CHAPTER 3. BIOCHEMICAL CHARACTERIZATION

3.1. ABSTRACT

Reduviids are found to paralyze and predate on the other insect prey by using their venomous saliva (VS). In this study, *Rhynocoris fuscipes* (Fab.) VS protein profile was qualified and quantified after lyophilization. It was characterized using EDX spectra, FTIR, HPLC, GC-MS and MALDI-TOFMS analysis. The enzyme profile and protein profile of salivary gland parts and guts were also analyzed. Results showed that the VS, salivary gland parts and gut showed the presence of amylase, lipase, protease and phosphatase, hyalurindase, phospholipase, and trypsin like enzymes. The SDS-PAGE profile showed that VS has 19 polypeptide bands (41.2 kDa to 6.4 kDa). Various parts of the salivary gland showed the 18 polypeptide bands (49.6 to 2.4 kDa). The combination of acetone and methanol (1:1) was found to be a system for the VS purification. The EDX spectrum results showed that potassium (29.21%) constituted the major element in the VS. Presence of proteins and their acidic nature and disulfide bridges were observed in the VS by FTIR spectroscopy, while in both HPLC and GC-MS analysis, the presence of phospholipase, hyalurindase and lysolecithin have been observed. About 69 components have been recorded in the VS through MALDI-TOFMS analysis ranging from 2.4 to 59 kDa and molecular mass at 3.8 kDa (RFIT1) have resulted in a higher intensity. In conclusion, the VS of *R. fuscipes* have potent of toxic nature resembling the higher analysis venom and yet the presence of disulfide bridges showed to non-toxic nature towards the mammals.

3.2. INTRODUCTION

Assassin bugs are the predatory bugs found to be predaing on other insect using their VS for paralysis and pre-oral digestion (Cohen, 1998a; 2000). Generally predatory Hemipteran bugs use the solid to liquid feeding method (Cohen, 1995, Swart and Felgenhauer, 2003). The fundamental nature of the digestion is to render macromolecules into simple compounds that can be absorbed and circulated (Gilmour, 1961; House, 1974). The types of digestive enzymes, especially those of salivary origin, are highly correlated with the feeding habitats of Hemipteran (Miles, 1972; Hori, 1975; Agusti and Cohen, 2000; Hori, 2000; Zeng and Cohen, 2000a, b; Boyd *et al.*, 2002; Boyd, 2003). Proteinases are the most important liquefaction enzymes for predators (Rees and Offord, 1969; Miles, 1972; Cohen, 1993). Trypsin like enzymes acts on amino acid sites cleaving the protein at lysine and
arginine residues (Law et al., 1977; Cohen, 1993). Chymotrypsin like enzymes attack proteins at their aromatic sites (Gilmour, 1961; Law et al., 1977; Cohen, 1990, 1993). The presence of protease in the salivary secretions was reported in Blestomatid (Rastogi, 1962; Rees and Offord, 1969) and in the reduviid (Edwards, 1961).

The salivary gland as the source of proteolytic enzymes is injected by carnivorous insects’ into the prey (Law et al., 1977). Trypsin like protease was the only protease detected in salivary gland complex of Deracocoris nebulosis (Boyd et al., 2002). The presence of trypsin like enzymes demonstrates the insect’s ability to access structural or other insoluble proteins (Cohen, 1993; 1998b, 2000). In the reduviid, Zelus renardii salivary gland trypsin like enzyme was found to be predominant and it has the ability to digest the protein in the prey (Cohen, 1993). The activity of carbohydrases in the salivary gland of the Lygus disponsi, Lygus saundersi, Adelphocaris suturalis, Orthocephalus funestus, Palomera angulosa, Eurydena rigosum and Coreus marginatus have been studied (Hori, 1975). Takanona and Hori (1974) have studied the digestive enzymes of Stenotus binotatus salivary gland. Triacyl glycerol lipases were found in the saliva and salivary gland of heteropteran predators (Rastogi, 1962; Wigglesworth, 1972; Cohen, 1990).

In L. rugulipennis salivary gland, secretable enzymes such as polygalacturonase, amylase and alkaline proteinase and other enzymes such as acid proteinase, phosphatase, trehalase, invertase and phenol oxidase were found (Laurema et al., 1985). Phospholipase digests the phospholipids in cell membranes, disrupting neurons and muscle cells (Schmidt, 1982). Phospholipase were detected in the saliva of heteropteran and they digest the phospholipids (Edwards, 1961; Cohen, 1990). The extra-oral digestion secretions could reasonably be expected to contain hydrolases that digest these substances and only hyalurindase have been found (Edwards, 1961; Rees and Offord, 1969; Mommsen, 1978b). In insects, considerable amounts of proteoglycans, glycosano glycans, chondroitin sulfates, dermatan sulfates and hyaluronic acids that form connective tissues, basement membranes, peritrophic matrices, collagenous and fibrous tissues were found (Chippendale, 1978). Hyalurindase is also a spreading factor for venom (Edwards, 1961; Mommsen, 1978a; Foelix, 1982). The saliva of the hemipteran insects separates cells in intact tissue and reduces viscosity of hyaluronic acid and prey fluids (Edwards, 1961). There are only a few reports for the presence of acid phosphatase in the venom (Dani et al., 2005; Sahayaraj et al., 2010).
*Apis mellifera*, acid phosphatase might be a vestigial enzyme, which may have served the function of predigestion of prey before it was eaten or fed to the young (Benton *et al.*, 1963).

Midgut digestive enzymes are similar to vertebrate digestive enzymes have been reported from a large number of insects (Day and Waterhouse, 1953; House, 1974; Dadd, 1970; Eguchi and Iwamoto, 1976; Law *et al.*, 1977; Pritchett *et al.*, 1981). Where the anterior midgut has low levels of trypsin like, elastase like and chymotrypsins like proteases were identified in *D. nebulosis* (Boyd *et al.*, 2002). The α–amylase is a hydrolytic enzyme that is widespread in nature. These enzymes catalyze the hydrolysis of α-D–(1, 4)–glucan linkages in glycogen and other related carbohydrates (Strobi *et al.*, 1998; Franco *et al.*, 2000). Amylase was found to be useful in the glycogen digestion (Rees and Offord, 1969). Salivary amylase was present more consistently in the pentatomomorphs than in Cimicomorpha (Edwards, 1961; Laurema *et al.*, 1985; Cohen, 1998). The high amylase activity not only indicates its potential to digest the starch, the major glycoside reserve found in plants, but also other polysaccharides and dextrin’s from glycogen, the major cell glycoside reservoir (Cohen, 1990; Stamppoulos *et al.*, 1993). The plant feeding mirids usually have high levels of amylase in their salivary glands (Agusti and Cohen, 2000). The amylase secretions are ingested by the mirid, along with the partially digested starch to be used in the midgut to continue the starch break down (Hori, 1973; Takanohana and Hori, 1974; Wheeler, 2001). In insects, the abundance and activity of α-amylase are dependent on food sources (Slansky, 1982; Dow, 1986).

The activity of carbohydrates in midgut of *L. disponsi, L. saundersi, A. suturalus, O. funestus, P. angulosa, E. rigosum and C. marginatus* has been studied and all these bugs had amylase, phlenase, α and β–glucosidase and α and β–galactosidase in the midgut (Hori, 1975). Trehelase is an important enzyme that has all the functions throughout the life cycle in all tissues which rely upon glucose generated from trehalose reserves to meet their energy needs (Wyatt, 1967). The lipase action is believed to occur mainly in the midgut to digest triacyl glycerol from prey items (Terra, 1990). Acid phosphatase is also one of the major lysosomal enzymes found in the invertebrates and they are associated with degradation and cell death (Bowen and Lockshin, 1981; Anderson, 1981; Gregorc *et al.*, 1998).

The electrophoretic profiles of the venom have shown the protein profiles and the possible venom peptides were identified (Munekiyo and Mackessy, 1998). Azevedo *et al.*
(2007) have analyzed the protein of the salivary gland homogenates using SDS–PAGE and they observed 13 polypeptides, with one of them exclusive of the posterior lobe and molecular weight < 60 kDa. The SDS–PAGE of the crude venom of Black widow spider, *Labrodectus tredecunguttatus* showed that the venom proteins are distributed through a wide range of molecular mass, with abundant protein bands at molecular weight greater than 31.0 kDa and few proteins distributed in the range of 14.4 kDa (Duan *et al*., 2006). The venom of *Pimpla turionellae* is composed of highly complex mixture of polypeptides. It primarily consists of components with molecular weight from 106 kDa to 20 kDa.

The FTIR spectroscopy has become a well accepted and widely used method to characterize biological tissues. A wide range of biological studies have been covered by FTIR analysis (Movasaghi *et al*., 2008). The infrared spectroscopy of endoparasitoid wasp *P. turionellae* has been analyzed and the possible frequencies of 3410, 2361, 1648, 1547, 1398, 1125, 1050 and 618 cm\(^{-1}\) were observed. And from their results they have characterized the venomous nature having proteinous nature and also the carboxylic nature of the venom (Uçkan *et al*., 2004). Similar observations were found in the venom of *Nasonia vitripennis* (Rivers *et al*., 2006). Scorpions and wandering spiders evolved their venoms to contain structurally compact peptides due to the presence of disulfide bonds; and, these peptides are characterized by their high affinity for ion channels and/or nervous receptors, causing activation or blockage of the ion flux through the cellular membranes (Escoubas, 2006; Sollod *et al*., 2005).

Shikata *et al*. (1995) characterized the venom of Funnel web spider using HPLC and they have purified a novel peptide isomerase found to be having 29 kDa polypeptide that consists of an 18-residue light chain and a 243–residue heavy chain connected by a single disulfide bridge. The venom of the *Boiga irregularis*, HPLC analysis showed about 8 to 10 protein peaks and they have their enzyme activity. Thirteen different compounds have been identified by gas chromatography mass spectrometry in the Sydney funnel web spider, *Atrax robustus*. The Matrix assisted Laser desorption/ionization (MALDI) is used for the identification of proteins isolated through electrophoresis, affinity chromatography, strong/weak ion exchange (Wu *et al*., 1993). The venom components of *Liocheles australiasiae* were characterized by MALDI-TOFMS analysis and its toxicity has been analyzed (Eitan *et al*., 1990; Zlotkin *et al*., 1991; Moskowitz *et al*., 1998; Sautrere *et al*., 1998; Hamon *et al*., 2002). In the venom of *Tityus discrepans*, 205 components were identified by MALDI-TOFMS analysis and 70% were having molecular mass less than 5000
kDa (Batista et al., 2006). The crude venom of Lycosa singoriensis analysis by MALDI showed 10000 to 1000 Da molecular mass distributions (Liu et al., 2009). Schwartz et al. (2008) have analyzed the venom components of Brazilian scorpion, Opisthacanthus cayaparum and they have obtained 221 distinct components.

Only a few reports are available on the characterization of reduviids VS. Previously no one had studied the VS of R. fuscipes; hence I had proposed these objectives to study the VS and parts of salivary gland components by enzyme profile and protein profile. The VS have been biochemicaly characterized by using EDX spectra, FTIR spectroscopy, HPLC chromatogram, GC-MS analysis and MALDI-TOFMS analysis.

3.3. MATERIALS AND METHODS

3.3.1. Venomous saliva collection

The laboratory emerged adult R. fuscipes (n=80) with three-day starved predators have been used for the VS collection and the collected VS was pooled and it was stored at 0 – 4 ºC for 2 months. Similarly in another set of predators, collected VS was pooled and it was air dried in a sterile condition under a laminar air flow chamber. The powdered VS sample was stored at 0 ºC until further use.

3.3.2. Venomous saliva pH

The pH of the crude and lyophilized VS was determined using the discs of different pH paper (Himedia, India). And the animal VS was placed to find out the pH which was compared with a pH standard.

3.3.3. Total water content of the venomous saliva

The total water content (%) of VS of R. fuscipes was analyzed from three-day starved adult predators (n=10). The VS was collected by the method of Sahayaraj et al. (2006a) with slight modifications. The animal was allowed to milk in the glass slide and the weight of the crude VS was taken as VS initial weight (SIW) and after 24 h, glass slide weight was noted and the lyophilized VS weight was considered as VS final weight (SFW) and the water content was calculated using the formula:
3.3.4. Enzyme levels

3.3.4.1. Preparation of Enzyme extracts

Enzyme samples were prepared by the method of Cohen (1993) and later modified by Sahayaraj et al. (2010) using 20 adult insects (Male and female in 1:1 ratio) for each sample. Young adults (one week post adult eclosion) were starved for at least 15h before dissection to standardize the insects and allowed for the accumulation of the enzymes. The insects were placed at -20 °C for 5 min and then dissected out in the ice cold insect Ringers solution (NaCl – 6.5 g, KCl – 0.25 g; CaCl$_2$ – 0.25 g, Na$_2$Co$_3$ – 0.25 g in one litre distilled water) under a dissection microscope (Everest, India). The salivary gland and its parts including the principal and accessory glands were removed away from the abdomen using a fine dissection forceps (Tiger, India). Following by the insects gut was also dissected out and they were split into fore, mid and hindgut. All the samples, accessory gland (AG), anterior lobe of principal gland (ALPG), posterior lobe of principal gland (PLPG), foregut (FG), midgut (MG) and hindgut (HG) were weighed separately using a monopan balance (Dhona, India) (+ 0.1mg) and homogenized using a tissue homogenizer (Kemi, India) for 3 min at 4 °C in the ice cold phosphate buffer (pH 7.0). Homogenates were centrifuged using a micro centrifuge (Kemi, India) at 10000 g for 15 min at 4 °C. The supernatant was removed and made up to 10 ml with the above said buffer. Now the extracts were used for the protein estimation, qualitative and qualitative enzyme analyses.

3.3.4.2. Qualitative enzyme profile

3.3.4.2.1. Amylase

The reaction mixture containing 1ml of 1% starch solution with 100ml of enzyme source was incubated in a water bath at 25 °C for about 30 min. Every 10 min a drop of iodine was added. Reddish violet color appeared when starch was hydrolyzed to dextrin. No color appeared, when starch hydrolyzed to maltose (Nigam and Kumar, 2003).
3.3.4.2.2. Invertase

The reaction mixture containing 2 ml of 10% sucrose solution with 0.2 ml of enzyme extract was incubated at 37 ºC for about 24 h. One drop of Fehling’s A and B were added. It was kept in a water bath at 45 ºC. At the end of reaction, brown or brick red precipitate appeared (Nigam and Omkumar, 2003).

3.3.4.2.3. Lipase

The reaction mixture was prepared by using one ml of boiled milk, one ml of 1% phenol red and 0.5ml of enzyme extract. Now a drop of 0.025% Na₂CO₃ was added. Appearance of pink color was observed. It was incubated at 37 ºC for 24h. The disappearance of pink color shows the presence of lipase (Nigam and Omkumar, 2003).

3.3.4.2.4. Protease

One ml of casein solution along with 100 µl of enzyme extract and 500 µl of TCA were centrifuged at 5000 rpm for 10 min. The supernatant was mixed with 0.5 ml of saturated Na₂CO₃ and a few drops of folin phenol reagent were added. Formation of blue color or precipitate indicates the digestion of casein (Balogun and Fisher, 1970).

3.3.4.2.5. Trypsin

The reaction mixture containing 0.5 ml of alkaline casein with 0.5 ml of enzyme extract was incubated at room temperature for about 6 h. 1% acetic acid was added. Increased turbidity indicates the tryptic activity (Balogun and Fisher, 1970).

3.3.4.2.6. Pepsin

The reaction mixture containing 0.5 ml of casein (pH 2.0) with 0.5 ml of enzyme extract was incubated at room temperature for about 6 h. 10% sodium acetate was added. Increased turbidity indicates the presence of pepsin (Tonapi, 1996).

3.3.4.2.7. Polypeptidase

Incubate the reaction mixture containing 0.5 ml of 2% peptone solution with 0.5 ml of enzyme extract along with 0.5 ml of phosphate buffer, incubated in room temperature for
about 24 h. A few drops of diluted acetic acid and bromine water were added. Pink color
developed indicating the presence of polypeptidase activity (Tonapi, 1996).

3.3.4.3. Quantitative enzyme studies

3.3.4.3.1. Amylase and Invertase

Amylase and invertase activity was studied (Bernfield, 1955 and later modified by
Ishaaya and Suriski, 1970). The reaction mixture consisted of 1 ml of 0.2% soluble starch in
borate buffer (amylase) and 0.2% sucrose in phosphate buffer (invertase). To the above
mixture 250 µl of enzyme extract was added and incubated at 37 º C for 60 min. The enzyme
activity was terminated by adding 0.4 ml of DNS reagent. The reaction was maintained at
100 º C for 5min. Absorbance of the sample was measured using optical density units at 575
nm (amylase) and 550 nm (invertase) against a blank containing distilled water. The enzyme
activity was expressed in terms of the weight of the reducing sugars, glucose (amylase) and
sucrose (invertase) produced by the enzyme action per unit weight of a sample per unit time.

3.3.4.3.2. Protease

Protease activity was qualified spectrophotometrically as described by Morihara and
Tsuzuki (1977) and later modified by Soyelu (2007). The reaction mixture consisted of 1ml
of 1% casein and 0.5 ml of the enzyme extract. This was incubated at 35 º C for 30 min in a
water bath. The reaction was terminated by adding 3 ml of ice cold 10% TCA. The mixture
was then allowed to stand at 4 º C for 30min, and centrifuged at 3000 rpm for 10min. Now the
supernatant was collected for the determination of non-precipitated products of digestion.
This was determined following the Folin Ciocalteu’s phenol reagent (Lowry et al., 1951).
One ml of the TCA protein was mixed with 5 ml of Lowry’s reagent of C, mixed thoroughly
and incubated at room temperature for 5 min. 0.5 ml of three fold diluted Folin Ciocalteu’s
phenol reagent was added to the mixture with shaking and incubated at room temperature for
30 min. The optical density was taken at 670 nm in Elico spectrophotometer. The amount of
non precipitated TCA protein was estimated as tyrosine from a standard curve known as
concentrations of tyrosine. One unit of protease activity is defined as the quantity required
producing 100 mg of tyrosine in 1ml of TCA filtrate under the above concentrations.
3.3.3.3. Lipase

The lipase activity was carried out titrometrically as described by Cherry and Crandall (1932). One ml of enzyme extract (the control tube was placed in a boiling water for 15 min to destroy the enzyme activity and then cooled. 500 µl of phosphate buffer solution (pH 8.0) and 2ml of olive oil emulsion were added, and placed in the shaker, incubated at 37 °C. After 24h, 3 ml of 95% ethanol was added and two drops of phenolphthalein indicator was also added to each tube, the tubes were titrated separately with 0.05N NaOH and the end point was the formation of pink color. Lipase activity was calculated using the following formula:

\[
\text{Lipase (mequ/min/g/sample)} = \frac{\text{Volume of NaOH consumed} \times \text{alkali strength}}{\text{Sample weight in g} \times \text{Time in min}}
\]

3.3.4.3.4. Acid phosphatase

Acid phosphatase activity was quantified following Beaufay et al. (1954) method. The enzyme reaction mixture consists of 0.5 ml of substrate (ρ-nitro phenyl phosphate disodium), 1.1 ml of 0.2M sodium acetate buffer (pH 5.5) and 0.2ml of the enzyme extract was incubated for 10min at 37 °C. And after incubation, the reaction was terminated by adding 4.5 ml of 0.01N NaOH. The product ρ-nitrophenol was measured by spectrophotometer; absorbance at 420 nm against an enzyme blank was taken. The amount of ρ-nitro phenyl phosphate released as a result of the enzyme activity was determined from a standard curve drawn using standard ρ-nitro phenyl (10 µmoles/ml).

3.3.4.3.5. Hyalurindase

Hyalurindase activity was determined tubidometrically by the method of Pukrittayakamee et al. (1988). The assay mixture consists of 0.2M acetate buffer (pH 6.0) containing 0.15M NaCl, 50mg hyaluronic acid (0.5 mg/ml in acetate buffer) and enzyme source having a final volume of 1.0ml. The mixture was incubated for 15 min at 37 °C. Then the reaction was stopped by the addition of 2 ml of 2.25% acetyl trimethyl ammonium bromide in 2% NaOH. The absorbance of each reaction mixture was read at 410 nm against a blank containing 1 ml of acetate buffer and 2 ml of 2.25% acetyl trimethyl ammonium bromide in 2% NaOH. Turbidity reducing activity was expressed as a percentage of the remaining hyaluronic acid, taking the absorbance of a tube as 100% in which no enzyme
added. Turbidity reducing unit is expressed as the amount of enzyme required to hydrolyze 50% (25 mg) of the hyaluronic acid. Specific activity is expressed as turbidity reducing units/mg enzyme per min. One unit is defined as the amount of enzyme that will cause 50% turbidity reduction as 1.0 unit of international standard preparation.

3.3.4.3.6. Phospholipase A<sub>2</sub> activity

Phospholipase activity was determined as described by Santoro et al. (1999). Fifteen µl enzyme source was added to 1.5 ml of reaction solution (100 mM NaCl, 10 mM CaCl<sub>2</sub>, 7 mM Triton X-100, 0.265% egg lecithin, 98.8 µm phenol red, pH 7.6) in a spectrophotometer cuvette. The mixture was read at 558 nm. One unit of activity was defined as the quantity of venom protein (mg of protein/assay) producing a decrease of 0.001 units of absorbance in the conditions described.

3.3.4.3.7. Trypsin-like-enzyme

The assay of trypsin-like-enzyme activity was carried out as described by the methods of Stewart (1993) and Zheng et al. (2002). The substrate solution (1 mg/ml) was prepared by dissolving 100 mg of N<sub>α</sub>-benzoyl-L-arginine-p-nitroanilide (BApNA) (Sigma, India) in 5 ml of dimethyl sulfoxide (DMSO) and then 95 ml of 0.05M Tris–HCL buffer (pH 8.2) was added. Trypsin-like activity was monitored as BApNA hydrolysis by adding 100 µL of extract (diluted to 100 µl with buffer for the crude sample before the assay) to a well in an assay plate, and by adding 100 µl of the substrate solution. The absorbance, which is proportional to protease activity, was measured with a plate reader ELISA strip reader (SR 601-Qualisystems). The plate was read at 420 nm after incubation for 10 min at 37 ºC. The endogenous control (substrate solution and buffer only) was used. The reading was corrected by subtracting the reading from that of the endogenous control sample.

3.3.5. Total protein quantification

Total protein content (Lowry et al. (1951) of the venomous saliva and parts of the salivary gland were done using Bovine Serum Albumin (BSA) as standard which was previously described in Chapter 2 (page no.).
3.3.6. Electrophoresis (Tricine SDS-PAGE)

Tricine sodium dodecyl sulfate polyacrylamide gel electrophoresis (Tricine SDS-PAGE) was performed by the method described by Schägger and Von Jagow (1987) using 16% separating gel and 5% stacking gel in a mini electrophoresis unit (Biotech, India). The separating gel (16%) contained acrylamide stock (29:1 ratio of acrylamide and bisacrylamide), 1.5M Tris HCl (pH 8.25), glycerol, 10% APS and TEMED. The above mixture was mixed and poured in the gel cast and one ml of the overlaying solution (70% n-butanol) was poured. After the polymerization, the excess overlaying solution was poured off and washed with distilled water. Now the stacking gel (5%) containing acrylamide stock (29:1 ratio), Tris HCL (pH 8.25), 10% APS and TEMED was added and after the polymerization, the gel was placed in the tank containing two buffer systems (cathode buffer and anode buffer). To the 30 µl of the sample, an equal quantity of sample buffer was added and it was loaded in the gel. And ten µl of reference standard marker (Low molecular weight protein marker, Genei, India) was also added. Now the current was set at 30 mv and later it was raised to 90 mv until the dye front reached the end of the gel.

After the electrophoresis, the gel was stained with the staining solution (0.1% Commasie brilliant blue, 40% methanol and 10% glacial acetic acid) for 24 h followed by destaining (40% methanol and 10% glacial acetic acid). Gel image was captured using Biotech image analyzer system (Biotech, India) connected to an Intel Pentium computer (Samsung, India) equipped with DGel system (Biotech, India). Molecular weight of protein bands were estimated with reference to molecular weight marker (Genei, India).

3.3.7. Fourier Transfer Infrared (FTIR) spectroscopy

Infrared spectroscopic analysis was performed essentially as described previously (Uçkan et al., 2004). In brief, 15 µg of lyophilized VS was homogeneously ground with potassium bromide (Hi Media, India) before the infrared spectroscopic analyses. The FTIR was carried out at room temperature using Schimadzu FTIR (Model 8400S, Japan). The spectra were interpreted with available literature (Stuart, 1997; Kalsi, 1998; Uçkan et al., 2004).
3.3.8. High Performance Liquid Chromatography (HPLC)

The lyophilized VS was analyzed on a HPLC (Shimadzu LC/10AD, Japan) equipped with an injector – 20 µl loop. Experiments were conducted using a Shimadzu C-18 column (5 µm particle size, 250 mm x 4.6 mm I.D) with a flow rate of 1 ml min⁻¹ at room temperature. The mobile phase used was CH₃CN/H₂O (1:1, v/v) with elution isocratic and UV detection (SPD-10A/UV-Vis) at 280 nm. Acetonitrile used was HPLC grade (Sigma Aldrich, India) and degassed in an ultrasonic bath before use. The water was distilled using a Milli-Q system (Millipore) and the mobile phase was filtered through a 0.49 µm nylon filter (Corzo et al., 2001).

3.3.9. Gas Chromatography-Mass spectrometer (GC-MS)

Lyophilized fractions from HPLC were mixed in 10 µl of HPLC grade water. The fractions were further analyzed in gas chromatography. For GC/MS a Hewlett Packard gas chromatograph 6390 series II Plus was linked to Hewlett Packard mass spectrometer system equipped with a capillary column HP5-MS (64 m\0.34 mm, 0.21 µm film thickness) was used. The temperature was programmed from 240 °C to 350 °C at rate of 6 °C min⁻¹ with 10 min hold. Helium was used as a carrier gas with a constant flow at 0.8 mL min⁻¹. The ionization voltage was 80 eV. Fraction also was analyzed after silylation at the conditions given for the silylated polar compounds mentioned below. Quantitative analysis of sterols was performed on a Hewlett Packard gas chromatograph (Zhu et al., 2008) 5890 equipped with FID and capillary column HP5-MS (64 m\0.34 mm, 0.21 µm film thickness), at 230 °C and programmed to 300 °C at 4 °C minD1 and5 10 min hold (Munyiri and Ishikawa, 2004; Zhu et al., 2008). Injector and detector were at 280 °C. One µl of each sample was injected triplicate split/splitless and quantities represented as relative area percent as derived from the integrator. Calibration was established using hexane as standards.

3.3.10. Matrix Assisted Laser Desorption/Ionization –Time of Flight Mass spectrometry (MALDI-TOFMS)

MALDI-TOFMS spectra were obtained on a The Voyager-DE™ PRO Biospectrometry™ spectrometer (Applied BioSystems, Framingham, MA, USA) equipped with a model VSL-337ND nitrogen laser (Laser Science, USA). The accelerating voltage was 20 KV. The matrix was α-Cyano-4-hydroxycinnamic acid (Sigma Aldrich, India), which
was prepared at a concentration of 10 mg/ml in 1:1 CH$_3$CN/0.1% TFA. Lyophilized VS was analyzed as described in assassin bugs by Corzo et al. (2001) to get the exact molecular weight of the component. An equal amount of the sample and matrix were dropped onto the MALDI sample plate and allowed to dry at room temperature. Time-to-mass conversion was achieved by external and/or internal calibration using standards of bovine pancreatic beta insulin (m/z 3496.9), bovine pancreatic insulin (m/z 5734.6), and apomyoglobin (m/z 16,952.6) (Sigma Aldrich, India). Experiments were facilitated by the Voyager Version 5 with Data Explorer™ software.

3.4. RESULTS

3.4.1. pH and water content

The pH of VS immediately collected from *R. fuscipes* was 7.3 ± 0.1, whereas the lyophilized VS was 6.24 ± 0.08. The water content of crude VS of *R. fuscipes* was 24.3 ± 2%.

3.4.2. Qualitative enzyme profile

In the qualitative enzyme studies, the VS and the salivary gland complex (SGC) consist of amylase, invertase, lipase, protease and trypsin. In the SGC and VS do not show pepsin (except in male) and polypeptidase level activities (Table 3.1). In the gut, presence of amylase, invertase, protease, trypsin and pepsin activities was recorded, whereas lipase activity was not recorded in the foregut of the predator. In the VS, salivary gland complex and gut no polypeptidase activity was observed.

3.4.3. Quantitative enzyme levels

The amylase level was maximum in the female accessory gland (df = 2, 9; F = 8.376; p > 0.05) and female midgut (df = 2, 9; F = 218.49; p < 0.05). Higher level of lipase activity was recorded in the accessory gland of both male (41.68 mequ/min/g) and female (42.81 mequ/min/g) and foregut (49.70 mequ/min/g) possess maximum enzyme activity. The invertase activity was found to be higher in the posterior lobe of the principal gland (df = 2, 9; F=1.385, p < 0.05; df =2, 9; F=3.178; p < 0.05) for male and female respectively and the foregut of both male and female have enzyme activity. The posterior lobe of the principal gland possess high level of trehalase activity (df = 2, 9; F = 4.504; p < 0.05) and foregut possesses high trehalose activity (Table 3.2).
The high level of protease activity was recorded in the VS (23.41 ± 0.00 mg tyrosine/g protein/min), and in hindgut (df = 2, 9; F = 8.879; p < 0.05). The AG of both male (df=2, 9; F=1.009; p > 0.05) and female (df = 2, 9; F = 2.429; p < 0.05) have maximum acid phosphatase activity. The foregut of both male and female have high level of enzyme of about 7.62 ± 0.05 and 7.85 ± 0.02 mmol/g/hr respectively. The VS of female possesses maximum phospholipase A$_2$ enzyme activity of 18.32 ± 0.73 nm/min/mg protein. The anterior lobe of principal gland (df = 2, 9; F = 1.877; p < 0.05) has a high level of hyalurindase enzyme activity. The higher level of trypsin like enzyme activity was observed in the female VS (18.08 ± 0.03 units/mg) (Table 3.3).

3.4.4. Total protein in the VS and salivary gland complex

The mean protein content of venom reservoirs, whole SGC samples were significantly higher (df = 3, 36; F =18.00; p < 0.05) than that of VS, PG and AG (Figure 3.1). Female always possesses more protein than the males.

3.4.5. Tricine SDS-PAGE of VS and salivary gland complex

The electrophoretic analysis of VS of *R. fuscipes* showed a complex protein composition with molecular weight ranging from 41.2-6.4 kDa. The most characteristic protein band profile was observed in the region between 41.2 and 26.0 kDa. This region contains 14% of the total applied protein distributed in 19 bands as determined by densitometry (Plate 3.1). The band at 41.2 kDa showed a high intensity of occurrence. Regarding the number of bands in AG, ALPG, PLPG and WSG; 17, 18, 16 and 18 and their molecular weights ranged from 48.6 to 2.4 kDa, 48.6 to 7.8 kDa, 49.4 to 5.0 kDa and 49.6 to 5.0 kDa respectively (Table 3.4). There was no significant difference in the band pattern between VS and AG (df = 1, 34; F = 1.745, p > 0.05), ALPG (df = 1, 34; F = 0.004; p > 0.05), PLPG (df = 1, 34; F=1.875; p > 0.05) and WSG (df = 1, 34; F = 0.564; p > 0.05).

The mixture of acetone and methanol (1:1) showed that the VS have been purified and it showed about 11 polypeptide bands ranging from 33.1 to 9.4 kDa and considering the other solvent systems such as pure methanol which showed 8 polypeptide bands ranging from 27.7 to 10.3 kDa and for the acetone 9 polypeptide bands ranging from 31.1 to 10.5 kDa (Plate 3.2). The action of methanol + acetone was found to be insignificant to acetone (df = 1, 16; F =0.649; p > 0.05) and methanol (df = 1, 16; F = 0.028; p > 0.05) respectively (Table 3.5).
3.4.6. EDX spectra

The EDX spectra of lyophilized VS showed the presence of inorganic elements (Silicon – 2.20%, Sulphur – 21.27% Chlorine – 23.35%); alkaline earth metal ions (Calcium – 2.29%); transition metals (Copper-6.46%, Zinc – 3.47% and alkali metals (Potassium – 29.23%, Zirconium – 11.72%). Potassium was found to be high (29.23%) (Figure 3.2).

3.4.7. FTIR spectroscopy

An infrared spectrum analysis of the VS is shown in the Figure 3.3 and characteristic absorption bands are interrupted in Table 3.6. The absorption bands at 3441.12, 1647.26, 1545.03 cm\(^{-1}\) can be interrupted to indicate the presence of secondary amines and amide groups and to confirm showed the proteinous nature of VS. The observed band at 2964.69 cm\(^{-1}\) is characteristic for the presence of carbohydrate moiety. The vibration at 2378.31 and 522.73 cm\(^{-1}\) showed the presence of sulphur. The acidic nature of the VS was supported by the presence of vibration 1398.44 cm\(^{-1}\) (Table 3.6). The absorption bands at 1085.96, 983.73cm\(^{-1}\) suggest the presence of some venom components, possibly enzymes with phosphorous. The presence of alkyl halides (675.11 cm\(^{-1}\)) has found interpretation of potassium bromide during the experimental analyses.

3.4.8. HPLC analysis

The HPLC analysis of lyophilized VS of *R. fuscipes* (Figure 3.4) showed the presence hyalurindase (30%) lysolecithin (20%) phospholipase and masto 2, 2’- pranol (89%). (Table 3.7)

3.4.9. GC.MS analysis

GC – MS analysis shows (Figure 3.5) the presence of metho 5-primol (80%), hyal-2, urindase (30%), phospho-lipase (22%) and lysolecithin 2, 2’-ol (20%) were identified (Table 3.7).

3.4.10. MALDI – TOFMS analysis

The lyophilized VS of *R. fuscipes* contain a wide variety of components with molecular masses ranging from 2357 to 58261 Da (Figure 3.6). About 69 molecular mass
components were identified. From the molecular mass components, the peptide at 3802.2Da showed a maximum absorbance of 10039.3 mAU (RFITI) and two minor peptides were recorded, they were at 2358Da (2608 mAU) and 3423 Da (2836 mAU) and named as RFIT2 and RFIT3 respectively (Figure 3.7). From the total molecular mass components, the percentage of distribution data showed that about 17.39% components were located between 20.0 to 25.0 kDa when considering the maximum absorbance they were located between 1000 to 5000Da and they had a percentage distribution of 7.24% (Table 3.8).

3.5. DISCUSSION

This is the first time that VS and salivary gland complex of *R. fuscipes* have been chemically characterized. The prey paralysis may results from damage by certain digestive enzymes in neurons, muscles and storage tissues of the prey. Structural proteins are hydrolyzed and liquefied inside the prey by endopeptidases such as trypsin like (14.64 and 18.1 µ/g) for male and female respectively) injected by the *R. fuscipes*, while cell membranes, storage tissues and reproductive system are affected by phospholipase (12.4 and 18.3 g/min/g protein for male and female respectively), lipase and amylase (Azevedo et al., 2007). The enzymes such as phospholipase and hyalurindase are important enzymes which were responsible for the toxin nature of the VS (Cohen, 2000) of *R. fuscipes*. The enzymatic action of phospholipase was already detected in the heteropteran saliva (Edwards, 1961; Cohen, 1990), and also in the spiders (Mommsen, 1978a). *R. fuscipes* use their VS for their extra oral digestion for liquefaction the solid prey into a liquid one and reduce the viscosity of intractable liquid (Cohen, 2000). Similar observation was made in the reduviid, *Catamirus brevipennis*, higher level of protease activity was observed in the salivary gland complex than the other enzymes (Sahayaraj et al., 2007, 2010). Later, the hydrolyzed material was ingested by the predator and additional processing occurs in the gut allowing its absorption by the predator (Cohen, 1993, 1995). The maximum protease activity in the gut showed that the several arthropods use the EOD, whose digestive enzymes all originate from the gut rather than the salivary glands (Cohen, 2000). Boyd (2003) observed that trypsin like; chymotrypsin like activity was high in the salivary gland of *Deraeocois nigritulus* rather than the gut, having a high level of α-amylase, α–glycosidase and elastase activity. Moreover, carbohydrates are as evident from the presence of significantly high trehalose and invertase here (Table 3.2). Protein and lipids are digested in the hindgut and foregut respectively.
Nuorteva (1954) reported that five species of miridae and two species of pentatomidae had amylase and protease in their salivary gland and in *Lygus disponsi* (Hori, 1970 a, b, c) and in the *Dydercus koeningii*, amylase, sucrase, protease and esterase (Saxena, 1955), and in the *D. fasciatus*, α-glucosidase, β-glucosidase, α–glactosidase, aminopeptidase, carboxypeptidase and lipase (Ford, 1962; Khan and Ford, 1967). Presence of invertase, amylase, protease and lipase indicates VS has digestive function. In Heteroptera, proteolytic activity has been found in the salivary glands of several carnivorous species, (Baptist, 1941; Kretovich *et al.*, 1943; Nuorteva, 1954; Edwards, 1961). The alkaline proteinase in the salivary glands of *Lygus* is certainly a secretable salivary enzyme, which facilitates the utilization of food proteins (Laurema *et al.*, 1985). In *R. fuscipes*, the enzyme activity was found to be high in the female than the male. This might be due to the egg production, higher predation rate, and larger size of animal. Salivary gland complex is an important source of general proteinase and endopeptidase activity, as observed in *Zelus renardii* by Cohen (1993). Trypsin like endopeptidase was observed in the salivary glands of the terrestrial heteropterans (Cohen, 1993; Cohen and Wheeler, 1998). Similarly in *R. fuscipes*, the general protease and trypsin like activity and phospholipase activity were found to be high in the VS. These enzymes help the animal to liquefy the prey (Cohen, 1995; Swart *et al.*, 2006; Silva Cardoso *et al.*, 2010). The spreading behavior of phospholipase makes the other EOD enzymes to spread very rapidly.

The SDS-PAGE of VS and SGC of *R. fuscipes* showed molecular mass ranged from 41 to 2.4 kDa. These small peptides may generally be neurotoxins in higher Hymenoptera (Schmidt, 1982). This is consistent with the paralyzing function of *Pimpla turionellae* (Kilincer, 1975; Uçkan *et al.*, 2004). However, these molecular weight proteins/peptides are not recorded from the MALDI-TOF MS studies, indicates that during lyophilization process these molecular weight components are denatured. Posterior lobe of *Acanthispis pedestris* had more number of protein fractions (11) than the anterior lobe (6) and accessory gland (2) (Morrison, 1989). An opposite trend has been recorded in this study. The presence of identical protein in the VS as well as anterior (41231 Da), posterior (35244 Da) and accessory gland (26068 and 18179 Da) presumably indicate the transport of salivary gland protein/peptides into the VS. SDS-PAGE analysis of snake venom showed that the molecular weight of phospholipases is 16-17 kDa (Ramírez-Avila *et al.*, 2003). Similar molecular weight protein was detected in VS (16724 Da) and ALPG (17452 Da) of *R. fuscipes* which is
supported by our HPLC (Figure 3.4), GC-MS (Figure 3.5) and enzyme quantification (Table 3.2) studies. However, MALDI-TOFMS does not support these observations.

The EDX spectra results of R. fuscipes revealed that about 29.23% of potassium and 21.27% of sulphur showed the disulfide bridges in the VS. Similar observations were recorded in the cobra venom (Miyashita et al., 2007) and also in reduviids (Corzo et al., 2001). The presence of sulphur indicates the disulfide bridges in the VS of R. fuscipes which was confirmed in the FTIR spectroscopy. The FTIR spectrum of the VS of R. fuscipes indicates that it is acidic in nature (1398 cm⁻¹) and it was confirmed by Leonard (1972) in the sawfly toxins. Absorption bands at 3441, 1639 and 1545 cm⁻¹ are expected for the proteinaceous nature of the VS (Stuart, 1997; Rivers et al., 2006) Alkanes characters was observed (2964 cm⁻¹). The presences of phosphorous in the VS confirm the presence of enzymes, as suggested by Schimdt (1982); Piek and Spanjier (1986); Parkinson et al. (2001, 2002 a, b); Moreau et al. (2004); Uçkan et al., (2004) from the venom of endoparastie species and social Hymenoptera species. The Silicon observed in the EDX spectra of VS of R. fuscipes was also characterized in the FTIR spectra (2378 cm⁻¹). The disulfide nature of VS in R. fuscipes has been previously observed in the cobra venom and also in the reduviids by Corzo et al. (2001).

The HPLC and GC–MS analysis of VS of R. fuscipes showed the presence of compounds such as hyalurindase, lysolecithin, phospholipase which were found common in both the analyses. Metho 5 –primol (80%) and Mastro 2, 2’-pranol (89%) was found to be abundant in the VS of R. fuscipes. The MALDI–TOFMS analyze of VS of R. fuscipes indicated that VS consists of 69 components predominantly composed of components with molecular mass of 20 to 25 kDa (17.4%), 25 to 30 kDa (16%), 30 to 35 kDa (13%) and 50 to 66 kDa (6%). Corzo et al. (2001) observed that molecular mass of less than 30 kDa (56.5%) in reduviids like Peirates turpis, Agriosphodrus dohrni and Isyndus obscurus. However, low molecular weight proteins and peptides are typical of VS of R. fuscipes 3802 Da (10039) (RF1T1). The peptides such as 3802 Da (RF1T1) (10039 mAU) major one and two minor bards RF1T2 (3423 Da) and RF1T3 (2358 Da) were identified. The venom of Lycosa sugouensis was characterized by MALDI-TOF and it was characterized into three groups based on their molecular weight group ranging from 8.0 to 2.0 kDa (Liu et al., 2009).
3.6. CONCLUSION

The VS of *R. fuscipes* has been characterized by using enzyme studies, protein profiling and also using various biochemical markers such as EDX spectra, FTIR, HPLC, GC-MS and MALDI–TOFMS analysis. In this study, the VS of *R. fuscipes* has the toxic nature of the venom and due to the presence of disulfide bridges it might not be toxic towards the mammals. The VS has the components like hyalurindase, phospholipase and lysolecithin. Hence, this can be used in the study for insecticidal activity. Clearly, additional studies are needed to further characterization and isolation of VS proteins.