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STRUCTURE OF THE D-GALACTO-D-MANNAN ISOLATED FROM THE SEEDS OF *Melilotus indica All**

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ABSTRACT

A D-galacto-D-mannan ($[\alpha]_D$ +72.0 and D-galactose-to-D-mannose ratio 1:1.14) was isolated from the seeds of *Melilotus indica All., syn. M. parviflora Desf.* The ^1H- and ^{13}C-n.m.r., and i.r. spectra indicated the presence of α-D-galactopyranosyl and β-D-mannopyranosyl residues. Methylation of the polysaccharide, followed by hydrolysis, afforded 2,3,4,6-tetra-, 2,3,6-tri-, 2,3-di-, and 3,4-di-*O*-methyl-D-mannose, and 2,3,4,6-tetra- and 2,3,6-tri-*O*-methyl-D-galactose in the molar ratios of 1:2:22:6:27:3. Periodate oxidation of the polysaccharide, followed by reduction and hydrolysis, gave erythritol (1 mol) and glycerol (1.24 mol). Partial acid hydrolysis of the polysaccharide afforded *O*-β-D-mannopyranosyl-(1→2)-D-mannopyranose, *O*-β-D-mannopyranosyl-(1→4)-D-mannopyranose, *O*-α-D-galactopyranosyl-(1→6)-D-mannopyranose, *O*-α-D-galactopyranosyl-(1→4)-D-galactopyranose, and *O*-α-D-galactopyranosyl-(1→6)-*O*-β-D-mannopyranosyl-(1→4)-D-mannopyranose. A highly branched structure having a mannan backbone composed of 36% of (1→4)- and 10% of (1→2)-linked β-D-mannopyranosyl units is proposed for the galactomannan.

INTRODUCTION

Melilotus indica All., syn. M. parviflora Desf. (Leguminosae) is one of the species cultivated in India[1] as a forage crop, together with the other Indian species *M. alba* and *M. officinalis*. The seeds of the plant are found as a winter weed of cultivation almost throughout India. The seeds are rich in mucilagenous matter and recommended as a remedy for bowel complaints and infantile diarrhoea. The present communication is concerned with the structure of a D-galacto-D-mannan isolated from the seed of *M. indica All.*

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TABLE I

IDENTIFICATION OF THE NEUTRAL SUGARS OBTAINED FROM *M. indica* D-GALACTO-D-MANNAN

Component[a]	R_T[b]		*M.s.* (m/z)[c]	*Molar ratio*[d]
	SE-30	*OV-225*		
D-Mannose	5.7	8.70	43, 73, 103, 115, 145, 157, 217, 289, 361, 375	1.14
D-Galactose	6.0	9.77	43, 73, 103, 115, 145, 157, 217, 289, 361, 375	1.00

[a]As alditol acetates. [b]Retention times of neutral sugars are for columns of SE-30, and 3% of OV-225; the values were identical with those of standard sugars. [c]Characteristic fragments are reported in the literature[8]. [d]Relative to D-galactose.

RESULT AND DISCUSSION

The crude polysaccharide was isolated from the aqueous extracts of the powdered and defatted seeds of *M. indica* by precipitation with ethanol. Purification of the polysaccharide by dialysis followed by ion-exchange resin treatment, and also by fractionation with Fehling's solution furnished a white, fibrous material. The homogeneity of the purified polysaccharide was established by moving-boundary electrophoresis[2] and ultracentrifugation[3]. The pure compound was water soluble, and nonreducing. It was free from nitrogen, sulfur, halogens, methoxyl, pentosan, and uronic acid. The ^{1}H-n.m.r. spectrum displayed a signal at δ 4.76 ($J_{1,2}$

TABLE II

IDENTIFICATION OF THE METHYLATED SUGARS OBTAINED FROM PERMETHYLATED *M. indica* D-GALACTO-D-MANNAN

Component[a]	R_T *rel.*[b]	*M.s.* (m/z)	*Molar ratio*[c]
2,3,4,6-Me_4-D-Man	0.98	43, 45, 71, 87, 101, 117, 129, 145, 161, 205	1
2,3,4,6-Me_4-D-Gal	1.17	43, 45, 71, 87, 101, 117, 129, 145, 161, 205	27
2,3,6-Me_3-D-Man	1.90	43, 45, 87, 99, 101, 113, 117, 233	2
2,3,6-Me_3-D-Gal	2.04	43, 45, 87, 99, 101, 113, 117, 233	3
2,3-Me_2-D-Man	3.62	43, 101, 117, 261	22
3,4-Me_2-D-Man	4.01	43, 87, 99, 129, 189	6

[a]As alditol acetates. [b]Retention times relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol; the values are reported in the literature[10–12]. [c]Relative to 2,3,4,6-tetra-*O*-methyl-D-mannose.

~0.9 Hz) for β-D-mannopyranosyl and doublet at δ 5.02 ($J_{1,2}$ ~1 Hz) for α-D-galactopyranosyl units[4]. The proton-decoupled ^{13}C-n.m.r. spectrum showed two anomeric carbon signals at δ 99.0 and 100.3. From these and literature data[5,6], the two signals were assigned to α-D-galactopyranosyl residues linked at two different positions. The i.r. spectrum showed absorption bands at 820 and 875 cm^{-1}, thus indicating the presence of α-D-galactopyranosyl and β-D-mannopyranosyl units, respectively[7]. The sedimentation coefficient ($S_{20,w}$) and diffusion coefficient ($D_{20,w}$) were found to be 2.3×10^{-13} and $3.2 \times 10^{-7} \cdot cm^2 \cdot sec^{-1}$, respectively, as determined by ultracentrifugation. The partial specific volume of the polysaccharide was estimated to be 0.6401 by pyknometry at 20°, and the solvent density at 20°, 1.008. Acid hydrolysis of the polysaccharide gave galactose and mannose in the molar ratio of 1:1.14, as analyzed by g.l.c. and g.l.c.–m.s. (see Table I). Methylation analysis showed a galactose-to-mannose ratio of 1:1.03. The difference between these molar ratios is probably due to inadvertent losses during evaporation[3].

The galactomannan was exhaustively methylated, and the permethylated polysaccharide was hydrolyzed with sulfuric acid[9] to afford 2,3,4,6-tetra-, 2,3,6-tri-, 2,3-di-, and 3,4-di-*O*-methyl-D-mannose, and 2,3,4,6-tetra-, and 2,3,6-tri-*O*-methyl-D-galactose, identified by the retention times of the alditol acetates relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol in g.l.c. analysis and by the characteristic mass fragments obtained during g.l.c.–m.s. analysis. Quantitative determination of the methylated sugars by g.l.c. analysis of the corresponding alditol acetates showed that the six methylated sugars were present in the molar proportions of 1:2:22:6:27:3, respectively (see Table II).

These results indicated the presence, per repeating unit, of 28 mannosyl and galactosyl end groups and 2 and 3 residues of D-mannose and D-galactose, respectively linked at O-4. The presence of 2,3,6-tri-*O*-methyl-D-galactose has previously been shown in the hydrolyzate of the methylated galactomannan of *Gleditsia triacanthos*[13]. Thus, the presence, in the present study, of this D-galactose derivative in the proportion of 4.9% (3 mol), as determined by g.l.c. and g.l.c.–m.s., is not an artifact of partial demethylation or undermethylation. Further, the occurrence of 22 mol of 2,3-di-*O*-methyl-D-mannose indicated that these mannose residues are joined through O-4 and -6. Similarly, the mannose residues that gave rise to 6 mol of 3,4-di-*O*-methyl-D-mannose are linked at O-2 and -6. The occurrence of O-2 and -6-linked D-mannose residues has previously been reported for the galactomannans isolated from *Medicago sativa*[14] and oak lichen *Evernia prunastri (L) Ach.*[15]. The foregoing results suggest a highly branched structure having 28 end groups in an average repeating unit of 61 hexosyl residues for the *M. indica* galactomannan.

The galactomannan consumed 1.44 mol of sodium metaperiodate, with concomitant liberation of 0.43 mol of formic acid, per "anhydrohexose" unit. The oxidized polysaccharide was reduced with sodium borohydride, and the derived polyalcohol was hydrolyzed with acid[7] (Smith degradation[16]) to give erythritol and glycerol in the molar ratio of 1:1.24. All the sugar units were oxidized by periodate.

Partial, acid hydrolysis of the galactomannan with dilute sulfuric acid gave

TABLE III

DATA OF THE OLIGOSACCHARIDES **1–5**

Oligosaccharide	*D.p.*	$[\alpha]_D^{28a}$ *(degrees)*	*Sugar component*	IO_4^- *consumed*[b]	HCO_2H *liberated*	*Glycol composition*
1	1.93	−29.5	D-Mannose	3.10	1.04	Glycerol
2	2.05	−8.8	D-Mannose	2.95	1.00	1:1 Erythritol–glycerol
3	1.89	+121.5	1:1 D-Galactose–D-mannose	4.17	2.05	Glycerol
4	2.16	+178.3	D-Galactose	3.11	1.09	Erythritol–glycerol
5	3.08	+31.5	1:2 D-Galactose–D-mannose	5.14	2.08	Erythritol–glycerol

[a]For a solution in water. Except for **1**, the values of optical rotation of oligosaccharides **2–5** are similar to literature data[17–19]. [b]Mol/mol of oligosaccharide.

oligosaccharides **1, 2, 3, 4,** and **5** (see Table III), in addition to galactose and mannose. Disaccharide **1** appears not to have been reported up to now and the presence of a β-D-(1→2) linkage in this disaccharide was further confirmed by formation of 2-*O*-β-D-mannopyranosylglycerol, $[\alpha]_D^{25}$ −42.3° (water) according to the procedure of Gorin and Perlin[20,21].

Based on the above findings, a tentative structure (**6**) showing one of the possible arrangements of the sugar residues was assigned for the average repeating unit of the galactomannan. The molecular weight of the galactomannan was found to be ~49 500, as determined by ultracentrifugation[22]. Since the average repeating unit of this polysaccharide consists of 61 hexosyl residues, it is obvious that the average repeating unit is repeated five times in the full structure of *M. indica* galactomannan.

The characteristic features of *M. indica* galactomannan, as shown in the tentative structure **6**, indicate a D-mannan backbone composed of 36% of (1→4)- and 10% of (1→2)-linked β-D-mannopyranosyl residues. Infrequent, short chains of (1→4)-linked α-D-galactopyranosyl residues (composed of two galactose units) are attached to the D-mannan backbone, in addition to single α-D-galactopyranosyl groups attached by an (1→6) linkages. The galactomannan also carries one short chain of (1→4)-linked β-D-mannopyranosyl units (consisting of three mannose residues) attached at O-4 of an α-D-galactosyl residue. This proposed structure for *M. indica* galactomannan differs from the classical structure of D-galacto-D-mannans not only in the D-mannan backbone but also in the mode of linkage of the side chains. The simultaneous occurrence of (1→4)- and (1→2)-linked β-D-mannopyranosyl units in a D-mannan backbone is rare, but has previously been reported for the D-galacto-D-mannans of *Crotalaria mucronata*[3] and *Caesalpinia pulcherima*[23]. The presence of infrequent, short chains of (1→4)-linked α-D-galactopyranosyl residues connected to the D-mannan backbone by (1→6) linkages has previously

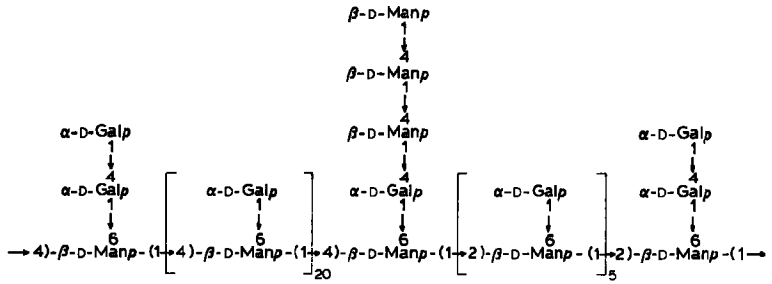
O-β-D-Mannopyranosyl-(1→2)-D-mannopyranose
1

O-β-D-Mannopyranosyl-(1→4)-D-mannopyranose
2

O-α-D-Galactopyranosyl-(1→6)-D-mannopyranose
3

O-α-D-Galactopyranosyl-(1→4)-D-galactopyranose
4

O-α-D-Galactopyranosyl-(1→6)-*O*-β-D-mannopyranosyl-(1→4)-D-mannopyranose
5



6

been shown in *Gleditsia triacanthos*[13] D-galacto-D-mannan. Thus, it appears that the highly branched galactomannan occurring in *M. indica* seeds is unique in its structural features.

EXPERIMENTAL

General methods. — Optical rotations were determined with a JASCO model DIP-SL automatic polarimeter. Moving boundary electrophoresis was carried out in an electrophoresis apparatus model LKBII. All the centrifugations were carried out in a model Centriscan 75 ultracentrifuge (M.S.E., Crawley, England) using the Schlieren optics at 550 nm; a 20-mm optical path and single-sector cells for sedimentation; and a 20-mm optical path, underfilling boundary-forming cell for the diffusion run. I.r. spectra were recorded with a Perkin–Elmer model 137-B infrared spectrophotometer. ^{13}C- (at 100.6 MHz) and ^{1}H-n.m.r. (at 400 MHz) spectra were recorded in F.t. mode with a Bruker WH-400 spectrometer. Conditions for ^{13}C-n.m.r. were: spectral width, 20 000 Hz; acquisition time, 0.8192 s; and pulse width, 3.0 μsec; and for ^{1}H-n.m.r.: spectral width, 4000 Hz; acquisition time, 2.0480 s; and pulse width, 2.0 μsec; for solutions at 29 and 90°, respectively, in D_2O (50 mg/mL); chemical shifts (δ) are given relative to Me_4Si as internal standard.

Paper chromatography (p.c.) for analytical purposes was performed on Whatman No. 1 paper, and for preparative purposes on Whatman No. 3 MM paper with the following solvent systems (all v/v): (*A*) 4:1:5 (upper layer) butanol–

ethanol–water[13], and (*B*) 4:3:2:2 ethyl acetate–acetic acid–butanol–water[24]. The spots were detected with the spraying reagents: (*a*) $AgNO_3$–NaOH[25], (*b*) *p*-anisidine phosphate[26], and (*c*) benzidine periodate[27]. G.l.c. analyses were carried out on a Hitachi model 063 gas chromatograph equipped with a H_2 flame-ionization detector and a column (0.3 × 200 cm, spiral glass) packed with 3% of OV-225 on Gas Chrome Q (100–200 mesh) at 220° and a flow rate of He of 40 mL/s for neutral sugars and at 180° and a flow rate of N_2 of 30 mL/min for methylated sugars. G.l.c.–m.s. of neutral sugars was performed with a Hewlett–Packard model 5985 equipped with a column (0.63 × 153 cm, stainless steel) packed with SE-30 and He at a flow rate of 40 mL/s programmed from 180 to 220° at 4°/min. The methylated sugars were analyzed by g.l.c.–m.s. with a JEOL model JGC-20K gas chromatograph and JMS-D100 mass spectrometer under similar conditions. Paper electrophoresis was conducted in a Laboratorium Felzerclsk model DE-201 apparatus on Whatman No. 1 paper strips (45 × 4 cm) at 400 V and 19mA in 50mM $Na_2B_4O_7$ buffer (pH 9.2). All evaporations were conducted at 40–50° with a rotary vacuum evaporator.

Isolation and purification of the polysaccharide. — The seeds were cleaved with a low-speed grinder in order to obtain the endosperm with the seed coat broken off from the cotyledons. The finely powdered, dried, and defatted *M. indica* seeds (100 g) were extracted four times with water at 50–60° for 16 h. The extract was cooled, centrifuged, made acid with acetic acid, and precipitated with ethanol. The crude polysaccharide was obtained in the form of a slightly-colored precipitate, centrifuged, and dried by solvent exchange with acetone and then absolute ethanol. The crude polysaccharide was further purified by dialysis, followed by treatment through successive columns of Dowex 50W-X8 (H^+) and Dowex 3 (OH^-) ion-exchange resins, and also *via* its copper complex to give a white, fibrous material (5.95 g), $[\alpha]_D^{45}$ +72.0° [*c* 0.06, buffer 0.2M NaOH and 0.2M KCl (pH 13.0)], which did not reduce Fehling's solution. The purified polysaccharide dissolved slowly in water to form a neutral, viscous solution (pH 6.5). No nitrogen, sulfur, halogens, methoxyl, pentosan, or uronic acid content was observed.

Moving-boundary electrophoresis. — The polysaccharide (1% solution) was analyzed by moving-boundary electrophoresis in 0.2M Tris · HCl buffer (pH 8.5) for 5 h at 12 V and 5 mA. The free boundary migration, recorded with a Schlieren optical system, showed only one sharp symmetrical peak, both in the descending and the ascending arm[2].

Ultracentrifugation. — The measurements were carried out on 0.18, 0.35, and 0.51% solutions in 40mM K_3PO_4–0.2M KCl buffer, pH 7.8), at 20°, 55 000 r.p.m., and various time intervals. The sedimentation patterns showed a single symmetrical peak, thereby confirming the homogeneity of the polysaccharide[3]. The measurement of the diffusion coefficient was performed on 0.26, 0.32, 0.35, 0.45, and 0.48% solutions in buffer at 5500 r.p.m., and the values were plotted against concentration.

Identification and estimation of constituent sugars. — Acid hydrolysis of the purified polysaccharide was performed either with H_2SO_4 or trifluoroacetic acid on

the basis of pilot experiments monitored by iodometric titration[28]. The pure polysaccharide (40 mg) was hydrolyzed with 2M H_2SO_4 (2 mL) for 10 h at 100° in a sealed tube, the solution made neutral with $BaCO_3$, centrifuged, concentrated to dryness, the residue extracted with absolute methanol, and the extract filtered and concentrated to a syrup. In another experiment, the pure polysaccharide (20 mg) was hydrolyzed with 2M trifluoroacetic acid (1.5 mL) in a sealed tube for 12 h at 100°, the solution filtered, and the acid removed by evaporating the syrup to dryness under a stream of N_2. The residue was heated at reflux with dry methanol, and the solution filtered and concentrated to a syrup. The components of each hydrolyzate were converted[29] into the alditol acetates, and analyzed by g.l.c. and g.l.c.–m.s. The results are given in Table I.

Methylation analysis of the galactomannan. — The galactomannan (100 mg) was methylated successively with dimethyl sulfate and alkali[30], and with methyl iodide and Ag_2O (ref. 31) to yield a syrup {49.4 mg; $[\alpha]_D^{25}$ +60.7° (*c* 1.3, chloroform); OCH_3, 45.41%} which showed no hydroxyl absorption in the i.r. spectrum. Further methylation of the product with Purdie reagents did not increase its methoxyl content. The methylated galactomannan (28.4 mg) was hydrolyzed according to the method of Croon *et al.*[9]. The hydrolyzate was made neutral ($BaCO_3$), filtered, and concentrated to a syrup which was reduced in water with $NaBH_4$ for 2 h. After neutralization with Dowex 50W-X8 (H^+) cation-exchange resin, the filtrate was evaporated and H_3BO_4 removed by repeated addition and evaporation of methanol. The alditols were converted[29] into the peracetates with 1:1 acetic anhydride–pyridine for 20 min at 100°. After evaporation of the solution, the residue was dissolved in 1:1 chloroform–methanol and the solution applied to g.l.c. and g.l.c.–m.s., which were carried out under similar conditions. The results are given in Table II.

Smith degradation of the galactomannan. — A solution of galactomannan (1.23 g) in water (230 mL) was mixed with 0.5M $NaIO_4$ (20 mL) and kept at 5° in the dark. Aliquots (5 mL) were withdrawn at various time-intervals and analyzed for periodate consumption[32] and formic acid liberation[33], which reached a plateau after 240 h. The residue of the mixture was successively treated with 1,2-ethanediol (5 mL) and sodium borohydride (1.2 g) for 8 h at room temperature, and made neutral by addition of 50% acetic acid. The resulting solution was dialyzed and evaporated to dryness, and H_3BO_4 was removed from the residue by repeated distillations with methanol. The residue was hydrolyzed with 0.5M sulfuric acid in a sealed tube for 8 h at 100°. The hydrolyzate was made neutral with $BaCO_3$, and then de-ionized with freshly regenerated cation- (Dowex 50W-X8) and anion-exchange (Dowex 3) resins. Analysis by p.c. using solvent (*A*) and spraying reagent (*a*) showed the presence of erythritol and glycerol; no sugars were detected. Erythritol and glycerol were separated quantitatively on paper and estimated by the chromotropic acid method[34].

Partial, acid hydrolysis of the galactomannan. — Based on the results of trial experiments, the galactomannan (2 g) was hydrolyzed with 50mM H_2SO_4 (80 mL)

for 12 h at 95°, the solution made neutral ($BaCO_3$) and filtered, and the filtrate evaporated to a syrup. This was heated at reflux with absolute methanol, and the solution filtered and evaporated. Examination of the resulting syrup by p.c. in solvent (*B*) with spray reagents either (*a*) or (*b*) revealed five oligosaccharides, in addition to galactose and mannose.

For resolution of the oligosaccharides in quantitative yield, the syrup was applied to a column of 1:1 charcoal–Celite and fractionated by successive elution with water, and water containing 0.5, 5, and 10% of ethanol. The fraction eluted with water contained only monosaccharides, and that eluted with ethanol in water contained oligosaccharides, which were fractioned by further preparative p.c. in solvent system (*B*) to give oligosaccharides **1–5** in a chromatographically pure form. The oligosaccharides were also found to be homogeneous by paper electrophoresis. The d.p. of the five oligosaccharides was determined by the anthrone–H_2SO_4 method[35].

The oligosaccharides were hydrolyzed with 0.5M H_2SO_4 for 6–7 h at 100°. After the usual treatment, the component sugars were identified by p.c. in solvent (*A*) with spray reagent (*a*). The molar ratio of the sugars was determined by the phenol–H_2SO_4 method[36]. The oligosaccharides were converted into their methyl glycoside derivative by treatment with 2%, Dry ice-cold, methanolic HCl under strictly anhydrous conditions[24]. The homogeneity of the methyl glycoside derivative was controlled by p.c. Periodate oxidation of the oligosaccharides was conducted with 0.1M $NaIO_4$ solution in the dark, at 5°, followed by iodometric titration[32,33]. Periodate consumption and formic acid liberation were determined in mol/mol of methyl glycoside derivative. The periodate oxidized product was reduced with $NaBH_4$ and hydrolyzed with 0.5M H_2SO_4 for 5 h at 100°. After the usual treatment, the resulting glycols were identified by p.c. in solvent (*A*) with spraying reagent (*c*) and quantitatively determined by the chromotropic acid method[34]. The linkages of the trisaccharide **5** were determined by methylation with the method of Hakomori[37], followed by that of Purdie and Irvine[31], and hydrolysis of the methylated product by the method of Croon *et al.*[9]. After the usual treatment, the mixture of methylated sugars obtained from **5** was separated by preparative p.c. in solvent (*A*), to give three methylated sugars which were identified as 2,3,4,6-tetra-*O*-methyl-D-galactose, and 2,3,4-tri- and 2,3,6-tri-*O*-methyl-D-mannose by their migration rates ($R_{2,3,4,6\text{-OMeGlc}}$ 0.90, 0.85, and 0.81) on p.c. and by co-chromatography with authentic samples in solvent (*A*) with spraying reagent (*b*). Their quantitative determination by a modified hypoiodite oxidation method[38] in buffer [NaOH–Na_3PO_4 (pH 11.40)] showed equimolar proportions. The d.p., optical rotation, sugar composition, periodate consumption, formic acid liberation, and glycol composition of each oligosaccharide are given in Table III.

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