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Glucans of lichenized fungi: significance for taxonomy of the genera *Parmotrema* and *Rimelia*

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Abstract

The glucans of lichenized fungi are an important class of polysaccharides with structural and chemotaxonomic roles. The water-insoluble glucans of the genus *Parmotrema* (*P. austrosinense*, *P. delicatulum*, *P. mantiqueirense*, *P. schindleri*, and *P. tinctorum*) and those of *Rimelia* (*R. cetrata* and *R. reticulata*), were investigated in order to evaluate the significance in chemotyping, with nigeran [(1 → 3),(1 → 4)-α-glucan] and lichenan [(1 → 3),(1 → 4)-β-glucan] characterized using ^{1}H and ^{13}C NMR, methylation analysis, and controlled Smith degradations. Results from all species were similar, suggesting that glucan chemistry does not support separation of *Rimelia* from *Parmotrema*.

Keywords: *Parmotrema*; *Rimelia*; Parmeliaceae; Lichen; Chemical structure; Glucans

1. Introduction

Lichenized fungi have been extensively investigated in terms of their carbohydrate components. Among the polysaccharides, two classes are present, one being heteropolysaccharides, including mannose-containing polysaccharides, and the other glucans (Gorin et al., 1988, 1993; Olafsdottir and Ingólfsdottir, 2001).

In terms of chemical structure, the glucans of lichenized fungi are mostly linear and can be classified according to the following structural types, namely nigeran [α-D-(1 → 3),(1 → 4) in a ratio of ∼1:1], lichenan [β-D-(1 → 3),(1 → 4)], isolichenan [α-D-(1 → 3),(1 → 4)], pullulan [α-D-(1 → 4),(1 → 6)], with varying ratios of glycosidic linkages, pustulan [β-D-(1 → 6)], pseudonigeran [α-D-(1 → 3)], laminaran [β-D-(1 → 3)], and amylose [α-D-(1 → 4) glucan]. Branched β-glucans containing (1 → 3)- and (1 → 6)-linkages can also be present (Gorin et al., 1988; Olafsdottir and Ingólfsdottir, 2001; Sassaki et al., 2002; Olafsdottir et al., 2003).

The lichenan-type glucan is suggested to be primarily a structural element of the fungal wall, with important functions in thallus–water relations, rather than a storage compound of lichen-forming ascomycetes (Honegger and Haish, 2001). The glucans of lichens have also been shown to have interesting biological properties and in a review covering the previous 30 years, Olafsdottir and Ingólfsdottir (2001) showed that lichen polysaccharides, including structurally different glucans, can have antitumoral, antiviral, and immunological activities, as well as a low level of toxicity. Glucans are also useful in terms of lichenized fungi taxonomy, based on their wide distribution throughout the lichens and the diversity of chemical structure (Sassaki et al., 2002; Woranovicz-Barreira et al., 1999; Carbonero et al., 2001, 2002).

Lichens of the Parmeliaceae have been examined cytochemically by Common (1991) using a series of

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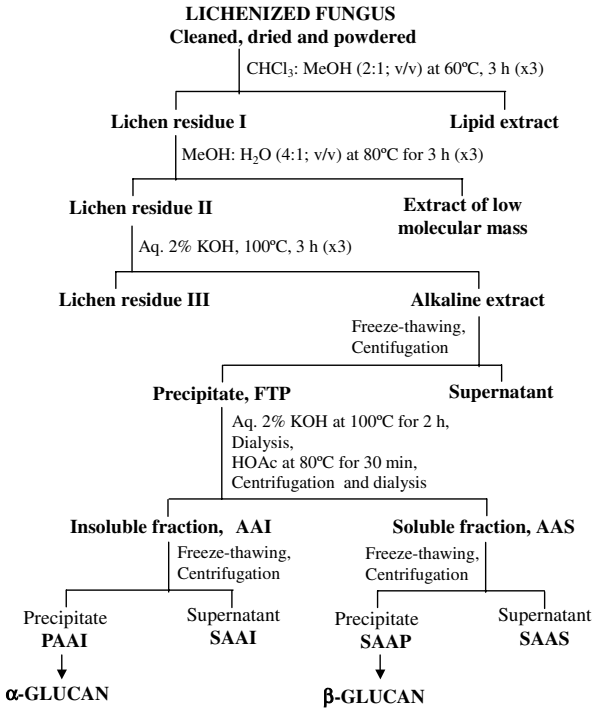
iodine tests. These gave various colorations, which were attributed to the presence of lichenan and isolichenan (red and blue, respectively). However, in our laboratory no color was formed with lichenan obtained as a precipitate of a hot-water extract of *Cetraria islandica* (Gorin and Iacomini, 1984). Also, Meyer and Gürtler (1947a) prepared lichenan in a similar way and obtained isolichenan from the mother liquor and although it gave rise to a blue coloration (Meyer and Gürtler, 1947b), it was later shown to arise from traces of amylose. *Ramalina usnea* similarly contained amylose (Gorin and Iacomini, 1984). Hence, these color reaction are not being utilized in this investigation.

Examination of lichens of the Parmeliaceae, belonging to several species of the genera *Parmotrema* and *Rimelia*, showed them to contain water-insoluble glucans. These were investigated in a chemo taxonomic perspective to determine if they provide any distinguishing features between the two genera.

2. Results

As shown in Scheme 1, lichenized fungi of *Parmotrema* and *Rimelia* spp. were extracted with $CHCl_3$–MeOH and then MeOH–H_2O to remove low-molecular weight compounds. Each resulting residue was next extracted thrice with hot 2% aq. KOH, which was neutralized with HOAc, dialyzed against tap water for 2 days, and freeze-dried. The freeze-dried extract was then resuspended in water, frozen, and thawed gently, resulting in the formation of a freeze–thawing precipitate (FTP) and soluble material. The FTP was subjected to a similar alkaline treatment, dialyzed and treated with 4% aq. HOAc at 80 °C for 30 min to give acetic acid insoluble (AAI) and acetic acid soluble (AAS) fractions. The AAI and AAS were treated again to further freeze–thawing. The precipitate from AAI, designated PAAI, is an α-glucan and that from AAS (PSAA) a β-glucan. All carbohydrate fractions, from FTP on, contained ~99% glucose as the main component (GC–MS of alditol acetates).



Scheme 1. Extraction and purification of the glucans obtained from *Parmotrema* and *Rimelia* spp.

In a preliminary examination, the ^{13}C NMR spectra of all FTP fractions (Fig. 1) contained 5 signals in the anomeric region, three of them from a β-glucan (δ 103.5, 102.6, and 102.5) and two from the α-glucan (δ 100.4 and 99.6). Those of the α-glucan (Fig. 2), together with resonances at δ 82.6 (*O*-substituted C-3) and 79.2 (*O*-substituted C-4) show it to be alternately (1 → 3)- and (1 → 4)-linked (Woranovicz-Barreira et al., 1999), indicative of a minor nigeran-like structure.

As shown above and in Scheme 1, purified α-glucan (PAAI) and β-glucan fractions (PAAS) were obtained. Fig. 2 shows the α-glucan spectrum of *R. cetrata* representing all PAAI fractions, since all lichens gave rise to indistinguishable ^{13}C spectra. Similar spectra were also observed for all of the PAAS fractions (Fig. 3). In this case, the anomeric signal at δ 103.5 arose from 3-*O*-substituted, and the predominant ones at δ 102.6, and 102.5 from 4-*O*-substituted β-Gl*cp* units. The β-configuration was confirmed by H-1 resonances at high-field of δ 4.38 and 4.28. These were assigned, respectively, to 3-*O*- and 4-*O*-substituted glucosyl units, since the area of the former was greater, as in the C-1 region.

The ^{13}C signals of the β-glucan can be assigned with the aid of the spectroscopic analyses of laminaran and cellulose (Gorin, 1981). The signals at δ 80.1 and 80.0 are from C-4 of 4-*O*-substituted glucosyl units, while that at δ 60.4 arises from non-substituted CH_2 group of the same units. The signals at δ 87.0, 76.3, 68.4, and 60.8 correspond to C-3, C-5, C-4, and C-6 of 3-*O*-substituted units, respectively. The β configuration, together with the signals referring to substitutions at O-3 and O-4 denotes the presence of lichenans in all studied species.

As the spectra of β-glucans from all of the lichens were virtually identical (Fig. 3), further analyses were carried out with that of *P. austrosinense*, as a representative species. An accurate ratio of glycosidic linkages was determined by comparing the peak areas of H-1 signals at δ 4.28–4.38. These were present in a 1:3.1 ratio for (1 → 3)- and (1 → 4)-linkages, respectively. This was identical to the linkage ratio obtained on methylation analysis incorporating measurement of peak areas obtained by GC–MS of derived alditol acetates of 2,3,6- and 2,4,6-Me_3Glc.

A Smith degradation incorporating mild hydrolytic conditions (Abdel-Akher et al., 1952; Hay et al., 1965)

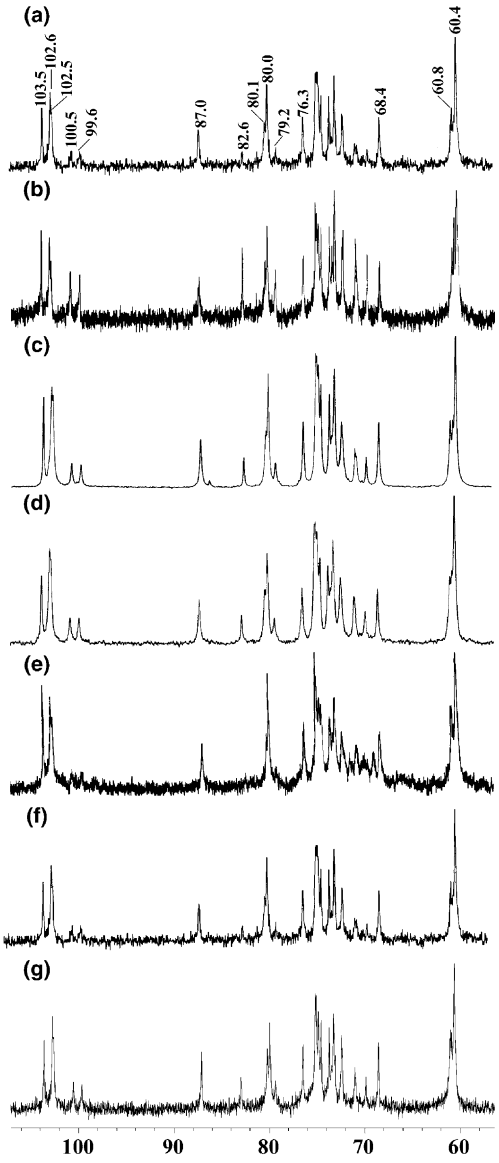


Fig. 1. ^{13}C NMR spectra of freeze–thawed precipitate fraction (FTP) obtained from *Parmotrema austrosinense* (a), *P. delicatulum* (b), *P. mantiqueirense* (c), *P. schindleri* (d), *P. tinctorum* (e), *Rimelia cetrata* (f), and *R. reticulata* (g).

was carried out to determine the sequence of linkages in this β-glucan. HPLC of the product gave only peaks corresponding to 2-*O*-β-D-glucopyranosyl-D-erythritol (9.6 min) and erythritol (13.1 min). The absence of

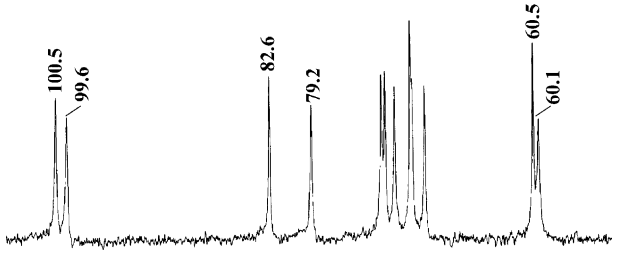


Fig. 2. ^{13}C NMR spectrum of PAAI fraction obtained from *Rimelia cetrata* in Me_2SO-d_6 at 50 °C (chemical shifts are expressed as δ, ppm).

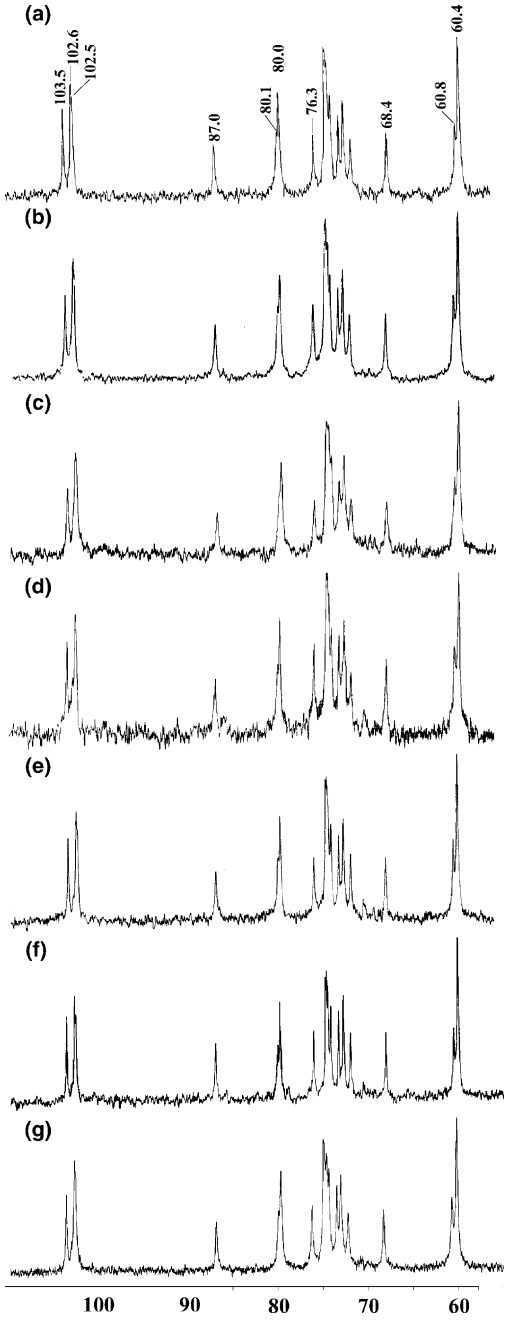


Fig. 3. ^{13}C NMR spectra of SAAP fraction obtained from *Parmotrema austrosinense* (a), *P. delicatulum* (b), *P. mantiqueirense* (c), *P. schindleri* (d), *P. tinctorum* (e), *Rimelia cetrata* (f), and *R. reticulata* (g).

glucobiosyl erythritol showed that most of the 3-*O*-substituted glucosyl residues are interspersed between the 4-*O*-substituted units, with no consecutive (1 → 3)-linkages.

3. Discussion

Both *Parmotrema* and *Rimelia* genera contain both water-insoluble α- and β-glucans, corresponding to

nigeran and lichenan, respectively, lichenan being present in a much higher quantity than nigeran.

The nigerans with a linkage ratio of 1:1 are well-known polysaccharides, widely found in lichens, including *Cladonia* (Woranovicz-Barreira et al., 1999), *Cladina* (Carbonero et al., 2002), and *Ramalina* (Stuelp et al., 1999; Cordeiro et al., 2003) among others. Woranovicz-Barreira et al. (1999) have suggested that nigerans could be used as chemotyping molecules in *Cladonia* spp. In the Parmeliaceae, they have been described in *Letharia vulpina* (Iacomini et al., 1988), and some species of *Parmelia* s. lat. (Shibata, 1973), among others.

Lichenans have also been characterized from several species of lichenized fungi, but are distinguished from the nigerans in that there is a species variation in the ratio of (1 → 3)- to (1 → 4)-linkages. Although this ratio is constant between some species of Parmeliaceae, such as *Cetraria islandica* (3:7) (Meyer and Gürtler, 1947a), *C.* (=*Masonhalea*) *richardsonii* (3:7) (Yokota et al., 1979), and *Usnea rubescens* (3:7) (Nishikawa et al., 1974), it differs in an *Usnea* sp. (1:3) (Iacomini et al., 1988). Species from the genus *Parmelia* s. lat. are also known to contain lichenans (Shibata, 1973), but the ratios are not yet cited in the literature.

Olafsdottir and Ingólfsdottir (2001) suggested that some polysaccharides from lichenized fungi are of taxonomic importance at the genus and family level. That is the glucan pustulan is characteristic for the Umbilicariaceae, the lichenan-type glucans are the only β-glucans found in Parmeliaceae, and the only α-glucan isolated from Cladoniaceae is of the nigeran type.

In Parmeliaceae, the genus *Parmotrema* is known to have a *Cetraria*-type lichenan (3:7) in the cell wall, while the segregated genus *Rimelia* contains intermediate-type lichenan, a term used for a range of coloration with iodine (Common, 1991; Elix, 1994). Another important difference, according to lichenologists, on polysaccharides from Parmeliaceae concerns the iodine reactions. The genus *Parmotrema* is known to give a characteristic color (red) with the lichen thalli when using iodine solutions, while the segregated genus *Rimelia* does not show the same response. These differences were considered to be due to a distinct lichenan content.

However, our findings show that species of *Parmotrema* and *Rimelia* (*P. austrosinense*, *P. delicatulum*, *P. schindleri*, *P. mantiqueirense*, *P. tinctorum*, *R. cetrata* and *R. reticulata*) contain similar nigerans and lichenans, even to the extent of their relative proportions. That is, lichenans having a (1 → 3)- to (1 → 4)-linkage ratio of 1:3.1 were found in all studied species after methylation analysis, this being confirmed by ^{1}H NMR spectroscopic analyse of *P. austrosinense*, and *Rimelia cetrata* (=*Parmotrema cetratum*) had a ratio of 1:2 when first analyzed (Corradi da Silva et al., 1993). The ratio difference, when compared to our data, could be due to contaminants in the sample, since the authors cited that the fractionation procedure was not fully successful.

We can thus conclude that our results do not support segregation of *Rimelia* from *Parmotrema*, but do agree with the DNA analyses of Crespo and Cubero (1998) and Louwhoff and Crisp (2000), which also support maintaining these genera as one genus.

4. Experimental

4.1. Lichenized fungi (Parmeliaceae)

Parmotrema austrosinense (Zahlbr.) Hale, *P. delicatulum* (Vain.) Hale, *P. schindleri* Hale, *P. mantiqueirense* Hale, *P. tinctorum* (Nyl.) Hale, *Rimelia cetrata* (Ach.) Hale & Fletcher and *R. reticulata* (Taylor) Hale & Fletcher were examined. *Parmotrema* spp. were collected in 1996, in the town of Lapa, State of Paraná, Brazil, while *Rimelia* spp. were from the city of Curitiba, State of Paraná, and have their voucher specimens (No. 33886, 33354, 33890, 33355, 28838, 38057, 38118, respectively), deposited in the UPCB (Herbarium name follows that assigned by Holmgren et al., 1990).

4.2. Isolation and purification of polysaccharides

Lichenized fungus samples (*Parmotrema austrosinense*, 41 g; *P. delicatulum*, 32 g; *P. schindleri*, 35 g; *P. mantiqueirense*, 43 g; *P. tinctorum*, 60 g; *Rimelia cetrata*, 31 g; and *R. reticulata*, 26 g) were successively extracted by refluxing in solution of $CHCl_3$–MeOH (2:1 v/v; 300 ml) and MeOH–H_2O (4:1, 300 ml), in order to solubilize low molecular weight components. The residual material lichen residue II for each species was then extracted three times with 2% aq. KOH containing traces of $NaBH_4$ at 100 °C for 3 h, with the resulting alkaline extract individually neutralized with HOAc, dialyzed against tap water, and after 48 h freeze-dried. The crude fractions obtained from each alkaline extraction were then individually submitted to freeze–thawing, which furnished insoluble (FTP) and soluble supernatant materials, these being separated by centrifugation (15 min, 9000 rpm, 25 °C). The insoluble fractions (FTPs) were then each submitted to a additional purification process, which was extracted once more with 2% aq. KOH at 100 °C for 2 h, dialyzed against tap water to neutral pH (48 h) and treated with 4% aq. HOAc at 80 °C for 30 min. After centrifugation, dialysis of each acetic acid to soluble (AAS) and acetic acid insoluble (AAI) fraction in HOAc was carried out against tap water for 2 days, when the last freeze–thawing step was performed to yield the final precipitates of acetic acid insoluble (PAAI) and corresponding supernatant with acetic acid solubles (SAAS), with the former consisted of purified glucans.

4.3. Monosaccharide composition

Hydrolysis of the individual fractions was carried out with 1 M TFA at 100 °C for 8 h the hydrolyzates were evaporated to dryness, reduced with $NaBH_4$ and acetylated with Ac_2O–pyridine (1:1, v/v; 2 ml) at room temperature for 12 h (Wolfrom and Thompson, 1963a,b). The resulting alditol acetates 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 helium as carrier gas. Each analysis was carried out from 50 to 220 °C at 40 °C/min for maintaining a temperature constant for 18 min.

4.4. Methylation analysis

The PAAS fraction from *P. austrosinense* (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 hydrolyzed with 50% v/v sulfuric acid (1 h, 0 °C), followed by dilution to 5.5% v/v (18 h, 100 °C), neutralized ($BaCO_3$) and filtered (Saeman et al., 1954). The resulting mixture of *O*-methylaldoses was reduced with $NaBH_4$ and acetylated as above to give a mixture of partially *O*-methylated alditol acetates, which was analyzed by GC–MS. The analysis was carried out from 50 to 215 °C at 40 °C/min with the final temperature maintained for 31 min; the resulting partially *O*-methylated alditol acetates were identified by their typical electron impact breakdown profiles and retention times (Jansson et al., 1976).

4.5. Nuclear magnetic resonance spectroscopy

NMR spectra were obtained using a 400 MHz Bruker model DRX Avance spectrometer with a 5 mm inverse probe. ^1H and ^{13}C NMR spectroscopic analyses were performed at either 50 or 30 °C, with samples being dissolved in Me_2SO-d_6. Chemical shifts of samples are expressed in ppm (δ) relative to Me_2SO-d_6 at δ 39.70 and 2.40 for ^{13}C and ^1H resonances, respectively.

4.6. Controlled Smith degradation

The SAAP fraction from *P. austrosinense* (100 mg) was oxidized to polyaldehydes with aqueous 0.05 M $NaIO_4$ (50 ml) for 72 h at room temperature in the dark (Abdel-Akher et al., 1952; Hay et al., 1965). Excess of oxidant was destroyed by addition of ethylene glycol (1.0 ml), and the solution was dialyzed against tap water for 48 h. The solution was treated with $NaBH_4$ for 18 h at 25 °C, with excess reducing agent destroyed by Dowex 50 × 8 (H^+ form) ion-exchange resin and the solution dialyzed for 48 h. The oxidized–reduced material was successively hydrolyzed (TFA pH 2, 100 °C for 40 min), evaporated to dryness, and the residual analyzed by HPLC.

4.7. High performance liquid chromatography (HPLC)

Experiments were carried out using a Shimadzu HPLC chromatograph equipped with a refractive index detector (Shimadzu 10A) at 40 °C. Samples were analyzed using an Aminex® ion exclusion (Bio Rad Inc.) column (HPX 87-H, 300 × 7.8 mm) after passing through a Micro-Guard (Bio Guard Inc.) cartridge filled with material similar to that of the main column. The material was eluted with degassed solution of sulfuric acid (8 mmol) in deionized water at 65 °C with a 0.6 ml min^{-1} flow rate. Samples (20 μl) were injected into the column using an injection manual valve. The samples were identified by their retention times compared with those of standards erythritol and 2-*O*-β-D-glucopyranosyl-D-erythritol.

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