Characterization of the hemagglutinin in the hemolymph of the freshwater crab *Travancoriana charu* (Bahir and Yeo, 2007)

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Abstract: The hemolymph agglutinin of the freshwater crab *Travancoriana charu* recognized with high avidity the simple sugar galactose and the amino sugar N-acetyl glucosamine. The sialoglycoprotein BSM was the strongest glycoprotein inhibitor. Hemagglutination activity (HA) was characterized using several kinds of mammalian erythrocytes and the highest hemagglutination titer was observed against dog erythrocytes containing NeuGc / NeuAc. Maximum hemagglutination of the calcium dependent hemagglutinin was observed at pH 7.5 and temperature 30°C. The agglutinin was sensitive to di- / tetra sodium EDTA and trisodium citrate. Protease treatment enhanced the hemagglutinability. However, treatment with sialidase and denaturing agents caused a decline in the HA titer. Further, cross adsorption studies suggested the presence of a single agglutinin in the hemolymph.

Key words **- Hemagglutination, lectin, *Travancoriana charu*, sialoglycoprotein, agglutinin**

I. INTRODUCTION

All living organisms from protists to humans, in their struggle to survive and thrive must defend themselves from infections. So host-response against invading pathogens is a basic physiological reaction in all living organisms. Even prokaryotes protect themselves by the use of restriction enzymes and clustered regularly interspaced palindromic repeats (CRISPs), being able to degrade invading foreign pathogens (Dunin-Horkawicz *et al*., 2014). Agglutinins are typically polyvalent proteins with multiple binding sites for one or more moieties that are found on the surface of microbes and other parasites (Fred Brewer, 2002). Some invertebrate agglutinins are capable of binding specifically to sugar residues which are not or cannot be synthesized by invertebrates. Lectins are (glyco) proteins possessing at least one non-catalytic domain that recognize and bind reversibly to specific carbohydrates inside and outside cells (Drickamer, 1988; Kilpatrick, 2002; Sharon and Lis, 2004). These are multivalent carbohydrate binding proteins that agglutinate erythrocytes, bacteria, and other cells through interaction with appropriate complementary ligands (Vazquez *et al*., 1988; Bayne, 1990). Lectin-carbohydrate recognition represents a ligand-receptor interaction that is universal in living organisms (Feizi, 2000; Cambi and Figdor, 2003).

In invertebrates, the role of lectins as mediators of nonself recognition in the innate immune response has been well documented (Epstein *et al*., 1996). Lectins have been isolated and characterized in various invertebrates, including sponges (Pajic *et al*., 2002), tunicates (Nair *et al*., 2000), crustaceans (Ravindranath *et al*., 1985; Vazquez, *et al*., 1993), echinoderms (Giga *et al*., 1987; Matsui *et al*., 1994) and clams (Bulgakov *et al*., 2004). Lectins that exhibit specificity for sialic acid or sialoglycoconjugates are ubiquitous in the body fluids of arthropods. These lectins are involved in defense against microorganisms that express sialic acid or its analogs on their surface (Tunkijjanukij *et al*., 1998) as sialic acids are reportedly absent in invertebrates phylogenetically below echinoderms (Schauer, 1985; Suzuki and Mori, 1990).

II. MATERIALS AND METHODS

Erythrocyte collection and preparation

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.

Hemplymph 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.

Hemagglutination (HA) assay

Hemagglutination assays were performed in 'U' bottom microtiter plates as described by Ravindranath and Paulson (1987). 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. HA titer was recorded as the highest dilution that still caused agglutination.

pH and thermal stability

To determine the optimum pH for agglutination, the crab hemolymph (25 µl) was serially diluted with equal volume of Tris Buffered Saline (TBS) of different pH (5 to 10) in microtiter plates and incubated at room temperature (30±2ºC) for 1 hour, before adding 1.5% dog erythrocyte suspension.

The optimum temperature for lectin activity was evaluated by incubating the hemolymph samples of *Travancoriana charu* at various temperatures (0ºC to 100ºC) for 1 hour and hemagglutination activity was determined using 1.5% dog erythrocyte suspension.

Effect of cations and EDTA on hemagglutination activity

To assess the effect of cations and EDTA on the HA activity, 25 µl of the crab hemolymph was serially diluted in microtiter plates with equal volume of Tris Buffered Saline (TBS), different concentrations of cations (Ca^{2+}, Mg^{2+}, Mn^{2+}) or disodium / tetrasodium EDTA and trisodium citrate (0-100 mM), and incubated at room temperature (30±2ºC) for 1 hour before adding the erythrocyte suspension.

Hemagglutination inhibition (HAI) assay

Hemagglutination inhibition assay was done to determine the sugars / glycoproteins that would inhibit agglutination. Inhibitors of known (25 µl) concentration were serially diluted with 25 µl of TBS in microtiter plate. Then to each well, 25 µl of hemolymph diluted to subagglutination concentration in TBS (to give a HA titer of 2) was added and incubated for 1 hour. To this 25 µl of 1.5% dog erythrocyte suspension was added, mixed and incubated. Hemagglutination inhibition titer was reported as the reciprocal of the highest dilution of inhibitor giving complete inhibition after 1 hour.

Enzyme Treatment

Protease treatment

Following the procedure of Pereira *et al.* (1981), dog erythrocytes were washed five times with TBS (pH 7.5) by centrifugation at 4000 x g for 5 minutes at room temperature (30 ±2ºC) and resuspended in the same buffer. Equal volume of trypsin, pepsin and neutral protease (1 mg/ml) were added, mixed and incubated at (30±2ºC) for 1 hour. The enzyme treated erythrocytes were washed five times in TBS and then used for hemagglutination assay.

Neuraminidase treatment

Asialo erythrocytes were prepared following the procedure of Ravindranath *et al*. (1988) and Mercy and Ravindranath (1993). A reaction mixture (total 5 ml) containing 10% washed dog erythrocytes in PBS-BSA (pH 7.0) and 140 mU (milliunits) neuraminidase of *Clostridium perfringens* (type X: Sigma) was incubated for 4 hours at room temperature. The treated cells were washed with PBS–BSA three times and pelleted by low speed centrifugation. Finally they were washed in TBS–BSA (pH 7.0) and tested for HA activity.

Chemical stability

To test the chemical stability of the agglutinin, the crab hemolymph was mixed with chloroform in 1:3 ratio and incubated for about five minutes. The precipitate was removed and the supernatant was dialyzed against distilled water at 4ºC for 24 hours and then assayed with dog erythrocytes.

To study the effect of denaturing agents, 0.1 ml of aliquot of crab hemolymph was mixed with 0.1 ml of denaturing agent such as HCl (0.1 N) and NaOH (0.01 N) and was allowed to react for 2 hours at room temperature (30±2ºC) and then checked for hemagglutination assay.

Cross adsorption assay

Cross adsorption assay was carried out as described by Hall and Rowlands (1974 a, b) and Mercy and Ravindranath (1992). To 1 ml of washed and packed erythrocytes, 1 ml of crab hemolymph was added and mixed well. This hemolymph erythrocyte mixture was incubated at 10ºC overnight (18 hours) with gentle occasional shaking. After centrifugation, the supernatant was tested against the selected erythrocytes for hemagglutination assay. The supernatant that gave hemagglutination activity was readsorbed in equal volumes of their respective washed and packed erythrocytes till it showed no agglutination activity or no change in agglutinability with the tested erythrocytes.

III. RESULTS

HA assay

Hemolymph of *Travancoriana charu* had the capacity to agglutinate a variety of vertebrate erythrocytes. The agglutination titer in a chronological order: dog > rat = mice > rabbit > buffalo = pig = guinea pig = camel > human AB = O = A = B erythrocytes. The weakest was towards horse > cow = goat erythrocytes.

Table 1: Hemagglutination titer of the hemolymph of the freshwater crab *Travancoriana charu* with different mammalian erythrocytes

Erythrocytes (n =25)	HA titer
Dog	256-512
Rat	128
Mice	128
Rabbit	64
Buffalo	16
Pig	16
Camel	16
Guinea pig	16
Human A	8
Human B	8
Human AB	8
Human O	8
Horse	4
Cow	2
Goat	2
Donkey	0

n= number of crabs tested

pH and thermal stability

The hemagglutinating activity of *Travancoriana charu* was highest at pH 7.5 and was temperature dependent. The hemolymph agglutinin was highly active at temperature 30ºC. At temperatures higher than 50ºC, the hemagglutination titer decreased markedly.

Table 2: Hemagglutination titer of the hemolymph of the freshwater crab *Travancoriana charu* in relation to changes in pH and temperature

pH (n =10)	HA titer	Temperature(n =10)	HA titer
5.0	64	0	128
5.5	64	10	128
6.0	128	20	256
6.5	128	30	256-512
7.0	256	40	256
7.5	256- 512	50	64
8.0	128	60	32
8.5	128	70	8
9.0	64	80	0
9.5	64	90	0
10.0	64	100	0

n= number of crabs tested

Effect of divalent cations and chelators

The hemolymph agglutnin of *Travancoriana charu* was dependent on Ca^{2+} ions for its activity. Hemagglutination was highest at 10 mM concentration of Ca^{2+}, 10-40 mM Mg^{2+} and 0.01-5 mM Mn^{2+}. Further increase in cations concentration showed a fall in hemagglutinating activity.

Hemagglutination of the hemolymph agglutinin of the freshwater crab *T. charu* was sensitive to EDTA. High HA titer was observed at 1-5 mM concentration of EDTA disodium and tetrasodium salts. At 10 mM concentration, there was a steep decrease in HA activity. Trisodium citrate also had an apparent influence on the HA titer at low concentration.

Table 3: Effect of cations on the hemagglutinating activity of the hemolymph agglutinin of the crab *Travancoriana charu*

Concentration of cation (mM) (n =10)	**HA titer**		
	Ca^{2+}	**Mg^{2+}**	**Mn^{2+}**
0	128	128	128
0.01	256	128	512
0.1	256	128	512
1.0	256	256	512
5	256	256	512
10	512	512	256
20	256	512	256
30	256	512	128
40	128	512	128
50	128	128	64
100	128	128	64

n= number of crabs tested

Table 4: Effect of EDTA and trisodium citrate on the hemagglutinating activity of the freshwater crab *Travancoriana charu*

Concentration (mM) (n =10)	**Calcium chelators**		
	EDTA		**Trisodium citrate**
	Disodium salt	**Tetrasodium salt**	
0	128	128	128
0.01	128	256	128
0.1	256	256	128
1.0	512	512	128
5	512	512	128
10	64	16	32
15	8	8	32
17	4	8	32
20	4	4	32
30	4	2	32
40	4	2	8
50	4	2	2
100	2	2	2

n= number of crabs tested

Hemagglutination inhibition

Sugars

Galactose and N-acetyl-D-glucosamine (1.56 mM) were the most powerful inhibitor of hemagglutinating activity. HA was also inhibited by relatively lower concentrations of N-glycolyl neuraminic acid, glucose-6-phosphate, L-fucose and D-

galactosamine. Trehalose, α–lactose, raffinose, D-fucose, N-acetyl neuraminic acid, glucose-3-phosphate, mannosamine hydrochloride, N-acetyl-D-mannosamine, melibiose and N-acetyl-D-galactosamine were the other sugar inhibitors of *Travancoriana charu* hemolymph agglutinin.

Table 5: Hemagglutination inhibition (HAI) of the hemolymph agglutinin of the freshwater crab *Travancoriana charu* by various sugars

Sugars (n =10)	**HAI titer**	**Minimum concentration for inhibition (mM)**	**Relative inhibitory potency (%)**
Galactose	64	1.56	100
N-acetyl-D-glucosamine	64	1.56	100
N-glycolyl neuraminic acid	32	3.12	50
Glucose-6-phosphate	32	3.12	50
L-fucose	32	3.12	50
D-galactosamine	32	3.12	50
Trehalose	16	6.25	25
α-lactose	16	6.25	25
Raffinose	16	6.25	25
N-acetyl neuraminic acid	8	12.5	12.5
D-fucose	8	12.5	12.5
Glucose-3-phosphate	8	12.5	12.5
Mannosamine hydrochloride	4	25	6.25
N-acetyl–D-mannosamine	2	50	3.12
Melibiose	2	50	3.12
N-acetyl-D-galactosamine	2	50	3.12

n= number of crabs tested

Glycoproteins

Among the glycoproteins tested, BSM (19.53 µg/ml) was the most powerful inhibitor. Bovine thyroglobulin (39.06 µg/ml) was also a good inhibitor. Lactoferrin, fetuin, PSM, apotransferrin and transferrin were able to inhibit the hemagglutination activity with a relatively low HAI titer.

Table 6: Hemagglutination inhibition (HAI) of the hemolymph agglutinin of the freshwater crab *Travancoriana charu* by various glycoproteins

Glycoproteins (n =10)	**HAI titer**	**Minimum concentration for inhibition (µg/ml)**	**Relative inhibitory potency (%)**
Bovine submaxillary mucin	256	19.53	100
Bovine thyroglobulin	128	39.06	50
Lactoferrin	32	156.25	12.5
Fetuin	16	312.5	6.25
Porcine stomach mucin	16	312.5	6.25
Apotransferrin	8	625	3.12
Transferrin	8	625	3.12

n= number of crabs tested

Enzyme treatment

An increase in hemagglutination activity was noticed when tested with protease treated (trypsin, pepsin and neutral protease) dog erythrocytes, however there was a decrease in hemagglutination titer when the hemolymph was checked with neuraminidase treated dog erythrocytes.

Table 7: Effect of enzyme treatment of dog erythrocytes on the hemagglutination titer of the crab *Travancoriana charu*

Enzymes (n=5)	HA titer
None	256-512
Trypsin (1 mg/ml)	4096
Pepsin (1 mg/ml)	2048
Neutral protease (1 mg/ml)	1024
Neuraminidase	16

n=number of crabs tested

Effect of denaturing agents

Denaturing agents like chloroform, HCl, and NaOH decreased the hemagglutination activity of the hemolymph agglutinin of *Travancoriana charu*.

Table 8: Effect of denaturing agents on the hemagglutinating activity of the freshwater crab *Travancoriana charu*

Denaturing agents (n=5)	HA titer
None	256-512
Chloroform	8
HCl (0.1N)	16
NaOH (0.01N)	4

n=number of crabs tested

Cross adsorption assay

Cross adsorption studies were carried out to assess the nature of the agglutinin, whether the agglutination activity is due to one or more agglutinins. HA activity was lost after adsorption with dog, rat, mice, rabbit, buffalo, camel and guinea pig erythrocytes. Although pig erythrocytes continued to agglutinate mice erythrocytes up to second and third adsorption the agglutinability was lost with the third adsorption, confirming the presence of a single agglutinin.

Table 9: Hemagglutination titer of the hemolymph of the freshwater crab *Travancoriana charu* after adsorption with different erythrocytes

	HA titer (n=5)							
Erythrocytes adsorbed	**Dog**	**Rat**	**Mice**	**Rabbit**	**Buffalo**	**Pig**	**Camel**	**Guinea pig**
None	256-512	128	128	64	16	16	16	16
Dog	0	0	0	8(0)	0	0	0	2(0)
Rat	4(0)	0	2(0)	4(0)	4(0)	0	4(0)	3(0)
Mice	4(0)	0	0	4(0)	4(0)	0	0	3(0)
Rabbit	0	0	0	0	0	0	0	0
Buffalo	0	0	0	0	0	0	0	0
Pig	0	0	16(4)(0)	0	0	0	0	0
Camel	0	0	0	0	0	0	0	0
Guinea pig	0	0	0	0	0	0	0	0

n=number of crabs tested

IV. DISCUSSION

The definition of the term lectin has evolved from being an aggregator of different kinds of erythrocytes (Boyd and Shapleigh, 1954), to the definition of lectins as proteins or glycoproteins carrying at least one binding site, possessing a reversible specific interaction with a particular carbohydrate moiety (Peumans and Van Damme, 1995). Lectins are capable of recognizing and non-covalently binding to specific saccharide moieties and therefore agglutinate cells by binding to cell surface glycoprotiens and glycoconjugates (Lis and Sharon, 1998; Cerenius *et al*., 2010). In the present study, the hemolymph of the freshwater crab *Travancoriana charu was* found to possess a naturally occurring agglutinin which agglutinted dog, rat, mice as well as several other mammalian erythrocytes. The hemolymph showed the highest reactivity with dog erythrocytes similar to the hemagglutination titer in the crab *Episesarma tetragonum* (Devi *et al*., 2013). It showed moderate reactivity with buffalo, pig,

camel and guinea pig erythrocytes. The weakest activity was against cow and goat erythrocytes. The hemagglutination activity of the hemolymph of *Travancoriana charu* was highest at pH 7.5. Basic-neutral pH represents the natural environment of animals. Hemagglutinability was stable between temperatures 20°C and 40°C, but the activity was completely abolished beyond 70°C, similar to *F. chinensis* plasma lectin activity which was inhibited at temperatures greater than 75°C (Sun *et al*., 2008). Lectins are known to be heat labile and their activity can be decreased by heat treatment (Liener, 1994).

Addition of Ca^{2+} resulted in an increase in the agglutination. On this basis, *Travancoriana charu* lectin may be classified as a C-type lectin. C-type lectins are calcium-dependent lectins which comprise a large superfamily of membrane and extracellular proteins that share disulphide-rich Ca^{2+}-binding carbohydrate recognition domains (CRDs). The C-type CRDs contain one or two Ca^{2+}-binding sites (Weis and Drickamer, 1996). This big gene family mediates sugar binding with diverse architecture contained homologous CRDs, by which they discriminate specific oligosaccharides at cell surfaces, attach to circulating proteins in the extracellular matrix (Drickamer and Taylor, 1993; Weis *et al*., 1991; Zelensky and Gready, 2005). C-type lectins specifically bind pathogen associated molecular patterns (PAMPs) on the surface of many pathogens, which provide them with the ability to recognize a wide variety of pathogens (Kilpatrick, 2002; Devi *et al*., 2010).

EDTA is a chelating agent which binds divalent cations such as calcium and magnesium. Presence of EDTA upto 5 mM enhanced the hemagglutination activity of *Travancoriana charu* hemolymph agglutinin. However, 10 mM EDTA reduced the HA titer suggesting that the hemaggltinin of *T. charu* is dependent on divalent cations. Such cation-protein linkage has been previously reported for lectins of other arthropods (Grubhoffer and Matha, 1991; Smith and Chisholm, 1992). Enzyme (proteases) treated dog erythrocytes were greatly recognized by the hemolymph agglutinin of *T. charu*. The increase in titer value may be due to the exposure of the cryptic receptor sites on the surface of the cell membrane (Hammarstrom and Kabat, 1969) and the removal of close interfering structures by possible rearrangement of receptor sites on the erythrocytes (Nicolson, 1971). Trypsin acts specifically on the peptide bonds of basic aminoacids (Murray *et al*., 1990). This is added to increase the susceptibility of erythrocytes to agglutination without affecting the total number of lectin binding sites (Lis and Sharon, 1986). However, neuraminidase-treated erythrocytes were 16-fold less agglutinated than the untreated ones, suggesting a preferential affinity of the activity towards sialoglycoconjugates.

The sugars that inhibit agglutination are the sugars specific for the lectin. This inhibitory effect of sugars can be attributed to their ability to compete for binding sites on the lectin molecule, which can interfere with the attachment of the lectin to the sugar units on the surface of erythrocytes. Complete inhibition of hemagglutination was seen at 1.56 mM of the monosaccharide galactose and N-acetyl-D-glucosamine (GlcNAc). Detection of lectins that react with galactose and N-acetyl-substituted sugars is common in invertebrate species. Inhibition was also seen at 3.12 mM N-glycolyl neuraminic acid, glucose-6-phosphate, L-fucose, D-galactosamine, 6.25 mM trehaloae, α-lactose, raffinose, 12.5 mM N-acetyl neuraminic acid, D-fucose, glucose-3-phosphate, 25 mM mannosamine hydrochloride, 50 mM N-acetyl-D-mannosamine, melibiose and N-acetyl-D-galactosamine. The serum agglutinating activity to recognize a wide range of carbohydrates will potentially help the animal to recognize a wide variety of pathogens based on their surface molecules (Zhang *et al*., 2009).

The hemagglutination inhibitior BSM contains N-acetyl-neuraminic acid, N-glycolyl-neuraminic acid, N-acetyl 9-O-neuraminic acid and N-acetyl 8, 9-di-O-acetyl-neuraminic acid (Schauer, 1982). BSM is composed of sialylated T and Tn (Neu Acα2, 3GalNAcβ1-0 Ser/Thr) antigens (Schauer *et al*., 1998). T/Tn antigen occurs on the surface of tumor cells as a mucin-associated antigenic marker and is one of the few chemically well-defined tumor antigens with a proven link to malignancy (Springer, 1995). They are good marker for several cancerous tissues, including the prognosis of colorectal cancer (Ono *et al*., 1994). Repeated adsorption of the hemolymph with the erythrocytes entirely removed the agglutinability of the hemolymph, suggesting the presence of a single hemagglutinin as reported in *Cancer antennarius* (Ravindranath *et al*., 1988). Sialic acid residues are commonly encountered in the cell membrane of all deuterostome animals but absent in protostomes including crustaceans (Schauer, 1982).

In decapods, many lectins have been reported, most of them recognize the N-acetyl groups of carbohydrates, such as N-acetyl-neuraminic acid (NeuAc or sialic acid), N-acetyl-D-galactosamine (GalNAc), and N-acetyl-D-glucosamine (GlcNAc), although they vary in molecular weight, structure and divalent cation requirements, suggesting that, in spite of their structural variability, the binding properties that determine their biological functions have been preserved. This study provides the necessary information for the purification of the agglutinin in the hemolymph of the freshwater crab *Travancoriana charu* using affinity chromatography for further studies on the lectin properties and possible biological applications.

V. CONCLUSION

Applications of lectins is based on its properties. The hemolymph agglutinin of the freshwater crab *Travancoriana charu* was inhibited by sialoglycoproteins and sugars like galactose, GlcNAc, N-glycolyl neuraminic acid, glucose-6-phosphate, L-fucose, D-galactosamine etc. The above investigations reveal the sugar and sialic acid specificity of the hemolymph agglutinin. The ability of lectins to recognize sialic acids and their derivatives on cell surfaces has great significance in the field of cancer research and therapy. The sialic acid specific hemolymph agglutinin can be further purified and characterized to be used as a potential tool in the in biomedical applications.

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