

Serine proteases represent the major proteases in the gut of larvae of *Spodoptera Mauritia* Boisid. (Lepidoptrea: Noctuidae)

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Abstract

Insects use a variety of proteases for the digestion of their food proteins. In this study we identified the major type of protease present in the larval gut extract of *Spodoptera mauritia*, a major pest of paddy, using commercially available protease inhibitors. We found that Phenyl Methane Sulphonyl Fluoride (5mM) and Benzamidine Hydrochloride (10 mM) inhibited larval gut proteases to the extent of $72.39 \pm 1.37\%$ and $39.41 \pm 2.04\%$ respectively. N-Ethyl Maleimide, a cysteine protease inhibitor, inhibited the gut protease activity up to $10.80 \pm 0.33\%$ and Pepstatin, an aspartyl protease inhibitor, inhibited the gut protease activity up to $2.67 \pm 0.22\%$ only. Thus it is clear that the predominant protease present in the larval gut of *S. mauritia* is serine proteases. On SDS-PAGE three major and two minor protein bands were identified in the gut extract of *S. mauritia*. Further characterization of the major gut proteases of *S. mauritia* will be helpful in targeting the gut proteases for the control of the pest.

Keywords: proteases, protease inhibitors, *spodoptera mauritia*, pest control

Introduction

A large number of insect species are adapted to feed over a variety of plants causing severe damage to our economy Live Stock and public health and such destructive insects are called pests. Pest is an insect (or organism) that causes harm to humans, their livestock, crops or possessions [1]. In general insects that cause less than 5% damage are not considered as pests. The insects which cause damage between 5 - 10% are called minor pests and those that cause damage above 10% are considered as major pests [2]. Yunus and Ho reported that about 187 species of insects on rice [3], but a few have ever become serious pests. *Spodoptera mauritia* (rice armyworm), *Chilo polychrysus* (stem borer), *Scotinophora coarctata* (Malayan black bug), *Cnapalocrocis medinalis* (leaf folder), *Nephotettix virescens* (green leafhopper) etc are some of the major insect pests of rice [4].

Spodoptera mauritia commonly called "rice swarming caterpillar", or "paddy armyworm" belongs to the order Lepidoptera, and family Noctuidae. It has wide distribution throughout the Oriental and Australian region. In south India it is common in all the rice growing tracts, especially along the west coast and the delta tracts. In some parts of India it is considered as major pest of paddy and causes serious damage while in other parts it occasionally causes severe damage. The caterpillars feed on the leaves and defoliate the plant completely. Different pest control strategies were used to control *S. mauritia* like Cultural, mechanical, biological and chemical control methods.

Proteases are the enzymes that catalyze the hydrolytic cleavage of specific peptide bonds in their target proteins [5]. Proteases are important for the digestion of proteins in the gut of insects and higher animals. Proteases can be found in animals, plants, bacteria, archaea and viruses. They are classified based on the active residues involved in catalysis into Serine proteases, Cysteine proteases, Aspartyl proteases

and Metallo proteases [6]. Some of the serine proteases Include trypsin, chymotrypsin and elastase etc.

Protease inhibitors (PIs) are molecules that inhibit the function of proteases and they are common in nature. Based on the type of protease inhibited, protease inhibitors are classified into Serine, Cysteine, Aspartic and Metallo protease inhibitors [7]. Synthetic protease inhibitors are used to identify the type of protease present in the gut of insects. Amrutha *et al* showed that serine proteases represent the major protease in the gut of mosquito larvae [8]. Phenyl methane sulfonyl fluoride (PMSF) is a widely used serine protease inhibitor, effective against enzymes such as chymotrypsin, thrombin, and trypsin. It acts as an inhibitor via sulfonation of the hydroxyl group of serine residues at the reactive sites of serine proteases. Benzamidine hydrochloride is also a serine protease inhibitor. Pepstatin A is an aspartic protease inhibitor and it forms complex with pepsin, renin, cathepsin D etc [9]. The cysteine protease inhibitor, N-Ethylmaleimide, inhibits enzymes like papain by inhibition of the enzyme's catalytic activity by chemical modification of the essential cysteine residue [10].

Plant protease inhibitors (PPIs) act as defence molecules and inhibit the larval gut proteases of various insect pests [11, 12, 13, 14, 15, 16, 17]. It has been proposed that protease inhibitors as an effective method for controlling pests as it can inhibit insect midgut proteases resulting in indigestion of proteins in the pest affecting their growth and development [16, 18]. Remya and Kannan screened several plants to identify plant extract containing protease inhibitor against larval gut proteases of *Spodoptera mauritia* [19, 20, 21]. The effect on the pest is not only through a direct inhibition of digestive proteases, but also through the induction or over synthesis of digestive enzyme leading to depletion of sulfur containing amino acids [22]. It is possible to enhance the inherent resistance in plants by transferring to the plant protease inhibitor genes taken from another plant species.

Hilder *et al* first demonstrated this by transferring *Vigna unguiculata* trypsin inhibitor gene to tobacco plant [23]. Insects adapt to the PPIs in different ways either by over expressing native proteases or by expressing novel proteases resistant to the inhibitor. In some cases the inhibitor was degraded by other non- targeted proteases present in the insect midgut. The identification of protease in the gut of pest is necessary to target proteases for the control of the pest. In this study we identified the major type of protease present in the gut extract of the larvae of *Spodoptera mauritia* using synthetic protease inhibitors.

Materials and Methods

Chemicals

Azo-casein, Phenyl Methyl Sulfonyl Fluoride (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 mauritia* larvae

Spodoptera mauritia adult moths 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. Initially the larvae were reared in glass beakers and later transferred to plastic troughs as they grew in size. The culture was maintained at room temperature (25 ± 5 °C), relative humidity $90 \pm 3\%$ and 12:12 light: dark photoperiodic regimen 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 six larval instars. Pupae were kept in beakers for adults to emerge. Collected adults were used for maintaining the culture.

Preparation of gut extract

Midgut of several 6th instar larvae of *Spodoptera mauritia* were dissected out and weighed. It is homogenized in sodium bicarbonate buffer, pH 9.0 (1ml/1g of tissue). The homogenates were centrifuged at 9,800 xg at 4 °C for 10 minutes. The supernatant was collected and frozen until use.

Protease Assay

The total protease activity was assessed by incubating 5µl of gut extract with 5.2 µl of azocasein (2.5µg/µl) at 37 °C for 30 minute in a total volume of 20.2 µl in bicarbonate buffer pH 9.0. After incubation, the reaction was stopped by adding 80 µl of 5% TCA. The mixture was centrifuged at 9,800 x g at 4 °C for 10 minutes. Fifty µl of supernatant mixed with 150 µl of 500mM NaOH and absorbance measured at 440 nm in a microplate reader. All assays were done in duplicates and repeated three times.

Protease Inhibition Assay

For protease inhibition assay, commercially available protease inhibitors were used Phenyl Methyl Sulfonyl Fluoride (2 mM and 5 mM), Benzamidine hydro chloride (10mM), Pepstatin A (7 µM), and N-ethyl maleimide (0.5 mM) were used. The inhibitor dissolved in suitable solvent (10µl) was pre incubated with 5µl of larval gut extract for 10 minutes at 37 °C and then the substrate was added. The assay was 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 absorbance of the control (without inhibitor) as 100% activity.

SDS-PAGE

SDS-PAGE was done according to the method described by Laemmli using 10 % acrylamide in the separating gel and stained with Coomassie brilliant blue dye [24].

Protein Estimation

Protein concentration of gut extract was estimated by Bradford's Dye binding method using Bovine serum albumin as standard [25].

Statistical Analysis

Statistical analysis was done using R- program.

Results and Discussion

To identify the type of proteases present in the larval gut extract of *Spodoptera mauritia* different synthetic protease inhibitor were used.

Treatment of gut extracts with Serine protease inhibitors

When the gut extract of the *Spodoptera mauritia* 6th instar larvae were treated with 10mM BHC, a serine protease inhibitor, the total protease activity was inhibited to the extent of 39.41 ± 2.04 % (Table 1). Larval gut extract on treatment with 2 mM PMSF and 5mM PMSF, the inhibition of total protease activity was up to $45.04 \pm 0.29\%$ and 72.39 ± 1.37 % respectively (Table 1). As the extent of inhibition by PMSF, a serine protease inhibitor, goes up to 72%, it indicates that majority of the total protease activity of the gut extract of *Spodoptera mauritia* is contributed by the serine proteases. Though PMSF can also inhibit some cysteine proteases, the contribution by cysteine protease is minimal as seen from the inhibition by N-Ethyle Maleimide, a cysteine protease inhibitor (Table 1).

Table 1: Percentage inhibition of larval gut proteases of *Spodoptera mauritia* treated with synthetic protease inhibitors.

Treatment	Average % of inhibition \pm SE
Gut extract + PMSF (2 mM)	45.04 ± 0.29
Gut extract + PMSF (5 mM)	72.39 ± 1.37
Gut extract +BHC (10 mM)	39.41 ± 2.04
Gut extract +NEM (0.5 mM)	10.80 ± 0.33
Gut extract + Pepstatin (7µM)	2.67 ± 0.22

Lepidopteran and dipteran insects have alkaline midguts and use serine proteases to degrade proteins in the diet [26, 27]. Christeller *et al.*, surveyed the larval gut extracts of 12 Lepidopteran species from 5 families and identified serine protease activity in all of them [28]. This is supported by our observation that the major proteases in the larval gut of *S. mauritia* are serine proteases. Anti-nutritional effects of serine protease inhibitors against several Lepidopteran insect species were reported in the literature [29, 30]. For example soybean contains serine protease inhibitors and feeding the larvae of many insect pests like *Helicoverpa armigera*, *Spodoptera litura*, *Spodoptera littoralis* etc. resulted in the reduced larval growth and development [31, 32, 33].

Treatment of Gut Extract with Cysteine protease inhibitor

Total protease activity in the gut extract of *Spodoptera*

mauritiae larvae is inhibited only to the extent of 10.80 ± 0.33 % when treated with 0.5 mM NEM, a cysteine protease inhibitor (Table 1). This indicates that the contribution of cysteine protease towards protein digestion in *Spodoptera mauritia* larvae is minimal. Coleopterans have mildly acidic midguts and they were known to use cysteine proteases to digest proteins [21, 23]. Cysteine proteases were found in the Coleopteran families like Tenebrionidae, Chrysomelidae, Coccinellidae, Curculionidae and Meloidae [21, 34]. In Colorado potato beetle (*Leptinotarsa decemlineata*), maize weevil (*Sitophilus zeamais*) cowpea bruchid (*Callosobruchus maculatus* (F)), and western corn rootworm (*Diabrotica virgifera virgifera*) [21, 35, 36, 37] cysteine proteases are the major digestive proteases [32, 33, 34, 35].

Treatment of gut extract with Aspartic protease inhibitor

When gut extract of *Spodoptera mauritia* was treated with 7 μ M pepstatin, an aspartic protease inhibitor, there was no appreciable inhibition of the total gut protease activity (Table: 1). This results indicate that aspartic protease activity in the gut extract of *Spodoptera mauritia* larvae is

negligible under these conditions. Larval midgut homogenates of cow pea burchids has been shown to have aspartic protease activity [38]. Novel aspartic proteases were purified from the larval gut extract of Diamondback moth (*Plutella xylostella*) [39].

SDS-PAGE

To analyze the protein profile of the *S. mauritia* larval gut extract, 75 μ g of protein from gut extract was loaded on to 10 % SDS PAGE and run under reducing conditions to separate the polypeptides. Three major bands, (53.70 kDa, 36.30 kDa, 24.54 kDa) and two minor bands (67.60 kDa, 60.25 kDa) were observed in the gel (Figure 1). Further, isolation of the polypeptides is necessary to identify and characterize the major protease from the gut of *S. mauritia* larvae. Two digestive trypsin like proteinases (molecular weight 24 kDa and 27 kDa) were purified by Novillo *et al.* from the larvae of stalk corn borer (*Sesamia nonagrioides* Lef. (Lepidoptera: Noctuidae)) [40]. Midgut extract from the *Helicoverpa armigera* was partially purified and showed serine protease activity [41]. Smrithy and Kannan isolated and characterized the major protease from the gut of *Aedes albopictus* mosquito larvae [42].

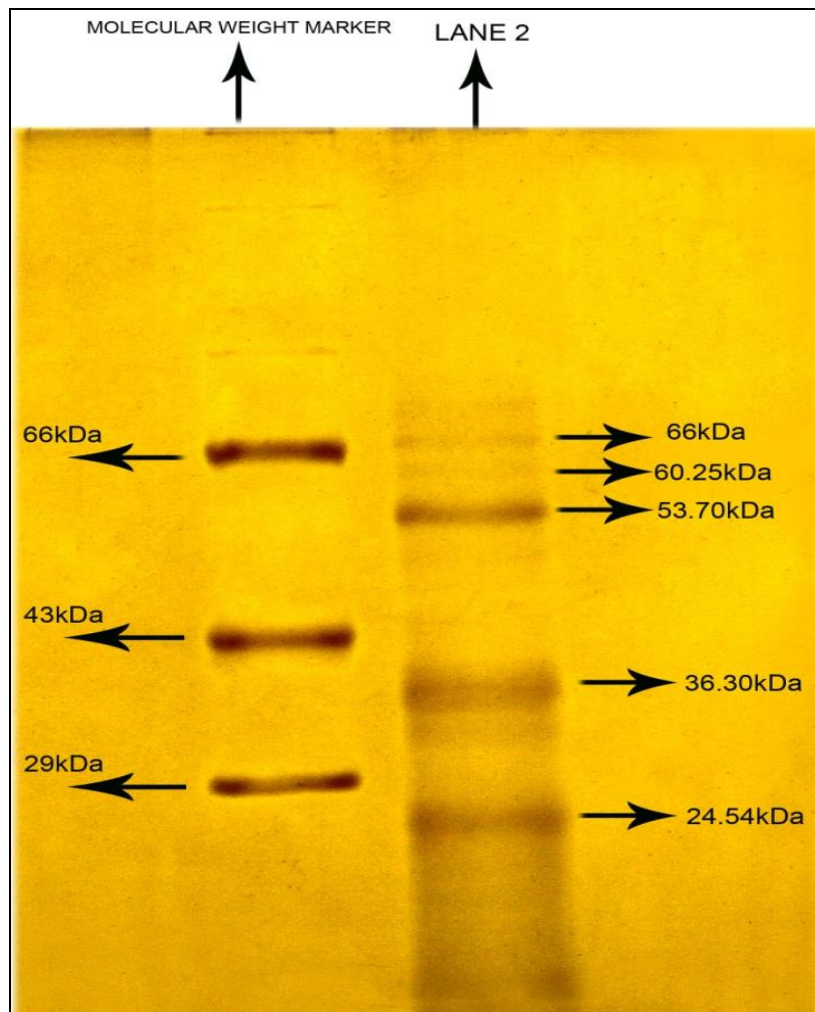


Fig 1: SDS-PAGE (10%) electrophoresis of *Spodoptera mauritia* gut extract: Lane 1: Molecular weight marker; Lane 2: gut extract (75 μ g protein).

Conclusions

Using synthetic protease inhibitors it is shown that the predominant protease in the gut of *Spodoptera mauritia* belongs to serine proteases. Cysteine proteases represent

second most active protease in the gut of *Spodoptera mauritia*. On SDS-PAGE the presence of three major and two minor polypeptide bands were identified in the gut extract. Further investigations are necessary to identify and

characterize the major serine proteases present in the gut of *Spodoptera mauritia* and this will help in designing better insect control strategies targeting gut proteases.

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