The Asian and Australasian Journal of Plant Science and Biotechnology ©2012 Global Science Books

Influence of Partially Purified Soybean Protease Inhibitor on Second Instar Larvae of *Bactrocera cucurbitae* (Coquillett) (Diptera:Tephritidae)

Amrit Pal Kaur • Satwinder K. Sohal*

Department of Zoology, Guru Nanak Dev University, Amritsar-143005 (India)

Corresponding author: * satudhillon27@hotmail.com

ABSTRACT

One approach that can be employed in integrated pest management is the use of proteins with anti-nutritional effects on insect metabolism and development. The antimetabolic properties of partially purified soybean protease inhibitors were evaluated against second instar larvae of melon fruit fly, *Bactrocera cucurbitae* (Coquillett) (Diptera: Tephritidae). Different concentrations (12.5, 25, 50, 100, 200 and 400 µg/mL) of the partially purified inhibitor had a detrimental effect on the growth and development of the *B. cucurbitae* larvae tested in laboratory feeding bioassays. A decrease was observed in the larval weight gain, mean relative growth rate, food assimilation and survival of the treated larvae. Inhibitory effect of the partially purified inhibitor was also observed on percentage pupation and emergence which decreased to a maximum of 42.22% at 50 µg/mL and 18.86% at 400 µg/mL, respectively. The activity of four proteases (trypsin, chymotrypsin, elastase, leucine amino-peptidase), two antioxidant (Superoxide dismutase, Catalase) and four detoxification enzymes (esterases, acid phosphatases, alkaline phosphatases, glutathione-*S*-transferases) assessed after an interval of 24, 48 and 72 h revealed a suppression in trypsin and chymotrypsin activity at all concentrations. Trypsin was maximally inhibited by 91.08% at 400 µg/mL while chymotrypsin showed a maximum inhibition of 62.02% at 200 µg/mL. The activity of elastase varied while that of leucine aminopeptidase increased after prolonged treatment. The activity of superoxide dismutase, catalase, phosphatases and glutathione-*S*-transferases also increased with treatment at most of the exposure intervals. The findings revealed the potential of the partially purified protease inhibitors to disrupt the development of the melon fruit fly.

Keywords: artificial diet, Diptera, enzyme assay, *Glycine max*, insect bioassay, melon fruit fly

Abbreviations: **AcP**, acid phosphatases; **AkP**, alkaline phosphatases; **ANOVA**, analysis of variance; **BSA**, bovine serum albumin; **BA*p*NA**, N-α-benzoyl-DL-arginine-*p*-nitroanilide; **BT*p*NA**, N-benzoyl-L-tyrosine-*p*-nitroanilide; **GST**, glutathione *S*-transferases; **LAP**, leucine amino-peptidase; **L*p*NA**, L-leucine-*p*-nitroanilide; **MRGR**, mean relative growth rate; **PI**, protease inhibitor; **SAAPL*p*NA**, N-succinyl-alanine-alanyl-prolyl-leucine-*p*-nitroanilide; **SBTI**, soybean trypsin inhibitor; **SOD**, superoxide dismutase

INTRODUCTION

Plant-derived protease inhibitors (PIs), which have evolved as part of the plant's natural defense system, are increasingly being recognised for their potential in pest management (Chauhan 2012). The defensive role of plant PIs relies on the inhibition of proteinases present in the insect gut or secreted by microorganisms, thus causing a reduction in the availability of amino acids necessary for their growth and development (De Leo *et al*. 2002).

Plant PIs are grouped primarily as serine, cysteine, aspartic, or metallo-PIs (Laskowski and Qasim 2000). Of these, the serine PIs are the most potent inhibitors of the normal functioning of the specific enzymes in insects. In insect gut, among serine proteases, trypsins, and chymotrypsins are the most commonly reported enzymes acting in a wide range of physiological processes including digestion, protein activation in the melanization cascade, antibacterial activity, and insect immune response (Nakajima *et al.* 1997; Gorman *et al.* 2000a, 2000b; Ma and Kanost 2000).

Serine PIs have been isolated from various leguminous plants (Giri *et al.* 2003) and their effect have been studied on various lepidopteran insects but a lacunae has been observed with respect to their influence on dipteran insect pests. Fruit flies are among the top tephritid pests of economic importance and their control with synthetic insecticides has led to increased environmental concerns necessitating that alternative strategies be sought for their control (Haq *et al*. 2010b). The melon fruit fly, *Bactrocera cucurbitae* (Coquillett), an economically important pest of fruits and vegetables is particularly considered as a destructive pest of cucurbits (White and Elson-Harris 1992; Koyama *et al*. 2004). It causes severe losses (up to 100% of crop loss) directly by damaging fruits and vegetables and also because of its quarantine status; its presence seriously interferes with the international marketing of these agricultural products (Haq *et al*. 2010a). As most dipteran pests largely depend on serine proteinases for digestion of food proteins (Gomes *et al*. 2005), the present study was envisaged to evaluate the biopesticidal potential of partially purified protease inhibitor from soybean against *B. cucurbitae* by monitoring their effect on growth, survival and enzyme system of the melon fruit fly. Their evaluation as a biopesticide would be the first step towards creating biologically and ecologically safe control measures against this fly.

MATERIALS AND METHODS

Sources of materials

Seed samples of soybean, (*Glycine max* L. cv. 'Merrill') were procured from the local market for the extraction and partial purification of protease inhibitors. Seeds were identified from the Department of Botanical and Environmental Sciences, Guru Nanak Dev University, Amritsar with accession no. 0405/HRB.

Bovine serum albumin (BSA) was obtained from Loba (India).

Received: 19 June, 2012. Accepted: 5 November, 2012.

Original Research Paper

Bovine trypsin, N-α-benzoyl-DL-arginine-*p*-nitroanilide (BA*p*NA), N-benzoyl-L-tyrosine-*p*-nitroanilide (BT*p*NA), N-succinyl-alanine-alanyl-prolyl-leucine-*p*-nitroanilide (SAAPL*p*NA) and L-leucine-*p*-nitroanilide (L*p*NA) were obtained from Sigma-Aldrich, Delhi, India.

Partial purification

Soybeans were soaked in 10 mM potassium phosphate buffer (pH 7.2) overnight (Duranti *et al*. 2003). Next day, the soaked material was filtered through multiple layers of surgical gauze and centrifuged at 10,000 rpm for 15 min at 4°C. Clear supernatant was stored at -20°C as crude extract and protease inhibitor activity was determined. It was then subjected to 0-80% direct ammonium sulphate saturation and kept overnight at 4°C. Centrifugation was done at 10,000 rpm for 15 min at 4°C on the next day. Precipitates were collected by dissolving in minimum known volume of buffer. The dissolved fractions were dialyzed in cold for about 96 hours against distilled water. The dialyzed partially purified protease inhibitor from soybeans was stored at -20°C for further experimental work.

Protein estimation

Protein estimation was done in crude as well as partially purified preparations by the method of Lowry *et al.* (1951) using bovine serum albumin (BSA; Loba, India) as the standard, for preparing various test concentrations for bioassay and enzyme studies.

Trypsin inhibition assay

Trypsin residual activity was estimated by hydrolysis of BA*p*NA in the presence of inhibitor (Paulino da Silva *et al*. 2001). The enzyme was pre-incubated with partially purified inhibitor as well as with crude extract for 15 min at room temperature in 50 mM Tris-Hcl buffer (pH 8.2). The assayed residual activities were followed by hydrolysis of 1 mM BA*p*NA and the liberation of *p*-nitroaniline, which was measured at 410 nm.

Insect rearing

The cultures of melon fruit fly were maintained on natural food in the insect culture room/B.O.D incubator with controlled temperature (25±2°C), relative humidity (70-80%) and photo phase (10L: 14D) (Gupta *et al.* 1978). Adult flies were provided 20% sugar solution and protinex (Pfizer, Delhi, India) in Petri dishes as food and pieces of pumpkin fruit, *Cucurbitae moschata* (Dusch.) for oviposition.

Insect bioassays

About 100 gravid females were released in wire mesh cages provided with fresh pumpkin pieces for 8 h. The pumpkin pieces were removed at an appropriate time interval from the cages and dissected in saline water for harvesting the second instar (64-72 h old) larvae. The harvested larvae were transferred to culture tubes (25 mm diameter × 100 mm length) containing artificial diet incorporated with various concentrations (viz. 12.5, 25, 50, 100, 200 and 400 µg/mL) of partially purified protease inhibitor. The artificial diet (control as well as treated) was prepared according to the standardized methodology given by Srivastava (1975). Observations were made after three time intervals (24, 48, 72 h) for the larval weight gain, mean relative growth rate (MRGR), food assimilated and their survival. There were six replications with 15 larvae in each replication for each concentration and each experiment was repeated twice.

The mean relative growth rate which provides a measure of the rate of change in weight in units of mg/mg/day was calculated according to the formula given by Martinez and Emden (2001). Food assimilated with respect to control was also assessed after the treatment of second instar larvae with a range of concentrations of partially purified soybean PI (Khan and Saxena 1985).

Enzyme assays

In order to determine the potential of protease inhibitors in reducing the digestibility of the insect gut proteinases, the effect of partially purified soybean PI was ascertained against *B. cucurbitae* gut proteinases by monitoring the activity of larval gut trypsin-like enzyme with BA*p*NA, chymotrypsin-like enzyme with BTPNA, elastase-like enzyme with SAAPL*p*NA and leucine-aminopeptidase-like enzymes using L*p*NA as substrate (Christeller *et al*. 1990, 1992). The activity of each enzyme was monitored for three consecutive time intervals (24, 48 and 72 h) after feeding second instar larvae of melon fruit fly on artificial diet incorporated with four concentrations (viz. 50, 100, 200 and 400 µg/mL) of partially purified PIs from soybean. The influence of partially purified soybean PI was also investigated on the activity of some detoxification (esterases, acid phospatases, alkaline phosphatases and glutathione *S*-transferases) and antioxidant enzymes (superoxide dismutase and catalase) in second instar larvae in order to ascertain their role in counteracting the metabolic stress produced by the ingestion of protease inhibitors. The larvae (64-72 h old) harvested from the pumpkin pieces were transferred to artificial diet containing different concentrations of the partially purified PI. The *ad libitum* feeding to these larvae was also given for three time intervals i.e. 24, 48 and 72 h. Activity determination of esterases was based on the methodology outlined by Katzenellenbogen and Kafatos (1971). 1 mM α-naphthyl acetate was used as a substrate and 0.1M sodium phosphate buffer (pH 6.5) as an extraction buffer. The activity of acid (AcP) and alkaline phosphatases (AkP) was assessed by the procedure given by McIntyre (1971) using the substrate 0.005M sodium α-naphthyl phosphate in 0.05M acetate buffer, pH 5.0. However, acid phosphatases were extracted in 0.05M acetate buffer (pH 5.0) and alkaline phosphatases were extracted in 0.05M Tris buffer (pH 8.6). Superoxide dismutase (SOD) was investigated by following the methodology provided by Kono (1978). Hydroxylamine hydrochloride (20 mM, pH 6.0) was used as a substrate solution and sodium carbonate buffer (50 mM, pH 10.0) as an extraction buffer. Catalase activity was determined by using 0.05% Hydrogen peroxide (H_2O_2) as a substrate solution and potassium phosphate buffer (0.05M, pH 7.0) as an extraction buffer as described by Bergmeyer *et al.* (1974). Glutathione-S-transferase activity (GST) was obtained by using sodium phosphate buffer (0.1M), pH 7.6 as an extraction buffer and 10 mM CDNB in 95% ethanol as a substrate solution according to the methodology given by Chien and Dauterman (1991). Each experiment had six replications and was repeated twice.

Statistical analysis

SPSS 10.0 computer program was used for the statistical analysis. The results were expressed as mean ± S.E. The means were compared using one way analysis of variance (ANOVA). Statistical differences were determined by Tukey post-hoc test. The means obtained for the enzymatic activities at all three exposure intervals were compared for their significance by *t*-test.

RESULTS AND DISCUSSION

Bioassay

1. Effect on growth

The effect of partially purified soybean PI (**Table 1**) on the larval weight gain, growth rate, food assimilated and survival of *B. cucurbitae* larvae was assessed by feeding the second instar larvae on artificial diet incorporated with different concentrations of PI. The larvae fed on control diet showed better development as compared to those fed on partially purified inhibitors from soybean when observed at different time intervals. A decrease in weight of larvae with treatment was observed which was significantly more at higher concentrations (**Table 2**). A similar effect was observed in the Mediterranean fruit fly, *Ceratatis capitata* (Wiedemann) where Soybean trypsin inhibitor affected larval mass at ED_{50} 3.01% (Silva *et al*. 2006). Likewise, the lepidopterans, African cotton leaf worm, *Spodoptera litto-*

Table 1 Partial purification of soybean protease inhibitor from seeds of *Glycine max* L.

Purification step	Total Activity	Total Protein (mg)	Specific Activity	Recovery (%age)	Purification fold
Crude	402.25	1560	0.26	100	1
Partially purified	380	331.1	1.15	94.47	4.42
Supernatant	-	1170	-	-	-

Table 2 Effect of partially purified soybean protease inhibitor on larval weight gain of second instar larvae of *Bactrocera cucurbitae.* Means within a column followed by the same letter are not significantly different, p>0.05; based on Tukey's test.

Concentration	Larval weight gain		
Used (µg/mL)	After 24 h	After 48 h	After 72 h
Control	14.75 ± 3.298 ab	41.43 ± 4.411 a	88.92 ± 6.066 a
12.5	14.63 ± 1.274 ab	33.82 ± 5.128 b	86.12 ± 6.040 a
25	15.67 ± 3.296 ab	28.85 ± 3.661 bc	63.40 ± 2.307 b
50	14.02 ± 2.396 ab	17.93 ± 1.743 d	58.63 ± 4.053 b
100	16.52 ± 1.663 a	23.22 ± 2.593 cd	63.52 ± 0.884 b
200	13.00 ± 1.112 ab	22.62 ± 1.353 d	50.37 ± 3.348 c
400	11.68 ± 1.347 b	18.33 ± 1.680 d	40.47 ± 1.972 d
F- value	3.15*	42.62**	118.49**

*significant at 0.05%, **significant at 0.01%

Table 3 Effect of partially purified soybean protease inhibitor on Mean relative growth rate (MRGR) of second instar larvae of *Bactrocera cucurbitae.* Means within a column followed by the same letter are not significantly different, p>0.05; based on Tukey's test.

Concentration	Mean Relative Growth Rate		
Used (µg/mL)	After 24 h	After 48 h	After 72 h
Control	0.12 ± 0.024 bc	0.15 ± 0.021 a	0.16 ± 0.010 b
12.5	0.13 ± 0.010 abc	0.14 ± 0.021 a	0.19 ± 0.012 a
25	0.16 ± 0.033 a	0.11 ± 0.014 b	0.13 ± 0.009 da
50	0.13 ± 0.019 abc	0.08 ± 0.015 bc	0.14 ± 0.005 cd
100	0.14 ± 0.016 ab	0.10 ± 0.009 bc	0.15 ± 0.005 b
200	0.12 ± 0.009 bc	0.09 ± 0.009 bc	0.12 ± 0.005 c
400	0.11 ± 0.010 c	0.07 ± 0.008 c	0.09 ± 0.011 d
F- value	4.56**	23.75**	70.20**

**significant at 0.01%

Table 4 Effect of partially purified soybean protease inhibitor on food assimilated with respect to control in second instar larve of *Bactrocera cucurbitae.* Means within a column followed by the same letter are not significantly different, p>0.05; based on Tukey's test.

Concentration	Food assimilated wrt control		
Used (µg/mL)	After 24 h	After 48 h	After 72 h
12.5	2.88 ± 0.264 ab	7.33 ± 0.618 ab	15.73 ± 1.151 a
25	2.73 ± 0.429 ab	7.55 ± 0.916 a	16.05 ± 1.049 a
50	2.77 ± 0.424 ab	6.24 ± 0.760 c	14.05 ± 1.237 b
100	3.05 ± 0.266 a	6.29 ± 1.032 c	14.24 ± 0.989 b
200	2.63 ± 0.274 b	6.83 ± 0.936 bc	13.59 ± 0.529 bc
400	2.54 ± 0.416 b	6.34 ± 0.642 c	12.88 ± 1.356 c
F- value	1.56NS	2.80*	7.80**

NSnon-significant, *significant at 0.05%, **significant at 0.01%

ralis (Boisduval), European corn borer, *Ostrinia nubilalis* (Hubner), and cluster caterpillar, *S. litura* (Fabricius) gained significantly less weight on Soybean protease inhibitor incorporated diet than the control (Steffens *et al*. 1978; McManus and Burgess 1995; Dorrah 2004). Casu *et al.* (1994) had also observed a strong reduction in larval weight when soybean Kunitz trypsin inhibitor (SKTI) was added at a concentration of 1.15% in artificial diet of sheep blow fly, *Lucilia cuprina* Wiedemann. The mean relative growth rate of second instar larvae of *B. cucurbitae* decreased significantly after 48 and 72 h of feeding on partially purified protease inhibitors from soybean (**Table 3**). Although the decrease in MRGR showed no correlation with increase in concentration but it was considerably more at higher concentrations. A decreased growth rate with soybean proteinase inhibitor has also been reported in the larvae of

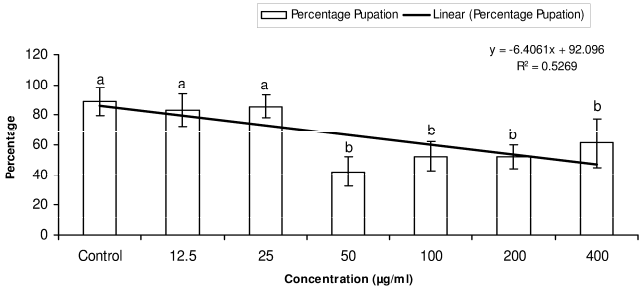

Fig. 1 Effect of partially purified soybean protease inhibitor on percent pupation of the melon fruit fly, when second instar larvae were fed on treated diet. The bars are the mean ± S.E. Different letters indicate mean values that are significantly different (p<0.01; Tukey test).

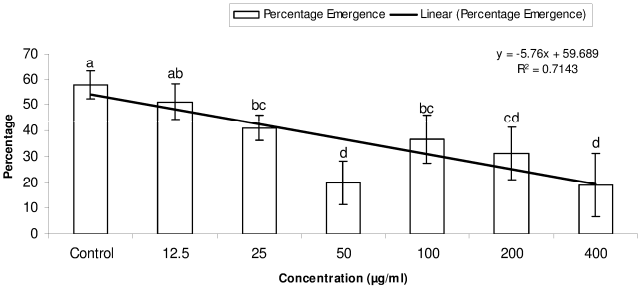

Fig. 2 Effect of partially purified soybean protease inhibitor on percent emergence of the melon fruit fly, when second instar larvae were fed on treated diet. The bars are the mean ± S.E. Different letters indicate mean values that are significantly different (p<0.01; Tukey test).

sugarcane borer, *Diatraea saccharalis* (Fabricius) (Pompermayer *et al*. 2001) and in European corn borer, *O. nubilalis* (Larocque and Houseman 1990). The observations made for the food assimilated showed that although the difference in the food assimilated with respect to control in treated larvae was not statistically significant at early 24 h exposure, but, thereafter, the difference in the means varied significantly (p<0.01) between different treatment groups at 48 and 72 h exposure interval (**Table 4**). After 72 h the food assimilated decreased almost in a consistent manner with increase in concentration. Adverse effects of partially purified protease inhibitors were also observed on percentage pupation and emergence which decreased significantly with treatment (**Figs. 1, 2**). Lower pupation and emergence has also been observed in the noctuid, *Helicoverpa armigera* (Hübner) fed on diets impregnated with soybean trypsin inhibitor (Shukla *et al*. 2005). In a previous study, Johnston *et al.* (1993) had observed inability of *H. armigera* larvae to survive on soybean trypsin inhibitor (SBTI) incorporated diet. The survival of sunn pest, *Eurygaster integriceps* Puton was also affected significantly by the presence of soybean trypsin inhibitor in the diet (Saadati and Bandani 2011).

Enzyme assay

1. Effect on larval digestive proteases

To effectively establish a novel insect control strategy based on proteinaceous inhibitors, knowledge of the digestive system of target insect is necessary. The digestive system of phytophagous pests is based mainly on serine and cysteine proteinases classes: serine proteinases are the major enzymes found in Lepidoptera and Diptera orders (Gomes *et al*. 2005). As the proteinase inhibitors in the seeds of plants of the leguminosae family primarily belong to the serine class of PIs, their ability to inhibit the activity of the digestive proteases of larvae of *B. cucurbitae* was investigated. Among the four digestive enzymes, a significant inhibition of trypsin (**Fig. 3A**) and chymotrypsin (**Fig. 3B**) was observed at all tested concentrations as well as at all exposure

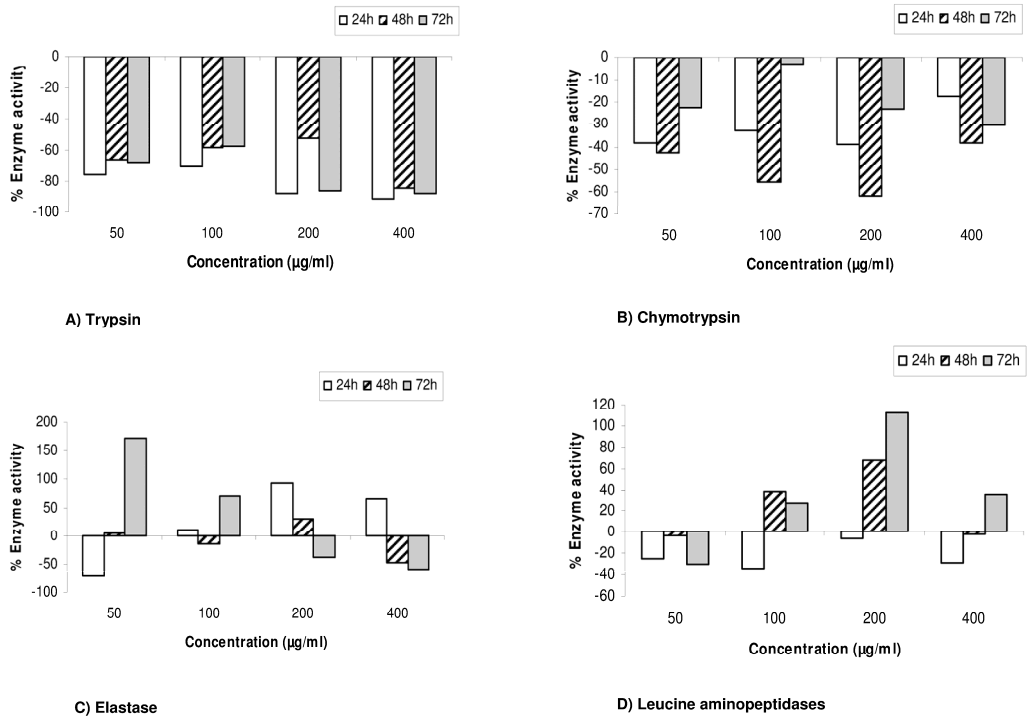

Fig. 3 The influence of partially purified soybean protease inhibitor on the activity of digestive enzymes (A-D), of second instar larvae (64-72 h) of *Bactrocera cucurbitae*.

intervals. Maximum inhibition in trypsin activity was observed at the highest concentration of 400 µg/mL where the activity was reduced to 91.08, 84.06 and 87.95% at 24, 48 and 72 h treatment interval, respectively with respect to the control. However, in case of chymotrypsin, maximum inhibition was observed after 48 h of treatment at all exposure intervals. The inhibition was more at 200 µg/mL concentration where the activity was inhibited by 62.02%. On the other hand, the activity of elastase showed a varied trend with increase in concentration (**Fig. 3C**). The activity which was less or not much different from control at lower concentrations (50 and 100 µg/mL) during the initial exposure intervals increased significantly after 72 h. However, at 200 and 400 µg/mL, the activity after showing an induction during the initial treatment given for 24h decreased more than control after 72 h of treatment. The Leucine aminopeptidase (LAP) activity was suppressed at all exposure intervals at 50 µg/mL but at higher concentrations the activity after showing a decrease at 24 h exposure interval, increased when the treatment was prolonged for another 48 h (**Fig. 3D**). Soybean trypsin inhibitor has also been shown to affect the growth and digestive physiology of *H. armigera* (Wang and Qin 1996). McManus and Burgess (1995) had also found that SBTI was particularly effective at inhibiting the trypsin-like activity, slightly effective against the elastase-like and chymotrypsin esterase-like activity and was ineffective against the exopeptidase, leucine amino peptidase in the larvae of *S. litura*. Dorrah *et al.* (2008) had also reported inhibition in the trypsin like activity of the anterior midgut of larvae of flesh fly, *Parasarcophaga hertipes* by SBTI.

2. Effect on antioxidant and detoxification enzymes

PIs through binding to the digestive proteases of phytophagous insects not only impair protein digestion (Broadway and Duffey 1986) but could also affect moulting and non-digestive enzyme regulation (Faktor and Raviv 1997). The investigations pertaining to the effect of partially purified soybean PI on other enzymes involved in antioxidant and detoxification mechanisms revealed a significant increase in the activities of SOD and Catalase. The SOD activity increased significantly with increase in treatment time up to 48 h but decreased on prolongation of treatment time to 72 h (**Fig. 4A**). The catalase activity which was either suppressed or not much different from control at the initial treatment interval of 24 h increased significantly after prolongation of the treatment time to 48 and 72 h in all the concentrations (**Fig. 4B**). SOD provides the first line of defense against toxicity from free radicals generated during metabolism (Paes *et al.* 2001; Wang 2001). SOD dismutates the free superoxide radical (O_2^-) to H_2O_2 which in turn is eliminated by catalase thereby indicating a quantitative relationship between SOD and catalase (Felton and Summers 1995).

The role of esterases in the growth and development of insects is very well established. They have been associated with insecticidal resistance in over 50 species of insects, where their functional role involves xenobiotic metabolism and sequestration (Devorshak and Roe 1999; Yu 2004). In the present study the esterase activity was suppressed significantly during the initial treatment interval of 24 and 48 h in almost all treatments and their induction only after prolonged treatment at 100 and 400 µg/mL (**Fig. 4C**) indicates that they might not be playing a significant role in the metabolism of plant protease inhibitors.

Phosphatases play an important role in the metabolism of nucleotides, carbohydrates, phospholipids and proteins (Rockstein 1956; Chaubey and Bhatt 1988). They have also been implicated in the development of resistance to insecticides (Oppenorth 1985). In the larvae of *B. cucurbitae*, the activity of acid phosphatase was found to be induced in most of the treatments but at 400 µg/mL the activity showed a maximum increase of 58.86% at 72 h treatment interval as compared to control (**Fig. 4D**). The alkaline phosphatase also showed an induction at most treatment intervals (**Fig. 4E**). The enzyme activity after showing an increase at 24 h got suppressed at 48 h, but again increased significantly after 72 h of treatment at 100, 200 and 400 µg/mL. Contrary to the present findings, the activity of acid and alkaline phosphatase was found to decrease in the larvae of *B. cucurbitae* treated with lectins (another plant defense protein) from *R. communis* and *G. max* (Singh *et al.* 2006a, 2006b). The increase in the activity of phosphatases in the melon fly larvae observed in the present study could be either due to the increased requirements of energy for counteracting the toxic effects of the PI or they might be involved in the metabolism of the inhibitors.

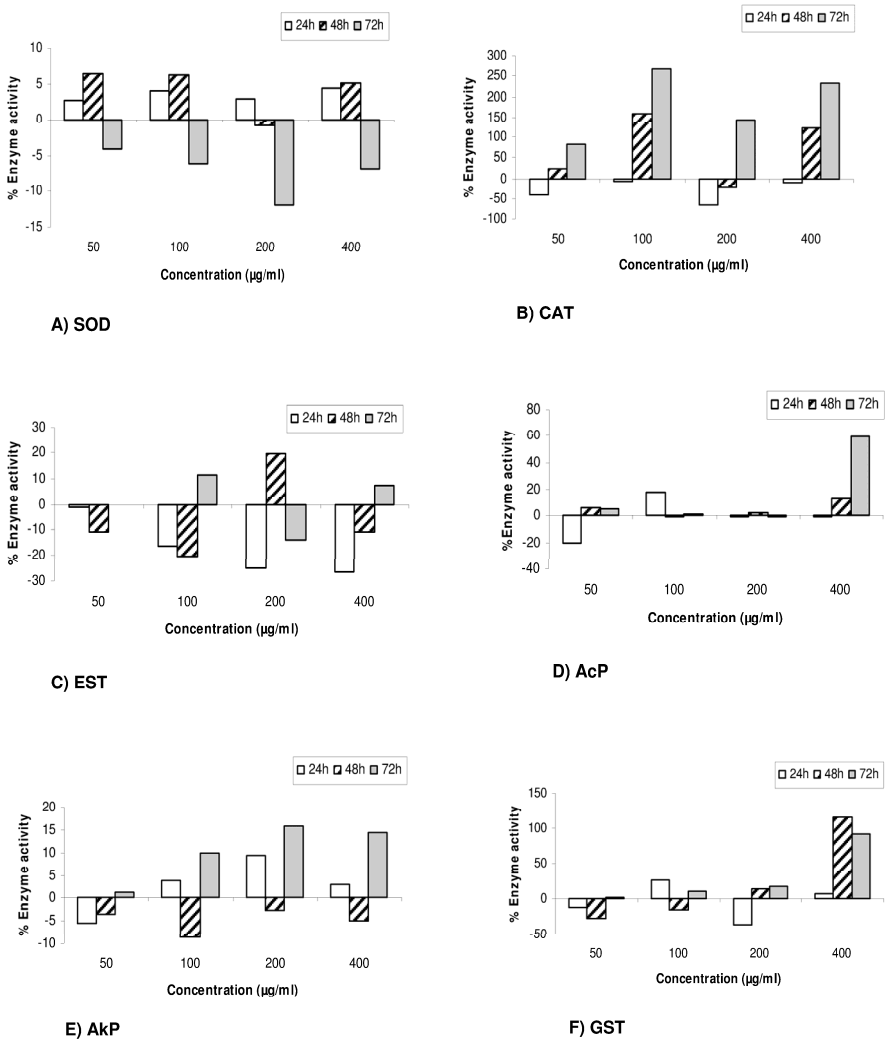

Fig. 4 The influence of partially purified soybean protease inhibitor on the activity of detoxification enzymes (A, B) and anti-oxidant enzymes (C-F) involved in growth and development of second instar larvae (64-72 h) of *Bactrocera cucurbitae*.

A significant induction in GST activity at all exposure intervals was observed only at the highest concentration of 400 µg/mL (**Fig. 4F**). At other concentrations, the enzyme activity was suppressed at the initial exposure interval but was induced with prolonged treatment suggesting that these enzymes which are generally involved in the detoxification of both endogenous and xenobiotic compounds might have some role in the metabolism of the plant protease inhibitor fed through artificial diet to the larvae of melon fruit fly. Singh *et al.* (2006b) had also reported an increase in the GST activity in the larvae of melon fruit fly when treated with legume lectin, *G. max*.

CONCLUSIONS

The retarded growth and development of the *B. cucurbitae* larvae caused by partially purified soybean PI could be due to their inhibitory action on insect digestive proteases, particularly trypsin and chymotrypsin. Since trypsin is involved in developmental processes such as molting and synthesis of neuropeptides, trypsin inhibitors can disrupt these processes thereby severely impairing the growth of the larvae (Lipke *et al*. 1954; Shukle *et al*. 1985; Broadway and Duffey 1986). Also, an increase in the activity of most of the other enzymes clearly showed an adaptation on part of the insect to counteract the metabolic stress generated due to nutritional imbalance created by ingestion of partially purified inhibitors.

It can be concluded that although partially purified PIs from soybeans ingested via artificial diet, were not acutely toxic to *B. cucurbitae*, the sublethal effects on life history parameters measured in this study must be considered in a broader context to determine their possible ecological significance.

ACKNOWLEDGEMENTS

The grant received from University Grant Commission (F. No. 34-472/2008 SR) is gratefully acknowledged.

REFERENCES

Bergmeyer HU, Gawehn K, Grassl M (1974) Enzymes as biochemical reagents. In: Bergmeyer HU (Ed) *Methods of Enzymatic Analysis*, Academic Press, New York, pp 425-522

Broadway RM, Duffey SS (1986) The effect of dietary protein on the growth and digestive physiology of larval *Heliothis zea* and *Spodoptera exigua*. *Journal of Insect Physiology* **32**, 673-680

Casu RE, Pearson RD, Jarmey JM, Cadogan LC, Riding GA, Tellam RL (1994) Excretory/secretory chymotrypsin from *Lucilia cuprina*: purification, enzymatic specificity and amino acid sequence deduced from mRNA. *Insect Molecular Biology* **3**, 201-211

Chaubey SN, Bhatt RS (1988) Changes in the levels of nucleic acid, protein, total free amino acid and glycogen and activity of Acid phosphatases in the eggs, during normal embryonic development of rice moth, *Corcyra cephalonica* Stainton (Lepidoptera: Pyralidae). *Insect Biochemistry* **18**, 443-447

Chauhan RD (2012) Protease inhibitors. *Functional Plant Science and Biotechnology* **6 (Special Issue 1)**, 107 pp

Chien C, Dauterman WC (1991) Studies on Glutathione *S*-transferases in *Helicoverpa* (=*Heliothis*) *zea*. *Insect Biochemistry* **21**, 857-864

Christeller JT, Laing WA, Shaw BD, Burgess EPJ (1990) Characterization and partial purification of the digestive proteases of the black field cricket, *Telleogryllus commodus* (Walker): Elastase is a major component. *Insect Biochemistry* **20**, 157-164

Christeller JT, Laing WA, Markwick NP, Burgess EPJ (1992) Midgut protease activities in 12 phytophagous lepidopteran larvae: Dietary and protease inhibitor interactions. *Insect Biochemistry and Molecular Biology* **22**, 735-746

De Leo F, Volpicella M, Licciulli F, Liuni S, Gallerani R, Ceci LR (2002) Plant-PIs: A database for plant protease inhibitors and their genes. *Nucleic Acids Research* **30**, 347-348

Devorshak C, Roe RM (1999) The role of esterases in insecticide resistance. *Reviews in Toxicology* **2**, 501-537

Dorrah MA (2004) Effect of soybean trypsin inhibitor on digestive proteases and growth of larval *Spodoptera Littoralis* (Boisd). *Efflatounia* **4**, 23-30

Dorrah MA, Elmelegi MK, Bassal TTM (2008) Trypsin- and chymotrypsin esterase-like activity in the midgut regions of the larval *Parasarcophaga hertipes* (Sarcophagidae, Cyclorrhapha, Diptera). *Efflatounia* **8**, 1-9

Duranti M, Barbiroli A, Scarafoni A, Tedeschi G, Morazzoni P (2003) One-step purification of Kunitz soybean tripsin inhibitor. *Protein Expression and Purification* **30**, 167-170

Faktor O, Raviv M (1997) Inhibition of molt in *Spodoptera littoralis* larvae treated with soybean Bowman-Birk protease inhibitor. *Entomologia Experimentalis et Applicata* **82**, 109-113

Felton GW, Summers CB (1995) Antioxidant systems in insects. *Archives of Insect Biochemistry and Physiology* **29**, 187-197

Giri AP, Harsulkara AM, Ku MSB, Gupta VS, Deshpande VV, Ranjekar PK, Franceschi VR (2003) Identification of potent inhibitors of *Helicoverpa armigera* gut proteinases from winged bean seeds. *Phytochemistry* **63**, 523-532.

Gomes APG, Dias SC, Broch Jr. C, Melo FR, Furtado Jr. JR, Monnert RG, Grossi-de-Sa MF, Franco OL (2005) Toxicity to cotton boll weevil *Anthonomus grandis* of a trypsin inhibitor from chick pea seeds. *Comparative Biochemistry and Physiology* **140**, 313-319

Gorman MJ, Andreeva OV, Paskewitz SM (2000a) Molecular characterization of five serine protease genes cloned from *Anopheles gambiae* haemolymph. *Insect Biochemistry and Molecular Biology* **30**, 35-46

Gorman MJ, Andreeva OV, Paskewitz SM (2000b) Sp22D: A multidomain serine protease with a putative role in insect immunity. *Gene* **251**, 9-17

Gupta JN, Verma AN, Kashyap RK (1978) An improved method for mass rearing for melon fruit fly *Dacus cucurbitae* Coquillett. *Indian Journal of Entomology* **40**, 470-471

Haq I, Cáceres C, Hendrichs J, Teal PEA, Wornoayporn V, Stauffer C, Robinson AS (2010a) Effects of the juvenile hormone analogue methoprene and dietary protein on male melon fly *Bactrocera cucurbitae* (Diptera: Tephritidae) mating success. *Journal of Insect Physiology* **56**, 1503-1509

Haq I, Mayr L, Teal PEA, Hendrichs J, Robinson AS, Stauffer C, Hood-Nowotny R (2010b) Total body nitrogen and total body carbon as indicators of body protein and body lipids in the melon fly *Bactrocera cucurbitae*: Effects of methoprene, a juvenile hormone analogue, and of diet supplementation with hydrolysed yeast. *Journal of Insect Physiology* **56**, 1807-1815

Johnston KA, Gatehouse JA, Anstee JH (1993) Effects of soybean protease inhibitors on the growth and development of larval *Helicoverpa armigera*. *Journal of Insect Physiology* **39**, 657-664

Katzenellenbogen B, Kafatos FC (1971) General esterases of silkmoth moulting fluid: Preliminary characterization. *Journal of Insect Physiology* **17**, 1139-1151

Khan ZR, Saxena RC (1985) Behavioral and physiological responses of *Sogatella furcifera* (Homoptera: Delphacidae) to selected resistant and susceptible rice cultivars. *Journal of Economic Entomology* **78**, 1280-1286

Kono Y (1978) Generation of superoxide radical during auto-oxidation of hydroxylamine and an assay for Superoxide dismutase. *Archives of Biochemistry and Biophysics* **186**, 189-195

Koyama T, Kakinohana H, Miyatake T (2004) Eradication of the melon fly *Bactrocera cucurbitae* in Japan: Importance of behaviour, ecology, genetics and evolution. *Annual Review of Entomology* **49**, 331-349

Larocque AM, Houseman JG (1990) Effect of ingested soybean, ovomucoid and corn protease inhibitors on digestive processes of the European corn borer, *Ostrinia nubilalis* (Lepidoptera: Pyralidae). *Journal of Insect Physiology* **36**, 691-697

Laskowski M, Qasim MA (2000) What can the structures of enzyme inhibitor complexes tell us about the structure of enzyme substrate complexes? *Biochimica et Biophysica Acta* **1477**, 324-337

Lipke H, Fraenkel GS, Liener IE (1954) Effects of soybean inhibitors on growth of *Tribolium confusum*. *Journal of the Science of Food and Agriculture* **2**, 410-415

Lowry OH, Rosebrough A, Lewis FA, Randall RJ (1951) Protein measurement with the Folin Phenol reagent. *Journal of Biological Chemistry* **193**, 263-275

Ma CC, Kanost MR (2000) A beta 1,3-glucan recognition protein from an insect, *Manduca sexta* agglutinates microorganisms and activates the phenoloxidase cascade. *Journal of Biological Chemistry* **275**, 7505-7514

Martinez SS, Emden HFV (2001) Growth distribution, abnormalities and mortality of *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) caused by azadirachtin. *Neotropical Entomology* **30**, 113-125

McIntyre RJ (1971) A method for measuring activities of acid phosphatases separated by acrylamide gel electrophoresis. *Biochemical Genetics* **5**, 45-50

McManus MT, Burgess EPJ (1995) Effects of the soybean (Kunitz) trypsin inhibitor on growth and digestive proteases of larvae of *Spodoptera litura*. *Journal of Insect Physiology* **41**, 731-738

Nakajima Y, Tsuji Y, Homma K, Natoris S (1997) A novel protease in the pupal yellow body of *Sarcophaga peregrine* (flesh fly). *Journal of Biological Chemistry* **272**, 23805-23810

Oppenorth FJ (1985) Biochemistry and genetics of insecticide resistance. In: Kerkut GA, Gilbert LI (Eds) *Comprehensive Insect Physiology, Biochemistry and Pharmacology* (Vol 12), Pergamon Press, Oxford, UK, pp 731-773

Paes MC, Oliveira MB, Oliveira PL (2001) Hydrogen peroxide detoxification in the midgut of the blood-sucking insect, *Rhodnius prolixus*. *Archives of Insect Biochemistry and Physiology* **48**, 63-71

Paulino da Silva L, Leite JRSA, Bloch Jr. C, de Freitas MS (2001) Stability of a black eyed pea trypsin/chymotrypsin inhibitor (BTCI). *Protein and Peptide Letters* **8**, 33-38

Pompermayer P, Lopes AR, Terra WR, Parra JRP, Falco MC, Silva-Filho MC (2001) Effects of soybean proteinase inhibitor on development, survival and reproductive potential of the sugarcane borer, *Diatrea sachharalis*. *Entomologia Experimentalis et Applicata* **99**, 79-85

Rockstein M (1956) Phosphatases of house fly, *Musca domestica* (L.). *Bulletin of Brooklyn Entomological Society* **51**, 9-17

Saadati F, Bandani AR (2011) Effect of serine protease inhibitors on growth and development and digestive serine proteinases of the sunn pest, *Eurygaster integriceps*. *Journal of Insect Science* **11**, 72

Shukle RH, Murdock LL, Gallun RL (1985) Identification and partial characterization of a major gut proteinase from larvae of the Hessian fly, *Mayetiola destructor* (Say) (Diptera: Cecidomyiidae). *Insect Biochemistry* **15**, 93-101

Silva FCBL, Alcazar A, Macedo LLP, Oliveira AS, Macedo FP, Abreu LRD, Santos EA, Sales MP (2006) Digestive enzymes during development of *Ceratitis capitata* (Diptera: Tephritidae) and effects of SBTI on its digestive serine proteinase targets. *Insect Biochemistry and Molecular Biology* **36**, 561-569

Singh K, Kaur M, Rup PJ, Singh J (2006a) Evaluation of the effect of *Ricinus communis* L. on melon fruit fly, *Bactrocera cucurbitae* (Coq.) (Diptera: Tephritidae). *Biopesticide International* **2**, 79-86

Singh K, Kaur M, Rup PJ, Singh J (2006b) Exploration for anti-insect properties of lectin from seeds of soyabean (*Glycine max*) using *Bactrocera cucurbitae* as a model. *Phytoparasitica* **34**, 463-473

Shukla S, Arora R, Sharma HC (2005) Biological activity of soybean trypsin inhibitor and plant lectins against cotton bollworm/legume pod borer, *Helicoverpa armigera*. *Plant Biotechnology* **22**, 1-6

Srivastava BG (1975) A chemically defined diet for *Dacus cucurbitae* (Coq.) larvae under aseptic conditions. *Entomology News Letter* **5**, 24

Steffens R, Fox FR, Kassell B (1978) Effect of trypsin inhibitors on growth and metamorphosis of corn borer larvae *Ostrinia nubilalis* (Hubner). *Journal of Agricultural and Food Chemistry* **26**, 170-174

Wang C, Qin J (1996) Effect of soybean trypsin inhibitor, gossypol, and tannic acid on the midgut protease activities and growth of *Helicoverpa armigera* larvae. *Acta Entomologica Sinica* **39**, 337-341

Wang X (2001) The expanding role of mitochondria in apoptosis. *Genes and Development* **15**, 2922-2933

White IM, Elson-Harris MM (1992) *Fruit Flies of Economic Significance: Their Identification and Bionomics*, CAB International, Wallingford, UK, 601 pp

Yu SJ (2004) Induction of detoxification enzymes by triazine herbicides in the fall armyworm, *Spodoptera frugiperda* (J.E. Smith). *Pesticide Biochemistry and Physiology* **80**, 113-122