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Extraction and Purification of Lectin from Soybean Seeds (*Glycine max*)

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Abstract: In this study, the effectiveness of the ion exchange column packed with anionic resin DEAE-Sephadex®-A-50 in purifying lectin isolated by conventional saline buffer method was investigated. After the lectin extraction, the purification efficacy of ion exchange cationic and anionic resins was compared. The purified lectin was freeze-dried to a solid state to characterize its physiochemical properties. The characterization included the presence and content of protein, sulfur, and carbohydrate, hemagglutination test, gel electrophoresis for molecular weight determination, and metal photometry for sodium content determination. The physiochemical characterization was carried out by Fourier-transfer infrared spectroscopy and X-ray diffraction studies. The test results were compared with the standard sample of soybean lectin. The purified lectin was amorphous, basic in nature, tetramer, galactose binding/N-acetylgalactosamine molecule in its sodium salt form, with a molecular weight of 120 kiloDaltons. Based on the results, the anionic resin DEAE-Sephadex®-A-50 can effectively purify lectin isolated from soybean seeds.

Keywords: soybean seeds; lectin; extraction; purification; freeze drying.

1. Introduction

Lectins are non-immunoglobulin proteins that bind different carbohydrates with a high degree of selectivity and stereo-specificity without changing the covalent structure of any recognized glycosyl ligands [1]. Lectin (also known as hemagglutinin or agglutinin) is a carbohydrate-binding protein that makes up 10% of total soybean protein [2]. Lectins differ in various properties, such as structure, molecular weight, composition, and the number of sugar-binding sites present per molecule [3]. Different glycan arrays are expressed in different cell types, especially in diseased cells such as transformed or cancerous cells [4]. The carbohydrate recognition groups on the surface of target cells encourage the application of carbohydrate epitopes as ligands for targeted drug delivery [5]. Based on their carbohydrate-binding specificity, lectins can be classified into groups such as mannose-binding, glucose-binding, galactose-binding, N-acetylglucosamine-binding, fucose-binding, sialic acid-binding, and N-acetylgalactosamine-binding [6].

Soybean is a short-day legume that is a staple in human food and animal feed due to its high nutritional content [7]. Raw, mature soybean seeds contain anti-nutritional factors, such as protease inhibitors, lectins, goitrogens, cyanogens, anti-vitamin factors, phytic acid, saponins, and estrogens [8]. Furthermore, soybean and its products find various non-food applications, such as in producing papers, plastics, pharmaceuticals, inks, paints, varnishes, pesticides, cosmetics, and, more recently, biodiesel [9]. Lectins possess anti-tumor, anti-proliferative, immune potentiating, antibacterial, antifungal, anti-insect, and antiviral activities [10,11]. Specific sugar molecules (such as lactose, galactose, and mannose) can recognize lectins over-expressed on numerous cancer cells' surfaces [12,13]. Due to the specific binding of lectins, they are used in prodrug synthesis [14]. Another approach is the development of lectin-grafted carrier systems [15]. A reservoir such as microparticles, nanoparticles, or liposomes containing the drug and lectins are immobilized at the reservoir's surface [16]. The lectins guide the drug container to the absorption site [15]. Lectins can mediate mucoadhesion, cytoadhesion, and cytoinvasion of the drug in lectin-grafted prodrug and biotechnological tool/carrier systems [15]. A higher percentage of lectin has a potentially deleterious effect on the body [17,18].

In the past, lectins were purified by size exclusion chromatography or affinity chromatography. Purification of lectin from Soybean seeds has been carried out using an affinity column with immobilized amino caproyl-β-Dgalactosylamine coupled to sepharose [19]. Lectin receptor-like kinases are one of the largest classes of cell surface RLKs that extensively participate in plant responses to biotic and abiotic stresses [20]. Lectin in the seed and root are similar but not identical [21]. In the present study, lectin purification from Soybean seeds was carried out through ion-exchange chromatography using anionic resin DEAE-Sephadex®-A-50. The lectin was extracted using the conventional saline buffer method. The purified extract was freeze-dried and tested for haemagglutination, sugar specificity, molecular weight, sodium content, and protein content. The freeze-dried lectin was characterized by Fourier transfer Infrared and X-ray diffraction [22].

2. Materials and Methods

2.1. Materials.

Soybean is among the most important oil and food protein crops grown worldwide [23]. Soybean (*Glycine max*) seeds were procured from a local market source (Green Heaven India Limited**,** Mumbai, India) and authenticated at Khalasa College Mumbai, India. Sepharose®-CM fast flow and diethyl amino ethyl (DEAE)-Sephadex®-A-50 resin were purchased from Ion Exchange Ltd., India, and Pharmacia Ltd., India, respectively. Polyacrylamide gel was purchased from Sisco Research Labs (SRL), Mumbai. Red blood cells (RBCs) from *Albino* rats were obtained from KEM Hospital, Mumbai, India. Double distilled water from Millipore system USA was used throughout the experiment. A soybean lectin (L1395) standard sample was purchased from Sigma-Aldrich, Germany.

2.2. Extraction of lectin.

The method for the extraction of lectin was reported earlier [24]. Briefly, dried and powdered soybean seeds (<1680 µm) were defatted in ice cold petroleum ether 70% v/v (maintained at 28°C) and dried in an oven for 15 min. This defatted meal was extracted in cold

phosphate buffer pH 7.2 in the ratio of 1:4 w/v for 4 h under stirring. The extract was centrifuged at 15000 revolutions per minute (rpm) for 15 min. The supernatant was separated and further centrifuged at 10000 rpm for 10 min. The residue was discarded, and the supernatant was treated with 80% v/v ammonium sulfate and stored at 4-8°C for 12 h. This solution was centrifuged at 15000 rpm for 15 min, and the supernatant solution was discarded. The residue was dissolved in a phosphate buffer of pH 7.2 and allowed to dialyze in two steps - firstly in distilled water and then in a phosphate buffer of pH 7.2. This isolated crude lectin was then subjected to purification. The process was repeated in duplicate for a batch size of 20 g and 375 g of soybean. The extract was obtained in the form of a viscous aqueous liquid and was evaluated for agglutination.

2.3. Purification of lectin.

The lectin extract was purified using ion exchange resins - ion exchange weak cationic (Sepharose®-CM Fast Flow) and anionic resin (DEAE-Sephadex®-A-50) were selected based on the nature of lectin, its solubility and stability, and swelling index of resin. The crude extract was loaded into cationic resin (Sepharose) and anionic resin (Sephadex), equilibrated with citric acid buffer pH 3.8 and sodium acetate buffer pH 6.1, respectively, and subjected to ion exchange chromatography. The columns were washed initially with the same buffer solutions to wash unbound proteins and then washed with linear salt gradient elution. Dialysis of obtained concentrate was carried out to remove the contaminants in double distilled water. The eluting fractions were analyzed for their protein content and tested for agglutination.

2.4. Freeze drying of lectin.

The purified lectin extract was in liquid form and freeze-dried to convert it into a solid powder form. The purified lectin extract solution was filled up to 30% of the capacity of the trays in the freeze dryer. Temperature probes were inserted into each of these trays. The initial freezing process was carried out at -42°C for 12 h before primary drying was carried out under vacuum (100 x 10^{-3} mbar) for 20 h for water removal. Secondary drying was carried out for 24 h at 25°C, and the trays were removed. The lyophilized lectin was removed from trays and sieved through a 40 # (400 µm) stainless steel sieve to break the agglomerate if any. This was stored in sealed vials at a temperature of 2-8°C**.**

2.5. Haemagglutination test.

In a 96-well microtitre round bottom plate, standard lectin (100 µl) and RBC suspension (100 µl) were added to each row's first well, which served as a positive control. In the last well of each row, blood RBC (100 µl) and saline solution (100 µl) were added, which served as a negative control. Blood RBC, saline solution, and lectin (100 µl each) were added between the positive and negative control. Normalized crude freeze-dried lectin (10 µl) was added to the first well and was serially diluted in the other wells. The same procedure was followed for the other samples. The plate was then placed at room temperature for an hour and then observed under 1000× magnification of a compound microscope (Scientific India Ltd., Mumbai) for any agglutination. The highest dilution and the lowest dilution for hemagglutination were noted. One hemagglutination unit is the reciprocal of the highest dilution causing visible agglutination.

2.5.1. Effect of pH on hemagglutination activity of soybean lectin.

The method has been reported previously [25]. Freeze-dried lectin solution equivalent to 30 IU was prepared in a phosphate buffer of pH 6.8 and diluted further to get 10 IU. This solution was incubated in various pH buffers for 48 h at room temperature (25^{o}C). Buffers of various pH were selected for this study; 10 mM glycine-HCl buffer of pH 3.0, 3.5,10 mM tris-HCl of pH 8.0, 8.5, 9.0, and phosphate buffer of pH 5.0, 6.2, and 7.2. ELISA reader measured hemagglutination at 280 nm.

2.5.2. Effect of temperature on hemagglutination activity.

The method has been reported previously [25]. Freeze-dried lectin solution equivalent to 30 IU solution was prepared in phosphate buffer of pH 6.8 and diluted further to get 10 IU. It was incubated at temperatures of 27 to 100°C. ELISA reader measured hemagglutination at 280 nm

2.6. Sugar specificity.

The procedure was carried out as reported earlier [25]. Briefly, D-galactose (100 mM) was serially diluted in phosphate buffer saline of pH 7.2 and mixed with an equal volume of freeze-dried lectin (30 IU). The mixture was allowed to interact with trypsinized *New Zealand White* rabbit erythrocytes suspension for 30 min at 37°C. The minimum titre/concentration of D-galactose inhibiting hemagglutination was determined by an ELISA reader from Birad Ltd., USA, at 280 nm by macroscopic observation.

2.7. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Homogeneity and molecular mass of the subunits of the lectins crude sample freeze dried obtained by conventional methodology was estimated by discontinuous polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) [26]. The SDS-PAGE was performed using a 13% separating and a 4% stacking gel.

2.8. Sodium content determination (Metal flame photometry).

Lectin (30 g) was placed in a silica crucible and heated for 5 min under stirring until the lectin turned into ash. Ten milligrams of this ash were dissolved in concentrated hydrochloric acid and diluted with distilled water. After calibration with distilled water, the standard graph was prepared with a lectin standard sample. Adjusting the flame, pressure readings were taken in triplicate for the sample lectin.

2.9. Protein content determination (Bradford method).

The procedure was carried out as reported earlier [26]. Lectin standard sample was serially diluted from a stock solution in phosphate buffer of pH 6.8 (1 mg/ml) to get concentrations of 0.2, 0.4, 0.6, 0.8 mg/ml. Bradford reagent (5 mL) was added to these solutions and mixed well-using cyclomixer. The absorbance of these solutions was determined at 595 nm using distilled water as blank. The concentration of the protein sample was determined from the graph plotted with a concentration of lectin against absorbance. The same procedure was followed for the freeze-dried lectin.

2.10. Fourier infrared spectroscopy.

The infrared spectrum of the freeze-dried lectin was carried out on FTIR Spectrum RXI spectrometer (Model-LM 500) Perkin Elmer Ltd., Germany, using potassium bromide pellet method at transmittance mode over wave number range of 4000 to 400 cm^{-1} for 4 mm/s at a resolution of 2 cm^{-1}. The spectrum was compared with the standard spectra of lectin.

2.11. X-ray diffraction.

The X-ray diffraction of freeze-dried lectin and standard lectin was carried out using a Bruker®D-8 advanced diffractometer in Spain. The X-ray diffractogram was scanned for 5 to 10 mg of the sample with a diffraction angle 2Ø range of 20 to 80° at a scan rate of 0.02°/min with a step angle of 0.02° and count time of 1 second at a constant room temperature 25°C. This XRD spectrum pattern obtained was compared with the standard.

3. Results and Discussion

This study presents a method to extract and purify lectin from Soybeans. The trials of lectin extraction were carried out in batches of 20 g, and 375 g of soybean, and the yield of extracted liquid was 15 ml and 290 ml, respectively. Defatting, dialysis, and re-precipitation with ammonium sulfate removed the extract's lipid, contaminants, and pigment molecules. The lectin was solubilized and extracted in a phosphate buffer of pH 7.2.

The extracted lectin contained soluble proteins and other impurities. Hence, it was purified by ion exchange resins. Ion exchange column Sepharose®-CM Fast Flow, a weak cationic resin, with binding buffer citric acid of pH 3 and DEAE-Sephadex®-A-50, a weak anionic resin with binding buffer sodium acetate of pH 6.1 for the stabilization of the resin was selected. The purification process of these columns is given in Table 1. During the purification process, sodium chloride solution obstructed flow in Sepharose®-CM Fast Flow, while the flow was uniform in DEAE-Sephadex®-A-50. This could be attributed to the higher degree of cross-linking and slower eluent flow rate in Sepharose®-CM Fast Flow.

Table 1. Purification of lectin by packed column.

Column and process details	**Ion exchange column Sepharose®-CM Fast Flow**	**Ion exchange column DEAE-Sephadex®-A-50**
Nature of resin	Cationic weak	Anionic weak
Resin make	Ion Exchange Ltd., India	Pharmacia Ltd., India
Packed column capacity	25 and 50 ml	25 and 50 ml
Preparation of resin solution	1 g in 100 ml double distilled water	1 g in 100 ml double distilled water
Volume of resin solution in column	For 25 ml column capacity -16 ml For 50 ml column capacity -32 ml	For 25 ml column capacity -16 ml For 50 ml column capacity -32 ml
Sample volume used for purification	For 25 ml column capacity -9 ml For 50 ml column capacity -18 ml	For 25 ml column capacity -9 ml For 50 ml column capacity -18 ml
Binding buffer	Citric acid buffer pH 3.8	Sodium acetate buffer pH 6.1
Equilibrium time	1.5-2 h	1.5-2 h
Elution condition	Gradient elution (0.05-1M sodium chloride (NaCl))	Gradient elution (0.05-1M NaCl)
Elution solution flow	Flow was obstructed	Uniform flow

The ionic capacity of the weak cationic resin was 90-130 µmol/ml of gel and had a larger mean particle size of 45-165 µm. Hence, DEAE-Sephadex®-A-50 cross-linked dextran resin with a total ionic capacity 2-2.6 mmol /g dry powder, dry particle size range of 40-120 µm, and with comparatively higher elution rate (45 cm/hr) was selected. The lectin was found to bind to this matrix reversibly and specifically and was eluted with D-galactose by affinity elution condition.

The purified lectin was in viscous form. Hence, it was subjected to freeze drying to convert to a solid powder. The freeze-dried product was a fine amorphous powder that was off-white to cream in color. The final yield was 0.0072 g in the 20 g batch and 1.33 g in the 375 g batch. Although the yield was very low, it was comparable with the similar processes carried out previously [25].

Lectins agglutinate erythrocytes [27]. Multivalent lectins identify and bind to sugar moieties present on the cell surface of erythrocytes leading to cross-linking of the cells and the formation of agglutinates [28]. In the hemagglutination test, agglutination of RBCs was observed with the freeze-dried lectin samples at all the concentrations studied. However, no agglutination was observed with saline solution.

Soybean lectin extracted by conventional method exhibited maximum hemagglutination activity between pH 5 to 8.0. At lower (pH < 5) and higher (pH > 8), hemagglutination activity was less. At a temperature of 27ºC to 60ºC, about 100 % haemagglutination was observed. However, a sharp decrease in agglutination was observed beyond 60°C, and a further decline was observed at a temperature of 80°C and 100°.

The loss of agglutination activity of lectin with increased temperature was due to the destabilization of sporadic weak interactions of the tertiary structure responsible for the native conformation of lectin. Similar results were obtained for lectin from Momardica Charantia by Willy J.P. *et al*. 1986. Thus, pH and temperature were found to influence haemagglutination activity. In all the studies, the sample was purified freeze-dried lectin obtained after the conventional methodology and Sigma-Aldrich Ltd., standard lectin.

The carbohydrate-binding specificity of lectins was evaluated by the ability of sugars to inhibit the agglutination of rabbit erythrocytes. D-galactose and its derivatives were found to be strong specific inhibitors of agglutination. The minimum inhibitory concentration of D-galactose to inhibit agglutination was found to be 3.585 mM. A previous study that isolated lectin from the Kashmir soybean variety found that D-galactose N-acetyl galactosamine was highly effective in inhibiting the agglutination of human RBCs [29].

The SDS-PAGE showed homogeneity of bands between purified and freeze-dried lectin and standard lectin. The bands were visualized by a gel documentation system. It showed that lectin was tetramer, galactose binding/N-acetylgalactosamine molecule in sodium salt form, with a molecular weight of 120 KiloDaltons. The electrophoresis migration pattern and a band of freeze-dried lectin were matched with the standard lectin sample (Fig. 1). This also suggested that the purification process was efficient in removing contaminants and provided pure lectin. The results were in line with a previous report [24].

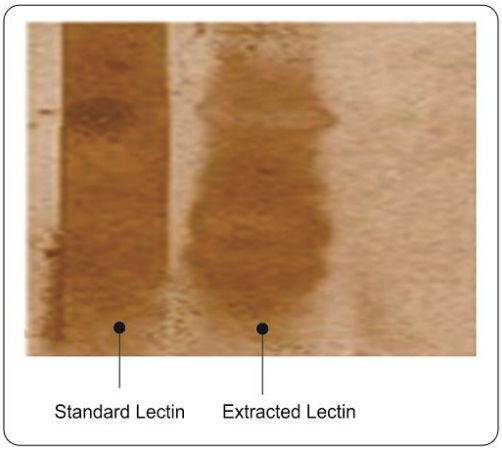

Figure 1. Gel electrophoresis pattern of standard and obtained lectin.

The lectin extracted in saline buffer was obtained in a complex with sodium. Flame photometry determined metal content by preparing a standard graph with sodium chloride solutions. The sodium content in the purified and freeze-dried lectin was 8.316% w/w.

Bradford method was used to determine the protein content in purified and freeze-dried lectin. In this method, a standard curve for standard lectin sample (fold purity 1 mg/ml; protein content 12 mg/ml) was prepared [29]. From the graph, the fold purity of purified lectin was found to be 0.94 mg/ml, corresponding to 11.28 mg/ml of protein. This was comparable to the standard lectin. Similarly, the specific activity of purified lectin was 1815.60 titre (determined by dividing standard lectin titre activity by protein content). This was comparable to the standard sample's protein content (1900.0 titre).

Figure 2 represents the infrared spectra of the purified lectin. In the infrared spectra, the characteristic strong and broad absorption area at 3415 cm^{-1} corresponded to OH stretching vibration due to intermolecular and intramolecular hydrogen bonds, 2925.91 cm^{-1} COOH group [30]. The absorption at 2925.91 cm^{-1} corresponded to the C H stretching vibration [31]. The absorption at 1630.76 cm^{-1} corresponded to an amino group, and the band at 1071 cm^{-1} corresponded to the ether groups' asymmetric and symmetric stretching vibration [30].

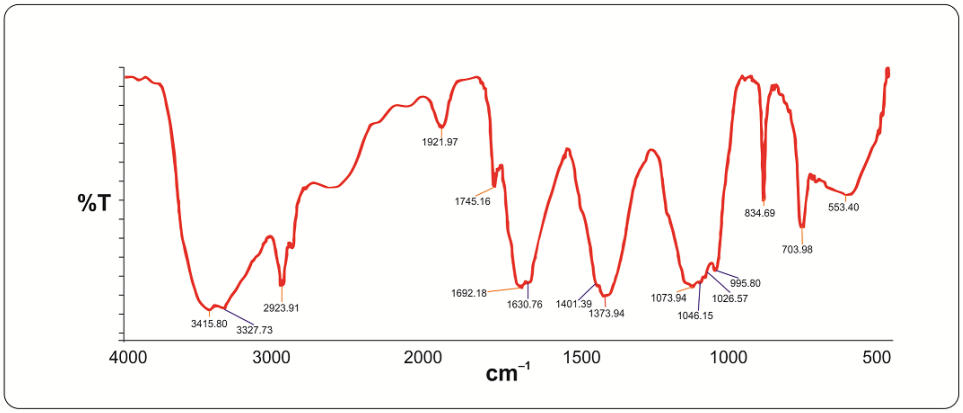

Figure 2. FTIR spectra of Soybean lectin.

Figure 3 represents the XRD pattern spectra of the purified and freeze-dried lectin, which does not show any crystallinity peaks, indicating its amorphous nature. XRD pattern of the extracted lectin complied concerning peak patterns, intensity, width, size, and position of steaks of the standard lectin.

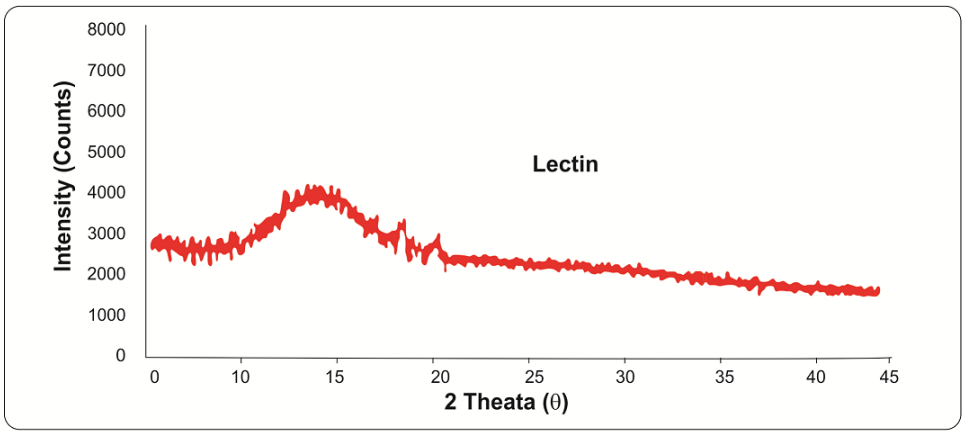

Figure 3. XRD pattern of Soybean lectin.

4. Conclusions

In conclusion, the results of this study suggest that anionic resin DEAE-Sephadex®-A-50 modified resin can be effectively used to purify lectin extracted from soybean (*Glycine max*).

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Conflicts of Interest

The authors declare no conflict of interest.

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