Characterization of the Agglutinin in the Hepatopancreas of the Freshwater Crab *Travancoriana Charu*

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Abstract- Hemagglutination assay of the extract from the hepatopancreas of the freshwater crab *Travancoriana charu*, using diverse mammalian erythrocytes revealed the presence of an agglutinin with specific affinity for rat > mice erythrocytes. Hemagglutinability was dependent on divalent cations and highly sensitive to the changes in the pH and temperature of the medium. Maximum agglutination was observed at pH 9.5 and temperature of 30ºC with the inclusion of 1-5 mM calcium or 1-10 mM manganese. HAI assay indicated the unique specificity of the agglutinin from the hepatopancreas to lactoferrin, a single subunit N-glycoprotein with Gal(α1-3) Gal(β1-4) GlcNAc (αGal), GalNAc(β1-4) GlcNAc (LacdiNAc) epitopes and N-glycolyl neuraminic acid (Neu5Gc).

Index Terms- Lectin, hemgglutination, hepatopancreas, agglutinin, *Travancoriana charu.*

I. Introduction

In Invertebrates, the physical barriers are the first obstacle to detain pathogenic micro-organisms [1]. Arthropods in general use a wide range of cellular and humoral defense mechanisms to protect themselves from disease agents that manage to gain access to their internal tissues by penetrating the exoskeleton/cuticle or alimentary canal. The hemocytes and hepatopancreas are crucial for the immune system in crustaceans as it is the main production site for immune recognition molecules, initates the humoral reaction, and takes part in the cellular reaction by the specialized cells and phagocytes [2]. In crustaceans, phagocytes can be found free in the hemocoel or on the surface of the hepatopancreas, and/or in the gills [3, 4]. The cells principally involved are the circulating and sessile body cells (termed hemocytes) and various other cell types, including those in the hepatopancreas and gills in crustaceans. Hepatopancreas is considered an important immune-related tissue in crustaceans and the place where most CTLs (C-type lectins) are generated [5]. The hepatopancreas is morphologically similar in most decapods [6, 7, 8].

The midintestinal glands (often called the "hepatopancreas") in molluscs and crustaceans are important source of molecules of the innate immunity [9, 10, 11, 12, 13, 14]. These molecules include hemocyanin, various lectins, ferritins, serine protease inhibitors, proteolytic enzymes, bacterial cell surface recognition molecules and nitric oxide [15, 16]. The midintestinal glands are the major digestive glands which are paired, tubular and associated with the midintestine of bivalves, gastropods and crustaceans. They are thus a multifunctional organ, with primary roles in nutrient uptake and intermediary carbon and nitrogen metabolism [17, 18] and also a source of molecules required for pathogen defense. The midintestinal gland of bivalve and crustacean species synthesize lectins such as C-type lectins and ficolins in response to infection [19, 20, 21]. The hemocoel sinusoids of the midintestinal gland allow the release and rapid distribution of the immune molecules that actively phagocytose cells and are also capable of removing foreign particles such as cellular pathogens from the hemolymph [22].

II. Materials and Methods

2.1 Animal collection and maintenance

The freshwater crab *Travancoriana charu* was collected from the freshwater streams of Ponmudi, Thiruvananthapuram 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.

2.2 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.

2.3 Preparation of erythrocytes

Blood from various mammalian species were obtained either by heart puncture (rat, mice) or venipuncture of the ear (rabbit), fore arm (dog) and blood bank (Human A, B, 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.

2.4 Hemagglutination assay (HA)

The hemagglutination assay was performed in U-bottom microtiter plates. The tissue extract of the hepatopancreas (25 µl) was added to equal volume of TBS and serially diluted. To this 25 µl of 1.5% of various mammalian erythrocyte suspension was added and mixed well. The last well in the microtiter plate with TBS alone and no tissue extract was treated as the control. The ability of the agglutinin to agglutinate erythrocytes at its highest dilution was considered as the positive result. A positive result was indicated by the formation of a uniform net like layer over the surface of the mixture. Formation of a discrete button at the bottom of the well indicated negative results.

2.5 pH and thermal stability

The stability of HA activity at different pH was determined by serially diluting 25 µl of the extract of the hepatopancreas with TBS of varying pH (5 to 10). After 1 hour incubation at room temperature (30±2ºC), 1.5% rat erythrocytes suspension was added and mixed well. To study the effect of temperature on hemagglutination activity, the extract of the hepatopancreas (25 µl), after incubation at various temperatures (0ºC to 100ºC) for 1 hour was serially diluted with TBS and then assayed for hemagglutination activity. HA titer value was determined as the reciprocal of the highest dilution of the extract giving complete agglutination.

2.6 Effect of cations and chelators

25 µl of the extract of the hepatopancreas was serially diluted with 25 µl of the divalent cations Ca^{2+}, Mg^{2+} or Mn^{2+} of varying concentration of 0 mM to 100 mM. After 1 hour incubation at room temperature, HA was determined using 1.5% rat erythrocyte suspension.

Disodium/tetrasodium EDTA and trisodium citrate in TBS were prepared. 25 µl of each of the EDTA/trisodium citrate in different concentrations from 0 mM to 100 mM were serially diluted with 25 µl of the extract of the hepatopancreas and incubated for 1 hour at room temperature. HA was checked after 1 hour incubation using 1.5% rat erythrocyte suspension.

2.7 Cross adsorption assay

Cross adsorption assay was carried out as described by Hall and Rowlands [23, 24] and Mercy and Ravindranath [25]. Extract from the hepatopancreas (1 ml) was incubated with 1ml of washed and packed erythrocytes of rat/mice. The mixture was incubated at 10°C over night (18 hours) with gentle occasional shaking. After centrifugation at 4000 x g for 5 minutes, the supernatant was used for hemagglutination against the selected erythrocytes. HA was observed after 60 minutes of incubation at room temperature.

2.8 Hemagglutination inhibition (HAI) assay

Glycoproteins and sugars were tested for their ability to inhibit hemagglutination. 25 µl of the various inhibitors were serially diluted with 25 µl of TBS. To this the extract of the hepatopancreas at sub-agglutination level (diluted tissue extract which gave a HA of 2 wells) was added. After incubation for 1 hour at room temperature, HAI was determined using 1.5% rat erythrocyte suspension.

III. Results

3.1 HA assay

Among the various erythrocytes tested, extract from the hepatopancreas of *Travancoriana charu* agglutinated only rat (HA=64) and mice (HA=32) erythrocytes.

Table 1: Hemagglutination titer of the extract from the hepatopancreas of *Travancoriana charu*

Erythrocytes (n = 25)	**HA titer**
Rat	**64**
Mice	32
Dog	0
Rabbit	0
Human A	0
Human B	0
Human O	0

n= number of animals tested

3.2 pH and thermal Stability

The extract of the hepatopancreas of *Travancoriana charu* showed maximum hemagglutination titer at the pH 9.5. The activity gradually increased from pH 5.5 and decreased beyond pH 10. The HA titer was highly thermostable as it retained high activity for 1 hour up to 60ºC, the activity decreased beyond 60ºC markedly. However maximum activity was observed at 30ºC

Table 2: Hemagglutination titer of the extract from the hepatopancreas of *Travancoriana charu* in relation to different pH and temperature using rat erythrocytes

pH (n=10)	**HA titer**	**Temperature (ºC) (n=5)**	**HA titer**
5.0	32	0	128
5.5	32	10	128
6.0	64	20	256
6.5	64	30	512
7.0	64	40	256
7.5	64	50	256
8.0	128	60	128
8.5	128	70	32
9.0	256	80	32
9.5	512	90	4
10.0	128	100	4

n=number of animals tested.

3.3 Effect of cations and chelators

The HA titer of the extract from the hepatopancreas of *Travancoriana charu* showed that hemagglutination was dependent on divalent cations. Maximum activity was observed when treated with TBS containing 1 mM and 5 mM concentration of $Ca^{2+}$and 1-10 mM Mn^{2+}. The chelators di- and tetrasodium EDTA and trisodium citrate showed an increase in HA titer with increase in concentrations up to 10 mM. However when tested with 50 and 100 mM EDTA (di- and tetra sodium) there was a marked decline in HA activity.

Table 3: Effect of cations (Ca^{2+}, Mg^{2+}, Mn^{2+}) on the hemagglutinating activity of the extract from the hepatopancreas of the freshwater crab *Travancoriana charu*

Concentration of cations (mM) (n=10)	HA titer		
	Ca^{2+}	Mg^{2+}	Mn^{2+}
0	128	128	128
0.01	128	128	128
0.1	256	128	256
1	512	256	512
5	512	256	512
10	128	64	512
50	32	32	8
100	8	16	8

n=number of animals tested

Table 4: Effect of chelators (Ca^{2+}, Mg^{2+}, Mn^{2+}) on the hemagglutinating activity of the extract from the hepatopancreas of the freshwater crab *Travancoriana charu*

Concentration of chelators (mM) (n=10)	HA titer		
	Disodium EDTA	Tetra sodium EDTA	Trisodium citrate
0	128	128	128
0.01	128	256	128
0.1	256	256	128
1	512	512	128
5	512	512	512
10	1024	1024	512
50	64	8	128
100	8	8	128

n=number of animals tested

3.4 Hemagglutination inhibition (HAI)

Among the glycoproteins tested, lactoferrin (HAI=128) was the most potent inhibitor. Fetuin and apotransferrin (HAI=2) were able to inhibit the hemagglutinatin activity very feebly. The hexose sugar glucose-6-phosphate (50 mM) was the only sugar inhibitor of the extract from the hepatopancreas.

Table 5: Hemagglutination inhibition of the agglutinin from the extract of the hepatopancreas of *Travancoriana charu* by various glycoproteins

Glycoroteins (n=5)	HAI titer	Minimum concentration for inhibition (µg/ml)	Relative inhibitory potency (%)
Lactoferrin	128	39.06	100
Fetuin	2	2500	1.56
Apotransferrin	2	2500	1.56
BSM	0	0	0

n=number of animals tested

Table 6: Hemagglutination inhibition of the agglutinin from the extract of the hepatopancreas of *Travancoriana charu* by various sugars

Sugars (n=5)	HAI titer	Minimum concentration for inhibition (mM)	Relative inhibitory potency (%)
Glucose-6-phosphate	2	50	100
N-acetyl-D-glucosamine	0	0	0
Trehalose	0	0	0
D-galactosamine	0	0	0
L-fucose	0	0	0
Galactose	0	0	0
N-glycolyl neuraminic acid	0	0	0
N-acetyl neuraminic acid	0	0	0

n=number of animals tested

3.5 Cross adsorption assay

Cross adsorption assay revealed the presence of a single agglutinin in the extract from the hepatopancreas as HA activity was completely lost after the fourth round of adsorption.

Table 7: Hemagglutination titer of the extract from the hepatopancreas of the freshwater crab *Travancoriana charu* after adsorption with different erythrocytes

Erythrocytes absorbed (n=5)	HA titer	
	Rat	Mice
None	256	32
Rat	64(32)(2)(0)	16(0)
Mice	16(0)	0

Values in the parenthesis refer to HA titer after successive adsorption

IV. Discussion

Invertebrates lack adaptive immune systems, but they have developed various defense systems of innate immunity that recognize antigens on the surface of potential pathogens, including a set of humoral and cellular immune reactions [26]. Some of the innate immune mechanisms in crustaceans are able to recognize specifically surface determinants on pathogens (pathogen-associated molecular patterns, PAMPs) [27] through the active production of lectins. Lectins have the property of recognizing specifically the carbohydrates from the membrane or surface of cells and consequently, are able to induce agglutination of these cells or can lead to diverse cellular events such as phagocytosis, acting as opsonins [28, 29]. Diverse crustacean C-type lectins with structural and functional variations are mainly expressed in the hepatopancreas and constitute a pathogen-recognition network against invading bacteria and viruses [30]. The ability of the tissue extract from the hepatopancreas of *Travancoriana charu* to specifically agglutinate rat erythrocytes indicates the presence of hemagglutinin. The receptor component of rat erythrocytes is NeuGc/NeuAc, O-AcSia [31].

The hemagglutinating agglutinin in the hepatopancreas of *Travancoriana charu* was heat sensitive and required divalent cations for activity, properties commonly observed for invertebrate lectins [32]. Most of the lectins reported in decapods are known to be dependent upon divalent cations, usually calcium, and they are sensitive to divalent chelators in a reversible or irreversible manner [33, 34]. Calcium dependent (C-type) lectins, which contain a characteristic carbohydrate recognition domain (CRD) with two or three pairs of disulphide bonds, mediate pathogen recognition and play an important role in the clearance of pathogens in innate immunity [35, 36, 37, 38]. Calcium is not only directly involved in the carbohydrate binding itself at the binding site [39] but contributes to the structural maintenance of the lectin domain that is essential for the lectin activity [40]. The crustacean hepatopancreas and hemocytes are the main source of many L-type lectins [41, 42] and C-type lectins [43]. C-type lectins are the most diverse and best studied among the lectin family. A C-type lectin in *Litopenaeus vannamei* with two CRDs (LvCTL) is expressed, only in the hepatopancreas [44]. In *Fenneropenaeus chinensis*, two C-type lectins (Fclectin (FcLec-1) and Fc-hsL (FcLec-2)) have been reported [45, 46]. FcLec-1 contains dual CRDs and is expressed in hemocytes, while Fc-hsL contains only one CRD and is expressed specially in the hepatopancreas. LvLectin-1 and LvLectin-2 mRNA transcripts of the shrimp *Litopenaeus vannamei* were mostly detected in hepatopancreas, and the result was consistent with the other C-type lectins such as MjLecA, MjLecB, MjLecC in *Marsupenaeus japonicas* [47], Fc-hsL (*Fenneropenaeus chinensis*), PmLT (*Penaeus monodon*), LvLT (*Litopenaeus vannamei*), PmAV (*Penaeus monodon)* [48, 49, 50, 51, 52]. The C-type lectins from the mud crab *Scylla paramamosain* SpLec1 and SpLec2 mRNA in the megalope stage was first increased and was expressed mainly in hemocytes, muscle and hepatopancreas, with the highest expression level found in hepatopancreas [53].

Concerning carbohydrate specificity, invertebrate lectins vary from specific molecules to others that exhibit a broader spectrum of recognition. The most potent inhibitor of the agglutinin from the hepatopancreas was lactoferrin, a member of the transferrin family. Lactoferrin is a sialylated N-glycosylated linked glycan [54]. In N-glycans, the linkage of a glycan to the protein is always formed by a GlcNAc-residue linked to an aspargine within the consensus sequence Asn-Xxx-Ser/Thr. Lactoferrin (LF) is an 80 kDa single subunit N-glycoprotein with multiple functions and is involved in many processes such as activation of the immune system [55, 56] inhibitory effects on tumourigenesis [57] and anti-metastatic activity [58, 59]. Lactoferrin complex-type N-glycans has Gal(α1-3)Gal(β1-4)GlcNAc(αGal),GalNAc(β1-4)GlcNAc(LacdiNAc) epitopes and N-Glycolyl neuraminic acid (Neu5Gc). Sialic acid-containing glycoproteins (eg, fetuin, human chorionic gonadotropin, α1-acid glycoprotein, serotransferrin, lactoferrin, fibrinogen etc.) and gangliosides have been found to be good ligands of sialic acid-binding proteins [60, 61, 62]. Sialic acid-binding lectins histochemically and cytochemically localize the sialo-glycoconguates in various cells and tissues. They can be used as specific probes to investigate the role of cell surface carbohydrates during development, differentiation and malignant transformation of cells, as indicated for other types of sugar receptors [63, 64, 65].

Repeated adsorption of the extract from the hepatopancreas with the agglutinating erythrocytes entirely removed the agglutinability, suggesting the presence of a single agglutinin. The simple sugar glucose-6-phosphate (50 mM) was capable of hemagglutination inhibition. Several functions have been attributed to the lectins detected in the hepatopancreas of crustaceans. In *Penaeus monodon*, PmLT distributes only in hepatopancreas and can enhance hemocyte encapsulation [66]. PmLec was purified and serves as an opsonin while PmAV is involved in virus resistanse [67, 68]. In the shrimp *Litopenaeus vannamei*, LvCTL-1 has direct activity against white spot syndrome virus (WSSV) and can prolong the survival of the WSSV-infected shrimps [69]. LvLec in *Litopenaeus vannamei* with one CRD can agglutinate Gram negative bacteria [70]. The C-type lectin, Fc-hsL in the shrimp *Fenneropenaeus chinensis* exhibits antimicrobial activity [71]. FcLec2 and FcLec3 were

constitutively expressed in the hepatopancreas of normal shrimp and were highly up-regulated following challenge with either bacteria or WSSV 72, 73, 74]. The C-type lectin, EsLecF in the Chinese mitten crab *Eriocheir sinensis* is involved in antimicrobial response [75]. The presence of agglutinin in the hepatopancreas of *Travancoriana charu* suggests that it may take part in the defense mechanism of this animal, which can be ascertained only after further tests. Independent of the organs where the agglutinins are expressed in crustaceans, these proteins seem to be constitutive and inducible active-phase proteins that might be involved in immune reponses to microbial infections and in virus resistance by inducing agglutination and opsonisation process.

V. Conclusion

Agglutinins/lectins are proteins or glycoproteins that have the ability to bind to specific carbohydrates expressed on different cell surfaces. Invertebrate immune system must rely on non-self-recognition molecules to ensure efficient defence responses against infectious pathogens that continuously threaten their survival. An agglutinin with specificity for N-glycolyl neuraminic acid was isolated from the hepatopancreas of the freshwater crab *Travancoriana charu*. The agglutinin was specific for glucose-6-phosphate, calcium dependent and sensitive to EDTA. The erythrocytes (rat, mice) agglutinated by the extract of the hepatopancreas of *Travancoriana charu* share N-glycolyl neuraminic acid as the common species if sialic acid. Considering the importance of sialic acids in the field of glycobiology and research, the sialic acid specific lectin in the hepatopancreas of the freshwater crab *Travancoriana charu* will play a significant role in biomedical research and therapy.

Author statement

All authors read, reviewed, agreed and approved the final manuscript.

Conflict of interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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