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Aminopeptidase-N from the *Helicoverpa armigera* (Hubner) Brush Border Membrane Vesicles as a Receptor of *Bacillus thuringiensis* Cry1Ac δ-Endotoxin

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Abstract. Brush border membrane vesicles (BBMVs) were prepared from the 2nd instar larvae of *Helicoverpa armigera*. Binding of the activated Cry1Ac of *Bacillus thuringiensis* (Bt) toxin was shown by immunoblot. A 120-kDa protein was identified as a receptor for the Cry1Ac type δ-endotoxin. The aminopeptidase-N activity of BBMVs was measured as the hydrolysis of L-leucine *p*-nitroanilide. The specific activity was 35 units/mg protein. The BBMV preparation also showed low level of alkaline phosphatase activity. Zn^{++} chelating agents 2,2′-dipyridyl and 1,10-phenanthroline inhibited aminopeptidase activity at 10 mM concentration, indicating the presence of zinc-dependent aminopeptidase in the brush border of *H. armigera*. The aminopeptidase activity was increased with increasing concentration of δ-endotoxin. The purified 120-kDa binding protein was N-terminally sequenced. The first 10-amino-acid sequence showed 60–77% similarity with human cysteine-rich secretory protein-1 precursor, inhibin alpha chain precursor. Salmonella flagellar hook protein and yeast carboxypeptidase S.

Bacillus thuringiensis produces insecticidal crystal proteins (ICPs) during sporulation. When these ICPs are ingested by the susceptible insect, they are first solubilized in the alkaline and reducing environment of the insect midgut and then processed by the midgut proteases to form the activated toxin. The activated toxin binds to receptors on the surface of the midgut epithelial cells of a susceptible insect and results in the rapid inhibition of K^+-dependent amino acid transport [18]. Changes in the permeability of the midgut membrane [9] and eventually lysis of the midgut cells result in death of the insect [15]. Brush border membrane vesicles are used to study the receptor binding and mode of action of Bt toxin in vitro [27]. It has been shown that *B. thuringiensis* toxins bind with high affinity to specific binding molecules present in the brush border membranes of susceptible insects [22]. In *H. armigera* the target of Bt insecticidal crystal protein is the brush border membrane of midgut cells. The bound toxin disrupts the midgut epithelial cells causing lysis [10]. Lepidoptcran insects have one or more toxin binding proteins. Cry lAc binding protein of 120 kDa and a Cry lAb binding protein of 205 kDa were shown in *Manduca sexta* [6, 23]. *Heliothis virescens* has toxin-binding proteins ranging from 45 to 170 kDa [4, 6, 12, 16]. The receptor for *B. thuringiensis* Cry lAc δ-endotoxin in the brush border membrane of the lepidoptera *Manduca sexta* was shown to be the metalloprotease aminopeptidase-N [11].

We report the brush border membrane receptor proteins of *Helicoverpa armigera* for *B. thuringiensis* Cry1Ac toxin. 120-kDa protein of BBmrs of *H. armigera* was observed as a major cry1Ac binding protein.

The protein sequence data reported in the paper will appear in SWISS-PROT protein Data Bank under the accession number P81731.

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Materials and Methods

Bacillus thuringiensis var. kurstaki HD-73 was a gift from the U.S. Department of Agriculture, Peoria, Illinois, USA. *Helicoverpa armigera* larvae were collected from the locally grown chick pea. Fresh field-collected larvae were used for all experiments.

Bacterial growth and toxin preparation. *Bacillus thuringiensis* was grown on Luria broth at 28 ± 2°C until sporulation. *B. thuringiensis* crystals were purified and solubilized as described earlier [1]. Solubilized protoxin was activated by incubation with trypsin (Sigma; 5% wt/vol) for 1 h at 37°C. Activated toxin was subjected to centrifugation

at 10,000 *g* for 10 min at 4°C. The activated toxin was used for binding studies.

SDS-PAGE and immunoblotting. Binding of Cry1Ac δ-endotoxin to the brush border membrane proteins was detected by immunoblot. Brush border membrane protein resolved on SDS-PAGE (10%) [13] was transferred onto Immobilon-P polyvynilidene difluoride (PVDF) membrane for immunoblotting [21]. The PVDF membrane was incubated with activated Bt toxin, followed by washing and incubation with primary rabbit anti-Cry1Ac antibody. Anti-Cry1Ac antibodies were raised in the rabbit against purified Cry1Ac toxin. After washing and incubation with peroxidase-conjugated goat anti-rabbit antibody, the blot was developed by diaminobenzidene (Sigma) as a substrate.

Preparation of brush border membrane vesicles. Brush border membrane vesicles from the midgut of *Helicoverpa armigera* were prepared according to the method of Wolfersberger [28]. Midgut collected from the third-instar larvae of *H. armigera* was used to purify the brush border membrane aminopeptidase-N. The brush border membrane vesicles were solubilized in 20 mM Tris-HCl, pH 7.6 buffer with 1% (3-3(3-chloramidopropyl) dimethylammonia)-1-propane-sulphonate) (Sigma). The suspension was centrifuged to remove insoluble materials. The supernatant was concentrated and fractionated on Sephadex G-150 column equilibrated with 20 mM Tris-HCl, 0.1 mM DTT, 1mM $MgCl_2$, and 50 mM KCl at pH 8.0. The fractions showing aminopeptidase activity were pooled and concentrated.

Enzyme assays. Aminopeptidase activity was assayed at 25°C in 250 mM Tris HCl, pH 7.8, 250 mM NaCl, with 1 mM L-leucine *p*-nitroanilide as a substrate. One unit of enzyme activity was defined as the amount enzyme catalyzing the hydrolysis of 1 μmol of L-leucine *p*-nitroanilide per min at 25°C. Specific activity was expressed as units/mg protein [8]. Alkaline phosphatase activity was assayed at 25°C in 10 mM diethanolamine at pH 9.8, with 1.25 mM 4-nitrophenyl phosphate as the substrate. One unit of enzyme activity was defined as the amount of enzyme catalyzing the hydrolysis of 1 μmol of 4-nitrophenyl phosphate per min at 25°C. Specific activity was expressed as units/mg protein [14].

N-terminal amino acid sequencing. N-terminal amino acid sequence was determined from the purified receptor resolved on 10% SDS-PAGE and electroblotting. The Coomassie Brilliant blue-stained, 120-kDa band was excised from the membrane and washed several times in distilled water, dried, and subjected to sequencing. Sequencing was carried out using gas-phase protein sequencer from Shimadzu Model PPSQ-10 at the biomembrane lab, Indian Institute of Technology, Mumbai, India. This system consists of an Edman reaction unit, an on-line PTH-analyzer, and a CR-7A data processor.

Results and Discussion

Brush border membrane vesicles were isolated and purified from the midgut of *Helicoverpa armigera* to study the binding ability of Bt. toxin. To demonstrate which protein of the BBMV is a putative receptor for the binding of Bt toxin, we carried out immunoblotting. The Coomassie blue-stained gel showed various molecular weight protein bands ranging from 150, 120, 96, 80, 66 to 60 kDa. The BBMV proteins were separated on SDS-PAGE and transferred onto the membrane. The membrane was incubated with the activated Bt toxin, followed by anti-Cry1Ac antibody and developed. A

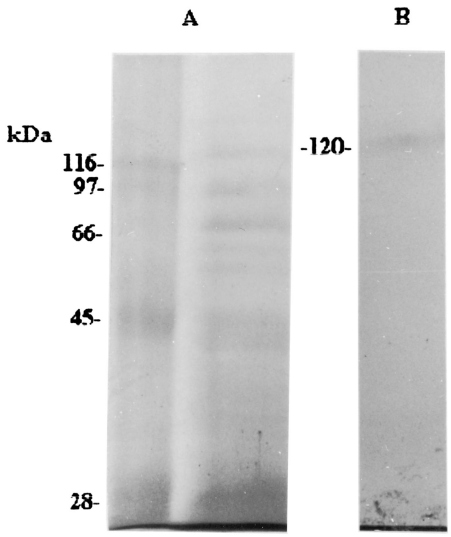

Fig. 1. SDS-PAGE profile of brush border membrane vesicles preparation and immunoblot showing binding of activated Bt δ-endotoxin to BBMV proteins. A. Coomassie blue-stained gel of BBMVs. Lane 1, molecular weight market, lane 2, BBMV protein. B. PVDF membrane incubated with Cry1Ac toxin, developed with anti-Cry1Ac antibodies followed by peroxidase-conjugated Goat anti-rabbit antibodies.

protein band corresponding to 120 kDa was observed on the blot. To confirm that the 120-kDa protein is a putative receptor for Bt toxin, BBMV proteins were separated by gel filtration, and the fraction having aminopeptidase activity was concentrated and resolved on SDS-PAGE (10%). The duplicate sample was transferred to PVDF membrane. The Coomassie blue-stained gel showed a single band of 120 kDa. The immunoblot showed binding of Cry1Ac δ-endotoxin to this protein. Hence, a 120-kDa protein of the BBMV was identified as a receptor for the Cry1Ac type Bt toxin, as shown in Fig 1 and 2. The enzymatic properties of this receptor protein were studied by the aminopeptidase assay. The specific activity of the purified protein was found to be 35 units/mg protein. The BBMV preparation showed alkaline phosphatase activity as well. The level of alkaline phosphatase activity was 5 units/mg protein. The purified protein did not show alkaline phosphatase activity [Table 1]. The effect of Zn^{++} chelating agents on the activity of aminopeptidase from BBMV was studied. The Zn^{++} chelating agents 2,2-dipyridyl and 1,10-phenanthroline inhibited 42 and 51% aminopeptidase activity respectively at 1 mM concentration. At 10 mM concentration of these inhibitors, there was complete inhibition of aminopeptidase activity [Table 2]. The 120-kDa protein has the characteristics of aminopeptidase-N. Aminopeptidase-N is a Zn^{++}-dependent dependent ectoenzyme existing in relatively high abundance in the brush border of many animals. Mammalian forms of intestinal aminopepti-

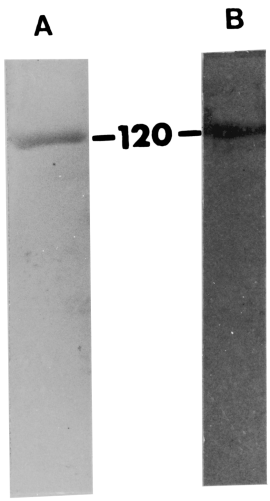

Fig. 2. SDS-PAGE and immunoblot of aminopeptidase-N from BBMVs of *H. armigera*. A. Coomassie blue-stained gel of purified aminopeptidase. (aminopeptidase purified by gel filtration. The fraction showing aminopeptidase activity was pooled, concentrated, and loaded on SDS-PAGE). B. Immunoblot showing binding of Cry1Ac δ-endotoxin.

Table 1. Aminopeptidase and alkaline phosphatase activities from brush border membrane vesicle preparation and purified protein

	Specific Activity (units/mg protein)	
Enzyme	BBMV	Purified
Aminopeptidase-N	6	35.0
Alkaline phosphatase	5.0	ND[a]

[a]ND, not detectable.

Table 2. Effect of inhibitors on aminopeptidase activity

Conditions	Specific activity (Units/mg protein)	% Inhibition
Control	36.0	—
2,2′-Dipyridyl (1 mM)	15.0	42
2,2′-Dipyridyl (10 mM)	ND[a]	100
1,10-Phenanthroline (1 mM)	18.0	51
1,10-Phenanthroline (10 mM)	ND	100

[a]ND, not detectable.

dase-N occur as monomers of 120–140 kDa proteins, which are associated with 95, 66, and 45 kDa breakdown products [17]. It was reported that toxin-binding protein acts by increasing the toxin concentration at the membrane surface microenvironment, resulting in pore formation at low concentration of toxin [5, 11]. The effect of Cry1Ac δ-endotoxin on the aminopeptidase activity was studied by adding increasing concentrations of δ-endotoxin in the assay system. The aminopeptidase activity was increased with increasing concentrations of δ-endotoxin up to 60 μg, but further concentrations lowered the enzyme activity to its original level. δ-Endotoxin by itself does not have aminopeptidase activity. Those δ-endotoxin which bind to the receptor enhance aminopeptidase activity. Inactive toxin did not show any increase in aminopeptidase activity [Fig. 3].

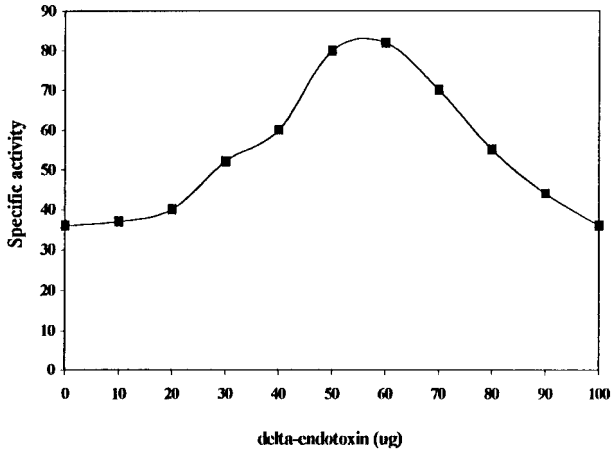

Fig. 3. Effect of δ-endotoxin of Bt toxin on the aminopeptidase activity. Increasing concentrations of δ-endotoxin were added in the assay system of aminopeptidase having specific activity of 35 units/mg protein, and change in the aminopeptidase activity was monitored.

The 120-kDa protein showing aminopeptidase activity was electroblotted onto PVDF and subjected to N-terminal amino acid sequencing. The first ten amino acid residues found were GMYTHEGSDP. This amino acid sequence was used to search the Swiss-Prot protein database. The multiple alignments of the N-terminal sequence of *H. armigera* aminopeptidase-N (AMPN_HELAM) showed 60–77% identity with four different N-terminal amino acid sequences, CRS1_HUMAN (cysteine-rich secretory protein-1 precursor), IHA_HUMAN (inhibin alpha chain precursor glycoprotein. Follitropin inhibitor), FLGE_SALTY (*Salmonella typhimurium* flagellar hook protein), and CBPS_Yeast (carboxypeptidase S precursor glycoprotein of *Saccharomyces cerevisiae*), as shown in Table 3. A multiple aminopeptidase-N protein sequence alignment showed that the first 40 residues of the human, rat, pig, and mouse enzymes have the characteristics of a signal sequence, which may function as a membrane anchor [7, 19, 20].

Aminopeptidase-N (EC.3.4.11.2) is a Zn^{++}-dependent enzyme existing in relatively high abundance in the brush border of many animals [3, 24, 25, 28]. Aminopeptidase catalyzes the removal of N-terminal neutral amino acids [8]. Aminopeptidase is a common component of lepidoptera larval midgut and is used as a marker

Table 3. The alignment of the N-terminal amino acid sequence of *H. armigera* AP-N

Source	N-terminal amino acid sequence										% Identity	Accession No.
H. armigera	G	M	Y	T	H	E	G	S	D	P	—	P81731
CRS1 human	C	H	Y	C	H	E	G	N	D	P	75	P54107
IHA Human	G	G	F	T	H	R	G	S	D	P	60	P05111
FLGE SALTY	A	V	Y	T	H	D	S	S	D	P	66.6	P16322
CBPS yeast	L	L	Y	T	W	E	G	S	D	P	77.77	P27614

enzyme to assess the purity of BBMVs from insect larval guts [26]. Correlation between toxicity and binding ability of BBMV to the Bt toxin was also reported [2]. The generally accepted theory for the mode of action of *B. thuringiensis* δ-endotoxin postulates that, after binding to a receptor in the insect midgut, the toxin undergoes a conformational change and inserts into the membrane to form a pore [11]. We report the presence of a 120-kDa protein of *H. armigera* brush border as the receptor for the Cry1Ac δ-endotoxin.

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