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THE GALACTOMANNAN SYSTEM FROM THE ENDOSPERM OF THE SEED OF *Gleditsia triacanthos*

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ABSTRACT

The galactomannan system from the endosperm of the seed of *Gleditsia triacanthos* is composed of four water-soluble galactomannans having a narrow range of distribution of sugar compositions. Nevertheless, molecular-weight and possible chain-distribution heterodispersion were observed. All of the galactomannan fractions obtained by fractional extraction and further stepwise precipitation with ethanol contained small proportions of xylose and arabinose, and their methylation analysis suggests that minor amounts of lateral chains are ended by arabinofuranose or xylopyranose residues. The variation of the rotations of the galactomannan fractions in solvents of increasing chaotropic power agrees with the suggestion that, in aqueous solution, these molecules exist in an extended, ribbon-like conformation. These entropically unfavorable conformations would be promoted by the spontaneous, noncovalent, and ordered binding of the molecules, producing lower free-energy aggregates; further interactions between these aggregates form gel networks that are maintained in solution by the galactose lateral chains. Methylation analysis shows that the galactomannans have the structure accepted for these polysaccharides. Only minor deviations from this structure were found, but even if they have minor importance as far as the primary structure of the polysaccharide is concerned, they could be important in determining the secondary and tertiary structures and, consequently, in the formation of aggregates and gel networks.

INTRODUCTION

The galactomannans extracted from the seeds of *Leguminosae* have been studied extensively[1]. Their gross structure consists of a backbone of β-(1→4)-linked D-mannopyranose units to which are attached single α-D-galactosyl stubs at O-6 of certain of the D-mannosyl residues.

The polysaccharides have been considered homogeneous in most of the

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cases, and this is probably true when the D-mannose:D-galactose molar ratio approaches[1] unity; but, for galactomannans having larger ratios, there is at least the possibility of a wide distribution of sugar compositions and, in fact, some galactomannans have been fractionated simply by stirring them with water at different temperatures[1,2].

The general structure of the galactomannans allows three types of molecular dispersion, namely, by (*a*) molecular weight, (*b*) D-mannose:D-galactose ratio, and (*c*) side-chain distribution.

The general structure of the galactomannan isolated from the seeds of *Gleditsia triacanthos*[3] agrees with the general one, and the average Man:Gal ratio was 3.2:1. Evidence of its homogeneity was provided by several procedures[3], although further work carried out on the isolated endosperm[4] suggested the presence in the sample of, at least, three galactomannans of different Man:Gal ratio.

We decided to subject the galactomannans of the endosperm of the seeds of *Gleditsia triacanthos* to further studies of homogeneity and dispersion, in order to provide some understanding of this system and of how intermolecular association determines the physical properties and the molecular cohesion.

EXPERIMENTAL

Materials. — The seeds of *Gleditsia triacanthos* were obtained from ripe pods collected at the Ciudad Universitaria (Buenos Aires). The separation of the endosperm was performed as previously described[5], only 1–3% of the weight of the seed being lost by solubilization during the treatment.

Extractions. — The milled endosperm (10 g) was extracted for 24 h with water (1 L) at room temperature, with constant, mechanical stirring. The insoluble matter was centrifuged off and extracted twice more, *i.e.*, until no more precipitate was obtained when an aliquot was added to three volumes of ethanol. The extracts were combined, and concentrated to 1 L. Ethanol was slowly added, with vigorous stirring, up to 10% concentration, and the small amount of insoluble material was discarded. Ethanol was added stepwise to the supernatant liquor until the concentration was 35% (turbidity was observed). Further addition of ethanol, to 38% concentration, precipitated the galactomannan; the mixture was kept overnight with constant stirring, and centrifuged, and the precipitate was dried in the usual way[5]. Ethanol was continuously added, and another three fractions were arbitrarily separated at 38–45, 45–50, and 50–55% ethanol concentration. Increase in the concentration of ethanol to 70–75% produced only a small, particulate precipitate. These galactomannan fractions were named Group A. The upper limit of precipitation was 85% ethanol concentration. The ethanol-soluble products were obtained by concentration and freeze-drying of the solutions.

The residue remaining after the extraction at room temperature was exhaustively extracted (twice) for 24 h with water (1 L) at 50°, and the extracts were worked up separately (Groups B and C). Particulate precipitates were obtained at

25–30, 35–40, and 45–50% ethanol concentration for the first extract, whereas the second one was arbitrarily fractionated at 20–25, 25–30, 30–35, and 35–40% ethanol concentration.

The insoluble material was further extracted (twice) for 24 h with water (1 L) at 95°. The extracts were combined and worked up as before. Particulate precipitates were obtained at 30–40 and 45–50% ethanol concentration (Group D).

Extractions with solutions of 7M urea at room temperature, 1% ammonium oxalate at 100°, and 10% potassium hydroxide at room temperature[6], were also conducted, consecutively, on the residue remaining after the exhaustive extraction with water. The ethanol-precipitable products constituted no more than 0.6% of the total endosperm, and were reserved for further studies.

Column chromatography. — All of the chemicals used were from Pharmacia, Ltd. The Sephadex G-100 columns (60 × 1.2 cm) were run at 4 mL/h, and 2-mL fractions were collected. Solutions (24 mg/mL, 0.3 mL) of the samples were applied at the top of the column. Solutions of 0.1M NaCl and 7M urea were used as eluants. The eluate was continuously monitored for carbohydrate by the phenol–sulfuric acid reaction[5] and for proteins by u.v. spectrophotometry[5] at 280 nm. Fractions corresponding to the same peak were pooled, concentrated, dialyzed, and freeze-dried. The columns were calibrated by using Blue Dextran and dextrans having molecular weight 9,400, 18,100, 40,000, and 80,700.

The anion-exchange column (15 × 1.2 cm) contained DEAE-Sephadex A-50 equilibrated with 0.05M Tris–hydrochloric acid buffer, pH 8.0. Solutions (16 mg/2 mL) of the samples were applied at the top of the column, and eluted with the same buffer at 25 mL/h. Fractions (2 mL) were collected, and carbohydrate and protein were monitored as before. Fractions corresponding to the same peak were pooled, concentrated, and desalted on a column (38 × 1.1 cm) of Sephadex G-10.

Analytical methods. — General procedures have been described elsewhere[5]. The optical rotations were measured at room temperature in a Perkin–Elmer 141 polarimeter with a photoelectric cell, using 0.05–0.35% solutions of the galactomannans in water, 0.1M NaCl, and 7M urea. The figures are equilibrium rotations; the insoluble materials (fractions 9, 10, and 11) were centrifuged off before the measurements, and the rotations calculated on the weight of the soluble product.

Monosaccharide analysis was performed according to the method of Reinhold[7], using a Hewlett–Packard 5840 A Gas Chromatograph equipped with a dual flame detector and a Hewlett–Packard 5840 A GC terminal, as described by Mazzini and Cerezo[5]. Neutral sugar analysis was also performed by the gas–liquid chromatographic method of Albersheim *et al.*[8], using a glass column (0.2 × 180 cm) of 3% of ECNSS-M on Gas Chrom Q (100–200 mesh) at 190°, with a nitrogen flow-rate of 24 mL/min. Hydroxyproline content was analyzed by the spectrophotometric method of Leach[9]. Mild, alkaline treatment was conducted as described[10].

Methylation analysis. — The galactomannans were initially methylated by the

method of Haworth, and further fully methylated according to Hakomori[11]. The samples (1.0–15.0 mg) were dissolved in water (0.2–0.6 mL) in a screw-capped (Teflon-lined) reaction-vial, and reduced with sodium borohydride (2.5–7.5 mg). Then, 60% sodium hydroxide was added to the vial, into which was placed a magnetic stirring-bar. The vial was flushed with nitrogen, and capped. The mixture was stirred, and 30% sodium hydroxide (sixteen 30-μL portions) and dimethyl sulfate (sixteen 10-μL portions) were added simultaneously, stepwise during 2 h, with vigorous stirring under nitrogen. After stirring for 16 h, the procedure was repeated twice at room temperature, and the solution dialyzed, and freeze-dried. The partially methylated product thus obtained (MeO 23.1%) was placed in the reaction vial, anhydrous, distilled dimethyl sulfoxide (0.1–0.5 mL) was added, and, after ultrasonication for 30–60 min, a solution (0.1–0.5 mL) of ~2M methylsulfinyl carbanion in dimethyl sulfoxide was added, and the vial flushed with nitrogen. After ultrasonication for 1 h, methyl iodide (0.1–0.5 mL) was slowly added with simultaneous cooling with ice–water. Ultrasonication was continued at room temperature for 2 h, and the procedure repeated once. The permethylated product was poured into water, dialysed, and recovered by freeze-drying. To ensure complete methylation of the samples, a third methylation, by the Kuhn method, was performed.

Permethylated polysaccharide samples (0.5–1.5 mg) were hydrolyzed for 2 h at 121° with 2M trifluoroacetic acid (0.1–0.3 mL). The resulting, partially methylated aldoses were converted into the corresponding alditol acetates as described[8]. Analytical g.l.c. was conducted on the same column as before, but at 170°. Peaks due to products of incomplete methylation were not observed.

Combined g.l.c.–m.s. was performed on a glass column (0.3 × 120 cm) of 3% of ECNSS-M on Gas Chrom Q (100–200 mesh) at 170°, with helium as the carrier gas (30 mL/min), and a Varian Series 1400 gas chromatograph connected to a Varian MAT 112 S mass spectrometer. Mass spectra were recorded over a mass range of 40–600 atomic mass units, using an ionizing potential of 70 eV. Scans were taken every 4 s. The methylated alditol acetates were identified by a combination of g.l.c. retention-times (known standards) and mass spectra.

Immunodiffusion. — The immunodiffusions were performed by the double-diffusion technique, using Agarosa Britania at 1.5% concentration in PBS, pH 7.4. Solutions (0.5%) of the galactomannans in the same buffer were used. The diffusions were conducted for 12, 24, 48, 60, and 72 h; after washing for 24 h in physiological solution, and drying for 24 h at 30°, they were stained with 0.5% Amido Schwarz 10B. A precipitin-like reaction between the galactomannans and *Ricinus communis* 120 lectin was obtained.

RESULTS

Fractionation and characterization of the galactomannans. — The endosperm was extracted with water at room temperature, at 50°, and at 95° (see Table I).

TABLE I

YIELDS AND PROPERTIES OF THE GALACTOMANNANS OBTAINED BY FRACTIONATION OF THE WATER-SOLUBLE GALACTOMANNANS OF THE ENDOSPERM OF THE SEED OF *Gleditsia triacanthos*[a]

Fraction	*Ethanol % (v/v)*	*Yield (%)*	*Man:Gal ratio*	$[\alpha]_D^{25}$ [b] *(degrees)*			*D.p.*[c]
				Water	*0.1M NaCl*	*7M urea*	
Group A							
1	35–38	2.2	2.5	+49.6(40)	+30.0(15)	+25.4(49)	80
2	38–45	10.6	2.0	+41.6(28)	+30.0(7)	+27.9(63)	59
3	45–50	18.3	2.0	+33.8(9)	+30.8(11)	+34.4(—)	60
4	50–55	2.4	2.0	+30.6(—)	+35.6(3)	+34.6(—)	100
5	70–75	0.3	2.4	+45.1(10)	+40.6(10)	+36.4(19)	62
Group B							
6	25–30	3.0	3.3	+28.2(16)	+23.6(29)	+16.8(40)	—
7	35–40	15.6	3.1	+38.6(25)	+29.1(54)	+13.5(65)	79
8	45–50	1.2	2.9	+41.2(30)	+28.9(62)	+11.1(73)	68
Group C							
9	20–25	3.1	4.1	+28.8(24)	+21.9[d](92)	+1.8(94)	139
10	25–30	1.9	4.2	+23.1(1)	+22.8[d](81)	+4.4(81)	127
11	30–35	2.8	4.2	+21.8(6)	+20.4[d](92)	+1.6(93)	159
12	35–40	3.4	4.2	+14.4(9)	+13.1(52)	+6.3(56)	144
Group D							
13	30–40	1.9	3.4	+28.4(22)	+22.3(—)	+22.5(21)	71
14	45–50	1.6	3.2	+29.3(8)	+26.9(14)	+23.7(19)	87

[a]The milled endosperm was extracted sequentially with water at room temperature (fractions 1–5, Group A), at 50° in a two-step procedure (fractions 6–8, Group B; and 9–12, Group C, respectively), and finally at 95°(fractions 13 and 14, Group D). The extracts were worked up separately, and each fraction was obtained by stepwise precipitation with ethanol. [b]Rotations were determined for dilute solutions (0.05–0.35%). Percentages of decrease in optical rotation on changing from one solvent to another are indicated in parentheses; the last parentheses corresponded to the change from water to 7M urea. [c]Determined on the basis of the nonreducing, endchain mannose content (see text). [d]Some 30–45% of the fraction was insoluble in 0.1M NaCl, and the rotations given were calculated by considering the soluble galactomannan. The insolubles of fractions 10 and 11 gave, in 7M urea, rotations of +12.8 and +3.1°, respectively.

High yields were obtained in the first two extractions (33.8 and 31.0%, respectively) and only a small proportion (3.5%) was solubilized with water at 95°. Part of the water-soluble endosperm was soluble in 85% ethanol (13.2% of the endosperm; 4.8, 7.6, and 0.8% for each extraction); these products were not galactomannans, and were reserved for further studies.

When the extracts were concentrated to a similar final concentration of galactomannans and then fractionated by stepwise addition of ethanol, a different precipitation pattern was obtained for each extraction. Of the galactomannan extracted at room temperature, 85.5% precipitated in the range of 38–50% ethanol concentration, with lower precipitations at both sides of this range (maximum

TABLE II

COMPOSITION OF THE GALACTOMANNAN FRACTIONS[a]

Fraction[b]	Sugar				Protein[c] (%)
	Ara	Xyl	Man	Gal	
Group A					
1	5.4	1.0	66.5	26.1	2.9
2	1.3	7.5	59.8	32.4	1.1
3	1.7	4.5	62.8	30.9	1.3
4	1.8	0.7	65.2	31.9	0.9
5	2.3	0.6	68.0	28.8	1.5
Group B					
6	5.0	0.6	73.3	22.3	1.9
7	2.2	2.4	71.8	23.5	1.3
8	5.1	—	65.3	22.6	1.2
Group C					
9	5.1	—	78.0	18.9	1.3
10	2.6	0.2	75.2	18.1	n.d.
11	2.6	0.7	76.0	18.2	0.7
12	1.5	0.4	68.9	16.5	n.d.
Group D					
13	4.0	0.3	73.7	21.9	n.d.
14	4.5	6.0	68.3	21.2	1.5
1a	1.6	1.5	60.0	36.0(1.7)[d]	n.d.
1b	2.9	0.3	66.7	29.4(2.3)[d]	n.d.
1c	1.6	2.8	62.4	33.3(1.8)[d]	n.d.

[a]The sugar composition is expressed in mol%. [b]Fractions 1, 8, 11, and 13 contained traces of fucose, and fractions 4, 11, and 12 showed small proportions of 2-acetamido-2-deoxy-D-glucose. Glucose was detected in fractions 8, 10, and 12 (7.6, 1.5, and 11.7%, respectively). Fractions 1a, 1b, and 1c were obtained from fraction 1 by gel chromatography on Sephadex G-100, using 7M urea as eluant. [c]n.d. = not determined. [d]Man:Gal ratio.

limits, 35–55%); fractions 1–5 (see Table I) were arbitrarily separated (Group A galactomannan). The extraction of the remaining residue was conducted at 50° in a two-step procedure, and the extracts were worked up separately. Of the galactomannan obtained in the first extraction, 79% precipitated at 35–40% ethanol concentration (fraction 7, Table I), and small, particulate precipitates were obtained at 25–30 and 45–50% ethanol concentration (fractions 6 and 8, respectively; Group B galactomannan, Table I). The galactomannan obtained in the second extraction precipitated over a wide range of ethanol concentration (20–40%) and was arbitrarily fractionated (fractions 9–12; Group C galactomannan, Table I). Finally, the galactomannans obtained at 95° gave two particulate precipitates, at 30–40 and 45–50% ethanol concentration (fractions 13 and 14, respectively; Group D galactomannan, Table I).

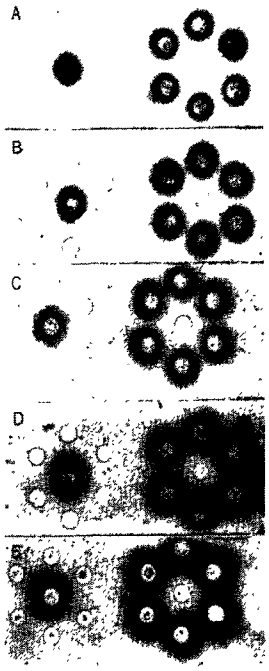

Fig. 1. Immunodiffusion of the galactomannans (fraction 3) against *Ricinus communis* 120 lectin. [Center wells, galactomannans (left) and lectin (right). Diffusion for A, 12 h; B, 24 h; C, 48 h; D, 65 h; and E, 72 h.]

The composition of the fractions is given in Table II. As expected, mannose and galactose are the major monosaccharide components. The results given in Table I show that the molar ratio of D-mannose to D-galactose is the same for fractions of the same group; thus, molar ratios of 2.0–2.5, 2.9–3.3, 4.1–4.2, and 3.2–3.4 were respectively obtained for Group A (extracted at room temperature), Group B (first extraction at 50°), Group C (second extraction at 50°), and group D (extracted at 95°). The increasing Man:Gal ratio in the first three cases accords with the more vigorous conditions used for the extraction, but it is curious that the small amounts of galactomannans solubilized at 95° have the same Man:Gal ratio as the galactomannans obtained in the first extraction at 50°. Sugar analysis of the galactomannans isolated by gel chromatography of fraction 1 on Sephadex G-100 showed a Man:Gal ratio of 1.7, 2.3, and 1.8 for subfractions 1a, 1b, and 1c, respectively (see Table II); this result indicates that the Man:Gal ratio in fraction 1 (2.5; see Table I) was not an average value, and shows that this gum exhibits a narrow range of distribution of sugar composition.

Galactomannans of leguminous seeds are co-precipitants for the *Ricinus communis* agglutinin. The reaction showed a general behavior for all of the samples, namely, a diffuse, precipitation zone without precipitation lines around the

TABLE III

AMINO ACID COMPOSITION OF THE GALACTOMANNAN FRACTIONS[a]

Fraction[b]	*Amino acids*														
	Asp	*Thr*	*Ser*	*Glu*	*Pro*	*Gly*	*Ala*	*Val*	*Ile*	*Leu*	*Tyr*	*Phe*	*Lys*	*His*	*Arg*
Group A															
1	13.2	4.5	7.0	14.3	4.0	3.6	7.8	6.1	7.3	8.4	5.9	4.8	5.2	1.6	6.2
2	13.8	4.9	13.0	15.1	—	8.1	9.5	5.2	4.7	6.6	5.7	3.7	7.8	1.9	—
3	10.2	5.1	15.1	12.8	3.6	8.8	9.1	4.7	3.6	5.2	2.6	2.4	10.1	6.2	—
4	14.3	4.5	15.5	11.7	4.1	8.3	9.5	4.8	2.9	5.2	2.8	1.6	10.9	1.2	2.6
Group B															
7	13.3	5.8	8.3	18.7	3.7	4.9	8.2	5.4	3.3	9.4	4.9	4.1	7.1	2.8	—
Group C															
9	11.3	3.9	8.9	18.9	3.1	6.0	7.0	7.1	4.5	8.3	5.1	4.6	9.2	2.1	—
11	9.5	3.6	7.5	15.4	14.5	6.6	6.5	5.4	2.8	4.7	2.7	4.1	10.4	4.7	1.6
12	10.0	5.0	13.7	17.5	4.1	7.9	7.3	6.8	3.1	7.2	3.0	2.7	8.6	2.9	—
Group D															
14	10.5	4.8	6.4	14.7	5.4	6.2	7.3	5.6	3.4	9.5	3.9	3.4	5.2	3.8	8.6

[a]The amino acid composition is expressed in g per 16 g of N. Percentages lower than 0.5% have not been considered. [b]See a, Table I. Hydroxyproline was not detected in any of the fractions. Fractions 5, 6, 8, 10, and 13 were not analyzed.

galactomannan wells (see Fig. 1); only for fractions 3, 4, and 8 was a precipitation line observed, slightly separated from the higher diffusion limit of the diffuse zone. These lines, which are not seen in the 12-h runs, are very faint in the 24-h runs (fraction 8), and neat in the longer developments (48–60 h), but disappear at higher diffusion-times (72 h). The precipitation lines are nearly straight, with a slight concavity to the well containing the lectin, and are situated nearest to the galactomannan wells, being different in the three cases. Different mixtures of (*a*) samples that only produce diffuse zones, (*b*) samples that also give rise to precipitation lines, and (*c*) both types of sample, showed only the diffuse zone around the galactomannan wells, but in no case were precipitation lines seen.

"Associated protein" of the galactomannan. — Nearly all of the fractions contained small proportions of protein (0.7–2.9%; see Table II), which could not be removed, either by gel chromatography on Sephadex G-100 with chaotropic eluants, or by anion-exchange chromatography on DEAE-Sephadex A-50. The amino acid composition of the "associated protein" is shown in Table III. Aspartic acid–asparagine, serine, and glutamic acid–glutamine are the major amino acids, and they constitute, together with variable proportions of some other amino acids, ~50% of the protein. Hydroxyproline was not detected. Attempts to eliminate, by mild, alkaline treatment, the protein of the possible *O*-glycosydic linkage between the polysaccharide chains and the hydroxyl groups of seryl or threonyl residues, or both, left the material unchanged.

Physicochemical properties. — All the fractions were completely soluble in water, most in 0.1M NaCl (not fractions 9, 10, and 11), and in 7M urea; dissolution was easier as the chaotropic power of the solvent was increased. Several relationships can be traced between the optical rotations (see Table I) and some parameters, namely: (*a*) rotations in water diminish along the sequence of extractions, with the exception of the galactomannan fractions extracted at 95° (Group D), the rotations of which are in the range of those of the products obtained in the first extraction at 50° (Group B); (*b*) different optical rotation values were obtained for galactomannans having similar Man:Gal ratios; (*c*) direct relationships were observed, for the products of each extraction (the galactomannans extracted at 95° again being the exception), between the optical rotations in water and the range of precipitation in ethanol–water, and, for group A and C fractions, the optical rotation decreases for the more-soluble products (not fraction 5), whereas for group B fractions, the optical rotation increases in that direction; and (*d*) in all cases, the optical rotation showed a decrease with increasing chaotropic power of the solvent (not fractions 3 and 4, which did not exhibit any change). For group A fractions, the optical rotations of 1 and 2 were significantly lower (30–40%) in 0.1M NaCl than in water, but very little difference was observed when passing from 0.1M NaCl to 7M urea solution. For the group B fractions, the decrease was significant in both media, but higher in the second one (16–30 and 29–62%, respectively). For the group C fractions, no decrease in rotation was observed in 0.1M NaCl (not fraction 9), but a very significant one is produced when 7M urea is used as the solvent (52–92%). Group D fractions did not show a definite tendency in the change of the optical rotations.

The molecular weight and the molecular-weight dispersion were analyzed by gel chromatography on calibrated columns of Sephadex G-100, using 0.1M NaCl and 7M urea as eluants. Fractions 1 (see Fig. 2) and 14 gave only one peak when eluted with 0.1M NaCl, and the apparent mol. wts. were 18,000 and 70,000, respectively; but, in the case of fraction 1, five subfractions were partially separated when the chromatography was conducted in 7M urea medium (fractions 1a–1e, Fig. 2). The calculated mol. wts. were 35,000, 23,000, 14,000, 5,700, and 3,500, and the yields of each subfraction were 29, 56, 12.2, 2.5, and 0.7%, respectively. Analysis of the sugar composition of the three main subfractions (see Table II) showed a similar Man:Gal ratio, also similar to that of the original product (fraction 1). It is noteworthy that, despite extensive dialysis to remove the urea, the galactomannans having a low mol. wt. (5,700 and 3,500) did not dialyze. Fraction 14 gave two peaks in the presence of 7M urea [mol. wts. 35,000 (92%) and 7,000 (8%), respectively]. Heterodispersion of mol. wt. was observed in fraction 7, even with 0.1M NaCl solution, and two peaks were detected [mol. wts. 53,000 (82%) and 34,000 (18%), respectively]. A similar elution-pattern was obtained in 7M urea solution, but the mol. wts. were lower [30,000 (82%) and 14,000 (18%), respectively].

Some other type of heterodispersion, Man:Gal ratio, or lateral-chain distribution is suggested for fractions 9, 10, and 11 by the fact that sub-fractional insolubilization is produced in 0.1M NaCl medium (see Table I).

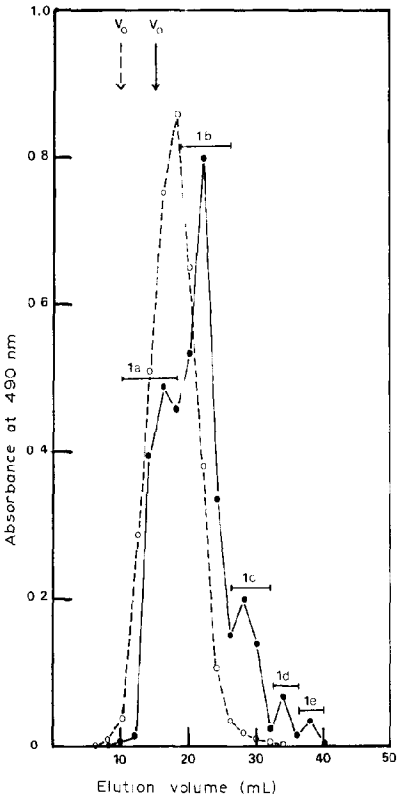

Fig. 2. Elution patterns of fraction 1 galactomannan on columns of Sephadex G-100. [The chromatography was conducted with 7M urea (———), and 0.1M NaCl (----). For yields and composition of the peaks, see text.]

Methylation studies. — The galactomannans were submitted to methylation analysis, and the permethylated derivatives were obtained through a two-step sequence of a Haworth and a Hakomori procedure on a small scale (see Table IV). A further methylation of some of the samples by the Kuhn method, with methyl iodide and *N,N*-dimethylformamide, did not change their compositions (data not shown) except for fractions 2, 9, and 10 (see Table IV).

All of the samples were composed, as usual, mainly of 2,3,4,6-tetra-*O*-methylgalactose. and 2,3,6-tri- and 2,3-di-*O*-methylmannose, in agreement with the accepted gross structure of the galactomannans[1]. Small amounts of 2,3,4,6-tetra-*O*-methylmannose were found in all the samples, and if it is accepted that they are produced by the nonreducing end-chain of the mannose backbone, the gross degree of polymerization (d.p.) of the galactomannans could be calculated from them (see Table I). The number-average d.p. value calculated for the group A, B, and C fractions is 97–100. The average d.p. of the products extracted at room temperature and of those obtained in the first and second extraction at 50° is

TABLE IV

RELATIVE PROPORTIONS OF METHYLATED SUGARS FROM THE GALACTOMANNAN FRACTIONS OF THE ENDOSPERM OF THE SEEDS OF *Gleditsia triacanthos* (MOL%)[a]

	Sugar residues						
	Mannose				*Galactose*		
	2,3,4,6-Tetramethyl	*2,3,6-Trimethyl*	*2,3-Dimethyl*	*2,6- + 4,6-Dimethyl*	*2,3,4,6-Tetramethyl*	*2,3,6-Trimethyl*	*2,4-Dimethyl*
	Deduced glycosidic linkages						
	Terminal	*4-*	*4,6-*	*3,4- + 2,3-*	*Terminal*	*4-*	*3,6-*
Group A							
1	1.2(0.05)	45.5(1.96)	23.2(1.00)	1.4(0.06)	23.2(1.00)	1.8(0.08)	1.4(0.06)
2[b]	1.7(0.06)	41.8(1.55)	26.4(0.98)	0.3(0.01)	27.0(1.00)	0.4(0.01)	0.9(0.03)
3	1.6(0.07)	42.1 (1.72)	23.1(0.94)	—	24.5(1.00)	3.6(0.15)	2.2(0.09)
4	1.0(0.04)	44.5(1.85)	22.7(0.95)	1.5(0.06)	24.0(1.00)	1.7(0.07)	1.6(0.07)
5	1.6(0.06)	40.3(1.55)	26.6(1.02)	0.3(0.01)	26.0(1.00)	0.2(0.01)	2.8(0.11)
Group B							
7	1.2(0.06)	49.4(2.27)	21.7(0.99)	1.6(0.07)	21.8(1.00)	0.9(0.04)	0.9(0.04)
8	1.4(0.06)	47.7(2.12)	21.7(0.96)	1.6(0.07)	22.5(1.00)	2.0(0.09)	2.1(0.09)
Group C							
9[b]	0.7(0.05)	58.8(4.11)	15.5(1.08)	1.0(0.07)	14.3(1.00)	2.3(0.16)	1.6(0.11)
10[b]	1.2(0.08)	58.8(3.82)	17.3(1.12)	0.2(0.01)	15.4(1.00)	1.4(0.09)	1.2(0.08)
11	0.6(0.04)	61.1(3.64)	17.3(1.03)	1.1(0.06)	16.8(1.00)	1.4(0.08)	0.7(0.04)
12	0.7(0.04)	56.8(3.46)	16.4(1.00)	3.7(0.23)	16.4(1.00)	0.7(0.04)	1.0(0.06)
Group D							
13	1.4(0.07)	53.3(2.79)	18.1(0.95)	1.3(0.07)	19.1(1.00)	2.9(0.15)	2.7(0.14)
14	1.1(0.07)	61.4(3.68)	15.8(0.95)	0.6(0.04)	16.7(1.00)	1.5(0.09)	2.3(0.14)

[a]Molar ratios, relative to 2,3,4,6-tetra-*O*-methylgalactose, are indicated in parentheses. Traces of 2,4,6-tri-*O*-methylgalactose were detected in fractions 7, 9, 10, 11, 13, and 14. Fractions 4, 7, 8, 9, and 12 also showed variable (0.1–2.4%) 2,4-di-*O*-methylmannose; 4,6- and 2,6-di-*O*-methylgalactose were detected in traces in all the fractions. [b]A third methylation, by the Kuhn method, was necessary (to obtain complete methylation).

consistent with the sequence of extraction. This is not the case for the d.p. of the galactomannans extracted at 95°, which is in the range of the d.p. of the products obtained in the first extraction at 50°. Minor amounts of 2,6- and 4,6-di-*O*-methylmannose, and 2,3,6-tri- and 2,4-di-*O*-methylgalactose were also detected (see Table IV).

Minor, non-usual, sugar components of the galactomannans. — Minor, but significant, proportions of arabinose, xylose, and fucose were detected in most of the galactomannan fractions. Fractions 8, 10, and 12 also showed glucose, and 2-acetamido-2-deoxy-D-glucose was found in traces in fractions 4, 11, and 12. When fraction 1 was chromatographed on Sephadex G-100, using 7M urea as the eluant,

TABLE V

METHYLATED DERIVATIVES OF NON-USUAL SUGAR COMPONENTS OF THE GALACTOMANNAN FRACTIONS[a]

	Sugar residues[b]		
	2,3,5-Tri-O-methylarabinose	*3,5-Di-O-methylarabinose*	*2,3,4-Tri-O-methylxylose (or -fucose, or both)*
	Deduced glycosidic linkages		
	Terminal	*2-*	*Terminal*
Group A			
1	tr	0.2	0.3
2	4.1	0.9	3.0
3	1.9	0.2	0.7
4	0.9	0.5	1.1
5	0.5	0.4	1.2
Group B			
7	0.5	—	0.9
8	—	0.2	1.0
Group C			
9	1.2	0.4	0.6
10	0.4	0.4	0.3
11	—	0.4	0.2
12	1.0	—	1.7
Group D			
13	—	0.5	0.3
14	—	0.2	—

[a]The sugar composition is expressed in mol% of the total methylated derivatives (see Table IV). Percentages lower than 0.1% were not considered. [b]Mass spectra of all the peaks were analyzed for monomethylated arabinose derivatives (without detecting them); traces of 2-*O*-methylfucose were detected (0.2% and 0.3% in samples 4 and 5, respectively). Mass spectra also indicated the absence of monomethylated xylose derivatives in detectable amounts.

the three subfractions isolated (see Table II) also contained significant proportions of arabinose, xylose, and fucose. Anion-exchange chromatography of the galactomannans on DEAE-Sephadex A-50 showed that the xylose, arabinose, and fucose components of the fractions are recovered unaltered after elution of the column, and are therefore not retained by it.

Analysis of the methylated galactomannans showed the presence of minor proportions of 2,3,5-tri- and 3,5-di-*O*-methylarabinose, and 2,3,4-tri-*O*-methylated xylose and fucose. It is noteworthy that no monomethylated derivatives of arabinose and xylose were detected, and only traces of 2-*O*-methylfucose were found (see Table V).

DISCUSSION

The endosperm of the seed of *Gleditsia triacanthos* contains 68.3% of water-soluble galactomannans, togeher with 13.2% of water-extractable, 85%-ethanol-soluble products. The total proportion extracted with water (81.5% of the endosperm) agrees with that reported by Anderson[12].

The endosperm contains, at least, four different galactomannans, respectively having Man:Gal ratios of 2.0–2.5, 2.9–3.3, 4.1–4.2, and 3.2–3.4, the second and the last differing in the temperature and sequence of extraction, and in the optical rotation in 7M urea (see Table I). The number-average Man:Gal ratio (3.1) of the products extracted at room temperature and at 50° agrees with that determined in previous work[3] for the galactomannan extracted directly at 50°.

The ethanol stepwise-fractionation proved to be difficult to reproduce, and this variability appears to be associated with temperature-, time-, and composition-dependent molecular associations[13,14].

The fractionation patterns showed that, if the Man:Gal ratio is considered, each galactomannan can be regarded as homogeneous and monodisperse, and this is also true for galactomannan A when the unimodal[15] precipitation pattern is taken into account. Particulate precipitation of galactomannans B, C, and D points to polydispersion, and possible molecular-weight or chain-distribution heterodispersion, even though the optical rotations of the fractions for a given galactomannan and solvent are similar. Apparent molecular-weight heterodispersion was determined in all of the fractions studied (1, 7, and 14), and, considering this, it is noteworthy that most of the fractions of each galactomannan showed similar average degrees of polymerization (see Table I).

The percentage of galactose in the galactomannan extracted with cold water was ~33% (see Table II), in agreement with McCleary *et al.*[16], but products having a lower galactose content (~20%) remained insoluble under those conditions, and were only solubilized by water at 50 or 95°. This property, together with the fact that galactomannans having similar Man:Gal ratios require different conditions of extraction (see Table I), agrees with the previous data[16], and shows that the extraction depends on the noncovalent associations of the molecules in the endosperm, which would, in turn, depend not only on the extent of galactose substitution[16] but also on the distribution and length of the galactose side-chain (see later) and on the total galactomannan composition of the endosperm.

All of the galactomannans contained small, but significant, proportions of protein (see Table II) that was evenly distributed in the fractions produced by ethanol precipitation and was not removed by gel filtration in a hydrogen-bond-breaking medium, by DEAE-Sephadex A-50 anion-exchange chromatography, or by mild, alkaline treatment. The composition of these proteins was similar in all of the fractions, and the major amino acids were glutamic and aspartic acids, serine, and lysine, which constitute 35–50% of the protein. They differ from the proteins of the embryo[5] in the lower percentages of glutamic acid and lysine and in the

higher one of serine. Similar proportions of protein had been found in galactomannan samples obtained from this[3,5] and other legume seeds[16] submitted to different procedures of purification[3,5,16]. This resistance to extensive efforts to resolve the carbohydrate and protein suggests that the associated material is a glycopeptide having a 2-acetamido-2-deoxy-D-glucose–asparagine linkage. This is consistent with the determination of small proportions of 2-acetamido-2-deoxy-D-glucose in some of the fractions (see Table II).

All of the fractions contained small proportions of xylose and arabinose, and traces of fucose, which also appeared in the subfractions produced by gel filtration in 7M urea of fraction 1, and were not eliminated by anion-exchange chromatography on DEAE-Sephadex A-50. Contaminant xylans had been detected in galactomannan samples extracted from cell walls of seeds of some legumes[16], but, in that case, they were eliminated by chromatography on DEAE-cellulose[16]. These facts, together with the presence of tri- and di-*O*-methyl derivatives of arabinofuranose and trimethyl ethers of xylose and fucose (see Table V), and the absence of monomethylated derivatives of these sugars in the hydrolyzates of permethylated galactomannans, suggest the possibility that a minor proportion of the lateral chains may be ended by arabinofuranose or xylopyranose residues.

The *Ricinus communis* agglutinin combines with the legume galactomannans *via* its (terminal) nonreducing galactosyl groups, and these combinations depend on the Man:Gal ratio of the polysaccharide[17] and, owing to steric effects, on the distribution of the lateral chains. Galactomannans having similar Man:Gal ratios and chain distributions would produce, in a double-diffusion gel-reaction with the lectin, precipitin lines whose position would depend on the diffusion[18], and, hence, on the size of the "molecules" of the galactomannans. The diffuse precipitation-zones found near the galactomannan wells are consistent with a nonspecific, reversible aggregation of the polysaccharide molecules, producing aggregates having variable amounts of reaction sites and aggregate sizes. In some cases, the composition of the sample, or its interaction with the agarose-gel matrix[19], or both, promotes the formation of a given aggregate, producing a definite precipitin-line in its reaction with the lectin. In these cases, the position and form of the precipitin lines suggested that the smaller aggregates have a "molecular weight" similar to that of the lectin[18] (120,000), at least ten times that obtained in the methylation analysis, and also higher than those obtained for other fractions in the gel chromatography.

The decrease of the optical rotation found when the solvent is changed from water to 0.1M NaCl and 7M urea is concomitant with changes from ordered forms to more-disordered ones[1], which is expected from the increase of the chaotropic power of the solvent. This is in agreement with the suggestion that, in aqueous solution, the galactomannan molecules exist in an extended, ribbon-like conformation[20]. These extended, entropically unfavorable conformations would be promoted by the spontaneous, noncovalent, and ordered binding of the molecules, producing lower free-energy aggregates[1,21]; further interactions between these

aggregates form gel networks that are maintained in solution by the galactose lateral chains.

The interactions between the galactomannan molecules are related to the lateral-chain content and their distribution along the mannan backbone[22], and, even if the side chains are presumed to have no apparent gross effects on the conformation of the molecules in solution[14], they presumably hold the main chains far enough apart to render less effective the noncovalent interactions, promoting penetration of the water molecules, and determining the facility with which galactomannans can be dissolved or dispersed.

X-Ray diffraction patterns of guar[23] and *Gleditsia amorphoides* galactomannans[24] indicated that, in the crystalline zones, the mannose backbone and the galactose lateral units are arranged in sheets, with the side chains and the planes of the pyranose rings lying in the planes of the sheets and the water molecules accommodated in the spaces between the sheets[24]. The side chains of all of the molecules in one sheet point in one direction, whereas the side chains in the adjacent sheets point in the opposite direction, and a direct relationship was found between the percentage of substitution and the separation of the sheets[24]. Fibers of *Gleditsia amorphoides* galactomannan obtained by precipitation from its aqueous solution with ethanol did not produce X-ray diagrams[24a], showing that the interchain associations can be produced irregularly.

Galactomannan A, extracted at room temperature (Man:Gal 2.0–2.5), showed no significant variation in optical rotation when the solvent was changed from 0.1M NaCl to 7M urea, suggesting that the extended conformations[20] and hence, some incipient aggregation[1,21], were maintained, even in a solvent that supposedly assists in breaking noncovalent bonds between adjacent chains[1]. Fractions 3 and 4 did not show rotational variations in water also, in agreement with the previous suggestion that molecules containing 25–38% of D-galactose do not form a three-dimensional-gel network[14]. Fractions 1 and 2 showed a significant change in optical rotation on passing from water to sodium chloride solution. In these molecules, the disposition of the lateral chains would permit not only an incipient aggregation able to convert the random-coil conformation into an ordered form[1,21], but also the formation of junction zones to produce a gel network, these junction zones being labile enough to be destroyed in a medium of high ionic strength. The formation of a gel network would explain the lower solubility of fractions 1 and 2 of galactomannan A in ethanol–water.

Galactomannan B, extracted at 50°, showed changes from more-ordered to less-ordered forms with increasing chaotropic power of the solvent, and these changes are more significant in the second solvent change and in the more-soluble samples. These changes in optical rotation indicated that galactomannans containing 23–26% of D-galactose can form gel networks having junction zones strong enough to be partially maintained in a medium of high ionic strength (0.1M NaCl). These associations might be formed between sections of the galactomannan backbone that are essentially unsubstituted with galactose[14], but it is probable that in

these molecules, having relatively high percentage of lateral chains, sections containing a regular arrangement of side chains play a role.

Galactomannan C, second extraction at 50°, showed little association changes in the first solvent change, but very important ones in the second change. This is in agreement with the stronger interactions expected for molecules having higher possibilities of unsubstituted backbones[14]. Most of the galactomannan C (fractions 9, 10, and 11) is heterodisperse with respect to the distribution of the lateral chains. These molecules are solubilized in water through the formation of soluble networks[1], but, when the ionic strength destroys the junction zones, they are reordered, producing insoluble aggregates.

Galactomannan D, extracted at 95°, has a Man:Gal ratio and optical rotations in water and sodium chloride solution similar to those of galactomannan B, but differs in the optical rotation in 7M urea, suggesting differences in the primary aggregation that influences the extraction.

Methylation analysis shows that the galactomannans obtained from the endosperm of the seed of *Gleditsia triacanthos* have the gross structure accepted for these types of polysaccharide[1], namely, a β-(1→4)-linked D-mannose backbone having single stubs of α-(1→6)-linked D-galactose. Only minor deviations from this structure were found, as shown by characterization of small proportions of 2,6- and 4,6-di-*O*-methylmannose (2.2% of the mannan chain) in the hydrolysis products of the permethylated galactomannan. The 4,6-di-*O*-methyl derivative was found in the permethylated galactomannan from *Cassia absus*[25] and shown to be β-(1→3)-linked in the backbone[25]. To the best of our knowledge, no 3,4-linked D-mannose units had been detected in galactomannans.

Some of the lateral chains contain more than one unit (see Table IV), as previously found in the galactomannans from *Gleditsia ferox*[26], *Gleditsia amorphoides*[27], and *Trifolium repens*[26]. If only one extra unit is considered in each lateral chain, the percentages of these longer side-chains go from 15% in fractions 3, 8, and 13 to 1% in fraction 5. Courtois and Le Dizet[26] demonstrated that the tri-*O*-methylated galactose was not an artifact produced by partial demethylation of tetra-*O*-methylgalactose.

The detection of 2,4-di-*O*-methylgalactose would suggest the presence of branching points formed by D-galactose units in the lateral chains or in the backbone.

These deviations from the classical structure have minor importance as far as the primary structure of the polysaccharide is concerned, but they could be important in determining the secondary and tertiary structures[21], and the formation of aggregates and gel networks of the galactomannans. Longer lateral chains, as well as xylose or arabinose residues as end chains, might modify the packing of the chains, increasing the distance between the sheets. This would, in turn, determine the facility with which galactomannans can be dissolved, and could be a mechanism for controlling the physical organization and the hydration of the endosperm. Mannose units having β-(1→3) or β-(1→2) linkages would determine the presence of[21]

"kinks" (residues in the backbone) and would modify the shape of the molecules, causing each chain to combine with more than one partner, as is necessary in order to form a network[21].

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