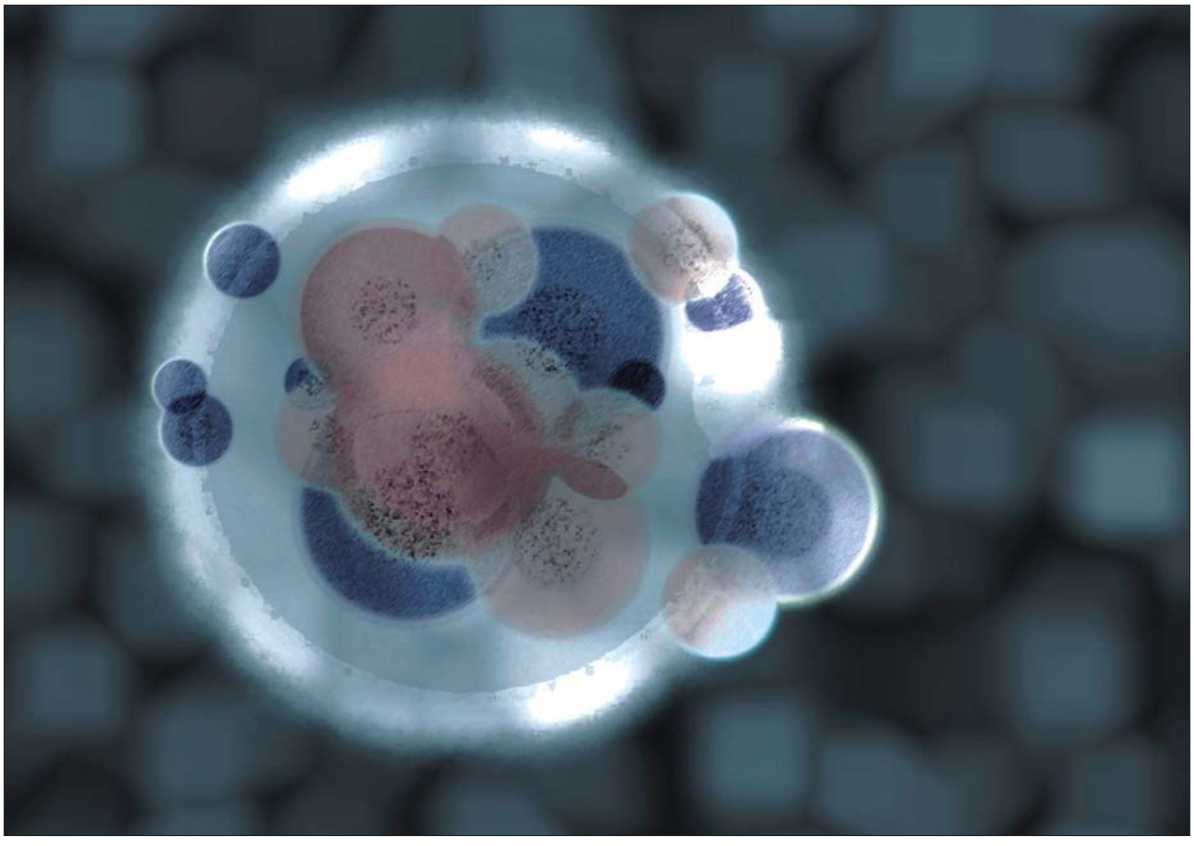

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Isolation of a New Lectin from the Bulbs of *Crinum latifolium* (L.)

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Abstract: The present study was a prerequisite for the evaluation of the biological properties of a new lectin, which was isolated from the bulbs of *Crinum latifolium* with carbohydrate specificity towards methyl-α-D-mannopyranoside. The lectin was purified by anion-exchange chromatography on DEAE-Sephacyl and gel filtration chromatography on Biogel P-200. The purified lectin showed a molecular mass of 24 kDa on gel filtration and a single peptide band with a molecular mass of 12 kDa on SDS-PAGE indicating it to be a dimeric protein composed of two identical subunits. The lectin agglutinated Red Blood Cells (RBCs) from rabbit and guinea pig, was non-glycosylated, labile above 60°C and did not require divalent ions for its activity. The heamagglutination activity of *Crinum latifolium* lectin was affected by denaturing agents like urea, thiourea and guanidine HCl. The lectin was virtually non-mitogenic like the other members of Amaryllidaeae.

Key words: Lectin, monocot, mannose, Amaryllidaceae

INTRODUCTION

Lectins are a heterogeneous group of proteins of non-immune origin that bind reversibly to mono- or oligo-saccharides but are devoid of any catalytic activity[1]. It is their unique ability to recognize and bind to specific carbohydrates that makes lectins invaluable tools in basic as well as applied biology. At present, monocot mannose binding lectins are being extensively studied because of their interesting biological properties. Popularly known as Monocot Mannose-Binding Lectins (MBLs), they are unique because of their exclusive specificity towards poly- and/or oligomannose. However, within this group there are distinct preferences for terminal and internal linkages and for different types of glycosidic linkages such as α (1-2), (1-3), (1-4) and (1-6)[2]. Mannose is an important cell surface sugar present in glycoconjugates that are implicated in a wide variety of important receptor mediated processes. The MBLs have been found to bind to mannosylated region of envelope glycoproteins of various reteroviruses such as HIV, SIV and FIV[3,4]. Some MBLs from the family Amaryllidaceae have been reported to possess anti-insect activity making these lectins as important component in crop management[5].

Keeping in view the importance of this class of lectins, much research has been focused on the search for the possible new members to evaluate their biochemical, physiological functions and biological activities. The present study deals with the purification, biophysical and biological characterization of a new lectin from the bulbs of *Crinum latifolium*, a member of Amaryllidaceae.

MATERIALS AND METHODS

Materials: Underground bulbs of *Crinum latifolium* were collected from the foothills of Jammu and Kashmir, India during the months of September-October, 2003. The plants were got identified from the herbarium of Regional Research Laboratory, Jammu. The carbohydrates, standard molecular weight markers for SDS-PAGE, Bovine serum albumin, Freunds complete adjuvant and agar were procured from Sigma, USA. Ampholine of pH range 3.0-10.5 and DEAE-Sephacyl were procured from Amersham Pharmacia, Sweden. Microtitre-plates were from Nunc, Denmark. Other reagents used were of reagent grade or higher.

Lectin isolation and purification: Bulbs of *C. latifolium* were washed, cut into small pieces, grounded and soaked overnight in 0.01M Phosphate Buffered Saline (PBS), pH 7.2 (1:5 w/v) at 4°C. The ground mixture was filtered through several layers of surgical gauze. The filtrate was centrifuged at 20,000×g for 20 min at 4°C. The supernatant was subjected to purification by DEAE-Sephacyl anion-exchange column (0.8×10 cm) equilibrated with Tris buffer, pH 8.3. Crude sample was dialyzed against Tris

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buffer, pH 8.3. The unbound proteins were washed off with Tris buffer, pH 8.3 and the proteins bound to the column were eluted with step-wise NaCl gradient (0.1-2 M). The lectin eluted at 0.5 M NaCl gradient was concentrated by 20% polyethylene glycol (PEG). The concentrated lectin was loaded onto Biogel P-200 column for further purification. The active fractions were stored at -20°C (Table 1).

Hemagglutination and hapten inhibition assays: Two fold serially double diluted lectin was incubated in the microtitre plate at 37°C for 30 min with an equal volume of 2% RBCs suspension (3.5×10^8 cells mL^{-1}) from 8 sources viz., human A, B and O blood groups, rabbit, guinea pig, sheep and goat. The reciprocal of the highest dilution of the lectin showing detectable agglutination was taken as titre of the lectin. Thirty-eight carbohydrates and 4-glycoproteins including their derivatives namely, Pentoses: D (-) arabinose, L (+) arabinose, D (-) ribose, Hexoses: β-D (-) fructose, D (+) galactose, D (+) glucose, D (+) mannose, L (-) sorbose, Deoxy sugars: α-L (-) fucose, α-L-rhamnose, Amino sugars: *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, *N*-acetyl-β-D-mannosamine, β-phenyl-D-glucosamine, Glycosides: α-methyl–D-glucoside, α-methyl–D-mannopyranoside, β-methyl-D- glucoside, I-O-methyl-α-D-galactoside, I-O-methyl-β-D-galactoside, β-phenyl-D-glucoside, Sugar alcohols: Adonitol, Myo-inositol, Disaccharides: β-gentiobiose, β-D (+) lactose, α-maltose, α-D (+) melibiose, D-trehalose, α-D-man (1,2)-D-man, α-D-man (1,3)-D-man, α-D-man (1,6)-D-man, *N*-acetyl-D-lactosamine, Sialic acid: *N*-acetyl neuraminic acid, Trisaccharides: melezitose, raffinose, Polysaccharides: chitin, inulin, yeast mannan, Chitin oligomers: N, N', N''-triacetyl chitotriose, Glycoproteins: asialofetuin, fetuin, mucin, thyroglobulin were tested in the agglutination inhibition assay. Each sugar was serially double diluted and mixed with the same volume (30 μL) of the lectin at twice the lowest concentration causing agglutination of rabbit erythrocytes. After incubation at 37°C for one hour, twice the volume of 2% erythrocyte suspension (60 μL) was added and examined after 30 min. The inhibitory activity was taken as the concentration of the hapten required for complete inhibition of hemagglutination.

Biochemical and biophysical characterization, protein and total neutral sugar estimation: Protein content of the lectin sample was quantified by the method of Lowry *et al.*[6] using BSA as standard. The carbohydrate content of the purified lectins was determined by Anthrone method with D-glucose as standard[7].

Table 1: Purification data for *Crinum latifolium*

Step	Total protein (mg)	Total activity (HU)	Specific activity (HU mg^{-1})	Purification Fold	Recovery (%)	MEAPC (μg mL^{-1})
Crude	300.0	800	2.67	1	100	375.0
Ion exchange chromatography						
Buffer profile	70.0	0	0	0	0	0
NaCl gradient						
0.10 M	10.66	0	0	0	0	0
0.25 M	29.98	0	0	0	0	0
0.50 M	83.97	384	4.57	1.17	48	13.12
0.75 M	35.09	0	0	0	0	0
1.00 M	26.31	0	0	0	0	0
1.50 M	16.66	0	0	0	0	0
2.00 M	7.33	0	0	0	0	0
Gel filtration	16.64	240	14.42	5.40	30	5.73

Data is for 50 g of fresh bulbs; HU= Hemagglutination Unit.
MEAPC= Minimal Erythrocyte Agglutinating Protein Concentration

Stability towards temperature, pH and denaturants: The stability of the purified lectin towards temperature was determined by incubating 1 mg of lectin at temperature ranging from 40-100°C for 15 min with 5°C increase at each step. The hemagglutination activity was determined after every temperature rise to check the effect of temperature on the lectin.

The stability towards pH was determined by dialyzing the purified lectin against buffers in the range of pH 1.0-13. The lectin was later centrifuged and the pH was adjusted to 7.0 with 0.1 N HCl or 0.1 N NaOH before testing the hemagglutination activity. Lectin was also treated with the denaturing agents like urea, thiourea and guanidine-HCl at a concentration ranging from 0.5-8.0 M. The effect of denaturants on the purified lectin was determined by comparing the agglutination activity of the treated with those of the untreated lectin.

Metal ion requirement: Purified lectin at a concentration of 1 mg mL^{-1} was dialyzed against 0.1 M Ethylenediamine Tetraacetic Acid (EDTA) for 72 h[8]. One part of the treated lectin was dialyzed against 0.1 M PBS, pH 7.2 while the other part was dialyzed against 0.1 M $CaCl_2$ and $MnCl_2$ for 48 h. Hemagglutination titre of the treated and untreated sample was compared to find out the role of the metal ions.

Electrophoretic analysis and M_r determination: Electrophoresis of the purified lectin was carried out using PAGE at pH 8.3 with 10 % (w/v) gel and PAGE at pH 4.5 using 7.5% (w/v) gel. The subunit molecular mass of the lectin was determined with SDS-PAGE, pH 8.3 using standard molecular weight markers such as phosphorylase (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20 kDa) and α-lactalbumin (14.4 kDa), both in the presence and absence of 2-mercaptoetanol using 10%

(w/v) slab gel. The native molecular mass was determined by gel filtration chromatography on Biogel P-200 column with 0.01 M, PBS pH 7.2. Isoelectric Focusing (IEF) was carried out in 5% polyacrylamide tube gels to determine the microheterogeneity of the lectin.

Biological characterization

Production of antiserum and Double Immunodiffusion (DID): Polyclonal antiserum against purified *C. latifolium* was raised by injecting rabbit with 1 mg of the lectin emulsified in Fruends complete adjuvant. Blood was collected by cardiac puncture 10 days after 3 booster doses each given at a week interval. Serum was separated by centrifugation at 10,000×g for 10 min at 4°C. Antisera raised against *C. latifolium* was laoded in the central well (A) while purified lectins *Crinum latifolium, Zephyranthes candida* and *Gloriosa superba* were added in wells 1, 2 and 3, respectively of petri dishes containing 1.25% noble agar and incubated for 48 h at 37°C in humidified chamber. The precipitin bands were stained with commassie blue.

Mitogenic assays: Mitogenic potential of the lectin was studied by MTT assay using human peripheral blood mononuclear cell suspension prepared in RPMI-1640 medium supplemented with 10% Fetal Calf Serum (FCS), L-glutamine, penicillin and streptomycin. Lymphocytes at a concentration of 1×10^6 cells mL^{-1} were incubated in various concentrations of the test lectins ranging from 0.1-10 μg mL^{-1} for 72 h at 37°C in an atmosphere of 5% CO_2 in air. The MTT assay was also performed by the method of Mosmann[9]. Four hours, before the termination of the reaction, 25 μL of 3,4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide (MTT) at a concentration of 2 mg mL^{-1} was added to each well. After 4 h 160 μL of acidified isopropanol was added to each well. The formation of blue color formazan was read at 570 nm using Labsystems Multiskan EX ELISA reader. Mitogenic lectin *Concanavalin* A was used as standard.

RESULTS AND DISCUSSION

The lectin under study was isolated from the bulbs of *Crinum latifolium*. With respect to its location in the plant tissue, *C. latifolium* resembled other MBLs, from family Amaryllidaceae, which have generally been isolated from the bulbs[10]. Interestingly, the lectin was not found to be specific towards mannose, however, it was inhibited by methyl α-D-mannopyranoside at a concentration of 10 mM in the hapten inhibition assay using 38 sugars and 4 glycoproteins. This is in conformity with the earlier findings that most of the mannose binding lectins from the family Amaryllidaceae demonstrate a complex sugar specificity in discriminating man-man linkages[11,12]. This property of the lectin to distinguish man-man linkages may be utilized as a tool to discriminate some glycoproteins in health and disease[4].

Table 2: Biological action spectra of *Crinum latifolium*

Types of RBC/ Lymphocytes	MEAPC/MLAPC (μg mL^{-1})	
	Untreated	Neuraminidase treated
Rabbit	5.73	1.43
Sheep	-	-
Goat	-	-
Guinea pig	133.50	4.17
Human A	-	-
Human B	-	-
Human AB	-	-
Human O	-	-
Human Lymphocytes	-	-

MEAPC = Minimal Erythrocyte Agglutinating Protein Concentration.
MLAPC = Minimal Lymphocyte Agglutinating Protein Concentration

The lectin was purified by anion-exchange chromatography followed by gel filtration on Biogel P-200. The crude extract with a specific activity of 2.67 was loaded on the anion-exchange column (Table 2). The complete absence of lectin activity in tris buffer, pH 8.3 indicated full adsorption of the lectin to the column. The bound lectin was eluted as multiple protein peaks using 0-2 M step-wise NaCl gradient, with only peak I showing lectin activity (Fig. 1). The other peaks were devoid of any lectin activity when tested against rabbit erythrocytes. In SDS-PAGE at pH 8.3, lectin positive ion-exchange fraction gave three bands of different intensities (Fig. 3) indicating co-elution of some non-lectin proteins. Therefore, ion-exchange fractions were pooled and loaded on Biogel P-200 column to get rid off unwanted proteins. Of the multiple peaks, only peak II (Fig. 2) showed the lectin activity with purification fold of 5.40. SDS-PAGE of the fractions with lectin activity gave single band with a subunit molecular mass of 12 kDa under reducing as well as non-reducing conditions (Fig. 3a) indicating absence of disulphide linkages. The low value of purification fold probably indicated the higher lectin content in the total extractable proteins present in the storage tissues. Similar findings have earlier been reported for other members of MBLs from Amaryllidaceae[10] where lectins constitute a major portion of the total extractable protein. Gel filtration with purified lectin gave a molecular mass of 24 kDa indicating it to be a dimer like most of the other members of Amaryllidaceae[10]. The lectin was acidic in nature as revealed by single band in PAGE, pH 4.5 (Fig. 3b). However, the appearance of multiple bands with different intensities in PAGE, pH 8.3 (Fig. 3c) suggested a probable variation in the amino acid composition thereby resulting the lectin to behave as isolectins/charged isomers, which was further corroborated by isoelectric focusing that

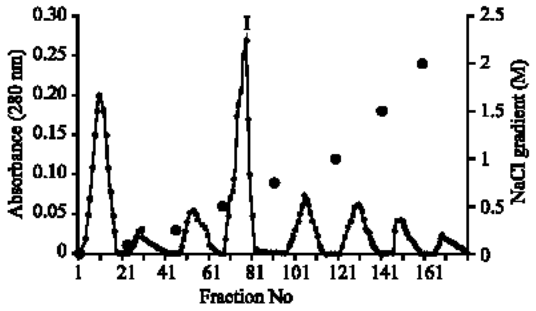

Fig. 1: *C. latifolium* lectin was purified on DEAE-Sephacyl anion exchange chromatography using a fraction size = 1 mL; flow rate = 10 mL h^{-1}; column temperature = 20°C; (○—○) at 280 nm

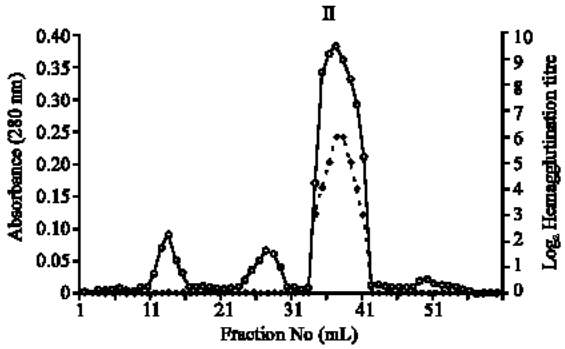

Fig. 2: Gel filtration of *C. latifolium* lectin on Biogel P-200 column using a fraction size = 1 mL; flow rate = 5 mL h^{-1}, column temperature = 20°C; (•---•) lectin activity

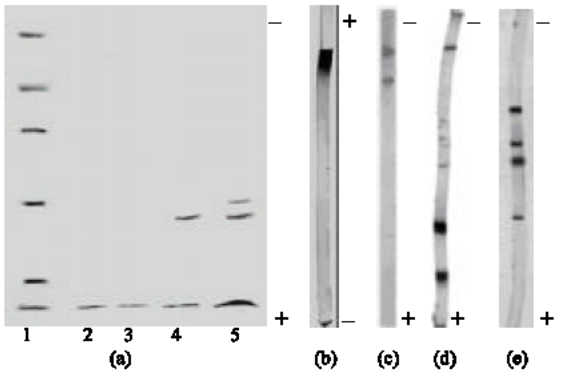

Fig. 3: Electrophoretic pattern of the purified *C. latifolium*. The gels were stained with coomassie blue. (a) SDS-PAGE with 10% gel in the presence and absence of 2% β-mercaptoethanol. About 30 μg of the lectin was loaded, current 30 mA, voltage 100 V and run time 7 h. Lane 1: molecular weight markers, Lane 2: with mercaptoethanol, Lane 3: without mercaptoethanol, Lane 4: anion-exchange purified lectin and Lane 5: crude extract (b) PAGE of the purified *C. latifolium* at pH 4.5 using 7.5% gel, current 10 mA/gel, run time 9 h and protein loaded 80 μg. (c) PAGE of the purified *C. latifolium* at pH 8.3 using 10% gel, current 2 mA/gel, run time 7 h and protein loaded 50 μg. (d) Polyacrylamide gel isoelectric focusing markerproteins; Trypsinogen (pI 9.3), Trypsin inhibitor (pI 4.6), Amyloglucosidase (pI 3.6). (e) Polyacrylamide gel isoelectric focusing of the purified *C. Latifolium*. Non-denatured lectin was loaded on 5% gel using carrier ampholine of pH range 3.0-10.5. Current 20 mA/gel, run time 12 h and protein loaded 20 μg

showed multiple bands in the pI range of 5.09-6.18 (Fig. 3d and 3e). Similar isoelectric focusing pattern has been observed for other MBLs[10,13].

The lectin agglutinated normal rabbit erythrocytes with a MEAPC value of 5.73 μg mL^{-} while erythrocytes from sheep, goat and human did not react at all (Table 2). The susceptibility of rabbit erythrocytes and refractory nature of other blood types to agglutination indicated the availability of the lectin receptors on the former RBCs and their complete absence on the latter. The MEAPC values were further reduced to 1.43 μg mL^{-1} after neuraminidase treatment, in the case of rabbit. A low value of MEAPC indicated more availability of lectin receptors on cells. On the other hand, the guinea pig erythrocytes agglutinated with a MEAPC value of 133.50 μg mL^{-1}, which was further reduced to 4.17 μg mL^{-1} after neuraminidase treatment suggesting unmasking of the receptors for desialylation. Neuraminidase treatment is known to allow better access to the receptors by removing the terminal sialic acid groups thus exposing lectin receptors, reducing the net negative charge on the cell surface and facilitating lectin binding[14]. *C. latifolium* showed optimum activity in the pH range of 5-9 (Fig. 4). The lectin activity decreased with increasing concentrations of urea, thiourea and guanidine HCl (Fig. 5). 50% activity was retained at 8 M urea, 6 M thiourea and 3 M guanidine-HCl, respectively. These denaturants are known to disrupt the three dimensional conformation and binding sites of lectins by disturbing the hydrophobic interactions that play a crucial role in stabilizing the native structure of these molecules. The lectin was stable up to 55°C for 15 min and lost 50% of its activity at 80°C (Fig. 6). The low thermal stability of this lectins is in consonance with the earlier observation about the absence of disulphide linkages and may also be attributed to the low carbohydrate content in the lectin. Similar behavior has also been observed in the case of

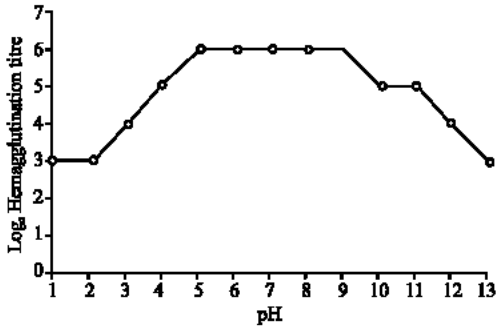

Fig. 4: Effect of pH on *C. latifolium* lectin

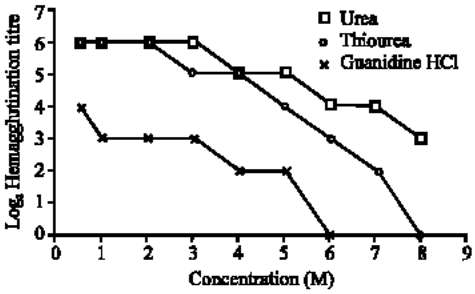

Fig. 5: Effect of denaturants on *C. latifolium* lectin

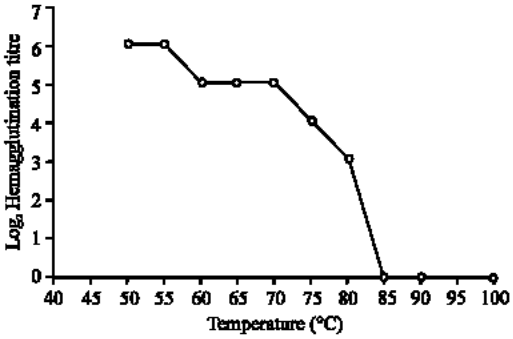

Fig. 6: Thermal stability of *C. latifolium* lectin

other members of Amaryllidaceae with respect to their thermal stability and treatment with denaturing agents[15]. The lectin also resembled other monocot lectins in non-requirement of divalent metal ions for their activity[1].

The purified lectin gave a precipitin line against its own antisera as well as with other lectins from Amaryllidaceae and Liliaceae (Fig. 7), thereby, suggesting that the lectins are serologically related to each other. As reported for other MBLs from Amaryllidaceae[2], the lectin was virtually non-mitogenic towards human peripheral blood mononuclear cells.

As mannose is an important cell surface sugar present in glycoconjugates that are implicated in a

Fig. 7: Double immunodiffusion of purified *C. latifolium* against rabbit antiserum: 25 µg of antisera of *C. latifolium* (A) Well 1: Purified lectin from *Zephyranthes candida* (Amaryllidaceae) Well 2: Purified lectin from *C. latifolium* (Amaryllidaceae) Well 3: Purified lectin from *Gloriosa superba* (Liliaceae). The precipitin bands were stained with coomassie blue

wide variety of important receptor mediated processes, *C. latifolium* lectin with exclusive specificity towards methyl α-D-mannopyranoside has provided an interesting line of investigations with respect to lectin dependent processes and warrants further studies to test its efficacy for biological activities.

REFERENCES

1. Lis, H. and N. Sharon, 1998. Lectins: Carbohydrate-specific proteins that mediate cellular recognition. Chem. Rev., 98: 637-674.
2. Van Damme, E.J.M., W.J. Peumans, A. Pustzai and S. Badocz, 1998. Handbook of Plant Lectins: Properties and Biomedical Applications. John Wiley and Sons, Chichester, UK.
3. Lam, Y.M. and T.B. Ng, 2001. A monomeric mannose-binding lectin from inner shoots of edible chive (*Allium tuberosum*). J. Protein Chem., 20: 361-366.
4. Davidson, E., J.M.S. Forrest, I.M. Morrison and D. Stewart, 2001. Mannose-specific plant lectins from plants as diagnostics, vaccines and tools for the elucidation of viral infection mechanisms in animals. In: Annual Report for the Scottish Crop Research Institute for 1999/2000, SCRI, Dundee, Scotland, pp: 125-128.
5. Murdock, L.L. and R.E. Shade, 2002. Lectins and protease inhibitors as plant defenses against insects. J. Agric. Food Chem., 50: 6605-6611.

6. Lowry, O.H., N.J. Rosebrough, A.R. Farr and R.J. Randall, 1951. Protein measurements with folin-phenol reagent. J. Biol. Chem., 193: 265-275.
7. Spiro, R.G., 1966. Analysis of sugars found in glycoproteins. Meth. Enzmol., 8: 3-26.
8. Paulova, M., G. Entlicher, M. Ticha, J.V. Kostir and J. Kocourek, 1971. Studies of phytohemagglutinins. VII. Effect of Mn^{2+} and Ca^{2+} on hemagglutinin of phytohemagglutinin of *Pisum sativum* L. Biochim. Biophys. Acta, 237: 513-518.
9. Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J. Immunol. Meth., 65: 55-63.
10. Van Damme, E.J.M., I.J. Goldstein and W.J. Peumans, 1991. A comparative study of mannose-binding lectins from Amaryllidaceae and Alliaceae. Phytochemistry, 30: 509-514.
11. Barre, A., E.J.M. Van Damme, W.J. Peumans and P. Rouge, 1996. Structure-function relationship of monocot mannose binding lectins. Plant Physiol., 112: 1531-1540.
12. Lopez, S., C. Codina, J. Bastida, F. Viladomat, E. Davidson and D. Stewart, 2002. Biodiversity of mannose specific lectins within *Narcissus* sp. J. Agric. Food Chem., 50: 2507-2513.
13. Ooi, L.S., T.B. Ng, Y. Geng and V.E. Ooi, 2000. Lectins from bulbs of the Chinese daffodil *Narcissus tazetta* (family Amaryllidaceae). Biochem. Cell Biol., 78: 463-468.
14. Piller, V., F. Piller and J.P. Cartion, 1986. Isolation and characterization of an *N*-acetylgalactosamine specific lectin from *Salvia sclarea* seeds. J. Biol. Chem., 261: 14069-14075.
15. Rudiger, H., 1998. Plant lectins-more than just tools for glycoscientists: Occurrence, structure and possible functions of plant lectins. Acta Anatomica, 161: 130-152.