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An Insight of the Naturally Occurring Agglutinins in a Few Species of Crabs and Characterization of the Agglutinin in the Crab *Travancoriana charu*

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Abstract: ***Three species of brachuran crabs were screened for the presence of agglutinins using mammalian erythrocytes as indicator cells. Hemagglutination assay results showed that the hemolymph of the freshwater crabs Travancoriana charu (Bahir and Yeo, 2007) and Oziotelphusa cf. hippocastanum (Muller, 1887) showed the highest HA titer with dog and buffalo erythrocytes respectively. The hemolymph of the marine crab Menippe rumphii (Fabricius, 1798) agglutinated with great avidity rat, mice and buffalo erythrocytes. Among the various tissues of Travancoriana charu analyzed for the presence of agglutinins, hemagglutination activity was observed in the hemolymph > hepatopancreas > muscles with rat and mice erythrocytes. HA was determined for both male and female crabs of Travancoriana charu ranging in size from 2.5-5 cm (weight 9.1-40 g). Hemagglutinating activity increased with increase in animal size and very significantly in the female crabs. Biochemical factors like water, protein and calcium content of the hemolymph did not have any conspicuous influence on the HA titer.***

Keywords: Agglutinin, lectin, hemagglutination, Travancoriana charu

1. Introduction

As invertebrates, crustaceans lack adaptive immune system and mainly rely on innate immunity to defend against invading pathogens [1]. Hepatopancreas and hemocytes of crustaceans are regarded as the most important tissues involved in crustacean immunity [2, 3]. The body fluid or hemolymph of almost all invertebrate species tested contains agglutinins [4, 5, 6, 7, 8]. Although there are more than 20,000 records of lectins from diverse groups of organisms [9], much remains to be elucidated about their precise physiological and ecological roles [10, 11]. Due to the probable functional similarities between agglutinins and vertebrate antibodies and the indications that agglutinins serve a defensive function [12], invertebrate agglutinins have been extensively studied.

2. Materials and Methods

Three species of crabs, *Travancoriana charu*, *Menippe rumphii* and *Oziotelphusa* cf. *hippocastanum* inhabiting different natural environments were selected for screening of hemagglutinins.

Animal collection and maintenance

The freshwater crab *Travancoriana charu* was collected from the freshwater streams of Ponmudi, Thiruvananthapuram District, Kerala, India and the freshwater field crab *Oziotelphusa* cf. *hippocastanum* was collected from the paddy fields of Kollengode, Palakkad District, Kerala, India. Crabs were maintained in plastic tubs with freshwater. Water was changed on alternate days and the crabs were fed with paddy grains.

The marine crab *Menippe rumphii* was collected from among the rocks adjoining the seashore of Muttom, Kanyakumari District, Tamilnadu, India. They were maintained in plastic tubs with sea water and fed with small fishes. Water was aerated continuously and changed daily.

Hemolymph collection

Hemolymph was collected from uninjured, non-autotomised adult male or female crabs. For larger crabs, after cutting the dactylus, the hemolymph was allowed to bleed directly in centrifuge tubes placed on ice. For smaller crabs the hemolymph was extracted using a sterile 1.0 ml syringe and 22 gauge needles from the hemocoel through the arthrodial membrane at the base of chelipeds and walking legs.

Preparation of tissue extract

Adult healthy crabs were dissected and the tissues were washed twice in cold tris buffered saline (TBS) to remove the adhering hemolymph. After weighing, the tissue extract was prepared by homogenizing 100 mg each of the tissue in 1 ml of cold TBS (Tris Buffered Saline: Tris HCl 50 mM, pH 7.5, NaCl 100 mM, $CaCl_2$ 10 mM) using a homogenizer. The extracts were centrifuged at 4000 x g for 10 minutes at 4ºC and the supernatant was used for hemagglutination activity.

Separatation of hemocytes

The hemocytes from the hemolymph were separated using the method of Soderhall and Smith [13]. The dactylus of the crab was cut and the hemolymph was collected in 1.35 ml of ice cold (4ºC) anticoagulant buffer, Citrate EDTA: (trisodium citrate 30 mM, citric acid 26 mM , NaCl 71 mM, glucose 100 mM and disodium EDTA 10 mM). The mixture of hemolymph and buffer was shaken gently to assist rapid mixing and centrifuged at 200 x g for 2 minutes at 4ºC. The hemocyte pellet was then resuspended in 1.5 ml of iso–osmotic buffer (Tris HCl 50 mM, NaCl 156 mM and $CaCl_2$ 1 mM, pH 7.5) used for HA assay.

Preparation of erythrocytes

Blood from various mammalian species were obtained either by heart puncture (guinea pig, rat, mice) or venipuncture of the ear (rabbit), fore arm (dog), neck (horse), slaughter houses (pig, buffalo, cow, goat, camel) and blood bank (Human A, B, AB, O). Erythrocytes were collected directly in sterile modified Alsevier's medium pH 6.1 (30 mM sodium citrate, 77 mM sodium chloride, 114 mM glucose, 100 mg neomycin sulphate and 330 mg chloramphenicol). Before use the erythrocytes were washed thrice by centrifugation at 1500 x g for 5 minutes and resuspended in TBS pH 7.5 as 1.5% erythrocyte suspension.

Hemagglutination (HA) assay

Hemagglutination assays were performed in 'U' bottom microtiter plates as described by Ravindranath and Paulson [14]. Serum samples or hemocyte suspension or tissue extract (25 µl) were serially diluted with 25 µl of TBS and mixed with 25 µl of 1.5% erythrocyte suspension, and incubated for one hour at room temperature (30 ± 2ºC). The hemagglutination titer or HA titer (the unit of agglutination activity) was considered as the reciprocal of the highest dilution of samples that gave positive agglutination. Positive hemagglutination was obtained when the erythrocytes did not sediment to the bottom of the well forming a red button. The titer was recorded as the highest dilution that still caused agglutination.

Effect of size and sex on hemagglutination assay

To understand the influence of size and sex on the hemagglutination titer, the hemolymph samples collected from the male and female crabs of various sizes and different stages of growth were analyzed for HA.

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 [15, 16].

Calcium content

Hemolymph calcium was measured following the procedure of Webster [17]. Chloranilic acid (0.1 ml) was added to 0.1 ml of hemolymph or 2 ml of ethanolic supernatant or 0.1 ml of calcium standard solution and allowed to stand for atleast one hour at room temperature. The suspension was centrifuged at 900 x g for 10 minutes and the supernatant was decanted. To the precipitate, 5 ml of 50% isopropyl alcohol was added, centrifuged at 900 x g for 5 minutes, and the supernatant was decanted. To the precipitate add 2 drops of 5% EDTA and the precipitate was broken by striking the bottom of the tube forcibly against a rubber stopper and mixed with 5 ml of 6% ferric chloride. After 5 minutes of incubation the absorbency was measured at 490 nm.

Estimation of protein

The protein concentration was estimated by Folin-Ciocalteau method [18]. Ethanolic precipitate of 50 µl of hemolymph was dissolved in 1 N NaOH. To this 5 ml of reagent mixture (50 ml of reagent A: 2 g of Na_2CO_3 in 100 ml of 0.1 N NaOH and 1 ml of reagent B: 500 mg of cupric sulphate solution in 1% sodium potassium tartarate) was added, mixed and incubated for 10 minutes. Then 0.5 ml Folin phenol reagent was added, mixed rapidly and incubated for 30 minutes and absorbency was measured at 500 nm.

3. Results

Hemagglutinating activity of hemolymph

The hemolymph from the studied species showed different hemagglutinating specificities and titer. *Travancoriana charu* showed the highest hemagglutinating activity against dog erythrocytes (HA 256-512), and a titer value of 128 against rat and mice erythrocytes. *Menippe rumphii* showed higher hemagglutinating activity with rat, mice and buffalo erythrocytes (HA 32); *Oziotelphusa* cf. *hippocastanum* hemolymph showed the highest hemagglutination titer (HA 256-512) for buffalo erythrocytes and a titer value of 256 against mice and rabbit erythrocytes. The hemolymph analyzed for HA from the collected species showed low hemagglutinating activity against the other erythrocytes (human A, B, O, cow and goat). The hemolymph of the freshwater crab *Travancoriana charu* had high titer value against dog erythrocytes, the crabs could easily acclimatize to the laboratory conditions and were available all throughout the year, and hence it was used for further studies.

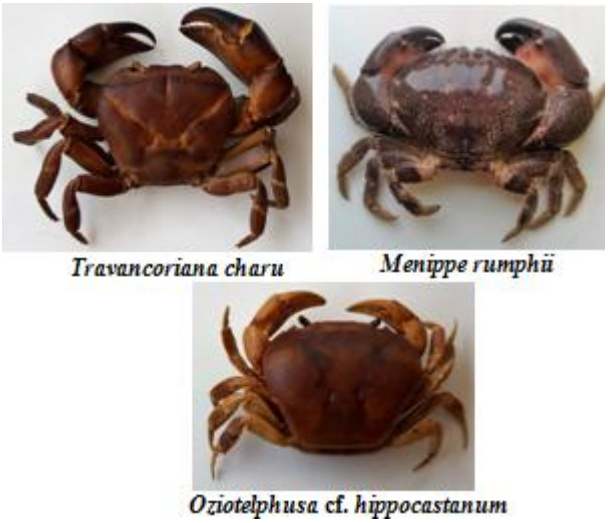

Figure 1: Crabs surveyed for agglutinins

Table 1: Hemagglutination titer of the natural hemagglutinins from the hemolymph of different species of crabs against mammalian erythrocytes

Erythrocytes (n = 25)	Hemagglutination titer		
	Travancoriana charu	*Menippe rumphii*	*Oziotelphusa* cf. *hippocastanum*
Dog	256-512	0	128
Rat	128	32	32
Mice	128	32	256
Rabbit	64	8	256
Buffalo	16	32	256–512
Pig	16	ND	32
Camel	16	8	ND
Human A	8	8	8
Human B	8	8	8
Human AB	8	8	8
Human O	8	8	8
Guinea Pig	16	4	32
Cow	2	ND	2
Goat	2	4	2
Horse	4	ND	32

n = number of animals tested

Hemagglutination titer of the hemolymph from the freshwater crab *Travancoriana charu* in relation to size and sex of the animal

The highest hemagglutination titer of *Travancoriana charu* was shown by male crabs with a carapace width of 4.6–5 cm and weight of 35.1 to 40 g and female crabs with a carapace width of 3.6-4.0 cm and weight 20.1-30 g. Smaller sized crabs with weight < 15 g showed significantly lower affinity towards dog erythrocytes.

Table 2: Hemagglutination titer of the hemolymph of *Travancoriana charu* in relation to sex and size against dog erythrocytes

Carapace width (cm) (n= 5)	Weight (g) (n= 5)	Hemagglutination titer with dog erythrocytes	
		Female	Male
2.5–3.0	9.1-15	16	32
3.1-3.5	15.1-20	256	64
3.6-4.0	20.1-30	512	64
4.1-4.5	30.1-35	256-512	128
4.6-5.0	35.1-40	256 - 512	512

n= number of animals tested

Agglutinins in the tissues of the freshwater crab *Travancoriana charu*

Extract from the hepatopancreas of *Travancoriana charu* agglutinated only rat (HA-64) and mice erythrocytes (HA-32), among the different types of mammalian erythrocytes tested. HA activity was also detected in the extract of muscles.

Table 3: Naturally occurring agglutinins in the tissues of the freshwater crab *Travancoriana charu*

Erythrocytes	Tissues (n=5)	
	Hepatopancreas	Muscles
Rat	64	8
Mice	32	8
Dog	0	0
Rabbit	0	0
Pig	0	0
Horse	0	0
Cow	0	0

n= number of animals tested

Tissues like gills, gut and eye stalk showed very little or no agglutinability with the various mammalian erythrocytes tested.

Hemagglutinating activity of the hemocytes from the hemolymph

The hemocytes from the hemolymph of *Travancoriana charu* agglutinated erythrocytes from several species with low HA titer.

Table 4: Hemagglutination titer of the hemocytes of the freshwater crab *Travancoriana charu* against various mammalian erythrocytes

Erythrocytes (n=5)	HA titer
Dog	32
Mice	16
Rabbit	8
Rat	8
Horse	4
Guinea Pig	2
Buffalo	2

n = number of animals tested

Effect of biochemical factors on hemagglutination titer

Biochemical factors such as water, protein and calcium content of the hemolymph did not influence the hemagglutinating activity of the hemolymph of *Travancoriana charu*.

Table 5: Biochemical analysis of the hemolymph of the freshwater crab *Travancoriana charu*

Characteristics analyzed (n= 10)	Quantity in the hemolymph
Water (%)	83.01 ± 0.43
Protein (mg/ml)	32.16 ± 0.19
Calcium (mM)	20 ± 0.20
HA titer	256-512

n = number of animals tested

4. Discussion

Agglutinins have a putative role in non-self recognition in vertebrate and invertebrate immunity [19, 20, 21]. Agglutinins / lectins are proteins or glycoproteins usually without catalytic activity that have the ability to bind to specific carbohydrates expressed on different cell surfaces. They agglutinate erythrocytes via cell surface glycoproteins and glycolipids [22, 23]. The lectin induced agglutination of cells has originally served as the most common assay to detect and quantify lectin activity in a variety of organisms [24, 25, 26]. In invertebrates, lectin have been detected in hemolymph and coelomic plasma [27, 28, 29].

The hemolymph of the three species of crabs *Travancoriana charu*, *Menippe rumphii* and *Oziotelphusa* cf. *hippocastanum* collected for analysis were found to possess naturally occurring agglutinins with hemagglutinating activity against various erythrocytes types tested, similar to those found in other screening of hemagglutinating / hemolytic activity of invertebrates [30]. The hemolymph of the freshwater crab *Travancoriana charu* was found to contain naturally occurring agglutinin which reacts with dog, rat, mice, rabbit, buffalo, pig as well as several other mammalian erythrocytes types. Similar findings were reported in other crustaceans, *Homorus americanus* [31], *Cancer antennarius* [32], *Scylla serrata* [33], *Macrobracuim rosenbergii* [34], *Liocarcinus depurator* [35], Litopenaeus *setiferus* [36], which suggests that the erythrocytes types agglutinated by the hemolymph of the crab *Travancoriana charu* probably share a common surface receptor, but with a quantitative difference in HA binding sites. The difference in agglutination activity could be attributed to several factors like nature, number, distribution, exposure and mobility of receptors; fluidity and surface charges of the membrane can also affect agglutination [37]. Agglutination of many different types of cells/glycoconjugates may actually reflect the ubiquity of the ligand.

The agglutinatinability of the hemolymph of *Travancoriana charu* depends on size and sex as reported in *Episesarma tetragonum* [38]. Sixteen fold lesser hemagglutination activity was observed in small female crabs compared to the larger crabs. Similar pattern of hemagglutination was reported in the prawn *Macrobrachium rosenbergii* where HA titer was three times lesser in juveniles than in adults [39]. This suggests that the physiological characteristics of the lectin are regulated through maturation.

Changes occur in the hemagglutinating activity during the course of development, or when influenced by the age, sex or size of the animal [40, 41, 42]. The spider crab *Maio squinado*, suffers a spontaneous loss of lectin titer prior to moulting [43]. In the coconut crab *Birgus latro*, the young apparently have no lectin, and the titer increases with size (presumably age) [44]. The lectin concentration and hemagglutinating activity could be influenced, such as in *Pieris brassicae* (insect), crabs and horseshoe crabs where the lectin contents of tissues and body fluids vary during certain development stages [45].

The extracts from the hepatopancreas and muscles of *Travancoriana charu* agglutinated rat and mice erythrocytes. The inefficacy of the agglutinin in the hepatopancreas to agglutinate dog erythrocytes as well as several other erythrocytes agglutinated by the hemolymph agglutinin may be due to the masking of the agglutinin specific receptors of the erythrocytes by certain components of the extract or may be due to the flexibility of the sugar binding requirements that permits the binding of structurally related carboydrates. Occurrence of agglutinins in the hepatopancreas has also been reported in other invertebrates such as *Fenneropenaeus chinensis* [46], *Penaeus monodon* [47], *Fenneropenaeus chinensis* [48]. Hepatopancreas, equivalent to fat body of insects and liver of mammals, was considered the most important tissue synthesizing proteins involved in the immune system of crustaceans [49]. The presence of agglutinins has been reported in the extracts of the gut and salivary glands [50], in the albumin gland of certain gastropod molluscs [51].

Hemocytes play a central role in the immune defense of crustaceans [52, 53]. The most important role of the circulating hemocyte is the protection of the animal against invading microorganisms by participating in recognition, phagocytosis, melanization and cytotoxicity [54, 55]. Lectins with structural characteristics and identical specificity to cell free hemolymph have been identified in the hemocyte membrane and cytoplasmic granules [56]. The hemocytes from the hemolymph of *Travancoriana charu* agglutinated mammalian erythrocytes. Several hemagglutinins have been detected on hemocyte surface [57, 58] suggesting that they act as receptors which bind directly to the surface sugars of foreign particles.

Modifications in serum component levels have been observed in invertebrates under different physiological conditions. Environmental factors such as temperature and salinity may influence hemolymph protein and carbohydrate concentration in crustaceans [59]. The biochemical constituents of the animal are known to vary with season, size of the animal, stage of maturity and availability of food [60, 61]. In the freshwater crab *Travancoriana charu*, water, protein and calcium content did not have any significant influence on the HA titer as observed in the blue shrimp *Penaeus stylirostris* [62].

5. Conclusion

Crustaceans have a unique host defense system differing from vertebrates. Due to the lack of adaptive immunity, the host defense against infection in crustaceans solely depends on innate immune systems. Some of the innate mechanisms in crustaceans are able to recognize specifically surface determinants on pathogens (pathogen associated molecular patterns, PAMPs) through the active participation of lectins. Lectins / hemagglutinins are proteins or glycoproteins, which have at least one non-catalytic domain that exhibits reversible binding to specific monosaccharides or oligosaccharides. Hemagglutinins were present in all the three species of crabs collected for analysis. In the freshwater crab *Travancoriana charu* selected for further studies, the hemagglutination titer of the hemolymph was higher than the other tissues tested. Sex and size influenced the hemagglutination titer. Hemagglutinin was also observed in the hemocytes from the hemolymph. Biochemical factors did not have any influence on the hemagglutination titer.

In invertebrates lectins have been reported to contribute in innate immune responses, including prophenoloxidase activation, enchancement of encapsulation, module formation of hemocytes, opsonisation, antibacterial activity, antifungal activity and injury healing. In crustaceans, a large number of lectins with different molecular weights and functions have been described. Most of these lectins are specific for N-acetylated sugars, sialic acid and their N or O–acetylated derivatives. Lectins that specifically recognize various sialic acids and their carbohydrate binding patterns can be used as a tool for identifying various sialyl epitopes in the field of cancer research and therapy.

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