# STUDY OF A LECTIN FROM *GANODERMA LUCIDUM*

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By

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*DEDICATED TO*

*MY PARENTS AND*

*BROTHERS*

CONTENTS

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# Certificate

**Certified that the work incorporated in the thesis entitled "Study of a lectin from *Ganoderma lucidum*" submitted by Mr. Atul Kumar Thakur was carried out under my supervision. Such material as 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 "Study of a lectin from *Ganoderma lucidum*" submitted by me for the degree of Doctor of Philosophy is the record of work carried out by me during the period from 19th Nov, 2001 to 29th Aug, 2007 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 . . . .

Atul Kumar Thakur

# ABSTRACT

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

*Ganoderma lucidum* is a well-known medicinal mushroom and many health-promoting and therapeutic effects have been attributed to this mushroom. Lectins from several pathogenic fungi have also been studied in detail but their physiological role has not been ascertained or remains uncertain. It is therefore essential to know the specificity of the lectins to explore their physiological role. In this thesis, we describe the isolation and characterization of a novel 114 kDa hexameric lectin from the fruiting body of *G. lucidum*.

**Chapter 1: General Introduction**

This part comprises a literature survey of lectins with reference to their purification, properties and applications.

**Chapter 2: Purification and characterization of *Ganoderma lucidum* lectin.**

*G. lucidum* lectin was purified approximately 13-fold with final recovery of 35%. The purified lectin migrated as a single band in native- as well as SDS- polyacrylamide gels indicating homogeneity. The molecular mass of the lectin determined by gel filtration is 114 kDa and it is made up of six identical subunits of 18.5 kDa, which are held together by non covalent bonds. *G. lucidum* lectin is a glycoprotein containing 9.3% neutral sugar. It is a basic protein with a pI of 9.7. G. lucidum lectin was active between pH 5-9, temperature up to 50 °C and did not require divalent cations for its

activity. N-terminal sequence of *G. lucidum* did not show similarity with any known lectin. Purified lectin agglutinated neuraminidase/ pronase treated human erythrocytes (A, B and O). Its hemagglutinating activity was inhibited by glycoproteins containing N-linked or O-linked glycans and polysaccharide Gum Karaya.

**Chapter3: Chemical modification studies on *Ganoderma lucidum* lectin**

Arginine, tyrosine, cysteine, aspartate, and glutamate were not found to be involved in hemagglutinating activity of the *G. lucidum* lectin. Purified lectin, when incubated with NBS, completely lost its activity and six tryptophan residues were modified indicating presence of one tryptophan per monomer of lectin. Number of tryptophan residues modified in urea denatured lectin was also six. Modification of lectin with NBS in presence of ligand asialo-triantennary glycan resulted in only 50% loss of activity indicating the presence of tryptophan at active site. Modification of lysine with TNBS, succinic anhydride, citraconic anhydride and reductive methylation also resulted in 100% loss of the hemagglutinating activity of the lectin. Analysis revealed that 11 out of the total 54 lysine residues modified were involved in activity indicating involvement of 2 lysine residues per monomer of lectin. Decitraconylation of the lectin completely restored its activity. Native lectin modified with DTNB resulted in modification of 6 free cysteine residues. Modification of lectin with DTNB after denaturation with urea and reduction with sodium borohydride resulted in modification of 18 residues indicating presence of 6 disulphide bonds in lectin. Disulphide bonds are not involved in holding the subunits together as *G. lucidum* lectin did not show any difference in molecular weight in the presence and absence of DTT.

**Chapter 4: Carbohydrate binding and Solute quenching studies of the *Ganoderma lucidum* lectin by steady state and time resolved fluorescence spectroscopy.**

The intrinsic fluorescence intensity of *G. lucidum* lectin was quenched upon binding to disaccharides and glycans, without any change in the emission maximum (336 nm) The *G. lucidum* lectin bound poorly with monosaccharides and disaccharides. The binding with disaccharides were close to binding with monosachharides indicating that the lectin probably does not discriminate between mono- and di-saccharides. *G. lucidum* lectin showed highest affinity for asialo-triantennary glycan among the sugars and glycans tested ($K_a$ 1.07 x $10^5$ $M^{-1}$). Energetically ligand-lectin interaction was found to be enthalpicaly driven. Solute quenching studies of the lectin using acrylamide, succinimide, potassium iodide and cesium chloride were carried out in the absence and presence of asialo-triantennary glycan and at different pH. These studies showed that the single tryptophan residue of the lectin (per monomer) is relatively buried, and could be in the vicinity of positively charged amino acid residues. Time resolved fluorescence studies of the native lectin, in the presence of ligands and acrylamide showed biexponential decay with a shorter life time ($\tau_1$) and longer life time ($\tau_2$) of fluorescence which indicated the existence of tryptophan in two different conformers. For the native lectin biexponential fits yielded lifetime values of 1.27 ns ($\tau_1$) and 4.66 ns ($\tau_2$). In the presence of ligand (triantennary N- glycan) shorter lifetime increases to 1.41 ns and longer lifetime decreases to 4.56 ns. In the presence of acrylamide a relative decrease in both lifetimes was observed, longer lifetime ($\tau_2$) decreases to 3.12 ns and shorter lifetime ($\tau_1$) to 0.98 ns.

**Chapter 5: Glycan and glycoprotein binding to the *Ganoderma lucidum* lectin: Surface Plasmon Resonance Studies.**

Sugar binding of *Ganoderma lucidum lectin was* lectin was also explored by surface plasmon resonance. *G. lucidum* lectin was immobilized on CM5 sensor chip by amino group coupling. The glycan and glycoprotein

solutions were passed over the chip at the flow rate of 50 µl/min. The binding of the sugar to the immobilized lectin resulted in the change in SPR signal. The change was recorded and the data were analyzed to determine different binding parameters as described by Langmuir and others. The glycans used were Galβ1→3GalNAc-O-Ser, Asialo-N-linked triantennary glycan of fetuin, Asialo-N-linked biantennary glycan of fibrinogen. Asialo-N-linked triantennary glycan of fetuin was most potent ligand of the lectin among glycans tested ($K_a = 3.52 \times 10^5$). The values of $\Delta H$ and $K_a$ determined by SPR and fluorescence also shows good agreement, indicating $K_a$ and enthalpy changes are related to the total binding process and rule out the occurrence of any intermediate that contributes appreciably to these parameters for the saccharide binding. The Arrhenius plots ($\ln k_{\ddagger}$ *vs* 1/T) were also linear, ruling out the occurrence of dramatic conformational changes in the lectin molecule in the temperature range studied. Glycoproteins were also checked for binding to *Ganoderma lucidum* lectin. Glycoproteins used were fetuin and fibrinogen. SPR data for glycoproteins also fits very well with Langmuir's 1:1 model and linear Arrhenius plots were obtained. Compared to glycans, their corresponding glycoproteins showed several fold higher affinity with significant contribution from enthalpy. Increase in affinity also resulted in increase in negative entropy which could be compensated by higher enthalpy.

**Chapter 6: General Discussion and Conclusion**

This part compares the properties of *Ganoderma lucidum* lectin with other lectins with respect to their biophysical properties.

**List of abbreviations used**

| | |
|---|---|
| DEPC | : Diethylpyrocarbonate |
| DTNB | : Dithiobisnitrobenzoic acid |
| EDC | 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide |
| Fuc | : L-Fucose |
| Gal | : 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 |
| Glc | : D-Glucose |
| GlcNAc | : N-acetyl-D-glucosamine |
| $k_1$ | : Association rate constant |
| $k_{-1}$ | : Dissociation rate constant |
| $K_a$ | : 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 | 3-nitro-L-tyrosine ethyl ester |
| PAGE | : Polyacrylamide gel electrophoresis |
| PMSF | : Phenylmethyl-sulphonilfluoride |
| SPR | : Surface plasmon resonance |
| TBS | : Tris buffer saline |
| TNBS | Trinitrobenzenesulphonic acid. |

*Chapter: 1*

# General Introduction

## Definition

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 wide spread in nature (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 non-immune origin which agglutinate cells and/or precipitate glycoconjugates. However, with the discovery of lectins with toxic or harmone 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 non-immunoglobulin 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".

# Classification

lectins are 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 structurally similar sugars. Since hololectins have

multiple binding sites they are fully capable of agglutinating cells and precipitating glycoconjugates. Most plant lectins belong to this subgroup (9-11).

***Chimerolectins:*** These are fusion proteins composed of a carbohydrate-binding domain 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).

**Historical perspective**

The lectin was first discovered by Stillmark in 1888 as an agent that can agglutinate animal erythrocytes from extract of castor bean (14). 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 (15-17). Hellin in 1891 discovered abrin (18), 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 (19) found some lectins showing specificity for the red blood cells of certain species, while in 1945 Boyd and Reguera (15) established blood group specificity of the lectin. One of the first lectins to be crystallized was con-A from jackbean by Sumner (20). Sugar specificity and inhibition of hemagglutination of the lectin was discovered by Watkins and Morgan in 1952 (21). Researches on lectin gained momentum when Nowell's laboratory at the University of Pennsylvania,

Philadelphia reported the mitogenicity of lectins, wherein the lectins stimulated mitosis in the dead-end lymphocyte cells (22).

Lectins are wide spread in distribution and have been isolated from microorganisms, insects, plants and animals (9,11,23-29). Although most of the well characterized lectins are from plants, mushroom lectins are receiving increased attention due to their importance from basic as well as applied aspects. This compilation gives comprehensive account of mushroom lectins with respect to their occurrence, purification, physicochemical properties, biological roles and applications.

## MUSHROOM LECTINS:

Mushroom can be defined as macrofungi with distinctive fruiting bodies which are either epigeous or hypogeous and sufficiently conspicuous to the naked eye to be hand picked (30). Occurrence of lectins in traditionally edible mushrooms *Lactarius deliciosus* and *Boletus edulis* prompted researchers to look further in this field (31). The first mushroom lectin 'phallin' was reported by Kobert in 1891 from *Amanita phalloides*, which was a hemolytic agent (32).

Haemolysins present in mushrooms are rarely responsible for accidental poisoning, because they are heat sensitive, and generally mushrooms are never eaten raw (33). *Laetiporus sulphureus* lectin shows both haemolytic and haemagglutinating activites inhibited by same sugars (34). It may be that in some instances the high rate of haemolysis masks agglutination causing such molecules to be termed as haemolysins rather than lectins (35). *Aleuria aurantia* was the first mushroom lectin, for which the crystal structure was solved by Wimmerova *et al.* in 2003 (36).

### Occurrence and localization

The occurrence of lectins in mushroom is wider than in higher plants (37). Mushroom lectins have been isolated from mycelia, caps, stipes, sporomes, basidiomes and fruiting bodies (31,35,37-40). Most of the

mushroom lectins have been isolated from fruiting bodies, however lectins have been found in cultured mycelia of the mushrooms also (41). The lectins in mycelium and basidiome can be identical or similar as in *Lactarius deliciosus* (42), *L. deterrimus* (31), *Fomes fomentarius* (43), *Kuehneromyces mutabilis* (44), *Rigidoporus lignosus* (45), or differ in structure and specificity as with *Pholiota squarrosa* and *Agrocybe aegerita* (46).

**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 animal as well as human erythrocytes (47-50). Sometimes erythrocytes are treated with enzymes like pronase, trypsin, papain and neuraminidase or any other proteolytic enzyme (50-53). Other types of cell lymphocytes, sperm, and yeast have also been used for lectin detection (49,54). Lectins also form cross-links between polysaccharide (55-58) or glycoproteins (59-63) in solution and induce their precipitation.

**Purification**

Most of the purification procedures, involves steps like lysis of cells, isolation of different parts, concentration of the crude extract by salt precipitation, ultrafiltration 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 mushroom lectins (64-66). In addition, hydrophobic matrix like Phenyl-Sepharose has been employed for the purification of lectin from fungi *A. fumigatus* (52) and *Beauveria Bassiana* (76).

Affinity chromatography has been extensively used for the purification of mushroom lectins. The ability of lectins to agglutinate

erythrocytes has been utilized for their purification on erythrocytes and erythrocyte stromas embedded in polyacrylamide (67-69). The other affinity absorbents include chitin column (70), BSM-Toyopearl (71) and gal-Sepharose/ sepharose (72-74)). 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 *Ischnoderma resinosum*, specific for methyl β-galactoside (73). Immobilized horse red blood stroma has been used for the purification of lectin from Psilocybe berrerae (75).

Modern purification techniques like HPLC and FPLC have been successfully used for purification of lectins from *Volvariella volvacea* (76), *Hygrophorus hypothejus* (77), *Ganoderma capense* (78) and *Peziza sylvestris* (79).

**Molecular mass and subunit structure**

Mushroom lectins generally exhibit variable molecular masses (16-190 kDa), could have more than one subunits which may or may not be identical. Except for few lectins as from *Lactarius lignyotus* (80) and *Phallus impudicus* (81) the subunits of which are linked by disulphide bridges, the other di-, tetra-, or polymeric lectins studied, namely those of *Agaricus edulis* (82), *Agaricus campestris* (66), *aleuria aurantia* (83), *Flammulina velutipes* (64), *Hericium erinaceus* (84), *Lactarius deliciosus, L. deterrimus, L. Salmonicolor, Pholiota aurivella, Pleurotus cornucopiae* and *Xerocomus chrysenteron* have subunits held together by non- covalent interactions (31).

**Table 1.2. Physical properties of mushroom lectins**

| Source | Mole-cular mass (kDa) | Sub-unit type | pI | Carbo-hydrate content (%) | References |
|---|---|---|---|---|---|
| *Agaricus blazei* | 70 | $\alpha_4$ | | 11 | (85) |
| *Agaricus bisporus* (ABA I) | 64 | $\alpha_4$ | 6.70 | 2.4 | (86) |
| *Aleuria aurantia* | 72 | $\alpha_2$ | 9.1 | 0 | (83) |
| *Auricularia polytricha* | 23 | $\alpha$ | 10.6 | 3.5 | (87) |
| *Boletus satanus* | 63 | $\alpha$ | 8.3 | - | (88) |
| *Chlorophyllum molybdites* | 32 | $\alpha_2$ | 3.75 | 12 | (89) |
| *Clitocybe nebularis* | 30 | $\alpha_2$ | 4.3 | 10 | (90) |
| *Flammulina velutipes* | 22 | $\alpha_2$ | 5.4 | 0 | (91) |
| *Hericium erinaceus* | 54 | $\alpha_2\beta_2$ | 5.0 | 1.5 | (84) |
| *Hygrophorus hypothejus* | 68 | $\alpha_4$ | 5.0 | 0 | (77) |
| *Ischnoderma resinosum* | 32 | $\alpha_2$ | 5.5 | 4 | (73) |
| *Laccaria amethystea* | 16 | $\alpha$ | 9.5 | 0 | (67) |
| *Lactarius deliciosus* | 37 | $\alpha\beta$ | 6.7 | 0 | (42) |
| *Lactarius lignyotus* | 100 | $\alpha_4$ | - | 4 | (80) |
| *Laetiporus sulphureus* | 190 | $\alpha_2\beta_2$ | 5.2 | 0 | (34) |
| *Macrophomina phaseolina* | 34 | $\alpha$ | - | 16.4 | (92) |
| *Marasmius oreades* | 50 | $\alpha\beta$ | 5.2 | 5.4 | (93) |
| *Phallus impudicus* | 75 | $\alpha_4$ | 6 | 13.9 | (94) |
| *Pleurotus cornucopiae* | 31 | $\alpha\beta$ | - | - | (95) |
| *Pleurotus ostreatus* | 81 | $\alpha\beta$ | 7.6 | 0 | (96) |
| *Pleurotus ostreatus* | 72 | $\alpha\beta$ | - | - | (97) |
| *Peziza sylvestris* | 20 | $\alpha$ | - | - | (79) |
| *Rigidoporus lignosus* | 150 | $\alpha_4$ | | 30 | (45) |
| *Xerocomus spadiceus* | 32 | $\alpha_2$ | - | - | (80) |

**Isoelectric point**

The mushroom lectins show very high diversity in the pI (Table 1.2). Lectins from *Clitocybe nebularis* (90) and *Chlorophyllum molybdites* (98) are highly acidic proteins with a pI of 4.3, and 3.75, respectively whereas, the lectins from *Laccaria amethystea* (67) and *Auricularia polytricha* (87) are highly basic proteins with pI values of 9.5 and 10.6 respectively.

**Carbohydrate content**

In mushrooms some lectins show very high sugar content as lectins from *Rigidoporus lignosus, Fomes fomentarius, Agaricus edulis, Phallus impudicus Psilocybe berrerae* contain 30%, 25%, 18%, 13.9% and 9.5% neutral sugar respectively (31,75,81,82). However lectins from *Aleuria aurantia*, *Lactarius deterrimus*, *Laccaria amethystea*, *Laetiporus sulphureus* and *Pleurotus cornucopiae* are not glycosylated (34,45,67,69,83,91) (Table 1.2).

**Metal ion requirement**

Mushroom lectins in general do not require metal ions for their activity. The activity of *Lactarius lignyotus* and *Xerocomus chrysenteron* lectins increase in the presence of $Ca^{++}$ ions. *Xerocomus chrysenteron* lectin also shows increase in activity in the presence of $Mn^{++}$ ions (80). *Polyporus adusta* lectin showed two fold increase in titre value in presence of 10 mm $FeCl_3$ (40). On the contrary, activity of Armillaria luteo-virens lectin is inhibited by $Fe^{3+}$, $Al^{3-}$ and $Zn^{2+}$ (99).

**Carbohydrate specificity**

Mushroom lectins exhibit a broad specificity varying from simple sugars to glycoproteins. *Peziza sylvestris* lectin is the only lectin specific for arabinose while *Hericium erinaceus* lectin is specific for sialic acid (Table 1.2). Lectins specific for glucose and mannose have not been

purified from mushrooms till now. Mikiashwili, 2006 had reported mannose specific lectin from *Trametes hirsuta* and glucose specific lectin from *Leucoagaricus leucothitus*, however these lectins were not purified and characterized (41).

Most of the lectins purified from mushrooms show specificity for galactose or di or oligosaccharides containing galactose i.e. lactose, N-acetyl galactosamine / lactosamine and Galβ1→3 GalNAc. Gallagher classified fungal lectins into two groups according to their carbohydrate specificity (99).

a) **Exolectins:** Which bind to appropriate external, nonreducing sugars in complex saccharides and their agglutination can be inhibited by low concentrations of the respective free sugar or their methyl glycosides. The first reported exolectins were PAL-I and PAL-II from *Phaeolepiota*. Other exolectins reported include those from *Agrocybe aegerita* (100), *Grifola frondosa* (101), *Clitocybe nebularis* (102), *Laccaria amethystina* (78), *Psathyrella velutina* (103) Table 1.3.

b) **Endolectins:** Which recognize complex oligosaccharides and their agglutination can be inhibited only by specific sugar sequences. Activity of Lectins from *Cordeceps militaris*, *Flammulina velutipes*, *Kuehneromyces mutabilis*, *Mycoleptodonoids aitchisonii*, *Paxillus involutus*, *Russula nigricans* and *Volvariella volvacea* is not inhibited simple sugars and inhibited only by glycoproteins so these lectins would be specific for complex oligosccharides exhibited on glycoproteins (31,64,76,100,101), Table 1.4. Hence these lectins can be defined as endolectins

**Table 1.3: Carbohydrate specificity of mushroom lectins**

| Sugars | Lectin | References |
|---|---|---|
| Fucose | *Aleuria aurantia* | (83) |
| | *Laccaria amethystea (LAF)* | (67) |
| | *Laccaria laccata* | (102) |
| | *Melastiza chateri* | (103) |
| | *Pholiota squarrosa* | (102) |
| Mannose | *Trametes hirsuta* | (41) |
| Arabinose | *Peziza sylvestris* | (79) |
| Rhamnose | *Agaricus arvensis* | (41) |
| | *Omphalotus olearius* | (41) |
| Glucose | *Leucoagaricus leucothitus* | (41) |
| Glucosamine | *Lentinula edodes* | (104) |
| *N*- Acetylglucosamine | *Ganoderma ramnosissimum* | (41) |
| | *Pleurotus tuber-regium* | (105) |
| | *Psathyrella lacrymabunda* | (70) |
| | *Psathyrella velutina* | (70) |
| | *Trametes versicolor* | (41) |
| Galactose | *Agrocybe aegerita* | (46) |
| | *Amanita ovoidea* | (41) |
| | *Boletus satanas* | (106) |
| | *Clitocybe nebularis* | (90) |
| | *Fomes formentarius* | (90) |
| | *Lepista nuda* | (41) |
| | *Marasmius oreades* | (107) |
| | *Melanoleuca brevipes* | (41) |
| | *Panus conchatus* | (26) |
| | *Psilocybe berrerae* | (75) |
| | *Coprinopsis cinerea* | (108) |

| **Sugars** | **Lectin** | **References** |
|---|---|---|
| *N*- Acetylgalactosamine | *Agaricus blazei* | (85) |
| | *Ciborinia camelliae* | (109) |
| | *Clitocybe geophyla* | (41) |
| | *Grifola frondosa* | (110) |
| | *Inocybe fastigiata* | (111) |
| | *Oudemansiella platyphylla* | (112) |
| | *Lactarius rufus* | (102) |
| | *Lactarius vellereus* | (113) |
| | *Pleurotus serotenus* | (26) |
| | *Pleurotus ostreatus* | (72) |
| Lactose | *Agrocybe cylindracea* | (114) |
| | *Auricularia polytricha* | (87) |
| | *Boletus edulis* | (115) |
| | *Boletus subtomentosus* | (115) |
| | *Hygrophorus hypothejus* | (77) |
| | *Coprinus atramentarius* | (115) |
| | *Corpinus comatus* | (41) |
| | *Ischnoderma resinosum* | (73) |
| | *Laccaria amethystea (LAL)* | (67) |
| | *Macrolepiota rachodes* | (41) |
| | *Pleurotus ostreatus* | (116) |
| | *Pleurotus spodoleucus* | (116) |
| | *Tricholoma fractum* | (41) |
| Galβ1→3 GalNAc | *Agaricus bisporus* | (86) |
| | *Lactarius deliciosus* | (42) |
| | *Lactarius deterrimus* | (69) |
| | *Lactarius salmonicolor* | (31) |
| | *Russula nigricans* | (31) |
| Galβ1→4 GlcNAc | *Laetiporus sulphureus* | (34) |
| Sialic acid | *Hericium erinaceus* | (84) |

**Table 1.4: Glycoprotein specificity of mushroom lectins***

| Lectin | Specificity | Reference |
|---|---|---|
| *Cordeceps militaris* | Fetuin> BSM> PSM** > porcine thyroglobulin | (100) |
| *Flammulina velutipes* | Asialo *Rana japonica* egg surface glycoprotein> asialofetuin> asialo human glycophorin> human transferrin> asialo BSM** | (64) |
| *Kuehneromyces mutabilis* | EBN glycoprotein**>asialo PSM**> asialofetuin> fetuin > $\alpha_1$-acid glycoprotein | (31) |
| *Mycoleptodonoids aitchisonii* | Asialo-BSM > BSM > asialo- Fetuin> Fetuin | (101) |
| *Paxillus involutus* | Asialo-PSM> asialo EBN glycoprotein> asialofetuin> $\alpha_1$-acid glycoprotein> human transferrin | (31) |
| *Russula nigricans* | Asialofetuin>asialo EBN glycoprotein> ovomucoid> $\alpha_1$-acid glycoprotein | (31) |
| *Volvariella volvacea* | Thyroglobulin | (76) |

* Carbohydrate specificity of these lectins is not defined

** EBN: Collcalia mucin from edible bird nests; BSM: bovine submaxillary mucin; PSM: porcine submaxillary mucin

**Biological properties of mushroom lectins**

*Specificity towards cells*

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 red blood erythrocytes:* Some of the mushroom lectins showed discrimination between erythrocytes of different blood groups. Treating erythrocytes with proteolytic enzymes can enhance their activity or modify specificity. The anti-H activity of *Laccaria proxima* lectin can be enhanced in this way, without modifying its specificity, by prior treatment of the cells with papain (39). Similar observation was made in *Clathrus cancellatus,* where enhancement in its anti-O effect was observed by papain treatment (117). In some cases higher specific activity was observed with trypsinized erythrocytes (118).

Sometimes, presence of macromolecules can facilitate agglutination and raise lectin activity, with or without alteration in specificity. Dextran and bovine albumin together with ficoll have been the most widely used (39,117).

*Animal erythrocytes:* It has been observed that certain mushroom lectins can discriminate both between human and animal erythrocytes, as well as among different animal erythrocytes. The lectin isolated from *Mycena galericulata* agglutinates pig erythrocytes exclusively (118). Whereas an extract from *Irpex sinuosus* agglutinates only trypsinized horse and rabbit erythrocytes (119). Erythrocytes from several other mammals, birds, reptiles and amphibians have also been used for Hemagglutination (31).

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

observed for one agglutinin of *Russula nigricans* that is active on the yeast *Candida albicans* (120). Similarly, the presence of a non-erythroagglutinant lectin in *Clitocybe geotropa* has been demonstrated in a study of the glycocalix of the protozoan *Crithidia oncopelti* (121).

*Effect of mushroom lectins 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.

*Effect on lymphocytes:* Tsuda in 1979 reported the existence of a stimulating activity on the *in vitro* multiplication of mouse spleen lymphocytes by high doses of the lectin of *Flammulina velutipes* (64). The lectin isolated from *Lentinula edodes* was mitogenic towards murine splenic lymphocytes (104). More recently, it was shown that the lectin from *Boletus satanas* is mitogenic for human T lymphocytes with secretion of interleukins 1 a and 2 by mononucleate cells in culture (122). The L-fucose specific lectin isolated from *Aleuria aurantia* has mitogenic activity on mouse T lymphocytes (Thy-$1^+$ splenocytes and cortisone-resistant thymocytes) (123). The lectin from *Lentinula edodes* is a potent mitogen towards murine splenic lymphocytes (104). A lectin from *Agaricus bisporus* abolishes the activation of T and B lymphocytes obtained by various stimulants (31).

*Effect on pancreatic cells :* The lectin from *Agaricus campestris* (PHA-A) and from *Agaricus bisporus* (PHA-B) can stimulate the production of insulin and glucagon by rat pancreatic Langerhans cells and raise glucose consumption by adipocytes (124,125).

*Effect on tumor cells :* Certain mushroom lectins showed inhibitory action on the *in vitro* growth of tumor cells. *Volvariella volvacea* lectin has a moderate effect on sarcoma-180 (126), and *Agaricus bisporus* lectin reversibly inhibited the proliferation of various lines of

malignant epithelial cells, with no specific toxic effect (127). Moreover, some lectins, such as that of *Aleuria aurantia,* exert a selective toxic action on certain modified cell lines (128).

**Structure of mushroom lectins**

*Aleuria aurantia* was the first mushroom lectin, for which the crystal structure was solved. The crystal structure of the lectin complexed with fucose revealed that each monomer consists of a six-bladed β-propeller fold and a small antiparallel two-stranded β-sheet that plays a role in dimerization (fig. 1.1 a). The six-bladed β-propeller of lectin from *Aleuria aurantia* (AAL), has been crystallized with either five (36) or three bound fucose residues (129), suggesting that the binding sites, although all very similar in geometry, do not have the same affinity for ligands. The ligand-binding site of AAL is characterized by numerous hydrogen bonds to the side chains of polar amino acids and by strong hydrophobic interactions between aromatic residues (fig. 1.1 b). However, the lectin structures based on a β-propeller architecture are rare. The five- to eight-fold pseudo-symmetry offered by this architecture is very favorable for multivalent sugar binding.

*Flammulina velutipes* lectin showed unique folds never before observed in lectins (130), and showed structural similarity to human fibronectin. Lectins from *Xerocomus chrysenteron* (131) and *Agaricus bisporus* (132), resembling actinoporins, a family of pore-forming toxins from sea anemones. *Agaricus bisporus* lectin is a tetramer and each monomer presents a novel fold with two β sheets connected by a helix-loop-helix motif. Agaricus bisporus lectin possesses two binding sites with two different specificities on same domain which can discriminate between two monosaccharides differing in the configuration of a single epimeric hydroxyl (LacNAc and T-antigen) (132).

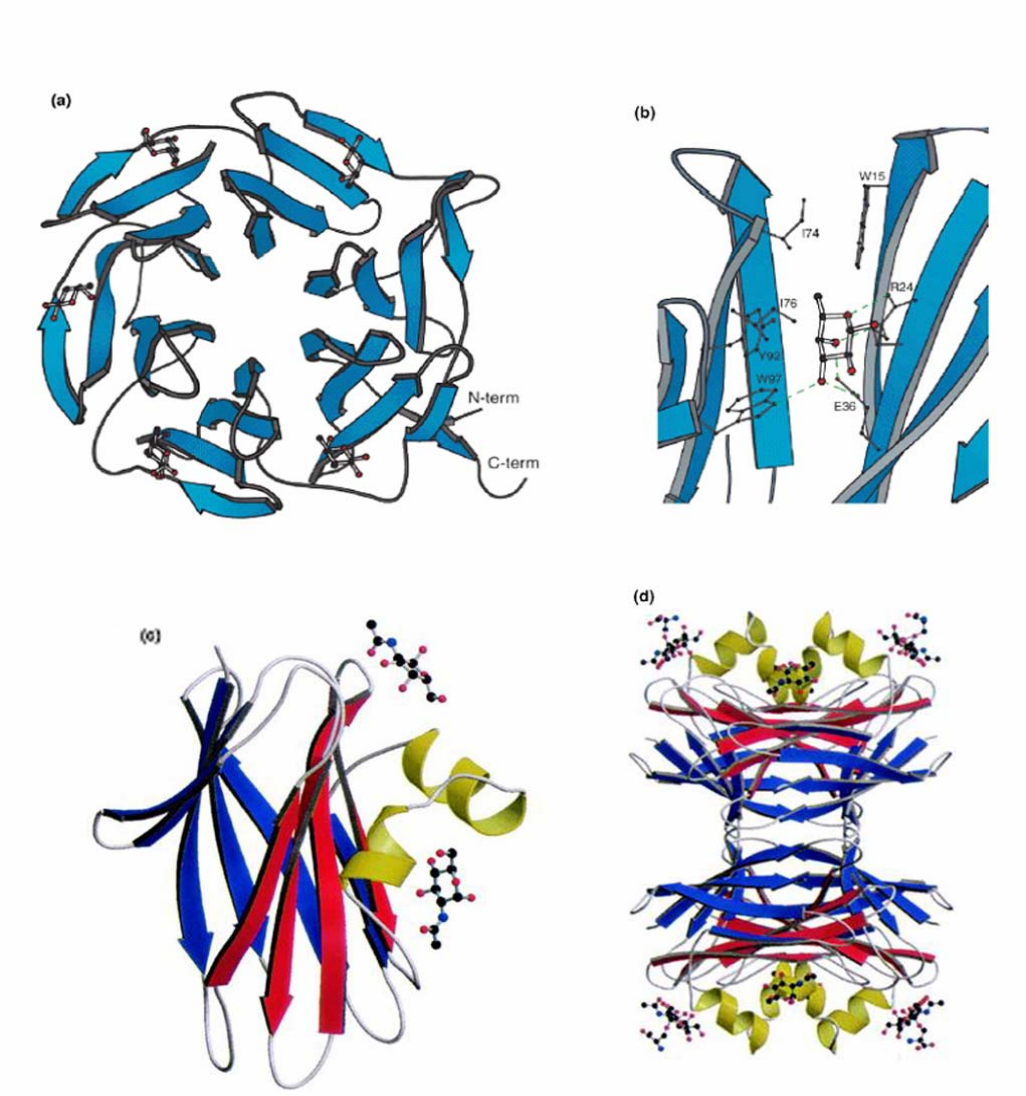


**Figure 1.1:** (a) Crystallographic structure of the six-bladed β propellers formed by one monomer of *A. aurantia*, (b) AAL complexed with fucose (PDB code 1OFZ), (c) Ribbon representation of ABL monomer. The six stranded β sheet is shown in *blue*, and the four stranded β sheet is shown in *red*. The two short helices are yellow and the connections are *grey*. N-acetylglucosamine is represented by a *ball- and- stick* model at the T-antigen binding site. (d) Stereogram of crystallographic dyads, top two (or bottom) monomers represent the dimer present in the crystallographic asymmetric unit. N-acetylglucosamine is represented by a *ball- and- stick* model. Figures adapted from (36) and (132).

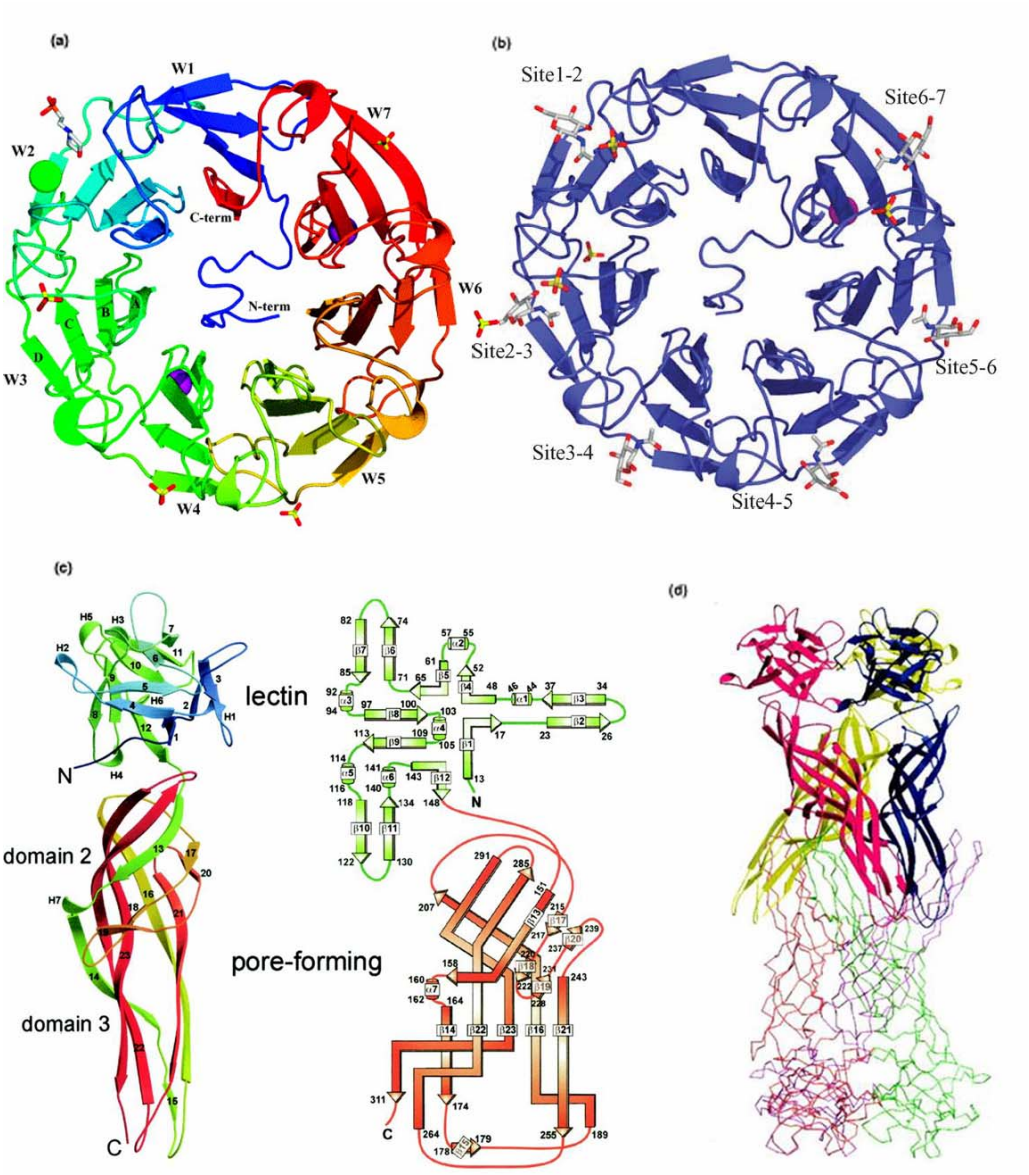


**Figure 1.2:** (a) Orthogonal view of crystal structure of *Psathyrella velutina* lectin. Calcium and chlroride ions are represented by magenta and green respectively. (b) Orthogonal view of crystal structure of *Psathyrella velutina* lectin complexed with N-acetylglucosamine. (c) Three dimensional structure and topology of *Laetiporus sulphureus* lectin. Ribbon model is shown on left and topology diagram on right. β-strands and $3_{10}$ helices are represented by arrows and cylinders respectively. (d) Oligomeric structure of *Laetiporus sulphureus* lectin. Monomers are shown as ribbon models (upper trimer) and as polypeptide traces (lower trimer). Figures are adapted from (133) and (134).

Lectin from *Psathyrella velutina* also showed binding sites with differing specificities, however this lectin has six binding sites four of which are high affinity binding sites which are specific to N-acetylglucosamine and two low affinity binding sites specific to N-acetylneuraminic acid (133). *Psathyrella velutina* showed seven bladed β-propellar fold with N-terminal region tucked into the central cavity of around the pseudo 7-fold axis (fig. 1.2 a and b). Tyrosine is present in the high affinity binding sites and tryptophan in low affinity binding sites (133). CGL2 from *Coprinus cinerea* showed fold similar to galectins (135), a large family of lectins from all classes of vertebrates (136). The lectins from *Laetiporus sulphureus* (134) and *Marasmius oreades* (137) showed resemblance with the ricin-B domain, a trefoil-based fold observed in many lectins and carbohydrate-binding domains, and referred to as the (QxW)3 domain (138). *Laetiporus sulphureus* is a hexamer, each monomer consisting of a ricin-B domain and an elongated C-terminal domain with structural similarity to aerolysin, a bacterial β-pore-forming toxin. This domain could be directly involved in pore formation because the lectin displays hemolytic activity (139). The ricin-like domain displayed structural similarity to similar domains from plant toxins (ricin, abrin and mistletoe lectin), but was even more similar to the hemagglutinin component of bacterium *Clostridium botulinum* (140). Structural comparison of the (QxW)3 domains from mushroom toxin, clostridial hemagglutinin and ricin illustrated their high similarity. During evolution, this domain has been conserved, and often duplicated and/or combined with other domains. It has been identified in bacteria, fungi and plants, and also in sponge, insects and mammals, generally conserving its role of targeting a sugar-coated substrate (138). *Agrocybe aegerita* lectin showed structural similarity with galectins, the carbohydrate recognition domain (CRD) of which contains consensus sequence motif, which consists of His-44, Arg-48, Val-59, Asn-61, Trp-68, Glu-71 and Arg-73 (141).

**Roles of lectins in mushrooms**

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. Mushroom lectin are reported to participate in the formation of primordia, creation of mycelium structures to facilitate, penetration of parasitic fungi into the host organism as well as mycorrhization (142,143).

*1. Involvement in growth and morphogenesis*

The lectins, *via* acting directly on carbohydrates by their specific binding sites, or indirectly by enzyme systems, possibly control depolymerization of stored material and intense movement of sugars, resulting initiation and often explosive growth of the sporomes. Lectin can repress the activity of the glycoprotein enzymes by binding them at their carbohydrate moieties. The release of simple sugars could thus redirect metabolism through competition for lectin binding (35).

A lectin may be involved in ensuring cohesion between hyphae during the development of the basidiome as seen in *Pleurotus cornucopiae* (143). However, the discovery in a strain of this species of a protein related to the lectin and regulated in the same way but not erythroagglutinant, makes the exact role of the lectin in this fungus uncertain. Either the lectin is not involved in the morphogenesis, or is but cannot be revealed by a hemagglutinating activity (144).

*2. Involvement in dormancy*

In some perennial fungus, variable lectin levels have been reported in different seasons, indicating possible role of lectin in the dormancy cycle, as in *Aegopodium podagraria* (145) or *Eranthis hyemalis* (146). Studies done on *Helianthus tuberosus* established that lectins are directly involved in maintaining and ending dormancy (147,148).

*3. Involvement in morphological changes in the host after parasitic infection*

During their growth, many fungi build specialized organs that require mycelial aggregation. These organs, cords, fans of mycelium, strands, rhizoids *etc*. are found particularly in parasitic and wood-rotting species, which use these organs to force their way amongst the host cells. *Rigidoporus lignosus,* a parasite of many tropical trees infects roots by the vegetative mycelium, but propagates *via* the fans of mycelium. A lectin seems to be involved in the building of the latter structures, since it has been found in significant amounts only in the hyphae that are able to aggregate and in the fans of mycelium themselves (45,149). This lectin seems therefore to be a cell wall lectin involved in a recognition mechanism.

*4. Molecular recognition during mycorrhization*

Presence of lectins, with strict specificities, in mushroom cells of ectomycorrhizal symbiosis, suggests that lectins might be involved in recognition between the tree and its symbiont. The hypothesis has been further exemplified by study of lectins from three species of fungi *Lacterius deliciosus, L. deterrimus* and *L. salmonicolor,* though very close morphologically, are specifically associated with different trees *Pinus, Picea* and *Abies,* respectively. Three essential conditions have been verified experimentally: (i) In spite of being present in closely related species the three lectin differ significantly in structures and their microspecificities towards oligosaccharides; (ii) It has been demonstrated, by shedding from cell walls by enzyme degradation or location by means of polyclonal antibodies, that the lectin is present at the surface of cultured hyphae; (iii) The receptors, born by the root cell surfaces of each of the conifers, are exclusively for the lectin of their own associated fungus (69).

Some of the molecules present in the soil can combine with mushroom lectins and block their specific binding sites and in the rhizosphere, *e.g.* certain phenolic acids that can modify carbohydrate

receptors on the roots of the host trees, will prevent recognition and consequently mycorrhization (150).

**Applications of mushroom lectins**

The ability of lectin to interact with simple, aminated, acetylated, sialated 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. They have also been successfully utilized as epidemiologic as well as taxonomic markers of specific microorganism (31,151).

*1. Use in taxonomic studies*

*Protozoa*

Agglutinating extracts from several fungi have been used for the taxonomic study of protozoa in the genera *Crithidia, Blastocrithidia, Leishmania* and *Trypanosoma* (121,152,153). The lectins of *Laccaria amethystea* and *Boletus edulis* have been used to study cysts and trophozoites of *Toxoplasma gondii* (154).

*Yeasts*

Striking diversity of the carbohydrates on the surface of *Candida albicans* was observed. The lectin of *Russula nigricans* showed exclusive specificity towards *Candida albicans,* and can therefore be used in identification tests. It was also suggested that this lectin may also interact to structures involved in the pathogenecity of *Candida albicans* (155).

*Anaerobic rumen fungi*

The mushroom lectins from *Laccaria amethystea, Clitocybe geotropa* and *Pholiota squarrosa* have proved to be useful reagents in the taxonomy of rumen micromycetes and also utilized in monitoring changes in the cell walls occurring during their life cycle (156-158).

*2. Use in food industry*

The lectin of *Agaricus bisporus* was immobilized on magnetic beads and successfully used to remove *Listeria monocytogenes* bacteria added experimentally to milk (154)

.

*3. To study role of glycoconjugates in embryo development*

Mushroom lectin have been used to study glycoconjugates, involved in the cellular interactions during the uro-genital morphogenesis in bird embryos, to understand the mechanisms responsible for the migration of germ cells. In birds, the primordial germ cells (PGCs), localized at the primitive line stage in the Swift crescent, subsequently migrate *via* the bloodstream towards future genital ridges. There are many arguments in favor of involvement of membrane glycoproteins in the mechanisms of recognition between PGCs and tissues during the migration. FITC-labeled lectins and NC-1/HNK-1 mono-clonal antibodies used in indirect fluorescence reactions bind to PGCs and afford ready visualization. The lectin of *Laetiporus sulphureus,* which recognizes N-acetyllactosamine residues is one of the most specific to this cell type (159-161).

*4. Isolation and purification of serum glycoconjugates*

Immobilization of lectin to an unreactive support also allows separation of glycoconjugates possessing lectin-specific glycan structures. The lectin isolated from *Aleuria aurantia* has been used for purification of fucosylated glycoproteins, such as H activity glycoconjugates from erythrocytes membrane (162), tumor antigens (163), Bence-Jones proteins (164), normal and pathologic human immunoglobulin-G (165) and brain glycoproteins (166,167).

*5. In cancer research*

Cancer cells exhibit altered specific properties such as loss of contact inhibition and ability to migrate and form metastases. Much work has been done to understand the modification occurring in membrane

glycoconjugates, glycoproteins and glycolipids during cancer induction. Modification to 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 from *Lacterius deliciosus,* which is specific to D-Galβ1→3D-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)* (35). The residue, D-Galβ1→3D-GalNAc, in cancerous cells results from an improper sialylation in the glucid part of M or N blood group antigens and related structures (31).

*6. Separation of cells*

The property of lectin to differentiate surface sugars/oligosaccharides 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 (22) 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 (31).

*7. 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 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 protein is missing or functionally impaired (35).

The other area of application is the detection, in a population of cells modified by oncogenesis, of lines displaying resistance to particular lectins. As above, this resistance is linked to alterations to surface glycans, which very often form the essential structures of receptor molecules or are involved in mechanisms of recognition between the cell and its environment. In this way it is possible to investigate how modifications to particular glycan structures affects the tumor-forming capacity of the cells and their metastatic potency (31).

The lectin of *Aleuria aurantia* has been used to study correlations between lectin-resistance and growth characteristics on cell lines from Lewis-type (LL2) mouse lung cancers (106,138,191,192) and line Mm B16 of mouse melanoma (168).

**FEW IMPORTANT MUSHROOM LECTINS**

The mushroom lectins have been localized on different parts as the caps, stipes and mycelia. The lectin content varies in the mushroom and depends on carpophore age, and the time and place of harvest. Lectins have been reported in several edible and non-edible mushrooms. In mushrooms, lectins probably play an important role in dormancy, growth, morphogenesis, morphological changes consequent on parasitic infection and molecular recognition during the early stages of mycorrhization (31). A detail survey of mushroom lectin is documented in the review articles by Kawagishi (38), Guillot and Konska (31) and Wang *et al.* (37).

***Agaricus sp.:*** Several species of mushroom *Agaricus* were found to contain lectins. It appears that *A. bisporus* produces more than one lectin (86). So far, two lectins PHA and PHB have been reported from the mushroom. The lectins are tetrameric and all have molecular mass of 64 kDa and 58 kDa (169). Hemagglutinating activity of the lectins can be inhibited by bovine and porcine submaxillary mucins (169). Both the lectins have similar specificities for cell surface carbohydrate receptors and have been purified by chromatography on DEAE-cellulose, Sephadex G-150, phosphocellulose and Sephadex G-150. PHA and PHB could be separated by phosphocellulose column chromatography. PHA is a glycoprotein with 2.5% carbohydrate. The lectins have been shown to stimulate the release of both insulin and glucagon from the pancreatic islets of Langerhans (124,125). The *A. bisporus* lectin has been reported to have an antiproliferative activity toward various tumor cell lines (170). The binding properties of ABA was investigated in detail by enzyme-linked lectinosorbent assay and lectin-glycan inhibition assay. The results indicated that polyvalent Galβ1→ related glycotopes, GalNAcα1→Ser/Thr (Tn), and their cryptoforms, were the most potent ligand for the lectin (171). Lectin from *A. blazei* has a molecular mass of 64 kDa and composed of four subunits each with a molecular mass of 16 kDa same as *A. bisporus*

and it was glycosylated. Its hemagglutinating activity was inhibited by glycoproteins *viz*. asialo bovine submaxillary mucin, asialo-fetuin and fetuin; and monosaccharides as *N*-acetyl-D-galactosamine (85).

A lectin reported from *A. campestris* has similar molecular mass and subunit as lectin from *A. bisporus* and *A. blazei*. But the lectin from *A. campestris* was more thermostable (65,66). *A. edulis* also produced two lectin as reported by Eifler, both the lectins were different in molecular mass and carbohydrate specificity (82). One of them was tetrameric with a molecular mass of 60 kDa while the other was dimeric with a molecular mass of 32 kDa. The lectins showed very high thermostability and could withstand high concentration of urea and extreme pH. One of the lectins has complex specificity and its hemagglutinating activity can not be inhibited by common simple sugars.

Lectin has been isolated from *A. cylindracea* by Yagi *et al.* (114) and Wang *et al.* (172) The lectin purified by Yagi *et al.* is homodimeric with a blocked N-terminus while that of Wang *et al.* is heterodimeric with distinct N-terminal sequences for the two subunits. Both the lectins were sialic acid specific. Lactose was a poor inhibitor of the hemagglutinating activity of the lectin purified by Yagi *et al.* but effectively counteracted the activity of the lectin purified by Wang *et al.*

The *A. cylindracea* lectin exhibited mitogenic response. A six-fold increase was observed in mitogenic response when mouse splenocytes were incubated with 2 μM *A. cylindracea* lectin. A dose-dependent increase in mitogenic response could be observed when the lectin concentration was elevated from 0.5 to 1 μM and then to 2 μM. When the lectin concentration was further increased to 4 and 8 μM there was a decline in mitogenic response. The mitogenic responses at these concentrations were, however, over 200% higher than the control value (172).

***Armillaria luteo-virens :*** From the dried fruiting bodies of the mushroom *A. luteo-virens*, a dimeric lectin with a molecular mass of 29.4 kDa was isolated. Its hemagglutinating activity could be inhibited only by polysaccharide inulin but not by simple sugars. The activity was stable up to 70 °C but was sensitive to high and low pH. The striking feature was the inhibition of activity by salts including $FeCl_3$, $AlCl_3$, and $ZnCl_2$ but not by $MgCl_2$, $MnCl_2$, and $CaCl_2$. The lectin stimulated mitogenic response of mouse splenocytes. Proliferation of tumor cells including MBL2 cells, HeLa cells, and L1210 cells was inhibited by the lectin. However, proliferation of HepG2 cells was not affected (99).

***Amanita pantherina :*** A dimeric lectin with a molecular mass of 43 kDa was purified from *A. pantherina* by hydrophobic chromatography on Butyl-Toyoperls, affinity chromatography on submaxillary mucin-Toyopearl and gel filtration on Superose-12. Its hemagglutinating activity was inhibited by a number of oligosaccharides, bovine submaxillary mucin and asialo-bovine submaxillary mucin. The lectin was active in the pH range 4-9.5 and a temperature below 40 ºC. The lectin did not show requirement of any metal ion for its activity. It was found to be a glycoprotein with carbohydrate content of 4.3 % and characterized by high content of Gly, Glx, Asx, and Ser, and low content of Met, Lys, Tyr. The N-terminal sequence of the lectin was Ile-Phe-Ala-Val-Gly-Glu-Thr-Gln-Gly-Glu (173).

***Agrocybe cylindracea:*** An antimushroom peptide, designated as agrocybin, with a molecular mass of 9 kDa was isolated from fresh fruiting bodies of the mushroom *A. cylindracea*. It showed antifungal activity against several fungal species but not antibacterial activity. The activity of HIV-1 reverse transcriptase was attenuated in the presence of agrocybin. It exhibited weaker mitogenic activity than

Con-A on isolated murine splenocytes, but was devoid of antiproliferative activity on HepG2 (hepatoma) cells (174).

***Boletus satanas:*** The lectin of *B. satanas,* designated as bolesatine was found to be a toxic glycoprotein with hemagglutinating activity. It was purified by ammonium sulfate precipitation and anionic exchange chromatography on FPLC and found to have a molecular mass of 63 kDa. It exhibited mitogenic activity toward lymphocytes and elicited the release of interleukins and tumor necrosis factor from mononuclear cell cultures (175). It inhibited DNA and protein synthesis in Maidn Darby canine kidney cells with an $IC_{50}$ of 0.62 and 0.14 μM respectively and the inhibitory effects could be reversed by addition of galactose (106). These biological activities of bolesatine might contribute to the toxicity of *B. satanas.*

***Coprinopsis cinerea:*** Two different dimeric lectins (CGL1 and CGL2) were purified by ion exchange and affinity chromatography on lactosyl-Sepharose from *C. cinerea* (formerly *Coprinus cinereus*) during fruiting body formation. The lectins were found to be structurally similar to galectins, a group of galactose specific animal lectins, and demonstrated 83 % sequence homology and conservation of all key residues present in galectins. They are different from most galectins in the absence of cysteine residues and N-terminal post-translational modification (108).

The role of these lectins in tissue development was studied by Walser *et al.* (176). The developmentally regulated ligands for galectins were co-localized with galectin expression, in the veil surrounding the developing primordium and the outer cells of the young stipe. In addition, galectin ligand were observed in the hymenium. The subcellular localization of the galectin ligands suggested these to be present in cellular compartment distinct from galectin transport. The sensitivity of the *in situ* interactions with

exogenous galectins towards detergents and organic solvents inferred that these ligands were lipid-borne. Accordingly, lipid fractions from primordia were shown to contain galectin-binding compounds. Based on these observations it was hypothesized that β-galactoside-containing lipids (basidiolipids) found in mushrooms are physiological ligand for the galectins in *C. cinerea* (176).

The crystal structure of CGL2 was solved and showed fold similar to vertebrate galectins. The 2.1 Å crystal structure of CGL2 and five substrate complexes revealed that this prototype galectin achieves increased substrate specificity by accommodating substituted oligosaccharides of the mammalian blood group A/B type in an extended binding cleft. Kinetic studies on wild-type and mutant CGL2 proteins demonstrated that the tetrameric organization is essential for functionality. The geometric constraints due to orthogonal orientation of the four binding sites have important consequences on substrate binding and selectivity (136).

***Flammulina velutipes:*** *F. velutipes* exhibited some peculiar properties compared to other mushroom lectins. It was found to be a heterodimer of 12 kDa and 8 kDa subunits. The lectin was purified by zinc acetate treatment and CM-cellulose column chromatography. The lectin was devoid of carbohydrate, half-cysteine, methionine, and histidine. Upon gel filtration on Sepharose 6B in the presence of 6 M guanidine-HCl the subunits of the lectin could be separated. Only the larger subunit of the lectin had hemagglutinating activity; and the hemagglutinating activity was not inhibited by any of the simple sugars. The lectin supposedly has affinity for more complex carbohydrate structures on the cell surface. The larger subunit also promoted proliferation of mouse splenocytes and elicited mitogenic activity (64).

***Ganoderma capense:*** The lectin showed similarity to fungal immunomodulatory proteins and very high temperature stability (stable

at 100 °C for 60 min). Its mitogenic activity was more potent than Con-A towards mouse splenocytes and showed antiproliferative activity towards leukemia (L 1210 and M1) cells hepatoma (HepG2) cells. However, no antifungal and HIV-1 reverse transcriptase activity was observed (78).

***Grifola frondosa:*** An *N*-acetylgalactosamine specific lectin was purified by affinity chromatography on acid-treated Sepharose CL-4B and subsequently on GalNAc-Toyopearl from fruiting bodies of *G. frondosa.* Its molecular mass determined by gel filtration using various buffers and matrices, ranges from 30 to 52 kDa. On the other hand, SDS-PAGE of the lectin in the presence or absence of 2-mercaptoethanol showed three major bands with molecular mass of 33, 66 and 100 kDa and a faint band of 65 kDa. The lectin contained high amount of acidic and hydroxyl amino acids, glycine and alanine, and low amount of methionine, phenylalanine, isoleucine and histidine residues. *G. frondosa* lectin was glycosylated and contained 3.3 % neutral sugar. Dialysis of the lectin with chelating agent, divalent metals including $CaCl_2$, $MgCl_2$, $ZnCl_2$ or $MnCl_2$ did not alter the hemagglutinating activity of the lectin. The lectin exhibited cytotoxic activity against the HeLa cells, the minimum concentration required to induce death of all the cells was 25 μg/ml. The toxicity could be inhibited by pre-incubating the lectin with the haptenic sugar *N*-acetylgalactosamine (38).

***Hericium erinaceum:*** The *H. erinaceum* lectin was a heterotetramer with molecular mass of 54 kDa as determined by gel filtration. It has two different subunits with molecular mass of 15 and 16 kDa. It contained substantial amount of glycine, glutamic acid or glutamine, serine, alanine and a small amount of tyrosine, histidine and methionine. It was a glycoprotein with 1.5 % carbohydrate and showed specificity

towards neuraminic acid and especially with *N*-glycolylneuraminic acid. Considerable thermal stability was observed in the lectin as it was stable upto 70° C. Its hemagglutinating activity was not affected by demetalization with EDTA or by addition of $CaCl_2$, $MgCl_2$, $ZnCl_2$ or $MnCl_2$ (84).

***Ischnoderma resinosum:*** From the fruiting body of *I. resinosum* a lectin was purified by affinity chromatography on Sepharose-4B. The lectin is made up of two identical subunits, each with a molecular mass of 16 kDa. It contained substantial amounts of acidic and hydroxy amino acids, glycine, valine and leucine, but minute quantities of methionine, histidine and arginine. The hemagglutinating activity of the lectin can be inhibited by Methyl β-galactoside, L-Fucose and L-arabinose (73).

***Laetiporus sulphureus*:** The parasitic mushroom *L. sulphureus* produced a lectin in carpophore, and was purified by affinity chromatography on Sepharose. The lectin also showed hemolytic activity. Some microheterogeneity was observed in isoelectric focusing although the HPLC profile exhibited homogeneity. Its molecular mass, as determined by gel filtration and also by electrophoresis under non-denaturing conditions, was around 190 kDa. The lectin was tetrameric, consisting of two distinct types of subunits (with molecular mass of 60 and 36 kDa respectively). It contained threonine, valine, and glycine residues in abundance, while aspartic acid and sulfur containing amino acids occurred in extremely small quantities. No carbohydrate was detected. Hapten inhibition assays indicated that lectin is specific for *N*-acetylglucosamine residues and the same site of the lectin molecule contributes to both hemagglutinating and hemolytic activities (34).

***Lacterius deterrimus*:** A lectin from carpophores of *L. deterrimus*, a specific symbiont of spruce, was isolated using steps including, affinity, hydroxylapatite, and gel filtration chromatographic

techniques. It showed molecular mass of 37 kDa in gel filtration. Although it was apparently homogeneous in HPLC gel filtration, isoelectric focusing showed microheterogeneity with a main band in the zone near pH 6.5. Amino acid analysis showed that the lectin contained high amount of glycine and methionine. Carbohydrate could not be detected in the lectin. Hapten inhibition assays indicated that *L. deterrimus* lectin was most specific for β-D-galactosyl(1-3)-D-*N*-acetylgalactosamine residues. The lectin was produced by cultured mycelia, and could be localized by immuno-fluorescence in the cell wall. Receptors for the lectin were localized on the root hairs of axenically grown spruce seedlings. This inferred a role of the mushroom lectin in recognition and specificity during the early stages of mycorrhiza formation (69).

***Lyophyllum shimeiji:*** A hemagglutinin, with a molecular mass of 30 kDa was isolated from fresh fruiting bodies of the edible mushroom *L. shimeiji*. However, the hemagglutinating activity could not be inhibited by simple sugars and glycoproteins. The hemagglutinin exhibited a novel N-terminal sequence not found in any lectin and hemagglutinin (177).

***Pleurotus ostreatus:*** A dimeric lectin, from fresh fruiting bodies of the edible mushroom *P. ostreatus,* was purified by ion-exchange chromatography. It was composed of two subunits with a molecular mass of 40 and 41 kDa, respectively, and demonstrated similarity in N-terminal sequence to each other and to *Aleuria aurantia* lectin. The lectin was sensitive to extreme pH and heat. Mellibiose, lactose, D-galactose, α-methyl-D-galactopyranoside, N-acetylneuraminic acid, raffinose, and inulin were capable of inhibiting its hemagglutinating activity and mellibiose was the most potent inhibitor. The lectin exerted potent antitumor activity in mice, bearing sarcoma S-180 and

hepatoma H-22 cells. Survival in these mice was prolonged and body weight increase reduced after lectin treatment (178).

The activation of an endogenous phosphatase by the lectin was observed for ADP and ATP. The lectin was also found to have α-galactosidase activity, the enzyme activity and carbohydrate binding property was not due to same site since the lectin accepts both α- and β-glycosides whereas the enzyme activity was restricted to the α-anomer only. Moreover, the α-galactosidase activity was inhibited by α-galactose but not by β-galactose. Therefore, lectin and enzymatic activities were either properties of two tightly associated proteins, or of just one molecule (96). Crystals of *P. ostreatus* lectin were grown by the hanging-drop technique using ammonium sulfate as the precipitant at 293 °K. Over a period of between two and three weeks, crystals of hexagonal bipyramidal morphology grew to maximum dimensions of 0.2 × 0.2 × 0.5 mm (179).

***Pleurotus tuber-regium :*** The fresh sclerotia of the edible mushroom *P. tuber-regium* showed presence of an N-acetylglucosamine-binding lectin, with a molecular mass of 32 kDa. Its N-terminal sequence showed some similarity to that of *Agaricus bisporus* lectin. The lectin exhibited hemagglutinating activity toward trypsinized rabbit erythrocytes but not toward untrypsinized rabbit erythrocytes (105).

***Polyporus adusta :*** The lectin from *P. adusta* was purified by ion-exchange chromatography on DEAE-cellulose, Q-Sepharose and CM-Sepharose. The hemagglutinating activity of the lectin was inhibited by turanose and several other carbohydrates as D(+)-mellibiose, D-fructose, L-arabinose and glucose. Hemagglutinating activity of the lectin showed 100% increase in the presence of $FeCl_3$ (10 mM). The lectin also showed antiproliferative activity towards tumor cell lines and mitogenic activity towards splenocytes (40).

***Peziza sylvestris :*** The lectin isolated from *P. sylvestris* was the first arabinose specific mushroom lectin with mitogenic activity towards splenocytes. It was a single chained protein with a molecular mass of 20 kDa. The N-terminal sequence showed only slight resemblance to other mushroom lectins. Its hemagglutinating activity was inhibited by arabinose, but not by a large variety of other carbohydrates (79).

***Schizophyllum commune:*** A homodimeric lactose-binding lectin with a molecular mass of 64 kDa was isolated from fresh fruiting bodies of the split gill mushroom *S. commune*. The N-terminal sequence of the lectin showed similarity to a part of the sequence of the cell division protein from *Gleobacter violaceus*. The hemagglutinating activity of the lectin was stable at temperatures up to 40 °C, and in concentrations of NaOH and HCl solution up to 125 and 25 mM, respectively. The lectin exhibited potent mitogenic activity toward mouse splenocytes, antiproliferative activity toward tumor cell lines, and inhibitory activity toward HIV-1 reverse transcriptase (180).

***Tricholoma mongolicum :*** Two lectins were purified from edible mushroom *T. mongolicum* and designated as TML-1 and TML-2. The lectins were homodimers, with subunit molecular mass of 17.5 kDa. Hydroxyproline was present in the lectins but no carbohydrate was detected. The hemagglutinating activities of the lectins were inhibited by α-lactose, β-lactose, lactose, *N*-acetyl-D-galactosamine and D-galactose, and were abolished in extreme pH values. EDTA, $CaCl_2$, $MgCl_2$ did not affect the hemagglutinating activity of the lectin, reduction of the activities occurred, however, after addition of $ZnCl_2$ or $MnCl_2$. Thermal stability between 10° C to 80° C was observed. The two lectins manifested antiproliferative activity against mouse monocyte-macrophage cells and mouse mastocytoma cells *in vitro* (35). Both of them were able to inhibit the growth of implanted

sarcoma 180 cells by 68.84% and 92.39% respectively. The growth of tumor cells in the mouse peritoneal cavity was also inhibited by the two lectins with TML-2 expressing a greater potency (181).

These lectins stimulated the production of nitrite ions by macrophages in normal and tumor-bearing mice. Both lectins exhibited negligible mitogenic activity towards T-cells or splenocytes from normal mice *in vitro* when compared with Con-A. Similarly, T-cells from sarcoma-bearing mice treated with the lectins did not exhibit a mitogenic response different from that of the control. However, T-cells from normal lectin-treated mice demonstrated a reduced mitogenic response when compared with the control. In mice, the growth of sarcoma 180 cells in the peritoneal cavity was inhibited and the life-span was prolonged by the two lectins (182).

***Volvariella volvacea :*** A lectin (VAG) was extracted from fruiting bodies of *V. volvacea* by ammonium sulfate fractionation, DEAE-C-52 and CM-C-52 column chromatography. The molecular mass was estimated to be 26 kDa comprising two non-identical subunits. The lectin was rich in aspartic acid, glutamic acid, tyrosine and leucine but lacking free-cysteine, methionine and histidine. Its hemagglutinating activity was preserved even after incubation at 80 °C for 2 h. The hemagglutinating activity of the lectin was not inhibited by any of the common simple sugars. It is likely that the lectin has an affinity for complex carbohydrate structures on the cell surface. The *V. volvacea* lectin showed toxicity, its $LD_{50}$ in mice was 17.5 mg/kg body weight. It has a mild retarding effect on the growth of tumor cells (126).

One more lectin (VVL) was isolated from the fungus by She (212). The lectin was purified from the fruiting bodies as well as cultured mycelia. It was a homodimeric protein with a molecular mass of 32 kDa as demonstrated by gel filtration and SDS-PAGE. Its hemagglutinating activity was inhibited by thyroglobulin but not by simple carbohydrates such as monomeric or dimeric sugars. The

immunomodulatory activity of the lectin was demonstrated by its potent stimulatory activity toward murine splenic lymphocytes. It was also found to markedly enhance the transcriptional expression of interleukin-2 and interferon-gamma by reverse transcriptase-polymerase chain reaction. As revealed by its N-terminal amino acid sequence, it was distinct from other immunomodulatory proteins previously reported (183) and VAG (126) from the same fungus.

***Xerocomus spadiceus :*** The lectin was capable of eliciting an approximately 4 fold stimulation of mitogenic response in murine splenocytes. The hemagglutinating activity was stable up to 60 °C. The hemagglutinating activity increased in the presence of $AlCl_3$ or $ZnCl_2$ and reduced in the presence of HCl and NaOH. Among the large number of carbohydrates tested, only inulin was able to inhibit the hemagglutinating activity of the lectin (184).

**Lectins from Ganoderma lucidum**

***GLL-M:*** GLL-M is a lectin isolated from mycelia of *Ganoderma lucidum.* It was purified by anion exchange chromatography and affinity chromatography on bovine submaxillary mucin-Toyopearl. GLL-M showed specific activity of 5600 units / mg of lectin. It is a monomer of 18 kDa. Amino acid analysis of GLL-M indicated showed high content of Gly, Ala, Asx and Thr and a low content of Arg, Lys, Met and His. It has acidic pI of 4.5 and carbohydrate content of4.0 %. Haemagglutination activity of GLL-M is not affected by metal ions. GLL-M did not agglutinate normal human red blood erythrocytes and agglutinated only pronase treated erythrocytes. Haemagglutination activity of GLL-M was not inhibited by mono-or oligosaccharides and could be inhibited only b y Asialo-BSM, Asialofetuin, BSM and fetuin, asialo-BSM being the most potent inhibitor (71).

***GLL-F:*** GLL-F is lectin isolated from fruiting body of *Ganoderma lucidum*. It was purified by the same procedure as that of GLL-M. SDS-PAGE of the lectin showed molecular weight of 16 kDa but gel filtration on superpose 12 showed molecular weight of 19 kDa. It might be monomer in native state or can exist as dimer which interacts with superpose 12 so that the estimated molecular weight is smaller than the true one. Haemagglutination activity of GLL-F is inhibited by high concentration (200mM) of glucosamine and galactosamine. Among glycoproteins Asialo-BSM, BSM and fetuin were the most potent inhibitors (71).

***LZ-8:*** LZ-8 is an immunomodulatory protein was isolated from *G. lucidum* mycelia and is homodimer of 24 kDa, having monomer molecular mass of 12.4 kDa (185). The protein designated as LZ-8 was a small protein of 110 amino acid residues/monomer. The N-terminal was found to be blocked by *N*-acetylation. It was glycosylated. The

lectin activity was observed with ovine but not with human erythrocytes (186). It displayed many biological activities characteristic of lectins including mitogenic and hemagglutinating activities. Other biological activities of LZ-8 included mitogenic activity on mouse splenocytes and human peripheral lymphocytes, and suppression of anaphylaxis induced by bovine serum albumin in CFW mice (187,188).

The two lectins isolated by Kawagishi *et al*. were distinctly different from LZ-8 reported by Tanaka *et al*. (186), in their molecular masses and sugar-binding activities.

***Other lectins:*** Mikiashvili (2006) has reported two lectins from *Ganoderma lucidum* biomass grown on malt extract agar (41). These lectins agglutinated normal rabbit erythrocytes. One lectin showed specific activity of 24986 units/mg and showed specificity towards arabinose. Other lectin showed specific activity of 5035 units/mg and showed specificity towards N-acetylglucosamine. These lectins are not yet purified and characterized and specific activity is shown for partially purified lectins.

**Present Investigation**

In last few years mushroom-lectins have attracted increased attention due to their antitumor, antiproliferative and immunomodulatory activities (76,189,190). Lectins from several pathogenic fungi have also been studied in great detail but their physiological role has not been ascertained or remains uncertain (74,191-194). Recognition is a key event in biological functionality of the lectins. Lectins play their roles in mushrooms by recognizing different carbohydrate structures present on the surface of their own or host cells. Some of these roles seem to concern the mushroom metabolism itself, while other activities are implicated in symbiotic or parasitic relationships with other organisms (31). *Ganoderma lucidum* is a well-known medicinal mushroom and many health-promoting and therapeutic effects have been attributed to this mushroom. Although some lectins have been purified or reported from this mushroom (described earlier in this chapter), complete characterization of the lectins has not been done. 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 understanding of lectin interaction with cell bound carbohydrates. Hence the present investigation was carried out to purify and characterize the lectin from *Ganoderma lucidum* to understand its structure-function correlations. Binding studies were carried out with different ligands using spectrofluorimetry and SPR, which provides vital information regarding subtle nature of its carbohydrate specificity.

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*Chapter: 2*

# Purification and characterization of *Ganoderma lucidum* lectin

**SUMMARY**

A lectin from the fruiting body of *Ganoderma lucidum* was purified to homogeneity by 0-30% ammonium sulphate precipitation and hydrophobic chromatography on Phenyl-Sepharose column with an overall yield of 35 %. The molecular mass of the lectin determined by gel filtration is 114 kDa and it is made up of six identical subunits of 18.6 kDa. It is a basic protein with a pI of 9.7. The purified lectin is a glycoprotein and contains 9.3 % carbohydrate. Lectin is stable in the pH range 5-9, and temperature up to 50 °C. The partial N-terminal sequence did not show similarity with any known lectin. Purified lectin agglutinated pronase treated human erythrocytes (A, B and O), with very low titre values. The hemagglutinating activity was inhibited by glycoproteins containing N-linked and O-linked glycans.

**INTRODUCTION**

Lectins are multivalent proteins or glycoproteins of non-immunogenic origin, which recognize and bind reversibly to diverse sugar structures in a highly stereo-specific and non-catalytic manner (1,2). Lectins have been isolated from various organisms such as- animals, plants, bacteria, viruses and fungi; though lectins from plant and animal sources have been extensively studied (3,4), very little information is available on lectins from fungal sources (5,6). In last few years mushroom-lectins have attracted increased attention due to their antitumor, antiproliferative and immunomodulatory activities (7-9). Lectins from several pathogenic fungi have also been studied in great detail but their physiological role has not been ascertained or remains uncertain (10-14).

The role of lectins in fungi seems to be more diverse than that in plants. In higher fungi, lectins probably play differential roles in different circumstances. Various roles have been postulated for fungal lectins which do not appear to be mutually exclusive - some of these seem to concern fungal metabolism itself, while other activities are implicated in symbiotic or parasitic relationships with other organisms (5).

Lectins from mushrooms viz *Agaricus bisporus, Boletus satanus, Flammulina velutipes, Ganoderma lucidum, Grifola frondosa, Tricholoma mongolicum, and Volvariella volvacea* show immunomodulatory and/or antitumor/ cytotoxic activities (6). *Ganoderma lucidum* is a well-known medicinal mushroom and many health-promoting and therapeutic effects have been attributed to this mushroom. An immunomodulatory protein with a molecular mass of 12.4 kDa, designated LZ-8, has been isolated from *G. lucidum* mycelia. Its amino acid sequence was homologous to that of a known immunomodulatory protein from the straw mushroom and it also showed hemagglutinating and mitogenic activities (15) . An 18 kDa lectin was isolated from *G. lucidum* mycelia and another 16 kDa lectin was purified from *G. lucidum* fruiting bodies (16), both of which were distinct from LZ-8 as judged from the molecular mass and sugar binding activity.

**MATERIALS**

N-Acetylimidazole, 2,4,6-trinitrobenzenesulphonic acid, phenylglyoxal, diethylpyrocarbonate, phenylmethylsulfonyl fIuoride, N-bromosuccinimide, 5,5' dithiobis-(2-nitrobenzoic acid), sodium borohydride, hydroxylamine hydrochloride, galactose, galactosamine, 2-deoxygalactose, L-fucose, glucose, mannose, methyl-α-D-galactose, methyl-β-D-galactose, mellibiose, lactose, Phenyl-Sepharose CL-4B, pronase-E, all glycoproteins, gums and the molecular weight markers (Sigma Chemical Co. St. Louis, U.S.A); Sephadex G-25 and Sephacryl S-200 (Amersham Bioscience, Uppasala, Sweden); trypsin and neuraminidase (Sisco Research Laboratories, Mumbai, India) and dextrose, peptone, malt extract, agar and yeast extract (HiMedia Laboratories Pvt. Ltd, Mumbai, India), were used. All other reagents were of analytical grade.

## METHODS

### Identification of *Ganoderma lucidum* fruiting bodies

Identification of *Ganoderma lucidum* was based upon the following characteristic features*:* Basidiocarps stipitate, stipe central, excentric or lateral, upper surface red, laccate, shiny fruiting bodies, leathery to tough woody, clamp connection present, spores broadly ellipsoid, with an apical thickening, truncate, brown, 8.3 – 10 x 5.8 – 6 µm, minutely vernucase, context brown, 2-10 mm, thick, hymenial surface whitish or creamish, later turning brown (17-22).

### Erythrocyte preparation

Human erythrocytes of A, B and O blood groups were washed 5 to 6 times with 20 mM Tris-HCl buffer pH 7.2 containing 150 mM NaCl. A 3% (v/v) suspension of the erythrocytes in the above buffer was treated with different enzymes (pronase 0.05%, trypsin 0.05% and neuraminidase 0.1 U/ml) at 37 ºC for 1 h, washed 3 times with the same buffer and used for further studies.

### Hemagglutination assays

Human erythrocytes of A, B and O blood groups were prepared as above, treated with 0.05% (w/v) pronase at 37 ºC for 1 h, and washed 4 times with the same buffer. Hemagglutination assays were performed in standard microtitre plates by the two-fold serial dilution method. A 50 µl aliquot of the erythrocyte suspension was mixed with 50 µl of serially diluted lectin and agglutination was examined visually after incubation for one hour. The unit of hemagglutination activity (U) was expressed as the reciprocal of the highest dilution (titre) of the lectin that showed complete agglutination. The specific activity of the lectin was defined as the number of hemagglutination units per milligram of the protein (U/mg).

**Hemagglutination inhibition assays**

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

**Protein determination**

Protein concentrations were determined according to Bradford (23) using BSA as standard.

**Extraction and purification of lectin**

All the steps involved in purification of the lectin were carried out at 4–8 °C. *Ganoderma lucidum* fruiting body was homogenized in liquid $N_2$. The homogenized powder was suspended (10% w/v) and crushed in 10 mM MOPS, 0.1% Tween20 (pH 6.5) for 5-6 h. Supernatant was collected by centrifugation at 10000g (20 min). The extract was then precipitated with ammonium sulphate (0-30% saturation). The precipitate was redissolved in 10 mM MOPS, 200 mM NaCl (pH 6.5).

The partially purified extract thus obtained was dialyzed against 10 mM MOPS, 200 mM NaCl (pH 6.5) and loaded on Phenyl Sepharose column pre-equilibrated with the same buffer at a constant flow rate of 12 ml/h. The column was washed with the same buffer and washing collected as fraction of 5 ml until $OD_{280}$ was <0.05. The elution was done by 10 mM MOPS (pH 6.5), 10% v/v ethylene glycol at constant flow rate (12 ml/h) and 3mL fractions were collected, checked for $A_{280}$ and hemagglutination activity.

**Electrophoresis**

Native PAGE of the purified lectin was carried out in 10% (w/v) polyacrylamide gel, pH 8.8, and the gels were stained with Coomassie Brilliant Blue R-250. SDS-PAGE was performed in 10% (w/v) polyacrylamide gel at pH 8.8 as described by Laemmli (24) and the protein visualized by staining with silver nitrate. Isoelectric focusing (IEF) in polyacrylamide gels was done according to Vesterberg (25) over the pH range 3-10.

**Carbohydrate content**

Purified lectin (400µg in 400 µl water) was incubated with 400 µl of 5 % (w/v) phenol for 10 min at room temperature. Two ml of sulphuric acid was then added and the mixture was allowed to cool for 20 min at room temperature. The color developed was then measured spectrophotometrically, at 490 nm by using galactose-mannose (4:3) as standard (26).

**Molecular mass determination**

**Gel filtration *:*** The molecular mass of the purified lectin was determined by HPLC (Waters Corporation) on a gel permeation column (Protein-PAK SW300, 300x7.8 mm) at pH 6.0 and 27 °C using β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa) and cytochrome C (12.4 kDa) as reference proteins.

**SDS-PAGE *:*** SDS-PAGE was performed in 10% (w/v) polyacrylamide gel at pH 8.8 as described by Laemmli (24) using glutamate dehydrogenase (55 kDa), ovalbumin (45 kDa), glyceraldehyde 3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsin inhibitor (20 kDa), α-lactalbumin (14.2 kDa).

**MALDI-ToF***:* Molecular mass of the purified lectin was determined by matrix-assisted laser desorption ionization time-of-flight (MALDI-ToF) mass spectrometry using a Voyager DE-STR (Applied Biosystems) equipped with a 337-nm nitrogen laser.

**Amino acid analysis**

The amino acid content of the purified lectin was analyzed using a commercial kit (AccQ-Fluor, Waters Corporation). Salt free lyophilized lectin (50 µg) was hydrolyzed under 6N constant boiling HCl, in vacuum sealed hydrolysing tubes for 24 h at 110 °C. The sample was then derivatized by 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) in borate buffer at pH 9 and 10 picomoles of the hydrolysate was loaded on AccQ-Ta9 column equipped with a fluorescent detector. Total cysteine was determined according to Cavallini et al. (27) and total tryptophan according to Spande and Witkop (28).

**N-terminal sequence analysis**

N-terminal sequence was determined by automatic Edman degradation by use of a Procise$^{TM}$ protein sequencer (Applied Biosystem) at the protein sequencing facility of IIT, Mumbai, India. The sample (700 pmol) was applied to a glass-fiber filter and then cycled through a conditioning process of fifteen repetitions of Edman chemistry.

**Effect of metal ions**

The activity of the purified lectin was determined in the presence of 10 mM each of $Ca^{++}$, $Mn^{++}$, $Mg^{++}$ 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 10 °C to 80 °C by incubating 25 µg of the lectin for 15 minutes at the

respective temperature, rapidly cooling in ice and assaying for hemagglutinating activity.

The pH stability of the lectin was determined by incubating 25 µg of lectin at different pH (pH 2-12) for 24 h. Buffers used – glycine-HCl (pH 2-3), acetate (pH 4-5), citrate-phosphate (pH 5), phosphate (6-7), Tris-HCl (pH 8-9) and glycine-NaOH (pH 10-12). The hemagglutinating activity was checked after 6, 12 and 24 h.

**Preparation of plant polysaccharides (gums)**

A number of plant gums were tested for hemagglutination inhibition of the lectin. The suspension of plant gums, gum arabic, gum tragacanth, gum guar, gum ghatti, gum karaya, gum locust, gum arabinogalactan, gum rosin and gum pontianac 0.5 g each in 10 ml of distilled water and gum mastic, gum elemi and gum storax 0.5 g each in 10 ml of 0.1 M NaOH were boiled for 2 h, centrifuged at 7,800 *g* for 30 min at 25 °C and the supernatants were subjected to alcohol precipitation (1:2 v/v). The precipitate formed was re-dissolved in 10 ml of hot water and reprecipitated with alcohol (1:2 v/v). The precipitate obtained was dissolved in distilled water and dialysed against the same. The concentration of sugar solution were determined in terms of neutral sugar by phenol sulphuric acid method (26)..

## RESULTS AND DISCUSSION

### Identification of *Ganoderma lucidum* fruiting bodies

*Ganoderma lucidum* fruiting bodies were identified based on the features described in materials and methods. The mushroom *Ganoderma lucidum* has been said to be a complex species and suggestions have been made to identify and designate its strains separately. In our study fruiting bodies of *Ganoderma lucidum* have been obtained repeatedly from the same site and the same tree to maintain uniformity of the samples.

### Lectin characterization

The results of a typical procedure for the purification of *G. lucidum* sp. lectin is given in Table 2.1. *G. lucidum* lectin eluted as single large peak of $OD_{280}$ and coincident single hemagglutination activity peak from Phenyl Sepharose column (Fig. 2.1). It was purified approximately 13-fold with final recovery of 35% (Table 2.1).

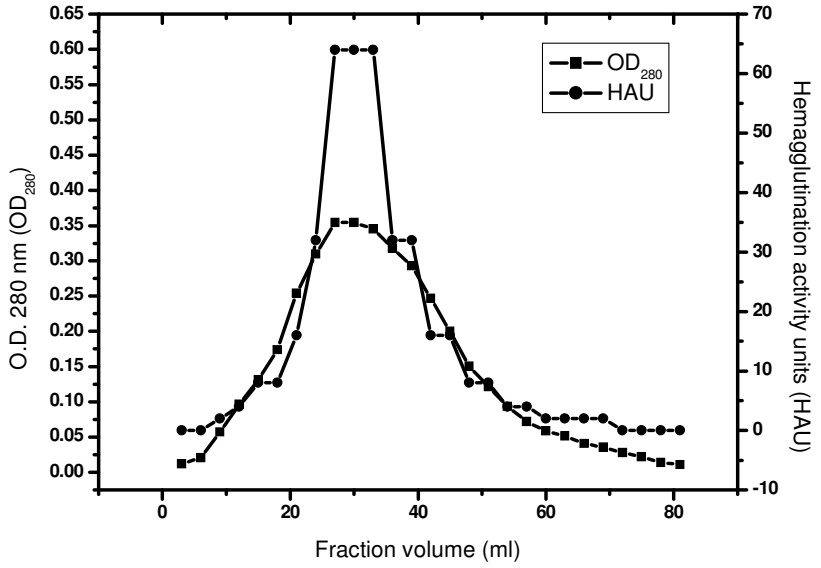


Figure 2.1**:** Elution profile of *G. lucidum* lectin on Phenyl Sepharose column.

The purified lectin migrated as a single band in native- as well as SDS-polyacrylamide gels indicating homogeneity (Fig. 2.2). The molecular mass of the lectin as determined by gel filtration in the presence and absence of dithiothreitol (DTT) was 114 kDa (Fig. 2.3), whereas that determined by SDS-PAGE was 18.5 kDa (Fig. 3).

The molecular mass of lectin was also determined by MALDI-ToF, which showed a peak corresponding to *m/z* 18.6 kDa (Fig. 2.4). The results of SDS-PAGE, gel filtration, and MALDI-ToF mass analysis indicated that this lectin is a homo hexamer of 18.6 kDa subunits which are held together by non covalent bonds. The lectin showed resemblance in being polymeric with other mushroom lectins reported from *Grifolia frondosa* and *Pholiota aurivella* (6).

**Table 2.1: Purification of *Ganoderma lucidum* lectin.**

| | Total Protein (mg) | Total Activity[a] (units) | Specific Activity[b] (units/mg) | Recovery of Activity (%) | Fold Purification |
|---|---|---|---|---|---|
| Crude Extract | 44.8 | 44800 | 1000 | 100 | 1 |
| 0-30% $(NH_4)_2SO_4$ precipitation | 5.67 | 38400 | 6772 | 85.71 | 6.77 |
| Hydrophobic interaction Chromatography | 1.2 | 16000 | 13333 | 35.71 | 13.33 |

[a] The reciprocal of the highest dilution (titre) of the lectin that showed complete agglutination was expressed as a unit of hemagglutinating activity.

[b] The specific activity of the lectin is defined as units of the hemagglutinating activity per milligram of lectin.

Except for few lectins as from *Lactarius lignyotus* and *Phallus impudicus* the subunits of which are linked by disulphide bridges, the other di-, tetra-, or polymeric lectins studied, namely those of *Agaricus edulis, Agaricus campestris, aleuria aurantia, Flammulina velutipes, Hericium erinaceus, Lactarius deliciosus, L. deterrimus, L. salmonicolor, Pholiota aurivella, Pleurotus cornucopiae* and *Xerocomus chrysenteron* have subunits held together by non- covalent bonds (5).

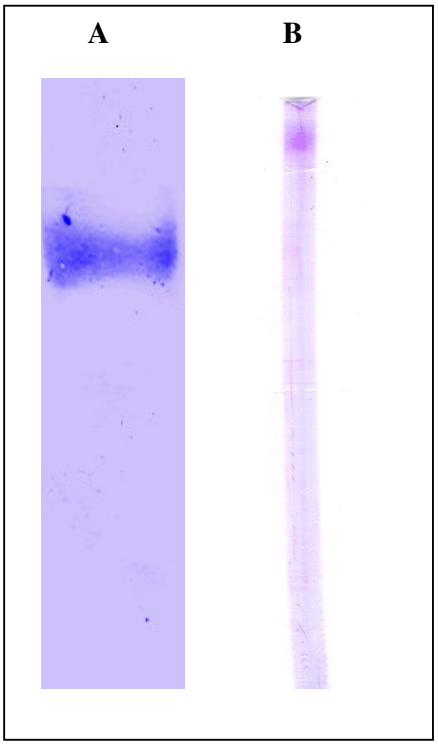


Figure 2.2: **Electrophoresis of purified lectin.**

(A) Native electrophoresis of *G. lucidum* lectin at pH 8.8.

(B) IEF-PAGE of *G. lucidum* lectin. A 50 µg of purified lectin was loaded and carrier ampholines of the range 3-10 were used.

*G. lucidum* lectin is a glycoprotein containing 9.3% neutral sugar. It is a basic protein with a pI of 9.7 (Fig 2.2). *G. lucidum* lectin was active between pH 5-9, and temperature up to 50 °C. However, incubation at 60°C and 70°C for 1 hour led to 50% and 100% loss in activity of the lectin, respectively. The lectin also did not require divalent cations for its activity since extensive dialysis against 10 mM EDTA, 20 mM MOPS, 200 mM NaCl (pH 6.5) followed by dialysis against plain buffer (to remove EDTA) did not affect the hemagglutinating activity and there was no increase in hemagglutinating activity on the addition of 1, 5 and 10 mM $Ca^{++}$, $Mn^{++}$, $Mg^{++}$.

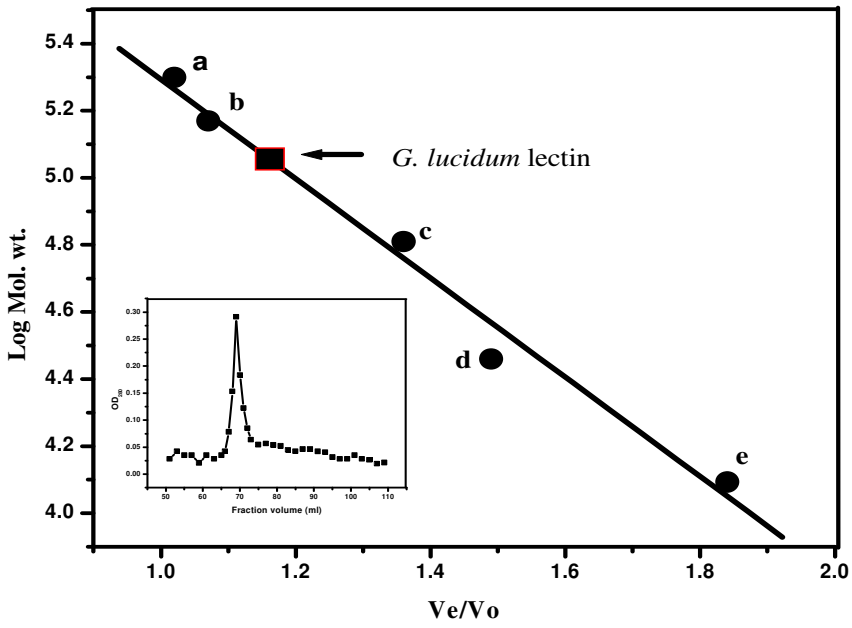


**Figure 2.3: Molecular mass determination of *G. lucidum* by gel filtration chromatography on Sephacryl S-200 column.** (a) b-amylase (200 kDa), (b) alcohol dehydrogenase (150 kDa), (c) bovine serum albumin (66 kDa), (d) carbonic anhydrase (29 kDa) and (e) cytochrome-C (12.4 kDa). *G. lucidum* lectin (114 kDa). Inset represents elution profile of *G. lucidum* lectin on Sephacryl S-200 column.

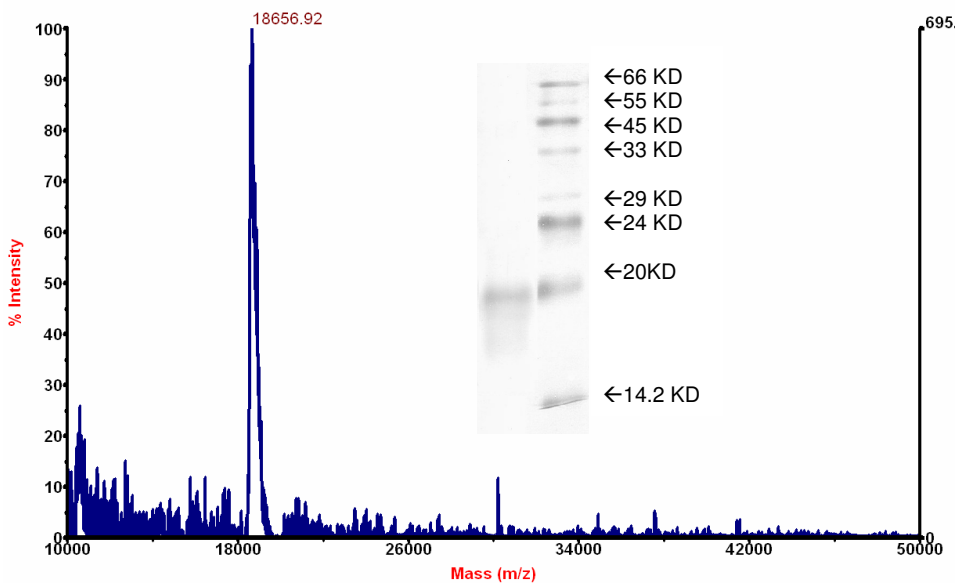


**Figure 2.4: Molecular mass determination of *G. lucidum* lectin by MALDI- ToF showed single sharp peak at 18.6 kDa.** Inset represents SDS-PAGE of the *G. lucidum* lectin; Lane 1: Purified *G. lucidum* lectin. Lane 2: (molecular weight markers). Molecular weight of lectin by SDS PAGE. (a)Bovine serum albumin (66,000) (b) Glutamic dehydrogenase (55000), (c) Ovalbumin (45000), (d) Glyceraldehyde 3-phosphate dehydrogenase (36000), (e) Carbonic anhydrase (29000), (f) Trypsinogen bovine pancrease (24000), (g) Trypsin inhibitor (20000), (h) α-Lactalbumin (14200) (i) *Ganoderma* Lectin (18600)

The amino acid composition of *G. lucidum* lectin showed that it contained a large amount of Gly (11.7%), Ala (10.0%), AsX (8.8%), a moderate amount of Thr (7.6%), GlX (7.1%), Ser (7.6%), Leu (6.4%), Tyr (6%), Phe (5.9%), Lys (5.9%), Arg (4.7%), and low amount of Pro (2.4%), Val (2.4%), Ile (3.1%), His (2.4%), Cys (1.77%), Trp (0.6%) (Table 2.2). N-terminal sequence (first twenty residues) of the lectin was QFIYNGKFNWLNYALNETIT which did not show similarity to any known lectin.

**Table 2.2: Amino acid composition of the *G. lucidum* lectin**

| Amino acid residue | No. of residues per molecule in *Ganoderma lucidum* lectin | Mol % in *Ganoderma lucidum* lectin |
|---|---|---|
| AsX | 90 | 8.8 |
| Thr | 78 | 7.6 |
| Ser | 78 | 7.6 |
| GlX | 72 | 7.1 |
| Pro | 24 | 2.4 |
| Gly | 120 | 11.7 |
| Ala | 102 | 10.0 |
| Cys[#] | 18 | 1.77 |
| Val | 24 | 2.4 |
| Ile | 42 | 3.1 |
| Leu | 66 | 6.4 |
| Tyr | 108 | 6.0 |
| Phe | 60 | 5.9 |
| His | 24 | 2.4 |
| Lys | 60 | 5.9 |
| Trp[$] | 6 | 0.6 |
| Arg | 48 | 4.7 |

[#] Determined according to Cavallini et al. (27)

[$] Determined according to Spande and Witkop (28)

Two other lectins have been isolated from *Ganoderma lucidum*, GLL-M, an 18 kDa lectin from mycelia and GLL-F, a 16 kDa lectin from the fruiting body (29). GLL-F and GLL-M exist as monomers in native state in contrast to 114 kDa lectin we have isolated which exists as hexamer in its native state. This lectin is different from GLL-M in terms of pI (4.5), carbohydrate content (9.3%). Both 114 kDa lectin from *Ganoderma lucidum* and GLL-M have a higher amount of Gly, Ala, AsX, Thr, GlX, Ser and Leu but remarkable differences can be observed in composition of other amino acids (16). This lectin is also different from LZ-8, an immunomodulatory protein, isolated from *Ganoderma lucidum* which shows hemagglutinating activity but is not characterized as lectin and is a homodimer of 24 kDa (30).

The lectin did not agglutinate normal or trypsinized erythrocytes but did agglutinate only pronase-E treated erythrocytes and showed titre value of 78 ng (Table 2.3). Pronase-E, a cocktail of different proteolytic enzymes, removes all protruding polypeptide from the erythrocyte membrane exposing GPI-anchors, glycoproteins and glycolipids, which may serve as better ligands for *G. lucidum* lectin

**Table 2.3: Specific activity of *G. lucidum* lectin with different blood group erythrocytes treated with different enzymes.**

| **Enzymes** | **Hemagglutinating activity with different erythrocytes (U/mg)** | | |
|---|---|---|---|
| | A | B | O |
| Untreated | 0 | 0 | 0 |
| Neuraminidase | 0 | 0 | 0 |
| Pronase | 13300 | 13300 | 13300 |
| Trypsin | 0 | 0 | 0 |

**Hemagglutination inhibition**

Simple sugars glucose, mannose, lactose, galactose, rhamnose, xylose, fucose, raffinose, glucosamine, mannosamine, galactosamine, N-acetyl-mannosamine, N-acetyl-galactosamine and N-acetyl-glucosamine failed to inhibit the hemagglutinating activity of *G. lucidum* lectin even at 500 mM, whereas the plant polysaccharide gum karaya exhibited inhibitory activity at 7.81 μg (Table 2). The hemagglutinating activity was also inhibited by glycoproteins, *viz.*, fetuin, asialofetuin, fibrinogen, asialo-fibrinogen, thyroglobulin, holotransferrin, ovalbumin and invertase. Among these glycoproteins, asialofetuin was the best inhibitor with a minimum inhibitory concentration of approximately 2.47 μg (Table 2.4).

Glycoproteins possess different N-linked and O-linked glycans, as ligands for lectins to interact. Asialofetuin has three triantennary N-linked core structure with a terminal Galβ1→4GlcNAc and three O-linked structure Galβ1→3GalNAcα1→*O*Ser/Thr ($T_\alpha$ Antigen), while the asialofibrinogen has a biantennary N-linked structure with terminal Galβ1→4GlcNAc residues (31). The lectin activity was also inhibited by plant polysaccharide gum karaya. Gum karaya consists of D-galactose, D-glucoronic acid and L-rhamnose but the details of molecular structures are still not known completely (32).

Very few lectins from mushrooms have been reported with hemagglutination activity unaffected by simple sugars and inhibited only by glycoproteins as from *Volvariella volvacea* (7,33) and *Mycoleptodonoids aitchisonii* (34). Other mushroom lectins with hemagglutination activity unaffected by either simple sugars or glycoproteins are from *Flammulina velutipes* (35) and *Lyophyllum shimeiji* (36).

Protein carbohydrate interactions between fungal lectins and carbohydrates could be the basis of various molecular processes such as growth and morphogenesis, molecular recognition and parasitic or symbiotic relationships with other organisms (5).

**Table 2.4: Inhibition of hemagglutinating activity of *G. lucidum* lectin with different glycoproteins and polysaccharides.**

| Inhibitor | Minimum inhibitory amount* (μg) |
|---|---|
| Fetuin | 4.95 |
| Asialofetuin | 2.47 |
| Fibrinogen | 39.06 |
| Asialofibrinogen | 19.53 |
| Thyroglobulin | 9.90 |
| Holotransferrin | 19.53 |
| Invertase | 54.68 |
| Gum karaya[$] | 7.81 |

Simple sugars, gum elemi, gum arabinogalactan, gum rosin, gum mastic, gum arabic, gum tragacanth, gum locust bean and gum xanthan were not inhibitory.

* Amount of inhibitor that can cause inhibition of 8 U hemagglutinating activity of neuraminidase treated type O human erythrocytes.

[$] In terms of neutral sugar content.

The lectins, *via* acting directly on carbohydrates by their specific binding sites, or indirectly by enzyme systems, possibly control depolymerization of stored material and intense movement of sugars, resulting initiation and often explosive growth of the sporomes. Lectin can repress the activity of the glycoprotein enzymes by binding them at their carbohydrate moieties. The release of simple sugars could thus redirect metabolism through competition for lectin binding (37). A lectin may be involved in ensuring cohesion between hyphae during the development of the basidiome as seen in *Pleurotus cornucopiae* (38).

During their growth, many fungi build specialized organs that require mycelial aggregation. These organs, cords, fans of mycelium, strands, rhizoids *etc*. are found particularly in parasitic and wood-rotting species, which use these organs to force their way amongst the host cells. *Rigidoporus lignosus,* a parasite of many tropical trees infects roots by the vegetative mycelium, but propagates *via* the fans of mycelium. A lectin seems to be involved in the building of the latter structures, since it has been found in significant amounts only in the hyphae that are able to aggregate and in the fans of mycelium themselves (5). This lectin seems therefore to be a cell wall lectin involved in a recognition mechanism. Presence of lectins, with strict specificities, in fungal cells of ectomycorrhizal symbiosis, suggests that lectins might be involved in recognition between the tree and its symbiont. The hypothesis was further exemplified by study of lectins from three fungi *Lacterius deliciosus, L. deterrimus* and *L. salmonicolor,* though very close morphologically, are specifically associated with different trees *Pinus, Picea* and *Abies,* respectively. Three essential conditions were verified experimentally: (i) In spite of being present in closely related species the three lectin differ significantly in structures and their microspecificities towards oligosaccharides; (ii) It was demonstrated, by shedding from cell walls by enzyme degradation or location by means of polyclonal antibodies, that the lectin is present at the surface of cultured hyphae; (iii) The receptors, bore by the root cell surfaces of each of the conifers, were exclusively for the lectin of their own associated fungus (5).

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*Chapter: 3*

# Chemical modification studies on *Ganoderma lucidum* lectin

**SUMMARY**

Chemical modification on purified *Ganoderma lucidum* lectin revealed the involvement of two lysine and one tryptophan residues per monomer of lectin in sugar binding activity of the lectin. Lysine residues were modified by using four different reagents. Protection from inactivation by asialo triantennary N glycan as well as recovery of activity by decitraconylation confirmed the involvement of lysine in sugar binding property of the lectin. Tryptophan modification by N-bromosuccinimide resulted in 100% loss of activity and protection with asialo triantennary N glycan resulted in only 50% loss of activity indicating presence of tryptophan at the active site of the lectin. Chemical modification of Arginine, tyrosine, cysteine, aspartate, and glutamate did not lead to inactivation of the lectin indicating that these amino acids are not involved in hemagglutinating activity of the *G. lucidum* lectin. Treatment of the lectin with DEPC resulted in 100% loss of activity but no histidine residue was modified as no increase in absorbance at 240 nm and no reactivation with hydroxylamine hydrochloride (0.5 M) was observed. Loss of activity after DEPC treatment was due to modification of lysine as studied by modification of DEPC modified lectin with TNBS.

**INTRODUCTION**

To understand the phenomenon of interaction between lectins and carbohydrates, knowledge of the reactive groups involved in the interaction is essential. Identification of specific amino acids involved in the biological activity of proteins elucidates the relationship between its structure and the role played by 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 of macromolecules. Site directed mutagenesis is also a useful technique to identify amino acids present at active site of a protein molecule but it is rather difficult procedure where complete information of the gene, its

cloning and expression of a functional protein are a prerequisite for modification of amino acids.

The use of amino acid side chain reactive reagents (i.e., those which under specified conditions react with a single or at least with a limited number of side chain groups in a fairly predictable manner) is, however a simpler approach. Modification of amino acid side chains by various reagents is a direct method for identifying amino acids required for the biological activity of proteins and protein sample in the absence of reagents serving as control. Removal of these reagents from reaction mixture is also relatively simple and can be easily done by dialysis or gel filtration. At least for initial screening, it is widely used to identify amino acid side chains required for biological activity of proteins.

Many mushroom lectins have been purified and studied for their sugar binding or biological properties i.e. antitumour (1,2), immunomodulatory (1,3,4) and mitogenic (5-7) properties but very little information is available on active site characterization of mushroom lectins. GLL-M, an 18 kDa lectin from mycelia, GLL-F, 16 kDa lectin from the fruiting body (8), an immunomodulatory protein LZ-8, which shows hemagglutinating activity from *Ganoderma lucidum* (9) and an 18 kDa lectin from *Ganoderma capense* (10) have been isolated, but amino acids involved in their sugar binding and immunomodulatory activityhave not been determined. We have purified and characterized a novel 114 kDa lectin from fruiting body of *G. lucidum* as described in previous chapter. In this chapter we describe chemical modification of *G. lucidum* lectin and its active site characterization.

**MATERIALS**

N-Acetylimidazole, 2,4,6-trinitrobenzenesulphonic acid, phenylglyoxal, diethylpyrocarbonate, phenylmethylsulfonyl fIuoride, N-bromosuccinimide, 5,5' dithiobis-(2-nitrobenzoic acid), sodium borohydride, hydroxylamine hydrochloride, succinic anhydride, citraconic anhydride, formaldehyde, sodium dodecyl sulphate, urea, octyl alcohol,

Phenyl-Sepharose CL-4B, pronase-E, fetuin and the molecular weight markers (Sigma Chemical Co. St. Louis, U.S.A); Sephadex G-25 and Sephacryl S-200 (Amersham Bioscience, Uppasala, Sweden); All other reagents were of analytical grade. Triantennary N-glycan from fetuin was purified and the homogeneity was checked on HPLC (11)

## METHODS

### Erythrocyte preparation

Human erythrocytes of A, B and O blood groups were washed 5 to 6 times with 20 mM Tris-HCl buffer pH 7.2 containing 150 mM NaCl (TBS). A 3% (v/v) suspension of the erythrocytes in the above buffer was treated with pronase (0.05%) at 37 ºC for 1 h, washed 4 times with the same buffer (TBS) and used for further studies.

### Hemagglutination assays

Hemagglutination assays were performed in standard microtitre plates by the two-fold serial dilution method. A 50 µl aliquot of the erythrocytes suspension was mixed with 50 µl of lectin serially diluted with TBS and agglutination was examined visually after incubation for one hour. A unit of hemagglutination activity (U) is expressed as the reciprocal of the highest dilution (titre) of the lectin that showed complete agglutination. The specific activity of the lectin is defined as the number of hemagglutination units/mg of the protein.

### Protein determination

Protein concentrations were determined according to Bradford (12) using BSA as standard.

### Purification of *G. lucidum* lectin

Extraction and purification of the lectin from fruiting body of *G. lucidum* was carried out as described earlier (Chapter 2).

**Chemical modification studies**

During chemical modification studies, the residual activity of the modified lectin was determined by hemagglutination assay. Lectin samples incubated in the absence of modifying reagents served as the control.

**Modification of serine with Phenylmethylsulphonyl fluoride (PMSF):**

The lectin (100 µg) in 50 mM Tris-HCl buffer, pH 8.0 was incubated with 5 mM PMSF, at 27±1 °C, for 60 min (13). Aliquots were removed at 15 min intervals, the excess reagent removed by dialysis and residual activity determined. Lectin sample incubated in the absence of PMSF served as the control.

**Modification of arginine with phenylglyoxal:**

Arginine residues were modified with phenylglyoxal 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 varying concentrations of phenylglyoxal (0.5-3.0 mM) for 30 min at 25 °C. Excess reagent was then removed by dialysis, and the residual hemagglutination activity determined. Lectin sample incubated in the absence of phenylglyoxal 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-nitrobenzoic acid) (DTNB):**

*Determination of free cysteine*: The lectin (200 µg) in 50 mM phosphate buffer, pH 8.0, was incubated with 0.1 mM DTNB at 27±1 °C for 1 h. Aliquots were removed at different time intervals and the residual activity determined. The cysteine residues were also estimated by taking the lectin (200 µg) in 2% SDS, 80 mM phosphate buffer, 0.1 M EDTA and by incubating with 0.1 mM DTNB at 27±1 °C for 1 h. The modification

reaction was also followed by monitoring the increase in absorbance at 412 nm and the number of sulphahydryl groups modified were calculated using a molar absorption coefficient of 13,600 $M^{-1}cm^{-1}$ (15).

*Determination of disulphide bonds*: The lectin (200 µg) in 8 M urea, 50 mM Na-EDTA, 1.25% sodium borohydride and a drop of octyl alcohol was added to a make final volume of the reaction to 3ml. Reduction was allowed to proceed for 30 min at 25 $^oC$ under stirring. Then 0.5 ml of 1 M $KH_2PO_4$ containing 0.2 M HCl was added. After 5 min 2 ml acetone was added and nitrogen was bubbled for 5 minutes. Then 0.5 ml of 10 mM DTNB was added and total volume was adjusted to 6 ml with water. Nitrogen was bubbled for 2 minutes and reaction mixture was kept at 25 $^oC$ for 15 minutes before determining the number of sulphahydryl groups modified as mentioned above (16).

**Modification of carboxylate with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide / 3-nitro-L-tyrosine ethyl ester (EDC/NTEE):**

The lectin solution (200µg), in 1 ml 50 mM MES/HEPES buffer, pH 6.0 was incubated with 50 mM EDC and 30 mM NTEE at 27±1 °C for 45 min. After the incubation period, a 100 µl aliquot was taken and the excess reagent was removed by gel filtration on Sephadex G-25 column and the residual hemmaglutinating activity was determined. The lectin sample in the same buffer was incubated under same conditions without EDC and NTEE served as control. Subsequently the reaction was arrested by the addition of 10 % (w/v) TCA 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 $M^{-1}cm^{-1}$ (17).

**Modification of lysine**

*Estimation of lysine with Trinitrobenzenesulphonic acid. (TNBS) :*

The reaction mixture containing 0.25 ml of lectin (50 µg) and 0.25 ml 4% (w/v) sodium bicarbonate was incubated with 25 µl of 0.5 % (w/v) TNBS at 37 °C, in the dark, for 2 h. The reaction was terminated by adding 0.125 ml HCI (1 N), and 0.25 ml of 10 % SDS was added to dissolve the precipitate formed (18). The number of free amino groups was determined, spectrophotometrically by assuming a molar absorption coefficient of 9950 $M^{-1}cm^{-1}$ for trinitrophenylated lysine at 335 nm.

*Citraconylation and decitraconylation:* The amino groups of *G. lucidum* lectin was reversibly blocked by citraconic anhydride by method of Dixon and Perham (19). Citraconic anhydride was diluted in dioxane and concentration of the diluted reagent was 100 mM. Purified *G. lucidum* (200µg) in 100 mM sodium carbonate buffer pH 8.5, was incubated with varying concentrations of citraconic anhydride (0.1-1mM) 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 free amino group as determined earlier. Decitraconylation was achieved by incubating the modified lectin samples at pH 4.0 and $30^{o}$ C for 1 hour followed by determining the hemagglutination activity.

*Succinylation* : This was carried out by the method of Habeeb (20). 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.

*Reductive methylation:* This was carried out as described by Means and Feeney (21). To 1 ml of the lectin (0.3 mg/ml) 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 min interval. The procedure was repeated using 3.5 (v/v) % 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 tryptophan with N-bromosuccinimide (NBS):**

The lectin (300 µg), in 100 mM sodium acetate buffer pH 5.0, was titrated with a total of 0.02 mM NBS, prepared in the same buffer. The reagent was added in five installments and the reaction was monitored spectrophotometrically by monitoring the decrease in absorbance at 280 nm. The number of tryptophan residues modified were determined by assuming a molar absorption coefficient of 5500 $M^{-1}cm^{-1}$ (22). The residual activity was determined by hemagglutination.

**Modification of tyrosine with N-acetylimidazole (NAI):**

This was performed as described by Riordan *et al.* (23). The lectin (300 µg) in 50 mM phosphate buffer pH 7.5 was incubated with different concentrations of NAI (0.1-10 mM) at 27±1 °C for 60 min followed by estimation of the residual activity. The excess reagent was removed by gel filtration on Sephadex G-25 column (1×10 cm) pre-equilibrated in the 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.

**Modification of histidine with diethyl pyrocarbonate (DEPC)**

The lectin in one ml of 20 mM phosphate buffer, pH 7.0 was treated with varying concentrations of DEPC (1-10 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 Ovaldi et al using a molar absorption coefficient for carbethoxyhistidine of 3200 $M^{-1}cm^{-1}$ (24). Ethanol concentration in reaction mixture did not exceed 2% (v/v) and had no effect on the activity and stability of the lectin for the incubation period.

DEPC concentration in the stock was determined by mixing an aliquot of diluted stock 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 was 100 mM.

**Reactivation with hydroxylamine hydrochloride**

Decarbethoxylation of the DEPC modified protein was carried out according to Miles (25). DEPC treated samples were incubated with hydroxylamine hydrochloride (500 mM) at pH 5.0 and 25$^{o}$C for 1-6 h and the hemagglutination activity was determined.

**Ligand protection**

This was carried out by pre-incubating the lectin with asialo triantennary N glycan (400 fold molar excess) followed by treatment with NBS and TNBS. Both modified and unmodified protein samples were dialysed, the residual activity estimated and the number of residues modified determined.

## RESULTS AND DISCUSSION

**Modification of Arginine, serine, tyrosine, cysteine and carboxylate:**

Modification of arginine residues by phenylglyoxal and serine residues by PMSF did not result in any loss of activity (Table 3.1). Modification of carboxylate residues by NTEE in the presence of EDC did not show any effect on the hemagglutination activity of *G. lucidum.* Number of nitrotyrosyl groups incorporated as determined by increase in O. D. at 430 nm showed modification of 24 carboxylate residues per molecule of the lectin. In the present studies acetylation by NAI did not result in modification of lysine and tyrosine residues of the *G. lucidum* lectin, instead only tyrosine residues were modified. Extensive modification of lysine residues by NAI has been reported in lentil lectin (26) contrary to Con A (27). In *G. lucidum* lectin, NAI modified 18 tyrosine residues per molecule and there was no loss of hemagglutination activity. Loss of sugar binding activity with the modification of tyrosine residues have been reported for lectin from *Ischnoderma resinosum*, where *O*-acetylation of tyrosine leads to complete loss of hemagglutination activity (28). Modification of cysteine residues by DTNB in native denatured *G. lucidum* lectin resulted in modification of 6 free cysteine residues and no loss of hemagglutination activity was observed. The total cysteine modification of SDS denatured protein also resulted in modification of 6 cysteine residues. However modification of cysteine residues of *G. lucidum* after denaturation by 8 M urea and subsequent reduction with sodium borohydride resulted in modification of 18 cysteine residues. As there are 6 free residues in *G. lucidum* lectin, hence 6 disulphide bonds are present in the lectin. Disulphide bonds are not involved in holding the subunits together as *G. lucidum* lectin did not show any difference in molecular weight in the presence and absence of DTT as determined by gel filtration on sephacryl S-200 column. Thus, there are intrasubunit disulphide bonds in the native lectin.

**Table 3.1: Effect of different modifying reagents on the activity of the *G. lucidum* lectin.**

| Reagent | Residues modified | No. of residues modified per molecule | Residual hemagglutination activity (%) |
|---|---|---|---|
| Phenylglyoxal | Arginine | ND* | 100 |
| PMSF | Serine | ND* | 100 |
| EDC/NTEE | Carboxylate | 24 | 100 |
| NAI | Tyrosine | 18 | 100 |
| DTNB | Cysteine | | |
| a. Native | | 6 | 100 |
| b. Urea + Sodium borohydride | | 12 | ND* |
| NBS | Tryptophan | | |
| a. Native | | 6 | 0 |
| b. Urea denatured | | 6 | ND* |

* ND = Not determined.

**Modification of histidine:**

Carbethoxylation of *G. lucidum* lectin by DEPC resulted in complete loss of activity but there was no increase in O. D. at 240nm (Table 3.2). As DEPC also modifies on lysine and tyrosine residues, only reversal of inactivation by hydroxylamine hydrochloride can confirm the involvement of histidine residues in sugar binding activity. There was no change in the absorbance at 278 nm due to formation of *O*-acetyl tyrosine, ruling out the tyrosine modification and treatment with hydroxylamine hydrochloride (0.5 M) did not result in reactivation of the modified lectin ruling out loss of activity due to histidine modification. Similar effects have been observed in case of *Artocarpus hirsuta* lectin (29) where DEPC treatment had resulted in loss of lectin activity although histidine was not found in its active site. Activity loss after DEPC treatment was due to modification of lysine because TNBS treatment of DEPC modified lectin resulted in modification only 18 residues indicating a total modification of the remaining 36 residues by DEPC.

**Table 3.2: Effect of histidine modification on *G. lucidum* lectin.**

| Reagent | Residues modified | No. of residues modified | Residual/Restored hemagglutination activity (%) |
|---|---|---|---|
| DEPC | Histidine | 0 | 0 |
| Reactivation with hydroxylamine hydrochloride | Tyrosine/Lysine | ND* | 0 |
| TNBS | Lysine | 18 | 0 |
| DEPC | Lysine | 36 | 0 |

* ND = Not determined.

**Modification of tryptophan residues:**

Purified lectin, when titrated with NBS, completely lost its initial activity with simultaneous decrease in O.D. at 280 nm and the number of modified tryptophans was six indicating presence of one tryptophan per monomer of lectin as *G. lucidum* lectin is homohexamer of 18.6 kDa subunits (Table 3.1). Number of tryptophans modified in urea denatured lectin was also six. Modification of lectin with NBS after ligand protection with asialo-triantennary glycan resulted in only 50% loss of activity indicating the presence of tryptophan in active site. Tryptophan has been reported to be involved in activity of fungal lectins from *Macrophomina phaseolina* (30) and S*treptomyces sp*(31) and plant lectins from winged bean (32) and soybean (33) etc.

**Modification of Lysine residues:**

Modification of lysine with TNBS resulted in complete loss of activity and number of lysine residues modifified was 54 (Table 3.3). The role of lysine was also evaluated by modifying the lectin with succinic anhydride and citraconic anhydride as these reagents show preference for lysine residues over acetic anhydride (34). Both succinilation and citraconylation of *G. lucidum* lectin resulted in inactivation of the lectin and number of lysine residues modified was same as with TNBS modification. A plot of residual activity of the lectin versus the number of amino groups modified by citraconylation and succinylation indicated that 11 out of 54 lysine residues were involved in activity indicating involvement of 2 lysine residues per monomer of the lectin (Figure 3.1). Decitraconylation of inactivated lectin at pH 4.0 completely restored the activity of lectin in one hour. Modification of lysine residues with TNBS in the presence of asialo triantennary N glycan resulted in only 50% loss of activity indicating involvement of these residues in sugar binding to the lectin.

**Table 3.3: Effect of lysine modification on the activity of G. lucidum lectin.**

| Reagent | Residues modified | No. of residues modified | Residual/Restored hemagglutination activity (%) |
|---|---|---|---|
| Succinic anhydride | Lysine | 54 | 0 |
| Citraconic anhydride | Lysine | 54 | 0 |
| Decitraconylation | Lysine | ND* | 100 |
| Reductive methylation (Formaldehyde) | Lysine | 54 | 0 |

* ND = Not determined.

Conversion of lysine groups to N-monomethyl and N, N'-dimethyl lysine by reductive methylation also resulted in complete loss of hemagglutination activity of the lectin. Chemical modification study of lysine suggested that positive charge of amino group (suggested by activity loss by succinylation and citraconylation), as well as its $pK_a$ (reductive methylation of amino group leads to activity loss), are necessary for the sugar binding property of the of the *G. lucidum* lectin. Lysine residues have been reported to be involved in activity of *Ricinus communis* agglutinin (35) and *Artocarpus hirsuta* lectin (29).

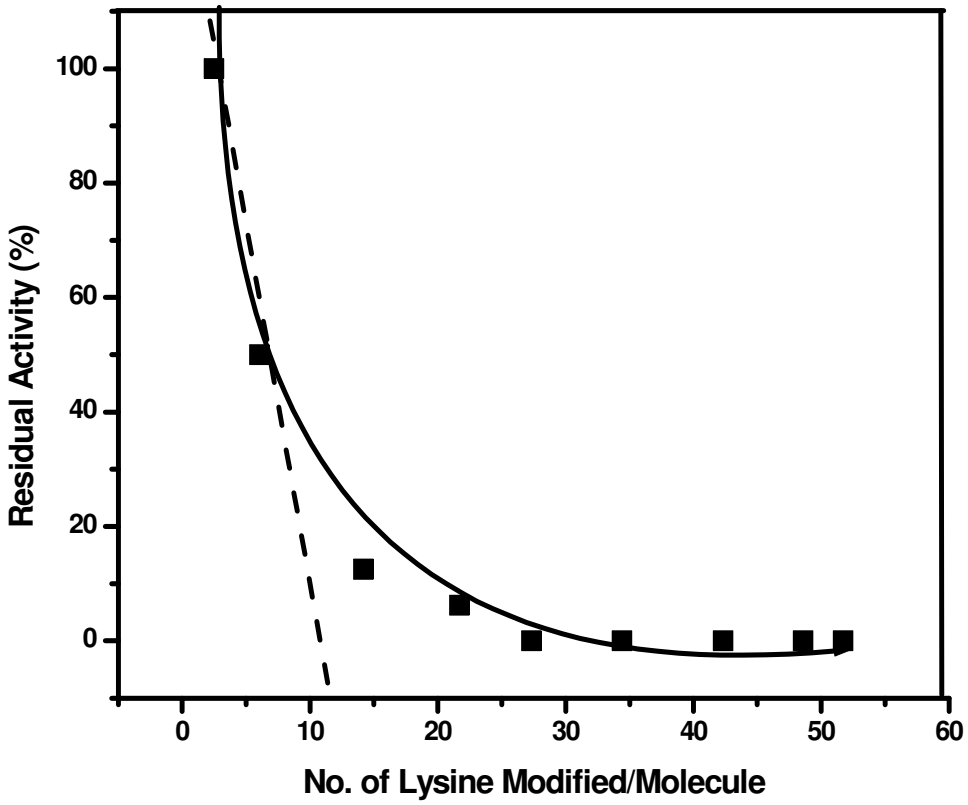


Figure 3.1: Plot of percent residual activity versues number of lysine residues modified.

In conclusion positive charge as well as pKa of lysine residues and indole ring of tryptophan residue are essential for sugar binding property of *G. lucidum* lectin. Two lysine residues and one tryptophan residue per monomer are involved in sugar binding property of the lectin. There are very few reports on the active site characterization of mushroom lectins. Tryptophan has been reported to be involved in the activity of lectin from mushroom *Hericium erinaceum* (36) and lectin from *Ischnoderma resinosum* (28) shows involvement of tyrosine residue in its sugar binding

activity. To our knowledge this is the first mushroom lectin which shows involvement of both tryptophan and lysine in sugar binding activity of the lectin.

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Chapter 4

# Carbohydrate Binding and Solute Quenching Studies of the *Ganoderma lucidum* Lectin by steady state and time resolved Fluorescence Spectroscopy

## SUMMARY

The intrinsic fluorescence intensity of *G. lucidum* lectin was quenched upon binding to disaccharides and glycans, without any change in the emission maximum (336 nm). The quenching observed with asialo-triantennary was 7%. The lectin interacted very poorly with monosaccharides but well with disaccharides indicating an extended binding site. Thermodynamic studies revealed that the binding of the lectin with all the saccharides is enthalpically driven and exothermic in nature. The asialo-triantennary glycan showed 5 fold higher affinity than asialo-biantennary glycan without any significant increase in binding enthalpy, pointing towards the importance of multivalency in the lectin-ligand interactions.

Solute quenching studies of the lectin using acrylamide, succinimide, potassium iodide and cesium chloride were carried out in the absence and presence of asialo-triantennary glycan and at different pH. These studies showed that the single tryptophan residue of the lectin (per monomer) is relatively exposed, and could be in the vicinity of positively charged amino acid residues. Time resolved fluorescence studies of the native lectin, in the presence of ligands and acrylamide showed biexponential decay with a shorter life time ($\tau_1$) and longer life time ($\tau_2$) of fluorescence which indicated the existence of tryptophan in two different conformers. For the native lectin biexponential fits yielded lifetime values of 1.27 ns ($\tau_1$) and 4.66 ns ($\tau_2$). In the presence of ligand (triantennary N-glycan) shorter lifetime increases to 1.41 ns and longer lifetime marginally decreases to 4.56 ns. In the presence of acrylamide a relative decrease in both lifetimes was observed, longer lifetime ($\tau_2$) decreases to 3.12 ns and shorter lifetime ($\tau_1$) to 0.98 ns.

## INTRODUCTION

Recognition is a key event in biological functionality of the lectins. In fungi by recognizing different carbohydrate structures present on the surface of their own or host cells, lectins play their roles. Some of these roles seem to concern the fungal metabolism itself, while other activities are implicated in symbiotic or parasitic relationships with other organisms (1). The overall biological activities of the lectins are manifestation of their specificities.

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 understanding of lectin interaction with cell bound carbohydrates. Determination of association constants with a series of ligands provides considerable insight into the spatial features of a lectin's combining site. Complementary thermodynamic data offers information on the forces involved in the binding and explains affinity differences encountered.

Since hemagglutination-inhibition studies provide semi-quantitative information, fluorescence spectroscopy has been used to obtain more quantitative information about a lectin's binding process. The advantage of using fluorimetry in studies of carbohydrate-protein interactions is that the binding can be studied at equilibrium without physical separation of the bound complex from the free ligand and the protein (2). Fluorescence of tryptophan is influenced by its microenvironment, hence changes which affect the tryptophan environment can cause changes in fluorescence properties (3).

Fluorescence quenching is a process, which decrease the intensity of the fluorescence emission. The accessibility of tryptophan in a protein molecules can be measured by use of quenchers perturbing fluorescence. Quenching by small molecules either in the solvent or bound to the protein in close proximity to the fluorophore can greatly decrease the quantum yield of a protein. Solute quenching of tryptophan fluorescence provides information on the microenvironment of these residues.

In this chapter, determination of the binding constants for the association of ligand with the *G. lucidum* lectin was carried out by ligand-induced quenching of the protein. Thermodynamic parameters for saccharide binding have also been determined and the accessibility of tryptophan residue of the lectin has been studied by steady state and time resolved fluorescence.

## MATERIALS

Galactose, galactosamine, 2-deoxygalactose, L-fucose, glucose, mannose, methyl-α-D-galactose, methyl-β-D-galactose, mellibiose, lactose, pronase-E, carboxypeptidase, aminopeptidase, all glycoproteins, acrylamide, CsCl, KI and succinimide (Sigma Chemical Co. St. Louis, U.S.A); Galβ1→3GalNAc, Galβ1→3GalNAcα1→*O*Ser and Galβ1→4GlcNAc (Dextra Labs, London, UK); Sephadex-G25 (Amersham Bioscience, Uppasala, Sweden); were used. All other reagents were of analytical grade.

## METHODS

### Protein determination

Protein concentrations were determined according to Bradford (4) using BSA as standard.

### Purification of *G. lucidum* lectin

Extraction and purification of the lectin from fruiting body of *G. lucidum* was carried out as described earlier (Chapter 2).

### Neutral sugar estimation

The glycan solutions (400µl) were incubated with 400 µl of 5% (w/v) phenol for 10 min at room temperature. Two 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 (5).

**Preparation of glycans**

One gm of the glycoprotein fetuin/fibrinogen was dissolved in 100 ml of 20 mM Tris-HCl (containing 150 mM NaCl, 0.5 % w/v sodium azide), pH 7.2 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 (10000 ***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 (6,7). Desialation of glycopeptides was carried out by incubating with 5 U of neuraminidase in 20 mM Tris-HCl 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 measurements and ligand-binding data analysis**

Fluorescence measurements were carried out using a Perkin Elmer LS-50B spectrofluorimeter, with slit width of 7 nm for both the monochromators and scan speed 100 nm/min. *G. lucidum* lectin samples (2 μM) in 10 mM MOPS buffer, pH 6.5 (containing 200 mM NaCl) were placed in a quartz cuvette maintained at desired temperature (± 0.1 °C) by means of a Julabo circulating cryobath. The sugar solution was added in 10-12 aliquots (5 to 10 μl each). Concentration of the stock solutions was in the range of 20-

100 mM. Samples were excited at 280 nm and the emission spectra were recorded for wavelength ranging from 300 nm to 400 nm. Each spectrum was an average of 5 accumulations. The fluorescence intensity at 348 nm ($\lambda_{max}$ of the lectin) was considered for further analysis. Corrections were also made to compensate the dilution effect upon addition of sugar to lectin. At the highest concentration of the saccharide to lectin, volume change was less than 5 % of the solution in the cuvette. Each data point was an average of three independent sets of experiments with SD less than 5%.

The association constants were calculated according to the method described by Chipman *et al.*(8). 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 p$K_a$ value for lectin-ligand interaction according to the relationship (8),

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

Where $F_c$ is the fluorescence intensity of the lectin at any point during the titration, $[P]_t$ is the total protein concentration, $\Delta F_\infty$ 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 ($\Delta G$) 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 ($\Delta H$) were determined from the Van't Hoff plots by using the equation,

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

Where $\Delta H$ is enthalpy change, R is gas constant, $\Delta S$ is entropy change and T is the absolute temperature. The entropy change was obtained from the equation,

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

**Solute quenching**

Titration of *G. lucidum* lectin with acrylamide, CsCl, KI and succinimide were performed in the absence and presence of 100 μM asialo-triantennary glycan. The iodine solution contained sodium thiosulfate (200 μM) to suppress tri-iodate formation. Defined amounts of the quencher (5-10 μl) were added from a stock of 5 M to 2 ml (2μM) lectin solution. The titrations were carried out at pH 5 (acetate buffer), pH 7 (phosphate buffer) and pH 9 (glycine-NaOH buffer), 27°C. The fluorescence spectra were recorded after 3 minutes and each spectrum was an average of 3 accumulations. The excitation wave length was 280 nm and slit width was 7.5 nm, the emission spectra were recorded in the range of wavelength 300 to 400 nm with slit width of 7.5 nm and scan speed 100 nm/min. Quenching data for all the quenchers used in this study were analyzed by the Stern-Volmer equation (6) as well as by the modified Stern-Volmer equation (9-11).

$$F_0/F_c = 1 + K_{SV}[Q] \quad (6)$$

$$F_0/\Delta F = f_a^{-1} + (K_a f_a)^{-1}[Q]^{-1} \quad (7)$$

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 obtained by modified Stern-Volmer plots. 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**

Fluorescence lifetime measurements were performed using FLS920 single photon counting spectrofluorimeter from Edinburgh 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 $\lambda_{max}$ (336 nm) 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 Edinburgh Instruments.

The quenching data was determined by equation (8), which allows resolution of the static and dynamic components (12):

$$F_0/F_c = (1 + K_{SV}[Q])\ (1 + K_S[Q]) \qquad (8)$$

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, $K_s$ is the static quenching constant.

Dynamic portion of the quenching can be determined by lifetime measurements, according to the equation (13),

$$\tau_0/\tau = 1 + K_{SV}[Q] \qquad (9)$$

where $K_{sv}$ is dynamic quenching constant $\tau_0$ is average lifetime in the absence of quencher.

The average lifetimes $\tau$ and $\tau_0$ were calculated by following expression (14-16):

$$\tau = \Sigma\ \alpha_i\ \tau_i / \Sigma\ \alpha_i \qquad (10)$$

$$\langle\tau\rangle = \Sigma\ \alpha_i\ \tau_i^2 / \Sigma\ \alpha_i\ \tau_i \quad (i = 1,2) \qquad (11)$$

where $\tau_i$ are the values of different lifetimes and $\alpha_i$ are the corresponding weighting factors obtained by bi-exponential fitting of decay curves.

## RESULTS AND DISCUSSION

### Ligand Binding

Titration of the *G. lucidum* lectin with sugars and glycans resulted in quenching of the lectin fluorescence without any shift in the emission maximum. The maximum quenching of the intrinsic fluorescence of the lectin, on binding with sugar, was 7% (Fig. 4.1). The slope of the plot of log $[F_0\text{-}F_c/F_c\text{-}F_\infty]$, versus log [C] was near unity for the ligands used, indicating one binding site per monomer of the lectin (Fig. 4.1). Association constants obtained for the binding of various sugars at 25 °C are listed in Table 4.1. Decrease in $K_a$ with increasing temperature was observed for all the ligands used. Van't Hoff plots for different sugars were linear ($r > 0.9$) in the range of temperatures studied (Fig. 4.2). Free energy of the binding ($\Delta G$) was negative at all the temperatures, suggesting the spontaneous nature of binding. Enthalpy change ($\Delta H$) of binding was also negative for all the sugars, indicating exothermic and enthalphically driven nature of binding (Table 3). The *G. lucidum* lectin bound very poorly with monosaccharides, and did not show any binding with Glc/Man or their derivatives. Very low binding was observed with GalNAc and galactosamine. The binding with disaccharides such as Galβ1-3GalNAc and Galβ1-4 Glc NAc, stachyose and mellibiose were close to binding affinity of monosachharides indicating that the lectin probably does not discriminate between mono- and di-saccharides. Among disaccharides, the lectin interacts primarily with the non-reducing sugar Gal; the stoichiometry and orientation of the reducing sugar did not significantly influence the binding. Addition of a methyl group at α or β position as in Met-α-Gal and Met-β-Gal did not result in increase in binding. Most of the mushroom lectins show well defined monosaccharide or disaccharide specificity as from *Aleuria aurantia* (L-Fucose), *Lentinula edodes* (D-Glucosamine), *Psilocybe berrerae* (D-Galactose), *Agaricus blazei* (GalNAc),

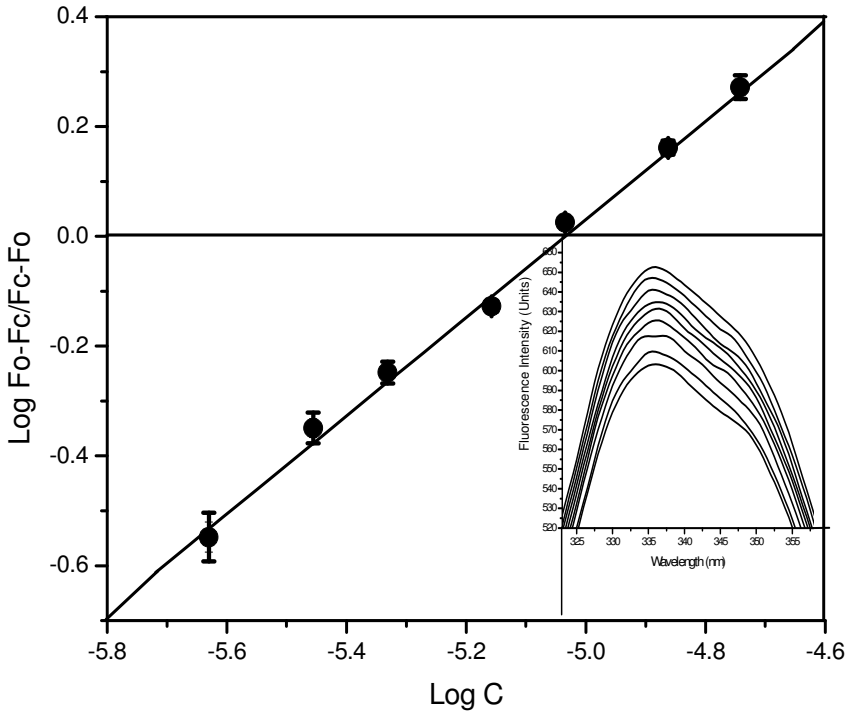


**Fig. 4.1: Determination of the association constant for asialo-triantennary glycan and lectin interaction by fluorescence spectroscopy.** $F_0$ and $F_\infty$ are the fluorescence intensity of free lectin and that bound to the ligand at infinite ligand concentration. $F_c$ is the corrected value of fluorescence intensity at any point of titration, [C] is the ligand concentration in molarity. Inset represents fluorescence quenching of *G. lucidum* lectin on addition of aliquots of asialo-triantennary N glycan to the lectin solution.

*Boletus edulis* (Lactose), *Agaricus bisporus* (Galβ1→3GalNAc), Laetiporus *sulphureus* (LacNAc) and *Hericium eraniceus* (Sialic acid) (1). Very few mushroom lectins have been reported which do not show specificity for monosaccharides or disaccharides and show specificity only for complex glycans as lectins from *Volvariella volvacea* (17) and *Mycoleptodonoids aitchisonii* (18).

**Table 4.1: Association constants of *G. lucidum* lectin with different sugars and glycans determined by fluorescence spectroscopy.**

| | Sugar | $K_a$ ($M^{-1}$) | $-\Delta G$ ($kJ \cdot mol^{-1}$) |
|---|---|---|---|
| 1. | D- GalNAc | 489.77 | -15.33 |
| 2. | Galactosamine | 251.18 | -13.67 |
| 3. | α Lactose | 479.43 | -15.28 |
| 4. | Gal β 1-3 GalNAc | 851.13 | -16.60 |
| 5. | Gal β 1-4 GlcNAc (LacNAc) | 891.21 | -16.82 |
| 6. | Stachyose | 812.83 | -16.60 |
| 7. | Mellibiose | 691.83 | -16.17 |
| 8. | Asialo-triantennary N glycan | $1.07 \times 10^5$ | -28.69 |
| 9. | Sialated- Triantennary N- Glycan | $7.76 \times 10^4$ | -27.70 |
| 10. | Asialo-biantennary N –glycan | $3.38 \times 10^4$ | -25.89 |
| 11. | Sialated- Biantennary N- glycan | $3.16 \times 10^4$ | -25.69 |

Methyl-α-D-Gal, methyl-β-D-Gal, glucose, N-acetylglucosamine, glucosamine, methyl-α-D-Glc, methyl-β-D-Glc, mannose, mannosamine, N-acetylmannosamine, methyl-α-D-Man, methyl-β-D-Man, 6-deoxy-α-(L)-mannose (rhamnose), Galβ1→3GlcNAc (Lacto-N-Biose) and mellibiose did not show considerable change in the fluorescence spectrum of *G. lucidum* lectin.

**Table 4.2: Association constants and thermodynamic parameters for the binding of glycans to *G. lucidum* lectin.**

| **Glycans** | **Ka ($M^{-1}$)** | | |
|---|---|---|---|
| | 20 °C | 25 °C | 30 °C |
| Asialo-triantennary N glycan | $1.31 \times 10^5$ | $1.07 \times 10^5$ | $5.28 \times 10^4$ |
| Sialated- Triantennary N-Glycan | $1.17 \times 10^5$ | $7.76 \times 10^4$ | $4.89 \times 10^4$ |
| Asialo-biantennary N –glycan | $5.17 \times 10^4$ | $3.38 \times 10^4$ | $2.51 \times 10^4$ |
| Sialated-Biantennary N- glycan | $4.27 \times 10^4$ | $3.16 \times 10^4$ | $2.09 \times 10^4$ |

Camparison of association constants for asialo triantennary N glycan with mono- or di- saccharides showed approximately 200 and 125 fold higher affinity respectively (Table 4.1). Lectin-carbohydrate interactions are generally characterized by a low affinity for monovalent ligands. In general, millimolar affinity is observed for lectins binding to monosaccharides. However, when longer oligosaccharides act as ligands, this corresponds to an extended binding site on the lectin surface, hence increased affinity up to micromolar range can be observed. Branched complex glycans, due to the clustering effect, offer multiple binding sites leading to several fold increase in the affinity (19), as was observed in case of *G. lucidum* lectin with complex glycans.

**Table 4.3: Thermodynamic parameters for the binding of glycans to *G. lucidum* lectin***

| Glycan | $-\Delta H$ (kJ·mol$^{-1}$) | $-\Delta G$* (kJ·mol$^{-1}$) | $-\Delta S$ (J·mol$^{-1}$·K$^{-1}$) |
|---|---|---|---|
| Asialo-triantennary N-glycan | -66.93 | -28.69 | -128.32 |
| Sialated-triantennary N-glycan | -64.18 | -27.70 | -122.41 |
| Asialo-biantennary N-glycan | -53.37 | -25.89 | -92.21 |
| Sialated-biantennary N-glycan | -52.29 | -25.69 | -89.25 |

* Values at 25 °C.

*G. lucidum* lectin showed highest affinity for asialo-triantennary glycan among the sugars and glycans tested (Fig.5). Lectins from *Agaricus bisporus* (20) and *Aleuria aurantia* (21) show increased affinity for multivalent Galβ1→3GalNAc and L-fucosyl ligands respectively than their monomeric counterparts. Asialo-triantennary glycan showed higher affinity for the lectin than asialo-biantennary glycan which can be attributed to increase in branching from biantennary to triantennary. The N-glycan of fetuin has a triantennary structure with ultimate Galβ1→4GlcNAc, whereas fibrinogen has a biantennary structure with ultimate Galβ1→4GlcNAc (7,22).

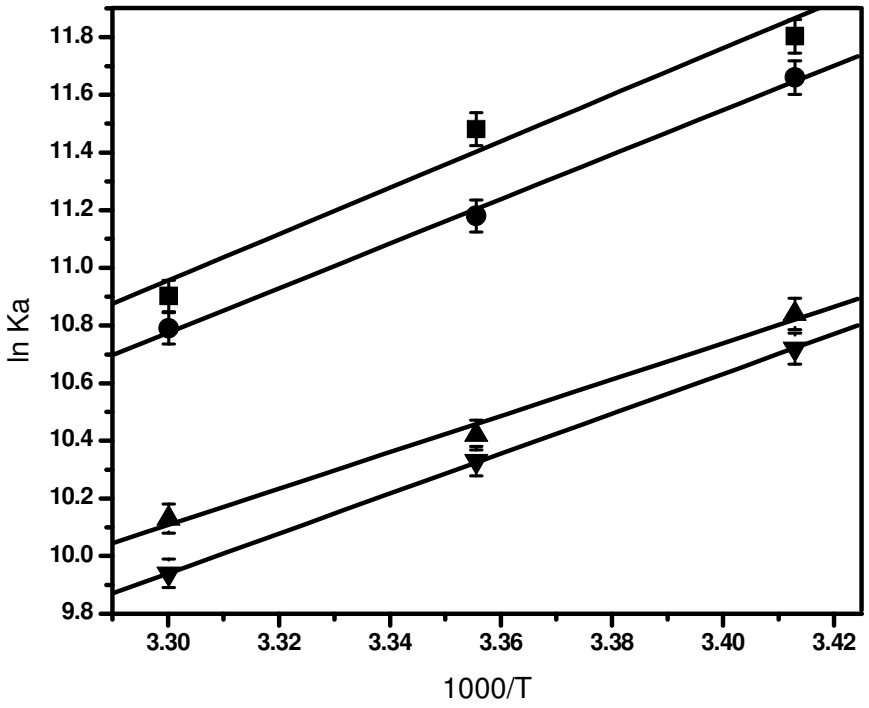


Fig. 4.2: Van't Hoff plots for the association of various glycans to *G. lucidum* lectin are drawn according to the regression equation ($r > 0.9$, $n = 3$). (■) asialo-triantennary glycan; (●) sialylated triantennary glycan; (▲) asialo-biantennary glycan; (▼) sialylated biantennary glycan

The asialo-triantennary glycan exhibited approximately 3 fold higher binding affinity than asialo-biantennary glycan (Table 3).The higher binding affinity observed with the former was also accompanied by coincidental enthalpy increase, suggesting increased polar interaction between glycan and lectin, which indicates that the asialo-triantennary glycan fits more tightly to the ligand binding pouch of the lectin. However there was increase in unfavorable entropy with the increased affinity which was compensated by increased favorable enthalpic contribution.

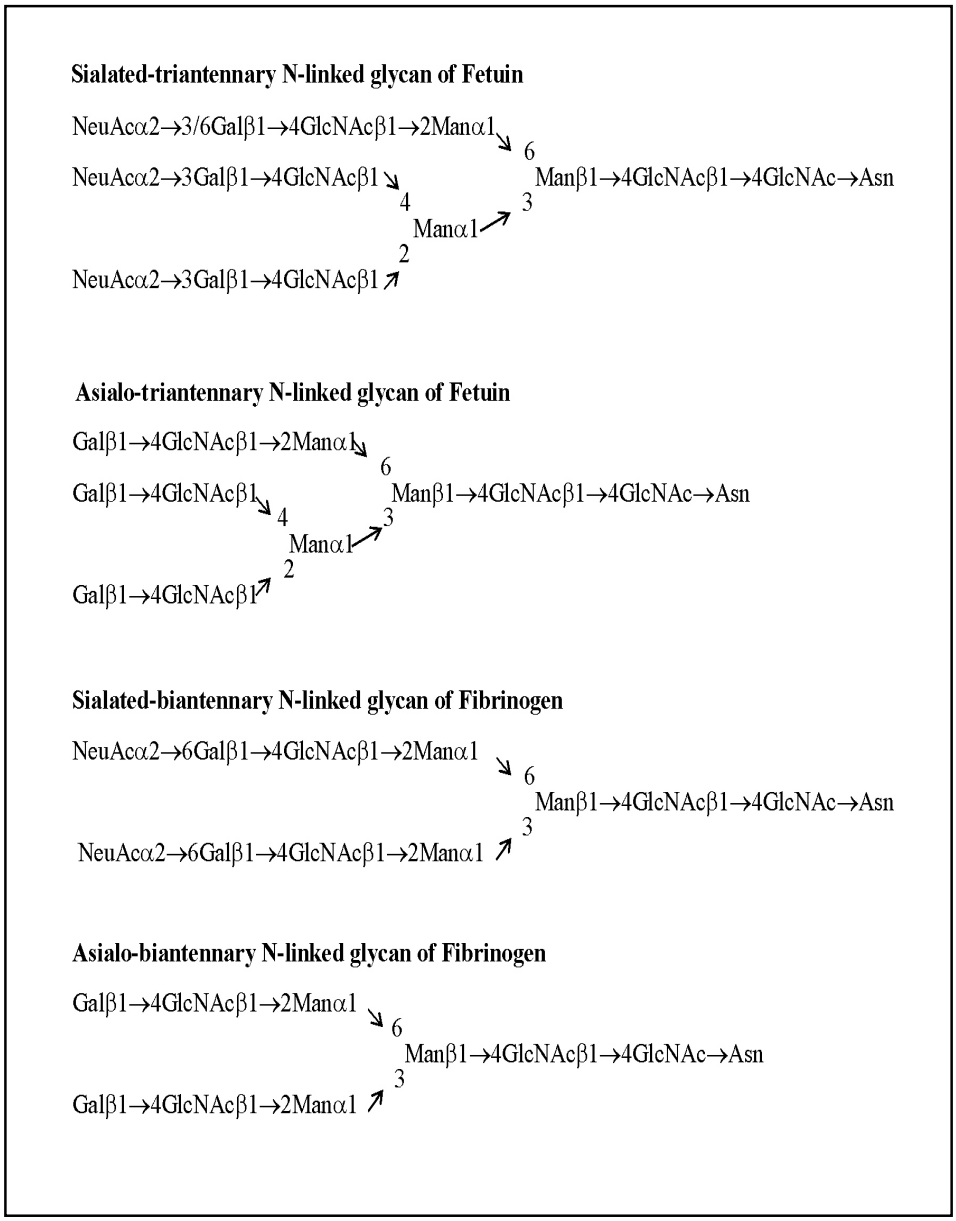


**Fig. 4.3: Structure of different glycans**

The glycans were prepared from fetuin and fibrinogen as described in Methods.

The sialylated form of the glycan has a terminal sialic acid as in NeuAcα2→3/6Gal.The removal of bulky and negatively charged NeuAc from C-3/6 position in the ultimate Gal did not significantly affect the binding and the lectin showed closer value of the affinity with asialo and sialylated glycans. The slight decrease in binding affinity of sialated glycan is probably due to some unfavorable interaction between NeuAc and lectin or the addition of the bulky NeuAc might be creating some steric hindrance by reducing the accessibility of penultimate Gal to the ligand binding pouch of the lectin. Moreover, probability of unfavorable ionic interaction between the NeuAc and lectin cannot be excluded. Simultaneous reduction of binding enthalpy is also obvious due to loss of contributory hydrogen bonding or van der Walls interaction. There is not much difference in the enthalpy of sialated and asialo glycans indicating that reduction in binding affinity is not due to loss of hydrogen bonding and van der Waal's interactions.

**Solute Quenching Studies**

The tryptophan exposure and its microenvironment in proteins can be investigated by fluorescence quenching, using small molecules as acrylamide, succinimide, iodide and cesium termed as quenchers. Titration of the lectin with acrylamide, succinimide, KI and CsCl at pH 7.0 resulted in 74 % (0.125 M), 25 % (0.062 M), 45 % (0.250 M) and 35 % (0.250 M) quenching, respectively (Table 4.4). The *G. lucidum* lectin was found to have six tryptophan residues per molecule (hexamer) in native as well as after denaturation (Chapter 3). One tryptophan residue is present at active site per monomer of the lectin. The *G. lucidum* lectin showed $\lambda_{max}$ of emission at 336 nm indicating presence of tryptophan residues in slightly hydrophobic environment. As all the tryptophan residues are present at the active site of the lectin hence all the tryptophans should be equally exposed and present in the homogenous environment. Stern-Volmer plots with different quenchers were monophasic in nature in the case of *G. lucidum* lectin and no downward curvature was observed for any of the quenchers studied (Fig 4.4).

Acrylamide was the most effiecient quencher among all the quenchers and showed 100% accessibility at all the pH studied (Table 4.4). A fluorescence spectrum of G. lucidum lectin with the increasing concentration of acrylamide is shown in figure 4.5. Acrylamide did not show considerable difference in accessibility ($f_a$) at different pH, but there was decrease in quenching from 74% to 66% (0.125 M) in the presence of ligand (Table 4.4). Total accessible fluorophore fraction was near unity. Direct Stern-Volmer plot of acrylamide quenching did not show considerable change at different pH and in the presence of ligand, but showed a clear upward curvature, indicating significant contribution from static quenching in the total quenching (Fig. 4.5). The static quenching is shown when a quencher deactivates an excited fluorophore at the instant of excitation, by forming a nonfluorescent or dark complex in the ground state, or by being present within a certain “sphere of action” at the time of excitation (23).

Succinimide, having more molecular radius than acrylamide, showed only 42% quenching of the total available fluorescence. Acrylamide and succinimide both are polar but neutral quenchers so the presence of local charges in the vicinity of tryptophan microenvironment does not affect their quenching ability. The $K_{sv}$ for succinimide (3.15 $M^{-1}$) as compared to acrylamide (25.69 $M^{-1}$) at pH 7.0 reflected both the inefficiency of quenching by the former as well as its restricted accessibility to the tryptophan (Table 4.4).

Succinimide has a larger molecular radius than acrylamide, and sterically much more rigid. Hence, its approach to buried tryptophan could be much more hindered. As *G. lucidum* lectin has tryptophan at its active site, so it should be relatively exposed to the polar environment, hence should be equally accessible to the succinimide. But it has been reported that succinimide is a poorer quencher than acrylamide for certain tryptophan residues in proteins not only due to critical size dependence for a penetration process but also due to less effieciency of the quenching by succinimide (24). *G. lucidum* lectin also has two lysine residues present at its active site. Tryptophan can also be probably sandwiched /buried between these two lysine residues, hence having reduced accessibility by succinimide. Slightly hydrophobic environment of tryptophan residue also supports this argument. But this suggestion cannot be held true unless three dimensional structure of the *G. lucidum* lectin active site is confirmed by X-ray crystallography.

The ionic quenchers, $I^-$ and $Cs^+$ being charged, cannot penetrate into the protein interior, unlike the neutral quenchers and thus do not probe buried tryptophan. The ionic quenchers are useful to explore the microenvironment of relatively exposed tryptophan. Their accessibility and quenching efficiency depend on the local charges of tryptophan microenvironment (25,26).

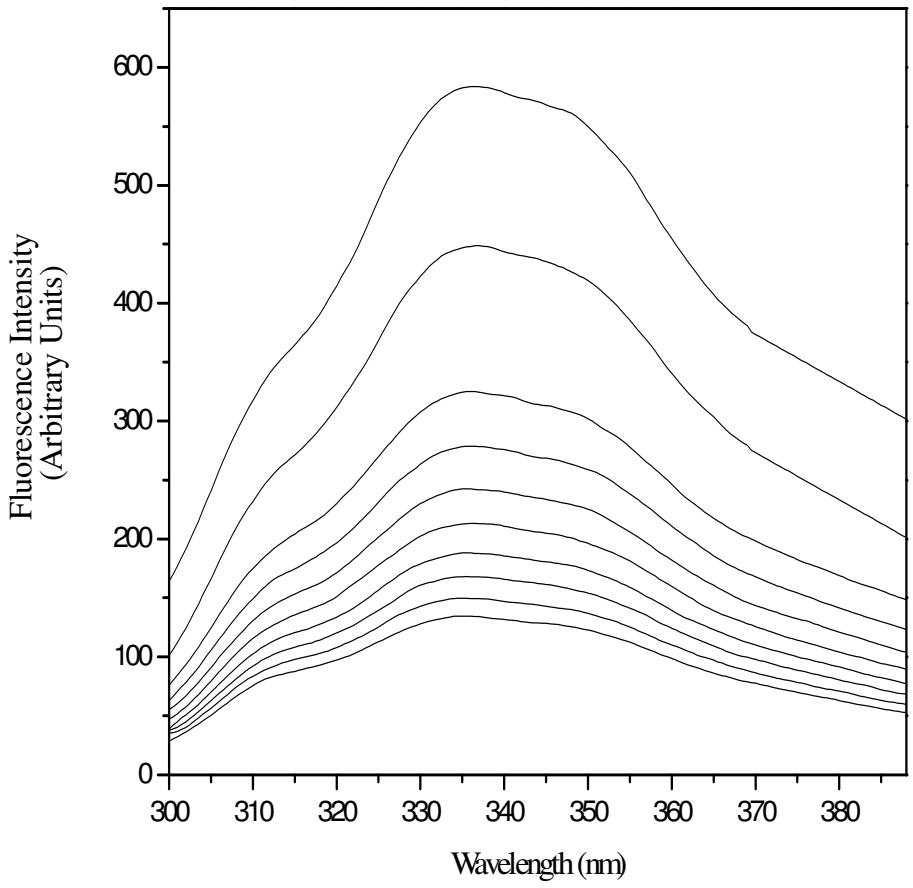


**Figure 4.4: Fluorescence spectra of native *Ganoderma lucidum* lectin with increasing concentration of acrylamide at pH 7.0.**

**Table 4.4: Summary of parameters obtained from the intrinsic fluorescence quenching with different quenchers.**

| Quencher and condition | $K_{sv}$ $M^{-1}$ | $K_a$ $M^{-1}$ | $f_a$ | % quenching |
|---|---|---|---|---|
| Acrylamide | | | | 0.125 M* |
| pH 5.0 | 25.74 | 15.00 | 1 | 74 |
| pH 7.0 | 25.69 | 16.92 | 1 | 74 |
| pH 9.0 | 26.56 | 18.93 | 1 | 76 |
| With ligand | 21.56 | 15.51 | 1 | 66 |
| Succinimide | | | | 0.062 M* |
| pH 5.0 | 3.76 | 7.53 | 0.47 | 23 |
| pH 7.0 | 3.15 | 7.02 | 0.42 | 20 |
| pH 9.0 | 4.17 | 6.54 | 0.46 | 21 |
| With ligand | 2.98 | 6.30 | 0.40 | 16 |
| KI | | | | 0.250 M* |
| pH 5.0 | 2.54 | 9.26 | 0.47 | 45 |
| pH 7.0 | 2.31 | 7.82 | 0.45 | 40 |
| pH 9.0 | 1.94 | 6.40 | 0.44 | 33 |
| With ligand | 2.09 | 7.50 | 0.44 | 34 |
| CsCl | | | | 0.250 M* |
| pH 5.0 | 0.57 | 3.69 | 0.34 | 16 |
| pH 7.0 | 0.66 | 4.36 | 0.35 | 14 |
| pH 9.0 | 0.74 | 5.89 | 0.36 | 11 |
| With ligand | 0.61 | 3.97 | 0.34 | 10 |

* Final concentration of the quenchers used.

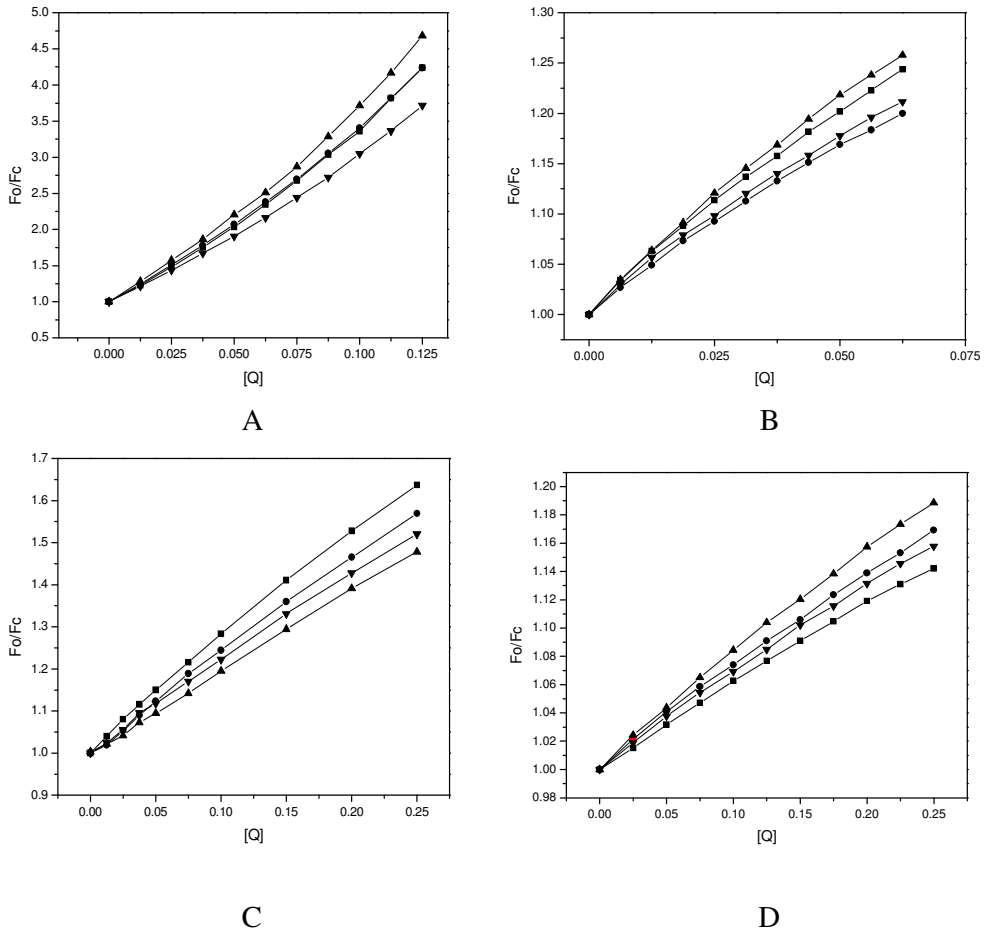


**Fig 4.4 : Stern-Volmer plots of fluorescence quenching for *G. lucidum* lectin.** The different quenchers used are (A) acrylamide (B) succinimide (C) iodide ion and (D) cesium ion. Lectin at (■) pH 5.0, (●) pH 7, (▲) pH 9 and (▼) in the presence of ligand.

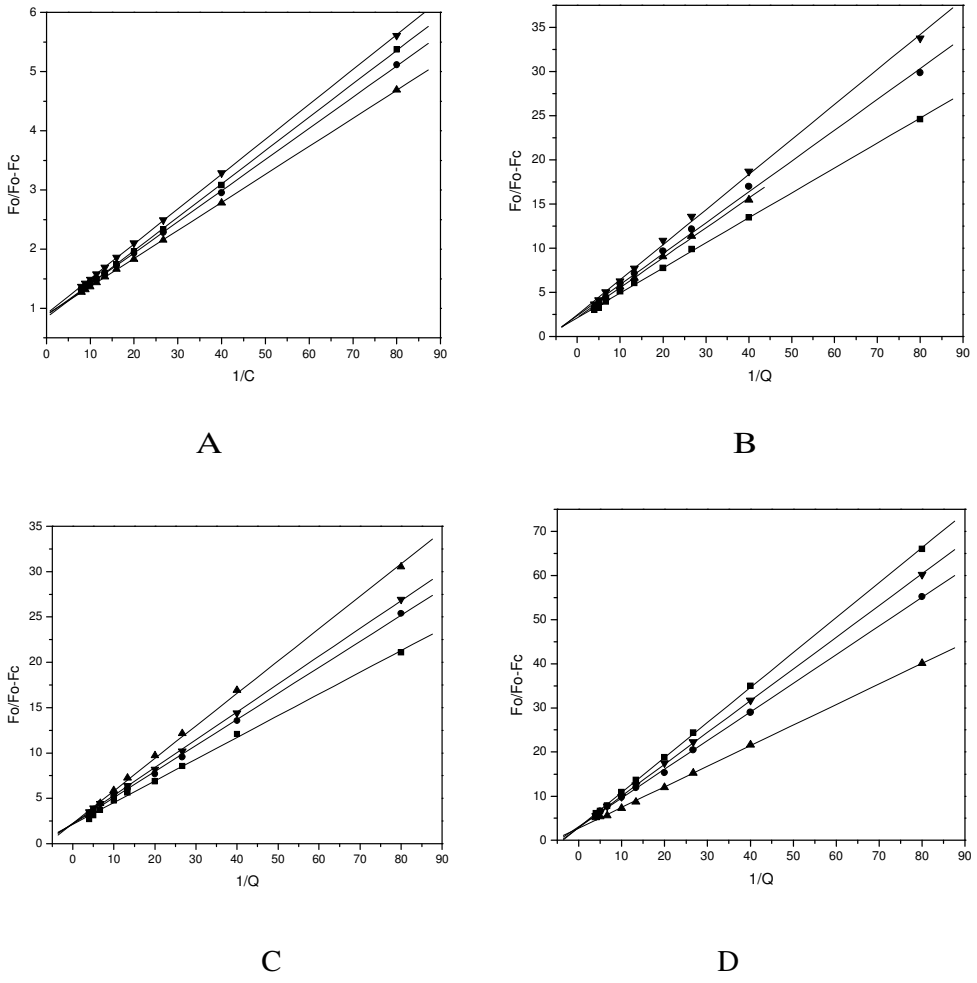


**Fig 4.5 : Modified Stern-Volmer plots of fluorescence quenching for *G. lucidum* lectin.** The different quenchers used are (A) acrylamide (B) succinimide (C) iodide ion and (D) cesium ion. Lectin at (■) pH 5.0, (●) pH 7, (▲) pH 9 and (▼) in the presence of ligand.

Quenching of *G. lucidum* lectin with $Cs^+$ and $I^-$ was carried out at pH 5, 7 and 9. Considerable difference in the quenching and the accessibility was observed with $Cs^+$ and $I^-$ at different pH (Fig 4.4 and 4.5). $K_{sv}$ value for $I^-$ (2.31 $M^{-1}$) and $Cs^+$ (0.66 $M^{-1}$), indicates iodide has more accessibility to the fluorophore than cesium (Table 4.4). $Cs^+$ and $I^-$ showed 40 % (0.125 M) and 14 % (0.125) quenching of the total fluorescence, respectively. In case of $Cs^+$ quenching, raising the pH resulted in increased quenching (11% at pH 5 and 16 % at pH 9) and accessibility ($K_{sv}$ = 0.57 $M^{-1}$ at pH 5 and 0.74 $M^{-1}$ at pH 9). Iodide a negatively charged quencher resulted in opposite effect than $Cs^+$ as quenching (33 % at pH 5 and 45 % at pH 9) and $K_{sv}$ decreased with increase in pH ($K_{sv}$= 2.54 $M^{-1}$ at pH 5 and to 1.94 $M^{-1}$ at pH 9). Accessibility and quenching efficiency of $Cs^+$ and $I^-$ with effect of pH suggest that the microenvironment of tryptophan is probably positively charged. This is in good agreement with chemical modification results which showed presence of two positively charged lysine residues at the active site besides tryptophan.

Quenching with acrylamide, succinimide, $Cs^+$ and $I^-$ in the presence of the asialo triantennary N-glycan resulted in decrease in quenching and $K_{SV}$ of *G. lucidum* lectin fluorescence. This showed interaction of tryptophan with the asialo triantennary N-glycan and is in good agreement with the chemical modification results (Chapter 3) which showed presence of tryptophan at the active site.

Time resolved fluorescence quenching studies were carried out in the presence of acrylamide to resolve dynamic and static component of quenching by acrylamide and in the presence of ligands to see effect of ligand binding on the fluorescence lifetime.

**Time resolved fluorescence Studies**

Fluorescence decay curve of native *G. lucidum* lectin is shown in Fig. 4.6. The decay curves of quenching of intrinsic fluorescence of native *G. lucidum* lectin, in the presence of asialo bi- and tri- antennary N glycans and acrylamide quenching could be fitted well to bi-exponential function ($\chi^2 \leq 1.1$). Average lifetimes ($\tau$ and $< \tau >$) were obtained by two different approaches (equations 10 and 11). Mono-exponential fits gave significantly larger errors ($\chi^2 \geq 1.7$) whereas tri-exponential fits did not result in the reduction of errors as compared to biexponential fits. For native *G. lucidum* lectin two lifetimes obtained were 1.27ns ($\tau_1$) and 4.66 ns ($\tau_2$) with relative contribution of 23.67% and 76.33% to the total fluorescence respectively.

Increase in shorter lifetime and decrease in longer lifetime was observed in presence of asialo biantennary N glycan ($\tau_1$=1.39, $\tau_2$=4.56) and asialo triantennary N glycan ($\tau_1$=1.41, $\tau_2$=4.45). Both the calculated average lifetimes ($\tau$ and $< \tau >$) showed a decrease in lifetimes in the presence of glycans (Table 4.5). This change in fluorescence lifetime in the presence of ligands suggests that tryptophan is at the active site and some interaction is involved between glycans and tryptophan. Relative contribution of shorter lifetime increased in the presence of glycans (Table 4.5).

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 (for this reason even single tryptophan proteins exhibit multiexponential fluorescence decay) (27,28) or due to heterogeneity of the environments of various tryptophan residues in multitryptophan proteins. In *G. lucidum,* biexponential decay can be explained on the basis of existence of tryptophan in different rotamers/conformers, although there are six tryptophan residues per molecule but single tryptophan per monomer of

the lectin is present at its active site ruling out the heterogeneity of the tryptophan residues.

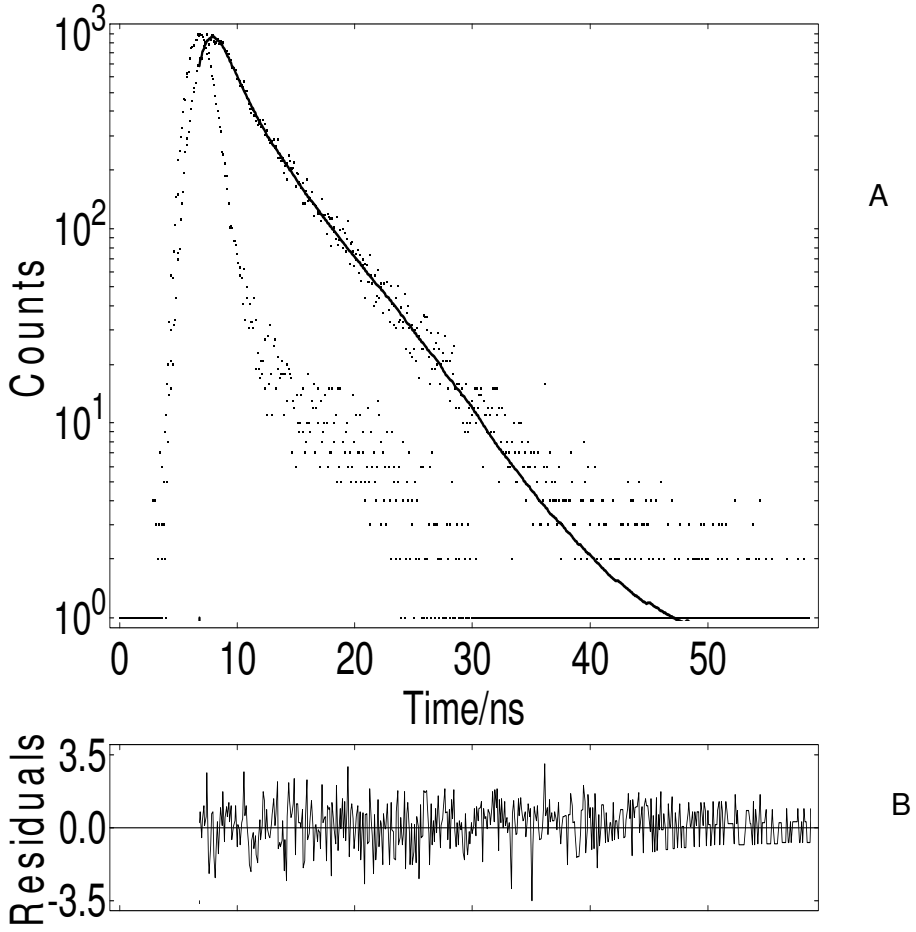


**Figure 4.6: (A) Time resolved fluorescence intensity decay of *G. lucidum* lectin under native conditions ($\chi^2$ = 1.023).** Typical fluorescence sdecay data was obtained at 25 $^{o}$C and protein concentration of 200 μg/ml in phosphate buffer, pH 7.0. Y-axis represents photon counts in logarithmic scale. The fast decaying, noisy line represents the IRF (Instrument response function) used as the excitation source. The slower decaying line represents the experimental fluorescence decay curve. (B) Plot of the auto correction function of the weighted residual used to judge the goodness of fitting.

**Table 4.5: The lifetimes of fluorescence decay of *G. lucidum* lectin and the corresponding pre-exponential factors along with calculated average lifetimes in the presence of ligands.**

| | α1 | τ1 (ns) | Rel % | α2 | τ2 (ns) | Rel % | τ (ns) | $\chi^2$ |
|---|---|---|---|---|---|---|---|---|
| Native lectin | 0.055 | 1.27 | 23.67 | 0.028 | 4.66 | 76.33 | 2.4136 | 1.023 |
| 0.2 mM Triantennary-N-glycan | 0.059 | 1.41 | 25.86 | 0.025 | 4.45 | 74.14 | 2.3148 | 1.001 |
| 0..2 mM Biantennary-N-Glycan | 0.06 | 1.39 | 25.78 | 0.026 | 4.56 | 74.22 | 2.3484 | 1.092 |

This lectin shows similarity with *Fusarium solani* lectin in biexponential decay of tryptophan fluorescence where two tryptophan residues per dimer (one tryptophan residue per monomer) showed biexponential decay (29). Many multitryptophan proteins show biexponential decay due heterogeneity in tryptophan environment in contrast to *G. lucidum* lectin e.g. snake gourd (*Trichosanthes anguina*) seed lectin (26) where biexponential decay of tryptophan fluorescence is due to differences in microenironment of the tryptophan.

**Table 4.6: The lifetimes of fluorescence decay of *G. lucidum* lectin and corresponding pre-exponential factors along with calculated average lifetimes of acrylamide quenching**

| [Q] ($M^{-1}$) | $\alpha_1$ | $\tau_1$ (ns) | Rel % | $\alpha_2$ | $\tau_2$ (ns) | Rel % | $\tau$ (ns) | $<\tau>$ (ns) | $\chi^2$ |
|---|---|---|---|---|---|---|---|---|---|
| 0.00 | 0.055 | 1.27 | 23.67 | 0.028 | 4.66 | 76.33 | 2.4136 | 3.478 | 1.082 |
| 0.025 | 0.058 | 1.19 | 24.59 | 0.026 | 4.29 | 75.41 | 2.1495 | 3.105 | 1.076 |
| 0.050 | 0.06 | 1.13 | 25.82 | 0.029 | 3.82 | 74.18 | 2.0065 | 2.7987 | 1.003 |
| 0.075 | 0.062 | 1.08 | 26.98 | 0.029 | 3.61 | 73.02 | 1.8863 | 2.6231 | 1.009 |
| 0.10 | 0.064 | 1.04 | 28.12 | 0.03 | 3.47 | 71.88 | 1.8155 | 2.5223 | 1.050 |
| 0.125 | 0.065 | 1.01 | 29.36 | 0.027 | 3.27 | 70.64 | 1.6733 | 2.3062 | 1.082 |
| 0.150 | 0.066 | 0.98 | 30.72 | 0.029 | 3.12 | 69.28 | 1.6333 | 2.2279 | 1.049 |

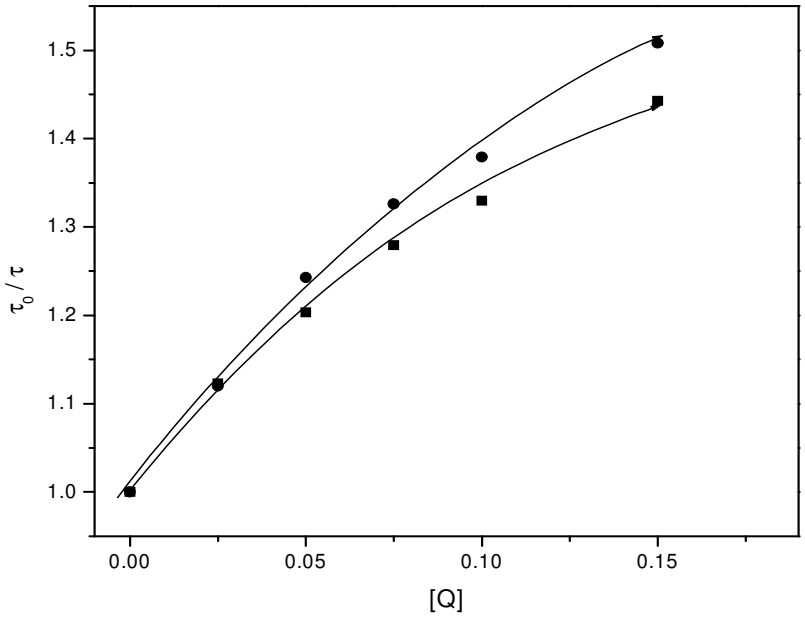


**Figure 4.7: Stern Volmer plots for $\tau_o/\ \tau$ and $<\tau_o>/\ <\tau>$ versus [Q].** Symbols used are (■) for $\tau_o/\ \tau$ and (●) for $<\tau_o>/\ <\tau>$. $Ksv_\tau$ and $Ksv_{<\tau>}$ obtained by slope of linear fit.

*G. lucidum* lectin when titrated with acrylamide showed decrease in shorter as well longer lifetime (Table 4.6). Both the calculated average lifetimes also showed decrease in lifetimes. Relative contribution of shorter lifetime increased to 30.72% and longer lifetime decreased to 69.28% (Table 4.6).

Quenching profile obtained by steady state fluorescence showed that quenching by acrylamide has both dynamic and static components. Dynamic portion ($K_{SV}$) of the observed quenching was obtained from plots of $\tau_o/\ \tau$ vs [Q]. Stern – Volmer constants ($K_{SV}$) obtained from two calculated average lifetimes ($\tau$ and $<\tau>$) were 2.85 ($\tau_o/\ \tau$ vs [Q]), 3.33 ($<\tau_o>/\ <\tau>$ vs [Q]) (Figure 4.7 and Table 4.7). Plots of $\tau_o/\ \tau$ do not show upward curvature because in fluorescence lifetime measurements total quenching is reflected only by collision quenching and

static quenching does not lead to decrease in lifetime. $\tau_o/\tau$ plots show downward curvature. As *G. lucidum* lectin has six tryptophan residues per molecule (hexamer), different accessibilities can be expected for the multitryptophan proteins due to existence of tryptophan in different conformers in collisional quenching.

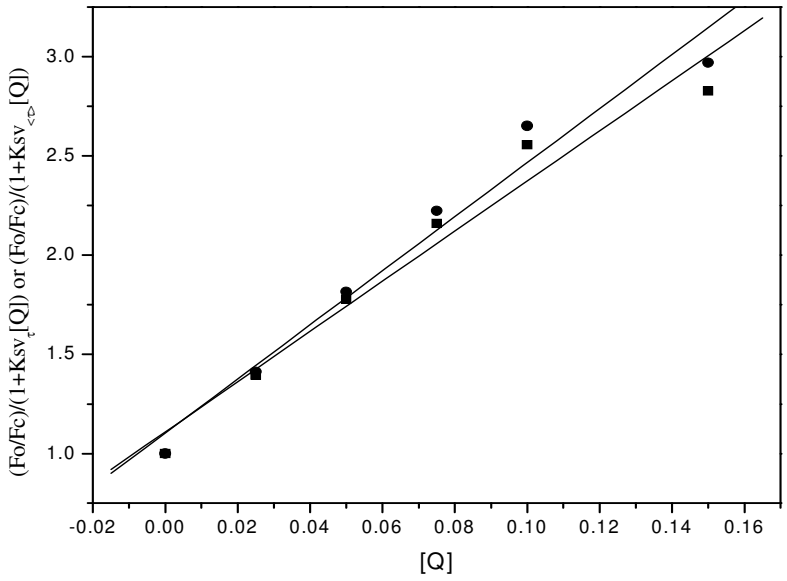


**Figure. 4.8:** Plot of (Fo/Fc)/(1+Ksv$_\tau$ [Q]) and (Fo/Fc)/(1+Ksv$_\tau$ [Q]) vs [Q], where Ksv$_\tau$ and Ksv$_{<\tau>}$ are Stern-Volmer constant for dynamic quenching and [Q] is concentration of the quencher. Slope of this plot gives static quenching constant Ks$_\tau$ and Ks$_{<\tau>}$. Symbols used are (■) for (Fo/Fc)/(1+Ksv$_\tau$ [Q]) and (●) (Fo/Fc)/(1+Ksv$_\tau$ [Q]).

Bimolecular quenching constants Kq$_\tau$ (Kq = Ks$_\tau$ / $\tau$) and Kq$_{<\tau>}$ (Kq$_{<\tau>}$ = Ksv$_{<\tau>}$ / $\tau$ ) obtained from the two calculated average lifetimes were 1.18 x $10^9$ $M^{-1}s^{-1}$ ($\tau$) and 0.95 x $10^9$ $M^{-1}s^{-1}$ (Table 4.7). Kq can measure the degree of exposure of tryptophan residue, which is the frequency at which probing

molecule encounters a fluorophore. High values of Kq indicate that frequency with which acrylamide collides with tryptophan is significantly high. Acrylamide is generally known to quench via a collisional mechanism (30) and does not normally interact with proteins, although at high concentrations weak interaction with some proteins has been shown (31).

Static portion of quenching was calculated by equation (8) by plot of $(Fo/Fc)/(1+Ksv_{\tau}[Q])$ and $(Fo/Fc)/(1+Ksv_{<\tau>}[Q])$ vs [Q], where $Ksv_{\tau}$ (obtained from plot of $\tau_0/\tau$ vs [Q]) and $Ksv_{<\tau>}$ (obtained from plot of $\tau_0/\tau$ vs [Q]) are Stern-Volmer constants for dynamic quenching obtained from two calculated average lifetimes and [Q] is concentration of the quencher (Fig. 4.8). Value of $Ksv_{\tau}$ and $Ksv_{<\tau>}$ were incorporated in equation (9) and by fitting static quenching constants $Ks_{\tau}$ (13.61 $M^{-1}$) and $Ks_{<\tau>}$ (12.61 $M^{-1}$) were obtained. Relatively high values of Ks showed that only a little fraction of excited state is quenched by collisional mechanism (Dynamic quenching). Some of the excited states are deactivated almost instantaneously after being formed because a quencher molecule is randomly positioned in their proximity at the time they are excited.

In conclusion, the major forces for the binding of different saccharides to *G. lucidum* lectin are hydrogen bonding and van der Waals interactions. The lectin does not discriminate between $\beta1\rightarrow3$ and $\beta1\rightarrow4$ linked disaccharides. Lectin shows high specificity for multiantennary glycans. The higher affinity accompanied by a large enthalpic contribution observed with complex N-glycans suggested the importance of multivalency in binding. The single tryptophan residue per monomer of the lectin (hexamer) of the lectin is relatively exposed and accessible to neutral as well as ionic quenchers and is present in vicinity of positively charged amino acids. Time resolved fluorescence studies showed that tryptophan lifetime show two components one with the higher contribituon and one with the lower contribution. Presence of ligands increase the shorter lifetime and decrease the longer lifetime. Lifetime fluorescence decay in the presence of acrylamide showed decrease in

shorter as well longer lifetime and both the calculated average lifetimes. Acrylamide quenching shows presence of static as well as dynamic components.

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*Chapter: 5*

# Glycan Binding to the *Ganoderma Lucidum* Lectin: Surface Plasmon Resonance Studies

## SUMMARY

The thermodynamics and kinetics of binding of glycans and glycoproteins to *Ganoderma lucidum* lectin was studied using surface plasmon resonance. The lectin showed highest affinity for asialo triantennary N glycan (Ka = 3.52 x $10^5$) among the glycans tested. There was several fold increase in affinity for glycoproteins compared to their corresponding glycans and coincident increase in contribution from enthalpy (ΔH), suggesting the involvement of hydrogen bonding in the interaction as well as involvement of protein-protein interactions. Increased affinity also showed increase in unfavorable negative binding entropy (ΔS) which was compensated with higher enthalpy. The glycoproteins showed faster association rates ($k_1$) and the activation energy ($E^{\ddagger}_1$) in the association process was much lower for the glycoproteins than glycans, resulting in their faster associations. These observations elaborate the role of protein matrix in lectin-glycoconjugate interaction.

## INTRODUCTION

Lectins are known as molecules possessing the capability to recognize different mono-, oligo- or polysaccharide structures. Lectins have been isolated from various organisms such as- animals, plants, bacteria, viruses and fungi; though lectins from plant and animal sources have been extensively studied (1,2), very little information is available on lectins from fungal sources (3,4). In last few years mushroom-lectins have attracted increased attention due to their antitumor, antiproliferative and immunomodulatory activities (5-7). Protein-carbohydrate interactions underlie many aspects of cellular recognition including cell adhesion, growth and morphogenesis, molecular recognition and pathogenesis etc (3,8). These specific interactions occur through glycoprotein, glycolipid, and polysaccharide displays found on cell surfaces. Lectins typically possess shallow binding pockets that are solvent-exposed; therefore, the interaction between the carbohydrate and the protein is typically weak

(9,10). To provide interaction strength and specificity, many carbohydrate-binding proteins are oligomeric, consisting of several similar or identical monomers that each bind to a carbohydrate (8). It is possible for the lectins to participate in multivalent binding, or the formation of several simultaneous binding events that provide an apparent binding affinity (functional affinity) that is greater than the sum of the individual interactions (11). Many aspects of the interactions between lectins and glycoproteins have been studied, especially regarding the recognition of molecules. These interactions have been used for the detection of the oligosaccharides of glycoproteins. The interaction of lectins and carbohydrates and their kinetics can be monitored in real time by surface plasmon resonance. We have purified and characterized a 114 kDa lectin from *Ganoderma lucidum* and carbohydrate specificity of the lectin has been studied by hemagglutination inhibition assay and fluorimetry in previous chapters. In this chapter, we describe the detailed binding kinetics of *G. lucidum* lectin with various glycans and glycoprotein by surface plasmon resonance (SPR).

## MATERIALS

Fetuin, fibrinogen, (Sigma Chemical Co. St. Louis, U.S.A); Galβ1→3GalNAcα1→*O*Ser (Dextra Labs, London, UK); Certified grade CM5 sensor chip and amine coupling kit (*N*-ethyl-*N'*-(dimethylaminopropyl-) carbodiimide hydrochloride and *N*-hydroxysuccinamide) (Pharmacia Biosensor AB, Uppsala, Sweden) were used. All other chemicals used were of analytical grade. *G. lucidum* fruiting bodies were obtained from H.P. University Shimla.

### METHODS

#### Protein determination

Protein concentrations were determined according to Bradford (2) using BSA as standard.

**Glycan preparation**

Asialo-triantennary glycans from fetuin and asialo-biantennary glycan from fibrinogen were prepared as described in Chapter 4.

**Purification of *Ganoderma lucidum* lectin**

*Ganoderma lucidum* lectin was purified by 0-30% ammonium sulphate precipitation and hydrophobic chromatography on phenyl Sepharose column as described in chapter 2

The purified lectin was dialysed extensively against deionized water and used for immobilization on the sensor chip.

**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. One hundred µg of *Ganoderma lucidum* lectin in 1 ml of 10 mM sodium acetate buffer, pH 4.0 were coupled to a certified grade CM5 chip at a flow rate of 5 µl/min for 50 min using the amine coupling kit. The unreacted groups, on the surface of the chip, were blocked with ethanolamine. All measurements were done using 10 mM phosphate buffer, pH 6.0, (containing 100 mM NaCl, 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 and glycoprotein solutions (0.1-500 µM) over the chip at a high flow rate 50µl/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.. The dissociation rate constants, on the other hand, were determined in a similar manner by passing plain buffer at a flow rate of 50 µl/min for 300 s. After every cycle, chip was regenerated by treating with 200 mM sodium carbonate, pH 9.5, for 3 min.

**Data Analysis**

Association ($k_1$), dissociation ($k_{-1}$) rate constants and association constants were obtained by nonlinear fitting of the primary sensogram by 1:1 (Langmuir) binding model using the BIAevaluation software version 3.1 with SD less than 5%.

Free energy changes of association ($\Delta G$) were determined by the equation,

$$\Delta G = - \mathrm{RT} \ln K_a \qquad (1)$$

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

$$\ln K_a = (-\Delta H/\mathrm{RT}) + \Delta S/\mathrm{R} \qquad (2)$$

Where $\Delta H$ is enthalpy change, R is gas constant, $\Delta S$ is entropy change and T is the absolute temperature. The entropy change was obtained from the equation,

$$\Delta G = \Delta H - \mathrm{T}\Delta S \qquad (3)$$

Activation enthalpies ($\Delta H^{\ddagger}$), entropies ($\Delta S^{\ddagger}$) and energies ($E_A$) were calculated using the following equations (Arrhenius plots):

$$\Delta H^{\ddagger} = E_A - \mathrm{RT} \qquad (4)$$

$$\ln (k^{\ddagger}/\mathrm{T}) = - \Delta H^{\ddagger}/\mathrm{RT} + \Delta S^{\ddagger}/\mathrm{R} + \ln (k'/h) \qquad (5)$$

$$\Delta G^{\ddagger} = \Delta H^{\ddagger} - \mathrm{T}\Delta S^{\ddagger} \qquad (6)$$

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

## RESULTS AND DISCUSSION

*Ganoderma lucidum* lectin was coupled to CM5 sensor chip corresponding to 3500 response units by amine coupling. Surface Plasmon Resonace studies on *Ganoderma lucidum* lectin showed significant change in RUs with the increasing concentration of glycans (Fig. 5.1) and glycoproteins (Fig. 5.2). Asialo triantennary N glycan showed highest affinity for the lectin (Ka = 4.51 x $10^5$ $M^{-1}$) among the glycans tested. Affinity for asialo triantennary N glycan was approximately 10 fold higher than asialo biantennary N glycan (Ka = 6.15 x $10^4$ $M^{-1}$). Asialo triantennary N glycan and asialo biantennary N glycan showed 63- and 38- fold higher affinity respectively for the lectin than O-linked glycan (Ka = 1.60 x $10^3$ $M^{-1}$) (Table 5.1). Increase in affinity for asialo triantennary N glycan and asialo biantennary N glycan over O-linked glycan was also accompanied with increase in negative enthalpy (ΔH) indicating the increase in hydrogen bonding and van der Waals interactions in the their binding, compared to O-linked glycan (Table 5.2).

Low affinity for O-linked glycan over asialo triantennary N glycan and asialo biantennary N glycan can be explained on the basis of valency since asialo biantennary N glycan is bivalent, asialo triantennary N glycan is trivalent whereas O-linked glycan is a monovalent glycan. Lectin carbohydrate interactions are generally characterized by a low affinity for monovalent ligands, which is balanced by multivalency, which provides high avidity for substrates with several potential ligands available, such as complex glycans or cell surfaces (12). Longer oligosaccharides bind to lectin by extended binding site present on the lectin hence leading to a higher affinity. In general these interactions are typified by a favorable enthalpy (ΔH) change due to high number of hydrogen bonds. These interactions are usually accompanied with an unfavorable entropy (ΔS) contribution, attributed either to solvent rearrangement (13) or to loss of ligand conformational flexibility (14).

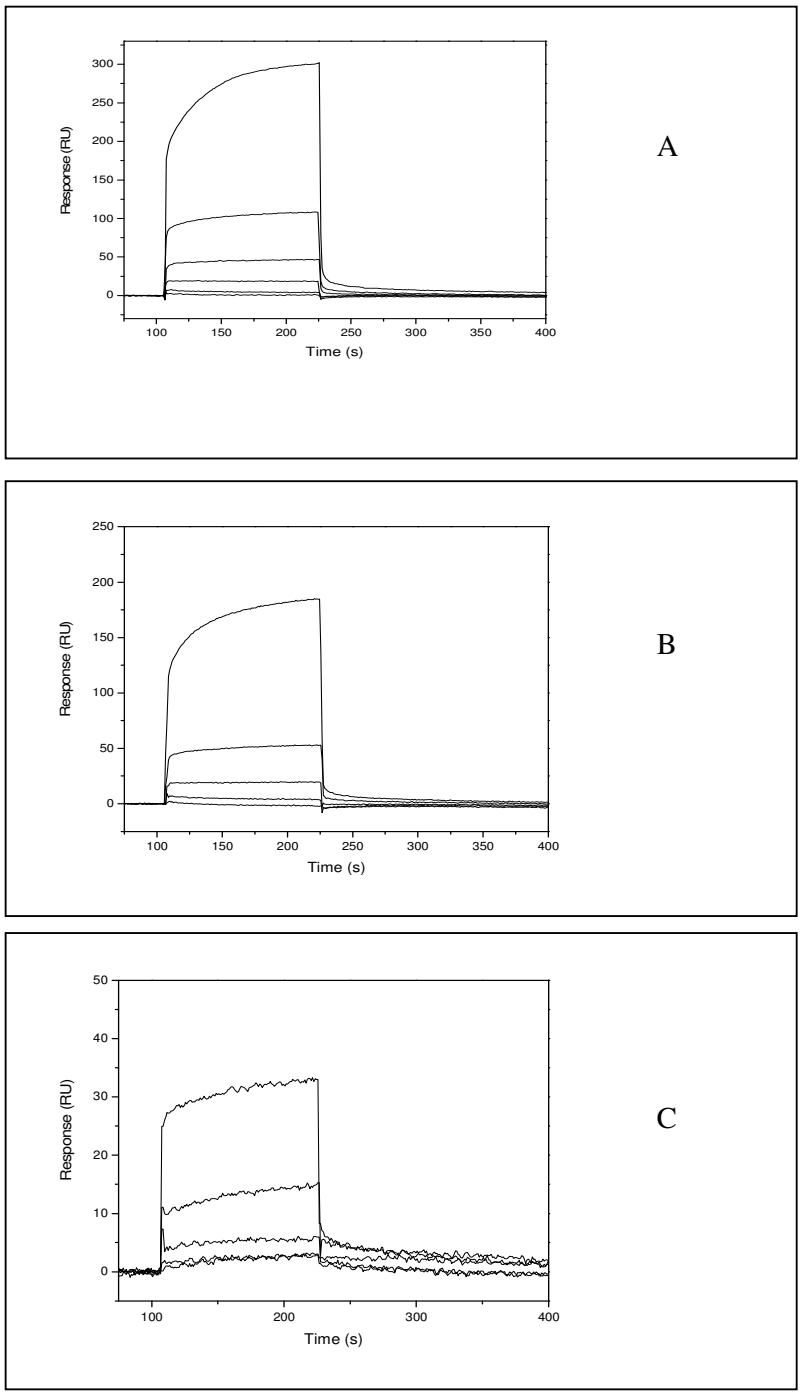


**Figure 5.1: The representative sensograms depicting interactions of increasing amounts (0.62-20.0 µM) of Glycans to the immobilized *G. lucidum* lectin at 25 °C.** (A) Asialo triantennary N- glycan, (B) Asialo biantennary N- glycan and (C) Gal β 1→ GalNAcα 1→O-Ser.

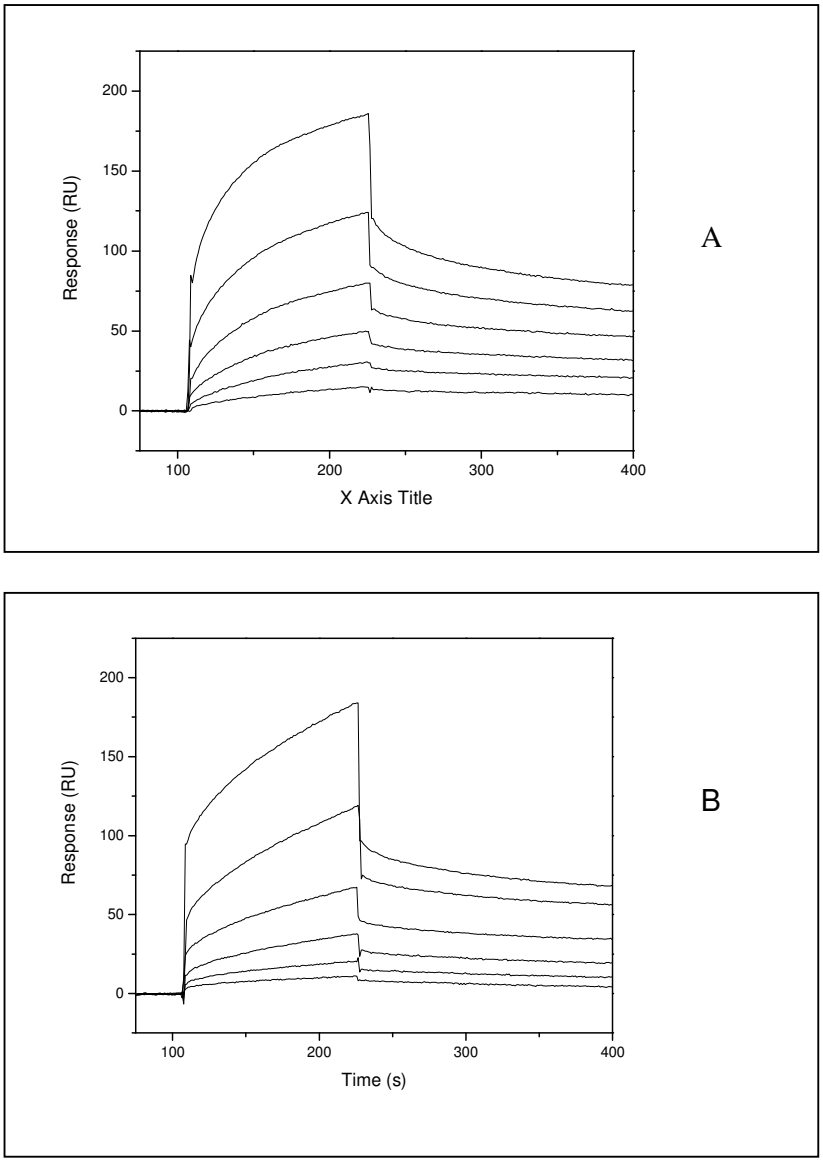


**Figure 5.2: The representative sensograms depicting interactions of increasing amounts of asialo triantennary N glycan (3.25-100 µM) to the immobilized *G. lucidum* lectin at 25 °C.** (A) Fetuin and (B) Fibrinogen

**Table 5.1: Association constants for the binding of different glycans and glycoproteins to immobilized *Ganoderma lucidum* lectin at different temperatures:** Determined by SPR (Kinetic analysis)

| **Glycans** | **Ka ($M^{-1}$)** | | |
|---|---|---|---|
| | 20 °C | 25 °C | 30 °C |
| Asialo-triantennary N glycan | $5.62 \times 10^5$ | $3.52 \times 10^5$ | $2.24 \times 10^5$ |
| Asialo-biantennary N –glycan | $8.91 \times 10^4$ | $6.15 \times 10^4$ | $4.24 \times 10^4$ |
| O-linked glycan | $1.99 \times 10^3$ | $1.60 \times 10^3$ | $1.22 \times 10^3$ |
| Fetuin | $1.74 \times 10^7$ | $9.34 \times 10^6$ | $6.12 \times 10^6$ |
| Fibrinogen | $8.61 \times 10^6$ | $5.20 \times 10^6$ | $3.11 \times 10^6$ |

Increase in affinity has been observed with the increase in valency of glycans in case of many other lectins for e.g. *Agaricus bisporus* lectin showed lower affinity for Gal β 1-4 GlcNAc (LacNAc) and Gal β 1-3 GalNAc than their multivalent counterparts (15), *Fusarium solani* lectin which showed higher affinity for asialo bi- and tri- antennary N glycan than O-linked glycan (16) and *Allium sativum* lectin which showed higher affinity with increase in valency, where substitution of α-1,6 branch of Man-5 by two α-1,2-linked mannosyl residues resulted in higher affinity for the latter (17).

**Table 5.2: Thermodynamic parameters for the binding of different glycans and glycoproteins to immobilized *Ganoderma lucidum* lectin at different temperatures:** Determined by SPR (Kinetic analysis)

| $-\Delta H$ ($kJ \cdot mol^{-1}$) | $-\Delta G^*$ ($kJ \cdot mol^{-1}$) 25 °C | $-\Delta S$ ($J \cdot mol^{-1} \cdot K^{-1}$) 25 °C |
|---|---|---|
| -67.89 | -32.25 | -121.65 |
| -54.81 | -27.76 | -92.31 |
| -36.11 | -18.50 | -60.10 |
| -77.12 | -40.61 | -124.61 |
| -75.16 | -38.89 | -123.76 |

* Determined at 25 °C

*G. lucidum* Lectin showed higher affinity for glycoproteins than glycans (Table 1). It showed approximately 2 fold higher affinity for fetuin (9.34 x $10^6$ $M^{-1}$) than fibrinogen (5.20 x $10^6$ $M^{-1}$). The difference in binding affinity of fetuin and fibrinogen is not very large although fetuin possesses three trivalent N-linked and three O-linked glycan whereas fibrinogen has only one bivalent N-linked glycan, which could be due to increased protein - protein interactions involved between *G. lucidum* lectin and fibrinogen. Higher affinity for glycoproteins than glycans was also manifested by subsequent increase in enthalpy (ΔH) indicating higher hydrogen bonding involved in lectin-glycoprotein interactions than lectin-glycan interactions (Table 1).

Thermodynamic parameters viz. free energy (ΔG), enthalpy (ΔH) and entropy (ΔS) of binding determined by Ka at different temperatures are given in Table 1. The Van't Hoff plots (Figure 3) were linear (r>0.9) for all the glycans and glycoproteins in the temperature range studied (Figure 2). A decrease in association constants was observed with an increase in temperature indicating negative enthalpy (ΔH) change. This suggested that the binding is exothermic in nature and driven by enthalpy.

Low affinity for O-glycan than N-glycans was also manifested by lower enthalpy (ΔH = -36.11 KJ $M^{-1}$) probably caused by loss of some favorable polar interactions.

The higher affinity was accompanied by a large and negative change in free energy and enthalpy. However there was increase in unfavorable entropy with the increased affinity which was compensated by increased favorable enthalpic contribution. In this study, thermodynamic studies provide an insight into the role of protein-protein interaction in the lectin – ligand interactions. All the glycans tested showed lower negative binding enthalpy than glycoproteins which indicated significant contribution of enthalpy in free energy change during the binding of these glycoproteins to the *G. lucidum* lectin, ultimately leading to their higher affinity. Lectin - sugar interactions can be visualized by enthalpy (ΔH)/entropy (ΔS) compensation where longer oligosaccharides bind with more hydrogen bonding, giving rise to a more favorable binding enthalpy, but lose their flexibility in the process, generating a high entropy barrier (12). This process probably explains the basis for low affinity range (milli/micro-molar) of lectin- monosaccharide interactions.

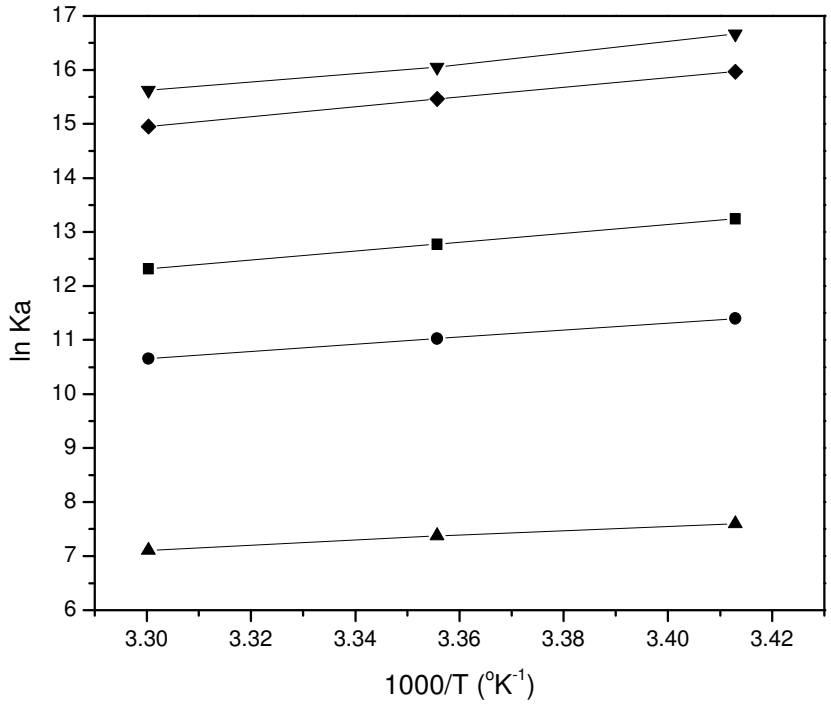


**Figure 5.3: Van't Hoff plots for association of various sugar glycans and glycoproteins to *G. lucidum* lectin are drawn to regression equation.** The symbols used are asialo triantennary N glycan (■), asialo biantennary N glycan (●), O-linked glycan (▲),fetuin (▼) and fibrinogen (♦).

***Kinetic analysis***

Asialo triantennary N glycan ($k_1$ = 60.5 x $10^3$ $M^{-1}s^{-1}$) and asialo biantennary N glycan ($k_1$ = 23.0 x $10^3$ $M^{-1}s^{-1}$) showed much faster association rates than O-linked glycan whereas dissociation rate of asialo triantennary N glycan ($k_{-1}$ = 172 x $10^{-3}$ $s^{-1}$) was lower than asialo biantennary N glycan ($k_{-1}$ = 374 x $10^{-3}$ $s^{-1}$) and O-linked glycan ($k_{-1}$ = 277 x $10^{-3}$ $s^{-1}$). Difference in binding affinity of the *G. lucidum* lectin to the different glycans was determined by their association rates. The association and dissociation rate constants determined at different temperatures are given in Table 5.3 and 5.4. The Arrhenius plots for all the glycans and glycoproteins were linear ($r>0.9$) in the temperature range studied. Glycoproteins showed significantly higher association and slower dissociation rate constants than glycans. Fetuin ($k_{-1}$ = 3.83 x $10^{-3}$ $s^{-1}$) and fibrinogen ($k_{-1}$ = 3.29 x $10^{-3}$ $s^{-1}$) showed similar dissociation rates and difference in the association rates resulted in higher affinity of the *G. lucidum* lectin for fetuin than fibrinogen.

**Table 5.3: Rate constants and activation parameters for the association process of glycans and glycoproteins to lectin from *Ganoderma lucidum***

| Ligand | Association | | | | |
|---|---|---|---|---|---|
| | $k_1 \times 10^{-3}$ $M^{-1} s^{-1}$ | $E^{\ddagger}_1$ kJ $mol^{-1}$ | $\Delta H^{\ddagger}_1$ kJ $mol^{-1}$ | $\Delta G^{\ddagger}_1$ kJ $mol^{-1}$ | $\Delta S^{\ddagger}_1$ $Jmol^{-1}K^{-1}$ |
| Asialo-triantennary N-glycan | 60.5 | 8.20 | 5.72 | 45.02 | -134.12 |
| Asialo-biantennary N-glycan | 23.0 | 15.96 | 13.48 | 47.53 | -116.20 |
| O- linked Glycan | 0.44 | 34.93 | 32.45 | 57.48 | -85.41 |
| Fetuin | 358 | 7.12 | 4.64 | 46.26 | -142 |
| Fibrinogen | 171 | 11.31 | 8.83 | 48.14 | -134 |

**Table 5.4: Rate constants and activation parameters for the dissociation process of glycans and glycoproteins to lectin from *Ganoderma lucidum.***

| Ligand | Dissociation | | | | |
|---|---|---|---|---|---|
| | $k_{-1} \times 10^3$ $s^{-1}$ | $E^{\ddagger}_{-1}$ kJ $mol^{-1}$ | $\Delta H^{\ddagger}_{-1}$ kJ $mol^{-1}$ | $\Delta G^{\ddagger}_{-1}$ $Jmol^{-1}K^{-1}$ | $\Delta S^{\ddagger}_{-1}$ $Jmol^{-1}K^{-1}$ |
| Asialo-triantennary N-glycan | 172 | 76.10 | 73.62 | 77.27 | -12.46 |
| Asialo-biantennary N-glycan | 374 | 70.77 | 68.29 | 75.29 | -23.88 |
| O- linked Glycan | 277 | 71.05 | 68.57 | 75.98 | -25.30 |
| Fetuin | 3.83 | 84.25 | 81.77 | 86.87 | -17.41 |
| Fibrinogen | 3.29 | 86.47 | 83.99 | 87.04 | -10.39 |

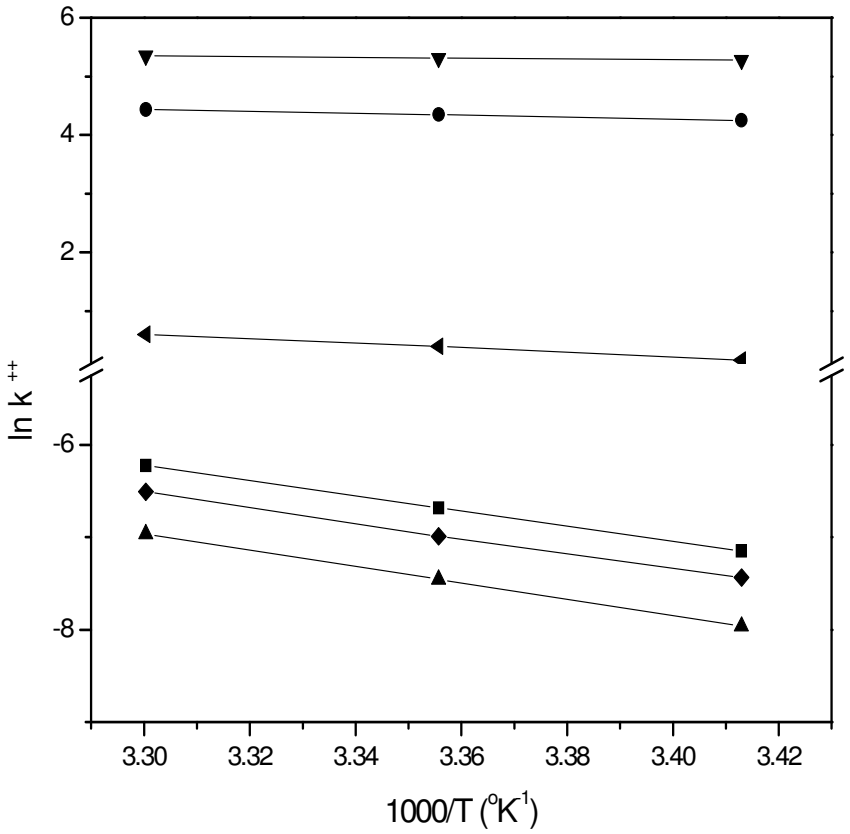


**Figure 5.4: Arrhenius plots for the association and dissociation kinetics of various sugar glycans to *G. lucidum* lectin.** The symbols used are association of asialo triantennary N glycan (▼), asialo biantennary N glycan (●) and O-linked glycan (◄), dissociation of asialo triantennary N glycan (▲), asialo biantennary N glycan (■) and O-linked glycan (♦).

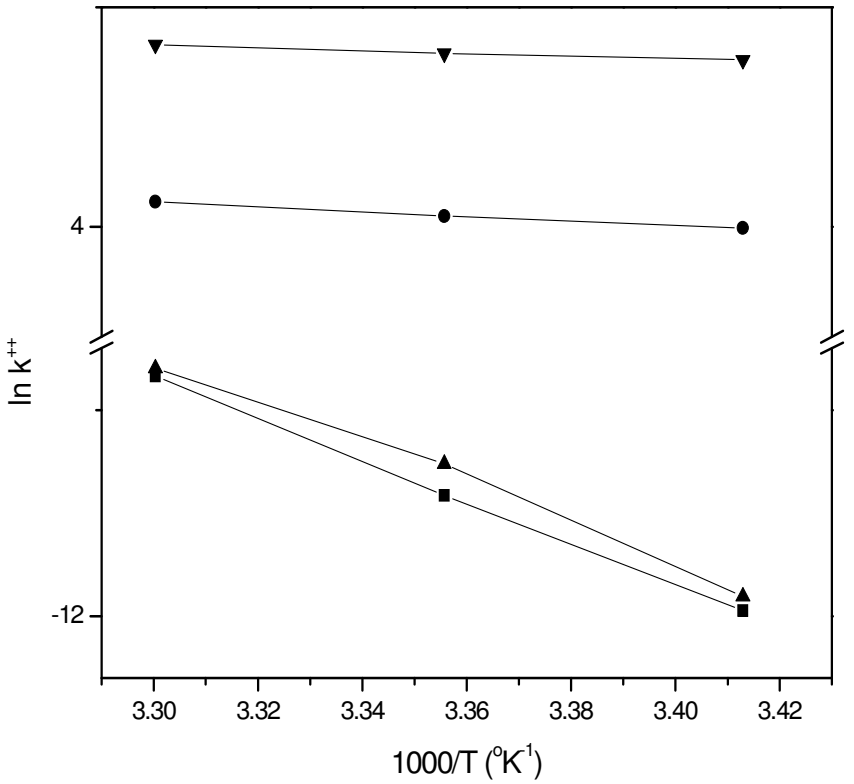


**Figure 5.5: Arrhenius plots for the association and dissociation kinetics of glycoproteins to *G. lucidum* lectin.** The symbols used are association of fetuin (▼), fibrinogen (●), dissociation of fetuin (▲), fibrinogen (■).

The association rate constants (Table 5.3) for the ligand binding to *G. lucidum* lectin follow the trend observed for lectins from *Ulex europeas* (18), *Ricinus communis* (19), soybean (20) and *Fusarium solani* (21). They are of several orders of magnitude slower than diffusion controlled reactions. 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 equilibrium analysis indicates 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. Linearity of Arrhenius plots (Figure 5.4 and 5.5), however, also rules out to a great extent, the formation of such an intermediate(s) and the occurrence of dramatic conformational changes in the lectin molecule at least in the temperature range studied. So the kinetics of ligand binding to *G. lucidum* lectin can be summarized with a single-step binding mechanism, and can be depicted by the equation:

$$P + L \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} PL \quad ; \quad K_a=k_1/k_{-1}$$

Most of the lectins studied so far follow single-step binding mechanism and stoichiometry kinetics can be fitted with 1:1 binding.

Asialo triantennary N glycan binds 3 times faster than asialo biantennary N glycan and the activation energy ($E^{\ddagger}_1$) for the association process of asialo triantennary N glycan is slower than asialo biantennary N glycan indicating that association of asialo triantennary N glycan to the *G. lucidum* lectin is energetically favorable as less amount of energy is required to for its association to the lectin. The entropy ($\Delta S^{\ddagger}_1$) of activation is more negative for the association process with asialo triantennary N glycan than asialo biantennary N glycan which indicates that association of the former to the lectin is more ordered than latter. This suggests that asialo biantennary N glycan can approach the lectin in more ways than asialo

triantennary N glycan but higher activation energy ($E^{\ddagger}_1$) required for its association to the lectin results in its lower association rate. Similarly low association rate of O-linked glycan ($k_1 = 0.44 \times 10^3\ M^{-1}s^{-1}$) to the lectin also requires much more activation energy ($E^{\ddagger}_1$) than asialo tri- and bi-antennary N glycans resulting in lower association rate.

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 ($k_1$) (22). In the present studies, higher association rate constant ($k^1$) was observed for binding of asialo-triantennary glycan to the lectin than asialo-biantennary. Moreover, the slower association rate constant and lower activation energy ($E^{\ddagger}_1$) of association process appears to be main factors responsible for lower affinity of asialo-biantennary glycan than asialo-triantennary in case of *G. lucidum* lectin.

Similar dissociation rate constants were observed with fetuin ($k_{-1} = 3.83 \times 10^{-3}\ s^{-1}$) and fibrinogen ($k_{-1} = 3.29 \times 10^{-3}\ s^{-1}$) despite differences in association constants, suggesting that the different association rates are solely responsible for the differences in the affinity of these glycoproteins. However, the lower affinity of glycans than glycoproteins is due to their faster association as well as slower dissociation. The higher activation energy ($E^{\ddagger}_1$) required for the dissociation of glycoproteins result in their slower dissociation than glycans. Association of various glycans and glycoproteins to *G. lucidum* lectin showed highly ordered transition state indicated by large negative entropy values (Table 2). This lectin showed similarity with peanut agglutinin (23), where a highly ordered transition state has been implicated in binding reaction ($\Delta S^{\ddagger}_1 = -160\ J \cdot mol^{-1} \cdot K^{-1}$).

In conclusion, the present studies show that the higher affinity of *G. lucidum* lectin for glycoproteins is accompanied by enthalpic contribution and highly negative unfavorable entropy ($\Delta S$) can be compensated by favorable enthalpy ($\Delta H$). In addition higher association and lower

dissociation rates as well as low activation energy ($E^{\ddagger}_1$) of association process contribute to its higher affinity for glycoprotein compared to glycans and association of various ligands to lectin show a highly ordered transition state. As carbohydrate specificity underlies all the functions mediated by lectin, hence our study can add valuable insight in understanding the structure-function relationship of lectins from mushrooms.

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*Chapter: 6*

# General Discussion and Conclusion

## DISCUSSION

Lectins have been isolated from various organisms such as- animals, plants, bacteria, viruses and fungi; though lectins from plant and animal sources have been extensively studied (1,2), comparatively less information is available on lectins from fungal sources (3,4). In last few years mushroom-lectins have attracted increased attention due to their antitumor, antiproliferative and immunomodulatory activities (5-7). Lectins from several pathogenic fungi have also been studied in great detail but their physiological role remains uncertain (8-12).

The role of lectins in fungi seems to be more diverse than that in plants. In higher fungi, lectins probably play differential roles in different circumstances. Various roles have been postulated for fungal lectins which do not appear to be mutually exclusive - some of these seem to concern fungal metabolism itself, while other activities are thought to be involved in symbiotic or parasitic relationships with other organisms (3).

Lectins from mushrooms viz *Agaricus bisporus, Boletus satanus, Flammulina velutipes, Ganoderma lucidum, Grifola frondosa, Tricholoma mongolicum, and Volvariella volvacea* show immunomodulatory and/or antitumor/ cytotoxic activities (4). *Ganoderma lucidum* is a well-known medicinal mushroom and many health-promoting and therapeutic effects have been attributed to this mushroom. An immunomodulatory protein with a molecular mass of 12.4 kDa, designated LZ-8, has already been studied from *G. lucidum* mycelia. Its amino acid sequence was homologous to that of a known immunomodulatory protein from the straw mushroom and it also showed hemagglutinating and mitogenic activities (13) . An 18 kDa lectin (GLL-M) was isolated from *G. lucidum* mycelia and another 16 kDa lectin (GLL-F) was purified from *G. lucidum* fruiting bodies (14), both of which were distinct from LZ-8 as judged from the molecular mass and sugar binding activity.

In the present work a lectin has been isolated from the fruiting bodies of *Ganoderma lucidum*. The lectin was purified to homogeneity by conventional procedures involving 0-30% ammonium sulphate precipitation followed by hydrophobic chromatography on phenyl sepharose. Mushroom

lectins have been purified by conventional procedures as well as by affinity chromatography (3,4). Sometimes when binding is too strong, it is difficult to elute the lectins from affinity columns, hence, we tried conventional methods. We were able to purify *G. lucidum* lectin with a simple procedure.

*Ganoderma lucicum* lectin was found to be a homohexamer of 114 kDa with subunit molecular mass of 18.6 KDa. Mushroom lectins generally exhibit variable molecular masses (16-190 kDa), could have more than one subunits which may or may not be identical. Except for few lectins as from *Lactarius lignyotus* and *Phallus impudicus* the subunits of which are linked by disulphide bridges, the other di-, tetra-, or polymeric lectins studied, namely those of *Agaricus edulis, Agaricus campestris, aleuria aurantia, Flammulina velutipes, Hericium erinaceus, Lactarius deliciosus, L. deterrimus, L. salmonicolor, Pholiota aurivella, Pleurotus cornucopiae* and *Xerocomus chrysenteron* have subunits held together by non- covalent bonds (3).

Two other lectins have been reported from *Ganoderma lucidum*, GLL-M, an 18 kDa lectin from mycelia and GLL-F, a 16 kDa lectin from the fruiting body (15). GLL-F and GLL-M exist as monomers in native state in contrast to 114 kDa lectin we have isolated which exists as hexamer in its native state. This lectin is also different from LZ-8, an immunomodulatory protein, isolated from *Ganoderma lucidum* which is a homodimer of 24 kDa (16).

*G. lucidum* lectin is a glycoprotein containing 9.3% neutral sugar. Lectins from *Rigidoporus lignosus, Fomes fomentarius, Agaricus edulis, Phallus impudicus Psilocybe berrerae* contain 30%, 25%, 18%, 13.9% and 9.5% neutral sugar respectively (3,17-19). But the lectins from *Aleuria aurantia*, *Lactarius deterrimus*, *Laccaria amethystea*, *Laetiporus sulphureus* and *Pleurotus cornucopiae* are not glycosylated (20-25).

*G. lucidum* lectin is active between pH 5-9, and temperature up to 50 °C. Most of the mushroom lectins are thermostable as from *Agaricus edulis*, *A. blazei*, *A. campestris*, *Tricholoma mongolicum*, *Volvariella volvacea Polyporus adusta* and *Ganoderma capense* which are stable above 60 °C (17,26-31). Lectins from *A. campestris*, *Hericium erinaceum* and *Ganoderma*

*capense* are stable in the pH range of 4-10, 5-10 and 4-11 respectively (27,31,32).

*Ganoderma lucidum* lectin does not agglutinate untreated and trypsinized human ABO type erythrocytes but agglutinates pronase treated ABO erythrocytes. Lectins, GLL-M and GLL-F, reported from *Ganoderma lucidum* also agglutinates only pronase treated erythrocytes (15). However, most of the mushroom lectins show hemagglutination activity with untreated human ABO type erythrocytes. Many mushroom lectins show blood group specificity and lectins binding to all the blood group types of ABO RBCs have also been isolated. Blood group specificity of some mushroom lectins is shown in table 6.1 (3).

*G. lucidum* is a basic protein with a pI of 9.7. The mushroom lectins show very high diversity in the pI, ranging from a minimum pH 4.3 for *Clitocybe nebularis* (33) to maximum upto pH 10.65 for *Auricularia polytricha* (34).

The lectin did not require divalent cations for its activity Most of the mushroom lectins do not require metal ions for their activity. The activity of *Lactarius lignyotus* and *Xerocomus chrysenteron* lectins increase in the presence of $Ca^{++}$ ions. *Xerocomus chrysenteron* lectin also shows increase in activity in the presence of $Mn^{++}$ ions (35). *Polyporus adusta* lectin showed two fold increase in titre value in presence of 10 mm $FeCl_3$ (30).

The amino acid composition of *G. lucidum* lectin showed that it contained a large amount of Gly (11.7%), Ala (10.0%), AsX (8.8%), a moderate amount of Thr (7.6%), GlX (7.1%), Ser (7.6%), Leu (6.4%), Tyr (6%), Phe (5.9%), Lys (5.9%), Arg (4.7%), and low amount of Pro (2.4%), Val (2.4%), Ile (3.1%), His (2.4%), Cys (1.77%), Trp (0.6%). GLL-M isolated from mycelia of *Ganoderma lucidum* also have a higher amount of Gly, Ala, AsX, Thr, GlX, Ser and Leu but remarkable differences can be observed in composition of other amino acids (14).

**Table 6.1: Specificity of mushroom lectins towards human red blood erythrocytes*.**

| | |
|---|---|
| **Anti-O (H)** | *Clathrus cancellatus* |
| | *Phallus impudicus* |
| | *Pholiota squarrossa* |
| | *Pleurotus ostreatus* |
| **Anti-A** | *Agrocybe aegerita* |
| | *Coprinus domesticus* |
| | *Entoloma sinuatom* |
| | *Tricholoma pessundatum* |
| **Anti-B** | *Fomes fomentarius* |
| | *Lentinus squarrosulus* |
| | *Lepista nuda* |
| | *Marasmius oreades* |
| **Anti A + B** | *Cortinarius anomalus* |
| | *Hygrophorus hypothejus* |
| | *Psilocybe spadicea* |
| | *Tricholoma albobrunneum* |
| **Anti- B + H** | *Cortinarius brunneus* |
| | *Cortinarius caerulescens* |
| | *Lepiota gracilenta* |
| | *Marasmius hariolorum* |

* This table has been adapted from Guillot and Konska, 1997 (3)

N-terminal sequence (first twenty residues) of the lectin was QFIYNGKFNWLNYALNETIT which did not show similarity to any known lectin. Mushroom lectins generally show high diversity in the N-terminal sequence, followed by some conserved sequences.

Hemagglutinating activity of *G. lucidum lectin* was not inhibited by any of the simple mono- and di- saccharides but only glycoproteins and plant polysaccharide Gum karaya. The desialated forms of the glycoproteins are more efficient inhibitors than the sialated counterpart. Most of the mushroom lectins show well defined simple sugar specificity. Very few lectins from mushrooms have been reported with hemagglutination activity unaffected by simple sugars and inhibited only by glycoproteins as from *Volvariella volvacea* (5,36) and *Mycoleptodonoids aitchisonii* (37).Other mushroom lectin with hemagglutination activity unaffected by either simple sugars or glycoproteins is from *Lyophyllum shimeiji* (38).

Active site characterization studies of *G. lucidum* lectin have shown involvement of two lysine and one tryptophan residues per monomer in sugar binding site. There are very few reports on the active site characterization of mushroom lectins. Tryptophan has been reported to be involved in the activity of lectin from mushroom *Hericium erinaceum* (39) and lectin from *Ischnoderma resinosum* (40) shows involvement of tyrosine residue in its sugar binding activity.

The sugar binding property of the *G. lucidum* lectin was explored in details using spectrofluorimetry and surface plasmon resonance. Agreement between the data obtained by these two techniques substantiate the monophasic nature of binding. *G. lucidum* lectin was found to interact with Gal/GalNAc residues present in glycopeptides. Wu *et al.* have (41) have 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)s

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 this classification the *G. lucidum* lectin belongs to class T as well as Class I/II, and binds to these structures only in multiantennary form. Many mushroom lectins bind to class T and I/II in their disaccharide form as from *Agaricus bisporus*, *Lactarius deterrimus*, *L. deliciosus* and *Laetiporus sulphureus* (24,25,42,43). The lectin from mushroom *Marasmius oreades* shows specificity for class B structures .

In the SPR studies, *G. lucidum* lectin showed several fold higher affinity for the glycoproteins ($K_a \approx 10^6$ $M^{-1}$) than glycans ($K_a \approx 10^4$-$10^5$ $M^{-1}$). Increase in affinity for glycoproteins than their corresponding glycans showed involvement of protein-protein interactions in the binding of lectin to glycoproteins. SPR study on the lectin from mushroom *Psathyrella velutina* showed Ka in the range of $10^7$-$10^8$ $M^{-1}$ with fetuin and asialo-agalactofetuin (44) and mushroom *Pleurotus ostreatus* lectin showed Ka in the range of $10^7$-$10^9$ $M^{-1}$ with asialo-fetuin and asialo-BSM (45). The interaction between *Psathyrella velutina* lectin and fetuin or transferrin implicated the involvement of terminal sialic acid residues in the lectin binding, although it also exhibits higher affinity for asialo-agalactofetuin than fetuin (44).

*G. lucidum* lectin showed increased affinity with the increase in valency of glycans. Which has also been observed in case of *Agaricus bisporus* lectin showing lower affinity for Gal β 1-4 GlcNAc (LacNAc) and Gal β 1-3 GalNAc than their multivalent counterparts (46)

The kinetics of binding of glycans and glycoproteins to *G. lucidum* lectin followed by SPR reveals that the binding process follows a bimolecular one step mechanism. The association rate constants are much slower than that for diffusion controlled reaction. High affinity of lectins for glycoproteins is accompanied by their faster association rates ($k_1 \sim 10^4$ $M^{-1}\cdot s^{-1}$) and slower

dissociation rates ($k_{-1}$ ~ $10^{-3}$ $s^{-1}$). Association and dissociation rates of glycoprotein binding to *G. lucidum* lectin were similar to lectins from mushrooms *Chlorophyllum molybdites* and *Psathyrella velutina* (44,47).

Lectins are a family of proteins of unknown function and the challenge of identifying the physiological function for these proteins still remains. In last few years mushroom lectins have attracted increased attention due to their antitumor, antiproliferative and immunomodulatory activities (5-7). Protein-carbohydrate interactions underlie many aspects of cellular recognition including cell adhesion, growth and morphogenesis, molecular recognition and pathogenesis etc (3,48). To understand various biological functions performed by lectins it is necessary to understand its molecular characters and detailed carbohydrate binding specificity. Biochemical approaches have resulted in 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 mushroom lectin from *Ganoderma lucidum* with different glycans and glycoproteins would be helpful in understanding the structure - function relationship of lectins from mushrooms and it might be helpful in understanding the biological role of lectin in *Ganoderma lucidum*.

**CONCLUSIONS**

1. A lectin was isolated from fruiting bodies of mushroom *Ganoderma lucidum.*
2. The lectin is a homohexamer, glycoprotein with a molecular mass 114 kDa and pI 9.63.
3. The lectin is stable up to 50 °C and is active between the pH range of 5-9.
4. Active site characterization studies showed the involvement of two lysine and one tryptophan residues per monomer in sugar binding.
5. Hemagglutination activity of the G. lucidum lectin is inhibited only by glycoproteins and plant polysaccharide gum karaya.
6. The lectin exhibits specificity for complex glycans and glycoproteins containing Galβ1→3GalNAc or Galβ1→4GlcNAc structures.
7. The interaction of the lectin with complex glycans is enthalpically driven

**List of publications:**

1. Purification and characterization of lectin from fruiting body of *Ganoderma lucidum*.
   Atul Thakur, Monika Rana, Lakhan Pal, Absar Ahmad, M. I. Khan (2007) Biochim. Biophys. Acta, **1770**, 1404-1412
2. Complex carbohydrate specificity of lectin from fruiting body of *Ganoderma lucidum*. A surface plasmon resonance study.
   Atul Thakur, Lakhan Pal, Absar Ahmad, M. I. Khan (Communicated).
2. Fluorescence quenching and time resolved fluorescence studies on lectin from fruiting body of *Ganoderma lucidum.*
   Atul Thakur, Lakhan Pal, Absar Ahmad, M. I. Khan
   (Communicated).

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