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Note

A neutral seed-gum from *Crotalaria verrucosa*

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Seed gums are of much industrial importance[1,2]. They have been used in a wide range of industries, sush a paper, pharmacy, textiles, foods, paints and plastics, explosives, and photography. They have been described as beneficial-fiber polysaccharides; seed gums are considered, to be dietary fibers. Some of them variously interact with milk protein, plant lectins[3], and protein antibodies[4], and act as inhibitors of viruses[2].

Species of Crotalaria are well known for their medicinal usage[5], and are rich sources of polysaccharide. Earlier studies on *Crotalaria cunninghamii*[6] and *Crotalaria mucronata*[7] showed the presence of a similar type of galactomannan. Due to the high medicinal importance of the plant *Crotalaria verrucosa*, a systematic phytochemical investigation of the polysaccharide from its seeds was undertaken with a view to studying the nature of the seed gum.

RESULTS AND DISCUSSION

The polysaccharide was extracted from the milled and extractive-free seeds (that had been extracted with petroleum ether, b.p. 40–60°, and 90% ethanol) with 1% aqueous acetic acid, and isolated by repeated precipitation from its solution therein with ethanol. It was purified by the Sevag method and by complexation with Fehling solution. Compounds of lower molecular weight were removed by extensive dialysis against water. The polysaccharide was further purified by ion-exchange chromatography. Its homogeneity was verified by zone electrophoresis and *via* acetylation and deacetylation. Acid hydrolysis of the polysaccharide (mol. wt. 22,000) gave D-galactose and D-mannose in the molar ratio of 1:4. Mild hydrolysis over time showed the removal of α-linked, peripheral D-galactose units.

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The way in which these building units are mutually linked to form the parent molecule was ascertained by methylation studies. On hydrolysis, methylated galactomannan, $[\alpha]_D^{25}$ $+9.2^0$ (chloroform), yielded 2,3,4,6-tetra-*O*-methyl-D-galactose (2 mol%), 2,3-di-*O*-methyl-D-mannose (2 mol%), and 2,3,6-tri-*O*-methyl-D-mannose (6 mol%), indicating ~20% of end groups.

Quantitative conversion of all of the D-galactosyl groups into the tetra-*O*-methyl derivative indicated its exclusive presence as nonreducing terminals. The di-*O*-methyl derivatives of mannose constitute the branching points in the main chain, whereas tri-*O*-methyl derivatives of mannose indicated the presence of (1→4)-linked-β-D-mannosyl units forming the main chain.

On periodate oxidation, the galactomannan liberated 124 mmol of formic acid, supporting earlier evidence of the presence of ~20% of end groups per repeating unit. On prolonged exposure to periodate, the oxopolysaccharide showed the presence of traces of D-mannose, but no D-galactose. The considerable differences in their rates of oxidation are probably attributable to the steric effect resulting from the highly ramified structure of the galactomannan, in which mannose units form all of the branching points. This behavior had been observed for other galactomannans[8,9]. Smith degradation gave glycerol and erythritol, indicating the presence of (1→4) and (1→6) linkages. The anomeric nature of glycosidic bonds and the sequential arrangement of the sugar units were determined by characterization of the following oligosaccharides formed on controlled acid hydrolysis: α-D-Gal*p*-(1→6)-D-Man*p*, β-D-Man*p*-(1→4)-D-Man*p*, α-D-Gal*p*-(1→6)-β-D-Man*p*-(1→4)-D-Man*p*, β-D-Man*p*-(1→4)-β-D-Man*p*-(1→4)-D-Man*p*, and β-D-Man*p*-(1[→4)-β-D-Man*p*(1-]$_2$→4)-D-Man*p*.

The weight-average molecular weight (22,000) was determined by laser light-scattering. Based on these findings, a tentative structure was assigned to the statistical units of the polysaccharide, namely,

The seed gum from *Crotalaria verrucosa* appears to possess a close structural resemblance to the galactomannans obtained from the seeds of *Crotalaria cunninghamii*[6] and *Crotalaria mucronata*[7]. Interestingly, the well-known clover[8], fenugreek[9], guar[10], and carob[11,12] seed galactomannans have similar structural characteristics. Methylated polysaccharides of the aforementioned seed-gums yield the same three partially methylated monosaccharides, but in various proportions, on hydrolysis. This difference may be due to the different degree of branching in each polysaccharide, or may be attributed to the presence in various proportions of D-mannose units forming the main chain of the polysaccharide.

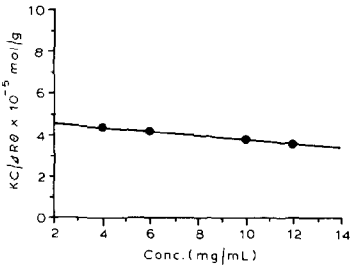

Fig. 1. Determination of the molecular weight of the polysaccharide.

EXPERIMENTAL

General. — All specific rotations are equilibrium values, and all melting points are uncorrected. Paper chromatography (p.c.) was carried out by the descending technique at room temperature, using the following solvent systems (v/v): (S1) (5:1:4) 1-butanol–ethanol–water[13], (S2) (11:6:3) 1-butanol–2-propanol–water[14], (S3) (10:4:3) ethyl acetate–pyridine–water[15], (S4) (2:1:2) ethyl acetate–pyridine–water[16], and (S5) (9:2:2) ethyl acetate–acetic acid–water[17].

Sepharose Cl-6B and Dowex-50 (H^+) were respectively used for purification of the galactomannan and decationizing the hydrolyzates. The spray reagents used for detecting the sugar spots were: (R1) aniline hydrogenphthalate and (R2) sodium metaperiodate–benzidine.

Light-scattering[18] measurements were performed by using laser light of wavelength 632 nm and a Bl 240 goniometer. Intensity data were analyzed by using a Debye plot. Benzene was used for the calibration, and dn/dc was determined to be 0.12. The weight-average molecular weight of the galactomannan was determined in 0.01M NaCl, 10mM phosphate (pH 7.0) at 90^0. $KC/\Delta R\theta$, values were plotted *vs*, various concentrations (mg/ml) and then extrapolated to C = O. The reciprocal of the intercept gives the molecular weight (see Fig. 1).

Isolation and purification of the polysaccharide. — Crushed seeds were successively extracted with light petroleum and ethanol, and then stirred with 1% aqueous acetic acid. The crude polysaccharide was precipitated by using 95% ethanol. The aqueous, mucilaginous solution was then shaken with chloroform to separate the denatured proteins[19]. The solution was centrifuged at ~6000 r.p.m., to remove the insoluble material. The treated mucilaginous solution was then poured slowly, with stirring, into Fehling solution. The precipitated copper complex[20] was decomposed with M hydrochloric acid. The polysaccharide was regenerated by slowly adding ethanol. The product so obtained was reprecipitated from its solution in 1% aqueous acetic acid by ethanol, to yield a nonreducing, white, amorphous material. The polysaccharide had $[\alpha]_D^{25}$ + 30° (water) and was eluted as a single peak on Sepharose Cl-6B in 0.01M NaCl, 10mM PO_4 (pH 7.0). Carbohydrate in

different fractions from the column was assayed by the phenol–sulfuric acid assay[21]. The polysaccharide was further purified by use of an anion-exchange resin in 10mM phosphate, pH 7.0, where it was completely excluded from the column, showing its neutral nature.

Homogeneity of the polysaccharide was further established by zone electrophoresis[22] in 0.1M sodium tetraborate (pH 9.3) for 6 h at constant current. A plot of the absorbance against segment number showed only a single sharp peak, indicating the homogeneity of the polysaccharide.

The polysaccharide was acetylated[23], giving a peracetate. Deacetylation of the peracetate regenerated the polysaccharide ($[\alpha]_D^{25}$ + 29.9°), showing the homogeneity of the polysaccharide.

Structural investigation of the polysaccharide

Complete hydrolysis. — The polysaccharide was hydrolyzed with M sulfuric acid for 28 h at 80°. P.c. (solvent S_2) revealed the presence of galactose and mannose. These sugars were quantified by hydrolyzing the polysaccharide (300 mg), with D-ribose (30 mg) as an internal standard, and fractionating the hydrolyzate on Whatman No. 3MM paper. Quantification of the sugars was done by periodate oxidation[24].

Graded hydrolysis. — The polysaccharide (200 mg) was hydrolyzed with 25mM sulfuric acid at 100°. The hydrolyzate was subjected to p.c. (S2, R1 or R2) at various intervals during 4 h. Galactose was found to be liberated first, followed by mannose.

Periodate oxidation. — Periodate oxidation of the polysaccharide by the method of Andrews *et al.*[8] liberated 124 mmol of formic acid with the comsumption of 742 mmol of periodate per 100 g of the polysaccharide. After 84 h, an excess of ethylene glycol was added, and the solution was evaporated and the contents hydrolyzed. Fractionation of the hydrolyzate by p.c. revealed the presence of mannose, whereas, after 96 h, the hydrolyzate showed its complete absence.

Smith degradation[25]. — The oxopolysaccharide was treated with an excess of ethylene glycol to decompose the excess of periodate. The solution was decationized on Dowex-50 (H^+) resin, and then concentrated. Sodium borohydride was then added along with a few drops of octanol to prevent foaming. The excess of borohydride was removed by the addition of 50% acetic acid. The boric acid formed was removed as methyl borate by the addition of methanol and evaporation. On hydrolysis of the reduction product, p.c. revealed the presence of glycerol and erythritol.

Methylation analysis. — The polysaccharide (8 g) was subjected to Hakomori methylation[26], followed by two Purdie[27] methylations. Methylated polysaccharide did not show any absorption in the hydroxyl region of the i.r. spectrum. The completely methylated polysaccharide had $[\alpha]_D^{25}$ +19.5° (chloroform); it was hydrolysed by the method of Croon *et. al.*[28]. The hydrolyzate was subjected to preparative p.c. (S1, R1, R2) using 2,3,4,6-tetra-*O*-methyl-D-glucose[29] as an internal

standard. Three products were identified.
2,3,4,6-Tetra-O-*methyl*-D-*galactose*[13,30] (OMe 51.5, Calc, OMe 52.5); R_{TMG} 0.87; m.p. 72–73°, $[\alpha]_D^{32}$ + 120° (water): anilide $[\alpha]_D^{32}$ + 43° (acetone).
2,3-Di-O-*methyl*-D-*mannose*[29,31] (OMe 29.5, Calc. OMe 29.8); R_{TMG} 0.53 (syrup), $[\alpha]_D^{32}$ − 15°; 1,4,6-tris-*p*-nitrobenzoate, m.p. 190°; $[\alpha]_D^{32}$ + 65° (chloroform).
2,3,6-tri-O-*methyl*-D-*mannose*[8,12] (OMe 42.1, Calc. OMe 41.8); R_{TMG} 0.81 (syrup),; $[\alpha]_D^{32}$ − 9.8° (water). The derived hydrazide had m.p. 122–129°; 1,4-di-*p*-nitrobenzoate, m.p. 185–186°, $[\alpha]_D^{25}$ + 30° (chloroform).

The methylated polysaccharide (2.0 g) together with an internal standard of D-glucose was hydrolyzed with 0.75M sulfuric acid for 18 h at 100°. The resulting methylated sugars were separated by p.c. (S1), and quantified by akaline hypoiodite[32]. The molar ratios of fractions of these methylated sugars were found to be 1:1:3.

Partial hydrolysis with acid. — The polysaccharide (8 g) was hydrolyzed with 0.05M sulfuric acid for 12 h at 100°. The hydrolyzate was subjected to preparative p.c. (S6), and elution of fractions with distilled water gave D-galactose, D-mannose, and the following oligosaccharides.
1: *Epimelibiose*[33]: m.p. 200°, $[\alpha]_D^{25}$ + 119° (water). Acid hydrolysis gave D-galactose and D-mannose. During 48 h, the disaccharide consumed 5.8 mol of oxidant and liberated 4.2 mol of formic acid. Smith degradation gave glycerol. High positive optical rotation and resistance to emulsin supported the presence of an α linkage. The derived phenylosazone had m.p. 172°.
2: *Galactosyl mannobiose*[33b]: m.p. 227°, $[\alpha]_D^{25}$ + 92–93° (water). Controlled hydrolysis with acid gave epimelibiose, mannobiose, mannose, and galactose. During 48 h the trisaccharide consumed 6.9 mol of metaperiodate and liberated 4.03 mol of formic acid. Smith degradation gave glycerol and erythritol.
3: *Mannobiose*[33b,34]: m.p. 200°, $[\alpha]_D^{25}$ − 6.2° (water). Acid hydrolysis gave mannose. High negative optical rotation and susceptibility to emulsin hydrolysis are consistent with the findings. Periodate oxidation showed the consumption of 5.2 mol of metaperiodate and the simultaneous liberation of 2.01 mol of formic acid. Smith degradation gave erythritol. The derived phenylosazone had m.p. 201°.
4: *Mannotriose*[34,35]: m.p. 211–213°. $[\alpha]_D^{25}$ − 20° (water). Acid fragmentation gave mannobiose and mannose. Periodate oxidation consumed 6.06 mol of periodate and liberated 2.92 mol of formic acid. Methylation analysis gave 2,3,4,6-tetra- and 2,3,6-tri-*O*-methylmannose. High negative optical rotation and susceptibility to emulsin confirmed β linkages.
5: *Mannotetraose*[34,35]: m.p. 231°, $[\alpha]_D^{25}$ − 28° (water). Controlled acid hydrolysis gave mannobiose, mannotriose, and mannose. During 48 h, the oligosaccharide consumed 6.98 mol of sodium metaperiodate and liberated 2.96 mol of formic acid. Methylation and hydrolysis gave 2,3,4,6-tetra- and 2,3,6-tri-*O*-methylmannose. Negative optical rotation, and hydrolysis by emulsin, supported the β configuration.

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