RESEARCH ARTICLE

Interaction of *Allium sativum* leaf agglutinin with midgut brush border membrane vesicles proteins and its stability in *Helicoverpa armigera*

Santosh Kumar Upadhyay[1], Manisha Mishra[1], Harpal Singh[1], Amol Ranjan[1], Krishnappa Chandrashekar[1], Praveen Chandra Verma[1], Pradhyumna Kumar Singh[1] and Rakesh Tuli[2]

[1] National Botanical Research Institute, Council of Scientific and Industrial Research, Rana Pratap Marg, Lucknow, Uttar Pradesh, India
[2] National Agri-Food Biotechnology Institute, Department of Biotechnology, Government of India, Mohali, Punjab, India

Allium sativum leaf agglutinin (ASAL) binds to several proteins in the midgut of *Helicoverpa armigera* and causes toxicity. Most of these were glycosylated. Six ASAL-binding proteins were selected for identification. PMF and MS/MS data showed their similarity with midgut aminopeptidase APN2, polycalins and alkaline phosphatase of *H. armigera*, cadherin-N protein (partial AGAP009726-PA) of *Acyrthosiphon pisum*, cytochrome P450 (CYP315A1) of *Manduca sexta* and alkaline phosphatase of *Heliothis virescens*. Some of the ASAL-binding midgut proteins were similar to the larval receptors responsible for the binding of δ-endotoxin proteins of *Bacillus thuringiensis*. *Galanthus nivalis* agglutinin also interacted with most of the ASAL-binding proteins. The ASAL showed resistance to midgut proteases and was detected in the larval hemolymph and excreta. Immunohistochemical staining revealed the presence of ASAL in the body tissue also.

Received: March 8, 2010
Revised: August 27, 2010
Accepted: September 7, 2010

Keywords:
Allium sativum leaf agglutinin / Animal proteomics / *Bacillus thuringiensis* / Glycosylated / *Helicoverpa armigera* / Midgut

1 Introduction

A number of plant and microbial proteins toxic to insect pests are possible alternatives to the chemical pesticides. Plant-derived lectins is a promising group of such proteins for the control of insect pests [1–7]. The mannose-binding *Allium sativum* leaf agglutinin (ASAL) (MW = 12 kDa) is considered safe because garlic is a widely consumed food crop. ASAL is toxic against not only the plant sap sucking homopteran insects but also the coleopteran and lepidopteran pests [8–13]. Transgenic tobacco plants expressing ASAL show insect resistance against *Spodoptera litura*. Severe retardation of larval growth and 100% mortality at the pupal stage are observed when larvae are fed on transgenic plant leaves [9]. Resistance to midgut proteolysis and binding to the midgut epithelial cells or to specific receptors is a prerequisite, although not sufficient for lectins to be toxic to insects. Lectins show resistance to gut proteolysis and are subsequently detected in hemolymph [14–16] and other insect tissues [17, 18]. Binding of ASAL to gut epithelial receptors in homopteran insects has been demonstrated through immunolocalization [19]. Putative receptors of 55 and 45 kDa have been identified in the brush border membrane vesicles (BBMV) preparation of the aphid *Lipaphis erysimi* and red cotton bug *Dysdercus* sp.,

Correspondence: Dr. Pradhyumna Kumar Singh, National Botanical Research Institute, Council of Scientific and Industrial Research, Rana Pratap Marg, Lucknow 226001, India
E-mail: pksingh@nbri.res.in; pradhyumnasingh@hotmail.com
Fax: +91-522-205836, +91-522-205839

Abbreviations: ALP, alkaline phosphatase; **ASAL,** *Allium sativum* leaf agglutinin; **BBMV,** brush border membrane vesicles; **GNA,** *Galanthus nivalis* agglutinin

respectively [18]. *Arum maculatum* tuber lectin binds to the receptors of 40 and 35 kDa in BBMV of aphids *L. erysimi* and *Aphis craccivora*, respectively [20].

ASAL is also toxic to cotton bollworm *Helicoverpa armigera* [8, 21], one of the most serious lepidopteran pests of cotton, pigeon pea, chickpea, sunflower, tomato, maize, *etc*. However, the mode of action of ASAL and other lectins has not been established. This study describes the purification and identification of the BBMV proteins responsible for the binding of ASAL in the midgut of *H. armigera* larvae, stability of ASAL to gut proteases and its immunolocalization. Since ASAL can be isolated only in limited quantity from garlic leaves, recombinant ASAL was expressed in *Escherichia coli* and purified in sufficient quantity, as described earlier [21].

2 Materials and methods

2.1 Isolation of BBMV from midgut of *H. armigera*

BBMV was isolated from the fifth instar larvae of *H. armigera* by repeated $MgCl_2$ differential centrifugation [22]. Larvae were immobilized by placing on ice for 5 min and dissected in MET buffer (300 mM Mannitol, 5 mM EGTA, 17 mM Tris-Cl, pH 7.4). The midguts were removed, rinsed in MET buffer, powdered under liquid nitrogen and homogenized in MET buffer in the ratio of 1:9 w/v. Equal volume of 24 mM $MgCl_2$ was added to the homogenate. The suspension was kept on ice for 30 min, followed by centrifugation ($2500 \times g$, 15 min). The pellet was discarded and the supernatant was centrifuged ($30\,000 \times g$, 30 min). The pellet was resuspended in half of the original volume of MET-$MgCl_2$ and the above steps were repeated. The final pellet was solubilized in PBS (pH 7.4) containing 0.1% Tween-20 by overnight incubation at 4°C with gentle shaking. The BBMV preparation was assayed for activity of the marker enzymes alkaline phosphatase (ALP) and leucine aminopeptidase. The enrichment in the specific activity of ALP in BBMV as compared with the crude extract was 5.08-fold (176.5 ± 7.43 to 898.23 ± 28.95 μmol/min/mg protein) and that in case of leucine aminopeptidase was 4.6-fold (49.42 ± 0.018 to 228.6 ± 0.10 μmol/min/mg protein).

2.2 Preparation of ASAL affinity column and purification of ASAL binding midgut BBMV proteins

The recombinant ASAL was expressed in *E. coli* (N-terminal fusion with SUMO peptide) and purified [21]. The purified protein was incubated with CNBr-activated sepharose 4B matrix and the affinity column was prepared as *per* the manufacturer's protocol (GE Healthcare, USA). BBMV proteins solubilized in PBS were centrifuged ($2500 \times g$, 15 min). The supernatant was filtered through 0.22 μm filter and loaded on the above column (at a flow rate of 0.5 mL/min) pre-equilibrated with PBS (pH 7.4). Nonspecifically bound proteins were removed by washing the column with PBS till OD_{280} became stable at 0.002. The BBMV proteins bound with the ASAL affinity column were eluted with different buffers (1 M mannose in PBS, 100 mM glycine buffer (pH 3.0) and 100 mM sodium carbonate (pH 11.6)). Mannose could elute the protein but in large volume and therefore was very dilute. In case of glycine buffer, the eluted proteins lost their marker enzyme activities. Unbuffered sodium carbonate eluted ASAL-bound protein in sharp peak along with the marker enzyme activity. This preparation was analyzed further.

2.3 Glycoprotein-specific staining of purified midgut BBMV proteins and deglycosylation

To detect glycosylation in the purified BBMV proteins, glycoprotein-specific staining was performed by using glycoprofile™ III fluorescent glycoprotein detection kit (Sigma, USA). The proteins were resolved on SDS-PAGE and fixed in 50% methanol containing 3% acetic acid for 1 h. The gel was washed repeatedly in water and incubated in oxidation reagent containing periodic acid for 20 min. The gel was further washed and submerged in glycoprotein-specific fluorescent staining solution for 1 h. The gel was washed again and the glycoproteins were detected by scanning at 312 nm under Flour-S™ multi-imager (BioRad, USA). The gel was further stained with Coomassie Brilliant Blue for the detection of total proteins.

To study the role of glycosylation in the interaction of ASAL with *H. armigera* BBMV proteins, the proteins were deglycosylated by deglycosylation enzyme PNGase F (New England Biolabs, USA) as *per* the manufacturer's protocol. The deglycosylated proteins were analyzed by glycoprotein-specific staining and used for ligand blot analysis.

2.4 Identification of purified midgut BBMV proteins by PMF and MS/MS analysis

Purified midgut BBMV proteins were resolved on SDS-PAGE. The protein bands were excised, reduced with 10 mM DTT (56°C, 30 min), alkylated with 50 mM iodoacetamide (room temperature, 30 min) and digested with trypsin (Sequencing grade, Porcine, Promega, USA) in 50 mM ammonium bicarbonate buffer (pH 8.0) at 37°C for overnight [23]. The digested peptides were extracted by sonication in extraction solution (60% ACN and 1% TFA) for several times. The extracted peptides were dried by centrifugal evaporation and suspended in 5 μL resuspension solution (50% ACN, 0.1% TFA). Briefly, 0.5 μL of the suspended peptides were spotted on MALDI plate followed by 0.5 μL CHCA matrix (10 mg/mL in 50% ACN, 0.1% TFA). Spots were dried completely and used for PMF on

MALDI-TOF-TOF platform (model 4800, ABsciex, USA). The PMF data were analyzed on the MASCOT search using MSDB, Swiss-Prot and NCBI database. The search parameters were peptide tolerance of 100 ppm, one missed cleavage, carbamidomethyl modification of cysteines and possible methionine oxidation. The peptides with higher signal intensity were selected for MS/MS analysis. The results obtained from MS/MS were analyzed by searching against the MSDB, Swiss-Prot and NCBI database with the following parameters: fixed precursor ion mass tolerance of 20 ppm, fragment ion mass tolerance of 0.05 Da, calibration error of 0.005 Da, one missed cleavage, carbamido methylation of cysteines and possible oxidation of methionine. The spectra of common contaminants were removed by searching against contaminant database, and the remaining data were used for the identification of proteins.

2.5 Digestion of native and recombinant ASAL by midgut proteases and trypsin

Native and recombinant ASAL were digested with trypsin and midgut protease preparation as described in Supporting Information method 1. Digested samples were analyzed on SDS-PAGE with Coomassie staining or immunoblotting as desired.

2.6 Detection of ASAL in hemolymph and excreta

Hemolymph and excreta were collected from the fifth instar larvae after 24 h of feeding on semi-synthetic diet supplemented with native ASAL, recombinant ASAL or BSA (100 $\mu g/cm^2$ concentration). Samples were prepared (described in Supporting Information method 2), resolved on SDS-PAGE (Fig. 3D) and used in immunoblot analysis.

2.7 Ligand blot and Western blot analysis

Ligand blot analysis was performed to further confirm the interaction of the midgut BBMV proteins of *H. armigera* with ASAL. The method was also used for the study of interaction of ASAL with the deglycosylated midgut proteins. Purified BBMV proteins separated on SDS-PAGE were transferred onto the PVDF membrane (Millipore, USA) by using I-Blot gel transfer apparatus (Invitrogen, USA). The membranes were blocked with TBST containing 5% nonfat dry milk. The membranes were incubated separately with ASAL and BSA at a concentration of 4 nM for 2 h in TBST containing 1% nonfat dry milk. BSA was taken as a negative control. The membranes were washed repeatedly in TBST and incubated with anti-ASAL antibody (1:1000 dilutions) for 2 h. The membrane was washed again and incubated with HRP-conjugated secondary antibody (1:15 000) for 2 h. After washing, blots were developed using DAB system (Bangalore Genei, India). *Galanthus nivalis* agglutinin (GNA) was also used in ligand blotting to study the interaction of an alternative mannose-binding protein with the midgut BBMV proteins. Binding of GNA was detected using digoxigenin-labeled GNA of DIG Glycan Detection Kit (Roche, Switzerland) by following the manufacturer's protocol.

Western blot analysis was performed to detect the native and recombinant ASAL in excreta and hemolymph of *H. armigera* and to analyze digested product of ASAL. The protein samples were resolved on SDS-PAGE and transferred onto the PVDF membrane as described above. The membranes were blocked and incubated with anti-ASAL antibody followed by HRP-conjugated secondary antibody and the blots were developed with DAB system.

2.8 Detection of ALP activity in SDS-PAGE blot

The presence of ALP in the purified proteins was detected by ''in gel'' assay [24]. Purified and crude midgut BBMV proteins, solubilized in sample buffer, were resolved on 12% SDS-PAGE without heat denaturation. Proteins were transferred onto PVDF membrane. The membrane was washed with water and colour was developed with Western blue-stabilized substrate for ALP (Promega, USA).

2.9 Immuno-localization of ASAL in *H. armigera*

One micrometer thick sections were prepared from fifth instar larvae of *H. armigera* fed for 24 h on semi-synthetic diet supplemented with 100 $\mu g/cm^2$ of native ASAL (set 1), recombinant ASAL (set 2), BSA (set 3) or PBS (set 4) (Supporting Information method 3). Some section of set 4 were incubated with 4 nM of native and recombinant ASAL designated as set 4a and 4b to serve as positive control. The few sections of set 4 were also processed through all the steps except incubation with ASAL, to serve as null control. All the sections were incubated for 2 h with the anti-ASAL antibody labeled with fluorescent Atto 488 dye (ReaMetrix, India). Sections were washed in PBST and water. The fluorescence signal in the sections was observed under confocal microscope.

3 Results

3.1 Purification of ASAL interacting midgut BBMV proteins of *H. armigera* and ligand blotting

Total BBMV proteins were isolated from the midgut of *H. armigera* and solubilized in PBS containing 0.1% Tween-20. The soluble BBMV proteins were purified by affinity chromatography as described in Section 2.2 and analyzed on denaturing PAGE (Fig. 1A). Several ASAL interacting

proteins were visualized on gel. Ligand blot analysis with purified proteins showed that almost all the affinity-purified proteins interacted with the native ASAL (Fig. 1D). They also showed nearly similar interaction with GNA (Fig. 1E).

3.2 Identification of ASAL interacting midgut BBMV proteins by MS analysis

The protein bands were excised from SDS-PAGE, digested with trypsin and digested fragments were analyzed by PMF and MS/MS analysis. PMF could identify all the six proteins. MS/MS could confirm four proteins only due to the limitation in database. Two proteins identified on the basis of PMF analysis were identical to cadherin-N protein (partial, AGAP009726-PA) of *Acyrthosiphon pisum* (band II) and cytochrome P450 (CYP315A1) of *Manduca sexta* (band VI). MS/MS data for band I matched with the midgut aminopeptidase APN2 and band III with polycalins of *H. armigera*. Band IV and V matched with ALP of *H. armigera* and *H. virescens* (Table 1). The data were also used to search the database described by Campbell *et al.* [25], but no further sequences were identified. The presence of ALP in the purified proteins was also confirmed by in-gel assay (Fig. 2). The presence of ALP and APN in affinity-purified proteins was further confirmed by enzymatic assay and the specific activities were 2534 ± 37.31 and 754 ± 0.17 μmol/min/mg, respectively. The detail of the identified proteins is given in Supporting Information file 1.

3.3 Characterization of purified proteins as a glycoprotein

Most of the purified midgut BBMV proteins were stained with glycoprotein-specific stain (Fig. 1C). Subsequent Coomassie Blue staining of the gel showed the presence of all glycosylated and nonglycosylated proteins (data not shown). Mobility shift was observed in SDS-PAGE analysis of purified midgut BBMV proteins after deglycosylation (Fig. 1B). It became evident that almost all the proteins were glycoproteins. Loss in binding of ASAL to deglycosylated midgut BBMV proteins on ligand blot analysis further confirmed that the interaction of ASAL with midgut BBMV proteins was glycans mediated (Fig. 1D). Ligand blot analysis of ASAL affinity-purified midgut BBMV proteins with GNA also showed nearly similar interaction (Fig. 1E).

3.4 Digestion of native and recombinant ASAL with gut proteases and trypsin

To study the stability of ASAL in the gut of *H. armigera*, both the proteins were treated with gut proteases and trypsin. BSA was taken as control. Gut proteases digested the BSA and additional part (*i.e.* SUMO peptide) of the recombinant ASAL (Fig. 3A and B). Trypsin digested the BSA and cleaved the recombinant ASAL into two constituent parts, *i.e.* SUMO and ASAL (18 and 12 kDa on denaturing PAGE, Fig. 3C and D). SUMO peptide was used for high level expression of ASAL in *E. coli* [21] with a trypsin cleavage site at the junction of SUMO and ASAL (data not shown). The digested products were confirmed by MS/MS analysis (data

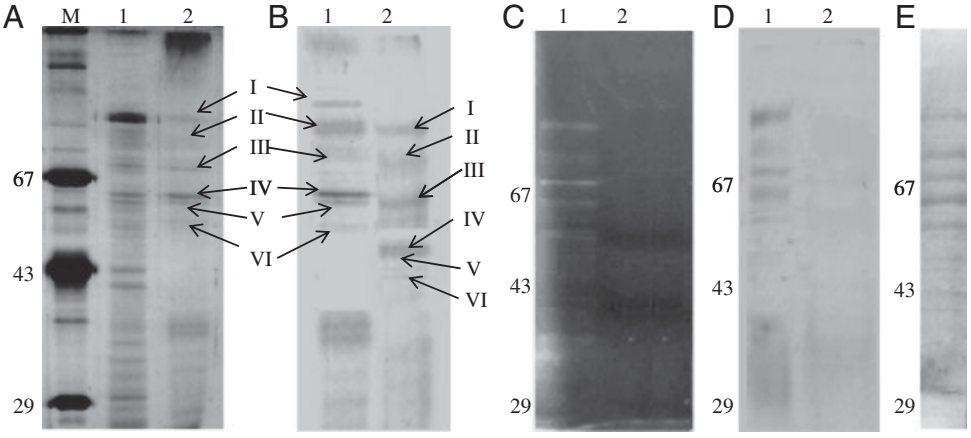

Figure 1. BBMV protein profile in SDS-PAGE. All the results were confirmed by three independent biological replicates. (A) Purification of total BBMV proteins on ASAL affinity column (Silver-stained 10% SDS-PAGE): lane M, molecular weight marker; lane 1, total BBMV proteins; lane 2, eluted BBMV proteins. Arrows show bands used for PMF and MS/MS analysis. (B) Deglycosylation of BBMV proteins purified by affinity chromatography: lane 1, affinity-purified BBMV proteins; lane 2, deglycosylated BBMV proteins showing band shift. Arrow marks on deglycosylated lanes show probable band shift. (C) Glycoprotein-specific staining of the ASAL affinity-purified BBMV proteins: lane 1, purified BBMV proteins; lane 2, deglycosylated BBMV proteins. (D) Ligand blot analysis of the affinity-purified BBMV proteins with ASAL: lane 1, with glycosylated (native) proteins; lane 2, with deglycosylated proteins. (E) Ligand blot analysis of the affinity-purified BBMV proteins with GNA shows nearly similar binding pattern as ASAL. Figure quality (sharpness, contrast and brightness) was improved by using adobe Photoshop.

Table 1. Predicted ASAL interacting midgut proteins of *H. armigera* larvae by PMF and MS/MS analysis (results were confirmed by three independent biological replicates)

Band	Method of identification	Gene bank accession	Description	Organism	Molecular weight (kDa)		Number of peptide hits	Coverage (%)	Score
					Matched protein	Observed on SDS-PAGE			
I	MS/MS	gi|25814968	Midgut aminopeptidase APN2	*H. armigera*	113 773	≈ 114	5	6	192
II	PMF	gi|193654903	AGAP009726-PA, partial (cadherin-Nprotein)	*A. pisum*	104 020	≈ 104	19	25	119
III	MS/MS	gi|171740913	Polycalin	*H. armigera*	102 732	≈ 81.8	2	2	65
		gi|171740923	Polycalin	*H. armigera*	82 522	≈ 81.8	2	2	65
IV	MS/MS	gi|194295558	ALP 2	*H. armigera*	59 223	≈ 60	6	14	331
		gi|194295556	ALP 1	*H. armigera*	59 265	≈ 60	5	14	309
V	MS/MS	gi|227462438	ALP	*H. virescens*	59 716	≈ 59.6	4	6	170
VI	PMF	gi|86440315	Cytochrome P450 CYP315A1	*M. sexta*	57 479	≈ 57	16	40	104

Figure 2. Detection of alkaline phosphatase activity in SDS/PAGE blot. Lane 1; crude BBMV proteins, lane 2; affinity purified BBMV proteins.

not shown). Neither trypsin nor *H. armigera* gut proteases were able to digest ASAL, confirming the stability of ASAL in the larval gut.

3.5 Detection of ASAL in excreta and hemolymph

ASAL was detected in hemolymph and excreta of *H. armigera*, collected after 24 h of feeding on either native or recombinant ASAL. The lectin was detected by Western blot analysis. However, the SUMO part of the recombinant ASAL was not detected in hemolymph and excreta (Fig. 4A–C). This suggests that SUMO was digested completely in the gut of *H. armigera*, leaving ASAL intact. A part of ASAL got absorbed through the gut and caused toxicity to the insect. The rest was excreted out.

3.6 Immuno-localization

To study the distribution of ASAL in the body/tissue of *H. armigera* larvae, immunohistochemical staining was done as described. Native and recombinant ASAL were detected by fluorescence Atto 488-labeled anti-ASAL antibody (Supporting Information Fig. 1). Although significant fluorescence was detected in all sets of experiments (Supporting Information Figs. 1C–F), some fluorescence was observed in case of the negative control (set 3, Supporting Information Fig. 1A) and null control (set 4, Supporting Information Fig. 1B) also. This might be due to nonspecific interactions of antibody. In case of the positive controls (set 4A and 4B, Supporting Information Figs. 1C and D), fluorescence was detected in gut wall, hemolymph and epidermal cells. The experimental sets 1 and 2, where

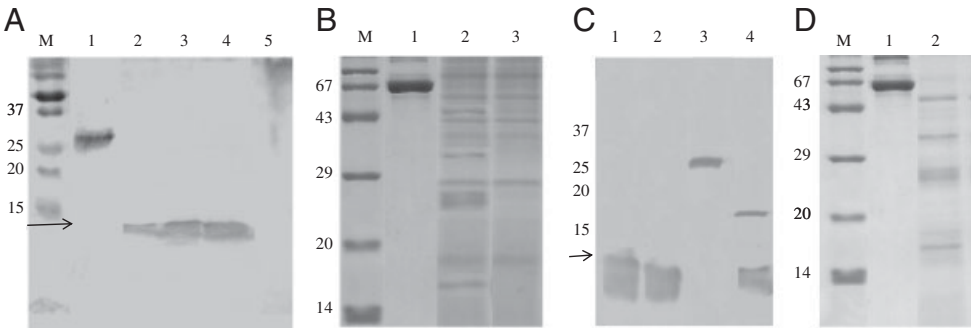

Figure 3. Digestion of native and recombinant ASAL with gut proteases and trypsin. (A) Western blot analysis after digestion with gut proteases: lane M, prestained molecular weight marker; lane 1, recombinant SUMO-ASAL undigested (30 kDa); lane 2, recombinant SUMO-ASAL digested; lane 3, ASAL undigested (12 kDa); lane 4, ASAL digested; and lane 5, total gut proteases. SUMO part of SUMO-ASAL was digested. (B) Coomassie-stained analysis of digestion product of BSA with gut proteases: lane M, molecular weight marker; lane 1, BSA undigested; lane 2, BSA digested; lane 3, total gut proteases. (C) Western blot analysis of digestion product of native and recombinant ASAL with trypsin: lane 1, ASAL undigested; lane 2, ASAL digested; lane 3, recombinant SUMO-ASAL undigested; lane 4, recombinant SUMO-ASAL digested. Trypsin cleaves the ASAL from SUMO (D) Coomassie-stained analysis of digestion product of BSA with trypsin: lane M, molecular weight marker; lane 1, BSA undigested; lane 2, BSA digested. Arrow shows the position of ASAL band.

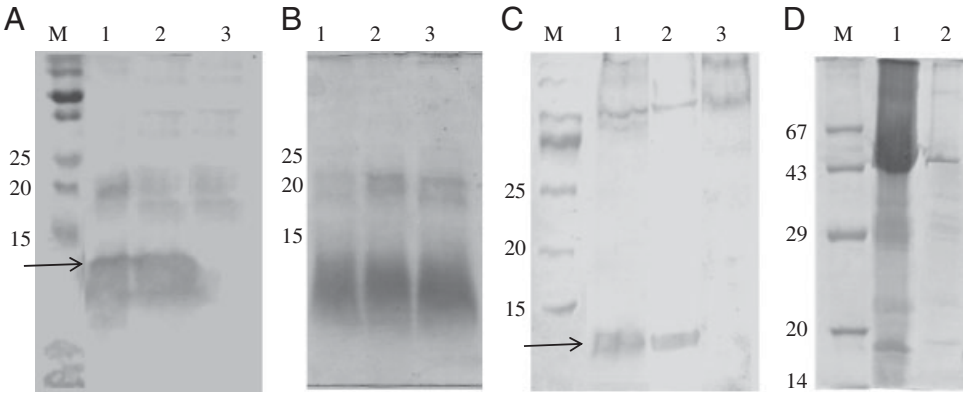

Figure 4. Detection of ASAL in the excreta and hemolymph of *Helicoverpa armigera* collected after 24 h of feeding on different proteins. (A) Western blot analysis of excreta: lane M, pre-stained molecular weight marker; lane1, recombinant SUMO-ASAL; lane 2, ASAL; lane 3, BSA. SUMO part of SUMO-ASAL was digested. (B) Coomassie staining of excreta: lane M, molecular weight marker; lane1, recombinant SUMO-ASAL; lane 2, ASAL; lane 3, BSA. (C) Western blot analysis of hemolymph: lane M, pre-stained molecular weight marker; lane1, recombinant SUMO-ASAL; lane 2, ASAL; lane 3, BSA. (D) Coomassie staining of hemolymph: lane M, molecular weight marker; lane 1, hemolymph total protein; lane 2, haemolymph after processing by 50 and 10 kDa cutoff filter. Arrow shows the position of ASAL band.

native and recombinant ASAL were fed to insect larvae, showed fluorescence in gut and hemolymph (Supporting Information Figs. 1E and F). Relatively less fluorescence was observed in case of feeding on recombinant ASAL.

4 Discussion

4.1 Midgut glycoprotein receptors

Binding of lectins to glycoproteins in the insect gut surface is necessary for its toxicity [26, 27]. ASAL recognizes several proteins in the midgut of *H. armigera* and some of them might be receptors. Glycoprotein-specific staining confirmed the glycosylated nature of the ASAL-binding proteins. ASAL failed to bind with the deglycosylated midgut BBMV proteins. These observations suggested that the ASAL binding requires the sugars on the surface of midgut BBMV proteins. It has been reported earlier that ASAL binds to the carbohydrate residues of 55 and 45 kDa brush border membrane receptor protein in the gut of the aphid and red cotton bug *Dysdercus cingulatus*, respectively [18]. *A. maculatum* tuber lectin also binds to the glycosylated receptors of 40 and 35 kDa in aphids *L. erysimi* and *A. craccivora* [20]. Similarly, several glycoproteins have been reported as receptors for δ-endotoxins of *B. thuringiensis* in the midgut of *H. armigera* [24, 28–30].

4.2 PMF and MS/MS analysis of putative midgut BBMV proteins

ASAL binds to proteins such as midgut aminopeptidase APN2, cadherin-N protein, polycalins, ALP and cytochrome P450 in the midgut of *H. armigera*. Several midgut proteins have been reported as receptors in *H. armigera* for the δ-endotoxins of *B. thuringiensis*, which includes cadherin-like proteins [29], aminopeptidase N [30] and ALP [24]. The third domain of the δ-endotoxin behaves like a lectin, which binds to specific sugars present on the receptors [26, 31]. Therefore, it might be possible that ASAL or other lectins interact with similar receptors. ASAL is a mannose-binding protein [10]. Since mannose is a common component in both *N*- and *O*-glycans of insect midgut proteins, the binding of ASAL with several proteins in midgut of *H. armigera* is understandable.

The cadherin is highly diverse superfamily of proteins with a variety of functions, such as cell adhesion, migration, cytoskeletal organization and morphogenesis [32, 33]. The cadherin expression is highly regulated, both temporally and spatially and sometimes unique to a particular cell type. The cadherin proteins contain repeated calcium-binding domains or cadherin repeats. Classical cadherins include five cadherin repeats [32, 34] but as many as 34 repeats have been reported [35]. Mucin [36], laminin or epidermal growth factor-like repeats [37] are also reported from cadherins. These are glycosylated proteins and are generally attached to the membrane by a single transmembrane domain, although seven transmembrane [38] or GPI-anchored variants have also been reported [39]. Role of cadherin receptors is crucial in larvicidal action of Cry1A toxin [40]. The first Cry1A cadherin receptor protein was isolated and cloned from the midgut epithelium of *M. sexta* [29]. Since then, several other lepidopteran cadherins have been identified [41, 42]. All reported cadherin receptors have similar domain organization, which consists of an ectodomain formed by 9–12 cadherin repeats, a membrane proximal extracellular domain, a transmembrane domain and a cytoplasmic domain [43]. The MASCOT search of PMF data of band II matched to AGAP009726-PA, a partial cadherin-N protein of *A. pisum*. The blast analysis against NCBI and Butterfly database showed similarity with the neural cadherin of different insects and BM1 cadherin of *Bombyx mori* (data not shown). Conserved domain blast analysis of this cadherin showed that it contains leminin G, EGF and cadherin domains and it was different from 12-cadherin domain protein reported as a receptor for δ-endotoxin proteins. However, none of the reported sequences of *Helicoverpa* showed any similarity with this protein. It might have been due to insufficient amount of sequence information for *Helicoverpa* in the data base. This is the first report for the affinity of ASAL with cadherin-like proteins.

The protein identified as cytochrome P450 CYP315A1 of *M. sexta* belongs to the protein encoded by halloween genes, which is highly conserved among insects and is involved in the biosynthesis of the insect-moulting hormone, 20-hydroxyecdysone (20E) [44–46]. The presence of these genes in other insect species has also been reported [47, 48], although their function has been demonstrated only in *Drosophila melanogaster* and lepidopteran insects. The occurrence of cytochrome P450 CYP315A1 (also known as shadow/sad) has been reported in the midgut microsomes of *M. sexta* [48, 49]. The cytochrome P450 has also been reported in fat body and midgut of *H. armigera* [50]. The role of cytochrome P450 CYP315A1 in *M. sexta* has also been reported in ecdysone biosynthesis, which plays role throughout the development and in reproduction [51]. The cytochrome P450 is a cytosolic protein and would not be expected to act as an extracellular receptor for ASAL. The interaction of ASAL with this protein has not been reported earlier. Since cytochrome P450 CYP315A1 is localized in the midgut microsomes, it could be isolated along with BBMV. There is a good possibility that some other proteins present in the midgut function as an extracellular primary receptor of ASAL and then ASAL interacts with this cytochrome p450 at the time of internalization (from insect gut to hemolymph or upon reaching hemolymph or by some other unknown mechanism). It will be interesting to study the effect of the interaction of ASAL with cytochrome P450, on the synthesis and degradation of ecdysone (moulting hormone) and the moulting process in insects.

Aminopeptidase N is predominantly associated with microvillar membrane in insect midgut. These have been suggested to function as receptor for δ-endotoxin in lepidopteran larvae [30, 31]. Aminopeptidase N binds the mannose and glucose-specific lectin Con A [1, 52]. It has also been reported as a receptor for *A. sativum* agglutinin in pea aphid *A. pisum* [15]. We suggest that aminopeptidase N might be a receptor for ASAL in the midgut of *H. armigera*.

MS/MS analysis of ASAL affinity-purified midgut proteins identified two variants of polycalins and three isozymes of ALP of *Helicoverpa* sp. The NCBI blast analysis of the identified polycalins sequences revealed that they have multiple lipocalin-like domains. Mauchamp *et al.* [53] coined the term polycalin to describe the proteins from *B. mori* with multiple lipocalin-like domains and characterized as chlorophyllide-A-binding protein. The polycalin sequences of *B. mori* match with a protein that binds Cry1Aa, Cry1Ab and Cry1Ac δ-endotoxin in heterologous competition binding studies [54, 55]. The interaction of Cry1Ac with polycalin from BBMV of *H. armigera* is also reported [56, 57]. Polycalins have been reported from the peritrophic matrix of *H. armigera* [25]. Several *N*- and *O*-linked glycosylation sites have also been reported from the polycalins of *H. armigera* [56]. The presence of polycalins in the midgut, occurrence of glycosylation sites, binding to the ASAL on affinity column and in ligand blotting indicate that polycalins play an important role in the interaction of ASAL with midgut of *H. armigera*.

The interaction of ASAL with ALP was also observed in this study. Membrane-bound ALP has been reported as a

putative receptor for Cry1Ac toxin in the BBMV of *H. armigera* [28], *H. virescens* [24], *B. mori* [58] and *M. sexta* [59]. ALP is a glycoprotein and Cry1Ac is known to bind to it through carbohydrate-based interactions [28] and inhibits the phosphatase activity. Binding of different agglutinins including mannose-specific lectin Con A to ALP has been reported [28], which indicates the presence of mannose residues in ALP. Contribution of the interaction of ASAL with ALP, APN and other proteins in toxicity against *H. armigera* will be studied in future.

4.3 Detection of ASAL in hemolymph, body tissue and excreta

The insecticidal lectins have been reported to be resistant to midgut proteolysis and they reach the hemolymph [14] and other tissues. ASAL was also detected in both hemolymph and excreta samples, which confirmed its resistance to midgut proteolysis. Immunohistolocalization depicted the binding of ASAL to the inner epithelial membrane of gut lumen. Lectins have been reported to bind to the epithelial membrane of insect gut, the peritrophic membrane and sugar moiety of some of the glycosylated digestive enzymes [14, 16]. Following the feeding of native and recombinant ASAL to insect larvae, fluorescence was detected in gut and internal tissues. This confirmed the presence of proteins in hemolymph and other body tissues. Relatively less fluorescence was observed in larvae fed on recombinant ASAL in comparison to the native ASAL. This might be due to cleavage and digestion of additional part (SUMO, which constituted half of the recombinant protein) by the gut proteases, resulting in reduced content of ASAL. In the positive control sections, fluorescence was detected in gut wall, hemolymph and epidermal cells, indicating interaction of ASAL with gut wall and other tissues.

4.4 Conclusions

This study identifies several proteins responsible for the interaction of ASAL in the midgut of *H. armigera*. Some of the ASAL-binding proteins showed similarity to the receptors responsible for the interaction of δ-endotoxin proteins of *B. thuringiensis*. This might be due to the lectin-like behavior of third domain of δ-endotoxins. Ligand blotting with GNA also showed similar binding pattern. It indicates that the mannose-binding lectins might interact with more or less common proteins in midgut of *H. armigera*. Lectins are being considered for gene pyramiding (use of two or more gene together) with δ-endotoxin proteins for the control of broad spectrum insects. Since ASAL also binds to receptors of δ-endotoxins, former may interfere in the binding of later to the gut receptors. Therefore, it will be necessary to evaluate combined toxicity of ASAL and δ-endotoxins before developing transgenic plants by gene pyramiding of these toxins. Further, it will be interesting to study the fate of interaction of ASAL with cytochrome P450 and polycalins.

The authors are thankful to Council of Scientific and Industrial Research (CSIR), Government of India, NMITLI program for funding, Peter Campbell and Michelle Colgrave of the Commonwealth Scientific and Industrial Research Organization, Australia, for their assistance in searching MS/MS data with the CSIRO database of cDNA sequences from H. armigera [25]. S. K. U. is thankful to CSIR for senior research fellowship. The authors are also thankful to Rajesh Kumar Srivastava for Mass Spectrometric Analysis, and SMH Abidi, Moti Lal and Aquila Bano for technical support. RT is grateful to DST for J C Bose fellowship.

The authors have declared no conflict of interest.

5 References

[1] Sauvion, N., Charles, H., Febvay, G., Rahbe, Y., Effects of jackbean lectin (ConA) on the feeding behaviour and kinetics of intoxication of the pea aphid, *Acyrthosiphon pisum*. *Entomol. Exp. Appl.* 2004, *110*, 31–44.

[2] Gatehouse, A. M. R., Powell, K. S., Van Damme, E. J. M., Peumans, W., Gatehouse, J. A., Insecticidal properties of plant lectins: their potential in plant protection, in: Pusztai, A. J., Bardocz, S. (Eds.), *Lectins: Biomedical Perspectives*, Taylor & Francis, London, UK 1995, pp. 35–57.

[3] Rao, K. V., Rathore, K. S., Hodges, T. K., Fu, X. *et al.*, Expression of snowdrop lectin (GNA) in transgenic rice plants confers resistance to rice brown plant hopper. *Plant J.* 1998, *15*, 469–477.

[4] Powell, K. S., Antimetabolic effects of plant lectins towards nymphal stages of the planthoppers *Tarophagus proserpina* and *Nilaparvata lugens*. *Entomol. Exp. Appl.* 2001, *99*, 71–78.

[5] Fitches, E., Gatehouse, A. M. R., Gatehouse, J. A., Effects of snowdrop lectin (GNA) delivered via artificial diet and transgenic plants on the development of tomato moth (*Lacanobia oleracea*) larvae in laboratory and glasshouse trials. *J. Insect Physiol.* 1997, *43*, 727–739.

[6] Hilder, V. A., Powell, K. S., Gatehouse, A. M. R., Gatehouse, J. A. *et al.*, Expression of snowdrop lectin in transgenic tobacco plants resulted in added protection against aphids. *Transgenic Res.* 1995, *4*, 18–25.

[7] Vasconcelos, I. M., Oliveira, J. T. A., Antinutritional properties of plant lectins. *Toxicon* 2004, *44*, 385–403.

[8] Arora, A., Sharma, H. C., Dreissche, E. V., Sharma, K. K., Biological activity of lectins from grain legumes and garlic against the legume pod borer, *Helicoverpa armigera*. *SAT eJournal/ejournal.icrisat.org.* 2005, *1*, 1–3.

[9] Sadeghi, A., Smagghe, G., Broeders, S., Hernalsteens, J. P. *et al.*, Ectopically expressed leaf and bulb lectins from garlic (*Allium sativum*) protect transgenic tobacco plants against

cotton leafworm (*Spodoptera lituralis*). *Transgenic Res.* 2008, *17*, 9–18.

[10] Smeets, K., Van Damme, E. J. M., Verhaert, P., Barre, A. *et al.*, Isolation, characterization and molecular cloning of the mannose-binding lectins from leaves and roots of garlic (*Allium sativum* L.). *Plant Mol. Biol.* 1997, *33*, 223–234.

[11] Saha, P., Dasgupta, I., Das, S., A novel approach for developing resistance in rice against phloem limited viruses by antagonizing the phloem feeding hemipteran vectors. *Plant Mol. Biol.* 2006, *62*, 735–752.

[12] Dutta, I., Saha, P., Majumder, P., Sarkar, A. *et al.*, The efficacy of a novel insecticidal protein, *Allium sativum* leaf lectin (ASAL), against homopteran insects monitored in transgenic tobacco. *Plant Biotech. J.* 2005, *3*, 601–611.

[13] Dutta, I. P., Majumdar, P., Saha, P., Ray, K., Das, S., Constitutive and phloem specific expression of *Allium sativum* leaf agglutinin (ASAL) to engineer aphid (*Lipaphis erysimi*) resistance in transgenic Indian mustard (*Brassica juncea*). *Plant Sci.* 2005, *169*, 996–1007.

[14] Fitches, E., Woodhouse, S., Edwards, J. P., Gatehouse, J. A., *In vitro* and *in vivo* binding of snowdrop (*Galanthus nivalis* agglutinin; GNA) and jackbean (*Canavalia ensiformis*; Con A) lectins within tomato moth (*Lacanobia oleracea*) larvae; mechanisms of insecticidal action. *J. Insect Physiol.* 2001, *47*, 777–787.

[15] Fitches, E., Wiles, D., Douglas , E. A., Hinchliffe, G. *et al.*, The insecticidal activity of recombinant garlic lectins towards aphids. *Insect Biochem. Mol. Biol.* 2008, *38*, 905–915.

[16] Powell, K. S., Spence, J., Bharathi, M., Gatehouse, J. A., Gatehouse, A. M. R., Immuno-histochemical anddevelopmental studies to elucidate the mechanism of action of the snowdrop lectin on the rice brown plant hopper, *Nilaparvata lugens* (Stal). *J. Insect Phys.* 1998, *44*, 529–539.

[17] Down, R. E., Fitches, E. C., Wiles, D. P., Corti, P. *et al.*, Insecticidal spider venom toxin fused to snowdrop lectin is toxic to the peach–potato aphid, *Myzus persicae* (Homoptera: Aphididae) and rice brown plant hopper, *Nilaparvata lugens* (Hemiptera: Delphacidae). *Pest. Mang. Sci.* 2006, *62*, 77–85.

[18] Bandyopadhyay, S., Roy, A., Das, S., Binding of garlic (*Allium sativum*) leaf lectin to the gut receptors of homopteran pests is correlated to its insecticidal activity. *Plant Sci.* 2001, *161*, 1025–1033.

[19] Majumder, P., Banerjee, S., Das, S., Identification of receptors responsible for binding of the mannose specific lectin to the gut epithelial membrane of the target insects. *Glycoconj. J.* 2004, *20*, 525–530.

[20] Majumder, P., Mondal, H. A., Das, S., Insecticidal activity of *Arum maculatum* tuber lectin and its binding to the glycosylated insect gut receptors. *J. Agric. Food Chem.* 2005, *53*, 6725–6729.

[21] Upadhyay, S. K., Saurabh, S., Rai, P., Singh, R. *et al.*, SUMO fusion facilitates expression and purification of garlic leaf lectin but modifies some of its properties. *J. Biotechnol.* 2010, *146*, 1–8.

[22] Wolfersberger, M. G., Luthy, P., Maurer, A., Parenti, P. *et al.*, Preparation and partial characterization of amino acid transporting brush border membrane vesicles from the larval midgut of the cabbage butterfly (*Pieris brassicae*). *Comp. Biochem. Physiol.* 1987, *86*, 301–308.

[23] Shevchenko, A., Wilm, M., Vorm, O., Mann, M., Mass spectrometric sequencing of proteins from silver stained polyacrylamide gels. *Anal. Chem.* 1996, *68*, 850–858.

[24] Jurat-Fuentes, J. L., Adang, M. J., Characterization of a Cry1Ac receptor alkaline phosphatase in susceptible and resistant *Heliothis virescens* larvae. *Eur. J. Biochem.* 2004, *271*, 3127–3135.

[25] Campbell, P. M., Cao, A. T., Hines, E. R., East, P. D., Gordon, K. H. J., Proteomic analysis of the peritrophic matrix from the gut of the caterpillar, *Helicoverpa armigera*. *Insect Biochem. Mol. Biol.* 2008, *38*, 950–958.

[26] Harper, S. M., Crenshaw, R. W., Mullins, M. A., Privalle, L. S., Lectin binding to insect brush border membranes. *J. Econ. Entomol.* 1995, *88*, 1197–1202.

[27] Zhu-Salzman, K., Shade, R. E., Koiwa, H., Salzman, R. A. *et al.*, Carbohydrate binding and resistance to proteolysis control insecticidal activity of *Griffonia simplicifolia* lectin II. *Proc. Natl. Acad. Sci. USA* 1998, *95*, 15123–15128.

[28] Sarkar, A., Hess, D., Mondal, H. A., Banerjee, S. *et al.*, Homodimeric alkaline phosphatase located at *Helicoverpa armigera* midgut, a putative receptor of Cry1Ac contains α-GalNAc in terminal glycans structure as interactive epitope. *J. Proteome Res.* 2009, *8*, 1838–1848.

[29] Vadlamudi, R. K., Weber, E., Ji, I., Ji, T. H., Bulla, L. A., Jr., Cloning and expression of a receptor for an insecticidal toxin of *Bacillus thuringiensis*. *J. Biol. Chem.* 1995, *270*, 5490–5494.

[30] Knight, P. J., Crickmore, N., Ellar, D. J., The receptor for *Bacillus thuringiensis* CrylA(c) delta-endotoxin in the brush border membrane of the lepidopteran *Manduca sexta* is aminopeptidase N. *Mol. Microbiol.* 1994, *11*, 429–436.

[31] Burton, S. L., Ellar, D. J., Li, J., Derbyshire, J., N-Acetylgalactosamine on the putative insect receptor Aminopeptidase N is recognized by a site on the domain III lectin-like fold of a *Bacillus thuringiensis* insecticidal toxin. *J. Mol. Biol.* 1999, *287*, 1011–1022.

[32] Angst, B. D., Marcozzi, C., Magee, A. I., The cadherin superfamily: diversity in form and function. *J. Cell Sci.* 2001, *114*, 629–641.

[33] Gumbiner, B. M., Cell adhesion: the molecular basis of tissue architecture and morphogenesis. *Cell* 1996, *84*, 345–357.

[34] Nollet, F., Kools, P., Roy, V. F., Phylogenetic analysis of the cadherin superfamily allows identification of six major subfamilies besides several solitary members. *J. Mol. Biol.* 2000, *299*, 551–572.

[35] Dunne, J., Hanby, A. M., Poulsom, R., Jones, T. A. *et al.*, Molecular cloning and tissue expression of FAT, the human homologue of the *Drosophila* fat gene that is located on chromosome 4q34-q35 and encodes a putative adhesion molecule. *Genomics* 1995, *30*, 207–223.

[36] Goldberg, M., Peshkovsky, C., Shifteh, A., Al-Awqati, Q., m-Protocadherin, a novel developmentally regulated proto-

cadherin with mucin-like domains. *J. Biol. Chem.* 2000, *275*, 24622–24629.

[37] Nakayama, M., Nakajima, D., Nagase, T., Nomura, N. *et al.*, Identification of high-molecular-weight proteins with multiple EGF-like motifs by motif-trap screening. *Genomics* 1998, *51*, 27–34.

[38] Usui, T., Shima, Y., Shimada, Y., Hirano, S. *et al.*, Flamingo, a seven-pass transmembrane cadherin, regulates planar cell polarity under the control of Frizzled. *Cell* 1999, *98*, 585–595.

[39] Vestal, D. J., Ranscht, B., Glycosyl phosphatidylinositol-anchored T-cadherin mediates calcium-dependent, homophilic cell adhesion. *J. Cell Biol.* 1992, *119*, 451–461.

[40] Gómez, I., Sánchez, J., Miranda, R., Bravo, A., Sánchez, M., Cadherin-like receptor binding facilitates proteolytic cleavage of helix α-1 in domain I and oligomer pre-pore formation of *Bacillus thuringiensis* Cry1Ab toxin. *FEBS Lett.* 2002. *513*, 242–246.

[41] Nagamatsu, Y., Toda, S., Yamaguchi, F., Ogo, M. *et al.*, Identification of *Bombyx mori* midgut receptor for *Bacillus thuringiensis* insecticidal CrylA(a) toxin. *Biosci. Biotechnol. Biochem.* 1998, *62*, 718–726.

[42] Hua, G., Zhang, R., Abdullah, M. A., Adang, M. J., *Anopheles gambiae* cadherin AgCad1 binds the Cry4Ba toxin of *Bacillus thuringiensis israelensis* and a fragment of AgCad1 synergizes toxicity. *Biochemistry* 2008, *47*, 5101–5110.

[43] Pigott, C. R., Ellar, D. J., Role of receptors in *Bacillus thuringiensis* Crystal toxin activity. *Microbiol. Mol. Biol. Rev.* 2007, *71*, 255–281.

[44] Gilbert, L. I., Warren, J. T., A molecular genetic approach to the biosynthesis of the insect steroid molting hormone. *Vitam. Horm.* 2005, *73*, 31–57.

[45] Rewitz, K. F., Rybczynski, R., Warren, J. T., Gilbert, L. I., Identification, characterization and developmental expression of halloween genes encoding P450 enzymes mediating ecdysone biosynthesis in the tobacco hornworm, *Manduca sexta*. *Insect Biochem. Mol. Biol.* 2006, *36*, 188–199.

[46] Rewitz, K. F., Rybczynski, R., Warren, J. T., Gilbert, L. I., Developmental expression of *Manduca* shade, the P450 mediating the final step in molting hormone synthesis. *Mol. Cell. Endocrinol.* 2006, *247*, 166–174.

[47] Warren, J. T., Petryk, A., Marques, G., Parvy, J. P. *et al.*, Phantom encodes the 25-hydroxylase of *Drosophila melanogaster* and *Bombyx mori*: a P450 enzyme critical in ecdysone biosynthesis. *Insect Biochem. Mol. Biol.* 2004, *34*, 991–1010.

[48] Rewitz, K. F., Rybczynski, R., Warren, J. T., Gilbert, L. I., The Halloween genes code for cytochrome P450 enzymes mediating synthesis of the insect moulting hormone. *Biochem. Soc. Trans.* 2006, *34*, 1256–1260.

[49] Weirich, G. F., Williams, V. P., Feldlaufer, M. F., Ecdysone 20-hydroxylation in *Manduca sexta* midgut: kinetic parameters of mitochondria1 and microsornal ecdysone 20-monooxygenases. *Arch. Insect Biochem. Physiol.* 1996, *31*, 305–312.

[50] Cai, H. Y., Xi, W. G., Partial purification of cytochrome P450 from *Helicoverpa armigera* (Hübner). *Insect Sci.* 2005, *12*, 313–317.

[51] Rewitz, K. F., Connor, M. B., Gilbert, L. I., Molecular evolution of the insect Halloween family of cytochrome P450s: phylogeny, gene organization and functional conservation. *Insect Biochem. Mol. Biol.* 2007, *37*, 741–753.

[52] Cristofoletti, P. T., Mendonça de Sousa, F. A., Rahbé, Y., Terra, W. R., Characterisation of a membrane-bound aminopeptidase purified from *Acyrthosiphon pisum* midgut cells. A major binding site for toxic mannose lectins. *FEBS J.* 2006, *273*, 5577–5588.

[53] Mauchamp, B., Royer, C., Garel, A., Jalabert, A. *et al.*, Polycalin (chlorophyllid A binding protein): a novel, very large fluorescent lipocalin from the midgut of the domestic silkworm *Bombyx mori* L. *Insect Biochem. Mol. Biol.* 2006, *36*, 623–633.

[54] Hossain, D. M., Shitomi, Y., Moriyama, K., Higuchi, M. *et al.*, Characterization of a novel plasma membrane protein, expressed in the midgut epithelia of *Bombyx mori*, that binds to cry1a toxins. *Appl. Environ. Microbiol.* 2004, *70*, 4604–4612.

[55] Pigott, C. R., Ellar, D. J., Role of receptors in *Bacillus thuringiensis* crystal toxin activity. *Microbiol. Mol. Biol. Rev.* 2007, *71*, 255–281.

[56] Angelucci, C., Barrett-Wilt, G. A., Hunt, D. F., Akhurst, R. J. *et al.*, Diversity of aminopeptidases, derived from four lepidopteran gene duplications, and polycalins expressed in the midgut of *Helicoverpa armigera*: identification of proteins binding the delta-endotoxin, Cry1Ac of *Bacillus thuringiensis*. *Insect Biochem. Mol. Biol.* 2008, *38*, 685–696.

[57] Malik, K., Mahmood, T., Riazuddin, S., The receptor for *Bacillus thuringiensis* Cry 1Ac delta-endotoxin in the brush border membrane of the lepidopteran *Helicoverpa armigera* is aminopeptidase N. *J. Biol. Sci.* 2001, *1*, 782–784.

[58] Takesue, Y., Yokota, K., Miyajima, S., Taguchi, R., Ikezawa, H., Membrane anchors of alkaline phosphatase and trehalase associated with the plasma membrane of larval midgut epithelial cells of the silkworm *Bombyx mori*. *J. Biochem.* 1989, *105*, 998–1001.

[59] Sangadala, S., Walters, F. S., English, L. H., Adang, M. J., A mixture of *Manduca sexta* aminopeptidase and phosphatase enhances *Bacillus thuringiensis* insecticidal CrylA(c) toxin binding and 86Rb+-K+efflux *in vitro*. *J. Biol. Chem.* 1994, *269*, 10088–10092.

