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Isolation, Purification and Characterization of a Lectin from a Local Kashmiri Variety of Soybean (*Glycine max*)

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Abstract: A purification scheme was developed to purify a lectin from the seeds of a local Kashmiri variety of *Glycine max* (soybean). The lectin that specifically binds to N- acetyl galactosamine was purified to electrophoretic homogeneity by affinity chromatography on CNBr activated Sepharose 6B column. Human blood group A, B, O and AB erythrocytes were used for agglutination assay and the sugar specificity was determined by hemagglutination inhibition assay. Protein estimation was done by Lowry's method and analysis was done by PAGE both under native conditions and in presence of SDS. The purified soybean lectin (SBL) showed equal agglutination with all four types of blood groups i.e., A, B, O and AB. Hemagglutinating activity of the lectin is inhibited by N- acetyl galactosamine and galactose and other carbohydrates containing the galactopyranosyl residue. The purified lectin gave a single symmetric protein peak on gel filtration chromatography showing a molecular weight of 110 kDa and when subjected to native PAGE, showed a single protein band. A single band of 30 kDa was obtained upon SDS-PAGE, establishing that the lectin is composed of similar subunits i.e., it is a homotetramer. A tetrameric galactose specific lectin that shows equal activity with all blood type human erythrocytes was purified and characterized from a Kashmiri variety of soybean.

Key words: Affinity chromatography, ligand, N-Acetyl-galactosamine, Soybean lectin

INTRODUCTION

Lectins are carbohydrate-binding proteins and are known for their ability to agglutinate erythrocytes *in vitro* (Van Damme *et al.*, 1998; Rudiger *et al.*, 2000). It is their unique ability to recognize and bind reversibly to specific carbohydrate ligands without any chemical modification that distinguishes lectins from other carbohydrate binding proteins and enzymes and makes them invaluable tools in biomedical and glycoconjugate research. They are widely distributed in nature and are most abundant in plants (Lis and Sharon, 1986). Plant lectins, a unique group of proteins and glycoproteins with potent biological activity, occur in foods like wheat, corn, tomato, peanut, kidney bean, banana, pea, lentil, soybean, mushroom, rice and potato. However, majority of studies on lectins have been carried out on legume seeds where they comprise up to 15% of total protein (Loris *et al.*, 1998; Van Damme *et al.*, 1998). Lectins may exist in various tissues of the same plant and have

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different cellular localizations and molecular properties. Lectins have been found performing diverse biological functions including cellular recognition and interaction etc. (Weis and Drickamer, 1996; Lis and Sharon, 1998). Their ability to specifically recognize and reversibly bind to soluble or cell bound complex carbohydrate structures make them special candidates to play different roles in many biological processes such as cell to cell interaction, ligand-receptor signaling or cellular activation (Wu *et al.*, 1995; Gorocica *et al.*, 1998; Sáez *et al.*, 1999; Peumans *et al.*, 2000) as well as in plant defense (Peumans and Damme, 1995).

Studies show that lectins have been recognized as useful probes for structural investigations of polysaccharide and complex carbohydrates on cell surfaces. Some lectins have been used for blood group typing because of their specificity to particular blood group. Certain lectins differentiate malignant from normal cells (Sharon and Lis, 1989, 2004; Sela *et al.*, 1970). Several lectins have been found to possess anticancer properties *in vitro*, *in vivo* and in human case studies; they are used as therapeutic agents, preferentially binding to cancer cell membranes or their receptors, causing cytotoxicity, apoptosis and inhibition of tumor growth. They also affect the immune system by altering the production of various interleukins, or by activating certain protein kinases. Lectins can bind to ribosomes and inhibit protein synthesis. They also modify the cell cycle by inducing non-apoptotic G1-phase accumulation mechanisms, G2/M phase cell cycle arrest and apoptosis and can activate the caspase cascade. Lectins can also downregulate telomerase activity and inhibit angiogenesis (Elvira de and Prisecaru, 2005). Plant lectins have also been used for cell separation and bone marrow transplantation (Reisner *et al.*, 1978, 1983). A very recent advance is the introduction of plant lectins in the form of microarrays as a unique means for high throughput analysis of protein glycosylation (Rosenfeld *et al.*, 2007) and for profiling global changes in mammalian (Tateno *et al.*, 2007) and bacterial (Hsu *et al.*, 2006) cell surface glycomes.

Availability of gram quantities of purified soybean lectin (SBL) to scientists will foster discovery of novel biomedical applications of the lectin and provide the opportunity to investigate the antinutritional effects of SBL in soybean-consuming food animals and poultry. Therefore, in the present study, successful efforts for isolating, purifying and characterizing SBL from a local Kashmiri variety of soybean was designed.

MATERIALS AND METHODS

Soybean lectin was isolated and purified from the seeds of a local cultivar of *Glycine max* (soybean) purchased from a local food store (Srinagar J and K India) using a modified published procedure of Entlicher *et al.* (1970). All the chemicals and sugars were purchased from sigma (USA). The present study was carried out at the Department of Biochemistry, University of Kashmir Srinagar, in 2006 and it took about six months to purify and characterize the lectin.

Preparation of Crude Extract of SBL

About 15 g of dry soybean seeds were swollen in water at 40°C overnight. The seed coat and embryo was removed carefully. The remaining cotyledons were homogenized for 5 min in a REMI auto mix blender in 100 mL of 20 mM phosphate buffer, pH 7.2 (containing 1 mM CaCl₂, 1 mM MgCl₂ and 0.02% NaN₃). The homogenate thus produced was filtered through double-layered cheesecloth and the filtrate centrifuged at 23500x g in a REMI-C-24 centrifuge for 20 min. The supernatant was treated with 1M acetic acid until pH 5.0 was reached. The suspension was again centrifuged at 23500x g for 20 min and the

supernatant dialyzed against 20 mM phosphate buffer, pH 7.2 for 24 h. The viscous extract thus obtained was the crude extract of SBL and was subjected to further processing.

Estimation of Hemagglutination Activity

The hemagglutination activity of the lectin was determined by a slight variation of the method devised by Peumans *et al.* (1985). The fresh human group A, B, O and AB erythrocytes were suspended in normal saline (Final concentration 5%). The lectin activity was expressed as E.U (erythrocyte agglutinating unit). One EU is defined as the minimum amount (in microgram) of lectin per ml that could cause agglutination of 1 mL of a 5% suspension of unmodified human erythrocytes.

Hemagglutination Inhibition Assay

Hemagglutination inhibition of the lectin was performed by observing the inhibition of lectin induced hemagglutination by various sugars namely D-glucose, D-galactose, D-mannose, lactose, maltose, sucrose, fructose, ribose and sugar derivatives like N-acetyl galactosamine and N-acetyl glucosamine. Sugar solutions of known concentrations were serially diluted with 0.15 M NaCl and 20 mM phosphate buffer. The minimum concentrations of each sugar capable of fully inhibiting hemagglutination after 1 h at RT were noted.

Affinity Purification of SBL

Crude SBL was subjected to affinity chromatography on CNBr activated Sepharose- 6B column, pre-equilibrated with 20 mM phosphate buffer, pH 7.2 (containing 1 mM CaCl₂, 1 mM MgCl₂ and 0.02% NaN₃). A 1-2 mL of the extract was layered on the gel and the unadsorbed proteins were washed off. The non-retained fraction of protein material was pooled and the column washed until A₂₈₀ approached zero. The bound SBL was eluted with 1M N-acetyl galactosamine and finally SBL was pooled, desalted by extensive dialysis first against 20 mM phosphate buffer, pH 7.2 (containing 1mM CaCl₂, 1 mM MgCl₂ and 0.02%NaN₃) and then against double-distilled water. All the fractions were concentrated by air drying and conserved at -20°C. This was labeled as purified Soybean Lectin (SBL) and used for characterization.

Estimation of Protein

Protein was estimated by the method of Lowry using bovine serum albumin as the standard protein (Lowry *et al.*, 1951).

Protein Analysis

The subunit molecular mass of the lectin was estimated by discontinuous SDS-PAGE. Denaturing polyacrylamide gel electrophoresis (SDS-PAGE) was done according to modified method of Lammeli (1970) on a 12.5% gel. For native PAGE, 8% polyacrylamide was employed and SDS and reductants were omitted. About 40 µg of the affinity purified Soybean lectin was electrophoresed. Gels were stained with Coomassie Brilliant Blue. Native molecular mass of the lectin was determined by gel filtration on Sephadex G-200 column using 0.01 M PBS, pH 7.2.

RESULTS

Purification

Soybean lectin was purified by affinity chromatography on CNBr activated Sepharose-6B column equilibrated with 20 mM phosphate buffer, pH 7.2 (containing 1 mM

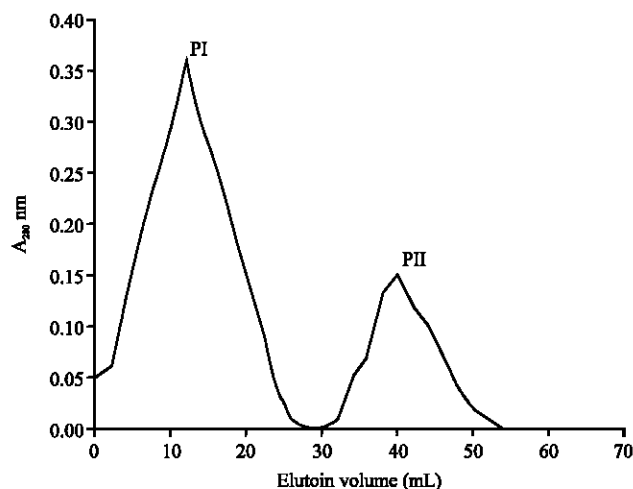


Fig. 1: Elution profile of Soybean lectin on CNBr activated Sepharose 6B column (1.5×10). PI shows the flow of unbound proteins eluted with the equilibration buffer-20 mM phosphate buffer, pH 7.2 (containing 1 mM CaCl₂, 1 mM MgCl₂ and 0.02% NaN₃). PII shows the elution of bound protein with the equilibration buffer containing 1 M N-acetyl galactosamine. Fractions of 2 mL were collected and their A₂₈₀ determined

Table 1: Purification of Soybean lectin

Purification step	Volume (mL)	Protein conc. (mg mL ⁻¹)	Total protein (mg)	Activity (EU)*	Species activity (E.U mg ⁻¹)	Purification factor
Crude	80	32	2560	500	15.625	-
Affinity Chromatography	2	0.3	0.6	26.0	86.7	5.55

*One erythroagglutinating unit (EU) is defined as the minimum amount of the lectin per ml required to give positive agglutination of 1 mL of a 5% erythrocyte suspension (μg mL⁻¹). EU is expressed in microgram of lectin/mL of the protein solution. Purification factor is the ratio of sp. Activity after to the sp. Activity before affinity chromatography

CaCl₂, 1 mM MgCl₂ and 0.02% NaN₃). Major portion of the protein in crude extract passed unbound through the column. This fraction did not carry any hemagglutinating activity. The bound protein when eluted with the equilibration buffer containing N-acetyl galactosamine emerged as a single peak showing hemagglutinating activity. Typical profile is shown in Fig. 1. The data on purification of soybean lectin is summarized in Table 1. Volumes of crude extract containing 500 EU mL⁻¹ of lectin gave a fair hemagglutination, however a 26.0 EU mL⁻¹ of purified lectin showed equal agglutinating activity. The soy lectin was purified to a purification factor of 5.55. Fractions collected were pooled and concentrated and simultaneously subjected to native PAGE until a dense single band was observed.

Chromatographic and Electrophoretic Analysis

The homogeneity of the purified SBL preparation was tested by various parameters. The affinity purified lectin when run on 8% gel under native conditions, pH 8.2 moved as a single band (Fig. 2). In SDS-PAGE, pH 8.3, under both reducing and non-reducing conditions, SBL migrated as a single band of 30 kDa (Fig. 3). The native molecular mass of SBL as determined by the position of the band in native PAGE (Fig. 2) and gel filtration chromatography was 110 kDa. The results of native PAGE and SDS-PAGE under reducing

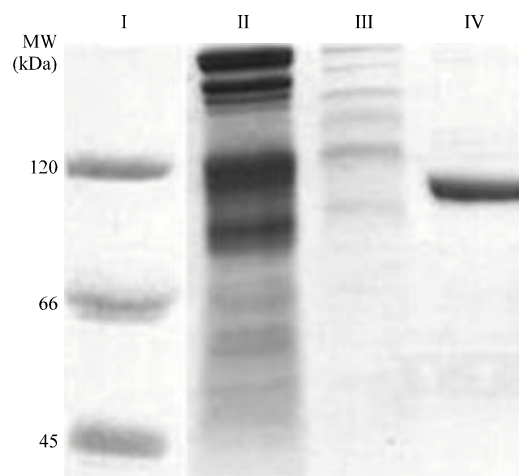


Fig. 2: PAGE profile of lectin from soybean. About 40 μg of the affinity purified soybean lectin was applied on polyacrylamide gel at pH 8.2, using 8% gel. Current of 2.5 mA per gel was applied and run time was 2 h. The gels were stained with Coomassie Blue. Lane I: Marker proteins from top downwards; β -galactosidase (120 kDa), bovine serum albumin (66 kDa) and ovalbumin (45 kDa), Lane II: Crude extract, Lane III: Dialysate, Lane IV: Affinity purified protein

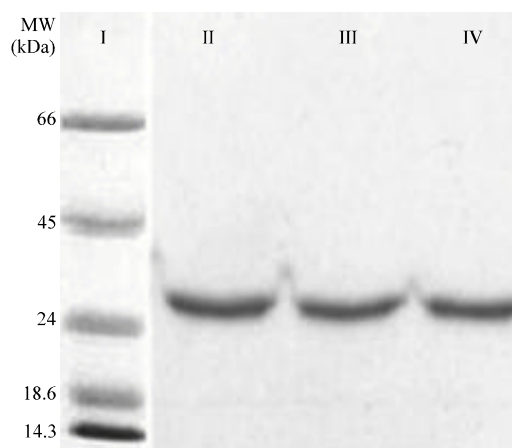


Fig. 3: SDS-PAGE, pH 8.3 purified SBL using 12.5% gel with (lane II) and without (lanes III and IV) 2% 2-mercaptoethanol (running time 3 h at 3 mA/well). The amount of purified lectin loaded is 40 μg . Lane I, Molecular weight markers (from top to bottom): bovine serum albumin (66 kDa); ovalbumin (45 kDa); trypsinogen (24 kDa); β -lactalbumin (18.6 kDa) and α -lactalbumin (14.3 kDa). The gels were stained with Coomassie brilliant blue

and non-reducing conditions together with the gel filtration data revealed that the lectin exists as a homotetramer of four identical subunits which are not held together by disulphide linkages.

Table 2: Blood group specificity of purified Soybean lectin (SBL)

Blood group	Specific activity (EU mg ⁻¹)*
O	8.67
A	8.67
B	8.67
AB	8.67

*One erythroagglutinating unit (EU) is defined as the minimum amount of the lectin mL⁻¹ required to give positive agglutination of 1ml of a 5% erythrocyte suspension

Table 3: Inhibition of Soybean lectin by different sugars

Sugar	Minimum conc. (mM)*
N-acetyl galactosamine	0.01
D-galactose	0.01
Lactose	No inhibition
Mannose	No inhibition
Glucose	No inhibition

*Minimum sugar conc. necessary for complete inhibition of agglutination of erythrocytes by a soybean lectin solution of 6 µg mL⁻¹

Biological Properties

Hemagglutination

Soybean lectin (SBL) does not show any blood group specificity (Table 2). With all blood group type erythrocytes the lectin showed a specific activity of 86.67 units. Extent of hemagglutination was found to be same, when human erythrocytes of blood group A, B, AB and O were incubated with purified SBL overnight at 7°C and for 6 h at 27 and 37°C. From these observations, it can be concluded that the purified protein is lectin in nature and is relatively stable to varying temperatures.

Hemagglutination Inhibition Assay

Hemagglutination inhibition assay was performed by observing inhibition and reversal of inhibition in the presence of different sugars and the results are depicted in Table 3. N-acetyl galactosamine and D-galactose are found to be highly effective for inhibiting the agglutination of human red blood cells.

DISCUSSION

Despite the great number of reports describing many biochemical and structural aspect of plant lectins and their increasing utilization as biotechnological tools, many questions concerning their role in the plant kingdom still remain open. As a starting point, lectin distribution in plants and their ultra structural localization addressed the first hypothesis regarding their functions. Accordingly, earlier investigations explored the role of lectins in plant defense against seed predators (insects or fungi) or during seedling development (Quinn *et al.*, 1987; Naeem *et al.*, 2001). Legumes are still considered as the most important source of lectins. In seeds, considerable amounts of lectin accumulates in the protein bodies forming a package together with well-characterized storage proteins, although lectins are also found in the root hair as in pea plants (Diaz *et al.*, 1989).

In their investigation Spilatro and Anderson (1989) reported several studies regarding isolation and purification of soybean lectin. Soybean lectin (SBL) was originally purified by Leiner and Pallansch in 1952 and the same lectin was purified on affinity column with immobilized aminocaproyl-β-D- galactosylamine coupled to Sepharose by Sharon and Lis (2004). The molecular weight (Mr) was found to be 110,00 dalton consisting of four identical subunits of around 30,000±500 (Lis and Sharon, 1973). Lectins have also been characterized

from the soybean vegetative tissues and roots. A lectin was reported to be present in the leaves of soybean that possessed structural and immunological similarities to SBA. Like SBA, the soybean leaf protein is a 120 kD glycoprotein with a tetrameric arrangement of subunits. The subunits of the leaf protein have molecular masses of 28 and 33 kD and react with antibodies raised against the seed lectin (Spilatro and Anderson, 1989).

In the present study, a lectin was isolated from a local Kashmiri variety of Soybean. To accomplish this, seeds of Soybean (*Glycine max*) were investigated for the presence of lectins. As presented above, SBL seemed to bind to some components of the erythrocytes. Crude extract from Soybean seeds were shown to contain lectin(s), evident by cross reacting with 5% erythrocyte suspension as explored by hemagglutination. Each step of the purification was monitored for lectin activity by measuring agglutination of these specifically prepared red blood cells. This test was of prime importance since it determined whether the carbohydrate binding characteristics of the final product have survived the procedures utilized thus far in the purification of the lectin. Carbohydrate specificity of the lectin was investigated by monitoring the displacement of the bound lectin from erythrocytes by various sugars. The purified protein was found to bind N- acetyl galactosamine, galactose and other carbohydrates containing the galactopyranosyl residue. Further, the protein was found to agglutinate human erythrocytes of all the four blood groups; A, B, AB and O revealing its non-specificity.

The purified SBL obtained gave a single peak in gel filtration chromatography and when subjected to electrophoresis under native conditions, moved as a single band showing a native molecular mass of 110 kDa. Under denaturing and reducing conditions in the presence of sodium dodecyl sulphate (SDS) and 2- β , mercaptoethanol according to Laemmli (1970), the lectin moved and again a single band was obtained at a position of 30 kDa, establishing the fact that the lectin is composed of four identical subunits. Present results were in consistence with the results obtained by Franco-Fraguas *et al.* (2003) and Lis and Sharon (1973) however, in contrast to the results obtained by Lis *et al.* (1966) mannose did not show any inhibition of agglutination. A single band in PAGE and a single peak in gel-filtration chromatography confirmed the purity of the lectin preparation.

In summary, the results presented here provide evidence of the isolated soybean lectin, obtained from the seeds and show that this lectin possesses carbohydrate-binding site for a specific sugar i.e., galactose and its sugar derivative N-acetyl galactosamine and oligosaccharides containing terminal N-acetyl galactosamine. Also the soybean lectin (SBL), did not show any blood group specificity i.e., reacts equally with all the four human blood group type erythrocytes.

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