J. Biochem. **139**, 223–233 (2006)
doi:10.1093/jb/mvj024

A Novel 96-kDa Aminopeptidase Localized on Epithelial Cell Membranes of *Bombyx mori* Midgut, Which Binds to Cry1Ac Toxin of *Bacillus thuringiensis*

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Received August 9, 2005; accepted October 31, 2005

Proteins in the brush border membrane (BBM) of the midgut binding to the insecticidal Cry1Ac toxin from *Bacillus thuringiensis* were investigated to examine the lower sensitivity of *Bombyx mori* to Cry1Ac, and new aminopeptidase N that bound to Cry1Ac was discovered. DEAE chromatography of Triton X-100–soluble BBM proteins from the midgut revealed 96-kDa aminopeptidase that bound to Cry1Ac. The enzyme was purified to homogeneity and estimated to be a 96.4-kDa molecule on a silver-stained SDS-PAGE gel. However, the native protein was eluted as a single peak corresponding to ~190-kDa on gel filtration and gave a single band on native PAGE. The enzyme was determined to be an aminopeptidase N (APN96) from its substrate specificity. Antiserum to class 3 *B. mori* APN (BmAPN3) recognized APN96, but peptide mass fingerprinting revealed that 54% of the amino acids of matched peptides were identical to those of BmAPN3, suggesting that APN96 was a novel isoform of the APN3 family. On ligand blots, APN96 bound to Cry1Ac but not Cry1Aa or Cry1Ab, and the interaction was inhibited by GalNAc. K_D of the APN96–Cry1Ac interaction was determined to be 1.83 ± 0.95 μM. The lectin binding assay suggested that APN96 had an *N*-linked bi-antennal oligosaccharide or an *O*-linked mucin type one. The role of APN96 was discussed in relation to the insensitivity of *B. mori* to Cry1Ac.

Key words: *Bacillus thuringiensis*, *Bombyx mori*, Cry1Ac resistance, insecticidal Cry1A toxin, novel Cry1Ac-binding aminopeptidase N.

Abbreviations: APN, aminopeptidase N; BBMV, brush border membrane vesicle; amino acid-*p*NA, amino acid-*p*-nitroanilide.

Insecticidal Cry toxins of *B. thuringiensis*, when ingested by susceptible insects, become solubilized in the alkaline midgut juice and are subsequently activated by resident proteases (*1*). Activated Cry toxins have been shown to bind to specific receptor proteins on the epithelial cell brush border membrane (BBM) (*2*, *3*). Domain I of Cry toxins consists of 7 α-helices and is thought to form a pore, whereas domains II and III are involved in receptor binding (*4*, *5*). A 120-kDa aminopeptidase N (APN120) [EC 3.4.11.2] has been reported to be a receptor for Cry1Ac in *Manduca sexta* (*6*, *7*), *Lymantria dispar* (*3*, *5*), and *Heliothis virescens* (*8*). APN120 has also been reported to be a receptor for Cry1Aa in *B. mori* (*9*).

On the other hand, cadherin-like proteins (CadLP) of 175- and 210-kDa have been identified as putative receptors for Cry1Aa and Cry1Ab in *B. mori*, hybrid Kinshu × Showa, *M. sexta* (*10–13*), and *Pectinophora gossypiella*, pink bollworm (*14*). Recently, Gómez *et al.* (*15*) determined the specific binding site for Cry1A on 175- and 210-kDa CadLP(s). Disruption of the CadLP gene with transposon conferred Cry1Ac resistance on *H. virescens* (*16*). These results support the theory that CadLP is an authentic Cry toxin-binding receptor.

However, APN is still a very interesting candidate for a binding receptor for Cry1A toxins since it has protease activity with the potential to modify these toxin molecules. Furthermore, it comprises various isoforms, and therefore trimming of the N-termini of various Cry toxins is possible.

We found various isoforms of APN in BBM vesicles (BBMVs) of *B. mori*, which bound to Cry1Aa, Cry1Ab and Cry1Ac with different intensities on ligand blot analysis. These isoforms were such as 110-, 105-, 100-, 93-, and 96-kDa aminopeptidases (Figs 1 and 4, lane 1), and the former 4 APNs have been partially characterized (*17*, *18*). Among them, even though the occurrence was not high in BBMV compared to the other 4 APN, 96-kDa aminopeptidase N (APN96) was of special interesting since it bound to only Cry1Ac, and the binding was inhibited by *N*-acetylgalactosamine (GalNAc). APN96 was recognized by anti-BmAPN3 antibodies, whereas major aminopeptidase APN120 was recognized by anti-BmAPN1 antibodies.

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Here we present various characteristics of APN96 and evidence showing it is a novel APN that can be grouped in the APN3 family. We also briefly discussed the role of this unique APN96 localized in BBM of *B. mori* showing high resistance to Cry1Ac.

MATERIALS AND METHODS

Insects and Bacteria—The silkworm, *B. mori* (hybrid of Shunrei × Shogetsu), was reared on a artificial diet, Silk mate (Nosan Kogyo, Yokohama, Japan), and 4th or 5th instar larvae were used in all experiments.

B. thuringiensis sotto T84A1, a gift from Professor M. Ohba, Kyushu University, Japan, and *B. thuringiensis kurstaki* HD-73 were used to prepare Cry1Aa and Cry1Ac, respectively, as described before (*19*). The bacteria were cultured in 500 ml bottom-baffled flasks containing 100 ml of NYS medium as in Ref. *19*. Cry1Ab was purified from *E. coli* JM109 harboring pYD4.0 containing the active portion of *cry1Ab* donated by Professor K. Kanda, Saga University, Japan (*20*).

Preparation of Cry1A Toxins—*B. thuringiensis* cells (200 ml, two culture flasks) were precipitated by centrifugation and the pellets were washed twice with 1 M NaCl three times with water (each suspension was centrifuged at 10,000 × *g* for 10 min at 4°C). The final precipitate was suspended in 10 ml of water, and the suspension was adjusted to pH 10.5 with 50 mM Na_2CO_3 containing 10 mM dithiothreitol and allowed to stand for 2 h at 37°C. The resulting alkali-soluble fraction was separated from insoluble materials by centrifugation at 15,000 × *g* for 10 min at 4°C. The crystal produced in the *cry1Ab*-transformed *E. coli* was obtained as in (*1*, *2*) and solubilized as above. Solubilized Cry toxins were activated with immobilized trypsin (*21*) and purified by DEAE Sepharose chromatography as described before (*22*).

Preparation of Brush Border Membrane Vesicles (BBMVs) from B. mori—Seven hundred 5th instar *B. mori* larvae were dissected and the prepared midguts were cut longitudinally. Each midgut was immersed in 1 ml of pre-chilled 50 mM Tris-HCl, pH 8.0, containing 5 mM EGTA and 300 mM mannitol (Buffer A). The buffer was then removed and each midgut was resuspended in buffer A containing 1 mM phenylmethyl sulphonyl fluoride and 6.3 μM leupeptin (Buffer B), and then homogenized with a Polytron homogenizer (DIAX-900; Heidolph Instruments, Schwabach, Germany) at 11,000 rpm for 4 min with 2-min intervals at 4°C. The homogenate was centrifuged at 1,000 × *g* for 10 min at 4°C to recover the supernatant. The pellet was homogenized as above and centrifuged. The two supernatants were pooled and used to prepare BBMVs as described before (*23*).

Solubilization of BBMVs—BBMVs from 700 silkworms were solubilized by gentle rotation at 4°C for 1 h in Triton X-100 (1% v/v final concentration in buffer B). About 700 mg of solubilized protein was recovered on ultracentrifugation at 100,000 × *g* for 1 h at 4°C.

DEAE Sepharose, and Sephacryl S-200 and S-300 Column Chromatography—The solubilized protein, 700 mg, was divided into four portions, each of which was applied to a DEAE Sepharose column (2.5 cm ϕ × 20 cm *h*) equilibrated with 20 mM Tris-HCl, pH 8.0, containing 0.05% (v/v) Triton X-100. Elution was performed with a linear gradient of NaCl, from 0 to 0.3 M, in 20 mM Tris-HCl buffer, pH 8.0, containing 0.05% Triton X-100 at the flow rate of 1 ml/min. Fractions (2 ml) were collected and assayed for aminopeptidase activity and the optical absorbance at 280 nm was determined.

The DEAE Sepharose fraction containing the 96-kDa aminopeptidase was further chromatographed on Sephacryl S-200 and S-300 columns (2.5 cm ϕ × 100 cm *h*). The running buffer was 100 mM Tris-HCl, pH 8.0, containing 0.05% Triton X-100 and 100 mM NaCl at the flow rate of 10 ml/h, 2-ml fractions being collected.

Aminopeptidase Activity Assay, and Determination of Effects of Divalent Cations and Chelators on APN96 Activity—Aminopeptidase activity was assayed with 85 μl of 100 mM Tris-HCl, pH 8.0, and 5 μl of 25 mM leucine *p*-nitroanilide (Leu-*p*NA). The 10-min reaction was initiated by the addition of 10 μl of a sample solution and terminated by the addition of 400 μl of 0.5 M glycine/HCl, pH 3.0. The reaction was measured spectrophotometrically at 405 nm, and one unit (U) of activity was defined as the amount of aminopeptidase required to hydrolyze 1.0 μmol Leu-*p*NA per minute.

For the substrate saturation curve experiments, Ala-*p*NA was used at 62 μM–16.25 mM instead of Leu-*p*NA.

To determine the effects of divalent cation and chelators, APN96 was preincubated with different concentrations of various cations, such as Zn^{2+}, Co^{2+}, Mn^{2+}, Cu^{2+}, Mg^{2+} or Ca^{2+}, and a metal chelator, EDTA or *o*-phenanthroline, in 100 mM Tris-HCl, pH 8.5, for 10 min at 30°C prior to initiating the reaction with the substrate, Ala-*p*NA. Activity was expressed as a percentage of that determined in the absence of the effector.

SDS- and Native PAGE—SDS-PAGE was performed according to (*21*, *24*) and the protein bands were visualized by silver or Coomassie Brilliant Blue (CBB) staining. Native PAGE was performed done as described before (*25*).

Ligand and Western Blot Analyses of APN96 with Cry1A Toxins and Anti-BmAPN Antisera—Proteins separated on SDS-PAGE gels were transferred to a PVDF membrane by means of an electric blotter at 2 mA/cm^2 for 2 h. The membrane was blocked overnight with 1% (w/v) skim milk in PBST [8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 130 mM NaCl, 2.7 mM KCl and 0.05% (v/v) Tween 20].

Ligand blotting of the proteins on the membrane was performed with 100 nM Cry1Aa, Cry1Ab or Cry1Ac for 60 min reaction. Cry1Aa and Cry1Ab binding was detected with anti-Cry1Aa antiserum, and Cry1Ac binding was detected with anti-Cry1Ac antiserum. The bound antibodies were recognized by goat anti-rabbit IgG conjugated with peroxidase and visualized with a enhanced chemiluminescence Western blotting detection system (ECL) (Amersham Biosciences Corp., Piscataway, NJ, USA). Bound proteins, toxins or antisera were quantified with an image analyzer (Quantity One; Bio-Rad Lab., Hercules, CA, USA).

For Western blotting, the membranes were incubated with anti-BmAPN antisera raised against 4 classes of BmAPN (*18*) for 1 h, and the bound antibodies were detected as above.

Effects of Sugars on Cry1Ac Binding to APN96—The effects of various sugars on Cry1Ac–APN96 binding were evaluated. A hundred mM each sugar was incubated

with 100 nM Cry1Ac for 30 min and then the mixture was reacted with protein on a PVDF membrane as above.

Lectin Binding Assay—Concanavalin A (ConA), *Lens culinaris* lectin (LCA), *Phaseolus vulgaris* lectin (PHA-E_4), wheat germ hem agglutinin (WGA), peanut hem agglutinin (PNA), and soybean agglutinin (SBA) were purchased from Seikagaku Corporation (Nihonbashi, Tokyo Japan). All lectins purchased were conjugated with peroxidase and detected by means of ECL (Amersham Biosciences Corp.).

K_D *Determination*—A resonant mirror–based biosensing system (=surface plasmon resonance, SPR), IAsys (Affinity Sensors, Cambridge, UK), was used to determine dissociation equilibrium constant K_D. Purified APN96 was placed in a carboxymethyl dextran cuvette according to the method recommended by the manufacturer, and 6.81 ng/mm^2 of APN96 was immobilized. Cry1Aa, Cry1Ab and Cry1Ac were diluted with 50 mM Tris-HCl, pH 8.0, and then reacted for 10 min at various concentrations (250, 500, 750, 1,000, 1,250 and 1,500 nM). The buffer was replaced with 50 mM Tris-HCl, pH 8.0, followed by incubation for 5 min to dissociate the Cry1A–APN96 complex. After each reaction, APN96 was regenerated by rinsing with 50 mM Tris-HCl, pH 8.0, containing 0.5 M NaCl and 0.5% CHAPS, and finally plain 50 mM Tris-HCl buffer. The dissociation rate constant (k_{diss}) and association rate constant (k_{ass}) were calculated, and the K_D value was calculated from k_{diss}/k_{ass} using the computer-aided software FASTfit (Affinity Sensors, UK).

Protein Quantification—BBMV proteins solubilized or chromatographed were quantified either as the absorbance at 280 nm or by the method in (*26*).

Peptide Mapping and Amino Acid Sequencing of APN96—The CBB-stained band of SDS-PAGE separated APN96 was excised, destained and in-gel digested with trypsin (Promega, Madison, WI) as described before (*27*). The tryptic peptides eluted from gel pieces were desalted with ZipTipC18 (Millipore, Bedford, MA, USA). The eluate was analyzed by matrix-associated laser desorption ionization time of flight (MALDI-TOF) mass spectrometry with an AXIMA-CFR plus mass spectrometer (Shimadzu, Kyoto, Japan) in the reflectron mode. Peptide mass-fingerprinting (PMF) was performed for protein identification by measn of a MASCOT search (http://www.matrixscience.com) based on the entire NCBInr protein database. To determine the amino acid sequences of non-matched fragments, one of them was further analyzed by MS/MS using an AXIMA-QIT, MALDI-quadrupole ion trap-TOF mass spectrometer (Shimadzu). α-Cyano-4-hydroxycinnamic acid and 2,5-dihydroxybenzoic acid were used as the matrices for MS and MS/MS analyses, respectively.

Determination of Optimum pH for APN96 Activity—The optimum pH for APN96 activity was determined as described for the aminopeptidase activity assay using Na_2HPO_4/citric acid buffer (pH 3.0–8.0), Tris-HCl (pH 7.5–8.5), boric acid/KCl/Na_2CO_3 (pH 8.0–11.0), and Na_2HPO_4/NaOH (pH 11.0 and 12.0).

RESULTS

Bioassay for Insecticidal Cry1A Toxins Using the Silkworm, Hybrid Shunrei x Shogetsu—The LC_{50} of Cry1Aa, Cry1Ab and Cry1Ac toward 4th instar larvae of *B. mori* were estimated on PROBIT analysis to be 0.23 (95% confidence values: lower, 0.158; upper, 0.333), 8.13 (lower, 5.104; upper, 13.653), and >734 μg/g diet, respectively. Cry1Aa was the most effective toxin and its LC_{50} was more than 3,000 times lower than that of Cry1Ac.

Ligand Blot Analysis of BBMV Proteins—To investigate the different sensitivities of *B. mori* to Cry1A toxins cited above, BBMVs which had been thought to be the site of receptors for Cry1A, were prepared and about 60% of the proteins were solubilized on 1% Triton X-100 treatment (data not shown). The whole BBMVs and Triton X-100–soluble proteins were ligand-blot analyzed with Cry1Aa, Cry1Ab and Cry1Ac toxins (Fig. 1, lanes 1 and 2, respectively). On whole BBMV analysis, two major binding

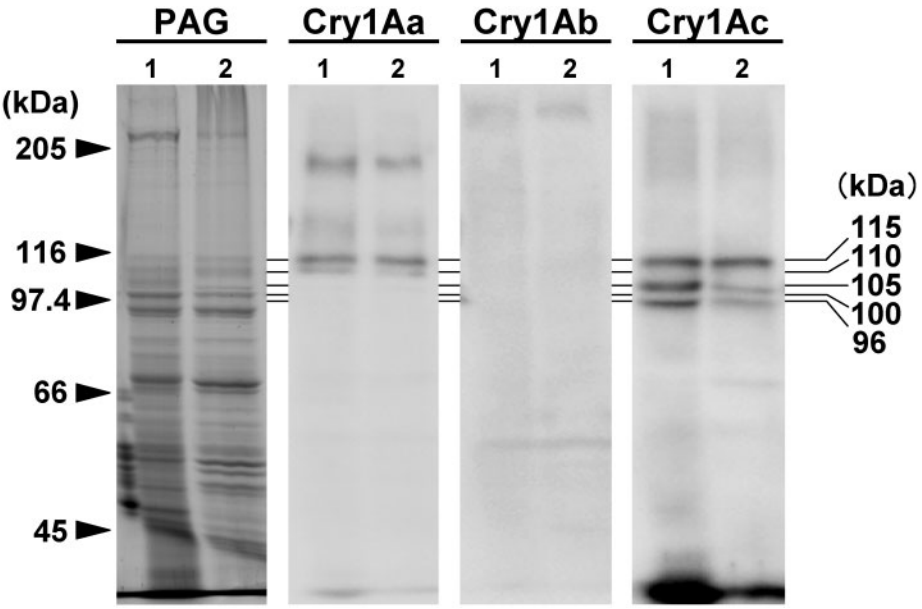

Fig. 1. **Ligand blot analysis of BBMV proteins with Cry1Aa, Cry1Ab and Cry1Ac.** Whole BBMVs or Triton X-100–soluble proteins from BBMVs were subjected to SDS-PAGE and then the proteins were blotted onto a PVDF membrane. Cry1Aa and Cry1Ab bound were detected with anti-Cry1Aa antiserum, and Cry1Ac was detected with anti-Cry1Ac antiserum. Toxin binding antibodies were detected with secondary antibodies by means of ECL. Lane 1: Whole BBMV proteins, lane 2: Triton X-100–soluble BBMV proteins. For the experimental details, see "MATERIALS AND METHODS."

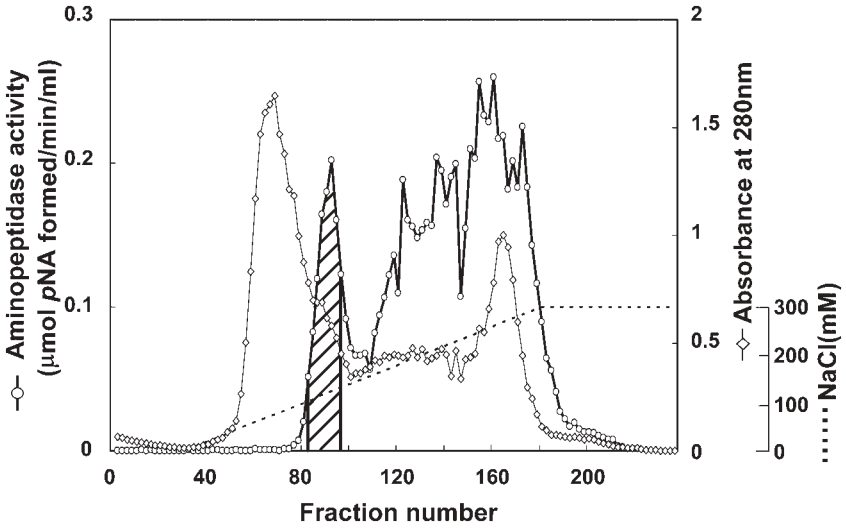

Fig. 2. **DEAE Sepharose column chromatography of Triton X-100–soluble BBMV proteins of *Bombyx mori*.** BBMVs from the midgut of *B. mori*, hybrid Shunrei × Shogetsu, were solubilized with 1% (v/v) Triton X-100, and then the soluble proteins were fractionated on a DEAE Sepharose column. Aminopeptidase activity (open circles) and protein (280 nm absorbance; open diamonds) were monitored. The fractions hatched were pooled and subjected to further purification. For the details, see "MATERIALS AND METHODS."

proteins were observed to be 115- and 110-kDa proteins on a ligand blot with Cry1Aa (Fig. 1, panel Cry1Aa, lane 1), and three clear binding signals were observed at 115-, 105- and 96-kDa on analysis with Cry1Ac (Fig. 1, panel Cry1Ac, lane 1). The binding signal of Cry1Ac with the 96-kDa protein was also detected in the Triton X-100–soluble fraction of BBMVs, even though the signal intensity was weak (Fig. 1, panel Cry1Ac, lane 2), and furthermore the 96-kDa protein seemed to bind only to Cry1Ac.

Purification of the 96-kDa Aminopeptidase by DEAE and Sephacryl Gel Filtration Chromatography—Various aminopeptidases were solubilized from BBMVs with 1% Triton X-100 and then fractionated on a DEAE Sepharose column eluted with a linear NaCl gradient. The eluate was monitored for aminopeptidase activity with Leu-*p*NA and the optical density at 280 nm. About 6–8 peaks or shoulders of aminopeptidase activity could be distinguished (Fig. 2). The peak of aminopeptidase activity that was eluted at ~0.1 M NaCl (Fig. 2, hatched shading) was found to bind to Cry1Ac only (data not shown).

The fractions comprising this peak were pooled, dialyzed, and re-chromatographed on a DEAE column under the same conditions (data not shown). Similar fractions containing amino peptidase activity were further chromatographed on columns of Sephacryl S-200 (once) and S-300 (twice). The final purified sample was chromatographed on a standardized Sephacryl S-300 column, which yielded a single symmetrical peak of amino peptidase activity corresponding to ~190 kDa (Fig. 3, panel A). The yield and specific activity of the aminopeptidase throughout the purification are summarized in Table 1.

SDS-PAGE of this purified protein followed by silver staining revealed a single band (Fig. 3, panel B), the molecular weight of which was estimated to be 96.4 ± 2.4 kDa (N = 6) with an image analyzer. The aminopeptidase migrated as a single band in a native gel and this band corresponded to Leu-*p*NA hydrolyzing activity (Fig. 3, panel C). Together, the purification and PAGE analyses suggested that the 96-kDa protein was a subunit of the ~190-kDa aminopeptidase.

Substrate Specificity of the 96-kDa Aminopeptidase—The substrate specificity of the aminopeptidase was determined using the *p*NA derivatives of various amino acids. Among the substrates tested, Ala-, Leu- and Met-*p*NA were hydrolyzed most efficiently, whereas Gly-, Pro- and Val-*p*NA exhibited negligible hydrolysis (Table 2). These substrate specificities exactly matched those of aminopeptidase N (APN), and thus we designated the 96-kDa peptidase as APN96.

Western Blot Analyses of APN96 with Antisera Raised against Four Groups of B. mori *APNs*—Western blotting of APN96 and Triton X-100–soluble BBMV proteins was performed using various anti-BmAPNs antisera. APN96 was only recognized by the anti-BmAPN3 antiserum (Fig. 4, panel D, lane 4). BBMV proteins that were recognized by anti-BmAPN1, 2 and 4 were only evident in the Triton X-100–soluble fraction (Fig. 4, in each panel, lane 2). Proteins of 110 and 105 kDa were recognized by anti-BmAPN1. Anti-BmAPN4 recognized only 100-kDa proteins. Proteins of 93, 73, 70 and 60 kDa were recognized by anti-BmAPN2. Major proteins detected by anti-BmAPN1, -BmAPN2 and -BmAPN4 were 110-, 93- and 100-kDa ones, respectively. On the other hand, about six different proteins, *i.e.*, 96- and 66-kDa ones in the detergent–soluble fraction (Fig. 4, panel D, lane 2), and 105-, 90-, 75- and 62-kDa ones in the detergent-insoluble fraction (Fig. 4, panel D, lane 3), were recognized by anti-BmAPN3. But in this case, the clear major detected protein was the 105-kDa one for the detergent-insoluble fraction of BBMVs. The sizes of the above-mentioned proteins positive to anti-BmAPN3 resembled those reported for strain Kinshu × Showa of *B. mori*, with the exception of the 96-kDa protein (*18*).

Peptide Mapping and Amino Acid Sequencing of Internal Peptides of APN96—Due to blocking of the N-terminus, APN96 was digested with trypsin and PMF was performed by MALDI-TOF-MS. Forty-one peaks matched those of BmAPN3, and the amino acid sequences of these peak materials for APN96 matched those for BmAPN3 with 54% sequence coverage (Table 3). One of the non-matched peptides (*m/z* 2,878.5) was subjected to further MS/MS analysis to determine amino acid sequence, which was shown to correspond to that of the BmAPN3 peptide stretching from the 140th to the 166th residue, but the 164th arginine of BmAPN3 was replaced with proline in the peptide of APN96 (Table 3).

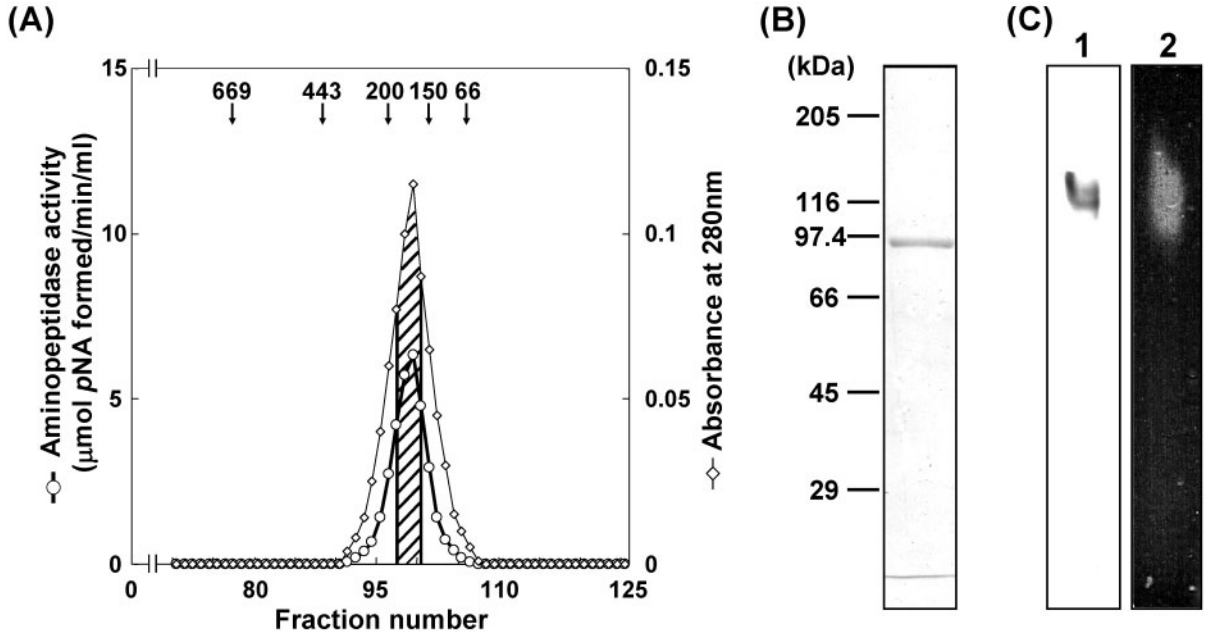

Fig. 3. **Sephacryl S-300 gel filtration chromatography of purified APN96 and PAGE analysis.** Panel A: The fractions indicated by the hatched region in Fig. 1 were pooled, and further fractionated sequentially by DEAE chromatography (a second time), and Sephacryl S-200 and S-300 (twice) column chromatography. A single peak was eluted from the second Sephacryl S-300 column. Aminopeptidase activity (open circles) and protein (280 nm absorbance; open diamonds) were monitored. Numbers/arrows above of the chromatogram indicate molecular size markers: thyroglobulin (669 kDa), apoferritin (443 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), and bovine serum albumin (66 kDa). Panel B: The purified APN96 was analyzed by SDS-PAGE in a 7.5% gel and visualized by silver staining. Panel C: The native PAGE gel was silver stained (lane 1) or submerged in 100 mM Tris-HCl containing 200 μM Leu-*p*NA, pH 8.0, for 30 min (lane 2). A reverse black and white image is shown since the enzyme activity, visualized as a yellow dye, was difficult to show in a monochrome photograph.

Table 1. **Summary of APN96 purification at each step.**

Step	Total activity (mol *p*NA/min)	Total protein (mg)	Specific activity (mol *p*NA/min/mg)	Yield (%)
Triton X-100–soluble BBMV proteins	23,843.4	669.3	35.6	100.0
First DEAE chromatography	545.5	6.5	84.6	2.3
Final sephacryl S-300gel filtration	39.0	0.3	144.5	0.2

Table 2. **Substrate specificity of APN96.**

Substrate	Relative activity (%)
Leu-*p*NA	100
Ala-*p*NA	133
Arg-*p*NA	23.4
Gly-*p*NA	0
Lys-*p*NA	37.6
Met-*p*NA	72.3
Pro-*p*NA	0
Val-*p*NA	3.6

Binding of Cry1A Toxin with APN96 and Effects of Sugars on It—Binding between Cry1A toxins and APN96 was checked by ligand blot analysis and only Cry1Ac was shown to bind to it (Fig. 5, panel A). These ligand blot analyses were performed with 100 nM each Cry1A toxin for 60 min reaction.

The effects of various sugars, such as GalNAc, GlcNAc, Gal, Fuc and Man, on the binding of APN96–Cry1Ac were examined. Only GalNAc was able to completely inhibit the binding (Fig. 5, panel B).

Binding of Various Lectins to APN96—We examined the binding of various lectins to APN96. ConA, LCA, PHA-E_4 WGA, PNA and SBA were used, and as shown in Fig. 5, panel C, ConA, LCA and SBA bound to APN96 but PHA-E_4, WGA and PNA did not. These binding characters were very unique and we discuss a plausible structure in the discussion section.

Characteristics of Binding of APN96 with Cry1A Toxins and the K_D *Value*—As shown above (Fig. 5, panel A), ligand blot analysis showed that APN96 bound to only Cry1Ac toxins, and this observation was confirmed by SPR analysis with an affinity biosensor system, IAsys (Fig. 6). Cry1Aa and Cry1Ab were shown to react with immobilized APN96 only at higher concentrations, such as 1,000, 1250 and 1500 nM, but the bound toxins were washed off with the buffer (Fig. 6, panel A, only the results with 1000 nM are shown). Compared to these two toxins, Cry1Ac was shown to be significantly retained on the membrane after the washing (Fig. 6, panel A). Thus,

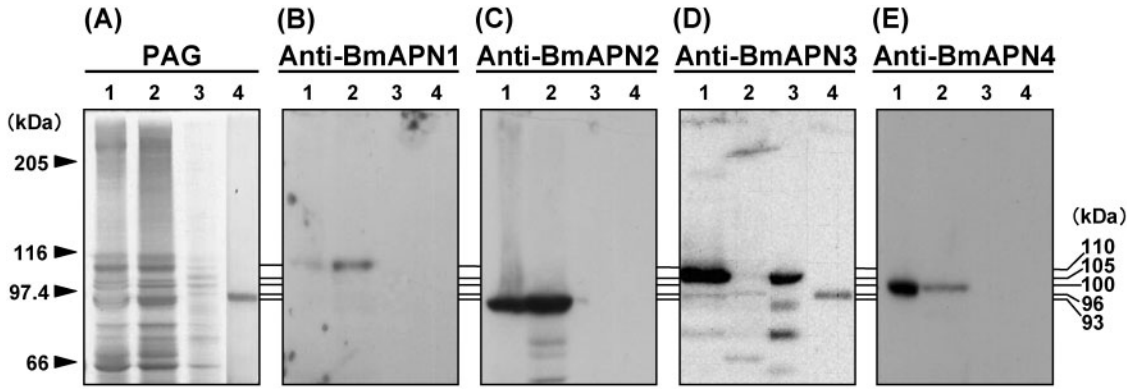

Fig. 4. **Western blotting analyses of purified APN96 with various antisera against BmAPN.** BBMVs (lane 1), and Triton X-100–soluble (lane 2), and –insoluble (lane 3) fractions, and purified APN96 (lane 4) were separated by SDS-PAGE on a 7.5% gel that was subsequently silver stained (panel A). After blotting onto a PVDF membrane, the bands were incubated with antiserum against *B. mori* APN class 1 (BmAPN1) (panel B), anti-BmAPN2 (panel C), anti-BmAPN3 (panel D), or anti-BmAPN4 (panel E). The bound antibodies were detected with goat anti-mouse IgG. The details are given under "MATERIALS AND METHODS."

only in the case of Cry1Ac, apparent rate constants of k_{ass} = 1.57 (±0.19) × 10^4 M^{-1} s^{-1} and k_{diss} = 2.86 (±1.22) × 10^{-2} s^{-1} were calculated with IAsys. Therefore, the calculated K_D value for the APN96–Cry1Ac interaction was 1.83 ± 0.95 μM (Fig. 6, panel B).

Substrate Saturation Curve for APN96 and K_m—The hydrolysis of Ala-*p*NA by APN96 gave an ordinary Michaelis-Menten-type substrate saturation curve up to ~0.7 mM, and K_m was estimated to be 0.13 mM, but interestingly beyond this concentration, the reaction was enhanced. The K_m was 4.88 mM for this higher substrate concentrations, and V_{max} increased from 1.99 μmol *p*NA formed/min/ml with lower substrate concentrations to 11.68 with higher concentrations (Fig. 7). Acceleration with higher substrate concentrations is not rare even for a homogeneous enzyme (*28*). But to elucidate the activation kinetics, more details of the enzyme/substrate complex are necessary, therefore we briefly determined K_m and V_{max} from a double reciprocal plots (Fig. 7, superimposed picture).

pH Optimum and Effects of Divalent Cations on APN96 Activity—The optimum pH range of 8–8.5 for APN96 activity was determined using Ala-*p*NA as the substrate (data not shown).

The effects of various divalent metal cations on APN96 activity were examined. Zn^{2+} inhibited APN96 activity by 80% at 1 mM, but none of the other cations tested were effective at this concentration (Table 4). EDTA had no inhibitory effect, thus the APN96 activity does not require metal ions. Interestingly, *o*-phenanthroline, which is chelator for Fe^{2+}, Ni^{2+}, Ru^{3+}, Ag^{+}, *etc.*, increased the APN96 activity by 50% at 10 mM.

DISCUSSION

The efficacy of Cry1Aa as to *B. mori*, Shunrei × Shogetsu, was 3,000 times greater than that of Cry1Ac, however, these toxins did not show an equivalent difference in receptor binding on ligand blot analysis with either whole BBMVs or detergent-soluble BBMV proteins (Fig. 1). This phenomenon has been observed for other insects as well. Thus, it has been reported that Cry1Ac exhibited the same binding characteristics with Triton X-100–solubilized BBMV proteins from both Cry1Ac-susceptible and -resistant *Plutella xylostella* (*29*, *30*). Therefore, some or all of the binding interactions in resistant insects seem to have no insecticidal consequences.

Triton X-100–solubilized BBMV proteins from *B. mori*, hybrid Shunrei × Shogetsu, have been shown to contain major Cry1A-binding proteins of 115, 110, 105 and 100 kDa (calculation with Quantity One). Each of the proteins reacted positively with anti-APN120 (BmAPN1) antiserum or other anti-BmAPN antisera, and bound to Cry1Aa, Cry1Ab or Cry1Ac (*17*, *18*). However, we found APN96, which bound only to Cry1Ac and was positive as to anti-BmAPN3 (Fig. 1, panel Cry1Ac, and Fig. 5, panel A). Cry1Ac-binding proteins are very important for explain the different sensitivities of *B. mori*. It is possible that some minor receptor proteins contribute to this different sensitivity, and therefore complete biochemical characterization of both major and minor binding proteins, irrespective of their contribution to the eventual toxicity, is essential for understanding of the specificity of Cry toxins.

APN and CadLP are two major candidate receptors for Cry1A. Recent papers support that CadLP is a receptor for Cry1A toxins (*15*, *16*, *31–33*). APN(s), however, is still a very interesting candidate receptor for Cry1A toxins (*5*, *31*, *34*, *35*). APNs will be able to alter toxin conformation *via* proteolysis, thereby facilitating the penetration of toxin α-helices into the host's plasma membrane. It is of interest that midgut juices of insects like *B. mori*, *P. xylostella*, *Adoxophyes* sp., and *Musca domestica* digested Cry1Ac up to residue 27 from its N-terminus. On the other hand, gut juices from the common cutworm, *Spodoptera litura*, which is insensitive to Cry toxins, removed about 65 residues from the N-terminus of Cry1Ac. That Cry1Ac reduced the insecticidal activity toward *P. xylostella* (*36*) and these findings suggest that limited proteolysis at the N-termini of Cry toxins is required for further minor processing that yields an insecticidally competent toxin. Thus, although, we have not succeeded yet in *in vitro* digestion, it is interesting to speculate that, in Cry-susceptible insects, APN activity has replaced that of midgut proteases to finely trim each toxin molecule to a length that

Table 3. **Amino acid sequences of peptides of APN96 matching those of BmAPN3.**

Residue Numbers of BmAPN3	Sequence	Observed *m/z*	M_r	
			Experimental[a]	Calculated[b]
54–61	SIAASTYR	868.43	867.42	867.44
62–81	LPTTTRPLHYNVLWAIDISR	2,366.26	2,365.26	2,365.29
82–96	LTFSGTVEIQLYATR	1,698.95	1,697.94	1,697.90
132–137	ELQFLR	805.41	804.41	804.45
167–174	TDMYGIYR	1,018.46	1,017.46	1,017.46
187–199	WMASTQFQATSAR	1,484.73	1,483.73	1,483.69
200–211	YAFPCYDEPSFK	1,523.65	1,522.64	1,522.64
212–219	ATFDITIR	936.48	935.48	935.51
220–225	RPTTHR	767.60	766.59	766.42
226–233	SWSCTNIK	995.46	994.46	994.45
237–250	VSTVTGYQDDIYNR	1,630.80	1,629.80	1,629.76
310–323	HTAMDFYSIHPNLK	1,673.79	1,672.79	1,672.80
324–346	MTQASIPDFSAGAMENWGLLTYR	2,559.17	2,558.16	2,558.19
347–362	EAYLMYDENHTNGYFK	1,994.84	1,993.83	1,993.85
399–417	YYQYFLTDWVEDYMGLGTR	2,420.08	2,419.07	2,419.08
467–478	MTEHFLGFEVHR	1,502.75	1,501.74	1,501.71
479–488	QGLNNYLIER	1,219.67	1,218.66	1,218.64
489–507	SFDTALPIHLFQTLEVSAR	2,145.15	2,144.14	2,144.13
508–526	AAGALSAYGPDFSFVDYYK	2,041.92	2,040.92	2,040.95
527–553	SWTEQSGHPVLNVQVNHQTGDMTIYQR	3,125.29	3,124.28	3,124.48
554–578	RFNINTGYSNVNTNYIVPITFATAR	2,846.35	2,845.34	2,845.45
555–578	FNINTGYSNVNTNYIVPITFATAR	2,690.29	2,689.29	2,689.35
579–593	NPNFANTKPTHVLTK	1,681.89	1,680.89	1,680.89
594–600	AVTVINR	772.41	771.40	771.46
601–612	GSVGDEWVIFNK	1,350.62	1,349.61	1,349.66
613–619	QQTGFYR	899.41	898.40	898.43
620–635	VNYDDYTWNLIVIALR	1,968.04	1,967.03	1,967.02
620–639	VNYDDYTWNLIVIALRGPQR	2,406.22	2,405.21	2,405.25
640–647	TQIHEYNR	1,060.50	1,059.49	1,059.51
648–659	AQIVNDVFQFAR	1,407.76	1,406.76	1,406.73
660–667	SGLMTYNR	941.42	940.42	940.44
698–713	LVGTAHLTTLNNLIAR	1,707.03	1,706.03	1,705.98
714–734	WSSNLMNQLTYSPIPNESFMR	2,515.13	2,514.12	2,514.17
739–754	YQLAPLLCNINVAACR	1,876.00	1,874.99	1,874.95
755–765	TAATTQFQALR	1,207.66	1,206.65	1,206.64
766–776	VNGQEVPVDNR	1,226.70	1,225.69	1,225.61
777–785	NWVYCNALR	1,195.58	1,194.57	1,194.56
786–798	DGTEADFNFLYQR	1,575.74	1,574.73	1,574.70
799–808	FQSHDVYTEK	1,253.54	1,252.53	1,252.57
864–889	FIQNNLAAVTAAFQSVATPLSYVSSR	2,755.36	2,754.35	2,754.43
892–919	TEAEIVSFQNWATQNQVALGDAYQAVFR	3,156.54	3,155.53	3,155.53
MS/MS analysis of APN96 peptide not matching BmAPN3				
140–166	LNTGTLVFNAASPVIYTLTIDFAA**P**LR	2,878.45	2,877.44	2,877.56

[a]Experimentally observed *m/z* transformed to the relative molecular mass. [b]Relative molecular mass was calculated from the matched peptide sequence.

allows the appropriate conformation required for plasma membrane penetration.

Gel filtration column chromatography and PAGE analyses (Fig. 3) suggested that APN96 is most likely a subunit of the 190-kDa APN. This assertion is supported by the fact that various APNs form dimers or oligomers in mammalian cells (*37*), and thus this putative dimer of an insect APN is not surprising. If APN96 is in fact a dimer, it will be very interesting to determine how the subunits form the Cry1Ac binding site and how they become anchored to the epithelial membrane, since some oligomeric APs do not have a GPI anchor (*37–39*).

Sato and colleagues cloned 8 APNs from *B. mori* (Kinshu × Showa) and *P. xylostella*, and grouped them into 4 categories (*18*). Although they did not characterize an anti-BmAPN3-positive 96-kDa protein from BBMVs, we detected APN96 in a detergent-soluble BBMV fraction. In their case, Cry1Aa was used to detect APNs belonging to the APN3 family, thus it was impossible to detect the occurrence of APN96. Even though the 105-kDa protein

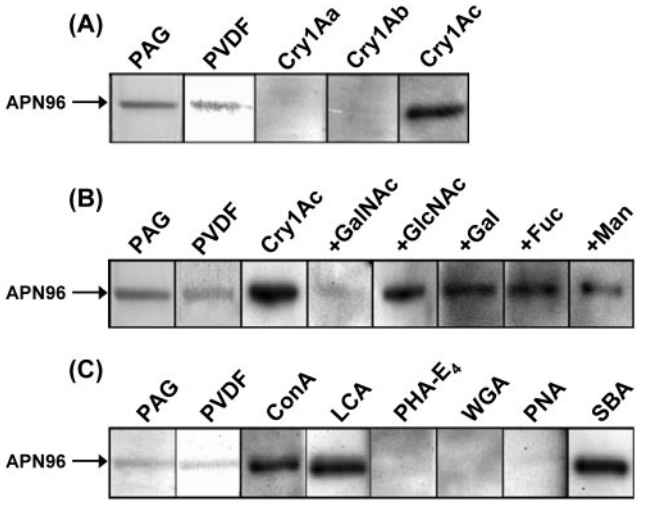

Fig. 5. **Ligand blot analyses of APN96-binding with Cry1A toxin, effects of sugars on the binding and the lectin binding assay.** Panel A: Purified APN96, 1 μg, was subjected to SDS-PAGE and then blotted onto a PVDF membrane. Proteins in the gel (PAG) and the membrane (PVDF) were stained with silver and CBB, respectively. Ligand blot analysis was performed with the Cry1A toxins indicated above the lanes. Bound Cry1Aa and Cry1Ab were detected with anti-Cry1Aa antiserum, whereas bound Cry1Ac was detected with anti-Cry1Ac. The secondary antibody was peroxidase-conjugated goat anti-rabbit IgG, and bands were visualized by means of ECL. Panel B: Purified APN96, 1 μg, was subjected to SDS-PAGE (PAG) and then blotted onto a PVDF membrane (PVDF). The blots were pre-incubated for 30 min with a sugar such as GalNAc, GlcNAc, Gal, Fuc or Man at 100 mM, and subsequently reacted with Cry1Ac and then analyzed as above. Panel C: The purified APN96, 1 μg, was subjected to SDS-PAGE (PAG) and then blotted onto a PVDF membrane (PVDF) and then stained with CBB. Lectin binding was visualized using peroxidase-conjugated lectins such as ConA, LCA, PHA-E_4, WGA, PNA and SBA, and the bound lectins were detected by means of ECL.

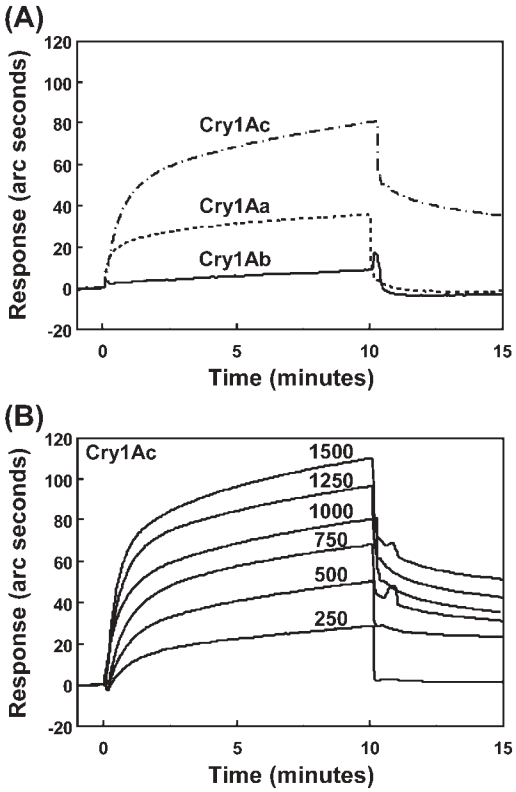

Fig. 6. **Surface plasmon resonance analysis of binding between APN96 and Cry1Aa, Cry1Ab or Cry1Ac.** Panel A: Cry1Aa, Cry1Ab and Cry1Ac, each at 1000 nM, were reacted; Panel B: Various concentrations of Cry1Ac, 1,500, 1,250, 1,000, 750, 500 and 250 nM, were reacted with APN96. See "MATERIALS AND METHODS" for the details.

was major among those sensitive to anti-BmAPN3 antiserum, the amount of APN96 in the APN3 family was significant. APN96 appears to be a novel class 3 APN isoform in *B. mori* hybrid Shunrei × Shogetsu. Indeed, one of the tryptic peptides of APN96, 2,877.56 Da, was shown to contain proline instead of arginine, which has been shown for BmAPN3. These results clearly suggested that, although the amino acid sequences of all non-matched peptides have not been determined yet, APN96 is a novel isoform belonging to class 3 APN.

B. mori hybrid Shunrei × Shogetsu was more than 3,000 times resistant to Cry1Ac compared with Cry1Aa. However, fluorescence microscopic observation revealed that the binding of Cy3-labeled Cry1Ac to BBMV was strong and comparable to that of Cy3-labeled Cry1Aa (*40*). Therefore, it is clear that the avidity of Cry1Ac-binding to BBMVs does not correlate with the insecticidal activity in *B. mori*. Such "non-toxic binding" may be one possible mechanism conferring Cry1Ac resistance. Although we cannot at present draw a firm conclusion regarding the physiological significance of Cry1Ac binding to APN96, we postulate that the binding may serve as a decoy to quench the Cry1Ac toxicity. As is evident from the hypothesis above, the K_D value of APN96 is very important. We estimated the K_D for the APN96–Cry1Ac interaction was 1.83 ± 0.95 μM on SPR analysis. In this analysis we

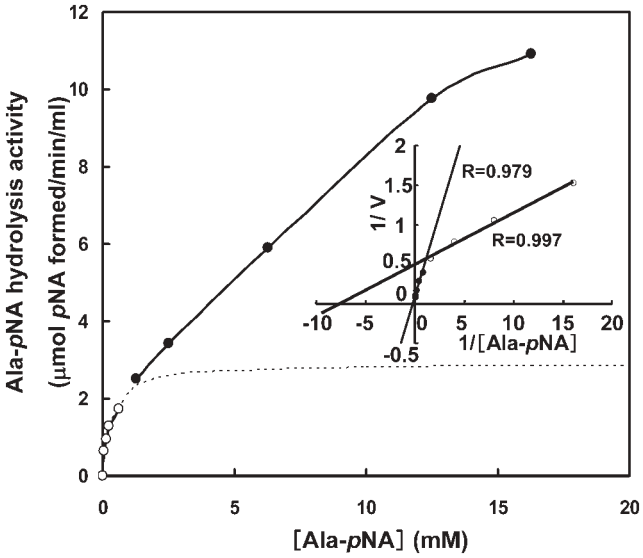

Fig. 7. **Substrate saturation curve for APN96.** APN activity was assayed as described under "MATERIALS AND METHODS" except that Ala-*p*NA was used instead of Leu-*p*NA at 62 μM-16.25 mM. The superimposed graph is a double reciprocal plot of the saturation curve. The reaction was measured spectrophotometrically at 405 nm, and one unit (U) of activity was defined as the amount of APN required to hydrolyze 1.0 μmol Ala-*p*NA per minute.

Table 4. **Effects of divalent metal ions on APN activity.**

Metal or Chelator	Conc. (mM)	Relative activity (%)
None		100
EDTA	1	94 ± 0.003
	100	92 ± 0.005
	100 (12 h)	97 ± 0.004
o-Phenanthroline	0.1	103 ± 0.003
	10	157 ± 0.012
Zn^{2+}	0.05	82 ± 1.7
	0.1	71 ± 2.4
	0.5	32 ± 0.4
	1	19 ± 0.0
Co^{2+}	0.5	104 ± 2.5
	1	100 ± 3.3
Mn^{2+}	0.5	95 ± 1.6
	1	91 ± 2.1
Cu^{2+}	0.5	97 ± 2.4
	1	94 ± 1.3
Mg^{2+}	0.5	99 ± 1.5
	1	101 ± 1.1
Ca^{2+}	0.5	99 ± 1.5
	1	98 ± 0.3

confirmed APN96 bound only to Cry1Ac, but the K_D value was about 100 times less compared to that of other plausible candidates, APN120 (*9*, *31*) and 175-kDa CadLP (*31*), and 252-kDa BBMV protein, which was discovered in our laboratory as a new candidate receptor for Cry1A (*22*). Therefore from the criterion of the K_D value, APN96 may not play a role as a decoy. But as mentioned above, the majority of the Cry1Ac binding to BBM is non-insecticidal, therefore, some of these APNs grouped into 4 families must play roles as decoys to quench the Cry1Ac toxicity.

The binding between Cry1Ac and APN120 detected in *M. sexta* (*6*, *7*) and *L. dispar* (*3*, *5*) was inhibited by GalNAc, but not by the other monosaccharides tested. The binding of Cry1Ac to APN96 was also inhibited by GalNAc. These findings suggest that APN96 and APN120 share similar sugar side chains or subsites that mediate binding to Cry1Ac, and that furthermore APN96 has an oligosaccharyl side chain containing GalNAc. Thus, a full understanding of the APN96–Cry1Ac interaction will require elucidation of the oligosaccharyl structure on APN96. A lectin binding assay will provide a useful view of the oligosaccharide structure with easy and simple methods, even though the results have not been fully described. APN96 was recognized by ConA, suggesting that the oligosaccharide moiety has a bi-antennal high-mannose-, hybrid- or complex-type structure containing -Manα1-2Man1-. APN96 was also recognized by LCA, which interacted strongly with oligosaccharides containing a fucosylated GlcNAc located at the reducing end of the N-linked side chain. On the other hand, WGA and PHA-E_4 did not bind to APN96. All these results suggested that APN96 contained a complex-type fucosylated N-linked bi-antennal oligosaccharide chain. SBA binding with APN96 strongly suggested the occurrence of an O-linked mucin type oligosaccharide containing GalNAcα1-3Gal. But PNA, which recognized the Galβ1-3GalNAc mucin type structure, did not bind to APN96. Taken together, these data suggest that APN96 contains not only an *N*-linked bi-antennal fucosylated oligosaccharide, but also an *O*-linked sugar side chain such as GalNAcα1-3Gal-Ser/Thr. *N*-linked oligossacharides containing GalNAc at the non-reducing terminus with an α-linkage were identified in the mosquito, *Anopheles stephensi* (*41*). Also this structure was shown to be recognized by SBA (*42–44*). If this is true, there is a possibility that APN96 has *N*-linked oligosaccharides with a GalNAc residue at the non-reducing end instead an O-linked mucin type side chain. The involvement of APN-linked sugar side chains in the recognition of Cry1A is a complex issue, particularly since the binding of Cry1Ac to 110-kDa APN from *H. virescens* was not inhibited by GalNAc (*45*). Further detailed studies of the effect of oligomerization of APN molecules, the precise function of each monomer, the exact role of sugar side chains, and the relationship between enzymatic activity and Cry1A-mediated conformation changes will help delineate the mechanism(s) underlying the specificity and toxicity of Cry1A toxins for *B. mori*.

This work was supported, in part, by research grants from the Ministry of Education, Culture, Sports, Science and Technology, 13306006 and 12558069. A grant for Promotion of the Niigata University Research Project (2004) also partially supported this work.

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