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Variation in brush border membrane vesicle receptors and activity of proteolytic enzymes in relation to toxicity of *Bt* toxins to legume pod borer, *Helicoverpa armigera* (Lepidoptera: Noctuidae) larvae

Inakarla Paramasiva, Pulipaka Venkata Krishnayya and Hari Chand Sharma

Abstract
Mechanism involved in development of resistance in *Helicoverpa armigera* larval populations from nine locations was evaluated at International Crops Research Institute for the Semi Arid Tropics (ICRISAT), Patancheru, Telangana, India during 2011 and 2012 by performing; a) *in-vitro* binding assays with Brush border membrane vesicle receptors and trypsin activated Bt toxin Cry 1Ac and b) by performing SDS-PAGE with activated toxin and gut juices of *H. armigra* populations form nine locations.
The present study results demonstrated that resistance/lower sensitivity of Mahabubnagar and Parbhani *H. armigera* larval populations (LC_{90} values 10.2851 and 15.6230 µg ml^{-1}) to *Bt* toxins was due to reduction in proteolytic activity of gut extract results in production of less activated toxin and degradation of the activated toxin to a relatively less toxic product. Resistance of Nanded population (LC_{90} value 13.7219 µg ml^{-1}) was due to low amount of the activated toxin produced from the protoxin. In case of Bidar and Kurnool populations, their relative sensitivity to *Bt* (LC_{90} values 4.0434 and 5.7445 µg ml^{-1}, respectively) was due to high binding ability of activated toxin to Brush border membrane vesicle receptor proteins of midgut epithelial cells (137 7 135 µg g^{-1}).

Keywords: *Helicoverpa armigera, Bacillus thuringiensis, Bt* toxins, BBMV receptors, protelytic enzymes

1. Introduction
The legume pod borer, *Helicoverpa armigera* (Hübner) is one of the most important constraints to crop production globally [1]. It is a polyphagous pest, and attacks more than 300 plant species [2]. The δ-endotoxin genes (Cry genes) of *Bacillus thuringiensis* (*Bt*) have been deployed for pest management in several genetically modified crops. In India, transgenic cotton has been adopted in a large scale, and more than 80% of the area (nearly 5 million ha) is under *Bt* cotton [3]. However, large-scale cultivation of insect-resistant transgenic crops may lead to development of resistance to *Bt* toxins. Hence, strategies need to be developed to manage resistance to *Bt* toxins in the target insect species, taking into account the various resistance and/or detoxification mechanisms involved.
Certain physiological and genetic features of Lepidopteran hosts are known to contribute to differences in susceptibility to *B. thuringiensis*. Activation of *B. thuringiensis* δ-endotoxin is a prerequisite for toxicity and insufficient processing or over digestion of a toxin may render it inactive. A number of reports have suggested that δ-endotoxin proteolysis is a major determinant of toxin potency. The midgut lumina of lepidopteran larvae contain a variety of alkaline proteases, mainly members of the serine proteases class, that exhibit predominantly trypsin-like and chymotrypsin-like protease activities [4, 5]. A strain of *Plodia interpunctella* (Hübner), (Indian meal moth) resistance to the δ-endotoxins of *B. thuringiensis* ssp. *entomocidus* HD-198 exhibited a lower protoxin activation rate than the susceptible insects due to decrease in the total proteolytic activity of the gut extract [6].
A critical step for insecticidal activity of *Bt* toxins is the binding of activated toxins to Brush border membrane vesicle receptors (BBMV) located on the apical microvilli membrane of epithelial midgut cells [7, 8]. The interaction of activated toxin with the receptor protein triggers

the formation of ionic channel [9], which allows a net uptake of ions and water, leading to midgut-cell swelling and eventual lysis. A direct correlation between toxicity and receptor binding has been reported in several studies [10].

Because either reduced binding or reduced conversion of protoxin to active toxin appear to be major mechanisms of resistance development in *H. armigera* larvae to Bt cry toxins, this study was conducted to determine which of the above two mechanisms involved in development of resistance to Cry 1Ac in *H. armigera* larvae collected from nine locations.

2. Materials and Methods

2.1 Estimation of LC_{90} Values of Cry1Ac Toxin towards *H. armigera* Populations Collected from Different Locations

To determine resistance levels in *H. armigera* larvae collected from nine locations, we conducted diet impregnated bio-assay and estimated the lethal concentration 90 (LC_{90}) levels of Cry 1Ac at International Crops Research Institute for the Semi Arid Tropics (ICRISAT), Patancheru, Telangana, India during 2011 and 2012.

Bacillus thuringiensis ssp. *kurstaki* (*Btk*) δ-endotoxin Cry1Ac (obtained from Dr. Marianne P. Carey, Case Western Reserve University, Department of Biochemistry, Cleveland, OH, USA) was used to determine lethal concentration 90 (LC_{90}) levels against of *H. armigera* larvae collected from Kurnool, Mahabubnagar, Guntur, and Medak (Andhra pradesh); Bidar, Raichur, and Gulburga (Karnataka); and Parbhani and Nanded (Maharashtra). Stock solution of Cry1Ac was prepared by dissolving the protein in distilled water. Subsequently, various volumes of the solution were mixed into *H. armigera* diet with a magnetic stirrer to obtain six serial dilutions of Cry1Ac (0.5, 1, 2, 4, 8, and 16 µg ml^{-1} diet). One and half ml of this diet was dispensed in each cup of 7 ml capacity and one neonate *H. armigera* larva was released in each cup. Each treatment (dilution) had 10 larvae per replication, and there were three replications per treatment. One set of larvae was fed on untreated artificial diet as a control. The mortality data were corrected according to [11] and data were was subjected to Log dose-Probit analysis (SPSS 13) to get LC_{90} doses.

2.2 Variation in Binding of Cry Toxins to BBMV Receptor Proteins of *H. armigera* from Different Locations

To test the mechanism of reduced binding of Cry 1Ac to BBMV receptor contributes to resistance development, we use *in-vitro* binding assay with BBMV from *H. armigera* larvae gut and trypsin activated cry toxins and developed correlation between toxicity and binding of Cry 1Ac to BBMV receptors.

Fourth to fifth-instar larvae were collected from Kurnool, Guntur (Andhra Pradesh); Mahabubnagar, Medak (Telangana); Bidar, Raichur and Gulburga (Karnataka); Parbhani, Nanded (Maharashtra) used for preparation of BBMV. BBMV were prepared according to the protocol used by [13]. Binding of trypsin activated Cry1Ac to freshly prepared BBMV was performed in 100 µl of Tris-BSA buffer (binding buffer). Hundred microgram of BBMV protein was incubated with trypsin activated Cry1Ac (50 µg ml^{-1} in binding buffer) for 60 min. at room temperature. The unbound toxin (supernatant) was removed by centrifugation for 15 min. at 14,000 g at 4 ºC. The BBMV were resuspended in 100 µl of ice-cold Tris-BSA buffer, and washed twice with the same buffer. Finally, the BBMV were suspended in 150 µl of Tris-BSA buffer. The protein contents of the BBMV preparations were determined by the method of [12] by using BSA as the standard.

2.3 Activity of proteolytic enzymes in relation to toxicity of *Bt* toxins to *H. armigera* larvae from different locations

To test the mechanism of reduced conversion of Cry 1Ac pro-toxin (around 130 kDa) to active toxin (60 kDa) contributes to resistance, we performed SDS-PAGE electrophoresis with digested/activated protoxin by the gut juices of *H. armigera* larvae from nine locations.

2.3.1 Insect gut-juice preparation

To prepare gut-juice, larvae were chilled on ice for 30 min before they were dissected following a 24 h starvation period to allow gut clearing. The peritrophic membrane of the containing the food bolus was isolated, homogenized and centrifuged at 30,000 rpm at 4 °C for 20 min. The supernatant was removed and recentrifuged for 20 min. at the same speed, and the resulting supernatant was subjected to acetone precipitation. Equal volume of chilled acetone was added to the supernatant, and kept for 2 h at -20 ºC. Then, the supernatant centrifuged at 14,000 rpm for 30 min. at 4 ºC. The supernatant was discarded and the pellet was air-dried to remove the traces of acetone. The pellet (gut juice) was reconstituted by adding 0.5 M Tris-HCl buffer, and used for further processes or stored at -80 °C.

2.3.2 Pro-toxin preparation

The methodology of [14] was follwed to prepare protoxin from the commercial *Bt* formulation. And the amount of protein present in the preparations was estimated by [12]. The concentration of protein present in the protoxin was adjusted to 1mg/ml

2.3.3 Activation of δ-endotoxin *in-vitro*

Gut-juice was added to the solubilized protoxin at a concentration of 5% (v/v) to start the reaction, and incubated the digestion mixture at room temperature (37 °C) for about 12 h. After 12 h the samples were removed and separated into soluble and insoluble fractions by centrifugation at 30,000 rpm at 4 °C for 20 min, and then each pellet was washed twice in ice-cold phosphate buffered saline (PBS). The activated protoxin sample (100 µl) was boiled with sodium Dodecyl Sulphate - Polyacrylamide Gel Electrophoresis (SDS-PAGE) sample buffer (20 µl) for 3 min at 100 ºC in a water bath before being loaded onto a 13% (w/v) polyacrylamide slab-gel containing 0.1% SDS, via a 5% stacking gel [15]. About 5 µl of 2-mercaptoethanol was added to each sample before the electrophoresis.

2.3.4 Sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to [15], using 13% polyacylamide gels. The stacking and resolving gel, staining and destaining solutions were made according to the protocol. Apparatus was assembled and the gels were polymerized in between glass slabs with 1.5 mm thickness spacers. Comb was cleaned with ethanol prior to use. It was placed just prior to polymerization of the gels. Comb was removed from the plates. The wells made in the stacking gel were washed with distilled water to wash out any unpolymerized gel.

Activated protoxin samples (40 µl which contains 40 µg of protein per well) were loaded in wells carefully to avoid the mixing of samples from adjacent wells. Apparatus was set in gel tank and filled with running buffer (3 g Tris, 14.4 g Glycine, and 1g SDS in 1000 ml water pH 8.3 with HCl). Gel run was carried out at 80 V; once the bands were crossed the staking gel, the voltage was increased up to 120 V to run the

proteins in resolving gel till the dye front reached to the base of the gel. The protein molecules according to the molecular weight traveled towards the opposite charge in the electric field. As soon as the dye reached the bottom of the gel, power supply was stopped and the glass slabs separated from each other carefully to avoid the breakage of gels. Thereafter, the gel was subjected to staining with 0.05% w/v coomassie brilliant blue in 25% v/v methanol and 10% v/v acetic acid by shaking with a mechanical shaker for 30 - 40 min to get uniform staining. After completion of staining, the gel was transferred to destaining solution (25% methanol and 10% acetic acid). Destaining was done till the bands were clearly visualized.

3. Results

3.1 Variation in susceptibility (LC_{90} values) of *H. armigera* populations from different locations to Cry1Ac toxins

The LC_{90} values of Cry1Ac toxins towards neonates of *H. armigera* from different locations ranged from 0.5798 to 1.9760 µg ml^{-1} diet (Table 1). The *H. armigera* populations from Bidar were most susceptible to *Bt* (LC_{90} value 4.0434 µg ml^{-1} diet), followed by the *H. armigera* population from Raichur (5.5899 µg ml^{-1} diet), Kurnool (5.7445 µg ml^{-1} diet), Gulburga (7.6795 µg ml^{-1} diet), and Medak (9.3343 µg ml^{-1} diet). The *H. armigera* population from Parbhani showed maximum tolerance to *Bt* toxins (LC_{90} value 15.623 µg ml^{-1} diet), followed by the populations from Nanded (13.7219 µg ml^{-1} diet), Guntur (10.9401 µg ml^{-1} diet), and Mahabubnagar (10.2852 µg ml^{-1} diet).

3.2 Variation in binding of Cry toxins to BBMV receptor proteins of *H. armigera* from different locations

The amounts of protein present in the BBMV preparations of *H. armigera* larvae collected from Kurnool, Mahabubnagar, Guntur, Medak, Bidar, Raichur, Gulburga, Parbhani, and Nanded ranged from 108 to 137-µg g^{-1} (Table 2). Binding of Cry1Ac toxins to BBMV was recorded in all strains of *H. armigera*. The highest amount of protein (maximum binding) was present in the BBMV preparations of the larvae collected form Bidar (137 µg g^{-1}), followed by the insects collected from Kurnool (135 µg g^{-1}), Raichur (126 µg g^{-1}), Guntur, and Gulburga (120 µg g^{-1}). The lowest amount of protein was present in the BBMV preparations of the insects collected from Nanded (108 µg g^{-1}), followed by the insects from Parbhani (109 µg g^{-1}), Medak (112 µg g^{-1}), and Mahabubnagar (119 µg g^{-1}).

3.3 Activity of proteolytic enzymes in relation to toxicity of *Bt* toxins to *H. armigera* larvae from different locations

The electrophoresis patterns from the digests of HD-1 protoxin with *H. armigera* gut juice are shown in Fig. 2. Incubation of HD-1 protoxin in gut juice turned the activation mixture to milky, indicating that precipitation had occurred. However, the clarified mixture follows denaturation in SDS-PAGE sample buffer, produced no precipitate when centrifuged.

There were no fragments larger than the 60 to 65- kDa toxin fraction, indicating that activation of the protoxin was complete in all *H. armigera* populations from Kurnool, Mahabubnagar, Guntur, Medak, Bidar, Raichur, Gulburga, Parbhani and Nanded. SDS-PAGE analysis showed that a large amount of activated toxin was produced from protoxin by the mid gut juice of the insect collected from Guntur, Medak, Raichur, Gulburga. While little was produced by the *H. armigera* populations from Kurnool, Mahabubnagar, Bidar, Parbhani, and Nanded.

Further degradation of activated toxin was observed in populations from Mahabubnagar, Guntur, and Parbhani. After 12 h incubation period, most of activated toxin was present as a single product having a molecular mass of ~64-kDa, with midgut proteases of populations from Kurnool, Bidar, Raichur, Gulburga, and Nanded, whereas with midgut proteases of Mahabubnagar, Guntur, and Parbhani populations, the activated toxin was degraded as a product having a molecular mass of ~53-kDa (Fig. 1).

4. Discussion

There are two essential factors in the pathway of toxicity of *B. thuringiensis* ICPs towards insects. Proteolytic activation of the pro-toxin (around 130 kDa) to the active toxin (around 66 kDa), and binding of the toxin to receptors on the brush border membrane of the midgut epithelium [7]. Binding of *B. thuringiensis* Cry toxins to their specific receptors is critical for toxicity, and a key element of species-specificity [16, 17]. ICPs will not be toxic if it does not bind to the cell membrane. Variation in binding of Cry toxins to BBMV proteins was studied in *H. armigera* populations collected from Kurnool, Mahabubnagar, Guntur, Medak, Bidar, Raichur, Gulburga, Parbhani, and Nanded. There is a positive correlation between binding of Cry1Ac toxin to the BBMV receptors of the midgut epithelium and sensitivity of *H. armigera* larvae to Cry1Ac toxins (Fig.1). The amount of proteins present in these populations ranged from 108 to 137 µg ml^{-1}. Maximum binding was observed in the insects collected from Bidar (137 µg g^{-1}), which were found to be more sensitive to Cry1Ac toxin (LC_{90} values of 4.0434 µg ml^{-1}), followed by the Kurnool population (135 µg g^{-1}) with the LC_{90} values of 5.7445 µg g^{-1}, and Raichur (126 µg g^{-1}) with LC_{90} values of 5.5899 µg ml^{-1}. Greater sensitivity of insects from these locations might be due to increase in Cry1Ac binding to the brush border of midgut epithelial cells, which may be due to increase in the Cry1Ac binding site concentration or due to increase in binding affinity of the toxin or both [7,10]. The minimum (lowest) binding was observed in the BBMV preparations of the insects collected from Nanded (108 µg g^{-1}), which were less sensitive to *Bt* with an LC_{90} value of 13.7219, followed by the Parbhani population (109 µg g^{-1}) with LC_{90} values of 15.6230 µg ml^{-1}. Low sensitivity of these populations might be due to reduced binding affinity of the receptor proteins or reduced in Cry1Ac binding site concentration. The aminopeptidase N has been identified as a Cry1Ac receptor. Evidence suggests that Ga1NAc (N-acetylgalactosamine) residues on aminopeptidase N play an important role in the reaction with the Cry1Ac toxin [18, 19]. The correlation between ICPs binding to the intestinal microvilli and toxicity in different insect species (*M. sexta*, *Plutella xylostella*, *L. decemlineata*, and *Ostrinia nubilalis*) has been reported by [20] Denolf *et al*. (1993). Resistance to Cry1Ab toxin in *Plodia interpunctella* L. is correlated with a reduction in affinity of Cry1Ab toxin binding, whereas increased sensitivity to Cry1Ac toxin is reflected in an apparent increase of Cry1Ac binding site concentration [8]. Similarly, the resistance of field population of *P. xylostella* (200-fold compared with the laboratory strain) is due to reduced binding of Cry1Ab to the BBMV of the field population, either because of strongly reduced binding affinity or because of the complete absence of the receptor molecule[21]. The populations of *P. xylostella* in various regions of the world have become resistant as a result of crop treatment with *Bt*. The resistance development is mainly due

to change in the binding characteristics of the Cry toxin [22]. Thus membrane receptors play a key role in determining the specificity of *B. thuringiensis* ICPs, strongly suggests that this mechanism will probably apply to other instances of resistance to *B. thuringiensis* ICPs.

Correct activation of a *B. thuringiensis* δ-endotoxin is likely to be a prerequisite for toxicity, and insufficient processing or over digestion of a toxin may render it inactive. The midgut proteases that an insect possesses are likely to be a major determinant of toxin potency. The midgut lumina of Lepidopteran insect larvae been shown to contain a variety of alkaline proteases, mainly members of the serine protease class, that exhibit predominantly trypsin like and chymotrypsin-like protease activities [4, 23, 24]. Such midgut proteases are likely to be responsible for δ-endotoxin activation.

The electrophoresis patterns from *H. armigera* midgut digests of HD-1 pro-toxin showed that there were no fragments larger than the 60 to 65-kDa toxin fraction, indicating that activation of the protoxin was complete in all *H. armigera* populations collected from Kurnool, Mahabubnagar, Guntur, Medak, Bidar, Raichur, Gulburga, Parbhani, and Nanded. Large amount of activated toxin were produced from pro-toxin by the midgut juice from Guntur, Medak, Raichur, and Gulburga, which indicated their high susceptibility to *Bt* (Fig. 2). Possibly because of higher protease levels in the midgut juice or higher proteolytic activity of gut juice. Little amount of activated toxin was yielded by the midgut juices of insects collected from Kurnool, Mahabubnagar, Bidar, Parbhani, and Nanded indicated their relative resistance to *Bt*, which might be due to a decrease in total proteolytic activity. Similarly, the high protease levels in *Pieris brassicae* have been found to be responsible for its sensitivity to *B. thuringiensis* subsp. *thuringiensis* as compared to *Mamestra brassicae* (L.) and *Spodoptera littoralis* Boisduval with lower proteases and lack of susceptibility to *Bt* [25]. A strain of Indian meal moth, *Plodia interpunctella* resistant to the δ-endotoxins of *B. thuringiensis* subsp. *entomocidus* is due to a decrease in the total proteolytic activity of the gut extract [26, 14] also reported that a large amount of activated toxin yielded from pro-toxin by *Bombyx mori* gut juice, which was found to be more sensitive to the protoxins of HD-1 a *H. armigera*, while little was obtained from *H. armigera* gut juice.

Further degradation of activated toxin (~64 kDa) to the low molecular weight of ~53 kDa was also observed in midgut juices of insects collected from Mahabubnagar, Guntur, and Parbhani populations, indicating their least susceptibility to *Bt*. Further degradation of activated toxin in *H. armigera* gut juice has also reported by [14]. With proteases from *P. brassicae*, the Cry1Ac - susceptible insect, Cry1Ac was processed to an insoluble product with a molecular mass of ~56 kDa, whereas with proteases from *M. brassicae*, the non-susceptible insect, generated products with molecular masses of ~58, ~40, and, ~20 kDa [27]. Similarly, [28] observed that the reduced sensitivity of fifth-instar larvae of *S. littoralis* to Cry1Ac could be attributed to increased degradation of the toxin in the less susceptible larvae [29]. also reported that the gut juice of *Anomala cuprea* (Hope) might due to both activation and degradation of *Bt* endotoxin. The proteolytic activity of gut juice, which is vital for activation of *Bt* δ-endotoxin in the first place, also plays an important role in subsequent detoxification.

Table 1: Log-dose Probit response of *H. armigera* populations collected from different locations to Cry1Ac toxins

District	LC_{50}	Fiducial limits (95%)	LC_{90}	Fiducial limits (95%)	Heterogenity (x^2)	Slope ± S.E	Regression equation
Kurnool	0.5798	0.34689 – 0.81998	5.7445	4.04604 – 9.68015	1.5718	1.2867 ± 0.1721	0.3046 + 1.2867x
Mahabubnagar	1.3898	1.05602 – 1.75408	10.2851	7.26422 – 16.94140	3.5767	1.4743 ± 0.1614	-0.2107 + 1.4743x
Guntur	1.3687	1.02684 – 1.74132	10.9401	7.60875 – 18.53734	3.2272	1.4197 ± 0.1591	-0.1935 + 1.4197x
Medak	1.2321	0.91853 – 1.56839	9.3343	6.62307 – 15.27130	1.8869	1.4572 ± 0.1618	-0.1321 + 1.4572x
Bidar	0.6454	0.43999 – 0.85058	4.0434	3.03801 – 6.05385	0.5982	1.6082 ± 0.1972	0.3058 + 1.6082x
Raichur	1.0979	0.85820 – 1.35051	5.5899	4.26354 – 8.09891	7.7766	1.8130 ± 0.1872	-0.0735 + 1.8130x
Gulburga	1.2328	0.94620 – 1.53932	7.6795	5.65327 – 11.77996	1.2340	1.6131 ± 0.1705	-0.1466 + 1.6131x
Parbhani	1.9760	1.53874 – 2.48846	15.6230	10.60867 – 7.36839	7.4702	1.4271 ± 0.1538	-0.4221 + 1.4271x
Nanded	1.6990	0.79099 – 2.96714	13.7219	6.47446 – 97.27309	10.7502	1.4126 ± 0.2559	-0.3252 + 1.4126x

LC_{50} and LC_{90} values are expressed as µg per ml of diet

Table 2: Variation in binding of *Cry1Ac* toxin (50 µg ml^{-1}) to BBMV (100 µg) receptor proteins of *H. armigera* collected from different locations

Location	OD Value	Protein (µg g^{-1})
Kurnool	0.56	135
Mahabubnagar	0.49	119
Guntur	0.5	120
Medak	0.47	112
Bidar	0.58	137
Raichur	0.52	126
Gulburga	0.5	120
Parbhani	0.46	109
Nanded	0.45	108
Blank	0.00	0.00

OD = Optical density

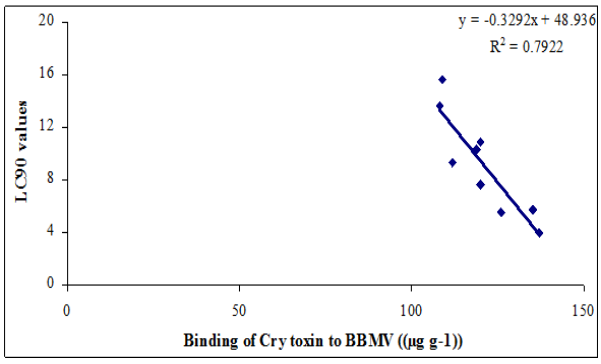

Fig 1: Relationship between binding to BBMV receptors and susceptibility of *H. armigera* larvae collected from different locations to Cry1Ac toxins

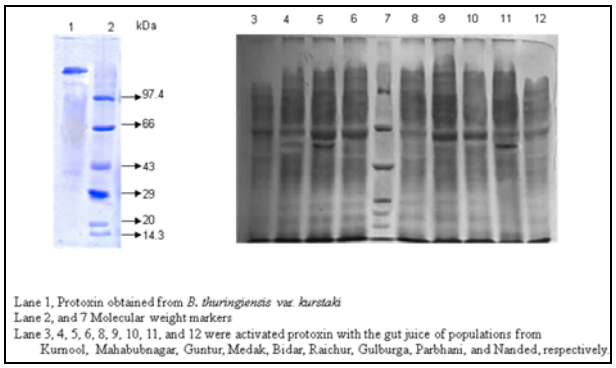

Fig 2: SDS-PAGE of *B. thuringiensis* sunsp. *kurstaki* HD-1 δ-endotoxin activated in *H.armigera* gut juice from different locations

5. Conclusion

Less sensitivity of Mahabubnagar and Parbhani populations with high LC_{90} values of may be due to combined effect of reduction in proteolytic activity of gut extract, and also due to further degradation of the activated toxin (60 to 65 kDa) to a relatively less toxic product of molecular mass of ~53 kDa. In case of Guntur population, even though large amounts of activated toxin was produced, it showed less sensitivity to *Bt* which may be due to further degradation to less toxic product of molecular mass of ~53 kDa. Low sensitivity of Nanded population may be due to less amount of activated toxin produced from the protoxin. In case of Bidar and Kurnool populations their sensitivity to *Bt* may be due to high binding ability of activated toxin to BBMV receptor proteins of midgut epithelial cells. High sensitivity of Medak, Raichur, and Gulburga populations may be due to large amount of activated toxin produced from pro-toxin, as well as their binding to brush border membranes of midgut epithelial cells at moderate level.

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