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Characterization of a novel O-acetyl sialic acid specific lectin from the hemolymph of the marine crab, *Atergatis integerrimus* (Lamarck, 1818)

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ABSTRACT

An O-acetyl sialic acid specific lectin was purified from the hemolymph of the marine crab *Atergatis integerrimus* by affinity chromatography using BSM (Bovine Submaxillary Mucin) coupled to cyanogen bromide activated Sepharose 4B and biospecific adsorption using formalinized buffalo erythrocytes. The purified AiL (*Atergatis integerrimus* lectin) showed an 1218 fold increase in specific activity when compared to the crude hemolymph agglutinin. The lectin, on non - denaturing PAGE showed a single band of 216 kDa and when subjected to SDS - PAGE, the lectin resolved into three subunits of molecular weight 70, 72 and 74 kDa. Physico chemical characterization revealed the lectin as pH and temperature sensitive, calcium dependent and sensitive to calcium chelators. Based on the calcium dependency of the lectin, AiL could be classified as a C-type lectin. The purified lectin agglutinated buffalo erythrocytes with greater avidity and was inhibited by the glycoproteins BSM, thyroglobulin, fetuin, PSM, and sugars raffinose, trehalose, L - fucose, α - Lactose, melibiose and GluNAc suggesting the affinity of the lectin to sialic acid. Reduction in HA with asialo buffalo erythrocytes and HAI titer with desialylated BSM, confirms the sialic acid specificity of the lectin. The reduction in HAI following de - O - acetylation confirms the specificity of the lectin for O - acetyl sialic acid. FTIR analysis confirms the purified lectin as a glycoprotein with spectral bands corresponding to amide bands and saccharides. Thus this study paves way to assess the therapeutic application of this lectin that could be targeted to modified sialic acid moieties that are expressed on the malignant cells and pathogenic microbes and also deduce the crystal structure of the lectin.

1. Introduction

Lectins are ubiquitous glycoproteins of non - immune origin that recognize specific carbohydrate structures and agglutinate a variety of animal cells by binding to cell surface glycoproteins and glycolipids [1]. Lectin - carbohydrate interaction represents a ligand - receptor interaction that is universal in all living organisms [2] and such interactions aid in different biological roles like cellular and tissue transport of carbohydrates, glycoproteins and calcium [3], cytolytic and cytotoxic [4] and cell adhesion, migration and apoptosis [5]. They are capable of inducing cell proliferation, cell arrest or apoptosis and have been implicated in organ morphogenesis, tumor cell metastasis, leukocyte trafficking, immune response and inflammation, as well as recognition of extracellular matrix [6]. Lectins isolated from animal tissues were investigated as apoptotic agents, immunomodulatory, antiviral and anticancer drug targets [7]. Among the different classes of lectins isolated from invertebrates, sialic acid specific lectins have gained much importance owing to its function which includes induction of apoptosis, negative regulation of B cell signaling, induction of cytokine secretion [8] and inhibition of bacterial and viral sialidases by altering the immunopotency of sialoconjugates expressed on the microbial surface [9]. Sialic acids are a family of sugars with more than twenty derivatives which differs only in the acyl substitution of the C-5 amino group and O-substituted sialic acids exhibit species and tissue specific distribution [10]. O-acetylation of sialic acids may change with transformation or alteration in the environment of the cell [11] and modified sialic acids such as 9-O-acetyl sialic acid, N-glycolyl neuraminic acid and α-2,6 sialic acids have been detected in human malignant cells [12]. Among arthropods, crustaceans were found to be rich source of sialic acid specific lectins. Lectins have been purified from many brachyuran crabs that are sialic acid specific [13,14]. A 9-O-acetyl and 4-O acetyl sialic acid specific lectin was purified from the hemolymph of marine crab *Cancer antennarius* which can recognize the modified sugar moieties expressed on tumour cells [15]. A sialic acid specific lectin having a unique

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specificity for N-glycolyl neuraminic acid has been isolated from marine crab *Scylla serrata* [16] and multiple agglutinins were detected form *S. serrata* that agglutinated bacteria [17]. Lectin highly specific to O-acetyl sialic acid and mannose residues expressed on bacterial pathogens was purified from marine crab *Erimacrus isenbeckii* [18]. A calcium dependent lectin has been isolated from the hemolymph of marine crab *Trichopeltarion nobile* [19], and *Atergatis ocyroe* [20] and antimicrobial property of lectin has been demonstrated in marine crabs *Portunus pelagicus* [21,22]. Lectin that recognizes N-glycolyl neuraminic acid and exhibits antiproliferative property against cancer cells was isolated from the Korean marine crab *Philyra pisum* and is used as a diagnostic and an anticancer agent [23,24]. The information on isolation and characterization of sialic acid specific lectins from marine crabs is limited. The present investigation reveals purification of novel sialic acid specific lectin from marine crab *A. integerrimus* that can be used in targeted delivery against microbes and malignant cells.

2. Materials and methods

2.1. Collection of hemolymph

The hemolymph from the crab *Atergatis integerrimus* was collected following the procedure of Mercy and Ravindranath [16]. Marine crabs were collected from the coastal regions of Kanyakumari District and the 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 −20 °C for further study.

2.2. Purification of A. integerrimus lectin

Purification of lectin by biospecific adsorption using formalinized buffalo erythrocytes were prepared as previously described Nowak and Barondes [25] with slight modification. Briefly, buffalo erythrocytes collected in Alsever's solution were washed three times in 20 vol of PBS, pH 7.5 (75 mM NaCl, 75 mM Na_2HPO_4) per packed cell volume by centrifugation at 4000 rpm for 5 min. The cells were suspended at a concentration by volume of 8% PBS (pH 7.2) and an equal volume of formalin (3% solution in PBS with pH adjusted to 7.2 with 0.1 M NaOH) was added. The mixture was incubated at 37 °C for 16 h with moderate shaking. The cells were then washed four times in five volumes of PBS (pH 7.2) per packed cell volume and stored at 4 °C as 10% suspension in this buffer. The stored formalinized cells were prepared for use as an affinity reagent by washing six times in 10 vol of TBS, pH 7.2 (50 mM Tris - HCl; 100 mM NaCl). The packed cells were then incubated with 20 vol of clarified serum in plastic tube for 2 h with moderate shaking at 4 °C and then washed 3 times with 20 vol of TBS, pH 7.2, containing 0.01 M $CaCl_2$. Elution of adsorbed hemagglutinin was accomplished by incubation of the cells with 10 vol of 10 mM disodium EDTA in TBS, pH 8.2. The elution was continued for 2 h with moderate shaking at 4 °C and the elution mixture was then centrifuged for 10 min at 28,000 × g to remove any residual particulate material and the resultant supernatant was dialyzed and tested for hemagglutination with 1.5% buffalo erythrocytes in TBS, pH 7.5. The protein concentration of the lectin was estimated by Folin-Ciocalteau method [26].

The initial step in the purification of the hemagglutinin from crab hemolymph is the removal of most of the respiratory pigment, hemocyanin which makes up approximately 95% of the hemolymph protein. This is achieved by high speed centrifugation. However, this centrifugation step appears to remove 50% of the hemagglutination activity. Following centrifugation, the clarified serum (20 ml) was applied to 3.5 ml of BSM-Sepharose 4B in an econo column (Bio - Rad) previously equilibrated with TBS at 4 °C. The eluant was collected at a rate of 0.6 ml/min. The column was washed with HSB until A_{280} of the effluent was <0.002 and the effluent was tested for HA activity. The column was further washed with LSB at 4 °C until the A_{280} of the effluent was <0.002, and it was then washed with warm LSB (30 ± 2 °C) until the A_{280} of the effluent was ˂0.002. This step further eluted additional inert proteins and was necessary for obtaining homogenous lectin. All the buffers so far used contained the calcium required for lectin binding to BSM-Sepharose 4B. The elution of lectin was done with EB that contained 10 mM disodium EDTA and collected as 1 ml fractions on ice in polypropylene tubes containing 10 μl of 100 mM calcium chloride at the rate of 0.3 ml/min. The fractions were vortexed immediately after collection and kept on ice. Fractions containing lectin were pooled on the same day and dialyzed against 10 mM $CaCl_2$, at 4 °C for 30 min to 1 h and the dialysate was then aliquoted, lyophilized (speed - vac, Sawant) and stored at −20 °C. The protein concentration of the lectin was estimated by Folin Ciocalteau method [26]. The "specific lectin activity", was obtained by dividing the lectin titer with protein concentration of the sample.

2.3. Hemagglutination (HA) and HAI assay

Hemagglutination assays and Hemagglutination inhibition assays were performed in 96 well, 'U' bottomed microtiter plates (Tarson, India) as described by Ravindranath and Paulson [27]. The purified lectin (25 μl) was serially diluted with TBS-BSA (25 μl, pH 7.5) and mixed with 25 μl of 1.5% erythrocyte suspension and incubated for 1 h at room temperature (30±2 °C). HA titre was reported as the reciprocal of the highest dilution of lectin giving complete agglutination after 1 h.

Hemagglutination inhibition assays were carried out by serially diluting 25 μl of known concentration of inhibitor (glycoprotein: 5 mg/ml or sugar: 100 mM) in microtiter plate with 25 μl of TBS - BSA. To each dilution, was added 25 μl of the purified lectin at subagglutination concentration (HA = 2) and mixed. After mixing, the lectin inhibitor solution was incubated for 1 h at room temperature (30 ± 2 °C), and was mixed with 25 μl of 1.5% suspension of buffalo erythrocytes. The HAI titers were reported as the reciprocal of the highest dilution of inhibitor giving complete inhibition of agglutination after 1 h.

2.4. Physico chemical characterization of lectin

The physicochemical properties were determined by hemagglutination assays with purified lectin samples under conditions of varying pH (5–14), temperature (0° to 100 °C), bivalent cations calcium, magnesium and manganese (1 mM–100 mM) and calcium chelators like di and tetra sodium EDTA and trisodium citrate (1 mM–100 mM).

2.5. Sialidase treatment of buffalo erythrocytes and sialoglycoproteins

Sialidase treatment of erythrocytes and sialoglycoproteins of the lectin was carried out following the procedure of Mercy and Ravindranath [16]. A reaction mixture (total 1.0 ml) containing 10% washed buffalo erythrocytes in PBS - BSA (pH 7.0) and 140 milliunits of neuraminidase of *Clostridium perfringens* (Type X: Sigma) was incubated at 37 °C for 4 h. Neuraminidase treated and untreated erythrocytes were washed with PBS - BSA three times and pelleted by low speed centrifugation and finally washed in TBS - BSA (pH 7.0). HA assays were performed against the native and desialylated erythrocytes using purified lectin. For sialidase treatment of glycoproteins, Asialo BSM was prepared by incubating 2 mg of glycoprotein with 0.1 unit of *Clostridium perfringens* sialidase (Type X: Sigma) in 400 μl of 5 mM acetate buffer, pH 5.5 for 2 h at 37 °C. As a control, BSM was treated similarly without sialidase. HAI assay of the purified lectin was performed using sialidase treated and untreated BSM against 1.5% buffalo erythrocyte suspension.

Table 1
Purification of lectin from the hemolymph of the marine crab *Atergatis integerrimus*.

Sample	Volume (ml)	Protein (mg)	Total activity (HA units)	Specific activity (HA units/mg)	Purification (fold)
Crude hemolymph	30	1447	3.07×10^5	209	1
Clarified Serum	20	281	2.04×10^5	728	3
Purified using formalinized Buffalo RBC	10	3.3	1.02×10^5	3.05×10^4	146
Purified using BSM Sepharose affinity column	10	0.20	5.12×10^4	2.54×10^5	1218

2.6. De - O - acetylation of sialoglycoproteins

De - O – acetylation of BSM was performed following the procedure of Schauer [10]. A solution of 750 μl of glycoprotein (5 mg/ml) was added to 250 μl of 0.04 N NaOH, vortexed and incubated on ice for 45 min and neutralized with 1 ml of 0.01 N HCl. For control, untreated BSM was reconstituted in TBS. The HAI assay was performed using base treated and untreated BSM against purified lectin and 1.5% suspension of buffalo erythrocytes.

2.7. Determination of molecular weight by SDS-PAGE and native PAGE

The homogeneity of the purified lectin was analyzed by native PAGE and the molecular weight was evaluated using SDS-PAGE. SDS-PAGE 12.5% slab gel electrophoresis was performed according to Laemmli [28].

2.8. FTIR analysis

FTIR analysis of the purified sample was done by forming lectin KBr pellet in 1:100 ratio and the spectrum was recorded using Shimadzu IR affinity – 1 in the range of 400–4000 cm^{-1} at a resolution of 4 cm^{-1} [29].

3. Results

3.1. Purification of Atergatis integerrimus lectin (AiL)

The crude agglutinin *Atergatis integerrimus* hemolymph was found to have strong agglutinating activity against buffalo erythrocytes. AiL, a lectin from *A. integerrimus* was purified by bio adsorption using formalinized buffalo erythrocytes and affinity purification (Table 1, Figs. 1 and 2). The specific activity of the affinity purified lectin showed 1218 purification fold and 2.54 × 10^5 increase in specific activity and

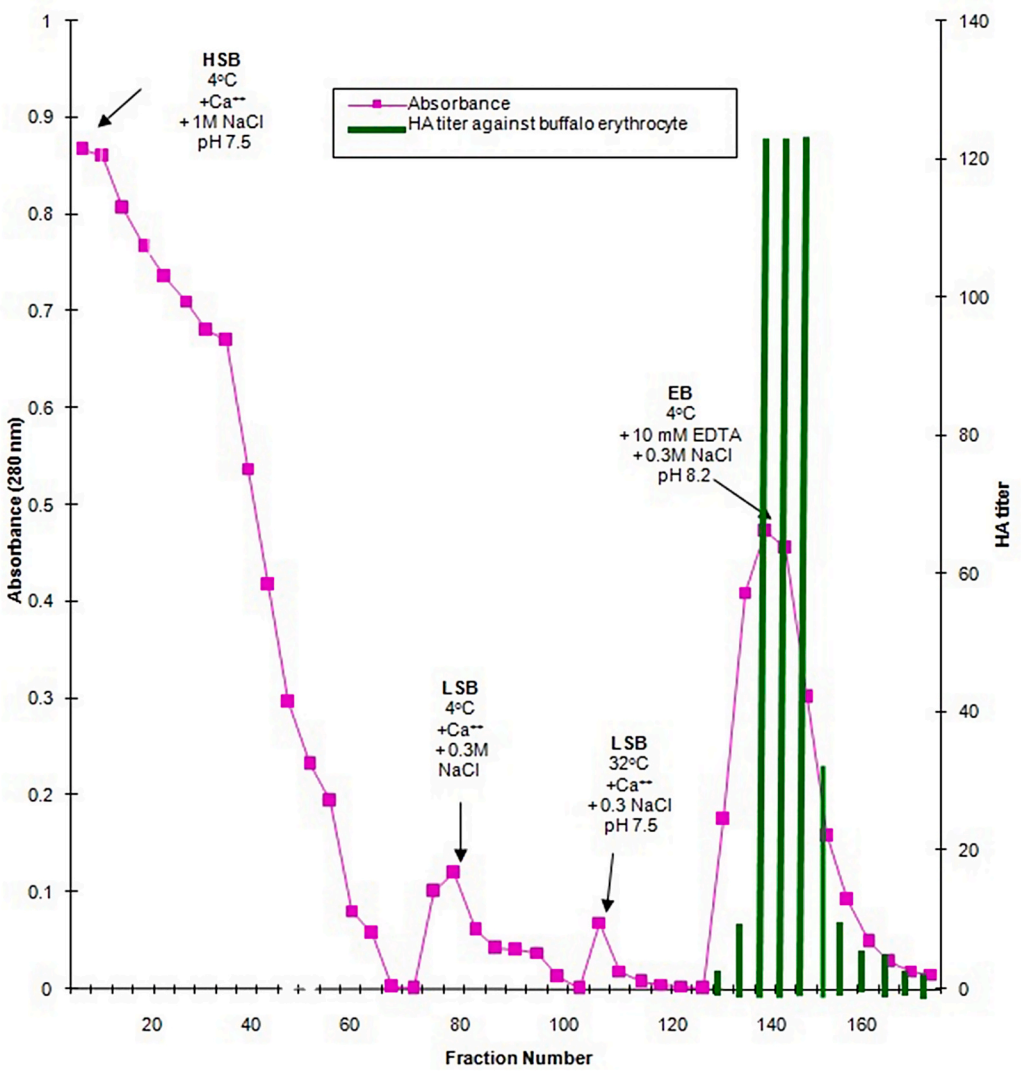

Fig. 1. BSM-affinity column profile for the purification of lectin. A major peak was observed and HA titre was maximum at fractions 115–125.

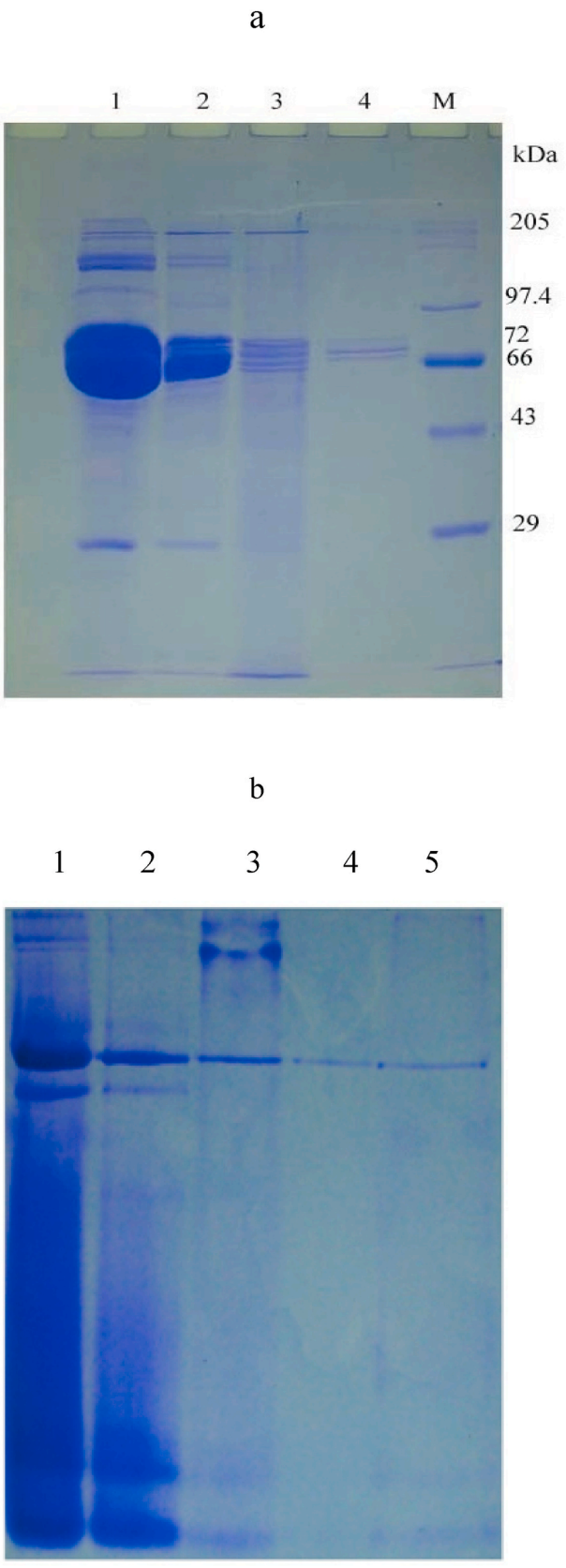

Fig. 2. SDS-PAGE (a): Lane 1- Crude hemolymph, Lane 2- Clarified hemolymph, Lane 3 - Formalinized buffalo erythrocytes adsorption purified lectin, Lane 4- BSM - Sepharose 4B affinity chromatography purified lectin, Lane M: Protein marker. Native gel electrophoresis (b): Lane 1, 2 and 3: Crude haemolymph, Lane 4 and 5: Lectin.

formalinized erythrocyte adsorption technique yielded 146 fold purification with the specific activity of 3.05×10^4. 10 ml of clarified serum following affinity purification yielded 0.20 mg of lectin. Native PAGE analysis of the purified lectin suggested AiL as a single lectin. The apparent molecular mass of the purified AiL on SDS-PAGE under reducing conditions resolved into three subunits 70, 72, 74 kDa.

3.2. FTIR analysis

Fig. 3a and b illustrate the FTIR spectrum of formalinized and affinity purified lectin respectively. FTIR spectra of the purified lectin of marine crab *Atergatis integerrimus* exhibited characteristic bands which included both proteins and sugars. The peptide group gave 2 bands named Amide I and II. The amide I band (between 1500 and 1700 cm^{-1}) leads to C═O stretching and N–H bending vibrations. A key peak was obtained at 1637 cm^{-1} suggesting the proteinaceous nature of the lectin. A small peak was observed at 1408 cm^{-1} which lies in the absorption spectra of Amide II band with N–H bending and C–N stretching vibrations (1300 cm^{-1} to 1400 cm^{-1}). FTIR studies showed that the spectral region between 1500 cm^{-1} to 1000 cm^{-1} would contain most of the characteristic bands relevant to major sugars. Absorption peaks include 1330, 1280, 1062 cm^{-1} and the key peak was observed at 1062.78 cm^{-1}. It corresponds to C–O bending and C–OH stretching and the sugars may correspond to glucose, fructose and galactose. Small peaks were also observed from 3400 to 3900 cm^{-1} which suggests the O–H stretch pertaining to alcohol assignments.

3.3. Physicochemical properties of hemolymph lectin

The purified lectin was pH and temperature sensitive. The HA was stable between pH 6.5 to 8.5 (Table 2a) and temperature ranging from 10 to 20 °C (Table 2). Addition of divalent cations (Ca^{2+}, Mg^{2+} and Mn^{2+}) increased the HA titer at 10 mM Ca^{2+} which on further increase showed a decrease in hemagglutinating activity (Table 2b). Calcium chelators di and tetra sodium EDTA and trisodium citrate reduced the HA at 5 mM concentration and a decrease was noted with further increase in concentration (Table 2c).

3.4. Hemagglutinating activity of AiL

The purified lectin agglutinated dog, buffalo and mice erythrocytes with high affinity. Moderate activity was recorded with rat, rabbit, guinea pig, Human A, B and O erythrocytes (Table 3). Maximum agglutination was observed with erythrocytes that expressed N-acetyl and N-glycolyl neuraminic acid on the cell surface.

3.5. Hemagglutination inhibition (HAI) assay

The agglutinability of the purified lectin was inhibited by glycoproteins, BSM = thyrolobulin > fetuin > PSM > lactoferrin > transferrin > Apo - transferrin (Table 4a). Sugars: raffinose > trehalose = α - lactose = melibiose = L - fucose = GluNAC > Glu - 6 - PO_4 > Glu - 3 - PO_4 = ManNAc = D – fructose = glucose = D – galactosamine > D - galactose > GalNAc = fucose inhibited the hemagglutinability of the lectin with varying titre. The other sugars tested failed to inhibit the lectin (Table 4b).

3.6. Binding specificity of lectin before and after desialylation and de-O acetylation

To identify the nature of the binding determinant of the purified lectin BSM and buffalo erythrocytes were enzymatically modified by treating with neuraminidase and a marked reduction in hemagglutination was observed. Base treatment, specific for hydrolysis of the O - acetyl groups of sialic acids considerably reduced the ability of BSM to inhibit hemagglutination suggesting the possible role of O-acetyl sialic

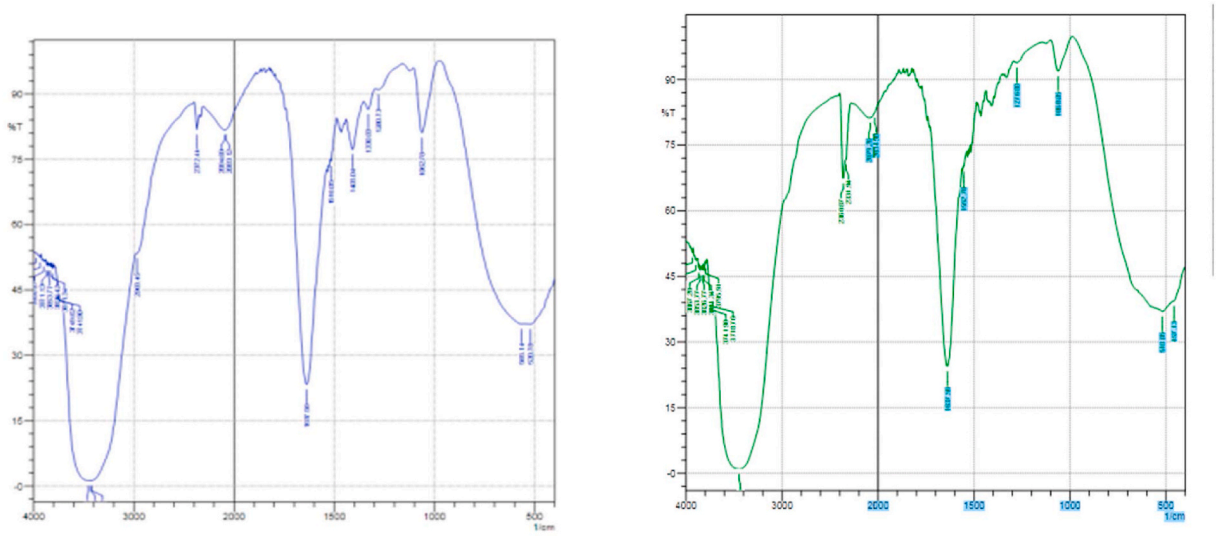

Fig. 3. a and b. FTIR analysis of formalinized RBC adsorption and affinity chromatography purified lectin.

acid in the interaction of lectin with BSM (Table 5a and b).

4. Discussion

A sialic acid specific natural lectin - AiL from marine crab *Atergatis integerrimus* was purified by affinity chromatography using BSM - linked sepharose 4B and bioadsorption using formalinized buffalo erythrocytes. FTIR spectra of the purified lectin of marine crab *Atergatis integerrimus* exhibited characteristic bands which included both proteins and sugars. The peptide group gave two major bands amide I and amide in the infrared spectrum. The amide I band is associated with the C═O stretching vibration and is directly related to the backbone conformation while amide II results from the N–H bending and C–N stretching vibration [30]. The parallel beta sheet structure leads to an amide I absorption near 1640 cm^{-1}. The spectral region between 1500 cm^{-1} to 1000 cm^{-1} represents the bands relevant to sugars and it corresponds to C–O bending and C–OH stretching. Further studies on the lectin conformation may pave way to analyse the α helix and β-sheet and there by deduce the lectin crystal structure by X-ray crystallography or NMR spectroscopy [31].

Lectins are proteins or glycoproteins present in invertebrates as defense molecules. Though capable of agglutinating a variety of erythrocytes, the agglutinin showed high affinity for dog, buffalo and mice erythrocytes that express sialic acid moieties on their surface. The purified lectin had a molecular mass of 216 kDa with three subunits of 70, 72 and 74 kDa on SDS - PAGE. Subunit heterogeneity is a characteristic feature of many lectins [32] and each subunit may have different biological properties with different affinities for lymphocytes and erythrocyte membrane receptors [33]. Lectins with subunits have been reported from marine crab *S. serrata* of Indian Oceanic region with molecular mass of 55 kD and is a heterodimeric lectin of subunits 30 kD and 25 kD respectively [34]. Murali et al. [35] reported a natural agglutinin purified from the serum of hermit crab, *Diogenes affinis* which possessed four subunits (51, 49, 42 and 39 kDa). Divalent cations are concerned with holding the lectin subunits together [36] and it serves to orient protein functional groups for ligand coordination [37] and

Table 2a
Hemagglutination titer of the hemolymph lectin of the marine crab, *Atergatis integerrimus* in relation to pH and temperature.

pH (N = 25)	HA titer	Temperature^{o} C (N = 25)	HA titer
5	32	0	128
5.5	64	10	128
6	64	20	128
6.5	128	30	64
7	128	40	8
7.5	128	50	8
8	128	60	4
8.5	128	70	2
9	64	80	0
9.5	64	90	0
10	32	100	0

Table 2b
Effects of cations on the hemaglutinating activity of the hemolymph lectin of the marine crab *Atergatis integerrimus.*

Cation conc.in mM (N = 25)	HA titer		
	Ca^{2+}	Mg^{2+}	Mn^{2+}
0	64	64	64
0.01	64	64	128
0.1	128	128	128
1.0	128	128	128
5.0	128	128	128
10	128	128	128
20	128	128	128
30	64	64	128
40	64	64	128
50	32	64	64
100	32	64	64

Table 2c
Hemagglutination titer of the *Atergatis integerrimus* hemolymph lectin against mammalian erythrocytes.

Erythrocyte N = 5	HA titer
	Purified lectin
Buffalo	128
Dog	128
Mice	64
Rat	32
Rabbit	16
Guinea Pig	8
Human A	4
Human B	4
Human O	16
Camel	ND
Goat	0
Pig	0
Horse	2
Donkey	ND
Cow	0

Table 3
Effect of chelators on the hemagglutinating activity of the hemolymph lectin of the marine crab *Atergatis integerrimus*.

Concentration (N = 20)	EDTA		Trisodium citrate
	Disodium	Tetrasodium	
0	64	64	64
0.01	64	64	64
0.1	64	64	64
1.0	128	128	64
5.0	128	128	128
10	16	64	128
15	4	16	128
20	4	8	64
30	2	2	64
40	2	2	16
50	0	0	8
100	0	0	8

Table 4a
Hemagglutination inhibition of the hemolymph lectin of the marine crab *Atergatis integerrimus* by glycoproteins.

Glycoproteins N = 5	HA titer	Nature of sialic acid	Minimum conc. Required (mg/ml)	Relative inhibitory potency (%)
BSM	128	NeuAc/ NeuGc	39.06	100
Thyroglobulin	64	NeuGc	78.125	50
Fetuin	16	NeuGc	156.25	25
PSM	16	NeuGc	312.5	12.5
Lactoferrin	16	NeuAc	312.5	12.5
Transferin	8	NeuGc	625	6.25
Apo-transferin	4	NeuGc	1250	3.125

maintain the functional integrity of lectin molecules [38]. Characterization of the purified *A. integerrimus* lectin revealed it to be calcium dependent and it was clearly evident from the reduction in HA titre on treatment of lectin with EDTA and restoration of the original activity on addition of Ca^{2+}. Lectins which have been strongly implicated in defense are calcium dependent and show binding to saccharide on bacterial cell surfaces indicating their role in defense [39]. Crustacean agglutinins although show marked variability in their molecular structure and sugar specificity, are calcium dependent [40]. Very few studies on crustacean lectins had shown them to be independent of divalent cations and also insensitive to EDTA [41].

Lectins are proteins/glycoproteins that can get denatured under extreme physical conditions and the purified lectin, AiL was also found to be pH and temperature sensitive. Thermal, acidic and alkaline pH or chemical induced denaturation may result in partial or complete loss of structure by disruption of the non-covalent interactions that stabilize a protein structure and the native dimeric state is more stable at pH 7 than at acid induce monomeric state [42]. Variation in HA titre at extreme temperatures may be due to either the structural transition of the lectin or modification of cell surface properties [43] and may cause protein unfolding by exposure of its hydrophobic core [42].

The specificity of the lectin is related to the carbohydrate to which it shows highest affinity and lectins that are specific for one saccharide may also bind although with low affinity to other saccharide that are structurally related. The hapten inhibition study shows that the agglutinin of the *A. integerrimus* is best inhibited by simple hexoses especially raffinose which is a trisaccharide made of galactose, glucose and fructose. The purified lectin was inhibited by disaccharides α - lactose and melibiose which is linked by glucose and galactose and trehalose by two glucosidic linkages. The lectin also recognized L-fucose which is a monosaccharide and is a common component of many N- and O-linked glycans and glycolipids produced by mammalian cells. N - acetyl derivatives (GluNAc, GalNAc, ManNAc), which contain the acetyl group on c - 2, inhibited the lectin there by demonstrating that an acetyl group is essential for agglutinin - ligand interaction. In decapods, the specificity of lectins towards carbohydrates is mainly related to N - acetylated carbohydrates, such as NeuAc, GluNAc, and N - acetyl - D - galactosamine (GalNAc), N – acetyl glucosamine (GluNAc), N - acetyl – mannosamine (ManNAc). The results show the ability of the lectin to recognize a wide range of carbohydrates that will potentially help the animal to recognize pathogens based on their surface molecules [44] and agglutinate cells that express specific carbohydrates on the cell surface [3].

Table 5a
Effect of Neuraminidase treatment of buffalo erythrocytes on hemagglutination assay of the hemolymph lectin from a marine crab *Atergatis integerrimus*.

Enzyme used	Site of enzyme activity	HA titer
None	–	128
Neuraminidase (*C.perfringenus*)	Neu Ac-D-Gal Neu Ac-D-Gal NAc	16

Table 5b
HAI of purified lectin of *A. integerrimus* by BSM before and after de-o-acetylation and desialylation.

Sl.No	Glycoprotein treatment	HAI titer
1	De-O-acetylation	
	BSM untreated	128
	BSM treated	4
2	Desialylation	
	BSM (without sialidase)	128
	BSM + Sialidase (4 h)	8

Table 4b
Hemagglutination inhibition of the hemolymph lectin of the marine crab *Atergatis integerrimus* by various sugars.

Sugars	HAI titer	Minimum con.required (mM)	Relative inhibitory potency (%)
Raffinose	64	1.56	100
Trehalose	32	3.12	50
α- Lactose	32	3.12	50
Melibiose	32	3.12	50
L-Fucose	32	3.12	50
Glu NAc	32	3.12	50
Glu-6-PO_4	16	6.25	25
Glu 3-PO_4	8	12.5	12.5
Man NAc	8	12.5	12.5
D-Fructose	8	12.5	12.5
Glucose	8	12.5	12.5
D-galactosamine	8	12.5	12.5
GalNAC	4	25	6.25
D- galactose	2	50	3.12
Fucose	2	50	3.12

Inhibition study with glycoproteins documented the sialic acid specificity of the humoral agglutinin. The agglutinability of the lectin was best inhibited by bovine sub maxillary mucin (BSM) which contains the sialic acids, N - acetyl neuraminic acid (85.5%), N – glycolyl neuraminic acid (14.5%), N - acetyl 9 - O - acetyl neuraminic acid and 8, 9 - di - O - acetyl neuraminic acid [45]. PSM that contains 90% N – glycolyl neuraminic acid and 10% NeuAc and traces of O - acetyl neuraminic acid [46], thyroglobulin NeuGc −10% of total sialic acid [47], fetuin (NeuGc - 7% of the sialic acid [48] and transferrin that contained NeuGc [49] showed weak inhibitory potency. The sialic acid affinity of the lectin was further established when sialidase treated BSM failed to inhibit hemagglutination of purified lectin in *A. integerrimus*. Sialidase treatment results in the release of 97% of the sialic acid from bovine submaxillary mucin [15] which causes a reduction in HAI. De - O – acetylation of such sialoglycoproteins by base treatment specifically hydrolyses O - acetyl groups of sialic acids without cleavage of peptide bonds [10], suggesting that the inhibitory potency of BSM and other sialoglycoproteins may be due to similar O - acetyl sialic acid. Thus our observations suggests that the inhibitory properties were mediated by 9 (7,8) O-acetyl sialic acids by α 2–6 GalNAc linkage [15]. Hence it can be inferred that the lectin shows specific binding to O - acetyl neuraminic acid containing glycoproteins. O – acetyl sialic acid specific lectins are reported from brachyuran crabs, *Cancer antennarius* [15], and *Travancoriana charu* [50].

Although the recognition of NeuAc is a common feature among crustacean lectins some of them exhibit a particular pattern of specificity towards O - acetylated sialic - acid derivatives [51]. The report of Ravindranath et al. [15] has shown the specificity of the marine crab *Cancer antennarius* hemolymph lectin to 9–0/4 - O - acetyl sialic acid and the marine crab, *Liocarcinus depurator* have been shown to specifically recognize 9 - O – acetyl sialic acid [52]. On the other hand, a sialic acid binding lectin with specificity for N – glycolyl neuraminic acid (Neu Gc) was purified from the hemolymph of the marine crab *Scylla serrata* [16]. As the common receptor component of the glycocalyx of buffalo, dog and rat erythrocytes is NeuGc/NeuAc [53] and mouse erythrocytes is 9-O-Ac NeuAc/NeuGc [11] and the specific inhibitors, bovine submaxillary mucin, thyroglobulin and fetuin have NeuAc/NeuGc in their composition [45] it can be considered that marine AiL has preference for sialic acid. Since sialidase treatment of erythrocytes and sialoglycoproteins resulted in a tremendous reduction in the affinity of the lectin to desialylated erythrocytes and glycoproteins, sialic acid specificity is confirmed.

Sialic acids a family of 9 - carbon α - keto acid amino sugars are recognized as ubiquitous proteins in nature that play a key role in human health and diseases. Sialylation of glycoproteins changes under pathological conditions as well as during developmental stages, and altered sialylation often has significant implication in the physiological role of glycoproteins [54]. Upregulation and downregulation of O-acetylation on Sias is associated with specific carcinomas [55]. In human malignant melanoma and acute lymphoblastic leukaemia there is a modification of 9 - O - acetyl - NeuAc [56] and N – glycolyl neuraminic acid and α 2 → 6 linked sialic acid in human colon carcinoma [57]. Modified sialic acids may alter cell and tissue interaction with viruses, bacteria and parasites [58]. Lectins exhibit growth inhibitory effects towards many carcinoma cells [59]. Hemolymph of the marine crab *Atergatis integerrimus* is a potential source of lectin - a defense molecule which owing to its specificity to modified sialic acid moieties expressed on the cell surface can be used to target malignant cells. Thus the results of the present investigation strongly suggest that *A. integerrimus* has a sialic acid specific lectin in the hemolymph with a strong affinity for O - acetyl group, which can be used as a valuable diagnostic tool for recognizing O - acetyl sialic acid on the cell surface of microorganisms and tumor cells.

CRediT authorship contribution statement

T. Elayabharathi: Writing - original draft. **J. Vinoliya Josephine Mary:** Supervision. **S. Mary Mettilda Bai:** Formal analysis.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2020.07.039.

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