AGGLUTININ IN THE HEMOLYMPH OF *BARYTELPHUSA CUNICULARIS*

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Abstract

Four different crustacean species collected from four different habitats, showed the presence of agglutinins when tested against various mammalian erythrocytes at varying by heamagglutination assay. Though the hemolymph of all the four species showed a preferential affinity for rabbit erythrocytes, maximum HA titer was observed in the hemolymph of the freshwater crab, *Barytelphusa cunicularis.* The hemagglutinating activity of *Barytelphusa cunicularis* was unaffected by sex and was greatly influenced by the size of the crabs. Bio-chemical factors such as water, calcium and protein content of the hemolymph of *Barytelphusacunicularis* had no effect on HA titer. Among the various tissues of *Barytelphusa cunicularis* analyzed for HA, high agglutinability was found in the hemolymph. The most potent inhibitor of haemagglutination proved to be bovine submaxillary mucin.

Key words: *Barytelphusa cunicularis,* agglutinin, hemolymph, hemagglutination.

Introduction

Studies on crustacean lectins commence with the discovery of erythrocyte agglutinating factor in the hemolymph of *Homarus americanus* (1). Lectins / agglutinins are a group of proteins that recognize a specific carbohydrate structure and agglutinate various cells by binding to cell surface glycol-conjugate (2). They are responsible for surface recognition and they aid in the attachment of microorganisms and other types of antigens to phagocytes and other host cells (3). While a monoclonal antibody can recognize three to six sugars an a glycocojugate, a lectin may recognize a part of sugar such as acetyl groups, N or O - linked glycerol side chain of salic acids (4) or whole sugar like N-acetyl neuraminic acid (5) or their glycosidic linkages (gal-GIc; NeuAc α 2, 3 gal; NeuAc α 2,6 gal; NeuAc α2-8 NeuAc) (6) or a sequence of sugars (7). It is this specificity that makes lectin as potential agents to study expression of cell surface glycoconjugates in normal and pathological mammalian tissues, including those in different kinds of human cancers (8). The most preferred ligand of the arthropod lectins are the family of sugars called sialic acids, which encompass more than 18 members (9). Sialic acids play an important in certain biological processes including malignancy (10). Lectins that recognize the linkages or modification of sialic acid are therefore indispensable as reagents in biochemical research and diagnostic analysis. Identification and purification of potent and novel lectins expand the scope of lectins as the diagnostic tool.

Materials and methods

The crustacean representatives namely *Artemia franciscana, Macrobrachium lamarrei, Oniscus asellus* and *Barytelphusa cunicularis* inhabiting four different habitats such as saltpan, freshwater ponds,

decaying woods and freshwater mountain streams respectively were used for the survey of agglutinins.

Collection and maintenance of the experimental animals

Artemia franciscana cysts, purchased from San Francisco Bay Brand INC., California. USA were rinsed with tapwater and incubated in 1 liter transparent cylinder at a concentration of 1.5 g/1 of seawater (35ppt). Hatching temperature was maintained at 28±1°C. The pH was adjusted to 8.0 throughout the hatching period and the tank was provided with strong aeration. Light was provided by placing a fluorescent lamp near the hatching cylinder. After 25 hours of incubation the naupli hatched. They were fed with fine grains of rice bran and dried algae.

Macrobrachium lamarrei were collected from the ponds and were reared in 45 cm x 75 cm tanks, with 6 cm depth of pond water with artificial aeration. These prawns were fed with boiled leaves, organic detritus and kept at room temperature (30^0C). Matured prawns of about 2.5 - 5.0 cm length were used for experiments.

Oniscus asellus, which resides beneath the stone, wood and bark were carefully collected by gently lifting the stone / bark / wood and were maintained in the laboratory in decaying plant material. The humidity was maintained throughout the experimental period by occasional sprinkling of water.

Barylelphysa cunicularis were obtained from the streams of mountain areas and were reared in cement tanks. They were fed with small fishes and bits of animal flesh. Animals of either sex with a carapace width of 4.5 x 6.5 cm were used throughout this investigation.

Collection of hemolymph

Hemolymph (0.1 ml) from adult *Macrobrachium lamarrei* was collected from each animal by inserting a 22 - gauge needle into the pericardial sinus. The collected hemolymph was poured in plastic tubes kept on ice and the serum was cleared by centrifugation and stored at – 20^0C until use. The hamolymph was collected and stored (12).

Preparation of body extract

The whole body extracts of *Oniscus* and *Artemia* were prepared by homogenizing 100 mg of the sample in 1 ml of cold TBS (tris buffered saline. Tris HCl pH 7.2 (50 mM), NaCl (100 mM), $CaCl_2$ (10 mM). The suspension was centrifuged at 4000 x g for 1 min. at 4^0C and supernatant was tested for HA activity.

To prepare the tissue extract of *B. cunicularis,* adult healthy specimens were dissected and the tissues were weighed and rinsed twice in cold Tris buffered saline (TBS) to remove the adhering

hemolymph. A portion of washed tissue (0.1 g) was placed in 1 ml of the buffer minced with scissors and then homogenized by a hand homogenizer, in 1 ml of cold TBS (TBS-HC1 50 mM; NaCl 100 mM; $CaCl_2$ 10 mM; pH 7.5). The homogenate was certrifuged and the supernatant was taken for experiments.

Erythrocyte preparation

Various mammalian erythrocytes were collected and 1.5% suspension was prepared and used for HA assay (12).

Hemagglutination Assay

Hemagglutination assays were performed as recommended for the crab hemolymph lectin (11). To study the effect of pH and temperature on hemagglutinting activity, hemolymph was incubated at specific pH and temperature for an hour before adding erythrocyte suspension.

Hemagglutination inhibition

The glycoproteins (5mg/ml) and Sugars (100 mM), were reconstituted in TBS, serially diluted in microtiter plates and mixed with the hemolymph previously adjusted to 2 HA units. After 60 minutes of incubation at room temperature (30-32^0 C), 25 µl of 1.5% suspension of rabbit erythrocytes were added to each microtiter well and mixed. The values of HAI titer were determined after 60 minutes of incubation and expressed as the highest dilution of glycoprotein that inhibited the agglutination of erythrocytes

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 (I2) (13).

Calcium content

Hemolymph calcium was measured following the procedure of Webster (14).

Protein Estimation

The protein concentration was estimated by Folin-Ciocalteu method (15).

Results

Agglutinins in the crustacean species surveyed

Hemagglutinins were observed in the whole body extract of *Oniscus asellus* and *Artemia franciscana* and the hemolymph of *Macrobrachium lamarreii* and *Barylelphusa cunicularis.* It was

interesting to note that among the fifteen types of erythrocytes tested for HA, all the four crustacean species showed strong affinity for rabbit erythrocytes (Table 1). However, the HA activity in the hemolymph of *Barytelphusa cunicularis* was comparatively higher than the other crustacean species tested. Hence the hemolymph of *Barytelphusa cunicularis* was chosen for further investigations.

Table 1 : Natural agglutinin in four different crustaceans

Erythrocytes	*Macrobrachium lamarrei*	*Barytelphusa cucicularis*	*Oniscus asellus*	*Artemia franciscana*
Rabbit	64	256	64	16
Rat	0	16	2	8
Horse	0	8	0	0
Dog	0	8	0	0
Mice	2	4	0	0
Pig	0	4	0	0
Guinea Pig	0	2	ND	ND
Cow	0	4	0	0
Goat	0	2	0	0
Sheep	0	4	ND	0
Buffalo	0	2	0	0
Human A	0	2	2	0
Human B	0	2	2	0
Human 0	0	2	2	0
Donkey	0	2	0	0

Distribution of hemagglutinins in *B. cunicularis*

A survey of agglutinins in the various tissues of the crab *B. cunicularis* revealed the presence of maximum activity was also observed in the hemolymph > hepatopancreas. Negligible quantity of agglutinins was observed in hemocytes, faeces, heart, mantle, intestine and muscle and no HA activity was detected in the extract of gills and ovary (Table 2).

Table 2 : Distribution of agglutinin in various tissues of the freshwater crab *B.cunicularis*

Tissues n=15	HA titer
Hemolymph	256

Hepatopancreas	128
Hemocytes	8
Faeces	4
Heart	2
Intestine	2
Mantle	2
Muscle	2
Gills	0
Ovary	0

n= number of animals tested

Table 3: Hemagglutination titer of the hemolymph of the freshwater crab, *Barytelphusa cunicularis* in relation to changes in pH

pH 6.0 to 8.0 was the optimum pH for the activity of hemolymph of the freshwater crab, *Barytelphusa cunicularis.*

pH (n=20)	**HA titer**
4.5	128
5.0	128
5.5	128
6.0	128
6.5	256
7.0	256
7.5	256
8.0	256
8.5	128
9.0	128
9.5	64

n= number of animals tested

Table 4: Hemagglutination titer of the hemolymph of the freshwater crab, *Barytelphusa cunicularis* in relation to changes in temperature

Temperature 30-40^0 C was the optimum temperature for the activity of hemolymph of the freshwater crab, *Barytelphusa cunicularis*.

Temperature (^{0} C) (n=20)	HA titer
10	16
15	16
20	32
25	128
30	256
35	256
40	256
45	128
50	128
55	16

n= number of animals tested

Effect of biological and biochemical factors on HA titer of the hemolymph of the freshwater crab *B. cunicularis*

Though hemagglutinability of haemolymph agglutinin was unaffected by sex of the animals, there was a significant increase in HA activity with increase in size of both male and female crabs (Table 5).

Table 5 : Effect of sex and size on the HA titer of the hemolymph of the intermoult freshwater crab *B.cunicularis*

Size (Carapace)	HA titer	
	Male n (n= 5)	Female (n = 5)
Small 2x2 cm	64	64
Medium 6.5x4.5 cm	256	256
Big 7.5x5cm	2048	2048

n = number of animals tested

The biochemical parameters such as water, calcium and protein content of hemolymph had no influence on HA activity of humoral agglutinin of the freshwater crab, *B.cunicularis* (Table 4).

Table 6 : Bio-chemical study of the hemolymph of the freshwater crab, *B. cunicularis*

Chemical parameters (n =10)	Hemolymph	HA titer
Water (mg/ml)	89.3	256
Calcium (mM/l)	14.8	256
Protein (mg/ml)	41.5	256

n = number of animals tested

Hemagglutination inhibition of the hemolymph of the freshwater crab, *Barytelphusa cunicularis* by glycoproteins

The agglutinating activity against rabbit erythrocytes was inhibited by glycoproteins BSM > α – acid glycoprotein > lactoferrin = fetuin > PSM, and sugars GalNAc= GluNAc = ManNAc> lactose> galactose> fructose> glucose = sucrose. (Table 7 and 8).

Table. 7: Hemagglutination inhibition assay of the hemolymph of a freshwater crab, *Barytelphusa cunicularis* by glycoproteins

Sialoglycoproteins (n =10)	Nature of sialic acid	HAI Titer
BSM	NeuAc/NeuGc	64
α-acid glycoprotein	NeuAc	16
Lactoferrin	NeuAc	8
Fetuin	NeuGc	8
PSM	NeuGc	2
Bovine thyroglobulin	NeuGc	0
Porcine thyroglobulin	NeuGc	0
Apotransferrin	NeuGc	0
Holotransferrin	NeuGc	0

n = number of animals tested

Table. 8: Hemagglutination inhibition of the hemolymph lectin of the freshwater crab, *Barytelphusa cunicularis* by sugars

Sugars (n =10)	HAI	Minimal conc. Required for inhibition mM	Relative inhibitory potency %
GalNAc	16	6.25	100
GlcNAc	16	6.25	100

ManNAc	16	6.25	100
Lactose	8	12.5	50
Galactose	4	25	25
Fructose	4	25	25
Glucose	2	50	12.5
Sucrose	2	50	12.5

n = number of animals tested

Discussion

In the present investigation, naturally occurring agglutinins are identified in four different crustacean species *Oniscus ascellus, Macrobrachium lamarrei, Artemia franciscana* and *Barytelphusa cunicularis* by way of hemagglutination assay. The agglutinins agglutinated a wide array of erythrocytes with specific affinity for rabbit erythrocytes. Numerous reports reveal the presence of naturally occurring agglutinins with a wide range of mammalian erythrocyte specificity (16).

Presence of high agglutinating activity in the hemolymph can be attributed to its physiological function. Hemolymph, being the circulating fluid of the crab may be involved in the defence strategy of the organism as it was stated that though the biological role of the agglutinin was not well known, they participate in the self defence mechanism of the organism (17). So also there are reports suggesting that the hemolymph in association with the hemocytes perform an essential role in immune defence and wound healing in insects and other invertebrates. It would appear that both cellular and humoral elements are involved in the reaction to non-self and that they combine to elicit a full range of defence reaction (18).

Analysis of the various tissues of *B. cunicularis* revealed the presence of high concentration of agglutinins in the hemolymph and hepatopanceras and negligible quantity in hemocytes feces, heart, mantle and muscles. Presence of agglutinin in the hepatopanceras of crab suggested that it may be the site of synthesis of storage of these agglutinins. Agglutinin is present in various tissues like hemolymph (19), hemocytes (20) and hepatopancreas (21) were also reported in other crustacean species.

Though reports on the hemolymph lectin of *Emerita emeritus* (22) and millipede *T. descriptus* (23) suggested the existence of variation in hemagglutinability based on sex/stages of maturity of the female, the present study suggested that sex had no influence on humoural agglutinin activity of the freshwater crab *B. cunicularis.* However, agglutinability increase with increase in the carapace size of the organism as it was reported in *Emerita emeritus* (22) and *Macrobrachium rosenbergii* (24). As the animal grew in size they may be exposed to more and more pathogens and the crab had to defend itself by way of producing large quantities of agglutinins, the defence molecule.

The ability to agglutinate different types of erythrocytes with varying HA titer argues for the specific recognition of the sugars constituting the glycocalyx of these erythrocytes which serve as

receptors to ligands as in the eukaryotic cells (25). The unique affinity for the rabbit erythrocytes suggested that the receptor determinants preferentially recognized by the agglutinins were either abundant or more accessible on rabbit erythrocytes, rather than in any other erythrocyte type tested. The receptor found on the glycocalyx of the rabbit erythrocytes were reported to be NeuAc, 9-O-acetyl NeuAc. NeuGc and 9-O-acetyl NeuGc (26). A NeuGc specific lectin with specific affinity for rabbit erythrocytes was purified from the hemolymph of marine crab *Scylla serrata* (20).

The presence of naturally occurring agglutinins in the hemolymph of several crustaceans has been well known since the beginning of the 20th centuary (Cantacuzene, 1912). An evaluation of the literature revealed that purification of lectin from the hemolymph of crustaceans was most successful by affinity chromatograph as it give a higher fold of purification and percentage of recovery (Mercy and Ravindranath, 1993; Ravindranath and Cooper, 1984; Ratanapo and Chulavatnatol, 1990; Frakiadiakis Stratakis 1997). BSM contains N-acetyl neuraminic acid and N-glycolyl neuraminic acid, N-acetyl 90% N-glycolyl neuraminic acid, 10% NeuAc, and traces of N-acetyl O-acetyl acid (Schoop and Faillard, 1967) showed weak inhibitory potency. Moreover, free NeuAc could inhibit haemagglutionation but NeuGc had no inhibitory potency of the NeuAc containing glycoprotein, BSM. Fetuin, PSM, Bovine and porcine thyroglobulin Holo and Apotansferrin has more NeuGc and relatively . So the inhibitory potency is very weak when compared with BSM. The strong inhibitory potency of BSM may be was due to NeuAc.

Based on the affinity of the hemolymph agglutinin of *B.cunicularis* to rabbit erythrocytes, we felt that the hemolymph agglutinin may have specific binding receptor for N-acetyl neuraminic acid or N-Glycolyl neuraminic acid or 9-O-acetyl neuraminic acid which was abundantly present in rabbit erythrocytes. However, this can be confirmed only after purification and subsequent characterization.

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