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Characterization of Insect Proteinases and Their Inhibition by Finger and Little Millet Inhibitors

S Sivakumar, M Mohan and B Thayumanavan*
Centre for Plant Molecular Biology, Tamil Nadu Agricultural University,
Coimbatore 641 003, India

Empirical approach was adopted to examine the interaction of a little and finger millet inhibitors with digestive proteinases of different field and storage pests. Accordingly, gut proteinases of four storage and phytophagous pests were characterized and their inhibition by finger millet (*Eluesine coracana* Gaertneri) and little millet (*Panicum sumatrense* Roth) inhibitors has been investigated. The C*allosobruchus* sp showed acid proteinase with pH optimum of 3.5 and *Sitophilus oryzae* showed pH optimum of 4.5 apart from their alkaline proteinases with pH 8.5. The proteinases of *Tribolium castaneum* had wider pH optimum from 5.5 to 8.5. However, all lepidopteran insect proteinases had pH optima ranging from 8.5 to 10.5. The optimum temperature was found to be 30 to 40 ^{o}C. The inhibitory activities of little and finger millet inhibitors towards the insect proteinases are very low except notable level inhibition of proteinases of some insects. The gut proteinase zymogram of different insects revealed 2 – 6 isozymes and the inhibitors moderately inhibited all the isozymes of insects tested.

***Key words*:** *Eleusine coracana, Panicum sumatrense,* proteinase inhibitors, insect proteinase assay, proteinase zymogram.

Plant proteinase inhibitors have long been proposed to play an important role in defense against insect attacks (1) and in regulation of endogenous proteinases (2). Many plant seeds contain relatively large amount of proteinase inhibitors (PIs) for defense mechanism of plants against various pests and parasites (3). They inhibit digestive proteinases of insects and even proteinases from microbial origin (4). These proteinaceous inhibitors have considerable variation in their efficiency towards insect proteinases (5). Christeller and Shaw (6) suggested that *in vitro* screening can be used to select the most effective proteinase inhibitors for particular insects. This requires knowledge of the type of proteinases present in the insect gut. Insect proteinases strongly interact with various inhibitors and these results would provide to select best inhibitor for a particular insect from a wide range of inhibitors before the crop plant is transformed with proteinase inhibitor genes (3). These plant genes that protect against herbivors and may be useful for heterologus expression into food and fibre crops (7). Moreover, these trangenes in crop plants would inhibit proteolytic enzymes of animal and fungal origin but rarely of plant origin and therefore thought to act as protective agents (8). Several studies have demonstrated that PIs might provide adequate protection against variety of economically important lepidopteran insects (9-11). Pulliam *et al* (12) also reported that the cabbage derived inhibitor BoPI (*Brassica oleracea* proteinase inhibitor) proved as a natural insecticide against certain lepidopteran pests. BoPI effectively reduced the survivorship of the Bt-resistant and Bt-susceptible strains of tobacco budworm. Since PIs are derived from plant genes and are easily inactivated by cooking, the introduction of PI gene into new host crops can be regarded as a safe strategy from the food safety standpoint. Another important factor is that the gene transfer to other species will not create a new environmental hazard (11). The complete amino acid sequence of bifunctional α-amylase / trypsin inhibitor from seeds of finger millet was reported by Campos and Richardson (13). In present study, digestive proteinases of field and storage pests were characterized by the proteinases inhibition assays and activity staining for the effect of proteinase inhibitors on the isozymes.

*Corresponding author. E-mail: thayumanavanb@yahoo.com

Materials and Methods

Test insects — The midgut proteinases of some important stored grain as well as field crop pests were taken for study. The stored grain pests used were rice weevil (*Sitophilus oryzae*), red flour beetle (*Tribolium castaneum*), pulse beetle (*Callosobruchus chinensis*) and rice moth (*Carcyra cephalonica*). Field crop pests viz., tobacco caterpiller (*Spodoptera litura*), gram pod borer (*Helicoverpa armigera*),

castor semilooper (*Acaea janata*) and diamondback moth (*Plutella xylostella*) were used. Stored grain pests were reared under laboratory conditions with suitable grains as feed. *S. litura* and *H. armigera* were reared on artificial diet (14), whereas *A. janata* and *P. xylostella* were maintained on natural diet viz., castor and cauliflower leaves, respectively. Lepidopteran adult moths were provided with 10% honey solution as feed. All insects were maintained at 28±2 °C, 60% RH and photoperiod of 14 h light and 10 h dark.

Extraction and partial purification of proteinases from larval midguts — Final instar larvae of uniform size were selected from cultures. The selected larvae were then starved for six hours and the midgut dissected under cold iso-osmotic saline, 0.15 M NaCl. Midguts of 5-50 insects were taken as one replicate depending upon the insect species, and homogenized in an ice cold pestle and mortar with 20 mM sodium phosphate buffer (pH 7.2) containing 20mM NaCl and 0.1mM $CaCl_2$. The content was centrifuged at 10,000 x g for 20 min at 4 °C. The supernatant was saturated to 55% with ammonium sulphate, kept for 12 h at 4 °C and then centrifuged at 10,000 x g for 10 min. The precipitate was dissolved and dialyzed using the same buffer. The buffer was changed thrice during 12 h period. The dialyzed sample was lyophilized and stored at -20 °C (15).

Extraction of protease inhibitors from seeds — Seeds of finger millet (*cv* CO II and CO I3) and little millet (*cv* CO 3) were used as sources for proteinaceous inhibitors. The method adopted by Oliveira *et al* (16) was followed for the extraction of inhibitor. Seeds (100g each) were initially powdered using the grinding machine. Finely ground meal was extracted with 0.05M Tris-HCl buffer, pH 7.5 for 30 min and centrifuged at 8000 x g at 4 °C. The supernatant was incubated at 70 °C for 20 min to inactivate any endogenous proteinases present in the inhibitor sample and then cooled. The supernatant was saturated to 80% ammonium sulphate concentration and the contents were centrifuged at 10,000 x g for 15 min. The precipitate was dissolved and dialyzed using water, and freeze-dried sample stored at -20 °C.

Proteinase assay — The proteinase activities of various insect midgut preparation and their optimum reaction conditions, such as temperature, pH and incubation time were studied by azocasein digestion method (17) with little modifications. Trypsin standard curve was constructed according to the method given by Kuntiz (18). The assay mixture contained: 100 µl of 1% azocasein prepared in 0.75 M NaCl and 200 µl of 100 mM Glycine-NaOH buffer (pH 9.5) containing enzyme. After incubation for 15 min at 30 °C, the reaction was stopped by addition of 500 ml of 10% trichloro acetic acid. Samples were mixed well, kept in ice for 30 min and then centrifuged at 12,000 x g for 10 min. From this, 600 ml supernatant was taken, mixed with 600 ml of 1 M NaOH and the absorbance was read at 420 nm after correction for enzyme blanks. Inhibition assays were done by incubating the gut enzyme (20 mg) with the partially purified inhibitor (60 mg) for 15 min at 30 °C (16). The reaction was started by adding the substrate and allowed for 15 min at 30 °C. The total protein content was measured by adopting the procedure of Bradford (19). Assays were done at a specific pH determined for each of the proteinases of insects being investigated. The common incubation time of 15 min and temperature 30 °C were followed for all the reactions. Bovine trypsin stock (0.1 mg ml^{-1}) was prepared using 2.5 mM HCl. Various amounts of trypsin were used to perform enzyme assay as stated below. The specific activity of the sample of trypsin used, was obtained by drawing straight line to the first part of the curve. The slope 0.20 / 1.4 divided by 15 min is the specific activity, i.e. activity per microgram trypsin protein

$$\text{i.e. TU} = \frac{0.2}{1.4 \times 15} = 9.52 \times 10^{-3}$$

$$= \frac{0.4}{2.9 \times 15} = 9.95 \times 10^{-3}$$

Average= 9.73×10^{-3} TU

A new curve was then plotted, the ordinates as absorbance, while the abscissae as tryptic units (TU). The amount of tryptic units present in insect samples were conveniently obtained using the standard curve.

Optimum conditions for proteinase activity — The buffer systems used for optimum pH for proteinase activity were 50mM buffers of acetate-pH 3.5, 4.5 and 5.5; phosphate-pH 6.5 and 7.5; Tris-HCl pH 8.5; glycine-NaOH - pH 9.5 and 10.5; carbonate pH 11.5 (20). All the pH study measurements were carried out at 30 °C. For optimum incubation time and temperature studies, Glycine-NaOH buffer (100 mM), pH 9.5 was used. One per cent azocasein was used as substrate and the assay details were same as mentioned above for proteinase assay. The optimum

incubation period was determined by incubating the assay mixture for 5,10, 15, 20, 30 and 40 min at 30 °C. The optimum temperature reaction was carried out at 10,20,30,40 and 60 °C for 15 min.

Trypsin activity in poyacrylamide gel — A rapid, sensitive and generally applicable non-denaturing polyacrylamide gel electrophoresis (native PAGE) was performed for detection of midgut digestive proteinases using casein as substrate (21). The bioactive bands would appear colourless upon staining with Coomassie brilliant blue R-250 due to hydrolysis of casein by the proteinases. Accordingly, the pre-incubated midgut proteinase and the inhibitor (20 µg of enzyme + 60 µg trypsin inhibitor, CO 11 and CO 13) and the control without inhibitor were run simultaneously in the 10% native PAGE. The gel was run at 4 °C to preserve enzyme activity. At the end of the run, the gel was carefully removed and immersed for 1 h in 1% casein solution prepared with Glycine-NaOH buffer, pH 9.0. Then, the gel was briefly washed in distilled water and stained with 0.25% Coomassie brilliant blue R-250 solution overnight. The gel was briefly destained with methanol:acetic acid:water (4:1:5 v/v/v) to view the proteinase bands and their inhibition by inhibitors.

Results

The optimum pH studies indicated (Fig.1) that the coleopteran insects possessed pH optima at acidic as well as alkaline conditions. Midgut proteinases of *S. oryzae* and *C. chinensis* showed two pH optima one at acidic (pH 3.5-4.5) and another at neutral to alkaline range (pH 7.7-10.5). However midgut proteinases of *T. castaneum* exhibited pH optima between 6.5 and 8.5. This indicated the presence of more than one kind of proteinases such as acid proteinases / cysteine proteinases (acidic pH optimum) and serine proteinases (alkaline pH optimum). However, all lepidopteran insect proteinases had pH optima from 8.5 to 10.5 which indicated the alkaline nature of their proteinases, probably serine proteinases. The optimum temperature was found to be 30 °C across the different insects (results not shown).

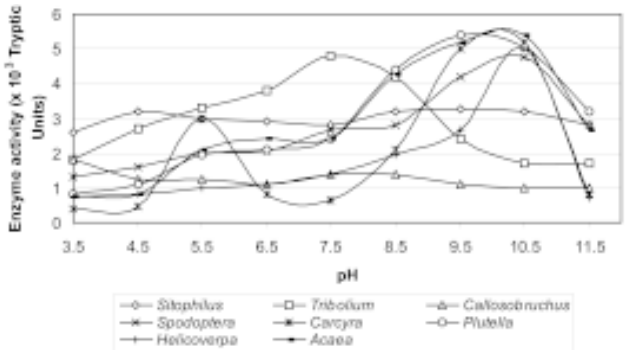

Fig. 1. Gut proteinases activities of various insects as affected by pH.

The inhibitory activities of little and finger millet inhibitors towards the insect proteinases were very low (Table 1). However, all three inhibitors (CO 3, CO 11 and CO 13) showed good inhibition of proteinases of *C. cephalonica*. The inhibition per cent was 32.6 for CO 3 inhibitor, 44.9 for CO 11 and 40.8 for CO 13. The CO 11 inhibitor inhibited the *S. oryzae* proteinase to the tune of 37.5 % and CO 13 inhibited the enzymic activity of *P. xylostella* by 22.2%. The proteinases of all other insects were poorly inhibited by all three inhibitors.

Table 1. Trypsin inhibitory activity of little millet *cv* CO 3 and finger millet *cv* CO 11 and CO 13 inhibitors

Insect sp	Per cent inhibition		
	Little millet	Finger millet	
	CO 3	CO 11	CO 13
Tribolium castaneum	3.6 ± 0.16	0.0	0.0
Sitophilus oryzae	5.0 ± 0.16	37.5 ± 4.08	20.0 ± 2.04
Callosobruchus chinensis	8.3 ± 0.48	0.0	0.0
Carcyra cephalonica	32.6 ± 0.48	44.9 ± 3.26	40.8 ± 4.0
Plutella xylostella	0.0	0.0	22.2 ± 1.87
Helicoverpa armigera	6.0 ± 0.24	16.0 ± 1.6	0.0
Spodoptera litura	0.0	10.8 ± 1.46	12.1 ± 0.98
Acaea janata	0.0	13.2 ± 0.98	0.0

The gut proteinase zymogram revealed 2 to 6 isozymes (Figs.2, 3), but all the isozymes were moderately inhibited by the inhibitors of CO 11 and CO 13 (Table 1). However, there was some inhibitor specificities noted on the isozymes of *H. armigera, S. oryzae* and *A. janata* (Fig.2). The proteinase isozyme "E" in *H. armigera* was well inhibited by the CO 11 inhibitor (lane 2), "A" in *A. janata* by CO 11 inhibitor (lane 9), "A" and "C" in *S. litura* by the both inhibitors (lanes 11,12). There was specific inhibition of isozyme "F" of *C. cephalonica* (Fig.3) by the inhibitors. Other insects zymogram showed negligible level of proteinase activity and its inhibition on the gel is not shown.

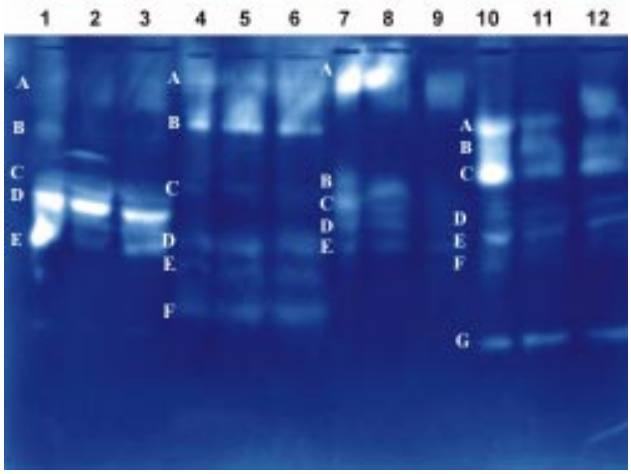

Fig. 2. Proteinase zymogram of field pests. Lanes 1-3, Proteinases of *H. armigera,* lanes 4 - 6, Proteinases of *P. xylostella,* lanes 7-9, Proteinases of *A. janata,* and lanes 10-12, Proteinases of *S. litura.* lanes 1,4,7,10, control; lanes 2,5,9,11 with CO 11 inhibitor; and lanes 3,6,8,12 with CO 13 inhibitor. Letters A - G indicate the isozyme of test insects.

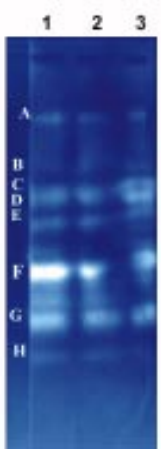

Fig. 3. Proteinase zymogram of *C. cephalonica.* Lane 1 - Proteinase (control) and lanes 2-3, proteinase with CO 11 and CO 13 inhibitor. Letters A - H indicate the isozyme of test insect.

Discussion

The response of proteinase activity at different pH ranges gives information on the type of proteinases present in the insect guts (22). The midgut proteinases activity was abundant at alkaline pH range (9.5-10.5) in *S. litura, C. cephalonica, P. xylostella, A. janata* and *H. armigera.* This is consistent with the high pH of midgut lumina of Lepidopterous larvae as reported (23,24). For instance, three trypsin –like proteinases with pH optima of 9.0, 10.5 and 11.0 respectively, were found in larval midguts of the army worm (*S. litura*) (25), where as, 24 kD trypsin-like proteinase with pH optimum of 9.5-10.0 was isolated in *H. armigera* (26). Midgut proteinases from larvae belonging to the lepidopteran families, Sphingidae, Noctuidae, Bombycidae, Pieridae, Pyralidae, Gelechiidae, Tortricidae and Hepialidae were found to contain serine proteases like, trypsin, chymotrypsin, elastase etc. with alkaline pH optima (27). The digestive physiology of coleopteran larvae are different from Lepidoptera larvae. The midgut proteinases of Coleoptera larvae are of thiol or acidic and cysteine proteinases with acidic to neutral pH optima. pH optima for *T. castaneum* and *S. oryzae* were found to be between 5.2 and 6.8 (28) and supports the findings from present investigation. The pH optimum for proteolytic activity of Colorado potato beetle midgut proteinase ranged from pH 3.0 – 8.0 suggesting presence of many isoforms of cysteine proteinases (29).

The midgut enzyme of lepidopteran pests *C. cephalonica* and *S. oryzae* was well inhibited by all three inhibitors. However, maximum inhibition by CO 11 inhibitor was, probably due to the matching specificities of the inhibitor with the gut enzyme. Similarly, inhibition specificity was observed for gut enzyme of *P. xylostella* by CO 13 inhibitor. The zymogram results are also consistent with the moderate inhibition of all proteinase isozymes across the insects tested by the finger millet inhibitors. The specificity of inhibition by inhibitors was also reported against few isozymes of the insects (3). Hence, the results obtained in the present study will be useful in identifying the inhibitor specificities and in further attempt for cloning of inhibitor genes from different species.

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