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PHYSICO-CHEMICAL CHARACTERIZATION OF THE HEMOLYMPH AGGLUTININ OF THE MARINE CRAB *ATERGATIS INTEGERRIMUS* (LAMARCK, 1818)

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ABSTRACT

A natural hemagglutinin with specific affinity for buffalo erythrocytes was detected in the hemolymph of the marine crab, Atergatis integerrimus. Physico chemical analysis of the hemolymph agglutinin demonstrated high hemagglutinin activity between pH 6.5-7.5 and temperature 20° - 30°C. The agglutinin activity was dependent on divalent cations calcium, magnesium and manganese but showed high affinity towards calcium. It was reversibly sensitive to di and tetra sodium EDTA and trisodium citrate. Heamagglutin activity was inhibited by sialic acid containing glycoproteins: BSM > bovine thyroglobulin > apo transferrin = transferrin > PSM = fetuin = lactoferrin and sugars (raffinose > trehalose = α-lactose). Buffalo erythrocytes treated with protease showed a high HA titre whereas neuraminidase treatment reduced the HA titre when compared to native erythrocytes. The hemagglutinability of the hemolymph agglutinin was significantly reduced when treated with denaturing agents like HCl and NaOH. Disappearance of agglutinability following cross adsorption revealed the presence of a single agglutinin. Thus, the preliminary characterization of the hemolymph agglutinin would provide strategies for purification of a lectin from the marine crab, Atergatis integerrimus.

KEY WORDS

natural hemagglutinin, trehalose

INTRODUCTION

Animal lectins are a heterogeneous group of molecules, which exhibit a high structural diversity and are capable of agglutinating molecules, enabling binding to more than one erythrocyte, bacteria or cell at a time [1]. Since agglutinins are proteins and can reversibly bind to the interacting sugar, the binding affinity to the saccharide moieties is influenced by the physico-chemical factors of the medium. pH and temperature of the medium determines the ionization state and the thermal tolerance of the agglutinin [2]. Lectins are diverse oligomeric proteins of varying molecular size, amino acid composition, three dimensional structures, metal requirement [3] and are inhibited by a spectrum of different carbohydrate and noncarbohydrate molecules [4].

Lectins reported in decapods are reported to be dependent upon divalent cations, usually calcium, and they are sensitive to divalent chelators in a reversible or irreversible manner [5]. The serum lectins of *P. indicus* [6] and that of *P. paulensis* do not require cations (Ca^{2+} and Mg^{2+}) for the hemagglutinin activity, a characteristic uncommon among crustacean lectins. However, in some cases, the behaviour of the molecule in the presence of chelators can suggest a partial dependency, as observed in some species of crayfishes [7].

Lectins are proteins that recognize specifically the carbohydrate moieties on the cell surface based on the ligand-receptor interaction. Therefore, different

biological roles have been proposed for these molecules, including the cellular and tissue transport and storage of carbohydrates, glycoproteins and calcium [8], cytolytic and cytotoxic activity [9], cell adhesion, migration and apoptosis [10], pathogenic interaction between parasite and host [11], differentiate benign and malignant tumors [12] and identification of cell surface receptors in pathogens [13].The specificity of a lectin is related to the carbohydrate for which it shows the highest affinity, but most lectins can bind to other carbohydrates, which are structurally related. The agglutination of many different types of cells / glycoconjugates may actually reflect the ubiquity of the ligand [14]. The binding affinity of the agglutinin is defined by the sugars, which inhibit their activity [15] and therefore provides with the ability to recognize pathogens [16]. A significant property of arthropodan agglutinins/lectins is its ability to bind to sialic acids found predominantly on glycoprotein and glycolipids [17]. Because of the inherent property of lectins to bind to various carbohydrates, especially sialic acids it can be used as tools for identifying the sialyl epitopes expressed on the surface of pathogens or the malignant tumors [18]. Since identifying a lectin with sialic acid specificity would be of immense value in biomedical application, the present study was carried to partially characterize the agglutinin of crab *Atergatis integerrimus* and to provide strategy for purification of the lectin.

MATERIALS AND METHODS

Animal collection and maintenance: The crabs, *Atergatis integerrimus* were collected from the coastal areas of Arockyapuram, Kanyakumari District, Tamilnadu, India. Crabs were maintained in plastic containers filled with marine water, fed with anchovy variety of fish and the water was renewed daily.

Collection and preparation of mammalian erythrocytes: Buffalo, mice, rat, guinea pig, rabbit, pig, dog, Human, A, B, O, camel, cow, goat, horse, donkey erythrocytes were prepared following the standard method of Ravindranath and Paulson [19].

Collection of hemolymph: The hemolymph from the crab *Atergatis integerrimus* was collected following the procedure of Mercy and Ravindranath [20].

Hemagglutination (HA) and Hemagglutination inhibition (HAI) Assay: Hemagglutination assay and Hemagglutination inhibition assay were performed in "U" bottomed 96 well microtiter plates (Tarson) as described by Ravindranath and Paulson [19].

pH and thermal stability: pH and temperature dependence of the agglutinin was tested by pre-incubating the hemolymph at different pH (5-10) and temperature (0 ^{o}C – 100 ^{o}C) for 1 hour before adding erythrocyte suspension and was checked for HA activity.

Cation dependency and EDTA sensitivity: To assess the effect of cations and EDTA on the HA activity, 25 µl of hemolymph was serially diluted with equal volume of TBS of different concentrations (0 -100 mM) of divalent cations (Ca^{2+}, Mg^{2+}) and calcium chelators (EDTA and trisodium citrate). After incubation, the HA activity of each sample was determined against buffalo erythrocytes.

Trypsin and protease treatment: Equal volumes each of trypsin (1 mg/ml of buffer pH 7.5) and neutral protease (0.25 mg/ml of buffer pH 7.5) with washed erythrocytes were incubated at 37 ^{o}C for 1 h. After incubation, erythrocytes were washed five times in TBS and used for hemagglutination assay.

Neuraminidase treatment: Asialo erythrocytes were prepared following the method of Mercy and Ravindranath [20]. The desialylated erythrocyte were used for hemagglutination assay.

Cross adsorption assay: The cross-adsorption assay was carried out following the method of Mercy and Ravindranath [21].

Chemical stability: The chemical stability of the agglutinin and the effect of denaturing agents was performed following the method of Bai [22].

RESULTS

Hemagglutination assay

The hemolymph agglutinin of the marine crab, *A. integerrimus* agglutinated a wide variety of mammalian erythrocytes with varying HA titre: dog > buffalo = mice > rat > rabbit > guinea pig > human A = B = O > camel > goat = cow = pig = horse = donkey (Table-1). The agglutinin agglutinated dog, buffalo and mice erythrocytes with much efficiency when compared to other erythrocytes.

Table-1: Hemagglutination titer of hemolymph agglutinin of *Atergatis integerrimus* with different mammalian erythrocytes

Erythrocytes (n=10)	HA titer
Dog	256 - 512
Buffalo	256
Mice	128
Rat	64
Rabbit	32
Guinea pig	16
Human A	16
Human B	16
Human O	16
Camel	8
Goat	4
Pig	4
Horse	4
Donkey	4
Cow	4

Effect of pH and temperature on hemagglutination

Hemagglutinating activity of the serum agglutinin of *A. integerrimus* against buffalo erythrocytes was stable between pH 6.5 to 7.5. A decrease in HA titre was observed below pH 6.5 and above pH 8 (Table- 2). Influence of temperature on agglutination was studied and the results showed an increase in HA at temperatures ranging from 0-30 °C. When the samples were heated above 40 °C, a progressive reduction in HA titer was observed and a total disappearance at 70 °C (Table- 2).

Table-2: Hemagglutination titer of hemolymph of *Atergatis integerrimus* in relation to change in pH and temperature

pH (n=5)	HA titer	Temperature °C (n=5)	HA titer
5	32	0	256
5.5	64	10	256
6	64	20	256
6.5	256	30	256
7	256	40	64
7.5	256	50	16
8	128	60	4
8.5	128	70	2
9	64	80	0
9.5	64	90	0
10	32	100	0

Effect of cations and chelators on agglutination

Hemagglutinability of the hemolymph of the marine crab *A. integerrimus* was unaffected by lower concentrations (0 - 1 mM) of cations - calcium and magnesium and an increase was noted up to 20 mM. However, high concentration of these cations reduced the HA titer. The HA activity remained unaffected even at 40 mM concentration in the case of manganese (Table-3). Calcium chelators tetra sodium EDTA and di sodium EDTA reduced the HA titer of the crab hemolymph above 10 mM concentration whereas reduction in HA was observed only above 40 mM concentration with trisodium citrate (Table-3).

Table-3: Effects of cations on the hemaglutinating activity of the agglutinin of the marine crab *Atergatis integerrimus.*

Cation conc. in mM (n=10)	HA titer		
	Ca^{2+}	Mg^{2+}	Mn^{2+}
0	128	128	128
0.01	128	128	128
0.1	128	128	128
1.0	128	128	128
5.0	256	256	128
10	256	256	128
20	256	256	128
30	128	256	128
40	128	128	128
50	128	64	64
100	128	32	64

Hemagglutination inhibition (HAI) assay

Binding determinant of the agglutinin was assessed using glycoproteins and sugars. The agglutination of serum with buffalo erythrocytes was inhibited by BSM > bovine thyroglobulin > fetuin > PSM > transferrin > apo – transferrin > lactoferrin (Table- 5). Of all the sugars tested for HAI assay the hemolymph agglutinin, was inhibited by raffinose > trehalose = melibiose = L-fucose = D– galactosamine = D- galactose = D- glucose and weakly inhibited by Glu – 6 – PO_4 = GluNAc = ManNAc = Glu -3- PO_4 = GalNAc > D-fucose > D – fructose (Table - 4).

Table- 4: Effect of chelators on the hemagglutinating activity of the naturally occurring agglutinin in the hemolymph of marine crab *Atergatis integerrimus*.

Concentration in mM (n=10)	EDTA		
	Disodium	Tetrasodium	Trisodiumcitrate
0	128	128	128
0.01	128	128	128
0.1	128	128	128
1.0	256	256	128
5.0	256	256	128
10	8	32	128
15	2	32	128
20	2	8	128
30	2	2	128
40	2	2	64
50	0	0	32
100	0	0	16

Effect of enzyme treatment on HA

Treatment of buffalo erythrocytes with neuraminidase tremendously reduced the HA titer, whereas treatment of buffalo erythrocytes with trypsin and neutral protease enhanced the HA titer of the hemolymph agglutinin (Table- 6).

Cross adsorption test

Cross adsorption studies revealed the presence of a single agglutinin in the hemolymph of the marine crab *A. integerrimus* (Table -7). The serum when adsorbed to any one of the seven erythrocytes (buffalo, dog, mice, rat, rabbit, guinea pig, human A) lost its ability to agglutinate the erythrocytes after either first or second or third or fourth adsorptions suggesting the presence of a single agglutinin.

Effect of chemicals on HA activity

Treatment of the hemolymph with chloroform and denaturing agents such as HCl and NaOH significantly reduced its agglutinability (Table- 8).

Table-5: Hemagglutination inhibition of the hemolymph agglutinin of the marine crab *Atergatis integerrimus* by various glycoproteins.

Glycoproteins (N=5)	HAI	Minimum conc. Required (mM)	Relative inhibitory potency (%)
BSM	512	19.53	100
Bovine Thyroglobulin	128	39.06	50
Fetuin	64	78.25	25
PSM	64	78.125	25
Transferin	32	156.26	12.5
Apo – transferin	32	156.25	12.5
Lactoferrin	16	312.5	6.25

Table- 6: Hemagglutination inhibition (HAI) of the hemolymph agglutinin of the marine crab *Atergatis integerrimus* by various sugars

Sugars	HAI	Minimum conc. Required (mM)	Relative inhibitory potency (%)
Raffinose	32	3.12	100
Trehalose	32	3.12	100
α-Lactose	16	6.25	50
Melibiose	16	6.25	50
L-Fucose	16	6.25	50
D-galactosamine	16	6.25	50
D-galactose	16	6.25	50
D-glucose	16	6.25	50
Glu-6-PO_4	8	12.5	25
GluNAc	8	12.5	25
Man NAc	8	12.5	25
Glu-3-PO_4	8	12.5	25
GalNAc	8	12.5	25
D-Fucose	4	25	12.5
D-fructose	2	50	6.25

Table-7: Effect of enzyme treatment of buffalo erythrocytes on the hemagglutination assay of the hemolymph agglutinin of the marine crab *Atergatis integerrimus*.

Enzyme (N=5)	Site of enzyme activity	HA titer
None	-	256
Neuraminidase (*Clostridium perfringens* Type X)	Neu Ac-D-Gal; Neu Ac-D-GalNAc	16
Trypsin (1mg/ml)	Arg-Lys-S	512
Neutral protease (1 mg/ml)	-	512

Table-8: Hemagglutination titer of the hemolymph of marine crab *Atergatis integerrimus* after adsorption with different erythrocytes.

Erythrocytes adsorbed (N=10)	HA Titer						
	Buffalo	Dog	Mice	Rat	Rabbit	Guinea pig	Human A
None	256	128	128	64	32	32	16
Buffalo	0	0	0	0	0	0	0
Dog	2 (0)	0	64,32,8 (0)	16,8,4 (0)	0	0	0
Mice	0	0	0	4 (0)	0	0	0
Rat	2 (0)	8 (0)	0	0	0	0	0
Rabbit	0	0	0	0	0	0	0
Guinea pig	0	8 (0)	0	16 (0)	0	0	0
Human A	0	0	0	0	0	0	0

Table-9: Effect of denaturing agents on the hemagglutinating activity of the marine crab *Atergatis integerrimus.*

Denaturing agents N=10	HA titer
None	256
Chloroform	4
0.1N Hcl	8
0.1N NaoH	8

DISCUSSION

Agglutinins are defense molecules ubiquitously present among invertebrates as soluble or membrane bound molecules [23] and are capable of agglutinating erythrocytes. The hemolymph agglutinin of the marine crab, *A. integerrimus* agglutinated all the erythrocytes tested at varied capacities with HA titer ranging from 4 to 512. The agglutination pattern with different mammalian erythrocytes demonstrated a high specificity for dog, buffalo and mice erythrocytes, moderate affinity towards rat and rabbit erythrocytes and very poor affinity towards the other erythrocytes tested. The specific affinity may be due to the common or similar membrane receptors expressed on the cell surface and varying agglutinabilty is because of the quantitative difference in their binding sites.

The erythrocyte specificity of the serum agglutinin 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 [24]. It has been found that different animal species have characteristic receptor determinants on their erythrocyte surface [25] and intra-specific variations [26]. The receptor component NeuGc on the glycocalyx of buffalo erythrocytes [27] NeuGc/ NeuAc on dog erythrocyte [28] and NeuGc expressed on horse, mouse and rat erythrocyte) [29] are best recognized by the agglutinin. Thus, it can be concluded that the agglutinin may bind to the sialic acid moieties of the glycocalyx of these erythrocytes.

pH and temperature are the key factors that determine the sensitivity of the agglutinin during extreme conditions. Hemagglutinin activity of the hemolymph of *A. integerrimus* was stable at an optimum pH ranging from 6.5 to 8.5 while highly acidic and basic pH reduced the agglutinin activity. At high acidic pH the lectin may display an open structure than at neutral pH making it more sensitive to degradation [30]. The pH changes would have either dissociated the agglutinin from hemocyanin or it would have dissociated into smaller subunits resulting in a high HA titer when subjected to optimum pH as reported in limulus [31], lobster [32] and squi.[33] The agglutinin was thermo sensitive and retained its maximum activity between 0-30^{o}C,

gradually reduced above 40ºC and totally disappeared at 70ºC. Reduction in agglutination of lectins may be due to a structural transition of the lectin itself or due to the modification of cell surface properties [34].

The proteinaceous nature of the agglutinin was confirmed by its sensitivity to chloroform, that precipitates protein [35] and denaturing agents like HCl and NaOH which denatures protein. Reduction in HA may be due to the disruption of the hydrophobic interactions in the interior of the lectin that support its native conformation [36]

Divalent cations maintain the structural integrity and stability of the agglutinin. Agglutination of the humoral agglutinin was reduced in the absence of calcium ions in the buffer and addition of calcium ions enhanced hemagglutination. The HA titer increased with increase in Ca^{2+}, Mg^{2+} and Mn^{2+} up to 10 mM indicating that the agglutinin depends on cations for its activity. The reduction in the agglutinating activity after treatment of serum with calcium chelator EDTA was indeed due to the chelation of calcium ions. These observations demonstrated that the serum agglutinin of *A. integerrimus* is reversibly sensitive to EDTA. This confirms that divalent cations are important in stabilizing the primary structure of hemagglutinins [37]. Metal ions play a major role in stabilizing the conformation of the key loop regions of the protein by coordination of the metal ions with a cluster of negatively charged amino acids. Therefore upon removal of the cations this conformation of the ion binding loop becomes energetically unfavourable, due to strong electrostatic repulsion and the stability is altered [38].

Repeated adsorption with buffalo, dog, mice, rat, rabbit, guinea pig and Human A erythrocytes removed agglutinability of the crab serum suggesting the presence of a single hemagglutinin as reported in *Scylla serrata* [20,21], *Lamella lamellifrons* [22].

Hemagglutination – inhibition tests performed in this study revealed that saccharides like raffinose, trehalose, α – lactose, Melibiose and L - fucose inhibited the agglutinating activity of *A. integerrimus* serum when compared to other sugars. The agglutinin recognized with low affinity the N-acetyl derivatives (GluNAc, GalNAc and ManNAc), which contain the acetyl group thereby demonstrating that an acetyl group might be necessary for agglutinin – ligand interaction. The hemagglutination activity of *A. integerrimus* agglutinin was inhibited by glycoproteins: BSM > bovine thyroglobulin > fetuin = PSM > transferrin = apotransferrin > lactoferrin. The results revealed that the preferred sugar may be NeuAc/NeuGc which is evident from the glycoprotein assay. The agglutinin recognized the receptor components on BSM which contains mainly 9-O acetyl and 8, 9- di – O – acetyl – N-acetyl neuraminic acid [39], thyroglobulin and fetuin that contains NeuGc [40] as the sialic acid moiety and PSM that contains large quantities of GalNAc as their terminal molecules [41]. Thus our findings suggest that hemagglutination may be the result of the binding of agglutinin to sialic acid which was evident when it failed to agglutinate desialylated buffalo erythrocytes and was not inhibited by desialylated BSM. This may be due to the removal of sialic acids on erythrocytes and glycoprotein which react with the agglutinin, by neuraminidase treatment [42]. The enhancement in HA titer with protease treatment of buffalo erythrocytes could be due to the removal of cell surface proteins that exposes most of the agglutinin binding sites, possibly the sugar residues of gangliosides recognized by the agglutinin. It is known that susceptibility of red blood cells to agglutinins generally increases when they are treated with trypsin [22].

CONCLUSION

The hemolymph of marine crab *Atergatis integerrimus* has a potent lectin which could be further purified for various applications.

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