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Biological Properties of Bacterial and Haemagglutinins from the Serum of Estuarine Crab *Charybdis lucifera*

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Abstract: A naturally occurring haemagglutinin (HA), with activity against bacteria and yeast cells were detected in the serum of *Charybdis lucifera* using mammalian erythrocytes (RBC), various bacteria and yeast as indicator cells. The serum gave highest HA titer with rabbit RBC, tripsinized yeast and *Vibrio` fluvialis*. An analysis of the biological properties of the HA showed it to be specifically dependent on the presence of Ca^{2+} for its activity. Further studies demonstrated that the HA- inhibition assays performed with carbohydrates revealed that the serum HA was specific for non-reducing terminal glucose with 1-2 glucosidic linkages. Thus this agglutinin appears to be unique among all the known crustacean agglutinins.

Key words: Haemagglutinin · Bacterial agglutinin · Yeast agglutinin · Serum · *Charybdis lucifera*

INTRODUCTION

Lectins are carbohydrate-binding proteins and in invertebrates, lectins are vital means for non-self recognition and clearance of invading microorganisms. In invertebrates, phagocytosis is considered to be the primary mechanism of innate defense against foreign invaders [1, 2, 3]. In this process, an intimate interaction of humoral substances, particularly as recognition factors, has been implicated [4, 5]. A variety of humoral factors, naturally occurring and/or formed after antigenic stimulation, have been detected in the serum of invertebrates and they include agglutinins [6-11], lysins [12], antibacterial [13] and antifungal proteins [14], phenoloxidase system [15], LPS binding protein [16] and β-1, 3 glucan binding protein [17]. Due to the probable functional similarities between agglutinins and vertebrate antibodies and the indications that agglutinins serve a defensive function [18], invertebrate agglutinins have been extensively studied.

Agglutinins (=lectins) are di- or multivalent carbohydrate-binding proteins with the ability to agglutinate cells with complementary carbohydrates on their surfaces [19, 20]. They are known to specifically recognize the whole sugar [21], a specific site in a sugar [22], a sequence of sugars [23], or their glycosidic linkages [24]. The agglutinating molecules are widely distributed in microorganisms [25], plants and animals [26].

The body fluid or hemolymph of almost all invertebrate species tested contains agglutinins [3, 7-11, 27]. The presence of agglutinins has also been detected in the mucus as well as in certain tissues of invertebrates [7, 28, 29]. However, its immunological role is best understood in the hemolymph and recent studies have shown that purified, hemolymph-derived agglutinins served as opsonin in a few insects and molluscs [4, 30-32]. Although a number of studies have demonstrated the presence of humoral agglutinins in several crustacean species, it can be noted that the immunological roles of these agglutinins remain largely unknown and that the carbohydrate specificity of serum agglutinins from crustaceans have been elucidated only in a few species [8, 10, 33-35]. This study thus describes RBC and bacterial binding activities, biological properties and carbohydrate specificity of a naturally occurring agglutinin in the serum of the marine crab *Charybdis lucifera*.

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MATERIALS AND METHODS

Experimental Animals and Laboratory Maintenance: The estuarine crab *Charybdis lucifera* were obtained from Vellar estuarine, Southeast coast of India. In the laboratory, these crabs were maintained in plastic tanks containing aerated seawater and the medium was changed every day.

Preparation of Serum: Haemolymph were collected by cutting each walking legs of the animal with a fine sterile scissor. The samples were collected in clean polystyrene plastic tubes held on ice and allowed to clot at room temperature (RT: 28 ± 2°C for 20 min). Serum was separated by centrifugation (400 x g, 10 min, RT) and the resulting clear supernatant (=serum) was used immediately.

Preparation of Erythrocyte (RBC) Suspension: Human and other mammalian blood samples were obtained by venous or cardiac puncture and collected in sterile Alsever's solution [36] containing 10 μg/ml of streptomycin. Prior to use, the RBCs were washed thrice with 0.9% saline and once with TBS-I (50 mM tris-HCl, 115 mM NaCl, 10 mM $CaCl_2$, 300 mOsm) by centrifugation (400 X g, 5 min, RT). Unless specified, the RBC pellet was finally resuspended in TBS-I as 1.5% suspension (v/v).

Preparation of Yeast Cell Suspension: 100 mg commercial grade baker's yeast (*Saccharomyces cerevisiae*) purchased from local market were suspended in 10 ml of 0.9% saline, washed extensively with saline by centrifugation (400 x g, 5 min, RT) and suspended in the same medium. The yeast cell suspension was heat-inactivated by autoclaving the suspension for 15 min at 15 psi. After cooling the suspension to room temperature, the heat-inactivated yeast cells were washed extensively with 0.9% saline and finally resuspended in TBS-I as 0.5% (v/v) suspension.

Trypsinization of Yeast Cells: 5 μl of washed yeast cells were suspended in 1 ml of TBS-I containing trypsin (0.5%) to give a final concentration of 0.5% yeast. This suspension was incubated for 1 h at 37°C with occasional gentle shaking. After incubation, the trypsinized yeast cells were washed once with TBS-I by centrifugation (400 x g, 5 min, RT) and finally resuspended in TBS-I as 0.5% (v/v) suspension.

Haemagglutination (HA) Assay: HA assays were performed in V-bottom microtiter plates (Greiner, Nürtingen, Germany) by serial two-fold dilution of a 25 μl serum sample with an equal volume of TBS-I. After dilution, 25 μl RBC suspension was added to each well and incubated for 45 min at RT. The HA titers were recorded as the reciprocal of the highest dilution of the sample causing complete agglutination of RBC [36]. Controls for all assays consisted of the substitution of the sample by TBS-I. All the HA assays were performed in duplicate.

Yeast Agglutination Assay: The agglutinating activity of serum against yeast cells was performed in V-bottom microtiter plates by serial two-fold dilution of 25 μl serum with an equal volume of TBS-I. After dilution, 25 μl of 0.5% native or trypsinized yeast cell suspension was added to each well and incubated for 45 min at 26°C. Control consisted of substitution of serum with TBS-I. The agglutination of yeast cells by serum was assessed under microscope (40 x) and the agglutination titers were recorded as the reciprocal of the highest dilution of the sample causing complete agglutination.

Bacterial Agglutinating Activity: Frozen stock culture of bacteria were inoculated in TBS-I and incubated for 6 h. The broth cultures were then centrifuged (5,000 x g, 10 min). The pellet was collected and washed 3 times by centrifugation with TBS-I. The final concentration was adjusted to 1 x 10^8 cells ml^{-1} in TBS-I before use. Two-fold serial dilutions of serum samples were made in TBS-I. Then, 25 μl of each serum dilution was incubated with 25 μl bacterial suspension. The reaction mixture was incubated at 20 ±2°C for 1h. The appearance of clumps of bacteria was then recorded by microscopic examination (40 x). Agglutination titer was defined as the reciprocal of the last dilution giving evidence of agglutination after incubation. The negative controls comprised mixed equal volumes of bacterial suspension and TBS-I.

Divalent Cation Dependency and EDTA Sensitivity: Serum samples (each 300 μl) were dialysed (MW exclusion limit <10,000) extensively at 20°C against divalent cation-free TBS-II (50 mM tris-HCl, 135 mM NaCl, 300 mOsm) to examine cation dependency or in TBS-III containing 50 mM EDTA (50 mM tris-HCl, 72 mM NaCl, 40 mM $CaCl_2$, 300 mOsm) to test EDTA sensitivity of the agglutinating activity of serum. The samples dialysed against TBS-III

were subsequently re-equilibrated by dialysis in TBS-II. All the resulting dialysates were centrifuged (400 x g, 10 min, 20°C). The supernatant was tested for hemagglutinating activity using rabbit RBC in the presence of TBS that did or did not contain 10 mM $CaCl_2$, $MgCl_2$ (or) $MnCl_2$. A serum sample (300 µl) concurrently dialysed against, TBS containing 10 mM $CaCl_2$ (TBS-I) was also tested for the haemagglutinating activity against rabbit RBC in TBS-I.

HA -Inhibition Assays: Several carbohydrates were tested for their ability to inhibit serum HA activity. They were dissolved in TBS-III (50 mM tris-HCl, 115 mM NaCl, 50 mM EDTA, 300 mOsm) and if necessary, the pH was adjusted to 7.5 using concentrated NaOH. Serum samples were diluted with TBS-IV (50 mM tris-HCl, 5 Mm NaCl, 30 mM $CaCl_2$, 135 mOsm) to a HA titer of 4 against rabbit RBC. The inhibitor to be tested (25 µl) was serially diluted two-fold with an equal volume of diluted sample in microtiter plates and incubated for 1 h at RT. Rabbit RBC suspension (25 µl) was added to each well and kept for 3 h at RT. The minimal concentration of carbohydrate that completely inhibited HA activity was recorded.

Protein Determination: Total protein concentration was measured using bovine serum albumin (BSA) as a standard [37].

RESULTS

Serum HA Profile: The serum of estuarine crab *Charybdis lucifera* agglutinated a variety of mammalian RBC types. Among the various RBC types tested, the highest titer of 64 was obtained with rabbit erythrocytes. Sheep and goat RBC were agglutinated at relatively low titers (Table 1). However serum did not agglutinate ox RBC and the serum showed highest agglutinating activity against tripsinized yeast cells when compare to native yeast cells (Table 2).

Bacterial Agglutination: The serum strongly agglutinated *Vibrio fluvialis* (titer 8), weekly agglutinated *V. parahemolyticus*, *V. mimicus*, *Escherichia coli* and *Aerobacter aerogenes*. The serum did not agglutinate *Pseudomonas sp*, *Bacillus subtilis* and *Aerobacter aerogenes*. The results of bacterial agglutination was assessed using a phase-contrast microscope (Table 3).

Table 1: Haemagglutinating (HA) activity of serum from the estuarine crab *Charybdis lucifera* against various mammalian erythrocyte (RBC) types

RBC types tested	HA titer*
Rabbit	64
Mice	32
Rat	32
Human B	32
Buffalo	16
Human A	8
Human O	8
Horse	4
Goat	2
Sheep	2
Ox	0

* Based on 20 determinations for each RBC type

Table 2: Agglutinating activity of *Charybdis lucifera* serum against native and trypsinized yeast cells

Yeast cells tested	HA titer*
Native	8
Trypsinized	32

* Based on 20 determinations for native and trypsinized yeast cells

Table 3: Agglutinating activity of *Charybdis lucifera* serum against various bacterial species

Bacterial species tested	Bacterial agglutination* (O.D: 0.8)
Vibrio fluvialis	8
Vibrio alginolyticus	4
Vibrio vulnificus	4
Vibrio anguillarum	4
Vibrio parahemolyticus	2
Vibrio mimicus	2
Escherichia coli	2
Pseudomonas sp	0
Bacillus subtilis	0
Aerobacter aerogenes	0

* The assay was repeated six times for each bacterial species with identical results using samples from different preparations

Divalent Cation Dependency and EDTA Sensitivity: The serum tested in TBS containing 10 mM $CaCl_2$ (TBS-I) gave a haemagglutianation titer of 64 against rabbit RBC. When the serum was dialysed against TBS- I and then tested in the absence of divalent cation, the agglutination titer reduced to 8. But, this serum sample recovered it's HA activity only upon addition of Ca^{2+} to the reaction mixture. Further, substitution of Ca^{2+} with Mg^{2+} showed a considerable improvement in HA titer, while Mn^{2+} could not reverse the effect of EDTA treatment. The serum dialyzed against TBS-III containing 50 mM EDTA and

Table 4: Effect of divalent cations and EDTA on the haemagglutinating (HA) activity of serum of *Charybdis lucifera*

Serum sample tested	Cation (10 mM) in sample diluting and RBC suspension	HA titer*
1. Before dialysis	$CaCl_2$	64
2. After dialysis against divalent cation free TBS (TBS-II).	None	8
	$CaCl_2$	64
	$MgCl_2$	32
	$MnCl_2$	8
3. After dialysis against TBS+10 mM $CaCl_2$ (TBS-I)	$CaCl_2$	64
4. After dialysis against TBS+50 mM EDTA (TBS-III)	None	8
followed by dialysis against TBS-II	$CaCl_2$	32
	$MgCl_2$	4
	$MnCl_2$	4

* Determination using rabbit RBC and the results based on six determinations

Table 5: Inhibition of agglutinating activity (titer = 4) of serum from the estuarine crab *Charybdis lucifera* by various carbohydrates

Carbohydrates tested	Maximum concentration tested (mM)	Minimum inhibitory concentration (mM)*
Monosaccharides		
Simple sugars		
D-mannose	200	50
L-sorbose	100	100
D-fucose	100	100
L-fucose	100	50
Deoxy sugars		
L-rhamnose	200	50
N-acetyl sugars		
N-acetyl-D-glucosamine (GlcNAc)	200	50
N-acetyl-D-galactosamine (GalNAc)	200	100
N-acetyl-D-mannosamine (ManNAc)	200	100
Disaccharides		
Trehalose (glcα1 → 1 glc)	200	100
Cellobiose (glcβ1 → 4 glc)	200	100
β-gentiobiose (glcβ1 → 6 glc)	200	50
Sucrose	200	100
Palatinose (glcα1 → 6 fruc)	200	100
Melibiose (galα1 → 6 glc)	200	50
Lactose (galâ1 → 4 glc)	200	100

The following carbohydrates also did not inhibit the agglutinating activity and unless otherwise stated, all carbohydrate was tested at concentrations upto 200 mM: D-glucose, D-galactose, β-allose, D-fructose, D-glucosamine (GlcN), D-galactosamine (GalN), mannosamine (ManN), maltose (glcα1 → 4 glc), turanose (glcα1 → 3 fruc)

* The assay was repeated five times for each carbohydrate with identical results

Table 6: Agglutination–inhibition of *Charybdis lucifera* serum (agglutination titer = 4) by polysaccharides against rabbit RBC

Polysaccharides tested	Structural linkages	Maximum concentration tested (mg. ml^{-1})	Minimum inhibitory concentration (mg. ml^{-1})*
Mannan	(α 1-6 homopolymer of mannose)	1	0.25
Laminarin	(β 1-3 homopolymer of glucose)	1	0.50
Dextran T_{70}	(α 1-6,3,2 homopolymer of glucose)	1	0.50
Dextran T_{500}	(α 1-6,3,2 homopolymer of glucose)	1	0.50
Inulin	(α 2-6 homopolymer of fructose)	5	NI
Colominic acid	(α 2-8 homopolymer of Neu5Ac)	5	NI

* The assay was repeated three times for each polysaccharide with identical results using samples from different preparations

NI: No inhibition

tested in the absence of divalent cation, considerably lost its agglutinating activity against rabbit RBC (Table 4). Further the addition of Mg^{2+} or Mn^{2+} to this sample could not restore the original HA activity and addition of Ca^{2+}, rescued the activity to 8 (Table 4).

Carbohydrate Binding Specificity: Among the 24 carbohydrates tested, as many as 15 carbohydrates were found to inhibit serum haemagglutinating activity at concentrations ranging from 50 to 100 mM. All the three acetylated hexosamines (GlcNAc, GalNAc and ManNAc), but not their hexoses and hexosamine counterparts, were inhibitory at 50 or 100 mM. But the few sialic acids examined in this study and 9 other carbohydrates were not inhibitory when tested up to concentrations from 20 to 200 Mm (Table 5). Among the six different polysaccharides tested (Table 6), only mannan, laminarin, Dextran T_{70} and Dextran T_{500} inhibited the HA activity at 0.25 and 0.50 $mg.ml^{-1}$, respectively. Among all the inhibitory Carbohydrates, mannan was found to be most potent.

DISCUSSION

The serum of the estuarine crab *Charybdis lucifera* was found to possess naturally occurring agglutinating activity which showed the highest reactivity with rabbit RBC among other RBC types tested. These results also suggest that the RBC types agglutinated by the serum of *C.lucifera* probably share a common surface receptor but with a quantitative difference in its HA binding sites. The serum agglutinated a variety of bacteria including Gram +ve and –ve types and the species of *Vibrio* tested are known to be the most frequent opportunistic pathogens of aquatic crustaceans [38, 39] and the plasma showed highest agglutinating activity against tripsinized yeast cells [17]. The ability of the serum of *C.lucifera* to agglutinate bacteria, particularly the potential pathogens, implicates a possible involvement of the humoral agglutinins in host defense response.

The serum lost most of it's HA activity after dialysis against cation-free TBS and when tested in the absence of cations. However, the activity in this sample completely regained only upon addition of Ca^{2+} and the HA titer of serum did not change after dialysis against TBS containing Ca^{2+}. These observations demonstrated that the serum agglutinin of *C.lucifera* specifically requires Ca^{2+} for it's HA activity. Furthermore, the activity was sensitive to EDTA treatment, since dialysis of serum against TBS containing EDTA resulted in a significant reduction in the HA activity. None of the cations tested could restore the HA activity, albeit Ca^{2+} moderately rescued the activity in these samples, thereby indicating that the HA of *C.lucifera* appears to be irreversibly sensitive to EDTA which is in contrast with other crustacean agglutinins [40, 41, 42].

Crustacean serum agglutinins were shown to be specific for fucose [43], glucose [44], galactose [42, 44], GalNAc [39, 43], or sialic acids such as NeuAc [33, 45, 46, 47, 48], 4- and 9-0-acetyl NeuAc [41], 9-0-acetyl NeuAc [49] and NeuGc [50]. The haemagglutination-inhibition test performed in this study using different carbohydrates, encompassing several diverse unrelated monosaccharides and their derivatives as well as di- and oligo-saccharides inhibited the serum agglutinating activity. Furthermore, all the three acetylated hexosamines tested consistently inhibited the HA activities of crab serum [50]. The serum HA activity of *C. lucifera* was not inhibited by the amino sugar tested. But it was inhibited by the simple hexoses namely mannose, L-sorbose, D-fucose and L-fucose. The C-l position of these hexoses is essential for interaction with the agglutinin. The amino derivatives (GlcN, GalN and ManN) did not inhibit the HA activity. However, their N-acetyl derivatives (GlcNAc, GalNAc and ManNAc) were able to inhibit the serum HA activity. The disaccharides D-maltose and turanose failed to inhibit the HA activity but all other disaccharides were inhibitory. All these observations clearly demonstrate that the presence of acetyl group at C-2 position of hexosamines does not favour the interaction with agglutinin whereas this position with a free hydroxyl group or its substitution with amino group is essential for the interaction.

HA inhibition tests employing polysaccharides indicated that only laminarin and mannan inhibited the serum agglutinating activity. This indicates that the agglutinin molecules in crab serum tend to exhibit affinity for extended structures particularly for polysaccharides with β-linked hexoses. Thus, all the results obtained from the inhibitory effects of various carbohydrates and glycoproteins taken together clearly indicate that the agglutinins present in the serum of *C. lucifera* interact with a wide range of carbohydrates including acetylated hexosamines, acetylated or non-acetylated sialic acids and several other carbohydrates and their preference for a specific carbohydrate structure, therefore, could not be ascertained. However, these findings in turn strongly suggest the natural occurrence of multiple agglutinins in the serum of this crab.

The agglutinins in several crustaceans have been characterized [6, 8, 10, 33, 48, 51-54]. This agglutinin appears to be unique among all the known crustacean agglutinins. Thus, based on the haemagglutination and bacterial agglutinating activity of the serum agglutinin, it is possible that this component of the crab is probably involved in non-self recognition and eliciting immune response in the mud crab *C. lucifera,* against invading pathogens. The identification of immune effectors like agglutinin and the understanding of their regulation in response to infection will open the way to the selection of pathogen resistant animals. This can be achieved through the characterization and purification of this novel agglutinin of *C. lucifera* as a prerequisite to elucidate the immunological roles of crustacean agglutinins.

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