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ORIGINAL PAPER

Kinetics and Thermodynamics of Glycans and Glycoproteins Binding to *Holothuria scabra* Lectin: A Fluorescence and Surface Plasmon Resonance Spectroscopic Study

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Abstract *Holothuria scabra* produces a monomeric lectin (HSL) of 182 kDa. HSL showed strong antibacterial activity and induced bacterial agglutination under *in vitro* conditions, indicating its role in animals' innate immune responses. Very few lectins have been reported from echinoderms and none of these lectins have been explored in detail for their sugar-binding kinetics. Affinity, kinetics and thermodynamic analysis of glycans and glycoproteins binding to HSL were studied by fluorescence and surface plasmon resonance spectroscopy. Lectin binds with higher affinity to *O*-linked than *N*-linked asialo glycans, and the affinities were relatively higher than that for sialated glycans and glycoproteins. T-antigen α-methyl glycoside was the most potent ligand having the highest affinity (*K*a 8.32 $\times 10^7$ M^{-1}). Thermodynamic and kinetic analysis indicated that the binding of galactosyl Tn-antigen and asialo glycans is accompanied by an enthalpic contribution in addition to higher association rate coupled by low activation energy for the association process. Presence of sialic acid or protein matrix inhibits binding. Higher affinity of HSL for *O*-glycans than *N*-glycans had biological implications; since HSL specifically recognizes bacteria, which have mucin or *O*-glycan cognate on their cell surfaces and play a major role in animal innate immunity. Since, HSL had higher affinity to T-antigen, makes it a useful tool for cancer diagnostic purpose.

Keywords *Holothuria scabra* · Lectin · Thermodynamic properties · Kinetic analysis · Surface plasmon resonance spectroscopy

Abbreviations

HSL	*Holothuria scabra* lectin
Galactosyl Tn-antigen (Gal β1-3 GalNAc α-1-*O*-L-Ser)	Thomsen-Friedenreich antigen
T-antigen α-methyl glycoside	Gal β1-3 GalNAc α-1-*O*-Me
T-antigen β–methyl glycoside	Gal β1-3 GalNAc β-1-*O*-Me
SPR	Surface plasmon resonance spectroscopy

Introduction

Marine invertebrates rely solely on innate immunity, which includes both humoral and cellular responses, as they lack an adaptive immune system. Various methods employed to counteract infectious agents include, hemolymph coagulation, melanization, cell agglutination, encapsulation, nodule formation and phagocytosis [1, 2]. The microbial load in natural marine habitat can number up to 10^6 bacteria and 10^9 virus·mL^{-1} of seawater [3]. It is therefore imperative that animals develop a robust innate immune system for survival.

Lectins are proteins with specificity for simple sugar, a sequence of sugars or their glycosidic linkages. Such ligands are known to occur on the surface and the capsule of bacteria. In invertebrates, lectins have been suggested to participate in innate immune response (humoral defense reaction) by inducing bacterial agglutination or by acting as opsonins to enhance phagocytosis by coelomycetes [2, 4]. Cell lysates and cell-free plasma of several invertebrates also expressed antibacterial activity, although the activity of the latter may possible be due to small antimicrobial proteins [5]. These

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results strongly support the contention that invertebrates possess "immune-like" defense mechanisms.

Many biological recognition and adhesion processes involve the formation of saccharide-protein complexes. To understand the selectivity and origin of the association energy, it is important to know the nature of forces controlling the saccharide-protein interaction. One of the best-characterized carbohydrate-mediated recognition of the cell surface receptor is the association between lectins, and the carbohydrate moiety of the glycolipids and glycoproteins present on the cell membrane [6–8]. Because of the integration of carbohydrate moieties in cell membranes, lectins can affect intercellular recognition, cell growth, and differentiation. They are, therefore, widely used in cell biology, biochemistry, and histochemistry to isolate and/or to characterize cell surface carbohydrates and glycoproteins [6–8]. Lectins require conformational and structural complementarity of sugars for interaction to occur and have been employed as tools for exploring the structure and dynamics of cell surfaces [7, 9].

Holothuria scabra, a sea cucumber belongs to phylum echinodermata, that found mostly in the littoral zone of tropical waters, produces a 182 kDa monomeric lectin (HSL: *Holothuria scabra l*ectin) in the coelomic fluid [10]. Exposing the animal to human bacteria enhances HSL protein levels in the coelomic fluid and its hemagglutination activity by 8–10 folds [11]. HSL also showed strong antibacterial activity and induced bacterial agglutination under *in vitro* conditions, indicating its involvement in animals' innate immune responses. The lectin is specific for Thomsen-Friedenreich (T)-antigen, a tumor associated antigen of oncofetal origin and it's probably one of the few chemically well-defined antigens with a proven link to malignancy, therefore anti-T probes have enormous potentials in cancer research [12, 13]. A number of lectins have been isolated from marine sources, however very few lectins are reported in phylum echinoderms. Among echinoderms, few lectins have been reported from sea urchin [14–18] and sea cucumbers [19–25]. However, as of our knowledge none of these marine lectins have been explored in detail for their sugar-binding kinetics and role in innate immune responses.

The sugar /ligand binding studies can be carried out by applying methods of hemagglutination inhibition, fluorescence spectroscopy and surface plasmon resonance, each one having its own advantages. Hemagglutination inhibition assay provides only semi-quantitative information about sugar specificity. Using fluorescence spectroscopy, we can study carbohydrate-protein interactions at equilibrium without physical separation of the bound ligand from free ligand and the protein [26]. Since, fluorescence of tryptophan which is, influenced by its local environment; changes in the tryptophan microenvironment upon ligand binding can change fluorescence properties of the protein [27]. However, surface plasmon resonance spectroscopy (SPR) measures the binding event between the immobilized molecule on sensor chip and the molecule carried in a flow buffer solution through a miniature flow cell in real time, leads to change in refractive index recorded as sensograms. Sensograms not only provide binding information but also contain kinetics and the strength of the interaction [28]. Determination of association-constants with a series of ligands provides considerable insight into the spatial features of a lectin's binding site. Complementary thermodynamic data offers information on the forces involved in the binding and explains affinity differences encountered.

In order to use HSL as a sensitive probe, it is not only necessary to elucidate its carbohydrate specificity in detail but also to delineate the forces involved in its interaction with ligands. With these objectives in mind, we report here the thermodynamics, kinetics and activation parameters for the binding of HSL with several glycans and glycoproteins using a combination of fluorescence and surface plasmon resonance spectroscopy.

Materials and Methods

Materials

Coelomic fluid extracted fro*m Holothuria scabra,* a sea cucumber distributed mostly in the littoral zone of tropical waters, comprise material of the present study. The chemicals and reagents like pronase-E, carboxypeptidase, aminopeptidase, fetuin (bovine), fibrinogen (human) were obtained from Sigma (St. Louis, U.S.A); certified grade CM5 sensor chip and amino coupling kit (*N*-ethyl-*N'*-(dimethylaminopropyl-) carbodiimide hydrochloride and *N*-hydroxysuccinimide) from Amersham Biosciences (Sweden); and Gal β 1–3 GalNAc α 1-*O*-L-Serine from Dextra Labs (London, UK). All other chemicals used were of analytical grade. Triantennary *N*-glycan from fetuin and biantennary *N*-glycan from fibrinogen were purified and the homogeneity of the preparation was checked by HPLC as indicated in Fig. 1. [29, 30].

Methods

Purification of Holothuria scabra Lectin

Lectin was purified from the coelomic fluid of sea cucumber, *Holothuria scabra*, following methods described by Gowda et al.[10]. Total neutral sugar content of the lectin was estimated by phenol-sulphuric acid method of Dubois

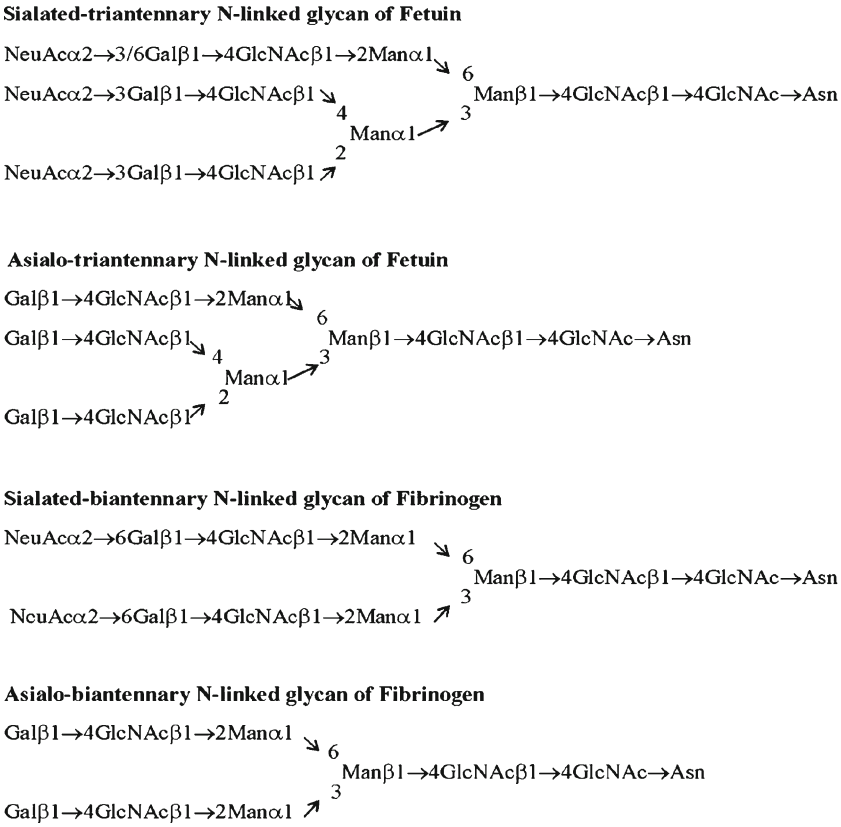

Fig. 1 Structures of different sialated- and asialo-glycans. Sialated and asialo glycans were prepared from fetuin and fibrinogen as described in methods section

et al. [31], using galactose-mannose (4:3) as standard. Protein concentration was determined according to the method of Bradford [32] using bovine serum albumin as standard.

Fluorescence Titration

Fluorescence measurements were carried out using a LS50B spectrofluorimeter (Perkin Elmer, MA, USA) with a slit width of 7 nm for both the monochromators and a scan speed of 100 nm/min. Samples were placed in a quartz cuvette maintained at constant temperature (± 0.1 °C) by means of a Julabo circulating cryobath. The sugar solution was added in 10 aliquots (5 to 20 μl each). Concentration of the sugar /glycan 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 dilutions due to addition of sugar solutions. Important point to be noted that, the ratio of ligand:protein used in this study is more than 10. This higher ligand ratio and the dilution correction factors can take care of inner-filter affects [33, 34]. At the highest concentration of the saccharide to lectin, the 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] and van de Weert [33, 34]. The abscissa intercept of the plot of log $[C]_f$ against log $\{(\Delta F)/(F_c - F_\infty)\}$, yielded p$K_a$ value for lectin-ligand interactions according to the relationship,

$$\log[F_0 - F_c / F_c - F_\infty] = \log K_a + \log\{[C]_t - [P]_t(\Delta F/\Delta F_\infty)\} \quad (1)$$

where F_c is the fluorescence intensity of the lectin at any point during the titration, $[P]_t$ is the total protein concentration, ΔF_∞ is the change in fluorescence intensity at saturation binding, $[C]_t$ is the total ligand concentration, and $[C]_f$ is the free and added ligand concentrations given by,

$$[C]_f = \{[C]_t - [P]_t(\Delta F/\Delta F_\infty)\} \quad (2)$$

Free energy changes of association (ΔG) were determined by the equation,

$$\Delta G = -\mathrm{RT}\ln K_a \quad (3)$$

Temperature dependence of the association constants was used to determine the thermodynamic parameters. Changes in enthalpy (ΔH) were determined from the Van't Hoff plots by using the equation,

$$\ln K_a = (-\Delta H/\mathrm{RT}) + \Delta S/R \quad (4)$$

where ΔH is enthalpy change, R is gas constant, ΔS is entropy change and T is the absolute temperature. The entropy change was obtained from the equation,

$$\Delta G = \Delta H - T\Delta S \quad (5)$$

BIAcore Biosensor Assays

Biospecific interaction studies were performed on a BIAcore 2000 (Pharmacia Biosensor AB, Uppsala, Sweden) biosensor system based on the principle of surface plasmon resonance. Fifty μg of HSL in 1 ml of 10 mM sodium acetate buffer, pH 4.0 were coupled (corresponding to 877 response units), to a certified grade CM5 chip at a flow rate of 5 μl/min for 50 min using amine coupling kit. Unreacted groups were blocked with ethanolamine. All measurements were done using TBS, pH 8.0 (containing 0.02 % (w/v) sodium azide and 0.05 % (v/v) tween-20). Prior to injection, sugar and protein samples were dissolved and diluted in the above buffer to avoid buffer mismatch. The association rate constants, at different temperatures, were determined by passing the glycan solutions (0.1-500 μM) over the chip at a flow rate of 5 μl/min for 300 s. The dissociation rate constants, on the other hand, were determined in a similar manner by passing plain buffer at a flow rate of 5 μl/min for 300 s. After every cycle, chip was regenerated by treating with 200 mM sodium carbonate, pH, 9.5, for 3 min. The glycoproteins were passed at a high flow rate 50 μl/min for 120 s to reduce the mass transport effect and dissociation was followed by passing buffer at a flow rate of 50 μl/min for 300 s.

Association (k_1) and dissociation (k_{-1}) rate constants were determined by nonlinear fitting of the primary sensogram data using the BIA evaluation software version 3.1. The dissociation rate constants were derived using the equation,

$$R_t = R_{t0}e^{-k-1}(t-t_0) \quad (6)$$

where R_t is the response at time t and R_{t0} is the amplitude of the initial response. The association rate constant k_1 was derived by Eq. 2 using the measured k_{-1} values,

$$R_t = R_{\max}\left[1-e^{-(k1C+k-1)(t-t0)}\right] \quad (7)$$

where $R_{\max}$ is the maximum response and C is the concentration of the analyte (ligand) in the solution. K_a (k_1/k_{-1}) is the association constant.

Free energy changes of association (ΔG), changes in enthalpy (ΔH) and entropy (ΔS) were determined by the Eqs. 4 and 5. Activation enthalpies ($\Delta H^{\ddagger}$), entropies ($\Delta S^{\ddagger}$) and energies (ΔG) were calculated using the following equations:

$$\Delta H^{\ddagger} = E_A - RT \quad (8)$$

$$\ln\left(k^{\ddagger}/T\right) = -\Delta H^{\ddagger}/RT + \Delta S^{\ddagger}/R + \ln(k'/h) \quad (9)$$

$$\Delta G^{\ddagger} = \Delta H^{\ddagger} - T\Delta S^{\ddagger} \quad (10)$$

where $k^{\ddagger}$ is the appropriate rate constants, k' is Boltzman's constant and h is Planck's constant.

Circular Dichroism (CD) Measurements

CD spectra were recorded at 25 °C on a J-715 spectropolarimeter (Jasco Analytical Instruments, MD, USA) at a scan speed of 20 nm/s with a response time of 4 s and a slit width of 1 nm. A cylindrical quartz cell of 1.0 mm path length was used for the measurement in the 200–250 nm range with a protein concentration of 1.9 μM, while the cell path length was 10 mm for measurements in the range of 260–300 nm range with a protein concentration of 11.98 μM. HSL was incubated with 200 μM of T-antigen and spectrum was taken in both the ranges. Each spectrum was an average of 20 accumulations; buffer (PBS, pH 8.0) scans recorded under the same conditions were subtracted from the protein spectra before analysis.

Results

The affinity and thermodynamics of binding of various glycans and glycoproteins to HSL was studied using fluorescence spectroscopy and affinity and kinetics of binding was studied by surface plasmon resonance. Upon excitation at 280 nm, HSL emits fluorescent radiation mostly centering at 343 nm. Incremental addition of carbohydrate ligand resulted in progressive quenching of intrinsic fluorescence without any shift in the emission peak, due to change in the tryptophan microenvironment of the protein after ligand binding [33, 34]. The proportionality between quenching and binding strength was used to measure the affinity of HSL for various ligands. The binding affinity of HSL for mono and disaccharides was reported earlier [10]. In this article we present the binding profile of HSL with various glycans and glycoproteins and their interaction. The maximum quenching of the intrinsic fluorescence on ligands binding was 15 %. Affinity for glycans and glycoproteins was also determined by surface plasmon resonance, wherein addition of glycans (Fig. 2) and glycoproteins resulted in significant increase in response units.

The coupling of HSL was carried out using amino coupling kit since, direct coupling of the lectin to CM5 chips led to inactivation. This was not surprising since modification of lysine ε-NH_2 leads to loss of activity (data not shown). The association constants (K_a) determined by SPR and fluorescence spectroscopy were similar; for instance, K_a of galactosyl Tn-antigen (one of the high affinity ligand) was 22.0 $\times 10^6$ M^{-1} and 20.1 $\times$ 10^6 M^{-1} by SPR and fluorescence methods respectively. The association constants determined for the various ligands are listed

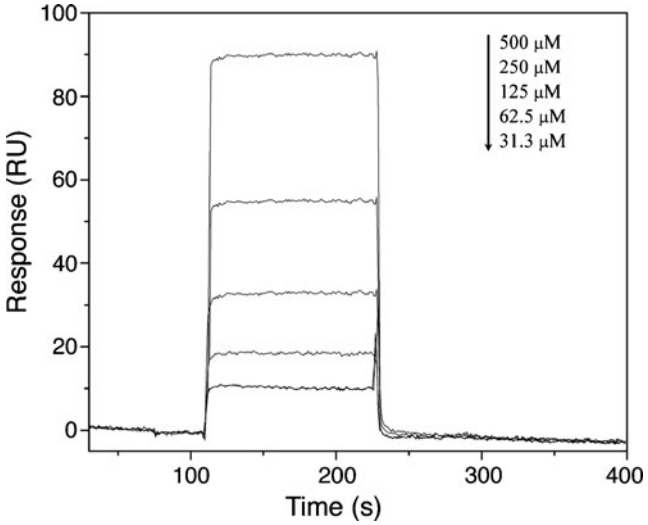

Fig. 2 The representative surface plasmon resonance sensograms. The relative responses depicted as sensograms obtained using surface plasmon resonance spectroscopy. The binding of increasing amounts of galactosyl Tn-antigen ranging from 31.3 μM (bottom trace) to 500 μM (top trace) was injected for 300 s at a flow rate of 5 μl/min. The dissociation reaction was recorded by following TBS buffer at 5 μl/min. The reaction was measured at 25 °C. Fifty μg of HSL was immobilized onto chip, corresponding to 877 responsive units

in Table 1. Thermodynamics of the binding process was determined by analyzing the thermal dependence of association constant for equilibrium binding of ligand by van't Hoff's method.

The binding of *N*-linked asialo-triantennary and -biantennary glycans was 3- and 30- fold lower than that of galactosyl Tn-antigen (*O*-linked glycan). The diminution in affinity of *N*-linked glycans over *O*-linked glycan, may be due to changes in sugar moieties. *O*-linked glycan comprised of Gal β 1–3 GalNAc, whereas *N*- linked glycans have Gal β 1–4 GlcNAc moieties. We had previously reported that HSL binds preferentially and with higher affinity to Gal β 1–3 GalNAc than Gal β 1–4 GlcNAc (10). The variation in the terminal amino acid substitution may also contribute to the observed differences in affinity. Thermodynamically, the equilibrium binding of *O*- and *N*-linked glycans differ mostly in enthalpy (3–10 $kJmol^{-1}$). Thus the binding differences appear to arise from the reduction in the number of favorable interactions in *N*-linked glycans. Furthermore, the valency of sugar moieties (Gal β 1–4 GlcNAc) in the *N*-linked asialo glycans appear to be critical for binding as the affinity for asialo triantennary glycan (trivalent) is 9-fold higher than that for asialo biantennary glycan (bivalent). The improvement in binding is mostly enthalpic in nature (5 kJ mol^{-1}), thus highlighting the involvement of sugar moiety in forming stabilizing interactions with the binding site. These results reflect the extended nature of the sugar-binding site [36, 37]. Furthermore, sialated glycans bind with 10–15 times lower affinity than their asialo counter parts accompanied by unfavorable change in entropy (11 $Jmol^{-1}K^{-1}$). Thus, the addition of bulky and negatively charged NeuAc at C-3/6 position in ultimate Gal appears to restrict the conformational freedom of the ligand while approaching the binding site. The binding of glycoproteins like fetuin and fibrinogen was 114- and 24-fold weaker than their respective asialo glycans respectively. Also, the unfavorable entropy change for equilibrium binding of glycoproteins was relatively high (60–70 $Jmol^{-1}K^{-1}$) indicating that the protein matrix reduces the accessibility of the ligands for binding to the lectin.

Table 1 Association constants and thermodynamic parameters for the binding of HSL with different ligands by fluorescence and surface plasmon resonance spectroscopy

Sugar	$K_a \times 10^6\ M^{-1}$			ΔG*	ΔH	ΔS
	20 °C	25 °C	30 °C	($kJmol^{-1}$)	($kJmol^{-1}$)	($Jmol^{-1}\cdot K^{-1}$)
Gal β1-3 GalNAc α-1-*O*-Ser	30.28	20.1	15.89	−41.8	−46.8	−16.7
	(31.20)	(22.01)	(16.30)	(−41.8)	(−47.2)	(−17.9)
Gal β1-3 GalNAc α-1-*O*-Me	ND	83.2	ND	−45.2	–	–
Gal β1-3 GalNAc β-1-*O*-Me	ND	0.176	ND	−29.9	–	–
Asialo-triantennary N- glycan	9.77	7.58	5.29	−39.2	−44.9	−19.2
	(9.72)	(7.42)	(5.42)	(−39.2)	(−44.2)	(−16.9)
Asialo-biantennary N- glycan	1.05	0.84	0.61	−33.8	−39.8	−20.2
	(1.03)	(0.83)	(0.62)	(−33.7)	(37.6)	(−12.8)
Sialated-triantennary N- glycan	0.603	0.468	0.345	−32.3	−41.3	−30.0
Sialated-biantennary N- glycan	0.186	0.148	0.112	−29.5	−37.6	−27.2
Fetuin	ND	ND	ND	ND	ND	ND
	(0.085)	(0.057)	(0.043)	(−27.2)	(−40.9)	(−89.8)
Fibrinogen	ND	ND	ND	ND	ND	ND
	(0.043)	(0.032)	(0.023)	(−25.6)	(−41.7)	(−71.5)

The values mentioned in the parenthesis are determined by SPR

ND not determined

*Values were calculated at 25 °C

Kinetics of Carbohydrate Binding

Kinetic analysis of all glycans and glycoproteins binding were studied by SPR and data indicated that, asialo triantennary *N*-glycan (k_1=13.35 ×10^3 $M^{-1}s^{-1}$), had faster association rate than galactosyl Tn-antigen (k_1=8.59 ×10^3 $M^{-1}s^{-1}$) and much faster than asialo biantennary *N*-glycan (k_1=2.31 ×10^3 $M^{-1}s^{-1}$). Whereas the dissociation rate of galactosyl Tn-antigen (0.049 s^{-1}) is much lower than asialo triantennary (0.0018 s^{-1}) and asialo biantennary (0.0025 s^{-1}) glycans. Thus, the difference in binding affinity of the HSL to the different glycan is determined by their difference in the association and dissociation rates. The lower binding affinity of glycoproteins is governed by slower association rate constant, for fetuin (k_1=1.66 ×10^3 $M^{-1}s^{-1}$) and fibrinogen (k_1=6.06 ×10^3 $M^{-1}s^{-1}$) as compared to glycans (Table 2).

The thermal dependence of the association and dissociation rate constants was determined at various temperatures and the activation energy for the respective process was determined from Arrhenius plots (Fig. 3). The values of activation energy ($E^{\ddagger}_1$) for the association reaction for the slowest ligand, fibrinogen is 36.14 kJ mol^{-1} and that of the fastest ligand, galactosyl Tn-antigen is 21.66 kJ mol^{-1} (Table 2). Galactosyl Tn-antigen binds 3-times faster than asialo triantennary glycan and the activation energy for the association process is much lower than asialo triantennary glycan (39.11 $kJmol^{-1}$). The entropy of activation for association process increased negatively to 53 $Jmol^{-1}$ K^{-1} indicating that the activation process involves highly ordered transition state. Asialo triantennary glycan binds 9-times faster than asialo biantennary glycan and the activation energy during the association process of the asialo triantennary (39.11 kJ mol^{-1}) is higher than asialo biantennary glycan (18.29 kJ mol^{-1}). However, the higher affinity of asialo triantennary is accompanied by lower entropy of activation for association process (−58.07 J mol^{-1} K^{-1}) as compared to biantennary glycan (−122.70 J mol^{-1} K^{-1}) indicating that association process does not involve highly ordered transition state. This suggests that the ligand can approach the binding pocket in several ways, leading to faster association.

Circular Dichroism Studies

Circular dichroism spectra of HSL in the native state and in the presence of T-antigen are shown in Fig. 4. The CD spectra of both native and in ligand bound HSL at far UV region remained identical, whereas in the near UV region (250–300 nm), the CD spectrum is characterized by a maximum around 293 and 266 nm and a minimum around 283 nm (Fig. 4). The band centered around 293, 283 and 266 nm can be assigned to Trp, Tyr and Phe respectively [38, 39]. Presence of ligand at near-saturation concentration led to an increase in the intensity of the native band in the region of 293 and 283 nm.

Discussion

Holothuria scabra, a sea cucumber that lives in the littoral regions and relatively shallow coastal areas, produces a high molecular weight monomeric lectin in the coelomic fluid [10]. This lectin levels in the fluid has increased to 8–10 fold upon challenge with specific human pathogenic bacteria. The role of this lectin in animals' innate immune response in controlling the pathogenic bacteria by antibacterial and agglutination activity has been reported earlier by the same authors [11]. The increase in hemagglutination and antibacterial activity of lectin only with specific bacteria and its high molecular weight, give rise to two important questions to be answered. Firstly, was there any relationship in the recognition of specific glycans or glycoproteins on particular bacterial surface by this lectin (HSL)? Secondly, since it had a high molecular size, what was its interaction with the protein component of the different glycoproteins and their role in recognizing different carbohydrate moieties on bacterial cell surface?

To answer above questions, a systematic thermodynamic and kinetic analysis of the interaction of HSL with different

Table 2 Rate constants and activation parameters for the interaction of glycans and glycoproteins to immobilized HSL by surface plasmon resonance spectroscopy (kinetic analysis)

Ligand	Association					Dissociation				
	$k_1 \times 10^{-3}$ M^{-1} s^{-1}	$E^{\ddagger}_1$ kJ mol^{-1}	$\Delta G^{\ddagger}_1$ kJ mol^{-1}	$\Delta H^{\ddagger}_1$ kJ mol^{-1}	$\Delta S^{\ddagger}_1$ $Jmol^{-1}$ K^{-1}	$k_{-1} \times 10^3$ S^{-1}	$E^{\ddagger}_{-1}$ kJ mol^{-1}	$\Delta G^{\ddagger}_{-1}$ kJ mol^{-1}	$\Delta H^{\ddagger}_{-1}$ kJ mol^{-1}	$\Delta S^{\ddagger}_{-1}$ $Jmol^{-1}$ K^{-1}
Galβ1-3GalNAcα1-*O*-Ser	8.59	21.66	52.11	19.18	−111.60	49.10	83.22	93.27	80.74	−41.89
Asialo-triantennary N-glycan	13.35	39.11	53.76	36.63	−58.07	1.81	84.06	92.93	81.59	−38.45
Asialo-biantennary N-glycan	2.31	18.29	52.02	15.82	−122.70	2.54	59.78	88.45	57.30	−105.59
Fetuin	1.66	37.93	55.02	34.93	−68.11	0.21	42.97	82.79	40.49	−143.40
Fibrinogen	6.06	36.14	52.07	33.66	−62.40	0.75	49.57	79.93	47.09	−111.33

Values of K_1, K_{-1}, $\Delta G^{\ddagger}_1$ and $\Delta G^{\ddagger}_{-1}$ are calculated at 25 °C

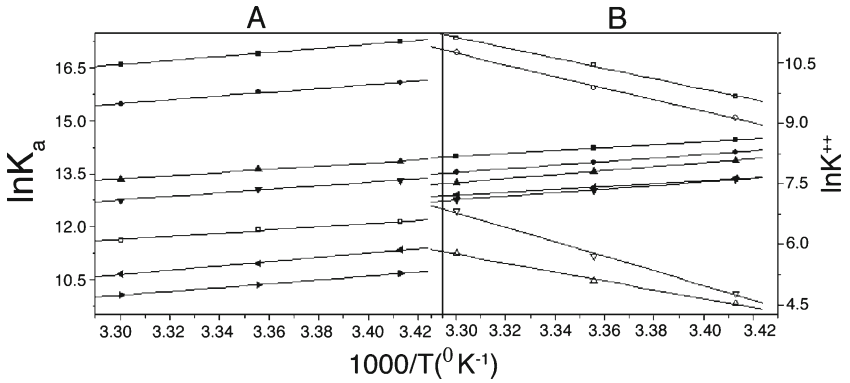

Fig. 3 van't Hoff and Arrhenius plots. The van't Hoff (**a**) and Arrhenius plots (**b**) for the association of glycans and glycoproteins to HSL are plotted according to the regression equation. Symbols used for van't Hoff plots are; galactosyl Tn-antigen (■), asialo-triantennary *N*-glycan (●), asialo-biantennary *N*- glycan (▲), sialated triantennary *N*-glycan (▼), sialated biantennary *N*-glycan (□), fetuin (◀) and fibrinogen (▶). Symbols used for Arrhenius plots are; associations of Gal β1-3 GalNAc α-1-*O*-Ser (■), asialo-triantennary *N*- glycan (●), asialo-biantennary *N*- glycan (▲), fetuin (▼), fibrinogen (◀), dissociations of Gal β1-3 GalNAc α-1-*O*-Ser (□), asialo-triantennary *N*- glycan (○), fetuin (▽) and fibrinogen (△). The association and dissociation constants were determined at three different temperatures, 20 °C, 25 °C and 30 °C

glycans and glycoproteins was carried out using fluorescence and surface plasmon resonance spectroscopy. In conjunction with the knowledge of HSLs' carbohydrate specificity which was studied earlier [10], and that of present study together, revealed several interesting features pertaining to its ligand binding properties. Decrease in association constant (K_a) with increasing temperature was observed in all ligands tested. van't Hoff plots for all ligands were linear ($r > 0.98$, see Fig. 3a) in the temperature range tested with negative values for changes in free energy, enthalpy and entropy. Thus the equilibrium binding of ligands to HSL is spontaneous and exothermic in nature.

From the present study it is revealed that, HSL is specific for *O*-linked glycans rather than *N*-linked glycans and glycoproteins. T-antigen α–methyl glycoside was found to be the best among the ligands tested. Among mono and disaccharides relatively higher affinity was observed with MeαGal and T-antigen, respectively [10]. In comparison, the equilibrium binding affinity of T-antigen α–methyl glycoside is ~19400 and ~1200 fold higher than that of MeαGal and T-antigen respectively (compare Table 1 with Table 5 in Ref 10). The higher affinity of T-antigen α–methyl glycoside may be a cumulative effect of differences in the orientation of the reducing sugar, axial orientation of the hydrophobic methyl group at C-1 position and C-2 acetamido group.

The above observed preferential recognition of HSL for *O*-glycans answers our previous finding that, the HSL binds to strains of *E. coli*, *Shigella sp. etc.* which are known to contain mucin or *O*-linked cognate glycan antigens on their cell surfaces, thereby agglutinating these strains and removing the infection from the host [40–42]. Even we cannot rule out the role of *N*-glycans on the bacterial surface in lectins recognition, since HSL binds to *N*-glycans with low magnitude. HSL hates to bind to glycoproteins; fetuin and fibrinogen due to their protein matrix, which might interfere with the access of ligands to the lectin, indicated by increase in entropy. Hence, we can rule out any protein-protein interactions between the HSL and the glycoproteins involved in the recognition of specific bacteria.

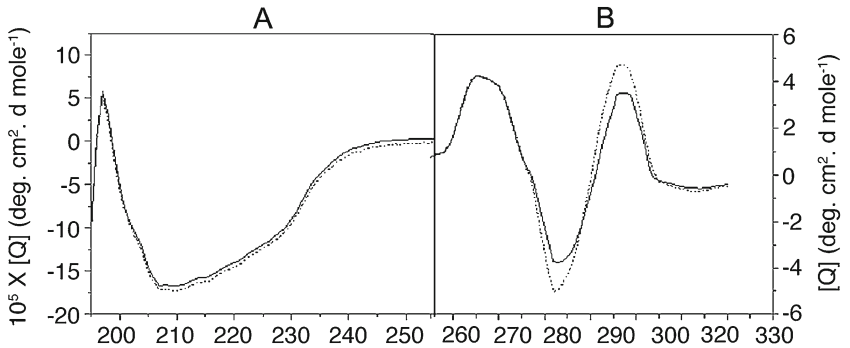

Fig. 4 Circular dichroism spectra of HSL. Circular dichroism spectra of HSL were recorded in the absence (—) and presence (—) of 200 μM of T-antigen at far UV (**a**) and near UV (**b**) regions. HSL was used at 1.9 μM and 11.98 μM for far UV and near UV scans respectively. Each spectrum was an average of 20 accumulations. Buffer (PBS, pH 8.0) scans were similarly recorded as controls and subtracted from the protein spectra for analysis. Scans were recorded at 25 °C

The value of *K*a determined by k_1/k_{-1} can increase either by increase in association rate constant (k_1) or decrease in dissociation rate constant (k_{-1}). For most protein-ligand interactions, the increase in affinity arises due to decrease in k_{-1} rather than increase in k_1 [43]. In the present study, higher binding affinity of galactosyl Tn-antigen over other glycans is associated with faster association rate compensated with slower dissociation rate. Also, the slower dissociation rate constant and higher entropy of both association and dissociation process appears to be the main factors responsible for lower affinity of *N*-linked glycans and glycoproteins.

Generally, these second order rate constants for the ligand (L) binding to a protein (P) are slower by several orders of magnitude than those seen in diffusion-controlled process and therefore the binding is presumed to involve a putative intermediate complex PL*, which then isomerizes into a final complex PL.

$$P + L \underset{k_{-1}}{\overset{k_2}{\rightleftharpoons}} PL^* \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} PL$$

where $K_1=k_1/k_{-1}$ and $K_2=k_2/k_{-2}$. Our inability to detect the formation of PL* complex could be due to an unobservable signal change in faster step. If the first step is always kinetically uncoupled from the second step, PL*→PL is rate-controlling *i.e.*, $k_{-1} >> k_2$, then the k_{obs} should progress from a first order dependence to a zero-order dependence as the concentration of the excess component (which is P in our case) increases from a value much lower than $1/K_1$ to $P >> 1/K_1$ (see Table 2). The agreement between kinetically determined values of association constants (k_1/k_{-1}) and changes in enthalpies indicate that the K_a and the enthalpy changes are related to the total binding process and not due to any intermediate that contributes appreciably to these parameters for the saccharide binding [43, 44]. Also, linearity of Arrhenius plots rules out to a great extent the formation of such an intermediate and the occurrence of dramatic conformational changes in the lectin molecule at least in the temperature range studied. Thus, it can be concluded that the binding of glycans and glycoproteins to the HSL follows a single step bimolecular association reaction, which is of several orders of magnitude slower than diffusion controlled reaction.

The activation energy for the association of various glycans and glycoproteins are high, indicating that sufficiently large amounts of energy have to be expended for the binding process to occur. This energy may be utilized in overcoming some steric constraints, such as conformational changes of the protein and/or in breaking hydrogen bonds between the solvent molecules and the sugar, as well as between the protein and the solvent molecules, and for the formation of newer ones between the sugar and the protein in the complexes. The activation entropy involved in the binding of *O*-linked glycan to HSL can be utilized in overcoming some steric constraints, such as some conformational changes in the protein and protein-ligand complex. These appear very probable in view of the considerable ligand induced changes in the CD spectra. Furthermore, these conformational changes occur only in the tertiary structure and not at the secondary structural level, as no change in the CD spectra was observed in far UV region (see Fig. 4). The change in the intensity of the near UV CD spectra in presence of ligand, suggest that the Trp and Tyr side chains are perturbed by saccharide binding. This is consistent with the chemical modification studies, which indicates the presence of Trp at the sugar-binding site (data not shown). Additionally, this term may also reflect perturbation release or shift in the state of water upon ligand binding. The higher affinity of HSL for galactosyl Tn-antigen and asialo triantennary glycan is due to enthalpic contribution, in addition to higher association rate as well as low activation energy for association process. The low binding affinity of asialo biantennary glycan, fetuin and fibrinogen glycoproteins are accompanied by increase in the negative entropy for both association and dissociation process.

In addition to underlining the unique ability of HSL to distinguish between α- and β-anomers of saccharides, our studies also provide differential binding affinity to Thomsen-Friedenreich-antigen. The non-binding of this lectin to Gal β1-3 GalNAc β -1-*O*-Methyl and its exquisite recognition of Gal β1-3 GalNAc α-1-*O*-Ser makes the lectin a valuable probe for monitoring the expression of Thomsen-Friedenreich-antigen on cell surfaces. It is well known that structural alterations of cell surface carbohydrates occur during transition from non-malignant to malignant state (12, 13). It has also been demonstrated that modifications of carbohydrate chains of glycoproteins, glycolipids and proteoglycans leads to cellular interactions, which are crucial for the metastasis development [12, 13]. Since HSL specifically recognizes Tn-antigen; one of the well-known tumor antigen of oncofetal origin, HSL can be used to identify normal cells from malignant ones.

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