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Development and mechanisms of resistance to *Bacillus thuringiensis* endotoxin Cry1Ac in the American bollworm, *Helicoverpa armigera* (Hübner)

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The American bollworm, *H. armigera*, evolved 31-fold resistance to selection pressure of *B. thuringiensis* endotoxin Cry1Ac within six generations. The Cry1Ac selected larvae of *H. armi*gera showed cross-resistance to Cry1Aa and Cry1Ab both in terms of mortality and growth reduction. Studies on mechanisms of resistance to Cry1Ac showed that proteases of resistant insects degraded Cry1Ac faster than those of susceptible insects, which led to the relative unavailability of toxin of about 58 kDa for binding and perforation of midgut epithelial membrane of the target insect. Besides, resistant and susceptible populations of *H. armigera* differed in the binding of their receptors with Cry1Ac toxin. These results suggest the possibility of both mechanisms existing in imparting resistance. These findings mandate the necessity of *B. thuringiensis* resistance management for usage of *B. thuringiensis* either as a conventional insecticide or through transgenic crops.

Keywords: American bollworm, *Bacillus thuringiensis*, Cry1Ac, *Helicoverpa armigera*, Resistance

The ever-increasing area of transgenic *Bacillus thuringiensis* (*Bt*) cotton, about 4.6 million hectare in 2002 in USA, China, Australia and other countries, including India, from that of about 1.7 million hectares in 1996 in USA, justifies utility of *Bt* transgenes against bollworm complex, a major constraint in cotton productivity. Transgenic *Bt* cotton has further helped in minimizing use of conventional insecticides and thereby reducing environmental pollution and associated health hazards[1,2]. One of ecological concerns of cultivation of transgenic *Bt* crops, especially evolution of resistance in insects to *Bt* toxins, has mandated implementation of *Bt* resistance management tactics legally in the developed countries like USA[3] and Australia[4]. The *Bt* resistance management essentially consists of high level expression of *Bt* Cry1Ac toxin, maintenance of refugia (non-*Bt* crop) and suitable alternate host crops and recently, the use of *cry1Ac* and *cry2A* genes as in Bollgard II. In China, because of complexity of small farm holdings and mixed cropping, *Bt* resistance management tactic like maintenance of structured refugia is considered unnecessary and impractical to enforce, and hence is non-mandatory for *Bt* cotton cultivation, in spite of lack of high dose of *Bt* toxin in the currently available transgenic varieties[5]. In India, however, maintenance of structured refugia is considered necessary for *Bt* cotton. Further, cotton being a cash crop, farmers may be tempted to discard refugia to favour economics of its cultivation. Since *Bt* resistance management has to be based on the nature of resistance in the test insect, the study of its characteristics in the insect populations is essential to fine-tune management tactics for location specific needs.

American bollworm, *Helicoverpa armigera* (Hübner) is an important polyphagous pest of worldwide occurrence. In India alone, it causes about US $ 1 billion worth of crop damage every year[6]. Being a key pest of cotton, many insecticides are used for its control. As a result of indiscriminate use, many insecticides have failed to achieve a desired level of *H. armigera* control due to its ability to evolve resistance[7].

Cry1Ac toxin in transgenic *Bt* cotton has successfully proved lethal to the larvae of *H. armigera* due to its constitutive availability in various parts, especially terminal leaves, flowers and bolls. Contrarily, target insect populations have also shown an ability to tolerate a wide range of toxicity of *Bt* strains and toxins *per se*[6,8,9] or *in situ* in cotton plants[10-14]. This variation in susceptibility of *H. armigera* may be due to genetic variation in the populations, cross-resistance between xenobiotics, agroecological conditions, and variation in expression of Cry1Ac toxin in *Bt* cotton itself. This ability of *H. armigera* to tolerate higher concentrations of Cry1Ac toxin was

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further confirmed by development of resistance under selection pressure in the laboratory[15,16]. The mechanism of *Bt* resistance in insects mainly centers around altered processing of Cry1Ac protoxin and the binding of Cry1Ac toxin to receptors on brush border membrane vesicles (BBMV) of midgut epithelium in many insects[17]. Extensive investigations on mechanisms of resistance in insects to conventional insecticides have helped in developing diagnostic tools and appropriate resistance management tactics. However, very little information is available on mechanisms of resistance in *H. armigera* to *Bt* toxins. This communication therefore reports studies on evolution of resistance, cross-resistance, and the importance of proteases and toxin-receptor binding in Cry1Ac resistance in *H. armigera*.

Materials and Methods

Chemicals and Bt Cry1A toxins—Azocasein, N-α-*p*-Tosyl-L-lysine chloromethyl ketone (TLCK), N-Tosyl-L-phenyl alanine chloromethyl ketone (TPCK), soybean trypsin inhibitor (SBTI), bovine serum albumin (BSA) (electrophoresis-marker grade) and bovine trypsin were purchased from Sigma Chemical Company, St Louis, MO, USA. Other chemicals and reagents were purchased from Bangalore Genie Pvt. Ltd., Bangalore, and Qualigens Fine Chemicals, Mumbai. *Escherichia coli* JM 103 strains (hyper-expressing recombinant plasmid vectors pKK223-3; BGSC ECE52 *cry1Aa,* ECE53 *cry1Ac* and ECE54 *cry1Ab*) producing Cry1Aa, Cry1Ac and Cry1Ab toxins were kindly provided by *Bacillus* Genetic Stock Center, Ohio State Univ., Columbus, USA.

Toxins of Cry1Aa, Cry1Ab and Cry1Ac were prepared from recombinant *E. coli* strains as per Lee *et al*[18]. Cells were grown in nutrient broth containing 50 µg/ml ampicillin for 72 hr, harvested by centrifugation at 4500 *g* at 4°C and the pellet suspended in lysis buffer (50 m*M* Tris, *p*H 8; 50 m*M* EDTA; 15% sucrose, lysozyme @ 2 mg/ml) and incubated for 4 hr. After incubation, lysis buffer was replaced with crystal wash-1 (0.5 *M* sodium chloride and 2% Triton X-100) and sonicated for 3 min on ice. The pellet was collected by centrifugation at 4500 *g* and washed thrice with crystal wash-1, crystal wash-2 (0.5 *M* sodium chloride) and then, with distilled water. Finally, the pellet was dissolved in solubilizing buffer (50 m*M* sodium carbonate, 10 m*M* dithiothreitol, *p*H 10.5) at 37°C for 6 hr. Supernatant containing toxin was collected following centrifugation at 4500 *g* for 10 min and stored at −20°C till further use. The proteins in the supernatant were separated by SDS-PAGE[19] and toxin band identified as per Mohan and Gujar[20].

Insect rearing and bioassay—Larvae of *H. armigera* originally collected from chickpea on the Institute's farm in Delhi were reared on a chickpea based meridic diet similar to that of Nagarkatti and Prakaash[21] at 27° ± 2°C and 60-80% RH. Adults were fed with 10% honey solution and allowed to lay eggs on markin cloth used for covering jars. The eggs were collected daily by changing cloth and keeping them in humid conditions till they hatched.

Bioassays of Cry toxins were carried out by diet incorporation method as per Gujar *et al*[6]. A series of dilutions of stock solution of Cry toxins (in water) was prepared and added to an aliquot of meridic diet, mixed thoroughly with pestle and mortar, and transferred into small plastic containers. Each container served as a replication. About 6 concentrations, ranging from 0.01 to 100 µg toxin g^{-1} diet, were used for each bioassay, with 3 to 5 replications per concentration. Neonate larvae (10) were released on treated diet per replication. The control consisted of normal meridic diet (without toxin). A minimum of 210 neonate larvae were used for each bioassay. The mortality was then pooled for each concentration. The concentrations showing corrected mortality between 20 and 80% at 96 hr were used for calculation of median lethal concentration (LC_{50}). The experiments with mortality of above 10% in control were discarded and repeated. All bioassays were carried out at 27° ± 2°C and 60-80% RH. Ten 96 hr old surviving larvae from each of treatments and control were weighed individually and growth reduction was estimated for each treatment in comparison to that of control.

Resistance development under selection pressure—Selection of larvae of *H. armigera* originally collected from chickpea fields in Delhi for evolution of resistance was initiated with the release of 400 F_1 neonate larvae on a meridic diet treated with 0.5 µg Cry1Ac g^{-1} for 4 days till about 90% of larvae died. The surviving larvae were then transferred to the normal diet and maintained till pupation. Adults were allowed to lay eggs and the above process of selection was continued with the neonate larvae of next generation until 6 generations. First 3 generation larvae were fed on diet treated with 0.5 µg toxin g^{-1} for 4 days that proved lethal to about 90% larvae. For the fourth generation, the selection pressure was increased to 0.7 µg toxin g^{-1} for 6 days from egg-

hatch. For the fifth and sixth generations, toxin concentration was increased to 1.5 and 3.25 μg toxin g^{-1} diet, respectively for 6 days. Bioassays of Cry1Ac with a full range of concentrations were carried out on neonate larvae of F_1, F_3, F_4 and F_6 generations to estimate LC_{50}. The neonate larvae of the Cry1Ac selected F_6 generation were used for their response to Cry1Aa and Cry1Ab to determine development of cross-resistance.

Protease extraction and characterization—Ten larvae of final instar of *H. armigera* were immobilized on ice for 15 min and then dissected in an ice-cold 20 m*M* Tris-HCl buffer (*p*H 7) for midguts, which were later rinsed with Tris-HCl buffer to remove food particles. The midguts were, then, homogenized in Tris-HCl buffer and centrifuged at 4°C at 13,200 *g* for 15 min. The supernatant was made up to 1 ml with Tris-HCl buffer and stored at −20°C until use. The midgut protease activity was measured by azocasein digestion method[22]. Midgut homogenate (20 µl) was mixed with (130 µl) of Tris-HCl buffer (*p*H 9). To the above mixture, 100 µl of 2% azocasein was added and incubated for 1 hr at 35 °C. The reaction was stopped by adding 500 µl of 5% ice-cold trichloroacetic acid (TCA). The mixture was centrifuged at 13,200 *g* for 15 min at 4 °C. Supernatant (500 µl) was mixed with 500 µl of 1 *N* NaOH and absorbance was estimated at 420 nm. The protease activity of sample was calculated using trypsin standard curve in terms of tryptic unit (TU) as per Kunitz[23]. For studies on inhibition of midgut proteases, PMSF, TPCK and TLCK were dissolved in methanol and SBTI in distilled water. Azocaseinolytic assay was carried out with gut homogenate (5×10^{-3} TU) incubated with different concentrations of each protease inhibitor separately in a total volume of 150 µl of 20 m*M* Tris-HCl (*p*H 9) for 1 hr followed by addition of 100 µl of 2% azocasein and 1 hr incubation[24].

Midgut protease of test insects was subjected to SDS-PAGE analysis on 12% gels under non-denaturing condition and processed for zymogram analysis[25]. Midgut homogenate with a known protease activity (2×10^{-3} TU) was dissolved in non-reducing sample buffer without boiling (2% SDS, 25% glycerol, 60 m*M* Tris-HCl, *p*H 6.8, 0.1% bromophenol blue), and electrophoresis carried out at 4°C. Thereafter, the gel was washed for 10 min in 2.5% Triton X-100 to remove SDS and cut into strips. One gel strip was incubated with 100 m*M* Glycine-NaOH buffer (*p*H 10) containing 2% casein for 1 hr. The second gel strip was incubated with Cry1Ac toxin (5 mg/ml) to identify the midgut protease responsible for its degradation. The gel strips were also stained with 0.5% Coomassie brilliant blue R250 and destained briefly with methanol-acetic acid-water (40:10:50) followed by treatment in 5% acetic acid overnight. Protease activity was revealed as zone of white clearing in a dark blue background.

Cry1Ab and Cry1Ac protoxins were incubated with protease preparations of resistant and susceptible populations of *H. armigera*. The samples (10 µl) were drawn at different time intervals, immediately boiled for a min, then SDS sample buffer added and boiled for 2 more min. The proteins of the samples processed at different time intervals were separated on SDS-PAGE.

Cry1Ac protoxin was converted into toxin by treating with trypsin. The complete conversion of protoxin to toxin was verified by loading the sample on SDS-PAGE and visualizing a single toxin band of molecular weight of 58 kDa. The midgut protease (1 TU) preparations from resistant and susceptible population were incubated with different concentrations of Cry1Ac toxin (4 to 30 µg) for 1 hr. After 1 hr incubation, samples were boiled for 1 min and then mixed with SDS sample buffer and boiled for 2 more min, and then separated on 10% SDS-PAGE. The protein content of toxin band was estimated by eluting Coomassie brilliant blue R250 dye, and comparing with a standard curve of BSA prepared by dye elution as above. The amount of toxin was then subtracted from the substrate to obtain the amount of toxin degraded.

BBMV extraction and characterization—BBMV from midguts of final instar larvae of *H. armigera* were prepared as per Wolfersberber *et al*[26]. Alkaline phosphatase[27] and leucine aminopeptidase[28] of BBMV preparations were estimated. The specific activity of alkaline phosphatase was 184.1 and 241.3 µmoles/min/mg protein, and that of leucine aminopeptidase was 0.48 and 0.52 µmoles/min/mg protein, respectively for resistant and susceptible populations. BBMV (10 µg) was boiled with SDS sample buffer at 95°C for 5 min and separated on 8% PAGE at a constant voltage of 70 mA (Mini-dual model Electrophoresis system of Bangalore Genei). The BBMV, thus separated, was transferred and immobilized onto nitrocellulose membrane of 0.45 mm pore size using electrotransfer system at 25 V for 1 hr as per standard protocol (Genei Blot Electro-Transfer System of Bangalore Genei). The membrane was

removed and floated in Tris buffered saline (TBST) (10 m*M* Tris-HCl, 150 m*M* NaCl, *p*H 8 and 0.05% Tween-20) followed by brief rinsing. The membrane was incubated with blocking buffer (TBST +1% BSA) for 2 hr, followed by incubation in Cry1Ab toxin in TBST buffer (20 μg/ml) for 2 hr, which were later challenged with toxin specific polyclonal antibodies in TBST buffer (1: 20,000 dilution) for overnight at 27°C. The unbound antibodies were removed by washing the membrane thrice with TBST buffer, 5-10 min each time, and then incubating in Goat's anti-IgG-alkaline phosphate conjugate at 1:50,000 dilution for 1 hr. The membrane was washed with TBST buffer thrice as above and then incubated with BCIP/NBT for 10 min and then washed with distilled water. The BBMV binding with Cry toxin was detected as a purple band and its molecular weight estimated as per Southern[29].

Cry1Ac toxin (50 μg) was labelled with I^{125} using I^{125} labelled sodium iodide (Na I^{125}) (in dilute NaOH) by following chloramine-T method[30]. The specific activity of labelled toxin was determined as per Karim *et al*[31]. Labelled toxin was mixed with 100 μl each of 2% BSA and 20% TCA and the mixture incubated on ice for 10 min, and then, centrifuged at 13,500 *g* for 10 min. The radioactivity of the pellet was counted in a gamma counter (I^{125} counter, ECIL, Hyderabad). The protein content of pellet was determined as per Bradford[32].

Saturation binding assays were performed using a fixed amount of labelled toxin for varying amounts of BBMV. Labelled Cry1Ac (1 μg) was incubated with an increasing concentration of BBMV (2.5 to 70 μg) in 100 μl of phosphate buffered saline (PBS)/binding buffer (58 m*M* $Na_2H_2PO_4$, 17 m*M* NaH_2PO_4, 6.8 m*M* NaCl and 0.1% BSA). After 1 hr of incubation at 27°C, bound toxin was separated from unbound toxin by centrifugation at 13,500 *g* for 10 min. The pellet was washed thrice with binding buffer to remove unbound toxin and the remaining radioactivity associated with pellet was then counted in a gamma counter.

Statistical analysis — The mortality and growth reduction data were analyzed by using maximum likelihood programme[33] for estimation of their respective LC_{50} and EC_{50} in terms of μg toxin g^{-1} diet for 96 hr period. The difference between two LC_{50} or EC_{50} was considered significant only if their 95% fiducial limits did not overlap[34].

Results

Development of resistance and cross-resistance — Larvae of *H. armigera* population were susceptible to Cry1Ac with LC_{50} of 0.21 μg toxin g^{-1} before selection. LC_{50} of Cry1Ac increased 4.2- and then 31.4-fold in the F_3 and F_6 generations, respectively (Table 1). Concurrently, LC_{50} of Cry1Aa and Cry1Ab, after 6 generations of selection with Cry1Ac, were 21.94 and 17.56 μg toxin g^{-1} diet, respectively which were 8.4-and 25.4-fold higher than their respective LC_{50} of 2.6 and 0.69 μg toxin g^{-1} diet before selection. Similarly, growth reduction was also affected with development of resistance. The EC_{50} for growth reduction increased from 0.21 to 4.29 μg toxin g^{-1} diet for Cry1Ab (20.5-fold) and from 0.359 to 4.56 μg toxin g^{-1} diet for Cry1Ac (12.8-fold). Cry1Ac selected insects of the F_6 generation did not show any growth reduction to Cry1Aa (Table 2).

Cry1Ac was about 12- and 3-fold more toxic to the neonates of unselected *H. armigera* than Cry1Aa and Cry1Ab, respectively. On selection, Cry1Ac was about 3-fold more toxic than Cry1Aa and Cry1Ab.

Characterization of midgut proteases — Six midgut proteases, 2 major bands with molecular weights of approximately 71.6 and 31.6 kDa and 4 minor bands of 44.6, 40.5, 35.7 and 29.7 kDa were observed in

Table 1 — Resistance development in *H. armigera* to selection pressure of Cry1Ac toxin

Generation	No. larvae treated	Cry1Ac (μg g^{-1} diet)	No pupated	LC_{50} (μg g^{-1} diet)	Slope± S.E	Fold resistance
1	400	0.5	25	0.21 (0.051–0 .354)	1.08 ± 0.37	—
2	500	0.5	40	—		—
3	500	0.5	50	1.04 (*)		4.2
4	635	0.7	60	2.60 (*)	1.04 ± 0.53	12.4
5	560	1.5	50	—		—
6	700	3.23	56	6.59 (3.87 – 28.78)	1.0 ± 0.29	31.4

*Not possible to calculate

both susceptible and resistant populations of *H. armigera*, while only one protease band was visible with Cry1Ac protoxin incubation (Fig. 1).

The general protease inhibitor, PMSF and trypsin specific inhibitors, TLCK and SBTI inhibited protease activity significantly. No inhibition of protease activity

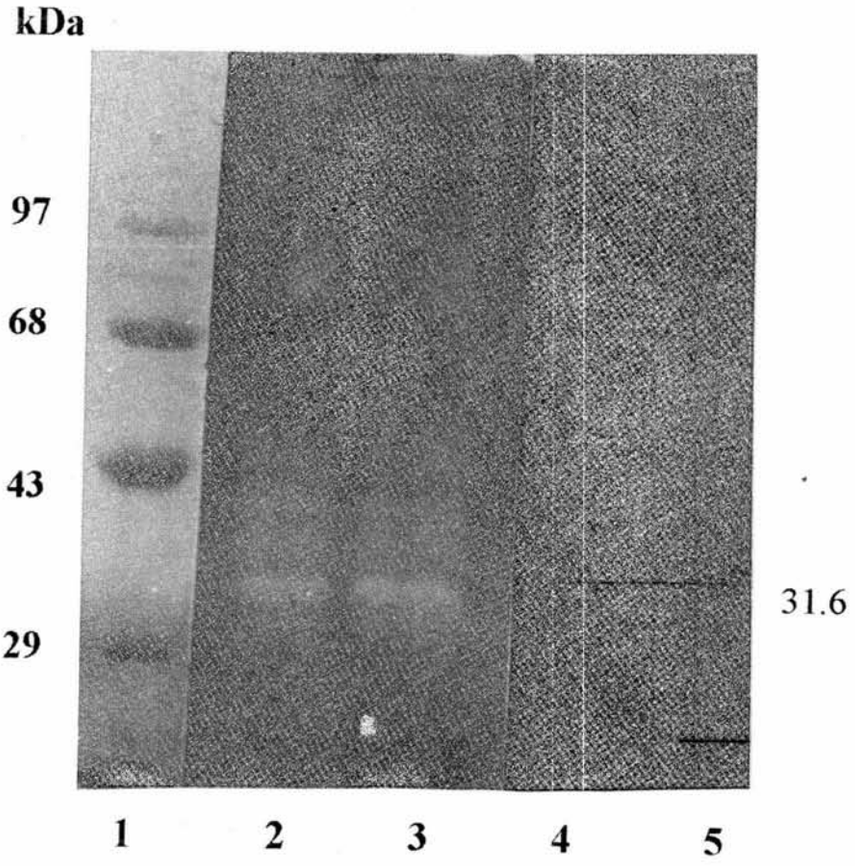

Fig. 1 — Zymogram of midgut proteases of *H. armigera* on 12 % non-denaturing SDS-PAGE. [Lane 1: Protein molecular weight marker; Lane 2 and 3, midgut proteases of resistant and susceptible insects, respectively with casein as a substrate; Lane 4 and 5, midgut proteases of resistant and susceptible insects, respectively with Cry1Ac as a substrate]

Table 2 — Response of the Cry1Ac selected strain of *H. armigera* to *B. thuringiensis* toxins

Toxin	Period of selection	LC_{50}/EC_{50} ($\mu g\ g^{-1}$ diet)	Slope ± SE.	Fiducial limits	Fold resistance
			Larval mortality (LC_{50})		
Cry1Aa	Before selection	2.59	1.29 ± 0.46	1.35-45.87	
	After selection	21.94	0.39 ± 0.30	8.2-1955.0	8.4
Cry1Ab	Before selection	0.69	1.63 ± 0.53	0.48-1.35	
	After selection	17.56	0.39 ± 0.30	*	25.4
Cry1Ac	Before selection	0.21	1.08 ± 0.37	0.05-0.35	
	After selection	6.59	1.00 ± 0.29	3.87-28.78	31.4
			Growth reduction (EC_{50})		
Cry1Aa	Before selection	0.67	1.68 ± 0.24	0.54-0.92	
	After selection		No growth reduction was observed		**
Cry1Ab	Before selection	0.21	1.35 ± 0.20	0.15-0.26	
	After selection	4.29	0.78 ± 0.19	2.70-12.90	20.5
Cry1Ac	Before selection	0.36	1.76± 0.18	0.30-0.41	
	After selection	4.56	1.33 ± 0.20	3.47-7.10	12.8

**Not possible to calculate

was observed with chymotrypsin specific inhibitor, TPCK even at a maximum concentration of 500 μ*M* (Table 3).

Activation of protoxins of Cry1Ab and Cry1Ac — Incubation of Cry1Ab protoxin with midgut proteases of susceptible population and also with trypsin for 2 and 4 hr led to the production of toxin. However, similar incubation even for only 2 hr with midgut proteases from resistant population did not produce toxin suggesting the possibility of its rapid degradation (Fig. 2a). Similarly, the toxin of Cry1Ac protoxin was formed on the susceptible population and not in the resistant one on incubation for 2 and 4 hr with their respective midgut proteases (Fig. 2b).

The degradation of toxin was higher in treatment of toxin with protease from resistant insects than that from susceptible ones (Table 4).

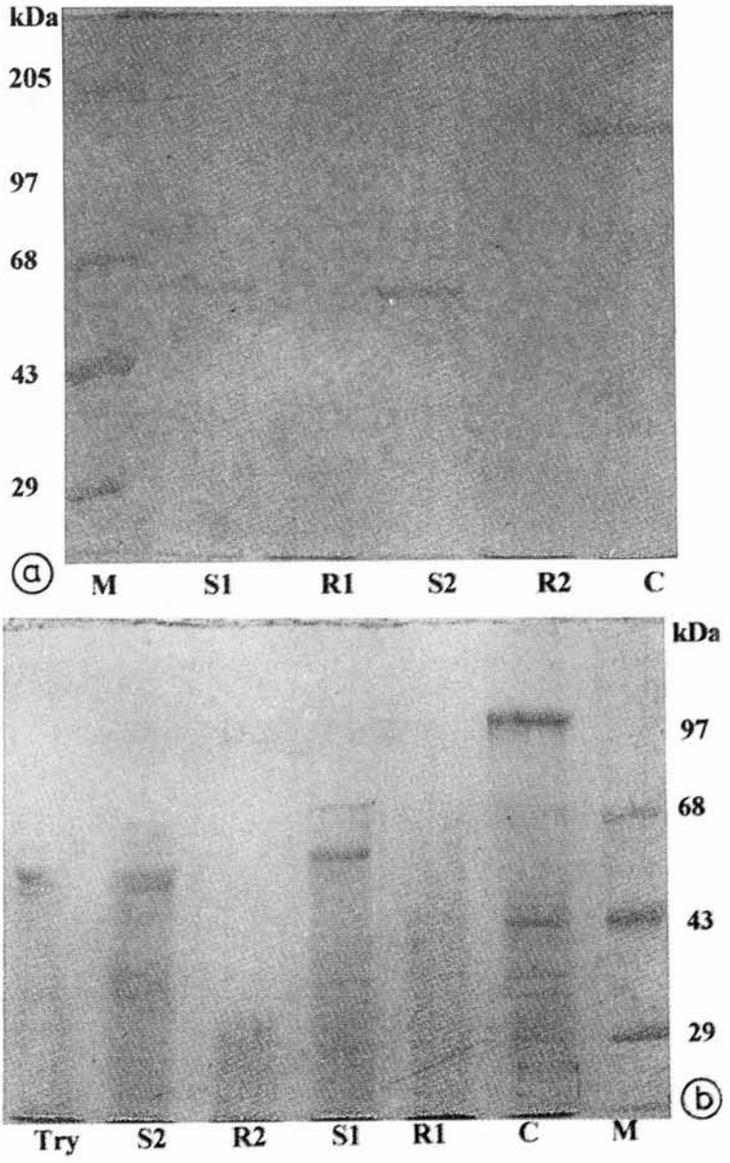

Fig. 2(a) — Proteolysis of Cry1Ab protoxin by midgut proteases of *B. thuringiensis* resistant and susceptible *H. armigera* [(Enzyme: 1 TU/2.5 μg protoxin) M, Protein molecular weight marker, S1 and S2 Cry1Ab incubated with midgut protease of susceptible population for 2 and 4 hr respectively; R1 and R2 Cry1Ab incubated with midgut protease of resistant population for 2 and 4 hr respectively; C, Control without any protease incubation for 4 hr]. (b) — Proteolysis of Cry1Ac protoxin by midgut proteases of *B. thuringiensis* resistant and susceptible *H. armigera* [(Enzyme: 1 TU/2.5 μg protoxin) Lane Try, trypsin; S1 and S2 Cry1Ac incubated with midgut protease of susceptible population for 2 and 4 hr respectively; R1 and R2 Cry1Ac incubated with midgut protease of resistant population for 2 and 4 hr respectively; C, Control without any protease incubation for 4 hr and M, Protein molecular weight marker]

Table 3 — Inhibition of midgut protease activity of the *H. armigera* with protease inhibitors

Protease inhibitor	Inhibitor concentration	% inhibition of total proteolysis
PMSF	10 μ*M*	59.7 ± 4.7
	1 μ*M*	15.7 ± 4.72
	0.1 μ*M*	2.6 ± 2.1
TPCK	500 μ*M*	No inhibition
	50 μ*M*	No inhibition
	1 μ*M*	No inhibition
TLCK	500 μ*M*	34.2 ± 5.3
	50 μ*M*	15.3 ± 5.9
	1 μ*M*	No inhibition
SBTI	0.1%	58.7 ± 5.8
	0.05%	45.1 ± 8.1
	0.01%	24.3 ± 4.5

Table 4 — Degradation of Cry1Ac toxin by midgut proteases of resistant and susceptible strain of *H. armigera*

Substrate (μg) (Cry1Ac toxin)	Amount degraded (μg /TU Enzyme/hr)	
	Resistant	Susceptible
4	Not detectable	0.17
8	5.2	0.32
12	6.2	1.15
16	5.4	1.82
24	4.7	1.56
30	4.0	0.95

Characterization of BBMV receptors — The Cry1Ab specific antibodies recognized a single receptor of about 77 kDa in BBMV preparation from both resistant and susceptible populations. Similar receptor was observed in both BBMV treated with Chaps buffer and untreated BBMV (Fig. 3). A difference was observed in binding of I^{125} labelled Cry1Ac toxin with receptors of resistant and susceptible population with resistance population showing saturation relatively at a lower concentration of BBMV (Fig. 4).

Discussion

Larvae of *H. armigera* developed many fold resistance to Cry1Ac depending up on the quantum and duration of its selection pressure[16,35]. About 7-fold resistance in *H. armigera* was reported after 17-generations of selection of larvae fed on *Bt* cotton leaves *per se*[12]. This ability of *H. armigera* to evolve resistance and lack of cotton varieties with high dose of Cry toxin expression may lead to evolution of resistance under field conditions. This possibility, simulated in northern China[5], suggested the expected life of *Bt* cotton to be of about 7-10 years in areas under *Bt* cotton exceeding 70%.

H. armigera selected for resistance to Cry1Ac showed cross-resistance to Cry1Ab and Cry1Aa. The

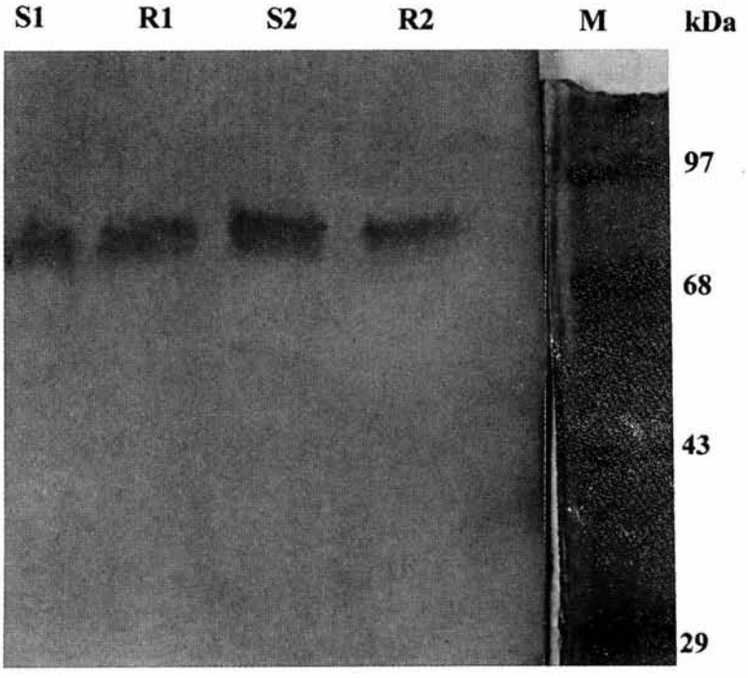

Fig. 3 — Western blot of BBMV receptors of *H. armigera* for Cry1Ab. R1 and R2 BBMV of resistant population solubilized in Chaps buffer and non-solubilized, respectively, S1 and S2 BBMV of susceptible population solubilized in Chaps buffer and non-solubilized, respectively; M, Protein molecular weight marker

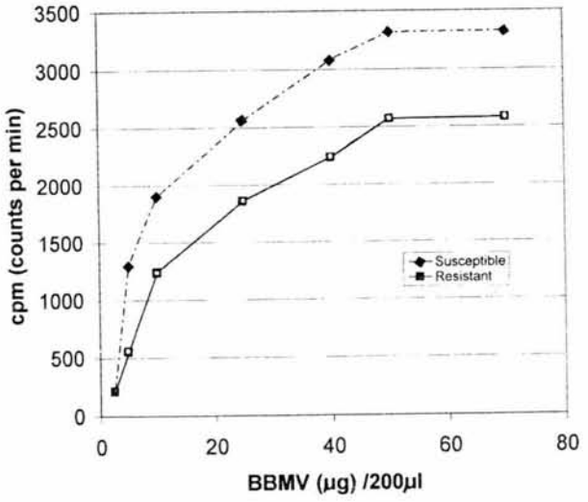

Fig. 4—Saturation binding of Cry1Ac with BBMV of resistant and susceptible populations of *H. armigera*

Cry1Ac selected resistant *H. armigera* showed little cross-resistance to Dipel® which contains Cry1Ab, Cry1Ac and Cry2Aa toxins but showed high resistance to MVP formulation which contains only one Cry1Ac toxin[15]. However, this cross-resistance was not extended to Cry1C and Cry2Aa[36]. Liao *et al.*[37] reported significant differences in susceptibility of 3 strains of *H. armigera* to Cry1Ac and Cry2Aa. There was a small but significant negative interaction between these two Cry toxins. However, Cry1Ac selected strain of *Heliothis virescens* was found to be broadly cross-resistant to Cry1Aa, Cry1Ab, Cry1Ba, Cry1Ca and Cry2Aa[38].

Growth reduction closely followed pattern of toxicity for Cry1Ab and Cry1Ac. However, Cry1Ac resistant insects showed very high tolerance to Cry1Aa. The cross-resistance between Cry1A type toxins may be due to the presence of a common binding site on BBMV, separate from that of Cry2A in *H. armigera*[31]. This is in contrast to non-saturable Cry2A binding with BBMV and subsequent inhibition of labelled Cry1Ac in *Helicoverpa zea*[39]. Similarly, Cry2A competed for the binding of Cry1Aa and Cry1Ab only slightly at high concentration of competitor, and Cry1Ac showed partial competition for binding of Cry2A only at high concentrations of the competitor in *H. virescens*[40].

Activation of Cry1A protoxin with the midgut proteases of *H. armigera* is an essential step of mode of action, and also associated with resistance. Incubation of Cry1A protoxin with midgut proteases at the ratio of 1 TU: 150 µg Cry1Ac protoxin for as long as 8 hr led to the formation of a stable toxin of about 58 kDa, similar to that of 65 kDa stable toxin reported on incubation of protoxins of Cry1Aa, Cry1Ab, Cry1Ac and Cry2Aa with gut juice of *H. armigera*[31]. Of 6 proteases, the present studies report only one protease of about 31.6 kDa for Cry1Ac protoxin activation, probably in view of its high concentration, in contrast to 4 proteases of about 71, 49, 36 and 30 kDa sizes in the larvae of *H. armigera*[41]. Activation of Cry1A protoxin with midgut proteases of *H. armigera* with 30 kDa protease[42] and 46.6 kDa protease[43] has been reported. The midgut proteases of *H. armigera* were of serine kind, few chymotrypsin-like[44], but most trypsin-like, as they were inhibited by TLCK and SBTI and not by TPCK[41]. Involvement of midgut proteases in insect resistance has been reviewed[45]; slower activation of Cry1Ac protoxin and faster degradation of Cry1Ac toxin in *H. virescens*[46,47]. Liang *et al.*[48] found higher trypsin-like activity in the third instar larvae of resistant insects over the susceptible ones of *H. armigera*. Forcada *et al.*[49] reported that qualitative differences related to the presence or absence of proteolytic activity bands using PAGE could be responsible for the observed resistance in *H. virescens*. The present findings suggest that the absence of toxin formation with proteases of resistant insects may contribute to resistance in *H. armigera*. However, at a high concentration of Cry toxin; a toxin band was found in resistant insects too; which suggest that resistance may be partially due to the proteolytic degradation of toxin.

BBMV receptors, important sites for the binding of Cry toxins, are found to vary in insects; 5 binding sites with molecular weights ranging from 63 to 155-kDa in *H. virescens* and *H. zea*[30], 7 receptors for Cry1Ac with molecular weights ranging from 50 to 170 kDa in *H. virescens*[50], while the present study showed only one of about 77 kDa in the last instar larvae. The 120 kDa glycoprotein of BBMV of *H. virescens* was found to have high homology with aminopeptidase N[51], whereas 170 kDa aminopeptidase N purified fraction was considered functional receptor of *H. virescens* for Cry1Aa, Cry1Ab and Cry1Ac toxins[52]. The reduced binding of toxin with BBMV of midgut of resistant population is often a cause of insect resistance[17]. Binding of BBMV with Cry1Ab and Cry1Ac of the resistant strain was similar to that of susceptible strain but totally lacked for Cry1Aa in resistant strain of *H. virescens*[40]. The differences in BBMV binding in the present investigations may not be completely responsible for resistance. The report of post-binding event like transport of ions across the epithelial membrane in

H. zea may also contribute to resistance[53]. The present studies call for more detailed investigations on the mechanisms of resistance and other characteristics, so as to develop *Bt* resistance management for *H. armigera* regardless of the fact that *Bt* is either used conventionally or through transgenic technology.

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