PURIFICATION AND CHARACTERIZATION OF A LECTIN FROM MARINE INVERTEBRATE, SEA CUCUMBER *(HOLOTHURIA SCABRA)*

A THESIS

SUBMITTED TO THE

UNIVERSITY OF PUNE

FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

(IN BIOTECHNOLOGY)

BY

NAGARAJ M. GOWDA

UNDER THE GUIDANCE OF

DR. M. I. KHAN

DIVISION OF BIOCHEMICAL SCIENCES

NATIONAL CHEMICAL LABORATORY

PUNE -411 008 (INDIA)

MAY 2008

DEDICATED TO

MY MOTHER

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Acknowledgement

I take this opportunity to gratefully acknowledge my research mentor Dr. M.I. Khan for his invaluable guidance, constant inspiration, unending support and keen interest during the course of this investigation. He has given me the freedom to think and work; and I shall cherish my learning experience under him. His scientific temperament, innovative approach, dedication towards profession and down to earth nature has inspired me the most. Although this eulogy is insufficient, I preserve on everlasting gratitude for him. I am also grateful to Mrs. Khan for love, care and treating me like part of family. I will miss delicious food they served during numerous dinner parties at their home especially chicken biryani.

I am equally obliged to Dr. Usha Goswami for helping me to work with Dr. Khan and all the support she had given me in course of time.

I am grateful to Dr. S. M. Gaikwad for her valuable suggestions, help in learning many techniques and devoting her valuable time in putting this thesis into perfect shape.

I am also grateful Dr. M. V. Krishnasastry, Dr. C. G. Suresh, Dr. S.S. Deshmukh, Dr. Mala Rao and Dr. A. Prabhune for their valuable suggestions during the course of investigation.

I would also like to extend my sincere thanks to: Dr. B. M. Khan, Plant Tissue Culture, NCL, for providing lab facility for molecular biology work, Dr. Arvind Sahoo, National Centre for Cell Sciences, for providing SPR facility and help in data analysis. Dr. K. N. Ganesh, Organic Chemistry (S), NCL, for permission to use CD facility, Dr. Mahesh Kulkarni, Centre for Material Characterization, NCL, for help in MALDI-ToF facility. Dr. C.G.Naik, Bioorganic Chemistry lab, NIO, for help in LC-MS/MS and data analysis.

I express my deep feelings and love for my friends and my lab mates Feroz, Anil, Atul, Siddharth, Shashi, Sajid, JP, Rohtas, Sreekanth, Ansari, Avinash, Madhurima, Deepti, Asad, Ali, Shabab, Shadab, Ashutosh, Santosh, Apeksha and Ravi for their help, cooperation and maintaining pleasant and healthy atmosphere throughout my doctorate course. I would like to express my deep felt gratitude to my friends, Aarohi, Abhishek, Ajay, Abhilash, Ambrish, Amit, Anish, Arun, Ashish, Ashwini, Bhuban, Bhushan, Chetan, Elan, Fazal, Kishore, Manish, Nishant, Nitin, Nookaraju, Noor, Pallavi, Pandey, Pathange, Pawan, Poonam, Poorva, Rhodu, Samir, Santosh, Sarvesh, Satya, Sachin, Samir, Sharath, Shailendra, Sridevi, Suhas, Sumita, Suresh, Sushim, and Zafar for help, support and charming company.

Sharath, Shashi, Prasanna, Manje, Sumanth, Satish, Uma, Suresh Poojari, Govinda raju, Anish, Nitin, Arun, Rohtas, Atul, Rakesh, Hemant, Satya and Suresh for countless things they have done for me and for always being there with me whenever I needed them.

I find no words for my parents, my brother and sisters who have been a constant inspiration for me and this work would not have been possible without their constant support and sacrifices.

I would also like to thank my dear love, Ranju for her love, care, understanding and standing by me and boosting my morale in times of stress.

Finally, I thank Heads, Division of Biochemical Sciences and Division of Biological Oceanography and the Directors of National Chemical Laboratory and National Institute of Oceanography for permitting me to submit this work in the form of the thesis and Council of Scientific and Industrial Research, India for financial assistance. The Department of Biotechnology and HRDG-CSIR, India for financial support for attending conference in Australia.

Nagaraj M. Gowda

CERTIFICATE

Certified that the work incorporated in the thesis entitled **"Purification and characterization of a lectin from marine invertebrate, sea cucumber, *Holothuria scabra*"** submitted by Mr. Nagaraj M. Gowda was carried out under my supervision. Such materials has been obtained form other sources has been duly acknowledged in the thesis.

Dr. M. I. Khan

Research Guide

DECLARATION OF THE CANDIDATE

I declare that the thesis entitled **"Purification and characterization of a lectin from marine invertebrate, sea cucumber, *Holothuria scabra*"** submitted by me for the degree of Doctor of Philosophy is the record of work carried out by me during the period from **7rd March, 2003 to 6th April, 2008** under the guidance of **Dr. M. I. Khan** and has not formed the basis for the award of any degree, diploma, associateship, fellowship, titles in this or any other University or other institute of Higher learning.

I further declare that the material obtained from other sources has been duly acknowledged in the thesis.

Signature of the Candidate Date . . . . . .

Nagaraj M. Gowda

ABSTRACT

Lectins are carbohydrate binding proteins found in virtually all groups of living organisms. They find applications in (1) defense mechanism, (2) isolation of glycoproteins, (3) structural studies of carbohydrates, (4) identification of cell surface sugars and (5) detection of cell surface changes during differentiation, development and malignancy. The biological role of lectins is a consequence of their biding to the cell surface carbohydrates.

Holothuria belongs to phylum Echinodermata and relatives of sea star and sea urchin, all echinoderms are marine and are bottom-dwellers. Several pharmaceutically important biological compounds have been isolated from this genus. High specificity of *Holothuria scabra* lectin (HSL) for Tn-antigen and glycans makes them useful in the biochemical study of membrane and cell wall glycoconjugates. Investigation was carried out to purify and characterize the HSL and its involvement in the animal innate immunity and to determine its structure-function relationship. The thesis is divided into seven chapters.

Chapter 1 : General Introduction

This part comprises a literature survey of lectins especially from marine organisms with reference to their isolation, purification, properties and applications.

Chapter 2 : Induction, purification and characterization of HSL

In invertebrates, the mechanism evolved to maintain their body immunity and integrity includes cellular and humoral components. Both these factors respond to different antigens such as microorganisms, vertebrate erythrocytes and foreign proteins. We report a detailed study of a lectin involved in immune response in the echinoderm, sea cucumber (*Holothuria scabra*). Correlative studies indicate that the expression of this defensive lectin is induced by bacterial challenge. Fractional purification using hydrophobic interaction chromatography identified the lectin activity to a monomeric glycoprotein of a mass 182 kDa. Under *in vitro* conditions, purified *Holothuria scabra* lectin (HSL) could mediate agglutination of the test bacteria, thereby indicating a possible mode of action in physiological

situation. HSL was highly thermostable and tolerates extreme pH conditions (2-11). Spectroscopic studies indicated the presence of calcium, magnesium and copper metal ions in the protein; however, biochemical studies reveal that presence of divalent cations do not influence hemagglutination activity. Hemagglutination inhibition studies indicate specificity for MeαGal, T-antigen and desialylated glycoproteins like, asialo fetuin and asialo fibrinogen.

Chapter 3: Characterization of active site and tryptophan microenvironment of HSL

Sugar binding site of *H. sca*bra lectin was characterized by modifying with residue specific reagents, which revealed presence of positive charge of the ε-amino group of lysine, indole group of tryptophan and carboxylate groups for the glycan-binding. Fluorescence quenching and time-resolved fluorescence studies were carried out to study tryptophan microenvironment of HSL using two neutral (acrylamide, succinimide) an anionic (I^-) and a cationic (Cs^+) quenchers. The Trp were found to be differentially exposed to the solvent and were not fully accessible to the quencher indicating heterogeneity in the microenvironment. The presence of 200 μM of T-antigen led to slight decrease in the quenching with all the quenchers, suggesting presence of Trp at the sugar-binding site. Acrylamide quenching yielded positive upward curving of Stern-Volmer plots, indicating that the quenching mechanism involves both static and dynamic components. Time-resolved fluorescence measurments yield bi-exponential decay curves with lifetimes of 1.77 and 4.98 ns in the absence of ligand, and 2.08 and 5.10 ns in its presence. The Trp present in the sugar-binding site are mostly buried and show for long duration lifetime decay.

Chapter 4: Equilibrium binding studies of HSL: Fluorescence spectroscopy and surface plasmon resonance

The affinity and kinetics of sugar binding of the lectin was studied by fluorescence and surface plasmon resonance spectroscopy. The lectin binds with higher affinity to MeαGal, T-antigen and galactosyl Tn-antigen with a distinction between β 1-4 and β 1-3 linkages. T-antigen α-methyl glycoside was the most potent ligand having the highest affinity (*K*a 8.32 x10^7 M^{-1}).

Monosaccharide binding is enthalphically driven while disaccharide binding involves both entropic and enthalpic contributions. The HSL preferentially binds to *O*-linked than *N*-linked asialo glycans, and the affinities were relatively higher than for sialylated glycans and glycoproteins. Thermodynamic and kinetic analysis indicates 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.

Chapter 5: Analysis of carbohydrate binding domain structure of HSL

Hemagglutination activity is a unique property of lectins brought about by cross linking of the ligand by the lectin. As cross bridges are necessary for agglutination only multivalent lectins (>one CBD) can support this process. In general, a single CBD occurs in a polypeptide chain (subunit) and the multivalence is achieved by association of the chains into oligomers. However, HSL is a monomeric lectin and still supports hemagglutination. Limited proteolysis with trypsin resulted in a single 25 kDa fragment, which has the carbohydrate binding activity but no hemagglutination. Thus, HSL belongs to the rare class of monomeric multivalent lectins having two homomeric domains. We observed enhanced carbohydrate binding affinity of HSL-CBD for mono/disaccharides. Lys, Trp and carboxylate groups are present in the carbohydrate binding site.

Chapter 6: Structural stability study of HSL

HSL has unique properties in terms of stability. The binding function of HSL is independent of pH and shows similar activity from pH 2-12. Also, the lectin is unaffected by temperature up to 80 °C. We have explored the exceptional conformational stability of HSL by fluorescence spectroscopy and circular dichroism. The secondary and tertiary structure of HSL was analyzed as a function of pH. Our results indicate that pH induces structural changes at the tertiary level without any impact on the secondary elements. Treatment of HSL with chemical perturbants like urea and guanidium hydrochloride did not induce denaturation. Instead, the lectin underwent extensive structural changes (both secondary and tertiary) without affecting the activity. Complete

unfolding of the lectin could be achieved by including 5 mM DTT along with the perturbants. HSL has six disulphide bonds and our present observations appear to indicate that these covalent bonds are crucial for maintaining the stability of the functional domain of HSL.

Chapter 7 : General Discussion and Conclusion

This part compares the properties of HSL with other lectins with respect to their biophysical properties.

List of abbreviations used

ANS	: 8-anilino-1-naphthalene sulfonate
BSM	: Bovine submaxillary mucin
CD	: Circular dichroism
DEP	: Diethylpyrocarbonate
DTNB	: Dithiobis 2-nitrobenzoic acid
DTT	: Dithiothreitol
EDC	: 1-ethyl-3 (3-dimethyl amino propyl) carbodiimide
EDTA	: Ethelenediaminetetra acetic acid
Fuc	: L-Fucose
Gal	: D-Galactose
2dgal	: 2-deoxy-D-galactose
Galβ1→ 3GlcNAc	: 2-acetamido-2-deoxy-3-O-(β-D-galactopyranosyl)-D-glucopyranose;
Galβ1→3GalNAc	: 2-acetamido-2-deoxy-3-O-(β-D-galactopyranosyl)-D-galactopyranose
Galβ1→4GlcNAc	: 2-acetamido-2-deoxy-4-O-(β-D-galactopyranosyl)-D-glucopyranose
GalNAc	: N-acetyl-D-galactosamine
GdnHCl	: Guanidine hydrochloride
Glc	: D-Glucose
GlcNAc	: N-acetyl-D-glucosamine
HA	: Hemagglutination activity
HSL	: *Holothuria scabra* lectin
IEF	: Iso electric focusing

K_1	: Association rate constant
k_{-1}	: Dissociation rate constant
Ka	: Affinity constant
K_q	: Modified Stern Volmer quenching constant
K_{sv}	: Stern Volmer quenching constant
MALDI-ToF	: Matrix-assisted laser desorption ionization time-of-flight
Man	: D-Mannose
Me	: Methyl
NAI	: *N*-acetylimidazole
NBS	: *N*-bromosuccinimide
NTEE	: Nitrotyrosine ethyl ester
PAGE	: Polyacrylamide gel electrophoresis
PMSF	: Phenylmethyl-sulphonilfluoride
SPR	: Surface plasmon resonance
SDS	: Sodium dodesyl sulphate
T-antigen	: Thomsen-Friedenreich antigen
TB	: Tris buffer, pH 8.5
TBS	: Tris buffer saline, pH 7.2
TNBS	: Trinitrobenzenesulphonic acid
WRK	: Woodward's reagent K

CHAPTER : 1

GENERAL INTRODUCTION

Lectins, a well-known class of multivalent carbohydrate binding proteins of non-immune origin which recognize diverse sugar structures with a high degree of stereospecificity in a non-catalytic manner, are widespread in distribution [1]. They have been implicated in cellular signaling, malignancy, host pathogen interactions, scavenging of glycoproteins from the circulatory system, cell-cell interactions in the immune system, differentiation and immune responses and protein targeting to cellular compartments [1-3]. Goldstein [4] defined lectins as di- or multivalent carbohydrate binding proteins or glycoproteins of nonimmune origin which agglutinate cells and/or precipitate glycoconjugates. However, with the discovery of lectins with toxic or hormone like activities, this definition was found to be inadequate. To overcome these shortcomings, Kocourek and Horejsi [5] proposed a modified version of the definition as "Lectins are proteins of nonimmunoglobulin nature capable of specific recognition and reversible binding to carbohydrate moieties of complex carbohydrate without altering the covalent structure of any of the recognized glycosyl ligands". However, in the light of data obtained mainly from molecular cloning of lectin and lectin related proteins, this broad definition was still inadequate. Hence, to overcome these shortcomings lectins were classified into three groups based on their overall structure and properties.

Merolectins: These are small proteins, incapable of precipitating glycoconjugates or agglutinating cells because of their monovalent nature. Monomeric mannose binding proteins from orchid [6, 7] and class I chitinases that possess a chitin binding domain and a catalytic domain separated by hinge region [8] come under this family.

Hololectins: They are exclusively made up of carbohydrate-binding domains. However, unlike merolectins, they contain two or more carbohydrate-binding domains, which are identical or very homologous and bind either the same or the structurally similar sugars. Since hololectins have multiple binding sites they are fully capable of

agglutinating cells and precipitating glycoconjugates. Most plant lectins and invertebrate lectins belong to this subgroup [9-11].

Chimerolectins: These are fusion proteins composed of a carbohydrate-binding domain (CBD) tandemly arrayed with an unrelated domain with well-defined catalytic activity which functions independently of the former [5]. Depending upon the number of binding sites, chimerolectins behave as merolectins or hololectins. For example, the type 2 Ribosome inactivating proteins (RIPs) ricin and abrin consist of a toxic A chain (which has the *N* glycosidase activity characteristic of all RIP's) and a carbohydrate binding B chain [12] with two carbohydrate binding sites to agglutinate cells function as hololectins whereas class I chitinases with a single chitin binding domain that do not agglutinate cell behave as merolectin [13].

Lectins can be divided into at least six families (Table 1.1) by sequence comparison of carbohydrate recognition domain (CRD) [14-16].

Table 1.1: Different lectin families.

Lectin type	Subunits	Subunit MW (kDa)	CRDs per subunit	Ca^{2+} dependent	Disulfide bonds	Specificity
Legume lectins	2 or 4	25-30	1	+	-	diverse
Cereal lectins	2	18	2	-	++	GlcNAc, NeuAc
P-type lectins	1-4*	46 or 275	1 or 15	-	+	Man-6-P
S-type lectins	1or 2	14-35	1 or 2	-	-	Gal
C-type lectins	variable	14-165**	1 or 8	+	+	diverse
Pentraxins	5 or 6	20-25	1	+	+	diverse

*It remains speculative whether P-type lectins are monomeric or associate in the membrane to form dimmers or tetramers.

**The subunits of C-type lectins are composed of CRDs of ~120 amino acids (~14 kDa), which may occur in isolation (invertebrates) or linked to other domains (mammals, birds).

Historical perspective

The lectin was first discovered by Stillmark in 1888 as an agent that can agglutinate animal erythrocytes from extract of castor bean [17]. Boyd and Shapleigh in 1949 coined the term lectin (Latin, 'legere', to select or choose) based on their observation that some plant seed extracts could distinguish among human blood-groups [18-20]. Hellin in 1891 discovered abrin [21], the toxic protein from jequirity beans. During the 1890's, Ehrlich worked with ricin and abrin, and discovered immunospecificity and reversibility of the antigen-antibody reaction as some of the fundamental principles of immunology. Landsteiner and Raubitschek in 1908 [22] found some lectins showing specificity for the red blood cells of certain species, while in 1945 Boyd and Reguera [18] established blood group specificity of the lectin. One of the first lectins to be crystallized was con-A from jack bean by Sumner [23]. Sugar specificity and inhibition of hemagglutination of the lectin was discovered by Watkins and Morgan in 1952 [24]. Researches on lectin gained momentum when Nowell's laboratory at the University of Pennsylvania, Hershey, reported the mitogenicity of lectins, wherein the lectins stimulated mitosis in the dead-end lymphocyte cells [25]. During the same period, Joseph demonstrated that WGA and Con A lectin preferentially agglutinated the malignant cells.

Lectins are wide spread in distribution and have been isolated from microorganisms, plants and animals (Table 1.2). Although most of the well-characterized lectins are from plants, marine invertebrate lectins are receiving increased attention due to their importance from basic as well as applied aspects. This compilation gives a comprehensive account of marine invertebrate lectins with respect to their occurrence, purification, physicochemical properties, biological roles and applications.

Table 1.2: Occurrence of lectins in different organisms.

Lectin	Source	Reference
Virus		
Influenza viral lectin	Influenza virus	[26]
Myxo and paramyxo lectin	Myxo and paramyxo virus	[26]
Bacteria		
PA-IL	*Pseudomonas aeruginosa*	[27]
RSL and	*Ralstonia solanacearum*	[28]
Hemagglutinin B (HagB)	*Porphyromonas gingivalis*	[29]
HBHA	*Mycobacterium tuberculosis*	[30]
FHA	*Bordetella pertussis*	[31]
Fungi		
PHA and PHB	*Agaricus bisporus*	[32]
BSL	*Boletus satanas*	[33]
CGL1 and CGL2	*Coprinopsis cinerea*	[34]
Ganoderma lectin	*Ganoderma capense*	[35]
Grifola lectin	*Grifola frondosa*	[36]
Hericium lectin	*Hericium erinaceum*	[37]
TML-1 and TML-2.	*Tricholoma mongolicum*	[38]
Plant		
TDSL	*Trichosanthes dioica*	[39]
Artocarpus lectin	*Artocarpus hirsuta*	[40]
Phaseolus vulgaris lectin	*Phaseolus vulgaris*	[41]
--	*Musa basjoo*	[42]

--	*Vicia sativa*	[43]
EspecL	*Erythrina speciosa*	[44]
Animal		
Anthocidaris lectin	*Anthocidaris crassipina*	[45, 46]
Electricus lectin	*Electricus electricus*	[47]
--	*Craniella australiensis*	[48]
Anguilla lectin	*Anguilla rostrata*	[49]
Marine lectins		
CEL-I, II, III, IV	*Cucumaria echinata*	[50]
Holothuria lectin	*Holothuria scabra* Jaeger	[51]
PjLec	*Penaeus japonicas*	[52]
Modiolus lectin	*Modiolus modiolus*	[53]
Clarias lectin	*Clarias batrachus*	[54]
Zhikong scallop lectin	*Chlamys farreri*	[55]
CVL	*Chaetopterus variopedatus*	[56]
Tunicate lectin	*Polyandrocarpa misakiensis*	[57]
Starfish lectin	*Asterina pectinifera*	[58]
Manila calm lectin	*Ruditapes philippinarum*	[59]
Crab lectin	*Cancer antennarius*	[60]
CvL	*Cliona varians*	[61]
Echinonectin	*Lytechinus variegatus*	[62]

MARINE LECTINS

The first hemagglutinins from marine animals was from *Holothuria polii* and *Holothuria tubulosa* coelomic fluid by Parrienello et al., in 1976 [63]. Naturally occurring hemolysins from coelomic fluid of *Holothuria polii* were reported by Parrinello et al., in 1979 [64]. Upon sheep erythrocyte injection, the levels of hemagglutinins and hemolysins levels were increased from baseline level in *Holothuria polii* by Canicatti and Parrinello in 1985 [65]. CEL-III, a hemolytic lectin from *Cucumaria echinata* was the first marine invertebrate lectin for which the primary crystal structure was solved by Fujisawa et al., in 1997 [66].

Occurrence and localization

The occurrence of lectins /hemolysins in marine animals is wider than higher plants and animals. Marine lectins have isolated from coelomic fluid [67], internal organs [48], whole body tissue [47, 54], plasma [68], hemolymph [69, 70] etc.

Detection and assay

Lectins were earlier discovered as hemagglutinating agents; hence, the technique is universally used to detect the lectin in the past and even today. The hemagglutination is carried out using animals like, rat, mice, hamster, guinea pig, rabbit, chicken and goat as well as human erythrocytes [71]. Sometimes erythrocytes are treated with enzymes like pronase, trypsin, papain and neuraminidase or any other proteolytic enzyme [72-75]. Bacterial cells were also used for agglutination [76]. Hemolysins were reported from different animals [77] and CvL agglutinated *Leishmania chagasi* promastigotes [78].

Purification

Majority of marine invertebrate lectins are secreted into the circulating body fluid and/or present in the body tissue or in circulating cells. Most of the purification procedures involve steps like lysis of cells/tissue and concentration of the crude extract by salt precipitation, ultra filtration or

lyophilization followed by conventional purification methods, such as ion-exchange chromatography and gel filtration.

Ion-exchangers like DEAE-, CM-cellulose and QAE-Toyopearl have been used for the purification of some of echinoderm lectins [54, 68, 77]. Gel filtration has been used as one of the purification steps for lectins from *Cucumaria echinata* [50] and *Polyandrocarpa misakiensis* [79]. In addition, hydrophobic matrix like phenyl-sepharose has been employed for the purification of lectin from *Stichopus japonicas* [80].

Affinity chromatography has been extensively used for the purification of lectins. The ability of lectins to agglutinate erythrocytes has been utilized for their purification on erythrocytes and erythrocyte stromas embedded on polyacrylamide [81], like Fetuin-CNBr-activated sepharose-4B [52, 82], BSM-CNBr coupled [53, 76], mucin-sepharose-4B [73], lactosyl-sepharose-4B [56], cross-linked guar gum [74], GalNAc-sepharose-CL4B [58]. In some cases, sephadex and sepharose or acid treated sepharose have been used to purify glucopyranosyl/mannopyranosyl and galactopyranoside binding lectins respectively, where they serve as affinity matrix and not as molecular sieves. Sepharose was used for the isolation of a lectin from *Halocynthia roretzi*, specific for galactose [68].

Molecular mass and subunit structure

The molecular mass of marine lectin range from 17-400 kDa, but majority of them are between 45-65 kDa (Table 1.3). Most of them are homo-multimeric proteins and the subunits are held together by disulfide linkages.

Isoelectric point

The isoelectric points of the majority of marine lectins are in the range of 4-6 (Table 1.2). Lectins from *Aplysina archeri* and *A. lawnosa* [83] are highly acidic proteins with a pI of 4.1 and 4.5 respectively, whereas lectin from *Urechis unicinctus* [81] has pI 5.5.

Table 1.3: Physical properties of marine lectins.

Source		MW (kDa)	Subunit type	pI	Carbohydrate content (%)	References
Stichopus japonicas		400	α_8			[80]
C. *echinata*	(CEL-I)	32.0	α_2	-	-	[77]
	(CEL-II)	35.0	α	-	-	[47]
	(CEL-III)	45.0	α		-	[47]
	(CEL-IV)	68.0	α_4	-	-	[47]
Clarias batrachus		200	α	-	-	[54]
Holothuria scabra Jaeger		355	6 het.	-	1.33	[51]
Gracilaria ornate		17.4	α	5.4	2.9	[73]
Penaeus japonicus		452	α_{12}	-	-	[52]
Aplysina archeri		63	α_4	4.1	3.5	[83]
Aplysina lawnosa		63	α_4	4.5	5.0	[83]
Lytechinus variegates		220	α_2	-	5.6	[62]
Serpula vermicularis		50	α_4	-	1.9	[75]
Urechis unicinctus (1 &2)		31,34	α	5.0	8.4	[84]
Pterocladiella capillacea		5.8	α	5.5	0	[85]
H. *okadai*	HOL-I	21.0	α_4	4.5	2.0	[86]
	HOL-II	42.0	α	4.5	7.5	[86]

Carbohydrate content

The carbohydrate content varies from lectin to lectin and in some cases it could be as high as 8.4% in *Urechis unicinctus* (1&2), while in some lectin it is totally absent as in *Pterocladiella capillacea* [85] (Table 1.2).

Metal ion requirement

Marine lectins are generally identified by their metal ion requirement for their hemagglutination activity. Majority of the marine lectins belongs to C-type lectin family of Ca^{2+} dependent [51, 52, 54, 55, 72, 77, 79, 80, 83], while Mg^{2+} stimulated the activity of *Modiolus modiolus* lectin [83, 87]. *Ptilota filicina* require the divalent cations, Ca^{2+}, Mg^{2+} and Mn^{2+} for its activity [74]. While few of the lectins does not require any metal ions for their activity [73, 75]

Specificity

Table 1.4: Current categories for classification of various marine lectins.

Family	Structural motif	Carbohydrate ligand	Modular arrangement
C-type	conserved CRD	variable (Man, Gal, Fuc, heparin tetrasaccharide)	yes
I-type	immunoglobulin-like CRD	variable ($Man_6GlcNAc_2$), HNK-1 epitope, hyaluronic acid	yes
Galectin (S-type)	conserved CRD	β-galactosides	variable
Pentraxins	Pentameric subunit rearrangement	4,6-cyclic acetal of β-galactose, galactose, sulfated and phosphorylated monosaccharides	yes
P-type	Similar but not yet strictly defined CRD	Mannose-6-phosphate-conatining glycoproteins	yes

Carbohydrate specificity

Marine lectins exhibit a broad specificity varying from simple sugars to glycoproteins. Based on their specificity marine lectins can be classified into different groups (Table 1.4). Most of them are Gal/GalNAc specific lectins while; *Crenomytilus grayanus* lectin is specific for mucin (Table 1.5). *Penaeus monodon* lectin showed specificity for bacterial lipopolysaccharide [88], lobster agglutinins (LAg-1, LAg-2) binds to human and animal erythrocytes [89].

Table 1.5: Carbohydrate specificity of marine lectins

Lectin /Source	Specificity	Reference
Crenomytilus grayanus	Glycoproteins of mucin type	[90]
Didemnum ternatanum DTL	GlcNAc	[71]
DTL-A	BSN, ds-BSM and heparin, dextran sulphate	[91]
Serpula vermicularis SVL-1	Mannan	[90]
SVL-2	GlcNAc	[75]
Tachypleus tridentatus	GlcNAc/GalNAc	[92]
Crenomytilus grayanus	Gal/GalNAc	[93]
Halichondria okadai HOL-I	GlcNAc β 1-4(GlcNAc β 1-2)Man α1-*O*$(Ch_2)_2CH_3$	[86]
HOL-II	N-acetyllactosamine	[86]
Lytechinus variegates	Gal	[62]
Urechis unicinctus	D-gal	[84]
Penaeus monodon	*O*-antigen of bacterial polysaccharide	[88]

Styela plicata	Gal	[94]
Litopenaeus vannamei	GalNAc/GlcNAc/NeuAc	[95]
Pterocladiella capillacea	Avidin, mucin	[85]
Didemnum ternatanum	GlcNAc	[96]
Codakia orbicularis	Mannose	[97]
Cucumaria echinata (CEL-I, II, III and IV)	GalNAc/Gal	[77]
Polyandrocarpa misakiensis	Gal	[79]
Halocynthia roretzi	Gal	[68]
Modiolus modiolus	Sialic acid	[72]
Clarias batrachus	Gal	[54]
Gracilaria ornata	Glycoproteins	[73]
Penaeus japonicus	Mucin	[52]
Aplysina archeri	Me-β-Gal/thiodigal	[83]
Chaetopterus variopedatus	β-gal	[56]
Ptilota filicina	Gal	[74]
Asterina pectinifera	GalNAc	[58]
Hypnea japonica	Desialylated glycoproteins	[98]
Ruditapes philippinarum	GalNAc/Mucin	[59]
Cancer antennarius	*O*-Acetylsialic acid	[99]
Cliona varians	Gal/sucrose	[78]

Cellular specificity

Lectins show specificity for certain cell types, *e.g.* erythrocytes, belonging to specific human or animal phenotypes and certain microbial cells. This property of the lectins has been used in the taxonomy of animal and single-cell plants.

Human erythrocytes: Some of the marine lectins showed discrimination between erythrocytes of different blood groups. Treating erythrocytes with proteolytic enzymes can enhance their activity or modify specificity, hemagglutinating activity of *Didemnum ternatanum* (DTL) [91] and *Crenomytilus grayanus* [93] can be enhanced by prior treatment of the cells with trypsin, without modifying its specificity. A lectin from *Pterocladiella capillacea* showed specificity for rabbit erythrocytes, after treatment with trypsin, bromelain, or subtilisin [85]. Similar observations were made in *Modiolus modiolus,* where enhancement in its hemagglutinating activity was observed by glutaraldehyde treatment followed by neuraminidase and pronase treatment [53]. Whereas a lectin from coelomycetes of the Echiuran, *Urechis unicinctus* agglutinated human erythrocytes regardless of any blood group equally well indicating, blood group non-specificity [84, 95]. A galactose specific serum lectin from *Clarias batrachus* agglutinated human, rabbit, rat and mice erythrocytes only after pronase E treatment [54]

Animal erythrocytes: It has been observed that certain marine lectins can discriminate between human and animal erythrocytes, as well as among different animal erythrocytes. The lectin isolated from *Penaeus monodon* agglutinates rabbit erythrocytes exclusively [88], whereas a lectin from *Ruditapes philippinarum* agglutinates only trypsinized human erythrocytes [59]**.** Several other erythrocytes from mammals, birds, reptiles and amphibians have also been used for hemagglutination.

Other types of cell: It has been reported that lectins isolated from few marine animals do not find complementary structures for their binding sites on any type of erythrocytes tested but bind to other cells. A lectin

from *Penaeus monodon* had agglutinated four gram negative (*E.coli, Pseudomonas fluorescens, Aeromonas hydrophilia* and *Vibrio alginolyticus*), a gram positive (*Micrococcus lysodeikticus*) bacteria and a yeast (*Candida albicans*), suggesting that this lectin was a type of immune-relevant protein in *Penaeus* [88]. Interestingly, a lectin from *Clarias batrachus* agglutinated gram-negative *Aeromonas* strains [54]. A recombinant lectin from *Chlamys farreri* showed larger agglutinants of *E. coli* upon incubation [55]. Lectin from Manila clams, *Ruditapes philippinarum* binds to the surface of the protozoa, *Perkinsus atlanticus* [59]. CVL lectin from *Cliona varians*, agglutinated *Leishmania chagasi* promastigotes [78].

Effect of marine lectin on different cells

Lectins are found to show other cell activities besides agglutination *e.g.* the binding of a lectin on a membrane receptor can trigger some specific cell reaction, or have a cytotoxic effect.

Cytotoxicity: The phagocytosis appears to be the cell mediated defense involved in cytotoxic reactions in a mixture of coelomycetes. When phagocytes from the sea urchin *S. droebachiensis* were co-cultured with phagocytes from either *E. esculentus* or *Strongylocentrotus pallidus*, 90% of the cells were killed, and in allogeneic mixtures of *S. droebachiensis* phagocytes, 70% of the cells were killed [100]. Pagliara et al., [101] have isolated cytolytic granules from phagocytes of *P. lividus*, suggesting that these cells mediated their killing function through the release of their cytolytic material.

The cytotoxic activities of some of marine lectins on HIV cell lines were isolated from *Crenomytilus grayanus* (CGL) mussel [93], *Didemnum ternatanum* (DTL, DTL-A) ascidia [71, 91] and from *Serpula vermicularis* (SVL-1, SVL-2) worm [90]. These lectins are examined on the cells of the C8166 T-lymphoblastoid cell line that are the target cells for HIV are listed in Table.1.6.

Table.1.6: Cytotoxicity and inhibition of the HIV-1 replication and hemagglutination activity of lectins.

Lectin	EC^{a}_{50} µg/ml	CC^{b}_{50} µg/ml	EC_{50} nm	CC_{50} µm	A^{c} µg/ml	AI^{d}	M kDa
DTL	0.006	>500	0.2	>18.5	0.9	83.33	27
DTL-A	0.59	123	42	8.8	0.007	209	14
SVL-2	0.23	>500	9.0	>19.6	62.5	>2178	25.5
CGL	45.7	263	2539	14.6	0.45	>6	18
SVL-1	89.1	>500	2874	>16.1	62.5	>6	31
Azidothymidine	0.012	>5	45	>18.7	-	>416	0.27

[a]EC_{50} is an effective concentration of compounds necessary for 50% inhibition of the HIV-1 replication.

[b]CC_{50} is a cytotoxic concentration of compounds necessary for 50% decrease in the number of viable cells of the C8166 cell line.

[c]Minimum concentration required for the agglutination.

[d]Antiviral index (AI) is the CC_{50}/EC_{50} ratio.

A 30 kDa lectin (CVL) from *Chaetopterus variopedatus* inhibited cytopathic effect induced by HIV-1 and the production of viral p^{24} antigen. Time-of-addition analysis of anti-HIV-1 activity indicated its action was at the early stage of infection. CVL could block the cell-to-cell fusion process of HIV infected and uninfected cells.

Phagocytosis: Subpopulation of coelomycetes from echinoderms is defined by their amoeboid behavior and their abilities to engulf foreign cells and particles. Phagocytes from two sea urchin species, *Strongylocentrotus franciscanus* and *S. purpuratus*, were noted to chemotax towards marine bacteria, where gram-positive bacteria being phagocytosed more readily than gram-negative bacteria [102]. Human and sheep RBCs were taken up within 30 min in vitro by phagocytes from the sea urchin, *Strongylocentrotus nudus*, and RBCs opsonized with

coelomic fluid from animals that had been pre-injected with RBCs enhanced the phagocytic rate compared to non-opsonized red cells [103].

Effect on lymphocytes: Nowell in 1960 observed that some phyto-hemagglutinin from bean and other plants including legumes, in addition to agglutinating activities towards lymphocytes, can also induce their conversion into lymphoblast forms, sometimes with mitotic induction [25]. The lectin from *Clarias batrachus* was able to induce the proliferation of head kidney lymphocytes of *Clarias* sp. and helped in the release of 'IL-1'like cytokines from head kidney macrophages [54].

Effect on tumor cells: Certain marine lectins showed inhibitory action on the in vitro growth of tumor cells. CEL-I lectin from *Cucumaria echinata* has a potent cytotoxic effect on MDCK, HeLa and XC cell lines and killed in a dose-dependent manner [104]. The sialic acid binding lectin from *Rana catesbeiana* oocyteis known to have antitumor activity was dependant on the net positive charge of the amino acids present in the active site [105].

Effect on adhesion and growth of cells: The lectin from ascidian *Didemnum ternatanum* seems to have characteristic of both adhesion and growth factors for cultivates of molluscan and echinoderm cells. The lectin was found to stimulate the growth or the differentiation of cultivated marine invertebrate cells depending on the stage of the embryonic development. The degree of attachment is considerably increased when collagen or polylysine substrates are used [96].

Antibacterial activity: Many marine lectins had shown significantly higher-level bactericidal activity against both gram-negative and gram-positive bacteria. The lectin isolated from *Clarias batrachus* could significantly reduce the viability of different *Aeromonas* strains and resulted in a dose dependent increase in the bactericidal activity of *C. batrachus* macrophages, resulted in loss of viability and pathogenicity of a bacterial pathogen [54]. A C-type lectin from tunicate, *Polyandrocarpa misakiensis* displayed strong antibacterial activity even at the

concentration of 1 μg ml^{-1} [79]. The recombinant protein from *Chlamys farreri* displayed remarkable inhibition effect on gram-negative *Micrococcus luteus* and weak lytic activity against *E. coli* [55].

Effect on insects: Insect pests are major constraints to increased grain legume production in tropical and subtropical regions. Few marine lectins had shown the insecticidal activity like, lectin from *Gracilaria ornata* at 1% (w/w) caused a 65.1% reduction in *Calosobruchus maculates* larval survival [73].

Hemolytic activity on erythrocyte membrane: CEL-III from *Cucumaria echinata* is a novel lectin that exhibits strong hemolytic activity [106], which is mediated by binding of the protein to the specific carbohydrate chains on the target cells, followed by the formation of ion-permeable pores in the cell membrane through oligomerization of the protein. After formation of the pores, erythrocytes are ruptured by colloid osmotic shock.

Structure and function

Figure1.1. shows a comparison of the amino acid sequence of CEL-I from *Cucumaria echinata* [107] with those of other C-type lectins from marine invertebrates. The highest similarity was found with starfish [58] for an identity of 36.4%. Similarity among C-type CRDs is generally low, and even CEL-IV [108] has an identity of only 30.2% with CEL-I. The C-terminal region after position 74 was relatively well conserved. While Cys31-Cys135 corresponds to one of the two disulfide bonds highly conserved among the C-type lectins. This was the same case even for C-type lectins from Holothuroidea such as CEL-IV [108] and SJL-I [109].

The AAA (*Anguilla anguilla*, agglutinin) fold has no similarity to other fucose-recognizing proteins, including selectins (C-lectin fold) and plant fucolectins (legume lectin fold), and represents a novel fold within the entire lectin family. However, a search for similar structures in the DALI database reveals three proteins with a fold similar to AAA, C1 and C2 repeats of blood

coagulation factor V [110] (FVa-C1 and -C2; FVa-C2), the C-terminal domain of sialidase [111] (CSIase and neuraminidase) and the *N*-terminal domain of galactose oxidase [112] (NGOase). An alignment of CSIase, NGOase, FVa-Ca and AAA sequences on the basis of the superposition of structurally similar domains indicates sequence identities with AAA in the 2−14% range. This alignment shows that only three residues, Pro, Asp and Arg are strictly conserved; these are equivalent to AAA residues Asp 64, Pro 106 and Arg 131. In the four structures, Pro residue is in different conformations, but the Asp and Arg form an equivalent salt bridge.

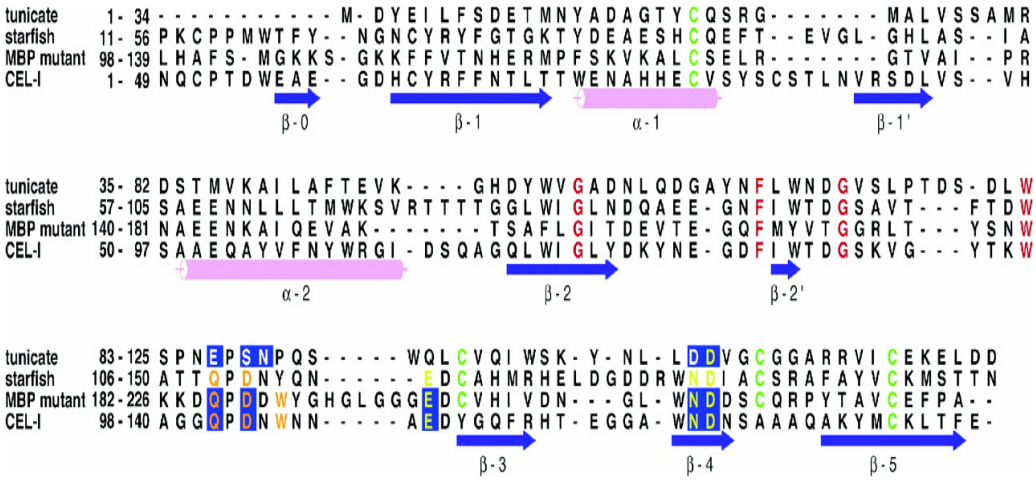

Fig. 1.1. Sequence alignment and secondary structure of CEL-I and other C-type lectins. Three invertebrate CRDs (CEL-I [113], tunicate [92], and starfish [58] lectins) and a GalNAc-binding (QPDWGH) mutant of MBP [114] were aligned. Conserved residues are illustrated in *red*. Conserved residues among both the galactose- and mannose-binding types and the galactose-binding type only of the C-type lectins are shown in *yellow* and *orange*, respectively. Cysteine residues widely conserved among CTLDs are shown in *green*. The structure-based sequence alignment was performed by DALI, except for the GalNAc-specific binding starfish lectin. The residues that coordinate with carbohydrate-binding Ca^{2+} ions are *boxed* in *blue*. This figure was drawn by the program ALSCRIPT and manually modified.

Homology search revealed that the ascidian plasma lectin showed little significant similarity to other known lectins or to carbohydrate binding proteins [68]. However, *N*-terminal region was homologous to the central part of fibrinogen β chain (31, 28 and 21% identities to human, lamprey and sea cucumber fibrinogen chains respectively). The C-terminal region was homologous to C-type lectin family. The amino acid sequence consists of a two-repeated sequence, a fibrinogen-related sequence and C-type lectin-homologous sequence. With respect to the fibrinogen-related sequence (51-105 residues), two conserved sulfhydral groups in this region of the fibrinogen β chain are cross-linked intramolecularly between Cys53 and Cys82 in plasma lectin. The secondary structure of this region is predicted to be composed of three successive β structures. In the C-terminal homologous sequence (216-270 residues) show a low similarity to the C-type lectin family (30% homology to barnacle plasma lectin-3), although the ascidian lectin seems to have little of the typical C-type CRD [115]. In addition, the central parts (121-170 and 177-204 residues) of the ascidian lectin show slight similarity (18 and 21%) to sea urchin plasma C-type lectin [67] and barnacle plasma lectin-3 [116] respectively. The starfish *Asterina pectinifera* lectin [58] exhibited 39%, 35% and 34% identities with echinoidin, BRA-3 and brevican C-type lectins.

CEL-III, a hemolytic lectin from *Cucumaria echinata* is the first marine lectin whose crystal structure was solved. It is a Ca^{2+}-dependent animal lectins with two *β*–trefoil domains, which were originally found in the B-chain of ricin. The *β*–trefoil domains of ricin and similar proteins have the characteristic sequence $(QXW)_3$ and are referred to as the $(QXW)_3$ family. In contrast to the other *β*–trefoil lectins, domains 1 and 2 of CEL-III bound five $Ca^{2+}$and two Mg^{2+} ions, and Ca^{2+}may be associated with recognition of specific carbohydrates. On the other hand, domain 3 contains characteristic *β*–sheets and two *α*–helices. These *α*–helices are located at the interface between carbohydrate binding domains (1 and 2) and domain 3, and are assumed to make conformational change upon binding to the cell surface carbohydrates. The hemolysis by CEL-III proceeds through cooperative action of its

functional domains; the binding of domains 1 and 2 to carbohydrates on the cell surface promotes association of domain 3, followed by its insertion into the membrane [117].

The structure of CEL-I/GalNAc complex was solved by the molecular replacement method using native CEL-I as a search model. Recognition of GalNAc by CEL-I is basically done through coordinate bonds between 3- and 4-OH of the sugar and one of the two Ca^{2+} ions in the CEL-I protomer as well as hydrogen bonds of these hydroxyl groups with Gln101, Asp103 and Asn123. In addition to these bonds, high affinity binding of GalNAc to CEL-I is achieved by formation of two hydrogen bonds between the side chains of Arg115 and carbonyl oxygen of the acetamido group of GalNAc [113].

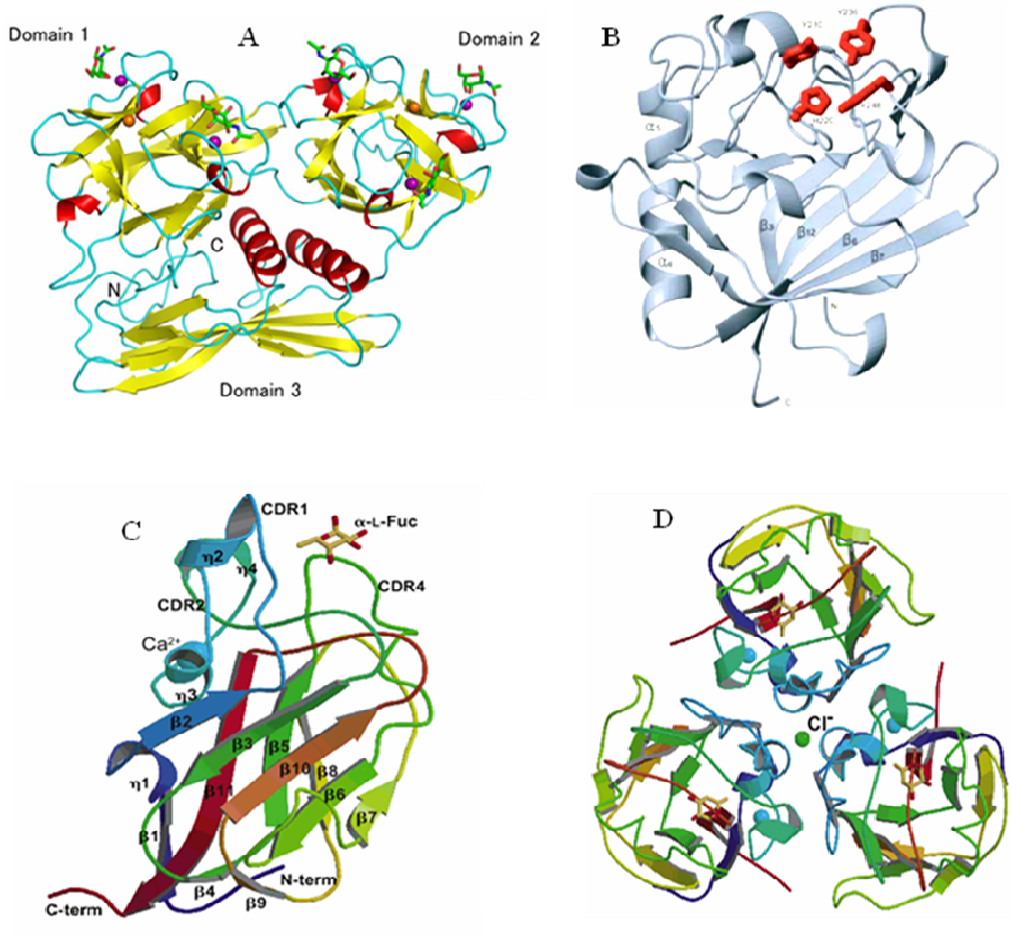

Fig 1.2. Crystallographic structures of common C-type lectins. A. CEL-III from *Cucumaria echinata*, B. FBG domain of TL5A from *Tachypleus tridentat*us complexes with Fuc. C. AAA lectin from *Anguilla anguilla*. D, Physiological trimer of AAA.

AAA folds as a β-barrel with jellyroll topology. The bulk of the fold consists of eight major antiparallel β-strands arranged in two β-sheets of five (β2, β3, β10, β6 and β7) and three (β11, β5 and β8) strands packed against each other. Two short antiparallel strands (β4 and β9) close one end of the barrel. The *N*- and *C*-terminal strands (β1 and β11) protrude 15 Å from this end of the barrel, forming an antiparallel two-stranded β-sheet. Five loops connect the two main β-sheets at the other end of the barrel; are the complementarity determining regions (CDR) 1−5 by analogy to the immunoglobulin. The CDR loops encircle a heavily positively charged hollow that binds the carbohydrate. These loops are spanned by the following residue ranges: Gln 22−Ser 31 (CDR1), Arg 41−Cys 50 (CDR2), Ser 53−Asn 58 (CDR3), Arg 79−Ala 90 (CDR4) and Pro 138−Ser 142 (CDR5).

The crystal structure of the AAA complex with fucose showed the bound fucose resting on a highly positively charged depression encircled by the CDRs. AAA recognizes the fucose ring O5 and 3-OH and 4-OH hydroxyls using the guanidinium of His 52 and the Nε groups of Arg 79 and Arg 86. A network of hydrogen bonds maintains this triad of residues in optimal positions to provide all the polar interactions to the carbohydrate: His 144 interacts with Arg 86, the main chain oxygen of Ser 53 interacts with His 52 and Asp 81 interacts with Arg 79 to form a salt bridge. The triad of residues at the center of the binding site forms hydrogen bonds with the fucose axial 4-OH with perfect tetrahedral geometry. Completing the list of polar interactions between AAA and the fucose, Arg 79 hydrogen bonds to O5 and Arg 86 hydrogen bonds to the equatorial 3-OH. The network of hydrogen bonds, together with one of the disulfide bridge (Cys 82−Cys 83) makes CDR4 a rigid and wide loop [118].

Crystal structure of tachylectin-2, a GlcNAc/GalNAc binding lectin is the first example of a 5-fold β-propeller structure. These 5 β-sheets are arranged in consecutive order and with 5-fold pseudosymmetry around a central tunnel. Each twisted β-sheet is built by four antiparallel β-stands with a W-like topology. Lectin in complex with GlcNAc reveals five quasi-

equivalent binding sites, with virtually identical occupancy and geometry in the crystal. The binding sites are all located between the connecting segment of adjacent β-sheets and the 3-4 loop of the following β-sheet, perfectly reflecting the 5-fold symmetry of lectin [92].

Biological role

Lectins, due to their ability to recognize different structures, constitute an important element of biological system. Their physiological role is connected with the identification of glycosylated structures at the level of cells, tissues and the whole organisms.

1. Involvement in animal defense mechanism

Unlike vertebrates, invertebrates are believed to lack adaptive immunity and to rely completely on their innate immune system including a set of humoral and cellular immune reactions. Recognition of non-self materials in the innate immune system is mediated by a group of proteins named pattern recognition proteins (PRPs), which recognize and bind to different molecules on the surface of invading microorganisms. Accordingly, these surface molecules are designated as pathogen-associated molecular patterns (PAMPs). Binding of PRPs to PAMPs triggers a series of immune responses, leading to the activation of the host-defense system. PRPs include lectins, lipopolysaccharide (LPS)-binding proteins, peptidoglycan-binding proteins, and β-1,3-glucan-binding proteins, and so on [119, 120]. Lectins have been known to play a crucial role in non-self recognition and clearance of invaders in invertebrate immunity [121, 122]. Some lectins from invertebrates were reported to be involved in various biological responses, for instance promotion of phagocytosis [123-125], antibacterial activity [126-128], activation of the proPO system [129].

2. Cell adhesion and growth promotion

The lectins, *via* acting directly on carbohydrates by their specific binding sites, can help in attachment of different cells. A lectin from *Didemnum ternatanum* (DTL) was found to increase the attachment of molluscan, and

echinoderm cells and to result in intensive cell spreading and an alteration of cell shape only at the gastrula stage, when the genes responsible for differentiation began to work. In addition, it was shown to stimulate the growth or the differentiation of cultivated marine invertebrate cells depending on the stage of embryonic development at which primary cell cultures were obtained.

The ability of DTL to interact with collagen (type I); this interaction is inhibited by Arg–Gly–Asp (RGD)-peptide and peptides obtained from collagen. It is possible, that the collagen-binding site or RGD-sequence may be important for specific adhesive activity of DTL for embryonic cells. Earlier, RGD-sequence, which has been assumed to be a cell binding signal in cell-adhesive molecules [130], was found in echinoidin, a C-type lectin from the coelomic fluid of the sea urchin [67]. Echinoidin-induced cell adhesion was only inhibited by peptide GRGDS [45], suggesting that human cancer cells adhered to the echinoidin-coated substrate only through the RGD sequence.

Glycoproteins may have a role not only as adhesion molecules but also as growth-promoting molecules with a role of bio-regulators [131-133]. Mitogenic lectins from tissues of sponges were described earlier for human peripheral blood lymphocytes [134, 135] and spleen lymphocytes from mice [136]. Moreover, several lectins and a mussel matrix protein contains EGF-like domains [137, 138]. In addition to adhesive activity, DTL has some characteristics of a growth factor stimulating the DNA synthesis in embryonic cells of sea urchins and mussel cells. The most intensive stimulation of the DNA synthesis was observed in the cell cultures obtained at the blastula stage when the adhesive effect of DTL was minimal. It is interesting that the other lectin, Con A, was shown to stimulate the DNA synthesis in only non-adherent cells of axial organ from the starfish *Asterias rubens* [139].

3. Settlement and metamorphosis of invertebrate larvae

Lectins present on the invertebrate larvae recognize a specific carbohydrate molecule from the microbial biofilm inducing settlement. Glucose present on the *Janua brasiliensis* helps in settlement of molluscs [140].

Applications

The ability of lectin to interact with simple, aminated, acetylated, sialylated and complex carbohydrates has been exploited for typing blood cells, carrier for chemotherapeutic agents, mitogens, fractionation of animal cells, or for studying cellular surfaces. They have also been used for the identification and differentiation of various microorganisms, also epidemiologic as well as taxonomic markers of specific microorganism [141, 142].

1. Identification of different strains of microorganisms

The lectin specifically binds to the sugar present on the cell surface, this property made the lectin as a marker tool for identification and classification of several microorganisms. Agglutinating extracts from several marine lectins have been used for the taxonomic study of protozoa in the genera *Crithidia, Blastocrithidia, Leishmania* and *Trypanosoma* [61]. A lectin from marine molluscs can distinguish serotypes and biotypes of *Vibrio cholerae* [143].

2. Lectin-parasite interactions

Lectins have been valuable tools for studying the insertion, fate, distribution and function of glycoconjugates on and in parasites; for example, lectins are useful in defining various developmental parasite cycles. A lectin from *Cliona varians* (CvL), galactose specific lectin binds to the galactose on the *L. chagasi* parasite cell surface [61]. Parasite lectin specificity was studied in detail in *Trypanosomatide* with respect to its lectin receptors [144], like 1B 4 of *Griffonia simplicifolia* has been utilized to define terminal β–galactosyl-bearing glycoconjugates on *T. cruzi*, *Leishmania braziliensis* and *L. mexicana* [145]. This is important because

of the presence of anti-galactose antibodies that are found in increased amounts in the sera of patients infected with these parasites.

3. Isolation and purification of serum glycoconjugates

Immobilization of lectin to an unreactive support also allows separation of glycoconjugates possessing lectin-specific glycan structures. The biological importance of oligosaccharide sequences in many different settings is undeniable. Glycan histochemistry has brought together the histological and biochemical approaches and provided insight into the mutual importance of both approaches [146].

4. Separation of cells

The property of lectin to differentiate surface molecules can be used for the separation of certain cell types either from complex media or from a mixture of different cell categories by exploiting difference in surface sugars. The cell sorting, using lectin was first attempted by Nowell [25] to separate red cells from white in human blood using phytohemagglutinins that led to the discovery of the lymphocyte-modifying properties of bean lectin. The lectin is immobilized on inert supports, in columns or magnetic bead followed by passing of the mixture over beads. Another approach is to exploit the toxic properties of lectins, whereby binding to specific sites causes cell death, only cells devoid of such sites surviving the treatment [142]. The property of specificity for glycoconjugates made the lectins to select and complex with microbial cell surface molecules to employ as a probes and sorbents for whole cells, mutants and numerous cellular constituents and metabolites.

5. In cancer research

Cancer cells exhibit altered specific properties such as loss of contact inhibition and ability to migrate and form metastasis. Much work has been done to understand the modifications occurring in membrane glycoconjugates, glycoproteins and glycolipids during cancer induction. Modification of glycoconjugates also includes the emergence of antigens

that do not normally belong to the cells of the tissues concerned, or which are present in the membranes of the embryo cells and are then repressed in differentiated cells. These antigenic neo-expressions are generally linked to impairment of glycosyltransferase and glycosidase activities, and in some cases concern blood group antigens. A lectin specific for D-Gal β1-3 D-GalNAc can be used to follow the expression of the T antigen (Thomsen-Friedenreich antigen) in the same way as the lectin of peanut *(Arachis hypogaea)* [147]. The residue, D-Gal β1-3 D-GalNAc in cancerous cells results from an improper sialylation in the glucid part of M or N blood group antigens and related structures [142]. Two marine lectins, *Bryothamnion seaforthii*-BSL and *Bryothamnion triquetrum*-BTL can recognize carbohydrate antigens of the human colon carcinoma cell variant (EB3) like fucosilate glycans structures around the cell surface and that their affinity was stronger. Preliminary results obtained by the use of BSL and BTL with another human colon carcinoma cell variants have shown that both lectins may be used to discriminate routes of metastatic propagation and for diagnostic propose [148].

6. Sorting of mutant and tumor cells

This technique involves toxic property of the lectins. In case of non-toxic lectins, chain A of ricin (effectomer) or diphtheria toxin can be attached to them to render toxicity. Another approach is to treat the cells, after lectin binding with an anti-lectin antibody and complement. The cell sorting has been applied in two separate areas: the selection of modified cell lines and the sorting of cells derived from artificially induced mutations. The selection of such mutant cells involves alteration in glycan chain of the surface glycoconjugates. Such alterations are caused by mutations affecting enzymes, such as glycosyltransferases and glycosidases or enzymes involved in the synthesis of intermediates such as nucleotide-sugars or lipid-bound sugars. In another approach, from ‘wild’ phenotype (lectin sensitive) to resistant lectin are created. In this way it is possible to select a wide range of mutants using a large number of lectins, also

identical mutants can be sorted out using lectins with different specificities. The main value of this selection is to understand structure-activity relations, since non-binding of the lectin implies that an enzyme is missing or functionally impaired [147].

The other area of application is the detection, in a population of cells modified by oncogenesis of lines displaying resistance to particular lectins. It is possible to investigate how modifications to particular glycan structures affects the tumor-forming capacity of the cells and their metastatic potency [142].

FEW IMPORTANT MARINE INVERTEBRATE LECTINS

Lectins from phylum Echinodermata

Few lectins have been isolated from different parts of sea cucumber, starfish, sea urchin etc., and showed their involvement in either animal's defense or their economical importance like, antimicrobial, antiviral and anticancer agents.

Sea cucumber lectins

Cucumaria echinata

Four Ca^{2+}-dependent, GalNAc/galactose-specific lectins were purified from the marine invertebrate, *Cucumaria echinata* (Holothuroidea) by column chromatography on lactosyl-Sepharose 4B, Sephacryl S-200, and Q-Sepharose. The molecular masses of these lectins were estimated to be 27 kDa (CEL-I), 35 kDa (CEL-II), 45 kDa (CEL-III), and 68 kDa (CEL-IV) on SDS-PAGE under non-reducing conditions [50]. Among these lectins**,** CEL-I and CEL-IV strongly agglutinated rabbit and human erythrocytes, and were found to recognize GalNAc and Gal-containing carbohydrates from the results of a hemagglutination inhibition assay. In contrast, CEL-II failed to agglutinate any erythrocytes tested, although its carbohydrate-binding ability was confirmed by a carbohydrate-binding assay involving asialofetuin-horseradish

peroxidase. CEL-I and CEL-IV were found to have apparent homology with C-type CRDs, like the other invertebrate Ca^{2+}-dependent lectins whose primary structures have been determined. Therefore, CEL-III can also be expected to belong to the C-type lectin family because of the Ca^{2+}-dependent nature of its hemolytic and hemagglutinating activity.

CEL-I

Lectin is composed of two identical subunits held by a single disulfide bond. The complete amino acid sequence of CEL-I was determined by sequencing the peptides produced by proteolytic fragmentation of *S*-pyridylethylated CEL-I. A subunit of CEL-I is composed of 140 amino acid residues. Two intra-chain (Cys3-Cys14 and Cys31-Cys135) and one inter-chain (Cys36) disulfide bonds were also identified from an analysis of the cystine-containing peptides obtained from the intact protein. The similarity between the sequence of CEL-I and that of other C-type lectins was low, while the C-terminal region, including the putative Ca^{2+} and carbohydrate-binding sites, was relatively well conserved. When the carbohydrate-binding activity was examined by a solid-phase microplate assay, CEL-I showed much higher affinity for GalNAc than for other galactose-related carbohydrates. The association constant of CEL-I for *p*-nitrophenyl *N*-acetyl-β-D-galactosaminide (NP-GalNAc) was determined to be 2.3×10^4 M^1, and the maximum number of bound NP-GalNAc was estimated to be 1.6 by an equilibrium dialysis experiment [107]. The crystal structures of CEL-I and its complex with GalNAc was studied, the acetamido group of GalNAc appeared to be recognized more strongly by the combination of hydrogen bonds to Arg^{115} and van der Waals interaction with Gln^{70}. Mutational analyses, in which Gln^{70} and/or Arg^{115} were replaced by Ala, confirmed that these residues contributed to GalNAc recognition in a cooperative manner [149].

CEL-III

The hemolytic activity of CEL-III was also Ca^{2+}-dependent and was found to be inhibited by Gal/GalNAc-containing carbohydrates, suggesting that the hemolysis was caused by CEL-III binding to specific carbohydrates on the

erythrocyte membrane by Ca^{2+}-dependent lectin activity, followed by partial destruction of the membrane. The hemolytic activity of CEL-III increased with increasing pH from neutral to 10, but almost no hemolysis was observed below pH 6.5. When erythrocytes were incubated with CEL-III in the presence of dextran with molecular masses greater than 4 kDa, lysis was impeded considerably, while a concomitant release of ATP was detected from these osmotically protected cells. It was found that CEL-III released carboxyfluorescein from artificial globoside-containing lipid vesicles, and it is suggested that CEL-III is a novel pore-forming protein with the characteristics of a Ca^{2+}-dependent lectin, which may act as a toxic protein to foreign microorganisms [106]. The addition of CEL-III to sensitive MDCK cells pre-incubated with 8-anilino-l-naphtha-lenesulfonate (ANS) caused an increase in the fluorescence intensity of the probe. The oligomerization may be accompanied by an enhancement of the hydrophobicity of CEL-III molecules, which in turn provides new ANS-binding sites. The difference in susceptibility of MDCK and CHO cells to CEL-III cytotoxicity may be due to a difference in oligomerization of bound CEL-III [150].

CEL-IV

The complete amino acid sequence of a Ca^{2+}-dependent lectin, CEL-IV showed that, it comprises 157 amino acid residues with a molecular mass of 17,098 Da (without disulfide bonds). From comparison with other proteins, CEL-IV was apparently homologous with the C-type lectin family. The identity was relatively high with a sea cucumber (*Stichopus japonicus*) lectin SJL-I (40%) and a sea urchin (*Anthocidaris crassispina*) lectin echinoidin (32.6%). In CEL-IV, one inter-chain and two intra-chain disulfide bonds were identified. Interestingly, one of the two intra-chain disulfide bonds that were highly conserved among the other C-type lectins was missing, suggesting that this might be a characteristic feature of C-type lectins in the Holothuroidea [108].

Cucumaria japonica

A new 44-kDa, C-type mannan-binding lectin (MBL-C) consisting of two identical subunits was isolated from the coelomic fluid of the holothurians, *Cucumaria japonica*. The lectin was effectively inhibited by highly branched mannans similar in structure to yeast mannans and composed of α(1-2) and α(1-6) bound D-Man residues. The lectin reaction depends on Ca^{2+}-concentration: maximum activity of MBL-C is observed at 10 mM Ca^{2+}. The activity of MBL-C increases in the pH range from 5 to 7 and reaches maximum at pH 7.0. Carbohydrate specificity, Ca^{2+}-dependence, and amino acid composition indicate that MBL-C belongs to the C-type mannan-binding lectins. Polyclonal antibodies against MBL-C revealed its immunochemical similarity to a mannan-binding lectin from another holothurian species, *Stichopus japonicus*; this provides evidence for structural homology between these proteins [151].

Apostichopus Japonicus

A new C-type lectin (CTL) specific for high-mannose glycans (MBL-AJ) was isolated from the coelomic plasma of the holothurians, *Apostichopus japonicus.* MBL-AJ has oligomeric forms with identical 17-kDa subunits on SDS-PAGE. Among natural ligands, lectin hemagglutination activity was competitively inhibited by extracellular low-branched, but not high-branched, α-D-mannans isolated from marine halophilic bacteria and composed of α(1-2) and α(1-6) linked D-mannose residues. The activity of the lectin was Ca^{2+}-, pH-, and temperature-dependent. MBL-AJ cDNA was cloned from a holothurian coelomocyte cDNA library. The subunit of the mature protein has 159 amino acids and a single carbohydrate-recognition domain (CRD) of CTL. More interesting finding was cross-reactivity of MBL-AJ and human serum MBL detected by the antibody against MBL-AJ. Taking into consideration such MBL-AJ peculiarities as its carbohydrate specificity, the presence of a conserved region forming the mannose-binding site, common antigenic determinants with human MBL, and participation in defense reactions, it is

possible that MBL-AJ belongs to the family of evolutionary conserved mannan-binding proteins.

Stichopus japonicus

Two structurally distinct lectins were purified from the coelomic plasma of holothurian, *Stichopus japonicus*, by affinity chromatography on a porcine stomach mucin-conjugated agarose column. The two lectins showed apparent molecular masses of about 400 kDa (SPL-1) and 60 kDa (SPL-2) on gel filtration, but about 17 kDa on SDS-PAGE under reducing conditions. Both lectins showed hemagglutination activity toward rabbit erythrocytes in the presence of Ca^{2+} ions. The *N*-terminal amino acid sequences were highly homologous to but distinct from those of a Ca^{2+}-dependent (C-type) lectin named SJL-I purified from the same species. In addition to porcine stomach mucin, the hemagglutination activity of SPL-1 was strongly inhibited by uronic acids such as galacturonic acid, and glucuronic acid, while the activity of SPL-2 was inhibited by GalNAc and galactosides [152].

Two more distinct Ca^{2+}-dependent lectins were purified from the sea cucumber *Stichopus japonicus* by affinity chromatography on lactosyl-sepharose 4B and ion-exchange chromatography on Q-sepharose. Their molecular masses were estimated to be 13 kDa (SJL-I) and 15 kDa (SJL-II) on SDS-PAGE. SJL-I agglutinated rabbit erythrocytes as well as human A, B, and O-type erythrocytes, but SJL-II agglutinated only rabbit erythrocytes. Hemagglutination by SJL-I was competitively inhibited by GalNAc and galactose-containing carbohydrates. SJL-II was activated at ten times lower Ca^{2+}-concentration than SJL-I. Both lectins lost activity in acidic pH, while SJL-I appeared more stable down to pH 4.5 [153]. SJL-I consists of 143 amino acid residues and comparison of the sequence revealed that SJL-I exhibits apparent homology with C-type lectins, especially with those of marine invertebrates. The highest homology (identity 28.6%) was found with echinoidin [109].

Holothuria polii

Hemagglutinin and hemolysins are isolated from coelomic fluid of *Holothuria polii*. After injection of formalinized sheep erythrocytes into the coelomic cavity the activity of the naturally occurring hemagglutinins remained constant, while the hemolysin level rose over an eight day period. The kinetics of the response was the same after a further injection, although the hemolytic titers reached higher levels over a longer period. Results obtained using rabbit erythrocytes indicate that this response can be considered a secondary one: higher titers were demonstrated over a 24 h period [65].

Holothuria scabra Jaeger

A lectin isolated and purified by gel chromatography from the internal organs of *Holothuria scabra* Jaeger with 355 kDa mass under native and having 5 subunits. It agglutinated all blood types of human blood as animal erythrocytes. The activity was trypsin, calcium ions, pH and temperature dependent [154]. The lectin was found to have hemolytic and mitogenic activities. It was also found to be a good inhibitor of seed germination and inhibited the germination of radish seeds. LC_{50} against *Artemia salina* is lower than commercial therapeutic agents [155].

Sea urchin lectins

Toxopneustes pileolus

A novel sea urchin lectin-1 (SUL-1) isolated from the large globiferous pedicellariae of *T. pileolus*. Dendritic cells (DC) play a pivotal role in controlling immune responses. The expression levels of CD10, CD80, CD83, CD86 and HLA-DR as expressed by mean fluorescence intensity (MFI) on DC differentiated from immature DC after culture with 1.0 μg/ml of SUL-1 for 1 day were decreased endocytic activity. SUL-1-treated DC also displayed enhanced T-cell stimulatory capacity in an MLR, as measured by T-cell proliferation. Cell surface expression of CD80, CD83 and CD86 on SUL-1-treated DC was inhibited by anti-DC-SIGN mAb, while anti-DC-SIGN mAb had no influence on allogeneic T-cell proliferation by SUL-1-treated DC. DC

differentiated with SUL-1 induced the differentiation of naïve T cell towards a helper T cell type 1 (Th1) response at DC/T (1:5) cells ratio depending on IL-12 secretion. In CTL assay, the production of IFN-γ and ^{51}Cr release on SUL-1-treated DC were more augmented than of immature DC or LPS-treated DC [156].

Toxopneustes pileolus

A new sea urchin lectin was isolated from *Toxopneustes pileolus*, is Gal-, Fuc-specific. Incubation of rat peritoneal mast cells with the lectin in the presence of 0.3 mM $CaCl_2$ for 10 min significantly and dose-dependently inhibited the histamine release induced by GlcNAc-specific *Datura stramonium* agglutinin (DSA), an activator of the Gi-protein-dependent pathway in mast cells. This inhibition of sugar-specificity was reversed in the presence of Gal or D-Fuc but not L-Fuc. It is suggested that the lectin binds to Gal residues of DSA to interfere with mast cell activation induced by DSA, a glycoprotein with arabinose and Gal residues [157].

Anthocidaris crassispina

The complete amino acid sequence of echinoidin, a lectin from the coelomic fluid of the sea urchin *Anthocidaris crassispina*, has been determined by sequencing the peptides obtained from tryptic, *Staphylococcus aureus* V8 protease, chymotryptic, and thermolysin digestions [158]. Echinoidin is a multimeric protein consists of a total of 147 amino acid residues and one carbohydrate chain attached to Ser38. Each polypeptide chain contains seven -cystines, and six of them form three disulfide bonds in the single polypeptide chain (Cys3-Cys14, Cys31-Cys141, and Cys116-Cys132), while Cys2 is involved in an inter-polypeptide disulfide linkage. From secondary structure prediction the protein appears to be rich in beta-sheet and beta-turn structures and poor in alpha-helical structure. This COOH-terminal region of echinoidin is also homologous to the central portion of the lectin from the flesh fly *Sarcophaga peregrina*. Moreover, echinoidin contains an Arg-Gly-Asp sequence which has been proposed to be a basic functional unit in cellular recognition proteins [67].

Lytechinus variegates

A Gal specific carbohydrate binding protein, named echinonectin has been identified in eggs and embryos of the sea urchin, *Lytechinus variegates*. This lectin has been described as a cell-substrate adhesion protein functioning during embryonic development. It is 220 kDa in size and exists as dimer. The lectin specificity was studied by competition assays, specific for Gal and Fuc and higher affinity for polymers of Gal or Gal sulfate such as carrageenan [62].

Echiuran, *Urechis unicinctus*

Two lectins specific for Gal with molecular weight of 31 kDa and 34 kDa were purified from coelomycetes of the echiuran, *Urechis unicinctus*. They were eluted together from a Biogel P-100 column as a single peak with an apparent molecular weight of 35 kDa. They closely resemble in their sugar specificity, pI and electrophoretic mobility under nonreducing conditions. Hemagglutination activity of both lectins was inhibited by a glycoconjugates fraction obtained from the pronase digest of coelomycetes [84].

Star fish lectin

Asterina pectinifera

A novel GalNAc specific lectin from starfish, *Asterina pectinifera* was purified, characterized, and cDNA cloning was done. The purified lectin showed 19-kDa, 41-kDa, and 60-kDa protein bands on SDS–PAGE, possibly corresponding to a monomer, homodimer, and homotrimer. The hemagglutination activity of the lectin was specifically inhibited by GalNAc, Tn antigen, and blood group A trisaccharide, but not by GlcNAc, galactose, galactosamine, or blood group B trisaccharide. The lectin was found to bind Gb5Cer, but not Gb4Cer, Gb3Cer, GM1a, GM2, or asialo-GM2, indicating that the lectin specifically binds to the terminal α-GalNAc at the nonreducing end. The hemagglutination activity of the lectin was completely abolished by chelation with EDTA or EGTA and completely restored by the addition of $CaCl_2$. cDNA cloning of the lectin showed that the protein is composed of 168

amino acids, including a signal sequence of 18 residues, and possesses the typical C-type lectin motif. These findings indicate that the protein is a C-type lectin. The recombinant lectin, showed binding activity for asialomucin in the presence of Ca^{2+} but no hemagglutination [58].

Tunicate lectins

Polyandrocarpa misakiensis

A lectin was isolated from the homogenate of the tunicate, *Polyandrocarpa misakiensis* and it is a monomeric protein with a molecular mass of approximately 15 kDa. The lectin bound to an immobilized Gal column in the presence of calcium ion with a threshold of 500 μM and eluted completely with 5 mM EDTA. *Polyandrocarpa* lectin was found to be a calcium-dependent galactose-binding lectin. The lectin composed of 125 residues, contains no carbohydrate group. The lectin contains four-cystines, Cys-21, Cys-119 and Cys-96, Cys-111 form intra-chain disulfide bridges, respectively. The amino acid sequence of lectin shows 20-30% homology with those of fly, barnacle, sea urchin, and several vertebrate lectins that belong to C-type lectin. Although the physiological role of *Polyandrocarpa* lectin is not clear, preliminary experiments suggest that the lectin may be related to defense mechanisms because it has a strong antibacterial activity [57].

Styela plicata

A C-type lectin from the tunicate, *Styela plicata*, that can stimulate tunicate and mammalian cell proliferation, activate phagocytosis, increase interleukin 2 (IL-2) secretions by mammalian peripheral blood mononuclear cells and enhance IL-2 receptor (IL-2R) expression by mammalian EL-4 was isolated and purified. Partial amino acid sequence data showed that the *S. plicata* protein resembles three C-type lectins (TC14, TC14-1 and TC14-2) from a closely related tunicate species, *Polyandrocarpa misakiensis*. Its similarity to CRDs from *P. misakiensis* lectins suggests that the *S. plicata* protein modulates the activities of mammalian immune-competent cells by interacting with carbohydrate moieties of glycosylated cell surface receptors [159].

Another 90 kDa lectin from *Styela plicata* was isolated and purified. The 90 kDa lectin was localized within a single type of hemocyte (morula cells), but was secreted from those cells when tunicates were challenged with the inflammatory elicitor, zymosan. Functional studies showed that, it binds to galactose-based sugars in a divalent cation-dependent manner. Amino acid composition analysis and *N*-terminal amino acid sequencing indicated that it is related to a collagenous lectin from *S. plicata*, splic43. However, peptide mass fingerprinting identified numerous differences between the two proteins. This suggests that the 90 kDa molecule represents a novel protein that is involved in host defense [94].

Didemnum candidum

The affinity-purified major galactosyl-specific lectin (DCL-I) from the tunicate *Didemnum candidum* binds and induces blastogenesis in untreated and *Vibrio cholerae* neuraminidase (VCN)-treated murine splenocytes. Binding and stimulation of VCN-treated splenocytes and thymocytes is specifically inhibited by Gal. Binding of lectin to splenocytes is highly increased by the VCN treatment, this suggests that additional DCL-I carbohydrate acceptors are uncovered by VCN treatment of the splenocytes, the ones responsible for triggering blastogenesis are already exposed on the untreated cells [160].

Didemnum ternatamim

A GlcNAc specific lectin was purified by affinity-chromatography and characterized from the ascidian, *Didemnum ternatamim* (DTL). Native molecular weight was 28 kDa and its hemagglutination is independent of any divalent cations. GlcNAc, di- and tri-acetyl chitobiose were the most potent inhibitors. Hapten inhibition with various sugars indicated that DTL require the presence of OH groups at the C-3 and C-4 of GlcNAc. The presence of the hydroxyl *p*-nitrophenyl aglycone significantly enhanced inhibitory power of GlcNAc. Among glycoproteins ovomucoid and ovalbumin were the best inhibitors. Using UV difference spectroscopy binding constants were calculated. CD analysis suggest that 4% α-helix, 57% β-sheet, 24% β-turn and

15% unorder [71]. DTL was found to stimulate the growth or the differentiation of cultivated marine invertebrate cells depending on the stage of embryonic development at which primary cell cultures were obtained [96].

A new GlcNAc/GalNAc-specific lectin DTL-A from the same ascidian was isolated and purified. DTL-A binds to BSM, asialo-BSM as well as heparin and dextran sulfate. The binding of DTL-A to BSM was inhibited by monosaccharides GlcNAc and GalNAc, their α-but not β-anomers. Among polysaccharides and glycoconjugates, DTL-A binding to BSM was effectively inhibited by BSM, asialo-BSM, pronase-treated BSM and synthetic α-D-GalNAc-PAA. Fetuin and asialofetuin showed a much lower inhibitory potency, heparin and dextran sulfate were non-inhibitory. On the other hand, DTL-A binding to heparin was effectively inhibited by dextran sulfate, fucoidan, whereas BSM showed insignificantly inhibitory effect. DTL-A binding to heparin was not inhibited by GlcNAc and GalNAc [161].

Halocynthia roretzi

Galactose-specific lectin isolated from the hemolymph of solitary ascidian, *Halocynthia roretzi*, has been further characterized. The hemagglutinating activity of the lectin is Ca^{2+}-dependent. The lectin shows a stimulatory effect on the superoxide anion production by guinea-pig polymorphonuclear leukocytes, and the effect is inhibited strongly by melibiose [69]. The *H. roretzi* plasma lectin is a single-chain protein consisting of 327 amino acids and four disulfide bonds, one of which was found to be cross-linked intra-molecularly. A comparison of the amino acid sequence of the *H. roretzi* plasma lectin with the sequences of other proteins reveals that it has a structure consisting of a twice-repeated sequence, a fibrinogen-related sequence and a C-type lectin-homologous sequence. The deduced amino acid sequences in the three cDNA clones contain the same sequence of the mature lectin molecule and the same putative signal sequence. In addition, it was demonstrated that this lectin can enhance phagocytosis by *H. roretzi* hemocytes. Thus, the plasma lectin is constructed into an oligomer structure

via intermolecular disulfide bonds and plays a role in the biological defense of *H. roretzi* as a defense molecule [68].

Shrimp lectin

Litopenaeus schmitti

The agglutinating activity of the hemolymph of *Litopenaeus schmitti* is insensitive to calcium and specific for acetylated sugars, particularly sialic acid (Neu5Ac) and *O*-sialoglycoconjugates (bovine submaxillary mucin) and has varying specificity for different LPS, which may suggest a putative role in microorganism recognition. Affinity chromatography on fetuin–agarose of the agglutinin resulted in a 220 kDa band (lectin), and an 82.5 kDa band, which probably is hemocyanin. The 220 kDa protein consists of 31 and 34 kDa subunits, suggesting that this lectin is multimeric. The lectin molecular mass was estimated by gel filtration to be 153±10 kDa. The hemolymph of *L. schmitti* comprises at least another soluble lectin, with distinct chemical and carbohydrate specificity than the 220 kDa lectin [162].

Present Investigation

Sea cucumbers are members of the phylum Echinodermata and relatives of the sea stars and sea urchins. All echinoderms are marine and are bottom-dwellers. Sea cucumbers differ from other echinoderms in having an elongate, cylindrical body with the mouth and anus at opposite ends. Sea cucumbers either gather detritus (bits of plant and animal matter) and bacteria from seafloor sediments or filter particles from the water. They lack spines and the protection of a rigid skeleton, but they have many different means of defense from predators. With soft, flexible bodies, they are able to crawl under rocks and into reef crevices. Some species have repellant or toxic chemicals in the skin that makes them distasteful. Others eject sticky threads (cuvierian tubules) from the anus, these threads entangle and immobilize potential predators and the animal crawls to safety. Still other sea cucumbers can eject (eviscerate) part of the digestive system as a defensive mechanism, regenerating lost parts later. These animals are having very little intestinal

bacterial population, we wanted to find out its defense mechanism against different bacterial population, and to isolate bioactive compound from coelomic fluid. Upon bacterial challenge, we had observed increase in the basal level of a protein which is showing hemagglutination, broad spectrum antibacterial activity and bacterial agglutination. Hence the objective of the present investigation was to isolate, purify and characterize this lectin and to study its structure-function relationship. Its binding studies were carried out with different ligands using spectrofluorimetry and SPR, which provides vital information regarding subtle nature of specificity. Structural organization of the lectin was studied using trypsin digestion and conformational stability was also determined for different chemical and physical chaotropes.

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CHAPTER : 2

INDUCTION, PURIFICATION AND CHARACTERIZATION OF HSL

SUMMARY

In invertebrates, the mechanism evolved to maintain their body immunity and integrity includes cellular and humoral components. Both these factors respond to different antigens such as microorganisms, vertebrate erythrocytes and foreign proteins. In this chapter, we report a detailed study of a lectin involved in immune response in the echinoderm, sea cucumber (*Holothuria scabra*). Correlative studies indicate that the expression of this defensive lectin is induced by bacterial challenge. Fractional purification using hydrophobic interaction chromatography identified the lectin activity to a monomeric glycoprotein of a mass 182 kDa. Under in vitro conditions, purified *Holothuria scabra* lectin (HSL) could mediate agglutination of the test bacteria, thereby indicating a possible mode of action in physiological situation. HSL was highly thermostable and tolerates extreme pH conditions (2-11). Spectroscopic studies indicated the presence of calcium, magnesium and copper metal ions in the protein; however, biochemical studies reveal that presence of divalent cations do not influence hemagglutination activity. Hemagglutination inhibition studies indicate specificity for MeαGal, T-antigen and desialylated glycoproteins, asialo fetuin and asialo fibrinogen.

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]. The microbial load in natural marine habitat can number up to 10^6 bacteria and 10^9 virus·ml^{-1} of seawater [2]. 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 [3]. 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 [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 results strongly support the contention that invertebrates possess "immune-like" defense mechanisms.

A large number of lectins have been isolated and characterized from marine sources, some of them are from sponges [6-9], tunicates [10-13], crustaceans [14-16], and molluscs [17]. However very few lectins are reported in phylum echinodermata in comparison with those of other marine invertebrates, including few lectins from sea urchin, a sialic acid binding lectin belonging to heat shock protein family [18], endogenous echinonectin from *Lytechinus variegates* [19], Lectin from *Asterina pectinifera* [20] and the lectin involved in the complement homologous immune system [21].

Sea cucumbers are echinoderms inhabitating relatively shallow coastal areas experiencing little or no currents. Some lectins have been isolated from different species of sea cucumbers. Four Ca^{2+} dependent, Gal/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, the nature of interaction of echinoderm lectin and bacterial cells, and their significance in innate immunity has not been elucidated in detail. In this chapter, we report our studies on a defensive lectin, named HSL, its induction, purification and characterization from the coelomic fluid of the sea cucumber, *H. scabra*, and its interaction with both gram positive and gram negative human pathogenic bacteria and demonstrate the involvement of HSL in innate immunity.

MATERIALS

D-glucose, D-mannose, D-galactose, D-galactosamine, 2-deoxy-D-galacose, L-fucose, methyl α–D-galactose, methyl β–D-galactose, D-maltose, α-lactose, α-D-mellibiose, raffinose, stachyose, phenyl-sepharose CL-4B, pronase-E, carboxypeptidase, aminopeptidase, fetuin (bovine), fibrinogen (human), thyroglobulin (bovine), phenylmethylsulphonylfluoride, 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; all other chemicals used were of analytical grade.

METHODS

Collection of sea cucumber animals

Adult sea cucumbers (*H. scabra*) were collected from Anjuna coast of Goa, India and maintained in circulating seawater for at least 3-4 days. During exposure animals were kept in fiber-plastic tanks containing 50 L seawater; with two changes of water everyday, care was taken to ensure that the animals were not much disturbed during the experiment. Except for the treatment under consideration, all other general environmental conditions were similar (salinity of seawater, 35 ± 5 PPT and temperature 28 ± 2 ^{0}C).

Immune challenge and preparation of coelomic fluid

Bacteria used in this experiment are human isolates *Staphylococcus citreus* and Group D *Streptococci* (gram positive), *Escherichia coli*, *Klebsiella pneumonia*, *Serratia marganii*, *Pseudomonas aeruginosa* and *Proteus vulgaris* (gram negative) obtained from Goa Medical College, Goa, India. Marine isolates *Vibrio cholerae*, *Shigella* sp. (gram negative) and *Bacillus* sp (gram positive), were isolated in the lab. All bacterial cultures were grown in nutrient broth, cells were harvested separately by centrifugation at 6,000 g for 8 min, washed thrice, and resuspended in brine saline (0.85% NaCl, w/v). Bacterial count was estimated by serial dilution method, to know the exact amount of bacteria used for challenging. About 10^{14} CFUs of each isolate was separately

injected into the coelomic cavity of the 24 animals. On each day 3 animals are sacrificed and coelomic fluid was collected from each challenged animal separately at regular time intervals (1, 2, 3, 4, 5, 6, 7 and 8^{th} day) in 1:1 ratio with alseiver solution as anticoagglutinant (dextrose 20.5 gm, tri sodium citrate 8 gm, citric acid 0.55 gm and sodium chloride 4.2 gm in 1000 ml water). The resulting fluid was centrifuged (5,000 g, 15 min at 4 ^{0}C) to remove coelomycetes and the clear supernatant was treated with serine proteinase inhibitor (PMSF, 20 $\mu g \cdot ml^{-1}$) and stored at -20 ^{0}C. Coelomic fluid from unchallenged animals served as control. After the 8^{th} day, the same sets of animals were rechallenged as before to determine the presence of any immune memory.

Estimation of residual bacterial load

Prior to processing, an aliquot was removed from the coelomic fluid preparation to estimate the residual bacterial load by serial dilution method. This was used as an index for in vivo anti-bacterial activity.

Protein concentration estimation

Protein concentration was determined according to the method of Bradford [28] using bovine serum albumin as standard.

Hemagglutination assay (HA)

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 pronase (0.5 $mg \cdot ml^{-1}$), incubated at 37 ^{0}C for one hour and later washed 4 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.

Agglutination and phagocytosis of bacteria

Above mentioned bacterial strains were grown in nutrient broth at 37 ^{0}C for 16 h, harvested by centrifugation at 2000 g for 10 min, washed thrice with TBS and suspended in same buffer. Bacterial count was calculated by serial dilution method and A_{620} was maintained around 1.0. Bacterial agglutination was tested by mixing 100 µl of coelomic fluid or HSL (10 µg·ml^{-1}) to an equal volume of bacterial suspension. In other set HSL was incubated with 20 mM of MeαGal for 30 min and later mixed with equal volume of bacterial suspension. Results were observed microscopically after incubating the mixture for 12 h and comparison was made with bacterial suspensions incubated in the absence of coelomic fluid or HSL.

Adsorption by bacteria

In order to associate agglutination with lectin activity, two-fold dilution of all the bacterial suspensions were added with 1:1, 1:2, 1:4 ratios of coelomic fluid or HSL (lectin: bacteria), incubated for 1 h, centrifuged for 3 min at 12,000 g and the supernatant tested for residual hemagglutination activity.

Bacterial glycoconjugates agglutination test

Glycoconjugate agglutination test was performed by modification of the method described by Kellens *et al.* [29]. In brief, bacterial cells were grown in three sets of 20 ml each in nutrient broth for 6 h at 37 ^{0}C. Cells were collected by centrifugation at 2,000 g for 20 min, washed thrice and suspended in TBS. A_{620} of the suspensions were maintained at 1.0. The cells were used in 3 sets of experiment. A set of cells was left untreated as a control. Second set was treated with trypsin (0.1 mg·ml^{-1}, incubated at 37 ^{0}C for 1 h) for partial hydrolysis of proteoglycans and thereby enhancing the surface availability of glycan residues. Third set was subjected to drastic condition (cell pellets was resuspended in 250 mM glycine-HCl buffer, pH 2.0 and boiled for 15 min) so as to degrade surface structures and dissolve more cell wall glycoconjugates. The treated and untreated samples assayed for agglutination under standard assay conditions.

Hemagglutination inhibition assays

Hemagglutination inhibition assays were performed similarly, except that serially diluted sugar solutions (25 µl) were pre-incubated for 15 min at 27 °C with 25 µl of the lectin (8 U). Erythrocyte suspension (50 µl) was then added, mixed and the plates read after one hour. The glycoproteins were desialylated by incubating in 0.1 M H_2SO_4 at 60 °C for 1 h followed by dialysis against distilled water.

Antibacterial activity

Antibacterial activity of coelomic fluid and HSL was checked under in vitro conditions by the agar adsorption technique, bacteria were cultured in nutrient broth for 24 h, and for each test the culture medium was diluted 10-fold for adjusting A_{660} 1.0. Direct adsorption technique was performed by making wells in the agar plates and known quantity of HSL (5, 10, 25, 50 and 100 µg, a positive control (ampicillin, 10 µg) and a negative control), was poured on to each well, and allowed for absorption of sample by the agar. Sterile cotton bud was then dipped in a bacterium culture, and the inoculums were spreaded uniformly on the surface of the agar media to produce a bacterial field. Each test was performed in duplicates.

Antibacterial activity was also measured by the method as previously described [30]. Each bacterial cell suspension was diluted to 10^4 cells ml^{-1} with nutrient broth. Several concentrations of HSL were added into a 2 ml broth, incubated for 6 h at 37 ^{0}C. The growth of bacteria was expressed as the turbidity, as measured at A_{620}. Comparison of antibacterial activity against different bacteria was performed using the ratios of different concentrations of HSL giving 50% inhibition of bacterial growth.

Purification of the lectin

Coelomic fluid preparation was concentrated over a 10 kDa ultra filtration membrane (to remove <10 kDa contaminants) and heat treated at 60 ^{0}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 ^{0}C until further use.

Electrophoresis

Purity of the protein was determined on a 7% (w/v) polyacrylamide gel, pH 4.3, according to Reisfeld *et al.* [31] and the protein bands were visualized by Coomassie Brilliant Blue R-250 as well as by silver staining separately.

Determination of molecular mass

Gel Filtration: The relative mass of the lectin was determined by gel filtration on sephacryl S-300 (1.5 cm x 180 cm) according to the method of Andrews [32]. The column was equilibrated with TBS and calibrated with appoferritin (443,000), β- amylase (200,000), alcohol dehydrogenase (150,000), bovine serum albumin (66,000) and ovalbumin (45,000).

***SDS-PAGE*:** Molecular mass of HSL was determined by SDS-PAGE according to Weber and Osborn [33] in 7% slab gel, followed by visualization with silver staining. Along with high molecular weight markers including, myosin (220,000), α–2-macroglobulin (170,000), β–galactosidase (116,000), transferrin (76,000), glutamate dehydrogenase (53,000), were used as reference proteins.

Isoelectric focusing

Isoelectric focusing in polyacrylamide gels was performed according to Vesterberg [34], over the pH range of 8-11. The gels were stained with

Coomassie Brilliant Blue R-250. The isoelectric point was confirmed by checking the pH of the gel slices of 1.5 cm each.

Carbohydrate content

The total neutral sugar content was estimated by the phenol sulfuric acid method of Dubois et al. [35] using galactose and mannose (4:3) as standard. 300 μg of HSL in 200 μl water was mixed with 200 μl of 5% (w/v) phenol and incubated for 10 min at room temperature. One milliliter of sulfuric acid then added to the mixture, allowed to cool for 20 min. The color developed was then measured spectrophotometrically at 490 nm.

Effect of metal ions

The activity of the purified lectin was determined in the presence of 1, 5 and 10 mM of Ca^{++}, Mg^{++}, Mn^{++} and also after dialysis against 10 mM of EDTA.

Temperature and pH stability

Effect of temperature on lectin stability was monitored in the range of 20 ^{0}C to 100 ^{0}C by incubating 20 μg of the lectin for 60 min at the respective temperature, rapidly cooling in ice and assaying for hemagglutination activity. Effect of pH on lectin stability was determined with 20 μg lectin and adjusting to different pH in the range of 2-11 by adjusting the lectin volume to 1 ml by adding different buffers of 100 mM and checking activity after different duration of incubation (upto 8 days). Buffers used were glycine–HCl (pH 2.0-3.0), sodium acetate (pH 4.0-5.0), sodium phosphate (pH 6.0-7.0), tris-HCl (pH 8.0-9.0), glycine-NaOH (pH 9.0-11.0). The hemagglutination activity was checked after 6, 12, 24, 48, 72 and 96 h.

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 8,000 g for 5 min to remove any particulate matter.

Circular dichroism (CD) measurments

CD spectra were recorded at 25 ^{0}C on a Jasco J-715 spectropolarimeter at a scan speed of 20 nm·s^{-1} 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 measurments 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 (TBS) scans recorded under the same conditions were subtracted from the protein spectra before analysis. Secondary structure was calculated by using CDpro software [36].

Amino acid and *N*-terminal analysis

50 μg of HSL was digested with 6 N HCl at 110 ^{0}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. 5 pmol of the sample was loaded onto AccQ-tag column and eluted with acetonitrile gradient (5-95%). The eluate was monitored with a florescence detector. The amino acid peaks were compared with standards run under identical conditions. Total cysteine and tryptophan were estimated with denatured protein according to the method of Cavallini *et al.* [36] and Spande and Witkop [37] respectively. N-terminal sequence was determined by automated Edman degradation method in Procise^{TM} protein sequencer (Applied Biosystem, Canada) at the protein sequencing facility, NII, New Delhi, India.

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. Two μL of in situ-gel tryptic digest of HSL was mixed with two μl matrix and one μl 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 $\mu l \cdot min^{-1}$ 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.

RESULTS

Table 2.1: Residual bacterial count as function of time.

Bacterial strains	**Total injected** (CFU x 10^{14})	**Day 1** (CFU x10^{6})	**Day 5** (CFU x 10^{3})
Control	--	0.0001	0.10
Serratia sp.	0.56	2.02	0.41
Klebsiella sp.	0.51	0.13	0.83
Shigella sp.	0.50	0.13	0.31
E. *coli*	0.49	0.96	0.10
Vibrio sp.	0.52	0.07	0.07
Pseudomonas sp.	0.48	0.02	0.02
Streptococcus sp.	0.52	0.05	0.06
Proteus sp.	0.51	0.07	0.61
Bacillus sp.	0.54	1.18	0.14
Staphylococcus sp.	0.49	0.11	0.09

Sea cucumbers, maintained in seawater-aquaculture, were challenged separately with 10^{14} CFUs of ten different bacteria and their coelomic fluid examined over a period of eight days. Except in case of *Serratia* sp. and *Bacillus* sp., the bacterial load dropped to ~0.3 x 10^6 CFU's within one day and to negligible level by the fifth day of post-inoculation (Table 2.1), indicating progressive clearance of bacterial load in the coelomic cavity of the animal.

Hemagglutination activity in coelomic fluid

Hemagglutinating activity of the coelomic fluid was monitored over a period of 8 days in both challenged and unchallenged animals. Agglutination activity was determined by measuring the lowest titre producing hemagglutination activity. The activity is expressed as the reciprocal of the titer of each fraction. On day one, the fluid from challenges with *Serratia sp*., *Klebsiella sp*., *Shigella* sp, *E. coli*, *Staphylococcus sp.* and Group D *Streptococci* showed an increased hemagglutinating activity (~2.25 x10^3 HA.units mg^{-1}) as compared to rest of the challenges (~0.65 x10^3 HA.units mg^{-1}).

Table 2.2: Hemagglutinating activity expressed (x10^3 HA.units mg^{-1}) as function of time.

Bacterial strains	Day 1	Day 5	Day 8
Control (unchallenged)	0.38	0.51	0.54
Serratia sp.	2.04	25.00	1.19
Klebsiella sp.	2.27	33.33	1.44
Shigella sp.	1.05	50.00	1.07
E.*coli*	1.05	25.00	1.20
Vibrio sp.	0.61	0.57	0.51
Pseudomonas sp.	0.68	1.09	0.51
Streptococcus sp.	1.32	50.00	1.88
Proteus sp.	0.62	0.61	0.49
Bacillus sp.	0.95	1.47	0.57
Staphylococcus sp.	1.88	100.00	1.45

The hemagglutination activity progressively increased till the 5th day (~50.0 x10^3 HA.units mg^{-1}), and thereafter returning to the basal level by the 8th day (Table 2.2). The protein content increased in the coelomic fluid from challenged animals as compared to that of control. These results clearly show that the hemagglutinating activity in the coelomic fluid of the challenged animals increased greatly after bacterial injection**.** The test animals were subjected to a second round of challenge on the 9th day and the bacterial load in the coelomic fluid monitored as before. No change was observed in the reduction pattern. As in the first challenge, complete clearance was achieved in duration of 5 days.

Bacterial agglutination

Coelomic fluid and purified lectin were tested for their ability to agglutinate the test bacteria. Microscopic observation revealed that co-incubation induced agglutination of bacterial cells. Sets of samples were subjected to trypsinization and acid-heat treatment to obtain uniform dispersion. For quantitation, the samples were centrifuged and the hemagglutination activity of the supernatant determined. Residual activity was inversely proportional to the amount of lectin adsorbed on bacterial cells.

Bacterial agglutination due to coelomic fluid and HSL was observed in untreated, trypsinized and acid-heat treated cells. Out of the ten bacteria tested, HSL mediated agglutination was evident with untreated cells of *E. coli*, *Staphylococcus* sp., and Group D *Streptococcus* (Fig.2.1). Agglutination with *Serratia* sp., *Klebsiella* sp. and *Shigella* sp. was observed only when subjected either to trypsinization or to acid-heat treatment. No agglutination was noticed with *Pseudomonas* sp., *Proteus* sp, *Bacillus* sp. and *Vibrio* sp., despite of pre-treatment. Reduction in bacterial agglutination was observed when lectin was pre-incubated with 20 mM, MeαGal.

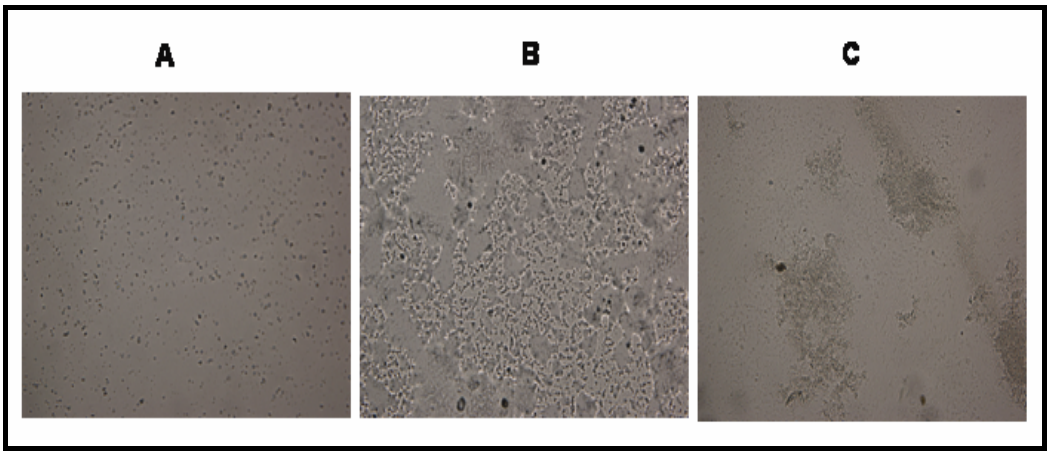

Fig.2.1. Agglutination of Group D Streptococci. Agglutination test for Group D *Streptococci* was done by incubating with equal volume of purified HSL, (A) Control cells with TBS buffer; (B) early stage of agglutination after incubating with equal volume of HSL; (C) complete agglutination after 12 h of incubation.

Antibacterial activity

Assay of antibacterial activity with purified HSL and cell free coelomic fluid of sea cucumber, revealed that the HSL exhibited strong antibacterial activity against both gram positive and gram negative bacteria. 5 μg of HSL was able to inhibit efficiently the growth of *Staphylococcus* sp. as indicated by decrease in 50% of turbidity, whereas 15 μg inhibited complete growth.

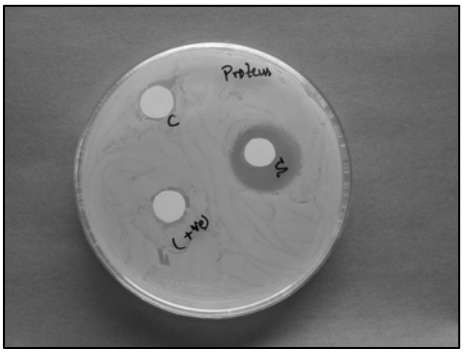

Fig.2.2. Antibacterial activity against *Proteus* sp. Antibacterial activity against *Proteus vulgaris* was done using zone inhibition method. HSL (S, 10 μg), standard amphicillin (5 μg) as a positive control (+ve) and negative control (c, buffer) was used. Plates were incubated for 24 h and the results were measured as zone of inhibition.

HSL also inhibited effectively the growth of other gram negative (*Serratia* sp., *Proteus* sp. (Fig.2.2), *Shigella* sp., and *E. coli*) and gram positive (Group D *Streptococci*) bacteria, indicating its broad spectrum antibacterial effect. These results are in accordance with the bacterial agglutination and residual hemagglutination assays and in vivo antibacterial activity.

Purification of lectin

The retentate of 10 KD ultra filtration membrane passed fraction showing hemagglutination activity, was loaded on hydrophobic column chromatography (phenyl sepharose). The active fraction was eluted in TB buffer of single sharp peak. These fractions showed some cross contamination of other proteins in the native-PAGE, so the active fraction were pooled and reloaded on phenyl sepharose-II, which gave a single sharp peak protein of A_{280} and hemagglutination activity (Fig. 2.3).

Lectin activity eluted as single peak corresponding to a pure protein of mass 190 kDa (Fig. 2.4A), having both antibacterial and agglutination activity. This indicated that the antibacterial activity and agglutination was due to a single species of lectin in the coelomic fluid. The lectin was purified by 28.30 fold with a yield of 53% corresponding to ~2.9 mg (Table 2.3).

Table 2.3: Purification of HSL.

Purification step	Total Protein (mg)	Total activity (HA units)	Specific activity (HA unit.mg^{-1})	Fold purification	Recovery (%)
Coelomic fluid	152.82	1.7 x 10^5	1130	1	100.00
Ultra filtration (>10 kDa)	44.32	1.5 x 10^5	3595	3.18	92.00
Phenyl sepharose-I	5.55	0.9 x 10^5	17297	15.31	55.55
Phenyl sepharose-II	2.88	0.9 x 10^5	32000	28.30	53.33

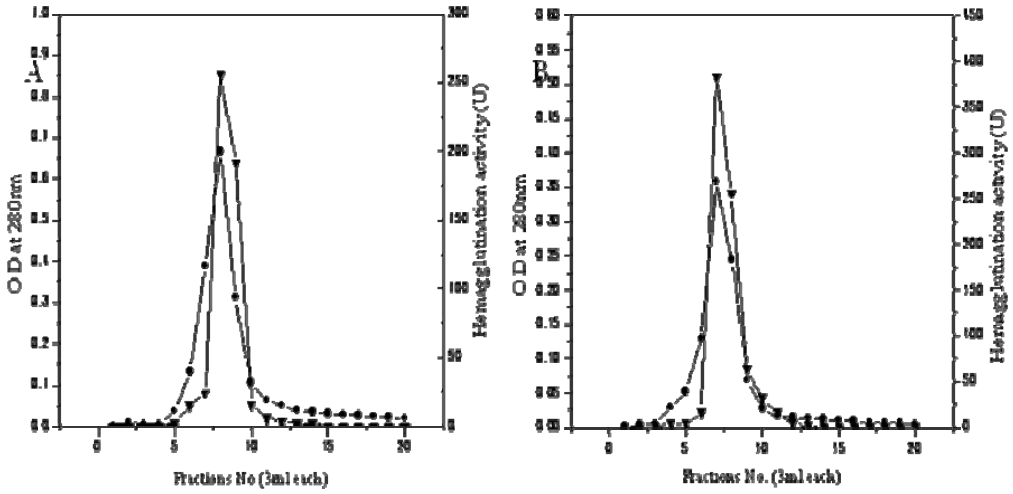

Fig. 2.3. Elution profile of HSL: (---) A_{280} nm and (—) hemagglutination titre

A) Coelomic fluid loaded on phenyl sepharose column, equilibrated with TB containing 3% ammonium sulphate, the active protein fractions were eluted with TB.

B) Pooled active fractions from earlier column was reloaded on to phenyl sepharose equilibrated with TB containing 1% ammonium sulphate, the active protein fractions were eluted with TB and each fraction was collected of 3 ml each.

The molecular mass of the native lectin was 190 kDa as determined by gel filtration on sephacryl S-300 chromatography (Fig. 2.5). The lectin showed a single band of molecular mass 182 kDa on SDS-PAGE, both in presence and absence of 2-mercaptoethanol (Fig.2.6). This indicated that HSL exists as monomer.

Sugar content analysis revealed that HSL is a glycoprotein containing 8% of neutral sugars. The protein is basic in nature with a pI of 10.3 (Fig. 2.4B). Atomic absorption spectroscopy indicated that HSL contains 3 moles of Ca^{2+}, 1 mole of Mg^{2+} and 0.01 mole of Cu^{2+} per mole 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 hemagglutination activity.

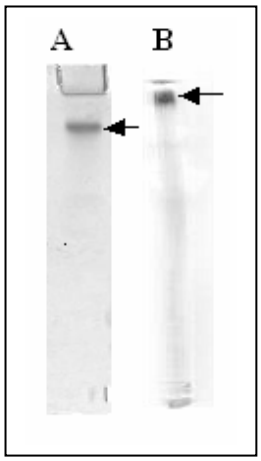

Fig.2.4. Electrophoresis of the HSL

A) **Native-PAGE:** The lectin (20 μg) was electrophoresed in a 7% (w/v) polyacrylamide gel, at pH 4.3; protein was visualized with Coomassie Brilliant Blue R-250.

B) **IEF-PAGE:** The lectin (100 μg) was focused in an 8% (w/v) polyacrylamide gel using narrow range of ampholines (pH range 8.0-11.0) and the gels were stained with Coomassie Brilliant Blue R-250.

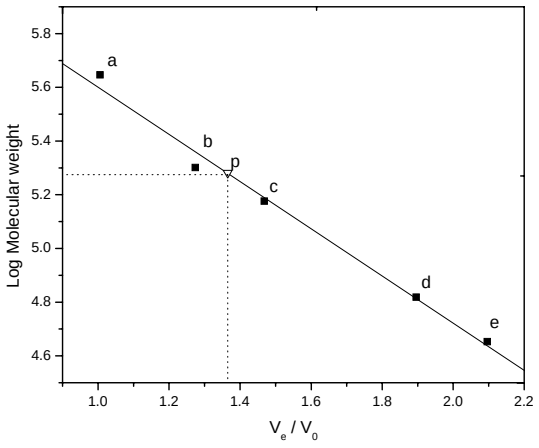

Fig. 2.5. Molecular weight determination of the HSL. Gel filtration: sephacryl S-300 column (1.5 cm x 180 cm) was equilibrated with TBS (pH 8.5), and calibrated with Appoferritin (443,000), β- Amylase (200,000), Alcohol dehydrogenase (150,000), Bovine serum albumin (66,000) and Ovalbumin (45,000). V_0 is the void volume, V_e the elution volume and (P) indicates the HSL.

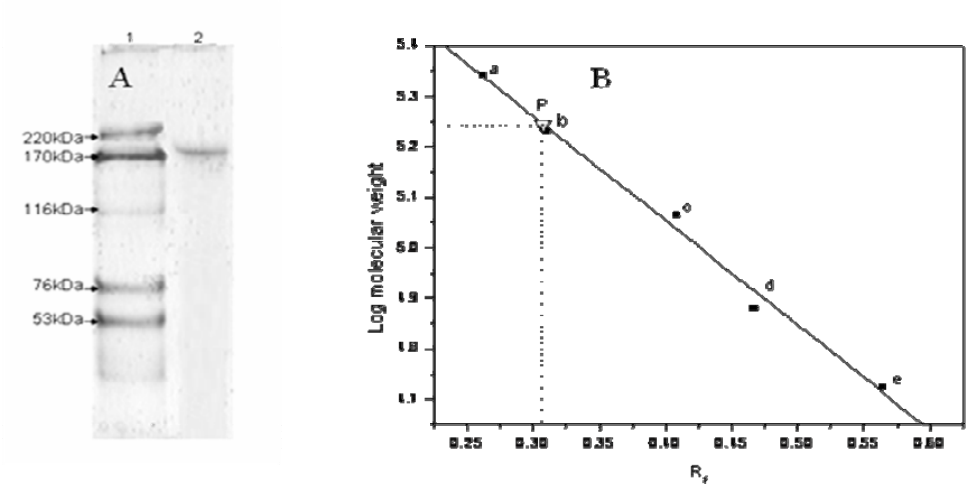

Fig.2.6. SDS-PAGE of purified *H. scabra* lectin.

A) The HSL (20 μg) was electrophoresed using 7% (w/v) polyacrylamide gel containing 0.1% SDS and stained with silver staining. Lane 1- Molecular weight markers, Lane 2- purified HSL.

B) Relative mobilities of the reference proteins were plotted against the log molecular weight. Reference proteins used were (a) myosin (220,000), (b) α–2-macroglobulin (170,000), (c) β–galactosidase (116,000), (d) transferrin (76,000), (e) glutamate dehydrogenase (53,000), and (P) protein (182,000).

HSL is highly thermostable as it retains full activity on heating at 80 ^{0}C for 1 h; complete loss of activity occurred at 100 ^{0}C. The hemagglutinating activity was not affected by incubation of the protein in the pH range 2-11 for a period of 48 h; incubation at pH 2-7 for 72 h resulted in 50% loss in activity. The lectin was completely stable at pH 8 and above. HSL agglutinated only pronase treated human erythrocytes but not trypsinized, desialylated and untreated erythrocytes.

Amino acid analysis reveals that HSL contains remarkably high level of Asx (14.97 mol %), 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 (Table 2.4). HSL contains 16 cysteine residues of which four cysteines were free and 12 were involved in six disulfide bonds. *N*-terminus found to be blocked.

Table 2.4: Amino acid composition of HSL.

Amino acid	Mol (%)	Amino acid	Mol (%)
Asx	14.97	Cys[a]	1.10
Ser	6.53	Tyr	1.42
Glx	8.32	Val	5.36
Gly	13.43	Met	1.35
His	2.58	Lys	13.12
Arg	6.53	Ile	3.33
Thr	6.22	Leu	3.69
Ala	6.16	Phe	1.84
Pro	2.65	Trp[b]	1.35

Determined spectrophotometrically by: [a] Spande and Witkop [37] and [b]Cavallini *et al.* [36].

Circular Dichroism measurments

Circular dichroism spectra of HSL in the native state and in the presence of T-antigen are shown in Fig.2.7. The CD spectra in far UV region remain identical both in presence and absence of ligand. CD spectrum of far UV region was characterized by a maximum negative absorption at 208 nm. 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 15% unordered coil. Whereas in the near UV region (250-300 nm), the CD spectrum of HSL is characterized by a maximum around 293 and 266 nm and a minimum around 283 nm. The band centered around 293, 283 and 266 nm can be assigned to Trp, Tyr and Phe respectively. Presence of ligand at near-saturating concentration led to a increase in the intensity of the native band in the region of 293 and 283 nm.

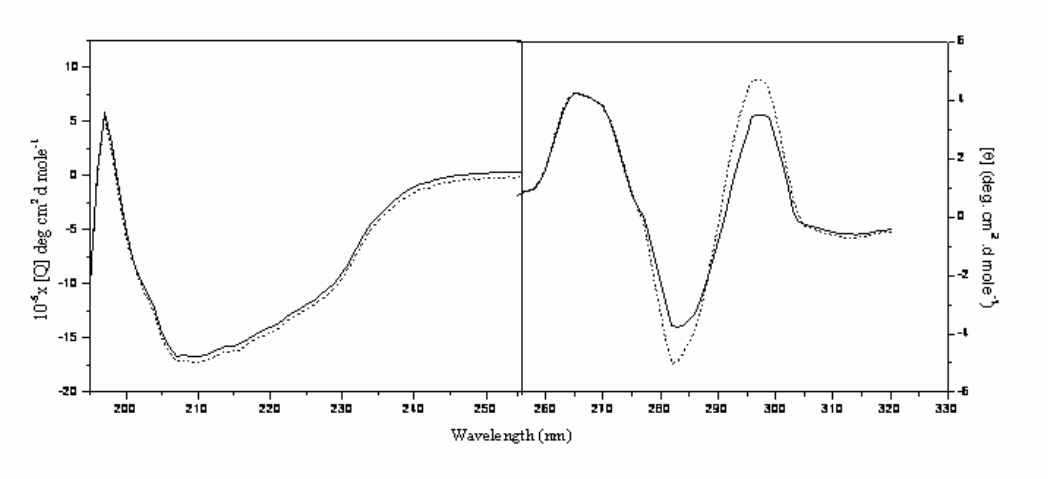

Fig.2.7. C.D. spectra of the HSL. Circular dichroism spectra of far UV and near UV of the native HSL (—) and in the presence (…) of 200 μM T-antigen sugar.

Tryptic peptide sequencing and mapping

Peptide mapping of tryptic digest of HSL by MALDI-TOF method did not result in any reasonable positive hit despite presence of resolved peaks in the spectrum. 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-icon and b-icon series according to known fragmentation mechanism [12].

Table 2.5: Tryptic digest peptide sequences of HSL.

M/z	Sequence	M/z	Sequence
467.3	(I/L)TPR	816.3	QTWTDGK
494.3	PGA(I/L)R	905.4	NCRFG(I/L)SK
564.2	TDGYK	1041.3	ATHSCEG(I/L)SR
661.4	LYVDR	1134.5	SAGRPDVAPGAR
667.3	DCGVHR	1248.5	CDPGKTDRGYR
794.5	CRY(I/L)FK	1320.8	NCKFARS(I/L)TPFR
845.3	SCPTDWR		

Peptide sequences so obtained (Table 2.5) were used in homology search to identify related proteins among the echinoderm lectins. Homologous motifs were found in SCPTDWR (CEL-I, 1WMZD), ATHSCEGLRS (CEL-III, BAC75827) and NCRFGLSK (CEL-IV, AAB35250), which are Ca^{2+} dependent lectins from the sea cucumber *Cucumaria echinata* [8].

Table 2.6: Hemagglutination inhibition of HSL by sugars/glycoproteins.

Inhibitor	MIC[a]	Inhibitor	MIC[a]
MeαGal	62.5 mM	Fetuin	15.63 µg
LacNAc	50.0 mM	Asialo Fetuin	0.19 µg
T-antigen	5.0 mM	Mucin	5.0 µg
Avidin	6.25 µg	Holotransferrin	15.63 µg
Fibrinogen	125.0 µg	Thyroglobulin	62.52 µg
Asialo fibrinogen	0.2 µg	Ovalbumin	23.43 µg

[a] represents minimum inhibitory concentration

Hemagglutination inhibition assay

Inhibition assay was performed with a variety of sugars to ascertain the binding specificity of the purified HSL. The activity was inhibited only by MeαGal (62.5 mM), LacNAc (50 mM) and T-antigen (5 mM) but not by alpha linked galacto-oligosaccharides such as mellibiose, 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 µg), asialo fibrinogen (0.2 µg), mucin (5.0 µg), avidin (6.25 µg), fetuin (15.63 µg), holotransferin (15.63 µg), ovalbumin (23.43 µg), thyroglobulin (62.52 µg) and fibrinogen (125 µg) but not with invertase (Table 2.6).

DISCUSSION

Humoral or natural lectins are believed to participate in immune response in invertebrates. They serve to opsonize the invading bacterium or foreign particle and thereby enhancing its phagocytosis by coelomycetes. Only few experiments have shown a lectin's precise mechanism of recognition of bacterial glycoconjugates, such as the horseshoe crab lectin, *Carcinoscorpius rotundacaudia,* which recognizes the 2-keto-3-deoxyoctonate group in the cell wall polysaccharide of *E. coli* [15]. *Sambucus nigra* agglutinin can agglutinate 1, 1/2, 2, 14, 15 and 16 stereotypes of *Streptococcus suis* out of 35 strains tested due to the presence of possible Neu5Ac-α(2,6) GalNAc sequence [29], even wheat germ agglutinin, v.gr., has agglutinated P. *haemolytica* biotype T and capsular sereotype 3, 4 and 10, due to the presence of *N*-acetylated residues or their oligomers on their cell wall determinants [37].

A number of lectins have been isolated from echinoderms that are capable of interacting with erythrocytes. However, little is known about the mechanism of interaction with bacteria and their role in innate immunity. In our extensive studies on sea cucumber, we observed upregulation in the expression of a humoral lectin in response to artificial challenge with bacteria. Similar observation was reported from pacific oyster (*Crassostrea gigas*) [30].

Sea cucumbers, maintained in seawater-aquaculture, were separately challenged with ten different bacterial types. Analysis of coelomic fluid over a period of 8 days indicated progressive clearance of bacteria reaching maxima after 5 days of inoculation. Hemagglutination assay of the coelomic fluid revealed progressive increase in lectin activity, reaching a high level at 5^{th} day and thereafter return to basal level by 8^{th} day. This clearly demonstrated a strong correlation between lectin expression and bacterial clearance. All the test bacteria were cleared in about the same time frame ascribing broad specificity to the induced lectin. *Serratia* sp., *Klebsiella* sp., *Shigella* sp., *E. coli*, *Staphylococcus* sp. and Group D *Streptococci* induced higher level of lectin expression when compared to rest of the inoculated bacteria. These results are in accordance with bacterial agglutination and residual

hemagglutination assays. The enhancement of lectin expression might be due to the presence of cognate glycan antigen on the cell surface of inducing bacteria. For instance, D-galactose is known to occur as a moiety in the *O*-antigen of *E. coli* [38]. It is possible that bacterial challenge induced higher production of coelomycetes and thereby synthesizing and releasing higher quantity of lectin into coelomic fluid [39].

Immune system in vertebrates is characterized by the unique property of memory, whereby a second antigen challenge results in immediate response. In order to examine the involvement of memory in the induction of this immune lectin, we rechallenged the test animals after the 8^{th} day; by this time, primary infection had been fully cleared and the associated increase in hemagglutination activity had subsided to basal level. However, the pattern of clearance and the corresponding increase in hemagglutination activity was as in primary challenge. This is indicative of absence of memory-based immunity in sea cucumbers. Previous studies have indicated such an absence in echinoderms [40].

Purification of HSL from coelomic fluid was carried out by hydrophobic interaction, phenyl sepharose column chromatography. This associated the lectin activity to a monomeric protein weighing 182 kDa. The purification process resulted in a yield of 2.1 mg of protein from 180 ml of coelomic fluid with 28.3 fold purification. HSL is a thermostable protein and active upto one hour at 80 ^{0}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. [41] found that thermostable proteins have an increased content of charged amino acid residues (i.e., K, E and R). Presence of three disulfide bonds in *Moringa oleifera* seem to be responsible for high thermostability of the lectin [42]. Though four free sulfhydral 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. HSL could hemagglutinate only pronase treated erythrocytes indicating specificity for desialylated glycoconjugates. This confirms the higher hemagglutination inhibition by desialylated glycoproteins. Pronase-E, a cocktail of different proteolytic enzymes, removes all protruding polypeptides from the erythrocytes membrane, there by exposing GPI-anchors and glycolipids. This might serve as better ligand for the HSL than asialo-glycoconjugates on erythrocyte membrane. 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 using ClustalW revealed that, HSL is homologous with lectins isolated from other genera of sea cucumber [43, 44].

Hemagglutination inhibition assay was employed to elucidate the preliminary carbohydrate binding specificities of the purified HSL. A range of monosaccharide, disaccharide and glycoproteins were used. Among monosaccharides, inhibition was evident only with MeαGal. Higher specificity shown for the α-anomer of the galactose derivative could be due to the axial orientation of the aglycone at C-1 position being accessible to the lectin-binding site. Whereas aglycone moiety in the β-anomer (MeβGal) occurs in the equatorial position, which could impose steric hindrance to lectin-carbohydrate interaction. Among the disaccharides studied, T-antigen was found to be a better inhibitor than LacNAc and other sugars. The affinity for T-antigen can be explained as a consequence of the presence of galactose at the reducing end at second sugar position. This demonstrates the extended nature of the carbohydrate-binding site. No inhibition was observed with alpha-linked galacto-saccharides like mellibiose, raffinose and stachyose indicating that the lectin might not bind to the sugars like glucose or fructose at the reducing end linked with alpha galactose.

HSL preferentially recognized glycoproteins containing desialylated *N*-linked glycans, such as asialofibrinogen and asialofetuin. These were better inhibitors as compared to their sialylated counter part. The minimum inhibitory concentration of desialylated glycoproteins was about 150-200 times lower than the sialylated once. However inhibition due to fetuin and mucin could be due to the presence of T-antigen in the backbone as compared to fibrinogen. Reduced inhibition with thyroglobulin and ovalbumin could be due to the presence of high mannose type oligosaccharides on these glycoproteins. Mucin also showed considerably good inhibition.

In conclusion, the present studies show that sea cucumber upon bacterial challenge produces a high molecular weight monomeric glycoprotein, which is involved in the innate immunity. HSL can agglutinate both gram +ve and gram –ve bacteria and pronase treated human erythrocytes. It exhibited strong antibacterial activity both under *in vivo* and *in vitro* conditions. The hemagglutination activity is independent of any metal ions and hemagglutination activity is inhibited by MeαGal, T-antigen and desialylated glycoproteins.

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CHAPTER : 3

CHARACTERIZATION OF ACTIVE SITE AND TRYPTOPHAN MICROENVIRONMENT OF HSL

SUMMARY

Sugar binding site of *H. sca*bra lectin was characterized by modifying with residue specific reagents, which revealed involvement of positive charge of the ε-amino group of lysine, indole group of tryptophan and carboxylate groups in the glycan-binding site. Fluorescence quenching and time-resolved fluorescence studies were carried out to study tryptophan microenvironment of HSL using two neutral (acrylamide, succinimide) an anionic (I^-) and a cationic (Cs^+) quenchers. The Trp were found to be differentially exposed to the solvent and were not fully accessible to the quencher indicating heterogeneity in the microenvironment. The presence of 200 μM of T-antigen led to slight decrease in the quenching with all the quenchers, suggesting presence of Trp at the sugar-binding site. Acrylamide quenching yielded positive upward curving of Stern-Volmer plots, indicating that the quenching mechanism involves both static and dynamic components. Time-resolved fluorescence measurments yield bi-exponential decay curves with lifetimes of 1.77 and 4.98 ns in the absence of ligand, and 2.08 and 5.10 ns in its presence. The Trp present in the sugar-binding site are mostly buried and show for long duration lifetime decay.

INTRODUCTION

To understand the phenomenon of interaction between lectins and carbohydrates, knowledge of the reactive groups that participate in the interaction is essential. Identification of specific amino acids within the active site of a biologically active protein elucidates the relationship between its structure and the role played by the amino acid side chains in its activity. Single crystal X-ray diffraction is an excellent method to determine these residues but it is often difficult to obtain good quality crystals from macromolecules and limited by the amount of pure protein available for the crystallization. Other than site directed mutagenesis, chemical modification is an alternative method, which provides reasonably reliable information about the residues involved in the saccharide binding. A numbers of lectins have

been isolated from marine invertebrates but very little information is available about the amino acid residues involved in their sugar-binding site [1, 2].

The fluorescence of the indole side chains of the tryptophan (Trp) residues in a protein is extremely sensitive to its environment, and hence changes which affect the tryptophan environment can cause changes in fluorescence properties of the protein [3-5] and such changes in the intrinsic fluorescence of proteins have been used to obtain information regarding structure, specificity and conformation of the proteins [6, 7]. The accessibility of tryptophan in protein molecule can be measured by use of small molecular-weight quenchers perturbing fluorescence [3, 5]. Fluorescence quenching by these molecules proceeds mainly via physical contacts of the quenchers with the fluorophores and hence is directly dependent on the extent to which they can approach the fluorophores in the protein. This in turn makes the process extremely dependent on the nature of the solvent, the degree of exposure or burial of the Trp residues as well as the nature of the quenchers themselves [8, 9]. In cases, where Trp residues are at the ligand-binding site, this technique has been used to study the changes resulting from ligand binding. Lifetime studies of fluorescence decay have likewise been used to obtain information regarding the Trp environment and its interaction with various quenchers [10, 11]. The characterization of active site and the tryptophan microenvironment of HSL were reported in this chapter.

MATERIALS

Succinic anhydride, citraconic anhydride, acetic anhydride, *N*-acetyl imidazole, 2,4,6-trinitrobenzenesulphonic acid, diethylpyrocarbonate, phenylmethylsulphonylfluoride, *N*-bromosuccinimide, phenyl glyoxal, 5,5'-dithiobis 2-nitrobenzoic acid, imidazole, 1-ethyl-3 (3-dimethyl amino propyl) carbodiimide, nitrotyrosine ethyl ester, cesium chloride, acrylamide, succinimide, guanidium hydrochloride, sodium borohydride and urea were purchased from Sigma Chemical Co. St. Louis, U.S.A. Molecular weight markers from Amershm Biosciences, England. Holotransferrin (bovine) was from Calbiochem, CA, USA, while Gal β 1-3 GalNAc (T-antigen) was

purchased from Dextra Labs, London, UK. Potassium iodide from S.D. Fine Chemicals. All other chemicals used were of analytical grade.

METHODS

Hemagglutination assay

The lectin samples were serially diluted in 20 mM tris-HCl buffer pH 7.2, containing 150 mM NaCl (TBS), in a microtitre plate and equal quantity of a pronase treated and washed 3% suspension of human erythrocytes was added. After incubation for one hour at room temperature, the agglutination was scored visually. Taking the titre value of native lectin to be 100%, percentage residual agglutination of the totally modified lectin samples were calculated from their respective titre values.

Purification of HSL

Induction and purification of HSL was carried out as described earlier (Chapter 2).

Protein determination:

Protein concentration was determined by the method of Bradford [12] using BSA as standard.

Active site characterization

Modification of serine with phenylmethylsulphonyl fluoride (PMSF)

The lectin (300 μg) in 20 mM tris buffer pH 8.5 was incubated with 5 mM PMSF, at room temperature, for 60 minutes [13]. Aliquots are removed at 15 min intervals, the excess reagent removed by dialysis and residual activity was determined. Lectin sample incubated in the absence of PMSF served as control.

Modification of arginine with phenylglyoxal

Arginine residues were modified with phenyl glyoxal by the method of Takahashi [14], the reagent was prepared in methanol. The lectin (100 μg), in 50 mM phosphate buffer, pH 8.0, was treated with phenylglyoxal (3.0 mM) for 30 minutes at 27°C. Excess reagent was then removed by dialysis and the

residual hemagglutination activity determined. Lectin sample incubated in the absence of phenyl glyoxal served as control. The methanol concentration in the reaction mixture did not exceed 2% (v/v) and had no effect on the activity and stability of the lectin during the incubation period.

Modification of cysteine with 5, 5'-dithiobis 2-nitrobenzic acid (DTNB)

One ml of the lectin (300 μg) in 100 mM sodium phosphate buffer, pH 8.0, was incubated with 1 mM of DTNB at room temperature for an hour [15]. Aliquots were removed at different time intervals and the residual activity was determined. The modification reaction was also followed by monitoring the increase in absorbance at 412 nm and the numbers of sulfhydral groups modified were calculated using a molar absorption coefficient of 13,600 $M^{-1}cm^{-1}$.

Determination of total protein sulfhydral groups

The sulfhydral groups in proteins exhibit variable reactivity toward DTNB owing to steric factors. Therefore, denaturation of total sulfhydral content requires that the protein be denatured, preferably with sodium dodecyl sulfate. One ml of lectin (300 μg) in 80 mM sodium phosphate buffer, pH 8.0, is containing 2% SDS, 0.5 mg/ml EDTA. Sample was incubated with 50 μl of DTNB (40 mg in 10 ml of 100 mM sodium phosphate buffer pH 8.0), at room temperature for an hour and read at 412 nm against protein solution in SDS to give apparent absorbance. A reagent blank was subtracted from the apparent absorbance to give net absorbance. The numbers of sulfhydral groups modified were calculated using a molar absorption coefficient of 13,600 $M^{-1}cm^{-1}$ [16].

Determination of disulfide bonds with DTNB after reduction with sodium borohydride

One ml of the lectin (300 μg) in 80 mM sodium phosphate buffer, pH 8.0 containing 8 M urea, 0.1 M sodium EDTA was added to, 1 ml of 2.5% sodium borohydride and a drop of octyl alcohol as an antifoaming agent. The reduction was allowed to proceed for 30 minutes at 38 ^{0}C, after which 150 μl

of 1 M potassium phosphate containing 0.2 N HCl was added [17]. After 5 min, 0.5 ml of acetone was added, and then nitrogen was bubbled through for 5 min. Then 0.5 ml of 10 mM DTNB was added, final volume was made upto 3 ml. Nitrogen was bubbled for 2 minutes and the tube was stoppered, ensuring that the gas space was filled with nitrogen. After incubation for 15 min, the absorbance was read at 412 nm. Blanks contained all reactants except the protein solution. A molar absorptivity of 12,000 $M^{-1}cm^{-1}$ was used for calculating the number of sulfhydral groups formed after reduction.

Modification of tryptophan with N-bromosuccinimide (NBS)

The lectin (300μg) in 100 mM sodium acetate buffer pH 5.5, was titrated with a total of 0.1 mM of NBS prepared in the same buffer [18]. The reagent was added in five installments and the reaction was monitored spectrophotometrically by measuring the decrease in absorbance at 280 nm. The numbers of tryptophan residues modified were determined by assuming a molar absorption coefficient of 5500 $M^{-1}cm^{-1}$. The total number of tryptophan residues was also determined after denaturation of lectin in 6 M GdnHCl for 24 h both in presence and after absence of 5 mM DTT as a disulfide reducing agent. The residual activity was determined by hemagglutination. The tangent plot of percent residual activity and number of tryptophan residues modified gave the number of essential residues for sugar binding.

Modification of Lysine

a) Estimation of free amino groups using trinitrobenzenesulphonic acid (TNBS)

The reaction mixture containing 0.5 ml of lectin (150 μg) and 0.5 ml of 4% (w/v) sodium bicarbonate was incubated with 50 μl of 0.1% TNBS at 37 °C, in the dark for 2 h. The reaction was terminated by lowering the pH to 4.5 with 1 N HCl and 0.5 ml of 10% SDS was added to dissolve the precipitate formed [19]. The numbers of free amino groups were determined spectrophotometrically by assuming a molar absorption coefficient, 9950 M^{-1} cm^{-1} for trinitrophenylated lysine at 335 nm.

b) Acetylation

Acetylation was performed as described by Fraenkel–Conrat [20]. To 500 μg lectin, in 1 ml saturated sodium acetate, known aliquots of acetic anhydride (0.1-1 mM) were added over the course of one hour, after each addition, an aliquot was removed and assayed for hemagglutination activity. The numbers of amino groups modified at the end of the reaction by acetic anhydride were estimated by determining the number of residual free amino group using TNBS as described earlier.

c) Succinylation

This modification was carried out following the method of Habeeb [21]. Lectin (500 μg), in 100 mM sodium carbonate buffer pH 8.5, was incubated with varying concentration of succinic anhydride (0.5-3.0 mM) prepared in dioxane. The residual activity and the number of amino groups modified were determined as described above. Lectin incubated in the absence of succinic anhydride served as control. The tangent plot of percent residual activity and number of lysine residues modified gave the number of essential residues for sugar binding.

d) Citraconylation and decitraconylation

The amino groups of HSL were reversibly modified by citraconic anhydride according to the Dixon and Perham [22]. Citraconic anhydride was diluted in dioxane and the concentration of the diluted reagent was estimated to be 110 mM. Purified HSL (500 μg) in 1 ml of sodium carbonate buffer (100 mM), pH 8.5, was treated with a total of 25 μl of citraconic anhydride at room temperature. After each addition, an aliquot was removed and assayed for hemagglutination activity and free amino groups by TNBS. Control consisted of lectin sample incubated with same conditions without citraconic anhydride. Decitroconylation was achieved by incubating the modified lectin samples at pH 4.0, 30 ^{0}C for an hour followed by determining the amount of recovered residual activity and free amino groups.

e) Reductive methylation

This was carried out as described by Means and Feeney [23]. To 1 ml of the lectin (500 μg) in 200 mM borate buffer pH 9.0, at 0 °C, 0.1 ml of sodium borohydride solution (0.5 mg/ml) was added, followed by 6 aliquots (5 μl each) of 0.35% (v/v) formaldehyde at 10 minutes interval. The procedure was repeated using 3.5% formaldehyde. At the end of the reaction, the residual hemagglutinating activity and the number of amino groups modified were determined as described above. Lectin incubated in the absence of formaldehyde served as control.

Modification of tyrosine using N-acetylimidazole

This was performed as described by Riordan *et al* [24]. The lectin (1.5 mg) in 50 mM phosphate buffer pH 7.5 was incubated with 1000 fold molar excess of NAI at 30 ^{0}C for 30 min followed by estimation of the residual hemagglutination activity by removing the excess reagent by gel filtration, sephadex G-25 column (1x10 cm, dimension) pre-equilibrated in the same 50 mM phosphate buffer pH 7.5. The lectin incubated in the absence of NAI served as control. The tyrosine residues modified were determined spectrophotometrically, using a molar absorption coefficient of 1160 $M^{-1}cm^{-1}$ at 278 nm. The NAI mediated modification of lysine residues was determined by estimating the number of amino groups before and after the modification reaction, by the TNBS method of Habeeb [19].

Modification of histidine using diethylpyrocarbonate

300 μg of lectin in 1 ml of 20 mM phosphate buffer, pH 7.0 was treated with varying concentrations of DEP (1-5 mM), freshly prepared in absolute ethanol. Modification of histidine residues was monitored spectrophotometrically by measuring the increase in absorbance at 240 nm as described by Ovadi et al [25], using a molar absorption coefficient for carbethoxyhistidine 3200 M^{-1} cm^{-1}. The ethanol concentration in the reaction mixture did not exceed 2% (v/v) and had no effect on the activity and stability of the lectin during the incubation period. Lectin samples incubated in the absence of DEP served as control.

The DEP concentration in the diluted samples was determined by mixing an aliquot of the diluted sample with 3 ml of 10 mM imidazole buffer, (pH 7.5) followed by monitoring the increase in absorbance at 230 nm. The amount of *N*-carbethoxyimidazole formed was calculated using a molar absorption coefficient of 3000 $M^{-1}cm^{-1}$. The concentration of the diluted stock of DEP was 100 mM. The tangent plot of percent residual activity and number of histidine residues modified gave the number of essential residues for sugar binding [26].

Decarbethoxylation of the DEP modified protein was carried out according to miles [27]. The DEP treated sample was incubated with hydroxylamine hydrochloride (50 mM-1000 mM) at pH 5, 6 and 7 at 25 ^{0}C for 1-16 h and the recovered residual hemagglutination activity was determined.

Modification of carboxylate with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide / nitrotyrosine ethylester.

The HSL sample (400 μg, 1 ml) in 50 mM MES/HEPES buffer (72:25 v/v), pH 6.0. was incubated with a effective concentration of 10 mM EDC and varying concentrations of NTEE at 30 ^{0}C for 45 min. subsequently the reaction was arrested by the addition of 10% (w/v) trichloroacetic acid and the precipitated protein was collected by centrifugation, washed extensively with chilled acetone, air dried and dissolved in 100 mM sodium hydroxide. The number of nitrotyrosyl groups incorporated was determined spectrophotometrically at 430 nm, using a molar absorption coefficient of 4600 M^{-1} cm^{-1} [28].

After the incubation period, a 100 μl aliquot was taken and the excess reagent was removed by gel filtration on sephadex G-25 column. The residual haemagglutinating activity was determined. The lectin sample in same buffer incubated without EDC and NTEE served as control. The tangent plot of percent residual activity and number of carboxylate groups modified gave the number of essential residues for sugar binding.

Circular dichroism

C.D. spectra, in the range of 190-250 nm were recorded using Jasco J-710 spectropolarimeter at 25 ^{0}C with a 0.1 cm path length for native and modified lectins at a protein concentration of 2.5 μM. Modified samples were passed through gel filtration (sephadex G-25 column, 1x10 cm dimension, pre-equilibrated with the 20 mM phosphate buffer pH 7.5), for the purpose of removing the excess reagent.

Steady-state fluorescence measurments

Fluorescent measurments were recorded on a Perkin Elmer LS 50B spectrofluorimeter, with slit width of 7 nm for both excitation and emission monochromators and scan speed 100 nm /min. Measurments were recorded by irradiating a protein sample (0.3 μM) at 280 nm wavelength light and emission spectra were recorded from 300 to 400 nm. In fluorescence quenching experiments, small aliquots of 5 M stocks (acrylamide, potassium iodide and cesium chloride) and 2.5 M of succinimide of defined amounts (5-10 μL) were added to protein samples and fluorescence spectra was recorded after 3 min and each spectra was an average of 3 accumulations. The potassium iodide solution contained 0.2 mM sodium thiosulphate to prevent the formation of tri-iodide (I^-_3). The fluorescence intensity at 343 nm (λ_{max} of the lectin) was considered for all the data analysis and calculations, unless otherwise mentioned. For experiments with sugar bound lectin, sample was incubated with 200 μM of T-antigen (Gal β1-3 GalNAc), a disaccharide specifically recognized by the lectin. Fluorescence intensities were corrected for dilution factor, before analysis of the quenching data. All experiments were conducted in duplicates at 25 ^{0}C and yielded reproducible results, average values were taken and reported here.

Denaturation experiments

For quenching studies with denatured lectin, the protein was incubated with varying concentrations of GdnHCl and urea for 16 h at room temperature. 5 mM DTT was used as a disulfide bond reducing agent with different concentrations of denaturants, to ensure that whole protein structure was

opened for solute quenching studies. Quenching data for all the quenchers used in this study were analyzed by the Stern-Volmer equation (1) as well as by the modified Stern-Volmer equation (2) [29].

$$F_0/F_c = 1 + K_{sv}[Q] \quad (1)$$

$$F_0/\Delta F = f_a^{-1} + (K_a f_a)^{-1}[Q]^{-1} \quad (2)$$

where F_0 and F_c are the respective fluorescence intensities, corrected for dilution, in the absence and presence of quencher. [Q] is the resultant quencher concentration, K_{sv} is the Stern-Volmer quenching constant of the lectin for given quencher, f_a refers to the fraction of the total fluorescence that is accessible to the quencher and K_a is the corresponding quenching constant. Slopes of Stern-Volmer plots yield K_{sv} values, whereas the slopes of modified Stern-Volmer plots give $(K_a f_a)^{-1}$ and their ordinate give values of f_a.

Time-resolved fluorescence measurments

Fluorescence lifetime measurements were performed using FLS920 single photon counting spectrofluorimeter from Edinberg Instruments, Livingston, United Kingdom. A nanosecond flash lamp (nF900) was used for excitation. Samples were excited at 280 nm and emission intensities were recorded at the 343 nm (λ_{max}) of the lectin. Slit widths of 15 nm were used on both the excitation and emission monochromators. The resultant decay curves were analyzed by a multiexponential iterative fitting program supplied by Edinburg Instruments.

The average life times were calculated by force fitting the decay curves to a single exponential function and were found to be comparable to the decay times obtained from following equations,

$$\tau = \Sigma_i \alpha_i \tau_i / \Sigma_i \alpha_I \quad (3)$$

$$\langle\tau\rangle = \Sigma_i \alpha_i \tau^2_i / \Sigma_i \alpha_i \tau_i \quad (4)$$

where τ and $\langle\tau\rangle$ are the average fluorescence lifetimes obtained by two different approaches.

RESULTS

Modification of tryptophan residues

Modification of tryptophan residues with NBS led to complete loss of hemagglutination activity of HSL. The loss of activity was concentration dependent. A plot of percent residual activity against number of tryptophan residues modified showed that the modifications of two tryptophan residues per molecule of the lectin are essential for the activity (Fig. 3.1A). Moreover the C.D. spectra of the native and modified lectins (Fig. 3.1B), indicated no change in the secondary structure of the lectin.

Modification of lysine residues

Modification of amino groups of HSL by acetic, succinic and citraconic anhydrides led to concentration dependent loss of hemagglutination activity. A plot of residual activity of the lectin versus the number of amino groups modified by succinylation and citraconylation indicated that six amino groups per molecule were essential for activity (Fig. 3.2A). Decitraconylation of the inactivated lectin, at pH 4.0, brought about 75% of the restoration of activity after an hour (Table 3.1).

Table 3.1: Effect of amino group modification of HSL.

Reagent	No. of residues modified /molecule	Residual hemagglutination activity (%)	M_r
Acetic anhydride	177	0	Unchanged
Succinic anhydride	161	0	Unchanged
Citraconic anhydride	165	0	Unchanged
Decitraconylation	00	75	-
Reductive methylation	162	100	-

Molecular mass of the succinylated and citraconylated lectin, determined by gel filtration showed no significant change (Fig. 3.3). The peptide backbone structure of the lectin on lysine modification did not show any gross change, as indicated by C.D. analysis (Fig. 3.2B). However, reductive methylation of the lectin did not result in any loss of hemagglutination activity even after 162- lysine residues modification. Conversion of lysine groups to N-monomethyl and N, N'-dimethyl lysine by reductive methylation had no effect on the agglutination activity. Reductive methylation did not lead to any drastic change in the pK_a of the ε-amino group of lysine nor did it lead to neutralization / reversal of the positive charge as with acetylation, succinylation and citraconylation.

Modification of carboxylate group

The carboxylate groups were modified with EDC and estimated using NTEE, resulted in complete loss of hemagglutination activity, because of modification of two active residues per molecule of the protein (Fig. 3.4). The peptide backbone did not undergo any change after modification as indicated by the C.D.

Modification of histidine residues

Modification of lectin with DEP resulted in the total loss of activity on modification of two histidine residues per molecule of the lectin. However, the lost activity could not be recovered even after incubating with different concentrations of hydroxylamine hydrochloride (0.05 –1 M), with pH ranging from 5.0-7.0 for 1-16 h. However, the estimation of residual amino groups with TNBS following DEP treatment indicated the modification of lysine residues. The loss in activity due to DEP modification is attributed to lysine modification but not histidine residues.

Modification of arginine, serine, cysteine and tyrosine residues:

Modification of arginine residues by phenyl glyoxal and serine with PMSF did not cause any loss of the lectin activity (Table 3.2). Cysteine residues modified by DTNB, and tyrosine with NAI also not leads to any loss in activity.

Table 3.2: Chemical modification of the HSL.

Reagent	Conc. of reagent used	Residue modified	Residual activity (%)
HSL-native	-	-	100
NBS	100 μM	Trp	0
NAI	10.0 mM	Tyr	100
PMSF	5.0 mM	Ser	100
DTNB	1.0 mM	Cys	100
DEP	5.0 mM	His	0
EDC	10.0 mM	Carboxylate group	0
Phenylglyoxal	3.0 mM	Arg	100
TNBS	0.1%	Lys	0
Acetic anhydride	1.0 mM	Lys	0
Succinic anhydride	3.0 mM	Lys	0
Citraconic anhydride	2.0 mM	Lys	0
Decitraconylaion	pH 4.0	Lys	75
Formaldehyde	3.5%	Lys	100

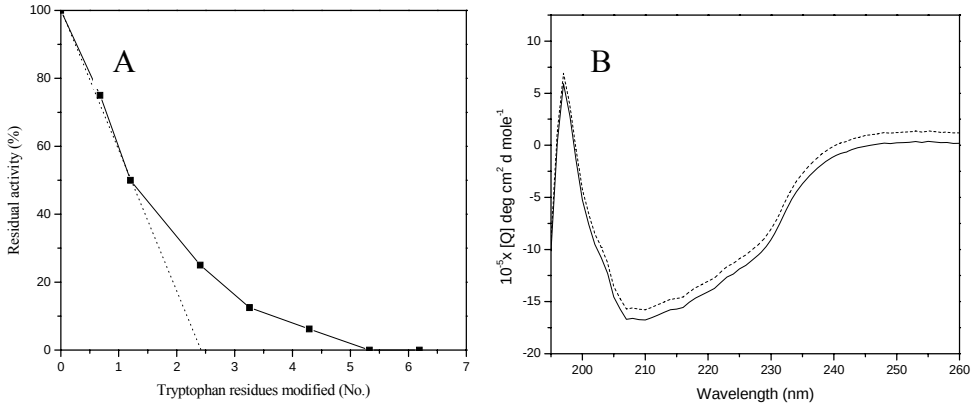

Fig. 3.1. Modification of tryptophan residues by NBS.

A) Residual hemagglutination activity of HSL after modification with NBS. Changes in the activity of HSL as a function of modified tryptophan residues.

B) Far UV C.D. spectra of native and NBS modified lectin. Spectra of 2.5 μM modified lectin in phosphate buffer, pH 7.5 was recorded on a Jasco-710 spectropolarimeter. Native lectin (—) and NBS modified lectin (…).

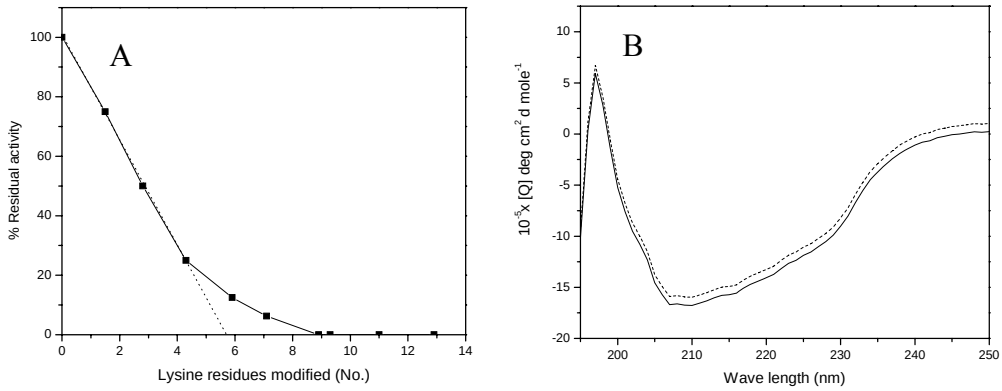

Fig. 3.2. Modification of lysine residues by succinic anhydride.

A) Effect of succinylation on HSL hemagglutination activity. Changes in the activity of HSL as a function of modified lysine residues.

B) Far UV C.D. spectra of native and succinic anhydride modified lectin. Spectra of 2.5 μM modified lectin recorded on a Jasco-710 spectropolarimeter. Native (—) and succinylated lectin (….).

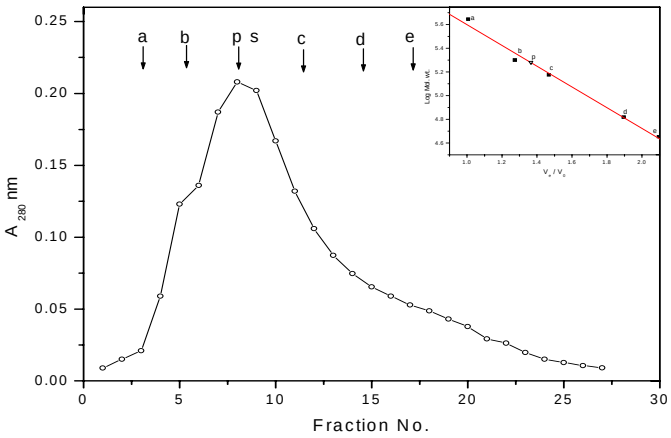

Fig.3.3. Elution profile of native and modified HSL on gel filtration (sephacryl S-300 column, 1.5 cm x 180 cm): Inset shows the molecular plot. The column was equilibrated with THS (pH 8.5), and calibrated with (a) appoferritin (443,000), (b) β- amylase (200,000), (p) native protein and (s) succinylated protein, (c) alcohol dehydrogenase (150,000), (d) BSA (66,000) and (e) ovalbumin (45,000).

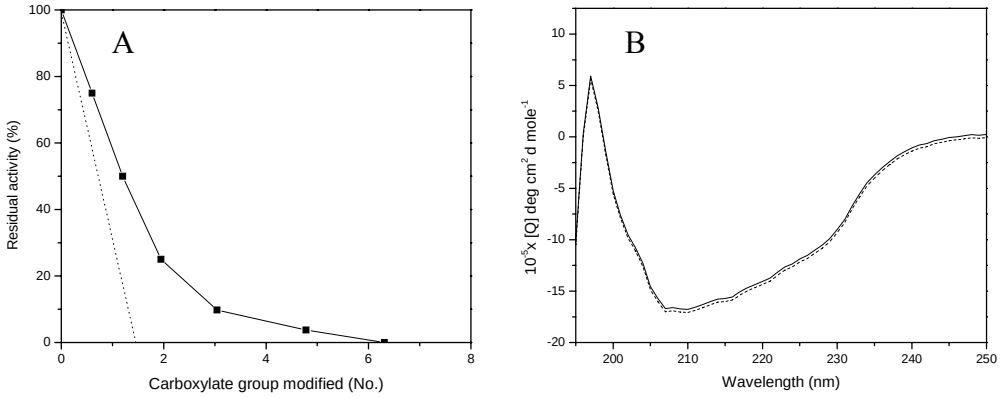

Fig. 3.4. Modification of carboxylate groups by EDC and NTEE.

A) **Effect of EDC and NTTE on HSL hemagglutination activity.** Changes in the activity of HSL as a function of modified carboxylate groups.

B) **Far UV C.D. spectra of native and EDC and NTTE modified lectin.** Spectra of 2.5 μM modified lectin in phosphate buffer, pH 7.5 was recorded on a Jasco-710 spectropolarimeter. Native lectin (—) and EDC modified lectin (….).

Solute Quenching Studies

We observed that the modification of tryptophan residues with *N*-bromosuccinimide led to a complete loss of the hemagglutination and sugar binding activity of the HSL. Also ascertained by spectroscopic studies that NBS specifically oxidized only indole side chain of the tryptophan residues, and the modification does not impose any gross conformational change of the lectin. These experiments, however, suggested a change in the exposure of the tryptophan residues that were accessible for the oxidation before and after ligand binding. Tryptophan residues appear to be uniquely sensitive to quenching by a variety of solutes because of propensity of the excited indole nucleus to donate electrons. There are 12 tryptophan in native, 16 on denaturation with 6 M GdnHCl and 22 residues upon denaturation and reduction with 6 M GdnHCl containing 5 mM DTT.

Quenching of intrinsic fluorescence emission of HSL

Quenching of the intrinsic fluorescence of proteins by a neutral quencher (acrylamide and succinimide), anionic quencher (iodide ion I^-) and cationic quencher (cesium ion, Cs^+) has been used as a probe to study the tryptophan microenvironment in the proteins. The data were analysed by the Stern-Volmer equation. The fluorescence emission spectra of native lectin and denatured lectin with different concentration of denaturant was recorded with increasing concentrations of acrylamide are shown in Fig 3.5, in both the cases spectrum one is native lectin in absence of the quencher and where as 2-11 are with increasing concentration of acrylamide, with last spectrum corresponding to an final acrylamide concentration of 0.5 M.

A comparison of the spectra shows that the emission λ_{max} of the native lectin was at 343 nm and red shifted to 354 nm with 6 M GdnHCl and to 356 nm on complete denaturation with 6M GdnHCl or 8 M urea containing 5 mM DTT. Furthermore, spectra also indicate increased percentage of quenching upon complete denaturation of HSL.

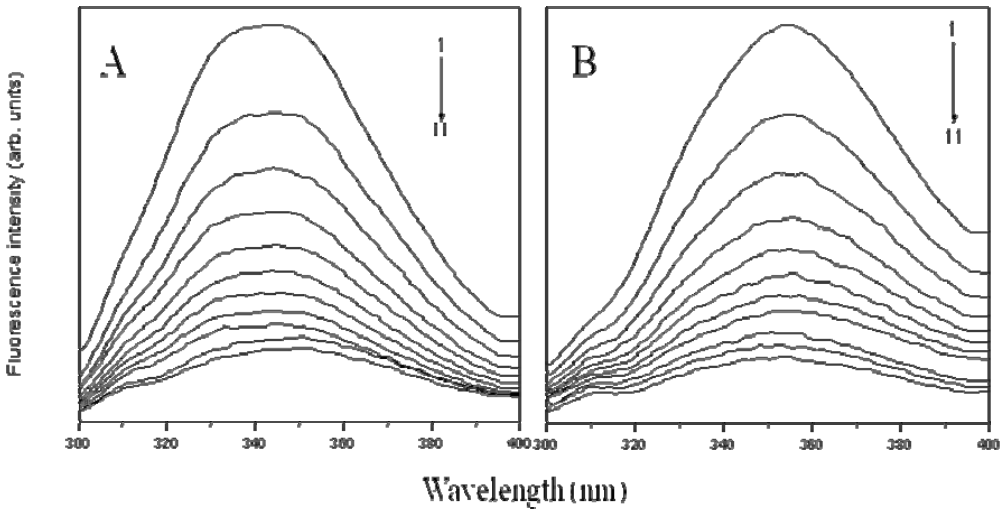

Fig: 3.5. Fluorescence emission spectra of HSL in the absence and presence of acrylamide: (A) under native condition (B) under denaturing condition (6 M GdnHCl). Spectrum 1 corresponds to the native lectin and spectra 2-11 corresponds to the lectin in the presence of increase in concentrations of acrylamide. The final concentration of the quencher in both A and B is 0.5 M.

Table 3.3: Extent of quenching of tryptophan fluorescence of HSL with different quenchers under different conditions.

Quencher	Quenching (%)[a]		
	Native	With ligand	In 6 M GdnHCl +5 mM DTT
Acrylamide (0.46 M)	68	62	94
Succinimide (0.30M)	42	38	82
CsCl (0.30 M)	22	19	52
KI (0.30 M)	29	24	68

[a]Quenching (%) was calculated from raw data.

Table 3.4: Summary of quenching parameters obtained from the intrinsic fluorescence quenching and time-resolved fluorescence of HSL.

Quencher and condition	K_{sv} (M^{-1})	K_q x10^9 (M^{-1})	K_s (M^{-1})	f_a	K_a
Acrylamide					
Native protein	6.16	1.43	1.6	1	14.44
With ligand	5.23	1.29	1.49	1	17.40
In 1M GdnHCl	7.84	2.08	2.14	1	17.39
In 6M GdnHCl +5 mM DTT	12.38	4.02	3.03	1	29.58
Succinamide					
Native protein	5.73	1.33		0.62	8.25
With ligand	4.62	1.14		0.59	9.01
In 1M GdnHCl	7.12	1.89		0.71	7.45
In 6M GdnHCl +5 mM DTT	9.14	2.96		0.91	13.44
Cesium ion					
Native protein	3.03	0.70		0.46	7.14
With ligand	2.81	0.69		0.40	7.86
In 1M GdnHCl	3.73	0.99		0.52	11.40
In 6M GdnHCl +5 mM DTT	4.28	1.39		0.75	18.34
Iodide ion					
Native protein	4.19	0.97		0.56	8.13
With ligand	3.86	0.95		0.53	8.89
In 1M GdnHCl	5.19	1.38		0.63	12.60
In 6M GdnHCl +5 mM DTT	6.71	2.17		0.88	17.76

Under native conditions, of the four quenchers used, acrylamide was the most effective quencher, causing 68% of the total intrinsic fluorescence quenching of the protein, whereas bulkier succinimide quenched only 42%. The ionic quenchers, cesium and iodide ions, which can not penetrate into the protein matrix and can access only surface exposed tryptophan, were found to quench only 22.5% and 29% respectively (Table 3.3). In the presence of 200 μM T-antigen, which specifically binds to HSL, quenching with acrylamide, succinimide, Cs^+ and I^- decreased marginally to 62%, 38%, 19% and 28% respectively.

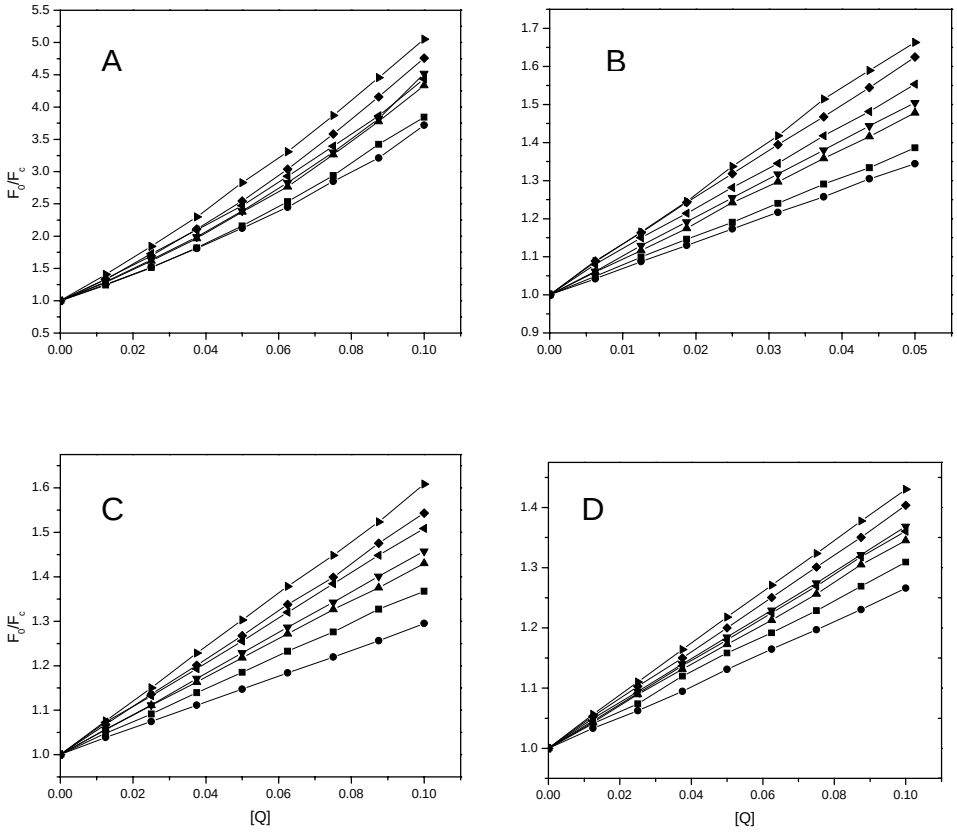

Fig 3.6. Stern-Volmer plots of fluorescence quenching for HSL. The different quenchers used are (A) acrylamide (B) succinimide (C) iodide ion and (D) cesium ion. Native lectin (●), with ligand (■), 1 M GdnHCl (▲), 3 M GdnHCl (▼), 6 M GdnHCl (♦), 3 M GdnHCl +5 mM DTT (◄) and 6 M GdnHCl +5 mM DTT (►).

Denaturation with guanidium or urea resulted in a significant increase in quenching with all the quenchers studied. Quenching with acrylamide was increased to 73% (1 M GdnHCl) to 94% with 6 M GdnHCl containing 5 mM DTT. Even with succinimide, cesium and iodide the quenching was increased to 82%, 52% and 68% respectively (Table 3.3). After denaturation, some residual confirmation was still present in the lectin, which could be preventing the access of the neutral and charged quenchers to the tryptophan.

Stern-Volmer analysis of quenching data

Quenching data for all the quenchers used in this study were analysed by the Stern-Volmer equation (1) as well as modified Stern-Volmer equation (2). The Stern-Volmer plots for the quenching of the HSL in absence and presence of 200 μM T-antigen with acrylamide, succinimide, cesium and iodide ions are shown in Fig. 3.6. The quenching profiles obtained with, succinimide, cesium and iodide ions follow linear dependence on the quencher concentration (Fig 3.6 B-D) from the slopes of which the Stern-Volmer quenching constants were determined. This linear type of quenching profile indicates collisional type of quenching. On the other hand, the profile obtained with acrylamide both in native and under denaturing conditions exhibit biphasic pattern or positive curvature (Fig 3.6A), indicating quenching has both dynamic and static components. The quenching data then can be analysed by equation (3), which allows resolution of the static and dynamic components [30];

$$F_0/F_c = (1 + K_{sv}[Q])\,(1 + K_s[Q]) \qquad (5)$$

where K_{sv} is the Stern-volmer (dynamic) and K_s is the static quenching constants.

The dynamic portion of the observed quenching can be determined by lifetime measurments, according to the equation [30],

$$\tau_0/\tau = (1 + K_{sv}[Q]) \qquad (6)$$

where τ_0 is the average lifetime in the absence of the quencher and τ is the average lifetime in the presence of the quencher at a concentration [Q]. Using the average lifetimes obtained from analysis of the time-resolved

fluorescence measurments described in Eq. (3), the K_{sv} value for acrylamide was obtained as 6.16 M^{-1} in native and 12.38 M^{-1} under denaturation condition. The static quenching constant K_s, was then obtained by incorporating the K_{sv} value in Eq. (5) and fitting the quenching data, as 1.6 and 3.03 M^{-1} respectively. The bimolecular quenching constant, K_q was calculated as $K_q = K_{sv} / \tau_0$ [31] and was found to be 1.43 and 4.02 x10^9 s.M^{-1} respectively. K_{sv} and K_s values were incorporated in the expression (1 + K_{sv} [Q]) (1+ K_s [Q]), the values obtained were plotted against [Q]. It was observed that the values of F_0/F_c and (1 + K_{sv} [Q]) (1+ K_s [Q]) match very well.

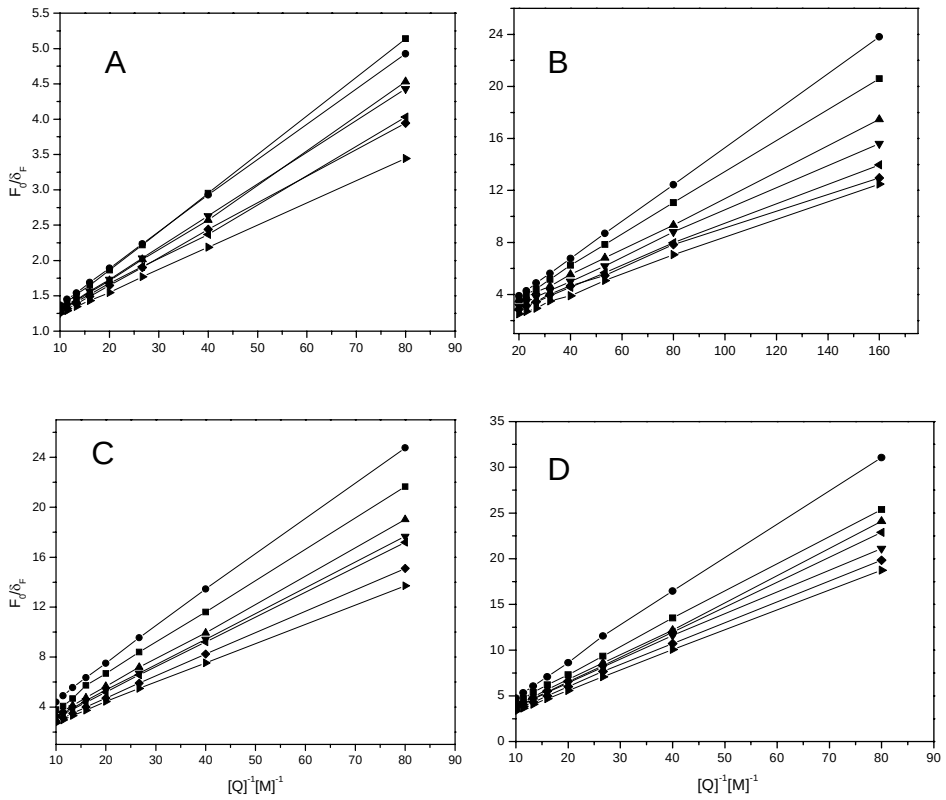

Fig 3.7. Modified Stern-Volmer plots of fluorescence quenching for HSL. The different quenchers used are (A) acrylamide (B) succinimide (C) iodide ion and (D) cesium ion. Native lectin (●), with ligand (■), 1 M GdnHCl (▲), 3 M GdnHCl (▼), 6 M GdnHCl (♦), 3 M GdnHCl +5 mM DTT (◄) and 6 M GdnHCl +5 mM DTT (►).

From the slopes of the linear components of the Stern-Volmer plots, collisional quenching constant K_{sv} was obtained for succinimide, Cs^+ and I^- and are listed in table 3.4. Under native condition, the K_{sv} for succinimide was 5.73 M^{-1} and increased to 9.14 M^{-1} under denaturation condition. Among ionic quenchers, I^- showed K_{sv} value of 4.19 M^{-1} and Cs^+ as 3.03 M^{-1} under native condition, indicating comparatively iodide ion to be a better quencher than cesium cation, but the differences are not significant.

The modified Stern-Volmer plots obtained for all the four quenchers are given in Fig.3.7. From the *Y*-intercept of all these plots, f_a, the fraction accessibility and K_a, value of corresponding quenching constant were calculated using Eq.(2) and listed in Table 3.4. Based on the f_a values of the four quenchers used, in native HSL 100% fluorescence intensity was accessible to acrylamide, while 62% was available for succinimide. I^- and Cs^+ could access only 56% and 46% respectively. Complete denaturation of HSL with 6 M GdnHCl containing 5 mM DTT led to 91% accessibility with succinimide, whereas with iodide and cesium ions the fraction of accessibility was increased to 88% and 75% respectively.

Lifetime measurments of fluorescence emission

Fluorescence decay curves of native lectin, with ligand (T-antigen) bound, with 0.5 M acrylamide and acrylamide quenching in presence of ligand were studied using time resolved fluorimetry (Fig. 3.8). The decay curves were analysed by a multi-exponential iterative program, in all the cases the decay profiles could be best fitted to a biexponential function ($\chi^2 \leq 1.05$), monoexponential fits gave significantly larger errors ($\chi^2 \geq 2.0$).

Biexponential fits for HSL showed two decay times τ_1 (1.77 ns) with 22.80% and τ_2 (4.98 ns) with 77.20% contribution for Trp fluorescence and the corresponding relative amplitudes α_1 and α_2, indicating presence of more than one type of fluorophores differentially emitting the energy. The longer duration lifetime contributes to the major fluorophores (Table 3.5). In the presence of ligand (T-antigen), marginal increase in both lifetimes (τ_1=2.08 ns and τ_2=5.10) was observed, indicating the presence of Trp at the ligand-

binding site, influences the accessibility for fluorescence. On quenching with 0.5 M acrylamide the shorter lifetime decreased substantially (1.01 ns), while longer lifetime decreased drastically from 4.98 to 1.07 ns. Whereas under ligand protection the shorter lifetime decreased to 0.96 ns but the longer lifetime decreases marginally (2.76 ns).

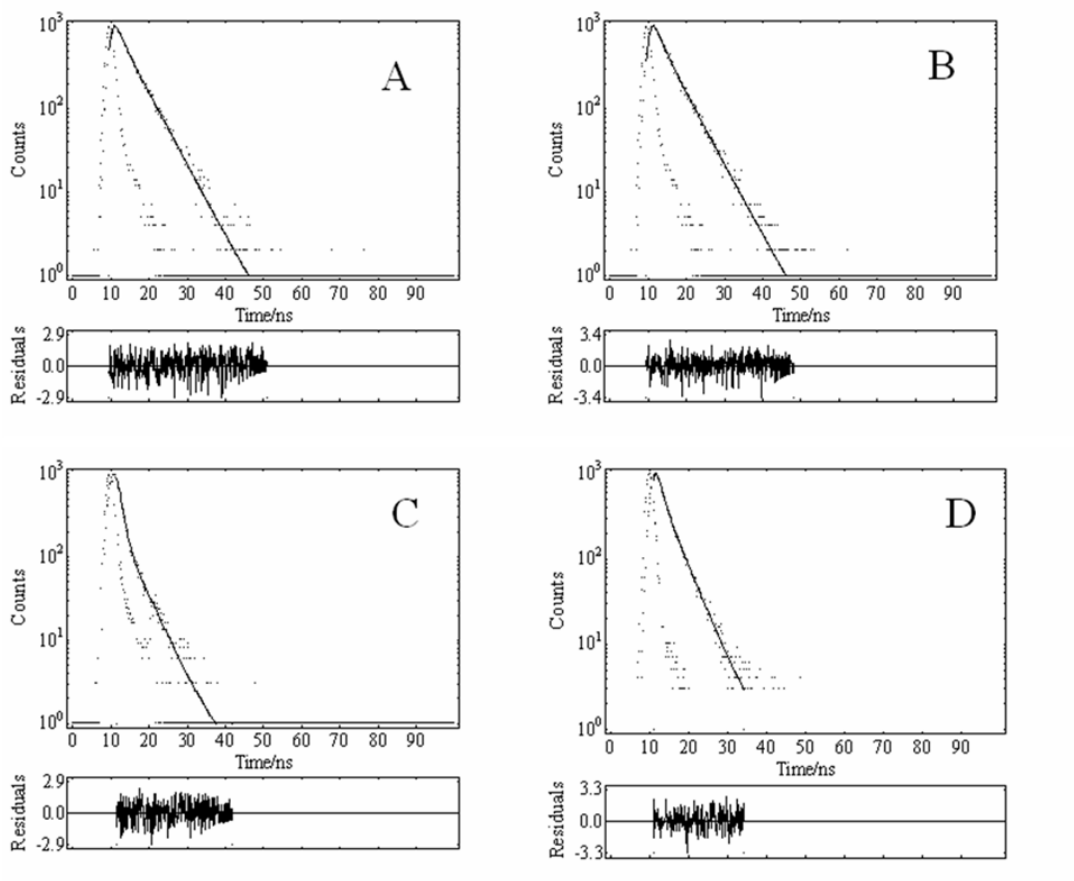

Fig.3.8. Representative time resolved fluorescence intensity decay of HSL. (A) Native HSL, (B) HSL coupled with ligand (T-antigen), (C) HSL quenched with 0.5 M acrylamide and (D) *N*-bromosuccinimide modified HSL. Y-axis represents photon counts in logarithmic scale. The fast decaying line represents the IRF (instrument response function) used as the excitation source. The slower decaying line represents the experimental fluorescence decay curve.

Table 3.5: The lifetime measurments of fluorescence decay of HSL and the corresponding pre-exponential factors along with calculate average lifetimes for acrylamide quenching under native condition and under ligand protection.

	HSL (native)					HSL with 200 μM bound T-antigen				
[Q] (M^{-1})	α_1	τ_1 (ns)	α_2	τ_2 (ns)	$<\tau>$	α_1	τ_1 (ns)	α_2	τ_2 (ns)	$<\tau>$
0.0	0.032	1.77	0.039	4.98	4.30	0.037	2.08	0.034	5.10	4.05
0.01	0.032	1.67	0.042	4.61	4.05	0.039	2.05	0.032	4.78	3.69
0.02	0.044	1.55	0.035	4.32	3.54	0.043	1.97	0.033	4.57	3.49
0.05	0.040	1.43	0.035	3.99	3.36	0.043	1.83	0.031	4.18	3.18
0.10	0.050	1.21	0.032	3.28	2.69	0.061	1.68	0.022	3.86	2.48
0.25	0.044	1.19	0.033	2.98	2.51	0.054	1.45	0.026	3.28	2.38
0.50	0.041	1.01	0.021	1.19	1.11	0.064	0.96	0.020	2.76	2.07

The average lifetime ($<\tau>$) of the fluorescence decay of the native protein under acrylamide quenching reduces to 1.11 ns from 4.30 ns, whereas under ligand protection reduces to 2.07 ns. It clearly indicates that Trp present at the sugar-binding site are of the longer duration lifetimes, which are protected for quenching or modification. Denaturation with 6 M GdnHCl containing 5 mM DTT the relative lifetime contributions are varied drastically, the τ_1 contribution increased from 22.80% to 45%, while τ_2 decreases to 55% from 77.20%. This can be due to the change in the confirmation of the protein, under denaturing condition and increasing the fluorophores accessibility.

Table 3.6: The lifetime of fluorescence decay of HSL and the corresponding pre-exponential factors along with calculate average lifetimes for NBS quenching of native protein and under denatured condition.

	NBS modified HSL					Denatured with 6 M GdnHCl + 5 mM DTT				
[Q] (nM^{-1})	α_1	τ_1 (ns)	α_2	τ_2 (ns)	$<\tau>$	α_1	τ_1 (ns)	α_2	τ_2 (ns)	$<\tau>$
0.0	0.055	1.77	0.024	4.98	3.44	0.059	1.69	0.029	4.33	3.08
0.01	0.054	1.65	0.026	4.74	3.43	0.054	1.62	0.033	4.02	3.04
0.02	0.053	1.62	0.029	4.68	3.51	0.050	1.59	0.035	3.78	2.94
0.03	0.052	1.58	0.030	4.51	3.44	0.053	1.53	0.040	3.62	2.89
0.04	0.055	1.38	0.030	4.46	3.49	0.053	1.40	0.032	3.32	2.58
0.05	0.056	1.29	0.032	4.32	3.47	0.055	1.33	0.035	3.05	2.42
0.06	0.059	1.17	0.035	4.16	3.45	0.050	1.29	0.040	2.86	2.38
0.07	0.059	1.12	0.038	4.05	3.44	0.056	1.15	0.038	2.18	1.82
0.08	0.061	1.10	0.036	3.89	3.26	0.059	1.09	0.036	1.78	1.51

The lectin with one Trp modified (with NBS) and 50% residual hemagglutination activity was analysed for the lifetime measurments of fluorescence decay. The shorter lifetime was reduced to 1.62 ns from 1.77 ns, while longer lifetime reduced to 4.68 ns from 4.90 ns. Whereas modifications of 7 Trp residues resulted in drastic decrease in shorter lifetime and longer one reduces marginally (Table 3.6). After denaturation and modification of 12 Trp residues both the lifetimes decreased drastically.

DISCUSSION

The carbohydrate-binding site(s) of a lectin contains amino acids with side chains that contributed to the association of specific saccharide groups with that lectin, in a manner similar to an enzyme-substrate association. Chemical

modifications of active-site amino acid chains will alter their properties and bring about a change in the biological activity of the protein, if they contributed to it. This change in activity can be measured for a variety of modifications of different amino acid side chains, and thus an idea of those involved in the biological activity can be obtained.

Oxidation of tryptophan by addition of highly reactive bromonium ions (Br^+) by the NBS [32] in the lectin leads to a complete loss of hemagglutination activity. Which suggests that tryptophan may have a role in lectin activity. The role of tryptophan was evaluated by modifying the lectin with *N*-bromosuccinimide, which resulted in the modification of two Trp residues with concomitant inactivation of lectin. Furthermore, the C.D. spectra of the NBS modified lectin did not show any change in its peptide backbone. Modification of tryptophan and histidine residues of sialic acid binding lectin from hemolymph of *Achatine fulica* led to a complete loss of hemagglutination activity [33].

Amino group substitution of HSL with TNBS led to complete loss of the activity, indicating involment of lysine in the activity of the lectin. Acetylation of lectin with acetic anhydride also led to total loss of hemagglutination activity. As acetic anhydride is not specific for lysyl residues but also modifies tyrosine, the role of lysine was evaluated by modifying with succinic and citraconic anhydrides since these reagents show a preference for lysyl residues [34]. Both succinylation and citraconylation leads to inactivation of the lectin with modification of six lysine residues per molecule of the lectin. Decitraconylation of the modified lectin leads to a 75 % restoration of activity. Moreover, the C.D. spectra of the modified lectin were similar to that of the native lectin, which indicated that the loss of activity was due to lysine modification and not as result of any structural changes. Modification of amino groups of hemolytic lectin CEL-III by succinic anhydride showed their involvement in oligomerization of lectin [1].

Treatment with formaldehyde converts the ε-NH_2 group of Lys to *N*-monomethyl and *N*, *N'*-dimethyl lysine by reductive methylation. The process

does not affect the positive charge on the nitrogen atom. On the contrary, acetylation renders the amino group electrically neutral while succinylation and citraconylation converts it to anionic derivatives. The agglutination activity was not affected by reductive methylation but could be abolished by treatment with acetic, succinic and citraconic anhydrides. These results indicate the importance of positive charge on the Nε of the essential Lys.

In concanavalin A, lysine modification resulted in the dissociation of the lectin from dimer to monomers [35]. Substitution of the positive charge of amino groups by a negative charge often leads to changes in the electosatic interactions and frequently dissociation into oligomers [36]. Whereas, even though HSL is a monomer, the elution profile of the native and lysine modified lectin samples on sephacryl S-300 (Fig. 3.3), did not show any change in the molecular size.

The reaction of histidine (imidazole group) with the diethyl pyrocarbonate, led to total loss of the lectin agglutination activity, from the modification of two histidine residues per molecule of the protein. However, DEP also acts on cysteine, lysine and tyrosine. Hence, only reversal of the inactivation by hydroxylamine hydrochloride could confirm the involment of histidine residues in the sugar binding activity. Attempts made to reverse the DEP mediated inactivation using varying concentrations of hydroxylamine hydrochloride, at range of pH values at different time intervals proved futile, suggesting that the loss of activity may be due to the modification of other residues. Moreover, the cysteine modification was ruled out since no increase in absorbance at 412 nm of DEP modified lectin was observed. The chance of tyrosine modification by DEP was ruled out by observing that there was no net decrease in the absorbance at 278 nm. However, the estimation of free amino groups with TNBS following DEP treatment indicated the modification of lysine residues. The failure of hydroxylamine hydrochloride to reactivate a DEP modified protein could be ascribed to the modified lysine residues (21). Furthermore reversible blocking of amino groups by citraconic anhydride prior to carbethoxylation resulted in no loss of activity; confirming that histidine is not involved in the sugar binding activity of the lectin.

The conversion of protein carboxyl group into amides in near-quantitative yield provides a convenient means for determining number of carboxyl groups in proteins [37]. The carboxylate groups were modified using EDC, which resulted in complete loss of hemagglutination activity. Two carboxylate groups per molecule of the protein, estimated using NTEE are essential for hemagglutinating activity. The secondary structure of the lectin was not disturbed as seen in C.D. spectrum.

In the present studies, acetylation by NAI did not lead to a concomitant modification of lysine and tyrosine residues in HSL, instead only tyrosine was modified. Extensive modification of lysine residues by NAI has been reported in the lentil lectin [38] contrary to Con A [39] where NAI modified two tyrosine residues with no loss of agglutination activity.

The dynamics of the steady state fluorescence of HSL was resolved to correlate the photophysical parameters of the lectin to its structural properties. The spectrum of the native lectin shows maximum fluorescence intensity between 338 and 346 nm indicating different populations of Trp exposed differentially to the polar environment. Quenching of the intrinsic fluorescence of proteins by a neutral quencher (acrylamide and succinimide), anionic quencher (iodide ion I^-) and cationic quencher (cesium ion, Cs^+) has been used as a probe for tryptophan microenvironment in proteins. Significantly lower quenching observed with the neutral quencher succinimide, may be due to the bulkier nature of the molecule. The lower quenching of HSL fluorescence intensity with charged quenchers indicated most of the tryptophan residues in the protein are buried in hydrophobic core. The difference in quenching percentages between the ionic quenchers was not significant, which indicates that the exposed or partially exposed Trp residues are surrounded equally with positive and negatively charged residues. The quenching profiles of succinimide, cesium and iodide ions follow linear dependence of quencher concentration, indicating collisional quenching, whereas acrylamide quenching with both static and dynamic components are reflected in biphasic or positive curvature.

HSL saturated with T-antigen led to marginal decrease in accessibility of Trp residues to all the four quenchers, indicating Trp is present in the sugar-binding site of the lectin and few of these residues are protected upon ligand binding. This is consistent with the result from chemical modification with NBS and the CD spectra, wherein tertiary structure of HSL at the 293 nm Trp region was significantly altered. Also in lifetime fluorescence decay wherein marginal increase in both lifetimes was observed. The complete accessibility of Trp residues to acrylamide and increased access to other quenchers indicated the exposure of buried Trp residues after denaturation of the protein. Even then, some residual confirmation was present in the lectin, which prevents the access of the neutral and charged quenchers to the tryptophan completely.

The intrinsic emission decay of the lectin was studied in nanosecond domain and could be described by two decay components τ_1 and τ_2 and the corresponding relative amplitudes α_1 and α_2 were obtained from reconvolution fit. HSL showed two decay times indicating the presence of more than one fluorophores differentially emitting the energy, and the longer lifetime contributes to the major quantum yield. Multiexponential decay of tryptophan can be either due to the presence of tryptophan in several different rotamers that are quenched to different extent by functional groups (peptide backbone and amino acid side chains) in the microenvironment surrounding the indole ring or due to heterogeneity of the environments of various tryptophan residues in multitryptophan proteins. In HSL, biexponential decay can be explained based on existence of Trp in different rotamers/ conformers. The Trp present in the sugar-binding site are mostly buried and available for long duration decay only.

In conclusion, the indole group of tryptophan, positive charge of the ε-amino group and negative charge of the carboxylate are essential for the hemagglutination activity of HSL. Together these residues may be involved in the docking and bonding of sugars to lectin with both electrostatic and hydrophobic interactions. HSL, a multi-tryptophan protein shows maximum fluorescence intensity between 338-346 nm, indicating presence of several

populations of Trp, few of which are differentially exposed to the polar environment and remaining are in the hydrophobic environment. The presence of T-antigen resulted in marginal differences in the quenching profiles and in the fluorescence decay lifetimes, indicating presence of Trp at the sugar-binding site. Mostly these Trp residues are buried in the hydrophobic core of the protein where they exhibit longer lifetime of decay. The quenching of the fluorescence by acrylamide showed involvement of both static and dynamic components. Trp present on the surface of the protein are equally surrounded with both positively and negatively charged residues. The heterogeneity of the Trp environment was reflected into the lifetime of the fluorescence of the native as well as after treatment of the enzyme with denaturant or quenchers.

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CHAPTER : 4

EQUILIBRIUM BINDING STUDIES OF HSL: FLUORESCENCE SPECTROSCOPY AND SURFACE PLASMON RESONANCE

SUMMARY

The affinity and kinetics of sugar binding of the lectin was studied by fluorescence and surface plasmon resonance spectroscopy. The lectin binds with higher affinity to MeαGal, T-antigen and galactosyl Tn-antigen with a distinction between β 1-4 and β 1-3 linkages. T-antigen α-methyl glycoside was the most potent ligand having the highest affinity (*K*a 8.32 x10^7 M^{-1}). Monosaccharide binding is enthalphically driven while disaccharide binding involves both entropic and enthalpic contributions. The HSL preferentially binds to *O*-linked than *N*-linked asialo glycans, and the affinities were relatively higher than for sialylated glycans and glycoproteins. Thermodynamic and kinetic analysis indicates 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.

INTRODUCTION

Many biological recognitions 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 the forces controlling the saccharide-protein interaction. One of the best-characterized carbohydrate-mediated recognition of the cell surface receptor is the association between lectins, the highly specific carbohydrate-binding proteins, and the carbohydrate moiety of the glycolipids and glycoproteins present on the cell membrane. 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 [1-3]. 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 [4, 5].

The sugar /ligand binding studies can be carried out by hemagglutination inhibition, fluorescence spectroscopy and surface plasmon

resonance. As hemagglutination inhibition study provide semi-quantitative information, whereas fluorescence of tryptophan is influenced by its environment, hence changes in the tryptophan micro-environment upon ligand binding can change fluorescence properties, which can be measured by fluorescence spectroscopy [6]. In addition to the advantage of studying carbohydrate-protein interactions at equilibrium without physical separation of the bound ligand from the free ligand and the protein [7]. Surface plasmon resonance spectroscopy 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 and also the kinetics and the strength of the interaction [8]. 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 binding affinity, thermodynamic, kinetic and activation parameters for the binding of HSL with several ligands using a combination of fluorescence and surface plasmon resonance spectroscopy.

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-mellibiose, 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, certified grade CM5 sensor chip and amine coupling kit (*N*-ethyl-*N'*-(dimethylaminopropyl-) carbodiimide hydrochloride and *N*-hydroxysuccinamide) 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; triantennary *N*-glycan from fetuin and biantennary *N*-glycan from fibrinogen were purified and the homogeneity of the preparation was checked by HPLC [9, 10]. All other reagents were of analytical grade.

METHODS

Protein determination

Protein concentrations were determined according to Bradford [11] using BSA as standard.

Purification of HSL

The lectin was purified from coelomic fluid of *Holothuria scabra* as described earlier (Chapter 2). All fluorimetry experiments were carried out in TBS buffer pH 8.5 and all solutions including protein were filtered through 0.45 μm filter before using.

Neutral sugar estimation

The sugar solutions (200 μl) were incubated with 200 μl of 5% (w/v) phenol for 10 min at room temperature. One ml of sulfuric acid was then added and the mixture was allowed to cool for 20 min at room temperature. The colour developed was then measured spectrophotometrically, at 490 nm, using galactose-mannose (4:3) as standard [12].

Preparation of glycans

One gm of the glycoprotein was dissolved in 100 ml of 20 mM TBS buffer pH 7.2 containing 0.5% w/v sodium azide and digested by 50 mg of pronase-E at 37 °C for 72 h, 20 mg of pronase was added after every 24 h. The digest was lyophilized, dissolved in 5 ml of 100 mM acetic acid, centrifuged (10,000 *g*, 20 min), supernatant was collected. The pellet was re-extracted five times in 1

ml of 100 mM acetic acid. Two ml of clear supernatant was loaded on sephadex G-25 column (1.5 × 100 cm) pre-equilibrated with 20 mM acetic acid, and eluted with the same buffer at the flow rate of 20 ml/h. The fractions (2 ml) were collected and those showing presence of sugar were pooled and further digested by carboxypeptidase (10 U at pH 7.0 and 25 °C for 24 h) and aminopeptidase (10 U at pH 8.5 and 25 °C for 24 h). The residual peptides were removed by chromatography on Dowex-50 column (1.5 × 4 cm) in 20 mm acetic acid [10, 13]. Desialylation of glycopeptides was carried out by incubating with 5 U of neuraminidase in TBS buffer, pH 7.2 at 37 °C for 4 h, the enzyme and sialic acid were removed by successive chromatography on Sephadex G-25 (1.5 × 10 cm) and Dowex-50 as described above.

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 (± 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 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.

The association constants were calculated according to the method described by Chipman et al. [14]. The abscissa intercept of the plot of log $[C]_f$ against log $\{(\Delta F)/(F_c - F_\infty)\}$, where $[C]_f$ is the free ligand concentration, yielded pK_a value for lectin-ligand interaction according to the relationship [14],

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

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 ligand concentration, 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,

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

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,

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

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 interactions 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 the amine coupling kit. Unreacted groups were blocked with ethanolamine. All measurements were done using TBS (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 BIAevaluation software version 3.1. The dissociation rate constants were derived using the equation,

$$R_t = R_{t0}\, e^{-k_{-1}} (t - t_0) \qquad (6)$$

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 equation 2 using the measured k_{-1} values,

$$R_t = R_{\max} [1 - e^{-(k_1 C + k_{-1})(t - t_0)}] \qquad (7)$$

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 change (ΔS) were determined by the equations 4 and 5. Activation enthalpies ($\Delta H^{\ddagger}$), entropies ($\Delta S^{\ddagger}$) and energies were calculated using the following equations:

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

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

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

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

RESULTS

Ligand Binding

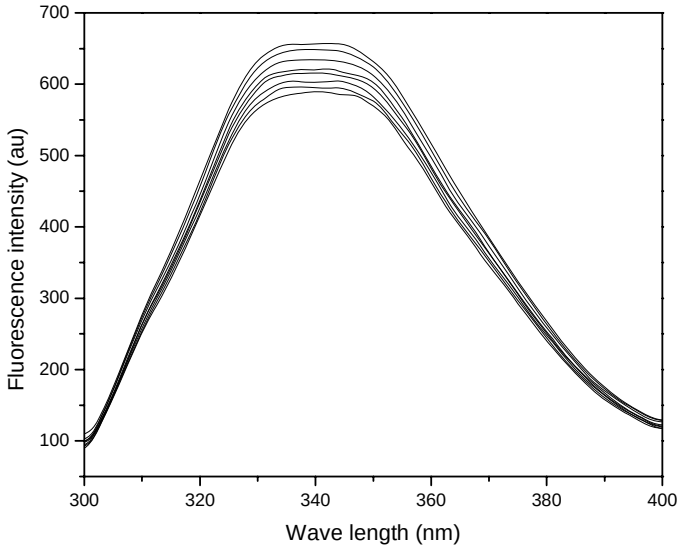

Fig.4.1. Fluorescence quenching profile of HSL on addition of aliquots of MeαGal to the lectin solution at 25 ^{0}C.

Addition of sugars and glycans resulted in quenching of the lectin fluorescence without any shift in the emission maximum (Fig. 4.1). The maximum quenching of the intrinsic fluorescence on sugar binding was 15%. Among monosaccharides Man, Glc and their derivatives showed very weak binding with association constants ranging from 200-500 M^{-1} (Table 4.1). Reasonably good binding was observed with Gal and its derivatives including, GalNAc (K_a 954 M^{-1}) and MeαGal (K_a 3630 M^{-1}) (Fig. 4.2). MeαGal showed 10 fold higher affinity as compared to its β anomer. No binding was observed with fucose (6-deoxygalactose) and L-arabinose (Table 4.2).

Table 4.1: Association constants of HSL with monosaccharides determined by fluorescence spectroscopy.

Sugars	K_a at 25 ^{0}C (10^3 M^{-1})	ΔG (k J mol^{-1})
D+ glucose	0.316	-14.48
D+ glucosamine	0.403	-15.11
N-acetyl glucosamine	0.535	-15.82
N-acetyl-Me-α-D+ glucose	0.501	-15.64
Me-α-D+ glucose	0.446	-15.36
Me-β-D+ glucose	0.488	-15.59
D+ mannose	0.436	-15.29
D+ mannosamine	0.478	-15.54
N-acetyl mannosamine	0.488	-15.59
Me-α-D+ mannose	0.774	-16.75
Me-β-D+ mannose	0.891	-17.1

Alpha linked galactose disaccharides Gal α1-3 Gal (K_a 1050 M^{-1}), Gal α1-4 Gal (K_a 1300 M^{-1}) and Gal α1-6 Glc (K_a 380 M^{-1}) had poor affinity (Table 4.3). Whereas beta-linked galactose disaccharides Gal β1-3 GlcNAc (K_a 5010 M^{-1}), Gal β1-4 GlcNAc (K_a 5620 M^{-1}) and Gal β1-3 GalNAc (K_a 53,700 M^{-1}) (Fig. 4.3) had higher affinity for the lectin, however lactose (Gal β1-4 Glc) (K_a 440) was a poor binding ligand (Table 4.2).

Table 4.2: Association constants of HSL with galactose derivatives determined by fluorescence spectroscopy.

Sugars	**K_a at 25 ^{0}C (10^3 M^{-1})**	**ΔG (k J mol^{-1})**
D+ galactose	0.48	-15.32
D+ Galactosamine	0.64	-16.00
N-acetyl galactosamine	1.25	-17.66
Me-α-D galactose	4.29	-20.72
Me-β-D galactose	0.64	-16.04
D+ fucose	NB	-
L-arabinose	NB	-
2-d-galactose	0.20	-13.12
Lactose	0.57	-15.73
Raffinose	0.48	-15.54
Stachyose	0.52	-15.74
Gal β1-3 GlcNAc	5.98	-21.54
Gal β1-4 GlcNAc	6.38	-21.70
Gal β1-3 GalNAc	68.79	-27.59

NB-no binding

Table 4.3: Association constants of HSL with disaccharides and oligosaccharides determined by fluorescence spectroscopy.

Sugars	***Ka* at 25 ^{0}C (10^3 M^{-1})**	**ΔG (k J mol^{-1})**
Gal α (1-3) Gal	1.05	-17.23
Gal α (1-4) Gal	1.30	-17.76
Gal α (1-6) Glc	0.88	-16.77
Gal β (1-3) Gal β (1-4) GlcNAc	19.0	-24.40
Gal β (1-3) GalNAc	68.79	-27.59
Gal β (1-3) GalNAc α-1-*O*-L-Serine	20,100	-41.67
Gal β (1-3) GalNAc α-1-*O*-Methyl	83,200	-45.19
Gal β (1-3) GalNAc β -1-*O*-Methyl	176	-29.93

T-antigen α-methyl glycoside (Gal β1-3 GalNAc α-1-*O*-Methyl) was the most potent ligand for the HSL, which binds 1569-, 4- and 472- times stronger than T-antigen, galactosyl Tn-antigen and T-antigen β-methyl glycoside (Gal β1-3 GalNAc β -1-*O*-Methyl) respectively (Tables 4.3 and 4.4).

The association constants in all the sugars and glycans decreased with increase in temperature. Van't Hoff plots for all sugars were linear ($r > 0.98$), in the range of temperature tested (Fig.4.4). 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 4.5).

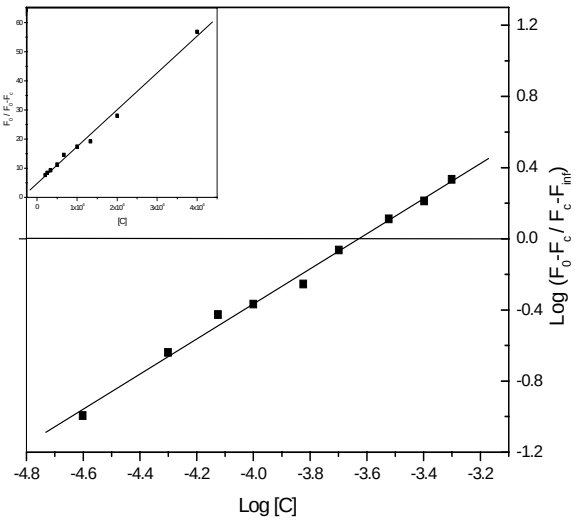

Fig. 4.2. Evaluation of the association constants for the binding of MeαGal to HSL by intrinsic fluorescence at 25 ^{0}C: Plot for the determination of the association constant. Inset: Quenching of the intrinsic fluorescence of HSL upon titration with MeαGal.

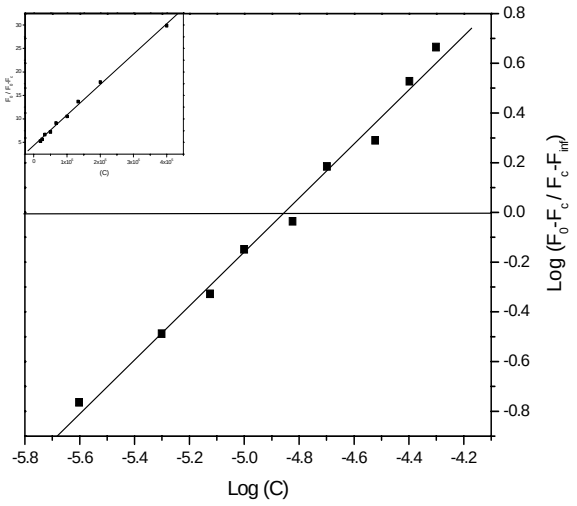

Fig. 4.3. Evaluation of the association constant for the binding of T-antigen to HSL by intrinsic fluorescence at 25 ^{0}C: Plot for the determination of the association constant. Inset: Quenching of the intrinsic fluorescence of HSL upon its titration with T-antigen sugar.

Table 4.4: Association constants for the binding of HSL at different temperatures to galactose and its derivatives determined by fluorimetry.

Sugar	K_a (10^3 M^{-1})		
	20 ^{0}C	**25 ^{0}C**	**30 ^{0}C**
Galactose	0.61	0.48	0.39
Galactosamine	0.79	0.64	0.52
N-acetyl galactosamine	1.64	1.25	0.95
Me α-galactose	5.12	4.29	3.63
Me β-galactose	0.85	0.64	0.46
Lactose	0.72	0.57	0.44
Gal β1-3 GlcNAc	7.03	5.98	5.01
Gal β1-4 GlcNAc	7.24	6.38	5.62
Gal β1-3 GalNAc	81.28	68.79	53.70
Asialo-triantennary *N*-glycan	9770	7580	5290
Sialated-triantennary *N*-glycan	603	468	345
Asialo-biantennary *N*-glycan	1050	840	610
Sialated-biantennary *N*-glycan	186	148	112

Table 4.5: Thermodynamic parameters for the binding of HSL to galactose and its derivatives determined by fluorimetry.

Glycans	**ΔG*** **(kJ M^{-1})**	**ΔH** **(kJ M^{-1})**	**ΔS** **(JM^{-1} K^{-1})**
Galactose	-15.32	-33.14	-59.78
Galactosamine	- 16.00	-30.79	-49.56
N-Acetyl Galactosamine	-17.66	-40.22	-75.68
Me α-Galactose	-20.72	-25.46	-15.91
Me β-Galactose	-16.04	-44.17	-94.41
Lactose	-15.73	-35.70	-67.03
Gal β1-3 GlcNAc	-21.54	-25.08	-11.86
Gal β1-4 GlcNAc	-21.70	-18.66	-10.20
Gal β1-3 GalNAc	-27.59	-30.51	-9.82
Asialo-triantennary *N*- glycan	-39.24	-44.96	-19.22
Sialated-triantennary *N*- glycan	-32.35	-41.29	-30.02
Asialo-biantennary *N*- glycan	-33.79	-39.80	-20.17
Sialated-biantennary *N*- glycan	-29.50	-37.59	-27.16

* Values were calculated at 25 ^{0}C.

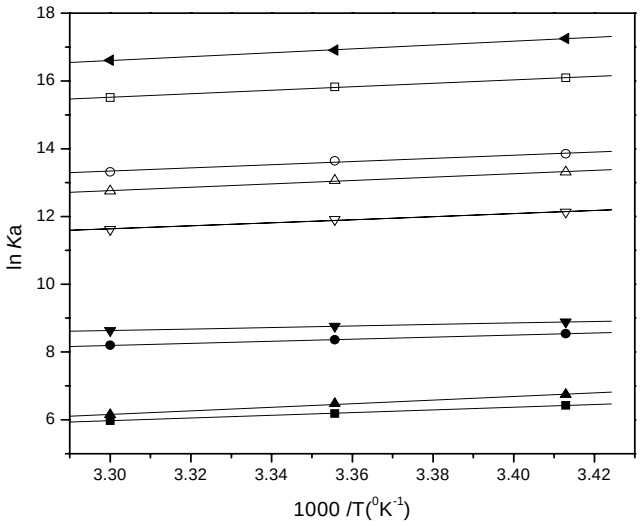

Fig.4.4. Van't Hoff plots for the association of sugars to HSL. The symbols used are; Gal (■), MeαGal (●), MeβGal (▲), LacNAc (▼), T-antigen (◀), asialo-triantennary *N*-glycan (□), asialo-biantennary *N*-glycan (○), sialo-triantennary *N*-glycan (△) and sialo-biantennary *N*-glycan (▽). The association constants were determined at 20 ^{0}C, 25 ^{0}C and 30 ^{0}C.

Among glycans tested (Fig. 4.5), asialo triantennary glycan binds 9-times stronger than asialo biantennary glycan, with an increase in enthalpic change from -39.80 kJ mol^{-1} to -44.96 kJ mol^{-1}, whereas asialo glycans bind with 10-15 times higher affinities over sialylated counter parts accompanied by favorable entropy change (7-10 J mol^{-1} K^{-1}) (Table 4.4, 4.5).

Surface plasmon resonance

Surface plasmon resonance studies on glycan binding to HSL showed significant change in responsive units with the increasing concentration of glycans (Fig.4.5) and glycoproteins. Van't Hoff plots for all glycans and glycoproteins were linear (r >0.98) in the range of temperature tested (Fig.4.7). The association constant (K_a) for galactosyl Tn-antigen (*O* linked glycan) is 22.01 x10^6 M^{-1} with a change in enthalpy of -47 kJ mol^{-1} and a change in entropy of -17.97 J mol^{-1} at 25 ^{0}C. *O*-linked glycan has 3- and 30-times higher binding over *N*-linked asialo triantennary and biantennary

glycans respectively (Table 4.6). The higher affinity of *O*-linked glycan was accompanied by increase in enthalpy change from -37.59 kJ mol^{-1} to -47.24 kJ mol^{-1} (Table 4.7).

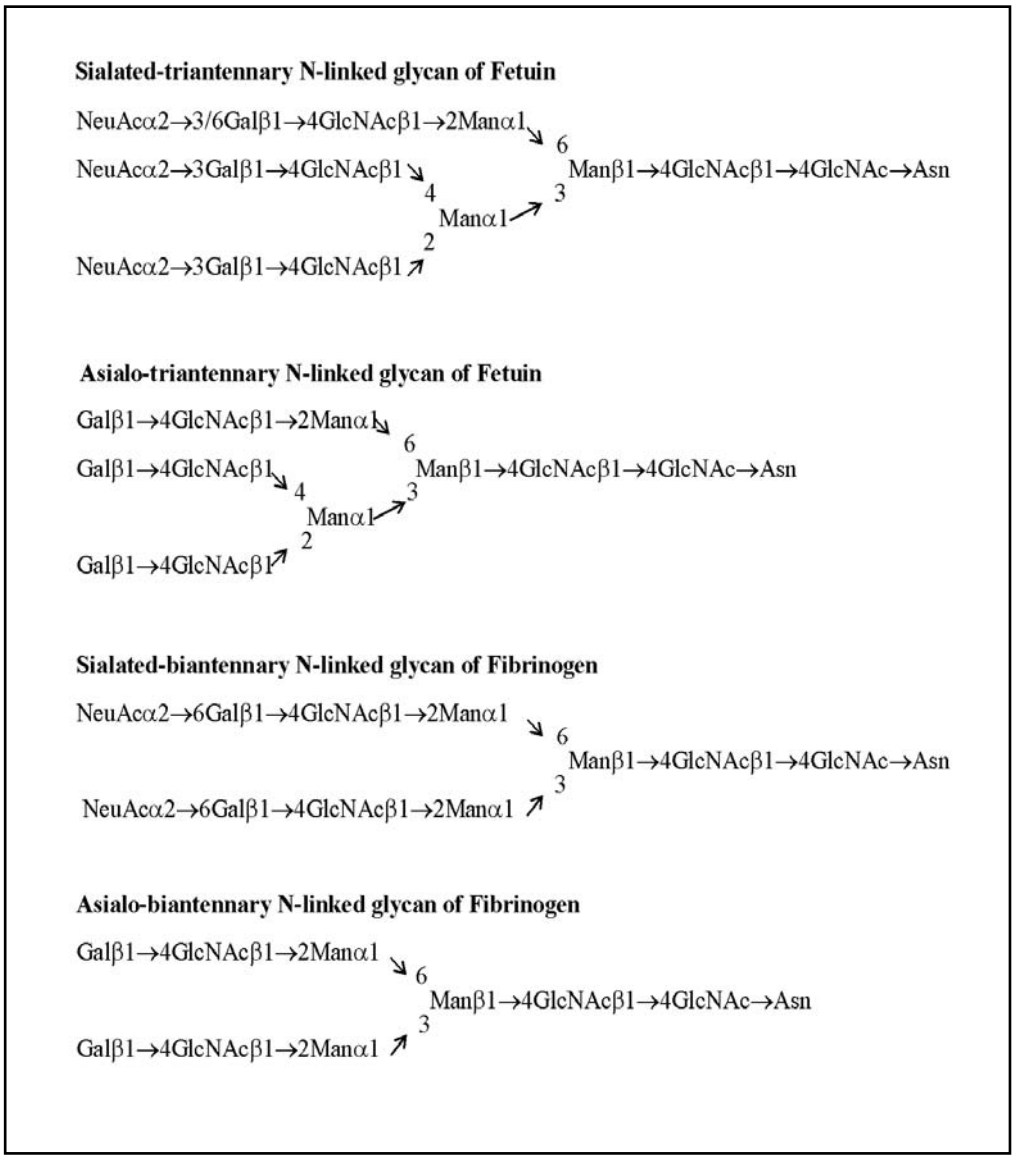

Fig. 4.5. Structure of different glycans. The glycans were prepared from fetuin and fibrinogen as described in methods.

The asialo triantennary glycan binds 9-times stronger than asialo biantennary glycan. The higher affinity was accompanied by increase in enthalpic. The HSL showed lesser affinity for the glycoproteins, fetuin (0.085 $\times 10^6$ M^{-1}) and fibrinogen (0.043 $\times 10^6$ M^{-1}). Fetuin is 114-times and fibrinogen is 24-times weaker ligands than their asialoglycans and fetuin binds with 2-fold higher affinity as compared to fibrinogen. The binding enthalpy was negative with both the glycoproteins with a value of ΔH= -50.95 and -45.75 kJ mol^{-1} for fetuin and fibrinogen respectively. The entropy was also negative with -77.86 and -67.50 J mol^{-1} K^{-1} respectively (Table 4.7).

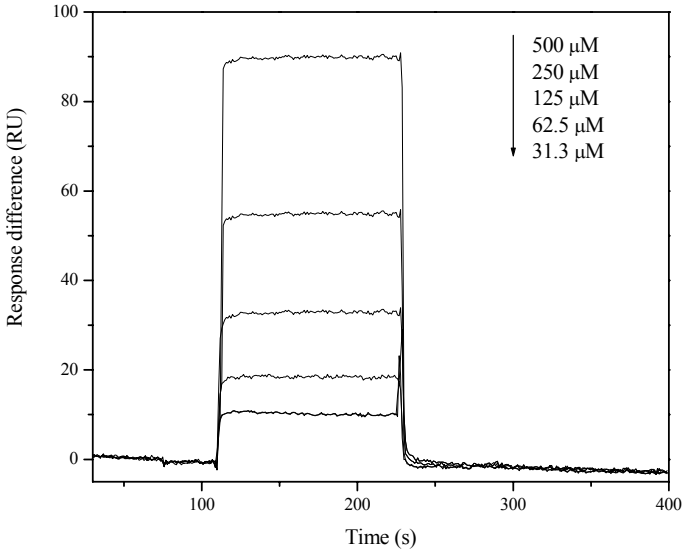

Fig. 4.6: The representative sensograms depicting interactions of increasing amounts of glycans to the immobilized HSL at 25 ^{0}C. Galactosyl Tn-antigen solutions were injected for 300 s at a flow rate of 5 µl/min. The dissociation reaction was recorded by flowing the plain buffer at flow rate of 5 µl/min for 300 s. The surface of the chip was regenerated by 10 s pulse of bicarbonate buffer pH 9.5.

Table 4.6: Association constants for the binding of glycans and glycoproteins to immobilized HSL at different temperatures: Determined by SPR (kinetic analysis).

Ligand	**K_a x10^6 M^{-1}**		
	20 °C	**25 °C**	**30 °C**
Gal β (1-3) GalNAc α-1-*O*-L-Ser	31.20	22.01	16.30
Asialo-triantennary N-glycan	9.72	7.42	5.42
Asialo-biantennary N-glycan	1.03	0.83	0.62
Fetuin	0.085	0.057	0.043
Fibrinogen	0.043	0.032	0.023

Table 4.7: Thermodynamic parameters for the binding of different glycans and glycoproteins to immobilized HSL at different temperatures: Determined by SPR (kinetic analysis).

Ligand	**ΔG***	**ΔH**	**ΔS**
	(kJ mol^{-1})	**(kJ mol^{-1})**	**(J mol^{-1} K^{-1})**
Gal β 1-3 GalNAc α 1-*O*-Ser	-41.89	-47.24	-17.97
Asialo-triantennary *N*-glycan	-39.19	-44.24	-16.95
Asialo-biantennary *N*-glycan	-33.76	-37.59	-12.86
Fetuin	-27.15	-50.95	-79.86
Fibrinogen	-25.64	-45.75	-67.50

* Determined at 25 ^{0}C

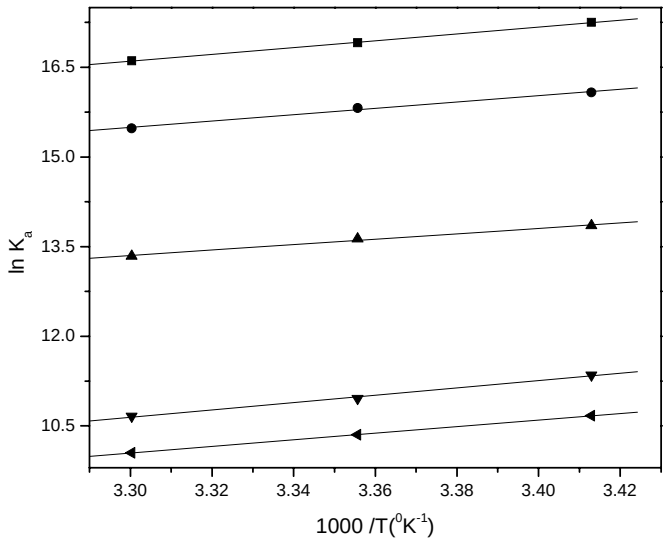

Fig.4.7. Van't Hoff plots for the association of various glycans and glycoproteins by SPR. The symbols used are; Gal β1-3 GalNAc α1-*O*-Ser (■), asialo triantennary *N*-glycan (●), asialo biantennary *N*-glycan (▲), fetuin (▼) and fibrinogen (◄). The association constants were determined using SPR at 20 ^{0}C, 25 ^{0}C and 30 ^{0}C.

Kinetic analysis

Kinetic analysis of all glycans and glycoproteins indicated that, asialo triantennary *N*-glycan (k_1= 13.35 $\times 10^3$ $M^{-1}s^{-1}$) had much faster association rate than galactosyl Tn-antigen (k_1= 8.59 $\times 10^3$ $M^{-1}s^{-1}$) and asialo biantennary *N*-glycan (k_1= 2.31 $\times 10^3$ $M^{-1}s^{-1}$). Whereas the dissociation rate of galactosyl Tn-antigen (0.049 s) is much lower than asialo triantennary (0.0018 s) and asialo biantennary (0.0025 s) glycans. Difference in binding affinity of the HSL to the different glycan was determined by their difference in the association and dissociation rates. The association (Table 4.8) and dissociation rate constants (Table 4.9) were determined at different temperatures. Arrhenius plots for association (Fig. 4.8) and dissociation kinetics (Fig. 4.9) for all the glycans and glycoproteins were linear (r>0.98) in the temperature range studied.

Table 4.8. Rate constants and activation parameters for the association process of glycan and glycoproteins to HSL.

Ligand	Association				
	$k_1 \times 10^3$	$E^{\ddagger}_1$	$\Delta G^{\ddagger}_1$	$\Delta H^{\ddagger}_1$	$\Delta S^{\ddagger}_1$
	$M^{-1}s^{-1}$	kJ mol^{-1}	kJ mol^{-1}	kJ mol^{-1}	$Jmol^{-1}K^{-1}$
Gal β1-3GalNAc α 1-*O*Ser	8.59	21.66	52.11	19.18	-111.60
Asialo-triantennary *N*-glycan	13.35	39.11	53.76	36.63	-58.07
Asialo-biantennary *N*-glycan	2.31	18.29	52.02	15.82	-122.70
Fetuin	1.66	37.93	55.02	34.93	-68.11
Fibrinogen	6.06	36.14	52.07	33.66	-62.40

Values of k_1 and $\Delta G^{\ddagger}_1$ are determined at 25 ^{0}C

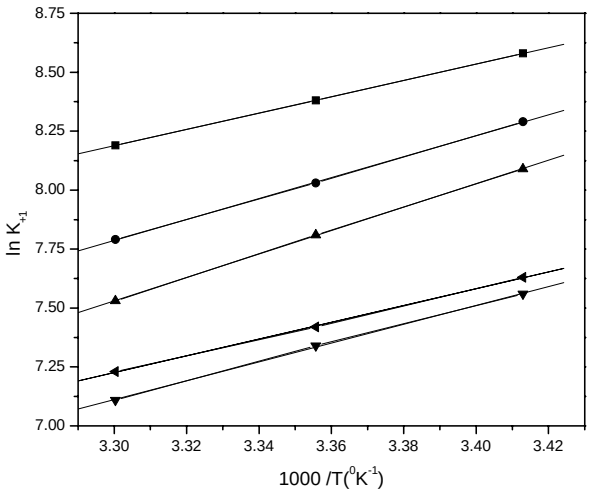

Fig.4.8. Arrhenius plots for the association kinetics of various glycans and glycoproteins to HSL: The symbols used are association of Gal β1-3GalNAc α1-*O* Ser (■), asialo triantennary glycan (●), asialo biantennary glycan (▲), fetuin (▼) and fibrinogen (◄). The association constants were determined at 20 ^{0}C, 25 ^{0}C and 30 ^{0}C.

Table 4.9. Rate constants and activation parameters for the dissociation process of glycan and glycoproteins to HSL.

Ligand	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}	$\Delta S^{\ddagger}_1$ $Jmol^{-1}K^{-1}$
Gal β1-3 GalNAc α1-*O* Ser	49.10	83.22	93.27	80.74	-41.89
Asialo-triantennary *N*-glycan	1.81	84.06	92.93	81.59	-38.45
Asialo-biantennary *N*-glycan	2.54	59.78	88.45	57.30	-105.59
Fetuin	0.21	42.97	82.79	40.49	-143.40
Fibrinogen	0.75	49.57	79.93	47.09	-111.33

Values of k_{-1} and $\Delta G^{\ddagger}_1$ are determined at 25 ^{0}C

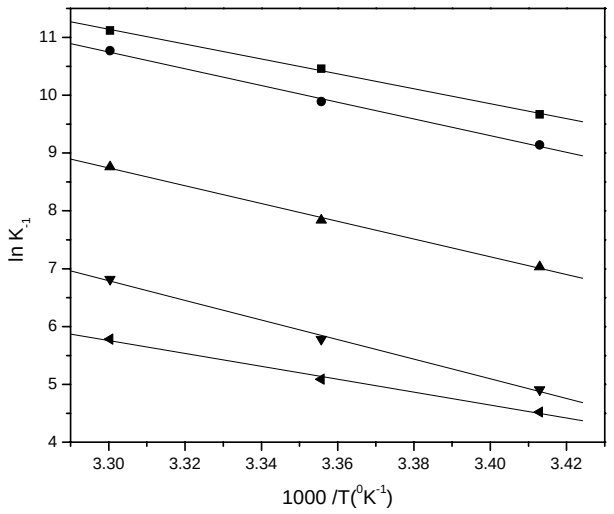

Fig.4.9. Arrhenius plots for the dissociation kinetics of various glycans and glycoproteins to HSL: The symbols used are dissociation of Gal β1-3GalNAc α1-*O* Ser (■), asialo triantennary glycan (●), asialo biantennary glycan (▲), fetuin (▼) and fibrinogen (◄). The dissociation constants were determined at 20 ^{0}C, 25 ^{0}C and 30 ^{0}C.

The glycoproteins showed much slower association rate constant for fetuin (k_1 = 1.66 $\times 10^3$ $M^{-1}s^{-1}$) and fibrinogen (k_1 = 6.06 $\times 10^3$ $M^{-1}s^{-1}$) as compared to glycans, whereas the dissociation rate constant was faster in fetuin (0.00021 s) and fibrinogen (0.00075 s) than glycans.

DISCUSSION

Detailed carbohydrate binding specificities of the HSL was studied by a combination of hemagglutination inhibition, fluorimetry and surface plasmon resonance spectroscopy. Due to the low molecular weight of mono-, di- and oligosaccharides, their binding to the lectin cannot be studied by surface plasmon resonance hence the studies were carried out by fluorescence spectroscopy. Negligible binding was observed for glucose, mannose, and their methyl and amine derivative. Affinity among monosaccharides was in the order MeαGal >GalNAc >GalN ≅MeβGal >2dGal ≅Gal. The better affinity was governed by the favourable enthalpic change.

Among monosaccharides affinities, GalN is twice better than Gal and 2dGal. Removal of OH group in C-2 (2dGal) 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 for the binding of Gal and GalN were almost equal (-33.14 and -30.79 kJ mol^{-1}), indicating that the contribution of enthalpy to the differences in the free energy of binding of these free sugars was not significant. The affinities therefore were a manifestation of a favourable entropic factor.

GalNAc is 4.5-, 5- and 2- times stronger binding a ligand when compared with Gal, 2dGal 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 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 does exhibit strong

hemagglutination inhibition against lectins from *Cucumeria echinata* (CEL-I, III and IV) [15] and from *Crenomytilus grayanus* [16].

MeαGal is the strongest ligand among the monosaccaharide with an 9-fold higher association constant for the HSL than that of galactose and 7- fold higher than that of MeβGal. A positive increase in entropy of MeαGal (-15.91 $Jmol^{-1}K^{-1}$) over Gal (-59.78 $Jmol^{-1}K^{-1}$), was observed indicating a strong and favourable association between the MeαGal 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 from -15.91 $Jmol^{-1}K^{-1}$ to -94.41$Jmol^{-1}K^{-1}$. However, the presence of a methyl group at C-1 position in the α-anomer alone is not sufficient to cause effective binding since MeαGlc and MeαMan bind to the lectin with very low affinity. The preference for the α-anomer over the β-anomer of galactose was also observed for other lectins including a lectin from *Didemnum ternatanum* [17], whereas lectin from marine worm, *Chaetopterus variopedatus* prefers to bind to MeβGal than MeαGal [18].

Mellibiose binds to HSL poorly, when compared to MeαGal indicating that the replacement of 1-*0*-methyl group in MeαGal 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 latter 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 MeαGal, GalNAc, MeβGal and Gal, respectively. The increase in the affinity for disaccharides over monosaccharides is accompanied by a positive increase in entropy.

Whereas among disaccharides, T-antigen has a significantly higher K_a (10-, 11- and 170- fold higher) than that for Gal β1-4 GlcNAc, Gal β1-3 GlcNAc and lactose respectively, which could be explained by their minimum energy confirmations. The confirmation for the nonreducing residue in all the four disaccharides is identical. However, the orientation of the residue for β(1-4) linked disaccharides is different from that of β(1-3) linked disaccharides. The C-3 hydroxyl groups of the reducing residue in β(1-4)-linked disaccharides and the C-4 hydroxyl group in Gal β1-3 GlcNAc are in equatorial configuration, whereas the C-4 hydroxyl group of the reducing residue in Gal β1-3 GalNAc is in the axial configuration. This difference alone seems to be responsible for weak binding of other three sugars. The major stabilizing force for the binding of Gal β1-3 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 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, galactosyl Tn-antigen showed 379- times stronger binding to the HSL than T-antigen, suggesting the role of alpha linked serine in binding. T-antigen α-methyl glycoside is the best ligand as it binds HSL 1209-, 4- and 472- times better than T-antigen, galactosyl Tn-antigen and T-antigen β-methyl glycoside respectively. The higher affinity of T-antigen α-methyl glycoside can be explained as 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.

Galactosyl Tn-antigen, *O*-linked glycan showed 3- and 30- times stronger affinity over *N*-linked asialo triantennary and asialo biantennary glycans. The higher affinity was achieved by increase in enthalpy over *N*-linked glycans, suggesting interactions of terminal serine at the alpha position with the active site of the lectin. Lectins from *Crenomytilus grayanus* mussel [19] and from *Crustacea decapoda* [20], exhibits very high affinity for sialylated *O*-glycosilated proteins than sialylated *N*-glycosylated proteins.

Asialo triantennary glycan binds 9-times stronger than asialo biantennary glycan. The increased binding is accompanied by an enthalpy change (-5 kJ mol^{-1}) suggesting formation of extra hydrogen bonds and van der Waals' interactions. Higher binding can also be due to the differences in valency, since asialo triantennary glycan is trivalent, having ultimate three Gal β (1-4) GlcNAc, whereas asialo biantennary glycan is bivalent with only two Gal β (1-4) GlcNAc residues. Increased affinity of these glycans (upto micro molar range) indicates the extended nature of the lectin's sugar binding site.

The addition of bulky and negatively charged NeuAc at C-3/6 position in ultimate Gal reduced the binding affinity by 16- and 5- fold with triantennary and biantennary *N*-glycans respectively and 25-100 folds less affinity for respective glycoproteins. The reduction in the binding affinity is probably due to some unfavorable interaction between NeuAc and lectin or due to steric hindrance reducing the accessibility of penultimate Gal to the ligand binding pouch of the lectin. The probability of unfavorable ionic interaction between the NeuAc and lectin cannot be excluded, which leads to simultaneous reduction of binding enthalpy due to loss of contributory hydrogen bonding or van der Walls' interaction. The lower affinity of glycoproteins to lectin, due to increase in the unfavorable entropy as compared to asialo glycans. Such type of reduction in the affinity with sialylated glycoconjugates was also reported from marine red alga *Gracilaria ornata* [21], However, higher affinity with sialylated glycoconjugates also be found in lectins, where primary interacting counterpart is NeuAc itself, rather than penultimate Gal residue [22].

Generally, when the 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 reactions, the binding is presumed to involve a putative intermediate complex PL*, which then isomerizes into a final complex PL.

$$P + L \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} PL^* \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} PL$$

where $K_1=k_1/k_{-1}$ and $K_2=k_2/k_{-2}$. Since the first step is too rapid to measure by SPR, it is possible that we are observing only the second step. The agreement between kinetically determined values of association constants (k_1/k_{-1}) and changes in enthalpies with those determined by scatchard analysis 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. 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 is consistent with a single step bimolecular association reaction, which is of several orders of magnitude slower than diffusion controlled reaction.

O-linked glycan binds 3-times faster than asialo triantennary glycan and the activation energy ($E^{\ddagger}_1$) for the association process (21.66 $kJmol^{-1}$) 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. This suggests that the ligand can approach the binding pocket in an ordered way, leading to faster binding. The higher affinity of asialo triantennary glycan is associated with lower entropy of activation for association process (76 $Jmol^{-1}K^{-1}$) than asialo biantennary; indicate that association process does not involve highly ordered transition state.

The value of K_a determined by k_1/k_{-1} can increase either by increase in k_1 or decrease in k_{-1}. For most protein-ligand interactions decrease in dissociation rate constants (k_{-1}) has been shown to be responsible for increased binding affinity and not increase in the association rate constants (k_1). 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. Asialo triantennary glycan binds nine times better than asialo biantennary glycan, associated with slower association and faster dissociation rate. Moreover the slower dissociation rate constant and higher entropy of both association and dissociation processes appears to be the main factors responsible for lower affinity of asialo biantennary glycan. Lower affinity of glycoproteins is associated with higher activation energy for association process and higher negative entropy for both association and dissociation processes.

In conclusion, the present studies show that the higher affinity of HSL for β (1-3) GalNAc linked Tn antigen and asialo triantennary glycan in order is accompanied by enthalpic as well as entropic contribution. In addition, higher association and lower dissociation rate as well as low activation energy for association process contribute to their higher affinity. HSL is a Tn antigen specific lectin having both primary and extended binding sites.

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CHAPTER: 5

ANALYSIS OF CARBOHYDRATE BINDING DOMAIN OF HSL

SUMMARY

Hemagglutination activity is a unique property of lectins brought about by cross linking of the ligand by the lectin. As cross bridges are necessary for agglutination only multivalent lectins (>one CBD) can support this process. In general, a single CBD occurs in a polypeptide chain (subunit) and the multivalence is achieved by association of the chains into oligomers. However, HSL is a monomeric lectin and still supports hemagglutination. Limited proteolysis with trypsin resulted in a single 25 kDa fragment, which has the carbohydrate binding activity but no hemagglutination. Thus, HSL belongs to the rare class of monomeric multivalent lectins having two homomeric domains. We observed enhanced carbohydrate binding affinity of HSL-CBD for mono/disaccharides. Lys, Trp and carboxylate groups are present in the carbohydrate binding site.

INTRODUCTION

Lectins bind specific sugars because of a cognate site called the carbohydrate binding domain (CBD). Based on the number of CBDs, lectins can be classified into two categories: monovalent and multivalent. Multivalency of lectins, as is also the case of antibodies (which are divalent), permits simultaneous binding to more than one target (blood or bacterial cells with specific surface sugars). The cross linking cause agglutination of the target. Agglutination is therefore a property exclusive to multivalent lectins. Also, the increase in avidity improves the strength of interaction [1].

In general, only one CBD occurs in a polypeptide chain and the apparent multivalency is due to oligomeric association of such chains. In this light it was surprising that HSL could induce hemagglutination despite being a monomer. Our analyses presented here shows that, this property is due to two CBDs located on the same chain. Surprisingly, very few multivalent monomeric lectins have been reported till date. However, functional and evolutionary importance for the non-involvement of quaternary structure is not known. Some of the previously reported monomeric multivalent lectins include macrophage mannose receptor [2], immulectins from *Manduca sexta*

[3], F-type lectin from Striped bass [4] and tandem-type soluble lactose-lectin [5] and a lectin from the fall webworm, *Hyphantria cunea* [6]. This study was conducted to know the structural makeup of the HSL for hemagglutination activity.

MATERIALS

D-galactose, D-galactosamine, 2-deoxy-D-galacose, L-fucose, methyl α–D-galactose, methyl β–D-galactose, pronase-E, trypsin, chymotrypsin, proteinase K, carboxypeptidase, aminopeptidase, fetuin (bovine), fibrinogen (human), succinic anhydride, 2,4,6-trinitrobenzenesulphonic acid, diethylpyrocarbonate, *N*-bromosuccinimide, 1-ethyl-3 (3-dimethyl amino propyl) carbodiimide, nitrotyrosine ethyl ester and guanidium hydrochloride were obtained from Sigma chemical Co. St. Louis, U.S.A; while 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. Triantennary *N*-glycan from fetuin and biantennary *N*-glycan from fibrinogen were purified and the homogeneity of the preparation was checked by HPLC [7, 8].

METHODS

Purification of HSL-native

Purification of HSL-native from the coelomic fluid of sea cucumber has already been described in chapter 2. Total neutral sugar content of the lectin was estimated by phenol-sulphuric acid method of Dubois et al. [9], using galactose-mannose (4:3) as standard.

Preparation of carbohydrate binding domain

HSL-native was digested with different proteolytic enzymes; trypsin, chymotrypsin and proteinase K in 0.1 M NH_4HCO_3 buffer at 37 ^{0}C for 20 min using an enzyme to substrate ratio of 1:50 (w/w). Proteolysis was stopped by adding PMSF to a final volume of 5 mM. The samples was dried and dissolved in 15 µl of water and mixed with appropriate amount of gel loading

buffer and the entire sample was loaded on tricine-polyacrylamide gel to confirm the proteolysis. The digested mixture was loaded on to a sephadex G-100 column (1.5 cm x 180 cm), equilibrated with 20 mM tris-HCl buffer pH 8.5 containing 0.15 M NaCl at the flow rate of 10 ml/h, one ml fractions were collected. The purity of the preparation of different fractions were analysed by SDS-PAGE and preserved at -20 ^{0}C.

Hemagglutination assay

Hemagglutination assay was done with pronase treated human erythrocytes of blood group A, B and O as mentioned in chapter 2.

Circular dichroism (CD) measurements

CD spectra were recorded for HSL-CBD at 25 ^{0}C on a Jasco J-715 spectropolarimeter as mentioned in chapter 2.

Amino acid and N-terminal sequence analysis

50-µg of HSL-CBD was digested with 6 N HCl at 110 ^{0}C for 24 h in vacuum-sealed tubes and derivatized with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AccQ-Fluor kit, Waters Corporation, USA). Other methods were followed as mentioned in chapter 2. Total cysteine and tryptophan were estimated with denatured HSL-CBD according to the method of Cavallini et al [10] and Spande and Witkop [11] respectively. PVDF blotted sample of HSL-CBD was used to determine the *N*-terminal sequence by automated Edman degradation method in Procise^{TM} protein sequencer (Applied Biosystem, Canada) at the protein sequencing facility, NII, New Delhi, India.

Active site characterization

The presence of various amino acid residues at the sugar binding site was determined by modification with a suitable reagent. HSL-CBD was incubated with and without the modifier and the effect was analysed by sugar binding activity with MeαGal. The sugar binding activity of the control sample was considered as 100%. Structural integrity of the modified samples were analysed by CD after removing the excess reagent by gel-filtration with sephadex G-25 column. Reagents and conditions used for the modification of

the various amino acid residues are shown in Table 5.1, and the same protocols were followed as mentioned in the chapter 3. The number of critical residues involved in carbohydrate binding was determined by a plot of percent residual hemagglutination activity versus the number of critical residues modified. Extrapolation of the plot to the ordinate gives the number of residues/molecule required for binding.

Fluorescence titration

Sugar binding affinity of HSL-CBD was studied by fluorescence titration method. The measurements were recorded in 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 (± 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 335 nm (λ_{max} of the CBD) 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 initial level.

The association constants were calculated according to the method described by Chipman et al. [12]. 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.

RESULTS

Preparation and characterization of HSL-CBD

Treatment of HSL-native with trypsin results in the liberation of a ~25 kDa fragment (Fig.5.1), which was then purified to homogeneity by gel filtration chromatography. The 25 kDa fragment could bind to MeαGal but fails to agglutinate pronase treated human erythrocytes of all the blood groups. The sugar binding activity of lectins is due to a cognate site present in the

carbohydrate binding domain (CBD). Presence of more than one CBD in a lectin (multivalency) facilitates cross linking of the blood cells to form an agglutinate. Thus individual CBDs are capable of binding sugars but do not support hemagglutination. This behavior in the 25 kDa fragment compelled us to designate it as HSL-CBD.

Table 5.1: Effect of various chemical modifiers on MeαGal binding activity of HSL-CBD.

Reagent	Buffer used	Conc. of reagent used	Residue modified	Residual activity (%)
HSL-CBD	20 mM tris-HCl pH 8.5 (TB)	-	-	100
NBS	100 mM sodium acetate pH 5.5	100 μM	Trp	0
EDC	50 mM MES/HEPES pH 6.0	10.0 mM	Carboxylate group	0
TNBS	TB	0.1%	Lys	0
Acetic anhydride	TB	1.0 mM	Lys	0
Succinic anhydride	TB	3.0 mM	Lys	0

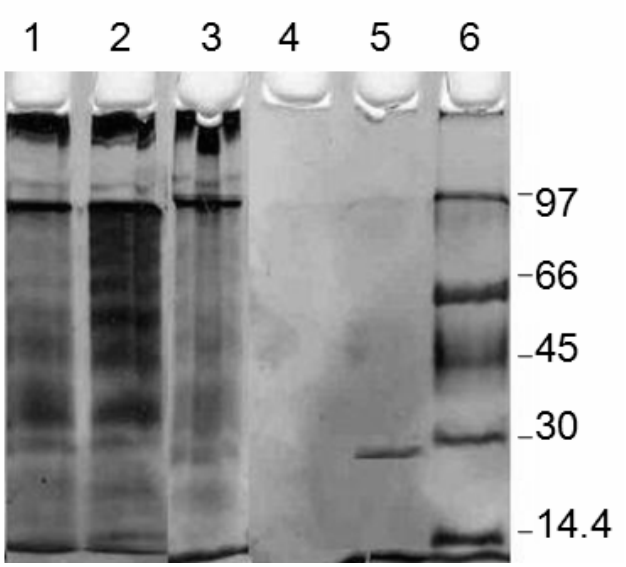

Fig.5.1. SDS-PAGE gel pattern of products obtained in limited trypsin digestion of HSL-native. Lane 1, 2, 3; tryptic digest at different duration of time, lane 4; trypsin at concentration used in the experiment, lane 5; purified HSL-CBD and lane 6; markers.

Table 5.2. Amino acid composition analysis of HSL-CBD

Amino acid	Mol (%)	Amino acid	Mol (%)
Asx	12.18	Cys	2.03
Ser	2.53	Tyr	5.07
Glx	14.72	Val	7.10
Gly	18.27	Met	-
His	2.53	Lys	9.13
Arg	2.53	Ile	2.03
Thr	5.07	Leu	4.56
Ala	6.04	Phe	2.03
Pro	2.53	Trp	3.01

Amino acid composition analysis

The amino acid analysis revealed that HSL-CBD also contains remarkable high amount of same amino acids, which are present in HSL-native like, Gly (18.27%), Glx (14.72%), Asx (12.18%) and Lys (9.13%), essential proportions of hydrophobic amino acids Ala, Leu, Val and hydroxyl containing amino acids Thr, Ser, Tyr and Trp, Ile, Cys and Phe are present in low amount, whereas Met is not present. HSL-CBD contains 6 Trp and 4 Cys residues out of which two are involved in forming a disulfide bond (Table 5.2). *N*-terminal sequence is AIQTNEV. Sugar content analysis revealed that HSL-CBD is a glycoprotein containing 1.0% neutral sugars, whereas HSL-native has 8% neutral sugars.

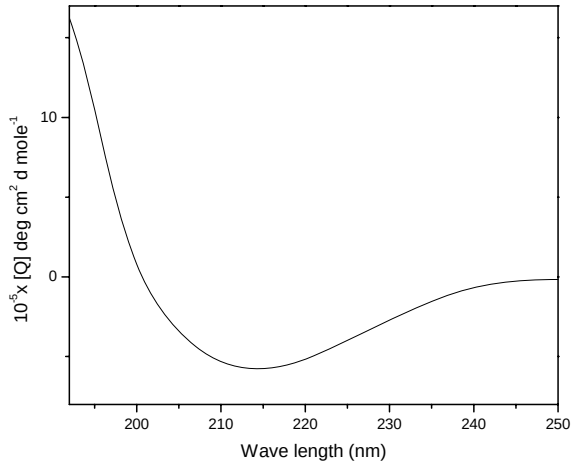

Fig. 5.2. Circular dichroism spectra of purified HSL-CBD. The spectrum was recorded on a Jasco J-715 spectropolarimeter.

Circular dichroism of HSL

CD spectrum of HSL-CBD was characterized by a maximum negative absorption at 211 nm in the far UV region. CDSSTR algorithm yielded the best-fit values (NRMSD=0.10) indicating that the secondary structure is comprised of 51% of β-sheet, 34% of α- helix and 15% unordered coil (Fig.5.2).

Active site characterization

The presence of different amino acid residues at the sugar binding site was determined by modification with a suitable reagent. HSL-CBD was incubated with different amino acid modifier and the effect was analysed by residual sugar binding activity with MeαGal. The binding property of HSL-CBD and chemically modified HSL-CBD was measured by fluorescence spectroscopy. Progressive loss in sugar binding affinity towards MeαGal of HSL-CBD by succinic anhydride due to modification of increasing number of lysine residues showed that three residues per sugar binding site are essential (Fig. 5.3).

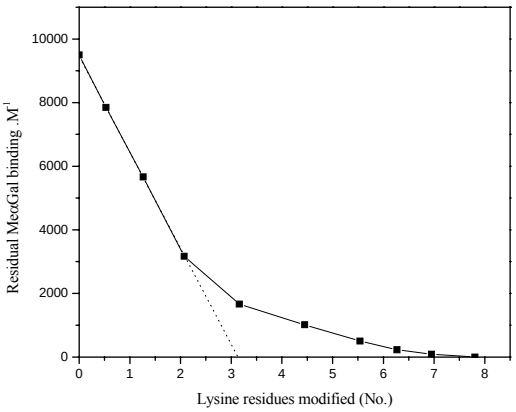

Fig. 5.3. Effect of modification of lysine residues on the MeαGal binding affinity of HSL-CBD.

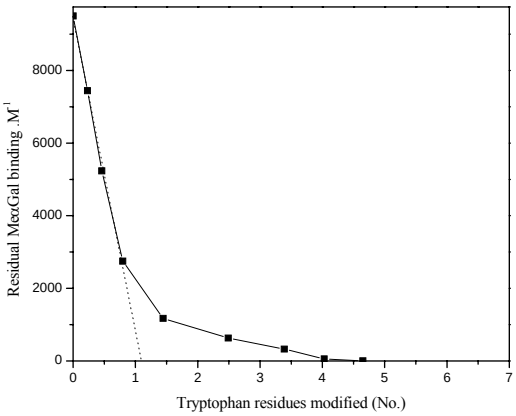

Fig. 5.4. Effect of modification of tryptophan residues on the MeαGal binding affinity of HSL-CBD.

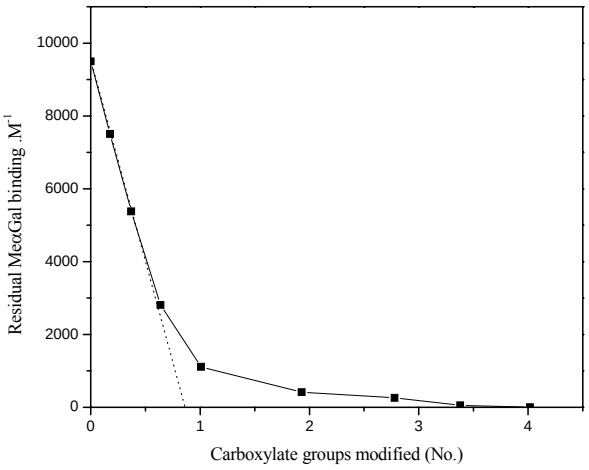

Fig. 5.5. Effect of modification of carboxylate groups on the MeαGal binding affinity of HSL-CBD.

Treatment with NBS resulted in complete loss of sugar binding activity accompanied by decrease in absorbance at 280 nm, indicating presence of tryptophan at the sugar binding site. The number of critical tryptophanyl residues was estimated to be one (Fig. 5.4). The modification of one carboxylate group per sugar binding site using EDC resulted in the complete loss of sugar binding activity (Fig. 5.5), suggesting presence of either glutamate or aspertate at the sugar binding site (Table 5.3). Structural integrity of all the modified HSL-CBD was analyzed by CD spectroscopy; the spectra were identical with that obtained from HSL-CBD. This indicates that the loss in activity due to modifications of specific residues and not as a consequence of structural changes.

Table 5.3: Effect of various chemical modifiers on MeαGal binding activity of HSL-CBD.

Chemical reagent	Residue modified	HSL-native	HSL-CBD
NBS	Trp	2	1
TNBS	Lys	6	3
EDC	Carboxylate group	2	1

Binding properties of HSL-CBD

Addition of sugars and glycans resulted in quenching of the HSL-CBD fluorescence without any shift in the emission maximum. The maximum quenching of the intrinsic fluorescence on sugar binding was 20%. Reasonably good binding was observed with Gal (K_a 890 M^{-1}), GalN (K_a 1140 M^{-1}), GalNAc (K_a 2680 M^{-1}) and MeαGal (K_a 9500 M^{-1}) at 25 ^{0}C. No binding was observed with MeβGal (Table 5.4).

Table 5.4: Association constants of HSL-CBD with different mono/disaccharides determined by fluorescence spectroscopy.

Sugar	**$K_a \times 10^3$ M^{-1}**		
	20 ^{0}C	**25 ^{0}C**	**30 ^{0}C**
Galactose	1.16	0.89	0.71
Galactosamine	1.52	1.14	0.89
N-Acetyl Galactosamine	3.52	2.68	2.01
Me α-Galactose	13.4	9.5	7.3
Me β-Galactose	NB	NB	NB
Lactose	NB	NB	NB
Gal β1-3 GlcNAc	18.21	14.62	12.33
Gal β1-4 GlcNAc	14.91	12.33	10.09
Gal β1-3 GalNAc	151.75	118.18	94.8

The strength of binding of mono/disaccharides to HSL-CBD was two times higher than HSL-native. The binding constant of HSL-CBD for MeαGal is 9.5 x10^3 M^{-1}, contributed by enthalpy (-45.04 kJ mol^{-1}) (Table 5.5), whereas *K*a is 4.29 x10^3 M^{-1} for HSL-native contributed by favourable entropy (-44 J mol^{-1} K^{-1}) as compared to Gal and GanNAc.

Table 5.5: Thermodynamic parameters for the binding of HSL-CBD with different mono/disaccharides determined by fluorescence spectroscopy.

Sugar	ΔG*	ΔH	ΔS
	(kJ·mol^{-1})	(kJ·mol^{-1})	(J·mol^{-1}·K^{-1})
Galactose	-16.82	-36.24	-65.27
Galactosamine	-17.44	-39.49	-74.02
N-Acetyl Galactosamine	-19.55	-41.39	-73.28
Me α-Galactose	-22.69	-45.04	-74.99
Me β-Galactose	-	-	-
Lactose	-	-	-
Gal β1-3 GlcNAc	-23.75	-28.77	-16.85
Gal β1-4 GlcNAc	-23.33	-28.75	-18.23
Gal β1-3 GalNAc	-28.93	-34.69	-19.31

*values were calculated at 25 ^{0}C.

Increased binding was observed with beta-linked galactose disaccharides Gal β1-3 GlcNAc (K_a 14.62 x10^3 M^{-1}), Gal β1-4 GlcNAc (K_a 12.33 x10^3 M^{-1}) and Gal β1-3 GalNAc (K_a 118.18 x10^3 M^{-1}); however Lactose (Gal β1-4 Glc) did not bind to HSL-CBD (Table 5.4). Galactosyl Tn-antigen, a *O*-linked glycan was the most potent ligand for the HSL-CBD with a Ka value of 42.30 x10^6 M^{-1}, with a change in enthalpy of -48.71 k J M^{-1} and a change in entropy of -17.49 J mol^{-1}K^{-1} at 25 ^{0}C (Table 5.6, 5.7).

Table 5.6: Association constants of HSL-CBD with different glycans determined by fluorescence spectroscopy.

Glycan	$K_a \times 10^6$ M^{-1}		
	20 ^{0}C	**25 ^{0}C**	**30 ^{0}C**
Gal β1-3 GalNAc α-1-*O*-Ser	60.20	42.30	31.19
Asialo-triantennary *N*-glycan	10.01	7.49	5.41
Asialo-biantennary *N*-glycan	1.09	0.82	0.60
Sialated-triantennary *N*-glycan	0.68	0.52	0.39
Sialated-biantennary *N*-glycan	0.18	0.14	0.11

Table 5.7: Thermodynamic parameters for the binding of HSL-CBD with different glycans determined by fluorescence spectroscopy.

Glycan	ΔG*	ΔH	ΔS
	(kJ mol^{-1})	**(kJ mol^{-1})**	**(J mol^{-1} K^{-1})**
Gal β1-3 GalNAc α-1-*O*-Ser	-43.50	-48.71	-17.49
Asialo-triantennary *N*-glycan	-39.21	-44.98	-19.37
Asialo-biantennary *N*-glycan	-33.74	-42.78	-30.35
Sialated-triantennary *N*-glycan	-32.57	-41.32	-29.35
Sialated-biantennary *N*-glycan	-29.25	-39.11	-33.07

*values were calculated at 25 ^{0}C.

O-linked glycan binds to HSL-CBD with 7-and 50-times higher affinity than of *N*-linked asialo triantennary and biantennary glycans respectively. Asialo biantennary glycan binds with 10-times lower affinity as compared to asialo triantennary glycan, lower binding is associated with increase in unfavorable entropy from -19.37 to -30.35 J mol^{-1}K^{-1}. Whereas asialo glycans binds to HSL-CBD with 5-15 times higher affinity as compare to sialylated, accompanied by increase in negative entropy (Table 5.6, 5.7).

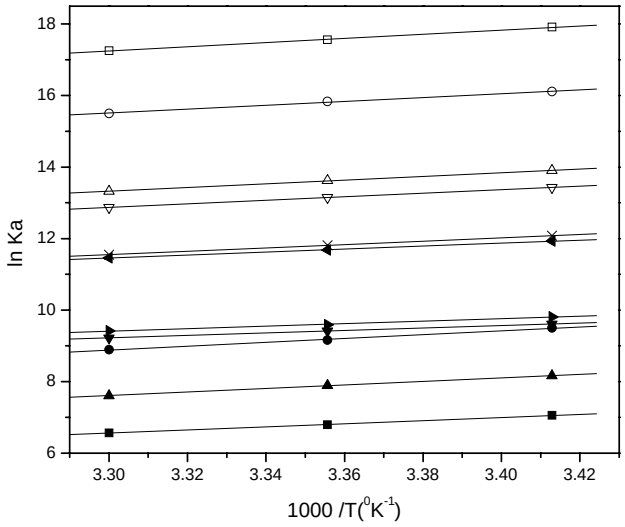

Fig.5.6. Van't Hoff plots for the association of sugars to HSL-CBD. The symbols used are; Gal (■), MeαGal (●), GalNAc (▲), LacNAc (▼), T-antigen (◄), Lactone N-biose (►), Gal β1-3 GalNAc α1-*O*-Ser (□), asialo-triantennary *N*-glycan (○), asialo-biantennary *N*-glycan (△), sialo-triantennary *N*-glycan (▽) and sialo-biantennary *N*-glycan (x). The association constants were determined at 20 ^{0}C, 25 ^{0}C and 30 ^{0}C.

DISCUSSION

Hemagglutination activity is a unique property of lectins. Agglutination is brought about by cross linking of the ligand by the lectin. As cross bridges are necessary for agglutination only multivalent lectins can support this process. In contrast to monovalent proteins, multivalent lectins have more than one CBDs. In general, a single CBD occurs in a polypeptide chain (subunit) and the multivalence is achieved by association of the chains into oligomers. However, HSL is a monomeric lectin and still supports hemagglutination. This situation can arise only if more than one CBD occurs in the same polypeptide chain. To test this hypothesis we carried out limited proteolysis with trypsin. This resulted in a single 25 kDa fragment, which has carbohydrate binding activity but not hemagglutination, indicating characteristic feature of carbohydrate binding domain (CBD).

The 25 kDa fragment was purified to homogeneity using gel filtration chromatography and biochemical properties were examined along with the binding affinity. Lys, Trp and Carboxylate group were found to be critical for sugar binding in HSL-native. These residues were also identified in HSL-CBD. However, the number of critical residues were half that found in HSL-native thus indicating the presence of two CBDs. Therefore, the hemagglutination activity of HSL is due to single chain localized bivalence. Interestingly, some of the monomeric hemagglutinating lectins were found to be divalent [3-6]. However, eight CBDs occur in macrophage mannose receptor [2].

The HSL-CBD preparation was homogenous as no overlapping peaks were produced during *N*-terminal sequencing. Thus, the two HSL-CBDs appear to be homologous. In fucose binding lectin from striped bass, the two CBDs are homologous and appear to be arise as a consequence of gene duplication [4]. Homologous divalency is also seen in the immulectins from *Manduca sexta* [3]. In contrast, lactose-binding protein from rat intestine contains two homologous but distinct domains. Domain-I showed the same affinity for lactose and the same general specificity characteristics of the other S-Lac lectins. The relative interaction of 4 and 6 hydroxyls of Gal and the 3 hydroxyl of Glc with domain-I makes it different from domain-II where these ligands won't bind to later one [5]. The lectin from the fall webworm contains two CBDs, located near the C and N termini respectively. These CBDs are heterologous as rCRD-N can bind both D-Man and GlcNAc, while rCRD-C binds only D-Man [6]. Also, the eight CBDs in macrophage mannose receptor protein are heterologous. CBD 1-3, 5 and 6 have very weak carbohydrate-binding activity; CBD-4 can mimic the monosaccharide binding properties of the whole receptor, but binds poorly to glycoconjugates; CBD 8 does not have any sugar binding affinity; therefore, CBD-4, 5 and 7 together are involved in binding to glycoconjugates, suggesting that the multiple CBDs are arranged spatially to accommodate the geometric configuration of natural oligosaccharides [13].

The binding profile of HSL-CBD is similar to that of HSL-native. The domain retains the preference for Gal and Gal derivatives. In general, the affinity is higher for glycans than disaccharides which in turn are better than for monosaccharides. The order of affinity among monosaccharides was MeαGal >GalNAc >GalN >Gal and Galβ1-3GalNAc > Galβ1-3GlcNAc ≅ Galβ1-4GlcNAc among disaccharides. However, the binding properties of HSL-CBD differ from that of HSL-native in the strength of interaction. The affinity of HSL-CBD for mono and disaccharides is nearly twice stronger than that of HSL-native. On the contrary, the binding strengths of *N*-linked sialylated and asialo glycans are similar. Van't Hoff plots for all ligands were linear (r >0.98, Fig. 5.3), in the range of temperature tested. *O*-linked glycan binds to HSL-CBD with 7-and 50-times higher than *N*-linked asialo triantennary and biantennary glycans respectively. The mono- and di-saccharide binding is contributed by both entropy and enthalpy.

In conclusion, it is tempting to suggest that native lectin is having two homomer carbohydrate binding domains, each weighing 25 kDa. The trimmed domain showed an increased binding affinity, which might play a role in physio (patho)-logical conditions in the animal defense system.

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CHAPTER : 6

STRUCTURAL STABILITY STUDIES OF HSL

SUMMARY

Holothuria scabra lectin (HSL) has unique properties in terms of stability. The saccharide binding function of HSL is independent of pH and shows similar activity from pH 2-12. Also, the lectin is unaffected by temperature up to 80 °C. We have explored the exceptional conformational stability of HSL by fluorescence spectroscopy and circular dichroism. The secondary and tertiary structure of HSL was analyzed as a function of pH. Our results indicate that pH induces structural changes at the tertiary level without any impact on the secondary elements. Treatment of HSL with chemical perturbants like urea and guanidium hydrochloride did not induce denaturation. Instead, the lectin underwent extensive structural changes (both secondary and tertiary) without affecting the activity. Complete unfolding of the lectin could be achieved by including 5 mM DTT along with the perturbants. HSL has six disulphide bonds and our present observations appear to indicate that these covalent bonds are crucial for maintaining the stability of the functional domain of HSL.

INTRODUCTION

The three dimensional structure assumed by a protein, is unique and suitable for its function, can in general be considered to be thermodynamically the most stable confirmation adopted by the polypeptide chain. This stable structure of a protein is held together by non-covalent interactions *viz*. hydrogen bonds, ionic interactions, hydrophobic interactions, van der Waals' forces and covalently by disulfide linkages. Conditions, which disturb these stabilizing forces, affect the native conformation of the protein by changing its physical properties and biological activity. A polypeptide can also adopt a less rigid or more flexible conformation, different from its functional native form, responding to changes in environment. Thus, proteins are only marginally stable because of the functional requirement for their inherent dynamic state and due to delicate balancing of interactions involved in stabilizing or destabilizing particular structure. Determination of the conformational stability

of a protein is critical for understanding the physical interactions that stabilize the protein [1-4].

In this context, detailed denaturation studies of partially folded conformation of a protein or a family of proteins is important in understanding the general principles that govern protein-folding pathways [5]. By recording changes in intrinsic tryptophan fluorescence and the secondary and tertiary structural features of protein in response to tailored changes in surroundings, one can establish presence of interesting structural intermediates relevant to structure-function relationship of the protein.

It has been known for many years that proteins can be unfolded in aqueous solution by high concentrations of certain reagents such as guanidium hydrochloride or urea. Denaturation with these chemicals is one of the primary ways of measuring the conformational stability of the proteins. The use of these two denaturants is extremely widespread [6], even though the exact nature of the molecular interaction of denaturant molecule with protein surface is not well understood. It is known from solubility and transfer experiments with model compounds that the interaction of urea and GdnHCl with the constituent groups of proteins is more favourable than the interaction of those groups with water [7]. The roles and magnitudes of specific electrostatic interactions in determining the stability of a protein can be studied by measuring the dependence of the stability on pH, or by perturbing the interaction through site-directed mutagenesis. pH is know to influence the stability of a protein by altering the net charge of the protein. The aim of this study is to understand the conformational stability of HSL as a function of temperature, pH and chemical denaturants using intrinsic fluorescence and circular dichroism.

MATERIALS

Sea cucumber animals were collected from Anjuna coast of Goa, India. Urea, guanidine hydrochloride, dithiothreitol, methyl α–D-galactose and 8-anilino-1-naphthalene sulfonate (Sigma Chemical Co., St. Louis, MO, USA), Gal β1-3

GalNAc from Dextra Labs, London, UK. All other chemicals used were of analytical grade. Solutions prepared for spectroscopic measurments were in MilliQ water. The buffers used at different pH were: glycine-HCl (pH 2-3), sodium acetate (4-5), sodium citrate-phosphate (5), sodium phosphate (6-7), tris-HCl (8-9) and glycine-NaOH (10-12). pH of the reaction remained stable till the end when it was checked. The urea and GdnHCl stock solutions were prepared fresh in respective buffers on the day of use as described by Pace et al [8].

METHODS

Purification of HSL

The lectin was purified from coelomic fluid of *Holothuria scabra* as described earlier (Chapter 2). The confirmation of the homogeneity and activity of the lectin were done.

Protein determination

Protein concentrations were determined according to Bradford [9] using BSA as standard.

Hemagglutination assay

Hemagglutination assay was carried using pronase treated human erythrocytes of blood group A, B and O as described in chapter 2.

Fluorescence studies

The protein samples (15 μg/ml) are equilibrated with 50 mM buffers of pH 2-12, incubated for 24 h at 25 ^{0}C and also in a different set of experiment the HSL was incubated with various concentrations of the denaturant (urea/GdnHCl) in 50 mM buffers of various pH, at 25 °C for 24 h. The changes in the conformation were monitored by intrinsic tryptophan fluorescence emission spectra recorded on a Perkin Elmer LS-50B spectrofluorimeter, equipped with Julabo F25 water bath. The protein was excited at 280 nm using a cell of 1.0 cm path length and both excitation and

emission slit widths were set at 7 nm. In the thermal unfolding experiments, the spectra were recorded 15 min after the desired temperature was attained.

Circular dichroism studies

CD spectra were recorded at 25 ^{0}C on a Jasco J-715 spectropolarimeter connected to a circulating water bath, at a scan speed of 20 nm·s^{-1} with a response time of 4 s and a slit width of 1 nm. Near (260-300) and far (200-250) UV spectra were collected in a path length of 10.0 and 1.0 mm respectively. The samples were used at 12 μM and 1.9 μM concentrations for near and far UV measurement. In thermal unfolding experiments, sample was incubated for 15 min after the desired temperature was attained. 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 using CDpro software [10].

Refolding of HSL

Two hundred micro liters aliquot was taken out from the samples treated with different concentrations of urea and GdnHCl, diluted 10 times with 50 mM buffer of the same pH at which the unfolding was done. After 4 h, the fluorescence spectra and hemagglutinating activity of the original (treated with urea or GdnHCl) as well as diluted samples were recorded. Protein samples without denaturant under identical conditions were taken as control. Also, all the GdnHCl and urea treated samples were thoroughly dialyzed in pH 8.0 buffer for 48 h and fluorescence spectra and hemagglutinating activity were checked. The renaturation of thermally denatured protein was followed by cooling the heated samples to 20 °C, removing any particulate matter by centrifugation, and then monitoring the fluorescence spectra and the activity.

Hydrophobic dye binding studies

A stock solution of ANS was prepared in methanol and the concentration was measured using an extinction coefficient of 5000 $M^{-1}\cdot cm^{-1}$ at 350 nm [11]. ANS emission spectra were recorded in the range of 400-550 nm with excitation at 375 nm using slit widths of 5 nm for emission and excitation

monochromators. Changes in the ANS fluorescence, induced by the binding to the lectin, were recorded at constant concentration of protein (1.0 μM) and ANS (25 μM). The spectrum of ANS alone in buffer was subtracted from the protein-ANS spectrum to yield the final spectrum.

Light scattering studies

Rayleigh light scattering experiments were carried out with the spectrofluorimeter to follow protein aggregation at different pH and temperature. Both excitation and emission wavelength were set at 400 nm, slit widths to 5 and 2.5 nm respectively and scattering was recorded for 120 sec. The time dependent changes in scattering intensity were followed.

Data analysis

The denaturation curves were plotted with the ratio of fluorescence intensities at 343 nm (λ_{max} of the native lectin), 352 nm (λ_{max} of the native lectin in presence of 5 mM DTT) and completely denatured lectin fluorescence maxima against denaturant concentration, and further analysis of the data was performed as described by Pace et al [8]. From the denaturation curves, a two state ($F \rightleftharpoons U$) unfolding mechanism was assumed and consequently for any of the points, only the folded and unfolded conformations were present at significant concentrations. Thus, if f_F and f_U represent the fraction of protein present in the folded and unfolded conformations, respectively then

$$f_F + f_U = 1$$

f_U was calculated using the following equation

$$f_U = (F_F - F_0) / (F_F - F_U) \qquad (1)$$

where, F_F is the fluorescence intensity of completely folded or native protein, F_0 is the observed fluorescence intensity at any point of denaturant concentration or temperature, F_U is fluorescence intensity of the completely denatured or unfolded protein.

For a two state F⇌U unfolding mechanism, the equilibrium constant K and ΔG_U, the free energy of unfolding was calculated using Equation 4 and 5 respectively.

$$K = f_U / (1 - f_U) \quad (2)$$

$$\Delta G_U = - RT \ln K \quad (3)$$

where, R is the gas constant, and T is the absolute temperature. It is assumed that the free energy of unfolding, ΔG_U, has a linear dependence on the concentration of the denaturant [D].

$$\Delta G_U = \Delta G^{H_2O} + m[D] \quad (4)$$

ΔG^{H_2O} and m are therefore the intercept and the slope respectively, of the plot of ΔG_U versus [D]. ΔG^{H_2O} corresponds to the free energy difference between the folded and unfolded states in the absence of any denaturant and m is a measure of the co-operativity of the unfolding reaction. The concentration of denaturant at which the protein is half unfolded (when $\Delta G_U = 0$) is given by $D_{1/2}$ and from equation (4), $\Delta G^{H_2O} = - m D_{1/2}$.

RESULTS

Effect of pH on structural stability

The influence of pH on the structure was analyzed by fluorescence spectroscopy. At pH 7.0, the emission maximum (λ_{max}) was 342 nm. In acidic pH (2.0), the emission maximum shifted to shorter wave length (λ_{max} =336 nm) while in alkaline pH (12.0) the maximum shifted towards longer wavelength (λ_{max}=355 nm) without any quenching or enhancement of the fluorescence intensity (Fig. 6.1A). The lectin retains complete hemagglutination and MeαGal binding activity in all the pH upto 48 h and later on start slowly decreasing in pH <8.0. The far UV CD spectra at pH 2.0, 6.0, 8.0 and 12.0 were super-imposable indicating the conservation of secondary structure across the pH spectrum (Fig. 6.1B).

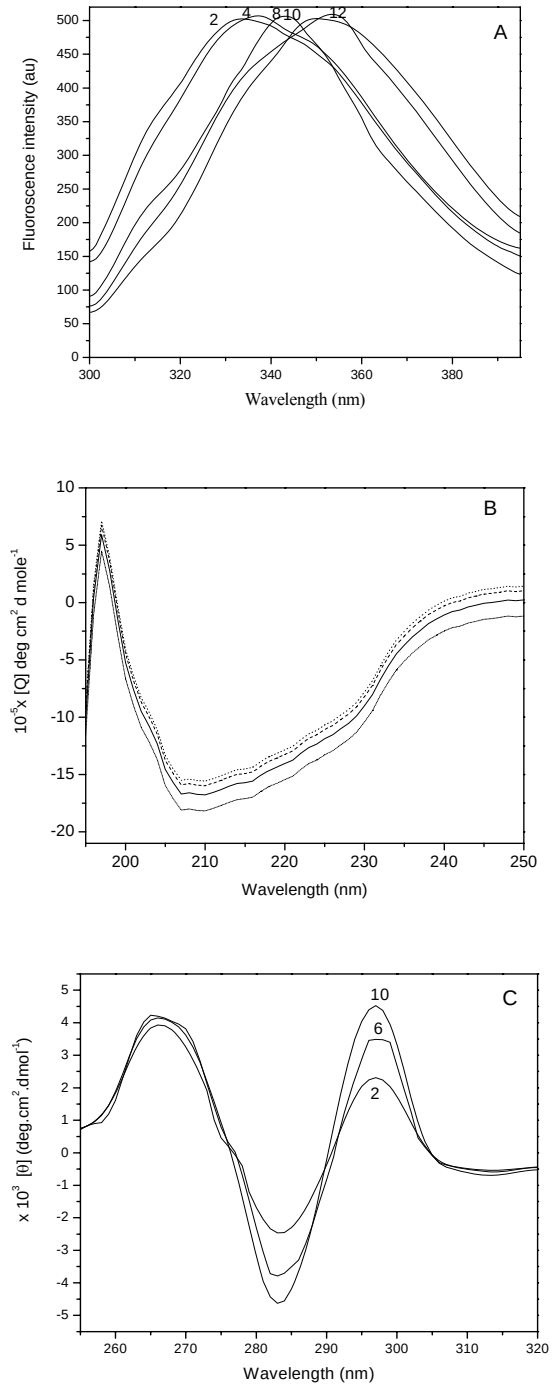

Fig. 6.1: Effect of pH on structure of HSL. (A) Fluorescence emission spectra (B) far-UV CD spectra, the symbols used are (····) pH 2, (---) pH 6, (—) pH 8, and (·-·-·) pH 12. (C) Near-UV CD spectra of the lectin. The spectra were recorded at different pH (as denoted by numbers) after 24 h incubation at 25 °C.

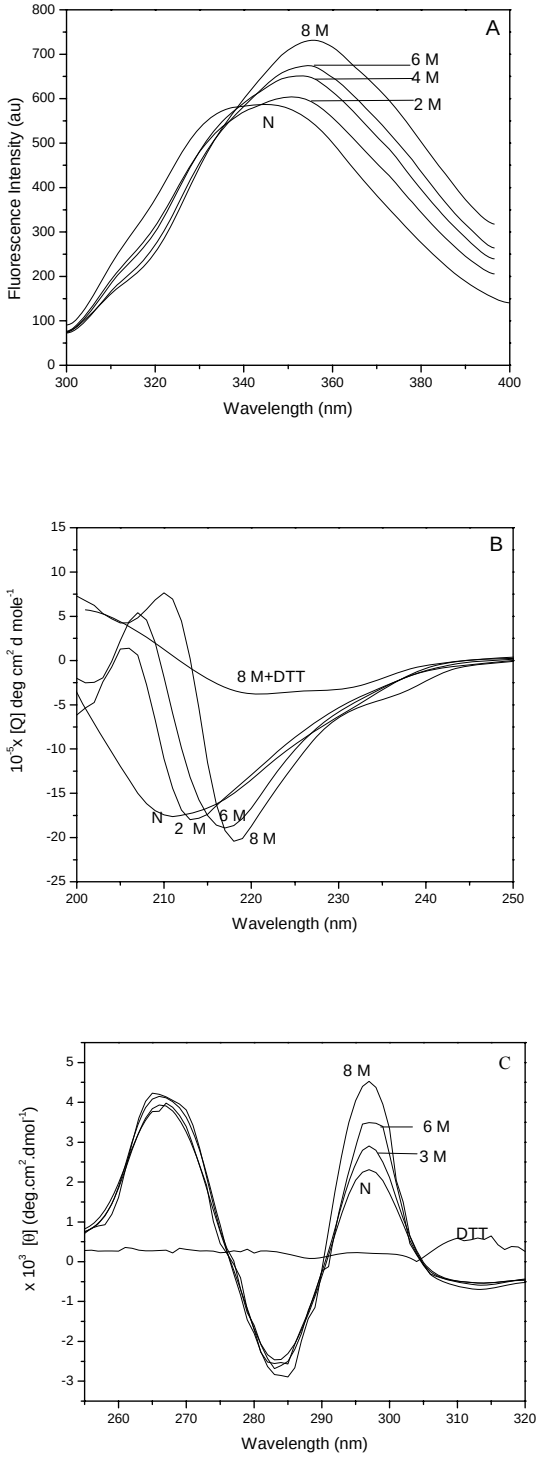

Fig. 6.2: Equilibrium unfolding of HSL in the presence of urea and DTT at pH 8.0. (A) Fluorescence emission spectra, (B) far-UV CD spectra, (C) near-UV CD spectra. Concentrations of denaturants are indicated in figure.

The near-UV CD spectrum showed distinct positive peaks at 266 and 296 nm and a negative peak 283 nm for phenylalanine, tryptophan and tyrosine, respectively (Fig. 6.1C). The magnitude of the positive peak at 296 nm and a negative peak at 283 nm were more prominent at pH 12.0 than at pH 2.0. No changes were observed in the peak at 266 nm.

Unfolding of HSL with chemical denaturants

Attempts were made to unfold HSL by denaturation using urea. Intensity of intrinsic fluorescence increase proportionately with the concentration of the urea. Also, the emission maximum (λ_{max}) was red shifted from 343 to 356 nm (Fig. 6.2A). The effect of denaturant on the secondary structure was analyzed by far UV CD (Fig. 6.2B).

Table 6.1. Secondary structure analysis of HSL[a].

Particulars	α-helix (%)	β-sheet (%)	Unordered coil (%)
Native protein	36.0	49.0	15.0
Native +1 M urea	32.7	51.3	16.0
Native +2 M urea	23.2	55.9	20.9
Native +3 M urea	23.7	55.3	21.0
Native +4 M urea	19.4	57.9	22.7
Native +6 M urea	18.0	61.2	20.8
Native +8 M urea	15.5	63.0	21.5

[a]Secondary structural parameters were calculated based on the far-UV CD spectra in the given urea concentration using CDpro software [10] .

The secondary structural composition of native HSL was 36% α-helix and 49% β-sheets. Increasing concentration of the denaturant was found to induce a gradual increase in the amount of β-sheets and turns while simultaneously decreasing the level of α-helices (Table 6.1). The percent composition of α-helix and β-sheets of HSL in the presence of 8 M urea was 15.5 and 63.0 respectively. Changes in tertiary structure was monitored by near UV CD (Fig. 6.2C), wherein enhancement of the Trp peak (296 nm) was observed in the presence of urea (8 M).

Guanidium hydrochloride (GdnHCl) at low concentrations inhibited lectin activity with 50% activity loss at <2.0 M, whereas lectin retains complete activity at higher concentration of GdnHCl (2-6 M). The structural changes induced by GdnHCl were similar to that of urea (Fig. 6.3). The denaturation experiments were repeated in the presence of 5 mM DTT. Near and far UV CD spectra of HSL in the presence of urea (8 M) and DTT (5 mM) showed complete collapse of structure (Fig. 6.2B,C). In addition, the hemagglutination and MeαGal binding activities were fully lost.

The emission maxima of the native (N) was 343 nm and shifted to 352 nm in presence of 5 mM DTT and fully denatured HSL (D) was 356 nm. Their ratios (*F*352/356) were plotted as a function of urea concentration to obtain the denaturation curve (Fig. 6.4B). The ratio decreased from curve was 1.01 to 0.992. The denaturation of HSL was also monitored by following the changes in the far-UV CD spectra. The denaturation curves were prepared by plotting θ_{208} as a function of denaturant concentration and equilibrium unfolding curve was calculated using equation 3 (Fig. 6.4C). The unfolding plots obtained from CD data were in good agreement with that obtained from fluorescence spectroscopy.

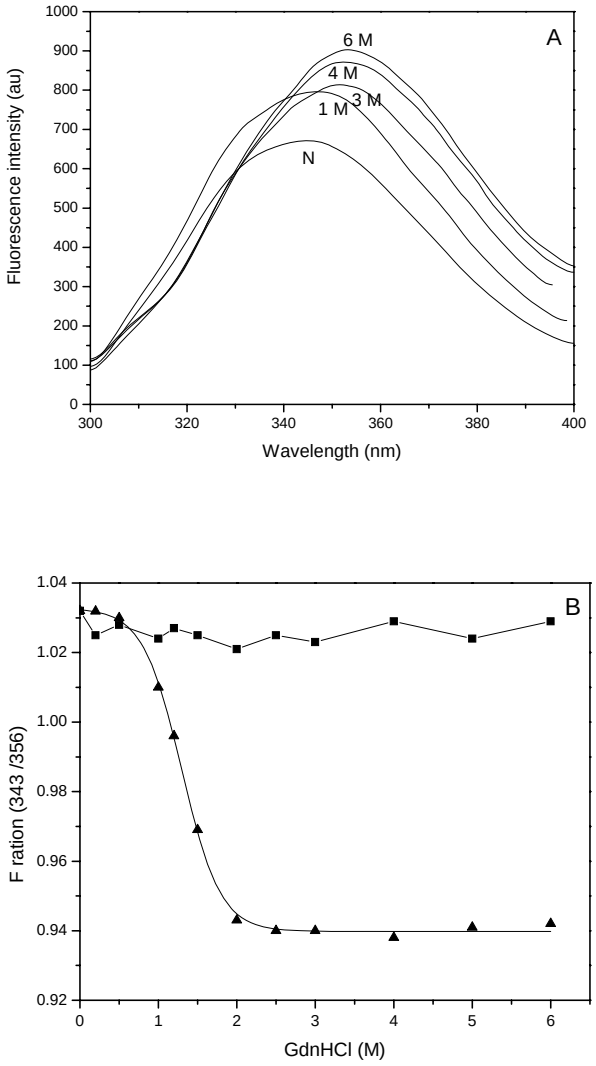

Fig. 6.3: Effect of GdnHCl on structure of HSL at pH 8.0. (A) Fluorescence emission spectra (B) Denaturation curve obtained by plotting ratio of fluorescence intensities at wavelengths 343 and 356 nm as a function of GdnHCl concentration. The symbols used are, denaturation (▲), and renaturation (■). The spectra were recorded after 24 h incubation of the lectin in different concentration of GdnHCl at 25 °C. Renaturation was carried out by diluting the above samples 10 times and incubating for 4 h.

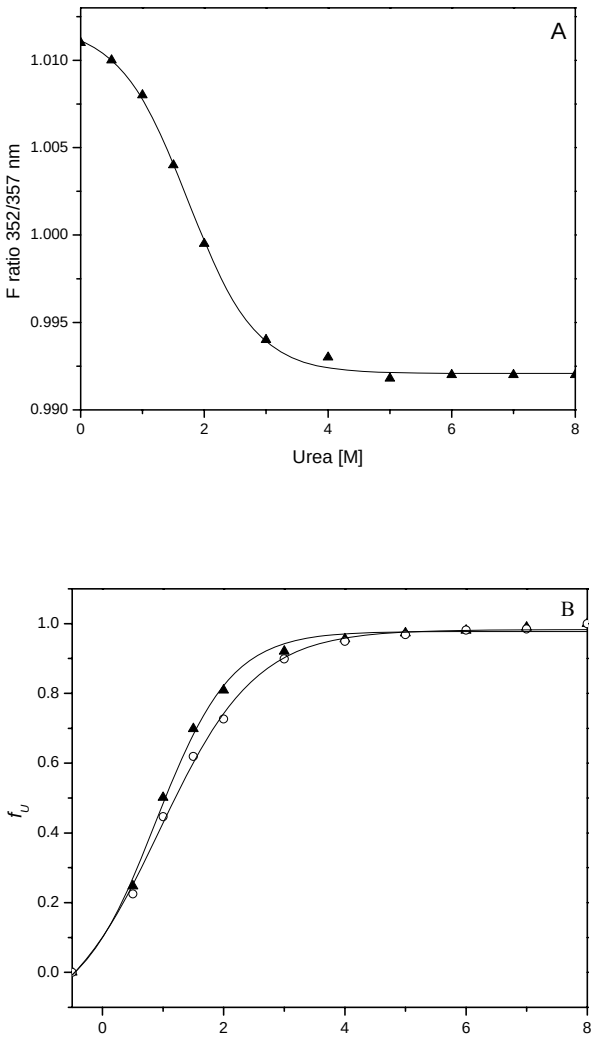

Fig. 6.4: Equilibrium unfolding curves of HSL in presence of urea and DTT. Denaturation curve obtained by plotting ratio of fluorescence intensities at wavelengths (A) 352 and 357 nm as a function of urea in presence of DTT. The symbols used are, denaturation (▲) and renaturation (■). (B) The fraction unfolded (f_U) from fluorescence (■), fluorescence in presence of DTT (○) and far-UV CD (▲) were calculated using Eq. 1 and plotted as a function of urea concentration.

The unfolding curve was used to obtain ΔG_U, using equations 2, 3 and 4. ΔG_U was plotted as a function of denaturant concentration to obtain $D_{1/2}$ and ΔG^{H2O} (Fig. 6.5A). The change in free energy (ΔG), associated with HSL unfolding varied linearly ($r > 0.98$) with the increase in urea concentration. From this, the concentration of denaturant required to unfold half of the protein ($D_{1/2,}$) was determined to be 1.1 M. Also, the difference in the free energy between the folded (N) and unfolded (D) states (ΔG^{H2O}) was found to be 2.3 kJ·mol^{-1} (Table 6.3).

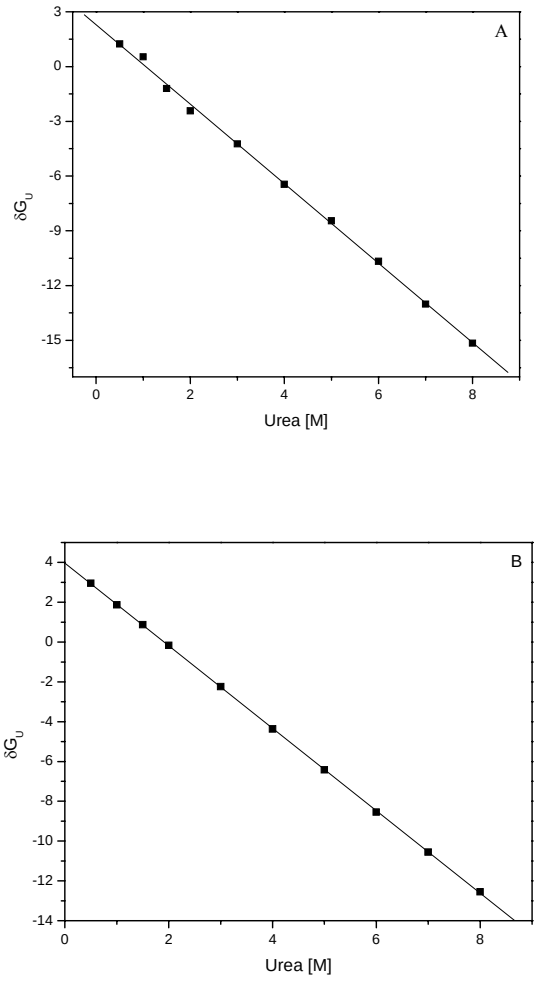

Fig. 6.5 : ΔG_U as a function of urea concentration at pH 8.0. (A) ΔG_U curve in presence of DTT, (B) in absence of DTT. ΔG_U was calculated using Equation 3, intercept and slope of the graph equals to ΔG^{H20} and *m,* respectively.

Table 6.2: Parameters for guanidine hydrochloride denaturation of HSL

pH	ΔG^{H2O} (kJ.mol^{-1})	*-m* (kJ.mol^{-1}. Deg^{-1})	$D_{1/2}$ (M)
8	3.1	1.7	1.8

Table 6.3. Parameters for urea denaturation of HSL at different pH.

pH	ΔG^{H2O} (kJ.mol^{-1})	*-m* (kJ.mol^{-1}. Deg^{-1})	$D_{1/2}$ (M)
2	6.6	1.4	4.6
4	6.2	1.9	3.2
6	5.9	2.1	2.7
8	4.6	1.9	2.3
10	3.9	2.1	1.9
12	2.1	1.8	1.1
8*	2.3	2.2	1.1

$D_{1/2}$, m, and ΔG^{H2O} values were obtained with linear extrapolation from plots of ΔG_U as a function of urea concentration using Equation 4.

*In presence of DTT at pH 8.0.

The structural transition induced by urea in the absence of DTT [N⇌X $_{(Urea/GdnHCl)}$] was examined at various pH conditions. The equilibrium curve (*F*343/356) was sigmoidal. At pH 8.0, GdnHCl was found to be a better transformer than urea as the corresponding $D_{1/2}$ were 1.8 M and 2.3 M respectively (Table 6.2). The transition in the structure was observed at lower concentrations of urea at pH 12 than at pH 2, 4, 6, 8 and 10 and the values of ΔG^{H2O} (2.05 kJ·mol^{-1}) and $D_{1/2}$ (1.12 M) were lowest at pH 12 (Table 6.3). The maximum value of ΔG^{H_2O} (6.58 kJ·mol^{-1}) with urea was obtained at pH 2.0 (Fig. 6.5B).

Refolding of the protein

Renaturation or refolding of lectin was carried out by dilution of the denaturant by 10 times and incubating it for 4 h and also by extensive dialysis for 48 h. Refolding of the protein was measured as the extent of reappearance of the original spectra (*F*343/356) and recovery of the activity (Fig.6.4A).

Temperature induced denaturation of HSL

The intrinsic fluorescence of HSL was monitored at different temperatures. The emission maximum (λ_{max}) of native HSL was 343 nm at 30 °C and pH 8.0. Increase in temperature caused gradual decrease in the fluorescence intensity without any shift in the emission maximum (Fig. 6.6A). This might be due to deactivation of the singlet-excited state by non-radiative processes. There was no loss in activity up to 80 ^{0}C. However, complete loss was observed when the lectin was incubated at 100 ^{0}C for 10 min. No ANS binding and light scattering intensity was observed upto 80 ^{0}C. The secondary structure was intact up to 80 ^{0}C but was completely lost at 100 ^{0}C (Fig. 6.6B) as analysed in far UV CD spectra.

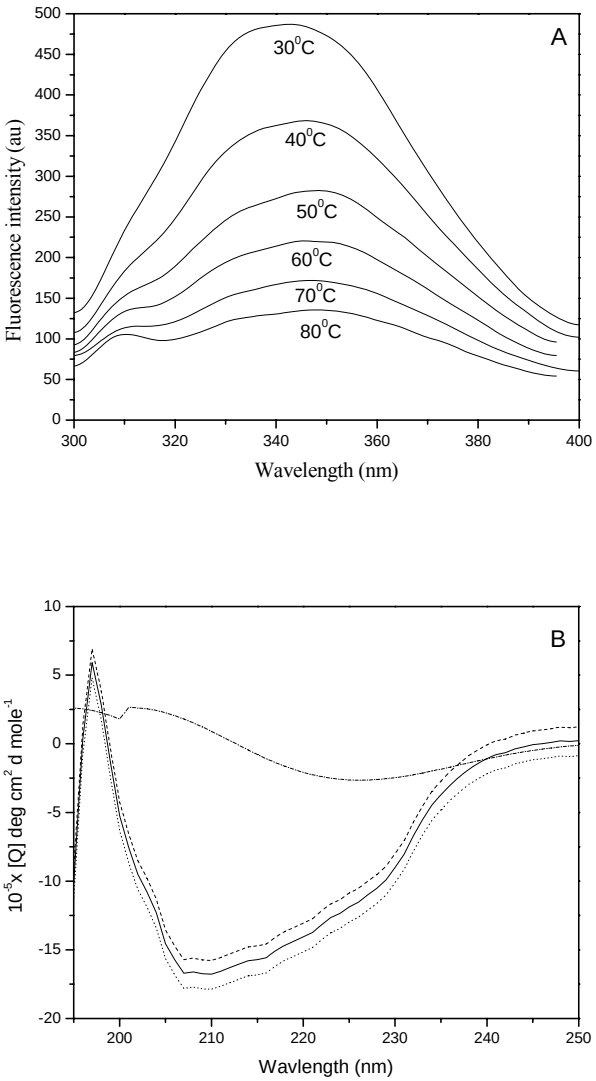

Fig. 6.6. Effect of temperature on the structure of HSL. (A) Fluorescence emission spectra of the lectin incubated at different temperatures (as denoted by numbers), (B) Far-UV CD spectra of the lectin at various temperature. The symbols used are (····) 30 ^{0}C, (—) 50 ^{0}C, (---) 80 ^{0}C, and (·-·-·) 100 ^{0}C, recorded in Jasco J-715 spectropolarimeter connected to water bath.

DISCUSSION

Denaturation of HSL was followed by monitoring the changes in hemagglutination activity and physical properties of the protein including, intrinsic fluorescence and circular dichroism. The HSL is a monomer of 182 kDa having 22 Trp and 23 Tyr. Tryptophan fluorescence has been used to study protein denaturation for several proteins by measuring intrinsic fluorescence [13-16].

The hemagglutination and MeαGal binding activities of HSL are exceptionally stable. It retains complete activity upto 48 h in both acidic and alkaline pH. However, after this the activity of the samples in acidic conditions decreases gradually while those in alkaline conditions are stable upto 5 days. The influence of pH on the structure was analyzed by fluorescence spectroscopy. The change in fluorescence emission maxima across the pH range without quenching or enhancement in the fluorescence intensity was observed, this pH induced unfolding of the proteins is achieved by protonation or deprotonation of relatively few discrete sites (-OH, -COOH and $-NH_2$) [17]. Thus, unlike temperature and chaotropes, pH induced unfolding of the proteins is achieved by perturbance of few residues and a complete unfolding of the protein may not be expected. The pH induced changes in the emission maximum indicates tertiary structural changes especially around the aromatic residues.

The structural changes were also analyzed by near and far UV CD. The magnitude of the positive peak in near UV CD spectrum at 296 nm and a negative peak at 283 nm were more prominent at pH 12.0 than at pH 2.0. No changes were observed in the peak at 266 nm. This indicates that the tertiary structure in the neighborhood of Trp and Tyr are affected by the pH. The reduction in molar ellipticity in the near-UV region at pH 2 might be due to flexibility of the environment of the aromatic residues [18]. Thus marked decrease of tertiary structure without any major effect on the lectin activity upto 48 h might be ascribed to the conformational changes of HSL without affecting local environment of the active site. The structural changes induced

by acidic pH are reversible as samples incubated for 48 h at pH 2.0 regained both activity and structure (CD and fluorescence spectra) after changing the sample to pH 8.0 for 24 h. These results indicate that pH induced changes are restricted to tertiary level without any impact on the secondary elements. Also, these changes appear to occur outside the functional domain of the lectin.

The mechanism of action of urea in unfolding of proteins is still unclear. It has been suggested that the urea molecules permeate the dynamic structure of the protein molecule and bind to the hydrophobic cavities resulting in perturbation of the closely packed interior [19,20] or displace and disrupts the dynamic network of hydrogen bonds that exist in the water molecules bound to the protein [21]. A relatively fragile and a flexible active site, easily perturbed by ligands such as urea, has been envisaged for full expression of catalytic activities of proteins [22].

Attempts were made to unfold HSL by denaturation using urea. Interestingly the lectin retained complete hemagglutination and MeαGal binding activities even in the presence of 8 M urea. The red shift and fluorescence enhancement is usually due to the perturbants induced exposure of Trp residues to the polar environment [15]. The presence of residual structure (as seen in far and near UV CD spectra) indicates that HSL retains extensive structural elements in the presence of urea (8 M). Conservation of binding and hemagglutination activities despite changes in the CD and fluorescence spectra indicates transformation of the structure both at secondary and tertiary levels in such a manner that the binding sites are protected against deformation. Thus urea alone is incapable of unfolding HSL. Similar results were observed in stem bromelain. The cysteine proteinase retains complete enzymatic activity up to 9 M urea though the alpha helical content was altered [23].

Interesting results were obtained when guanidium hydrochloride (GdnHCl) was used as the denaturant. The effect of activity was dependent on the concentration of GdnHCl. Low concentration of GdnHCl (<2.0 M) inhibited lectin activity by about 50%. The inhibition is likely due to

interaction of the positively charged guanidium groups with a critical carboxyl group that occurs at the binding site of HSL (Chapter 3). However, no loss in activity was observed when GdnHCl was used at more than 2 M concentration. It is possible that the structural changes induced at more than 2 M concentration protect the active domains from modification.

We presumed that the inability of urea and GdnHCl to denature HSL could be due to the presence of six disulfide bridges. The loss in activity and structure in presence of DTT indicates that HSL was completely unfolded. Hence, mechanism of conformational alteration of HSL by urea /GdnHCl in presence of DTT may therefore be represented as:

$$N \rightleftharpoons D_{\text{(Urea/GdnHCl + DTT)}} \quad (5)$$

and the equivalent reaction in the absence of DTT as,

$$N \rightleftharpoons X_{\text{(Urea/GdnHCl)}} \quad (6)$$

where N and D are the native and denatured state of HSL respectively and X is the chemically induced conformation with distinct secondary and tertiary structure that exhibits complete hemagglutination and MeαGal binding activity.

The majority of the proteins exhibit a single step denaturation curve and so are assumed to follow a two-state N⇌D mechanism in which only the native state and denatured states are present at significant concentrations in the transition region [8,24,25]. Superimposition of f_U curves (Fig. 1C), obtained by fluorescence and CD measurements for urea-induced denaturation of HSL in presence of DTT supports this assumption. Moreover, the $D_{1/2}$ value obtained with GdnHCl at pH 8.0 was twice lower than that of urea indicating that GdnHCl is twice more efficient denaturant than urea. In general, proteins are most stable at their isoelectric pH since the electrostatic interactions among the charged groups will tend to favor the maximum conformational stability occurring near the pI [26] but not with the HSL. The structural transformation induced by urea in the absence of DTT was examined at

various pH conditions. The equilibrium curve was sigmoidal, indicating that the transformation was biphasic without the involvement of an intermediate. The transformation was found to dependent on the pH with alkaline condition being the most favourable. $D_{1/2}$ for transformation at pH 12 was nearly four times lower than that required at pH 2.

After renaturation, HSL showed considerable blue shift in emission spectra and even after dialysis, the secondary structure also returned to its native conformation (Fig.3B). The lost hemagglutination activity at low concentration of GdnHCl was regained after dialysis. Urea and GdnHCl probably change the conformation of a protein in such a way that substantial interactions are reformed after removal of the denaturants, leading to the significant reformation of the structure and regaining activity. Only partial renaturation and reactivation was observed in case of *Artocarpus hirsuta* lectin when 3-5 M GdnHCl treated protein was diluted [27]. No ANS binding was observed in either sample incubated different pH or at different chemical denaturants, indicating that under all the above conditions hydrophobic patches are not being exposed.

The lectin is highly thermostable losing neither its structure nor its hemagglutination activity. At none of the temperatures, the lectin showed ANS binding indicating that hydrophobic patches are not exposed during temperature incubation. Even light scattering intensity was not affected at different temperature, indicating that there was no aggregation on thermal incubation of the lectin.

In conclusion, our results clearly indicate that the functional domain of HSL is stabilized against the deleterious effects of pH and denaturants by disulphide bonds. Looking into the unusual pH stability, ability to change its conformation under high concentrations of denaturants and its ability to refold back after chemical denaturation strongly suggests that HSL probably can survive extreme environmental conditions, thus playing an important physiological role in the animal system.

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CHAPTER: 7

GENERAL DISCUSSION AND CONCLUSION

DISCUSSION

Lectins, a well-known class of multivalent carbohydrate binding proteins of non-immune origin which recognize diverse sugar structures with a high degree of stereospecificity 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. These lectins from a similar group vary in their molecular weights, carbohydrate specificity and the physiological role played by them. In our studies, we induced, purified and characterized a lectin from coelomic fluid of sea cucumber, *Holothuria scabra*. It belongs to phylum Echinodermata and relatives of the sea stars and sea urchins.

All echinoderms are marine and are bottom-dwellers. Sea cucumbers differ from other echinoderms in having an elongate, cylindrical body with the mouth and anus at opposite ends. Sea cucumbers either gather detritus (bits of plant and animal matter) and bacteria from seafloor sediments or filter particles from the water. They lack spines and the protection of a rigid skeleton, but they have many different means of defense from predators. Some species have repellant or toxic chemicals in the skin that makes them distasteful, others eject sticky threads (cuvierian tubules) from the anus, these

threads entangle and immobilize potential predators and the animal crawls to safety. Still other sea cucumbers can eject (eviscerate) part of the digestive system as a defensive mechanism, regenerating lost parts later.

A novel lectin was isolated from the coelomic fluid of the sea cucumber *Holothuria scabra*, named as HSL. Its involvement in the animal immune response has been demonstrated. Correlative studies indicate that the expression of this defensive lectin was induced by bacterial challenge, wherein cell wall glycoconjugates of bacteria are involved in lectin induction. The lectin was purified to homogeneity by conventional procedures involving hydrophobic interaction column chromatography (phenyl sepharose). Most of the reported lectins like those from *Cucumaria* sp. [19] and *Polyandrocarpa sp.* [20] have been purified by conventional procedures. Even affinity column was extensively used for the purification including Fetuin-CNBr-activated sepharose-4B [21,22] etc.

HSL was found to be a high molecular weight (182 kDa) monomeric protein. The molecular mass of the marine lectins ranging from 17-400 kDa, but majority of them are between 45-65 kDa. Most of them are homo-multimeric proteins and disulfide linkages hold the subunits together. The lectins from *Clarias batrachus* is a monomer of molecular weight 200 kDa [23], whereas lectins from *Cucumaria* sp. CEL-I (32 kDa) is a dimer and CEL-IV is a tetramer [19] and a lectin from *Penaeus japonicas* is a polymeric lectin containing 12 subunits [22].

HSL is a glycoprotein with 8% neutral sugar content. Lectins from *Urechis unicinctus* contains 8.4% neutral sugars [24] but the lectin from *Pterocladiella capillacea* [25] is not glycosylated. The isoelectric point of majority of marine lectins is acidic ranging from 4-6, whereas HSL is highly basic with a pI of 10.3. Marine lectins are generally identified by their metal ion requirement for their hemagglutinating activity. Majority of the marine lectins are C-type lectin family of Ca^{2+} dependent proteins [20,22,23,26-31], while Mg^{2+} stimulated the activity of *Modiolus modiolus* lectin [31,32]. *Ptilota filicina* require the divalent cations, Ca^{2+}, Mg^{2+} and Mn^{2+} for its

activity [33], while few of the lectins not require any metal ions for their activity [34,35].

HSL does not agglutinate untreated, trypsinized and desialylated human ABO type erythrocytes but agglutinates pronase treated ABO erythrocytes. However, the lectin from *Penaeus monodon* agglutinates rabbit erythrocytes exclusively [36], in contrast to *Clarias batrachus* agglutinated human, rabbit, rat and mice erythrocytes [23]. The agglutinating activity of *Didemnum ternatanum* (DTL) [37] and *Crenomytilus grayanus* [38] can be enhanced by prior treatment of the cells with trypsin, without modifying its specificity. Apart from erythrocytes agglutination, HSL also agglutinated both gram positive and negative bacteria and specifically binds to glycan moiety present on the cell wall components of bacteria. A lectin from *Penaeus monodon*, had agglutinated four gram negative (*E.coli, Pseudomonas fluorescens, Aeromonas hydrophilia* and *Vibrio alginolyticus*), a gram positive (*Micrococcus lysodeikticus*) bacteria and a yeast (*Candida albicans*), suggesting that this lectin was a type of immune-relevant protein in *Penaeus* [36]. HSL showed strong broad-spectrum antibacterial activity against both gram-positive and gram-negative bacteria.

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. A lectin from *Penaeus* sp. is stable only between pH 6-8 [36]. HSL is highly thermostable and retains full activity on heating at 80 ^{0}C for 1 h; complete loss of activity occurred on incubation at 100 ^{0}C for 10 min. Most of the other marine lectins are stable upto 20-50 ^{0}C.

Amino acid analysis revealed that 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). HSL contains 16 cysteine residues of which four cysteines were free and 12 were involved in six disulfide bonds. Similarly ascidian

lectin DTL-A contains relatively high amount of Gly, Asx, and Glx and basic amino acids such as Lys and Arg [37]. The amino acid composition of DTL-A and DTL, other lectin from same species are considerably distinguishable [14].

The hemagglutination activity of HSL was inhibited by MeαGal, LacNAc and T-antigen 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 desialylated glycoproteins like asialo fetuin, asialo fibrinogen. Lectins isolated from other genera of sea cucumber, *Cucumeria echinata* (CEL-I, III and IV) [19] interacts well with GalNAc. The preference for the α-anomer over the β-anomer of galactose was also observed for other lectins including a lectin from *Didemnum ternatanum* [37], whereas lectin from marine worm, *Chaetopterus variopedatus* prefers to bind to MeβGal than MeαGal [39].

Active site characterization studies of HSL have shown the involvement of tryptophan, lysine and carboxylate residue in the sugar-binding site. Modification of tryptophan and histidine residues of sialic acid binding lectin from hemolymph of *Achatine fulica* led to a complete loss of hemagglutination activity [40]. Modification of amino groups of hemolytic lectin CEL-III by succinic anhydride showed their involvement in oligomerization of lectin [41].

The sugar binding properties of the HSL was explored in detail using spectrofluorimetry and surface plasmon resonance. Agreement between the data obtained by these two techniques substantiates the monophasic nature of binding. HSL was found to interact with MeαGal and Gal/GalNAc residues present in disaccharides and glycopeptides. Wu *et al.* have [42] grouped Gal/GalNAc specific lectins according to their specificity for the disaccharides (as they are part of bigger structures):

Class F- Forssman specific disaccharide (GalNAcα1→GalNAc)

Class A- Human blood group A specific disaccharide

(GalNAcα1→3Gal)

Class Tn- GalNAcα1→*O*Ser/Thr

Class I/II-Human blood group type I/II precursor sequences (Galβ1→3/4GlcNAc)

Class T- Galβ1→3GalNAc

Class B- Blood group B specific disaccharide (Galα1→3Gal)

According to the classification the HSL belongs to class Tn as well as Class T, and the order of reactivity is galactosyl Tn-antigen > Galβ1→3GalNAc > Galβ1→4GlcNAc.

In the SPR studies, HSL showed several fold higher affinity for the *O*-linked glycan (*K*a = 8.32 M^{-1} x10^7 M^{-1}) than *N*-linked glycans (*K*a = 7.58 M^{-1} x10^6 M^{-1}) and glycoproteins (K_a = 10^3-10^4 M^{-1}). A lectin (CGL) from *Crenomytilus grayanus* mussel [43] and from *Crustacea decapoda* [44], exhibits a very high affinity for sialylated *O*-glycosilated proteins than sialylated *N*-glycosylated proteins. Reduction in the affinity with sialylated glycoconjugates was also reported from *Gracilaria ornata* [34], However, higher affinity with sialylated glycoconjugates can also be found in lectins, where primary interacting counterpart is NeuAc itself, rather than penultimate Gal residue [22]. The kinetics of binding of glycans and glycoproteins to HSL followed by SPR reveals that the binding process follows a bimolecular one-step mechanism. The higher affinity for 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. Increase in negative entropy for both association and dissociation process leads to decrease in affinity for asialo biantennary glycan, fetuin and fibrinogen.

Hemagglutination activity is a unique property of lectins brought about by cross-linking of the ligand by the lectin. As cross bridges are necessary for agglutination and only multivalent lectins (>one CBD) can support this

process. In general, a single CBD occurs in a polypeptide chain (subunit) and association of the chains into oligomers achieves the multivalence. However, HSL is a monomeric lectin and still supports hemagglutination. Limited proteolysis with trypsin resulted in a single 25 kDa fragment, which has the carbohydrate binding activity but no hemagglutination. Thus, HSL belongs to the rare class of monomeric multivalent lectins having two homomeric domains. Some of the previously reported monomeric multivalent lectins include macrophage mannose receptor [45], immulectins from *Manduca sexta* [46], F-type lectin from Striped bass [47] and tandem-type soluble lactose-lectin [48] and a lectin from the fall webworm, *Hyphantria cunea* [49].

Effect of different chemical and physical denaturants such as urea, guanidine hydrochloride, temperature and pH on structural stability of HSL was monitored by fluorescence spectrometry and circular dichroism. Changes in fluorescence emission maxima (λ_{max}) and the enhanced fluorescence intensity measured in the presence of urea and guanidium hydrochloride under different pH, indicated changes in microenvironment of the Trp residues, suggesting alterations in tertiary structure. The secondary structural composition of native HSL was 36% α-helix and 49% β-sheets. Increasing concentration of the denaturant was found to induce a gradual increase in the amount of β-sheets and turns while simultaneously decreasing the level of α-helices. The percent composition of α-helix and β-sheets of HSL in the presence of 8 M urea was 15.5 and 63.0 respectively. Changes in tertiary structure was monitored by near UV CD, wherein enhancement of the Trp peak (296 nm) was observed in the presence of urea (8 M). The presence of residual structure (as seen in far and near UV CD spectra) indicates that HSL retains extensive structural elements in the presence of urea (8 M). Conservation of binding and hemagglutination activities despite changes in the CD and fluorescence spectra indicates transformation of the structure both at secondary and tertiary levels in such a manner that the binding sites are protected against deformation. Similar results were observed in stem

bromelain. The cysteine proteinase retains complete enzymatic activity up to 9 M urea though the alpha helical content was altered [50].

At pH 7.0, the emission maximum (λ_{max}) was 342 nm and in acidic pH (2.0), the emission maximum shifted to shorter wavelength (λ_{max} =336 nm) while in alkaline pH (12.0) the maximum shifted towards longer wavelength (λ_{max}=355 nm) without any quenching or enhancement of the fluorescence intensity. This pH induced unfolding of the proteins is achieved by protonation or deprotonation of relatively few discrete sites (-OH, -COOH and -NH_2) [51]. Moreover, thermal denaturation did not result in the loss of activity or aggregation or precipitation or loss in structure even at 80 ^{0}C.

We presumed that the inability of urea and GdnHCl to denature HSL could be due to the presence of 6 disulfide bridges. So the denaturation experiments were repeated in the presence of 5 mM DTT. Near and far UV CD spectra of HSL in the presence of urea (8 M) and DTT (5 mM) showed complete collapse of structure. Also, the hemagglutination and MeαGal binding activities were fully lost. These results indicate that HSL was completely unfolded under the said conditions. The mechanism of conformational alteration of HSL by urea /GdnHCl in presence of DTT may therefore be represented as:

$$N \rightleftharpoons D_{(Urea/GdnHCl + DTT)} \quad (1)$$

and the equivalent reaction in the absence of DTT as,

$$N \rightleftharpoons X_{(Urea/GdnHCl)} \quad (2)$$

where N and D are the native and denatured state of HSL respectively and X is the chemically induced conformation with distinct secondary and tertiary structure that exhibits complete hemagglutination and MeαGal binding activity.

The concentration of denaturant required to unfolding half of the protein ($D_{1/2,}$) in presence of DTT was determined to be 1.1 M. Also, the difference in the free energy between the folded (N) and unfolded (D) states (ΔG^{H2O}) was

found to be 2.3 kJ·mol^{-1} (Table 2). $D_{1/2}$ for transformation at pH 12 was nearly four times lower than that required at pH 2. At pH 8.0, GdnHCl was found to be a better transformer than urea as the corresponding $D_{1/2}$ were 1.8 M and 2.3 M respectively. The free energy (2-6.5 kJ·mol^{-1}) required for HSL unfolding was comparatively low than other lectins. Frutalin (*Artocarpus incise* lectin) a tetrameric lectin showed conformational stability of 17.12 kJ·mol^{-1} and 12.34 kJ·mol^{-1}, in the presence and absence of D-galactose, respectively [53]. Unfolding process of HSL was irreversible, whereas the unfolding process of galectin-1 (from porcine spleen) was reversible [54]. Surolia *et al.* [55] and Schwarz *et al.* [56] investigated thermal unfolding transition of galectin-1, describing tetrameric and octameric folding intermediates. However, such intermediates were not observed in the unfolding studies of HSL.

Biochemical approaches have resulted in generation of extensive data on the structure and molecular properties of lectins. The elucidation of lectin function will require synthesis and correlation of data obtained from the study of many lectins. In this way, information regarding specificity of a marine invertebrate lectin from *Holothuria* with different glycans and glycoproteins would be helpful in understanding the role of the lectins in the animal defense mechanism.

CONCLUSIONS

1. Humoral lectin level in the coelomic fluid of sea cucumber, *Holothuria scabra*, was increased by challenging with ten different human pathogenic bacteria.
2. Lectin showed selective bacterial agglutination, broad-spectrum antibacterial activity and hemagglutinating activity against pronase treated human erythrocytes.
3. Lectin was isolated and purified from coelomic fluid by re-chromatogram on phenyl sepharose column.
4. The lectin is a monomeric, glycoprotein with a molecular mass 182 kDa and basic pI of 10.3.

5. The lectin is highly thermostable, retains its full hemagglutination activity up to 80 °C for 1h and wider pH stability over 2-12.
6. Active site characterization studies show the involvement of Trp, Lys and carboxylate groups at the sugar binding.
7. The lectin exhibits specificity for MeαGal and galactosyl-Tn-antigen and asialo N-glycans.
8. HSL is a monomeric lectin having two homomer carbohydrate binding domains.
9. HSL exhibits functional stability against guanidine hydrochloride upto 6 M, urea upto 8M. Temperature does not have any effect on stability, whereas acidic pH has marginal effect.

List of publications:

1. Nagaraj M. Gowda, Usha Goswami and M. Islam Khan, T-antigen binding lectin with antibacterial activity from marine invertebrate, sea cucumber (*Holothuria scabra*): Possible involvement in differential recognition of bacteria, J. Invertebr. Pathol., ***(In Press).***
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3. Nagarajappa, A. Ganguly and U. Goswami, DNA damage in male gonad cells of green mussel (*Perna* sp.), upon exposure to tobacco products, J. Ecotoxicol., 2006, **15**:365-369.
4. S. R. Desai, X. N. Verlecar, Nagarajappa and U. Goswami, Marine diatom *Chaetoceros tenuissimus* as a test organism for cadmium toxicity using DNA damage by comet assay, J. Ecotoxicol., 2006, **15**:359-363.

5. Nagaraj M. Gowda, Usha Goswami and M. Islam Khan, Monomeric lectin from marine invertebrate, *Holothuria scabra* and its carbohydrate binding domain: glycan binding properties, *Glycoconjugate J, (**Under review)***.
6. Nagaraj M. Gowda, Usha Goswami and M. Islam Khan, Steady state and time-resolved fluorescence studies of *Holothuria scabra* lectin, J. Fluorescence, (***Under review***).
7. Nagaraj M. Gowda, Usha Goswami and M. Islam Khan, Studies on conformational stability of *Holothuria scabra* lectin by fluorescence spectroscopy and circular dichroism, FEBS. J. (***Under Review***)

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