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 (*Cajava 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. *ligosus*). 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 (116,000), 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 arm 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 4°C. 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 4°C.

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

All operations were carried out at 4°C. 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 coomassie 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) \( \beta \)-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 erythrocyte 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 necessary 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 \( \mu \text{M} \) p-mercaptoethanol. Cell cultures containing \( 2 \times 10^5 \) cells in 200 \( \mu \text{l} \) of medium were kept in triplicates with different concentrations of purified lectin (10-100 \( \mu \text{g} \)) 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 \( \mu \text{Ci} \) of tritiated thymidine (low specific activity, 5-20 \( \text{Ci/mmole} \)) 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 \( \text{ml} \) 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 formed 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<sub>280</sub>. Unreacted sites in the gel were blocked using 200 µl of 0.1 M ethanolamine hydrochloride pH 8.0 for 1 h 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 pre-equilibrated 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 4°C. 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 4°C. 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 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 and dialysed against the same buffer.
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 coomassie blue and destained.

**Isolation and purification of the galactose specific lectin**

All operations were carried out at 4°C. 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 dialysed 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 dialysed against 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 coomassie brilliant blue and destained.

**Haemagglutination** assay

This was performed as described earlier for mannose specific lectin.

**Immunodiffusion**

This was performed 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-divinylsulfone-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 chromatography 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.
**Table 1:** Purification of the glucose/mannose specific lectin on Sepharose-divinylsulfone-mannose matrix.

<table>
<thead>
<tr>
<th>STEP</th>
<th>VOLUME (ml)</th>
<th>PROTEIN (mg)</th>
<th>ACTIVITY (U)</th>
<th>SPECIFIC ACTIVITY (U/mg)</th>
<th>RECOVERY (%)</th>
<th>FOLD PURIFICATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>36</td>
<td>710</td>
<td>47360</td>
<td>86</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Dialysed Amm SO4 Pellet (80% saturation)</td>
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<td>421</td>
<td>46080</td>
<td>109</td>
<td>97</td>
<td>1.26</td>
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<tr>
<td>Affinity Chromatography</td>
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<td>13</td>
<td>37128</td>
<td>2834</td>
<td>78</td>
<td>33</td>
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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.
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.

<table>
<thead>
<tr>
<th>STEP</th>
<th>VOLUME (ml)</th>
<th>TOTAL PROTEIN (mg)</th>
<th>TOTAL ACTIVITY (U)*</th>
<th>SPECIFIC ACTIVITY (U/mg)</th>
<th>RECOVERY (%)</th>
<th>FOLD PURIFICATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
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<td>9600</td>
<td>68</td>
<td>100</td>
<td>—</td>
</tr>
<tr>
<td>Dialysed pH 7.4 Supn.</td>
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<td>90</td>
<td>8967</td>
<td>99.6</td>
<td>93</td>
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<tr>
<td>Afinity Chromatography</td>
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<td>2.18</td>
<td>5120</td>
<td>2348</td>
<td>53</td>
<td>34.5</td>
</tr>
</tbody>
</table>
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 M₀. Wts. 15 kDa and 12 kDa in SDS-PAGE (Fig. 3B).

Affinity chromatography of purified glucose/mannose specific lectin on Sepharose–divinyl sulfone-methyl α-D-mannopyranoside gel.

Fig. 4 shows the specific binding of the glucose/mannose specific seed lectin on Sepharose- divinylsulfone-methyl mannose 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 of 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.
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.
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.
**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.

**Immunoadfinity** 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 apparent 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 up to concentrations of 0.1M.

<table>
<thead>
<tr>
<th>Sugar</th>
<th>Inhibitory concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>5.0</td>
</tr>
<tr>
<td>Mannose</td>
<td>5.0</td>
</tr>
<tr>
<td>Methyl α-D-glucoside</td>
<td>&lt; 0.5</td>
</tr>
<tr>
<td>Methyl α-D-mannoside</td>
<td>&lt; 0.5</td>
</tr>
<tr>
<td>3-O-methyl glucose</td>
<td>2.5</td>
</tr>
<tr>
<td>Phenyl α-D-mannose</td>
<td>&lt; 2.5</td>
</tr>
<tr>
<td>Phenyl α-D-glucose</td>
<td>&lt; 2.5</td>
</tr>
<tr>
<td>N-Acetyl glucosamine</td>
<td>10.0</td>
</tr>
<tr>
<td>3-O-D-mannopyranosyl mannopyranose</td>
<td>NI</td>
</tr>
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<td>Mannosamine</td>
<td>NI</td>
</tr>
<tr>
<td>Melizitose</td>
<td>NI</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume (ml)</th>
<th>Protein (mg)</th>
<th>Activity (U)</th>
<th>Recovery (%)</th>
</tr>
</thead>
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<td>70</td>
<td>4800</td>
<td>100</td>
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<tr>
<td>pH 7.4 supn. (S0% Amm SO₄ fractionation)</td>
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<td>39.5</td>
<td>2560</td>
<td>53</td>
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<td>1.5</td>
<td>1280</td>
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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 respectively.

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

The purified lectin was found to be 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 corresponding 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.
Fig. 9. Isolation of lectin receptors on Lectin-Affigel column. Protein precipitated by acid at pH 5.0 from the crude extract of the lablab bean seeds was solubilised and dialysed against Tris-HCl buffer pH 8.0. Clear supernatant (76 mg protein) was passed through Sephroose-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.
Fig. 10. SDS-PAGE pattern of Lectin binding proteins from lablab beans under reducing conditions. 10% gel.
Purification of the galactose specific lectin

The galactose specific lectin was purified by conventional methods since the lectin activity failed to bind on the Scpharose-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 0.1 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.
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.
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.
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 apparent 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 lablab* 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 $\alpha$-D-mannopyranoside gel. As high as 4 mg of purified lectin could be bound on 1 ml 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 Sepharose-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-Affigel 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. Endogenous 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 for 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-III.