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Characterization of naturally occurring agglutinin in the crab *Varuna litterata*

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Abstract
Naturally occurring agglutinin was analyzed in the hemolymph of crab *Varuna litterata* by hemagglutination assay (HA). Hemolymph of *V. litterata* had the capacity to agglutinate a variety of mammalian erythrocytes, rat > rabbit = guinea pig = horse = buffalo = human A = human B > human O > dog > donkey = goat. Among the various tissues tested for HA, maximum HA titer was observed in the hemolymph of *V. litterata* with rat erythrocytes. Agglutinin capable of agglutinating rat erythrocytes was observed in tissues like hemolymph > hepatopancreas > hemocytes = fat body > gills > muscle. Significant variation in HA titer was observed in the hemolymph in relation to sex and body size. Biochemical parameters like hemolymph protein, calcium and water had no significance influence on HA.

Keywords: Lectins, agglutinins, erythrocytes, *Varuna litterata,* hemolymph.

1. Introduction
Hemagglutinins often called “agglutinins” or “lectins” are proteins or glycoproteins that specifically recognize carbohydrate structures [1], capable of agglutinating erythrocytes, bacteria and other normal and malignant cells [2]. Invertebrate lectin seems to participate in innate immune response by inducing bacterial agglutination or activation of phagocytosis through binding to sialic acids on foreign cells [3]. Invertebrate innate immunity relies on both cellular and humoral components. Invertebrate humoral immunity involves the presence of biologically active molecules that occur naturally or that may be induced. These molecules, by their lytic or agglutinating properties are able to act on the antigens responsible for their induction; only in this respect do they resemble vertebrate antibodies. The humoral factors (native, induced) in invertebrates include LPS binding proteins [4, 5, 6], phenoloxidase system [7], antibacterial proteins [8], antifungal proteins [9], lysins [10] and agglutinins. According to Iwanaga and Lee [11], the defense molecules include phenoloxidases, clotting factors, complement factors, protease inhibitors, antimicrobial peptides, toll-free receptors, lectins and other humoral factors found mainly in hemolymph plasma and cell hemocytes. Sialic acids play an important role in certain biological processes including malignancy [12, 13], signaling, apoptosis, ion transport, growth, differentiation and ageing [14]. The sialic acid specific lectins are also useful in distinguishing highly pathogenic strains of bacteria [15]. Among invertebrates hemolymph of arthropods and mollusc contain agglutinins that recognize sialic acid [16]. This study was therefore undertaken to preliminarily characterize the lectins in the serum of *Varuna litterata* as a prerequisite for isolation, purification and pharmacological studies.

Materials and Methods
Animal collection and maintenance
The animals used in this study include an estuarine crab *Varuna litterata* (Fabricius) collected from Manakudy, Kanyakumari District, Tamil Nadu. *Varuna litterata*, the free swimming estuarine crabs were collected from the fishing nets and maintained in plastic tubs with estuarine water. The crabs were fed with fine paddy grains, and the water was changed on alternate days.

Collection of hemolymph
Hemolymph was collected from healthy, uninjured, non-autotomised crabs. The hemolymph of *V. litterata* was collected by cutting the dactylus of third walking leg. The hemolymph was

allowed to bleed directly into the pre-chilled centrifuge tubes placed on ice with moderate shaking. Soon after collection the hemolymph was centrifuged at 1500 rpm for 5 minutes to collect the serum and was stored in eppendorf tubes at -20 °C.

Hemagglutination assay (HA)

Hemagglutination assays were performed in 'U' bottomed microtiter plates (Falcon 3910) (Ravindranath and Paulson, 1987) by two-fold dilution of 25 µl of serum/ whole body extract/tissue extract with an equal volume of TBS. After dilution, 25 µl of erythrocyte suspension (1.5%) was added to each well and incubated for one hour at room temperature. The hemagglutination titers were recorded as the reciprocal of the highest dilution of the test sample causing complete agglutination of tested RBCs.

Separation of hemocytes

The hemocytes were separated using the method of Soderhall and Smith (1983) [17]. The dactylus of the crab was cut and the hemolymph was collected in 1.35 ml of ice-cold (4 °C) anticoagulant buffer (Citrate-EDTA: trisodium citrate (30 mM), citric acid (26 mM), glucose (100 mM) and disodium EDTA (10 mM). The mixture of hemolymph and buffer was shaken gently to assist rapid mixing and centrifuged (200 x g) for 2 minutes at 4°C. The hemocyte pellets were resuspended in 1.5 ml of iso-osmotic buffer (Tris-HCl (50 mM), NaCl (156 mM), and $CaCl_2$ (1 mM) pH 7.5).

Collection of mammalian erythrocytes

Human and other mammalian blood samples were directly collected in modified Alsevier's medium (sodium citrate 30 mM, NaCl 77 mM, glucose 114 mM, neomycin sulfate 100 µg/ml, chloramphenicol 330 µg/ml, pH 6.1). Erythrocytes were obtained by heart puncture (guinea pig, rat) or venipuncture of ear (rabbit), or fore arm (man, dog, cat) or neck (horse, buffalo, cow, donkey) or from slaughterhouse (pig, goat).

Preparation of erythrocyte suspension

For hemagglutination assay, the erythrocytes were washed three times with ten volumes of tris buffered saline (TBS) pH 7.5 (Tris-HCl 50 mM, NaCl 100 mM, $CaCl_2$ 10 mM) at 1500 rpm for 5 minutes and resuspended in the same buffer as 1.5% suspension.

Preparation of tissue extract

The adult, healthy and non-autotomised intermoult male crabs of *V. litterata* were dissected and the tissues were removed. Tissues were then rinsed in cold Tris Buffered Saline (TBS) pH 7.5 to remove the hemolymph. The tissue extracts were prepared by homogenizing 100 mg of tissue in 1 ml of cold Tris Buffered Saline (TBS) pH 7.5 (Tris-HCl 50 mM, NaCl 100 mM, $CaCl_2$ 10 mM). Homogenized extracts were centrifuged and the supernatant was used for HA assay.

Biochemical analysis

Water content

Known quantity of hemolymph was dried in a desiccator. The difference between the wet weight and dry weight gives the amount of water present in the hemolymph [18, 19].

Calcium content

Hemolymph calcium was measured following the procedure of Webster (1962) [20]. Chloranilic acid (2, 5 dichloro, 3, 6 dihydroxy quinine compound L 111) precipitates the hemolymph calcium and forms a calcium chloraniliate complex. The precipitate was dissolved in tetra sodium EDTA which liberates the chloranilic acid which in turn combines with ferric chloride to form a colored complex that was measured at 490 nm in a spectrophotometer. The amount of liberated chloranilic acid is directly proportional to the amount of calcium precipitated.

Protein estimation

The protein concentration was estimated by Folin-Ciocalteu method [21].

Results

Hemagglutinin in crustaceans

The hemolymph of the crab *V. litterata* agglutinated a wide range of erythrocytes like rat > rabbit = guinea pig = horse = buffalo = Human A = B > O > dog > donkey = goat. The highest HA titer was observed in the hemolymph of the crab *V. litterata* with rat RBCs. (Table 1)

Influence of size and sex on HA

HA titer increased along with increase in body weight of both male and female crab *V. litterata*. However male crabs showed higher agglutinating activity than the females of similar body weight (Table 2.Figure.1).

Hemagglutinating activity in the tissues of crab *V. litterata*

In addition to hemolymph, agglutinating activity was also found in hepatopancreas, hemocytes, fat body, gills and muscle. However, agglutinating activity was observed to be very high in the hemolymph (Table.3, Figure 2.).

Biochemical factors and HA activity

Studies on the role of biochemical parameters such as water, protein and calcium content of the hemolymph showed no significant influence on hemagglutinating activity (Table.4).

Discussion

The hemagglutination assay revealed the presence of agglutinin in the hemolymph of crab *V. litterata*. The agglutinins showed differential affinity with different species of erythrocytes. The inability of the hemolymph agglutinin to agglutinate erythrocytes of some mammalian species suggests that these erythrocyte membranes may express different types of cell surface receptors which were not recognized by the agglutinins. The specific preference of the hemolymph of crab *V. litterata* to rat erythrocytes suggests that the receptor determinants of rat erythrocytes are specifically recognized by the hemolymph agglutinin.

Among the various tissues of *V. litterata* screened for HA activity, hemolymph showed remarkably high HA titer with rat erythrocytes. Presence of hemagglutinins in the hemolymph was reported in a number of crustacean species *Homarus americanus* [21], *Macrobrachium rosenbergii* [22], *Cancer antennarius* [23] and *Scylla serrata* [24]. However, agglutinins were also observed in the hepatopancreas, hemocytes, fat body, gills and muscle. The agglutinability of the hemolymph in *V. litterata* as reported in *Emerita emeritus* [25] and *Episesarma tetragonum* [26] depends very much on size and sex. These findings however strongly suggest the natural occurrence of multiple agglutinins in the serum of this crab. It is also interesting to note that serum agglutinated a variety of erythrocytes tested are known to be the model for most frequent opportunistic pathogens of aquatic crustaceans. The

ability of the serum of *V. litterata* to agglutinate the erythrocytes, particularly the rat erythrocytes implicates a possible involvement of the humoral agglutinins in host defense response. It is therefore concluded that hemolymph extract of the crab *V. litterata* exhibits properties indicative of multiple lectins and further studies are recommended to isolate and characterize the individual lectins.

Conclusion

Hemolymph of the crab *V. litterata* showed highest agglutinating activity with rat erythrocytes. Size and sex have an impact on the hemagglutinating activity of the hemolymph of the crab *V. litterata.* Hemolymph showed the highest HA titers, compared to other tissues. Water, calcium and protein content of the hemolymph had no influence on HA.

Table 1: Hemagglutination titer of the hemolymph of *V. litterata* against mammalian erythrocytes

Erythrocytes (N=25)	Hemagglutination titer of *V. litterata*
Rat	128
Rabbit	32
Guinea pig	32
Goat	2
Cow	0
Dog	8
Buffalo	32
Pig	0
Donkey	2
Cat	0
Horse	32
Human A	32
Human B	32
Human O	16

Table 2: Hemagglutination titer of the hemolymph of *V. litterata* in relation to sex and size against rat erythrocytes

Weight (gm) (N=20)	Hemagglutination titer with rat erythrocytes	
	Male	Female
5	32	16
20	64	32
30	64	32
50	128	64
65	128	64

Table 3: Naturally occurring agglutinin in the tissues of *V. litterata* against rat erythrocytes

Tissue (N=10)	HA titer
Hemolymph	128
Hepatopancreas	32
Hemocytes	16
Fat body	16
Gills	8
muscle	4

Foregut, midgut, hindgut and eyestalk showed no hemagglutination titer with rat erythrocytes. N= Number of animals tested

Table 4: Biochemical study on the hemolymph of intermoult adult male crab of *V. litterata*

Characteristics analyzed (N=10)	Hemolymph
Water (%)	91±0.5
Total Protein (mg/ml)	28±0.7
Total Calcium (mM)	10±0.2
HA titer	128

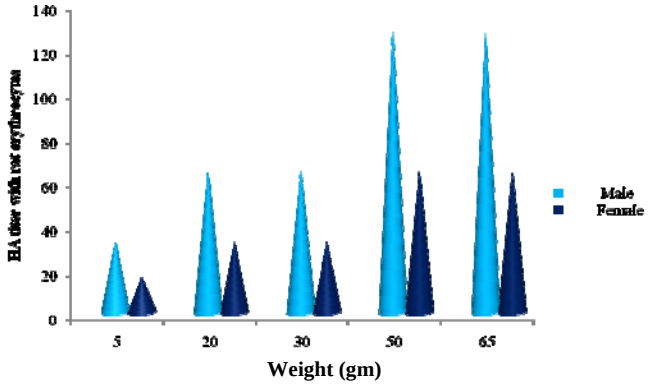

Fig 1: Influence of sex and size on the hemolymph HA titer of *Varuna litterata* against rat erythrocytes

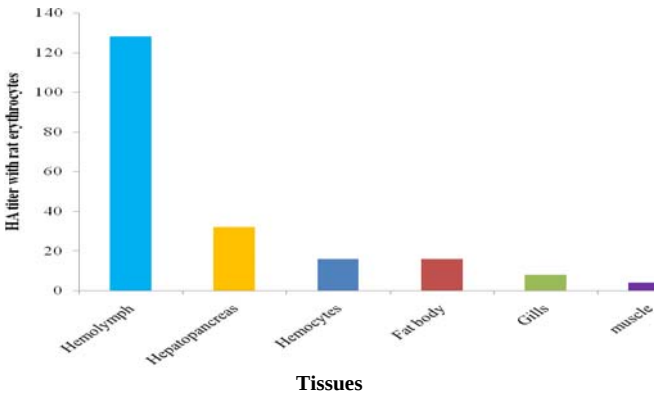

Fig 2: Hemagglutination titer of the tissues of *V. litterata* against rat erythrocytes

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