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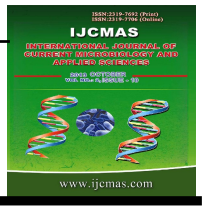

Original Research Article

Characteristics of serum agglutinins in marine crab *Scylla serrata* (Forskal) and their interaction with various bacteria species

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Keywords

Multiple-agglutinins; hemagglutination; bacterial agglutination; serum agglutinin; crustacean; divalent cation dependent; carbohydrates; glycoproteins; host defense.

A B S T R A C T

Agglutinins or lectins are conventionally defined as proteins/glycoproteins of non-immune origin with a remarkable ability to specifically and reversibly interact with carbohydrate ligands. Although lectins are produced by a myriad of organisms, some of which are phylogenetically remote, and therefore exhibit distinct physicochemical and biochemical characteristics, lectins from different sources may essentially exhibit common biological activities. This study was therefore undertaken to preliminarily characterize the lectins in the serum of *S. serrata* as a prerequisite for isolation, purification and biological activity evaluation. Multiple agglutinins were detected in the serum of the marine crab *Scylla serrata*. The serum agglutinins agglutinated a wide range of mammalian erythrocytes (RBC). The hemagglutinating activity was high within a pH range of 7.0 to 8.5, and was dependent on divalent cations Ca^{2+}, Sr^{2+} & Mg^{2+}, with preference for Ca^{2+}. An agglutinating activity independent of Ca^{2+} was also demonstrated, indicating the presence of heterogenous agglutinins .The serum HA activity was inhibited by a wide range of related and unrelated carbohydrates, suggesting the presence of multiple agglutinins. The serum agglutinins reacted/interacted with a variety of bacteria including the *Vibrio* species known to be the most frequent opportunistic pathogens of aquatic crustaceans.

Introduction

Agglutinins or lectins are conventionally defined as proteins/glycoproteins of non-immune origin with a remarkable ability to specifically and reversibly interact with carbohydrate structures present on cell surfaces, extracellular matrices or secreted glycoproteins (Goldstein *et al.*, 1980; Barondes, 1988; Sharon and Lis, 1995; Weis,. 1997; Goldstein 2002). They have also been demonstrated to additionally interact or recognize non-carbohydrate ligands bearing an appropriate structural

determinants (Gabius, 1997; Kawagishi *et al*., 1994; Zhang *et al*., 1995; Gokudan *et al*., 1999; Murali *et al*., 1994; Maheswari *et al*., 2002). The lectins may possess mono-, di-, or multi-valent carbohydrate binding sites, whereas agglutinins possess di- or multi-valent sites. The latter structural characteristic is an essential prerequisite for agglutinin molecules to cause agglutination of cells displaying on their surfaces more than one saccharide of sufficient complementarity (Barondes, 1981).

Lectins are produced by a myriad of organisms, some of which are phylogenetically remote, and exhibit distinct physicochemical and biochemical characteristics although lectins from different sources may essentially exhibit common biological activities. Various studies with lectins at molecular levels are in progress all with ultimate goal of not only the identification and structural characterization of all of their natural ligands, and the elucidation of the nature of the relevant binding interactions at atomic resolution, but also a full understanding of the biological roles of these molecules. The latter must eventually be confirmed by natural or genetically manipulated mutations in the expression of the lectins and/or their ligands in intact animals.

The biological functions of agglutinins are attributed primarily to their ability to recognize and bind to specific carbohydrate structures (Sharon and Lis. 1989). Invertebrate agglutinins have been implicated in a variety of endogenous biological functions. These include feeding, larval settlement, fertilization, and diverse endogenous functions such as cell aggregation, embryonic development, metamorphosis, regeneration, wound repair and transport of carbohydrates (Yeaton, 1981; Rögener and. Uhlenbruck. 1984; Olafsen, 1986; Vasta, 1991). Based on these inherent properties, lectins are not only already being exploited in identifying cells with specified glycan signatures, but also in quantifying these carbohydrate motifs. New and efficient techniques for cell separation/enrichment based on the conjugation of lectins to magnetic particles and affinity columns have been developed (Galvez *et al*. 2002; Shinya *et al*. 2004; Bakalova & Ohba 2003; Putnam et al. 2003). Lectins are also major components of the innate immune system of several decapod crustaceans, including lobster, crabs, shrimps and freshwater crayfish (Bang, 1962; Cornick, and Stewart. 1968; Ratcliffe, *et al*. 1985; Richards, and. Renwrantz. 1991; Mercy and Ravindranath 1994; Wilson, *et al*. 1999) where they defend these invertebrates from invading bacterial, fungal and viral pathogens (Stewart and Zwicker, 1972; Noga *et al*., 1996).

Naturally occurring agglutinins in the serum of the marine crab *S. serrata* have been studied previously by different investigators, which mainly involved detection and isolation of single humoral agglutinins. In these independent studies, the isolated agglutinins were reported to express specificity for Neu5Gc (Mercy, and. Ravindranath. 1993) and sialoglycoconjugates such as fetuin (Chattopadhyay and Chatterjee, 1993) or BSM (Kongtawelert, 1998). Although the actual carbohydrate structures recognized by the two agglutinins reactive with sialoglycoconjugates remain unresolved, these findings, from different laboratories suggest the possible occurrence of at least three agglutinins in the serum of *S. serrata*. The role of Neu5Gc-specific agglutinin in hemocoelic clearance of

foreign materials has been investigated by Mercy and Ravindranath (1994), but the involvement of two other agglutinins in immuno-defense functions in *S. serrata* has not been examined. This study was therefore undertaken to preliminarily characterize the lectins in the serum of *S. serrata* as a prerequisite for isolation, purification and pharmacological studies.

Materials and Methods

Acquisition and Preparation of Reagents and buffers

Carbohydrates (mono-, di-, tri- & polysaccharides) were products of s.d. Fine, BDH, CDH & Qualigens (Bombay, India) or Sigma (St. Louis, Missouri, USA). Glycoproteins (bovine submaxillary mucin (BSM- Type II), asialo-mucin, fetuin, asialo-fetuin, porcine thyroglobulin, porcine stomach mucin (PSM- Type III) and ovalbumin), Ethylene glycol-bis (β-aminoethyl ether) N, N, N^1, N^1 - tetraacetic acid (EGTA) and bacterial species (*Vibrio anguillarium, V.alginolyticus, V.parahaemolyticus, V.fluvialis, V.vulnificus, V.mimicus, Escherichia coli, Pseudomonas sp Bacillus subtilis and Aerobacter aerogenes*- Type I) were purchased from Sigma All other chemicals and reagents used were of the highest analytical grade and were supplied by local agencies. The chemicals were used to prepare tris-buffered saline specified in table 1.

All these buffer solutions contained 0.02% NaN_3, and the osmolarity were determined using a cryoscopic osmometer (osmomat 030, gonotec, Germany) and adjusted if necessary by either dissolving a few crystals of NaCl or diluting with distilled water. All buffers were stored at 10^0C, and used within a week. Other solutions used were saline (0.9% NaCl & 0.02% NaN_3) and alsever's solution (Garvey et al. 1979). Alsever's solution was autoclaved at 15 psi for 15 min, cooled to room temperature (RT = 26 $\pm$ 2^0C), then 50 mg streptomysin were dissolved in the solution and stored at 10^0C until use.

Crabs and preparation of serum

Agile, uninjured, non-autotomized and intermoult male marine crabs (*Scylla serrata)* weighing 200-300 g were purchased from a local fish market in Chennai- India. The crabs were maintained in plastic tanks containing filtered sea water (salinity; 30%) and fed *ad libitum* with the surface clam *Donax sp*. The sea water was aerated continuously and changed daily. The tip of the dactylus region of the walking leg was wiped with ethanol–soaked cotton, dried with tissue paper and cut with a sharp pair of scissors. The exuding hemolymph was collected in tubes held on ice and allowed to clot at room temperature (RT). The clot was vigorously disrupted using a glass rod, and centrifuged (450 x g, 10 min, RT). The resulting clear supernatant (serum) was used immediately.

Preparation of erythrocyte (RBC) suspension

Human A, B, O, sheep, goat, ox, buffalo, horse, rabbit, rat and mouse blood samples were obtained by venous or cardiac puncture where applicable. All the blood samples were collected in sterile Alsever's solution (Garvey *et al*. 1979) containing 10 μg/ml of streptomycin, stored at 10^0C and used within five days. The Alsever's solution was decanted and replaced daily. RBC in the blood samples were washed thrice with 0.9% saline and once with TBS-IV (containing 10 mM $CaCl_2$) by

centrifugation (400 x g, 5 min, RT). Unless otherwise specified, the washed RBC pellet was finally re-suspended in 5 ml TBS-IV (containing 10 mM $CaCl_2$) as 1.5% (v/v).

De-O-acetylation of bovine submaxillary mucin (BSM)

De-O-acetylation of BSM was performed following the procedure of Sarris & Palade (1979). Briefly, 5 mg of BSM were dissolved in 375 µl of TBS-IV (containing 10 mM $CaCl_2$) mixed with 125 µl of 0.4 N NaOH (10 mg BSM.ml^{-1}) and incubated on ice for 45 minutes. The solution was brought to room temperature and then neutralized by adjusting the pH to 7.2 using 0.1 N HCl. Finally, the solution was dialysed (MW exclusion limit < 3000-5000 Da) extensively against the same buffer and the dialysate represented de-O-acetylated BSM.

Hemagglutination (HA) assays

The hemagglutination assays were performed in V-bottomed microtiter plates (Greiner, Nürtigen, Germany) by serial dilution of 25 µl serum samples with an equal volume of appropriate TBS. 25 µl of RBC suspension was added to each well and incubated for 1 hr at RT. The HA titers were recorded as the reciprocal of the highest dilution of the samples causing complete agglutination of RBC (Garvey *et al*. 1979). Controls for all assays consisted of the substitution of the sample by TBS. Each experiment was performed in duplicate for at least three times using samples from different preparations, and the hemagglutination activities were analyzed based on the median titer values. The assay was repeated with TBS- IV containing 10 mM $CaCl_2$ at different pH (ranging between 6.5 and 8.5).

Divalent cation dependency and EDTA/EGTA sensitivity

Serum samples (each 250 µl) were dialysed (MW exclusion limit < 10,000 Da) extensively against cation-free TBS (TBS –I) at 15^oC to examine cation dependency, or against TBS-VI containing 50 mM EDTA to test its sensitivity to EDTA. The samples dialysed against TBS-VI were subsequently re-equilibrated by dialysis against TBS-I. All the resulting dialysates were centrifuged (400 x g, 5 min, RT) and the hemagglutinating activity in the supernatant was determined using human B RBC in the presence of buffer that did (TBS-IV) or did not (TBS-I) contain 10 mM $CaCl_2$ or $SrCl_2$ or $MgCl_2$. EDTA sensitivity was also analysed by two-fold serial dilution of 25 µl serum sample in TBS-VI containing 25 or 50 mM EDTA and mixing with an equal volume of human B RBC in the corresponding buffer. For control, the EDTA-containing buffers were substituted by TBS-IV (containing 10 mM $CaCl_2$). The effect of EGTA was similarly analysed by serial dilution of 25 µl serum samples in TBS containing 25 or 50 mM EGTA (TBS-VII). For controls, the EGTA containing buffers were substituted by TBS-IV containing 10 mM $CaCl_2$.

Effect of different concentrations of divalent cations on HA activity of the serum

HA activity was assayed as described above. The effect of different concentrations of divalent cations was assayed by serially diluting the serum sample and adding human B RBC suspensions containing different cations at various concentrations (TBS-IV and TBS-V). The assay was repeated using rabbit, mouse and rat RBC.

Hemagglutination - Inhibition (HAI) assays

Several simple sugars and their derivatives (dissolved in TBS-VIII), polysaccharides and glycoproteins (dissolved in TBS-IV containing 10 mM, $CaCl_2$, pH 7.2.) were tested for their ability to inhibit serum hemagglutinating activity. The pH of the test substances was adjusted, wherever necessary to 7.2 using small crystals of NaOH. Serum was diluted with TBS-V containing 10 mM $CaCl_2$, pH 7.2 or 50 mM $CaCl_2$ pH 8.0 to hemagglutination titer of 4 against human B RBC. 25 µl of the diluted serum was added to each well and an equal volume (25 µl) of the test substance was serially diluted with the serum in the titer plate. After 1 hr incubation at RT, 25 µl of human B RBC suspension (prepared in TBS-IV containing 10 or 50 mM $CaCl_2$) was added to each well and incubated further for 1 hr at RT. The inhibitory potency of the test substance is expressed as the minimal concentration of the substance causing inhibition of the hemagglutinating activity.

Bacterial agglutination assays

Cultures of eight bacterial species; namely *Vibrio anguillarium, V.alginolyticus, V.parahaemolyticus, V.fluvialis, V.vulnificus, V.mimicus, Escherichia coli* and *Pseudomonas sp.* isolated from sea water and harvested at their stationary phase were used. An aliquot (3 ml) of each bacterial culture in nutrient broth was washed thrice in 0.9% saline by centrifugation (4,000 x g, 20 min, 4^oC). The pelleted bacteria were resuspended in 3 ml of saline, heat-killed (1hr, 60^0C) and then washed once with TBS-IV (containing 10mM $CaCl_2$, pH 7.2) by centrifugation as described above. Other two bacterial species *Aerobacter aerogenes*-Type I and *Bacillus subtilis* were suspended in appropriate volume of the same buffer. The optical density of all the bacterial suspensions were adjusted to 0.8 (=1 x 10^7 cells. ml^{-1}) at 580 nm.

Bacterial agglutination assay was performed in slides. 50 µl of serum sample mixed with an equal volume of each bacterial suspension in the cavity of glass slide. These slides were kept in moist chamber and incubated overnight at RT. The agglutination of bacteria was examined under phase optics (400 x) in a Carl Zeiss (Axiolab) phase contrast microscope, and the reaction was scored qualitatively based on the size and the number of bacterial aggregates observed.

Result and Discussion

Hemagglutination profile of the serum

The serum agglutinated all the mammalian RBC types tested, except ox RBC. It gave moderate to high HA titers for buffalo, rat, human B, mouse and rabbit RBC (titers =16 to 128). Sheep, goat, horse, human O and A RBC were weakly agglutinated (titers = 2 to 8).

Effect of pH, divalent cation and EDTA/EGTA on serum HA activity

The HA titer of serum was 32 or 64 within a pH range of 7.0 to 8.5 and the activity decreased to a titer of 8 at pH 6.5 (Figure 1). The HA sample dialysed against TBS-I and then tested in the absence of divalent cations did not show any HA activity (Table 2). However, the HA activity was restored in the presence of $CaCl_2$, $SrCl_2$ or $MgCl_2$, but it was completely restored only with $CaCl_2$. Similar results were obtained with serum samples dialyzed against TBS-VI containing 50 mM EDTA. In serial

Table.1 Types of tris-buffered saline (TBS) used to characterize the serum agglutins from the marine crab *Scylla serrata*

Type	**Specifications**
TBS-1	50 mM Tris-HCl, 125 mM NaCl (pH 7.2, 300 mOsm)
TBS –II:	100 mM Tris- HCl, 230 mM NaCl (pH 7.2, 560 mOsm);
TBS – III	100 mM Tris HCl, 200 mM NaCl (pH 7.2, 500 mOsm);
TBS – IV	Several buffers were prepared consisting of 50 mM Tris-HCl, and different concentrations of NaCl, $CaCl_2$, or $MgCl_2$ or $SrCl_2$ at different pH values and all at 300 mOsm such as: 115 mM NaCl, 10 mM $CaCl_2$ (pH 6.5, 7.0, 7.5, 8.0, 8.5); 90 mM NaCl, 25 mM $CaCl_2$ or $MgCl_2$ or $SrCl_2$ (pH 7.2); 50 mM NaCl, 50 mM $CaCl_2$(pH 7.2 or 8.0); 50 mM NaCl, 50 mM $MgCl_2$ or $SrCl_2$ (pH 7.2); 115 mM NaCl, 10 mM $CaCl_2$ or $SrCl_2$ or $MgCl_2$ (pH 7.2);
TBS – V	50 mM Tris-HCl, 10 mM NaCl, 100 mM $CaCl_2$ or $SrCl_2$ or $MgCl_2$ (pH 7.2, 370 mOsm);
TBS VI	50 mM Tris-HCl, 115 mM NaCl, 25 mM EDTA or 50 mM Tris-HCl, 100 mM NaCl, 50 mM EDTA (pH 7.2, 300 mOsm)
TBS- VII	50 mM Tris-HCl, 115 mM NaCl, 25 mM EGTA or 50 mM Tris HCl, 100 mM NaCl, 50 mM EGTA (pH 7.2, 300 mOsm)
TBS- VIII	50 mM Tris-HCl, 30 mM NaCl, 10 mM $CaCl_2$ (pH 7.2, 135 mOsm).

Table.2 Divalent cation dependency and EDTA sensitivity of hemagglutinating activity of *Scylla serrata* serum.

Treatment of serum	**Divalent cations tested**[@]	**HA titer***
Untreated serum (before dialysis)	$CaCl_2$	32
Serum dialysed against cation-free TBS	None	0
(TBS-I)	$CaCl_2$	32
	$SrCl_2$	16
	$MgCl_2$	16
Serum dialysed against TBS + 50 mM	None	0
EDTA (TBS-VI) and re-equilibrated by	$CaCl_2$	32
dialysis against TBS-I	$SrCl_2$	16
	$MgCl_2$	16
Control (untreated sample) held for 48h at 10^0C	$CaCl_2$	32

*Data represent median values from three determinations with human B RBC using serum samples from different preparations.

Table.3 Hamagglutination-inhibition of *Scylla serrata* serum (hemagglutination titer = 4) by glycoproteins against human B RBC

Glycoproteins tested	**Maximum Concentration tested (mg.ml^{-1})**	**Predominant terminal sugar residues**	**Minimum inhibitory concentration (mg.ml^{-1})***
Porcine thyroglobulin	10	Neu5Gc	1.250
Fetuin	10	Neu5Ac α(2-3) Gal	0.156
Asialo-fetuin	5	Gal	0.312
Bovine submaxillary mucin (BSM)	10	O-IN-AcNeu5Ac α(2-6) GalNAc	0.156
Asialo-BSM	5	GalNAc	0.312
De-0-acetylated BSM	5	N-AcNeu5Ac α(2-6) GalNAc	0.312
Porcine stomach mucin (PSM)	10	Neu5Gc	0.625
Ovalbumin	10	GlcNAc	2.500

*The assay was performed three times for each glycoprotein

Figure. 1 Effect of pH on hemagglutinating activity of the serum of *Scylla serrata*.

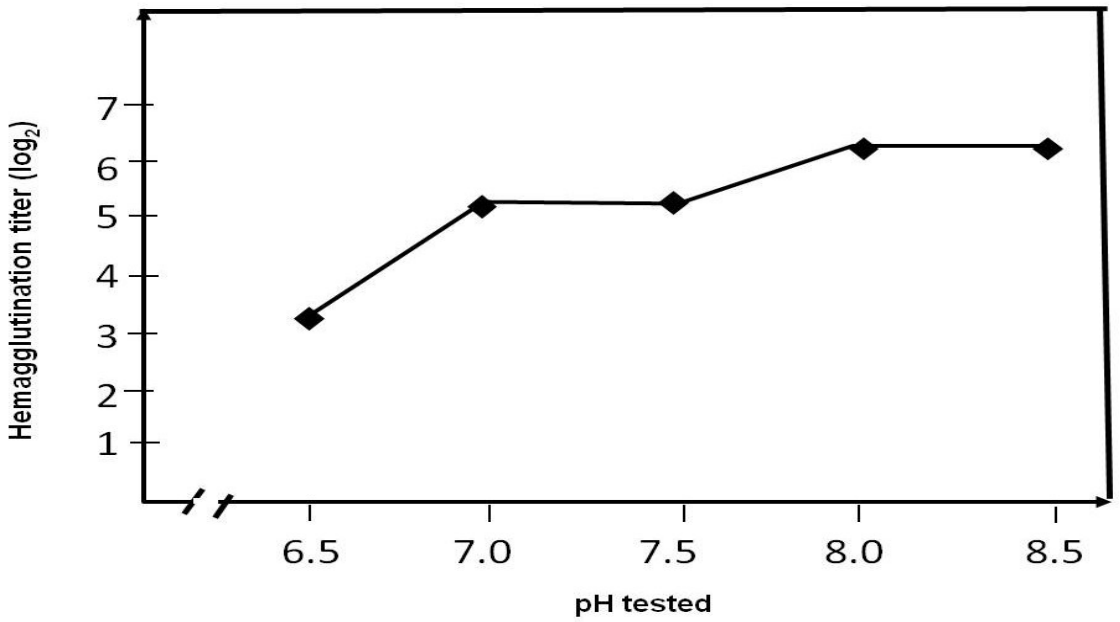
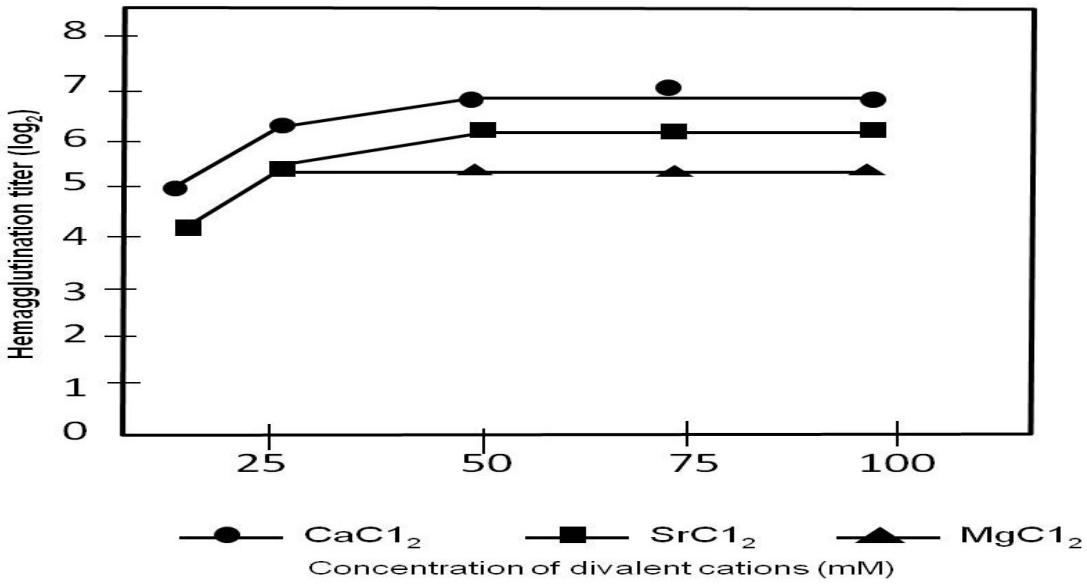


Figure.2 Effect of different concentrations of divalent cations on hemagglutinating activity of the serum of *Scylla serrata.*



dilution experiment, both 25 and 50 mM EDTA completely abolished the serum HA activity whereas a residual activity (titer = 4) was observed with EGTA tested at identical concentrations. When tested in the presence of other cations, it was observed that HA activity of serum against human B RBC increased steadily from a titer of 32 or 16 in the presence of 10 mM $CaCl_2$ or $SrCl_2$ to 128 or 64 at 50 mM $CaCl_2$ (Figure 2). However, further increase in the concentration of these two divalent cations up to 100 mM did not enhance the HA activity of the serum. On the other hand, the HA activity increased only marginally from 10 mM to 25 mM $MgCl_2$ but in the presence of 50 mM $CaCl_2$, HA of the serum was enhanced from a HA titer of 128 to 256 (rabbit RBC), 64 to 128 (mouse RBC) and 32 to 128 (rat RBC).

Hemagglutination - inhibition (HAI) assays

Among the 41 carbohydrates tested, 25 carbohydrates were found to inhibit serum hemagglutination activity at concentrations ranging from 12.5 to 100 mM (Table 3). Only the acetylated hexosamines (GlcNAc, GalNAc and ManNAc) were inhibitory at 50 or 100 mM. Among the six different polysaccharides tested [i.e Laminarin (β 1-3 homopolymer of glucose), Mannan (β 1-6 homopolymer of mannose) Dextran T_{70} (α 1-6,3,2 homo- polymer of glucose), Dextran T_{500} (α 1-6,3,2 homo- polymer of glucose), Inulin (α 2-6 homopolymer of fructose) and Colominic acid (α 2-8 homopolymer of Neu5Ac)], only mannan and laminarin inhibited the HA activity at 0.25 and 0.50 $mg.ml^{-1}$, respectively. In contrast, all the eight glycoproteins tested were inhibitory at various concentrations in which fetuin and BSM were the most potent inhibitors (0.156 $mg.ml^{-1}$). The patterns of inhibition observed with various simple sugars and their derivatives, polysaccharides, and glycoproteins were essentially same in the assays performed with TBS-IV (containing 10 or 50 mM $CaCl_2$, pH 7.2 or 8.0)

Bacterial agglutination profile of the serum

All the ten bacterial species tested, comprising both Gram -ve and Gram +ve bacteria, were agglutinated by serum of *S. serrata*. The highest degree of agglutination was observed with *Vibrio fluvialis* whereas moderate agglutination was seen with *V.alginolyticus, V.vulnificus, V.anguillarum,* and *Bacillus subtilis*. Other bacterial species including *V.parahemolyticus, V.mimicus, E.coli, Pseudomonas sp.* and *A. aerogenes,* were weakly agglutinated by the crab serum.

The serum was found to agglutinate various mammalian erythrocytes with high reactivity against rabbit, mouse, human B and rat RBC. These results concur with those from the previous study (Mercy and. Ravindranath. 1992). The serum agglutinating activity was high within a pH range of 7.0 to 8.5, and lacks specificity for human blood groups, a feature that appears to be common among crustaceans (Sarris and Palade, 1979). Since cross adsorption tests demonstrated the efficiency of human B RBC in adsorbing from serum all agglutinating activity for this and other RBC types (Mercy and. Ravindranath. 1992), this RBC was used as a suitable indicator cell type for all subsequent experiments performed.

Several/Related studies on humoral agglutinins in crustaceans revealed that their activity is dependent on divalent cations, usually calcium ions, and sensitive to divalent cation chelators such as EDTA and EGTA (Smith and. Chisholm. 1992; Marques and Barraco. 2000). However, few exceptions to these features have been reported (Imai *et al*., 1994; Maheswari *et al*. 1997; Kondo *et al*,. 1992). Dialysis experiments performed in the current study against cation-free TBS demonstrated that the HA activity in the crab serum is dependent on divalent cations such as Ca^{2+}, Sr^{2+} or Mg^{2+} since the activity was not detected in the absence of these divalent cations. Moreover, the activity was recovered upon addition of these cations, but complete recovery was achieved only with $CaCl_2$, suggesting that the serum HA activity exhibits requirement for divalent cations with a preference for Ca^{2+}. Similar results were obtained from dialysis experiments performed with EDTA, thereby indicating that the serum HA activity is reversibly sensitive to EDTA, a divalent cation chelator. Furthermore, serial dilution experiments performed with both EDTA and EGTA indicated that the serum HA activity is sensitive to EDTA, whereas it is only partially sensitive to EGTA (Ca^{2+} chelator). This finding suggests the possible occurrence of an agglutinating activity independent of Ca^{2+}, thus indicating the presence of at least two agglutinins, which are heterogeneous with regard to Ca^{2+}requirement. The results obtained with various concentrations of different divalent cations, demonstrated that HA activity increased in the presence of especially $CaCl_2$ or $SrCl_2$ in a dose-dependent manner, with a maximal activity detectable at 50 mM a further indication of the presence of multiple agglutinins.

In hemagglutination–inhibition assays performed in this study, several diverse and unrelated monosaccharides and their derivatives as well as di- and oligosaccharides inhibited the serum agglutinating activity. In addition, all the three acetylated hexosamines (GlcNAc, GalNAc and ManNAc), but not their hexose and hexosamine the counterparts

tested, consistently inhibited the HA activity of crab serum, thereby suggesting affinity of the serum agglutinin(s) for acetylated aminosugars. It is also notable that the two sialic acids Neu5Ac and Neu5Gc tested were not inhibitory even upto concentrations of 40 and 20 mM, respectively. By contrast, Mercy & Ravindranath (Mercy, and. Ravindranath. 1992) demonstrated the inhibitory activity of Neu5Gc at 0.6 mM as well as inability of all the three acetylated hexosamines to inhibit the serum HA activity of *S. serrata*. The exact reason for these discrepancies between the two studies is not clear at present. Further studies with polysaccharides indicated that only laminarin and mannan inhibited the serum agglutinating activity, albeit weakly, while free glucose and mannose were non-inhibitory, indicating that the agglutinin molecules in crab serum tend to exhibit affinity for extended structures particularly for polysaccharides with β-linked hexoses.

Further inhibition tests were performed with glycoproteins comprising eight sialo- and asialo-glycoconjugates, whose oligosaccharide side chains, as reported earlier (Schauer, 1982; Tsuji and. Osawa. 1986; Corradi *et al*.,1995), contain predominantly terminal Neu5Gc (thyroglobulin and PSM) and Neu5Ac (fetuin, BSM and de-O-acetylated BSM), galactose (asialo-fetuin) and GlcNAc (ovalbumin). In this inhibition assay, the serum agglutinin(s) appear to show higher affinity to sialoglycoproteins terminated with Neu5Ac (fetuin and BSM) or Neu5Gc (PSM and thyroglobulin) as well as desialylated glycoproteins, namely, asialofetuin and asialo-BSM terminated with Gal and GalNAc respectively. These observations suggest that the serum agglutinin(s) recognize two types of sialic acids Neu5Ac or Neu5Gc which is apparent in inhibition studies with glycoproteins, but not with free sialic acids. Thus all the results obtained from the inhibition studies clearly indicate that the serum agglutinins of *S. serrata* interact with a wide range of carbohydrates including acetylated hexosamines, acetylated or non-acetylated sialic acids and several other carbohydrates, and therefore their specificity for a specific carbohydrate structure could not be ascertained. These findings however strongly suggest the natural occurrence of multiple agglutinins in the serum of this crab.

It is also interesting to note that serum agglutinated a variety of bacteria including Gram +ve and Gram -ve types and the species of *Vibrio* tested are known to be the most frequent opportunistic pathogens of aquatic crustaceans (Egidius, 1987). The ability of the serum of *S. serrata* to agglutinate the bacteria, particularly the potential pathogens implicates a possible involvement of the humoral agglutinins in host defense response. It is therefore concluded that heamolymph extract of the marine crab S*cylla serrata* exhibits properties indicative of multiple lectins and further studies are recommended to isolate and characterize the individual lectins.

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