Purification and characterization of a galactose specific seed and vegetative lectin from a legume plant: *Dolichos lablab*

Thesis submitted for the Degree of

Doctor of Philosophy

By

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Enrollment No: 03LBPH16 **February 2008**

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CERTIFICATE

This is to certify that this thesis entitled "**Purification and characterization of a galactose specific seed and vegetative lectin from a legume plant: *Dolichos lablab"*** submitted to the University of Hyderabad, Hyderabad by **Mr. R. NAGENDER RAO** for the degree of Doctor of Philosophy, is based on the studies carried out by him under my supervision. I declare to the best of my knowledge that this work has not been submitted earlier for the award of degree or diploma from any other University or Institution.

Dr. N. Siva Kumar
Supervisor

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DECLARATION

I hereby declare that the work presented in my thesis is entirely original and was carried out by me in the Department of Biochemistry, University of Hyderabad, Hyderabad, under the supervision of **Dr. N. Siva Kumar**. I further declare that this work has not been submitted earlier for the award of degree or diploma from any other University or Institution.

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CONTENTS

ABBREVIATIONS

APS	Ammoniumperoxodisulfate
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
Con A	Concanavalin A
CD	Circular dichrosim
cDNA	complementary Deoxyribonucleic acid
CHCA	α- Cyano-4-hydroxycinnamic acid
DEPC	Diethyl pyrocarbonate
DLL	Glc/Man specific lectin from seeds of *Dolichos lablab*
DLL-II	Galactose-specific lectin from seeds of *Dolichos lablab*
DLL-VL	Galactose-specific lectin from vegetative tissues of *Dolichos lablab*
DTT	Dithiothreitol
DVS	Divinyl sulfone
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme linked immunosorbant assay
ESI	Electron spray ionisation
et al	et alii (Latin: and others)
Gdn–HCl	Guanidium Hydrochloride
Glu 6-P	glucose 6-phosphate
HEPES	(N-(2-Hydroxyethyl)-piperizine-N'-(2-ethane sulfonic acid))
HPLC	High performance liquid chromatography
IBA	2-Iodosobenzoic acid
IgG	Immunoglobulin G
kDa	Kilo Dalton
MALDI	Matrix assited laser desorption ionisation
Man 6-P	Mannose 6-phosphate
min	Minute
mL	Milli Litre
MS	Mass Spectrometry

nm	Nanometer
OD	Optical density
PAGE	Polyacrylamide Gel electrophoresis
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
pH	-log (H^+) concentration
PM	Phosphomannan
PMF	Peptide mass Fingerprints
PMSF	Polymethylsulfonylfluoride
pmol	Picomole
pNP	para nitro phenol
pNPP	para nitro phenyl phosphate
PPM	Parts per million
PVDF	Polyvinyldifluoride
rpm	Rotations per minute
rRNA	Ribosomal RNA
SDS	Sodium dodecyl sulfate
S/N	Sound to noise ratio
TBS	Tris-buffered saline
TCA	Trichloroacetic acid
TEMED	N,N,N',N'-Tetramethylendiamine
TFA	Trifluroacetic acid
Tris	Tris-(Hydroxymethyl) aminoethane
Tof	Time of flight
UV	Ultraviolet
β-M.E	β–mercaptoethanol
µCi	micro Curie

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CHAPTER I

INTRODUCTION

Lectins, also called agglutinins or haemagglutinins, have the longest history of plant proteins. The history of lectins starts in 1888, when Stillmark discovered the cell agglutinating properties of ricin, a toxic protein isolated from the seeds of castor beans [*Ricinus communis*]. The term haemagglutinin was introduced for the first time by Elfsrand in1898 for plant proteins that cause clumping of cells, due to the striking similarity of their activity to that of human and animal serum agglutinins. At that time toxicity was thought of as an intrinsic property of lectins. This idea was abandoned after the discovery of non toxic lectins in legume seeds- *Phaseolus vulgaris*, *Pisum sativum, Lens culinaris* and *Vicia sativa* [Landsteiner and Raubitschek, 1907]. Though the majority of lectins represented plant kingdom a number of lectins was reported outside the plant kingdom also for example in fungi, bacteria, viruses, invertebrates and vertebrates.

The next milestone in the history of plant lectins was that they could distinguish human blood groups specifically [Renkonen, 1948]. Based on the new interest initiated by this result systematic screening of plant seeds for specific blood group lectins has been reported. Investigations on lectins as recognition molecules were undertaken. The term 'lectin' was originated from a latin word 'legere' which means 'to select'. At present the term ‘lectin’ is the most commonly used name but the terms ‘agglutinin’ and 'hemagglutinin' still persist as synonyms. The name ‘lectin’, however is the appropriate one because the specific carbohydrate binding activity and not the agglutination activity is the functional criterion of these proteins [some lectins are monovalent, and just for this reason they can not agglutinate cells, and hence can not be called agglutinins. Yet, they can bind specific sugar structures in a selective manner]. Thus, although the term ‘lectin’ was introduced to describe the blood group specific agglutination activity of some members of this class of proteins, it is much more appropriate to describe the specificity of their interaction with mono and oligosaccharides. Moreover names like ‘[hem]agglutinin’ may cause some confusion with elements of other system, e.g., in the precipitation of antigens by their specific antibodies, the antibody is called 'agglutinin' in general and 'hemagglutinin' in case of erythrocyte antisera [Treffers, 1992].

Definition: The following definition of lectins is more flexible than all proposed before and covers a broad range of proteins which behave differently with respect to their agglutination and precipitation properties: *lectins are proteins of non immune origin possessing at least one non catalytic domain which binds reversibly to a specific mono- or oligosaccharide.* The original definition [Peumans and Van Damme, 1995] does not include the condition ‘*of non immune origin*’ because it considers only plant lectins. This definition is applicable to the

monovalent lectins [merolectins, see below] which can not agglutinate cells or precipitate glycoconjugates such as hevein, a small chitin binding lectin from the latex of rubber tree, *Hevea brasiliensis* [Van Parijs *et al*., 1991], and the monomeric mannose binding lectin from *Listera ovata* [Van Damme *et al*., 1994]. This definition also includes some chimeric plant enzymes with genuine lectin domains [chimerolectins, see below] such as type 2 RIP and class I chitinases, which are also monovalent lectins [Beintema, 1994].

Classification: lectin families

Lectins represent a heterogeneous group of proteins with wide diversity in size, structure, physicochemical properties, biological activities, sugars specificities and composition and architecture of binding sites. However, based on the sequence information and structural motifs, most lectins can be classified into a fairly small number of distinct families [Van Damme *et al*., 1998; Drickamer, 1994]. This classification is dealt with very briefly below:

Plant lectins: The evolutionary relationships traced by similarities in the amino acid sequence and structural features among over 300 plant lectins known at present allow their classification into seven families:

1. Amaranthin lectins
2. Chitin binding lectins [Hevein-like]
3. Cucurbitaceae phloem lectins
4. Monocot mannose binding lectins
5. Type 2 RIP
6. Legume lectins
7. Jacalin-related lectins

Lectins that do not fall into any of the above families are referred to as unclassified lectins. Three-dimensional structure and the binding site[s] fold of representative members of all these families, with the exception of Cucurbitaceae phloem lectins, are now available at atomic level and have been shown to be conserved within each family. This structural conservation is reflected in the fact that each one of the families mentioned above [except legume and jacalin-related lectins] shows very similar specificity among its members. Thus, each of these lectin families has its own typical fold and binding site motif and specificity [Van Damme *et al*., 1998; Peumans *et al*., 2000], as shown below:

Amaranthin lectins: This family is a small group of Gal*N*Ac specific lectins. Members are approximately 66 kDa homodimeric unglycosylated proteins. They are found only in the seeds of the genus *Amaranthus* species. The prototype member of this lectin family is amaranthin, the lectin from *A. caudatus*, which binds preferentially to T-antigen disaccharide [Rinderle *et al*., 1989]. The crystal structure of amaranthin bound to benzylated T-antigen disaccharide has been solved at 2.2 Å resolution [Transue *et al*., 1997]. This structure shows that amaranthin monomer consists of two homologous *N*- and *C*-domains which are associated by extensive non-covalent forces in head-to-tail manner to form the dimer, resulting in the formation of a binding site at each interface between the *N*- and *C*-domains of the two facing monomers. The sugar binding activity of amaranthin depends on a complex network of H-bonds and some of them [those involve in binding of the non-reducing part of the disaccharide] are mediated by water. No hydrophobic contact is involved in the binding process.

Chitin binding lectins [Hevein-like]: Not all chitin binding lectins belong to this family, but only those which have one or more hevein-domain[s] as the chitin binding domain. However, this family is widespread in many plant species of different taxonomy. Hevein-domain is a polypeptide structural unit of about forty amino acids. It binds chitin and lower oligomers of Glc*N*Ac specifically and exhibits sequence and structure similarities to hevein [a 43 amino acid chitin binding merolectin from the latex of rubber tree, *Hevea brasiliensis*]. By definition, class I chitinases are chimerolectins belonging to this family because they have a single *N*-terminal hevein-domain linked to the chitinase domain [Beintema, 1994]. Most of these family members are hololectins such as WGA and the other lectins of Gramineae species which have essentially the same structure as that of WGA [Stinissen *et al*., 1983]. WGA was the first member of this family to be isolated and fully characterized. The monomer of WGA is built up of four identical hevein domains and two such monomers are non-covalently associated in a head-to-tail manner to form a dimer having four pairs of hevein domains [one from each monomer]. Hence there are eight binding sites in WGA; only four of them were shown by solution, crystal and modeling studies to be of high and roughly equivalent affinities whereas the remaining four are at least 2-fold weaker [Wright and Kellogg, 1996].

Monocot mannose binding lectins: This family consists of a large number of lectins with restricted specificity to mannose and oligomannose, and found only in some of the monocot plant families. GNA [the mannose specific lectin from snowdrop bulbs] is the first member of this family to be isolated and characterized [Van Damme *et al*., 1987] Detailed specificity,

cloning and structural studies proved GNA to be different from any previously characterized lectin in its sequence, sugar binding activity and 3-D structure [Shibuya *et al*., 1988; Van Damme *et al*., 1991; Hester *et al*., 1995]. Thus GNA became the first representative of a new lectin family which has grown rapidly since then by the isolation and characterization of similar lectins from several monocot plant families to form the super family of monocot mannose binding lectins [Barre *et al*., 1996]. The molecular structure of these proteins involves, in most cases including GNA, one, two or four non-covalently associated small protomers [11-14 kDa]. The main secondary structural element of this protomer is β-sheet. It is built up of three tandemly arrayed domains and three mannose binding sites [Peumans *et al*., 2000].

Type 2 RIPs: A typical type 2 RIP consists of a C-terminal domain [called B-chain] with a specific sugar binding activity [mostly to Gal/Gal*N*Ac] linked by a disulfide bridge to an *N*-terminal domain [called A-chain] with catalytic ribosome inactivation activity of eukaryotic ribosome. Thus, members of this family are chimeric proteins, i.e., they are built up of two structurally and functionally independent domains, and since one of these domains is a lectin, they are classified as chimerolectins [see below].

RIPs which are built up of only the A-chain are termed 'type 1 RIP'. Both type 1 RIP and A-chain of type 2 RIP share a highly similar sequence and once in the cytoplasm of an eukaryotic cell both can inactivate its ribosome by catalytic cleavage of the *N*-glycosidic bond of a single, highly conserved adenosine residue from the 28S subunit of ribosomal RNA [rRNA], resulting in the inhibition of protein synthesis, which leads to cell death. Most RIPs are also able to cleave such adenine *N*-glycosidic bonds at several positions on other polynucleotides as well [Barbieri *et al*., 1997].

Some type 2 RIPs, like ricin, are potent cytotoxic agents, where the B-chain [divalent lectin] serves to bind to the cell through the Gal/Gal*N*Ac moiety of the cell surface glycoconjugates. This binding facilitates the endocytosis of the toxin into the cytoplasm where the A-chain can start its role as mentioned above [Stirpe *et al*., 1992].

Type 2 RIPs have been found in the seeds and various vegetative tissues in at least ten plant families. The native type 2 RIPs consist of one, two or four similar protomers. The protomer consists of the A- and B-chain [usually both are glycosylated] linked by a disulfide bond as mentioned above and called A-s-s-B pair. The classical potent toxins, ricin from *Ricinus communis* seeds and abrin from *Abrus precatorius* seeds, are monomeric type 2 RIP i.e., they consist of a single A-s-s-B pair, whereas the lectins RCA and APA from the same sources,

respectively and also the lectin from *Momordica charantia* seeds, MCL, are dimeric type 2 RIPs built up by noncovalent association of two A-s-s-B pairs [Van Damme *et al*., 1998]. Inspite of the high sequence homogeneity between monomeric and dimeric type 2 RIPs, the later seem to have no or weak cytotoxicity and contain only two sugar binding sites instead of four.

Three-dimensional structure of several type 2 RIPs were solved. The crystal structures of ricin and abrin [Rutenber *et al*., 1991; Tahirov *et al*., 1995] show similar overall fold, where the B-chain is built up of tandem repeat of two similar domains and is devoid of extended regular secondary structures but stabilized by four intrachain disulfide bonds, unlike A-chain which consist of eight α-helices and six β-sheets. Modeling studies, on the other hand, on type 2 RIPs from *Sambucus nigra* showed that the structure of their B-chain is very similar to that of ricin and abrin [Van Damme *et al*., 1998].

Cucurbitaceae phloem lectins: Members of this lectin family also bind chitin but their structure is not made of hevein domains. So far they have been found only in the phloem exudates of Cucurbitaceae species. They are very different from galactose-specific lectins found in seeds and roots of the same plant family. They are dimers composed of about 25 kDa unglycosylated subunits. Like most chitin binding lectins, they are inhibited only by oligomers of Glc*N*Ac [Sabnis and Hart, 1978; Anantharam *et al*., 1986; Sanadi and Surolia, 1994; Peumans *et al*., 2000].

Legume lectins: The well-characterized lectins belong to this family of proteins. Therefore, it is the largest family of plant lectins comprising of proteins found only in legume species. It may be noted that not all lectins from legume plants belong to this family; for example, the type 2 RIPs abrin and APA, from the legume *Abrus precatorius*, and the class 1 chitinase from red kidney beans do not fall into this lectin family. All native legume lectins at physiological pH are tetramers [or dimers] of about 30 kDa protomers [mostly *N*-glycosylated] with one sugar binding site and one each for Mn^{2+} and Ca^{2+}, which are essential for sugar binding activity [Sharon and Lis, 2003]. Demetalisation of legume lectins leads to large changes in their structures and loss of the sugar binding activity. In most cases the protomers are identical or very similar; however, some legume species express two different protomers in their cells which may associate randomly to form different homogeneous and heterogeneous isolectins.

The classical example for this is the PHA isolectins, E_4, E_3L_1, E_2L_2, E_1L_3 and L_4, from the common beans, *Phseolus vulgaris* [Feldsted *et al*., 1977].

In some lectins, which are called two-chain legume lectins, e.g., pea lectin [Trowbridge, 1974], the protomer polypeptide is fragmented unequally into light chain [α] and heavy chain [β]. The protomers in all legume lectins have very similar 3-D structure which is built up of two antiparallel β-sheets: one is a six-stranded flat sheet [back face] and the other is a seven-stranded concave sheet [front face]. The β-sheets are interconnected by turns and loops to form a flattened dome. The β-folds of the seven-stranded sheet form the sugar and metals binding sites. The protomers associate noncovalently to form dimers or tetramers. The most common dimerization mode involves side-by-side association of two monomers using their flat β-sheets in a two-fold symmetry plane to form a twelve-stranded β-sheet. As a result, the two binding sites are located at both ends of the dimer. This mode of dimerization in legume lectins is called the canonical dimerization. The 3-D structure of Con A dimer complexed with MeαGlc [Harrop, 1996], clearly depicting the canonical dimerization. Some other modes of dimerization in legume lectins [called noncanonical dimerization] are also known [Elgavish and Shaanan, 2001]. All tetrameric lectins are formed by association of two dimers either by loop interactions using the central parts of the dimers, as in Con A, or by interactions between the outermost strands of the two twelve-stranded β-sheets creating a channel between them, as in SBA. PNA is unusual not only in its noncanonical dimer but also in being an asymmetric tetramer unlike any other homotetramer protein [Banerjee *et al*., 1994, 1996].

In spite of the high sequence homology and very similar 3-D structure among them, legume lectins cover almost all the monosaccharide specificities. This is because few changes in the amino acid sequence and lengths of loops involved in the sugar binding lead to binding sites of different conformations and hence of different specificities [Sharma and Surolia, 1997].

Jacalin-related lectins: This family includes all lectins which show high similarity to jacalin, a lectin from jackfruit [*Artocarpus integrifolio*] seeds, in structure and sequence. The jackfruit seeds also contain another lectin very similar to jacalin [jacalin-related lectin] called artocarpin. Both lectins are homotetramers of very similar 3-D crystal structure in which the protomer has a novel fold [called β-prism fold], which consists of three four-stranded β-sheets in three-fold symmetry arrangement [Sankaranarayanan *et al*., 1996; Pratap *et al*., 2002]. Like jackfruit seeds, the black mulberry tree [*Morus nigra*] bark also contains two jacalin-related lectins: one is galactose specific [moringaG] and the other is mannose specific [moringaM]

[Van Damme *et al*., 2002]. In fact, jacalin-related lectins are classified into two subfamilies: galactose specific lectins [like jacalin] which are found only in some *Moraceae* species and mannose specific lectins [like artocarpin] which are widespread among flowering plants [Peumans *et al*., 2000].

Jacalin's monomer is synthesized as a preproprotein which, after removal of the signal peptide and a propeptide, is cleaved into two chains, a heavy [α] chain and a light [β] chain. Although it was suggested that carbohydrate specificity of jacalin is probably generated by the cleavage of the single chain polypeptide [Jeyaprakash *et al*., 2003], recent studies indicate that the carbohydrate specificity pattern of recombinant jacalin expressed in *E. coli*, which is not cleaved, is unaltered but its affinity is about 100-fold lower for a battery of sugars as compared to native jacalin [Sahasrabuddhe *et al*., 2004]. Therefore, it appears that post-translational cleavage of the jacalin polypeptide is required for the increase in the affinity for the saccharides that it binds. Artocarpin, the other lectin present in the jack fruit seeds, which is mannose-specific, is not cleaved.

Jacalin and artocarpin are promiscuous lectins. Different agglutination/precipitation inhibition assays of jacalin by sugars showed that in addition to galactose and its derivatives, other unrelated sugars, especially mannose and MeαMan also inhibit the hemagglutination activity of this lectin [Dalmau and Freitas, 1989]. On the other hand, surface plasmon resonance hapten inhibition experiments demonstrated that artocarpin interacts with a wide range of unrelated monosaccharides even though it displays a higher affinity for mannose [Barre *et al*., 2004]. Crystal structure of jacalin complexed with MeαMan at 2.0 Å resolution has been determined by Bourne *et al*. [1992], who attributed the ability of jacalin to bind monosaccharides with different hydroxyl conformations to its relatively large binding site and the flexibility of the β-prism fold.

Microbial lectins: The viral and bacterial surface lectins initiate infections by mediating the adhesion of these pathogenic agents to the target cells. This adhesion is a prerequisite for infections to occur and it is the result of specific binding of these lectins to the carbohydrate moieties on the target cells since it is inhibited [and hence infection prevented] by sugars to which these lectins are specific [Lis and Sharon, 1998]. This illustrate that carbohydrates can be used in prevention of infections caused by organisms which express surface lectins. This is the basis for development of *anti-adhesion therapy* [Ofek and Sharon, 2002]. Controversially, microbial lectins [mainly bacterial mannose specific lectins] may act against microbial infections via what is known as *lectinophagocytosis* [Sharon 1987; Ofek and Sharon, 1988] in

which lectins on either the host's [man, mice] phagocytes or the infectious agent interact [in the absence of opsonins] with specific carbohydrate on the apposing cells, leading to the activation of phagocytes and ingestion and killing the infectious agent. The presence of the specific sugar [e.g. methyl α-mannoside in case of type 1 fimbriated *E. coli,* which express mannose binding domain on its surface] prevents the lectinophagocytosis of this bacteria

Unclassified lectins: Lectins which can not be yet confidently considered to belong to any of the above mentioned families, due to the lack of structure and sequence information about them, are referred to as 'unclassified lectins'. Among those unclassified lectins, Cucurbitaceae galactose-specific lectins [CGL] are of special interest because all members studied so far among them resemble type 2 RIPs in that they are glycosylated, galactose/Gal*N*Ac specific lectins built up of one or more disulfide bridge-linked subunits [M_r 25-35 kDa] pairs, and also their amino acid composition, if known, is very similar to that of type 2 RIPs.

Animal lectins: In spite of the diversity of the overall structure of animal lectins, the carbohydrate binding activity of each lectin is ascribed to a limited portion of it called the carbohydrate recognition domain, CRD [Drickamer, 1988, 1994]. On the basis of the sequence motif of these CRDs, most animal lectins can be classified into a number of distinct types; the most notable of them are C-, S- and P-type lectins. CRDs in each lectin type share invariant and highly conserved amino acids. In addition to the sequence similarity of their CRD, each animal lectin type shows the following properties among its member:

1. C-type lectins are multidomain cell surface and extracellular lectins with diverse specificities and require Ca^{2+} ions for sugar binding activity [hence the name C-type].
2. S-type lectins [better known nowadays as galactins] are both intra- and extracellular soluble lectins with specificity for β-galactosides [mainly lactose and *N*-acetylactosamine] and require cysteine residues with free -SH groups for sugar binding activity [hence the name S-type].
3. P-type lectins are mannose 6-phosphate recepters [hence the name P-type] of either high molecular weight [~300 kDa] and Ca^{2+}-independent activity or low molecular weight [~45 kDa] and Ca^{2+}-dependent activity.

Physicochemical Properties

Molecular Structure: The amino acid sequences of several hundreds of lectins and three-dimesional structures of a few dozen of them have been elucidated and new sequences and structures are being added at an increasing rate. At present over 100 members have been charecterized from the largest and most thoroughly studied legume family alone [Goldstein *et al*., 1997; Sharon & Lis, 1990; Konami *et al*., 1995; Pusztai *et al*., 1990, 1991]. Con A was the first plant lectin to be purified and crystallized [Summer & Howell, 1936] and was also the first lectin whose primary structure and three-dimensional structure are resolved [Edelman *et al*., 1972; Hardman & Ainsworth, 1972]. Typically legume lectins consist of two or four identical or almost identical subunits of 25-30 kDa each with a single carbohydrate-combining site with the same specificity. They also contain a tightly bound Ca^{2+} and a transition metal ion, predominantly Mn^{2+}, per subunit [Emmerich *et al*., 1994]. In addition to their carbohydrate-binding site, several of the legume lectins possess a hydrophobic site that binds non polar compounds such as adenine and indole acetic acid. The first report of lectin sequence came from Edelman and coworkers, who determined the primary structure of Con A. Subsequently Cunningham *et al*. [1979] determined the structure of *Vicia faba* lectin [Favin] and showed that the amino acid sequences of Con A and favin are related to each other in an unusual fashion. This study has stimulated interest for a detailed analysis of primary structure for several other legume lectins. The primary structures of more than 40 of these lectins have been established by chemical or molecular genetic techniques till today. They have been found to exhibit remarkable homologies, with 20 % of amino acid residues invariant. The subunits of legume lectins are commonly made up of single polypeptide chains of about 250 amino acids that may carry one or two N-linked oligosaccharides. Circular dichroic spectroscopy has given valuable information on the secondary structure of lectins in solution and the effect of saccharide binding on lectin conformation. β-pleated sheet is the predominating structure among lectins, particularly those from legumes. Studies have shown that the lectin from red kidney bean [Jirgensons, 1979], peanut, lentil and soyabean [Jirgensons, 1978] and *Dolichos biflorus* [Pere *et al*., 1975] contain significant amounts of β-pleated sheet and no α- helix. The three dimensional structure of ten legume lectins, most of them in complex with mono- or oligosaccharides, has been elucidated by X-ray crystallography. The first reported structure is for ConA followed by pea, peanut, Abrus precatorius, soyabean etc. They are in the shape of a half-dome, with carbohydrate-binding

sites forming a shallow depression at its apex. Metal ions are located close to the combining site but do not bind directly to the carbohydrate. In each of the legume lectins, 4-10 hydrogen bonds, some of them bridged by water molecules, as well as a few hydrophobic interactions, hold the monosaccharide in the combining site. A single carboxyl group of the sugar may be linked to the protein by more than one hydrogen bond [as reviewed in Lis & Sharon, 1998, and Sharon & Lis, 1995].

Being the oldest of all known lectins, the lectins of euphorbiaceae family are very well characterized. Two structurally similar lectins have been charecterized from the castor bean, one of which is a toxin [ricin] and the other an agglutinin [Olsnes, 1978]. Ricin is a hetro dimeric toxic protein with a molecular weight of 60 kDa, made up of two S-S linked chains, A and B. The B chain contains three carbohydrate-binding sites according to a recent report by Frankel and co-workers [1996]. The cytotoxic activity resides in the A chain which acts by enzymatically inactivating the RNA involved in protein synthesis. The B chain is made up of two globular domains, each of which comprises a link domain and three homologous 40-residue sub domains. The three dimensional structure of ricin has been determined by X-ray crystallography [Rutenber *et al*., 1991]. The A-chain is a globular protein with extensive secondary structures, both β pleated sheet and α helix, and a reasonable prominant cleft, assumed to be the active site responsible for the toxic action of ricin. The complete amino acid sequences of both the chains have been reported. The B chain folds into two topologically similar domains, each binding lactose in a shallow cleft. RCA [*Ricinus communis* agglutinin] isolated from same plant is a tetramer of the type $\alpha_2\beta_2$ which exist as two noncovalently associated hetro-dimers, each of which with a structure analogous to ricin but it is not toxic [Nicolson *et al*., 1974; Olsnes *et al*., 1978]. Preliminary crystallographic charecterization of of RCA has shown that it forms an elongated molecule with two A chains at the center and a B chain at each end. The A chains are covalently associated, with a disulfide bridge between the chains.

The lectins isolated from cereals consist mostly of two identical subunits. They are rich in cystein. The WGA lectin, the only member of this family characterized in detail, is a mixture of three isolectins that differ slightly in their amino acid composition. The isolectins have two identical subunits, has four carbohydrate-binding sites located at the inter face of subunits and are devoid of metals [Goldstein *et al*., 1997; Emsley *et al*., 1994]. Each subunit is made up of four homologous sub domains of 43 amino acids held in a compact stable conformation by four interlocking disulfide bridges [Wright, 1987] that appear to be essential for the activity.

There are altogether 16 disulfide bonds. The polypeptide chain of each domain is characterized by irregular folding, devoid of commonly occurring secondary structural elements such as α-helices or β- sheets. The specificity of cereal lectins is somewhat unusual, in that they interact with both sialic acid and N-acetylglucosamine. They are also unusual in having a very high content of half-cystine residues and unlike other lectins it is not a glycoprotein [Allen *et al*., 1973]. In the crystalline complex of WGA with sialyl-lactose, examined by X-rays at high resolution, the sialic acid is bound to lectin by a number of hydrogen bonds as well as by non-polar contacts with aromatic amino acids. The aminoacids involved in the binding are not located in the same subunit, as found in other lectins, but belong to different subunits of the WGA dimer [Wright, 1990].

The lectins of amaryllis, orchid, and garlic families exhibit 80-90% sequence homology [Van Damme *et al*., 1991; Van Damme *et al* 1994]. These mannose specific lectins are distinguished by their small monomer size, presence of 3-fold internal repeats of 36 amino acids, lack of metal ion requirement and weak affinity for the monosaccharide ligand [Chervanak and Toone, 1995]. The snowdrop [*Galanthus nivalis*] lectin from this family whose three dimensional structure has been elucidated is tetrameric, each subunit is composed of three pseudo-symmetrically related beta sheet domains, with a conserved mannose-binding site. There is one inter-domain disulfide bond, between the second and third subdomains, and the interior of the monomer is stabilized by conserved hydrophobic residues. The tetramer exists as two pairs of dimers. The lectin is unusual in that it has one carbohydrate-combining site per subdomain i.e., the tetramer is dodecavalent [Hester *et al*., 1995; Wright, 1996]. Crystal structures of monosaccharide and disaccharide complexes of GNA have revealed that all 12 binding sites of the tetramer are functional, and that the degree of occupancy is dependent on the availability of availability of subsidiary interactions from neighboring subunits. Two unique mannopentose binding modes co-exist in the tetragonal structure [1 subunit/asymmetric unit] of the complex [Wright & Hester, 1996]. The two distinctly different binding modes observed show that the high affinity mannose binding occurs only at the two domain sites located near dimer interfaces.

The galactose specific lectin, Jacaline, represents moraceae family. It is a homo tetrameric galactose specific protein with a mol.weight of 66kDa. Each subunit of this lectin has a cabohydrate-binding site that recognises the α-anomer of galactose or N-acetyl galactosamine [Gupta *et al*., 1992]. Jacaline has attracted a great deal of attention due to its specific recognition of the tumour associated T- antigenic disachharide, Galβ13GalNAcα [Sastry *et al*.,

1986; Mahanta *et al*., 1990]. Each of its subunits consists of a heavy chain [α] of 133 aminoacids and light chain [β] of 20 residues [Kabir & Daar, 1994]. X-ray crystallographic studies have shown that subunits of jacaline are made up of three four-stranded antiparallel β sheets, arranged like the faces of triangular prism, with loops connecting the strands in the sheets. It is stabilized by hydrophobic interactions in the core of subunit and a small number of hydrogen bonds involving main chain, as well as side chain atoms. This kind of arrangement called β-prism fold is not found in any other lectin [Sankaranarayan *et al*., 1996]. Apart from moraceae family lectins, several lectins evolutionary related to jacaline have been found in taxonamically distant species. For example rhizomes of hedge bindweed [Calystegia sepium, Convolvulacea] contain a lectin called calsepa that shares sequence similarity with jacaline [Van Damme *et al*., 1996]. These lectins do not recognise Gal, GalNAc or the T-antigen, but exhibits a clear preference for mannose/ maltose [Peumans *et al*., 1997]. Similar mannose/maltose binding lectins have also been found in other Convolvulacea species and in Jerusalem artichoke [*Helianthus tuberosus*, family, Asteraceae].

A high content of L-arabiose and also the presence of rare amino acid hydroxyproline are the charecteristics of solanaceous lectins [eg. thorn apple, tomato and potato] [Allen *et al*., 1978; Nachbar *et al*., 1980; Desai *et al*., 1981; Ashford *et al*., 1982]. Most of these lectins are glycoproteins composed of equal amounts of protein and carbohydrates, the latter consisting of 85% L-arabinose and 15% galactose. The lectins are specific for chitin oligosaccharides and exist as dimers of two identical subunits. Each subunit consists of two evolutionary autonomous domains: a carbohydrate-binding region fused to a hydroxy proline rich, highly glycosylated module. The former shares sequence similarity with other chitin-binding plant proteins and also with platelet-aggregation inhibitors from snake venoms [Kieliszewski *et al*., 1994]. The hydroxyproline-rich domain, in turn, is similar to extensins, a family of glycoproteins that are components of plant cell walls. Three dimensional structures of none of these lectins is known. Potato [*Solanum tuberosum*] lectin, chimeric chitin-binding protein from this family, is comprised of a lectin domain fused to a hydroxyproline-rich glycoprotein domain. Accurate determinations of the molecular weight of the lectin by a MALDI mass spectrometer have shown that the subunit molecular weight is 65,500 [+/- 1100] and that of a totally deglycosylated sample is 31,250 [+/- 30]. This means that the lectin is 52.3 [+/-1] % carbohydrate with a considerable number of glycoforms being present. Partial sequences and other analyses confirm the existence of three distinct domains in the lectin. These are: [1] an N-terminal region which is rich in proline but poor in hydroxyproline; [2] a glycosylated

region with a glycosylated molecular weight of 45,300 [+/- 1100] and a deglycosylated molecular weight of 11,050 [+/- 50] which is extremely rich in glycosylated hydroxyproline residues with a similar sequence to extensins; and [3] a cystine-rich domain which has the sugar binding site shows partial conservation of a repeated motif common to many chitin-binding proteins of the hevin family including wheat-germ agglutinin. Sequence similarities identify potato lectin as a member of both the hevein and extensin families of plant proteins**.**

Carbohydrate Binding Specificity

It is of vital importance to establish the carbohydrate-binding specificity of a lectin in order to make use of it as a tool in biochemical and immunochemical studies. Sugar-lectin complementarity is determined most generally by hapten-inhibition technique.

Binding of Monosaccharides: The carbohydrate specificity of lectins varies greatly with respect to the binding of simple saccharides. The affinity of lectins for monosaccharides is usually weak, with association constants in the millimolar range, yet it is highly selective. Lectins exhibit a wide range of variations with respect to the configeration and substitution patterns at different carbon atoms of monosaccharides. Lectins are often classified into five groups based on the specificity for simple sugars: mannose binding lectins, galactose/ N-acetylgalactosamine binding lectins, N-acetyl glucosamine binding lectins, fucose binding lectins and N-acetyl neuraminic acid binding lectins [Lis and Sharon, 1998]. Certain lectins belonging to the same specificity group combine preferentially, or almost exclusively, either with the α- or β- glycosides of the respective monosaccharide, where as others lack anomeric specificity. While the lectin from *Giffonia simplicifolia* [B4] [Hayes and Goldstein, 1974] and lima bean lectins [Roberts and Goldstein, 1984] are specific for α- anomers of galactose, snakegourd lectins [Komath *et al*., 1998] and *P. vulgaris* lectins [Kornfeld and Kornfeld, 1969,1970] are specific for β- anomer of galactose. On the other hand, lectins from soyabean, *M. charentia* and castor bean are almost indefferent in their anomeric specificity. Many lectins tolerate some variations at the C - 2 of the sugar to which they bind. Lectins, which show a primary specificity for mannose such as ConA [Goldstein *et al*., 1965; So and Goldstein, 1967], lectins from pea [Van Wauwe *et al*., 1975], lentil [Young *et al*., 1971; Toyoshima *et al*., 1970] and fava bean will also bind to glucose, and to a lesser extent, N-acetyl glucosamine. Similarly several lectins specific for N-Acetylgalactosamine also bind to galactose. Soyabean agglutinin [Pereira *et al*., 1974] and limabean lectin [Galbraith *et al*., 1972] are examples for

this. Lectins tolerate very little variation at C-3 of the sugars they bind though one or two exceptions are there. The C-4 hydroxyl group of carbohydrates is crytically involved in lectin binding. Mannose/ glucose binding lectins do not ineract with galactose and vice versa. Similarly, N-acetylglucosamine binding lectins do not interact with N-acetylgalactosamine binding lectins [Lis and Sharon, 1984]. The properties of aglycon may markedly influence interaction of a glycoside with a lectin. Aromatic glycosides bind to many lectins much more strongly than aliphatic ones, attesting to the presence of hydrophobic region close to the carbohydrate-binding site. The hydrophobic effect is at times so strong that lectins that show a marked preference for methyl α-glycosides over the corresponding β anomers exhibit an inverse specificity when tested with corresponding p-nitrophenyl glycosides [Lis *et al*, 1984]. The lectins within each group may also differ markedly in their affinity for other derivatives. Polar interactions between carbohydrate hydroxyl goups and polar side chains of amino acid residues within a lectin's hydrophobic binding site appear to be ideal model for specific carbohydrate-lectin interactions.

Binding of oligosaccharides: Lectins often exhibit a an exquisite specificity for di-, tri- and tetra saccharides with association constants up to 1000 fold higher as compared with monosaccharides. Certain lectins interact only with oligosaccharides. *Datura stramonium* lectin for example is inhibited only by the oligosaccharides of GlcNAc [Horejsi *et al*., 1978; Kilpatrick *et al*., 1979]. The binding of oligosaccharides is of special significance as they are most likely the natural ligands of lectins. The selectivity of lectins towards their natural targets, usually oligosaccharides, is assumed to be acheived through multiple binding by mechanisms of additional binding in subsites [or extended sites] and subunit multivalency. The usually low affinity for monosaccharides is elicited during this process [as reviewed in Elglavish & Shaanan, 1997]. In sub-site binding, one monosaccharide, usually the terminal one is bound at the primary binding site of the lectin. And additional monosaccharides along the carbohydrate chain are bound to secondary sub-sites on the lectin. This kind of selectivity enhancement is demonstrated in the binding of the Glc/ Man- specific *Lathyrus ochrus* lectin [LOL] to a series of mannose containing oligosaccharides [Bourney *et al*., 1992] and that of cholera toxin to GM1 ganglioside through the terminal sialic acid and galactose [Merritt *et al*., 1994]. Subunit multivalency is exhibited when several units of the same lectin bind to different extensions of a branched oligosaccharide as in case of asialoglycoprotein receptor [Rice *et al*., 1990] or to separate carbohydrate chains as in case of the trimeric mannose-

binding protein [Sheriff *et al*., 1994; Weis and Drickamer, 1994]. Oligosaccharides are flexible molecules with considerable freedom of rotation around glycosidic bonds connecting the individual monosaccharide constituents. Because of their flexibility oligosaccharides that differ in their chemical structure may have substantial topographic features in common and, as a result of this similarity, will bind to the same lectin [for eg. $Lewis^b$ and $Lewis^y$ blood group determinants bind to Griffolia simplicifolia lectin, Spohr *et al*., 1985]. On the other hand, different lectins specific for the same oligosaccharide may recognize different regions on its surface.

Blood group specificity: The studies by Boyd [1949] and Renkonen [1948] on phyto hemagglutinins can be considered as the first approach to investigate the blood group specificity of plant lectins. Boyd observed that saline extract prepared from dried lima beans agglutinated erythrocytes of some human individuals specifically and not those of others. He found that the differences were correlated with blood goups. Following their studies a lot of lectins were discovered with blood group specificity.

Hydrophobic Binding: Lectins bind hydrophobic sugar derivatives more strongly than the analogous non-hydrophobic derivatives. This suggests the existence of hydrophobic regions near the carbohydrate binding sites of lectins. In addition, several lectins bind hydrophobic compounds devoid of sugar moieties. Such bindings are not inhibited by specific sugars indicating that hydrophobic ligands bind to lectins at sites distinct from the carbohydrate binding sites. Some of the hydrophobic compounds that interact with lectins are indoleacetic acid, 1, 8-anilinonaphthalenesulfonic acid, 2, 6-toluidinosulfonic acid, adenine and adenine-derived plant hormones, i.e. cytokinins [Roberts and Goldstein, 1982 and 1983; Maliark and Goldstein, 1988; Gegg *et al*., 1992; Puri and Surolia, 1994]. The binding affinity of some of the lectins for these ligands is sufficiently high [in the range 10^3- 10^6 M^{-1}]. The exact function of these bindings is unknown, but adenine/ cytokinins-binding lectins must be involved in the storage of phytohormones or plant growth regulation. It has been suggested that lectins may function not only by virtue of their ability to bind carbohydrates but also by serving as binding proteins for biologically active hydrophobic ligands. The fact that for most of the lectins, particularly those of plant origin, a physiologically relevant carbohydrate ligand has not been found to date adds credence to this belief. Though hydrophobic contacts are known extremely important in stabilizing protein-protein and protein-membrane interactions, only recently such contacts are shown to be present in lectins also. For example, the cryoprotective galactose-

specific lectins from mistletoe bind to head groups of digalactolipids in thylakoid membranes and efficiency of this binding depends on hydrophobicity [Hincha *et al*., 1997]. A number of animal lectins have multifunctional domains and some of these are endogenously involved in hydrophobic contacts with receptors/ ligands [Asperg *et al*., 1997; Barondes, 1981; Hinek *et al*., 1988; Kuroki *et al*., 1997].

Anomeric preference: Certain lectins show anomeric specificity, i.e., within the same specificity group they bind one of the sugar anomers, α or β, stronger than the other. For example, the mannose/glucose specific legume lectins bind α-mannosides and α-glucosides with greater affinity than the corresponding β-anomers. Similarly, the galactose/Gal*N*Ac specific lectins from the seeds of Cucurbitaceae species prefer β-galactosides over α-galactosides.

The aromatic effect: The nature of the aglycon of the glycoside ligand may affect the strength of the glycoside-lectin interaction. In particular, the binding of glycosides with aromatic aglycon to many lectins is stronger than that of the corresponding sugars with aliphatic aglycon, although opposite cases were also noticed, attesting to the presence of a hydrophobic pocket at or near the sugar binding site on the lectin. This aromatic [or hydrophobic] effect is sometimes so strong that it results in inverse anomeric specificity as is the case with *Griffonia simplicifolia* isolectin I-B4 which shows α or β anomeric preferential specificity with methyl or aromatic galactosides, respectively [De Boeck *et al*., 1981]. Aromatic substitutions at positions other than the anomeric one also leads to a much greater affinity of a lectin to its specific sugars, e.g., the dansyl group in Gal*N*Dns, increases the affinity of soybean agglutinin to this sugar by about 160 fold over that observed for Gal*N*Ac [Swamy *et al*., 1986].

Quantitative study of lectin-carbohydrate interaction: Due to the crucial role of lectin-carbohydrate interaction in many biological recognition processes it is important to determine the thermodynamic parameters associated with such interaction [n, K_b, ΔH_b and ΔS_b]. These parameters are used generally to predict the extent of binding [affinity] in a given system under certain conditions. In case of lectin-carbohydrate interaction, the differences in binding parameters of various sugars to a lectin help to predict the contribution of each substituent and functional group on the sugar to the overall binding process which provides information on the forces that govern the binding and the nature of binding site [see e.g., Schwartz *et al*., 1996].

Several physical methods can be used to obtain the binding parameters of a protein-ligand system. Among these, isothermal titration calorimetry [ITC] yields the most accurate information on the stoichiometry of binding and the thermodynamic parameters associated with the interaction, whereas fluorescence spectroscopy, due to its high sensitivity, is very widely used. In the studies reported in this thesis these methods have been used for investigating carbohydrate binding to the *Dolichos lablab* seed lectin [DLL-II].

In ITC the heat evolved [or absorbed] as the macromolecule is gradually saturated with the ligand is accurately measured, which allows simultaneous determination of all binding parameters [n, K_b, ΔH_b and ΔS_b] in a single run [Wiseman *et al*., 1989]. Additional useful parameters, heat capacity, ΔC_p, and van't Hoff enthalpy, $\Delta H_{b[v]}$, can also be obtained by repeating the ITC run at different temperature[s]. Fluorescence spectroscopy is a sensitive and reliable method to study protein-ligand interactions. It is often the method of choice when ITC facility is not accessible or to overcome some technical difficulties [such as protein/ligand concentration required]. In cases where ligand binding to a protein can significantly perturb the intrinsic fluorescence of the latter [usually when tryptophan/tyrosine exists in the binding site] this effect can be exploited to study the interaction [See e.g., Das *et al*., 1981; Sastry and Surolia, 1986]. Otherwise, fluorescently-labeled sugar probes are required. 4-Methylumbelliferyl glycosides are among the most commonly used fluorescence probes for protein-carbohydrate interaction [see e.g., Loontiens *et al*., 1977; Khan *et al*., 1981; Gupta *et al*., 1992]. The emission intensity of the umbelliferyl moiety is very sensitive to its microenvironment and usually changes significantly upon binding to the lectin, thus providing a handle to follow the binding process.

Biological Properties

Agglutination activity: The ability to agglutinate cells distinguishes lectins from other sugar-binding molecules. Though lectins are discovered without agglutination activity also majority of them show agglutination activity. The observation that some lectins preferentially agglutinate tumour cells as compared to normal cells has stimulated much interest in this particular property of lectins. Some Gal/GalNAc specific lectins recognize the T-antigen disaccharide Galβ13GalNAc [for example, Puri *et al*., 1992; Sastry *et al*., 1986]. The tumour selectivity of lectins has resulted in attempts to use lectins for targeted drug delivery in chemotherapy [Gabaius and Gabius, 1991].

Mitogenic Stimulation of Lymphocytes: Certain lectins can stimulate the triggering of quiescent, nondividing lymphocytes into a state of growth and proliferation. The first mitogenic agent to be described was PHA, the lectin from red kidney bean [Nowell, 1960]. Now many lectins have been recognized to have mitogenicity for the T-cells, B-cells or both. However, PHA and ConA remain the most widely used mitogens. Almost all of these mitogens are inhibited by simple sugars. Lectins can stimulate a large number of polyclones and this greatly facilitates the detection and study of changes associated with proliferation. The exact mechanism of mitogenic stimulation by lectins is not known, but the phenomenon has clinical applications such as in the production of polyclonal antibodies [Kilpatrick, 1991].

Insulinomimetic Activity: ConA, wheat germ agglutinin and several other lectins mimic the effects of insulin on adipocytes. The receptor for insulin is a glycoprotein and these lectins are able to compete with insulin in binding with fat cells. So it is suggested that lectins also bind to the insulin receptor [Lis & Sharon, 1986].

Lectin-induced cytotoxicity: In the presence of mitogenic lectins, cytotoxic T-lymphocytes could lyse a wide variety of cells that are not their corresponding target cells. This phenomenon is called lectin-induced cytotoxicity. Several lectins including ConA, wheat germ agglutinin and phytohaemagglutinin are toxic to mammalian cells [Lis and Sharon, 1981, 1984]. Toxic lectins are generally selective in their action on cells. Transformed cells are usually more sensitive to cytotoxic effects of lectins than normal ones.

Lectins with enzymatic properties: Galactose specific mung bean lectin is the first lectin reported to have enzymatic properties. Later on a few more lectins from legume family were reported to exhibit glycosidase activity. Of these most of the lectins have sugar binding and enzyme activity closely related such that they also get defined as glycosidases with lectin like behavior. For example, mung bean lectin binds for short while to erythrocytes because upon binding to the carbohydrate on the cell surface, they proceed to hydrolyse them as well. The lectin from *Vicia faba* alone clearly distinguishes between enzymatic specificity and its carbohydrate binding specificity, indicating the presence of multiple domains with different function in the same protein [Dey *et al*., 1982].

Lectin-induced agglutination of glycolipid vesicles/membrane fusion: Lectins cross-link with glycolipid receptors as like glycoprotein receptors leading to agglutination of membranes. This property has been used to study the liposome-lectin interactions [Surolia *et al*., 1975, Surolia and Bachhawat, 1978, Grant and peters, 1984]. Their studies have shown that receptor concentration in the membrane is very critical and sensitive determinant of agglutinability when the lectin receptors are glycolipids. Receptor density in the membrane is also a critical factor in lectin agglutination via glycolipid as receptors. Researchers have shown that WGA agglutinate model membranes that have lectin receptors on their surface and induce fusion between the vesicles.

RIP activity: Some lectins have the capacity to inactivate eukaryotic ribosomes and are called ribosome-inactivating proteins. Recently they are considered as polynucleotide: adenosine glycosidases that can use various polynucleotides as a substrate for deadinylation [Barbieri *et al*., 1993]. RIPs are subdivided into type1 and type 2 RIP. Type 1 RIP consist of a single polypeptide of about 30 kDa with polynucleotide: adenosine glycosidase-activity, where as type 2 RIP contain an enzymatically active A chain and a carbohydrate binding B chain with lectin activity. The A and B chains remain covalently linked by a disulfide bond.

Animal lectins: Animal lectins are the most understood lectins in terms of their biological functions. Some of these functions are:

1. *Traffic of lysosomal enzymes:* P-type lectins mediate the traffic of lysosmal enzymes from cell surface and golgi network to lysosomes. This traffic depends on binding of Man6P moieties of the oligomannose glycans of these enzymes to Man6P receptors [P-Type lectins] [Drickamer, 1994].
2. *Clearance of glycoproteins:* Most mammalian serum glycoprotein glycans terminate in sialic acid. When these sialic acid residues are cleaved, the galactose residues become exposed and bind to hepatocyte asialoglycoprotein receptors [C-type galactose specific lectin]. These glycoproteins are then rapidly internalized into hepatocytes and digested by lysosomes. It seems that desialylation of serum glycoproteins is a signal to indicate that these glycoproteins have become useless and must be cleared from circulation. Glycoprotein receptors which recognize terminal sugars other than galactose are also known [Drickamer, 1994].
3. *Defense:* Many animal lectins [mainly C-type lectins] are involved in immune response. In addition to the macrophage mannose receptor's role in lectinophagocytosis [see *5.2*], liver and serum MBPs bind in a carbohydrate-specific manner to several bacterial and fungal pathogens

and act as opsonins, thus bypassing the need for antibody recognition step in the immune response aganist these pathogens [Weis *et al*., 1998].

4. *Cell adhesion and recognition:* Selectins, a class of C-type lectins, mediate the adhesion of circulating leukocytes to the endothelial cell of blood vessels. This adhesion is the result of selectin-carbohydrate interaction and is the first step in targeting leukocytes to lymphoid organs or to sites of inflammation [McEver, 2002].

Some Biological Functions of plant lectins

Lectins as plant storage proteins: As more and more information became available on the developmental control of plant lectins, their spatial and temporal distribution, as well as on their cellular and sub cellular location, evidence increased to assume that quite a lot of lectins behave as typical storage proteins. Especially those lectins, which are abundantly present in either seeds or different kind of vegetative storage tissues exhibit such a storage protein like behavior, it was proposed that they function as reserve proteins. These lectins are synthesized during seed development together with the seed storage proteins. During germination and seedling growth, both storage proteins and lectins are broken down to provide amino acids for the growing seedling. Advantage of these sugars- binding, storage protein over the normal storage protein is that they perform double function. As long as they reside in the plant, they act as storage proteins but once they are released from the plant they play a role in plant defence. This is evidenced from the fact that PHA causes severe harm upon oral administration to animals. Another example is several lectins such as WGA are toxic for particular insects.

Lectins in plant defense: The toxicity of various plant lectins for animals and their growth inhibitory effect on fungi are the basis for the assumption that they function in the defence of plants against pathogenic fungi and predatory animals. Much of the information about the toxic effects of plant lectins on animals comes from feeding experiments with PHA and accidental poisoning of humans by raw or insufficiently cooked beans. Ingested PHA binds to the border cells of the intestine, where it is rapidly endocytosed. Upon entering the cells, the lectin enhances their metabolic activity, which eventually leads to hyperplasia and hyperthrophy of small intestine [Pusztai *et al*., 1990]. Moreover, ingestion or PHA or raw beans causes acute nausea followed by vomiting and diarrhea. The bark lectins of black locust and elderberry provoke similar toxic effects. Type 2 RIPs are known to be potent cytotoxic agents. They are

toxic to all eukaryotes if they reach cytoplasm. While fungi seem to be unaffected by them except when chitinase is present, bacteria, viruses, certain insects and higher animals show great sensitivity towards these proteins [as reviewed in Peumans & Van Damme, 1995]. These examples illustrate the potential of lectins in protection against predators. Another argument in favor of defence role of plant lectins is their marked stability under unfavorable conditions. Most of these lectins are stable over wide range of pH, are able to withstand heat, and are resistant to animal and insect proteases. Their role in defence mechanism has also been inferred from the fact that many of these lectins show remarkable specificity for oligosaccharides not found within plant system but abundant in other organisms [for example, chitin in the cell wall of fungi].

Lectins as cryoprotective agents: Some cryoprotective lectins in plants have been correlated with cryoprotective properties in the tissues that they accumulate in [Hincha *et al*., 1993]. For example the leaves of mistletoe contain three Gal/GalNAc specific lectins, two of which show strong cryoprotectivity during freezing and thawing of isolated spinach thylakoid membranes [Hincha *et al*., 1997]. It has also been shown that accumulation of these lectins in the leaves is seasonally regulated.

Lectins as metabolic signals for gut: It has been known for a while that red kidney bean is toxic to a veriety of animals and humans when eaten raw. Further research has shown that lectins such as those from snowdrop bulb and the elderberry tree bark have effects similar to that of red kidney bean. This has led to interesting research on lectin receptors and the consequences of lectin binding to epithelium in mammals. The results suggest that lectins in the gut may act via either class-I or the class-II receptors. In the former case the binding of the lectin triggers off a second messenger molecule that acts as a signal to elicit response from the system. This action is weak compared to that of latter. In the latter case, subsequent to lectin binding, the lectin itself gets endocytosed. The anti nutritional effect appears to be proportional to their ability to stimulate growth of the gut at the cost of growth of the animal itself and the ones endocytosed appear to be more efficient at this stimultion [Peumans & Van Damme, 1995; Pusztai *et al*., 1991; Pusztai, 1993].

Lectins in Rhizobium-legume interactions: Lectins function in the establishment of symbiosis between nitrogen fixing bacteria, mainly rhizobia, and leguminous plants which is

of importance in both the nitrogen cycle of terrestrial life and in agriculture. When rhizobia encounter root hairs in the soil, several profound developmental events take place in the infected roots. The invasion into root hair requires a highly specific association between the bacteria and root hair surface. It is assumed that rhizobial attachment to plant roots occurs by the interaction between and rhizobial surface carbohydrates and lectins present in the roots of legume plants. This is known as 'lectin recognition hypotheses'. Molecular genetic experiments favor this hypothesis.

Applications

Lectins, because of their carbohydrate-binding specificity, are used for the isolation and characterization of glycoconjugates. The ready availability of large number of lectins with different carbohydrate specificities has led to their extensive utilization as reagents for study of simple and complex carbohydrates in solution and on cell surfaces. Lectins have been used for the study of carbohydrate-binding sites in proteins. Lectins derivatized with fluorescent dyes, gold particles or enzymes are employed as histochemical and cytochemical reagents for detection of glycoconjugates in tissue sections, on cells and sub cellular organelles, and in investigation of intracellular pathways of protein glycosylation [Rhodes and Milton, 1998]. They are used for the isolation, purification and structural studies of carbohydrate containing polymers. Lectin binding has been used to demonstrate that membrane receptors for hormones, growth factors, neurotransmitters and toxins are glycoconjugates. Immobilized lectins, such as those covalently bound to Sepharose, are indispensable for the purification and isolation by affinity chromatography of glycoproteins, glycopeptides, and oligosaccharides [Carlson, 1994; Debray, 1991; Hasselbeck and Hosel, 1993; Yamamoto *et al*., 1993]. The use of Con A for resolving mixtures of closely related glycopeptides such as those found in proteolytic digests of glycoproteins is well established.

Hemagglutination, the earliest of all properties discovered, by lectins which show ability of distinguishing between different blood groups, has been popular as a means of achieving blood typing [Kilpatrick and Green, 1992]. Thus lectins from *Lotus tetragonobus* and *Ulex europaeus*, both specific for fucose, are employed to identify blood type O cells, and for the identification of secretors of blood group substances. The lectin from *Dolichos biflorus* is used to distinguish between A_1 and A_2 subgroups and that from Vicia graminae, specific for blood group type N, to differentiate between M and N cells. In addition PNA, specific for gal [β1-3] GalNAc is employed in the detection of “polyagglutination” [or “polyagglutinability”], a condition accompanying certain bacterial and viral infections, in which human erythrocytes

become agglutinable by antibodies normally present in the sera of nearly all adults. If not diagnosed in time, it may lead to serious complications and death. In addition lectins have been used to resolve location and structure of blood group - active molecules in the erythrocyte membrane.

Mouse and human cortical [immature] thymocytes differ from the medullatory [mature] ones in their surface carbohydrates, as evidenced by the fact that the former are bound and agglutinated by peanut agglutinin [PNA^+ cells] [Sharon, 1983]. Separation with PNA provides access to individual thymocyte subpopulations and makes it possible to examine *invitro* their developmental and functional relationship.

Selective agglutination of SBA permits separation of B and T mouse splenocytes. The main application of this lectin is purging human bone marrow for transplantation [Aversa *et al.,* 1994]. It is employed routinely for transplantations into children born with severe combined immune deficiency with close to 70% success. SBA purging is also used experimentally in bone marrow transplantation of leukemic patients, as an alternative to other accepted techniques for T cell depletion, such as monoclonal antibodies. The ability of some lectins to interact preferentially with certain transformed cells has led to use of these compounds as carriers for chemotherapeutic agents. Examples of such conjugates are the chimeric toxins, consisting of Concanavalin A and the α chain of diphtheria toxin [Gilliland *et al*., 1978] or ricin [Yamaguchi *et al*., 1979], in which the lectin serves to direct the cytotoxic agent to target cells. There have been attempts at the saccharide-assisted delivery of cytotoxic liposomes to human tumor cells [Vodovozova *et al*., 1998] and it remains to be seen whether similar attempts will be successful with other lectins. Certain lectins such as peanut lectin or jacalin have been shown to bind T-antigen structural determinant, Galβ13GalNAc, with high selectivity. Site directed mutagenesis could be used to enhance this specificity as demonstrated in the case of peanut lectin [Sharma *et al*., 1996]. There have been attempts to replace toxic chain of lectin with polypeptide chains of the drug, which can then be endocytosed into tissue [Pusztai *et al*., 1991]. When toxic lectins are coupled to monoclonal antibodies raised against tumor cells they can be directed selectively to the tumor and destroy the transformed cells.

Certain lectins are potent mitogens, activating and inducing them to divide. PHA and concanavalin A, for example stimulate T lymphocytes, while pokeweed mitogen stimulates both T and B cells [Borrebaeck & Carlsson, 1989; Di Sabato *et al*., 1987]. The mitogenic lectins are polyclonal activators, in that they activate lymphocytes irrespective of their

antigenic specificity. Prior to the advent of monoclonal antibodies to cell surface antigens, lectins were the major tools for studies of mechanism of cell activation. Mitogenic stimulation by lectins provides an easy and simple means to the immunocompetence of patients suffering from a diversity of diseases, including AIDS, and to monitor the effects of various immunosuppressive and immunotheraputic manipulations. It has been used to examine the effect of stress, both physical and psychological, on the immune system. It is also employed for the preparation of chromosome maps for different purposes, such as karyotyping, sex determination, and detection of chromosome defects, since the chromosomes are easily visualized in the stimulated cells.

Horseradish peroxidase conjugated lectins have proved to be useful markers in mapping central neuronal pathways, since the conjugate is taken by neurons and transported within the axons [Mesulam, 1982]. When ricin conjugates were used as tracers, morphological lesions and cell death in neurons were observed. Such "suicide transport" offers new approach for tackling neurobiological problems, for example, by deserving target organs in peripheral nervous system.

Motivation and focus of the present work

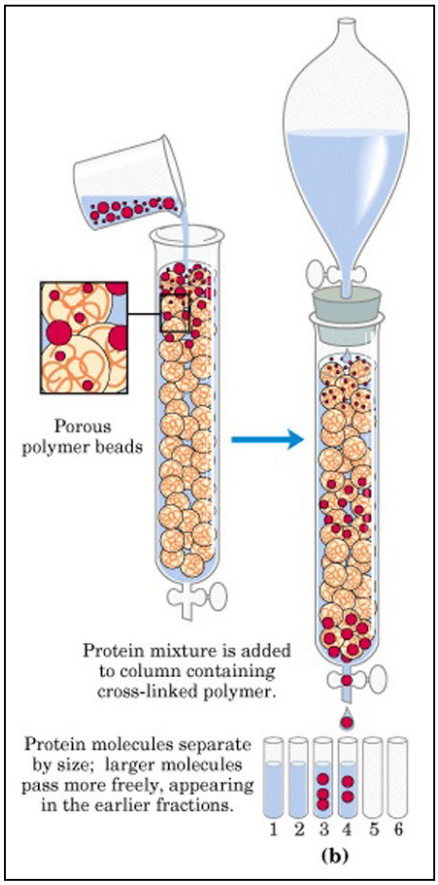
Since their identification, great strides have been made in lectin research and it is firmly established now that lectins are ubiquitous. In particular, plant lectins have been widely studied and among these the legume lectins have been extensively investigated. Some, such as legume lectins have received considerable attention over years and a great deal of information including sequence information and crystal structure for some of them are now available. These lectins therefore are now widely used in applications such as targeted drug delivery, membrane fusion or glycobiology. It has also been possible to get a detailed understanding of the similarities between them in terms of primary, secondary and tertiary structure as well as at the level of three-dimensional structure resulting in the unraveling of evolutionary relationships between them. It is hoped that by isolating new lectins and undertaking systematic physicochemical characterization of these will eventually lead to similar understanding and exploitation of lectins from other plant families as well. Therefore, in this study we have chosen to carry out investigations on *Dolichos lablab* lectins.

In the first part, detailed studies were carried out on a new galactose-specific lectin was purified from the seeds of *Dolichos lablab* by affinity chromatography and characterized with respect to biochemical, immunological and macromolecular properties like secondary structure

and carbohydrate specificity. The amino acid residues important for the sugar binding activity were identified by chemical modification studies using group-specific reagents. Finally, fluorescence quenching studies were carried out to understand the environment of the tryptophan residues of this protein.

In the second part, studies were carried out on determination of the sequence of the lectin using the advances of mass spectrometry and further to understand the immunological relationship between the lectins from the same plant. The primary structure and quantification technology developed during the study is expected to shed further light on our current understanding of *Dolichos lablab* lectins. Finally we have also explored to identify lectins in the vegetative parts of the *Dolichos lablab* plant that are homologous to the seed lectins.

CHAPTER II



PURIFICATION, BIOCHEMICAL AND IMMUNOLOGICAL CHARCTERIZATION OF DLL-II

PART A

2.1.0. Introduction

Lectins have been the subject of intense investigation over the last few decades owing to the variety of interesting biological properties exhibited by them making them very useful tools in biochemistry, biology and medicine (described in Chapter 1). Among the different lectins studied so far, those isolated from the legume seeds are the most widely studied as these seeds have been found to be very rich in lectin content [Sharon *et al*., 2003]. For several years, research in this area focussed on plant lectins. During this period, many lectins had been purified by affinity chromatography on immobilized carbohydrates, which laid the foundation to study their structure - function relation. Thus, owing to be readily available plant resources, lectins from this kingdom has laid important evolutionary relationship. The first definitive work on lectin binding proteins was reported from the seeds of jack bean and fava bean (Gansera etal., 1979). The amino acid sequences and 3-dimensional structures of many legume lectins have been determined, resulting in a detailed understanding of the evolutionary relationships in the primary, secondary, tertiary and quaternary structure among these proteins [Sharon and Lis, 2003; Strosberg *et al*., 1986; Rougé *et al*., 1991; Rini 1995; Van Damme *et al*., 1998a]. Additionally these studies have resulted in the development of a variety of applications for the legume lectins [Sharon and Lis, 2003]. However, lectins from the other plant families have not been studied in such detail and there is a need to purify and characterize the molecular properties and sugar-binding specificity of lectins from other plant families also.

Most of the legume lectins contain usually one type of lectin with a distinct sugar specificity. However, some leguminous seeds, like the common vetch, have two lectins with different sugar specificities. These two lectins lack immunological cross reactivity and have been designated as products of two distinct genes (Bauman *et al*., 1979). Research work in our laboratory is focused on the large-scale isolation, purification and characterization of animal and plant lectins (Siva Kumar and Von Figura, 2002; Rajasekhar *et al*., 1998; Rajasekhar *et al*., 1997) with a long term objective to understand their structure-function relationships. Seeds of the *Dolichos lablab* obtained in India contain two distinct varieties, field bean and lablab beans (Siva Kumar and Rajagopal Rao, 1986). Seeds of the field bean, *Dolichos lablab var. lignosus*, also contain two lectins, the glucose/mannose specific lectin (Siva Kumar and Rajagopal Rao, 1986) and galactose specific lectin (Mo *et al*., 1990). Seeds of the lablab bean, *Dolichos lablab var typicus*, contain the glucose/mannose specific lectin that exhibits similar properties to the

field bean lectin and has been well characterized. The complete primary sequence of the glucose/mannose specific lectin from the field bean seeds has been published. This lectin has a native molecular mass of 60,000 Daltons and is a tetramer consisting of two types of sub-units with apparent molecular mass of 15 kDa and 12 kDa respectively (Gowda *et al*., 1994). A cDNA of a lectin from the field beans has been cloned and has been shown to preserve the hematopoeitic factors in culture for one month (Colucci *et al.*, 1999). Several authors described the existence of glucose/mannose lectins from a variety of the *Dolichos lablab* (field bean) seeds (Favero *et al*., 1988; Guran *et al*., 1983; Rao *et al*., 1976; Silva-Lima *et al.*, 1988) whose properties differ apparently due to the different varieties of seeds used. Compared to the extensive studies carried out on the field bean seeds, work on the Indian lablab beans is limited. The glucose/mannose specific lectin from the Indian lablab beans was also affinity purified on goat IgM-Sepharose in our laboratory (Rajasekhar *et al*., 1997). In addition to the glucose/mannose specific lectin these seeds were also found to contain another lectin whose activity is inhibited by galactose. This lectin has been isolated by us employing conventional chromatography techniques as it failed to bind on different affinity matrices tested (Rajasekhar and Siva Kumar, 1998). The lectin exhibited a native molecular mass of 1, 20,000 Daltons and is possibly a tetramer. The stems and leaves of this plant were also found to contain the galactose lectin that cross reacts with the same antibody, suggesting that these two lectins may be related immunologically.

In the present study we have set the objectives to establish conditions that allow binding and affinity purification of the galactose-specific lectin from the seeds of *Dolichos lablab* (Wipro Seeds, Hyderabad, Lot No.KR 306) on Sepahrose-galactose gel. To separate the subunits and raise antibody for the larger subunit and to analyze its specificity and cross reactivity with the smaller subunit as well as with extensively characterized glucose/mannose lectin. To characterize the purified lectin biochemically and immunologically and to identify the important amino acids in the lectin that are essential for the sugar binding and lectin activity.

PART B

2.2.0. Materials and Methods

Seeds of *Dolichos lablab* (Indian lablab beans) were obtained from the local market. Sepharose 6B, galactose, divinyl sulfone, bicinchoninic acid and other chemical modification

reagents were all procured from Sigma Chem. Co., USA. Con A-Sepharose gel was obtained from Pharmacia (Sweden). All the chemicals and the reagents used in the present study were of high purity and obtained from reputed firms.

2.2.1. Preparation of Sepharose- DVS- Galactose affinity gel

25 mL of Sepharose 6B was washed thoroughly with double distilled water on a sintered glass funnel and the wet cake was suspended in 0.5 M Na_2CO_3 pH 11. 2.5 mL of DVS was added and rotated for 70 minutes at room temperature followed by washing with double distilled waster. It was later washed with 0.5 M Na_2CO_3 pH 10. Then, 5 gm of galactose (20% w/v) was added and rotated for 72 hrs at 4°C. The gel was washed with water and suspended in 0.5 M $NaHCO_3$ pH 8.5. To this, 0.5 mL of β-mercaptoethanol was added to block the unreactive sites and incubated for three hours at 4°C. Finally, the gel was washed with double distilled water and suspended in TBS until further use.

2.2.2. Extraction and purification of the lectin

100 g of seed powder was extracted overnight with 700 mL of 25 mM Tris buffered saline pH 7.4 (TBS) at 4 °C. The suspension was clarified by centrifugation and the clear supernatant (crude extract) was subjected to 0-60% and 60%-80% ammonium sulfate fractionation. This fractionation allowed the separation of the glucose/mannose lectin in the 0-60% fraction and the galactose lectin in the 60-80% fraction (Rajasekhar, 1998). The fraction containing the galactose lectin was dialyzed against TBS and passed through Sepharose-mannose gel to deplete the fraction of any glucose/mannose lectin. The unbound protein from the Sepharose-mannose gel was made to 1.5 M ammonium sulfate and applied on a Sepharose-galactose gel equilibrated with TBS containing 1.5 M ammonium sulfate (column buffer). The gel was thoroughly washed with the column buffer until the A_{280} was 0.05 and bound protein was eluted using 0.3 M galactose in column buffer (This concentration was necessary to completely desorb the lectin). Protein containing fractions were pooled, concentrated and dialyzed against TBS and stored at 4 °C. Purified lectin was used in all further studies.

2.2.3. Hemagglutination assay

This was carried out according to Siva Kumar and Rajagopal Rao (1986). By ear vein puncture, rabbit blood was collected into Alsevier's solution. It was centrifuged at 3000 rpm for 10 minutes at 4°C. The sedimented erythrocyte pellet was washed thrice with 0.9% saline

and the pellet was made to 4% suspension with saline. Processed erythrocytes were treated with 0.1% trypsin and incubated at 37°C for one hour. Erythrocytes were centrifuged at 3000 rpm for 10 minutes at 4°C and then made upto their original volume. To 200 µL of sample serially diluted in 200 µL of saline, 200 µL of the trypsin treated erythrocytes were added separately in a plexiplate and incubated at 37°C for one hour and the hemagglutination was visually observed (Fig. 2.1).

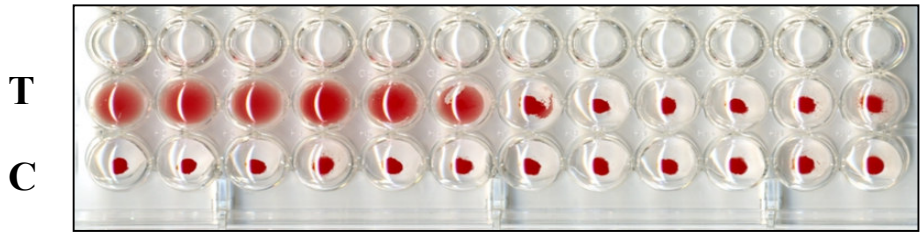


Figure: 2.1

"Carbohydrate binding proteins of non-immune origin which agglutinate cells and/or precipitates glycoproteins", a phenomenon referred to as Hemagglutination.
This figure shows general agglutination pattern of lectins. This is not from the lectin used in the study. [@] *One HU is defined as the amount of protein required to cause visible agglutination using rabbit erythrocytes. Figure displays the test and control lanes.*

2.2.4. Sugar inhibition studies

Hemagglutination inhibition assays with the purified lectin were performed as follows: 45 µL of different sugar solutions (0.1 M) were placed in the plate and serially diluted. Then, 10 µL of the purified lectin (1mg/ml) was added to each well. Later, 45 µL of 4 % erythrocyte suspension was added and the plate was incubated for 1 hour at 37 °C. Hemagglutination inhibition titer was scored visually.

2.2.5. Chromatography on Con A- Sepharose gel

Con A-Sepharose gel (0.5 mL) was equilibrated with TBS pH 7.4 containing 5 mM $CaCl_2$ and 5 mM $MnCl_2$ (column buffer). 1 mg/mL of the purified lectin was applied on Con A-Sepharose and washed with column buffer till the A_{280} was zero. The bound lectin was eluted sequentially using 0.15 M Methyl α-mannoside in column buffer.

2.2.6. Molecular weight determination

The native molecular mass of the lectin was determined using Sephadex G-200, gel filtration column (60 cm x 1.2 cm) equilibrated with 0.9% NaCl. The column was calibrated with proteins of known molecular weight viz., lactose specific lectin from *unio* (Mr 120 kDa), peanut agglutinin (Mr 110 kDa), bovine serum albumin (Mr 66 kDa) and ovalbumin (45 kDa). 2.0 mL fractions were collected; peak fractions were pooled, concentrated, dialyzed and used for further studies. The homogeneity of the protein was further confirmed by using a Pharmacia FPLC on a column of Superose-12 (30 × 1 cm) gel filtration column that was already calibrated with standard proteins [This experiment was carried out in the laboratory of Prof. M. J. Swamy, School of Chemistry, University of Hyderabad]. About 1 mg of the protein in 1 ml of PBS containing 0.1 M galactose was loaded onto the column pre-equilibrated with the same buffer. Chromatograms were run at a flow rate of 30 ml/hour and the elution was monitored by A_{280}. Elution volumes were determined from the retention times obtained from the chromatographic traces. The molecular mass of the *Dolichos lablab* seed lectin was determined by calibrating with standard proteins.

2.2.7. Protein estimation by the Bicinchoninic acid (BCA) method

Protein estimation was done using BCA reagent with BSA as standard following manufacturer's instructions.

BCA reagent: 10 mL of BCA and 0.5ml of 4% copper sulfate.

The volume of the protein sample was made upto 500 µL with distilled water and mixed with 1.0 mL of BCA reagent and incubated for 30 minutes at 37^0C. After incubation the absorbance was measured at 562 nm

2.2.8. Carbohydrate estimation

Carbohydrate content in the lectin was determined by the phenol - H_2SO_4 method (Dubois *et al*., 1956). Glucose was used as the standard.

2.2.9. Temperature stability

The lectin (1 mg/mL concentration) was incubated at different temperatures of 4 °C, 30 °C, 40 °C, 50 °C, 60 °C, 70 °C, 80 °C and at 90 °C for a period of 30 minutes in the presence and absence of the inhibitory sugar. The samples were brought back to room temperature and their activity of agglutinating erythrocytes was determined.

2.2.10. pH stability

The lectin (1 mg/mL concentration) was incubated at different pH's of 3-12 for a period of 60 minutes in the presence and absence of the inhibitory sugar. The samples were tested for their activity of agglutinating erythrocytes.

2.2.11. Native -Polyacrylamide gel electrophoresis

Native PAGE was performed using Tris - glycine buffer pH 8.3 on a 7.5% gel (Reisfeld *et al*., 1966).

2.2.12. Sodium dodecyl sulphate -Poly Acrylamide Gel Electrophoresis

The electrophoretic separation of proteins was done by discontinuous SDS-PAGE according to Laemmli (1970).

Tank buffer: 3g Tris, 14g Glycine and 1g SDS dissolved in 1 litre of double distilled water

2x Sample buffer: (Reducing) 0.5M Tris-HCl pH 6.8 2.5 mL, 10% SDS 4.0 mL, 100% glycerol 2.0 mL, β-mercaptoethanol 1.0 mL, bromophenol blue 0.05% and made up the volume to 10 mL

Resolving gel buffer: 1.5 M Tris-HCl pH 8.8

Stacking gel buffer: 0.5M Tris-HCl pH 6.8

Acrylamide: 30% (w/v) Acrylamide, 0.8% (w/v) N N' methylenebisacrylamide

Ammoniumperoxide sulfate (APS): 10% (w/v) in water

Resolving gel and stacking gels are made as shown in the **Tables** respectively. APS and TEMED must be added at the end to resolving gel and poured into the sealed glass plates (mini gel). After polymerization the gel was rinsed with water. Stacking gel solution was poured, a comb with required number of wells was inserted and allowed to polymerize for 30 min. Wells were rinsed with water. The samples were cooked at 95^0C for 5 minutes with sample buffer mixed in 1:1 ratio, centrifuged briefly and the supernatant loaded into the wells. These were overlaid with tank buffer and placed in the electrophoretic chamber. Samples were subjected to electrophoresis for 2-3 h at 50 100 V at room temperature.

% of PAA gel	7.5%	10%	12.5%
H_2O (mL)	3.650	3.025	2.400
Resolving gel buffer (mL)	1.875	1.875	1.875
Acrylamide (mL)	1.875	2.500	3.125
10% SDS (µL)	75	75	75
APS (µL)	40	40	40
TEMED (µL)	10	10	10

Table: Reagents used for the preparation of resolving gel with different polyacrylamide concentration.

H_2O (mL)	1.875
Acrylamide (mL)	0.375
Stacking gel buffer (mL)	0.3125
10% SDS (µL)	25
APS (µL)	40
TEMED (µL)	8

Table: Reagents used for the stacking gel.

2.2.13. Separation of the subunits by Electroelution

The subunits were separated on a SDS-PAGE and visualized by colloidal coomassie blue staining. The subunits were excised from the gel and cut into rectangular pieces, transferred to a dialysis membrane containing the running buffer *(25 mM Tris-Glycine, pH 8.5 with 0.1 % SDS; 50 mM Tris-Acetate, pH 7.8 with 0.1 % SDS; 100 mM Sodium bicarbonate, pH 7.8 with 0.1 % SDS)* and was electrophorese for 2 hours at 30-50 Volts. The Sample in the dialysis tube was pipette out carefully and estimated the protein concentration.

2.2.14. Silver Staining of the gel

This was carried out according to Blum *et al*., (1987).

Reagents: Methanol, Ethanol, $AgNO_3$, Hypo, Na_2CO_3, Formaldehyde and Acetic acid.

All the following steps were carried out at room temperature by placing the gel on a rocking platform.

1. Fixative: 50% methanol, 12% glacial acetic acid, 50 µL of formaldehyde (HCHO) was taken in a flask and the volume made up to 50 mL with double distilled water. The gel was soaked for 45min to overnight. (Fixative can be stored in a brown bottle and can be reused 4-5 times).
2. The gel was transferred to 50% ethanol and incubated for 1 hour.
3. The gel was rinsed 3 times with double distilled water.
4. 10 mg Hypo was dissolved in 50 mL of double distilled water. The gel was soaked exactly for 1 min. in the above solution with constant shaking.
5. The gel was rinsed 3 times with double distilled water.
6. 100 mg $AgNO_3$ was dissolved in 50 mL of double distilled water. To this 18 µL of formaldehyde was added. The gel was soaked in the above solution for 20 min. with constant shaking.
7. The gel was rinsed 3 times with double distilled water
8. Developer: 3 g of Na_2CO_3 was dissolved in 50 mL of distilled water. To this 26 µL of formaldehyde was added. The gel was soaked in this solution to visualize the protein bands.
9. The gel was rinsed with double distilled water.
10. The developing reaction was stopped by the addition of 12% glacial acetic acid.

The gel was washed twice with double distilled water.

2.2.15. Per iodic acid Schiffs staining

To determine the carbohydrate nature of the protein qualitatively, periodic acid - schiffs staining was carried out following the method of Zacharius *et al.,* 1969, with little modifications. Schiffs reagent was prepared as follows: 1 gm of Basic Fuschin was added to 200 mL of water at 70 °C. This was boiled for few minutes, cooled and filtered. The temperature was adjusted to 50 °C. To this 5 mL of HCl and 2 gm of potassium metabisulphite were added and incubated overnight. The solution turns colorless or pale straw yellow. To decolourise completely 0.25 gm to 0.5 gm of activated charcoal was added and filtered. This stain should be tightly stoppered and stored at 4 °C. SDS-PAGE gel was stained in 1% periodic acid in 3% acetic acid for one hour. The gel was washed for one hour with water and stained in Schiffs reagent for 30 minutes in dark. It was then destained with 10% acetic acid and finally stored in 3% acetic acid.

2.2.16. Raising antibodies to the purified lectin and to the isolated 31 kDa subunit

In an earlier work, for the purified lectin antibodies were raised and in the present study, the two subunits of the lectin corresponding to 31 kDa and 29 kDa, respectively were separated by SDS-PAGE and the 31 kDa protein band was excised from several gels and the protein electroeluted. After confirming its mobility on the SDS-PAGE to be 31 kDa, the protein was injected into a rabbit as follows: 1 mg/mL of the purified lectin was emulsified with an equal volume of Freund's complete adjuvant and was injected subcutaneously into a rabbit. At subsequent intervals of 3 weeks and 5 weeks a booster dose of the protein in Freund's incomplete adjuvant was given. Ten days after the second booster injection, blood was collected from the ear vein, allowed to clot and the serum was collected by centrifugation. The specificity of the antiserum was checked by Western blot analysis as described above using the intact lectin, as well as the isolated subunits of the lectin.

2.2.17. Western blot analysis:

This was carried out according to Towbin *et al*., (1979). A 12 % SDS-PAGE gel was run with the purified lectin and the proteins were transferred to a nitrocellulose membrane. The membrane was blocked with 5% defatted milk powder in TBS buffer pH 7.4 for 1 hour. The membrane was incubated with antiserum of galactose specific lectin as the primary antibody (1:1000 dilution) for one hour in TBS buffer pH 7.4 containing 1% defatted milk powder. The membrane was washed with TBS, TBS Tween followed by TBS for 5 minutes each and blocked for 5 minutes with 5% defatted milk powder in TBS buffer. The membrane was later incubated with secondary antibody, goat-anti rabbit IgG conjugated with alkaline phosphatase for 1 hour in TBS buffer containing 1% defatted milk powder. The membrane was washed with TBS, TBS Tween followed by TBS and then with distilled water. The blot was developed with BCIP/NBT substrate until the bands were visualized and then the blot was washed with water to stop the reaction. The membrane was air dried and photographed.

2.2.18. Chemical modification studies

Purified lectin was modified by using various group specific reagents that alter the amino acid side chains. Lysine, arginine, histidine, tyrosine and tryptophan residues were modified as described [Latha *et al*, 2006]. After the modifications, in each case, the modified protein was separated from rest of the reagents on a Sephadex G-50 gel filtration column (0.5 cm x 10 cm). Protein containing fractions were pooled and analyzed for the extent of modification, hemagglutinating activity and its ability to react with the native lectin antibody. Since purified

lectin binds on Con A-Sepharose gel even in the absence of high salt concentration, this gel was used to test the binding ability of the modified lectins. Unmodified protein sample served as control where specified protection experiments were carried out using 0.1 M galactose during modification.

Modification of the Histidine residues

This was carried out according to Melchior and Fahrney (1970). To 3 mg/mL of protein in 10 mM phosphate buffered saline pH 7.4, 40 µL of DEPC (10 µL of DEPC diluted to 600 µL with distilled ethanol) was added in aliquots for two hours at room temperature. The reaction was stopped by the addition of 20 mM histidine. The absorbance of the modified protein was then measured at 250 nm and 280 nm. Modification was reversed by incubating the modified lectin at 37 °C for two hours. The lectin was also pre-incubated with 0.1 M galactose and modification was performed as described above in order to substantiate the role of histidine residues in the lectin binding.

PART C

2.3.0. Results

2.3.1. Extraction and purification of the lectin

Seed extracts of the *Dolichos lablab* (Indian lablab beans) obtained in India have been shown to contain two distinct lectins a glucose/mannose specific lectin and a galactose specific lectin. In the present study the 60-80 % ammonium sulphate fraction obtained from the seeds contained the galactose lectin. This was passed through the Sepharose-galactose gel equilibrated with 25 mM TBS pH 7.4, in the presence of high salt concentration (1.5 M ammonium sulfate). The lectin was bound on the gel specifically and could be completely desorbed with 0.3 M galactose in the same buffer (Fig. 2.2). From 100 g of the seed powder 150 mg of purified lectin was obtained as estimated by bicinchoninic acid method.

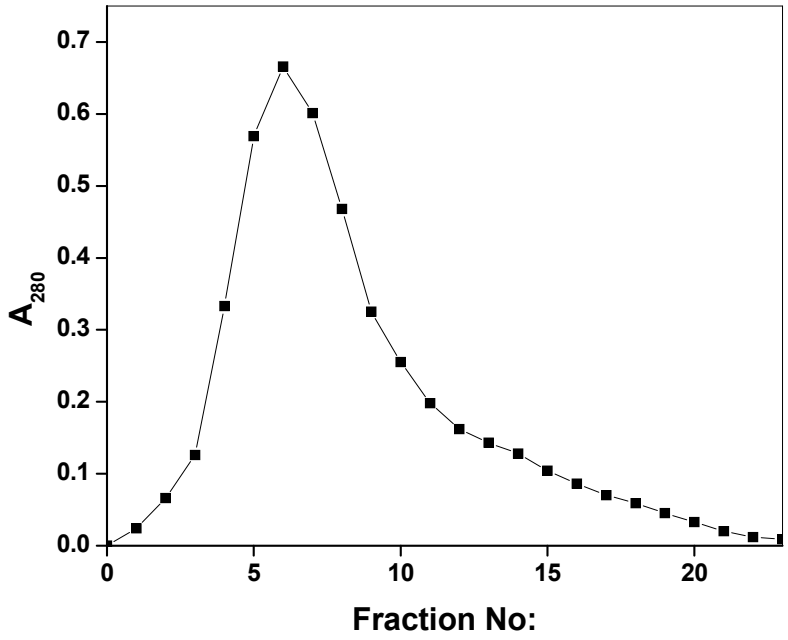


Figure: 2.2

Affinity purification of the galactose-specific lectin
To the unbound fraction from Sepharose-DVS-Mannose gel, 1.5 M ammonium sulphate at a flow rate 20 mL/hour. 0.3 M galactose was used for elution. 5 mL fractions were collected and their absorbances were recorded at 280 nm.

2.3.2. Con A-Sepharose chromatography

Purified *Dolichos lablab* lectin was found to be a glycoprotein consisting of about 5 % carbohydrate as estimated colorimetrically. The glycoprotein nature of the lectin was further confirmed by its ability to bind on Con A-Sepharose gel and its specific elution using 0.15 M methyl α-mannoside sugar. In a typical experiment, 0.45 mg protein could be specifically bound to 100 μL of gel.

2.3.3. Sugar inhibition studies

The carbohydrate binding specificity of the *Dolichos lablab* seed lectin was studied by carrying out sugar inhibition assays as described under methods. Among a number of monosaccharides tested for inhibition of lectin activity, galactose and its derivatives like N-acetylgalactosamine and MeβGal were found to inhibit the lectin activity. Other sugars such as glucose, mannose, their methyl glycosides, and N-acetylglucosamine were non-inhibitory upto 100 mM concentrations. The results of the sugar inhibition data are given in Table 2.1.

Sugar	Minimum Concentration for Inhibition (mM)	Relative Inhibitory potency (Galactose=1.0)
Galactose	2.82	1.0
MeβGal	1.40	2.0
MeαGal	11.3	0.25
GalNAc	5.63	0.5
Galactosamine	22.5	0.12
2-DeoxyGal	45.0	0.06
pNPαGal	5.63	0.5
pNPβGal	1.40	2.0
Lactose	5.63	0.5
Lactulose	11.3	0.25
Melibiose	5.63	0.5

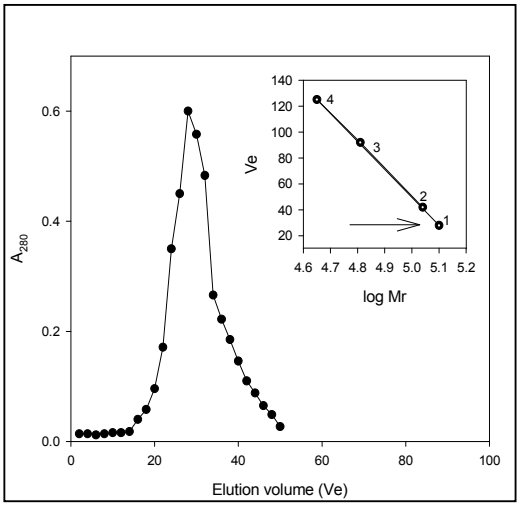
Table: 2.1

Experiment was performed with trypsin treated erythrocytes with a final lectin concentration of 50 µg/mL. Glucose, mannose and GluNAc did not inhibit the hemagglutinating activity of Galactose-specific lectin up to 100mM concentration. Values shown are the average of 2 experiments.

2.3.4. Native and subunit molecular weight determination

The *Dolichos lablab* lectin eluted as a single peak from Sephadex G-200 (Fig. 2.3-A) suggesting it to be homogeneous and was further confirmed by applying it on superpose-FPLC chromatography, where the protein eluted as a single peak (Fig. 2.3-B). Calibrating both gels with proteins of known molecular weights the native molecular mass of the affinity purified lectin was found to be 120 ± 5 kDa. However, in SDS-PAGE, the lectin dissociated into two subunits with molecular masses of 31 kDa and 29 kDa, respectively (Fig. 2.4-A), suggesting the lectin to be tetrameric in nature. The separated and electroeluted subunits migrate to same as the intact protein (Fig. 2.4-B).

A



B

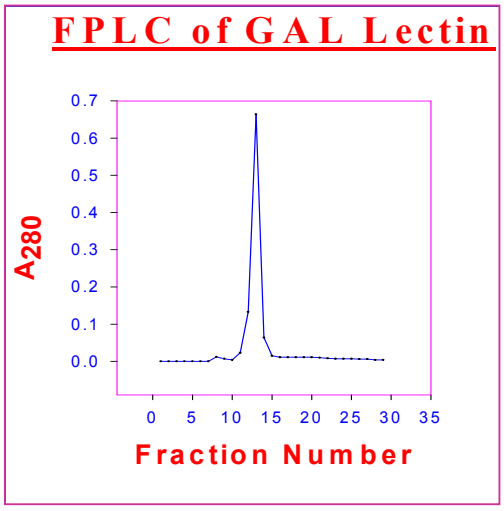


Figure: 2.3

(A) Molecular weight determination of the galactose specific seed lectin
Insert shows the proteins of known molecular weight used on the column - 1. Lactose specific lectin from unio (Mr 120 kDa), 2. Peanut agglutinin (Mr 110 kDa), 3. Bovine serum albumin (Mr 66 kDa) and 4. Ovalbumin (Mr 45 kDa). Arrow indicates position of the lablab bean lectin (Mr 120 kDa) that elutes at the same elution volume as the lactose specific lectin from unio.
(B) Fast Performance Liquid Chromatography of GAL Lectin
The lectin eluted as a single peak on a Superose- 12 FPLC column suggesting it to be homogenous.
Specifications
Column Volume -30 ml
Flow rate -0.5 ml/min
Fraction volume -1 ml

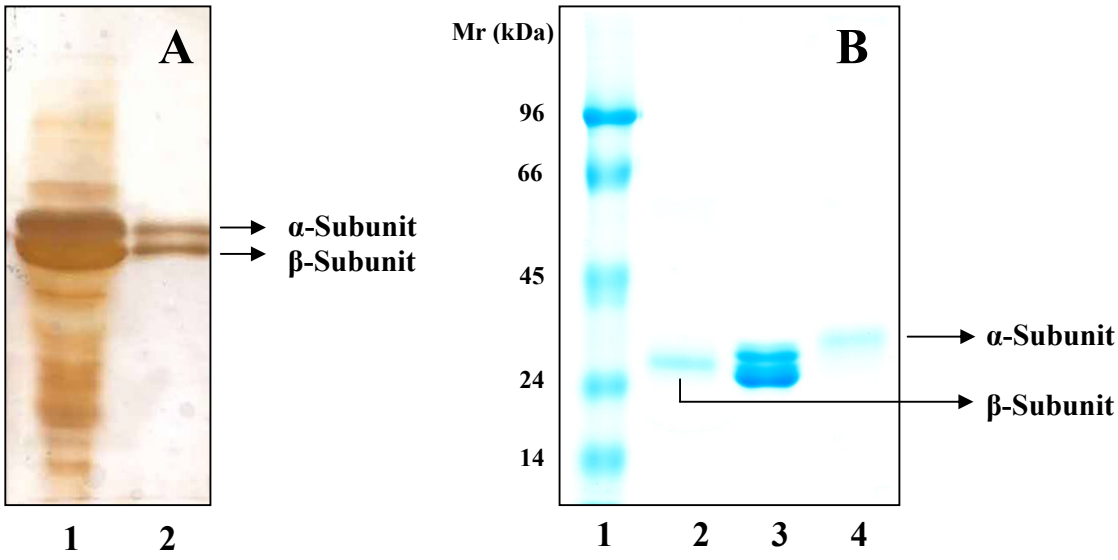


Figure: 2.4

(A) 12% SDS-PAGE analysis of the Galactose specific lectin.
Lane 1: Affinity purified lectin, (In some preparation, at very high concentrations of the lectin, minor additional bands could seen). Lane 2: Sephadex G-200 eluate. Arrow indicates the position of the galactose specific lectin of larger sub-unit of molecular mass 31 kDa and the smaller sub-unit of molecular mass 29 kDa. For all studies, the lectin obtained after gel-filtration was used.
(B) 12% SDS-PAGE analysis of the Separated Subunits.
Lane 1: Molecular weight marker, lane 2: Beta Subunit (29kDa); lane 3: Intact Lectin; lane 4: Alpha Subunit (31kDa).

2.3.5. Raising antibodies to the isolated 31 kDa subunit

Antibodies to the isolated 31 kDa subunit were raised as described under methods. The specificity of the antiserum was tested in a western blot experiment. In a western blot experiment, both native lectin (Fig. 2.5-A) and subunits reacted with the antisera suggesting the specificity of the antibody and the subunit nature of the protein (Fig. 2.5-B). Since both

subunits react with the antibody raised for the native protein it is logical to conclude that these subunits together form the protein. Further the subunits stained with the per iodic schiff base, suggesting the glycoprotein nature of the protein (Fig.2.5-C).

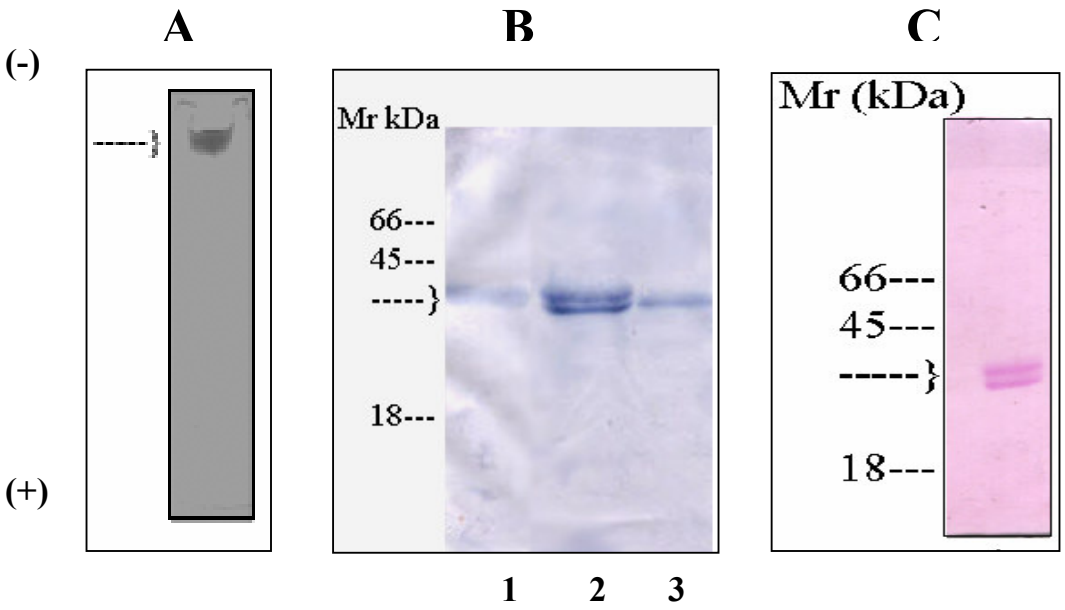


Figure: 2.5

(A) Native PAGE analysis of Galactose-specific lectin
(B) 15% SDS-PAGE and Western blot analysis of the Galactose-specific lectin
The purified protein and separated subunits were probed with the antibody raised to the 31 kDa subunit. Lane 1 shows the immuno-reactivity of the larger subunit (31 kDa) to the antibody, lane 2 indicates the native protein with both the subunits, and lane 3 shows the cross-reactivity of the smaller subunit (29 kDa) to the antibody. Arrow indicates the position of the galactose specific lectin.
(C) Periodic acid Schiff's (PAS) staining of the Galactose-specific lectin

2.3.6. Temperature stability and pH optimum of the purified lectin

The hemagglutination activity of the lectin was shown to maximum at 4 ºC. Increase in temperature till 40 ºC displayed the same activity suggesting it to be stable till 40 ºC. Beyond 40 ºC, the stability of the purified lectin decreased and at 90 ºC the lectin had negligible activity (Fig. 2.6-A).

The purified lectin showed optimal hemagglutination activity at pH 7.4, suggesting this to be the pH optimum for the lectin. At pH values of 4.0 and 5.0 there was mere activity but at pH 8.0 and 9.0, there was 80% and 20% activity, respectively (Fig. 2.6-B).

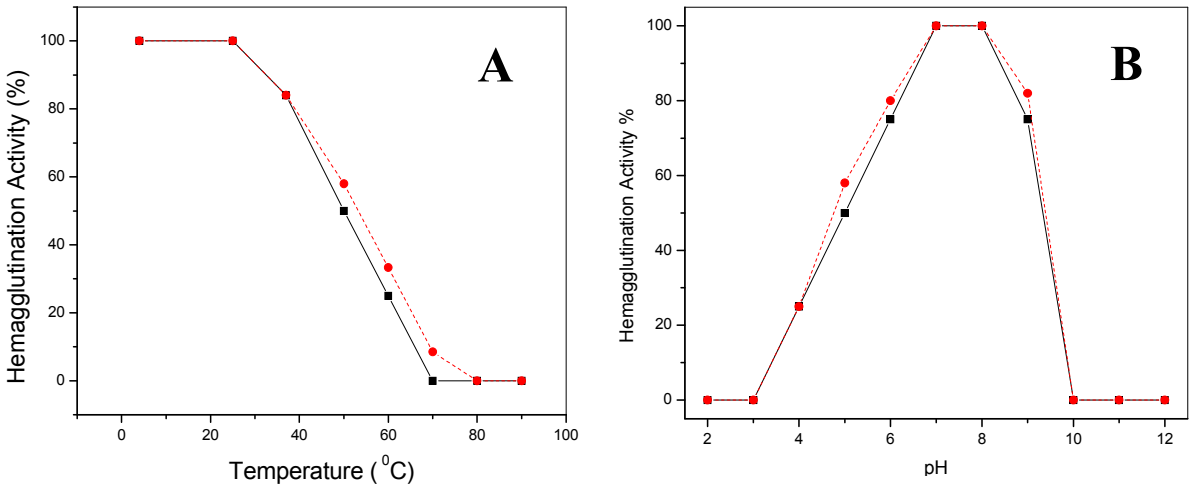


Figure: 2.6

(A) The thermal stability, the lectin (1 mg/ mL concentration) was incubated at different temperatures of 4, 30, 40, 50, 60, 70, 80, and 90 °C for a period of 30 min in presence and absence of sugars (0.1 M) and the hemagglutination activity was further tested. Solid lines represent lectin in absence of sugar and the dotted lines represent in presence of sugar.
(B) The purified lectin (4 mg/mL) was dissolved in and dialyzed against buffers of different pHs in presence and absence of sugars (as described above) and the hemagglutination activity was further tested to determine the pH optimum.

2.3.7. Chemical modification studies

In the present study various chemical modifications of the purified galactose lectin were carried out in the absence as well as in the presence of the inhibitory sugar to demonstrate the involvement of specific amino acids in the sugar binding site of this lectin. In each case the extent of modification and the effect of modification on the biological activity of the lectin, its immuno-reactivity, as well as, its ability to bind on Con A-Sepharose gel was assessed. Modified lectin showed 75 % loss in hemagglutinating activity and about 70 % loss in its binding ability to Con A-Sepharose gel. Reversal of modification was achieved by incubating the modified sample at 37 °C for 2 hours. However, modification performed in presence of the inhibitory sugar 0.1 M galactose, revealed that only 7 histidine residues were modified suggesting that the binding sugar protected the histidine residues in the active site of the lectin [Latha *et al*., 2006].

From the protection experiments with galactose as the ligand, it is evident that the imidazole groups of histidine are present in the sugar binding pocket of the lectin, as non-inhibitory sugar glucose did not show any protection (Fig 2.7).

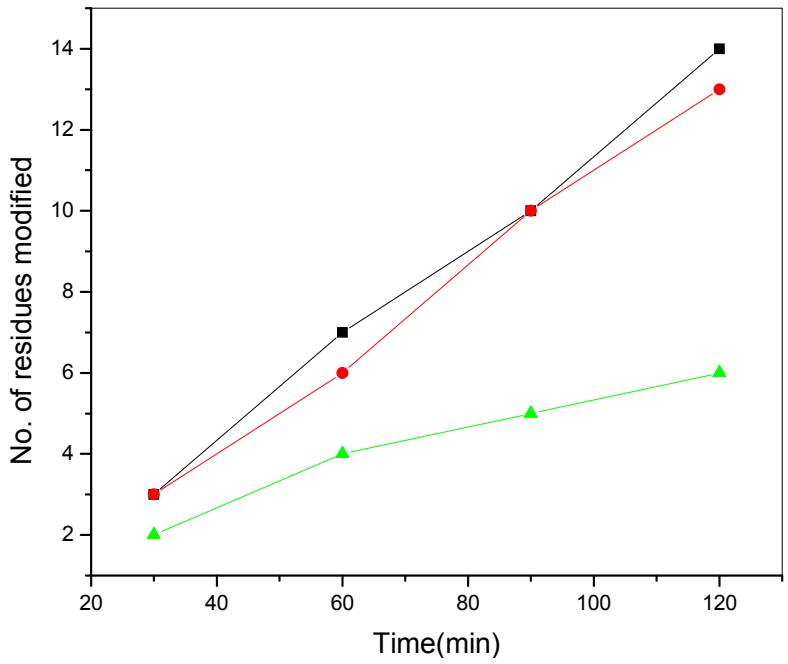


Figure: 2.7

Protection of histidine modification of *Dolichos lablab* seed lectin by 0.1 M Galactose
The lectin without sugar (squares), with 0.1 glucose (circles), and with 0.1 M galactose (triangles) have been modified with DEPC and the number of histidine residues modified at specific time intervals has been determined.

PART D

2.4.0. Discussion

The glucose/mannose specific lectin has been well characterized [Gowda *et al*., 2004, Siva Kumar, 1999]. Though the galactose-specific lectin was identified and isolated by conventional methods of protein purification [Rajasekhar and Siva Kumar, 1998] it has not been well characterized and no affinity method was established earlier for the purification of this lectin. In the present study an affinity methodology has been developed for purification of lectin in presence of 1.5 M ammonium sulfate, in absence of which the lectin binding to galactose matrix was not seen. In addition we also found that the same galactose matrix binds other galactose-specific lectins such as those from the seeds of Momordica *charantia* and soyabean in the absence of salt. *Erythrina crystagalli* galactose-specific lectin was also purified using Sepharose-galactose gel [Iglesias *et al*., 1982]. Since the bound lablab bean lectin could be eluted using 0.3 M galactose, taken together these observations suggest that the gel matrix used in this study contains sufficient added galactose and the binding of the lablab

bean lectin to the matrix, is possibly sugar specific. However we cannot rule out the possibility that the galactose lectin studied here also binds to the affinity gel in presence of high salt through hydrophobic interactions. Several glycosidase enzymes have been shown to specifically bind to phenyl Sepharose gel in presence of high salt [Rajasekhar and Siva Kumar, 1997]. Sepharose gel alone was not useful to bind the lectin in the presence or absence of high salt. The fact the lectin bound on Sepharose-galactose gel can be eluted specifically only with the galactose sugar suggests that the lectin interacts predominantly with the sugar on the gel. In our laboratory several monosaccharides such as mannose, N-acetylglucosamine, and galactose, were coupled to Sepharose via divinyl sulfone and successfully used for the isolation of the corresponding sugar specific lectins by affinity chromatography. Additionally, Sepharose-lactose and Sepharose-phosphomannan gels were also prepared and have been used for the affinity purification of some animal lectins [Siva Kumar *et al*., 2004]. The glycoprotein nature of the lablab lectin was clearly understood by colorimetric assay and binding of the lectin to Con A Sepharose gel.

Inhibition of hemagglutination activity by galactose and its derivatives like N-acetylgalactosamine and MeβGal, and no alteration in the activity with other monosaccharides such as glucose, mannose, their methyl glycosides, and N-acetylglucosamine upto 100 mM concentrations explains the specificity of the lectin for galactose sugar. Moreover it is apparent from the studies that 2-deoxygalactose is 16 fold weaker compared to galactose in inhibiting the lectin activity, indicating that the equatorial hydroxyl group on C-2 of galactose is an important locus for carbohydrate binding of the lectin. The inhibitory potency of MeβGal is 8 times greater when compared to it's α anomer indicating that the equatorial position at the anomeric position is better recognized. Substitution at the C-2 with an amino group does not have any significant inhibitory effect as compared to galactose. These results suggest that the activity of the *Dolichos lablab* lectin is best inhibited by galactose.

On the other hand a hydrophobic moiety attached at the anomeric position seems to make it a better inhibitory sugar, as pNP α-galactoside was found to be better inhibitory as compared to the methyl α-galactoside. However, there was no difference between pNP β-galactose and methyl β-galactose in their inhibitory effects. Disaccharides such as lactose (Galβ1-4Glc), lactulose (Galβ1-4Fru) and melibiose (Galα1-6Glc) were less inhibitory compared to galactose. These results are comparable to other galactose specific lectins such as the *Trichosanthes dioica* seed lectin described recently [Sultan *et al*., 2004].

The native molecular mass of the affinity-purified lectin was found to be 120 $\pm$ 5 kDa. However, in SDS-PAGE, the lectin dissociated into two subunits with molecular masses of 31

kDa and 29 kDa, suggesting the lectin to be tetrameric in nature. The glucose/mannose specific lectin from the same seeds exhibits a native molecular mass of 60 kDa and is a tetramer composed of two types of subunits, 15 kDa and 12 kDa, respectively [Gowda et al., 2004]. An antibody to this lectin also cross-reacts with the affinity purified galactose lectin.

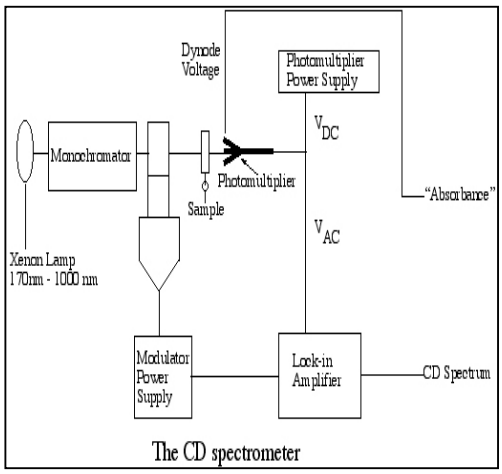
Antigenic homology was seen for both subunits as the antibody raised against the specific 31 kDa subunit was cross reacting with both the subunits. This suggested, structural similarity among both subunits, and it was already known that the two subunits had identical amino acid sequences at the amino terminal end (The first 10 residues in both the subunits). It is therefore not surprising that the antibody raised to the 31 kDa subunit cross-reacts with the 29 kDa subunit. When these sequences were aligned with some known legume lectin sequences, some amino acids were found to be highly conserved. The fact that the sequence of the galactose-specific lectin studied here, is not exactly identical to the glucose/mannose lectin or the related lectins (FRIL), suggests that it is distinctly a new protein. Elution of lectin to homogeneity from Sepharose galactose gel at 4˚C and pH 7.4 demarks as the optimum conditions for purification and alterations in these conditions showed weak binding.

Chemical modification studies carried out on different lectins suggests the importance of histidine residues in sugar binding particularly for the galactose-specific lectins. For example, there was a total loss in activity when the histidine residues in the lectin isolated from *Saccharomyces cerevisiae* were modified [Kundu *et al*., 1987]. Chemical modification studies on the glucose/mannose specific lectins from the field and lablab beans showed 95 % loss in activity upon lysine modification and a total loss in activity when tyrosine and tryptophan residues were modified. Histidine modifications led to 50 % loss in activity. Further, there was no change in the immunological property of the modified lectin [Siva Kumar, 1999]. Histidine modification led to a complete loss of activity of the snake gourd seed lectin (SGSL) [Komath *et al*., 1998]. In a recent report chemical modification studies on *Trichosanthes dioica* lectin revealed that tyrosine residues are important for carbohydrate binding and hemagglutinating activity [Sultan *et al*., 2004]. Four lines of evidence suggest the involvement of histidine residues in the sugar binding site of the lablab bean galactose lectin. First, histidine modified sample exhibits only 25 % hemagglutinating activity as compared to the control. Second, there is only 32 % binding of the modified lectin to the affinity gel. Third, there is partial reversal of modification, which leads to better biological properties than the modified protein [Latha *et al*., 2006]. Fourth, protection of modification was seen when the lectin was preincubated with 0.1 M galactose prior to modification while glucose had no effect. The galactose-specific lectin shows involvement of histidine residues in the sugar binding unlike the glucose/mannose

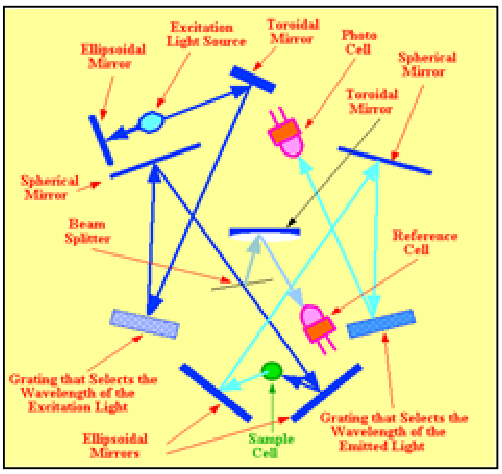
specific lectin which shows the involvement of tyrosine and tryptophan residues in the sugar binding.

In summary, detailed studies carried out on the affinity purified galactose-specific lectin clearly demonstrate that the buffer conditions used, facilitated affinity purification of the lectin. Several lines of evidence suggest that the affinity purified protein is a galactose-specific lectin representing a second lectin from the seeds of lablab beans which shares some properties with the well characterized glucose/mannose lectin, such as its ability to agglutinate rabbit erythrocytes, glycoprotein nature etc,. However, it can effectively be purified to homogeneity on Sepharose-galactose gel only in the presence of 1.5 M ammonium sulfate in buffer. The lectin is a glycoprotein with about 5 % carbohydrate content and binds to Concanavalin A specifically. It exhibits typical tetrameric nature possibly consisting of $\alpha_2\beta_2$ subunits. The amino terminal sequences of both the lectin subunits are identical and also exhibit some degree of homology to the other legume lectins. Chemical modification studies (protection experiments) carried out suggests the importance of histidine residues in sugar binding. Only a few legume seeds have been shown to contain more than one type of lectin, which differ in their sugar specificity [Baumann *et al*., 1979].

CHAPTER III



The CD spectrometer



BIOPHYSICAL CHARACTERIZATION OF DLL-II

PART A

3.1.0. Introduction

Due to their presence in large quantities in plant seeds, the plant seed lectins, especially a large number from the legume seeds have been characterized well with respect to physico-chemical properties and carbohydrate-binding specificity and the forces that govern their interaction with various saccharides. Structural studies on legume lectins also led to a deeper understanding on the evolutionary relationships among the legume species [Sharon, 1993; Elgavish *et al*., 1997; Sharma *et al*., 1997; Vijayan *et al*., 1999]. Much of this may be attributed to the fact that many legumes are commercially important and are produced in large quantities. Studies characterizing the lectins from other plant families have been considerably fewer and correspondingly the knowledge available on them is also much less.

Studies on the intrinsic fluorescence properties have been widely used to obtain information about protein structure and conformational changes induced by alteration of environment and/ ligand binding [Lakowicz, 1999; Eftink *et al*., 1981; Grinvald *et al*., 1976]. Intrinsic fluorescence of proteins is dominantly due to the presence of tryptophan residues present in the protein. A valuable feature of this protein fluorescence is the high sensitivity of tryptophan residues to its local environment. Also tryptophan appears to be uniquely sensitive to collisional quenching, due to a tendency of indole to donate electrons while in the excited state. Tryptophan can be quenched by externally added quenchers or by nearby groups in the protein. The intrinsic fluorescence of proteins arises primarily from the side chains of tyrosine and tryptophan residues. By exciting the protein sample at 295 nm or above, where tyrosine residues do not absorb, it is possible to study the fluorescence due to tryptophan alone [Lakowicz, 1999]. Changes in the fluorescence characteristics of tryptophan residues are used widely to obtain information about conformational transitions in proteins, association of subunits in oligomeric proteins, protein unfolding or ligand binding to proteins [Lakowicz, 1999; Eftink and Ghiron, 1981; Das *et al*., 1981; Kakitani *et al*., 1987]. Hence there are many reports on the study of tryptophan fluorescence. Tryptophan residues present in the active site of protein or on the exterior are relatively easy to study and a large number of studies using chemical modifications and/fluorescence techniques have been published, elucidating the environment and role of these residues in such proteins [Privat *et al*., 1980; Peterman *et al*., 1979, 1980; Patanjali *et al*., 1984]. However, buried residues are generally much more difficult to study. All the tryptophan residues of DLL-II appear to be buried deep within the protein matrix, as they were not accessible, and thus provide an interesting problem to study.

Although as indicated in chapter 2, DLL-II has been extensively characterized biochemically and immunologically, it is not studied in detail with respect to the structural aspects. In the present study the secondary structure of this protein has been investigated by circular dichroism and the environment and accessibility of the tryptophan residues in it have been probed by fluorescence quenching studies. Systematic studies have been carried out on the intrinsic fluorescence of DLL-II in the native state, in the presence of ligand (Lactose), and upon denaturation. The accessibility of the fluorescent tryptophan residues has been investigated by quenching studies employing a neutral quencher, acrylamide, a cationic quencher, cesium ion (Cs^+) and an anionic quencher, iodide ion (I^-). In addition thermal unfolding of the protein has been studied by CD spectroscopy.

PART B

3.2.0. Materials and Methods

Dolichos lablab seeds [Chapter 2]. Potassium iodide was obtained from Qualigens (Mumbai, India). Cesium chloride was obtained from Loba-Chemie (Mumbai, India). Acrylamide (spectroscopic grade), lactose, MeβGal, mannose, Tris and bicinchoninic acid reagent used in protein assay were obtained from Sigma (St. Louis, MO, USA). Seralose 4B (which is similar to Sepharose 4B) was purchased from SRL Chemicals (Mumbai, India) and Sephadex G-200 was obtained from Pharmacia (Uppsala, Sweden). All other chemicals used were obtained from local suppliers and were of the highest quality available. Double distilled water was used for preparing all the buffers. Galactose and mannose were coupled to Seralose 4B as reported earlier

3.2.1. Purification of *Dolichos lablab* galactose specific lectin (DLL-II)

The DLL-II galactose-specific lectin was purified by affinity chromatography as reported previously [Chapter 2]. All experiments were performed in TBS unless otherwise stated.

3.2.3. Steady state fluorescence spectroscopy

All emission spectra were recorded on a Spex Fluoromax-3 fluorescence spectrometer from Jobin-Yvon (Edison, NJ, USA, website: http://www. jobinyvon.com). Slit widths of 3 and 6 nm were used on the excitation and emission monochromators, respectively. Measurements were performed by irradiating lectin samples (OD280 $\leq$0.1) with 295 nm wavelength light, in

order to selectively excite tryptophan residues of the protein and emission spectra were recorded above 300 nm. In fluorescence quenching experiments, small aliquots of 5 M quencher stocks (acrylamide, potassium iodide, or cesium chloride) were added to protein samples and fluorescence spectra were recorded after each addition. The final quencher concentration attained in each case was 0.52 M. The iodide stock solution contained 0.2 mM sodium thiosulphate to prevent the formation of triiodide (I_3^-). For quenching studies with denatured lectin, the protein was incubated with 6M Gdn–HCl overnight at room temperature. For experiments with sugar-bound lectin, the lectin samples and the quencher stocks were made 0.1 M in lactose, a sugar that is specifically recognized by the lectin. Fluorescence intensities were corrected for volume changes before further analysis of the quenching data. All measurements were performed in duplicate at 25 °C and yielded reproducible results. The average values obtained from these are reported.

3.2.4. Circular dichrosim spectroscopy

Circular dichrosim (CD) spectra were recorded on a Jasco J-810 spectropolarimeter (Jasco International Co., Ltd., Tokyo, Japan, website: http:// www. jascoint.co.jp) equipped with a Peltier thermostat supplied by the manufacturer. Samples were placed in a 2-mm pathlength rectangular quartz cell. Lectin concentration was 0.8–1.6 μM for measurements in the far UV region (250– 190 nm) and 8–15 μM for measurements in the near UV region (300–250 nm). In order to study thermal unfolding of the protein, CD spectra were recorded in the near UV region at different temperatures. In addition, temperature scans were performed between 30 and 90 °C at a scan rate of 1° per minute, by monitoring the ellipticity (Θ) at 289 nm as well as 205 nm, corresponding to the near UV and far UV regions, respectively.

PART C

3.3.0. Results

3.3.1. Quenching of the intrinsic fluorescence emission of DLL-II

Quenching studies were carried out with the lectin using a neutral quencher (acrylamide), an anionic quencher (iodide ion, I^-) and a cationic quencher (cesium ion, Cs^+). The fluorescence emission spectra of native lectin, sugar bound and lectin denatured with 6 M guanidine hydrochloride (Gdn–HCl) recorded in the absence and in the presence of increasing concentrations of acrylamide are shown in Fig. 3.1 (A-C), respectively. In all cases, spectrum 1

is that of the native lectin in the absence of quencher and spectra 2–20 correspond to DLL-II in the presence of increasing concentrations of acrylamide, with spectrum 20 corresponding to a resultant quencher concentration of 0.52 M. A comparison of the spectra shows that the emission λ_{max} of the native lectin seen at 328 nm (Fig. 3.1, spectrum A) is red-shifted to about 352 nm upon denaturation with 6 M Gdn–HCl (Fig. 3.1, spectrum C). The fluorescence emission spectra of native lectin, sugar bound and lectin denatured with 6 M guanidine hydrochloride (Gdn–HCl) recorded in the absence and in the presence of increasing concentrations of two charged quenchers, cesium ion and iodide ion are shown in Fig. 3.2 (A-C) and Fig. 3.3 (A-C) respectively. Furthermore, the spectra also indicate that the extent of quenching is higher in the presence of Gdn–HCl, clearly indicating that unfolding results in a significant increase in the accessibility of the tryptophan residues to the quencher. Denaturation with Gdn–HCl also led to a significant increase in the extent of quenching with the other quenchers used in this study, namely I^- and Cs^+ (Fig. 3.2-C and 3.3-C). These observations indicate that the indole side chains of tryptophan residues in DLL-II become significantly more exposed to the bulk aqueous medium upon denaturation.

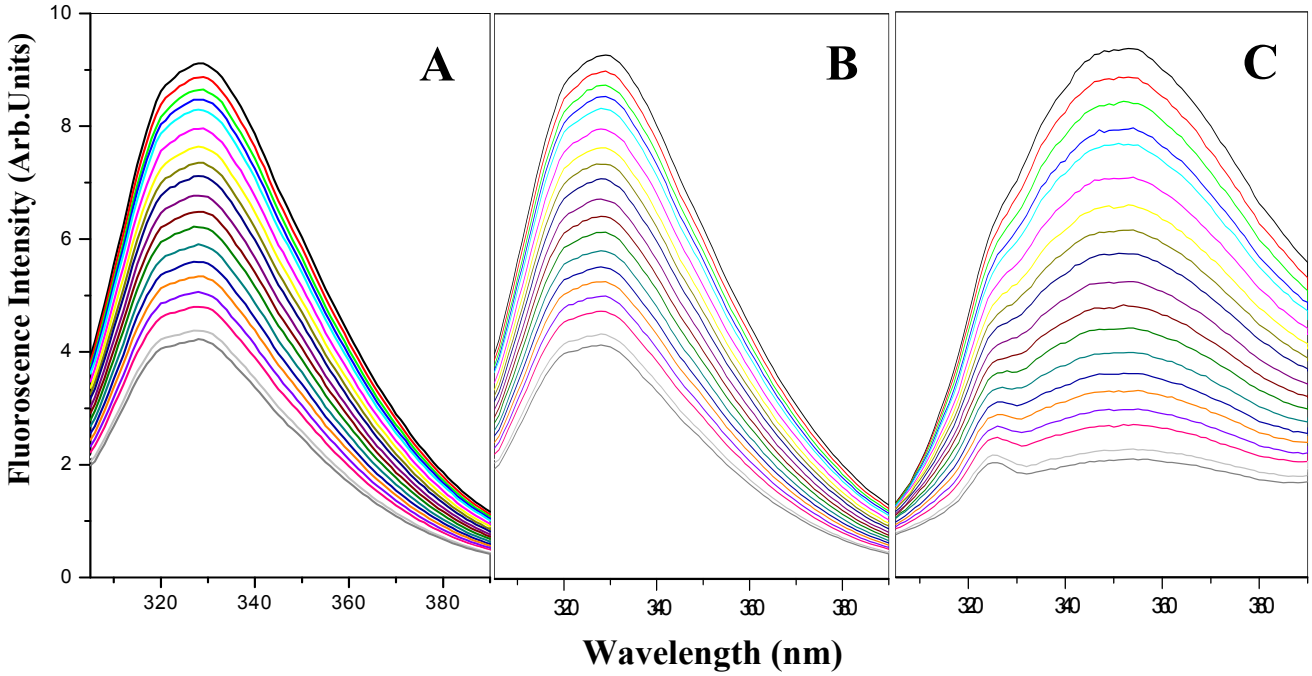


Figure: 3.1

Fluorescence spectra of DLL-II in the absence and in the presence of Acrylamide
(A) Under native conditions; (B) under sugar (Lactose) bound conditions (C) under denaturing conditions (6MGdn–HCl). Spectrum 1 corresponds to the lectin alone and spectra 2–20 correspond to the lectin in the presence of increasing concentrations of acrylamide. The final concentration of the quencher in both A, B and C is 0.52 M.

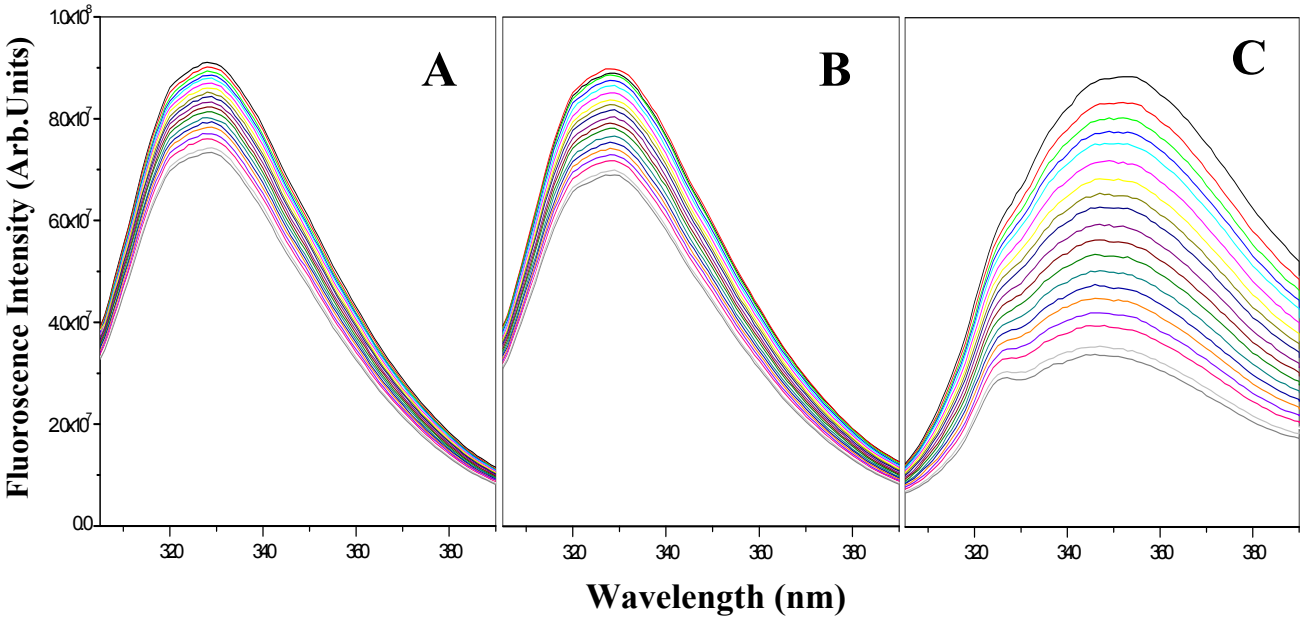


Figure: 3.2

Fluorescence spectra of DLL-II in the absence and in the presence of Iodide

(A) Under native conditions; (B) under sugar (Lactose) bound conditions (C) under denaturing conditions (6MGdn–HCl). Spectrum 1 corresponds to the lectin alone and spectra 2–20 correspond to the lectin in the presence of increasing concentrations of Iodide. The final concentration of the quencher in both A, B and C is 0.52 M.

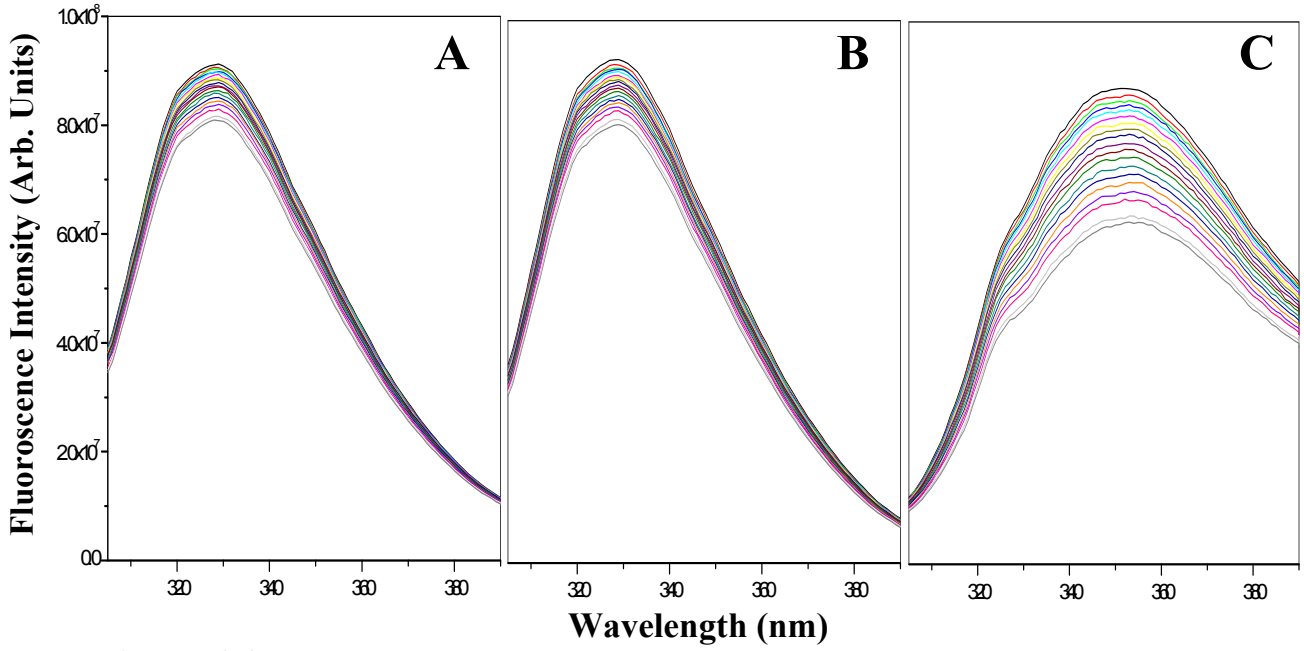


Figure: 3.3

Fluorescence spectra of DLL-II in the absence and in the presence of Cesium

(A) Under native conditions; (B) under sugar (Lactose) bound conditions (C) under denaturing conditions (6MGdn–HCl). Spectrum 1 corresponds to the lectin alone and spectra 2–20 correspond to the lectin in the presence of increasing concentrations of cesium. The final concentration of the quencher in both A, B and C is 0.52 M.

The extent of quenching observed with different quenchers under different conditions and at a final quencher concentration of 0.52 M is shown in Table 3.1. From the data presented in this Table it is clear that among the three quenchers employed in this study acrylamide is the most effective, quenching about 48.9% of the total fluorescence of the native protein at a quencher concentration of 0.52 M whereas I^- and Cs^+ could quench only 10.6 and 1.9%, respectively, at the same concentration. In the presence of 0.1 M concentration of the disaccharide lactose, which binds specifically to DLL-II, the extent of quenching observed with acrylamide remained almost unchanged whereas with I^- and Cs^+ it increased marginally to 14.4% and 2.6%, respectively. These observations suggest that ligand binding does not significantly alter the exposure/accessibility of the tryptophan residues of DLL-II. Denaturation resulted in a significant increase in the quenching by all the three quenchers, with the extent of quenching observed at 0.5 M concentration being 74.7%, 57.5% and 21.0%, with acrylamide, iodide ion and cesium ion (Table 3.1).

Extent of quenching of fluorescence of DLL-II observed with different quenchers

	Quenching %		
Quencher	Native	With 0.1 M Lactose	In 6M Gdn-HCl
Acrylamide	48.9 (±0.5)	48.4 (±1.4)	74.7 (±0.1)
Iodide ion	10.6 (±0.5)	14.4 (±0.3)	57.5 (±0.4)
Cesium ion	1.9 (±0.1)	2.6 (±0.3)	21.0 (±0.3)

Table: 3.1

The final quencher concentration in each case was 0.5 M. Values are averages from three independent experiments with the estimated errors given in parentheses.

3.3.2. Analysis of fluorescence quenching data

The steady-state fluorescence quenching data obtained with different quenchers were analyzed by Stern-Volmer and modified Stern–Volmer Equations (Eqs. (1) and (2), respectively) in order to obtain quantitative quenching parameters [Lehrer, 1971]:

$$F_o/F = 1 + K_{SV}[Q] \quad (1)$$

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

where F_o and F are the relative fluorescence intensities in the absence and presence of the quencher, respectively, [Q] is the quencher concentration, K_{SV} is Stern–Volmer quenching constant, ΔF (=F_o−F) is the change in fluorescence intensity at any point in the quenching titration, f_a is the fraction of the total fluorophores accessible to the quencher and K_a is the corresponding Stern–Volmer constant for that fraction of the fluorophores. The values of K_{SV} for different quenchers and under different conditions were obtained from the slopes of plots of F_o/F versus [Q], according to Eq. (1), whereas the slope and intercept of plots of $F_o/\Delta F$ against $[Q]^{-1}$ yielded values of f_a and K_a according to Eq. (2).

Stern–Volmer plots for the quenching of the lectin with different quenchers are shown in Fig. 3.4. From these plots it can be seen that with the lectin in the native state as well as in the presence of 0.1M lactose both acrylamide and Cs^+ yield biphasic quenching patterns, whereas with I^- triphasic patterns were observed under the same conditions. From the slopes of the different phases of these plots, values of the corresponding Stern–Volmer quenching constants, K_{SV1}, K_{SV2} and K_{SV3} were obtained and listed in Table 3.2.

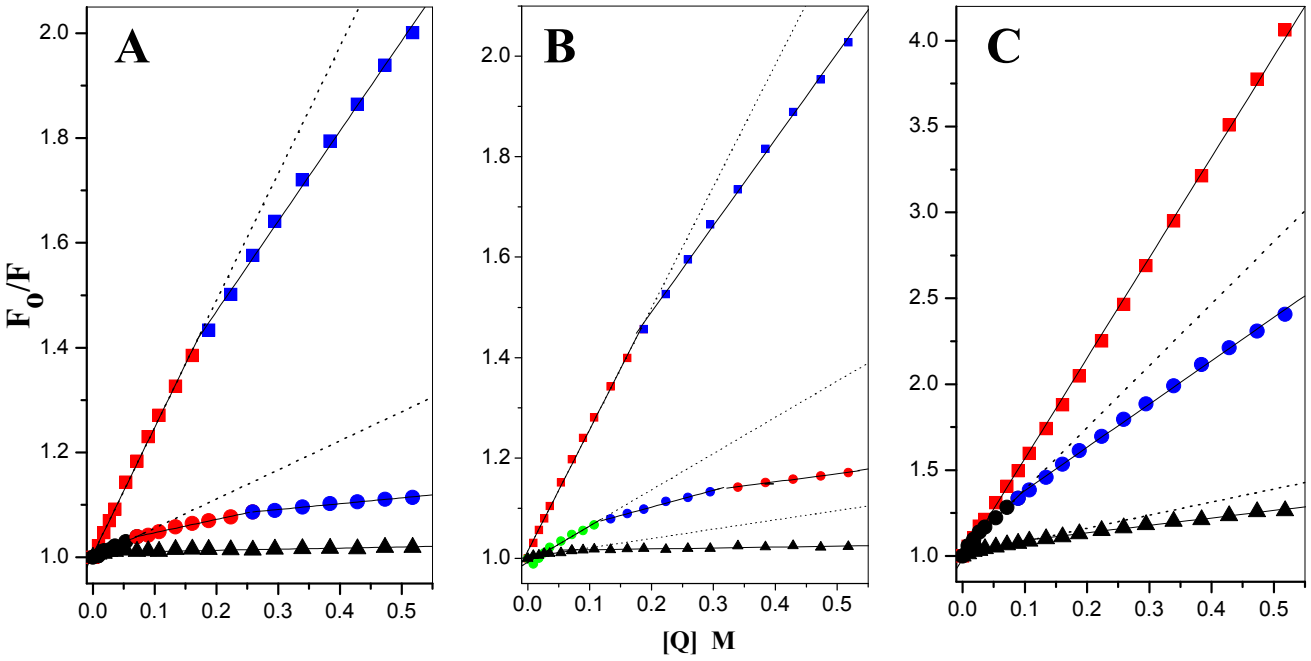


Figure: 3.4

Stern–Volmer plots of fluorescence quenching of DLL-II
(A) Under native conditions, (B) under native conditions in the presence of 0.1 M lactose and (C) under denaturing conditions (6 M Gdn–HCl). The different quenchers used are: acrylamide (■), iodide ion (●), cesium ion (▲). The dotted lines are linear extrapolations from the initial linear part of the plot.

Summary of parameters obtained from the intrinsic fluorescence quenching of DLL-II with different quenchers

Quencher & condition	K_{SV1} (M^{-1})	K_{SV2} (M^{-1})	K_{SV3} (M^{-1})	f_a	K_a (M^{-1})
Acrylamide					
Native	2.339	1.688		0.765	3.482
With 0.1 M lactose	2.276	1.631		0.652	4.905
In 6 M GdnCl	5.854			0.988	5.836
Iodide ion (I^-)					
Native	0.626	0.279	0.120	0.126	6.932
With 0.1 M lactose	0.722	0.335	0.159	0.249	3.007
In 6 M GdnCl	3.458	2.480		0.622	8.386
Cesium ion (Cs^+)					
Native	0.182	0.012		0.024	13.675
With 0.1 M lactose	0.264	0.020		0.028	27.931
In 6 M GdnCl	0.835	0.441		0.222	6.538

Table: 3.2

The modified Stern–Volmer plots obtained with all three quenchers are shown in Fig. 3.5 from which fa and Ka were obtained according to Eq. (2) and listed in Table 3.2. It can be seen from this table that 77%, 13%and 2.4% of the total fluorescence of the native lectin is accessible to acrylamide, I^- and Cs^+ respectively. The corresponding values obtained in the presence of 0.1M lactose are 65.2%, 24.9%and 2.8%. Denaturation with 6 M Gdn–HCl led to nearly 100% accessibility with acrylamide. For I^- and Cs^+, the fraction accessible increased to 62% and 22%, respectively.

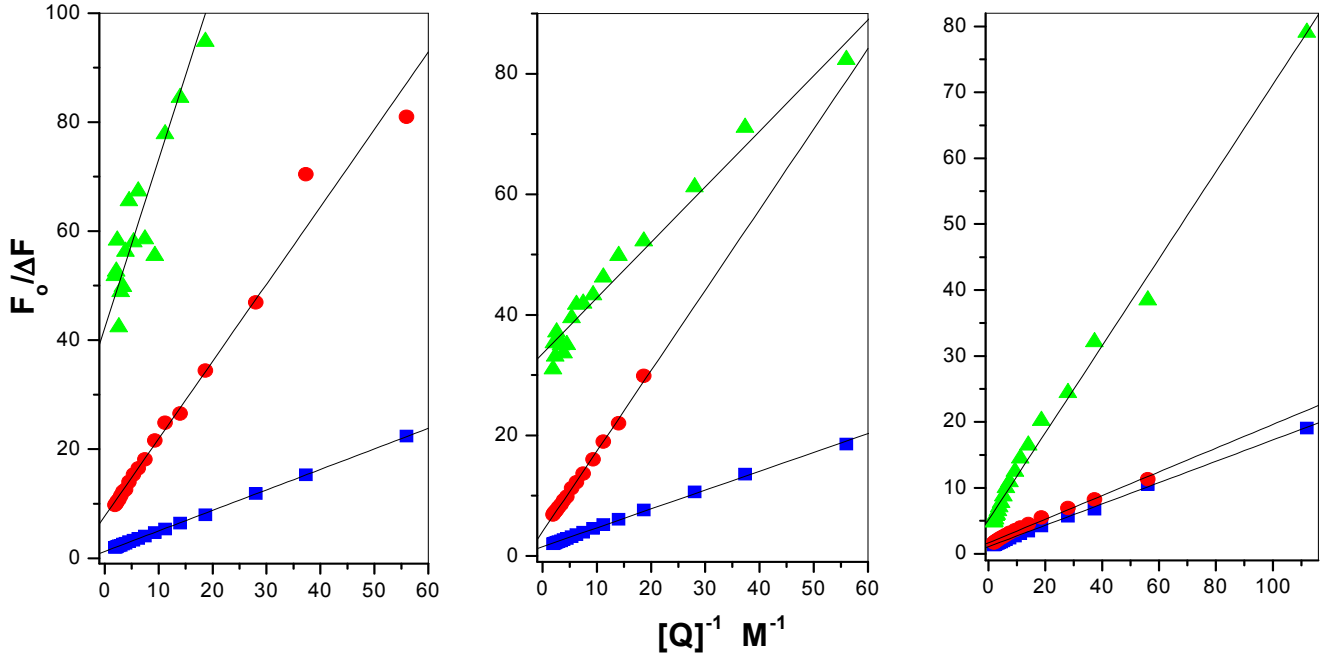


Figure: 3.5

Modified Stern–Volmer plots of fluorescence quenching of DLL-II
(A) Under native conditions, (B) under native conditions in the presence of 0.1 M lactose and (C) under denaturing conditions (6 M Gdn–HCl). The different quenchers used are: acrylamide (■), iodide ion (●), cesium ion (▲).

3.3.3. CD spectroscopy and secondary structure

Circular dichroism spectra of native DLL-II in the far UV and near UV regions are shown as solid lines in Fig. 3.6-A and B respectively. In addition, CD spectra obtained in the presence of 100 mM lactose, both in the near UV region and far UV region, are also shown (dotted line). In order to estimate the content of different types of secondary structures in DLL-II, the far UV CD spectrum of the native protein in the wavelength range 240–190 nm has been analyzed by the CDSSTR program using the routines available at DICHROWEB (www.cryst.bbk.ac.uk/cdweb/html) [Compton *et al*., 1986; Lobley *et al*., 2001; Lobley *et al*., 2002]. Reference set 4 containing 43 proteins was used for fitting the experimental spectra. The calculated fit obtained, shown as a dashed line in Fig. 3.6, is in excellent agreement with the experimentally obtained spectrum of DLL-II indicating high accuracy in the estimates obtained from this analysis. The values obtained for the different types of secondary structures are: α-helix (57%), β-sheet (21%), β-turns (7%) and unordered structures (15%). The agreement between the experimental and calculated spectra was high with an NRMSD value of 0.001 (to know more about the meaning and significance of NRMSD see [Whitmore *et al*., 2002]).

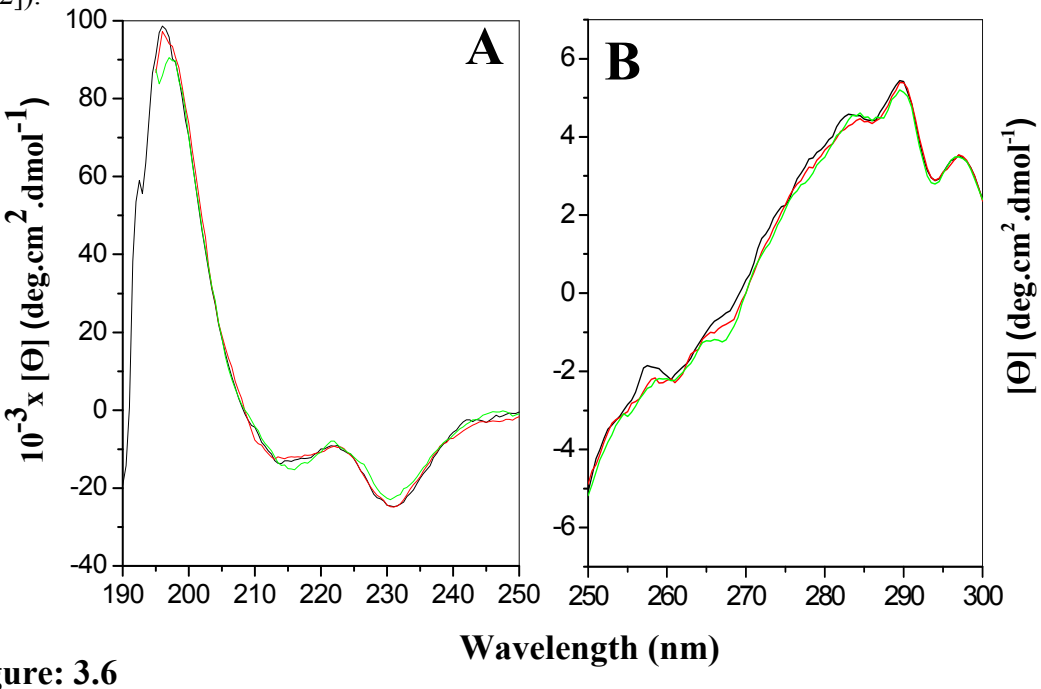


Figure: 3.6

Circular dichroic spectra of DLL-II
(A) Far UV region. (B) Near UV region. (—) lectin alone, (·····) lectin +100 mM lactose. The spectra were recorded at 25 °C. Calculated fit (— — — —) obtained by using the CDSSTR program is also shown. See text for details.

3.3.4. Thermal stability and unfolding of DLL-II

To investigate the thermal unfolding of DLL-II, near UV CD spectra of the lectin were recorded at different temperatures. Spectra recorded at 30, 45, 60, 70, 75 and 80 °C are shown in Fig 3.7-A. While only moderate changes in the spectral features are seen in the spectra recorded at 45, 60 and 70 °C, including small decrease in the spectral intensity at 289 nm and 297 nm, significantly larger changes were observed in the spectra recorded at 75 and 80 °C, suggesting the occurrence of a thermally-induced unfolding phase transition between 70 and 80 °C. This is clearly seen in the inset of Fig. 3.7-A which gives a plot of the ellipticity at 289 nm as a function of the temperature.

In order to determine the unfolding temperature of DLL-II more accurately and to understand the nature of the unfolding transition, the change in the CD signal at 289 nm, corresponding to the positive maximum in near UV CD spectrum (see Fig. 3.7-B), was monitored as a function of temperature. In this experiment, the protein sample was heated at a scan rate of 1° per minute in the temperature range of 30 and 90 °C. A temperature scan thus obtained is given in Fig. 3.7-B. From this figure, it is seen that the CD signal intensity decreases very marginally between 30 and 70 °C, but experiences a steep drop between 70 and 80 °C (centered around 75 °C) and remains low upon further increase in temperature. These observations are consistent with a cooperative unfolding of the polypeptide chains of DLL-II. In addition, the steep decrease in the ellipticity seen around 75 °C is accompanied by an irreversible precipitation of the lectin as observed visually, clearly indicating that unfolding results in heavy aggregation of the polypeptide chains. In the presence of a saturating concentration of MeβGal (Fig. 3.8, dotted line), the unfolding transition was found to occur at approximately 2–3 degrees higher temperature, reflecting the stabilizing effect of the bound carbohydrate ligand on the lectin structure. Duplicate experiments yielded very similar results, strongly suggesting that the lectin structure is somewhat stabilized by the presence of the ligand. When this temperature scan was carried out by monitoring the ellipticity at 205 nm in the far UV region, a similar sharp transition was observed in the same temperature range (Fig 3.8, inset).

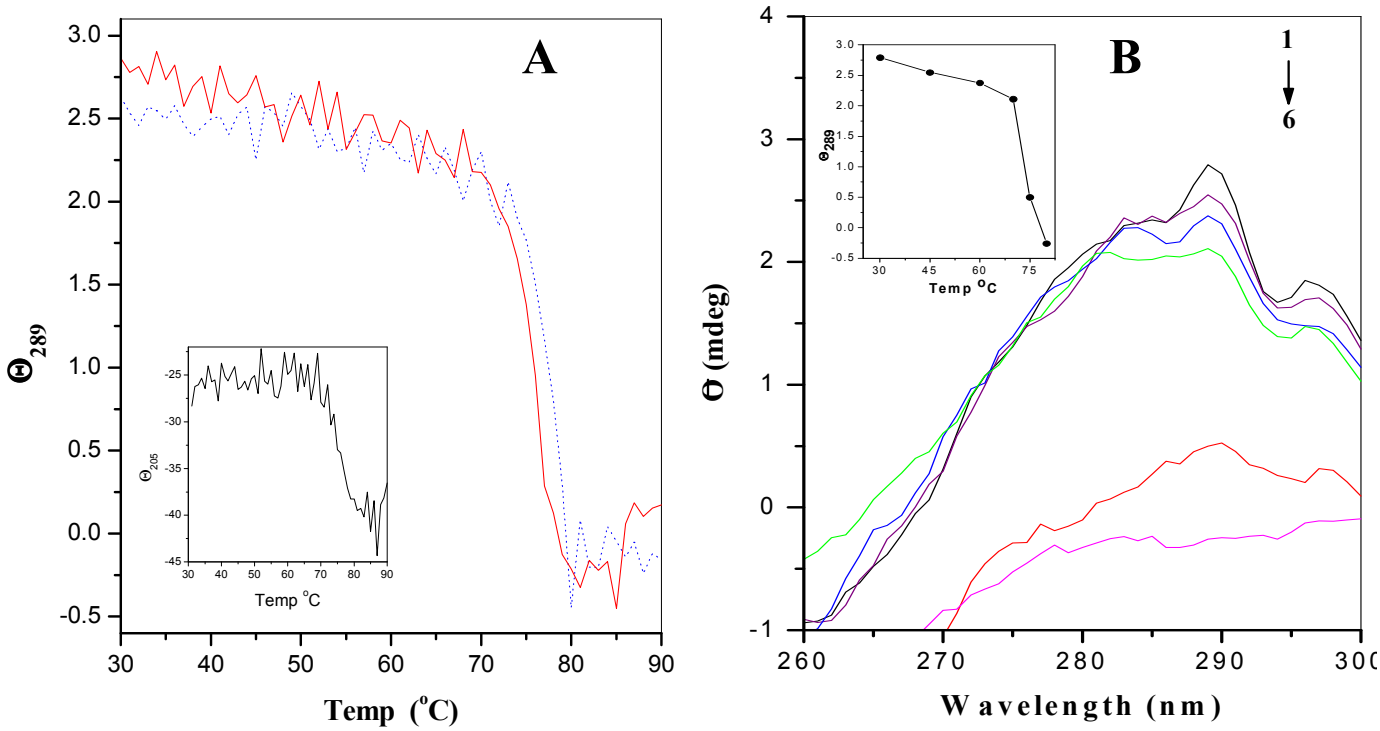


Figure: 3.7

(A) Thermal unfolding of DLL-II monitored by CD spectroscopy
The ellipticity (Θ) in the near UV region of the native protein (—) was monitored at 289 nm as a function of temperature. (.....) Lectin – MeβGal complex. The sharp decrease in the ellipticity around 75 °C indicates the transition from folded structure to the unfolded state. Inset shows the ellipticity change in the far UV region (205 nm) versus temperature, which also indicates a transition centered around 75 °C. See text for details.

(B) Near UV CD spectra of DLL-II at different temperatures
Spectra numbered 1 to 6 correspond to those recorded at 30, 45, 60, 70, 75 and 80 °C, respectively. A plot of the ellipticity (Θ) at 289 nm is shown in the inset.

PART D

3.4.0. Discussion

The significantly lower quenching observed with the two charged quenchers with native DLL-II indicates that most of the fluorescent tryptophan residues in this protein are buried in the hydrophobic core of the protein. This is also consistent with the emission maximum of the protein at 328 nm, which is indicative of a significantly non polar environment around the indole side chains of Trp residues. Additionally, the very low quenching observed with Cs^+ (even when compared with I^-, which is also charged) appears to be due to the inability of this quencher to access the fluorophores. This may be due to the presence of positively charged residues in the vicinity of some of the exposed (or partially exposed) tryptophan residues,

which repel the positively charged cesium ion, but allow the neutral acrylamide and the negatively charged iodide ion to approach the indole moieties of the tryptophan residues in their neighborhood. Besides, the inherently low quenching efficiency of Cs^+ may also be partly responsible for the lower quenching observed with it. The size of different quenchers does not seem to affect the extent of quenching because the highest quenching was seen with acrylamide which is larger than I^- (ionic radius 2.2 Å) and Cs^+ (ionic radius 1.88 Å), whereas Cs^+ which is the smallest of the three quenchers, exhibited the lowest extent of quenching. Presence of positively charged residues near tryptophan residues was also suggested for several cucurbitaceae seed lectins based on the results from fluorescence quenching studies with neutral and ionic quenchers [Sultan *et al*., 2005; Komath *et al*., 1999; Kenoth *et al*., 2003].

The yield of biphasic quenching patterns for both acrylamide and cesium ion, whereas triphasic patterns were seen iodide ion were consistent with the pattern expected from dynamic fluorescence quenching of proteins with more than one tryptophan residues in different accessible environments. These observations are also in good agreement with results of chemical modification experiments, where only 6 tryptophan residues in DLL-II tetramer could be modified under native conditions, whereas 10 tryptophan residues could be modified upon denaturation of the protein, clearly indicating heterogeneity of tryptophan environment.

The sugar binding probably tightens the protein structure a little bit, leading to a slight decrease in the accessibility of some of the residues to acrylamide; some of the residues that are partially accessible to I^- become more exposed, resulting in a somewhat higher extent of quenching being observed with it. This interpretation is consistent with the results of CD spectral studies, which indicated that the secondary and tertiary structures of DLL-II are not significantly altered by ligand binding.

The complete accessibility of the tryptophan residues to acrylamide clearly indicated that the protein completely unfolds upon treatment with 6 M Gdn–HCl. Since the protein lacks any sulfur containing amino acids [Latha *et al*., 2006], the possibility of any local order being present due to the presence of disulfide bonds does not exist. In view of this, the lower accessibility observed with I^- and Cs^+ must be due to the presence of charged residues in the vicinity of some of the tryptophan residues. Since complete unfolding of the polypeptide chains of the protein does not seem to render all the tryptophan residues accessible to these two quenchers, it appears that the charged residues are proximal to the tryptophan residues by virtue of their presence near the Trp residues in the primary structure of the polypeptide chains.

Furthermore, because the extent of quenching achieved with Cs^+ is significantly lower than that observed with I^-, it is quite likely that a larger fraction of the tryptophan residues have positively charged residues in their close proximity. On the other hand, fewer tryptophan residues are expected to have negatively charged residues in their vicinity.

The far UV CD spectrum of native DLL-II shown in Fig. 3.6-A, exhibits two minima centered around 214 nm and 231 nm, suggesting the presence of helical structure along with other secondary structural elements. A comparison of the spectra of DLL-II alone with those obtained in the presence of lactose clearly indicates that the spectra obtained in the presence of lactose are nearly indistinguishable from those of DLL-II alone, suggesting that carbohydrate binding induces insignificant changes in the secondary and tertiary structures of the protein. Similar results were also obtained in the presence of MeβGal, another sugar that is specifically recognized by DLL-II.

However, specific assignment of the fraction of different types of secondary structures required a detailed quantitative analysis in the study, for which the routines available with DICHROWEB have been used. The high α-helical content in the secondary structure of DLL-II is quite unusual for a legume lectin. All the legume lectins known so far contain predominantly β-sheet structure and have negligible or no α-helical content [Sharon *et al*., 2003; Loris *et al*., 1998]. Comparison of the N terminal amino acid sequence of DLL-II with that of DLL-I and FRIL (another glucose/mannose-specific lectin isolated from *Dolichos lablab* seeds, which exhibits high homology to DLL-I) indicated that only two of the first 10 residues are identical. In addition, comparison with a number of other legume lectins suggested very low sequence homology between DLL-II and other legume lectins [Latha *et al*., 2006]. These observations suggest that DLL-II differs considerably from other legume lectins in its amino acid sequence, secondary structure, and possibly in the tertiary and quaternary structures. Currently, we are carrying out single crystal X-ray diffraction studies on DLL-II in order to determine its 3-dimensional structure and to understand its mode of interaction with carbohydrate ligands [Latha, Kulakarni *et al*., 2006].

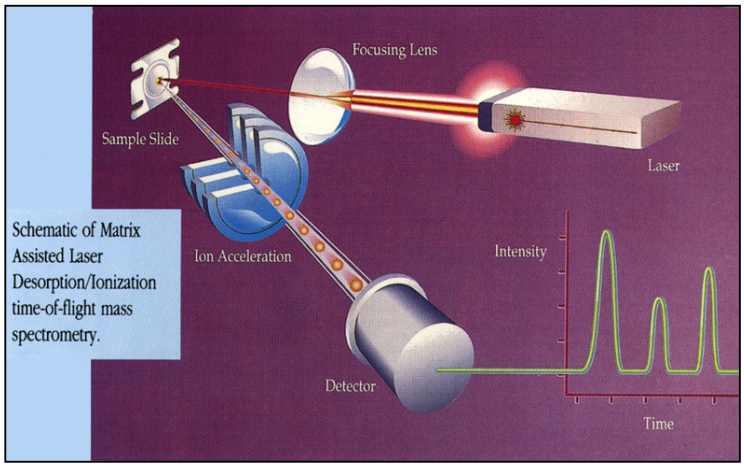
The near UV CD spectrum of DLL-II is characterized by the presence of a maximum around 289 nm and two peaks of somewhat lesser intensity around 297 nm and 283 nm. These features are likely to arise due to contributions from the side chains of tryptophan and tyrosine residues, which absorb in the 270– 300 nm region. However, ligand binding does not seem to perturb these signals to any detectable level. Since chemical modification studies have implicated the side chains of tyrosine residues in the carbohydrate binding activity of DLL-II,

it appears that the side chains of tyrosine residues involved in the interaction do not undergo significant reorientation, at least when the protein binds mono- or disaccharides.

The unfolding temperature of ~75 °C indicates that DLL-II is a rather stable protein. Most legume seed lectins investigated have been found to be generally stable to thermal denaturation with the midpoint of transition being found between 56 and 92 °C for different lectins [Srinivas *et al*., 2005]. However, different legume lectins were found to adopt different unfolding mechanisms. For example, the tetrameric Con A undergoes a two-state unfolding transition (folded tetramer↔unfolded monomer) with the midpoint of the transition at 87–92 °C, whereas peanut agglutinin, another tetrameric legume lectin exhibits a more complex unfolding process, with two separate transitions with midpoints at 56–61 °C (corresponding to dissociation of the tetramer into monomers) and 63 °C (corresponding to unfolding of the monomers), respectively [Schwartz *et al*., 1993; Reddy *et al*., 1999]. Although the studies reported here appear to suggest that thermal unfolding of DLL-II tetramer involves a two-state transition as found with Con A, further experiments are required to draw firm conclusions on this. Preliminary differential scanning calorimetric studies have also indicated that DLL-II precipitates during thermal unfolding due to aggregation of the denatured protein and we are currently trying to use additives such as spermine and spermidine, which have been shown, prevent protein aggregation [Kudou *et al*., 2003] in the DSC experiments. The present data do not reveal the nature of the unfolded protein or its aggregation state (such as monomer, dimer or tetramer). Since the unfolded protein aggregates to yield a turbid suspension, it was not possible to investigate its oligomeric state by gel permeation chromatography.

In summary, the studies reported here indicate that the tryptophan residues of the *Dolichos lablab* galactose-specific lectin are in a heterogeneous environment that is predominantly hydrophobic. Quenching with the charged quenchers I^- and Cs^+ indicate that some of the tryptophan residues have positively charged residues close to them in the primary structure, while some other tryptophan residues have negatively charged residues close to them. Circular dichroism spectral studies indicate that the secondary structure of DLL-II is predominantly α-helical with relatively less β-sheet structure and very little unordered structure. The protein is thermally quite stable and undergoes an unfolding transition at ~75 °C, which is shifted to higher temperatures by ca. 2–3 degrees in the presence of 0.1M MeβGal, indicating a slight stabilization of the protein structure by carbohydrate binding.

CHAPTER IV



PRIMARY STRUCTURE OF DLL-II - A PROTEOMICS APPROACH

PART A

4.1.0. Introduction

Lectins isolated from the seeds of leguminous plants are composed of two types of subunits, which can display different functional properties [Etzler, 1985]. The subunits can originate from different genes, as in the case of *phaseolus vulgaris* lectin [Hoffman *et al*., 1985, Goldstein *et al*., 1986], or by proteolytic splitting of a single gene product as has been found in the biosynthesis of *Dolichos biflorus* seed lectin, DB 58 lectin [Etzler 1994], favin [Hemperley *et al*., 1982], the pea [Foriers *et al*., 1981] and lentil [Higgins *et al*., 1983] lectins.

Plant lectins are mostly found in the vegetative and storage parts of the plant where they presumably play an important role which is not clearly established. Legume plant, *Dolichos lablab* (Indian lablab beans) seeds contains two types of lectins specific for selected carbohydrates: glucose/mannose and galactose. Glucose/Mannose lectin (DLL-I), which has a native molecular mass of 60 kDa, is made of two types of subunits 15 and 12 kDa. It was completely characterized and is virtually identical to the lectin from field beans whose primary structure had been deduced by conventional protein sequencing methodology [Gowda *et al*., 1994]. We successfully affinity purified the DLL-II galactose-specific lectin which is a glycoprotein with an apparent molecular mass of 120 $\pm$ 5 kDa and is made of two types of subunits with molecular weights of 31 kDa (α subunit) and 29 kDa (β subunit), respectively suggesting the lectin to be tetrameric in nature ($\alpha_2\beta_2$ type) [Sultan *et al*., 2006]. Interestingly the lectin shows cross-reactivity with the antibody for the galactose specific lectin (DLL-II) [Latha *et al*., 2006]. The stems and leaves of this plant were also found to contain the galactose-specific lectin that cross-reacts with the same antibody, suggesting that these two lectins may be related immunologically [Rao *et al*., 2008]. In literature there are only a few legume plant seeds that have been shown to contain two distinct types of lectins. Since we extensively characterized the newly identified DLL-II, and the available evidence suggested that it may be related to the DLL-I it became important to obtain the primary sequence of the DLL-II. Therefore we used a mass spectrometry centered proteomics approach to achieve the following objectives. i) deduce its complete primary structure and ii) make a structural comparison of the sequences obtained to the DLL-I, and also to other known legume lectins. Later on the crystal structure of the protein in conjunction with the primary sequence will be used to reveal structure-function relationships of the protein.

Since the early 1980's, when mass spectrometry was first employed for the measurement of the molecular weight of short peptides, great advances have been made in the uses of MS for

protein and peptide identification and MS is now a routine method for rapid identification and characterization of proteins. It is now commonly used in areas as diverse as genotyping for molecular medicine and cancer research to food quality evaluation, as well as in the analysis of plant proteomes and discovery of novel plant viruses [Shadforth *et al*., 2005]. Current methodologies generally combine two stages: fractionation of proteins or peptides followed by ionization of the sample using either MALDI or ESI. The first of these, the separation step, is necessary to reduce complexity allowing comprehensive coverage of even complex mixture by the subsequent mass spectrometric analysis. Separation protocols include: protein separation using 1- or 2-DE followed by digestion of the products using a cleavage agent, most often sequence specific proteases such as trypsin. Typically the experimental data generated by MS are then compared with theoretical peptide masses or fragment ion mass values, derived by in-silico digestion of entire sequence databases with the same enzymes/chemicals. Corresponding matches are scored with probability based methods in a way that allows the peptide or protein that best matches the data to be identified. However, if the protein of interest is not part of the existing sequence databases, the aim would be to identify those entries that exhibit closest homology, often-equivalent related species [Perkins *et al*., 1999]. Alternatively, the sequence of the protein could be determined by de-novo sequencing. Here, we report in this chapter the determination of the primary sequence of the DLL-II galactose-specific lectin from *Dolichos lablab* by a mass spectrometry based approach.

PART B

4.2.0. Materials and Methods

Ammonium bicarbonate was obtained from Merck (USA). Acetonitrile, acetic acid *trypsin, chymotrypsin, Staphylococcus V8 protease, Aspartase N*, trifluroacetic acid Carboxypeptidases, α- Cyano-4-hydroxy cinammic acid (CHCA) was obtained from Sigma (St. Louis, MO, USA). C18 ziptips was purchased from Millipore (Bedford, MA, USA). 2-Iodosobenzoic acid (IBA) and Hydroxylamine was obtained from Aldrich (USA). All other chemicals used were obtained from local suppliers and were of the highest quality available. Millipore water was used for preparing all the buffers.

4.2.1. Purification of DLL-II

The *Dolichos lablab* seeds were obtained from the local market. The lectin was purified from the saline extracts of defatted lablab bean flour by fractional precipitation with ammonium sulphate, followed by affinity chromatography as described earlier [Chapter 2].

4.2.2. Separation and confirmation of subunits

Purified lectin has been characterized. It is a glycoprotein with an apparent molecular mass of 120 kDa and dissociates into 31 kDa and 29 kDa on denaturing electrophoresis indicating probably that it is tetrameric in nature. The subunits were separated on SDS-PAGE and electroeluted from the gel. The electroeluted purified subunits were collected manually, stored at 4°C and subunit total masses were determined by mass spectrometry using an API-QSTAR PULSAR instrument.

4.2.3. Preparation of proteins for analysis by mass spectrometry

The subunits were separated on a 12% SDS-polyacrylamide gel and visualized by colloidal coomassie blue staining. α and β subunits were excised from the gel, destained with 25 mM ammonium bicarbonate in 50% acetonitrile, dehydrated with 100% acetonitrile and vacuum dried in a speedvac. Dried gel slices were in gel-digested separately with *trypsin, chymotrypsin*, *Staphylococcus V8 protease* and *aspartase N* overnight at 37º C. In gel digestion with different proteases were done according to manufacturer´s description. Peptides were extracted twice with 50% acetonitrile containing 0.1% acetic acid in a water bath sonicator for 15 min each. The digests of each enzyme were pooled, vacuum dried in a speedvac and redissolved in water containing 0.1% trifluroacetic acid (TFA) for desalting using C18 ziptips (Millipore, Bedford, MA, USA). Finally, peptides were dissolved in α- Cyano-4-hydroxy cinammic acid (CHCA) for MALDI-MS measurements (see below).

For limited acid hydrolysis the purified subunits were desalted using C18 ziptips (Millipore, Bedford, MA, USA) (10µl), hydrolyzed with 3 and 6 N HCl (1:10) separately and incubated for 2 and 5 minutes, respectively, at 100° C. Then, the samples were transferred on ice to stop the reaction and vacuum dried. The peptide containing pellet was reconstituted in 4-6 µl of CHCA.

For formic acid treatment, excised vacuum dried gel slices were swollen with 0.1 ml of 70% formic acid, which was completely absorbed, and were then incubated in tightly closed eppendorf tubes at 37°C for 18 to 24 h. Digestion was stopped by removing the formic acid in

a speedvac. Finally, peptides were dissolved in CHCA for MALDI MS measurements (see below).

2-Iodosobenzoic acid (IBA) cleavage was performed on SDS-PAGE-purified subunits as described for formic acid cleavage, except that SDS was removed from the gel slices before treatment by washing in 10% (vol/vol) acetic acid- 25% (vol/vol) isopropanol or by colloidal coomassie blue staining. This was necessary because solubilization of IBA requires 4 M guanidine, and guanidine and SDS tend to form an insoluble gel. After removal of SDS, protein containing gel slices were dried, and 0.1 ml of 10mg per ml IBA dissolved in 80% acetic acid - 4 M guanidine was added. The gel slices were then incubated in tightly sealed tubes at room temperature for 24 h. Guanidine was removed by washing with acetic acid; the gel slices were vacuum dried in a speedvac. Peptides were dissolved in CHCA for MALDI-MS measurements (see below).

Hydroxylamine treatment was carried out as described by Saris *et al*., 1983. The vaccum dried digests were reconstituted in 4-6 µl of CHCA matrix and spotted directly onto MALDI targets. The peptides mixtures generated were analyzed with positive-ion matrix-assisted laser desorption/ionisation-mass spectrometry on a 4800 MALDI TOF/TOF Analyzer (Applied Biosystems, Foster City, CA, USA).

4.2.4. C- Terminal sequencing

The purified α and β subunits were dissolved in 10 µl of sodium citrate buffer (50mmol/l, pH 6.0). Carboxypeptidase Y was added to give an enzyme: protein ratio of 1:100 by weight. After 10 min incubation the same amount of Carboxypeptidase P was added and incubated for 60 min. Aliquots of 1.5 µl digests were mixed with the CHCA matrix solution and spotted directly on MALDI target and analyzed with positive-ion matrix-assisted laser desorption/ionisation-mass spectrometry 4800 MALDI TOF/TOF Analyzer (Applied Biosystems, Foster City, CA, USA) following the protocol of Thiede *et al*., 1995.

4.2.5. Mass Spectrometric analysis of the peptides

A 1/10 fraction of the peptides dissolved in 0.1% TFA and mixed with equal volumes of the MALDI matrix, a-cyano-4-hydroxycinnamic acid (CHCA), was analyzed with positive-ion matrix-assisted laser desorption/ionisation-mass spectrometry using a 4800 MALDI TOF/TOF Analyzer (Applied Biosystems, Foster City, CA, USA). The spectra were recorded in reflector mode in a mass range from 800 to 3700 Da with a focus mass of 2000 Da. For one main spectrum 25 sub-spectra with 100 shots per sub-spectrum were accumulated using a random

search pattern. If the autolytical fragments of trypsin with the mono-isotopic $(M+H)^+$ m/z at 1045.5 and/or at 2211.1 reached a signal to noise ratio (S/N) of at least 10, an internal calibration was automatically performed using at least one peak for one- or both peaks for a two-point-calibration. Calibration was performed manually for the less than 1% samples for which the automatic calibration failed.

Additionally MALDI-MS/MS analyses were performed for the five strongest peaks of the TOF-spectrum after subtraction of peaks corresponding to background, keratin and trypsin fragments. For one main spectrum 20 sub-spectra with 125 shots per sub-spectrum were accumulated using a random search pattern. The internal calibration was automatically performed as one-point-calibration if the mono-isotopic arginine (M+H)+ m/z at 175.119 or lysine (M+H)+ m/z at 147.107 reached a signal to noise ratio (S/N) of at least 5. After calibration a combined database search of MS and MS/MS measurements was performed using the GPS Explorer software (Applied Biosystems, Foster City, CA, USA) with the following settings: (i) MS peak filtering: mass range from 800 to 3700 Da; minimum S/N filter of 10; peak density of 50 peaks per range of 200 Da and maximal 200 peaks per protein spot; mass exclusion list contained background peaks and trypsin fragments with an exclusion tolerance of 100 ppm (ii) MS/MS peak filtering: mass range from 60 Da to a mass that was 20 Da lower than the precursor mass; peak density of 50 peaks per 200 Da and maximal 65 peaks per MS/MS; minimum S/N filter of 10 (iii) database search: precursor tolerance 35 ppm and MS/MS fragment tolerance 0.65 Da. The peptide search tolerance was 35 ppm but the actual RMS value was between 5 and 15 ppm. Peak lists were compared with a SwissProt database (R46) using the Mascot search engine (Matrix Science Ltd, London, UK). Peptide mixtures that yielded a mowse score of at least 49 (p-value=0.05) were regarded as positive identifications.

4.2.6. Peptide sequencing by MS/MS

De novo sequencing was carried out on a 4800 MALDI TOF/TOF Analyzer (Applied Biosystems, Foster City, CA, USA). Nitrogen was used as collision gas for MS/MS analysis. All samples were dissolved in 50% acetonitrile containing 0.1% v/v TFA and analyzed in the positive-ion mode. Using the *De novo* Explorer™ software (Applied Biosystems, Foster City, CA, USA) candidate amino acid sequences were generated from MS/MS data through a de novo sequencing algorithm. Subsequently, the candidate amino acid sequences were submitted for MS Blast database searching for protein identification. Additionally, all MS/MS spectra

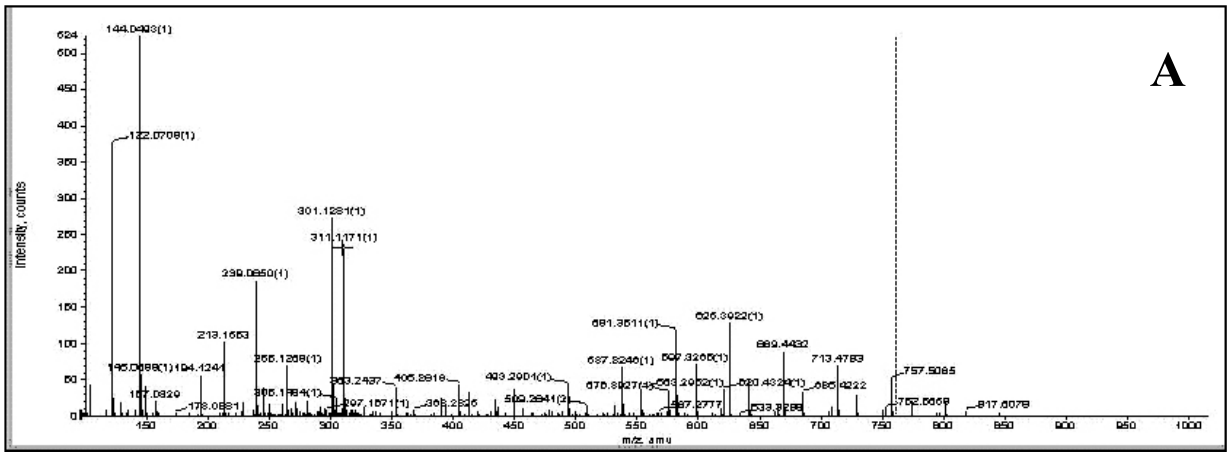
were analyzed manually using the sequencer tool of the Data Explorer software (Applied Biosystems, Foster City, CA, USA). Resulting sequence tags were also submitted for MS Blast database searching, charting the proteomes of organisms with unsequenced genomes by MALDI-quadrupole time-of-flight mass spectrometry and BLAST homology searching [Shevchenko *et al*. 2001].

PART C

4.3.0. Results

4.3.1. Characterization of α and β subunits

Our earlier studies on DLL-II have shown that the protein has a native molecular mass of 120 ± 5 kDa and is composed of two subunits (α and β subunits). Here, the subunits were separated by denaturing electrophoresis, electro eluted from the gel and their total molecular masses were determined by ESI-MS. The ions generated by ESI of larger peptides/ proteins usually carry multiple protons and thus the ESI mass spectra of the two subunits displayed multiple charged species, these signals appeared in different parts of the mass spectrum, so All signals were used to calculate the MW of the protein, and this resulted in improved mass accuracy of the subunits as shown in Fig 4.1-A, B. Deconvolution of the spectrum was done using the vendor specific Analyst QS software. The Bayesian protein reconstruct tool was used to calculate the molecular masses of the α and β subunits to be 30.746 kDa and 28.815 kDa, respectively, which is in good agreement to the calculated MW (31,000 Da and 29,000 Da) based on SDS-PAGE [Chapter 2].



B

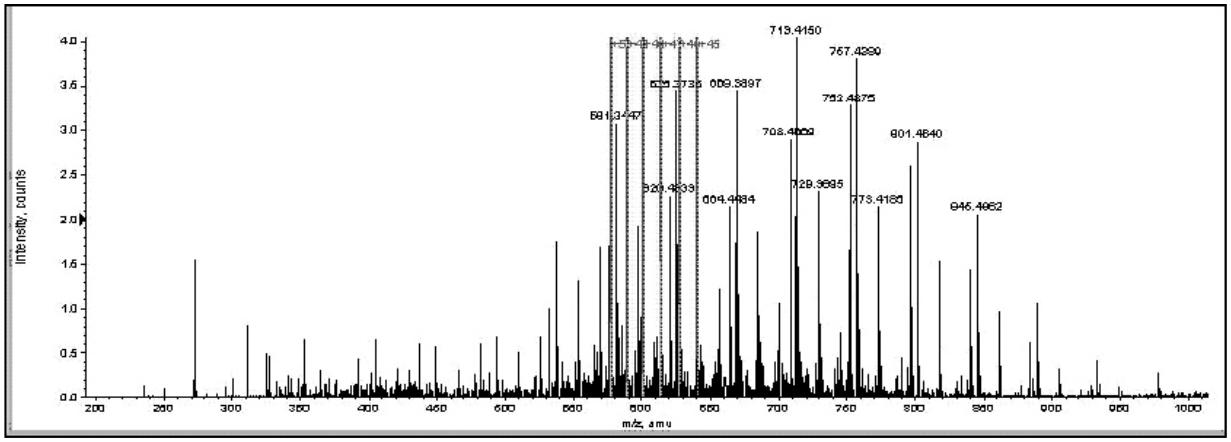


Figure: 4.1

Determination of the molecular mass of the lectin subunits
The molecular mass of the individual subunits was determined by ESI-MS with an API-QSTAR PULSAR triple quadrupole mass spectrometer. Mass spectra of the purified α subunit [31 kDa] (A) and β subunit [29 kDa] (B) are displayed.

4.3.2. The primary structure of the DLL-II

Mass spectrometry is the method of choice for the rapid analysis of complex peptide/protein mixtures in proteomics. These modern techniques of proteomics that have been developed during the last decade allow the fast and reliable identification and characterization of proteins even from complex mixtures. Currently, proteomics technologies are primarily applied for the identification and characterization of proteins from organisms with sequenced genomes but with the constant introduction of new bioinformatics tools in the future will also be applicable as a routine procedure for organisms, the sequence of which has not been determined yet. Here we applied a combination of traditional biochemical, mass spectrometry and bioinformatics tools to reveal and analyze the primary sequence of a affinity purified galactose-specific lectin subunits from *Dolichos lablab*. The flowchart for the determination of sequence of DLL-II used in this study is illustrated in Fig. 4.2.

Schematic presentation of the workflow for the determination of the primary sequence of DLL-II

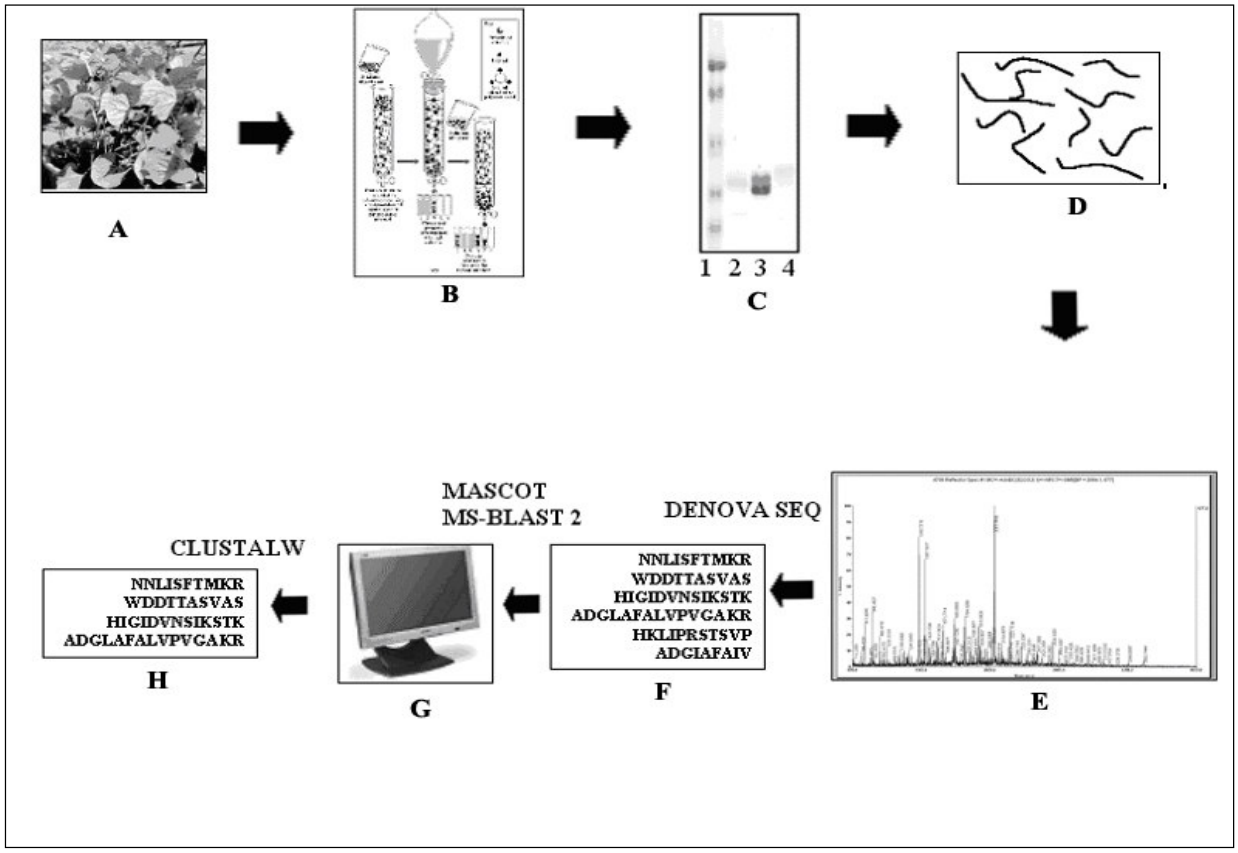


Figure: 4.2

The workflow for determination of the primary sequence of lectin from the legume Dolichos lablab (A) Legume Dolichos lablab (B) Purification of the DLL-II with different chromatographic columns (C) Separation by SDS-PAGE of purified lectin and subunits and protein markers (M). (D) Digestion of the proteins with Trypsin (E) Analysis of peptides by MALDI-MS/MS. (F, G) Database search with MASCOT, MS-BLAST 2, and PROTEIN PROSPECTOR programs H) Multiple Alignment with Clustal W.1.82 algorithm.

After separation by SDS-PAGE a fraction of the peptides obtained by enzymatic and chemical digestion of the lectin subunits were analyzed by MALDI-TOF-MS to obtain complementary peptide mass fingerprints. The digestion of the both subunits with protease trypsin displayed almost similar peptide mass fingerprints when analyzed by MALDI-TOF-MS and MS/MS analysis that are shown in Fig. 4.3 (A - C). Similarly, PMF of both subunits with other proteases are shown in Fig. 4.4 (A - C) [*Chymotrypsin]*, Fig. 4.5 (A - C) [*Aspartase N*], Fig. 4.6 (A - C) [*Staphylococcus V8 protease*]. Chemical treatment with 70% formic acid has also displayed similar PMF for both subunits Fig. 4.7 (A - C).

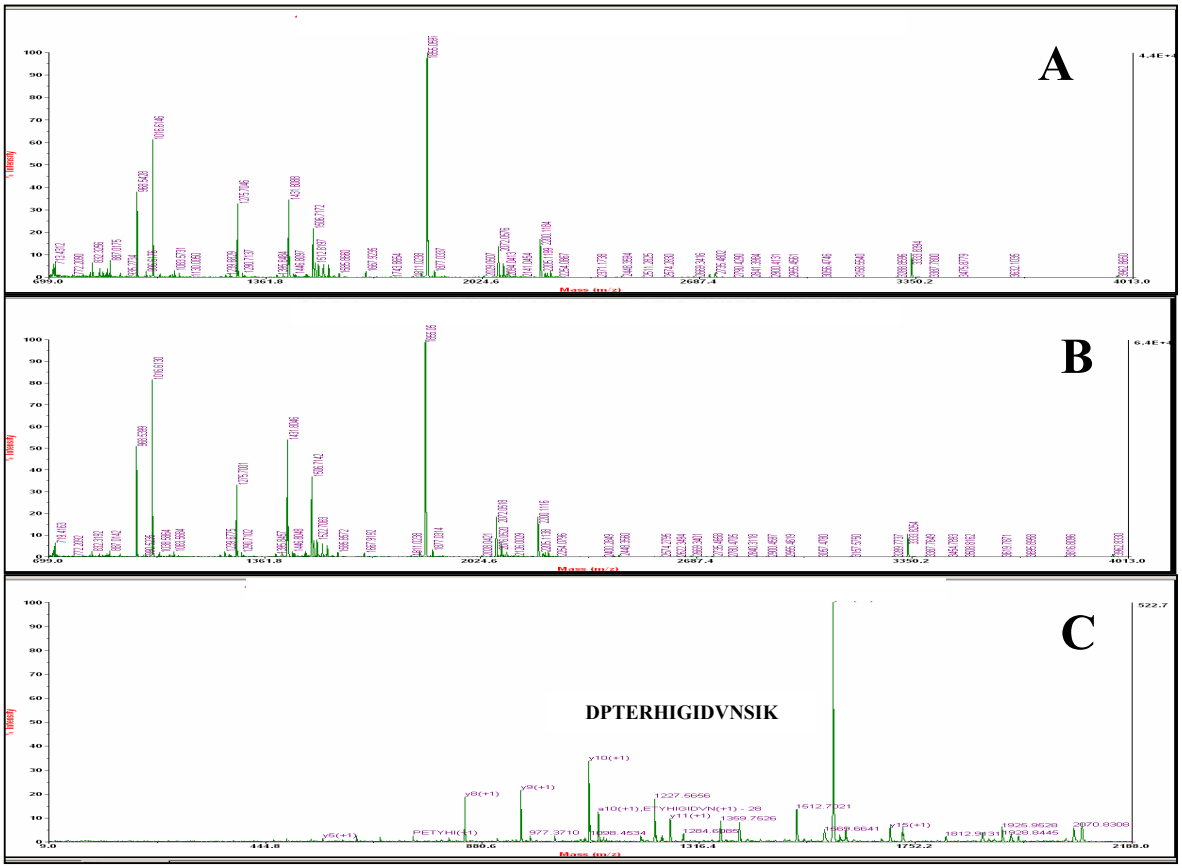


Figure: 4.3

Determination of peptides sequences by MALDI-MS/MS

In order to accomplish complete coverage of the sequence, MALDI –MS/MS analysis was performed with proteolytic digests of the Dolichos lablab Galactose-specific lectin, generated by digestion of the subunits overnight with the proteases. Figures A through B display exemplarily peptide mass fingerprints generated with trypsin (A) α - subunit (B) β – subunit and (C) MS/MS fragmentation spectra of one selected peptide [DPTERHIGIDVNSIK] of these peptide mass finger prints. De novo sequencing was carried out on a 4800 MALDI TOF/TOF Analyzer (Applied Biosystems, Foster City, CA, USA). Nitrogen was used as collision gas for MS/MS analysis. All digests were dissolved in 50% acetonitrile containing 0.1% v/v TFA and analyzed in the positive-ion mode.

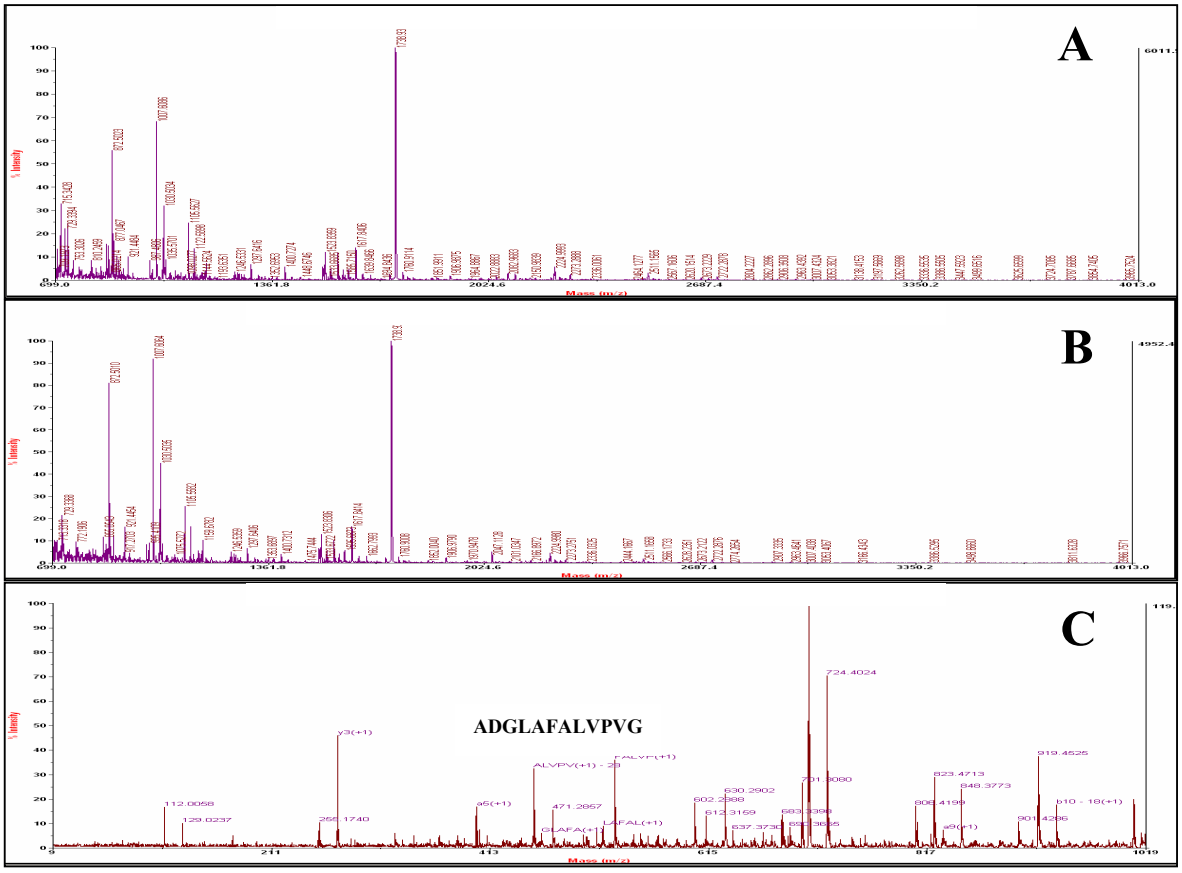


Figure: 4.4

Peptide mass fingerprints generated with Chymotrypsin (A) α - subunit (B) β – subunit and (C) MS/MS fragmentation spectra of one selected peptide [ADGLAFALVPVG] of these peptide mass finger prints.

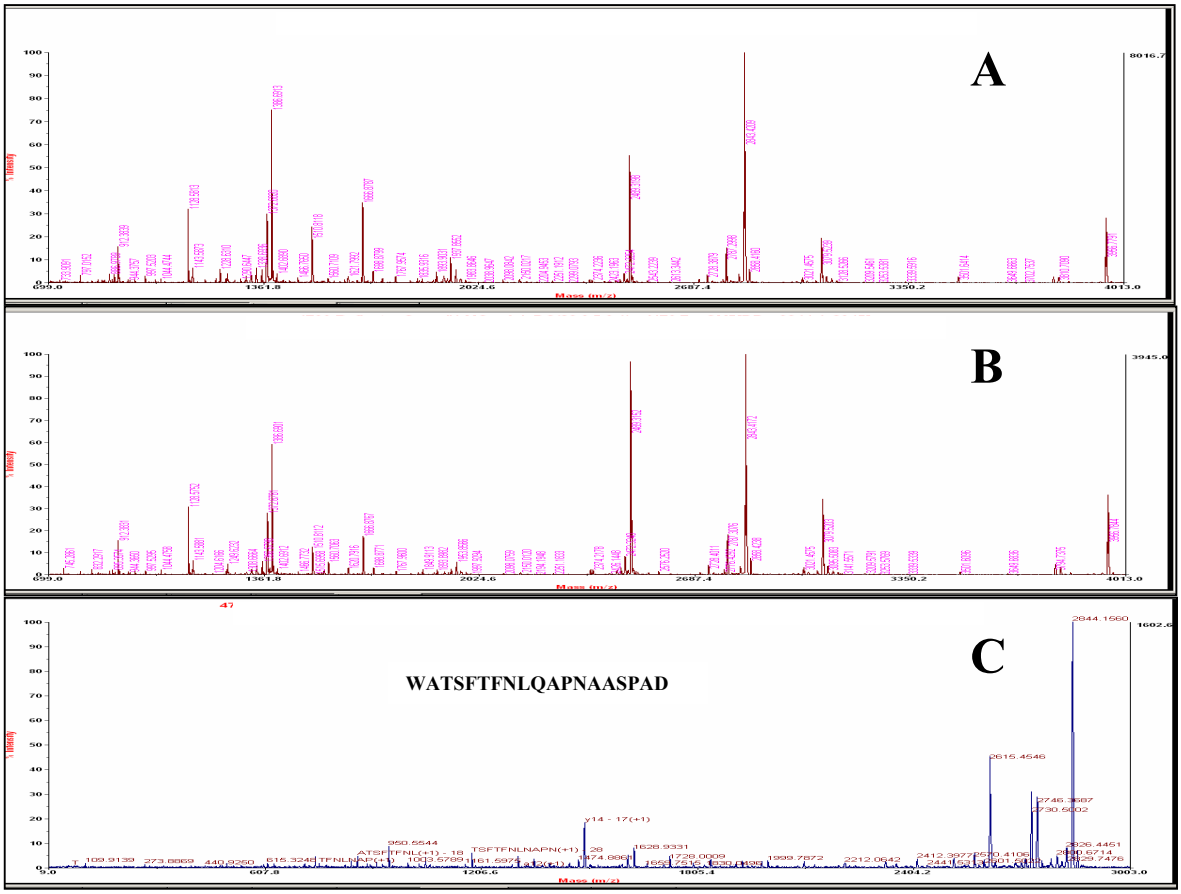


Figure: 4.5

Peptide mass fingerprints generated with Aspartase-N (A) α - subunit (B) β – subunit and (C) MS/MS fragmentation spectra of one selected peptide [WATSFTFNLQAPNAASPAD] of these peptide mass finger prints.

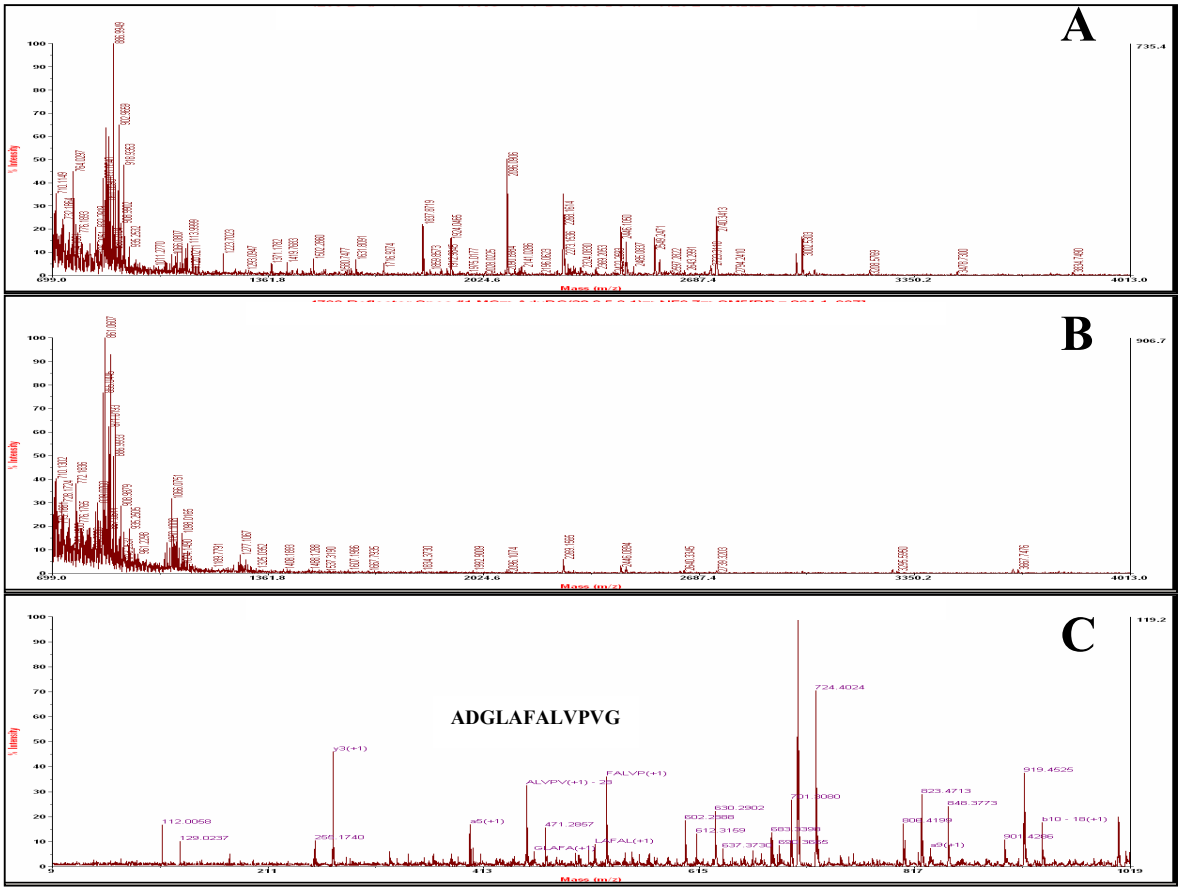


Figure: 4.6

Peptide mass fingerprints generated with Staphylococcus V8 protease (A) α - subunit (B) β – subunit and (C) MS/MS fragmentation spectra of one selected peptide [ADGLAFALVPVG] of these peptide mass finger prints.

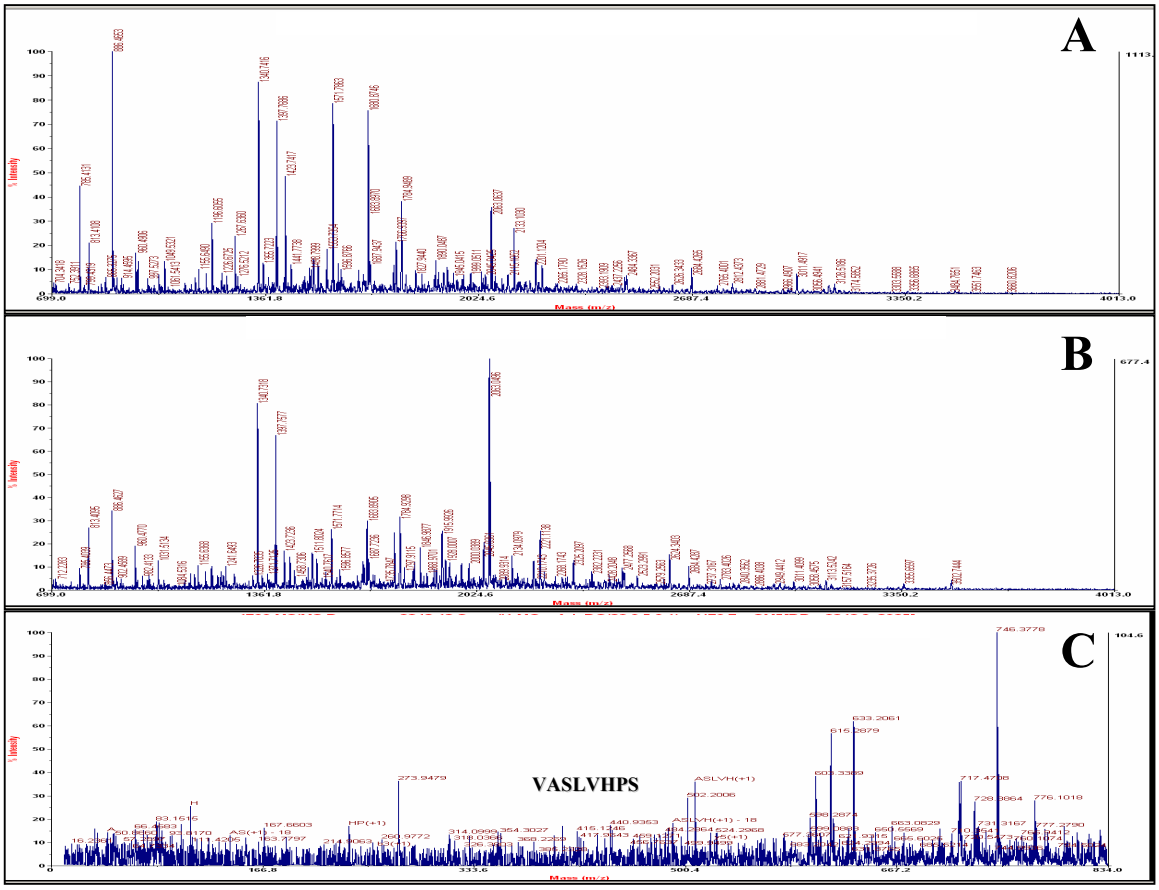


Figure: 4.7

Peptide mass fingerprints generated with 70 % Formic Acid treatment (A) α - subunit (B) β – subunit and (C) MS/MS fragmentation spectra of one selected peptide [VASLVHPS] of each of these peptide mass finger prints.

Experimentally obtained masses were compared to peptide mass sets obtained from the in silico theoretical digests of lectin proteins in databases. Using this approach, however, only a very small fraction of the peptides could be assigned which was due to the fact that the sequence was novel and thus only sequences absolutely conserved among the lectins from different legume species could be assigned. Therefore, the next step was *de novo* sequencing of peptides by low-energy CID on a 4800 MALDI TOF/TOF Analyzer (Applied Biosystems, Foster City, CA, USA). Using this strategy we obtained clear-cut – manually interpretable – peptide fragmentation spectra displaying the expected consecutive runs of b^- and y^- ions. In this study the MASCOT, MS-BLAST 2, and PROTEIN PROSPECTOR programs were used for the analysis of the MS data allowing us to utilize the complete set of peptide sequences of unknown order as determined by MS/MS from one protein in the same query. Particularly, we made use of the complementary sequence information obtained from the different enzymatic and chemical digests. This complementary sequence information was partially overlapping permitting deduction of the complete primary structure for lectin. In order to distinguish between the two subunits the MS-aided determination of the lectin sequence was performed for the affinity purified lectin with isolated, separated intact subunits.

In Table 1 the details of the various peptide sequences that were derived from the proteolytic digestion of the subunits with different proteases and chemicals have been summarized. Digestion of the α subunit with trypsin and chymotrypsin yielded 22 peptides ($T^1 – T^{22}$) (Table 4.1) and 28 peptides (C^1 - C^{28}) (Table 4.2) respectively, from which the backbone of the sequence was established. Additional overlap in sequences for the tryptic and chymotryptic peptides was then provided with 13 peptides that resulted from cleavage at the Asp - NH_2 terminal peptide bond ($A^1 – A^{13}$) (Table 4.3) by proteolytic digestion with the Asp-N endopeptidase. To close the remaining gaps and to support the sequence even further, 25 peptides generated by proteolytic digestion of the α subunit with *Staphylococcus V8 protease* that specifically cleaves at Glu – COOH terminal ($G^1 – G^{25}$) (Table 4.4) were added to the analysis. Chemical cleavage with 70 % formic acid yielded thirteen fragments resulting from hydrolysis of the peptide bond between Asp-Pro (Table 4.5). Cleavage between Asn^{60}-Gly^{61}, Asn^{152}-Gly^{153} and Asn^{181}-Gly^{182} peptide bonds by treatment of hydroxylamine generated four large fragments. IBA treatment hydrolyzed the tryptophanyl peptide bonds at positions (I^{80}, I^{90}, I^{155}, I^{177}, I^{226}, I^{250}), which resulted in generation of seven large fragments. Using the peptides generated by digestion with trypsin, chymotrypsin, Asp-N and *Staphylococcus V8 protease* as well as the data generated by chemical degradation with formic acid or IBA the sequence of the α subunit was assembled (Fig. 4.8).

Peptide No:	Trypsin Peptides	Monoisotopic Mass
T^{1}	NNLISFTMK	1067.5554
T^{2}	RIVLFLILLTK	1172.8016
T^{3}	KAASANLISFTFK	1257.6473
T^{4}	RFNETNLILQRDATVSSGK	1893.9552
T^{5}	RDATVSSGK	764.3784
T^{6}	KLR	288.2030
T^{7}	RITK	361.2445
T^{8}	KAAENGVPTAGSLGR	1299.6651
T^{9}	RAFYSTPIQIWDNTTGTVASW ATSFTFNLQAPNAASPADGL AFALVPVGSQPK	5426.7007
T^{10}	KDK	262.1397
T^{11}	KGGFLGLFDSK	1040.5411
T^{12}	KNYASSNQTVAVEFDTFYNGG WDPTER	2968.3020
T^{13}	RHIGIDVNSIK	1095.6156
T^{14}	KSIK	347.2289
T^{15}	KTTSWDFANGENAEVLITYDS STNLLVASLVHPSQK	3807.8711
T^{16}	KTSFIVSER	938.4941
T^{17}	RVDLTSVLPEWVSVGFSATTGLSK	2393.2598
T^{18}	KGYVETNEVLSWSFASK	1816.8752
T^{19}	KISINK	574.3559
T^{20}	KEDEENK	763.3104
T^{21}	KLLISNLEGK	986.5880
T^{22}	KAINNLA	615.3460

Table: 4.1

Peptides derived from trypsin digestion

Purified subunits were subjected to proteolytic (Trypsin) digestion and were spotted on MALDI target, followed by MS/MS analysis and De-novo sequencing on a 4800 MALDI TOF/TOF Analyzer (Applied Biosystems, Foster City, CA, USA).

Peptide No:	Chymotrypsin Peptides	Monoisotopic Mass
C^{1}	NNLISF	707.3722
C^{2}	FTMKRIVLF	1007.6070
C^{3}	FLILLTKAASANLISF	1574.9516
C^{4}	FTF	267.1339
C^{5}	FKRF	450.2823
C^{6}	FNETNLILQRDATVSSGKLRITKAAENGVPTAGSLGRAF	3956.1311
C^{7}	FY	182.0812
C^{8}	YSTPIQIW	844.4563
C^{9}	WDNTTGTVASW	1051.4691
C^{10}	WATSF	425.2031
C^{11}	FTF	267.1339
C^{12}	FNLQAPNAASPADGLAF	1556.7703
C^{13}	FALVPVGSQPKDKGGF	1499.8216
C^{14}	FLGLF	449.2758
C^{15}	FDSKNY	626.2780
C^{16}	YASSNQTVAVEF	1152.5531
C^{17}	FDTF	382.1609
C^{18}	FYNGGW	615.2642
C^{19}	WDPTERHIGIDVNSIKSIKTTSW	2497.3045
C^{20}	WDF	281.1132
C^{21}	FANGENAEVLITY	1293.6321
C^{22}	YDSSTNLLVASLVHPSQKTSF	2131.1029
C^{23}	FIVSERVDLTSVLPEW	1742.9323
C^{24}	WVSVGF	508.2765
C^{25}	FSATTGLSKGY	984.4996
C^{26}	YVETNEVLSW	1076.5258
C^{27}	WSF	253.1183
C^{28}	FASKISINKEDEENKLLISNLEGKAINNLA	3168.7110

Table: 4.2

Peptides derived from Chymotrypsin digestion

Purified subunits were subjected to proteolytic (Chymotrypsin) digestion and were spotted on MALDI target, followed by MS/MS analysis and De-novo sequencing on a 4800 MALDI TOF/TOF Analyzer (Applied Biosystems, Foster City, CA, USA).

Peptide No:	Aspartase - N Peptides	Monoisotopic Mass
A^{1}	NNLISFTMKRIVLFLILLTKAASANLISFTFKRFNETNLILQR	5012.8637
A^{2}	DATVSSGKLRITKAAENGVPTAGSLGRAFYSTPIQIWD	3863.0449
A^{3}	DNTTGTVASWATSFTFNLQAPNAASPAD	2740.2849
A^{4}	DGLAFALVPVGSQPKD	1498.8263
A^{5}	DKGGFLGLFD	953.5090
A^{6}	DSKNYASSNQTVAVEFD	1759.8133
A^{7}	DTFYNGGWD	959.3893
A^{8}	DPTERHIGID	1037.5374
A^{9}	DVNSIKSIKTTSWD	1478.7849
A^{10}	DFANGENAEVLITYD	1555.7274
A^{11}	DSSTNLLVASLVHPSQKTSFIVSERVD	2814.4995
A^{12}	DLTSVLPEWVSVGFSATTGLSKGYVETNEVLSWSFASKIS INKED	4776.4294
A^{13}	DEENKLLISNLEGKAINNLA	2198.1662

Table: 4.3

Peptides derived from Aspartase – N - Endopeptidase digestion
Purified subunits were subjected to proteolytic (Aspartase – N - Endopeptidase) digestion and were spotted on MALDI target, followed by MS/MS analysis and De-novo sequencing on a 4800 MALDI TOF/TOF Analyzer (Applied Biosystems, Foster City, CA, USA).

Peptide No:	Staphylococcus V8 protease Peptides	Monoisotopic Mass
G^{1}	NNLISFTMKRIVLFLILLTKAASANLISFTFKRFNE	4174.3612
G^{2}	ETNLILQRD	972.5472
G^{3}	DATVSSGKLRITKAAE	1531.8802
G^{4}	ENGVPTAGSLGRAFYSTPIQIWD	2350.1826
G^{5}	DNTTGTVASWATSFTFNLQAPNAASPAD	2740.2849
G^{6}	DGLAFALVPVGSQPKD	1498.8263
G^{7}	DKGGFLGLFD	953.5090
G^{8}	DSKNYASSNQTVAVE	1497.7180
G^{9}	EFD	281.1132
G^{10}	DTFYNGGWD	959.3893
G^{11}	DPTE	332.1452
G^{12}	ERHIGID	710.3944
G^{13}	DVNSIKSIKTTSWD	1478.7849
G^{14}	DFANGE	537.2303
G^{15}	ENAE	333.1405
G^{16}	EVLITYD	723.3923
G^{17}	DSSTNLLVASLVHPSQKTSFIVSE	2444.3031
G^{18}	ERVD	389.2143
G^{19}	DLTSVLPE	758.4294
G^{20}	EWVSVGFSATTGLSKGYVE	1887.9487
G^{21}	ETNE	363.1510
G^{22}	EVLSWSFASKISINKED	1708.9268
G^{23}	DEE	392.1299
G^{24}	ENKLLISNLE	1043.6095
G^{25}	EGKAINNLA	800.4624

Table: 4.4

Peptides derived from *Staphylococcus V8 protease* digestion

Purified subunits were subjected to proteolytic (Staphylococcus V8 protease) digestion and were spotted on MALDI target, followed by MS/MS analysis and De-novo sequencing on a 4800 MALDI TOF/TOF Analyzer (Applied Biosystems, Foster City, CA, USA).

Peptide No:	70% Formic Acid Peptides	Monoisotopic Mass
F^{1}	NNLISFTMKRIVLFLILLTKAASANLISFTFKRFNETNLILQR	5012.8637
F^{2}	DATVSSGKLRITKAAENGVPTAGSLGRAFYSTPIQIWD	3863.0449
F^{3}	DNTTGTVASWATSFTFNLQAPNAASPAD	2740.2849
F^{4}	DGLAFALVPVGSQPKD	1498.8263
F^{5}	DKGGFLGLFD	953.5090
F^{6}	DSKNYASSNQTVAVEFD	1759.8133
F^{7}	DTFYNGGWD	959.3893
F^{8}	DPTERHIGID	1037.5374
F^{9}	DVNSIKSIKTTSWD	1478.7849
F^{10}	DFANGENAEVLITYD	1555.7274
F^{11}	DSSTNLLVASLVHPSQKTSFIVSERVD	2814.4995
F^{12}	DLTSVLPEWVSVGFSATTGLSKGYVETNEVLSWSFASKIS INKED	4776.4294
F^{13}	DEENKLLISNLEGKAINNLA	2198.1662

Table: 4.5

Peptides derived from 70 % Formic Acid digestion

Purified subunits were subjected to Chemical (70 % Formic Acid) digestion and were spotted on MALDI target, followed by MS/MS analysis and De-novo sequencing on a 4800 MALDI TOF/TOF Analyzer (Applied Biosystems, Foster City, CA, USA).

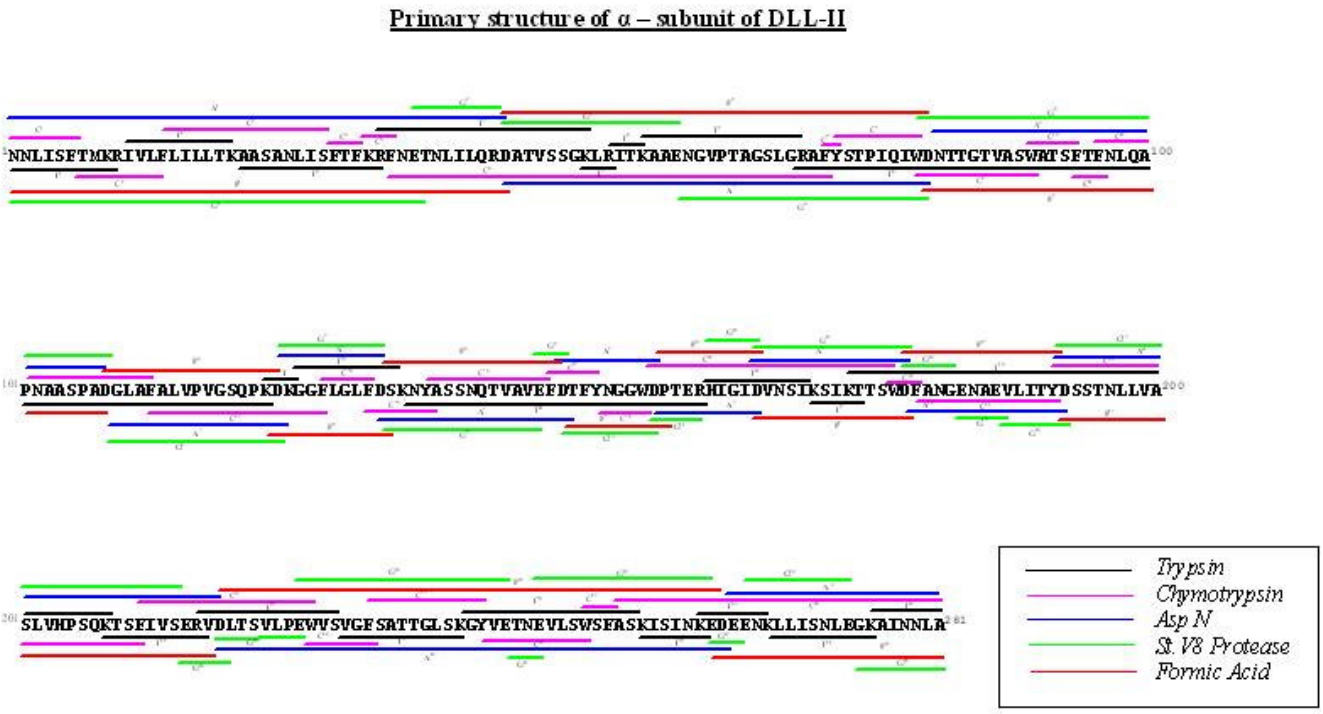


Figure: 4.8

Assembly of the primary structure of DLL-II from the various digestions
*MALDI-MS/MS analysis of the different enzymatic and chemical digestion of the two subunits of lectin yielded overlapping data sets that were then used to assemble the primary sequence of both subunits. The figure displays the primary sequence obtained as well as the contribution of the different complementing digests. Peptides supporting the α subunit are indicated in the amino acid sequence. Peptides obtained by Trypsin (**T**), Chymotrypsin (**C**), Staphylococcus V8 protease (**G**), Aspartase N endopeptidase (**A**) and 70% formic acid (**F**) are indicated with different colours.*

In a separate effort, the isolated β subunit was also subjected to the proteolytic and chemical treatments similar to the α subunit. MS-analysis and interpretation of the MS/MS-fragmentation data of these different digests revealed 20 tryptic peptides, 28 chymotryptic peptides, 12 Asp-N peptides, 22 *Staphylococcus V8 protease* peptides. Peptides generated from chemical treatments with formic acid, hydroxylamine and 2-iodosobenzoic acid was also aligned as for α subunit. Performing the same analysis as for the α subunit, the peptides of the β subunit were assembled to yield a complete primary structure (Fig. 4.9). Using the Data Explorer software - all MS spectra of both subunits were analyzed: Most of the spectra showed no differences in the peak distribution, allowing us to conclude that both subunits were highly similar. Additionally, we analyzed manually all differences between corresponding spectra to reveal any differences in the sequence.

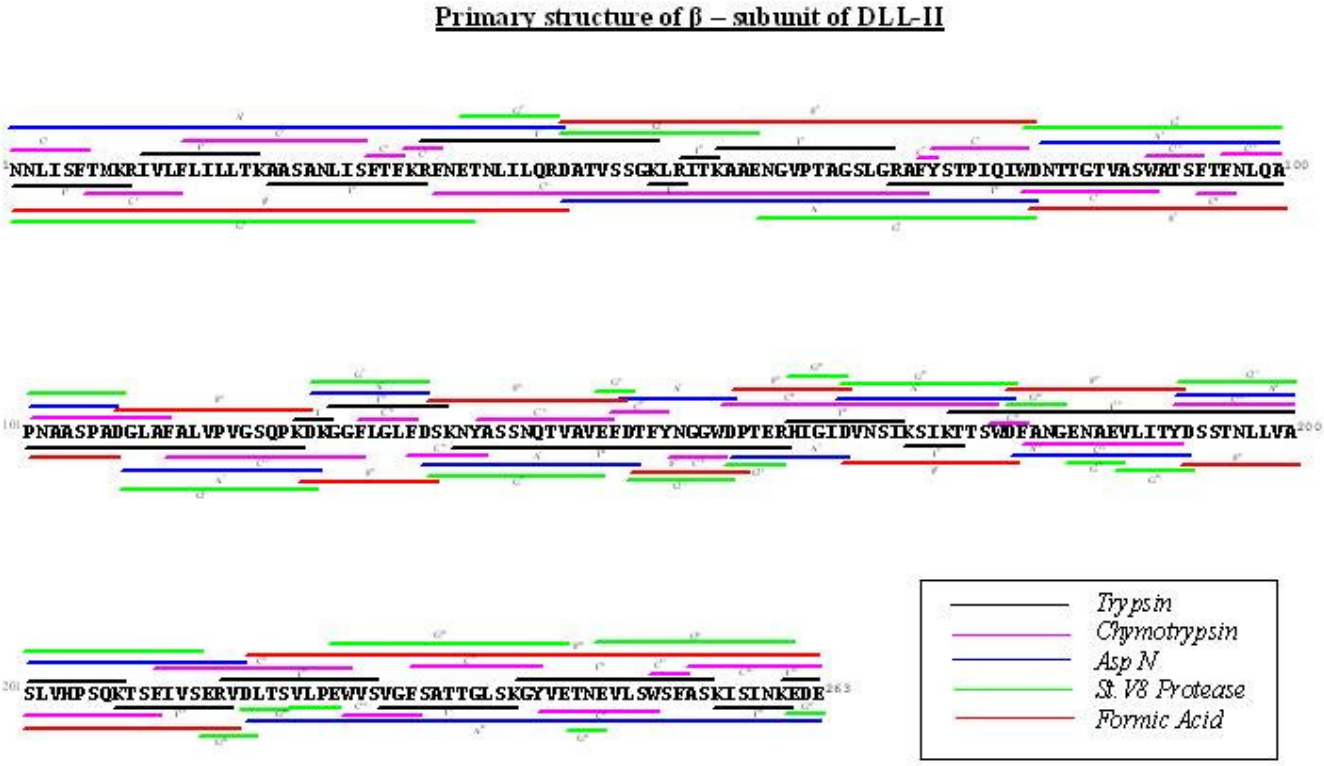


Figure: 4.9

*The figure displays the primary sequence obtained as well as the contribution of the different complementing digests. Peptides supporting the β subunit are indicated in the amino acid sequence. Peptides obtained by Trypsin (**T**), Chymotrypsin (**C**), Staphylococcus V8 protease (**G**), Aspartase N endopeptidase (**A**) and 70% formic acid (**F**) are indicated with different colours.*

Since both subunits displayed identical primary structure but yet differed in size the determination of the N - and C - termini was of particular importance. From the previous studies we learnt from the N terminal edman sequencing that the N terminus of the α and β subunits was similar (Latha *et al*., 2006). Thus the determination of the carboxy-terminus was of special interest and was performed here by carboxypeptidase treatment followed by MALDI TOF MS/MS. Carboxypeptidase treatment released 18 carboxy terminal amino acids from the α subunit. Initially, we determined the mass of the α subunit by ESI-MS to be 30,746 Da which fits well to the initial rough estimation that was first based on SDS-PAGE (31 kDa). The molecular mass calculated on the basis of sequence analysis (30,420 Da) compares well with the total molecular mass determined by ESI-MS. By identifying peptides by MS covering the individual N-termini of each of the subunits isolated from the SDS-PAGE we had

reestablished that each of the subunits has identical NH_2 termini. Furthermore, the primary structure of the β subunit obtained from the mass spectrometry analysis of the proteolytic and chemical digests revealed that the sequence of the β subunit is identical in 263 amino acids and differs from the α subunit only in the absence of 19 amino acids at the C – terminus.

Comparison of the carboxy termini of α and β subunits shows that the α subunit contains at its C-terminus four leucines, four aspargines, three isoleucines, two lysines, two alanines, two glutamic acid and an extra serine and glycine. An analysis of carboxy-terminus of the β subunit by carboxypeptidase treatment indicated EDE to be the COOH terminal sequence, which is also that of the C-terminal tryptic peptide. This assignment of the C-terminus of the β subunit was also supported by the molecular mass that calculated on the basis of the derived sequence. This calculated value of 28,485 Da compares well with that estimated by SDS-PAGE (29,000 Da) and the total mass of the subunit determined by ESI-MS (28,815 kDa).

4.3.3. Comparison of the DLL-II sequence with other legume lectins

The sequence obtained from the de novo sequencing and manual sequencing using GPS explorer from Applied biosystems was compared with the Glu/Man lectin from the *Dolichos lablab* and the other legume lectins. Structural comparison of the glu/man lectin sequences with other legume lectins, e.g. from *Dolichos biflorus*, Phytohemagglutinin, and Soybean agglutinin etc, suggests high degree of conservation/ identity. On the other hand the galactose lectin sequenced in the present study showed only limited homology to the Glu/Man lectin but showed a higher degree of homology with other legume lectins described above, which illustrates to understand the evolutionary relatedness of these two lectins from the same plant.

The sequence of the *Dolichos lablab* galactose-specific lectin was aligned with 14 other legume lectins of varying sugar specificity using the CLUSTAL W 1.82 (www.expasy.org) program. Both subunits of the galactose specific lectin were identical except at the carboxy terminal end. Glucose/mannose lectin purified from the same seed material displayed a non-identical subunit pattern compared to DLL- II with a difference of 2000 Da. Direct sequence comparison of the glucose/mannose lectin with the related galactose lectin from *Dolichos lablab* revealed that both lectins showed only 36.4 % identity, proving that the lectin analyzed in this study is novel and displays a different subunit heterogeneity. Comparison of the sequence with other legume lectins showed higher sequence conservation for the *Dolichos biflorus* seed lectin (59.3 % identity) *Dolichos biflorus* stem and leaf lectin (62.6 %) and *Phaseolus vulagaris* (55.3 %). The extensive sequence conservation exhibited by both subunits of the galactose specific lectin encouraged us to investigate the similarity with other legume

lectins. Indeed it is known in literature that the *Dolichos biflorus* lectin, favin and lentil lectins also exhibit this property [Foriers *et al*., 1981; Hemperley *et al*., 1982; Higgins *et al*. 1983; Etzler 1994]. Fig. 4.10 shows the similarity / identity of the β subunit in its amino terminal region to other legume lectins.

```
    DLL-II      --------------NNLISFTMKRIVLFLILLTKAASANLISFTFKRFN------ETNLILQRDATVSS-GKLRITKAAE
LEC1_DOLBI      ---------------MASSTVSVVLSLFLLLLTQANSANIQSFSFKNFN------SPSFILQGDATVSS-GKLQLTKVKE
LEC5_DOLBI      ---------------MASSTVSVVLSLFLLLLTQAYSADIQSFSFKNFN------SSSFILQGDATVSS-SKLRLTKVKG
PHAE_PHAVU      ----------------MASSNLLSLALFLVLLTHANSASQTSFSFQRFN------ETNLILQRDATVSSKGQLRLTNVND
LEC_PEA         -------MASLQTQMISFYAIFLSILLTTILFFKVNSTETTSFLITKFS----PDQQNLIFQGDGYTT-KEKLTLTKAVK
LECS_SOPJA      MATSNSRPHLLQTHKPFSVVLAISITFFLLLLNKVNSAEILSFSFPKFA----SNQEDLLLQGDALVSSKGELQLTTVE-
LEC_LENCU       -------MASLQTQMISFYLIFLSILLTTIFFFKVNSTETTSFSITKFS----PDQKNLIFQGDGYTT-KGKLTLTKAVK
LEC1_LABAL      ------------------------------------LNELSFNFDKFV----PNQNNILFQGVASVSTTGVLQVTKVT-
LEC_VICFA       ----------------------------------TDEITSFSIPKFR----PDQPNLIFQGGGYTT-KEKLTLTKAVK
LEC_ERYCG       -----------------------------------VETISFSFSEFE----PGNDNLTLQGAALITQSGVLQLTKINQ
LECA_DOLLA      ----------------------------------AQSLSFSFTKFD----PNQEDLIFQGTATS---------KLDS
LEC_BOWMI       ---------------------------------ANSVCFTFTDFE----SGQQDLIFQGDASVGSNKALQLTKVDS
LEC_ERYCO       -----------MATYKLCSVLALSLTLFLLILNKVNSVETISFSFSEFE----PGNDNLTLQGAALITQSGVLQLTKINQ
LEC_SOYBN       -----MATSKLKTQNVVVSLSLTLTLVLVLLTSKANSAETVSFSWNKFV----PKQPNMILQGDAIVTSSGKLQLNKVDE
ARC2_PHAVU      ----------------MASSNLLTLALFLVLLTHANSSNDASFNVETFN------KTNLILQGDATVSSEGHLLLTNVKG

    DLL-II      NGVPTAGSLGRAFYSTPIQIWDN--TTGTVASWATSFTFNLQAPNAAS----PADGLAFALVPVGSQPK--DKGGFLGLF
LEC1_DOLBI      NGIPTPSSLGRAFYSSPIQIYDK--STGAVASWATSFTVKISAPSKAS----FADGIAFALVPVGSEPR--RNGGYLGVF
LEC5_DOLBI      NGLPTLSSLGRAFYSSPIQIYDK--STGAVASWATSFTANIFAPNKSS----SADGIAFALVPVGSEPK--SNSGFLGVF
PHAE_PHAVU      NGEPTLSSLGRAFYSAPIQIWDN--TTGAVAASPTSFTFNIDVPNNSG----PADGLAFVLLPVGSQPK--DKGGLLGLF
LEC_PEA         N------TVGRALYSSPIHIWDR--ETGNVANFVTSFTFVINAPNSYN----VADGFTFFIAPVDTKPQTG--GGYLGVF
LECS_SOPJA      NGVPIWNSTGRALYYAPVHIWDK--STGRVASFATSFSFVVKAPVAS----KSADGIAFFLAPPNNQIQ-GPGGGHLGLF
LEC_LENCU       S------TVGRALYSTPIHIWDR--DTGNVANFVTSFTFVIDAPSSYN----VADEFTFFIAPVDTKPQTG--GGYLGVF
LEC1_LABAL      N-----TGIKRALYAAPIHAWDDDSETGKVASFATSFSFVVKEPPIQSRKADGVDGLAFFLAPANSQIPSGSSAGMFGLF
LEC_VICFA       N------TVGRALYSLPIHIWDS--ETGNVADFTTTFIFVIDAPNGYN----VADGFTFFIAPVDTKPQTG--GGYLGVF
LEC_ERYCG       NGMPAWDSTGRTLYTKPVHMWDS--TTGTVASFETRFSFSIEQPYTRP---LPADGLVFFMGPTKSKP--AQGYGYLGVF
LECA_DOLLA      AGNPVSSSAGRVLYSAPLRLWED---SAVLTSFDPTIYIFTN---YTS----RIADGLAFIAPPDSVIS--YHGGFLGLF
LEC_BOWMI       KGNPQGGSVGRALYTAPIRLWQS---SSLVASFETTFTFSISQGSSTP----AAALTFFIASPDTKIPS--GSGGRLLGL
LEC_ERYCO       NGMPAWDSTGRTLYAKPVHIWDM--TTGTVASFETRFSFSIEQPYTRP---LPADGLVFFMGPTKSKP--AQGYGYLGIF
LEC_SOYBN       NGTPKPSSLGRALYSTPIHIWDK--ETGSVASFAASFNFTFYAPDTKR----LADGLAFFLAPIDTKPQTHAG--YLGLF
ARC2_PHAVU      NEE---DSMGRAFYSAPIQINDR--TIDNLASFSTNFTFRINAKNNEN----SAYGLAFALVPVGSRPK--LKGRYLGLF

    DLL-II      DSKN--YASSNQTVAVEFDTFYNG---GWDP-------TERHIGIDVNSIKSIKTTSWDFANGE---NAEVLITYDSSTN
LEC1_DOLBI      DSDV--YNNSAQTVAVEFDTFSNS---GWDP-------SMKHIGIDVNSIKSIATVSWDLAN---GENAEILITYNAATS
LEC5_DOLBI      DSDV--YDNSAQTVAVEFDTFSNT---DWDP-------TSRHIGIDVNSIKSIRTASWGLAN---GQNAEILITYNAATS
PHAE_PHAVU      NNYK--YDSNAHTVAVEFDTLYNV---HWDP-------KPRHIGIDVNSIKSIKTTTWDFVK---GENAEVLITYDSSTK
LEC_PEA         NSA--EYDKTTQTVAVEFDTFYN---AAWDP-----SNRDRHIGIDVNSIKSVNTKSWKLQNGE---EANVVIAFNAATN
LECS_SOPJA      HSS--GYNSSYQIIAVDFDTHI----NAWDP------NTR-HIGIDVNSINSTKTVTWGWQNGE---VANVLISYQAATE
LEC_LENCU       NSK--EYDKTSQTVAVEFDTFYN---AAWDP-----SNKERHIGIDVNSIKSVNTKSWNLQNGE---RANVVIAFNAATN
LEC1_LABAL      CSS--DYNSSNQIIAVEFDTYFGKAYNPWDP------DFK-HIGVDVNSIKSIKTVKWDWRNGD---VANVVITYRAPTK
LEC_VICFA       NGK--DYDKTAQTVAVEFDTFYN---AAWDP-----SNGKRHIGIDVNTIKSISTKSWNLQNGE---EAHVAISFNATTN
LEC_ERYCG       NNS--KQDNSYQTLAVEFDTFS----NPWDP------PQVPHIGIDVNSIRSIKTQPFQLDNGQ---VANVVIKYDAPSK
LECA_DOLLA      PNAAESGIAESNVVAVEFDTDYLN-PDYGDP-------NYIHIGIDVNSIRSKVTASWDWQNGK---IATAHISYNSVSK
LEC_BOWMI       FGSSNNAGSDNGVVAVEFDT-YPN-TDIGDP-------NYRHIGIDVNSIRSKAASKWDWQNGK---TATAHISYNSASK
LEC_ERYCO       NNS--KQDNSYQTLGVEFDTFS----NPWDP------PQVPHIGIDVNSIRSIKTQPFQLDNGQ---VANVVIKYDASSK
LEC_SOYBN       NEN----ESGDQVVAVEFDTFR----NSWDP------PNP-HIGINVNSIRSIKTTSWDLANNK---VAKVLITYDASTS
ARC2_PHAVU      NTAN--YDRDAHTVAVVFDTVSNR----------------IEIDVNSIRPIATESCNFGHNN-GEKAEVRITYYSPKN

    DLL-II      LLVASLVHPS-----QKTSFIVSERVDLTSVLPEWVSVGFSATTGLSKGYVETNEVLSWSFASKISINKEDE----------
LEC1_DOLBI      LLVASLVHPS-----RRTSYILSERVDITNELPEYVSVGFSATTGLSEGYIETHDVLSWSFASKLPDDSTAEPLDLASYLVRNVL-
LEC5_DOLBI      LLVASLVHPS-----RRTSYIVSERVDITNELPEYVSIGFSATTGLSEGYTETHDVLSWSFASKLPDDSTTEPLDIASYLVRNVL-
PHAE_PHAVU      LLVASLVYPS-----LKTSFIVSDTVDLKSVLPEWVIVGFTATTGITKGNVETNDILSWSFASKLSDGTTSEALNLANFALNQIL-
LEC_PEA         VLTVSLTYPNSLEEENVTSYTLSDVVSLKDVVPEWVRIGFSATTG---AEYAAHEVLSWSFHSELSGTSSSKQAADA---------
LECS_SOPJA      TLTVSLTYPS----SQTSYILSAA-VDLKSILPEWVRVGFTAATGLTTQYVETHDVLSWSFTSTLETGDCGAKDDNVHLVSYAFI-
LEC_LENCU       VLTVTLTYPNSLEEENVTSYTLNEVVPLKDVVPEWVRIGFSATTG---AEFAAHEVHSWSFHSELGGTSSSKQAADA---------
LEC1_LABAL      SLTVSLSYPS----DQTSNIVTAS-VDLKAILPEWVSVGFSAGVGN-AAKFN-HDILSWYFTSNLEPNNPAVNQAQ---------
LEC_VICFA       VLSVTLLYPN------LTGYTLSEVVPLKDVVPEWVRIGFSATTG---AEYATHEVLSWTFLSELTGPSN----------------
LEC_ERYCG       ILHVVLVYPS-----SGAIYTIAEIVDVKQVLPDWVDVGLSGATGAQRDAAETHDVYSWSFQASLPE-----------------
LECA_DOLLA      RLSVTTYYPG------RGKPATSYDIELHTVLPEWVRVGLSASTG---QNIERNTVHSWSFTSSLWTNVAKVGVASISG-------
LEC_BOWMI       RLSVVSSYPN------SSPVVVSFDVELNNVGPPDVRVGFSATTG---QYTQTNNILAWSFRSSLMGYQAN----------------
LEC_ERYCO       ILHAVLVYPS-----SGAIYTIAEIVDVKQVLPEWVDVGLSGATGAQRDAAETHDVYSWSFQASLPETNDAVIPTSNHNTFAI---
LEC_SOYBN       LLVASLVYPS-----QRTSNILSDVVDLKTSLPEWVRIGFSAATG-LDIPGESHDVLSWSFASNLPHASSNIDPLDLTSFVLHEAI
ARC2_PHAVU      DLRVSLLYPS-----SEEKCHVSATVPLEKEVEDWVSVGFSATSGSKKETTETHNVLSWSFSSNFIN-FEGKKSERSNILLNKIL-
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Figure: 4.10

Comparison of the complete amino acid sequence of DLL- II β subunit with those of other leguminous lectins from (LEC5_DOLBI, LEC1_DOLBI) Dolichos biflorus, (PHAE_PHAVU, ARC2_PHAVU) Phaseolus vulgaris, (LEC_PEA, LEC_VICFA) Vicia faba, (LECS_SOPJA,) Sophora japonica, (LEC_LENCU) Lens culinaris, (LEC1_LABAL) Laburnum alpinum, (LECA_DOLLA) Dolichos lablab, (LEC1_LABAL) Laburnum alpinum, (LEC_ERYCO) Erythrina corallodendron, (LEC_BOWMI) Bowringia mildbraedii (LEC_SOYBN) Glycine max.

Table 4.6, summarizes the percentage of the DLL-II sequence identity with lectins of the same tribe *Phaseoleae* and with other legume lectins with different sugar specificity.

S.No	Species	Abbreviation	Sugar Group Specificity	Tribe	Identity
1	*Dolichos biflorus* (Horse gram)	----	I & VII	*Phaseoleae*	62.6%
2	*Dolichos biflorus* (Horse gram)	SL	I VII	*Phaseoleae*	59.3%
3	*Leucoagglutinating phytohemagglutinin*	PHA-L	II	*Phaseoleae*	58.1%
4	*Erythroagglutinating phytohemagglutinin*	PHA-E	II	*Phaseoleae*	55.3%
5	*Leucoagglutinating phytohemagglutinin*	PHA-L	II	*Phaseoleae*	56.8%
6	*Glycine max* (Soybean)	SBA	I & VII	*Phaseoleae*	50.8%
7	*Cytisus scoparius* (Scotch broom)	CSII	IV	*Genisteae*	50.6%
8	*Lathyrus ochrus* (Yellow-flowered pea)	----	II	*Vicieae*	49.5%
9	*Pisum sativum* (Garden pea)	PSL1	II	*Vicieae*	46.3%
10	*Lens culinaris* (Lentil)	----	II	*Vicieae*	45.9%
11	*Vicia faba* (Broad bean)	Favin Lectin	V	*Vicieae*	45.7%
12	*Griffonia simplicifolia*	GS4	V	*Cercideae*	44.0%
13	*Erythrina corallodendron* (Coral tree)	ECorL	I & VII	*Phaseoleae*	41.9%
14	*Laburnum alpinum* (Scotch laburnum)	LAA-I	III	*Genisteae*	41.9%
15	*Cladrastis lutea* (Yellow wood)	LecClAII	II	*Sophoreae*	40.8%
16	*Bowringia mildbraedii*	BMA	X	*Sophoreae*	39.2%
17	*Dolichos lab lab* (Field bean)	----	II	*Phaseoleae*	36.4%
18	*Phaseolus vulgaris* (Kidney bean) (French bean)	----	V	*Phaseoleae*	46.5%
19	*Sophora japonica* (Japanese pagoda tree)	LECSJABG	VII	*Sophoreae*	49.2%
20	*Canavalia ensiformis* (Jack bean)	Con A	II	*Diocleae*	35.7%

Table: 4.6

Comparative display of the sequence identity of DLL- II lectin with other legume lectins
The lectin has been compared with the other legume lectins for the sequence homology and identity using the Clustal W algorithm.

PART D

4.4.0. Discussion

The proteomics approach used in the present study illustrates a novel method for the characterization of lectins. Thus far, sequencing of many lectins has been exclusively performed using the conventional Edman degradation method [Shadforth *et al*., 2005] and

cDNA cloning [Kaku *et al*., 1996]. Unsequenced proteomes are now being well characterized using the advances of the mass spectrometry [Matis *et al*., 2005]. In the present study we successfully deduced the complete primary sequence of the newly characterized galactose lectin (DLL-II) from the *Dolichos lablab* seeds which allowed us to make a comparison of the DLL-II with the glucose/mannose specific lectin that has been sequenced from field beans as well as other legume lectins whose sequences are known [Gowda *et al*., 1994]. Most lectins contain subunits that are identical or nearly identical in molecular weight; these are called one-chain lectins, which are mostly tetramers. Some lectins are composed of two subunits, one heavy and light chain, forming tetramers of the type $\alpha_2\beta_2$ and are called two chain lectins. Subunit architecture of *Dolichos lablab* lectins revealed that they are two chain lectin, DLL- I with non-identical subunit pattern of molecular mass (α, 15,000; β, 12,000) [Gowda *et al*., 1994] and DLL- II with a pattern (α, 31,000; β, 29,000) differing at the carboxy terminus and being probably present as tetramers of the type $\alpha_2\beta_2$. Subunit heterogeneity is a common feature to a number of legume lectins [Goldstein, 1986; Etzler, 1992] and must be considered in studies concerning the analysis of their structure and function. The isolation of the subunits of DLL-II in the present study has enabled the direct characterization of these two very similar polypeptides. The strategy adopted in this study clearly demonstrates the close identity of both subunits of DLL-II. This finding is supported by the almost identical amino acid composition of the two subunits and our previous report wherein we have shown that an antibody raised against the α-subunit also crossreacts with the β-subunit suggesting sequence similarities between the two subunits [Latha *et al*., 2006]

De novo and manual sequencing of the spectra's of the proteolytic and chemical digests of the α and β subunits and subsequent database searches allowed us to look for the conserved regions of the lectin; sequences such as the HIGIDVNSIK, AFYTTPI, ADGLAFVPVG, which are significantly conserved in almost all legume lectins listed in Fig.4 and Table 2 above, which confirms the protein in this study has the conserved regions and falls in the family of legume lectins. As reported earlier, both subunits showed identical amino terminal sequences as determined by Edman sequencing [Latha *et al*., 2006].

From literature it is known that several other legume lectins, including concanavalin A and the pea lectin have been found to undergo the posttranslational processing of the amino acids from their COOH termini during biosynthesis [Etzler 1992]. In concanavalin A, this processing accompanies the posttranslational ligation that results in the formation of the circularly permuted mature form of this lectin [Bowles *et al*., 1986]. The seed lectin from *Dolichos biflorus* is a hetrotetramer and these heteromeric subunits 1 and 2 appear to differ only at the

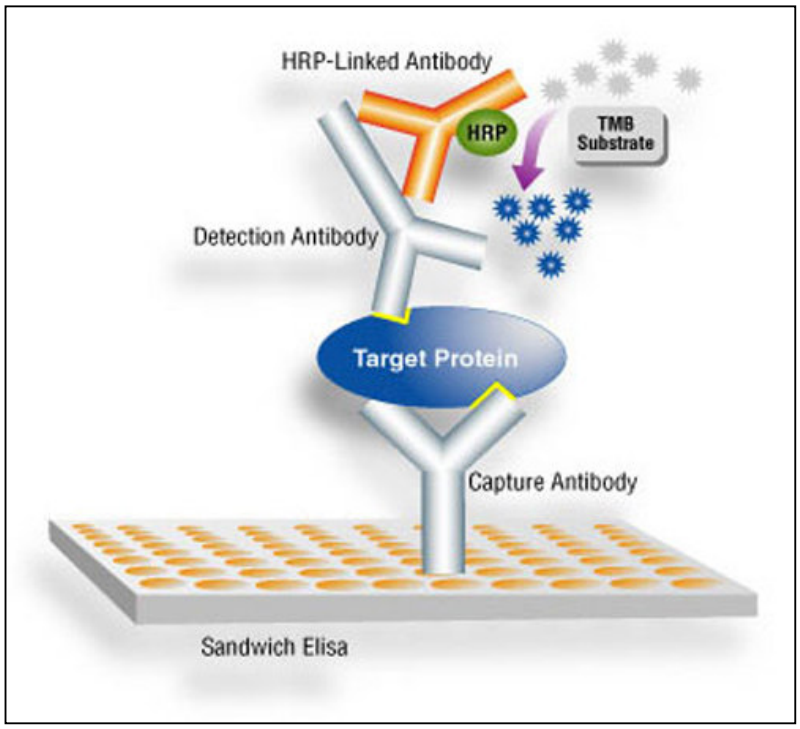
carboxyl termini, and subunit 2 arises by proteolytic cleavage of the subunit 1. It has been suggested that differential cleavage of the COOH terminus of the α chain of the pea lectin may give rise to the molecular forms of this lectin that are commonly found in peas [Stubbs *et al*., 1986]. DLL-II might also undergo posttranslational processing as, addition of protease inhibitors during isolations and variations in time and type of extraction procedures have failed to alter the subunit stoichiometry, leading to the conclusion that the conversion of these subunits occurs *in vivo.* The derivation of the β subunit of DLL-II from its α subunit resembles the origin of subunit II of the *Dolichos biflorus* seed lectin and stem/leaf lectins from subunit I [Carter *et al*.,1975a, Carter *et al*., 1975b, Etzler *et al*., 1981; Roberts *et al*., 1982; Schnell *et al*., 1987; Quinn *et al*., 1989]. In the case of both lectins, only about half of the subunits are converted resulting in the production of the heterooligomeric proteins [Carter *et al*., 1975a, Talbot *et al*., 1978]. These results suggested that the β subunit was derived from the α subunit by posttranslational modification/processing. Truncation at the carboxyl end is well known in certain leguminous lectins, resulting in a mixture of isoforms with different chain lengths. In these cases, cleavage performed by carboxypeptidase(s) and the digested products are sometimes heterogeneous. The present data support this interpretation, and heterogeneity of the COOH terminus of the β subunit suggests that proteolytic conversion occurs by sequential removal of individual amino acids from its COOH terminus rather than a single endoproteolytic cleavage.

Previous studies have shown that both subunits of the purified galactose-specific lectin showed positive staining for the carbohydrate when analyzed by PAS reagent, suggesting that both subunits contain carbohydrate. In addition, the partial amino acids sequences of both α and β subunits indicate that they are identical at the amino terminal region with a sequence NNLISFTMKR [Latha *et al*., 2006]. Trypsin treatment of the α and β subunits derived the same peptide NNLISFTMK that was overlapped with the C^1 peptide which was similar to the NH_2 terminal sequence reported earlier. Chemical modification studies with native lectin carried out earlier revealed a role of specific amino acids such as histidine, to be important for the lectin activity [Latha *et al*., 2006]. Carbohydrate binding activity of the lectin from the sequence *motif scan* using the sources of www.expasy.org showed two N-glycosylation consensus sites in each subunit Asn^{25} – Tyr^{28} and Asn^{139} – Asn^{142}. A comparison of the amino acid sequences of a variety of legume lectins belonging to Gal/GalNAc specific category has extended COOH-terminal sequences [Sharon *et al*., 1990]. Although both subunits have identical N-glycosylation sites and covalently bound carbohydrate, isolated subunits showed

much weaker agglutinating activity with untreated and trypsin treated rabbit erythrocytes than the intact protein, thus confirming the importance of both subunits for this activity and other functions of the lectin.

A recent study reported that the *Dolichos biflorus* seed lectin also exhibited lipoxygenase activity [Roopashree *et al*., 2006]. This prompted us to analyze the purified galactose lectin for lipoxygenase activity and preliminary studies revealed that it exhibits this enzyme activity too [Since this is only a preliminary result, it has not been presented here]. In summary, the present study is the first report on the primary sequence determination of the newly characterized galactose lectin from the seeds of *Dolichos lablab*. The study presents the potential application of utilizing the advances in mass spectrometry, proteolytic digestions, MS/MS analysis for *de novo* sequencing and database search with different programs as powerful tools for the identification, characterization and determination of the primary sequences of proteins.

CHAPTER V



PURIFICATION AND QUANTIFICATION OF GALACTOSE-SPECIFIC LECTIN FROM VEGETATIVE TISSUES OF *DOLICHOS LABLAB*

PART A

5.1.0. Introduction

Plant lectins are proteins/glycoproteins that cause agglutination of a variety of erythrocytes. They exhibit distinct sugar specificity and have been isolated and characterized from the seeds of different plants. Many of these hemagglutinins have been shown by hapten inhibition studies to react with specific saccharides on erythrocyte surface [Lis *et al*., 1970]. Lectins serve as valuable reagents in glycobiology research and can be employed for the detection and preliminary characterization of glycoconjugates on the surface of cells [Tateno *et al*., 2003]. Among the different lectins studied so far, those isolated from the legume seeds are the most widely studied as these seeds have been found to be very rich in the lectin content [Sultan *et al*., 2004]. Some of the biological roles proposed for plant lectins are carbohydrate transport, stimulation of cell division, cell wall extension, storage of seed protein reserves or the packaging or mobilization of these storage materials, specific attractants for rhizobial symbiosis and protection against plant pathogens [Roberts *et al*., 1984]. Although plant lectins are primarily found in the protein bodies of seeds [Ruediger, 1998], there are reports on the identification, purification and characterization of lectins from vegetative tissues of some legume plants. The well characterized DB58 lectin purified from stems and leaves of *Dolichos biflorus* is a heterodimer composed of two closely related subunits, alpha and beta, which have been separated and purified by high-performance anion-exchange chromatography [Etzler, 1994]. Bowles and Marcus identified lectin receptors from seed extracts of soybeans and jack beans. Lectin like proteins has also been obtained from lentil and pea; these do not possess any hemagglutinating activity but are potent mitogens for lymphocytes [Kummer *et al*., 1988]. A protein of soybean (*Glycine max*) leaves was shown to possess properties similar to the seed lectin whose N-terminal amino acid sequence shares 63% identity with the seed lectin. Immunoblot analysis indicated that the protein occurs in leaves, petioles, stems, and cotyledons of seedlings but not in seeds [Spilatro *et al*, 1996].

Seeds of *Dolichos lablab* grown in India contain two distinct sugar specific lectins (i) DLL-I (glucose/mannose specific) (ii) DLL-II (galactose-specific). In the preceeding chapters, the purification, biochemical, biophysical characterization of the DLL-II and its primary structure have been described. DLL-II has also been crystallized [Latha *et al*., 2006]. The purified lectin is a glycoprotein, with an apparent molecular mass of 120 kDa and is possibly a tetramer, composed of non-covalently associated subunits of apparent molecular masses of 31 kDa and 29 kDa respectively. The lectin activity was inhibited by galactose sugar and its derivatives

clearly suggesting the specificity of lectin towards galactose. Antibody raised to 31 kDa subunit, showed reactivity with the two subunits of DLL-II and also with the DLL-I seed lectin.

Preliminary studies carried out with the crude extracts of the vegetative tissues (Stems, leaves and roots) of *Dolichos lablab* plant revealed that the extracts of stems and leaves exhibit hemagglutinaing activity that is inhibited by galactose and the 31 kDa antibody of the DLL-II seed lectin was able to recognize the vegetative lectin in a western blot experiment. The present study was undertaken with the following objectives (i) to isolate and affinity purify the lectin from the stems and leaves of *Dolichos lablab* and determine its subunit character, (ii) to purify the seed lectin-specific antibodies on a lectin affinity gel (iii) to develop an immuno-affinity gel for the single step purification of the galactose-specific lectin from stem and leaf extracts, (iv) to develop a simple and efficient ELISA method to quantify the lectin in vegetative extracts of *Dolichos lablab*, using the available antibodies.

PART B

5.2.0. Materials and Methods

ELISA plates (96 wells) were purchased from Tarsons (India). Affigel-10 was purchased from Bio-rad laboratories, USA. Galactose, other sugars used in the study, p-nitro phenyl phosphate, manganese chloride, Triton X-100, Sepharose 4B, divinyl sulfone, acrylamide, N N' methylene bis-acrylamide and Freund's complete adjuvant were purchased from Sigma, USA. All other chemicals and reagents used in the study were of high quality and purchased from reputed firms.

5.2.1. Growing of plants and collection of plant material

Dolichos lablab seeds were processed as described earlier [Chapter 2] and sowed in pots and maintained in a plant nursery for 3 weeks (University of Hyderabad). Stems, leaves and roots were collected from these plantlets, frozen in liquid nitrogen and stored at -80˚C until use.

5.2.2. Extraction of proteins and purification of the lectin from the vegetative tissues

All operations were carried out at 4˚C. The frozen stems and leaves of the plant were thawed on ice, and 100 grams of the tissue was extracted with 1000 ml of 25 mM TBS pH 7.4 containing 2 mM PMSF. This extract was further centrifuged at 17,226 × g for 20 min and to

the clear supernatant solid ammonium sulphate was added to 60 % saturation. The suspension was centrifuged as above and the protein pellet was dissolved in 20 mM TBS pH 7.4 and dialyzed extensively against same buffer. This (stem and leaf extract) was used for the purification of the lectin. To obtain the total proteins from the roots, they were extracted following the protocol described for the stems and leaves. Protein concentration in these was determined according to Lowry *et al*, using BSA as standard.

5.2.3. Purification of the galactose-specific lectin from the seeds and stems/leaves of *Dolichos lablab*

Sepharose-divinyl sulfone-galactose was prepared in our laboratory and the galactose-specific lectin was extracted from the seed meal and purified to homogeneity on this gel by affinity chromatography [Chapter 2]. In the present study the lectin activity detected from the stems and leaf extracts obtained above could also be isolated on this affinity gel following the protocol described for the seed lectin.

5.2.4. Raising antibodies to the purified 31kDa subunit of DLL-II

The antibody to the 31 kDa subunit of the DLL-II was prepared and its specificity tested as described [Chapter 2].

5.2.5. Affinity purification of lectin specific antibodies on DLL-II lectin affinity gel

Coupling of the purified DLL-II to Affigel-10 was carried out following manufacturer's instructions. Affigel-10 (3 mL) was thoroughly washed with chilled isoproponal followed by cold-water and 0.1 M HEPES buffer pH 7.4. To this 10 mg/mL lectin (preincubated with 0.1M galactose) was added and coupling reaction allowed to proceed at 4˚C for 24 hours by end over end rotation. At the end of this incubation period, the unreacted sites in the gel were blocked with 0.1M-ethanolamine-HCl pH 8.0 (200µl/1mL Affigel-10) for one hour at 4˚C. The gel was finally washed with PBS and equilibrated with TBS. Antiserum to purified 31 kDa subunit of DLL-II was dialyzed against 10 mM Tris–HCl buffer pH 7.4 containing 150 mM sodium chloride (column buffer) and then applied to the lectin affigel at 4˚C equilibrated with the same buffer. After the gel was extensively washed with column buffer, bound IgG was specifically eluted with six column volumes of 100 mM Glycine-HCl buffer pH 2.65. The eluted protein was immediately neutralized with 2M Tris, analyzed on a 7.5% SDS-PAGE under non-reducing conditions and was stored at 4˚C.

5.2.6. Western blot analysis

To detect the specificity of the antibodies purified DLL-II and the ammonium sulphate precipitated proteins from the stems, leaves and root extracts [designated as the *Dolichos lablab* vegetative lectins, DLL-VL], were separated on a 10% SDS-PAGE and the proteins were transferred to a nitrocellulose membrane and it was processed for blotting as described earlier [Chapter 2]. The membrane was incubated with affinity-purified lectin-specific antibodies prepared above (1:1000) as the primary antibody. The lectin bands were finally detected by incubating the membrane with the secondary antibody (goat anti-rabbit IgG conjugated to alkaline phosphatase) followed by incubation with the substrate BCIP/NBT (Bangalore Genei, Bangalore).

5.2.7. An Immuno affinity method for purification of the DLL-VL

Affinity purified lectin-specific antibodies obtained above was dialyzed against 10 mM HEPES buffer pH 7.4 and was concentrated. Protein (10 mg/mL, 2.0 mL) was allowed to couple onto 2.0 mL Affigel-10 as described above and designated as the immuno-affinity gel. The dialyzed vegetative extracts (60% ammonium sulphate pellet of stem and leaf) were separately passed through immuno-affinity gel pre-equilibrated with 25 mM TBS pH 7.4. After washing the gel with the buffer, bound lectin was eluted with 0.1 M glycine - HCl buffer pH 2.65 and absorbance at A_{280} was recorded, the protein containing fractions were pooled, extensively dialyzed and analyzed for lectin activity and subjected to 10 % SDS-PAGE. Protein bands were detected by coomassie blue staining.

5.2.8. An ELISA method for the quantification of the galactose-specific lectin from the Stems, leaves and roots of the plant

Affinity-purified antibodies against 31 kDa subunit of DLL-II were adsorbed to micro titer wells of an ELISA plate (96 wells) for 4 h at 37°C (250 ng of affinity-purified IgG in 50 µL of 25 mM Tris–HCl, pH 7.4). The wells were washed with 200 µl of 25 mM Tris–HCl, 150 mM NaCl buffer pH 7.4 and incubated overnight at 4°C/1 h at room temperature with 200 µl of buffer C (5% lipid free milk powder, 0.05% Triton X-100, 10 mM sodium phosphate, 150 mM NaCl pH 7.4). 50 µL of solution (0.5-50 ng protein) containing purified DLL-II, extracts of root, stem/leaf and seed (0.5-50 ng protein concentration diluted in Buffer C; quantitation of protein was done according to Lowry *et al* [12], was bound for 2.5 h at 37°C. The wells were washed four times with 200 µl of buffer D (0.05% Triton X-100, 10 mM sodium phosphate,

37°C. Subsequently, 50 µL of diluted rabbit antiserum against 31 kDa subunit of DLL-II (1:1000 dilutions in buffer C) was added and the plate incubated for 1 h at 37°C. After washing four times with buffer D, goat anti-rabbit IgG conjugated to alkaline phosphatase (Bangalore Genei) (dilution 1:6000 in buffer C) was added and incubated for 1 h at 37°C. The wells were washed four times with buffer D and one time with 200 µL of buffer E (0.1 M Tris–HCl, 0.1 M NaCl, 2 mM $MgCl_2$, pH 9.5). The colour was developed with p-nitro phenyl phosphate (1 mg/mL in buffer E) for 10 to 20 min at room temperature and the absorbance was measured at 405 nm in a micro plate ELISA reader. All experiments were carried out in triplicates and data presented represent the average values.

5.2.9. Mass Spectrometric analysis

The protein was separated on a SDS-polyacrylamide gel and visualized by colloidal coomassie blue staining. 31 kDa and 29 kDa subunits were excised from the gel, destained with 50 mM ammonium bicarbonate in 50% acetonitrile, dehydrated with 100% acetonitrile and vacuum dried in a speedvac. Dried gel slices were in gel-digested separately with trypsin overnight at 37º C according to manufacturer´s (sigma) description. Peptides were extracted twice with 50% acetonitrile containing 0.1% TFA for 15 min each. The digests were pooled, vacuum dried in a speedvac and redissolved in water containing 0.1% trifluroacetic acid (TFA) for desalting using C18 ziptips (Millipore, Bedford, MA, USA). A 1/10 fraction of the peptides dissolved in 0.1% TFA and mixed with equal volumes of the MALDI matrix, a-cyano-4-hydroxycinnamic acid (CHCA), was analyzed with positive-ion matrix-assisted laser desorption/ionisation-mass spectrometry on a 4700 MALDI TOF/TOF Analyzer (Applied Biosystems, Foster City, CA, USA). The spectra were recorded in reflector mode in a mass range from 800 to 3900 Da with a focus mass of 1500 Da. Peptide mass calibration was performed with external mass standards (Calmix 1 and 2; Applied Biosystems, an internal calibration was automatically performed using at least one peak for one - or both peaks for a two-point-calibration. Calibration was performed manually for the less than 1% samples for which the automatic calibration failed. (Calibration error in PPM is 10). Additionally MALDI-MS/MS analyses were performed for the five strongest peaks of the TOF-spectrum after subtraction of peaks corresponding to background, keratin and trypsin fragments (calibration for MS/MS was done using 1570 Da peptide from Calmix 1 Applied Biosystems). After calibration a combined database search of MS and MS/MS measurements was performed using the GPS Explorer software (Applied Biosystems, Foster City, CA, USA) with the following

settings: (i) MS peak filtering: mass range from 800 to 3900 Da; minimum S/N filter of 25; peak density of 50 peaks per range of 200 Da and maximal 200 peaks per protein spot; mass exclusion list contained background peaks and trypsin fragments with an exclusion tolerance of 0.2 Da (ii) MS/MS peak filtering: mass range from 50 Da to a mass that was 20 Da lower than the precursor mass; peak density of 50 peaks per 200 Da and maximal 65 peaks per MS/MS; minimum S/N filter of 10 (iii) database search: precursor tolerance 50 ppm and MS/MS fragment tolerance 0.25 Da. Peak lists were compared with NCBInr database using the Mascot search engine (Matrix Science Ltd, London, UK). Peptide mixtures that yielded a mowse score of at least 49 (p-value=0.05) were regarded as positive identifications.

PART C

5.3.0. Results

5.3.1. Purification of the lectin from the vegetative tissues

The galactose specific lectin from the seeds of *Dolichos lablab* has recently been extensively characterized by us [Chapter 2]. In the present study an attempt was made to first identify the lectin activity from the vegetative tissues of the *Dolichos lablab* plant. Therefore from the stems, leaves and the roots of the plant, total proteins were extracted and they were analyzed for the agglutinating activity using trypsin treated rabbit erythrocytes. The agglutinating activity from the stem and leaf extracts was inhibited by galactose among other sugars tested, and therefore the lectin was purified on a Sepharose-galactose gel. In the present study from the extracts of stems and leaves of the plant the galactose specific lectin was affinity purified on a Sepharose-galactose gel employing the same conditions as described for the seed lectin (25 mM TBS pH 7.4, containing 1.5 M ammonium sulphate [Chapter 2]). The ammonium sulphate precipitated proteins from the stems and leaves after dialysis was first passed through the Sepharose-mannose gel in order to deplete the extracts of any other glycoproteins. To the unbound fraction solid ammonium sulphate was added to 1.5 M concentration and was then applied on Sepharose- galactose gel equilibrated with column buffer and the bound protein was eluted from the gel using 0.3 M galactose in column buffer (Fig 5.1). From the Table 5.1, it is apparent that from 100 g of the stems and leaves, about 15 mg of the purified lectin could be obtained. [***Footnote:*** *The lectin activity was weak in the root extracts*].

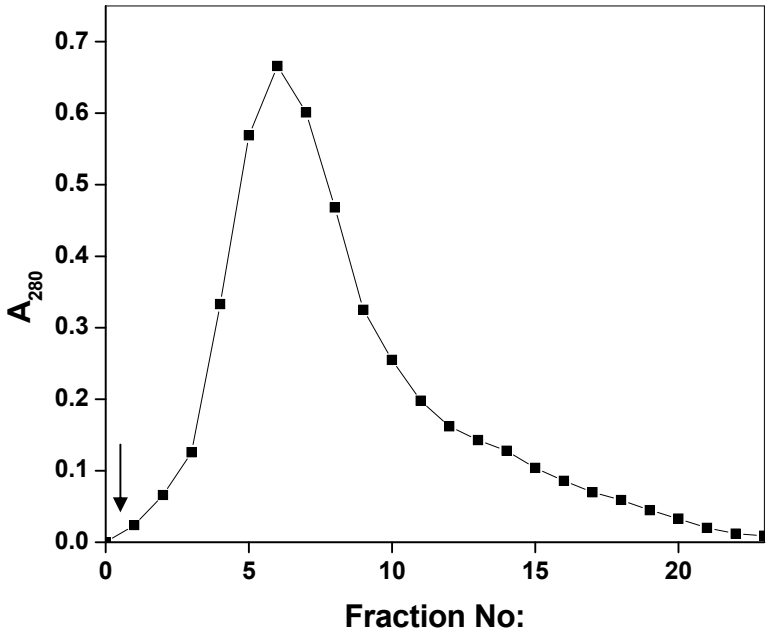


Figure: 5.1

Affinity purification of the DLL-VL on Sepharose-Galactose gel
The 0-60 % $(NH_4)_2SO_4$ fraction corresponding to the total proteins from the vegetative tissues was processed as described under methods. After washing the gel, the lectin bound on the galactose-gel was eluted using 0.3 M galactose. Arrow indicates point of application of the sugar.

S. No	Step	Total Protein (mg)	Total Activity @ HU	Specific Activity (Units/mg)	Purification Fold
1	Crude	2000	3600	1.8	1
2	0-60% $(NH_4)_2SO_4$ fraction	420	1680	4	2.2
3	Sepharose mannose gel unbound	190	1250	6.6	3.6
4	Sepharose galactose gel eluate	15	810	54	30

[@] One HU is defined as the amount of protein required to cause visible agglutination using rabbit erythrocytes.

Table: 5.1

Purification of the galactose-specific lectin from the vegetative extracts of Dolichos lablab *(100g of the vegetative extract was used in the study)*

5.3.2. SDS-PAGE analysis of DLL-VL

In SDS-PAGE analysis the vegetative lectin migrated to the same extent as the seed lectin displaying the two subunit patterns corresponding to molecular masses 31 and 29 kDa respectively (5.2-A).

5.3.3. Western Blot Analysis

In order to understand if the seed lectin and the vegetative lectin are immunologically related, the purified seed and vegetative lectin were separated on a 10% SDS-PAGE, and the proteins transferred to a nitrocellulose membrane. When this was processed for western blot analysis with the antiserum to the 31 kDa subunit of the seed lectin, both subunits in the seed lectin and the vegetative lectin were reactive (Fig 5.2-B), suggesting the immunological relatedness among these two lectins.

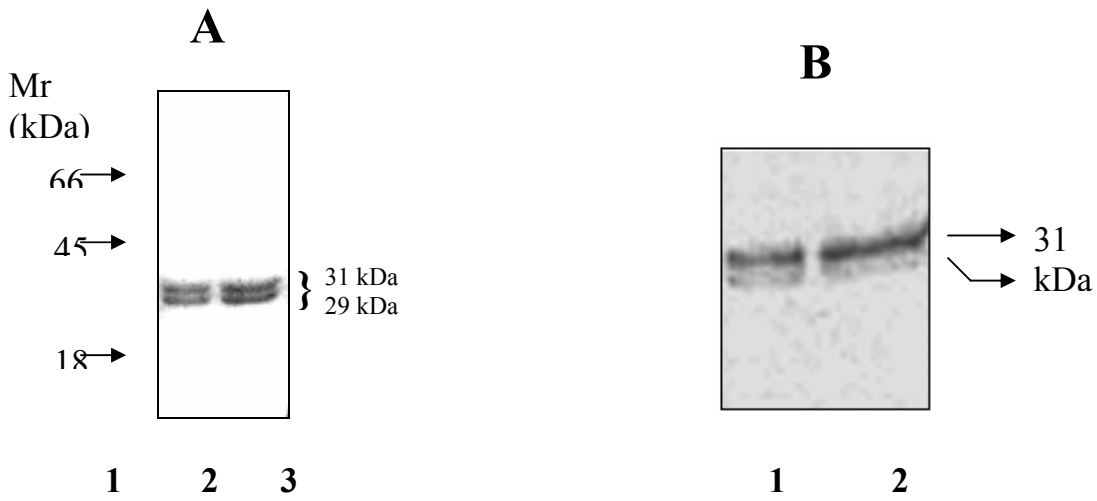


Figure: 5.2

(A) **10 % SDS-PAGE analysis of DLL-VL lectin.** *Lane1: Molecular weight markers - bovine serum albumin (Mr 66 kDa), ovalbumin (Mr 45 kDa) and β-lactoglobulin (Mr 18 kDa); lane 2: DLL-VL; lane 3: DLL-II. Arrow (}-) indicates the position of larger subunit of molecular mass 31 kDa and the smaller subunit of molecular mass 29 kDa of the galactose-specific lectins from Dolichos lablab.*

(B) **Western blot analysis of the DLL-VL lectin.** *After transfer of proteins to the nitrocellulose membrane, it was probed with the DLL-II, 31 kDa subunit antibody at a dilution of 1:1000. Goat anti-rabbit IgG-ALP conjugate (1:1000) was used as the secondary antibody. The blot was developed with BCIP/NBT substrate. Lane 1 shows the purified DLL-VL lectin and lane 2 shows DLL-II lectin, recognized by the antiserum.*

Sugar	Minimum Concentration for Inhibition (mM)	Relative Inhibitory potency (Galactose=1.0)
Galactose	2.82	1.0
Lactose	11.3	0.25
MeβGal	1.40	2.0
MeαGal	11.3	0.25
Galactosamine	22.5	0.12
2-DeoxyGal	45.0	0.06
pNPβGal	1.40	2.0
pNPαGal	11.3	0.25

Table: 5.2

Inhibition of agglutinating activity of DLL-VL lectin by various saccharides

Experiment was performed with trypsin treated erythrocytes with a final lectin concentration of 50 μg/mL. Glucose, mannose and GluNAc did not inhibit the hemagglutinating activity of DLL-VL lectin up to 100mM concentration. Values shown are the average of 2 experiments.

5.3.4. Immuno-affinity Chromatography

In the present study we have also explored the possibility of purifying the DLL-VL by a single step from the vegetative tissue extracts employing immuno-affinity chromatography. To prepare the immuno-affinity gel, the 31 kDa specific IgG was obtained as follows. First, lectin-affigel containing 20 mg purified seed lectin was prepared as described under methods. The antiserum to the 31 kDa subunit of the DLL-II was passed through this gel to get the specific IgG, which eluted as a single peak (Fig 5.3). The protein containing fractions were concentrated and the specific IgG was coupled to 3 mL of affigel at a concentration of 5 mg/mL.

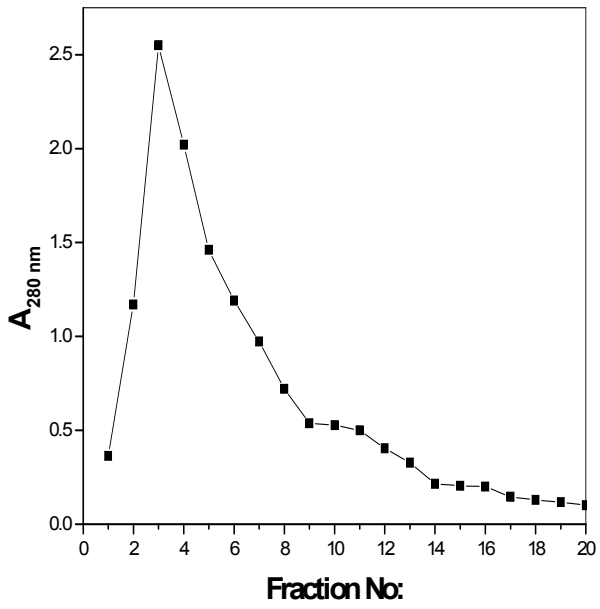


Figure: 5.3

Lectin Affinity chromatography

Anti-lectin antibodies were purified on DLL-II lectin-Affigel equilibrated with 0.1M HEPES buffer. Glycine-HCl pH 2.65 was used to elute Specific anti-lectin antibodies. 1mL fractions were collected and absorbance at 280 nm was measured.

Fig 5.4-A shows the chromatogram for the purification of the lectin using immuno-affinity gel prepared as described under methods from different vegetative extracts, the lectin activity was isolated by passing the vegetative extracts separately on the immuno-affinity gel. After washing extensively, the bound protein was eluted using 100 mM glycine-HCl buffer pH 2.65, and the eluates were immediately neutralized. From 100 g of the stems and leaves we were able to obtain 15 mg of the purified lectin in a single step. A western blot experiment proved the immunological relatedness of the lectin in various vegetative extracts. The lectin purified from different vegetative extracts on a immuno-affinity gel were probed with the lectin specific IgG which was able to detect the two subunits from all the extracts (Fig 5.4-B).

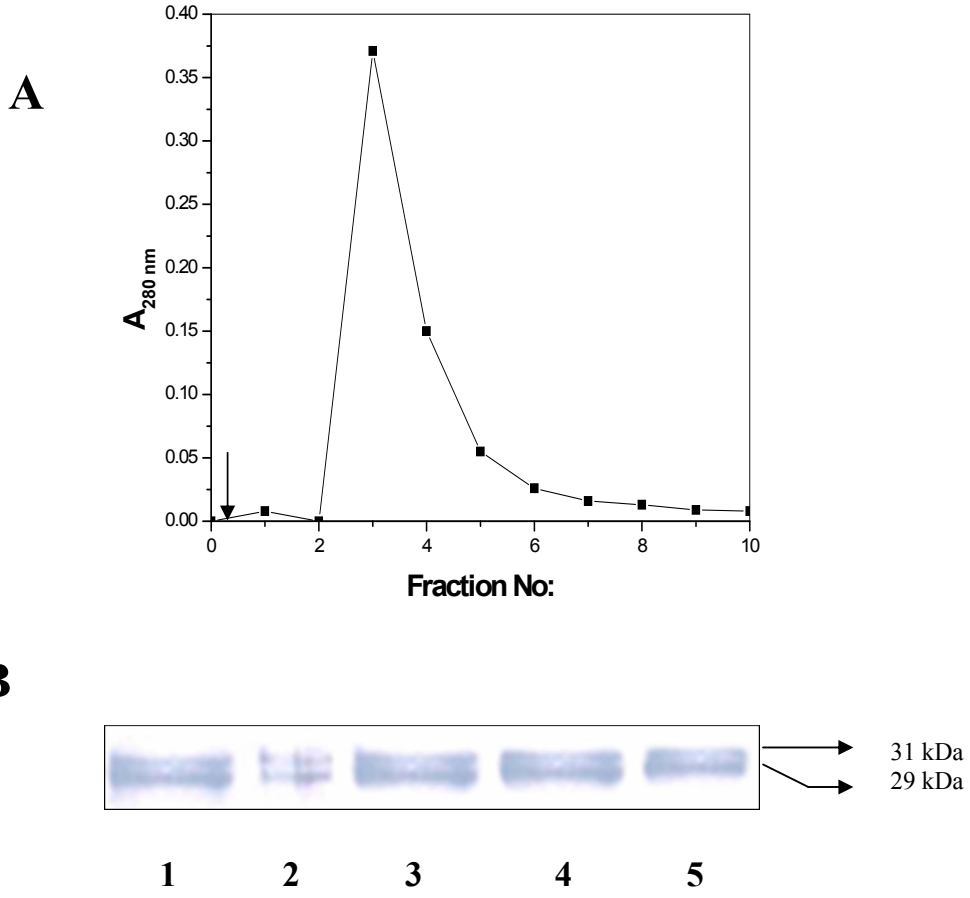


Figure: 5.4

(A) Immunoaffinity method for purification of DLL-VL lectin

DLL-II anti-lectin antibodies were coupled to Affigel-10 in presence of 0.1M HEPES buffer and DLL-VL lectin was purified from this immuno-affinity gel, where the protein eluted as a single peak with 0.1M Glycine-HCl pH 2.65, suggesting it to be homogenous.

(B) Western blot analysis of the vegetative extracts of *Dolichos lablab*

Proteins on SDS-PAGE were transferred to the nitrocellulose membrane; blot was probed with the DLL-II anti-lectin antibody at a dilution of 1:1000. Goat anti-rabbit IgG-ALP conjugate (1:1000) was used as the secondary antibody. The blot was developed with BCIP/NBT substrate. Lanes 1 shows 0-60% Root Extract, Lane 2 - 0-60% Stem/Leaf Extract, Lane 3 - 60-80% Seed Extract, Lane 4 - Affinity purified DLL-II, Lane 5 - Affinity purified DLL-VL that is recognized by specific lectin-specific antibody.

5.3.5. Quantification of lectin from various vegetative extracts

Since the results obtained with the affinity chromatography and immuno-affinity purification of the stem and leaf lectin was encouraging, we wanted to develop a sensitive assay method that could allow us to quantify the lectin at nanogram level from the vegetative tissues of the plant. Therefore, in the present study, an ELISA method was developed to quantify the *Dolichos lablab* vegetative lectins (stems, leaves and roots). The details of the results are presented in Fig 5.5. Fig 5.5-A shows the standard curve where different concentrations of the purified DLL-II were taken. When the 60 - 80 % ammonium sulphate fraction containing the DLL-II was used in an ELISA experiment, the lectin could be detectable in the concentration range of 10 - 50 ng (Fig 5.5-B). Similar results were obtained for the stem/leaf lectin and the root lectin (Fig 5.5-C and 5.5-D). The method allowed detection of the lectin at concentrations as low as 0.5 ng with linearity until 50 ng concentration.

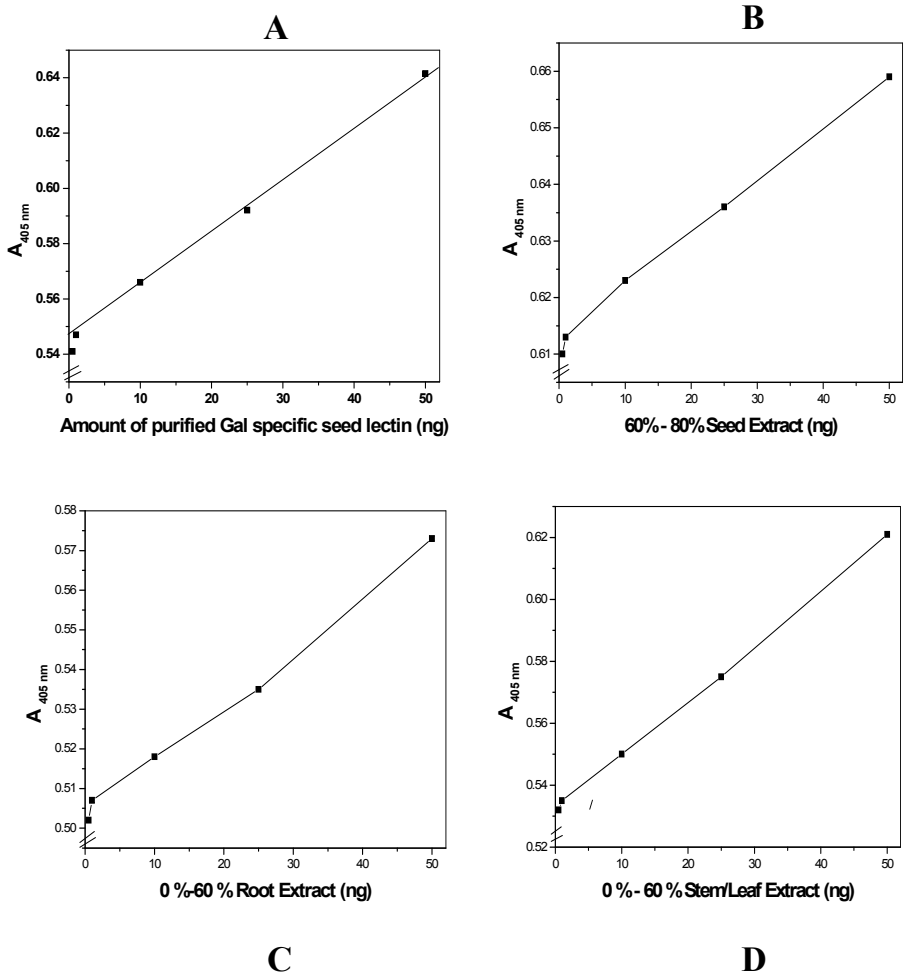


Figure: 5.5

ELISA to detect the Galactose-specific lectin in the vegetative extracts of *Dolichos lablab*

(A) Represents the affinity purified DLL-II, (B, C and D) represent the extracts of seeds, roots and stems/leaves respectively. Assay was performed as described in materials and methods. An aggregate of three experiments is shown here.

5.3.6 MS analysis

The DLL-VL lectin as described above is made of two subunits which are recognized by the DLL-II antibody described [Chapter 2] suggesting possible sequence homology between the two subunits of the DLL-VL. This prompted us to understand more about the similarities between the two subunits. Mass spectrometry has become a major tool for identification of the

novel proteins, whose sequences have not been determined to study the function of these proteins. After separation of the protein by SDS-PAGE the subunits have been digested with trypsin and a fraction of the peptides obtained by enzymatic digestion of the lectin subunits were analyzed by MALDI-TOF-MS to obtain peptide mass fingerprints that are shown in Fig. 5.6-A-B. Experimentally obtained masses were compared to peptide mass sets obtained from the theoretical digests of lectin proteins in databases.

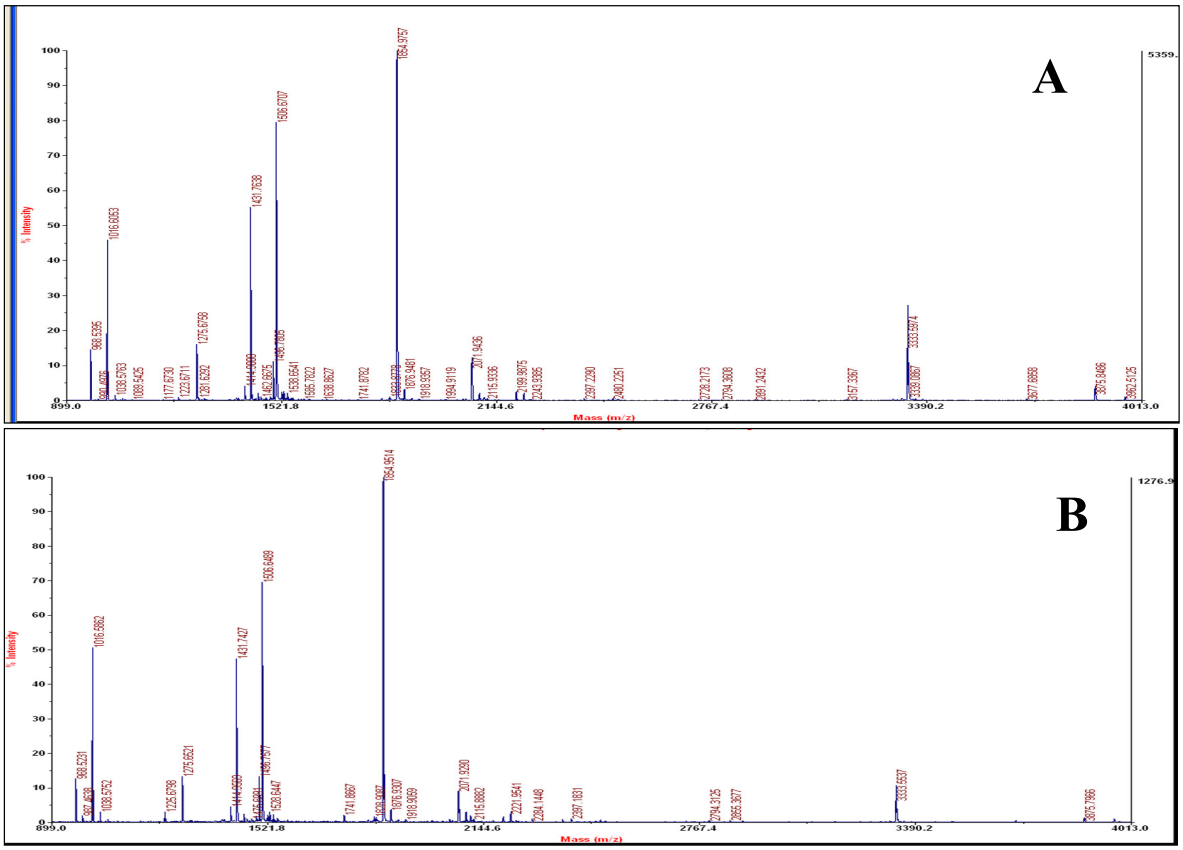


Figure: 5.6

Mass spectrometry analysis of the DLL-VL

MALDI – MS analysis was performed with proteolytic digests of the DLL-VL Galactose-specific lectin, generated by digestion of the subunits overnight with the protease trypsin. Figures A and B display exemplarily peptide mass fingerprints generated with trypsin (A) 31 kDa subunit (B) 29 kDa subunit.

PART D

5.4.0. Discussion

Legume lectins are the most widely studied proteins. More often, they are identified and detected by their specific property of agglutinating erythrocytes. A detailed study was undertaken to characterize the purified stem and leaf lectin and to examine its relatedness to the seed lectin. Furthermore, the antibodies to the seed lectin provided a useful tool to develop methods that allowed purification of the stem and leaf lectin and to quantify by ELISA the stem, leaf lectin as well as the root lectin. The seeds of the Indian lablab have been shown to contain two distinct sugar specific lectins designated as the DLL-I (glucose/mannose specific), and DLL-II (galactose-specific). Both lectins share only a partial sequence homology. DLL-II is a glycoprotein with a native molecular mass of 120 kDa, consisting of two types of subunits, 31 kDa and 29 kDa respectively that are noncovalently associated.

Among a number of sugars tested for inhibition of agglutination of the purified DLL-VL (*Dolichos lablab* vegetative lectin) galactose and its derivatives like N-acetyl galactosamine and Methyl-β-Gal were found to inhibit the lectin activity. However, sugars such as glucose, mannose, their methyl glycosidases, and N-acetylglucosamine were non-inhibitory up to 100 mM concentration. Sugar inhibition data presented in Table 5.2 shows that 2-deoxygalactose is 16 fold weaker compared to galactose in inhibiting the activity, indicating that the equatorial hydroxyl group on C-2 of galactose is an important locus for carbohydrate binding of the lectin. The inhibitory potency of Methyl β Gal is eight times greater when compared to the α-anomer, indicating that the equatorial position at the anomeric position is better recognized. Substitution at the C-2 with an amino group does not have any significant inhibitory effect as compared to galactose. These results confirm that the activity of the DLL-VL lectin is best inhibited by galactose. In addition, a hydrophobic moiety attached at the anomeric position as pNP-β-galactose was found to be a better inhibitory sugar than Methyl α Gal. These results are comparable to the other galactose-specific lectins, including the seed lectin, DLL-II purified from *Dolichos lablab* [Chapter 2].

This antiserum for 31 kDa subunit of DLL-II has already been shown by us to react with both the 31 and 29 kDa subunits of the seed lectin [Chapter 2]. It is therefore interesting to note that in the vegetative lectin also both the subunits which exhibit similar molecular masses as the seed lectin show reactivity suggesting that it is related to the seed lectin. The specificity of the antibodies played a crucial role in understanding the antigenic relatedness of the lectins isolated from the plantlet and seeds. Previously only few reports have been shown to develop

such a methodology (immuno-affinity chromatography) where antibodies to the seed lectin are employed for the purification of second lectin from the same plant, and our experiment proves to be an efficient one as we succeeded to obtain the DLL-VL lectin from the various extracts from single step. Very few reports are there on the development of an ELISA for quantification of the vegetative lectins.

Reports on the other vegetative lectins where the lectin was purified by affinity and other modes for the purification and characterized has been cited in the literature. Although lectin activity has been detected in wheat leaves [Mishkind *et al*., 1980], soybean leaves [Pueppke *et al*., 1978] phloem exudates [Sabons *et al*., 1978], peanut leaves [Bowles *et al*., 1979], flowers of *Datura stramonium* [Kilpatrick *et al*., 1979] and in sieve-tube sap of various trees [Giem *et al*., 1979] only few of these lectins have been thoroughly characterized. Suzuki *et al* isolated two lectins from the leaves of *Aloe arborescens*. These lectins (P-2 and S-1) had a molecular mass of 18,000 and 24,000, respectively. Each lectin was believed to be a dimer. A more extensive characterization of the lectin from *Dolichos biflorus (DB)* leaves and stems (CRM) was performed by Talbot and Etzler. *DB* stems and leaves contained a glycoprotein which cross-reacted with antibodies raised against the seed lectin.

Dolichos lablab seed lectin that has been characterized and reported to cross- react with the DLL-VL laid a scope for the characterization of the vegetative lectin. Furthermore, in literature there are examples where the lectins from the same plant did not show immunological cross-reactivity. The antibodies to the *Robinia* bark lectin did not cross-react with proteins from *Robinia* seeds and leaves. On the other hand, the antibody for the *Robinia* bark lectin cross-reacted with polypeptides from the extracts of the seeds and bark of *Sophora japonica* suggesting immunological relatedness of the lectins from two different plants [Kiyoshi *et al*., 1992]. Hemagglutinating activity was identified in the roots and vegetative tissues of Bengal gram using rabbit erythrocytes. The activity in the roots appears to be similar to that of the seeds in respect to their sugar inhibition property [Nair *et al*., 2000].

A very small fraction of the peptides could be assigned using mass spectrometry approach which was due to the fact that the sequence was novel and thus only sequences absolutely conserved between lectins from different legume species could be assigned. In this study the MASCOT and MS-BLAST 2 programs were used for the analysis of the MS data allowing us to utilize the complete set of peptide sequences of unknown order as determined by MS/MS from one protein in the same query. The MS spectra of both subunits showed no differences in the peak distribution suggesting high similarity of both subunits and additionally, we analyzed manually all differences between corresponding spectra to find any differences in the sequence

using Data explorer software. The lectin sequence achieved from the MS data is limited and database search could achieve the conserved regions of the lectin on comparison with other legume lectins. Primarily after observing the spectra's of the digests and comparing with the subunits it was clear to understand that the both subunits share maximum amount of identity in peak distribution, so we assume maximum sequence homology among them. This further is in good agreement with the complete primary sequence we obtained recently for the two subunits of the DLL seed lectin that showed extensive sequence homologies among the two subunits
In summary, we present here a detailed investigation on the isolation and affinity purification of the vegetative lectin from the DLL plant that agglutinates rabbit erythrocytes and compare its properties with the purified seed lectin. An affinity method was developed to purify the galactose-specific lectin from *Dolichos lablab* vegetative tissues (stem and leaf) 15mg of the purified lectin was obtained from 100g starting material. The levels of the vegetative lectin are thus lower when compared to the seed lectin [Latha *et al*., 2006].

Seed and vegetative lectin exhibited similar molecular masses and subunit pattern. Furthermore, the seed lectin antibody coupled to affigel, has been found to be very efficient tool, to purify the lectin from the vegetative extracts and seeds in a single step with a protein yield that compares well to the affinity method (Sepharose-galactose gel). The DLL-VL and the root lectin show immunological cross-reactivity with DLL-II antibody. An ELISA method was developed that allowed quantification of the lectin present in different vegetative tissues (stems, leaves and roots) of the plant at very low concentrations (0.5 to 10 ng). MS/MS analysis and database search of the tryptic peptide maps generated from the subunits of the lectin suggests that the protein is a lectin with sequence homology in the conserved regions when compared with other legume lectins, we understand here the protein on work shares homology in the sequence between both subunits and also to the seed lectin (DLL-II).

BIBLIOGRAPHY

**** For some references, titles could not be indicated.***

References

- **Allen, N. K**., Neuberger, A., Sharon, N. (1973). *Biochem. J*. **131**: 155-162.
- **Allen, N. K**., Desai, N. N., Neuberger, A., Creeth, J. M. (1978). Properties of Potato Lectin and the Nature of its Glycoprotein Linkages. *Biochem. J*. **171**: 665-674.
- **Anantharam, V**., Patanjali, S. R., Swamy, M. J., Sanadi, A. R., Goldstein, I. J. and Surolia, A. (1986) Isolation, macromolecular properties, and combining site of a chito-oligosaccharide-specific lectin from the exudate of ridge gourd (*Luffa acutangula*). *J. Biol. Chem.* **261**: 14621-14627.
- **Ashford, D**., Desai, N. N., Allen, N. K., Neuberger, A., O'Neill, M. A., Selvendran, R. R. (1982).*Biochem. J*. **201**: 199-208.
- **Asperg, A**., Muira, R., Bourdoulous, S., Shimonaka, M., Heinegard, D., Schachner, M., Rouslahti, E., Yamaguchi, Y. (1997). The C-type lectin domains of lecticans, a family of aggregating chondroiting sulfate proteoglycans, bind tenascin-R by protein-protein interactions independent of carbohydrate moiety. *Proc. Natl. Acad. Sci*. USA. **94:** 10116-10121.
- **Banerjee, R**., Das, K., Ravishankar, R., Suguna, K., Surolia, A. and Vijayan, M, (1996) Conformation, protein-carbohydrate interactions and a novel subunit association in the refined structure of peanut lectin-lactose complex. *J. Mol. Biol.* **259**: 281-296.
- **Banerjee, R**., Mande, S. C., Ganesh, V., Das, K., Dhanaraj, V., Mahanta, S. K., Suguna, K., Surolia, A. and Vijayan, M. (1994) Crystal structure of peanut lectin, a protein with an unusual quaternary structure. *Proc. Natl. Acad. Sci. USA* **91**: 227-231.
- **Barbieri, L**., Batelli, G. B., Stripe, F. (1993). Ribosome-inactivating proteins from plants. *Biochim. Biophys. Acta*. **1154**: 237-282.
- **Barbieri, L**., Valbonesi, P., Bonora, E., Gorini, P., Bolognesi, A. and Stirpe, F. (1997) Polynucleotide:adenosine glycosidase activity of ribosome-inactivating proteins: effect on DNA, RNA and poly(A). *Nucleic Acids Res.* **25**: 518-522.
- **Barondes, S**. H. (1981). Lectins: their multiple endogenous cellular functions. *Annu. Rev. Biochem*. **50**: 207-231.
- **Barre, A**., Peumans, W. J., Rossignol, M., Borderies, G., Culerrier, R., Van Damme, E. J. M., and Rougé, P. (2004) Artocarpin is a polyspecific jacalin-related lectin with a monosaccharide preference for mannose. *Biochimie* **86**: 685-683.

- **Barre, A**., Van Damme, E. J. M., Peumans, W. J. and Rougé, P. (1996) structure-function relationship of monocot mannose-binding lectins. *Plant Physiol.* **112**: 1531-1540.
- **Baumann, C**., Rüdiger, H., and Strosberg, A.D. (1979) A comparison of the two lectins from *Vicia cracca*, FEBS *Lett.* **102:** 216-218.
- **Beintema, J. J**. (1994) Structural feature of plant chitinases and chitin-binding proteins. *FEBS Lett.* **350**: 159-163.
- **Borrebaeck, C. A. K**. and Carlsson, R. (1989).*Adv. Lectin. Res*. **2**: 10.
- **Bourne, Y**., Rouge, P., cambillau, C. (1992). X-ray structure of a Biantinnary Octasaccharide-Lectin Complex Refined at 2.3 A^o Resolution. *J. Biol. Chem*. **267**: 197-203.
- **Boyd, W. C** and Reguera, R. M. (1949). Hemagglutinating substances in various plants. *J. Immunol*. **62:** 333-339.
- **Boyd, W.C** and Shapleigh, E., (1954). Specific precipitating activity of plant agglutinins (Lectins). *Science.* **119:** 419.
- **Bowles, D. J**., Lis, H., Sharon, N. (1979) *Planta*. **145:** 193-198.
- **Bowles, D. J** and Marcus, S. (1981) Characterization of receptors for the endogenous lectins of soybean and jackbean seeds. *FEBS Letters*. **129:** 135-138.
- **Bowles, D. J**., Marcus, S. E., Pappin, D. J. C., Findlay, j. B. C., Eliopoulos, E., Maycox, P. R., Burgess, J., (1986). Posttranslational processing of concanavalin A precursors in jack bean cotyledons. *J. Cell. Biol.* **102:** 1284-1297.
- **Carter, W.G** and Etzler, M.E., (1975a). Isolation and characterization of subunits from the predominant form of *Dolichos biflorus* lectin. *Biochemistry.* **14:** 2685-2689.
- **Carter, W.G** and Etzler, M.E., (1975b). Isolation, characterization, and subunit structures of multiple forms of *Dolichos biflorus* lectin. *J. Biol. Chem.* **250:** 2756-2762.
- **Carlson, S. R**. (1994). In *Glycobiology, a practical aproach*; Fukuda, M., Kobata, A., Eds.; IRL Press: Oxford. pp 1.
- **CLUSTAL W**, 1.82 programs, www.expasy.org, proteomics tools.
- **Colucci, G.,** Moore, J.G., Feldman, M. and Crispeels, M. J. (1999). cDNA cloning of FRIL, a lectin from *Dolichos lablab*, that preserves hematopoietic progenitors in suspension culture. *Proc. Natl. Acad.Sci., U.S.A.* **96:** 646-650.

- **Compton, L.A**., Johnson Jr. W.C. (1986) Analysis of protein circular dichroism spectra for secondary structure using a simple matrix multiplication, *Anal. Biochem.* **155:** 155–167.
- **Cunningham, B**. A., Hemperly, J. J., Hopp, T. P., Edelman, G. M. (1979). *Proc. Natl. Acad. Sci. USA*. **76**: 3218-3222.
- **Dalmau, S. R**. and Freitas, C. S. (1989) Sugar inhibition of the lectin jacalin: comparison of three assays. *Braz. J. Med. Biol. Res*. **22**: 601-610.
- **Das, M. K**., Khan, M. I. and Surolia, A. (1981) Fluorimetric studies of the binding of *Momordica charantia* (bitter gourd) lectin with ligands. *Biochem. J.* **195:** 341-343.
- **Debray, H** and Montreuil, J. (1991). *Adv. Lectin. Res*. **4**: 51.
- **De Boeck, H**., Loonteins, F. G., Delmotte, F. M. and De Bruyne, C. K. (1981) The use of 4-methylumbelliferyl glycosides in binding studies with the lectins BS I-A_4, BS I-B_4 and BS II from *Bandeiraea (Griffonia) simplicifolia. FEBS Lett.* **126**: 227-230.
- **De Miranda-Santos**, I.K.F., Mengel Jr, J.O., Bunn-Morino, M.M., Campos-Neto, A. (1991) Activation of T and B cells by a crude extract of Artocarpus integrifolia is mediated by a lectin distinct from jacalin, *J. Immunol. Methods.* **140:** 197–203.
- **Desai, N. N**., Allen, A. K., Neuberger, A. (1981). *Biochem. J*. **197**: 345-353.
- **Dey, P. M**., Pridham, J. B. and Sumar, N. (1982). Muliple forms of Vicia faba α-galactosidases and their relationships. *Phytochemistry*. **21**: 2195-2199.
- **Di Sabato, G**., Hall, J. M., Thompson, L. (1987). *Methods Enzymol*. **150**: 3.
- **Dixon, H.B.F**., and Perham, R.N. (1968) Reversible blocking of amino groups with Citraconic anhydride, *Biochem. J.* **109:** 312-314.
- **Drickamer, K**. (1988). Two distinct classes of carbohydrate-recognition domains in animal lectins. *J. Biol. Chem.* **263**: 9557-9560.
- **Drickamer, K**. (1994) Molecular structure of animal lectins. *In* Molecular Glycobiology. (Fukuda, M. and Hindsgaul, O. eds.), pp 53-87. Oxford University Press. Oxford.
- **Dubois, M**., Gilles, K. A., Hamilton, J. K., Rebers, P.A., and Smith, F. (1956) Colorimetric method for the determination of sugars and related substances, *Anal. Chem*. **28:** 350-355.
- **Edelman, G. M**., Cunningham, B. A., Reeke, G. N.Jr., Becker, J. W., Waxdal, M. J., Wang, J. L. (1972). *Proc. Natl. Acad. Sci. USA*. **69**: 2580-2584.

- **Elglavish, S**., Shaanan, B. (1997). Lectin-carbohydrate intercations: different folds, common recognition principles. *Trends Biochem. Sci*. **4**: 462-467.
- **Elgavish, S**. and Shaanan, B. (2001) Chemical characteristics of dimer interfaces in the legume lectin family. *Protein Sci.* **10(4)**: 753-761.
- **Emmerich, C**., Helliwell, J. R., Redshaw, M., Naismith, J. H., Harrop, S. J., Raftery, J., Kalb (Gilboa), J. A., Yariv, J., Dauter, Z., Wilson, K. S. (1994). *Acta Crystallogr.D*. **50**: 749.
- **Emsley, J**., White, H. E., O'Hara, B. P., Oliva, G., Srinivasan, N., Tickle, I. J., Blundell, T., Pepys, M. B., Wood, S. P. (1994). *Nature*. **367**: 338.
- **Etzler, M.E.,** Gupta, S., Borrebaeck, C. (1981). Carbohydrate binding properties of the *Dolichos biflorus* lectin and its subunits *J. Biol. Chem*. **256**: 2367-2370.
- **Etzler, M.E**. (1985). Plant lectins: molecular and biological aspects. *Annu. Rev. Plant Physiol.* **36**: 209-234.
- **Etzler, M.E**., Liener, I.E., Sharon, N. and Goldstein, I.J. (Eds.). (1986) The Lectins: Properties, functions and applications in biology and medicine, Academic press, Orlando, FL, 371-435.
- **Etzler, M.E**., (1992). *Glycoconjugates: Composition, Structure, and Function* (Allen, H.J, and Kisailus, E. C, Eds), Marcel-Dekker, Inc., New York: 521-539.
- **Etzler, M.E**. (1994). Isolation and Characterization of Subunits of DB58, a Lectin from the Stems and Leaves of *Dolichos biflorus*. *Biochemistry*. **33**: 9778-9783.
- **Favero, J**., Miquel, F., Dornand, J., and Mani, J.C. (1988) Determination of mitogenic properties and lymphocyte target sites of *Dolichos lablab* lectin (DLA): comparative study with concanavalin A and galactose oxidase cell surface receptors, *Cell Immunol*. **112:**302-314.
- **Feldsted, R. L**., Egorin, M. J., Leavitt, R. D. and Bachur, N. R. (1977) Recombination of subunits of *Phaseolus vulgaris* isolectins. *J. Biol. Chem.* **252**: 2967-2971.
- **Foriers, A**., Lebrum, E., van Rapenbusch, R., de Neve, R., Strosberg, A. D., (1981). The structure of the lentil (*Lens culinaris*) lectin. Amino acid sequence determination and prediction of the secondary structure. *J. Biol. Chem*. **256**: 5550-5560.
- **Gabius, H. J** and Gabius, S. (1991). Tumour lectinology: a glycobiological approach to tumour diagnosis and therapy. In *Lectin Reviews*; Kilpatrick, D.C., Van Driessche, E. and Bog Hansen, T. C., ed., Sigma Chem.Co. **Vol 1**: 91-102.

- **Gabriella, C**., Jeffrey, G. M., Michael, F., and Crispeels, M.J. (1999) cDNA cloning of FRIL, a lectin from *Dolichos lablab*, that preserves hematopoietic progenitors in suspension culture, *Proc.Natl.Acad.Sci.* U.S.A., **96**: 646-650.
- **Galbraith, W**., Goldstein, I. J. 1972. *Biochemistry*. **11**: 3976-3984.
- **Gansera, R**.*,* Schurz, H. and Rudiger, H. *(1979).* Lectin –associated proteins from the seeds of leguminosae.*Hoppe Seylers Z Physiol Chem.* **360 (11):**1579-85.
- **Gegg, C. V**., Roberts, D. D., Segel, I. H., Etzler, M. E. (1992). Characterization of adenine binding sites of two *Dolichos biflorus* lectins. *Biochemistry*. **31**: 6938-6942.
- **Giem, C**., Kauss, H., Ziegler, H. (1979) *Planta*. **144:** 367-371.
- **Gilliland, D. G**., Collier, R. J., Moehring, J. M.and Moehring, T. J. (1978). Chimeric toxins: Toxic, disulfide-linked conjugate of Concanavalin A with fragment A from diphthereia toxin. *Proc. Natl. Acad. Sci. U. S. A*. **75**: 5319-5323.
- **Goldstein, I. J**., Hollerman, C. E., Smith, E. E. (1965). *Biochemistry*. **4**: 876-883.
- **Goldstein, I.J**., Hughes, R.C., Monsigny, M., Osawa, T., Sharon, N., (1980). What should be called a lectin? *Nature.* **285**: 66-66.
- **Goldstein, I.J**. and Etzler, M.E. (1983) Chemical taxonomy, molecular biology and function of plant lectins. Alan, R. Liss, Inc., New York.
- **Goldstein, I.J** and Poretz, R.D. (1986). In *The Lectins. Properties, Functions and applications in Biology and Medicine* (Liener, I.E, Sharon, N, and Goldstein, I.J. Eds.), Academic Press, Inc, New York.
- **Goldstein, I. J**., Winter, H. C., Poretz, R. D. 1997. In *Glycoproteins II*; Vliegenthrat, J. F. G., Montreuil, J., Schachter, H., Eds. Elsevier Science B.V: Amsterdam; p 403.
- **Gowda, L. R.,** Savithri, H. S., Rajagopal Rao, D. R., (1994). The complete primary structure of a unique mannose/glucose specific lectin from the field bean (*Dolichos lablab*). *J. Biol. Chem*. **269**: 18789-18793.
- **Grant, C. W. M**., Peters, M. W. (1984). Lectin-membrane interactions: Information from model systems. *Biochim. Biophys. Acta*. **779**: 403-422.
- **Gupta, D**, Rao, N. V., Puri, K.D., Matta, K. L. and Surolia, A. (1992) Thermodynamic and kinetic studies on the mechanism of binding of methylumbelliferyl glycosides to jacalin. *J. Biol. Chem.* **267**: 8909-8918.
- **Guran, A**., Ticha, M., Filka, K. and Kocourek, J. (1983) Isolation and properties of a lectin from the seeds of Indian field bean (*Dolichos lablab L*.), *Biochem. J.* **209**: 653-657.

- **Hamelryck, T.W**., Moore, J.G., Chrispeels, M.J., Loris, R., Wyns, L. (2000) The role of weak protein–protein interactions in multivalent lectin–carbohydrate binding: crystal structure of cross-linked FRIL, *J. Mol. Biol*. **299:** 875–883.
- **Hardman, K. D** and Ainsworth, C. F. (1972). Structure of concanavalin A at 2.4 Å resolution. *Biochemistry*. **11**: 4910-4919.
- **Harrop, S. J**. (1996) Structure solution of a cubic crystal of concanavalin A complexed with methyl α-D-glucopyranoside. *Acta Crystallogr. D Biol. Crystallogr*. **52**: 143-155.
- **Hasselbeck, A** and Hosel, W. (1993). In *Glycoprotein analysis in Biomedicine*. Hounsell, E. L. Ed. *Mothods. Mol. Biol*. **14**: 161.
- **Hayes, C. E** and Goldstein, I. J. (1974). An α-D-galactosyl-binding lectin from *Bandeiraceae simplicifolia* seeds. Isolation by affinity chromatography and charecterization. *J. Biol. Chem*. **249**: 1904-1914.
- **Hemperley, J. J**., Mostov, K.E., Cunningham, B. A., (1982). In vitro translation and processing of a precursor form of Favin, a lectin from Vicia faba. *J. Biol. Chem*. **257**: 7903-7909.
- **Hester, G**., Kaku, H., Goldstein, I. J. and Wright, C. S. (1995) Structure of mannose-specific snowdrop (*Galanthus nivalis*) lectin is representative of a new plant lectin family. *Nat. Struct. Biol.* **2**: 472-479.
- **Higgins, T. J. V**., Chandler, P. M., Zurawski, G., Button, S. C., Spencer, D., 1983. The biosynthesis and primary structure of pea seed lectin. *J. Biol. Chem* **258**: 9544-9549.
- **Hincha, D. K**., Bakaltcheve, I. and Schmitt, J. M. (1993). Galactose specific lectins protect isolated thylakoids against freeze-thaw damage. *Plant Physiol*. **103**: 59-65.
- **Hinek, A**., Wrenn, D. S., Mecham, R. P., Barondes, S. H. (1988). The elastin receptor: a galactose binding protein. *Science*. **239**: 1539-1541.
- **Hincha, D. K**., Pfuller, U., Schmitt, J. M. (1997). The concentration of cryoprotective lectins in mistletoe (*Viscum album L*.) leaves is correlated with leaf frost hardiness. *Plant Phisiol*. **203**: 140-144.
- **Hoffman, L.M** and Donaldson, D.D., (1985). Characterization of two Phaseolus vulgaris phytohemagglutinin genes closely linked on the chromosome. *EMBO J.* **4**: 883-889.
- **Horejsi, V** and Kocourk, J. (1978). *Biochim. Biophys. Acta*. **532**: 92-97.
- **Hortan, H.R**. and Koshland, D.E. (1972) Modification of proteins with active benzyl halides, *Meth. Enzymol.* **25:** 468-482.

- **Iglesias, J.L**., Lis, H., and Sharon, N. (1982) Purification and properties of a D-galactose/N-acetyl-D-galactosamine- specific lectin from *Erythrina cristagalli*, *Eur. J Biochem.* **123:** 247-252.
- **Jeyaprakash, A. A**., Katiyar, S., Swaminathan, C. P., Sekar, K., Surolia, A. and Vijayan, M. (2003) Structural basis of the carbohydrate specificities of jacalin: an X-ray and modeling study. *J. Mol. Biol.* **332**: 217-228.
- **Jirgensons, B.** (1978). *Biochim. Biophys Acta*. **536**: 205-211.
- **Jirgensons, B.** (1979). *Biochim. Biophys Acta*. **577**: 307-313.
- **Kabir, S** and Daar, A. S. (1994). The composition and properties of jacalin, a lectin of diverse applications obtained from jackfruit (*Artocarpus heterophyllus*) seeds. *Immunol.Invest*. **23**: 167.
- **Kaku, H**., Tanaka, Y., Kiyoshi, T., Minami, E., Mizuno, H., Naota, S., 1996. Sialylated Oligosaccharide-specific Plant Lectin from Japanese Elderberry (*Sambucus sieboldiana*) Bark Tissue Has a Homologous Structure to Type II Ribosome-inactivating Proteins, Ricin and Abrin**.** *J. Biol. Chem.* **271**: 1480-1485.
- **Kenoth, R** and Swamy, M.J. (2003) Steady-state and time-resolved fluorescence studies on Trichosanthes cucumerina seed lectin, *J. Photochem. Photobiol. B Biol.* **69:** 193–201.
- **Khan, M. I**., Mazumder, T., Pain, D., Gaur, N. and Surolia, A. (1981) Binding of 4-methylumbelliferyl-β-D-galactopyranoside to *Momordica charantia* agglutinin: Fluorescence quenching studies. *Eur. J. Biochem.* **113**: 471-476.
- **Kieliszewski, M. J**., Showalter, A. M., Leykam, J. F. (1994). Potato lectin: a modular protein sharing sequence similarities with the extensin family, the hevein lectin family, and snake venom disintegrins (platelet aggregation inhibitors). *Plant J.* **5**: 849-861.
- **Kilpatrick, D. C**., Yeoman, M. M. (1978). *Biochem. J*. **175**: 1151-1153.
- **Kilpatrick, D. C.,** Yeoman, M. M., Gould, A. R. (1979) *Biochem J*. **184:** 215-219.
- **Kilpatrick, D. C**. (1991). Lectin interactions with human leukocytes: mitogenicity, cell seperation, clinical applications. In *Lectin Reviews*; Kilpatrick, D. C., Van Driessche, E. and Bog Hansen, T. C., ed. Sigma Chem. Co. **Vol. 1**: 1-32.
- **Kilpatrick, D. C**. and Green, C. (1992). *Adv. Lectin. Res*. **5**: 51.
- **Kiyoshi, T** and Kazumasa, Y. (1992) Plant *and Cell Physiology*. **33 (2):** 125 – 129.
- **Komath, S. S** and Swamy, M. J. (1998). Further characterization of the saccharide specificity of snake gourd (*Trichosanthes aguina*) seed lectin. *Current Sci*. **75**: 608-611.

- **Komath, S.S**., Nadimpalli, S.K., and Swamy, M.J. (1998) Identification of Histidine residues in the sugar binding site of snake gourd (*Trichosanthes anguina*) seed lectin. *Biochem.Mol.Biol.Int.* **44:** 107-116.
- **Komath, S.S** and Swamy, M.J. (1999) Fluorescence quenching, time-resolved fluorescence and chemical modification studies on the tryptophan residues of snake gourd (*Trichosanthes anguina*) seed lectin, *J. Photochem. Photobiol. B Biol.* **50:** 108–118.
- **Konami, Y**., Yamamoto, K., Osawa, T., Irimura, T. (1995). *Glycoconjugate J.* **12**:128.
- **Kornfeld, S** and Kornfeld, R (1969).*Proc. of. Nat. Acad. Sci. USA*. **63**: 1439.
- **Kornfeld, R** and Kornfeld, S. (1970). *J. Biol. Chem*. **245**: 2536.
- **Kudou, M**., Shiraki, K., Fujiwara, S., Imanaka, T., Takagi, M. (2003) Prevention of thermal inactivation and aggregation of lysozyme by polyamines, *Eur. J. Biochem.* **270:** 4547–4554
- **Kumar, N.S**. and Rao, D.R. (1986) The nature of lectins from *Dolichos lablab*, *J.Biosci.* **10:** 95-109.
- **Kummer, H**., Rudiger, H. (1988) Characterization of lectin binding storage protein from pea (*Pisum sativum*). *Biol Chem Hoppe Seyler*. **369 (8):** 639-46.
- **Kundu, M**., Basu, J., Ghosh, A., and Chakrabarti, P. (1987) Chemical modification studies on a lectin from *Saccharomyces cerevisiae* (Baker's yeast), *Biochem. J.* **244:** 579-584.
- **Kuroki, Y**., Honma, T., Chiba, H., Sano, H., Saitoh, M., Ogasawara, Y., Sohma, H., Akino, T. (1997). A novel type of binding specificity to phospholipids for rat mannose binding proteins isolated from serum and liver. *FEBS Lett*. **414**: 387-392.
- **Laemmli, U.K**. (1970) Cleavage of structural proteins during the assembly of the head of T4 bacteriophage, *Nature.* **227:** 680-685.
- **Landsteiner, K**. and Raubitschek, H. (1907). Beobachtungen uber Hamolyse und Hamagglitination. *Zentralbl. Bakteriol. Parasitenk. Infektionskr. Hyg.Abt.I: Org.* **45**: 660-667.
- **Latha, V.L**., Rao, R.N., Nadimpalli S.K., (2006). Affinity purification, physicochemical and immunological characterization of a galactose-specific lectin from the seeds of *Dolichos lablab* (Indian lablab beans). *Prot. Exp. Pur.* **45**: 296-306.
- **Latha, V.L**., Kulkarni, K.A., Rao, R.N., Kumar, N.S., Suguna, K. (2006) Crystallization and preliminary X-ray crystallographic analysis of a galactose-specific lectin from *Dolichos lablab, Acta Crystallogr*. **F62:** 163–165.

- **Lehrer, S.S**. (1971) Solute perturbation of protein fluorescence: the quenching of tryptophyl fluorescence of model compounds and of lysozyme by iodide ion. *Biochemistry*. **10:** 3254–3263.
- **Lis, H**., Sela, B. A., Sachs, L., Sharon, N. (1970) *Biochim. Biophys*. *Acta*. **211:** 582-585.
- **Lis, H** and Sharon, N. (1981). In: *The Biochemistry of Plants*; (Marcus, A., Ed.); Academic Press, New York. **Vol. 6**: pp 371-447.
- **Lis, H** and Sharon, N. (1984). In *Biology of carbohydrates*: Ginsberg, V., Robbins, P. W., Eds.; John Wiley & Sons: NewYork. **Vol.2**: p 1.
- **Lis, H** and Sharon, N. (1986). In *The Lectins: Properties, Functions and Applications in Biology and Medicine*; Liener, I. E., Sharon, N., Goldstein, I. J., Eds; Academic Press, Inc.: Orlando. p 293.
- **Lis, H**. and Sharon, N. (1998) Lectins: carbohydrate-specific proteins that mediate cellular recognition. *Chem. Rev.* **98**: 637-674.
- **Lobley, A**., Wallace, B.A. (2001) DICHROWEB: a website for the analysis of protein secondary structure from circular dichroism spectra, *Biophys. J.* **80:** 373a.
- **Lobley, A**., Whitmore, L., Wallace, B.A. (2002) DICHROWEB: an interactive website for the analysis of protein secondary structure from circular dichroism spectra, *Bioinformatics.* **18:** 211–212.
- **Loontiens, F. G**., Clegg, R. M. and Jovin, T. M. (1977) Binding of 4-methylumbelliferyl α-D-mannopyranoside to tetrameric and unmodified or derivatized dimeric concanavalin A: equilibrium studies. *Biochemistry.* **16**: 159-66.
- **Loris, R**., Hamelryck, T., Bouckaert, J., Wyns, L. (1998) Legume lectin structure, *Biochim. Biophys. Acta.* **1383:** 9–36.
- **Lowry, O. H**., Rosebrough, N. J., Farr, A. L., Randall, R. J. (1951) *J. Biol. Chem*. **193:** 265-271.
- **Majumdar, T**., and Surolia, A. (1979) A general method for isolation of galactopyranosyl specific lectins, *Indian J. Biochem. Biophys.* **16:** 200-203.
- **Majumdar, T**., and Surolia, A. (1981) The physiochemical properties of the galactose-specific lectin from *Momordica charantia*, *Eur. J. Biochem.* **113:** 463-470.
- **Maliark, M. J**., Goldstein, I. J. (1988). Photoaffinity labelling of the adinine binding sites of the lectins from lima bean, *Phaseolus lunatus* and red kideney bean, *Phaseolus vulgaris*. *J. Biol. Chem*. **263**: 11274-11279.

- **Matis, M**., Mavric, M. Z., Katalinic, J.P. (2005). Mass spectrometry and database search in the analysis of proteins from the fungus *Pleurotus ostreatus. Proteomics*. **5**: 67-75.
- **McEver, R. P**. (2002) Selectins: lectins that initiate cell adhesion under flow. *Curr. Opin. Cell Biol.* **14:** 581-586.
- **Melchior, W.B**., and Fahrney, D. (1970) Ethoxy formylation of proteins. Reaction of ethoxy formic anhydride with α-chymotrypsin, pepsin and pancreatic ribonuclease at pH 4. *Biochemistry.* **9:** 251-258.
- **Mishkind, M**., Palevitz, B. A. (1980) *Plant Physiol*. **66:** 950-955.
- **Misquith, S**., Rani, P.G., Surolia, A. (1994) Carbohydrate-binding specificity of the B-cell maturation mitogen from Artocarpus integrifolia seeds, *J. Biol. Chem*. **269:** 30393–30401.
- **Mo, H**., Meah, Y., Moore, J.G., and Goldstein, I.J. (1999) Purification and characterization of *Dolichos lablab* lectin. *Glycobiology*. **9(2):** 173-179.
- **Nachbar, M. S**., Oppenheim, J. D., Thomas. J. O. (1980). Lectins in the US diet. Isolation and charecterization of a lectin from tomato (*Lycopersicon esculentum*). *J. Biol. Chem*. **255**: 2056-2061.
- **Nair, G. N**., Das, H. R. (2000) *Plant Foods for Human Nutrition (Formerly Qualitas Plantarum)*. **55 (3):** 243 – 253.
- **Nowell, P. C**., (1960). Phytohemagglutinin: An initiator of mitosis in cultures of normal human leukocytes. *Cancer Research*. **20:** 462-466.
- **Ofek, I**. and Sharon, N. (1988) Lectinophagocytosis: a molecular mechanism of recognition between cell surface sugars and lectins in the phagocytosis of bacteria. *Infect. Immun.* **56**: 539-547.
- **Ofek, I**. and Sharon, N. (2002). A bright future for anti-adhesion therapy of infectious diseases. *Cell Mol. Life Sci.* **59**: 1666-1667.
- **Olsnes, S**. (1978). Toxic and nontoxic lectins from *Abrus precatoius*. *Meth. Enzymol*. **50**: 232-330.
- **Parkin, S**., Rupp, B., Hope, H., (1996). Atomic Resolution Structure of A at 120 K. Concanavalin. *Acta Crystallogr*, D. **52**: 1161–1168.
- **Patthy, L**., and Smith, E.L. (1975) Reversible modification of arginine residues, *J.Biol.Chem.* **250:** 557-564.
- **Pere, M.,** Bourillon, R., Jirgensons, B. (1975). *Biochim. Biophys. Acta*. **393**: 31-36.

- **Periera, M. E. A**., Kabat, E. A., Sharon, N. (1974). *Carbohydr. Res*. **37**: 89-102.
- **Perkins, D. N**., Pappin, D. J. C., Creasy, D. M., Cottrell, J. S., (1999). Probability-based protein identification by searching sequence databases using mass spectrometry. *Electrophoresis*. **20**: 3551-3567.
- **Peumans, W. J**. and Van Damme, E. J. M. (1995) Lectins as plant defense proteins. *Plant Physiol.* **109**: 347-352.
- **Peumans, W. J**., Winter, H. C., Bemer. V., Van Leuvan, F., Goldstein, I. J., Truffa-Bachi, P., Van Damme, E. J. M. (1997). Isolation of a novel plant lectin with an unusual specificity from *Calystegia sepium*. *Glycoconjugate. J*. **14**: 259-265.
- **Peumans, W. J**., Barre, A., Hao, Q., Rougé, P. and Van Damme, E. J. M. (2000) Higher plants developed structurally different motifs to recognize foreign glycans. *Trends Glycosci. Glycotechnol.* **12**: 83-101.
- **Pratap, J. V**., Jeyaprakash, A. A., Rani, P. G., Sekar, K., Surolia, A. and Vijayan, M. (2002) Crystal structures of artocarpin, a Moraceae lectin with mannose specificity, and its complex with methyl-alpha-D-mannose: implications to the generation of carbohydrate specificity. *J. Mol. Biol***. 317**: 237-247.
- **Pueppke, S. G**., Wolfgand, D. B., Keegstra, K., Ferguson, A. L. (1978) *Plant Physiol.* **61:** 779-784.
- **Puri, K. D.,** Gopalakrishnan, B., Surolia, A. (1992). Carbohydrate binding specificity of the T_n -antigen binding lectin from *Vicia villosa* seeds (VVLB4). *FEBS Lett*. **312**: 208-212.
- **Puri, K. D**., Surolia, A. (1994). Amino acid sequences of winged bean (*Psophocarpus tetragonolobus*) basic lectin. *J. Biol. Chem*. **269**: 30917-30926.
- **Pusztai, A**., Ewen, S. W. B., Grant, G., Peumans, J., Van Damme, E. J. M., Rubio, L. and Bardocz, S. (1990). The relationship between survival andbinding of plant lectins during small inestine passage and their effectiveness as growth factors. *Digestion*. **46**: 308-316.
- **Pusztai, A**., Ewen, S. W. B., Grant, G., Peumans, W. J., Van Damme, E. J. M., Rubio, L. A. and Barodocz, S. (1991). Plant (food) lectins as signal molecules: effects on the morphology and bacterial ecology of the small intestine. In *Lectin Reviews*: Kilpatrick, D. C., Van Driessche, E. and Bog-Hansen, T. C., ed., Sigma Chem. Co. **Vol.1**: pp. 1-15.
- **Pusztai, A**. (1993). Dietary lectins are metabolic signals for the gut and modulate immune and hormone functions. *Eur. J. Clin. Nutr*. **47**: 691-699.

- **Quinn, J.M**., Etzler, M.E. (1989). *In Vivo* Biosynthetic Studies of the *Dolichos biflorus* Seed Lectin. *Plant Physiol*. **91**: 1382-1386.
- **Rao, D.N**., Hariharan, K., and Rao, D.R. (1976) Purification and properties of a phytohaemagglutinin from *Dolichos lablab* (field bean), *Lebensm.-Wiss. Technol*. **9:** 246-250.
- **Rao, R. N**., Nadimpalli, S.K. (2008). An efficient method for the purification and quantification of a Galactose-specific lectin from vegetative tissues of *Dolichos lablab*. *Journal of Chromatography, B*. **861:** 209-217.
- **Radha, Y**. (2002) Studies on some biologically important proteins from the invertebrate *unio* Ph.D. thesis, Biochemistry Department, University of Hyderabad, Hyderabad, India.
- **Rajasekhar, B.T**., Padma, P., Kumar, N.S. (1997) Purification of a lectin in high yield from the Indian lablab beans on goat IgM-Sepharose and by immunoaffinity chromatography. Evidence for the presence of endogenous lectin receptors, *Biochem. Arch.* **13:** 237–244.
- **Rajasekhar, B.T**. and Siva Kumar, N. (1997) Purification of α mannosidase activity from Indian lablab beans, *Biochem. Mol. Biol.Int.* **41:** 925-931.
- **Rajasekhar, B.T**., Padma, P., and Siva Kumar, N. (1997) Purification of a lectin in high yield from the Indian lablab beans on Goat IgM-Sepharose and by immunoaffinity chromatography: Evidence for the presence of endogenous lectin receptors, *Biochem. Arch*. 13 237-244.
- **Rajasekhar, B.T**. and Siva Kumar, N. (1998) A new unusual galactose specific lectin from the seeds of Indian lablab beans.*Curr. Sci*. **75**: 840-842.
- **Reddy, G.B**., Bharadwaj, S., Surolia, A. (1999) Thermal stability and mode of oligomerization of the tetrameric peanut agglutinin: a differential scanning calorimetry study, *Biochemistry* **38:** 4464–4470.
- **Renkonen, K. O**. (1948). Studies on hemagglutinin present in seeds of some representatives of Leguminoseae. *Ann. Med. Exp. Biol. Fenn*. **26**: 66-72.
- **Reisfeld, R**. A., Lewis, U. J. and Williams, D. E. (1962). Disc electrophoresis of basic proteins and peptides on polyacrylamide gels. *Nature*. **195:** 281-283
- **Rhodes, J. M.** and Milton, J. D.1998. Eds.; *Lectin methods and protocols*; Humana Press Inc.: Totowa, NJ. pp 616.

- **Rice, K. G**., Weisz, O. A., Barthel, T., Lee, R. T., Lee. Y. C. 1990. Defined geometry of Binding between Triantinnary Glycopeptide and the Asialoglycoprotein Receptor of Rat hepatocytes. *J. Bio. Chem*. **265**: 18429-18434.
- **Rinderle, S. J**., Goldstein, I. J., Matta, K. L. and Ratcliffe, R. M. (1989) Isolation and characterization of amaranthin, a lectin present in the seeds of *Amaranthus caudatus*, that recognizes the T- (or cryptic T)-antigen. *J. Biol. Chem.* **264**: 16123-1631.
- **Riordan, J.F**., Wacker, W.E.C., and Vallee, B.L. (1965) N acetyl imidazole: A reagent for the determination of "free" tyrosyl residues of proteins, *Biochemistry*. **4:** 1758-1765.
- **Roberts, D. D**., Goldstein, I. J. 1982. Hydrophobic binding properties of lectin from lima beans (*Phaseolus lunatus*). *J. Biol. Chem*. **257**: 9198-9204.
- **Roberts, D. D**., Goldstein, I. J. 1983a. Binding of hydrophobic ligand to plant lectins: titration with arylaminonaphthalenesulfonates. *Arch. Biochem. Biophys*. **224**: 479- 485.
- **Roberts, D.M.,** Walker, J., Etzler, M.E., 1982. A structural comparison of the subunits of the *Dolichos biflorus* Seed lectin by peptide mapping and carboxyl terminal amino acid analysis. *Arch. Biochem. Biophys*. **218**: 213-219.
- **Roberts, D. M**., Etzler, M. E. (1984) *Plant physiol*. **76:** 879-884.
- **Roopashree, S**., Singh, S.A., Gowda, L.R., Rao, A.G., (2006). Dual-function protein in plant defence: seed lectin from *Dolichos biflorus* (horse gram) exhibits lipoxygenase activity. *Biochem J*. **395(3)**: 629-639.
- **Rudiger, H.** (1988) Preparation of plant lectins, in: H. Franz (Ed.), Advances in Lectin Research, vol. 1, Springer, Berlin, pp. 26–72.
- **Rudiger, H.** (1998) Plant lectins – more than just tools for glycoscientists: occurrence, structure and possible functions of plant lectins. *Acta Anal* (Basel). **161:**130-52.
- **Rutenber, E**., Katzin, B. J., Ernst, S., Collins, E. J., Mlsna, D., Ready, M. P. and Robertus, J. D. (1991) Crystallographic refinement of ricin to 2.5 Å. *Proteins.* **10**: 240-250.
- **Sabins, D. D**. and Hart, J. W. (1978) The isolation and some properties of a lectin (haemagglutinin) from *cucurbita* phloem exudates. *Planta.* **142**: 97-101.
- **Sahasrabuddhe, A. A**., Gaikwad, S. M., Krishnasastry, M. V. and Khan, M. I. (2004) Studies on recombinant single chain Jacalin lectin reveal reduced affinity for saccharides despite normal folding like native Jacalin. *Protein Sci.* **13**: 3264-3273.
- **Sabons, D. D**., Hart, J. W. (1978) *Planta*. **142:** 97-101.

- **Sanadi, A. R**. and Surolia, A. (1994) Studies on a chitooligosaccharide-specific lectin from *Coccinia indica*. Thermodynamics and kinetics of umbelliferyl glycoside binding. *J. Biol. Chem.* **269**: 5072-5077.
- **Sastry, M.V.K**., Banerjee, P., Patanjali, S.R., Swamy, M.J., Swarnalatha, G.V., Surolia, A. (1986) Analysis of saccharide binding to Artocarpus integrifolia agglutinin reveals specific recognition of T-antigen (Galβ1→GalNAc), *J. Biol. Chem*. **261:** 11726–11733.
- **Sankaranarayanan, R.,** Sekar, K., Banerjee, R., Sharma, V., Surolia, A. and Vijayan, M (1996) A novel mode of carbohydrate recognition in jacalin, a Moraceae plant lectin with a β-prism fold. *Nat. Struct. Biol.* **3**: 596-603.
- **Saris, C.J.M**., Eenbergen, J.V., Jenks, B.G., Bloemers, H.P.J. (1983) Hydroxylamine cleavage of proteins in polyacrylamide gels. *Analytical Biochemistry.* **132**: 54-67.
- **Sastry, M. V. K**. and Surolia, A. (1986) Intrinsic fluorescence studies on saccharide binding to *Artocarpus integrifolia* lectin. *Biosci. Rep.* **6**: 853-860.
- **Sastry, M. V. K**., Banerjee, P., Patanjali, S. R., Swamy, M. J., Swarnalatha, G. V., Surolia, A. (1986). Analysis of sacharide binding to *Artocarpus integrifolia* lectin reveals specific recognition of T-antigen (β-D-Gal (1→3) D-GalNAc). *J. Biol.Chem*. **261**: 11726-11733.
- **Schnell, D.J**., Etzler, M.E. (1987). Primary structure of the *Dolichos biflorus* seed lectin. *J.Biol.Chem.* 262: 7220-7225.
- **Schwartz, F.P**., Puri, K.D., Bhat, R.G., Surolia, A. (1993) Thermodynamics of monosaccharide binding to Concanavalin A, pea (Pisum sativum) lectin and lentil (Lens culinaris) lectin, *J. Biol. Chem.* **268:** 7668–7677.
- **Schwartz, F. P**., Misquith, S. and Surolia, A. (1996) Effect of substituents on the thermodynamics of D-galactopyranoside binding to concanavalin A, pea (*Pisum sativum*) lectin and lentil (*Lens culinaris*) lectin. *Biochem. J.* **316:** 123-129.
- **Shadforth, I**., Todd, K., Crowther, D., Bessant, C. (2005). Protein and peptide identification algorithms using MS for use in high-throughput, automated pipelines. *Proteomics*. **5**: 1787-1796.
- **Sharma, V**., Vijayan, M. and Surolia, A. (1996). Imparting exquisite specificity to peanut agglutinin for the tumour-associated Thomsen-Friendenreich antigen by redesign of its combination site. *J. Biol. Chem*. **271**: 21209-21213.

- **Sharma, V**. and Surolia, A. (1997) Analyses of carbohydrate recognition by legume lectins: size of the combining site loops and their primary specificity. *J. Mol. Biol.* **267:** 433-445.
- **Sharon, N.** (1987) Bacterial lectins, cell-cell recognition and infectious disease. *FEBS Lett.* **217**: 145-157.
- **Sharon, N** and Lis, H. (1990). Legume lectins- a large family of homologous proteins. *FASEB J*. **4**: 3198-3208.
- **Sharon, N** and Lis, H. (1995). Lectins-proteins with a sweet tooth: functions in cell recognition. *Essays in Biochem*. **30**: 59-75.
- **Sharon, N**. and Lis, H. (2003) Lectins, 2nd Edition. Kluwer Academic Publishers, Dordrecht, The Netherlands.
- **Sheriff, S**., Chang, C. Y. Y., Ezekowitz, R. A. B. (1994). *Nat. Struct. Biol*. **1**: 789-794.
- **Shevchenko, A**., Sunyaev, S., Loboda, A., Shevchenko, A., Bork, P., Ens, W., Standing, K. G. (2001). Charting the Proteomes of Organisms with Unsequenced Genomes by MALDI-Quadrupole Time-of-Flight Mass Spectrometry and BLAST Homology Searching. *Anal Chem.* **73(9)**: 1917- 26.
- **Shibuya, N.,** Goldstein, I. J., Van Damme, E. J. M. and Peumans, W. J. (1988) Binding prosperities of a mannose-specific lectin from the snowdrop (*Galanthus nivalis*) bulb. *J. Biol. Chem*. **263**: 728-734.
- **Siva Kumar, N**. (1999) Chemical modification studies on the glucose/mannose specific lectins from Indian lablab beans. *Biochem. Mol.Biol.Int.* **47 (5):** 825-834.
- **Siva Kumar, N** and von Figura, K. (2002) Identification of the putative mannose 6-phosphate receptor (MPR 46) protein in the invertebrate mollusc, *Biosci.Rep.* **22**: 513-521.
- **Siva Kumar, N**., Nirmala, P., and Suresh, K. (2004) Biochemical and immunological characterisation of a glycosylated α-fucosidase from the invertebrate *Unio*. Interaction of the enzyme with its *invivo* binding partners, *Protein expression and purification.* **37:** 279-287.
- **So, L. L** and Goldstein, I. J. (1967). *J. Immunol*. **99**: 158-163.
- **Spande, T.F**., and Witkop, B. (1967) Determination of the tryptophan content of proteins with N-Bromosuccinimide, *Meth. Enzymol.* **11:** 498-506.
- **Sphor, U.,** Hindsgaul, O., Lemieux, R. U. (1985). *Can. J. Chem*. **63**: 2644-2652.

- **Spilatro, S.R**., Cochran, G.R., Walker, R.E., Cablish, K.L., Bittner, C.C. (1996) Plant *Physiology*. **110 (3):** 825-34.
- **Srinivas, V.R**., Reddy, G.B., Ahmad, N., Swaminathan, C.P., Mitra, N., Surolia, A. (2001) Legume lectin family, the 'natural mutants of the quaternary state', provide insights into the relationship between protein stability and oligomerization, *Biochim. Biophys. Acta.* **1527:** 102–111.
- **Stinissen, H. M**., peumans, W. J. and Carlier, A. R. (1983) Occurrence and immunological relationships of lectins in gramineous species. *Planta.* **159**: 105-111.
- **Stirpe, F**., Barbieri, L., Battelli, M. G., Soria, M. and Lappi, D. A. (1992) Ribosome-inactivating proteins from plants: present status and future prospects. *Biotechnology (N. Y.)* **10**: 405-412.
- **Stubbs, M.E.,** Carver, J. P., Dunn, R.J. (1986). Production of pea lectin in Escherichia coli. *J. Biol. Chem*. **261**: 6141-6144.
- **Sultan, N.A.M**., Roopa, K., Swamy, M.J. (2004) Purification, physicochemical characterization, saccharide specificity and chemical modification of a Gal/GalNAc specific lectin from the seeds of *Trichosanthes dioica*, *Arch. Biochem. Biophys.* **432:** 212-221
- **Sultan, N.A.M**., Swamy, M.J. (2005) Fluorescence quenching and time-resolved fluorescence studies on Trichosanthes dioica seed lectin, *J. Photochem. Photobiol. B Biol.* **80:** 93–100.
- **Sultan, N. A. M**., Rao, R. N., Nadimpalli S.K., Swamy, M.J. (2006). Tryptophan environment, secondary structure and thermal unfolding of the galactose-specific seed lectin from *Dolichos lablab*: Fluorescence and circular dichroism spectroscopic studies. *Biochim. Biophys. Acta.* **1760**: 1001-1008.
- **Summer, J. B**. and Howell, S. F. (1936). The identification of haemagglutinin of the Jack bean with Concanavalin A. *J. Bacteriol*. **32**: 227-237.
- **Surolia, A**. and Bachhawat, B. K. (1978). The effect of lipid composition on liposome-lectin interaction. *Biochim. Biophys. Acta*. **83**: 779-785.
- **Surolia, A**., Bachhawat, B. K. and Podder, S. K. (1975). Interaction between lectin from *Ricinus communis* and liposome containing gangliosides. *Nature*. **257**: 802-804.
- **Suzuki, I**., Saito, H., Inoue, S., Migita, S., Takahashi, T. (1979) J *Biol Chem*. **85:** 163-171.

- **Swamy, M. J.,** Sastry, M. V. K., Khan, M. I. and Surolia, A. (1986) Thermodynamic and kinetic studies on saccharide binding to soya-bean agglutinin. *Biochem. J.* **234**: 515-522.
- **Tahirov, T. H**., Lu, T. H., Liaw, Y. C., Chen, Y. L. and Lin, J. Y. (1995) Crystal structure of abrin-a at 2.14 Å. *J. Mol. Biol.* **250**: 354-367.
- **Talbot, C. F**., Etzler, M.E. (1978). Isolation and Characterization of a Protein from Leaves and Stems of *Dolichos biflorus* that Cross Reacts with Antibodies to the Seed Lectin. *Biochemistry.* **17**: 1474-1479.
- **Tateno, H**., Winter, H. C., Petryniak, J., Goldstein, I. J. (2003) *J. Biol. Chem.* **278:** 10891-10899.
- **Tayoshima, S**., Osawa, T., Tonomura, A. (1970). *Biochim. Biophys. Acta*. **221**: 514-521.
- **Thiede, B**., Wittmann-Liebold, B., Michael, B., Krause, E. (1995). A method to define the carboxyl terminal of proteins. *FEBS Letters.* **357**: 65-69.
- **Towbin, H**., Staehelin, T., and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications, *Proc. Natl. Acad. Sci.* **76:** 4350-4354.
- **Transue, T. R**., Smith, A. K., Mo, H., Goldstein, I. J. and Saper, M. A. (1997) Structure of benzyl T-antigen disaccharide bound to *Amaranthus caudatus* agglutinin. *Nat. Struct. Biol.* **4**: 779-783.
- **Treffers, H. P**. (1992) Agglutination reaction. In Encyclopedia of Science and Technology. 17th Edn. p 172. McGraw-Hill, Inc.
- **Trowbridge, I. S**. (1974) Isolation and chemical characterization of a Mitogenic lectin from *Pisum sativum. J. Biol. Chem.* **249**: 6004-6012.
- **Van Damme, E. J. M.,** Allen, A. K. and Peumans, W. J. (1987) Isolation and characterization of a lectin with exclusive specificity toward mannose from snowdrop (*Galanthus nivalis*) bulb. *FEBS Lett.* **215:** 140-144.
- **Van Damme, E. J. M**., Kaku, H., Perini, F., Goldstein, I. J., Peeters, B., Yagi, F., Decock, B and Peumans, W. J. (1991) Biosynthesis, primary structure and molecular cloning of snowdrop (*Galanthus nivalis*) lectin. *Eur. J. Biochem.* **202**: 23-30.
- **Van Damme, E. J. M**., Goldstein, I. J., Peumans, W. J. (1991). *Phytochemistry*. **30**: 509.

- **Van Damme, E. J. M**., Balxarini, J., Smeets, K., van Leuven, F., Peumans, W. J. (1994). The monomeric and dimeric mannose binding proteins from Orchidaceae species *Listera ovala* and *Epipactis helleborine*: Sequence homologies and differences in biological activities. *Glycoconjugate J.* **11**: 321-332.
- **Van Damme, E. J. M**., Barre, A., Verhaert, P., Rouge, P., Van Leuvan, F., Peumans, W. J. (1996). Molecular cloning of the mitogenic mannnose/maltose-specific rhizome lectin from Calystegia sepium. *FEBS Lett*. **397**: 352-356.
- **Van Damme, E. J. M**., Peumans, W. J., Barre, A. and Rougé, P. (1998) Plant lectins: a composite of several distinct families of structurally and evolutionarily related proteins with diverse biological roles. *Crit. Rev. Plant Sci.* **17**: 575-692.
- **Van Damme, E. J. M**., Hause, B., Hu, J., Barre, A., Rouge, P., Proost, P. and Peumans, W. J. (2002) Two distinct jacalin-related lectins with a different specificity and subcellular location are major vegetative storage proteins in the bark of the black mulberry tree. *Plant Physiol.* **130**: 757-769.
- **Van Parijs, J**., Broekaert, W. F., Goldstein I. J. and Peumans, W. J. (1991) Hevein: an antifungal protein from rubber tree (*Hevea brasiliensis*) latex. *Planta.* **183**: 258-262.
- **Vodovozova, E. L**., Gayenko, G. P., Razinkov, V. I., Korchagina, E. Y., Bovin, N. V., and Molotkovksy, J. G. (1998). Sacharide assisted delivery of cytotoxic liposomes to human malignant cells. *Biochem. Mol. Biol. Int*. **44**: 543-553.
- **Weis, W. I**., Drickamer, K. (1994). Trimeric structure of a C-type mannose-binding protein. *Structure*. **2**: 1227-1240.
- **Weis, W. I**., Taylor, M. E. and Drickamer, K. (1998) The C-type lectin superfamily in the immune system. *Immunol. Rev.* **163**: 19-34.
- **Whitmore, L**., Wallace, B.A. (2004) DICHROWEB, an online server for protein secondary structure analyses from circular dichroism spectroscopic data, *Nucleic Acids Res.* 32 W668–W673 (Web Server Issue).
- **Wiseman, T**., Williston, S., Brandts, J. F. and Lin, L. N. (1989) Rapid measurement of binding constants and heats of binding using a new titration calorimeter. *Anal. Biochem.* **179**: 131-137.
- **Wright, C. S**. (1987). *J. Mol. Biol*. **194**: 501
- **Wright, C. S**. (1990). 2.2 Å resolution structure analysis of two refined N-acetylneuraminyl-lactose wheat germ agglutinin isolectin complexes. *J. Mol. Biol*. **215**: 635-651.
- **Wright, C. S**., Hester, G. (1996). *Structure*. **4**: 1339.

- **Wright, C. S**. and Kellogg, G. E. (1996) Differences in hydropathic properties of ligand binding at four independent sites in wheat germ agglutinin-oligosaccharide crystal complexes. *Protein Sci.* **5**: 1466-1476.
- **Yamaguchi, T**., Kato, R., Beppu, M., Terao, T., Inoue, Y., Ikawa, Y. and Osawa, T. (1979). Preparation of Concanavalin A- Ricin A-chain conjugate and its biological activity against various cultured cells. *J. Natl. Cancer Inst*. **62**: 1387-1395.
- **Yamamoto, K.,** Tsuji, T., Osawa, T. (1993) In *Glycoprotein analysis in Biomedicine*. Hounsell, E. L. Ed. *Mothods. Mol. Biol*. **14**: 17.
- **Young, N. M**., Leon, M. A., Takahashi, T., Howard, I. K., Sage, H. J. (1971). *J. Biol. Chem*. **246:** 1596-1601.
- **Zacharias, R**. **M**., Zell, T. E., Morrison, J. H. And Woodlock, J. J. (1969). Glycoprotein staining following electrophoresis on acrylamide gels. *Anal Biochem.* 30 (1): 148-152.

PUBLICATIONS

Publications:

- **Lavanya latha, V, *Rameshwaram Nagender Rao* and Siva Kumar, N.** Affinity purification, physico-chemical and immunological characterization of a galactose specific lectin from the seeds of *Dolichos lablab* (Indian lablab beans)**, *Protein Expr Purif*.** 2006; 45(2):296-306
- ***Rameshwaram Nagender Rao,* Ali Mohammed Sultan N, Nadimpalli SK, and Swamy MJ**: Tryptophan environment, secondary structure and thermal unfolding of the galactose-specific seed lectin from *Dolichos lablab*: Fluorescence and circular dichroism spectroscopic studies. ***Biochim Biophys Acta***. 1760: 1001-1008
- **Latha VL, Kulkarni KA, *Rameshwaram Nagender Rao*, Kumar NS, Suguna K.:** Crystallization and preliminary X-ray crystallographic analysis of a galactose-specific lectin from *Dolichos lablab*. ***Acta Crystallograph Sect F Struct Biol Cryst Commun*.** 2006, 62(Pt 2):163-5
- ***Rameshwaram Nagender Rao* and Nadimpalli Siva Kumar:** An efficient method for the purification and quantification of a galactose-specific lectin from vegetative tissues of *Dolichos lablab*. (***Journal of Chromatography B.*** 2008, 861: 209-217

Under Communication:

- ***Rameshwaram Nagender Rao*, Karanam Narasimha Kumar, Christian Scharff, Uwe Voelker and Nadimpalli Siva Kumar:** Primary structure of the galactose-specific lectin from *Dolichos lablab* - A Proteomics approach. (Under communication in ***Phytochemistry Journal***)

Under Preparation:

- **Nitin Kumar Singhal, Amit Kumar, *Rameshwaram Nagender Rao,* Nadimpalli Siva Kumar and C.P. Rao:** Synthesis and characterization of C1- Imino-derivatives of D-galactose, D-lactose and D-ribose, and C2-Imino-derivatives of D-glucose and their binding and inhibition towards glycosidase activities isolated from soybean and jack bean. (Under preparation – ***Glycoconjugate Journal***)

- ***Rameshwaram Nagender Rao,*** **Ramesh, CH, Kiran, T and Nadimpalii Siva Kumar:** Affinity Purification, Biophysical characterization of a lactose-specific lectin from *Unio*. (Under preparation – ***BBRC Journal***)