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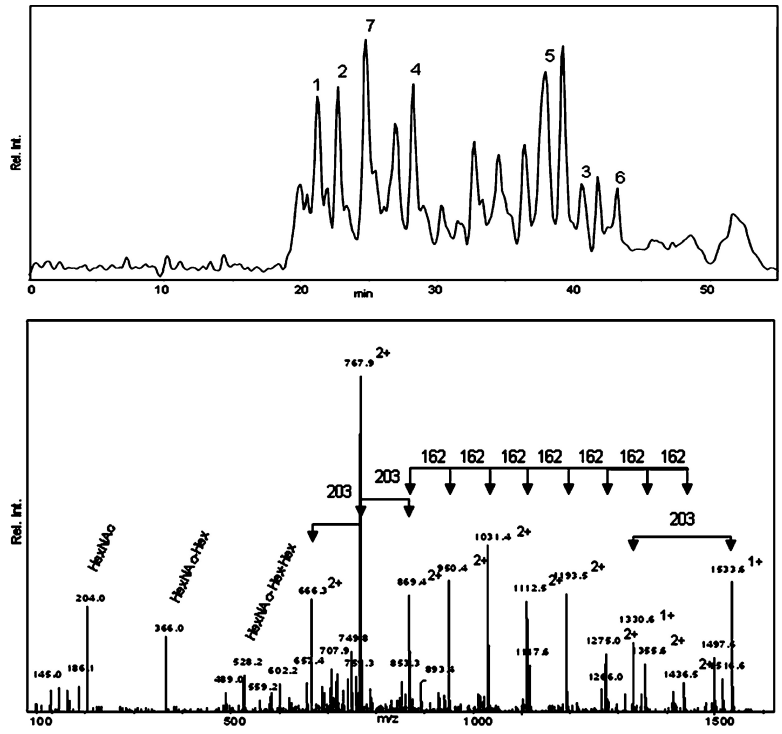


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# Homodimeric Alkaline Phosphatase Located at *Helicoverpa armigera* Midgut, a Putative Receptor of Cry1Ac Contains α-GalNAc in Terminal Glycan Structure as Interactive Epitope†

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*Helicoverpa armigera* causes massive yield loss of various crops globally. *Bacillus thuringiensis* coded Cry1Ac toxin is effective against the pest. Through the present investigation, an alkaline phosphatase type putative receptor of the toxin was identified in the target insect gut BBMV. Lectin-ligand immunoblot assay detected the presence of α-GalNAc residue at the nonreducing terminal of the glycan structure in the membrane bound HaALP protein which mediates the toxin-receptor interaction.

**Keywords:** Alkaline phosphatase • Cry1Ac • *Helicoverpa armigera* • Putative receptor • Toxin receptor interaction

## Introduction

*Helicoverpa armigera* (Hübner) is one of the most serious and widespread lepidopteran pests in the world that infests cotton, pigeonpea, chickpea, pea, cowpea, tobacco, sunflower, and tomato among others[1] and destroys pulses and cotton worth over US$ 530 million per annum in India and worldwide. Yield loss of chickpea has been estimated at over US$ 330 million annually.[2] To overcome this problem, a number of insecticidal genes have been analyzed and used for the development of insect-resistant transgenic plants throughout the world. Among them, *cry* genes produce environment friendly Insecticidal Crystal Proteins (ICP), Bt toxins or δ-endotoxins, coded by the spore-forming bacteria *Bacillus thuringiensis* during its sporulation stage which makes up 90% of the world biopesticide market.[3]

These bioinsecticides (ICPs) are mainly used to control insect species from the orders Lepidoptera, Diptera and Coleoptera.[4] Among all of these ICPs, Cry1Ac toxin in particular has been established as efficient controlling agent against *H. armigera*.[5] Subsequently, Cry1Ac coding sequence, in synthetic, modified and codon-optimized forms, has been expressed in cotton and many other crops. But the big concerns regarding the use of transgenic crops for insect control are the possibility of developing resistance in the insect populations[6] as well as the biosafety related issues of the toxins. Hence, a proper understanding of the machinery of the toxins acting on target insects is highly solicited. The information regarding the specific interactive processes taking place between the receptors and the toxins followed by their binding at the molecular level is still incomplete. Till date it has been known that insertion of the toxin into the midgut membrane after binding to the specific receptors leads to the disruption of the electrical, $K^+$ and pH gradients and osmotic balance leading to the irreversible damage to insect midgut wall through cell lysis and consecutive death of the insect.[7] In one of the recent studies, it has been indicated that the binding to the receptors further activates the intracellular signaling cascades which results in apoptosis.[8] Recently, Gómez et al. reported that Cry toxins interact sequentially with multiple receptors.[9] The Cry1A monomeric toxins primarily interact with a receptor which further triggers the oligomerization of the toxins and these toxin oligomers then bind to a second receptor inducing insertion into the membrane microdomains and lead ultimately to larval death. Whatever may be the phenomenon, the foremost requisite is the efficient binding between the toxin and the gut membrane receptor. In due course of time, a number of receptors were detected in *H. armigera*[10−14] and some of them have been identified as aminopeptidase and cadherin-like receptors. Similar aminopeptidase and cadherin-like receptors as well as alkaline phosphatase type of receptor have also been identified in another important insect pest, *Heliothis virescens*[15−17] from the same *Heliothine* group.

During the present study, few more putative receptors of Cry1Ac toxin were detected in the midgut membrane of *H.*

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*armigera* by ligand-binding approach. We are reporting here one of the important detected putative receptors of Cry1Ac toxin from the brush border membrane vesicles (BBMV) of the midgut of this insect that has been identified by LC-MS/MS analyses as a membrane bound form of Alkaline phosphatase (ALP; EC 3.1.3.1), which has similarity with the membrane bound form of ALP (PPB_BOMMO) from *Bombyx mori*. This presently observed alkaline phosphatase-type putative receptor molecule has been designated as HaALP. The presently identified putative receptor molecule has been studied through extensive biochemical and biophysical experiments and was characterized as a homodimeric membrane bound protein of molecular weight ~138 kDa. A unique lectin competitive binding study between HaALP and Cry1Ac revealed that Cry1Ac may affect *H. armigera* by binding to the insect membrane-bound vital enzyme alkaline phosphatase through a glycosylation mediated reaction. An α-GalNAc residue was found to be the key molecule in the terminal glycan structure of HaALP which mediates the specificity of the Cry1Ac toxin for binding to this putative receptor, though earlier it was reported that Cry1Ac can bind to $\beta$-GalNAc residue of the HvALP receptor purified from *H. virescens*.[17] The presence of glycosylation was further confirmed by LC-MS/MS analyses. The presently described membrane bound enzyme has various important roles in insect metabolism in the uptake of nutrients. In other experiments, we have also demonstrated that Cry1Ac toxin has been found to be a potent blocker of the enzymatic activity of the HaALP molecule which further implicates the importance of the toxin in insect control and high productivity of food crops. This phenomenon of effective blocking of the enzymatic activity of the HaALP after Cry1Ac binding may provide us new information about the plausible cause of antagonistic activity of the Cry1Ac toxin on *H. armigera*.

## Experimental Section

**Cry Toxin Purification.** *B. thuringiensis* var. *kurstaki* HD73 was cultured and the spores were collected. Parasporal toxic crystals were separated from spores using 50−75% sucrose density gradient centrifugation at 25 000*g* for 2 h.[18] Isolated, ~133 kDa protoxin was dissolved in 50 mM $Na_2CO_3$, 10 mM DTT, pH 10 at 37 °C for 1 h and then activated with 2% trypsin (Sigma) at 37 °C for 2 h.[19] The trypsinized sample was dialyzed overnight at 4 °C against 20 mM Tris-HCl, 50 mM NaCl, pH 8.5. Further toxin purification was achieved using size-exclusion Sephadex G-75 column chromatography (Amersham Biosciences) with a flow rate of 0.633 mL $min^{-1}$. The eluted ~66 kDa toxin was finally dialyzed against 50 mM Tris-HCl, 10 mM EDTA, pH 8.5.

**Preparation of Brush Border Membrane Vesicles.** Second to third instar larva of *H. armigera* selected for this study were collected from the laboratory culture of ICRISAT, Patancheru, India. After dissection of the larvae, the midgut was taken out and homogenized in a hand-held Potter homogenizer in a minimum volume of isolation buffer (300 mM D-Mannitol, 5 mM EGTA in 12 mM Tris-HCl, pH 7.4). In presence of $MgCl_2$, differential centrifugation steps were applied to prepare the BBMV.[20] The pellet V (P-V) fraction containing BBMV was verified by marker enzyme assay and SDS-PAGE analysis. All centrifugation steps were carried out in a Hettich tabletop refrigerated centrifuge at 4 °C and BBMV proteins were quantified by Bradford assay.[21]

**Purification of Membrane-Bound Alkaline Phosphatase.** The P-V fraction was suspended at 5 mg $mL^{-1}$ concentration in a buffer containing 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.2 mM PMSF, and 0.2% 3-[(3-Cholamidopropyl) dimethyl ammonio]-1-propane sulfonate and subjected to gel filtration chromatography using a pre-equilibrated column containing Sephadex G-150 (Amersham Biosciences) and eluted in a buffer containing 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, and 0.2 mM PMSF. The purified protein was dialyzed against a buffer containing 20 mM Tris-HCl, pH 7.4, 0.2 mM PMSF, with two to three changes. The protein was concentrated using a Centricon-30 ultrafiltration device (Amicon). The purified protein was analyzed using 10% SDS-PAGE,[22] two-dimensional gel electrophoresis[23] and subsequent ligand blot experiment.

**Ligand Blot Assay.** Approximately, 50 $\mu$g of BBMV samples solubilized in 1% sodium deoxycholate was separated in duplicate lanes of 10% SDS-PAGE. After electrophoresis, one of the lanes was stained with Coomassie brilliant blue and the other was transferred electrophoretically to a Hybond-C (Amersham Biosciences) membrane in a Hoeffer submerged electroblot apparatus. The membrane was transiently stained with Ponceau-S (Sigma). The blotted membrane was blocked with 5% BSA (Sigma) in 1× TBST (Tris Buffered Saline containing Tween 20) for 1 h and further incubated in approximately 1 nM Cry1Ac in 1× TBST for 1 h. The membranes was washed with three changes of TBST each for 2 min and incubated with anti-Cry1Ac polyclonal antibody (1:4000 dilution) for another hour. After thorough washing with TBST as done before, the membrane was then incubated in anti-rabbit IgG horseradish peroxidase (HRP) conjugate (Sigma) at a dilution of 1:10 000 for 1 h. The bound antibody was detected using an ECL kit (Amersham Biosciences) on Kodak X-Ray film.

**Identification of the Putative Receptor Using LC-MS/MS.** The protein spot detected in 2-D PAGE was excised, reduced with 10 mM DTT, alkylated with 55 mM iodoacetamide, and cleaved with trypsin (Promega, sequencing grade, porcine) in 50 mM ammonium bicarbonate buffer (pH 8.0) at 37 °C for overnight.[24] The extracted peptides were analyzed by capillary liquid chromatography tandem mass spectrometry (LC-MS/MS) using a 100 $\mu$m × 10 cm Magic C18 HPLC column (Spectronex, Switzerland) connected on-line to a 4000 Q Trap (MDS Sciex, Concord, Ontario, Canada). A linear gradient from 15% to 45% buffer B (0.1% formic acid, 80% acetonitrile in $H_2O$) in buffer A (0.1% formic acid, 2% acetonitrile in $H_2O$) was delivered using a 1100 Nano-HPLC system (Agilent, Palo Alto, CA) at 300 nL $min^{-1}$ in 30 min. The peptides were loaded for 5 min at a flow rate of 10 $\mu$L $min^{-1}$ in 5% buffer B onto a peptide captrap (Michrom BioResources, Inc.) which was then switched into the nanoflow system. The eluting peptides were ionized by electrospray ionization. The masses of the peptide ions were measured in the linear ion trap; then, the detected ions were automatically selected in Quadrupol 1 and fragmented in Quadrupol 2, and the generated ions were measured in the linear ion trap. Individual MS/MS spectra, containing sequence information for a single peptide, were compared using the program Mascot[25] against the protein sequence database Uniprot from April 2006 (2 952 845 sequences). Uniprot is the combined Swiss-Prot and TrEMBL protein sequence database. This resulted in the identification of the peptides and, by association, of the protein in the spot. After the identification

of the protein, an error-tolerant search was performed allowing the identification of additional homologous peptides.

**Kinetic Study of Alkaline Phosphatase Activity.** For spectrophotometric assay of alkaline phosphatase (AP), the known marker enzyme of BBMV, *p*-nitrophenyl phosphate (*p*-NPP) was used as the substrate. The enzyme activity was monitored at 405 nm by chasing the increasing OD of *p*-nitrophenol.[26] 0.5 units (432 ng) of commercially available alkaline phosphatase (Sigma) was used as standard and the change in OD ($\Delta OD_{405nm}$) was recorded at every 30 s up to 15 min. To determine the enzyme activity of all the fractions in the process of BBMV preparation, aliquots of various fractions were added to the reaction cocktail and kept for 10 min at room temperature and $OD_{405nm}$ was monitored. The specific activities of all fractions were regressed from the specific activity of the commercially available AP. $K_m$ and $V_{max}$ values of the HaALP toward *p*-NPP substrate were calculated using seven different substrate concentrations (0, 2.7, 5.4, 10.8, 16.2, 21.6, and 26.9 mM) at pH 10.3, incubated for 10 min at 37 °C. The pH and temperature optima were calculated using buffers containing different pH (8.05, 8.51, 8.9, 9.4, 10.14, 10.6, and 11.5) with 10.8 mM substrate concentration and with buffer containing 10.8 mM substrate having pH 10.3, at seven different temperature conditions (0, 10, 25, 37, 60, 80, and 100 °C). The enzymatic activity of HaALP after Cry1Ac binding was also studied by incubating 1.5 nM purified HaALP with 0.75, 1.5, 3, and 6 nM purified Cry1Ac using seven different buffers, having *p*-NPP concentrations of 0, 2.7, 5.4, 10.8, 16.2, 21.6, and 26.9 mM at pH 10.3 and 37 °C. The $K_I$ values of the different reactions at different stoichiometric ratios were calculated. The percent of alkaline phosphatase activity of midgut homogenate (MGH) bound ALP was also measured after incubating the same without and with Cry1Ac at increasing weight/weight ratios of MGH to Cry1Ac which were 1:0, 1:0.1, 1:0.2 and 1:0.4 with 10.8 mM substrate concentration at pH 10.3 and 37 °C.

**Alkaline Phosphatase Activity in SDS-PAGE and Membrane Blot.** To detect the alkaline phosphatase activity in BBMV proteins from the P-V fraction, 100 $\mu$g of solubilized protein was taken in the SDS-PAGE sample buffer and were not heat denatured before gel loading in 10% SDS-PAGE. The proteins were transferred to Hybond C membranes and were subjected for the alkaline phosphates activity according to the protocol described by Jurat and Adang.[17]

**Glycoprotein-Specific Staining.** The total protein extracted from the BBMV was dissolved in 1% sodium deoxycholate and was separated using 10% SDS-PAGE and stained specifically for covalently bound oligosaccharide moieties according to the methods of Moller and Poulsen.[27] This method involves periodic acid oxidation of the fixed proteins in SDS-PAGE, staining with Alcian blue, and subsequent silver enhancement staining at high temperatures to specifically stain the glycoproteins. Finally, the stain was developed with 2.5% sodium carbonate for 30−60 s. The staining profile was recorded and the gel was further stained with Coomassie brilliant blue according to standard procedures to stain the total proteins, including the nonglycosylated ones.

**Deglycosylation of HaALP Protein and Ligand Blot.** The total BBMV proteins from *H. armigera* were subjected to deglycosylation, using the *N*-glycosidase F (Roche) and *O*-glycosidase (Sigma) according to the protocol described in the supplied enzyme manual. The deglycosylated samples were boiled with SDS-PAGE sample buffer and run in a 10% SDS-polyacrylamide gel. Nondeglycosylated BBMV proteins were loaded onto a separate well of the same gel. Ligand blot analysis of the samples separated in the gel was carried out as mentioned before.

**Lectin Ligand Immunoblotting Assay.** HaALP proteins (1 $\mu$g each) were separated using 10% SDS-PAGE and electroblotted onto Hybond-C membrane. After transfer, the membrane was blocked for 1 h at room temperature with 1× TBST containing 0.1% Tween-20 and 5% BSA. Blocked membranes containing HaALP protein were incubated independently with lectins from *Canavalia ensiformis* [ConA (Sigma) at 0.05 $\mu$g $mL^{-1}$], *Ricinus communis* [RCA-I (Sigma) at 5 $\mu$g $mL^{-1}$], *Vicia villosa* [$VVLA_4$ (Sigma) at 5 $\mu$g $mL^{-1}$], *Triticum vulgare* [WGA (Sigma) at 5 $\mu$g $mL^{-1}$], and *Lotus tetragonolobus* [LTA (Sigma) at 5 $\mu$g $mL^{-1}$] for 1 h in blocking buffer (1× TBST with 2.5% BSA). The membranes were then washed with three changes of TBST for 2 min each time and further incubated with 5 $\mu$g $mL^{-1}$ Cry1Ac in 1× TBST containing 2.5% BSA for 1 h. After that, the membranes were washed as described previously and then incubated with anti-Cry1Ac polyclonal antibody (1:4000 dilutions) for 1 h. After thorough washing with 1× TBST as mentioned before, the membranes were further incubated with anti-rabbit IgG HRP conjugate (Sigma Immunochemicals) at a dilution of 1:10 000 for 1 h and finally detected using the ECL kit (Amersham Biosciences) on Kodak X-Ray film. Three other membrane strips containing HaALP were incubated, after blocking, with ConA, RCA-I, and $VVLA_4$ lectins presaturated with specific hapten sugars: α-D-mannose, β-D-galactose and *N*-acetylgalactosamine (GalNAc) for 30 min at room temperature before probing with the Cry1Ac antigen, and primary and secondary antibodies. Ligand-binding experiment on other set of membranes was performed using Cry1Ac preincubated with three different hapten sugars: α-D-mannose, β-D-galactose, and GalNAc, without any lectin, for 30 min at room temperature.

## Results

**Purification and Detection of the ~138 kDa HaALP.** The P-V fraction of the insect BBMV protein collected during purification process was run in 10% SDS-PAGE. The total protein profile was monitored through Coomassie brilliant blue staining (Figure 1A). Subsequently, ligand-positive binding proteins of Cry1Ac were detected as shown in Figure 1B. The total BBMV protein was subjected to gel filtration chromatography and a fraction corresponding to ~138 kDa was separated in 10% SDS-PAGE (Figure 1C) as well as two-dimensional gel electrophoretic analyses (Figure 1E). The fraction was further subjected to ligand blot analyses. This fraction showed a signal in the region of ~68 kDa in the ligand blot experiment of SDS-PAGE (Figure 1D), and a single spot in the isoelectric focusing (Figure 1F) that clearly indicated the homodimeric nature of the fraction comprising putative receptor protein (Gel filtration profile of the purified HaALP calibrated with known molecular weight markers is available in the Supporting Information, Figure S1).

**LC-MS/MS and BLASTP Analyses.** The tryptic peptide fragments of the putative receptor protein which was previously excised from the 2-D PAGE was analyzed using MS/MS. Figure 2A shows the base peak chromatogram from the MS/MS analysis of the tryptic digests of the protein. The spectra were analyzed with MASCOT, and three peptides from the putative receptor protein matched with a 59 235 Da membrane-bound alkaline phosphatase [Precursor] or EC 3.1.3.1 mALP isolated from *B. mori* (silk moth) (Swiss-Prot identifier PPB_BOMMO,

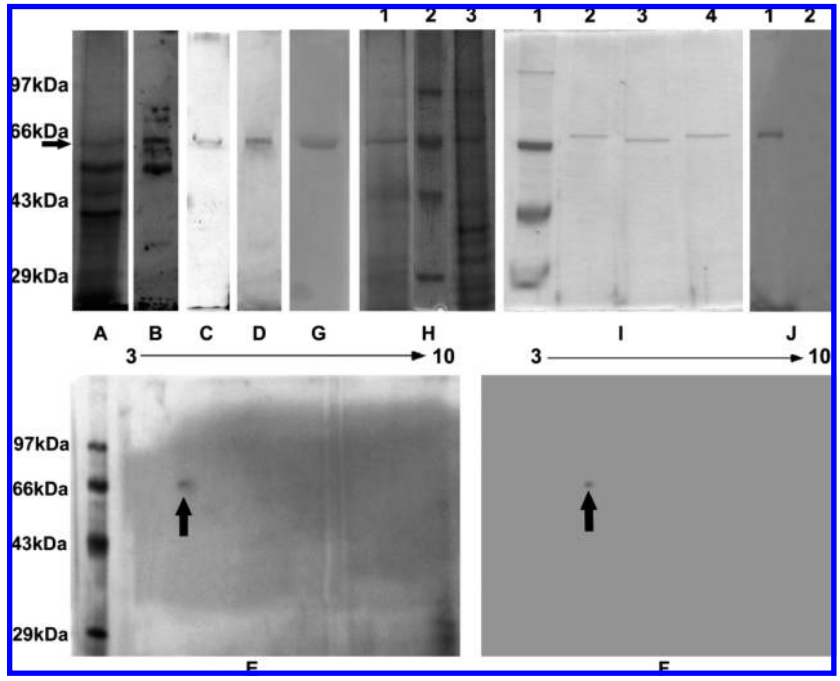


**Figure 1.** BBMV protein profile in SDS-PAGE and its ligand blots. (A) Coomassie-stained total BBMV protein. (B) Ligand blot analysis of the total BBMV protein detected by Cry1Ac toxin; arrowhead indicates 68-kDa HaALP protein. (C) Purified HaALP showing single band. (D) Ligand blot analysis of the purified HaALP. (E) Two-dimensional PAGE. Arrow shows the position of the spot. The HaALP protein separated at a pH range of 5.5−5.8. Isoelectric focusing was run with 3−10 pH range carrier ampholytes in the first dimension. (F) Ligand blot analysis from a replica of the 2D gel shown in E. (G) Alkaline phosphatase activity of blotted HaALP challenged by Cry1Ac toxin. (H) Glycospecific and Coomassie-stained profile of total BBMV protein: lane 1, the glycoprotein-specific staining of *H. armigera* total BBMV protein; lane 2, Coomassie-stained molecular weight marker; lane 3, Coomassie-stained total proteins after glycoprotein-specific staining. (I) Purified HaALP before and after deglycosylation: lane 1, Coomassie-stained molecular weight marker; lane 2, nondeglycosylated purified HaALP; lane 3, deglycosylated HaALP using *N*-glycosidase F; lane 4, deglycosylated HaALP using *O*-glycosidase. (J) Ligand blot of HaALP detected by Cry1Ac: lane 1, ligand blot of purified HaALP; lane 2, ligand blot of *N*-glycosidase F treated HaALP.

primary accession number: P29523). Besides this, an error-tolerant MASCOT search, allowing single amino acid mutations, identified three additional peptides. The alignment of the peptides is shown in Figure 2B. The sequences of the peptides were identified with MASCOT using standard scoring (Table 1). On the other hand, the BLASTP tool (Basic Local Alignment Search Tool) of the NCBI server (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to detect the homology between the LC-MS/MS generated HaALP peptides with the recently published two full-length sequences of *H. armigera* alkaline phosphatases which have been published in NCBI database with accession numbers ACF40806 and ACF40807. The percentage of identities between the LC-MS/MS generated peptides and reported sequences have been calculated (Table 2). It was found that three out of six peptides of HaALP have 100% identities; one shows 94% identity and the other two are different from that of the published sequences. This gives further evidence that the currently identified HaALP may be somewhat different from that of the reported ALPs.

**Analysis of Alkaline Phosphatase Activity of HaALP, the Putative Receptor.** A total of 100 $\mu$g of nonheat-denatured P-V fraction of BBMV preparation was run in 10% SDS-PAGE and blotted onto a Hybond-C membrane. The blotted membrane was treated according to the protocol described by Jurat and Adang.[17] A purple-red precipitate was generated in the region of ~68 kDa (Figure 1G) which was further verified for alkaline phosphatase activity. The kinetic study was performed with the commercially available alkaline phosphatase from Sigma, the P-V fraction of the *H. armigera* BBMV and the column purified ~138 kDa protein. The specific activity for the commercially available alkaline phosphatase was calculated to be of 1170 U $mg^{-1}$ protein. The specific activity of the enzyme was found to be 14.02 and 343 U $mg^{-1}$ protein for the P-V fraction and the purified protein, respectively. The $K_m$ and $V_{max}$ were calculated as 1.58 ± 0.02 mM and 384.63 ± 1.71 $\mu$mol $min^{-1}$ $mg^{-1}$ (Figure 3A). The catalytic constant or turnover number ($K_{cat}$) of HaALP was calculated as $19.2 \times 10^4$ $min^{-1}$. The pH optimum of HaALP was calculated as 10.14 (Figure 3C) and the temperature optimum for the enzyme activity was detected at 37 °C (Figure 3D). The ratio of $K_{cat}$ and $K_m$ for HaALP was determined to be $12.2 \times 10^4$ $mM^{-1}$ $min^{-1}$. The inhibition kinetics was studied with a specific concentration of HaALP with different concentrations of Cry1Ac at different stoichiometric ratios and inhibition was found to be maximum at 1:2 molar ratio (HaALP/Cry1Ac), depicted from the lowest $K_I$ value ($0.316 \times 10^{-6}$ mM) and was found to be competitive (Figure 3B). The association/interaction phenomenon between the two molecules is highest at a molar ratio of 1:2. The effective binding of the Cry1Ac toxin to the enzyme molecule may block the enzyme active site of the enzyme and interferes with the activity of the vital enzyme in the insect midgut. Thus, the inhibition of enzymatic activity due to Cry1Ac binding has been found to be competitive. ALP activity of MGH of *H. armigera* has further been investigated in absence and presence of Cry1Ac. Normally, the percentage of activity was recorded as (98.91 ± 0.65)%, whereas the percentage of the same was reduced from (98.91 ± 0.65)% to

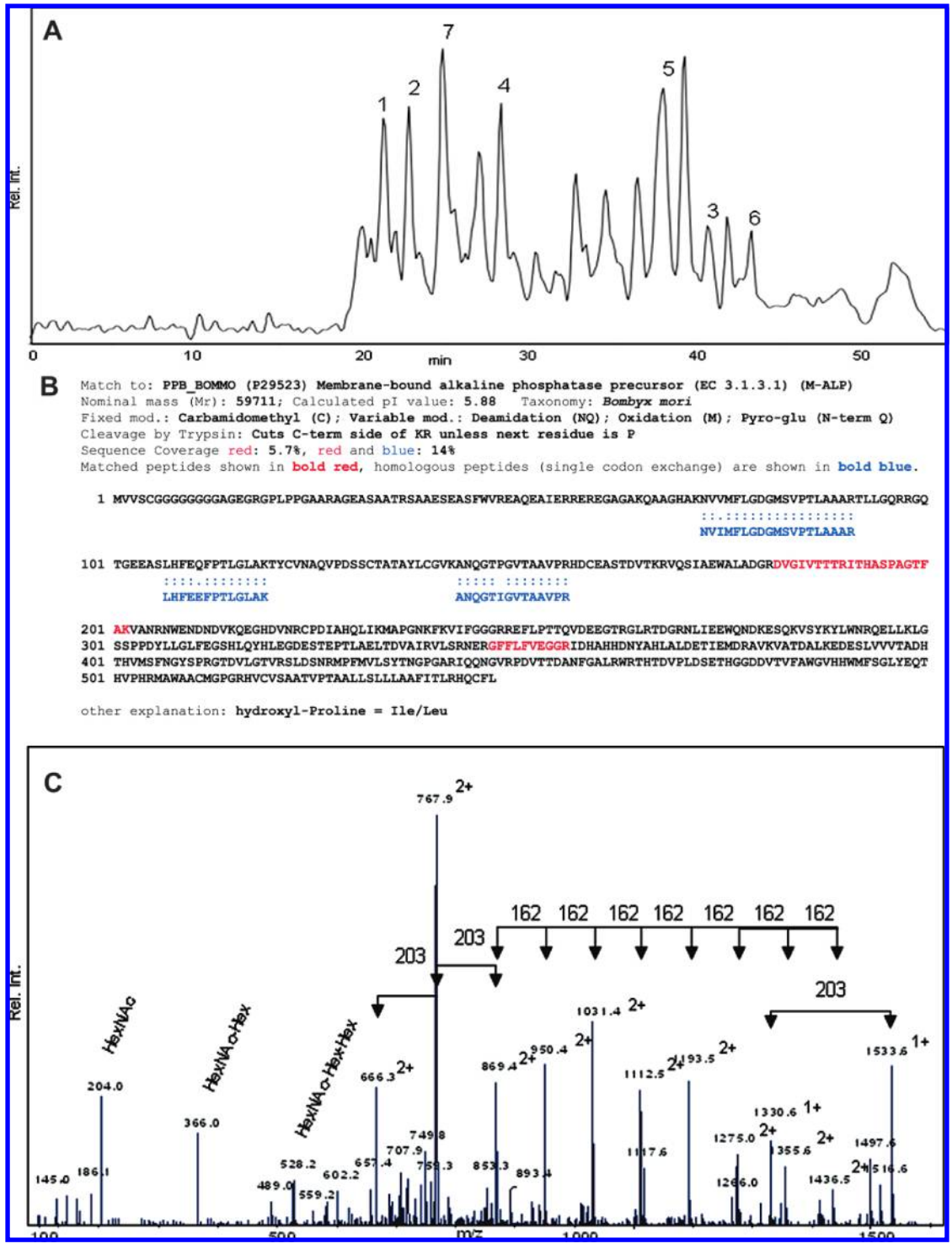


**Figure 2.** Identification of the putative receptor protein through LC-MS/MS after tryptic digestion of HaALP. (A) The base peak chromatogram of the MS is shown. Peptides identified with MASCOT are marked 1−6 (Table 1). (B) Sequence alignment of the peptides identified with MASCOT to the homologous protein from *B. mori*. (C) Product ion spectra of 1066 (fraction 7) showing the glycopeptide obtained in this analysis.

(39.18 ± 0.26)%, (34.57 ± 0.23)% and (16.84 ± 0.26)%, respectively, in reactions where MGH was prior incubated with Cry1Ac at varying weight/weight ratios of 1:0.1, 1:0.2 and 1:0.4. The result confirms that increasing concentration of Cry1Ac can decrease the ALP activity of midgut membrane bound HaALP by approximately 60%, 65% and 82% as shown in Supporting Information Figure 3.

**Characterization of HaALP as Glycoprotein.** Following the glycospecific staining protocol, the glycoprotein bands in the gel were stained within 30 s (Figure 1H, lane 1) where the nonglycosylated proteins remained unstained (Figure 1H, lane 1). Subsequent Coomassie brilliant blue staining of the gel detected all glycosylated, as well as the nonglycosylated, proteins (Figure 1H, lane 3). It became evident that the ligand-

**Table 1.** Sequenced Peptides of HaALP as a Result of LC-MS/MS Analysis[a]

| peptide identified using MASCOT | peptides in Figure 2A | precursor mass | charge state | $M_r$ (expt) | $M_r$ (calc) | score |
|---|---|---|---|---|---|---|
| ITHASPAGTFAK | 1 | 600.89 | 2 | 1199.77 | 1199.63 | 54 |
| DVGIVTTTR | 2 | 481.24 | 2 | 960.47 | 960.52 | 38 |
| GFFLFVEGGR | 3 | 564.84 | 2 | 1127.67 | 1127.58 | 53 |
| ANQGT**L**GVTAAVPR | 4 | 677.86 | 2 | 1353.71 | 1353.70 | 79 |
| LHFE**E**FPTLGLAK | 5 | 751.57 | 2 | 1501.13 | 1500.8 | 38 |
| NV**L**MFLGDGMSVPTLAAAR | 6 | 655.02 | 3 | 1962.04 | 1962.01 | 74 |

[a] Peak 7 corresponds to the analyzed glycopeptides.

**Table 2.** BLASTP Analyses with the Identified Peptides of HaALP through LC-MS/MS Analysis with the Recently Published *H. armigera* ALP Sequences from NCBI Database[a]

| peptides identified using MASCOT | NCBI accession numbers | peptides matched | % of identities |
|---|---|---|---|
| ITHASPAGTFAK | ACF40807 | ITHASPAGTFAK | 100 |
| | ACF40806 | ITHASPAGTFAK | 100 |
| DVGIVTTTR | ACF40807 | DVGIVTTTR | 100 |
| | ACF40806 | DVGIVTTTR | 100 |
| GFFLFVEGGR | ACF40807 | GFFLFVEGGR | 100 |
| | ACF40806 | GFFLFVEGGR | 100 |
| **A**N**Q**G**TL**GV**TAA**V**P**R | ACF40807 | NNYGAIGVDGTVRR | 46 |
| | ACF40806 | NNYGAIGVDGTVRR | 46 |
| LHFE**E**FPT**L**GL**A**K | ACF40807 | LHFETFPTIGLVK | 76 |
| | ACF40806 | LHFETFPTIGLVK | 76 |
| NV**L**MFLGDGMSVPTLAAAR | ACF40807 | NVIMFLGDGMSVPTLAAAR | 94 |
| | ACF40806 | NVIMFLGDGMSVPTLAAAR | 94 |

[a] Analyses were done by the server http://blast.ncbi.nlm.nih.gov/Blast.cgi.

positive ~138 kDa HaALP is a glycosylated protein which got stained immediately (Figure 1H, lane 1) (Color figure is available in the Supporting Information, Figure S2). Subsequently, the tryptic peptide generated from the purified protein when analyzed using LC-MS/MS showed a high hexose-type signature within it (Figure 2C). The purified HaALP was digested separately using *N*-glycosidase F and *O*-glycosidase enzymes to investigate the type of linked oligosaccharides present in the side chains. The untreated and treated HaALP were run in 10% SDS-PAGE (Figure 1I). Analysis of HaALP after digestion with two different enzymes demonstrated that the protein is an N-linked glycosylated one, which is evident from the shifting of the protein band after treatment with *N*-glycosidase F and staining with Coomassie brilliant blue (Figure 1I, lane 3). After ligand blot analysis of the deglycosylated HaALP, no visible band could be detected (Figure 1J, lane 2). The result indicated that Cry1Ac binding to this HaALP is mediated by the N-linked glycosyl moieties of the protein (Figure 1J).

**Ligand Blot Analyses of HaALP Intermediated by Lectins and Sugar Saturated Lectins.** Six different lectins having specificity to different sugar residues (Table 3) were used to identify the glycan structure of the N-linked glycosylated HaALP. Ligand positive signals were found in cases of WGA and LTA, but there were no signals in cases of ConA, RCA-I, or $VVLA_4$, suggesting that HaALP contains specific oligosaccharide moieties (Figure 4A) that were recognized by ConA, RCA-I, and $VVLA_4$. In the next experiment, ConA, RCA-I and $VVLA_4$ were preincubated with their specific hapten sugars: α-D-mannose, β-D-galactose, and GalNAc, respectively, and subjected to a ligand binding experiment as mentioned above. The positive signals (Figure 4B) pointed to the fact that Cry1Ac as well as ConA, RCA-I, and $VVLA_4$ lectins bind to HaALP which may be mediated by respective glycan moieties. Three independent sets of HaALP-blotted membranes were treated with sugar saturated Cry1Ac, which had been independently preincubated with three previously mentioned sugars. The blot in which Cry1Ac preincubated with *N*-acetylgalactosamine was used generated no signal but others gave signals (Figure 4C), indicating that the interaction between toxin and HaALP is mediated through the *N*-acetylgalactosamine.

## Discussion

Cry1Ac is a potent controlling agent against *H. armigera* and its toxicity is primarily dependent upon the stringent binding between insect receptors and the toxin. Unraveling the underlying mechanism of such a strict and specific interaction between receptor and toxin molecules needed identification and characterization of the relevant receptors from the target insect gut tissue. Immunofluorescence localization of Cry1Ac toxin on *H. armigera* midgut epithelial cells indicated about the presence of various such specific receptors of the toxic protein on the insect midgut cell surface (data not shown). These receptors get involved in toxin binding followed by oligomerization, interaction, membrane insertion, ion channel disbalance and finally death of the insect. Determination of mechanism of interaction between newly identified receptors and their specific toxin is absolutely essential for the insect resistant crop developmental programs.

**Membrane Bound Alkaline Phosphatase as Putative Receptor of Cry Toxin Detected in *H. armigera*.** Recently, a 120 kDa aminopeptidase-N was identified in the BBMV of *H. armigera* as receptor of Cry1Ac.[10−12] In the same year, Malik et al. reported the presence of 70 kDa aminopeptidase receptors of Cry1Ac in the BBMV of *H. armigera* in addition to the earlier detected 120 kDa protein.[14] Subsequently, few more receptors were also identified through ligand blot analysis using a Cry1Ac-specific antibody in the membrane proteins of the gut cells of *H. armigera*[28] including a cadherin type of receptor.[13] In the present investigation, a homodimeric membrane-bound ALP of molecular weight ~138 kDa was detected in the BBMV of *H. armigera* as putative receptor of Cry1Ac. The protein was

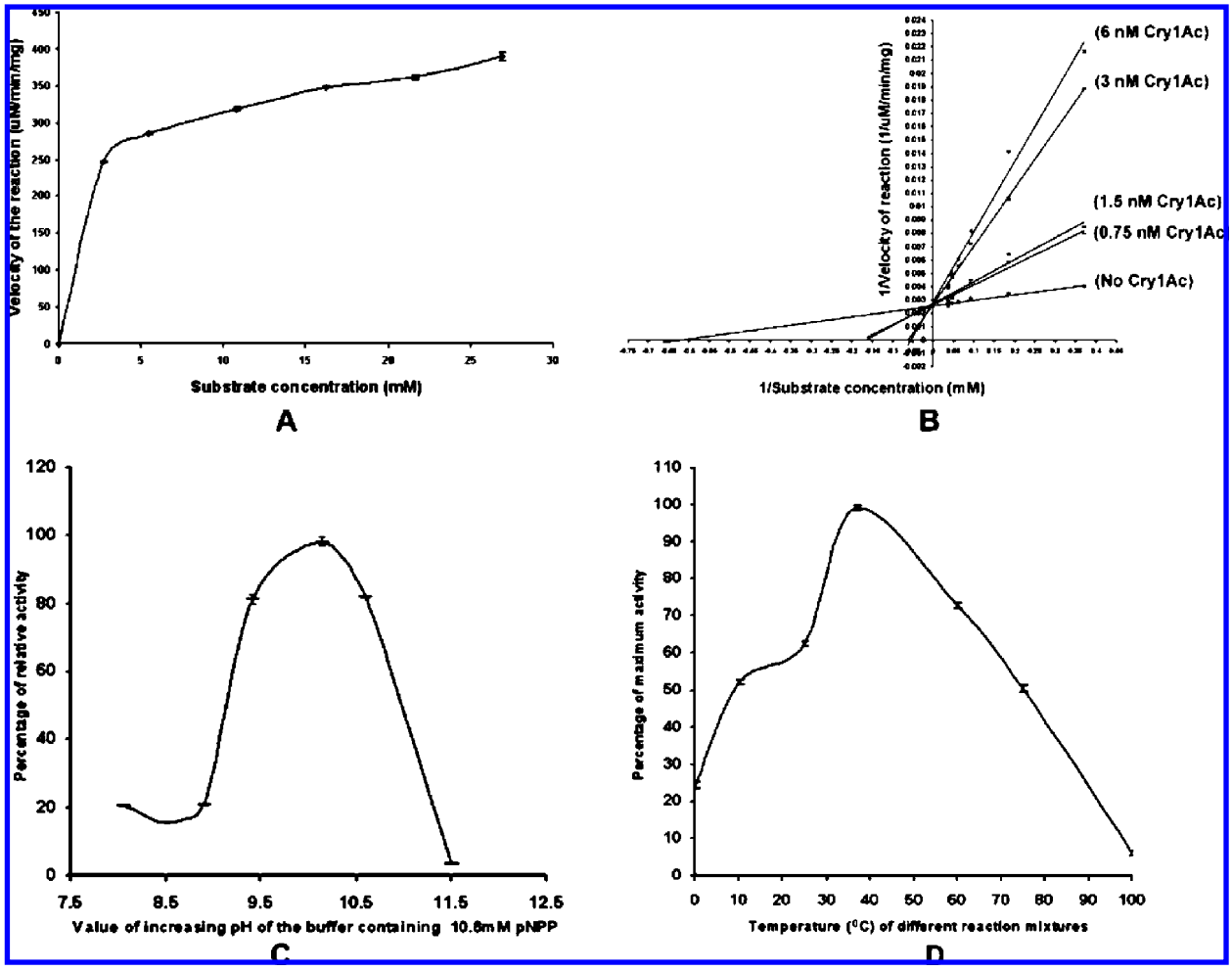


**Figure 3.** Kinetic study of HaALP. (A) Substrate saturation kinetics at pH 10.3 and 37 °C. (B) Double reciprocal plot showing inhibition of HaALP enzymatic activity in the presence of different concentrations of Cry1Ac. (C) Determination of the optimum pH for HaALP activity. (D) Determination of the optimum temperature for HaALP activity.

**Table 3.** Sugar Specificities [Gal, *N*-acetylgalactosamine (GalNAc), *N*-acetylglucosamine (GlcNAc), Man or Glc] of Different Lectins Used in Blots, and Respective Hapten Sugars Used for Lectin Specificity Controls and for Cry1Ac Specificity

| lectins used | sugar specificity of therespective lectin | hapten sugars forthe respective lectins |
|---|---|---|
| *C. ensiformis* (ConA) | α-Man,<br>α-Glc | 0.2 M α-Man/α-Glc |
| *R. communis* (RCA-I) | Gal $\beta$1-4GlcNAc-linked units at nonreducingends,<br>Gal$\beta$1-4GlcNAc$\beta$1−6Gal,<br>Gal$\beta$1-4GlcNAc,<br>Gal$\beta$1-3GlcNAc,<br>Gal$\alpha$1-3Gal,<br>Gal$\beta$1-3GalNAc$\alpha$1-Ser/Thr,<br>NeuAc$\alpha$2-6Gal$\beta$1−4GlcNAc | 0.2 M Gal |
| *V. villosa* (VVLA$_4$) | GalNAc$\alpha$1-3Gal,<br>GalNAc$\alpha$1-6Gal,<br>GalNac$\alpha$1-3Gal$\beta$1-3GlcNAc,<br>GalNAc$\alpha$1-3GalNAc,<br>GalNAc$\alpha$1-3[LFuc$\alpha$1-2]Gal$\beta$1-4GlcNAc$\beta$1-6R,<br>Gal$\alpha$1-3Gal | 0.2 M GalNAc |
| *T. vulgare* (WGA) | Three $\beta$-(1−4) linked GlcNAc residues<br>(GlcNAc$\beta$1-4GlcNAc$\beta$1-4GlcNAc),<br>α-NeuAc at thenonreducing terminalcontaining<br>(NeuAc$\alpha$2-6Gal$\beta$1-4GlcNAc) | 0.2 M GlcNAc |
| *L. tetragonolobus* (LTA) | α-(1−6) linked fucose residue to GlcNAc,<br>α-Fuc-(1−3)-$\beta$-GlcNAc | 0.2 M L-fucose |

characterized using chromatographic, electrophoretic and LC-MS/MS studies, and designated as HaALP, a well-studied vital enzyme found in most organisms. Similar enzyme has been reported in the past as Cry1Ac receptor in other insect species

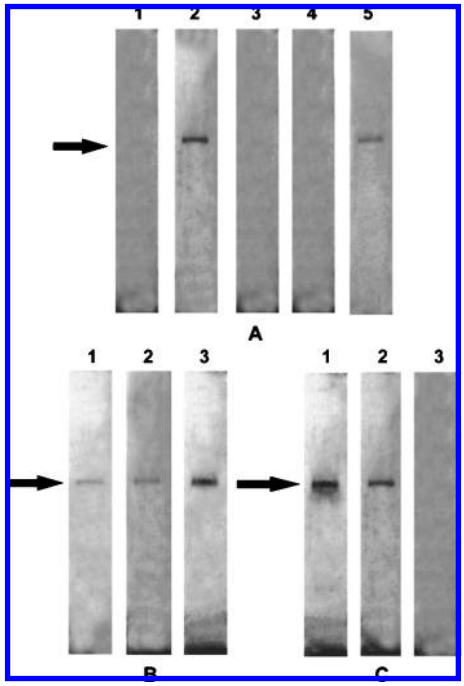


**Figure 4.** Immunoblot analyses of HaALP to identify which carbohydrate moieties are present in the oligosaccharide side chain. (A) Immunoblots of HaALP challenged with Cry1Ac after incubating with lectin: lane 1, blotted membrane treated with ConA; lane2, blotted membrane treated with WGA; lane 3, blotted membrane treated with RCA-I; lane 4, blotted membrane treated with $VVLA_4$; lane 5, blotted membrane treated with LTA. (B) Immunoblots of HaALP challenged with Cry1Ac after incubating with lectin-sugar complex: lane 1, lectin immunoblot using ConA-Man mixture; lane 2, lectin immunoblot using RCA-I-Gal mixture; lane 3, lectin immunoblot using $VVLA_4$-GalNAc mixture. (C) Immunoblots of HaALP challenged with sugar-bound Cry1Ac: lane 1, Cry1Ac-mannose mixture; lane 2, Cry1Ac-galactose mixture; lane 3, Cry1Ac-GalNAc mixture.

(59 kDa in *B. mori*, 65 kDa in *Manduca sexta*, and 68 kDa in *H. virescens*) by previous authors where it has also been mentioned that Cry1Ac toxin inhibits the phosphatase activity of the respective ALP receptors.[17,29−31] An alkaline phosphatase-type receptor against the Cry11Aa toxin has also been identified previously in *Aedes aegypti* larvae.[32] Interestingly, the alkaline phosphatase (ALP) receptors isolated from *B. mori* and *H. virescens* were reported as monomeric, whereas human ALPs of 120−170 kDa have been found to be dimeric or tetrameric proteins.[33] The presently described HaALP, the putative receptor of Cry1Ac which is homodimeric, has been characterized to be a glycoprotein, homologous to the 59 235 Da membrane-bound alkaline phosphatase [Precursor] or EC 3.1.3.1 mALP of *B. mori* (Swiss-Prot identifier PPB_BOMMO, primary accession number: P29523) identified through LC-MS/MS analysis.

**Biochemical Studies of Enzymatic Properties of HaALP.** The ALP enzyme kinetics of the HaALP was studied and specific activity of purified fraction and the partially purified P-V fraction of BBMV was determined which was compared to that of the commercially available alkaline phosphatase. The ratio of $K_{cat}$ and $K_m$ for HaALP was determined to be $12.2 \times 10^4$ $mM^{-1}$ $min^{-1}$. The optimum pH and temperature for HaALP enzymatic activity were also calculated as 10.14 and 37 °C. The results of the kinetic studies were quite comparable with that of the membrane bound ALP from *B. mori*.[34] Interestingly, the stoichiometric ratio of the two interacting molecules (HaALP/Cry1Ac) was found to be 1:2 M, depicted from the lowest $K_I$ value where the chance of dissociation of the two molecules is minimum. In other words, the association/interaction phenomenon between two molecules is highest at the molar ratio 1:2. It has also been found that Cry1Ac can decrease the ALP activity of MGH bound HaALP by approximately 60%, 65% and 82%, respectively, when incubated with different ratios of ALP/Cry1Ac as 1:0.1, 1:0.2 and 1:0.4. The effective binding of the Cry1Ac toxin to the enzyme molecule may block the active site of the enzyme and interferes with the activity of the vital enzyme in the insect midgut. Thus, the inhibition of enzymatic activity due to Cry1Ac binding has been found to be competitive.

ALP, the abundant metalloproteins are known to play a role in phosphate uptake and in secretory processes in mammalian epithelia. In insects, the presence of functional ALP has been found in *Leptinotarsa decemlineata* (Colorado potato beetle), *B. mori* gut, *Bemisia tabaci* (whitefly) salivary glands, *Drosophila melanogaster* brain and lower Malpighian (renal) tubules, as well as in several mosquito species.[35] ALPs also play a crucial role in the active transport of metabolites, fluid transport processes, maintain resting rates of transport[35] and participate in cell adhesion and cell differentiation,[36] indicating about the importance of the enzyme in maintaining the insect cell physiology. The binding of Cry1Ac to this putative receptor and also the competitive inhibition of the enzymatic activity of HaALP by the toxin further confirms that Cry1Ac toxin may create blockage in one or more of the above-mentioned important activities of the enzyme and forms pores on the cell linings in the insect midgut resulting in the ionic imbalance of the cells and or the initiation of intracellular signaling cascade which further leads to cell apoptosis as has been indicated earlier by Jurat and Adang.[8]

**Glycosylation, Key Regulator of the Receptor−Toxin Interaction.** The extensive lectin mediated ligand blot analyses determined the presence of terminal glycosylated moieties in the HaALP which gets involved in Cry1Ac toxin binding and subsequent insect mortality through ionic imbalance followed by cellular destruction. Treatment with *N*-glycosidase F and *O*-glycosidase further proved the presence of an N-linked glycosyl moiety in the HaALP, and the glycosylation does not contain any fucose substitution at the asparagine-linked GlcNAc residue. The glycosylated and deglycosylated proteins were further subjected to ligand binding assay with Cry1Ac and no binding was found in case of deglycosylated HaALP, confirming that the interaction or binding of HaALP with Cry1Ac is specifically mediated by an N-linked glycosylated sugar moiety. This feature of HaALP was further authenticated by analyzing its binding profile through immunoligand blots using different sugar-binding lectins, namely, ConA, RCA-I, WGA, LTA, and $VVLA_4$. Trimannosidic core structure-binding ConA[37] and RCA-I as well as $VVLA_4$ lectins[38] showed binding to HaALP, indicating the presence of specific hapten sugars (Table 3) in the N-linked oligosaccharide side chains of HaALP. Possibly because of the absence of α-NeuAc at the nonreducing terminal, or NeuAcα2-6Galβ1-4GlcNAc- in the peripheral region of HaALP, WGA could not bind to HaALP. Likewise, the lack of LTA binding feature suggested that there is no α-(1−3)- or α-(1−6)-linked fucose residue in the glycosyl moieties of HaALP.[37] Two commonly known sugar substitutes, GalNAcα1-3Gal and Galβ1-4GlcNAc (Table 3), may be responsible for binding to $VVLA_4$ and RCA-I, respectively. Furthermore, $VVLA_4$ binding suggests the presence of α anomeric carbon atom at the terminal GalNAc residue

which is a common feature of O-linked glycosylation. $VVLA_4$ and RCA-I usually bind with decreasing specificity to the carbohydrate structural units A >$A_h$; Tn antigen family and II ⩾ I > E (blood group $p^k$ and $P_1$ active disaccharide); B > T family.[39] The GalNacα1-3Galβ1-4GlcNAc- binding domain is found in the carbohydrate side chains of Hog A + H stomach glycoproteins and Hog gastric mucin no. 4.[40] Both $VVLA_4$ and RCA-I binding strongly indicates the presence of core GalNacα1-3Galβ1-4GlcNAc- structure in HaALP. Nevertheless, the Galβ1-4GlcNAc- type of core structure is present in many N-linked glycosylated proteins[21] as evident in human N-linked glycopeptides isolated from human small intestinal epithelial cells of individuals with blood group A.[41] RCA-I and $VVLA_4$ also bind to other peripheral sugar substitutions (Table 3) found in the O-linked glycosylated proteins. Thus, HaALP may have GalNacα1-3Galβ1-4GlcNAc-, GalNacα1-3Galβ1-3GlcNAc-, and/or Galβ1-4GlcNAc-linked units at the nonreducing end of the peripheral domains at the terminal side chain in its N-linked glycosylated unit. Additionally, the ligand immunoassay data (Figure 4B) by incubating the specific hapten sugars along with the respective lectins (ConA, RCA-I, and $VVLA_4$) indicates the possibility of Cry1Ac-HaALP binding, mediated by individual or combination of three hapten sugars (Man, Gal, α-GalNac). No signal was detected after ligand blotting using α-GalNAc preincubated with Cry1Ac, suggesting the absolute binding of Cry1Ac to the α-GalNAc residue present in HaALP. This also supports the previous observation where *H. armigera* BBMV and Cry1Ac binding was strongly inhibited by GalNAc and sialic acid, and not by GlcNAc.[42] Combining both the results of previous and present studies, it can be interpreted that Cry1Ac binds to the terminal α-GalNAc residue at the peripheral region of the glycan structure present in HaALP. On the other hand, the LC-MS/MS results demonstrated the presence of a high hexose-type signature in HaALP (Figure 2C). From the results of above-mentioned two independent experiments, it may be interpreted that probably more than one type of N-glycosylation exist in HaALP, but the interaction of Cry1Ac toxin is mediated by α-GalNAc residue.

Cry1Ac toxin contain a galactose binding-like domain with an ID of IPR008979 at the C-terminal end from 462−609 amino acids which was confirmed by the server InterPro (http://www.ebi.ac.uk/cgi-bin/iprscan/) and the motif sequence is in the Galactose binding like superfamily confirmed through Superfamily 1.69, HMM library and genome assignments server (http://supfam.org/SUPERFAMILY/index.html) having the SCOP fold ID SSF 49785. This data matched those of Burton et al.[43] who explicitly stated that the lectin-like domain or galactose binding-like domain of Cry1Ac is located at and adjacent to the β sheet (β−16) of the β-sandwich structure with a "jelly roll" topology at the C-terminal end (domain III), which shares 80% overall sequence similarity with the Cry1Aa toxin, and the N506, Q509, and Y513 amino acids are responsible for GalNAc binding using *M. sexta* aminopeptidase N receptor. It has been elucidated from the current findings that the nonreducing terminal glycosylated residue of putative receptor, HaALP, is the important binding epitope of Cry1Ac which is constituted by α-anomeric carbon, although the presence of β-*N*-acetylgalactosamine residue in HvALP has been shown by previous authors as the general recognition epitope of Cry1Ac binding in *H. virescens*.[17] Relying on two independent experiments, it may be concluded that interaction between Cry1Ac and its putative receptor (HaALP) is mediated through the binding epitope the terminal GalNAc, irrespective of the presence of the anomeric carbon, α or β.

Recently, two full-length sequences of *H. armigera* alkaline phosphatases have been published in NCBI database with accession numbers ACF40806 and ACF40807. The BLASTP tool (Basic Local Alignment Search Tool) of the NCBI server (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to detect the homology between the LC-MS/MS generated HaALP peptides with the reported sequences (Table 2). It was found that out of six detected peptides of HaALP three have 100% identities, one showing 94% identity and remaining two peptides are different from that of the published sequences. This gives further evidence that the presently identified HaALP is somewhat different from that of the reported ALPs. Moreover, LC-MS/MS analyses predicted that HaALP is homologous to the mALP from *B. mori* to a certain degree, and not with its soluble analogue. Therefore, it can be predicted that this protein is a membrane-bound analogue of alkaline phosphatase, and toxin oligomerization and insertion into the cell membranes on the surface of the midgut of *H. armigera* is mediated through proper binding of Cry1Ac to the glycosyl moiety present in HaALP molecule. In conclusion, in the present investigation, a membrane bound homodimeric ∼138 kDa alkaline phosphatase enzyme was identified as a putative receptor of Cry1Ac in *H. armigera* which also provided information regarding the nature of glycosylation present in the HaALP, that mediates toxin−receptor interaction. Furthermore, detail lectin ligand immunoblot analyses suggested the possibility of the presence of α-GalNAc residue at the nonreducing terminal of HaALP, responsible for Cry1Ac binding and the presence of the "Galactose-binding domain-like" sequence at the C-terminal end of the toxin from the previous and present bioinformatics studies further confirm the molecular mechanism of toxin−receptor interaction. Additionally, this HaALP-Cry1Ac binding was found to be inhibited by three important lectins, namely, ConA, RCA-I and $VVLA_4$. Incidentally, these lectins have specificity to different classes of hapten sugar moieties present in the N-glycan structure of HaALP. Surprisingly, in spite of such binding characteristics, the above-mentioned lectins have never shown insecticidal activity against *H. armigera*. The presently described alkaline phosphatase type of putative receptor is being reported for the first time from *H. armigera* against the potential Bt toxin, Cry1Ac. This important information may be used in future to combat the problem of resistance development among insect populations as well as addressing the biosafety related issues of different Bt toxins.

## Conclusion

The study of molecular mechanisms of the interaction of the insecticidal toxins with their specific receptors in the insect midgut has significance in the context of the insect management research. In the present observation, we identified a membrane bound homodimeric ∼138 kDa alkaline phosphatase in *H. armigera* (Hubner) as the putative receptor of Cry1Ac in the insect midgut. Differential binding and competitive binding studies revealed that Cry1Ac effectively blocks the enzymatic activity of the HaALP molecule upon binding to the putative receptor protein. α-GalNAc residue in the nonreducing terminal of the glycan chain of the N-glycosylated HaALP was recognized as the key epitope for Cry1Ac interaction. In this way, the correlation between the binding of toxin to the glycosylated moieties of the receptors and possible mechanism of insecticidal activity of this Bt

toxin was established. Such understanding could be utilized in future for developing better insect pest management strategies.

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**Supporting Information Available:** HPLC gel filtration chromatograms, glycospecific and Coomassie-stained profile of total BBMV protein, and kinetic study of alkaline phosphatase activity in midgut homogenate (MGH). This material is available free of charge via the Internet at http://pubs.acs.org.

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