# Purification, characterization and partial cDNA cloning of a Galactose specific lectin from *Dolichos lablab* seeds

Thesis submitted for the Degree of

**DOCTOR OF PHILOSOPHY**

**by**

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## CERTIFICATE

This is to certify that this thesis entitled **"Purification, characterization and partial cDNA cloning of a Galactose specific lectin from *Dolichos lablab* seeds"** submitted to the University of Hyderabad by V. **LAVANYA LATHA** for the degree of Doctor of Philosophy, is based on the studies carried out by her 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.

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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, under the supervision of **Prof. N. Siva Kumar** and **Prof. M. Ramanadham.** (Part of the work on crystallization of the lectin was done in I1SC, Bangalore). 1 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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# Abbreviations

| | |
|---|---|
| ALP | Alkaline Phosphatase |
| APS | Ammonium per Sulfate |
| BCA | Bicinchoninic acid |
| BC1P | 5-Bromo, 4-Chloro indolyl Phosphate |
| bp | base pair |
| BSA | Bovine serum albumin |
| cDNA | Complementary DNA |
| Con A | Concanavalin A |
| cpm | Counts per minute |
| CRD | Carbohydrate recognition domain |
| CTP | Cytidine triphosphate |
| dATP | Deoxyadinosine triphosphate |
| dCTP | Deoxycytidine triphosphate |
| DFPC | Diethyl pyrocarbonate |
| dGTP | Deoxyguanosine triphosphate |
| DLL | *Dolichos lablab* |
| DBL | *Dolichos biflorus* |
| DNA | Deoxyribonucleic acid |
| DTT | Dithiothreitol |
| dTTP | Deoxythymidine triphosphate |
| DVS | Divinyl sullbne |

| | |
|---|---|
| ECO | *Erylhrina corollodemiron* |
| EDTA | Ethylene diamine tetra acetic acid |
| *et al* | et alii (Latin: and others) |
| EtBr | Ethidium bromide |
| FBL | *Fava bean* lectin |
| FCS | fetal calf serum |
| FITC | Fluorescein Isothiocyanate |
| FRIL | Flt3 receptor interacting lectin |
| GAFP-1 | *Gastrodiu* antifungal protein |
| GalNAc | N-acetyl galactosaminc |
| HEPES | (N-(2-Hydroxyethyl)-piperizine-N'-(2-ethane sulfonic acid)) |
| Kb | Kilo basepair |
| kDa | Kilo Dalton |
| LB | Luria Bertani |
| LBE | Lima bean lectin |
| LCL | Lentil lectin |
| min | Minute |
| MOPS | Morpholino propane sulfonic acid |
| MPR | Mannose 6-phosphate receptor |
| Mr | Molecular mass |
| NBS | N-bromo succinimide |
| NBT | Nitro blue tetrazolium |

| | |
|---|---|
| nm | Nanometer |
| OD | Optical density |
| PAGE | Polyacrylamide Gel electrophoresis |
| PAS | Periodic acid Schiff s |
| PHA | Phytohemagglutinin |
| PNA | Peanut agglutinin |
| PBM's | Protein body membranes |
| PBS | Protein body supernatant |
| PCR | Polymerase chain reaction |
| PEG | Polyethylene glycol |
| pH | -log ($II^1$) concentration |
| pmol | Picomole |
| rpm | Rotations per minute |
| rRNA | Ribosomal RNA |
| RT | Reverse Transcription |
| SB A | Soybean agglutinin |
| SDS | Sodium dodecyl Sulfate |
| SL | Sainfoin lectin |
| Taq | *Thermophilus uquaticiis* |
| TBS | Iris-buffered saline |
| TCA | Trichloroacetic acid |
| TEL | *Talisia esculenfa* lectin |
| TEMED | N, N. N\ N"-Tetramethylcndiamine |

| | |
|---|---|
| "ITA | Trifluroacetic acid |
| TNBS | 2, 4. 6 Tri nitro benzene sulphonic acid |
| TM | Melting temperature |
| Tris | Tris-(IIydroxymethyl) aminoethane |
| UDP | Uridine diphosphate |
| UDA | *Urtica dioica* agglutinin |
| UV | Ultraviolet |
| WGA | Wheat germ agglutinin |
| [3-M. E | ‘p[1]-mercaptoethanol |
| \|.iCi | micro Curie |

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# Chapter 1

# Introduction

In 1888, Herman Stillmark in his doctoral thesis reported the toxicity of the Castor beans, *Ricimis communis* and described that its extract could agglutinate erythrocytes. Paul Ehrlich (1890) then realized that these plant hemagglutinins would be more useful model antigens for the solution of immunological problems. Karl Landsteiner working together with Raubitchek in 1908 discovered that the relative hemagglutinating activities of various seed extracts were quiet different when tested with red blood cells from different animals. The discovery of sugar specificity of these agglutinins was credited to James B. Sumner, well known for crystallization of *urease* en/yme from Jack bean, *Canavaliu ensiformis.*

Later, Boyd and Shapleigh (1954) coined the term "lectin" for these molecules. During the period 1960-1980's several lectins have been purified from a variety of sources and have been well characterized. Then followed an era when glycoprotein lectins from animals were discovered and were functionally characterized (Gabius *et al.,* 1997). In the last few years, the structural analysis of lectins is being studied for drug designing and therapeutics (Leonidas *et al.,* 1998).

Lectins bind specifically and non-covalently to carbohydrate residues (Van Damme *et al.,* 1998). Each lectin molecule typically contains two or more binding sites, that is, they are divalent or polyvalent. They bind to sugars on the surfaces of cells and thus cause their agglutination. The hemagglutinating activity is a major property of these proteins and is commonly used for their detection and characterization. The hemagglutinating activity is generally expressed as a titre that is measured visually or microscopically (Sharon and Lis, 2004). This activity of the lectins is inhibited by the sugar ligands for which the lectins are specific. Many lectins agglutinate animal, as

well as human erythrocytes and some of them exhibit blood group specificity. Polar interactions such as hydrogen bonds and dipole interactions could play a dominant role in this carbohydrate - protein binding abilities. The carbohydrate specificity is examined by hapten-inhibition technique. Their ability to specifically recognize and reversibly bind to soluble or cell bound complex carbohydrate structures make them special candidates to play different roles in many biological processes such as cell to cell interaction, ligand receptor signalizing or cellular activation among other important biological events (Wu *et al.,* 1995; Gorocica *et al.,* 1998; Saez *et al.,* 1999).

Lectins have wide spread occurrence in most organisms such as the plants, animals, viruses, bacteria and fungi (Vandamme *et al.,* 1998; Ruediger *et al.,* 2000). They arc-readily obtainable in purified form, mostly by affinity chromatography and more recently by recombinant DNA techniques. They represent a heterogeneous group of oligomeric proteins that vary in size, structure and molecular organization. They are thus defined as "Protein or glycoprotein substances, of non-immunoglobulin nature, capable of specific recognition of and reversible binding to, carbohydrate moieties of complex glycoconjugates without altering the covalent structure of any of the recognized glycosyl ligands[1]". Based on the specificity of the lectin to monosaccharides they are classified as follows (Sharon and Lis, 1990):

> **The glucose / mannose binding lectins:** Ex: Con A from *Canavalia emiformis;* glucose / mannose specific lectin from *Do/ichos lablab* seeds. These comprise a large group of lectins in the Lcguminosac family. On the basis of their molecular structure these are classified into two groups - those that contain identical subunits (Con A with four identical subunits, CX4) and those that contain two light and two

heavy chains having the general composition o^p: (the pea lectin and the lentil lectin). These lectins require metal ions, especially $Mn^{2+}$ and $Ca^{2+}$ ions for their activity. They are rich in acidic or hydroxyl amino acids but devoid or low in sulphur containing amino acids.

> N-acetyl glucosamine binding lectins: Ex: Lectins from potato (Kilpatrick *et al.,* 1980); Wheat germ lectin (Peumans *et al.,* 1983). These lectins specifically bind to N-acetyl glucosamine or its $\beta^{1}$ 1,4 linked oligomers. The glucose / mannose binding lectins also bind N-acetyl glucosamine, but weakly and only if this sugar occurs in a anomeric linkage at oligosaccharides chain ends.

> N-acetyl galactosamine / galactose-binding lectins: Lectins from *Ricinus communis* and *Abrus precatorius* were the first hemagglutinins to display blood group specificity. Studies on *Ricinus Communis* (Stillmark, 1888) led to the understanding of the toxic properties of galactose binding lectins. These lectins show considerable similarities in their sequence homology, protein structure polymorphism and carbohydrate binding specificities.

> L-fucose binding lectin: Ex: Eel hcmagglutinin, Anguilla and Ulex lectins. These lectins have been isolated from various sources such as plants, animals, fungi etc., and have shown little sequence homology. They are widely used in clinical serological laboratories. Ex: *VI ex curopeus-X* lectin exhibits anti-blood group O activity.

> Sialic acid binding lectins: Ex: lectins isolated from horseshoe crabs, lobsters, tunicates (Yeaton, 1981). These are found mainly in the invertebrate hemolymph or

sera. These lectins bind to sialo glycoproteins and usually consist of a large number of subunits.

## Biological properties of lectins

The biological properties of the lectins serve as a basis for the application of the lectins to the investigation of some chemical and biological problems. Some of these effects **are**

*** **Agglutination of erythrocytes and other types of cells:** This property is useful in studying the membrane changes that occur during physiological and pathological conditions. Agglutinibility caused between normal and malignant cells, embryonic and adult cells, or between mitotic and interface cells are quiet different (Burger, 1973; Nicolson, 1976 a, b; Rapin and Burger, 1974). This particular property of the lectins helps in revealing their presence in biological sources (Ruediger, 1993). Agglutination is affected by a number of factors such as, the number of saccharide binding sites, molecular size, number and accessibility of receptor sites, membrane fluidity and metabolic state of cells (Nicolson, 1976 a). It is also affected by external conditions of the assay such as temperature, cell concentration and mixing of the lectin. When agglutination does occur and is inhibited by a particular sugar, it is an indication that the sugar for which the lectin is specific is present on the cell surface. Agglutination activity of the lectins is mostly carried out using animal cells. Few incidences have been reported where non-animal cells were agglutinated by lectins. Ex: mycoplasma (Schiefer *et al,* 1974); fungi (Cassone *et al,* 1978). Tissue sections can be stained with glycoproteins or neoglycoproteins for lectin detection. Another

method of detection of lectins is based on sequence similarities by searching the database at the protein or cDNA level (Drickamer and Dodd, 1999). Recently, carbohydrates coupled to the wells of a microtitre plate have been developed to detect the presence of lectins (Gargir, 2001; Bryan *et al.*, 2002).

- **Mitogenic stimulation of lymphocytes:** Lectins have the ability to stimulate mitogenesis that is, triggering of quiescent, non-dividing lymphocytes into a state of growth and proliferation (Kilpatrick, 1998). The first mitogen to be studied was phytohemagglutinin (PUA), a lectin from red kidney bean, *Phaseolus vulgaris* (Nowell, I960). Later, lectins from other sources like *Wistaria floribwula* and *Canavalia ensifonnes* (Lis and Sharon, 1977) have been shown to have mitogenic activity. An interesting protein is the hepatic binding protein (HBP) isolated from rabbit liver cell membranes that act as a mitogenic lectin to sialidase-treated lymphocytes (Novogrodsky and Ashwell, 1977). It participates in the recognition and uptake of asialoglycoproteins from the circulatory system into the liver (Ashwell and Uarford, 1982). Jacalin, a plant lectin is found to completely block the 1J1V-1 infection of lymphoid cells. This property of the lectins is due to the induction of proliferation of CD4+ T lymphocytes in humans. Many lectins differ markedly in their ability to stimulate lymphocytes of different species. This activity is affected by modification of the cell surface, as well as of the lectin molecule. The mitogenic activity of the lectins is inhibited by specific sugars for which the lectin is specific. The mechanism of mitogenic stimulation is described further in the thesis (Chapter 4).

***l*** **Induction of suppressor cells:** Lectins also induce the generation of suppressor cells capable of inhibiting the activities of the T and the B cells *invitro.* In patients suffering from immunodeficiency diseases generation of suppressor cells is decreased (Kaufman and Bostwiek, 1979) in the peripheral blood lymphocytes. Upon treatment with the lectin, the immune response is normalized. This property of the lectins is useful clinically for assessing the level of immuno-competence of patients.

- **Lectin dependent cytotoxicity of lymphocytes and macrophages:** Sometimes, in the presence of lectins, a large number of antigcnically unrelated target cells are lysed by cytotoxic T lymphocytes, a phenomenon known as lectin dependent cytotoxicity (Asherson *et al.,* 1973). Lectins are effective in mediating lectin dependent cytotoxicity by having a dual role (1) in bridging target and effector cells (2) activating the effector cells to kill the target cells non-specifically (Green, 1982; Parker and Martz, 1980) or modifying the target cells to express structures essential for cell lysis (Bonavida and Katz, 1985). It has been proposed that some of the target cell structures that are affected by lectins are products of Major Histocompatibility Complex. Lectins also have the ability to induce the killing of tumor cells by macrophages. Ex: wheat germ lectin (Kurisu *et al.,* 1980).

***t*** **Lectin mediated phagocytosis of target cells:** Lectins cause phagocytosis of other types of cells (Sharon, 1984). Wheat germ agglutinin causes the phagocytosis of *Staphylococcus aitreus* by the macrophages.

- **Insulinomimetic activity:** Lectins bind to insulin receptors and thus mimic the activities of insulin such as stimulation of lipogenesis, oxidation and inhibition of lipolysis.

***t*** **Lectin toxicity:** Several lectins, for example WGA and PHA are toxic to mammalian cells, both *invitro* and *invivo.* Toxicity is affected by binding of lectins to cell surface sugars. As transformed cells are much more sensitive to the cytotoxic effects of lectins than the normal cells, these are used to inhibit tumor growth *invivo* (Nicolson, 1974; Brown and Hunt, 1978).

## Plant lectins

Lectins have been first discovered in plants. Although plant lectins are primarily found in the storage organs (Ruedigcr, 1998), they are also found in other plant tissues (Cavada *et al.,* 2000). The precise functions of plant lectins are still unknown and are a matter of debate. Most of the results suggest that plant lectins may take part in plant defense system together with chitinase, glucosidase and protease inhibitors (Etzler, 1998). Some of the well studied lectins are listed below

**Legume lectins:** The largest family of plants where the lectins have been well characterized is the Leguminosae (Sharon and Lis, 1990). Though they differ in the carbohydrate specificities, they resemble each other in their physiochemical properties. They usually consist of 2 or 4 subunits, each with a carbohydrate-binding site. The subunits are in the shape of a half-dome with the carbohydrate-binding site forming a shallow depression at its apex. One major property of the lectins is their specific saccharide binding sites. In the early 1980's and the 1990\s a German group devoted some attention to the possible occurrence of endogenous lectin binding components

following the idea that the legume lectins which are vacuolar proteins stored in the protein bodies in seeds, could be the natural ligands for other glycocompounds located in the protein bodies, including their membranes (Einhoff *et al.,* 1986; Ruediger and Schecher, 1993; Gers barlag *et al.,* 1993; Schecher and Ruediger, 1994; Wenzel and Ruediger, 1995).

## Primary sequences of legume lectins

The primary sequences of most of these lectins have been determined and it is shown that they exhibit sequence homologies with a significant number of invariant amino acid residues, most of which are involved in metal binding. Lectins have highly conserved amino acids that are involved in metal binding. The amino acids involved in carbohydrate binding sites are highly conserved in all legume lectins. All glycoprotein lectins contain a peptide sequence Asn-X-threonine / serine that is a characteristic feature of glycosylation sites. Peptide sequences, around the glycoside side chains in glycoproteins arc not necessarily conserved in the other glycoprotein lectins. This suggests that the biological activity of the lectins may not be determined by carbohydrate part of their structure.

The 3 D structures are also similar, mostly containing (3 sheets and a lack of a helix. This paved the way for the investigation of the carbohydrate binding sites of the lectins and to establish the relationship between the primary, secondary, tertiary and quaternary relationship between these proteins (Rini, 1995; Van Damme, 1998). Several of the lectin genes have been cloned and expressed in heterologous systems. This may provide us information on the role of lectins in nature. The Fig. 1 shows the

**Figure 1.**

**Relationships of the amino acid sequences of various lectins**

The arrangement of Con A (the innermost circle); the two subunit lectins - favin, pea and lentil lectins and the single subunit lectins - soybean, sainfoin and peanut is based on the amino acid sequences proposed by Hemperly and Cunningham. 1983. The *Dolichos lablab* lectin u-subunit is arranged along the NHi terminus of Con A followed by the (3-subunit starting of 123 of Con A, which then completes the circle.

alignment of the primary sequences of Fava bean, Con A and SBA and the glucose/mannose specific lectin from the seeds *of Dolichos lablab.*

**Figure 1**

Examination of the 3 D structure of legume lectins in complex with their ligands and the results of site directed mutagenesis studies revealed, that irrespective of their specificity, these lectins bind monosaccharides in their primary combining site mainly through the side chains of invariant residues, an asparatic acid, an asparagine and an aromatic amino acid. In few cases, asparagine is replaced by asparatic acid (Imberty *et al.*, 2000). A fourth conserved amino acid that participates in ligand binding is glycine, which in Con A is replaced by arginine. Proof that the invariant aromatic amino acid is involved in the combining site of the lectins comes from mutagenesis experiments showing that its replacement with smaller non-aromatic residue afforded always an inactive residue. The structure of the metal binding region of the legume lectins may have an important role in determining their specificity (Yamamoto *et al,* 2000). In the 3 D structures of complex legume lectins with disaccharides or linear oligosaccharides, the non-reducing residue of the saccharide occupies usually the primary combining site, with the same contacts to the protein as in complexes with the corresponding free

monosaccharides. A well studied case is the complex of Con A with Man cx-2 Man, refined to 1.2 $A^0$ (Sanders *et al.,* 2001). The disaccharide was shown to bind to the lectin with its non-reducing moiety in the primary combining site and the reducing one in an extension of this site formed by Tyr 12, Asp 16 and Arg 228.

**Non legume lectins:** A well studied lectin of the monocotyledonous family is the wheat germ agglutinin (WGA). It is a dimer of two identical, 18 kDa subunits and has four carbohydrate binding sites, located at the interlace of the subunits. The specificity of the cereal lectins is unusual since they interact with both the sialic acid and N-acetyl glucosamine. The amino acids involved in carbohydrate binding are not present on the same subunit but in two different subunits of the dimer.

Some examples of the Solanaceous lectins are tomato and potato lectins. These are specific for chitin oligosaccharides (P 1, 4 linked oligomers of N-acetyl glucosamine) and exists as a homodirner. Each subunit consists of two evolutionary autonomous domains: a carbohydrate-binding region fused to a hydroxy proline rich, highly glycosylated module, flic former shares sequence similarity to other chitin binding plant proteins and also with platelet-aggregation inhibitors from snake venom. The hydroxy proline rich domain is similar to extensins, a family of glycoproteins that are components of plant cell walls.

In the Cucurbitaceae family, the lectins from the seeds of Trichosanthes (Kenoth *et al.,* 2001) and bitter gourd, *Momordica charantia* (Sultan *et al.,* 2004) have been well studied.

## Role of lectins in plants

> **Defense mechanisms:** Lectins are used to defend plants against bacteria and viruses and therefore may be termed as plant antibodies. Seed lectins may help to protect cotyledons from bacteria that degrade the seed coats (Jones *et al.*, 1964). Lectins have thus been proposed to protect plants during imbibition, germination and early growth of seedlings (Mirlcman *et al.*, 1975; Barkai Golan *et al.*, 1978). The carbohydrate binding properties of the lectins make them useful probes in distinguishing the microorganisms. Their defense roles determine the extent of pathogenicity of the organism and the potential sites of infection of the plant. Suggestive evidence for their defense role had come from their interactions with the fungi, which are the causative agents for crop losses of cultivated plants (Peumans *et al.*, 1995; Ruediger, 1998; Ciopraga *et al.*, 1999). Investigators explored the role of lectins in plant defense against seed predators (insects or fungi) or during seedling development (tvtzler, 1998; Naeem, 2001).

*> **Pathogenesis:** The infection of sugarcane by the fungus *Helminthosporiwn sacehari* is an example where lectins may aid in pathogenesis (Strobel, 1973). Thus, apart from their defense role, lectins may also aid in the invasion of a pathogen into a plant by serving as a receptor for phytotoxins or for attachment of the pathogen (Sequeira, 1978).

*y* **Symbiosis:** Lectins play a role in the binding of the bacteria to the root hairs of plants. This was first tested by Makela in 1957. Trifoliin (from white clover) has been shown to bind a nodulating strain *oi~ Rhizobium* (Dazzo *et al.*, 1978). Several others later proposed that the lectin recognition of *Rhizohia* might account for the

specificity in the initiation of nitrogen fixing symbiosis (Hirsch et al., 2001; Murdock and Shade, 2002).

> **Other cell recognition functions:** The interaction of the pollen and the pistil is thought to be due to the interaction of the glycoproteins on the cell surface of the stigma or the style. Example, Con A was found to react specifically with receptors on the surface of the stigma and to block the penetration of the stigma cuticle by the pollen tube (Knox *et al.,* 1976). This interaction leads to either the retardation of growth, penetration of the incompatible pollen tubes or the promotion of the growth of the compatible pollen tube through the style to the ovary (Heslop and Harrison, 1978). Lectins were also found to be useful for embryogenesis and development (Brill *ci al.,* 2001)

*V* **Cell wall elongation:** The finding that the Mung bean seedlings lectin is non-covalently associated with the cell walls led Kauss and Glaser (1974) to propose that lectins may serve as a non-covalent 'glueing' substance in the cell wall and play a role in cell wall extension by participating in the breaking and reformation of non-covalent cross links with other wall components.

> **Enzymes:** Lectins may be carbohydrate specific enzymes that bind certain substrates with limited catalytic activity, under particular conditions or they may represent precursors that modify carbohydrate-specific enzymes. Hankins and Shannon (1978) isolated a hemagglutinin from mung bean seeds that had strong cc-galactosidase activity. This hemagglutinin was heat labile and sensitive to the treatment with sulfhydryl reagents. Antisera against the hemagglutinin showed cross-reaction of this preparation with several other galactose specific lectins.

- **Other roles:** Boyd *et al.,* (1958) proposed that lectins are 'carbohydrate catchers' and thereby help in the transport of carbohydrates and their immobilization in the seeds. Howard *et al.,* (1972) suggested that lectins may function in the maturation and germination of seeds. Yoshida (1978) has suggested that the lectins may play a role in the binding of ribosomes and inhibit protein synthesis. The membrane lectins may function in stabilizing membrane protein complexes or in regulating the compartmentalization of internal membranes. The binding of lectins to specific ligands may result in the expression of other activities of these molecules (Olsnes andPihl, 1982).

## Animal lectins

Much of the earlier research on lectins was focused on their isolation and characterization from plants. With the advent of modern tools, several lectins have been purified from animal sources and their functions are being understood. Table 1 shows some of the key discoveries in the history of animal lectins.

**Table 1: Key discoveries in animal lectin history**

| Year | History |
|---|---|
| 1853 | First description of charcot - leyden crystals |
| ] 860-1886 | Weir Mitchell's studies on rattle snake venom |
| 1902 | Horse crab agglutinins first reported |
| 1906 | Conglutinin, first animal lectin to be associated with the immune system |
| 1935- 1946 | Eel agglutinins and their application as blood typing reagent |
| 1973- 1975 | Discoidins and related lectins in cellular slime molds |
| 1974 | Rabbit hepatic lectin (asialoglycoprotein receptor) reported |
| 1975 | Electrolectin and beginning of research into galectins |
| 1989 -1991 | Selectins identified as new subfamily of C -type lectins after publication of primary sequence data from various adhesion molecules |
| 1991 - | X ray crystallography and structural analysis of lectins |

Given below is the classification of animal lectins based on the carbohydrate recognition domains (Gabius, 1997).

***r* C type lectins:** This class of proteins has been so named because they require $Ca^{++}$ for their activity (Drickamer, 1988). These arc all characterized by an extra cellular carbohydrate recognition domain (C-CRD) consisting *of* 115-130 amino acids, of which 14 are invariant and 18 are highly conserved. This class has again been sub divided into three groups depending on the nature of the domains. They are the (1) endocytic lectins - ex: the galactose / N-acetyl galactosamine specific lectin from rabbit hepatocytes which is also known as the hepatic asialoglycoprotein receptor.

(Ashwell *et al,* 1982) (2) collectins – these are soluble proteins with an amino terminal cysteine rich domain followed by a collagen like repeat, an a helical neck region and a carboxy terminal CRD, ex : pulmonary surfactant apoproteins A and D, collectin Cl- 43 from bovine serum (Epstein *et al.,* 1996). (3) selectins - these are highly asymmetric membrane bound proteins (Drickamer, 1988) that mediate selective contact between cells. Each contains in addition to CRD, an adjoining epidermal growth factor like domain ex: E- selcctin, P- selectin.

> **1 type lectins:** These are characterized by innumerable immunoglobulin like domains and are thus members of immunoglobulin super family (Powell *et al.,* 1995). The amino terminal extracellular domain is similar to the variable region of immunoglobulin G (Ig G). The remaining regions are similar to the constant region of Jg G. A conserved arginine was implied, by site directed mutagenesis studies to play a key role in the affinity of these lectins to glycoproteins containing sialic acid (Vander Merwe, 1996).

> **Galectins** (S **type lectins):** This is a family of galactoside binding proteins comprising of bioactive molecules with powerful immuno-regulatory functions. Different members *of* this family have been shown to modulate positively or negatively, the multiple steps of inflammatory response such as cell matrix interactions, cell trafficking, cell survival, cell growth regulation, chemotaxis and proinflammatory cytokine secretion.

> **Pentraxins:** This is a family of oligomeric plasma proteins with the capacity of calcium dependent ligand binding, named for the pentameric arrangement of subunits. C reactive protein (CRP) is the most studied protein of this group.

> **P type lectins:** This group comprises of two closely related lectins designated as the mannose 6 phosphate receptors - MPR 300 and MPR 46. Of these, MPR 300 does not require divalent ions for its activity where as MPR 46 requires divalent ions for its activity. Both are type-1 transmembrane glycoproteins.

Some of the functions of the animal lectins are summarized in the Table 2.

**Table 2: Functions of animal lectins**

| Likely function | Examples |
|---|---|
| Self/ non self recognition | Collectins, ficolins, complement factor II, CD1 lb / CD18 (CR3), tachylectins |
| Intracellular routing of glycoconjugates | T type lectins, ERGIC - 53, VIP -36 |
| Molecular chaperones during glycoprotein synthesis | Calnexin, calreticulin |
| Mediation of endocytosis | Asialoglycoprolein receptors, macrophage mannose receptor |
| Cellular growth regulation | Galectins, sarcolectin, cytokines |
| Extracellular molecular bridging | Galectins, interleukin -2 |
| Cell - cell interactions for homing and trafficking | Selectins, CD22, CD31, CD44 |
| Scavenging of cellular debris; anti - inflammatory action | Pentraxins, galectins |

* Hoflack, B and Lobel, P. (1993). Functions of the mannose 6 phosphate receptors. Advances in cell and Mol. Biol. of membranes, 1, 51-80.

## Uses of lectins

Lectins serve as invaluable tools in diverse areas of biological and medical research. Because of their ubiquitous nature they play a role in cancer, immune response and signal transduction (Gabius and Gabius, 1997). Lectins are useful for

- **Isolation, purification and structural studies of carbohydrate containing polymers:** Studies with lectins have expanded our knowledge of the tissue bound carbohydrates in histology and Histopathology (Hwen, 1998). The individual glycoproteins in a mixture can be detected in a SDS-Polyacrylamide gel electrophoresis and stained with radiolabeled (Burridge, 1978) or fluorescent (Furlan *et al,* 1979) lectins. The carbohydrate content of the lectins can be detected qualitatively on SDS-PAGE by Periodic acid - Schiff staining. Enzyme linked lectin assay, (ELLA), is conceptually used for detecting the carbohydrate content of a glycoprotein quantitatively. Immobilized lectins covalently bound to Sepharose or other carriers are indispensible for affinity chromatography of glycoproteins, glycopeptides and oligosaccharides. Affinity chromatography of glycoproteins on lectins provides a useful technique for the purification of membrane receptors. Purification of glycoproteins on immobilized lectin-affinity columns also provides a useful method for resolving murine IgM and human $a_2$- macroglobulin on immobilized GNA (Shibuya *et al.,* 1988). Table 3 shows the different glycoproteins purified on lectin-chromatography column. Since Con A has a wide range of specificity for a number of different lectins. Con A- Sepharose affinity column is used frequently for the purification of lectins. The chromatographic technique can also be used for the isolation and puriilcation of N-linked oligosaccharides.

**Table 3: Membrane glycoproteins purified on immobilized lectins**

| Glycoprotein | Source | Lectin used |
|---|---|---|
| Asialoglycocalicin | Sialidase-treatcd human platelets | Peanut |
| Asialoglycophorin | Sialidase-treated human erythrocytes | Peanut |
| Glycophorin | Human erythrocytes | Wheat germ |
| Human histocompatibility antigen | Lymphoblastoid cells | Lentil |
| Laminin | Mouse sarcoma | Griffonia simplicifolia I |
| Rhodopsin | Bovine retina | Concanavalin A |

- **Studies of cellular and sub cellular membranes:** Lectin receptors on cells are demonstrated by the use of radiolabelled lectins or fluorescent lectins. Quantitatively, the number of receptors can be estimated by microscopic methods using small molecules like territin and hemocyanin that can be used as conjugates coupled to lectin molecules. Intracellular binding sites can be visualized by using non-ionic detergents (Laurila *et ciL* 1978) or by staining of subcellular organelles (Brasitus *et al,* 19X2; Rittman and Mackenzie, 1983). The binding of lectins to surface receptors is affected by modification *oi^* the cell surface sugars by glycosidase, glyeosyl transferases, galactosc oxidases etc. Some studies also proved

the receptors to be glycolipids (Surolia *et al,* 1975; Curatolo *et al,* 1978). Lectins specific to one particular sugar bind to a specific sub cellular organelle depending upon the glycosylation steps in the maturation of N-linked oligosaccharide chains (Kornfeld and Kornfeld, 1985). Ex: mannose specific lectins bind specifically to the endoplasmic reticulum and to the proximal cisternae of the golgi appartus (Tartakoff and Vassalli, 1983). Lectins also bind to tissues and organs, such as the skin, muscle, kidney etc. Con A and WGA bind uniformly to the surfaces of rods and cone of photoreceptor cells (Peters and Goldstein, 1979). Lectins are increasingly employed in the investigations of membrane structure of intracellular pathways of protein glycosylation and *oi'* the changes that occur in cell glycoconjugates during differentiation, growth and development (Spicer and Schulte, 1992; Hwen, 1998). They are used to study the distribution of glycoconjugates in epithelial cells of normal human colon (Brinck *et al.,* 1998). In a recent development, lectins were also used in determining the functions of N-linked oligosaccharides (Stanley and loffe, 1995). Peanut agglutinin binds most exclusively to the CL)8 receptor of mouse and humans where the immunologically immature cells of the thymic cortex reside, but not to the medullary regions where the mature cells are present (Wu *et al.,* 1996). Based on the structural changes in the cell surface saccharides, lectins are being used to determine if they could act as reliable markers for normal and malignant cells (Boland *et al,* 1982). In lysosomal storage disease characterized by deficiencies in specific hydrolases that catabolise tissue constituents, undegraded substrates primarily glycoconjugates accumulate in patient's lysosomes. This accumulation can be revealed by lectin staining (Alroy *et*

*al,* 1994). MPA was reported to recognize a marker of breast cancer associated with both high metastatic potential and aggressive tumor behavior in young women (Dwek *et al.,* 2001). The same lectin was also found to be a prognostic indicator of the adenocarcinoma of the lung (Laack *et al.,* 2002).

- Cell separation: Lectins have the ability to separate viable cells having different carbohydrate specificities into sub populations (Sharon, 1983). This property can be used for the purification of glycoproteins and for the histoehemical characterization of tissues. By using this method, it is possible to get a high yield of the lectin reactive and the non-reactive cells. PI1A was first used for the separation of leukocytes from erythrocytes in human blood. Separation is carried out cither by selective agglutination, mixed rosetting, affinity chromatography or flow micronourimetry. In selective agglutination, the cell aggregates are separated from the unagglutinated cells by sedimentation at unit gravity in a viscuous medium and are then dissociated into single cells by suspension in a solution of sugar for which the lectin is specific (Reisncr *et* a/., 1976 a, b). Rosetting method is used when the number of cells is relatively low or when the lectin receptors on the cells are low. Lectins immobilized to solid support can also be used for the separation of cells (Hellstrom *et al.,* 1976). Murine thymocytes have been separated into mature and immature cells on a column of anti-peanut agglutinin - Sepharosc (Irle *et al.,* 1978). Flow micronourimetry is used to sort cells based on their size or the amount of fluorescent lectin bound to the cells (Parks and Herzenberg, 1984). Another example is the soybean agglutinin that is used for the fractionation of cells for bone marrow transplantation in humans. PNA was used to bind to terminally

differentiated lymphoid cells for purging bone marrow of plasma cells (Rhodes, 1998). Recently, GSL-1 served for the isolation of viable murinc eosinophils in good yields from the lavage fluid of the inflamed lung (Shinagawa and Anderson, 2000). Table 4 lists some of the plant lectins that have been used for cell separation.

**Table 4: Plant lectins used for cell separation**

| Source of lectins | Source of cells | Examples of cells separated |
|---|---|---|
| Dolichos biflorus | Human | Ai and O(H) erythrocytes |
| Griffonia simplicifolia J | M urine | Stimulated and resident macrophagcs |
| Helix pomatia | Human | Peripheral B and T lymphocytes |
| Limulus polyphemus | Murine | Spleen T helper cells |
| Peanut | Human | Cortical and medullary thymocytes, suppressor spleen T cells |
| Pokeweed | Murine | Granulocyte macrophage progenitor cells |
| Soybean | Human | Helper and suppressor lymphocytes |

- **Identification of microrganisms:** *Ncisscria gonorrhocac* can be differentiated from the other species by wheat germ agglutinin (Schaefcr *et al.,* 1979). *Bacillus anthracis* and *Bacillus mycoides* were agglutinated with soybean agglutinin. This is because lectins have been used for the identification *oi'* microorganisms from a primary isolate that confirms their identification (Doyle, 1994). They are selective to the pathogenic strains *o\^ Entamocba lustolytica* rather than the non-pathogenic

forms. Lectins were also shown to be useful probes for the detection and characterization of glycoconjugates in bacterially originating biofilms (Neu, 2001).

•> **Lectin resistant cells:** Lectins act as agents in the selection of cell variants with altered cell surface carbohydrates. Increase in lectin resistance correlates with decreased lectin binding ability, although some mutants have unaltered lectin binding capacity. In order that the cells become resistant to lectins, the surface oligosaccharides may be sterically hindered or the sugar residues may be deleted. Masking of receptors has been encountered in ricin resistant baby hamster kidney cells and mouse L cells (Stanley, 1980; Briles, 1982). These variants have more amounts of sialic acid residues and therefore, block the galactose residues that serve as ricin binding sites. A variant of the mouse myeloma cell line resistant to wheat germ agglutinin exhibits an increased sensitivity to the *Lotus tetragonolobus* lectin (Finne *et al.,* 1980, 1982). Deficiency of a particular sugar, specific for a lectin accounts for the resistance of these variants to the lectin. Lectin resistant variants, for example, Chinese hamster ovary cell line, have been used to characterize certain aspects of various virus-cell interactions and virus production (Briles, 1982). This cell line cannot be fused by Sendai virus.

* **Lectins as drug carriers:** Lectins can act as chemotherapeutic agents by binding to transformed cells. Example, conjugates of Con A - antitumor drugs such as duanomycin (Kitao and Hatton, 1977) had a higher activity against various cultured cell lines than the equivalent dose *o(* the free drug and lectin. Another example is the chimeric toxin consisting *oi'* Con A and the *a* chain of the Diphtheria toxin (Gilliland *et al.,* 1978). Doxorubin conjugated to mannose derivatised human serum

albumin was found to be effective in suppressing the growth of *Leishmania donovani* in infected peritoneal macrophages (Sett *et al.,* 1993)

- **Clinical uses:** Lectins distinguish erythrocytes of different blood types. Lectins are used in the rare blood type Cad (Cajal *et al.,* 1968). Cad erythrocytes are polyagglutinable by all ABO compatible sera except their own and can be distinguished from other forms of polyagglutination by their interaction with the lectin from *Sa/via hormimum* (Bird and Wingham, 1974). They are used in blood banks as an aid in blood typing (Malsui *et al.,* 2001). They are also used in the differential diagnosis of polyagglutination that accompanies certain bacterial and viral infections (Beck, 2000). Table 5 lists some of the blood group specific lectins.

**Table 5: Blood type specific lectins**

| Specificity | Source of lectin |
|---|---|
| **Anti** - A | *Griffonia simplicifolia* 1(A4), *Helix pomatia, Phaseolus lunatus* |
| Anti - A i | *Dolichos hiflorus* |
| Anti-B | *Griffonia simplicifolia* 1 (B4), *Anguilla anguilla* |
| Anti - **0(11)** | *Anguilla anguilla, Lotus tetragonolohus, Ulex europeus* |
| Anti - N | *Vicia graminea* |
| Anti -A+ N | *Mollucella laevis* |
| Anti-A+B | *Saphora japoniea* |
| Anti - T | *Arachis hypogea* |
| Anti - Tn | *Sal via set area* |
| Rh antigen | *Phaseolus vulgaris* **E4PHA** |

Lectins also help in the detection of fetal neural tube defects and other malformations. Mitogenic lectins are also used to monitor the effects of various immunosuppressive and immunotherapeiitie manipulations and in the diagnosis of genetic diseases with chromosomal defects (Oppenheim *et al.,* 1975). Lectins are used in bone marrow transplantations. Patients with leukemia (O'Reilly *et al.,* 1985; Aversa, 1998) or with severe combined immunodeficiencies (Friedriek *et al.,* 1984; Myers, 2002)) have been successfully transplanted with bone marrow, depleted of T cells, by agglutination with soybean agglutinin. These transplanted cells were capable of reconstituing durable hematopoietic and lymphoid function in the recipients. In another approach, a ricin linked anti-CD45 antibody has been recently shown to kill CD4+ T cells latently infected with HIV in the blood of the HIV positive patients (Saavedra Lozano *et al.,* 2002).

•> **Nutritional significance:** Earlier, it was thought that some legume seeds were toxic to humans. With the growth of science, it was found that the lectins which were considered to be toxic long ago were now treated as nutritive. This impact had its effect only after cooking the plant material, where the heat produced denatures the toxic material. Lectins are widely distributed in the food items commonly consumed by man like the fruits, spices, cereals and roasted nuts. The hemagglutinins found in *Phaseolus vulgaris* are responsible for the toxicity of the raw bean. This toxicity could inhibit the growth of the organism (Jaffa, 1955). Some of the lectins cause reduced growth, diarrhoea and interference with nutrient absorption. H.\: the bright red seeds *o( Abrus prccatorms* contain abrin that is a toxic glycoprotein. Several lectins are resistant to proteolytic digestion, for

example, the WGA. Similar agglutinins have been reported in castor bean, jack bean and other edible plants. Pathological lesions occur in animals infected with red kidney bean extracts. Parenchymatous fatty degeneration, edema, necrosis, hemorrhages, goitrogenesis are some of the effects. Research done by Ehrlich, considered to be the Father of Immunology, has shown that feeding small amounts of lectin containing seeds to rabbits caused partial immunity to the toxicity, demonstrating lectins are antigenic.

**3 D structures of some legume lectins.**

Among lectins, the two most important proteins Concanavalin A (Reeke et al.. 1971) and wheat germ agglulinin. (Wright, 1977) were the first to be studied by X-ray crystallography, among which ConA belongs to the legume lectin family. The primary structural analyses and X-ray crystallographic studies report the structural similarities of the legume lectins. X-ray studies have shown that the tertiary fold of the polypeptide chains is the same in legume lectins. In the region of the carbohydrate-binding sites, significant differences in the primary and tertiary structures have been found to occur in one *of* the four loops that interact with the carbohydrate. The legume lectins have a number of other interesting attributes. Some undergo post, translational modifications thereby show evidence of close relatedness by circular permutation. In order to express their agglutination property, they form dimers and tetramers, but despite their relatively close homology, more than one kind of quaternary structure has been observed.

The quaternary structure *of* DIU, shows that the two dimers associate to form a tetramer. One dimer is formed from intact subunits and the other is formed from

truncated ones. The (3 sheets of each dimer interact forming a channel between the dimers which harbors adenine binding site of the lectin (1 Iamelryck *et al.,* 1999).

FRIL (FU3 receptor interacting lectin) is a glucose/mannose specific lectin isolated from the seeds *ofDo/ichos lahlah* (Ilyacinlh bean) (Colucci *et a*\, 1999). This lectin has 78% homology with another glucose/mannose specific lectin isolated from a different *Doliehos lahlah* cultivar (Gowda *et al.,* 1994). It has 52% identity with Con A and 50% with *Saphora japoniea* bark lectin (Van damme *et al.,* 1998). This lectin was crystallized in the presence of the tnsaccharide Man (al-3) [Man (al-6)| Man (al-o-Me) M3M6M. The monomer resembles a typical legume lectin and the quaternary structure resembles that of Con A. The two distinct sheets on the back \\ sheet ofthe subunit are invoked in dimer-dimer contacts including (3 strands 4 and 5 (Mis 181-Ser 200) and a cluster of residues around the C terminus (Val 64, Val 65. Trp 246 and Asn 248). The same regions in Con A are involved in intei-dimer contacts, but with additional residues. This makes the total buried surface lower for Con A than for FRIL. There are only two ionisable residues present at the interface in FRIL (His 181. Argl90) compared to 7 in ConA. These crystals were obtained by hanging drop method at room temperature in PFd 8K. pH 6.5. The crystals were hexagonal and diffracted to a resolution of 3.5 °A (llamelryck *et al.*. 2000).

DB58. the stem and the leaf lectin of *Doliehos hiflorus* is a noncanonical dimer (Dao-Thi *et al.,* 1998). Its subunit consists *ol*~ 253 residues that is truncated between 241-242 and associates with their Hat six-strnmled $\beta^1$ sheets facing each other. The C terminal

part of the intact subunit forms a-helix which is sandwiched between p sheets of the facing monomers.

PNA has a unique quaternary structure (Banerjce *et al.,* 1996) which does not possess either 4-fold or 222 symmetry. It has been shown thai the primary structure of legume lectins has an influence on its quaternary structure (Prabu *et al.,* 1999). The N terminal strands are intercalated by a series of six water bridges leading to lack of symmetry in the tetramer.

The structure *o\' Rohinia pseudoacacia* bark lectin has been isolated in the free form (1.80 °A) and eoinplexed with N-acetyl galaclosamine (2.05 A) (Rabijns *et al.,* 2001). The polypeptide chains *oi'* the two structures exhibit the characteristic legume lectin tertiary fold. The quaternary structure resembles that of the *Phaseolus vulgaris* lectin, the Soybean lectin and the *Doliehos hijlonis* lectin, but displays some unique features leading to the extreme stability of this lectin.

## Scope of the present investigation

Research in the laboratory where this work has been carried out focuses on the development of new affinity methods for the large scale isolation, purification and characterization of lectins from plants and animals. Among the plants emphasis is on the legume lectins. Among animals, emphasis is on establishing the evolution of Mannose 6- phosphate receptor proteins that mediate transport of lysosomal enzymes to lysosomes in eukaryotes. Several new lectins with different sugar specificities from plants and animals have been purified employing new affinity methods developed. In addition, work is being carried out on the glycosidascs such as a - mannosidasc, fucosidase and hexosaminidases. The long term objective is to understand the structure - biological activity and the functions of these proteins.

The genus *Dolichos* (family: Leguminoseae) consists of a large number of plants among which the *Dolichos lablab* var *typicus* (lablab bean) and *Dolichos lablab* var *lignosis* (field bean) are widely grown in South India. The food form of the field bean is largely used as a seed whereas that of the lablab bean is used as a vegetable. Both these varieties contain identical glucose / mannose specific seed lectins that have been affinity purified. They also exhibit similar physiochemical and biological properties. The primary structure of the field bean lectin has been completely elucidated (Gowda *et al.,* 1994).

The lablab bean seed extracts in addition to containing the glucose/mannose specific lectin also contains a second lectin that is specific to galactose. This was isolated in our laboratory by conventional methods of protein purification. This protein had a native mass of 120 kDa and it cross-reacted to the antibody raised to the well characterized

glucose / mannose specific lectin. This lectin failed to bind on the Sepharose -galactose affinity gel and hence, was thought to be an unusual protein. This lectin was also found to be present in the stems and leaves of the same plant. An ex - mannosidase enzyme has also been purified from these seeds.

The objectives of the present study were

a. To develop an affinity matrix for the large scale purification of this galactose specific lectin from a variety of *Dolichos lablab* seeds (Wipro Hybrid seeds company, Hyderabad, Lot No: K.R. 306, Fig. 2). Further, a systematic study was undertaken to characterize its biological and immunological properties. Extensive chemical modifications have been carried out to substantiate the role of amino acids involved in lectin binding.

b. To purify the [3 N - acetyl hexosaminidase by affinity chromatography from the lablab beans and to biochemically characterize the same. Further, it was envisaged to identify the site of the lectins and the enzyme within the protein bodies in the seeds.

c. To determine the possible functions of the different lectins purified in our laboratory. Viz., glucose / mannose specific lectin and the galactose specific lectin, the lactose specific lectin from *unio* species and the wheat germ lectin. Their antifungal nature, mitogenicity to the lymphocytes and their ability to act as tracers in detecting the myeloma cells has been analyzed. Purified lectins immobilized to affigels were used as tools to understand their functions.

d. To obtain a partial cDNA sequence for the galactose lectin (Since both the lectins ulucosc/mannose and ulaactosc specific lectins from the *Dolichos lablab*

seeds were found to be related immunologically) and to compare its sequence to the glucose/mannose lectin and other known legume lectins.

e. To crystallize the galactose specific lectin.

*Dolichos lablab*

Family: Leguminosae

FIG : 2

# Chapter 2

# Affinity purification and characterization of galactose specific seed lectin from the seeds of *Dolichos lablab*

## Introduction

Lectins constitute a very large group of glycoproteins that agglutinate cells and/or precipitate glycoproteins. The carbohydrate specificity of a given lectin is established by the method of fixation - site saturation in which different sugars are tested for **their** ability to inhibit agglutination or precipitation of polysaccharides and/or glycoconjugatcs by the lectin. Since the 1880's, it has been known that the seed extracts of certain plants could agglutinate red blood cells. Since then, research work was focused on the large scale isolation and characterization of lectins. The choice of the purification of lectins is largely by affinity chromatography employing biospecific adsorbents containing covalently bound carbohydrates. The ready availability of the pure lectins from different plant sources (predominantly from legume seeds) laid the foundation to study their structure - function relationships. This allowed understanding the evolutionary characteristics of these lectins. In addition the legume seeds have also been known to contain proteins designated as lectin receptor proteins that bind to the constitutive lectins. The first definitive work on lectin binding proteins was reported from the seeds of jack bean and lava bean (Gansera *et al.,* 1979). Bowles and Marcus (1981) identified lectin receptors from seed extracts of soybeans and jack beans. Lectins have also been obtained from lentil and pea which have been shown to be potent mitogens for lymphocytes (Kummer and Ruediger, 1988).

Legume lectins are the most thoroughly studied proteins. Most of these lectins contain usually one type of lectin exhibiting 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 plant lectins and animal lectins (Rajasekhar, 1997 and 1998; Siva Kumar, 2002) 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. Both contain identical glucose/mannose specific lectins (Siva Kumar, and Rajagopal Rao, 1986). 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 kDa 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 (Rao *et al.,* 1976 Guran, *et al.,* 1983., Favero, *et al.,* 1988; 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 *et al.,* 1998). The lectin exhibited a native molecular mass *of* 120 kDa and is possibly a

tetramer. An antibody to the glucose/mannose lectin was found to cross-react with this galactose lectin. 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 order to develop an efficient affinity method for the large scale isolation and purification of the galactose lectin, in the present study we have used a particular variety of the seed material *(Dolichos lablab).* The objectives were to

(i) affinity purify the galactose specific lectin from the seeds of *Dolichos lablab* to homogeneity using an affinity matrix, Sepharose-divinyl sulfone-galactose.

(ii) study its biochemical properties, such as temperature stability, amino acid and carbohydrate content.

(iii) identify the amino acids involved in lectin binding by chemical modification studies

(iv) *in vitro* translation studies of lablab bean mRNA in wheat germ lysate to know whether it is synthesized as a precursor protein.

**Materials**

Seeds of *Dolichos lablab* (Indian lablab beans) were obtained from the local market (Wipro Hybrid seeds company, Hyderabad, Lot No: K.R. 306). Sepharose 6B, galactose, divinyl sulfone (DVS), bicinchoninic acid and other chemical modification reagents were all procured from Sigma Chem. Co., USA. Con A-Sepharose gel was obtained from Pharmacia (Sweden). $^{35}S$ methionine was obtained from BRIT, BARC, India. All the chemicals and the reagents used in the present study were of high purity and obtained from reputed firms. Wheat germ lysate was kindly provided by Prof. K.V.A. Ramaiah, Department of Biochemistry, University of Hyderabad.

**Methods**

**Preparation of Sepharose- Divinyl sulfone - Galactose affinity gel**

Our laboratory has already standardized the preparation of the Sepharose-mannose gel for the purification of the glucose/mannose specific lectin from the *Dolichos lablab* seeds. For the preparation of the Sepharose-galactose gel, briefly, 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:CO; pH 11. 2.5 mL of DVS was added and rotated for 70 minutes at room temperature followed by washing with double distilled water. It was later washed with 0.5 M $Na_2CO_3$ pH 10. Then, 5 g of galactose (20% w/v) was added and the gel suspension was allowed to rotate for 72 hrs at 4 °C. The gel was washed with water and suspended in 0.5 M Nai ICCh pH 8.5. To this, 0.5 mL of [3-mercaptoethanol was added to block the unrcactive sites and gel was incubated for three hours al room temperature. Finally, the gel was washed with double distilled water and suspended in TBS until further use.

**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 (10,000 rpm at 4 °C) 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 as assessed by the sugar inhibition studies. The fraction containing the galactose lectin was dialyzed against TBS and passed through Scpharose-mannose gel to deplete the fraction of any glucose/mannose lectin. To the unbound protein from the Sepharose-tnannose gel 1.5 M ammonium Sulfate was added 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 Aigo 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.

**Hcmagglutination** assay

This was carried out according to Siva Kumar and Rajagopal Rao (1986). Rabbit blood was collected by ear vein puncture, into Alsevier's solution containing 2.05% glucose, 0.89% sodium citrate, 0.42% sodium chloride and 0.05% citric acid. It was centrifuged at 3000 rpm for 10 minutes at 4 °C. The sedimented crythrocyte 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 eentrifuged at 3000 rpm for 10 minutes at 4 °C and then made up to their original volume. To 200 JIL of the protein sample serially diluted in 200 j.iL of saline, 200 ^L 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.

**Sugar inhibition studies**

Hemagglutination inhibition assays with the purified lectin were performed as follows: 45 ,uL of different sugar solutions (0.1 M of Galactose, GalNAc, Galactosamine, 2-DeoxyGal, Lactose) were placed in the plate and serially diluted. Then, 10 ,ul, of the purified lectin (1 mg/ml) was added to each well. Later, 45 ,uL of 4 % erythrocyte suspension was added and the plate was incubated for 1 hour at 37 °C. Hemagglutination inhibition titer was scored visually.

**Chromatography on Con A- Sepharosc gel**

Con A-Sepharose gel (0.5 mL) was equilibrated with IBS pH 7.4 containing 5 mM CaCL and 5 mM MnCL (column buffer). 1 mg/mL of the purified lectin was applied on Con A-Sepharose and the gel washed with column buffer till the A^o was zero. The bound lectin was elutcd using 0.15 M Methyl u-mannosidc in column buffer.

**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 vi/.., lactose specific lectin from *unio* (Mr 120 kDa), peanut agglutinin (Mr 1 10 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.

**Protein estimation by Bicinchoninic acid** (BCA) **method**

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

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

Aliquots of the protein solution in water was taken and the volume was made upto 500 µL with distilled water and mixed with 1.0 mL of BCA reagent and incubated for 30 minutes at 37 $^{0}$C. After incubation the absorbance was measured at 562 nm.

**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.

**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. The samples were brought back to room temperature and their binding ability on Sepharose-galactose gel was determined.

**Native -Polyacrylamide gel electrophoresis**

Native PAGF. was performed using Tris - glycine buffer pH 8.3 on a 7.5% gel (Reisfeld *et al.,* 1962).

**Sodium dodecyl sulphate -Poly Acryiamidc Gel Electrophoresis:**

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

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

2x Sample buffer: 0.5M Tris-HCl pH 6.8 2.5 mL, 10% SDS 4.0 mL, (reducing) 100% glycerol 2.0 mL, (3-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-1IC1 pi I 6.8

Acryiamidc: 30% (w/v) Acryiamidc 0.8%. (w/v) N N' methylencbisacrylamide

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

Resolving gel and stacking gels are made as shown in the Tables 6 and 7, respectively. APS and THMED 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 °C 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 |
| Acryiamidc (mL) | 1.875 | 2.500 | 3.125 |
| **10% SDS** (uX) | **75** | **75** | 75 |
| APS (uL) | **40** | **40** | 40 |
| TEMED (uL) | **10** | **10** | **10** |

**Table 6: Reagents used for the preparation of resolving gel with different polyacrylamide concentration.**

| **Reagents** | **Volume** |
|---|---|
| 11:0 (mL) | 1.875 |
| Acryiamidc (mL) | 0.375 |
| Stacking gel buffer (mL) | 0.3125 |
| 10% SDS (^L) | 25 |
| APS (uL) | 40 |

**Table 7: Reagents used for the stacking gel**

**Silver Staining of the gel**

This was carried out according to Blum *et al.,* (1ᶜ^7).

Reagents: Methanol, Ethanol, AgNO, Hypo, Na.CCK Formaldehyde and Acetic acid.

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

1. Fixative: 50% methanol, 12% glacial acetic acid, 50 *[\L* of formaldehyde (MCHO) were taken in a flask and the volume made up to 50 mL with double distilled water. After the electrophoresis, 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 AgNCh was dissolved in 50 mL of double distilled water. To this 18 juL 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^CO^ was dissolved in 50 mL of distilled water. To this 26 *yiL* 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.

**Periodic 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 ni,* (1969). Schiffs reagent was prepared as follows: 1 g 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 11C1 and 2 g of potassium metabisulphite were added and incubated overnight. The solution turns colorless or pale straw yellow. To decolourise completely 0.25 g to 0.5 g of activated charcoal was added and filtered. This stain solution is stored in a stoppered brown bottle at 4 °C. Purified lectin was separated by SDS-PAGE and the gel was incubated with 1% periodic acid in 3% acetic acid for one hour. It 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.

**Preparation of the antisera to the lectin**

The purified protein (500 ug in 0.5 mL TBS) was emulsified with an equal volume of Freund's complete adjuvant and injected subcutaneously into a rabbit. At subsequent intervals of 3 and 5 weeks, a booster dose of the protein in Freund's incomplete adjuvant was given. After a week of the booster injection, blood was collected from the ear vein, allowed to clot and the serum was collected by centrifugation.

**Iinmunodiffusion**

The specificity of the lectin was tested by Ouchterlony's double immunodiffusion technique against antisera obtained for the affinity purified protein. Immunodiffusion was carried out on 1 % auar (in PBS) plates lor 24-48 hours at 4 °C and visualized for

precipitin arcs (Oucterlony, 1948). Antiserum was placed in the central well and the **glucose/mannose** specific lectin and galactose specific lectin were placed in the outer well.

**Western blot analysis:**

This was carried out according to Towbin *et al.,* (1979). 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 to 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 BC1P/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.

The cross-reactivity of the galactose specific lectin to the antiserum of the glucose/mannose specific lectin was checked by Western blot analysis. Similarly, the cross -reactivity of the antisera of the galactose specific lectin to the glucose/mannose specific lectin (this lectin was purified following the method of Siva Kumar and Rajagopal Rao, 1986), was also tested.

**Amino acid analysis**

Purified lectin (5 mg) was hydrolyzed with 6 N HC1 for 24 hours at 110 °C (Siva Kumar and Rajagopal Rao, 1986). Following hydrolysis, the sample was analysed on a Beckman 119 CL automatic amino acid analyzer. The analysis was performed according to the manufacturer's instructions.

The separated subunits of the protein were identified on SDS-PAGE and the corresponding bands were transferred to PVDF membrane. The membrane was stained with coomassie blue and destained. The two subunit bands were cut separately and subjected to partial amino acid sequencing on a 477 A pulsed liquid-phase protein/peptide sequencer (Applied Hiosystems) and standard protocols according to manufacturer's instructions.

**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 below. 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, therefore 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 ofLysine residues*

3 mg/mL of lectin in 0.05 M borate buffer pH 9.5 was modified using 400 molar excess of Citraconic anhydride (Dixon and Perham, 1968) for 1 hour at 4 °C. Similar reaction was carried out with the lectin incubated with 0.1 M galactose. The extent of modification was estimated by using 2, 4, 6-tri nitro benzene sulphonic acid (TNBS) (Habeeb, 1966).

*Modification of arginine residues*

This was carried out according to the method of Patthy and Smith (1975) by the addition of 0.05 M 1, 2 cyclohexanedione to 3 mg/mL lectin in 0.2 M borate buffer pH 9.0. The reaction tube was flushed with nitrogen and kept at 37 °C for 4 hours. The reaction was terminated by the addition of 5% acetic acid. Extent of modification was not determined.

*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 uL of DHPC (10 fiL of DEPC diluted to 600 |.iL 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 modi (led 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.

*Modification of tyrosine residues*

This was done according to Riordan *et a/.,* (1965). Acelylation of tyrosine side chain phenoxy groups was done at room temperature by incubating the lectin in 25 mM Tris-HCl buffer pH 7.5 with 60 fold molar excess N-acetyl imidazole for an hour. Its absorbance was measured at 278 nm and 280 nm.

*Modification of tryptophan residues*

Tryptophan residues were modified using 2-hydroxy 5-nitro benzylbromide (Horton and Koshland, 1972). To 3 mg/mL lectin in 0.1 M sodium acetate buffer pH 3.5, a 60 fold molar excess of the reagent in dry acetone was added and stirred for 1 hour at room temperature. The degree of modification was calculated according to Spandc and Witkop(1967).

**Preparation of poly $(A)^+$ mRNA on oligo (clT) cellulose column**

15 gm of dried seed flour was homogenized in 100 mL of aqueous SDS buffer (150 mM NaCl, 5 mM EDTA, 10 mM sodium acetate pH 8.0 and 8%SDS) and 100 mL of organic buffer (chloroform: isopentyl alcohol: phenol - 50:1:50) for 3 minutes (llemperly *et al.,* 1982). The mixture was allowed to stir for 10 minutes at room temperature and the phases were separated by centrifugation. The organic phase was re-extracted with aqueous buffer and the aqueous phases were combined. To this clear aqueous phase 3 volumes of absolute alcohol was added and left at -20 °C overnight to precipitate the nucleic acids. The precipitated nucleic acids were dissolved in KNase free water and the total RNA was precipitated by placing the sample in 3 M LiCl and 0.2 M NaCl. The precipitate was washed twice with ethanol. Oligo (dT) cellulose column was equilibrated with 0.1 M NaOll, 1 M Tris-HCl buffer pH 7.5 and then with

high salt SDS buffer containing 10 mM Tris-HCl buffer pH 7.5, 0.5 M NaCl and 0.5% SDS. Total RNA in high salt SDS buffer was applied on the gel. The column was first washed with high salt SDS buffer and then with the same buffer without SDS. mRNA was eluted with DEFC water. To this, 0.1 M NaCl and 2.5 volumes of chilled ethanol were added and the tubes were placed at -20 °C. mRNA that was precipitated was collected by centrifugation and the pellet was washed in absolute ethanol twice. It was finally suspended in DEPC water. The $A_{260}/A_{280}$ ratio was checked.

***In vitro* translation using wheat germ lysate**

*In vitro* translation was carried out for a 25 uL reaction by adding the following components serially : 2 JJL DEPC water, 5 uT. 5X translation mix (mixture of all amino acids except methionine, 0.5 mM GTP, 150 |Lig/mL Spermine, 40 mM Creatine Phosphate, 350 j-ig/mL Creatine phosho kinase and 70 mM Hepes pH 7.6), 2.5 pL 1 M Pot.acetate, 1 uL 25 mM Magnesium acetate, 1 |aci/uL $^{35}$S methionine, 20 units RNasin, 10 ^iL wheat germ lysate, 2 uL mRNA. The sample was incubated at 25 °C - 27 °C for 60 minutes and the reaction was finally terminated by the addition of 2X sample buffer. 12% SDS-PAGE analysis was done and the translated product was analysed by a Western blot analysis using an antibody that was raised for the affinity purified galactose lectin.

**Results**

In the present study when the 60%-80% ammonium sulphate fractionated extract containing the galactose lectin was passed through the Sepharose-galactose gel equilibrated with 25 mM Tris-HCl buffer pH 7.4, in presence of high salt 1.5 M ammonium Sulfate, at 4 °C this lectin was bound on the gel. It was completely desorbed with 0.3 M galactose in the same buffer as shown in Fig. 3. However, in the absence of high salt there was no binding of the lectin to this matrix. Table 8 shows the purification of the galactose specific lectin from 100 g of seeds.

The lectin agglutinated trypsin treated rabbit erythrocytes. The results of the sugar inhibition data are given in Table 9. From the results it is apparent that GalNAc is two fold weaker and 2 deoxy galactose 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. Substitution at the second position 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. Disaccharide, lactose (Galβ1M-4Glc) was less inhibitory compared to galactose.

The lectin eluted as a single peak from Scphadex G-200 (Fig. 4) suggesting it to be homogeneous. The native molecular mass of the lectin was found to be 120 ± 5 kDa as determined by gel filtration employing proteins of known molecular weights for calibration. The purified lectin moved as a single band in native gel electrophoresis as shown in Fiu. 5. From 100 g of the seed powder 175 mg of purified lectin was

obtained. Purified lectin contained about 5% carbohydrate as estimated colorimetrically by the phenol-sulphuric acid method.

The glycoprotein nature of the lectin was further determined by its ability to bind on Con A-Sepharose gel and its specific elution using 0.15 M methyl-mannoside sugar (Fig. 6). In a typical experiment 0.45 mg protein could be specifically bound to 100 uL of gel.

In SDS-PAGE the lectin dissociated into two subunits with molecular masses of 31 kDa and 29 kDa, respectively (Fig. 7 A). Both the bands stained positive for carbohydrate on periodic acid sell if fs staining as shown in Fig 7 B.

Antibodies to the purified galactose specific lectin were raised as described under methods. The specificity of the antibodies was tested in Western blot experiment, as well as by immunodiffusion. In Western blot experiment, both subunits of the galactose specific lectin reacted with the antisera suggesting the specificity of the antibody and the subunit nature of the protein (Fig. 8 A). In an immunodiffusion experiment, this antibody formed precipitin arcs to the glucose/mannose specific lectin and to the galactose specific lectin (Fig. 8 B). This cross-reactivity was further confirmed by Western blot analysis where both the subunits of the glucose/mannose specific lectin were recognized by the galactose specific lectin antiserum (Fig. 8 C) and the both the subunits of the galactose specific lectin were recognized by the glucose/mannose specific lectin antiserum (Fig.8 D).

The purified lectin showed complete binding to the affinity gel at room temperature suggesting it to be stable upto 40 "C. Beyond 40 T, the stability of the purified lectin decreased and at 90 "C the lectin had negligible activity (Fig. 9). The amino acid

sequence of the first 10 residues in both the subunits as determined by an automatic protein sequenator revealed that the sequences in both subunits are identical (Fig. 10). Amino acid analysis of the purified lectin showed high content of acidic and hydrophobic amino acids (Table 10). Methionine and half cystine could not be **detected.**

**In** the present study various chemical modifications of the purified galactose specific lectin were earned 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 the affinity gel were assessed. The results of the various modification studies have been summarized in Table **1** 1.

***Modification of lysine residues:*** Citraconylation of the lectin resulted in modification of 12 residues in the absence of the sugar and 4 residues in the presence of the sugar. Modification in the absence of the sugar resulted in decreased binding on Con A-Sepharose gel by 31% and its agglutinating activity by 43%. Protection of the lectin binding sites with galactose showed only 4 residues to be modified. However, the Con A-Sepharose binding ability and hemagglutinating activity decreased by 15 % and 10% respectively.

***Modification of histidine residues.*** 15 histidine residues out of 18 were modified in the absence of 0.1 M galactose. Modified lectin showed 75% loss in hemagglutinating activity and about 70% loss in its binding ability to Con A-Scpharose gel. Reversal of modification was achieved by incubating the modified sample at 37 °C for 2 hours.

This reversed 5 out of 15 histidine residues. However, modification performed in presence of inhibitory sugar 0.1 M galactose, revealed thai only 7 histidine residues were modified (Fig. 11 A) suggesting that the binding sugar protected the active site of the lectin.

***Modification of tyrosine residues.*** Modification with N-acetyl imidazole led to the acetylation of all the tyrosine residues. There was a decrease in biological activity by 20% upon tyrosine modification. In the presence of 0.1 M galactose, there was no significant protection observed.

***Modification of tryptophan and arginine residues:*** Modification of tryptophan and arginine residues with 2-hydroxy 5-nitro benzyl bromide and 1,2 cyclohexane dionc, respectively, did not alter the biological activity of the lectin, as well as its binding ability to Con A- Sepharose gel

The histidine (Fig. 1 1 B) and lysine modified (Fig. 11 C) samples were also subjected to Oucterlony's immunodiffusion using the unmodified sample as the control. Precipitin arcs have been observed in all the cases.

The protocol followed for the isolation of RNA has yielded mRNA with A260/A2S0 ratio of 1.4. Translation of the oligo (dT) purified lablab bean mRNA in a wheat germ derived system (this is a good translating system and is routinely used to translate different mRNA's) in the presence of ^ S methionine followed by analysis of the translated products by SDS-PAGF and Western blot transfer with the antiserum against the whole purified protein yielded a single polypeptide chain of molecular mass of 33 kDa (Fig. 12).

**Figure 3: Affinity purification of the galactose specific lectin**

To the unbound fraction from Sepharose - DVS - mannose gel, 1.5 M ammonium sulphate was added and applied on Sepharose - DVS - galactose column equilibrated with TBS and 1.5 M ammonium sulphate at a flow rate of 20 mL/hr. 0.3 M galactose was used for elution. 5 mL fractions were collected and their absorbance was measured at 280 nm.

**Table 8: Purification of the galactose specific lectin from the seeds of *Dolichos lablab*. (lOOg of the seed flour was used in the study)**

Elution profile of the lectin on Sepharose-DVS-Galactose columr

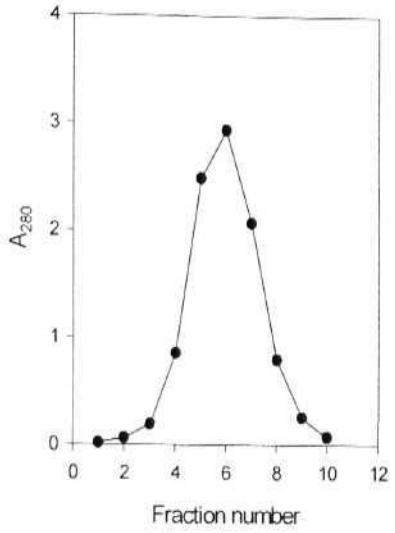



**Figure 3**

| S.No | Step | Total Protein (mg) | Total activity (HU)[@] | Specific activity (units/mg) | Purification fold |
|---|---|---|---|---|---|
| 1. | Crude | 13500 | 30600 | 2.2 | 1 |
| 2. | (*) 60 -80% $(NH_4)_2SO_4$ fraction(galactose-lectin) | 3690 | 16400 | 4.4 | 2 |
| 3. | Sepharose mannose gel unbound | 1750 | 14000 | 8 | 3.6 |
| 4. | Sepharose galactose gel eluate | 175 | 12000 | 68 | 31 |

(*) The 0-60 % $(NH_4)_2SO_4$ fraction representing the glucose/mannose lectin was processed on Sepharose -mannose gel for the purification of the glucose/ mannose specific lectin. [@]One HU is defined as the amount of protein required to cause visible agglutination using rabbit erythrocytes.

Table 8

Table 9: Inhibition of agglutinating activity of *Dolichos lablab* seed lectin by galactose and its derivatives

| Sugar | Minimum Concentration for Inhibition ( mM ) | Relative inhibitory potency (galactose = 1.0) |
|---|---|---|
| Galactose | 2.81 | 1.0 |
| GalNAc | 5.63 | 0.5 |
| Galactosamine | 22.5 | 0.12 |
| 2-DeoxyGal | 45.0 | 0.06 |
| Lactose | 5.63 | 0.5 |

All the sugars used were of'D configuration.

Table 9

**Figure 4: Molecular weight determination of the galactose specific seed lectin.**

The affinity purified lectin eluted from the Sepharosc-galactose gel (-10 A2X0 units) was applied on Sephadex G-200 (60 cm x 1.2 cm) gel filtration column equilibrated with 0.9% NaCl. 2.0 mL fractions were collected. Inset 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 that clutes at the same elution volume as the lactose specific lectin from *unio.*

**Figure 5: Native page pattern of the galactose specific lectin**

Arrow indicates the position of the pure protein.

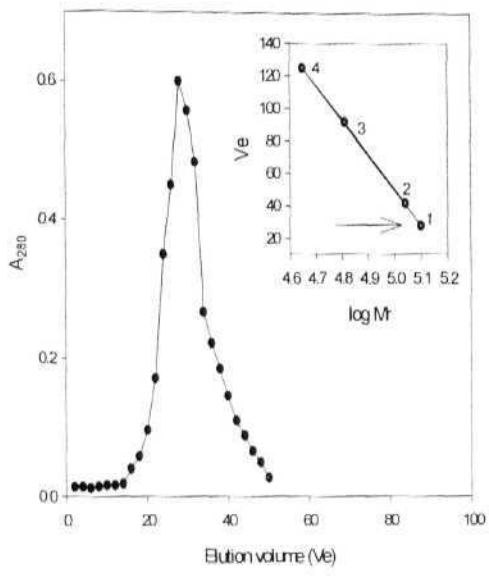



**Figure 4**

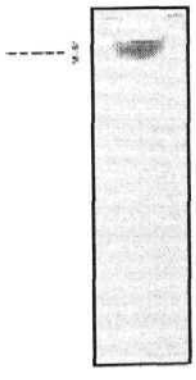


**Figure 5**

**Figure 6: Binding of lectin on Con A - Sepharose gel:**

0.5 mg of galactose specific lectin was loaded on Con A - Sepharose gel (200µL) prc equilibrated with TBS. 0.3 M methyl **a** - mannoside was used for elution.

**Figure 7: A. 12% SDS-PAGE analysis of the galactose specific lectin**

Lanel: Molecular weight markers - bovine serum albumin (Mr 66 kDa), ovalbumin (Mr 45 kDa) and (3-lactoglobulin (Mr 18 kDa); Lane 3: Affinity purified lectin; lane 5: Sephadex G-200 eluatc and Lane 7: Con-A Sepharose eluate. Arrow indicates the position of the galactose specific lectin of larger subunit of molecular mass 31 kDa and the smaller subunit of molecular mass 29 kDa.

**B. Periodic acid Schiff s staining of the galactose specific lectin**

The two bands of the galactose specific lectin were detected by Periodic acid Schiff s reagent (PAS), which stains glycocompounds. Arrow indicates the position of the lectin at 31 kDa and 29 kDa.

Binding of the lectin on Con-A Sepharose gel

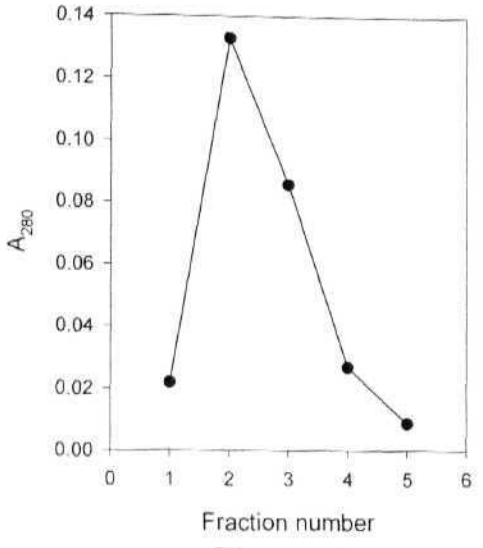



**Figure 6**

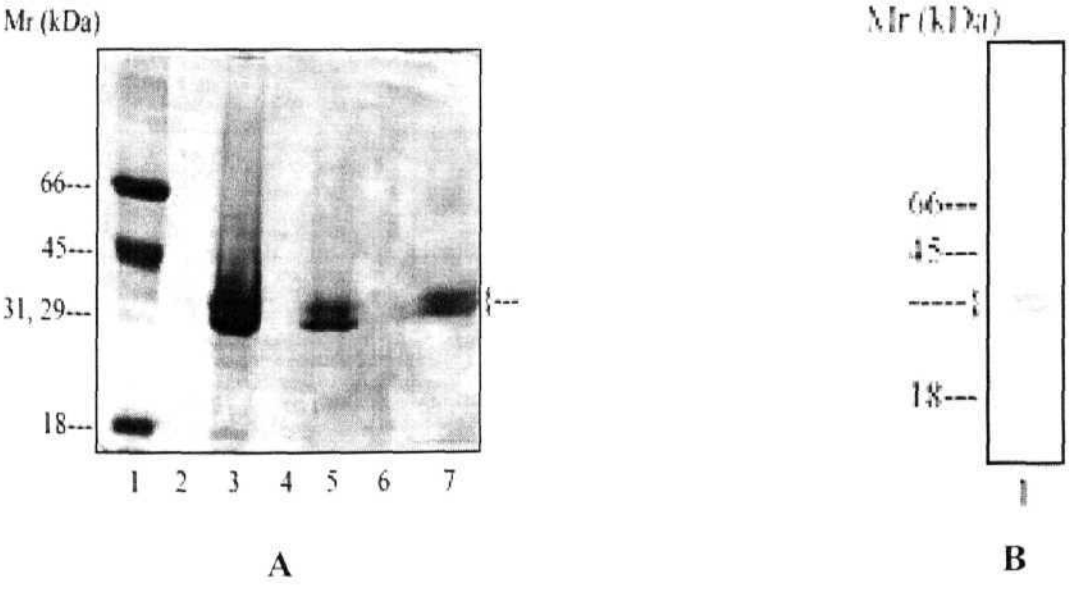



**Figure 7**

**Figure 8: A. Western blot analysis of the lectin.**

After transfer of proteins to the nitrocellulose membrane, it was probed with the galaetose specific 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 BC1P/NBT substrate. Lane 1 shows the molecular weight markers of 66 kDa, 45 kDa and 18 kDa. Lane 2 shows the purified lectin recognized by the antiscrum. Arrow indicates the position of the galaetose specific lectin.

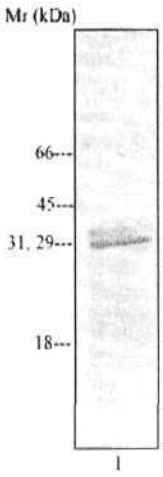
**B. Immunodiffusion of the purified galaetose specific lectin**

Well A contains the galaetose specific lectin; well B contains glucose/mannose specific lectin and well X contains the purified galaetose specific lectin atisera.

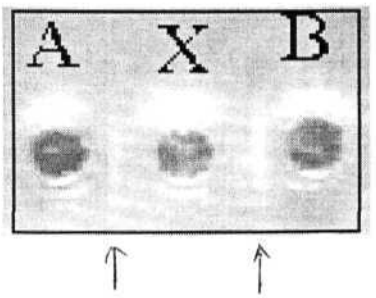
**C. Western blot analysis of the glucose/mannose specific lectin with the antisera for the galaetose specific lectin and**

**I). Galaetose specific lectin probed with the glucose/mannose specific lectin antisera.**

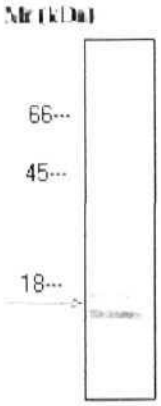
The purified lectins were transfen-cd to a nitrocellulose membrane and probed with tIr-respective antisera. The blots were developed with BCIP/NBT substrate. Arrow indicates the position of the (C) glucose/mannose specific lectin 15 kDa and 12 kDa and (D) Galaetose specific lectin (31 kDa and 29 kDa).



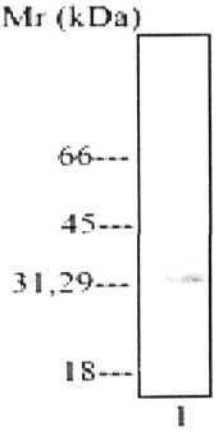

A




B




C




D

**Figure 8**

**Figure 9: Effect of temperature on the lectin activity**

**Figure 10: N-terminal sequence determination of the galactose specific lectin.**

The first 10 residues of both the subunits are identical and they were aligned to the N-terminal regions of other legume lectins. These legume lectins were *Dolichos lablab* (DLL (Man)), *Dolichos biflorus* (DBL), *Glycine max* (SBA) lectin. *Erythrina corallodendron* (ECO) lectin. *Pi sum sativwn* (PSL) lectin. *Vicia faba* (FBL) lectin. *Onbrychis viciafolia* (SL)1 ectin. *Lotus letragonolohus* (LTA) lectin. *Lens culinaris* (LCL) lectin. Identical residues are in bold face letters. FRIL is also aligned

Effect of temperature on the lectin

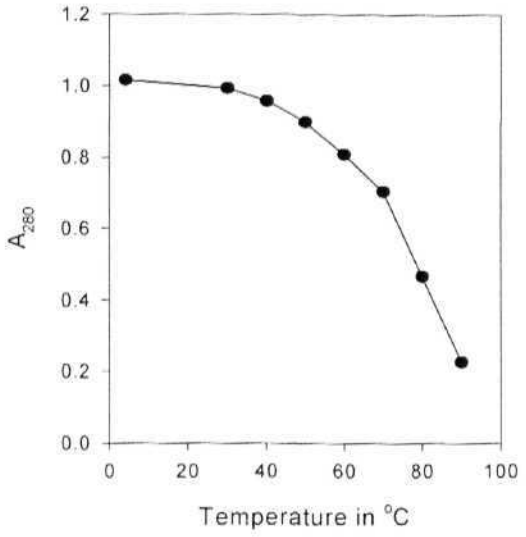



**Figure 9**

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| **DLL (Gal)** | N | **N** | L | **I** | **S** | **F** | T | M | **K** | R |
| **FRIL (Man)** | A | Q | S | L | **S** | **F** | S | F | T | K |
| DLL (Man) | A | Q | S | L | **S** | **F** | S | F | T | K |
| DBL | A | **N** | I | Q | **S** | **F** | S | F | **K** | N |
| SBA | A | E | T | V | **S** | **F** | S | W | N | K |
| ECO | V | E | T | **I** | **S** | **F** | S | F | S | E |
| PSL | T | E | T | T | **S** | **F** | L | I | T | K |
| FBL | T D | E | I | T | **S** | **F** | S | I | P | K |
| SL | A E | **N** | T | V | **S** | **F** | D | F | S | K |
| LTA | | | | V | **S** | **F** | X | Y | T | E |
| LCL | T | E | T | T | **S** | **F** | S | I | T | K |

Figure 10

**Table 10:** Amino acid composition of the purified galactosc specific lectin

| Amino acid | * Relative amino acid residues/mole |
|---|---|
| Asparatic acid | 11.0 |
| Threonine | 3.7 |
| Serine | 2.9 |
| Glutamic acid | 3.9 |
| Proline | 3.9 |
| Glycine | 6.6 |
| Alanine | 7.0 |
| Valine | 3.8 |
| Isoleucine | 4.4 |
| Leucine | 7.7 |
| Tyrosine | 1.5 |
| Phenylalanine | 5.1 |
| Lysine | 2.9 |
| Histidine | 1.8 |
| Arginine | 2.4 |
| Tryptophan | 1.0 |

* The relative amino acid composition has been calculated normalizing tryptophan value as 1.0. No corrections have been made for loss of serine and threonine during acid hydrolysis. Aspartic acid and glutamic acid include asparagine and glutamine, respectively. Methionine and half cystine could not be detected.

**Table 10**

Table 11: Chemical modification studies of the purified galactose lectin

| Serial No. | Reagent used | Residue modified | Number of residues modified | % binding ability on ConA - Sepharose | % of Hemagglutinating activity |
|---|---|---|---|---|---|
| 1. | DEPC[a] | Histidine | | | |
| | | - S[b] | 15 | 32% | 25% |
| | | Reversal | 10 | 43% | 43% |
| | | + S[c] | 7 | 75% | 100% |
| 2. | Citraconic anhydride | Lysine | | | |
| | | - S | 12 | 69% | 57% |
| | | + S | 4 | 85% | 90% |
| 3. | N-acetyl imidazole | Tyrosine | | | |
| | | - S | 15 | 81% | 50% |
| | | + S | 12 | 100% | 60% |
| 4. | 2-hydroxy 5-nitro benzyl bromide | Tryptophan | | | |
| | | - S | n.d.[e] | 100% | 100% |
| | | + S | n.d. | 100% | 100% |
| | NBS[d] | - Urea | 6 | 100% | n.d. |
| | | + Urea | 10 | 100% | n.d. |
| 5. | 1,2cyclo hexane dione | Arginine | | | |
| | | - S | n.d. | 100% | 100% |
| | | + S | n.d | 100% | 100% |

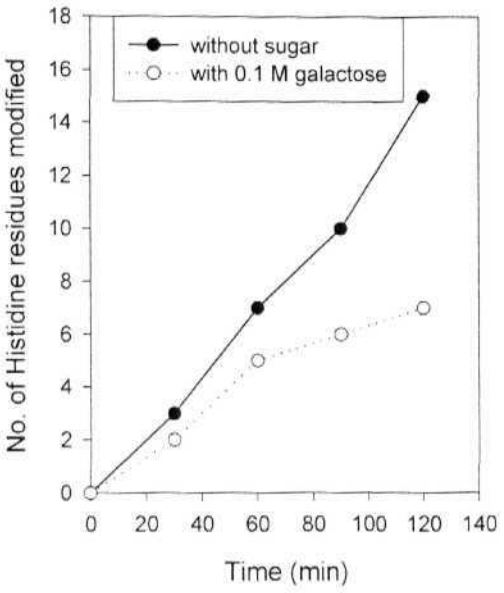
a - Diethyl Pyroearbonate, b - in the absence of galaetose sugar, c - in the presence of 0.1M galaetose sugar, d - N-bromosuccinimide, e - not determined. Results are the average values of two experiments.

**Table 11**

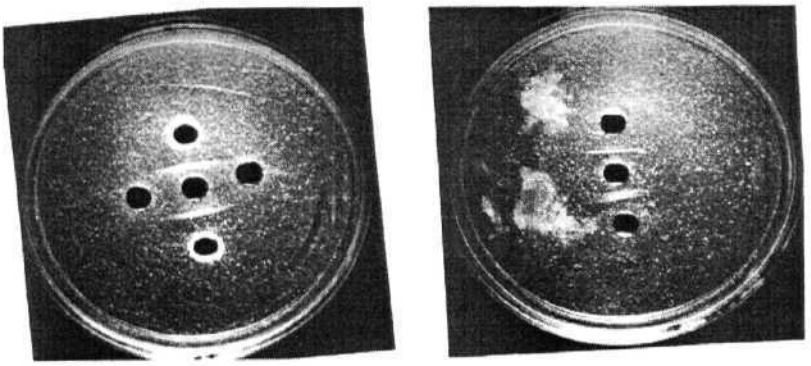
**Figure 11: A. Number of histidine residues modified with time**

**B. Immunodiffusion of the native and histidine and C, lysine modified lectin tested with anti-galactose specific lectin antiserum.**

Central well: 20 |aL antisera; well A: Unmodified lectin; well B: Modified sample.




A



B C

**Figure 11**

**Figure 12: Western blot analysis of the *in vitro* translated product of the lablab bean mRNA.**

Lane 1: molecular weight markers; Lane 2: wheat germ lysate; Lane 3: *in vitro* translated product of the galactose lectin mRNA; Lane 4: purified lectin

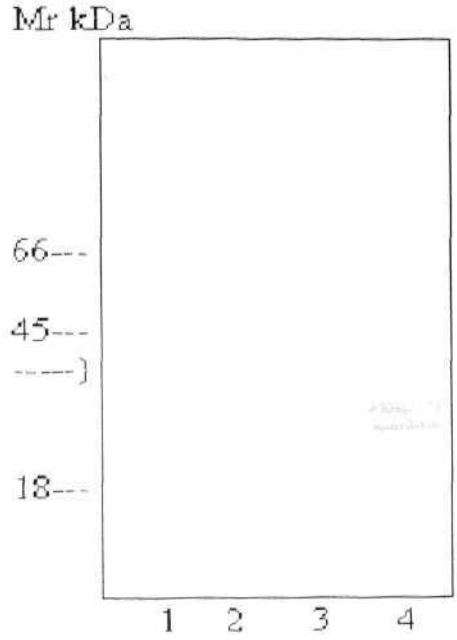



Figure 12

**Discussion**

Several authors described the existence of glucose/mannose specific lectins from the seeds of *Do/ichos lablab.* Our laboratory has affinity purified the glucose/mannose specific lectin from the Indian lablab beans and also identified a galactose specific lectin from these seeds. However, this lectin failed to bind on the Sepharose-galactose gel prepared in the lab. In the present study we have selected a distinct seed material (details given under methods) of the Indian lablab beans and analyzed it for the presence of both lectins. The results indicate that it contains the glucose/mannose lectin and the galactose lectin. The glucose/mannose lectin exhibited identical properties to that reported earlier (Siva Kumar and Rajagopal Rao, 1986). Compared to the information on the glucose/mannose lectin, the information on the galactose lectin from *Do/ichos lablab* seeds is limited. Therefore, to characterize this lectin in detail, it was necessary to obtain it in a pure form in large quantities. We have in the present study established conditions that allowed the affinity purification of the galactose lectin. The lectin was bound on the affinity gel only in the presence of TBS containing 1.5 M ammonium Sulfate. The bound lectin can be completely desorbed using 0.3 M galactose in the same buffer. Although binding of proteins to gels at high salt concentrations, may be considered as the hydrophobic interaction, as has been well established for the binding of some glycosidase enzymes to Phenyl Sepharose gel, (Siva Kumar et al., 2004) in the present study the lectin exhibits preferential binding to the galactose matrix only at high salt concentrations, and the bound lectin can be eluted using only 0.3 M galactose sugar (glucose or mannose does not elute the sugar).

Hence, the interaction is more likely to be a specific interaction, although it is possible that hydrophobic interaction might play a role in binding of the lectin to the affinity gel. Further, the fact that the biospecific adsorbent prepared contains galactose sugar was further substantiated by testing the binding ability of other galactose specific lectins on the gel, *Momordica charantia* and the Soyabean in the absence of high salt concentration. Some researchers have already established the use of Sepharose divinyl sulfone galactose gels for the purification of galactose specific lectins from *Erythrina eristagal/i* (Iglesias *et al.,* 1982). Taken together these observations suggest that the gel matrix used in this study contains sufficient added galactosc and the binding of the lablab bean lectin to the matrix, is possibly sugar specific. In our laboratory several monosaccharides such as mannose, N-acetylglucosamine, galactose, were coupled to Sepharose via divinyl sulfone and successfully used for the isolation of the corresponding sugar specific lectins by affinity chromatography. In addition, Sepharose-lactosc gel and Sepharose-phosphomannan gels were also prepared in our laboratory and have been used for the affinity purification of some animal lectins (Siva Kumar *et al.,* 2004). Thus, conditions were established for the first time to isolate the galactose lectin from the Indian lablab beans by affinity chromatography.

Among different sugars tested for inhibition of lectin activity, galactose and its derivative, N-acetyl galactosamine, were found to inhibit the lectin activity. These sugar inhibition results are comparable to other galactose specific lectins such as the *Trichosanthes dioica* described recently (Sultan *et al.,* 2004).

The glycoprotein nature of the lectin was confirmed by its ability to bind on Con A-Sepharose gel and by the reaction of the two sub-units of the lectin to periodic acid

staining. It is interesting to note that the affinity purified galactose lectin, does not require 1.5 M ammonium Sulfate to bind on Con A-Sepharose gel. All glycoprotein lectins have been shown to interact with Con A via multiple terminal non-reducing and 2 -*O*- substituted a-mannopyranosyl units (Goldstein, 1974).

The galactose lectin is a tetramer with a native molecular mass *oi"* 120 kDa as detemiined by gel filtration chromatography with two subunits of molecular masses of 31 kDa and 29 kDa, respectively. Since both sub-units react with the antibody as shown by the Western blot analysis and the immunodiffusion experiment it is logical to conclude that these subunits together form the protein. The well characterized glucose/mannose specific lectin from the *Dolichos lablab* seeds exhibits a native molecular mass of 60 kDa and is composed of two types of subunits 15 kDa and 12 kDa, respectively (Siva Kumar and Rajagopal Rao, 1986). The subunits of the galactose lectin were also shown to react and form precipitin arcs with the antibody raised to the glucose / mannose specific lectin and hence it may be concluded that the galactose specific lectin shows some homology to the glucose / mannose specific lectin. The amino acid sequence of the first 10 residues in both the sub-units as determined by an automatic protein sequenator revealed that the sequences in both subunits are identical (The sequence information was provided by Prof.Dr. B. Schmidt, Institute fur Biocheinic Und Molekulare Zellbiologie, Universitaet Goettingen, Goettingen, Germany). 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 lectin studied here, is not exactly identical to the glucose/mannose lectin, suggests that it is distinctly a new protein.

Amino acid analysis of the purified lectin showed high content of acidic and hydrophobic amino acids. Many purified legume lectins have high content of acidic amino acids (Rajsekhar *et al.,* 1997). Thus, the general properties of the lectin such as sugar specificity, native molecular size, tetrameric and glycoprotein nature seem to be similar to other well characterized galactose specific seed lectins (Majumdar and Surolia, 1979 and 1981), thus, indicating the protein isolated is a lectin.

Chemical modifications of lectins serve as useful tools to identify the amino acid residues involved in the sugar binding site. Modification of the side chain amino acids in a lectin leads to either reversible or irreversible change in the conformation of the protein thereby affecting the biological activity of the protein. The loss in biological activity on modification implies that these amino acid residues are possibly essential for the biological activity. However, modifications carried out in the presence of the inhibitory sugar selectively establish the involvement of a particular amino acid in the sugar binding site of the lectin. In the present study various chemical modifications of the purified galactose lectin were earned 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 the affinity gel was assessed.

Chemical modification studies, thus, conclude that the imidazole groups of histidine residues are involved in lectin binding site. Presence of a specific ligand, galactose, resulted in partial protection of the modification reaction. Since modification of the side chain amino groups of lysine and the hydroxyl groups of tyrosine led to partial loss of

biological activity and did not show significant protection of modification in presence of the binding sugar, it is logical to conclude that these residues are not directly involved in the sugar binding site of the lectin, but might be necessary to maintain the conformation of the protein. Since, the modifications of the tryptophan and arginine residues did not alter the hemagglutinating activity or the binding ability to Con A-Sepharose gel, these modified samples were not further analyzed.

Several chemical modification studies carried out on different lectins suggest the importance of histidine residues in sugar binding. There are studies that report histidine residues to have a major role in the biological activities of most of 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). A similar case was observed in a lectin isolated from *TrichosaiUhes anguina* (Sneha Sudha Komath *et al.,* 1998). 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 (Siva Kumar, 1999). Involvement of histidine residues in the biological activity of the lectins have also been reported recently for a lactose specific lectin from the invertebrate *unio* (Radha, 2002). 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 and this lectin exhibits better biological properties than the modified

protein. Fourth, Protection of modification was seen when the lectin was preincubated with 0.1 M galactose prior to modification.

Since a molecular mass of 33 kDa has been obtained for the *in vitro* translated product and this reacts with the antibody to the purified lectin, it is possible that this product is the precursor of the lectin containing the signal peptide, followed by the lectin. Similar *in vitro* translation studies carried out with the fava bean mRNA, isolated from *Vicia faba* seeds, yielded a single polypeptide chain of molecular mass 29,000 Da with a 29 amino acid hydrophobic signal peptide followed by the \\ chain and the a chain. Fava bean lectin purified by affinity chromatography from the seeds has been shown to contain two types of sub-units, 2 a and 2 [3 chains (Mr a-5,571 Da and Mr fi-20,700 Da) (Hemperly *et al.,* 1982). Thus, it was reported that the two chains arise by post translational cleavage of the single polypeptide precursor. Several lectins isolated from the pea and the lentils are homologous to favin in their subunit structure and sequence (Strosberg *et al.,* 1976). In an earlier study, it was found that the glucose/mannose specific lectin from the *Doliehos lablab* seeds also is synthesized //; *vitro* as a precursor protein with an apparent molecular mass of 18 kDa (Siva Kumar and Rajagopal Rao, unpublished information). It is established that some proteins synthesized within the cells are synthesized as precursors co-translationally translocated across the rough endoplasmic reticulum. The signal sequences are cleaved and asparagine linked core oligosaccharides are added (Blobel, 1980). These processes can be carried out *in vitro* using the appropriate components. Further studies on the lablab lectin are necessary to define whether the precursor formed could be similarly processed.

# Chapter 3

# Studies on the localization of the galactose specific lectin and its interaction with other other proteins in the protein bodies

## Introduction

To understand the functions of the DLL lectins it is essential to explore their localization in the seeds and their specific interactions with other components at the site of their localization. In 1856 protein bodies were first isolated from the oil seeds by Hartig who named them as "aleurone grains", after the Greek word meaning flour. Later, several scientists studied these internal inclusions which are small, spherical organelles. They were found to vary in their size from species to species and have diameters ranging from 0.1 to 25 ,urn. Protein bodies are characterized by a specific density of 1.20 - 1.30 g/cm".[3] (Maschcrpa, 1975). These are surrounded by a single limiting membrane (13) and are composed of storage proteins (70-80% dry weight), salts of phytic acid (10% of dry weight), lectins, acid hydrolases, cations and ribonucleic acid. Small amounts of carbohydrates are found in the protein bodies in association with the storage proteins to form the glycoproteins (Ashton, 1976). Starch is never associated with the protein bodies except by contamination. Lipids are sometimes detected in significant amounts within the protein bodies. Their membranes are generally rich in Phosphatidyl choline and Phosphatidyl ethanolamine and contains smaller amount of phospahatidyl inositol (Mettler and Beevers, 1979).

In cotyledons of leguminous seeds, formation of protein bodies occurs during seed ripening. Reserve proteins are synthesized on rough endoplasmic reticulum; accumulate in the central vacuole and later in the protein bodies formed denovo with the loss of water during seed maturation. The protein bodies get degraded during seed germination. During this process, the protein bodies swell and the matrix proteins are

released due to small changes in osmotic or ionic composition of the media (Pusztai *et al.,* 1978). The remnants of the protein bodies become smaller which later fuse to form the central vacuole. Such a process if it occurs *invivo* would provide an osmotically self-regulating system for the continuous supply of nitrogen for growth. In seeds of the legumes, most of the lectin is localized in the cotyledons in protein bodies, the subcellular organelles related to eukaryotic lysosomes where it may be interacting with endogenous proteins, named "lectin binders" (Ruediger, 1998).

Thus, understanding the architecture of the membranes and the identification of the membrane proteins might facilitate tracing the ontogeny of the protein bodies. Further, very little research has been carried out to study the characteristics of the protein body membrane from the dry seeds. It would be interesting to know whether all protein bodies in the same tissue contain all the storage proteins or whether these proteins are differentially distributed among these organelles. The primary goal of this work was to isolate the protein bodies from the seeds *ofDolichos lablab* and to study the interaction between the protein body membranes and the DLL lectins, (the glucose/mannose specific lectin, galactose specific lectin). It is of great importance to study the interaction of lectin with any endogenous component as it would contribute significantly to understand the role of these proteins in plants. The method for isolation of protein bodies described here is quick, reliable and reproducible.

Glycosidases such as a-mannosidases, p-N-acetyl hexosaminidases and fucosidases have **been** purified to homogeneity from plants and animals (Andree *et* a/., 1980; Muramatsu, 1966). a-mannosidase deficiency in mammals leads to severe hereditary diseases referred to as mannosidosis. This enzyme, with a native molecular mass of 195

kDa was purified from the seeds of *Dolichos lablab* in our laboratory (Rajasekhar *et al*, 1997) and has been well studied from other legume plants by Einhoff *et al.*, (1986). Compared to the vast information on the mannosidases from legumes, very little work has been carried out on the hexosaminidases, although some studies indicated that this enzyme is localized in the protein bodies (Colucci *et al*, 1980). Since we have already purified the mannosidase from the *Dolichos lablab* seeds, and have also characterized the glucose/mannose lectin and the galactose lectin, we have undertaken this study to achieve the following objectives.

(i) to isolate and purify the (3-N-acetyl hexosaminidase enzyme from the seeds

(ii) to isolate the protein bodies from the seeds and identify the important constituents such as the lectins and glycosidases

(iii) prepare protein body membranes by sucrose-density gradient centrifugation

(iv) to study the interaction of the various constituents such as the lectins and enzymes with the protein body membranes

(v) to identify an endogenous receptor for the galactose specific lectin in the seeds.

**Materials**

Para nitrophenyl a-D-mannoside and para nitrophenyl N-acetyl-(3-D-glucosaminide substrates were obtained from Sigma Chemical Company, USA. Sephadex G-200 was obtained from Pharmacia, Uppsala, Sweden. Seralose CL 4B was procured from Sisco Research Laboratory, India. The antibody to the human placental J3- N acetyl hexosaminidase was a kind gift from Prof. Dr. K. von Figura, University of Goettingen, Germany. All other chemicals and reagents used in the present study were of high quality and were procured from local firms.

**Methods**

**Enzyme** assays

The activities of a-mannosidase and (3-N-acetyl hexosaminidase the marker enzymes of protein bodies were measured using the respective p-nitro phenyl derivatives of the glycosidases as substrates in 50 mM sodium acetate buffer pH 5.0 (Rajasekhar *et al.,* 1997). Absorption of the released p-nitro phenol was read at 405 nm. Absorption co-efficient of 1.81 x 104 cm" M" was used for calculation. One enzyme unit is defined as the amount of enzyme able to release 1 uM of p-nitro phenol per minute.

**Isolation, purification and characterization of the P-N-acetyl hexosaminidase enzyme from the seeds**

***Preparation of the crude extract:*** 50 g of the seed powder was extracted overnight with 300 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 % ammonium Sulfate fractionation. This fraction was dialyzed against TBS and

passed through Sepharose-mannose gel to deplete the fraction of any glucose/mannose lectin.

***Phenyl*** *Sepharose column:* The unbound protein from the Sepharose-mannose gel was dialysed against 25 mM Tris -HC1 pH 8.0 containing 1 M ammonium sulphate and applied on Phenyl Sepharose column (10 mL) pre-equilibrated with the same buffer. The column was washed extensively with the same buffer until the absorbance at 280 nm was 0.05. Bound protein was desorbed with 25 mM Tris -HC1 pH 8.0. All the eluates were assayed for the hexosaminidase and mannosidase activities. Fractions exhibiting higher activity of hexosaminidase were pooled and concentrated.

*Gel filtration and native molecular mass determination of the enzyme:* The fraction containing P-N-acetyl hexosaminidase activity was applied on a Sephadex G-200 gel filtration column (60 cm x 1.2 cm) which was pre-equilibrated with 0.9% saline. The column was eluted with the same buffer at a flow rate of 2.0 mL per 10 min. The absorbance of the fractions was read at 280 nm. The enzyme activities of a-mannosidase and the p-N-acetyl hexosaminidase were measured. Those fractions containing the P-N-acetyl hexosaminidase activity were pooled and concentrated. The native molecular mass of the enzyme was determined by calibrating the column with proteins of known molecular weight viz., peanut agglutinin (Mr 110 kDa), concanavalin A (Mr 104 kDa), bovine serum albumin (Mr 66 kDa) and ovalbumin (45 kDa).

***Con*** *A-Sepharose gel chromatography:* Con A-Sepharose gel was equilibrated with 50 mM sodium acetate pH 5.0 containing 5 mM $CaCl_2$ and 5 mM $MnCl_2$ (column buffer). 0.5 mg/mL of the G-200 eluate was applied on Con A-Sepharose gel and washed with

column buffer. After extensive washing, the bound lectin was eluted using 0.15 M Methyl a-mannoside and 0.75 M sodium chloride in column buffer.

***Subunit composition of the purified** enzyme:* The molecular weight of the sub-units of P-N-acetyl hexosaminidase enzyme was determined by 12 % SDS-PAGE analysis using the standard reference proteins.

The specificity of the P-N-acetyl hexosaminidase to the antibody raised to the human placental P-hexosaminidase was tested by Western blot analysis.

*Temperature stability:* The purified enzyme (0.5 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. The samples were brought back to room temperature and their activity was determined.

*Carbohydrate estimation:* The carbohydrate content of the P-N-acetyl hexosaminidase enzyme was determined by Phenol-sulphuric acid method.

**Isolation of protein bodies**

Protein bodies from the seeds were isolated according to Pusztai *et al.,* (1968). 50 g of seed flour was homogenized in 50 % sucrose solution and was centrifuged at 3,500 rpm for 10 minutes. The supernatant was rc-centrifuged at 12,000 rpm for 30 minutes. The supernatant obtained here was discarded and the pellet was applied on a linear sucrose gradient of 40 -84 % (40 %, 50 %, 60 %, 70 % and 84 %) in 10 mM sodium phosphate buffer pH 7.6. Tubes were centrifuged in a Beckmann centrifuge for 2 hours at 20,000 rpm using a swing out rotor. White bands at the interfaces of the gradient, 50 -60 %, 60 -70 % and 70 -84 % were carefully aspirated and stored. Aliquots were visualized under a light microscope.

**Preparation of the protein body membranes**

The isolated protein bodies were sonicated in 1 M NaCl at 12 amps for 30 sec. The suspension was diluted in 18 % sucrose in phosphate buffer pH 7.6 and centrifuged at 1,00,000 x g for 1 hour. Supernatant was saved and the membrane pellet was washed and re-suspended in 25 mM Tris-HCl buffer pH 8.0 containing 1 M NaCl. This step was repeated twice and the pellet was suspended in 25 mM Tris-HCl buffer pH 8.0. The supernatant was tested for the presence of the glucose/mannose specific lectin and the galactose specific lectin by Western blot analysis using the respective antibodies raised in rabbit.

The membrane pellet was applied on a linear sucrose gradient of 20 -60 % prepared as described above. Protein body membranes (PBM's) found at 30 -40 %, 40 -50 % and 50 -60 % interfaces of the gradient were carefully aspirated and stored. These PBM's (20 mg) were physically entrapped in Seralose CL 4B equilibrated with 50 mM sodium acetate buffer pH 5.0.

**Interaction of the lectins and (3- N-acetyl hexosaminidase enzyme with the protein body membranes**

The affinity purified galactose lectin [1 mg], the glucose/mannose lectin [1 mg] and p-N-acetyl hexosaminidase enzyme [0.5 mg] were separately applied on the PBM gel at pH 5.0 and pH 8.0. The gel was washed with the respective pH buffers and elution was performed using different conditions, viz., 0.3 M glucose, 0.3 M galactose, 0.5 M and 1.0 M NaCl, and a change in pH. [At pH 8.0 (Tris-HCl), there was no binding of either lectin to the PBM].

Further, the histidine, lysine and arginine residues of the galactose lectin were chemically modified as described earlier, in chapter 2, and applied on the PBM - gel at pH 5.0 to analyze if modification of these amino acids alters the binding ability of the lectin to protein body membranes. For elution pH 8.0 buffer was used.

**Isolation of an endogenous lectin receptor from the seeds**

The method was carried out as described by Ramos *et al.*, (2002). Briefly, 50 g of dehulled seeds were ground to a fine powder and dissolved in 0.5 M sodium chloride (1: 10 w/v) for 2 hours under shaking, followed by centrifugation at 20,000 g at 4°C. The pellet was reextracted and the new supernatant combined to the previous one. The supernatant designated as crude extract was dialyzed against distilled water and centrifuged again under the same conditions. The supernatant was named albumins and the precipitate was named as globulins. The pellet obtained initially in the first step was extracted with 70% v/v ethanol. This fraction was centrifuged and the supernatant was designated as prolamines. The pellet was extracted with 0.1 M HC1 and centrifuged. The supernatant was named acidic glutelins and the pellet was extracted in 0.1 M NaOH and centrifuged. This supernatant was dialyzed against water and was termed as basic glutelins.

These different fractions were assayed for carbohydrate content, protein content using the BCA method and agglutination activities. These different fractions were separated by SDS-PAGE and the proteins were transferred to nitrocellulose membrane. The membrane was first incubated with the galactose lectin and then with the antiserum to the galactose lectin. The blot was developed with BCIP/NBT substrate. The acidic glutelin fraction only was applied on galactose specific lectin coupled to affigel, which

was equilibrated with 0.15 M NaCl. After washing the gel, elutions were done with 0.3 M galactose in saline. The eluate was analysed on 10% SDS-PAGE. The gel was transferred to a nitrocellulose membrane and probed with the galactose specific lectin antisera.

**Chemical cross-linking of lectins with PBMs**

Cross-linking studies were carried out as described (Causin *et al.*, 1989). 2 to 3jxg of the galactose specific lectin was dialyzed against 50 mM-sodium phosphate buffer, pH 7.4, containing 150 mM-NaCl/ 0.1% Triton X-100 and incubated for 3 hours at 4 °C with 13 uL of PBMs dialyzed against the same buffer. DSS was added to the cross-linking mixture to a final concentration of 1 mM and the reaction was carried out for 15 minutes in ice. The reaction was stopped with 5 JJL of 1 M-Tris/HCl pH 7.4, 2.75 uL of 20% SDS and 3 uL of glycerol (100%). The reaction mixture was heated at 95 °C for 5 min and the sample was subjected to 10% SDS-PAGE and the protein bands were transferred onto a nitrocellulose membrane. The membrane was probed with the galactose lectin antiserum (1:500 dilution). The cross-linked product was visualized by incubating the blot with the secondary antibody (goat anti rabbit IgG conjugated to alkaline phosphatase) followed by incubation with BC1P/NBT substrate.

**Results**

The total seed extracts were found to contain reasonable activity for the enzyme β-N-acetyl hexosaminidase. This was isolated and purified by a combination of ammonium Sulfate fractionation, hydrophobic affinity chromatography, gel filtration and Con A-Sepharose chromatography. When the ammonium Sulfate fraction containing the enzyme activity was applied on phenyl Sepharose gel, the enzyme was completely bound to this gel in presence of 1 M ammonium Sulfate (Fig. 13) and could be desorbed together with another glycosidase, the α-mannosidase from the phenyl-Sepharose gel in the absence of the salt. The enzyme containing fractions were applied on a Sephadex G-200 gel filtration column, both the enzyme activities could be separated and the molecular mass of the β-N-acetyl hexosaminidase was found to be 104 kDa (Fig. 14). Owing to the higher molecular mass of the α-mannosidase enzyme, it was eluted from the gel much before the β-N-acetyl hexosaminidase. The β-N-acetyl hexosaminidase enzyme containing fractions from the gel filtration column were applied on a Con-A Sepharose gel, and the bound enzyme was eluted using 0.15 M methyl α-mannoside. This fraction together with the gel filtration eluted fraction was analyzed on SDS-PAGE and the results are shown in (Fig 15). From the figure it is apparent that the protein eluted from G-200 gel showed more than 3 bands. However, the Con-A Sepharose gel eluate showed only three bands corresponding to molecular masses of 32 kDa, 30 kDa and 29 kDa.

Since an antibody to a plant β-N-acetyl hexosaminidase is not available with us, we wanted to check if this enzyme from the *Dolichos lablab* seeds shows any similarity to

the mammalian enzyme. When the Con-A Sepharose eluted sample was separated on SDS-PAGE, and the proteins analyzed by Western blot using the human placental (3-N-acetyl hexosaminidase, only one of the subunits of the plant enzyme showed reactivity with this antibody (Fig. 16). Table 12 shows the purification of the p-N-acetyl hexosaminidase enzyme obtained through different steps. The enzyme obtained after Con A-Sepharose gel was completely active at 4 "C upto 40 °C. Beyond 40 °C, the stability of the purified enzyme decreased and at 90 °C the lectin had negligible activity (Fig. 17).

We isolated the protein bodies from the *Dolichos lablab* seeds using sucrose-density gradient centrifugation as described under methods. The protein bodies were separated and focused as white layers at the interfaces of 50-60 %, 60-70 % and 70 -84 % sucrose concentrations. These three layers were aspirated from the centrifuge tubes and examination of a small fraction of the isolated protein bodies under the light microscope showed it to be a homogenous preparation of small round organelles (Fig. 18). Since the three layers showed similar pattern, these were all pooled and used in further experiments.

The membrane pellet obtained by sonication was processed as described under methods to remove any glycosidase activities. This sample contains negligible amounts of the lectins and was separated further on sucrose density gradient centrifugation. White bands appeared at the interface of 30 -40 %, 40 -50 %, and 50 -60 % gradients. From Fig 19, it is apparent that the protein body membrane preparations (lane 3) are mostly devoid of any lectin. However, several other protein bands could be detected. This final preparation of the protein body membranes equivalent to 20 mg protein,

(designated as PBM) was physically entrapped in Seralose gel as described under methods.

From the protein bodies obtained above, the activities of some glycosidases such as the a-mannosidase and p-N-acetyl hexosaminidase were assayed in the supernatant obtained after lysis of these bodies. Total enzyme activities were detectable in the range of 3.5 units and 3.3 units for both the enzymes, respectively. The activities of other glycosidases such as the (3-galactosidase, p-glucosidase and (3-xylosidase were found to be very low and therefore were not characterized further. The clear supernatant was collected after centrifugation and designated as PBS (protein body supernatant). The clear supernatant was analyzed by SDS-PAGE in separate lanes in order to identify the protein constituents. The proteins separated on SDS-PAGE were transferred to a nitrocellulose membrane and the lanes were probed with the antibodies for galactose lectin, the glucose/mannose lectin, a-mannosidase and P-N-acetyl hexosaminidase. When the blot was developed with BCIP/NBT substrate, the specific proteins were recognized (Fig. 20).

The protein body membranes prepared in this study was physically entrapped in Seralose CL 4B gel. The ability of these purified proteins to interact with protein body membranes was tested at pH 5.0 and pH 8.0. At pH 8.0, there was no interaction of either of them with the PBM's (data not shown). However, at pH 5.0, these proteins could be bound to the PBM's. The results of the interaction of the lectins and the enzyme with the PBM's are summarized in Table 13. While there was no binding of either lectin to this gel at pH 8.0, the lectins applied completely bound to the PBM gel at pH 5.0. Only about 15 % of galactose specific lectin could be desorbed using 0.3 M

galaetose. Shifting the pH to 8.0 completely desorbed the bound galaetose specific lectin. On the other hand, in presence of NaCl, there was practically no elution. The glucose/mannose specific lectin however, showed a different profile with different eluting conditions. Very little lectin could be eluted with 0.3 M glucose. 90% of the bound lectin could be completely desorbed with pH 8.0 buffer, while partial elution (8 % only) could be achieved with 0.5 M NaCl. Similarly, [3-N-acetyl hexosaminidase enzyme also exhibited binding to protein body membranes at pH 5.0 and not at pH 8.0. About 85 % of the bound enzyme was desorbed with a change in pH to 8.0. 8% of the enzyme was desorbed using 0.5 M NaCl and 10% with sugar, N-acetyl glucosamine.

Chemical modification of the galaetose specific lectin was shown to have some effect on its binding properties to the PBM gel. Modification of the histidine, lysine and arginine residues in the lectin decreased the binding abilities of the lectin to protein body membranes to 40 %, 45 % and 75 % respectively, as shown in the Table 13.

Protein fractions obtained from the crude saline extract of the seed flour were characterized for the protein content and lectin activity. Although there were other proteins recognized by the galaetose specific lectin (Fig. 21 A), a significant protein band at 97 kDa was detected in the acidic glutelin fraction, which could not be detected in the other fractions. Fig. 21 B shows that when the acidic glutelin fraction was applied on the galaetose specific lectin affigel and eluted with galaetose, a protein band (with apparent molecular mass of 97 kDa) that reacted with the galaetose lectin antiserum in Western blot experiment was identified.

Cross-linking studies using protein body membrane proteins and the galaetose lectin were carried out as described under methods. The cross-linked product of the galaetose

specific lectin with the PBM's showed higher mobility (Mr 205 kDa) compared to the pure protein. Fig. 22 shows the cross-linked product of galactose specific lectin in lane 1 and the affinity purified galactose specific lectin in lane 2, detected by Western blot using galactose lectin antiserum.

**Figure 13: Phenyl Sepharose elution profile of P- N acetyl hexosaminidase enzyme.**

**Figure 14: Sephadex G-200 separation of a-mannosidase and P-N-acetylhexosaminidase.** Elution profile of the [3-N-acetylhexosaminidase enzyme from Sephadex G-200 gel filtration column (60 cm x 1.2 cm), a- mannosidase and P-N-acetylhexosaminidase activities are shown as peak 1 and peak 2, respectively. Inset shows the proteins of known molecular weight used to calibrate the column -1. Peanut agglutinin (Mr 110 kDa), 2. Concanavalin A (Mr 104 kDa), 3. Bovine serum albumin (Mr 66 kDa) and 4. Ovalbumin (45 kDa). Arrow indicates elution volume (Ve) of the enzyme on the column.

Bution profile of the enzyme on Phenyl - sepharose gel

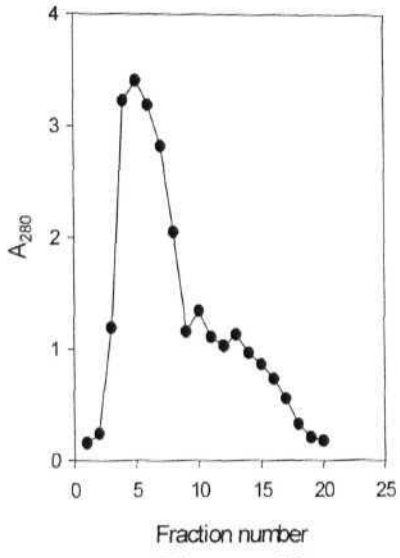



**Figure 13**

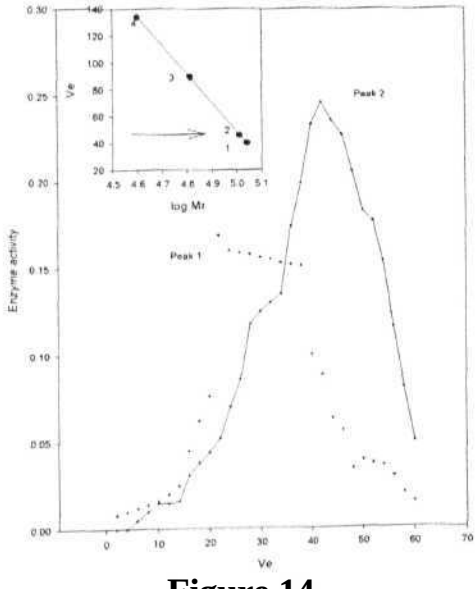



**Figure 14**

**Table 12: Purification table of P-N acetyl hexosaminidase en/yme**

**Figure 15: Subunit composition of the purified p-N-acetylhexosaminidasc on a 12 % SDS-gel** (Silver staining). Lane 1: molecular weight markers, myosin (Mr 205 kDa), [3- galactosidase (Mr 116 kDa), phosphorylase (Mr 97 kDa), bovine serum albumin (Mr 66 kDa), ovalbumin (Mr 45 kDa) and P-lactoglobulin (Mr 18 kDa); Lane 2: phenyl Sepharosc eluate; Lane 3: Sephadex G-200 eluate; Lane 4: Con A- Sepharose eluate. Arrow indicates the position of the three subunits of the enzyme at 32 kDa, 30 kDa and 29 kDa. *Possible contaminant.

| Fraction | Total protein (mg) | Total enzyme units | Specific activity (units/mg) | Enzyme recovery (%) | Purification fold |
|---|---|---|---|---|---|
| Crude | 3400 | 12.97 | 0.003 | 100 | 1 |
| Sepharose-Mannose gel (unbound) | 1500 | 10.273 | 0.006 | 79 | 2 |
| Phenyl-Sepharose eluate | 113.5 | 4.67 | 0.0411 | 36 | 13.7 |
| Sephadex G-200 eluate | 20 | 1.05 | 0.0525 | 8.0 | 17.5 |
| Con A- Sepharose eluate | 1 | 0.808 | 0.808 | 6.2 | 269.3 |

**Table 12**

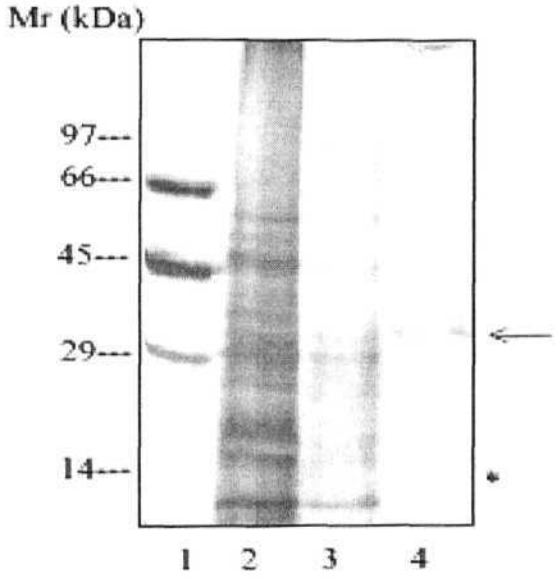



**Figure 15**

**Figure 16: Immuno-reactivity of the human placental P-N-acctylhexosaminidase jjntisera with the plant enzyme analyzed by Western blot analysis on a 10 % SDS gel.** Arrow indicates the position where 32 kDa subunit band of the enzyme reacts with the antibody.

**Figure 17: Effect of temperature on the activity of the enzyme p-N acetyl hexosaminidase.**

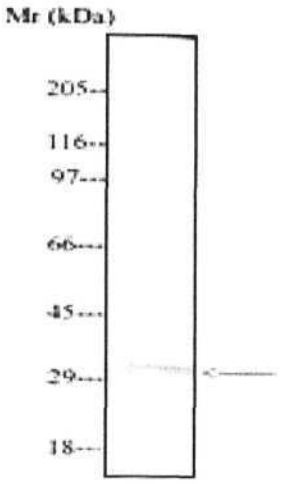



**Figure 16**

Effect of temperature on the activity of enzyme

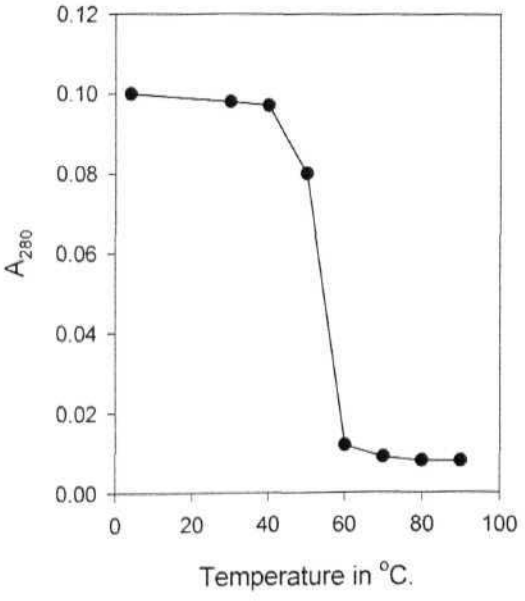



**Figure 17**

**Figure 18: Microscopic view of the Protein Bodies**

v

**Figure 19: 15% SDS-PAGE analysis of the polypeptides associated with protein bodies**

Lanel: Molecular weight markers bovine serum albumin (Mr 66 kDa), ovalbumin (Mr 45 kDa) and p-lactoglobulin (Mr 18 kDa); Lane 2: protein bodies, Lane 3: PBS and Lane 4: PBM's.

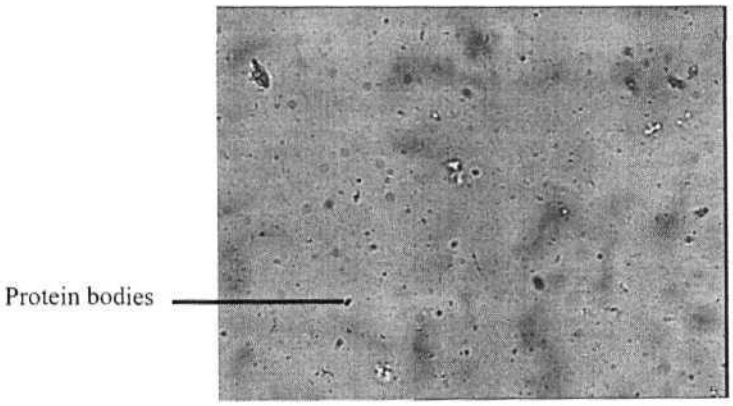



**Figure 18**

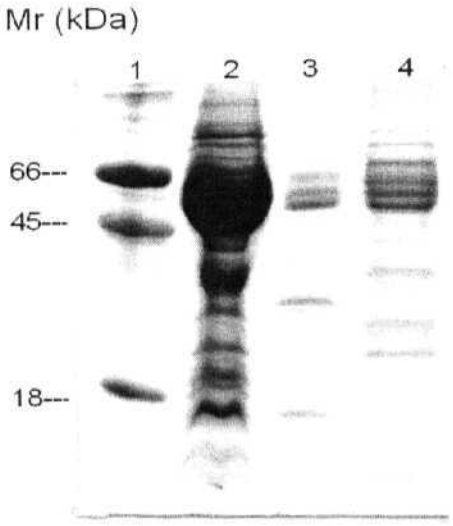
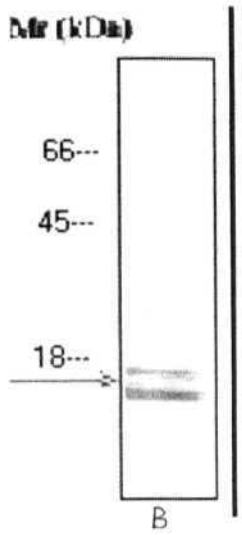
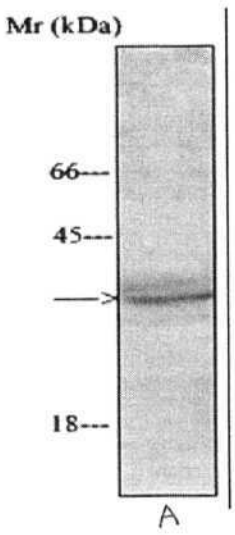


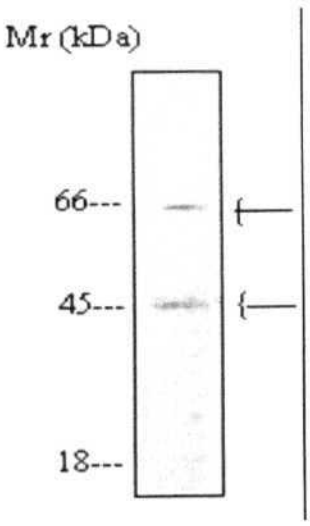

**Figure 19**

**Figure 20: 15 % SDS-PAGE followed by Western blot analysis of the protein bodies.**

Protein bodies were lysed as described in the text. Aliquots of the PBS were applied on a PBM gel at pH 5.0. The bound proteins eluted at pH 8.0 were subjected to Western blot analysis which were probed with (A) galactosc specific lectin antibody (31 and 29 kDa galactose lectin) (B) glucose/mannose specific lectin antibody (15 and 12 kDa glucose /mannose lectin) and (C) oc-mannosidase (66 and 45 kDa) and (D) (3-N acetyl hexosaminidase. Goat anti-rabbit Ig G ALP conjugate was used as the secondary antibody. The membranes were developed with BCIP/NBT substrate. Arrow indicates the presence of the respective proteins in A, B , C and D.


Mr (kDa)
66---
45---
18---
B


Mr (kDa)
66---
45---
18---
A


Mr (kDa)
66---
45---
18---
C

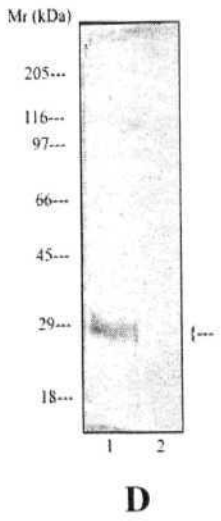

Mr (kDa)
205---
116---
97---
66---
45---
29---
18---
1
2
D

**Figure 20**

Tahle 13: Interaction of the glucose/mannose, the galactose specific lectin and |i-N-acetyl hexosaminidase with the PBIVTs

| Purified protein body components | Binding at pH 8.0 | Binding at pH 5.0 | (%)Elution at pH 8.0 | (%)Elution with sugar | (%)Elution with 0.5 M NaCl |
|---|---|---|---|---|---|
| **Glucose/Mannose Lectin** | - | + | 90 | 2 | 8 |
| **Galactose lectin** | - | + | 80 | 15 | 5 |
| **Histidine** | | + | 40 | - | - |
| **Lysine** | | + | 45 | - | - |
| **Arginine** | | + | 75 | - | - |
| **ß N-acetyl hexosaminidase** | - | + | 85 | 10 | 8 |

**Table 13**

**Figure 21. Identification of an endogenous lectin receptor for the galactose specific lectin.**

**A.Western blot of the different protein fractions from the seed extract of the *Dolichos lablab.*** Lane 1 shows the molecular weight markers, Lane 2 crude extract, Lane 3 albumins, Lane 4 globulins, Lane 5 prolamins, Lane 6 acidic glutelins, Lane 7 basic glutelins. Arrow indicates the position of the endogenous receptor in the albumin and acidic glutelin fraction.

**B.Western blot of acidic glutelin fraction eluted from galactose specific lectin affigel.** Lane 1: acidic glutelin fraction applied on Galactose specific lectin affigel. Lane 2: eluate from galactose specific lectin affigel

**Figure 22: Western blot of the presence of the lectin-receptor cross-linked product**

Lane 1 shows the cross-linked receptor and Lane 2 indicates the galactose specific lectin cross-linked to the receptor. Arrow indicates the position of the cross-linked product.

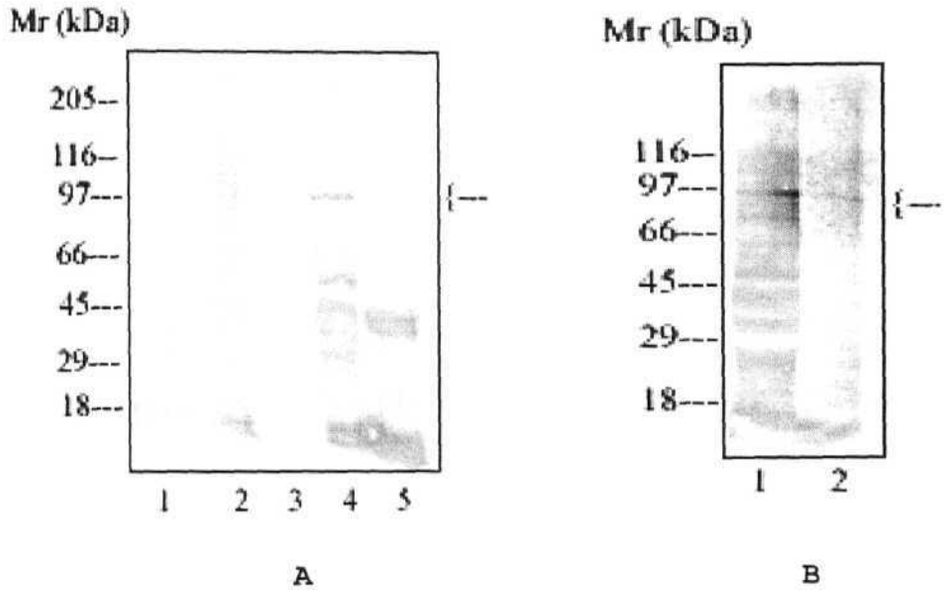



Figure 21

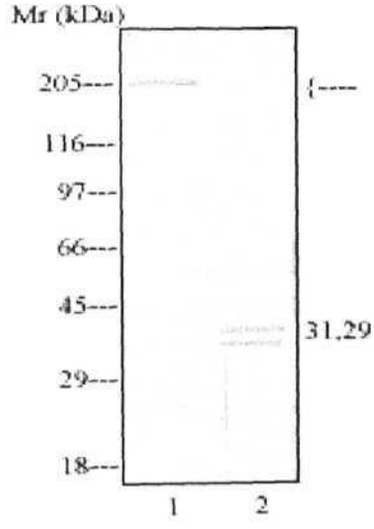



Figure 22

**Discussion**

The p-N-acetyl hexosaminidase enzyme bound to phenyl Sepharose column at a high ammonium sulphate concentration and was desorbed together with another hydrophobic glycosidase, the ot-mannosidase from the gel in the absence of the salt. This is similar to the desorption of hydrophobic glycosidases from Jackbean (Einhoff *et al,* 1986). In contrast P-N-acetyl hexosaminidase from soybean can be desorbed at a medium ionic strength (Gers Barlag *et al.,* 1988). In addition to the p-N-acetyl hexosaminidase enzyme, the seeds of *Dolichos lablab* were found to contain several other glycosidases, whose activitites were very low. The activities of mannosidase and hexosaminidase only were significant. In an earlier study, the a-mannosidase enzyme from the *Dolichos lablab* seeds has been isolated and characterized. The enzyme exhibited some properties similar to the well characterized jack bean enzyme Rajasekhar, and Siva Kumar, N (1997).

The presence of different glycosidases causes a difficulty in obtaining the hexosaminidase in pure form. Among several methods tested the method described here, although involves a substantial sacrifice in the quantitative yield of the enzyme, is relatively simple and reproducible. For some unknown reason, a-mannosidase was found to be retarded on the Scphadex G-200 gel. The molecular weight of a-mannosidase is in the range of 200 kDa in many of the legume sources (Einhoff *et al.,* 1986; Rajsekhar *et al.,* 1997). In *Phaseolus vulgaris,* this enzyme is a dimer composed of two 110 kDa sub-units (Pauss, 1977). p-N-acetyl hexosaminidase enzyme was

purified from Indian lablab beans on Con A-Sepharoe gel and this preparation was used in the studies.

Unlike the Jack bean a-mannosidase and the Soybean P-N-acetyl hexosaminidase, the *Dolichos lablab* P-N-acetyl hexosaminidase enzyme was not only desorbed by sugars that are specific for Con A but also by salts. But, when the protein analyzed by SDS-PAGE, and the sub-units transferred to a nitrocellulose membrane and probed with an antibody raised to the human placental P-N-acetyl hexosaminidase, only one of the sub-units reacted, indicating that the mammalian and plant enzyme are immunologically related.

Earlier studies carried out by various workers have postulated that the protein bodies contain the lectins, glycosidases and storage proteins. Protein bodies in seeds are analogous to the lysosomes in eukaryotes. Though protein bodies were isolated from some legume seeds by other workers, there has been no report on the isolation and characterization of the protein bodies from the *Dolichos lablab* seeds. Therefore, in the present study, since two distinct sugar specific lectins from the Indian lablab beans were already purified in our lab, we made an attempt to isolate the protein bodies from these seeds in order to understand the localization of the lectins, as well as to study the interaction of these lectins with the protein body membranes. Protein bodies from seeds can be isolated employing different conditions such as Ficoll gradients or by sucrose density gradient centrifugation. We isolated the protein bodies from the seeds of *Dolichos lablab* using sucrose-density gradient centrifugation. The inherent difficulties in obtaining pure and undamaged protein storage organelles from plant tissues have been discussed by Pusztai *et* «/., (1978). The instability of these protein bodies is

because of the limiting membranes of these organelles. The yield of protein bodies is very much dependent on the initial homogenisation step which has to be handled with care. Protein bodies can be lysed by a variety of buffers. Water alone or buffers at low pH are unsuitable. Sonication followed by incubation with alkaline buffer containing 1 M NaCl solubilises the storage proteins leaving the membranes intact.

It is known that the protein bodies differ in their densities and get separated on sucrose density gradient (Pusztai *et al.,* 1968). Assay of the marker enzymes from the supernatant revealed that both a-mannosidase and (3-N-acetyl hexosaminidase activities could be detected in the supernatant obtained after lysis of the protein bodies supporting the fact that we isolated intact protein bodies from the seeds.

The protein body membranes were prepared from the protein bodies as described under methods. It was necessary to separate the PBM preparation on sucrose density gradient centrifugation, in order to ensure that the PBM are obtained as a homogeneous preparation. These protein body membranes were also analyzed by SDS-PAGE analysis and found to contain several proteins. In order to study the interactions of various protein body constituents with the PBIVTs, the PBM's were physically entrapped in Seralose CL 4 B gel, and this was packed in a glass column as described under methods. PBM's prepared from the pea seed protein bodies were used to examine such interactions (Gers barlag *et al.,* 1993).

Since the putative lectins and p-N-acetyl hexosaminidase enzyme were identified in the isolated protein bodies, in order to understand the possible physiological function of these proteins, we wanted to examine whether these proteins show specific interaction with the protein body membranes. Only a few examples are known in literature, which

indicate the possible interaction of the lectins and enzymes with their protein body membranes (Ruediger, *et al.,* 1993).

These results suggest that the proteins purified in this study show specific interactions with their protein body membranes at pH 5.0, a pH that exists in the protein bodies *in vivo.* These interactions can be removed by employing different conditions though the shift in pH to 8.0 is the best condition for removing these interactions. It is interesting to note that the proteins though localized together in the protein bodies, show distinct modes of interactions with their protein body membranes. While the galactose specific lectin binds to the membranes in a sugar dependent and pH dependent manner, the glucose/mannose specific lectin binds only in a pH dependent manner. Studies carried out by other workers on the pea lectin and soybean lectin suggests that the pea lectin interacts with the protein body membranes in a pH dependent manner while the soybean lectin interacts in a sugar dependent manner (Ruediger *et al.,* 1993).

In order to further understand if specific amino acids of the galactose lectin might be interacting with the protein body membranes, the histidine, lysine and arginine residues of the lectin were modified as described under methods, and the modified lectin was applied onto the gel. The positively charged side chains of these amino acids might play a role in interacting with the negatively charged phosphate groups of the lipids. Gers-Barlag *et al.,* (1993), reported that modification of these side chains in the pea lectin impairs the interaction of the lectin with protein body membranes. Thus, pH seems to play a critical role in the interaction of the lectins with the protein body membranes. However, different lectins seem to exhibit characteristic interactions. It remains to be established why these differences exist with respect to different lectins.

We have investigated here the presence of an endogenous lectin receptor for the galactose specific seed lectin from the seeds of *Dolichos lablab.* It should contribute to extend results showing that lectins may recognise endogenous components through interactions mediated by their carbohydrate binding site or by the whole molecule. Assuming that the researched ligand is present in very low concentrations, the crude extract was fractionated into albumins, globulins, prolamins, acidic and basic glutelins. This particular receptor protein for the galactose specific lectin is found only in the acidic glutelin fraction, which when applied on a galactose lectin-affigel, binds on the gel and can be specifically eluted with 0.3 M galactose, suggesting the interaction to be sugar specific. The nature of the lectin interaction with the endogenous molecules is of great interest as these proteins are grouped based on their ability to specifically bind carbohydrates. Another legume lectin from *CratyHa floribunda* was found to interact with an endogenous receptor that is found in all the protein fractions mentioned above, except for prolamin fraction (Ramos *et al.,* 2002).

The cross-linking experiments suggest that the galactose specific lectin from *Dolichos lablab* has an endogenous receptor that is also present in the protein body membrane. Since the native molecular mass of the galactose specific lectin is 120 kDa and by its interaction with the 97 kDa receptor protein with the protein body membranes, a cross-linked product of 205 kDa is obtained. This product was recognized by the antibody to the galactose lectin suggesting that the cross-linked product contains the lectin. From these experiments, it can be concluded that the receptor is a component of the protein body membranes. Similar cross-linking experiments were carried out to determine the

specificity of interaction between a lysosomal enzyme a-fucosidase and the purified goat Mannose 6-phosphate receptor protein (Siva Kumar et al., 2004).

Chapter 4

# Functional characterization of Glucose/mannose specific lectin and Galactose specific lectin

## Introduction

The major function of lectins appears to be in cell recognition. This is due to the complementarity shown between lectins and the structures present on the cell surfaces. This concept represents the "Lock and Key" hypothesis introduced by Emil Fischer to represent the specificity between enzymes and substrates.

Legume lectins associate symbiotically with the soil bacteria of *Rhizobia* family, thus making them independent from soil nitrogen supplies. When *Rhizobia* encounter root hairs in the soil, its attachment to the root hairs occurs by interaction between the rhizobial surface carbohydrates and the lectins present in the roots of the legume plants. This is known as the "lectin recognition hypothesis". The toxicity of various plant lectins for animals and their growth inhibitory effect on fungi (Mirelman *et al.,* 1975) are the basis for the assumption that they function in the defense of plants against phytopathogenic fungi, predatory animals, insects and bacteria (Keen, 1992; Ayoubo, 1994).

The immature lymphocytes of the mouse and human cortical lymphocytes differ markedly from the niedullary mature lymphocytes in their surface carbohydrates, as evidenced from the fact that the former arc bound and agglutinated by pea nut agglutinin (PNA+ cells) whereas the latter are not (PNA-) (Sharon, 1983). Separation with peanut agglutinin provides facile access to the individual thymocyte sub-populations and makes it possible to examine *invitro* their developmental and functional relationship. Selective agglutination by SBA permits separation of B and T mouse splenocytes. The main application of this lectin is for purging human bone

marrow for transplantation (Aversa *et al,* 1994). It is employed routinely for the transplantation into children born with severe combined immune deficiency. It is also used for leukemic patients as an alternative for T cell depletion such as monoclonal antibodies. The lectin from *Dolichos bijlorus* is used to distinguish between Al and A2 subgroups.

Certain lectins are potent mitogens, activating lymphocytes and inducing them to divide. PHA and Con A for example, stimulate T lymphocytes, while pokeweed mitogen stimulates both the T and the B cells (Di Sabato *et al,* 1987; Borrebaeck *et al,* 1989). 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 the mechanism of cell activation. Mitogenic stimulation of lymphocytes by plant lectins is routinely made use in cell culture techniques. It also provides an easy and simple means to assess the immunocompetence of patients suffering from diseases such as AIDS and to monitor the effects of various immunosupprcsive and immunotherapeutic manipulations. It has also been used to examine the effect of stress, both physical and psychological, on the immune system. It is employed for the preparation of chromosome maps for different purposes, such as karyotyping, sex determination, and detection of chromosome defects since chromosomes are easily visualised in the stimulated cells.

The mitogenic lectins interact with unique cell surface sugars that may act as "triggering receptors" present on the membranes (Lis and Sharon. 1977). The earliest detectable changes are seen in the membrane. These include increased permeability for a variety of metabolites such as glucose, amino acids, *C* and $Ca^{n+}$ ions and an

accelerated turnover of membrane phosholipids. This leads to the stimulation of acetylation of histones, phosphorylation of nuclear proteins and modification of lipid and carbohydrate metabolism. After a day, RNA and protein synthesis accelerate and morphological changes become apparent. At about 48 hr, DNA synthesis starts and the cells enter mitosis. This causes reversion of the postmitotic cells to small lymphocytes. The stimulated lymphocytes release biologically active lymphokines (Cohen *et al,* 1979) like the 1L-2 (Robb, 1984) and *y* interfcron (Epstein, 1981). 76 hours after stimulation, other differentiated functions of the activated lymphocytes, such as imrnunoglobulin production by B cells and cytotoxicity of T cells are detected (O'Brien *et al.,* 1978).

For numerous purposes lectin derivatives are required. Lectins conjugated to flourescent dyes, gold particles or enzymes are employed as histochemical and cytochemical reagents for detection of glycoconjugates in tissue sections, on cells and subcellular organelles, and in investigations of intracellular pathways of protein glycosylation (Rhodes *et a/.,* 1998). The binding of the lectins to the membrane receptors of hormones, growth factors, neurotransmitters and toxins indicate that they are glycoconjugates. Immobilized lectins that are covalently bound to Sepharose are indispensable for the isolation and purification by affinity chromatography, glycoproteins, glycopeptides and oligosaccharides (Debray, 1991; Hasselback *et al.,* 1993; Yamamoto, *et al.,* 1993; Carlson, 1994).

In the present study it was envisaged to see whether the glucose/mannose specific lectin and the galactose specific lectin purified from Indian lablab beans can act as antifungal

agents, as mitogens and whether they could be used in the purification of some glycoproteins.

**Materials**

All the fungi used in the present study were a kind gift from Prof. Appa Rao Podile, Department of Plant Sciences, University of Hyderabad. Blood samples were collected from healthy donors. $^{3}$H Thymidine was obtained from BRIT, BARC, India. Mannose 6-phosphate receptor deficient cells MPR minus (-) cells were a kind gift from Prof.Dr.Dr.h.c. Kurt von Figura, Gottingen, Germany. All the chemicals and the reagents used in the present study were of high purity and obtained from reputed firms.

**Methods**

**Preparation of biospecific adsorbents**

The following ligands have also been coupled to Sepharose that has been activated with divinyl sulfone as described in chapter 2: N- acetylglucosamine and Lactose

**Purification of lectins**

All operations were carried out at 4 °C. Centrifugations were done at 12,000 rpm using a Sorvall regfrigerated centrifuge. The following lectins were purified using published procedures and the purity of the lectins confirmed by SDS-PAGE and then these were used in the studies.

*Purification of Concanavalin A lectin from Jack Bean Meal (legume)*

100 g of Jack Bean Meal *(Canavalia ensiformis)* was suspended in 1 M sodium chloride containing 5 mM calcium chloride and 5 mM manganese chloride (extraction buffer). This was stirred overnight at 4 °C and the crude extract obtained after centrifugation was subjected to 80% ammonium Sulfate saturation. The pellet was dissolved in extraction buffer and extensively dialyzed against the same buffer. The

suspension was centrifuged and the supernatant was applied on G-100 gel equilibrated with the same buffer. After extensively washing the gel with buffer, bound protein was eluted with 0.1 M glucose and the absorbance was measured at 280 nm. Peak fractions were pooled and dialyzed against water, lyophilized and stored at -20 °C.

*Purification of Wheat germ agglutinin (non-legume)*

100 g of wheat germ defatted seed powder was stirred overnight with 0.05% sodium acetate buffer at pH 4.5 at 4 °C. To the crude extract obtained after centrifugation, 40% ammonium Sulfate was added and the protein pellet obtained was dissolved and dialyzed against phosphate buffered saline (PBS, pH 7.4). The protein was applied on an affinity gel Sepharose-DVS-N-acetylglucosamine. The gel washed with buffer and eluted using 0.5 M N-acetyl glucosamine in PBS. Absorbance was measured at 280 nm. Peak fractions were pooled, dialyzed against water, lyophilized and stored at -20 °C.

*Purification ofunio lectin from whole animal tissue (animal lectin)*

200 g of *unio* tissue was homogenized in 25 mM Tris-HCl buffer pH 8.0 containing 50 mM sodium chloride (TBS) and the extract clarified by centrifugation in cold. To the clear supernatant solid ammonium Sulfate was added to 80% saturation. The suspension was clarified by centrifugation and the pellet so formed was dialyzed against TBS and applied on Sepharose-lactose gel equilibrated with TBS. After extensively washing the gel with TBS, bound lectin was eluted with 0.2 M lactose in buffer. Protein containing fractions were pooled, dialyzed against water and stored at -20 °C.

*Purification of Mannose specific lectin from Dolichos lablab seeds*

100 g of defatted bean powder was suspended in TBS and stirred overnight at 4 °C. The crude extract obtained after centrifugation was subjected to 60% ammonium Sulfate saturation. The suspension was clarified by centrifugation and the pellet was dissolved and dialyzed against TBS. This was applied on Sepharose-mannose gel equilibrated with TBS. After extensively washing the gel with TBS, bound lectin was eluted with 0.25M glucose in TBS. The lectin was dialyzed against water and lyophilized and stored at - 20 °C.

The Galactose specific lectin from these seeds was purified as described in chapter 2.

**Preparation of lectin-Affigels**

Affinity purified lectins obtained above were immobilized to Affigels (Bio-Rad labs) following the instructions of the manufacturer. Coupling of purified lectins to affigels was carried out at 4 °C. For each lectin 4 mL of affigel -10 was washed successively with ice cold isopropanol, water and then with 0.1 M Hepes buffer.

Purified lectins (10 mg/mL) were dialyzed against 0.1 M Hepes and the corresponding ligand (0.1M) that is inhibitory to the lectin was added. The lectins were separately coupled overnight to affigeI-10. The unbound fraction was then collected and stored. To **the** gel, 0.2 mL of 0.1 M ethanolamine~HCl pH 8.0 was added, followed by Hepes **buffer** to attain a final concentration of 0.1 M. The gel was allowed to rotate for 1 hour at 4 °C. The gel was finally washed with phosphate buffered saline and stored at 4 °C until use. The amount of lectin bound to each of the affigels was calculated by measuring the protein concentration in the unbound fraction according to manufacturer's instructions.

**Inhibition of fungal growth by the lectins**

**Petri** plates containing potato dextrose agar were inoculated with *Thchodenna viridae, Aspergillus flavus* and *Aspergillns niger.* Simultaneously, wells were drilled in the plates about 3 cm from the centre and filled with 100 jig of Glucose/mannose specific lectin, Galactose specific lectin. Wheat germ lectin, Concanavalin A and the *imio* lectin. The plates were kept in dark at 25 °C. The inhibition of fungal growth was assessed by the growth of the fungal colony on the plates.

**Mitogenic stimulation of lymphocytes by the lectins**

***Cell preparation:*** Lymphocytes were isolated from heparinised human peripheral blood by centrifugation over histopaque solution (specific gravity 1.077) (Parish *et al.,* 1974). 8 mL of venous blood was collected and was put into a 30 mL tube containing 6 units of heparin per mL of blood and was mixed gently. The blood sample was kept under refrigeration until use. The blood sample was diluted with an equal volume of normal saline. 8 mL of diluted blood was layered over 3 mL of histopaque solution. Care should be taken to prevent the mixing of the two solutions. The tubes were centrifuged for 25 minutes at 2000 rpm. Supernatant containing the plasma was carefully removed with a pasteur pipette without disturbing the interface which contains the lymphocytes and platelets. Then, the interface was collected. The cell suspension was then transferred to RPM1-1640 containing 5% FCS. The cells were sedimented by centrifugation at 2000 rpm for 10 minutes and washed once more with the medium and then stored in ice until use.

***Lymphocyte count:*** Lymphocyte suspension was diluted appropriately in Turk's solution. Using a micropipette a small quantity of the diluted cell suspension was

loaded into the counting chamber. The cells were allowed to settle for a couple of minutes and then counting was done under 10X magnification.

$$\text{Number of cells/mL} = \frac{\text{average number of cells/large square} \times 10^4}{\text{Dilution}}$$

*Determination of cell viability:* Four parts of 0.2% Trypan blue is mixed with one part of 5 X saline. To one part of the Trypan blue - saline solution, one part of cell suspension was added (1:2 dilution). The cells were loaded on a hemocytometer and the number of unstained and stained cells was counted separately. The viable cells were counted using the formula

$$= \frac{\text{Average number of viable cells in large square} \times 10^4/\text{mL}}{\text{Dilution}}$$

$$\%\text{ of viability was counted as } \frac{\text{number of viable cells} \times 100}{\text{number of viable cells + dead cells}}$$

*Mitogenic stimulation:* This was done following the method of Bradley (1979). Peripheral blood lymphocytes ($2 \times 10^5$/ 0.2 mL) were cultured in triplicate in RPMI-1640 medium supplemented with 5% fetal calf scrum. 5 (ig of the lectins (the galactose specific lectin and the glucose / mannose specific lectin) were added to these cells. In addition, wheat germ agglutinin was also taken as the control. The cultures were incubated in an atmosphere of 5% $CO_2$ for 72 hours. For the last 24 hours of culture, the cells were pulsed with 1 uCi of $^3$H-thymidine. The cells were harvested onto glass-fiber filters using Skatron automatic cell harvester. The incorporation of radioactivity

into DNA was determined in a Beckman scintillation counter. The values are expressed as cpm per $10^6$ cells.

In order to determine the sugar specificity of the mitogenic stimulation by the glucose / mannose specific lectin and the galactose specific lectin, they were prc-incubated in the presence of specific sugars, that is 0.2 M mannose and 0.3 M galactose, respectively, for 24 hours. Then, the lectins were assessed for mitogenic activity as described above.

*Glucose consumption during mitogenic stimulation:* Mitogenic stimulation on the lymphocytes by the lectins was also determined by a modification of the glucose consumption method described by Hori *el al.,* (1987). Similar cultures were set up as described above. At 24 hour intervals, the glucose concentration in the supernatant in each well was estimated by the Phenol - sulphuric acid assay. The glucose consumption by the lymphocytes is determined by subtracting the concentrations of glucose obtained at different intervals of time from that obtained initially.

Interaction of the galactose specific lectin with normal human peripheral **blood** lymphocytes and multiple myeloma cells

Lymphocytes have been prepared as described as above.

*Preparation of FITC labeled lectin:* This was prepared according to Clark and Shepard (1963). About 1% of the galactose specific lectin in 0.025 M $Na_2CO_3$ and 0.025 M $NaHCO_3$ was dialyzed against fluorescein isothiocyanate (0.1 mg/mL) in the same buffer. After 24 hours the conjugated galactose specific lectin was dialyzed against phosphate buffered saline, pH 7.3 until fluorescence was no longer detected in the dialysate. The conjugate was then ready for use.

***FITC - lectin binding*** *assay:* Human peripheral multiple myeloma cells (U266 cells) and normal human peripheral blood lymphocytes (0.1 mL, 3x $10^6$) were incubated with FITC conjugated to galactose specific lectin (0.1 mL) for 15 minutes at room temperature (Reisner *el al.,* 1979). The cells were then washed with PBS and finally suspended in 0.1 mL of the same buffer. The percentage of fluorescent cells was determined for the two sets of lymphocytes under a fluorescent microscope.

**Interaction of the lysosomal enzymes secreted from cultured MPR (-) cells with the lectins coupled to affigels**

**MPR** (-) cells were cultured in Dulbecco's modified eagle medium, DMEM, containing 10% fetal calf serum. Cells were grown in standard flasks, as well as in 3 cm petri plates in an incubator, at 37 °C, in an atmosphere containing 5% CO2 as described by Matzner *et al.,* (1996). Later, the cell secretions were concentrated by ammonium sulphate and the protein pellet dialyzed extensively against TBS, pH 7.4. This protein (2 mg/mL) was loaded on the different lectin affigels pre-equilibrated with TBS, pH 7.4. The gels were then washed with the same buffer and the bound protein was eluted using specific sugars that is: 0.3 M mannose on glucose/mannose lectin affigel; 0.3 M galactose on galactose lectin affigel column; and 0.3 M N acetyl glucosamine on wheat germ lectin column.

Aliquots of the eluted fractions obtained from the different columns were analyzed for (3-N -acetyl hexosaminidase and cx-mannosidase activities. 10% SDS - PAGE was carried out for all the peak fractions obtained from the above columns and the protein bands identified by silver staining. In another experiment, the eluted fractions were separated on a 10 % SDS-PAGE, the proteins transferred to a nitrocellulose membrane,

and the membrane blotted using Con A-Biotin. The membrane was developed using Avidin-ALP conjugate and the bands detected using the substrate BC IP/NBT. The eluates from the galactose lectin-affigel and the glucose/mannose lectin affigel were also analyzed on a 10 % SDS-PAGE, and the proteins transferred to a nitrocellulose membrane. The membrane was probed separately with the bovine a-mannosidase antibody and the p N-acetylhexosamindase antibody. The blot was developed using standard protocols as described earlier in the thesis for the detection of protein bands.

**Interaction of the glycoproteins presents in normal human serum with the lectin - affigels**

Normal human scrum was dialyzed against TBS pH 7.4 and was applied separately on the galactose affigel, glucose/mannose lectin affigel, wheat germ lectin affigel equilibrated with TBS. After washing the gels with TBS, the gels were eluted specifically with 0.3 M galactosc and 0.3 M glucose and 0.5 M N-acetylglucosamine respectively. The protein in the eluates from the gels was monitored at 280 nm and aliquots from the peak fractions were separated on 10 % SDS-PAGE and identified by silver staining. In a separate experiment the eluted fractions from the galactose lectin affigel and the glucose/mannose lectin affigel were separated by SDS-PAGE and then proteins transferred to nitrocellulose membrane. The membrane was probed with antibodies to bovine a-mannosidase and the (3 N-acetylhexosamindase to identify if there are any glycoproteins that bind to these lectins specifically.

**Results**

Table 14 shows the different lectins purified by affinity chromatographic procedures and the concentration of the lectins bound to the affigels. Fig. 23 shows the growth of the fungi, *Trichoderma viridae, Aspergillns niger* and *Aspergillus flavus* in the presence of the lectins - Con A, glucose/mannose specific lectin, galactose specific lectin, wheat germ lectin and *imio* lectin. None of the lectins have exerted any inhibitory effect on the fungi.

From Fig. 24, it is evident that the galactose specific lectin obtained from the seeds of the *Dolichos lablab* did not exhibit any mitogenic activity to the human peripheral **blood** lymphocytes. In contrast, the glucose / mannose specific lectin from the same seeds exhibits mitogenic stimulation several fold at a concentration of 5 fig. This stimulation by the glucose / mannose lectin is 10,000 fold higher in activity (cpm / $10^6$ cells) when compared to the control mitogenic lectin, wheat germ agglutinin (Greene *et al.,* 1981). In the presence of 0.2 M mannose, the mitogenic stimulation of the glucose / mannose lectin reduced considerably, indicating the specificity of the mitogenicity. Further in the presence of glucose/mannose specific lectin, there was an increase in the glucose consumption with the increase in the time of incubation. On the **other hand the** galactose specific lectin showed negligible amount of glucose consumption. The control, wheatgerm agglutinin showed glucose consumption to the same extent as the glucose/mannose specific lectin. (Fig. 25).

Fig. 26 shows the microscopic view under the fluorescence microscope of the F1TC labeled galactose specific lectin that is bound to the normal human peripheral

lymphocytes and to the human peripheral multiple myeloma cells, U266. The galactose specific lectin binds to few normal human peripheral blood lymphocytes (40%) (Fig. 26 A), but it binds to most of the multiple myeloma cells (95%) (Fig. 26 B).

The results of the interaction of the lysosomal enzymes secreted from cultured MPR (-) cells with the lectins coupled to affigel are shown in Fig. 27. (A) shows a 10% SDS-PAGE of the proteins obtained from the MPR (-) cell secretions that were specifically bound to the lectin-affigels and eluted from these gels using the corresponding sugar ligand. Protein bands were detected by silver staining. These proteins in the eluates were also identified by Western blot analysis, using Con A-biotin (Fig. 27 B).

The eluates from the galactose lectin affigel and the glucose/mannose lectin affigel were also analyzed by Western blot analysis using the bovine ot-mannosidase antiserum (Fig. 27 C). While no protein band could be detected from the galactose lectin affigel, a single protein band corresponding to Mr 200 kDa was detectable in the glucose/mannose lectin affigel eluates and Fig. 27 D shows the results with p N-acetyl hexosaminidase antiserum. With the galactose lectin affigel eluate, a single protein band at Mr 66 kDa was detectable and no protein band could be detected in the eluates from the glucose/mannose specific lectin affigel.

The results on the interaction of the glycoproteins present in normal human serum with the lectin affigels are shown in Fig 28. (A) shows a 10% SDS-PAGE of the proteins (silver staining) from the normal human serum that bound on the lectin affigels. Two distinct protein bands were detectable in the molecular range of 66 and 55 kDa that were eluted from the galactose lectin affigel and glucose//mannose lectin affigel. However, only a single protein band corresponding to Mr 66 kDa was detectable in the

eluates from wheat germ lectin affigel. Fig. 28 B shows the blot of the eluted fraction from the galactose-afflgel and glucose lectin affigel column probed with a-mannosidase antibody. While, three protein bands in the range of 66 kDa, 40 kDa and 35 kDa were detectable in the galactose lectin aftlgel eluate, no protein bands could be detected in the glucose/mannose lectin-affigel eluates. Fig. 28 C shows the blot of the eluted fraction from the galactose lectin specific affigel and glucose/mannose specific lectin affigel column probed with p-N acetyl hexosaminidase antibody. While a single protein band in the region of 66 kDa was detectable in the galactose lectin affigel, no protein band was detectable in the glucose-mannose lectin affigel eluate.

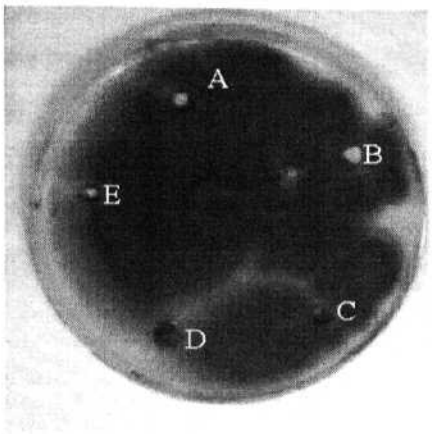
**Table 14:** Affinity purified lectins and the concentration of lectins bound to affigels

| Lectins | Sugar specificity | Native molecular mass and sub unit structure | Affinity matrix | Cone, of protein bound to the affigel (per mL gel) |
|---|---|---|---|---|
| *Canavalia ens if or mis* | Glucose/ mannose | 106 kDa $tx_4$(25) | G-100 | 6.75 mg |
| *Dolichos lablab* | Glucose/mannose | 60kDa $oc_2$(15), (32(12) | Scpharose -Mannose gel | 7 .75 mg |
| *Dolichos lablab* | Galactose | 120kDa $a_2$(31). $P_2$(29) | Sepharose -galactose gel | 4.67 mg |
| Wheat germ lectin | N-acetyl glucosamine | 36 kDa $cx_2$(18) | Sepharose-N-acetyl glucosamine gel | 5.9 mg |
| *Unio* lectin | Lactose | 105 kDa $a_2$(28), P2(23) | Sepharose-Lactose gel | 7.43 mg |

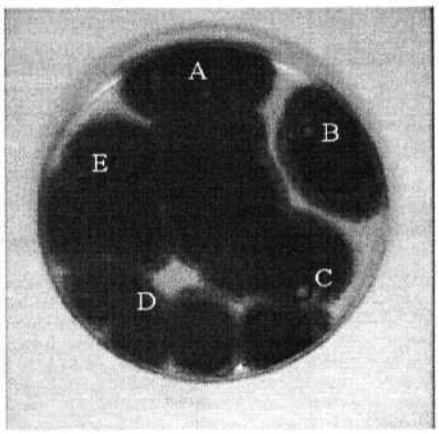
Table 14

**Figure 23: Effect of lectins on the growth of the fungi**

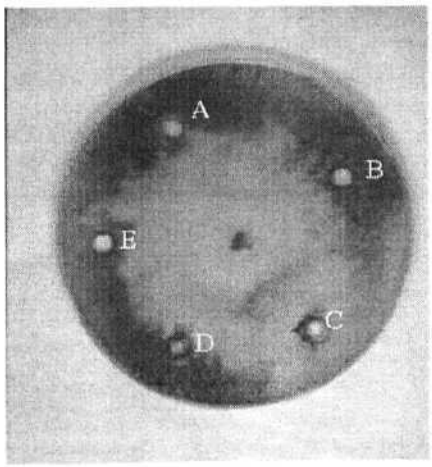
A) *Aspergillus jlavus* B) *Aspergillus niger* C) *Trichoderma viridae* Well A contains Con A, well B contains glucose/mannose specific lectin, well C contains galactosc specific lectin, well D contains wheat germ lectin and well E contains *unio* lectin




A




B




C

**Figure 23**

**Figure 24: Mitogenic stimulation of lymphocytes by the lectins**

The amount of $^{3}$H-Thymidine incorporated into the lymphocytes is measured in a scintillation counter. Panel A indicates the response of the lymphocytes in the presence of Galactose specific lectin, B in the presence of Glucose/mannose specific lectin , C in **the** presence of Wheat germ lectin

**Figure 25: Consumption of glucose by the lymphocytes in the presence of lectins**

Circles indicate the glucose consumption by the lymphocytes in the presence of Galactose specific lectin, squares indicate the glucose consumption by the lymphocytes in the presence of Glucose/mannose specific lectin, triangles indicate the glucose consumption by the lymphocytes in the presence of Wheat germ lectin.

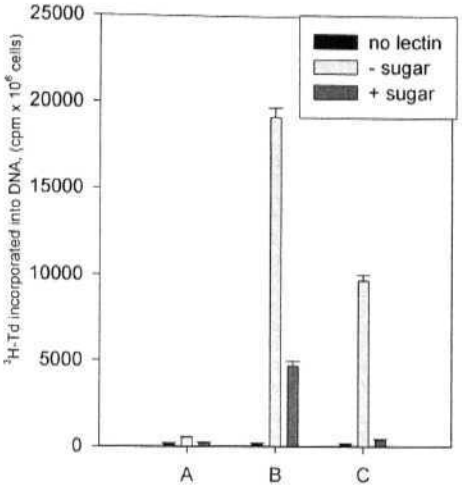



**Figure 24**

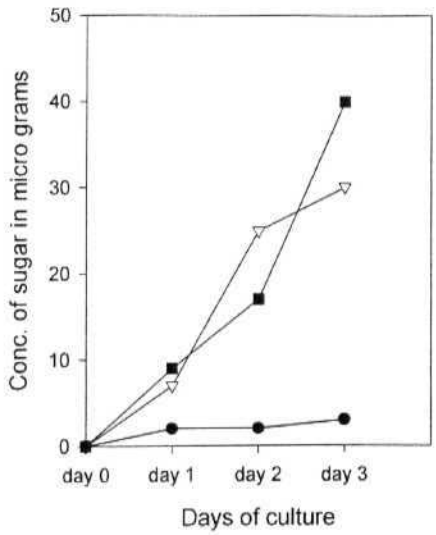
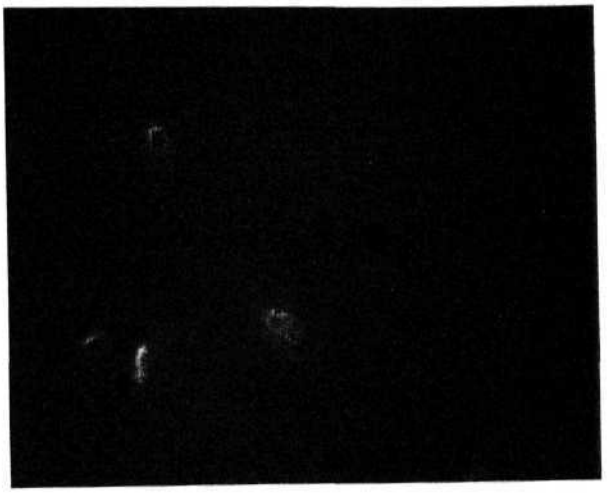



**Figure 25**

Figure 26: Flourcscence microscopy of FITC-galactose specific lectin in sections of A) Normal peripheral blood lymphocytes and B) Human peripheral Multiple myeloma cells (1)266).



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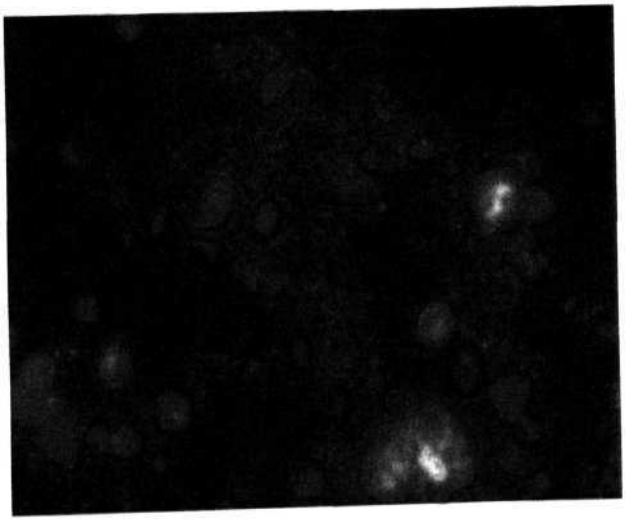


B

**Figure 26**

**Figure 27: Detection of glycoproteins in the secretions of Mannose 6-phosphate deficient cells.**

**A. 10% SDS-PAGE of the Mannose 6-phosphate deficient [MPR (-)] cell secretions, bound and eluted from the lectin afigels.** Lane 1: molecular weight markers, Lane 2 : Galactose lectin affigel eluate, Lane 3 : Glucose/mannose lectin affigel eluate, Lane 4 : Wheat germ lectin affigel eluate.

**B. Con** A - **Biotin blot of the MPR (-) cell secretions eluted from the lectin affigels.** Lane 1: Galactose lectin affigel eluate, Lane 2: Glucose/mannose lectin affigel eluate, Lane 3 : Wheat germ lectin affigel eluate.

**Western blot of the MPR (-) cell secretions bound and eluted from the lectin affigels to detect for the presence of**

**C) a-mannosidase, using bovine mannosidase antibody and D) B N-acetyl hexosaminidase.**

Lane 1: Galactose lectin affigel eluate. Lane 2: Glucose/mannose lectin affigel eluate.

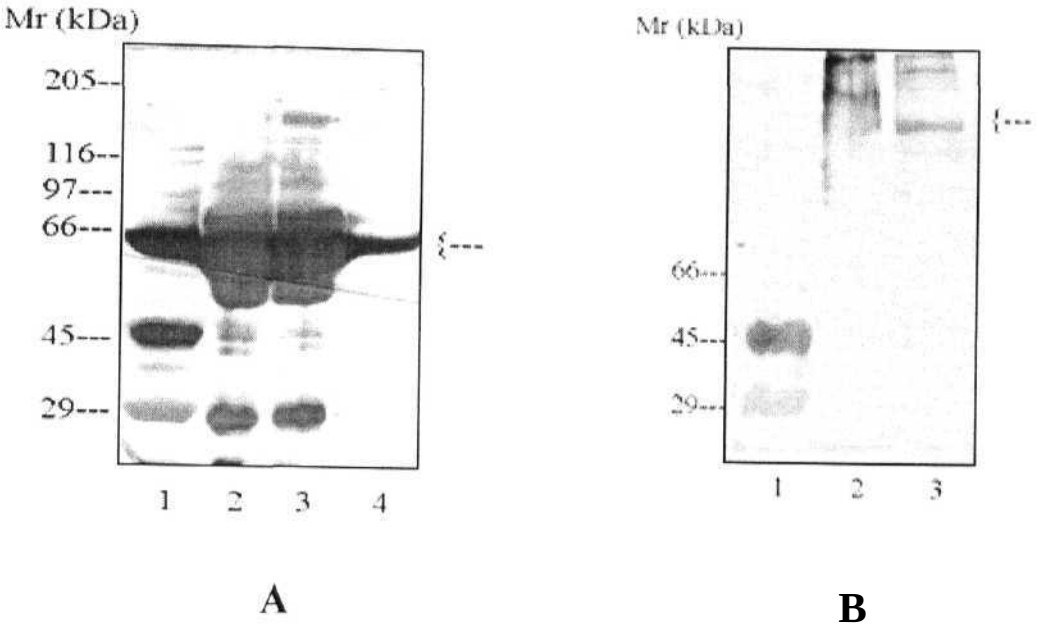

Mr (kDa)
205---
116---
97---
66---
45---
29---
1 2 3 4
A
Mr (kDa)
66---
45---
29---
1 2 3
B
Mr (kDa)
205---
116---
97---
66---
45---
29---
18---
1 2
C
Mr (kDa)
66---
45---
18---
1 2
D

**Figure 27**

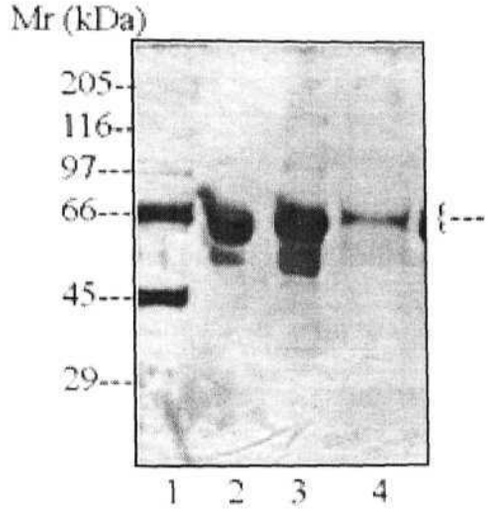
**Figure 28: Detection of glycocomponents in normal human serum.**

**A. 10% SDS-PAGE of the human serum eluted from the lectin afigels.**

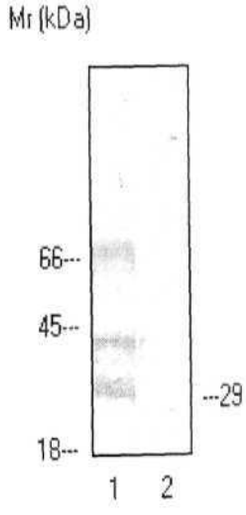
Lane 1: molecular weight markers, Lane 2 : Galactose lectin affigel eluate, Lane 3: Glucose/mannose lectin affigel eluate. Lane 4 : Wheat germ lectin affigel eluate.

**Western blot of the normal human serum eluted from the lectin affigels to detect for the presence of B) a-mannosidase and C) 15 N-acetyl hexosaminidase.**

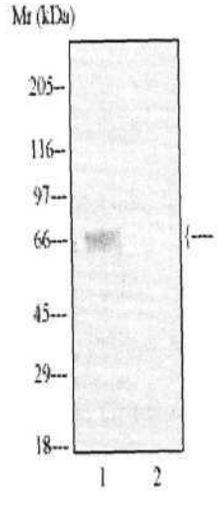
Lane 1: Galactose lectin affigel eluate, Lane 2: Glucose/mannose lectin affigel eluate.




A




B




C

Figure 28

**Discussion**

Mannan, chitin and other saccharides are important components of most of the fungal cell walls. It is believed that most of the antifungal lectins cross-link with these carbohydrates preventing cell expansion at the growing hyphae. This binding could slow hyphal growth as a **first** line of integrated defense mechanism (Chrispeels and Raikhel, 1991). Some known examples of antifungal lectins are WGA, (Mirelman, 1975; Ciopraga *et al.,* 1999) TEL (Maria das Gracas *et al.,* 2002), GAFP-1, from *Gastrodia elata* (Xu *et al.,* 1998); UDA from Stinging nettle (Van Parijs *et al.,* 1992). WGA binds to all fungi, except for those that are lacking chitin. Con A, however, binds poorly to these residues which are present on the cell walls. SBA and PNA bind to *Pencillia* and *Aspergilli* strongly suggesting the presence of D-galactose on their surfaces. These studies show that the lectins in plants are a part of their protection system helping them to combat attack by fungal pathogens.

Since none of the lectins tested in this study show any antifungal activity towards any of the fungi studied, it is thereby concluded that the lectins tested are poor binders of polysaccharides present on the cell walls of fungi.

Lectins have the ability to trigger quiescent, non-dividing cells into a state of growth and proliferation. Several lectins purified have been shown to be potent mitogens (Lis and Sharon, 1977; Tsuda, 1979). In 1990, lentil seed storage proteins that have been affinity purified on immobilized lentil lectin have been found to be strong stimulators of murine B lymphocyte proliferation (Freier *et al.,* 1990). The galactose specific lectin from *Dolichos lablab* is found to be unusual as it does not stimulate proliferation of

lymphocytes, unlike other lectins. The mitogenic stimulation by the lectin is also confirmed by the glucose consumption by the lymphocytes, which is a measure of blastogenesis of the cells. In the presence of the galactose specific lectin, the lymphocytes consume negligible amount of glucose, indicating that there was no appreciable mitogenesis. But, in the presence of the glucose / mannose specific lectin, there was an increase in the glucose consumption with the increase in number of days. This increase was almost similar to that observed by the wheat germ agglutinin, a known mitogen. This shows that the glucose / mannose lectin does act as a mitogenic lectin.

FITC conjugated lectins bind more to multiple myeloma cells than to normal human lymphocytes. This indicates that they are more of carbohydrate binding sites in the myeloid series than in the normal lymphocytes. Thus, lectins may serve as targets for drug binding and enable a selective destruction of the pathologic cells. The lectins if bound to a drug may direct and concentrate the drug on the target cells. The use of Duanomycin bound to Con A has been demonstrated in a mouse model system (Kittao and Hattori, 1977). The validity of PNA as a marker of immature blood cells and its potential clinical application have been discussed by Reisner *et al.*, (1979). FITC conjugated PNA binds to exposed carbohydrate surfaces present on the malignant colonic mucin epithelium. (Boland *et al.*, 1982). Thus, from the present study using FITC, the galactose specific lectin can be used as tracers of malignant formations.

Lectins in free form are relatively crude probes for detecting differences in the fine structure of glycoconjugates. Immobilization of lectins covalently bound to affigels are useful tools for the purification of glycoproteins. As mentioned earlier, the laboratory

where this work has been carried out has been studying the evolution of mannose 6 phosphate receptors that mediate the transport of lysosomal enzymes to lysosomes and the cells lacking these receptors [MPR (-) cells] secrete their lysosomal enzymes. So, these cells were cultured in the lab following the standard published protocols. All lysosomal enzymes are known to be glycoproteins and contain N-glycan structures that can be recognized by Con A. In order to analyze if these secretions contain some proteins that can specifically bind to the *Dolichos lablab* lectins, we allowed the secretions containing the proteins to be passed through the lectin affigels prepared. As an additional lectin we chose wheatgerm lectin-affigel. From the results it is apparent that the lectins from the *DoUchos lablab* and the whealgerm can preferentially bind some proteins that are present in the MPR (-) cell secretions. It is apparent from the results that the proteins eluted can be recognized also in a Con A-biotin blot suggesting them to be glycosylated. On the other hand, the eluted protein from the galactose lectin affigel could be recognized by [3 N-acetylhexosaminidase antibody. The glucose/mannose lectin affigel eluate however could be recognized by the bovine a-mannosidase antibody.

When normal human serum was applied on the galactose lectin affigel, glucose/mannose affigel and wheatgerm agglutinin-affigel, some proteins bound on these gels which could be specifically eluted with the respective sugar ligands. The eluted protein peak fraction from both the gels was analyzed by SDS-PAGE as duplicates, and the proteins transferred to nitrocellulose membrane. One portion of the membrane containing eluates from both the gels, was probed with the bovine a-mannosidase antibody, while the other with the p N-acetyl hexosaminidase antibody.

Interestingly, when the a-mannosidase antibody was used, some protein bands could be detected on the blot. Their molecular sizes are much smaller than the lysosomal enzyme. Since these experiments were performed using human serum, it is likely that these proteins may be the constituents of normal human serum that are being recognized by this antibody. From the results obtained it is difficult to ascertain the nature/function of these proteins now. On the other hand, the P N-acetyl hexosaminidase antibody recognized a single protein species that elutcd from the galactose lectin affigel. From these different results, it is logical to conclude that the *Dolichos lablab* lectins can have potential applications in the isolation and characterization of some glycoproteins.

# Chapter 5

# Molecular cloning and partial sequencing of galactose specific lectin

**Introduction**

The complete primary structures of a number of well characterized lectins have been determined. From these studies, it appears that most of the leguminous lectins exhibit extensive sequence homologies. The metal binding sites have been highly conserved among the various lectins studied. All the amino acids that interact directly with the metal ions $Ca^{2+}$ and $Mn^{2+}$ in Con A are among the identical residues ($Glu^{8}$, $Asp^{10}$, $Asn^{14}$, $Asn^{19}$, $His^{24}$, $Ser^{84}$). The amino acids that contribute to the 3 D structure of the hydrophobic cavity in the homologous positions within the sequences have been highly conserved through evolution. This supports an essential role in the function of plant lectins. For example, it has been seen that Con A can bind plant auxins (Edelman and Wang, 1978). It has also been noted that the glycosylation sites in some lectins have been conserved.

The amino acids that constitute the sugar binding site in Con A have been poorly conserved in other lectins. (Hardman and Einsworth, 1976). For instance, $Asp^{208}$, is the only amino acid conserved. Based on the homologous sequences, it is presumed that there may be a close resemblance in the folding and 3 D structure of the proteins. It appears that most of the legume lectins are similar in their 3 D structure (Olsen, 1983). Thus, lectin structures have been highly conserved in evolution presumably for ensuring the maintenance of important physiological functions yet to be characterized.

As mentioned earlier in this thesis, the seeds of *Dolichos lablab* used in this study has two distinct sugar specific lectins. They are glucose/mannose specific lectin and the galactose specific lectin. The properties of the glucose/mannose specific lectin from these seeds are identical to those studied earlier (Siva Kumar and Rajagopala Rao,

1986). The complete primary structure of the glucose/mannose specific lectin has been determined (Gowda et al., 1994). The peptides used for sequencing were obtained by enzymatic and chemical cleavage. The galactose specific lectin shows cross-reactivity to the antisera raised to the glucose/mannose specific lectin and vice versa. This indicates that there may be conserved homologous sequences between the two lectins. In order to detect these sequences the present study was undertaken to determine a partial cDNA of the galactose specific lectin. Availability of the full length sequence of this lectin at a later date would be useful in the determination of amino acids involved in sugar binding as well as in the determination of the 3 D structure of this lectin.

## Materials

### Instruments

| | |
|---|---|
| Eppendorf thermocycler | |
| Transilluminator Model IL-400-M | Bachofer, Reutlingen |
| UV-Hand lamp (312 nm and 254 nm) | Bachofer, Reutlingen |
| 373 A DNA sequencing system | Applied Biosystems |

### Chemicals

| | |
|---|---|
| Agar | Sigma |
| Agarose (Electrophoresis grade) | G1BCO/ BRL |
| Bacto Yeast extract | Difco |
| Bacto Tryptone | Difco |
| Dextransulfate | Pharmacia |
| Diethyl pyrocarbonate | Sigma |
| Ethidiumbromide | Serva |
| Formamide | Fluka |
| p-Formaldehyde | Merck |

### Kits Used for Molecular Biological Work:

| | |
|---|---|
| QIAgen plasmid mini kit | Qiagen |
| QIAquick gel extraction kit | Qiagen |
| TA cloning kit | lnvitrogen |
| Hot star *Taq* polymerase PCR QIAgen kit | Qiagen |
| Random primer DNA labeling kit | Amersham, |

| | |
|---|---|
| QIAgen RNeasy total RNA isolation kit | Qiagen |
| First strand cDNA synthesis kit | Qiagen |

**Enzymes for Molecular Biological Work:**

| | |
|---|---|
| Alkaline phosphatase | Sigma |
| $T_4$ DNA ligase | New England Biolabs |
| Restriction enzymes | New England Biolabs |

**Plasmid DNA Vector:**

| | |
|---|---|
| pCR 2.1 Topo cloning vector | Invitrogen |

**DNA Standards:**

| | |
|---|---|
| DNA-Ladder | G1BCO/BRL |

**Reagents Used for Molecular Biology Work:**

All the following reagents were prepared according to Sambrook *et al.,* 1989.

*50 x TAE:* 2 M Tris-Base, 0.1 M EDTA, pH was adjusted to 8.0 with acetic acid.

*TE:* 10 mM Tris-HCl pH 7.5 and 1 mM EDTA.

***50 x Denhardt Solution:*** 5 g of Ficoll, 5 g of Polyvinylpyrrolidine and 5 g of BSA, Volume was adjusted to 500 mL with deionized water.

*/ x* ***SM Buffer:*** 5.8 g of NaCl, 2.0 g of $MgSO_4.7H_2O$, 50 mL of 1 M Tris-HCl (pH 7.5) and 5.0 mL of 2% (w/v) gelatin. Volume was made up to 1 litre with deionized water, autoclaved and used.

***LB medium:*** 10 g of NaCl, 10 g of Tryptone, 5 g of Yeast extract and pH was adjusted to 7.0 with 5 N NAO11. Final volume was made up to 1 litre with deionized water, autoclaved and used.

*LB agar (per liter):* 10 g of NaCl, 10 g of Tryptone, 5 g of Yeast extract, 20 g of agar. The pH was adjusted to 7.0 with 5 N NaOH and the final volume was made up to 1 litre with deionized water. This was autoclaved and poured into petri dishes (25 mL/10 cm plate or 70 mL/ 14.5 cm plate).

*20 x SSC Buffer:* 175.3 g of NaCl, 88.2 g of Sodium citrate, 800 mL of deionized $H_2O$. The pH was adjusted to 7.0 with a few drops of 10 N NaOU and finally made upto 1 litre with deionized water.

*Preparation of Antibiotics:* 25 mg/ mL stock solution of the sodium salt of Ampicillin in water; pH was adjusted to 8.0 with 2 N NaOH. This was sterile filtered and stored in aliquots at -20 °C.

*Plates with Appropriate Antibiotic:* LB-Agar was autoclaved and cooled to 55 $^0$C. Ampicillin was added to final 200 fig / mL concentration and poured into petri plates.

*Denaturation Buffer:* 1.5 M NaCl, 0.5 M NaOH, 81.6 g NaCl and 20 g of NaOH were dissolved in deionized water and the final volume was made up to 1 liter.

*Neutralization Buffer:* 1.5 M NaCl, 0.5 M Tris-HCl, pH 7.2; 1 mM EDTA. lOx Tris-buffer (100 mL) was prepared. To this NaCl and EDTA (pH 8.0) were added to a final concentration of 1.5 M and 1 mM concentration, respectively. The volume was made upto to 1 liter.

***High Stringency Prehybridization Buffer:*** 48% Formamide, 4.8x SSC, 10 mM Tris-HCl pH 7.4, 1% SDS, 1% Denhardt solution, 10% Dextran Sulfate, 100 ug/ mL of Salmon sperm DNA. This was stored at 4 °C.

*Low Stringency Prehybridization Buffer* 35% formamide, 6x SSC, 1% SDS, 1% Denhardt solution, 10% Dextran Sulfate and 100 ^ig/ mL Salmon sperm DNA. This was stored at 4 °C.

*3 M **Sodium** acetate:* 408.1 g of sodium acetate **(tli** hydrated) was dissolved in 800 mL water and pH was adjusted to 5.2 with acetic acid. Volume was made up to 1 liter with water.

***Denatured Salmon Sperm DNA;*** 10 mg / mL of Salmon sperm DNA (sodium salt) was dissolved in water and the solution was stirred on a magnetic stirrer for 2-4 hours at room temperature to dissolve the DNA. The DNA was passed through a 20 G gauze needle, boiled for 10 min (at 100°C), sonicated for 1-2 minutes and stored at -20 °C in small aliquots.

*20% **SDS** :* 20 g of Sodium dodecyl Sulfate was dissolved in 100 mL water at 65 °C and sterile filtered.

***lOx MOPS Buffer:*** 20.93 g of MOPS (200 mM), 2.05 g of NaAc (50 mM), 1.86 g EDTA (10 mM), were dissolved in 300 mL DEPC-ILO and pH was adjusted to 7.0 with 2N NaOH in DEPC-$H_2O$ (for 500 mL, 15-16 mL of NaOH required). After each step of addition of base, pH was controlled by taking an aliquot in an eppendorf tube and final volume was made up to 500 mL with DEPC-water.

***5x Loading Buffer:*** 16 uL of saturated bromophenol blue, 80 L of 500 mM EDTA, pH 8.0, 720 uL of 37% (-12.3 M) formaldehyde, 2 mL of 100% glycerol, 3.084 mL of formamide, 4 mL of lOx MOPS buffer were made upto 10 mL with RNase-free water.

***SOC medium*** *(pH 7.0):* 2.0 g of Bacto-Tryptone, 0.5 g of Bacto-Yeast Extract, 1 mL of sterile filtered 2 M $Mg^{2+}$ stock (1 M $MgCk6H_2O$/ 1 M $MgSO_4.7H_2O$) and 1 mL of sterile filtered 2M glucose.

*IPTG (isopropylthiogalactoside) stock solution (QAM):* 240 mg of IPTG was dissolved in 10 mL of deionized water, sterile filtered and stored at 4 °C.

***X-Gal:*** 100 mg of 5-bromo-4-chloro-3-indolyl-p-D-galactoside was dissolved in N, N'-dimethyl formamide, covered with aluminum foil and stored at -20 °C.

**Methods**

**Total RNA Isolation Using QIAgen Kit:**

**Important points to be considered while handling RNA:**

**Glassware:** Glassware was treated as follows before use to ensure that it is RNase free. They were filled with IN NaOH and left overnight or a minimum of l-2hours, rinsed with Millipore water followed by 0.1% DEPC in water (*Diethyl pyrocarbonate-carcinogen) and then autoclaved at 100°C for 15min to remove residual DEPC.

**Nondisposable Plasticware:** Plasticware were incubated overnight in 1 N NaOH, thoroughly rinsed with Millipore water and then with RNase free water to ensure that it is RNase free.

**Solutions:** Solutions (water and other solutions) were treated with 0.1% DEPC. DEPC is a strong, but not absolute inhibitor of RNases. It is commonly used at a concentration of 0.1% to inactivate RNases on glass or plasticware or to create RNase free solutions and water. DEPC inactivates RNases by covalent modification. 0.1 mL DEPC was added to 100 mL of the solution to be treated, vigorously stirred to bring the

DEPC into solution, and the solution was allowed to stand for 12 h at 37 °C. They were autoclaved for 15 min to remove any traces of DEPC.

**Lysis** of **tissue:** Seeds were soaked in DEPC water for an hour and the coat was removed and the cotyledons were lysed in 0.6 mL lysis buffer.

**Homogenization:** A homogenous suspension of the tissue was made by pipetting up and down and passing 5 times through a 20-G needle fitted to a syringe.

600 µL (1 volume) of 70% ethanol was added (to provide appropriate binding conditions) to the homogenized lysate and mixed well by pipetting up and down (A precipitate may form after the addition of ethanol). 700 µL of sample was applied onto RNeasy spin column sitting in a 2 mL collection tube, centrifuged for 15 seconds at 13,000 rpm (Eppendorf centrifuge). The above step was repeated for rest of the sample. The column was washed with 700 µL of wash buffer RW1 by applying the buffer on column and centrifuging for 15 seconds at 13,000 rpm. Flow through and collection tube was discarded. RNeasy spin column was transferred to a new 2 mL collection tube and washed as above with 500 µL of buffer RPE. Column was rewashed with 500 fiL of buffer RPE and centrifuged for 2 min at 13,000 rpm to dry the RNeasy membrane (*lt is important to dry the RNeasy membrane since residual ethanol may interfere with subsequent elution). Column was transferred into a new sterile 1.5 mL collection tube (supplied). 50 jiL of RNase free water was pipetted directly onto the membrane, allowed to stand for a minute and centrifuged for 1 min at 10,000 rpm to elute RNA.

**Quantitation of Nucleic acids (RNA / DNA):**

The concentration and purity of RNA/DNA was determined by measuring absorbency at 260 nm ($A_{260}$) and 280 nm ($A_{280}$) in a spectrophotometer.

* $A_{260}$=1 corresponds to 40 g/ mL of RNA or 50 g/ mL double stranded DNA. The ratio between the absorbency readings at 260 nm and 280 nm gives an estimate of purity. Pure RNA/DNA preparation will have an $A_{260}/A_{280}$ ratio of 2.0.

**Denaturing Agarose Gel Electrophoresis for RNA:**

**1%Agarose gel:**

| | |
|---|---|
| Agarose (electrophoresis grade) | 2 g |
| lOxMOPS-buffer | 20 mL |
| DEPC-water | 180 mL |

Boiled in microwave, cooled to 65°C, 1-2 uL of EtBr (10 mg/ mL) and 3.3 mL of formaldehyde (37%) added, mixed well and poured into the sealed electrophoresis trough fitted with a comb. Solidified gel was cut to the required size, placed in the electrophoresis chamber.

**Sample Preparation and Gel Run:**

RNA sample (1-5 fig) was mixed with loading buffer (1 vol. of sample buffer to 4 vol. of RNA sample), cooked at 65 °C for 5 min, chilled on ice and used for loading. Gel was run at 70 V for 3 hours.

**Agarose Gel Electrophoresis for DNA:**

**DNA** fragments were subjected to agarose gel electrophoresis for resolution

| | |
|---|---|
| Sample buffer (Loading buffer IV) | 0.25 %(w/v) Bromophenolblue |
| | 40 % (w/v) Saccharose in TAE |
| Ficoll-Marker | 0.05 % (w/v) Bromophenolblue |
| | 0.05% (w/v) Xylenecyanol |
| | 15%(w/v)Ficoll |

Depending on the percentage of the gel, agarose was weighed and added to 300 mL of TAE, cooked in microwave and cooled to 55 °C. Ethidium bromide was added to final 0.5 ng/ mL, mixed and poured into the gel trough fitted with the combs, allowed to cool to room temperature. The gel was cut into the required size with required number of wells. Sample was mixed with sample buffer (10-20% (v/v)) and loaded in the wells; gel was run for 1 to 2 hours in 1 x TAE buffer at 120-240 V depending on the size. The gel was viewed under UV transilluminator.

**Gel Documentation:**

Nucleic acids intercalated with the fluorescent dye ethidium bromide were visualized under UV light using transilluminator.

**Primer Designing:**

The parameters to be considered during primer selection are,

**Length:** 18-30 nucleosides.

**G/C Content:** 40-60%

$T_m$: $T_m$= 2 °C x (A+T) + 4 °C x (G+C). If possible design primer pairs with a difference of ± 2 °C Tm values. Optimal annealing temperature may be calculated as 5 °C below the estimated melting temperature.

Complementarity of two or three bases at the 3'ends of primer pairs was avoided to reduce primer dimer formation.

Complementary sequences within a primer sequence were avoided to reduce hairpin formation.

Primer with A or T at 3' end is avoided, as it has greater tolerance of mismatch. It is always advantageous to have G/C at the 3' end.

**The** compute program "Oligo analysis" was used for primer designing.

Once the primers were selected, blast search was done to see percentage homology with the known sequences.

**Reverse Transcription or First Strand cDNA Synthesis (Pharmacia kit):**

This was prepared using the kit and following manufacturer's instructions. The denatured RT product was used for PCR amplification using specific sense and anti-sense primers.

**PCR Amplification (Q1AGEN Hot Star Taq DNA Polymerase Kit):**

**Hot Star *Taq* DNA Polymerase**

Hot Star *Taq* DNA Polymerase is a recombinant 94 kDa *Taq* DNA Polymerase. Hot Star *Taq* DNA Polymerase is provided in an inactive state with no Polymerase activity at ambient temperatures. This prevents the formation of misprimed products and primer-dimers at low temperatures. Hot Star *Taq* Polymerase is activated by 15 min incubation at 95 °C. Hot Star *Taq* DNA Polymerase provides high PCR specificity and often increases the yield of the specific PCR product.

**PCR Reaction:**

Polymerase chain reaction is a method used to amplify DNA fragments of interest from the template DNA with the use of sense and anti-sense primers of specific interest

Following were used for one PCR reaction:

| | |
|---|---|
| 10 x PCR buffer | 2 \iL |
| dNTP Mix | 0.4 µL |
| Primers | 1.2 uL each of sense and anti-sense primers |
| *Hoi star Taq* DNA polymerase | 0.2 µL |

Template DNA

(First strand cDNA (5 µL) or DNA) 1. µL

Master mix was prepared as above. Template DNA was added at the end, volume was made up to 20 µL with sterile water. The PCR reaction was carried out using the thermal cycler program shown in the Table 15. The annealing temperature and others were modified depending on the $T_m$ and specificity of the primers used for amplification.

| **Reaction Step** | **Time (min)** | **Temperature (°C)** |
|---|---|---|
| Initial activation step | 15 | 95 |
| Repeated Number of Cycles | 30-35 | |
| Denaturation | 1 | 94 |
| Annealing | 45 sec | 55 |
| Extension | 1 | 72 |
| Final extension | 10 | , ..^y^. •;•;;;."J:-^ |
| Mold | a | **4** |

**Table 15: Thermal Cycler Program**

**Gel Purification of PCR Product (QIAquick Cel Extraction Kit):**

A preparative agarose gel was run; cDNA band of expected length was cut with a clean sharp scalpel. Gel slice was weighed, 3 volumes of buffer QG (solubilization and binding buffer) was added to one volume of gel (100 mg = 100 µL). The maximum amount of gel slice per Ql A quick column is 400 mg. Incubated at 50 °C for 10 min (or until the gel slice was completely dissolved). During incubation the tube was vortexed

for every 2-3 min to dissolve the gel. After the gel slice has dissolved completely, the color of the solution was similar to QG buffer colour.

1 volume of isopropanol was added to the sample and mixed. (This step increases the yield of DNA fragments between 500 bp and 4 kb). QIAquick spin column was placed in a 2 mL collection tube. To bind DNA, the sample was applied to the column, and centrifuged for 1 min. (The maximum volume of column reservoir is 800 µL). Flow through was discarded, QIAquick column placed back into the same collection tube. 0.5 mL of buffer QG was added to the column and centrifuged for 1 min (This step will remove all traces of agarose). 0.75 mL of buffer PE was added to the column and centrifuged for 1 min at 13,000 rpm. Flow through was discarded and the column centrifuged as above. Column was placed into a clean 1.5 mL microfuge tube. To elute DNA, 50 µL of sterile water was added to the center of the QIAquick column, allowed to stand for 1 min and centrifuged for 1 min at maximum speed. Eluted DNA was analyzed by analytical agarose gel electrophoresis.

TA Cloning *(Invitrogen):*

*Taq* Polymerase has a non template-dependent activity, which adds a single deoxyadenosine (A) to the 3' ends of PCR products. The linearized vector supplied in this kit has single 3' deoxythymidine (T) residues. This allows PCR inserts to ligate efficiently with the vector. The kit provides a quick, one step cloning strategy for the direct insertion of a polymerase chain reaction (PCR) product into a plasmid vector. Advantages using the kit are, one can eliminate any enzymatic modifications of the PCR product.

**Topo cloning:**

| | |
|---|---|
| Gel purified PCR product | 3.5 uL |
| Salt solution | 0.5 uL |
| pCR 2.1 Topo vector | 1.0 JIL |

The above mixture was incubated 5 minutes at room temperature for ligation.

**Transformation:**

**Before Start:**

Water bath was equilibrated to 42 °C.

A vial of SOC medium was thawed and incubated at 37°C.

Preparation of agar plates: Ampicillin LB agar plates (two plates for each ligation/ transformation) were equilibrated at 37 °C for 30 minutes. Each plate was coated with 40 UL of 40 mg/mL X-Gal (chromogenic substrate for (3-galactosidase) 60 uL of 100 mM IPTG (isopropylthiogalactoside-used to maximize the expression of genes cloned in expression vectors). Liquid was allowed to soak into the plates by incubating at 37 °C for 20-30 min.

* Plates may also be made ahead of time (2-3 hours).

**Procedure for Transformation:**

The vials containing the ligation reactions were centrifuged briefly to bring the contents down and placed on ice. To 3 uL of it, 25 uL of One Shot competent cells (TOPI OF' provided in the kit) were added and incubated on ice for 30 minutes. The remaining ligation mixture was stored at - 20 °C. Heat shock was given exactly for 30 seconds in a 42 °C water bath (*Do **not mix or** shake). The vials were removed from the water bath and placed on ice for 2 minutes. 250 uL of SOC medium (at room temperature)

**was** added to each tube. The vials were kept for horizontal shaking at 37 °C for 1 hour at 180 rpm in a rotary-shaking incubator. The vials with the transformed cells were placed on ice. 50 uL and 200 uL from each of the transformation vial was spread on separate, labeled LB agar plates containing 50 u^/ml of ampicillin, preadsorbed with X-Gal and IPTG. The liquid was allowed to be absorbed, and then the plates were inverted and placed in 37°C incubator for atleast 18 hours. Plates were then shifted to cold room for proper colour development.

The transformed cells appear white in contrast to the untranslbrmed blue colonies.

**Plasmid DNA Isolation (Q1A prep plasmid DNA Isolation kit):**

Bacterial colony (white) was picked up with a sterile toothpick and inoculated into 5 mL medium (LB medium containing 50 *ig/* mL ampicillin). From an overnight culture plasmid DNA was prepared and digested with restriction enzymes. 2 mL of overnight culture was taken into a sterile microcentrifuge tube, centrifuged at 6,000 rpm (1800 x g) for 5 min. Supernatant aspirated and pellet was recentrifuged briefly to remove all liquid. Bacterial cell pellet was resuspended in 250 u,L of buffer PI. 250 uL buffer P2 was added and the tube was gently inverted 4-6 times (solution becomes viscous and slightly clear). 350 uL of buffer N3 was added and the tube was inverted immediately but gently 4-6 times to avoid localized precipitation (solution becomes cloudy), centrifuged at 13,000 rpm for 10 min. Meanwhile, QIAprep spin column was placed in **a** 2 mL collection tube.

The supernatant from the above step was applied to the column, briefly centrifuged and flow through discarded. Column was washed by adding 0.5 mL of buffer PB and centrifuged at 13,000 rpm for 30-60 sec, flow-through was discarded. Column was

washed by adding 0.75 mL of buffer PE, centrifuged at 13,000 rpm for 60 sec, flow through discarded and centrifuged for an additional 1 min to remove residual wash buffer. QIAprep column was placed in a clean 1.5 mL microfuge tube. 50 uL of sterile water was dropped exactly in the middle, over the membrane, incubated for a minute and centrifuged for a minute at 13,000 rpm for eluting DNA.

**Digesting DNA with Restriction Endonucleases:**

About 0.5 to 1.0 ug of plasmid DNA was used for restriction analysis with restriction enzyme as specified. Volume of the DNA sample was made upto 8 uL. To this, 1 uL of Eco RI enzyme and 1 uL of 10 x buffer were added. The sample was incubated at 37 $^{0}$C for 1-2 hours. Product was analyzed by analytical agarose gel electrophoresis.

**Random Primer Labeling (Redivue random primer labeling kit):**

25 ng of gel purified DNA probe was dissolved in 45 uL of sterile water. DNA was denatured by heating at 95 °C for 5 min in boiling water bath followed by chilling on ice. Denatured DNA was added to the labeling mix (contains dATP, dGTP, dTTP, Klenow-Fragment of DNA Polymerase I, random primers), mixed gently by flicking the tube, (a $^{32}$P) dCTP (2 uL-5 uL (20-50 u€i)) offered and incubated at 37 °C for 10 min. The reaction was stopped by adding 150 ul of TE (10 mM Tris-HCl pH 8.0 and lmM EDTA). Labeled probe was separated from un-incorporated dNTPs by passing over Sephadex G-50 (500 uL) packed in lmL blue tip, centrifuged for 2 min at 3000 rpm. Second elution was done with 100 uL of TE buffer. 1/100 of eluted volume was used for cerenkov counting.

**% Incorporation**

'x'cpmx 100 = Total counts.

Calculate total Ci (1 Ci = 2.22 xl $0^6$ cpm)

%lncorporation = .Total.counts obtained (in ,i.Ci)

Radioactivity offered

1 juiCi / mL of hybridization buffer used.

**Northern Blotting (RNA Transfer):**

**Pretreatment of formaldehyde-agarose gel for RNA transfer:**

After the electrophoresis, the gel was soaked in 20 x SSC buffer for 40 min.

Note: Before assembling the gel for transfer, the gel picture with scale was taken by exposing briefly to UV at higher wavelength (312 nm).

During the transfer, gel was inverted so that the wells are facing down.

During the transfer, sides of the gel were covered with polythene cover (cut into narrow pieces) to prevent the buffer from spreading to the sides

The transfer of nucleicacids from agarose gel to hybond-N nylon membrane was done just by capillary action by assembling the gel for transfer as shown in the Overnight transfer was done at room temperature. Filter was air dried, exposed to UV light for 15 seconds, oven baked at 60 °C for 1 hour and used for hybridization.

**Hybridization:**

**Prehybridization:**

The filters or membranes (Northern or Southern blots) were placed in a cylinder, 10-20 mL of prehybridzation solution added according to the number of filters, tightly

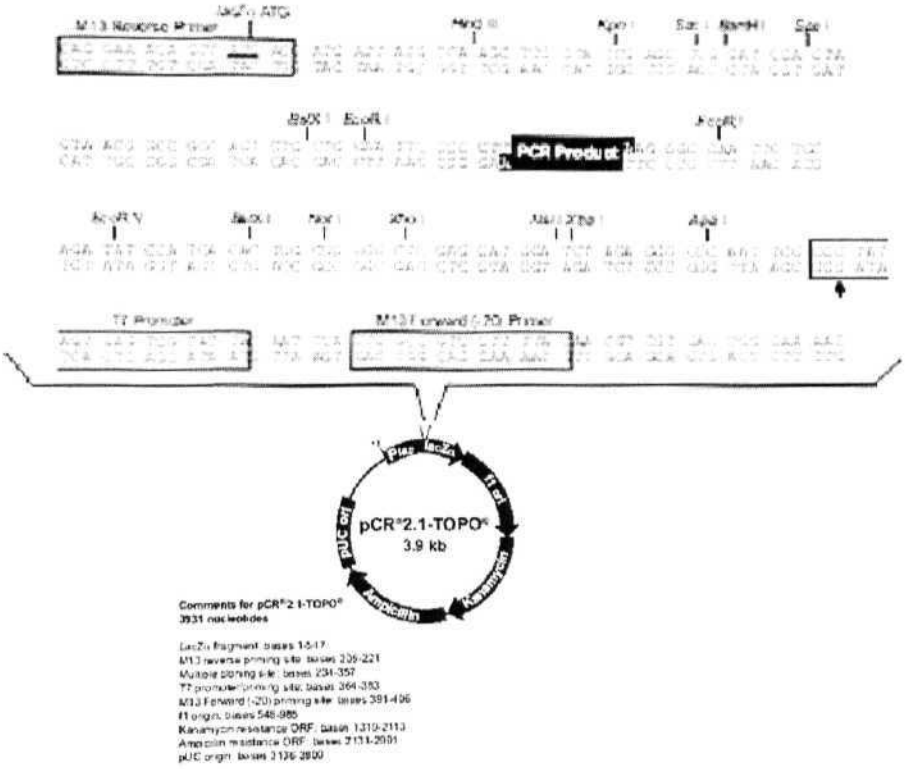

M13 Reverse Primer
PCR Product
T7 Promoter
M13 Forward (-20) Primer
pCR®2.1-TOPO®
3.9 kb
lacZα
f1 ori
Kanamycin
Ampicillin
pUC ori

**Figure 32**

**Figure 33: Undigested plasmid isolated from a positive clone**

**Figure 34: Restriction Digestion of the plasmid DNA**

Lane 1: DNA markers, Lane 3 : F2R2 insert, Lane 4 : Undigested plasmid, Lai F1R1 insert.


**Figure 33**

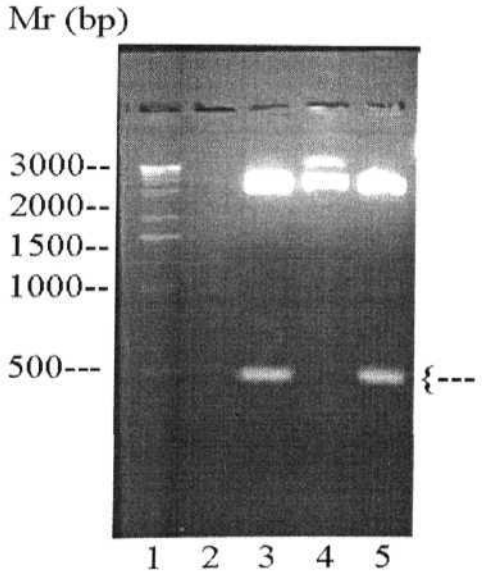



**Figure 34**

Figure 35: Sequence of FlRl fragment

Q E D L 1 F Q G H A T S T N N V F

5' CAA GAG GAT CTT ATC TTC CAA GGT CAT GCC ACT TCT ACA AAC AAT GTC TTA

N L Y K F D S A G N P V S S S A G

CAA CTC ACC AAG TTA GAC AGT GCA GGA AAC CCT GTG AGT TCT AGT GCG GGA

R V L Y S A P L R L W E D S A V L

AGA GTG TTA TAT TCT GCA CCA TTG CGC CTT TGG GAA GAC TCT GCG GTA TTG

T S F D T I I N F E I S S P Y T S

ACA AGC TTT GAC ACC ATT ATC AAC TTT GAA ATC TCA ACA CCT TAC ACT TCT

R I A D G L A F F I A P P D S A I

CGT ATA GCT GAT GGC TTG GCC TTC TTC ATT GCA CCA CCT GAC TCT GCC ATC

S Y H G G F L G L F P N X E H S

AGT TAT CAT GGT GGT TTT CTT GGA CTC TTT CCC AAC NCG GAA CAC TCT

N Q T T T K A A S S N V V A V E

AAC CAA ACC ACC ACT AAG GCT GCA TCA AGC AAC GTT GTT GCT GTT GAA

F D T Y L N P D Y G D P N Y I H I

TTT GAC ACC TAT CTT AAT CCC GAT TAT GGT GAT CCA AAC TAC ATA CAC ATC

G I D V N S'

GGA ATT GAC GTC AAC TCA 3'

**Figure 35**

Figure 36: Sequence of F2R2 fragment

G R V L Y S A P L R L W E D S

5 'GGA AGA GTG TTA TAT TCT GCA CCA TTG CGC CTT TGG GAA GAC TCT

A V L T S F D T I I N F E I S

GCG GTA TTG ACA AGC TTT GAC ACC ATT ATC AAC TTT GAA ATC TCA

S P Y T S R I A D G L A F F I A

TCA CCT TAC ACT TCT CGT ATA GCT GAT GGC TTG GCC TTC TTC ATT GCA

P P D S A I S Y H G G F L G L F

CCA CCT GAC TCT GCC ATC AGT TAT CAT GGT GGT TTT CTT GGA CTC TTT

P N A N T L N N S S T S E N Q

CCC AAC GCA AAC ACT CTC AAC AAC TCT TCC ACC TCT GAA AAC CAA

T T T K A A S S N V V A V E F

ACC ACC ACT AAG GCT GCA TCA AGC AAC GTT GTT GCT GTT GAA TTT

D T Y L N P D Y G D P N Y I H

GAC ACC TAT CTT AAT CCC GAT TAT GGT GAT CCA AAC TAC ATA CAC

I G I D V N S I R S K V T A K

ATC GGA ATT GAC GTC AAC TCT ATT AGA TCC AAG GTA ACT GCT AAG

W D W 0 N G K I A T A II I S Y

TGG GAC TGG CAA AAT GGG AAA ATA GCC ACT GCA CAC ATT AGC TAT

N S V S K R L S V T T Y Y P G S

AAC TCT GTC TCT AAA AGA CTA TCT GTT ACT ACT TAT TAT CCT GGG AGT

K P A T L S Y I D 1 E L H T V L P

AAA CCT GCG ACT CTC TCC TAT GAT ATT GAG TTA CAT ACA GTG CTT CCT

E W V R V G

GAA TGG GTC AGA GTA GGG 3 '

**Figure 36**

**Figure 37: Overlapping sequences of F1R1 and F2R2 fragments of galactose specific lectin**

**Figure 38: Partial amino acid sequence of galactose specific lectin**

Highly conserved regions have been highlighted

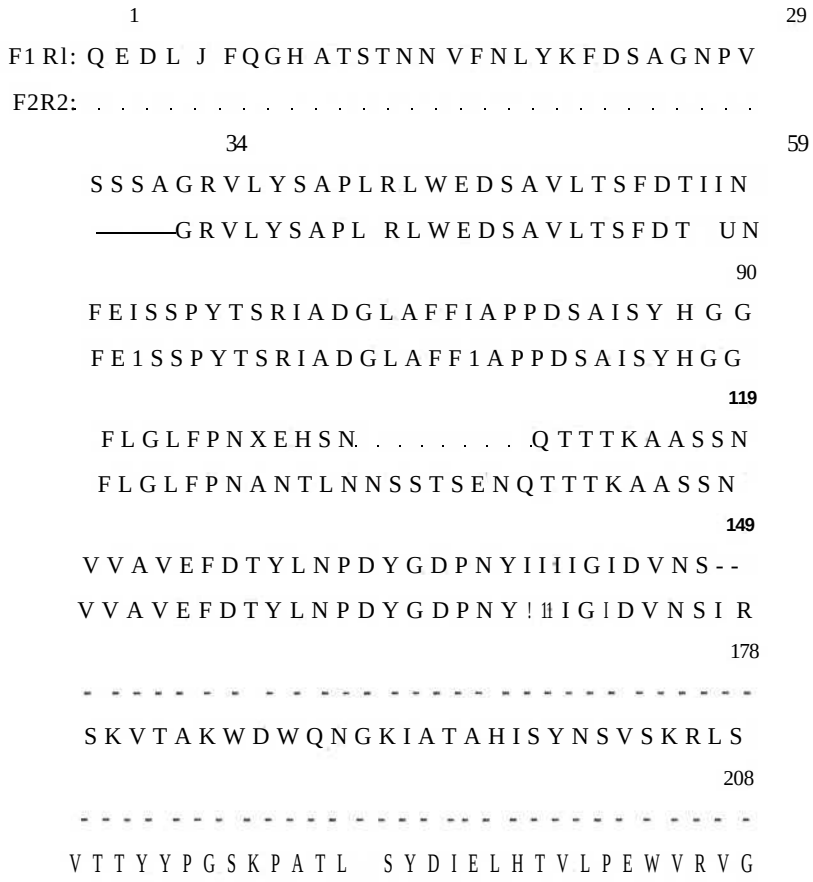



**Figure 37**

'Q E D L 1 F $^{7}$Q $^{S}$G H A T S T N N V F N L Y K F D S A G N P V S S S A $^{14}$G $^{3S}$R V L Y S A P L R L W E D S A V L T S F D T I I N F E I S S P Y T S R I A D G L A F F I A P P D S A I S Y U G G F L G L F P N A E H S N N S S T S E N Q T T T K A A S S N V V A V$^{124}$ E$^{125}$ F D T Y L N P D Y G D P N Y I H I G I D V N S I R S K V T A K W D W Q N G K I A T A H I S Y N S V S K R L S V T T Y Y P G S K P A T L S Y D I E L H T V L P E$^{204}$W V R V G$^{208}$

**Figure 38**

**Figure 39: Comparison of the Galactose specific lectin sequence (GAL)** with **other legume lectins - DLL,** *Dolichos lablab* (glucose/mannose lectin); **FBL,** *Fava bean* lectin; **PNL,** Pea nut agglutinin; **DBL,** *Dolichos biflorus* lectin; SBA, Soybean agglutinin; ECO, *Erythrina corollodendron* lectin; SL, Sainfoin lectin; **LBL,** Lima bean lectin; **LCL,** Lentil lectin; **Con** A, Concanavalin A. Multiple sequence alignment was performed according to Combet *et al,* (2000). The cDNA sequence of another *Dolichos lablab* lectin, (glucose/mannose specific) FRIL, that was found to be greater then 95 % homologous to the glucose/mannose lectin of DLL has not been included in the figure. **[Colucci,** *et al.,* (1999). cDNA cloning of FRIL, a lectin from *Dolichos lablab,* that preserves hematopoietic progenitors in suspension culture. *Proc. Nail. Acad Sci.,* U.S.A., 96, 646-650]. The DLL, galactose lectin also showed homology with the FRIL sequences, and since the complete sequence of the galactose lectin is not available here, this was not included in the figure.

```
GAL  QEDLIFQGHATS-TNNVFNl.YKFDSAGNPVSSSA
DLL  QEDLIFQCTATS. . . . . .KLDS AGNPVSSSA
FBL  QPNLIFQGGGY-TTKEKLTLT K. . . . - AV K N TV
PSL  QQNL1FQGDGY-TTKEKLTLTK. . . . .AV K N TV
DBL   SFSFILQGDATV-SSGKLQLTKVKF.NG FPLRFPS
SBAOPNM1LQGDAIVTSSGKLQI.NKD-ENGTPKPSSL
ECO NDNLTLQGAALITQSGVLQLTK1 N QN G MP A WD S T
 SL  QEN I. ILQGDTVTDDSNKRCL VI. T R HNNG R P V Q DS V
LBL AANL1LQGNAVSSK GII-LLI.TNVTHNGEPSVASS
LCL  QQNLIFQGDGYTGKEGI.TI.TKVSK1-:. . . . . .TG
CON  QKDLILQGDATVSSEGPLRLTSSVSSNGSPQGSS

GAL  GRVLYSAPl.RLWE- DSAVI.TSF
DLL  GRVLYSAPI.RLWE-DSAVl.TSF
FBL  GRALYSLPIH1 WDSETGNVADF
PSL  GRALYSSP1H1 W DRETGNVANF
DBL  GRAFYSSPIQ 1Y DKFTGAVASW
SBA  GRALYSTPIHI WDKE'ICiSVASF
ECO GRIL YAKPVHIWDMTTGTVASF
 SL  GRVLYQTPIHLWDKQ1DKEASF
LBL  GRALYSAP1Q IR D-STGNAS ST
LCL  GRALYSTPIHIWDRDTVNVANF
CON  GRALFYAPVHIWE-SSAVVASF

GAL  DT 1 INFE1SSPYTSR1-AOGLAFIIAPPDSAISY—HGGFLGLF-PNA
DLL   DPTI-YIFTNYTSR1--ADGLAF-1APPDSVISY- - HGGFLGLF-PNA
FBL   TFIFVIDAPNGYNV-ADGFTFF1APVDTKPQTG-GGYLGVF-NGK
PSL   SFTFV1NAPNSYNV-AOGFTFFIAPVDTK.PQTG-GGYLGVF-NSA
DBL  SFTVK.1SAPSKASF- A DG IA F Al.VPVGSEPRRNGGYLCJVFDSD
SBA   SFNFTSYAPDTRRL-ADGl.AFFLAPIDTYPQTHAGYI.GLF--NEN
ECO   RFSFS1EQPYTRPLP-ADGLVFFMGPTKSKPAQG-GYL.GIF--NNS
 SL   SFTFFIYRENINRG--GDGITFFLAPTDTQPKSGG-GYLGIF-KDA
LBL   HSYTLQQ1FQNVTDDPAWLFALVPVDSQPKKKGRLLGLF-NKS
LCL   GSQVFRESPNGYNVADGFTFFIAPVDTKPQTG--GGYLGVFYNGK
CON   EATFTFLIK.SPDSHP-ADG1AFF1SNIDSSIPSGST-GRLLGLF-PDN
```

**Figure 39**

**Figure 40: Northern blot analysis**

15 |ig of total RNA isolated from the seeds of *Dolichos lablab* was subjected to 1% denaturing agarose gel electrophoresis, transferred to hybond-N nylon membrane and hybridized with $^{32}$P labeled F1R1 fragment. After processing, two bands corresponding to 3.7 Kb and 1.9 Kb were obtained. The size was determined comparing the mobilities based on a published standard RNA sample data.

GAL EHSNNSSTSENOTT-KAASSNVV-AVEFDT--I.NP-DYGDPNYIH1G1DVNS1RSKVTAKWDWQN-GK1AT
DLL AES-G1AESNVVAVEFDTDYLNP-DYGDPNYIH1GIDVNSIRSKVTASWDWQN-GK1AT
FBL DYD_K1AOTV--AVEFDTFYNAAWDP-SNGKRHIG1DVNTIKS1STKSWN1.QN--GEEAH
PSL KYD-K-TTQ~-1VAVEFDTFYNAAWDP-SNRDRHIG1DVNS1KSVSTKSWNI.QN-GEEAN
DBL VYN-NSAQ^ -TVAVEFDTI.SNSGWDP--SM— K.H1GIDVNSIK.SIATVSWDLAN--GENAE
SBA --E-SGDQ -VVAVEFDTFRNS-WDP-PN - PHIGINVNSIRSIK.TTSWDLAN--NKVAK.
ECO KQD-NSYQ- -TLGVEFDTFSNQ-WDP-PQV-PHIG1DVNSIRSIKTQPFQLDN-- -GQVAN
SL --E-SNET—VVAVEFDTTSNR-WDP-AN---SH1G1NVNSVKSKITTPWGI.KN—DYFT
LBL END-rNAL —TVAVEFDTCHNI.DWDK—NS1AVNL—G1GSVPWNFRDYDGQNAD
LCL YD-KTSQ - - TVAVEFDTFYNAAWDP-SNKERH1GIDVNSIKSVNTKSWNLQN--G
CON -AD--T--IVAVEI.DTY- -PNT-DIGDPSYPH1GID1K.SVRSKKTAKWNMQN GKVGT

GAL AH1SYNSVSKRLSVTTYYPG———SKPATI.SYDir.LHTVLPEWVRVG
DLL AHISYNSVSKRLSVTTYYPG—RGKPAT-SYDIKLH'I'VI.PEWVRVG
FBL VA1SFNATTNVLSVTLLPYN—! TGY- -TLSF.VVPLKDVVPEWVRIG
PSL FNAATNV1.TVSLTYPNS1.EEENVTSY-TLSDVVSLKDVV PEWVRIG
DBL TYNA ATSLLV VSLVHPS—RRTSYI-LSER VD1TNEL PEYVGVG
SBA VLITYDASTSLLVASL.VYPS-QRTSNII.-SUVVnLK'rSI.PEWVRI G
ECO VVIKYDASSK1LI1AVLVYPS—SGA1Y-TIAEIVDVK.QVI.PEWVDVG
SL V1TIYDATRSLSVSSFYRNK—DDIF-TVKASV1ILRDALPQWVRIG
LBL VI.1TYDSSTKFLAVSL.FYP1—TGKRNNV-SANVELEKVLDDWVSVG
LCL VTSY-ILNF.VVPLKDVVPEWVRIG
CON AHIIYNSVDK.RLSAVVSYPN. . . . .ADSA TVSYDVDLDNVI.PEWVRVG

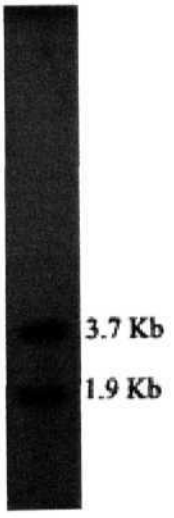



**Figure 40**

**Discussion**

Most legume seeds contain only one type of sugar specific lectin. However, there are a few examples such as *Vicia cracca* seeds that have been shown to contain two distinct sugar specific lectins (Bauman *et al.,* 1979). *Dolichos lablab,* seeds used in this study is a typical example as that of *Vicia cracca* where there exist two distinct sugar specific lectins, a glucose/ mannose and a galactose specific lectin. As the antisera of one lectin cross-reacts with the other it would be interesting to compare the primary structure of the galactose specific lectin from the Indian lablab beans with that of the glucose/mannose specific lectin whose sequence is known. This would help us to understand the evolutionary relatedness between the two proteins.

The primary structures of a number of legume lectins have been determined. Most of them have two subunits of the type otipV The seed lectin from the *Dolichos biflorus* is a heterotetramer (Quinn *et al.,* 1989). The glucose/mannose specific lectin from the seeds of the *Dolichos lablab* var *lignosis* (field bean) is composed of two non-identical subunits of sizes 15 kDa and 12 kDa. The complete primary structure of this lectin has been established by sequential Edman analyses of the intact subunits and the peptides derived from enzymatic and chemical cleavage. This lectin shows homology to other glucose/mannose specific lectins, especially to Con A (Gowda *et al.,* 1994). Both the subunits of the glucose/mannose lectin are different as indicated by their amino acid composition.

An RT-PCR approach used in the study allowed us to obtain two positive clones for the galactose specific lectin gene. When two positive clones obtained were sequenced, and their amino acid sequences aligned, they showed overlapping residues, indicating that

both the clones are part of the same gene. Since, the sequence is not exactly similar to the glucose/mannose specific lectin, it is logical to conclude that the sequence obtained here is indeed a partial sequence of the galactose specific lectin (the second lectin from the *Dolichos lablab* seeds). When one of the amplified fragment (Fl Rl) was used as a probe for detection of the RNA transcript size of the *Dolichos lablab* lectins, two transcripts of sizes, 3.7 Kb and 1.9 Kb were identified. Since the sequence of the F1R1 is homologous to the glucose/mannose lectin sequences, it is possible that this fragment is recognizing two RNA transcripts in Northern blot analysis. From this preliminary observation, it is likely to be noted that the 3.7 Kb transcript recognized, possibly represents the galactose specific lectin and the 1.9 Kb transcript represents the glucose/mannose lectin. Since we arc able to isolate specifically from the seeds two distinct lectins which differ in their properties, but show some sequence similarities, the results obtained here suggest that these two proteins in the seeds are the products of two independent genes.

Further the galactose specific lectin sequence was aligned with other sequences of legume lectins with varying sugar specificity using the CLUSTALW program. The galactose specific lectin shows about 85% homology to the glucose/mannose specific lectin isolated from the same species. Most of the conserved residues include several of those that participate in hydrogen bonding or hydrophobic interactions with the monosaccharide in the primary combining site and almost all these residues co-ordinate with the metal ions.

The subunits of most legume lectins are composed of a single polypeptide chain of about 250 amino acids. Often, they are truncated at the carboxy end resulting in a

mixture of isoforms with different chain lengths (Young *et al.*, 1995). Some lectins are cleaved into two, resulting in the formation of a larger N-terminal chain and a smaller C terminal chain, which are referred to as two-chain lectins. With a few exceptions (Con A, Lentil and PNA) lectins are glycosylated, carrying one or two N-linked oligosaccharides per subunit, attached at different positions of the protein, for example Asn 17 and Asn 114 in ECO. These glycosylated plant lectins exhibit microhetergeneity, that is, individual molecules of a given glycoprotein carry different saccharides at the same attachment site in the polypeptide chain.

It is interesting to note that the galactose specific lectin also shows considerable sequence homology with the other known legume lectins and some amino acids that have been highly conserved are Gin 7, Gly 8, Gly 34, Arg 35, Glu 124, Phe 125, Trp 204 and Gly 208. Once the complete sequence of the galactose lectin is determined, then it would be useful to determine the location of the precise sugar binding sites of this lectin, to identify the conserved regions as well as the potential glycosylation sites. The sequence information can further be used to solve the crystal structure of the protein.

# Chapter 6

# Protein crystallization

## Introduction

Crystallography is the most powerful tool for the elucidation of the detailed structure of large biomolecules. The most popular methods for crystallization are slow evaporation, vapour diffusion by the hanging drop, the sitting drop methods and microdialysis. Automated machines for setting up crystallization experiments have been developed by Cox *et al.,* (1987).

Factors such as availability of the protein, purification procedure, yield of the **protein,** the presence of cofactors and metal ions required for its activity, conformational changes due to binding the substrates, etc. play a role in the success of obtaining protein crystals.

The recent revival of protein crystallography is due to the increased availability of protein as a result of cloning techniques in addition to the development in computer technology and the availability of synchrotron radiation. Through crystallography high resolution structures have been obtained for single polypeptides larger than 100 kDa such as the photosynthetic reaction centre (Deisenhofer, *et al.,* 1985), medium resolution has been obtained for histone octamer and low resolution for molecules such as the nucleosome (Richmond *et al.,* 1984). At present the crystal structures of more than 30,000 proteins are available in the Protein Data Bank (http://www.rcsb.org).

**X-ray** crystallography is an experimental technique that exploits the fact that X-rays are diffracted by crystals. It is not an imaging technique. X-rays have the proper wavelength (in the Angstrom range, ~$10^{-8}$ cm) to be scattered by the electron cloud of

density can be reconstructed. Crystals are generally formed from a pure homogenous preparation of a protein. True crystals often feature sharp edges. These appear as needles, blades, spherulites etc. There are also crystals formed from proteins after the removal of small irregular domains by proteolysis, and these cleaved proteins have all the activities of the intact protein. A protein crystal should have the size of 0.5 x 0.5 x 0.3 mm, without cracks or defects, that is ideal to diffract X rays to high resolution. Phase information must be extracted either from the diffraction data or from supplementing diffraction experiments to complete the reconstruction (the phase problem in crystallography). The methods used to determine the phases are the molecular replacement method, the multiple isomorphous replacement method and the multiwavelength anomalous dispersion method. The electron density maps are calculated and the protein model is then progressively built into the experimental electron density, refined against the data and the result is a quite accurate molecular structure.

The knowledge of accurate molecular structures is a prerequisite for rational drug design and for structure based functional studies to aid the development of effective therapeutic agents and drugs. Crystallography can reliably provide the answer to many structure related questions, from global folds to atomic details of bonding. In contrast to NMR, no size limitation exists for the molecule or complex to be studied by X-ray crystallography.

**Materials**

*Dolichos lablab seeds*, affinity purified lectin and different crystal screen kits

**Methodology**

Purification of the galactose specific bean lectin has been carried out as described in Chapter I. Suitable crystallization conditions were screened using different methods at 20 °C. The concentration of protein taken was 20 mg/mL. The hanging drop consisted of 2 µL of protein containing 20 times molar excess of galactose and 2 µL of the well solution (precipitant). The drops were equilibrated against 0.5 mL of the precipitant.

Crystallization was set up using different screening kits. In Grid screening 24 different PEG 6K conditions were used, in Hampton crystal Screen 1, 48 conditions were used, in Hampton Screen 2, 48 conditions were used and Wizard Screen kit with 96 conditions.

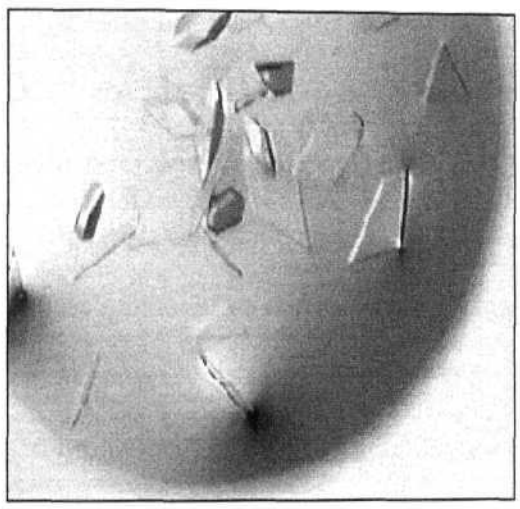
**Results**

Small crystals were obtained in conditions in which polyethylene glycol (PEG, 6K) of different conditions was used as precipitant al pH 6.0 (Fig. 41). Optimization of these conditions resulted in obtaining bigger crystals which diffracted to about 3.0 A. The crystals were mounted in glass capillaries and exposed to X-rays generated by a rotating anode X-ray generator. The diffraction images were recorded with a mar imaging plate. Attempts are being made to improve the size and the quality of the crystals to obtain diffraction data to higher resolution for structure determination and analysis.

**^Figure 41: Crystals of galactose specific lectin obtained in PEG 6K**

A: Crystals obtained in the presence of 20% PEG 6K, pH 6.0.

B: Crystals obtained in the presence of 30% PEG 6K, pH 6.0.



A

B

**Figure 41**

**Discussion**

The 3 D structures of the legume lectins are dimers or tetramers made of dimmers. Each subunit is formed of 3 p sheets: an antiparallel back sheet that is almost flat, a concave 7 stranded front sheet and a smaller five stranded sheet that plays a role in holding the two large sheets together. Most of the other residues form loops and bends forming the "lectin fold" (Srinivasan *et al.,* 1996). The carbohydrate binding sites of the legume lectins are located mostly in the p strands of the curved seven chain sheet. The $Ca^{2+}$ and $Mir^{2+}$ are in close proximity to the carbohydrate binding site. Crystals have also been obtained from demetallised legume lectins (Bouckaert *et* «/., 2000) which differ from their native lectins by lacking carbohydrate binding ability. Lectins dimerise and oligomerise in several modes (Elgavish and Shaanan, 2001) and small differences in the primary structure may lead to large changes in the quaternary structures and thus its biological properties (Prabu, Suguna & Vijayan, 1999; Manoj & Suguna, 2001).

The galactose specific lectin purified in this study was found to be more stable in 30% PEG 6 K, pH 6.0 when this was used as the precipitant. Further analysis of the 3 dimensional structure of the galactosc specific lectin has to be determined to elucidate the structural and functional relationship of this lectin with the glucose/mannose specific lectin.

# Conclusions

## CHAPTER 2

- A galactose specific lectin has been purified from *Dolichos lablab* seeds by affinity chromatography on Sepharose-galactose gel, in the presence of 1.5 M ammonium sulphate.
- The activity of the lectin was inhibited by galactose at concentrations of 2.8 mM and above.
- From 100 g of seeds 175 mg of affinity purified lectin could be obtained.
- Purified lectin is a glycoprotein containing 5 % carbohydrate and binds to Con A - Sepharose gel.
- It was found to be homogenous by native gel electrophoresis and exhibits a native molecular mass of 120 ± 5 kDa in gel filtration. In SDS-PAGE it dissociates into two bands with molecular masses of 31 kDa and 29 kDa.
- Both the bands stained positive for carbohydrate (PAS staining).
- Partial amino temiinal sequence analysis of the two subunits revealed that they are identical suggesting the oligomeric nature of the lectin.
- Antibodies raised to the purified lectin showed specific reactivity with the protein in immunodiffusion and Western blot analysis. The lectin was also found to cross-react with the antibody raised to the well characterized glucose/mannose specific lectin from the same seeds and vice-versa.
- Purified lectin exhibited its activity upto 40 °C. Beyond this temperature its activity reduced considerably.

- The amino acid composition of this lectin shows high concentrations of acidic and hydrophobic amino acids. Cysteine and methionine could not be detected.
- Modification of tryptophan and arginine residues did not alter the agglutination property and the binding ability of the lectin to the affinity gel.
- Modification of the lysine residues resulted in decreased binding on the affinity gel by 31 % and its agglutinating activity by 43 %.
- Modification of tyrosine residues led to the acetylation of all the tyrosine residues. There was a decrease in biological activity only by 20 %.
- Modification of histidine residues showed 75 % loss in hcmagglutinating activity and about 70 % loss in its binding ability to the affinity gel. Reversal of histidine modification regained 5 out of 15 histidine residues. Histidine modification performed in presence of the inhibitory sugar showed protection against modification, suggesting the possible involvement of histidine residues in the biological activity of the lectin.
- However, modification of the amino acids did not alter the immunological property of the lectin, indicating that the loss of activity is not due to alterations in the gross changes in the overall structure of the protein.
- *In vitro* translation of the lablab bean mRNA yielded a single polypeptide precursor of 33 kDa that was recognized by the seed lectin antibody.

**CHAPTER 3**

- |3-N-acetyl hexosaminidase was purified from the seeds and its native molecular mass by gel filtration was calculated to be 104 kDa ± 5 kDa. The enzyme contains 4 % carbohydrate. In SDS-PAGE the enzyme dissociated into three sub-units of 32

kDa, 30 kDa and 29 kDa. One of the three sub-units cross-reacted with the human placental p-N-acetyl hexosaminidase antisera.

- The enzyme was fully active at 4 °C and upto 40 °C. Beyond 40 °C, the stability of the purified enzyme decreased.
- Intact, protein bodies have been isolated from the seeds successfully by sucrose density gradient centrifugation.
- Protein bodies were lysed and the supernatant obtained contained both the glucose/mannose specific lectin and the galactose specific lectin. It also showed the presence of enzymes like a-mannosidase and P-N-acetyl hexosaminidase.
- The pellet was processed by sucrose density gradient centrifugation to prepare the protein body membranes. The lectins were able to bind to these protein body membranes at pH 5.0 and can be desorbed at pH 8.0.
- The P-N-acetyl hexosaminidase enzyme also bound to the protein body membranes at pH 5.0 and was desorbed with a change in pH to 8.0.
- Modification of the histidine, lysine and arginine residues in the galactose specific lectin decreased the binding abilities of lectin to the protein body membranes to 45 %, 40 % and 75 %, respectively.
- An endogenous lectin receptor has been detected in the acidic glutelin fraction of proteins obtained from the seed extract. Preliminary studies revealed that this receptor is a component of the protein body membrane fraction as determined by the cross-linking studies.

- Neither of the lectins under study exhibited antifungal activity when tested with *Trichoderma viridae, Aspergillus niger* and *Aspergillus flaws.*
- Galactose specific lectin obtained from the seeds of the *Dolichos lablab* did not exhibit any mitogenic activity to the human peripheral blood lymphocytes.
- In contrast, the glucose / mannose specific lectin from the same seeds exhibits mitogenic stimulation to the human peripheral blood lymphocytes.
- Estimation of glucose levels during the course of mitogenesis reveal that the cells incubated with galactose specific lectin consume a negligible amount when compared to the cells incubated with glucose/mannose specific lectin.
- FITC labeled galactose specific lectin binds more to the human peripheral multiple myeloma cells than to the normal human peripheral lymphocytes, thus suggesting that this lectin might act as tracers in detection of multiple myeloma cells.
- a-Mannosidase and p-N acetyl hexosaminidase lysosomal enzymes present in the cell secretions of MPR (-) cells interacted well with the lectins. The cx-mannosidase enzyme was recognized by glucose/mannose lectin and p- N acetyl hexosaminidase enzyme could be recognized by the galactose lectin. These enzymes were also recognized by Con-A biotin blotting suggesting them to be glycoproteins.
- a-Mannosidase and (3-N-acetyl hexosaminidase enzymes present in the normal human serum interacted only with the galactose specific lectin. In Western blot analysis of the lectin affigel eluates, a single protein band of (5-N acetyl hexosaminidase (66 kDa) was detected with human placental (3-N acetyl hexosaminidase antisera.

## CHAPTER 5

•**An RT-PCR** approach was used to amplify the galactose specific lectin gene in two cDNA fragments that were cloned and sequenced. The sequence of the fragments showed overlapping residues.

•Since a partial sequence of 208 amino acids obtained here was homologous but different from the glucose/mannose specific lectin, it is logical to conclude that this represents the sequence of the galactose specific lectin.

• When the sequence of the galactose specific lectin was aligned with other legume lectins, it showed considerable homology.

• In the preliminary Northern blot, there were two mRNA transcripts detectable of the sizes of 3.7 Kb and 1.9 Kb corresponding possibly to the galactose specific lectin and the glucose/mannose specific lectin, respectively.

## CHAPTER 6

- Preliminary experiments on the crystallization of the galactose specific lectin revealed that the lectin can be obtained as stable crystals.

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