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Structure of two glucans and a galactofuranomannan from the lichen *Umbilicaria mammulata*

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Abstract

The three main polysaccharides from the lichenized fungus *Umbilicaria mammulata* were isolated and characterized. Their structures were determined using GC-MS of derived alditol acetates, GC-MS-methylation analysis and ^{13}C NMR spectroscopy. Two of them were (1→3)- and (1→6)-linked β-glucans, namely laminaran and pustulan, respectively. The other was a galactofuranomannan, which was analyzed as well as a galactose-free polysaccharide formed on partial hydrolysis. The former consisted of a main chain of (1→6)-linked α-mannopyranosyl residues, part of them being substituted at O-2, O-4, and O-2,4 by complex, branched side chains containing α-mannopyranosyl and β-galactofuranosyl units. This heteropolysaccharide is related to those of other Umbilicariaceae, and its chemical characterization presents a useful tool for the systematics of lichenized fungi.

Keywords: Lichen; *Umbilicaria mammulata*; Galactofuranomannan; Laminaran; Pustulan; Chemotaxonomy

1. Introduction

Lichen symbiosis is an evolutionary strategy that has resulted in a rich diversity of incorporated fungi (Grube & Kroken, 2000), with 13.500 distinct species growing worldwide (Galun, 1988). Of this great number, only about 100 lichens have so far been investigated to determine their polysaccharide components. The best known classes of their polysaccharides are glucans, galactomannans, and galactoglucomannans (Gorin, Baron, da Silva, Teixeira, & Iacomini, 1993; Woranovicz, Pinto, Gorin, & Iacomini, 1999), and recently reported complex heteroglycans, such as a rhamnogalactofuranan (Olafsdottir, Omarsdottir, Smestad Paulsen, Jurcic, & Wagner, 1999) and galactomannoglucans (Carbonero, Montai, Woranovicz-Barreira, Gorin, & Iacomini, 2002; Woranovicz-Barreira, Gorin, Sassaki, Marcelli, & Iacomini, 1999).

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 and Iacomini, 1985). These generally include monosubstituents at O-2 with units of α-D-Man*p* or α-D-Gal*p*, at O-4 by β-Gal*p* and sometimes with disubstituents at O-2 and O-4 by units of α-D-Gal*p* and β-D-Gal*p*, or α-D-Man*p* and β-D-Gal*p*, respectively, although in several cases, some of the main-chain units are not substituted. A recent study on *Lasallia pustulata* demonstrated the presence of a galactomannan with complex side-chains of variable structures containing of α-(1→2)-linked Man*p* and β-Gal*f* units (Pereyra, Prieto, Bernabé, & Leal, 2003). Galactomannans have been isolated from several lichens, such as *Cladonia* spp., *Collema leptosporum*, and *Ramalina usnea* among others (Gorin et al., 1993; Olafsdottir & Ingólfsdottir, 2001). Since the structures of lichen polysaccharides are often typical of the parent genus and family level, they are also an important as an aid in chemotaxonomic studies. These have been aided particularly by ^{13}C-NMR studies, which provide fingerprints typical of each lichen species (Gorin et al., 1993; Teixeira, Iacomini, & Gorin, 1995).

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We now examine the polysaccharides of *Umbilicaria mammulata* and compare them to those from other members of the Umbilicariaceae family.

2. Experimental

2.1. Collection of Umbilicaria mammulata

The source of the material of *Umbilicaria mammulata* collected and analyzed: USA WI, Lincoln County, Rock Falls, Grandfather Dam, 2002, coll. Teuvo Ahti no. 61176; voucher specimens preserved in Botanical Museum, Helsinki (H) and Herbarium of the Federal University of Paraná (UFPR, no. 49053).

2.2. Extraction and fractionation of lichen polysaccharides

The thalli of *U. mammulata* (100 g) were cleaned, dried, and powdered, and then processed according to the summary in Fig. 1. The sample was successively extracted with H_2O (600 ml) at 100 °C for 3 h (×3), the residue isolated, treated with 2% aq. KOH (600 ml) at 100 °C for 3 h (×3), and the extract neutralized with HOAc. Each extract was added to excess EtOH (3:1; v/v) to form a precipitate. After dialysis, a freeze-thawing process was carried out on each supernatant and repeated until precipitates no longer appeared.

The precipitated PW formed following an aq. extraction (19.4% yield) was isolated by centrifugation (4680*g* for 20 min, at 25 °C) and was ready for further analysis. The solution prepared from precipitated PK (7.9% yield) and the supernatant PS formed following KOH extraction (5.7% yield) were each treated with Fehling solution (100 ml) (Jones & Stoodley, 1965) and the resulting insoluble Cu^{2+} complex formed was isolated by centrifugation (4680*g* for 20 min, at 25 °C). The respective complexes (FP-SK and FP-PK) and supernatants (FS-SK and FS-PK), were each neutralized with HOAc, dialyzed against tap water and deionized with Dowex 50×8 (H^+ form) ion-exchange resin.

The FS-SK fraction was further purified by ultrafiltration through a M_r 10 kDa cut-off membranes (Millipore), furnishing retained (TrFS) and eluted (TeFS) fractions. The latter was then submitted to ultrafiltration with a M_r 300 kDa cut-off membrane, giving rise to retained (TZrFS) and eluted (TZeFS) fractions.

2.3. Preparation of polysaccharide (PM) containing O-acetyl groups

In order of check the presence of acetyl groups, the lichen sample (7.0 g) was treated with Me_2SO (150 ml) at

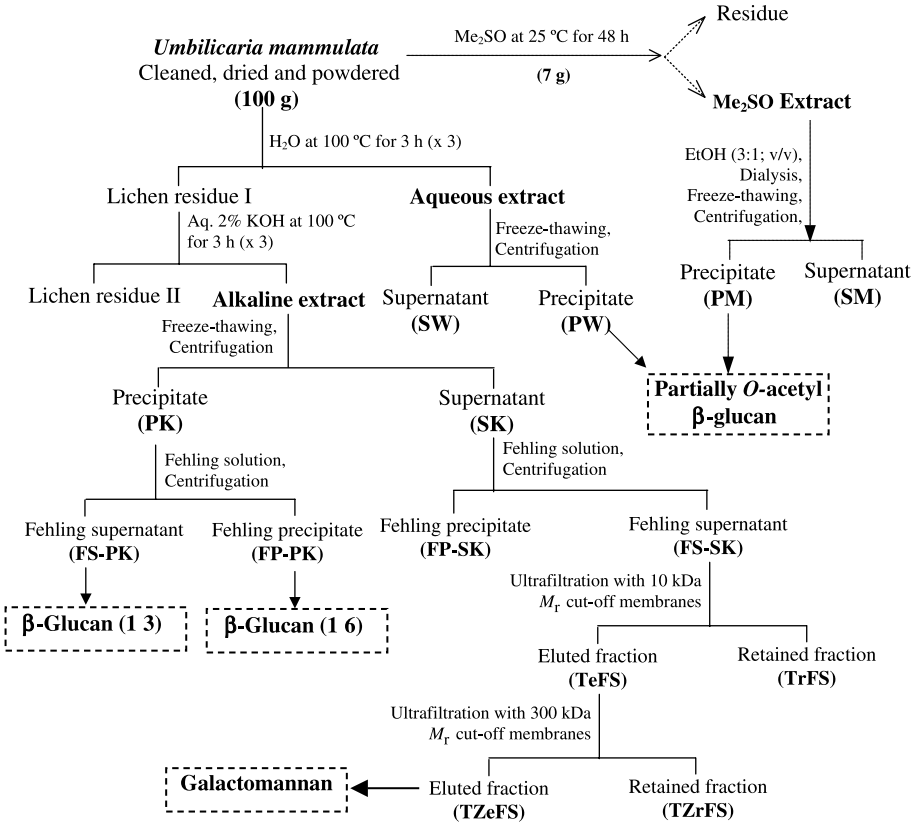

Fig. 1. Extraction, fractionation, and isolation of polysaccharides from *U. mammulata*.

room temperature for 48 h. The extract obtained was precipitated with excess EtOH (3:1; v/v) and the resulting precipitate was centrifuged (as above), isolated, dialyzed against distilled water, and freeze-dried. The extract was then resuspended in water, frozen, and then thawed gently, resulting in the formation of a precipitate (PM; 19.8% yield) and soluble material.

2.4. Analysis of monosaccharides present in polysaccharides

Monosaccharide components and their ratios were determined by hydrolysis of polysaccharides (~1 mg) with 2 M TFA at 100 °C for 8 h. The hydrolyzates were evaporated to dryness and the residues submitted to successive reduction with $NaBH_4$ and acetylation with Ac_2O-pyridine (1:1, v/v; 2 ml) (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 MAT, model ITD 800 mass spectrometer, using a DB-225 capillary column (30 m × 0.25 mm i.d.), with helium as carrier gas. The column was programmed from 50 °C (1 min) at 40 °C/min to 230 °C (const. temp.). The products were identified by their typical retention times and electron impact profiles (Jansson, Kenne, Liedgren, Lindberg, & Lönngren, 1976).

2.5. Methylation analysis of polysaccharides

Fractions TZeFS and PHTZeFS (5 mg) were *O*-methylated according to Ciucanu and Kerek (1984), using powdered NaOH in Me_2SO-MeI The per-*O*-methylated derivatives were treated with 50% v/v sulfuric acid (1 h, 0 °C) and the resulting solution diluted with H_2O to 5.5% v/v and completely hydrolyzed at 100 °C for 16 h. (Saeman, Moore, Mitchell, & Millet, 1954). The solution was neutralized ($BaCO_3$), filtered and the filtrate, following evaporation to a small volume, was treated with $NaBD_4$ to convert the partially *O*-methylated aldoses to alditol derivatives. The mixtures were analyzed by GC-MS, the DB-225 column (as above) being programmed from 50 °C (1 min) at 40 °C/min to 220 °C (const. temp.). The resulting partially *O*-methylated alditol acetates identified by their typical electron impact breakdown profiles and retention times (Carpita & Shea, 1987; Jansson et al., 1976).

2.6. Partial acid hydrolysis of galactofuranosylmannan

Fraction TZeFS (300 mg) was partially hydrolyzed with a solution adjusted to pH 2.0 (20 ml) with aq. trifluoroacetic acid, at 100 °C, for 18 h. After neutralization with NaOH, a polymeric product (PHTZeFS) was obtained by precipitation with excess EtOH (3:1; v/v) from a small volume of water, and then retained on dialysis with a M_r 2 kDa cut-off membrane (86 mg).

2.7. Determination of homogeneity and molar mass of water-soluble polysaccharides

A Waters size exclusion chromatography (SEC) apparatus coupled to a differential refractometer (RI) was used for examination of soluble fractions (FS-SK and TzeFS). Four Waters Ultrahydrogel 2000/500/250/120 were connected in series and coupled with a multidetection equipment. Samples, previously filtered through a membrane (0.22 μm; Millipore), were injected (250 μl loop) at a 2 mg/ml concentration. The eluent was 0.1 mol/l aq. $NaNO_3$ with 200 ppm aq. NaN_3.

The specific refractive index increment was determined by using a Waters 2410 refractive index detector. The value of d*n*/d*c* (differential refractive index increment of the solvent-solute solution with respect to a change in solute concentration), measured at 633 nm, was 0.133.

2.8. Nuclear magnetic resonance spectroscopy

^{13}C NMR spectra were obtained using a 400 MHz Bruker model DRX Avance or 500 MHz Bruker Avance™ 500 spectrometer. The ^{13}C NMR (100.6 MHz) analyses were performed at 50 or 30 °C, with the samples being dissolved in D_2O or Me_2SO-d_6, depending on solubility characteristics. Chemical shifts of the samples, dissolved in D_2O, are expressed in ppm (δ) relative to acetone at δ 30.20 for ^{13}C and at δ 39.70 (^{13}C) for those dissolved in Me_2SO-d_6.

3. Results and discussion

The lichenized fungus *Umbilicaria mammullata* was submitted to hot aqueous and aq. 2% KOH extractions, followed by purification of component polysaccharides (Fig. 1). As one of the component glucans contained a small proportion of *O*-acetyl groups, the lichen was extracted with Me_2SO at 25 °C to avoid acetyl migration or removal. (Reicher, Corrêa, & Gorin, 1984).

3.1. Isolation and characterization of glucan components

The lichen was extracted with Me_2SO and the extract was treated with ethanol to give a precipitate. This was subjected to freeze-thawing process, the water-insoluble fraction PM was obtained in a high yields (19.6% yield) containing 97% of glucose and consisted of a lightly *O*-acetylated (1→6)-linked glucan (pustulan) with a typical ^{13}C NMR spectrum (Fig. 2), as recorded by Iacomini, Gorin, and Baron (1988). It contained six major signals of the glucan at δ 103.1 (C-1), 76.4 (C-3), 75.4 (C-5), 73.3 (C-2), 69.9 (C-4), and 68.4 (*O*-substituted C-6). Minor signals were at δ 20.8 and 175.0 from $-CH_3$ and $-C{\equiv}O$ of *O*-acetyl groups respectively, while those at δ 77.7 and 71.2 were from *O*-acetylated carbons.

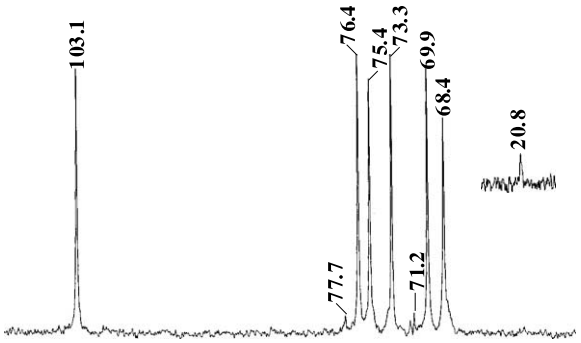

Fig. 2. ^{13}C NMR spectrum of pustulan (PM fraction) in Me_2SO-d_6 at 50 °C: chemical shifts are in δ PPM.

The alkaline extract of the lichen, following neutralization, was submitted to several freeze-thawing processes, followed by neutralization, until no more precipitate was found. The water-insoluble fraction (PK, 7.9% yield) was further purified via precipitation with Fehling solution. Both the precipitate (FP-PK, 7.1% yield) and supernatant (FS-PK, 0.5% yield) provided water-insoluble glucans. Fraction FP-PK had the same pustulan structure, except that the *O*-acetyl groups were removed by saponification. Fraction FS-PK was found to be a (1→3)-linked β-glucan (laminaran), since its ^{13}C NMR spectrum (Fig. 3) contained six typical signals at δ 102.8 (C-1), 86.0 (*O*-substituted C-3), 76.2 (C-5), 72.7 (C-2), 68.3 (C-4), and 60.7 (C-6) (Carbonero et al., 2001).

Glucans have been found to date in all species of lichenized fungi. Partially *O*-acetylated pustulans are typical of Umbilicariaceae, and have been described for several *Umbilicaria* spp., such as *U. pustulata* and *U. hirsuta* (Drake, 1943), *U. angulata*, *U. caroliniana*, and *U. polyphylla* (Nishikawa, Tanaka, Shibata, & Fukuoka, 1970). They have also been found in *Lasallia pustulata* (Pereyra et al., 2003) and *U.* (*Actinogyra*) *muehlenbergii* (Iacomini et al., 1988). Structurally distinct laminarans are extensively distributed throughout lichenized fungi and are present in several families (Olafsdottir & Ingólfsdottir, 2001), and also in free-living fungi (Gorin & Barreto-Bergter, 1983), and algae (Painter, 1983).

Of interest is whether the *O*-acetyl groups are introduced during biosynthesis on to several hydroxyl groups of the pustulans, or specifically on to one hydroxyl group and then undergoing migration to other groups during the growth of the lichen? It is significant that the *U. mammulata* pustulan contains acetates only at C-3 and C-4, but not C-2 (Fig. 2), whereas that of *U. muehlenbergii* has acetates at C-2, C-3 and C-4 (Iacomini et al., 1988). The migration mechanism thus appears unlikely.

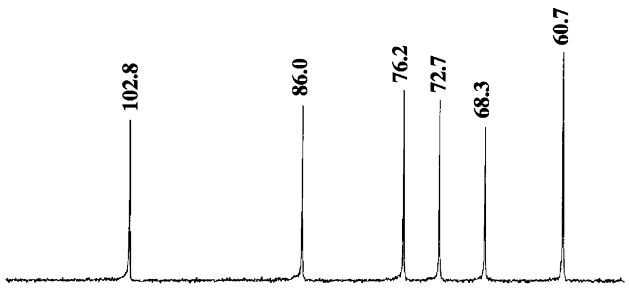

Fig. 3. ^{13}C NMR spectrum of laminaran (FS-PK fraction) in Me_2SO-d_6 at 50 °C: chemical shifts are in δ PPM.

3.2. Isolation and characterization of galactofuranomannan component

As can be seen from Fig. 1, the mother liquor of the freeze-thawing process following alkaline extraction (SK) was further fractionated. It was treated with Fehling solution to give a precipitate (FP-SK), which was discarded and material regenerated from the mother liquor FS-SK (4.6% yield) was subjected to membrane ultrafiltration. The polysaccharide (TeFS), which passed through a 10 kDa cut-off membrane, was fractionated with a 300 kDa cut-off membrane to give an eluted fraction (TZeFS) (3.1%). It gave a homogeneous profile when analyzed by HPSEC with *Mw* 48×10^3 and d*n*/d*c* = 0.133 (Fig. 40. It contained Man, Gal, and Glc in a 65:33:2 molar ratio, showing a galactomannan structure (Fig. 4).

Methylation analysis of TZeFS (Table 1) showed a highly branched structure based on derived partially *O*-methylated alditol acetates (GC-MS) with a high proportion of nonreducing end-units of Man*p* (15%) and Gal*f* (25%). All the galactosyl units were in a furanosyl form, and also included 2-*O*- (2%) and 5-*O*- (1%), 6-*O*- (2%), and 5,6-di-*O*-substituted units (1%). Other α-Man*p* units were non-substituted (3%), 2- (6%) and 6-*O*- (4%), 2,3- (4%), 4,6- (8%), 2,6- (13%) and 3,6-di-*O*- (3.5%), and 3,4,6- (1%), 2,4,6- (14%), and 2,3,6-tri-*O*-substituted (3%).

The ^{13}C NMR spectrum of the galactofuranomannan (Fig. 5A) corresponded to a highly complex polysaccharide with many C-1 signals. Low field resonances from β-Gal*f* units were present from δ 108.8, the main signal, to δ 105.2. To agree with the methylation data, the signal at δ 108.8 probably arises from non-reducing-end units, while

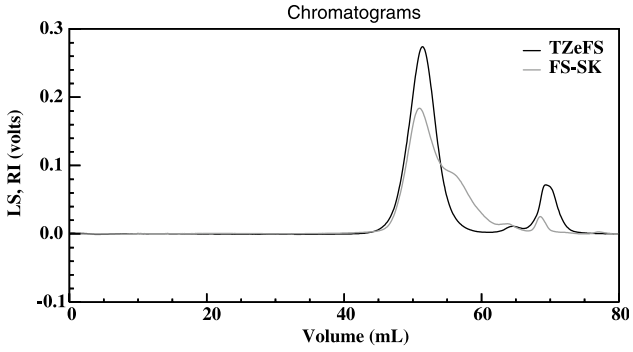

Fig. 4. Elution profiles of fractions FS-SK and TZeFS, as determined by HSPEC with refractive index detector.

Table 1
Partially *O*-methylated alditol acetates formed on methylation-GC-MS analysis of heteropolyssaccharide (TZeFS) and its partial hydrolysis product (PHTZeFS)

O-Me-alditol acetate[a]	Fractions (molar %[b])		Linkage type[c]
	TZeFS	PHTZeFS	
2,3,4,6-Me_4Man	15	16	Man*p*-(1→
2,3,5,6-Me_4Gal	25	3	Gal*f*-(1→
3,4,6-Me_3Man	6	39	→2)-Man*p*-(1→
3,5,6-Me_3Gal	2	–	→2)-Gal*f*-(1→
2,3,6-Me_3Man	–	2	→4)-Man*p*-(1→
2,3,6-Me_3Gal	1	–	→5)-Gal*f*-(1→
2,3,4-Me_3Man	4	23	→6)-Man*p*-(1→
2,3,6-Me_3Glc	–	1	→4)-Glc*p*-(1→
2,3,5-Me_3Gal	2	–	→6)-Gal*f*-(1→
4,6-Me_2Man	4	1	→2,3)-Man*p*-(1→
2,3-Me_2Man	8	1	→4,6)-Man*p*-(1→
3,4-Me_2Man	13	14	→2,6)-Man*p*-(1→
2,4-Me_2Man	1		→3,6)-Man*p*-(1→
2,3-Me_2Gal	1	–	→5,6)-Gal*f*-(1→
2-MeMan	1	–	→3,4,6)-Man*p*-(1→
3-MeMan	14	–	→2,4,6)-Man*p*-(1→
4-MeMan	3	–	→2,3,6)-Man*p*-(1→

[a] *O*-Me-alditol acetates obtained by methylation analysis, followed by successive hydrolysis, reduction with $NaBD_4$ and acetylation, analyzed by GC-MS (column DB-225).
[b] % of peak area relative to total peak area.
[c] Based on derived *O*-methylalditol acetates.

the others are *O*-substituted. (Gorin, 1981; Leal, Prieto, Gómez-Miranda, Jiménez-Barbero, & Bernabé, 1993; Nagaoka et al., 1996). The other C-1 signals are of α-Man*p* units and probably correspond the non-reducing-end groups linked (1→2)- to another α-Man*p* unit (δ 103.3) which gives rise to a signal at δ 101.6). The signal at δ 100.1 should be from 6-*O*-substituted α-Man*p* units and that at δ 99.2 from 2,6-*O*-substituted units (Gorin, 1981). These occur at slightly lower field since the temperature of the D_2O is 30 °C is lower that that of the reference, which was 70 °C. It was also possible to observe signals from C-2, C-5 and C-6 of the nonreducing end-units of Gal*f* in δ 83.9, 82.0 and 63.8, respectively (Gorin & Mazurek, 1975).

In order to elucidate to obtain the core of the galactofuranomannan, a partial acid hydrolysis was carried out to remove the majority of the Gal*f* units. The product PHTZeFS, retained on dialysis, contained Man (94%), Gal (3%) and Glc (3%). Methylation-GC-MS analysis indicated (Table 1) that the Man*p* units were present as nonreducing end- (16%), 2- (39%), 4- (2%), and 6-*O*- (23%), 2,3- (1%), 4,6- (1%), and 2,6-di-*O*-substituted (14%). Non-hydrolyzed Gal*f* nonreducing end-units (3%) were detected as were 4-*O*-substituted Glc*p* units (1%). ^{13}C-NMR examination (Fig. 5B) agreed with the removal of β-Gal*f* units, as their low-field C-1 signals were barely detectable. The C-1 region of PHTZeFS can be interpreted in terms of a principal structure with a (1→6)-linked α-Man*p* main-chain partially substituted at O-2 with (1→2)-linked α-Man*p* side-chains. The key signals (Fig. 5B), as in the parent galactofuranomannan, are δ 103.2 (α-Man*p*-(1→2)-α-Man*p*), 101.6 (α-Man*p*-(1→2)-α-Man*p*-), 100.5 (non-subsituted (1→6)-linked α-Man*p*) and 99.2 (2,6-di-*O*-substituted α-Man*p*). Such structures are common in yeast mannans (Gorin & Barreto-Bergter, 1983). C-2 signals arising from 3 types of 2-*O*-substituted α-Man*p* units are present at 79.1, 79.6, and 79.8.

The results of our investigation show the presence of two β-glucans, pustulan and laminaran, and a galactofuranomannan with a (1→6)-linked α-Man*p* main-chain mainly substituted at O-2, O-4, and O-2,4 with β-Gal*f*- and α-Man*p*-containing side chains. The content of pustulan was higher when compared to those of the other polysaccharides and it apparently represents a significant chemotaxonomic marker, as it is a typical polymer from Umbilicariaceae. It is noteworthy that in recent phylogenetic DNA analyses (e.g. Peršoh, Beck, & Rambold, 2004) the Umbilicariaceae have an uncertain, isolated position when compared to most of the other lichens, which are now often classified as Lecanoromycetes within Ascomycota. The above structure of the galactofuranomannan with a high content of Gal*f* units and a varying length of its α-Man*p* side chains, seems to be typical of Umbilicariaceae, having been found in *U. pustulata*, *U. spodochroa* (Kjølberg & Kvernheim, 1989), and *Lasallia*

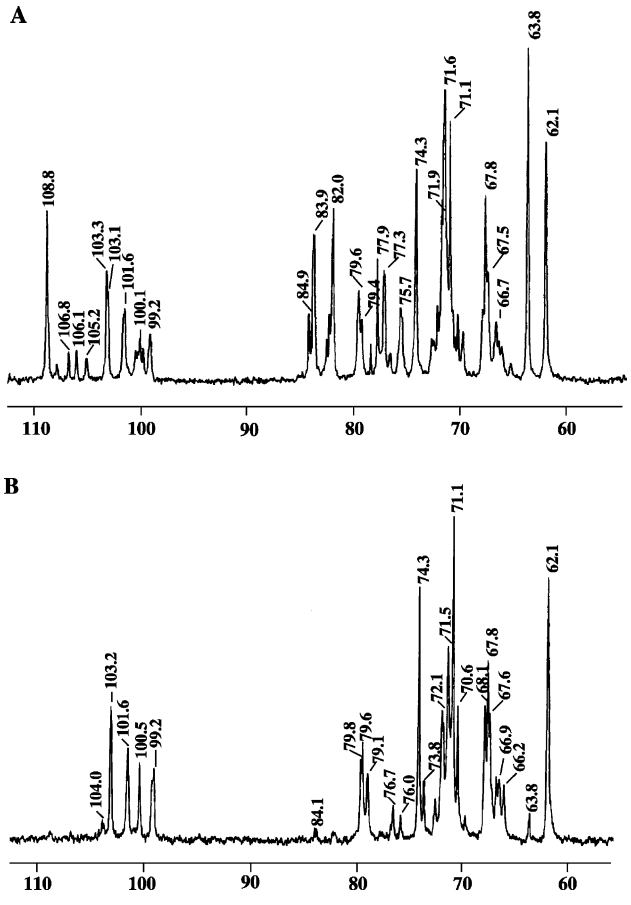

Fig. 5. ^{13}C NMR spectra of fractions TZeFS (A; galactomannan) and PHTZeFS (B; acid degraded galactomannan) with D_2O at 30 °C as solvent: chemical shifts are in δ PPM.

pustulata (Pereyra et al., 2003). Such heteropolysaccharides from lichenized fungi are being extensively investigated in our laboratory due to their chemotaxonomic significance and possible biological activity.

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