Articles

Isolation and Characterization of a Soybean Lectin Having 4-*O*-Methylglucuronic Acid Specificity

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ABSTRACT: A new lectin from soybeans having specificity toward the extracellular 4-*O*-methyl-D-glucurono-L-rhamnans produced by certain strains of *Rhizobium japonicum* has been purified and characterized. Isolation was accomplished initially by isoelectric precipitation of contaminating globulins and subsequently by affinity chromatography on partially hydrolyzed glucuronorhamnan covalently coupled to aminohexylagarose. Residual globulins were removed by adsorption of the lectin on concanavalin A–agarose and elution with methyl α-mannoside. The lectin is a glycoprotein (3–5% carbohydrate) with a molecular weight of approximately 175 000. It is a tetramer with subunit molecular weights of 45 000 when dissociated with sodium dodecyl sulfate. Reverse-phase high-pressure liquid chromatography analysis indicates the presence of two types of subunits, both having equivalent molecular weights. According to amino acid analyses, the lectin is rich in acidic but low in sulfur-containing amino acids. The carbohydrate portion of the lectin contains mannose; no hexosamines could be detected. Chemical modification of the lectin indicated that neither sulfhydryl groups nor amino groups participate in binding. Quantitative binding studies of the lectin with various carbohydrate haptens showed that specificity was directed toward 4-*O*-methyl-D-glucuronic acid, D-glucuronic acid, and their methyl glycosides with 4-*O*-methyl-D-glucuronic acid 3–4-fold more effective. In each instance, the methyl glycoside is a more effective hapten.

Soybean lectin from *Glycine max* (*L.*) Merr. is a carbohydrate binding protein specific to terminal nonreducing *N*-acetyl-D-galactosamine and to lesser extent to D-galactose (Pereira et al., 1974). Although many functions have been suggested for this lectin, one hypothesis that has received much attention is that soybean lectin plays an integral role in the initial recognition of *Rhizobium japonicum* by the plant, which leads to a strain-specific, nitrogen-fixing symbiosis (Bohlool & Schmidt, 1974). A difficulty with this proposal is tht *R. japonicum* strains belong to two major subspecies that differ in physiology and DNA homology (Hollis et al., 1981; Huber et al., 1984). These subspecies also differ in the nature of the exopolysaccharide formed, with one forming a capsular, cell-bound heteropolysaccharide that contains both D-galactosyl and 4-*O*-methyl-D-galactosyl nonreducing end groups (Dudman, 1976; Mort & Bauer, 1982) and the other subspecies forming a soluble, extracellular 4-*O*-methyl-D-glucurono-L-rhamnan (Dudman, 1978) which does not interact with soybean lectin (Dombrink-Kurtzman et al., 1983). Other contradictions to this proposal are that some varieties of soybeans are preferentially nodulated by certain subspecies of *R. japonicum* (Caldwell & Vest, 1968) and "lectinless" soybean varieties are readily nodulated (Pull et al., 1978). The earlier report from this laboratory (Dombrink-Kurtzman et al., 1983) presented evidence for a protein in soybean seeds, including those of lectinless varieties, that specifically interacts with the 4-*O*-methyl-D-glucurono-L-rhamnan (MGR)[1] from *R. japonicum* strain 61A76.

In this paper, we report the isolation and characterization of the lectin from soybean var. Pomona by use of an affinity support prepared from the extracellular polysaccharide of *R. japonicum* strain 61A76. Although this lectin has many properties common to other legume lectins, its unusual uronic acid specificity may help resolve some of the inherent difficulties of the "lectin recognition" hypothesis of Bohlool and Schmidt (1974).

Experimental Procedures

Bacterial Polysaccharide Production and Preparation for Affinity Coupling. Bacterial exopolysaccharides from *R. japonicum* strains were prepared as previously described (Dombrink-Kurtzman et al., 1983). A partial hydrolysate of the MGR was prepared by treatment of a 0.4% solution with 0.5 N trifluoroacetic cid at 70 °C for 5 h. Degraded preparations were reduced with sodium borohydride, and number-average molecular weights were calculated from molar ratios of L-rhamnose:L-rhamnitol obtained after hydrolysis with 2 N trifluoroacetic acid (100 °C, 4 h). Semilogarithmic first-order reaction plots of degradation time vs. molecular weight were constructed for more highly degraded preparations; molecular weights of less highly degraded preparations were estimated by extrapolation of hydrolysis times from these plots. The degraded polysaccharide was neutralized and lyophilized before use.

Synthesis of MGR–Agarose. Partially hydrolyzed MGR was coupled to aminohexylagarose by a modification of the procedure of Baues and Gray (1977). Swollen aminohexylagarose (8 mL) was added to 200 mL of a 0.5% solution of

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[1] Abbreviations: PBS, phosphate-buffered saline (0.01 M NaH_2PO_4 and 0.12 M NaCl, pH 7.2); HPLC, high-pressure liquid chromatography; SDS, sodium dodecyl sulfate; MGR, 4-*O*-methyl-D-glucurono-L-rhamnan; Con A, concanavalin A.

the degraded exopolysaccharide dissolved in 0.2 M sodium phosphate (pH 7.0). Sodium cyanoborohydride (Aldrich) was added to a final concentration of 1.5 mg/mL, and the slurry was incubated with shaking for 10 days at 37 °C. The gel was washed several times with PBS and stored at 4 °C in PBS containing 0.05% sodium azide.

Ring Precipitin Assay. Ring precipitin activity was detected in 6 × 50 mm tubes or in capillary tubes (Dombrink-Kurtzman et al., 1983). A positive test was considered the formation of a visible precipitin band in 2 h at room temperature. Titer is defined as the highest dilution of lectin giving a visible precipitin band with a 1% polysaccharide solution in 2 h.

Extraction and Purification of the D-*Glucuronic Acid Specific Lectin.* Soybeans [*Glycine max* (L.) Merr. var. Pomona] were purchased from commercial sources, and extracts were prepared by stirring suspensions of hexane-defatted flour in PBS (1 g/20 mL) overnight at 4 °C. The suspension was filtered through cheesecloth and the pH adjusted to 6.4 to remove the bulk of the 11S globulin (Thanh & Shibasaki, 1976). The extracts were clarified by centrifugation (20000*g*, 15 min, 4 °C) and readjusted to pH 7.2. Unless otherwise noted, all procedures were carried out at 25 °C.

Crude extract (100 mL) was applied to a 0.7 × 15 cm column containing MGR–agarose. The gel was washed with PBS at a flow rate of 50 mL/h until the A_{280} reached, the base line. Adsorbed lectin was eluted with either 0.3 M D-glucuronic acid (sodium salt) or 0.5 M NaCl. The eluate was dialyzed overnight against PBS at 4 °C.

Dialyzed eluate was applied to a 1.2 × 10 cm column of concanavalin A–Sepharose[2] (Pharmacia) previously equilibrated with 0.1 M sodium phosphate (pH 7.4) containing 0.15 M NaCl and 0.1 mM $MgCl_2$, $MnCl_2$, and $CaCl_2$. The column was washed with this buffer until the A_{280} reached the base line and then eluted with 0.1 M methyl α-D-mannoside. The eluate was dialyzed against PBS overnight at 4 °C.

This dialyzed eluate was concentrated 10-fold in an Amicon ultrafiltration cell containing a PM-30 membrane and then applied to a 1.2 × 95 cm column of Ultrogel AcA 34 (LKB) equilibrated with PBS. Protein was eluted at a flow rate of 7 mL/h, and fractions of 3 mL were collected. The fractions with absorbance at 280 nm were tested for precipitin activity. Active fractions were pooled and stored in PBS at 4 °C or dialyzed against distilled water at 4 °C and lyophilized.

Protein was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Electrophoresis. Disc electrophoresis on polyacrylamide gels was performed at pH 8.4 according to Ornstein (1964) and at pH 4.5 by the method of Reisfield et al. (1962). Discontinuous polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was performed according to Laemmli (1970). The gels were stained with Coomassie Blue R-250.

HPLC Analysis. Lectin preparations were analyzed by HPLC using absorbance detection at 210 nm. Protein separations on a Syncropak RP-P 300-Å reverse-phase C_{18} column (250 × 4.1 mm) were achieved by gradient elution with acetonitrile–water in the presence of 0.1% trifluoroacetic acid. Samples of 50 μL were injected and eluted from the column with a linear gradient of acetonitrile (21.5–58.5%) over 70 min.

Molecular Weight Determination by Gel Filtration. Lectin (15 mg/2 mL) was applied to a 1.2 × 95 cm column of Ultrogel AcA 34 equilibrated with PBS. The column was eluted with PBS at room temperature at a flow rate of 5 mL/h. Molecular weights were estimated according to the relative elution volume after calibration of the column with standards of known molecular weight (in parentheses): catalase (210 000), bovine IgG (156 000), bovine serum albumin (68 000), ovalbumin (45 000), and ovomucoid trypsin inhibitor (28 500). Blue dextran was used to estimate the void volume.

Amino Acid Analysis. Amino acid analysis was performed after hydrolysis in 6 N HCl for 24 h at reflux. Analysis was done with a Glenco MM100 amino acid analyzer. Ninhydrin was used to detect amino acids. Methionine and cysteine were detected after performic acid oxidation by the method of Moore (1963).

Carbohydrate Analysis. The carbohydrate content of the lectin was estimated by the phenol–sulfuric acid method of Dubois et al. (1956) using D-mannose as a standard. Uronic acids were determined by the colorimetric method of Blumenkrantz and Asboe-Hansen (1974) using D-glucuronic acid as a standard.

Analysis of the carbohydrate component of the lectin was by gas–liquid chromatography after methanolysis, hydrolysis of methyl glycosides, and conversion of the carbohydrates to the per-*O*-acetylaldononitriles (Jackson et al., 1982).

Lectin Modification. Lectin (15 mg in 5 mL of 0.1 M sodium phosphate buffer, pH 8.0) was succinylated with a 30-fold molar excess of succinic anhydride by adding the solid anhydride over a 30-min period while maintaining the pH at 8.0. All reaction mixtures were dialyzed (PBS, 16 h, 4 °C) prior to use.

Acetylation with acetic anhydride was performed essentially as described above while the reaction mixture was stirred in an ice bath.

Amidation of carboxyl groups with glycine methyl ester (Hassing et al., 1971) was carried out at pH 6.5 on 15 mg of lectin.

Carboxymethylation of sulfhydryl groups with iodoacetic acid (Crestfield et al., 1963) was attempted at pH 8.0 in the absence of urea.

Haptenic Interaction Assay. Carbohydrates were tested as equilibrated, aqueous solutions in PBS. Uronic acids, whether reducing or glycosides, were tested as the sodium salts. Potential haptens were prepared as previously described (Dombrink-Kurtzman et al., 1983) or purchased commercially.

To test the effectiveness of various haptens, solutions of 1% exopolysaccharide and lectin were made to the appropriate concentration in hapten and tested in the precipitin assay. The lowest concentration which resulted in a 50% reduction in the precipitin titer was used to compare the effectiveness of the haptens.

Results and Discussion

Characterization of the MGR Affinity Support and Isolation of the 4-O-Methylglucuronic Acid Specific Lectin. Immobilization of a partial acid hydrolysate of MGR on aminohexylagarose provided an affinity support which was used to isolate the 4-*O*-methylglucuronic acid specific lectin from soybean seeds. Because the reductive amination with cyanoborohydride was performed with a large excess of reducing end groups, the reactive amino groups on the aminohexylagarose were nearly saturated. The degree of substitution was measured at ca. 350 μmol of uronic acid/mL of gel. On the basis of extrapolated molecular weight estimates of the partially hydrolyzed polysaccharide (ca. 3000), this measurement represents greater than 90% substitution of the available amino groups. To completely block any remaining amino groups in the gel, excess L-rhamnose and additional cyanoborohydride

[2] The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

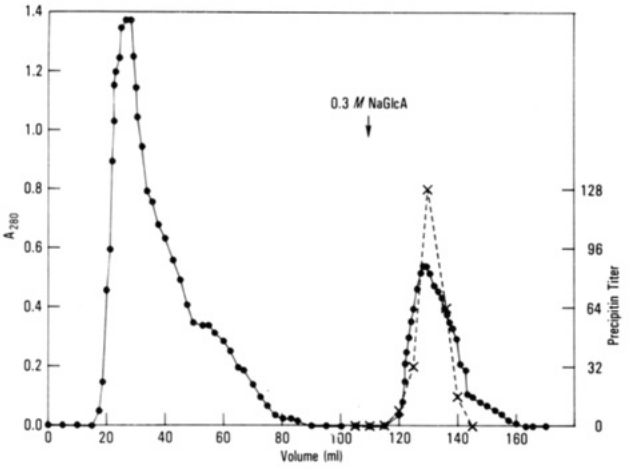

FIGURE 1: Affinity chromatography of the D-glucuronic acid specific lectin from soybeans on MGR–agarose. Conditions are described under Experimental Procedures. (●) A_{280}; (×) precipitin titer.

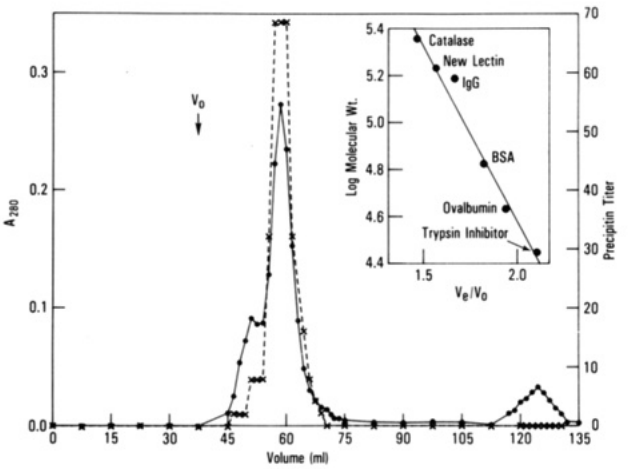

FIGURE 2: Gel filtration of the D-glucuronic acid specific lectin from soybeans on Ultrogel AcA 34. Conditions for chromatography are described under Experimental Procedures. The inset shows calibration of the column with proteins of known molecular weight. (●) A_{280}; (×) precipitin titer. V_0 indicates the position of the void volume as determined by the elution of Blue Dextran 2000.

were added at the end of the incubation period, and the gel was incubated for an additional 48 h. This modification of the procedure gave an affinity support which yielded less contaminated lectin preparations, possibly due to the removal of hydrophobic effects arising from underivatized spacer arms. The reaction conditions for the coupling were mild and unlikely to cause any modification of the polysaccharide.

D-Glucuronic acid specific lectin in 100 mL of crude soybean extract was completely bound to a 0.7 × 15 cm affinity column. This amount corresponds to approximately 6 mg of lectin/mL of gel. The unbound fraction (Figure 1) showed precipitin activity with *R. japonicum* USDA 123 exopolysaccharide but not with MGR. This result indicates that the galactose-specific soybean lectin does not bind to the affinity column and that the new lectin is quantitatively removed from the extract. After unbound protein was eluted with PBS, the column was routinely washed with 0.1 M solutions of glucose, galactose, and mannose to eliminate any other carbohydrate binding proteins which may have bound either to the affinity support or to the new lectin. The lectin was eluted by washing the column with 0.3 M D-glucuronic acid or 0.5 M NaCl. Washing with 0.1 N acetic acid or raising the pH to 9 also eluted the lectin. These nonspecific elution methods gave lectin preparations of greatly diminished activity.

The major drawback to the use of the affinity support is the charge-bearing 4-*O*-methylglucuronic acid residue. Broadening of the elution peak in Figure 1 was reduced only slightly by a reduction in flow rate to 10 mL/h. This behavior is likely due to an ionic exchange effect arising from the 4-*O*-methylglucuronic acid residues in the immobilized polysaccharide. The conditions necessary for binding of the lectin to the exopolysaccharide, i.e., low ionic strength and neutral pH, did not allow suppression of ionic effects in the column.

Initial attempts to isolate the new soybean lectin involved preliminary removal of galactose-specific lectin from crude extracts as described by Dombrink-Kurtzman et al. (1983). This step was found to be unnecessary, as the capacity of the affinity column remained unchanged in either the presence or the absence of the hemagglutinin. No precipitin activity with the exopolysaccharide from *R. japonicum* USDA 123, which has D-galactopyranosyl nonreducing end groups, was observed in either D-glucuronic acid or NaCl eluates from the affinity column. The typical affinity column eluate had a precipitin titer of 2^7–2^8 with native MGR.

Gel electrophoresis of the affinity column eluate under alkaline conditions showed the presence of several distinct

FIGURE 3: Discontinuous polyacrylamide gel electrophoresis under alkaline conditions (pH 8.3) of the purified D-glucuronic acid specific lectin from soybean seeds; 25 μg was loaded and run at 3 mA/tube for 4 h.

bands and a small amount of high molecular weight material migrating very near the top of the gel. The bands were not resolvable by gradient elution of the affinity column. Much contaminating protein was removed by passage of the crude affinity column eluates through a column of Con A–Sepharose. Protein bound to the column was eluted with 0.2 M methyl α-D-mannoside. Even after this treatment, several unidentified proteins appeared in the electrophoresis gels. Almost all of these proteins subsequently were removed by gel filtration through Ultrogel AcA 34. This step removed not only the contaminating proteins but also much of the high molecular weight material present in the Con A affinity eluates (Figure 2). To remove the final traces of contaminating protein, active fractions from the gel filtration were reapplied to a 0.5 × 5 cm column of MGR affinity gel and eluted with 0.5 M NaCl. Gel electrophoresis under alkaline conditions of the second affinity eluate gave a single diffuse band of protein with a small amount of high molecular weight material near the top of the gel (Figure 3). Electrophoresis at pH 4.3 showed a smear of staining material in the upper part of the gel rather than

Table I: Purification of Glucuronic Acid Specific Lectin from Soybeans

purification step	volume (mL)	protein (mg/mL)	titer[a] (dilution)	sp act.[b]	total titer (units)	purification (x-fold)	yield (%)
crude	100	6.95	32	4.6	3200		100
MGR affinity chromatography	44	0.56	128	228.5	5630	50	175
Con A–Sepharose	40	0.57	128	224.6	5120	48	160
Ultrogel AcA 34	24	0.07	16	228.5	384	50	12
MGR affinity chromatography	2	0.51	128	251.0	256	55	8

[a] Titer measurements have an error of ±1 tube; titer measurements from crude extracts may be higher due to higher ionic strengths or inhibitory substances. [b] Specific activity = titer per milligram of protein.

distinct bands. Repeated attempts to remove the aggregated material by gel filtration at high ionic strengths, by gel filtration in the presence of several monosaccharides, including D-glucuronic acid, and by chromatography on DEAE- and CM-Sephadex were not successful.

Table I shows the results of a typical purification of the 4-*O*-methylglucuronic specific lectin from soybean seeds. The initial purification step using the MGR affinity column results in a 50-fold purification. Even though subsequent steps gave insignificant changes in specific activity and large losses of protein, they were necessary to obtain a homogeneous lectin preparation. The increase in specific activity, as well as the large titer unit increase during the initial purification step, suggests that inhibitory substances are removed during affinity chromatography. Only small changes in the specific activity throughout the remainder of the purification likely reflect difficulties in determining the exact titer end point. This factor possibly accounts for the apparent discrepancy in total activity recovered. The eluate from the first affinity separation was routinely used for studies of carbohydrate binding specificity, because no difference in specificity was observed between these preparations and those following the extensive purification scheme outlined in Table I. The purity of the first affinity column eluate was judged by gel electrophoresis to be greater than 80%.

Properties of the Glucuronic Acid Specific Lectin. Purified lectin could be detected at concentrations as low as 10 μg/mL by ring precipitin assay with partially degraded MGR and was stable in PBS for up to 3 weeks at 4 °C. The lectin was also stable at room temperature for 48 h with only slightly diminished activity. Lectin preparations stored for periods at 4 °C slowly formed insoluble aggregates. These aggregates could not be redissolved by increasing the ionic strength or by warming. Preparations stored at high ionic strength (I = 0.5) also aggregated, but not as rapidly. Aggregation was enhanced by low ionic strength (I = 0.05) and below pH 6.5. The stability of the purified lectin was not significantly increased by the presence of salt or D-glucuronic acid.

Slow aggregation has also been observed with other legume lectins, most notably concanavalin A (McKenzie et al., 1972) and galactose-specific soybean lectin (Lotan et al., 1975). In both examples, the rate of aggregation is higher at a low pH and is enhanced by a low ionic strength. Lyophilization did not increase the amount of aggregation as has been observed for the galactose-specific soybean lectin and concanavalin A. It has also been noted that once aggregates of concanavalin A and galactose-specific soybean lectin are formed, treatment with 2% sodium dodecyl sulfate only partially dissociates the aggregates and leads to heterogeneity on sodium dodecyl sulfate–polyacrylamide gels (McKenzie et al., 1972; Lotan et al., 1975). The aggregates formed by the D-glucuronic acid specific lectin are completely dissociated by this treatment and show the same migration as the nonaggregated preparations.

Ring precipitin activity of the lectin with MGR was eliminated upon heating to 50 °C; pretreatment of the lectin with

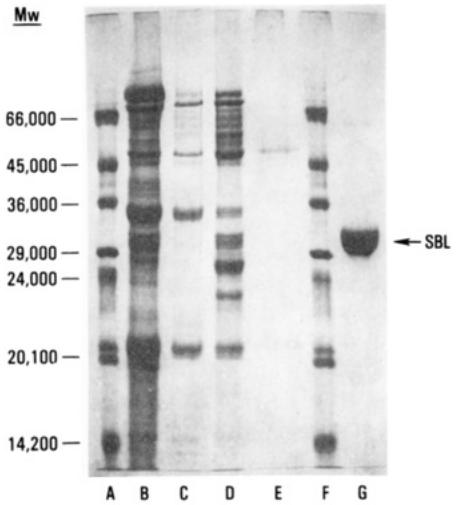

FIGURE 4: SDS–polyacrylamide gel (12%) of the D-glucuronic acid specific lectin from soybean. Lanes A and F, molecular weight standards (bovine serum albumin, 66 000; ovalbumin, 45 000; glyceraldehyde-3-phosphate dehydrogenase, 36 000; carbonic anhydrase, 29 000; trypsinogen, 24 000; soybean trypsin inhibitor, 20 100; and α-lactalbumin, 14 200); lane B, crude seed extract, 15 μg; lane C, 61A76 exopolysaccharide–agarose affinity column eluate, 20 μg; lane D, Con A–Sepharose affinity column eluate, 20 μg; lane E, Ultrogel AcA 34 and 61A76 exopolysaccharide affinity column eluate, 10 μg; lane G, galactose-specific soybean lectin (var. Lee), 20 μg.

0.5% sodium dodecyl sulfate also inactivated the protein. Partial stability to pretreatment with 2 M urea was observed at 0 °C but not at higher temperatures. Pretreatment of the lectin with 2% Triton X-100, 0.1 M 2-mercaptoethanol, 0.1 M ethylenediaminetetraacetic acid (EDTA), or 0.2 M sodium citrate (pH 7.2) did not affect subsequent precipitin activity. Precipitin activity remained unchanged at pH values between 6 and 8, and no metal ion involvement could be detected. Exhaustive dialysis against 1 N acetic acid or acidification with 0.1 N HCl followed by dialysis against PBS only slightly reduced activity. This diminution in activity was not restored by the addition of Mn^{2+}, Mg^{2+}, Ca^{2+}, Zn^{2+}, Co^{2+}, Fe^{2+}, or Fe^{3+}, either alone or in combination. These conditions have been used to demetalize concanavalin A to give inactive preparations in which activity is restored by the addition of Ca^{2+} and Mn^{2+} (Agrawal & Goldstein, 1968; Sumner & Howell, 1936).

The molecular weight of the glucuronic acid specific lectin from soybeans was estimated from gel filtration experiments to be 175 000 ± 10 000 (Figure 2). During storage of purified preparations, the amount of material eluting near the void volume increases, and the elution peak of the lectin broadens. Gel filtration at high ionic strengths did not change the molecular weight estimates, whereas low ionic strengths resulted in a small increase in the apparent molecular weight and a large increase in the amount of aggregated material in the void volume. Gel filtration in the presence of several monosaccharides, including D-glucuronic acid, was without effect.

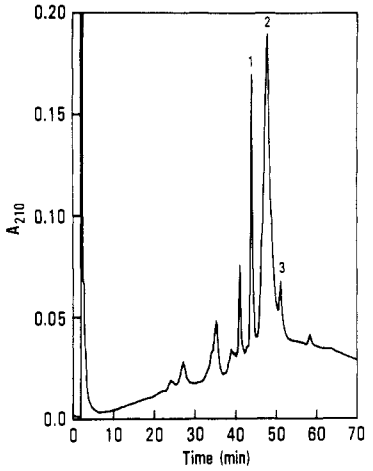

FIGURE 5: HPLC analysis of purified lectin on a 250 × 4.1 mm Syncropak RP-P 300 300-Å (C_{18}) column. Elution conditions are described under Experimental Procedures.

Gel electrophoresis of purified lectin in the presence of sodium dodecyl sulfate shows a single diffuse band of estimated molecular weight 45 000 ± 1000 (Figure 4E). Lectin preparations stored for long periods often contained a second protein band of estimated molecular weight ca. 100 000. This band may represent a dimeric form of the lectin subunits not completely dissociated by the SDS treatment. The sulfhydryl reducing reagents mercaptoethanol and mercaptopropionic acid did not affect molecular weight estimates.

Comparison of the molecular weight estimates under native and denaturing conditions suggests that the native lectin exists as a tetramer. Because the lectin is a glycoprotein, estimates of the molecular weight by sodium dodecyl sulfate gel electrophoresis may be greater than the actual molecular weight. This factor would explain the slight discrepancy between the calculated tetrameric molecular weight from subunit weights and from gel filtration of the native protein. No effects of sulfhydryl reducing reagents in the presence of 2% sodium dodecyl sulfate suggest that the tetramer is not covalently linked by disulfide bonds.

Isoelectric focusing of the lectin showed a smear of staining material near the acidic end of the gel. Inclusion of urea in the gel only slightly improved the resolution. It was possible to distinguish two separate regions of staining material on some of the gels containing urea. This indication that the lectin was composed of two different types of subunits of similar molecular weight was further investigated by reverse-phase HPLC.

Figure 5 illustrates the HPLC analysis of purified lectin. Several small peaks of UV-absorbing material were present and well separated. Throughout this study, it was observed that a small amount of pigment, present in crude extracts, copurifies with the lectin. This pigment was removed only by repeated washing of precipitates with acetone. The only components in the purified sample not removed by acetone washing were peaks 1–3.

Almost all legume lectins that have been characterized are composed of subunits that have molecular weights of 25 000–30 000 which are associated to form either dimers of about M_r 50 000 or tetramers of about M_r 100 000–120 000 (Strosburg et al., 1983). The glucuronic acid specific lectin from soybeans also appears to be a tetramer but is slightly larger than other legume lectins. Subunits within the same lectin have been observed for several other lectins, but most are of different sizes (Goldstein et al., 1983). The subunits of some lectins show different primary carbohydrate specificity.

Table II: Amino Acid Analysis of the Glucuronic Acid Specific Lectin

residue	g/100 g of protein	residues/ subunit[a]	residue	g/100 g of protein	residues/ subunit[a]
Asp	12.14	48	Leu	9.09	36
Thr	3.06	13	Tyr	3.56	10
Ser	6.75	34	Phe	6.65	21
Glu	20.49	73	His	2.23	7
Pro	4.48	20	Lys	5.90	21
Gly	5.83	41	Arg	8.38	25
Ala	3.78	22	Cys	0.46	2
Val	4.53	20	Met	0.31	1
Ile	4.81	19	NH_2	4.72	

[a] Residues/subunit were calculated on the basis of four equal-size subunits (M_r 45 000) with correction for 4% carbohydrate and assuming 1 methionine residue/subunit.

In some, most notably from *Banderaea simplicifolia* (Murphy & Goldstein, 1977; Goldstein, 1981), subunits associate to form isolectins having varying proportions of the two subunits. No evidence for isolectins was found in the present study.

Chemical Composition and Modification of the Glucuronic Acid Specific Lectin. Amino acid analysis of the purified lectin (Table II) shows a protein rich in acidic amino acids with glutamic acid accounting for more than 20% of the residues. Because of the large amount of ammonia recovered, it is likely that many of the glutamic and aspartic acid residues exist in the amide form. The recoveries of sulfur-containing amino acids upon performic acid oxidation were very low; they accounted for less than 1% of the total amino acid residues, i.e., one to two residues per subunit.

The lectin is a glycoprotein composed of 3–5% carbohydrate by weight. D-Mannose was the only identifiable sugar detected after methanolysis of the lectin and likely accounts for the strong binding with Con A, which has high affinity for either terminal or 2-*O*-linked α-D-mannosyl residues. Hexosamines were not detected either by gas–liquid chromatography or during the amino acid analysis. The chemical composition of the lectin is similar to those reported for other legume lectins (Goldstein & Hayes, 1978), which are rich in acidic and hydroxylic amino acids and have low contents of sulfur-containing amino acids. The carbohydrate content appears to be average for legume lectins, but it is unusual that no hexosamine is present.

A number of group-specific modifications were made in an attempt to ascertain functional groups important in the carbohydrate binding activity. Neither carboxymethylation of free sulfhydryl groups nor succinylation of free amino groups affected activity. Acetylation with acetic anhydride precipitated the lectin, which was inactive when resolubilized. Inactivity of the lectin after acetylation is due likely to the powerful denaturing properties of acetic anhydride. Amidation of free carboxyl groups in the presence of a water-soluble carbodiimide and glycine methyl ester gave inconclusive results. Some preparations were completely inactive whereas others retained full activity. Hassing et al. (1971) have shown that the activity of carboxyl-modified concanavalin A is dependent on the pH during modification. No evidence of a pH dependence was observed with this lectin. These inconclusive results do not permit a conclusion to be drawn regarding the participation of free carboxyl groups. As also found for concanavalin A (Agrawal et al., 1968) and galactose-specific soybean lectin (Liener & Wada, 1956), free amino and sulfhydryl groups do not appear to be involved in binding activity.

Carbohydrate Binding Specificity. Binding specificity of the lectin appears to be limited to *R. japonicum* exopoly-

Table III: Inhibition of 4-*O*-Methyl-D-glucurono-L-rhamnan Precipitation by Glucuronic Acid Specific Lectin (Ring Precipitin Test)

D-glucuronic acid derivative	concn giving 50% inhibn (mM)
4-*O*-methyl-α,β-D-methylglucuronopyranoside	2.5
4-*O*-methyl-α,β-D-glucuronic acid	5
α,β-D-methylglucuronopyranoside	10
D-glucuronic acid	15

saccharides that contain 4-*O*-methyl-D-glucopyranuronsyl resides (Dombrink-Kurtzman et al., 1983). Ring precipitin activity with native MGR is detectable when concentrations of purified lectin as low as 50 μg/mL are used. Partial hydrolysis of the native polysaccharide lowers this limit to 10 μg/mL. This effect may be due to the removal of steric hindrance in the high molecular weight exopolysaccharide. There were no interactions with capsular heteropolysaccharides from *R. japonicum* strains USDA 123, 138, and 110. These polysaccharides contain D-galactosyl nonreducing end groups but no D-glucuronic acid or 4-*O*-methyl-D-glucuronic acid. Reduction of the terminal L-rhamnosyl residues in the partially hydrolyzed MGR did not affect interaction with the lectin.

Attempts to quantitate precipitin activity by analyses of precipitated nitrogen or protein were unsuccessful because the solubility of the lectin–polysaccharide complex is high. Centrifugation of the mixture did allow the recovery of a precipitate, but washing resulted in the loss of variable amounts of protein. It was possible, however, to determine the effectiveness of various haptenic sugars by measuring the minimal concentration necessary to reduce the precipitin titer by 50%.

As previously reported, the only effective monosaccharide inhibitors were D-glucuronic acid, 4-*O*-methyl-α,β-D-glucuronic acid, methyl-α,β-D-glucopyranosiduronic acid, and 4-*O*-methyl-α,β-methyl-D-glucuronopyranoside. D-Glucuronic and methyl-α,β-D-glucopyranosiduronic acids were effective at concentrations between 10 and 15 mM, with the methyl glycoside being slightly more effective. The 4-*O*-methyl derivatives of these sugars were effective at concentrations of 2.5–5 mM, again with the methyl glycosides being slightly more effective (Table III). NaCl and D-gluconic and D-galacturonic acids inhibit the precipitin reaction only at 0.3 M concentrations or greater. This effect is likely an inhibition of precipitation of the complex because clouding of the precipitin assays was seen even though there was no distinct precipitin band. No haptenic inhibition was observed with D-glucose, 4-*O*-methyl-D-glucose, methyl 4-*O*-methyl-α,β-methyl-D-glucuronopyranoside, methyl α,β-methyl-D-glucurono-pyranoside, or D-glucurono-3,6-lactone.

These results suggest that the C6 carboxyl of D-glucuronic acid and also the correct configurations at the C3 and C4 positions are necessary for binding. Glucose, the methyl ester of glucuronic acid, and lactones of glucuronic acid are ineffective. Apparently, some modification of the C4 position can be tolerated because both D-glucuronic and 4-*O*-methyl-D-glucuronic acids are effective inhibitors.

Earlier work indicated the presence of an activity in crude soybean extracts that precipitated MGR and caused a polar aggregation of *R. japonicum* 61A76 cells (Dombrink-Kurtzman et al., 1983). We have now isolated and characterized the protein responsible for this specific interaction. The presence of this lectin in soybeans resolves some of the difficulties with the lectin recognition hypothesis. The existence of D-galactose-specific and D-glucuronic acid specific lectins allows inclusion of both major subspecies of *R. japonicum*. Detection of this lectin and small amounts of soybean hemagglutinin (Tsien et al., 1983) in lectinless varieties suggests that this proposal may be valid for all soybean varieties. It remains to be seen whether the relative abundance of these lectins accounts for preferential nodulation of certain soybean varieties by some subspecies of *R. japonicum* (Caldwell & Vest, 1968).

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Registry No. 4-*O*-Methyl-α,β-D-methylglucuronopyranoside, 85718-42-9; α,β-D-methylglucuronopyranoside, 99094-38-9; D-glucuronic acid, 6556-12-3; 4-*O*-methyl-α,β-D-glucuronic acid, 4120-73-4.

REFERENCES

Agrawal, B. B. L., & Goldstein, I. J. (1968) *Can. J. Biochem. 46*, 1147.
Agrawal, B. B. L., Goldstein, I. J., Hassing, G. S., & So, L. L. (1968) *Biochemistry 7*, 4211.
Baues, R. J., & Gray, G. R. (1977) *J. Biol. Chem. 252*, 57.
Blumenkrantz, N., & Asobe-Hansen, G. (1973) *Anal. Biochem. 54*, 484.
Bohlool, B. B., & Schmidt, E. L. (1974) *Science (Washington, D.C.) 185*, 269.
Caldwell, B. E., & Vest, G. (1968) *Crop Sci. 8*, 680.
Crestfield, A. M., Stein, W. H., & Moore, S. (1963) *J. Biol. Chem. 238*, 622.
Dombrink-Kurtzman, M. A., Dick, W. E., Burton, K. E., Cadmus, M. C., & Slodki, M. E. (1983) *Biochem. Biophys. Res. Commun. 111*, 798.
Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956) *Anal. Chem. 28*, 350.
Dudman, W. F. (1976) *Carbohydr. Res. 46*, 97.
Dudman, W. F. (1978) *Carbohydr. Res. 66*, 9.
Goldstein, I. J. (1981) *Recent Adv. Phytochem. 15*, 25.
Goldstein, I. J., & Hayes, C. E. (1978) *Adv. Carbohydr. Chem. Biochem. 35*, 127.
Goldstein, J. J., Lamb, J. E., Roberts, D. D., & Poola, I. (1983) in *Chemical Taxonomy, Molecular Biology, and Function of Plant Lectins* (Goldstein, I. J., & Etzler, M. E., Eds.) pp 21–29, Alan R. Liss, New York.
Hassing, G. S., & Goldstein, I. J. (1971) *Biochim. Biophys. Acta 243*, 90.
Hollis, A. B., Kloos, W. E., & Keister, D. L. (1981) *J. Bacteriol. 158*, 1168.
Huber, T. A., Agarwal, A. K., & Keister, D. L. (1984) *J. Bacteriol. 158*, 1168.
Jackson, L. K., Slodki, M. E., Plattner, R. D., Burton, K. A., & Cadmus, M. C. (1982) *Carbohydr. Res. 110*, 267.
Laemmli, U. K. (1970) *Nature (London) 227*, 680.
Liener, I. E., & Wada, S. (1956) *J. Biol. Chem. 222*, 695.
Lotan, R., Lis, H., & Sharon, N. (1975) *Biochem. Biophys. Res. Commun. 62*, 144.
Lowry, O., Rosebrough, N., Farr, A., & Randall, R. (1951) *J. Biol. Chem. 193*, 265.
McKenzie, G. H., Sawyer, W. H., & Nichol, L. W. (1972) *Biochim. Biophys. Acta 263*, 283.
Moore, S. (1963) *J. Biol. Chem. 238*, 235.
Mort, A. J., & Bauer, W. D. (1982) *J. Biol. Chem. 257*, 1870.
Murphy, L. A., & Goldstein, I. J. (1977) *J. Biol. Chem. 252*, 4739.
Ornstein, L. (1964) *Ann. N.Y. Acad. Sci. 121*, 321.
Pereira, M. E. A., Kabat, E. A., & Sharon, N. (1974) *Carbohydr. Res. 37*, 89.

Pull, S. P., Pueppke, S. G., Hymowitz, T., & Orf, J. H. (1978) *Science (Washington, D.C.) 200*, 1277.
Reisfeld, R. A., Lewis, U. J., & Willis, D. E. (1962) *Nature (London 195*, 281.
Strosburg, A. D., Lauwerys, M., & Forieis, A (1983) in *Chemical Taxonomy, Molecular Biology, and Function of Plant Lectins* (Goldstein, I. J., & Etzler, M. E., Eds.) pp 7-20, Alan R. Liss, New York.
Sumner, J. B., & Howell, S. F. (1936) *J. Biol. Chem. 115*, 583.
Thanh, V. H., & Shibaski, K. (1976) *J. Agric. Food Chem. 24*, 1117.
Tsien, H. C., Jack, M. A., Schmidt, E. L., & Wold, F. (1983) *Planta 158*, 128.

In Vitro Collagen Fibril Assembly in Glycerol Solution: Evidence for a Helical Cooperative Mechanism Involving Microfibrils

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ABSTRACT: Glycerol inhibits the in vitro self-association of monomeric collagen into fibrils and induces the dissociation of fibrils preassembled from $NaBH_4$-reduced collagen. These effects were investigated in an effort to understand the mechanism of fibril assembly of the protein. In PS buffer (0.03 M NaP_i and 0.1 M NaCl, pH 7.0) containing 0.1-1.0 M glycerol, the self-association of type I collagen from calf skin took place only if the protein concentration was above a critical value. This critical protein concentration increased with increasing glycerol concentration. Velocity sedimentation studies showed that below the critical protein concentration and under fibril assembly conditions, the collagen was predominantly in a monomeric state. Electron microscopic examinations revealed that the collagen aggregates formed above the critical concentration consisted mostly of microfibrils of 3-5-nm diameter along with some banded fibrils. Upon removal of glycerol from the solution through equilibrium dialysis, only banded fibrils were found. Collagen treated with pepsin to remove its nonhelical telopeptides also self-associated into microfibrils and fibrils in the presence of glycerol, but the reaction did not exhibit any critical concentration. These results are consistent with a mechanism of in vitro collagen fibril assembly which involves the initial formation of microfibrils through a helical cooperative mechanism. They also suggest that contacts of the nonhelical telopeptides of each collagen with its neighboring molecules provide the necessary negative free energy change for the cooperativity and that subsequent lateral association of the microfibrils leads to banded fibrils.

The interstitial collagens are fibrous proteins with a unique amino acid composition rich in glycine, proline, and hydroxyproline. Their secondary structures differ substantially from those of globular proteins in that they consist of three polypeptides, each of which is a left-handed helix, intertwined into a right-handed triple helix. In extracellular matrices, the triple-helical collagen exists in fibril form. Little is known regarding the mechanism of formation of collagen fibrils from the monomers. In vitro, monomeric collagen can be induced to self-associate into fibrils by incubation at 25-30 °C near neutral pH. The kinetics of the in vitro collagen fibril assembly can be divided into two stages, initiation and growth (Wood, 1960a,b; Wood & Keech, 1960; Comper & Veis, 1977a,b; Williams et al., 1978; Gelman et al., 1979a). During the fibril initiation period, the solution does not display any turbidity, indicating that large fibril structure has not yet formed. The growth of the fibrils follows the initiation period and is characterized by a sigmoidal increase of the solution turbidity and the appearance of collagen fibrils with a 67-nm repeat band pattern. In addition to the banded fibrils, unbanded microfibrils of 2-4-nm diameter have been observed during in vitro fibril assembly (Gelman et al., 1979a). The exact time of the formation of the microfibril and its structural relationship with the banded fibrils are not clear.

There are few reported equilibrium studies of in vitro collagen fibril assembly. Piez and co-workers showed that the in vitro collagen fibril assembly entails no critical concentration (Williams et al., 1978; Gelman et al., 1979a,b). In addition, their kinetic studies showed a linear plot with a slope of -1 of the logarithm of the time required for half of the collagen to assemble into fibrils vs. the logarithm of the total collagen concentration. On the basis of these observations, it was suggested that the fibril assembly reaction proceeds through an accretion mechanism and does not involve the Oosawa helical cooperative assembly mechanism as has been shown in the actin filament and microtubule formations (Oosawa & Kasei, 1962; Gaskin et al., 1974). However, it was pointed out that from their equilibrium data, a very low critical concentration of 7 μg/mL or less cannot be ruled out due to the limited sensitivity of the technique (Williams et al., 1978). As far as the kinetic evidence is concerned, the interpretation of the slope of the double logarithmic plot is uncertain because of the possible presence of collagen oligomers in the initial solution (Chandrakasan et al., 1976; Purich & Kristofferson, 1984). Consequently, the possibility remains that the assembly reaction proceeds through a cooperative mechanism but has a critical concentration which is too low to be detected by available techniques.

In an attempt to further understand the mechanism of collagen fibril assembly, we added glycerol to the assembly buffer to weaken the intercollagen interaction and examined its effect on the chemical equilibrium of in vitro collagen fibril