

Purification and characterization of a T-antigen specific lectin from the coelomic fluid of a marine invertebrate, sea cucumber (*Holothuria scabra*)

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Abbreviations used: HSL, *Holothuria scabra* lectin; 2dGal, 2-deoxy-D-galactose; Me α/β Gal, methyl α/β -D-galactopyranoside; T-antigen, Gal β 1-3 GalNAc; T (α -ser)-antigen (Gal β 1-3 GalNAc α -1-O-L-Ser), Thomsen-Friedenreich antigen; Me α -T-antigen, Gal β 1-3 GalNAc α -1-O-Me; Me β -T-antigen, Gal β 1-3 GalNAc β -1-O-Me.

Abstract

A novel lectin was purified from the coelomic fluid of the sea cucumber *Holothuria scabra* (HSL), subjected to bacterial challenge. HSL is a monomeric glycoprotein of molecular mass 182 kDa. The lectin is highly thermostable as it retains full activity for 1 hr at 80 °C. Further, the hemagglutination activity of HSL is unaffected by pH in the range 2-11. Unlike other lectins purified from marine invertebrates, the hemagglutination activity of HSL does not require any divalent metal ions. The affinity profile of HSL was studied by a combination of hemagglutination inhibition and fluorescence spectroscopy. HSL binds to desialylated glycoproteins, Me α Gal, T-antigen and T (α -ser)-antigen with a distinction between β 1-4 and β 1-3 linkages. Me α -T-antigen was a potent ligand having highest affinity (K_a 8.32 x 10⁷ M⁻¹). Monosaccharide binding is enthalpically driven while disaccharide binding involves both entropic and enthalpic contributions.

Keywords: Marine invertebrate; *Holothuria scabra*; Lectin; Fluorescence spectroscopy; Thermodynamic properties.

1. Introduction

Lectins are mono- or multivalent, proteins or glycoproteins of non-immunogenic origin, which recognize specific carbohydrate structures reversibly in a non-catalytic manner [1]. They are ubiquitous in nature, and are found in plants, microorganisms and animals including marine invertebrates. In marine invertebrates, lectins have been suggested to participate in innate immune response by inducing bacterial agglutination or by acting as opsonins to enhance phagocytosis by coelomycetes [2-5]. Besides role in cell recognition and host defense, lectins have long been used as probes to determine sugar composition of glycan and glycoconjugates like bacterial lipopolysaccharide, cell surface glycoproteins and glycolipids [6]. Therefore, it is essential to understand the mechanism of ligand binding to lectin, in order to facilitate their use as an analytical tool and for better characterization of lectin interaction with cell bound carbohydrates.

A large number of lectins have been isolated and characterized from marine sources, some of them are from sponges [7-10], tunicates [11-14], crustaceans [15-17], and molluscs [18]. However very few lectins are reported in echinoderms in comparison with those of other marine invertebrates, including few lectins from sea urchin, a sialic acid binding lectin belongs to heat shock protein family [19], endogenous echinonectin from *Lytechinus variegates* [20], Lectin from *Asterina pectinifera* [21] and also shown the involvement of lectin in the complement homologous immune system [4]. Sea cucumbers are echinoderms inhabiting relatively shallow coastal areas experiencing little or no currents. Some lectins have been isolated from different species of sea cucumbers. Four Ca^{2+} dependent, galactose/GalNAc specific (CEL I, II, III and IV) lectins from *Cucumaria echinata* [22]; a mannan-binding lectin from coelomic fluid of *Cucumaria japonica* [23]; four structurally distinct Ca^{2+} dependent lectins; uronic acid (SPL-1), GalNAc (SPL-2, SJL-I) and lactose/melibiose (SJL-II) specific lectins were isolated from *Stichopus japonicus* [24,25]; Hemagglutinins and hemolysins from coelomic fluid of *Holothuria polii* [26,27]. However, none of the marine invertebrate lectins have been explored for their sugar-binding kinetics and thermodynamics in detail. In this article, we report our extensive studies on isolation and characterization of a lectin, named HSL, from the coelomic fluid of a sea cucumber, *Holothuria scabra*. The

specificity profile and thermodynamic properties of HSL were studied by a combination of hemagglutination inhibition and fluorescence spectroscopy.

2. Materials and methods

2.1 Materials

D-glucose, D-mannose, D-galactose, D-galactosamine, 2-deoxy-D-galactose, L-fucose, methyl α -D-galactose, methyl β -D-galactose, D-maltose, α -lactose, α -D-melibiose, raffinose, stachyose, phenyl-sepharose CL-4B, pronase-E, carboxypeptidase, aminopeptidase, fetuin (bovine), fibrinogen (human), thyroglobulin (bovine), were obtained from Sigma chemical Co. St. Louis, U.S.A; molecular weight markers and Sephacryl S-300 from Amersham Biosciences, Sweden; holotransferrin (bovine) was from Calbiochem, CA, USA; while Gal α 1-3 Gal, Gal α 1-4 Gal, Gal β 1-3 GlcNAc, Gal β 1-4 GlcNAc, Gal β 1-3 GalNAc, Gal β 1-3 GalNAc α 1-O-L-Serine, Gal β 1-3 GalNAc α 1-O-methyl and Gal β 1-3 GalNAc β 1-O-methyl were purchased from Dextra Labs, London, UK, all other chemicals used were of analytical grade.

2.2 Purification of lectin

Adult sea cucumbers (*Holothuria scabra*) were collected from Anjuna coast of Goa, India and maintained in running seawater facility at National Institute of Oceanography, Goa. Lectin accumulation was induced by challenging about 5-6 animals with a cocktail of six different bacterial strains that include, *Staphylococcus citreus*, Group D *Streptococci*, *Escherichia coli*, *Klebsiella pneumoniae*, *Serratia morganii* and *Shigella* sp. Coelomic fluid was collected from the challenged animal on 5th day, in 1:1 ratio with Alsever's solution as anticoagulant (dextrose 20.5 gm, tri sodium citrate 8 gm, citric acid 0.55 gm and sodium chloride 4.2 g in 1000 mL water). The resulting solution was centrifuged (5000 x g, 15 min at 4 °C) to remove coelomycetes and the clear supernatant was treated with serine proteinase inhibitor (PMSF, 20 $\mu\text{g mL}^{-1}$) and stored at -20 °C.

Coelomic fluid preparation was concentrated over a 10 kDa ultra filtration membrane (to remove <10 kDa contaminants) and heat treated at 60 °C for 2 h to inactivate proteases. Tris-HCl buffer, pH 8.5 (TB) was added to a final concentration of 20 mM. Solid ammonium sulphate was added to the preparation to a final concentration of 3 % (w/v). The sample was then passed through hydrophobic interaction column (phenyl sepharose), pre-equilibrated with TB containing 3 % (w/v) ammonium sulphate. The column was washed with loading buffer until A_{280} of the eluate returned to baseline. The bound lectin was eluted with TB. Fractions showing hemagglutination activity were pooled together, treated with ammonium sulphate to a final concentration of 1 % (w/v) and re-loaded on phenyl sepharose column, pre-equilibrated with TB containing 1 % (w/v) ammonium sulphate. Bound protein was eluted with TB. Fractions with lectin activity were pooled together and stored at 4 °C until further use.

Protein concentration was determined according to the method of Bradford [28] using bovine serum albumin as standard. Total neutral sugar content of the lectin was estimated by phenol-sulphuric acid method of Dubois et al [29], using galactose-mannose (4:3) as standard.

2.3 Erythrocyte preparation, hemagglutination and inhibition assays

Human erythrocytes of blood group A, B and O were washed 4-5 times with 20 mM Tris-HCl buffer pH 7.2 containing 150 mM NaCl (TBS) and a final suspension of 3 % (v/v) was prepared in TBS and treated with different enzymes, pronase (0.5 mg.mL⁻¹), trypsin (2.5 U.mL⁻¹) and neuraminidase (0.1 U.mL⁻¹) at 37 °C for 1 h. Enzyme treated erythrocytes were washed four times with TBS before use.

Hemagglutination tests were performed in standard microtitre plates with U-bottom wells by two-fold serial dilution method. A 50 µl aliquot of erythrocyte suspension was mixed with 50 µl of serially diluted coelomic fluid, incubated for 1 h and visually examined for agglutination. The unit of activity (HA units) was expressed as the reciprocal of the highest dilution (titre) of the lectin that showed complete agglutination.

The specific activity of the lectin is defined as the hemagglutination units per milligram of protein (HA unit.mg⁻¹).

Hemagglutination inhibition assays were performed essentially as hemagglutination assay, except that serial dilution of various sugar solutions (25 µL) were pre-incubated for 15 min at room temperature with 25 µL of HSL (concentration 8 HA). 50 µL of erythrocyte suspension was added and the plates examined after 1 h. Glycoproteins were desialylated by incubating in 0.1 M H₂SO₄ at 60 °C for 1 h followed by dialysis against distilled water.

2.4 Biochemical characterization of the purified lectin

The relative mass of the purified HSL was determined by gel filtration on sephacryl S-300 (1.5 cm x 180 cm), equilibrated with TB, pH 8.5 and calibrated with apoferritin (443,000), β- amylase (200,000), alcohol dehydrogenase (150,000), bovine serum albumin (66,000) and ovalbumin (45,000). Molecular mass of HSL under denaturing condition was determined by SDS-PAGE [30] in 7 % gel, followed by visualization with silver staining. Isoelectric focusing (IEF) in polyacrylamide gels was carried out using ampholines in the pH range of 8-11 [31].

2.5 Effect of pH, temperature and divalent cations

The pH dependence of the lectin was determined by incubating 20 µg of HSL with buffers in different pH: 0.1 M glycine /HCl (pH 2-3), 0.05 M sodium acetate /acetic acid (pH 4-5), 0.05 M Potassium phosphate (pH 6-7), 0.05 M Tris-HCl (pH 8-9) and 0.1 M glycine-NaOH (pH 10-11) for 5 days at 25 °C and pH was adjusted to 7.2 just prior to hemagglutination assay. Thermal stability of HSL was monitored in the range of 20-100 °C by incubating the lectin for 60 min at the respective temperature, followed by cooling on ice and determination of residual activity under standard conditions. The hemagglutination activity of the HSL was also determined in the presence of divalent cations, Ca⁺⁺, Mg⁺⁺ and Mn⁺⁺ (1, 5 and 10 mM) and also after extensive dialysis against 10 mM of EDTA.

2.6 Atomic absorption spectroscopy (AAS)

Presence of magnesium, calcium, iron, copper and manganese in HSL was determined by atomic absorption spectroscopy with ATI UNICAM 929 AA spectrometer at 285.10, 422.7, 248.3, 324.8 and 279.48 nm respectively. Sample preparation involved extensive dialysis against deionised water, followed by centrifugation at 8000 g for 5 min to remove any particulate matter.

2.7 Circular dichroism (CD) measurements

CD spectra were recorded on a Jasco J-715 spectropolarimeter in the range of 190–250 nm at a scan speed of 100 nm.s⁻¹ with a response time of 1 s and slit width of 1 nm. HSL (1.9 µM) in TB was used with a path length of 1.0 mm. Each spectrum was an average of 20 accumulations; buffer scans recorded under the same conditions were subtracted from the protein spectra before analysis. Secondary structure was calculated by using CDpro software [32].

2.8 Amino acid and N-Terminal sequence analysis

50 µg of HSL was digested with 6 N HCl at 110 °C for 24 h in vacuum-sealed tubes. The hydrolysate was then used for amino acid analysis with AccQ-Fluor kit (Waters Corporation, USA). The digested sample was derivatized with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) following manufacturer's instruction. Five pmol of the sample was loaded onto AccQ-tag column and eluted with acetonitrile gradient (5-100 %). The eluate was monitored with a fluorescence detector. The amino acid peaks were compared with standards running under identical conditions. Total cysteine and tryptophan were estimated with denatured protein according to the method of Cavallini et al [33] and Spande and Witkop [34] respectively.

N-terminal sequence was determined by automated Edman degradation method in ProciseTM protein sequencer (Applied Biosystem, Canada) at the protein sequencing facility, NII, New Delhi, India.

2.9 MALDI-TOF MS and Nano ESI-ion trap MS/MS

Saturated solution of α -cyano-4-hydroxycinnamic acid in aqueous 50 % (v/v) acetonitrile /0.1 % (v/v) trifluoroacetic acid was used as a MALDI matrix. 2- μ L of in situ-gel tryptic digest of HSL was mixed with two μ L matrix and 1 μ L of this mixture was placed on the plate. Positive ion MALDI mass spectra were measured on a Voyager DE-STR (Applied Biosystem, Canada) system equipped with a 337 nm nitrogen laser. The sample was dissolved in a 1:1 (MeOH: Water) containing 0.1 % (v/v) TFA and was directly infused at a constant flow rate of 10 μ L min⁻¹ into the ion spray source using an integrated syringe pump. Positive ionization modes were measured on an ESI-QTOF MS/MS (Applied Biosystem, Canada), equipped with MDS Sciex Analyst software. The spray voltage was held at 5.7 kV, declustering potential (DP) 120 V, focusing potential (FP) 365 V, declustering potential (DP2) 14 V and collision gas (CAD) 3 (arbitrary units). Full scan spectra were recorded in positive mode over the mass range 350-2000 atomic mass units. MS/MS data were automatically acquired on the most intense precursor ion in each full-scan spectrum. Acquired MS/MS spectra were interpreted manually.

2.10 Fluorescence titration

Fluorescence measurements were carried out using a Perkin Elmer LS 50B spectrofluorimeter, with a slit width of 7 nm for both the monochromators and scan speed of 100 nm/min. Samples were placed in a quartz cuvette maintained at constant temperature (\pm 0.1 °C) by means of a Julabo circulating cryobath. The sugar solution was added in 10 aliquots (5 to 20 μ l each). Concentration of the sugar stock solution was in the range of 1-500 mM. Samples were excited at 280 nm and the emission spectra were recorded between 300 - 400 nm. Fluorescence intensity at 343 nm (λ_{max} of the lectin) was considered for all the data calculations. Corrections were made to compensate the dilution due to addition of sugar. At the highest concentration of the saccharide to lectin, volume change was less than 5 % of the solution in the cuvette.

The association constants were calculated according to the method described by

Chipman et al [35]. Temperature dependence of the association constants was used to determine the thermodynamic parameters, changes in enthalpy (ΔH), entropy (ΔS) were determined from the Van't Hoff plots.

3. Results

3.1 Purification of lectin

The bacterially induced coelomic fluid of sea cucumber showed lectin activity, which on successive purification by phenyl sepharose column chromatography resulted in a pure protein of mass 190 kDa. Purification resulted in 53 % recovery with 2830 fold purification (Table 1).

3.2 Bio-chemical properties of HSL

SDS-PAGE revealed a single homogeneous band with a molecular size of 182 kDa (Fig.1). A sharp symmetrical peak with volume corresponding to molecular mass of 190 kDa was obtained by gel filtration (Fig. 2), confirming the monomeric nature of HSL. It is basic in nature with a pI of 10.3. It agglutinated only pronase treated human erythrocytes but not trypsinized, desialylated and untreated erythrocytes.

3.3 pH and temperature stability and effect of divalent cations

The hemagglutinating activity was not affected by incubation in the pH range 2-11 for a period of 48 h. However prolonged incubation for a period of 52 h in the pH range of 2-7 resulted in 50 % loss in activity. HSL was completely stable at pH 8 and above. Lectin is highly thermostable and retains full activity on heating at 80 °C for 1 h; complete loss of activity occurred on incubation at 100 °C for 10 min (Fig. 3). Atomic absorption spectroscopy indicated the presence of 3 moles of Ca^{2+} , 1 mol of Mg^{2+} and 0.01 mol of Cu^{2+} per mol of protein. However dialysis against EDTA containing buffer or inclusion of divalent cations such as Ca^{2+} , Mg^{2+} and Mn^{2+} did not affect the hemagglutination activity.

3.4 Amino acid composition and carbohydrate content

Amino acid analysis revealed that HSL contains remarkably high level of Asx (14.97 %), Gly (13.43 %) and Lys (13.12 %), essential proportions of hydrophobic amino acids, Ala, Ile, Leu and Phe and hydroxyl containing amino acids Ser and Thr, while Tyr, Met and Trp were present in low amount. HSL contains 16 cysteine residues of which four cysteines were free and 12 were involved in six disulfide bonds (Table 2). Sugar content analysis revealed that HSL is a glycoprotein containing 8 % neutral sugars.

3.5 Circular dichroism of HSL

CD spectrum of HSL was characterized by a maximum negative absorption at 208 nm in the far UV region. CDSSTR algorithm yielded the best-fit values (NRMSD=0.10) indicating that the secondary structure is comprised of 49 % of β -sheet, 36 % of α -helix and 17 % unordered coil (Fig.4).

3.6 Mass spectrometry and data processing of HSL

Peptide mapping of tryptic digest of HSL by MALDI-TOF method resulted in presence of resolved peaks in the spectrum. Peptides masses were used to identify the protein using mascot search analysis [36], no homology was found with any known proteins. Subsequently, the positive spectrum of peptide mixture was analysed by LC-MS/MS on an ion trap mass spectrometer. The sequences were read out from y-ion and b-ion series according to known fragmentation mechanism [37]. Peptide sequences so obtained were analysed for homology with known echinoderm lectins using ClustalW software. Homologous motifs were found in SCPTDWR (CEL-I, 1WMZD), ATHSCEGLRS and TNSDQWTWDGSS (CEL-III, BAC75827), NCRFGLSK (CEL-IV, AAB35250) and lectins from the sea cucumber, *Cucumaria echinata* [38,39]. Some of these peptides were used in designing degenerative primers to isolate and amplify the gene coding for the lectin.

3.7 Hemagglutination inhibition assay

The hemagglutination activity was inhibited by Me α Gal (62.5 mM), LacNAc (50 mM) and T-antigen (5 mM) but not by alpha linked galacto-oligosaccharides such as melibiose, raffinose, stachyose and other simple saccharides like glucose, galactose, mannose, fructose, fucose and lactose. Higher level of inhibition was observed with glycoproteins like asialo fetuin (0.19 μ g), asialo fibrinogen (0.2 μ g), mucin (5.0 μ g), avidin (6.25 μ g), fetuin (15.63 μ g), holotransferrin (15.63 μ g), ovalbumin (23.43 μ g), thyroglobulin (62.52 μ g) and fibrinogen (125 μ g) but not with invertase (Table 3).

3.8 Fluorescence titration

Addition of sugars and glycans resulted in quenching of the lectin fluorescence without any shift in the emission maximum. The maximum quenching of the intrinsic fluorescence on sugar binding was 15 %. Among monosaccharides mannose, glucose and their derivatives showed very weak binding ranging from 200-500 M⁻¹. Reasonably good binding was observed with Gal (K_a 390 M⁻¹), 2dgal (K_a 200 M⁻¹), GalN (K_a 523 M⁻¹), GalNAc (K_a 954 M⁻¹) and Me α Gal (K_a 3630 M⁻¹) at 30 °C. Me α Gal showed 10 fold higher affinity as compared to its β anomer. No binding was observed with fucose (6-deoxygalactose) and L-arabinose (Table 4, 5).

Alpha linked galactose disaccharides Gal α 1-3 Gal (K_a 1050 M⁻¹), Gal α 1-4 Gal (K_a 1300 M⁻¹) and Gal α 1-6 Glc (K_a 380 M⁻¹) (Table 4) had poor affinity. Whereas beta-linked galactose disaccharides Gal β 1-3 GlcNAc (K_a 5010 M⁻¹), Gal β 1-4 GlcNAc (K_a 5620 M⁻¹) and Gal β 1-3 GalNAc (K_a 53,700 M⁻¹) (Fig. 5) had higher affinity for the lectin, however Lactose (Gal β 1-4 Glc) (K_a 440) was a poor binding ligand (Table 5). Me α -T-antigen (Gal β 1-3 GalNAc α -1-*O*-Methyl) was the most potent ligand for the HSL, which binds 1569-, 4- and 472- times stronger than T-antigen, T(α -ser)-antigen and Me β -T-antigen (Gal β 1-3 GalNAc β -1-*O*-Methyl) respectively (Table 5).

4. Discussion

We noticed a significant rise in lectin activity in the coelomic fluid of sea cucumber (*H. scabra*), when challenged with various bacterial strains. By fractional purification, the lectin activity was assigned to a monomeric glycoprotein of mass 182 kDa. The hemagglutinating property of HSL is unique as it does not require any divalent metal ions. Marine lectins are generally identified by their metal ion requirement for their hemagglutination activity. Majority of the marine lectins are from C-type lectin family, Ca^{2+} dependent [9,13,15,18,22,24,25,40,41], however Mg^{2+} stimulated the activity of *Modiolus modiolus* [42] and Aplysina [10] lectins. *Ptilota filicina* lectin requires all these divalent cations, Ca^{2+} , Mg^{2+} and Mn^{2+} for its activity [43]. A few of the lectins do not require any metal ions for their activity [14,44].

Another interesting property of HSL was with respect to its thermostability. The lectin was stable upto one hour at 80 °C. Origin of thermostability can be found in the lectin's amino acid composition. It contains high amount of Lys (13.12 %), Glx (8.32 %) and Arg (6.53 %). Vieille et al. [45] found that thermostable proteins have an increased content of charged amino acid residues (i.e., K, E and R). Though four free sulfhydryl groups could be detected by reaction with DTNB, no difference in migration in SDS-PAGE was observed in the presence of 2-mercaptoethanol, thereby indicating absence of intermolecular disulfide bridges. HSL contains high amount of acidic (28.4 %), basic (22.23 %) and hydrophilic (12.75 %) and reasonable amount of aromatic amino acids (accounting for the high extinction coefficient of $^{0.1\%}A_{280}=1.20$), large amounts of lysine (13.12 %), (contributing to the basicity of the HSL; pI=10.3). It remains active at alkaline pH up to 11.0 and the higher stability could be due to the presence of six disulfide bonds, metal ions and higher proportion of β -content in secondary structure. Tryptic digest peptide profile of MALDI-TOF was analysed by mascot search, but it did not yield any reasonable homology hit with any known protein. Later analysed by MS/MS, homology search for sequenced peptides revealed that HSL is homologous with lectins isolated from other genera of sea cucumber [38,39].

HSL could hemagglutinate only pronase treated erythrocytes indicating specificity for desialylated glycolipids. Pronase-E, a cocktail of different proteolytic enzymes, removes all protruding polypeptides from the erythrocytes membrane, there by exposing GPI-anchors, cryptantigen and glycolipids. This might serve as better ligand for the HSL than asialo-glycoconjugates on erythrocyte membrane.

Detailed carbohydrate binding specificities of the HSL was studied by a combination of hemagglutination inhibition and fluorescence spectroscopy. Among monosaccharides the primary specificity of lectin is directed towards galactose and the affinities for galactose derivatives are in good agreement with those obtained by hemagglutination inhibition studies using human erythrocytes. Negligible binding was observed with glucose, mannose, and their methyl and amine derivatives, indicating that these sugars did not bind to the lectin. Affinity among monosaccharides was in the order $\text{Me}\alpha\text{Gal} > \text{GalNAc} > \text{GalN} \cong \text{Me}\beta\text{Gal} > \text{dGal} \cong \text{Gal}$. The better affinity is governed by the favourable enthalpic change.

The association constants in all the sugars decreased with increase in temperature. Van't Hoff plots for all sugars were linear ($r > 0.95$), in the range of temperature tested (Fig.6). A decrease in association constants with increase in temperature was observed indicating free energy of the binding (ΔG) and enthalpy change (ΔH) to be negative. This suggested that binding is exothermic in nature and driven by enthalpy (Table 5).

Among monosaccharides affinities, GalN is twice better than Gal and dGal. Removal of OH group in C-2 (dGal) had no influence in binding, which indicated that the C-2 hydroxyl group is not critical for binding; whereas substitution at the same position has considerable influence on its binding. Addition of hydrophilic amino group at C-2 position of Gal resulted in a two-fold increase in the affinity. The change in enthalpy ($-\Delta H$) for the binding of Gal and GalN were almost equal (-33.14 and $-30.79 \text{ kJ mol}^{-1}$), indicating that the contribution of enthalpy to the differences in the free energy of binding of these free sugars were not much significant. The affinities therefore were a manifestation of a favourable entropic factor.

GalNAc is 2.5-, 6- and 2- times stronger binding a ligand when compared with Gal, dGal and GalN respectively. The better affinity of GalNAc over Gal is presumably due to a favourable enthalpy contribution of the acetamido group for the binding. This amounts to $-7.08 \text{ kJ mol}^{-1}$ and probably reflects additional van der Waals' interactions or hydrogen bonding between the acetamido group of the sugar and the protein or potentiation of the effectiveness of the existing ones. GalNAc exhibited strong hemagglutination inhibition against lectins from *Cucumeria echinata* (CEL-I, III and IV) [22] and from *Crenomytilus grayanus* [46].

Me α Gal is the strongest ligand among the monosaccharide with an 9-fold higher association constant for the HSL than that of galactose and 7- fold higher than that of Me β Gal. A positive increase in entropy (ΔS) of Me α Gal over Gal was observed ($33.87 \text{ Jmol}^{-1}\text{K}^{-1}$), indicating a strong and favourable association between the Me α Gal and the lectin due to the axial orientation of the hydrophobic methyl group at C-1 position. The same group in the β -position had a destabilizing effect on binding to lectin due to its equatorial position, which could impose steric hindrance to lectin-carbohydrate interaction, as indicated by negative increase in entropy ($78.50 \text{ Jmol}^{-1}\text{K}^{-1}$). However, the presence of a methyl group at C-1 position in the α -anomer alone is not sufficient to cause effective binding since Me α Glc and Me α Man fails to bind to the lectin. The preference for the α -anomer over the β -anomer of galactose was also observed for other lectins including a lectin from *Didemnum ternatanum* [47], whereas lectin from marine worm, *Chaetopterus variopedatus* prefers to bind to Me β Gal than Me α Gal [48].

Melibiose binds to HSL poorly, when compared to Me α Gal indicating that the replacement of 1-*O*-methyl group in Me α Gal with glucopyranosyl residue could lead to projections of hydrophilic hydroxyl groups in the combining site of the lectin. No monosaccharide with a modified substitute at C-3 of gal was available. At C-4, inversion of the hydroxyl group as in glucose is not allowed. The C-6 hydroxyl group acts as important binding locus as shown by the observation that fucose (6-deoxygalactose) and L-arabinose did not bind to the HSL.

Between mono- and disaccharides, the later were found to have better affinity for HSL. The affinity for T-antigen is 16-, 55-, 107- and 143- fold higher than that observed for Me α Gal, GalNAc, Me β Gal and Gal, respectively. The increase in the affinity for disaccharides over monosaccharides is accompanied by a positive increase in entropy.

Among disaccharides, T-antigen has a significantly higher K_a (10-, 11- and 170-fold higher) than that for Gal β 1-4 GlcNAc, Gal β 1-3 GlcNAc and lactose respectively, which could be explained by their minimum energy conformations. Although these disaccharides have same nonreducing sugar (β -Gal), but have significant differences in the orientation of the reducing sugar [49]. The major stabilizing force for the binding of Gal β 1-3 GalNAc to the lectin is apparently provided by the C-4 hydroxyl group as well as the C-2 acetamido group of the reducing sugar moiety of this saccharide, which are attributed to positive change in enthalpy (5.0 kJ mol⁻¹) and entropy (3.0 Jmol⁻¹ K⁻¹).

Among beta linked galactose disaccharides, T (α -ser)-antigen showed 379- times stronger binding to the HSL than T-antigen, suggesting the role of alpha linked serine in binding. Me α -T-antigen is the best ligand as it binds HSL 1209-, 4- and 472- times better than T-antigen, T(α -ser)-antigen and Me β -T-antigen respectively. The higher affinity of Me α -T-antigen could be due to cumulative effect of presence of hydrophobic methyl group at C-1 alpha position and C-2 acetamido group as well as *O*-linked serine groups.

In conclusion, the present investigation shows that sea cucumber produces a high molecular weight monomeric glycoprotein with T-antigen specificity. It agglutinates pronase treated human erythrocytes and the activity is independent of any metal ions. Lectin hemagglutination activity is inhibited by Me α Gal, T-antigen and desialylated glycoproteins. The monosaccharide binding is enthalpically driven while disaccharide binding involves significant entropic contribution along with enthalpy.

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Figure legends:

Fig. 1. Molecular weight estimation of HSL by SDS-PAGE. HSL was electrophoresed using 7 % polyacrylamide gel containing 0.1% SDS and silver staining was done. Lane 1; molecular weight markers: myosin (220 kDa), α -2-macroglobulin (170 kDa), β -galactosidase (116 kDa), transferrin (76 kDa) and glutamate dehydrogenase (53 kDa), lane 2; purified HSL.

Fig.2. Molecular weight determination by gel filtration chromatography. HSL was passed through a sephacryl S-300 column (1.5 cm x 180 cm), was calibrated with (a) appoferritin (443,000), (b) β - amylase (200,000), (c) alcohol dehydrogenase (150,000), (d) bovine serum albumin (66,000) and (e) ovalbumin (45,000). V_0 is the void volume, V_e the elution volume and (P) indicates the purified lectin.

Fig. 3. Effect of temperature on the hemagglutinating activity of the HSL. 70 °C (-7-), 80 °C (-B-), 90 °C (-A-) and 100 °C (- Ω -).

Fig. 4. Circular dichroism spectra of purified HSL. The spectrum was recorded on a Jasco J-715 spectropolarimeter.

Fig. 5. Representative association constant curve for the binding of T-antigen to HSL by intrinsic fluorescence AT 25 °C. Inset: Quenching of the intrinsic fluorescence of HSL on titration with T-antigen.

Fig. 6. Van't Hoff plots for the association of sugars to HSL are drawn according to the regression equation. Symbols used are; galactose (!), methyl α -galactose (μ), methyl β -galactose (7), LacNAc (B), Gal β 1-3 GlcNAc (Ω) and T-antigen (Λ). The association constants were determined at 20 °C, 25 °C and 30 °C.

Table legends:

Table 1. Purification of lectin from *Holothuria scabra*.

Table 2. Amino acid composition of HSL.

Table 3. Hemagglutination inhibition activity of HSL by different sugars and glycoproteins.

Table 4. Association constants of HSL with different sugars determined by fluorescence spectroscopy.

Table 5. Association constants and thermodynamic parameters for the binding of HSL with different sugars by fluorescence spectroscopy.

Figure 1

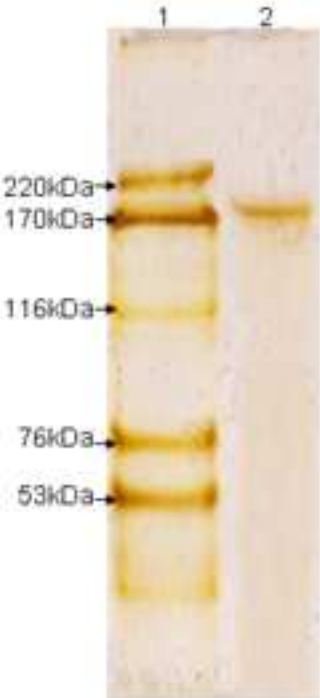


Figure 2

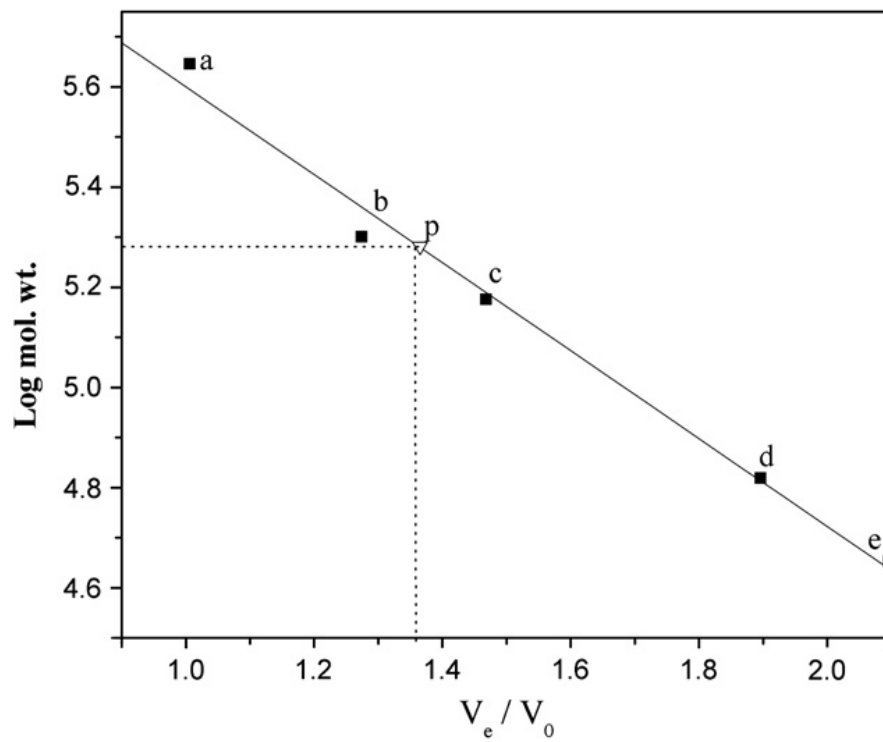


Figure 3

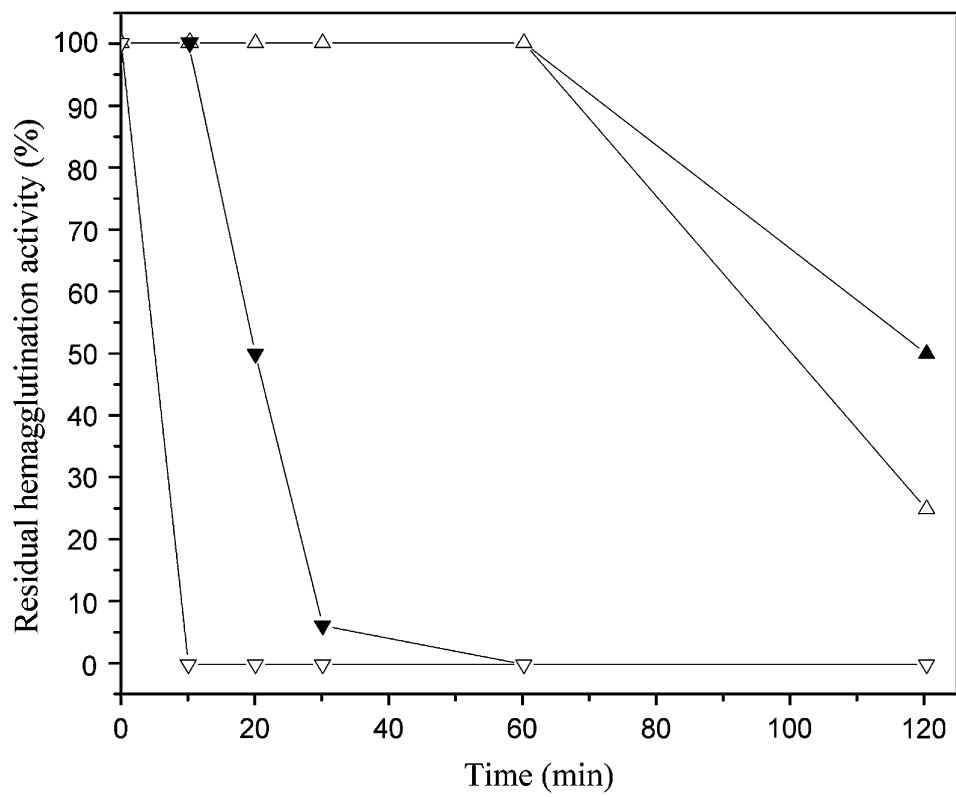


Figure 4

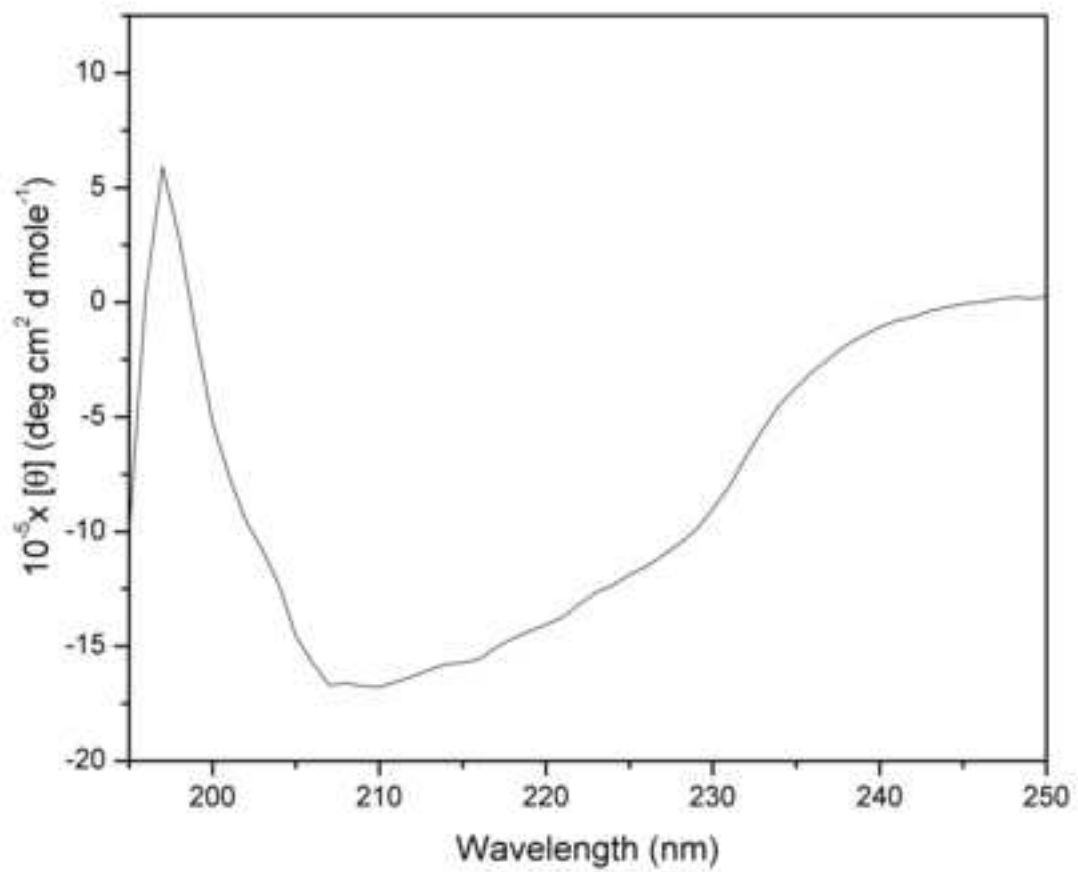


Figure 5

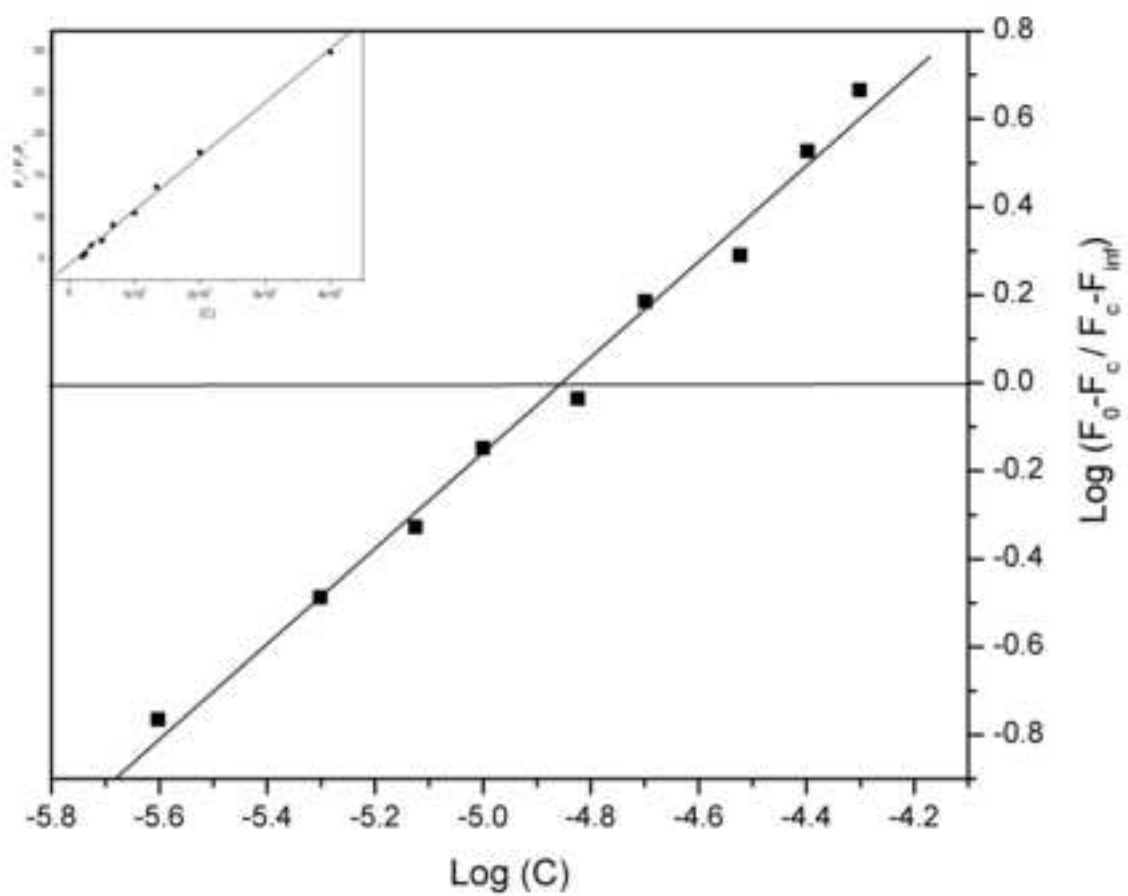


Figure 6

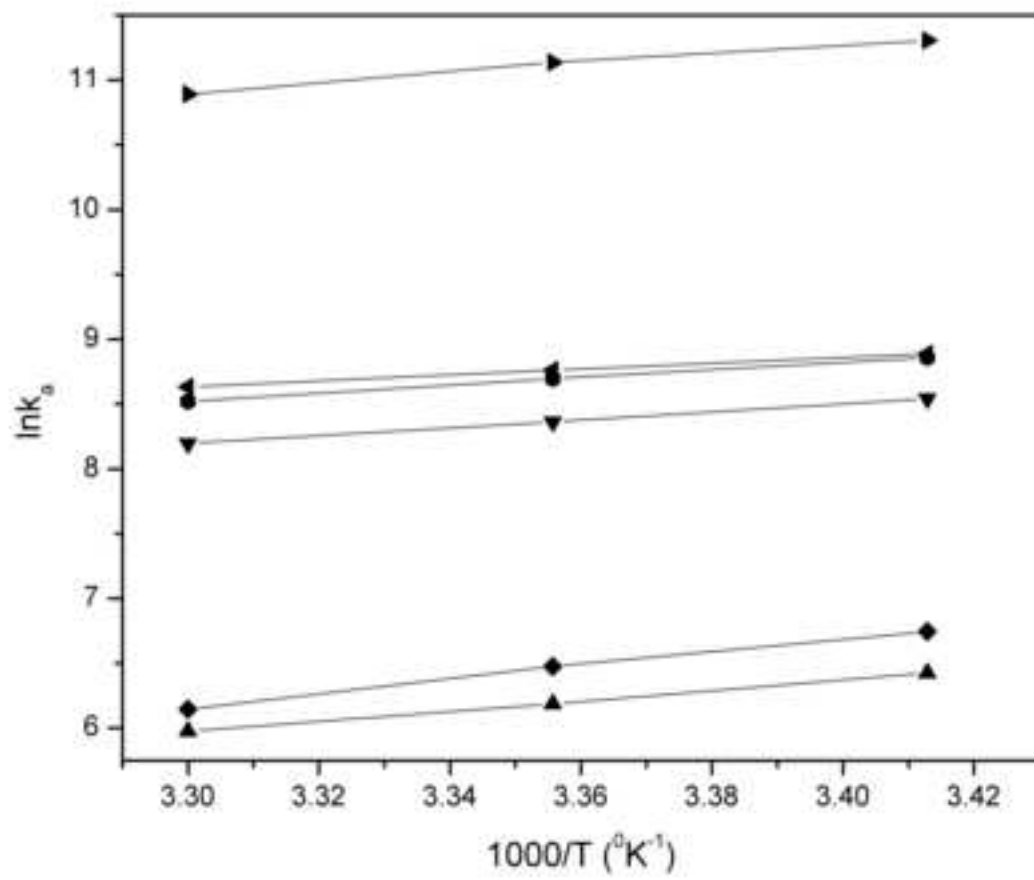


Table 1

Purification step ^a	Total Protein (mg)	Total activity (HA units x 10 ⁵)	Specific activity (HA unit.mg ⁻¹)	Fold purification	Recovery (%)
Crude coelom	152.82	1.7	1130	1	100.00
Ultra filtration (>10 kD)	44.32	1.5	3595	318.01	92.00
Phenyl Sepharose-I	5.55	0.9	17297	1529.75	55.55
Phenyl Sepharose-II	2.88	0.9	32000	2830.05	53.33

^a starting from 180 mL of coelomic fluid.

Table 2

Inhibitor	Minimum Inhibitory concentration
Methyl- α Galactose	62.5 mM
LacNAc	50.0 mM
T-antigen	5.0 mM
Avidin	6.25 μ g
Fibrinogen	40.0 μ g
Asialo fibrinogen	0.2 μ g
Fetuin	15.63 μ g
Asialo Fetuin	0.19 μ g
Holotransferrin	15.63 μ g
Mucin	5.0 μ g
Thyroglobulin	62.52 μ g
Ovalbumin	23.43 μ g

Table 3

Sugars	K_a ($10^3 M^{-1}$)	ΔG ($k J \cdot mol^{-1}$)
2-d-Galactose	0.20	-13.12
D+ Fucose	NB	-
L- Arabinose	NB	-
Lactose	0.44	-15.31
Raffinose	0.48	-15.54
Stachyose	0.52	-15.74
Gal α 1-3 Gal	0.11	-17.23
Gal α 1-4 Gal	0.13	-17.76
Gal α 1-6 Glc	0.38	-16.45
Gal β 1-3 Gal β 1-4 GlcNAc	1.90	-24.40

NB; No binding.

Table 4

Sugar Name	$K_a (10^3 M^{-1})$			ΔH (kJ mol ⁻¹)	ΔG^* (kJ mol ⁻¹)	ΔS (J mol ⁻¹ K ⁻¹)
	20 °C	25 °C	30 °C			
D-Gal	0.61	0.48	0.39	-15.32	-33.14	-59.78
Galactosamine	0.79	0.64	0.52	-16.00	-30.79	-49.56
N-Acetyl Galactosamine	1.64	1.25	0.95	-17.66	-40.22	-75.68
Me- α -Galactose	5.12	4.29	3.63	-20.72	-25.46	-15.91
Me- β -Galactose	0.85	0.64	0.46	-16.04	-44.17	-94.41
Lactose	0.72	0.57	0.44	-15.73	-35.70	-67.03
Gal β 1-4 GlcNAc	7.24	6.38	5.62	-21.70	-18.66	-10.20
Gal β 1-3 GlcNAc	7.03	5.98	5.01	-21.54	-25.08	-11.86
Gal β 1-3 GalNAc	81.28	68.79	53.70	-27.59	-30.51	-9.82
Gal β 1-3 GalNAc α -1- <i>O</i> -Ser	-	20100	-	-	-41.67	-
Gal β 1-3 GalNAc α -1- <i>O</i> -Me	-	83200	-	-	-45.19	-
Gal β 1-3 GalNAc β -1- <i>O</i> -Me	-	176	-	-	-29.93	-

* Values were calculated at 25 °C.