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RESEARCH ARTICLE

DEVELOPMENTAL IMPAIRMENT IN *BACTROCERA CUCURBITAE* INDUCED BY PARTIALLY PURIFIED PROTEASE INHIBITOR FROM CHICK PEAS

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ABSTRACT

Melon fruit fly, *Bactrocera cucurbitae* (Coquillett) is an important pest of cucurbit crops that can cause severe damage to its host species and thus reduce drastically the crop production. Use of digestive serine proteinases by dipterans has led to the present investigation in which the influence of partially purified chick pea proteinase inhibitors on the survival and digestive processes of melon fruit fly was determined. The artificial diet bioassay using various concentrations (*viz*. 12.5, 25, 50, 100, 200 and 400µg/ml) of the partially purified chick pea inhibitor on the second instar (64-72old) larvae revealed that the larval weight gain and mean larval growth rate were significantly inhibited ($p<0.01$) while the food assimilated by the larvae varied with treatment. Enzymatic assays at different concentrations (50, 100, 200 and 400µg/ml) of partially purified chick pea PI significantly reduced the activities of digestive enzymes (trypsin, chymotrypsin and elastase) and significantly induced the activities of other enzymes *viz.* esterases, acid and alkaline phosphatases, glutathione-S-transferases and catalase.

INTRODUCTION

The sustainability of ecosystems is increasingly being threatened by mankind's exclusive reliance on pesticides for control of insect pests. It is therefore imperative that alternative compounds be found which are not only effective but at the same time safe and biodegradable. Among the various pest management strategies being explored, botanical pesticides remain the safest and most ecofriendly approach for pest management. Plants provide an untapped reservoir of compounds which, if explored for their anti-insect activity, could be manipulated for development of resistance in plants to insect pests. The plant's defense arsenal comprises of a number of proteins which play a role in resisting herbivory (Chen *et al*. 2007; Zhu-Salzman and Liu 2012). Protease inhibitors are one such group of plant proteins which are receiving considerable attention due to their ability to inhibit proteases present in the insect gut, thereby reducing the availability of amino acids necessary for their growth and development (Zhu salzman *et al*. 2003).

The melon fruit fly, *Bactrocera cucurbitae* (Coquillett) is an economically important pest of fruits and vegetables and 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*. 2010).

The digestive system of phytophagous pests is based mainly on serine and cysteine proteinase classes. Serine proteinases are the major enzymes found in lepidopteran and dipteran orders (Gomes *et al*. 2005). Keeping this in mind the present study was envisaged to investigate the influence of protease inhibitors partially purified from chick peas on the development of the larvae of *B. cucurbitae*. The legumes are a rich source of serine protease inhibitors which belong to either kunitz or Bowman-birk inhibitor family (Prasad *et al*. 2010). It is hoped that the present study would help in exploring the possibility of using these PIs for imparting resistance in crops to melon fruit fly.

MATERIALS AND METHODS

Sources of materials

Seed samples of Chick peas (*Cicer arietinum* L.) were procured

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from the local market for extraction and partial purification of protease inhibitors. Seeds were identified from the Department of Botanical and Environmental Sciences, Guru Nanak Dev University, Amritsar.

Bovine serum albumin (BSA) was obtained from Loba (India). Bovine trypsin, N-a-benzoyl-DL-arginine-p-nitroanilide (BA*p*NA), Benzoyl-tyrosine-p-nitroanilide (BTPNA), Succinyl-alanine-alanyl-prolyl-leucine-p-nitroanilide (SAAPLpNA), Leucine-p-nitroanilide (LpNA) were obtained from Sigma-Aldrich, Delhi, India.

Partial purification

Chick Peas were soaked in 0.2M Sodium phosphate buffer (pH 8.3) and left overnight. Next day, the soaked material was filtered through multiple layers of surgical gauze, and the filtrate was centrifuged at 14,000 rpm for 20 minutes at 4ºC (Smirnoff *et al*. 1976). Clear supernatant was stored at -20ºC as crude extract. After ascertaining the protease inhibitory activity, the crude extract was dissolved in 0-80 percent ammonium sulphate saturation and was kept overnight to ensure complete precipitation. The precipitates obtained after the centrifugation at 14,000 rpm for 20 minutes at 4ºC were collected by dissolving them in minimal known volume of distilled water. The dissolved fractions were dialyzed (cut off 14kDa) in cold for about 12-14 hours against distilled water. The dialyzed partially purified Protease inhibitor from chick peas was again checked for protease inhibitory activity and stored at -20ºC 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 N-a-benzoyl-DL-arginine-p-nitroanilide (BAPNA) 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 50mM Tris-Hcl buffer (pH 8.2). The assayed residual activities were followed by hydrolysis of 1mM BAPNA and the liberation of p-nitroaniline, which was measured at 410nm. One unit of inhibitor activity corresponded to its amount that produced a decrease in absorption at 410 nm by 0.1 unit in the inhibitor activity assay at 50% inhibition.

Insect rearing

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

Bioassays

About 100 gravid females were released in wire mesh cages provided with fresh pumpkin pieces for 8h. The pumpkin pieces were removed at an appropriate time interval from the cages and dissected in saline water for harvesting the second instar (64-72h old) larvae. The harvested larvae were transferred to culture tubes (25mm diameter x 100mm length) containing artificial diet incorporated with various concentrations (viz. 12.5, 25, 50, 100, 200 and 400µg/mL) of partially purified chick pea protease inhibitor. The artificial diet (control as well as treated) was prepared according to the standardized methodology given by Srivastava (1975). Observations were made for the larval weight gain, mean relative growth rate (MRGR) and food assimilated after three time intervals (24h, 48h, 72h). 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 according to the formula given by Khan and Saxena (1985).

Enzyme assays

The potential of partially purified chick pea protease inhibitors in reducing the digestibility of the gut proteinases of *B. cucurbitae*, was ascertained by estimating the activity of larval gut trypsin-like enzyme with BAPNA, chymotrypsin-like enzyme with BTPNA, elastase-like enzyme with SAAPLpNA and leucine-aminopeptidase-like enzymes using LpNA as substrate (Christeller *et al*. 1990, 1992). The activity of each enzyme was monitored for three consecutive time intervals (24h, 48h and 72h) after feeding second instar larvae of melon fruit fly on artificial diet incorporated with four concentrations (viz. 50, 100, 200 and 400µg/mL) of partially purified protease inhibitor from peas. The influence of partially purified pea PI was also investigated for its influence 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 (64-72h old) of *B. cucurbitae* in order to ascertain their role in counteracting the metabolic stress produced by the ingestion of protease inhibitors. The larvae harvested from the pumpkin pieces were transferred to artificial control diet as well as diet containing different concentrations of the partially purified PI and enzymatic activity was assessed after three time intervals i.e. 24h, 48h and 72h. Activity determination of esterases was based on the methodology outlined by Katzenellenbogen and Kafatos (1971). α-naphthyl acetate (1mM) 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 Mc Intyre (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 (20mM, pH 6.0) was used as a substrate solution and sodium carbonate buffer (50mM, 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 10mM 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

Statistical analyses were performed using SPSS 10.0 computer program. One way analysis of variance (ANOVA) was used to analyse the effect of partially purified chickpea protease inhibitor on growth and development parameters of melon fruit fly, *B. cucurbitae*. The results were expressed as mean ± S.E. If the variable was significant, Tukey's multiple range test was used for pairwise comparison of the difference between treatments for mean separation ($p<0.05$). Significance of the effect of the partially purified chickpea protease inhibitor on activity of various digestive, detoxification and anti-metabolic enzymes of *B. cucurbitae* at three exposure intervals was determined by t-test.

RESULTS

Growth and Development

The partially purified chick pea PI (Table 1) was assessed for its inhibitory influence on the larval weight gain, growth rate and food assimilation of *B. cucurbitae* larvae. The larvae fed on control diet showed better development as compared to those fed on partially purified inhibitors from chick peas when observed at different time intervals. The observations made on larval weight gain showed a significant increase after 24h of treatment, but after 48h and 72h of treatment, the larval weight gain was significantly inhibited (Table 2). The inhibition was more prominent at 100µg/ml concentration where the larval weight gain was inhibited by 47.78% after 48h and by 27.51% after 72h feeding interval. The mean relative growth rate of *B. cucurbitae* decreased significantly ($p<0.01$) after 48h and 72h of feeding the second instar larvae on partially purified protease inhibitors from chick peas (Table 3). However, the decrease in MRGR showed no correlation with increase in concentration. The food assimilated showed an almost invariable trend with increase in concentration (Table 4).

Table 1 Partial purification of pea protease inhibitor from seeds of *Cicer ariarietinum* L

Purification step	Total Activity	Total Protein (mg)	Specific Activity	Recovery (%age)	Purification fold
Crude	385	774	0.49	100	1
Partially purified	310	260	1.19	80.52	2.43
Supernatant	-	514	-	-	-

Enzyme Assay

Among the serine proteases, the activity of trypsin (Fig. 1A) and elastase (Fig. 1C) was inhibited at almost all the concentrations while the activity of chymotrypsin (Fig. 1B) was inhibited only at the higher concentrations of 200 and 400µg/ml. Maximum inhibition was observed for trypsin (77.54%) at 400µg/ml concentration after 24h of treatment (Fig. 1A). However, the elastase activity was not affected. The observations on leucine aminopeptidase showed an inhibition in the activity at most of the concentrations during the initial treatment intervals (Fig. 1D), but the enzyme activity increased as the feeding of the larvae on the treated diet was prolonged to 72h.

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

Concentration Used (µg/ml)	Larval weight gain		
	After 24 h	After 48 h	After 72 h
Control	17.45 ± 1.08^{c}	82.50 ± 4.87^{b}	127.95 ± 3.05^{a}
12.5	17.97 ± 0.98^{c}	74.87 ± 5.26^{bc}	122.05 ± 10.39^{ab}
25	9.37 ± 1.01^{d}	73.12 ± 0.99^{bcd}	112.25 ± 9.56^{bcd}
50	22.52 ± 2.32^{b}	105.70 ± 7.76^{a}	107.18 ± 7.23^{cd}
100	28.10 ± 2.54^{a}	43.08 ± 3.43^{e}	92.75 ± 2.33^{e}
200	23.70 ± 2.02^{b}	63.12 ± 4.30^{d}	99.15 ± 10.46^{de}
400	19.25 ± 1.73^{c}	69.72 ± 8.66^{cd}	113.07 ± 1.44^{bc}
F- value	66.51**	69.59**	16.71**

** Significant at 1%

Protease inhibitors not only affected the digestive proteases of *B. cucurbitae* larvae but also influenced the activity of antioxidant and detoxification enzymes in the larvae of melon fruit fly. Among the two antioxidant enzymes, the superoxide dismutase (SOD) activity after showing a slight increase during the initial treatment interval, decreased considerably after 72h of treatment at 100, 200 and 400µg/ml (Fig. 2A). On the other hand, a significant induction in catalase activity was observed after 24h of feeding the larvae on 50, 100 and 200µg/ml and after 48h of feeding on 400µg/ml concentration of protease inhibitors contained in artificial diet (Fig. 2B). However, at 72h, a decrease in enzyme activity was observed.

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

Concentration Used (µg/ml)	Mean Relative Growth Rate		
	After 24 h	After 48 h	After 72 h
Control	0.110 ± 0.006^{c}	0.190 ± 0.009^{b}	0.165 ± 0.005^{a}
12.5	0.112 ± 0.008^{c}	0.185 ± 0.005^{b}	0.167 ± 0.005^{a}
25	0.058 ± 0.008^{d}	0.167 ± 0.005^{c}	0.153 ± 0.005^{b}
50	0.127 ± 0.010^{bc}	0.225 ± 0.012^{c}	0.150 ± 0.006^{bc}
100	0.153 ± 0.012^{a}	0.110 ± 0.011^{e}	0.132 ± 0.004^{de}
200	0.135 ± 0.105^{ab}	0.143 ± 0.005^{d}	0.125 ± 0.010^{e}
400	0.108 ± 0.015^{c}	0.150 ± 0.015^{cd}	0.140 ± 0.006^{cd}
F- value	50.56**	86.44**	36.86**

** Significant at 1%

The analysis of detoxification enzymes revealed a significant induction in the activity of both esterases and GST's at almost all concentrations. Maximum induction in esterase activity was observed at 400µg/ml where the activity increased by 8.32 fold after 48h feeding interval (Fig. 3C). In case of GSTs, the induction was maximum (5.13 fold) at 100µg/ml after 72h feeding interval (Fig. 3D). The activity of both AcP and AkP

was completely inhibited at the highest concentration of 400µg/ml (Fig. 2E and 2F). At lower concentrations, the AcP activity was inhibited during the initial feeding intervals but was induced significantly with prolonged feeding of the larvae on PI incorporated artificial diet. On the other hand, the AkP activity at lower concentration was induced during the initial feeding interval but was inhibited with prolonged feeding.

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

Conc. (µg/ml)	Food assimilated wrt control		
	After 24 h	After 48 h	After 72 h
12.5	3.64 ± 0.208^{c}	15.36 ±1.279^{b}	24.28±2.150^{abc}
25	2.82 ± 0.132^{d}	16.03 ±0.515^{b}	23.78 ±0.743^{abc}
50	4.17 ± 0.257^{b}	18.83 ±1.158^{a}	23.17 ±0.499^{bc}
100	4.75 ±0.227^{a}	13.28 ±0.615^{c}	22.34 ± 0.861^{c}
200	4.29 ± 0.332^{b}	15.99 ±0.983 ^{b}	24.76 ±1.804^{ab}
400	3.95 ± 0.218^{bc}	16.73 ± 0.882^{b}	25.94 ±0.882^{a}
F- value	47.07**	20.73**	5.57**

** Significant at 1%

DISCUSSION

An anti-insect influence of partially purified chick pea protease inhibitor can be well judged through regular monitoring the growth rate of the tested larvae. Congruent with the present results, a progressive decline in larval weight was also reported in *H. armigera* larvae fed on diet supplemented with increasing concentration of chickpea trypsin inhibitor (Kansal *et al*. 2008). Sudheendra and Mulimani (2002) had also observed a significant reduction in larval growth of *H. armigera* fed with chick pea and mungbean inhibitors.

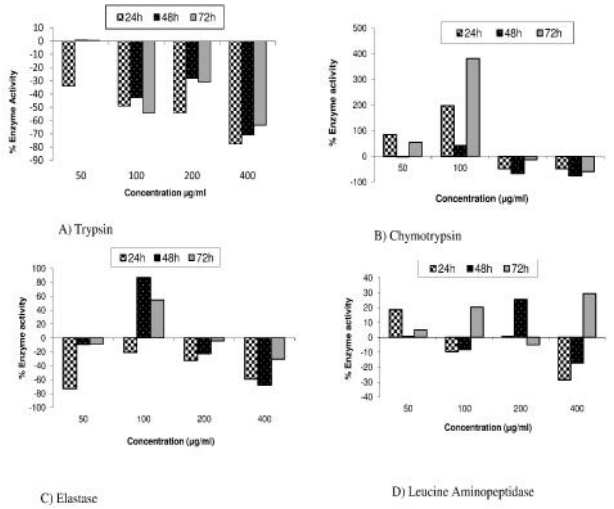

Figure 1 Influence of partially purified Chick pea PI on the activity of digestive enzymes (A, trypsin; B, chymotrypsin; C, elastase; D, leucine aminopeptidase) of second-instar (64–72 h old) *Bactrocera cucurbitae* larvae.

Serine proteinases inhibitors are the most potent inhibitors of the normal functioning of the specific enzymes in insects (Nakajima *et al.* 1997). The present study showed the inhibition of major proteases responsible for wide range of physiological processes in insects. A similar kind of inhibition in trypsin, chymotrypsin and elastase activity has been reported in *B. cucurbitae* under the influence of protease inhibitor partially purified from peas (Kaur and Sohal 2013). An inhibition in trypsin and chymotrypsin activity has also been reported in the melon fruit fly larvae with protease inhibitors partially purified from soybean (Kaur and Sohal 2012).

The fact that PIs could affect moulting and non-digestive enzyme regulation (Faktor and Raviv 1997) is well evident from the present results for antioxidant and detoxification enzymes. The SOD activity after showing an increase during the initial treatment interval decreased with prolonged treatment. Quick induction of SOD that rapidly removes superoxide radicals is an initial response of phytophagous insects to plant pro-oxidants (Ahmad 1992). SOD provides the first line of defense against toxicity from free radicals generated during metabolism (Paes *et al*. 2001; Wang 2001). The catalase activity which plays an important role in removing toxic H_2O_2 produced by the action of SOD by dismutating the free superoxide radicals (O_2^-) (Felton and Summers 1995) was induced significantly in the initial feeding intervals. It seems that SOD and catalase in the melon fruit fly larvae could be playing a protective role against the reactive oxygen species which might have been produced due to metabolic impairment caused by PIs.

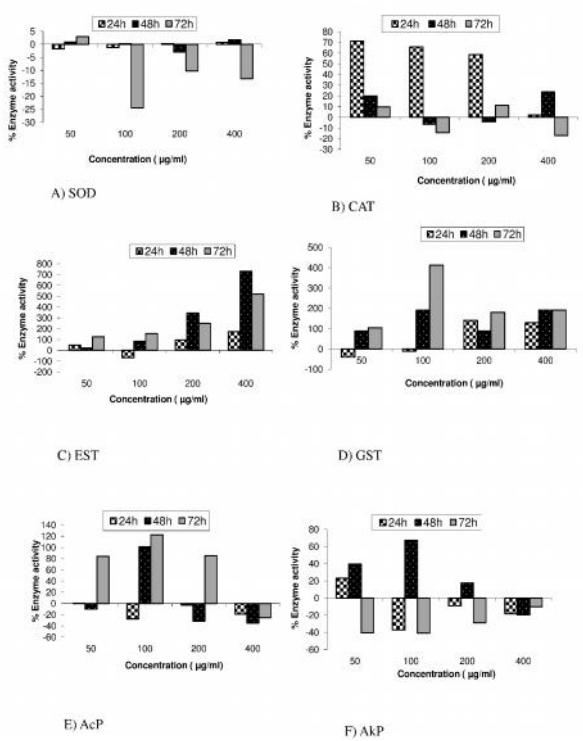

Fig 2 Influence of partially purified Chick pea PI on the activity of antioxidant enzymes (A, superoxide dismutase; B, catalase) and detoxification enzymes (C, esterase; D, glutathione-S-transferase; E, acid phosphatase; F, alkaline phosphatase) involved in growth and development of second instar larvae (64–72 h) of *Bactrocera cucurbitae.*

Both esterase and GSTs are generally involved in the detoxification of xenobiotics (Oppenorth 1985; Enayati *et al.* 2005).The ability to detoxify xenobiotics plays an important role in the survival of insects. The increase in the activity of both the enzymes in the larvae of *B. cucurbitae* reflects a biochemical adaptive mechanism on part of the insect to

counteract the stress caused by PI ingestion. Phosphatases play an important role in the metabolism of carbohydrates, phospholipids and nucleotides (Hollander 1971; Govindwar and Bhawane 1989; Bhanot and Bhawane 1991). Acid phosphatase is found mainly in the cytosol of midgut tissue cells of Diptera (Ferreira and Terra 1980) and Lepidoptera (Santos and Terra 1984). In silkworm larvae, the acid phosphatases activities in the blood and midgut tissues have been strongly related to silk protein synthesis, digestion and absorption of phosphorylating substances (Wu 1993). The Akp is a brush border membrane marker enzyme and is thought to be involved in the digestion and absorption of nutrients by columnar epithelial cells of the insect midgut (Terra and Ferreira 1994; Azuma and Eguchi 1989). Changes in the activity of both enzymes in the larvae of *B. cucurbitae* indicate that these enzymes might have got affected by the changes in the physiological balance of the midgut caused by the ingestion of partially purified chickpea PI.

The partially purified chickpea PI caused a developmental impairment in the larvae of *B. cucurbitae* which might be the result of changes in the normal physiological metabolism of the larvae caused by variations in the activities of key enzymes such as trypsin, chymotrypsin, elastase, esterase, GSTs and phosphatases.

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