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Purification and Characterization of Mannose/Glucose-Specific Lectin from Seeds of *Trigonella foenumgraecum*

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Abstract—A lectin present in seeds of *Trigonella foenumgraecum* was isolated and purified by acid precipitation, salt fractionation, and affinity chromatography on mannan cross-linked agarose. SDS-PAGE revealed a single band corresponding to a molecular weight of 27,350 daltons. The lectin agglutinated trypsin-treated rat erythrocytes. Sugar specificity as determined by hemagglutination inhibition assay indicated that the lectin belongs to a glucose/mannose-specific group. The reaction of the lectin with glycoprotein was affected by pH changes. The carbohydrate binding specificity of the lectin was investigated by turbidity and activity measurements. As the lectin belongs to the Leguminoceae family, the specificity of the lectin for glucose/mannose renders it a valuable tool for *Rhizobium*–legume symbiosis.

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Lectins are proteins or glycoproteins of non-immune origin that agglutinate cells and precipitate complex carbohydrates or polysaccharides [1-3]. The recognition of carbohydrate determinants by lectins plays an important role in mediation of intercellular binding and elicitation of biosignaling processes [4-7]. Because of their characteristic carbohydrate binding properties, plant lectins have been widely used for the detection, isolation, and characterization of glycoconjugates. The increasing use of lectins in chemical and biological aspects has prompted their purification [8-13]. Extensive study of sequence homology and 3-D structure of various lectins suggest that they are significantly conserved and thus may play an important role in the physiology of plants. In plants they have been implicated in pathogenesis, cell elongation, defense against fungal attack, and *Rhizobium*–legume symbiosis [10, 14]. Thus, lectins are believed to interact with invading microorganisms and provide a line of defense against various bacteria and fungi.

Investigations of *Rhizobium*–legume recognition have generally made use of seed rather than root lectin because of the relatively high concentrations and readily availability of lectin in most legume seeds. The root lectins of leguminous plants are usually similar to their seed counterpart except in the case of *Dolichos biflorus* [5, 8, 15, 16].

Trigonella foenumgraecum is known to have symbiotic relationship with certain bacteria; these bacteria form nodules on the roots and fix atmospheric nitrogen. Though *T. foenumgraecum* is widely used in traditional Indian systems of medicine as an anticholesterolemic, antiinflammatory, and diuretic agent, the lectin from this source has gained little attention. Searching for new lectins having unique carbohydrate-binding specificities with potential application to the isolation and characterization of glycoconjugates and *Rhizobium*–legume symbiosis is one of the objectives of our laboratory. We have also studied the minimum structure requirement of concanavalin A for carbohydrate binding and specificity [17]. This paper deals with the purification of a novel lectin from a new leguminous source, *T. foenumgraecum* (*Trigonella foenumgraecum* agglutinin (TFA)), and its carbohydrate binding properties.

MATERIALS AND METHODS

Mannan cross-linked (mannan-CL) agarose was purchased from Bangalore-Genei Pvt Ltd. (India).

Abbreviations: mannan-CL) mannan cross-linked; TFA) *Trigonella foenumgraecum* agglutinin.

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Sugars and proteins were purchased from Sigma (USA). *T. foenumgraecum* seeds were procured from the local market.

Determination of protein and neutral sugars. Protein was assayed by the method of Lowry et al. [18] using bovine serum albumin as standard, and carbohydrate content was determined by the method of Dubios et al. using glucose as a standard carbohydrate [19].

Preparation of crude extract. *T. foenumgraecum* seeds were thoroughly washed with distilled water and soaked overnight in Tris-HCl buffer, pH 7.0, containing 0.15 M NaCl and 1 mM each of $CaCl_2$ and $MgCl_2$ (TM buffer). The seeds were homogenized in a blender, and the homogenate was filtered through cheesecloth. The resulting suspension was treated with 0.3 M acetic acid, pH 4.0, and allowed to stand overnight at 4°C. The acid-precipitated fat and extraneous proteins were removed by centrifugation. To the clear supernatant, ammonium sulfate was added to 30% saturation. The suspension was incubated for 4-5 h and then centrifuged. The supernatant was further precipitated to 50% saturation. The pellet was dissolved in an appropriate amount of the TM buffer and dialyzed overnight against the same buffer.

Affinity chromatography. Ammonium sulfate precipitated lectin solution was loaded on a mannan-CL agarose affinity column. The column was washed thoroughly with Tris-HCl, pH 7.0, at a flow rate of 12 ml/h, and bound lectin was eluted with 50 mM mannose in the TM buffer. Protein content in the respective fractions was monitored by absorbance at 280 nm. The peak fractions were dialyzed against TM buffer. The activity was assayed by its ability to agglutinate erythrocytes and to interact with goat IgM. The specificity was checked by measuring inhibition of hemagglutination.

SDS-PAGE. SDS-PAGE was carried out according to the method of Laemmli et al. [20] in 10% gel with electrophoresis buffer containing 0.1% SDS in Tris-glycine buffer (pH 8.53). The purified lectin was in a sample buffer containing 2% SDS and 2.5% 2-mercaptoethanol that was subsequently treated in a boiling water bath for 5 min.

Size-exclusion chromatography (SEC). SEC experiments were performed on a Sepharose 6B (74 × 1.1 cm) column. The column was standardized with bovine serum albumin (66 kD), ovalbumin (45 kD), lactoglobulin (35 kD), lysozyme (14 kD), and cytochrome *c* (12 kD). The column was pre-equilibrated with 60 mM sodium phosphate buffer, pH 7.0. Two milliliters of 3 mg/ml native protein was applied to the column and eluted at 20 ml/h. The eluted fractions were read at 280 nm.

Measurement of lectin activity. The hemagglutinating activity of the lectin was determined in microtiter plates. The ability of the lectin to interact with glycoprotein and polysaccharides was also assayed in 10 mM Tris-HCl, pH 7.0, containing 1 mM each of $CaCl_2$ and $MgCl_2$. IgM was extracted from goat plasma by the method of Fahey and Terry [21].

For hemagglutinating activity, equal volumes of seed extract were mixed separately with equal volume of 4% rabbit erythrocytes in normal saline. For lectin–ligand assay, an equal volume of lectin (100 μg/ml) was incubated with increasing concentration of ligand (1-20 mg) at 37°C. Sugar specificity was checked by precipitation-inhibition assay and hemagglutination-inhibition assay in the presence of monosaccharides such as fucose, mannose, glucose, lactose, fructose, sucrose, maltose, and α-methyl mannose.

The buffers used in the pH dependence study were glycine-HCl (pH 2), sodium acetate (pH 3-6), sodium phosphate (pH 7-8), Tris-HCl (pH 9-10), and glycine-NaOH (pH 11-12). The pH was measured on an Elico digital pH meter (model LI 610) with a least count of 0.01 pH unit. The lectin (0.1 mg/ml) was preincubated in respective buffer solutions at 37°C for 30 min, and aliquots were then pipetted into tubes containing glycoprotein IgM (0.6 mg). The volume of the reaction mixture was adjusted to 1 ml with the respective pH buffers.

Spectroscopic studies. Absorption spectra were recorded on a Hitachi (Japan) U-1500 spectrophotometer in the wavelength range 240-340 nm using a protein concentration of 0.5 mg/ml in a 10 mm path length quartz cell. Fluorescence spectra were recorded with a Shimadzu RF 540 spectrofluorometer (Japan) in a 10 mm path length quartz cell. The excitation wavelength was 280 nm and emission wavelength range was 300 to 400 nm. Protein concentration used was 15 μM. CD was measured with a JASCO (Japan) J-720 spectropolarimeter calibrated with ammonium D-10-camphorsulfonate. Cells of path length 0.1 and 1 cm were used for scanning between 250-200 and 300-250 nm, respectively. Protein concentration of the samples was typically 20 and 50 μM in 60 mM phosphate buffer, pH 7.2, for the far-UV and near-UV CD studies, respectively.

RESULTS AND DISCUSSION

Purification of lectin. A summary of the purification of the lectin from *T. foenumgraecum* is given in the table. Several affinity matrices containing terminal D-glucose residues including Sephadex, divinylsulfone-activated glucose, or mannose-derivatized Sepharose were assessed for their use in the isolation of the hemagglutinin. However, all these attempts failed. Finally, the hemagglutinin was found to be adsorbed to mannan-CL agarose, perhaps due to its strong binding to polysaccharide of mannose (spacer ligand). The elution profile of mannose-specific lectin from *T. foenumgraecum* is illustrated in Fig. 1. In a typical preparation, about 25 g dry weight of seeds was extracted and homogenized. After ammonium sulfate fractionation, the redissolved pellet was loaded onto a mannan-CL agarose column. The elution profile gave a symmetrical peak that coincided with the peak of hemag-

glutination activity. Hemagglutination activity titer value 4 corresponded to the concentration of 40 μg/ml of TFA required for agglutination. The reciprocal of the highest dilutions of the lectin giving complete agglutination was taken as hemagglutination titer. One hemagglutination unit is defined as the minimum amount of TFA required for complete agglutination of the cells. The final purified lectin (10 mg) amounted to 0.04% of the initial protein.

Molecular weight of lectin. The affinity-purified lectin was subjected to electrophoresis in the presence of 2-mercaptoethanol. The lectin moved as a single band as revealed by SDS-PAGE (lane *3*) as compared to crude lectin (lane *2*) (Fig. 2). The molecular weight of *T. foenumgraecum* lectin determined by SDS-PAGE using six marker proteins (lane *1*) is 27.4 kD.

SEC. Gel filtration equipments indicated that in phosphate-buffered saline TFA has a molecular weight of 52.7 kD either in the presence or absence of 0.2 M mannose. In both cases, the lectin eluted as a single symmetrical peak, an indication that the TFA sample was homogeneous (data not shown). Taken together, these observations suggest that the lectin exists as a homodimer with an apparent molecular weight of 52.7 kD.

Lectin–ligand interaction and inhibition. The property of lectins to interact with glycoproteins was used to measure the amount of lectin present in the seed homogenate. However, to form detectable precipitate, both the lectin and the glycoconjugate must be multivalent as well as in an appropriate stoichiometric ratio. So lectin was allowed to interact with glycoproteins and polysaccharides. Figure 3 shows the precipitin reaction curve of ovalbumin, IgM, glycogen, and dextran obtained by a turbidity method at 350 nm using a spectrophotometer. An increase in the absorbance at 350 nm was observed due to lectin–ligand interaction [14]. To rule out the possibility of aggregation, proper blanks of TFA and glycoproteins or polysaccharide were taken into consideration.

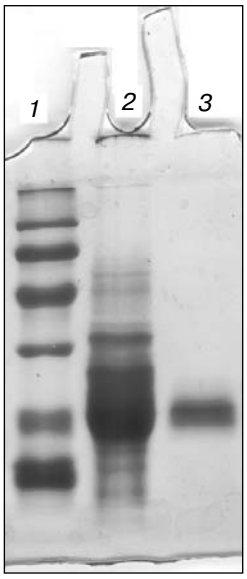

Fig. 2. SDS-PAGE (10%, pH 8.3) of *T. foenumgraecum* lectin isolated by affinity chromatography. Lanes: *1*) marker proteins; *2*) crude lectin; *3*) 30 μg of purified lectin.

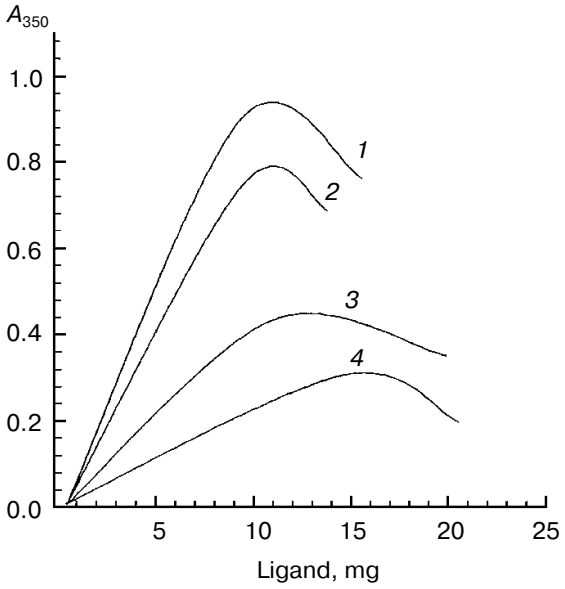

Fig. 3. Interaction of TFA with glycoproteins. Each tube contained 0.1 mg/ml lectin and varying amounts of glycoprotein (1-20 mg). After 48 h at 4°C, the absorbance at 350 nm was monitored against respective blanks of glycoprotein and lectin. Curves: *1-4*) glycogen, ovalbumin, IgM, and dextran, respectively.

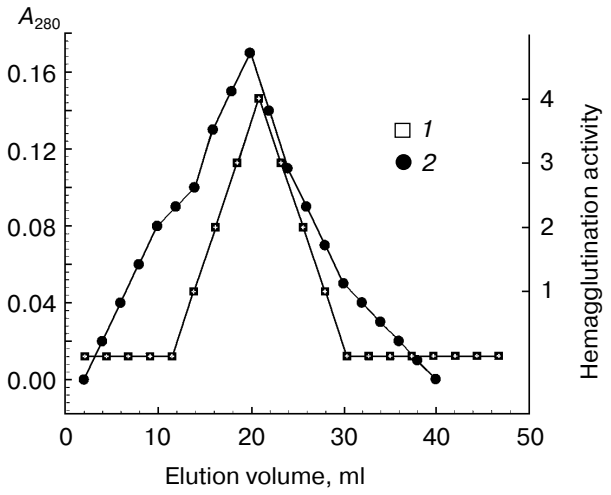

Fig. 1. Isolation of crude *T. foenumgraecum* lectin on a mannan-CL agarose column. The active fraction obtained from crude TFA by $(NH_4)_2SO_4$ fractionation was applied onto the mannan column (0.9 × 5 cm; 5 ml bed volume). The column was washed with TM buffer, and the bound lectin desorbed by 50 mM mannose. Fractions of 0.5 ml were collected and monitored by the absorbance at 280 nm (*1*) and by hemagglutination activity (*2*).

Purification of *T. foenumgraecum* agglutinin

Step	Total protein, mg	Hemagglutination activity*	Specific activity	Yield, %	Purification degree
Crude homogenate from 25 g of seeds	1300	40,324	31.2	100	1
Acid precipitation	500	n.d.	n.d.	38.5	n.d.
Ammonium sulfate (0-30%) fractionation	312	29,615	95	24	3
Ammonium sulfate (30-50%) fractionation	292	39,961	136	22	4.3
Affinity chromatography on mannan-CL agarose	10	3,241	324.1	0.04	10.1

Note: n.d., not determined.

* Hemagglutinating unit corresponds to TFA concentration of 40 μg/ml required for complete agglutination of the cells.

The turbidity developed due to interaction of TFA with the above-mentioned ligands was maximal in glycogen, followed by ovalbumin, IgM, and dextran. As ovalbumin and IgM possesses mannose residues on their surfaces, whereas glycogen and dextran are the polymers of glucose, *T. foenumgraecum* agglutinin showed a significant increase in turbidity, thus indicating its specificity towards mannose residues. The results of sugar hapten inhibition are shown in Fig. 4. Among the monosaccharides tested, only D-mannose and its derivatives were inhibitory, whereas epimers of D-galactose were non-inhibitory. Mannose (the C-4 epimer) showed maximum inhibition followed by glucose and its disaccharide maltose. The specific inhibition of lectin–ligand interaction further proved the lectin to be mannose-specific (Fig. 5).

Sugar specificity. The crude homogenate obtained from seeds was assayed for its ability to agglutinate erythrocytes in Tris-HCl buffer, pH 7.0, containing 1 mM each of $CaCl_2$ and $MgCl_2$. The *T. foenumgraecum* homogenate failed to show hemagglutination activity towards erythrocytes of rabbit, human, or mouse but was able to agglutinate formaldehyde-treated erythrocytes of human blood group A and formaldehyde-treated rat erythrocytes. The reciprocal of the highest dilutions of the lectins giving complete agglutination was taken as hemagglutination titer. The hemagglutination titer of the affinity-purified lectin was found to be 4.

Sugar specificity of crude extracts and purified TFA in the presence of various monosaccharides was assayed by inhibition of hemagglutination. Only the following commonly available sugars were taken: D-glucose, sucrose, D-mannose, D-fructose, D-fucose, D-galactose, lactose, N-acetyl-D-muramic acid, methyl-β-D-mannopyranoside, D(+)-galactosamine, and N-acetyl-

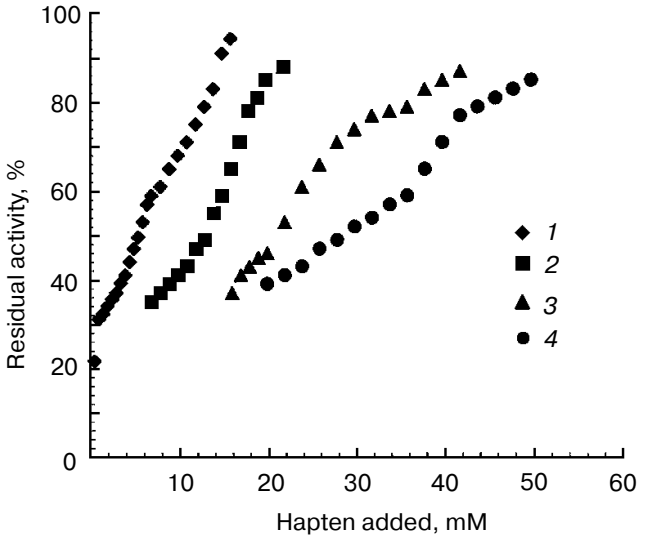

Fig. 4. Hapten inhibition of TFA/IgM interaction. Curves: *1-4*) mannose, α-methylmannose, maltose, and glucose, respectively.

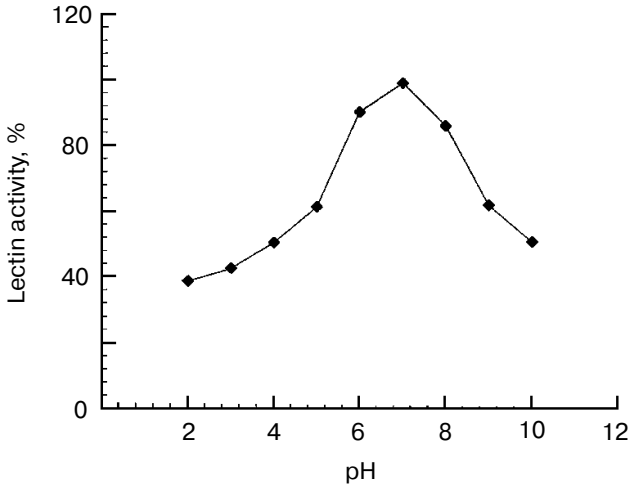

Fig. 5. The pH dependence of TFA/IgM precipitation monitored by turbidity. Each tube contained 0.1 mg/ml lectin and 0.6 mg/ml IgM.

D-galactosamine. Mannose showed maximum inhibition followed by glucose, and its derivatives like maltose and fructose were weakly inhibitory. D-Galactose (the C-2 epimer), sucrose, D-fructose, N-acetyl-D-glucosamine, and N-acetyl-D-muramic acid were all non-inhibitory. The crude lectin was inhibited by simple sugars, whereas varying inhibitory activities were obtained with purified TFA. The lectin was thus found to be glucose/mannose specific. These results indicate that specificity of the lectin is similar to that of *Cajanus cajan* seed and root lectin as reported earlier [8, 14, 15].

Spectral properties. The ultraviolet absorption spectra of the native purified lectin showed absorption maximum at 280 nm and fluorescence emission spectrum at 337 nm, indicating the presence of aromatic amino acids [22]. A positive band at 278-280 nm and a trough at 275 nm observed at neutral pH in the near-UV CD are generally due to the absorption of phenylalanine, tyrosine, and tryptophan residues [23]. A typical β-sheet protein secondary structure in far-UV CD was obtained for TFA at neutral pH, like in α-chymotrypsinogen (data not shown).

Further studies on this lectin involving immunological aspects would be useful in proving it as a valuable biological and medicinal tool. Biophysical studies involving mild denaturation and refolding can give information about the minimal structure requirement of the lectin for its carbohydrate binding.

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