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An efficient method for the purification and quantification of a galactose-specific lectin from vegetative tissues of *Dolichos lablab*☆

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Abstract

The affinity purified galactose-specific seed lectin from *Dolichos lablab*, designated as DLL-II, is a tetrameric protein with an apparent native molecular mass of 120 kDa that is composed of two non-identical subunits of 31 and 29 kDa, respectively, associated non-covalently. The stems and leaves of the *D. lablab* plant also contain a galactose-specific lectin that cross-reacts with the seed lectin antiserum (antiserum raised against the 31 kDa subunit of DLL-II). Anti-lectin antibodies have been purified from this antiserum using a gel containing purified DLL-II lectin. Lectin specific antibodies have been used to develop simple and efficient immuno-affinity matrix, which allowed the purification of the lectin from stems and leaves of the *D. lablab*. The vegetative lectin (DLL-VL) exhibits similar electrophoretic properties as the seed lectin. Using these antibodies, an ELISA method was developed that allowed quantification of the lectin in the vegetative tissues (stems, leaves and roots) at concentrations of 0.5–50 ng. MS and database analysis of the tryptic peptides of the purified subunits of the DLL-VL suggested the purified protein to be a lectin.

Keywords: *Dolichos lablab*; Vegetative lectin; Affinity chromatography; ELISA; Galactose-specific

1. Introduction

Plant lectins are proteins/glycoproteins that cause agglutination of a variety of erythrocytes. They exhibit distinct sugar specificity and have been isolated and characterized from the seeds of different plants. Many of these hemagglutinins have been shown by hapten inhibition studies to react with specific saccharides on erythrocyte surface [1]. Lectins serve as valuable reagents in glycobiology research and can be employed for the detection and preliminary characterization of glycoconjugates on the surface of cells [2]. Among the different lectins studied so far, those isolated from the legume seeds are the most widely studied as these seeds have been found to be very rich in the lectin content [3]. Some of the biological roles proposed for plant lectins are carbohydrate transport, stimulation of cell division, cell wall extension, storage of seed protein reserves or the packaging or mobilization of these storage materials, specific attractants for rhizobial symbiosis and protection against plant pathogens [4]. Although plant lectins are primarily found in the protein bodies of seeds [5], there are reports on the identification, purification and characterization of lectins from vegetative tissues of some legume plants. The well characterized DB58 lectin purified from stems and leaves of *Dolichos biflorus* is a heterodimer composed of two closely related subunits, alpha and beta, which have been separated and purified by high-performance anion-exchange chromatography [6]. Bowles and Marcus [7] identified lectin receptors from seed extracts of soybeans and jack beans. Lectin like proteins has also been obtained from lentil and pea; these do not possess any hemagglutinating activity but are potent mitogens for lymphocytes [8]. A protein of soybean (Glycine max) leaves was shown to possess properties similar to the seed lectin whose N-terminal amino acid sequence shares 63% identity with the seed lectin. Immunoblot analysis indicated that the protein occurs in leaves, petioles, stems, and cotyledons of seedlings but not in seeds [9].

Seeds of *Dolichos lablab* grown in India contain two distinct sugar specific lectins: (i) DLL-I (glucose/mannose specific) and (ii) DLL-II (galactose-specific). In a recent study we have

Abbreviations: DLL-II, *Dolichos lablab* galactose-specific seed lectin; DLL-VL, *D. lablab* galactose-specific stem/leaf lectin; ELISA, enzyme-linked immunosorbant assay.

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affinity purified the galactose-specific lectin from the seeds of *D. lablab* and characterized the same [10] and also obtained crystals for this lectin [11]. The purified lectin is a glycoprotein, with an apparent molecular mass of 120 kDa and is possibly a tetramer, composed of non-covalently associated subunits of apparent molecular masses of 31 and 29 kDa, respectively. The lectin activity was inhibited by galactose sugar and its derivatives clearly suggesting the specificity of lectin towards galactose. Antibody raised to 31 kDa subunit, showed reactivity with the two subunits of DLL-II and also with the DLL-I seed lectin. Our long-term objective is to completely characterize this lectin and identify a precise function for this protein.

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

2. Experimental

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

2.1. Growing of plants and collection of plant material

D. lablab seeds (Wipro seeds, Hyderabad, Lot No. K.R. 306) were obtained from the local market. Seeds were processed as described earlier [10] and sowed in pots and maintained in a plant nursery for 3 weeks (University of Hyderabad). Stems, leaves and roots were collected from these plantlets, frozen in liquid nitrogen and stored at −80 °C until use.

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

All operations were carried out at 4 °C. The frozen stems and leaves of the plant were thawed on ice, and 100 g of the tissue was extracted with 1000 mL of 25 mM TBS pH 7.4 containing 2 mM PMSF. This extract was further centrifuged at 17,226 × *g* for 20 min and to the clear supernatant solid ammonium sulphate was added to 60% saturation. The suspension was centrifuged as above and the protein pellet was dissolved in 20 mM TBS pH 7.4 and dialyzed extensively against same buffer. This (stem and leaf extract) was used for the purification of the lectin. To obtain the total proteins from the roots, they were extracted following the protocol described for the stems and leaves. Protein concentration in these was determined according to Lowry et al. [12] using BSA as standard.

2.3. Agglutination assay

The stem, leaves and root extracts of the plant were analyzed for the lectin activity by serially diluting in 100 μL of saline in a plexiplate. About 100 μL of the trypsin treated erythrocytes were added separately in a plexiplate, incubated at 37 °C for 1 h and the hemagglutination was observed. One hemagglutination unit is defined as the minimum amount of protein required to cause visible agglutination.

2.4. Purification of the galactose-specific lectin from the seeds and stems/leaves of D. lablab

Sepharose-divinyl sulfone-galactose was prepared in our laboratory and the galactose-specific lectin was extracted from the seed meal and purified to homogeneity on this gel by affinity chromatography [10]. In the present study the lectin activity detected from the stems and leaf extracts obtained above could also be isolated on this affinity gel following the protocol described for the seed lectin.

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

The antibody to the 31 kDa subunit of the DLL-II was prepared and its specificity tested as described [10].

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

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

2.7. Western blot analysis

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

2.8. An immuno-affinity method for purification of the DLL-VL

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

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

Affinity-purified antibodies against 31 kDa subunit of DLL-II were adsorbed to micro-titer wells of an ELISA plate (96 wells) for 4 h at 37 °C (250 ng of affinity-purified IgG in 50 μL of 25 mM Tris–HCl, pH 7.4). The wells were washed with 200 μL of 25 mM Tris–HCl, 150 mM NaCl buffer pH 7.4 and incubated overnight at 4 °C/1 h at room temperature with 200 μL of buffer C (5% lipid free milk powder, 0.05% Triton X-100, 10 mM sodium phosphate, 150 mM NaCl pH 7.4). About 50 μL of solution (0.5–50 ng protein) containing purified DLL-II, extracts of root, stem/leaf and seed (0.5–50 ng protein concentration diluted in buffer C; quantitation of protein was done according to Lowry et al. [12]) was bound for 2.5 h at 37 °C. The wells were washed four times with 200 μL of buffer D (0.05% Triton X-100, 10 mM sodium phosphate, 37 °C. Subsequently, 50 μL of diluted rabbit antiserum against 31 kDa subunit of DLL-II (1:1000 dilution in buffer C) was added and the plate incubated for 1 h at 37 °C. After washing four times with buffer D, goat anti-rabbit IgG conjugated to alkaline phosphatase (Bangalore Genei) (dilution 1:6000 in buffer C) was added and incubated for 1 h at 37 °C. The wells were washed four times with buffer D and one time with 200 μL of buffer E (0.1 M Tris–HCl, 0.1 M NaCl, 2 mM $MgCl_2$, pH 9.5). The colour was developed with *p*-nitro phenyl phosphate (1 mg/mL in buffer E) for 10–20 min at room temperature and the absorbance was measured at 405 nm in a micro-plate ELISA reader. All experiments were carried out in triplicates and data presented represent the average values.

2.9.1. Mass spectrometric analysis

The protein was separated on a SDS-polyacrylamide gel and visualized by colloidal coomassie blue staining. The 31 and 29 kDa subunits were excised from the gel, destained with 50 mM ammonium bicarbonate in 50% acetonitrile, dehydrated with 100% acetonitrile and vacuum dried in a speedvac. Dried gel slices were in gel-digested separately with trypsin overnight at 37 °C according to manufacturer's (Sigma) description. Peptides were extracted twice with 50% acetonitrile containing 0.1% TFA for 15 min each. The digests were pooled, vacuum dried in a speedvac and redissolved in water containing 0.1% trifluroacetic acid (TFA) for desalting using C18 zip-tips (Millipore, Bedford, MA, USA). A 1/10 fraction of the peptides dissolved in 0.1% TFA and mixed with equal volumes of the MALDI matrix, a-cyano-4-hydroxycinnamic acid (CHCA), was analyzed with positive-ion matrix-assisted laser desorption/ionisation-mass spectrometry on a 4700 MALDI-TOF/TOF Analyzer (Applied Biosystems, Foster City, CA, USA). The spectra were recorded in reflector mode in a mass range from 800 to 3900 Da with a focus mass of 1500 Da. Peptide mass calibration was performed with external mass standards (Calmix 1 and 2; Applied Biosystems, an internal calibration was automatically performed using at least one peak for one- or both peaks for a two-point-calibration). For less then 1% samples, where automatic calibration failed, calibration was performed manually (calibration error in PPM is 10).

Additionally MALDI-MS/MS analyses were performed for the five strongest peaks of the TOF-spectrum after subtraction of peaks corresponding to background, keratin and trypsin fragments (calibration for MS/MS was done using 1570 Da peptide from Calmix 1 Applied Biosystems). After calibration a combined database search of MS and MS/MS measurements was performed using the GPS Explorer software (Applied Biosystems, Foster City, CA, USA) with the following settings: (i) MS peak filtering: mass range from 800 to 3900 Da; minimum S/N filter of 25; peak density of 50 peaks per range of 200 Da and maximal 200 peaks per protein spot; mass exclusion list contained background peaks and trypsin fragments with an exclusion tolerance of 0.2 Da; (ii) MS/MS peak filtering: mass range from 50 Da to a mass that was 20 Da lower than the precursor mass; peak density of 50 peaks per 200 Da and maximal 65 peaks per MS/MS; minimum S/N filter of 10; (iii) database search: precursor tolerance 50 ppm and MS/MS fragment tolerance 0.25 Da. Peak lists were compared with NCBInr database using the Mascot search engine (Matrix Science Ltd., London, UK). Peptide mixtures that yielded a mowse scoring algorithm of at least 49 (p-value = 0.05) were regarded as positive identifications.

Table 1
Purification of the DLL-VL lectin from the vegetative tissues of *Dolichos lablab*

S. no.	Step	Total protein (mg)	Total activity (HU)[a]	Specific activity (Units/mg)	Purification fold
1	Crude	2000	3600	1.8	1
2	0–60% $(NH_4)_2SO_4$ fraction	420	1680	4	2.2
3	Sepharose-mannose gel unbound	190	1250	6.6	3.6
4	Sepharose-galactose gel eluate	15	810	54	30

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

3. Results and discussion

Legume lectins are the most widely studied proteins. More often, they are identified and detected by their specific property of agglutinating erythrocytes. The galactose-specific lectin from the seeds of *D. lablab* has recently been extensively characterized by us [10]. In the present study an attempt was made to first identify the lectin activity from the vegetative tissues of the *D. lablab* plant. Therefore from the stems, leaves and the roots of the plant, total proteins were extracted and they were analyzed for the agglutinating activity using trypsin treated rabbit erythrocytes. The agglutinating activity from the stem and leaf extracts was inhibited by galactose among other sugars tested, and therefore the lectin was purified on a Sepharose-galactose gel (Table 1). A detailed study was undertaken to characterize the purified stem and leaf lectin and to examine its relatedness to the seed lectin. Furthermore, the antibodies to the seed lectin provided a useful tool to develop methods that allowed purification of the stem and leaf lectin and to quantify by ELISA the stem, leaf lectin as well as the root lectin.

The seeds of the Indian lablab have been shown to contain two distinct sugar specific lectins designated as the DLL-I (glucose/mannose specific), and DLL-II (galactose-specific). Both lectins share only a partial sequence homology. DLL-II is a glycoprotein with a native molecular mass of 120 kDa, consisting of two types of subunits, 31 and 29 kDa, respectively, that are non-covalently associated [10]. In the present study from the extracts of stems and leaves of the plant the galactose-specific lectin was affinity purified on a Sepharose-galactose gel employing the same conditions as described for the seed lectin (25 mM TBS pH 7.4, containing 1.5 M ammonium sulphate). The ammonium sulphate precipitated proteins from the stems and leaves after dialysis was first passed through the Sepharose-mannose gel in order to deplete the extracts of any mannose binding proteins. To the unbound fraction solid ammonium sulphate was added to 1.5 M concentration and was then applied on Sepharose-galactose gel equilibrated with column buffer and the bound protein was eluted from the gel using 0.3 M galactose in column buffer (Fig. 1). From Table 1, it is apparent that from 100 g of the stems and leaves, about 15 mg of the purified lectin could be obtained.

In SDS-PAGE analysis the vegetative lectin migrated as the seed lectin displaying the two subunit pattern corresponding to molecular masses 31 and 29 kDa respectively (Fig. 2A). In order to understand if the seed lectin and the vegetative lectin are immunologically related, the purified seed and vegetative lectin were separated on a 10% SDS-PAGE, and the proteins transferred to a nitrocellulose membrane. When this was processed for Western blot analysis with the antiserum to the 31 kDa subunit of the seed lectin, both subunits in the seed lectin and the vegetative lectin were reactive (Fig. 2B), suggesting the immunological relatedness among these two lectins. This antiserum has already been shown by us to react with both the 31 and 29 kDa subunits of the seed lectin. It is therefore interesting to note that in the vegetative lectin also both the subunits which exhibit similar molecular masses as the seed lectin show reactivity suggesting that it is related to the seed lectin. Among a number of sugars tested for inhibition of agglutination of the purified DLL-VL (*D. lablab* vegetative lectin) galactose and its derivatives like *N*-acetyl galactosamine and methyl-β-Gal were found to inhibit the lectin activity. However, sugars such as glucose, mannose, their methyl glycosidases, and *N*-acetylglucosamine were non-inhibitory up to 100 mM concentration. Sugar inhibition data presented in Table 2 shows that 2-deoxygalactose is 16-fold weaker compared to galactose in inhibiting the activity, indicating that the equatorial hydroxyl group on C-2 of galactose is an important locus for carbohydrate binding of the lectin. The inhibitory potency of methyl-β-Gal is eight times greater when compared to the α-anomer, indicating that the equatorial position at the anomeric position is better recognized. Substitution at the C-2 with an amino group does not have any significant inhibitory effect as compared to galactose. These results confirm that the activity of the DLL-VL lectin is

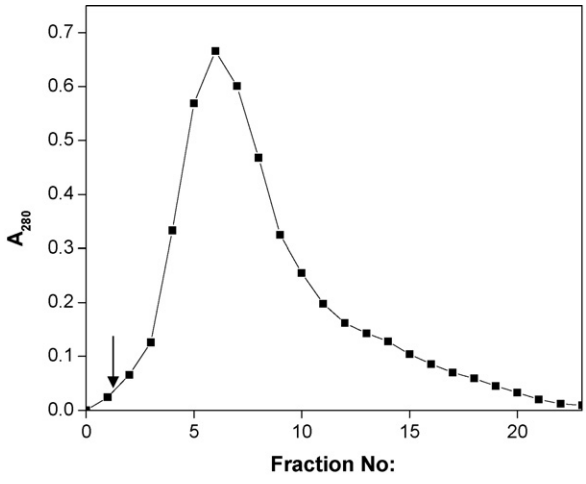

Fig. 1. Affinity purification of the DLL-VL on Sepharose-galactose gel. The 0–60% $(NH_4)_2SO_4$ fraction corresponding to the total proteins from the vegetative tissues was processed as described in Section 2. After washing the gel, the lectin bound on the galactose gel was eluted using 0.3 M galactose. Arrow indicates point of application of the sugar.

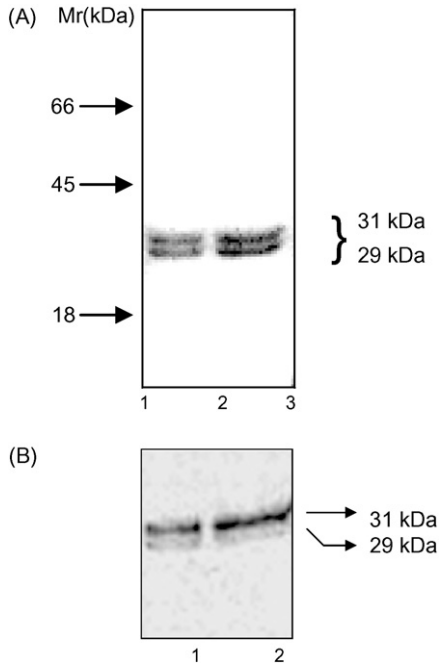

Fig. 2. The 10% SDS-PAGE analysis of DLL-VL lectin. (A) Lane 1: molecular weight markers—bovine serum albumin (Mr 66 kDa), ovalbumin (Mr 45 kDa) and β-lactoglobulin (Mr 18 kDa); lane 2: DLL-VL; lane 3: DLL-II. Arrow (→) indicates the position of larger subunit of molecular mass 31 kDa and the smaller subunit of molecular mass 29 kDa of the galactose-specific lectins from *Dolichos lablab*. (B) Western blot analysis of the DLL-VL lectin. After transfer of proteins to the nitrocellulose membrane, it was probed with the DLL-II, 31 kDa subunit antibody at a dilution of 1:1000. Goat anti-rabbit IgG-ALP conjugate (1:1000) was used as the secondary antibody. The blot was developed with BCIP/NBT substrate. Lane 1 shows the purified DLL-VL lectin and lane 2 shows DLL-II lectin, recognized by the antiserum.

best inhibited by galactose. In addition, a hydrophobic moiety attached at the anomeric position as pNP-β-galactose was found to be a better inhibitory sugar than methyl-α-Gal. These results are comparable to the other galactose-specific lectins, including the seed lectin, DLL-II purified from *D. lablab* [10].

In the present study we have also explored the possibility of purifying the DLL-VL by a single step from the vegetative tissue extracts employing immuno-affinity chromatography. To make the immuno-affinity gel, the 31 kDa specific IgG was obtained

Table 2
Inhibition of agglutinating activity of DLL-VL lectin by various saccharides

Sugar	Minimum concentration for inhibition (mM)	Relative inhibitory potency (galactose = 1.0)
Galactose	2.82	1.0
Lactose	11.3	0.25
Me(Gal	1.40	2.0
Me(Gal	11.3	0.25
Galactosamine	22.5	0.12
2-DeoxyGal	45.0	0.06
pNPβGal	1.40	2.0
pNPαGal	11.3	0.25

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

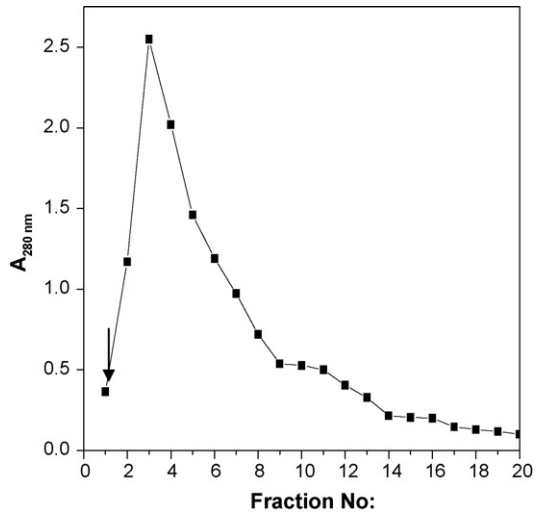

Fig. 3. Lectin affinity chromatography. Anti-lectin antibodies were purified on DLL-II lectin-affigel equilibrated with 0.1 M HEPES buffer. Glycine-HCl pH 2.65 was used to elute specific anti-lectin antibodies. About 1 mL fractions were collected and absorbance at 280 nm was measured.

as follows. First, lectin-affigel containing 20 mg purified seed lectin was prepared as described in Section 2. The antiserum to the 31 kDa subunit of the DLL-II was passed through this gel to get the specific IgG, which eluted as a single peak (Fig. 3). The protein containing fractions were concentrated and the specific IgG was coupled to 3 mL of affigel at a concentration of 5 mg/mL.

Fig. 4 shows the chromatogram for the purification of the lectin using immuno-affinity gel prepared as described in Section 2 from different vegetative extracts, the lectin activity was isolated by passing the vegetative extracts separately on the immuno-affinity gel. After washing extensively, the bound protein was eluted using 100 mM glycine-HCl buffer pH 2.65, and the eluates were immediately neutralized. From 100 g of the

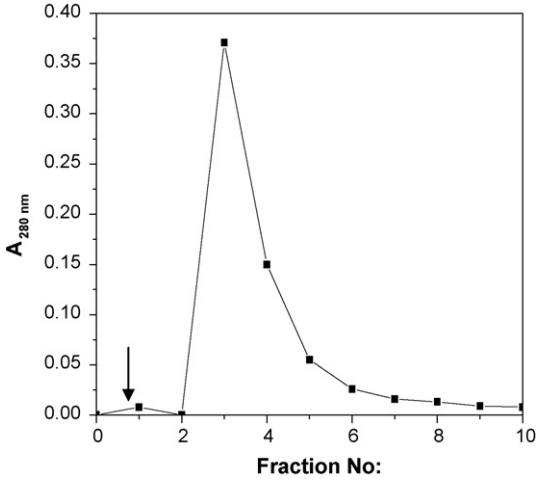

Fig. 4. Immuno-affinity method for purification of DLL-VL lectin. DLL-II anti-lectin antibodies were coupled to Affigel-10 in presence of 0.1 M HEPES buffer and DLL-VL lectin was purified from this immuno-affinity gel, where the protein eluted as a single peak with 0.1 M glycine-HCl pH 2.65, suggesting it to be homogenous.

stems and leaves we were able to obtain 15 mg of the purified lectin in a single step (data not shown). In literature, legume lectins are largely purified employing affinity matrices containing the ligand which inhibits the agglutinating activity of the lectin. We have earlier purified the DLL-II lectin using Sepharose-galactose gel (affinity gel). The same matrix was also found useful to purify the DLL-VL. Furthermore, since our initial studies with the antibodies allowed us to develop an ELISA for the DLL-VL lectin, we explored the use of these antibodies to immunopurify the DLL-VL. The immuno-affinity gel that was developed, also proved to be a good affinity support to isolate the DLL-VL. From our results it is apparent that the yields of the DLL-VL using the affinity gel and the immuno-affinity gel are similar and therefore the immuno-affinity gel is yet another method to isolate the lectin. This matrix in future could also be explored for the isolation and purification of the root lectin. A Western blot experiment proved the immunological relatedness of the lectin in various vegetative extracts. The lectin purified from different vegetative extracts on a immuno-affinity gel was probed with the lectin specific IgG which was able to detect the two subunits from all the extracts (Fig. 5).

Reports on the other vegetative lectins where the lectin was purified by affinity and other modes for the purification and characterized has been cited in the literature. Although lectin activity has been detected in wheat leaves [13], soybean leaves [14] phloem exudates [15], peanut leaves [16], flowers of *Datura stramonium* [17] and in sieve-tube sap of various trees [18] only few of these lectins have been thoroughly characterized. Suzuki et al. [19] isolated two lectins from the leaves of *Aloe arborescens*. These lectins (P-2 and S-1) had a molecular mass of 18,000 and 24,000, respectively. Each lectin was believed to be a dimer. A more extensive characterization of the lectin from *D. biflorus* (DB) leaves and stems (CRM) was performed by Talbot and Etzler [20]. DB stems and leaves contained a glycoprotein which cross-reacted with antibodies raised against the seed lectin.

D. lablab seed lectin that has been characterized and reported to cross-react with the DLL-VL laid a scope for the

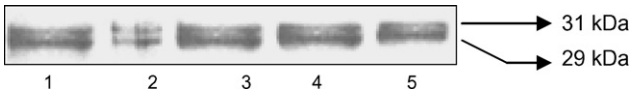

Fig. 5. Western blot analysis of the vegetative extracts of *D. lablab*. Proteins on SDS-PAGE were transferred to the nitrocellulose membrane; blot was probed with the DLL-II anti-lectin antibody at a dilution of 1:1000. Goat anti-rabbit IgG-ALP conjugate (1:1000) was used as the secondary antibody. The blot was developed with BCIP/NBT substrate. Lane 1 shows 0–60% root extract; lane 2, 0–60% stem/leaf extract; lane 3, 60–80% seed extract; lane 4, affinity purified DLL-II; lane 5, affinity purified DLL-VL that is recognized by specific lectin-specific antibody.

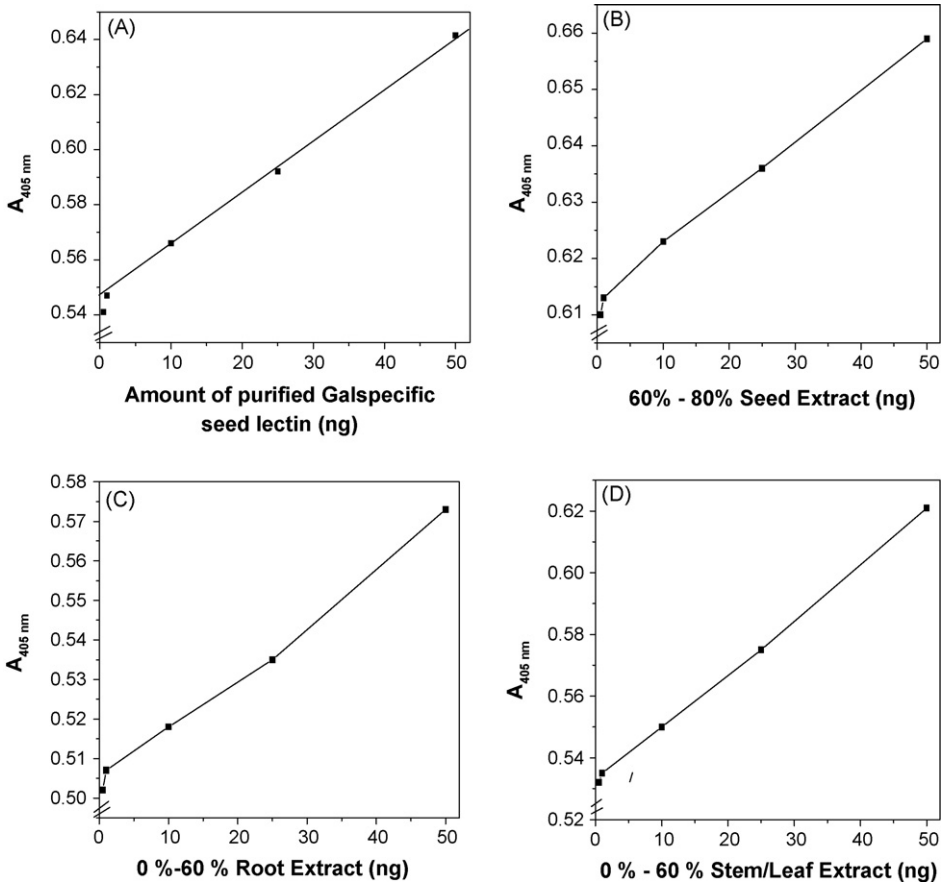

Fig. 6. Quantification of the galactose-specific lectin in vegetative extracts by ELISA. ELISA to detect the galactose-specific lectin in the vegetative extracts of *D. lablab*. (A) represents the affinity purified DLL-II; (B), (C) and (D) represent the extracts of seeds, roots and stems/leaves, respectively. Assay was performed as described in Section 2. An aggregate of three experiments is shown here.

characterization of the vegetative lectin. Furthermore, in literature there are examples where the lectins from the same plant did not show immunological cross-reactivity. The antibodies to the *Robinia* bark lectin did not cross-react with proteins from *Robinia* seeds and leaves. On the other hand, the antibody for the *Robinia* bark lectin cross-reacted with polypeptides from the extracts of the seeds and bark of *Sophora japonica* suggesting immunological relatedness of the lectins from two different plants [21]. Hemagglutinating activity was identified in the roots and vegetative tissues of Bengal gram using rabbit erythrocytes. The activity in the roots appears to be similar to that of the seeds in respect to their sugar inhibition property [22].

Since the DLL-VL could be purified by affinity, immunoaffinity chromatography, and the initial ELISA experiments were promising, we wanted to develop a sensitive assay method that could allow us to quantify the lectin at nanogram level from the vegetative tissues of the plant. Therefore, in the present study, an ELISA method was developed to quantify the *D. lablab* vegetative lectins (stems, leaves and roots).

Very few reports are there on the development of ELISA for quantification of the vegetative lectins. The details of the results are presented in Fig. 6. Fig. 6A shows the standard curve where different concentrations of the purified DLL-II were taken. When the 60–80% ammonium sulphate fraction containing the DLL-II was used in an ELISA experiment, the lectin could be detectable in the concentration range of 10–50 ng (Fig. 6B). Similar results were obtained for the stem/leaf lectin and the root lectin (Fig. 6C and D). The method allowed detection of the lectin at concentrations as low as 0.5 ng with linearity until 50 ng concentration. This methodology should prove to be useful to further trace the function of the lectin in the respective vegetative tissues, in particular to identify with what other proteins this lectin is interacting at the site of localization in the plant.

The DLL-VL lectin as described above is made of two subunits which are recognized by the DLL-II antibody described [10] suggesting possible sequence homology between the two subunits of the DLL-VL. This prompted us to understand more

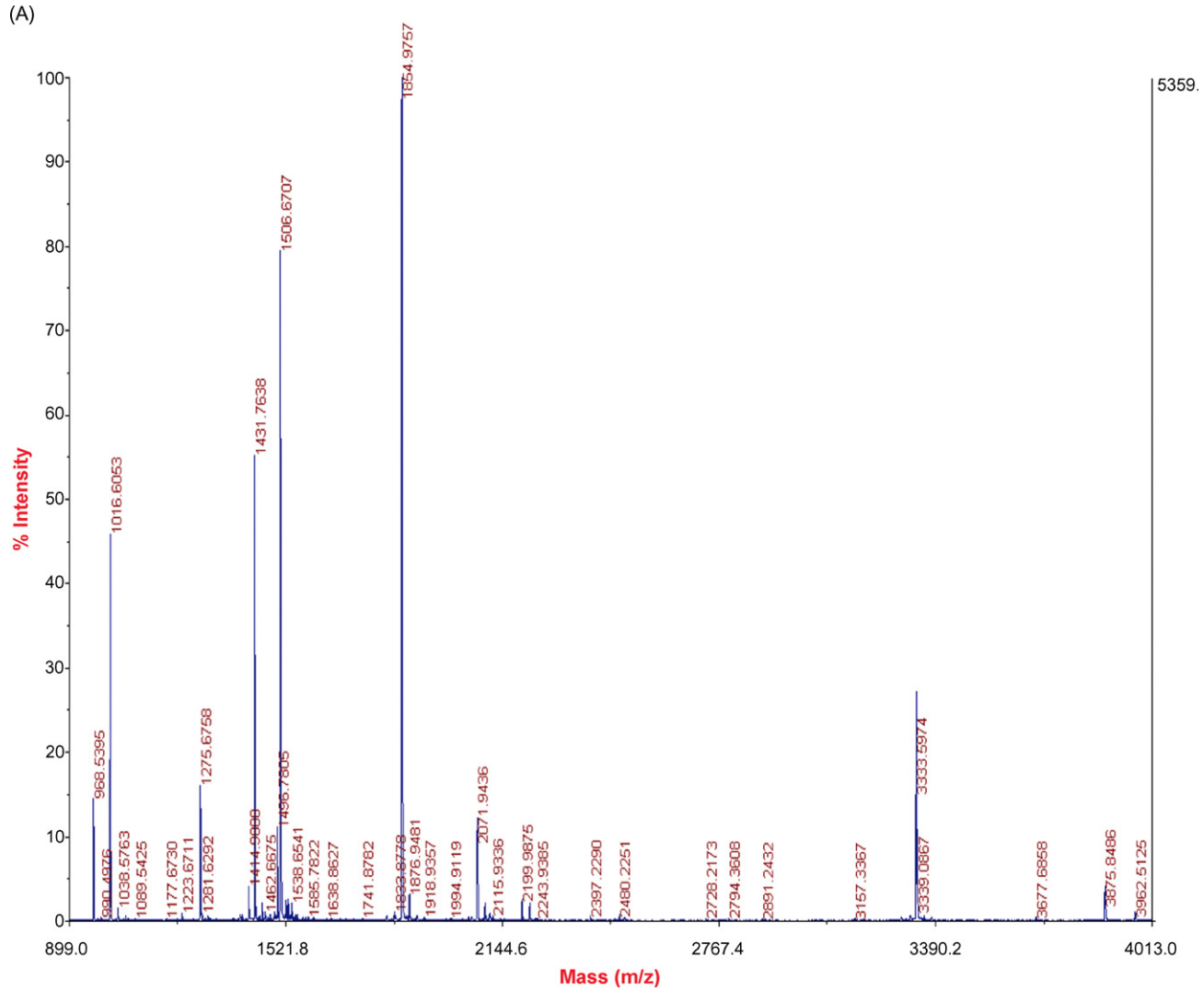

Fig. 7. Mass spectrometry analysis of the DLL-VL. MALDI-MS analysis was performed with proteolytic digests of the DLL-VL galactose-specific lectin, generated by digestion of the subunits overnight with the protease trypsin. (A) and (B) display exemplarily peptide mass fingerprints generated with trypsin: (A) 31 kDa subunit (B) 29 kDa subunit.

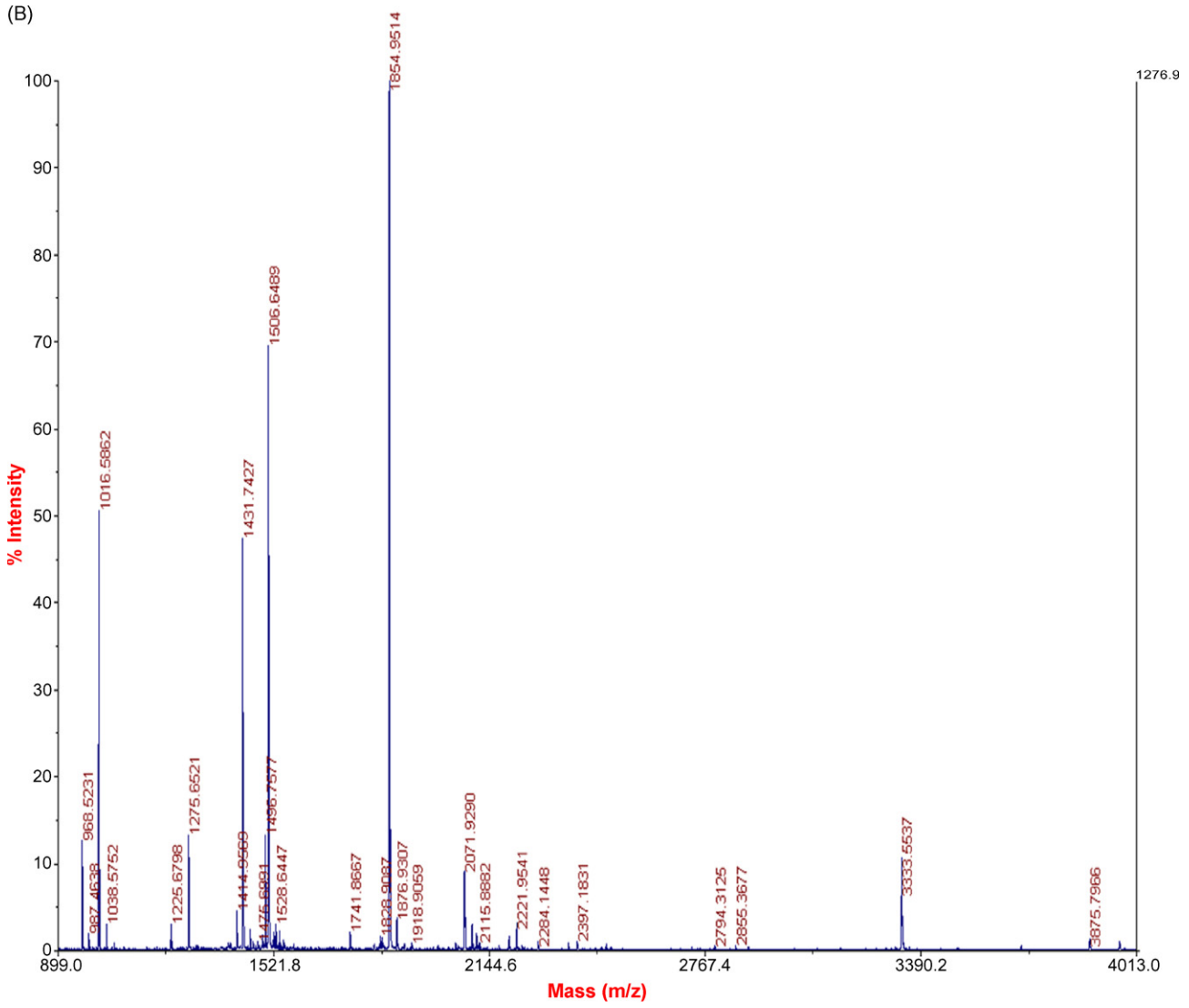

Fig. 7. (*Continued*).

about the similarities between the two subunits. Mass spectrometry has become a major tool for identification of the novel proteins, whose sequences have not been determined to study the function of these proteins. After separation of the protein by SDS-PAGE the subunits have been digested with trypsin and a fraction of the peptides obtained by enzymatic digestion of the lectin subunits were analyzed by MALDI-TOF-MS to obtain peptide mass fingerprints that are shown in Fig. 7A and B. Experimentally obtained masses were compared to peptide mass sets obtained from the theoretical digests of lectin proteins in databases. Using this approach, however, only a very small fraction of the peptides could be assigned which was due to the fact that the sequence was novel and thus only sequences absolutely conserved between lectins from different legume species could be assigned (data not shown). In this study the MASCOT and MS-BLAST 2 programs were used for the analysis of the MS data allowing us to utilize the complete set of peptide sequences of unknown order as determined by MS/MS from one protein in the same query. The MS spectra of both subunits showed no differences in the peak distribution suggesting high similarity of both subunits and additionally, we analyzed manually all differences between corresponding spectra to find any differences in the sequence using Data explorer software. The lectin sequence achieved from the MS data is limited and database search could achieve the conserved regions of the lectin on comparison with other legume lectins. Primarily after observing the spectra's of the digests and comparing with the subunits it was clear to understand that the both subunits share maximum amount of identity in peak distribution, so we assume maximum sequence homology among them. This further is in good agreement with the complete primary sequence we obtained recently for the two subunits of the DLL seed lectin that showed extensive sequence homologies among the two subunits (unpublished data).

4. Conclusions

In summary, we present here a detailed investigation on the isolation and affinity purification of the vegetative lectin from

the DLL plant that agglutinates rabbit erythrocytes and compare its properties with the purified seed lectin. An affinity method was developed earlier to purify the galactose-specific seed lectin which was also useful to purify the *D. lablab* vegetative lectin (DLL-VL) from the stems and leaves of the plant. The fact that only 15 mg of the purified lectin was obtained from 100 g starting material, suggests that the level of the DLL-VL is far less compared to the seed lectin [10].

Seed and vegetative lectin exhibited similar molecular masses and subunit pattern. Furthermore, the seed lectin antibody coupled to affigel, has been found to be an efficient tool, to purify the lectin from the vegetative extracts and seeds in a single step with a protein yield that compares well to the affinity method (Sepharose-galactose gel). The DLL-VL and the root lectin show immunological cross-reactivity with DLL-II antibody. An ELISA method was developed that allowed quantification of the lectin present in different vegetative tissues (stems, leaves and roots) of the plant at very low concentrations (0.5–50 ng). MS/MS analysis and database search of the tryptic peptide maps generated from the subunits of the lectin suggests that the protein is a lectin with sequence homology in the conserved regions when compared with other legume lectins. We have already sequenced the DLL-II lectin using a proteomics approach that suggests extensive homologies between the two subunits (unpublished information). Future studies involving the complete sequence of the stem and leaf lectin should further throw light on the structural similarities between the DLL-II and DLL-VL which is the future direction of our work.

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