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THE GALACTOMANNAN-LIKE OLIGOSACCHARIDES FROM THE ENDOSPERM OF THE SEED OF *Gleditsia triacanthos*

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ABSTRACT

The endosperm of the seed of *Gleditsia triacanthos* contains 4.8% of 85% ethanol-soluble, galactomannan-like oligosaccharides having Man:Gal ratios of 1.5–2.6:1 and an average degree of polymerization of 15. They have a narrow distribution of molecular weights and of ratio of components. The oligosaccharides have the gross structure accepted for the galactomannans, namely, a β-(1→4)-linked D-mannopyranosyl backbone having single stubs of α-(1→6)-linked D-galactopyranosyl groups. Some of the lateral chains contain more than one unit, and a minor proportion of the branches are ended by arabinofuranose or fucopyranose residues. Unusual branching points formed by 3,4-linked D-mannosyl, or 3,6-linked D-galactosyl units, or both, were also found. Despite their low molecular weight, the oligosaccharides form aggregates with a structure similar to that of the aggregates of the related galactomannans, but having a lower association energy. This fact, together with the difficulty of combining with more than one partner (due to the short, central chain), results in an increased solubility and in nonviscous solutions. The ^{13}C-n.m.r. spectrum differentiated clearly the five structural units of the oligosaccharides, namely, the reducing and nonreducing end-chains of the D-mannosyl backbone; substituted and nonsubstituted, internal β-(1→4)-linked mannopyranosyl units of the backbone; and the galactosyl nonreducing end-chain of the lateral chains. The C-4 signal of the (1→4)-linked D-mannose and the C-6 signal of the same, but substituted, units showed splitting into three lines. The first has been attributed to sequence-related heterogeneity, whereas the latter is tentatively explained by assuming that this resonance is sensitive to whether the mannosyl units linked to that residue are also branched, or not.

INTRODUCTION

The water-extracted, 85% ethanol-insoluble galactomannans of the seed of Leguminosae have been extensively studied[1]. In contrast, little is known of the

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85% ethanol-soluble products; a previous report[2] showed that the supernatant liquor from the precipitation of the galactomannans contained mannose and galactose in the ratio of 1.2:1.0, and it was suggested that, if they were forming galactomannan-like molecules, the compounds would have low molecular weights, or would contain another sugar(s) in the molecules[2], or both.

We now report the presence, in the 85% ethanol-soluble fraction extracted with water at room temperature from the endosperm of the seed of *Gleditsia triacanthos*, of galactomannan-like oligosaccharides having an average degree of polymerization of 15, and arabinofuranose or fucopyranose, or both, end-chain residues. Their structure and molecular interactions were studied by chemical and spectroscopic methods.

EXPERIMENTAL

General methods. — (*a*) *Analytical methods.* General procedures have been described[2]. Monosaccharide analysis was performed according to the method of Reinhold[3], in a column (0.3 × 180 cm) of 3% of OV-17 on Chromosorb W HP (80–100 mesh), with a nitrogen flow-rate of 30 mL/min and a temperature program from 100 to 180° (8°/min), using a Hewlett–Packard 5840 A Gas Chromatograph equipped with a dual-flame detector and a Hewlett–Packard 5840 A GC terminal. Neutral-sugar analysis was also performed by the gas–liquid chromatographic method of Albersheim *et al.*[4], using a glass column (0.2 × 180 cm) of 3% ECNSS-M on Gas Chrom Q (100–200 mesh) at 190° with a nitrogen flow-rate of 24 mL/min. Estimation of hexosamines was also conducted by the colorimetric method of Johnson[5].

Combined g.l.c.–m.s. was performed in a glass column (0.3 × 120 cm) of 3% of ECNSS-M on Gas Chrom Q (100–200 mesh), with helium as the carrier gas and a temperature program from 100 to 180° (4°/min), and a Varian Aerograph Series 1400 Chromatograph connected to a Varian MAT CH 7 A mass spectrometer, using an ionizing potential of 70 eV, a trap current of 100 A, and a source pressure of 0.1 mPa (10^{-9} atm).

Optical rotations at equilibrium were measured at room temperature in a Perkin–Elmer 141 polarimeter equipped with a photoelectric cell, using 0.7–1.0% solutions of the samples in water, 0.1M NaCl, and 7M urea.

Paper electrophoresis was performed on Whatman No. 3 paper, with borate buffer, pH 9.1. Sample solutions (1%, 0.8 μL) were run at 900 V during 3 h, or at 600 V during 4 h. Detection was effected by the periodate–cuprate reagent[6].

(*b*) *Column chromatography.* The Sephadex G-15 column (1.2 × 60 cm) was run in water at 16 mL/h, and the Sephadex G-100 analytical columns (1.2 × 70 cm) were run in 7M urea at 4 mL/h; in all cases, 2-mL fractions were collected. Solutions (20 mg/mL, 0.3 mL) of the samples were applied at the top of the column. Preparative columns of Sephadex G-100 and G-200 (3.5 × 80 cm) were also run in 7M urea at 8 mL/h; solutions (30 mg/mL, 5 mL) of the samples were applied, and 10-mL fractions were collected. The eluates were continuously monitored for carbo-

hydrate by the phenol–sulfuric acid method, and for proteins by u.v. spectrophotometry at 280 nm. Fractions corresponding to the same peak were pooled, dialyzed, and freeze-dried. The analytical columns were calibrated by using Blue Dextran and dextrans having weight-average molecular weights of 9,400, 18,100, 40,000, and 80,700. The Sephadex G-25 column (1.8 × 120 cm) was run at 50 mL/h, using 1% acetic acid as the eluant; fractions (2 mL) were collected, and carbohydrate and protein were monitored as before. The column was calibrated with Blue Dextran and a standard mixture of D-glucose and oligomers thereof having d.p. 2–6, 9, and 10.

The anion-exchange, analytical (0.8 × 10 cm) and preparative (2.5 × 30 cm) columns of DEAE-Sephadex A-50 (CO_3^{2-}) were equilibrated with water, and then solutions [50 mg/mL (0.5 mL) and 135 mg/mL (10 mL), respectively] of the samples were applied at the top of the column. Elution was first achieved with water, and then with solutions of ammonium carbonate of (step-wise) increasing ionic strength. Fractions (2 and 10 mL) were respectively collected for the analytical and preparative columns, and carbohydrate and protein were monitored as before. Fractions corresponding to the same peak were pooled, desalted, and freeze-dried.

(*c*) *Spectroscopic methods.* The i.r. spectra were recorded for Nujol mulls, in a Perkin–Elmer 710-B infrared spectrophotometer. The ^{1}H-n.m.r. spectrum of fraction N was obtained for a solution of 35 mg in D_2O (0.4 mL) (after three dissolutions in this solvent, each followed by freeze-drying) at room temperature, using an XL-100 Varian spectrometer, and external tetramethylsilane as the standard. The ^{13}C-n.m.r. spectra were recorded with the same sample dissolved in 1:3 D_2O/H_2O (0.4 mL) in the same equipment (25.2 MHz for ^{13}C), with complete proton-decoupling; chemical shifts were again referred to external tetramethylsilane.

Material. — The seeds of *Gleditsia triacanthos* were obtained from ripe pods collected at the Ciudad Universitaria (Buenos Aires). The separation of the endosperm was conducted as previously described[2].

Extraction. — The endosperm (10 g) was exhaustively extracted with water (1 L, three times) at room temperature, as already described[7], and the extracts were combined. The galactomannans were precipitated by stepwise addition of ethanol up to 85% concentration, as described[7]. The ethanol-soluble products were obtained by concentration, and freeze-drying, of the solutions.

Attempts at fractionation of subfraction N by stepwise precipitation with ethanol. — Subfraction N (see later, 100 mg) were dissolved in 20 mL of distilled water (0.5% concentration), and ethanol was added stepwise, in 2-mL portions, with constant mechanical stirring, up to 85% concentration. Because precipitation was not observed, the ethanolic solution was kept at 4° and, after two weeks, only a small precipitate (I) appeared, which was centrifuged off, redissolved in water, and the solution freeze-dried (yield 1.2%). Subfraction N was recovered by freeze-drying.

Determination of the degree of polymerization. — (*A*) *Measurement of the*

formaldehyde produced by periodate oxidation[8]. The sample (15.0 mg of subfraction N) was dissolved in distilled water (3 mL), sodium borohydride (10 mg) was added, and the mixture was kept for 96 h at room temperature. A second addition of sodium borohydride (2.5 mg) was made and the mixture was kept for 168 h at room temperature, to complete the reduction. Acetic acid was added stepwise to decompose the excess of sodium borohydride, and the pH was then adjusted to 7.5 by addition of sodium hydrogencarbonate. The solution was made to 10.0 mL, sodium metaperiodate (30 mg) was added, and the mixture was kept for 96 h in the dark at room temperature. The sodium iodate formed, and the excess of periodate, were removed by addition of lead formate (10 mg) to an aliquot (2 mL) in an ice–water bath. The mixture was kept overnight at 4°, centrifuged, and the supernatant liquor dialyzed in a closed system against distilled water (2 mL) for 48 h. The external solution (1 mL) was used for the determination of formaldehyde by the chromotropic acid method[9].

(*B*) *Determination of reducing end-chain residues.* Fraction N (2.0 mg) was treated as described by Somogyi[10].

Periodate oxidation. — The sample (N-2, 2.98 mg) was dissolved in 15mM sodium metaperiodate solution in 0.1M acetate buffer, pH 4.9, and made up to 5.0 mL. The reaction was conducted in the dark, at room temperature. The periodate consumption was determined by the Guthrie method[11]. After 50 h, the uptake of periodate was constant, corresponding to 1.16 mol of sodium metaperiodate per glycosyl unit. The excess of periodate was decomposed by addition of ethylene glycol (0.1 mL). The polyaldehyde was reduced with sodium borohydride for 20 h at room temperature, employing a 35-fold excess over the periodate used. After acidification, the solution was evaporated to dryness, and boric acid was removed as the methyl ester by addition and distillation of methanol (5 × 1.0 mL). The resulting product was desalted on a Sephadex G-15 column (0.5 × 20 cm) eluted with water. The aliquots collected (0.5 mL each) were analyzed for carbohydrate-containing material (spot test: alkaline permanganate reagent on glass-fiber paper). The fractions giving positive reaction (only one peak) were pooled, and freeze-dried. After hydrolysis, the products were analyzed as their respective alditol acetates by g.l.c., as described for neutral sugars; a temperature program from 100 to 180° (4°/min) was used in this case. Combined g.l.c.–m:s. was performed as described under Analytical methods.

Methylation analysis. — The purified galactomannan-like oligosaccharides (7.0–15.0 mg) were methylated by the method of Hakomori[12]. The reaction mixture was extracted with chloroform (4 × 8.0 mL) and the combined extracts were washed with water (3 × 4.0 mL). The methylation product was isolated in the usual way, as a white solid. This product was dissolved in anhydrous *N,N*-dimethylformamide, and methylated by the Kuhn method[13]. Permethylated samples (0.5 mg) were hydrolyzed with 0.25M sulfuric acid in 95% acetic acid (0.4 mL) overnight[14] at 80°. The hydrolyzate was desalted on a column (0.9 × 3 cm) of Amberlite IR-45 (acetate) resin by elution with methanol (5 vol.). The eluate was evaporated to

dryness under diminished pressure at 40°, M ammonium hydroxide (0.4 mL) and $NaBD_4$ (5 mg) were added, and the mixture was kept for 2 h. Another methylation of N-2 (13.2 mg) was conducted by the method of Haworth, and the product further methylated according to Hakomori, as described[7]. Analytical g.l.c. of the

TABLE I

SUGAR COMPOSITION OF THE WATER-EXTRACTABLE (AT ROOM TEMPERATURE), 85% ETHANOL-SOLUBLE FRACTION OF THE ENDOSPERM OF THE SEED OF *Gleditsia triacanthos*, AND OF SOME OF ITS GALACTOMANNAN-LIKE OLIGOSACCHARIDE COMPONENTS

Fraction[a]	*Sugar*						*Man:Gal molar ratio*
	Fuc	*Ara*	*Xyl*	*Man*	*Gal*	*Glc*	
S	1.3	5.9	2.0	39.3	25.8	25.3	1.5
A	1.1	9.5	2.4	50.4	32.5	4.2	1.5
A-1	tr	6.6	tr	64.6	19.8	9.0	3.3
A-2	tr	5.9	tr	48.1	31.6	14.0	1.5
ND	tr	2.6	tr	55.5	35.4	5.5	1.6
D	tr	2.6	tr	57.6	34.3	4.3	1.7
N	tr	2.1	—	67.9	29.9	tr	2.3
I	tr	2.6	—	68.2	26.6	2.5	2.6
N-1	tr	6.4	—	55.6	30.2	7.9	1.8
N-2	tr	1.6	—	62.3	31.7	4.2	2.0
N-3	tr	1.3	—	30.0	20.8	47.9	1.4

[a]S: Room temperature-water extractable, 85% ethanol-soluble fraction; A: higher-molecular-weight fraction from gel chromatography; A-1: higher-molecular-weight fraction separated in the gel chromatography of A; A-2: lower-molecular-weight fraction separated in the gel chromatography of A; ND: non-dialyzable fraction; D: dialyzable fraction; N: neutral fraction from the anion-exchange chromatography; I: insoluble fraction from N; N-1, N-2, and N-3: fractions obtained by gel chromatography of N on Sephadex G-200 in 7M urea. Fraction S also contains 3.1% of 2-acetamido-2-deoxy-D-glucose; traces of this sugar were also found in N.

TABLE II

PROPERTIES OF SOME OF THE GALACTOMANNAN-LIKE OLIGOSACCHARIDES AND OF THE WATER-EXTRACTABLE (AT ROOM TEMPERATURE), 85% ETHANOL-SOLUBLE FRACTION FROM THE ENDOSPERM OF THE SEED OF *Gleditsia triacanthos*

Fraction	*Yield (%)*	$[\alpha]_D^{25}$ *(degrees)*			*Protein (%)*
		H_2O	*0.1M NaCl*	*7M urea*	
S	5.1[a]	+51.0	+32.3	+46.4	7.5[b]
A	52[c]	+59.6	+18.3	+17.6	2.4
D	52[c]	+34.0	+33.0	+19.6	3.8[d]
ND	47[c]	+58.3	+29.0	+36.2	2.5
N	46[c]	+33.8	+28.5	+18.1	3.3[d]

[a]Yield relative to total endosperm. [b]The amino acid composition, expressed as g per 16 g of N, for fraction S, is Asp, 19.9; Thr, 4.1; Ser, 6.0; Glu, 4.2; Pro, 3.6; Gly, 5.7; Ala, 7.6; Val, 5.0; Ile, 3.4; Leu, 5.5; Tyr, 5.4; Phe, 3.5; Lys, 7.9; and His, 8.2. Percentages lower than 0.5% have not been considered. [c]Yields relative to fraction S. [d]Protein, calculated as N × 6.25, gave 12.3% for fraction D, 3.1% for fraction ND, and 8.4% for fraction N.

methylated alditol acetates, obtained as before, was conducted in the same column as before, but at 170°. Combined g.l.c.–m.s. was performed as described under Analytical methods.

RESULTS

The endosperm was extracted exhaustively with water at room temperature, the extract was separated, and the galactomannans were precipitated with ethanol up to 85% concentration. The supernatant liquor was concentrated, and freeze-dried (yield, 4.8% of the endosperm).

The sugar composition of the 85% ethanol-soluble products (S) is given in

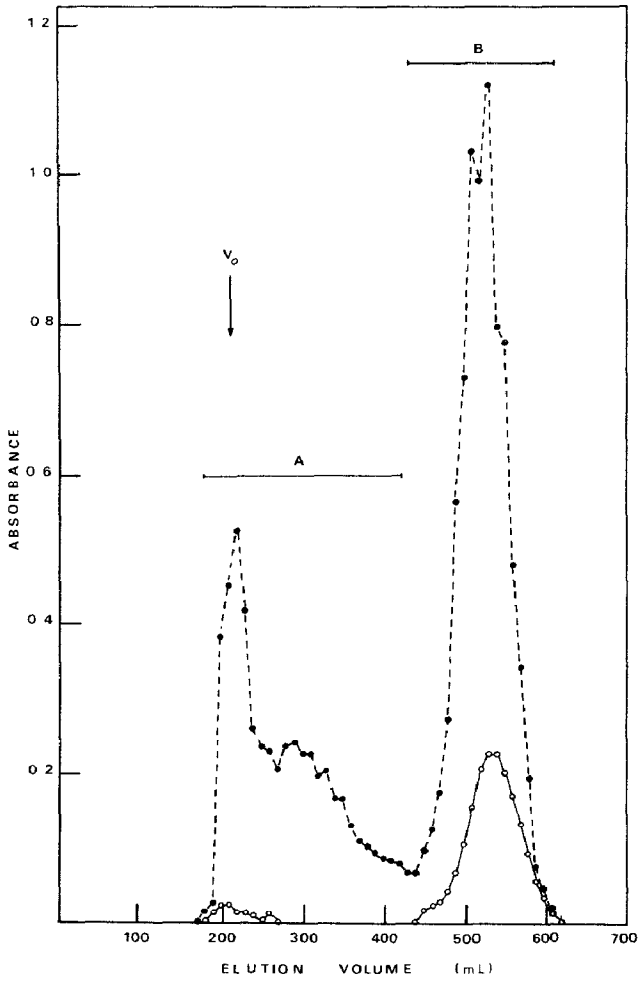


Fig 1. Elution pattern, on a preparative column (3.5 × 80 cm) of Sephadex G-100, of fraction S, using 7M urea as eluant. [Key: (——), A_{280}; (----), A_{490}.]

Table I, and some of their properties in Table II. The sample contained not only mannose (33.3%), and galactose (21.5%) but also glucose (21.3%), together with minor proportions of arabinose (4.9%), xylose (2.0%), fucose (1.3%), and 2-acetamido-2-deoxy-D-glucose (3.1%). The amino acid composition of the "associated" protein is given in Table II; aspartic acid/asparagine and glutamic acid/glutamine are the major amino acids, and they constitute ~35% of the protein.

Attempts at fractionation. — (*a*) *Gel chromatography.* Chromatography of S on Sephadex G-25 in an aqueous medium produced only one peak, at the void volume. When the same sample was chromatographed on Sephadex G-100, using 7M urea as the eluant, several peaks were obtained which, according to the patterns of diffe-

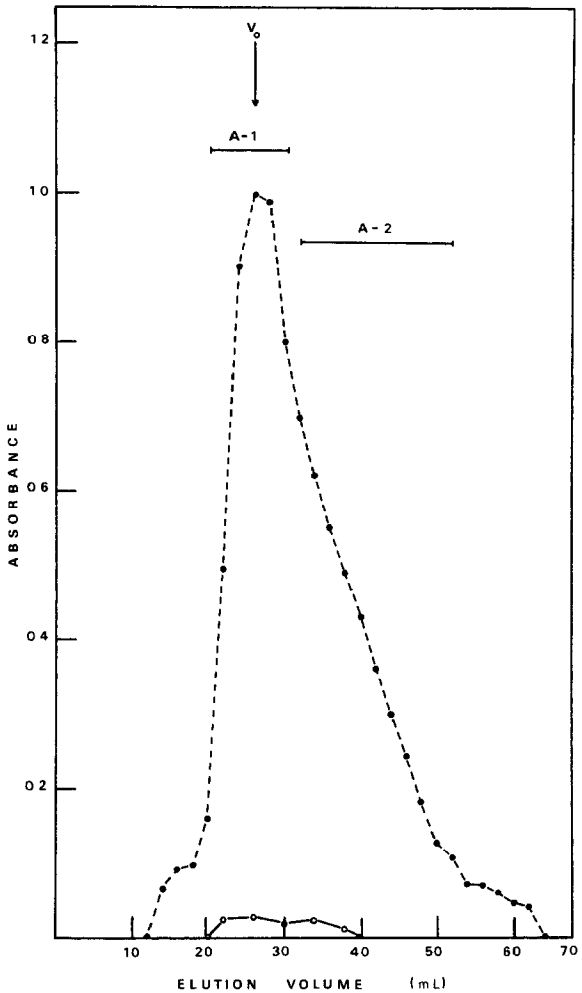


Fig. 2. Elution pattern, on a preparative column (3.5 × 80 cm) of Sephadex G-200, of subfraction A, using 7M urea as eluant. [Key: (——), A_{280}; (----), A_{490}.]

rent chromatographic procedures, were grouped into (*i*) a broad and unsymmetrical peak, with a maximum at 40,000, or several, well defined peaks; in the latter case, the elution volume of the major one suggested a molecular weight in the range of 25,000; only traces of protein were detected in this zone; (*ii*) a well defined peak, the elution volume of which suggested a molecular weight in the range of 1,600, with an appreciable proportion of protein eluted with the carbohydrate. Preparative chromatography under the same conditions produced the elution pattern shown in Fig. 1. The two subfractions were separated, dialyzed, and freeze-dried. The first (A) was obtained in 52% yield; its sugar composition is shown in Table I, and some of its properties in Table II. The second subfraction (B) was lost in the dialysis step. As the composition of A compared to that of the initial product (S) suggested that no sharp fractionation had been obtained, A was further chromatographed in the same medium, but using Sephadex G-200, producing a very broad and asymmetrical peak (see Fig. 2) which showed the apparent heterogeneity of the sample. The peak was arbitrarily subdivided into fractions A-1 and A-2 (see Fig. 2), which were isolated as before (yields, 27 and 18%, respectively). The carbohydrate compositions, shown in Table I, indicated that no sharp fractionation was obtained by this method.

(*b*) *Dialysis.* On the basis of the apparent difference in molecular weights produced in the Sephadex G-100 chromatography, fractionation of S through dialysis was attempted. Sugar analysis of the dialyzable (D) and nondialyzable (ND) subfractions are shown in Table I, and some properties of both subfractions are given in Table II.

(*c*) *Ion-exchange chromatography.* The sample (S) was chromatographed on DEAE-Sephadex A-50 (CO_3^{2-}), and eluted sequentially with water and solutions of ammonium carbonate of stepwise increasing concentration.

The elution with water produced only one fraction (N, 42% yield), and that with ammonium carbonate gave 11 fractions which contained most of the protein, and which were reserved for further work. The sugar composition of N is given in Table I, and some of its properties, in Table II. It has the composition of a galactomannan, and the present work was conducted on this product.

Determinations of homogeneity. — Paper electrophoresis of N in borate buffer produced only one, rounded, spot.

Gel-permeation chromatography on Sephadex G-15, in 1% acetic acid, gave only one peak, at the void volume. Only one, symmetrical peak was also obtained in Sephadex G-200 in 7M urea, corresponding to a molecular weight of ~2,500 (d.p. 15). This chromatography was repeated in a preparative column, and three subfractions were separated (see Fig. 3). It is noteworthy that, after elimination of the urea by dialysis, the total yield was 34.2%, and the subfractions contained 5.2 (N-1), 26.0 (N-2), and 3.0% (N-3) of the starting material. Analysis of the subfractions showed that the Man:Gal ratios as well as their specific rotations were maintained throughout the chromatographic and dialysis steps. Also noteworthy are the high (N-3), and significant (N-1 and N-2), percentages of glucose, which

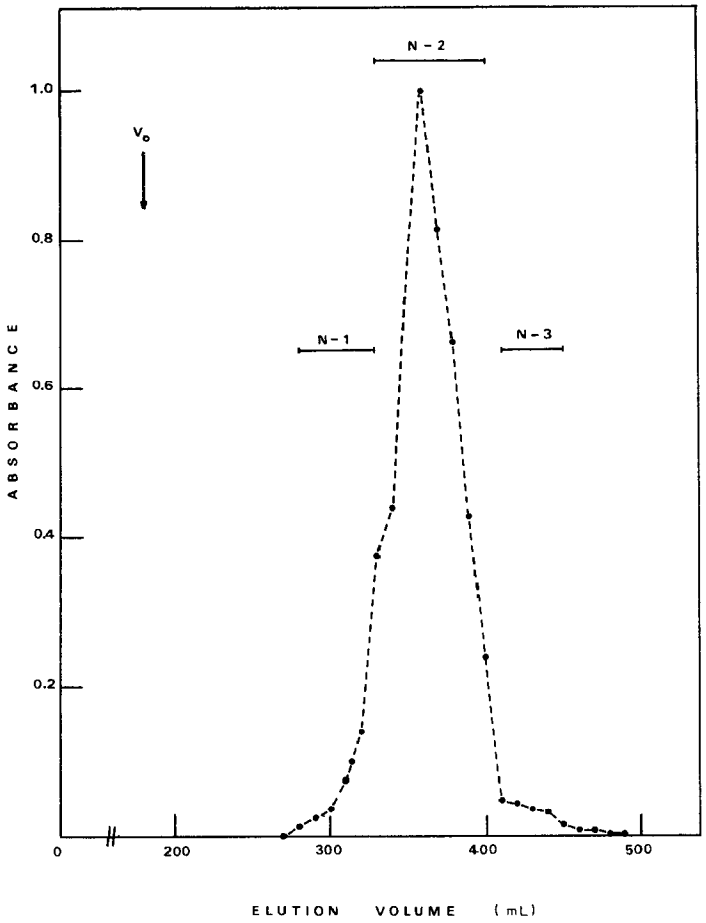


Fig. 3. Elution pattern, on a preparative column (3.5 × 80 cm) of Sephadex G-200, of subfraction N, using 7M urea as eluant. [Key: (----), A_{490}.]

were also found in different proportions in samples obtained by other procedures (see Table I).

Precipitation with ethanol. — A 0.5% aqueous solution of the oligosaccharide (N) was taken to 85% ethanol concentration without any precipitation. After two weeks at 4°, a small precipitate appeared (I, 1.2% yield), whose composition was similar to that of the starting material (see Table I).

Methylation analysis. — The oligosaccharides were methylated by the method of Hakomori[12]; further methylation by the Kuhn[13] procedure did not change their composition. The sample was also methylated by following the methylation sequence of the Haworth and Hakomori procedures used for the related galactomannans[7]. Hydrolysis of the permethylated derivatives produced the mixtures of partially methylated sugars shown in Table III. This Table also shows the

TABLE III

RELATIVE PROPORTIONS (MOL %) OF METHYLATED SUGARS FROM THE GALACTOMANNAN-LIKE OLIGOSACCHARIDES OF THE ENDOSPERM OF THE SEED OF *Gleditsia triacanthos*

	Methylated sugar residues									*Man:Gal ratio*[a]	*D.p*[a]
	Mannose				*Galactose*			*Arabinose*	*Fucose*		
	2,3,4,6-Tetra Deduced glycosidic linkages	*2,3,6-Tri*	*2,3-Di*	*2,6-Di*	*2,3,4,6-Tetra*	*2,3,6-Tri*	*2,4-Di*	*2,3,5-Tri*	*2,3,4-Tri*		
	term	4-	4,6-	3,4-	term	4-	3,6-	term	term		
Fraction											
N-2[b]	5.2	40.0	22.3	0.2	23.3	2.2	4.1	1.9	tr	2.1	18
N-2[c]	6.8	36 8	20.6	0.7	26.4	7.0	0 5	0.5	tr	1.9	14
A[b]	2.8	40.0	20.8	0.3	25.8	1.7	4.8	2.9	0 8	1.8	34

[a]Calculated from the data in this Table. [b]Methylated according to Hakomori. [c]Methylated by a sequence of Haworth and Hakomori procedures.

methylated monosaccharides resulting from the methylation of the less-purified oligosaccharide sample (A). The composition patterns in the three cases were similar. The Man:Gal ratios (1.9–2.1:1) and the degrees of polymerization (14–18) calculated from the data of Table III for N-2 agree with those of the original sample (2.0:1, and 15, respectively). The degree of polymerization of oligosaccharide A (34) was higher than for the other samples (~15), but it was still considerably lower than that obtained for the same sample by gel-filtration chromatography (~150). Only trimethylated derivatives of arabinofuranose or fucopyranose were detected. Combined g.l.c.–m.s. did not disclose any derivative of xylose or glucose.

Degree of polymerization. — The d.p. of N-2 determined by the periodate method was 14.8 considering that two mol of formaldehyde were produced per mol of sample, but 7.4 if only one mol is considered. The determination of reducing end-groups, in the same sample, indicated a d.p. of 14.7, whereas that calculated by using the percentage of tetra-*O*-methylmannose was 14–18, and that obtained by gel-permeation chromatography in 7M urea was 15.

Periodate oxidation. — The consumption of periodic acid was 1.16 mol per mol of glycosyl unit, and the analysis of the alditols obtained by reduction of the polyaldehyde with sodium borohydride showed the presence of erythritol and glycerol in the molar ratio of 2.1:1.0. Nonreducing mannose (3%) and galactose (0.6%) were detected. No glucose survived oxidation.

I.r. and n.m.r. spectroscopy. — The infrared spectrum showed two absorption bands, at 870 and 810 cm^{-1}, characteristic of the deformation of the equatorial C-2–H bond in manno-oligosaccharides, and of the ring-breathing frequency of the

D-mannopyranosyl residues of the same unit, respectively[15]. It is noteworthy that the i.r. spectrum of sample N in the range of 1200–900 cm^{-1} is more similar to the spectrum of β-(1→4)-mannotetraose[15] than to those of the galactomannans.

The ^{1}H-n.m.r. spectrum of N (see Fig. 4) showed, in the anomeric region, three absorptions, namely, two doublets, at δ 4.78 (J ~1.0 Hz) and 5.06 (J ~3.0 Hz), corresponding to the anomeric protons of β-D-mannopyranosides and α-D-galactopyranosides, respectively, and a singlet at δ 5.20 assigned to the anomeric proton of α-L-arabinofuranoside[16]. The ratios of these absorption peaks were 22.5:10.2:1.0.

The ^{13}C-n.m.r. spectrum of oligosaccharide N is shown in Fig. 5. All the

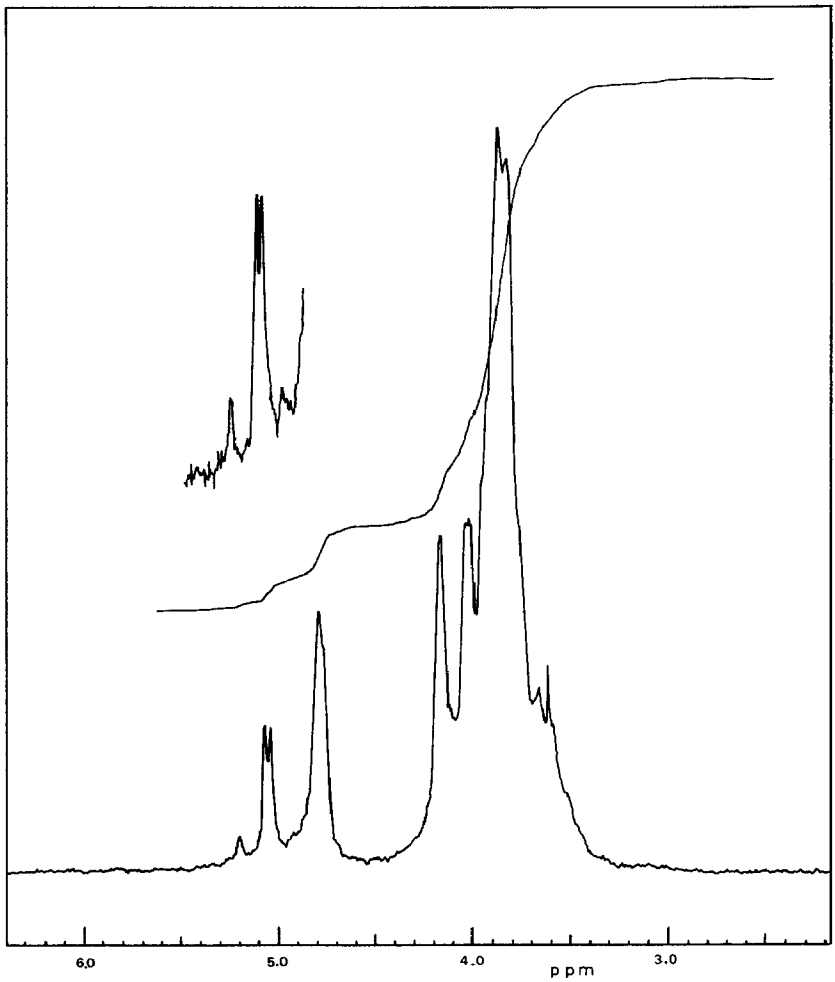


Fig. 4. ^{1}H-N.m.r. spectrum (100.1 MHz) of a solution (87 mg/mL) in D_2O (at pD 7 and room temperature) of subfraction N. [Top: 4.9–5.3-p.p.m. region, expanded × 2.]

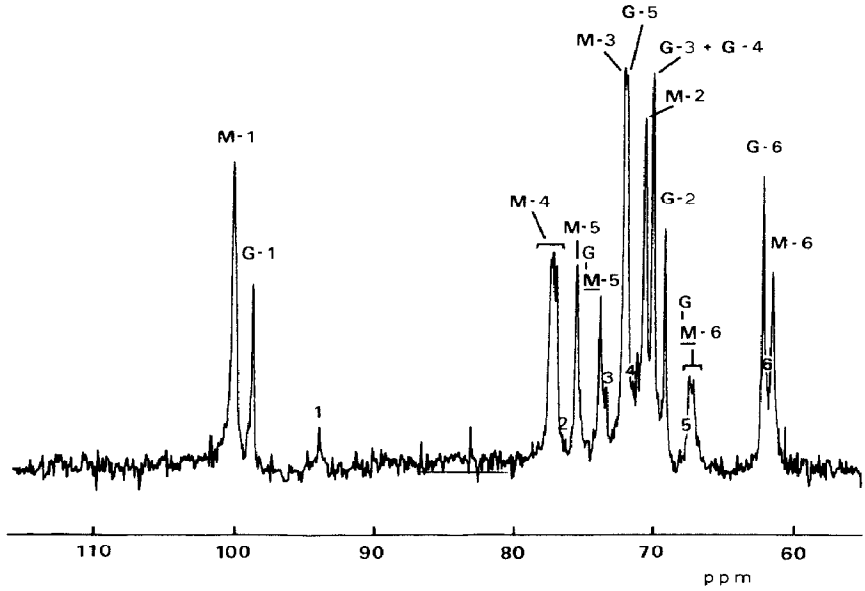


Fig. 5. ^{13}C-N.m.r. spectrum (25.2 MHz) of a solution (87 mg/mL) in 1:3 D_2O–H_2O (at pD 7 and room temperature, with 210.2 k transients) of subfraction N. [M = Mannose; G = Galactose. 1: M-1, 2: M-5, 3: M-3 and 4: M-2 of the reducing-end chain-residues; 5: M-4 of the nonreducing end chain-residues, and 6: M-6 of the reducing or the nonreducing end chain residues.]

different carbon lines are resolved, and their chemical shifts are recorded in Table IV. The resonances are narrower than in the spectra of legume-seed galactomannans[17–19], possibly due to the fact that the small size of the molecule allows higher reorientational mobility.

Five different types of unit could be identified, namely, (*i*) the α-D-galactopyranosyl (nonreducing) end-chain groups, whose resonances agreed with those of the monomeric methyl α-D-galactopyranoside[20]; (*ii*) the 4-linked β-D-mannopyranose residue forming the reducing end of the mannosyl backbone, whose carbon resonances accorded well with those of 4-*O*-methyl-β-D-mannopyranose[21]; (*iii*) the (1→4)-linked β-D-mannopyranosyl residues forming the interior units of the mannosyl backbone, whose signals were in agreement with those calculated by addition of the α, β, and γ effects produced by the O-1 and O-4 glycosidic linkages to the absorptions of β-D-mannopyranose; (*iv*) the β-D-mannopyranosyl group forming the nonreducing end of the mannosyl backbone, whose resonances agreed with those of methyl β-D-mannopyranoside[21]; and (*v*) the O-6-substituted, (1→4)-linked β-D-mannopyranosyl interior residues of the central chain, and, in this case, the positions of the C-6 and C-5 resonances are shifted, relative to the corresponding resonances of unbranched D-mannosyl residues, by 6 p.p.m. downfield, and 1.6 p.p.m. upfield, respectively.

The corresponding resonances in the ^{13}C-n.m.r. spectra of the oligo-

TABLE IV

ASSIGNMENTS OF PEAKS IN THE ^{13}C-N.M R SPECTRUM OF THE GALACTOMANNAN-LIKE OLIGOSACCHARIDES[a]

Type of unit	*C-1*	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*
α-D-Galactopyranosyl	99.54	69.33	70.22	70.22	72.17	62.15
β-D-Mannopyranosyl residue, unbranched at O-6	100.96	70.81	72.35	77.37[b] 77.60[c]	75.83	61.45
β-D-Mannopyranosyl residue, branched at O-6	100.96	70.81	72.35	77.60[b] 77.77[c]	74.10	67.57[d] 67.47[e] 67.26[f]
β-D-Mannopyranosyl (nonreducing) end-chain group	100.96	70.81	72.35	67.83	75.83	61.92[g]
β-D-Mannopyranose (reducing) end-chain residue	94.74	71.73	73.71	77.37	76.18	61.45[g]

[a]Shifts (p.p.m.) downfield from external tetramethylsilane; δ 1.96–2.16 p.p m. upfield from sodium 4,4-dimethyl-4-silapentanoate-d_4. [b]When the preceding D-mannosyl residue is unbranched. [c]When the preceding D-mannosyl residue is branched. [d]Intermediate unit from groups of three, contiguous, substituted D-mannosyl residues. [e]Superposition of signals from triads wherein two contiguous units are substituted. [f]Blocks of three contiguous D-mannosyl units wherein only the intermediate unit is substituted. [g]These assignments may have to be interchanged.

saccharides agree with those in the spectra of guaran[17], carob[17,18], and the galactomannans from clover seeds[17] and *Trigonella foenum-graecum*[18].

DISCUSSION

The endosperm of the seed of *Gleditsia triacanthos* contains 4.8% of 85% ethanol-soluble, galactomannan-like oligosaccharides having a Man:Gal ratio of 1.4–2.6:1 and an average degree of polymerization of 15. The fractionations showed that, if the Man:Gal ratio is taken into account, the oligosaccharides can be considered to be monodisperse. In this sense, as well as in the Man:Gal ratio, the oligosaccharides are similar to the galactomannans extracted, at the same time, at room temperature[7]. The elution patterns of the gel-permeation chromatography in 7M urea, and the fact that the weight-average molecular weights determined by this procedure agreed with the number-average molecular weights obtained by the analysis of the end-chain units, suggest that major proportions of the oligosaccharides are molecular-weight monodisperse.

The decrease of the optical rotation with the increase of the chaotropic power of the solvent[22] indicates that the oligosaccharides, as well as the related, high-molecular-weight galactomannans[7], exist in aqueous solution in ordered forms, producing lower free-energy aggregates. They are differentiated from the galactomannans extracted under the same conditions[7], in that the oligosaccharide molecules dissociate completely in 7M urea, and this easy dissociation can be

ascribed to the shorter backbone, with concomitant decrease of cooperative interactions. The small decrease in the optical rotation in sodium chloride solution may reflect an incipient dissociation of the aggregates. The shorter central-chain also precludes the possibility of combination with more than one partner, and consequently, of the formation of soluble networks, and this results in an increase in solubility, and in nonviscous solutions. This shorter backbone, together with the presence of abundant and longer lateral chains (with some end-chain residues different from D-galactose) could explain the lack of gelation in borate solutions[23].

The results of gel chromatography of the 85% ethanol-soluble fraction of the endosperm (S) suggest that, even in 7M urea, interactions exist between the oligosaccharides and the non-galactomannan components of the fraction.

The oligosaccharide samples contained small proportions of "associated" protein, as the related galactomannans[7], but the determinations of end-chain groups, as well as the presence of 2-acetamido-2-deoxy-D-glucose in only traces, showed that most of the molecules are true carbohydrates. It is not yet possible to determine whether a small part of the molecules are oligopeptides, or if the protein binds noncovalently to the carbohydrate chains.

Methylation analysis showed that the oligosaccharides have the gross structure accepted for the galactomannans, namely, a β-(1→4)-linked D-mannose backbone having single stubs of α-(1→6)-linked D-galactose. Minor deviations from this structure were found in some molecules, namely, (*i*) lateral chains having more than one galactose unit, with the "extra" residue having a (1→4) linkage, and this had been found in the galactomannans from *Gleditsia ferox*[24], *Gleditsia amorphoides*[25], *Gleditsia triacanthos*[7], and *Trifolium repens*[24]; (*ii*) lateral chains ended by arabinofuranose or fucopyranose residues, as was found in the galactomannans of the same endosperm[7]; and (*iii*) unusual branching-points formed by 3,4-linked D-mannose or 3,6-linked D-galactose units, or both.

No evidence for the presence of xylose or glucose residues in the oligosaccharide molecules was found by methylation or any other structural method. This is consistent with the existence of a small proportion of xylo-(1→4)-linked glucan or cellulose microfibril segments solubilized through noncovalent binding with the backbone of the oligosaccharides[26]. Glucose had also been found in some galactomannan samples from the endosperm of *Gleditsia triacanthos*[7], but no structural significance could be attributed to it[7].

Data from periodate oxidation, as well as those from the ^{1}H-n.m.r. spectrum, agreed with the structure obtained by methylation analysis.

The ^{13}C-n.m.r. spectrum clearly differentiated five structural units in the oligosaccharide, namely, the mannose backbone, reducing and nonreducing end-chains, substituted and nonsubstituted, internal, β-(1→4)-linked mannopyranose units of the backbone, and the galactose nonreducing end-chains of the lateral chains. The C-4 signal of the (1→4)-linked mannose residues showed a splitting into three lines that had previously been observed in the spectrum of the galactomannans from clover seeds[17], guaran[17], and carob gum[17], and that was attributed

to sequence-related heterogeneity, and explained by assuming that this resonance is sensitive to whether or not the residue linked to O-4 (*i.e.*, the preceding residue in the chain) is branched. This provided the basis for determining the nearest-neighbor probabilities in the central chain[17]. According to this concept, the absorption at the lowest field (77.75 p.p.m.) originates from groups of two contiguous, substituted D-mannose residues, the intermediate peak (77.60 p.p.m.) represents the superposition of signals originating from diads in which only one of the two mannose residues is substituted, and the peak at high field (77.37 p.p.m.) must be due to unsubstituted mannose residues that are adjacent to another residue of the same kind.

The three lines have the same intensities, and therefore the distribution of the diad frequencies is 0.33, 0.17, and 0.33, respectively. The theoretical values, calculated for a random distribution (from the Man:Gal ratio measured from the spectrum), are 0.26, 0.25, and 0.24.

The C-6 absorption of the (1→4)-linked D-mannose residue substituted by a lateral chain also showed a splitting into three lines of similar intensities; this splitting, which is incipient in the 25-MHz spectrum of carob gum[17], is clearly shown in the 75-MHz spectrum of the degraded galactomannan of the endosperm of the seed of *Gleditsia triacanthos*[19]. It can be explained by assuming that this resonance is sensitive to whether or not the mannose units linked to it are also branched. The reasoning of Grasdalen and Painter[17], applied to this pattern of lines and to that shown in the 75-MHz spectrum of the aforementioned galactomannan of *Gleditsia triacanthos*[19] (see Fig. 6), suggested that the peak at the lowest field (67.57 p.p.m.)

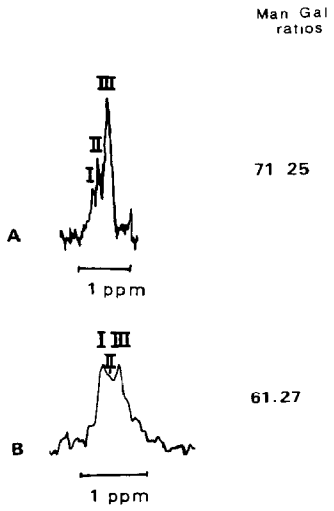
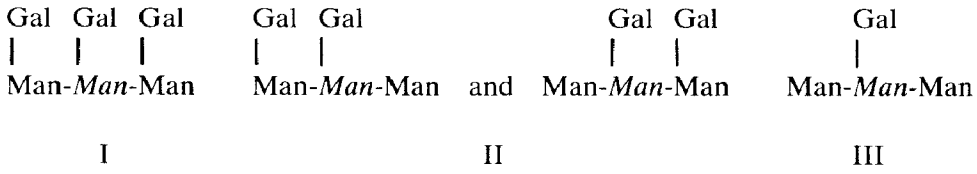


Fig. 6. ^{13}C-N.m.r.-spectral region of C-6 of the substituted D-mannosyl residues in: A, depolymerized galactomannan from *Gleditsia triacanthos*, at 75 MHz, and B, galactomannan-like oligosaccharides (N) from *Gleditsia triacanthos*, at 25.2 MHz. [I, II, and III correspond to the triads shown in the text.]

originates from the C-6 resonance of the intermediate unit from groups of three contiguous, substituted D-mannose residues (I), that the absorption at higher field (67.26 p.p.m.) is due to blocks of three contiguous, mannose units, where only the intermediate residue is substituted (III), and that the intermediate peak (II) must represent a superposition of signals from triads where two contiguous units are substituted.



The intensities of the absorption lines are similar, and consequently, the distribution of the aforementioned triads will be 0.18, 0.07, and 0.17, respectively*. The theoretical values calculated for a random distribution of the lateral chains with the Man:Gal ratio of 1.96:1, measured from the C-6 absorptions in the spectrum of the oligosaccharides, are 0.13, 0.13, and 0.12* for structures I, II, and III, respectively.

In this case, as well as in that of the C-4 absorption lines of the D-mannose units, the discrepancies between the experimental and calculated frequencies preclude a random arrangement of the side chains. Also, both sets of experimental values permit discarding of limit structures with a strictly alternating arrangement of branched and unbranched D-mannose residues, or that in which a block of branched D-mannose units is contiguous to a block of nonsubstituted D-mannose residues. The experimental data are consistent with a more-complex, nonrandom arrangement, as is found in guaran and locust-bean gum[27].

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