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Protease Mediated Resistance Mechanism to *Cry1C* and *Vip3A* in *Spodoptera litura*

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ABSTRACT

The present study was undertaken to understand the protease mediated resistance mechanism of *Cry1C* and *Vip3A* in *S. litura.* Proteases play an important role for convertion of protoxins to active toxin. Hence laboratory selection of *Spodoptera litura* (Fab.) with *Cry1C* and *Vip3A* was done for eight generations to develop resistance, which developed 30.32 and 285.47 fold resistance respectively. Fourth instar larvae from nine generation of *S. litura* were dissected in an ice-cold 20 mM Tris-HCl buffer (pH7). Major midgut proteases viz, azocaseinolytic, tryptic and chymotryptic were quantified by using azocasein, trypsin and chymotrypsin as substrate. Results revealed that lowest azocaseinolytic activity 0.896 U/gut was observed in *Cry1C* resistant strain of *S. litura* which was 2.57 fold less than susceptible. Similarly, *Vip3A* resistant strain recorded 1.08 U/gut azocaseinolytic activities, which was 2.13 fold less than susceptible strain of *S. litura*. There was 2.26 and 3.35 fold decrease in activity respectively in *Cry1C* resistant and *Vip3A* resistant *S. litura* over susceptible strains. Chymotryptic activity in midgut of susceptible stain of *S. litura* was 1.13 U/gut, whereas in *Cry1C* resistant strain it was 0.512 U/gut which was 2.20 fold less over susceptible strain. Similarly, in *Vip3A* resistant strain, lower chymotryptic activity was recorded as compared to susceptible, which was 1.66 fold less than susceptible strain. Maximum five protease isoforms were identified in the electrophoretic profile of susceptible *S. litura*. Remarkable variation was observed between susceptible and resistant *S. litura* for protease isoform. The *Cry1C* resistant *S. litura* homogenate showed three bands viz., Pro3, Pro4 and Pro5 while only two bands viz., Pro3 and Pro5 in *Vip3A* resistant homogenate of *S. litura* whereas five bands were observed in susceptible strain of *S. litura*.

Key words: Protease, resistance, *Cry1C* , *Vip3A, S. litura*

INTRODUCTION

Mode of action of *B.thuringiensis* in the gut is a complex process, involving many steps in the conversion of ICPs to toxins (Gill *et al*. 1992). ICPs interact through hydrogen bonding, disulfide linkages, and hydrophobic interactions. In lepidopteran insects, ICPs are released in the alkaline gut and hydrolyzed to toxins by proteases. Toxin binds to brush border membrane cells in the midgut, receptor toxin aggregation leads to pore formation, ionic imbalance, cell lysis and septicemia. Traditionally Bt toxicity has been attributed mainly to its ICPs. Therefore, most studies of Bt over the past two decades have focused on the discovery of new ICPs and elucidation of their mode of action in the insect midgut by proteases. Proteases involved in protoxin activation are described as trypsin or chymotrypsin like proteases in several insect species. Proteases are defined as peptide hydrolases and include all enzymes that hydrolyze peptide bonds (Beynon and Bond, 1993). Proteinases refer to a specific class of proteases and are synonymous with the term endopeptidases, which cleave internal bond in a peptide. Most of the proteases that degrade Bt ICPs are Proteinases. Proteases are involved in crystal dissolution and protoxin activation and contribute to toxin specificity. Reduced protoxins processing due to decreased activities of proteinases may be associated with resistance to Bt toxin in *Ostrinia nubilalis* (Huraong *et al.*, 2004). *Platella interpunctella* resistant to *B.thuringiensis* subsp. *entomocidus* HD-198 was found to process Bt protoxin at a slower rate (more than 300 times slower) than the parental susceptible strain (Oppert *et al.*, 1994). Serine proteinases, such as trypsin, chymotrypsin and elastase, are important in both solublization and activation of Bt protoxins (Dai and Gill, 1993). Proteinase mechanism was responsible for about 90% of the total resistance to Cry1Ac (Herrero *et al.*, 2001). Extensive investigations on mechanism of resistance in insects have helped in developing diagnostic tools and appropriate resistance management tactics.

MATERIALS AND METHODS

Insect

S. litura eggs, larvae were collected from the field of soybean, castor and sunflower in and around Akola and reared in the laboratory under controlled conditions of temperature 25 ± 2°C, 75 ± 5 per cent relative humidity and photo period of 13 hrs light : 11 hrs dark. The larvae were reared in the plastic jars on fresh castor leaves washed with water, and fed up to last instar larvae. Final instar larvae were transferred to jars containing sand for pupation. The pupae were collected and disinfected with 0.02 per cent sodium hypochloride solution. Disinfected pupae were separated by sex determination (Krickpatrick, 1961) and transferred to adult emergence chamber. The adults emerged were transferred into mating chamber by maintaining male female ratio (1:1) and were provided with adult diet. Two pairs of moths were released in each mating chamber. The neonates emerged from the eggs were transferred on fresh castor leaves. In this way continuous rearing was done up to 12 generations for bioassay studies. Toxin of *Cry1C* was prepared from recombinant *E. coli* strains as per Lee *et al.* (1992). Serial dilutions of *Cry1C* (in distilled water) were prepared and the bioassays were carried out using leaf dipping method. About 5 concentrations of toxin with 3 replications were used for each bioassay. One sq.cm. of castor leaf disc dipped in *Cry1C* toxin solution and allowed to air dry. Leaf disc was kept in petriplate. Ten neonate larvae were released on treated leaf disc per replication. The control consisted of leaves dipped in distilled water (without toxin). A minimum 180 neonate larvae were used for each bioassay. All the bioassays were carried out at 25^0±2°C and 60 to 80% RH. To maintain the leaf turbid, moist tissue paper was placed at the bottom of the plate. Moribund larvae not responding to probing were considered as dead. Observations on mortality of larvae were recorded after 72 hours post treatment.

Toxin

Toxin of *Cry1C* and *Vip3A*3A was prepared from recombinant *E. coli* strains as per Lee *et al*. (1992). Cells were grown in Luria broth containing 50 µg/ml ampicillin for 72 hrs., harvested by centrifugation at 4500 g at 4°C and the pellet suspended in lysis buffer (50 mM Tris, pH 8; 50 mM EDTA, 15 % sucrose, lysozyme @ 2 mg/ml) and incubated for 4 hr. After incubation, lysis buffer was replaced with crystal wash I (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 II (0.5 M sodium chloride) and then with distilled water. Finally the pellet was dissolved in solubilizing buffer (50 mM sodium carbonate 10 mM dithiothreitol, pH 10.5) at 37°C for 6 hrs. Supernatant containing toxin was collected after centrifugation at 4500 g for 10 minutes and stored at -20°C till further use. The proteins in the supernatant were quantified by Bradford method.

Selection of resistant strain to *Cry 1C* and *Vip3A*

Field collected population of *S. litura* were reared for four generations. Fifth generation homogenized *S. litura* neonates were exposed to a dose corresponding to the LC_{50}.of *Cry1C* and *Vip3A* toxin by using leaf dip method. Surviving larvae were allowed to complete their life cycle upto adult and placed in a mating chamber for oviposition. Progeny larvae and subsequent generations were reexposed to *Cry1C* and *Vip3A* toxins to select for resistance. After twelve generations, the strain exhibiting high resistance level was considered as resistant strain and use for further study. The strain which was found to be most susceptible to *Cry1C* and *Vip3A* having the lowest LC_{50} was maintained in the laboratory for several generations without exposure to *Cry1C* and *Vip3A* toxin and considered as susceptible population

Protease activity assays

Azocaseinolytic activity

The midgut azocaseinolytic activity was measured by azocasein digestion method (Marcheti *et al*. 1998). Midgut homogenate was mixed with 130 µl of Tris HCl buffer (pH 9). To the above mixture, 100 µl of 2% azocasein was added and incubated for 1 hr at 37°C. The reaction was stopped by adding 500 µl of 5% ice-cold trichloroacetic acid (TCA). The mixture was centrifuged at 14000 rpm for 15 minutes at 4°C. Supernatant (50 µl) was mixed with 50 µl of 1 N NaOH and absorbance was estimated at 420 nm by using microplate reader. The protease activity of sample was calculated using trypsin standard curve in terms of tryptic unit (TU). Increasing OD by one unit was considered as one unit activity (Chandrashekar and Gujar, 2003).

Tryptic activity

Homogenized midgut supernatant containing soluble gut enzymes was used in assays. Samples were diluted 1:100 in buffer containing 200 mM Tris, $CaCl_2$ and 50 µl were added to microplate well. N-α-Benzyl-L-arginine p nitroanilide (BApNA, Sigma 100 mg /ml in dimethyl sulfoxide) was diluted 1:100 in buffer and 50 µl were added to each well to initiate the reaction. After 30 second incubation at 37°C, absorbance was monitored at 405 nm (Oppert *et al*., 1997). Increasing OD by one unit was considered as one tryptic unit activity.

Chymotryptic activity

Midgut supernatant were diluted (1:100) in buffer containing 200 mM Tris, pH 8.0, 20 mM $CaCl_2$ and 50 µl were added to microplate well N-succiyl-ala-ala-pro-leucine p-nitranilide (SAAPLpNA), Sigma 100 mg / ml in dimethyl sulfoxide) was diluted 1:100 in buffer A and 50 µl were added to each well to initiate the reaction. After 30 second incubation at 37°C absorbance was monitored at 405 nm (Oppert *et al* 1997). Increasing OD by one unit was considered as one chymotryptic unit activity.

Qualitative protease activity profile

Midgut protease of test insects was subjected to SDS-PAGE analysis on 12% gels under non denaturing condition and processed for zymogram analysis. Midgut homogenate with known protease activity was dissolved in non reducing sample buffer without boiling (2% SDS, 25% glycerol, 60 mM Tris-HCl, pH 6.8, 0.1% bromophenol blue), and electrophoresis was carried out at 4°C. Thereafter, the gel was washed for 10 min in 2.5% Tritox X-100 to remove SDS. Gel was incubated with 100 mM Glyicine NaOH buffer (pH10) containing 2% casein for 1hr. Then gel was stained with 0.5% commassie brilliant blue R250. Protease activity was revealed as zone of white clearing in dark blue background (Garcia-Carreno *et al*., 1993).

RESULTS

Total protease activity (Azocaseinolytic activity) in *S. litura* midgut

The data regarding the azocaseinolytic activity are presented in Table1. Lowest azocaseinolytic activity 0.896 U/gut was observed in *Cry1C* resistant strain of *S. litura* which was 2.57 fold less over susceptible. Similarly, *Vip3A* resistant strain recorded 1.08 U/gut azocaseinolytic activity, which was 2.13 fold less over susceptible strain of *S. litura*.

Table 1: Azocaseinolytic activity in *Spodoptera litura* midgut using azocasein as a substrate

Strain	Activity U/gut	Decreased over susceptible
Cry1C resistant	0.896	2.57
Vip3A resistant	1.080	2.13
Susceptible	2.310	--

Tryptic activity in *S. litura* midgut

Data presented in Table 2 showed that specific activity of trypsin like proteases in the soluble fraction of *Cry1C* resistant and *Vip3A* resistant *S. litura* larvae was significantly lower than that of susceptible larvae. There was 2.26 and 3.35 fold decrease in activity respectively in *Cry1C* resistant and *Vip3A* resistant *S. litura* over susceptible.

Table 2: Tryptic activity in *Spodoptera litura* midgut using BApNA as substrate

Strain	Activity U/gut	Decreased over susceptible
Cry1C resistant	0.564	3.35
Vip3A resistant	0.834	2.26
Susceptible	1.890	--

Chymotryptic activity in *S. litura* midgut

Chymotryptic activity in midgut of susceptible stain of *S. litura* was 1.13 U/gut, whereas in *Cry1C* resistant strain it was 0.512 U/gut which was 2.20 fold less over susceptible strain. Similarly, in *Vip3A* resistant strain, lower chymotryptic activity was recorded as compared to susceptible, which was 1.66 fold less than susceptible strain.

Table 3: Chymotryptic activity in *Spodoptera litura* midgut using SAAPLpNA as a substrate

Strain	Activity U/gut	Decreased over susceptible
Cry1C resistant	0.512	2.20
Vip3A resistant	0.680	1.66
Susceptible	1.130	--

Electrophoretic profile of midgut protease activity in *S. litura*

The electrophoretic pattern of proteases from midgut homogenate of *Cry1C* resistant, *Vip3A* resistant and susceptible strains of *S. litura* larvae is presented in Table 4 and Plate 1. In this study five protease isoforms (Pro1 to Pro5) were observed having molecular weight 295.43, 40.66, 32.44, 27.09, 23.01 kDa according to its mobility towards anode. Variation in protease activity profile of *Cry1C* resistant, *Vip3A* resistant and susceptible strains of *S. litura* were observed. In midgut homogenate of susceptible *S. litura* showed five protease isozymes (Pro1 to Pro5) with Rf values 0.20, 0.38, 0.44, 0.52 and 0.58 respectively, were observed. This differed significantly from electrophoretic profile of midgut homogenate from *Cry1C* resistant and *Vip3A* resistant *S. litura*. Three protease isozymes viz, Pro3, Pro4 and Pro5 were present in *Cry1C* resistant strain, out of which Pro3 and Pro5 showed medium intensity and pro4 showed light intensity. In *Vip3A* resistant strain, only two protease isozymes viz, Pro3 and Pro5 were observed having Rf value 0.44 and 0.58, respectively.

Table 4: Electrophoretic analysis of midgut proteases activity in *Spodoptera litura*

Isozymes	Molecular weight (kDa)	Rf value	Susceptible (Intensity)	*Cry1C* Resistant (Intensity)	*Vip3A* Resistant (Intensity)
Pro1	295.43	0.20	+ (D)	-	-
Pro2	40.66	0.38	+ (M)	-	-
Pro3	32.44	0.44	+ (M)	+ (M)	+ (M)
Pro4	27.09	0.52	+ (L)	+ (L)	-
Pro5	23.01	0.58	+ (M)	+ (M)	+ (D)

+ (D)= Low intensity + (M)= Medium intensity + (L)= Light intensity

DISCUSSION

Total protease activity (Azocaseinolytic activity)

Azocaseinolytic activity was less in *Cry1C* resistant strain of *S. litura* which was 2.57 fold less over susceptible. Similarly, *Vip3A* resistant strain recorded 1.08 U/gut azocaseinolytic activity, which was 2.13 fold less over susceptible strain of *S. litura*. Our findings are in agreement with the previous workers. Bai *et al*. (1990) analysed the midgut juices of *S. littoralis* resistant to Bt which contained only 50% of total protease activity, compared with that in gut juice of susceptible strain. Oppert (1996) also studied the protease activity of midgut extract of HD-198 resistant insect which showed lower proteolytic activity toward several substrates than midgut extracts of susceptible insects.

Tryptic activity

Tryptic activity was 2.26 and 3.35 fold decrease respectively in *Cry1C* resistant and *Vip3A* resistant *S. litura* over susceptible. Similar, results were reported by Bai *et al*. (1990) who analyzed the midgut juices of *S. littoralis* resistant to Bt which contained about five times less trypsin activity compared with those in the gut juice of susceptible. Houseman and Chin (1995) also quantified the digestive trypsin proteases in the midgut of *Ostrinia nubilalis,* resistant to *B. thuringiensis.* They found that trypsin activity was higher in midgut sap of susceptible 5th instar larvae as compared to resistant population. Huarong *et al*. (2004) also found trypsin like protease activity in Bt resistant strain compared to that in which was approximately half of that susceptible strain of *Ostrinia nubilalis.*

Chymotryptic activity

Chymotryptic activity in midgut of susceptible stain of *S. litura* was 2.20 fold less over susceptible strain. Similarly, in *Vip3A* resistant strain, lower chymotryptic activity was recorded than susceptible strain. Bai *et al*. (1990) analyzed the midgut juices in *S. littoralis* resistant to *B. thuringiensis* which contained about six times less chymotryptic activity as compared to susceptible. Huang *et al*. (1999) also reported the reduced chymotryptic activity in resistant strain of European corn borer as compared to susceptible strain.

The present results indicated that there was less total protease, tryptic and chymotryptic activity in *S. litura* resistant to *Cry1C* and *Vip3A* toxins as compared to susceptible strain. These results suggested that there is protease mediated mechanism of resistance against *Cry1C* as well as *Vip3A* toxins. Midgut proteases found to play an important role for the proteolytic processing of protoxin. Protease convert the protoxin into toxin by proteolytic processing, which is essential for binding to brush border membrane vesicle. In the present study, there is no conversion of *Cry1C* as well as *Vip3A* protoxin into toxin, which is essential for binding to brush border membrane vesicle. This is one of the mechanism of Bt resistance in *S. litura*. The other mechanism such as alteration in binding site, reduced binding need to be studied for better understanding of Bt resistance mechanism in insect.

Electrophoretic Protease activity analysis

Variation in protease activity profile of *Cry1C* resistant, *Vip3A* resistant and susceptible strains of *S. litura* were observed. In Vip3A resistant strain only three protease isoforms were observed, and in Cry1C resistant strain two protease isoforms detected whereas in susceptible strain five isoforms were detected. Similarly Chandrashekar and Gujar (2003) also studied the mechanism of resistance to Bt endotoxin Cry1Ac in *H. armigera*. Midgut proteases of *H armigera* were subjected to SDS-PAGE analysis. It was further observed that 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, 29.7 kDa visible in susceptible and resistant populations of *H. armigera,* while only one protease band was visible with Cry1Ac protoxin, suggesting the possible role of protease in development of resistance to Cry1Ac in *H. armigera.* Huarong *et al*. (2004) who studied the comparative analysis of proteases activities of *B.thuringiensis* resistant and susceptible *Ostrinia nubilalis* (Lepidoptera) using casein as a substrate. Further zymogram analysis indicates six proteases activities (C1 to C6) in soluble extracts of susceptible larvae. Activities C5 and C6 were the most prominent in susceptible larvae. However, isozyme C6 was not detected in soluble extracts of resistant larvae. The relative intensities of all proteases in the resistant strains were weaker than the corresponding activities in the susceptible strain.

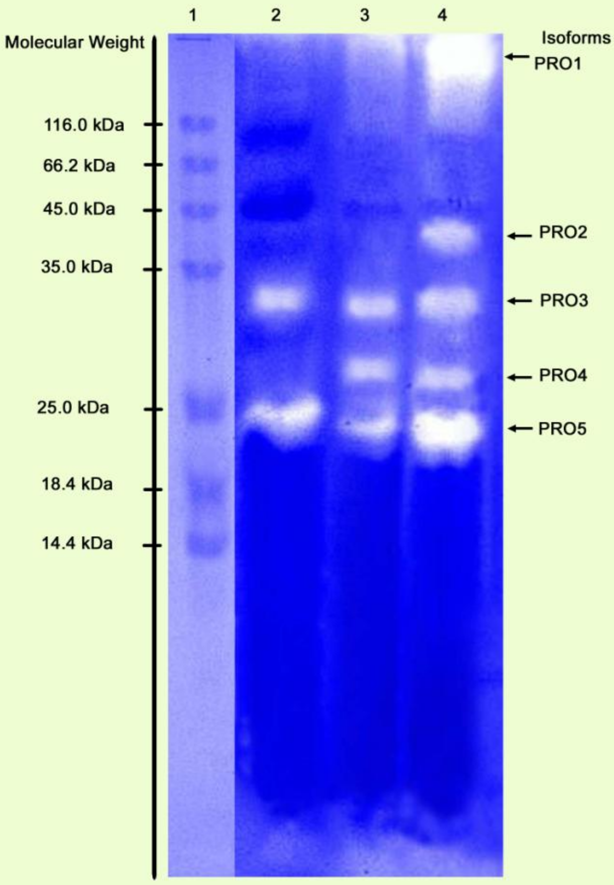

Plate 1: Protease activity profile in midgut of *S. litura*

Lane 1 - Standard Molecular Weight Marker
Lane 2 - Protease activity in midgut of Vip resistant *S. litura*
Lane 3 - Protease activity in midgut of Cry1C resistant *S. litura*
Lane 4 - Protease activity in midgut of susceptible *S. litura*

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