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# Biochemical and electrophoretic analyses of saliva from the predatory reduviid species *Rhynocoris marginatus* (Fab.)

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**The saliva of *Rhynocoris marginatus* consists of amylase, invertase, trehalase, protease, acid phosphatase, alkaline phosphatase, phospholipase, lipase, trypsin, hyaluronidase, and esterase. All enzyme activities were significantly higher in the saliva of female *R. marginatus* when compared to the saliva of male individuals. The saliva was analyzed by tricine SDS/PAGE, sephadex column chromatography, FT-IR, and MALDI-TOF. The pH of the saliva was slightly alkali. The SDS/PAGE revealed a few proteins with molecular masses greater than 29.5 and 36.2 kDa for male and female predator saliva respectively. The FT-IR spectrum confirmed the acidic, proteinaceous, enzymatic, and aromatic nature of the saliva. The MALDI-TOF-MS revealed the presence of enzymes, proteins, peptides, and other biomolecules. The most prominent peptides were named as RmIT-1 (3.79kDa), RmIT-2 (9.7kDa), and RmIT-3 (10.94kDa) (*Rhynocoris marginatus* Insect Toxin). Further studies are underway to isolate and identify these biomolecules.**

**Key words**: Biochemical characterization, enzymes, paralysis, reduviid predator, saliva, venom proteins

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## INTRODUCTION

Arthropod venom constitutes a potential source of bioactive substances. Several are known to display insecticidal activity towards an array of pest insects, operating through a variety of mechanisms including paralytic, apoptotic and non-apoptotic programmed cell death, and oncotic pathways (Moreau & Gulloit, 2005; Rivers *et al.,* 2010; Asgari & Rivers, 2011). Salivary secretions produced by some predatory true bugs (Hemiptera: Reduviidae) contain toxins that induce paralysis and cause death in susceptible prey species. The immobilized prey, in turn, poses little physical threat to reduviids during feeding, and is also partially digested by the enzymatic activity of the saliva (Edward, 1961; Cohen, 1996; Ambrose, 1999; Sahayaraj, 2007). Other presumed venom proteins present in the saliva demonstrate antimicrobial activity (Sahayaraj *et al.,* 2006a).

In fact, an array of proteins (Edward, 1961; Maran, 2000; Morrison, 1989), enzymes (Cohen, 1996; Sahayaraj *et al.,* 2007), and peptides (Corzo *et al.,* 2001) have been identified and partially characterized in saliva collected from several predatory reduviids. Despite the qualitative reports on the biological activity of the saliva from a number of predatory reduviid species, very little is known about the chemical composition of these true bugs' saliva (Corzo *et al.,* 2001). One species of particular interest is *Rhynocoris marginatus,* a polyphagous predator found in semiarid zones, scrub jungles and tropical rainforests in India. This predator is an effective biological control agent against more than 25 important agricultural pests. Like other predatory reduviids, it hunts prey, and then uses salivary toxins to induce paralysis that precedes partial tissue digestion (Sahayaraj, 2007). Anecdotal observations suggest that adult females require less time to inject venom and consume more prey than males.

This may be consistent with reports indicating that saliva composition varies between the sexes of some true bug species. This study focuses on characterizing the biochemical composition of venomous saliva from *R. marginatus* of adult males and females. Isolated saliva was compared using SDS/PAGE, and chemical constituents were partially characterized using FT-IR, and MALDI-TOF-MS.

## MATERIALS AND METHODS

**Insect rearing.** Laboratory colonies of *R. marginatus* were established from individuals collected from cotton fields of Tamil Nadu, India. Adults and nymphs were reared in round plastic containers (7 cm height and 6 cm diameter) under laboratory conditions (29 ± 2ºC, 70–80% RH and 11 D: 13 L) and fed fifth instar larvae of *Corcyra cephalonica* (Stainton) *ad libitum* as described previously (Sahayaraj *et al.,* 2006b).

**Saliva collection**. Saliva was collected from 10-day old freshly emerged male and female *R. marginatus* (n = 50 for each sex) as described by Sahayaraj *et al.* (2006b). Isolated venom was stored at –4ºC until use. In a separate set of experiments, the saliva (n = 50 for each sex) was collected as described and then used immediately in MALDI-TOF-MS analyses.

**Protein determination**. The total protein content of the *R. mariginatus* male and female saliva was estimated spectrophotometrically at 280 nm (Lowry *et al.,* 1951). Bovine serum albumin (BSA) served as the standard.

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**Abbreviations**: ACP, acid phosphatase; ALP, alkaline phosphatase; ANOVA, analysis of variance; CID, Collision Induced Dissociation; D, dark; SDS/PAGE, Sodium dodecyl sulfate polyacrylamide gel electrophoresis; FT-IR, Fourier Transform Infrared analysis; MALDI-TOF-MS, matrix-assisted laser desorptioin/ionization time of flight Mass spectroscopy; L, light; BSA, Bovine serum albumin; Tricine-SDS/PAGE, Tricine-Sodium dodecyl sulfate polyacrylamide gel electrophoresis; EDTA, Ethylene Diamine Tetra-Acetic acid; SPSS, Statistical Package for the Social Sciences; kDa, kilo Dalton; UV, ultra violet.

**Analysis of venom pH.** The pH of the freshly collected venom (0 day or 0 h) was measured using disks of narrow range pH paper (Himedia, India). The collected venom was dropped on the pH paper and any color change was compared to a standard strip. The predator was deprived of food continuously up to 3 days (72 hs) and 7 days (168 hs), and the pH of the saliva was measured in a similar manner from different groups of predators. In each group, saliva from 8–10 predators was tested.

**Enzyme assays.** Amylase, invertase (Bernfeld, 1955; Ishaaya & Swirski, 1970), trehalase (Ishaaya & Swirski, 1976), acid phosphatase (ACP) and alkaline phosphatase (ALP) (Beaufay *et al.,* 1954), protease (Soyelu *et al,,* 2007), lipase (Cherry & Crandall, 1932), esterase (van Asperen, 1962; Cho *et al.,* 1999) hyaluronidase (Pukrittayakamee *et al.,* 1988), phospholipase A2 (Santoro *et al.,* 1999), trypsin-like-enzyme (Zheng *et al.,* 2002) were quantified using saliva preparations from adult males and females. Each enzyme assay was replicated three times.

**Protein electrophoresis of saliva**. Tricine-SDS discontinuous polyacrylamide gel electrophoresis (Tricine-SDS/PAGE) was performed using a vertical electrophoresis kit (model 05-01-00, BioTech, India), and a 16% acrylamide separating gel with a Tris-glycine buffer system (Schägger & von Jagow, 1987). Prior to the electrophoresis, samples (saliva) were diluted with the sample buffer (40% sucrose, 0.154% dithiothreitol, 0.0372% EDTA and 0.2% Triton X-IOO in Tris glycine buffer, pH 8.3) at 1:1 ratio. Fifty microliters of each sample was loaded into each well and electrophoretic separation was performed at a constant voltage of 150-mV for 1 hour and 30 min at room temperature. Broad range (3000–43000 Dalton) molecular weight markers (Geni, Bangalore) were used for the estimation of molecular weights and gels were stained with 0.2 M sodium phosphate buffer (pH 6.5) containing 1% α-naphthyl acetate, 1% β-naphthyl acetate and 0.13% fast blue RR salt (4-Benzoylamino-2,5-dimethoxybenzenediazonium chloride hemi(zinc chloride) salt). Destaining was performed using a solution of 10% acetic acid and 50% methanol. The gels are visualized and images captured using gel documentation system (Biotech, Tamil Nadu).

**Fourier Transform Infrared (FT-IR) analysis**. Isolated saliva was analyzed by Fourier Transform Infrared analyses over a range of 500–4000-$cm^{-1}$ using a Shimadzu FT-IR Model 8400S. A solid film of saliva was obtained by evaporating an aliquot of an aqueous solution (10-µl) on to a plate (13-mm) in a vacuum desiccator at room temperature. The plate was then subjected to FT-IR and the resulting IR spectra were compared with available references (Leonard, 1972; Areas *et al.,* 1987; Arrondo *et al.,* 1993; Yoshida *et al.,* 1997; Uçkan *et al.,* 2004).

**Sephadex gel chromatography**. Collected saliva was kept in petri dishes (3-cm diameter and 1-cm height) and placed in sterile laminar airflow chamber for 5–10 minutes at room temperature, as mentioned above. Then, the dried contents were scrapped out and ground with fine glass mortar and pestle. The resultant fine powder was considered as lyopholized saliva (17.5 mg). It was dissolved in 500 µl 0.05 M Tris buffer (pH 6.8) and loaded on to a Sephadex G-75 (Sigma-Aldrich) (2.5 × 10 cm) gel filtration column equilibrated with the same buffer. The column was eluted using 0.05 M Tris buffer with a flow rate at 4 ml/h and 5 ml fractions were collected and monitored by UV absorption at 280 nm. Maximum absorption was recorded for fractions 7–8, which then were further analyzed in by SDS/PAGE (Schägger & von Jagow, 1987).

**MALDI–TOF-MS analysis**. The venomous saliva was analyzed by matrix-assisted laser desorptioin/ionization time of flight (MALDI-TOF) mass spectrophotometer. MALDI-TOF-MS measurements were carried out on a Voyager-DE™ PRO Biospectrometry™ spectrometer (Applied BioSystems, Framingham, MA, USA) equipped with a VSL-337ND nitrogen laser (Laser Science, USA). The accelerating voltage was 20-KV. Argon gas was used for the Collision Induced Dissociation (CID)/ Post-source decay (PSD) experiment. A matrix, α-cyano-4-hydroxycinnamic acid (Aldrich) was prepared at the concentration of 10 mg/mL in 1:1 CH3 CN/0.1% TFA (Corzo *et al.,* 2001). The saliva was dissolved in a matrix solution (10 mg-1 2, 5-dihydroxybenzoic acid, 50% acetonitrile and 0.1% TFA), and 1 µl of the solution was spotted on to the MALDI sample plate along with equal amount each of sample and matrix, and then allowed to dry at room temperature. The time-to-mass conversion was achieved by external and/or internal calibration using standards such as bovine pancreatic beta insulin ($m/z$ 3496.9), bovine pancreatic insulin ($m/z$ 5734.6), and apomyoglobin ($m/z$ 16,952.6) (Sigma-Mumbai). The measurements were carried out in a positive ion mode.

**Statistical analysis**. Total protein and enzyme level data of male true bugs were compared with females using the analysis of variance (ANOVA) by means of the SPSS statistical software (Version 11.5). Significance differences were determined at $\alpha = 0.05$. Correlation was analyzed for the starvation period and saliva pH using the same software.

## RESULT

**Protein contents of the saliva**. Newly emerged 12-h old males of *R. marginatus* weighed from 100 to 162 mg (mean value 139.7 ± 2.07-mg), whereas female weights ranged from 146 to 322 mg (mean value 184.4 ± 1.92 mg). Despite the size differences our observations indicate that males produce larger volumes of saliva than females. However, the total protein content of the pooled saliva of male reduviids was significantly less ($F = 3.4$; df = 2, 12; $P < 0.05$) (351.25 ± 28.1 µg/mg saliva, n=20) than for females (420.00 ± 30.1 µg/mg, n = 20). However, when the saliva protein content was corrected by the body weight of the individuals, males yielded significantly more saliva (1.16 ± 0.03-mg/100 mg body weight) ($P < 0.05$) than the females (0.92 ± 0.02 mg/100 mg body weight).

**Table 1. *Rhynocoris marginatus* male and female salivary venom pH in relation to starvation (n = 20, mean ± S.E.)**

| Starvation (days) | pH | |
|---|---|---|
| | Male predator | Female predator |
| 01 | 7.35 ± 0.10 | 7.50 ± 0.00* |
| 02 | 7.10 ± 0.10 | 7.05 ± 0.50$^{Ns}$ |
| 03 | 7.07 ± 0.13 | 7.06 ± 0.06 $^{Ns}$ |
| 04 | 6.52 ± 0.21 | 6.53 ± 0.01* |
| 05 | 5.50 ± 0.05 | 5.60 ± 0.16* |
| 06 | 5.50 ± 0.00 | 5.50 ± 0.10* |
| 07 | 5.07 ± 0.07 | 5.08 ± 0.10* |

$^{Ns}$, Indicates insignificant *, Indicates the significant at 95% level in paired sample T test

**Table 2. Quantitative enzyme analysis of saliva of male and female *Rhynocoris marginatus***

| Enzyme | Male | Female |
|---|---|---|
| Amylase (µg maltose released/min/mg protein) | 0.37±0.01[a] | 0.42±0.05[a] |
| Lipase (meq /min/g) | 27.95± 3.1[b] | 29.43±2.8[a] |
| Protease (µg tyrosine/mg protein/min) | 10.01±3.8[a] | 11.18±2.5[a] |
| Acid Phosphatase (µmol /mg/h$^{-1}$) | 3.82±1.14[a] | 4.19±1.20[a] |
| Invertase (µg glucose released/min/mg protein) | 0.29±0.01[b] | 0.40±0.01[a] |
| Alkaline Phosphatase (µmol /mg/h$^{-1}$) | 14.14±0.19[b] | 16.56±0.19[a] |
| Trehalase (µg glucose released/min/mg protein) | 1.06±0.03[a] | 1.17±0.03[a] |
| Esterase (mmoles/min/mg protein) | 16.40±1.02[b] | 21.79±1.28[a] |
| Trypsin (U/mg) | 10.35±0.70[b] | 13.40±0.19[a] |
| Hyaluronidase (units/mg protein) | 25.39±0.21[b] | 27.60±1.14[a] |
| Phospholipase (nM/min/mg protein) | 21.08±0.11[b] | 40.49±0.79[a] |

Mean ±S.E., The mean difference significance at 5% level ($P<0.05$); same alphabets between male and female are not significant at 5% level ($P>0.05$).

**pH measurements of adult saliva**. Saliva pH of adult females was slightly alkaline ($t=-6.66$; $df=19$; $P<0.05$) when isolated from prey-fed individuals. However, the saliva gradually became neutral following 3 days of food deprivation ($t=0.604$; $df=19$; $P<0.05$) and continued to become more acidic with increasing length of the starvation period of adults up to 7 days ($t=0.609$; $df=19$; $P<0.05$) (Table 1). Correlation between starvation period and pH of saliva was negative for males ($r=-0.965$) and females ($r=-0.977$).

**Saliva enzymatic activity**. Isolated saliva collected from adults was analyzed for the activity of a range of enzymes commonly found in insect saliva. Saliva from both males and females contain at least 11 enzymes based on substrate specificity assays (Table 2). However, the activity of the enzymes was not the same among the sexes: the saliva from female *R. marginatus* displayed higher activity than from male for all enzymes (Table 2).

**Protein electrophoresis of saliva**. One-dimensional SDS/PAGE was performed to examine the protein profiles of saliva samples collected from both males and females of *R. marginatus*. For both sexes, proteins detected following the separation on 16% tricine-SDS gels ranged from low to midrange molecular weight (Fig. 1). Eight major protein bands were evident in saliva from both sexes. However, band intensity for nearly all proteins was more intense in female saliva than that collected from male individuals (Fig. 1).

**Sephadex G75- column fractionation**. Isolated saliva samples from both males and females were cumulatively subjected to fractionation using column chromatography. Fifty-one fractions were obtained from passing saliva through a Sephadex G-75 column monitoring absorption at 280 nm (Fig. 2). Maximum absorbance (0.621) was detected in fractions 7-8 for the saliva. These fractions were further analyzed using 1-D SDS/PAGE. Three distinct bands (22, 20 and 4 kDa) were observed for both male and female saliva samples (Fig. 3).

**Fourier Transfer Infrared analysis**. The FT-IR spectrum of *R. marginatus* male and female crude saliva is shown in Fig. 4a and b with the interpretation of characteristic absorption bands in Table 3. The comparison of *R. marginatus* male and female saliva FT-IR spectra shows that there is an apparent change in relative intensity of the bands (Fig. 4), and that there appears to be unique IR banding with female saliva. For example, a peak at 897-cm$^{-1}$ (presumed to an aliphatic nitro group), 1315 (amide III), and 3080 (amino acid) and 3296 (amide A) were unique to saliva from females (Fig. 4, Table 2). By contrast, a peak at 2370-cm$^{-1}$ (amine salts) was found in saliva from males only (Fig. 4, Table 3).

**Mass spectrometric analysis**. As shown in Fig. 5, the *R. marginatus* SV contained a wide variety of components with molecular masses ranging from 3.0 to 50.0

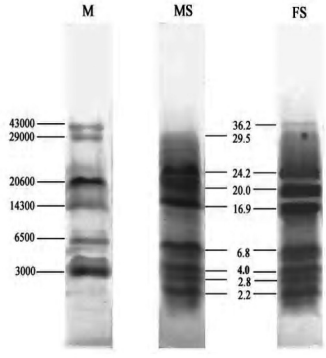


**Figure 1. SDS/PAGE protein profile of *Rhynocoris marginatus* venomous saliva.**
Samples: MS, male venomous saliva; FS, female venomous saliva; M, marker.

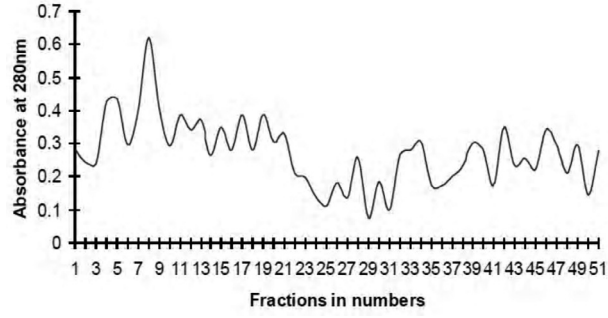


**Figure 2. Gel filtration chromatography of *Rhynocoris marginatus* saliva.**
Sephadex G-75 column (2.5 x 10 cm) 0.05 M Tris buffer a flow rate of 4 ml/h

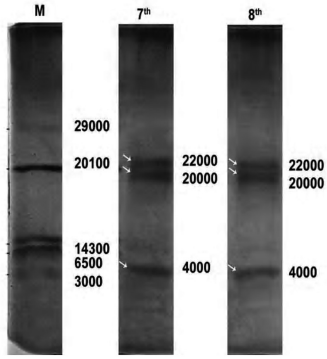


**Figure 3. SDS/PAGE protein profile of the seventh (7th) and eight (8th) fractions of *R. marginatus* venomous saliva obtained by Sephadex G75 column chromatography.**
Samples: M, marker, M7, male venomous saliva seventh fraction, F8, female venomous saliva eighth fraction

kDa. The number of components with molecular masses over 10.0 kDa was much lower in case of female *R. marginatus* saliva (Fig. 5a and b). MALDI-TOF analyses of crude saliva allowed the fluid to be categorized into three groups according to the proportions of amino acid residues (says Liu *et al.,* 2009). Using the classification of Liu *et al.* (2009), 1000 to 4000 Da, 5000 to 10 000 Da and 17000 to 58 000 Da, we categorized as group I (10 to 40 amino acid residues), group II (50–100 amino acid residues) and group III (170–580 amino acid residues), respectively. Variation was observed between male (3000–58 000 Da) and female (3 000 to 54 000 Da) predator saliva. However, 11 components (3, 4, 6, 7, 9, 10, 21 and 23 kDa) were common in both sexes. Among the components, 3.79, 7.5 and 10.94 kDa peptide showed highly prominent peaks in male and female. They were designated as RmIT-1, RmIT-2 and RmIT-3 (*Rhynocoris marginatus* insecticidal toxin). Among the three peptides, 4 kDa peptide was detected in Tris-SDS/PAGE analysis. Moreover, 2 (3 and 21 kDa) and 5 (7, 9, 17, 18 and 21kDa) peptides for male and female saliva of the predator observed in MALDI-TOF confirmed the SDS/PAGE analysis.

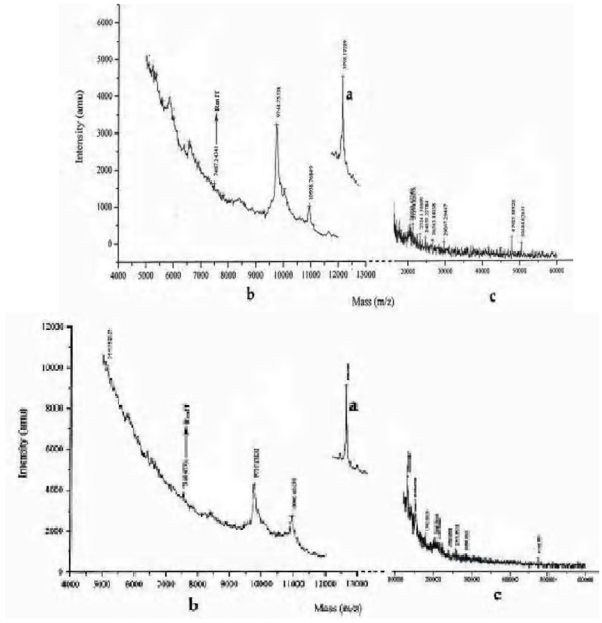


**Figure 5. MALDI-TOF spectrum of saliva of male (upper) and female (lower) Rhynocoris marginatus.**
a, indicates the peptides with 40.0 to 13.0 kDa, b, indicates the peptides with 3.0 to 4.0 kDa and c, indicates the peptides with 10.0 to 50.0 kDa

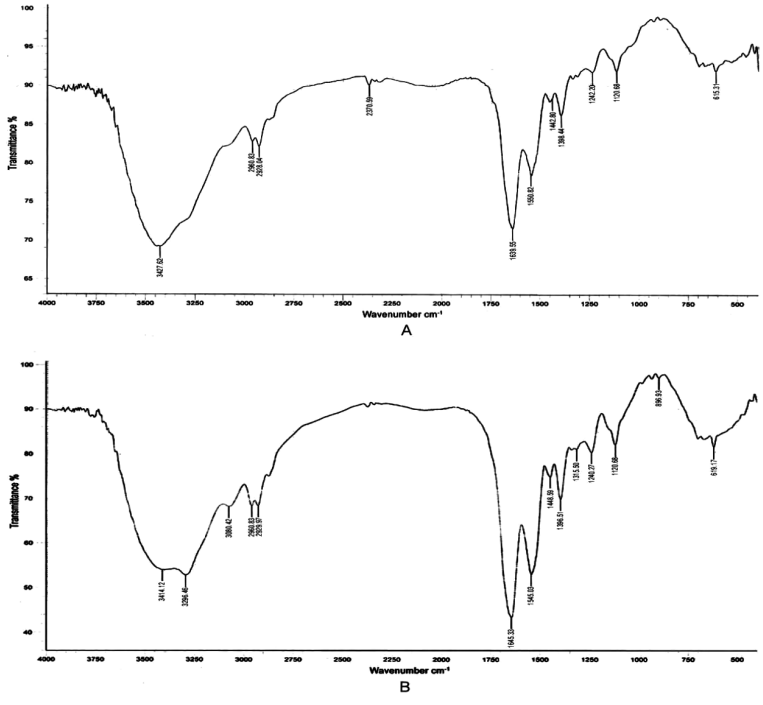


**Figure 4. Fourier Transform Infrared spectroscopy (FT-IR) was obtained for both male (A) and female (B) venomous saliva of Rhynocoris marginatus.**
The arrows show the modifications observed on both spectra. Note that these modifications are evidenced by alternations in the wave number parameters.

## DISCUSSION

This study is the first to biochemically characterize the saliva of *R. marginatus* using an array of methods. We have applied the milking method (Sahayaraj *et al.,* 2006b) to collect saliva by inserting only the tips of the rostrum into a glass capillary tube during the collection process. Previously it was reported that prevention of venom contamination by non-venom enzymes, particularly digestive enzymes (mainly from gastric juice), is an important consideration during venom milking (Rash & Hodgson, 2002). Sexual dimorphism in size, shape, color and behavior is a widespread phenomenon among reduviid taxa (Ambrose, 1999; Sahayaraj, 2007); studies that explicitly demonstrate the sexual dimorphism in saliva are almost nonexistent. For instance, Sahayaraj (2007) reported that the female reduviid paralyses the prey more rapidly than the male. This observation suggests that biochemical differences exist in terms of the composition of saliva in adult male and female *R. marginatus*.

Consistent with such speculation are our observations that saliva of male

**Table 3. Peak ($cm^{-1}$), mode of vibration and peak assignment in the male and female venomous saliva**

| S. Number | Peak (cm-1) | Mode of vibration | Possible assignment |
|---|---|---|---|
| | | Male Venomous Saliva | |
| 1 | 3428 | N-H stretching | Secondary amines |
| 2 | 2961 | $CH_3$ asymmetric stretching | Alkanes |
| 3 | 2928 | $CH_3$ asymmetric stretching | Alkanes |
| 4 | 2370 | $NH^+_2$ asymmetric stretching | Amine salts |
| 5 | 1640 | C=O stretching | Amide I |
| 6 | 1551 | $NO_2$ asymmetric stretching | Amide II (α helix) |
| 7 | 1443 | $CH_2$ bending | Alkanes |
| 8 | 1398 | C-O-H | Carboxyl compounds |
| 9 | 1242 | $PO_2^-$ asymmetric | Amide III |
| 10 | 1121 | Phosphodiester | Enzyme with phosphorus |
| 11 | 615 | C-H bending | Alkynes |
| | | Female Venomous Saliva | |
| 1 | 3414 | N-H stretching | Secondary amine |
| 2 | 3296 | N-H stretching | Amide A |
| 3 | 3080 | $NH^+_3$ stretching | Amino acids (in hydrochlorides) |
| 4 | 2961 | $CH_3$ asymmetric stretching | Alkanes |
| 5 | 2930 | $CH_3$ asymmetric stretching | Alkanes |
| 6 | 1645 | $NH^+_3$ asymmetric bending | Amide I (unordered form or random coiling) |
| 7 | 1545 | $NO_2$ asymmetric stretching | Amide II (α helix) |
| 8 | 1448 | $CH_2$ bending | Alkanes |
| 9 | 1396 | C-O-H | Carboxyl compounds |
| 10 | 1315 | C-CO-C skeletal Amid III | Amide III |
| 11 | 1240 | $PO_2^-$ asymmetric | Amide III |
| 12 | 1121 | Phosphodiester | Enzyme with phosphorus |
| 13 | 897 | C-N stretching | Aliphatic nitro group |
| 14 | 619 | C-H bending | Alkynes |

and female *R. marginatus* displayed different FTIR, SDS/PAGE and MALDI-TOF MS patterns. These observations indicate that sex is a substantial factor in the interspecific variation of *R. marginatus* saliva. The saliva pH of *R. marginatus* was alkaline and became acidic while the reduviid was subjected to prolonged starvation. Previous studies have shown that reduviid venoms represent a complex mixture of proteins (Morrison, 1989; Maran, 2000), peptides (Corzo *et al.,* 2001) and enzymes (Sahayaraj *et al.,* 2007; Sahayaraj & Muthukumar, 2011). The range of molecular masses observed after SDS/PAGE and MS analysis indicated that *R. marginatus* saliva contained numerous components, especially low molecular weight molecules, although some high molecular mass bands were also detected. Edward (1961) first reported six distinct protein fractions in *Platymeris rhadamanthus* salivary gland homogenates. Similarly, multiple protein bands were detected in saliva from *R. marginatus* when subjected to SDS/PAGE, with molecular weights ranging from $\leq 2$ to $>21$ kDa in male and $\leq 4$ to $>30$ kDa in female. Differences in protein composition between male and female saliva may lead to different physiological affects on target prey. To our knowledge, this possibility has not been tested experimentally.

Enzymes are common components of several venomous arthropods including bees, wasps, scorpions, and spiders. In this respect, the saliva of reduviid predators is no different; it contains a wide variety of enzymes. Tricine SDS/PAGE analysis of Sephadex column seventh and eighth fractions showed three distinct proteins (4-22 kDa). Though these molecular weight bands are considered as enzymes (de Lima & Braga, 2003), the 22 kDa (Parkinson *et al.,* 2002) and 20 kDa (de Lima & Braga, 2003) proteins may be paralytic factors, and the band estimated to be 4 kDa is consistent with the mass of several peptide neurotoxins (Zeng *et al.,* 2003). Salivary amylase, lipase, hyaluronidases and phospholipase are found in *Cimicomorpha* (Reduviidae) (Edward, 1961; Cohen, 1998), and as expected, were detected in saliva based on substrate specific enzyme assays. The Trypsin-like enzyme in the saliva permits extra-oral digestion for use of protein in animal food. Trypsin-like enzymes and amylase are very active in the salivary glands of the reduviid predator *Z. renardii* (Cohen, 1993). *Rhynocoris marginatus* saliva shows acid and alkaline phosphatase activity, which is consistent with venom from the parasitic wasp *Pimpla hypochondriaca* (Dani *et al.,* 2005).

High phospholipase A2 activity was observed in fractions 7 and 8 collected by gel filtration, corresponding to a protein with a predicated mass of 22.4 kDa. This is lower than the 30.00 kDa of purified enzyme, possibly indicating that the fractions contain subunits of either phospholipase A1 or A2 (Cohen, 1998) (Table 1). The saliva also displayed a substantial hyaluronidase activity (Table 1). Hyaluronidase is common in arthropod venoms, especially spiders (Silveira *et al.,* 2007), and may facilitate the spreading of saliva toxins by enhancing the absorption and diffusion rates of the venom through the prey's tissues (Cohen, 1998; Morey *et al.,* 2006). Edwards (1961) also reported the presence of hyaluronidase in *Platymeris rhadamantus* Gaerst, a reduviid predator.

The amide I and II, amine modes in the vibrational spectra, are correlated with the polypeptide secondary structure of the protein molecule. In the FT-IR spectrum, the amide I band is intense and broad, with the maximum intensity at 1640 and 1645-cm$^{-1}$ in males and females, respectively. The vibration at 1640 cm$^{-1}$ indicates the presence of elastase (Arrondo *et al.,* 1993). The amide II band is of lower intensity than in case of amide I band, which indicates that the proportion of amide I is higher than amide II. The band at 1550.82 (for male) and 1545-cm$^{-1}$ (for female) are assigned to α-helix secondary structure (Areas *et al.,* 1987), which confirms the presence of polypeptide backbone with a mixture of secondary structures and the proteinaceous nature of the saliva. However, the band at 3428 and 3414 cm$^{-1}$ for male and female saliva respectively are disproportionally large, and could be caused in part by trace amounts of ethanol (Uckan *et al.,* 2004). The vibrations at 1396 and 1398-cm$^{-1}$; 618-cm$^{-1}$ and 1120-cm$^{-1}$ can be associated with acidic (carboxylic), alkynes and aromatic compounds in venom, respectively (Uçkan *et al.,* 2004). The absorption band at 1120-cm$^{-1}$ suggests that these may also be structures containing mannose-6-phosphate or phosphorylated saccharide residue in the venom (Yoshida *et al.,* 1997).

Comparative FT-IR spectrum revealed that spectra for male and female saliva were very similar. Saliva from adult males did display unique peaks at 2961 and 2371-cm$^{-1}$ indicative of secondary amines. Female spectra revealed unique peaks at 3296, 3080, 316 and 897-cm$^{-1}$, which is consistent with the presence of sulphonamides, amino acids, and carbonyl compounds, respectively. An alternation in the absorbance at wave number = 2370-cm$^{-1}$ in the FT-IR spectrum for the $NH^{+2}$ groups of male saliva suggests an ionic interaction and similar alternations for N-H (3296-cm$^{-1}$) and $NH^{+3}$ (3080-cm$^{-1}$) of female saliva indicates structural difference between male and female saliva. Peaks observed at 897 and 1315-cm$^{-1}$ in female saliva indicate the presence of amino acid and amide-III not detected in males (Fig. 4B). FT-IR spectrum also revealed the presence of phosphorus containing structure, typical of enzymes, in the saliva, which is confirmed by the observations of different enzyme activity (amylase, invertase, trehalase, protease, acid phosphatase, alkaline phosphatase, phospholipase, lipase, trypsin, hyaluronidase and esterase) in this study.

The MALDI-TOF spectrum of reduviid saliva showed constituents with molecular weights ranging from 1 to 30 kDa (Corzo *et al.,* 2001). We recorded 15 and 21 peptides by MALDI-TOF spectrum of male and female respectively. Peptides or small proteins with masses of 3, 4, 5, 6, 7, 9, 10, 17, 21, 23, 24, 25, 26, 29, 33, 36, 41, and 44 kDa were present in saliva collected from both sexes. Gel filtration and SDS/PAGE analyses detected only some of the molecules revealed by MALDI-TOF, suggesting that many of saliva components are not proteinaceous.

To conclude, like the venoms of snakes, scorpions, or spiders, the saliva of reduviids is heterogeneous, not only between species but also within male and female individuals. The major constituents of reduviid saliva are protein, polypeptides and enzymes. Our findings provide new information about the composition and biochemical characteristics of venomous saliva from adult *R. marginatus*. *Rhynocoris marginatus* enzymes in the saliva are used initially to kill the prey and enhance the pre-oral digestion. The presence of hyaluronidase in the venom is presumed to function as a spreading agent to facilitate the dissemination of other venom components through the prey. This study clearly shows that saliva composition is a complex mixture containing enzymes, peptides, proteins and other constituents that have yet to be identified. Even though the saliva from adult females has more proteins than in case of males, saliva from both sexes has similar effects on prey insects. This work opens new research directions aimed at understanding the physiology, toxicology, and evolutionary relatedness of predatory venoms and toxins.

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