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Carbohydrate Polymers 40 (1999) 101–106

Carbohydrate Polymers

The glucans of *Ramalina celastri*: relation with chemotypes of other lichens

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Received 28 November 1998; received in revised form 6 January 1999; accepted 15 January 1999

Abstract

Several structurally different glucans were characterized as components of *Ramalina celastri*. Aqueous KOH extraction of the lichen at 100°C, followed by dialysis provided amylose (0.02%), identified and quantified by its blue coloration with iodine. The extract was frozen and thawed and the resulting precipitate (2% yield) shown to be a mixture of two insoluble D-glucans, with (1 → 3)- and (1 → 3),(1 → 4)-linkages, respectively, as shown by ^{13}C NMR spectroscopy. On treatment with 0.5% aqueous NaOH at 50°C, the material which remained insoluble was a linear β-glucan with regularly distributed (1 → 3)- and (1 → 4)-linkages in a 1:1 molar ratio (nigeran, 1.2% yield), whereas that which solubilised was a linear β-glucan with (1 → 3)-linkages (laminaran, 0.8% yield). The mother liquor of the KOH extraction was treated with Fehling solution to give a precipitate and the supernatant contained an α-D-glucan (28% yield) with (1 → 3)- and (1 → 4)- linkages in a molar ratio of 3:1, and which were distributed irregularly. The structures of these two (1 → 3),(1 → 4)-linked β-glucans were characterized by methylation, controlled Smith degradation and ^{13}C and ^{1}H NMR spectroscopic analyses.

Keywords: *Ramalina celastri*; α-D-glucans; β-D-glucan; Amylose

1. Introduction

α- and β-D-glucans with many chemical structures occur in lichens. Most of them contain (1 → 3)- and (1 → 4)-linkages with varying ratios: (1 → 6)-linked β-D-glucans are also known (Gorin, Baron & Iacomini, 1988; Gorin, Baron, Silva, Teixeira & Iacomini, 1993). All glucans present in ascomycetous lichens are linear, but that of the basidiomycetous lichen *Dictyonema glabratum* (formerly *Cora pavonia*) is a branched β-D-glucan with (1 → 3)- and (1 → 6)-linkages (Zanin, Fontana, Hogge, Gorin & Iacomini, 1987). We now describe the unprecedented number of four glucans of *Ramalina celastri* (formerly *Ramalina ecklonii* (Spreng.) Mey. and Flot.). The structures of these glucans are compared with those of other lichens from a chemotyping standpoint.

2. Materials and methods

2.1. General methods

Specific rotations were determined at 25°C, with an Acatec model PDA-8200 polarimeter. Gas liquid chromatography–mass spectrometry (GC–MS) was performed on a Varian model 3300 gas chromatograph linked to a Finnigan Ion-Trap, model 810 R-12 mass spectrometer, using He as a carrier gas. Paper chromatography (PC) was carried out by the descending method using Whatman no. 1 filter paper (solvent: *n*-BuOH–pyridine–H_2O, 5:3:3), and 2-*O*-α-D-glucopyranosyl-D-erythritol (GE) as a standard, sugars being detected by the acetone–$AgNO_3$ dip reagent. Acetylation of alditols was carried out with Ac_2O–pyridine (1:1) for 12 h at room temperature. Quantitative determination of carbohydrate was performed by the phenol:H_2SO_4 method (Dubois, Gilles, Hamilton, Rebers & Smith, 1956).

2.2. Extraction and purification of glucans from R. celastri

R. celastri (50 g) was collected in the region of Curitiba, PR, Brazil. It was dried, cleaned, and extracted with 500 ml 2:1 (v/v) $CHCl_3$–MeOH at 60°C for 2 h, followed by reflux in 500 ml 80% aq. MeOH at 60°C for 3 h, in order to remove low-molecular weight material. The residue was isolated, treated with 2% aq. KOH at 100°C for 2 h (Iacomini, Schneider & Gorin, 1985), filtered, and then dialysed after neutralisation (HOAc). The extract was centrifuged rapidly, the supernatant evaporated to 50 ml, precipitated by addition to excess EtOH (200 ml). It was isolated, dried,

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0144-8617/99/$ - see front matter
PII: S0144-8617(99)00048-X

dissolved in H_2O (100 ml), the solution frozen, and then allowed to thaw at 4°C overnight, resulting in the formation of insoluble material (yield 2%). This was stirred for 2 h in aq. 0.5% NaOH at 50°C (10 ml). After neutralisation (HOAc), centrifugation and dialysis, the solubilised material was a β-D-glucan (Fraction B; yield 0.8%) with $[\alpha]_D$ + 10° (c, 0.3, 0.5% aq. NaOH), whereas the insoluble material was an α-D-glucan (Fraction A; yield 1.2%) with $[\alpha]_D$ + 155° (c, 0.3, 1% aq. NaOH).

An aliquot of a solution of the remaining polysaccharides present in the supernatant of the KOH extraction was tested with iodine solution (Krisman, 1962), giving rise to a royal blue colour, corresponding to that typical of amylose (yield 0.02%). A solution of the polysaccharides was then treated with Fehling solution (60 ml). The resulting insoluble Cu^{++} complex of galactomannan was removed by centrifugation, and after decomplexation and dialysis was obtained in a 5.8% yield. The supernatant was dialysed against tap water, deionized with a mixed-bed ion-exchange resin, evaporated to a small volume and added to an excess of EtOH, which precipitated an α-D-glucan (Fraction C; 28% yield) with $[\alpha]_D$ + 213° (c, 0.4 H_2O).

2.3. Monosaccharide composition of the polysaccharides

The presence of glucose as the only component in Fractions A, B and C was confirmed by hydrolysis with 1 M TFA for 8 h at 100°C, evaporation, and conversion of the resulting aldose into glucitol hexaacetate by successive $NaBH_4$ reduction and acetylation with Ac_2O–pyridine (1:1 v/v) at 25°C for 14 h. The products were analysed by GC–MS using a capillary column of DB-225 (30 m × 0.25 mm i.d.), held at 50°C during injection, and then programmed at 40°C/min to 230°C (const. temp.) or with a column of OV-225 (30 m × 0.25 mm i.d.), injection at 50°C, but with programming at 40°C/min to 220°C (then hold).

2.4. Homogeneity tests on the water-soluble α-D-glucan: molecular weight and intrinsic viscosity ([η]) determinations

The molecular weight (M_r) and molecular weight distribution of the α-glucan (Fraction C) were determined by steric exclusion chromatography (SEC; Waters 150 ACL/GPC), using multidetection equipment in which a differential refractometer (Waters), a multiangle laser light scattering (MALLS; Dawn DSP-F, Wyatt Technology), and a viscosimeter (home-made, CERMAV) were adapted online (Tindland, Mazet & Rinaudo, 1988). The eluant was 0.1 M $NaNO_3$ containing 0.5 g/l NaN_3, with an OHpak B804 column (Shodex) at 30°C. The polysaccharide solution (1.0 g/l) was filtered through a 0.2 μm membrane. The d*n*/d*c* of the glucan was found to be 0.135.

2.5. Determination of homogeneity and molecular weight (M_r) of water-insoluble glucans (Fractions A and B)

A sample of each polysaccharide (2.0 mg) was dissolved in 1.0 M NaOH at 60°C and the soluble material (0.5 ml) was applied to a column of Sepharose CL 4B (21.5 × 1.8 cm i.d). This was then eluted with 0.2 M NaOH and the resulting fractions of 2 ml were each tested for carbohydrates (Dubois et al., 1956). The void volume of the column was determined by elution with a standard dextran of $M_r = 5 \times 10^6$ and each glucan was eluted after the void volume, showing only one peak for each polysaccharide.

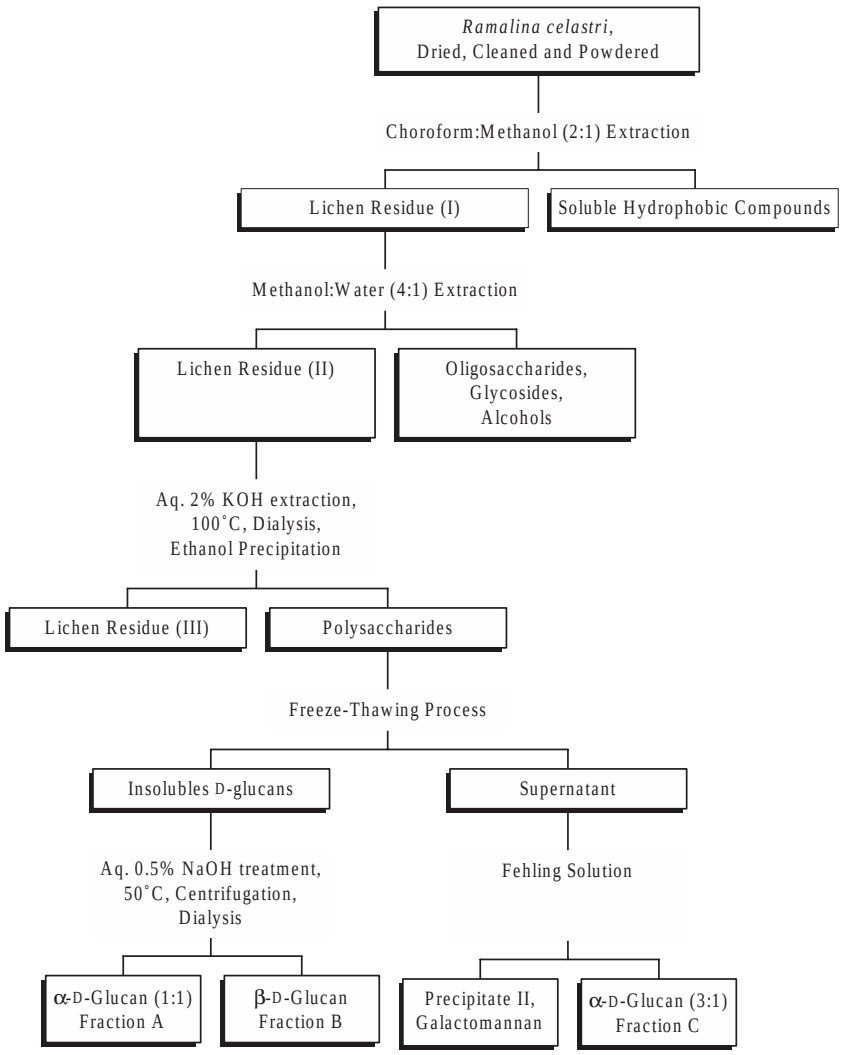
2.6. Per-O-methylation of the polysaccharides

The glucans (50 mg) were *O*-methylated twice by the Haworth procedure (1915), the reaction mixtures being heated at 100°C for 20 min to destroy excess Me_2SO_4, desalted by dialysis against running tap water and then distilled water, and freeze-dried. The partially methylated polysaccharides were then submitted twice to the method of Kuhn, Trischmann and Löw (1955) to methylate fully. The per-methylated polysaccharides were converted into partially *O*-methylated alditol acetates by successive treatments with 3% MeOH–HCl for 3 h under reflux, 1 M H_2SO_4 for 14 h at 100°C, reduction with $NaBH_4$, and acetylation with Ac_2O–pyridine. The products were examined by capillary GC–MS under the conditions described above.

2.7. Controlled Smith degradation of the water-soluble polysaccharide (Fraction C)

The polysaccharide (200 mg) obtained by alkaline extraction was oxidised in 0.5 M $NaIO_4$ (100 ml) for 3 days in the dark at 0–2°C. 1,2-Ethanediol was then added, the solution dialysed, reduced with $NaBH_4$, neutralised with HOAc, and after 24 h, the solution redialysed. It was then concentrated to 20 ml and partial hydrolysis was effected by adjustment to pH 2.0 with dil. H_2SO_4 and heating at 100°C for 30 min. Following deionisation and evaporation of the solution, it was examined by PC (solvent: *n*-BuOH–EtOH–H_2O, 40:11:19 v/v; developer: $AgNO_3$–NaOH–acetone dip), which showed the presence of erythritol, and three other spots with mobilities compared with that of 2-*O*-α-D-glucopyranosyl-D-erythritol, R_{GE}, of 0.37, 0.66 and 1.00. Cellulose-column chromatography using acetone–H_2O mixtures as eluants, gave erythritol with 10:1 v/v, the materials having R_{GE} 1.0 and 0.66 with 7:1 v/v, and R_{GE} 0.37 (with 4:1 v/v). Each oligosaccharide gave glucose and erythritol on acid hydrolysis.

The polyalcohol obtained from the glucan via successive treatments with $NaIO_4$ and $NaBH_4$ was hydrolysed with 1 M TFA for 7 h at 100°C. The hydrolysate was then successively reduced with $NaBH_4$, treated with Ac_2O–pyridine, and the resulting acetates examined by GC–MS as described above.



Scheme 1.

2.8. Smith degradation of water-insoluble α-D-glucans (Fraction A)

The polysaccharides (20 mg) were solubilised in 5.0 ml of 0.1 M NaOH and shaken at 60°C. The resulting solutions were adjusted with HOAc to pH 8.0, 0.1 M $NaIO_4$ (5.0 ml) added, and the solutions shaken at 25°C for 72 h. Ethylene glycol was then added, reductions carried out with $NaBH_4$, and the products partially hydrolysed, as described above for the water-soluble glucan. Paper chromatography of the products from the α-D-glucan showed the exclusive presence of the α-form of 2-*O*-D-glucopyranosyl-D-erythritol (Gorin & Iacomini, 1984).

2.9. ^1H and ^{13}C nuclear magnetic resonance spectroscopy

^1H and ^{13}C NMR spectra were obtained using a 400 MHz Bruker model DRX NMR spectrometer incorporating Fourier transform. Samples were dissolved in D_2O, 1% NaOD in D_2O, or DMSO-d_6, and examined at 70°C. Chemical shifts (δ) are expressed relative to the resonance of Me_4Si (TMS; $\delta = 0$), obtained in a separate experiment (Gorin & Iacomini, 1984). Quantification of (1 → 3)- and (1 → 4)-linkages was carried out by measurement of the areas of relevant H-1 signals in the ^1H NMR spectrum with δ 5.04 and δ 4.98 for the α-glucan, nigeran (Fraction A; solvent DMSO-d_6), which gave a 1:1 molar ratio, and δ 5.02 and δ 4.98 for the α-glucan, isolichenan (Fraction C; solvent D_2O), which gave a 3:1 molar ratio. These results agreed with methylation data. For the β-glucan, lichenan (Fraction B; solvent NaOD–D_2O), the relevant H-1 signal was at δ 4.539, $J_{1,2}$ 6.8 Hz.

3. Results and discussion

The lichen of *R. celastri* was extracted with hot aqueous potassium hydroxide at 100°C, and the resulting solution neutralised, dialysed and freeze-thawed. The resulting precipitate was isolated and the process was repeated (Scheme 1 (Process of extraction and fractionation of polysaccharides from the lichen *R. celastri*)). The insoluble

D-glucans obtained by freeze-thawing (Fractions A and B, 1.2 and 0.8% yield, respectively) were examined by ^{13}C NMR spectroscopy (Fig. 1(A), solvent NaOD–D_2O). Its C-1 region contained three signals, one at δ 102.8 (β-configuration), and at δ 100.2 and δ 99.3 (α-configuration), indicating a probable mixture. Others were present at δ 86.0 (*O*-substituted C-3_β), δ 82.7 (*O*-substituted C-3_α) and δ 79.1 (*O*-substituted C-4_a), and δ 60.9, δ 60.7 and δ 60.3 (unsubstituted C-6). This mixture was treated with 0.5% aq. NaOH at 50°C for 2 h, was then centrifuged and both insoluble and soluble fractions were dialysed. The soluble fraction (Fraction B), which thus had a laminaran structure, was homogeneous on Sepharose CL 4B and had M_r 62 kD, was analysed by ^{13}C NMR spectroscopy and

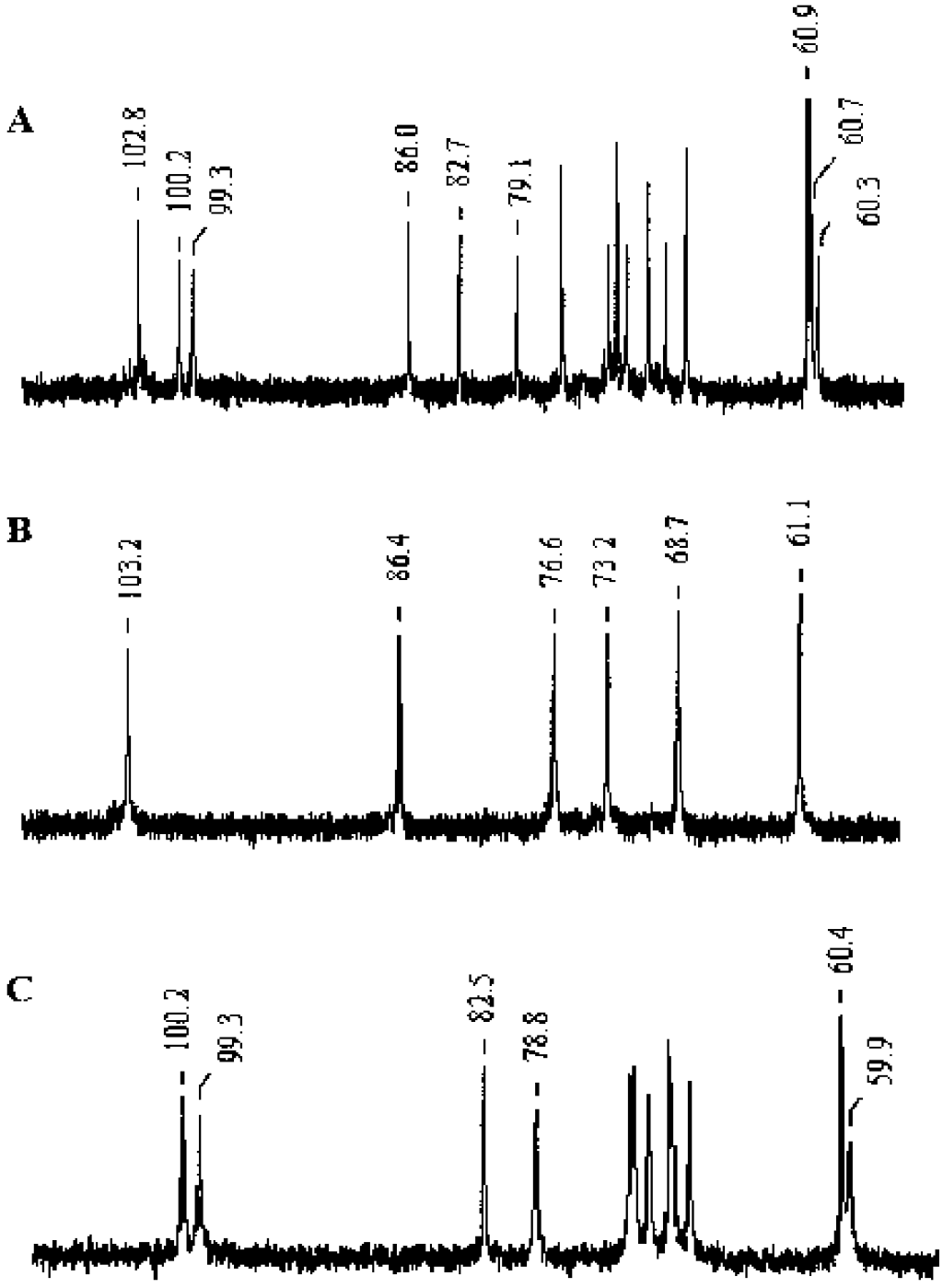


Fig. 1. ^{13}C NMR spectra of the insolubles D-glucans: (A) mixture of α and β-D-glucans with (1 → 3) and (1 → 3)- (1 → 4)- linkages, respectively (400 MHz, NaOD–D_2O, 343 K); (B) linear (1 → 3)- β-D-glucan purified (400 MHz, NaOD–D_2O, 303 K); (C) α-D-glucan purified with regular (1 → 3) and (1 → 4)-linkages along the linear chain (400 MHz. DMSO-d_6, 343 K).

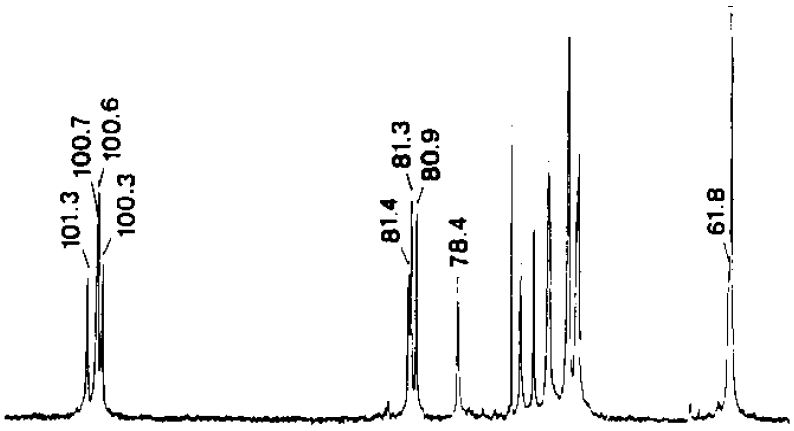


Fig. 2. ^{13}C NMR spectrum of α-D-glucan with (1 → 3)- and (1 → 4)- linkages in a molar ratio of 3:1 (400 MHz, D_2O, 343 K).

shown to be a linear (1 → 3)-β-glucan (6; Fig. 3) with typical signals δ 103.2 (C-1), δ 86.4 (C-3), δ 76.6 (C-5), δ 73.2 (C-2), δ 68.7 (C-4) and δ 61.1 (C-6) (Fig. 1(B), solvent NaOD–D_2O) and examination by ^1H NMR spectroscopy showed signals δ 4.61 (H-1), δ 3.38 (H-2), δ 3.58 (H-3), δ 3.33 (H-4), δ 3.34 (H-5) and δ 3.54 (H_α-6) and δ 3.78 (H_β-6). ^{13}C and ^1H correlations were obtained by HMQC, according to Delgobo, Gorin, Jones and Iacomini (1998).

The α-D-glucans (Fraction A, nigeran) was homogeneous on Sepharose CL 4B and had M_r 69 kD. On methylation analysis, it gave rise to 2,4,6 and 2,3,6-tri-*O*-methylglucitol acetates in a molar ratio of 1:1 (GC–MS), corresponding to (1 → 3)- and (1 → 4)-linkages, respectively. A Smith degradation of the glucan furnished acetates of glucitol and erythritol in a molar ratio of 52:48 (GC–MS), and a controlled Smith degradation gave 2-*O*-α-D-glucopyranosyl-D-erythritol only (PC). This result shows a regular distribution of the (1 → 3)- and (1 → 4)-linkages along the linear chain (5; Fig. 3). Analysis by ^{13}C NMR spectroscopy furnished high-field C-1 signals at δ 100.2 and δ 99.3, showing an α-configuration, with other signals at δ 82.5 and δ 78.9, corresponding to *O*-substituted C-3 and C-4, respectively, and δ 60.4 and δ 59.9 (unsubstituted C-6) (Fig. 1(C); solvent DMSO-d_6).

The supernatant solutions were combined and evaporated to a small volume, which was treated with Fehling solution to give an insoluble copper complex, which has been previously regenerated and characterized as a galactomannan (Miceno, Gorin & Iacomini, 1991). The supernatant of a Fehling precipitation (Fraction C, 28% yield), contained an α-D-glucan, now shown to have M_r 294 kD, [η] 1.66 dl/g, and to give a single peak when analysed by SEC.

Also present was a trace of amylose (0.02%), a (1 → 4)-linked linear α-glucan (1; Fig. 3), which was identified and quantified by its typical blue colour with iodine. In view of this very small content, the main α-glucan (Fraction C; 28% yield) gave rise to the relevant ^{13}C NMR signals (Fig. 2; solvent: D_2O), which were at δ 101.3, δ 100.7, δ 100.6 and δ 100.3, indicating an α configuration; and others at δ 81.4, δ 81.3 and δ 80.9 (*O*-substituted C-3s); δ 78.4 (*O*-substituted C-4), and δ 61.8 and δ 61.7 (C-6). A methylation analysis provided a mixture of partially *O*-methylated alditol acetates, which was examined by capillary GC–MS of OV-225, which showed acetates of 2,4,6- and 2,3,6-tri-*O*-methylglucitol in a molar ratio of 2.8:1.0, corresponding to the (1 → 3)- and (1 → 4)-linkages, respectively.

A Smith degradation of the present α-glucan (Fraction C) gave a mixture of glucose and erythritol, which was converted to acetates of glucitol and erythritol in a 3:1 molar ratio (GC). A controlled Smith degradation provided a mixture of erythritol and oligosaccharides with R_{GE} of 1.00, 0.66 and 0.37, which were isolated via cellulose-column chromatography in a w/w ratio of 1:5:14. Each component was subjected to methylation analysis and acetates of 2,3,4,6-tetra-*O*-methylglucitol (from R_{GE} 1.00), 2,3,4,6-tetra- and 2,4,6-tri-*O*-methylglucitol in a 1:1 ratio (from R_{GE} 0.66), and the same fragments in a 1:2 ratio (from R_{GE} 0.37), were detected. The products are therefore erythritol (from amylose, very little); 2-*O*-α-D-glucopyranosyl-D-erythritol; *O*-α-D-glucopyranosyl-(1 → 3)-*O*-α-D-glucopyranosyl-(1 → 2)-D-erythritol; and *O*-α-D-glucopyranosyl-(1 → 3)-*O*-α-D-glucopyranosyl-(1 → 3)-*O*-α-D-glucopyranosyl-(1 → 2)-D-erythritol, respectively. This corresponds to an irregular distribution of (1 → 3) and (1 → 4)-linkages along an α-D-glucopyranosyl chain, with structures 2, 3 and 4 (Fig. 3).

The presence of four different glucans has not been previously observed in a lichen. In terms of the α-glucans, the simplest is amylose which may be present in many lichens, but was possibly not detected since tests with iodine were not carried out. The only exceptions are with the related *R. usnea* (Gorin & Iacomini, 1984) and *Cetraria islandica*, where the blue colour was incorrectly considered to be a property of another α-glucan, isolichenan (Meyer & Gürtler, 1947).

The major α-glucan corresponding to Fraction C contains (1 → 3)- and (1 → 4)-linkages in a 2.8:1 molar ratio (Miceno et al., 1991), and is now found to have these linkages distributed in an irregular manner: a property common to the

$$-(\alpha\text{-D-Glc}p\text{-}(1\rightarrow4)\text{-})_n \quad (1)$$

$$-\alpha\text{-D-Glc}p\text{-}(1\rightarrow3)\text{-}\alpha\text{-D-Glc}p\text{-}(1\rightarrow4)\text{-} \quad (2)$$

$$-\alpha\text{-D-Glc}p\text{-}(1\rightarrow3)\text{-}\alpha\text{-D-Glc}p\text{-}(1\rightarrow3)\text{-}\alpha\text{-D-Glc}p\text{-}(1\rightarrow4)\text{-} \quad (3)$$

$$-\alpha\text{-D-Glc}p\text{-}(1\rightarrow3)\text{-}\alpha\text{-D-Glc}p\text{-}(1\rightarrow3)\text{-}\alpha\text{-D-Glc}p\text{-}(1\rightarrow3)\text{-}\alpha\text{-D-Glc}p\text{-}(1\rightarrow4)\text{-} \quad (4)$$

$$-(\alpha\text{-D-Glc}p\text{-}(1\rightarrow3)\text{-}\alpha\text{-D-Glc}p\text{-}(1\rightarrow4)\text{-})_n \quad (5)$$

$$-(\beta\text{-D-Glc}p\text{-}(1\rightarrow3)\text{-})_n \quad (6)$$

Fig. 3. Chemical structures of the glucans from the *R. celastri*: (1) structure of amylose; (2)–(4) segments present in the α-D-glucan 3:1 ratio; (5) structure of α-D-glucan 1:1 ratio; and (6) structure of β-D-glucan.

isolichenans which have ratios that can vary from 4:1 to 1:2.5 (Gorin et al., 1988). However, only the α-glucan of *R. usnea* has a confirmed ratio of (1 → 3)- to (1 → 4)-linkages of ∽ 3:1, indicating that the presence of this α-D-glucan perhaps could be used as a chemotaxonomic key for the genus *Ramalina*. The minor α-glucan contains similar linkages but which alternate in a ratio of 1:1 (nigeran = mycodextran). This glucan is typical of *Cladonia* spp. (Nishikawa, Ohki, Takahashi, Kurono, Fukuoka & Emori, 1974), and might be used as a marker, although it has been reported in *Parmelia caperata* (Takeda, Nishikawa & Shibata, 1970) and was also found in *R. usnea*, using conditions identical to those of the present preparation (unpublished data). The β-glucan component with only (1 → 3)-linkages (laminaran) has also been observed in *R. usnea* (Gorin & Iacomini, 1984) and *Stereocaulon ramulosum* (Baron et al., 1988) and its structure and low yield may indicate that it arose from the phycobionts.

Acknowledgements

The authors thank Mr Cesar A. Tischer for preparation of NMR spectra, Mr Guilherme L. Sassaki for GC–MS analyses, Elaine Carbonero for technical support, and Prof. Tania B. Bresolin, who carried out SECs at CERMAV/CNRS, Centre de Recherches sur les Macromolécules Végétales, Grenoble, France. We are also indebted to the Brazilian financing agencies CNPq, and PADCT II/SBIO and PRONEX, both of FINEP, without which the investigation would not have been possible.

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