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Note

Fungal cell-wall galactomannans isolated from *Geotrichum* spp. and their teleomorphs, *Dipodascus* and *Galactomyces*

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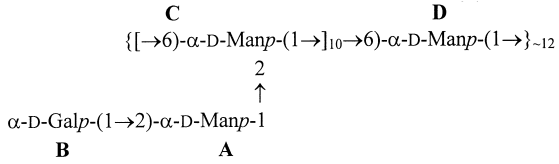
Dedicated to Professor Horton on occasion of his 70th birthday

Abstract

The alkali-extracted water-soluble galactomannan F1SS isolated from the cell wall of two species each of *Geotrichum*, *Galactomyces*, and *Dipodascus* have been studied by methylation analysis and NMR spectroscopy, and their structure is established as the following:



Keywords: Fungi; Geotrichum; Dipodascus; Galactomyces; Polysaccharides; NMR spectroscopy

The genus *Geotrichum* comprises human pathogenic species, and its distinction from *Candida*, another human pathogenic genus, is problematic. It is heterogeneous because it has two teleomorphs (*Dipodascus* and *Galactomyces*), and the phylogeny of the group is unsettled, since it has been related to the Onygenales and Zygomycetes, and it is considered to be the ancestor of the Ascomycetes.[1,2] A detailed revision of the history, systematics and phylogeny of the genus has been published.[3]

Continuing the search for new alkali-extractable water-soluble cell-wall polysaccharides (F1SS), which may be used as immunogenic, taxonomic and evolutive characters,[4] and as a source of smaller oligosaccharides, we now report the structure of a polysaccharide F1SS obtained from the cell wall of species of *Geotrichum*, *Dipodascus* and *Galactomyces*.

The polysaccharide F1SS amounted to ~5% of the dry cell-wall material in all species. Acid hydrolysis revealed the presence of mannose and galactose in a molar ratio of ~2:1. Absolute configuration analysis showed the D configuration for both sugars. Methylation analysis indicated the presence of nonreducing end-units, 2-*O*-, 6-*O*-, and 2,6-di-*O*-substituted mannopyranosyl units, in the proportion 33:33:2:32%, as identified by retention times in GC and MS analysis of each component.

The ^{1}H NMR spectra of the polysaccharide of all the species studied were very similar (see Fig. 1), and the polysaccharide from *Geotrichum citri-aurantii* was selected for further studies.

Its ^{1}H NMR spectrum contained, inter alia, four anomeric signals, with an area ratio of 1:1:1:0.1, which were labeled **A**–**D** from low to high field (Fig. 2(a)).

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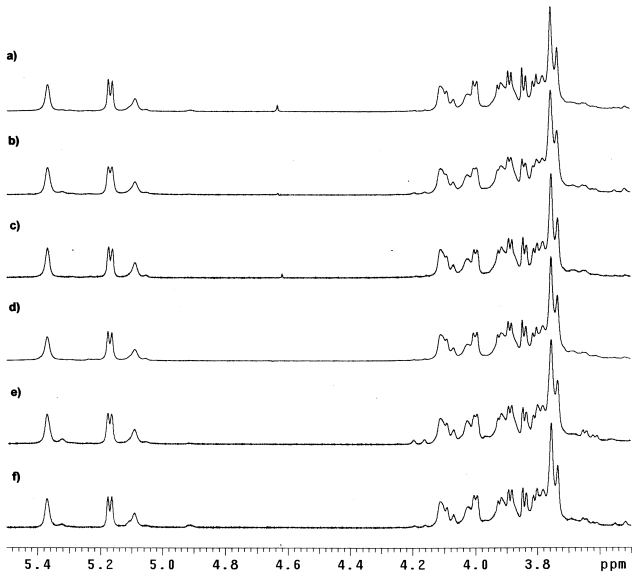


Fig. 1. ^{1}H NMR spectra (300 MHz) of alkali-extracted, water-soluble cell-wall polysaccharides isolated from: (a) *G. citri-aurantii*, (b) *G. fermentans*, (c) *G. geotrichum*, (d) *G. reesi*, (e) *D. armillariae*, and (d) *D. australiensis*.

The ^{13}C NMR spectrum (Fig. 2(b)), showed only three singlets in the anomeric region, probably due to the low proportion corresponding to the **D** signal.

2D-Shift correlation spectroscopy, i.e., DQF-COSY and TOCSY, allowed the assignment of most of the main signals of the proton spectrum. A HMQC experiment exhibited four crosspeaks in the anomeric zone, with a carbon crosspeak at 100.4 ppm corresponding to the signal **D** of the proton spectrum. HMQC also permitted assignment of most of the carbon signals. A HSQC–TOCSY experiment led to complete assignment of all the proton and carbon signals of the three main monosaccharide residues (**A**–**C**) (see Table 1). On comparison of the values obtained with that of model compounds,[5,6] it was clear that unit **A** was 2-*O*-substituted Man*p*, **B**, terminal Gal*p*, and **C**, 2,6-di-*O*-substituted Man*p*. The information obtained for residue **D** was very poor, due to its small proportion. However, from the data obtained for analogous mannans[6] and the methylation analysis results, it was safely assigned to 6-*O*-substituted Man*p* units.

Concerning the anomeric configuration, the coupling constant of unit **B** ($J_{1,2} = 3.7$ Hz) is demonstrative of the α configuration for Gal*p*. A coupled HMQC experiment revealed that $^{1}J_{\text{H-1,C-1}}$ of units **A**, **C**, and **D**, were 176 ± 0.5 Hz, which demonstrated the α configuration for all these units.

With respect to the glycosidic linkages of the units, a 2D NOESY experiment (mixing time = 300 ms) contained, inter alia, crosspeaks H-1**A**/H-1**B**, H-1**A**/H-1**C**, H-1**A**/H-2**C**, H-1**C**/H-6a**C**, and H-1**C**/H-6b**C**. In addition, a HMBC experiment (Fig. 3) showed crosspeaks H-1**A**/C-2**C**, H-1**B**/C-2**A**, and H-1**C**/C-6**C**, which unequivocally demonstrated the sequence **B** → 2**A** → 2**C** → 6**C**.

To further confirm the structure, the possibility of obtaining a trisaccharide from the repeating group was explored. Partial acetolysis of the polysaccharide produced a mixture of oligosaccharides. Isolation by column chromatography gave a main peracetylated trisaccharide fraction which was deacetylated, giving a single compound. The proton and carbon spectra of this material (Fig. 4) were assigned using techniques analogous to those described for the intact polysaccharide (Table 2). The structure **B** → 2**A** → 2**E** was assigned to the trisaccharide, **A** and **B** being units identical to those found in the native polysaccharide, and **E** a second 2-*O*-substituted Man*p* residue. A MALDI-TOF spectrum gave a molecular $m/z = 527$, corresponding to $[M + Na]^{+}$.

From the above data, the structure of the F1SS from *G. citri-aurantii* is indicated to be the following:

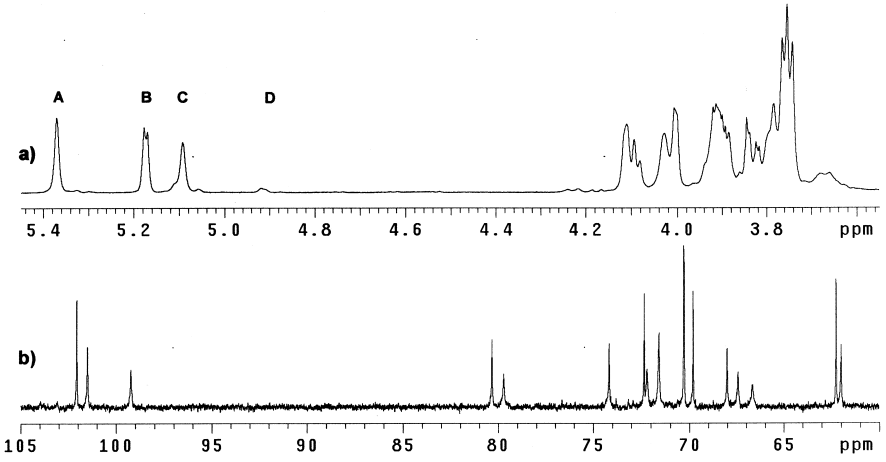
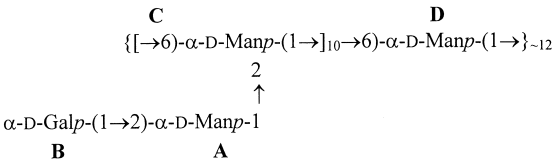


Fig. 2. (a) ^{1}H NMR spectrum (40 °C, 500 MHz) and (b) ^{13}C NMR spectrum (40 °C, 125 MHz) of the alkali-extracted water-soluble, cell-wall polysaccharide isolated from *G. citri-aurantii*. The anomeric peaks have been labeled.

Table 1
^{1}H and ^{13}C NMR chemical shifts (δ) for the alkali-extracted, water-soluble cell-wall polysaccharide F1SS isolated from *G. citri-aurantii*

Residue *		1	2	3	4	5	6a	6b
A	H	5.37	4.11	3.92	3.77	3.77	3.88	3.78
	C	101.5	**80.3**	71.6	68.0	74.2	62.0	
B	H	5.17	3.83	3.91	4.01	4.09	3.75	3.75
	C	102.1	69.8	70.3	70.3	72.3	62.3	
C	H	5.09	4.03	3.93	3.82	ca. 3.83	4.03	3.68
	C	99.2	**79.7**	71.6	67.4	72.2	**66.7**	
D	H	4.92	4.01					
	C	100.4						

* Underlined bold numbers represent glycosylation sites.



The average molecular mass of the polydisperse polysaccharide is in the range of 60–70 kDa, as calculated by gel-permeation chromatography on a Sepharose CL-6B column, previously calibrated with standard dextrans.

A similar polysaccharide, isolated from *Trichosporon fermentans*, has been described by Gorin and Spencer.[7]

These results add fresh support to the proposition that the polysaccharide F1SS from the fungal cell wall might display as many different structures as fungal genera exist. In addition to their immunological relevance, they may be useful as taxonomic characters and for establishing the relationship between mitosporic (anamorph) with meiosporic (teleomorph) fungi.[4] It is possible that these structures may have evolved from a linear mannan to different heteropolysaccharides,[4] in parallel to fungal evolution.[8]

1. Experimental

Microorganisms and growth conditions.—The isolates of *G. citri-aurantii* (CBS 175.89), *G. fermentans* (CBS 409.34), *Galactomyces geotrichum* (CECT 1902), *G. reesii* (CBS 179.60), *Dipodascus armillariae* (CBS 817.71), and *D. australiensis* (CBS 666.75) were maintained on slants of Bacto potato dextrose agar supplemented with Bacto yeast extract (Difco) 1 g/L. The culture medium and growth conditions were as previously described.[9]

Wall material preparation and fractionation.—Wall materials were obtained by soaking the mycelia with dodecyl sulfate and washing with water, as reported elsewhere.[10] Polysaccharide F1SS was obtained by extraction with 1 M NaOH, according to Ahrazem et al.[11]

Chemical analysis.—For analysis of neutral sugars the polysaccharides were hydrolyzed with 3 M TFA (1 h at 120 °C). The resulting monosaccharides were converted into their corresponding alditol acetates[12] and identified and quantified by gas–liquid chromatography (GLC) using an SP-2380 fused silica column (30 m × 0.25 mm i.d. × 0.2 μm film thickness) with a temperature program (210–240 °C, initial time 3 min, ramp rate 15 °C/min, final time 7 min) and a flame-ionization detector with He as carrier gas.

The monosaccharides released after hydrolysis were derivatized according to Gerwig et al.,[13] and their absolute configuration was determined by GC–MS of the obtained per-trimethylsilylated-(+)-2-butyl glycosides.

Methylation analyses.—The polysaccharide (1–5 mg) was methylated according to the method of Ciucanu and Kerek.[14] The methylated material was treated with

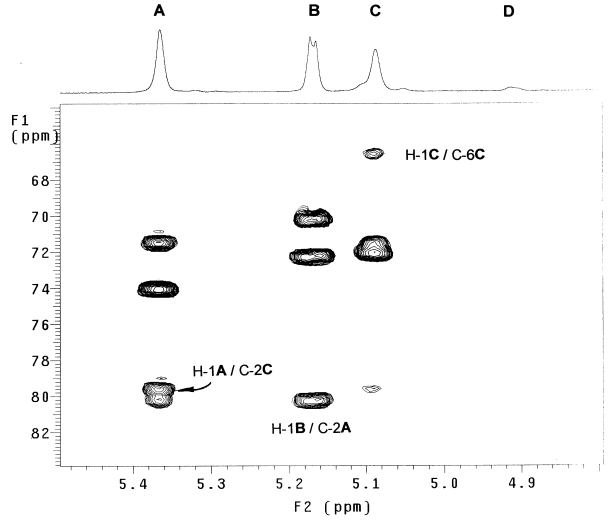


Fig. 3. Key region of the HMBC spectrum of the alkali-extracted, water-soluble cell-wall polysaccharide F1SS isolated from *G. citri-aurantii*. Relevant crosspeaks have been labeled.

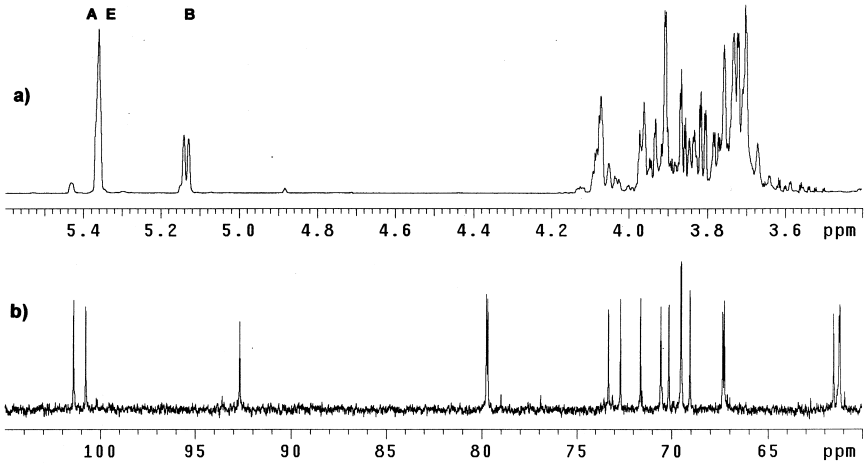


Fig. 4. (a) ^{1}H NMR spectrum (40 °C, 500 MHz) and (b) ^{13}C NMR spectrum (40 °C, 125 MHz) of the trisaccharide obtained by partial acetolysis of the polysaccharide isolated from *G. citri-aurantii*.

Table 2
^{1}H and ^{13}C NMR chemical shifts (δ) for the trisaccharide obtained by partial acetolysis of the polysaccharide F1SS isolated from *G. citri-aurantii*

Residue *		1	2	3	4	5	6a	6b
A	H	5.38	4.09	3.94	3.74	3.76	3.87	3.77
	C	101.4	**80.3**	70.8	68.0 [a]	73.9	61.8 [b]	
B	H	5.15	3.82	3.90	3.98	4.09	3.73	3.73
	C	102.0	69.7	70.1	70.1	72.2	62.2	
E	H	5.38	3.92	3.96	3.70	3.79	3.87	3.77
	C	93.3	**80.4**	71.2	67.9 [a]	73.3	61.9 [b]	

* Underlined bold numbers represent glycosylation sites.
[a,b] These values may be interchanged.

3 M TFA for 1 h at 120 °C, reduced with $NaBD_4$ and further acetylated with Ac_2O–pyridine, and the components identified by GLC–MS, according to Ahrazem et al.[15]

Partial acetolysis of the polysaccharide.—The polysaccharide (50 mg) was treated with 10:10:1 Ac_2O–acetic acid–sulfuric acid, according to the method of Stewart and Ballou.[16] The mixture of peracetylated oligosaccharides obtained was subjected to column chromatography on silica gel. The main fraction was deacetylated using the Zemplén reaction.[17]

MALDI-TOF analysis.—The oligomers obtained were analyzed on a Biflex III time-of-flight instrument (Bruker–Franzen Analytik, Bremen, Germany) operated in the reflectron mode. 2,5-Dihydroxybenzoic acid (10 mg/mL in water) was used as matrix. The sample in water (0.5 μL) and the matrix solution (0.5 μL) were spotted onto the target and allowed to dry. The mass of the oligosaccharides was observed as $[M + Na]^+$ ion.

NMR analysis.—Routine ^{1}H NMR spectra of the polysaccharides were recorded on a Varian INOVA-300 MHz spectrometer. 1D and 2D ^{1}H and ^{13}C NMR experiments were carried out at 40 °C using a Varian Unity 500 MHz spectrometer with a reverse probe and a gradient unit. ^{1}H NMR chemical shifts refer to residual HDO at δ 4.61 ppm. ^{13}C chemical shifts refer to internal acetone at δ 31.07 ppm. F1SS (ca. 20 mg) was dissolved in D_2O (1 mL) followed by centrifugation (10,000*g*, 20 min) and freeze drying. The process was repeated twice, and the final sample was dissolved in D_2O (0.7 mL, 99.98% D).

2D NMR experiments (DGF-COSY, TOCSY, NOESY, HMQC, HSQC-TOCSY and HMBC) were performed by using the standard Varian software, as described.[18]

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