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Effect of serine protease inhibitors on gut protease activity of *Aedes albopictus* fourth instar larvae

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Abstract
Mosquitoes are vectors of many viral, protozoan and parasitic diseases. Mosquitoes of genus *Aedes* transmit many viral diseases including dengue fever, chikungunya and yellow fever. The mosquito control strategies are not completely successful owing to the development of insecticide resistant mosquitoes. In this study we examined the effect of different serine protease inhibitors on the gut protease activity of *Aedes albopictus* larvae. In *in vitro* study, the serine protease inhibitor Aprotinin inhibited the gut protease activity of *Ae. albopictus* fourth instar larvae to the extent of 91.28 ± 0.95% followed by soybean trypsin inhibitor 90.44 ± 6.03%, PMSF 65.00 ± 2.59%, and Benzamidine Hydrochloride 40.04 ± 2.02%. When larvae were exposed to protease inhibitors, the highest percentage of mortality was observed on treatment with SBTi (16.67 ± 1.95%), and for PMSF (16.67 ± 6.68%) followed by Aprotinin (15 ± 4.09%).

Keywords: Gut protease, protease inhibitor, *Aedes albopictus*

1. Introduction
Mosquitoes are unquestionably the most significant blood feeding ectoparasites and medically important arthropod vectors of diseases. There are a number of viruses transmitted by mosquitoes which drastically affect the public health and is a major burden in terms of economy worldwide. Every year over a million people die from mosquito transmitted diseases worldwide. Mosquito born diseases include malaria, chikungunya, filariasis, dengue, encephalitis, Zika disease and yellow fever. The number of dengue cases reported increased from 2.2 million in 2010 to more than 3.34 million in 2016 [1]. Mosquito-borne diseases are transmitted when the infected blood meal is ingested by the female mosquito and the pathogens are passed from the vector on to its human host [2-4]. The transmissions of the pathogens that cause mosquito born diseases are dependent on the abundance of competent mosquito vectors.

Aedes mosquitoes are the vectors of viral diseases like Chikungunya, Dengue and Zika. The two species of *Aedes* genus, *Ae. albopictus* and *Ae. Aegypti*, have the vector status. Replication of viral pathogens occur first in midgut cells of the vector, followed by distribution to other organs including salivary glands. Soon after the viral infection of the salivary gland of the vector, it gets transmitted to the host during blood feeding [5-7]. Development of vector is dependent on various factors like vector competence, temperature, and viral dose at infection.

Increased *Aedes* vector preponderance and distribution is reported in Western Ghats region in Kerala and the reason for the population outbreak of mosquito may be due to increased ambient temperature [8]. Since the epidemiology and incidence of mosquito born diseases have dramatically increased in recent years and the traditional strategies for mosquito control is not effective, novel control strategies have to be developed for the management of mosquito population.

Trypsin-like enzymes play an important role in blood meal digestion in mosquitoes [9]. In *Aedes* mosquito, trypsin-like serine peptidases are the major enzymes expressed during various developmental stages [10]. Thus studying the effect of serine protease inhibitor on gut protease activity and its effect on the survival of the larvae will be useful in formulating better mosquito control strategies directed at the gut protease activity of the mosquito larvae. In the present study we examined the effect of serine protease inhibitors on the gut protease activity and mortality of 4th instars larvae of *Aedes albopictus* (Skuse)

(Insecta: Diptera: Culicidae). Protease inhibitors studied include serine protease inhibitors like Soya Bean Kunitz Trypsin Inhibitor (SBTi), Aprotinin, Phenyl Methyl Sulfonyl Flouride (PMSF) and Benzamidine Hydro Chloride (BHC).

2. Materials and methods

Aedes albopictus larvae were collected from University of Calicut campus, Malappuram, Kerala, India. The species identification was done based on the morphological features of the larvae, adults and DNA bar coding. The collected *Aedes albopictus* larvae were maintained in the laboratory and fed with yeast granules.

2.1 Estimation of Protein

Protein quantification of gut extract was done by Bradford's dye binding method [11]. Bovine serum albumin was used as standard.

2.2 Protease Assay

Gut from 30 numbers of fourth instar larvae of *Ae. albopictus* was dissected out and homogenized in 50 µl 0.1M bicarbonate buffer, pH 9.0 and centrifuged at 9400x g for 10 minutes at 4ºC. Supernatant was stored at -20ºC until use or directly used for protease assay. The total protease activity was assessed by incubating 5 µl of gut extract with 5.2 µl azocaesein (44.8µg/µl) at 37ºC for 30 minutes in a total volume of 20.2 µl in bicarbonate buffer pH 9.0. The reaction was stopped by adding 80 µl of 5% Trichloro Acetic Acid and centrifuged at 9400x g at 4ºC for 10 minutes. Fifty micro liter of supernatant was diluted to 200 µl with 0.5M NaOH. The absorbance was measured at 440 nm using a microplate reader. All assays were done in duplicates and repeated three times.

2.3 Conjugation of soybean trypsin inhibitor to yeast cells

Yeast cells were allowed to grow under YPD broth overnight in the shaking incubator at 37 °C.

Centrifuged at 2400×g for 5 minutes at 37ºC to collect the pellet and the pellet was washed with PBS 7.4. To 200 mg yeast cells, 1 ml of Soybean Kunitz Trypsin Inhibitor (1mg/ml) was added and kept at room temperature. A control was also prepared by adding 1 ml of PBS 7.4 to 200 mg of yeast cells. Glutaraldehyde (2%) was added as a cross-linking agent and kept at room temperature for two hours. The mixture centrifuged to remove the supernatant and pellet washed thoroughly to remove glutaraldehyde. The reactive sites were blocked with 50 mM Tris HCl and the pellet stored at 4 °C.

2.4 Protease inhibition assay

For the protease inhibition assay, protease assay mixture prepared as in protease assay except that the mixture was pre-incubated with protease inhibitor for 10 minutes before addition of substrate. The final concentration of protease inhibitors used were SBTi (50 µg/ml), Aprotinin (0.01µM), PMSF (0.5mM) and Benzamidine hydrochloride (1mM). The inhibition was calculated taking the absorbance of test as 100% activity. Appropriate controls such as protease inhibitor alone and gut extract alone were also done.

2.5 Zymography

Mosquito larval gut extract prepared from 30 larvae in 50 µL bicarbonate buffer was diluted four times with bicarbonate buffer. Two micro litre of diluted sample was loaded onto caseine (1%) impregnated polyacrylamide gel and Zymography was done as described by Martha Toth and Rafael Fridman (2001) [12].

2.6 Treatment of 4th instar *Aedes albopictus* larvae with protease inhibitors

For *in vivo* experiments, 20 mosquito larvae were treated with protease inhibitor in a total volume of 100ml well water. The larvae were fed with yeast granules and mortality assessed after 48 hours. A control with respective vehicle alone was also carried out. SBTi (0.10 mg/ml), Aprotinin (0.02µM), PMSF (1mM), were used in the study. Fresh PMSF was added at intervals to compensate for the loss due to degradation in aqueous medium.

3. Results and Discussion

Among the inhibitors tested the highest inhibition of gut protease activity was shown by Aprotinin (91.28 ± 0.95%). Soybean trypsin inhibitor inhibited the gut protease activity to the extent of 90.44 ± 6.03% followed by PMSF (0.5mM), 65.00 ± 2.59% and BHC(1mM), 40.04 ± 2.02%. (Table 1).

Table 1: Inhibition of gut protease activity in *Aedes albopictus* mosquito larvae.

Inhibitor	Percentage Inhibition *(Mean± SEM)
Aprotinin (0.01µM)	91.28±0.95
SBTi conjugated to yeast cells	53.62±0.73
SBTi (50 µg/ml)	90.44±6.03
PMSF (0.5mM)	65.76±2.59
BHC (1mM)	40.04±2.02

*Percentage inhibition was calculated by taking the activity of gut enzyme alone as 100%.

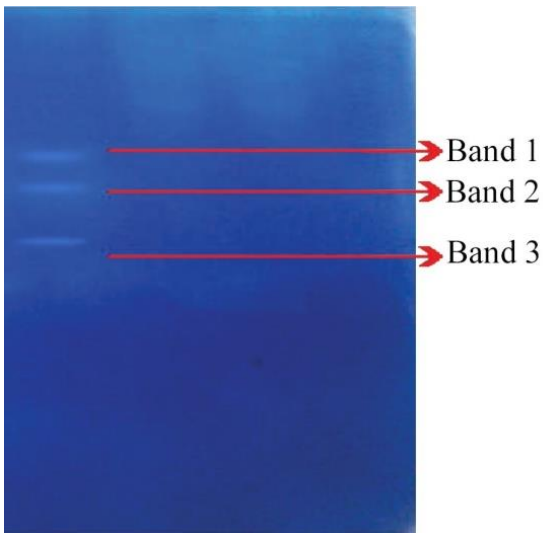

Fig 1: Zymography of gut extract *Aedes albopictus* larvae. Zymogram developed and stained with Coomassie Brilliant Blue stain.

Table 2: Percentage mortality of *Aedes albopictus* 4th instar larvae treated with different protease inhibitors.

Inhibitor	Percentage mortality* (after 48 hrs) (Mean±SE)
SBTi (100 µg/ml)	16.67 ± 1.95
PMSF (1 mM)	16.67 ± 6.68
Aprotinin (0.02µM)	15.00 ± 4.09

*Mortality expressed as the percentage of control.

3.1 Effect of protease inhibitors on gut protease activity of 4th instar larvae of *Aedes albopictus*

The highest inhibition of larval gut protease was shown by Aprotinin (91.28 ± 0.95%) (Table1). Aprotinin is a serine protease inhibitor and is known to inhibit insect protease. Carabid beetle *Nebria brevicollis* was treated with protease inhibitor Aprotinin and the Aprotinin fed beetles had a significantly lower levels of trypsin [13]. Gut protease activity of *Aedes aegypti* larvae was inhibited up to 90.44 ± 6.03% by SBTi in the present study. Soybean Trypsin inhibitor is a serine protease inhibitor and this high inhibition is in conformity with the fact that major proteases in the gut of mosquito larvae are serine proteases [14].

PMSF is a synthetic serine protease inhibitor which inhibited 65.00 ± 2.59% of the gut protease activity of *Ae. Albopictus* (Table 1). The extracts from the midgut of larvae of lesser mulberry moth, *Glyphodes pyloalis* Walker (Lepidoptera: Pyralidae), when treated with PMSF showed inhibition of larval gut protease activity up to 40.2% [15]. When SBTi conjugated yeast cells disrupted by sonication and used in the inhibition assay, it inhibited gut protease activity up to 53.62 ± 0.73%. Benzamidine Hydro Chloride inhibited (BHC) the gut protease activity to the extent of 40.04 ± 2.02% in the *in vitro* assay (Table 1). A trypsin- like serine protease was purified from a parasitic nematode *Steinernema carpocapsae* which showed high sensitivity to Benzamidine [16]. Effect of sub lethal dose of BHC on *Aedes* mosquito was studied and the observations showed that *Aedes* larvae are susceptible to it [17].

3.5 Zymography

In the present study the zymogram analysis of *Aedes albopictus* larval midgut extract showed three high intensity bands (Fig 1). These proteases represent the major proteases in *Ae. albopictus* larvae. In a previous study trypsin- like serine peptidases in the egg, larval and pupal stages of *Aedes albopictus* was investigated [18] and the protein profile consisted of 8 bands from 17 to 130 kDa molecular weight range.

3.6 Treatment of 4th instar *Aedes albopictus* larvae with protease inhibitors

Aedes albopictus fourth instar larvae when exposed to SBTi resulted in 16.67 ± 1.95% mortality (Table 2). A similar mortality (16.67 ± 6.68%) was also observed for PMSF treatment. The protease activity of both larval and pupal stages of *Aedes* were inhibited by PMSF (Leonardo Saboia *et al*., 2013) [18]. The percentage of mortality for larvae treated with Aprotinin (0.02 µM) was 15 ± 4.09% (Table 2). Although high enzyme inhibition is obtained in *in vitro* study for SBTi, Aprotenin and PMSF, the low mortality in *in vivo* experiments may be due to overcoming the inhibition by secreting other proteases or detoxification of the inhibitors *in vivo* or may be due to bioavailability issue. This aspect also should be taken into consideration when using protease inhbitiors for the control of mosquito larvae.

4. Conclusions

From this study, it is concluded that among the serine protease inhibitors tested, Aprotinin inhibited the gut protease activity of *Ae. albopictus* larvae to the extent of 91.28 ± 0.95% followed by soybean trypsin inhibitor 90.44 ± 6.03%, PMSF 65.00 ± 2.59%, and Benzamidine Hydrochloride 40.04 ± 2.02%. When larvae were exposed to protease inhibitors, the percentage of mortality was observed for SBTi was 16.67 ± 1.95%, PMSF 16.67 ± 6.68% and Aprotinin 15.00 ± 4.09%. Although *in vitro* enzyme inhibition is high, the mortality is low. This may be due to overcoming the inhibition by secreting other proteases which are insensitive to inhibition or detoxification of the inhibitors *in vivo* or reduced bioavailability of the inhibitor. This needs to be taken into account when considering protease inhibitors for the control of mosquito larvae.

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