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Cloning and characterization of the Cry1Ac-binding alkaline phosphatase (HvALP) from *Heliothis virescens*

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ABSTRACT

Membrane-bound alkaline phosphatases (mALPs, EC 3.1.3.1) in the insect midgut have been reported as functional receptors for Cry toxins from the bacterium *Bacillus thuringiensis*. We previously reported the identification of HvALP in the midgut of *Heliothis virescens* larvae as a Cry1Ac-binding protein that is down-regulated in Cry1Ac-resistant insects. To further characterize HvALP, we localized mALP protein to foregut and midgut tissues using anti-mALP serum and then cloned five mALPs from *H. virescens* larval midgut. All five clones displayed high levels of sequence identity (above 90%), suggesting that they may represent allelic variants, and grouped with other lepidopteran mALPs in sequence alignments. All these cloned ALPs were predicted to contain a glycosylphosphatidylinositol (GPI) anchor and were named HvmALP1–5. We expressed two of the most diverse HvmALPs in a heterologous system to test binding of Cry1Ac and recognition by HvALP cross-reacting antiserum. Our data highlight the importance of glycosylation for Cry1Ac binding to HvALP and suggest that, depending on glycosylation, all the identified HvmALPs may be synonymous with HvALP, the Cry1Ac-binding phosphatase identified in *H. virescens* midgut epithelium.

1. Introduction

The insecticidal Cry1Ac toxin from the bacterium *Bacillus thuringiensis* (Bt) is expressed in transgenic Bt cotton cultivars to effectively control *Heliothis virescens* larvae (Perlak et al., 2001). The current model for Cry1A intoxication in susceptible larvae includes toxin solubilization and activation in the insect midgut fluids, followed by binding to cadherin-like proteins. According to the model of Bravo et al. (2007), binding of activated toxin monomers to cadherin results in additional toxin processing by an unidentified protease. This additional cleavage results in the formation of toxin oligomers, which in the case of Cry1Ac display high affinity binding to N-acetylgalactosamine (GalNAc) residues on N-aminopeptidases (APNs) (Pardo-Lopez et al., 2006), and possibly alkaline phosphatases (ALPs) (Jurat-Fuentes and Adang, 2004). This second binding step is conducive to toxin insertion into the cell membrane and the formation of a pore that leads to cell death by osmotic cell lysis. Alternatively, it has also been proposed that binding of Cry1Ac monomers to cadherin activates an intracellular cell death pathway (Zhang et al., 2006).

We previously reported the identification of a membrane-bound form of alkaline phosphatase (HvALP) as a Cry1Ac-binding protein in the *H. virescens* midgut brush border membrane (Jurat-Fuentes and Adang, 2004; Krishnamoorthy et al., 2007). HvALP is attached to the cell membrane by a glycosylphosphatidylinositol (GPI) anchor and contains a terminal N-linked GalNAc residue recognized by Cry1Ac (Jurat-Fuentes and Adang, 2004). Levels of HvALP are reduced in larvae of the YHD2-B strain of *H. virescens*, which are highly resistant to Cry1Ac (Jurat-Fuentes and Adang, 2004). Furthermore, HvALP levels directly correlate with susceptibility in backcrosses (Jurat-Fuentes and Adang, 2007), suggesting a correlation between Cry1Ac binding to HvALP and toxicity.

The goal of this project was to further characterize HvALP and its interaction with Cry1Ac. HvALP was detected only in the foregut and midgut epithelia. Based on this information, we used mRNA purified from *H. virescens* larval gut and rapid amplification of cDNA ends (RACE) to obtain five clones encoding mALPs. Using heterologous expression of the two most diverse clones in *Escherichia coli*, we confirmed the importance of glycosylation for Cry1Ac binding and identified all mALP clones as synonymous with HvALP.

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2. Materials and methods

2.1. Insects

H. virescens larvae from a laboratory colony maintained at the Southern Insect Management Research Unit, USDA-ARS, (Stoneville, MS) were used as the source of genetic materials for this work. Eggs from Cry1Ac-susceptible (YDK) and Cry1Ac-resistant (YHD2-B) strains of *H. virescens* were generously provided by Dr. Fred Gould (North Carolina State University, Raleigh, NC). The selection and Cry1Ac susceptibility phenotypes of these strains have been described previously (Gould et al., 1995; Jurat-Fuentes and Adang, 2004). Larvae were reared on artificial diet (modified from Shaver and Raulston, 1971) and fourth instar larvae used for dissection to obtain specific tissues.

2.2. Cry1Ac toxin purification and labeling

B. thuringiensis strain HD-73 obtained from the *Bacillus* Genetic Stock Center (Ohio, USA) was used to produce Cry1Ac crystals. Crystalline inclusions were solubilized by incubation overnight at 30 °C in 50 mM Na_2CO_3 pH 9.8, 100 mM NaCl 0.1% 2-β-mercaptoethanol. After solubilization, samples were clarified by centrifugation at 30,000 × g for 20 min at 4 °C. Cry1Ac protoxin in the supernatant was activated by treatment with 0.1% (v/v) of midgut fluids obtained from actively feeding 4th instar *H. virescens* larvae. After incubation for 1 h at room temperature, the activated toxin sample was loaded on a HiTrap HP Q 5 ml anion exchange column (GE Healthcare) pre-equilibrated with 50 mM Na_2CO_3 pH 9.8 (buffer A). Activated Cry1Ac toxin was eluted from the column using a linear gradient of 50 mM Na_2CO_3 pH 9.8, 1 M NaCl (buffer B). Purified toxin samples (verified by reducing SDS-8%PAGE) were pooled, protein concentration determined using the method of Bradford (Bradford, 1976) with BSA as standard, and stored at −80 °C until used.

Purified Cry1Ac toxin (1 mg) was biotinylated using a 1:30 molar ratio of EZ link NHS–LC–biotin (Pierce) following manufacturer's instructions. After biotinylation, labeled toxin samples were extensively dialyzed in 20 mM Na_2CO_3 pH 9.8, 150 mM NaCl at 4 °C. Labeled toxins were quantified as above before use in ligand blotting.

2.3. Two-dimensional electrophoresis (2DE) separation of brush border membrane vesicle (BBMV) proteins

Brush border membrane vesicles (BBMV) were isolated as described elsewhere (Wolfersberger et al., 1987). BBMV proteins were extracted by precipitation and then solubilized as previously described (Krishnamoorthy et al., 2007). Solubilized proteins were quantified using the 2D Quant Kit (GE Healthcare) following manufacturer's instructions and kept at −80 °C until used.

BBMV protein samples (50 μg) were used to rehydrate 7 cm, pH 4–7 Immobiline DryStrips (GE Healthcare) overnight in rehydration buffer (solubilization buffer plus 0.018 M dithiothreitol [DTT] and 0.5% ampholytes). Following rehydration, strips were subjected to isoelectric focusing (IEF) using a Multiphor II unit (GE Healthcare) following manufacturer's recommendations. Temperature was maintained at 20 °C throughout focusing. Focused strips were equilibrated for 15 min in equilibration buffer (6 M urea [Plus-One; GE Healthcare], 2% SDS, 30% glycerol, 0.05 M Tris [pH 8.8], 0.002% bromophenol blue) containing 1% DTT followed by a second equilibration for 15 min in equilibration buffer plus 4% iodoacetamide. For second dimension separation we used an EttanDalt six electrophoresis system (GE Healthcare) and SDS-8%PAGE gels. Separated proteins were transferred to PVDF filters overnight and processed as described for Western and ligand blots.

2.4. Localization of HvALP expression in larval tissues

For tissue extract preparation, 4th instar *H. virescens* larvae were carefully dissected and the specific tissues (foregut, midgut, hindgut, muscle, Malphigian tubules, fat body, hemolymph, and gut fluid) were collected in centrifuge tubes containing 0.5 ml of PBS (135 mM NaCl, 2 mM KCl, 10 mM Na_2HPO_4, 1.7 mM KH_2PO_4, pH 7.5). Samples were homogenized using disposable pellet pestles and then 50 μl of 10%SDS was added to each sample. Protein extracts were clarified by centrifugation for 5 min at 21,000 × g at room temperature. Supernatants were collected and protein concentrations quantified using the 2D Quant Kit (GE Healthcare) with BSA as standard.

Tissue extracts (20 μg) or BBMV (15 μg) proteins were mixed with loading buffer (Laemmli, 1970), heat denatured or loaded directly and resolved using SDS-8%PAGE gels. After electrophoretic separation, gels were used for alkaline phosphatase (ALP) activity staining (Jurat-Fuentes and Adang, 2004), total protein staining with ProtoBlue™ safe stain (National Diagnostics, GA), or transferred overnight to polyvinylidene difluoride (PVDF) filters at 4 °C with 20 V constant voltage.

For ALP activity staining, samples were not heat denatured before gel loading. Following electrophoresis, gels were 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 visualized by the formation of a purple-red precipitate. Reactions were stopped by incubation of gels in 50 ml of PBS pH 7.5 containing 200 μl of 500 mM EDTA pH 8.0.

For Western blotting, following electrotransfer, filters were blocked for 1 h in PBST (135 mM NaCl, 2 mM KCl, 10 mM Na_2HPO_4, 1.7 mM KH_2PO_4, pH 7.5, 0.1% Tween-20) plus 3% BSA. After blocking, all incubations and washes were in PBST plus 0.1% BSA. For detection of HvALP, blocked filters were probed with a 1:10,000 dilution of rabbit antiserum developed against the full-length *Anopheles gambiae* membrane-bound alkaline phosphatase (mALP) expressed in *E. coli*, generously provided by Dr. Gang Hua (University of Georgia, Athens, GA). Goat anti-rabbit serum conjugated with horseradish peroxidase (HRP) was used as secondary antibodies and blots developed using Supersignal West Pico enhanced chemiluminescence substrate (Pierce).

For ligand blots 0.1 μg/ml of biotinylated Cry1Ac was used to probe blocked filters for 1 h at room temperature. After washing, blots were probed with streptavidin–HRP conjugate (Invitrogen) at a 1:30,000 dilution for 1 h at room temperature. Proteins binding Cry1Ac were detected with enhanced chemiluminescence substrate as above.

2.5. RNA extraction and cDNA synthesis

Midguts of fourth instar *H. virescens* larvae were dissected and food boli were carefully removed before snap freezing in a 1.5 ml micro-centrifuge tube kept on dry ice. Pools of 20 midguts were homogenized in 1 ml of Trizol reagent (Invitrogen) followed by the addition of 200 μl of chloroform and vortexing for 10 s. The mixture was incubated at room temperature for 15 min and centrifuged at 16,000 × g for 10 min at room temperature. The top aqueous phase was carefully transferred to a new 1.5 ml micro-centrifuge tube containing 600 μl of isopropanol and total RNA was precipitated by centrifuging at 16,000 × g for 15 min in a refrigerated centrifuge. RNA pellets were re-suspended in 200 μl of 10 mM Tris–HCl, pH 8.0.

Messenger RNA was recovered from the total RNA preparation using the poly(A)$^+$-Tract mRNA purification system (Promega) following manufacturer's instructions. Briefly, approximately 100 μg of total RNA was hybridized with 2 μl of 10 μM biotinylated oligo(dT) in a buffer containing 6× SSC (900 mM NaCl, 90 mM sodium citrate, pH 7.0) and 0.1% SDS. The biotinylated oligo(dT) annealed to mRNA was added to streptavidin coated paramagnetic beads pre-washed three times with 0.5× SSC, followed by incubation at room temperature for 10 min. with intermittent mixing by inversion to facilitate binding of biotinylated oligo(dT) to paramagnetic beads. The magnetic beads were captured using a magnetic tube stand and the liquid was aspirated out carefully. Three washes with 0.1× SSC were performed to remove contaminants and the mRNA was released from the magnetic beads by adding 50 μl of RNAse free water followed by magnetic capture of particles. The aqueous phase containing mRNA was transferred to a new tube and was used immediately in the synthesis of full-length cDNA using the GeneRacer™ RLM-RACE kit (Invitrogen) following manufacturer's instructions. Briefly, approximately 2 μg of mRNA was treated with calf intestinal phosphatase to remove 5′-phosphate in non-capped/degraded mRNA, followed by phenol extraction and precipitation. Dephosphorylated mRNA was treated with tobacco acid phosphatase to remove the cap structure from the full-length mRNA. Resulting full-length mRNA with a 5′-phosphate molecule was extracted with phenol and precipitated prior to ligation with the RNA oligonucleotide provided with the GeneRacer™ kit. Reverse transcription of the mRNA with a T7-Oligo(dT) primer yielded cDNA containing the T7 promoter sequence at the 3′-end and the synthetic GeneRacer™ 5′-primer sequence at the 5′-end. Double stranded cDNA production was carried out by amplification of the first-strand cDNA for 10 rounds using the GeneRacer™ 5′-primer and T7 primer in a 100 μl reaction containing 20 μl of the reverse transcription reaction, 10 μl of 10× PCR buffer, 4 μl of 50 mM $MgCl_2$, 2 μl of 10 mM dNTP mix, 30 pmoles each of 5′-RACE primer and T7 Primer, and 2 units of Platinum *Taq* DNA polymerase (Invitrogen).

2.6. Cloning of membrane-bound alkaline phosphatase from H. virescens larval gut tissue

Conceptually translated sequences for *Anopheles gambiae* and *Bombyx mori* membrane-bound ALP sequences from the National Center for Biotechnology Information (NCBI) Entrez server (accession numbers XM308522 and D90454, respectively) were aligned using AlignX module of Vector NTI Suite version 10.3 (Invitrogen). A degenerate primer ALP1-F (5′-GARACGCACGGYGGMGAYGAYGT-3′) and its reverse complement ALP1-R (5′-ACRTCRTCK-CCRCCGTGCGTYTC-3′) were designed to a conserved amino acid block and the corresponding area of the DNA sequence alignment. ALP1-R primer and GeneRacer 5′-primer were used to amplify the 5′-end of the *H. virescens* ALP gene from midgut cDNA. Amplified cDNA was cloned into PCR 2.1-TOPO TA cloning vector and Top10-MachT1® cells were transformed using 3 μl of the amplification reaction. Transformations were plated on Luria Bertani agar plates containing 50 μg/ml kanamycin and 40 μg/ml X-Gal. DNA sequences of recombinant clones were obtained by sequencing with the Big-Dye reagent system and ABI 3730xl instrument (Applied Biosystems). All sequence analyses were carried out at the USDA-ARS mid-South Area Genomics Laboratory, Stoneville, MS.

Primers designed using the sequence information from 5′ clones positive for ALP were used with the GeneRacer 3′ primer to amplify the 3′-end of the *H. virescens* ALP. Finally, all nucleotide sequences were assembled to generate full-length cDNA sequences. All nucleotide and amino acid analyses were carried out using Vector NTI Suite version 10.3. The PSORT program (http://psort.nibb.ac.jp/) was used to detect protein sorting signals and predict protein localization, the OGPET v1.0 with the default prediction constraint (http://ogpet.utep.edu/OGPET/) and the NetOGlyc 3.1 (http://www.cbs.dtu.dk/services/NetOGlyc/) servers were used to predict O-glycosylation sites. The NetNGlyc v1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/) program was used to determine potential N-glycosylation sites, and the big-PI predictor server (http://mendel.imp.ac.at/sat/gpi/gpi_server.html) was used to predict glycosylphosphatidylinositol (GPI) anchoring sites. For sequence alignments we used the AlignX software included in the Vector NTI Advance 10 package (Invitrogen).

2.7. Heterologous expression of selected alkaline phosphatase cDNA clones

Membrane-bound *H. virescens* alkaline phosphatase (HvmALP) clones displaying the highest sequence diversity (HvmALP1 and HvmALP2), were subcloned into the pET30a expression vector (Novagen) using PCR primers designed with *EcoRI* and *NotI* restriction sites at the 5′- and 3′-ends, respectively. To ensure proper protein expression, coding frame in transformants was verified by sequencing in forward and reverse directions (University of Tennessee Sequencing Facility).

For protein expression, purified vectors were transformed into *E. coli* strain BL21(DE3). Transformants were grown overnight with constant agitation at 37 °C in 5 ml of LB broth containing 30 μg/ml kanamycin. The following morning, 100 μl of this overnight culture were used to inoculate 100 ml of LB broth plus 30 μg/ml kanamycin in a 250 ml flask. This culture was allowed to grow at 37 °C with constant agitation until reaching OD_{600} 0.70 (typically about 4–5 h). After bacterial cultures reached an OD_{600} between 0.7 and 1, expression was induced with 1 mM IPTG. Aliquots (1 ml) were obtained at a series of time points (0, 2, 4, 6, and 20 h after induction) to determine optimal expression. This pilot study suggested that peak expression for both clones was observed 2–4 h after induction (data not shown). Consequently, in subsequent experiments, bacterial cultures were collected for analysis 3 h post-induction. After this time, bacterial cultures were collected by centrifugation. Pellets were solubilized in sample buffer (Laemmli, 1970) and 15 μl aliquots separated on a 10%SDS-PAGE gel. Expression of HvALP clones was detected using Western blotting as described above.

3. Results

3.1. Tissue expression of HvALP in H. virescens larvae

We previously reported on the identification of HvALP, a Cry1Ac-binding mALP in BBMV from guts of *H. virescens* larvae (Jurat-Fuentes and Adang, 2004). Fig. 1 shows the detection of *H. virescens* BBMV proteins from Cry1Ac-susceptible (YDK) or resistant (YHD2-B) larvae, cross-reacting with antiserum against mALP from *A. gambiae* or biotinylated Cry1Ac on 2DE blots. Both mALP antiserum and Cry1Ac toxin recognized the same chain of BBMV protein spots in samples from susceptible larvae. Even though the same protein spots were also detected for BBMV from resistant larvae, and as previously reported (Jurat-Fuentes and Adang, 2004), the levels of these HvALP protein spots detected by the antisera in YHD2-B samples were reduced when compared to YDK samples. Considering that HvALP can be defined as BBMV proteins displaying Cry1Ac toxin binding, recognition by anti-mALP antisera, and reduced levels of expression in BBMV from YHD2-B larvae, the lack of cross-reactivity with other BBMV proteins in 1D (data not shown) or 2D blots validated the specificity of the anti-mALP serum for HvALP spots.

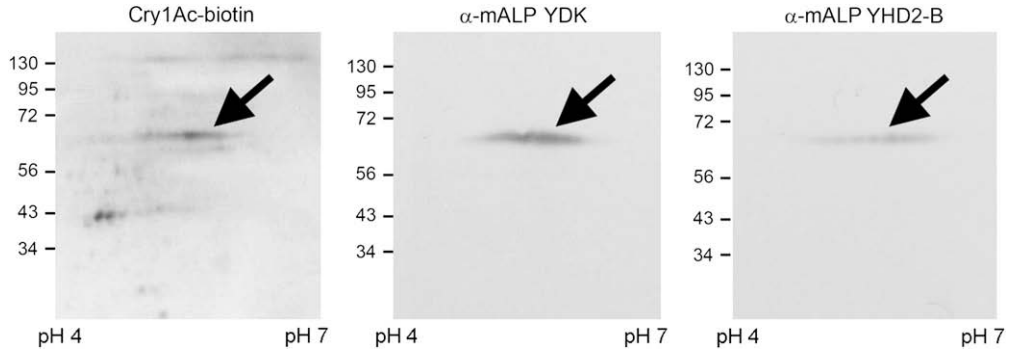

Fig. 1. Detection of Cry1Ac-binding proteins (Cry1Ac-biotin) and proteins cross-reacting with antiserum to *A. gambiae* mALP (α-mALP) in 2DE-resolved BBMV proteins from the YDK or YHD2-B strains of *Heliothis virescens* as indicated. Proteins (50 μg) were separated by isoelectrofocusing (IEF) on pH 4–7 Immobiline DryStrips in the first dimension and SDS-8% PAGE gels for the second dimension. 5 nM of biotinylated Cry1Ac and antiserum to *A. gambiae* mALP were used to probe blots. Arrows point to HvALP spots detected by both Cry1Ac and anti-mALP serum. Masses of molecular weight markers (kDA) are indicated at the left of each blot.

To localize expression of HvALP in *H. virescens* larvae, we screened protein extracts from several larval tissues for both ALP activity and protein detected by anti-mALP serum (Fig. 2). In ALP activity gels phosphatase activity bands of about 68-kDa in size were detected in all tested tissue samples, except for hemolymph and hindgut. These results suggest a broad distribution of alkaline phosphatases in *H. virescens* larval tissues. In comparison, mALP antiserum only detected 68-kDa proteins in tissue extracts prepared from foregut tissue, midgut tissue and BBMV (Fig. 2). The anti-mALP serum also detected a 68-kDa protein in gut fluid, although the signal was weaker than in tissues or BBMV. These results suggest that even though a number of alkaline phosphatase isoforms are present in various *H. virescens* larval tissues, expression of the 68-kDa Cry1Ac-binding HvALP is localized to the foregut and midgut, the latter being the target tissue for Cry1Ac intoxication (Bravo et al., 2007).

3.2. Cloning of full-length HvALP cDNA

Based on HvALP expression being exclusive to gut tissue, we designed an RT-PCR strategy to clone HvmALP cDNA from a larval midgut cDNA library. RT-PCR amplification with degenerate primers to HvALP sequence tags (Krishnamoorthy et al., 2007) and protein regions conserved among insect membrane-bound alkaline phosphatases yielded a partial ALP sequence that was used to design specific primers for Rapid Amplification of cDNA Ends (RACE). After extensive RACE cloning, we obtained clones representing a total of five different ALP sequences. This multiplicity of ALP isoforms was not the result of PCR errors, since all the clones were repeatedly amplified in independent PCR experiments.

All five sequences had high amino acid identity (more than 94%), which suggested that some of the clones represent allelic variants of the same gene. Most sequence divergence was observed in the 3′ UTR region of the clones. Protein sequence alignments (Fig. 3) also denoted this high identity, with most variability observed after the first 190 residues on the N-termini. Sequence alignments revealed that all the clones contained a predicted GPI-anchor site at ^{517}A. Based on this information, we named these clones predicted to be tethered to the cell membrane by a GPI anchor as HvmALP (*H. virescens* membrane-bound alkaline phosphatase) 1–5 (accession numbers FJ416470, FJ416471, FJ416472, and FJ416473 for HvmALP1 to HvmALP4, respectively, and EF531619 for HvmALP5).

All HvmALP cDNAs are predicted to encode full-length proteins of about 59 kDa in molecular size (Fig. 3), suggesting that glycosylation and other posttranslational modifications may make up for the mass difference between these mALP proteins and HvALP (estimated to be 68 kDa) detected in larval tissues (Fig. 2). All five mALP clones contain a signal peptide for localization to the cell surface, a predicted phosphatase domain and active site (^{122}IADSACTAT^{130}, with ^{125}S being the active site), and two potential N-glycosylation sites at residues ^{200}NRTW^{203} and ^{279}NVSH^{282}. While no potential O-glycosylation patterns were detected using the

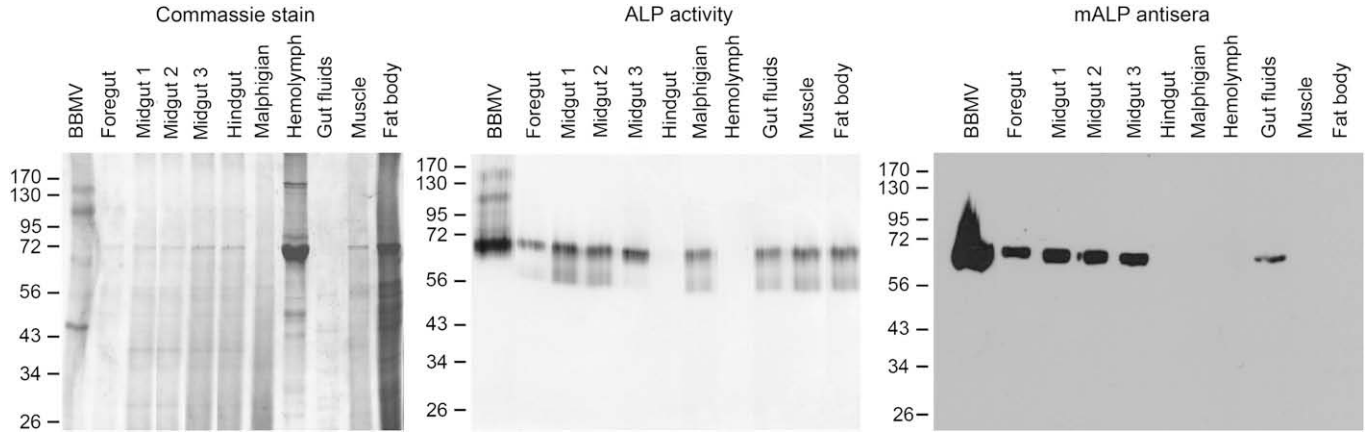

Fig. 2. Detection of ALP activity and HvALP protein in 4th instar *Heliothis virescens* larval tissues. Tissue protein extracts were prepared as described in Materials and methods. Extracts (20 μg) or BBMV proteins (10 μg protein) were separated by SDS-10% PAGE, and stained for total protein with Coomassie Blue, stained for ALP activity with NBT-BCIP, or transferred to PVDF filters and probed with anti-*A. gambiae* mALP serum, as indicated. Samples are as indicated at the top of each lane.

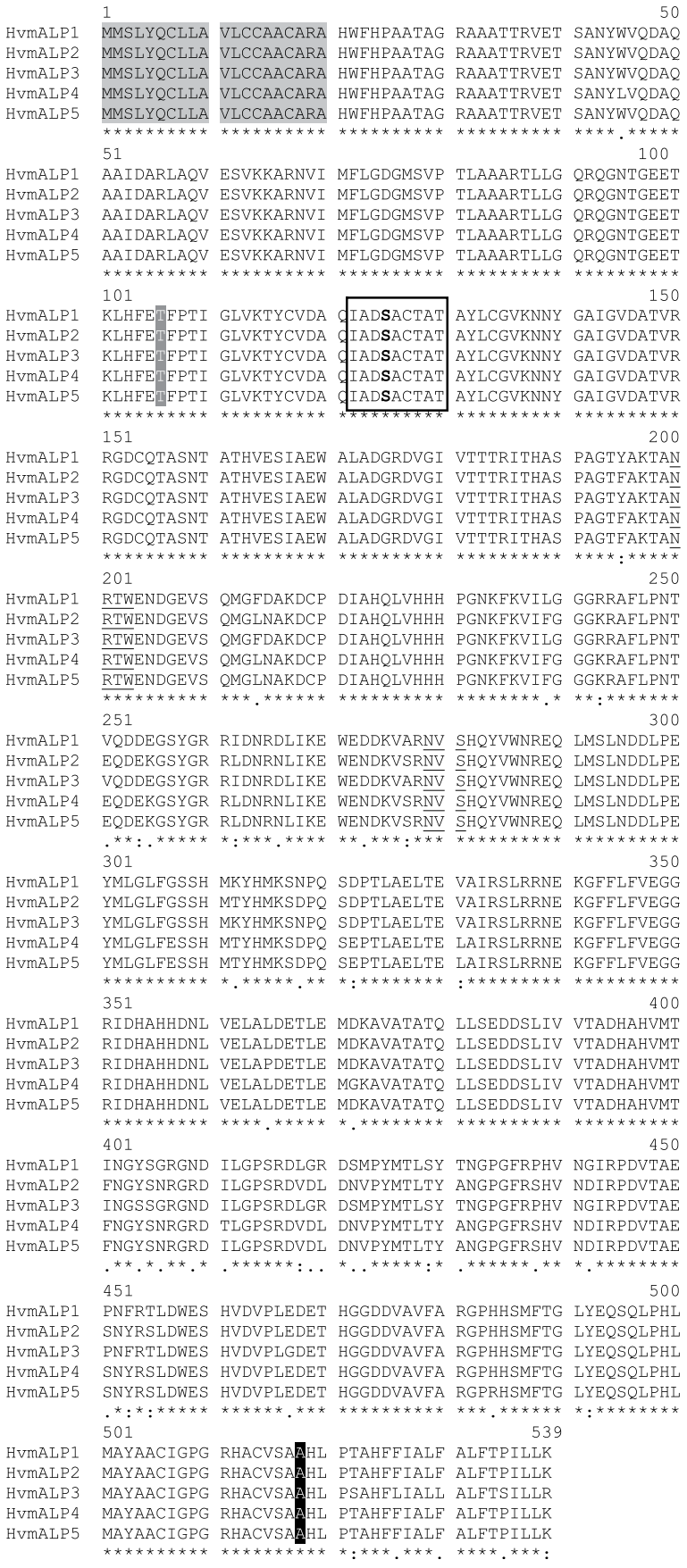

Fig. 3. Protein sequence alignment of membrane-bound midgut alkaline phosphatases from *Heliothis virescens* larvae. The presence of complete sequence identity (*), conservative changes (:), and non-conservative changes (.) is denoted below each residue. The signal peptide for expression on the cell surface is highlighted in gray. The predicted phosphatase domain is included in a square, with the predicted active site in bold. Putative N-glycosylation sites are underlined, while putative O-glycosylation site is highlighted in gray with white font. Predicted GPI-anchor sites are highlighted in black.

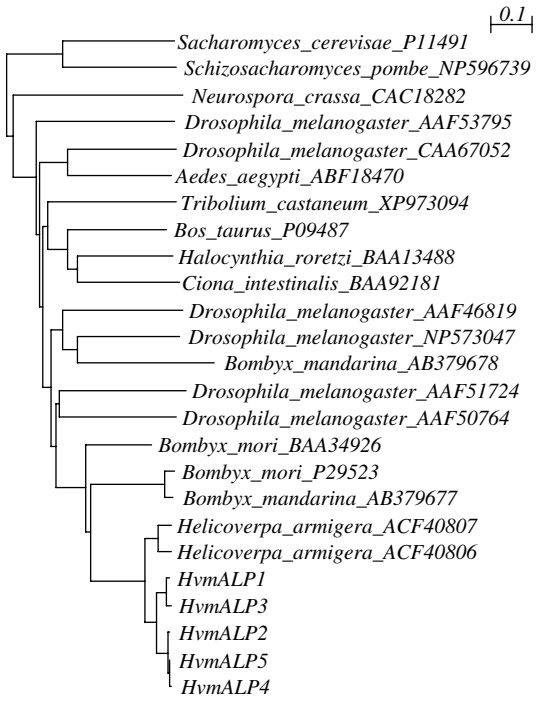

Fig. 4. Phylogenetic tree obtained by using complete protein sequences of selected and predicted alkaline phosphatases. ClustalX 2.0.9 was used to generate a basic sequence alignment to search for homology among protein sequences. After the general alignment was performed an unrooted Phylip tree was generated with the draw tree function in ClustalX 2.0.9, and then exported into NJPLOT to display the tree.

OGPET v1.0 prediction software, the NetOGlyc 3.1 server detected a low probability O-glycosylation site (^{109}T) for all HvmALP isoforms.

When comparing HvmALP protein sequences with other eukaryotic ALPs (Fig. 4), all clones grouped together with ALPs from *Helicoverpa armigera*, with up to 90% sequence identity (for *H. armigera* AFC40807). Sequence identity to membrane-bound forms of ALP from *B. mori* and *Bombyx mandarina* was lower (61%), and other insect ALPs were less similar to HvmALP sequences. Considering the grouping of the HvmALP clones in these sequence alignments (Fig. 4), HvmALP1 and HvmALP3 are probably allelic variants of one gene, while HvmALP2, HvmALP4, and HvmALP5 would represent allelic variants of an alternative ALP gene. All HvmALP protein sequences contained the predicted essential amino acids for alkaline phosphatase activity (Sowadski et al., 1985) in highly conserved protein sequence regions (Fig. 5). Interestingly, the two predicted N-glycosylation sites found in all isoforms of HvmALP (^{200}NRT^{203} and ^{279}NVS^{281}) were conserved in *H. armigera* ALP proteins, but did not align with N-glycosylation sequences in other eukaryotic ALPs, including *Bombyx* sequences.

3.3. Heterologous expression of mALP clones and identification of HvALP

Due to the number of HvmALP clones and the high sequence identity among them, we hypothesized that all of them would be recognized by the antisera to mALP from *A. gambiae*, and thus be synonymous with HvALP. To test this hypothesis, we selected the most sequence-diverse clones, HvmALP1 and HvmALP2 sharing 94% identity, and expressed them in *Escherichia coli* cultures. Protein bands of the expected mass for both isoforms were detected in stained gels and by probing Western blots with antisera to the 6× histidine tag added by the *E. coli* expression vector (Fig. 6). In ALP activity gels, alkaline phosphatase activity was not detected for these proteins (data not shown), suggesting incorrect folding or posttranslational modifications of the expressed phosphatases. As predicted, both expressed HvmALP proteins were recognized by antiserum to the *A. gambiae* mALP (Fig. 6). Analysis of the stained gels suggests that the HvmALP1 clone was expressed more efficiently, explaining the higher level of signal for this protein in the immunoblots. These results suggest that both isoforms are synonymous with HvALP. As expected from the reported binding of Cry1Ac to GalNAc residues on HvALP (Jurat-Fuentes and Adang, 2004), no binding of biotinylated Cry1Ac to any of the expressed HvmALP clones was detected in ligand blots (Fig. 6). We did observe Cry1Ac binding to small *E. coli* proteins, although these proteins were detected even for control samples, indicating that they do not represent truncated or degraded expressed mALP proteins. As a positive control, Cry1Ac bound to HvALP in *H. virescens* BBMV (Fig. 6, lane 5).

4. Discussion

Alkaline phosphatases (EC 3.1.3.1) are common abundant enzymes that are mainly involved in removing phosphate groups from organic molecules. In insects, ALPs have been reported to be involved in several biological processes (Eguchi, 1995; Chang et al., 1993). Physiological levels of midgut alkaline phosphatase activity have been reported to change during stress or pathogenesis in insects (Kucera and Weiser, 1974; Miao, 2002; Sukhanova et al., 1996), in some cases related to the low energy state associated with infection (Sujak et al., 1978). We have previously described the identification of HvALP, a membrane-bound alkaline phosphatase (mALP) that binds Cry1Ac toxin in midgut brush border membrane from *H. virescens* (Jurat-Fuentes and Adang, 2004). In addition to toxin binding, down-regulation of HvALP in midguts of larvae from Cry1Ac-resistant *H. virescens* larvae (Jurat-Fuentes and Adang, 2004, 2007), suggests a role for HvALP in Cry1Ac toxicity. Towards the characterization of the role of HvALP in Cry1Ac intoxication, we report the cloning of mALP isoforms from the *H. virescens* midgut through cloning and expression of two diverse mALP clones in a heterologous system, in an attempt to identify HvALP among the ALP isoforms.

In agreement with the reported pattern of localization for *M. sexta* mALP (Chen et al., 2005), HvALP expression was localized to the foregut and midgut regions of the *H. virescens* larval gut. The midgut region is where Cry1Ac toxin mode of action takes place (reviewed in Bravo et al., 2007). The posterior region of the larval midgut has been previously reported to display higher levels of Cry1Ac-induced pore formation than the anterior part of the midgut (Carroll et al., 1997; Rodrigo-Simon et al., 2008). Considering that the *H. virescens* cadherin (HevCaLP) is mostly localized to the anterior part of the gut (Aimanova et al., 2006), localization of HvALP throughout the midgut would favor the sequential participation of multiple receptors in Cry1Ac intoxication as proposed by the model of Bravo et al. (2007). Considering that Cry1Ac oligomers bind to GalNAc residues on aminopeptidase in *M. sexta* (Pardo-Lopez et al., 2006), and the similarities in the Cry1Ac mode of action between *M. sexta* and *H. virescens* (Zhuang et al., 2002), it is plausible that after toxin activation and binding to HevCaLP in the anterior midgut region, resulting Cry1Ac toxin oligomers would interact with GalNAc residues on GPI-anchored proteins on lipid rafts (aminopeptidases and alkaline phosphatases) and insert on the cell membrane to create a pore that would lead to osmotic cell lysis. This hypothesis does not exclude the possibility that binding to cadherin may also result in midgut cell death per se as proposed by an alternative model (Zhang et al., 2006).

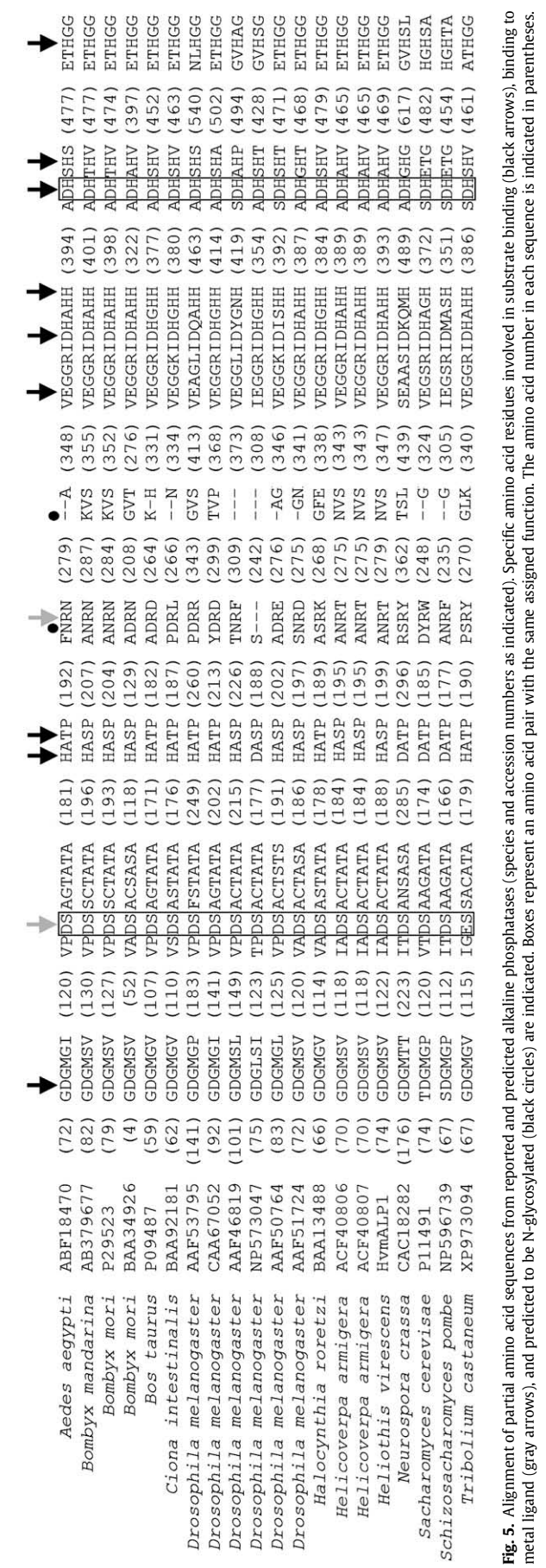

Fig. 5. Alignment of partial amino acid sequences from reported and predicted alkaline phosphatases (species and accession numbers as indicated). Specific amino acid residues involved in substrate binding (black arrows), binding to metal ligand (gray arrows), and predicted to be N-glycosylated (black circles) are indicated. Boxes represent an amino acid pair with the same assigned function. The amino acid number in each sequence is indicated in parentheses.

In contrast to the case of *B. mori* (Takesue et al., 1989), we did not find a soluble (sALP) alkaline phosphatase form in our cloning efforts. It is possible that the protein detected in midgut fluid by anti-mALP serum is a soluble isoform of ALP. Alternatively, it is also possible that mALP isoforms are released into the gut lumen by cleavage of the GPI anchors as is the case in *B. mori* (Takesue et al., 1989).

The high sequence identity observed between HvmALP isoforms suggests that they may represent allelic variants of a limited number of genes. In *B. mori*, one mALP and one sALP cDNA have been cloned (Itoh and Kanamori, 1999; Itoh et al., 1991). In this insect, the single *mALP* and *sALP* genes are arranged in tandem and seem to have been derived from duplication (Itoh et al., 2003). Sequence alignments clearly suggest that the five HvmALP sequences we obtained represent allelic variants of at least two mALP genes. Thus, the HvmALP1 and HvmALP3 proteins would be isoforms of HvmALP2, HvmALP4, and HvmALP5. Interestingly, the *sALP* gene from *B. mori* (Itoh and Kanamori, 1999) and the human intestinal ALP gene (Henthorn et al., 1988) were reported to be transcribed into a minimum of two mRNAs of different size. In contrast, we found multiple cDNAs of the same size but with slightly differences in sequence in *H. virescens* midgut. Further research is necessary to determine the number of mALP genes present in *H. virescens*.

When expressed in a heterologous system, clones displaying the highest diversity among the HvmALP clones were detected by antisera cross-reacting with HvALP in immunoblots. However, none of the sequences matched perfectly with the sequence tag (GFFLFVENR) previously obtained for HvALP (Krishnamoorthy et al., 2007) and used to design degenerate primers for cloning. Since this sequence tag was obtained from de novo sequencing with a 95% expected confidence, and due to potential post-translational modifications and mass inaccuracies, we believe this sequence tag was probably inaccurate. In support of this conclusion, the specific region matching this sequence tag in HvmALP sequences (^{342}GFFLFVEGGR^{351}) is highly conserved in all reported insect ALP sequences (data not shown). Additionally, none of the reported Eukaryotic ALP sequences contain this sequence tag. Considering the overall high sequence identity among all the HvmALP clones and the cross-reactivity of the most diverse HvmALP sequences with the HvALP-reacting antisera, we propose that all HvmALP isoforms are synonymous with HvALP. This observation may explain the long chain of protein spots detected by antiserum to mALP in 2D blots, with each isoform representing a number of spots. Considering the importance of N-glycosylation for Cry1Ac binding to HvALP (Jurat-Fuentes and Adang, 2004), and that the antiserum used to detect HvALP was developed against a non-glycosylated protein, it is possible that although all HvmALP proteins may be detected by the antisera, they may not contain the specific sugars necessary for Cry1Ac binding. In this regard, it is interesting to note that the N-glycosylation sites predicted to exist in HvmALP isoforms were preserved in *H. armigera* ALP sequences, but did not align to corresponding N-glycosylation sites in other insect ALPs (including *B. mori*). In *B. mori* mALP a single N-glycosylation site was predicted, and localized more proximal to the N terminus of the protein (Itoh et al., 1991). Although speculative, this observation may suggest that localization of these N-linked sugars on ALP may be crucial for Cry1Ac binding and may explain higher Cry1Ac susceptibility in *H. virescens* and *H. armigera* when compared to *B. mori* larvae (Van Frankenhuyzen and Nystrom, 2002). In contrast, *B. mori* larvae are more susceptible than *H. virescens* or *H. armigera* to other Cry1A toxins whose binding to gut proteins is not inhibited by N-acetylgalactosamine.

Previous reports have demonstrated interactions between Cry1Ac toxin and alkaline phosphatases in Lepidoptera resulting in decreased enzymatic activity (English and Readdy, 1989; Sangadala et al., 1994).

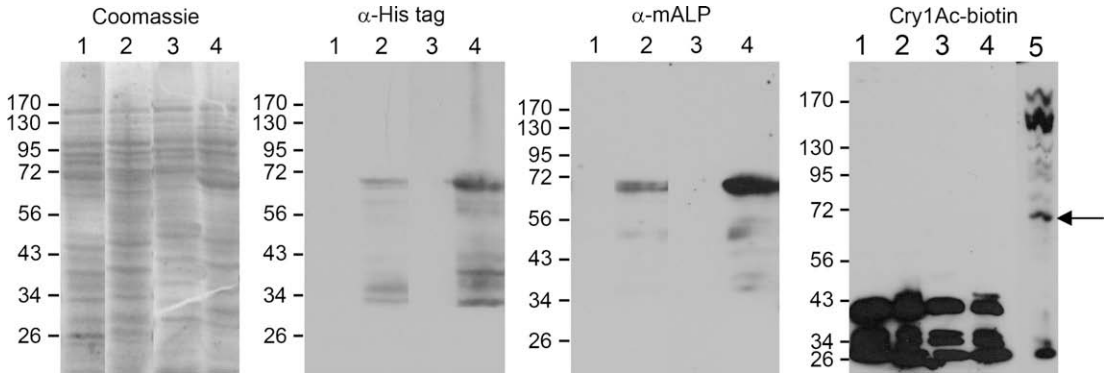

Fig. 6. Cry1Ac does not interact with HvmALP cDNAs expressed in *Escherichia coli*. Aliquots of *E. coli* cultures carrying pET vectors encoding specific full-length cDNA sequence were separated by SDS-10% PAGE. After electrophoresis, proteins were stained (Coomassie), or transferred to PVDF filters and probed with antiserum to His tag (α-His tag), antiserum against mALP of *Am. gambiae* (α-mALP), or biotinylated Cry1Ac toxin (Cry1Ac-biotin). Cross-reacting proteins and bound toxin were detected using enhanced chemiluminescence. Lane1: HvmALP2 before induction, lane 2: HvmALP2 2h. after induction, lane 3: HvmALP1 before induction, lane 4: HvmALP1 2h. After induction, lane 5: BBMV proteins (20 μg). Arrow indicates position of HvALP in BBMV lane.

We have previously reported on decreased HvALP levels in midguts of larvae from Cry1Ac-resistant strains of *H. virescens* (Jurat-Fuentes and Adang, 2004, 2007). In *Aedes aegypti* larvae, an mALP has been reported as a functional Cry11Aa toxin receptor (Fernandez et al., 2006). Taken together, this information suggests a direct role of HvALP in Cry1Ac intoxication, yet further research is needed to characterize the specific physiological functions of the HvmALP isoforms and their putative role as functional Cry1Ac receptors.

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