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Note

Structural study of a polysaccharide from the seeds of *Borassus flabellifer* Linn.

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Borassus flabellifer Linn. [1] is a tall dioecious palm with a stout trunk and is unbranched. It is cultivated throughout the plains of Bangladesh, India, Burma, Sri Lanka, Malaysia, and tropical Africa. Almost all parts of the palm are used medicinally [2]. Its fruit is a kind of drupe, large and fibrous with usually three to five nut-like portions, each of which encloses a seed when it is tender. The seeds contain a soft sweet gelatinous pulp with a little liquid in them. The tender pulp gradually hardens and develops a fibrous kernel. Subrahmanyan et al. [3] first reported the isolation of a galactomannan from soft kernel of palm by extracting with aqueous 10% sodium hydroxide and showed it to consist of mannose (78.32%) and galactose (27.64–28.06%).

Subsequently, Rao and Mukherjee [4] extracted a galactomannan from the kernel of palmyra palm nut (*B. flabellifer* Linn.) with 4% alkali and a mannan with 18% alkali. The galactomannan was reported to contain galactose and mannose in the molar ratio 1:2.4, consisting of a backbone of (1 → 4)-β-D-mannopyranosyl residues with galactose and mannose side-chains. The mannan was shown to be considerably branched; the backbone of the molecule was made up of (1 → 4)-linked β-D-mannopyranosyl residues with mannose residues attached as branches to O-6.

The aims of the present investigation were to study further the polysaccharides of the matured palmyra palm kernel, using conventional and spectroscopic methods. For this

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Table 1
Methylation analysis of the polysaccharide

Sugar and location of methoxyl groups	t_R (min)	T [a]	Mol%
1,5-Di-*O*-acetyl-2,3,4,6-tetra-*O*-methylmannitol	2.45	0.98	3.1
1,5-Di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol	2.9	1.16	3.2
1,4,5-Tri-*O*-acetyl-2,3,6-tri-*O*-methylmannitol	5.1	2.04	87.5
1,4,5,6-Tetra-*O*-acetyl-2,3-di-*O*-methylmannitol	9.2	3.68	6.2

[a] Retention time of the corresponding alditol acetate relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol on an OV-225 glass capillary column at 170°C.

purpose, the kernel was successively extracted with petroleum ether, alcohol, water, 3% alkali, and 10% alkali; the extracted material amounted to 0.24, 1.02, 4.62, 3.5, and 44.10%, respectively. This paper reports the structural study of a polysaccharide isolated from the 10% alkali extract, the major portion.

The aqueous 10% sodium hydroxide extract of palm seed kernel, after purification using Sepharose CL-2B, afforded a homogeneous polysaccharide, $[\alpha]_D^{30}$ −7.2° (*c* 0.05, aq 10% NaOH), indicating that its monosaccharide units were linked in the β form. The polysaccharide was hydrolyzed with CF_3CO_2H and the sugars were reduced with sodium borohydride and acetylated. The resulting alditol acetates were analyzed by GLC which indicated the presence of mannose (97%) and galactose (3%).

The polysaccharide was completely methylated by the Hakomori method [5], to give a product of methoxyl content, 44.5% (calcd for $C_8H_{16}O_5$; 45%); the IR spectrum showed absence of hydroxyl groups. The methylated polysaccharide was hydrolyzed with formic acid and CF_3CO_2H, and the products were reduced with sodium borohydride, and acetylated. The resulting alditol acetates were identified by GLC, from their retention times, as 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylmannitol, 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol, 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylmannitol, and 1,4,5,6-tetra-*O*-acetyl-2,3-di-*O*-methylmannitol, in the molar ratios 3:3:88:6 (Table 1). These hydrolyzed products of the methylated polysaccharide were also confirmed by GLC-MS spectroscopy. These findings provided evidence for the presence of structural units

$$\text{Man}p\text{-}(1\rightarrow,\ \underset{\displaystyle\downarrow 6}{\rightarrow 4)\text{-Man}p\text{-}(1\rightarrow},\ \rightarrow 4)\text{-Man}p\text{-}(1\rightarrow,\ \text{and Gal}p\text{-}(1\rightarrow\ .$$

The presence of 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylmannitol as the major product (88%) revealed a linear chain of D-mannosyl residues linked (1 → 4) in the polysaccharide, a general structural pattern of alkali-soluble mannans [6]. The backbone structural pattern and its branching position in the polysaccharide also appeared to be similar to that of the previously reported polysaccharide [4]. The possibility of (1 → 5)-linked mannofuranosyl residues was ruled out since (*a*) glycofuranosides are highly susceptible to acid hydrolysis [7] (the polysaccharide required 4–8 h to be hydrolyzed in 0.05 M sulfuric acid at 100°C) and (*b*) the partial hydrolysis products, oligosaccharides **i**, **ii**, and **iii**, all contained a mannopyranosyl group at the non-reducing terminus.

Table 2
^{13}C NMR spectral data[a] for the polysaccharide

δ	Assignment
101.7 ($J_{C\text{-}1,H\text{-}1}$ 160.5 Hz)	C-1
77.0	C-5
77.5	C-4
72.0	C-2
73.3	C-3
67.4	C-6-substituted
62.2	C-6

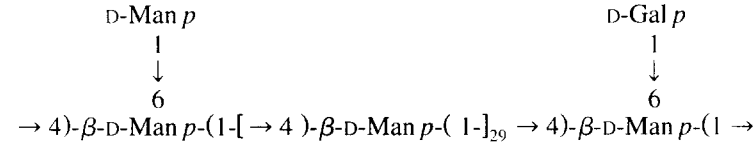
[a] Noise-decoupled spectrum, recorded with a Jeol FX 90 Q spectrometer at 89.55 MHz and 80°C, for a solution in D_2O (containing NaOD) with sodium 2,2-dimethyl-2-silapentane-5-sulfonate as reference.

The polysaccharide was subjected to periodate oxidation. Periodate consumption (0.98 mol per "anhydrosugar" residue) was indicative of either a (1 → 4) or a (1 → 2) linkage in the polysaccharide. The oxidized polysaccharide was reduced by sodium borohydride and hydrolyzed by sulfuric acid (Smith degradation), which revealed the presence of erythritol and glycolaldehyde, further confirming the linkage (1 → 4) rather than (1 → 2). The confirmation of the β form came from the coupling constant value $J_{C\text{-}1,H\text{-}1}$ 160.5 Hz (reported [6] 160 Hz) in the ^{13}C NMR spectrum (Table 2).

GLC analysis of the alditol acetates from the hydrolyzed methylated polysaccharide indicated that two mannosyl residues in the repeating unit carried substituents at C-6, D-mannose in one case and D-galactose in the other. Confirmation of the (1 → 6) linkage came from a minor signal (δ 67.4) in the ^{13}C NMR spectrum of the polysaccharide due to substituted C-6, deshielded by 5.2 ppm compared to the unsubstituted C-6 signal (δ 62.2) [8].

The ratio of the amounts of tetra-, tri-, and di-*O*-methyl sugars (Table 1) gave the molecular weight (5200) of the repeating unit in the polysaccharide.

Based on the foregoing discussion, the structure of the repeating unit of the polysaccharide is, therefore, proposed as **1**.



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1. Experimental

General methods.—Evaporations and concentrations were carried out under reduced pressure using a rotary vacuum evaporator at a bath temperature not exceeding 40°C.

Analytical or reagent grade (Sigma, E. Merck, or BDH) solvents and chemicals were used in all experiments, unless otherwise stated.

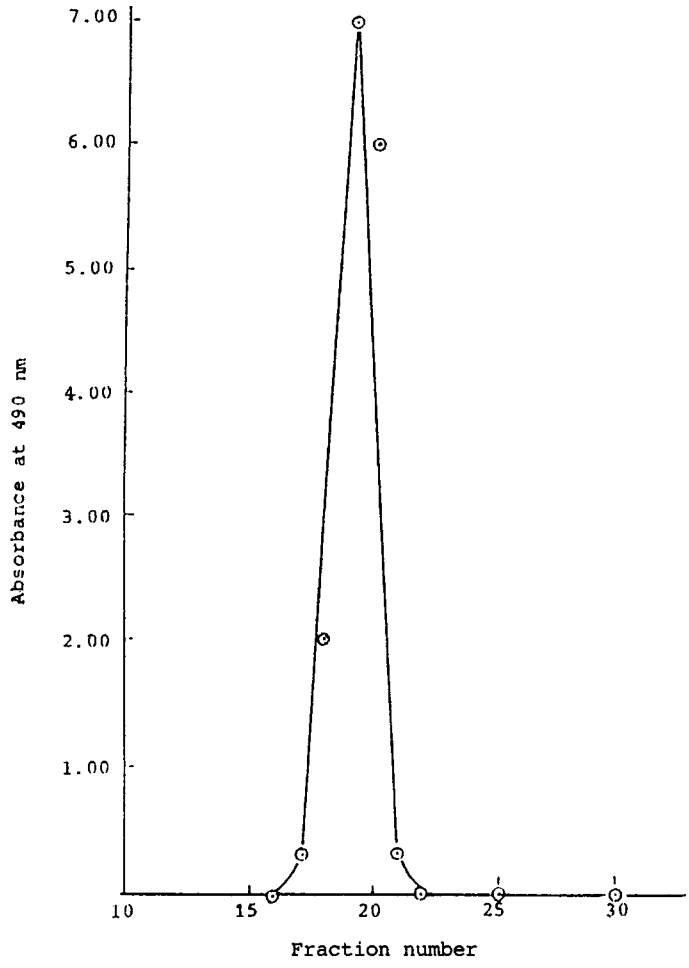

Fig. 1. Purification of the polysaccharide on a column of Sepharose CL-2B gel.

Dialysis of polysaccharide solutions was carried out in seamless cellulose tubing (Union Carbide, molecular weight cut off 10,000) against running water for 3 days. Toluene was added to prevent bacterial action.

All freeze-drying was carried out using a HETOSICC CD 52 (HETO Lab. Equipments, Denmark) freeze-dryer.

Isolation and purification of the polysaccharide.—Matured palm-seed kernel was extracted successively with petroleum ether, alcohol, water, and aq 3% NaOH. The pretreated material was then extracted with aq 10% NaOH, centrifuged, acidified (pH 5–6) in the cold (0.5°C), dialyzed, and precipitated with EtOH (44.1%). The polysaccharide was purified by chromatography on Sepharose CL-2B as described (Fig. 1).

Acid hydrolysis of the polysaccharide.—The samples (10–20 mg) were heated in a sealed tube on a boiling water bath with 0.5 M H_2SO_4 (1–2 mL) for 5–9 h. The

hydrolysates were neutralized with solid $BaCO_3$ and filtered, and the residue was thoroughly washed with water.

The filtrate and washings were combined, deionized with Amberlite IR-120 (H^+) resin and then with Amberlite IR-45 (OH^-) resin (in case of neutral sugars only), and concentrated to a syrup.

Paper chromatography.—Unless otherwise stated, paper chromatography was carried out by the descending technique, using the following systems [9], and the spots were visualized both by silver nitrate dip [10] and a spray reagent (aniline oxalate). *A*, EtOAc–AcOH–formic acid–water (18:3:1:4); *B*, EtOAc–pyridine–water (10:4:3 and 8:2:1); *C*, *i*-BuOH–EtOH–water (40:11:19); *D*, *i*-BuOH–AcOH–water (2:1:1); *E*, *i*-BuOH–pyridine–water (6:4:3); *F*, *i*-BuOH–EtOH–water (upper layer, 4:1:5).

Reduction.—All reductions were carried out with $NaBH_4$ in aqueous solution in an atmosphere of N_2. The alkaline solution was neutralized with AcOH. The boric acid was removed by repeated evaporation with MeOH.

GLC.—For GLC-MS, a Finnegan 4021 instrument was used. Separations were performed on a glass column (180 × 0.15 cm) containing 3% Sp 2430 on Supelcoport (100–120 mesh) at 170°C. The spectra were obtained at an ionization potential of 70 eV; ion source temperature, 160°C; He flow rate, 25 mL/min.

Gel chromatography.—A glass column (50 × 1.6 cm; c type column from Pharmacia AB, Sweden) was loaded with Sepharose CL-2B (Pharmacia); 0.1 M phosphate buffer (pH 6.5) was used as the eluent. The fractions were collected at 15-min intervals using an automatic fraction collector (Redirace Fraction Collector LKB, Sweden), and constant flow rate (7 mL/15 min) was regulated with a Peristaltic pump (Pharmacia). The carbohydrates in the collected fraction were monitored by the phenol–H_2SO_4 method [11], absorbance being measured at 490 nm.

Other experimental conditions.—The sample was converted into alditol acetates [12] for quantitative sugar analysis by hydrolyzing with 0.5 M CF_3CO_2H for 18 h and reducing with $NaBH_4$. The reduced product was acetylated with a 1:1 mixture of pyridine and Ac_2O.

A 0.30 M solution of the polysaccharide was oxidized with 0.05 M $NaIO_4$ [13] and periodate consumption was determined by a titrimetric procedure.

A portion of the homogeneous polysaccharide was hydrolyzed with 0.5 M H_2SO_4 on a boiling water bath for 9 h. The process of neutralization, deionization, and concentration of the hydrolysate was carried out. Examination of the sample by paper chromatography, using solvent *A* and the aniline oxalate spray, revealed the presence of mannose and galactose (trace).

Methylation analysis.—The polysaccharide was dissolved in Me_2SO and methylated by the Hakomori method [5]. The methylated product showed no hydroxyl absorption band in the IR spectrum.

The methylated polysaccharide was hydrolyzed by formic acid (90%) for 1 h on a boiling water bath followed by refluxing with 0.5 M CF_3CO_2H (after removal of formic acid) for 18 h on a boiling water bath. The hydrolysis products were reduced with $NaBH_4$ and then acetylated with a mixture of pyridine and Ac_2O. The resulting alditol acetates of the methylated sugars were analyzed by GLC and the mol% of various components, calculated from the peak areas, are recorded in Table 1.

The methylated alditol acetates were also studied by GLC-MS. The m/z values indicated the presence of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylhexitol, 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylhexitol, and 1,4,5,6-tetra-*O*-acetyl-2,3-di-*O*-methylhexitol.

Smith-degradation studies of the polysaccharide.—The polysaccharide (300 mg) was oxidized with an excess of 0.05 M $NaIO_4$ for 48 h at room temperature until the periodate consumption was constant.

The oxidized polysaccharide was reduced by using $NaBH_4$ (0.5 mg). The reduced polyalcohol was hydrolyzed with 0.5 M H_2SO_4 at room temperature for 24 h and on a boiling water bath for 12 h, which revealed the presence of erythritol, glycolaldehyde, and glycerol (trace).

Partial hydrolysis of the polysaccharide.—This was carried out with 0.05 M H_2SO_4 at 100°C for 4 h. Paper chromatographic studies of the hydrolysates indicated the presence of galactose, mannose, and three oligosaccharides. The oligosaccharides were separated by fractionation of the hydrolysate on a charcoal–Celite column (40 × 4 cm). The column was eluted first with water and then with aqueous EtOH (2.5, 5, 15, 20, and 30%), and the fractions were collected on an automatic fraction collector. Each fraction was analyzed by paper chromatography (solvent *A*). The aqueous fraction contained only monosaccharides. Fractions eluted by different concentrations of EtOH contained the oligosaccharides.

The major three oligosaccharides (**i**, **ii**, and **iii**) were isolated as pure entities after repeated separation on filter sheets [Whatman No. 1 (solvent *A*)]. The oligosaccharides were analyzed for their R_{Gal} (solvent *B*), dp, and structural components and linkages after methylation as shown below.

Oligosaccharide **i** (R_{Gal} 0.49; dp 2; 2,3,4,6-tetra-*O*-methylmannose and 2,3,6-tri-*O*-methylmannose in the molar ratio 1:1) is *O*-D-mannopyranosyl-(1 → 4)-D-mannopyranose.

Oligosaccharide **ii** (R_{Gal} 0.35; dp 3; 2,3,4,6-tetra-*O*-methyl-D-mannose and 2,3,6-tri-*O*-methyl-D-mannose in the molar ratio 1:2) is *O*-D-mannopyranosyl-(1 → 4)-*O*-D-mannopyranosyl-(1 → 4)-D-mannopyranose.

Oligosaccharide **iii** (R_{Gal} 0.14; dp 4; 2,3,4,6-tetra-*O*-methylmannose and 2,3,6-tri-*O*-methylmannose in the molar ratio 1:3) is *O*-D-mannopyranosyl-(1 → 4)-*O*-D-mannopyranosyl-(1 → 4)-*O*-D-mannopyranosyl-(1 → 4)-D-mannopyranose.

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