

**IDENTIFICATION, PURIFICATION, CLONING AND  
EXPRESSION OF A NOVEL RECEPTOR FOR *BACILLUS  
THURINGIENSIS* CRY1A DELTA-ENDOTOXINS IN THE  
BRUSH BORDER MEMBRANES OF THE *HELICOVERPA  
ARMIGERA* (LEPIDOPTERA: NOCTUIDAE)**

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**Abstract**

Insecticidal crystal proteins of *Bacillus thuringiensis* are effective in controlling agriculturally and biomedically harmful insects. However, little is known about the mechanism of insecticidal activity of these proteins. We report here a 65 kDa Protein present in the extract of the larval midgut membrane of *Helicoverpa armigera* as putative receptor for Bt Cry1A delta-endotoxin, on the basis of binding affinity to Cry1Aa, Cry1Ab and Cry1Ac but not to Cry2A. The protein has been highly purified by a combination of chromatography, electrophoresis and isoelectrifocussing techniques. The isolated protein exists as an oligodimer in its native form. The purified protein exhibits amino-peptidase activity. N-terminal sequence of the purified protein shows no homology to protein sequences in the Gen bank (NCBI) protein database. Degenerate primers were designed, based on N-terminal sequence of the purified protein and hybridization of Probe with mRNAs of *Helicoverpa armigera* indicated sequence complementarity. The structural gene of this purified protein was cloned in pGEX-4T-3 expression vector. The cloned Protein exhibited binding properties, aminopeptidase activity and other characteristics of native protein of *Helicoverpa armigera*. Larval mortality of *Helicoverpa armigera* to Cry1A toxin was considerably reduced when the larvae were pre-fed a diet containing antibodies to the 65 kDa protein, presumably due to blocking of the receptor sites in BBMVs.

**Introduction**

During sporulation, *Bacillus thuringiensis* (Bt) produces a variety of insecticidal crystal proteins (ICPs) that accumulate as parasporal crystals (Oddou *et al.*, 1991). A wide range of lepidopteron, dipterans and coleopteran larvae are susceptible to different Bt parasporal crystal (Cry) proteins (Hofte & Whiteley, 1989; Lereclus *et al.*, 1989). In native form, the crystal proteins are inactive, however, in the larval midgut, they are proteolytically cleaved to yield an active component of approx. 60 kDa. The larvicidal domain is located in the N-terminal half of the protoxin (Schnepf & Whiteley, 1985). The activated crystal protein binds with high affinity to receptors on the brush border membrane, that leads to its insertion into the epithelial membrane of midgut epithelial cells, resulting in toxin oligomerization and the formation of pores, that cause cell lysis (Gill *et al.*, 1992, Slatin *et al.*, 1990, Tabashnik *et al.*, 1994). A number of putative receptors have been identified (Vadlamudi *et al.*, 1993, 1995; Knight *et al.*, 1994). The host specificity of Bt parasporal crystal proteins is defined by its specific binding to different receptor proteins present on the brush border membrane. Further, it is believed that insect resistance build up emanates from mutational changes in the receptor protein (Aronson & Shai, 2001). *Bacillus thuringiensis* (Bt) is a valuable source of insecticidal proteins for use in conventional sprayable formulations and in transgenic crops. It is the most promising alternative to synthetic insecticides.

To elucidate the biochemical basis of toxin specificity, we have identified, purified, cloned and expressed a 65 kDa protein from the midgut of the American bollworm *Helicoverpa armigera*. The protein binds to Cry1Aa, Cry1Ab and Cry1Ac toxins, but not to Cry2A protein. The N-terminal sequence of the purified Protein (65 kDa) has been entered in the Gen Bank (NCBI) protein database (Accession # A59445).

### Materials and Methods

All the organic and inorganic chemicals used were from Sigma Chemical Company. Molecular weight protein markers, polyvinylidene difluoride (PVDF) membrane and Bradford protein assay reagents were from Bio-Rad Laboratories. *H. armigera* and *Earias vitella* larvae were obtained from the insectary of the CEMB. All protein concentrations were measured by Bio-Rad protein assay with bovine serum albumin as standard (Bradford, 1976).

Restriction enzyme (EcoRI), and other DNA active enzymes were from New England Biolabs ; and prestained protein markers from Invitrogen Life Technologies. *Escherichia coli* (*E. coli*) strains DH5 $\alpha$ , BL21 (DE3), and plasmid vector pGEM-T were obtained from Promega. Expression vector pGEX-4T-3 and GST antibody were obtained from Amersham Pharmacia Biotech. CryIAa, CryIAb, CryIAc and Cry2A clones were obtained from culture collection lab of CEMB.

**Purification of insecticidal crystal proteins:** CryIAa, CryIAb, CryIAc and Cry2A were purified by the procedure described by (Lee *et al.*, 1992). Purified proteins were solubilized in 50mM Na<sub>2</sub>CO<sub>3</sub> pH 9.5, containing 10mM dithiothreitol, treated with 5% trypsin at 37°C for 4-hours and stored in 2ml aliquots at 4°C.

**Preparation of brush border membrane vesicles:** Midguts of *Helicoverpa armigera* larvae were isolated and stored in MET buffer (300mM mannitol, 5mM EGTA in 17mM Tris pH 7.5) BBMVs were isolated from larval midgut as described by Wolfersberger *et al.*, (1987) and suspended at 5mg/ml in a buffer containing 20 mM Tris-HCl pH 7.4 150mM NaCl, 5mM EDTA, 1mM PMSF, 3-[(3-cholamidopropyl) dimethyl ammonia]-1-propane sulphonate (CHAPS). The suspension was centrifuged at 10,000 xg for 15 minutes and the clear supernatant was stored in 5ml aliquots at 4°C (Pool A).

### Purification of the protein

**Gel-filtration chromatography:** Fifty mg Crude proteins (Pool A) were applied to a sephacryl-300 column (4.91cmx5cm) and eluted with buffer I (20 mM Tris-HCl pH 7.4 150mM NaCl, 5mM EDTA, 1mM PMSF, 0.2% CHAPS). Fractions were monitored by measuring the absorbance at 280nm (Fig. 1) and by Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). Fractions containing major protein peak were pooled and designated as pool B fraction.

**Affinity column chromatography:** Pool B proteins were applied to a column of CNBr-activated Sepharose 4B coupled to CryIAc protoxin, pre-equilibrated with buffer II (10mM Tris-HCl pH 8.0 150mM KCl, 5mM EDTA, 5mM EGTA, 1mM PMSF, 8mM CHAPS). CryIAc binding proteins were eluted with 0.2M N-acetyl Galactosamine (GalNAc). Protein containing fractions were pooled (pool C).

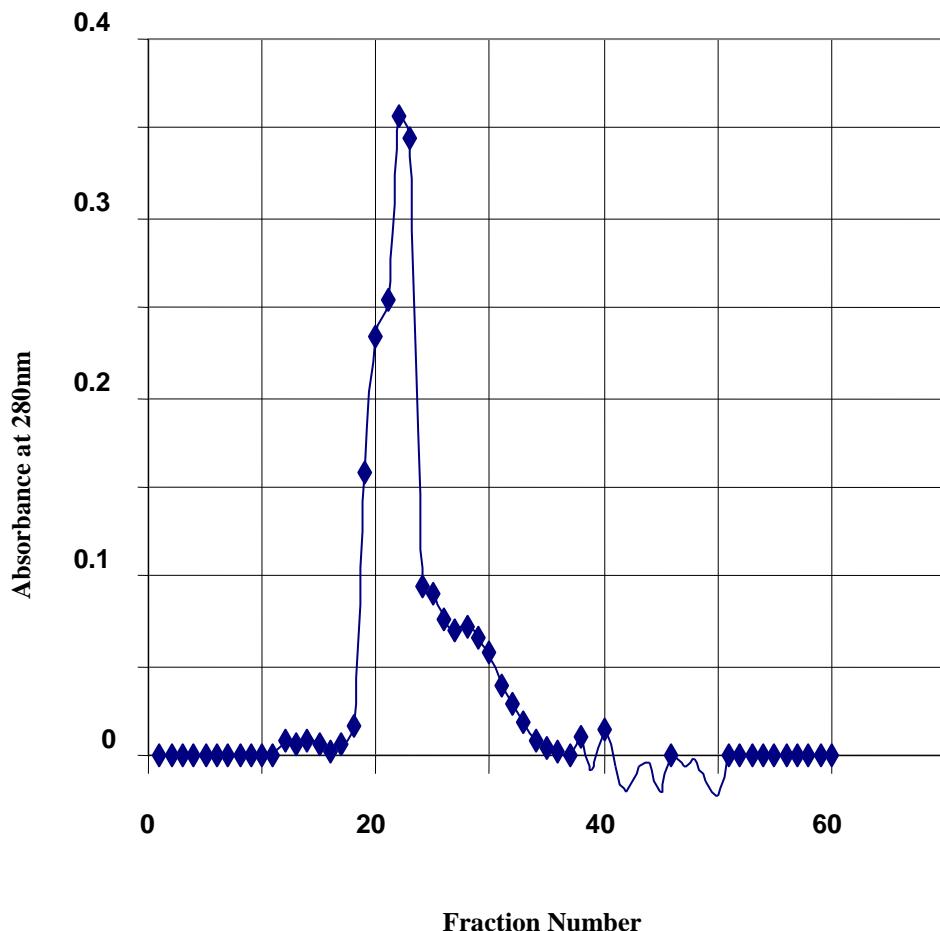


Fig. 1. Elution Pattern of protein from Sephadex 300 column

**Ion Exchange Chromatography:** Pool C was applied to a DEAE-52 column, pre equilibrated with buffer III (20 mM Tris-HCl pH 7.4, 0.2% CHAPS) at 4°C. The bound proteins were eluted with a linear gradient of 0-600 mM NaCl in buffer III. The major protein peak eluting at 180 mM NaCl was pooled, dialyzed and concentrated using centricon-30 ultra-filtration (Pool D).

**Preparative gel electrophoresis:** Pool D proteins were further resolved by Preparative gel electrophoresis. A major bands at 130 and 65 kDa was electroeluted and analyzed on native as well as denaturing PAGE (Pool E).

**Two dimensional-gel electrophoresis:** An appropriate aliquot of pool D proteins was resolved by isoelectric focusing in a 1.5x140 mm Tube gel, and then on SDS-poly acrylamide slab gel.

### Affinity for various *bt* crystal proteins

Specific affinity of the purified protein for CryIAa, Cry1Ab, Cry1Ac and CryIIA crystal proteins was determined by ligand blotting. Pool D protein was electrophoresed on 10% SDS-PAGE, transferred to PVDF-membrane by using transfer buffer (Trizma base 0.582 g, Glycine 0.293 g, Methanol 20ml/liter) and incubated with various *Bt* crystal proteins for one hour after blocking with 3% skimmed Milk. The ligand blots were processed as Western blots as described by Towbin *et al.*, (1979).

### Associated aminopeptidase, alkaline phosphatase activities

Any associated aminopeptidase or alkaline phosphatase activities were assayed by the method of Hafkenscheid (1984) and Lowry *et al.*, (1954) respectively. Effect of  $\delta$ -endotoxin of *Bt* on the aminopeptidase activity was determined by increasing concentrations of  $\delta$ -endotoxin Cry1Ac in the assay system of aminopeptidase having specific activities of 31-units/mg protein and 60 units/mg protein of *H. armigera* and *E. vitella*, respectively.

### Amino acid sequencing

Highly purified 65 kDa protein was transferred to PVDF-membrane, stained with Coomassie brilliant blue and used in N-terminal amino acid micro-sequencing by Edman degradation.

### Characterization of 65 kda protein

Degenerate primers were designed on the basis of N-terminal sequence of 65 kDa Protein, by insect codon usage (Wada *et al.*, 1991). The primer DNA was labeled with digoxigenin-11-dUTP according to the instructions of the manufacturers. Total RNA was isolated from midgut tissue of fourth instar *H. armigera* larvae, by using RNeasy QIAGEN kit, mRNA was separated from total RNA by using an oligo (dT) column. The purity of mRNA was examined on 1.2%FA gel. The 3' DIG labeled primers were used as probe in Northern dot blot analysis.

### Production of antibodies

Purified receptor protein was used to immunize rabbits to generate antibodies (Cooper & Patterson, 1992). Antibodies were further purified from one aliquot of antiserum by affinity purification by Immunoabsorbant technique (Rybicki *et al.*, 1990) with slight modification.

**Cloning and expression of the 65 kDa protein:** cDNA was synthesized by reverse transcription of *H. armigera* midgut mRNA. The forward degenerate primer (GARACYWSSGCBAACTACTGC) and poly-T reverse primer were used in PCR to amplify corresponding cDNA by reverse transcription (Saiki *et al.*, 1988). The recombinant pGEMT vector carrying the protein cDNA was digested with EcoRI and the resulting fragment was cloned into EcoRI digested pGEX-4T-3 Glutathione S-transferase

expression vector (Pharmacia). The construct was transformed into BL21 (DE3) cells. The identity of the selected Clone was confirmed by restriction digestion and by PCR. After IPTG-induction, transformants were screened for expression of the expected fusion protein by SDS-PAGE. GST fusion receptor protein expressed in *E. coli* strain BL21(DE3) was purified by using Glutathione Sepharose 4 Fast Flow column.

To confirm whether the 65 kDa protein expressed in prokaryotic system has binding properties similar to the native protein purified from the midgut, Ligand blotting and Western blotting experiments were performed. Aminopeptidase activity of purified protein after expression was also determined.

**Biotoxicity assay:** The antibody serum raised against purified receptor protein, was mixed in artificial diet and fed to first instar larvae prior to addition of Cry1Ac toxin to the diet. After three days, larvae were shifted to diet containing Cry1Ac toxin alone. The control larvae were fed on normal diet prior to Cry1A toxin diet. Larval mortality was determined after 72 hours.

## Results

**Purification of protein:** The protein profile eluting from Sephadryl-300 gel column revealed the presence of two proteins with molecular sizes 130 kDa and 65 kDa. Both proteins co-migrated on the sepharose affinity column as well as anion exchange (DEAE cellulose) column (Fig. 2, lane 2).

When the 130 kDa and 65 kDa proteins from preparative SDS-PAGE were run on native PAGE, both proteins exhibited a molecular size of 65 kDa, these observations suggested that 130 kDa protein is a dimer of 65 kDa protein. The suggestion that 130 kDa is a dimer of 65 kDa protein was also supported by two dimensional gel electrophoresis, in which only 65 kDa protein appeared with 7.3 isoelectric point (pI). Our data from expression and sequencing studies provide further support to the notion that 130 kDa protein is a dimer of 65 kDa protein.

**Binding affinity:** Ligand blot assays showed that the 65 kDa protein binds to Cry1Aa, Cry1Ab and Cry1Ac but not to Cry2A (Fig. 3). These results indicated that the 65 kDa Protein is the receptor Protein in the midgut of *H. armigera* (Table 1).

**Table 1. Affinity of *Bt* crystal proteins with receptor proteins of different insect species by ligand blotting technique.**

<i>Bt</i> crystal proteins	Receptor proteins of insects		
	<i>H. armigera</i>	<i>Earias. Vitella</i> (positive control)	<i>Tribolium castaneum</i> (negative control)
CryIAa	+	+	-
CryIAb	+	+	-
CryIAc	+	+	-
CryIIA	-	-	-

+

Sign indicate binding,

-

Indicate no binding

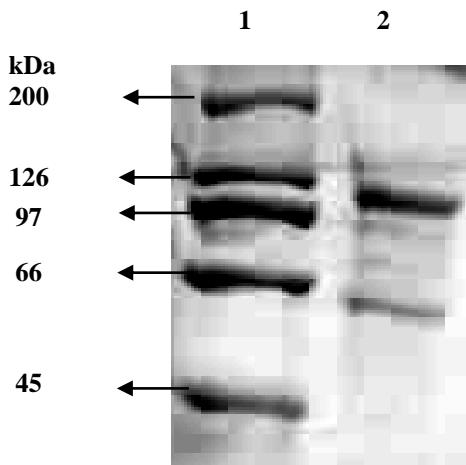


Fig. 2. Resolution pattern of proteins  
Lane 1 high molecular weight marker lane 2, purified protein.

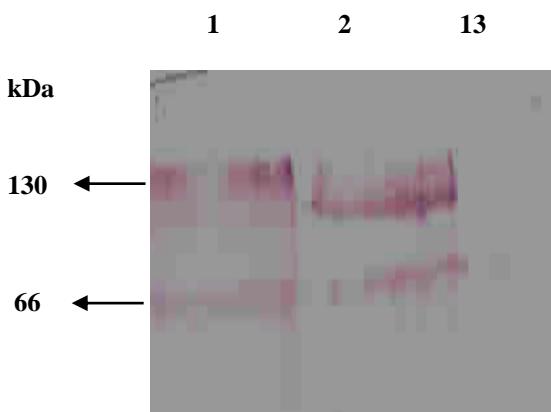


Fig. 3. Ligand blot of purified receptor proteins with CryIAa, CryIAb and CryIAc.  
Lane 1; *Helicoverpa armigera*, Lane 2; positive control; *Earias vitella*, Lane 3; negative control; *Tribolium castaneum*.

**Associated aminopeptidase and alkaline phosphatase activity:** *Helicoverpa armigera* BBMVs and the purified receptor hydrolysed L-Leucine-p-nitroanilide, a chromogenic substrate specific for aminopeptidase N. The specific activity was estimated at 13.1  $\mu\text{mol}/\text{min}/\text{mg}$  protein for BBMVs and 31.0  $\mu\text{mol}/\text{min}/\text{mg}$  protein in pool D. There is gradual enrichment of aminopeptidase activity in the purified protein, but there was no increase in alkaline phosphatase activity. It is therefore, concluded that purified protein is aminopeptidase in nature.

The effect of Cry1Ac  $\delta$ -endotoxin on the APN activity was studied by adding increasing concentrations of  $\delta$ -endotoxin in the assay system. The aminopeptidase activity was increased with increasing concentrations of  $\delta$ -endotoxin up to 40  $\mu\text{g}$ , but

further concentrations lowered the enzyme activity to its original level.  $\delta$ -endotoxin by itself does not have aminopeptidase activity. That  $\delta$ -endotoxin which binds to the receptor enhances aminopeptidase activity. Inactive toxin did not show any increase in aminopeptidase activity (Fig. 4).

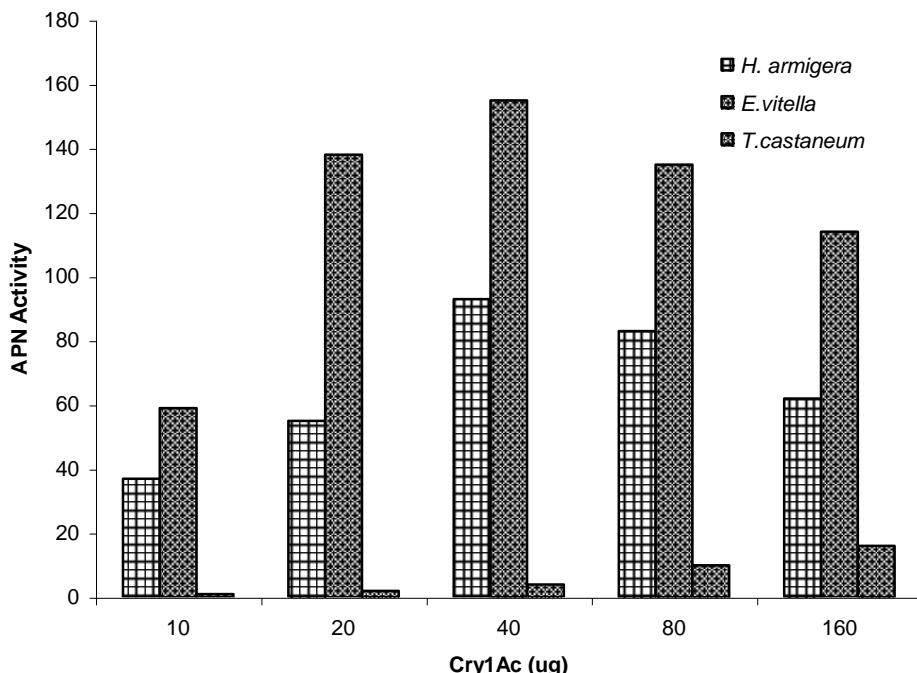


Fig. 4. Effect of Cry1Ac (two fold increasing concentrations 10ug to 160ug) on Aminopeptidase activity of purified receptor proteins of *Helicoverpa armigera*, *Earias vitella* (positive control), and *Tribolium castaneum* (negative control).

**Amino acid sequence comparison:** When the 65 kDa protein was sequenced by Edman degradation, it yielded the following N-terminal amino acid sequence:

A E T S A N Y W A Q D A Q A A I N A R L E (~65 kDa)

When this sequence was used to BLAST search in the Gen Bank (NCBI) protein database, there were no significant sequence similarities to any of the protein in the database, which shows the uniqueness of this N-terminal sequence of protein. This N-terminal sequence of the 65 kDa protein will appear in protein information resources (PIR), under the Accession number A59445.

**Primer-mRNA sequence complimentarity:** To examine sequence complementarity between oligonucleotides synthesized on the basis of the protein sequence and mRNA isolated from the midgut, the Probe was hybridized with mRNAs of *Helicoverpa armigera*. The results show the primer which was designed on the basis of N-terminal sequence of Cry1A binding protein, had sequence similarity with midgut mRNA of *Helicoverpa armigera*.

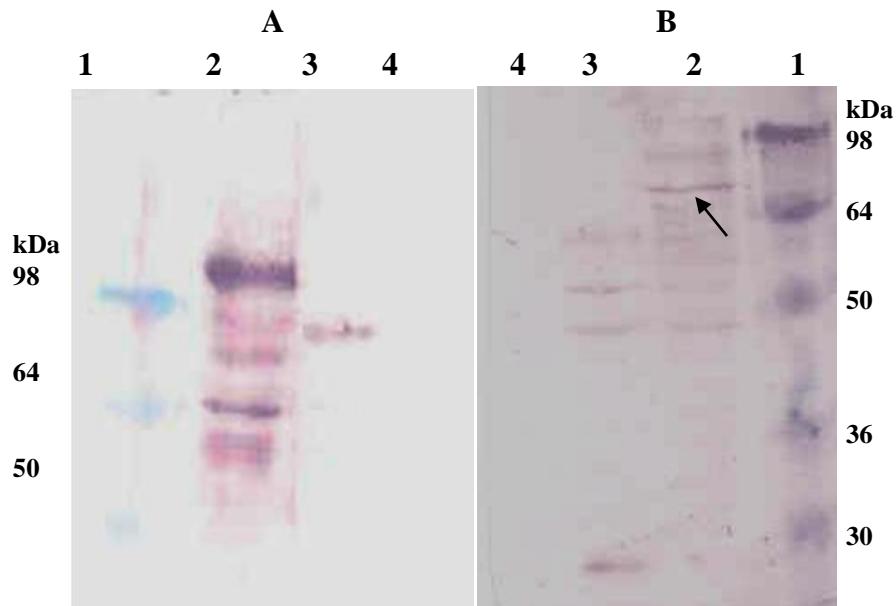


Fig. 5. Western Blot (A) and (B) showing expression of receptor protein gene in *E. coli*. Receptor antibodies raised against 120kDa/65kDa proteins were used to detect the expressed protein in Western Blot (A) and GST antibodies was used to detect the expression protein in the Western Blot (B).

(A) Lane 1: Pre-stained HMW protein standards (Invitrogen). Lane 2: Purified receptor (positive control). Lane 3: Cell extract from 2-3 hours induced recombinant clone. Lane 4: cell extract from non-transformed BL-21(DE3).

(B) Lane 1: Pre-stained HMW protein standards (Invitrogen), Lane 2: GST fusion receptor protein (92kDa) expressed in *E. coli* strain BL21(DE3) was purified by using Glutathione Sepharose 4 Fast Flow, Lane 3: Cell extract from 2-3 hours induced recombinant clone, Lane 4 : cell extract from non-transformed BL-21(DE3).

**Cloning and expression of the protein:** To determine whether the 65kDa protein is a *Bt*-receptor capable of binding to Cry1A toxins, its cDNA was cloned into the pGEX-4T3 vector. The recombinant protein was purified by using Glutathione Sepharose 4 Fast Flow, was used in Western blotting and Ligand blotting with the antibody generated against native 65 kDa protein, Fig. 5 shows that the results are positive. As expected, a 92 kDa fusion protein (GST-receptor protein) was detected in western blots by using receptor antibody and anti-GST antibody (Fig. 5 A; lane 3 Fig. 5 B; lane 2). Ligand blot of cloned, expressed receptor protein also gave positive results. Expressed receptor protein seems to be unstable in crude lysate in prokaryotic system of expression, as appeared in western blot with anti-GST (Fig. 5 B; lane 3).

These results clearly show that the 65 kDa protein may function as a receptor protein for the binding of Cry1A to the larval midgut which is presumably the first step in the larvicidal action of Cry1A *Bt* proteins. Expressed *Bt*- receptor protein exhibited similar properties both *in vitro* and *in vivo*.

**Aminopeptidase activity of Cloned protein:** Aminopeptidase activities of cloned, purified protein and cell lysate were determined by using L-leucine-p-nitroanilide. The purified protein has three time more activity (52 $\mu$ mole/min/mg) than cell lysate (14 $\mu$ mole/min/mg) indicating that the purified protein is aminopeptidase in nature.

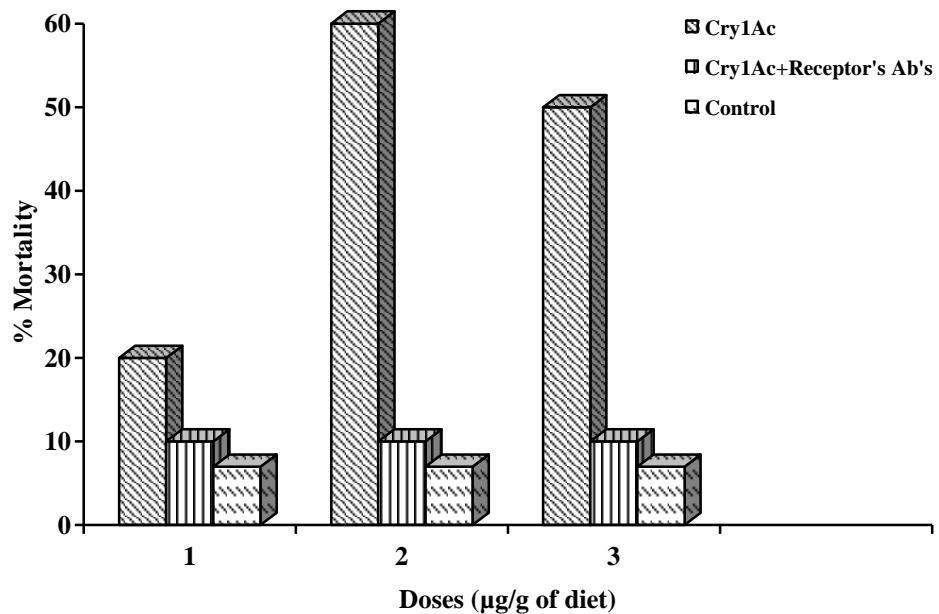


Fig. 6. Determination of change in mortality rate in 2nd instar larvae of *Helicoverpa armigera*, fed on anti-Receptor serum containing artificial diet prior to Cry1Ac containing artificial diet.

**Biotoxicity assay:** We assumed that antibodies produced to Bt-receptor will inhibit binding of the toxin to the receptor in BBMV. We therefore studied larval mortality and the results showed that antibody serum caused reduction in Cry1Ac activity against *Helicoverpa armigera*, when it was fed to larvae prior to toxin addition to the diet, only 10% larval mortality was observed which is near to control set larval mortality value (Fig. 6).

### Discussion

The present study showed that purified protein both in native and expressed forms, binds to Cry1Aa, Cry1Ab, Cry1Ac toxins but not to Cry2A. Similar studies in *Helicoverpa zea* have shown that Cry2A neither binds to BBMVs saturably nor inhibit subsequent binding of Cry1Ac. Further, Cry2A did not lead to the formation of voltage independent cation selective channels in planar lipid bilayers, a characteristic of Cry1Ac (English *et al.*, 1994). Obviously, therefore, Cry2A protein can be used in combination with Cry1Ac protein in a strategy to delay insect resistance build up during the continued use of Bt insecticidal proteins.

The 65 kDa protein from midgut of *Helicoverpa armigera*, exists in monomeric (65 kDa) and dimeric (130 kDa) forms. The expression of cloned gene of 65 kDa protein was studied in *E.coli*. The expressed GST fusion protein was purified through Glutathione Sepharose 4 Fast Flow column. Larval mortality of *H. armigera* to Cry1A toxin was considerably reduced when the larvae were prefed a diet containing antibodies to the 65

kDa protein, presumably due to blocking of the receptor sites in BBMVs. Similar results have been reported by Nagamatsu *et al.*, (1998) in *B. mori*.

In similar studies we have identified and purified a 90 kDa protein from *Earias vitella*, that displays binding with CryIA proteins. The 90 kDa purified protein of *Earias vitella* exhibited binding with antibodies of receptor protein of *Helicoverpa armigera* in Western blot experiment.

When our research paper was in preparation, a report appeared by Rajagopal *et al.*, (2003) that showed existence of two aminopeptidases (APN1, APN2) in *Helicoverpa armigera*, with molecular sizes of ~ 112kDa and ~113 kDa. We have found the 65 kDa protein as Bt Cry1A receptor in *Helicoverpa armigera* and it exhibits aminopeptidase activity, so it appeared that more than one APNs are present in *Helicoverpa armigera* as in *M. sexta* (Denolf *et al.*, 1997), *H. punctigera* (Emmerling *et al.*, 2001) and in *H. virescens* (Jurat-Fuentes & Adang 2001). All three aminopeptidases in *H. punctigera* are more closely related to APNs from other lepidopterans than they are to each other. This report of three different aminopeptidases N in *Helicoverpa punctigera* adds support to a recent suggestion that at least one gene duplication has taken place in ancestral lepidopterans. Chang *et al.*, (1999) and Garner *et al.*, (1999) suggested that gene duplication explains the existence of multiple aminopeptidases. Such aminopeptidase diversity could explain the heterogeneity observed with BBMV preparations used to determine toxin affinity. Clearly, more than one or two toxin-binding aminopeptidases are present in the insect midgut, and each enzyme may exhibit different affinities. Giordana *et al.*, (1989) revealed that for insects, the presence of multiple related aminopeptidases is important for hydrolysis of amino acids from peptides and subsequent transport of amino acids by amino acid transporters across the brush border membrane. It has been shown that Cry1Aa toxin specifically inhibits K<sup>+</sup>-dependent leucine transport either by acting on the transporter itself or by acting through an associated protein (Parenti *et al.*, 1995). Toxin insertion in the vicinity of the aminopeptidase that may be associated with the leucine transporter could lead to inhibition of leucine transport into the cell.

Understanding the molecular biology of insect receptors for Cry toxins will be a crucial part of gaining better control over the use of Bt biopesticides by allowing the engineering of more effective toxins in terms of longer persistence in the field, higher toxicity, customized spectrum of toxicity, and ultimately control of resistance development in crop pests. For these reasons, it is imperative to gain a complete understanding of toxin mode of action and the role that receptors play in this mechanism.

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