -BmAPN2, -BmAPN3, and -BmAPN4 antisera recognized 115-, 90-, 110-, and 100-kDa proteins in BBMV, respectively (Fig. 3A, lanes 3–6). After immunoblot with anti-BmAPN3 antiserum, immunoblots with anti-BmAPN1 or -BmAPN4 antiserum were performed using the same membrane. The result of this double immunoblot analysis confirmed that each antiserum recognized the different protein (data not shown). On the other hand, anti-APN antiserum, which was raised using the purified intact BmAPN1 as an antigen, recognized the 120- and 230-kDa proteins in BBMV, as well as the proteins recognized by anti-BmAPN1, -BmAPN2 and -BmAPN3 antisera (Fig. 3A, lane 2). The 115-, 110-, and 100-kDa proteins were also detected in Coomassie-stained SDS–PAGE gels, while the 90-kDa protein was not (Fig. 3A, lane 1). A ligand blot was performed to identify the Cry1Aa and Cry1Ab toxin-binding proteins in *B. mori* BBMV. Both toxins bound to the 115-kDa protein at 10 nM (Fig. 3A, lanes 7, 8). After immunoblot analysis with anti-BmAPN3 antiserum, ligand blot was performed, using the same membrane. The band of 110 kDa recognized with anti-BmAPN3 antiserum was certainly differ-

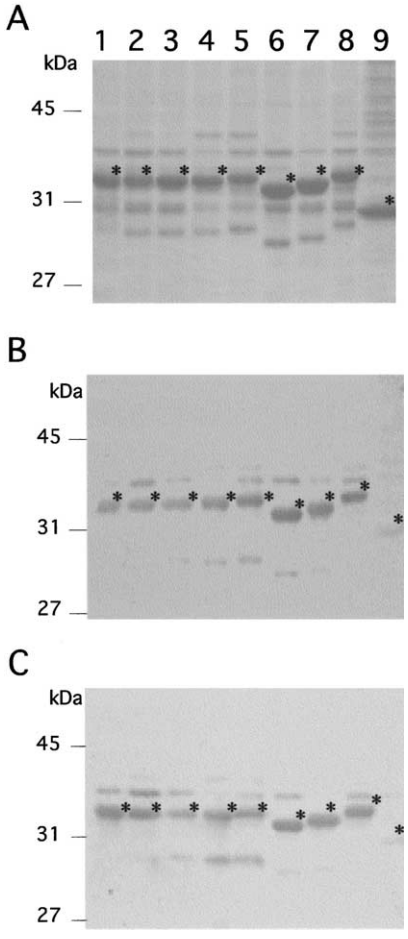

Fig. 4. Ligand blot analyses of Cry1Aa toxin-binding regions of *B. mori* and *P. xylostella* APN isoforms. Recombinant APN fragments corresponding to the Cry1Aa toxin-binding region of BmAPN1 were expressed as GST fusion proteins in *E. coli* cells. The recombinant APN fragments were separated by 12.5% SDS–PAGE and the Cry1Aa and Cry1Ab toxin-binding ability was analyzed using ligand blotting. Coomassie brilliant blue-stained gel (A), ligand blot with 10 nM Cry1Aa toxin (B) and Cry1Ab toxin (C). Lanes 1, GST–BmAPN1 fusion protein; lanes 2, GST–BmAPN2 fusion protein; lanes 3, GST–BmAPN3 fusion protein; lanes 4, GST–BmAPN4 fusion protein; lanes 5, GST–PxAPNA fusion protein; lanes 6, GST–PxAPN1 fusion protein; lanes 7, GST–PxAPN3 fusion protein; lanes 8, GST–PxAPN4 fusion protein; lanes 9, GST alone. The asterisks indicate recombinant proteins. The bands larger and smaller than the recombinant proteins are unidentified reactive proteins derived from the inclusion bodies only from the APN-transformed *E. coli*.

ent from the band of 115 kDa detected by the ligand blot (data not shown).

Using the class-specific antisera for *B. mori* APN isoforms, we conducted an immunoblot analysis of *P. xylostella* BBMV (Fig. 3B). The anti-BmAPN1 and anti-BmAPN3 antisera recognized the 120- and 110-kDa proteins in BBMV (Fig. 3B, lanes 3, 5). The anti-BmAPN2 and anti-BmAPN4 antisera were not bound to any proteins in BBMV (Fig. 3B, lanes 4, 6). Ligand blot analysis showed that both Cry1Aa and Cry1Ab toxins bound to the 110-kDa protein at 10 nM (Fig. 3B, lanes 7, 8).

3.5. Ability of Cry1A toxin to bind to the Cry1Aa toxin-binding region

Recombinant APN fragments corresponding to the BmAPN1 Cry1Aa toxin-binding region [27] from each APN class of *B. mori* and *P. xylostella* were expressed as GST fusion proteins in *E. coli* cells (Fig. 4A). The ability of Cry1A toxin to bind to the APN fragments was subsequently analyzed. Both Cry1Aa and Cry1Ab toxins bound to all four APN classes derived from *B. mori* and *P. xylostella* at 10 nM (Fig. 4B,C). Bands smaller than GST fusion proteins were visible in the ligand blot – these were likely degradation products of GST fusion proteins.

4. Discussion

BmAPN3 and PxAPN3 possess the characteristics of lepidopteran APNs, so they appear to belong to the APN family. Fig. 2 shows that BmAPN3 and PxAPN3 belong to class 3, and it is clear that all four APN classes are present in *B. mori* and *P. xylostella*. The third APN, a 106-kDa Cry1C-binding APN, was reported in *M. sexta* [8]. A 105-kDa APN was reported in the midgut of *L. dispar* [19]. Hence, unidentified APNs may exist in the midguts of lepidopterans. In addition to *B. mori* and *P. xylostella*, all lepidopterans may possess all four APN classes. Moreover, in *B. mori*, the 120- and 230-kDa proteins that were recognized by anti-APN antiserum (Fig. 3A, lane 2) might be unidentified APN isoforms. On the other hand, in *P. xylostella*, two novel cDNA fragments, which were different from the four APN isoforms cloned and had high similarity to lepidopteran class 3 and class 4 APNs, were cloned by RT-PCR using primers S1 and R1 (data not shown). Therefore, two more unidentified APN isoforms should be present in the *P. xylostella* midgut. Although PxAPN4 was tentatively grouped in the same class as BmAPN4, HpAPN2, and Hv110kDaAPN (Fig. 2), the similarity between these isoforms is not high. Thus, the class 4 APNs, which we identified as one group, might be subdivided into two or more classes. In conclusion, lepidopteran APN isoforms are grouped into at least four classes.

Using class-specific antisera, BmAPN1, BmAPN2, BmAPN3, and BmAPN4 were identified as 115-, 90-, 110-, and 100-kDa proteins, respectively (Fig. 3A, lanes 3–6). BmAPN1, which was reported to be a 120-kDa protein [13,21], was a 115-kDa protein in this study, which used 5% SDS–PAGE instead of 10% SDS–PAGE to separate APN isoforms. The molecular size of BmAPN2, identified by immunoblot, was consistent with a previous report [20]. Several partial amino acid sequences of a 100-kDa *B. mori* APN [20] were identical to the deduced amino acid sequence of BmAPN4. On the other hand, in *P. xylostella*, PxAPNA and PxAPN3 were considered to be 120- and 110-kDa proteins, respectively (Fig. 3B, lanes 3–6).

In *B. mori*, both Cry1Aa and Cry1Ab toxins were proposed to bind to BmAPN1 (Fig. 3A, lanes 3, 7, 8). In *P. xylostella*, both toxins bound to a 110-kDa protein the same size as the protein recognized by anti-BmAPN3 antiserum (Fig. 3B, lanes 5, 7, 8). Since PxAPN1 and PxAPN4 were not identified, Cry1Aa and Cry1Ab toxins were not concluded to bind to PxAPN3. However, it is certain that neither toxin binds to PxAPNA, as PxAPNA is a 120-kDa protein. Cry1Aa and Cry1Ab toxins bind to MsAPN1 and Hv170kDaAPN, which belong to class 1 [18,40]. Our results showed that both toxins

Fig. 5. Comparison of the Cry1Aa toxin-binding regions of *B. mori* and *P. xylostella* APNs. The sequences of BmAPN1, BmAPN2, BmAPN3, BmAPN4, PxAPNA, PxAPN1, PxAPN3 and PxAPN4 were compared. Perfectly conserved amino acid residues have black backgrounds. Highly conserved amino acid residues have gray backgrounds.

bind to BmAPN1, but not to PxAPNA, although BmAPN1 and PxAPNA also belong to class 1. Binding of Cry1Ab toxin to class 2 APNs has been reported only in the case of MsAPN2 [23], but binding of Cry1Ab toxin to class 2 BmAPN2 was not observed in this study (Fig. 3A, lanes 4, 7, 8). Therefore, it is suggested that each toxin does not necessarily bind to a specific class of APN isoform.

Cry1Aa toxin binds only to the Cry1Aa-binding region on BmAPN1 (Ile135–Pro198) [27] (Fig. 1). Although Cry1Aa and Cry1Ab toxins bound only to intact BmAPN1 in *B. mori*, both toxins bound in a similar way to all the fragments of the toxin-binding regions of the four classes of APN isoforms, from both *B. mori* and *P. xylostella* (Fig. 4B,C). Since the Cry1Aa toxin-binding regions have many conserved amino acid residues such as RXXFPXXDEP in eight APN isoforms (Fig. 5), Cry1Aa and Cry1Ab toxins may recognize and bind to a common structure in these regions. Most of the conserved amino acids within the *B. mori* and *P. xylostella* APNs are also conserved in other lepidopteran APNs, suggesting that both toxins might bind to this region of APN in insects other than the Bombycidae and Plutellidae.

BmAPN2, BmAPN3, and BmAPN4 had Cry1Aa toxin-binding regions whose Cry1Aa toxin-binding abilities were similar to that of BmAPN1. However, Cry1Aa toxin did not bind to intact BmAPN2, BmAPN3, or BmAPN4 in BBMV. Apparent differences in the Cry1Aa toxin-binding ability between APN isoforms in BBMV could be due to expression levels in conjunction with differences in binding affinity.

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