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Purification and characterization of two major lectins from *Vigna mungo* (blackgram)

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Abstract. Blackgram *(Vigna mungo* L. Hepper) seeds contain two galactose-specific lectins, BGL-I and BGL-II. BGL-I was partially purified into two monomeric lectins which were designated as BGL-I-1 (94 kDa) and BGL-I-2 (89 kDa). BGL-II is a monomeric lectin of 83 kDA. The purified lectins were associated with galactosidase activities. BGL-I-1 and BGL-II were copurified with α-galactosidase activity while BGL-I-2 was largely associated with β-galactosidase activity. These lectins agglutinate trypsin treated rabbit erythrocytes, but not the human erythrocytes of A, B or O groups. They were stable between pH 3·5 and 7·5 for their agglutination. The lectins did not show any metalion requirement. They were inactivated at 50°C. The lectin activity was inhibited by D-galactose (0·1 mM). The Scatchard plots of galactose binding to these lectins are nonlinear and biphasic curves indicative of multiple binding sites. The data show that the monomeric lectins have both lectin and galactosidase activities suggestive of a bifunctional protein.

Keywords. *Vigna mungo*; legiiminosae; blackgram; lectin; galactosidase.

1. Introduction

Lectins are a unique group of sugar binding proteins of non-immune origin, able to agglutinate cells and/or precipitate glycoconjugates (Goldstein *et al* 1980). Though lectins display a wide variety of unique and interesting properties (Lis and Sharon 1981), the common functions of lectins have been questioned due to their ubiquitous nature and varied chemical and physical properties (Hankins and Shannon 1978; Singh and Rao 1991). Most studies on legume lectins have been carried out by isolating them from the mature seeds where they constitute 2-10% of the soluble proteins (Liener 1976; Pusztai and Watt 1974; Osborn *et al* 1985). Some legume lectins display both lectin and galactosidase activity (Hankins and Shannon 1978; Dey *et al* 1982a,b). This bifunctional property of enzymatic lectins raises the need of updating the definition of lectins. Recently, Peumans and Van Damme (1995) have redefined 'lectin'. According to them the prerequisite for a protein to be named a lectin is that it should possess at least one non-catalytic domain that binds reversibly to a specific carbohydrate.

Vigna mungo L. Hepper (blackgram or urid) and *Vigna radiata* L. Wilczek (greengram or mungbean) are important pulse crops of the country. An enzymatic lectin from mungbean has been purified and extensively characterized (Hankins and Shannon 1978; Hankins *et al* 1979,1980; Haab *et al* 1981; Dey 1984). Lectins have been reported in blackgram (Singh and Rao 1991; Reddy *et al* 1982; Sharma and Salahuddin 1993).

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The present report describes characterization of lectins from *Vigna mungo* L. Hepper cv. TAU-1.

2. Materials and methods

2.1 *Materials*

Seeds of blackgram cultivar TAU-1 developed at this Research Centre were used as the source of lectin.

The chemicals and reagents used in this study were of analytical grade. N, N, N′, N'-tetramethyl ethylene diamine (TEMED) was obtained from Eastman Organic Chemicals, Rochester, New York, USA. Orthophthalaldehyde (OPA), phenylmethyl-sulfonyl fluoride (PMSF), trypsin (bovine, type XI), *p*-nitrophenyl-α- and *ß*-D-galactopyranosides, Coomassie brilliant blue (R-250) and sugars for inhibition studies were procured from Sigma Chemical Company, St. Louis, Mo, USA. Ultrogel (AcA44:4% acrylamide cross-linked to 4% agarose) was obtained from LKB, Sweden. CM-Sepharose and protein molecular weight markers for electrophoresis and gel filtration were obtained from Pharmacia AB, Uppsala, Sweden. D-galactose-^{14}C(U) was obtained either from Sigma Chemical Company, St. Louis, Mo, USA or BRIT, Bhabha Atomic Research Centre, Mumbai.

2.2 *Purification of lectin*

2.2a *Extraction and initial fractionation:* Cotyledons (100 g) were obtained after removing the testa from seeds soaked for 16 h in dist. water. They were homogenized in 1 litre of 25 mM Na-citrate buffer, pH 4·7 (CB), containing lmM PMSF, 0·5 Mm D-galactose and200 µg/ml NaN_3, using a Sorvall Omnimixer, for2-3 min. The extract was stirred for 4 h at 4°C andwas passed through two layers of cheese cloth. The filtrate was centrifuged at 12,000*g* for 30 min at 4°C. The resulting supernatant fraction was used for purification.

2.2b *Binding and elution of lectin using* CM-*Sepharose:* Regenerated CM-Sepharose (50 ml packed volume) was equilibrated with citrate buffer, pH 4·7 and mixed with the above supernatant fraction by stirring with a glass rod. This was allowed to stand for 1 h at 4°C and filtered through a sintered funnel G 3.

The CM-Sepharose with bound lectin was washed extensively with CB and then suspended in CB, containing 200µg/ml NaN_3 and 0·5 M NaCl for 18 h at 4°C. The eluted lectin was recovered by filtering through sintered funnel.

The lectin was precipitated from the filtrate by ammonium sulphate at 60% saturation. The precipitate was dissolved in the extraction buffer and dialyzed against CB containing 200µg/ml N a N_3 to remove ammonium sulphate.

2.2c *Ion-exchange chromatography:* The dialyzed protein solution was subjected to ion-exchange chromatography using CM-Sepharose. The protein was charged on CM-Sepharose column (60 × 15 mm) pre-equilibrated with CB containing 200 µg/ml NaN_3. After charging, the column was washed thoroughly with equilibration buffer in order to remove the unbound proteins. The CM-Sepharose-bound protein was; eluted

from the column using a salt gradient of 0-300 mM NaCl prepared in the equilibration buffer. The flow rate was adjusted to 12 ml/h using a peristaltic pump P-l (Pharmacia) and fractions of 3 ml each were collected on a fraction collector FRAC-100 (Pharmacia). The fractions for individual peaks were pooled after checking for agglutination activity and were rechromatographed under similar experimental conditions. The protein in these fractions was precipitated with 60 % saturation of $(NH_4)_2 SO_4$. The precipitate was dissolved in and dialyzed against CB and used for gel filtration.

2.2d *Gel filtration chromatography:* Lectin fractions obtained from CM-Sepharose column were further purified by gel filtration on Ultrogel (AcA44) column (560 × 16 mm), pre-equilibrated with elution buffer. The column was eluted with CB containing 0·5 M NaCl at a flow rate of 12 ml/h and 5 ml fractions were collected and were checked for agglutination as well as α- and *β*-galactosidase activities. The peak fractions (35-42) of BGL-I and peak fractions (35-44) of BGL-II were pooled, precipitated with 60% saturation of $(NH_4)_2SO_4$ and used for gel filtration on FPLC.

2.2e *Fast protein liquid chromatography:* The protein fractions obtained from AcA44 were further purified by gel filtration on fast protein liquid chromatography (FPLC) (Pharmacia) using Superose-12 column which was equilibrated with CB containing 0·5 M NaCl and 200 µg/ml NaN_3. The same buffer as in the case of Ultrogel (AcA44) was used for elution. The flow rate was kept at 0·7 ml/min. Fractions (0·5 ml size) were collected for BGL-I and BGL-II and checked for agglutination and α- and *β*-galactosidase activities.

2.3 *Agglutination assay*

Agglutination assay was carried out by using trypsin-treated (500*µ*g/ml) rabbit erythrocytes of 2% suspension in PBS (Hankins *et al* 1980). Two-fold serial dilutions of 50 µl of lectin solution (10-50 µg) with 0·3 M NaCl were incubated with 50 µl of erythrocyte suspension in a microtitre plate for 30 min at room temperature (25°C) and were examined under a microscope. The titre strength of lectin was determined as agglutination units (HAU: haemagglutination units) taking reciprocal of the last dilution which showed detectable agglutination. The specific activity of lectin was expressed as HAU per mg of protein.

2.4 *Protein estimation*

Protein was estimated by the method of Lowry *et al* (1951) or by the Biuret method (Layne 1957). Lipidfree bovine serum albumin was used as the standard protein.

2.5 *Assay for galactosidases*

The enzyme activities of a- and *β*-galactosidases were assayed following the method given by Murray (1983), using *p*-nitrophenyl-*α*- and *β*-D-galactopyranosides as substrates. The assay mixture contained 20-100 µl of enzyme solution and 1 ml of 3 mM substrate prepared in 0·1M Na-acetate buffer, pH 4·7. The mixture was incubated at

30°C for 45 min and the reaction was stopped by adding 2 ml of 0·2 M Na-carbonate. The enzyme activity was determined by measuring the absorption at 400 nm in a spectrophotometer (Perkin-Elmer, Lambda 1).

The amount of enzyme which hydrolyzes 1 µmol of substrate per min, per ml at 30°C was taken as one unit of enzyme activity. Specific activity was expressed as units per mg of protein.

2.6 *Assay for inhibition of agglutination*

Inhibition of agglutination was studied by using a variety of sugars according to the method described by Kurokawa *et al* (1976). Two-fold serial dilutions of various test sugar solutions (25 µl) in 25 mM CB, pH 4·7 were incubated with 25 µl (5–10 µg) of lectin solution for 30 min at room temperature. Agglutination assay was performed as described above. The inhibitory concentration was the one which failed to agglutinate erythrocytes.

2.7 *pH stability*

The pH dependence of BGL was examined using buffer ranging from pH 1-10. The buffers used were: 0·1N HCl for pH 1; 50mM glycine-HCl for pH 2 and 3; 50 mM Na-acetate-acetic acid for pH 4 and 5; 50 mM maleic acid-NaOH for pH 6; 50 mM Tris-HCl for pH 7 and 8; and 50 mm glycine-NaOH for pH 9 and 10. A volume of 50 µl lectin solution (40 µg) was incubated with 50 µl of buffer for 1 h at room temp. The agglutination as well as enzyme assay were performed as described earlier. A graph was plotted for pH vs. residual enzyme activity.

2.8 *Effect of temperature and thermal Inactivation*

Inactivation of BGL was studied by incubating samples at various temperatures. For both BGL-I and BGL-II, lectin solution (250 µl of 4 5 µg/µl) was taken in screw cap tubes containing 250 µl of 25 mM CB, pH 4·7. Each of the sample was heated for 10 min at 25, 35, 45, 55, 65, 75, 85 and 100°C and 25 µl aliquots were withdrawn from each treatment and were checked for residual agglutination and enzyme activities. The inactivation kinetics of BGL was checked by heating 225 µl (4·5 µg/µl) of each lectin solution taken in screw cap tubes containing 225 µl of 25 mM CB, pH 4·7 and was heated at 45°C in a Multi Block heater. At regular interval 25 µl aliquot was withdrawn and checked for agglutination and galactosidases activities.

2.9 *Effect of metal ions*

The metal ion requirement for lectin activity was examined by demetalizing the sample and then treating with different metal ions (Kawagishi *et al* 1990). Lectin solution (100 µl of 4·5 µg/µl) was taken in an eppendorf tube and incubated with 400 µl of 10 mM EDTA at pH 5·0 for 20 h at 4°C. The sample was then dialyzed against 25 mM CB, pH 4·7 and 50 µl aliquots were transferred to eppendorf tubes containing 50 µl of l mM $CaCl_2$, $MnCl_2$ or $MgCl_2$ and incubated for 2h. Activity of the samples were then examined by agglutination and enzyme assays as described above.

2.10 *Polyacrylamide gel electrophoresis*

Low pH, non-dissociating, discontinuous system (Reisfeld *et al* 1962) was used for native protein electrophoresis. The method given by Laemmli (1970) was followed for dissociating, discontinuous electrophoresis for polypeptide separation and molecular weight determination. Acrylamide concentration used for non-dissociating system was 10% and that of dissociating system was 12%. Molecular weight markers from Pharmacia were used as standard proteins. Electrophoresis was carried out at constant voltage (100 V) for 5 h on a Pharmacia electrophoretic apparatus (GE 2/4). After electrophoresis the gels were stained with 0 1 % Coomassie brilliant blue (R 250) prepared in destaining solution containing acetic acid: methanol: water (1:3:6 v/v).

2.11 *Isoelectrofocussing*

Isoelectrofocussing of native lectin was done on Polyacrylamide gel in tubes (14 × 4 mm) using ampholine of pH range 3–10, for 6000 Volt-hours. A solution of 10 µl (100 µg) lectin was treated with 20 µl of diluent (10 mg methyl red, 0·3 ml ampholine pH 3-10, 0·75 g sucrose in a final volume of 5 ml). Ethylenediamine 0·01 M and iminodiacetic acid 0·01 M were used as cathode and anode buffers, respectively. The gels were prefocussed for 30 min at 450 Volts (constant). The isoelectrofocussing was carried out for 6000 Volt-hours.

2.12 *Equilibrium dialysis*

Equilibrium dialysis studies on binding of D-galactose to BGL were made according to the method given by Colleen *et al* (1975) using ^{14}C-D-galactose at room temperature. Control experiments showed that equilibrium was reached in 16-20 h. The purified lectins (40-50 µg) were subjected to dialysis. The dialysis was done against varying concentrations of the radioactive sugar ($1{\cdot}7 \times 10^7$ cpm/µmol) in 25 mM CB, pH 4·7 for 24 h at room temperature in a sterilized dialysis cell with wells of 10 mm dia. and 4 mm depth. Two such pieces were kept face to face partitioned by a dialysis membrane. Total volume was made up to 200 µl in each compartment. Aliquots were withdrawn and radioactivity was measured using Packard Tri-Carb liquid scintillation spectrometer (Model 3255). A graphic analysis of the data was done by the Scatchard (1949) plot.

2.13 *Amino acid analysis*

Amino acid analysis of lectins were carried out by the method of Ashman and Bosserhoff (1985) on a Pharmacia HPLC system, using dual pump binary gradient system and C-18 column. Lectin samples were hydrolyzed in 1 ml of 6 N HCl at 110°C for 24 h and the dried, acid-free hydrolysate was dissolved in 100 µl of 0·25 M potassium borate buffer, pH 10·4 before derivatization. Tryptophan was determined by the spectrophotometric method of Spande and Witkop (1967).

3. Results

3.1 *Ion-exchange chromatography*

The elution pattern of lectin using a linear gradient of 0–300 mM NaCl is shown in figure 1. The graph was plotted by considering maximum agglutination activity as

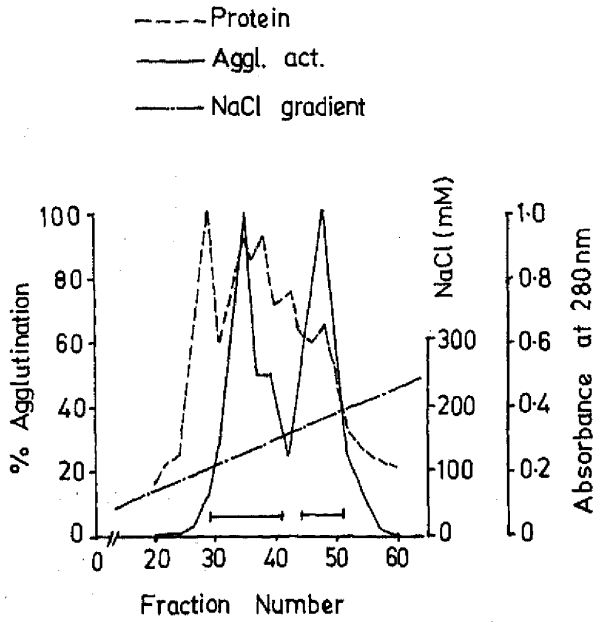


Figure 1. Ion-exchange chromatography of BGL on CM-Sepharose using NaCl gradient 0-250 mM. (---), Protein measured at 280 nm; (—), Agglutination activity considering highest activity as 100%. Bar indicates the number of fractions pooled for BGL-I and BGL-II.

100%. There are two peaks of agglutination activity (fractions 29 to 41 and 43 to 52). The peak which eluted first, at 130 mM NaCl concentration was designated as BGL-I and the latter, eluted at 180 mM NaCl concentration as BGL-II. All the fractions showing agglutination activity were pooled and subjected to gel filtration on AcA44.

3.2 *Gel filtration chromatography*

On gel filtration the BGL-I peak was broadly resolved into a peak with a shoulder having agglutination activity (figure 2). The BGL-II separated into a major peak of lectin activity (figure 3). In addition to lectin activity the former peak (BGL-I) showed α- and *β*-galactosidase activity (figure 2) and the latter (BGL-II) showed only α-galactosidase activity. Further purification of BGL-I on FPLC resulted into a symmetrical agglutination peak having both *α*- and *β*-galactosidase activities (figure 4). BGL-II on FPLC resulted into a single peak of agglutination activity which showed only α-galactosidase activity (figure 5). The extent of purification is shown in table 1. The final recovery of BGL-I was 16.6% with a purification fold of 114 and that of BGL-II was 5·5% with a purification fold of 110.

3.3 *Criteria of purification*

On non-dissociating, low-pH (acid) gel BGL-I showed a discrete band with a relative mobility (R_f) of 3·07 and BGL-II resolved into a single band with R_f value of 3·13 (data not shown). The R_f values were calculated from the mobility of protein bands with respect to the migration of the dye Front.

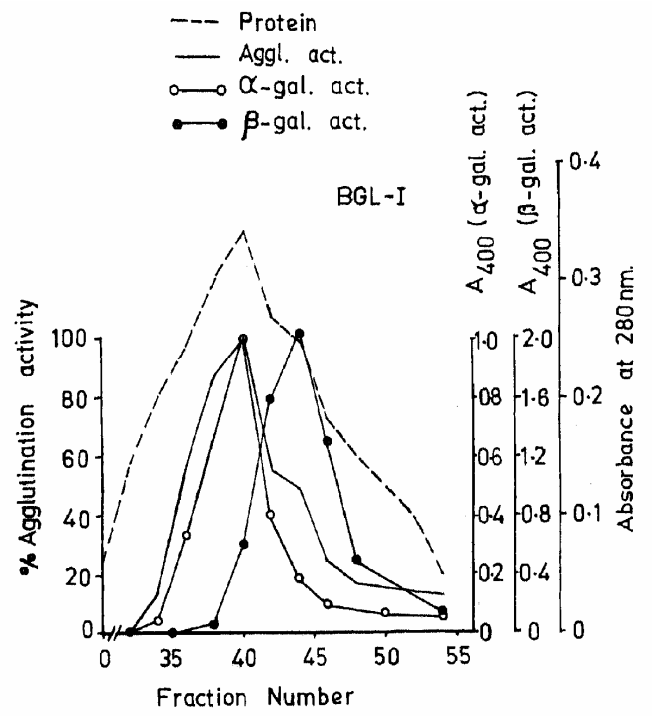


Figure 2. Gel filtration chromatography of BGL-I on Ultrogel (AcA44). (– – –), Protein measured at 280 nm; (——), % Agglutination activity; (O, ●) α– and *β*-galactosidase activity measured at 400 nm.

Table 1. Purification chart of blackgram lectin*.

Sample	Total Protein (P) (mg)	Total Aggl. unit (HAU)	Sp. Act. HAU/mg P	Purification fold	Recovery of Aggl. act. (%).
Crude extract	3036	2,709,200	892	1	100
0·5 M NaCl elute from CM-Sepharose	240	1,702,400	7093	8	62·8
60% $(NH_4)_2SO_4$ ppt dissolved and dialyzed	159·7	1,501,867	9404	11	55·4
Ion-exchange chromatography					
Peak 1(BGL-I)	15·7	863,500	55,176	62	31·9
Peak 2(BGL-II)	10·1	496,700	49,374	55	18·3
Gel filtration (AcA44)					
BGL-I	9·4	809,200	86,177	97	29·9
BGL-II	5·0	325,465	64,576	72	12·0
Gel filtration (FPLC)					
BGL-I	4·4	450,560	101,386	114	16·6
BGL-II	1·5	149,760	98,462	110	5·5

* 100 g cotyledons from soaked seeds of TAU-1 was used for extraction.

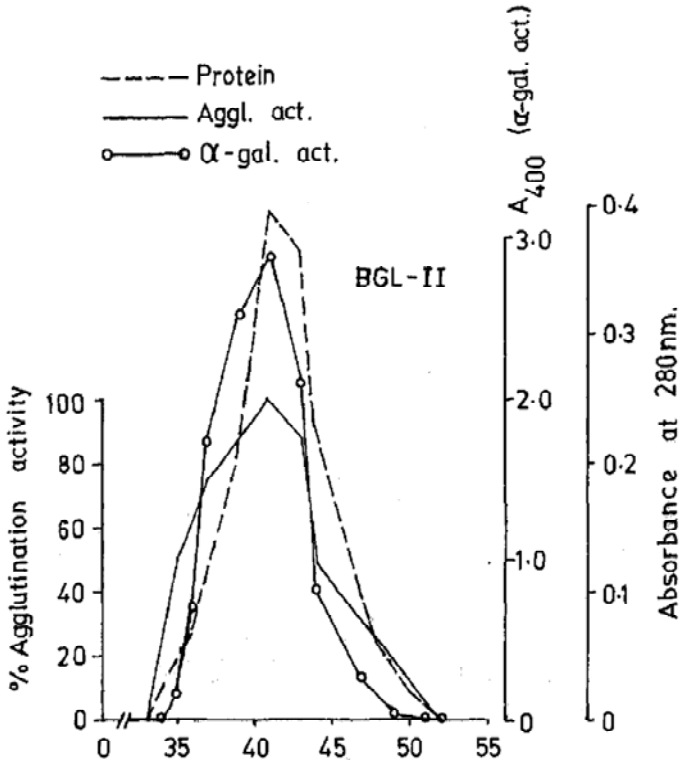


Figure 3. Gel filtration chromatography of BGL-II on Ultrogel (AcA44). (- - -), Protein measured at 280 nm; (——), % Agglutination activity units; (O), α-galactosidase activity measured at 400 nm.

On SDS-polyacrylamide gel BGL-I showed two bands of M_r 94 kDa and 89 kDa (figure 6, lane a). As the native M_r of BGL-Idetermined by gel filtration was 105 kDa (data not shown), the two polypeptides correspondingly appeared to be separate entities. BGL-II moved as a single band on SDS-PAGE (figure 6, lane b) and the M_r (83 kDa) was found to be same as native M_r estimated by gel filtration. The relative position of BGL-I and BGL-II did not change in presence or absence of β-mercaptoethanol indicating the absence of S-S bonds between the polypeptide components (data not shown). Attempts to separate two polypeptides of BGL-I were not successful, although repeated chromatography on CM-Sepharose and monitoring the individual fractions on SDS-PAGE indicated relative enrichment of each polypeptide (figure 7). The enriched fraction of 94kDa (BGL-I-1) was associated with 59·7 units of α-galactosides (72%) and 23 units of β-galactosidase (28%) per mg of lectin, while the enriched fraction of 89kDa (BGL-I-2) showed 113·6 units of β-galactosidase (86%) and 18 units of α-galactosidase (14%) per mg lectin. BGL-II had only α-galactosidase activity.

3.4 *Isoelectric points*

BGL-I showed two closely spaced, sharp bands with isoelectric points (p1) 6·3 and 6·2, where as BGL-II showed a single band with pl of 5·8 (figure 8).

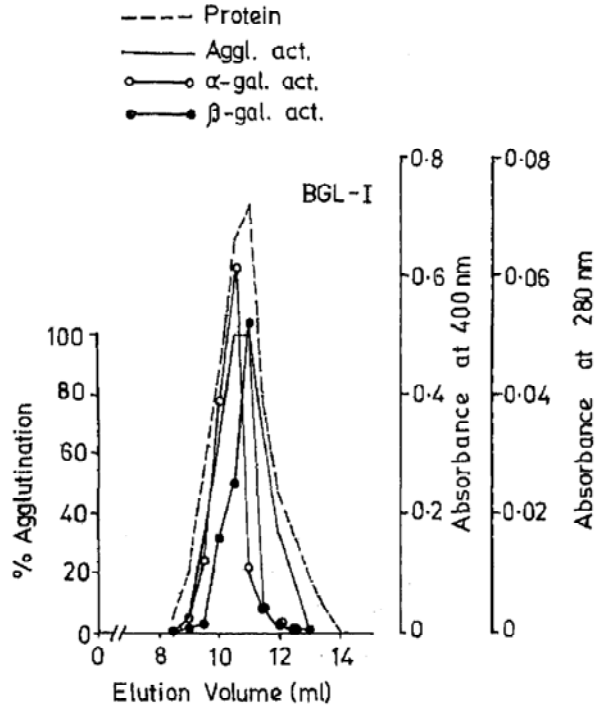


Figure 4. Gel filtration chromatography of BGL-I on FPLC. (- - -), Protein measured at 280 nm; (———), % Agglutination activity; (O, ●), α- and *β*-galactosidase activity measured at 400 nm.

3.5 *Physical and chemical properties of BGL*

3.5a *Thermostability, kinetics of heat Inactivation and fate of BGL at Inactivation temperature:* When BGL was heated from room temperature to 100°C at intervals of 10°C, a linear relationship was observed for per cent residual activity vs. temperature. When BGL was heated at 45°C, 50% of agglutination activity was lost in 10 min and more than 90% of activity was lost in 60 min. At Inactivation temperature of 50°C, BGL lost 50% of its agglutination activity at 5 min and was totally inactivated at 10 min. At this temperature 90% α-galactosidase activity was lost in 10 min, while *β*-galactosidase activity was reduced by 20% (data not shown).

3.5b *pH stability and pH optima:* The pH dependence of BGL was determined by incubating in buffers of varying pH. The lectin was found to be active between pH 3·5 and 7·5 (figure 9). The pH profile of associated *α*- and *β*-galactosidase activities were found to be similar to that of agglutination. The optimum pH for agglutination activity was between pH 4 and 5 and that of *α* and *β*-galactosidase was pH 4·5. BGL lost 50% agglutination activity between pH 6 and 7, and about 90% activity at pH 8. BGL was found to be totally inactive at pH 3 and 8.

3.5c *Michaelis constant:* The apparent Michaelis constants (K_M) of α-galactosidase for *p*-nitrophenyl-*α*-D-galactopyranoside was $0{\cdot}5 \times 10^{-3}$ M for both BGL-I and

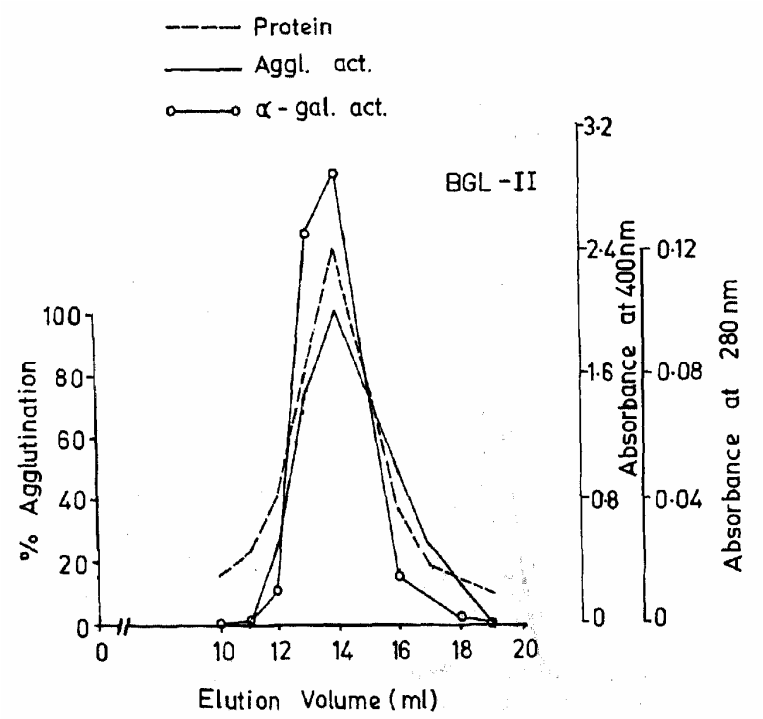
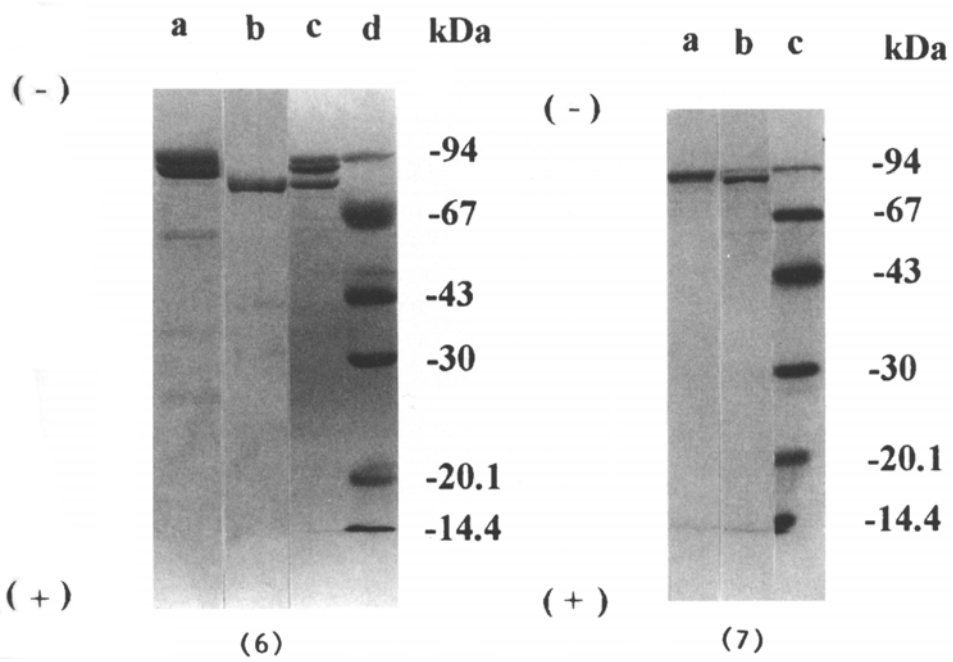


Figure 5. Gel filtration chromatography of BGL-II on FPLC. (- - -), Protein measured at 280nm; (———), % Agglutination activity; (O), α-galaqtosidase activity measured at 400 nm.

BGL-II. In presence of 5 mM D-galactose, the K_M was changed to $1{\cdot}00 \times 10^{-3}$ M and $1{\cdot}14 \times 10^{-3}$ M for BGL-I and BGL-II, respectively.

3.5d *Effect of metal ions:* When the EDTA treated BGL samples were incubated with metal ions like Ca^{2+}, Mg^{2+} and Mn^{2+} no change in the activities was observed before and after treatments. Heavy metals like Ba^{2+} and Pb^{2+} also did not inhibit activities of BGL.

3.5e *Inhibition with sugars:* Both BGL-I and BGL-II showed similar effect with different sugars (table 2). The agglutination activity was readily inhibited by D-galactose at 0·1 mM and by *p*-nitrophenyl-*α*-D-galactopyranoside at 1·5 mM concentration, α- and *β*-anomers of methyl-galactopyranoside derivatives, D-raffinose, α-L(-) fucose and α-*D*(+) melibiose inhibited agglutination at 12·5 mM concentration. The inhibitory concentrations of myo-inositol and D (+) xylose were 25 mM and that of L(+) arabinose and D(+)glucosamine was 50 mM. *α*-D(+)fucose, D-glucose, methyl-α-D-mannopyranoside, D(+)cellobiose, L-galactose, stachyose, *p*-nitrophenyl-*β*-D-galactopyranoside, N-acetyl-D-glucosamine, isopropyl-*β*-D-thio-galactopyranoside, lactose, D(+)galactosamine, *p*-nitrophenyl-*α*-D-glucopyranoside, *p*-nitrophenyl-*β*-D-glucopyranoside did not inhibit the agglutination activity up to 100 mM concentration.



Figures 6 and 7. (6) BGL on SDS-PAGE. Each lane contained 30 μg protein and the protein was visualized by Coomassie brilliant blue staining, (a), BGL-I; (b), BGL-II; (c), BGL-total; (d), molecular weight markers. (7) BGL-I on SDS-PAGE after being enriched by rechromatography on CM-Sepharose. Each lane contained 30 μg lectin and protein was visualized by Coomassie brilliant blue (R-250) staining, (a), BGL-I-1; (b), BGL-I-2; (c), molecular weight markers.

3.6 *Equilibrium dialysis*

The Scatchard plots of binding data show biphasic curves, indicative of multiple binding sites (figure 10). Both BGL-I and BGL-II seemed to have two types of binding sites of high and low affinity. The calculated values of association constants for high and low affinity sites were $9{\cdot}77 \times 10^3$ M^{-1} and $1{\cdot}32 \times 10^3$ M^{-1} for BGL-I and $8{\cdot}00 \times 10^3$ M^{-1} and $0{\cdot}56 \times 10^3$ M^{-1} for BGL-II, respectively.

3.7 *Amino acid analysis*

The amino acid compositions of BGL-I and BGL-II are shown in table 3. Both the lectin species were found to be rich in aspartic acid, glutamic acid, serine, glycine and alanine. Methionine was not detected in blackgram lectins. The number of tryptophan residues was calculated spectrophotometrically. The present method could not determine proline. No attempt was made to determine cysteine.

4. Discussion

The two lectins BGL-I and BGL-II are present both in dry as well as soaked seeds. We did not find any modification of these lectins upon seed imbibition as the lectin patterns

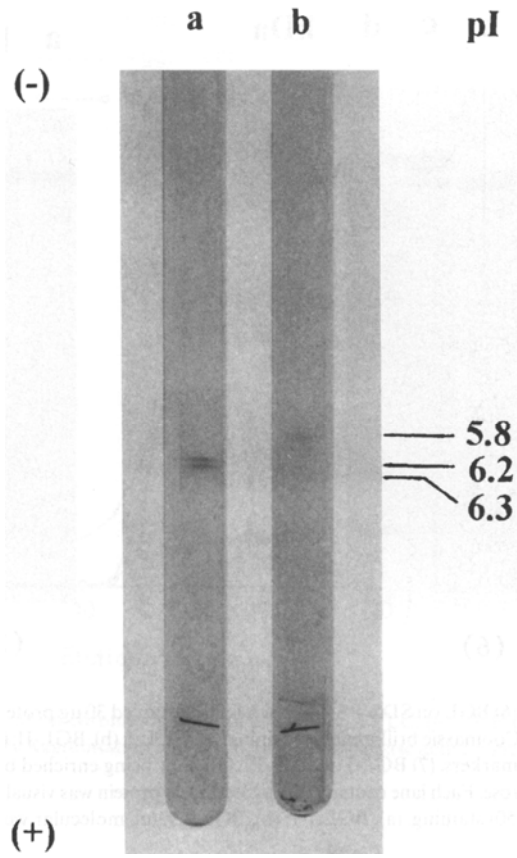


Figure 8. Isoelectrofocussing pattern of native BGL. 100 µg protein was used for each lectin species, (a), BGL-I; (b), BGL-II.

were same on the Western blot of the seed extracts, when a mixture of anti-BGL-I and BGL-II antibodies were used to detect the lectins (Suseelan 1995). The seeds were soaked in order to remove the seed coat which contains polyphenols.

Blackgram seeds contain mucilage which interfered with the protein purification procedures employed initially. This problem was overcome by extracting lectin with citrate buffer at pH of 4.7, which rendered lectin soluble and precipitated mucilage and other seed storage proteins. The soluble protein was adsorbed by CM- Sepharose at pH 4·7 and the eluted lectin was further purified by conventional methods. Although BGL was found to be galactose-specific, attempts to use affinity chromatography did not succeed. We tried several affinity matrices such as galactose-agarose, melibiose-agarose, stachyose-agarose, N-acetyl galactosamine-agarose, guar gum and fetuin-agarose. The possible reason for the failure could be the cleavage of the terminal galactose (to which this lectin was supposed to bind) by the lectin-associated α-galacto-sidase. In the present studies 114- and 110-fold purification of the two lectins with a recovery of 16·6 and 5·5% for BGL-I and BGL-II, respectively, was achieved. Both the lectins showed the dissolution of agglutinated blood cells after 1 h as observed by

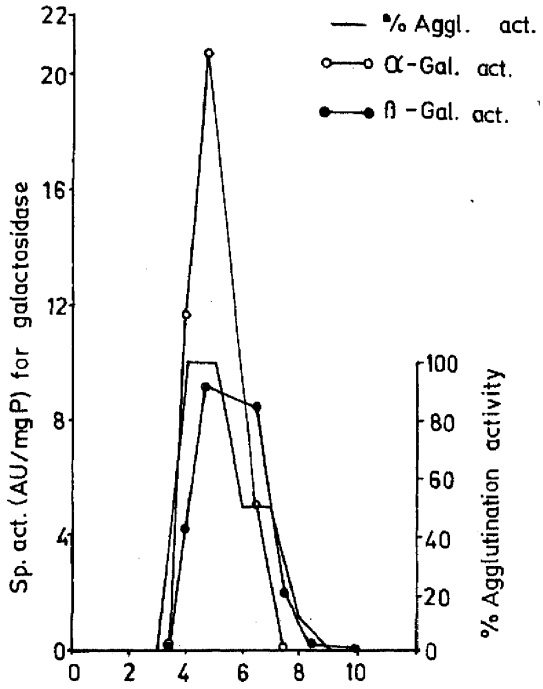


Figure 9. pH stability and pH optima of BGL. (———),Agglutination activity units; (O, ●), α- and *β*-galactosidase activity measured at 400 nm.

Table 2. Inhibition of agglutination of blackgram lectin by sugars.

	Inhibitory concentration (mM)	
Sugar	BGL-I	BGL-II
D-galactose	0·1	0·1
p-nitrophenyl-α-D-galactopyranoside	1·5	1·5
Methyl-α-D-galactopyranoside	12·5	12·5
D-raffinose	12·5	12·5
α-L(-) fucose	12·5	12·5
α-D(+) melibiose	12·5	12·5
myo-inositol	25	25
D(+)xylose	25	25
L(+)arabinose	50	50
D(+)glucosamine	50	50
D(+)mannose	100	100
N-acetyl-D-galactosamine	100	100

L-galactose, D-glucose,D-cellobiose,methyl-*α*-D-mannopyranoside, *p*-nitrophenyl-*β*-D-galactopyranoside, N-acetyl-D-glucosamine, lactose, α-D(+)fucose, D(+)galactosamine, stachyose, isopropyl -*β*-D-thiogalactopyranoside, *p*-nitrophenyl-*α*-D-glucopyranoside.*p*-nitrophenyl *β*-D-glucopyranoside and *p*-nitrophenyl-*α*-D-mannopyranoside did not inhibit even at 100 mM concentration.

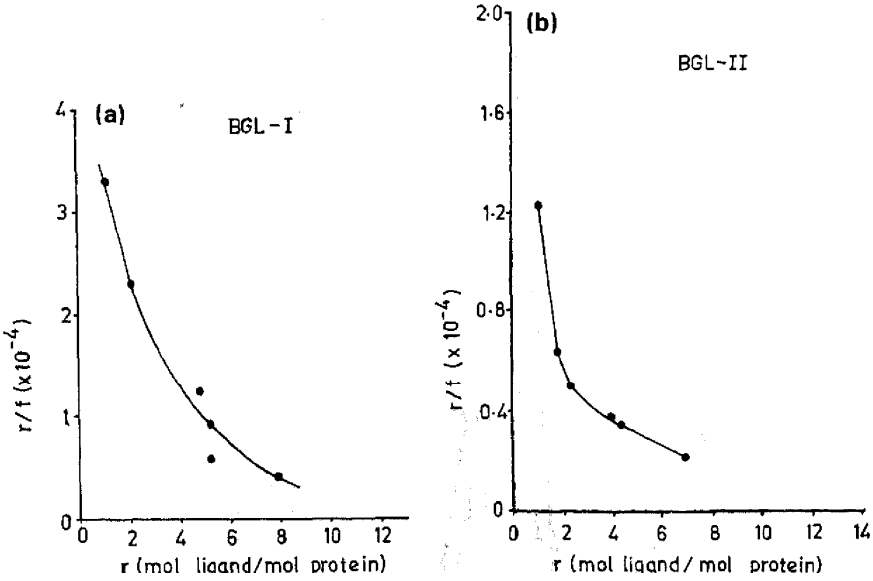


Figure 10. Scatchard plot of BGL **(a)** BGL-I and **(b)** BGL-II. X-axis indicate mol ligand bound per mol of protein and Y-axis indicate *r/f,* where *r* is the ligand bound per mol of protein and *f* is the free ligand.

Table 3. Amino acid composition of blackgram lectins.

	mol percentage	
Amino acid	BGL-I	BGL-II
Aspartic acid	17·1	9·8
Glutamic acid	23·3	14·7
Serine	12·4	13·5
Histidine	3·8	4·9
Glycine	10·5	8·6
Threonine	1·4	1·5
Arginine	1·9	3·7
Alanine	9·0	9·8
Tyrosine	1·4	2·1
Methionine	–	–
Valine	4·8	6·1
Phenylalanine	3·8	5·5
Isoleucine	1·9	3·7
Leucine	2·9	4·6
Lysine	5·7	11·6
Proline	ND	ND
Cysteine	ND	ND
Tryptophan*	3·4	3,4

ND, Not determined.

* Tryptophan was calculated spectrophotometrically.

Hankins and Shannon (1978) with mungbean agglutinin. The lectins were found to be inactivated on freezing and thawing. They could be preserved in 15 % glycerol at 20°C.

Both BGL-I and BGL-II are monomeric lectins, though native M_r of BGL-I determined by gel filtration chromatography (105 kDa) was different from the M_r on SDS-PAGE (94 kDa and 89 kDa). BGL-I on native PAGE gave a single band, indicating that the two polypeptides have very close mass and charge ratios and could not be separated by the present methods. In FPLC also the separation resulted into single peaks of activities for both BGL-I and BGL-II (figures 4 and 5). Such discrepancy of native M_r and M_r on SDS-PAGE was observed in other lectins also (Nicolson *et al* 1974; Shet and Madaiah 1988). The two lectins of BGL-I could be only partially enriched by rechromatography on ion exchange column as adjudged visually from the staining intensity (figure 7). The partially purified fractions of BGL-I-1 showed 72% of α-galactosidase activity, while BGL-I-2 snowed 86% of *β*-galactosidase activity, when expressed on the basis of total activity of both α- and *β*-galactosidase activities in enriched fractions of individual lectins.

Previously Singh and Rao (1991) isolated a 58 kDa monomeric agglutinin from blackgram, with *β*-galactosidase activity, but without any sugar specificity for simple as well as complex sugars. Later, Sharma and Salahuddin (1993) reported a 132 kDa dimeric blackgram lectin (66 kDa × 2) without any galactosidase activity. The present blackgram lectins are associated with galactosidase activities. After the discovery of an enzymatic lectin in *Vigna radiata* (Hankins and Shannon 1978), lectins with galactosidase activity have been reported from other legume species also (Dey *et al* 1982a, b). However, lectin with associated *β*-galactosidase activity has not been reported, except a mention of presence of *β*-galactosidase activity with M_r 58 kDa blackgram lectin by Singh and Rao (1991). Our results showed that BGL-I-2 *(*M_r 89 kDa) was associated predominantly with *β*-galactosidase activity. Since this lectin activity could be inhibited with D-galactose and not with *β*-anomer of galactoside, the protein could be a *β*-galactosidase with alternate sites for D-galactose binding for its lectin activity. We are not sure whether the lectin and galactosidase activities reside in the same molecule as the protein is monomeric. The possibility exists as the enzyme activities are copurified with the lectin protein with similar mass and charge characteristics. Most of the legume lectins and galactosidases reported in the literature are polymeric and the lectin and α-galactosidase activities could be located into separate subunits of the native protein (Liener 1976; Roberts *et al* 1982). Only a limited number of lectins consist of a single polypeptide (Suvachittanout and Peutpaiboon 1992; Utarabhand and Akkayanont 1995). No carbohydrate moiety was detected in any of the blackgram lectins (data not shown). The blackgram lectin reported by Sharma and Salahuddin (1993) contained 8 2% carbohydrate. The disaggregation property and the reactions towards human erythrocytes of BGL were similar to those of *Vigna radiata* observed by Hankins and Shannon (1978). D-galactose was found to be the most potent inhibitor of BGL with preference for the α-anomer.

The pH optima for agglutination and both α- and *β*-galactosidase activities were found to be similar. Temperature of Inactivation for agglutination and α-galactosidase activities were the same as reported by Hankins and Shannon (1978). However, *β*-galactosidase was not inactivated at the Inactivation temperature of lectin activity, which suggests that sites for lectin and *β*-galactosidase could be different. The apparent K_M for *p*-nitrophenyl-*α*-D-galactopyranoside was 0·5 mM for both BGL-I and BGL-II. The affinities were reduced by 2-fold when reaction was carried out in presence of

D-galactose. In mungbean lectin, Hankins and Shannon (1978) found K_m of 0·34 mM for pNP-α-Gal. The observed affinity for galactose is also reflected in the relative concentration required to inhibit enzyme activities (K_i = 1 mM) and complete inhibition of lectin activity (0·1 mM).

The Scatchard plots of BGL-I and BGL-II are nonlinear and biphasic in nature (figure 10). The objective of studying galactose binding to BGL-I and BGL-II was to determine the valency of these lectins as they are monomeric in nature. The biphasic Scatchard plots indicate that both lectins have two types of binding sites of high and low affinity. Similar observations were made with the monomeric lectin, the B chain of the toxin äbrin a and b, with two saccharide binding sites with differential affinities (Ohba *et al* 1993). In *Araucaria brasiliensis* (Gymnospermae), the two polymeric lectins also showed two binding sites with different affinities for sugars and each lectin subunit had more than one site for sugar (Datta *et al* 1993). The curved nature of the Scatchard plots obtained in this study indicate the existence of multiple binding sites on the monomeric lectins, BGL-I and BGL-II.

The amino acid composition of BGL is typical of a legume lectin (Lis and Sharon 1981), devoid of methionine (table 3), and a common genetic origin of these proteins has been suggested. Like other plant lectins which are abundant in storage organs, as plants accumulate part of their nitrogen reserve in the form of carbohydrate binding proteins, blackgram lectin also can be one such 'chimerolectin' (Peumans and Van Damme 1995) which have a galactose-specific carbohydrate binding domain and also catalytic domains. The functions of such fusion proteins in the plant life cycle are yet to be investigated.

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