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Structural characterization of a galactomannan from the cyanolichen *Leptogium azureum*

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Abstract

A galactomannan was isolated from the cyanolichen *Leptogium azureum* via successive alkaline extraction and precipitation with Fehling solution. The structure of the polysaccharide was investigated using NMR spectroscopy, methylation analysis, Smith degradation, and HPSEC-MALLS. As galactomannans from other lichens species, the polymer obtained presents a (1 → 6)-linked main chain of α-mannopyranose, substituted preferentially at O-2 by α-Man*p* or β-Gal*p* non-reducing ends. As observed in previous investigations, the C-1 region of the ^{13}C-NMR of these heteropolysaccharides are typical of some lichens species, and can be used as fingerprints in chemotaxonomy. However, in despite of the general structure in common, the substitution level of this structure and their content of mannose is higher than of the others galactomannans obtained of lichenized fungi contained the green alga of the genus *Trebouxia.*

Keywords: *Leptogium azureum*; Cyanolichen; Galactomannan

1. Introduction

Unlike the majority part of lichenized fungi, the 'jelly lichens' contain the alga as the dominant partner of symbiosis. Few studies on structural characterization of the polysaccharides have been carried out on cyanolichens, such as *Collema* spp. and *Leptogium* spp. These organisms grow in the proximity to wet habitats, and can contain cyanobacteria from the genus *Nostoc* (formerly called blue-green algae) as their photobionts (Purvis, 2000). Polymeric carbohydrates containing heteropolysaccharides based on mannose and glucans are among the most common polysaccharides of lichenized fungi (Gorin, Baron, da Silva, Teixeira, & Iacomini, 1993). The structural diversity of the galactomannans from several lichenized fungi depend on their side-chain substituents on (1 → 6)-linked α-D-mannopyranosyl main-chains (Gorin & Iacomini, 1985). These include, generally, monosubstituents at O-2 of α-D-Man*p* or α-D-Gal*p*, at O-4 by β-Gal*p* and sometimes with disubstitution occurring at O-2 and O-4 by α-D-Gal*p* and β-D-Gal*p*, or α-D-Man*p* and β-D-Gal*p*, respectively, although some of the main chains of units are frequently not substituted. Galactomannans have been isolated from several lichens, namely *Cladonia* spp. (Iacomini, Schneider, & Gorin, 1985; Woranovicz, Gorin, Marcelli, Torri, & Iacomini, 1997), *Newropogon aurantiaco-ater* (Baron, Iacomini, Fanta, & Gorin, 1991), *Collema leptosporum* (Prado, Gorin, Stuelp, Honda, & Iacomini, 1999), *Ramalina usnea* (Gorin & Iacomini, 1984), etc. We now characterize a highly substituted galactomannan, occurring in the lichenized fungus *Leptogium azureum.*

2. Experimental

2.1. Biological material

The lichenized fungus *L. azureum* (Collemataceae family) was collected in 2001, in the Serra da Graciosa, State of Paraná, Brazil. It was identified by Prof. Dra. Sionara Eliasaro (Department of Botanic of the Federal University of Paraná—UFPR) and has its voucher deposited in the Herbarium of the UFPR.

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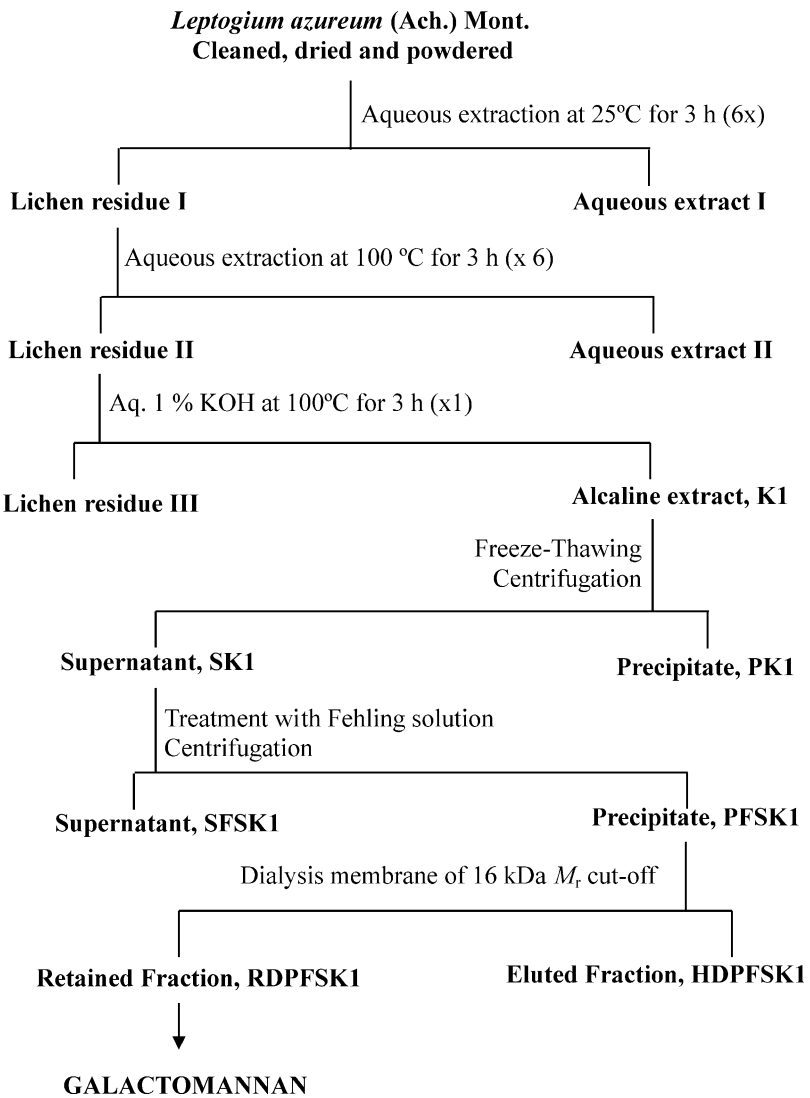
2.2. Isolation and purification of polysaccharides

Powdered thalli (100 g) were homogenized and extracted in a blender with 2 l of distilled water for 3 h at room temperature and then at 100 °C for 3 h (Scheme 1). Residual material was then extracted with 1% aq. KOH (600 ml) containing traces of $NaBH_4$ at 100 °C for 3 h. The alkaline extract was neutralized with HOAc, dialyzed against tap water, and after 48 h was lyophilized. The crude fraction obtained from alkaline extraction was submitted to a freeze thaw process, which furnished an insoluble (PK1) and a soluble material (SK1), which were separated by centrifugation (15 min, 9000 rpm, 25 °C). The SK1 fraction was submitted to a second purification process using Fehling solution (100 ml) (Jones & Stoodley, 1965), resulting in a precipitate (Cu^{2+} complex; PFSK1) and a soluble fraction (SFSK1) which were separated by centrifugation using the same conditions as above. Each fraction was neutralized with HOAc, dialyzed against tap water and deionized with mixed ion exchange resins. The PFSK1 fraction was analyzed by HPSEC and proved to be heterogeneous (Fig. 1). For further purification PFSK1 was submitted to dialysis through a membrane with 16 kDa M_r cut-off, which resulted into an eluted (HDPFSK1) and a retained fraction (RDPFSK1), and the latest was analyzed by HPSEC (Fig. 1), presenting now a homogeneous behavior.

2.3. Monosaccharide composition

Hydrolysis of PFSK1 and RDPFSK1 were carried out with 1 M TFA at 100 °C for 8 h and the hydrolyzates then evaporated to dryness, followed by successive reduction with $NaBH_4$ and acetylation with Ac_2O-pyridine (1:1, v/v; 2 ml) at room temperature for 12 h (Wolfrom & Thompson, 1963a,b). The resulting alditol acetates obtained were analyzed by GC–MS using a Varian model 3300 gas chromatograph linked to a Finnigan Ion-Trap, model 810 R-12 mass spectrometer, using a DB-225 capillary column (30 m × 0.25 mm i.d.), with He as carrier gas. The analysis



Scheme 1. Extraction and purification of the galactomannan from *Leptogium azureum*.

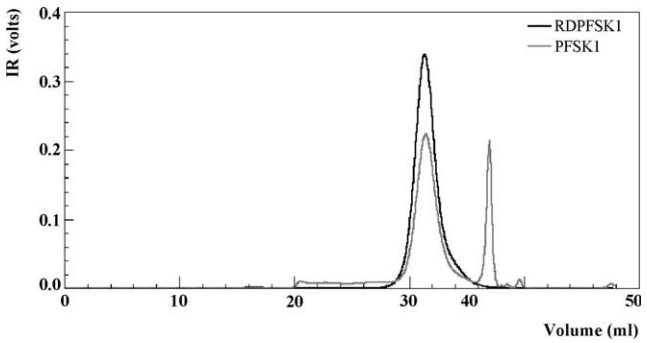


Fig. 1. Elution profiles of the fractions (PFSK1 and RDPFSK1) determined by HPSEC.

was carried out from 50–220 °C at 40 °C/min maintaining the temperature constant to the end of analysis (18 min).

2.4. Methylation analysis

Fraction RDPFSK1 (5 mg) was per-O-methylated according to the method of Ciucanu and Kerek (1984), using powdered NaOH in Me_2SO-MeI. The per-O-methylated derivatives were treated with refluxing 3% HCl–MeOH for 2 h at 80 °C, then 0.5 M H_2SO_4 at 100 °C for 14 h. The resulting mixtures of *O*-methylaldoses were reduced with $NaBH_4$ and acetylated as cited above to give a mixture of partially *O*-methylated alditol acetates, which was analyzed by GC–MS. The analysis was carried out from 50–215 °C at 40 °C/min maintained the temperature constant until to the end analysis (31 min), and the resulting partially O-methylated alditol acetates identified by their typical electron impact breakdown profiles and retention times (Jansson, Kenne, Liedgren, Lindberg, & Lönngren, 1976).

2.5. Periodate oxidation

Fraction RDPFSK1 (20 mg) was oxidized to polyaldehydes with aqueous 0.05 M $NaIO_4$ (20 ml) for 72 h at room temperature in the dark (Abdel-Akher, Hamilton, Montgomery, & Smith, 1952; Hay, Lewis & Smith, 1965). Excess of oxidant was then eliminated by addition of ethylene glycol (1.0 ml), and the solution was dialyzed against tap water for 24 h. The solution was treated with $NaBH_4$ for 10 h at 25 °C, and excess reducing agent was destroyed by Dowex 50 × 8 (H^+ form) ion exchange resin, and the solution dialyzed and then freeze-dried. The product was successively hydrolyzed, reduced with $NaBH_4$, acetylated, and the resulting polyol mixture analyzed by GC–MS (conditions as in item 2.3).

2.6. Determination of homogeneity and molar mass

The elution profiles of fractions (PFSK1 and RDPFSK1) were determined by high performance size-exclusion chromatography (HPSEC), using a WATERS 510 HPLC pump at 0.6 ml/min with four gel permeation columns in series with exclusion sizes of 1×10^6, 4×10^5, 8×10^4, and 5×10^3 Da, using a refraction index (RI) detector. The eluent was 0.1 mol/l aq. $NaNO_3$ containing 200 ppm aq. NaN_3. Samples, previously filtered through a membrane (0.22 μm; Millipore), were injected (250 μl loop) at 2 mg/ml.

The specific refractive index increment (d*n*/d*c*) was determined for fraction RDPFSK1. The sample was dissolved in 50 mM $NaNO_3$ and five increasing concentrations, ranging from 0.25 to 0.7 mg/ml, were used to determine the slope of the increment. Results were processed in software provided by the manufacturer (Wyatt Technologies).

2.7. Nuclear magnetic resonance spectroscopy

Nuclear magnetic resonance (NMR) spectra were obtained using a 400 MHz Bruker model DRX Avance spectrometer with a 5 mm inverse probe. ^{13}C-NMR (100.6 MHz) and ^{1}H-NMR (400.13 MHz) analyses were performed at 50 or 30 °C, with the samples being dissolved in D_2O. The OH groups were exchanged with D_2O followed by freeze-drying. Chemical shifts of samples are expressed in ppm (δ) relative to acetone at δ 30.20 and 2.22 for ^{13}C and ^{1}H signals, respectively.

The ^{13}C NMR coupled pulse of 90° was performed with delay time of 0.1 s and acquisition time of 0.6 s for decoupled spectra. For coupled spectra methyl α-D-mannopyranoside had a coupling constant $J_{C1,H1}$ of 172.0 Hz, and methyl β-D-galactopyranoside 160.0 Hz.

COSY, HMQC, ROESY, and DEPT spectra were obtained according to the Bruker manual.

3. Results and discussion

The precipitate obtained using Fehling solution (fraction PFSK1; 2.8% yield), contained xylose, mannose, galactose, and glucose in a molar ratio of 1:11:3:2. HPSEC analysis showed heterogeneity with two main peaks (Fig. 1). This fraction was dialyzed against distilled water (membranes of 16 kDa M_r cut-off) giving two pure fractions (RDPFSK1, 2.3% yield; and HDPFSK1, 0.4% yield). The former homogeneous fraction when analyzed by HPSEC (Fig. 1), had a M_r 4.6×10^5 kDa (d*n*/d*c* = 0.150). This fraction contained mannose and galactose in a molar ratio of 5:1. Methylation analysis (Table 1) showed a highly branched polysaccharide, containig non-reducing end-units of Man*p* (29.0%) and Gal*p* (17.2%), besides the 2,6 di-O-substituted (32.6%) and 2,4,6 tri-O-substituted units (4.5%). Periodate oxidation showed high content of glycerol (87%) obtained from this sample arose from the non-reducing end of Man*p* and Gal*p*, as well as 2-O-, 6-O- and 2,6 di-O-substituted units. The production of erythritol (8%) was due to 4-O-substituted units, and the mannitol acetate (5.0%) was

Table 1
Analysis of partially O-methylated alditol acetates obtained from methylated galactomannan

O-Me-alditol acetates	Molar %	Linkage type[a]
2,3,4,6-Me_4-Man	29.0	Man*p*-(1 →
2,3,4,6-Me_4-Gal	17.2	Gal*p*-(1 →
3,4,6-Me_3-Man	3.3	2 →)-Man*p*-(1 →
2,3,6-Me_3-Man	6.0	4 →)-Man*p*-(1 →
2,3,4-Me_3-Man	7.4	6 →)-Man*p*-(1 →
3,4-Me_2-Man	32.6	2,6 →)-Man*p*-(1 →
3-Me-Man	4.5	2,4,6 →)-Man*p*-(1 →

[a] Based on derived *O*-methylalditol acetates.

consistent with the presence of 2,4,6 tri-O-substituted Man*p* units. The methylation and periodate oxidation data are in agreement in determining the structure of this heteropolysaccharide.

The ^{13}C-NMR spectrum of the galactomannan (RDPFSK1; Fig. 2) contained signals in the C-1 region at δ (104.7, 103.5, 100.5, and 99.6, which correspond to non-reducing-end groups of β-D-Gal*p*, of α-D-Man*p*-(1 → 2)-α-Man*p*, 6-O- and 2,6-di-O-substituted units of α-D-Man*p* from the core, respectively (Gorin & Iacomini, 1985; Prado et al., 1999). The α and β configurations were determined by ^{1}H resonance characteristics in a ^{1}H (obs.), ^{13}C HMQC spectrum (Fig. 3). A coupled ^{13}C-NMR was prepared to determine glycosidic configurations (Perlin & Casu, 1969). The non-reducing end of Gal*p* has a β-configuration by virtue of a high-field H-1 signal in δ 4.42 (104.7), which is in agreement with the $J_{C\text{-}1,H\text{-}1}$ value of 158.9 Hz. The low-field H-1 signals in δ 5.07 (103.5 and 103.2; $J_{C\text{-}1,H\text{-}1} = 170.5$), 5.16 (100.5) and 5.11 (99.6; $J_{C\text{-}1,H\text{-}1} = 174.4$ Hz) indicated that the units of Man*p* had an α-configuration, agreeing with a typical high-field C-1 signals in its ^{13}C-NMR spectrum and the $J_{C\text{-}1,H\text{-}1}$ values obtained.

Its ^{1}H (obs.), ^{13}C HMQC (Fig. 3), COSY and DEPT analysis of the fraction RDPFSK1 gave information about the linkages. The inverted signal in the DEPT spectrum that appear as a doublet in the HMQC spectrum at δ 67.0 with H-6 at 4.02 and H6′ at 3.70 suggest a substituted CH_2 group and probably of C-6 of α-Man*p* units concerning

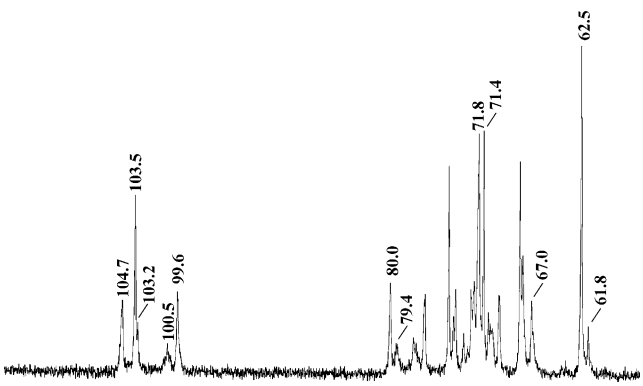


Fig. 2. ^{13}C NMR spectrum of the galactomannan in D_2O at 50 °C (chemical shifts are expressed as ppm).

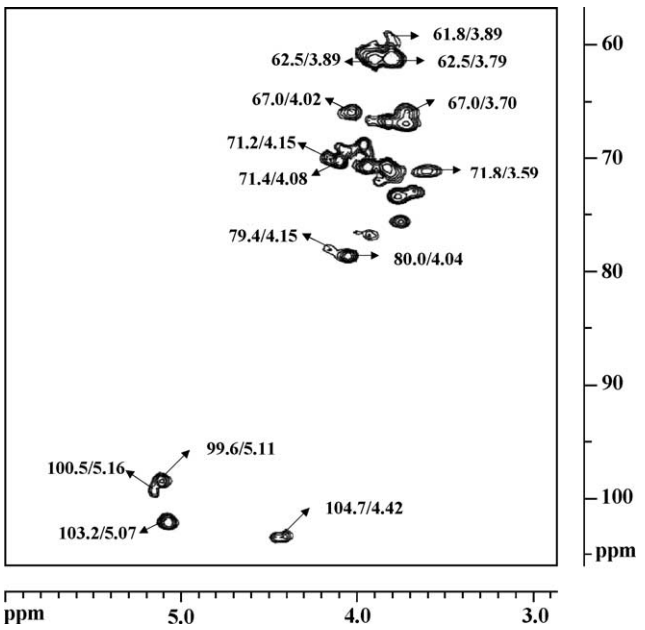


Fig. 3. ^{1}H, ^{13}C HMQC spectrum of the galactomannan in D_2O at 50 °C (chemical shifts are expressed as ppm).

the methylation analysis (3,4-*O*-Me_2-Man, 32.6%). The non substituted C-6's appear as, a doublet at 62.5 (3.89; 3.79) and, the signal 61.8/3.89 from the non reducing ends of the α-Man*p* and β-Gal*p* units; these were confirmed by inverted CH_2 signals at 62.5 and 61.8 in the DEPT spectrum. COSY examination showed coupling of the five anomeric protons with their respective H-2 resonances. Only the H-2 signal at δ 3.59 (C-2, δ 71.8) is coupled with δ 4.42 signal, the H-1 of the β-Gal*p* unit, that is typical to those obtained by Gorin to the Me-Galactosides (Gorin & Mazurek, 1975). Similarly, H-1 of the non-reducing end group of α-Man*p* units at δ 5.07 was coupled to H-2 at δ 4.08 (C-2, δ 71.4), was observed with the well-resolved signals of the HMQC and COSY spectra. The same to the couplings between the δ 5.11 to H-2 at δ 4.04 (C-2, δ 80.0) arising from Man*p* non reducing end 2-O-substituted Man*p* units of the (1 → 6)-linked main-chain. A small signal at δ 79.4 (4.15) can be attributed to the Man*p* units C-2 substituted by non reducing end units of β-Gal*p* concerning the intensity and the coupled H-1 proton that appear in COSY at δ 5.06. As confirmation, H-1 signal of the non reducing end Gal*p* at δ 4.42 showed ROESY correlation with the H-2 signal of the 2-O-substituted Man*p* units of the (1 → 6)-linked main-chain (δ 4.15). The methylation analyses have showed ~7.4% of 2,3,4-*O*-Me-Man*p* suggesting small amounts of the Man*p* that was not C-2 substituted, in agreement of these, we found the correspondent H-1 at δ 5.16 coupled with δ 4.15 (δ 71.2) (Gorin & Mazurek, 1975).

So, we conclude that the galactomannan (fraction RDPFSK1) presents a main chain of (1 → 6)-linked α-D-mannopyranosyl residues, almost all which preferentially branched at O-2 with side chains of different lengths. However, galactomannan with high substitution level and

elevated content of mannose was encountered only in the cyanolichen *Collema leptosporum* (Prado et al., 1999), suggesting that similar structures can be typical of lichenized fungi from the Collemataceae family.

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