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Anticoagulation activity of salivary gland extract of oriental blackfly *Simulium indicum*

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PEER REVIEW

Peer reviewer

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Comments

The mode of action of the anticoagulant of the salivary glands of *S. indicum* is clearly shown by proper bioassay methods. This study is the first for black flies in the Oriental region to be analysed for their anticoagulant activities, and highly evaluated.

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ABSTRACT

Objective: To study the morphology of the salivary gland of the female blackfly of the species *Simulium indicum* (*S. indicum*) along with protein profile and anticoagulant activity of the salivary gland extract.

Methods: Sodium dodecyl sulphate polyacrylamide gel electrophoresis was used to analyze the protein profile of the salivary gland extract (SGE) and anticoagulant activities against thrombin, and the extrinsic and intrinsic coagulation pathways were found in *S. indicum* SGE in the TT, PT and APTT assays, respectively.

Results: Results revealed that each gland consisted of a cylindrical U-shaped secretory lobe and a more or less spherical reservoir. The protein contents of whole salivary glands were also quantified and the amount of salivary gland proteins in the adult female *S. indicum* was found out to be approximately 1.12 ± 0.13 $\mu\text{g}/\text{female}$. At least 16 major and several minor protein bands were detected in the female salivary glands. The molecular masses of these major protein bands were estimated at 69, 65, 61, 58, 44, 42, 39, 33, 30, 28, 27, 26, 23, 21, 18 and 16 kDa, consecutively. Anticoagulant activities were found in *S. indicum* SGE in all the assays. It was found that SGE prolonged human plasma clotting time in a dose-dependent manner. Factor Xa inhibition was shown by the SGE of *S. indicum*. Percent inhibition value was 93.8. A positive correlation ($r=0.89$) was observed between total protein and percent inhibition of factor Xa.

Conclusions: The present study demonstrated that the mode of action of the anticoagulant(s) is mainly on the inhibition of thrombin and factor Xa along with other target factors of the coagulation cascade.

KEYWORDS

Anticoagulation, Blackfly, Morphology, Protein, Salivary glands extract, *Simulium indicum*

1. Introduction

Simulium, commonly known as blackfly or buffalo gnat or Turkey gnat, is a member of the family Simuliidae of the Culicomorpha infraorder^[1]. Rather than being fragile looking or elongated, they are small and compact, and have short but broad wings and thick legs.

The adaptation to blood feeding by blackflies involves evolution of a complex cocktail of salivary components that help the blood sucker to overcome host defenses against blood loss (hemostasis) as well as inflammatory reactions at the feeding site that disrupt blood flow or cause pain and itching. Accordingly, saliva of blood-sucking arthropods contains anticlotting, antiplatelet, vasodilatory,

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antiinflammatory, and immunomodulatory components, usually in redundant amounts[2].

Blackflies are different from other hematophagous insects described to date in possessing salivary inhibitors of coagulation factors Xa and V^[3–5] and utilizing these anticoagulants to prevent the formation of a fibrin clot.

Three anticoagulants have been identified in salivary gland extracts (SGE) of blackflies. Collectively, their inhibitory activities are targeted at 3 factors (II, V and X) in the common path of the coagulation cascade that either participate in or are end products of the prothrombinase complex. Based on information to date, this particular strategy appears to be unique among hematophagous arthropods.

In this study effort has been given to study the morphology of the salivary gland of the female blackfly of the species *Simulium indicum* (*S. indicum*) along with protein profile and anticoagulant activity of the SGE.

2. Materials and methods

2.1. Collection

Adult blackflies were collected by human baited traps. The collection times were according to the peak biting periods for each species[6]. Female blackflies were caught by an insect net while landing or flying around human baits. They were kept in a paper cup covered by a net. A pad of cotton wool soaked with 5% sucrose solution was placed on top of a cup and stored in a humid chamber.

2.2. Identification

Adult blackflies collected were identified by keys of Datta[7] and Takaoka and Suchitra[8], and by the original descriptions by Puri[9–11] and Takaoka and Davies[12].

2.3. Salivary gland dissection

The salivary glands of the adult female blackflies of the species, *S. indicum* were dissected individually using fine entomological needles under a dissecting microscope in 2-hydroxyethyl (HEPES) saline (10 mmol/L HEPES, 150 mmol/L NaCl, pH 7.4). Each pair of glands was stored separately in a microcentrifuge tube with a small volume of HEPES saline at -80 °C for further analysis.

2.4. Microscopy

The salivary glands of the female *S. indicum* Becher were dissected in HEPES saline and allowed to settle onto slides without drying out. Photographs of the glands were taken using a camera (Olympus, CAMEDIA C-5060) attached to a phase-contrast microscope (Olympus CX 31, Tokyo, Japan).

2.5. Extraction of salivary gland

Proteins were extracted from 100 pairs of salivary glands by homogenization of the tissue in phosphate buffer solution (PBS) using ultrasonication (Sartorius Stedim, BBI-8535108, Germany) followed by three freeze-thaw cycles (between liquid N₂ and 37 °C). The extracted suspension was centrifuged for 10 min, 14000 r/min at 20 °C, and the supernatant were collected for further analysis.

2.6. Quantification of total soluble protein

The protein content of each salivary gland pair was determined using a BCA Protein Assay Kit (Sigma-Aldrich, US) according to the manufacturer's instruction. The protein concentration was determined based on a bovine serum albumin (BSA) standard curve.

2.7. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out using the SDS-PAGE system (Bio-Rad, USA) according to standard techniques[13]. A 15% gel was run and molecular weight markers (Sigma-Aldrich, US) were applied in each gel. Salivary gland samples were thawed on ice and mixed in 1:2 1X SDS gel loading buffer [50 mmol/L Tris-HCl (pH 6.8), 100 mmol/L DTT, 2% SDS, 0.1% bromophenol blue, 10% glycerol]. Then, the samples were heated for 5 min in a boiling water bath and loaded on the gels.

2.8. Staining

Following the electrophoresis gels were Coomassie brilliant blue (CBB) stained. First the gels were fixed in 50% methanol and 10% acetic acid for 30 min, then stained with 1% CBB in 10% methanol and 5% acetic acid for 2 h and finally destained with 10% methanol and 5% acetic acid until dark protein bands were visible. Digital images of the gel were captured by scanning at 300 dpi using a color scanner.

2.9. Coagulation assays

Pooled human citrated plasma showing normal coagulation times was provided by the Department of Haematology, Base Hospital, Tezpur, Assam, India. Following assays were performed according to Kazimirova *et al.*[14] to determine the anticoagulant activities in blackfly SGE.

The assays were carried out with 50 µL [prothrombin time (PT, extrinsic pathway assay), activated partial thromboplastin time (APTT, intrinsic pathway assay)] or 100 µL [thrombin time (TT, thrombin activity assay)] plasma. The plasma was pre-incubated with 1-5 µL SGE, the same volume of PBS, pH 7.2 (control), was in a water bath at 37 °C for 1 min. The reaction was started with the addition of

the appropriate pre-warmed reagent: 100 μL thromboclotin reagent (lyophilized bovine thrombin, Sigma–Aldrich, USA) in the TT assay, 100 μL thromboplastin reagent (lyophilized thromboplastin from rabbit brain, Sigma–Aldrich, USA) in the PT assay, and 50 μL actin FS activated PTT reagent (purified soy phosphatides in 1.0×10^{-4} mol/L ellagic acid) + 50 μL of pre-warmed 0.02 mol/L CaCl_2 after 3 min of incubation in the APTT assay. The time required for the formation of the fibrin clot was determined in triplicate samples visually using a stopwatch. In all assays, fibrin clot formation was monitored for a maximum of 2 min. If no clot formation occurred by that time, SGE was considered to inhibit coagulation by 100%. Prolongation of coagulation in comparison with the control was expressed as percentage of inhibition in the range 0%–100%.

2.10. Chromogenic substrate assays for thrombin and factor Xa

The assays were carried out using 96-well microplates according to the method described by Kazimirova *et al.*[14]. For screening of antithrombin activity, 190 μL tris buffer (50 mmol/L tris, 227 mmol/L NaCl, pH 8.3, containing 0.1% BSA and 0.1% sodium azide), 10 μL tris buffer containing 0.029 NIH units of thrombin from human plasma (Sigma–Aldrich, USA), 10 μL PBS (control), 10 μL of SGE (6 μg protein), 8 μL PBS were pipetted into a well and incubated at room temperature for 10 min. Twenty microliters of substrate, 1.9 mmol/L N-p-tosyl-gly-pro-arg-p-nitroanilide (Sigma–Aldrich, USA), dissolved in redistilled water were added.

Chromogenic substrates S-222 (Sigma, US) specific for factor Xa was used for the enzyme inhibition assays[4]. The enzyme used was bovine factor Xa (Sigma, US). Enzyme inhibition assays were conducted as follows: about 10 μL (7.5 ng protein) of factor Xa and one gland equivalent, 4 μL SGE was added to 436 μL of 50 mmol/L tris-HCl, 0.15 mol/L NaCl and 0.1% BSA (pH 8.4 at 37 °C). Control tubes each received 4 μL of 0.15 mol/L saline. The mixture was then incubated for 80 min at 37 °C. After this time 10 μL of either S-222 were added. This was followed by a second incubation of 40 min. The reaction was then stopped with the addition of 25 mL of 50% acetic acid and the absorbance was determined at 405 nm using a spectrophotometer (Thermo Scientific, Multiscan Spectrum-51118650). Tests were conducted in triplicate.

3. Results

3.1. Salivary Glands of *S. indicum*

Each female blackfly had a pair of salivary glands located on the dorsal part of the prothorax. Using the terminology of Gosbee *et al.*[15], each gland consisted of a cylindrical U-shaped secretory lobe and a more or less spherical reservoir (Figure 1). Salivary ducts arising from each

reservoir united to form a common salivary duct that passed to the hypopharynx. The secretory lobe was about 90–120 μm wide at its proximal end, while the reservoir was about 120–200 μm at its widest diameter. The average length of the secretory lobe of *S. indicum* was 700 μm .

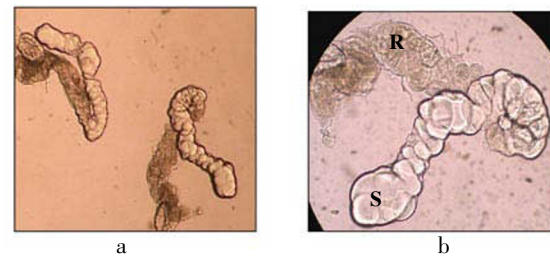


Figure 1. a) Pair of salivary gland detached from prothorax (40X); b) Single salivary gland showing secretory lobe (S) and spherical reservoir (R) (100X).

3.2. Total soluble protein content

The protein contents of whole salivary glands were quantified. The amount of salivary gland proteins in the adult female *S. indicum* was approximately (1.12 ± 0.13) μg /female (mean \pm SD, $n=10$).

3.3. SDS-PAGE

Total proteins in whole female salivary glands of *S. indicum* were examined in CBB stained SDS-polyacrylamide gels (Figure 2). At least 16 major and several minor protein bands were detected in the female salivary glands. The molecular masses of these major protein bands were estimated at 69, 65, 61, 58, 44, 42, 39, 33, 30, 28, 27, 26, 23, 21, 18 and 16 kDa, consecutively.

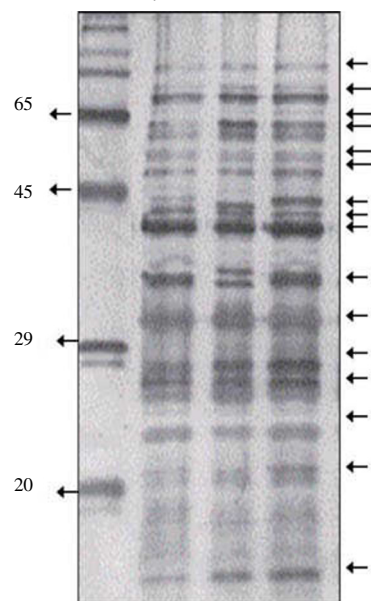


Figure 2. SDS-PAGE profile of salivary extract of *S. indicum*.

3.4. Coagulation assays

Anticoagulant activities against thrombin and the extrinsic and intrinsic coagulation pathways were found in *S. indicum*

SGE in the TT, PT and APTT assays, respectively (Figure 3). It was found that SGE prolonged human plasma clotting time in a dose-dependent manner. Values represent means±SD of three assays. Actual percentages are presented.

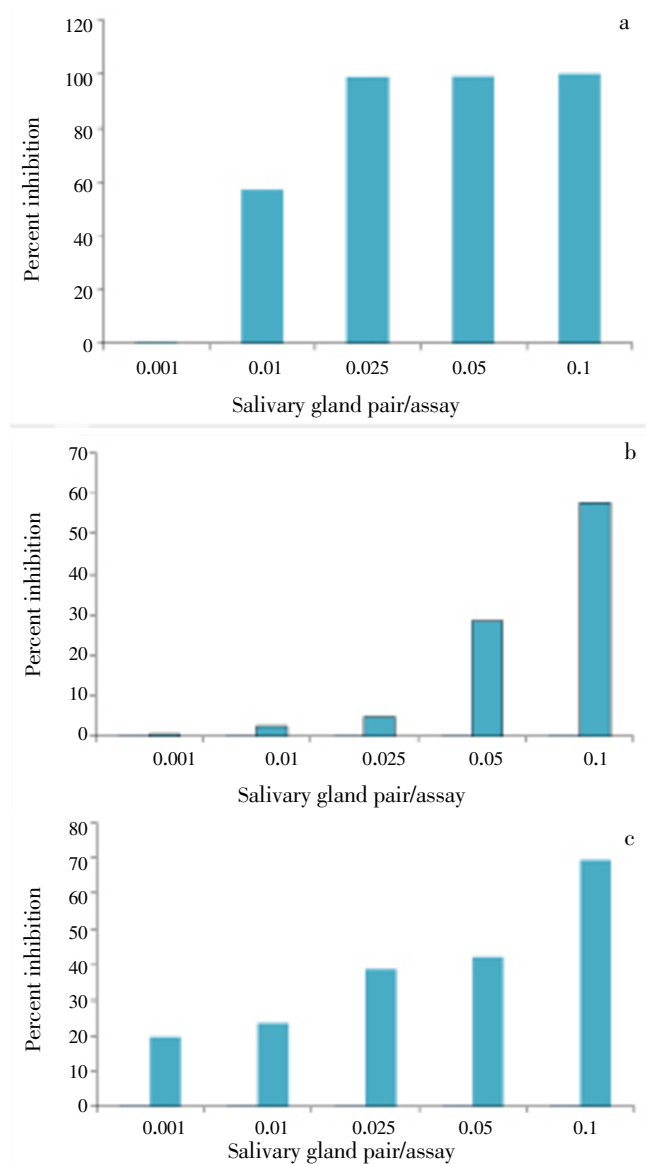


Figure 3. Effect of SGE of *S. indicum* on inhibition of fibrin clot formation of human plasma.

Values represent means±SD of three assays. Actual percentages are presented. a) TT-assay, b) PT-assay and c) APTT-assay.

3.5. Chromogenic substrate assays for thrombin and factor Xa

Chromogenic substrate assay for human thrombin activity showed inhibition of substrate hydrolysis by SGE of *S. indicum* (Figure 4).

Factor Xa inhibition was shown by the SGE of *S. indicum*. Percent inhibition value was 93.8. A positive correlation ($r=0.89$) was observed between total protein and percent inhibition of factor Xa.

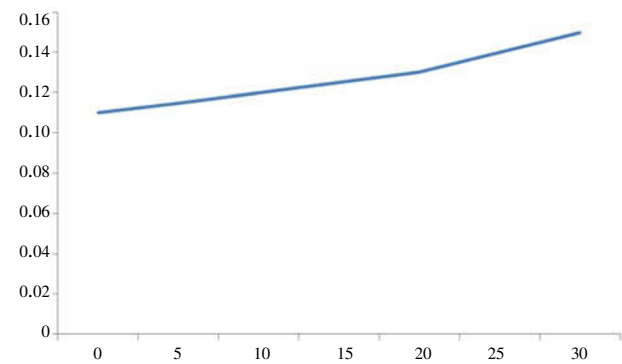


Figure 4. Inhibitory effect of blackfly salivary gland extracts on substrate hydrolysis by thrombin (human thrombin: 0.029 IU/well, substrate: 20 mmol/L Tosyl-glycyl-prolyl-arginine-4-nitroanilideacetate: 20 μL/well, Control: 10 μL PBS, SGE (6 μg protein in 10 μL PBS/well).

4. Discussion

4.1. Salivary gland morphology and protein profile of *S. indicum*

The results revealed that the morphology of the salivary glands of female *S. indicum* was similar and followed the same pattern as described for *Simulium vittatum* (*S. vittatum*)^[15], *Simulium nigroparvm*^[16] and *Simulium nigrogilvum*, *Simulium rufibasis*, *Simulium nodosum* and *Simulium asakoe*^[17]. An analysis of salivary gland proteins of *S. vittatum* and three New World Simuliid species, i.e., *Simulium argus* (*S. argus*), *Simulium ochraceum*, and *Simulium metallicum*, using SDS-PAGE was reported by Cross *et al*^[18]. After the analysis on SDS silver-stained gels, salivary gland profiles of *Simulium metallicum*, *Simulium ochraceum*, *S. vittatum*, and *S. argus* comprised individually separated proteins of 11, 12, 19, and 20 bands, respectively. Comparing the protein profiles of salivary gland extracts revealed that the two zoophilic species (*S. vittatum* and *S. argus*) were differed markedly both qualitatively and quantitatively from those observed from salivary gland extracts of the two anthropophilic species (*Simulium metallicum* and *Simulium ochraceum*). Furthermore, salivary glands extracted from the anthropophilic species contained higher levels of total soluble protein than those from the zoophilic species. Studies suggested that differences in saliva composition may represent modifications of this part of the more anthropophilic species because of evolutionary adaptation to feeding on hosts^[17]. In this study, the Oriental blackfly species, which was reported as anthropophilic^[19] had fewer number of major proteins detected in the salivary gland and this is in accordance with the results of Jaryapan *et al*^[17]. In addition, some molecular masses of the major protein bands detected in female salivary gland samples of *S. indicum* were similar to *Simulium nigrogilvum*, but were different from *Simulium rufibasis*, *Simulium nodosum* and *Simulium asakoe*. This is may be due to the fact that *S. indicum* and *Simulium nigrogilvum* belongs to the same

subgenus and both are severe pest on human. These results may support the hypothesis of evolutionary adaptation to feeding because different blackfly species live at different altitudes and/or regions where various vertebrate hosts and plants are found^[6,20]. However, the amount and profile of salivary gland proteins of these blackflies should be compared with other Simuliid species from different regions of the world.

4.2. Coagulation assays

The blood–coagulation cascade is launched by various mechanisms set by injury to blood vessels. It ends in the production of active thrombin, which cleaves fibrinogen to fibrin, the clot protein. The fibrin polymerizes and forms the blood clot, providing rigidity to the platelet plug. Salivary anticoagulants from blood–feeding arthropods seem to target specific proteases or complexes of the blood coagulation cascade, blocking or delaying the clot formation process until the blood feeder finishes the meal^[21]. Different insects have evolved diverse molecules responsible for these actions, which effectiveness also varies by species. Many of these salivary molecules are in different stages of molecular characterization. Most salivary anticoagulants target components in the final common pathway of the coagulation cascade, including factors V, Xa and II (thrombin).

In Diptera, inhibition of thrombin and factor Xa seems to be the most common mode of action of anticoagulants. Blackflies may use a variety of anti–hemostatic molecules to promote blood–feeding during probing and subsequent ingestion of a blood meal, which may include platelet anti aggregation factors^[22], vasodilators^[23] and anticoagulants. One of the advantages of the latter is to keep the food channel in the mouth parts relatively free of blood clots, thereby installing feeding fitness^[24]. Also, anticoagulants keep the blood meal in a fluid state as it passes into the midgut for digestion^[25]. As for other telmophagus invertebrates, blackflies are faced with the problem of clotting of the hematoma that is formed in the host's tissues during attempts at acquiring a blood meal. Thus the general approaches to inhibit clot formation taken by this group, although not identical in all the members of the genus, are similar to those of other pool feeding organisms. *Ornithodoros moubata* (Murray), an argasid tick, inhibits thrombin^[26] and factor Xa^[27] while the three–host ixodid tick, *Rhipicephalus appendiculatus* (Neumann), inhibits factor Xa^[28]. Among the Hirudinea, the European leech is known to possess anti–thrombin (hirudin) activity^[29] while antistasin from the Mexican leech, *Hementria officinalis*, inhibits factor Xa. The Indian leech, *Hemadipsa sylveltris*, possesses haemadin, a 5 kilodalton protein with antithrombin activity^[30]. Tsujimoto *et al.*^[31] reported that Simukunin preferentially inhibits factor Xa. The inhibition of elastase and cathepsin G further suggests this protein may modulate inflammation, which could potentially affect pathogen transmission.

Our results confirmed the existing knowledge on blackfly anticoagulants in routine coagulation assays (TT, PT, APTT). The results of the chromogenic substrate assay suggest that *S. indicum* prevent blood coagulation mainly through the

presence of a potent antithrombin, and through inhibition of factor Xa. Abebe *et al.*^[4], studied four species of *Simulium* and found that all four species have an anti–Xa factor, the highly anthrophilic species, *Simulium ochraceum*, produces greater quantities on a per gland basis than the three zoophilic taxa. This trend has also been noted for a potent vasodilator that induces a persistent erythema^[23] as well as increases in the salivary concentration of apyrase, an enzyme that prevents platelet aggregation by breaking down ATP and ADP to AMP^[32]. Thus when appraising the relative amount of soluble protein available from the salivary glands, particularly in the case of *S. indicum* and its relationship to the Xa inhibitor, it is necessary to consider that a wide variety of antihemostatic secretions are being processed and delivered during feeding as in the case of *Simulium ochraceum*^[4]. In addition it is also of interest in this regard that there is a significant size difference between the salivary glands of *Simulium ochraceum*, a highly anthrophilic species, and those of the other three zoophilic taxa that were examined by Abebe. When our results were compared with the results of Abebe *et al.*, it was found that *S. indicum*, which is an anthropophilic species of North–East India, particularly Arunachal Pradesh shows the same trend. In conclusion, our study demonstrated that the mode of action of the anticoagulant(s) is probably based mainly on the inhibition of thrombin and factor Xa along with other target factors of the coagulation cascade like platelet anti aggregation factors^[22], vasodilators^[23] and, as demonstrated here, anticoagulants.

Conflict of interest statement

We declare that we have no conflict of interest.

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Comments

Background

Anticoagulant activities in the salivary glands of biting insects or ticks are of medical importance.

Research frontiers

Anticoagulant activity in the salivary glands of *S. indicum*, the most dominant anthropophilic blackfly species in the Oriental region, is assessed in detail for the first time. No other Oriental blackfly species have been investigated for their mode of anticoagulant activities

Related reports

Related reports were present on North and Central American species of blackflies, showing the different mode of action of the anticoagulants between the anthropophilic

and zoophilic species.

Innovations and breakthroughs

This research represents the first report to prove the mode of action of the anticoagulant activity of the salivary gland of the Oriental species of the griseifrons species-group of the subgenus *Simulium*.

Applications

Proteins with anticoagulant or other functional activities contained in the salivary glands of blackflies will be applied in the research field of medicine.

Peer review

The mode of action of the anticoagulant of the salivary glands of *S. indicum* is clearly shown by proper bioassay methods. This study is the first for blackflies in the Oriental region to be analysed for their anticoagulant activities, and highly evaluated.

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