Indian Journal of Entomology, **80**(3): 960-970 (2018) DOI No.: 10.5958/0974-8172.2018.00145.1

INFLUENCE OF *BACILLUS THURINGIENSIS* TOXINS ON THE DEVELOPMENT AND MIDGUT PROTEASES IN DIFFERENT LARVAL INSTARS OF *HELICOVERPA ARMIGERA*

Visweshwar, R., Akbar, S.M.D**, Sharma, H.C.** and Sreeramulu, K.***

**Department of Biochemistry, Gulbarga University, Kalaburagi 585106, Karnataka*
***Department of Entomology, International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru 502324, Telangana*
**Email: ksramu@rediffmail.com (corresponding author)*

ABSTRACT

***Helicoverpa armigera* (Hubner) (Lepidoptera: Noctuidae) is one of the most important pest worldwide. *Bacillus thuringiensis* (Bt) toxins have used as a biopesticide or deployed in transgenic plants for controlling this pest. We examined the biological activity of Cry1Ac, Cry1Ab and Bt formulation in different larval instars of *H. armigera* with respect to larval development and proteolytic activity to pinpoint the most susceptible instar, and the insect response to ingestion of Bt toxins. In the presence of Bt toxins, the larval mortality and weight loss increased in a dose-dependent manner, and the maximum effect was observed in neonates. Active Cry1Ac toxin resulted in greater mortality and weight loss in all the larval instars. Total protease, trypsin and chymotrypsin activities declined in the presence of Bt toxins as compared to the untreated control in all the larval instars. Ten protease isozymes were observed in the untreated control larvae in second, third and fourth instars. Maximum protease isozymes inhibition was observed in Bt toxin fed neonates. Inhibition of protease activity increased with the concentration of Bt toxins. In presence of Bt toxins, aminopeptidase activity increased from II to IV instar and alkaline phosphatase activity decreased from II to III and then increased in IV instar. The pathogenicity of Bt was greater in early larval instars of *H. armigera* than the later instars, suggesting that application of Bt formulation or deployment of Bt toxins in transgenic plants should be directed against the early larval instars.**

Key words: *Helicoverpa armigera, Bacillus thuringiensis* toxins, larval growth and survival, proteases, aminopeptidases, alkaline phosphatases, pest control

The cotton bollworm, *Helicoverpa armigera* (Hubner) (Lepidoptera: Noctuidae), is one of the most damaging insect pest worldwide causing destruction to cotton, pulses, cereals, and vegetable and fruit crops (Fitt, 1989; Sharma, 2005). It is difficult to control this pest by synthetic pesticides as it has developed high levels of resistance to chemical insecticides (Kranthi et al., 2001a,b; McCaffery, 1998). Host plant resistance to insect pests is economically feasible and ecologically preferred alternatives to other pest management strategies, particularly the synthetic pesticides. Development of improved crop varieties with resistance or tolerance to *H. armigera* is highly desirable, particularly for the subsistence farming systems in the developing countries (Sharma, 2005). Therefore, *Bacillus thuringiensis* (Berliner) (Bt) toxins are generally deployed in genetically modified plants for controlling *H. armigera* (Sharma, 2009).

A wide range of lepidopteran larvae, including *H. armigera* are susceptible to Bt toxin proteins (Hofte and Whiteley, 1989; Lereclus et al., 1989; Surekha Devi et al., 2011).In the native form, the Bt crystal proteins are in inactive state (protoxin) and are non-toxic. After ingested by the larvae, the protoxins get solubilized in the alkaline environment of larval midgut and are activated to active toxins by midgut proteases. The active toxin binds to the receptors located on the midgut brush border membrane vesicles, which leads to insertion of the toxin protein into the midgut epithelial cells, resulting in toxin oligomerization, activation of cell signalling pathway, formation of pores that cause cell lysis, and ultimately leads to larval death (Gill et al., 1992; Tabashnik et al., 1994)

In some studies the endpoint for Bt toxins bioassay was mortality (Tapp and Stotzky, 1998), for others it was growth (Sims and Holden, 1996; Herman et al., 2001; Herman et al., 2002a), and for others it was a combination of growth and mortality (Herman et al., 2002b). Larval mortality and weight loss is directly proportional to the Bt toxins concentration. Cry1Ac

induced larval mortality and weight loss in *H. armigera* (Purushottam and Vandana, 2013; Visweshwar et al., 2015). Susceptibility of *H. armigera* (Anand et al., 2011; Paramasiva et al., 2014) and *Spodoptera litura* (L.) (Anand et al., 2011) larvae to Bt toxins varies across larval instars and the host plants. Insect proteases appear to be the key in determining toxin specificity. Depending on the insect species, protoxins proteolytically processed by trypsin like serine proteases, elastase like and chymotrypsin like midgut proteases (Peyronnet et al., 1997). Toxin oligomers bind to the soluble ectodomains of membrane-associated glycosyl-phosphatidylinositol anchored proteins such as aminopeptidase (APN) (Sangadala et al., 1994; Knight et al., 1994) and alkaline phosphatase (ALP) (Jurat-Fuentes and Adang, 2004; Arenas et al., 2010).

We studied the effect of Cry1Ac and Cry1Ab, and Bt formulation on survival and development of different larval instars in relation to the activity of proteolytic enzymes and binding efficiency of Cry1Ac and Cry1Ab with the brush border membrane vesicles (BBMVs) in the mid gut of *H. armigera.*

MATERIALS AND METHODS

Insect culture: The *H. armigera* larvae were reared on chickpea based artificial diet under laboratory conditions at 26 ± 2 °C, 60% RH and photoperiod of 16: 8 hr (L: D) (Chitti Babu et al., 2014) in the insect rearing laboratory at the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Telangana.

Active Cry1Ac toxin preparation: The Cry1Ac protoxin was prepared as described by Maa et al. (2008) and Shao et al. (1998). Cry1Ac toxin was prepared from Bt 4D4 strain (supplied by Daniel R. Zeigler, Bacillus Genetic Stock Center, Ohio State University, Ohio, USA). The strain was grown in LB medium (tryptone 1%, yeast extract 0.5%, NaCl 1%, pH 7–7.2) for 3-4 days at 28 °C. After 48 h fermentation, the culture medium was collected and placed at 4 °C until cells lysed. The spore and crystal mixture was precipitated by centrifugation at 10,000g x 10 min, and the pellet washed 3 times with 1 M NaCl to remove the endogenous proteases, followed by several washes with distilled water. The ä-endotoxins of Bt were selectively dissolved in 2% β-mercaptoethanol-NaOH buffer, pH 10.7, and centrifuged at 4 °C, 10,000g x 20 min. pH of the supernatant was adjusted to 4.4 with 2 M acetic acid, and centrifuged at 10,000g for 30 min; the precipitate of Cry1Ac protoxin was collected, dialyzed against water, lyophilized, and stored at -20 °C. All steps were conducted at 4 °C. The protoxin was treated with bovine trypsin at the ratio 1: 100 (w/w) for 12 h at 37 °C, and the active Cry1Ac toxin protein concentration was estimated.

Trypsin activated Cry1Ab was obtained from Dr Marianne P. Carey (Department of Biochemistry, Case Western Reserve University, Cleveland, Ohio, USA). The Bt formulation was obtained from Biolep®, Biotech International Ltd, New Delhi, India (spores 5-8% (w/w) and ä-endotoxins 5-8% (w/w)).

Larval feeding assays: Neonates were reared on artificial diet till they attain first-, second-, third- and fourth-instar larvae. Neonates, first-, second-, third- and fourth-instar larvae were fed on artificial diet, and the diet treated with Cry1Ac (0.4, 0.8, 1.6, 3.2, 6.4 and 13 µg/g diet), Cry1Ab (0.4, 0.8, 1.6, 3.2, 6.4 and 13 µg/g diet) and Bt formulation (0.005, 0.010, 0.025, 0.05, 0.1 and 0.2%). There were three replications, and each replicate had 10 larvae. Insects were maintained at 26 ± 2 °C, 60% RH and a photoperiod of 16:8 h (L: D). After 5 days, data were recorded on larval survival and larval weights.

Preparation of midgut extracts: To study the protease file of *H. armigera* larvae, the larvae were fed on diet containing trypsin activated Cry1Ac (1 and 10 µg/g diet), trypsin activated Cry1Ab (1 and 10 µg/g diet) and Bt formulation (0.02 and 0.10%). Whole body of second-instar, midguts from the third- and fourth-instar larvae, fed on the above mentioned diets and homogenized in ice-cold 0.1 M glycine-NaOH buffer, pH 10.0. All the homogenates were centrifuged at 10,000g at 4 °C for 15 min. The supernatants were collected and divided into small aliquots and stored at -20 °C until use.

Total protease, trypsin and chymotrypsin activity assay: Total protease activity in gut homogenates, from the larvae reared on Cry toxins, was determined by incubating gut extract (100 µl) with 500 µl of azocasein (1% in 0.1 M glycine-NaOH, pH 10.0) (Sigma-Aldrich, India) for 30 min (Vinod et al., 2010; Visweshwar et al., 2015). Then 200 µl of 5% TCA was added and centrifuged. To the supernatant, equal volume of 1N NaOH was added, and the absorbance read at 450 nm. Protease activity (UA) was computed as: Unit activity (UA) = ABS_{450} nm/ [time (min) x volume of enzyme (ml).

Trypsin and chymotrypsin activity assays were carried out using selective synthetic substrates Ná-benzoyl-DL-arginine-p-nitroanilide (BApNA) (Sigma-Aldrich, India) and N-succinyl-alanine-alanine-proline-phenylalanine-p-nitroanilide (SAAPFpNA) (Sigma-Aldrich, India), respectively, using UV-visible spectrophotometer (Hitachi U-2900, Japan) (Visweshwar et al., 2015). One unit of enzyme activity was defined as the amount of enzyme catalysing the hydrolysis of 1 µmol p-nitroanilide released per minute at 37 °C.

Zymogram analysis of *H. armigera* gut proteases: The *H. armigera* gut extracts, prepared from second-, third- and fourth-instar larvae reared on trypsin activated Cry1Ac (1 and 10 µg/g diet), trypsin activated Cry1Ab (1 and 10 µg/g diet), and Bt formulation (0.02 and 0.10%) were subjected to 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) (Laemmli, 1970) under non-reducing and non-denaturing conditions. After electrophoresis, the gel was treated with 2.5% triton X-100 for 10 min to remove SDS from gel. Then, the gel was incubated with 2% casein in 0.1 M glycine-NaOH buffer, pH 10.0 for 60 min. The gel was then washed with distilled water and stained with coomassie brilliant blue for 15 min and destained (Garcia-Carreno et al., 1993). Proteolytic bands appeared as clear zones against the blue background.

Preparation of BBMVs: BBMVs from second-, third- and fourth-instar larvae were prepared according to Wolfersberger (1993). Midguts were dissected from one hour starved fourth-instar larvae and homogenized in 10 volumes of MET buffer (300 mM mannitol, 5 mM EGTA and 17 mMTris-HCl, pH 7.5). An equal volume of 24 mM $MgCl_2$ solution was added and further homogenized. Samples were centrifuged at 2,500g for10 min. The supernatant was re-centrifuged for 30 min at 30,000 g. The final pellet was suspended in half-strength MET buffer. The protein concentration of BBMV fraction was estimated by the method of Lowry et al. (1951).

APN and ALP activities assay: In BBMVs prepared from Cry1Ac, Cry1Ab and Bt formulation treated larvae, APN and ALP activities were estimated by using leucine-p-nitroanilide (LpNA) and p-nitro phenyl phosphate (PNPP), respectively as substrates. The reaction was started by adding 20 µl of LpNA (50 mM) or 50 µl of PNPP (50 mM), to 50 µl of the homogenate in 0.1 M glycine-NaOH buffer, pH 10.0, and continued for 20 min at 37 °C. The reactions were terminated by adding 200 µl of 30% acetic acid for aminopeptidases, and 200 µl of 2M NaOH for alkaline phosphatase. The samples were centrifuged and absorbance was read at 410 nm using UV-visible spectrophotometer. One unit of enzyme activity was defined as the µmol of p-nitroaniline released per minute for APN and 4-nitrophenol for ALP at 37 °C.

ELISA binding assay: Cry1Ac and Cry1Ab toxins were incubated with BBMVs from second-, third- and fourth-instar larvae reared on artificial diet, for 1 h at 37 C. Samples were centrifuged at 10,000g for 10 min, pellet containing bound toxin was washed three times with distil water and dissolved in phospahate-buffered saline (PBS). Pellet dissolved in PBS (100 µl) was transferred to ELISA plates (EnviroLogix, USA) pre-coated with Cry1Ac/Ab primary antibodies and incubated for 20 min. Then, goat anti-rabbit–alkaline phosphatase secondary antibodies (EnviroLogix, USA) (1:5,000) were added to each well and incubated for 1 h and then wells were washed with blocking buffer (PBS-tween). Substrate tetra methyl benzidine (TMB)/ H_2O_2 was added to each well. After 20 min, 1 N HCl was added to each well to stop the reaction and absorbance was recorded at 450 nm.

Statistical analysis: The data were subjected to analysis of variance using the GenStat (14th edition, Version 14.1.0.5943, VSN International Ltd, United Kingdom) software. The significance of differences between the treatments was judged by F-test, while the treatment means were compared by least significant difference (LSD) at $p< 0.05$. Significance between the protease, APN and ALP activities between the treatments was judged by Duncan's multiple range test (DMRT). The LC_{50} values were determined by using EPA PROBIT analysis program (Version 1.5).

RESULTS AND DISCUSSION

Bioassays

The *H. armigera* larval mortality increased with an increase in the concentration of activated Cry1Ac, and Cry1Ab, and Btformulation. In Cry1Ac-, Cry1Ab- and Bt formulation-fed neonates, the larval mortality ranged from 0 to 100% ($y = 4.44x + 58.90$ ($R^2 = 32.54\%$), $y = 4.93x+53.54$ ($R^2 = 40.24\%$) and $y = 382.39x+37.98$ ($R^2 = 68.83\%$), respectively) (Fig. 1). Mortality of active Cry1Ac-fed first-, second- ,third-, and fourth-instar larvae of *H. armigera* ranged from 0 to 100% ($y = 5.61x + 43.93$ ($R^2 = 55.72\%$)), 0 to 80% (y =

4.71x+27.19 (R^2 = 71.66%)), and 0 to 70% (y = 4.16x+24.92 (R^2 = 65.76%)) and 0 to 60% (y = 3.92x + 18.62 (R^2 = 71.12%), respectively (Fig. 1A). Activated Cry1Ab treated first-, second-, third-, and fourth-instars, mortality ranged from 0 to 100% (y = 6.35x+31.94, (R^2 = 72.20%)), 0 to 70% (y = 4.20x+24.76 (R^2 = 67.22%)), 0 to 60% (y = 3.76x + 18.51 (R^2 = 72.91%)) and 0 to 50% (y = 3.34x + 12.87 (R^2 = 77.20%), respectively (Fig. 1B). In Bt formulation-fed first-, second-, third-, and fourth-instars, mortality ranged from 0 to 100% (y = 446.75x+23.68 (R^2 = 82.39%)), 0 to 75% (y = 332.31x+21.49 (R^2 = 72.52%)) and 0 to 65% (y = 293.45x+13.65 (R^2 = 83.52%)) and 0 to 55% (y = 273.28x+6.91 (R^2 = 90.79%), respectively (Fig. 1C). The LC_{50} values for activated Cry1Ac, Cry1Ab and the Bt formulation for neonates, first-, second- and third-instars increased with respect to larval age and weight (Table 1).

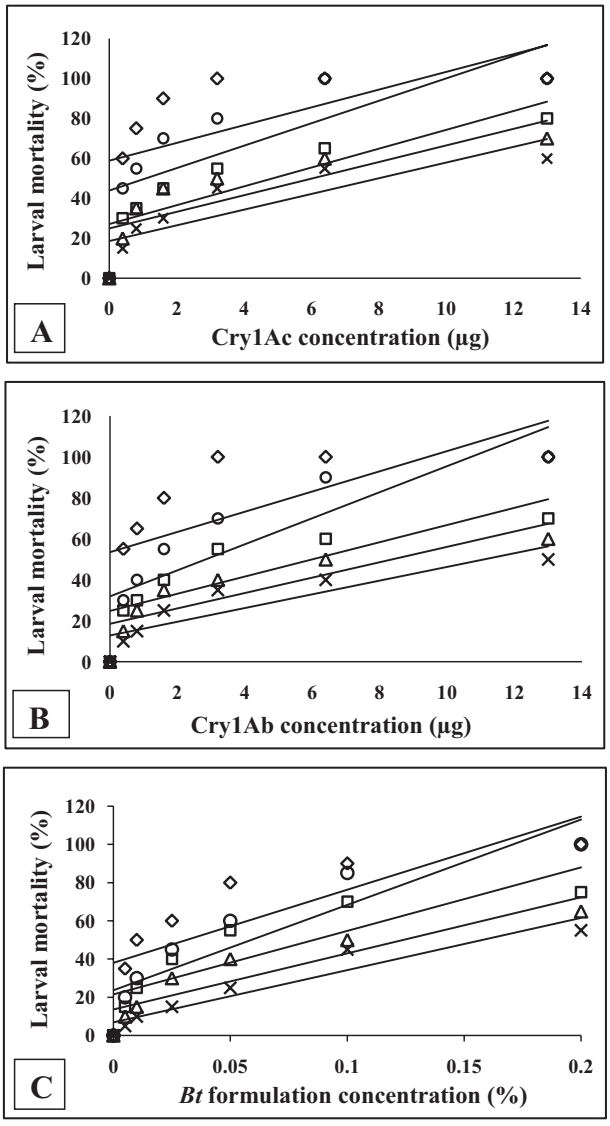

Fig. 1. Survival of different larval instars reared on Bt toxin fed diets. Larvae reared on (A) active Cry1Ac treated-, (B) active Cry1Ab treated- and (C) Bt formulation treated-diet. Neonates (◊), first (o), second (□), third (Δ) and fourth (×) instar larvae reared on respective toxin treated diets. Regression lines of % mortality plotted in the graph. The larvae reared on toxin treated diet for 5 days. Three replications performed per each treatment, and for each replication ten larvae used. The data represented as mean (n = 3).

Decrease in larval weights and increase in weight loss was observed with an increase in concentration of Cry1Ac-fed larvae [first- (23.17 to 100%), second- (21.39 to 85.28%), third- (20.15 to 74.14%) and fourth- (17.54 to 65.22%) instar larvae] (Fig. 2A). Weight loss increased in first- (20.16 to 100%), second- (18.99 to 70.05%), third- (15.37 to 68.44%) and fourth- (10.09 to 57.51%) instar larvae with increase in concentration of Cry1Ab in diet (Fig. 2B). Similar effects were exhibited by Bt formulation [weight loss increased in first- (24.55% to 100%), second- (20.11% to 73.15%) and third- (19.65% to 72.07%)) and fourth- (12.71 to 59.26%) instars] (Fig. 2C).

Protease activities

Protease activities increased in the larvae fed on untreated control diets with an increase in larval instars from II – IV. In second-instar larvae, non-significant decrease ($p>0.01$) in total protease activity, significant decrease ($p<0.01$) in trypsin and chymotrypsin activities were observed in larvae fed on diets containing Cry1Ac, Cry1Ab and Bt formulation compared to the larvae reared on untreated control diets (Table 2). In third- and fourth-instar larvae, with respect to control a significant decrease ($p<0.01$) in total protease, trypsin and chymotrypsin activities were observed in larvae fed on diets with Cry1Ac, Cry1Ab and Bt formulation.

Casein zymogram

There were ten protease bands throughout the larval instars as observed on casein zymogram (P1 to P10). Of the ten protease isozymes, one (P7) and two (P7 and P8) proteases were inhibited by Cry1Ab (in larvae fed on 1 and 10 µg Cry1Ab/g diet, respectively), one (P7) and four (P1, P7, P8 and P9) proteases were inhibited in larvae reared on diet containing 1 and 10 µg Cry1Ac (per g), respectively. One (P7) and three (P1, P7 and P8) proteases were inhibited in in Bt formulation-fed second instar larvae (at 0.02 and 0.10%, respectively) (Fig. 3A). One (P9) and two (P3

Table 1. The LC_{50} values of Bt toxins for different larval instars

Treatment	LC_{50}				
	Neonates	First instar	Second instar	Third instar	Fourth instar
Trypsin activated Cry1Ac (µg)	0.342 (0.027-0.601)	0.618 (0.175-1.089)	2.420 (0.303-12.111)	3.377 (0.879-15.740)	5.624 (1.156-25.118)
Trypsin activated Cry1Ab (µg)	0.431 (0.088-0.736)	1.109 (0.500-1.879)	2.926 (0.887-15.196)	4.425 (1.679-25.065)	5.927 (1.383-32.340)
Bt formulation (%)	0.011 (0.004-0.021)	0.023 (0.012-0.040)	0.038 (0.018-0.089)	0.112 (0.056-0.321)	0.191 (0.081-0.609)

Trypsin activated Cry1Ac and Cry1Ab were used. Control larvae fed on artificial diet without adding any toxin. Values in parentheses for lower and upper limit of 95% confidence; Values outside parentheses LC_{50} values.

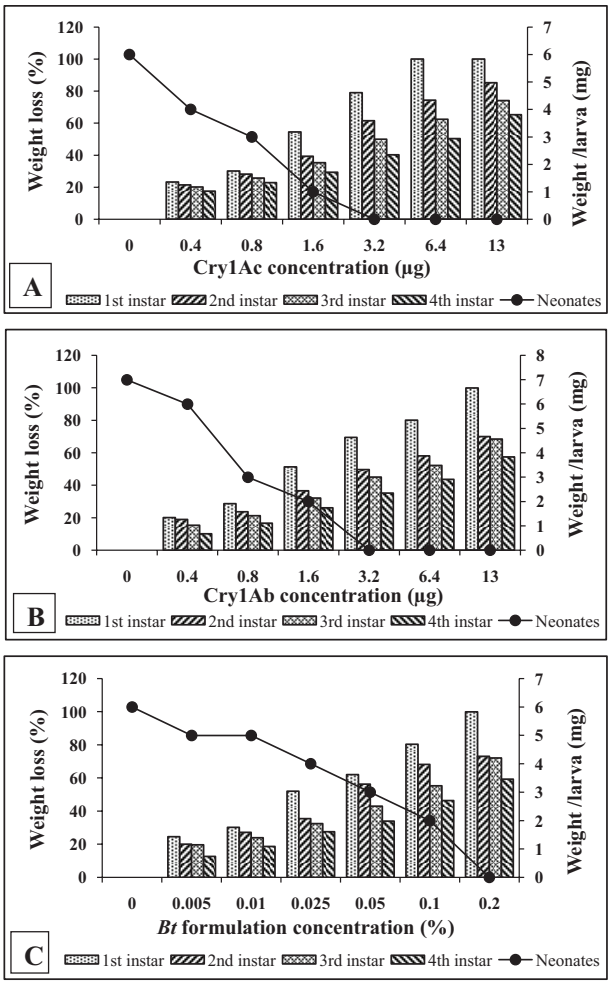

Fig. 2. Development of different instars of larvae reared on Bt toxin. Larvae reared on (A) active Cry1Ac treated-, (B) active Cry1Ab treated- and (C) Bt formulation treated-diet. Neonates, first, second, third and fourth instar larvae reared on respective toxin treated diets.The larvae reared on toxin treated diet for 5 days. Three replications performed per each treatment, and for each replication ten larvae used. The data represented as mean (n = 3).

and P9) in Cry1Ab-fed larvae (1 and 10 µg/g diet, respectively), three proteases (P1, P3 and P10) were inhibited in Cry1Ac-fed larvae (10 µg/g diet), and no protease isozyme was inhibited in 1 µg of Cry1Ac-fed third instar-larvae. One (P9) and three (P1, P3 and P9) were inhibited in Btformulation fed (0.02 and 0.10% respectively) third-instar larvae (Fig. 3B). Two (P1 and P2) proteases were inhibited in fourth-instar larvae reared on diet containing 1 and 10 µg Cry1Ab. Two (P1, P9) proteases were inhibited in 1 and 10 µg of Cry1Ac-fed fourth-instar larvae. One (P9) and two (P1 and P9) were inhibited in fourth-instar larvae reared on diet containing Bt formulation (0.02 and 0.10%, respectively) (Fig. 3C).

APN and ALP activities

APN activity was increased as the larvae develop from second to fourth-instar control larvae. In every instar, in presence of Cry1Ac, Cry1Ab and Bt formulation, the APN activity was increased significantly ($p < 0.01$). ALP activity was increased from second to third-instar larvae and then decreased in fourth-instar control larvae ($p < 0.01$). In Cry1Ac, Cry1Ab and Bt formulation treated second- and third-instar larvae, ALP activity was decreased significantly ($p < 0.01$) and in fourth-instar larvae activity was increased ($p < 0.01$) (Table 3).

Cry1Ac/Ab binding assays

BBMV-bound Cry1Ac and Cry1Ab concentrations were increased with the increase in larval instars. BBMV-bound Cry1Ac and Cry1Ab was increased in third- (60% and 75%, respectively), and fourth-instar (83.33% and 90%) with respect to second instar larvae (Table 4).

The Cry toxin proteins produced by Bt are widely

Table 2. Midgut protease activities in presence of Bt toxins

Treatment†	Total (UA)			Trypsin (U)			Chymotrypsin (U)		
	Second instar	Third instar	Fourth instar	Second instar	Third instar	Fourth instar	Second instar	Third instar	Fourth instar
Cry1Ac (1µg/g)	0.44d	0.45cde	0.43e	0.23d	0.31d	0.42cd	0.20b	0.20ab	0.30bc
Cry1Ac (10µg/g)	0.35abc	0.32a	0.25ab	0.15a	0.19a	0.17a	0.17a	0.17a	0.17a
Cry1Ab (1µg/g)	0.47cde	0.42bcd	0.55f	0.27e	0.35e	0.52e	0.24e	0.24bcd	0.36de
Cry1Ab (10µg/g)	0.34ab	0.36ab	0.35cd	0.23d	0.24c	0.41c	0.21cd	0.19ab	0.30bc
Bt formulation (0.02%)	0.40b	0.53f	0.34c	0.21c	0.35e	0.50e	0.20b	0.23bc	0.34cd
Bt formulation (0.10%)	0.30a	0.38abc	0.24a	0.18b	0.20ab	0.35b	0.17a	0.23bc	0.28b
Control	0.56f	0.58f	0.66g	0.34f	0.39f	0.53e	0.27f	0.33e	0.39e
Vr	16.18	21.25**	326.40**	386.76**	368.63**	74.42**	34.15**	11.43**	30.77**
Mean	0.40	0.43	0.40	0.23	0.29	0.41	0.21	0.22	0.31
SE	0.02	0.02	0.01	0.01	0.01	0.02	0.01	0.01	0.01
LSD	NS	0.07	0.03	0.01	0.02	0.05	0.02	0.05	0.04
CV%	7.50	6.60	3.00	1.90	2.00	4.80	4.00	9.20	5.80

† Larvae were reared on artificial diet containing respective toxin per g of diet. Control larvae were fed on artificial diet without adding any toxin; Data represented as mean (n = 3); Values with different letter in the columns significantly different; ** Significantly different from control at p d" 0.01.

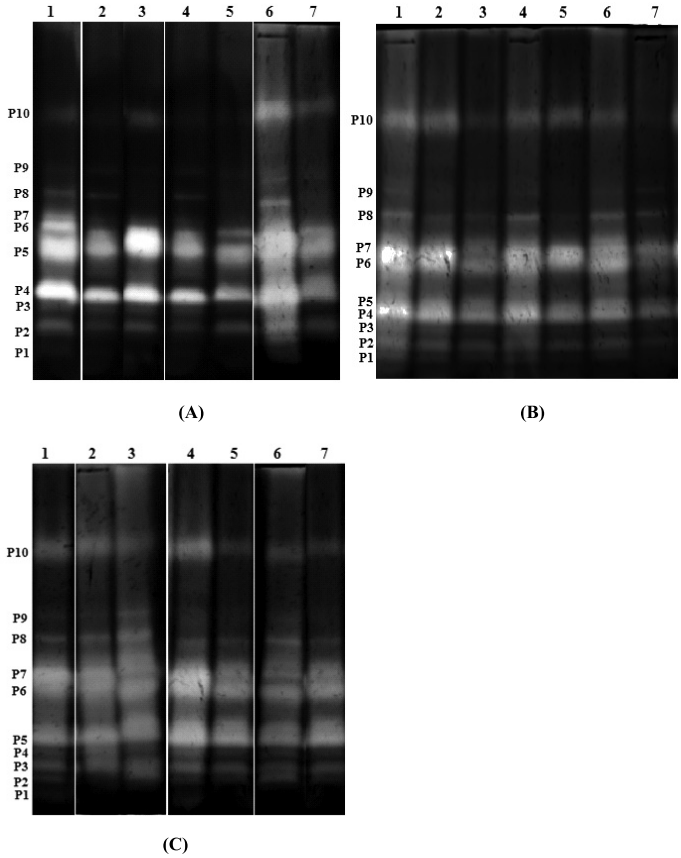

Fig. 3. Proteolytic profile of gut proteases on casein zymography. (A) second instar, (B) third instar and (C) fourth instar. The gut homogenates from respective larvae reared on active Cry1Ac, Cry1Ab and Bt formulation electrophoresed on 10% SDS-PAGE. After electrophoresis, gel treated with 2% casein. Control larvae fed on artificial diet without adding any toxin. Lane1- gut extract from larvae reared on artificial diet, lane 2- Cry1Ab (1 µg), lane 3- Cry1Ab (10 µg), lane 4- Bt formulation (0.02%), lane 5- Bt formulation (0.10%), lane 6- Cry1Ac (1 µg), lane 7- Cry1Ac (10 µg) fed larval gut extract.

Table 3. APN and ALP activities in larval BBMVs

Treatment†	APN activity (U)			ALP activity (U)		
	Second-instar	Third-instar	Fourth-instar	Second-instar	Third-instar	Fourth-instar
Cry1Ac (1µg/g)	2.67de	5.45bcd	7.90d	2.80e	5.64d	5.80bcd
Cry1Ac (10µg/g)	3.13g	6.88g	8.79g	2.20a	5.26a	6.18e
Cry1Ab (1µg/g)	2.50b	5.33b	7.63b	2.90f	5.80f	5.62b
Cry1Ab (10µg/g)	2.91c	6.48e	8.61e	2.35c	5.41c	5.99cde
Bt formulation (0.02%)	2.63d	5.39bc	7.77c	2.62d	5.71e	5.75bc
Bt formulation (0.10%)	3.04f	6.72f	8.69f	2.29b	5.33b	6.04cde
Control	2.07a	4.71a	7.26a	3.23g	6.55g	5.09a
Vr	537.38**	350.72**	1489.84**	1280.59**	2376.96**	1196.81**
Mean	2.71	5.85	8.10	2.63	5.672	5.79
SE	0.02	0.04	0.02	0.01	0.009	0.01
LSD	0.05	0.14	0.05	0.03	0.03	0.32
CV%	1.00	1.3	0.30	0.70	0.30	0.30

† Larvae reared on artificial diet containing respective toxin per g of diet. Control larvae fed on artificial diet without adding any toxin; Data represented as mean (n = 3); Values with different letter in the columns significantly different; ** Significantly different from control at p d" 0.01.

Table 4. Detection of BBMV-bound Cry1Ac/Cry1Ab by ELISA

Instar	Cry1Ac (ppm)	Cry1Ab (ppm)
Second	0.2 ± 0.02	0.1 ± 0.03
Third	0.5 ± 0.02	0.4 ± 0.01
Fourth	1.2 ± 0.03	1.0 ± 0.02

BBMVs prepared from the larvae reared on artificial diet. BBMVs (30 µg) incubated with 5 µg Cry1Ac and Cry1Ab for 1 hr; Samples centrifuged and the bound toxin detected in pellet; Data represents mean ± SE.

used as insecticides (DeMaagd et al., 2003; Pigott and Ellar, 2007) against Lepidoptera. These proteins have been recognized as pore-forming toxins and their primary action is to lyse midgut epithelial cells in target insects by forming pores in the apical microvilli membrane. The ä-endotoxins undergo proteolysis in the alkaline environment of insect midgut and produce an active toxin, which binds to specific receptors in the insect midgut epithelium leading to the pore formation and eventual death of the target insects Btbiopesticides are usually applied on early instar larvae because the older larvae are more tolerant (Sanahuja et al., 2011). Several studies have compared the susceptibility of neonate and third instar larvae of H. armigera to Bt products or Cry proteins (Bird and Akhurst, 2007; Cooper, 1984; Liao et al., 2002), but a consistent pattern in susceptibility is not apparent. .In present study, we focused on the susceptibility of the neonates, first-, second-, third- and fourth-instar larvae to the active Cry1Ac, Cry1Ab and Bt formulation.

The susceptibility of lepidopteran larvae to ä-endotoxins decreases with increase in larval instars (Griko *et al*., 2008). In our study, neonate larvae exhibited consistently lower LC_{50} values (Table 1) than first, second, third and fourth instar larvae. Mortality rate was more in neonates as compared to first, second and third instar larvae. Increase in Cry1Ac, Cry1Ab and Bt formulation increased mortality and weight loss in larvae. As *H. armigera* larvae develop, in presence of Bt toxins the mortality rate was decreased and LC_{50} values were increased. Cry1Ac, Cry1Ab and Bt formulations were positively and significantly correlated with mortality (correlation coefficient, r = 0.86 to 1.00) and weight loss (r = 0.99 to 1.00) in *H. armigera* larvae. This decreased susceptibility for first, second and third instar larvae towards Bt toxins was in agreement with earlier studies which showed that later larval instars of *H. zea* (L.) and *Plutella xylostella* (L.) were less susceptible to Cry proteins than early instar larvae (Ali and Young, 1996; Asano et al., 1993). In our study, the Cry1Ac exhibited more toxicity towards *H. armigera* than Cry1Ab and Bt formulation. Cry1Ac was slightly more toxic to *Manduca sexta* (L.) than Cry1Aa and Cry1Ab (Coux et al., 2001; Van Rie et al., 1989). Second-instar *H. armigera* and *Spodoptera litura* larvae reared on five different Bt cotton genotypes showed highest mortality than third instar larvae. In the presence of Bt toxins, larvae lost their weight, however, there was weight gain in control larvae. As the larval instars increased, the weight loss % decreased in the presence of Bt toxins. Cry1Ac alone reduced *H. zea* body weight by 62% after feeding for 5 days (Zhu et al., 2007).

In our study, proteases activities were increased in control larvae across larval instars i.e., from second to fourth-instar (r = 0.94 to 1.00) (Table 2). Protease activities increased from third to the fifth and then decreased (Kipgena and Aggarwala, 2014). Cry1Ac, Cry1Ab and Bt formulation reduced the total, trypsin and chymotrypsin activities in second, third, and fourth instar larvae. Cry1Ac, Cry1Ab and Bt formulations were negatively correlated with total protease, trypsin and chymotrypsin (r = -0.87 to -1.00) (Table 2). Maximum protease inhibition was exerted by Cry1Ac as compared to that of Cry1Ab and Btformulation. The protease activities were lower in Bt var. *israelensis* ä-endotoxins treated mosquito larvae than those of the control group (Qiu and Lei, 1986; Wu, 1986). Cry1Ac toxin reduced the protease activity in *H. zea* (Zhu et al., 2007) and in *H. armigera* (Visweshwar et al., 2015). Ten protease isozymes were observed in second, third and fourth instar larvae reared on artificial diet (control). Differential expression inhibition of proteases was observed when the different instars were fed on activated Bt toxins and Bt formulation (Fig. 3). In *H. armigera*, ten protease isozymes were detected on casein zymogram (Vinod et al., 2010; Visweshwar et al., 2015) and Cry toxins inhibited protease isozymes (Visweshwar et al., 2015). The larvae often undergo a decrease in sensitivity to Bt toxins as they age (Bai et al., 1993; Sneh et al., 1981) and this may be related to change in protease activity.

APN activities (r = 1.00) in controls increased with the larval development from II to IV instar and ALP activity (r = 0.56) increased from second to third instar and then decreased in fourth instar larvae (Table 3). APN activity was found to be significantly low in neonates than in third instar larvae (Gilliland et al., 2002) and ALP activity was expressed in early instars than

later (Upadhyay and Singh, 2011). In Cry1Ac, Cry1Ab and Bt formulation fed larvae, APN activity increased from second to fourth instar, and ALP activity decreased in second to third instar and increased in fourth instar. Cry1Ac, Cry1Ab and Bt formulations were significantly correlated with APN (r = 0.87 to 0.99) and ALP (r = -0.79 to 0.46) (Table 3). ALP and APN had been identified as a Bt toxin receptor in the midgut epithelium of insect pests (Gill et al., 1995; Denolf et al., 1997). The APN activity was increased in the presence of ä-endotoxin in *H. armigera* (Ingle et al., 2001; Visweshwar et al., 2015) and in gypsy moth, *Lymantria dispar* (L.) larvae (Valaitis, 2008). Binding of Cry1Ac toxin to ALP causes inhibition in phosphatase activity in several insect pests (Sangadala et al., 1994; English and Readdy, 1989; Sarkar et al., 2009). Higher expression of ALP during early larval instars in *M. sexta* is correlated to its susceptibility for Cry1Ab (Arenas et al., 2010). The BBMV-bound Cry1Ac and Cry1Ab concentration increased with the larval development from II to IV instar (Table 4). Binding of Cry1Ac with BBMV increased with the larval development (Upadhyay and Singh, 2011). It was shown that ALP could have a predominant role in Cry proteins toxicity since it was preferentially expressed in young instar larvae that are more sensitive to the toxin in contrast to APN that was expressed in later larval instars.

The activated Bt toxins (Cry1Ac and Cry1Ab) and Bt formulation resulted in reduced growth and development in all larval instars of *H. armigera* and this reduction was higher in early larval instars, indicate that Bt pathogenicity was maximum in early larval instars. Application of Bt on the early larval instars than older will gives better results in controlling the growth and development of *H. armigera*.

ACKNOWLEDGEMENTS

The authors thank the staff, Insect Rearing Laboratory, Entomology, for providing the insects throughout the study. This work was supported by UGC under UGC – SAP (DSR - I) and UGC-MRP scheme.

REFERENCES

Ali, A. and Young, S.Y. 1996. Activity of Bacillus thuringiensis Berliner against different ages and stages of *Helicoverpa zea* (Lepidoptera: Noctuidae) on cotton. *Journal of Entomological Science*, 31: 1-8.

Anand, H., Udikeri, S.S., Patil, S. B., Khadi, B. M., Biradar, D. P., Basavana Goud, K. and Bhat, A.R.S. 2011. Characterization of resistance of different cry toxins to early and late instar *Helicoverpa armigera* (Hub.) and *Spodoptera litura* (Fab.). *Karnataka Journal of Agricultural Science*, 24: 300-302.

Arenas, I., Bravo, A., Soberon, M. and Gomez, I. 2010. Role of alkaline phosphatase from *Manduca sexta* in the mechanism of action of *Bacillus thuringiensis* Cry1Ab toxin. *Journal of Biological Chemistry*, 285: 12497-12503.

Asano, S., Maruyama, T., Iwasa, T., Seki, A., Takahashi, M. and Soares Jnr. G.G. 1993. Evaluation of biological activity of *Bacillus thuringiensis* test samples using a diet incorporation method with diamond back moth, *Plutella xylostella* (Linnaeus) (Lepidoptera: Yponomeutidae). *Applied Entomology and Zoology*, 2: 513-524.

Bai, C., Degheele, D., Jansens, S. and Lambert, B. 1993. Activity of insecticidal crystal proteins and strains of *Bacillus thuringiensis* against *Spodaptera exempta* (Walker). *Journal of Invertebrate Pathology*, 62: 211-215.

Bird, L.J. and Akhurst, R.J. 2007. Variation in susceptibility of *Helicoverpa armigera* (Hübner) and *Helicoverpa punctigera* (Wallengren) (Lepidoptera: Noctuidae) in Australia to two *Bacillus thuringiensis* toxins. *Journal of Invertebrate Pathology*, 94: 84-94.

ChittiBabu, G., Sharma, H.C., Madhumati, T., Raghavaiah, G., Murthy, K.V.M.K. and Rao, V.S. 2014. A semi-synthetic chickpea flour based diet for long-term maintenance of laboratory culture of *Helicoverpa armigera*. *Indian Journal of Entomology*, 76: 336-340.

Cooper, D. J. 1984. The application of a model to achieve predicted mortality in a field trial using *Bacillus thuringiensis* to control *Heliothis punctigera*. *Entomologia Experimentalis et Applicata*, 36: 253-259.

Coux, F., Vachon, V., Rang, C., Moozar, K., Masson, L., Royer, M., Bes, M., Rivest, S., Brousseau, R., Schwartz, J.L., Laprade, R. and Frutos, R. 2001. Role of inter domain salt bridges in the pore-forming ability of the *Bacillus thuringiensis* toxins Cry1Aa and Cry1Ac. *Journal of Biological Chemistry*, 276: 35546-35551.

DeMaagd, R.A., Bravo, A., Berry, C., Crickmore, N. and Schnepf, H.E. 2003. Structure, diversity, and evolution of protein toxins from spore-forming entomopathogenic bacteria. *Annual Review of Genetics*, 37: 409-433.

Denolf, P., Hendrickx, K., Van Damme, J., Jansens, J., Pereroen, M., Degheele, D. and Van Rie, J. 1997. Cloning and characterization of *Manduca sexta* and *Plutella xylostella* midgut aminopeptidase N enzymes related to *Bacillus thuringiensis* toxin-binding proteins. *European Journal of Biochemistry*, 248: 748-761.

English, L. and Readdy, T.L. 1989. Delta endotoxin inhibits a phosphatase in midgut epithelial membranes of *Heliothis virescens*. *Insect Biochemistry*, 19: 145–152.

Fitt, G.P. 1989. The ecology of *Heliothis* species in relation to agroecosystem. *Annual Review of Entomology*, 34: 17-52.

Garcia-Carreno, F.L., Dimes, L.E. and Haard, N.F. 1993. Substrate-gel electrophoresis for composition and molecular weight of proteinases or proteinaceous proteinase inhibitors. *Analytical Biochemistry*, 214: 65-69.

Gill, S.S., Cowles, E.A. and Pietrantonio, P.V. 1992. The mode of action of Bt. endotoxins. *Annual Review of Entomology*, 37: 615-636.

Gill, S., Cowles, E.A. and Francis, V. 1995. Identification, isolation, and cloning of a *Bacillus thuringiensis* Cry1Ac toxin-binding

protein from the midgut of the lepidopteran insect *Heliothis virescens*. *Journal of Biological Chemistry*, 270: 27277-27282.

Gilliland, G., Chambers, C.E., Bone, E.J. and Ellar D.J. 2002. Role of *Bacillus thuringiensis* Cry1 endotoxin binding in determining potency during lepidopteran larval development. *Applied and Environmental Microbiology*, 68: 1509-1515.

Griko, N., Zhang, X., Ibrahim, M., Midboe, E.G. and Bulla, L.A.Jr. 2008. Susceptibility of *Manduca sexta* to Cry1Ab toxin of *Bacillus thuringiensis* correlates directly to developmental expression of the cadherin receptor BT-R1. Comparative Biochemistry and Physiology, *Biochemistry and Molecular Biology*, 151: 59-63.

Herman, R.A., Evans, S.L., Shanahan, D.M., Mihaliak, C.A., Bormett, G.A., Young, D.L. and Buehrer, J. 2001. Rapid degradation of Cry1F ä-endotoxin in soil. *Environmental Entomology*, 30, 642-644.

Herman, R.A., Scherer, P.N. and Wolt, J.D. 2002a. Rapid degradation of a binary, PS149B1, ä-endotoxin of *Bacillus thuringiensis* in soil, and a novel mathematical model for fitting curve-linear decay. *Environmental Entomology*, 31: 208-214.

Herman, R.A., Wolt, J.D. and Halliday, W.R. 2001b. Rapid degradation of the Cry1F insecticidal crystal protein in soil. *Journal of Agricultural and Food Chemistry*, 50: 7076-7078.

Hofte, H. and Whiteley, H.R. 1989. Insecticidal crystal proteins of *Bacillus thuringiensis*. *Microbiology Reviews*, 53: 242-255.

Ingle, S.S., Trivedi, N., Prasad, R., Kuruvilla, J., Rao, K.K. and Chhatpar, H.S. 2001. Aminopeptidase-N from the *Helicoverpa armigera* (Hubner) brush border membrane vesicles as a receptor of *Bacillus thuringiensis* CrylAc delta-endotoxin. *Current Microbiology*, *43*: 255-259.

Jurat-Fuentes, J.L. and Adang, M.J. 2004. Characterization of a Cry1Ac-receptor alkaline phosphatase in susceptible and resistant *Heliothis virescens* larvae. *European Journal of Biochemistry*, 271: 3127-3135.

Kipgena, L. and Aggarwala, K.K. 2014. Gut protease profile of different instars of *Helicoverpa armigera* (Lepidoptera: Noctuidae). *International Journal of Tropical Insect Science*, 34: 172-178.

Knight, P.J.K., Crickmore, N. and Ellar, D.J. 1994. The receptor for *Bacillus thuringiensis* CrylA(c) delta-endotoxin in the brush border membrane of the lepidopteran *Manduca sexta* is aminopeptidase N. *Molecular Microbiology*, 11: 429-436.

Kranthi, K.R., Jadhav, D.R., Wanjari, R.R., Ali, S.S. and Russel, D. 2001a. Carbamate and organophosphate resistance in cotton pests in India, 1995 to 1999. *Bulletin of Entomological Research*, 91: 37-46.

Kranthi, K.R., Jadhav, D.R., Wanjari, R.R., Ali, S.S. and Russel, D. 2001b. Pyrethroid resistance and mechanisms of resistance in field strains of *Helicoverpa armigera* (Lepidoptera: Noctuidae). *Journal of Economic Entomology*, 94: 253-263.

Laemmli, U.K. 1970.Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature*, 227: 680-685.

Lereclus, D., Bourgouin, C., Lecadet, M.M., Klier, A. and Rapoport, G. 1989. Regulation of prokaryotic development. (eds.): I. Smith, R. Stepecky and P. Setlow. American Society Microbiology, Washington D. C. 255-276 pp.

Liao, C.Y., Heckel, D.G. and Akhurst, R. 2002. Toxicity of *Bacillus thuringiensis* insecticidal proteins for *Helicoverpa armigera* and *Helicoverpa punctigera* (Lepidoptera: Noctuidae), major pests of cotton. *Journal of Invertebrate Pathology*, 8: 55-63.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. 1951. Protein measurement with the folin phenol reagent. *Journal of Biological Chemistry*, 193: 265-75.

Maa, X., Liu, X., Ning, X., Zhang, B., Han, F., Guan, X., Tan, Y. and Zhang, Q. 2008. Effects of *Bacillus thuringiensis* toxin Cry1Ac and *Beauveria bassiana* on Asiatic corn borer (Lepidoptera: Crambidae), *Journal of Invertebrate Pathology*, 99: 123-128.

McCaffery, R.A. 1998. Resistance to insecticides in Heliothine Lepidoptera: a global view. *Philosophical Transactions of the Royal Society*, London B, 353: 1735-1750.

Paramasiva, I., Krishnayya, P.V., War, A.R. and Sharma, H.C. 2014. Crop hosts and genotypic resistance influence the biological activity of *Bacillus thuringiensis* towards Helicoverpa armigera. *Crop Protection*, 64: 38-46.

Peyronnet, O., Vachon, V., Brousseau, R., Baines, D., Schwartz, J.L. and Laprade, R. 1997. Effect of *Bacillus thuringiensis* toxins on the membrane potential of lepidopteran insect midgut cells. *Applied and Environmental Microbiology*, 63: 1679-1684.

Pigott, C.R. and Ellar, D.J. 2007. Role of receptors in *Bacillus thuringiensis* crystal toxin activity. *Microbiology and Molecular Biology Reviews*, 71: 255-281.

Purushottam, R.L. and Vandana, K.H. 2013.Effect of *Bacillus thuringiensis* (Bt) Cry1Ac toxin and protease inhibitor on growth and development of *Helicoverpa armigera* (Hubner). *Pesticide Biochemistry and Physiology*, 105: 77-83.

Qiu,X.J. and Lei, B.Q. 1986. The crystals of *Bacillus thuringiensis* var. *israelensis* and their effect on the midgut trypsin and chymotrypsin activities of mosquito larvae. *Acta Entomologica Sinica*, 29: 126-130.

Sanahuja, G., Banakar, R., Twyman, R.M., Capell, T. and Christou, P. 2011. *Bacillus thuringiensis*: a century of research, development and commercial applications. *Plant Biotechnology Journal*, 9: 283-300.

Sangadala, S., Walters, F.S., English, L.H. and Adang, M.J. 1994. A mixture of *Manduca sexta* aminopeptidase and phosphatase enhances *Bacillus thuringiensis* insecticidal CryIA(c) toxin binding and (Rb^{+}-K^{+})-Rb^{86} efflux in vitro. *Journal of Biological Chemistry*, 269: 10088-10092.

Sarkar, A., Hess, D., Mondal, H.A., Banerjee, S., Sharma, H.C. and Das, S. 2009. Homodimeric alkaline phosphatase located at *Helicoverpa armigera* midgut, a putative receptor of Cry1ac contains a-GalNAc in terminal glycan structure as interactive epitope. *Journal of Proteome Research*, 8: 1838-1848.

Shao, Z., Cui, Y., Liu, X., Yi, H., Ji, J. and Yu, Z. 1998. Processing of ä-Endotoxin of *Bacillus thuringiensis* subsp. *kurstaki* HD-1 in *Heliothis armigera* midgut juice and the effects of protease inhibitors. *Journal of Invertebrate Pathology*, 72: 73-81.

Sharma, H.C. 2005.*Heliothis/ Helicoverpa* management. Emerging trends and strategies for future research. Oxford and IBH Publishing Co, New Delhi.

Sharma, H.C. 2009. Biotechnological approaches for pest management and ecological sustainability. CRC Press/ Taylor and Francis, New York, USA. 526pp.

Sims, S.R. and Holden, L.R. 1996. Insect bioassay for determining soil degradation of *Bacillus thuringiensis* subsp. *kurstaki* CryIA(b) protein in corn tissue. *Environmental Entomology*, 25: 659-664.

Sneh, B., Schuster, S. and Broza, M. 1981. Insecticidal activity of *Bacillus thuringiensis* strains against the Egyptian cotton leaf worm *Spodoptera littoralis* [Lep.:Nocutidae]. *Biocontrol*, 26: 179-190.

Surekha Devi, V., Sharma, H.C., and Arjuna Rao, P. 2011. Interaction between host plant resistance and biological activity of *Bacillus thuringiensis* in managing the pod borer *Helicoverpa armigera* in chickpea. *Crop Protection*, 30: 962-969.

Tabashnik, B.E., Finson, N., Groeters, F.R., Moar, W.J., Johnson, M.W., Luo, K. and Adang, M.J. 1994. Reversal of resistance to *Bacillus thuringiensis* in *Plutella xylostella*. *Proceedings of National Academy of Science,* United States of America, 91: 4120-4124.

Tapp, H. and Stotzky, G. 1998. Persistence of the insecticidal toxin from *Bacillus thuringiensis* subsp. *kurstaki* from soil. *Soil Biology and Biochemistry*, 30:471-476.

Upadhyay, S.K. and Singh, P.K. 2011. Role of alkaline phosphatase in insecticidal action of Cry1Ac against *Helicoverpa armigera* larvae. *Biotechnology Letters*, 33: 2027-2036.

Valaitis, A.P. 2008. *Bacillus thuringiensis* pore-forming toxins trigger massive shedding of GPI-anchored aminopeptidase N from gypsy moth midgut epithelial cells. *Insect Biochemistry and Molecular Biology*, 38: 611-618.

Van Rie, J., Jansens, S., Hofte, H., Degheele, D. and Mellaert, V. 1989. Specificity of *Bacillus thuringiensis* ä-endotoxins. Importance of specific receptors on the brush border membrane of the mid-gut of target insects. *European Journal of Biochemistry*, 186: 239-247.

Vinod, D.P., Sharma, H.C. and Kochole, M.S. 2010. In vivo inhibition of *Helicoverpa armigera* gut pro-proteinase activation by non-host plant protease inhibitors. *Journal of Insect Physiology*, 56: 1315-1324.

Visweshwar, R., Sharma, H.C., Akbar, S.M.D. and Sreeramulu, K. 2015. Elimination of gut microbes with antibiotics confers resistance to *Bacillus thuringiensis* toxin proteins in *Helicoverpa armigera* (Hubner). *Applied Biochemistry and Biotechnology*, 177: 1621-1637.

Wolfersberger, M.G. 1993. Preparation and partial characterization of amino acid-transporting brush-border membrane vesicles from the larval midgut of the gypsy moth (*Lymantria dispar*). *Archives of Insect Biochemistry and Physiology*, 24: 139-147.

Wu, Q.Y. 1986. A pathological study on mosquito larvae treated with *Bacillus thuringiensis* var. *israelensis*. *Acta Entomologica Sinica*, 29: 35-39.

Zhu, Y.C., Abel, C.A. and Chen, M.S. 2007. Interaction of Cry1Ac toxin (*Bacillus thuringiensis*) and proteinase inhibitors on the growth, development, and midgut proteinase activities of the bollworm, *Helicoverpa zea*. *Pesticide Biochemistry and Physiology*, 87: 39-46.

(Manuscript Received: July, 2016; Revised: December, 2017;
Accepted: January, 2018; Online Published; April, 2018)