INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
10–002/WIB/2010/12–3–447–450
http://www.fspublishers.org

Full Length Article

Partial Characterization of Hemolymph of Different Spider Species of Citrus Orchards

FATIMA JALAL[1], SHAKILA MUSHTAQ, SHOUKAT PARVEZ†, SHAHNAZ ATHTER RANA AND ANJUM SUHAIL‡
Department of Zoology and Fisheries, University of Agriculture, Faisalabad, Pakistan
†*Pakistan Atomic Energy Commission (PAEC), Islamabad, Pakistan*
‡*Department of Entomology, University of Agriculture, Faisalabad, Pakistan*
[1]Corresponding author's e-mail: fatima_jalal@hotmail.com; fatima_jalal222@yahoo.com

ABSTRACT

Erythrocyanin a toxic protein present in the hemolymph. The hemolymph is composed of low and high molecular weight components, while toxins constituting a very small portion. The spider species including in this study were *Myrmarachne maratha, M. orientales, M. bengalensis, M. laetus, M. japonica, Phidippus workmani, Thyene imperialis, Plexippus paykulli, Phintella sp*., *P. castriesiana, Pelegrina verecunda*, *Cheiracanthium mildei, Elaver sp.*, *Stegodyphus sarasinorum Heteropoda kandiana*, *Olios lutescens*, *O. flavidus* and *O. giganteus*. Free ions, biochemical properties were studies. *P. verecunda* and *C. mildei* showed lower ion concentration. Family Sparassidae (pooled) had Na^+ concentration and *S. sarasinorium* showed high Ca^{2+} concentration. Genus *Myrmarachne* (pooled) had lower protein concentration but high casein hydrolization. *Elaver sp.* had high protein contents but moderate casein and gelatin activity. SDS-PAGE revealed 200 kDa and 60 kDa bands were present in all species. This study can be helpful in further advancement in drug development and other pharmaceuticals. © 2010 Friends Science Publishers

Key Words: Spider species; Hemolymph; Biochemical; Free ion

INTRODUCTION

Hemolymph is transparent fluid, which transports nutrients, hormones, oxygen and cells. Mostly, the hemolymphatic cells contain copper instead of iron. Burton (1984) reported that the concentration of Ca^{2+} representative of each species of spiders and scorpions was depended on Na^+, K^+ and Mg^{2+}, while Cohen (1980) reported that the total K^+, Ca^{2+} and Mg^{2+} concentrations were 43 mg/L for *Araneus gemma* and 29 mg/L for *Argiope trifasciata*, respectively. Totals of K^+, Ca^{2+} and Mg^{2+} were 43 mg/L for *A. gemma* and 29 for *A. trifasciata* or 8% and 6% of the total osmolytes, respectively. Paul *et al*. (1994) reported that in *Eurypelma californicum* the concentrations of Na^+ was 188.5, K^+ 2.5, Ca^{2+} 4.3, Mg^{2+} 0.4 and Cl^- 215.6 mmol/L and in *Pandinus imperator* these vales were Na^+ 230.5, K^+ 2.9, Ca^{2+} 4.1, Mg^{2+} 1.1 and Cl^- 219.2 mmol/L, were estimated at resting stage. Punzo (1989) analyzed organic and inorganic constituents present in the cell-free hemolymph of spiders, *Dugeseilla echina*, *Botheriocyrtum californicum*, *Euagrus comstocki* and *Ariadna bicolor*. He measured the concentrations of Na^+, K^+, Mg^{2+}, Ca^{2+} and Cl^- along with osmolarity and pH values in the adult female of *Tegenaria atrica*.

Punzo (1990) estimated the chemical composition of different segments of the spider body. He found that gamma aminobutaric acid (GABA), taurine, Glycine, Glutamine and Alanine were most abundant in the brain tissues. This composition comprised 32% of the total ninhydrin-positive compounds. The arthropod hemocyanins were multipule subunits proteins with a molecular mass 70-75 kDa per peptide chain arranged as hexamers and multiple of hexamers, which were the species specific characteristics (Voit *et al*., 2000). Cunningham *et al.* (2007) reported that the composition of arachnid lipoproteins differs among species and hemocyanin performs the function of apolipoprotein in some arachnid species. It has been investigated that hemocyanin has the capacity to bind neutral and polar lipid classes, including ecdysteroids. Tillinghast and Townley (2008) reported that glycine, taurine, proline, histidine and alanine were founding both orb and non-orb builders five spider families; among those, glutamine was the most abundant. All the above information are indicated a wide scope of investigation in the research on hemolymph in order to use the identified its components in agriculture and medicine industries and check the biochemical composition. This may open new horizons in research in the future.

In Pakistan, this is the first time research done on valuable mixture (hemolymph) of spiders. The exploration of useful components from the hemolymph of spiders is likely to pave the ways of future potential applications in different industries of health, medicine and agriculture.

To cite this paper: Jalal, F., S. Mushtaq, S. Parvez, S.A. Rana and A. Suhail, 2010. Partial characterization of hemolymph of different spider species of citrus orchards. *Int. J. Agric. Biol.*, 12: 447–450

MATERIALS AND METHODS

Spiders: Spiders were collected from the Citrus orchards in Faisalabad District using jerrying method. Collection was done early in the morning from June, 2007 to July, 2008 and identified up to species.

Hemolymph collection: For the collection of hemolymph, the adult spiders were selected as described by Yigit and Benl (2008) with some modifications. The live spiders were anesthetized with alcohol; the forth waking legs were separated from the coax. The outwards flowing liquid was collected with micropipettes, which varied from 05-10 µL. It is a brownish white, viscous and odorless liquid. It was diluted in the ratio of ¼ in distilled water for further analysis.

Free ions determination: The concentration of ions such as K^+, Na^+ and Ca^{2+} were detected using flame photometer (Jenway, PFP 7), while Cl^- detected titration method with 0.0141 M silver nitrate.

Hydrolytic properties: These were determined according to the method recently modified by Nagaraju *et al*. (2006) with some modifications by 2% casein/gelatin in 0.02 M Tris-HCl buffer at pH 8.5. Spider hemolymph samples, 50 µL each, were incubated with 0.5 mL substrate for 2.5 h at 37°C. For the precipitation of undigested casein and gelatin 1.5 mL of trichloro-acetic acid (0.44 M) was added. The released tyrosine was determined from the supernatant of the reaction by using the Folin-Ciocalteu's reagent (Devaraja *et al*., 2008). The method was standardized by using the decreasing amount of hemolymph samples in the reactions. One unit of activity was defined as the amount of enzyme to liberate the amount of tyrosine at 550 nm/min.

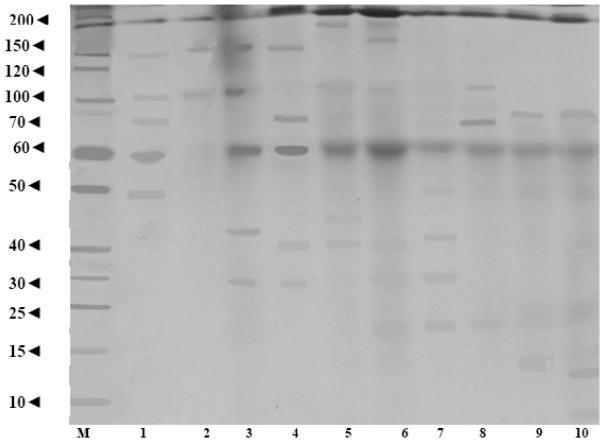
SDS-PAGE: Hemolymph proteins of selected spider species were separated by SDS (Yigit *et al*., 2004). For this purpose, clear supernatant was collected and concentration of proteins measured by the dye-binding method of Bradford (1976), using bovine serum albumin as standard. For electrophoretic separation of proteins, 10% acrylamide gels with 1 mm thickness were used following dissociating and discontinuous buffer system. Proteins bands were visualized by Commessei blue staining protocol described by Tambourgi *et al.* (2002).

RESULTS

Ionic concentrations: The average data for Na^+ contents within species were significant ($p \leq 0.015$) except in case of *Cheiracanthium mildei* Na^+ contents ♂♂ (6.1437 mg/L), while as family (pooled) *Sparassidae spp.* (13.417 mg/L) showed high concentration as compared to rest species followed by *Phintella castriesian* ♀♀ (Na^+ 6.55 mg/L) (Table I). *Phidipus workmani* (♀♀), hemolymph had a low concentration (5.627 mg/L), followed by *Phintella sp.* ♂♂ (5.87 mg/L). In some cases the values of Na^+ concentration was low as in *Stegodyphus sarasinorum* ♀♀, Na^+ concentration was (3.347 mg/L) followed by *Pelegrina verecunda* (♀♀), (2.198±0.1156 ppm) and in *C. mildei* ♂♂ the Na^+ quantity (1.637±0.1263 ppm).

Fig. 1: The analysis of proteins/ polypeptides of hemolymph of different spider species using 10 % SDS-PAGE

M=Marker, 1= *Thyne imperialis* 2= *Pelegrina verecunda*, 3= *S. sarasinorum,* 4= *P. workmani*, 5= *C. Mildei* 6= *P. castriesiana*, 7= *P. Paykulli,* 8= *Phentella sp* 9= *Elaver* sp., 10= *H. kandiana*



The average data (Table I) of K^+ showed that there was not a significance difference between various species ($p \geq 0.05$). *P.* workmani had a high K^+ as compared to the rest species, except, in case of *Phintella sp.* ♂♂ (0.068 ppm) *Thyene imperialis* (♀♀) (0.039 ppm) of K^+ as compared to *Phintella sp.* ♂♂ and in case of *Myrmarachne spp.* (♀♀) exhibited lower concentration (0.014).

Analyses of variability in quantity standardized Ca^{2+} present per milking and per venom gland among species within the families and species for which three or more samples, which indicated non-significant difference between various species of spiders ($p \geq 0.05$), but significant one in case of hemolymph ($p \leq 0.003$) (Table I). The total Ca^{2+} contents in species of spiders revealed that non-significant difference among the species of the spiders ($p \geq 0.05$). In case of *T. imperialis* (♀♀), the concentration of Ca^{2+} contents were (0.042 ppm), There was a non-significant difference in Ca^{2+} contents among the rest species, except in case of *S. sarasinorum* (♀♀) (0.24 ppm), which was highly significant and has higher concentration as compared to the rest of species. *Myrmarachne spp.* (♀♀) exhibited lower concentration (0.014). The comparison between genera and species showed that highly significant difference was present between genera but non-significant ($p \geq 0.05$) between the species (Table I).

The average data (Table I) of Cl^- contents showed that within species, the results were non-significant ($p \geq 0.05$). In case of *Phintella* sp. ♂♂ having high concentration (118.94 mg/L) followed by *T. imperialis* (♀♀) (103.27 mg/L), while *P. castriesiana* (♀♀) and *P. verecunda* (♀♀) had lower (79.77 & 46.29 mg/L) concentration (Table I). The comparison between venoms and body fluid showed significant results ($p \leq 0.01$) and concentration was greater

Table I: Free ions concentration profile of different spider species of Citrus orchards of Faisalabad district

Sr.#	Species	Na^+	K^+	Ca^{2+}	Cl^-
1	*Thyene imperialis*	3.21±2.49	0.032±0.01	0.04±0.03	103.27±25.67 ^{h}
2	*Cheiracanthium mildei*	1.64±0.13 ^{L}	0.028±0.01	0.04±0.03	103.98±29.94 ^{h}
3	*Elaver sp*	2.94±2.84	0.042 ±0.10	0.05±0.04	101.85±26.71
4	*Stegodyphus sarasinorium*	3.35±2.73	0.042±0.01	0.25±0.37 ^{h}	99.71±48.08
5	*Phidipus workmani*	5.63±7.42	0.050±0.02	0.03±0.02	65.52±19.74
6	*Phintella* sp	5.87±6.46	0.068±0.02 ^{h}	0.04±0.03	118.94±16.03
7	*Plexippus paykulli*	3.84±2.78	0.053±0.02	0.06±0.04	93.30±30.24
8	*Phentella castriesiana*	6.56±0.75	0.040±0.02	0.07±0.05	79.77±60.45
9	*Myrmarachne* .* Pooled	1.93±0.08 ^{L}	0.014±0.01 ^{L}	0.02±.020 ^{L}	95.44±28.45
10	*Sparassidae spp.** Pooled	13.41±1.20 ^{h}	0.046±0.01	0.07±0.04	106.12±63.11 ^{h}
11	*Pelegrina verecunda*	2.20±0.12	0.046±0.01	0.04±0.02	46.29±6.17^L

Table II: Biochemical Properties of different species of spider's haemolymph

Sr. No	Species	♀/♂	Protein contents of venom/50 spiders in U=mg/mL	Caseinolytic activity/50 spiders in U=µmole/mL	Gelatinolytic activity/50 spiders in U=µmole/mL
1	*Elaver spp.*	♂	10.69±0.34	6.73±0.0	4.63±0.51
		♀	12.04±0.05^h	6.01±0.006	5.05±1.35
2	*Stegodyphus sarasinorum*	♂	-	-	-
		♀	6.65±0.18	7.62±0.86	6.34±1.45 ^{h}
3	*Cheiracanthium mildei*	♂	8.25±0.30*	2.952±0.045 ^{L}	3.83±0.44
		♀	8.13±0.67*	6.01±0.01	5.14±0.63
4	*Phidippus workmani*	♂	7.65±0.32	9.09±.07 ^{h}	-
		♀	8.66±0.37*	9.09±0.09 ^{h}	4.36±1.84
5	*Plexippus paykulli*	♂	4.31±0.15	7.66±0.36	3.69±0.64
6	*Phentilla sp*	♂	4.26±0.31	7.44±1.13	2.19±0.72 ^{L}
7	*Myrmarachne spp.*	♂	0.79±0.09 ^{L}	8.30±0.43 ^{h}	-
		♀	2.26±0.34 ^{L}	8.30±0.43 ^{h}	7.69±0.83 ^{h}
8	*Phentella castriesiana*	♂	-	-	-
		♀	6.80±0.98	8.68±0.33 ^{h}	6.55±1.31 ^{h}
9	*Thyene imperialis*	♂	6.48±0.05	7.90±0.58	4.35±2.02
		♀	7.22±.067	8.03±0.40 ^{h}	4.89±0.17
10	*Sparasidae spp.*	♂	5.85±0.31	-	-
		♀	5.81±0.73	-	3.12±0.58

Note: Low concentration; L, High concentration; h

Cl^- in body fluid than venom of the same species.

Casein hydrolization: The data on caseinolytic activity showed a significant difference in the sexes of various species ($p \leq 0.01$). The females had high caseinolytic activity as compared to males except, in case of *Cheiracanthium mildei* (♀♀ & ♂♂), where both gender exhibited similar (♀♀ 8.82 & ♂♂ 9.07 1U/mL) activity although, the results were significant ($p \leq 0.01$). The hemolymph showed casein hydrolyzation in *Phetella castriesiana* ♀♀ (8.68 1U/mL) and *Myrmarachne* (♀♀ & ♂♂) (8.30 1U/mL, 8.30 1U/mL), respectively (Table II).

Gelatin hydrolyzation: Results regarding gelatinolytic activity showed no significant ($p \geq 0.05$) difference trend between sexes. In case of *S. sarasinorum* ♀♀ (venom (6.34 1U/mL) and pooled species of *Myrmarachne spp.* ♀♀ (7.688) exhibited gelatinolytic activity, while *Phintella sp.* ♂♂ (2.18 1U/mL) showed similar pattern. The Table II showed the proteolytic activity of body fluid on gelatin substrate, species exhibited significant ($p \leq 0.002$) difference (Table II).

In all species, the highest molecular weight of bands was between 200 and 60 kDa. Other similar bands were 120, 100, 50 and 40 kDa in most species; although between 60-70 kDa were most prominent (Fig. 1). A 30 kDa band was present with lighter intensity in *S. sarasinrum* ♀♀, *Phidipus workmani* ♀♀, *Cheiracanthium mildei* (♀♀),*Phintella sp.* ♂♂, *Phetella castriesiana* ♀♀, *P. paykulli* ♂♂and *H. kandiana* ♀♀. There were variations in bands in each lane present between 10 and 90 kDa, which is due to variation in species of spiders. Occasional bands were detected around 20 kDa.

For visible comparative analyses the integrated intensities of bands within the size regions <10, 20–30, >30–80 and >80 kDa were combined in order to analyzed the composition of different component of the samples. Comparisons of banding patterns of hemolymph among different spider species of citrus orchards yielded no obvious differences in electrophoretic patterns. However, none of the citrus spiders was significant if data for *T. imperialis* were excluded (<10, 10–30, 30–80, >80 kDa). Differences between pooled spiders (in the percent total concentration of <10 kDa & 30–80 kDa) persisted when *Myrmarachne, Plexippus* and *Phidippus* spp. data were excluded from the comparison. The hemolymph had higher molecular weight than that of venom and hemolymph of the Karakurt spider (*Latrodectus tredecinguttatus*) contained proteins with molecular weights from 8-300 kDa. This showed that hemolymph has low as well as high molecular weight proteins as are found in

venoms. The hemolymph of spiders also has biologically active components (Akhunov *et al*., 2001).

DISCUSSION

In this study partial characterization of hemolymph of selected spider species have been done. The availability of high activity components is an essential element in the successful process for the bio-activity of the selected constituent from the spider venoms. The spider hemolymph contained a mixture of components having toxicological and biological potentials. The synergistic effect of different components in a mixture of venom has been reported to modulate the bioactive potential of each component (Nentwig *et al.*, 2004; Kozlov *et al*., 2006).

The profile of K^+, Na^+, Ca^{2+} and Cl^- in free form indicated that *Phintella* sp. ♂♂ and *Sparassidae* spp. (pooled) had higher concentration of free ions as compared to rest of the species. Individually family *Sparassidae* spp. (pooled) had high Na^+, while *Phintella* sp. ♂♂ had high Cl^- (118.94), which is comparable with ion concentration of *E. californicum* (Paul *et al.*, 1994). In *S. sarasinorium* the concentration of Ca^{2+} was very high as compared to the investigated species reported that the concentration of Ca^{2+} was dependent on K^+, Na^+ and Mg^{2+}.

Biochemical properties indicated that the hemolymph showed hydrolytic of casein and gelatin. Overall casein hydrolysis was greater than gelatin. The natural substrate of the 32-35 kDa protease is unknown (since gelatin is denatured collagen), but based on gelatinolytic activity. This protease has properties like vertebrate gelatinases that appears to cleave connective components and is functionally related to the deleterious effects of the hemolymph (Foradori *et al.*, 2001). Native collagen can suffer an initial effect of collagenase from polymorphonuclear neutrophil leukocytes and seem to play a role in dermonecrosis, partially denaturing this molecule, which then can be sequentially degraded by these gelatinase-like proteases.

The hemolymph has higher molecular weight proteins than venom and haemolymph of the Karakurt spider (*L. tredecinguttatus*) contain proteins of molecular weights from 8-300 kDa. This comparison showed that the hemolymph has low as well as high molecular weight proteins are found in venoms. The haemolymph of spiders also has biologically active (Akhunov *et al*., 2001). A 200 kDa protein was found in all the selected species and 150–70 kDa found in *Thyne imperialis, P. verecunda*, *S. sarasinorum, P. workmani*, *C. Mildei*, *P. castriesiana*, *P. paykulli*, *Phintella sp*. and *Elaver* sp. Earlier studies show that arthropod hemocyanins are the multiple subunits of proteins with molecular mass 70–75 kDa and are species specific (Voit *et al*., 2000).

In conclusion, the functional diversity of hemolymph seems to be the most significant variable influencing spider abundance and diversity. The abundance of a species depends on seasons (weather, temperature, humidity, rain fall, etc.). Therefore hemolymph quantity and quality significantly varied with the due course of time and spiders physiological conditions (age, sex, fecundity, etc.). Future investigations on the molecular mechanisms would be of great help in the discovery of drugs using the toxins from spider hemolymph.

REFERENCES

Akhunov, A.A., Z. Golubenko, N.A. Abdurashidova, E. Ch. Mustakimova, F.A. Ibragimov and S. Mackessy, 2001. Comparative biochemistry of the physiologically active components of venom, hemolymph and eggs of the karakurt spider (*Latrodectus tredecimguttatus*). *Chem. Nat. Comp.*, 37: 562–565

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizating the principle of protein dye-binding. *Anal. Biochem.*, 72: 248–254

Burton, R.F., 1984. Haemolymph composition in spiders and scorpions. *Comp. Biochem. Physiol., Part: A: Pysiol.*, 78: 613–616

Cohen, A.C., 1980. Haemolymph chemistry of two species of araneid spiders. *Comp. Biochem. Physiol., Part: A: Pysiol.*, 66: 715–717

Cunningham, A., F. Garcia and R.J. Pollero, 2007. Arachnid lipoproteins: comparative aspects. *Comp. Biochem. Physiol. Part C.,* 146: 79–87

Devaraja, S., S. Nagaraju, Y.H. Mahadeswaraswamy, K.S. Girish and K. Kemparaju, 2008. A low molecular weight serine protease: Purification and characterization from *Hippasa agelenoides* (funnel web) spider venom gland extract. *Toxicon*., 52: 130–138

Foradori, M.J., L.M. Keil, R.E. Wells, M. Diem and E.D. Tillinghast, 2001. An examination of the potential role of spider digestive proteases as a causative factor in spider bite necrosis. *Comp. Biochem. Physiol. Part: C. Toxicol. Pharmicol*., 130: 209–218

Kozlov, S.A., A.A. Vassilevski, A.V. Feofanov, A.Y. Surovoy, D.V. Karpunin and E.V. Grishin, 2006. Latarcins, Antimicrobial and cytolytic peptides from the venom of the spider Lachesana tarabaevi (Zodariidae), exemplify biomolecular diversity. *J. Biol. Chem.*, 281: 20983–20992

Nagaraju, S., Y.H. Mahadeswaraswamy, K.S. Girish and K. Kemparaju, 2006. Venom from spiders of the genus *Hippasa*: Biochemical and pharmacological studies. *Comp. Biochem. Physiol. Part C: Toxicol. Pharmacol.*, 144: 1–9

Nentwig, L., J. Schaller and W. Nentwig, 2004. Biochemistry, toxicology and ecology of the venom of the spider Cupiennius salei (Ctenidae). *Toxicon.*, 43: 543–553

Paul, R.J., A. Pfeffer-Seidl, R. Efinger, H.O. Portner and H. Storz, 1994. Gas transport in the haemolymph of arachnids II. Carbondioxide transport and acidbase balance. *J. Exp. Biol.,* 188: 47–63

Punzo, F., 1989. Composition of the heamolymph of myglomorph spiders (Orthongatha). *Comp. Biochem. Physiol., Part: A: Physiol*., 93: 757–760

Punzo, F., 1990. The heamolymph composition and neurochemistry of the spiderwasp, *Pepsis Formosa* (say) (hymenoptera, pompilidae). *Comp. Biochem. Physiol., Part: A: Physiol.*, 96: 341–345

Tambourgi, D.V., F.C. Magnoli, C.W. Van Den Berg, B.P. Morgan, P.S. Araujo, A.E.W. De, Alves and W.D. Silva, 1998. Sphingomyelinases in the venom of the spider *Loxosceles intermedia* are responsible for both dermonecrosis and complement-dependent hemolysis. *Biochem. Biophys. Res. Commun.*, 251: 366–373

Tillinghast, E.K. and M.A. Townley, 2008. Free amino acids in spider hemolymph. *Comp. Biochem. Physiol, Part B.,* 151: 285–295

Voit, R., G. Feldmaier-Fuchs, T. Schweikardt, H. Decker and T. Burmester, 2000. Complete sequence of the 24-mer hemocyaninof the tarantula *Eurypelma californicum*. *J. Biol. Chem.,* 275: 39339–39344

Yigit, N. and M. Benli, 2008. The antibacterial activity of hemolymph of spider, Agelena labyrinthica (Araneae: Agelenidae). *J. For. Fac.*, 8: 120–124

Yigit, N., T.G. Ven, A. Bayram and K. Avupoulu, 2004. A Morphological Study on the venom apparatus of the spider *Agelena labyrinthica* (Araneae, Agelenidae). *Turkish J. Zool.*, 28: 149–153

(Received 04 January 2010; Accepted 27 March 2010)