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Note

A water-soluble galactomannan from the seeds of *Phoenix dactylifera* L.

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Abstract

A water-soluble polysaccharide, isolated from the seeds of dates, has been investigated using methylation, periodate and CrO_3 oxidation, NMR spectroscopy, and reaction with *Bandeiraea simplicifolia* lectin and α-D-galactosidase. The polysaccharide consists of a backbone composed of (1→4)-β-D-mannopyranosyl residues and carries a single (1→6)-α-linked D-galactopyranosyl residue.

Keywords: *Phoenix dactylifera*; Seed and cell wall; Galactomannan

The date palm (*Phoenix dactylifera* L.) is the major fruit tree and an economically important crop for many populations in North Africa and Arabian countries, and it also has an environmental impact in a desert climate.[1] During the pre-oil period and even today, in spite of the drastically changed socioeconomic conditions in the region, the fleshy part of dates always plays an essential role in the diet of the local inhabitants of the Arab countries, leaving a large quantity of seeds as the waste product from dates.[2,3] Much work has been done on the composition of dates and the influence of the stage of maturity on composition.[4–7] Water-insoluble polysaccharides from date seed have been extensively investigated, but data on the soluble polysaccharides are scarce and indicate only the general structural features.[8,9] In this paper we wish to report the water-soluble galactomanann from dry date seeds of the cv Aple variety. By using a different extraction procedure and modern chromatographic and structure elucidation techniques (see Experimental), we confirm the previous finding of a galactomannan from Indian dates.[10] Plant galacotomannans are of industrial importance,[11,12] most notably used in food, pharmaceuticals, cosmetics, paper products, and paints.[13] In addition, these compounds possess non-cytotoxic antitumor activity[14] and act as inhibitors of viruses.[12] As the industrial applications of the polysaccharides from the seeds are continually increasing all over the world, it is of interest to study whether the structures of polysaccharides from date seed are similar to those of the galactomannan that is already known.[10]

The crude product, extracted with water from date seeds, was fractionated by precipitation with ethanol and gave a polysaccharide fraction (25% yield) with 50% aqueous ethanol. The minor fractions obtained at lower

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and higher concentrations of ethanol were not further studied. The polysaccharide had $[\alpha]_D^{25} + 24°$ (H_2O), gave a single peak on elution from Sepharose 6B, and gave an asymmetric peak on analytical ultracenrifugation. The molecular weight, determined according to Yphantis,[15] was 26,400. Acid hydrolysis of the polysaccharides yielded galactose (26.6%) and mannose (71.8%) in a molar ratio 1:2.69, and their optical rotations indicated that they were D sugars.

The results obtained on methylation analysis of the polysaccharide are given in Table 1 and indicate that the backbone is composed of (1→4)-β-D-mannopyranosyl residues and carries a single (1→6)-α-linked D-galactopyranosyl residue (formation of 2,3-di-*O*-methylmannose).

Acid hydrolysis of the purified galactomannan was shown to give rise to D-galactose (26.6%) and D-mannose (71.8%) in a molar ratio of 1:2.69. Variable results were obtained in the periodate oxidation studies. With 0.01 N periodate, 4 mol equiv of periodate were consumed for every hexose unit, and hydrolysis of the corresponding reduced oxopolysaccharide gave rise to 1 mol equiv of mannose, as well as glycerol, erythritol and trace amounts of galactose for every four hexose units. However, when the oxidation was carried out with 0.01 N periodate, 4.8 mol equiv of the oxidant were consumed for every four hexose residues, and hydrolysis of the corresponding polyalcohol in this case gave glycerol (1 mol equiv) and erythritol (2.7 mol equiv). Thus, periodate oxidation of the backbone is hindered by the side chain. Methylation analysis of the periodate-oxidised (236 h) and borohydride-reduced polysaccahrides gave 2,3,6-tri-and 2,3-di-*O*-methylmannoses. Thus, not only the branch point mannose, but also some unsubstituted mannose, escaped oxidation. The resistance of mannosyl residues to periodate oxidation has been observed by others.[16–20] The fact that only a trace of mannose and galactose survived the periodate treatment indicates that no significant number of (1→3)-linkages are present. The large proportion of erythritol released upon acid hydrolysis of the polyalcohol serves as evidence that the main polymeric linkage is of the (1→4) type, and the ratio of erythritol to glycerol indicated the backbone. The molar proportions of periodate consumed and of formic acid produced (1 mol equiv/every four-hexose units) corroborate these findings.

The ^{1}H NMR spectrum of the polysaccharide contained signals for H-1 of Gal (δ 4.98 d, $J_{1,2} \sim 3.6$ Hz) and Man (δ 4.69) are compatible with the expected $^4C_1$ conformation of

Table 1
Retention times and mass spectral data of the partially methylated alditol acetates derived from date seed

Peak	t_R[a]	Ratio[b]	Characteristic mass ion (m/z)	Structure assigned
A	1.25	0.89	45, 117, 161, 205	1,5-Di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol
B	2.41	1.50	45, 117, 233	1,4,5-Tri-*O*-acetyl-2,3,6-tri-*O*-methylmannitol
C	4.81	1.00	117, 261	1,4,5,6-Tetra-*O*-acetyl-2,3-Di-*O*-methylmannitol

[a] Retention time in GLC relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgluctitol on SE-30.
[b] Calculated taking the amount of 1,4,5,6-tetra-*O*-acetyl-2,3-di-*O*-methylmannitol as unity.

Table 2
^{13}C NMR data for the galactomannan from date seed

Unit	Chemical shifts[a]					
	C-1	C-2	C-3	C-4	C-5	C-6
α-D-Galactopyranosyl	100.2	70.7	69.72	70.8	72.7	62.6
4-Linked β-D-mannopyranosyl	101.6	71.3	72.81	77.9	76.4	62.0
4,6-Linked β-D-mannopyranosyl	101.4	71.3	72.81	78.2	74.4	68.0

[a] In ppm downfield relative to the signal for Me_4Si.

the α-D-galactopyranose and β-D-mannopyranose rings.

The ^{13}C NMR data for the polysaccharide (Table 2) indicate substitution of mannose at C-4 and C-6 [α shifts: C-4, Δ10.7 (4-linked) and 11.0 (4,6-linked); C-6, Δ6.5 (4,6-linked)].[21] The chemical shift assignments are in accord with those reported.[22,23]

The anomeric configuration of the residues was determined by a gated decoupling experiment [α-Gal $J_{C\text{-}1,H\text{-}1}$ 169.8 Hz; β-Man $J_{C\text{-}1,H\text{-}1}$ 162.5 Hz] and by oxidation of the acetylated polysaccharide with chromium trioxide (β residues are oxidised at a higher rate than α residues[24]). Whereas $>70\%$ of the galacotose residues survived, $>97\%$ of mannose residues were destroyed, suggesting that the D-mannose was β and the D-galactose was α. That the D-galactose residues were α was confirmed (a) by the strong precipitin reaction of the polysaccharide and *Banderiaea simplicifolia* lectin, which is specific for α-D-galactosyl residues[25] and (b) by treatment of the polysaccharide with α-D-galactosidase, which hyrolysed almost all of the galactosyl residues and gave a mannan-type polymer.

Thus we can suggest that the galactomannan obtained from seeds of Libyan dates, variety cv Aple, consists of a backbone of (1→4)-linked β-D-mannopyranosyl residues with α-D-galactopyranosyl groups attached to the 6-positions, a feature that is common to most galactomannans of leguminous seed.[26] The polysaccharide contains one D-galactose residue per ~2.5 D-mannose residues. The distribution of the D-galactose residues remains to be determined.

1. Experimental

General.—Evaporations were conducted under diminished pressure at 50 °C (bath). Descending PC and TLC were done on Whatmann No.1 and 3MM papers and silica gel (E. Merck), respectively, using 6:4:3 1-butanol–pyridine–water. Sugars were detected by aniline hydrogenphthalate[27] and alkaline silver nitrate.[28] Constituent sugars were determined as alditol acetates[29] by GLC with ribose as the internal standard, using a Varian Aerograph series 1400 chromatograph fitted with an automatic recorder, a temperature programmer, a 3380 A Hewlett–Packard integrator, and a 3% ECNSS-M column. NMR spectra were recorded on a Bruker 500 instrument. For the ^{1}H NMR spectroscopy at 70 °C, the sample (10 mg) was repeatedly dissolved in D_2O (5 × 5 mL), and the solution was lyophilised. The final freeze-dried sample was dissolved in 1 mL of 99.99% D_2O. For ^{13}C NMR spectroscopy at 50 °C, the sample (65 mg/mL) was dissolved in D_2O (external sodium 3-trimethylsilylproionate-2,2,3,3-d_4). The ^{13}C chemical shifts were corrected likewise (-1.13 ppm) by reference to 1,4-dioxane (δ 67.4 relative to Me_4Si). The ^{1}H chemical shifts were corrected likewise (-0.07 ppm) with the dioxane signal at δ 3.7. Analytical centrifugation was carried out at 20 °C, using a Beckman Model E Spinco analytical ultracentrifuge. A solution (6 mg/mL) of the polysaccharide in 0.2 M ammonium acetate was placed in a single sector cell (12 mm, 4 °C). The sedimentation coefficient was calculated by method of Schachmann[30] and expressed in Svedberg units ($S = 10^{-13}$ s), and the molecular weight by the Yphantis method.[15] Optical rotations were measured with a Perkin–Elmer 141 polarimeter.

Plant material.—Dry dates used in this study (1 kg) (*Phonenix dactylifera* L. cv Aple which is in high commercial production in Libya[31]) were grown in Southern Libya. The seeds of dry fruits were ground and samples were analysed. The maturity of the fruits selected for study were in the 'tamr' stage (full ripeness), as in this stage the fruit contains a larger amount of polysaccarides than in green and yellow stages.[32]

Isolation and purification of the polysaccharide.—Date seeds (500 g) from dates were fed through a mill, which was not fitted with a screen. After several repetitions of milling, practically all the seeds were broken in to multiple pieces. The split seeds (250 g) were extracted with hot water (100 °C) with constant mechanical stirring during 48 h. This procedure was repeated until no further precipitate was obtained when the extract was added two volumes of ethanol. The combined extracts were filtered while warm through

glass wool and centrifuged, and the supernatant was poured with vigorous stirring into two volumes of ethanol, whereby the crude polysaccharide precipitated. The liquors were decanted, and the product was squeezed in fine cloth and left overnight under alcohol. The product was dried by solvent exchange (absolute ethanol and ether), and then finally in a vacuum desicator at room temperature; yields 25–28%.

The crude polysaccharide was purified by fractionation from a 0.6% aqueous solution by increasing the concentration of ethanol stepwise; nearly 90% of the initial weight precipitation at an ethanol contraction between 22 and 26% (wt. of ethanol/wt. of solution), no significant precipitation was obtained at lower or higher concentration (upper limit 50%). This purification was repeated twice without any change in the optical rotation.

Investigation of the polysaccharide

(a) Sugar compositional analysis. The polysaccharide was hydrolysed with M hydrochloric acid in a sealed tube for 11 h at 100 °C. The sugars were separated by descending paper (Whatmann No 1) chromatography using 6:4:3 butanol–pyridine–water and 60:25:20 ethylacetate–pyridine–water. The chromatograms were sprayed with aniline hydrogen phthalate[27] and alkaline silver nitrate.[28] Constituent sugars were determined as alditol acetates by GLC.

(b) Gel filtration. A solution of the polysaccharide (2 mg) in 0.2 M sodium chloride (1 mL) was applied to a column (1.5 × 45 cm) of Sepharose 6B and eluted with 0.2 M sodium chloride at 10 mL/h. Fractions (1 mL) were collected and analysed by the phenol–sulfuric acid method.[33]

(c) Methylation analysis. The polysaccharide (6 mg) was methylated by a modified Hakomori method[34], then hydrolysed with $HCOOH–H_2SO_4$, and the product was converted into partially methylated alditol acetates.[35] The mixture of methylated sugars was separated into its components by cellulose column chromatography.

(d) Periodate oxidation. A solution of polysaccharide (25 mg) and Sörensen phosphate buffer (pH 7.0) was oxidised with 0.06 M sodium metaperiodate at 4 °C in the dark for 100, 140, and 236 h. Excess periodate was reduced by ethylene glycol (2 mL), and the mixture was dialysed for three days against distilled water, then concentrated under reduced pressured (to ~30 mL). The products were reduced overnight with sodium borohydride (65 mg) at room temperature. The excess borohydride was destroyed by Dowex 50-X8 (H^+) resin, the solution was filtered, and the polyalchol (22 mg) was recovered by dialysis and freeze-drying. Samples of this material were subjected to sugar analysis and methylation analysis.

(e) Oxidation with chromium trioxide. The acetylated[36] polysaccharide (11 mg) and hexa-*O*-acetyl-*myo*-inositol (2.4 mg) were dissolved in chloroform (1 mL), and a portion (0.3 mL) of this solution was taken as a control. The remaining solution was concentrated to dryness, glacial acetic acid (4 mL) and chromium trioxide (60 mg) were added to the residue, and mixture was kept at 50 °C with sonication for 90 min. The product was recovered by partition between chloroform and water. Sugar analysis was carried out before and after oxidation. The ratio of *myo*-inositol, mannose, and galactose in the control and oxidised material were 1.0:0.49:0.2 and 1.0:0.013:0.14, respectively.

(f) Precipitin reaction. Aliquots (5, 10 and 20 μL) of a 3% solution of the polysaccharide in phosphate-buffered saline (PBS, pH 7.33) were applied to three wells in an agarose gel plate (1.2% of agarose in PBS). In the central well, 10 μL of a 0.3% solution of *Bandeirea simplicifolia* lectin[25] in PBS was applied. The plate was kept at 4 °C overnight, and the appearance of precipitin bands was observed.

(g) Treatment with α-D-galactosidase. To a solution of the polysaccharide (12 mg) in Sörensen phosphate buffer (pH 6.5, 2 mL) was added 40 μL of α-D-galactosidase suspension (5 mg/mL; coffee beans, Boehringer Mannheim). The mixture was stirred and dialysed at 37 °C overnight against the same buffer. The dialysed solution was heated to ~100 for 5 min to destroy the enzyme and then centrifuged, and the supernatant solution was dialysed against distilled water and freeze-dried. The dialysate was deionised using ion-exchange resin and concentrated to syrup.

TLC and PC of the syrup revealed only galactose.

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