Studies on Lectins, Lectin Receptors and Glycosidases from the seeds of Indian lablab beans

A Thesis submitted
to the Department of Biochemistry
School of Life Sciences
for the degree of

Doctor of Philosophy

By
B.T. Rajasekhar

Department of Biochemistry
School of Life Sciences
University of Hyderabad
Hyderabad - 500 046, **INDIA**

July 1997

Enrol. No. 91LSPH03

CERTIFICATE

University of Hyderabad
Department of Biochemistry
School of Life Sciences

This is **to** certify that the Thesis entitled ``**Studies on Lectins, Lectin Receptors and Glycosidases from the** seeds **of Indian lablab beans'' is** based on the results of the work done by Mr. Baru Tulasi Rajasekhar for the Degree of the Doctor of Philosophy under the supervision of **Dr. N. Siva Kumar.** This work has not been submitted for any Degree or Diploma of any other University or Institution.

N Siva Kumar 30th July 1997
Dr. N. Siva Kumar
Supervisor

T Suryanarayana 30/7/97
Professor T. Suryanarayana
Head
Department of Biochemistry
School of Life Sciences

A R Reddy 30/7/97
Professor A.R. Reddy
Dean
School of Life Sciences

DECLARATION

I, hereby, declare that the work presented **in** this Thesis entitled ` **Studies on Lectins, Lectin Receptors and Glycosidases from the seeds of Indian lablab beans"** has been carried out by me under the supervision of **Dr. N. Siva Kumar** and that this has not been submitted for any degree or diploma of any other University or Institution.

B.T. Rajasekhar
30/7/97.

B.T. Rajasekhar
Candidate
Enrol. No. 91LSPH03

ACKNOWLEDGEMENTS

I do not have adquate words to express my gratitude to my supervisor, Dr. N. Siva Kumar, who impressed upon me the beauty of plant lectin biochemistry. The fact that this thesis has been completed is mainly because of his constant encouragement and close supervision of my work. He has been unfailingly courteous, helpful and a tremendous motivator. It was because of him that the herculean task of research work never turned somber.

I place on record my gratitude to Prof. A.R. Reddy (Dean, School of Life Sciences), Prof. K. Subba Rao, Prof. P.R.K. Reddy, Prof. N.C. Subrahmanyam (former Deans) and Prof. T. Suryanarayana (Head, Department of Biochemistry) for providing me with the necessary central facilities to cany out my research work.

I thank Dr. M. Ramanadham and his colleagues for carrying out the mitogenicity experiments and also for helping me in several ways. I thank Dr. K.V.A. Ramaiah and Dr. K. Anand Kumar and Dr. M.J. Swamy for their constant encouragement.

I thank my labmates Mrs. K. Padma, Mrs. Usha Jyothi Miss. Y. Udaya Lakshmi for their constant help.

I wish to thank many other friends in the School of Life Sciences who extended their help whenever required

It is beyond words to thank my parents and my brother. The love, affection, encouragement and admonition extended by them provided me succor and solace, and made me stiong in my commitment to carry out my research work. I am extremely thankful to my uncles, aunts and cousins who have made my six year stay in Hyderabad, a memorable experience.

Finally, I acknowledge UGC for having financially supported me for five years in the form of JRF and SRF.

B. T. Rajasekhar

Rajasekhar

CONTENTS

ABBREVIATIONS

Con A	-	Concanavalin A
HU	-	Haemagglutination unit.
MPA	-	*Madura pumifera* agglutinin
NANA	-	N-acetyl neuraminic acid.
PBS	-	Phosphate buffered saline, pH 7.4.
PH A	-	Phytohaem agglutinin
PSA	-	*Pisum sativum* agglutinin.
PWM	-	Poke weed mitogen.
PAGE	-	Polyacrylamide gel electrophoresis.
NaCl	-	Sodium chloride.
SDS-PAGE		Sodium dodecyl sulphate polyacrylamide gel electrophoresis.
TBST	-	Tris buffered saline Tween.
WGA	-	Wheat germ agglutinin.

LIST OF TABLES

LIST OF FIGURES

8. Mitogenic activity of the purified glucose/mannose specific lablab bean lectin using rat and murine lymphocytes.
9. Isolation of lectin receptors on lectin-Affigel column.
10. SDS-PAGE pattern of the eluted protein from lectin-Affigel column.
11. Gel filtration of the lablab bean galactose specific seed lectin.
12. (A) Native PAGE pattern of the galactose specific seed lectin at pH 8.9.

    (B) SDS-PAGE pattern of the galactose specific seed lectin.
13. Immunodiffusion pattern of the galactose specific lectin and glucose/mannose specific lablab bean seed lectin.

CHAPTER-HI

1. Elution profile of the stem and leaf lectin from DE-52 cellulose gel-
2. Elution profile of the stem and leaf lectin from DE-52 cellulose gel.
3. Gel filtration of the stem and leaf lectin on Biogel P-200 gel.

   Inset: Native molecular size of the stem and leaf lectin.
4. SDS-PAGE pattern of the stem and leaf lectin.
5. Immunodiffusion of the stem and leaf lectin.
6. Immuno-affinity chromatography of the stem and leaf lectin.

CHAPTER-IV

1. Chromatography of lablab bean seed extract on DE-52 cellulose.

2. Hydrophobic chromatography of lablab bean α-mannosidase.
3. Gel filtration of the a-mannosidase on Biogel P-200.
   Inset: Native molecular size of the a-mannosidase.
4. (A) Native PAGE pattern of a-mannosidase.
   (B) SDS-PAGE pattern of a-mannosidase.
5. (A) Immunodiffusion pattern of the lablab bean a-mannosidase.
   (B) Western blot analysis of the lablab bean a-mannosidase.
6. Immuno-affinity chromatography of the lablab bean a-mannosidase.
7. Lectin-affinity chromatography of the purified a-mannosidase.

SYNOPSIS

Lectins are ubiquitous proteins/glycoproteins that have the ability to agglutinate cells and are highly specific to sugars. Owing to their ability to bind saccharides, they have been used as invaluable tools in the structural and functional investigations of glycoconjugates and are also employed for detection and isolation of glycoproteins. They are also used for cell identification and fractionation.

The principal source of dietary protein for a large section of population in India is legumes. The legume seeds have been known to contain antinutritional components like lectins (haemagglutinins) and proteolytic enzyme inhibitors. The genus *Dolichos lablab* contains two main varieties viz., *var. lignosus* (field bean) and *var. typicus* (lablab bean).

Seeds of the field bean, seeds and pods of the lablab bean form a part of the diet in some areas of India. The present investigation is concerned with the purification of the lablab bean seed lectin on different affinity matrices, its characterization, identification of proteins from the seed extracts (endogenous lectin receptors) that interact with the seed lectin, identification of a new galactose specific lectin from the seeds, isolation of the stem and leaf lectin from the plants and isolation and purification of α-mannosidase from the seeds. This thesis consists of four chapters.

Chapter-I

This chapter deals with the general introduction of lectins, their recent definition, occurence, biological and physico-chemical properties, sequence homologies of legume lectins, applications, distribution and functions of lectins. The scope of present investigation has also been indicated.

Chapter-II

This chapter deals with the purification and properties of the glucose/mannose specific lablab bean seed lectin and also on the identification of endogenous lectin receptors in the seeds. It also deals with the identification and isolation of a new galactose specific lectin from the seeds. This chapter has been divided into the following sections.

Section - A

This deals with the general introduction of the glucose/mannose specific lectins, about the endogenous lectin receptors and also about the leguminous seeds that contain more than one type of lectin.

Section - B

This deals with the materials and methods that have been used in the study. The methods of preparations of various affinity supports for the purification of the lablab bean seed lectin are given. Also, experimental methods for the physical characterization of the lectin such as the determination of homogeneity, molecular weight, subunit molecular weights have been described. Experimental details of haemagglutinating activity and sugar specificity are also given. Methods for raising antibodies to purified lectin and immunological characterization have been described. Methods to determine the mitogenic activity of the purified lectin have been described. Also the methodology for the preparation of seed extracts to isolate lectin binding proteins (endogenous lectin receptors) by lectin-affmity chromatography have been described. Also methods for isolation of the galactose specific seed lectin have been given.

Section - C

This section deals with the results of various experiments carried out for the isolation of the glucose/mannose specific lectin on different affinity matrices. The lectin has been purified in high yields using goat IgM-sepharose matrix. The purified lectin is found to be homogeneous in PAGE and showed a molecular size of 60000 in gel filtration. The lectin is a glycoprotein and is possibly made of two types of subunits (M_r 15 kDa and 12 kDa). The lectin agglutinates rabbit and human erythrocytes and its activity is best inhibited by glucose, mannose and its derivatives.

Further, the lectin is found to be mitogenic to murine lymphocytes. From the seed extracts, proteins that specifically interact with the immobilised glucose/mannose specific seed lectin have been identified. Results on the isolation of a new galactose specific lectin from the seeds have also been described. The molecular size of this lectin is found to be 120000 ± 5000. This lectin is found to be homogeneous in PAGE and dissociated into two subunits in SDS-PAGE (50 kDa and 20 kDa) and is possibly a tetramer. This lectin cross-reacts with the antiserum to glucose/mannose specific seed lectin.

Section - D

The results obtained above are discussed as compared to the information available in the literature.

Chapter-Ill

This chapter deals with growing of seeds of lablab beans into plants, collecting stems and leaves from 3 week old plants, identification of strong

haemagglutinating activity in extracts of stems and leaves, isolation and partial biochemical characterization of this activity. This chapter has been divided into the following sections.

Section - A

This deals with the occurence of lectin activities that have been identified from different parts of the plants among legumes and non-legumes.

Section - B

This deals with the materials and methods used in the isolation of stem and leaf lectin. Details of extraction procedure, determining the haemagglutinating activity, sugar specificity, isolation of the lectin by ion-exchange chromatography and gel filtration, methodology related to affinity chromatographic purification of the stem and leaf lectin on various affinity supports is also given. The native and subunit molecular weight determination of the isolated lectin is described. In addition, the immunological reactivity of this protein with the anti serum to glucose/mannose specific seed lectin has been described.

Section - C

This section describes the detailed results of the various experiments on the stem and leaf lectin. The isolated lectin showed a molecular size of 66 kDa in gel filtration and dissociates in SDS-PAGE into two bands corresponding to molecular sizes 48 kDa and 20 kDa. The lectin agglutinates only rabbit erythrocytes and the activity is inhibited by sugars such as galactose and its derivatives but not by glucose and mannose. The lectin cross-reacts with the antiserum raised against the glucose/mannose specific seed lectin, suggesting similar antigenic sites on these two proteins.

Section - D

The results obtained on this unusual lectin have been discussed.

Chapter-IV

a-mannosidase is one of the glycosidases that is found in the protein bodies of legume seeds along with lectins and storage proteins. This chapter deals with the isolation and purification of the a-mannosidase enzyme from the lablab beans and is divided into the following sections.

Section - A

This deals with the general introduction about the plant glycosidases particularly the a-mannosidases and their possible functions in the seeds.

Section - B

This deals with the materials and methods that have been used in the isolation and purification of a-mannosidase from lablab beans. These include the extraction of the enzyme, assay of enzyme activity, purification of the enzyme by a combination of ion-exchange, hydrophobic chromatography and gel filtration. The heat stability of the enzyme and requirement of metal ions has also been indicated. Further, methods to raise antibodies to the well studied jack bean a-mannosidase and its use in testing cross-reactivity with the lablab bean enzyme both by immunodiffusion and Western blot analysis have been described.

Section - C

This section deals with the results obtained on the a-mannosidase. The purified enzyme showed a molecular size of 195000±5000 in gel filtration and dissociates into two subunits of molecular sizes 66 kDa and 44 kDa in SDS-PAGE, both of them reacting with an antibody for the jack bean a-mannosidase suggesting antigenic similarities among these legume mannosidases. The purified lablab bean enzyme strongly interacts with the immobilized glucose/mannose specific lectin, the physiological significance of which is unclear.

Section - D

Results obtained above are discussed with the information available in literature.

Summary

The results on lectins, lectin receptors from the seeds, stem and leaf lectin and a-mannosidase from the seeds have been summarized here.

CHAPTER I

INTRODUCTION

Lectins constitute an extended and diverse group of proteins or glycoproteins that share the unique ability to bind specific sugars or sugar containing molecules such as glycoproteins (Liener, 1976). Though the lectins have been found in almost all classes of organisms, only plant lectins or phytohaemagglutinins are very well characterised biochemically and physicochemically (Goldstein and Hayes, 1978). Legume lectins are the most well studied among plant lectins and are also most abundant in the seeds which prompted scientists to use them as tools in diverse areas like biochemistry, cell biology, immunology, cancerology and medicine.

Lectins have become indispensable tools in biological research as they serve as recognition determinants in a variety of biological systems such as plants, microorganisms and animals because of their ability to bind and cross link monosaccharides and oligosaccharides.

Though most of the higher plant seed lectins have been thoroughly characterised, their role in these organisms is poorly understood. There is evidence suggesting that in certain legumes, lectins mediate the attachment of nitrogen fixing bacteria to form the symbiotic nodules in legume roots (Smit *et al.,* 1992). They may serve as defense agents against seed eating herbivores owing to their cytotoxic properties and their high concentration in certain seeds (Chrispeels and Raikhel, 1991). Membrane associated lectins appear to function in the clearance of glycoproteins from the circulating system and in

the intracellular translocation and targeting of glycoproteins in animals (Ashwell and Hartford, 1982).

The development in lectin research has been divided into three periods. In early period (1888-1918), the following seem to be the most important: establishing the protein nature of lectins (von Eisler and von Portheim, 1926) and demonstration of some of their properties analogous to those of antibodies, agglutinating as well as precipitating activities, varying selectivity of interaction with different cells and inhibition of the activity by certain substances. However, the finding by Stillmark (1888) of the haemagglutinating properties of ricin from castor bean seed extracts is usually considered to be the beginning of the actual lectin story. During the period 1935-1964 lectins stimulated new interests of hematologists and immunologists owing to their use in blood typing and serological diagnostics. A very important discovery was made by Nowell (1960) who observed the mitogenic properties of a lectin from the seeds of *Phaseolus vulgaris.* The use of lectins as mitogenic substances developed into a special branch of immunological research. Their application as structural probes to determine organization on the cell surface, its changes during cell cycle and malignant transformations brought very important innovations into the field of experimental biology. Different carbohydrate markers on cell surfaces permitted the application of lectins for separations of various biologically distinct cell populations. Furthermore the saccharide binding properties of lectins could advantageously be utilized in a number of special analytical and preparative techniques for characterization, sequencing and purification of carbohydrates, glycopeptides and glycoproteins (Sharon and Lis, 1990).

The term lectin (Latin Leger: to choose) was coined by Boyd and Shapleigh (1954 a,b) to a group of plant agglutinins, some of which were human blood group specific. Later several carbohydrate binding proteins were discovered from a variety of sources like bacteria, sponges, snails and hemolymph of lobsters. The term lectin was thus broadened to include sugar-binding proteins from any source (Ashwell, 1977). According to Goldstein *et al.* (1980), "Lectin is a carbohydrate binding protein (or glycoprotein) of non-immune origin which agglutinates cells and/or precipitates glycoconjugates". Kocourek and Horejsi (1983) defined lectins as; "proteins of non-immunoglobulin nature capable of specific recognition and reversible binding to carbohydrate moieties of complex carbohydrates without altering covalent structure of any of the recognised glycosyl ligands". Lectins significantly differ from antibodies in that there is significant structural diversity, variation in molecular size. amino acid composition, three dimensional structure and metal requirement. The latest definition of lectins is by Peumans and Van Damme (1995). They define lectins as "All plant proteins that possess at least one noncatalytic domain that binds reversibly to a specific mono or oligosaccharide".

In order to analyse a lectin in its usefulness as a tool in biochemical and immunological studies it is of vital importance to establish the carbohydrate binding specificity of a lectin. Sugar-lectin complementarity is determined generally by Landsteiner hapten-inhibition technique (Landsteiner, 1962). Also physical methods like equilibrium dialysis, fluorescence spectroscopy have been used widely to establish the carbohydrate specificity of lectin (Krishna

Sastry' *et al.,* 1986). Lectins not only interact with free sugars but also with polysaccharides and glycoproteins at their non reducing terminal glycosyl groups. Lectins with similar specificities to monosaccharides differ in their affinity towards di- and oligosaccharides (Debray *et al.,* 1981).

Based on their monosaccharide specificity, Goldstein and Poretz (1986) classified lectins into the following classes,

1) Mannose/glucose binding lectins.
2) N-Acetylgalactosamine binding lectins.
3) N-Acetylglucosamine binding lectins.
4) L-Fucose binding lectins.
5) Sialic acid - binding lectins.

The mannose/glucose binding lectins comprise a group of agglutinins found mostly in the Leguminoseae family. This group of lectins has been most thoroughly studied. ConA from jack beans (*Canavalia ensiformis*) was the first lectin whose primary and three dimensional X-ray crystallographic structure was determined (Hardman *et al.,* 1971). Table 1 shows some of the properties of glucose/mannose specific lectins.

The human blood group specificity for lectins was first discovered in the N-Acetyl galactosamine/galactose binding lectins. Galactose-binding toxins were identified in diverse plants including *Ricinus communis* (Euphorbiaceae). *Abrus precatorius* (Leguminoseae) and *Viscum album* (Loranthaceae).

The N-Acetyl glucosamine binding lectins comprise a diverse group of agglutinins that exhibit a primary specificity for their monosaccharide and/or its ($\beta.1\rightarrow4$) linked oligomeis (Chitin oligosaccharides) and in some instances glucosamine. This group includes lectins from three families namely Gramineae, Solanaceae and Leguminoseae.

The Fucose binding lectins have been found to be useful serological reagents. In clinical serological laboratories the *Ul ex europaeus* I lectin was widely used as it exhibits anti blood group O activity (Renkonen, 1948). Fucose binding lectins occur in diverse organisms like green plants, *Lotus tetragonolobus* and *Ulex europaeus,* fungus, *Aleuria aurantia* and animals, *Anguilla anguilla.*

The haemolymph and sera of most of the vertebrates is a rich source of the sialic acid binding lectins which are not found in plants.

Distribution of lectins

Although lectins were widely distributed in nature much of the research for nearly 100 years has been focussed on plant lectins. Among the plants, seeds of the legumes contain considerable amounts of lectins (Su *et al,* 1980). In the Leguminoseae family the lectins are largely localised in cotyledons of mature seeds (Howard *et al,* 1972). Appreciable amounts have also been reported from embryos (Pueppke *et al,* 1978) and small amounts were detected in seed coats (Rouge, 1975).

1. Entlicher, G , Kostir, J.K. and Kocourek, J. (1970). Biochim. Biophys. Acta. 221,272-281.
2. Allen, A.K., Desai, N.N. and Neuberger, A. (1976). Biochem. J. **155,** 127-135.
3. Baumann, C.M., Strosberg, A.D. and Ruediger, H. (1982). Eur. J. Biochem. **122,** 105-110.
4. Siva Kumar, N. and Rao, D.R. (1986). J. Biosci. 10, 95-109.
5. Ueno, M., Ogawa, H., Matsumoto, I. and Seno, N. (1991). J. Biol. Chem. 266, 3146-3153.
6. Kalsi, G., Das, H.R., Babu, C.R. and Das, R.H. (1992). Biochim. Biophys. Acta. 1117, 114-119.
7. Fomstedt, N. and Porath, J. (1975). FEBS Lett. **57,** 187-191.
8. Siddiqui, S., Hasan, S. and Salahuddin, A. (1995). Arch. Biochem. Biophys. 319, 426-431.
9. Correia, M.T. and Coelho, L.C. (1995). Appl. Biochem. Biotech. **55,** 261-273.

PROPERTIES OF SOME GLUCOSE/MANNOSE SPECIFIC LECTINS

SOURCE	PURIFICATION METHOD	NATURE OF PROTEIN	MOL.WT	DIMERIC/ TETRA-MERIC	Reference
Canavalia ensiformis	Sephadcx G-50, G-100	Not a glyco-protein	1,06,000	α_4	Entlicher *et al* 1970
Vicia faba	CH-Sepharose-3-O methyl glucose	glycoprotein	52,452	$\alpha_2\beta_2$	Allen *et al,* 1976
Vicia cracca	Sephadex G-100	glycoprotein	44,000	$\alpha_2\beta_2$	Baumann *et al* 1982
Dolichos lab lab var lignosus	Sepharose 6B-DVS-mannose	glycoprotein	60,000	α_4	Siva Kumar and Rajagopal Rao. 1986
Sophora japonica (bark)	I actamyl-sepharose, maltaniyl-sepharose	glycoprotein	65,800	$\alpha_2\beta_2$	Ueno *et al.,* 1991
Arachis hypogea root lectin	Sephadex G-50	glycoprotein	66,000	α_2	Kalsi *et al.,* 1992
Vicia ervilia	Sepharose-mannnose	glycoprotein	53.000	$\alpha_2\beta_2$	Fornstedt and Porath. 1975
Ca/anus cajan	Amm.SO_4 fractiona-tion. IgM-sepharose 6B chromatography	glycoprotein	39,000	α_2	Siddiqui *et al* 1995
Cratylia mollismart	Sephadex G-75	glycoprotein	31,000	—	Correia *et al.*. 1995.

Among the Euphorbiaceae members the lectin from castor bean seeds has been well characterized (Nicolson *et al.,* 1974). latex of several Euphorbiaceae plants has also been known to contain lectins. Among the Cucurbitaceae members, seeds of snake gourd (*Trichosanthes anguina)* contain galactose specific lectin that has been well characterized (Komath *et al.,* 1996). Among the monocotyledonous plants, distribution of lectins has been thoroughly studied for the Gramineae seed family members. Lectins with similar sugar specificity and structural properties to the WGA have been isolated from rye and barley embryos (Peumans *et al.,* 1982). Recently an N-acetyl glucosamine specific lectin was affinity purified from the seeds of Triticale (Siva Kumar and Padma, 1996).

From members belonging to Solanaceae family lectins have been isolated from potato tubers and tomato. There are only a few reports about agglutinating activities from the extracts of non-flowering and lower plants (Horejsi and Kocourek. 1978).

Biological Properties of Lectins

The binding of lectins to cells results in a variety of biological effects such as agglutination, mitogenic stimulation of lymphocytes, induction of suppressor cells, enhancement of phagocytosis of yeast and bacteria by macrophages, insulin like effects on fat cells, toxicity to cells and animals, inhibition of fungal growth, inhibition of growth of tumour cells etc. Some of these properties are discussed in detail below.

(i) Agglutination

The ability to agglutinate cells distinguishes lectins from other sugar binding molecules like glycosidascs and glycosyl transferases. For agglutination to occur, the bound lectin must form multiple cross bridges between opposing cells. Agglutination is affected by many factors, such as molecular properties of the lectin (for example, number of saccharide binding sites, molecular size), cell-surface properties (eg. number of receptor sites accessible, membrane fluidity), and metabolic state of cells (Nicolson, 1976). In addition agglutination is also affected by external conditions of assay such as temperature, cell concentration, mixing and so on. When agglutination is inhibited by an appropriate sugar, it can be taken as an indication that carbohydrate structures for which the lectin is specific are present on the surface of the cells.

Chemical or enzymatic modifications of lectins or modification on cell surfaces by enzymes or by proteolysis often have remarkable effects on cell-agglutination by lectins. For instance, cross linking of soybean agglutinin with glutaraldehyde increases the haemagglutinating activity of the lectin for human erythrocytes by 100 to 200 fold. Human erythrocytes are not agglutinated by peanut agglutinin even at a concentration as high as 1 mg of lectin/ml. Removal of sialic acid by treatment of cell with sialidase enables the lectin to bind and cause agglutination at very low concentrations (Lis and Sharon, 1981).

(ii) Mitogenic stimulation of Lymphocytes

One of the most dramatic effects of the interaction of lectins with cells is mitogenic stimulation, i.e., the triggering of quiescent, non dividing lymphocytes into a stale of growth and proliferation. The first mitogenic agent to be described was PHA, the lectin from red kidney bean, *Phaseolus vulgaris* (Nowell, 1960).

Many lectins differ markedly in their ability to stimulate lymphocytes of different species. Moreover the mitogenic activity is affected by chemical modifications of the cell surface, as well as of the lectin molecule itself. Most mitogenic lectins stimulate only the thymus-dependent population of lymphocytes (T cells) and are inactive or inhibitory for mitosis of other class of lymphocytes, the thymus-independent (or B) cells. There are however some exceptions to this. including the lentil lectin, which was previously considered to be solely a T-cell mitogen, has been shown to stimulate human B-cell proliferation as well (Miller, 1983). Recently a B-cell maturation mitogen has been purified from the seeds of jack fruit, *Artocarpus integrifolia* (Misquith *et al.,* 1994). It was also shown that lectins from peanut, *Arachis hypogea;* mushroom. *Agaricus bisporus* and coral tree. *Erythrina corallodendron,* stimulate vascular cell proliferation (Stanford and Hooker, 1990). In contrast to stimulation by antigens, in which specific clones of lymphocytes are induced to proliferate. lectins activate multiple lymphocyte clones irrespective of their antigen specificity, so that the percentage of responding cells is rather high, upto 70-80% of appropriate lymphocyte population (Hume and

Weidmann, 1980). This amplification facilitates the detection and study of the changes associated with proliferation. Several plant lectins such as PHA, PWM, ConA, MPA and PSA are potent mitogens for human lymphocytes. The pattern of activation used however is not uniform for all mitogenic lectins. The different biological effects following lectin activation of human lymphocytes might be due at least in part to a differential binding of the various lectins to lymphocyte subsets. In a study (Serke *et al*, 1989) studied the binding of five mitogenic plant lectins namely PHA, PWM, ConA, MPA and PSA to three major human lymphocyte subsets using flow cytometric analysis. The study revealed that all subsets uniformly show high binding of PHA, whereas two different populations, one high binding and the other low binding, can be detected with PWM, ConA, MPA and PSA.

(iii) Lectin toxicity

Several lectins, e.g., ConA, WGA, PHA and lectin from *Robinia pseudoacacia,* are toxic to mammalian cells, both *in vitro* and *in vivo.* Toxic lectins are generally selective in their action on cells. In particular, transformed cells are frequently much more sensitive to the cytotoxic effects of lectins than normal cells (Brown and Hunt, 1978; Nicolson, 1974). It is therefore not surprising that attempts were made to inhibit tumour growth by lectins *in vivo.* Rao (1987) found that feeding of alcoholic seed extracts of *Abrus precatorius,* to male rats caused deliterious effects on fertility. Other lectins such as *Viscum album* (Mistletoe) were found to cause decrease of viability of human leukemia cell cultures.

Sequence homology of lectins

To gain further insight into the structure - function relationship of lectins, several groups of investigators have initiated primary sequence determination of these proteins. It has been found that lectin structures have been highly conserved in evolution, presumably for ensuring the lnaintanance of important physiological functions yet to be characterized.

Most work on sequence homology in lectins has been done with leguminous lectins. All available evidence suggests that legume lectins are initially synthesized as single polypeptide chains of a molecule of about 30,000. After removal of a 20 residue hydrophobic leader sequence, this chain may be post synthetically cleared to remove a few amino acids. Non covalent interactions are involved in subunit binding. Only one legume lectin, the lima bean agglutinin has been reported to contain a disulphide bond which links two 31,000 Da subunits of the protein (Goldstein *et a!.*, 1983).

It appears that all leguminous lectins are homologous throughout their sequence, provided one introduces appropriate deletions. To maximize the homology, the ConA sequence has to be rearranged into 2 portions, one (residues 122 to 237 and 1 to 69) aligned with residues 1 to 185 of the soybean agglutinin and the other residues (70 to 121) aligned with residues 186 to 238 of the soya bean agglutinin and also with the peanut and sainfoin lectins. In a similar fashion, maximum homology was observed when the lectins from the lentil, the pea and *vicia faba* are aligned, in random, such that their α-chains

correspond to residues 70 to 119 of ConA and their β-chains beginning at residues 120 of ConA.

The diagrammatic representation of the circularly permuted sequence homology that related conA to other leguminous lectins is as shown in the Figure 1 (Gowda *et al,* 1994).

The amino acid residues involved in metal binding site and the hydrophobic cavity are highly conserved among the various lectins compared above, supporting the functional role of the lectin through evolution. The amino acid residues that constitute the sugar binding site in ConA (Becker *et al,* 1976; Hardman *et al,* 1971) are poorly conserved in other mannose/glucose binding lectins. However, the striking homologies between the sequences of the one and two chain lectins strongly suggest a close resemblance in the folding and three dimensional structure of these proteins.

The complete primary structure of many lectins has been worked out. The complete amino acid sequence of the lima bean *{Phaseolus lunatus)* lectin was deduced from the nucleotide sequence of the cDNA clone (Milligan *et al,* 1989). The complete primary structure of the lectin from *Dolichos lablab,* var. *lignosus* (field bean) was worked out (Gowda *et al,* 1994). Similarly the cDNA cloning and expression of the *Bauhinia purpurea* lectin has been studied (Kusui *et al,* 1991).

The tetrameric *Galanthus nivalis* agglutinin belongs to a super-family of alpha-D-mannose specific plant bulb lectins known to be potent inhibitors

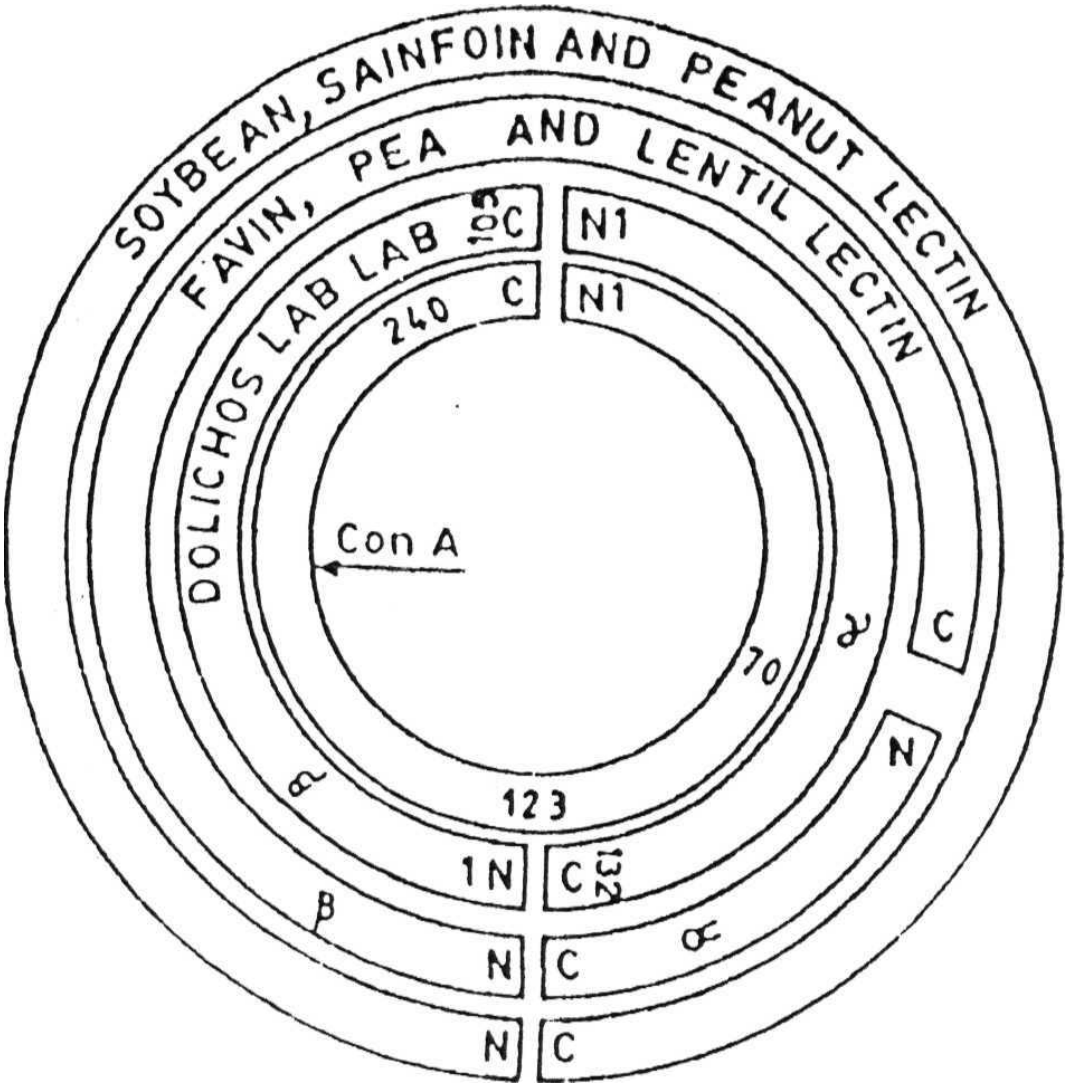


Fig.1: Schmatic representation of circular permutation of amino acid sequences among some legume lectins.

The arrangement for ConA (the innermost circle); the two subunit lectins, favin, pea, and lentil lectins and the single-subunit lectins soybean sainfoin, and peanut, is based on their amino acid sequence proposed by Hemperly and Cunnigham. The *Dolichos lab lab* lectin α-subunit is arranged along **the** NH_2 terminus of ConA, followed by the p-subunit starting at 123 of ConA, which then completes the circle. Taken from Gowda *et al*., (1994).

of retroviruses. The 2.3A crystal structure of this lectin complexed with methyl-α-D-mannose reveals a novel three fold symmetric beta-sheet polypeptide fold (Hester *et al,* 1995). The *Maackia amurensis* haemagglutinin (MAH) is a leguminous lectin which preferentially binds to a cluster of sialylated O-linked carbohydrate chains. A 950 bp cDNA clone encoding MAH was isolated from a cDNA library (Yamamoto *et al,* 1994). From the nucleotide sequence, MAH was predicted to consist of 285 amino acid residues. Homology searching as well as molecular modelling studies revealed that each subunit of the *Sambucus siebaldiana* lectin has a highly homologous structure to the galactose specific lectin subunit and ribosome inactivating subunit of plant toxic proteins such as ricin and abrin, indicating a close evolutionary relationship between these carbohydrate binding proteins (Kaku *et al,* 1996).

Physico-chemical properties of plant lectins

Legume lectins consist of 2 or 4 identical or very similar subunits with molecular sizes ranging from 25 to 30 kDa, each with a single small carbohydrate combining site and tightly bound Ca^{2+} and Mn^{2+} which are required for activity. Employing chemical or molecular biological techniques, the primary' structure of nearly 40 lectins has been established. These exhibit remarkable homologies. X-ray crystallographic studies carried out on 8 legume lectins revealed that the subunits are in the shape of a half dome with the carbohydrate binding site forming the shallow depression at its apex. The metal ions are located close to the combining site where they help to position amino acid residues for carbohydrate binding but do not bind directly to the

carbohydrate. Although several amino acids that are involved in the binding of carbohydrate are highly conserved in all legume lectins, the galactose specific lectins do not bind glucose nor the glucose specific lectins combine with galactose. This difference is possibly due to the orientation of the monosaccharide in the binding site of the glucose specific and galactose specific lectin apparently due to the differences in the structure of variable amino acids that line the binding pocket (Sharon and Lis, 1995). In case of WGA, the amino acids involved in the binding are not located in the same subunit as found in legume lectins but belong to two different subunits of the WGA dimer (Wright, 1990).

Lectins as cell recognition molecules: Functions and Applications

Although lectins have been purified from several sources in the last few decades, only recently most of the interesting data have been obtained to elucidate their application in biology and medicine. Based on these data, it has been indicated that lectins act as recognition molecules by binding to complementary carbohydrates either in cell-cell interactions or in cell-molecule interactions. Lectins thus play a key role in the control of various normal and pathological processes in living organisms.

Lectin-carbohydrate recognition in viruses

This has been well exemplified in the interaction of influenza viruses with target cells (Paulson, 1985; Wiley and Skehel, 1987). It has been shown that the human virus binds to erythrocytes and other cells by recognising N-

acetyl neuraminic acid (NANA), one of the sialic acids present on the cell surface and that this binding is a prerequisite for initiation of infection. Subsequently the viral haemagglutinin (lectin) that mediates the binding was extensively characterized to show its specific interaction with NANA containing oligosaccharides. The lectin is made of 2 types of subunits HA_1 and HA_2 with molecular masses of 36 and 26 kDa respectively covalently linked by disulphide bond. The lectin associates non-covalently to form trimers that are located on surface of viral membrane. The carbohydrate binding site forms a pocket located in a domain of the lectin protruding from membrane and is composed of amino acids that are largely conserved in the numerous strains of the vims.

The role of the lectin in initiating infection by influenza vims has been well demonstrated. The binding of lectin to sialic acid containing carbohydrates on the surface of target cells leads to attachment of vims to the cells. This results in fusion of viral and cellular membranes allowing release of viral genome in to the cytoplasm and subsequent application. Removal of sialic acid from cell membranes by sialidase abolishes binding and prevents infection whereas enzymatic rcattachment of sialic acid or insertion of sialic acid containing oligosaccharides into the membranes of sialidase treated cells restores the ability of the cells to find the virus and to be infected by it.

Bacterial lectins: recognition and infection

Bacteria containing lectins serve as **a** useful model to study the lectin-carbohydrate interactions that are due to the cell-cell recognition between the

bacteria and the eukaryotic host cells. Several bacterial strains produce surface lectins which are commonly in the form of submicroscopic hair like appendages known as fimbriae (pili) that protrude from the surface of the cells. These have been well documented in enterobacteria such as *Escherichia coli* and *Salmonellae* species (Sharon, 1987). Fimbriae are usually 5-7 nm in diameter and 100-200 nm in length. The well characterised fimbriae of *E. coli* are mannose-specific which preferentially bind oligo mannose and hybrid oligosaccharides of animal cell surface glycoproteins. Purified fimbriae consists of several hundred flmbrillin (or pilin) subunits of different sizes most of which have a molecular mass of 14-22 kDa. Most fimbriae are very stable structures being resistant to detergents and chaotropic agents. These are also termed as ``adhesins" or ``agglutinins". Bacterial surface lectins play a key role in the initiation of infection by mediation of bacterial adherence to epithelial cells of the host, for example in the urinary and gastrointestinal tracts. This has been well documented for type-I fimbriated *E. coli* and *klebsiella pneumonieae*. The flmbriated strains of these organisms are more effective. Sugars that inhibit binding bacteria to epithelial cells *in vitro* as well as antibodies to the lectin receptors significantly decrease the rate of urinary tract infection in experimental animals (Sharon and Lis, 1989). The galactose specific lectins produced by oral *Actinomyces* such as *Actinomycisnaeslundi* and *Actinomycis riscosus* facilitate initial colonization of epithelial surfaces of mouth and teeth by mediating the attachment of the bacteria to galactose residues, lined on the surface of epithelial cells or on the surfaces of other bacteria which are adsorbed to the enamel of the teeth (Cisar, 1987).

Lectins as recognition determinants in lectinophagocytosis

During late 1980s it has been shown that specific recognition between phagocytes and their targets can be accomplished by lectins on the surface of the type of cell that combine with complementary sugar on the surface of another cell, in a lock and key manner. This type of recognition has been described as lectinophagocytosis (Ofek and Sharon, 1988). Lectino-phagocytosis in bacteria can occur in two major modes. In the first mode, the bacteria which carry surface lectins bind to complementary carbohydrates on the surface of the phagocyte cells. In the second mode, lectins that are integral components of the phagocytic cell membrane bind to cabohydrates on the bacterial surfaces. Lectin carrying bacteria also bind readily to sugars on phagocytic cells, for example human polymorpho nuclear leucocytes or human and mouse peripheral macrophages.

Lectin-carbohydrate interactions in protozoa

Humans and animals are infected by numerous protozoa and among these the occurence of lectins has been best documented in the pathogenic amoeba *Entamoeba histolytica* which causes dysentry in humans by destruction and invasion of the colonic mucosa (Ravdin, 1989). Several sugars inhibit the amoebic adherence to enterocytes suggesting that the adherence is mediated by lectin-carbohydrate interactions. From *Entamoeba histolytica,* two distinct lectins, one specific for $\beta 1 \rightarrow 4$ linked oligomers of N-acetyl glucosamine and the other galactose and N-acetyl galactosamine have been

isolated. The purified lectins inhibit binding of amoebic trophozoites to Chinese hamster ovary (CHO) cells and to cultured human intestinal epithelial cells. Li *et al.* (1988) by their studies on the interaction of *Entamoeba histolytica* to wild type CHO cells and mutants with altered glycosylation pattern observed that the galactose specific lectin plays a key role in recognition of mammalian cells by *Entamoeba histolytica*.

Role of lectins in slime molds

Dictyostelium discoideum is a well known example for the involvement of lectins in cell-cell recognition. During the vegetative growth of this mold, there are no lectins expressed. However at the aggregating stage wherein the cells adhere to each other, a galactose specific lectin discoidin 1 is expressed. The lectin is present on the surface of aggregating cells and in its isolated form, it agglutinates aggregating slime mold cells but not vegetative cells (Barondes, 1986).

Role of lectins in plants

The lectins characterized to date have been largely isolated from plants. However little is known about their precise function. The hypothesis which is valid is that lectins serve as mediators of the symbiosis between the nitrogen fixing micro organisms, primarily rhizobia, and leguminous plants, a process of immense importance in both the nitrogen cycle of terrestrial life and in agriculture. The association between legumes and nitrogen fixing bacteria is highly specific. The bacteria that infect and nodulate soybeans can not

nodulate garden peas and vice versa. The soybean agglutinin was found to bind in a sugar specific manner to the corresponding rhizobial species and not to bacteria that are syrnbionts of other legumes (Bohlool and Schmidt, 1974). A similar specificity pattern was also observed with lectins from soybean, pea, red kidney bean and jack bean seeds and lipopolysaccharides from the respective syrnbionts (Etzler, 1985). Rhizobia contain several *nod* genes essential for post nodiilation. These *nod* genes are major determinants of host specificity. These might affect productions of bacterial cell surface carbohydrates. The carbohydrates in turn will be recognized by plant root lectins as signals to induce the events leading to nodiilation. Lectin recognition in symbiosis between white clover *(Trifolium repens)* and *Rhizobium trifolii* has been well studied (Dazzo and Hollingsworth, 1984). A lectin (Trifoliin A) specific for 2-deoxyglucose was isolated from extracts of clover seeds and seedling roots. It bound to infective but not to uninfective strains of rhizobia. Trifoliin was found to act as a bridge between similar carbohydrates on both the root hair tips and *Rhizobium trifolii*.

Wheat germ lectin has been shown to inhibit growth of specific fungal species by binding to the hyphal tips and preventing their growth (Mirelman *et al,* 1975). In a later study it was found that plant Chitinases and not chitin binding lectins are antifungal proteins (Schlumbaum *et al,* 1986). More recently it was also found that purified wheat germ agglutinin and Triticale lectin had no effect on the growth of the fungal species tested (Siva Kumar and Padma, 1996). Soybean lectin was found to inhibit mycelial growth of *phytophthora megasperma* (Gibson *et al,* 1982). Based on these lectins have been thought to protect plants against fungal pathogens.

Role of lectins in higher animals

Animals produce a number of lectins that are membrane bound and soluble which have been implicated in cell recognition phenomena. Ashwell and Morell (1974) first reported on the membrane bound galactose and N-acetyl galactosamine specific lectin from rabbit hepatocytes (known as the hepatic binding protein) and demonstrated that it may be involved in clearance of glycoproteins from circulatory system. A similar galactose specific membrane bound lectin was reported by Kelm and Schauer (1988). A surface lectin specific for galactose and N-acetyl galactosamine appears to be responsible for the ability of activated niacrophages to distinguish tumour cells from normal ones and to kill the tumour targets. The putative lectin was purified from activated mouse macrophages (Oda *et al.,* 1988). Membrane lectins with specificities other than for galactose have also been isolated from different animal cells.The macrophage lectin specific for mannose and N-acetylglucosamine has been involved in lectinophagocytosis (Sharon and Lis, 1989). Many vertebrates also contain two distinct membrane bound glycoproteins that are specific for mannose 6-phosphate. These proteins designated as mannose 6-phosphate receptors mediate the transport of several lysosomal enzymes to lysosomes (Siva Kumar *et al.,* 1997).

Selectins

The name selectins was proposed to the receptors as they promote selective cell-cell interactions and because they are hypothesized to mediate

adhesion by lectin type interactions with oligosaccharides on target cells. To date three selectins have been identified that are designated as LAM] (Leucocyte adhesion molecule 1), $ELAM_1$ (Endothelial leucocyte adhesion molecule 1) and GMP_{140} (Granule membrane protein 140). The selectins have been shown to contain lectin like domains at the amino tenninal end and have been implicated to play a key role in cell-cell interactions which require Ca^{2+}. The selectins mediate a variety of leucocyte interactions with the blood vessel wall. These cellular interactions are of fundamental importance for inflammatory responses (McEver, 1991).

Lectins for cell fractionation and bone marrow transplantation

Lectins have been used for identification and fractionation of immune cells especially of mouse thymocytes and human bone marrow cells (Reisner and Sharon, 1984). Since the binding of lectins to the cells can be reversed without damage to the cells by addition of an appropriate sugar, both the lectin reactive and non reactive cells are readily recovered resulting in high yields of fully viable cells. Peanut agglutinin has been used by Sharon and his co-workers for effective separation of murine thymocytes. This study provided access to both thymocyte sub populations (immature cortical cells, medullary thymocytes) separated from each other to examine *in vitro* the developmental and functional relationship between them. Selective agglutination by peanut agglutinin is also useful for isolation of lymphocyte subpopulations.

Soybean agglutinin has been first used in separation of mouse B and T splenocytes. Work carried out by Reisner and coworkers (1980) has shown

that treatment of human bone marrow with soybean agglutinin results in agglutination of bulk of cells responsible for graft-versus-host disease, the main cause of mortality in patients treated with allogenic bone marrow.

Scope of Present Investigation

Lectins are ubiquitous proteins/glycoproteins that are abundantly present in the eds of legumes. Owing to the ready availability and high protein content, seed of a number of legumes have become potential sources for the isolation and purii ;ation of lectins. These cell agglutinating sugar specific proteins have been pur ed by affinity chromatography on various affinity matrices, by covalently cor ing the inhibitory ligand.

The genus *Dolichos* (Family Leguminoseae; sub family; papilionaceae) cons ,ts of a number of plants among which *Dolichos lablab* var. *lignosus* (field bean and *Dolichos lablab* var. *typicus* (lablab bean) are grown fairly widely in India Austrialia and some African countiies for various food uses. (The wealth of Indi 1952, Tropical legumes, resources for the future, 1979., Duke, 1981). The food orm of the field bean is largely as seed while in the lablab bean the entire seed pod) is used as a vegetable.

Both varieties contain a glucose/mannose specific lectin that has been affinity purified on Sepharose-mannose gels and was found to have similar physico-chemical and biological properties (Siva Kumar and Rajagopal Rao, 1986). Work canned out by other workers outside India, was mainly on the field bean lectin. There are a number of differences in the properties of these lectins reported by other workers, presumably because of the varietal differences (Guran *et al*., 1983). Field bean seeds were also found to contain a galactose specific lectin (Mo *et al* 1990). More recently, the primary structure of the field bean lectin has completely been elucidated in India (Gowda *et al,* 1994).

However there has been a systematic study on only one variety of the lablab bean lectin (Siva Kumar and Rajagopal Rao, 1986). With a long term objective to understand the physiological significance of the lablab bean lectin, and to study its fine sugar specificity and to identify endogenous lectin receptors from the lablab bean seeds with which the lectin interacts to detect lectins in stems and leaves and to purify the α-mannosidase from the seeds, the present work was carried out.

At the first instance, the glucose/mannose specific lectin from the lablab beans was purified by altering the purification protocol and employing other affinity matrices.

i) Sepharose-divinylsulfone - mannose;

ii) Cyanogen Bromide Sepharose - goat IgM;

iii) Affigel-10-Lectin IgG matrix (immunoaffinity matrix).

The biological properties such as the haemagglutinating activity, blood group specificity, mitogenicity. sugar inhibition studies have been carried out using the purified lectin. As we had ready access to the Sepharose-mannose gels, this gel was largely used for the purification of the seed lectin. Antibodies for the purified lectin were raised in rabbits and the immunological properties of the lectin were studied. The seed extracts also contain a galactose specific lectin. This lectin was purified and some of its properties studied.

In addition seed extracts of the lablab beans contain a protein that interacts with the seed lectin. This protein has been identified as an endogenous receptor. Lectins although predominantly present in the seeds of the legumes, have also

been identified in the stems and leaves of a few leguminous seeds (Etzler, 1978) and from the roots of some legumes (eg. pea). Isolation of these proteins have led to some understanding about the function of lectins in plants.

To identify lectin activities in the vegetative parts of the plant, seeds were grown into plants and the stems and leaves were collected from 3 week old plants. Protein extracts of these showed strong haemagghitinating activity with trypsin treated rabbit erythrocytes. However the lectin activity is not inhibited by glucose/mannose or its derivatives like the seed lectin but is inhibited by lactose, galactose and methyl derivatives of galactose. However the stem and leaf lectin could not be bound on any of the affinity matrices prepared using different sugars, and hence it has been isolated to homogeneity by DE-52 cellulose and Biogel P-200. The biological properties of this lectin, the native and subunit molecular weights and its immunological cross-reactivity with the seed lectin have been studied.

Plant glycosidases particularly from the legume seeds are enzymes that are highly specific in their action to release sugars from glycoproteins. Based on what type of sugar they release they are called as mannosidases, galactosidases. Among these enzymes, α-mannosidase has been purified from some legume sources such as *Canavalia ensiformis* (Einhoff nad Reudiger. 1986), *phaseolous vulgaris* (Paus and Christensen, 1972). The enzyme from *Canavalia ensiformis*, is well studied (Einhoff aned Ruediger, 1988). However there has been no study on the immunological properties of different legume mannosidases.

Seed extracts of the lablab beans also exhibited a-mannosidase activity. With a view to isolate and purify this enzyme and to understand its possible interactions with the seed lectin, owing to the colocalisalion of lectins and glycosidases in the protein bodies, the mannosidase enzyme has been purified on DE-52 cellulose, phenyl Sepharose, and Biogel P-200. The native and subunit molecular weights of the purified enzyme have been determined and its inimunological properties have been compared to the well studied jack bean a-mannosidase.

CHAPTER II

SECTION -A

INTRODUCTION

Glucose/mannose specific lectins are a group of proteins that are abundantly found in the family Leguminoseae. Owing to the ready availability of the jack bean *{Caiiavalia ensiformis*), seeds a glucose/mannose specific lectin designated as Concanavalin A was purified and extensively characterized. Other lectins included in this group are those from pea *(Pisum sativum),* the lentil *(Lens culinaris),* the fava bean *(Vicia faba),* the common vetch, *(Vicia cracca*), the forage legume, sainfoin (*Onobrychis viciifolia*), the field bean (*Dolicos lablab* var. *lignosus*). On the basis of their molecular structure, these lectins are classified into two groups, the first group are those which are made of four identical subunits (Concanavalin A) and the second group are those that are made of two light (a) and two heavy (P) chains having the composition $\alpha_2\beta_2$ (pea, lentil, fava bean, fieldbean). Most of these lectins lack cysteine and methionine and agglutinate rabbit and human erythrocytes. Most of these are mitogenic for lymphocytes.The amino acid sequences of many of these lectins reveal extensive homology suggesting important evolutionary' relationships. Although many of these lectins have been well characterized, their physiological functions are not fully understood. Studies carried out by Prof. H. Ruediger's group in Germany have lead to the identification of endogenous lectin receptors from different legumes, that interact with the seed lectins. The first definitive work on lectin binding proteins was reported from the seeds of pea, jack bean, fava bean (Gansera *et al,* 1979). These proteins were isolated from the seed extracts by

affinity chromatography on a resin containing covalently bound homologous lectins from the seeds of the plants. The lectin receptor from pea was found to be an oligomeric glycoprotein with subunit molecular size of approximately 50000. The binding of these receptors to lectins is inhibited by the sugars for which the lectins are specific. Bowles and Marcus (1981) also identified lectin receptors from the seed extracts of soya beans and jack beans.

Subsequently the lectin receptors have been isolated, purified and well characterized from lentil and pea. These proteins do not possess any haemagglutinating activity but most of these are potent mitogens for B lymphocytes (Kummer and Ruediger, 1988).

Most of the legume seeds contain usually one type of lectin with distinct sugar specificity. However some leguminous seeds such as common vetch *(Vicia cracca)* contain two distinct lectins one of them is human blood group *A* specific and an N-acetyl galactosamine binding lectin. The second one is a blood group non-specific and glucose/mannose specific lectin. These two lectins lack immunological cross-reaction and have been designated as products of two distinct genes (Baumann *et al.,* 1979). Seeds of the field bean (*Dolichos lablab* var.*lignosus*) have been shown to contain two lectins, one of them is a glucose/mannose specific lectin (Siva Kumar and Rajagopal Rao, 1986) and the second one is a galactose specific lectin (Mo *et al.,* 1990).

In the present study, a glucose/mannose specific lectin has been affinity purified from the seeds of Indian lablab beans. Using this immobilized lectin, endogenous lectin receptors have been isolated from the seed extracts.

Additionally a new galactose specific lectin was also isolated, from the seed extracts.

SECTION - B

MATERIALS AND METHODS

Materials

Seeds of the lablab bean, (*Dolichos lablab* var.*typicus*) were obtained from local market. Various chemicals and other materials used in this study were purchased from different sources. Standard proteins such as myosin (205,000), p-galactosidase (116000), bovine serum albumin (66,000) and ovalbumin (45,000), sugars such as Phenyl α-D-glucoside, Phenyl α-D-mannopyranoside, 3-O-α-D-mannopyranosyl mannopyranose, glucose, mannose, melizitose, gentiobiose, 3-O-methyl glucose, cellobiose, raffinose, N-acetyl glucosamine, fucose, mannosamine, methyl α-D-glucopyranoside, methyl α-D-mannopyranoside and divinyl sulphone were from Sigma Chemical Company, U.S.A. Sepharose~-6B was from phannacia fine chemicals, Uppsala, Sweden. Biogel P-200, Affigel-10 were from Biorad labs. All other chemicals used were obtained from reputed local firms. Centrifugations were done in a Beckmann J2-21-M/E refrigerated centrifuge.

Methods

Preparation of the affinity matrix Sepharose--divinyl sulfone-mannose

The biospecific adsorbent Sepharose--mannose was prepared by linking the affinity ligand mannose to Sepharose~6B through an extension ann of divinyl

sulfone as had been described originally by Fornstedt and Porath (1975), Hapner and Robbins (1979).

Coupling reactions were carried out in 25 ml batches. 25 ml of Sepharose—6B was washed with distilled water on a sintered funnel. It was then washed with 25 ml of 0.5 M sodium carbonate buffer pH 11.0 and suspended in 25 ml of the same buffer. To this gel 2.5 ml of divinyl sulfone was added. The suspension was shaken for 70 min at room temperature and later washed with distilled water on a sintered funnel. To the activated gel, 25 ml of 20% (W/V) of D-mannose in 0.5 M sodium carbonate buffer pH 10.0 was added. The coupling was allowed to proceed for 24-72 hrs in cold with continuous rotation. The gel was then extensively washed with distilled water. The gel was finally suspended in 25 ml of 0.5M sodium carbonate buffer, pH 8.5 containing 0.5 ml of β-mercaptoethanol and the suspension was mixed for 3 hrs at room temperature. Later the gel was washed with distilled water and suspended in PBS and stored in cold until further use.

Preparation of Sepharose--divinyl sulfone-methyl α-D-mannopyranoside

Methyl α-D-mannopyranoside sugar was coupled to Sepharose essentially as described for the coupling of mannose to Sepharose, except that in the place of mannose, methyl a-D-mannopyranoside was used. The ability of this gel to bind the purified glucose/mannose specific lectin was tested.

Preparation of goat IgM-Sepharose matrix

Goat IgM was isolated from fresh goat serum (Ahmed, 1995) and was coupled to cyanogen bromide activated Sepharose, that was prepared as follows (Dean *et al.,* 1985). 5 ml of packed Sepharose--6B was washed with water and was suspended in 5 ml of carbonate buffer pH 10.8. The temperature of the flask was maintained at 10-15°C by addition of crushed ice. 500 mg of cyanogen bromide was added to the gel suspension and the pH maintained at 10.8±0.1 by the addition of a few drops of 4.0 M NaOH. The gel was cooled rapidly and poured into a large sintered glass funnel that has been pre-cooled with crushed ice. The suspension was filtered into a Buchner flask containing solid ferrous sulphate and the gel was washed thoroughly with water. The washed gel was suspended in carbonate buffer pH 8.5 and goat IgM (10 mg/ml) was added and the coupling allowed to proceed overnight at 4°C. At the end of the incubation period, the unbound protein was collected and the gel was washed thoroughly with water and the unreacted sites blocked using 1 M glycine at pH 8.0 for 6-8 hrs. Finally the gel was washed with water and stored in PBS until use.

Purification of **the lablab** bean **lectin**

The following two affinity matrices were used to purify the glucose/ mannose specific lectin from the Indian lablab beans.

(i) Sepharose--divinyl sulfone-mannose gel.

(ii) Goat IgM-Sepharose matrix.

(i) Purification of **the seed lectin** on **Sepharose--divinyl sulfone-mannose matrix**

All operations were carried out at 4oC. 5 gm of the defatted seed powder from lablab beans was stirred with 50 ml of phosphate buffered saline (PBS), pH 7.4 for 16 hours. The extract was centrifuged for 15 minutes at 7000 rpm and the clear supernatant collected. Solid ammonium sulphate was added to the supernatant to 80% saturation and the suspension stirred for 3 hrs in cold. This was centrifuged at 10,000 rpm for 20'. The pellet was dissolved in PBS and dialysed against PBS. The sample was centrifuged after dialysis and passed through a Sepharose-divinyl sulfone-mannose column pre-equilibrated with PBS. The unbound protein was thoroughly washed with PBS and the bound lectin was eluted using 0.25 M glucose in PBS. The sample was dialysed against water, freeze dried and stored at 4oC.

(ii) **Goat IgM-Sepharose matrix**

All operations were earned out at 4oC. 1 gm of the defatted seed meal was extracted for 2 hours with 10 ml PBS. The extract was centrifuged at 7000 rpm for 20 minutes. The pH of the supernatant was adjusted to 5.0 with 2 N acetic acid. This was centrifuged at 9000 rpm for 20'. The pH of the supernatant was brought back to 7.4 with 2 N NaOH. This sample was passed through the goat IgM-Sepharose gel that was pre-equilibrated with PBS. The column was thoroughly washed with PBS and the bound protein was eluted with 0.5M glucose in PBS.

Polyacrylamide gel electrophoresis (PAGE)

PAGE at pH 4.6 using β-alanine-acetic acid buffer was carried out according to Reisfeld *et al.,* (1962). Electrophoresis was earned out at room temperature on 7.5% aciylamide tube gels with different protein concentrations. Methyl green was used as marker. The gels were stained with coommassic brilliant blue and destained.

SDS-PAGE

This was carried out in tube gels using Weber and Osborn method (1969) and in slab gels by Laemmli method (1970).

Sepharose--divinyl sulfone-methyl α-D-mannopyranoside matrix

This matrix was packed into a column and equilibrated with PBS. About 4 mg of the purified lectin was dissolved in PBS and passed through this column slowly. The column was thoroughly washed with PBS and the lectin was eluted with 0.5 M methyl mannoside sugar.

Determination of molecular weight by gel **filtration**

The molecular weight of the lectin was determined by passing it through a Biogel P-200 gel filtration column according to the method of Andrews (1978).

The column was calibrated using appropriate standard proteins, myosin (205000) β-galactosidase (116000) bovine serum albumin (66000) and ovalbumin (45000).

Protein and carbohydrate estimation

Protein was estimated as described earlier (Lowry *et al.,* 1951). The absorbance of column fractions was routinely monitored for protein at 280 nm. Neutral sugars were colorimetrically estimated by phenol-sulphuric acid method of Dubois *et al.*, (1956).

Haemagglutinating activity

The haemagglutinating activity of the lectin was determined according to the method described by Paulova *et al.,* (1971). The following are the details of the preparation of erythrocytes for the assay.

(i) Preparation of erythrocytes

Rabbit blood was collected from the ear vein of healthy rabbits in Alsevier's solution (Bukantz *et al.,* 1946). The blood was centrifuged at 3000 rpm at room temperature for 15 minutes and the eiytlirocyte pellet thus obtained was washed with 0.9% saline for 3-4 times. The volume of the pelleted erythrocytes was measured and a 4% (v/v) suspension was made in 0.9% NaCl.

(ii) Preparation of standard trypsin treated erythrocyte suspension

The erythrocytes were treated with trypsin on the day of the assay. The 4% erythrocyte suspension was treated with 0.1% trypsin (w/v) for a period of 1 hour at 37°C. The trypsin treated erythrocytes were washed 4 times with 0.9% saline, A 4% suspension of erythrocytes was prepared in saline.

(iii) Agglutination assay

0.2 ml of saline was placed in each well of the haemagglutination plate. 0.2 ml of the lectin was placed in the first well, mixed thoroughly and diluted serially till the last well in a row. 0.2 ml of trypsin treated erythrocytes were added to each well and the plate was kept at 37°C and agglutination visualised after 1 hour. The highest dilution which showed positive haemagglutination was taken as the titre. The amount of protein present at this dilution represents the minimum quantity of protein necessaiy for agglutination and is defined as one unit. Specific activity is the number of units per mg protein.

Sugar inhibition assay

Solutions (0.1M) containing different sugars, prepared in saline were used in this assay. 1-10 μM concentrations of sugar solution were placed in the wells of haemagglutination plate, in a final volume of 0.1 ml. The sugar solution in each of the wells was incubated with 0.1 ml of lectin solution containing 4 haemagglutination units at room temperature for 1 hour. 0.2 ml of trypsin treated rabbit erythrocytes were added to each well. The plate was incubated at 37°C for 1 hour. The agglutination was visualized and the extent of inhibition by different sugars was expressed in millimoles.

Mitogenic activity of the lectin

The mitogenic activity of the lectin was tested using rat and mouse splenic lymphocytes as described (Bradley, 1980). These were isolated and cultured in RPMI-1640 medium containing 5% foetal calf serum and 50 μM p-mercaptoethanol. Cell cultures containing $2x10^5$ cells in 200 μl of medium were kept in triplicates with different concentrations of purified lectin (10-100μg) in 96 well flat bottomed microtitre plates. Cell cultures without lectin were always kept, and were incubated at 37°C in a CO_2 incubator using 5% CO_2 containing atmosphere. The cultures were pulsed with 0.5 μCi of tritiated thymidine (low specific activity, 5-20 ci/mmole) for the last 10 hours of the 48 hour culture period. The cultures were harvested using a Scatron cell harvester and the radioactivity was measured using Beckman Liquid Scintillation Counter.

Immunological methods

(a) Raising antibodies to the purified seed lectin

Antibodies to the lectin purified either on Sepharose--mannose or goat IgM Sepharose gels were raised in a healthy rabbit. The animals were immunised with 1 mg of the lectin in complete Freund's Adjuvant. 0.5 ml of the lectin in saline was mixed thoroughly with 0.5 ml of Freund's Adjuvant till a homogeneous suspension was obtained. In the first course, 1 ml of the antigen solution was injected subcutaneously. Subsequently one injection was given per every week

for 3 weeks. The animal was bled at the end of the third week to check for the antibodies. The next week a booster dose of the lectin was injected and the animal was bled in the subsequent week. The serum was collected and stored at -20°C.

(b) **Immunodiffusion** and Western **blot analysis`**

Immunodiffusion was performed according to the method of Ouchterlony (1948). Antiserum to the purified glucose/mannose specific lectin was placed in the central well. Seed lectin purified on Sepharose-mannose gel and goat IgM-Sepharose gel were placed in the neighbouring wells. The precipitin lines fonned were visualised. For Western blot analysis, the seed lectin was separated on SDS-PAGE and proteins were transferred on to a nitrocellulose sheet using a Biorad wet transfer unit. The nitrocellulose sheet was soaked in Tris buffered saline Tween (TBST, 10 mM Tris-HCl buffer pH 8.0, 150 mM NaCl with 0.05% Tween) containing 3% milk powder for 3 hrs at room temperature. The membrane was then washed twice with TBST and incubated overnight with a 1:1000 diluted antiserum to the seed lectin. The membrane was then washed thoroughly and the antigen-antibody complex was detected using a secondary' antibody conjugated to alkaline phosphatase and visualised by incubating the membrane with the substrate 5-bromo4-chloro3-indolyl phosphate/nitroblue tetrazolium (Bangalore Genei, India).

(c) Preparation of lectin-Affigel-10

Affigel-10 was processed following the manufacturer's instructions. 1 ml of Affigel-10 was packed in a sintered syringe and washed consecutively with cold

isopropanol, water and 0.1 M HEPES buffer pH 7.5. Purified glucose/mannose specific seed lectin (10 mg dissolved in 1 ml of 0.1 M HEPES buffer pH 7.5) was added to the washed Affigel. The column was closed and the gel suspension rotated for 24 h at 4°C. At the end of the incubation period, the unbound fraction was acidified and the protein measured at A_{280}. Unreacted sites in the gel were blocked using 200 µl of 0.1 M ethanolamine hydrochloride pH 8.0 for 1 hr at 4°C and the gel washed thoroughly with water followed by PBS.

(d) **Purification of the specific IgG from antiserum**

1 ml of antiserum was diluted with 1 ml PBS and passed through seed lectin coupled to Affigel-10 column that has been prc-cquilibrated with PBS. The column was thoroughly washed with PBS till no protein could be detected in washings. The column was then eluted with 0.1M glycine-HCl buffer pH 2.65. The eluted protein was immediately neutralized with 2 M Tris.

(e) **Coupling of seed lectin specific IgG to 1 ml of Affigel-10 (Immuno-affinity matrix)**

This was carried out as described above.

iv) Purification of seed lectin on Affigel- 10-seed lectin specific IgG (Immuno-affinity matrix)

All operations were carried at 4oC. 1 gm of the seed powder was extracted with 10 ml of PBS for 2 hrs. The extract was centrifuged at 7000 rpm for 20 min.

The pH of the supernatant was adjusted to 5.0 with 2N acetic acid. This was centrifuged at 9000 rpm for 20 minutes. The pH of the supernatant was brought back to 7.4 with 2N NaOH. Solid ammonium sulphate was added to this to 80% saturation. This was allowed to stir for sometime and centrifuged at high speed and the pellet was dissolved in PBS and dialysed against PBS. The dialysed sample, after centrifugation was passed through the immuno-affinity gel pre-equilibrated with PBS in two separate portions.. After the column was thoroughly washed with PBS, the bound protein was eluted with 0.1 M glycine-HCl buffer pH 2.65.

Purification of lectin binding proteins

(i) Preparation of lectin-Affigel-10 matrix

This was prepared as described under immunological methods.

(ii) Extraction of proteins from seeds and lectin-affinity chromatography

All operations were carried out at 4oC. 1 gm of seed meal was extracted with 10 ml PBS, pH 7.4 for 2 hours. This was centrifuged at 7000 rpm for 20 min. The pH of the supernatant was adjusted to 5.0 with 2N acetic acid. This was centrifuged for 20 min at 9000 rpm. The pellet was dissolved in 50 mM Tris-HCl buffer pH 8.0 and dialysed against the same buffer. This was passed through Sepharose--divinyl sulfone-mannose column to eliminate the mannose specific lectin. The unbound fraction was passed through Affigel-10 coupled to mannose specific seed lectin column, that has been pre-equilibrated with 50 mM Tris-HCl

buffer pH 8.0. The column was thoroughly washed with the same buffer and eluted with 20 mM sodium acetate buffer pH 4.0.

SDS-PAGE

The eluted protein was analysed on 10% gels in SDS-PAGE according to Laemmli (1970). The gels were stained with coommassic blue and dcstained.

Isolation and purification of the galactose specific lectin

All operations were carried out at 4oC. 7 gm of the defatted seed meal was stirred with 70 ml of PBS, pH 7.4 for 16 hours. The extract was centrifuged at 7000 rpm for 15 min. Solid ammonium sulphate was added to the supernatant to 30% saturation. This was allowed to stir for 4 hrs. This was centrifuged at 10000 rpm for 20 minutes. To the supernatant solid ammonium sulphate was added to 60% saturation. After stirring for 3 hours the suspension was centrifuged at 10000 rpm for 20 minutes. The pellet was diaiysed against 25 mM Tris-HCl buffer pH 7.4 and this contained the mannose specific lectin only. To the supernatant, solid ammonium sulphate was added to 80% saturation. This was centrifuged at 10000 rpm for 20 min. The pellet was dissolved in 25 mM Tris-HCl buffer pH 7.4 containing 150 mM NaCl and diaiysed agamst the same buffer. This fraction contained the galactose specific lectin only and was subjected to affinity chromatography on the following matrices (i) Sepharose-divinylsulfone-mannose, (ii) Sepharose-divinylsulfone-galactose that were sequentially connected. The protein could not be retained on these gels and was concentrated and passed through the Biogel P-200 column, pre-equilibrated with 25 mM Tris-HCl buffer

pH 7.4 containing 150 mM NaCl. Fractions of 2 ml were collected. Protein and activity were monitored in the column fractions.

PAGE and SDS-PAGE

PAGE was carried out on 7.5% slab gels without SDS and β-mercaptoethanol. SDS-PAGE was carried out on 10% gels (Weber and Osborn, 1969). The gels were stained with cooniinassie brilliant blue and destained.

Haemagglutination assay

This was performed as described earlier for mannose specific lectin.

Immunodiffusion

This was pcrfoiTned essentially as described earlier for mannose specific lectin. The immuno-reactivity of the galactose specific lectin with the antiserum to the mannose specific lectin was tested by immunodiffusion.

SECTION - C

RESULTS

Purification of the glucose/mannose specific seed lectin on Sepharose--divinylsulfone-mannose column

The glucose/mannose specific lectin was purified on Sepharose-divinyl-sulfone-mannose matrix. Fig. 1 shows the purification profile of the glucose/mannose specific seed lectin on Sepharose-divinylsulfone-mannose matrix. Table 1 gives the purification of this lectin from 5 gm of seed powder. As can be seen from the Table, about 13 mg of purified lectin can be obtained from 5 gm seed **powder.**

Purification of glucose/mannose specific lectin on goat IgM-Sepharose-column

The glucose/mannose specific lectin could be purified on goat IgM-Sepharose column. Fig. 2 shows the purification profile of glucose/mannose specific lectin on this matrix. Table 2 gives the purification of this lectin starting from 1 gm seed powder. From 1 gm of seed powder about 2.18 mg of purified lectin can be obtained using this matrix.

Fig.1. Affinity chromotography of lablab bean glucose/mannose specific lectin on Sepharose-divinyl sulfone-mannose column (1.5X3cm). Dialysed 80% ammonium sulphate pellet (421 mg protein) was loaded on the column, pre-equilibrated with PBS, pH 7.4. 2 ml fractions collected. Column eluted with 0.2 M glucose in PBS.

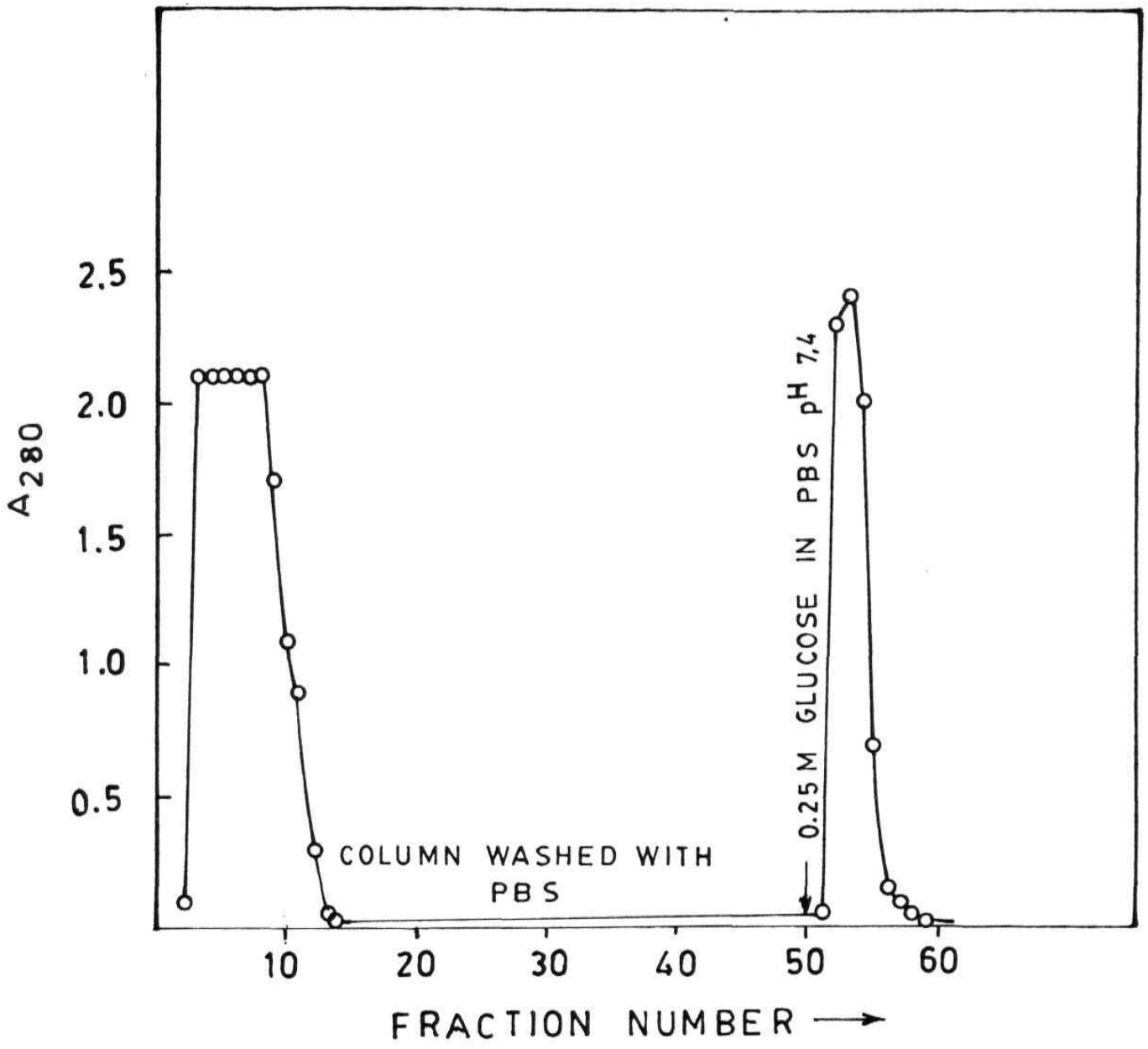

2.5
2.0
1.5
1.0
0.5
A_{280}
COLUMN WASHED WITH PBS
0.25 M GLUCOSE IN PBS pH 7.4
10
20
30
40
50
60
FRACTION NUMBER →

Table - 1: Purification of the glucose/mannose specific lectin on Sepharose-divinylsulfone-mannose matrix.

STEP	VOLUME (ml)	PROTEIN (mg)	ACTIVITY (U)	SPECIFIC ACTIVITY (U/mg)	RECOVERY (%)	FOLD PURIFICATION
Crude extract	36	710	47360	86	100	1
Dialysed Amm SO4 Pellet (80% saturation)	18	421	46080	109	97	1.26
Affinity Chromatography	29	13	37128	2834	78	33

Fig.2. Purification of Jablab bean lectin on goat IgM-Sepharose. 90 mg of protein was loaded on goat IgM-Sepharose gel (0.8 X 6 cm) equilibrated with PBS. Fractions 1.0 ml were collected and the absorbance monitored at 280 nm. Elution was performed using 0.5M glucose in PBS.

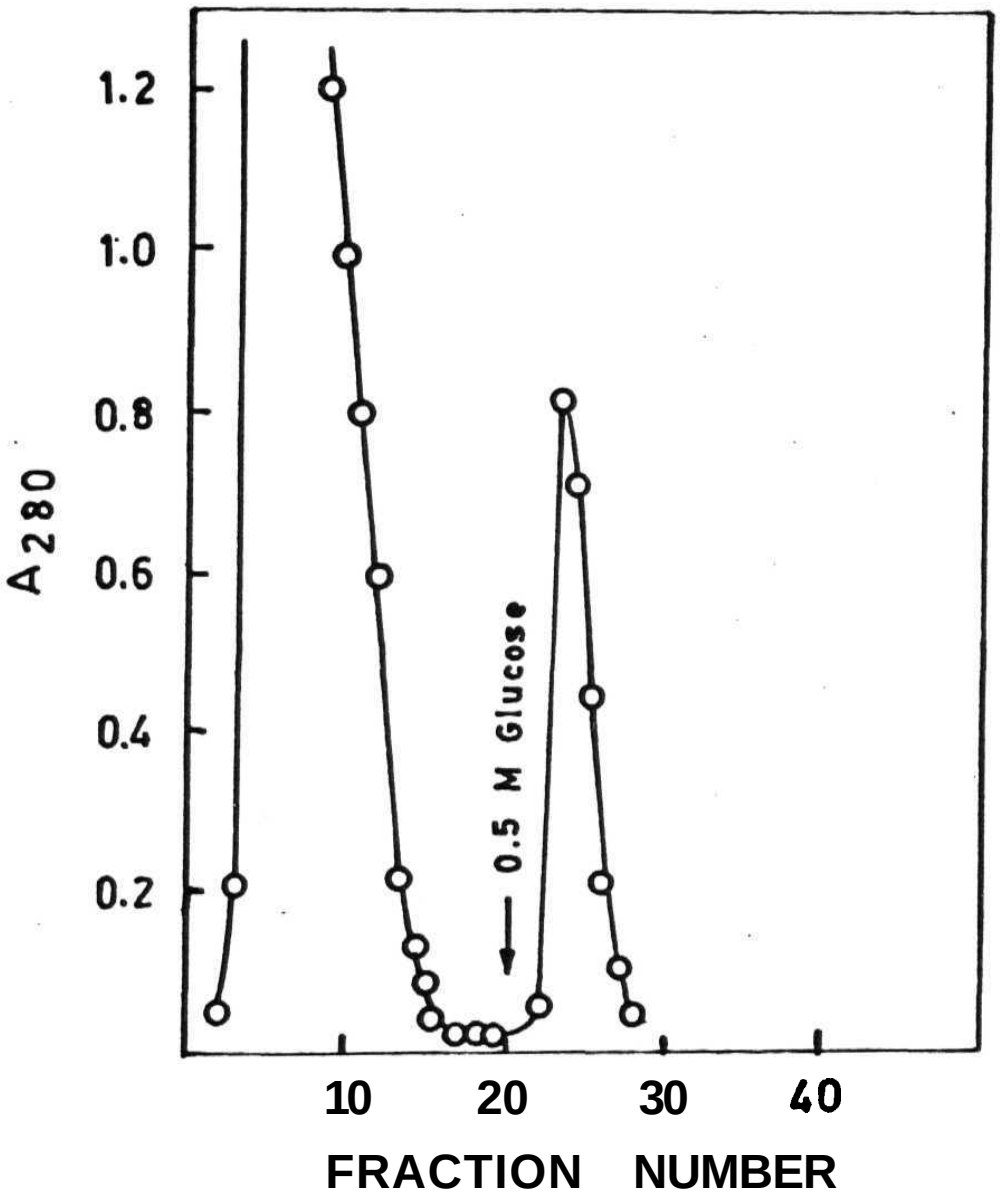

1.2
1.0
0.8
0.6
0.4
0.2
A280
0.5 M Glucose
10
20
30
40
FRACTION NUMBER

Table - 2: Purification of the lablab bean lectin on goat IgM-Sepharose gel. 1 gm of the seed meal was used for the purification. (U)* One HU. (Haemagglutinating unit) is defined as the minimum amount of protein required to cause visible agglutination.

STEP	VOLUME (ml)	TOTAL PROTEIN (mg)	TOTAL ACTIVITY (U)*	SPECIFIC ACTIVITY (U/mg)	RECOVERY (%)	FOLD PURIFICATION
Crude extract	7.5	140	9600	68	100	—
Dialysed pH 7.4 Supn.	7.0	90	8967	99.6	93	1.46
Affinity Chromatography	1.0	2.18	5120	2348	53	34.5

PAGE and SDS-PAGE

The lectin was found to be homogeneous when purified on either matrices, as can be seen from the PAGE profile (Fig. 3A) at pH 4.6. The lectin dissociated into two subunits of Mol. Wts. 15 kDa and 12 kDa in SDS-PAGE (Fig. 3B).

Affinity chromatography of purified glucose/mannose specific lectin on Sepharose--divinyl sulfone-methyl a-D-rnannopyranosidc gel.

Fig. 4 shows the specific binding of the glucose/mannose specific seed lectin on Sepharose- divinylsulfone-methyl mannoside column and its elution with 0.5 M methyl mannoside sugar.

Molecular weight determination

The molecular weight of the lectin was determined by passing the purified lectin on a gel filtration column (Biogel P-200). The molecular weight was found to be 64000 Da. Fig. 5 shows the elution profile of the lectin on gel filtration column.

Carbohydrate estimation

The lectin was found to be a glycoprotein with around 3% carbohydrate.

Fig. **3A.** PAGE pattern of purified mannose specific lectin from the seeds ot lablab beans at pH 4.6 (i) lectin purified on Sepharose-mannose gel, (ii) lectin purified on goat-IgM Sepharose gel.

B. SDS-PAGE pattern of purified mannose specific lectin.

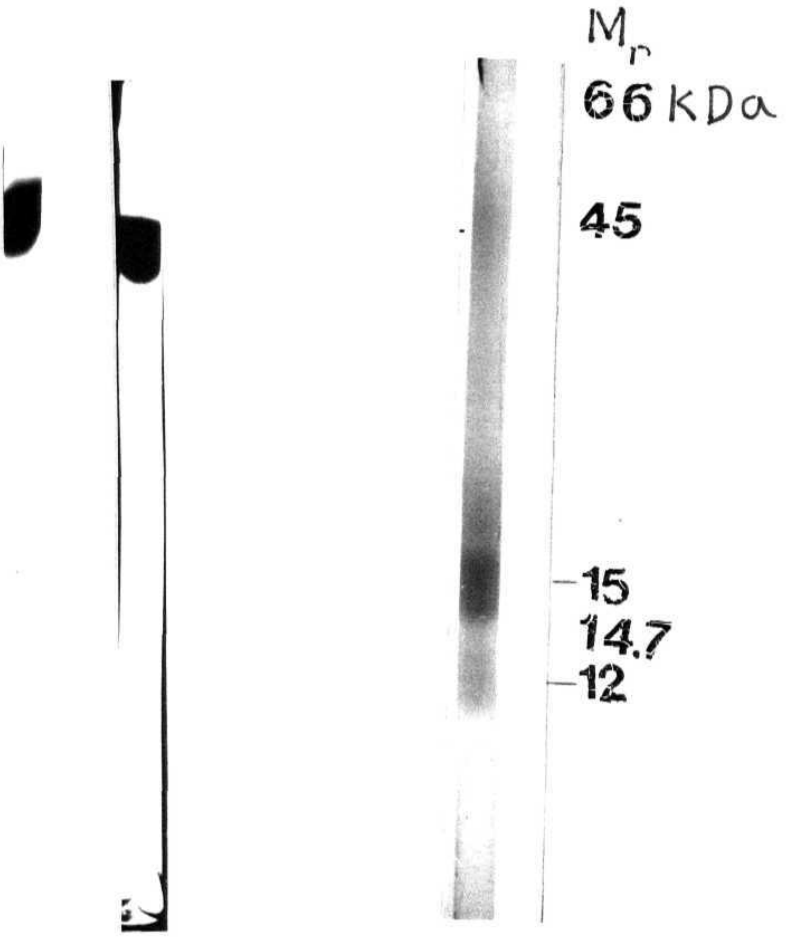

ii
A
M_r
66 KDa
45
15
14.7
12
B

Fig. 4. Binding and elution of mannose specific seed lectin on Sepharose-divinyl sulfone-methyl mannoside gel. 4.57 mg of purified lectin in PBS passed through the column and eluted with 0.5 M methyl mannoside sugar.

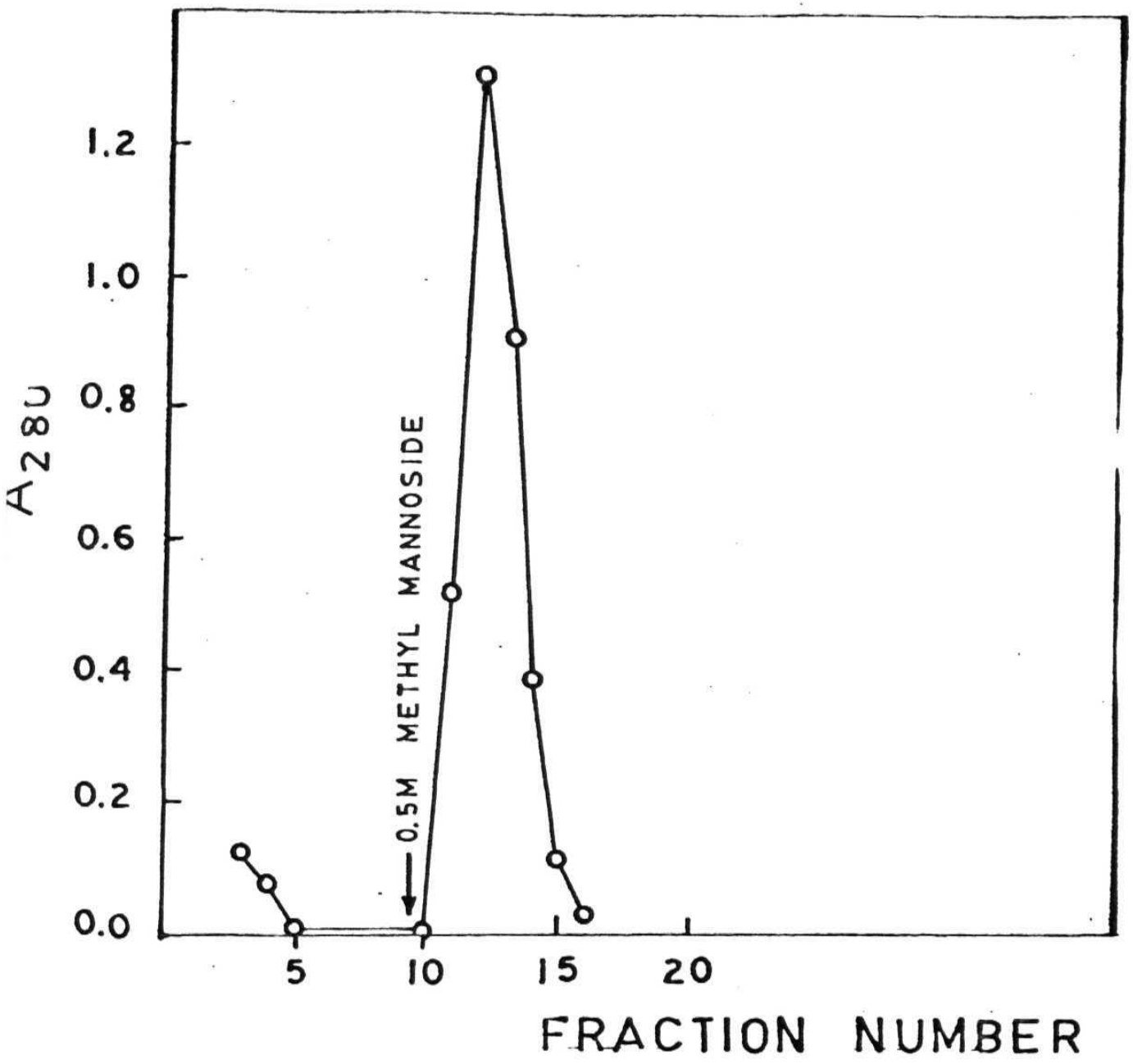
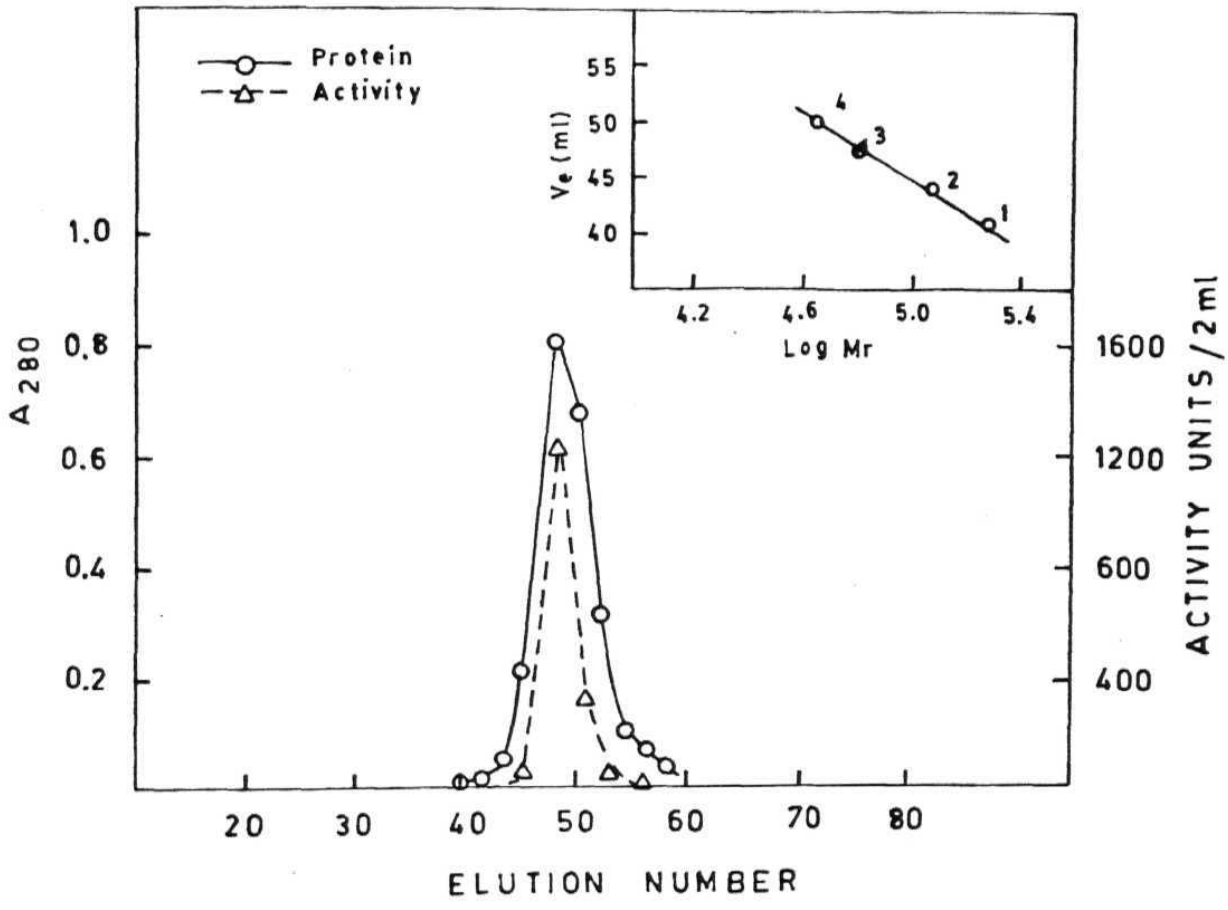

1.2
1.0
0.8
0.6
0.4
0.2
0.0
A280
0.5M METHYL MANNOSIDE
5
10
15
20
FRACTION NUMBER

Fig. 5. Gel filtration of the lablab bean mannose specific lectin. Mannose specific-seed lectin was dissolved in 25 mM Tris-HCl buffer pH 8.0 containing 0.15 M sodium chloride and was loaded on to a column of Biogel P-200 (1.4 X 86 cm). Column equilibrated with 25 mM Tris-HCl buffer pH 8.0 containing 0.15 M sodium chloride and eluted with the same buffer. Fractions 2 ml were collected and elution was followed by monitoring absorbance at 280 nm. Inset. Plot of elution volume (V_e) vs log Mr. (o) standards, 1. Myosin 2. β-galactosidase, 3. Bovine serum albumin and 4 Ovalbumin. • lablab bean mannose specific lectin.


Protein
Activity
A 280
1.0
0.8
0.6
0.4
0.2
20
30
40
50
60
70
80
ELUTION NUMBER
ACTIVITY UNITS / 2 ml
1600
1200
600
400
Ve (ml)
55
50
45
40
4.2
4.6
5.0
5.4
Log Mr
1
2
3
4

Haemagglutination and sugar inhibition studies

The glucose/mannose specific lectin is human blood group non-specific and also agglutinates rabbit erythrocytes. Various sugars inhibit the lectin activity. The results of the sugar inhibition studies are given in Table 3.

Immuno-affinity purification of glucose/mannose specific seed lectin

The glucose/mannose specific seed lectin could be purified using the immuno-affinity matrix. Fig. 6 shows the purification profile of this lectin on immuno-affinity matrix. The bound lectin was eluted with 0.1 M glycine-HCl buffer pH 2.65. The eluted protein was immediately neutralized with 2 M Tris. Table 4 shows the purification of this lectin from 1 gm seed meal. As can be seen from the Table, from 1 gm seed meal about 3 mg of the lectin could be purified using this matrix.

Immunodiffusion & Western blot analysis

The lectin cross-reacts with the antiserum raised against this lectin in rabbits. Fig. 7A shows the results obtained. The central well contained antiserum raised against this lectin. Well I contained lectin purified using goat TgM-Sepharose matrix. Well II contained lectin purified from immuno-affinity matrix. A single precipitin line was obtained when the lectin was purified by either method. Fig. 7B shows the Western blot of the seed lectin. It is apparant from the figure that both the 15 kDa and 12 kDa protein bands react with the antiserum.

Table - 3 Inhibition of haemagglutinating activity by various sugars by the lablab bean lectin, using trypsin treated rabbit erythrocytes. 0.01 to 0.1 ml of 0.1M stock sugar solutions were used in the assay. 4 haemagglutinating units of the lectin in 0.1 ml were incubated with the different sugars and then 0.2 ml of trypsin treated rabbit erythrocytes were added and agglutination visualised after incubation at 37°C for 90 minutes. Other sugars tested, galactose, lactose, cellobiose, gentiobiose, raffinose fucose, N-acetyl galactosamine and sucrose were non-inhibitory upto concentrations of 0.1M.

Sugar	Inhibitory concentration (mM)
Glucose	5.0
Mannose	5.0
Methyl α-D-glucoside	< 0.5
Methyl α-D-mannoside	< 0.5
3-O-methyl glucose	2.5
Phenyl a-D-mannose	<2.5
Phenyl α-D-glucose	<2.5
N-Acetyl glucosamine	10.0
3-O-D-mannopyranosyl mannopyranose	NI
Mannosamine	NI
Melizitose	NI

NI: non-inhibitory

Fig. 6. (i) Purification of mannose specific seed lectin on immuno-affinity matrix (seed lectin specific IgG-Affigel-10). 2 ml of 80% ammonium sulphate fraction (39 mg protein) loaded on the column (1.1X1 cm) equilibrated with PBS, pH 7.4. Column washed with PBS and eluted with 0.1 M glycine-HCl buffer pH 2.65.
(ii) Native PAGE of the purified lectin at pH 4.6.

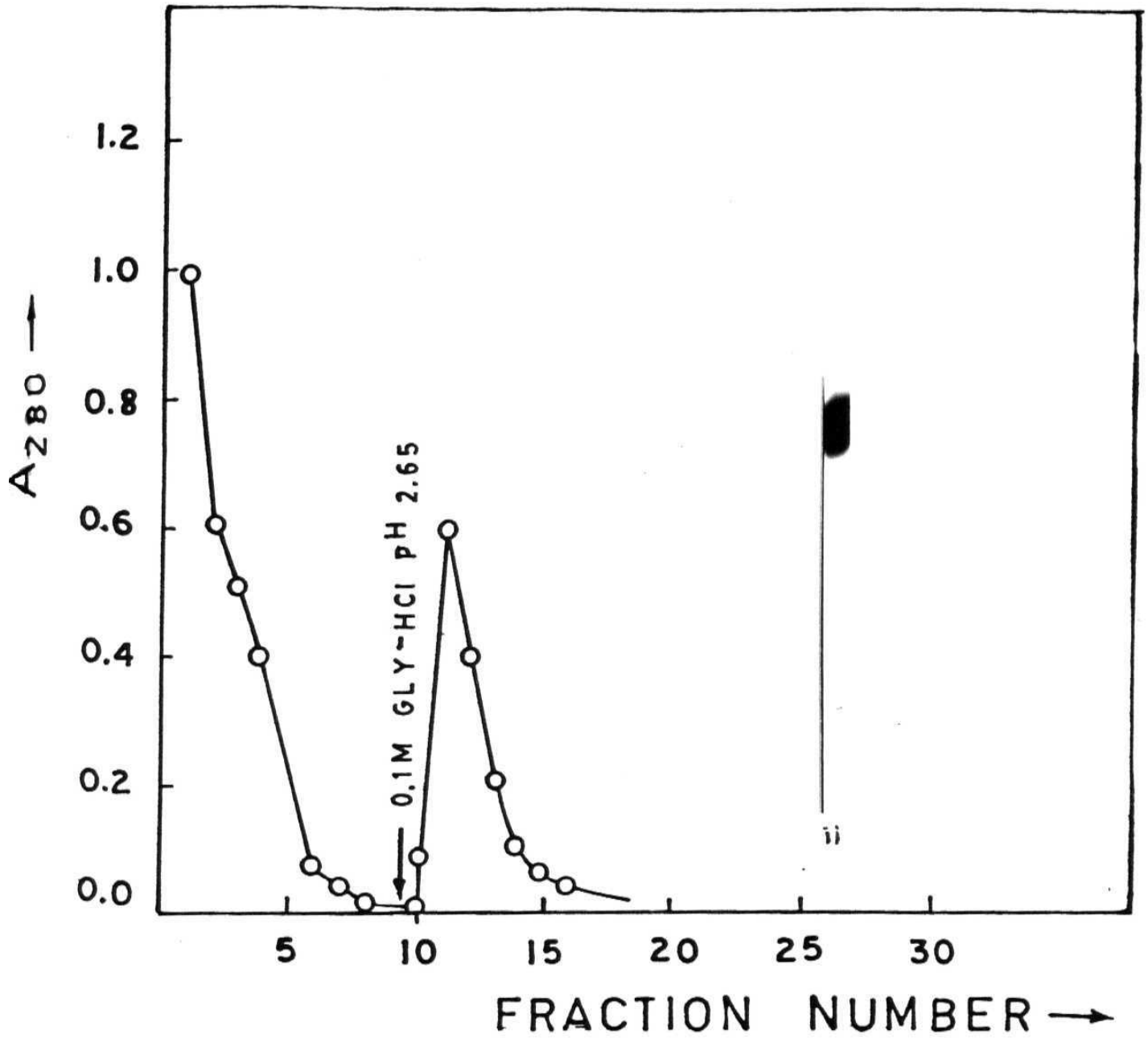
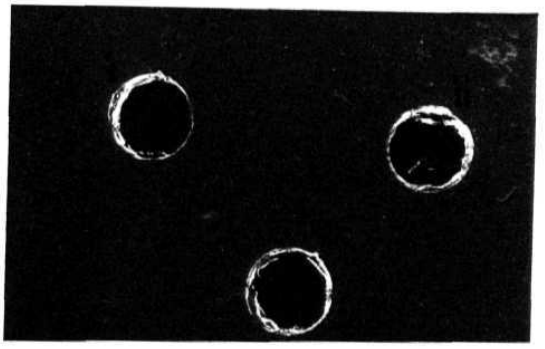

1.2
1.0
0.8
0.6
0.4
0.2
0.0
A_{280} →
0.1M GLY-HCl pH 2.65
ii
5
10
15
20
25
30
FRACTION NUMBER →

Table - 4: Purification of lablab bean Glucose/Mannose specific lectin on immuno-affinity column.

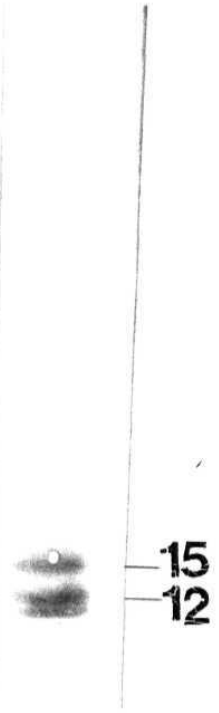
Step	Volume (ml)	Protein (mg)	Activity (U)	Recovery (%)
Crude extract	3.75	70	4800	100
pH 7.4 supn. (S0% Amm. SO_4 fractionation)	2	39.5	2560	53
Affinity chromatography	1	1.5	1280	26

Fig. 7A. Immunodiffusion experiment of the purified mannose specific seed lectin. Central well contained antiserum to the mannose specific seed lectin. Wells (i) and (ii) contained purified mannose specific lectin purified on goat IgM-Sepharose gel and immuno-affinity column repsectively.

B. Western blot analysis of the mannose specific seed lectin.



A



B

Mitogenic activity of the lectin

The purified lectin was found to *he* mitogenic **to** rat and murine lymphocytes **at** concentrations as low as 10 μg (Fig. 8).

Isolation of lectin binding proteins

Protein precipitated by acid at pH 5.0 from the crude extracts of the lablab bean seeds was solubilised and dialysed against Tris-HCl buffer pH 8.0. Clear supernatant (76 mg protein) was passed through Sepharose--divinylsulfone-mannose gel and the flow through was passed through lectin-Affigel column. **The** column was washed with the same buffer and the bound protein was eluted with 20 mM sodium acetate buffer pH 4.0. The purification profile can be seen from Fig. 9. From 76 mg of crude protein 1.0 mg of the lectin binding protein could **be** obtained. The lectin binder could not be eluted using sugar or by high **salt.**

SDS-PAGE

Fig. **10** shows the SDS-PAGE pattern of the lectin binder. The protein dissociated into one major band conesponding to molecular weight of 53 kDa and two minor bands of molecular sizes of 58 kDa and 52 **kDa.**

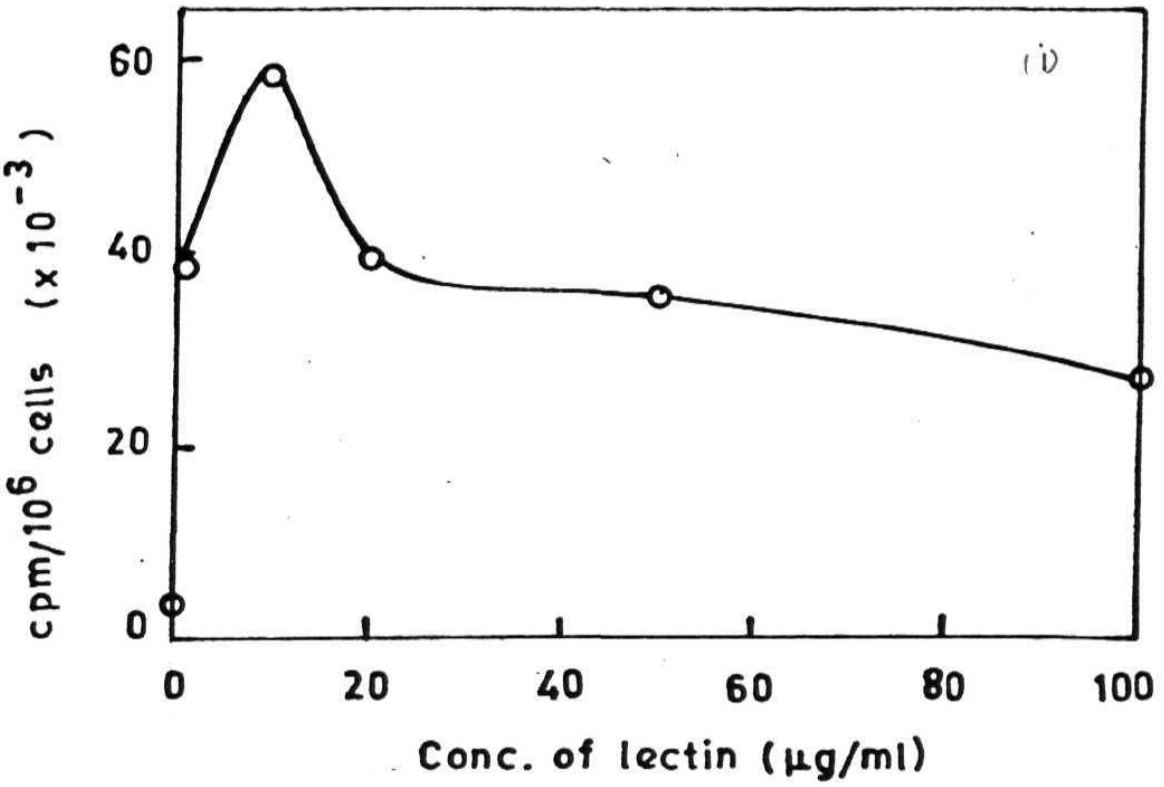
Carbohydrate estimation

The lectin binder was found to be a glycoprotein with around 1.1%, carbohydrate.

Fig. 8. Mitogenic activity of the purified mannose specific lablab bean lectin.

(i) rat lymphocytes

(ii) mouse lymphocytes.


(i)
60
40
20
0
cpm/10^6 cells (x10^-3)
0
20
40
60
80
100
Conc. of lectin (µg/ml)

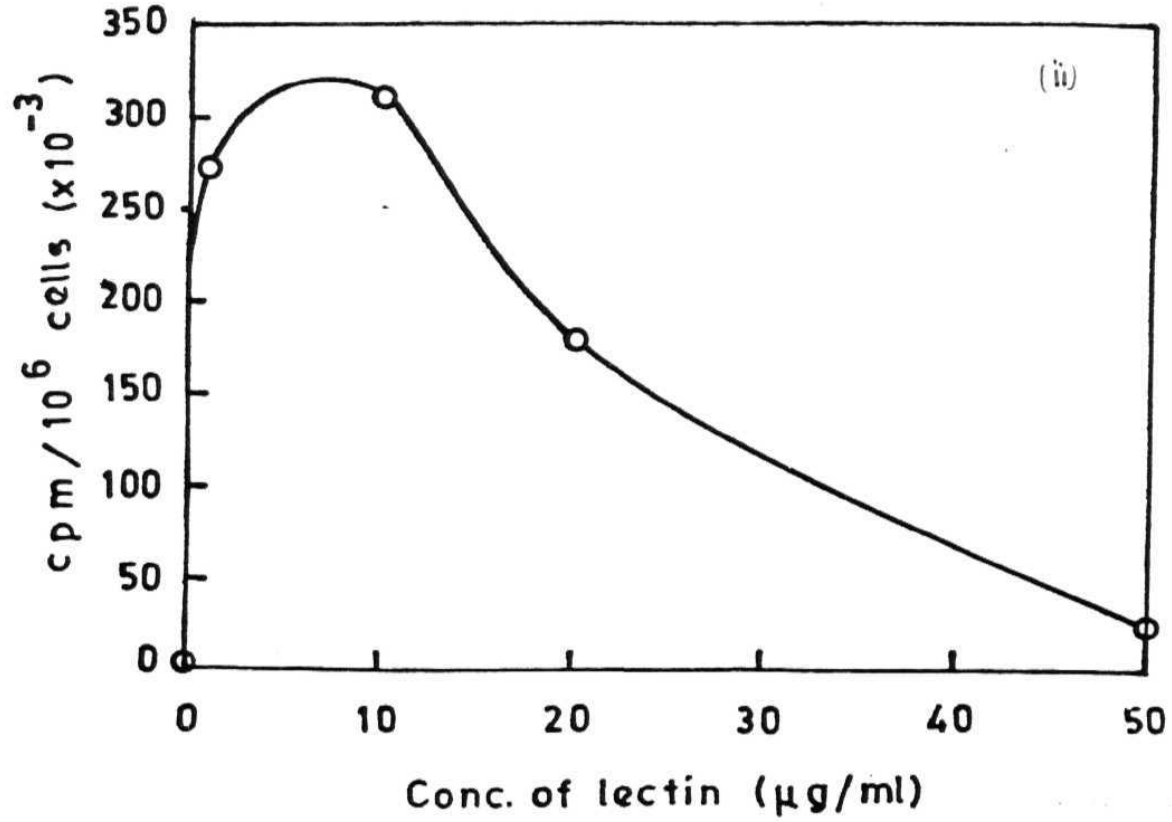

(ii)
350
300
250
200
150
100
50
0
cpm/10^6 cells (x10^-3)
0
10
20
30
40
50
Conc. of lectin (µg/ml)

Fig. 9. Isolation of lectin receptors on Lectin-Affigel column. Protein precipitated by acid at pH 5.0 from the crude extiact of the lablab bean seeds was solubilised and dialysed against Tris-HCl buffer pH 8.0. Clear supernatant (76 mg protein) was passed through Sephraose-mannose gel (1.2 X 1 cm) and the flow through was connected to a Lectin-Affigel column (1.3 X 1.3 cm) equilibrated with Tris-HCl buffer pH 8.0. Elution was performed using 20 mM sodium acetate buffer pH 4.0.

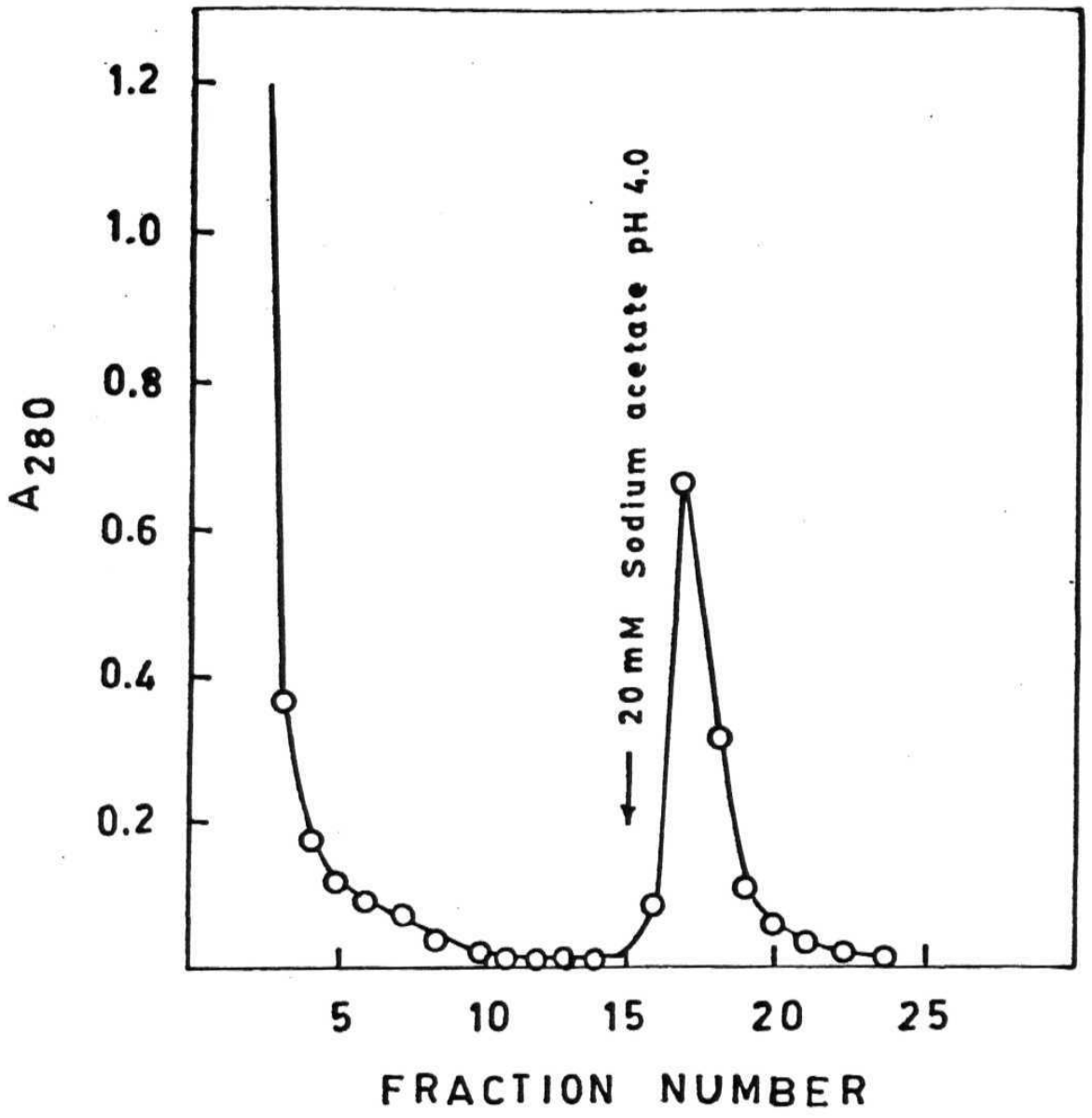
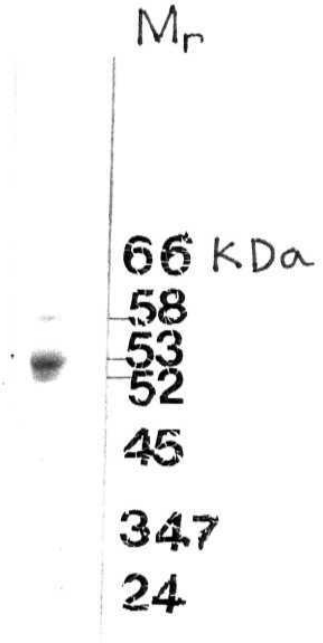

1.2
1.0
0.8
0.6
0.4
0.2
A280
20 mM Sodium acetate pH 4.0
5
10
15
20
25
FRACTION NUMBER

Fig. 10. SDS-PAGE pattern of Lectin binding proteins from lablab beans under reducing conditions. 10% gel.


Mr
66 KDa
58
53
52
45
347
24

Purification of the galactose specific lectin

The galactose specific lectin was purified by conventional methods since the lectin activity failed to bind on the Sepharose--galactose, Sepharose--lactose and Affigel-erythrocyte membrane protein matrices. The crude extract was fractionated with ammonium sulphate (0-30%, 30-60%, 60-80%). The 60-80% fraction containing the lectin activity was passed through Biogel P-200 gel filtration column. The lectin activity was eluted as a single peak (Fig. 11). About 4 mg of purified protein can be obtained from 7 gm seed meal. The fractions containing lectin activity were pooled and concentrated.

PAGE and SDS-PAGE

Native PAGE was performed for the galactose specific lectin at pH 8.9 and the lectin was found to be homogeneous (Fig. 12A). SDS-PAGE on Weber and Osboni tube gels revealed the presence of two subunits, 50 kDa and 20 kDa (Fig. 12B).

Immunodiffusion

Fig. 13 shows the immunodiffusion pattern of the galactose specific seed lectin. Central well contained antiserum to the mannose specific seed lectin. Wells I and HI contained purified mannose specific seed lectin and wells II and IV contained purified galactose specific seed lectin. The precipitin lines fuse with each other suggesting the presence of similar antigenic sites on the two proteins.

Haemagglutinating activity

The galactose lectin agglutinates only trypsin treated rabbit erythrocytes.

Fig. 11. Gel filtration of the lablab bean galactose specific lectin. The 60-80% ammonium sulphate fraction was passed through a column of Biogel P-200 (1.4 X 86 cm). Column equilibrated with 25 mM Tris-HCl buffer pH 8.0 containing 0.15 M sodium chloride and eluted with the same buffer. Fractions 2 ml were collected and elution was followed by monitoring absorbance at 280 nm. Inset. Plot of elution volume (V_e) vs log Mr. (o) standards, 1. Myosin 2. β-galactosidase, 3. Bovine serum albumin and 4 Ovalbumin. • lablab bean galactose specific lectin.

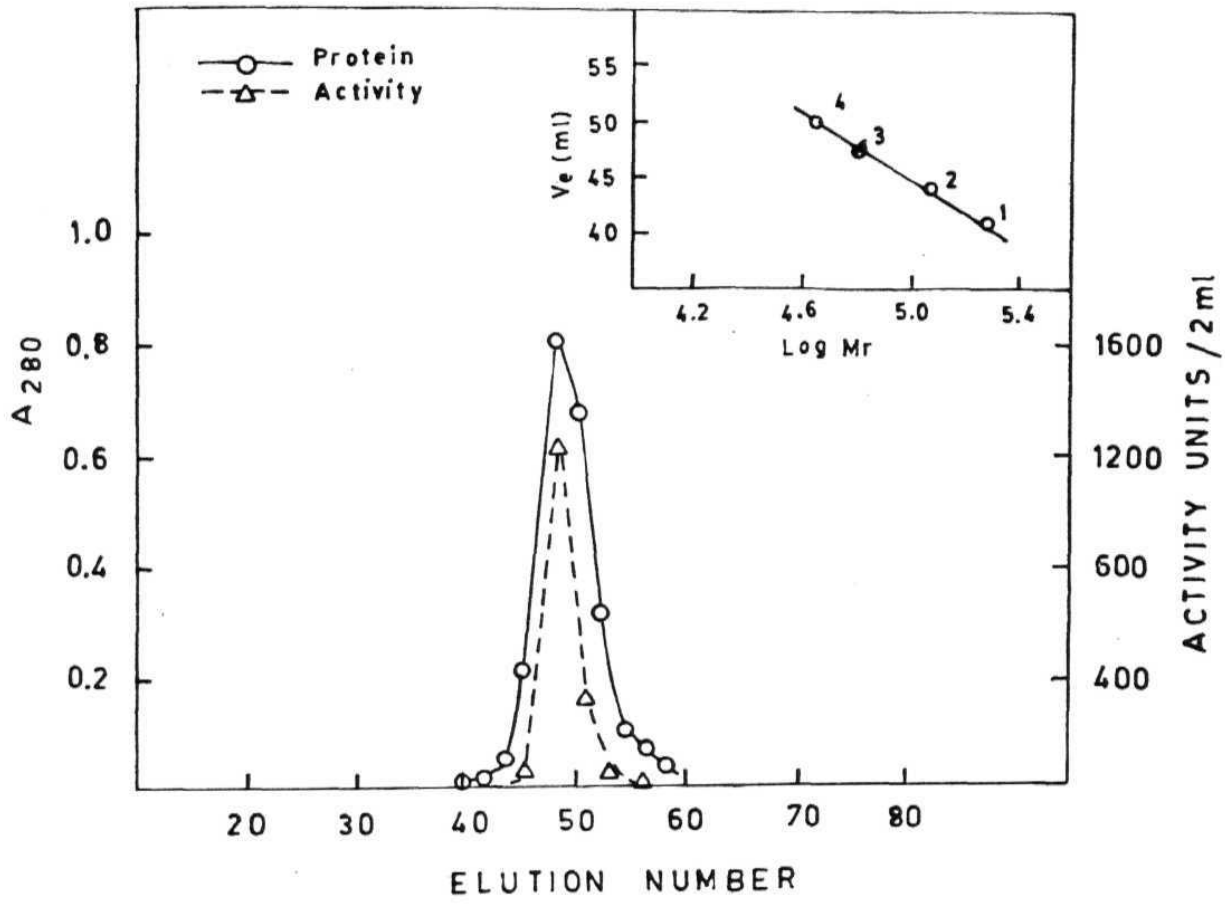

Protein
Activity
Ve (ml)
55
50
45
40
4
3
2
1
4.2
4.6
5.0
5.4
Log Mr
A 280
1.0
0.8
0.6
0.4
0.2
1600
1200
600
400
ACTIVITY UNITS / 2 ml
20
30
40
50
60
70
80
ELUTION NUMBER

Fig. 12A. PAGE pattern of galactose specific lectin from the seeds of lablab beans at pH 8.9. PAGE was performed on 7.5% native gel.

B. SDS-PAGE pattern of purified galactose specific lectin.

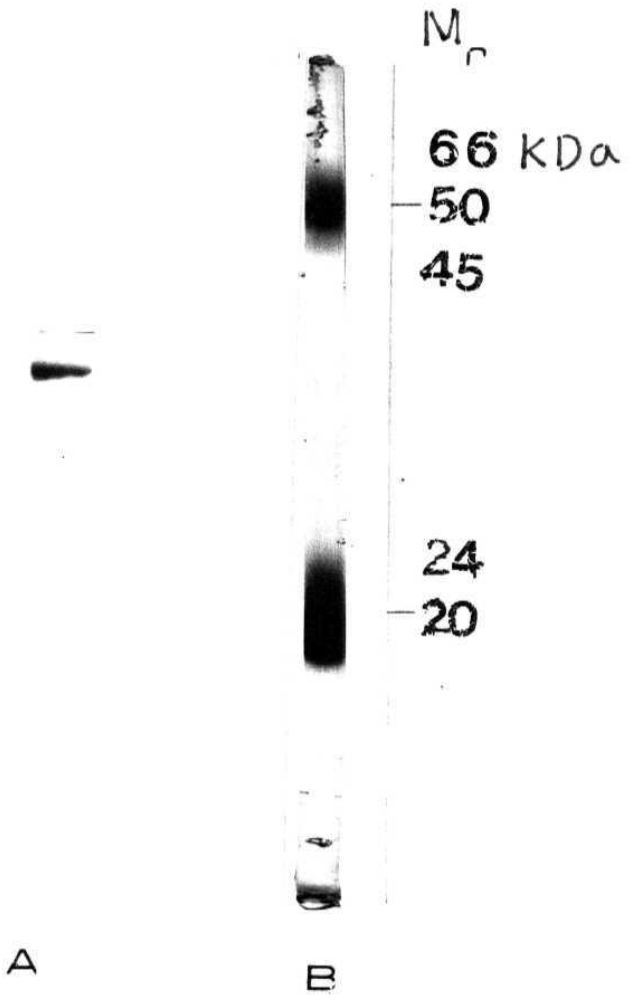
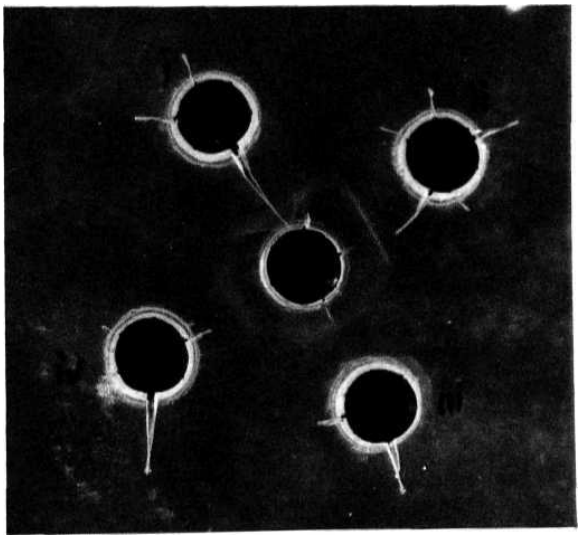

Mr
66 KDa
50
45
24
20
A
B

Fig. 13. Immunodiffusion of the galactose specific seed lectin. Central well contained 30 µl antiserum to the mannose specific seed lectin. Wells (i) and (iii) contained purified mannose specific seed lectin. Wells (ii) and (iv) contained purified galactose specific seed lectin.



SECTION - D

DISCUSSION

Seeds of the Indian lablab beans (*Dolichos lablab* var. *typicus*) were found to contain a glucose/mannose specific lectin that was earlier purified on Sepharose-divinylsulfone-mannose gel (Siva Kumar and Rajagopal Rao, 1986). In order to develop alternate affinity methods to isolate the lectin in large quantities and to study its fine sugar specificity, mitogenicity and to identify endogenous lectin receptors and other lectins with different sugar specificity, the present work was carried out. The glucose/mannose specific lectin could be purified on Sepharose-mannose gels, with yields of 13 mg from 5 gm of seed powder, with slight modifications in the purification protocol. Another affinity matrix, goat IgM-Sepharose yielded as high as 2.18 mg of purified lectin from 1 gm seed powder. Thus by modifying the extraction procedures and using these affinity matrices, higher amounts of purified lectin were obtained which enabled to study different properties of this purified lectin.

The lectin isolated from both the affinity matrices was found to be homogeneous in PAGE, at pH 4.6. However on SDS-PAGE in tube gels, using Weber and Osborn procedure, the lectin dissociated into two subunits corresponding to molecular sizes 15 kDa and 12 kDa respectively consistent with the earlier finding (Siva Kumar and Rajagopal Rao, 1986). The native molecular size of the purified lectin determined on Biogel P-100 and P-200 was found to be 64000 Da. The lectin was found to be a glycoprotein with 3% carbohydrate. From the native molecular size and SDS-PAGE analysis it is apparant that the lectin is

possibly made of two types of subunits with an $\alpha_2\beta_2$ structure. In a recent study, primary sequence analysis of the field bean lectin *(Dolichos lab/ab* var. *lignosus*) revealed that it has a $\alpha_2\beta_2$ structure (Gowda *et al,* 1994). The purified lectin agglutinates rabbit and human erythrocytes. The agglutinating activity of the purified lectin was inhibited by a number of sugars related to glucose and mannose. Among simple sugars tested, glucose and mannose were potent inhibitors. The results obtained indicate that the hydroxyl group at C-2 seems important for binding. Substitution at C-1 in glucose/mannose by methyl as well as by phenyl groups enhanced their ability to inhibit the lectin activity. The C-3 hydroxyl group in mannose seems important as blocking it with another mannose sugar abolishes inhibition. This is further confirmed by the fact that 3-O-methyl glucose is also a good inhibitor for lectin activity. The configuration around C-4 does not affect the binding as galactose and lactose were non-inhibitory. N-acetylglucosamine was inhibitory only at higher concentrations. Hydrophobic sugars such as methyl glucose and methyl mannose are better inhibitors for lectin activity. This fact was further reconfirmed by passing the purified lablab bean lectin on to Sepharose-divinylsulfone-methyl α-D-mannopyranoside gel. As high as 4 mg of purified lectin could be bound on 1ml of this gel. These different sugars were found to be effective inhibitors of several other glucose/mannose specific lectins reported (Allen *et al,* 1978).

Although the lablab bean lectin is a glycoprotein, it failed to bind on Con A-Sepharose gel, a property exhibited by many other glycoprotein lectins owing to their glycoprotein nature (Iglesias *et al,* 1982). The purified lectin was also found to be mitogenic to rat and murine lymphocytes at concentrations as low as

10 μg. Many of the glucose/mannose specific lectins reported were found to be mitogenic to other lymphocytes (Lis and Sharon, 1986).

Antibodies to the purified lablab bean lectin raised in a rabbit specifically interacted with the lectin, purified on a Sepharosc-mannose gel, goat IgM-Sepharose gel, giving a single precipitin line. From the antiserum, lectin specific IgG could be easily purified on lectin-Affigel matrix. This specific IgG when immobilized to Affigel was found to be a suitable immuno-affinity matrix for the direct purification of the lablab bean lectin. Upto three mg protein could be obtained on immuno-affinity matrix from 1 gm seed meal. The specificity of the antibody was further tested in a Western blot experiment, wherein the subunits are separated by SDS-PAGE in slab gels and the protein bands detected using primary and secondary antibodies. However, in this 3 protein bands appeared all of them reacting with the antibody suggesting that they are part of the native lectin. This suggests that the additional band detected is possibly due to proteolysis.

From the seed extracts of the Jablab beans, proteins that interact specifically with immobilized lectin in lectin-affinity chromatography have been isolated. These proteins bind to the lectin at pH 8.0 and can be desorbed by lowering the pH to 4.0. Neither glucose nor high salt desorbs the proteins. The protein eluted with pH 4.0 buffer had neither lectin nor glycosidase activity but was found to be a glycoprotein with 1.1% carbohydrate. When the eluted protein was concentrated, dialysed and reloaded on the same gel, upto 80% of protein could be rebound on the gel which could again be eluted with sodium acetate buffer pH 4.0. The same amount of protein could be bound on a lectin-Affigel

matrix that was pre-equilibrated with 0.5 M glucose at pH 8.0 suggesting that the lectin does not interact with the receptor through the sugar binding site. In SDS-PAGE, the protein eluted from the lectin-Affigcl matrix dissociated into one major band corresponding to molecular size 53 kDa and two minor bands of molecular sizes 58 kDa and 52 kDa. The two minor bands might possibly represent proteolytically derived fragments of the isolated protein. Fndogenous lectin receptors exhibiting similar electrophoretic mobilities have been identified from some legume seeds. However in these studies, the authors observed that **the** lectin interacts with the endogenous receptors by its sugar binding site or by ionic interactions as the lectin bound proteins could be eluted by inhibiting sugar or by high salt (Ganscra *et al.,* 1979). The data obtained with the lablab bean lectin and its endogenous receptors suggest that the lectin-receptor interaction is only pH dependent. Only a few Leguminous seeds have been known to contain more than one lectin which significantly differed in the properties and their sugar specificity (Baumann *et al.,* 1979). Seeds of field bean (*Dolichos lablab* var.*lignosus*) were found to contain a glucose/mannose specific lectin and a galactose specific lectin which differed largely in their native and subunit molecular sizes (Mo *et al.,* 1990). However an antibody for the glucose/mannose specific lectin cross-reacted with the galactose specific lectin. Seed extracts of lablab beans *(Dolichos lablab* var. *typicus*) when subjected to ammonium sulphate fractionation, (0-30%, 30-60%, 60-80%) clearly separated lectin activities as (i) 0-30% and 30-60% fraction, the activity being inhibited by glucose/mannose and not by galactose and lactose (ii) 60-80% fraction, the activity being inhibited only by galactose/lactose and not by glucose/mannose. This fraction containing the galactose specific seed lectin failed to be retained on Sepharose-mannose, Sepharose-galactose gels. However **the** protein eluted as two peaks on a gel filtration column, **the** major

protein peak alone contained lectin activity and was also found to be homogeneous in native PAGE, pH 8.9. It was found to have a native molecular size of 120000 Da and dissociated into two subunits in SDS-PAGE corresponding to molecular sizes 50 kDa and 20 kDa respectively. This data suggests that the lectin is possibly a tetramer with $\alpha_2\beta_2$ type of structure. In an immunodiffusion experiment, when an antiserum far the glucose/mannose specific lectin was placed in a central well and the mannose specific seed lectin and galactose specific seed lectin were placed in neighbouring wells, a clear cross-reactivity could be observed suggesting antigenic similarity between the glucose/mannose specific lectin and the galactose specific lectin.

These data clearly indicate that the seeds of the lablab beans contain two lectins that show specificity to different sugars and also differ in their native and subunit molecular size pattern. However, they show immunological cross-reactivity. Extensive biochemical characterisation of the galactose specific lectin should reveal its structural similarities to that of the glucose/mannose specific seed lectin, as well as to the galactose, specific stem and leaf lectin that has been discussed in Chapter-Ill.

CHAPTER III

SECTION - A

INTRODUCTION

Lectins are carbohydrate binding proteins that are widely distributed among the seeds of various dicots and monocots whose functions are still not clear (Etzler, 1986, Goldstein and Poretz, 1986, Kijne *et al.*, 1986, Pusztai, 1991). Lectins from the seeds of Leguminoseae members have been extensively studied owing to the relative abundance of these proteins in the seeds. However among the monocots, lectins have been purified from embryos of some cereals and from Triticale seeds (Siva Kumar and Padma, 1996).

Various workers have identified lectin activities from vegetative tissues such as stems, leaves, roots, flowers, tubers and bark (Bonebaeck, 1984; Etzler 1986; Van Damme and Peumans 1990; Ho Pak *et al,* 1992). The levels of the vegetative lectins are much lower as compared to their seed counterparts. While most of the vegetative lectins have been known to show strucrural/immunological identity to the corresponding seed lectin, very few showed strong haemagglutinating activities, a property that is characteristic to the lectins (Pusztai, 1991).

The stems and leaves of 3 week old *Dolichos biflorus* plant contain a protein that cross-reacts with antibodies to the seed lectin. This cross-reactive material was purified by conventional methods and has a

molecular size of 68,000-70,000. It however fails to agglutinate human A+ ciythrocytes and is incapable of binding to the affinity matrix that is used to purify the seed lectin (Talbot and Etzler, 1978; Etzler and Borrebaeck, 1980). Strong haemagglutinating activities were reported for protein extracts from the leaves and flowers of *sophora japonica* which were purified by affinity chromatography on lactose - agarose gels. Both lectins showed similar sugar specificity and amino acid composition (Ito, 1986). Two isolectins were also isolated from the leaves of *Psophocarpus tetragonolobus* (winged bean) whose molecular masses were estimated to be 60 kDa and each of the isolectin contained similar subunit pattern (Yagi *et al.*, 1994). Kammemura *et al.* (1996) have isolated a novel lectin from the leaves of *phaseolus vulgaris* by affinity chromatography on ovomucoid-Sepharose. The subunits of this protein share structural homology to the pod lectin purified from the same source.

Wheat germ agglutinin - like - lectins were also isolated from vegetative tissues of wheat (Peumans *et al.*, 1982). Among the other monocots, vegetative lectins have been isolated from cereals. A lectin from the leaves of *Agropyrum jepens* was found to differ in its sugar, blood group specificity and scrological properties to that of the embryo lectin. The embryo lectin is non-blood group specific and its activity is not inhibited by N-acetyl glucosamine (Cammue *et al.*, 1985). The leaves of *Listera ovata* (Tway blade) contain a D-mannose specific lectin which does not agglutinate human erythrocytes and is a dimeric protein composed of 2 subunits with molecular size of 12,500 (Saito *et al.*, 1993). Koike *et al,* (1995) isolated a lectin from the leaf skin of *Aloe arborescens miller.*

var. *Natalensis Berger* (Kidachi Aloe) by conventional methods. The purified lectin was found to be mitogenic to mouse lymphocytes and agglutinates only rabbit erythrocytes. It also showed structural homology to the mannose binding lectin from *Galanthus niv al is* (snow drop) bulbs.

The function of the vegetative lectins is poorly understood, although various workers have attributed that root lectins play a role in symbiosis (Pueppke *et al.*, 1978, Pusztai, 1991).

Lectins from the roots of some leguminous species have been isolated by conventional methods, affinity chromatography, immuno-affmity chromatography. The pea root lectin showed haemagglutinating activity and also cross-reacted with antibodies to the seed lectin (Gatehouse and Boulter, 1980). The soybean root lectin was found to have different subunit composition as compared to the seed lectin (Gade *et al*, 1981). A root lectin with completely different molecular weight, subunit stoichiometry and amino acid composition compared to the seed lectin was isolated from the *Dolichos biflorus* plant. This showed a weak cross-reactivity to the seed lectin antibody (Quinn and Etzler, 1987).

A glucose specific lectin was also isolated from *Arachis hypogea* roots that had a different amino acid composition as compared to its seed lectin (Kalsi *et al*, 1993). Root extracts of *Arummaculatum* were found to contain a lectin that agglutinated human spermatozoa (Mladenov *et al*, 1993). Vegetative lectins have also been identified from plants belonging

to families such as Solanaceae, Cucurbitaceae, Euplioibiaccae (Etzler, 1986).

SECTION - B

MATERIALS AND METHODS

Collection of Plant Material

Seeds of *Dolichos lablab* var. *typicus* (Indian lablab beans) were sown and grown in a well protected natural environment. Stems and leaves from 3 week old plants were separated and frozen in liquid nitrogen and stored at -80°C. Gels, sugars and other materials used in the study are as described in Chapter-II.

Extraction of protein from stems and leaves

All operations were carried out at 4°C. The protocol published by Talbot and Etzler (1978) was followed for the extraction of proteins-Typically 200 gm of the frozen material was homogenized in a blender with five fold excess (w/v) of 0.1 M potassium phosphate buffer pH 7.2 containing 0.15 M isoascorbic acid and 0.002 M thyoglycollic acid. After stilling for 2 hrs, the paiticulate matter was removed by eentrifugation at 8,000 rpm for 20 minutes and the clear supeniatant was subjected to 0-40% followed by 40-80% ammonium sulphate fractionation. Both the ammonium sulphate precipitated pellets were dissolved in 40 mM Tris-HCl buffer pH 7.4 and dialysed against the same buffer.

Haemagglutinating activity and sugar inhibition studies

The hacmagglutinating activity at all steps of purification was monitored by using trypsin treated rabbit erythrocytes as described (Siva Kumar and Rajagopal Rao, 1986). Agglutination was also tested using human A, B, O and AB erythrocytes.

Sugar inhibition studies were carried out using a wide variety of sugars (as listed in the Table under results) for the 40-80% ammonium sulphate precipitated proteins and Biogel P-200 eluate.

Chromatographic procedures

A. Preparation of Sepharose-lactose, Sepharose-galactose and rabbit erythrocyte membrane proteins - Affigel-10 gels

Sepharose-lactose and Sepharose-galactose gels were prepared by coupling lactose and galactose sugars separately to Sepharose activated by divinyl sulfone. Details for coupling sugars to this matrix are given in Chapter-II. Erythrocyte membrane proteins were extracted from rabbit erythrocytes and coupled to Affigel-10 following the methodology described earlier (Peumans *et al.*, 1985).

B. Two columns of DE-52 cellulose were packed and equilibrated with 40 mM Tris-HCl buffer pH 7.4. Ammonium sulphate precipitated samples 0-40% and 40-80% after dialysis were loaded on the two columns and the

columns were extensively washed with the column buffer. The bound proteins were eluted by a stepwise gradient of sodium chloride (0.1, 0.2, 0.3 and 0.4 M). 0.4 M NaCl eluates from both columns showing agglutination, were pooled separately, concentrated and dialysed against 25 mM Tris-HCl buffer pH 7.4 containing 150 mM NaCl.

Chromatography **on Biogel P-200**

As most of the protein and activity was found in the 0.4 M NaCl eluate of 40-80% fraction, this was applied to a Biogel P-200 column to separate the lectin activity. In order to determine the native molecular size of the lectin, standard proteins were chromatographed on the same gel.

Protein and Carbohydrate estimation

Protein in the column fractions, and carbohydrate in the purified protein were determined as described in Chapter-II.

SDS-PAGE

The Biogel P-200 eluates were subjected to SDS-PAGE analysis using Weber and Osborn procedure (1969).

Immunological methods

Antiserum to the mannose specific seed lectin from the seeds of Indian lablab beans, purified on Sepharose-divinyl sulfone-mannose gels was prepared as described in the methods section in Chapter-II. For immunodiffusion experiments, antiserum to the seed lectin was placed in a central well in an agar plate. Affinity purified mannose specific lectin, 40-80% protein fraction from the stems and leaves or protein from Biogel P-200 pooled eluate were used in the study.

Immuno-affinity chromatography

From the antiserum directed against the mannose specific seed lectin, specific IgG was purified by passing the antiserum on Affigel-lectin column as described under methods in Chapter-II. The specific IgG thus obtained from several chromatographic runs was pooled, concentrated and coupled to Affigel-10 (immuno-affinity matrix).

5 ml of the 40-80% ammonium sulphate precipitated protein from the stem and leaf extracts was loaded on this immuno-affinity matrix and after washing the unbound proteins, the bound protein could be eluted using 0.1 M glycine-HCl buffer pH 2.65. The eluates were neutralized with 2 M Tris and their lectin activity tested.

SECTION - C

RESULTS

Extraction and purification of the stem & leaf lectin

The biological activity of the stem and leaf lectin was monitored by haemagglutination assay employing trypsin treated rabbit erythrocytes. Untreated erythrocytes reacted weakly. The proteins from the crude extract were precipitated by ammonium sulphate fractionation (0-40% and 40-80%) and these were used for the isolation of the lectin. The 40-80% fraction which contained most of the lectin activity failed to bind on different affinity matrices tested, such as Sepharose-galactose, Sepharose-lactose and rabbit erythrocyte membrane proteins coupled to Affigel-10. When both fractions were passed through two separate DE-52 cellulose gels, the lectin activity was retained on the gels. This is desorbed from the gels by sequential increase in salt concentration (Figs. 1 & 2). It is apparent from these figures that most of the protein that was eluted with 0.4 M NaCl in the 40-80% fraction is the lectin.

Since most of the biological activity was recovered in the 0.4 M NaCl eluates of 40-80% fraction, this was further processed for purification. When 18 mg of this protein was applied to Biogel P-200 gel, the lectin eluted as a single symmetrical peak coinciding with its activity (Fig. 3.) Table 1 summarizes the purification scheme of these consecutive steps which leads to about 61-fold purification of the lectin. As can be seen

Fig. 1: Elution profile of the stem and leaf lectin from DE-52 gel. 0-40% ammonium sulphate fraction (13 mg protein) was loaded on DE-52 gel (2.1X10 cm) equilibrated with 40 mM Tris-HCl buffer pH 8.0. Fractions 4 ml collected. Absorbance monitored at 280 nm. Stepwise elution was performed using 0.1 M, 0.2 M, 0.3 M and 0.4 M NaCl in column buffer.

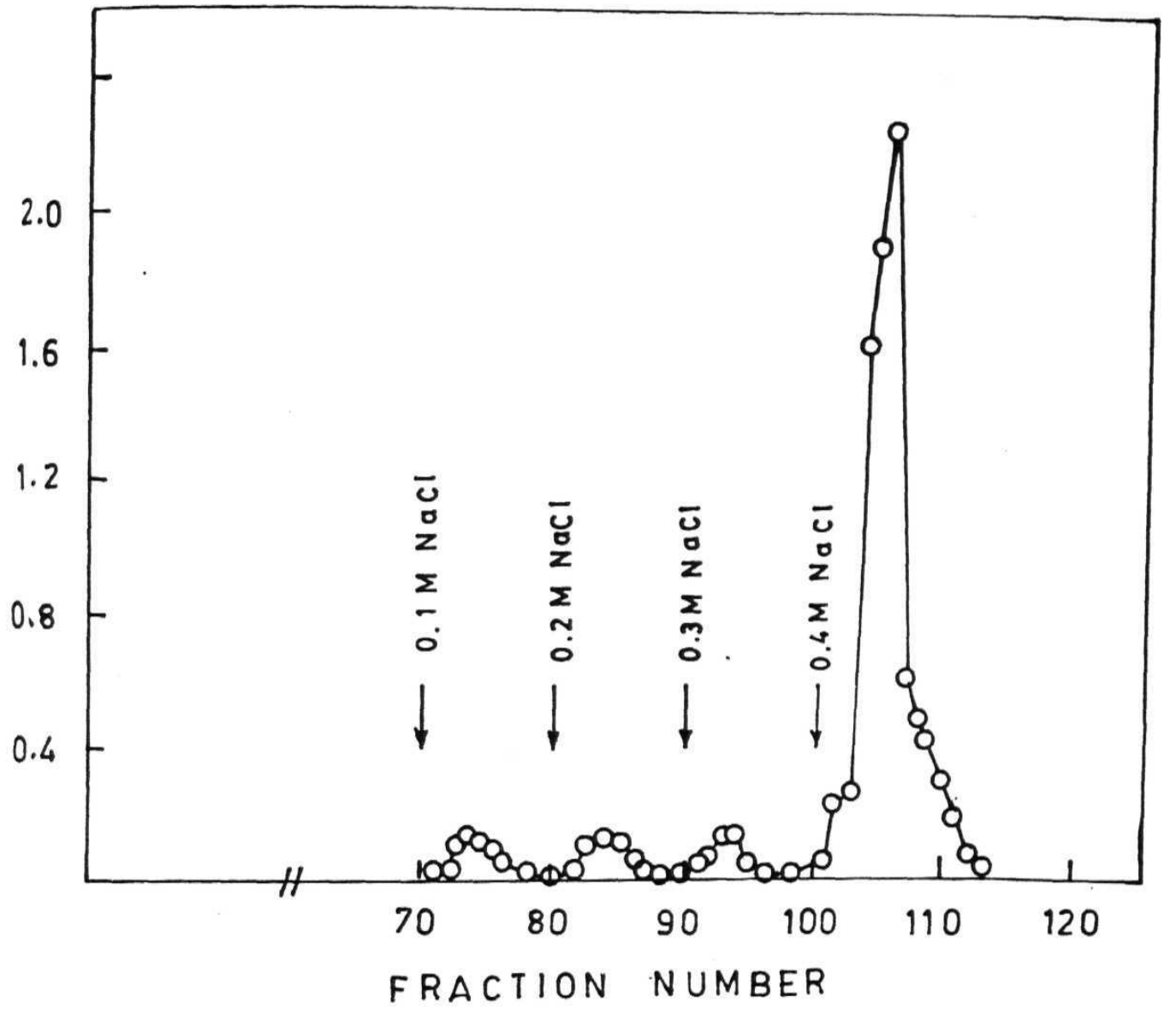

2.0
1.6
1.2
0.8
0.4
0.1 M NaCl
0.2 M NaCl
0.3M NaCl
0.4 M NaCl
70
80
90
100
110
120
FRACTION NUMBER

Fig. 2: Elution profile of the stem and leaf lectin from DE-52 gel. 40-80% ammonium sulphate fraction (47 mg protein) was loaded on DE-52 gel (2.1X](cm) equilibrated with 40 mM Tris-HCl buffer pH 8.0. Fractions 4 ml collected Absorbance monitored at 280 nm. Stepwise elution was performed using 0.1 M 0.2 M, 0.3 M and 0.4 M NaCl in column buffer.

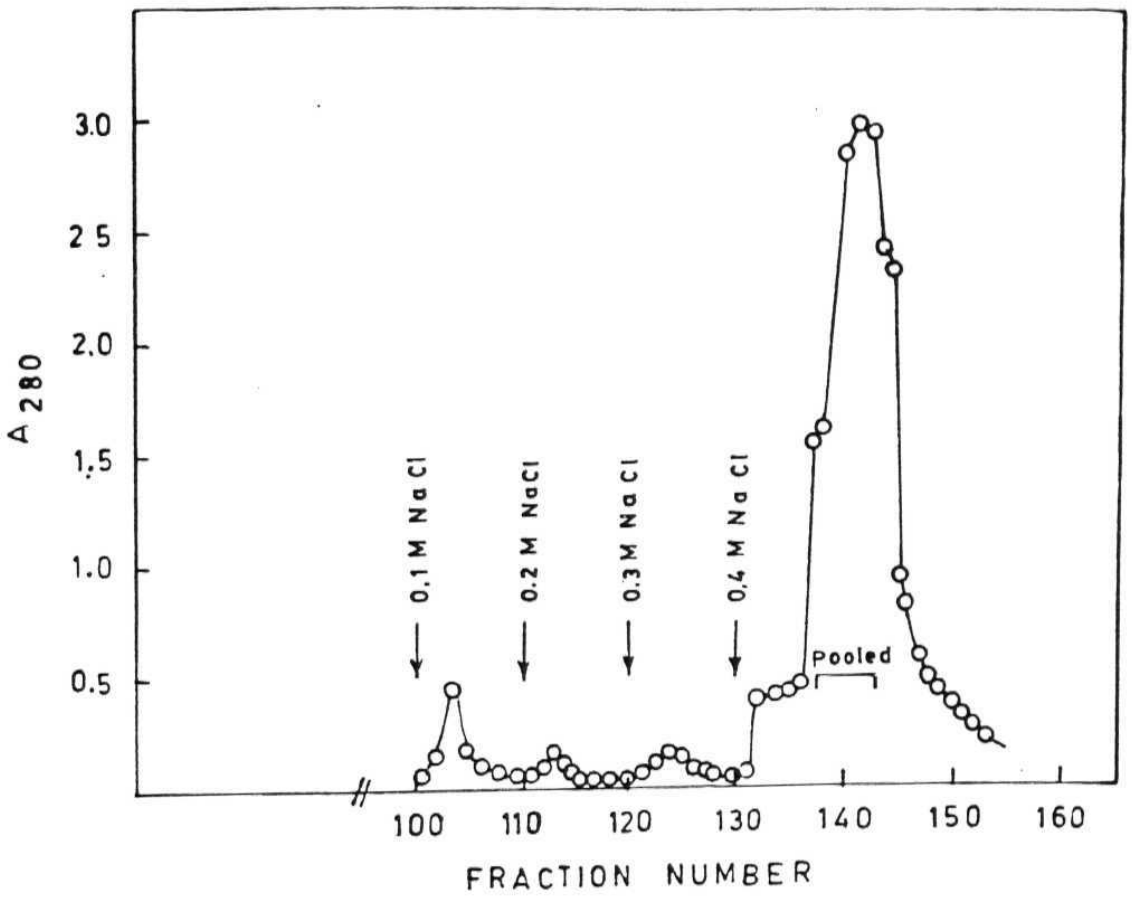

3.0
2.5
2.0
1.5
1.0
0.5
A 280
0.1 M Na Cl
0.2 M NaCl
0.3 M Na Cl
0.4 M Na Cl
Pooled
100
110
120
130
140
150
160
FRACTION NUMBER

Fig. 3. Gel filtration of the lablab bean stem and leaf lectin. DE-52 eluate (9 mg/ml) in 25 mM Tris-HCl buffer pH 8.0 containing 0.15 M NaCl was loaded onto a column of Biogel P-200 (1.4 X 86 cm) and eluted with the same buffer. Fractions 2 ml were collected and elution was followed by monitoring absorbance at 280 nm. Inset. Plot of elution volume (Ve) vs log Mr. (o) standards, 1. Myosin 2. β-galactosidase, 3. Bovine serum albumin and 4 Ovalbumin. o stem and leaf lectin.

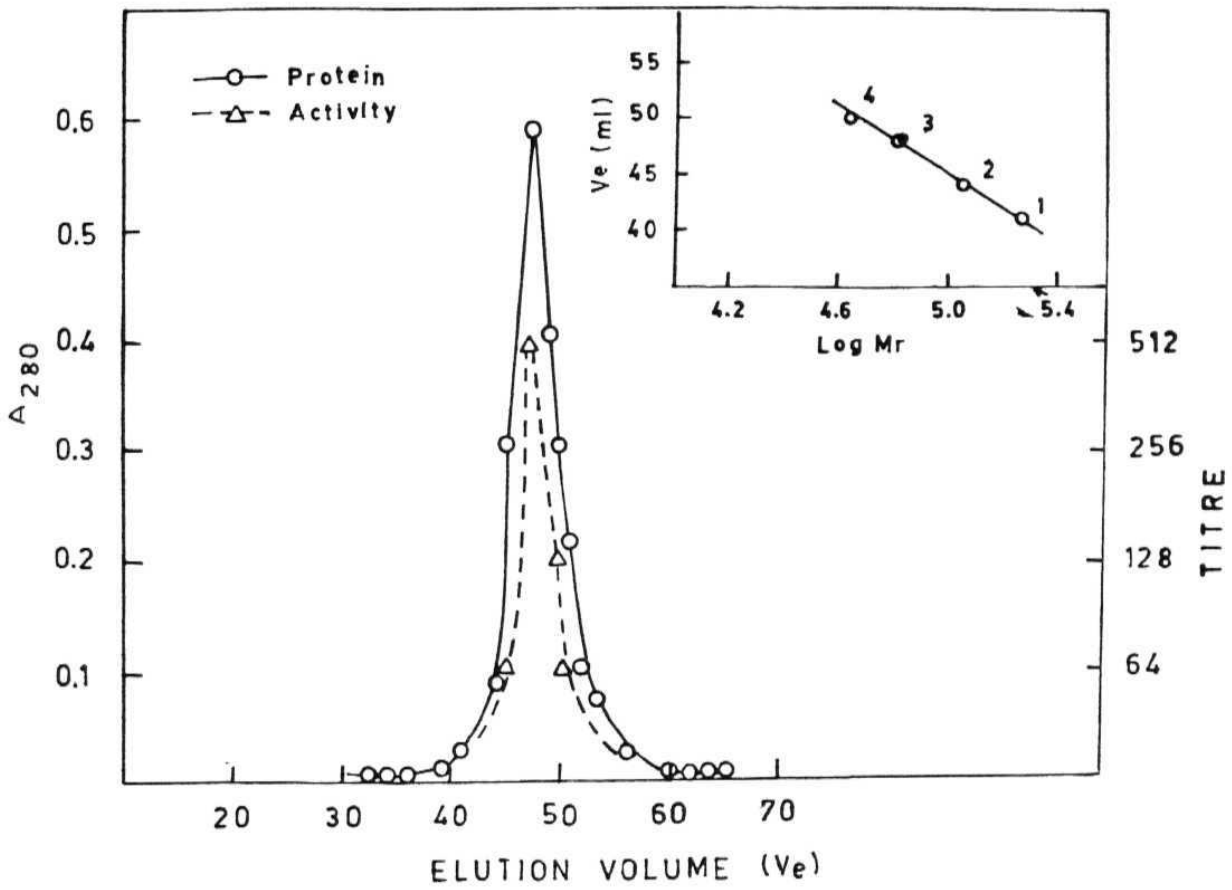

Protein
Activity
A_{280}
0.6
0.5
0.4
0.3
0.2
0.1
512
256
128
64
TITRE
20
30
40
50
60
70
ELUTION VOLUME (Ve)
Ve (ml)
55
50
45
40
4.2
4.6
5.0
5.4
Log Mr
4
3
2
1

Table - 1

purification of the lablab bean stem and leaf lectin. 200 gm of freshly frozen tissue was used.

Stem	Volume (ml)	Protein (mg)	Activity (U)	Specific Activity (U/mg)	Recovery (%)	1 old Purifica-tion
Crude Extract	901	317	36040	114	100	1
Ammonium sulphate 0 - 40%	5	13	400	31	11	0.3
40 - 80%	20	47	25600	545	71	4.8
DE-52 Eluate (0.4 M NaCl)	35	18	22400	1244	62	11
Biogel P-200	12	3	21000	7000	58	61

from the table, about 3 mg of the purified lectin can be obtained from 200 gm. of the stem and leaf material.

Native molecular size of the stem and leaf lectin

The native molecular size of this lectin on the Biogel P-200 column using a calibrated standard curve was found to be 66,000 (Fig. 3 inset).

Carbohydrate estimation

The purified lectin was found to be a glycoprotein with about 3% carbohydrate.

SDS-PAGE

On SDS-PAGE the protein dissociated into 2 bands with molecular weights of 48 kDa and 20 kDa respectively (Fig. 4).

Haemagglutinating activity and Sugar inhibition studies

Among a number of sugars tested for their inhibitory effect on the lectin, N-acetyl galactosamine, galactose, its derivatives and lactose were found to be potential inhibitors. Table 2 shows the inhibitory effect of various sugars on the lectin activity. The inhibitory effects were tested both with the 40-80% ammonium sulphate precipitated proteins and the Biogel P-200 pooled eluate.

Fig. 4. SDS-PAGE pattern of the purified stem and leaf lectin.

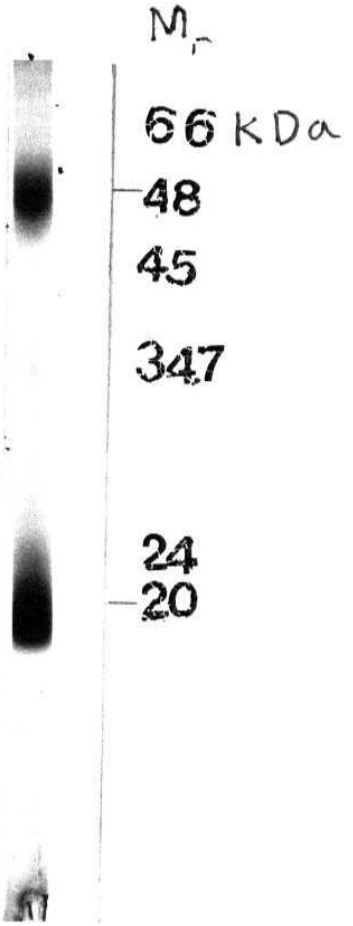

M,
66 KDa
48
45
347
24
20

Table - 2 : Inhibition of the **haemagglutinating** activity of the **stem** and **leaf** lectin with various sugars. A) 4 haemagglutinating units of the 40-80% ammonium sulphate precipitated proteins were used in the assay. B) 4 haeniagglutinating units of the P-200 eluate of purified lablab bean stem and leaf lectin were used in the assay. Inhibition is expressed as mM of the sugar concentration.

Sugar	Inhibitory concentration (mM)	
	40-80% ammonium sulphate fraction	Biogel P-200 pooled eluate
1. Galactose	5.0	2.5
2. Lactose	5.0	2.5
3. Methyl α-D-galactopyranose	2.5	2.5
4. 6-O-Methyl galactopyranoside	2.5	2.5
5. 4-0 D-galactopyranosyl-D-galactopyranose	NI	ND
6. N-Acetyl D-galactosamine	2.5	ND
7. Mannose	NI	NI
8. Glucose	NI	NI
9. Fucose	NI	ND
10. Cellobiose	NI	ND
11. N-Acetyl glucosamine	NI	NI

0.01 to 0.1 ml of the 0.1M stock solutions of the various sugars were used in the assay.

NI : Non-inhibitory.
ND : Not determined.

Immunological methods

The immunoreactivity of the stem and leaf lectin against an antiserum to the mannose specific seed lectin was studied by immunodiffusion analysis (Fig. 5). Under the same conditions, the mannose specific seed lectin gave a precipitin line that fused with the adjacent precipitin band. In addition a faint band appeared below the precipitin line in the agar plate where the stem and leaf lectin was included.

The stem and leaf lectin from the 40-80% ammonium sulphate precipitated proteins was also able to bind specifically to an immuno-affinity matrix and could be eluted specifically using 0.1 M glycine-HCl buffer pH 2.65. Typically when 408 mg protein were loaded on the gel, 1.4 mg protein which showed appreciable biological activity was specifically eluted (Fig. 6).

Fig. 5. Immunodiffusion of the stem and leaf lectin. Central well contained 20 μl of anti serum to the mannose specific seed lectin. Well (i) contained 25 μg of the purified mannose specific seed lectin and well (ii) contained 25 μg of the purified stem and leaf lectin.

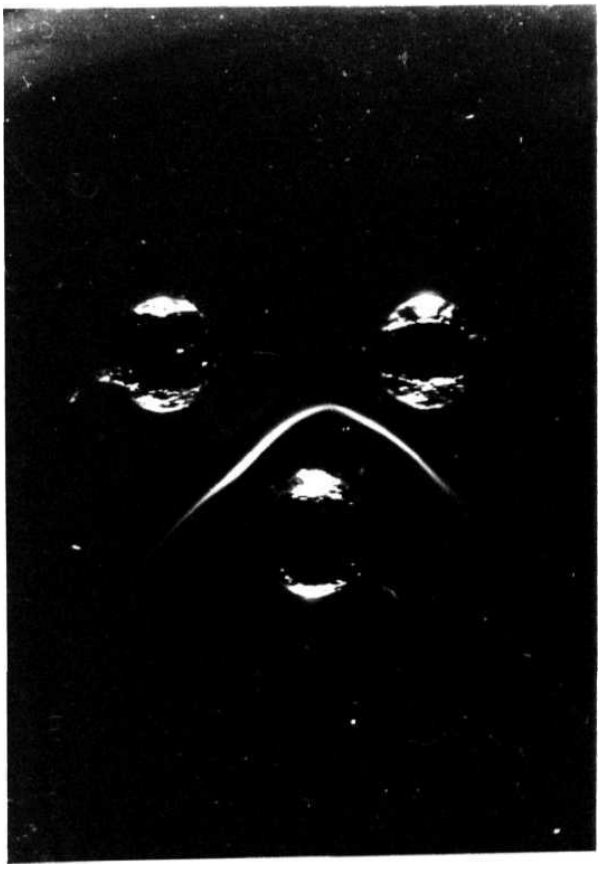
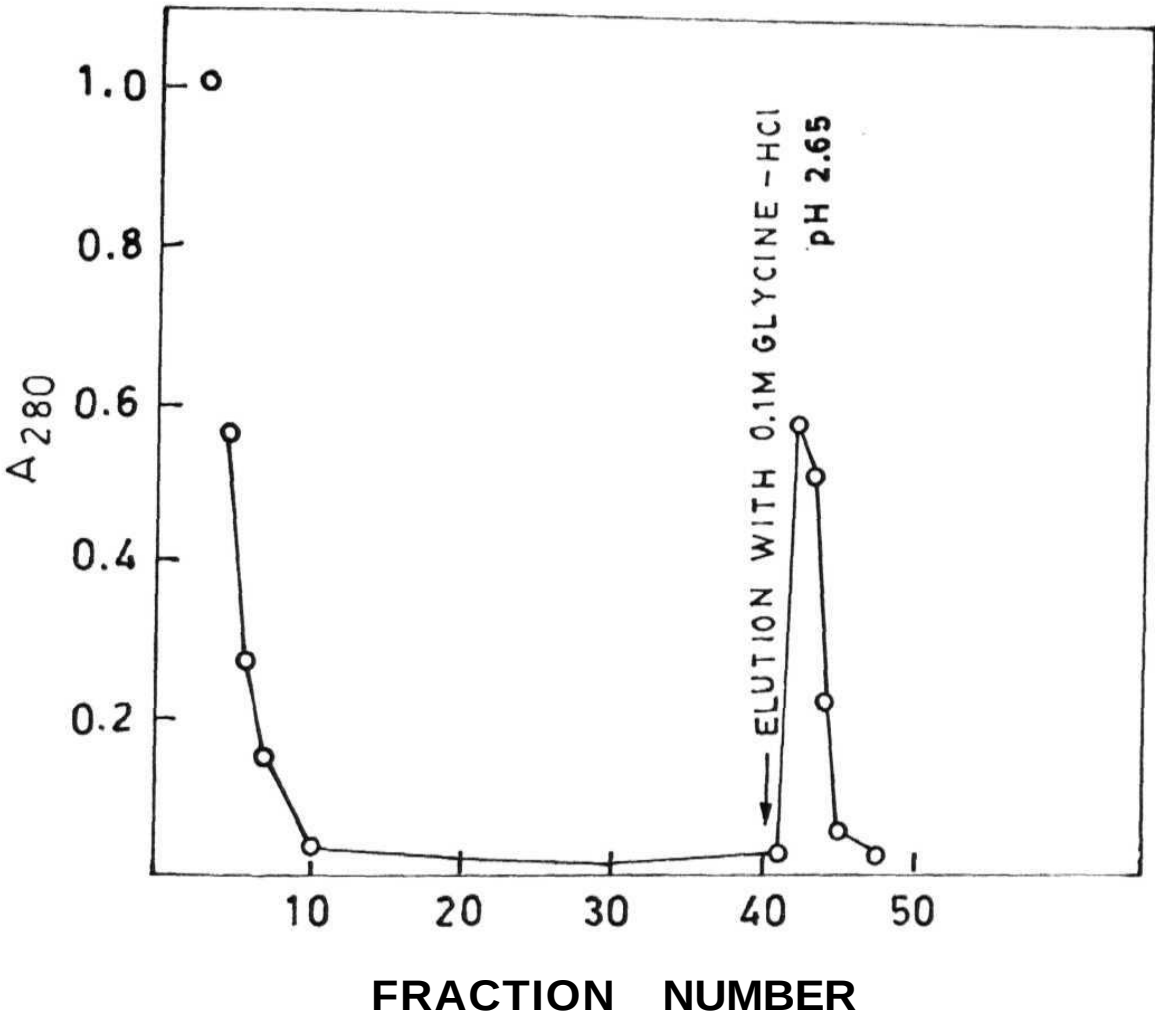


Fig. 6. Immuno-affinity chromatography of the stem and leaf lectin. 40-80% ammonium sulphate precipitated protein loaded on to a column of mannose specific seed lectin IgG-Affigel-10 (1.1X1 cm) pie-equilibrated with 25 mM Tris-HCl pH 7.4. Fractions 1 ml were collected and column eluted with 0.1 M glycine-HC1 buffer, pH 2.65.


1.0
0.8
0.6
0.4
0.2
A280
ELUTION WITH 0.1M GLYCINE-HCl
pH 2.65
10
20
30
40
50
FRACTION NUMBER

SECTION - D

DISCUSSION

In the present study, the stem and leaf extracts from 3 week old plants *of Doiichos lab/ab* var. *typicus* were found to contain proteins that showed strong haemagglutinating activity when tested with trypsin treated rabbit erythrocytes. Most of the protein and the lectin activity could be recovered in the 40-80% ammonium sulphate fraction which served as the source for purifying the lectin.

The lectin activity was inhibited by sugars such as N-acetyl galactosamine, galactose, its derivatives and lactose. Therefore an attempt was made to purify this lectin by affinity chromatography employing Sepharose-galactose and Sepharose-lactose gels. As the lectin agglutinates only rabbit erythrocytes, additionally another affinity matrix, rabbit erythrocyte membrane proteins-Affigel-10 was also employed for the purification of this lectin. None of these affinity matrices could retain the lectin and all the activity loaded on the matrix was recovered in the flow through fractions. The galactose, lactose matrices however retained the lectin from bittergourd seeds (Siva Kumar and Usha Jyothi, 1994). In this context it is essential to emphasize that some of the vegetative lectins reported in literature could not be purified by affinity chromatography on immobilized ligands which potentially bound the seed lectins from the same source (Talbot and Etzler, 1978).

Owing to the non availability of N-acetylgalactosamine sugar which is also expensive, a matrix containing this immobilized sugar was not tried for purification of the stem and leaf lectin.

The lablab bean stem and leaf lectin could be isolated using conventional methods as the lectin failed to bind to the affinity matrices prepared. On gel filtration this lectin exhibited a native molecular size of 66000. However on SDS-PAGE, the lectin dissociated to give two bands corresponding to molecular sizes 48 kDa and 20 kDa respectively. In addition to the glucose/mannose specific lectin, seeds of lablab beans also contain a galactose specific lectin that has been discussed in Chapter-II. This lectin has a native molecular size of 120000 and dissociates into two subunits in SDS-PAGE corresponding to molecular sizes 50 kDa and 20 kDa respectively. The galactose specific seed lectin thus seems to be a tetramer with an $\alpha_2\beta_2$ type of structure.

The native molecular size, subunit molecular size and the sugar specificity of the stem and leaf lectin suggest that it is a dimer and possibly made of a and β chains.

The isolated stem and leaf lectin agglutinated only rabbit erythrocytes and did not agglutinate human A, B, O and AB erythrocytes. The data obtained on the sugar inhibition studies using the 40-80% ammonium sulphate fractionated protein and the Biogel P-200 eluate clearly indicate that the lectin activity is best inhibited by N-acetyl galactosamine, galactose and its derivatives and lactose. It is **clear** from the data that an axial free hydroxyl group at C-4 is essential for lectin binding as the 4-0-galactopyranosyl α-D-galactopyranose did not show any

inhibition while lactose was inhibitory'. Galactose with methyl groups at either position 1 or 6 were found to be more inhibitory as compared to free galactose. Sugars containing hydrophobic groups were found to be potent inhibitors for a number of other lectins characterized (Allen *et al.*, 1978). Neither mannose nor glucose or their derivatives were inhibitory to the lectin activity.

An antiserum directed to the purified glucose/mannose specific seed lectin cross-reacts with the partially purified (40-80% ammonium sulphate fraction) and purified galactose specific stem and leaf lectin. When the seed and the stem and leaf lectin were placed in neighbouring wells in an immunodiffusion plate, the precipitin lines from both fused suggesting antigenic similarity among these two lectins despite the difference in their sugar specificity.

It has already been observed and discussed in Chapter-II that the galactose specific seed lectin also cross-reacts with the antiserum directed against the glucose/mannose specific seed lectin. In immunodiffusion experiment an additional faint band could be detected in the 40-80% ammonium sulphate fraction of the stem and leaf lectin. It is possible that this band may correspond to the small amount of mannose specific seed lectin which could not be detected by sugar inhibition and affinity chromatography, or it may represent some other cross-reactive material in the stems and leaves. Additionally, the stem and leaf lectin could also be bound specifically on to an immuno-affinity matrix (mannose specific seed lectin specific IgG-Affigel-10).

Purified stem and leaf lectin was found to be a glycoprotein with carbohydrate content of 3%. Vegetative lectins have so far been well

characterised from some Leguminous plants. The stems and leaves of *Dalit has biflorus* contain a glycoprotein that cross-reacts with the seed lectin antibody. The seed lectin is a tetramer composed of apparently equal amounts of two closely related subunits I and 11. Extensive biochemical studies carried out on this cross-reactive material suggest that the cross-reactive material is a dimer containing a subunit identical in electrophoretic mobility to subunit I of the seed lectin and another subunit of higher molecular weight than the seed lectin subunits. These subunits may represent different degrees of completion or modification of a common polypeptide chain (Roberts *et al.,* 1982). Work carried out by Lamb *et al.* (1983), on the leaves of *Griffonia simplicifolia* plants also suggests that the leaf lectins and seed lectins had similar properties. Borrebaeck (1984) isolated lectins from stems, leaves and roots of *Phaseolus vulgaris* and found that these were immunologically identical to that of the seed lectin. Strong haemagglutinating activities were detected and purified from the leaves and flowers *of Sophora japonica.* These lectins were also found to be inhibited by N-acetyl galactosamine, galactose and lactose and could be affinity purified on immobilised lactose gels (Ito, 1986). In a recent study a lectin from the stems and leaves of soybean plant designated as soybean vegetative lectin has been shown to be structurally and immunologically identical to the soybean seed lectin and has been classed as a member of legume lectin family (Spilatro *et al,* 1996).

The biochemical, sugar inhibition and immunological data obtained on the stem and leaf lectin, clearly indicate that this lectin is closely related to the newly identified galactose specific seed lectin and seems to be an unusual lectin, as it fails to bind on any of the affinity matrices tested for the purification of the lectin. Available evidence indicates that the seed lectin is possibly a tetramer while the

stem and leaf lectin is a dimer. However both these proteins cross-react with an antibody directed to the affinity purified glucose/mannose specific seed lectin suggesting antigenic similarity among these proteins despite the difference in sugar specificity.

Isolation of the stem and leaf lectin in large quantities and analysing its structural properties such as amino acid sequence, tryptic peptide maps and chemical modification studies in future should conclusively establish the relatedness of the stem and leaf lectin to that of the seed lectins.

CHAPTER IV

SECTION - A

INTRODUCTION

Glycosidases of varied specificities are widely distributed in plants and animals. In the plants, they have been studied in some legume seeds. Glycosidases are mostly localized in the protein bodies, some reside outside, either attached to the cell wall or in the cytoplasm (Dey, 1984). Artificial chromogenic substances, mostly the p-nitrophenyl glycosides are used to assay their activities. However, they also act on other substrates like oligosaccharides and glycoprotein side chains.

Among the different glycosidases that have been studied, α-mannosidase from jack beans *(Canavalia ensiformis)* is one of the most thoroughly studied enzymes. Like the lectin of this plant, ConA, this enzyme also occurs in large quantities (Li, 1966). Several workers used the purified enzyme for the study of glycoconjugate structures. (Kornfeld and Kornfeld, 1970; Tai *et al.,* 1975; Sharon, 1975; Liang *et al.,* 1979; Vitale *et al.,* 1984).

The jack bean mannosidase has been purified by several workers (Li, 1967; Snaith and Levvy, 1968; Chu and Turner, 1974; Wagh, 1978) by conventional methods. Table 1 summarises some properties of the α-mannosidase enzyme isolated from different sources.

1. Paus, E. and Christensen, J.B. (1972). Eur. J. Biochem. 25, 308-314.
2. Phillips, N.C., Robinson, D. and Winchester, B.G. (1976). Biochem. J. **153,** 579-587.
3. Curdel, A. and Petek, F. (1980). Biochem. J. **185,** 455-462.
4. Plant, A. and Moore, K.G. (1982). Phytochemistry, 21, 985-989.
5. Einhoff, W. and Ruediger, H. (1986). Biol. Chem Hoppe-Seyler, 367, 313-320.
6. Yashida, T., Inoue, T. and Ichischima, E. (1993). Biochem. J. 290, 349-354.
7. Nebes, V.L. and Schmidt, M.C. (1995). Biochim. Biophys. Res. Comm. **200,** 239-245.

Table -1

Some properties of purified a-mannosidases

Sl. No.	Name	Purification	Mol. Wt.	Metal ion requirement	Reference
1)	*Phaseolus vulgaris*	acetone extraction, water extraction, amm.SO_4 fractionation, DEAE-sephadex, gel-filtration, isoelectric focusing	a-mase I 220 kDa a-mase II 220 kDa	Zn2+	Paus and Christensen (1972)
2)	Human liver acidic α-mase	ConA-sepharose, mannosylamine substituted CH-amino hexanoyl fucosylamine	250,000 300,000	Zn2+	Phillips *et al.* (1976)
3)	*Medicago sativa*	Hydroxyapatitc chromatography, gel-filtration, preparatory electrophoresis	230 kDa	Zn^{2+}	Curdel and Petek (1980)
4)	*Lupinus angustifolius*	ion exchange, gel filtration chromatography	a-mase A 300,000 a-mase B 300,000	Zn2+	Plant and Moore (1982)
5)	*Canavalia ensiformis*	Ion exchange, hydrophobic and ConA-Sepharose chromatography	220,000	Mg^{2+},Ca^{2+},Zn^{2+}	Einhoff and Ruediger (1986)
6)	*Penicillium citrinum*	DEAE-TOYOPEARL, SP-TOYOPEARL chromatography	Two isoforms Ia-54,000 Ib-54,000		Yashida *et al.* (1993)
7)	Human lysosomes	DEAE-Cellulose, cellulose phosphate	107,644 Da	Zn^{2+}	Nebes and Schmidt (1994)

Most lectins bind glycosidases from the plant of their origin under appropriate conditions. Usually the glycosidases are bound to the sugar binding site of the lectin, with some exceptions as in the case of the jack bean α-mannosidase. Some authors explained that the interactions of glycosidases with lectins from the same plant are of physiological significance *in vivo* due to the colocalization of these proteins in the protein bodies of the seeds.

During the course of this investigation on the purification of glucose/mannose specific lectin from Indian lablab beans, *Dolichos lablab* var *typicus*, α-mannosidase activities were detected in the seed extracts. In order to understand the physiological significance of the lablab bean seed lectin, and its possible *in vivo* binding partners, the α-mannosidase from the lablab beans has been purified and characterised. The details of these are given below.

SECTION-B

MATERIALS AND METHODS

Materials

Seeds of *Dolichos lablab* var. *typicus* (Indian lablab beans) were purchased from the local market. P-nitrophenyl α-mannopyranoside, phenyl-sepharose and jack bean a-mannosidase were purchased from Sigma Chemical Company, USA. Sepharose 6B, DE-52 cellulose and Biogel P-200 were from Pharmacia, Whatman and Biorad labs respectively. Affigel-10 was obtained from Biorad labs. All other chemicals used in the study were obtained from reputed local firms.

Methods

Determination of enzymatic activity

a-mannosidase activity in the crude extracts and at all stages of purification was assayed as described earlier (Einhoff and Ruediger, 1986). 5 mM solution of the P-nitrophenyl a-D-mannopyranoside in 0.05M sodium acetate buffer pH 5.0 was incubated with aliquots of enzyme solutions in a total volume of 500 µl upto 15 min, mixed with an equal volume of 0.2 M sodium carbonate buffer pH 9.0 and the A405 read in a spectrophotometer. One enzyme unit (U) is defined as the enzyme amount able to release 1 µmol of 4-nitrophenol per minute.

Extraction and Purification of the α-mannosidase

All operations were carried out at 4°C. 10 gm of the lablab bean defatted seed meal was extracted with 50 ml of 50 mM Tris-HCl buffer pH 8.0 for 3h. The extract was centrifuged for 20 min at 7000 rpm. The crude extract was dialyzed extensively against 25 mM Tris-HCl buffer pH 8.0. (Buffer A). The dialysed crude extract was passed through a Scpharose-divinyl sulphone-mannose column that has been pre-equilibrated with buffer A to remove the seed lectin. The unbound fraction from this matrix was applied to a column of DE-52 cellulose which has been pre-equilibrated with buffer A. After washing the column extensively with buffer A, the bound enzyme was eluted with 0.1M NaCl in buffer A.

The fractions showing α-mannosidase activity were pooled and made 1 M with respect to ammonium sulphate and directly applied to a column of phenyl-Sepharose which has been pre-equilibrated with 50 mM Tris-HCl buffer pH 8.0 containing 1 M ammonium sulphate. After extensively washing the column with the same buffer, the bound enzyme was desorbed using buffer A.

The active fractions from this column were pooled and concentrated. This was applied to a Biogel P-200 gel filtration column which was pre-equilibrated with buffer A containing 150 mM NaCl. Active fractions from this column were pooled and concentrated.

Protein and carbohydrate estimation

Protein in the column fractions and carbohydrate in the purified enzyme were estimated as described in Chapter-II.

PAGE and SDS-PAGE

Native PAGE was performed at pH 8.3. SDS-PAGE was performed on 10% gels under reducing conditions (Laemmli, 1970). The gels were stained with coommassie brilliant blue and destained.

Molecular weight determination

The native molecular weight of the enzyme was determined by passing it through a gel filtration column of Biogel P-200. The phenyl-Sepharose eluate (7 mg protein) was loaded onto this gel. Myosin (Mr 205000), β-galactosidase (Mr 116000), bovine serum albumin (66000) ovalbumin (Mr 45000) were used as standards. The molecular size of the enzyme was determined from a plot *of* the elution volumes *vs* log molecular weight of the standard proteins.

Temperature stability of the purified enzyme and metal ion requirement

30 μg of the enzyme was incubated at 37°C, 40°C, 50°C, 60°C, 70°C and 80°C for 15 minutes. The samples were then cooled on ice, assayed for enzyme activity as described earlier.

In a separate batch the extraction of the enzyme was carried out in the presence of 1 mM $ZnCl_2$ and the activity assayed as described earlier.

Coupling of jack bean α-mannosidase to Affigel-10

1 ml of Affigel-10 was processed according to manufacturer's instructions. The gel was washed with 3-4 volumes of cold isopropanol, followed by 3-4 volumes of cold double distilled water. The washing was completed within 20 minutes. The gel was suspended in 0.2 ml of 0.5 M HEPES buffer pH 7.5. To this 2 mg of jack bean α-mannosidase (Sigma) was added and the volume was made upto 1 ml. The coupling was allowed to proceed for 24 h in cold with constant rotation. The extent of coupling was determined by measuring the absorbance of the unbound protein at A280 after adjusting it to pH 2.0. The unreacted sites in the gel were then **blocked with** 200 μl **of 1 M ethanolamine hydrochloride, pH 8.0 in cold for 1 h with rotation.** The gel was washed with 10 **ml** PBS, pH 7.4 and stored in **PBS.**

Raising antibodies to the jack bean α-mannosidase

Due **to** the ready availability of the jack bean a-mannosidase (Sigma), antibodies were raised to this enzyme in a healthy rabbit. 200 μg of the jack bean enzyme was mixed with 400 μl of PBS, pH, 7.4 and 0.6 ml of Freund's complete adjuvant till an emulsion was formed. This was injected into a rabbit subcufaneously. The rabbit received a second injection after 3 weeks at the same concentration. Subsequent booster doses were given using 150 μg protein in Freund's incomplete adjuvant. The rabbit was bled 10 days after each booster dose and the serum was collected and stored at -200C.

Purification of specific IgG to jack bean a-mannosidase

Anti serum was diluted with an equal volume of PBS pH 7.4 and passed through the Affigel-α-mannosidase column. **The** washings were recycled two times and the column was washed with PBS pH 7.4 until no protein was detectable in the washings. The column was then eluted with 0.1 M glycine-HCl pH 2.65 to dcsorb the specific IgG. The eluate was immediately neutralized with 2 M Tris. The protein eluted was confirmed as IgG by its mobility in SDS-PAGE in the absence of p-mercaptoethanol.

Coupling of specific IgG of a-mannosidase to Affigel 10 and Immuno-affinity chromatography

4 mg of the purified IgG obtained from 10 ml of antiserum was added to 1 ml of washed Affigel-10. After processing the gel as above, the extent of IgG bound was monitored as described earlier. 7 mg of enzyme eluted from phenyl-Sepharose matrix was passed through the immuno-affinity matrix prepared above. The matrix was thoroughly washed with 25 mM Tris-HCl buffer pH 8.0 (equilibration buffer) and eluted with 0.1 M Gly-HCl buffer pH 2.65. The eluates were immediately neutralized with 2 M Tris and assayed for activity.

Immunodiffusion

This was performed according to the procedure described earlier (Ouchterlony, 1948).

Western blot analysis

This was performed essentially as described earlier in Chapter-II. Purified lablab bean enzyme and the jack bean enzyme were separated on SDS-PAGE and transferred on to a nitrocellulose membrane, using a Biorad transfer unit. After the transfer the membrane was processed. The membrane was soaked in TBST [10 mM Tris pH 8.0, 150 mM NaCl, with 0.05% (v/v) Tween 20] containing 3% (w/v) milk powder for 3 h at room temperature. It was then washed twice with TBST and then soaked in

TBST containing (1:1000 dilution) of antibody raised in rabbit against purified jack bean a-mannosidase, overnight, at room temperature. After three more washes in TBST, the antigen-antibody complex was detected using a secondary antibody conjugated to alkaline phosphatase and visualized by incubating the membrane with the substrate 5-bromo4-chloro3-Indolyl phosphate/ Nitroblue tetrazolium (Bangalore Genei, India).

Lectin affinity chromatography

Purified lablab bean glucose/mannose specific lectin was immobilised to Affigel-10 as described in Chapter-II. Purified a-mannosidase was passed through this gel matrix that was pie-equilibrated with 50 mM sodium acetate buffer, pH 5.0. The column was sequentially eluted using 0.5 M glucose, 1 M NaCl and finally with 0.1 M Tris-HCl buffer pH 9.2 containing 0.5 M NaCl. Protein and enzyme activity in the washings and eluate were determined as described earlier.

SECTION - C

RESULTS

Purification of a-mannosidase

An optimal protocol was followed for the purification of the enzyme in which maximal purification efficiency was achieved with a minimum of steps. Affinity chromatography of the seed lectin on Sepharose-divinyl sulfone-mannose column facilitates its removal. Ion exchange using DE-52 cellulose facilitates the removal of most of the storage proteins and galactosidases. Most of the α-mannosidase activity that is bound on DE-52 cellulose is desorbed with 0.1 M NaCl in the buffer. Fig. 1 shows the clution profile of α-mannosidase on DE-52 cellulose. 73% of the enzyme activity could be recovered in this step.

The next step is the hydrophobic chromatography. The active fractions from the DE-52 column when passed through the phenyl-Sepharose gel. the enzyme was bound under the conditions used. It could be eluted using buffer A. Figure 2 shows the elution profile of α-mannosidase on phenyl-Sepharose matrix. 53% of the enzyme activity could be recovered in this step.

The active fractions from the phenyl-Sepharose column after concentration were chromatographed on a Biogel P-200 gel filtration

Fig.1: Chromatography of dialysed lablab bean seed extract on DE-52 cellulose (3X10 cm). Column was equilibrated with 25 mM Tris-HCl pH 8.0. Flow through from Sepharose-mannose gel, corresponding to 897 mg protein, 11 enzyme units was loaded on the gel. Fractions 4 ml were collected. Bound enzyme was eluted with 100 mM NaCl in column buffer.

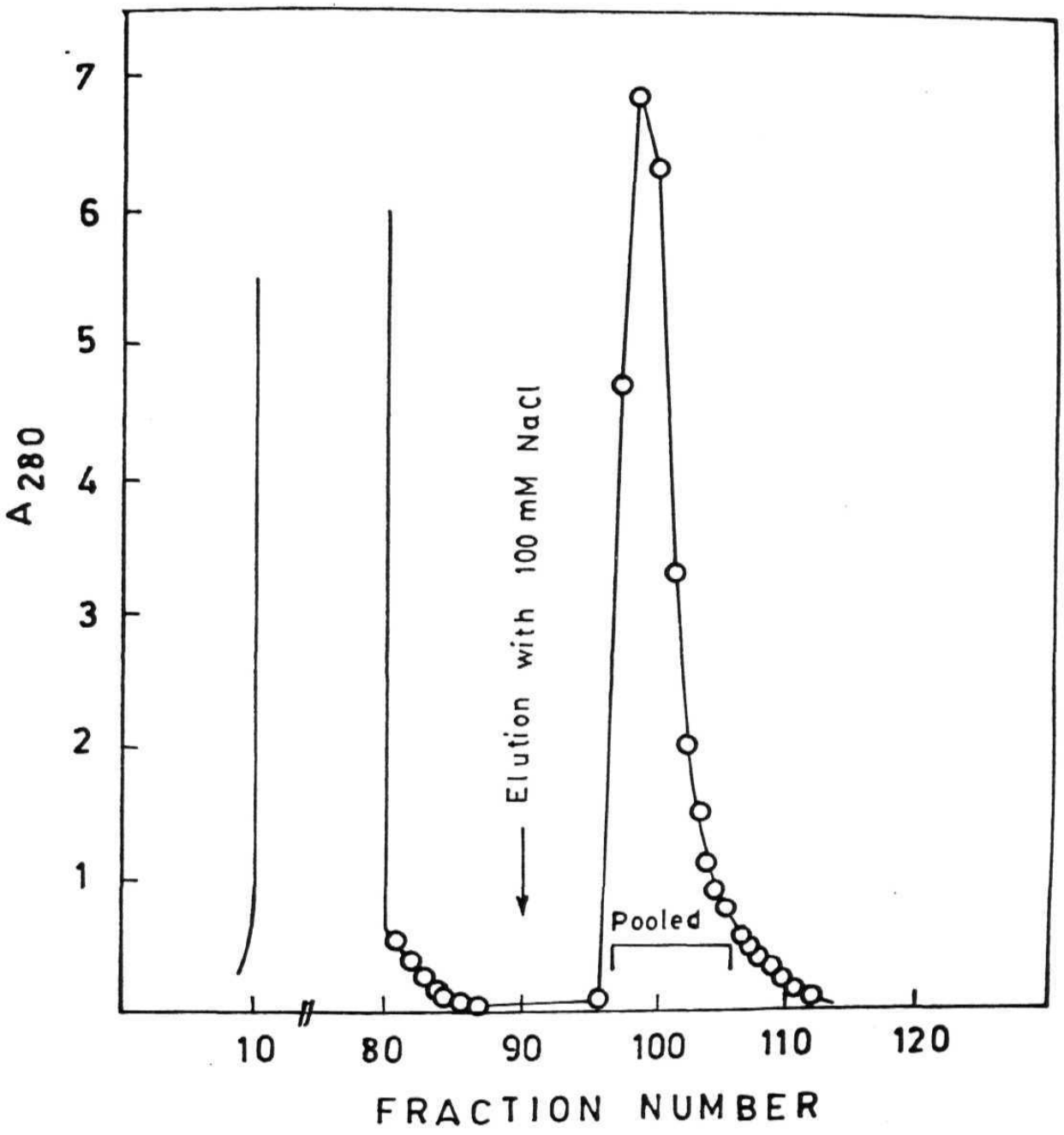
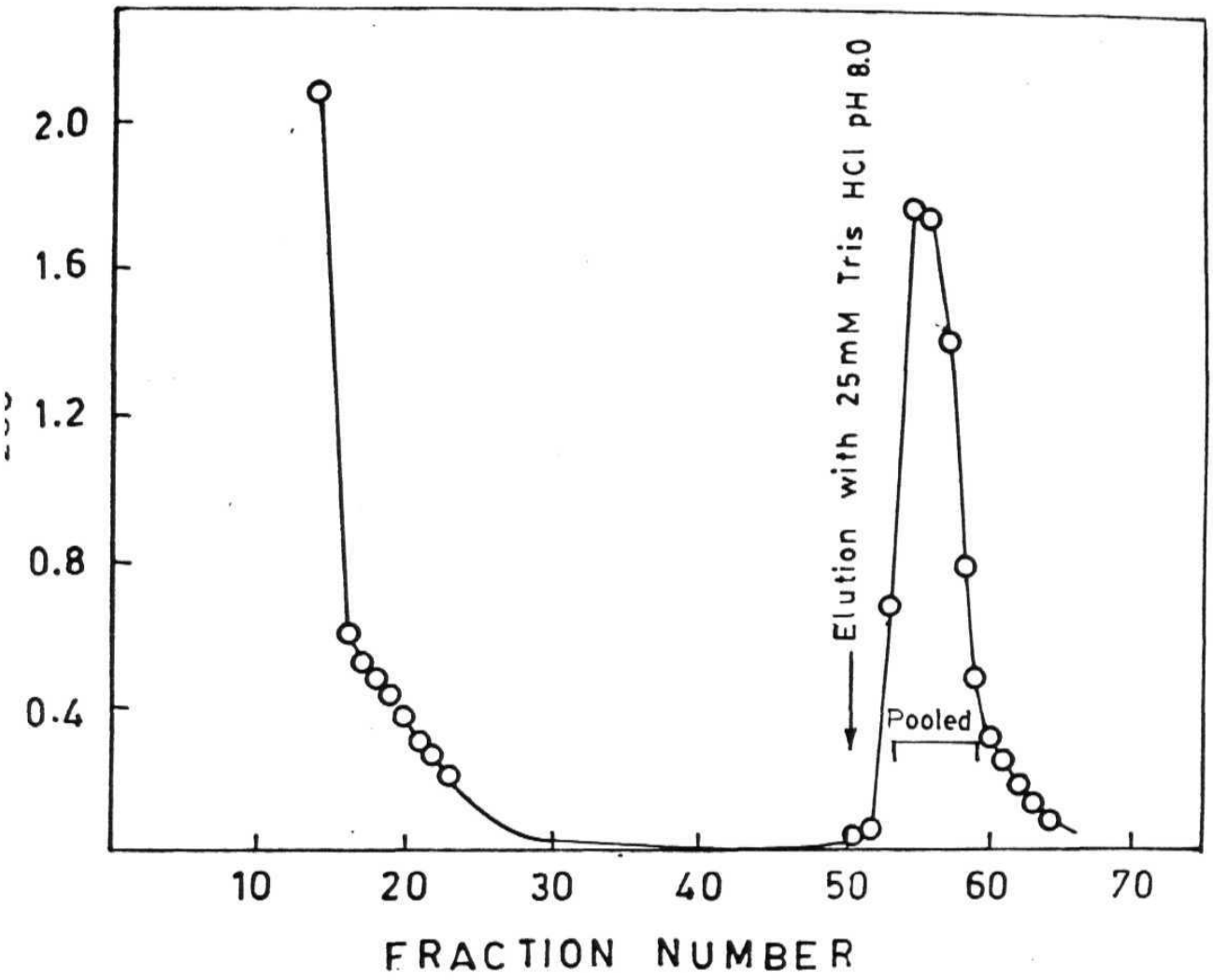

A 280
7
6
5
4
3
2
1
Elution with 100 mM NaCl
Pooled
10
80
90
100
110
120
FRACTION NUMBER

Fig. 2: Hydrophobic chromatography of lablab bean α-mannosidase. Pooled active fractions from DE-52 cellulose column were made to 1 M ammonium sulphate and 56 mg protein, 8 enzyme units were loaded on phenyl-Sepharose column (1.5 X 4.5 cm), equilibrated with 50 mM Tris/HCl pH 8.0 in 1 M ammonium Sulfate. Fractions 2 ml were collected. Bound enzyme was elutcd using 25 mM Tris/HCl pH 8.0.


2.0
1.6
1.2
0.8
0.4
Elution with 25mM Tris HCl pH 8.0
Pooled
10
20
30
40
50
60
70
FRACTION NUMBER

column.. Fig. 3 shows the elution profile of the enzyme on this column. The enzyme eluted as a single symmetrical peak.

Table 2 summarizes the purification scheme of these consecutive steps which lead to about 83-fold purification of the α-mannosidase. As can be seen from the Table, about 4 mg of the purified enzyme can be obtained from 10 gm of the seed meal.

PAGE and SDS-PAGE

The sample eluted from the Biogel P-200 column gave a single band in 10% native gels (Fig. 4Λ). In presence of SDS and under reducing conditions, two major bands corresponding to Mr 66 kDa and 44 kDa were obtained (Fig. 4B). These prominent bands could also be detected in the phenyl-Sepharose eluate. Taken together these results indicate that the enzyme from lablab beans is possibly made of two types of subunits, that are linked by disulfide bridges and may have $\alpha_2\beta_2$ type of structure similar to the jack bean enzyme.

Molecular weight determination

The apparent molecular size of the enzyme on gel filtration was found to be 195,000 ± 5,000 (Fig. 3, inset). Jack bean a-mannosidase when run on Biogel P-200 eluted at the same elution volume as myosin. The molecular size of the different mannosidases reported in the literature varies from 190.000 to 230,000 (Einhoff and Ruediger, 1988). Although

Fig.3: Gel filtration of lablab bean α-mannosidase phenyl-sepharose eluate (7 mg/.ml) in 25 mM Tris/H Cl pH 8.0 containing 150 mM NaCl was loaded onto a column of Biogel P-200 (1.4X86 cm) and eluted with same buffer. Fractions, 2 ml were collected and elution was followed by monitoring absorbance at A_{280} nm Inset plot of elution volume (V_e) vs log M_r. (0) standards, 1. Myosin, 2. β-galactosidase, 3. Bovine serum albumin and 4. ovalbumin.● lablab bean a-mannosidase.

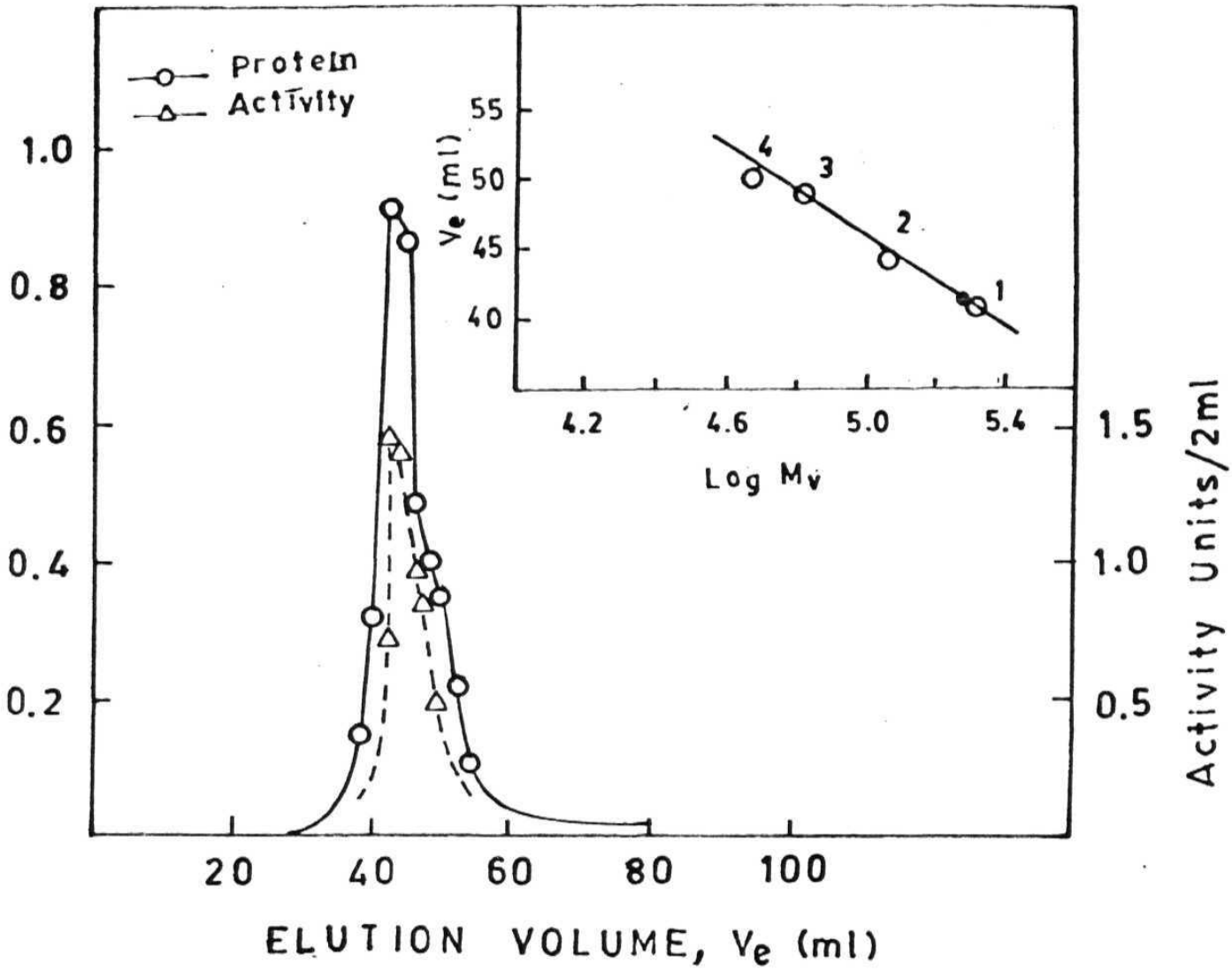

Protein
Activity
1.0
0.8
0.6
0.4
0.2
20
40
60
80
100
ELUTION VOLUME, Ve (ml)
Activity Units/2ml
1.5
1.0
0.5
Ve (ml)
55
50
45
40
4.2
4.6
5.0
5.4
Log Mv
1
2
3
4

Table 2

Purification of lablab bean α-mannosidase

Purification Step	Protein (ng)	Activity (U)*	Specific activity (U/mg)	Recovery (%)	Fold Purification
Crude extract	857	11	0.012	100	1
DE-52 eluate	56	8	0.140	73	12
Phenyl-Sepharose eluate	9	6	0.660	54	55
Biogel P-200 eluate	4	5	1.250	45	104

* One enzyme unit (U) is defined as the enzyme amount able to release 1 umol of 4-nitrophenol per minute.

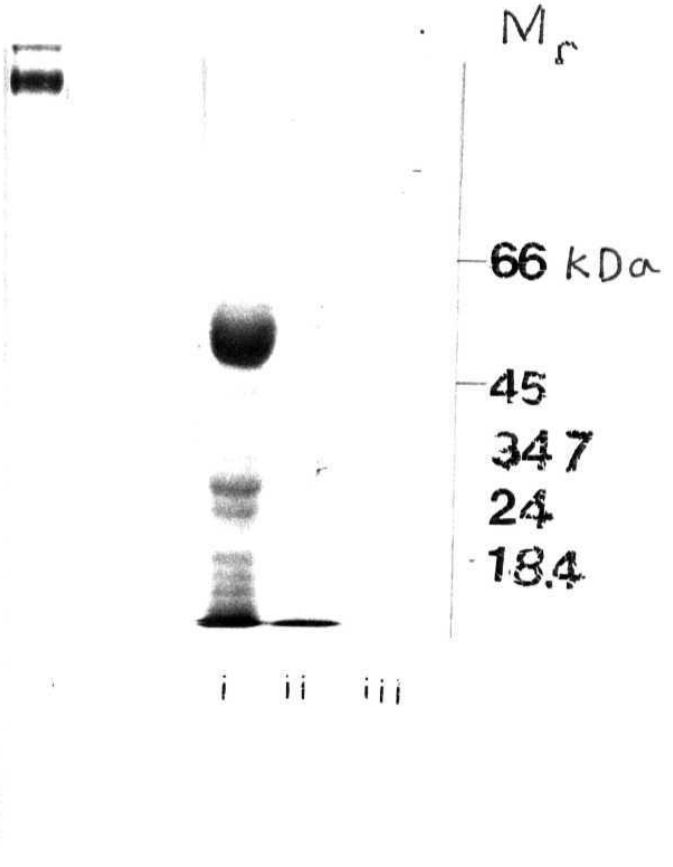
Fig.4: A. PAGE Pattern of Purified lablab bean α-mannosidase; under non-denaturing conditions was done in 10% gels. Purified enzyme 20 μg was loaded on the gel and proteins detected by coommassie stain.

B: SDS-PAGE pattern of the lablab bean a-mannosidase at different stages of purification under reducing conditions.

Lane 1. DE-52 cellulose cluate (30 μg protein).

Lane 2. Phenyl-Sepharose eluate (30 μg protein)

Lane 3. Biogel P-200 eluate (15 μg protein).


M_r
66 kDa
45
34.7
24
18.4
i
ii
iii
A
B

the lablab bean enzyme eluted 1 ml after the jack bean enzyme, the subunit pattern of both enzymes was similar.

Carbohydrate estimation

The purified enzyme was found to be a glycoprotein with 4.5% carbohydrate.

Temperature stability of (he purified enzyme and metal **ion** requirement

The lablab bean α-mannosidase is stable upto 40oC. At 50°C and upto 80°C (after 15 minutes) the enzyme activity decreased from 61% to 17%. The enzyme activity is not affected in the presence or absence of 1 mM $ZnCl_2$.

Coupling of jack bean α-mannosidase to Affigel-10

2 mg of the enzyme was offered to 1 ml of Affigel-10. About 1.4 mg was bound to the gel.

Immunological methods

When 2.5 ml of the antiserum was passed through Affigel-10 coupled to jack bean a-mannosidase about 1 mg of the specific IgG could be eluted with 100 mM glycine-HCl buffer. Additional quantities of the specific IgG were collected by repeating the above experiments. The specific IgG obtained was pooled, concentrated and immobilized to

Affigel-10 as described under methods. When 4 mg of specific IgG was offered, 3.3 mg was coupled to 1.0 ml Affigel-10.

The immunological cross-reactivity of the lablab bean enzyme with jack bean enzyme antisemm was established by immunodiffusion as well as Western blot analysis. From Fig. 5A, it is apparent that the antiserum to jack bean a-mannosidase cross-reacts with the lablab bean en/.yme suggesting immunological identity among these legume α-mannosidases.

Further confirmation of this identity was established in Western blot analysis wherein the subunits of both enzymes migrated to the same extent on SDS gels and also showed immunological reactivity with the antibody to the jack bean enzyme (Fig. 5B). Additionally the lablab bean enzyme could also be bound and eluted from an immuno-affinity gel prepared as described under methods (Fig. 6). When 7 mg of the enzyme eluted from phenyl-Sepharose gel was passed through the immuno-affinity matrix, 2 mg of protein could be bound and eluted from the matrix.

Lectin affinity chromatography

When 2 mg of the purified a-mannosidase was passed through the Affigel-10 coupled to lablab bean mannose specific seed lectin. around 1.1 mg of the enzyme could be bound on to the matrix and could be eluted only with 0.1 M Tris-HCl buffer pH 9.2 containing 0.5 M NaCl (Fig.7). The protein eluted was devoid of any enzyme activity, under the standard assay conditions.

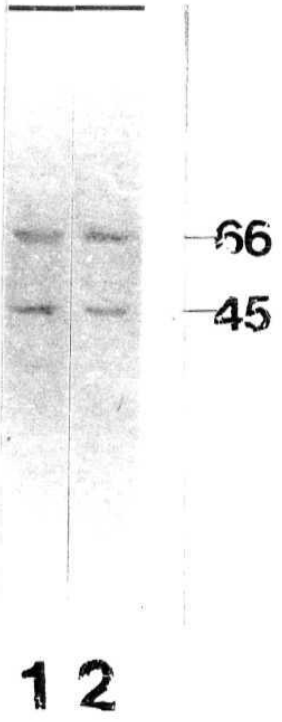
Fig. 5. A. Immunodiffusion of the lablab bean a-mannosidase. Central well contained 20 µl of antiserum to the jackbean mannosidase. Wells i and ii contained 20 µg of jack bean mannosidase and Biogel P-200 eluate of the lablab bean enzyme.

B. Western blot analysis of the lablab bean α-mannosidase.

Lane 1. Jack bean a-mannosidase.

Lane 2. Purified lablab bean a-mannosidase.

A



B

Fig.6: Immuno-affinity chromatography of lablab bean α-mannosidase. 7 mg of the phenyl-Sepharose eluate was passed through the immuno-affinity column (Jack bean α-mase specific IgG-Affigel-10). The column (1.1 X 1 cm) was equilibrated with 25 mM Tris-HCl. Column washed with same buffer and eluted with 0.1 M gly-HCl buffer pH 2.65. 1 ml fractions collected.

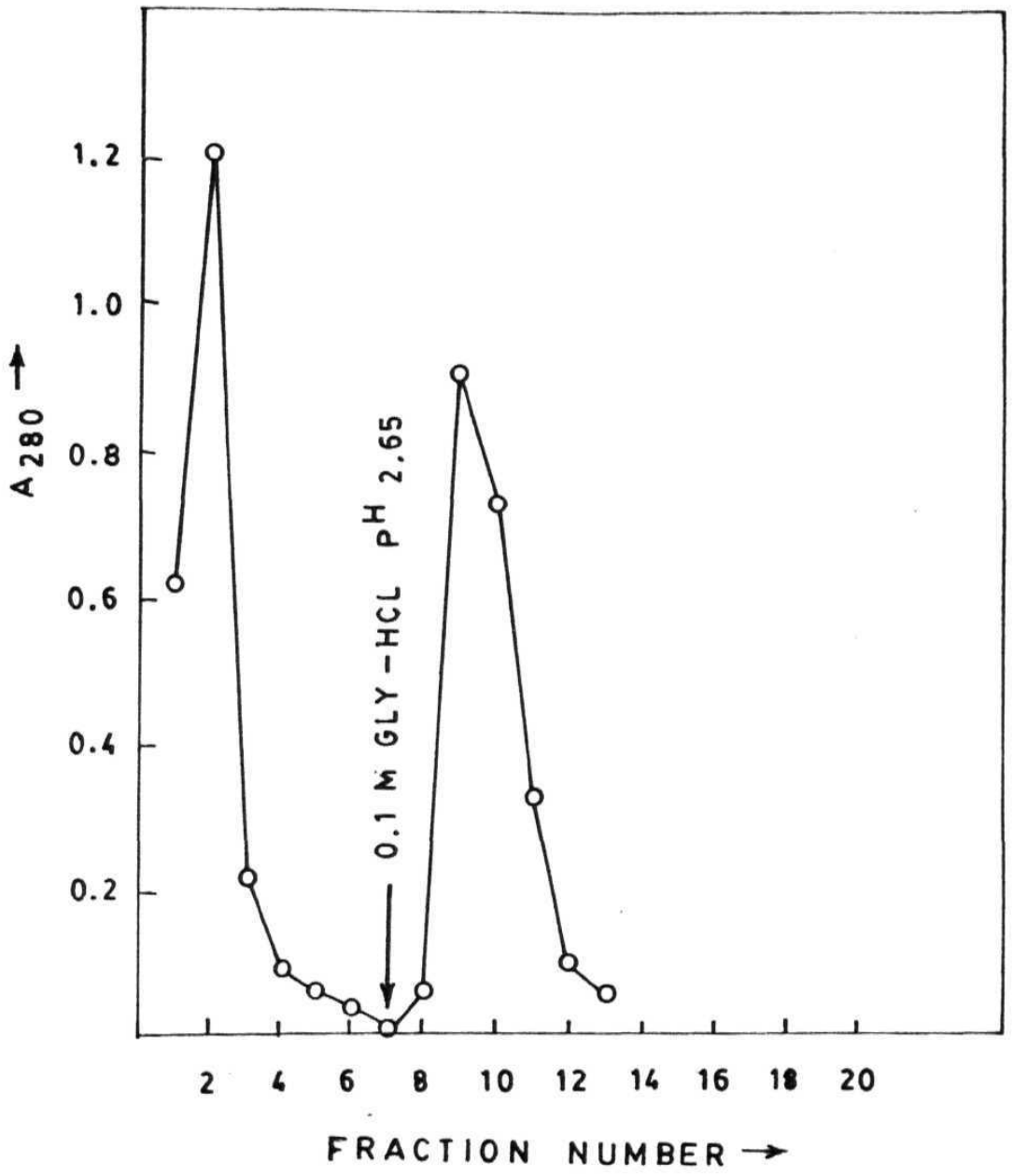
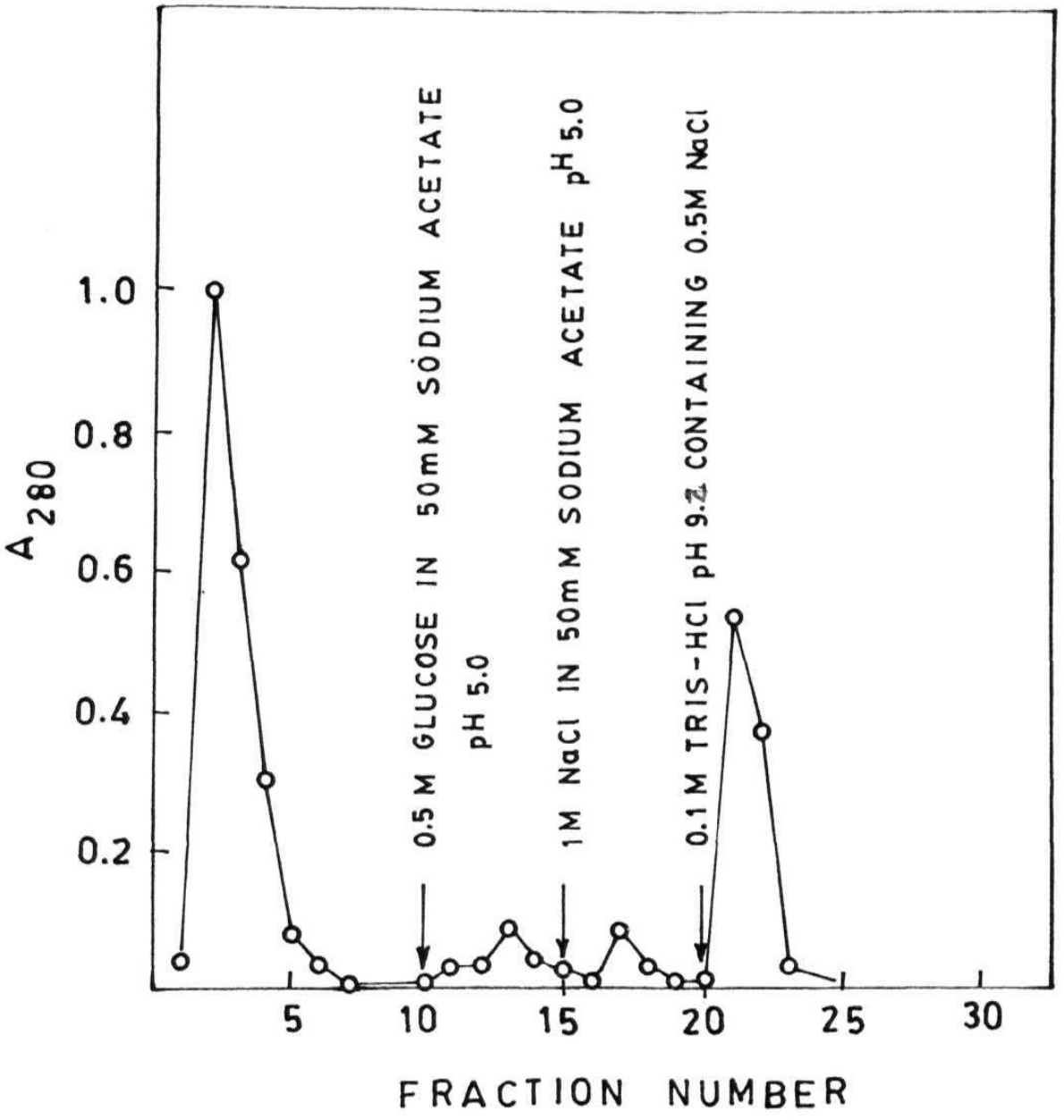

1.2
1.0
0.8
0.6
0.4
0.2
A280 →
0.1 M GLY-HCL pH 2.65
2
4
6
8
10
12
14
16
18
20
FRACTION NUMBER →

Fig.7: Lectin affinity chromatography of purified α-mannosidase.. Purified lablab bean a-mannosidase was passed through mannose specific seed lectin-Affigel-10 matrix (1.1 X 1 cm) that was equilibrated with 50 mM sodium acetate buffer pH 5.0. The column was eluted sequentially with 0.5 M glucose, 1 M NaCl and 0.1 M Tris-HCl buffer pH 9.2. 1 ml fractions collected.


1.0
0.8
0.6
0.4
0.2
A_{280}
0.5 M GLUCOSE IN 50mM SODIUM ACETATE pH 5.0
1M NaCl IN 50mM SODIUM ACETATE pH 5.0
0.1 M TRIS-HCl pH 9.2 CONTAINING 0.5M NaCl
5
10
15
20
25
30
FRACTION NUMBER

SECTION - D

DISCUSSION

α-mannosidase enzyme has been purified from the seeds of Indian lablab beans, *Dolichos lablab* var. *typicus* by conventional methods. Ion exchange using DE-52 cellulose not only removed most of the storage proteins but also the galactosidases. Most of the a-mannosidase activity was dcsorbed with 0.1 M NaCl. The next step, hydrophobic chromatography proved to be a very effective method for separating the a-mannosidase from other proteins. Einhoff and Ruediger (1986) purified the jack bean enzyme on phenyl-Sepharose gel. Subsequently the enzyme from other sources was also found to interact with phenyl-Sepharose gel. Einhoff and Ruediger (1989) tested the binding capacities of the a-mannosidase from several plants, *Glycine max, Lens culinaris, Pisum sativum, Phaseolus vulgaris* and *Sophora japonica* and found that all these mannosidases bind the hydrophobic matrix. The molecular weight of the lablab bean enzyme was found to be 190,000 + 5,000 in gel filtration. This is in close agreement with the molecular weight of the a-mannosidase from jack bean, reported earlier (Einhoff and Ruediger, 1986). The lablab bean enzyme also resembles the jack bean enzyme in its subunit molecular sizes (66 kDa and 44 kDa). The molecular weights of the enzyme from *Phaseolus vulgaris* and *Medicago saliva* are respectively 220,000 and 230,000. The lablab bean enzyme and the jack bean enzyme are immunologically similar. The enzyme from all these sources was found to be a glycoprotein. The lablab bean enzyme has about 4.5% carbohydrate and the jack bean enzyme has about 6% carbohydrate. The enzyme activity from Jack beans and other species was found to be Zn^{2+} dependent

(Table 1). However, the activity of the lablab bean enzyme was found to be Zn^{2+} independent. Thus the properties of the lablab bean enzyme are similar to that of the jack bean enzyme.

In an *in vitro* experiment the lablab bean enzyme strongly interacts with the immobilised glucose/mannose specific lectin. Neither glucose, mannose, their glycosides nor other carbohydrates are able to desorb the enzyme. The enzyme also does not get desorbed from the matrix even when a high ionic strength is applied. The only means to elute the enzyme is to apply high pH. But at this high pH, the enzyme loses its activity. It is surprising that the interaction between the lectin and the enzyme is not mediated through the sugar binding site of the lectin and the oligosaccharidc chains of the enzyme. Other glycosidases from jack bean and from other plants are bound to their lectins at pH 5.0 and desorbed using sugar (Einhoff and Rucdiger, 1986). Both proteins, the a-mannosidase and the lectins from other plants are localized in the protein bodies. This common occurence together with the fact that an interaction between them takes place at low pH and low ionic strengths, i.e., under conditions that are likely to exist in the seeds suggest that these proteins also interact *in vivo.* During deposition and degradation of the protein body contents, small cooperative alterations in pH and ionic strength may enable the cell either to release or to adsorb the enzyme and thereby to regulate its activity. Though the general properties of the lablab bean enzyme are similar to the jack bean enzyme, the nature of interaction of the lablab bean lectin and its enzyme seems to be different, as compared to the interaction of jack bean a-mannosidase and its lectin. The physiological significance of this unusual interaction needs to be further explored to understand precisely the function of the lablab bean lectin.

SUMMARY

SUMMARY

1. Literature regarding the lectins particularly from plants, their sugar specificity, their distribution and biological properties, sequence homologies among legume lectins, the functions and applications of lectins have been reviewed.

2. A glucose/mannose specific lectin from the seeds of Tndian lablab beans has been purified to homogeneity on Sepharose-mannose gel, goat IgM Sepharose gel and by immuno-afTinily chromatography.

3. The purified lectin is a glycoprotein with molecular weight 64000 and dissociates into two subunits 15000 and 12000 on SDS-PAGE analysis.

4. The lectin agglutinates rabbit and human erythrocytes and is mitogenic to rat and murine lymphocytes.

5. Seeds of Indian lablab beans also contain a second lectin that is specific for galactose/lactose.

6. The galactose/lactose lectin has a native molecular size of 120000 and is made of two types of subunits. This lectin also cross-reacts with an antibody to the glucose/mannose specific lectin.

7. Seed extracts of the lablab bean contain proteins that specifically interact with the immobilised glucose/mannose specific lectin.

8. Stems and leaves of the lablab bean plant contain strong haemagglutinating activities that are inhibited by galactose and lactose.

9. The stem and leaf lectin is unusual as it does not bind to any sugar affinity columns tested. The lectin has a native molecular size of 66000 and is possibly made of a and p-chains.

10. Seeds of the Indian lablab beans also contain a-mannosidase activity that has been purified to homogeneity by a combination of ion-exchange, hydrophobic chromatography and gel filtration.

11. The physico-chemical and immunological properties of the purified lablab bean enzyme seem to be similar to the well studied jack bean α-mannosidase.

REFERENCES

REFERENCES

Ahmed, S. (1995). 64th Annual Meeting of Society of Biological Chemists. October 6th, Lucknow, India, P 2 D 55.

Allen, A.K., Desai, N.N., Neuberger, A. and Crceth, J.M. (1978). Biochem. J. **171,** 665-674.

Andrews, P. (1978). In Methods Biochem. Analysis. **18**.

Ashwell, G. (1977). Trends. Bioehem. Sci. (Pers. Ed.). 2, N186.

Ashwell, G. and Hartford, J. (1982). Annu. Rev,. Bioehem. 51, 531-554.

Ashwell, G. and Morell, AG. (1974). Adv. Enzymol. 41, 99.

Barondes, S.H. (1986). The lectins: Properties, Functions and Applications in Biology and Medicine (Eds. Liener, I.E., Sharon, N. and Goldstein, T.J.) Academic Press, Orlando, 438-466.

Baumann, C.M., Ruediger. H. and Strosberg, A.D. (1979). FEBS Lett. **102,** 216-218.

Becker, J.W., Recke, G.N.. Cunningham, B.A. and Edelman, G.M. (1976). Nature (London). 259, 406-409.

Bohlool, B.B. and Schmidt. E.L. (1974). Science. **185,** 269.

Borrebaeck, C.A.K. (1984). Planta. **161,** 223-228.

Bowles, D.J. and Marcus. S. (1981). FEBS Lett. **129,** 135-138.

Boyd, W.C. and Shapleigh. E. (1954a). Science. **119,** 419.

Boyd, W.C. and Shapleigh. E. (1954b). J. Immunol. 73, 226-231.

Bradley, L.M. (1980). In Selected Methods in Cellular Immunology. (Eds. Mischell, B.B., Shigi. S.M.), W.H. Freeman and Company, USA, 156-161.

Brown, J.C. and Hunt, R.C. (1978). Int. Rev. Cytol. 52, 277-349.

Bukantz, S C , Rein, R.C. and Kent, J.F. (1946). J. Lab. Clin. **31,** 394.

Cammue, B., Stinissen, H.M. and Peumans, W.J. (1985). Eur. J. Biochem. **148,** 315-322.

Chrispeels, M.J. and Raikhel, N.V. (1991). In Lectin Reviews. 1, 183-194.

Chu, C.Y.T. and Turner, M.D. (1974). Biochem. J. 139, 469-471.

Cisar, J.O. (1987). Microbial Lectins and Agglutinins: Properties and Biological Activity. Wiley, New York.

Dazzo, F.B. and Hol lings worth, RE. (1984). Biol. Cell. 51, 267.

Dean, P.D.G., Johnson, W.S. and Middle, F.A. Eds. Affinity Chromatography, A Practical Approach, (1985). 31-34, ARL Press.

Debray, H., Decout, D., Strecker, G., Spik, G. and Montreuil, J. (1981). Eur. J. Biochem. **117,41-55.**

Dey, P.M. (1984). Phytochem. 23, 257-260.

Dubois, M., Gilles, K.A., Hamilton, J.K., Robers, P.A. and Smith, F. (1956). Anal. Chem. 28, 350.

Duke, J.A. (991). In Hand book of Legumes of world economic importance (New York, Plenum-press), `102.

Einhoff, W. and Ruediger, H. (1986). Biol. Chem. Hoppe-Sey Lr, **367,** 313-320.

Einhoff, W. and Ruediger, H. (1989). Phytochem. **28,** 355-357.

Etzler, M.E. (1985). Annu. Rev. Plant. Physiol. **36,** 209.

Etzler, ME. (1986). Distribution and function of plant lectins. In The Lectins: Properties, Functions and Applications in Biology and Medicine. (IE Liener, N. Sharon and I.J. Goldstein eds.). Academic Press, New York, PP: 371-435.

Etzler, ME. and Borrebaeck, C.A.K. (1980). Biochim. Biophys. Res. Comm. 96, 92-97.

Fornstedt, N. and Porath, J. (1975). FEBS Lett. 57, 187-191.

Gade, W., Jack, MA, Dahl, J.B., Schmidt, E.L. and Wold, F. (1981). J. Biol. Chem. 256, 12905-12910.

Gansera, R., Schurz, H. and Ruedigcr, H. (1979). Hoppe-Seyler's Z. Physial. Chem. 360, 1579-1585.

Gatehouse, J.A. and Boulter, D. (1980). Physiol. Plant. 49, 437-442.

Gibson, D.M., Stack, S., Krell, K. and House, J. (1982). Plant. Physiol. 70, 560-566.

Goldstein, I.J. and Hayes, C.E. (1978). The lectins: Carbohydrate binding proteins of plants and animals. Adv. Carb. Chem. Biochem. 35, 127-340.

Goldstein, I.J., Hughes, R.C., Monsigny, M., Osawa, T. and Sharon, N. (1980). Nature (London) 285, 66.

Goldstein, I.J., Lamb. J.E., Roberts, D.D. and Poola, I. (1983). In ``Chemical Taxonamy, Molecular Biology and Function of Plant Lectins" (I.J. Goldstein and ME. Etzler, eds.) 21-29. Alan. R. Lass, Inc., New York.

Goldstein, I.J. and Poretz, R.D. (1986). Isolation, Physiochemical characterization and carbohydrate binding specificity of lectins, In The Lectins: Properties,

Functions and Applications in Biology and Medicine. (IE Liener, N. Sharon, I.J. Goldstein, eds.), Academic Press, New York, PP: 33-245.

Gowda, L.R., Savithri, H. S. and Rao, DR. (1994). J. Biol. Chem. **269,** 18789-18793.

Guran, A., Ticha, M., Filka, K. and Kocourek, J. (1983). Biochem. J. **209,** 653-657.

Hapner, K.D. and Robbins, J.E. (1979). Biochim. Biophys. Acta. **580,** 186-197.

Hardmann, K.D., Wood, M.K., Schiffer, M., Edmundson, AB. and Ainsworth, C.F. (1971). Proc. Natl. Acad. Sci. (USA). **68,** 1393-1397.

Hester, G., Kaku, H., Goldstein, I.J. and Wright, G.S. (1995). Nat. Struct. Biol. 2, 472-479.

Ho pak, J., Handrickson, T. and Dobres, M.S. (1992). Plant. Mol. Biol. **18,** 857-863.

Horejsi, V. and Kocourek, J. (1978). Biochem. Biophys. Acta. **532,** 98-104.

Howard I.K., Sage, H.J. and Horton, C.B. (1972). Arch. Biochem. Biophys. **149,** 323-326.

Hume, D.A. and Weidmann, M.J. (1980). ``Mitogenic Lymphocyte Transformation". Hlsevier/North Holland Biomedical Press, Amsterdam.

Iglesias J.L.. Lis, H. and Sharon, N. (1982). Eur. J. Biochem. **123,** 247-252.

Ito, Y. (1986). Plant Science, 47, 77-82.

Kaku, H., Tanaka, Y., Tazaki, K., Minami, E., Mizuno, H. and Shibuya, H. (1996). J. Biol. Chem. 271, 1480-1485.

Kalsi, G., Das, H.R., Das, R.H. and Babu, C.R. (1993). Ind. J. Biochem. Biophys., 30, 400-404.

Kammemura, K., Furuichi, Y., Umekawa, H. and Takanashi, T. (1996). Biochim. Biophys. Acta, 1289, 87-94.

Kelm, S. and Schauer (1988). Biol. Chem. Hoppe-Seyler 369, 693.

Kijne, J.W., Diaz, C.L. and Bakuizen, R. (1986). The Physiological function of lectins. (T.C. Bog-Hanson, E. Van Driessche, Eds.), Lectins: Biology, Biochemistry and Clinical Biochemistry, Walter dc Gruyter Berlin, 5, PP: 45-52.

Kocourek, J. and Horejsi, V. (1983). In Lectins, Biology, Biochemistry. 3, 3-6.

Koike, T., Titani, K., Suzuki, M., Beppu, H., Kuzuya, H., Maruta, K , Shimoo, K. and Fujita, K. (1995). Biochim. Biophys. Res. Comm. **214,** 163-170.

Komath, S.S., Siva Kumar, N. and Swamy, M.J. (1996). Biochem. Molbiol. Int. 39, 243-252.

Kornfeld, R. and Kornfeld, S. (1970). J. Biol. Chem. **245,** 2536-2545.

Krishnasastry, M.V., Banerjee, P., Patanjali, S.R., Swamy, M.J., Swarnalatha, G.V. and Surolia, A. (1986). J. Biol. Chem. 261, 11726.

Kummer, H. and Ruediger, H. (1988). Biol. Chem. Hoppe-Seyler. 369, 639-646.

Kusui, K., Yamamoto, K., Konami, Y. and Osawa, T. (1991). J. Biochem. **109,** 899-903.

Laemmli, U.K. (1970). Nature, 227, 680.

Lamb. J.E., Shibata, S. and Goldstein, I.J. (1983). Plant. Physiol. 71. 879-887.

Landsteiner, K. (1962). In ``The specificity of serological reactions", Rev. ed. Dover, New York.

Li, E., Becker, A. and Stanley Jr., S.L. (1988). J. Exp. Med. **167,** 1725.

Li, Y.-T. (1966). J. Biol. Chem. **241,** 1010-1012.

Li, Y.-T. (1967). J. Biol. Chem. **242,** 5474-5480.

Liang, C l , Yamashita, K., Mullenberg, C.G., Shichi, H. and Kobata, A. (1979). J. Biol. Chem. **254,** 6414-6418.

Liener, I.E. (1976). Phytoagglutinins (Phytolectins) Annu. Rev. Plant Physiol. **27,** 291-319.

Lis, H. and Sharon, N. (1981). In ``The Biochemistry of Plants" (A. Marcus, ed.). 6, 371-447, Academic Press, New York.

Lis, H. and Sharon, (1986). Biological Properties of lectins. In The Lectins, Properties, Functions and Applications in Biology and Medicine (Eds. I.E. Liener, N. Sharon, I.J. Goldstein) Academic Press, 266-293.

Lo\\Ty, O.H., Rosebrough, NJ., Farr, AL. and Randall, R.J. (1951). J. Biol. Chem. **193.**

Maldenov, I., Bulanov, I. and Stamenova, M. (1993). Eksp-Med-Morfol., 31, 22-28.

McEver, R.P. (1991). Thrombosis and Haemostasis, **65,** 223-228.

Miller, K. (1983). Immunobiology, 165, 132-146.

Milligan, LC, Datta, P. and Goldstein, I.J. (1989). J. Biol. Chem. **264,** 16793-16797.

Mirelman, D., Galun, E., Sharon, N. and Lotan, R. (1975). Nature (London) **256,** 414-416.

Misquith, S., Rani, P.O. and Surolia, A. (1994). J. Biol. Chem. **269,** 30393-30401.

Mo, H , Lin, C. and Sun, C. (1990). Lectins: Biology, Biochemistry and Clinical Biochemistry. 7, 35-40.

Nicolson, G.L. (1974). Int. Rev. Cytol. 39, 84-190.

Nicolson, G.L., Blaustein, J. and Etzcler, ME. (1974). Biochemistry **13,** 196-204.

Nicolson, G.L. (1976). Biochim. Biophys. Acta. 457, 57-108.

Nowell, P.C. (1960). Cancer. Res. 20, 462-466.

Oda, S., Sato, M., Toyoshima, S. and Osawa, T. (1988). J. Biochem. **104,** 600.

Ofek, I. and Sharon, N. (1988). Tnfect. Immun., **56,** 539.

Ouchterlony, O. (1948). Acta. Pathol. Microbiol. Scand. 25, 186.

Paulova, M., Ticha, M., Entlicher, G., Koster, J. and Kocourek, J. (1971). Biochim. Biophys. Acta. 237, 513.

Paulson, J.C. (1985). Tn *The Receptors,* Conn. P.M. Ed. Academic Press, New York. 2, 131-219.

Paus, E. and Christensen, J.B. (1972). Eur. J. Biochem. 25, 308-314.

Peumans, W.J., Stinissen, H. and Carlier, A.P. (1982). Biochem. J. **203,** 239-243.

Peumans, W.J., Nsimba-Lubaki, M., Peeters, B. and Broekaert, W.F. (1985). Planta, **164,** 79-82.

Peumans, W.J. and Van Damme, E.J.M. (1995). Plant Physiol. **109,** 347-352.

Pueppke, S.G., Bauer, W.D., Keegstra, K. and Ferguson, A.L. (1978). Plant Physiol. **61,** 779-784.

Pusztai, A. (1991). Plant lectins. Cambridge University Press, New York.

Quinn, J.M. and Etzler, ME. (1987). Arch. Biochem. Biophys. 258, 535-544.

Rao, M.V. (1987). Acta. Eur. Fertil. 18, 217-220.

Ravdin, J.I. (1989). J. Infect. Dis. 159, 426.

Reisfeld, R.A., Lewis, J.J. and Williams, D.E. (1962). Nature, **195,** 281.

Reisner, Y. and Sharon, N. (1984). Methods. Enz.. Mol. **108,** 168-179.

Reisner, Y., Kapoor, N., O'Reilly, R.J. and Good, R.A. (1980). Lancet. **11**, 1320-1324.

Renkonen, K.O. (1948). Ann. Med. Exp. Biol. Fenn. 26, 66-72.

Roberts, D.D., Etzler, ME. and Goldstein, I.J. (1982). J. Biol. Chem. 257, 9198-9204

Rouge, P. (1975). C.R. Hebd. Seances. Acad. Sci., Ser,. D. 280, 2105-2108.

Saito, K., Komae, A., Kakuta, M., Van Damme, E.J.M., Peumans, W.J., Goldstein, I.J. and Misaki, A. (1993). Eur. J. Biochem. **217,** 677-681.

Schlumbaum, A. Mauch, F., Vogeli, U. and Boiler, T. (1986). Nature. **324,** 365-367.

Serke, S., Neubauer, A. and Van-Lessen, A. (1989). J. Immunol. Methods. **121,** 231-235.

Sharon, N. (1975). Complex Carbohydrates, Addison Wesley Publ. Comp. Reading Mass.

Sharon, N. (1987). FEBS Lett. **217,** 145.

Sharon, N. and Lis, H. (1989). Science, **246,** 227-234.

Sharon, N. and Lis, H. (1990). FASEB J. 4, 3198-3208.

Sharon, N. and Lis, H. (1995). Essays Biochem. 30, 59-75.

Siva Kumar, N. and Rao, DR. (1986). J. Biosci. 10, 95-109.

Siva Kumar, N. and Usha Jyothi, G. (1994). 16th IUBMB Meeting, New Delhi,

Siva Kumar, N. and Padma, K. (1996). Biochem. Molbiol. Int. 38, 1059-1066.

Siva Kumar, N., Hille-Rehfeld, A. and Von Figura, K. (1997). Comp. Biochcm. Physiol (in press).

Smit, G., Swart, S., Lugtenbarg, B.J. and Kijne, S.W. (1992). Mol. Microbiol. 6, 2897-2903.

Snaith, S.M. and Levvy, G.A. (1968). Biochcm. J. 110, 663-670.

Spilatro, S.R., Cochran, G.R., Walker, R.E., Cablish, K.L. and Bittner, C.C. (1996). Plant Physiol. 110, 825-834.

Stanford, G.L. and Hooker, RS. (1990). FASEB J. 4, 2912-2918.

Stillmark, H. (1888). Thesis, University of Dorpat, Dorpat (Tartu).

Su, L C, Pucppke, S.G. and Freidman, H.G. (1980). Biochim. Biophys. Acta. 629, 292-304.

Tai, T., Yamashita, K., Ugata, A., Koida, N., Muramatsu, T., Iwashita, S., Inoue, Y. and Kobata, A. (1975). J. Biol. chem. 250, 8569-8575.

The Wealth of India (1952). Counsil of Scientific and Industrial Research, New Delhi, 3, 104.

Talbot, C.F. and Etzler, M.E. (1978). Biochemistry, 16, 1474-1479.

Tropical Legumes, Resources for the future (1979). National Academy of Sciences, Washington D.C. 59-67.

Van Damme, E.J.M. and Peumans, W.J. (1990). Planta. 182, 605-609.

Vitale, A., Ceriotti, A., Bollini, R. and Chnspeels, M.J. (1984). Eur. J. Biochem. 141,97-104.

von Eisler, M. and von Portheim, L. (1926). Z. Immunitaetsforsch. Exp. Ther. 47, 59-82.

Wagh, P.V. (1978). J. Chromatogr. **152,** 565-568.

Weber, K. and Osborn, M. (1969). J. Biol. Chem. **244,** 4406-4412.

Wiley, D.C. and Skehel, J.J. (1987). Annu. Rev. Biochem. 56, 365.

Wright, C.S. (1990). J. Mol. Biol. **215**, 635-651.

Yagi, F., Sawada, R , Imada, T., Toyonaga, S., Tadera, K. and Ishihata, K. (1994). Plant Cell Physiol., 35, 1087-1095.

Yamamoto, K., Ishida, C, Saito, M., Konami, Y., Osawa, T. and Irimura, T. (1994). Glycoconj. J. **11,** 572-575.

PUBLICATIONS

Vol. 41, No. 5, April 1997 BIOCHEMISTRY and MOLECULAR BIOLOGY INTERNATIONAL
Poges 925-931

PURIFICATION OF α-MANNOSIDASE ACTIVITY FROM INDIAN LABLAB BEANS

RAJASEKHAR BARU TULASI AND SIVA KUMAR NADIMPALLI #

Protein Biochemistry Laboratory, Department of Biochemistry,
School of Life Sciences, University of Hyderabad,
Hyderabad 500 046, India, Fax 0091-40-3010120.

Received October 28. 1996
Received after revision January 2. 1997

Summary : Seeds of *Dolichos lablab* var. *typicus* (Indian lablab beans) contain a glucose/ mannose specific lectin that was affinity purified on Sepharose mannose columns in our laboratory. The unbound fraction from this matrix showed α-mannosidase activity. In the present study this has been purified to homogeneity by a combination of ion-exchange, hydrophobic chromatography and gel filtration. Purified α-mannosidase had an apparent molecular weightof 1 95,000 $\pm$ 5,000 with 4.5% carbohydrate. On SDS-PAGE under reducing conditions, the enzyme dissociated into two major bands corresponding to Mr 66,000 and Mr 44,000. An antibody to the well studied jack bean a-mannosidase cross-reacts with the enzyme from the lablab beans suggesting antigenic similarity between these two legume mannosidases.

Introduction

Seeds of leguminous plants contain lectins, storage proteins and glycosidases that are localised in the protein bodies. These have been purified and well studied from some legumes by H. Ruediger's group (1). By systematic *in vitro* studies these workers have shown that the jack bean lectin interacts with the jack bean a-mannosidase (2). These specific interactions have been found to be significant owing to the colocalisation of the lectins and glycosidases in the seeds, suggesting a possible role of lectins in the plants in the ordered storage and degradation of proteins during seed development and germination (3).

While working with seed extracts of the Indian lablab beans to isolate the glucose/mannose specific lectin, and to study its function, we detected that these extracts also exhibit α-mannosidase activity. With a long term objective to gain further insight into the physiological function of the purified lablab bean lectin, and to study its interaction with various protein body constituents such as the a-mannosidase and storage proteins, the present work was carried out. In this report we describe the purification of the lablab beans a- mannosidase.

Corresponding author : e-mail : nsksl@uohyd.ernet.in

1039-9712/97/050925-07$05.00/0

Materials and Methods

Seeds of *Dolichos lablab* var. *typicus* (Indian lablab beans) were purchased from local market and were ground into fine powder using a dry grinder and defatted using petroleum ether and the dried powder used as the starting material. Sepharose 6B. DE-52 cellulose, Biogel P-200 were from Pharmacia, Whatman and Biorad labs, respectively. Phenyl Sepharose, p-Nitrophenyl a-D-mannopyranoside, jack bean a-mannosidase, divinyl sulfone, standard proteins, were from Sigma Chemical Company, USA. All other chemicals used in the study were obtained from reputed local firms.

Preparation of affinity matrix (Sepharose-Mannose): This was prepared as described earlier (4). Seed meal was extracted with 25mM Tris-HCl buffer, pH 8.0 and passed through the affinity matrix. The bound lectin could be eluted using 0.25 M glucose in buffer.

Estimation of protein and Enzyme Assay : Protein was measured by absorbance at 280 nm and also by Lowry method (5). a-mannosidase (E.C.3.2.1.24) was assayed as described earlier (2).

Extraction of the seed meal and chromatography : All operations were carried out at 4°C. The dry seed powder(10g) was extracted with 50ml of 25mM Tris-HCl buffer pH 8.0 (buffer A) for 3 hours, and the extract clarified by centrifugation at 10,000 rpm for 20 minutes. The clear supernatant was dialysed extensively against buffer A and was passed through Sepharose-mannose gel. The flow through from this was connected to a DE-52 cellulose (3 x 10 cm) equilibriated with buffer A. After passing the sample the column was extensively washed with buffer A and eluted with 100mM NaCl in the same buffer. The active fractions were pooled, made to 1M Ammonium Sulfate and passed through Phenyl Sepharose gel (1.5 x 4.5 cm) equilibriated with 50mM Tris-HCl buffer, pH 8.0 containing 1M Ammonium Sulfate. After washing the unbound proteins, the bound enzyme was eluted using buffer A. The active fractions were pooled, concentrated by ammonium Sulfate and dialysed against buffer A containing 150mM NaCl and passed through Biogel P-200 column (1.4 x 86 cm) equilibriated with the same buffer.

Gel Electrophoresis : Polyacrylamide gel electrophoresis (PAGE) in the absence of SDS and under denaturing and reducing conditions was carried out on 10% gels as described (6) and proteins were detected by Coomassie blue staining.

Gel Filtration : Gel filtration was done on a column of Biogel P-200 using the conditions described above. The Phenyl Sepharose eluate (7 mg protein) was loaded onto this gel. Myosin (Mr 205,000), β-galactosidase (Mr 116,000), Bovine serum albumin (Mr 66,000), Ovalbumin (45,000), were used as standards.

Carbohydrate estimation : This was carried out using Phenol-Sulphuric acid method (7) using mannose as standard.

Raising antibodies to jackbean α-mannosidase : Antibodies were raised for the enzyme in the rabbit and their formation was detected by immunodiffusion as described (8). For Western Blot analysis, proteins at different stages of purification were separated on SDS gels under reducing conditions and then transferred to nitrocellulose paper and probed with the jackbean a-mannosidase antisera. Detection of the bands was done as described (9).

Results and Discussion

The data presented here is the first report on the purification of a-mannosidase activity from the seeds of the Indian lablab beans from which a glucose/mannose specific lectin was earlier purified in our laboratory (10). The enzyme has been purified from only a few legume species among which the enzyme from jack beans is well studied (2).

Enzyme activity : Crude extracts from the seeds of Indian lablab beans showed a-mannosidase activity when assayed under standard conditions with p-nitrophenyl a-D-mannopyranoside as the substrate. The extracts were passed through Sepharose-mannose gel in order to completely deplete the lectin and the flow through was used to purify the a-mannosidase. About 14 mg of the lectin could be obtained from 10 grams of the seed powder.

Purification and characterisation of the a-mannosidase : The overall purification of the α-mannosidase is shown in Table 1. Upto 73% of the activity from the crude extracts could be recovered on the DE-52 cellulose. The enzyme also was bound specifically to Phenyl Sepharose gel in presence of 1M Ammnonium Sulfate and could be easily eluted using buffer A. About 54% of the activity could be recovered in this step. In order to further purify the enzyme and also to determine its molecular size, the phenyl sepharose eluate was passed through the Biogel P-200 where the enzyme eluted as a single symmetrical peak (Fig. 1). Typically about 4mg of the enzyme could be obtained from 10g of the seed extract.

The sample eluted from the Biogel P-200 gave a single band in 10% native gels (Fig. 2A). The apparent molecular size of the enzyme on gel filtration was found to be 195,000 $\pm$ 5,000 (Fig. 1) In presence of SDS and under reducing conditions, two major bands corresponding to Mr 66kDa and 44kDa were obtained (Fig. 2B). These prominent bands could also be detected in the phenyl sepharose eluates. Taken together these results indicate that the enzyme from lablab beans is possibly made of two types of subunits, that are linked by disulfide bridges and may have $\alpha_2\beta_2$ type of structure similar to the jack bean enzyme. Jack bean a-mannosidase when run on Biogel P-200 eluted at the same elution volume as myosin. The molecular size of the

Table 1 : Purification of lablab bean a-mannosidase.

Purification step	Protein mg	Activity Units*	Specific activity	Recovery %	Fold purification
Crude extract	857	11	0.012	100	1
DE-52 eluate	56	8	0.14	73	12
Phenyl Sepharose eluate	9	6	0.66	54	55
P-200 eluate	4	5	1.25	45	104

* One enzyme Unit (U) is defined as the enzyme amount able to release 1μmol of 4-nitrophenol per minute, under the conditions used.

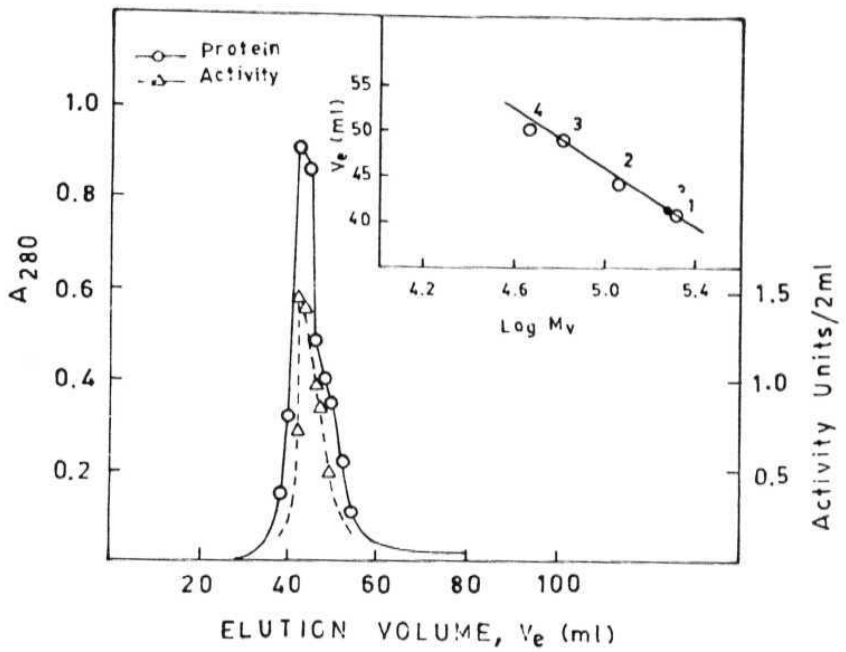


Fig. 1 : Gel Filtration of lablab bean α-mannosidase. Phenyl Sepharose eluates (7 mg/ml) in buffer A containing 1 50 mM NaCl was loaded onto a column of Biogel P-200 (1.4 x 86 cm) and eluted with same buffer. Fractions, 2 ml were collected and elution was followed by monitoring absorbance at 280 nm. *Inset* Plot of elution volume (Ve) *vs* Log Mr. (o) Standards, 1. Myosin, 2. β-galactosidase, 3. Bovine serum albumin, and 4. Ovalbumin. • lablab bean a-mannosidase

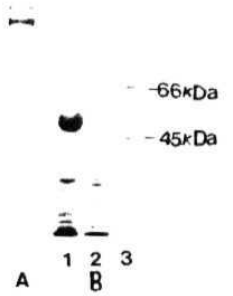


Fig. 2 : PAGE pattern of purified lablab bean a-mannosidase; A: PAGE under nondenaturing conditions was done in 10% gels. Purified enzymes 20 μg was loaded on the gel and proteins detected by Coomassie stain. B : SDS-PAGE pattern of the lablab bean *a*-mannosidase at different stages of purification under reducing conditions. Lane 1, DE-52 eluates (30 μg protein), Lane 2 Phenyl Sepharose eluates (30 μg protein), Lane 3, Biogel P-200 eluates (15μg protein).

different mannosidases reported in the literature varies from 190,000 to 230,000 (11). Although the lablab bean enzyme eluted 1ml after the jack bean enzyme, the subunit pattern of both enzymes was similar.

Immunodiffusion and Western blot analysis : Owing to the ready availability of the jackbean a-mannosidase (Sigma), antibodies were raised for this enzyme and were used to check if they are able to cross react with the lablab bean enzyme. In addition, the lablab bean enzyme could also be specifically bound and eluted from an immunoaffinity gel (jack bean α-mannosidase IgG coupled to Affigel 10; data not shown). From Fig. 3A it is apparent that the jack bean *a*-mannosidase and the lablab bean enzyme are immunologically identical. Further confirmation of this identity was established in Western blot analysis wherein the subunits of both enzymes migrated to the same extents on SDS gels and also showed immunological reactivity with the antibody to the jack bean enzyme (Fig. 3B). To our knowledge there is no report so far about the immunological properties of the legume mannosidases.

Similar to the jack bean mannosidase the lablab bean enzyme is a glycoprotein with 4.5% carbohydrate. The lablab bean enzyme is stable upto 40°C. At 50°C and upto 80°C (after 15 minutes) the enzyme activity decreased from 61 to 17%. The enzyme does not show any requirement of zinc for its activity nor does zinc inhibit the activity of the enzyme as has been observed for some mannosidases (2,14).

A large number of plant proteins have so far been purified and characterised from legume seeds. These include lectins, storage proteins and glycosidases. Owing to their colocalisation in the protein bodies in the seeds and the ability of the lectins to bind specifically to sugars, lectins have been attributed to play a key role in the ordered aggregation and degradation of proteins during seed development and germination (3). In an attempt to understand the physiological function of the lablab lectin and to study the type of interactions it might have with the mannosidase from the same source, as has been described for Con A lectin and the jack bean a-mannosidase we have purified the a-mannosidase from lablab beans.

In a preliminary experiment when the purifed lablab bean enzyme was passed through immobilised lablab bean lectin (7mg lectin per ml of Affigel-10) or Con A Sepharose gel, at pH 5 employing 50 mM sodium acetate buffer, the enzyme tightly bound to the matrix and no protein could be eluted using either high concentrations of glucose or mannose, 1M NaCl or increasing the pH to 8.0. Some protein could be eluted with Tris-glycine buffer pH 9.2 which

A 1 2 B

Fig. 3 : Immunodiffusion and Western Blot analysis of the lablab bean mannosidase. A : Immunodiffusion : Central well contained 20 μL of antiserum to the jack bean mannosidase. Wells 1 and 2 contained 20 μg of jack bean α-mannosidase and Biogel P-200 eluates of the lablab bean enzyme. B : Western Blot Analysis : Lane 1 jack bean α-mannosidase, Lane 2, purified lablab bean mannosidase.

was devoid of any appreciable enzyme activity. While it has been already reported that the jack bean enzyme is absorbed to Con A Sepharose gel at pH 5 and can be easily desorbed with 1 M NaCl (12), it is hard to arrive at any reasor.able conclusions as to why there is such strong interaction between the lablab bean enzyme and its lectin.

In summary, the lablab bean mannosidase exhibits similar abilities such as binding to phenyl sepharose gels as of other glycosidases studied (13). Further, the glycoprotein nature of the lablab bean enzyme, its subunit pattern and its immunological cross reactivity with the jack bean mannosidase antibody suggests that these two enzymes are closely related. Our future studies aim at understanding the physiological significance of the interactions between the purified lablab bean lectin and mannosidase.

Acknowledgements : This work was partly supported by a minor research grant of the UGC to SKN. RBT gratefully acknowledges receipt of Research Fellowship from the University Grants Commission, Govt. of India, New Delhi. Some of this work was presented at the annual meeting of the Society of Biological Chemists (India), Lucknow, India in October, 1995.

References

1. Einhoff, W., Fleischmann, G., Frier, T., Kummer, H., and Ruediger, H., (1986) Biol. Chem. Hoppe-Seyler, **367**, 15-25.
2. Einhoff, W., and Ruediger, H., (1986) Biol. Chem. Hoppe-Seyler, **367**, 943-949.
3. Einhoff, W., Fleischmann, G., Frier, T., Kummer, H., and Ruediger, H., (1986) in Lectins **Vol. 5, 45-52**. Walter de Gruyter & Co., Berlin, New York. Ed. Bog-Hansen, T.C., and Van Driessche, E.
4. Siva Kumar, N., and Rajagopal Rao, D., (1986) J. Biosci. 10. 95-109.
5. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J., (1951) J.Biol. Chem. **193**, 265-275.

6. Laemmli, U.K., (1970) Nature (London) **227.** 680-685.
7. Dubois, M., Galles, K.A., Hamilton, J.K., Rebers, P.A., and Smith, F. (1956) Anal. Chem. 28. 350-356.
8. Siva Kumar N., and Padma, K., (1996) Biochem. Mol. Biol. Int 38, 1059-1066.
9. Udaya Lakshmi, Y., and Siva Kumar, N., (1996) Biochem. Mol. Biol. Int. **40, 815-821**
10. Siva Kumar, N., Padma, P., Ramanadham, M., and Kasyapa, C.S.S., (1992) Proceedings, **Interlec 14.** 52 Darjeeling, India.
11. Einhoff, W., and Ruediger, H., (1988) Biol. Chem. Hoppe-Seyler. **369. 165-169.**
12. Einhoff, W., and Ruediger, H., (1986) Biol. Chem. Hoppe-Seyler, **367.** 313-320.
13. Einhoff, W., and Ruediger, H., (1989) Phytochem. 28, 355-357.
14. Curdel, A., and Petek, F., (1980) Biochem J. 185. 455-462.

BIOCHEMICAL ARCHIVES

M.B.R. PRESS, INC.
P.O. BOX P
KENYON, MINNESOTA 55946-OOOP
U.S.A.

July 7, 1997

Dr. N. Siva Kumar
Senior Lecturer
Biochemistry Department
University of Hyderabad
Hyderabad 500 046
INDIA

Dear Dr. Siva Kumar:

I am pleased to inform you that your manuscript, "Purification of a Lectin in High Yield from the Indian Lablab Beans on Goat IGM Sepharose and by Immunoaffinity Chromatography: Evidence for the Presence of Endogenous Lectin Receptors," reference number A-1558, by Tulasi, Potturi, and Siva Kumar, has been accepted for publication in BIOCHEMICAL ARCHIVES.

Please complete and return the enclosed Transfer of Copyright Agreement, immediately upon receipt, and the Reprint Order Form.

Please complete and return the enclosed shortage of research grant funds form to obtain the reduced rate.

With best regards,

Sincerely yours,

Robert Derr

Robert Derr, Ph.D.
Editor

PURIFICATION OF A LEGTIN IN HIGH YIELD FROM THE INDIAN LABLAB BEANS ON GOAT IGM SEPHAROSE AND BY IMMUNOAFFINITY CHROMATOGRAPHY : EVIDENCE FOR THE PRESENCE OF ENDOGENOUS LECTIN RECEPTORS

Rajasekhar Baru Tulasi, Padma Potturi@ and Siva Kumar Nadimpalli* Protein Biochemistry Laboratory, Biochemistry Department, University of Hyderabad, Hyderabad 500 046, India. Fax No. 0091-40-3010120. e-mail : nsksl@uohyd.ernet.in @present address;School of Chemistry University of Hyderabad, Hyderabad. India. *Corresponding Author

Abstract

Seeds of the *Dolichos lab lab* var. *typicus* (Indian lablab beans) contain a glucose/mannose specific lectin that has been affinitypurified on Sepharose-mannose columns (1). We report here on the affinity purification of this lectin in high yields using alternate methods i) goat IgM Sepharose., and ii) Immuno-affinity chromatography (Lectin specific IgG coupled to Affi-gel-10). The molecular size and subnunit pattern of the lectin purified by both methods was similar to that reported earlier. In addition the purified lectin could be quantitatively bound to Sepharose divinyl sulfone methyl α-mannopyranoside gel. Purified lectin was also found to be mitogenic to murine lymphocytes. Additionally Lectin-affinity chromatography (Lectin coupled to Affigel-10) indicated the presence of endogenous lectin receptors that specifically interact with the lectin without the involvement of the sugar binding site.

Lectins are ubiquitous proteins/glycoproteins that are abundantly present in the legume seeds and have also been purified from some cereal seeds (2). Owing to their unique property of binding sugars in a reversible manner, lectins have found wide spread applications in different areas of modern biology (3). The genus *Dolichos fab/ab* contains two varities namely, var. *lignosus* (Field bean) and var. *typicus* (lablab bean). Both seeds contain glucose/mannose specific lectin that has earlier been affinity purified on Sepharose-mannose gel. Purified lectins exhibited similar physicochemical and biological properties (4). The objective of the present study was to develop alternate affinity methods for the purification of the lablab bean lectin in high yields and to study its fine sugar specificity as well as its other biological properties such as the mitogenecity and to identify endogenous lectin receptors from the seeds in order to understand the physiological significance of the lectin. The results obtained on these are presented here.

Materials and Methods

Seeds of Indian lablab beans were purchased from the local market, ground into fine powder using a dry mill and the fine powder defatted using 40-80°C Petroleum ether. The defatted seed meal was used in all studies. Sepharose 6B was purchased from Pharmacia, Uppsala, Sweden. Affigel-10 was purchased from Bio-Rad laboratories, USA. All sugars used in the study,divinyl sulfone, Con A Sepharose were from Sigma Chemical Company, USA. All other chemical and reagents used in the study were of high quality and were purchased locally from reputed firms.

Preparation of affinity matrices : Sepharose - goat IgM : Goat IgM was prepared as described and was coupled to Cyanogen Bromide activated Sepharose following the protocol described (4). 8 mg of protein was coupled to one ml of the gel.

Sepharose divinyl sulfone Methyl α-D-mannopyranoside: This gel was essentially prepared as described (2), except that in place of N-Acetylglucosamine, methyl α-D-mannopyranoside was used. 5ml this gel was prepared.

Preparation of Lectin-Affigel column: Purified lectin was coupled to Affigel-10 (Biorad Labs) following the manufacturers instructions. (7mg lectin was coupled to 1 ml).

Preparation of Antibodies to the purified seed lectin and purifying the lectin specific IgG on Lectin Affigel : Purified seed lectin was injected into a rabbit to raise antibodies as described (1). Lectin specific IgG was purified by passing the serum on Lectin- Affigel equilibrated with 10mM PBS, and eluting the bound IgG with 100mM Glycine-HCL buffer pH 2.65. The eluted IgG was immediately neutralised with 2M Tris and concentrated and analysed on SDS-PAGE under nonreducing conditions.

Preparation of Immunoaffinity matrix : (Lectin Specific IgG coupled to Affigel-10). Lectin specific IgG obtained above was coupled to Affigel-10. Typically 7mg of the protein was coupled to 1 ml of Affigel.

Extraction of proteins and purification of the seed lectin on goat IgM Sepharose : 1g of the defatted lablab bean seed powder was extracted with 10ml of 20mM PBS for 3 hours at 4°C. The suspension was centrifuged at 7000rpm for 20 minutes and the clear supernatant was brought to pH 5.0 with 2N acetic acid. The suspension was clarified by centrifugation and the precipitate discarded. The clear supernatant was brought back to pH 7.4 using 2N sodium hydroxide and loaded on goat IgM sepharose gel at a flow rate of 10ml per hour at 4°C. The gel was washed extensively with the column buffer and the bound lectin was eluted from the gel using 0.5M glucose in column buffer. The eluted fractions were pooled, dialysed, concentrated and analysed for protein and activity. The pH 7.4 supernatant obtained from another 1g batch was passed through Sepharose-mannose gel as desccribed (1) and 1.4 mg of the lectin could be eluted from this matrix.

Extraction of proteins and purification of the lectin on the Immuno-affinity matrix : 1 g of the seed meal was processed as described above. To the pH 7.4 supernatant was added ammonium Sulfate to 80% saturation and stirred for 3 hours at 4°C. The suspension was centrifuged at 7000rpm and the pelleted protein was dissolved in small volume of PBS and dialysed extensively against PBS. This protein fraction (80 mg) was passed through the immunoaffinity gel, to purify the lectin.

Extraction of lectin binding proteins (lectin receptors) from the seeds : 1 g of the seed meal was extracted with 20mM PBS, for 3 hours and the suspension centrifuged at 7000rpm. The clear supernatant was adjusted to pH 5.0 using 2N acetic acid and the suspension centrifuged at 7000 rpm. The precipitated proteins were redissolved in 50mM Tris-HCl buffer pH 8.0 (buffer A) and dialysed against the same buffer. Any precipitate formed was clarified by centrifugation and the clear supernatant 76 mg protein was loaded on LectinAffigel at a flow rate of 10ml

per hour in buffer A. After washing the column with the same buffer until the absorbance at A280 was 0.05, the bound proteins were sequentially eluted using 0.5M glucose, 1M Sodium chloride., and 50mM sodium acetate bufferpH 4.0. Protein that was eluted with sodium acetate buffer was pooled, concentrated and analysed by SDS-PAGE analysis.

PAGE and SDS-PAGE Analysis : Native PAGE and SDS-PAGE of the purified lectin was performed as described (1). SDS-PAGE of the lectin receptors was performed as described (5).

Haemagglutinating activity and sugar inhibition studies : Haemagglutinating activity and sugar inhibition studies using various sugars was carried out with trypsin treated rabbit erythrocytes as described (1).

Results and Discussion

Purification of the lablab bean seed lectin on goat Igm-Sepharose: When the lablab bean seed extract obtained as described under methods was passed through the goat IgM gel, the lectin could be bound and specifically eluted using 0.5M glucose. Figure 1 shows the typical elution profile of the lectin from the gel. The fact that the protein eluted was the lectin itself was not only confirmed by its agglutinating activity but also by gel electrophoresis (Fig 1 A). From 1g of the seed powder, 2.18mg of the purified lectin could be obtained (Table 1). Goat IgM, contains large amounts of mannose residues and was earlier used as a ligand for purifying the glucose/mannose specific lectin from the seeds of *cajanus ca/an* (4). The lablab bean lectin could also be specifically bound onto goat IgM Sepharose and could be eluted using glucose. Purified lectin on gel filtration on Biogel P-200 eluted as a single peak corresponding to molecular size of 60,000 (data not shown). The subnunit pattern of the lectin (Fig 2A) was similar to that reported earlier (1). Using the same conditions for purifications, and employing Sepharose-mannose gel, 1.4mg of the lectin could be eluted from 1g of the seed meal.

Immunoaffinity purification of the lablab bean seed lectin : The lectin sepcific IgG isolated from the antiserum on lectin-Affigel column was found to be homogeneous in SDS-PAGE under nonreducing conditions. This was coupled to Affigel-10 and is referred to as the Immunoaffinity matrix. The immunoaffinity matrix prepared bound the seed lectin readily from the total proteins of the seed extract. From 80 mg of the ammonium sulfate precipitated protein 3.0 mg of purified lectin was obtained (data not shown). The specificity of the interaction between the antigen and antibody was further confirmed by immuno-diffusion experiment (Fig. 2).

Sugar inhibition studies : The agglutinating activity of the lablab bean lectin was found to be inhibited by a number of sugars related to glucose and mannose. Among the simple sugars, glucose and mannose were potent inhibitors. The hydroxyl group at C-2 seems important for binding. Substitution at C-1 in glucose/mannose by methyl as well as phenyl groups enhcanced their abililty to inhibit the lectin activity. These data suggests that the sugar binding site in the lectin contains a hydrophobic region as of other lectins studied (6). The C-3 hydroxyl group in mannose seems important as blocking it with another mannose sugar abolishes inhibition. This is further confirmed by the fact that 3-0 methyl glucose is also a good inhibitor for lectin activity. The configuration around C-4 does not affect the binding as galactose and lactose are noninhibitory. N-Acetylglucosamine was inhibitory only at high concentrations.

The fact that the methyl derivatives of glucose/mannose are better inhibitory for lectin activity,

was further reconfirmed by passing purified lablab bean lectin onto Sepharose-divinyl sulfone methyl α-D-mannopyranoside gel. 4 mg of lectin could be bound on 1.0ml of the gel. These sugars were found to be effective in inhibiting the lectin activities of several other glucose/ mannose specific lectins reported (1,6). The lablab bean lectin however failed to bind onto ConA Sepharose gel, a property exhibited by many other glycoprotein lectins owing to their glycoprotein nature. In this respect, the lablab bean lectin differs from other glycoprotein lectins (7).

When different concentrations (10 to 60 ug) of the purified lectin were tested for mitogenic acitvity against murine lymphocytes, the lectin was found to be mitogenic at 10ug concentration (data not shown). Several other lectins, particularly glucose/mannose specific lectins were found to be mitogenic to other lymphocytes (8).

Endogenous Lectin receptors : From the seed extracts of the lablab beans, proteins that interact specifically with immobilised lectin in Lectin affinity chromatography have been isolated. These proteins could be bound to the lectin at pH 8.0 and can be desorbed from the affinity matrix by lowering pH to 4.0 (Fig 3A). From 76 mg of the acid preciptated proteins , 1.0 mg of the receptor could be obtained. Elution with 0.5M glucose or methyl glucose, follwed by 1M sodium chloride did not desorb the receptor (data not shown). The protein eluted was found to be a glycoprotein with 1.1% carbohydrate and had neither glycosidase activity nor lectin activity. In SDS-PAGE analysis the protein dissociated into one major band corresponding to molecular size (53KDa) and two minor bands of molecular sizes (58 KDa, and 52 KDa, Fig 3B) The two minor bands might possibly represent proteolytically derived fragments of the isolated protein as similar observations have been made earlier for the lectin receptors from the peas. When the Lectin-affigel was preequilibriated with 0.5M glucose in column buffer, the same amount of the lectin receptors could be bound on the gel suggesting that the lectin doesnot interact with the receptor with the sugar binding site (data not shown).

When the sodium acetate eluted proteins were concentrated, dialysed and reloaded on the same gel, upto 80% of the protein could be rebound on the gel, which could be again eluted with sodium acetate buffer pH 4.0.

Endogenous lectin receptors exhibiting similar electrophoretic mobilities have been identified from some legume seeds (9). However in these seeds the authors observed that the lectin interacts with endogenous receptors by its sugar binding site or by ionic interactions as the lectin bound proteins could be easily eluted with the inhibitory sugar or by high salt. The endogenous lectin receptors from the lablab beans although showed comparable electrophoretic properties to other legume receptors studied, could be eluted from the lectin column only by decreasing the pH to 4.0. and not by sugar or high salt. These data suggests that the lectin - receptor interaction is pH dependent.

In summary, the present report describes alternate methods for the purification of a mitogenic lectin from the seeds of lablab beans with higher yields and also elaborates on its fine sugar specificity. Further the proteins that interact with the lectin *in vitro* have been identified and these interactions in the seed may be of physiological significance in view of the glycoprotein nature of the lectin as well as ofthe endogenous receptors and colocolisation of these in the protein bodies. Our future studies aim at understanding the significance of these interactions using biochemical and immunological techniques which would eventually define the function

of the lablab bean lectin which is poorly understood.

Acknowledgements

BTR acknowledges University Grants Commission, Govt. of India, New Delhi for a Research Fellowship.

References

1. SIVA KUMAR.N., AND RAJAGOPAL RAO, D. J. Biosci. 10, 99-105., 1996.
2. SIVA KUMAR, N., AND PADMA, K., Biochem.Mol.Biol.Int. 38, 1059-1066., 1996.
3. SHARON, N., AND US, H. FASEB.J. 4, 3198-3208, 1990.
4. AHMAD, S. 64th Annual Meeting of the Society of Biological Chemists, October 6-th, Lucknow, India, 1995. P2D55.
5. LAEMMLI, U.K., Nature (London) 227, 680-684. 1970.
6. ALLEN, A.K., DESAI, N.N., AND NEUBERGER, A., Biochem. J. 131, 155-162.
7. IGLESIAS,J.L., LIS, H., AND SHARON, N. Eur.J.Biochem. 123, 247-252, 1982.
8. LIS, H., AND SHARON, N. *in* Lectins, Properties, functions and applications in biology and medicine., 270-277 Academic Press, Liener, I.E., Sharon, N., and Goldstein, I.J. Eds.
9. KUMMER, H., and RUEDIGER, H. Biol. Chem. Hoppe-Seylers. 369, 639-646, 1988.

Table

Purification of the lablab bean lectin on goat IgM Sepharose gel

Step	Vol ml	Total protein (mg)	Total activity H.U.	Specific activity	Recovery %	fold purification
Crude extract	7.5	140	9600	68	100	-
Dialysed pH 7.4 supernatant	7.0	90	8967	99.6	93	1.46
Affinity chrom-atography.	1.0	2.18	5120	2348	53	34.5

1g of the seed meal was used for purification. One H.U. (Haemagglutinating unit) is defined as the minimum amount of protein required to cause visible agglutination.

Table II

Inhibition of Haemagglutinating activity by various sugars by the lablab bean lectin, using trypsin treated rabbit erythrocytes.

Sugar	Inhibitory concentration mM
Glucose	5.0
Mannose	5.0
Methyl α-D-glucoside	<0.5
Methyl α-D-mannoside	<0.5
3-0-Methyl glucose	2.5
Phenyl α-D-mannose	<2.5
Phenyl α-D-glucose	<2.5
N-Acetylglucosamine	10.0
3-0 α-D-mannopyranosyl α-D-mannopyranose	NI
Mannosamine	NI
Melizitose	NI

0.01 to 0.1ml of 0.1M stock sugar solutions were used in the assay. 4 Haemagglutinating units of the lectin in 0.1ml was incubated with the different sugars and then 0.2ml of trypsin treated rabbit erythrocytes were added and agglutination visualised after incubation at 37°C for 90 minutes. Other sugars tested, galactose, lactose, cellobiose, gentiobiose, raffinose, fucose, N-Acetylgalactosamine, sucrose were noninhibitory upto concentrations of 0.1M.

Legends for Figures

Fig 1

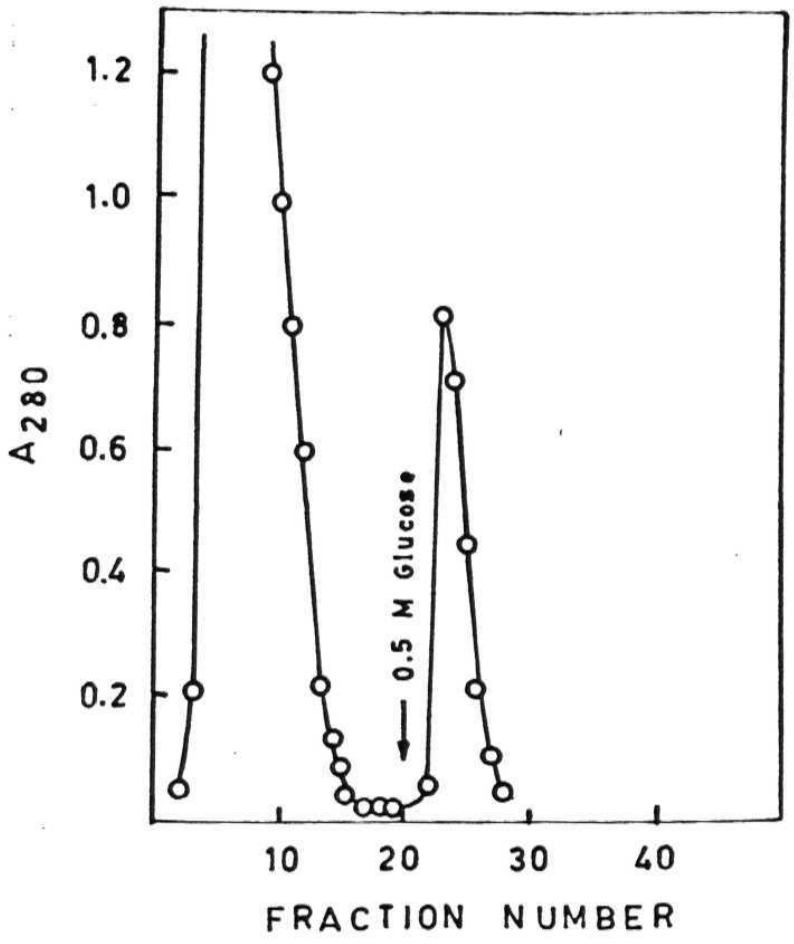
A. Purification of lablab bean lectin on goat IgM Sepharose. 90 mg of protein was loaded on goat IgM Sepharose gel (0.8 x 6 cm) equilibrated with PBS. Fractions 1.0ml were collected and the absorbance monitored at 280nm. Elution was performed using 0.5M glucose in PBS.

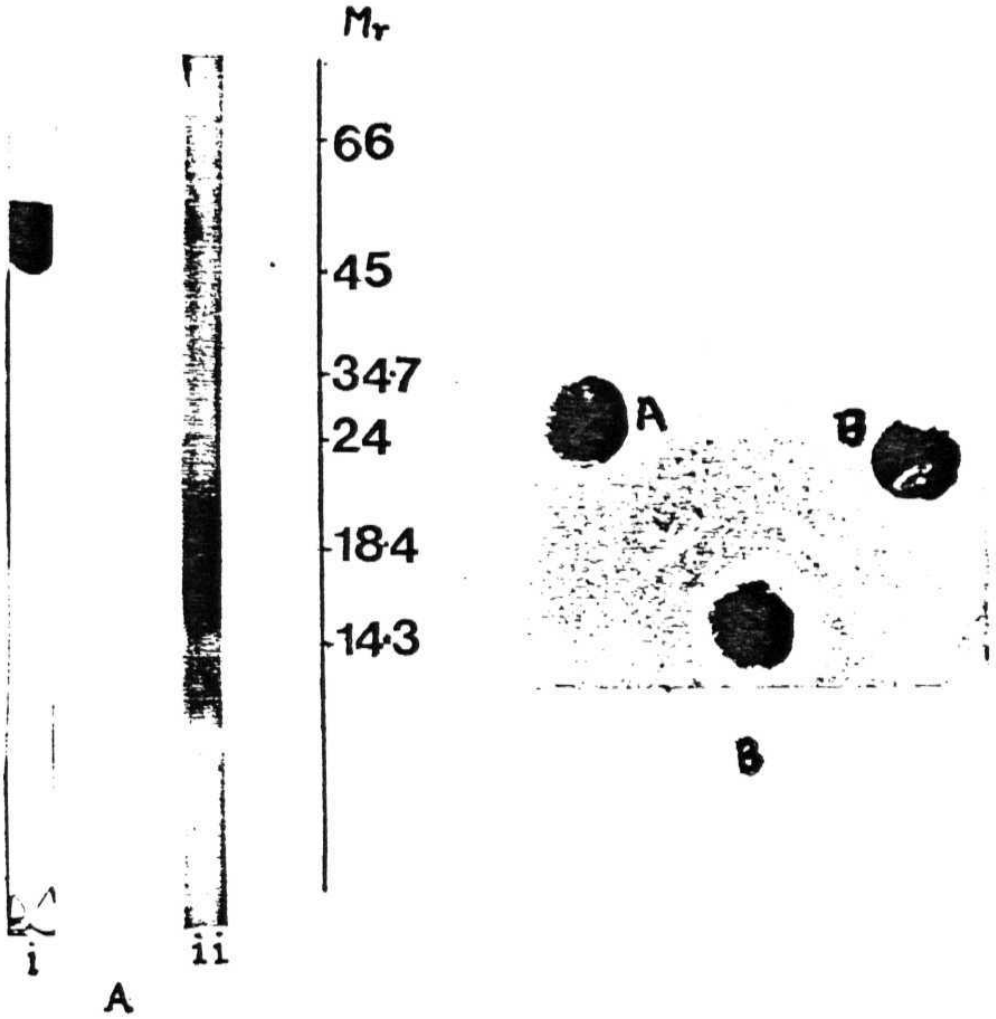
Fig 2

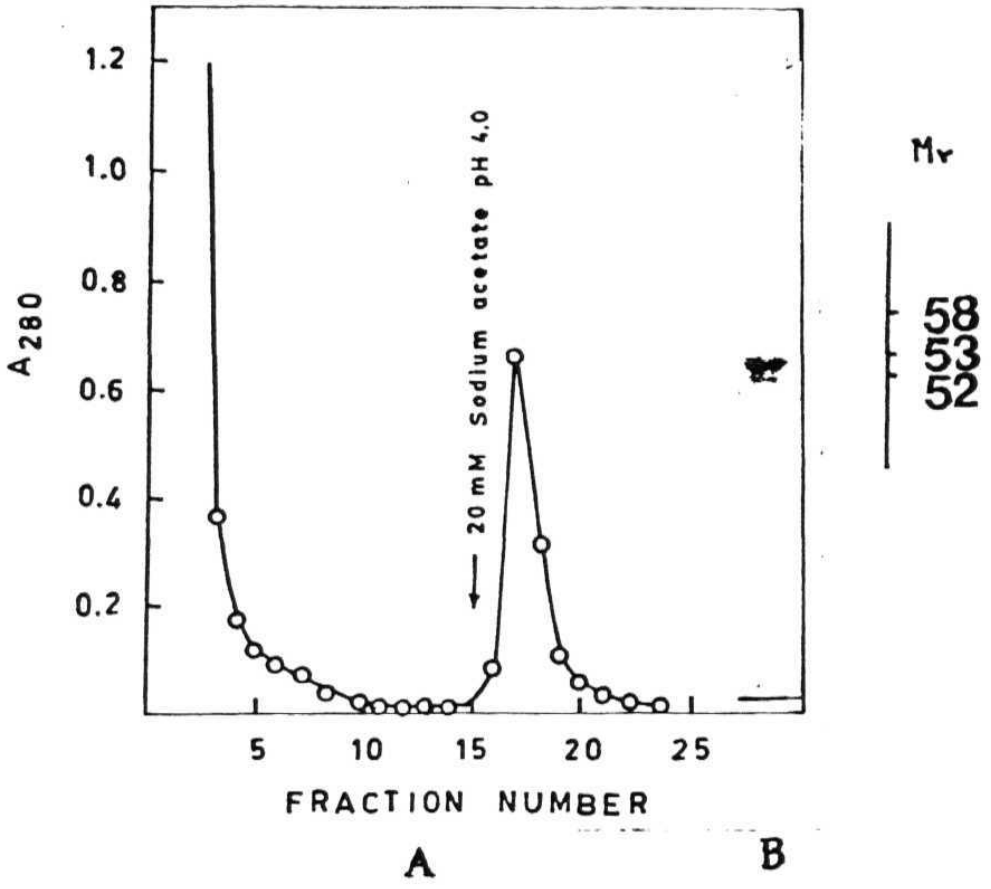
A. PAGE (lane i) and SDS-PAGE (lane ii) of the purified lablab bean lectin. B. Immunodiffusion of the lectin from lablab bean proteins. Central well contained 20uL of affinity purified antiserum to the lablab bean lectin. Well A and B contained 20ug each of the lectin purified on goat IgM gel and on Immunoaffinity matrix respectively.

Fig 3

A. Isolation of lectin receptors on Lectin-Affigel coulmn. Protein precipitated by acid at pH 5.0 from the crude extract of the lablab bean seeds were solubilised and dialysed in Tris-HCl buffer pH 8.0.. Clear supernatent (76 mg protein) was passed through Sepharose-mannose gel (1.2 x 1cm) and the flow through was connected to a Lectin-Affigel column (1.3 x 1.3 cm) equlilibrated with Tris-HCl buffer pH 8.0. Elution was performed using 20mM sodium acetate buffer pH 4.0. B. SDS-PAGE pattern of the eluted protein.


1.2
1.0
0.8
0.6
0.4
0.2
A280
0.5 M Glucose
10
20
30
40
FRACTION NUMBER


Mr
66
45
34·7
24
18·4
14·3
i
ii
A
A
B
B


1.2
1.0
0.8
0.6
0.4
0.2
A280
20 mM Sodium acetate pH 4.0
5
10
15
20
25
FRACTION NUMBER
A
B
Mr
58
53
52