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Carbohydrate Research 340 (2005) 1699–1705
Carbohydrate RESEARCH

Galactomannans with novel structures from the lichen *Roccella decipiens* Darb

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Received 1 December 2004; accepted 23 March 2005
Available online 3 June 2005

Abstract—Two homogeneous galactomannan fractions were isolated from the lichen, *Roccella decipiens*, one (FP) containing Man and Gal in an 81:19 molar ratio and the other (RFS), having Man, Gal, and Glc in a 43:56:1 molar ratio. FP consisted of a main chain with (1→4)-linked α-D-Man*p* units, most of which were substituted at O-2 with side chains consisting of nonreducing end-, 2-*O*- and 6-*O*-substituted α-Man*p* units. The latter appeared to be substituted by single-unit β-D-Gal*f* nonreducing ends. RFS contained a similar α-D-Man*p* core structure, but with side chains containing nonreducing end, 5-*O*-, 6-*O*-, and 5,6-di-*O*-substituted β-D-Gal*f* units. Such polysaccharide structures have not been previously reported.

Keywords: Lichen; *Roccella decipiens*; Galactomannans; Chemical structure

1. Introduction

Lichens are slow-growing symbiotic organisms consisting of a fungus and one or more algae: about 13,500 species grow worldwide. The chemistry of their secondary metabolism has been more extensively investigated, and over 200 different secondary compounds have been reported.[1,2] However, fewer than 100 species of lichens have been investigated for their polysaccharide constituents, and these have been divided into three main structural types, namely linear or lightly substituted α- and β-glucans and branched galactomannans.[3–6] Recently, new complex heteroglycans, such as rhamnogalactofuranan[7] and galactomannoglucans, have been described.[8,9]

The main chain of the galactomannans already investigated have generally consisted of (1→6)-linked α-Man*p* units, their structural differences arising from the degree and sequence in which these are unsubstituted, or substituted at O-2, O-4, and O-2,4 by various side chains containing units of α- or β-Gal*p*, α-Man*p*, or Glc*p*, and more rarely β-Gal*f*. The ratio of Man:Gal:Glc is variable.[3–5,10] These heteropolysaccharides have been isolated from a number of lichen species, belonging mainly only to the families Cladoniaceae, Parmeliaceae, Umbilicariaceae, Ramalinaceae, and Stereocaulaceae,[5,11] thus leaving a large research field to be exploited.

Lichen polysaccharides, which can be isolated in high yield, such as the glucans and galactomannans, are generally expected to have a fungal origin.[4] Cordeiro et al.[12] found for the cultured mycobiont of a *Ramalina* sp., that nigeran- and laminaran-type glucans and

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0008-6215/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carres.2005.03.022

galactomannans were present, whereas the water-soluble isolichenan-type glucan was found only in the native thallus. Moreover, the cell-wall polysaccharides of lichen-forming fungi can be more complex than those of free-living fungi, due to their co-evolution with algal symbionts and their longevity.[13,14] The majority of lichens investigated so far for their polysaccharide components are lichenized with trebouxioid photobionts, despite 32 genera of alga having been identified to date as photobionts.[15,16] The influence of the nature of the photobiont on the overall polysaccharide content of the lichen thallus is still unknown.

Galactomannans from the lichen *Roccella decipiens*, which has a *Trentepohlia* sp. as photobiont, have now been characterized. These have structures which have not been previously reported in lichens or other biological material.

2. Results and discussion

In order to remove lipids, pigments, and hydrophobic material, the lichen thallus (13 g) was extracted successively with $CHCl_3$–MeOH and MeOH–H_2O. The defatted lichen was then submitted to successive extraction with water and 2% aqueous KOH, both at 100 °C (Fig. 1), and after neutralization (HOAc) the extracted polysaccharides (fractions W and K, respectively) were recovered by ethanol precipitation. Fractions K and W both contained Man, Gal, and Glc in the same molar ratio 45:46:9, but fraction K was obtained in 8.1% yield, while that of fraction W was 2.5%, the former was chosen for further investigation. Fraction W was submitted to freeze-thawing treatment, resulting in a cold-water insoluble fraction (IK, 1.7% yield) and a soluble fraction (SK, 5.4% yield). SK was then treated with Fehling's solution, giving rise to a precipitate (FP, 3.4% overall yield) and supernatant (FS, 2.0% overall yield).

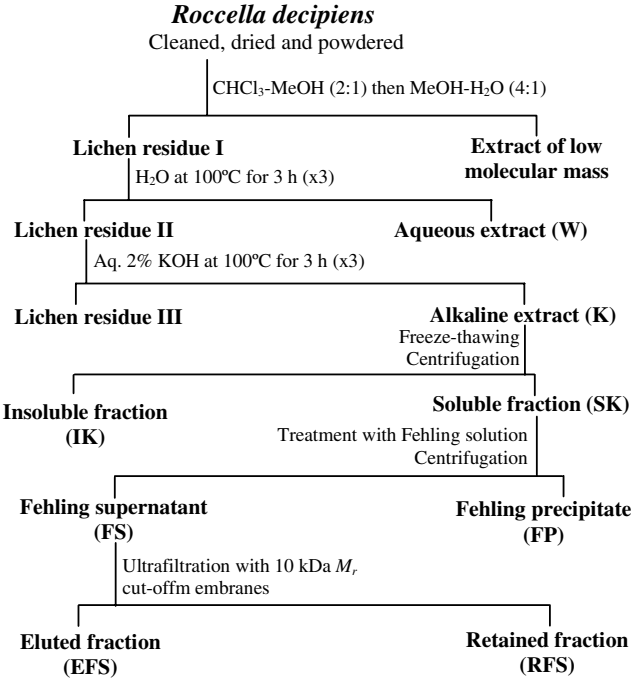


Figure 1. Scheme of extraction and purification of polysaccharides from the lichen, *Roccella decipiens*.

FP consisted of Man and Gal in an 81:19 molar ratio (Table 1) and gave a single peak on HPSEC analysis (Fig. 2a), with M_w of 20.5×10^4 (d*n*/d*c* 0.114). However, FS gave a heterogeneous elution profile (Fig. 2b), so it was then purified by ultrafiltration (10 kDa M_r cut-off membrane), giving rise to eluted (EFS) and retained (RFS) material. Comparison of their respective Man, Gal to Glc ratios (46:53:1 and 43:56:1; Table 1) and their identical ^{13}C NMR spectra, showed that they contained the same structural components, but with different molar masses (EFS: M_w 27.8×10^3; RFS: M_w 46.4×10^3; d*n*/d*c* 0.132 (Fig. 2c and d, respectively). Such mass differences can be explained as a result of the severe environmental conditions to which thalli are exposed during their long life, which can promote a partial degradation of their components.[14]

Methylation analysis of FP showed it to have a highly branched structure. Units of Gal*f* were detected as being mainly nonreducing ends, with small proportions of 5-*O*-, 6-*O*-, and 5,6-di-*O*-substituted units. The core contained nonreducing end, 2-*O*-, 4-*O*, 6-*O*-, and 2,4-di-*O*-substituted Man*p* units (Table 2).

The main-chain structure of FP was determined via a controlled Smith degradation, which gave rise to SmFP, sparingly soluble in water but soluble in dimethyl sulfoxide, and composed entirely of mannose (Table 1). Methylation analysis indicated a mannan with nonreducing end- (2%), 4-*O*- (96%), and 2,4-di-*O*-substituted units (2%) (Table 2). This linear structure was consistent with its ^{13}C NMR spectrum (Fig. 3c) with signals at δ 101.5, 73.8 (two overlapping), 71.9, 71.4, and 62.1 (C-6). For their assignment, incomplete connectivity was found in the COSY spectrum of the mannan (its α-configuration was shown by its low-field H-1 signal at δ 5.32). However, it occurred between H-1 (δ 5.32) and H-2

Table 1. Monosaccharide composition of polysaccharide fractions obtained from *Roccella decipiens*

Fraction	Monosaccharide composition[a] (mol %)		
	Mannose	Galactose	Glucose
FP	81	19	—
HFP	96	3	1
SmFP	100	—	—
EFS	46	53	1
RFS	43	56	1
HRFS	98	2	—
SmRFS	100	—	—

[a] Alditol acetates obtained on successive hydrolysis, reduction, and acetylation, analyzed by GC–MS (column DB-225).

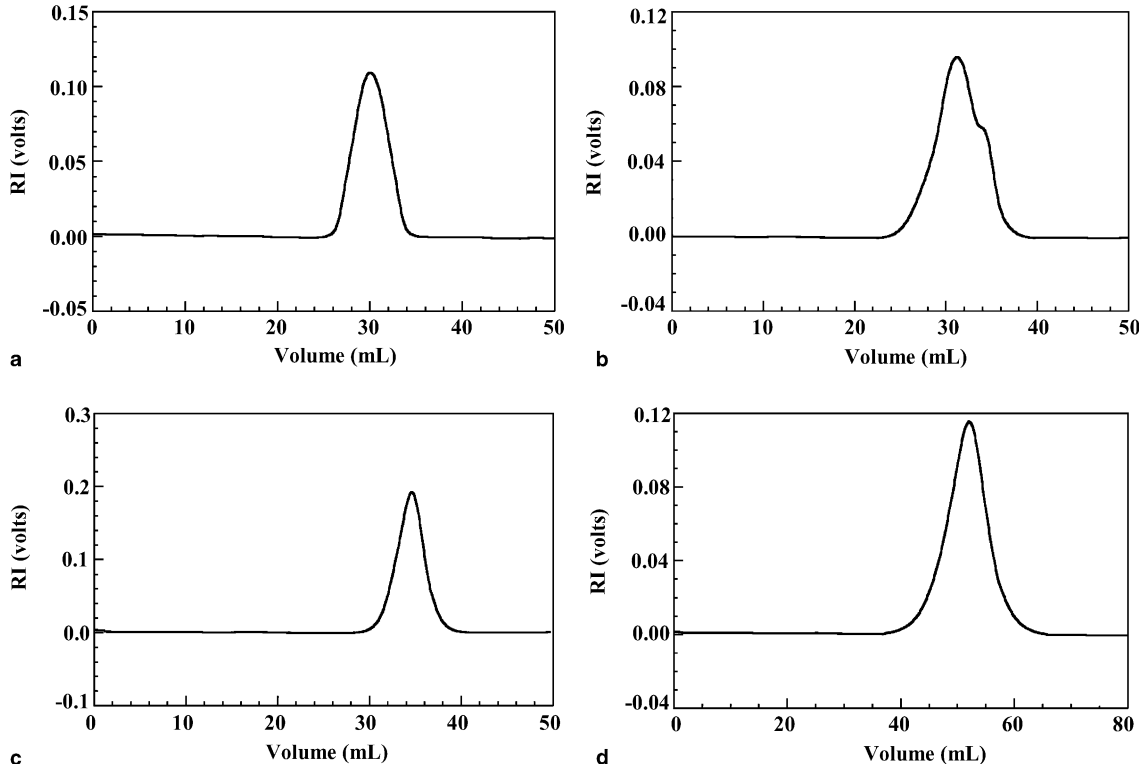


Figure 2. HPSEC elution profiles of FP (a), FS (b), EFS (c), and RFS (d), using a refractive index (RI) detector.

Table 2. Analysis of partially *O*-methylated alditol acetates obtained from methylated carbohydrate fractions isolated from the lichen *Roccella decipiens*

OMe-alditol acetate[a]	Fractions (% values)[b]						Linkage type[c]
	FP	HFP	SmFP	RFS	HRFS	SmRFS	
2,3,5,6-Me_4Gal	14	2	—	19	1	—	Gal*f*-(1→
3,5,6-Me_3Gal	1	—	—	Tr	—	—	→2)-Gal*f*-(1→
2,3,6-Me_3Gal	1	—	—	24	—	—	→5)-Gal*f*-(1→
2,3,5-Me_3Gal	1	—	—	8	—	—	→6)-Gal*f*-(1→
2,3-Me_2Gal	1	—	—	11	—	—	→5,6)-Gal*f*-(1→
2-MeGal	—	—	—	Tr	—	—	→3,5,6)-Gal*f*-(1→
2,3,4,6-Me_4Man	16	29	2	8	35	8	Man*p*-(1→
3,4,6-Me_3Man	11	11	—	5	15	—	→2)-Man*p*-(1→
2,3,6-Me_3Man	8	18	96	2	14	92	→4)-Man*p*-(1→
2,3,4-Me_3Man	12	3	—	5	3	—	→6)-Man*p*-(1→
4,6-Me_2Man	Tr	—	—	—	—	—	→2,3)-Man*p*-(1→
2,6-Me_2Man	—	—	—	Tr	—	—	→3,4)-Man*p*-(1→
3,6-Me_2Man	33	37	2	16	31	—	→2,4)-Man*p*-(1→
2,3-Me_2Man	Tr	—	—	Tr	—	—	→4,6)-Man*p*-(1→
3,4-Me_2Man	1	—	—	1	1	—	→2,6)-Man*p*-(1→
3-MeMan	1	—	—	Tr	Tr	—	→2,4,6)-Man*p*-(1→

Tr = trace.

[a] OMe-alditol acetates obtained on methylation analysis, followed by successive hydrolysis, reduction and acetylation, and analyzed by GC–MS (column DB-225).

[b] % of peak area relative to total peak area.

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

(δ 4.10), and H-6 (δ 3.89 and 3.94) and H-5 (δ 3.83), which was sufficient, with the aid of its HMQC spectrum, to correlate the H-2 and H-5 resonances with C-2 at δ 71.4 and C-5 at δ 73.8, respectively. Since the C-4 signal of Me α-D-Man*p* is at δ 68.4,[17] part of the overlapping mannan signal at δ 73.8 arose from 4-*O*-substituted units, due to an downfield α-shift of ~5 ppm (different solvents and temperatures were used).

The ^{13}C NMR spectrum of FP contained several signals in the C-1 region, consistent with a complex

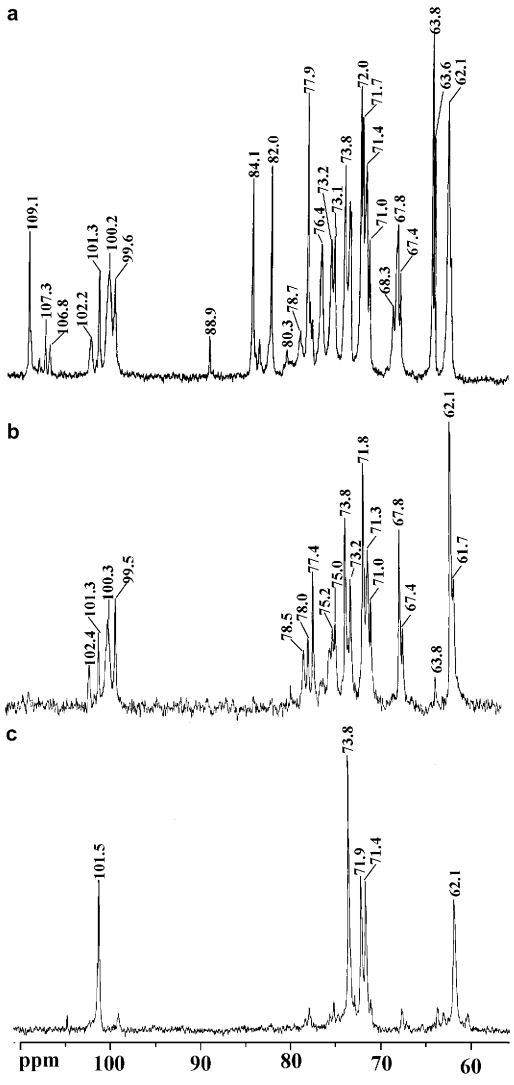


Figure 3. ^{13}C NMR spectra of FP obtained at 50 °C in D_2O (a), HFP in D_2O (b) and SmFP in Me_2SO-d_6 (c).

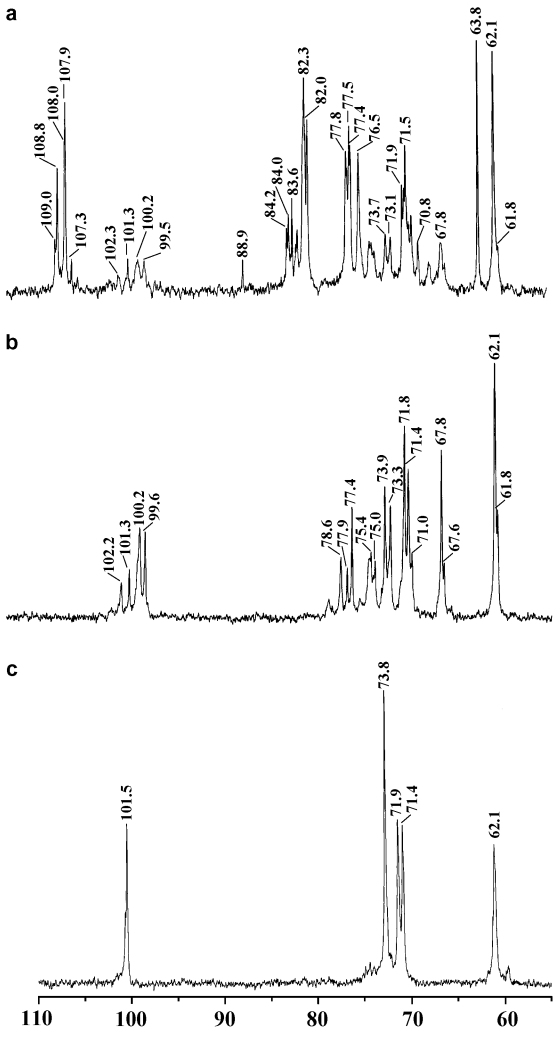
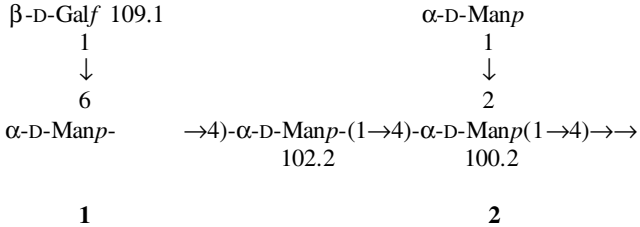


Figure 4. ^{13}C NMR spectra of FS obtained at 50 °C in D_2O (a), HRFS in D_2O (b) and SmRFS in Me_2SO-d_6 (c).

structure (Fig. 3a). The C-1 signals of nonreducing end units of Gal*f* units in a coupled spectrum had $J_{C\text{-}1,H\text{-}1}$ = 175.5 Hz, consistent with a β-D-glycosidic configuration, whereas Man*p* units from δ 99.6 to 102.2 had $J_{C\text{-}1,H\text{-}1}$ = 172.7 Hz from an α-D configuration.[18]

The C-1 signal of β-D-Gal*f* units at δ 109.1 (Fig. 3a) is probably from nonreducing end-units (14% by methylation analysis, Table 2) attached to O-6 of α-D-Man*p* units as in structure **1** (12% by methylation analysis, Table 2), since β-D-Gal*f*-(1→6)-Me α-D-Man*p* gave, in D_2O at 70 °C, a C-1′ signal at δ 109.7, lower than the more shielded 2-*O*- (δ 107.7) and 3-*O*-linked isomers (δ 106.5).[19] Two C-1 signals in the FP spectrum (Fig. 3a) can be assigned. One is the major α-D-Man*p* C-1 signal of δ 100.2, which is from 2,4-di-*O*-substituted units (structure **2**), based on the major 33% fragment obtained on methylation analysis (Table 2). This agrees with the shift of a signal at δ 100.3 found for an α-mannan from *Sporothrix schenckii*, having a (1→4)-linked main-chain partly substituted at O-2 with single-unit side chains.[20] The other is the small signal at δ 102.2 whose shift is close to those obtained for nonreducing units of (1→4)-linked α-mannotriose (C-1′ and C-1″ δ 102.5 and 102.8).[20] In an attempt to characterize sequences in FP by 2D NMR spectroscopy, its HMQC (Fig. 5a), COSY (Fig. 5b), and TOCSY spectra (Fig. 5c) were obtained. However, staring from H-1s of α-Man*p* and β-Gal*f* units, connectivity beyond H-2 was not detected, so that further ROESY or HMBC examination was not possible, due to the lack of assignable signals.



1 **2**

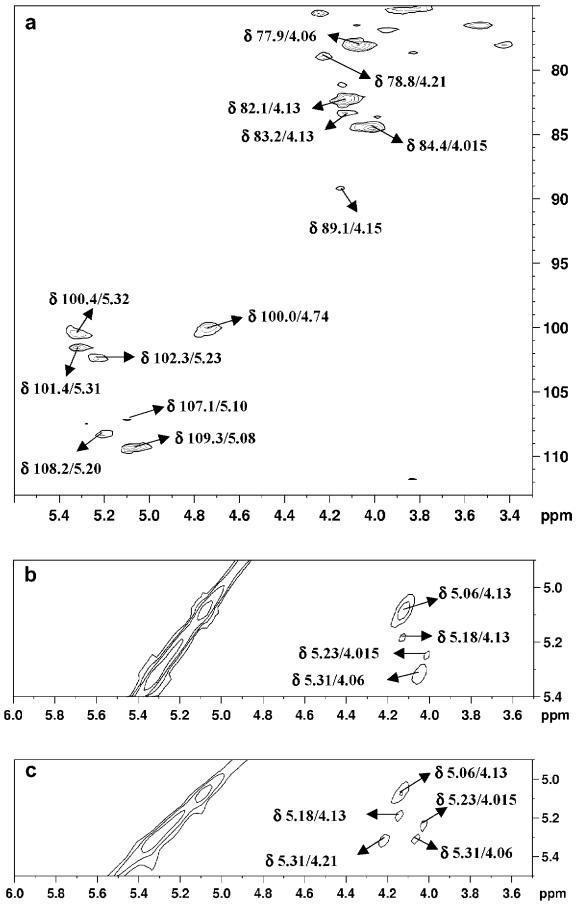


Figure 5. Partial HMQC (a), COSY (b), and TOCSY (c) NMR spectra of FP.

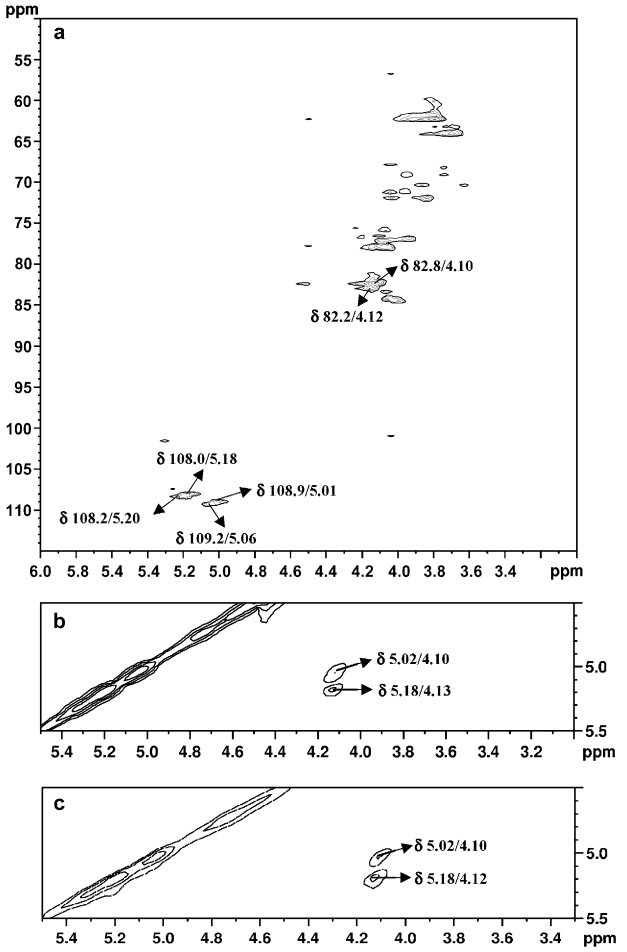


Figure 6. Partial HMQC (a), COSY (b), and TOCSY (c) NMR spectra of β-Gal*f*-rich RFS.

A partial acid hydrolysis eliminated almost all Gal*f* units from FP, as shown by monosaccharide (Table 1) and methylation data (Table 2). The C-1 region of the product (Fig. 3b) contained the same α-D-Man*p* signals as those of FP (Fig. 3a).

Fraction RFS had a higher Gal content than the galactomannan FP (Table 1). Methylation analysis (Table 2) showed a variety of Gal*f* structures in its side chains with nonreducing end- (19%), 5-*O*- (24%), 6-*O*- (8%), and 5,6-di-*O*-substituted units (11%). The Man*p* structures were the same as for FP, with predominant nonreducing (35%) and 2,4-di-*O*-substituted units (31%). RFS gave HMQC signals containing only signals of β-Gal*f* units (Fig. 6a) and in its COSY (Fig. 6b) and TOCSY spectra (Fig. 6c), the limit of connectivity from H-1 was H-2.

RFS also gave a (1→4)-linked α-D-mannan (SmRFS) on controlled Smith degradation, as shown by its monosaccharide composition (Table 1), and methylation (Table 2) and ^{13}C NMR data (Fig. 4c).

The C-1 portion of the ^{13}C spectrum of RFS (Fig. 4a) had signals at δ 108.8 > 109.0, and that of the latter that arise from some of the β-D-Gal*f* (19%: methylation data) could be linked to O-6 of α-D-Man*p* units (6%; methylation data), that at δ 108.8 could be from units linked (1→6)- to β-D-Gal*f* (8%: methylation data). The signals at δ 107.9 > 108.0, obtained at 50 °C, may be associated with 5,6-di-*O*-substituted (11%: methylation data) and/or consecutive (1→5)-linked β-D-Gal*f* as in structure **3** (24%: methylation data) and their nonreducing end units, by analogy with the oligosaccharide of *Penicillium charlesii* (δ 108.0 at 33 °C)[21] and the galactomannan of *Aspergillus niger* (δ 108.3 at 70 °C).[22]

β-D-Gal*f*-(1→5)-[β-D-Gal*f*-(1→5)]$_n$- 107.9 > 108.0

3

The partial hydrolysis product (HRFS) of RFS contained Man and Gal in a 98:2 molar ratio (Table 1), and methylation analysis showed the same structures as those present in HFP (Table 2) with nonreducing end- (35%) and 2,4-di-*O*-substituted Man*p* units (31%), as main components.

3. Conclusions

Although an α-D-mannan with a main chain of (1→4)-linked α-Man*p* units, partially substituted at O-2 with

single-unit Man*p* side chains, has been isolated from the pathogenic fungus *S. schenckii*,[20] we have not found any basic similarities of FP and RFS with other heteropolysaccharides previously isolated from lichens, which usually have main chains of (1→6)-linked α-Man*p* units, substituted mainly at O-2, O-4, or O-2,4 by units of α- or β-Gal*p*, α-Man*p* or Glc*p*, and more rarely by β-Gal*f*.[3–5,10] Mannose-containing heteropolysaccharides have been isolated from some species of lichens, mainly from the families Cladoniaceae, Parmeliaceae, Umbilicariaceae, Ramalinaceae, and Stereocaulaceae,[4,5,11] which are lichenized with a trebouxioid alga as primary photobionts.[15,23]

On the other hand, *R. decipiens* contains galactomannans with previously unreported structures, and it is lichenized with a photobiont of the genus *Trentepohlia*, a green filamentous alga.

Whether the nature of the photobiont exerts any influence on the polysaccharide components of the lichen thallus is still unknown. An extension of the present study to other genera having different photobionts could be useful to establish the relationship of polysaccharide structure and the nature of the mycobiont–photobiont association.

4. Experimental

4.1. General experimental procedures

Gas–liquid chromatography–mass spectrometry (GC–MS) was performed using a Varian model 3300 gas chromatograph linked to a Finnigan Ion-Trap model 810 R-12 mass spectrometer, with He as carrier gas. A capillary column (30 m × 0.25 mm i.d.) of DB–225, held at 50 °C during injection and then programmed at 40 °C min^{-1} to 220 °C (constant temperature) was used for quantitative analysis of alditol acetates and partially *O*-methylated alditol acetates.

^{13}C NMR spectra were obtained using a Bruker DRX 400 or Bruker Avance™ 500 spectrometer incorporating Fourier transform. Analyses were performed at 50 °C, samples being dissolved in D_2O or in Me_2SO-d_6. Chemical shifts of water-soluble samples are expressed in δ ppm relative to acetone at δ 30.20 and 2.22 for ^{13}C and ^{1}H signals, respectively, and at δ 39.70 (^{13}C) and 2.40 (^{1}H) for those soluble in Me_2SO-d_6. 1D (^{1}H and ^{13}C) and 2D NMR spectra (^{1}H (obsd.) ^{13}C, COSY, and TOCSY) were obtained by following the Bruker manual.

4.2. Collection of lichenized fungi

The foliose thalli of *R. decipiens* were collected by Dr. E. Stocker-Wörgötter in the Baja California peninsula (Mexico) in May of 2002. A voucher sample is deposited in the private collection of E. Stocker-Wörgötter, number 526, and a duplicate voucher sample in the UPCB (Herbarium name follows Holmgren et al.[24]), registration number 49052.

4.3. Extraction and purification of polysaccharides

The lichen thallus of *R. decipiens* was first extracted with 2:1 (v/v) $CHCl_3$–MeOH at 60 °C for 2 h (×3, 250 mL each) and then with 4:1 (v/v) MeOH–H_2O at 60 °C for 2 h (×3, 250 mL each), to remove low-molecular-weight material. The residue was submitted to sequential extraction (Fig. 1) with water at 100 °C for 3 h (×3, 300 mL each) and 2% aq KOH at 100 °C for 3 h (×3, 300 mL each). Each combined extract was neutralized (HOAc), added to ethanol (3 vol), and the resulting polysaccharide precipitates were dissolved in water and dialyzed, giving rise to fractions W and K, respectively. Fraction K was frozen and then allowed to thaw slowly, and the resulting insoluble material (fraction IK) was centrifuged off. The supernatant (SK) was treated with Fehling's solution,[25] and precipitated material (FP) centrifuged off. Both FP (precipitate) and FS (supernatant) fractions, were neutralized with HOAc, dialyzed against tap water, deionized with mixed ion-exchange resins, and then freeze dried.

Fraction FS was further purified by ultrafiltration through a membrane of 10 kDa M_r cut-off (Millipore-regenerated cellulose), giving rise to eluted (EFS) and retained (RFS) material.

4.4. Monosaccharide composition

Monosaccharide components of the polysaccharides and their ratios were determined by hydrolysis with 2 M TFA for 8 h at 100 °C, followed by conversion to alditol acetates (GC–MS) by successive $NaBH_4$ reduction and acetylation with 1:1 Ac_2O–pyridine for 12 h at room temperature.[26,27]

4.5. Determination of hetero- or homogeneity of polysaccharides and their molecular weight

The homogeneity and molar mass (M_w) of water-soluble polysaccharides were determined by high-performance steric-exclusion chromatography (HPSEC), using a refractive index (RI) detector. The eluent was 0.1 M $NaNO_3$, containing 0.5 g/L NaN_3. The polysaccharide solution was filtered through a membrane with pores of 0.2 μm diameter (Millipore). The specific refractive index increment (d*n*/d*c*) was determined, the samples being dissolved in 50 mM $NaNO_3$, and five increasing concentrations, ranging from 0.2 to 1.0 mg/mL, were used to determine the slope of the increment.

4.6. Methylation analysis of polysaccharides

Samples were *O*-methylated using NaOH–Me_2SO–MeI.[28] The per-*O*-methylated derivatives were hydrolyzed with 50% v/v H_2SO_4 (1 h, 0 °C), followed by dilution to 5.5% v/v (14 h, 100 °C), neutralization ($BaCO_3$), and filtration.[29] The resulting mixture of *O*-methylaldoses was reduced with $NaBD_4$ and acetylated as cited above to give a mixture of partially *O*-methylated alditol acetates, which was analyzed by GC–MS.

4.7. Partial acid hydrolysis of galactomannans

Fractions FP and RFS were submitted to partial acid hydrolysis with aq trifluoroacetic acid, pH 2.0, for 18 h at 100 °C. The neutralized solutions containing HFP and HRFS, respectively, were dialyzed (2 kDa cut-off membrane), and the retained material was freeze dried.

4.8. Isolation of the mannan main chains

FP and RFS (150 mg) were oxidized in 0.05 M $NaIO_4$ (20 mL) at room temperature, in the dark for 96 h. To stop the reaction, 1,2-ethanediol was added, the solution dialyzed, and the resulting polyaldehydes reduced with sodium borohydride.[30] After neutralization with HOAc followed by dialysis, the materials were partially hydrolyzed with aq trifluoroacetic acid, pH 2.0, for 30 min at 100 °C.[31] The neutralized solutions were dialyzed (2 kDa cut-off membranes), and the polysaccharide main chains were recovered by freeze drying (SmFP and SmRFS, respectively).

Acknowledgements

The authors would like to thank CAPES and Fundação Araucária for the financial support.

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