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Major protease activity in the larval gut of *Spodoptera litura* boisd. (Lepidoptera: Noctuidae) is contributed by serine proteases

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Abstract
Spodoptera litura or tobacco cut worm or army worm is a polyphagous pest and has a wide host range of up to 120 species. Insects use a variety of proteases for the digestion of food materials. Proper identification of the kind of protease present in the gut of the insect pest is important for targeting the gut proteases for the control of pests. In this study we identified the major proteases in the midgut of the larvae of *S. litura* using synthetic protease inhibitors. Phenyl Methane Sulphonyl Fluride (5mM) and Benzamidine Hydrochloride (10mM), the serine protease inhibitors, showed the highest protease inhibition of 68.12±1.42 and 60.25±0.79 % respectively. This indicates that serine proteases represent the major protease in the gut of the larvae of *S.litura*. Aspartyl protease inhibitor, pepstatin (7µM) and cysteine protease inhibitor, NEM (0.5mM) inhibits respectively 18.66 ±0.29% and 15.33 ± 1.74% of the gut protease activity of *S.litura* indicating that the contribution of aspartic protease and cysteine protease in protein digestion in the gut of *S.litura* is much less compared to serine proteases. *In vivo* feeding experiments with leaf dipped in PMSF followed by zymography of the gut extract revealed that the inhibitor is active under *in vivo* conditions. Further characterization of the major gut proteases in the midgut of the larvae of *S. litura* will help design strategies for the control of the pest targeting the gut proteases.

Keywords: proteases, protease inhibitors, *Spodoptera litura*, pest control

Introduction
Agriculture sector plays an important role in the economic development and pest control has become an inevitable part of any successful agricultural practices. Pests are harmful species whose population or density goes beyond the damage threshold level either throughout the year or during specific season [1]. The pests are reported to cause 20-30% total loss in the agriculture sector [2]. Most of the insect pests that attack the crops come under the order lepidoptera. *Spodoptera litura* or tobacco cut worm or army worm is a polyphagous defoliator of many crops and pose deleterious damage to them by their voracious feeding behaviour. *Spodoptera litura* has a wide host *range* of up to 120 species [3]. *Spodoptera litura* is widely distributed throughout tropical and temperate regions of South and East Asia, Europe, Africa and Oceania [4]. In India, the pest is widely distributed in almost all states and cause significant losses to economically important crops. The Life cycle of *S.litura* involve complete metamorphosis with four stages like egg, larva pupa and adult. The larvae are voracious feeders and there are six larval instars.

Biological or chemical pest control strategies were used for the control of *S. litura*. The mass releases of egg and larval parasitoids for the control of *S. litura* in different crops in different areas had achieved only partial success [5, 6]. Chemical pesticides were also used for the control of *S. litura*. Recently many pests including *S. litura* have developed resistance to many commercially available pesticides [7, 8]. The extensive use of conventional insecticides has led to several major concerns. The development of resistance to the pesticides, loss of natural enemies and effects on non-target species were the major concerns in the agricultural sector [9]. Thus alternate strategies for pest management are gaining momentum and insect gut proteases are new targets for pest control.

Proteases catalyze the hydrolytic cleavage of specific peptide bonds in their target proteins [10]. Larval gut proteases play a major role in growth and development in insects as they make available the amino acids from proteins in the diet which is crucial for development of the larvae to pupae and adult. The role of protease inhibitors in plant defence against insect pests was unveiled when Mickel and Standish observed the inability of the larvae of *Tribolium castaneum* to grow on soybean products [11]. Later on the presence of trypsin inhibitors in soybean and its toxic effect on the larvae was proved [12]. These findings suggest the ability of plant protease inhibitors to block protein digestion and retard the growth and development in insects [13]. Plants have developed mechanisms to fight the pests and pathogenic organisms. One important line of defence that plants use to fight against the pests and pathogens is through various inhibitors that act against the gut proteolytic enzymes. Exploiting this natural defence mechanism of plants, over expressing the plant protease inhibitors in certain transgenic plants to protect them against pest attack is tried. Proper identification of the kind of protease present in the gut of the insect pest is important for targeting the gut proteases for the control of pests. In many lepidopteran pests the major proteases in the gut are trypsin-like serine proteases [14]. In this study we identified the different kinds of proteases present in the gut of *S. litura* and their relative contribution towards protein digestion.

Materials and Methods

Chemicals

Azo-casein, Phenyl Methyl Sulfonyl Fluride (PMSF), Pepstatin A, Benzamidine hydro chloride (BHC) and N-ethyl maleimide (NEM) used for the study was purchased from Sigma- Aldrich Chemicals Company USA. All other chemicals used were of analytical grade.

Collection and rearing of *Spodoptera litura* larvae

The pupae of *Spodoptera litura* were purchased from NBAIR Bangalore, India. The emerged adult moths were kept in glass beakers covered with a muslin cloth and fed with the dilute solution of honey. They were allowed to mate and lay eggs. After 3-4 days, larvae hatched out. The larvae were reared in glass beakers. As they grew in size were transferred into plastic troughs fed with fresh *Ricinus communis* leaves. The larval culture was maintained at room temperature (25± 5^0C) and relative humidity of 90±3%%. There are six larval stages for *S.litura* and the life cycle is completed in 19-21 days.

Preparation of larval gut extract of *Spodoptera litura*

The mid-gut of the 5th instar larvae of *Spodoptera litura* was dissected out after ether/cold anaesthesia and weighed. It is homogenized in sodium bicarbonate buffer pH 9.0 (1ml/g of tissue). The homogenates were centrifuged at 9800 xg at 4ºC for 10 minutes. The supernatant containing gut protease was collected and frozen until use.

Protease Assay

Protease assay was done by incubating gut extract (5µl) with azocaesine ((11.48µg/µl)) as a substrate in bicarbonate buffer (pH 9.0) at 37ºC for 30 minutes in a total volume of 20.2µl. After incubation, the reaction was stopped by adding 80 µl of 50%TCA. The tubes were centrifuged at 9800 x g at 4°C for 10 minutes. Fifty microliter supernatant was diluted to 200 µl with 500 mM NaOH. The absorbance was measured at 440 nm in a microplate reader. All assays were done in duplicate and repeated 3 times.

Preparation of protease inhibitor

The required concentration of protease inhibitors was made by dissolving in appropriate solvents to make a stock and diluted to get the desired final concentration. Phenyl Methane Sulphonyl Fluoride (PMSF) 5mM, Benzamidine Hydrochloride (BHC) 10mM, Pepstatin7µM, and N-ethyl Maleimide (NEM) 0.5mM final concentration were used from a 10X stock dissolved in isopropanol, water, methanol, and alcohol respectively.

Protease Inhibition Assay

The inhibitors were dissolved in suitable solvents as mentioned and 10µl of it was pre incubated with 5µl of larval gut extract for 10 minutes at 37 ^{0}C. After pre incubation the substrate was added and the assay continued as described in protease assay. A control with solvent alone was also done. All assays were done in duplicates and repeated thrice. Percentage inhibition was calculated by taking the protease activity of gut extract alone as hundred percentage activity.

In vivo protease inhibitor treatment and Zymography

Ricinus communis leaves dipped in 5mM PMSF and fed to the fifth instar larvae. After twenty minutes the gut was removed and extract prepared as described earlier. Zymography was done with gelatine impregnated gel as per the protocol of Martha Toth and Rafael Fridman (2001) [15].

Statistical Analysis

Statistical analysis was done using R- Programme.

Results and discussion

Treatment of gut extracts with Serine protease inhibitors

Spodoptera litura, fifth instar day 0 larvae were treated with 5mM PMSF, a serine protease inhibitor. The inhibition of gut protease activity was 68.12±1.42% (Table 1). The lepidopteran larvae have trypsin like serine protease activity with its alkaline pH optima at pH 11.0 [16, 17]. Chymotrypsin and trypsin are the major proteinase present in pests [18, 19, 20]. Serine proteases have been partially purified from the gut extract of *S. litura* and all the three proteases are completely inhibited by PMSF [17]. Among the protease inhibitors tested, PMSF is giving the highest inhibition indicating that the major proteases in the gut of *S.litura* are serine proteases.

Table 1: Percentage inhibition of larval gut proteases of *Spodoptera litura* on *in vitro* treatment with PMSF, BHC, Pepstatin and NEM

Protease inhibitor (concentration)	% of inhibition Mean ± SE
PMSF(5mM)	68.12±1.42
BHC (10mM)	60.25±0.79
Pepstatin (7µM)	18.66 ±0.29
NEM (0.5mM)	15.33 ± 1.74

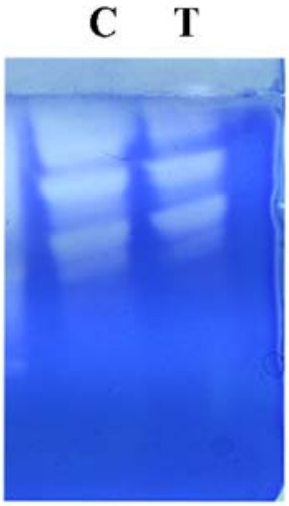

Fig 1: Zymogram showing the protease activity of the gut extracts of *S.litura* untreated (C) and fed with leaf dipped in 5 mM PMSF (T).

When late 5th instar larvae of *Spodoptera litura* gut extract was treated with 10 mM BHC, another serine protease inhibitor, the total protease activity was inhibited to the extent of 60.25±0.79 % (Table 1). Soybean trypsin inhibitor (SBTI), a serine protease inhibitor, when incorporated in the diet of neonate larvae of *S. litura* led to retardation in growth indicating the presence of serine protease in the larval gut [21]. From our results it is conformed that serine protease is the major type of protease in the gut of *S. litura* larvae. When *Spodoptera mauritia* larvae treated with serine protease inhibitor, BHC and PMSF similar results were observed [22].

Treatment of gut extract with Aspartic protease inhibitor

Pepstatin is an aspartic protease inhibitor. Total protease activity in the gut extract of *Spodoptera litura* larvae inhibited to the extent of 18.66 ±0.29 % when treated with 7µM pepstatin (Table 1). This indicates that the contribution of aspartic proteases towards the larval gut protease of *S. litura* is minimal. Similar results were obtained in *Spodoptera mauritia* larvae also [22]. Aspartic protease was partially characterized from the larval midgut of Lepidopteran diamondback moth, *Plutella xylostella* [23] and purified protease was inhibited by pepstatin.

Treatment of Gut Extract with Cysteine protease inhibitor

When *S.litura* gut extract was treated with 0.5 mM NEM, a cysteine protease inhibitor, there was 15.33 ± 1.74 % inhibition of the larval gut protease activity (Table 1). The result indicates that the cysteine protease activity is less in the gut extract of *Spodoptera litura*. Nimmi *et al* also reported negligible cysteine protease activity in the gut extract of *Spodoptera mauritia* when treated with NEM [22]. Cysteine proteases are predominant in Coleopteran midgut [24], but they also utilize serine as well as aspartic proteases [25, 26, 27].

***In vivo* protease inhibitor treatment and Zymography**

When *S.litura* larvae were fed with leaf dipped in 5mM PMSF and the gut protease activity assessed by zymography, it is found that the gut protease activity was reduced when compared to control (Fig 1). This indicates that the inhibitor is active *in vivo* but the activity was not reduced to comparable level to that of *in vitro* assay (68%).This may be due to detoxification/elimination of the inhibitor under *in vivo* conditions. Three alkaline proteases from the gut of the larva of army worm, *Spodoptera litura* is purified and characterized [17]. Also the effects of soybean trypsin inhibitor, a serine protease inhibitor, on growth and development of *Spodoptera litura* is studied [28]. The identification of the kind of proteases and their relative contribution towards protein digestion will help in design strategies for the control of the pests by targeting gut proteases.

Conclusions

In vitro experiments using synthetic protease inhibitor showed that the predominant proteases in the gut of *S. litura* are serine proteases as PMSF gave the maximum inhibition (68.12±1.42%) of the gut protease activity. Cysteine protease represents second most active proteases in the gut of *S. litura* followed by aspartic protease proteases. *In vivo* feeding experiments showed that the serine protease inhibitor, PMSF is active *In vivo*. Further characterization of the gut proteases will help identifying protease inhibitors, synthetic or naturally occurring plant protease inhibitors for the control of insect pests.

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