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A neutral β-D-glucan from dates of the date palm, *Phoenix dactylifera* L.

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Abstract

Polysaccharides extracted from Libyan dates with hot water and 0.05 M NaOH were fractionated and purified by ion-exchange and gel-filtration chromatography. According to the methylation and hydrolysis analyses, the results indicate the D-glucan to be linear and to contain both (1→3)- and (1→4)-linkages. The anomeric NMR measurements confirm that the sugar residues are β-glycosidically linked. This is the first report on the isolation of a neutral β-D-glucan from dates. © 2002 Elsevier Science Ltd. All rights reserved.

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The date palm (*Phoenix dactylifera L.*, 'Nakhla' in Arabic) is the 'tree of life' and is just one of many examples of a tree crop that can benefit immediately from applications of recently developed biotechnologies of plant tissue culture and plant molecular biology. Slow growth, diœcy (separate male and female trees), the slow offshoot-based propagation system, and the impossibilities of predicting adult characteristics of the seedlings have severely restricted improvement of this ancient tree crop. The total number of date palm trees is approximately 105 million covering an area of 800,000 ha. These are distributed throughout the Middle East, North Africa and South Sahel, areas of East and South Africa, Southwest USA, Central and South America and even Southern Europe (Spain and Italy). Date palms have yet to be developed in other suitable areas of the globe that experience harsh climates and in other areas where there is a desperate need to stabilise and create new sustainable environments, such as in desert climates.[1] Much work has been carried on the composition of dates and the influence of the stage maturity on composition.[2–5] The structure of the polysaccharide has been investigated,[6–8] and we now report on a D-glucan isolated from fruit of the date that has not been previously studied in detail.

Extraction of the fruit dates with water gave a polysaccharide that upon chromatography on DEAE-cellulose gave fractions I (eluted with water) and fraction III and IV (eluted with 0.05 M NaOH). The major fraction I, which constituted 43% of the polysaccharide mixture, had $[\alpha]_D^{26}$ +33° (*c* 0.1, water), gave a single peak on gel filtration through Sephadex G-100, and was homogeneous under high-voltage paper electrophoresis.

Complete acid hydrolysis of the polysaccharide gave only glucose (110 mg), and Hakomori[9] methylation, followed by conventional methylation analysis, gave 2,3,4,6-tetra-*O*-methyl- (3 mg), 2,3,6-tri-*O*-methyl- (3 mg) and 2,4,6-tri-*O*-methyl-D-glucose (26 mg). Analysis of the partially methylated sugars was conducted by GLC of their alditol acetates.[10] Collectively, the results indicate the D-glucan to be linear and to contain both (1→3)- and (1→4)-linkages.

The glucan consumed[11,12] 0.3 mol of periodate during 12 h, which accorded with the value expected on the basis of the results of methylation analysis. Upon Smith degradation of the glucan, 75% of the glucosyl residues survived. Smith degradation gave a product that could be precipitated with EtOH from an aq solution. These results indicated the presence of an extended sequence of (1→3)-linked glucosyl residues. The anomeric pro-

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ton single at δ 4.85 confirmed that the sugar residues were β-glycosidically linked,[13] which agrees with the presence of an IR band at 870 cm^{-1}.

Graded acid hydrolysis of the glucan gave five fractions, which were characterized conventionally (see Section 1). Fractions II–VI were designated as oligomers I–V. The oligomers were separately methylated by the Hakomori method,[9] and the product was isolated by extraction with chloroform. The extract was washed with water and dried (anhyd Na_2SO_4), the solvent was evaporated, and the final product was dried over P_2O_5 in vacuo. These methylated oligomers showed no OH-group absorption band at 3600–3300 cm^{-1} in their IR spectra, indicating complete methylation. The methylated oligomers were analyzed by the method described for methylation analysis of the glucan. Fractions I–V are shown to have the structures indicated in Table 1.

1. Experimental

Plant material.—Dried dates (500 g from *Phonenix dactylifera* L. cv Aple, which is in high commercial production in Libya) that were used in this study were grown in Southern Libya.[14] The dry fruits were uniform in shape, size, and color and were obtained from southern Libya at weekly intervals from July 15 to August 15, 2000. Dry dates were cut to small pieces after removal of the seed and calyx. The maturity of the date fruits selected for this study is in the Tamar stage (full ripeness) as the dates contain larger amounts of polysaccharides than at the green and yellow stages.[15]

General.—Solvents were evaporated under diminished pressure at 50 °C (bath). Optical rotations were determined with a Perkin–Elmer 141 polarimeter. Ascending paper chromatography (PC) was performed on Whatmann No.1 and 3 MM papers using A, the upper layer of 4:1:5 BuOH–HOAc–water; B, 4:1:5 BuOH–EtOH–water; C, 8:3:1 EtOAc–pyridine–water; and D, 6:4:3 BuOH–pyridine–water. Sugars were detected by alkaline silver nitrate and by aniline oxalate. TLC was performed on Silica Gel 60 (E. Merck), using solvents B and C with detection by anisaldehyde–sulfuric acid and aq 2% sulfosalicylic acid.[14] IR spectra were recorded with a Beckman Acculab 10 instrument as KBr pellets. High-voltage paper electrophoresis at 2 °C was conducted with a model No. OE201 instrument, on Whatman No.1 and 3 MM papers using borate buffer (pH 8). Periodate oxidation was monitored at 225 nm with a Beckman 26 UV–Vis spectrophometer. NMR spectra were recorded on a Bruker 500 instrument. For the ^1H 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. The GLC was conducted on a Packard Model 419 and Hewlett–Packard Model 5713 gas chromatographs, each equipped with flame-ionisation detectors, and columns of 1.3% of ECNSS-M on Gas Chrom Q (100–200 mesh); and 2.3% of OV-225 on GC-Q (100–200 mesh). GLC–MS was conducted with a Hewlett–Packard 5895 instrument, using a fused-silica capillary column (30 × 25 mm) coated with a 0.2 μm film of OV-1. The ionisation potential was 70 eV, and the temperature of the ion source was 200 °C.

Isolation and fractionation of the polysaccharide.—Dates (500 g) were cut in to small pieces, crushed by mechanical hammering, and dried. The material was then extracted with hot water (5 L) for 3 h. The process of extraction was repeated, and the extracts were combined, and than centrifuged for 20 min at 5000 rpm. The supernatant solution was diluted with EtOH, and the resulting precipitate was collected by centrifugation, washed four times with acetone, and dried. A solution of the polysaccharide in water (200 mL) was diluted with EtOH, and the precipitate was collected by centrifugation (5000 rpm, 15 min). This process was repeated several times. A portion (100 mg) of the

Table 1
Structure of the products of partial acid hydrolysis of the glucan from Libyan dates

Compound	$[\alpha]_D^{26}$ (water) (degrees)	t_{Glc}[a]	M_G	Products of methylation analysis (equiv)[b]			Proposed structure
				2,3,4,6,-Glc	2,3,6-Glc	2,4,6-Glc	
I	+53	1	1	1			D-Glucose
II	+22	0.70	0.65	0.93		1	Glc-(1 → 3)-Glc
III	−13	0.51	0.63	0.7	1	1	Glc-(1 → 4)-Glc-(1 → 3)-Glc
IV	+2	0.20	0.59	0.7		3	Glc-[(1 → 3)-Glc]$_2$-(1 → 3)-Glc
V	+5	0.1	0.23	0.7	1	4	Glc-[(1 → 3)-Glc]$_5$- → 4)-Glc

[a] 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 (1) ECNSS-M column at 170 °C, and (2) OV-225 column at 170 °C.

[b] 2,3,4,6-Glc = 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol, etc.

resulting polysaccharide (5 g) was then eluted from a column (2.8 × 25 cm) of DEAE-cellulose (Pharmacia) anion-exchange resin with water (500 mL), followed by 0.05 M NaOH (500 mL). Fractions (5 mL) were assayed for carbohydrate by the phenol–sulfuric acid method.[16] Four fractions were obtained, of which I, which had $[\alpha]_D^{26}$ + 33° (*c* 0.1, water) and constituted 43% of the polysaccharide, was used in the subsequent studies.

Gel filtration in borate buffer (pH 9.5), through a column (1 × 30 cm) of Sephadex G-100 (Phamacia), and high-voltage paper electrophoresis at 2 °C in the same borate buffer showed I to be homogeneous.

Acid hydrolysis.—The polysaccharide (0.05 g) was hydrolysed in 0.2 M sulfuric acid (2 mL) for 10 h at 100 °C in a sealed tube, and 15 mg of *myo*-inositol was added as internal standard. The hydrolysate was neutralized with barium carbonate, deionised with Amberlite IR-120 (H^+) resin, and concentrated. TLC (solvents B and C) and PC (solvents A, C, and D) of the hydrolysate revealed glucose, the identity of which was confirmed by GLC (columns 1 and 2) of the derived alditol acetates. The D-configuration was indicated by the $[\alpha]_D^{26}$ value (+ 53° (*c* 0.2, water)) of the sugar isolated by preparative PC (solvent D).

Methylation analysis.—The glucan (10 mg) was methylated by the method of Hakomori,[9] and the product showed no IR absorption for OH. The methylated polysaccharide was treated with 85% aq formic acid (5 mL) for 2 h at 100 °C, and the product was isolated and hydrolysed with 0.5 M sulfuric acid for 18 h at 100 °C. The partially methylated sugars were converted into their alditol acetates and analysed by GLC. The results are shown in Table 1.

Periodate oxidation.—(a) The glucan was treated with 0.04 M $NaIO_4$ in the dark at 4 °C, and the consumption of oxidant, monitored sepctorphotometrically[11,12] became constant (0.3 equiv per glucosyl residue) after 14 h.

(b) The glucan (80 mg) was oxidised with 0.04 M $NaIO_4$ (200 mL) in the dark for 16 h at 5 °C, and the product was isolated conventionally and reduced with $NaBH_4$ (800 mg). A part (6 mg) of the product (67 mg, with *myo*-inositol as internal standard) was hydrolysed with 0.5 M sulfuric acid (5 mL) for 10 h at 100 °C. GLC (Table 1) of the derived alditol acetates indicated that 70% of the glucose was resistant to periodate. The remaining part (61 mg) was treated with 0.5 M sulfuric acid for 16 h at room temperature. The hydrolysate was neutrlised with $BaCO_3$ and centrifuged. The supernatant solution was decationised with Amberlite IR-120 (H^+) resin, and concentrated. The residue was subjected to a second Smith degradation, and the final solution was added to 500 mL of cold EtOH. The resulting precipitate was collected by centrifugation and dried (yield, 1.2 mg).

Graded acid hydrolysis.—A solution of the glucan (200 mg) in 40% aq formic acid (50 mL) was heated for 4 h at 100 °C. The formic acid was removed under diminished pressure by co-distillation with water. PC (Whatman paper, solvent D*)* of the hydrolysate gave homogeneous fractions I–V (see Table 1). To establish the sequence of linkages in the oligsaccharides, they were subjected to paper electrophoresis in borate buffer. Index of movement of boric acid complexes in ionophoresis is designated by the term M_G, the mobility with respect to D-glucose and tetra-*O*-methyl-D-glucose. The distance between the spots of these two standards (D-glucose and 2,3,4,6-tetra-*O*-methyl-D-glucose, respectively) is taken as denominator and is divided in to the true distance migrated by D-glucose derivatives to give the M_G values recorded in Table 1[17,18]. The oligsaccharides were then subjected to conventional methylation analysis. The resulting partially methylated alditol acetates were analysed[9,18,19] by GLC (columns 1 and 2) and GLC–MS. (column 3). The results are presented in Table 1.

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