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Partial purification and characterization of hemolymph lectin of marine crab *Atergatis ocyroe* by adsorption on formalinized erythrocytes

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Abstract
Hemolymph lectin of marine crab *Atergatis ocyroe* was purified by biospecific adsorption using formalinized buffalo erythrocytes. SDS-PAGE of the lectin yielded two subunits of molecular masses 50 kDa and 25 kDa. The isolated lectin is a non-blood group specific lectin since it agglutinated both human and animal erythrocytes. The lectin showed high affinity towards buffalo and rabbit erythrocytes suggesting its specificity to bind to the sialic acids that are expressed on the erythrocytes. The hemagglutinin was calcium dependent and sensitive to pH, temperature and calcium chelator – EDTA. Hemagglutination inhibition assay revealed bovine thyroglobulin as the potent inhibitor followed by fetuin, BSM and PSM. Sugars like GlcNAc, Trehalose, D - Fructose, melibiose, L - Fucose and α – Lactose weakly inhibited the agglutinin. Based on the erythrocyte and glycoprotein specificity it could be concluded that the lectin is sialic acid specific which could be further purified using affinity chromatography and used in lectin targeted therapy.

Keywords: agglutinin, glycocalyx, hemagglutination, formalinized erythrocytes, sialic acid

Introduction
Immunity is the ability of an organism to distinguish self from non-self. Invertebrates which lack adaptive immune system rely on innate immunity to respond to non-self-material [1]. Marine crabs which belong to class Crustacea of phylum Arthropoda have an efficient defense system to fight against the intruding pathogens [2]. Among crustacean's innate immune compounds, lectins play an important role as a sensor and regulator of foreign organisms [3]. Lectins are proteins/glycoproteins capable of binding non-covalently to specific saccharide moieties and therefore agglutinate cells by binding to cell surface glycoprotein and glycol conjugates [4]. C-type lectins that dependent on calcium for legend binding have displayed remarkable antimicrobial activity [5] and have been reported to promote cellular encapsulation in invertebrates [6]. Lectins which recognize sialoconjugates at the terminal glycan of the cells and microbes [7] have immense biomedical applications. Crustaceans though they lack the ability to synthesize sialic acid [8], they have the affinity to bind to various structural forms of neuraminic acid and have been isolated from crustaceans [7, 9].
Lectins from marine organisms have attracted great interest because of their cytotoxic effect on malignant cells [10], anti-inflammatory [11], impairing the development of malaria parasite [12], suppression of HIV [13], antimicrobial [14], immune modulatory and anti-insect [15] and mitogenic [16] activities. While most of the agglutinins were purified by affinity chromatography, among arthropods few were also purified by conventional procedure like formalinized erythrocyte adsorption. This simple biospecific adsorption method of purification of carbohydrate binding proteins was first adopted by Reitherman [17]. Nowak and Barondes purified a hemagglutinin from the hemolymph of the horse shoe crab *L. polyphemus* using formalinized erythrocytes as an affinity reagent [18]. Fragkiadakis improved this biospecific adsorption procedure to isolate the hemolymph lectins of the decapods, *Liocarcinus depurator* and *Potamanpotamios* [19]. Formalinized erythrocyte adsorption purification technique was used for isolation of invertebrate lectins [9, 20, 21]. Hence the present study aims to isolate and characterize hemolymph lectins from marine crab *Atergatis ocyroe* by adsorption on formalinized RBC.

Materials and Methods
Animal Collection and Maintenance
Atergatis ocyroe crabs were collected from Kanyakumari Sea and maintained in plastic tubs filled with 6 cm of marine water and aerated with an aerator. The crabs were fed with anchovy variety of fish and the water was changed daily.

Collection of Hemolymph
Hemolymph was collected from uninjured, non- autotomised crabs, either by inserting a sterile 1.0 ml syringe with a 22 gauge needle into the arthrodial membrane of the base of third walking leg or by cutting the tip of third walking leg. The hemolymph collected in centrifuge tubes placed on ice was allowed to clot and then centrifuged for the collection of serum. The serum was aliquoted and stored at -20ºC for further study.

Erythrocyte Preparation
Buffalo, mice, rat, guinea pig, rabbit, pig, dog, Human, A, B, O, camel, cow, goat, horse, donkey erythrocytes were prepared following the standard method [22].

Preparation of formalinized buffalo erythrocytes

Buffalo erythrocytes were obtained in Alsevier's solution. Immediately upon receipt, the cells were washed 3 times in 20 volumes of phosphate – buffered saline (PBS) pH 7.2 (75 mM NaCl, 75 mM Na_2HPO_4) per packed cell volume by centrifugation at 1000 x g for 5 minutes. The cells were suspended at a concentration volume of 8% in PBS and an equal volume of formalin (3% solution in PBS with pH adjusted to 7.2 with 0.1M NaOH). The mixture was incubated at 37ºC for 16 h with moderate shaking. The cells were then washed 4 times in 5 volumes of PBS, pH 7.2 per packed cell volume and stored at 4ºC as 10% suspension in this buffer. This could be used in the agglutination assay for months after preparation.

Purification of Agglutinin

The stored formalinized rabbit erythrocytes were prepared for use as an affinity reagent by washing 6 times in 10 volumes of TBS (50 mM Tris HCl, 100 mM NaCl) pH 7.5. The packed cells were then incubated with 20 volumes of the hemolymph in plastic tubes for 2 h with moderate shaking at 4ºC and then washed 3 times with 20 volumes of TBS pH 6.5 containing 10 mM $CaCl_2$. Elution of the adsorbed hemagglutinin was accomplished by incubation of the cells with 10 mM EDTA in TBS. The elution was continued for 5 h with moderate shaking at 4ºC and the elution mixture was centrifuged for 10 minutes to remove any residual particulate material and the resultant supernatant was dialyzed and tested for further analysis.

Molecular mass determination

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in accordance with the procedure of Laemmli[23], using a 12% resolving gel and a 5% stacking gel. After electrophoresis, the gel was stained with Coomassie brilliant blue, destained and electrophoretic mobilities of the marker proteins and the molecular weight of the purified lectin were determined.

Results

Purification of *Atergatis ocyroe* Hemolymph Lectin

The lectin was desorbed from formalinized buffalo erythrocytes using Tris disodium EDTA. The lectin preparations obtained resulted in 302 fold increase in specific activity (Table - 1). SDS-PAGE analysis revealed two bands of 25 kDa and 50 kDa (Fig-1).

Erythrocyte Binding Specificity of ***A. ocyroe* Lectin**

Agglutination of mammalian erythrocytes by the lectin is represented in Table-2. The lectin agglutinated all the erythrocytes tested with varying HA titer, but maximum agglutinability was recorded with buffalo and dog erythrocytes.

pH and Thermal Stability

The hemolymph lectin was sensitive to pH and temperature (Table-3). The hemagglutinating activity was stable from pH 7 to 8 and remained unaffected at temperature ranging from 0˚C - 20˚C. The lectin activity gradually reduced at 30˚C and was completely abolished above 60˚.

Effect of calcium and calcium chelator

The purified lectin was treated with different concentrations of Ca^{++} and optimum HA activity was obtained from 0.1 to 10 mM (Table-4). The Ca^{++}dependence of the lectin was confirmed by the EDTA treatment. A drastic decrease in HA was noted on addition of 10 mM disodium EDTA and 20 mM tetrasodium. The lectin was unaffected by trisodium citrate (Table-5).

Hemagglutination Inhibition (HAI) Assay

The inhibitory potency of the lectin was tested with different glycoproteins and sugars**.** Among the glycoproteins tested, the agglutination of lectin with buffalo erythrocytes was inhibited by bovine thyroglobulin > BSM > fetuin > PSM (Table -6). Of the sugars tested for HAI the hemolymph lectin, agglutinability was reduced by GlcNAc = Trehalose = D. Fructose > melibiose = L - Fucose > α – Lactose (Table -7).

Table 1: Purification of lectin from the hemolymph of the marine crab *Atergatis ocyroe*

Sample	Volume (ml)	Protein (mg)	Total activity (HA units)	Specific activity (HA units/ mg)	Purification
Crude	20	500	102400	204	1
Clarified	10	13.4	51200	3820	18
Purified using formalinized Buffalo RBC	5	0.83	51200	61686	302

Table 2: Hemagglutination titer of hemolymph lectin of *Atergatis ocyroe* with different mammalian erythrocytes

Erythrocytes	HA titer
Buffalo	256
Dog	128
Guinea pig	32
Rabbit	32
Rat	32
Mice	32
Human A	8
Human B	8
Human O	8
Pig	8
Horse	2
Donkey	2

Cow and goat erythrocytes were not recognized by the hemolymph agglutinin of *Atergatis ocyroe*.

Table 3: Hemagglutination titer of hemolymph lectin of *Atergatis ocyroe* in relation to change in pH and temperature

pH	HA titer	Temperature	HA titer
5.0	64	0	128
5.5	64	10	256
6.0	128	20	128
6.5	128	30	32
7.0	256	40	8
7.5	256	50	2
8.0	256	60	0
8.5	128	70	0
9.0	128	80	0
9.5	64	90	0
10.0	64	100	0

Table 4: Effect of cations on the hemagglutination activity of the hemolymph lectin of *Atergatis ocyroe*

$CaCl_2$ (mM)	HA titer
0	128
0.01	128
0.1	256
1	256
5	256
10	256
20	128
30	128
40	128
50	128
100	64

Table 5: Effect of calcium chelators on the hemagglutination of hemolymph lectin of *Atergatis ocyroe*

Concentration (mM)	HA Titer		
	EDTA		Trisodium citrate
	Disodium	Tetra sodium	
0	256	256	256
0.01	128	256	128
0.1	128	128	128
1.0	16	128	128
10	8	64	128
20	4	8	128
30	4	4	128
40	2	2	128
50	0	2	128
100	0	0	128

Table 6: Hemagglutination inhibition (HAI) of hemolymph lectin of *Atergatis ocyroe* by various sugars

Sugar	HAI Titer	Minimum Conc. Required (mM)	Relative Inhibitory Potency (%)
N-acetyl D-Glucosamine	16	6.25	100
Trehalose	16	6.25	100
D-Fructose	16	6.25	100
Melibiose	8	12.5	50
L-Fucose	8	12.5	50
α - Lactose	4	25	25
N-acetyl D-Manosamine	4	25	25

Glucose-3-Phosphate, Glucose-6-phosphate, D-Fucose, Raffinose, Glucose and D-Glucuronic acid did not inhibit HA at concentration as high as 100 mM.

Table 7: Hemagglutination inhibition (HAI) of hemolymph lectin of *Atergatis ocyroe* by various glycoproteins

Glycoprotein	HAI titer	Minimum Conc. Required (µg/ml)	Relative Inhibitory potency (%)
Bovine thyroglobulin	32	156.25	100
BSM	16	312.5	50
Fetuin	16	312.5	50
PSM	8	625	25
Lactoferrin	0	0	0
Apo transferrin	0	0	0

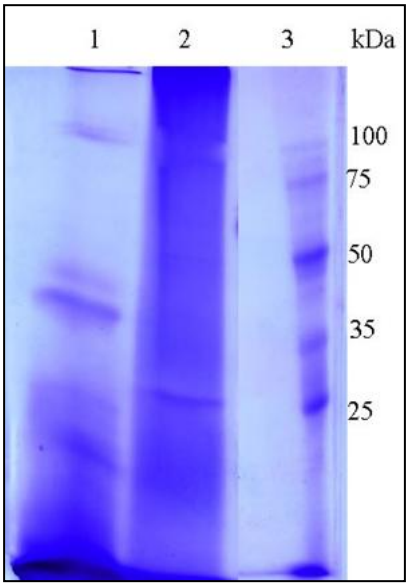

Fig 1: SDS–PAGE analysis: 1- Crude hemolymph, 2- purified lectin, 3-Marker

Discussion

A 25 kDa and 50 kDa lectin with 302 fold increase in specific activity was purified from the hemolymph of *Atergatis ocyroe* by formalinized erythrocyte adsorption technique. Homogeneous lectins with different molecular weights were reported from brachyuran crabs: C*ancer antennarius* [24] and *Trichopeltarionnobile* [25].

Hemagglutination assay of the lectin showed differential affinity with different mammalian erythrocytes. It was found to be non-blood type specific since it agglutinated both animal and human erythrocytes. The lectin preferentially agglutinated buffalo erythrocytes that suggest the specificity of the lectin to the sialic acid moieties expressed on the glycocalyx of the erythrocytes [26]. *A. ocyroe* lectin agglutinated guinea pig, rabbit, rat, mice, human, pig, horse and donkey erythrocytes with low affinity which may be due to the incompatibility of these receptor sites on the erythrocytes with the lectin binding sites [27]. Physico-chemical characterization revealed the lectin as pH and temperature sensitive. Maximum agglutinability was recorded between pH 7.0 and 8.0, which got slightly altered below pH 7.0 and above pH 8.0. Lectin with pH sensitivity is also reported in the hemolymph of *Atergatis*

reticulates [28]. Hemolymph lectin was highly sensitive to temperature because the purified lectin showed high HA titer only upto 20ºC and it gradually reduced and resulted in complete loss of activity at high temperature. This result is in contrast to the results obtained with crude agglutinin of *A. ocyroe* which could retain its activity at temperature ranging from 0-40ºC [29]. This may be due to the conformational changes that occur at the binding sites of the lectin at higher temperatures which may accelerate or suppress the agglutinability.

Hemagglutinin activity of the lectin was enhanced on addition of calcium and the calcium dependency of the lectin was confirmed by the reduction in activity when treated with calcium chelator-EDTA. On this basis, it could be confirmed that *A. ocyroe* lectin is a calcium dependent lectin and therefore be classified as a C-type lectin. Carbohydrate binding activity of C-type lectins is based on the function of the carbohydrate recognition domain whose structure is highly conserved [30]. Calcium is not only involved in the carbohydrate binding at the binding site [31] but contributes to the structural maintenance of the lectin domain that is essential for lectin activity [32, 33]. C-type lectin was also reported in the coastal crab, *Scylla serrata* [34].

The binding determinants of the purified lectin were identified by the hemagglutination inhibition test (HAI) using glycoproteins and sugars. The agglutinability of the purified lectin of the marine crab *A. ocyroe* was inhibited by glycoproteins Bovine thyroglobulin, BSM, Fetuin and PSM and sugars N-acetyl D-Glucosamine, Trehalose and D-Fructose. Inhibition of hemagglutination indicated that the lectin binds to NeuGc of Thyroglobulin[35] and Fetuin [35, 36], NeuGc/ NeuAc of PSM[37] and NeuGc/ NeuAc, N-acetyl 8-O-acetyl neuraminic acid and 7, 8-di-O-acetyl neuraminic acid of BSM [38] suggesting the sialic acid specificity of the agglutinin. The presence of sialic acid specific lectin was also reported in brachyuran crabs, *Scylla serrata* [34] and *Lamella lamellifrons* [9]. The affinity of the lectin to the sugar units can be related to its blood group specificity. Thus, from the present study it is clear that the hemolymph of marine crab *A. ocyroe* has a potent lectin which could be further purified by affinity purification and assessed for its therapeutic potential.

Conclusion

The present study indicates hemolymph lectin of marine crab *Atergatis ocyroe* as sialic acid specific which could be further purified by affinity purification using bovine thyroglobulin as the affinity matrix. Purification of these sialic acid specific lectins would be of interest because of their biomedical potential in the diagnosis and treatment of pathogenic bacteria and human tumor associated antigens.

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