Appl Microbiol Biotechnol (2012) 93:2365–2375
DOI 10.1007/s00253-011-3547-1

Compatibility of garlic (*Allium sativum* L.) leaf agglutinin and Cry1Ac δ-endotoxin for gene pyramiding

Santosh Kumar Upadhyay · Seema Singh · Krishnappa Chandrashekar · Rakesh Tuli · Pradhyumna Kumar Singh

Received: 14 June 2011 / Revised: 1 August 2011 / Accepted: 13 August 2011 / Published online: 26 August 2011

Abstract δ-Endotoxins produced by *Bacillus thuringiensis* (Bt) have been used as bio-pesticides for the control of lepidopteran insect pests. Garlic (*Allium sativum* L.) leaf agglutinin (ASAL), being toxic to several sap-sucking pests and some lepidopteran pests, may be a good candidate for pyramiding with δ-endotoxins in transgenic plants for enhancing the range of resistance to insect pests. Since ASAL shares the midgut receptors with Cry1Ac in *Helicoverpa armigera*, there is possibility of antagonism in their toxicity. Our study demonstrated that ASAL increased the toxicity of Cry1Ac against *H. armigera* while Cry1Ac did not alter the toxicity of ASAL against cotton aphids. The two toxins interacted and increased binding of each other to brush border membrane vesicle (BBMV) proteins and to the two important receptors, alkaline phosphatase (ALP) and aminopeptidase N (APN). The results indicated that the toxins had different binding sites on the ALP and APN but influenced mutual binding. We conclude that ASAL can be safely employed with Cry1Ac for developing transgenic crops for wider insect resistance.

Keywords Alkaline phosphatase · Aminopeptidase · *Allium sativum* leaf agglutinin · Brush border membrane vesicles · Cry1Ac · *H. armigera*

Electronic supplementary material The online version of this article (doi:10.1007/s00253-011-3547-1) contains supplementary material, which is available to authorized users.

S. K. Upadhyay · S. Singh · K. Chandrashekar · P. K. Singh (✉)
National Botanical Research Institute,
Council of Scientific and Industrial Research,
Rana Pratap Marg,
Lucknow 226001, UP, India
e-mail: pradhyumnasingh@hotmail.com

P. K. Singh
e-mail: pksingh@nbri.res.in

R. Tuli
Department of Biotechnology,
National Agri-Food Biotechnology Institute,
C-127, Phase VIII, Industrial Area, SAS Nagar,
Mohali 160071, Punjab, India

Introduction

The application of δ-endotoxins of *Bacillus thuringiensis* (Bt) has contributed substantially in enhancing the productivity of cotton and maize during the last decade. This is due to the control of *Helicoverpa armigera*, *Helicoverpa zea*, and other lepidopteran pests through the development of transgenic crops. This has also brought down the application of chemical pesticides. δ-Endotoxins are receptor specific and therefore toxic to selected insects only. The success of Bt crops is threatened by a concomitant increase in the population of secondary pests like whiteflies, aphids, and leafhoppers against which the δ-endotoxins are not effective (Dutt 2007; Virla et al. 2010). Pest management practices in the future will be based on transgenic crops which would express insecticidal proteins for the control of both lepidopteran and homopteran pests (Ferry et al. 2004; Wu and Guo 2005).

Garlic (*Allium sativum* L.) leaf agglutinin (ASAL) is toxic primarily to the sap-sucking homopteran pests like cotton and pea aphids, and also against a few lepidopteran pests. It is a 25-kDa homodimeric protein structurally and evolutionarily related to lectins like *Galanthus nivalis* agglutinin, GNA (Van Damme et al. 1992; Smeets et al. 1997; Bandyopadhyay et al. 2001; Saha et al. 2006;

Sadeghi et al. 2008). It has potential to complement the Bt technology for broadening the range of insect resistance in transgenic plants. Due to edible source of origin, ASAL may be safe to mammals. However, for successful complementation, the binding sites and mode of insecticidal action of ASAL must not compete with the partner δ-endotoxins. Only then, gene pyramiding for the control of lepidopteran and homopteran pests can succeed.

Mode of insecticidal action of δ-endotoxins includes a number of chronological steps. The protoxin forms of the δ-endotoxins are activated into the toxin by the midgut proteases. The activated toxins bind to the cadherin, get assembled into oligomers, then bind to proteins such as aminopeptidase N (APN) or alkaline phosphatase (ALP) on the cell membrane of midgut microvilli. This results in oligomer insertion, formation of pores, and cell death by metabolite leakage and osmotic shock (Bravo et al. 2004). Additional proposed mode of action involves activation of the intracellular signaling after binding of the monomeric toxins to cadherin, followed by the induced enterocyte death and septicemia (caused by midgut bacteria) leading to larval death (Zhang et al. 2005; Broderick et al. 2006). Membrane-bound ALPs have been proposed as a family of receptors for δ-endotoxins in *Manduca sexta* (McNall and Adang 2003), *H. armigera* (Upadhyay and Singh 2011), *Heliothis virescens* (Jurat-Fuentes and Adang 2004, 2007; Krishnamoorthy et al. 2007), *Aedes aegypti* (Fernandez et al. 2006), *Anopheles gambiae* (Hua et al. 2009), and *Anthonomus grandis* (Martins et al. 2010). APNs are another family of receptors for the δ-endotoxins in *M. sexta* (Knight et al. 1994), *H. virescens* (Luo et al. 1997), *H. armigera* (Ingle et al. 2001), and *A. gambiae* (Zhang et al. 2008).

The insecticidal lectins are resistant to digestive enzymes in the insect midgut, exhibit stable binding, and cause toxicity (Sauvion et al. 2004). Their insecticidal activity has been accredited to their binding with glycoproteins in the digestive tract (Fitches et al. 2001; Majumder et al. 2004). The strength of binding determines their anti-nutrient value (Vasconcelos and Oliveira 2004). The insecticidal lectins have been detected in hemolymph, body tissue, and excreta, which suggests their transport across the digestive tract (Fitches et al. 2001; Upadhyay et al. 2010a). However, the exact mechanism of their insecticidal action has not been elucidated.

In our earlier study, we reported the binding of ASAL to several proteins in the midgut of *H. armigera* larvae (Upadhyay et al. 2010b), and most of them were receptors of δ-endotoxins also. Both the insecticidal proteins had affinity for ALP and APN, the two major groups of known receptors. It was suspected that ASAL might negate the toxicity of Cry1Ac (δ-endotoxin used in the development of Bt cotton) if the two proteins are expressed together in transgenic plants. The purpose of the present study was to understand (a) the synergistic or antagonistic effects of ASAL on the toxicity of Cry1Ac to *H. armigera*, (b) the effect of Cry1Ac on the toxicity of ASAL to cotton aphids, and (c) the kinetics of binding of both the toxins with brush border membrane vesicle (BBMV) proteins of *H. armigera*.

Materials and methods

Expression and purification of Cry1Ac and ASAL

The Cry1Ac protein was cloned and expressed in *Escherichia coli* strain HB101 (pRT200) and purified as described earlier (Tuli et al. 1989; Chaturvedi et al. 2000). ASAL was purified from mature leaves of garlic (*A. sativum*) on mannose column by affinity chromatography following an established protocol (Smeets et al. 1997). The purified protein was concentrated on a 10-kDa cut-off filter (Millipore, USA). Both the proteins were stabilized in phosphate buffered saline (PBS), and used in insect bioassay and receptor binding studies.

Insects

H. armigera larvae were maintained in the laboratory on artificial diet at constant temperature of 26±2 °C with 80% relative humidity. Cotton aphids (*Aphis gossypii*) were maintained on cotton plants grown in a glass house.

Insect bioassay

Insect bioassay was carried out with neonatal larvae of *H. armigera* by feeding them on artificial diet. Different amounts of Cry1Ac and ASAL (0, 10, 20, 40, 60, and 80 pmol, separately and in 1:1 molar ratio) were mixed with 1 g of artificial diet and used for bioassay. Twenty individual larvae were released on diet for each concentration, and each treatment was taken in triplicate. Bioassay with cotton aphid (*A. gossypii*) was carried out by feeding them on artificial liquid diet mixed with different amounts of ASAL and Cry1Ac (0.5, 1, 2, and 4 nmol) separately and together (in 1:1 molar ratio). Test proteins were mixed with 1 ml of the artificial diet and bioassay was performed. BSA was taken as negative control. BSA was also mixed with the toxins for stabilization. Bioassays were carried out for 5 days and data collected every day. Entire set of experiments was repeated three times. The data was analyzed by one-way analysis followed by DMRT using SPSS program (version 10).

Preparation and solubilization of BBMV from the midgut of *H. armigera* larvae

BBMVs were prepared from the midgut of different larval instars by repeated $MgCl_2$ differential centrifugation

(Wolfersberger et al. 1987). Larvae were immobilized by placing them on ice for 5 min and dissected in MET buffer (300 mM mannitol; 5 mM EGTA; 17 mM Tris–Cl, pH 7.4). Midguts were removed, rinsed, powdered under liquid nitrogen, and homogenized in MET buffer at a 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 (2,500×*g*, 4 °C, 15 min). The pellet was discarded and the supernatant was centrifuged (30,000×*g*, 4 °C, 30 min). The pellet was re-suspended in half of the original volume of MET and $MgCl_2$, and the above steps were repeated. The final pellet was solubilized in PBS containing 0.1% Tween 20 by overnight incubation at 4 °C with gentle shaking (Upadhyay et al. 2010a). Protein content in BBMV preparations were measured by Bradford dye (Bio-Rad, USA), and BSA (Sigma, USA) served as standard. Quality of BBMV preparations were assessed on the basis of marker enzymes alkaline phosphatase and leucine aminopeptidase (Table 1). The BBMV preparations from each larval stage were used for the interaction studies with Cry1Ac and ASAL.

Purification of ALP and APN

ALP was purified from solubilized BBMV of third and APN from fourth instar larvae. In the respective instar stage, the expression of the target enzymes was maximum (Upadhyay and Singh 2011). BBMV solubilized in PBS containing 0.1% Tween 20 was dialyzed against 20 mM Tris–Cl (pH 8.0), centrifuged (2,500×*g*, 4 °C, 15 min), filtered through a 0.22-μM filter, and applied to an anion exchange column (MonoQ; GE Healthcare, USA) pre-equilibrated with 20 mM Tris–Cl (pH 8.0). The column was washed with 20 mM Tris–Cl (pH 8.0) till A_{280} became 0.002. The proteins were eluted with 0.0–1.0 M gradient of NaCl at a flow rate of 0.5 ml/min. The fractions with ALP or APN activity were analyzed on denaturing polyacrylamide gel. The fractions with more than 90% homogeneity (either ALP or APN) were pooled and stored at 4 °C for further studies. MS/MS analysis of ALP and APN was done on MALDI-TOF-TOF (model 4800, ABsciex, USA) for characterizing the enzymes.

ALP and APN assay

ALP activity was assayed with 3 mM *p*-nitrophenyl phosphate (pNPP) (Merck, India) as substrate in 50 mM glycine buffer (pH 10.4) containing 5 mM $MgCl_2$ and 0.1% Triton X-100 as described by Lowry et al. (1954) with some modifications. The enzyme activity was monitored by measuring the absorbance of *p*-nitrophenol at 405 nm after every 1 min up to 15 min. The initial rate was considered for the calculation of specific activity. One unit of specific ALP activity was defined as the amount of enzyme producing 1 μmol *p*-nitrophenol min^{-1} (mg protein)$^{-1}$ at 37 °C. *p*-Nitrophenol concentration was estimated on the basis of a standard curve. Alkaline phosphatase activity was further confirmed by "in gel" assay (Jurat-Fuentes and Adang 2004). The protein was resolved on 10% denaturing polyacrylamide gel without heat denaturation and transferred onto the PVDF membrane. Membrane was washed with water and color was developed with western blue stabilized substrate for alkaline phosphatase (Promega, USA).

APN activity was assayed with L-leucine-amide (Sigma–Aldrich, USA) following the protocol of Mitz and Schlueter (1958) with some modifications. The product of the reaction was monitored at 238 nm. The initial rate was used for the calculation of specific activity. The molar extinction coefficient of L-leucine was 9.8 M^{-1} cm^{-1}. One unit of specific APN activity was defined as the amount of enzyme catalyzing the hydrolysis of 1 μmol L-leucine-amide min^{-1} (mg protein)$^{-1}$ at 25 °C.

To determine the enzyme activity in purified fractions of ALP and APN and in BBMV preparations, the respective aliquots were added to the reaction mixture. The reaction velocity was monitored and specific activities were calculated. K_m and V_{max} of ALP for pNPP were calculated after

Table 1 Specific activity of aminopeptidase-N (APN) and alkaline phosphatase (ALP) for midgut brush border membrane vesicles (BBMV) preparations from different developmental stages of *H. armigera* larvae

Midgut homogenates				BBMV				
Larval stage	Total protein (mg)	Specific activity of ALP (μmol/min/mg)	Specific activity of APN (μmol/min/mg)	Total protein (mg)	Specific activity of ALP (μmol/min/mg)	Fold enrichment of ALP	Specific activity of APN (μmol/min/mg)	Fold enrichment of APN
1st	0.57	57.89	21.22	0.11	291.66	5.03	98.67	4.64
2nd	1.16	87.46	36.78	0.24	454.54	5.19	168.90	4.59
3rd	4.65	112.94	50.46	0.89	555.55	4.91	229.81	4.55
4th	11.8	98.71	103.21	2.14	490.93	4.97	478.22	4.63
5th	23.76	62.83	78.63	3.94	310.07	4.93	354.31	4.50

conducting the reaction (37 °C, 10 min, pH 10.4) with different substrate concentrations (0, 1, 3, 6, 12, 15, and 18 mM). K_m and V_{max} of APN for L-leucine-amide were calculated by conducting the reaction (25 °C, 5 min, pH 8.0) with the substrate concentrations 0, 25, 50, 100, 200, and 400 mM. The data were analyzed on Prism (3.0) software.

ELISA binding assays

ELISA was performed to study the (a) binding of the toxins with BBMV proteins (at different larval instars) and purified ALP and APN; (b) effect of one toxin on other in the binding to BBMV, ALP, and APN; and (c) interaction of the two toxins between themselves.

BBMV from larvae at different instar stages were diluted in bicarbonate buffer (pH 9.6), coated onto the 96-well ELISA plate (100 ng/well, Greiner Bio-One, Germany) and incubated overnight at 4 °C. The plate was washed and blocked with 1% BSA in PBS containing 0.05% Tween 20, washed again with PBST, and incubated with different concentrations of Cry1Ac or ASAL in PBST containing 0.25% BSA at 37 °C for 2 h. Excess BSA was washed and the plate incubated with respective primary antibody and peroxidase conjugated secondary antibody (Sigma, USA) as per standard protocol. Color was developed with the HRP substrate solution (Merck, India) and absorbance measured at 490 nm. Similar study was also performed with purified ALP and APN. All the steps were same except that the coating of ELISA plate was done with 10 ng purified enzymes/well.

To study the effect of one toxin on binding of the other to BBMV, ALP, and APN, ELISA plates were coated with BBMV, ALP, or APN. The plates were incubated with a mixture of Cry1Ac and ASAL in 1:1 molar ratio. For each experimental combination, either anti-Cry1Ac or anti-ASAL was used as primary antibody followed by the HRP-labeled secondary antibody.

Interaction of Cry1Ac with ASAL was studied by incubating different concentrations of Cry1Ac (0.062–30 nM) onto the ELISA plate, pre-coated with 1 nM ASAL. This was followed by incubation with anti-Cry1Ac antibody and then the secondary antibody.

Ligand and immuno-blot analysis

Ligand blot analysis was performed to study the interaction between Cry1Ac and ASAL and their binding with ALP and APN. Then 200 ng each of ASAL, Cry1Ac, ALP, and APN was resolved on SDS–PAGE and transferred onto the PVDF membrane (Millipore, USA) by using I-Blot gel transfer apparatus (Invitrogen, USA). The membranes were blocked with TBST containing 5% non-fat dry milk. Membrane having Cry1Ac, ALP, and APN was incubated with ASAL and the membrane having ASAL, ALP, and APN with Cry1Ac, each at 2 nM concentration in TBST containing 1% non-fat dry milk for 2 h. Incubation with BSA (2 nM) served as negative control. The membranes were washed repeatedly in TBST buffer and incubated with respective anti-ASAL and anti-Cry1Ac antibody for 2 h. The membrane was washed and incubated with HRP conjugated secondary antibody for 2 h. Color was developed with DAB system (Merck, India).

Effect of Cry1Ac and ASAL on enzymatic activity of ALP and APN

To study the effect of Cry1Ac and ASAL on enzyme activity of ALP, 1 nM enzyme was incubated with 1 and 5 nM of either Cry1Ac or ASAL or both. The enzymatic reaction was performed with substrate (pNPP) at different concentrations (0, 1, 3, 6, 12, 15, and 18 mM). A similar experiment with APN did not produce any meaningful result; therefore, some modifications were introduced. One nanomolar enzyme was mixed with higher concentrations (10–200 nM) of Cry1Ac or ASAL or both in 1:1 molar ratio, followed by the enzyme reaction with the substrate L-leucine-amide at 100 mM concentration.

Results

Insect bioassay

The efficacy of Cry1Ac and ASAL towards *H. armigera* cannot be compared directly, as the former caused mortality while the latter showed only growth retardation. In combination, the morbidity caused by ASAL enhanced the mortality caused by Cry1Ac. At higher levels (above 40 pmol), the two toxins showed significant synergy (Table 2). The mortality reached 97% to 100%, above 60 pmol, although the LC_{50} was not significantly different. ASAL showed significant mortality of aphids. Cry1Ac was neither toxic to aphids nor altered the toxicity of ASAL (Table 3). BSA as a control neither caused any toxicity to test insects nor affected the toxicity of any toxin.

Binding analysis of toxins to BBMV proteins prepared from different developmental stages of larvae

Since both the toxins were effective against *H. armigera*, their interaction with BBMV proteins was studied. The binding affinity of Cry1Ac and ASAL to BBMV was determined in terms of dissociation constant (K_d). The effect of Cry1Ac on the binding of ASAL with BBMV and vice versa was also examined (Table 4). Affinity of the

Table 2 Insect bioassay of Cry1Ac and ASAL with neonate larvae of *H. armigera*

Concentration (pmol)	Mean mortality (%)		
	ASAL	Cry1Ac	ASAL+Cry1Ac
Control	2.0±2.5a		
10	3.0±1.5a	27.5±2.5cd	20.0±5.0c
20	2.5±2.5a	35.0±5.0d	32.5±2.5d
40	3.0±2.0ab	72.5±2.50e	85.0±5.0f
60	4.5±2.5ab	85.0±5.0f	97.5±2.5g
80	3.5±0.0ab	93.7±1.2f	100.0±0.0g
LC_{50}		22.10	20.864
[Fiducial limit]		[19.20–25.03]	[18.89–22.89]

Means compared using DMRT (Duncan's multiple range test) at $P=0.05$; means followed with the same letter are not significantly different

toxins for BBMV increased with larval development. The K_d values for the binding of Cry1Ac to BBMV were maximum (5.647 nM) for the first and minimum (1.281 nM) for the fifth instar larvae. These were 30.32 and 2.142 nM in case of ASAL for the first and fifth instar larvae, respectively. The K_d values at different stages of larval growth followed a similar trend even when both the toxins were used in combination. ASAL did not show any significant impact on the binding of Cry1Ac to BBMV, whereas Cry1Ac increased the affinity of ASAL very significantly, especially at early larval development stages. None of the toxins showed any binding with BSA.

Purification and enzyme kinetics of ALP and APN

The two receptors, purified on MonoQ ion exchange column, were analyzed for enzymatic activities and visualized on 10% denaturing PAGE (Fig. 1, lanes 1 and 2). Although several fractions showed both the enzymatic activities (data not shown), only a few fractions were nearly homogenous and appeared as a single band on denaturing PAGE (APN ~110 and ALP ~66 kDa, respectively) and were in agreement with the earlier reports. The enzymes were also confirmed by MS/MS analysis and found similar to the midgut aminopeptidase APN2 (gi|25814968) and alkaline phosphatase 2 (gi|194295558), reported earlier as the Cry1Ac receptors of *H. armigera* (Upadhyay and Singh 2011). APNs are a family of proteins with size ranging from 110 to 180 kDa (Angelucci et al. 2008). APNs of different sizes were observed in different fractions during purifications (data not shown). Similarly, various sizes of ALPs were also observed. Only the fractions purified to near homogeneity were used for the interaction studies. ALP was analyzed by "in gel" activity assay also (Fig. 1, lane 3). The purified protein fractions were pooled and used in enzyme kinetic study. The specific activity of ALP in the midgut homogenate was 112.94 μmol min^{-1} mg^{-1} which increased to 555.55 μmol min^{-1} mg^{-1} in BBMV preparation and 4,226.21 μmol min^{-1} mg^{-1} in the purified enzyme (Supplementary Table 1a). The increase in specific activity was 4.919 and 37.41 folds, respectively. The specific activity of APN in the midgut homogenate was 103.2 μmol min^{-1} mg^{-1}, which increased by 4.75 and 35.59 folds at the two steps of purification (Supplementary Table 1b).

ALP and APN activities followed normal Michaelis–Menten enzyme kinetics. Velocity of the reaction increased with increase in the substrate concentration and reached maxima (Fig. 2a, c). The K_m, V_{max}, and K_{cat} of ALP were 1.9 mM, 75.7 μmol min^{-1} μg^{-1}, and 4.54×10^6 min^{-1}, respectively. The ratio of K_{cat} to K_m for the ALP was 2.389×10^6 mM^{-1} min^{-1}. The K_m, V_{max}, and K_{cat} of APN were 84.55 mM, 2,537 μmol min^{-1} mg^{-1}, and 2.1×

Table 3 Insect bioassay of Cry1Ac and ASAL with cotton aphids (*Aphis gossypii*)

Concentration (nmol)	Mean mortality (%)		
	ASAL	Cry1Ac	ASAL+Cry1Ac
Control	6.6±3.3a		
0.5	26.6±2.50ab	5.0±3.0a	26.5±2.5ab
1	36.7±3.3bc	4.5±2.5a	35.00±5.0bc
2	48.50±4.5c	5.5±3.5a	53.50±2.5c
4	73.24±2.5d	5.0±3.5a	70.50±2.5d

Means compared using DMRT (Duncan's multiple range test) at $P=0.05$; means followed with same letter are not significantly different.

Table 4 Binding (K_d in nM) of Cry1Ac, Cry1Ac+ASAL, ASAL, and ASAL+Cry1Ac with BBMV proteins prepared from different developmental stages of *H. armigera* larvae

Larval stage	Cry1Ac	Cry1Ac+ASAL	ASAL	ASAL+Cry1Ac
1st instar	5.64±1.12	4.94±0.95	30.20±7.28	7.00±1.06
2nd instar	2.81±0.42	2.97±0.42	25.56±6.22	2.10±0.37
3rd instar	2.52±0.39	2.46±0.27	9.18±1.41	2.49±0.26
4th instar	1.78±0.24	1.77±0.26	2.65±0.25	2.05±0.30
5th instar	1.28±0.14	1.07±0.11	2.14±0.26	2.11±0.29

100 ng BBMV was coated on ELISA plate, incubated with different concentrations of proteins and developed with anti-Cry1Ac antibody in case of Cry1Ac and Cry1Ac+ASAL, and anti-ASAL antibody in case of ASAL and ASAL+Cry1Ac

10^6 min^{-1}, respectively, and the ratio of K_{cat} to K_m was 2.48×10^4 mM^{-1} min^{-1}.

Binding analysis of Cry1Ac and ASAL to ALP and APN

Cry1Ac and ASAL showed binding to both the receptors. The former showed higher affinity towards ALP and the later towards APN. Further, the toxins enhanced each other's affinity towards the receptors. K_d values of Cry1Ac and ASAL for ALP were 0.62 and 13.27 nM, respectively, which decreased to 0.40 and 3.98 nM in the combined treatments. Similarly, K_d value of Cry1Ac and ASAL for APN were 7.12 and 3.42 nM, respectively, and decreased to 5.60 and 2.12 in the combined treatment (Fig. 3a–d).

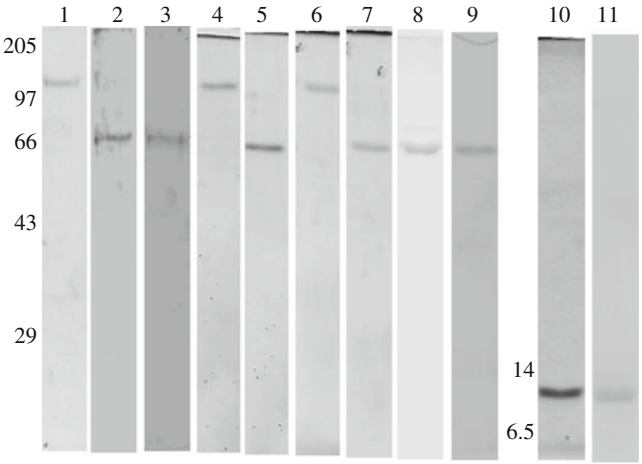

Fig. 1 Purification of ALP, APN, Cry1Ac, and ASAL and ligand blotting. APN (~110 kDa, *lane 1*) and ALP (~66 kDa, *lane 2*) purified on MonoQ column (Supplementary Table 1a and b) and electrophoresed on 10% SDS–PAGE. *Lane 3* shows "in gel" assay of ALP. Ligand blot of APN and ALP with Cry1Ac (*lanes 4* and *5*) and with ASAL (*lanes 6* and *7*). Purified Cry1Ac (~66 kDa) and its ligand blot with ASAL are shown in *lanes 8* and *9*, respectively. Similarly purified ASAL (~12 kDa) and its ligand blot with Cry1Ac are shown in *lanes 10* and *11*, respectively

Interaction of Cry1Ac with ASAL

Since the binding of ASAL to BBMV receptors improved in the presence of Cry1Ac and vice versa, interaction between these two proteins was studied. The results showed that both the proteins interacted with each other with the K_d value 2.05 nM (Fig. 4). None of the proteins showed any interaction with BSA.

Ligand blotting

Binding of Cry1Ac with ASAL on blot further established their affinity for each other (Fig. 1, lanes 9 and 11). Ligand blotting of the purified APN and ALP with Cry1Ac and ASAL reconfirmed the interaction of the receptors with the insecticidal proteins (Fig. 1, lanes 4–7).

Effect of Cry1Ac and ASAL on the enzyme activity of ALP and APN

Both Cry1Ac and ASAL inhibited the activity of ALP, competitively (Fig. 2b). ASAL caused comparatively higher inhibition than Cry1Ac. When incubated with both the toxins, the inhibition of ALP was less as compared to individual toxin. In the treatment of ALP with 5 nM each of Cry1Ac and ASAL, high K_i value (29×10^{-6} mM) was observed in comparison to 10.85×10^{-6} and 4.72×10^{-6} mM for Cry1Ac and ASAL, respectively, in individual treatment.

ASAL did not show any effect on the APN activity in individual and combined treatments. Cry1Ac modulated APN activity, which increased with increasing concentration of Cry1Ac (up to 20 nM). Further increase of Cry1Ac (till 100 nM) decreased the APN activity and stabilized (Fig. 2d).

The toxins did not show any ALP or APN activity themselves and BSA did not affect the enzymatic activity of the receptors.

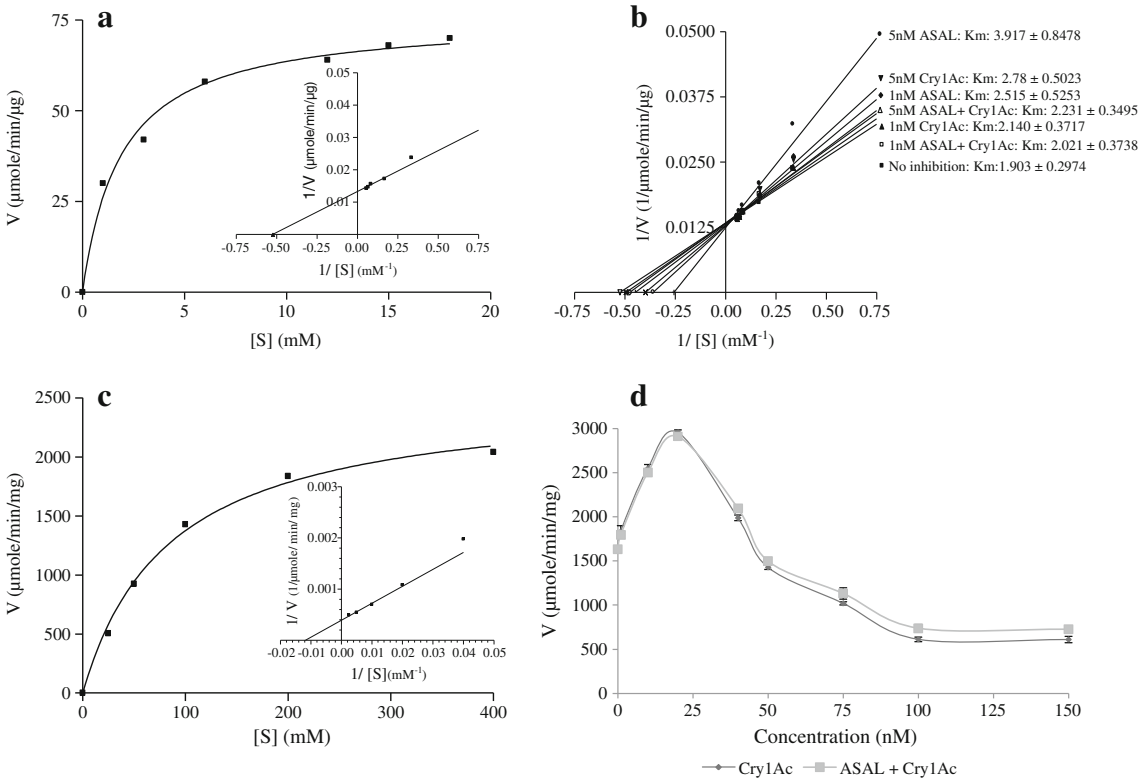

Fig. 2 Enzyme kinetics of ALP and APN. **a** Michaelis–Menten and Lineweaver–Burk Plot of ALP. **b** Effect of different concentrations of ASAL and Cry1Ac on enzymatic activity of ALP (1 nM), individually and in combination (Cry1Ac+ASAL). The inhibition is competitive. Both V_{max} and K_m are affected. **c** Michaelis–Menten and Lineweaver–Burk Plot of APN. **d** Effect of different concentrations of Cry1Ac and Cry1Ac+ASAL (1:1 molar ratio) on enzymatic activity of APN. ASAL alone did not influence APN activity

Discussion

Gene pyramiding in transgenic plants has been proposed as an important strategy (1) for the management of resistance development in insects and (2) to broaden the insect resistance in transgenic plants which cannot be achieved by a single toxin (Jackson et al. 2003; Manyangarirwa et al. 2006). δ-Endotoxins control lepidopteran pests effectively but are not effective against sap-sucking homopterans (Dutt 2007; Virla et al. 2010). Therefore, future pest management strategies will include stacking more than one gene in transgenic crop plants. One such strategy could be based on δ-endotoxins against lepidopteran pests and another toxin for homopteran pests (Ferry et al. 2004; Wu and Guo 2005).

ASAL is considered a good candidate protein for pyramiding with Cry1Ac δ-endotoxin in transgenic plants. It is significantly toxic to sap-sucking pests like aphids of cotton, pea, and mustard and also inhibitory to the growth of lepidopteran pests like *H. armigera* and *S. litura* (Bandyopadhyay et al. 2001; Saha et al. 2006; Sadeghi et al. 2008; Upadhyay et al. 2010a). Although efficacy of ASAL to lepidopteran pests is substantially lower as compared to δ-endotoxins, in pyramiding, the former can contribute to delaying resistance development against δ-endotoxins.

Earlier, we reported the binding of ASAL to several proteins in the midgut of *H. armigera*, and a majority of those were known receptors of Cry1Ac (Upadhyay et al. 2010b). This study was aimed at finding if both the insecticidal proteins can function together without interfering with each other. Knowledge of the combined effect of Cry1Ac and ASAL on *H. armigera* and cotton aphids is necessary before designing a strategy for pyramiding. Some lectins, like Con A (specific to mannose and glucose) and SBA (specific to GalNAc) inhibit the binding of Cry1Ac and Cry1Ab to the BBMV of *H. armigera* (Estela et al. 2004). Contrary to earlier reports, ASAL improved the binding of Cry1Ac to the BBMV and contributed to the toxicity, though marginally. Cry1Ac did not interfere with the toxicity of the ASAL on cotton aphids. We also noticed that the ASAL made a complex with Cry1Ac through protein–protein interaction, although ASAL is known for carbohydrate-mediated interactions only (Upadhyay et al. 2010a). This has not been reported earlier.

To understand the mechanism of action of the toxins, we studied their binding with BBMV proteins of *H. armigera*.

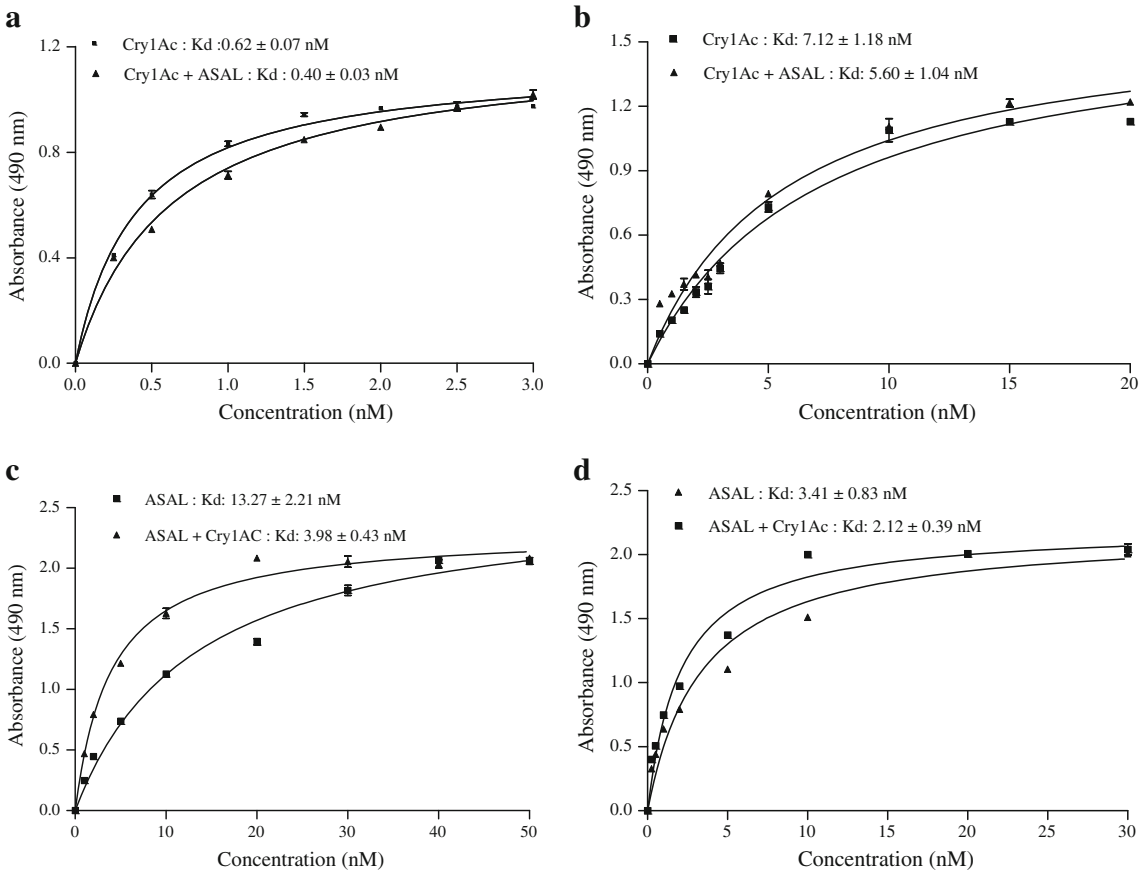

Fig. 3 Binding kinetics of Cry1Ac and ASAL with purified ALP and APN. The purified APN and ALP were coated on ELISA plate, incubated with different concentrations of the toxins and developed with respective antibodies. **a** Binding of Cry1Ac (0.25–3 nM) and Cry1Ac+ASAL (0.25–3 nM in 1:1 ratio) with ALP (10 ng) and development with anti-Cry1Ac antibody. **b** Binding of Cry1Ac (0.5–20 nM) and Cry1Ac+ASAL (0.5–20 nM in 1:1 ratio) with APN (10 ng) and development with anti-Cry1Ac antibody. **c** Binding of ASAL (1–50 nM) and ASAL+Cry1Ac (1–50 nM in 1:1 ratio) with ALP (10 ng) and development with anti-ASAL antibody. **d** Binding of ASAL (0.25–30 nM) and ASAL+Cry1Ac (0.25–30 nM in 1:1 ratio) with APN (10 ng) and development with anti-ASAL antibody. Figure shows that both the proteins interacted with both the receptors; one increased the affinity of other toward the purified receptors

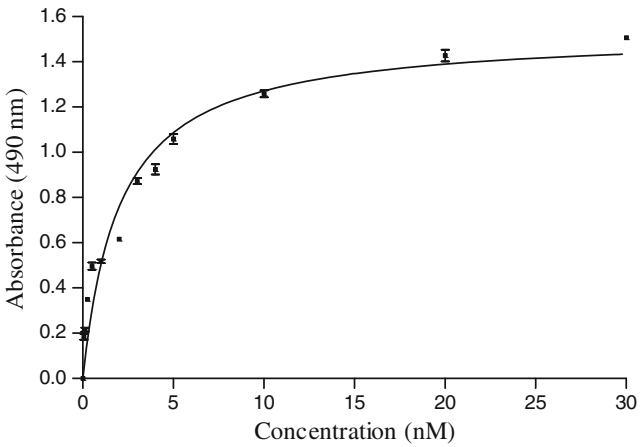

Fig. 4 Binding of Cry1Ac with ASAL. ELISA plate coated with 1 nM ASAL, was incubated with different concentrations of Cry1Ac (0.062–30 nM) and developed by using anti-Cry1Ac antibody. Figure shows the interaction between the two proteins

The toxins showed affinity towards BBMV proteins, which increased with larval development. The increase might be either due to increase in the abundance of the receptors or due to their modification, resulting in an increase in the affinity of the receptors to the toxins or both. The toxins either helped each other in binding to BBMV proteins or did not interfere. Earlier studies have shown that some lectins reduce the affinity of δ-endotoxins towards the BBMV proteins (Estela et al. 2004).

It was not possible to predict the ratio of the complex and free forms of the toxins in the midgut of larvae in the feeding experiments. There could be three possibilities for higher insecticidal actions in combination—(1) the toxins were in complex form at the time of feeding, but got free from each other at alkaline pH in the midgut and then bound to the BBMV proteins; (2) toxins bound to the BBMV proteins as a complex with higher affinity and caused toxicity; and (3) these may bind sequentially to the

receptors and enhance mutual affinity. Since the toxicity of Cry1Ac improved with ASAL, the latter two hypotheses appear more logical. The toxins improved each other's binding to the BBMV proteins; it seemed that the sites of interaction between them were different from their BBMV binding sites.

ALP and APN are important receptors of δ-endotoxins in the midgut of insects (Ingle et al. 2001; McNall and Adang 2003; Arenas et al. 2010; Upadhyay and Singh 2011). Our earlier study has shown the binding of ASAL to these receptors in *H. armigera* (Upadhyay et al. 2010b). The studies on the interaction of Cry1Ac and ASAL with purified ALP and APN reported here demonstrate significant affinity of both the toxins towards the two receptors and increase in the affinity of one toxin in the presence of the other. This indicated that each receptor had two types of independent or overlapping binding sites, one for each toxin. Binding at site A influenced the binding of other toxin at site B. ALP had maximum affinity for Cry1Ac–ASAL complex, followed by the affinity for Cry1Ac and ASAL. APN also had maximum affinity for the complex, followed by ASAL and Cry1Ac. However, the increase in the binding affinity of the toxin complex did not result in increase in the insecticidal activity. This suggested that the strength of the toxin–receptor interaction does not completely account for the insecticidal activity.

Cry1Ac, ASAL, and their complex inhibited the enzymatic activity of ALP. This has been reported for Cry1Ac (Sarkar et al. 2009) but not for ASAL. ALP activity was maximally inhibited by ASAL, followed by Cry1Ac and Cry1Ac–ASAL complex. This was opposite to the affinity of the toxins and their complex with ALP. This indicated that the binding of the toxins to ALP and inhibition of the ALP activity were unrelated. Only binding of toxins to the ALP contributed to insecticidal activity. Enzymatic activity of APN remained unaffected. The result was in agreement with Ingle et al. (2001).

ASAL showed some additive effect in the larvicidal activity of Cry1Ac against *H. armigera*. We propose two hypotheses for the result—(1) ASAL binds to the BBMV proteins and enhances the interaction of Cry1Ac with its receptors like Bt R_1, aminopeptidase N (Knight et al. 1994; Burton et al. 1999), and alkaline phosphatase (Jurat-Fuentes and Adang 2004); and (2) ASAL helps in internalization of Cry1Ac into the hemolymph and contributes to the increment in toxicity. ASAL has been reported to get internalized into the hemolymph of *H. armigera* (Upadhyay et al. 2010a). A similar lectin of *Allium* (ASA) is reported as a carrier in the transportation of attached protein to hemolymph (Fitches et al. 2008). Cry1Ac has been detected in hemolymph of *H. armigera* (Ding et al. 2009). δ-Endotoxins, which are toxic to insects through oral route, have been reported to be effective when injected directly also (Cerstiaens et al. 2001). These evidences indicate the possibility of internalization of Cry1Ac with the help of ASAL and, thus, enhancement in the toxicity. Nevertheless, the exact mechanism of combined toxicity is yet to be elucidated.

Gene pyramiding is one of the methods to develop broad-range insect-resistant transgenic plants and delay the development of resistance against a given toxin. It is based on three assumptions: (1) insects developing resistance against one toxin can be controlled by the second toxin (Jackson et al. 2003; Ferry et al. 2004), (2) insects with resistance against the two toxins (with independent mode of actions) cannot emerge through selection pressure of one toxin alone, and (3) a single gene in insect cannot confer resistance against two toxins if they are immunologically distinct and have different binding targets (Gahan et al. 2005). A species cannot easily develop resistance against two or more toxins because it would require simultaneous and autonomous mutations (Jackson et al. 2003). Second-generation Bt Cottons [Bollgard II® (Cry 1Ac+Cry 2Ab) and WideStrike™ (Cry1Ac+Cry 1F)] express two δ-endotoxins. These have been developed to increase the level of control for *H. zea*, which was not adequately controlled by the Cry1Ac alone (Jackson et al. 2003; Ferry et al. 2004; Bates et al. 2005; Gahan et al. 2005). Cry1Ac and Cry2Ab have different binding sites in the larval midgut; they make a reasonably good combination for delaying the resistance development.

ASAL is toxic to sap-sucking pests and also effective against lepidopteran pests like *H. armigera*. It does not interfere with the insecticidal action of Cry1Ac against *H. armigera.* If it is pyramided with Cry1Ac in cotton, transgenic plants will exhibit protection against two entirely different species of insects and also enhance protection against *H. armigera*. Our study shows that ASAL can be employed with Cry1Ac in the transgenic crop plants for protection against a wider range of insects. Other important observations which support pyramiding of these two toxins are as follows: (1) Cry1Ac increased the affinity of ASAL for BBMV proteins, (2) the two toxins enhanced the affinity of each other toward ALP and APN, (3) Cry1Ac and ASAL inhibited the enzymatic activity of ALP, and (4) Cry1Ac interacted with ASAL. Detailed examination of these aspects is in progress.

Acknowledgments Authors are grateful to the Council of Scientific and Industrial Research, Government of India, New Millennium Technology Leadership Initiative (NMITLI) program for the financial support. SKU is thankful to CSIR for Senior Research Fellowship and GB Technical University for PhD registration. RT is thankful to DST for JC Bose Fellowship. Authors are thankful to SMH Abidi for insect culture, Rajesh K Srivastava for mass spectrometric analysis, Moti Lal for protein extraction from garlic, and Aquila Bano for technical support.

References

Angelucci C, Barrett-Wilt GA, Hunt DF, Akhurst RJ, East PD, Gordon KH, Campbell PM (2008) 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 38:685–696

Arenas I, Bravo A, Sobero M, Gomez I (2010) Role of alkaline phosphatase from *Manduca sexta* in the mechanism of action of *Bacillus thuringiensis* Cry1Ab toxin. J Biol Chem 285:12497–12503

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

Bates SL, Zhao J, Roush RT, Shelton AM (2005) Insect resistance management in GM crops: past, present and future. Nat Biotechnol 23:57–62

Bravo A, Gomez I, Conde J, Munoz-Garay C, Sanchez J, Miranda R, Zhuang M, Gill SS, Soberon M (2004) Oligomerization triggers binding of a *Bacillus thuringiensis* Cry1Ab pore-forming toxin to aminopeptidase N receptor leading to insertion into membrane micro domains. Biochim Biophys Acta 1667:38–46

Broderick NA, Raffa KF, Handelsman J (2006) Midgut bacteria required for *Bacillus thuringiensis* insecticidal activity. Proc Natl Acad Sci USA 103:15196–15199

Burton SL, Ellar DJ, Li J, Derbyshire J (1999) 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 287:1011–1022

Cerstiaens A, Verleyen P, Van Rie J, Kerkhove EV, Schwartz JL, Laprade R, Loof AD, Schoofs L (2001) Effect of *Bacillus thuringiensis* Cry1 toxins in insect hemolymph and their neurotoxicity in brain cells of *Lymantria dispar*. Appl Environ Microbiol 67:3923–3927

Chaturvedi R, Bhakuni V, Tuli R (2000) The d-Endotoxin proteins accumulate in *Escherichia coli* as a protein±DNA complex that can be dissociated by hydrophobic interaction chromatography. Protein Exp Purif 20:21–26

Ding J, Lib J, Liua X, Zhanga Q (2009) The life parameters of a parasitoid *Microplitis mediator* (Hymenoptera: Braconidae), reared on cotton bollworm *Helicoverpa armigera* (Hübner) with Cry1Ac diet. Biocon Sci Technol 19:931–941

Dutt U (2007) Mealy bug infestation in Punjab: Bt cotton falls flat. Environment News Service, 21 August (countercurrents.org)

Estela A, Escriche B, Ferre J (2004) Interaction of *Bacillus thuringiensis* toxins with larval midgut binding sites of *Helicoverpa armigera* (Lepidoptera: Noctuidae). Appl Environ Microbiol 70:1378–1384

Fernandez LE, Aimanova KG, Gill SS, Bravo A, Soberón M (2006) A GPI anchored alkaline phosphatase is a functional midgut receptor of Cry11Aa toxin in *Aedes aegypti* larvae. Biochem J 394:77–84

Ferry N, Edwards MG, Mulligan E A, Emami K, Petrova A S, Frantescu M, Davison GM, Gatehouse AMR (2004) In: Christou P, Klee H (eds). Handbook of Plant Biotechnology. Wiley, Chichester 1:373–394

Fitches E, Woodhouse S, Edwards JP, Gatehouse JA (2001) *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 47:777–787

Fitches E, Wiles D, Douglas EA, Hinchliffe G, Audsley N, Gatehouse JA (2008) The insecticidal activity of recombinant garlic lectins towards aphids. Insect Biochem Mol Biol 38:905–915

Gahan LJ, Ma YT, Cobble MLM, Gould F, Moar WJ, Heckel DG (2005) Genetic basis of resistance to Cry 1Ac and Cry 2Aa in *Heliothis virescens* (Lepidoptera: Noctuidae). J Econ Entomol 98:1357–1368

Hua G, Zhang R, Bayyareddy K, Adang MJ (2009) *Anopheles gambiae* alkaline phosphatase is a functional receptor of *Bacillus thuringiensis* jegathesan Cry11Ba toxin. Biochemistry 48:9785–9793

Ingle SS, Trivedi N, Prasad R, Kuruvilla J, Rao KK, Chhatpar HS (2001) Aminopeptidase-N from the *Helicoverpa armigera* (Hubner) brush border membrane vesicles as a receptor of *Bacillus thuringiensis* Cry1Ac d-endotoxin. Curr Microbiol 43:255–259

Jackson RE, Bradley JR, Van Duyn JW (2003) Field performance of transgenic cottons expressing one or two *Bacillus thuringiensis* endotoxins against bollworm, *Helicoverpa zea* (Boddie). J Cotton Sci 7:57–64

Jurat-Fuentes JL, Adang MJ (2004) Characterization of a Cry1Ac-receptor alkaline phosphatase in susceptible and resistant *Heliothis virescens* larvae. Eur J Biochem 271:3127–3135

Jurat-Fuentes JL, Adang MJ (2007) A proteomic approach to study Cry1Ac binding proteins and their alterations in resistant *Heliothis virescens* larvae. J Invert Pathol 95:187–191

Knight PJK, Crickmore N, Ellar DJ (1994) The receptor for *Bacillus–Thuringiensis* Cryla(C) delta-endotoxin in the brush-border membrane of the Lepidopteran *Manduca sexta* is aminopeptidase-N. Mol Microbiol 11:429–436

Krishnamoorthy M, Jurat-Fuentes JL, McNall RJ, Andacht T, Adang MJ (2007) Identification of novel Cry1Ac binding proteins in midgut membranes from *Heliothis virescens* using proteomic analyses. Insect Biochem Mol Biol 37:189–201

Lowry OH, Roberts NR, Wu ML, Hixton WS, Crawford EJ (1954) The quantitative histochemistry of brain II. Enzyme measurements. J Biol Chem 207:19–37

Luo K, Sangadala S, Masson L, Mazza A, Brousseau R, Adang MJ (1997) The *Heliothis virescens* 170 kDa aminopeptidase functions as "receptor A" by mediating specific *Bacillus thuringiensis* Cry1A delta-endotoxin binding and pore formation. J Biochem Mol Biol 27:735–743

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

Manyangarirwa W, Turnbull M, McCutcheon GS, Smith JP (2006) Gene pyramiding as a *Bt* resistance management strategy: how sustainable is this strategy? Afr J Biotechnol 5:781–785

Martins ES, Monnerat RG, Queiroz PR, Dumas VF, Braz SV, De Souza Aguiar RW, Gomes AC, Sánchez J, Bravo A, Ribeiro BM (2010) Midgut GPI-anchored proteins with alkaline phosphatase activity from the cotton boll weevil (*Anthonomus grandis*) can be the putative receptors for the Cry1B protein of *Bacillus thuringiensis*. Insect Biochem Mol Biol 40:138–145

McNall RJ, Adang MJ (2003) Identification of novel *Bacillus thuringiensis* Cry1Ac binding proteins in *Manduca sexta* midgut through proteomic analysis. Insect Biochem Mol Biol 33:999–1010

Mitz M, Schlueter R (1958) Direct spectrophotometric measurement of the peptide bond: application to the determination of acylase. Biochim Biophys Acta 27:168

Sadeghi A, Smagghe G, Broeders S, Hernalsteens JP, DeGreve H, Peumans WJ, VanDamme EJ (2008) Ectopically expressed leaf and bulb lectins from garlic (*Allium sativum* L.) protect transgenic tobacco plants against cotton leaf worm (*Spodoptera littoralis*). Transgenic Res 17:9–18

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

Sarkar A, Hess D, Mondal HA, Banerjee S, Sharma HC, Das S (2009) Homodimeric alkaline phosphatase located at *Helicoverpa armigera* midgut, a putative receptor of Cry1ac contains α-GalNAc in terminal glycan structure as interactive epitope. J Prot Res 8:1838–1848

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

Smeets K, Van Damme EJM, Verhaert P, Barre A, Rouge P, Van Leuven F, Peumans WJ (1997) Isolation, characterization and molecular cloning of the mannose-binding lectins from leaves and roots of garlic (*Allium sativum* L.). Plant Mol Biol 33:223–234

Tuli R, Saluja J, Notani NK (1989) Cloning and expression in *Escherichia coli* of entomotoxic protein gene from *Bacillus thuringiensis* subspecies kurstaki. J Genet 68:147–160

Upadhyay SK, Singh PK (2011) Role of alkaline phosphatase in insecticidal action of Cry1Ac against *Helicoverpa armigera* larvae. Biotechnol Lett. doi:10.1007/s10529-011-0665-x

Upadhyay SK, Mishra M, Singh H, Ranjan A, Chandrashekar K, Verma PC, Singh PK, Tuli R (2010a) Interaction of *Allium sativum* leaf agglutinin (ASAL) with midgut brush border membrane vesicle proteins and its stability in *Helicoverpa armigera*. Proteomics 10:4431–4440

Upadhyay SK, Saurabh S, Rai P, Singh R, Chandrashekar K, Verma PC, Singh PK, Tuli R (2010b) SUMO fusion facilitates expression and purification of garlic leaf lectin but modifies some of its properties. J Biotechnol 146:1–8

Van Damme EJM, Smeets K, Torrekens S, Van Leuven F, Goldstein IJ, Peumans WJ (1992) The closely related homomeric and heterodimeric mannose-binding lectins from garlic are encoded by one-domain and two-domain lectin genes, respectively. Eur J Biochem 206:413–420

Vasconcelos IM, Oliveira JTA (2004) Antinutritional properties of plant lectins. Toxicon 44:385–403

Virla EG, Casuso M, Frias EA (2010) A preliminary study on the effects of a transgenic corn event on the non target pest *Dalbulus Maiid* (Hemitera: Cicadeliidae). Crop Prot 29:635–638

Wolfersberger MG, Luthy P, Maurer A, Parenti P, Sacchi VF, Giordana B, Hanozet GM (1987) 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 86:3001–3008

Wu KM, Guo YY (2005) The evolution of cotton pest management practices in China. Ann Rev Entomol 50:31–52

Zhang X, Candas M, Griko NB, Rose-Young L, Bulla LA Jr (2005) Cytotoxicity of *Bacillus thuringiensis* Cry1Ab toxin depends on specific binding of the toxin to the cadherin receptor BT-R1 expressed in insect cells. Cell Death Differ 12:1407–1416

Zhang R, Hua G, Andacht TM, Adang MJ (2008) A 106-kDa Aminopeptidase is a putative receptor for *Bacillus thuringiensis* Cry11Ba toxin in the mosquito *Anopheles gambiae*. Biochemistry 47:11263–11272