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The Receptor for Bacillus Thuringiensis Cry 1Ac Delta-endotoxin in the Brush Border Membrane of the Lepidopteran *Helicoverpa armigera* is Aminopeptidase N

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Abstract: 120 Kda and 70 Kda protein in the larval midgut membrane of the lepidopteran *Helicoverpa armigera* identified as putative receptor for Bt Cry 1Ac delta-endotoxin .Receptor proteins have been purified by a combination of gel-filtration and anion exchange chromatography .In ligand-blotting experiment ,the purified protein has binding capacity with Cry 1Ac and Cry 1Ab but not to Cry 2A .N-terminal sequence obtained from the protein show no homology to already existing sequences .When assayed for amino-peptidase and alkaline phosphatase activities , purified receptors preparations were enriched 2.5 fold in amino-peptidase activity compare to *Helicoverpa-armigera* brush border membrane vesicles . The 70 Kda protein seems to be the part of 120 Kda receptor protein.

Key words: Lepidopteran, *Helicoverpa armigera*Delta-endotoxin,Amino-peptidase,Alkaline-phosphatse Ligand-blotting, Homology

Introduction

The gram-positive spore-forming bacterium *Bacillus thuringiensis* synthesizes and intracellular parasporal glycoprotein crystal(s) during the sporulation cycle. The glycoprotein which is insecticidal ,is a protoxin that is activated after ingestion by an insect susceptible to the toxic product. For most glycoprotein crystals, the protoxins are processed proteolytically to yield smaller toxic componants in the alkaline midgut of the insect. There is variety of subspecies of *Bt* that exhibit highly specific toxic activity aginst lapidopteran (moth), dipteran(mosquito) and coleopteran (beetle) larvae. Considerable nucleotide sequence information is available on that has been termed Cry(crystal protein) genes of *Bt* which encode a family of 13 related insectisidal proteins. These genes are divided further into four major classes and several subclasses depending primarily on the insecticidal spectra of the encoded proteins. The four major classes of genes are I(lepidopteran-specific),II(lepidopteran and dipteran-specific), III(coleopteran-specific),and IV(dipteran-specific). Biopesticide based on the bacterium *Bt* have attracted wide attention as safe alternatives to chemical pesticides.

The target of lepidopteran active *Bt* insecticidal crystal δ-endotoxin is the apical Brush border membrane of larval midgut cells (Percy and Fast ,1983;Singh *et al.*,1986; Bauer and Pankratz, 1992),while different *Bt* toxins have significantly different insecticidal spectra *in vivo*, in most but not all cases so for studied, the activity spectrum corelates with the presence of specific receptors in the brush border membrane vesicals (BBMVs) prepartions from susceptible insects (Hofmann *et al.*, 1988; Van Rie *et al.*, 1989, Wolfersberger,1990). BBMVs from the insect guts thus costitude a powerful system in which receptor binding and mode of action of *Bt*. toxins can be studied in vitro. There is complete lack of information about the nature of the insect target receptors. In the present study, we have identified ,purified and partially characterized proteins of 120Kda and 70Kda to which the Cry 1A© toxin of *Bt* bind in BBMV of *H. armigera*. Our research work will help to understand the mechanism of resistance to *Bt* toxins and causes of resistance which are related to the disruption of steps involved in *Bt* toxin action such as solubilization, activation of protoxin by proteases , binding to receptor, insertion into the membrane and pore formation. The resistance is often related to a change in receptor binding properties on the BBMV of insect midgut.

We employed ligand blotting to visualize Cry 1Ac toxin binding protein in BBMVsfrom the susceptible lepidopteran *Helicoverpa armiregera* and identified 120 Kda, 70 Kda proteins as putative receptor for this toxin .

It is reported Cry 1Ac toxin binding protein in BBMVs from the suceptible Lepidopteran *Manduca sexta*,visualize by ligand blotting and identified a 120Kda glycoprotein as a putative receptor for this toxin (Knowles *et al.*, 1991).

We report the puification of 120 Kda and 70 Kda receptor by a combination of gel filtration and anion exchange chromatography .N terminal sequencing of 120 Kda reveal no amino acid similarity with the already known-sequencing in order to explore the possibility that the Cry 1Ac receptor is infact aminopeptidase ,purified receptor protein preparation were assayed for aminopeptidase activity.

Materials and Methods

Brush Border Membrane Vesicles Preparation: Mid guts of *Helicoverpa armiregera* larvae (5th instar)were isolated and stored in MET buffer(300mM mannitol, 5mM EGTA in 17mM Tris pH 7.5 adjusted with HCl) BBMVs were prepared as described byWolfersberger *et al.* (1987). The final BBMVs preparation was resuspended at 5mg ml^{-1} in a buffer containing 20 mM Tris-HCl pH 7.4 150mM NaCl ,5mM EDTA, 1mM PMSF,3-[(3-cholamidopropyldimethyle ammonio]-1-propane-sulphonate (CHAPS).

Purification of Recombinant Bt. Toxins: Crystal proteins were purified by the procedure described. Purified crystals were solubilized in 50mM Na_2Co_3 buffer pH 9.5 containing dithiothreitol at 37°C for 4 h. Solubilized protoxins were digested with 5%(wt/wt) trypsin at 37°C for 4 h.

Protein Estimation: Protein concentration was measured by Bio-Rad protein assay and bovine serum albumin(Sigma) as standard.

Gel-filtration chromatography: Gel-filtration chromatography was performed at 4°C in a cold room. In a typical run ,CHAPS-solubilized BBMVs were loaded on sephacryl-300 column. The column effluent was monitored by SDS-PAGE and fractions in buffer-A(20 mM Tris-HCl pH 7.4 150mM NaCl ,5mM EDTA, 1mM PMSF,0.2% CHAPS) were collected . The column was regenerated by washing with buffer-A.

Anion Exchange Chromatography: Samples of partially purified

Table 1: Specific activity and enrichment of enzymes in H. Armigera BBMVs and purified receptor

Proteins	BBMVs	Purified receptor	Enrichment
Aminopeptidase	13.1	31.0	2.5
Alkalinephosphatase	4.28	0.57	---

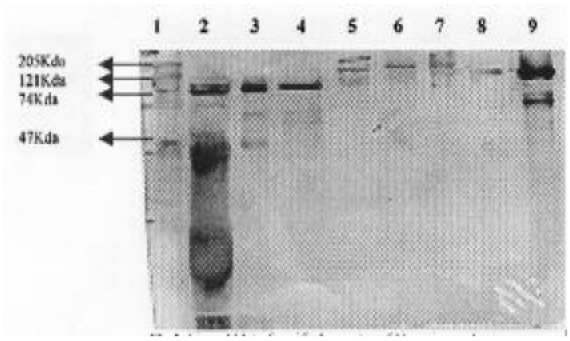

Fig. 1: Ligand blot of purified receptor of *H. armigera* along with Cry 1Ac and Cry 1Ab Lanel: Marker, Lane2, and 2,3 4,8: 4, 8: Cry 1Ac, Lane 5,6,7: Purified recepter Lane 9: Cry 1Ab

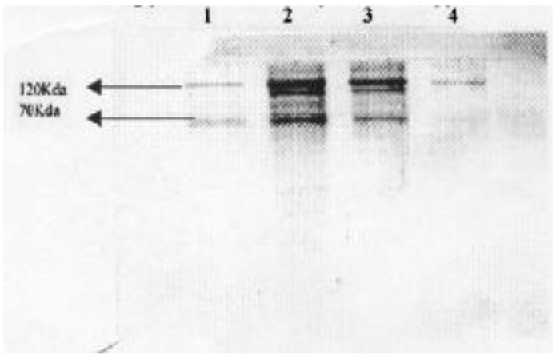

Fig. 2: Commassie brilliant blue stained 10% SDS-PAGE showing 120Kda and 70Kda bands of receptor proteins

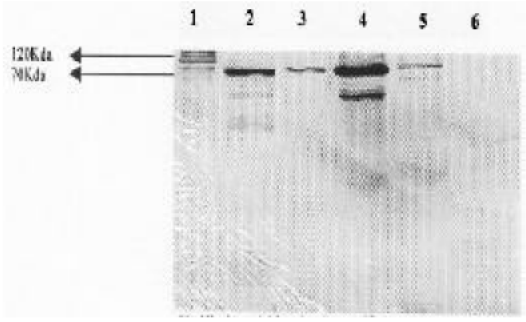

Fig. 3: Ligand blot showing purified receptor proteins along with psotive control (Cry 1Ab, Cry 1Ac) negetive control (Cry 2A) shows the reproducibility of results shown in Fig. 1 Lane 1: receptor, Lane 2,3,5: Cry 1Ac Lane 4: Cry 1Ab Lane 6: Cry 2A

receptor were loaded on to a DEAE-52 column which was pre equilibrated with buffer-B(20 mM Tris-HCl pH 7.4 ,0.2% CHAPS) at 4°C,then proteins were eluted 0-600mM NaCl in buffer-B.Protein eluted at 180 mM NaCl concentration and analyzed on SDS-PAGE . Receptor protein was dialysed and was concentrated using centricon -30 ultra-filtration devices(Amicon) (Fig. 2)

Ligand Blotting: Receptor protein resolved by SDS PAGE separated protein were transferred to PVDF-membrane by electrobloting using transfer buffer (Tris 2mM, Glycine 192mM pH 8.3, 20% methanol)and incubated with Cry 1Ac toxin for one hour after blocking with 3% skim Milk .Then incubated with anti-Cry 1Ac for 1 h washed the membrane extensively by TTBS-buffer(20mM Tris, 150mM NaCl pH 7.5, 0.05% tween-20% or 0.05% Triton-x-100) after secondary antibody treatment colour development by 33μl BCIP+33μl NBT in D. water (Fig. 1).

Enzyme assays: Amino peptidase activity was assayed at 25°C(Hafkenscheid, 1984)in 250mMTris HCl pH. 7.8,250mM NaCl using 1mM L-leucine-p-nitroanilide (Sigma)as a substrated. The initial rate of in crease in absorbance at 405nm was used to calculate specific enzymatic activity , the absorption coefficient of p-nitroanilide was taken to be 9.9×10^3mol/l.
Alkaline phosphatase activity was assayed at 25°C(Lowry *et al*., 1954) in 10mM diethanolamine pH. 9.8, using 1.25mM 4 -nitrophenyl phosphat e(Sigma) as the substrate the absorption coefficient of p-nitrophenol was taken to be 18.33×10^3mol/l.

Results and Discussion

Purification of receptors: *Helicoverpa armiregera*BBMVs were solubilized in the detergent CHAPS and applied to a gel filtration column in buffer-A .Fractions were collected and analyzed by SDS PAGE . Fractions showing protein band were pooled and applied to DEAE-52 column for anion exchange chromatography . Column was pre-equilibrated with buffer-B at 4°C proteins were eluted with o-600mM NaCl in buffer-B. protein s eluted at 180mM NaCl concentration as judged by SDS-PAGE and silver staining ,the final 120Kda and 70Kda receptor preparation is greater than 95% pure (Fig. 2).

Ligand Blotting : BBMVs and purified receptor from *Helicoverpa*armigera were resolved by SDS PAGE transferred to PVDF-membrane and characterized according to their ability to bind to toxin. Cry 1Ac toxin bound to a major band of 120 Kda and when present ,minor band 70Kda. In BBMVs 120Kda band protein degraded by freeze-thaw effect when 70Kda band become more prominent and show binding to Cry 1Ac toxin .Cry 1Ab used as positive control and Cry 2A as negative control (Fig. 3).

Amino acid sequencing: N-terminal amino acid sequence was determined from the purified receptor, resolved by SDS_PAGE and electroblotting the co massie brilliant blue -stained 120Kda band was excised from the membrane and sequenced .The sequence is given below:
V I Q T G Q C D Q S I A V V T N F N L S A

Amino acid sequence comparison: Amino acid sequence from the 120Kda receptor was used to search the Gen Bank(NCBI)protein data base .there are no similarities to already existing sequences, which shows novelity of this N-terminal sequence of receptor protein.

Assay of Aminopeptidase Activity: To confirm that 120 Kda and 70Kda receptor is aminopeptidase N, we assayed purified receptor preparation for enzyme activity by the method of Hafkenscheid(1984).Both H*elicovepa armigera*BBMVs and the purified receptor hydrolysed L-Leucine-p-nitroanilide, a chromogenic substrate specific for aminopeptidase N.The specific activity was 13.1 μmol/min/mg protein for BBMVs and 31.0μmol/min/mg protein the purified receptor (Table 1).This represent a 2.5 fold enrichment in amino peptidase activity in the purified receptor preparation compare to BBMVs .the purity of the receptor preparation was indicated by the absence of detectable alkaline phophatase activity , commonly used as a midgut brush border membrane marker (Wolfersberger, 1984).

Cry 1A toxin binds to the brush border membrane after that large portion of the molecule inserts into the membrane , forming ion channels (Luo *et al.*, 1999). The binding of the toxin to midgut apithelial cell receptor is essential for insecticidal activity . It is thought that structural differences iin the receptor molecules produce differences in the susceptibility of insect specie to insecticidal proteins . However it is still unclear how differences in the receptor molecules or their structures influence insect susceptibility . Studies of receptors are also important to understand the mode of action of insecticidal proteins and the development of resistant insects. (Nakanishi *et al.*, 1999).

It is reported that insect APNs contain conserved structure such as the zinc binding motif ,the catalytic domain and the toxin binding region in the course of molecular evolution, the conserved Cry 1Aa toxin binding structure of insect APNs has changed slightly ,

divergent Bt. Cry toxins might be the result of adaptations to these slight changes in the insect APNs.

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