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Structural aspects of water-soluble galactomannans isolated from the seeds of *Retama raetam*

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Abstract

Two homogeneous galactomannans were isolated from the seeds of *Retama raetam*, and investigated by using methylation analysis, periodate and CrO_3 oxidation, NMR spectroscopy, and reaction with *Bandeiraea simplicifolia* lectin and α-D-galactosidase. The polysaccharide had backbones of (1→3)- and occasional (1→4)-linked β-D-mannopyranosyl residues, and side chains, at positions 6, of single α-D-galactopyranosyl groups. One of the polysaccharides also had a few non-reducing terminal D-mannosyl groups.

Keywords: *Retama raetam*; Seed and cell wall; Galactomannan

1. Introduction

From ancient times, the Libyan population has extensively used plant medicines to control diabetes mellitus. In the last decade, there has been increasing interest in the medicinal uses of the local flora for treating various diseases including diabetes. *Retama raetam*, locally named as 'R'tm', is a wild plant belonging to the Fabaceae family. It is common to North and East Mediterranean regions. In Libya, it is largely located in regions Ejfarah south of the city Zawi. The plant flowers from April to May. The molecular and biochemical mechanisms associated with dormancy and drought tolerance in this desert plant have been elucidated. According to a recent ethnobotanical survey in the northeastern region of Libya, 10 traditional herbal healers among 25 prescribe *Retama raetam* for diabetes control and treatment and also prescribed it for treatment of hypertension. (Archer & Pyke, 1991; Izhaki & Neeman, 1997; Kassem, Mosharrafa, & Saleh, 2000; Taylor, 1981). However, in spite of this extensive exploitation of *Retama raetam*, no information has been published about the polysaccharide from *Retama raetam*. This paper reports the results of two homogeneous galactomannans isolated from the seeds of Libyan *Retama raetam*.

2. Results and discussion

The products extracted were cold-water-soluble (I) and hot-water-soluble (II) being obtained in yields of 62 and 24%, respectively. The crude polysaccharides were composed mainly of mannose and galactose (Table 1). Only 83% of (I) redissolved in water and the insoluble residue was an arabinogalactan (Table 1), probably originating from the hemicellulosic fraction (s) of the seed coat.

Fractionation of the water-soluble portion of (I) with aq 7% cupric acetate gave four sub-fractions of differing sugar composition (Table 1) in yields of 12–28%. Fractions 2

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Table 1
Sugar composition (%) of seeds of *Retama raetam*

	Native	I	II	Water insoluble residue	Fractions[a]			
					1	2	3	4
Yield (%)	(100)	64	23	16.4	13	27	16	11
Sugar identified[b]								
Rha	1.6	0.1	0.3	17.1	9.8	4.0	1.0	8.3
Ara	10.4	11.1	19.1	16.5	39.1	2.1	4.5	16.6
Xyl	3.5	3.1	5.7		5.0		0.5	15.5
Man	54.5	54.1	51.6	12.1	28.5	60.0	65.0	43.2
Gal	22.1	30.4	23.2	54.1	17.1	28.8	27.7	15.8

[a] Derived from the water-soluble portion of (A) (see Section 2.1).
[b] Quantified by GLC as alditol hexa-acetates using *myo*-insositol as the internal standard.

(27%) and 3 (15%) each gave a single symmetrical peak on elution from Sephacryl S-300 and also on ultracentrifugation and electrophoresis, had $[\alpha]_D$ values of $+30°$ and $+28°$ (water), respectively, and were composed mainly of D-galactose and D-mannose in the molar ratios 1:2.11 and 1:2.36, respectively. These ratios, deduced (Grasdalen & Painter, 1980) from the relative peak areas of the signals for H-1 (1:1.97 and 1:2.25) and C-1 (1:1.98 and 1:2.67), were reasonably comparable with those noted above, calculated from the GLC data. Molecular sieving on Sephacryl S-300 indicated fractions 2 and 4 to have molecular weights of 1.78×10^5 and 1.1×10^5, respectively.

The results of methylation analysis (Table 2) indicated fractions 2 and 3 each to contain a backbone mainly composed of (1→3)-(∼80%) and (1→4)-linked (∼20%) D-mannosyl residues. The side chains attached to positions 6 of D-mannosyl residues contained single D-galactosyl groups. Fraction 3, in addition, contained small amounts of non-reducing terminal D-mannopyranosyl groups, a feature not reported hitherto for seed galactmannans (Berit, Egil, & Elna, 1978).

Supporting the results of methylation analysis, mild hydrolysis with acid of the product obtained on borohyride reduction of the periodate-oxidised polysaccharides gave glycerol, erythritol and mannose. GLC quantification of the latter showed that ∼80% of the original mannose had survived oxidation.

The ^{1}H NMR spectrum of fraction 2 shows the resonances of the anomeric protons to be well separated. The doublet at 4.95 ppm from H-1of Gal, however, has $J_{1,2}\sim2.8$ Hz, and the signal for H-1 of Man ∼4.61 ppm ($J_{1,2}\sim2.8$ Hz) is close to the reported (Bociek, Izzard, Morrison, & Welti, 1981; Grasdalen & Painter, 1980; Ishurd, Zhid, Zhou, & Pan, 2001) values, and therefore, compatible with the expected $^4C_1$ conformation of the α-D-galactopyranose and β-D-mannopyranose rings. The ^{1}H NMR spectrum of fraction 3, although comparable to that of fraction 2, was not so sharp for reasons unknown at present.

The broad-band decoupled ^{13}C NMR spectrum confirms the substitution pattern of mannose revealed by the methylation studies. All of the resonances are resolved (Ishurd et al., 2003) and their chemical shifts are recorded in (see Table 3). The anomeric configuration of the residues was determined by a gated decoupling experiment, which gave $J_{C\text{-}1,H\text{-}1}$ values for α-D-Gal and β-D-Man of 172.5 and 155.5 Hz, respectively.

A similar inference was made from the effect of chromium on the acetylated polysaccharide (Hakomori, 1964; Ishurd et al., 2001) and by treatment of the polysaccharide with α-D-galactosidase. In the former,

Table 2
GLC MS of Date of the partially methylated alditol acetates derived from fractions 2 and 3

Peak	T_R[a]	Molar ratio[b]	Diagnostic ions (m/z)	Structure deduced
Fraction 2				
1	1.18	1.00	45,117,161,205	2,3,4,6-Tetra-*O*-methygalactitol
2	1.92	1.50	45,117,161,173,233	2,4,6-Tri-*O*-methylmannitol
3	3.71	0.69	117,161,201,261	2,3-Di-*O*-methylmannitol
Fraction 3				
1	1.00	0.16	45,117,161,205	2,3,4,6-Tetra-*O*-methylmannitol
2	1.17	1.00	45,117,161,205	2,3,4,6-Tetra-*O*-methylgalactitol
3	1.92	1.68	45,117,161,173,233	2,4,6-Tri-*O*-methylmannitol
4	3.70	0.92	117,161,201,261	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*-methygalactitol on OV-225.
[b] Relative to 2,3,4,6-tetra-*O*-methygalactitol

Table 3
^{13}C NMR date for the galactomannan (fraction 2) from seeds of *Retama raetam*

Type of unit	Chemical shifts[a]					
	C-1	C-2	C-3	C-4	C-5	C-6
α-D-Gal*p*	99.4	70.0	69.1	70.5	70.0	61.0
(1-3)-linked β-D-Man*p*	100.7	70.6	72.2	77.3	77.3	60.3
(1-3,6)-linked β-D-Man*p*	100.7	71.5	72.4	77.4	77.6	68.4

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

there was almost complete destruction of the D-mannose residues but >90% of the D-galactose residues survived, suggesting that the D-mannose was β and the D-galactose was α. In the latter, enzymic hydrolysis of almost all of the D-galactosyl residues confirm the presence in fraction 2 and 3 of α-D-galactosyl residues.The enzyme-treated polysaccharide was exclusively composed of D-mannose (Andrews, Hough, & Jones, 1952a,b; Cerezo, 1965). That the D-galactose was α was confirmed also by strong precipitin reaction of polysaccride and *Banderiae simplicifolia* lectin, which is specific for α-D-galactosyl residues

Thus, the galactomannans isolated from the seeds of *Retama raetem* have an 'anomalous' backbone of mainly (1→3)-linked together with a small proportion of (1→4)-linked β-D-mannopyranosyl resides with single α-D-galactopyranosyl units attached to positions 6. The distribution of these side chains remains to be determined.

2.1. Experimental

General. Descending paper chromatography and TLC were done on Whatman No.1 and 3MM papers and silica gel (E.Merck), respectively, using 6:4:3 1-butanol–pyridine–water as irrigant. Sugars were detected by aniline hydrogen phthalate and alkaline silver nitrate. Evaporations were conducted under diminished pressure at 50 °C (bath). Constituent sugars were determined as alditol acetates by GLC with ribose as the internal standard, using Avarian Aerograph series 1400 chromatograph fitted with an automatic recorder, a temperature programmer, a 3380A Hewlett-Packard integrator, and 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 lyophilized. 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-trimethylsilypropionate-2, 2,3,3-d_4). The ^{13}C NMR chemical shifts were corrected likewise (−1.13 ppm) by reference to 1,4-dioxane (δ67.4 relative to Me_4Si). The ^{1}H chemical shifts were corrected likewise (−0.07 ppm) with the dioxane signal at δ3.7. Analytical centrifugation was carried out at 20 °C using a Beckman Model E Spinco analytical ultracentrifuge. Optical rotation was measured with a Perkin-Elmer 141 polarimeter.

2.1.1. Plant material

The plant used in this study was collected from its natural habitat, from region Ejfarah (Libya) in May 2002 and dried with hot air (40–60 °C). The plant was identified and authenticated as *Retama raetam*.

2.1.2. Isolation and purification of the polysaccharide

Seeds of Libyan *Retama raetam* (2 kg) were powdered in a hand mill, and then defatted by successive treatments with boiling chloroform-hexane (1:1) followed thrice by extraction with aqueous 70% ethanol. The product was extracted with water at room temperature and 100 °C. Brief centrifugation of the viscous extracts followed by addition of ethanol to the supernatant solution precipitated the crude polysaccharides (I) and (II), respectively. Polysaccharide (I) (20 g) was insoluble in the water to the extent of 16.5%. To the soluble portion was added aqueous 7% cupric acetate (13 ml), and the precipitate formed was collected by centrifugation. Further additions of cupric acetate to the clear supernatant solution yielded three more fractions. Each time, the cupric complex was dissociated by macerating with acidic ethanol and the polysaccharide was recovered.

2.1.3. Homogeneity criteria

(a) *Gel filtration*. A solution of the polysaccharide (5 mg) in 0.2 M sodium chloride (1 ml) was applied to a column (1.5×85 cm) of Sephacryl S-300 and eluted with 0.2 M sodium chloride at 6.5 ml/h. Fractions (1 ml) were analysed by the phenol–sulfuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956).
(b) *Sedimentation*. The sedimentation behavior of a solution of the polysaccharide (2 mg) in 0.1 M sodium chloride (1.5 ml) at 56,000 rpm was determined in a Beckman analytical ultracentrifuge.
(c) *Electrophoresis*. Electrophoresis of the dyed (Procion Brilliant Red 2BS) polysaccharides on cellulose acetate membranes was carried out in a Beckman microzone cell in sodium acetate buffer (0.05 M, pH 4.8) at an applied voltage of ∼180 V.

2.1.4. Methylation analysis

The polysaccharides were methylated by the Hakomori method (Hakomori, 1964), and the products were purified

by passing through SEP-PAK C_{18} cartridges. The methylated polysaccharides was treated with 90% aq formic acid for 6 h at 100 °C, and the product was isolated by evaporation of the formic acid and was then hydrolysed with 0.75 M sulfuric acid for 10 h at 100 °C. The products were then converted into partially methylated alditol acetates and analysed by GLC-MS (Bjornal, Lindberg, & Svenndon, 1967; Perret, Bruneteau, Michel, Marais, Joseleau, & Ricci, 1992).

2.1.5. Periodate oxidation

A solution of the polysaccharide was oxidised with 0.06 M sodium metaperiodate for 100 h in the dark at 4 °C. The excess of periodate was consumed with ethylene glycol (2 ml) and the products were reduced with sodium borohydride (25 mg). The solution was then dialysed and freeze-dried. The resulting polyalcohol was hydrolysed with 0.5 M sulfuric acid for 48 h at room temperature followed by paper chromatography and GLC of the alditol acetates.

2.1.6. Chromium trioxide oxidation

The polysaccharide (20 mg) was acetylated with acetic anhydride (4 ml) in formamid (4 ml). To a portion of the product in glacial acetic acid (2 ml) was added chromium trioxide (50 mg), and the oxidation at room temperature was continued for 4 h. The products were recovered by partition between chloroform–water and sugars analysed by paper chromatography and GLC both before and after oxidation (Gupta & Jann, 1984).

2.1.7. Reaction with α-D-galactosidase

To a solution of the polysaccharide (10 mg) in 0.05 M sodium acetate buffer (pH 5.2; 2 ml) was added 40 μl of α-D-galactosidase (5 mg/ml; from coffee beans, Boehringer Mannheim). The mixture was stirred and dialyzed at 37 °C overnight against the same buffer. The dialysed solution was heated to ~100 °C 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 a syrup. TLC and paper chromatogaphy of the syrup revealed only galactose (Ishurd et al., 2001).

2.1.8. Precipitin reaction with lectin

Aliquots (5, 10 and 20 μl) of a 3% solution of the polysaccharide in phosphate-buffered saline (PBS, pH7.33) 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*in PBS were applied, the plate was kept at 4 °C overnight, and the appearance of precipitin bands was observed (Ishurd et al., 2001).

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