Protein J (2012) 31:259–264
DOI 10.1007/s10930-012-9402-0

Interaction of Salivary and Midgut Proteins of *Helicoverpa armigera* with Soybean Trypsin Inhibitor

Santosh Kumar Upadhyay · Krishnappa Chandrashekar

Published online: 14 March 2012

Abstract Feeding of *Helicoverpa armigera* larvae on semi-synthetic diet containing Soybean trypsin inhibitor (STI) resulted in disappearance of STI sensitive protease in salivary and midgut protease extract. This might be due to in situ inhibition by dietary STI. STI was largely degraded within 1 h of incubation with total salivary protease (1:1). Degradation was relatively low in midgut proteases. STI interacting proteins were isolated from saliva and midgut extracts of larvae fed on STI supplemented diet using affinity column. Most of the isolated proteins showed caseinolytic activity in zymogram. Denovo sequencing data of seven different peptides selected from trypsin digested total protein showed similarity to chymotrypsinogen, serine protease, aminopeptidase N, peroxidase, hypothetical protein and muscle specific protein.

Keywords Soybean trypsin inhibitor · *Helicoverpa armigera* · Protease · Affinity column · Zymogram

Abbreviations

STI	Soybean trypsin inhibitor
PI	Protease inhibitors
PMF	Peptide mass fingerprinting
ACN	Acetonitrile
TFA	Tri-fluoro acetic acid

Electronic supplementary material The online version of this article (doi:10.1007/s10930-012-9402-0) contains supplementary material, which is available to authorized users.

S. K. Upadhyay · K. Chandrashekar (✉)
National Botanical Research Institute, Council of Scientific and Industrial Research, Rana Pratap Marg, Lucknow 226001, UP, India
e-mail: kc_shekar2001@yahoo.com

1 Introduction

Plants display variety of defence mechanisms to kill, repel or minimize the damage due to phytophagous insects. Most of the studies on plant–insect interactions are mainly focused on plants, whereas herbivore side is seldom studied. One of the host plant defence mechanisms is disruption of nutrient acquisition system of insect pests. Plant protease inhibitors are involved in such mechanism [18]. Plants synthesize a variety of toxic proteins and enzyme inhibitors including inhibitors of insect gut proteases for their protection against insects [2, 12–14, 25]. Effect of PIs on insect growth and development has been demonstrated by feeding with artificial diet and transgenic plants containing PIs [16, 21]. Insects are able to adapt to protease inhibitors (PI) due to their ability of shifting the target proteolytic enzyme to the inhibitor insensitive isoforms [3–7, 11, 20, 22]. Gut trypsin: HzTrypsin-S isolated from *Helicoverpa zea* fed on plants expressing protease inhibitor could not be inhibited by 1,000-fold excess of inhibitors [30].

Three different types of strategies have been proposed for adaptation of insect to the protease inhibitors [33] viz., (1) enhanced expression of target proteases to maintain their activity level (2) production of PI insensitive proteases and (3) activation of PI detoxification mechanism [1, 17, 23, 32, 33]. Oryzacystatin I and Bowman-Birk inhibitors are rapidly digested by serine proteinases in association with leucine aminopeptidases [15]. Giri et al. [17] reported the in vitro degradation of chickpea trypsin inhibitor by *Helicoverpa* gut proteases. *H. armigera* larvae fed on soybean trypsin inhibitor showed lower azocaseinolytic activity whereas inhibition of their gut trypsin activity by STI was low [24]. It is also reported that potato protease trypsin inhibitor (PPTI) and STI does not affect in vivo protein digestion in *H. zea and S. exigua* [5]. Larvae

of *H. armigera* which is an important lepidopteran pest on several important commercial and food crops are known to frequently feed on plants expressing protease inhibitors.

Several studies reported the different mechanisms of adaptation of insects towards protease inhibitors. However, none of the study reported the STI interacting proteins from the midgut of *H. armigera.* Present study describes the isolation of STI interacting protein from the saliva and midgut of *H. armigera*. We also report the effect of STI on larval proteases and its degradation.

2 Materials and Methods

2.1 Feeding of *H. armigera* Larvae on Diet Containing STI

For inducing trypsin inhibitor inactivating mechanisms, 4 days old larvae of *H. armigera* were continuously reared on artificial diet containing STI (Sigma, USA; 2 mg/gm diet) till they reach fifth instar stage. Larvae were shifted to diet without STI 1 day before extraction of saliva and midgut proteases. The salivary and midgut extracts were extracted from induced (diet with STI) and uninduced (diet without STI) larvae.

2.2 Preparation of Midgut and Salivary Protein Extracts

2.2.1 Preparation of Salivary Proteins

Fifth instar larvae were gently pressed in the thorax-abdominal region to make insects to regurgitate (vomit). Regurgitates (containing mixture of saliva and food) were collected in an ice–cold Tris–HCl buffer (20 mM, pH 7.0) [8] and centrifuged (13,200×*g*, 4 °C, 15 min.). The supernatant was collected and stored at 4 °C.

2.2.2 Preparation of Midgut Proteases

Fifth instar larvae were immobilized on ice for 15 min and dissected in ice cold Tris–HCl buffer (20 mM, pH 7.0) [8]. The midguts were removed and food particles were eliminated by cutting open midgut and several times rinsing in buffer. Finally midguts were homogenized and centrifuged (13,200×*g*, 4 °C, 15 min). Supernatant was collected and stored at 4 °C.

2.3 SDS-PAGE Analysis

The electrophoresis was carried out in Mini Protein (II) Dual slab gel system (BioRed, USA) at constant current of 20 mA. Total 10 µg protein was loaded in each well for Coomassie staining. After electrophoresis, the stacking gel was removed and resolving gel was washed with water and stained using Coomassie brilliant blue R250 (0.25 % in water:methanol:acetic acid: 50:40:10). The staining was carried out for 15 min with continuous shaking followed by destaining in a destain solution (water:methanol:acetic acid: 45:45:10).

2.4 Preparation of STI Affinity Column and Isolation of STI Interacting Midgut Proteins of *H. armigera*

STI was incubated with CNBr-activated Sepharose 4B matrix and the affinity column was prepared as per the manufacturer's protocol (GE Healthcare, USA). The quantity protein in midgut and salivary preparation was less, therefore they were pooled together and loaded on the above column (at a flow rate of 0.5 ml/min) pre-equilibrated with 20 mM Tris–HCl buffer containing 150 mM NaCl, pH 7.0. The column was washed with the same buffer till OD_{280} became stable. The bound proteins were eluted with unbuffered Na_2CO_3 (100 mM, pH. 12.0) [29] and used for further analysis.

2.5 MS/MS Analysis and Denovo Sequencing

Quantity of STI affinity column eluted proteins was too less to isolate each protein band separately in sufficient quantity for MS/MS analysis. Therefore, total eluted proteins were accumulated at the interface of stacking and resolving portion of SDS–polyacrylamide gel by running for few minutes only. Accumulated protein band was excised, reduced with DTT (10 mM, 56 °C, 30 min), alkylated with iodoacetamide (50 mM, room temperature, 30 min) and digested with trypsin (Sequencing grade, Porcine, Promega, USA) at 37 °C for overnight in 50 mM ammonium bicarbonate buffer (pH 8.0) [27]. The digested peptides were extracted by sonication in 60 % acetonitrile (ACN) and 1 % trifluoroacetic acid (TFA), lyophilized, suspended in 5 µl 50 % ACN, 0.1 % TFA and 0.5 µl of the suspended peptides were spotted on MALDI plate followed by 0.5 µl alpha-cyano-4-hydroxycinnamic acid matrix (10 mg/ml in 50 % ACN, 0.1 % TFA). Spots were dried completely and used for MS/MS analysis on MALDI-TOF-TOF platform (model 4800, ABsciex, USA). The results obtained from MS/MS were analyzed by searching against the MSDB, Swiss-Prot and NCBI database with the following parameters (1) calibration error (0.005 Da), mass tolerance of fixed precursor ion (20 ppm), mass tolerance of fragment ion (0.05 Da), one missed cleavage, cysteines carbamidomethylation and possible oxidation of methionine. Common contaminants data was removed and remaining data was used in protein identification.

Extracted peptides were further used for de novo sequencing by N-terminal derivatization of trypsinized peptides [10]. For derivatization, trypsinized protein sample was recovered in 50 % acetonitrile containing 1 % trifluoroacetic acid (TFA), lyophilized and resuspended in 0.1 % TFA in water. ZipTip C18 (Millipore, USA) was wetted in 100 % acetonitrile (1–2×) and equilibrated with 0.1 % TFA (3–4×). Sample was adsorbed to the Zip Tip (10×) and washed with H2O (2×) and 0.5 M O-methyl iso-urea hydrogen sulphate (MIS) in 0.25 M Na_2CO_3 (pH 11.7, 1×). Tip was leaved at room temperature till MIS became aspirated and again kept into MIS solution for 3 h at 37 °C to completely saturate the C18 matrix. Tip was washed twice with H2O and 4-sulphophenyl iso-thiocyanate (SPITC) in 50 mM Tris–Cl (pH 8.2). SPITC was aspirated and tip was again saturated with SPITC solution by incubation at 50 °C for 2 h. Tip was washed four times with 0.1 % TFA, peptides were eluted in 10 ul of 80 % ACN containing 0.5 % TFA, lyophilized and dissolved in 50 % ACN, 0.1 % TFA. Derivetised and un-derivetised peptides were spotted in different wells of 384 well plate and used for peptide mass fingerprinting. Derivetised peptides (which were 215 or 257 Da more than their underivetised counterpart) were selected comparing the peptide mass fingerprinting of both the samples. Denovo sequencing of selected peptides was performed by using a laser intensity of 4200. Spectrum was speculated manually and sequence was derived by subtracting the mass of previous peak from next peaks automatically.

2.6 Proteases Activity Analysis on Zymograms

Proteases activity of saliva and midgut extract and STI interacting proteins was analysed by zymogram analysis [14]. Proteins were dissolved in non-reducing sample buffer (2 % SDS, 25 % glycerol, 60 mM Tris–HCl, pH 6.8, 0.1 % bromophenol blue). To prevent irreversible inactivation of protease, the samples were not boiled and electrophoresis was carried out at 4 °C. The gel was washed for 10 min in 2.5 % TritonX100 and rinsed in water. Gels were incubated in Glycine-NaOH buffer (50 mM, pH 10.0) containing 2 % casein for 1 h. Gels were rinsed again in water, stained with Coomassie brilliant blue R250 and destained briefly with destain solution followed by 5 % acetic acid for several hours. Protease activity was revealed as white clearing zone in a dark blue background.

2.7 Inhibition of Salivary and Midgut Proteases of Induced and Uninduced *H. armigera* Larvae by STI

Salivary and midgut extract of induced and uninduced larvae were separated in non-reducing condition and without heat denaturation on SDS-PAGE. Gel was washed with 2.5 % TritonX100, rinsed in water and incubated with STI (2 mg/ml) for 1 h. Gel was rinsed with water and used for zymogram analysis.

2.8 Degradation of STI by Salivary and Midgut Proteases

STI was incubated with the salivary and midgut extracts (1:1 total protein) in 20 mM, Tris–HCl buffer (pH 7.0) in two different sets. The reaction was stopped after 1 and 3 h by adding SDS-PAGE loading buffer followed by heat denaturation. Samples were analysed on SDS-PAGE. To study the protease bands responsible for the degradation of STI, zymogram analysis was carried by incubating gel with STI instead of casein [8].

3 Results

3.1 Salivary and Midgut Proteases of Induced and Uninduced *H. armigera* Larvae

Salivary and midgut proteases isolated from induced and uninduced insects were studied by zymogram analysis using casein as substrate (Fig. 1a, b). About 11 (S1–S11) and 8 (H1–H8) protease bands were observed in salivary and midgut extract of uninduced insects, respectively. The protease band S4 and H3 were found absent in case of saliva and midgut extracts of induced insects, respectively. No visible difference was observed between induced and uninduced with respect to other protease bands.

3.2 Inhibition of Salivary and Midgut Proteases of Induced and Uninduced *H. armigera* Larvae by STI

Zymogram analysis of induced and uninduced salivary and midgut proteases was performed after incubation with STI. The bands (S4 and H3) which were only visible in uninduced larvae vanished, while there was no effect on other bands that are common in induced and uninduced insects (Fig. 1c, d). The protease bands of induced insects were unaffected by the treatment with STI.

3.3 Isolation and Identification of STI Binding Proteins of Salivary and Midgut Extracts of *H. armigera* Larvae

STI binding proteins of salivary and midgut extracts of *H. armigera* larvae were isolated by affinity chromatography. SDS-PAGE analysis of eluted proteins showed several protein bands (Fig. 2a). Since, protein content in each

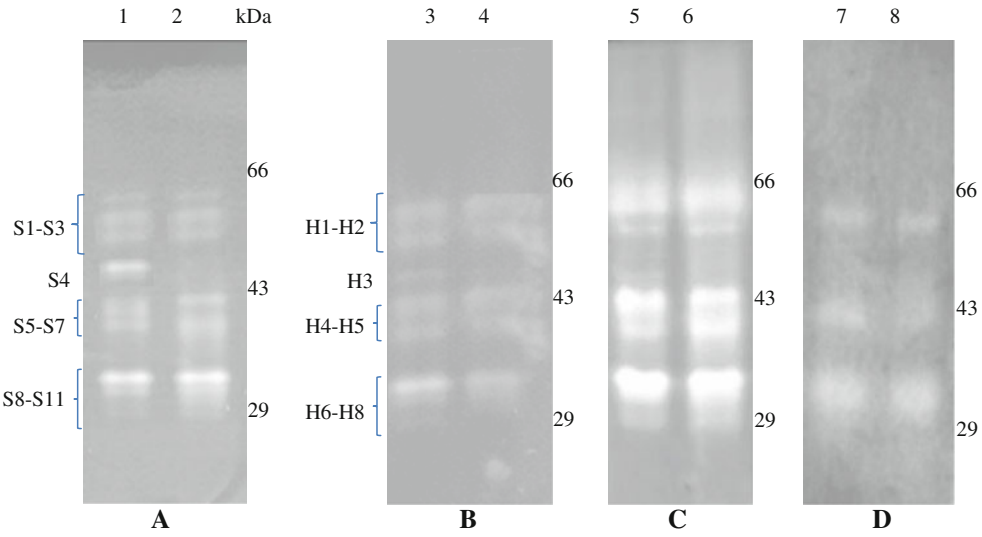

Fig. 1 Zymogram analysis of salivary and midgut proteases isolated from *Helicoverpa armigera* larvae. **a** Zymogram analysis of salivary proteases; *lane 1* uninduced; *lane 2* induced. **b** Zymogram analysis of midgut proteases; *lane 3* uninduced; *lane 4* induced. **c** Zymogram analysis of salivary proteases after soybean trypsin inhibitor (STI) treatment; *lane 5* uninduced; *lane 6* induced. **d** Zymogram analysis of midgut proteases after STI treatment; *lane 7* uninduced; *lane 8* induced

protein band was less; total eluted protein was used for MS/MS analysis and denovo sequencing on MALDI-TOF-TOF. MS/MS analysis did not show significant similarity with the reported database (data not included). This might be due to the limitation of database. Therefore, we proceeded for denovo sequencing of peptides. Total seven peptides were selected for denovo sequencing on the basis of intensity and N-terminal derivatization and used for protein sequencing (Fig. 3). The sequence obtained by denovo sequencing was used for protein identification by blast (blastp) analysis with lepidopteran database in Butterflybase (http://butterflybase.ice.mpg.de/blast/index.php).

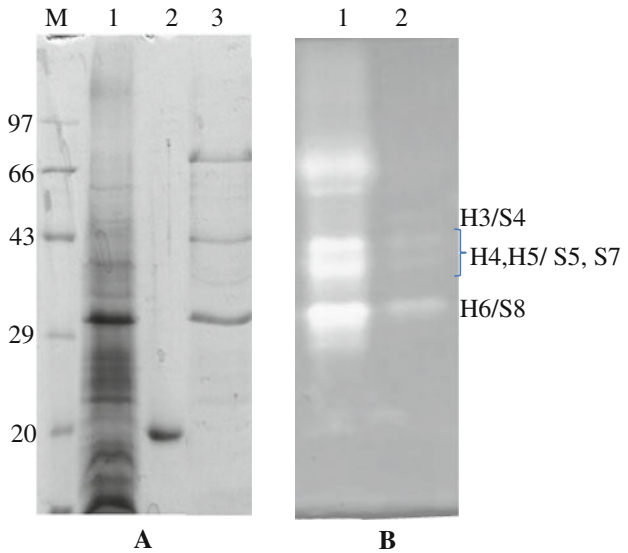

Fig. 2 SDS-PAGE and zymogram analysis of crude and eluted proteins from soybean trypsin inhibitor (STI) affinity column. **a** Coomassie stained SDS–polyacrylamide gel. LaneM; molecular weight marker, 1; crude protein, 2; STI, 3; eluted proteins. **b** Protease assay on zymogram. Lane 1; crude, 2; eluted

The matched ESTs were further blast against uniprot and used for Gene ontologies and functional analysis at the same server.

All the seven peptide sequences were matched with seven different sequences of lepidopterans (Table 1; Supplementary file 1). Blastp analysis of those protein sequences showed similarity with chymotrypsinogen, serine protease, aminopeptidase N, peroxidase, hypothetical protein and muscle specific protein.

3.4 Protease Activity of STI Binding Proteins

Proteases activity of STI binding proteins was analysed by caseinolytic activity on zymogram (Fig. 2b). Total four proteases bands corresponding to H3/S4, H4/S5, H5/S7 and H6/S8 were observed in zymogram.

3.5 Degradation of STI by Salivary and Midgut Proteases and Zymogram Analysis

STI was found almost completely degraded after 1 h of incubation with salivary proteases. However, degradation of STI was relatively less in case of treatment with midgut proteases (Fig. 4). At least four protease bands S5, S7, S8 and S9 of salivary preparation were found degrading the STI on zymogram (Supplementary Fig. 1).

4 Discussion

Feeding of *H. armigera* on diet containing STI did not cause any significant change in the mean weight of larvae (Supplementary Table 1) indicating its insensitivity to STI. Ineffectiveness of host plant protease inhibitor on larval development *H. armigera* has been reported [19]. Potato

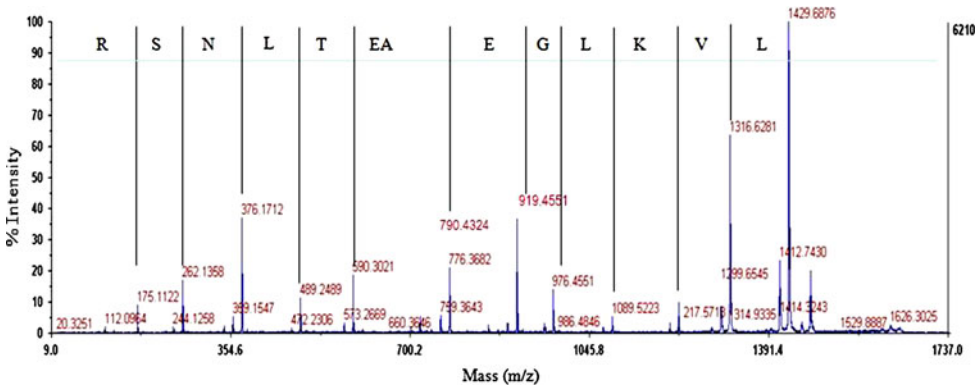

Fig. 3 Denovo sequencing of peptide 1. Amino acid sequences were obtained by subtracting the mass of a peak from the next peak mass. Similar procedure was followed for the sequencing of other peptides also

Table 1 Peptide sequences obtained after denovo sequencing, matched sequences and functional annotation

Peptide	Sequenced peptide	MW	Matched peptide	% Similarity	Matched sequences[a]	Functional annotation[b]
1	LVKLGEEATLNSR	1,473.69	LIKLSEEATLNSR	84	SFP07730_1 of *Spodoptera frugiperda*	Chymotrypsinogen
2	FTEWITTVETR	1,382.54	FTEWITTVETR	100	BMP015707_1 of *Bombyx mori*	Muscle-specific protein
3	FFETGFVDDVAR	1,402.53	FFETGFVDDVP	100	MSP01496_1 of *Manduca sexta*	Aminopeptidase
4	SPPYFQNVR	1,107.23	PPYFQNVR	88	HEP02564_1 of *Heliconius erato*	Serine protease
5	YPIPTEVANSPR	1,343.50	YPVPTEVANSPR	90	BMP013713_1 of *Bombyx mori*	Hypothetical protein
6	AVEVGNVR	830.90	AVEVGNSR	87	ONP00376_1 of *Ostrinia nubilalis*	Chymotrypsinogen
7	LKLTAGVR	857.06	LKLTAGVR	100	BMP042098_1 of *Bombyx mori*	Peroxidase

[a] Lepidopteran database at butterflybase server was used for blastp analysis of peptide sequences obtained in sequencing

[b] Functional annotation was determined after blastp analysis of matched sequences against uniprot database

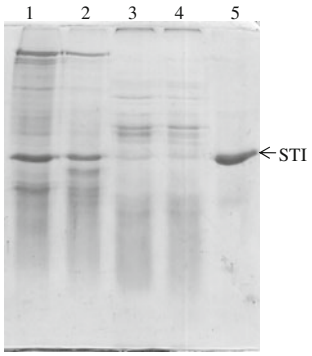

Fig. 4 SDS-PAGE analysis of degradation of soybean trypsin inhibitor (STI) by salivary and midgut proteases after 1 h of incubation. *Lane 1* Incubated with uninduced midgut proteases; *lane 2* Incubated with induced midgut proteases; *lane 3* Incubated with uninduced salivary proteases; *lane 4* Incubated with induced salivary proteases; *lane 5* STI without incubation

protease trypsin inhibitor (PPTI) and STI does not affect in vivo protein digestion in *H. zea* and *S. exigua* [5].

The protease band S4 and H3 were not detected in salivary and midgut extracts respectively in induced insects. Further, these were inhibited by trypsin specific inhibitors indicating their trypsin like nature. Several insects produce inhibitor insensitive proteases to counter protease inhibitors (PI) in their diet [3–7, 11, 20, 22]. Production of inhibitor resistant proteinases as an adaptive response to PI is also reported in *H. armigera* [3, 31]. Wu et al. [31] reported the induction of elastase like proteinases activity in the midgut of *H. armigera* larvae feed on transgenic tobacco plants expressing giant taro PI. *H. armigera* larvae fed on soybean cultivars L17 and Sahar which contain serine protease inhibitors, showed lowest tryptic activity but hyper production of chymotrypsin and elastase-like enzymes [24]. Gut trypsin (HzTrypsin-S) isolated form *Helicoverpa zea* raised on trypsin-inhibitor containing diet could not be inhibited by 1000 fold excess of inhibitors [30].

The proteins isolated from STI-affinity column predominantly consisted of protease other than the trypsin and showing similarity with proteases like, chymotrypsin, serine-type endopeptidase and aminopeptidase N. STI–binding proteases may be involved in degradation or inactivation of trypsin inhibitor. However, we did not find any degradation

of STI by STI-affinity column eluted proteins in spite of increase in time and amount eluted protein (data not shown). The STI binding proteins may be assisting serine proteases in degradation of STI. Serine proteinases in association with leucine aminopeptidases are reported to rapidly digest Oryzacystatin I and Bowman-Birk inhibitors [15].

Treatment of STI with salivary proteases resulted in its almost complete degradation; however, degradation of STI was relatively low in case of treatment with midgut proteases. The observation suggests that salivary proteases plays major role in degradation of STI as compared to midgut proteases in *H. armigera*. However, similar rate of degradation of STI was observed in both salivary and midgut protease of induced and uninduced insects. Since insects were fed with normal diet (without STI) for 1 day after feeding on STI, arthropods seem to be fast in recharging their protease pattern [26]. *H. armigera* gut proteases reported to proteolyse chickpea protease inhibitors (CPI) [17] and elastase like proteinases are responsible for inactivation of CPI [28]. PI degrading proteases are also reported in cowpea bruchid (*Callosobruchus maculatus*) [32]. Most of the reported studies isolated proteases from the midgut of *H. armigera*. However, in our results maximum degradation of STI was observed due to the salivary protease.

The gene encoding for inhibitors degrading protease can be isolated and cloned in heterologous expression system like *E. coli* for large scale production of such proteins. Trypsin inhibitors degrading protease may find their utility for the detoxification of human food items and feed for livestock where trypsin inhibitors function as anti-nutritional factor. Trypsin inhibitors have been reported in several food crops like cereals (wheat, oats, barley, and corn), pulses (mung beans and garden peas), oil seeds (soybeans and peanuts), potato and sweet potatoes which are important sources of dietary protein. Most of the PI's gets degraded in the rumen of ruminants [9] hence non toxic to them. However, in case of monogastric animals PI's causes adverse effects.

Acknowledgments Authors are grateful to the Council of Scientific and Industrial Research, Government of India. KC is thankful to CSIR-EMPOWER program for the financial support. SKU is thankful to CSIR for Senior Research Fellowship and GB Technical University for PhD registration. Authors are thankful to Rajesh K Srivastava for mass spectrometric analysis.

References

1. Ahn JE, Salzman RA, Braunagel SC, Koiwa H, Zhu-Salzman K (2004) Insect Mol Biol 13:649–657
2. Boulter D (1993) Phytochemistry 34:1453–1466
3. Bown DP, Wilkinson HS, Gatehouse JA (1997) Insect Biochem Mol Biol 27:625–638
4. Broadway RM (1997) J Insect Physiol 43:855–874
5. Broadway RM, Duffey SS (1986) J Insect Physiol 32:827–833
6. Brunelle F, Cloutier C, Michaud D (2004) Arch Insect Biochem Physiol 55:103–113
7. Brunelle F, Nguyen-Quoc B, Cloutier C, Michaud D (1999) Arch Insect Biochem Physiol 42:88–98
8. Chandrashekar K, Gujar GT (2004) Indian J Exp Biol 42: 164–173
9. Cheeke PR, Shull LR (1985). AVI Publishing Inc., West Port, Connecticut
10. Chen P, Nie S, Mi W, Wang X, Liang S (2004) Rapid Commun Mass Spectrom 18:191–198
11. Cloutier C, Jean C, Fournier M, Yelle S, Michaud D (2000) Arch Insect Biochem Physiol 44:69–81
12. Ehrlich PR, Raven PH (1964) Evolution 18:586–608
13. Felton GW (1996) Arch Insect Biochem Physiol 32:107–130
14. Garcia-Olmedo F, Salcedo G, Sanchez-Monge R, Gomez L, Roys J, Carbonero P (1987) Oxford Surv Plant Mol Cell Biol 4: 275–334
15. Girard C, Le Métayer M, Bonadé-Bottino M, Pham-Delègue MH, Jouanin L (1998) Insect Biochem Mol Biol 28:229–237
16. Giri AP, Chougule NP, Telang MA, Gupta VS (2005) Recent Res Dev Phytochem 8:117–137
17. Giri AP, Harsulkar AM, Deshpande VV, Sainani MN, Gupta VS, Ranjekar PK (1998) Plant Physiol 116:393–401
18. Green TR, Ryan CA (1972) Science 175:776–777
19. Harsulkar AM, Giri AP, Patankar AG, Gupta VS, Sainani MN, Ranjekar PK, Deshpande VV (1999) Plant Physiol 121:497–506
20. Jongsma MA, Bakker PL, Peters J, Bosch D, Stiekema WJ (1995) Proc Natl Acad Sci USA 92:8041–8045
21. Lawrence PK, Koundal KR (2002) EJB Elec J Biotechnol 5:1
22. Mazumdar-Leighton S, Broadway RM (2001) Insect Biochem Mol Biol 31:645–657
23. Michaud D, Cantin L, Vrain TC (1995) Arch Biochem Biophys 322:469–474
24. Naseri B, Fathipour Y, Moharramipour S, Hosseininaveh V, Gatehouse AMR (2010) Pest Manag Sci 66:1316–1323
25. Ryan CA (1990) Annu Rev Phytophathol 28:425–449
26. Schwarzenberger A, Zitt A, Kroth P, Mueller S, Von Elert E (2010) BMC Physiol 10:6
27. Shevchenk OA, Wilm M, Vorm O, Mann M (1996) Anal Chem 68:850–858
28. Telang MA, Giri AP, Sainani MN, Gupta VS (2005) J Insect Physiol 51:513–522
29. Upadhyay SK, Mishra M, Singh H, Ranjan A, Chandrashekar K, Verma PC, Singh PK, Tuli R (2010) Proteomics 10:4431–4440
30. Volpicella M, Ceci LR, Cordewener J, America T, Gallerani R, Bode W, Jongsma MA, Beekwilder J (2003) Eur J Biochem 270:10–19
31. Wu Y, Llewellyn D, Mathews A, Dennis ES (1997) Mol Breed 3:371–380
32. Zhu-Salzman K, Koiwa H, Salzman RA, Shade RE, Ahn JE (2003) Insect Mol Biol 12:135–145
33. Zhu-Salzman K, Zeng RS (2008) Insect Sci 15:477–481