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Note

A water-soluble galactomannan from the seeds of *Indigofera tinctoria linn.*

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The plant *Indigofera tinctoria* grows abundantly in India. In view of the constantly increasing demand by industry for plant gums[1], we were prompted to undertake a chemical investigation of the galactomannan isolated from the seeds of *Indigofera tinctoria*.

Hot-water extraction of the endosperms of the seeds of *Indigofera tinctoria*, followed by repeated precipitation with ethanol, yielded a crude polysaccharide that had $[\alpha]_{589.5}^{23}$ +54.1° (*c* 0.11, water). On hydrolysis it gave D-galactose (35.4%) and D-mannose (58.7%) in 1:1.66 mol ratio, along with L-arabinose (2.2%) and L-rhamnose (0.7%) (enantiomeric D or L configurations commonly encountered in plant polysaccharides were assumed). The material was further purified by gel-permeation chromatography on Sephadex G-200. The purified polysaccharide had $[\alpha]_{589.5}^{23}$ +56.4° (*c* 0.1, water), and contained D-galactose (38.5%) and D-mannose (58.5%) in the molar ratio of 1:1.52. The material was free of protein and uronic acid, but traces of arabinose and rhamnose were still present.

The polysaccharide was eluted as a broad peak in gel-permeation chromatography on Sepharose CL-4B. The molecular weight[2] was ~1.4–2.1 × 10^6, against a calibration curve using dextrans of known molecular weight.

The polysaccharide was fully methylated by the Hakomori method[3]. The permethylated polysaccharide was hydrolyzed and the partially methylated sugars were identified and estimated as their alditol acetates[4] by g.l.c.–m.s. and g.l.c., respectively. The results are summarized in Table I.

Periodate oxidation[5,6] of the polysaccharide consumed 0.84 mol of periodate per mol of hexose residues. The oxidized product was reduced with sodium borohydride. Sugar analysis of the resultant polyol, as the alditol acetates, showed glycerol and mannose. These results show that only galactose was oxidized by periodate and almost all of the mannose was protected, probably by hemiacetal

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TABLE I

METHYL ETHERS OF SUGARS FROM THE HYDROLYZATES OF METHYLATED, PURIFIED POLYSACCHARIDE (*A*), AND THE METHYLATED OLIGOSACCHARIDES (*B*)

Partially methylated sugars[a]	*Retention time* T[b]		*Mole proportions*[c]						*Mode of linkage*
	(*i*)	(*ii*)	*A*	*B*					
				I	*II*	*III*	*IV*	*V*	
2,3,4,6-Man	1.00	0.99	trace	1			1	2.0	Man*p*-(1→
2,3,4,6-Gal	1.15	1.18	2		1	1		2.2	Gal*p*-(1→
2,3,6-Man	1.98	2.02	1	0.7		0.7	1.7	2.2	→4)-Man*p*-(1→
2,3,4-Man	2.23	2.19			0.8	1		1.3	→6)-Man*p*-(1→
2,3-Man	4.52	4.13	2					1.0	→4,6)-Man*p*-(1→

[a]2,3,4,6-Man = 2,3,4,6-tetra-*O*-methyl-D-mannose etc. [b]Retention times of the corresponding alditol acetates, relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol on (*i*) 3% ECNSS-M column at 170°, and (*ii*) 3% OV-225 column at 170°. [c]Average value from three determinations.

TABLE II

SUGAR COMPOSITIONS OF ORIGINAL AND OXIDIZED POLYSACCHARIDE

Time of oxidation	*Galactose*	*Mannose*	myo-*Inositol*
0	8.7	13.1	10
1	8.7	6.2	10
2	7.7	3.6	10
3	6.9	2.5	10

formation. However, periodate oxidation of the polyol consumed 0.86 mol of periodate per mol of hexose residues, that is, ~90% of the total mannose was oxidized.

The anomeric configurations of the sugar residues were determined by chromium trioxide oxidation[7]. Oxidation of the fully acetylated polysaccharide and sugar analyses (Table II) indicated that the D-mannopyranosyl residues are β-linked and D-galactopyranosyl residues are α-linked.

Graded hydrolysis of the polysaccharide gave four oligosaccharides, which were separated and isolated as homogeneous fractions by paper chromatography. A mixture of tetrasaccharides could not be separated by paper chromatography with various solvents. The oligosaccharides were characterized by sugar analyses and methylation studies (Tables I and III).

From the experiments outlined here, it may be concluded that the galactomannan has a β-(1→4)-linked D-mannose backbone with 67% of the mannose units being substituted by single α-D-galactose units through O-6, a feature common to most galactomannans of leguminous seeds[12,13]. As, during the first periodate oxida-

TABLE III

CHARACTERIZATION OF OLIGOSACCHARIDES

Oligosaccharides	R_{Gal} *in solvents*		*Sugar composition*		$[\alpha]_{589.5}^{23\pm1}$
	B	*C*	*Gal*	*Man*	
βMan*p*1→4Man	0.78			1	−2.4° (*c* 0.43, water) lit.[8] −2.3°
αGal*p*1→6Man	0.65		1	1	+123.2° (*c* 0.51, water) lit.[8] +124.6°
αGal*p*1→6βMan*p*1→4Man	0.39	0.31	1	2	+96.5° (*c* 0.53, water) lit.[9] +98.4°
βMan*p*1→4βMan*p*1→4Man	0.34	0.23		1	−20.6° (*c* 0.32, water) lit.[10] −23.3°
βMan*p*1→4βMan*p*1→4Man *a* *b* *c* αGal*p*1→6) *a* or *b*, or *c* (mixture of three oligosaccharides)	0.15		1	3	+27.6° (*c* 1.6, water) lit.[11] +38°

tion, almost all of the mannose residues were protected by hemiacetal formation[14], it may be concluded that the galactopyranosyl residues are linked to O-6 of mannopyranosyl residues randomly rather than in blocks.

EXPERIMENTAL

General methods. — Optical rotations were measured with a Perkin–Elmer model 241 MC spectropolarimeter at 23 ±1° and 589.5 nm for solutions in water. I.r. spectra was recorded with a Perkin–Elmer model 177 grating infrared spectrophotometer. For g.l.c., a Hewlett–Packard 5730 A gas chromatograph with a flame-ionization detector was used. Resolutions were performed on glass columns (1.83 m × 6 mm) containing (*a*) 3% of ECNSS-M on Gas Chrom Q (100–120 mesh) at 190° (for alditol acetates of sugars), and at 170° (for partially methylated alditol acetates), or (*b*) 3% OV-225 on Gas Chrom Q (100–120 mesh) at 170° (for partially methylated sugars). A recording integrator 3390A (Hewlett–Packard) was used to determine the peak areas. Paper chromatography was performed on Whatman No. 1 and 3 MM paper, with the following solvent-systems; (*A*) 8:2:1 ethyl acetate–pyridine–water, (*B*) 6:4:3 butanol–pyridine–water, and (*C*) 10:4:3 ethyl acetate–pyridine–water. The sugars were detected with (*a*) alkaline silver nitrate or (*b*) 3% *p*-anisidine hydrochloride in ethanol at 120°. Evaporations were performed under diminished pressure at bath temperatures not exceeding 40°. Small volumes of aqueous solutions were lyophilized.

Isolation and purification of the polysaccharide. — The dry endosperms of seeds (15 g) were blended with water (1 L) in a Waring Blender for 5 min. The blended material was extracted 5 times by stirring in water (2 L) for 1 h at 80°.

After each extraction, the suspension was centrifuged. The combined clear solution was concentrated to low volume, acidified to pH 5 with acetic acid, and added to four times its volume of ethanol, dropwise with constant stirring. The precipitated polysaccharide was allowed to settle overnight at 4°, filtered, washed with ethanol and diethyl ether, and finally dried over phosphorus pentaoxide under vacuum. The polysaccharide (3.2 g, 21.3%) was obtained as a gray, amorphous powder.

The polysaccharide was further purified by precipitation with ethanol (twice) and finally by column chromatography on Sephadex G-200 (2.5 × 90 cm). Water containing sodium azide (0.05%) was used as eluant. The material was dialyzed against distilled water and was obtained as a white fluffy material in 90% yield after lyophilization.

Detection and estimation of sugars. — The polysaccharide was hydrolyzed with 2M trifluoroacetic acid[15] for 2 h at 120° and the acid was removed under vacuum. Traces of acid were removed by co-distillation with water. Sugars were then detected by paper chromatography using solvent *A* and spray reagents *a* and *b*, and by g.l.c. as their alditol acetates[4] using column *a*. For quantitative estimation, D-allose was added after hydrolysis as an internal standard[16]. Sugars were then analyzed by g.l.c. on column *a* as alditol acetates and estimated from the peak areas after appropriate corrections.

Molecular-weight determination[2]. — The polysaccharide (1.5 mg in 0.75 mL of water) was applied to a column of Sepharose 4B (1.6 × 90 cm) that was eluted with water containing 0.002% merthiolate at a flow rate of 10 mL/h. The elution of material was monitored by a refractive-index detector and by the phenol–sulfuric acid method. The molecular weight was estimated from a calibration curve prepared by using a dextran T-series (Pharmacia Fine Chemicals AB).

Methylation analysis[3]. — The polysaccharide (10 mg, dried over phosphorus pentaoxide) was methylated by the Hakomori procedure. The methylated sample was purified by passing it through a column (1.5 × 30 cm) of Sephadex LH-20 in 1:1 chloroform–acetone. The absence of the OH band in the i.r. spectrum indicated complete methylation.

The methylated sample was hydrolyzed with 90% formic acid for 2 h at 100°. The acid was removed by co-distillation with water. The product was further hydrolyzed with 0.5M sulfuric acid for 15 h at 100°, and the solution was made neutral with barium carbonate. The product was reduced with sodium borohydride, converted into the alditol acetates, and the latter were examined by g.l.c. using columns *a* and *b* and by g.l.c.–m.s. Results are shown in Table I.

Oxidation of the polysaccharide with sodium periodate[5,6]. — To a solution of the polysaccharide (25 mg) in water (50 mL) was added 0.08M sodium metaperiodate (20 mL), and the solution was stirred at 10° in dark. The periodate consumption was estimated iodometrically; consumption ceased after 72 h. The excess of periodate was then decomposed with ethylene glycol (1 mL) and the solution was dialyzed against distilled water. The solution inside the dialysis bag was concentrated (20 mL) and sodium borohydride (0.5 g) was added. After 10 h at

room temperature, the solution was made neutral with acetic acid, thoroughly dialyzed, and lyophilized. The polyol (2 mg) was hydrolyzed with 2M trifluoroacetic acid for 1 h at 120°, and the acid was removed under vacuum. The sugars were analyzed as their alditol acetates by g.l.c. on column *a*.

Periodate oxidation of the polyol was performed in the same way.

Acetylation of the polysaccharide and oxidation with chromium trioxide[7]. — The polysaccharide (100 mg) was dissolved in dry formamide (50 mL) and pyridine (5 mL) and acetic anhydride (5 mL) were added. The mixture was kept for 20 h at room temperature and then dialyzed against distilled water. The acetylated material obtained after lyophilization was reacetylated by the same procedure. The acetylated polysaccharide (160 mg) was further purified by passing it through a column (1.6 × 50 cm) of Sephadex LH-20 with 1:1 chloroform–acetone.

Oxidation by chromium trioxide was then performed by Lindberg's procedure[7]. The oxidized products formed after 0, 1, 2, and 3 h were then hydrolyzed with 2M trifluoroacetic acid for 2 h at 120°, and the sugars were estimated as their alditol acetates by g.l.c. using column *a* at 190°. The results are shown in Table II.

Graded hydrolysis. — The polysaccharide (1 g) was dissolved in 0.3M trifluoroacetic acid (1 L) and hydrolyzed in a boiling-water bath for 2 h (optimum condition). The mixture was then cooled, filtered, the filtrate evaporated, and traces of acid were removed by co-distillation with water.

The oligosaccharides thus formed were then separated and isolated by preparative paper chromatography with solvent *B*. The oligosaccharides were purified by re-chromatography with either solvent *B* or solvent *C*. Sugars were detected and estimated as alditol acetates[4] after hydrolysis of the oligosaccharides with 2M trifluoroacetic acid for 1 h at 120°. The oligosaccharides were methylated by the Hakomori procedure[3], hydrolyzed with 0.5M sulfuric acid for 12 h at 100°, and finally analyzed as partially methylated alditol acetates by g.l.c. with columns *a* and *b*. Results are summarized in Tables I and III.

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