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Characterization of a Cry1Ac toxin-binding alkaline phosphatase in the midgut from *Helicoverpa armigera* (Hübner) larvae

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ABSTRACT

Midgut membrane-bound alkaline phosphatases (mALP) tethered to the brush border membrane surface by a glycosylphosphatidylinositol (GPI) anchor have been proposed as crucial for Cry1Ac intoxication. In the present work, two full-length cDNAs-encoding alkaline phosphatases in the midgut of *Helicoverpa armigera* larvae were cloned and named HaALP1 (GenBank accession no. EU729322) and HaALP2 (GenBank accession no. EU729323), respectively. These two clones displayed high identity (above 94%) at the amino acid sequence, indicating that they may represent allelic variants, and were predicted to contain a GPI anchor. Protein sequence alignment revealed that HaALPs were grouped with mALP from the *Heliothis virescens* midgut. The HaALP1 and HaALP2 (~68 kDa) proteins were heterologously expressed in Sf9 cells using a baculovirus expression system and purified to homogeneity. Ligand blot and dot blot analysis revealed that the Cry1Ac bound to both denatured and native purified HaALPs. Data from lectin blots, competition assays with soybean agglutinin (SBA) lectin and GalNAc binding inhibition assays were indicative of the presence of GalNAc on HaALPs and binding of Cry1Ac toxin to this residue. This observation was further confirmed through N-glycosidase digestion of HaALPs, which resulted in reduced Cry1Ac binding. Our data represent the first report on HaALPs and their putative role as receptors for Cry1Ac toxin in *H. armigera*.

1. Introduction

Cry toxins are produced as crystalline inclusions by the bacterium *Bacillus thuringiensis* (Bt) during the sporulation phase. These toxins display highly specific insecticidal activity and are used in mixtures and expressed in transgenic Bt crops to control relevant lepidopteran pests. In 2007, Bt transgenic cotton expressing the Cry1Ac toxin was grown on 14 million ha worldwide, representing approximately 33.3% of the worldwide transgenic insecticidal crops, with 3.8 million ha grown in China (Wu et al., 2008).

Cry intoxication begins with toxin solubilization and activation by proteases in the insect host midgut fluids. Activated toxins traverse the peritrophic matrix and bind to cadherin receptors on the brush border membrane of the midgut epithelium. This binding facilitates further toxin processing, resulting in formation of a toxin oligomer that displays high binding affinity towards *N*-acetylgalactosamine (GalNAc) residues on glycosylphosphatidylinositol (GPI)-anchored proteins (Pardo-López et al., 2006), such as aminopeptidase-N (APN) and alkaline phosphatase (ALP). After this second binding step, the oligomer inserts on the cell membrane, forming a pore that leads to cell death by osmotic lysis (Bravo et al., 2007). In an alternative model, toxin monomer binding to cadherin activates intracellular oncotic pathways to result in cell death (Zhang et al., 2005, 2006).

Even though resistance to Cry toxins can develop by alterations in any of these steps, most cases of laboratory selection result in resistance due to alteration of toxin binding to midgut receptors (Ferré and Van Rie, 2002). A number of insect midgut proteins have been proposed as putative Cry toxins receptors, including cadherin, APN, and ALP (Pigott and Ellar, 2007). Alterations of cadherin gene have been reported to be associated with Bt resistance in a number of laboratory insect strains (Gahan et al., 2001; Morin et al., 2003; Tabashnik et al., 2005; Xu et al., 2005). In comparison, less is known on the interactions between toxin oligomers and other receptors, such as APN or ALP, that facilitate oligomer insertion on the membrane. Binding of Cry1Ac is dependent on the existence of *N*-acetylgalactosamine (GalNAc) residues in *Manduca sexta* APN (Pardo-López et al., 2006), and *Heliothis virescens* mALP (Jurat-Fuentes and Adang, 2004). Membrane-bound ALP has been proposed as receptor for Cry toxins in *M. sexta* (McNall and Adang, 2003), *H. virescens* (Jurat-Fuentes and Adang, 2004, 2007;

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Krishnamoorthy et al., 2007), *Aedes aegypti* (Fernandez et al., 2006), *Anopheles gambiae* (Hua et al., 2009), and *Anthonomus grandis* (Martins et al., 2010). Reduced levels of ALP correlated with high levels of resistance to Cry1Ac toxin in larvae of the YHD2 strain of *H. virescens* (Jurat-Fuentes and Adang, 2004). Taken together, these data suggest a role for mALPs in Cry1Ac intoxication and resistance.

The cotton bollworm, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae), is a major pest in cotton-growing regions. Transgenic Bt cotton expressing Cry1Ac has proved highly efficient in suppressing regional outbreaks of *H. armigera* (Wu and Guo, 2005; Wu et al., 2008), and in increasing yields of cotton (Pray et al., 2001; Wu et al., 2003). In this insect, a cadherin protein has been proposed as Cry1Ac receptor based on binding data and linkage between laboratory Cry1Ac resistance and mutations in the cadherin gene (Xu et al., 2005; Yang et al., 2006, 2007). Considering the potential role of alternative receptors in the Cry intoxication process and in resistance to Cry toxins, the goal of our research was to identify mALP as putative Cry1Ac-binding protein in the midgut of *H. armigera* larvae and characterize its interactions with the toxin. In the present work, two *H. armigera* alkaline phosphatase (HaALP) cDNAs were cloned and their heterologous expression were expressed in Sf9 cell cultures. Protein blot analysis revealed that Cry1Ac bound to both denatured and native forms of purified HaALPs. Lectin blots using soybean agglutinin (SBA), GalNAc inhibition assays and N-glycosidase digestion of HaALPs revealed that Cry1Ac recognizes GalNAc on HaALPs as binding epitope.

2. Materials and methods

2.1. Insects

The Cry1Ac-susceptible *H. armigera* strain 96S used in this work was collected from Xinxiang, Henan Province (China) in 1996, and has been reared for several years in the laboratory on an artificial diet without exposure to any Bt toxin. *H. armigera* was cultured at 27 ± 2 °C and 75 ± 10% relative humidity (RH) under a photoperiod of 14:10 h (L:D) (Liang et al., 1999).

2.2. Preparation of activated Cry1Ac toxin

B. thuringiensis strain HD-73 cultures were grown 2–4 days at 30 °C until sporulation and autolysis. The Cry1Ac crystal inclusions were separated from spores by discontinuous sucrose density gradient centrifugation (25,000 × *g* for 1.5–2 h). Crystals were solubilized in 50 mM Na_2CO_3, pH 10.0 plus 10 mM DTT or 1% β-mercaptoethanol, at 37 °C for 1–2 h. Solubilized protoxin was recovered from debris by centrifugation (30,000 × *g* for 30 min at 4 °C) and was incubated with 1/25 (w/w) trypsin (Sigma) at 37 °C for 2 h. After processing, the digested toxin was dialyzed against 20 mM Tris–HCl, pH 8.5, 50 mM NaCl, at 4 °C for 10–12 h.

The activated toxin was purified by FPLC (fast protein liquid chromatography) using a Superdex 200 HR 10/30 column (Amersham) in the carbonate buffer (30 mM Na_2CO_3, 20 mM $NaHCO_3$, pH 10.0). Purified Cry1Ac toxin (~66 kDa) was characterized by 10% SDS-PAGE, and the toxin concentration was determined using the Bradford assay (Bradford, 1976) with bovine serum albumin (BSA) as a standard. Activated toxin was stored at −70 °C until used.

2.3. Preparation of brush border membrane vesicles (BBMV)

Midguts from fifth instar larvae of *H. armigera* were dissected longitudinally, washed in ice-cold MET buffer1 (250 mM mannitol, 17 mM Tris–HCl, and 5 mM EGTA, pH 7.5), blotted on filter paper, flash-frozen in liquid nitrogen, and stored at −70 °C until used. BBMV were prepared from the midguts by the differential magnesium precipitation method of Wolfersberger et al. (1987). The midguts were homogenized in 9 times their weight of MET buffer2 (300 mM mannitol, 17 mM Tris–HCl, and 5 mM EGTA, pH 7.5). After adding an equal volume of 24 mM $MgCl_2$ buffer, the samples were incubated on ice for 15 min, and the mixture was centrifuged at 2500 × *g* for 15 min at 4 °C. The supernatant was centrifuged at 30,000 × *g* for 30 min at 4 °C. The pellet thus obtained was re-suspended in half-strength volume of MET buffer 2 and the centrifugation cycle was repeated as described above. The pellet was re-suspended in PBS buffer (pH 7.5), frozen and stored at −70 °C until used. The concentration BBMV proteins were measured as described above for toxin.

2.4. Cloning and sequencing of ALPs from the midgut of *H. armigera* larvae

Total RNA was extracted from the midgut of fifth instar *H. armigera* larvae with Trizol reagent (Invitrogen) according to the manufacturer's instructions. The final RNA was treated with DNase I (TaKaRa) and reverse-transcribed with SuperScript III $RNaseH^-$ reverse transcriptase (Invitrogen). The first strand cDNA was used as a template for PCR. Degenerate primers used for PCR amplification ALPS (5′-GGKATHGTGACSACSACBCG-3′) and ALPA (5′-TCRTCNCCGCCGTGMGTCTC-3′) were designed from a conserved stretch of amino acids between ALP from *Bombyx mandarina* (GenBank accession no. BAF62124), membrane-bound and soluble ALPs from *Bombyx mori* (GenBank accession nos. P29523 and BAB62746, respectively), and *H. virescens* HvmALP5 (GenBank accession no. ABR88230). The PCR products were subcloned into the pEASY-T1 Simple vector (Transgen, China), transformed into *Escherichia coli* TOP10 cells (Invitrogen), and transformants sequenced in an ABI3770XL sequencer. Rapid amplification of cDNA ends (RACE) was used to obtain the full-length ALP cDNA (5′-Full RACE kit, TaKaRa) following manufacturer's instructions. Specific primers for RACE were designed according to the sequence from the degenerate PCR product. Primers to amplify the full-length ALP cDNA were designed based on the RACE products.

The NCBI/BLAST database and the ClustalX 2.0.9 were used to analyse the homology of ALP among species. The SignalP 3.0 Server (http://www.cbs.dtu.dk/services/SignalP/) was used to test for the presence of a signal peptide, and the GPI Modification Site Prediction (http://mendel.imp.ac.at/sat/gpi/gpi_server.html) server was used to predict GPI-anchor signal sequence and GPI anchoring sites (Nielsen et al., 1997; Eisenhaber et al., 2001). The ExPASy Compute pI/Mw tool (http://ca.expasy.org/tools/pi_tool.html) was used to predict the molecular weight and isoelectric points of the predicted proteins. Presence of O- or N-glycosylation on the predicted protein sequences was tested using the ExPASy NetOGlyc 3.1 and NetNGlyc 1.0 servers (http://www.cbs.dtu.dk/services) (Gasteiger et al., 2003). The Myhits (http://myhits.isb-sib.ch/cgi-bin/motif_scan) server was used to predict the localization of the active phosphatase site (Pagni et al., 2001).

2.5. Preparation of antibody to HaALPs

A 780 bp cDNA fragment of *H. armigera* ALP (GenBank accession no. EU729322) was cloned with primers AntiALP-F (5′-CG*GGATCC*GCGAAGACGGCGAACCGCACCTG-3′ BamHI) and AntiALP-R (5′-CG*CTCGAG*AGTGCGATAGTTTGGCTCAAGGGT-3′ XhoI). The amplified fragment was sequenced as described above, cloned into the His-tagged expression vector pET28a+ (Novagen), and transformed into *E. coli* BL21 (DE3) cells (Transgen, China). The positive transformed cells were amplified and expression was induced with 1 mM IPTG for 10 h at 25 °C. For protein purification,

cells were pelleted by centrifugation (5000 × *g* for 10 min at 4 °C) and the pellet re-suspended in STE buffer (10 mM Tris–HCl, 1 mM EDTA, 150 mM NaCl, 20 mM imidazole, 1 mM DTT, 5% sodium lauroylsarcosine, pH 7.5). After sonication for 5 min on ice, debris was pelleted by centrifugation (25,000 × *g* for 20 min at 4 °C), and the supernatant was subjected to affinity purification using Ni-Sepharose beads (GE Healthcare). After washing with 500 mM imidazole in PBS buffer (pH 7.5), the recombinant protein was eluted with 1000 mM imidazole and gradually dialyzed against PBS buffer. The purified proteins were separated by 10% SDS-PAGE and concentration was measured as described above.

The purified ALP fragment was used as antigen to develop antisera in injected New Zealand white rabbits. Briefly, the purified ALP fragment (300 μg) was emulsified with an equal volume of Freund's complete adjuvant for the first injection and incomplete adjuvant for three additional injections. These four injections were performed at two-week intervals, and blood was collected 7 days after the last injection. The AntiALP serum was aliquoted and titer measured by ELISA (Sambrook et al., 1989).

2.6. Expression of HaALPs in Sf9 cell cultures using a baculovirus expression system

For generation of recombinant bacmid, two full-length cDNAs of HaALP were PCR cloned with primers ALPF (5′-CG*GGATC*-*C*ATGGTGACACTGTTCCCGT-3′ BamHI) and ALPR (5′-CCC*AAGCTT*T-TAGTGATGGTGATGGTGATGTCGCAGTAAAATGGAAGTG-3′ HindIII). The PCR products were first cloned into the pEASY-T1 Simple vector, then digested using the BamHI and HindIII sites and ligated to the pFastbac1 vector (Invitrogen). The recombinant plasmid (pFastbac1-HaALPs) was transformed into DH10Bac cells (Invitrogen), and the recombinant bacmid DNAs were isolated and identified by PCR.

For expression of HaALPs, the *Spodoptera frugiperda* cell (Sf9) cultures were grown in SF-900 SFM media (Gibco) with greater than 95% viability. Transfections were performed in a six-well format with HaALPs DNA constructs (1–2 μg bacmid DNAs) and cellfectin reagent (Invitrogen) following manufacturer's instructions. Four days after transfection or when signs of viral infection were visually clear, culture supernatants were collected as P1 viral stock and the titer was identified by plaque analysis. Using this viral stock Sf9 cells were infected at a multiplicity of infection (MOI) of 0.05. This infection resulted in 80% cell mortality, and the supernatants of these cell cultures were collected as P2 viral stock and the titer identified by plaque analysis. For protein expression, Sf9 cells were seeded at 1×10^6 cells/ml in a shaking flask, and were infected at MOI 5–10. Samples were taken out at different time intervals after infection (24, 48, 72 and 96 h) to assay for protein expression by 10% SDS-PAGE, Western blotting and Ligand blotting.

For purifications of expressed HaALPs, we took advantage of the 6× His tag contained in the C-terminus of the proteins and performed affinity purification using Ni-Sepharose beads (GE Healthcare) following manufacturer's instructions. Cells were collected by centrifugation (5000 × *g* for 10 min at 4 °C) and solubilized in lysis buffer (10 mM Na_2HPO_4, 30 mM NaCl, 0.25% Tween-20, 1 mM EDTA, pH 7.5) plus 0.1% proteinase inhibitor cocktail (Sigma) and 0.1 mM phenylmethylsulphonylfluoride (PMSF). Re-suspended cells were sonicated four times (10 s each) and centrifuged at 20,000 × *g* for 15 min at 4 °C. The supernatants were loaded on a Ni-Sepharose column. After washing the column with 15 mM imidazole in PBS buffer (pH 7.5), the recombinant protein was eluted with 50 mM imidazole and gradually dialyzed against PBS buffer. The final concentrations of purified proteins were measured as described above for toxin samples.

2.7. Detection of HaALP expression and Cry1Ac binding

Alkaline phosphatase activity of expressed HaALPs was detected as described elsewhere (Jurat-Fuentes and Adang, 2004). Briefly, purified proteins (5 μg) or the BBMV (10 μg) were solubilized in sample buffer (without DTT) for 15–30 min at room temperature, and separated in 10% SDS-PAGE. After electrophoresis, the gel was washed with ALP buffer (100 mM Tris–HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$) for 15 min at room temperature. After addition of 330 μg/ml of p-Nitro-Blue tetrazolium chloride (NBT) and 165 μg/ml of 5-bromo-4-chloro-3-indolyl phosphate (BCIP) to the ALP buffer, ALP activity was developed until purple-red bands were generated and was stopped by incubating the gel in PBS (pH 7.5) plus 2 mM EDTA.

For Western and ligand blotting, purified HaALPs (5 μg) or the BBMV (10 μg) were separated by 10% SDS-PAGE and transferred onto a polyvinylidene difluoride (PVDF) membrane filter (Millipore) (Towbin et al., 1979). The membrane filter was blocked for 2 h at room temperature in blocking buffer (135 mM NaCl, 2 mM KCl, 10 mM Na_2HPO_4, 1.7 mM KH_2PO_4, 5% skim milk powder, pH 7.5). For Western blotting, filters were probed with anti-HaALP antisera (1:10,000 dilution) for 2 h at room temperature in blocking buffer (0.1% Tween-20). Ligand blots were performed by probing blocked filters with Cry1Ac toxin (10 nM) for 1.5 h at room temperature in blocking buffer (0.1% Tween-20). The ligand blot filter was then washed three times (10 min each) with PBST (135 mM NaCl, 2 mM KCl, 10 mM Na_2HPO_4, 1.7 mM KH_2PO_4, 0.1% Tween-20, pH 7.5) and incubated with rabbit antisera to Cry1Ac antibody (1:10,000 dilution) for 1.5 h at room temperature in blocking buffer (0.1% Tween-20). After washing as previously, both Western and ligand blot filters were probed with an HRP-conjugated anti-rabbit secondary antibody (ZSGB-BIO, China) at a 1:20,000 dilution for 1 h. After another series of washes, filters were developed using an ECL chemiluminescence detection kit (Amersham) and exposed to film.

For dot blot analyses, purified HaALPs (2 μg) were spotted directly onto PVDF membranes and dried for 20 min with a vacuum (Bio-Rad). The filters were blocked, incubated and developed as described above.

2.8. Lectin blots and analysis of Cry1Ac binding to sugars on HaALPs

Purified HaALPs (6 μg) or the BBMV (10 μg) were separated, transferred onto a PVDF filter, and blocked as described above. After blocking, filters were probed with 0.5 μg/ml of HRP-conjugated soybean agglutinin (SBA) from *Glycine max* (Sigma) for 2 h at room temperature in blocking buffer (0.1% Tween-20). After washing, the filter was developed as described above.

To release the N-linked oligosaccharides from purified HaALPs, proteins were subjected to deglycosylation with N-glycosidase F (NGase-F, NEB) according to the manufacturer's instructions. Purified HaALPs were denatured in 1× glycoprotein denaturing buffer (5% SDS, 0.4 M DTT) at 100 °C for 10 min, followed by the addition of 1/10 volume of each 10× G7 buffer (0.5 M sodium phosphate pH 7.5) and 10% NP-40. The preparations were incubated with N-glycosidase F at 37 °C for 24 h. The deglycosylated samples were boiled with SDS-PAGE sample buffer, separated by 10% SDS-PAGE and transferred onto a PVDF membrane filter. The filter was blocked and probed with SBA lectin and Cry1Ac toxin as described above.

For SBA-Cry1Ac competitive binding assays, purified HaALPs (10 μg), were separated by 10% SDS-PAGE and transferred onto a PVDF membrane filter. The membrane filters were blocked as described above and then were incubated with SBA lectin (1 μg/ml) in the presence of increasing Cry1Ac toxin concentrations at room temperature in blocking buffer (0.1% Tween-20). After

washing, the filter was developed as described above for lectin blotting.

For the oligosaccharide binding inhibition assays, Cry1Ac toxin (10 nM) was pre-incubated with different concentrations of GalNAc or 400 mM galactose for 45–60 min at room temperature, then used to probed blots of HaALP proteins (8 μg) as described for ligand blots.

3. Results

3.1. Cloning of H. armigera ALP

Using degenerate primers and PCR, two full-length cDNAs-encoding alkaline phosphatases from midguts of *H. armigera* larvae were cloned. These clones were named HaALP1 (GenBank accession no. EU729322) and HaALP2 (GenBank accession no. EU729323), respectively. The two HaALPs displayed high identity (above 94%), with most sequence divergence localized to the 3′ untranslated region, indicating that they may represent allelic variants. The open reading frame (ORF) of both HaALPs was 1605 bp, encoding a predicted protein of 535 amino acid residues, with predicted molecular weight of 59 kDa and isoelectric point of 5.94 for HaALP1 and 5.8 for HaALP2. A hydrophobic signal sequence with 20 amino acids in the N-terminal region and a GPI-anchor signal sequence with 23 amino acids in the C-terminal region, suggest that both ALPs are tethered to the cell membrane with a GPI anchor (GPI-anchor site at ^{512}S). Both clones contain a predicted phosphatase domain and active site (^{118}IADSACTAT^{126}, with ^{121}S being the enzymatic active site). Two potential N-glycosylation sites (^{196}NRTW^{199} and ^{275}NVSH^{278}) and one potential O-glycosylation site (^{105}T) are common to both clones, although HaALP2 presents and additional potential N-glycosylation site at ^{442}NVTA^{445} (Fig. 1).

Previous protein sequence alignments revealed that HaALPs had high homology with other heliothine ALPs, particularly 94% identity to mALP1-5 from *H. virescens* midgut (GenBank accession nos. ACP39712, ACP39713, ACP39714, ACP39715 and ABR88230) (Perera et al., 2009). Lower identity (60%) was found when compared to other lepidopteran mALPs from *B. mori* (GenBank accession no. P29523) or *B. mandarina* (GenBank accession no. BAF62124).

3.2. Heterologous expression of HaALPs

To develop antisera to HaALP1, a cDNA fragment from this protein was cloned, expressed in *E. coli* cells, and purified to use as antigen (Fig. 2A). The developed antisera specifically recognized the HaALP1 fragment (Fig. 2A, lane 4) and the HaALP band in BBMV from *H. armigera* (Fig. 2B). This HaALP band in BBMV was also recognized by soybean agglutinin (SBA), a lectin that specifically recognizes GalNAc and galactose on proteins (Wu et al., 1997), and activated Cry1Ac (Fig. 2B).

Once we confirmed specificity of the HaALP1 antisera, we expressed both HaALP1 and HaALP2 in Sf9 cell cultures using a baculovirus system. After affinity purification to the C-terminal His tag contained in both HaALPs, proteins of about 68 kDa were observed in stained gels (Fig. 3). Both proteins displayed alkaline phosphatase activity and were detected by the HaALP1 antisera (Fig. 3), confirming expression of functional HaALP1 and HaALP2 proteins. The difference between the expected and observed molecular size suggested the presence of post-translational modifications on the proteins. To test for this possibility, and to establish glycosylation of the expressed HaALP proteins, we performed lectin blots with SBA. In agreement with results using BBMV (Fig. 2B), the purified HaALPs were specifically recognized by SBA lectin, indicating the presence of GalNAc and/or galactose on these proteins. To determine the linkage of oligosaccharides containing these sugars, we treated purified HaALPs with N-glycosidase F. This treatment resulted in lack of SBA binding (Fig. 3), suggesting that the GalNAc and/or galactose were part of N-linked oligosaccharide structures.

3.3. Cry1Ac binding to HaALPs

To test binding of Cry1Ac to heterologously expressed HaALPs, we performed ligand and dot blots. As shown in Fig. 4A, Cry1Ac bound to both HaALP1 and HaALP2 in ligand blots. Since denaturing conditions in ligand blots may affect binding specificity (Daniel et al., 2002), we also tested Cry1Ac binding to HaALPs using native conditions with dot blots (Fig. 4A). As observed in BBMV (Fig. 2B), Cry1Ac bound to both HaALPs, and this binding was dependent on the presence of N-linked oligosaccharides on these proteins, since digestion with N-glycosidase F eliminated toxin binding (Fig. 4A).

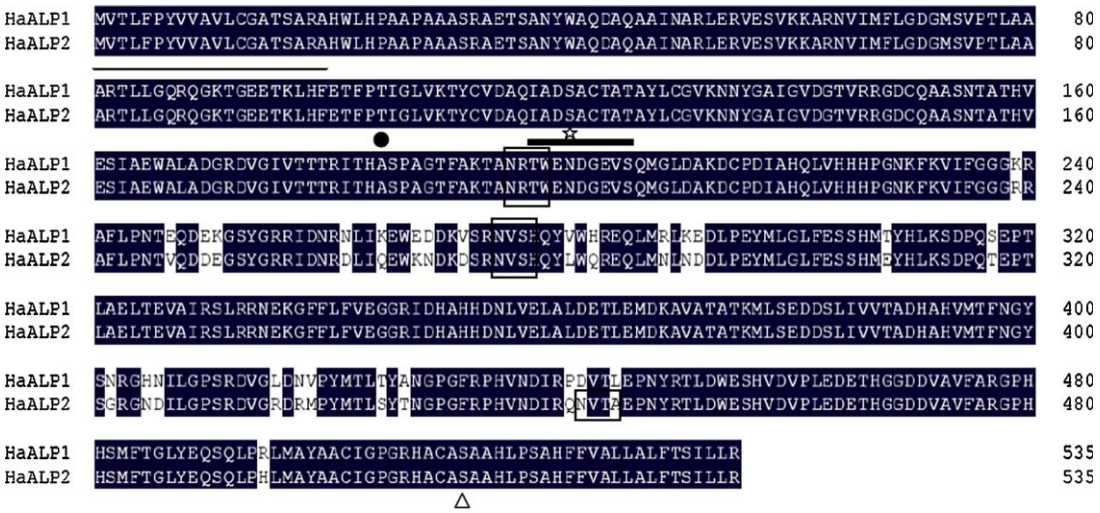

Fig. 1. Protein sequence alignment of ALPs from *H. armigera* larvae midgut. Letters with black shading indicate complete sequence conservation. Signal sequence is indicated by a thin underline. Alkaline phosphatase active domain is indicated by a thick underline and the predicted enzymatic active site within this domain is indicated by an asterisk. Potential N-glycosylation sites are shown inside a rectangle and the potential O-glycosylation site is indicated by a black dot underneath the residue. Predicted GPI-anchor site is indicated by a triangle. HaALP (*Helicoverpa armigera* alkaline phosphatase) 1–2 (GenBank accession nos. ACF40806 and ACF40807).

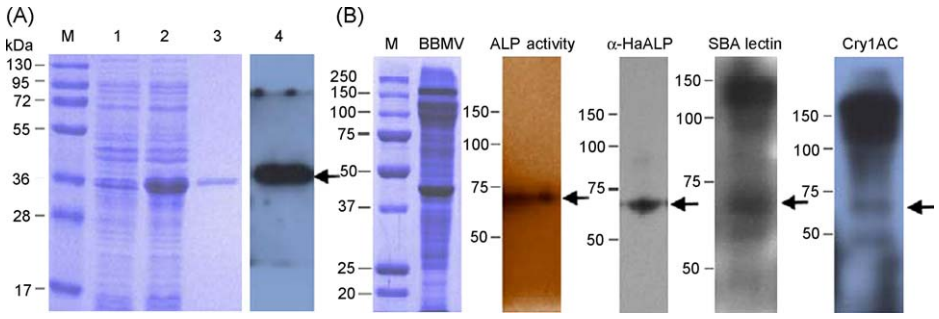

Fig. 2. Development of antisera to HaALP and detection of HaALP in BBMV from *H. armigera*. (A) SDS-PAGE and Western blot analysis of the expression of the HaALP1 fragment used for antisera production. Arrows indicate the 33.5 kDa HaALP1 fragment. Lane 1: IPTG-0 h; lane 2: IPTG-10 h; lane 3: purified HaALP1 fragment; lane 4: purified HaALP1 fragment detected with anti-HaALP antisera. (B) Identification and characterization of the 68 kDa glycoprotein of *H. armigera* BBMV as alkaline phosphatase. *H. armigera* BBMV proteins (10 μg) were separated by 10% SDS-PAGE and stained with Coomassie Brilliant Blue R-250 (left panel), stained for ALP enzymatic activity, or transferred onto a PVDF membrane and probed with anti-HaALP antisera, SBA lectin, or Cry1Ac toxin as indicated on the top of each figure. Arrows indicate the position of the 68 kDa HaALP in *H. armigera* BBMV.

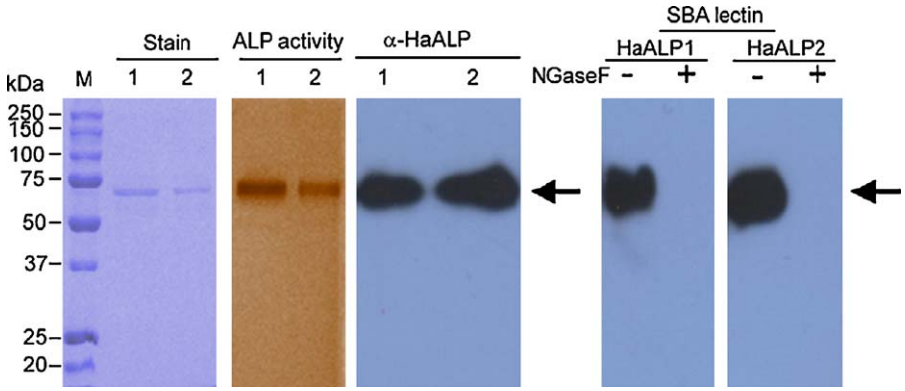

Fig. 3. Detection and characterization of HaALPs expressed in Sf9 cell cultures using a baculovirus expression system. Arrows indicate the 68 kDa HaALP proteins. Lane 1: HaALP1; lane 2: HaALP2. Purified HaALP proteins were stained with Coomassie Blue (stain), stained for enzymatic ALP activity (ALP activity), or detected with antisera to HaALP1 (α-HaALP) as indicated on the top of each image. Purified proteins were also probed with SBA lectin to detect the presence of GalNAc and/or galactose before of after digestion with N-glycosidase F (NGaseF), as indicated on top of each image. Arrows indicate the position of heterologously expressed HaALPs.

To test for Cry1Ac binding to the GalNAc or galactose on the N-linked oligosaccharides on HaALPs, we performed competition assays using these sugars as competitors (Fig. 4B). Cry1Ac binding was completely inhibited by 250 mM GalNAc, while no effects were detected after treatment with 400 mM galactose, suggesting that Cry1Ac binding was binding to GalNAc on HaALPs. To further test this hypothesis, we performed competition between SBA and Cry1Ac for binding to HaALPs. Because SBA binding affinity for GalNAc is much lower than the affinity of Cry1Ac for binding to BBMV (Jurat-Fuentes and Adang, 2004), we use Cry1Ac as competitor. As shown in Fig. 4C, binding of SBA to purified HaALPs was completely eliminated by addition of 200 μg/ml of Cry1Ac. These results further supported our hypothesis that Cry1Ac toxin bound to the GalNAc on N-linked oligosaccharides on HaALPs.

4. Discussion

We report, for the first time, on the identification, cloning and expression of two isoforms of membrane-bound alkaline phosphatase (HaALPs) from *H. armigera* larval midgut. Furthermore, we provide evidence that supports previous reports on the recognition of N-linked GalNAc on HaALPs by the Cry1Ac toxin (Das et al., 2009). Since Cry1Ac binding to specific receptors on the brush border membrane of the larval midgut is required for toxicity, our data suggest the potential participation of HaALPs in Cry1Ac intoxication.

Alterations in the interactions between Cry toxins and their receptors are reported as the most common mechanism leading to high levels of resistance to Cry toxins (Ferré and Van Rie, 2002). Recently, cadherin (Wang et al., 2005) and APN (Ingle et al., 2001; Liang et al., 2004; Liao et al., 2005) have been identified as Cry1Ac receptors in *H. armigera* and their alterations have been reported to result in resistance to Cry1Ac in *H. armigera* (Xu et al., 2005; Zhang et al., 2009). In comparison, little is known on the identity and role of alternative Cry1Ac receptors from *H. armigera* midgut on toxicity. In a phylogenetically related species, *H. virescens*, ALPs have been proposed as putative Cry1Ac receptors involved in resistance (Jurat-Fuentes and Adang, 2004, 2007; Krishnamoorthy et al., 2007). Furthermore, ALPs have been described as Cry toxin receptors in other Lepidoptera (McNall and Adang, 2003), Diptera (Fernandez et al., 2006; Bayyareddy et al., 2009; Hua et al., 2009), and Coleoptera (Martins et al., 2010) models.

The two cloned HaALP proteins displayed above 94% sequence identity, indicating that they may represent allelic variants of a single gene. In contrast, there is evidence supporting the existence of multiple ALP genes expressed in the insect midgut. Five isoforms probably derived from two genes were described in *H. virescens* larval midgut (Perera et al., 2009). In *Bombyx mori* larval midgut two genes encoding for different forms of ALP have also been reported (Itoh et al., 2003). In *Aedes aegypti* larvae, three ALPs sharing only 40% identity were reported as representing three different genes (Bayyareddy et al., 2009). Protein sequence alignment revealed that HaALPs were grouped with a subgroup of mALP from *H. virescens*, suggesting that additional ALP isoforms may be present in the *H. armigera* midgut. Nevertheless, common peptide sequences with previous reports (Das et al., 2009) and our

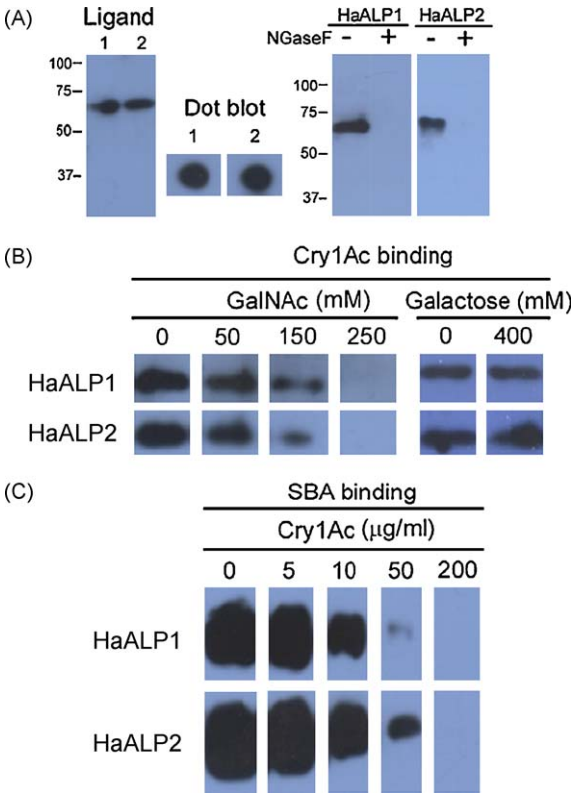

Fig. 4. Characterization of Cry1Ac binding to HaALPs. (A) Ligand blot and dot blot analysis testing binding of Cry1Ac and binding after N-glycosidase F digestion. Lane 1: HaALP1; lane 2: HaALP2. Cry1Ac binding to HaALPs under denaturing (ligand) or native (dot blots) conditions was tested. The role of N-glycosylation in Cry1Ac binding to HaALPs was tested by comparing binding with or without digestion with N-glycosidase F (NGase-F), as indicated on top of the right panel. Cry1Ac bound to purified HaALPs, but did not bind to N-glycosidase F treated HaALPs, indicating that binding was dependent on the presence of N-linked oligosaccharides (GalNAc or galactose) on HaALPs. (B) Inhibition of Cry1Ac binding to HaALPs by increasing concentrations of GalNAc, but not by galactose. (C) Cry1Ac competes for SBA binding to the HaALPs. Increasing concentrations of Cry1Ac as indicated decreased SBA binding to purified HaALPs. Taken together, these results indicated that Cry1Ac binding to HaALPs is mediated by GalNAc on a N-linked oligosaccharide.

Cry1Ac-binding data suggest that HaALP1 and HaALP2 represent Cry1Ac-binding proteins.

The predicted molecular weight of the cloned HaALPs (~59 kDa) was smaller than the detected here (Fig. 2A) and reported size for purified ALP from *H. armigera* midgut (~68 kDa), indicating the potential existence of glycosylation and/or other post-translational modifications (Das et al., 2009). When expressed in Sf9 insect cell cultures, HaALPs were 68 kDa in size, suggesting that heterologous expression produced mature proteins. Comparison of our Cry1Ac-binding data with previous reports on ALP from *H. armigera* BBMV further supports that our expressed HaALPs present similar glycosylation to *in vivo* forms.

In agreement with previous reports (Das et al., 2009; Estela et al., 2004), we found that Cry1Ac bound specifically to N-linked GalNAc on HaALPs, and that toxin binding could be prevented by changes in glycosylation or the presence of GalNAc. Binding of Cry1Ac to GalNAc on APN or ALP has been proposed as a crucial step in the intoxication process (Pardo-López et al., 2006). The importance of binding to GalNAc for Cry1Ac toxicity is highlighted by reports of inhibition of Cry1Ac binding to *H. armigera* BBMV by GalNAc resulting in lack of pore formation (Rodrigo-Simón et al., 2008). According to this hypothesis, changes in protein glycosylation could result in reduced Cry1Ac binding, lack of pore formation, and resistance. In support of this prediction, resistance to Cry toxins linked to genes involved in glycosylation pathways has been reported in *Caenorhabditis elegans* (Griffitts et al., 2001). Further work would be necessary to characterize the specific role of glycosylated ALPs and APNs in Cry intoxication and resistance.

Assessed collectively, data from the present and previous studies suggest that ALP in the insect midgut is involved in the mode of action of Cry1Ac. The identification of ALP as a Cry1Ac-binding protein in the midgut of *H. armigera* allows us to further test its role in Cry1Ac intoxication. Our future work will focus on characterization of HaALPs as functional Cry1Ac receptors, determine the potential role of HaALP in resistance to Cry1Ac, and the identification of HaALP genes in *H. armigera*.

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