Purification and Some Properties of *Phaseolus mungo* Lectin

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Phaseolus mungo lectin in pure form was isolated from seeds by affinity chromatography on a galactosyl Sepharose 6B column. The lectin contained 8.3% neutral carbohydrate. It absorbed maximally near 278 nm, and the corresponding maxima in fluorescence excitation and emission spectra occurred, respectively, at 278 and 337 nm. Its elution profile from an analytical Sephadex G-150 column was consistent with a molecular weight of 137 000 and a Stokes radius of 4.3 nm; the frictional ratio, f/f_0, of the lectin was calculated to be 1.26. The lectin moved as a single protein band of M_r 66 000 in SDS-PAGE under both reducing and nonreducing conditions. The results suggested that the lectin is a dimer whose overall native conformation is nearly globular. From the effect of 10 saccharides on the hemagglutinating activity of lectin against trypsinized rabbit erythrocytes, it was concluded that the lectin was galactose/*N*-acetylgalactosamine-specific, having significantly reduced affinity for *N*-acetylgalactosamine. The lectin showed preference for α-glycoside and probably does not contain a hydrophobic binding site.

Tissues of leguminous plants such as seeds, leaves, bark, and roots have been shown to contain lectins (Lis and Sharon, 1986; Strosberg et al., 1986; Sharon and Lis, 1990). However, legume lectins have been generally isolated from mature seeds, where they constitute as much as 10% of the total seed protein. The bulk of the seed lectins are located in protein bodies in cotyledons. Available sequence data on over 15 legume lectins show extensive sequence homology (Strosberg et al., 1986; Sharon and Lis, 1990). Further, cross-hybridization studies on cDNAs of several legume lectins suggest that the lectin genes must have evolved from a common ancestral gene (Sharon and Lis, 1990). Therefore, many molecular and structural properties of the legume lectins are expectedly similar. Accordingly, all legume lectins show subunit structure and almost all of them are glycoproteins and metalloproteins (Lis and Sharon, 1986; Strosberg et al., 1986; Sharon and Lis, 1990). Besides the saccharide binding site, they contain metal binding and hydrophobic binding sites. Available crystal structures of several legume lectins including concanavalin A reveal similar structural organization (Sharon and Lis, 1990). However, they differ markedly in carbohydrate binding specificity (Lis and Sharon, 1986; Strosberg et al., 1986; Sharon and Lis, 1990).

In this paper we report the isolation of *Phaseolus mungo* lectin in pure form, along with the results on some of its important physiochemical and carbohydrate binding properties, whose delineation would form an essential prerequisite for a proper understanding of the role of the lectin in plant physiology. Although the exact physiological role of legume lectins remains obscure, evidence exists to show that lectins may be involved in *Rhizobium*-legume interactions (Hamblin and Kent, 1973; Bohlool and Schmidt, 1974; Dazzo et al., 1976, 1978; Dazzo and Brill, 1977; Dazzo and Truchet, 1983; Halverson and Stacey, 1986; Diaz et al., 1989). Unlike many legume lectins that are specific for *N*-acetylgalactosamine and show little or no affinity for galactose, *P. mungo* lectin binds galactose and shows measurable but reduced affinity for *N*-acetylgalactosamine.

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MATERIALS AND METHODS

Marker proteins, sugar substrates, trypsin type II (lot T-8128), Sephadex G-150, and Sepharose 6B were purchased from Sigma Chemical Co., St. Louis, MO. Reagents used in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were the same as reported earlier (Waseem and Salahuddin, 1983). Divinyl sulfone was a Merck product (Darmstadt, Germany). *P. mungo* seeds were obtained from the local market. Other chemicals were of reagent grade.

Light absorption and fluorescence measurements were made on a Cecil CE 202 UV double-beam spectrophotometer and a Shimadzu R-540 spectrofluorometer, respectively. Protein concentration was routinely determined according to the method of Lowry et al. (1951) using crystalline bovine serum albumin as a standard. The neutral carbohydrate and sialic acid contents of the lectin were determined according to the methods of Dubois et al. (1956) and Warren (1959), respectively. SDS-PAGE was carried out in 7.5% gel according to the procedure of Weber and Osborn (1969).

The hemagglutinating activity of *P. mungo* lectin was measured against trypsinized rabbit erythrocytes (Ali and Salahuddin, 1989). Typically, the assay mixture consisted of 88 μg of lectin in 10 mM Tris-HCl buffer, pH 7.4, containing 0.25 M NaCl (operating buffer) and 1×10^6 cells in the 1 mL of the incubation mixture. The number of unagglutinated cells was counted and hemagglutinating activity of the lectin calculated.

Isolation of Lectin. To isolate the lectin from *P. mungo* seeds by affinity chromatography, first, affinity gel was prepared according to the method of Fornstedt and Porath (1975). Sepharose gel (50 mL) in 0.5 M carbonate buffer, pH 11.0, was treated with 5 mL of divinyl sulfone (final concentration, 10% v/v) for 70 min with continuous stirring. The activated gel slurry was treated overnight with 20% (w/v) galactose solution in 0.5 M carbonate buffer, pH 10.0. The affinity gel was washed with distilled water and treated with 0.14 M 2-mercaptoethanol in 0.5 M carbonate buffer, pH 8.5, for 2 h. Legume seeds (50 g) were homogenized in the operating Tris-HCl buffer and subsequently acidified with 0.3 M acetic acid (pH 4.0) overnight at 10 °C to remove extraneous proteins. The clarified seed homogenate (30 mL containing 153 mg of protein) was then applied on the galactosyl Sepharose 6B column. The column was washed with buffer alone, and the fractions were assayed for protein according to the method of Lowry et al. (1951) (Figure 1). The bound lectin was specifically eluted with 0.25 M galactose in the operating buffer.

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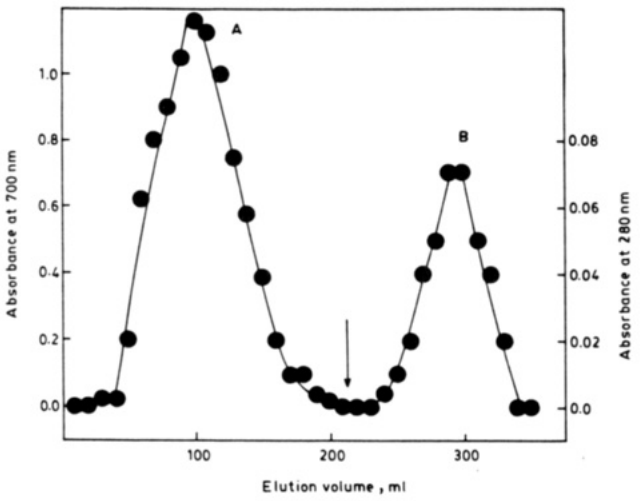

Figure 1. Isolation of *P. mungo* lectin by affinity chromatography on a galactosyl Sepharose 6B column (4 × 8.8 cm). Protein fractions under peak A were obtained by elution with the buffer alone. The bound lectin was specifically eluted with 0.25 M galactose in the Tris-HCl buffer and the protein content determined spectrophotometrically.

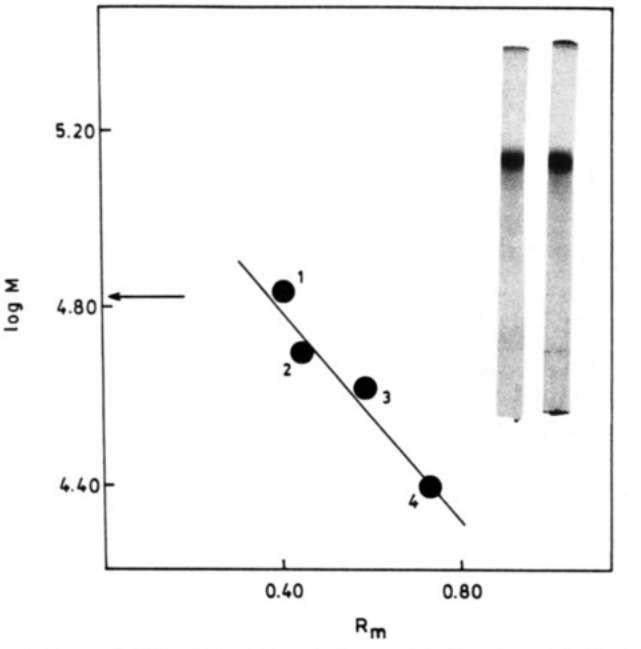

Figure 2. SDS–PAGE of *P. mungo* lectin under reducing (A, left) and nonreducing (B, right) conditions. Marker proteins or lectin (45 μg) was electrophoresed in 7.5% gel in the presence of 0.1 M sodium phosphate buffer, pH 7.0, containing 0.1% SDS according to the procedure of Weber and Osborn (1969). The gels were stained with 0.2% Coomassie brilliant blue R-250 and destained mechanically with 10% (v/v) acetic acid. The linear plot of log M vs R_m was obtained by the method of least-squares and fits eq 1: $\log M = -1.235R_m + 5.3$.

RESULTS

The affinity-purified lectin from *P. mungo* seeds constituted about 0.2% of the total soluble proteins in the seed homogenate. The lectin preparation was homogeneous with respect to size as evident from a single protein band in SDS–PAGE, both in the presence and in the absence of 2-mercaptoethanol (see Figure 2), and a single protein peak in gel filtration from a Sephadex G-150 column (see Figure 3). The purified lectin absorbed maximally near 277 nm in the operating buffer, and the corresponding fluorescence excitation and emission maxima occurred near 278 and 334 nm, respectively. The lectin was found to be a glycoprotein devoid of sialic acid residues; it contained 8.3% neutral carbohydrate.

The relative mobilities (R_m) of marker proteins as well as of *P. mungo* lectin in SDS–PAGE were determined (see Figure 2). The marker proteins (molecular weights) were BSA (68 000), heavy chain of goat IgG (50 000), ovalbumin (43 000), and light chain of goat IgG (25 000). The relative mobility of the lectin was measured to be 0.39 under both reducing and nonreducing conditions, which according to the equation given in the legend to Figure 2 would correspond to a molecular weight of 66 000.

The lectin was eluted from an analytical Sephadex G-150 column with an elution volume of 105 mL. The column was calibrated with the help of marker proteins (molecular weights; Stokes radii) which were goat IgG (150 000), bovine serum albumin dimer (138 000; 4.3 nm), bovine serum albumin monomer (69 000; 3.5 nm), ovalbumin (43 000; 3.0 nm), chymotrypsinogen A (25 700; 2.2 nm), and cytochrome *c* (12 400; 1.7 nm). The column characteristics were $V_t = 246$ mL, $V_0 = 78$ mL, and $V_i = 152$ mL. The molecular weight and Stokes radius of the lectin were calculated from its gel filtration behavior using equations given in the legend to Figure 3, and the respective values were 137 000 and 4.3 nm. With these values of molecular weight and Stokes radius the diffusion coefficient, D, and frictional ratio, f/f_0, of the lectin were calculated to be 5.18×10^{-7} cm^2 s^{-1} and 1.26, respectively.

Carbohydrate Binding Specificity. The affinity-purified *P. mungo* lectin readily caused agglutination of trypsinized rabbit erythrocytes in the operating Tris-HCl buffer, pH 7.4, containing 0.25 M NaCl. Preliminary results showed that the lectin did not require Ca^{2+} or Mn^{2+} for its hemagglutinating activity. Further, the hemagglutinating activity of the lectin was found to be sensitive to temperature (see Figure 4). The lectin retained its full activity when exposed to temperatures below 30 °C for 40 min. Above 30 °C the lectin underwent thermal inactivation, and at or above 60 °C the lectin was completely inactivated. The lectin-induced hemagglutination showed significant dependence on pH and ionic strength. Maximum activity was observed near pH 6.0 and at ionic strength of 0.6.

The hemagglutinating activity of the lectin was determined in the presence and absence of increasing concentrations of 10 sugars, and the results are summarized in Table I. From the curve (not shown) describing inhibition of hemagglutinating activity of the lectin as a function of increasing concentrations of a saccharide, the concentration of the saccharide required for 50% inhibition of the lectin activity, i.e., C_M, was computed. Thus, the C_M value for galactose was determined to be 9.5 mM. Likewise, C_M values for other saccharides were also determined (see Table I). Evidently glucose, sucrose, and lactose had virtually no effect on lectin-induced hemagglutination. Further, in the concentration range used in this study, galactosamine and methyl β-galactoside did not significantly affect the hemagglutinating activity of the lectin (see Table I). *P. mungo* lectin appears to have substantially higher affinity for galactose than for *N*-acetylgalactosamine. The lectin showed higher affinity for melibiose than for galactose. The fact that methyl α-galactoside was the most potent inhibitor and methyl β-galactoside did not inhibit lectin activity clearly suggests that the lectin showed preference for α-glycosides. Further, the observation that *p*-nitrophenyl α-galactoside was a slightly less effective inhibitor than methyl α-galactoside indicated the absence of any hydrophobic binding site adjacent to the saccharide binding site on the lectin.

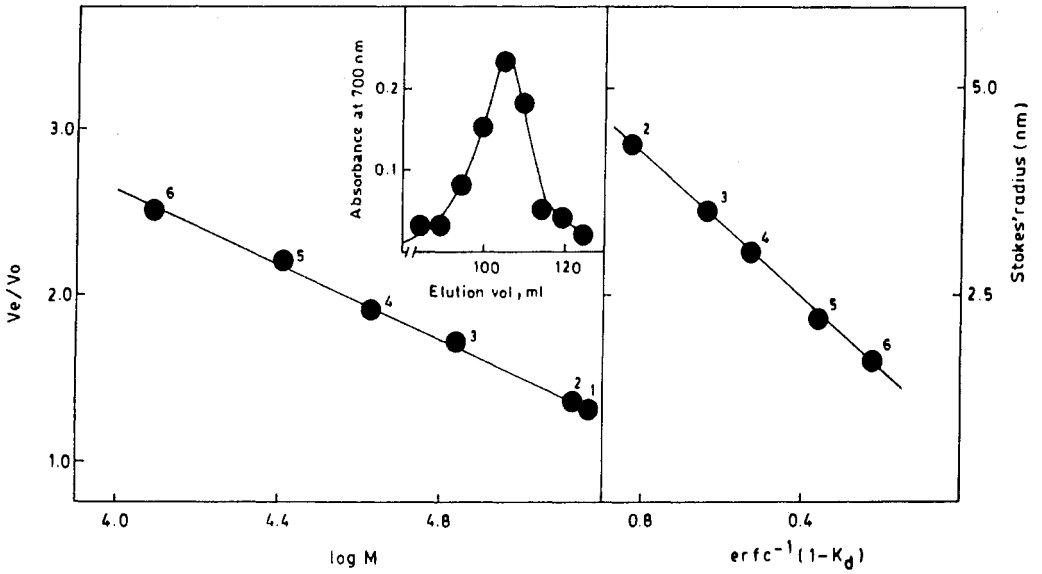

Figure 3. Gel filtration of marker proteins and *P. mungo* lectin on a Sephadex G-150 column (1.79 × 99 cm). The inset shows the elution profile of the lectin. Numerals 1, 2, 3, 4, 5, and 6, respectively, represent goat IgG, bovine serum albumin dimer, bovine serum albumin monomer, ovalbumin, chymotrypsinogen A, and cytochrome *c*. The results were analyzed according to the methods of Andrews (1970) and Ackers (1970) by the method of least-squares, and the two straight lines obey the following equations $V_e/V_0 = -1.135 \log M + 7.18$ (A); $r = 4.393\ \text{erfc}^{-1}(1 - k_d) + 0.682$ (B). Equation B was obtained without considering results on goat IgG.

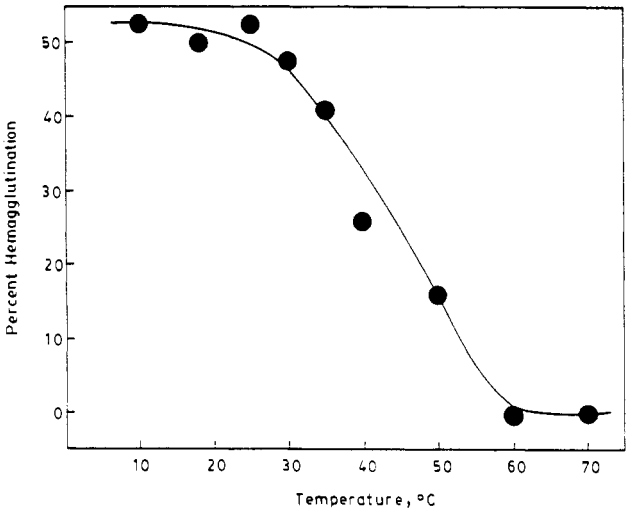

Figure 4. Thermal stability of *P. mungo* lectin. The lectin in the operating Tris-HCl buffer, pH 7.4, containing 0.25 M NaCl was kept for 40 min at the indicated temperature and subsequently incubated overnight at room temperature. The hemagglutinating activity of the heat-treated lectin was determined.

Table I. Effect of Carbohydrates on the Hemagglutinating Activity of *P. mungo* Lectin

carbohydrate	concn range, mM	relative inhibitory potency: *P. mungo* lectin[a]	relative inhibitory potency: *G. simplicifolia* B_4 isolectin[b]
(1) galactose	0–14.5	1.00	1.00
(2) methyl α-galactoside	0–9.5	3.27	3.65
(3) methyl β-galactoside	0–10.7	<0.89	<0.94
(4) melibiose	0–13.3	1.56	2.21
(5) *p*-nitrophenyl α-galactoside	0–18.5	2.30	2.58
(6) *N*-acetylgalactosamine	0–29.0	0.56	<0.89
(7) galactosamine	0–15.5	<0.61	
(8) lactose	0–188.0	<0.05	<0.73
(9) glucose	0–300.0	<0.03	
(10) sucrose	0–115.0	<0.08	

[a] In hemagglutination assay galactose is normalized to 1.0 (9.5 mM galactose is required to achieve 50% inhibiition of hemagglutination of trypsinized rabbit erythrocytes (1 × 10^6 cells) by 88 μg of lectin in 1 mL of mixture). [b] See Wood et al. (1979).

DISCUSSION

The procedure used in this study yielded a pure preparation of *P. mungo* lectin that was sensitive to heat treatment; the lectin was rapidly inactivated above 40 °C. The purified glycoprotein lectin contained about 8% neutral carbohydrate. The molecular weight of the native lectin as determined by gel filtration (137 000) taken together with that (66 000) of its subunit measured by SDS–PAGE showed that the lectin is a dimer whose two identical subunits are held together only by noncovalent forces. The hydrodynamic behavior ($f/f_0 = 1.26$) of the native lectin (MW 137 000) was identical to that of dogfish lactate dehydrogenase ($f/f_0 = 1.27$; MW 141 000), which is known to exist in a compact and globular conformation (Creighton, 1984). Interestingly, the measured frictional ratio of the lectin compares well with that ($f/f_0 = 1.299$) found for concanavalin A dimer, which is globular in shape (Creighton, 1984). Thus, like other legume lectins (Strosberg et al., 1986; Sharon and Lis, 1990) *P. mungo* lectin is a glycoprotein comprising two identical subunits, although its subunit molecular weight (66 000) is markedly higher than that (25 000–30 000) found for subunits of legume lectins (Sharon and Lis, 1990). The overall conformation of the native lectin appears to be similar to that of concanavalin A dimer.

Results on the inhibition of hemagglutinating activity of *P. mungo* lectin by sugars (see Table I) showed that the lectin had higher affinity for galactose than for *N*-acetylgalactosamine. The lectin preferentially interacted with α-glycosides. Therefore, the lectin from *P. mungo* appears to belong to the *N*-acetylgalactosamine/galactose group of lectins showing significantly more affinity for galactose than for *N*-acetylgalactosamine. In contrast, legume lectins from *Phaseolus lunatus*, *Dolichos biflorus*, *Vicia craca*, *Arachis hypogaea*, and *Glycine max* belonging to the *N*-acetylgalactosamine group show much greater affinity for *N*-acetylgalactosamine than for galactose.

The saccharide binding property of *P. mungo* lectin compares well with that of the isolectin B_4 form of *Griffonia simplicifolia* lectin I (Wood et al., 1979), which is tetrameric and contains two types of subunits, A (M_r = 32 000) and B (M_r = 33 000). Of the five forms of the lectin, A_4

possesses severalfold greater affinity for *N*-acetylgalactosamine than for galactose. In contrast, B_4 isolectin binds galactose but does not bind *N*-acetylgalactosaminyl-terminated oligosaccharides or *N*-acetylgalactosamine (Wood et al., 1979). It should be pointed out that the affinity of B_4 isolectin for *N*-acetylgalactosamine, galactose, and melibiose is similar to that of *P. mungo* lectin (see Table I).

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