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Physico-Chemical Characterization of a naturally occurring Hemagglutinin in the Serum of Sand Lobster, *Thenus orientalis* with Affinity for N-Acetylated Aminosugars

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A B S T R A C T

A naturally occurring serum hemagglutinin was detected in the sand lobster, *Thenus orientalis*. The serum hemagglutinating activity was highest with buffalo erythrocytes. Preliminary characterization of the serum hemagglutinating activity showed that the activity was independent of calcium ions. However, there was reduced activity when exposed to divalent cationchelator, such as, EDTA and the activity was effectively restored with calcium ions. The HA activity of the lobster serum was stable between pH 7 and 9 and showed thermal stability between 20 and 40°C. Haemagglutination-inhibition assays performed with several carbohydrates revealed that the serum agglutinin was specific for *N* - acetylated amino sugars and that the presence of the *N*-acetylation was essential for agglutinin-ligand interaction. Also, the haemagglutinating activity of the serum could be specifically inhibited by lipopolysaccharides from various gram negative bacteria.

Introduction

The immune system of vertebrates is capable of expressing adaptive immunity based on clonal expansion of activated lymphocytes. Invertebrates do not possess such an immune system, and these animals, therefore, rely on innate immune mechanisms for internal defense to protect themselves against various infectious agents (Rowley and Powell, 2007; Ghosh *et al.*, 2011).

Despite these limiting features, the internal defense system of invertebrates, especially arthropods and molluscs, have developed unique abilities to recognize rapidly and react effectively against a vast range of biotic and abiotic foreign materials (Coombe *et al.*, 1984; Mullaindhan and Renwrantz, 1986).

Agglutinins are proteins/glycoproteins that have the ability to recognize and bind reversibly to specific structural (usually a carbohydrate) determinants present on cell surfaces, extra cellular matrices, and secreted glycoproteins (Goldstein *et al.*, 1980; Barondes, 1988; Wu *et al.*, 1988; Sharon and Lis, 1995; Weis, 1997).In the invertebrate defense mechanism, lectins are considered to be molecules of immunological importance in

the discrimination of non-self from self. Agglutinins are common among all groups of living organisms. They are seen in microbes (Esko and Sharon, 2009), plants (De Hoff *et al.*, 2009), animals (Kilpatrick, 2002) and humans (Turner, 1996).The primary mode of action of agglutinins is attributed to their ability to recognize and bind to specific carbohydrate structures (Sharon and Lis, 1995). In invertebrates, agglutinins are involved in physiological functions such as, wound repair and immunological function such as opsonization (Vasta, 1991; Kondo *et al.*, 1992, Wang *et al.*, 2014; Denis *et al.*, 2015). In pharmaceutical industries, lectins are also used as diagnostic tool to detect conditions like cancer (Mody *et al.*, 1995)

Among invertebrates, crustaceans are being most extensively examined in recent years for their immune responsiveness, because of size, wide distribution in varying habitats, and potential for intensive aquaculture (Rowley and Pope, 2012).The internal defense system of crustaceans is known to recognize an array of foreign invaders and eventually express various types of immune responses mediated by both humoral and cellular immune components (Vázquez *et al.*, 2009). Humoral agglutinins in crustaceans are frequently studied due to their ubiquitous occurrence in circulating hemolymph, involvement in hemocoelic clearance of pathogens, and functional resemblance with pattern-recognition proteins or PRPs (Wang *et al.*, 2009; Wang and Wang, 2013). Therefore, agglutinins form a major component of the innate immune system of several decapod crustaceans.

Isolation of agglutinin in native form is a prerequisite to study their functional significance. This study was aimed at characterization of serum hemagglutinating activity of the sand lobster, *Thenus orientalis* in order to develop strategies to isolate the serum agglutinin in native form.

Materials and Methods

Experimental animal and preparation of lobster serum

Sand lobster, *Thenus orientalis* is available throughout the year. Live specimens of sand lobster, *Thenus orientalis* (body length of 12-15 cm), irrespective of sex, were purchased from fishermen in Royapuram fish landing centre, Chennai. Hemolymph samples from healthy lobsters were collected by inserting a sterile pre-chilled 2-ml polypropylene sterile syringe with a 26 G needle directly into the ventral sinus. The hemolymph sample drawn from each lobster was transferred to Eppendorf tube and allowed to clot for 10 min at room temperature (RT: 28 ± 2ºC). The clot was disturbed repeatedly using a clean glass rod and then centrifuged (400 x *g*, 5 min, 4ºC). The resulting clean supernatant (= serum) was used.

Collection of vertebrate blood samples

Human blood samples (A, B and O blood groups) were collected from voluntary donors in our laboratory. Blood samples of sheep, goat, ox and buffalo were obtained from a slaughter house in Perambur, Chennai. Hen blood was collected from a broiler shop. All blood samples were collected in Alsever's solution (prepared according to Garvey *et al.*, 1979), stored at 10ºC and used within 5 days.

Preparation of RBC suspension

RBC from each of the above mentioned eleven blood samples were washed three times with 0.9 % saline and once with TBS-I (50 mM Tris-HCl, 95 mMNaCl, 10 mM $CaCl_2$. $2H_2O$, pH 7.5, 300 mOsm) by centrifugation (400 × *g*, 5 min, RT). The washed RBC pellet was finally resuspended in 5 ml TBS-I as 1.5 % (v/v) RBC suspension.

Hemagglutination assays

The hemagglutination assays were performed in V-bottom microtitre 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 of RBC suspension was added to each well and incubated for 45 minutes at 26 ºC. The hemagglutination titres were recorded as the reciprocal of the highest dilution of the sample causing complete agglutination of RBC (Garvey *et al.,* 1979). Controls for all assays consisted of the substitution of the sample by TBS-I. Each experiment was performed in duplicate for at least three times using serum samples from different preparations, and the hemagglutinating activities were analysed based on the median hemagglutination titer values.

Cross adsorption tests

300 µl of serum samples from healthy *T. orientalis* were mixed with an equal volume of washed and packed native buffalo, human B, mouse or rabbit erythrocytes and incubated for 1 h with frequent shaking at RT. The RBC suspension was centrifuged (400 ×g, 5 min, RT), the supernatant was collected and adsorbed for a second and third time under the same conditions. The serum adsorbed thrice was finally tested for HA activity against all the four RBC types used for adsorption and sheep erythrocytes.

Divalent cation dependency and EDTA sensitivity

500 µl of lobster serum samples were dialysed (MWCO : 12-14 kDa) extensively against cation-free TBS-II (50 mMTris-HCl, 115 mMNaCl, pH 7.5, 300 mOsm) at 15ºC to examine divalent cation dependency, or in TBS containing 50 mM EDTA (TBS-III) 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 × *g*, 5 minutes, RT) and the hemagglutinating activity in the supernatant was determined using buffalo RBC in the presence of TBS that did or did not contain 10 mM $CaCl_2$, $MgCl_2$, or $CaCl_2$ +$MgCl_2$.

pH and thermal stability

Serum samples (250 µl) were dialysed against following buffers (200 mM) at pH ranging from 3 to 12 : acetate buffer (pH 3 to 6), Tris-HCl buffer (pH 7-9), and glycine-NaOH buffer (pH 10-12). After dialysis, all the samples were finally re-equilibrated by dialysis against TBS-I. The dialysates were centrifuged (400 × g, 10 minutes, RT) and the resulting supernatant was tested for hemagglutinating activity against buffalo RBC. Thermal stability of the serum agglutinating activity was examined by holding 150 µl serum samples for 30 minutes at temperature ranging from 10 to 80 ºC. All the samples were centrifuged (400 × g, 10 minutes, RT) and the clear supernatant was used to determine the agglutinating activity against buffalo RBC as described above.

Hemagglutination-inhibition assays

Carbohydrates and lipopolysaccharides (LPS) from various gram negative were tested for their ability to inhibit serum HA activity. The pH of the inhibitor solutions were adjusted, wherever necessary, to 7.5 using concentrated NaOH. The serum samples from healthy sand lobsters were diluted with TBS-I to a HA titer of 4 against buffalo RBC. The carbohydrate to be tested for inhibition (25 µl) was serially diluted two-fold with the serum samples in the microtiter plate. After incubation for 1 h at RT, 25 µl of the 1.5% buffalo RBC suspension was added to each well and

incubated up to 1 h, and the HA reaction was recorded. Inhibitory potency of the test carbohydrate is expressed as the minimum concentration of the carbohydrate that completely inhibited the HA activity of serum against buffalo erythrocytes.

Results and Discussion

Serum hemagglutination profile

The serum of *T. orientalis* agglutinated 10 out of 11 vertebrate RBC types tested with varying hemagglutination titers (Table 1). The highest hemagglutination titer of 128 was obtained with buffalo RBC. The serum moderately or weakly agglutinated 9 other erythrocyte types. Interestingly, mild hemolysis was observed with sheep erythrocytes in first 2 or 3 wells with no sign of hemagglutination in the following wells.

Cross-adsorption tests

In cross adsorption tests, RBC with high (buffalo RBC), moderate (human B and mouse RBC) and relatively low titer (rabbit RBC) were used for adsorption of lobster serum. The adsorbed serum was checked for residual activity with buffalo, human B, mouse, rabbit and sheep RBC. As shown in table 2, adsorption of serum thrice with any of the above mentioned RBC, resulted in complete removal of both agglutinating and lytic activity against all the erythrocytes used for the test. Since buffalo RBC gave a high titer with lobster serum, it was employed to determine the agglutinating activity of lobster serum in all the subsequent experiments.

Divalent cation dependency and EDTA sensitivity

Lobster serum, when dialysed against cation free buffer (TBS-II), the serum haemagglutinating activity against buffalo RBC did not alter. Whereas, when the serum was dialysed against a buffer containing EDTA (TBS-III), the serum heamagglutination activity declined in the absence of divalent cations and the activity was completely restored when calcium was supplied (Table 3).

pH and thermal stability

The effect of dialyzing serum samples against buffers at various pH is shown in figure 1. Lower pH (4 and below) and higher pH (11 and above) completely destroyed the serum haemagglutinating activity against buffalo RBC. The activity was stable between the pH of 7 – 9. As shown in the figure 2, the serum haemagglutinating activity was stable between $20^{\circ}C$ to $40^{\circ}C$. The activity sharply decreased after $40^{\circ}C$ and was completely destroyed at $70^{\circ}C$.

Hemagglutination-inhibition assays

Of the various carbohydrates tested, only 4 inhibited the agglutinating activity of the lobster serum against buffalo erythrocytes (Table 5). The three simple hexoses, namely glucose, galactose and mannose as well as their aminoderivatives (glucosamine, galactosamine and mannosamine) failed to inhibit the hemagglutinating activity of the serum against buffalo RBC.

By contrast, their *N*- acetyl derivatives such as, *N*-acetylglucosamine, *N*-acetylgalactosamine and N-acetylmannosamine inhibited the serum agglutinating activity with varying efficiency, with ManNAc showing highest inhibitory potency of 3.125 mM. *N*-acetyl neuraminic acid (Neu5Ac), a nine carbon sialic acid also effectively inhibited the serum HA activity against buffalo RBC. However, *N*-glycolylneuraminic acid (Neu5Gc), another sialic acid did not exhibit any inhibitory

activity towards serum HA activity. It was also observed that LPS from various gram negative bacteria's effectively inhibited the serum HA activity against buffalo RBC (Table 4).

Table.1 Hemagglutinating activity of the serum of *Thenus orientalis* against different types of vertebrate erythrocytes

RBC types tested	Hemagglutinationtiter*
Buffalo	128
Human B	64
Human O	64
Mouse	32
Rat	32
Human A	16
Rabbit	8
Ox	2
Goat	2
Hen	2
Sheep	Complete hemolysis observed in the first one or two wells; partial hemolysis in the subsequent well

* Data represent median values from 12 to 15 determinations for each RBC type using serum samples from different preparations.

Table.2 Cross-adsorption tests on *Thenus orientalis* serum

Serum adsorbed [@] with RBC of	Hemagglutinationtiter against RBC types tested*				
	Buffalo	Human B	Mouse	Rabbit	Sheep
None	128	64	32	8	Complete hemolysis observed in the first one or two wells; partial hemolysis in the subsequent well
Buffalo	0	0	0	0	0
Human B	0	0	0	0	0
Mouse	0	0	0	0	
Rabbit	0	0	0	0	0

[@] Three 60- min adsorption at room temperature.

* Data represent median values from three determinations for each RBC type using serum samples from different preparations.

Table.3 Divalent cation dependency and EDTA sensitivity of serum Hemagglutinating activity of *Thenus orientalis*

Treatment of serum samples	Divalent cations tested	Hemagglutinationtiter*
Starting sample (Freshly collected serum)- Control 1	10 mM$CaCl_2$	128
Undialyzed serum held at 5° degree Celsius for 48 hours- Control 2	10 mM$CaCl_2$	128
Serum dialyzed against divalent cation free TBS (48 hours, 5 degrees)	None 10mM $CaCl_2$ 10mM $MgCl_2$ 2mM $MnCl_2$	128 128 64 64
Serum dialyzed against 20mM EDTA (24 hours, 5 degrees); subsequently re equilibrated in divalent cation free TBS (24 hours, 5 degrees)	None 10mM $CaCl_2$ 10mM $MgCl_2$ 2mM $MnCl_2$	16 128 16 16

* Data represent median value from three to five determinations using serum samples from different preparations.

Table.4 Inhibition of serum hemagglutinating activity (titer = 4) of *Thenus orientalis* by various bacterial lipopolysaccharides

Bacterial lipopolysaccharide tested	Maximum concentration tested (mg/ml)	Minimum inhibitory concentration (µg/ml)[@]
Serratiamarcenscens	2	500
Pseudomonas aeruginosa	2	500
Salmonella abortusequi	2	25
Salmonella minnesota	2	500
Escherichia coli	2	500
Klebsiellapneumoniae	2	500

[@]The assay was repeated three times for each bacterial LPS with identical results using samples from different preparations.

Table.5 Hemagglutination-inhibition of *Thenus orientalis* serum (HA titer = 4) by carbohydrates against buffalo RBC

Carbohydrates tested	Maximum concentration tested (mM)	Minimum inhibitory concentration (mM)
Hexoses		
D-glucose	200	-
D-galactose	200	-
D-mannose	200	-
D-fucose	200	-
D-fructose	200	-
Hexosamines		
Glucosamine	200	-
Galactosamine	200	-
Mannosamine	200	-
***N*-acetyl hexosmines**		
N-acetylglucosamine (GlcNAc)	200	25
N-acetylgalactosamine (GalNAc)	200	12.5
N-acetylmannosamine (ManNAc)	200	3.125
Sialic acids		
N-acetyl neuraminic acid (Neu5Ac)	50	3.125
N-glycolylneuraminic acid (Neu5Gc)	10	-

* Data represent median values from three to five determinations using serum samples from different preparations against buffalo RBC No inhibition

Fig.1 Effect of different temperature on serum HA activity of the sand lobster

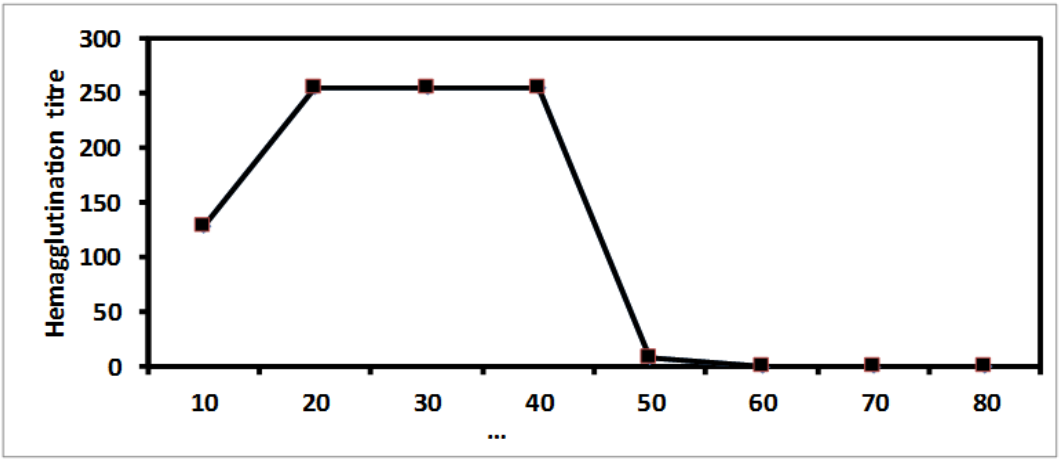
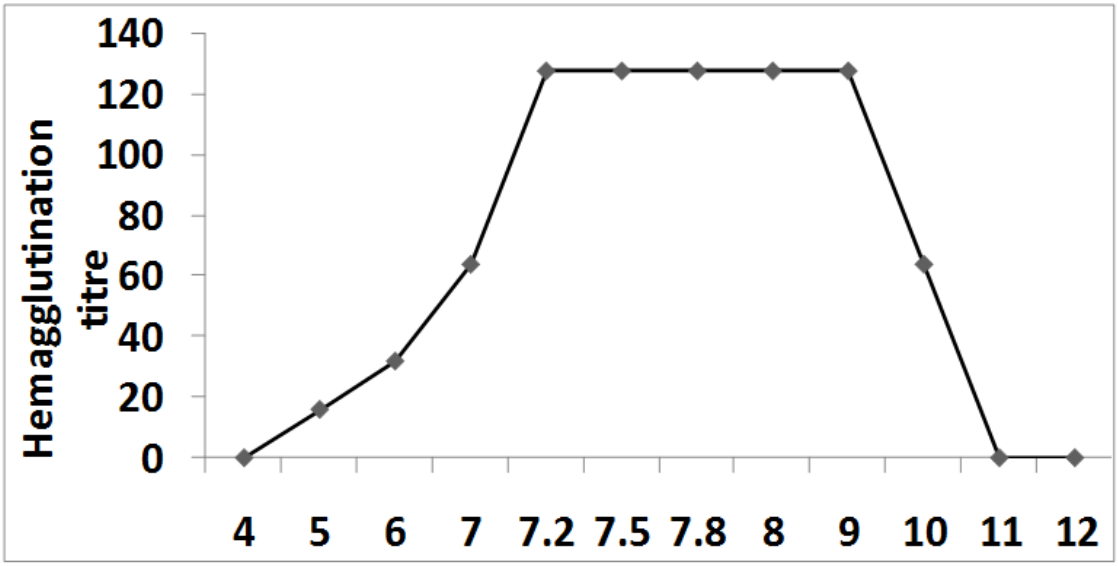


Fig.2 Effect of pH on serum HA activity of the sand lobster



Serum agglutination studies using various indicator cells such as RBC, bacteria, sperm cells provides initial indication about presence of agglutinins. Of all the indicator cells used, RBC is one of the most widely used targets in order to ascertain the presence of agglutinins (Ravindranath *et al.,* 1985;Murali*et al.,* 1999; Maheswari *et al.,* 2002;Alpuche*et al.,* 2005; Sun *et al.,* 2008; Sánchez-Salgado *et al.,* 2014). In our study, the serum hemagglutinating activity was screened with 11 different vertebrate erythrocytes, with 10 RBC showing agglutination with various titres. Out of the 10 RBC, buffalo RBC showed the highest reactivity with a titre of 128. A similar profile was observed with estuarine crab, *Portunus sanguinolentus* (Meena *et al.,* 2011). Another interesting observation in this study was that the lobster serum caused lysis of sheep RBC, with no sign of agglutination. Cross adsorption studies showed complete removal of agglutinating activity when adsorbed with any RBC tested, vaguely suggesting that there could be presence of only a single agglutinin in the lobster serum.

Dialysis experiments showed that the agglutinating activity of lobster serum against buffalo RBC showed no divalent cation dependency. However, dialysis of lobster serum against divalent cation chelators, such as, EDTA showed significant decrease in agglutinating activity, but was completely restored upon addition of calcium ions. From previous studies, it was found that predominant of crustacean lectins were dependent on divalent cations, more often calcium or magnesium ions and was sensitive to EDTA (Marques and Barracco, 2000; Denis *et al.,* 2016). Very few studies on crustacean lectins had shown them to be independent of divalent cations and also insensitive to EDTA (Imai *et al.,* 1994; Murali *et al.,* 1994; Maheswari *et al.,* 1997; Yang *et al.,* 2007). This could suggest that the agglutinin of our interest seen in the serum of sand lobster, has the ability to maintain its agglutinating activity with low or intrinsic level of divalent cations in the serum. However, prolonged exposure to EDTA hampered the agglutinating activity which was effectively restored when supplied with exogenous calcium ions, indicating that the agglutinating activity is mildly sensitive to EDTA.

The optimal pH and temperature for serum agglutinating activity of lobster serum was found to fall between the range of 7-9 (alkaline)

and 20-40˚ C respectively. This is in accordance with previous studies where most crustacean humoral agglutinins showed activity at alkaline pH range and temperature between 10 and 50˚C (Nalini *et al.*, 1994).

The serum hemagglutinating activity of the sand lobster was inhibited by acetyl group containing carbohydrates, such as *N*- acetyl hexosamines and *N*- acetyl neuraminic acid. Of these, Man NAc and Neu5Ac showed high inhibitory potency. In crustaceans, serum lectins specific for diverse carbohydrates such as, glucose (Umetsu *et al.,* 1991),galactose (Umetsu *et al.,* 1991), fucose (Amirante and Basso, 1984), aminosugars (Sivakamavalli and Vaseeharan, 2014), *N*- acetylated aminosugars (Zenteno *et al.,* 2000; Maheswari*et al.,* 2002; Alpuche *et al.,* 2005; Sun *et al.,* 2008), or sialic acids such as Neu5Ac (Ratanapo and Chulavatnatol, 1990; Sudhakar*et al.,* 2012), 4- and 9-0-acetyl neuraminic acid (Ravindranath *et al.,* 1985), and Neu5Gc (Mercy and Ravindranath, 1993) have been identified.

It is also notable that many crustacean lectins have special affinity towards *N*-acetylated aminosugars. Another striking observation was that LPS from gram negative bacteria inhibited serum HA activity, thus indicating the agglutinin also recognize some components of LPS. From previous studies, it is notable that many crustacean lectins that has affinity for *N*-acetylated hexosamines or Neu5Ac also have LPS binding activity (Murali *et al.,* 1999; Maheswari *et al.,* 2002; Luo *et al.,* 2006; Yang *et al.,* 2007; Sun *et al.,* 2008.

From these initial tests, strategies were developed to isolate the agglutinin from the serum of sand lobster using affinity column chromatography which is a prerequisite in understanding the functional significance.

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