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Studies of an acidic polysaccharide from *Encephalartos friderici guilielmi*

Matteo Adinolfi, Maria Michela Corsaro*, Lorenzo Mangoni, Michelangelo Parrilli,
Dipartimento di Chimica Organica e Biologica, Univerita' di Napoli, Via Mezzocannone 16, 80134 Napoli (Italy)

and Elia Poerio
Dipartimento di Agrobiologia e Agrochimica, Universita' della Tuscia, Via S. Camillo de Lellis, 01100 Viterbo (Italy)

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ABSTRACT

An acidic polysaccharide from *Encephalartos friderici guilielmi* has been shown by chemical degradative methods and ^{13}C-n.m.r. spectroscopy to comprise a (1→3)-β-D-galactan main chain with α-L-Rha*p*-(1→4)-β-D-Glc*p*A-, α-L-Ara*f*-(1→6)-β-D-Gal*p*, and α-L-Ara*f*-(1→6)-β-D-Gal*p*-(1→3)-β-D-Gal*p* branches at positions 6.

INTRODUCTION

Cycads are ancient plants that have remained relatively unchanged morphologically since the Mesozoic age and are characterised by an abundant system of mucilage ducts. The monosaccharide composition of the mucilages has been used in the chemotaxonomy of Cycads[1], and a preliminary study of the gum exudate of one female specimen of *Encephalartos longifolius* described the mono- and di-saccharides produced by graded acid hydrolysis[2]. The structure of a chemically homogeneous acidic polysaccharide of the mucilage from *Encephalartos friderici guilielmi* is now described.

EXPERIMENTAL

Origin and purification of polysaccharide B2. — A sample of mucilage (3 g), collected from a specimen of *Encephalartos friderici guilielmi* cultivated in the Botanic Garden of Naples, was pulverised and dispersed by stirring in water (90 mL) at 50°. The dispersion was centrifuged for 20 min at 13,000 r.p.m. and 20°, and the upper phase was filtered and freeze-dried. A solution of the resulting solid (1.9 g) in water (30 mL) was diluted with ethanol (40 mL), the insoluble material was collected by centrifugation, and more ethanol (30 mL) was added to the supernatant solution, which precipitated further

* To whom correspondence should be addressed.

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material. The carbohydrate nature of each precipitate was checked by the phenol–H_2SO_4 test[3]. The combined solids were dialysed against water, using a Spectrapor 4 cellulose tube with a cut-off of 12,000–14,000 Da, and freeze-dried. The product, which was shown to contain uronic acid by the *m*-hydroxybiphenyl test[4], was fractionated on Q-Sepharose equilibrated with buffer (20mM Tris–HCl, pH 7.5) (Fig. 1). Based on colorimetric assays for total and acidic sugars and on the absorption at 280 nm, the appropriate fractions were combined to give A and B. The more acidic fraction B was further chromatographed on Bio-Gel A 5m (Fig. 2). The appropriate fractions were combined to give the fractions B1–B3. Fraction B2, which showed almost negligible absorption at 280 nm, was further chromatographed on Bio-Gel A 1.5m and gave a homogeneous peak. Hydrolysis of fractions taken across the peak gave the same molar ratios of neutral monosaccharides. An apparent molecular weight of 1.2×10^6 for polysaccharide B2 was estimated by calibration of the column with dextran standards.

General. — The ^1H- and ^{13}C-n.m.r. spectra were obtained at 400 and 75 MHz, respectively, with a Bruker AM 400 spectrometer, equipped with a dual probe, in the F.t. mode. The DEPT experiment was performed using a polarisation transfer pulse of 135° and a delay adjusted to an average C,H coupling of 160 Hz. ^{13}C Chemical shifts were measured using 1,4-dioxane as the internal standard. Optical rotations were determined on a Perkin–Elmer 141 polarimeter. H.p.l.c. was performed with a Varian 5020 instrument, using a Waters R 401 differential refractometer as the detector. T.l.c. was carried out on Silica Gel F_{254} (Merck). G.l.c. was performed with a Carlo Erba instrument equipped with a flame-ionisation detector, and g.l.c.–m.s. with a Hewlett–Packard 5890 instrument.

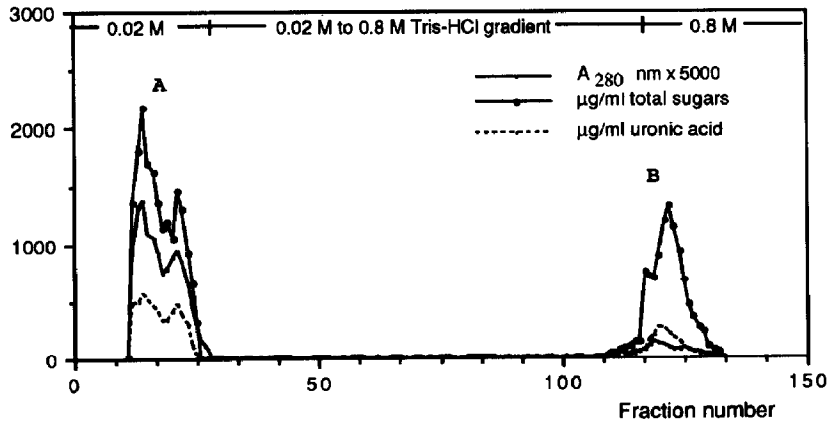


Fig. 1. Fast-flow (120 mL//h) ion-exchange chromatography on a column (2.3 × 70 cm) of Q-Sepharose (16-mL fractions) of the ethanol-precipitated mucilage from *Encephalartos friderici guilielmi*.

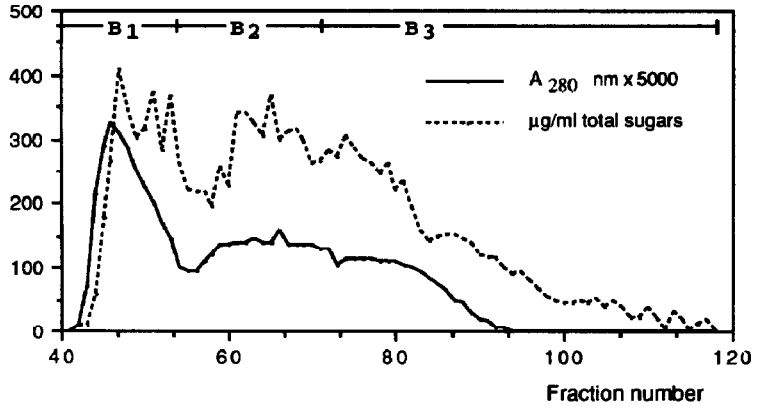


Fig. 2. Bio-Gel A 5m gel-filtration chromatography of fraction B (column, 2.5 × 110 cm; flow rate, 40 mL/h; fraction volume, 4.1 mL) from Fig. 1.

Samples of each polysaccharide were hydrolysed[5] with 2M trifluoroacetic acid at 120° for 1 h. Neutral sugars in the hydrolysates were analysed as the derived alditol acetates[5] by g.l.c. on an SP 2330 capillary column (Supelco, 15 m × 0.25 mm i.d.) at 238°, using N_2 as the carrier gas. The molar ratios of the sugars were evaluated by using *myo*-inositol as internal standard and the appropriate response factors.

Methanolysis was performed[6] with M HCl in MeOH for 5 h at 80° and, after removing the solvent in a stream of N_2, the crude product was treated overnight with M HCl in MeOH again. The resulting mixture of methyl glycosides was dried over P_2O_5, treated with Trisil (Pierce) for 20 min at 80°, and subjected to g.l.c. on an OV-1 capillary column (30 m × 0.32 mm i.d.) with the temperature programme: 160° for 3 min, 160°→200° at 2°/min, 200°→260° at 10°/min, 260° for 10 min. The trimethylsilyl derivatives were identified and quantified by comparison with appropriate standards.

Carboxyl reduction of polysaccharides was performed with 1-cyclohexyl-3-(2-morpholinoethyl)carbodi-imide metho-*p*-toluenesulfonate[7] at pH 4.75, followed by reduction with 2M $NaBH_4$ at pH 7.0. The reaction mixture was dialysed against water, freeze-dried, and again subjected to reduction under the same conditions.

The carboxyl-reduced polysaccharides were methylated by a modification of the Hakomori procedure[8,9], and the products were dialysed, freeze-dried, and hydrolysed with acid. The methylated products in the hydrolysates were reduced with $NaBD_4$, acetylated, and analysed by g.l.c.–m.s. on an SP-2330 capillary column (Supelco, 30 m × 0.25 mm i.d., flow rate 0.8 mL/min, He as carrier gas), with the temperature programme: 170° for 3 min, 170°→240° at 4°/min, 240° for 10 min[6]. G.l.c. of the methylated alditol acetates was carried out on a column identical with that used for g.l.c.–m.s., with a flow rate of 1 mL/min, using effective carbon response factors[10], and normalising the peak areas with respect to that of *myo*-inositol hexa-acetate, used as the internal stadard.

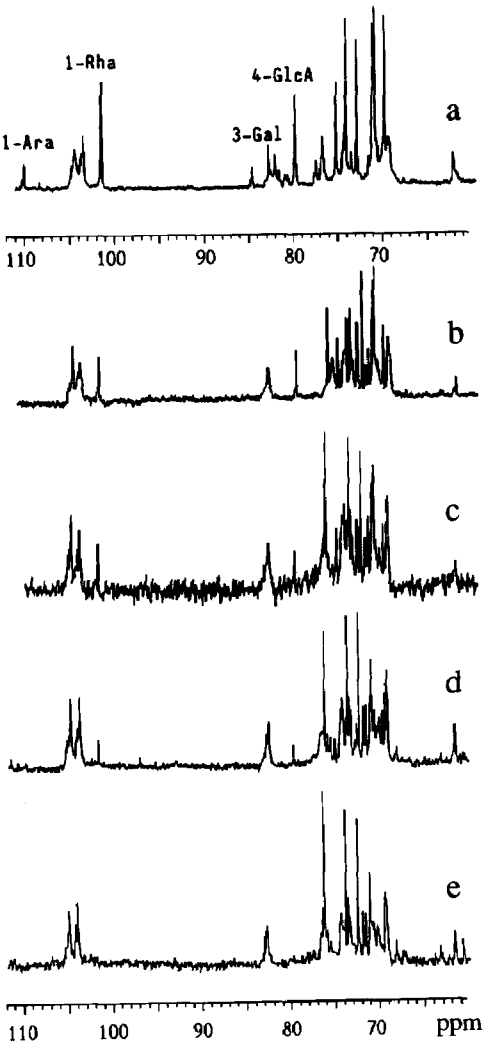


Fig. 3. ^{13}C-N.m.r. spectra (61–112 p.p.m.) at 75 MHz on solutions in D_2O of the native polysaccharide B2 (a), and the products of autohydrolysis (95°) after 16 (b), 32 (c), and 75 h (d), and of the B2 core (e).

Autohydrolysis and mild acid hydrolysis of B2. — A solution of B2 (50 mg) in water was stored at 95°. After 16, 32, and 75 h, portions of the solution were dialysed and freeze-dried, and the residue was submitted to ^{13}C-n.m.r. analysis. Finally, the sample was hydrolysed with 0.1 M H_2SO_4 at 95° for 2 h, dialysed, and freeze-dried to give the B2 core which was also analysed by ^{13}C-n.m.r. spectroscopy. The ^{13}C-n.m.r. spectra are shown in Fig. 3.

Partial acid hydrolysis of B2. — B2 (20 mg) was treated with M H_2SO_4 at 100° for 1 h, and the solution was neutralised ($BaCO_3$), centrifuged, de-ionised with Dowex 50W-X8 (H^+) resin, and freeze-dried. The product was eluted from a column (48 × 1

cm) of Bio-Gel P2 (Biorad) with 20mM AcONa (pH 5.2). Fractions were combined on the basis of t.l.c. analysis (2:1:1 *n*-BuOH–HOAc–H_2O). The only homogeneous fraction was identified as 6-*O*-*β*-(D-glucopyranosyluronic acid)-D-galactose on the basis of its ^1H- and ^{13}C-n.m.r spectra. ^1H-N.m.r. data (internal acetone, δ 2.23): δ 5.29 (d, 0.4 H, *J* 3.7 Hz, H-1α of Gal*p*), 4.62 (d, 0.6 H, *J* 8 Hz, H-1β of Gal*p*), 4.53 (d, 1 H, *J* 8 Hz, H-1β of Glc*p*A).

Smith degradation. — A sample of the carboxyl-reduced B2 (120 mg) was subjected to Smith degradation[11]. The homogeneity of the product was proved by gel-filtration chromatography on Bio-gel A 0.5m (80mM sodium acetate, pH 5.2). An apparent molecular weight of 23,000 for the product was estimated by calibration of the column with dextran standards. Total hydrolysis (trifluoroacetic acid) of the Smith-degraded B2 yielded galactose only. Methylation analysis showed that the ratios of the 3-substituted, 3,6-disubstituted, and unsubstituted Gal*p* residues were 7:1:1.

Determination of the absolute configuration of the monosaccharides isolated from B2. — Carboxyl-reduced B2 (10 mg) was hydrolysed with 2M trifluoroacetic acid (2 mL) at 120° for 1 h. The mixture of monosaccharides, obtained after evaporation of the solvent, was subjected to h.p.l.c. on a column (30 cm × 7.8 mm i.d.) of Aminex HPX-87H (Biorad) by elution with 0.004M H_2SO_4. The configuration of each monosaccharide, identified by h.p.l.c., was established by polarimetry.

RESULTS AND DISCUSSION

The main structural features of the acidic polysaccharide B2 were determined by chemical degradation and ^{13}C-n.m.r. spectroscopy of the native and degraded polysaccharides. Table I shows the monosaccharides and their molar ratios, obtained by acid hydrolysis and methanolysis of B2 and acid hydrolysis of carboxyl-reduced B2. The arabinose and rhamnose were L and the galactose and glucuronic acid were D.

Table II lists the products of hydrolysis of methylated carboxyl-reduced B2, which indicate a highly branched structure with 3,6-linked Gal*p* units at the branch-points. In addition to non-reducing terminal Rha*p* and Ara*p*, 3- and 6-substituted Gal*p* and 4-substituted Glc*p*A were present.

TABLE I

Molar ratios of the monosaccharides obtained by hydrolysis and methanolysis of B2 and the hydrolysis of carboxyl-reduced B2

Monosaccharide	*B2*		*Carboxyl-reduced B2*
	Hydrolysis	*Methanolysis*	*Hydrolysis*
Ara	1.0	1.0	1.0
Rha	2.9	3.0	3.0
Gal	4.6	4.7	4.8
GlcA(Glc)	–	2.6	(2.8)

TABLE II

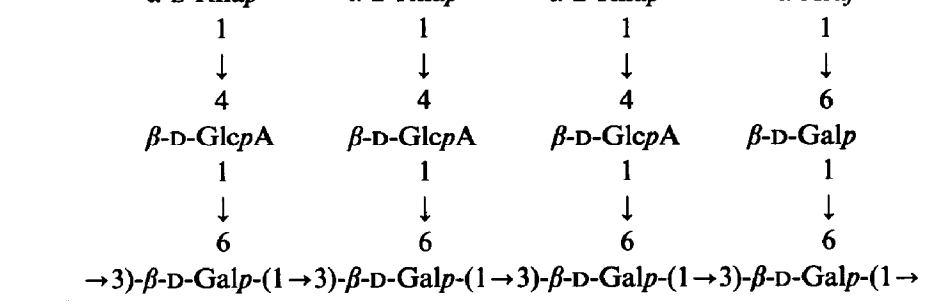
Molar ratios of methylated monosaccharides from the hydrolysis of methylated carboxyl-reduced B2

2,3,5-Tri-*O*-methylarabinofuranose	1.0	2,4,6-Tri-*O*-methylgalactopyranose	1.0
2,3,4-Tri-*O*-methylrhamnopyranose	3.0	2,3,4-Tri-*O*-methylgalactopyranose	0.8
2,3,6-Tri-*O*-methylglucopyranose	2.8	2,4-Di-*O*-methylgalactopyranose	3.7

The ^{13}C-n.m.r data for B2 (Fig. 3a) accord with these results. By comparison with data for the relevant methyl glycosides, the presence of the Ara*f* unit is indicated by the C-1 signal at 110.1 p.p.m., uronic acid by a signal for carbonyl carbon at 175.5 p.p.m., Rha*p* by the signals at 101.5 and 17.4 p.p.m., and 6-linked Gal*p* by the CH_2 signal shifted to 70.6 p.p.m. because of the glycosylation. In addition, the chemical shifts of the C-1 signals indicate the Gal*p* and Glc*p*A to be β and Ara*f* to be α. The Rha*p* was provisionally assigned as α because of the common occurrence of α-Rha*p* in plant polysaccharides[12]. Further information was obtained from the ^{13}C-n.m.r spectra (Fig. 3b–d) of the dialysed products of autohydrolysis of B2. These spectra show that after 16 h (Fig. 3b) all of the signals for terminal Ara*f* had disappeared and the intensity of those of the terminal Rha*p* (including the signal for CH_3, not shown in the Fig.) had decreased markedly. This situation accords with the ease of hydrolysis of furanose and deoxyhexose sugars[13]. In addition, the decrease of the signals for Rha*p* occurs together with a decrease of the signal at 79.7 p.p.m., assigned to the C-4 of Glc*p*A and shifted by glycosylation, which suggests that each terminal Rha*p* was 4-linked to Glc*p*A. Finally, the signals at 82.8 p.p.m., which became more evident as autohydrolysis proceeded, indicated the presence of many glycosidic linkages that involved C-3 of Gal*p* in the core structure. The total acid hydrolysis of the carboxyl-reduced B2 core gave the molar ratios of Rha:Ara-:Gal:Glc as 3:1:12:14, which indicates that, during the autohydrolysis, the bonds broken were mainly those involving Rha, Ara, and Gal, whereas those involving GlcA survived, as expected[13]. This conclusion was confirmed by the isolation of 6-*O*-β-(D-glucopyranosyluronic acid)-D-galactose, identified on the basis of its ^{13}C-n.m.r data[14], from the products of the partial hydrolysis of B2.

Smith degradation of the carboxyl-reduced B2 afforded an oligosaccharide with a molecular weight of ~23,000, methylation analysis of which indicated 3-substituted, 3,6-disubstituted, and unsubstituted Gal units in the proportions 7:1:1. The ^{13}C-n.m.r data for the Smith-degraded polysaccharide [δ 104.8 (C-1), 71.0 (C-2), 82.8 (C-3), 69.2 (C-4), 75.5 (C-5), 61.7 (C-6)] accord with a (1→3)-galactan structure.

Based on the above evidence, the following average structure is suggested for the repeating unit of the acidic polysaccharide of the *Encephalartos* gum, irrespective of the relative position of the branches:



In addition, of every two repeating polysaccharide units, one carries a further (1→3)-linked galactopyranose unit between the Gal*p* bearing the terminal Ara*f* and the branching point.

The structure of B2 is similar to those of the gum polysaccharides from some *Acacia* species[12].

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