Identification and physico-chemical characterization of a natural agglutinin from coelomic fluid of the sea urchin *Stomopneustes variolaris* (Lamarck)

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Abstract : Natural agglutinins with diverse specificity for diverse mammalian erythrocytes were identified in different tissues of sea urchin *Stomopneustes variolaris*. Of the various tissues analyzed for the distribution of agglutinin, maximum HA was observed in the coelomic fluid of the sea urchin *S. variolaris.* Physico-chemical properties of the coelomic fluid agglutinin showed that it specifically depends on Ca^{2+} for its activity, stable to wide range of pH and sensitive to temperature and EDTA. Disappearance of agglutinability following cross adsorption revealed the presence of a single agglutinin. The coelomic fluid agglutinin was inhibited by sugars such as GalNAc and GlcNAc and was effectively inhibited by the glycoprotein PSM. BSM, transferrin, α-acid glycoprotein and also animal body fluids such as saliva, milk and urine inhibited the hemagglutination of the coelomic fluid.

Keywords: Agglutinin, Coelomic fluid, lectin.

I. Introduction

Invertebrates are phylogenetically diverse and have evolved a multiplicity of efficient defense strategies to defend against microbial attacks (Mydlarz *et al*., 2006). They lack an adaptive immune system and depend on innate immune system for defense against invading microorganisms. 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 (Nalini *et al*., 1994; Murali *et al*., 1999). The body fluid or hemolymph of almost all invertebrate species tested contains agglutinins (Jayaraj *et al*., 2008).

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, 19814; Rogener and. Uhlenbruck. 1984; Olafsen, 1986; Vasta, 1991).

Agglutinins/ Lectins are glycoproteins possessing at least one non-catalytic domain that binds reversibly to specific carbohydrates inside and outside cells (Sharon and Lis, 2004) and the binding affinity of agglutinins are influenced by physico-chemical factors of the medium (Reeves and Rahn, 1979). It is well known that invertebrates have hemagglutinins for a variety of erythrocytes (Jeskin and Rowley, 1970) and have been isolated from the coelomic fluid and coelomocytes of invertebrates (Giga *et al*., 1987).

In echinoderms, agglutinating molecules have been demonstrated in species belonging to the Echinoidea, Asteroidea, and Holothuroidea (Ey and Jenkin, 1982). They have been detected in circulating fluids, whole body homogenate of female and male and internal organs (Drago *et al*., 2009). Presence of agglutinins have been recorded from sea urchins *Strongylocentrotus droebachiensis* (Brown *et al*., 1968), *Psammechinus miliaris* (Uhlenbruck *et al*., 1970), *Anthocidaris crassispina* (Giga *et al*., 1987), *Hemicentrotus pulcherrimus* (Maehashi *et al*., 2003), *Lytechinus variegates* (Alliegro *et al*., 1988), *Paracentrotus lividus* (Drago *et al*., 2009), *Strongylocentrotus purpuratus* (Multerer and Smith, 2004), *Toxopneustes pileolus*, *Tripneustes gratilla* (Nakagawa *et al*.,1996) *Pseudocentrotus depresses*, *Strongylocentrotus nudus* (Bulgakov *et al*., 2013) and *Echinometra lucunter* (Carneiro *et al*., 2015).

The present study attempts to elucidate, the physico-chemical properties of the agglutinin and to identify the potent sugar/ glycoprotein inhibitor of the *S. variolaris* coelomic fluid, so as to develop strategies for affinity purification.

II. MATERIALS AND METHODS

Experimental animal and sample collection: Live sea urchins, *Stomopneustes variolaris* (Lamarck) were collected from the dip nets of the fishermen of Kanyakumari, Muttom, and Colachel of Kanyakumari district of Tamilnadu, India. All the animals were maintained in sea water and transported to the laboratory. Coelomic fluid was withdrawn by inserting a sterile 23-gauge needle through the peristomium of the coelomic cavity. Gonad, gut, shell, spine and teeth were carefully dissected, and were thoroughly rinsed in cold Tris Buffered Saline (TBS) to remove the coelomic fluid. 100 mg each of gonad, gut, shell, spine and teeth were collected and grounded well, suspended in 1 ml of cold saline and centrifuged for 10 min at 4000 x g and the supernatant was assessed for hemagglutination activity.

Collection and preparation of mammalian erythrocytes: Buffalo, rat, rabbit, pig, dog, Human, A, B, O, AB, cow, goat erythrocytes were prepared following the standard method of Ravindranath and Paulson (1987).
Hemagglutination (HA) Assay: Hemagglutination assay were performed in 96 well, ‘U` bottomed microtiter plates (Tarson) as described by Ravindranath and Paulson (1987).
pH and thermal stability : pH and temperature dependence of the agglutinin was tested by pre incubating the coelomic fluid at different pH (5-12) and temperature (0ºC - 100ºC) 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 -50 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 human A erythrocytes.
Hemagglutination inhibition (HAI) Assay: Hemagglutination inhibition assay were performed in 96 well, ‘U` bottomed microtiter plates (Tarson) as described by Ravindranath and Paulson (1987).
HAI Assay with animal body fluids: Hemagglutination inhibition assay was performed to test the ability of animal body fluids (such as saliva from persons of A, B, AB, O blood group; milk of cow and goat, urine of cow, goat and human) to inhibit agglutination. Known concentration (25 µl) of inhibitor was serially diluted with 25 µl of TBS in microtiter plate. To each well, 25 µl of coelomic fluid diluted to subagglutination concentration in TBS (to give a HA titer of 2) was added and incubated for 1 hour. 25 µl of 1.5% human A erythrocyte suspension was added, mixed and incubated. Hemagglutination inhibition titer was reported as the reciprocal of the highest dilution of inhibitors giving complete inhibition of agglutination after 1 hour.
Trypsin and protease treatment: Equal volumes each of trypsin (1 mg/ ml) and neutral protease (0.25 mg/ ml) with washed erythrocytes were incubated at 37ºC for 1 hour. 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 (1993). The desialylated erythrocytes were used for hemagglutination assay.
Cross adsorption assay
The cross adsorption assay was carried out following the method of Mercy and Ravindranath (1992).

RESULTS

Natural hemagglutinins in the coelomic fluid of sea urchin

Hemagglutinating activity was observed in the coelomic fluid with a maximum HA titer of 512 with Human A erythrocytes. It also agglutinated other mammalian erythrocytes: Pig > Human AB > Human B = Human O = Rabbit = Rat > Goat > Cow (Table 1).

Distribution of hemagglutinins in tissues of sea urchin

The tissues of the sea urchin *S. variolaris* agglutinated only rabbit erythrocytes. The hemagglutinability of the various tissues can be listed as gut > shell = teeth > spine (Table 1).

Effect of pH and temperature

Treatment of coelomic fluid with the pH ranging from 5-8 showed a similar HA of 512, but on exposure to basic pH above 10.5 there was a gradual reduction in hemagglutination (Table 2). Maximum HA of 8192 was observed at temperature ranging from 0ºC to 10ºC. A gradual reduction in agglutination was noted with increase in temperature and agglutinin activity was seen even at 100ºC with a minimum HA of 4 (Table 2).

Effect of cations and calcium chelators an hemagglutinin

Addition of divalent cations (Ca^{2+}, Mg^{2+}) to the coelomic fluid enhanced the HA titer at lower concentrations up to 1 mM. However, at higher concentration of these cations a slight decrease in HA was noted. Calcium chelators di and tetra sodium EDTA and trisodium citrate revealed a decrease in HA with increase in concentration. However a fluctuation in HA was observed with trisodium citrate (Table 3).

Hapten inhibition studies

The agglutination of coelomic fluid with human A erythrocyte was inhibited by glycoproteins: PSM > BSM > Transferrin > α acid glycoprotein. PSM showed the highest inhibitory potency with coelomic fluid. HA against human A erythrocyte was weakly inhibited by sugars GlcNAc and GalNAc (Table 4).

HAI with body fluids (saliva, milk and urine)

Saliva obtained from four different blood group individuals (A, B, AB and O) were tested for HAI and the results revealed that the agglutination of coelomic fluid with human A erythrocytes was inhibited by human A and AB blood group saliva. Human O and B individuals' saliva failed to inhibit the agglutination activity. The coelomic fluid agglutinin was highly inhibited by cow and goat milk, while the coelomic fluid was weakly inhibited by human urine and was not inhibited by goat and cow urine (Table 5).

Enzyme treatment

An increase in HA titer was observed when coelomic fluid agglutinin was mixed with protease (trypsin and neutral protease) treated erythrocytes. Sialidase treatment of human A erythrocytes showed no difference in the HA titer when compared to untreated human A erythrocytes (Table 6).

Cross Adsorption test

Cross adsorption profile revealed the presence of a single agglutinin in the coelomic fluid of the sea urchin, *S. variolaris* as evidenced by the loss of HA activity after first or second or third adsorption with any of the erythrocytes species that showed agglutination with the coelomic fluid agglutinin of the sea urchin, *S. variolaris* (Table 7).

Table 1: Hemagglutination titer of coelomic fluid and different parts of the sea urchin *Stomopneustes variolaris* at pH 7.5

Erythrocytes (n=5)	HA titer				
	Coelomic fluid	Teeth	Spine	Shell	Gut
Rabbit	64	8	2	8	32
Human B	64	0	0	0	0
Human O	64	0	0	0	0
Human AB	128	0	0	0	0
Human A	512	0	0	0	0
Pig	256	0	0	0	0
Rat	64	0	0	0	0
Cow	4	0	0	0	0
Buffalo	0	0	0	0	0
Goat	8	0	0	0	0
Dog	0	0	0	0	0

Table 2: Impact of pH and temperature on the hemagglutination titer of coelomic fluid of the sea urchin, *S. variolaris*

pH (n=5)	HA Titer	Temperature	HA titer
5	512	0	8192
5.5	512	10	8192
6	512	20	4096
6.5	512	30	2048
7	512	40	2048
7.5	512	50	2048
8	512	60	1024
8.5	2048	70	64
9	2048	80	32
9.5	2048	90	16
10	2048	100	4
10.5	1024		
11	1024		
11.5	128		
12	64		

Table 3: Impact of divalent cations and chelators on the hemagglutinating activity of coelomic fluid of the sea urchin, *S. variolaris* at pH 9.5

Conc. in mM (n=5)	HA Titer				
	Cations		Chelators		
	Ca^{2+}	Mg^{2+}	Disodium EDTA	Tetra sodium EDTA	Tri sodium citrate
0	2048	2048	2048	2048	2048
0.1	4096	4096	2048	2048	256
1	4096	4096	2048	1024	512
10	2048	4096	2048	1024	1024
20	2048	4096	2048	512	2048
30	2048	2048	512	256	1024
40	1024	2048	512	16	512
50	1024	1024	64	8	256

Table 4: Hemagglutination inhibition (HAI) of the coelomic fluid agglutinin of *Stomopneustes variolaris* by various glycoproteins/sugars at pH 9.5

Glycoproteins (n=5)	HAI titer	Minimum conc. required (µg/ ml)	Relative inhibitory potency (%)	Sugars (n=5)	HAI titer	Minimum conc. required (mM)	Relative inhibitory potency (%)
PSM	4096	0.610	100	N-acetyl glucosamine	16	6.25	100
BSM	32	3.12	12.5				
Transferrin	4	25	1.56	N-acetyl galactosamine	2	50	50
α-acetyl glycoprotein	2	50	0.78				

Glycoprotein: Bovine thyroglobulin, Lactoferrin, Fetuin, Apotransferrin, thyroglobulin & sugars: D-galactose, α-methyl mannose amine, Fucose, L-fucose, Xylose, D-glucose-6- phosphate, Trehalose, Raffinose, Arabinose did not inhibit the HA titer.

Table 5: Hemagglutination inhibition (HAI) of the coelomic fluid agglutination of *Stomopneustes variolaris* by animal body fluids at pH 9.5

Animal body fluids (n=5)	HAI titer	Minimum concentration required (µl)	Relative inhibitory potency (%)
A group person's saliva	512	0.195	100
Cow milk	512	0.195	100
Goat milk	512	0.195	100
AB group person's saliva	128	0.78	25
Human urine	8	12.5	1.562

Cow urine, Goat urine, B and O group person saliva did not inhibit the HA titer.

Table 6: Effect of enzyme treatment of erythrocyte on hemagglutination titer of coelomic fluid of the sea urchin, *Stomopneustes variolaris* at pH 9.5

Enzymes (n=5)	HA titer
None	2048
Neuraminidase (*C. perfringens* type X)	2048
Trypsin (1 mg/ ml)	8192
Neutral Protease (0.25 mg/ ml)	4096

Table 7: Hemagglutination activity of coelomic fluid of *Stomopneustes variolaris* after adsorption with different erythrocytes at pH 9.5

Erythrocyte adsorbed (n = 5)	HA titer				
	Human A	Human B	Human O	Human AB	Rabbit
None	2048	256	256	512	256
Human A	0	0	0	0	0
Human B	0	0	0	(2) 0	0
Human O	0	0	0	(32) 0	0
Human AB	(32) 0	0	0	0	0
Rabbit	0	0	0	0	0

n= Number of animals tested

DISCUSSION

The body fluid or hemolymph of almost all invertebrate species tested contains agglutinins (Ratcliffe *et al.*, 1985). Survey of agglutinins by hemagglutination assay revealed the presence of agglutinin in the coelomic fluid of sea urchin *S. variolaris*. The agglutinins showed differential affinity with different species of erythrocytes, yielding the highest titer with human A erythrocyte. The agglutinin showed high affinity for pig, human AB, human B, human O, rabbit and rat erythrocytes and very poor affinity towards the other erythrocytes tested.

The possible reason for selective agglutination may be the recognition of the receptor component NeuAc expressed on the glycocalyx of human, pig rabbit and rat erythrocytes (Murayama *et al*., 1981) or the sugar determinants N-acetyl D galactosamine, D galactose and L fucose of blood types A, B and O, which can fit better on the binding sites of the lectin (Mojica *et al*., 2005). The titer values for animal blood are quite low in comparison with that of the human blood except for pig. The preferential agglutination may be either due to the presence of more or due to lesser number of receptor sites in animal red blood cells or incompatibility of these receptor sites with the lectin binding sites (Merca and Reyas, 1989). This suggests that the receptor determinants preferentially recognized by the coelomic fluid agglutinin are more abundant on human erythrocytes than the other erythrocytes tested.

Tissues such as gut, spine, teeth and shell showed agglutination only with rabbit erythrocytes. The tissue lectins are highly blood group specific, as it could bind only with rabbit RBC and did not bind to human A or other RBC's that were recognized by coelomic fluid. Thus our results suggest the possibility that tissue agglutinins may be different from coelomic fluid agglutinin. The tissue extracts of *Stichopus japonicus* (Matsui *et al*., 1994), seminal plasma of *Paracentrotus lividus* (Stabili *et al*., 1993), coelomic fluid of *Asteria amurensis* (Yoshitaka and Yoshihiro, 2013) also agglutinated only rabbit erythrocytes.

The *S. variolaris* agglutinin had a marked stability over a wide range of pH. Though the hemagglutinin was stable from pH 5 to 8, acidic and neutral and low alkaline pH reduced the HA activity, when compared to alkaline pH. Agglutinins sensitive to alkaline pH is also reported in the sea urchins *Anthocidaris crassispina, Pseudocentrotus depressus* and *Hemicentrotus pulcherrimus* (Ryoyama, 1974). The HA activity of the agglutinin was maximum at temperature ranging from 0°C to 10°C and it gradually decreased above 30°C, suggesting the sensitivity of the lectin to high temperature. Mojica *et al*. (2005) reported that the *Holothuria scabra* lectin was most stable from 20°C to 50°C and it can therefore be isolated at room temperature without denaturation of the lectin.

Divalent cations are known to be important in stabilizing the structure of hemagglutinins (Anderson and Good, 1975). In the present study, change was observed in the HA activity of the coelomic fluid agglutinin when tested with different concentration of cations, calcium and magnesium. The result implies that the coelomic fluid agglutinin could be a C-type lectin. Calcium is not only directly involved in the carbohydrate binding itself at the binding site (Weis *et al*., 1992) but also contributes to the structural maintenance of the lectin domain that is essential for the lectin activity (Kimura *et al*., 1995) and bound calcium may stabilize the native structure of proteins. Calcium dependent lectins were also observed in the sea urchins *Strongylocentrotus purpuratus* (Smith *et al*., 1996; Multerer and Smith, 2004), *Anthocidaris crassispina* (Giga *et al*., 1987), *Paracentrotus lividus* (Canicatti *et al*., 1992), *Tripneustes gratilla* (Nakagawa *et.al*., 1996). *Strongylocentrotus nudus* (Bulgakov *et al*., 2013).

Cross adsorption of coelomic fluid with human erythrocytes resulted in the disappearance of agglutinability with all the erythrocytes revealing the presence of single agglutinin. Serological studies have shown that agglutinability of the agglutinin to one type of erythrocytes can be adsorbed by that type of erythrocytes, leaving residual agglutinating activity to other type of erythrocyte (Mc Dade and Tripp, 1967; Mckay *et al.,* 1969; Noguchi, 1903). Presence of single agglutinin was also reported in coelomic fluid of the sea urchin *Apostichopus japonicus* (Bulgakov *et al*., 2007), *Linckia laevigata* (Santhiya *et al.,* 2013).

S. variolaris hemagglutinin recognised the trypsin and protease treated erythrocytes with greater avidity than the untreated ones. Addition of trypsin is done to increase the susceptibility of erythrocytes to agglutination without affecting the total number of lectin binding sites (Lis and Sharon, 1986). The increase in agglutinability may be either due to the exposure of the cryptic receptor sites on the surface of the cell membrane (Hammarstram and Kabat, 1969) or the removal of close interfering structures by possible rearrangement of receptor sites on the erythrocytes (Nicolson, 1971). Treatment with protease enzyme may remove the cell surface proteins that mask the agglutinin binding sites, possibly the sugar residues of ganglioside recognized by the agglutinin. The same has been suggested by Cohen (1968) who stated that protease alters the surface of the erythrocyte, so that cell-agglutinin cell- lattice formation is possible with subsequent clumping of cells and the same has been reported in the sea urchins *Paracentrotus lividus* (Drago *et al*., 2009), *Diadema* sp., *Tripneustes* sp. (Mojica *et al*., 2005).

Body fluids contain various sugar moieties which could inhibit the hemagglutination of the agglutinin. More than one sugar may inhibit an agglutinator and one or more of these may be present in the glycoproteins of the biological materials tested (Khalap *et al*., 1970). Certain hemagglutinins from marine sources react with human erythrocytes and hemagglutination by them can be inhibited by human saliva (Brain and Grace, 1968; Pemberton, 1971). Among the 4 blood group specific individual, the agglutination of coelomic fluid with human A erythrocytes was inhibited by human A and B blood group person's saliva. Similar result was obtained from *Vicia cracca* lectin that was strongly inhibited by human A group secretor saliva but not by B and O secretor saliva (Makela, 1957). The effect of human saliva on hemagglutination inhibition was also observed in the sea urchins *A. crassispina, P. depressus* and *H. pulcherrimus* (Ryoyama, 1974). Cow and goat milk also possessed remarkably high inhibiting titers which may probably be due to the presence of lactose and oligosaccharides (Kuhn and Gauhe,1962)

The agglutinability of the coelomic fluid of the sea urchin *S. variolaris* was inhibited by PSM, BSM, Transferrin and α-acetyl glycoprotein. PSM that contains the sialic acid N-glycolyl neuraminic acid (90%) and NANA (10%) (Schoop and Faillard, 1967) is the potent inhibitor. It also recognizes BSM that contains mainly 9-O-acetyl and 8,9-di-O-acetyl-N-acetyl neuraminic acid (Schauer, 1982) and transferrin which possess a terminal α, 2,6 linkage (Gottschalk and Graham, 1959).

The binding affinity of agglutinin is defined by the sugars, which inhibit their activity (Liener *et al*., 1986). The multiple binding sites in the lectin could interact with a wide variety of sugars or it is possible that the binding site is flexible on the size and shape of sugar residue (Lacsamana and Merca, 1994). Hemagglutination inhibition tests performed in this study revealed that the coelomic fluid agglutinin was inhibited by sugars like GalNAc, GlcNAc and D-fucose. GalNAc specificity is also reported in the coelomic fluid of *Holothuria atra* (Mojica and Merca 2004), *Cucumaria echinata* (Hatakeyama *et al*., 1994), *Toxopneustus pileolus* (Edo, 2014), and *Asterina pectinifera* (kakiuchi *et al*., 2002).

Thus from our findings it is clear that the *S. variolaris* coelomic fluid hemagglutinin is an unique agglutinin that is more specific to human erythrocyte binding, recognizes specific glycoproteins and sugars and hence can be used to understand the immunological role of the lectin in the animal as defense strategy and its therapeutic application. This preliminary characterization has paved way for the purification of a lectin and also to assess its biomedical potential.

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