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Serine proteases represent the predominant protease in the gut of *Culex pipiens* mosquito larvae

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Abstract
Many insects including mosquitoes digest proteins in the diet with the help of different kinds of proteases secreted in the gut. Different classes of protease inhibit the activity of different kinds of proteases. Thus the kind of proteases present in the gut can be identified using synthetic proteases of known specificity. In this study the contribution of different types of protease in the gut of *Culex pipiens* mosquito larvae towards protein digestion is identified using synthetic protease inhibitors. Benzamidine Hydrochloride (BHC), Phenyl Methane Sulphonyl Fluride (PMSF), both serine protease inhibitors, inhibited the gut protease activity of *Culex pipiens* mosquito larvae to the extent of 37.03±1.24% and 78.16±4.17% respectively. A synthetic cysteine protease inhibitor, N-Ethyle Maleimide (NEM), inhibited the gut protease activity of larvae of *Culex pipiens* up to 15.85±1.66 %. This indicates that the major protease in the gut of *Culex pipiens* belong to the class of serine proteases. Thus targeting serine proteases with synthetic or naturally occurring plant protease inhibitors will be an ideal strategy for the control of mosquitoes.

Keywords: *Culex pipiens*, mosquito larvae, benzamidine hydrochloride (BHC), phenyl methane sulphonyl fluride (PMSF)

Introduction
Mosquitoes that bite humans act as vectors of a number of diseases, affecting millions of people every year. The females of many species of mosquitoes are blood sucking pests and vectors of diseases. *Culex* mosquitoes are persistent biters and prefer to attack at dusk and after dark. *Culex* mosquitoes spread diseases like West Nile fever, lymphatic filariasis, Japanese encephalitis, and viral diseases of birds and horses.

Proteases are enzymes which catalyze the hydrolysis of proteins [1]. Proteases can be found in animals, plants, bacteria, archaea, and viruses [2]. Proteases are commonly classified in to 6 classes such as Serine proteases, Cysteine proteases, Aspartate protease, Threonine proteases, Glutamic acid proteases and Metalloproteases [3, 4, 5, 6]. Mid gut of many insects contain proteases which play a vital role in providing free amino acids essential for insect's normal growth and development. There are qualitative differences among the proteases from different species of insects [7, 8]. In the case of mosquitoes, the increased blood meal contributes to the maximal protease secretion and activity [9].

Protease inhibitors are the molecules that inhibit the functions of proteases. There are synthetic protease inhibitors as well as naturally occurring plant protease inhibitors with specificity towards different class of proteases. For example Benzamidine hydrochloride inhibits serine proteases and Pepstatin is an aspartate protease inhibitor [10]. Thus by using such specific synthetic protease inhibitors, we can identify the type of proteases present in the gut of mosquito larvae. Characterization of the kinds of proteases in the gut of mosquito larvae will be helpful in designing strategies for the control of mosquito using protease inhibitors either synthetic or plant protease inhibitors which are naturally occurring. In this study we analyzed the contribution of different types of proteases towards the protein digestion in the gut of larvae of *Culex pipiens* mosquito using synthetic protease inhibitors.

Materials and Methods
Collection of mosquito larvae
The larvae of *Culex pipiens* mosquito were collected from the Botanical garden of University of Calicut, Kerala, India. The identification of larvae was done using morphological characteristics and DNA barcoding. The collected larvae were kept in plastic beakers containing well water and fed with yeast granules until they reach fourth instar.

Preparation of gut extract
Alimentary canal of several 4th instar larvae of *Culex pipiens* were dissected out and weighed. It was homogenized in bicarbonate buffer (pH 9.0).The homogenates were centrifuged at 9390 Xg at 4°C for 10 minutes. The supernatant containing soluble gut proteases was frozen until use.

Preparation of protease inhibitors
Required concentration of protease inhibitors were made by dissolving in appropriate solvents as a 10X stock and added to the gut extract to get the final concentration used. Benzamidine Hydrochloride (BHC) stock was made by dissolving in water immediately before use and Phenyl Methane Sulphonyl Fluoride (PMSF) was dissolved in isopropanol. N-ethyl Maleimide (NEM) was dissolved in alcohol. An equivalent volume of vehicle alone was added to the control (gut extract).

Protease assay
Protease assay was done by incubating gut extract (5µl) with azocaesine (2.5µg/µl) as 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 9390X g at 4°C for 10 minutes. Fifty micro liter supernatant was diluted to 200 µl with 500 mM NaOH. The absorbance was measured at 440 nm in micro plate reader. All assays were done in duplicate and repeated 3-4 times.

In vitro protease inhibition assay

Protease inhibition assay was conducted as above except that the gut extract (5µl) was pre-incubated with protease inhibitor (in a volume of 2 µl) for 10 minutes before adding the substrate. All experiments were done in duplicates and repeated 3-4 times. The percentage inhibition was calculated taking the activity in the control as hundred percent.

Results

In vitro protease assay

Effect of Benzamidine Hydrochloride on gut protease activity

Larval gut (4th instar) extract of *Culex pipiens* mosquito when treated with 10mM BHC, the total protease activity was inhibited to the extent of 37.03±1.24% (Table 1). This highly significant inhibition (P<0.001) indicates that, about one third of the gut extract protease activity is contributed by serine proteases inhibitable by BHC.

Effect of Phenyl Methane Sulphonyl Fluride on gut protease activity

When 4th instar larvae of *Culex pipiens* mosquito larvae were treated with 2mM PMSF, gut protease activity was inhibited up to 52.72±1.53% and when the concentration of PMSF increased to 5mM, the inhibition increased up to 78.16±4.17% (Table 1). The extent of inhibition by PMSF, a serine protease inhibitor, is up to almost fifty percent at 2mM PMSF and three fourth of the total protease activity at 5mM PMSF.

Effect of N-Ethyle Maleimide on gut protease activity

Culex pipiens mosquito larval extract when treated with 0.5mM N-Ethyle Maleimide (NEM), the total protease activity was inhibited to the extent 15.85±1.66% (table 1) NEM is a Cysteine protease inhibitor and the result indicates that the cysteine protease activity is present in the gut extract of *Culex pipiens* mosquito larvae.

Table 1: Percentage inhibition of gut protease activity of *Culex pipiens* mosquito larval gut extract on in vitro treatment with BHC, PMSF, and NEM.

Treatments	Average % of inhibition ± SE	P Value
Gut extract + BHC (10mM)	37.03±1.24	p <0.001
Gut Extract + PMSF (2mM)	52.72±1.53	p <0.001
Gut extract + PMSF(5mM)	78.16±4.17	p <0.001
Gut extract+ NEM (0.5mM)	15.85±1.66	p <0.004

Discussion

Gut extract from 4th instar larvae of *Culex pipiens* mosquito when treated with 10mM BHC, the total protease activity was inhibited to the extent of 37.03±1.24% (Table 1). This indicates that, about one third of the gut extract protease activity is contributed by serine proteases inhibitable by BHC.

When the gut extract from the 4th instar larvae of *Culex pipiens* mosquito were treated with 2mM PMSF, gut protease activity was inhibited up to 52.72±1.53% and when the concentration of PMSF increased to 5mM, the inhibition increased to 78.16±4.17% (Table1). The extent of inhibition by PMSF, a serine protease inhibitor, is up to almost fifty percent at 2mM PMSF and three fourth of the total protease activity at 5mM PMSF. As PMSF inhibits to a certain extent cysteine proteases also, the inhibition may be the reflection of inhibition of serine and cysteine proteases in the gut of mosquito larvae. This indicates that up to three forth of the total protease activity of the gut extract of *Culex pipiens* mosquito larva is contributed by the serine and cysteine proteases inhibitable by PMSF. Gut extract of *Culex pipiens* mosquito larvae when treated with 0.5mM N-Ethyle Maleimide (NEM), the protease activity was inhibited to the extent 15.85±1.66% (P=0.004) (table 1). NEM is a cysteine protease inhibitor and the result indicates that the cysteinee protease activity is present in the gut extract of *Culex pipiens* mosquito larva and is much lower than that of serine protease activity. Considering the low activity of cysteine protease in the gut extract it can be concluded that the predominant protease in the gut of *Culex pipiens* mosquito larvae is represented by serine proteases.

Trypsin-like proteases account for the major share of blood meal digestion in mosquitoes and the trypsin is expressed in a biphasic manner during blood meal digestion [11]. Gene silencing of 5G1, which is associated with late phase protein digestion, or the addition of soybean trypsin inhibitor to the blood meals of *Aedes aegypti* mosquito significantly increased mid gut infection rates of dengue virus DENV-2 [12] indicating a role for gut protease in viral infection of mosquito. Using soybean trypsin inhibitor, it was shown that early trypsin enzyme activity is required for late trypsin gene transcription in the mid gut of *Ae. aegypti* [13, 14]. Also it has been shown that addition of synthetic protease inhibitor enhances the activity of Bt toxin in *H.*zea larvae. The role of protease inhibitors on the larvae of mosquito is not largely explored. The finding reported here that serine proteases is the predominant protease in the gut of *Culex pipiens* larvae makes serine protease an ideal target for mosquito control. This can be achieved by inhibiting the gut protease activity by synthetic molecules or naturally occurring plant protease inhibitor either by targeted delivery or activation inside the gut of mosquito larvae.

Conclusions

From in vitro studies carried out in the fourth instar larvae of *Culex pipiens*, it is clear that the major protease activity in the gut of *Culex pipiens* larvae is contributed by serine proteases. It may be concluded that targeting serine proteases with synthetic or naturally occurring plant protease inhibitors will be an ideal strategy for the control of mosquitoes.

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