Identification of plant extracts containing protease inhibitors against the gut proteases of *Spodoptera mauritia* Boisduval (Lepidoptera: Noctuidae)

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ABSTRACT

Plant protease inhibitors (PPIs) are defense proteins which protect plants from insect attack by inhibiting the gut protease activity of insects. In this study we screened plant extracts to identify extracts containing protease inhibitors against gut proteases of *Spodoptera mauritia* larvae. Plant extracts were made by homogenizing soaked seeds/other parts of plants in bicarbonate buffer, pH 9.0 (1ml/mg tissue). Gut extract was prepared by homogenizing the gut of 5th instar larvae of *Spodoptera mauritia* in bicarbonate buffer, pH 9.0 (1ml/g tissue). Protease assay was done by incubating 25 µl of casein (2%) with 10 µl of gut extract in a total volume of 60 µl bicarbonate buffer, pH 9.0 at room temperature for one hour in the presence or absence of 25 µl plant extract. The amino acids released as a result of protein digestion was estimated by Lowry's method. Out of the several plant extracts tested, 11of them exhibited greater than 20% inhibition of protease activity of the gut extract. The tender seeds of cashew (*Anacardium occidentale*) showed the highest inhibition (73.6±0.35%) followed by carrot (*Davcus carota*) (51.6 ± 0.37%) and seed of beans (*Phaseolus vulgaris*) (50.0±1.3 %). *Hopea ponga* and *Syzygium cumini* seed extracts inhibited the gut protease activity to 37.5±0.43% and 36.2±1.0%, respectively. To our knowledge no protease inhibitor was reported from *Hopea ponga* and *Syzygium cumini* seeds. Though the other plant extracts were reported to contain PPIs against protease from other insects or animals, in this study we showed that these extracts were able to inhibit the gut protease activity of S*podoptera maurita* larvae.

Keywords: Plant protease inhibitors, *Spodoptera mauritia*, insect gut protease, *Hopea ponga*, *Syzygium cumini*

INTRODUCTION

Plants synthesize various proteinaceous and non- proteinaceous compounds against insect attack. Among these Protease Inhibitors (PIs) are the most studied class of plant defense proteins. Plant protease inhibitors (PPIs) are ubiquitous in nature and are usually proteins restricted not only to storage tissues but reproductive and vegetative tissues of most plant families [1].The possible role of PPIs was investigated as early as 1947, when Mickel and Standish observed the larvae of certain insects were unable to develop normally on soybean products [2]. These inhibitors are proteins or peptides capable of inhibiting catalytic activities of proteases. They are grouped primarily as either serine, cysteine, aspartic or metallo protease inhibitors [3,4] based on the class of protease they inhibit. Among these the serine protease inhibitors are the most studied. Serine proteases have been identified in extract from the digestive tract of insects from many families, particularly those of

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Lepidoptera [5] and many of these enzymes are inhibited by plant protease inhibitors. Serine protease inhibitors have anti-nutritional effects against several Lepidopteran insect pests [6,7].

Plant protease inhibitors act by reducing the digestive capability of insects thereby arresting their growth and development [8-10]. Protease inhibitors have been made use of in plant defense improvement against insects through transgenic technology [11,12]. Many PPIs have been shown to act as defensive compounds against Lepidopteran insect pests by direct assay or by expression in transgenic crop plants. [13-17]. The protease inhibitor gene CpTi was successfully transferred producing transgenic tobacco with significant resistance against tobacco hornworm (*Manducta sexta*) [14]. The efficiency of transgenic tobacco plants expressing CpTi was also tested against armyworm (*Spodoptera litura*) in feeding trails under laboratory conditions. Reduction to the extent of 50 % was observed in the biomass of army worm larvae fed on transgenic leaves expressing 3-5 µg of CpTi/g of fresh leaves [18].

In the order Lepidoptera, which includes a number of crop pests, the p^H optima of the gut are in alkaline range of 9-11 [7] and reported to contain serine proteases. The larvae of *Spodoptera mauritia* is a pest of paddy causing considerable damage to the crops. The early growing stages of paddy are most susceptible to the attack of the caterpillar. It is estimated that the loss in yield caused by larval infestation of *S. mauritia* range from 10 to 20%. In this study we screened several plant extracts to identify the extracts containing protease inhibitors against larval gut proteases of *S. mauritia*.

MATERIALS AND METHODS

Casein was obtained from Nice Chemicals Pvt. Ltd., Cochin. All other chemicals used were of analytical grade. The plant seeds/ plant parts were collected from botanical garden, University of Calicut and from other local sources. Plant parts were stored at -20°C until use.

Collection and rearing of *spodoptera mauritia* larvae

The adult moths of the insect were collected at night using fluorescent light traps. They were kept in glass beakers covered with muslin cloth and fed with a dilute solution of honey. The moths were allowed to mate and lay eggs. Larvae hatched out after 3-4 days and were reared in glass beakers initially and later transferred to plastic troughs as they grew in size. The culture was maintained at room temperature (28±2^{o}C), relative humidity (RH, 90±3%) and larvae were fed with fresh leaves of the grass *Ischaemum aristatum*. The total larval period was found to range from 17-19 days and consisted of 6 larval instars. Pupae were kept in beakers for adults to emerge and produce the second generation.

Preparation of gut extract

Fifth instar larvae were anesthetised using Diethyl ether and dissected out the gut and stored at -20°C until use. The gut was homogenized in sodium bicarbonate buffer, pH 9.0 (1ml/g of tissue). The homogenates were centrifuged at 10,000 rpm at 4°C for 10 minutes (Eltek refrigerated centrifuge RC 4100 D). The soluble protein recovered from the supernatant was stored at -20°C until use.

Preparation of extract from plants

Seeds/other plant parts soaked in bicarbonate buffer, pH 9.0 (1 ml/g of tissue) overnight and homogenized using a mortar and pestle. The homogenates were centrifuged at 10,000rpm at 4°C for 10 minutes (Eltek refrigerated centrifuge RC 4100 D). The soluble protein recovered from the supernatant was used for protease inhibition assay directly or stored at -20°C until use.

Protease assay

The total protease activity was assayed by incubating 10µl of crude gut extract (protein concentration, 0.78 mg/ml) with 25 µl of 2% casein, in a total volume of 60µl in bicarbonate buffer, pH 9.0 at room temperature for one hour. After incubation the volume was made up to 1ml with water and reaction stopped by adding 0.5ml of 10% Trichloro acetic acid (TCA). The tubes were centrifuged at 10,000rpm at 4°C for 10 minutes. The amino acid released in the supernatant was assayed by Lowry's method [19]. All assays were done in triplicate.

Protease inhibition assay

For the protease inhibition assay 10µl (0.78mg/ml) of gut extract was pre-incubated with 25µl of plant seed extract (inhibitor) for 10 minutes, followed by addition of 25µl casein (2%) and incubated at room temperature for one hour. After incubation the volume made up to 1ml with water and reaction was stopped by adding 0.5ml of 10% Trichloro acetic acid (TCA). The tubes were centrifuged at 10,000rpm at 4°C for 10 minutes. The amino acids released in the supernatant were assayed using Lowry's Method [19]. All assays were done in triplicate. A protease inhibitor control was also done to account for the protease activity present in the seed extracts (inhibitor). At the end of incubation enzyme/inhibitor was added to the tubes such that all the tubes contain enzyme substrate and inhibitor.

Calculations

Absorbance of the control (casein alone) was subtracted from the absorbance of inhibitor control and the value thus obtained represents the protease activity present in the plant extract. This value was subtracted from the absorbance of the test in presence of the inhibitor to get the actual absorbance in the absence of any protease activity from the plant extract. The absorbance of the test was taken as 100% enzyme activity. Based on this the absorbance of the test in the presence of inhibitor was converted into percentage activity. This value was subtracted from 100 to get percentage inhibition.

Statistical analysis

Statistical analysis was done using the R-program.

RESULTS AND DISCUSSION

We tested 20 plant extracts for their capacity to inhibit the protease from gut of *Spodoptera mauritia* larvae. Out of the 20 extracts tested, 11of them were found to inhibit the gut protease activity of *Spodoptera mauritia* larvae to the extent of greater than 20% of the total enzyme activity (Table 1). Different plant extracts were used to test the inhibition of the gut protease activity of *Spodoptra*

mauritia larvae. Out of the 20 plant extracts tested, 11of them inhibited greater than 20% protease activity of the gut extracts (Table 1 and 2). Of these extracts, the seed of tender cashew (*Anacardium occidentale* L.) seeds showed the highest inhibition (73.6±0.35%). It has been already reported that cashew seed extracts inhibit trypsin and chymotrypsin activities [20]. The seed from beans inhibited the gut protease activity of *Spodoptera mauritia* larvae to 50%. Richa et al. isolated trypsin inhibitor from beans [21]. Carrot extracts also inhibited the activity to similar extent (51.56% inhibition). A trypsin inhibitor was purified from carrot cells by Irene carlberg et al. [22]. *Syzygium cumini* L. and *Hopea ponga* (Dennst.) seed extracts inhibited the gut protease activity to about 37%. To our knowledge no protease inhibitor was reported these seeds. Teak seeds, ladies finger seed, Water melon seed and Mamey Sapote seed inhibited gut protease activity of *Spodoptera mauritia* larvae to the extent of 32.5%, 27.45%, 21.59% and 19.81% respectively. Trypsin inhibitor has been reported from seeds of ladies finger and watermelon (*Citrullus vulgaris*) [23,24]. The extent of inhibition by extract from coffee seed was 45.58%. Genes coding for cysteine proteinase inhibitor was reported from coffee seeds by QRT-PCR [25].

Table 1. List of plants showing greater than 20% inhibition of gut protease activity of Spodoptera *mauritia* larvae.

Name of the plant	Mean % inhibition ± SE
Anacardium occidentale L.(Cashew)	73.6 ± 0.35
Daucus carota L. (Carrot)	51.56 ± 0.37
Phaseolus vulgaris L. (Beans)	49.96±1.28
Coffea arabica L.(Coffee)	45.58±0.25
Hopea ponga (Dennst.) Mabb. (Kambakam)	37.5±0.43
Syzygium cumini L. (Java pium)	36.2±1.00
Dioscoria alata L. (Purple yam)	35.58±1.27
Tectona grandis L. (Teak)	32.5±0.43
Abelmoschus esculentus L. (Ladies finger)	27.45±0.58
Citrullus lanatus (Thunb.) (Water melon)	21.6±0.18
Pouteria sapota (Jacq.) (Mamey sapote)	19.81±0.70

Table 2. List of plants showing no or less than 20% inhibition of gut protease activity of Spodoptera *mauritia* larvae.

Name of the plant
Calophyllum inophyllum L.(Alexandrian laura)
Moringa oleifera Lam. (Drum stick, Moringa)
Allium oschaninii O. Fedtsch. (Shallot)
Bixa orellana L. (Achiote)
Solanum melongena L. (Brinjal)
Cephalandra indica Naud. (Ivyguard)
Solanum lycopersicum L.(Tomato)
Saraca asoca (Roxb.) (Asoka)
Attalea cohune Mart. (Cohune palm)

To our knowledge this is the first report indicating the presence of protease inhibitor from the seeds of *Hopea ponga* and *Syzygium cumini*. The other plants containing protease inhibitors (showing greater than 20% inhibition) reported in this study are previously reported to contain protease inhibitor against protease from other insects or animals. But here we showed that the

protease inhibitors from these extracts were able to inhibit the gut protease activity of S*podopera maurita* larvae as well.

The genetically modified (GM) crops expressing protease inhibitors are being tried as an alternative to GM crops with toxin genes (like Bt toxins). It is advantageous to introduce protease inhibitor genes in plants as they are naturally present in plants, so the chances of adverse effects on human and other animals is less likely. But many of the GM crops expressing protease inhibitors are not very successful as the insects develop resistance by secreting proteases insensitive to inhibitors or by degrading protease inhibitors [26]. Available biochemical and molecular evidence indicates that some insects adapt to the presence of protease inhibitors by overproducing existing digestive proteases [27,28].

Hence, it is important to identify better protease inhibitors which are more potent and less likely to develop resistance. This can be achieved by modifying the already identified protease inhibitors or by isolating novel protease inhibitors. Thus it is worth further investigating the extracts containing protease inhibitors reported here to identify, purify, characterize and clone the gene encoding protease inhibitors. Studies in this direction are ongoing in our laboratory.

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