Recombinantly expressed isoenzymic aminopeptidases from *Helicoverpa armigera* (American cotton bollworm) midgut display differential interaction with closely related *Bacillus thuringiensis* insecticidal proteins

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Several investigators have independently identified membrane-associated aminopeptidases in the midgut of insect larvae as the initial interacting ligand to the insecticidal crystal proteins of *Bacillus thuringiensis*. Though several isoenzymes of aminopeptidases have been identified from the midgut of an insect and their corresponding cDNA cloned, only one of the isoform has been expressed heterologously and studied for its binding to Cry toxins. Here we report the cloning and expression of two aminopeptidases N from *Helicoverpa armigera* (American cotton bollworm) (HaAPNs). The full-length cDNA of *H. armigera* APN1 (*haapn1*) is 3205 bp in size and encodes a 1000-amino-acid protein, while *H. armigera* APN2 (*haapn2*) is 3116 bp in size and corresponds to a 1012-amino-acid protein. Structurally these

proteins show sequence similarity to other insect aminopeptidases and possess characteristic aminopeptidase motifs. Both the genes have been expressed in *Trichoplusia ni* (cabbage looper) cells using a baculovirus expression vector. The expressed aminopeptidases are membrane-associated, catalytically active and glycosylated. Ligand-blot analysis of both these aminopeptidases with bioactive Cry1Aa, Cry1Ab and Cry1Ac proteins displayed differential interaction. All the three toxins bound to HaAPN1, whereas only Cry1Ac interacted with HaAPN2. This is the first report demonstrating differential Cry-toxin-binding abilities of two different aminopeptidases from a susceptible insect.

Key words: Cry toxin, insect, receptor.

INTRODUCTION

The insecticidal crystal proteins (ICP) produced by the soil bacterium *Bacillus thuringiensis* (Bt) are effective in controlling a wide range of insect pests. The isolates of this bacterium produce different toxins, each of which are active only against a select group of insects. This specificity and potential utility in pest management has resulted in cloning of toxin encoding genes and their expression in bacteria and plants [1,2]. The American cotton bollworm (*Helicoverpa armigera*) is the principal cotton pest in Asia, including major areas in India, China, Pakistan, South and East Asia and parts of Australia [3]. With the indiscriminate use of chemical insecticides, *H. armigera* has developed manifold resistance and cross-resistance to various groups of chemicals, including organophosphates, carbamates and, now, synthetic pyrethroids [4,5]. Against this background, Bt and its formulations are being encouraged to control this pest and transgenic crops expressing Cry proteins are now grown in various parts of the world. Concerns have repeatedly been raised about the continued utility of these toxins in insect-pest-management programs specifically in relation to development of resistance by insects to these insecticidal proteins [6].

Following ingestion by a susceptible larva, ICPs are solubilized and activated in the midgut. Binding of the activated toxin to proteins present in the membrane of midgut epithelial cells is absolutely essential for toxicity. Studies on the mode of action of Bt insecticidal proteins have revealed the interacting ligands

primarily as aminopeptidase N (APN) and cadherin, which are located at the brush-border membranes in the midgut of susceptible larvae [7,8]. Molecular analysis of insecticidal protein–APN interaction have led to the cloning of the genes encoding for these proteins from different insects. The aminopeptidase cDNA has been cloned from *Manduca sexta* (tobacco hornworm), *H. virescens* (tobacco budworm), *H. punctigera* (Australian budworm), *Plutella xylostella* (diamondback moth), *Bombyx mori* (silkworm), *Plodia interpunctella* (Indian meal moth), *Lymantria dispar* (gypsy moth) and *Epiphyas postvittana* (light brown apple moth) [9–18].

Independent ligand-binding experiments on purified brush-border membrane-vesicle (BBMV) proteins of *H. virescens*, from different laboratories, have revealed that there is heterogeneity in Cry1Ac-interacting aminopeptidases [19,20]. The 170 kDa aminopeptidase (receptor A) purified from BBMV of *H. virescens* showed a high affinity towards Cry1Ac toxin by surface-plasmon-resonance (SPR) analysis. However, other investigators have independently identified aminopeptidases of 120 and 110 kDa from *H. virescens* that interact with Cry1A toxins [11,21]. Implications of existence of a single receptor in the development of resistance to insecticidal proteins are obvious.

Though molecular cloning of the cDNA for Cry toxin interacting aminopeptidases from different insects have been widely reported, only a very few reports describe the expression of these proteins in heterologous systems. Midgut-associated putative ICP-interacting aminopeptidases have been expressed in insect

Abbreviations used: AP, alkaline phosphatase; APN, aminopeptidase N; BBMV, brush-border membrane vesicle; BCIP, 5-bromo-4-chloroindol-3-yl phosphate toluidine; Bt, *Bacillus thuringiensis*; Cry, crystalline inclusion protein; GPI, glycosyl-phosphatidylinositol; HaAPN, *Helicoverpa armigera* APN; ICP, insecticidal crystal protein; NBT, Nitroblue Tetrazolium; ORF, open reading frame; RACE, rapid amplification of cDNA ends; SPR, surface plasmon resonance.

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cell lines from *P. xylostella* [16] and *M. sexta* [22]; however, a direct interaction between ligands could not be demonstrated, since they failed to show binding of expressed APN to Cry toxins. Recently, Simpson and Newcomb [23] expressed *Epiphyas* APN on Sf9 [*Spodoptera frugiperda* (fall armyworm)] cells and demonstrated its interaction with insecticidal proteins Cry1Ac and Cry1Ba by ligand blotting.

We have been intrigued by the reported presence of different Cry-protein-interacting aminopeptidases from different laboratories from the polyphagous pest *H. virescens* and have sought to examine the presence of aminopeptidase from homologous populations of *H. armigera*. We have cloned two different aminopeptidase-encoding genes from the midgut of the insect *H. armigera*. Both the genes have been expressed in *T. ni* cells using a baculovirus expression vector, and we go further to show that these proteins interact differentially with a set of insecticidal proteins.

EXPERIMENTAL

Cloning of APN from *H. armigera*

H. armigera larvae were reared on an artificial diet under a photoperiod of 14 h: 10 h (light/dark), at 70 % relative humidity and 27 °C. Midguts from 100 fifth-instar second-day larvae were dissected in diethyl pyrocarbonate-treated water, snap-frozen into liquid nitrogen and stored at -70 °C.

Total RNA was extracted from the midgut tissue using Trizol reagent (Life Technologies) according to manufacturer's protocol. Reverse transcription with oligo(dT) primer was done using 5 µg of total RNA as template and Superscript II (Life Technologies) and the cDNA was generated. Degenerate oligonucleotides were synthesized from the conserved regions of previously published insect aminopeptidases. They were APN1 (forward primer) coding for AFPCYDEP amino acids and APN2 (reverse primer) coding for MENWGLL amino acids. An approx. 450-bp fragment, obtained by PCR using these primers and cDNA as template, was cloned into pGEM-T easy vector. Sequencing of 20 different clones (T7 sequenase kit; Amersham Pharmacia Biotech) by Sanger's dideoxy-chain-termination method resulted in the identification of two clones that had different sequences but showed similarity to insect aminopeptidases. Sequence-similarity searches were carried out using the program BLAST (<http://www.ncbi.nlm.nih.gov/BLAST/>).

On the basis of the sequence of both the clones, gene-specific primers were designed in the reverse direction for 5' RACE (rapid amplification of cDNA ends) and in the forward direction for 3' RACE. The 3' and 5' ends of both the genes were amplified following the protocol described in the RACE kit manuals (Life Technologies). The PCR-amplified fragments were cloned into pGEM-T easy vector and sequenced.

On the basis of the sequence of 5' and 3' RACE amplified products of both the clones, gene specific end primers were synthesized. Using the respective set of end primers complete 3.2 kb *haapn1* and 3.1 kb *haapn2* were amplified by performing 30 cycles of PCR, each of 94 °C for 30 s, 54 °C for 30 s and 72 °C for 3 min. The PCR products were cloned into pGEM-T easy vector and sequenced by the 'primer-walking' method. Ten clones each for both the genes *haapn1* and *haapn2* were sequenced, which yielded identical sequence, thus confirming the fidelity of PCR reactions.

Sequence alignment and analysis

The complete cDNA sequence was analysed for multiple alignment with other reported insect APNs using CLUSTAL W

alignment and dendrogram analysis was performed by UPGMA method using the Mac Vector software suite (version 7.0) (Oxford Molecular Group, Oxford, U.K.). The sequence was also analysed for the presence of N-terminal signal sequence based on von Heijne scores using the program PSORT II (<http://psort.nibb.ac.jp>). Glycosyl-phosphatidylinositol (GPI) anchor prediction was carried using GPI prediction program DGPI (http://129.194.186.123/GPI-anchor/index_en.html). The sequence was also analysed for the presence of mucin-type galactose *N*-acetyl-*O*-glycosylation, using the program NetOGlyc 2.0 (<http://www.cbs.dtu.dk/services/netOGlyc>).

Expression of *H. armigera* APN1 in *Escherichia coli* and production of polyclonal antibodies

The 450 bp fragment of *haapn1*, obtained using degenerate primers and cloned in pGEM-T easy vector, was excised with *SphI* and *PstI* restriction enzymes and subcloned into *SphI*/*PstI*-digested *E. coli* expression vector pQE 32 (Qiagen). The pQE 32 carrying the 400 bp fragment was transformed into *E. coli* M15 strain. The expression of the truncated APN was induced by adding 1 mM isopropyl β-D-thiogalactoside. The 15 kDa protein was produced in the inclusion bodies. The inclusion bodies were solubilized in SDS loading buffer containing β-mercaptoethanol and resolved by SDS/10 %-(w/v)-PAGE. The gel was stained, destained, and the 15 kDa protein was excised from the gel and macerated using a pestle and mortar. An emulsion of the truncated APN (0.2 mg) was made in Freund's complete adjuvant (Sigma), which was injected, into a New Zealand White rabbit, followed by two boosters of the same protein. The rabbit serum was collected 10 days after the second boost and its reactivity was assessed by Western blotting.

Cloning of *H. armigera* apn genes in a baculovirus expression vector

Expression in *T. ni* cells was done by cloning the cDNA of both the genes into baculovirus expression vector pBlue Bac V5 HIS (Invitrogen Corporation). The PCR product comprising the open reading frame of the cDNA (including the four bases of Kozac sequence from the 5' untranslated region) was cloned into pBlue Bac V5 HIS that was adapted for TOPO® (Invitrogen Life Technologies) cloning. Care was taken while designing PCR primers for the gene to be cloned in-frame with the C-terminal His₆ and V5 epitope. Co-transfection and generation of the recombinant virus was carried out according to the manufacturer's protocol using the Bac-N-Blue transfection kit (Invitrogen Corporation). Plaque assay of both recombinant viruses using 5-bromo-4-chloroindol-3-yl β-D-galactopyranoside ('X-Gal') resulted in the production of blue plaques. Ten plaques were selected for each recombinant virus, amplified, and the presence of the gene was confirmed by PCR. One positive plaque for each of the recombinant viruses baculovirus-*H. armigera* APN1 (HaAPN1) and baculovirus-HaAPN2 was amplified to 1 × 10⁸ plaque-forming units/ml and used as a virus stock for protein expression.

Expression of recombinant HaAPN in *T. ni* cells

T. ni (High-five; Invitrogen Corp.) cells were grown and maintained at 27 °C in Excell-405 with L-glutamine and augmented with NaHCO₃ (JRH Biosciences, Lenexa, KS, U.S.A.). Cells were grown as a monolayer up to 70–80 % confluence in T-75 (Nunc) tissue-culture flasks. For protein-expression studies, *T. ni* cells were infected at a multiplicity of infection of 10 using the

Figure 1 Amino acid sequence of *H. armigera* APN1 and APN2

Deduced amino acid sequence of the ORF of clones *haapn1* (A) and *haapn2* (B). The membrane targeting signal sequence is denoted by broken underline (---) and the C-terminal hydrophobic region denoted by the long underline (—) starting from the GPI addition site. Predicted O-glycosylation sites are dot-underlined (.*.*) and the mucin like serine/threonine region indicated by the region with the black arrowheads at either end. The canonical metal (zinc)-binding site is boxed and the *GAMEN* motif characterizing gluzincin aminopeptidase is italicized. The four conserved cysteine residues in eukaryotic APN are shown in **bold** (positions 732, 739, 766 and 801 in HaAPN1, and positions 750, 757, 785 and 821 in HaAPN2).

virus stock. The infection was carried out for 56 h, after which the cells were harvested and the pellet washed with $1 \times$ PBS. The cells were resuspended in lysis buffer [100 mM potassium phosphate buffer (pH 7.4)/150 mM NaCl/1 mM dithiothreitol/5% (v/v) glycerol/1 mM $MgCl_2$], containing 10 mM aprotinin, 5 mM leupeptin and 1 mM PMSF, and sonicated (40 W power output) for four 5 s bursts, with intermittent cooling on ice. The resultant extract was ultracentrifuged at 100 000 g at 4 °C for 1 h and the pellet was washed once with buffer and re-centrifuged as above. Finally the pelleted cell membranes were resuspended in fresh lysis buffer, snap-frozen in liquid nitrogen and stored at -70 °C.

The membrane preparations (10 μ g) were resuspended in SDS loading buffer containing β -mercaptoethanol, boiled for 5 min, centrifuged at 10 000 g for 5 min and separated by SDS/7.5%-PAGE gel. The separated proteins were transferred on to a nitrocellulose membrane by electroblotting. Following transfer, the membrane was incubated for 60 min in blocking buffer (3% BSA in $1 \times$ PBS) and washed three times with $1 \times$ PBS. The membranes were probed with 1:2500 dilutions of anti-APN antibodies for 60 min and washed three times with $1 \times$ PBS. AP-conjugated goat anti-rabbit secondary antibodies (Calbiochem) 1:5000 in 1% BSA was overlaid on the membrane for 60 min. After three washings with $1 \times$ PBS, the blot was developed with Nitroblue Tetrazolium/5-bromo-4-chloroindol-3-yl phosphate toluidine (NBT/BCIP) substrates (Life Technologies) in alkaline phosphatase (AP) buffer.

Glycosylation status of the expressed APN

A 10 μ g sample of the membrane preparation of the *T. ni* cells expressing *H. armigera* APN1 and 2 along with wild-type-virus-

infected cells were separated by SDS/7.5%-PAGE and electroblotted to nitrocellulose membranes. After blocking for 1 h in 3% BSA, the blots were overlaid with 50 mg of biotinylated concanavalin A (Sigma) for 1 h. The blot was washed three times with PBS and incubated with streptavidin-conjugated AP (1:5000 dilution) for 1 h. The blot was developed with NBT/BCIP after washing three times with PBS containing 0.1% Tween-20.

Toxin-overlay assay

Membrane preparations (10 μ g) of *T. ni* cells expressing APN1 and APN2 of *H. armigera* along with wild-type-virus [*Autographa californica* (alfalfa looper) multicapsid nucleopolyhedrovirus ('AcMNPV')]-infected *T. ni* cell membranes were overlaid with activated Cry1Aa, Cry1Ab and Cry1Ac proteins. The inclusion bodies consisting of the Cry proteins were solubilized in 50 mM sodium carbonate buffer, pH 10.4, and activated with trypsin (Sigma) at 10:1 ratio for 30 min at 37 °C. Further, the activated toxin was purified by anion-exchange chromatography and eluted with 400 mM NaCl in 50 mM sodium carbonate buffer, pH 10.4. The activated toxins were biotinylated using the enhanced chemiluminescence ('ECL[®]') protein biotinylation module (Amersham Pharmacia Biotech.) according to the manufacturer's protocol. The activated and biotinylated toxin was observed as a near-homogeneous 65 kDa protein on SDS/10%-PAGE.

T. ni membranes resolved on SDS/7.5%-PAGE gels were transferred on to nitrocellulose membrane as described above. After blocking with 3% BSA in $1 \times$ PBS the membrane was incubated with activated and biotinylated toxin (50 ng/ml) for 60 min. The blot was washed three times with PBS and incubated with streptavidin-conjugated AP (1:5000 dilution) for 1 h. The

Figure 2 Comparison of HaAPNs with published insect APN sequences

Bootstrap (1000 replicates) dendrogram tree using the UPGMA ('Unweighted Pair Group Method with Arithmetic Mean') method was constructed by analysing the CLUSTAL W alignment of the reported APN sequences. GenBank® accession numbers of the APN are: *Plutella xylostella* APN1 (Px 1), X97878; *P. xylostella* APNA (Px A), AF020389; *P. xylostella* APN 3 (Px 3), AJ222699; *L. dispar* APN1 (Ld 1), AF126442; *L. dispar* APN2 (Ld 2), AF126443; *M. sexta* APN1 (Ms 1), X89081; *M. sexta* APN2 (Ms 2), X97877; *B. mori* APN1 (Bm 1), AF084257; *B. mori* APN2 (Bm 2), AB011497; *H. virescens* 170 kDa APN (Hv 170), AF173552; *H. virescens* 120 kDa APN (Hv 120), U35096; *H. punctigera* APN1 (Hp 1), AF217248; *H. punctigera* APN2 (Hp 2), AF217249; *H. punctigera* APN3 (Hp 3), AF217250; *P. interpunctella* APN1 (Pi 1), AF034483; *H. armigera* APN1 (Ha 1), AF521659; *H. armigera* APN2 (Ha 2), AF521660; *S. litura* APN1 (SI 1), AF320764; *E. postvittana* APN1 (Ep 1), AF276241. The percentage similarity shown by HaAPN1 and HaAPN2 to the reported APNs are indicated against each in parentheses. Branch lengths are arbitrary. The bootstrap values detected above 50% for 1000 replicates are given before the branch. Where no values are given, aminopeptidase gene sequences are not significantly different from each other.

blot was developed with NBT/BCIP after washing three times with PBS containing 0.1% Tween-20.

Aminopeptidase assay

The activity of aminopeptidases expressed in *T. ni* cells was assayed by following the protocol described in Denolf et al. [16] with some modifications. Recombinant-HaAPN-expressing *T. ni* cells (1×10^6) were resuspended in 500 μ l of $1 \times$ PBS and mixed with 500 μ l of substrate solution (5 mM alanine *p*-nitroanilide, leucine *p*-nitroanilide or valine *p*-nitroanilide prepared in 67 mM $\text{Na}_2\text{HPO}_4/67$ mM KH_2PO_4). The reaction was allowed to proceed at 37 °C for 20 min. The amount of product (*p*-nitroaniline) released was measured spectrophotometrically at 405 nm and

quantified by using $0.0099 \text{ M} \cdot \text{cm}^{-1}$ as the absorption coefficient of *p*-nitroaniline.

RESULTS

Cloning, sequencing and characterization of HaAPN

Using the single-stranded cDNA as template, a 450 bp fragment was amplified with degenerate primers APN1 and 2, and cloned into pGEM-T easy. Sequencing of the positive clones led to the identification of two different clones coding for *haapn1* and *haapn2*. The 3' RACE of both the genes yielded a 2.0 kb PCR product and the 5' RACE yielded a 700 bp PCR fragment. All

Figure 3 Expression of HaAPN1 and HaAPN2 in *T. ni* cells over various time intervals

Total cell extract prepared from cells harvested at different time intervals post-infection was resolved by SDS/7.5%-PAGE gels. Lane 1, uninfected cell; lanes 2, 3 and 4, HaAPN1 at 24, 48 and 72 h; lanes 5, 6 and 7, HaAPN2 at 24, 48 and 72 h following infection. The relative positions of high-molecular-mass prestained marker (Bio-Rad) are indicated on left. The gel was electroblotted to nitrocellulose membranes and probed with anti-APN antibodies followed by AP-conjugated goat anti-rabbit antibodies. The blots were developed with NBT/BCIP for visualizing the bands.

the PCR fragments were cloned in pGEM-T easy and sequenced. Analysis of 5' and 3' RACE clones led to the identification of start and stop codon of the respective open reading frames (ORFs). Gene-specific primers corresponding to 5' start codon and 3' stop codon of both the genes were used to amplify the full-length cDNAs.

The 3205 bp cDNA of *haapn1* (GenBank® accession no. AF521659) contained an ORF extending from 29 to 3031 bp. It encodes a 1000-amino-acid protein having a predicted molecular mass of 111 kDa. The 3116 bp *haapn2* cDNA (GenBank® accession No AF521660) has an ORF extending from 36 to 3074 nucleotides, which encoded a 1012-amino-acid-residue protein with a predicted molecular mass of 113 kDa (Figure 1).

Further computational analysis of these proteins for the presence of signature sequences revealed the presence of cleavable signal peptides at the N-terminus (amino acids 1–20 in HaAPN1 and 1–18 in HaAPN2). In HaAPN1, Gly⁹⁷⁹ and Asp⁹⁹⁰ in HaAPN2 were predicted to be the residues for GPI anchorage. The C-termini of the two aminopeptidases are significantly different from one another. HaAPN1 has a stretch of 46 threonine residues, which is absent from HaAPN2. The polythreonine stretch is characteristic of mucins, which form possible O-glycosylation sites (Figure 1).

The multiple sequence alignment of HaAPN1 with other published insect aminopeptidases using Clustal W alignment program showed 86 % similarity to *H. virescens* 170 kDa protein (GenBank® accession number AF173552) and 88 % similarity *H. punctigera* APN1 (AF217248). Similarly, analysis of APN2 protein revealed that it is 86 % similar to *H. virescens* 120 kDa protein (U35096) and 90 % similar to *H. punctigera* APN3 (AF217250). HaAPN1 and HaAPN2 showed 53 % similarity between them. Dendrogram construction clustered the different published insect APNs into four major groups (Figure 2), with HaAPN1 falling in group 2 and HaAPN2 in group 3.

Expression of HaAPNs

Both the APNs from *H. armigera* were expressed in *T. ni* cells using baculovirus expression vector. The amplified stock of both the recombinant baculovirus HaAPN1 and HaAPN2 was used to infect *T. ni* cells for checking the expression of the proteins. Analysis of the infected cell lysate (Figure 3) showed the

Figure 4 Immunoblot analysis of HaAPN1 (lane 1) and HaAPN2 (lane 2)

A 10 µg portion of recombinant *T. ni* cell membrane protein (lane 1, wild-type-virus infected; lane 2, HaAPN1-infected; and lane 3, HaAPN2-infected) was separated by SDS/7.5%-PAGE and electroblotted on to nitrocellulose membrane. (A) The membranes were overlaid with anti-His antibodies followed by AP-conjugated anti-mouse antibodies. The blots were developed with NBT/BCIP for visualizing the bands. (B) The membranes were overlaid with 50 µg of biotinylated concanavalin A followed by 1:5000 dilution of AP-conjugated streptavidin and developed with NBT/BCIP. Relative position of prestained (high-molecular-mass) markers (Bio-Rad) are indicated in left.

expression of a 120 kDa protein which was absent in uninfected cells. A time-course study on the expression of both the proteins revealed that maximum expression occurred between 48 and 72 h (Figure 3). The expression of the protein was further confirmed by Western analysis using anti-His antibodies (Figure 4A).

The expressed aminopeptidases were characterized for their catalytic efficiency by using baculovirus–HaAPN-expressing *T. ni* cells. For calculating the specific activity of the expressed aminopeptidases, care was taken to account for endogenous APN activity associated with host *T. ni* cells. The aminopeptidase activity was assayed using alanine *p*-nitroanilide, leucine *p*-nitroanilide or valine *p*-nitroanilide as substrates. The release of *p*-nitroaniline, monitored over a period of 20 min at 37 °C, increased linearly with time and protein concentration (results not shown). Incubation of reaction mixture with an inhibitor specific to aminopeptidase, amastatin, resulted in 70–100 % inhibition of the three substrates screened for catalytic efficiency of the two aminopeptidases. HaAPN-1 metabolized alanine more efficiently, followed by leucine and valine, while HaAPN2 metabolized leucine more efficiently, followed by alanine and valine (Table 1). The glycosylation status of expressed proteins was examined by incubating the proteins electroblotted on nitrocellulose membrane with biotinylated concanavalin A and

Table 1 Specific activity of recombinant HaAPN expressed in *T. ni* cells

The APN assay was performed with 1×10^6 *T. ni* cells following 48 h of infection with the respective recombinant baculovirus. Assay for aminopeptidase was done by calculating the production of *p*-nitroaniline after 20 min incubation of cells with alanine *p*-nitroanilide, leucine *p*-nitroanilide or valine *p*-nitroanilide. The absorbance was recorded at 405 nm. Absorbance of uninfected healthy cells was subtracted from HaAPN-expressing cells. The molar concentrations of product formed was calculated by an absorption coefficient of *p*-nitroaniline of 0.0099 M. All values represent an average of three replications. Values in parentheses indicate the percentage inhibition of APN activity in the presence of APN inhibitor amastatin.

Recombinant HaAPN	Substrate ...	Specific activity (nmol of <i>p</i> -nitroaniline/min per mg of total protein)		
		Alanine <i>p</i> -nitroanilide	Leucine <i>p</i> -nitroanilide	Valine <i>p</i> -nitroanilide
HaAPN 1		26.2 (100%)	22.91 (77%)	19.91 (65%)
HaAPN 2		28.94 (100%)	35.11 (91%)	16.82 (100%)

Figure 5 Ligand blot analysis of HaAPN1 and HaAPN2

A 10 µg sample of membrane protein of recombinant-virus- HaAPN1-infected *T. ni* cells (lane 1), HaAPN2 (lane 2) and wild-type-virus-infected *T. ni* cells (lane 3) were separated by SDS/7.5%-PAGE. (A) The gel was stained with Coomassie Brilliant Blue. The separated proteins were transferred to nitrocellulose membrane and the blots were incubated (B) with biotinylated Cry1Aa, (C) with biotinylated Cry1Ab and (D) with biotinylated Cry1Ac activated toxin (approx. 50 ng/ml). The blots were then incubated with 1:5000 dilution of AP-conjugated streptavidin and developed with NBT/BCIP. The relative positions of prestained (high-molecular-mass) markers (Bio-Rad) are indicated on left.

the results revealed that both the expressed proteins were highly glycosylated (Figure 4B).

Cry-toxin overlay assay

The interaction of the two recombinant APNs with different Cry toxins was studied by ligand-blot analysis. Although both the APNs were expressed at the same size (Figure 5A), they showed a distinct difference in the spectrum of toxins with which they interacted. The expressed receptor HaAPN1 was recognized by insecticidal Cry1Aa, Cry1Ab and Cry1Ac, while aminopeptidase HaAPN2 interacted only with Cry1Ac (Figures 5B and 5D).

DISCUSSION

The ICPs of Bt are activated inside the gut of the target insect and their interaction with receptors result in blebbing and pore formation in the gut epithelium, leading to death of the insect [24]. These events are mediated by specific receptors located at the brush-border membranes of the insect midgut. Initial ligand binding studies with BBMV proteins from *H. virescens* revealed that Cry1Aa, Cry1Ab and Cry1Ac bind to these proteins with different affinities. Results from competition binding experiments with these toxins on *H. virescens* BBMV proteins led to the prediction of a three-site toxin-binding model. Receptor A protein binds to all three Cry1A toxins – Aa, Ab and Ac, receptor B binds with Cry1Ab and Ac, while receptor C binds only with Cry1Ac. A direct correlation between *in vitro* binding experiments and level of toxicity *in vivo* gives justification to this model [1,25] and was confirmed by different independent studies later by Lee et al. [19] and Luo et al. [20]. Subsequently the receptors were identified as aminopeptidases [7,26–28].

Heterogeneity in aminopeptidase molecular masses (170, 120 and 110 kDa) from *H. virescens* has led to speculation about multiple APNs being involved in initial recognition event of toxin docking. Banks et al. [21] have described a 110 kDa APN, while Luo et al. [20] described a 170 kDa APN as Cry1Ac-binding protein in *H. virescens*. Other independent studies on *H. virescens* [11] and *H. armigera* [29] have identified a 120 kDa APN as the receptor to Cry1Ac. This heterogeneity could be because these are independent reports using different populations of the same insect *H. virescens*. The cDNA of 120 kDa and 170 kDa proteins have been isolated from *H. virescens* [9,11], while the partial cDNA encoding 110 kDa APN has been identified and found to be similar to *H. punctigera* APN2 [21]. On the basis of dendrogram analysis of so-far-reported insect APNs, APNs are clustered into three major groups and the *H. punctigera* APN2 could not be assigned to any particular group [18]. Our results on dendrogram analysis reveal that this *H. punctigera* APN2 is closely related to *P. xylostella* APN 3 (GenBank® accession no. AJ222699) and *Spodoptera litura* (cutworm) APN1 and together form a separate cluster of APN (Figure 2). The presence of multiple forms of APNs and their close similarity suggests that they could have arisen as a result of gene-duplication events [10]. Though APN-encoding genes have been isolated from *H. virescens* and *H. punctigera*, they have neither been expressed in a heterologous system nor has their interaction with insecticidal toxins been demonstrated.

HaAPN1 and HaAPN2 expressed in *T. ni* cells were catalytically active, and the three APN substrates used were hydrolysed at different rates by the two HaAPNs, thus showing that they are functionally different (Table 1). The physiological significance of these observations is not clear at present.

The Cry1Ac-binding 170 kDa APN (receptor A) of *H. virescens* is a protein of 1010 amino acids whose calculated molecular mass is 113 kDa [11]. It shows 86% similarity to HaAPN1 (a 111 kDa predicted protein) and both share similar toxin recognition spectrum. HaAPN1 is expressed as a 120 kDa protein in *T. ni* cells. Examination of the glycosylation status of the expressed protein reveals that both HaAPN1 and HaAPN2 are glycosylated and this may in part explain the discrepancy in the observed and predicted molecular mass. It is pertinent to point out here that the molecular-mass estimates for the *H. virescens* 170 kDa protein are based on BBMV preparations from insect midgut [20]. The native protein may also include other protein modifications, which results in higher apparent molecular mass of 170 kDa. Previous reports on the expression of insect APNs by baculovirus expression vectors in insect cell lines resulted in proteins of

molecular mass close to their predicted molecular masses [16,22,23]. Probably, the insect-cell-line environment does not fulfil the needs for higher-order protein modifications. Similar results were obtained when the midgut mucin gene from the malaria mosquito *Anopheles gambiae* was expressed through a baculovirus expression system in Sf21 cells [30]. The insect cell line when infected with baculovirus results in rapid expression of the protein. Hence it has been suggested that higher-order glycosylation is not possible in this rapid-expression environment [30]. Also, this protein (HaAPN1) has a serine/threonine-rich region at the C-terminus, showing characteristic signatures of mucin-like proteins, which are highly O-glycosylated membrane proteins [31]. It is possible that, in the insect-midgut environment, this APN is highly glycosylated, resulting in higher molecular mass of 170 kDa. Also it has been reported that glycosylation is a crucial requirement for the receptor protein to bind to Cry toxins [32].

Recombinant expression of insect aminopeptidase has been achieved for only one of the isoenzymes from *M. sexta* [22], *P. xylostella* [16], *B. mori* [12] and *E. postvittana* [23]. This is the first report in which two APN-encoding genes from the same insect, *H. armigera*, have been cloned and expressed in insect cells. Aminopeptidase cDNA isolated from the midgut of *Epiphyas* and expressed in Sf9 cells showed interaction with both Cry1Ac and Cry1Ba. This lack of selectivity in binding to toxins questioned the ability of APN to act as a specific ligand to Cry toxins [23]. Our results go further to show that APNs do not bind in a broad manner without specificity to all Cry toxins. Examination of the binding of the expressed aminopeptidases to different Cry1A toxins revealed that, while HaAPN1 acts as a receptor to all the three Cry1A toxins tested, viz. Cry1Aa, Cry1Ab and Cry1Ac, overlapping and meeting the binding specificities of receptor A; HaAPN2 interacted with Cry1Ac only, meeting the specificities of receptor C [19,20,25]. Of the different Cry1A toxins screened, Cry1Ac is the most effective in controlling *H. armigera* larvae. The same is the case with *H. virescens* and *H. zea* (corn earworm) larvae [1]. The higher toxicity of Cry1Ac could be due to its simultaneous recognition by different receptors in the insect midgut. Such overlapping recognition profile by different protein receptors in an insect to the same insecticidal protein will have a direct bearing on the development of resistance against these toxins and the sustainability of Bt proteins in crop protection.

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