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Isolation of soya bean trypsin inhibitor-binding protease from the gut of A*edes albopictus* (Diptera: Culicidae) larvae

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Abstract
Aedes albopictus is a mosquito widely distributed across tropical and sub-tropical regions of the world and is associated with the transmission of several arboviruses including that for Dengue fever, Yellow fever and Chikungunya. *Ae. albopictus* is increasingly relevant to public health due its ability to successfully colonize both urban and rural habitats, favouring the wide dispersion of viral infections. Trypsin-like enzymes play an important role in the *Aedes albopictus* digestive process. The trypsin-like enzymes present in adult mosquito were characterized previously, but little is known about enzymes in the larvae. In the present work, for the first time, from the gut of fourth instar larvae of *Ae.* a*lbopictus*, a protease was purified by affinity chromatography on Soya Bean Trypsin Inhibitor covalently linked to Sepharose. On gelatin zymography of the gut extract of fourth instar larvae of *Aedes albopictus*, there were four major protease bands of which one corresponds to the isolated protease. The isolated protease is a serine protease as it is inhibited by serine protease inhibitors, PMSF and Soya Bean Trypsin Inhibitor, to the extent of 88.3±0.002% and 87.2±0.003% respectively. On SDS-PAGE, the isolated protease runs as a 21.5 kDa single band indicating that it a pure protein. As serine proteases represent the major proteases in the gut of mosquito larvae, targeting the protease isolated from gut of the larvae of *Ae.* a*lbopictus* will be a promising strategy for the control of the mosquitoes.

Keywords: *Aedes albopictus*, gut proteases, soya bean trypsin inhibitor, mosquito larvae, zymography

1. Introduction
Many diseases affecting humans are spread by invertebrate vectors. A wide variety of pathogens, including viruses, bacteria, protozoans, and worms cause debilitating diseases and the transmission of these pathogens by vectors add impact on human and animal health [1]. More than 17% of all infectious disease are vector-borne diseases and account more than 700,000 deaths annually [2]. This is a major health concern worldwide and adds to the economic growth constraints by increasing expenses on health care system.
Many arthropods are dangerous vectors of deadly pathogens and parasites, which may hit as epidemics [1]. Of the arthropods, mosquitoes represents a key menace, since they act as vectors of many diseases like Malaria, Yellow fever, Dengue fever, Chikungunya, Lymphatic filariasis, Japanese encephalitis etc. The global incidence of dengue has grown dramatically in recent decades and about half of the world's population is now at risk as per World Health Organisation. The global estimate of the dengue infections may range from 100-400 million each year [2]. Dengue is mainly transmitted by the female *Aedes aegypti* mosquitoes but in recent years, the *Aedes albopictus* mosquito is also involved in dengue transmission in numerous countries around the world, especially in Southeast Asia [2, 3, 4].
The mosquito, *Aedes albopictus* (Skuse), has a wide geographic distribution and is reported from Europe, Middle East, Asia, North and Central America, South America, and Africa. Adult *Aedes albopictus* mosquito is with bold black shiny scales and silver white scales on the palpus and tarsi [5]. The scutum (back) is black with a white stripe down the center beginning at the dorsal surface of the head and continuing along the thorax. *Aedes albopictus* (Skuse), the Asian tiger mosquito, is an aggressive daytime-biter and in addition to spreading dengue it acts as vectors of Yellow fever virus, Chikungunya virus and Zika virus. In recent years, the relevance of this species to public health has increased because it is able to successfully colonize both urban and rural habitats [6].
An important step in food digestion is the hydrolysis of proteins to amino acid residues by proteolytic enzymes. Among the enzymes, trypsin-like and chymotrypsin-like proteases are the important digestive proteases for most insects, except for some hemipterans and coleopteran species [7]. Molecular characterization of genes encoding trypsin-like enzymes from *Aedes aegypti* larvae was carried out and purified a 28 kDa protease [8]. The main peptidases expressed during the egg, larval and pupal stages of *Ae. albopictus* were identified as trypsin-like serine peptidases [9] but no protease from the gut of *Ae. albopictus* larvae is purified. In this study we purified a serine protease from the gut of *Ae. albopictus* larvae by affinity chromatography on Soya Bean Trypsin Inhibitor coupled to Sepharose (SBTI-Sepharose).

2. Materials and methods
2.1 Materials
Azocaesin and Bovine Trypsin was purchased from HiMedia Laboratoris, India, Protein molecular weight Marker and cyanogen bromide (CNBr) were from Sisco Research Laboratories Limited, Mumbai. All other reagents used were of analytical grade.
2.2 Collection and maintenance of mosquito larvae
Mosquito larvae collected from the Campus of University of Calicut, Kerala, India and were brought to the laboratory and transferred to small plastic trays containing water. The larvae were fed with yeast granules. The larvae were

identified and fourth instar larvae of *Ae. albopictus* were used for experiments.

2.3 Preparation of gut extract.
The gut of 200 4th instar larvae of *Aedes albopictus* were dissected out after chilling the larvae on ice and the gut homogenized in 300 µl 0.1 M bicarbonate buffer pH 9.0 in a glass homogeniser. The homogenate was centrifuged at 9,400×g for 10 minutes at 4°C. The supernatant was collected and used for enzyme assays and for isolating the protease.

2.4 Isolation of gut protease
2.4.1 Purification of Soya Bean Trypsin Inhibitor
Soya Bean Trypsin Inhibitor (SBTI) was purified by the method of Rackis *et al.*, [10] with modifications. Twenty five grams of soya bean seed was soaked overnight in 25 ml 0.01 M phosphate buffer pH 7.6 and diluted with 175 ml of the same buffer and centrifuged at 9,400×g, 4ºC for 10 minutes. The SBTI was purified in single step using ion exchange chromatography on DEAE-Cellulose. Twenty milliliter of crude soya bean extract containing a total 215 mg protein was applied on to 2.2×10 cm column pre-equilibrated with 0.01 M phosphate buffer pH 7.6. The unbound proteins were washed out till protein free with the same buffer. SBTI was eluted with 0.01 M phosphate buffer pH 7.6 containing 0.25 M NaCl.

2.4.2 Coupling of SBTI to Sepharose
Sepharose 4B Gel was washed several times with distilled water under suction. Twenty grams of washed gel transferred to 40 ml 2M Sodium carbonate and 20 ml distilled water was added. The mixture was kept stirring at 4ºC. Then 800 mg Cynagen bromide was dissolved in 2 ml Dimethyl formamide and was added to the gel while stirring, and the activation continued for 5 minutes. The gel is immediately washed with cold 0.1 M Sodium bicarbonate using at least 20 times gel volume [11].
Cyanogen Bromide-activated Sepharose was equilibrated with 20 mM phosphate buffered saline pH 7.4 (PBS) and washed with 1 mM HCl for 30 minutes. To the 250 µl packed activated resin suspended in 250 µl PBS, 400 µg of SBTI was added and the coupling was carried out at room temperature for 4 hours with gentle agitation. The residual active sites were blocked by adding 600 µl of 50 mM Tris-HCl and kept for 2 hours and the gel washed till protein free.

2.4.3 Affinity chromatography
Affinity chromatography was carried out by batch method. Two hundred microliter of gut extract of 4th instar larvae of *Aedes albopictus* with a protein concentration of 0.85 µg/µl was incubated with 100 µl packed SBTI- Sepharose gel pre-equilibrated in 100 µl 0.1M bicarbonate buffer pH 9.0. The treated gel was kept initially for 10 mints at room temperature and then 4 hours at 4°C. The gel was washed with bicarbonate buffer, pH 9.0. The elution was carried out using 5 mM HCl. Hundred microliter of fractions were collected and immediately neutralized with 2.4 µl of 0.05 M NaOH to preserve the enzyme activity.

2.4.4 Estimation of protein
The protein concentration was estimated by measuring absorbance at 280nm in a UV-Spectrophotometer [12] for fractions from affinity chromatography and using Bradford dye binding method for gut extract [13].

2.4.5 Protease assay and Protease inhibition assay
For protease assay 5 µl of the gut extract or purified protease was mixed with 10 µl of 10 mM bicarbonate buffer pH 9.0 and incubated with 5.2 µl of azocasein (13.3 µg/µl) at 37°C for 30 min. The reaction was stopped by the addition of 80 µl of 5% TCA solution. Samples were centrifuged at 9400×g for 10 min at 4°C and 50 µl of the supernatant was mixed with 150 µl of 0.5 N NaOH solution and the absorbance was measured at 440 nm in a microplate reader. All the assays were performed in duplicate and repeated thrice.
For protease inhibition assay, 10 µl of the inhibitor was pre-incubated for 10 min at room temperature with 5 µl of gut extract (0.85 µg/µl) or purified protease (0.28 µg/µl) and proceeded with the protease assay as described earlier. All the assays were performed in duplicate and repeated thrice.

2.4.6 Protease inhibition assay with serine protease inhibitors
To confirm the nature of protease isolated, the enzyme activity in the presence of two serine protease inhibitors was investigated. The inhibition assay was done with the serine protease inhibitors, Phenyl Methyl Sulphonyl Fluoride (PMSF) and Soyabean Trypsin Inhibitor (SBTI), at a final concentration of 2 mM. Vehicle alone controls were also done.

2.4.7 Zymographic analysis
Mosquito larval gut extract (prepared from gut of 30 larvae of *Aedes albopictus* in 50 µl bicarbonate buffer pH 9.0), with a protein concentration of 0.85 µg/µl and the isolated protease (0.28 µg/µl) was diluted 50 times with bicarbonate buffer. From the diluted sample, 1 µl and 0.5 µl crude gut extract and isolated protease respectively were loaded onto gelatin impregnated gel (8%) and zymography was done as described by Toth *et al*, 2012 [14].

2.4.8 Determination of purity and subunit molecular weight of purified protease.
The purity and subunit molecular weight of isolated protease was analyzed by 10% SDS-PAGE as per the method of Laemmli [15].

3. Results and Discussion
3.1 Isolation of SBTI-binding protease from the gut of Aedes *albopictus* larvae
The crude extract from the gut of *Aedes albopictus* larvae showed protease activity comparable to that of trypsin when assayed at a concentration of 0.5µg/µl. The crude extract was loaded on to SBTI-Sepharose and eluted after washing with PBS. The total activity of protease eluted from affinity chromatography was found to be 54.3±0.005% that of crude extract. The remaining total protease activity in the crude extract, as expected, is due to the presence of other non-SBTI-binding proteases in the extract.
3.2 Effect of Serine protease inhibitors on isolated protease
As SBTI is a serine protease inhibitor, it is expected that the bound protease belongs to serine protease group. This was confirmed by treating the isolated protease with PMSF (2mM), a synthetic serine protease inhibitor, which inhibited the isolated protease activity to the extent of 88.3±0.002%.

Further the isolated protease was treated with SBTI (2mM), and found that it inhibited the isolated protease to the extent of 87.2±0.003%. This confirmed that the protease isolated from gut extract of fourth instar larvae of *Ae. albopictus* belongs to serine protease group.

Many studies have reported the presence of serine proteases that act in alkaline pH as predominant enzyme in the Lepidopteran and Dipteran gut responsible for the digestion of food [16]. Kannan *et al*., earlier reported that serine proteases represent the predominant protease in the gut of *Culex pipiens* mosquito larvae [17]. In the egg, larval, and pupal stages of *Aedes albopictus* presence of trypsin-like serine peptidases are reported [18]. The Effect of serine protease inhibitors on gut protease activity of *Aedes albopictus* fourth instar larvae showed that the major proteases in the gut of the *Aedes albopictus* larvae are serine proteases [19]. But so far no gut protease was isolated from the larvae of Ae. *albopictus.* In this study we isolated a serine protease from the gut of *Ae. albopictus* larvae.

3.3 Zymographic analysis of the purified protease

When the isolated protease was subjected to gelatin zymography only one band with protease activity was detected (Figure 1). This band corresponds to one of the four major protease bands observed in the zymogram of the crude gut extract of fourth instar larvae of *Aedes albopictus* indicating that only one protease bound and eluted from SBTI-Sepharose under the conditions of chromatography.

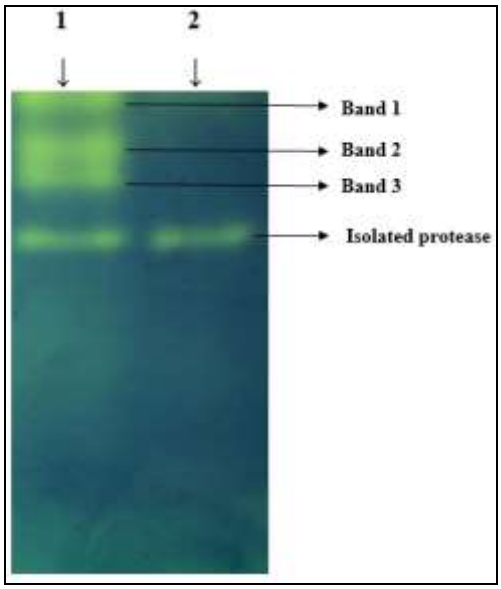

Fig 1: Gelatin zymography of gut extract of *Aedes albopictus* larvae and the isolated protease.

It was reported earlier that by zymographic analysis that the serine proteases in the larval stadia of *Ae. albopictus* presented 8 bands ranging the molecular mass from 17 to 130 kDa [9]. The zymographic analysis and by using serine protease inhibitors, serine peptidases were identified in the midgut of *A. albopictus* females [17].

3.4 Determination of purity and subunit molecular weight of purified protease by SDS-PAGE

On SDS-PAGE, the SBTI-Sepharose affinity purified protease from the gut extract of fourth instar larvae of *Aedes albopictus* showed a single band of molecular weight 21.5 kDa indicating the bound protein is a pure single protein (Figure 2).

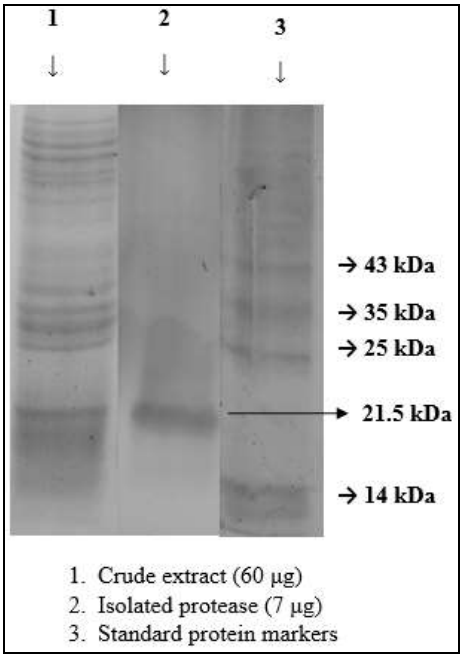

Fig 2: SDS-PAGE of protease isolated from the gut extract of *Aedes albopictus* larvae.

In *Ae.aegypti* 66 putative trypsin genes are identified in the larvae and adults [17]. Soares *et al.* purified a trypsin from *Ae. aegypti* larvae by reverse phase chromatography with a molecular weight of about 28 kDa on SDS-PAGE [8]. Although the genome of *Ae. albopictus* larvae has not yet been sequenced completely, mass spectrometric analysis of two trypsin-like serine peptidases and a spot corresponding to chymotrypsin were previously identified in the 2DE map of the *Ae. albopictus* female midgut [20]. By zymographic analysis and using protease inhibitors, presence of trypsin-like serine peptidase profiles in the egg, larval, and pupal stages of *Aedes albopictus* has been demonstrated [18]. No protease from the larvae of *Ae. albopictus* is purified. Here we report the purification of a serine protease from the gut of 4th instar *Ae. albopictus* larvae by affinity chromatography on SBTI-Sepharose. As serine proteases represent the major protease in the gut of mosquito larvae, targeting this protease will be helpful in designing strategies for the control of mosquito larvae. Small molecules or plant protease inhibitors or blocking the synthesis of the protease may be used to inhibit the activity of this enzyme. Effect of such inhibitors of on the activity or synthesis of the enzyme reported in this study will helpful for developing larvicides. Mosquito larvae are usually targeted using organophosphates, insect growth regulators, and microbial control agents [21], but targeting gut protease with specific inhibitors will be another promising strategy for the control of mosquitoes.

4. Conclusions

Aedes albopictus is one of the vectors of dengue representing an important threat to public health worldwide. Proteases play a key role in processes such as digestion, oogenesis, and metamorphosis. However, most of the detailed information on the mosquito proteolytic enzyme activities were based on the data collected from their adult stages based on blood digestion. For the first time, we isolated a serine protease from the gut extract of fourth

instar larvae of *Aedes albopictus* by affinity chromatography on Soyabean Trypsin Inhibitor coupled Sepharose. The molecular weight of the isolated protease is 21.5 kDa on SDS-PAGE. The protease activity of the purified protease is confirmed by protease assay and gelatine zymography. The purified protease was highly inhibited by phenyl-methyl sulfonyl-fluoride (PMSF) and Soyabean Trypsin Inhibitor (SBTI), with the percentage inhibition of 88.3±0.002 and 87.2±0.003 respectively, indicating that the isolated protease belongs to serine protease group.

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