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Unusual partially 3-*O*-methylated α-galactan from mushrooms of the genus *Pleurotus*

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Abstract

Indistinguishable partially 3-*O*-methylated galactans were isolated from the edible basidiomycetes *Pleurotus eryngii* and *Pleurotus ostreatoroseus*. They were obtained via successive aqueous extraction, freeze–thawing, precipitation with Fehling solution of soluble material, and ultrafiltration. Mono- and bidimensional ^{13}C and ^{1}H-nuclear magnetic resonance spectroscopy (HMBC, HETEROTOCSY, COSY, and HMQC), and methylation analysis were used to determine their structures. They were linear, partially 3-*O*-methylated, (1 → 6)-linked α-D-galactans containing Gal and 3-Me-Gal, in a 3:1 molar ratio (GC–MS of alditol acetates).

Keywords: Edible mushrooms; *Pleurotus eryngii*; *Pleurotus ostreatoroseus*; *O*-Methylated galactan; Chemical structure

1. Introduction

Mushrooms have been used in the orient for many years to prepare therapeutic teas (Smith et al., 2002). Recently, they have also become attractive as functional foods and a source for development of new drugs. As well as their medicinal and/or nutritional properties, they are especially appreciated for their texture and flavour (Manzi and Pizzoferrato, 2000; Leung et al., 1997; Manzi et al., 2004; Mallavadhani et al., 2006; Smiderle et al., 2006).

Pleurotus is an important genus of edible basidiomycetes, especially those occurring in the subtropics and tropics. The biotechnological potential of this genus has been exploited to enhance the digestibility of animal fodder, to be a good source of molecules such as polysaccharides that can act as antitumor, antifungal, and antiviral agents, as well as for their ability to lower cholesterol levels, to synthesize fine chemicals, and as bioremedies (Croan, 2004; Kashangura et al., 2006). The production of *Pleurotus* spp. has been increasing worldwide at a rapid rate, especially because of their ready cultivation incorporating adaptability, aggressiveness, and productivity, besides their nutritional value (Gunde-Cimerman, 1999; Silva et al., 2002).

Polysaccharides have previously been isolated from the fruit bodies of *Pleurotus* spp. Studies carried out on this genus have described in terms of the chemical structure of their homo- and heteropolymers. Glucans are the most common homopolymers in these organisms. A branched β-glucan, with a main chain of (1 → 3)-linked β-Glc*p*, substituted at O-6 by single β-Glc*p* units was isolated from fruiting bodies of *Pleurotus ostreatus* (Karácsonyi and Kuniak, 1994). A similar structure was found in the fruiting bodies of *Pleurotus eryngii* and *Pleurotus ostreatoroseus* (Carbonero et al., 2006). Rout et al. (2005) reported the presence of a glucan consisting of (1 → 3)-, (1 → 6)-linked Glc*p* units with both α- and β-configurations, in *Pleurotus florida*. Further studies have been carried out on heteropolymers, such as a partially *O*-methylated mannogalactan

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present in *P. ostreatus* "florida", *P. ostreatoroseus* (Rosado et al., 2003), and *P. ostreatus* (Jakovlević et al., 1998). A non-methylated heteropolymer was isolated from *Pleurotus sajor-caju*, being a polysaccharide consisting of D-glucose, D-galactose, and D-mannose (1:1:1) (Pramanik et al., 2005). We now describe structural features of an unusual linear, partially 3-*O*-methylated (1 → 6)-linked α-galactan isolated from the fruiting bodies of *P. eryngii* and *P. ostreatoroseus*.

2. Results and discussion

In order to remove low-molecular weight compounds, *P. eryngii* and *P. ostreatoroseus* fruiting bodies (64 and 66 g, respectively) were extracted with hot $CHCl_3$–MeOH and then MeOH–H_2O. Each residue was submitted to aqueous extractions at 100 °C, and the component polysaccharides were obtained by ethanol precipitation, followed by dialysis against tap water (fractions EPW-PE and EPW-PO for *P. eryngii* and *P. ostreatoroseus*, respectively). The solutions were freeze-dried to give EPW-PE (7.8% yield) and EPW-PO (7.7% yield) (Fig. 1).

EPW-PE and EPW-PO contained glucose as their main component, besides mannose, galactose and 3-*O*-methylgalactose, according to GC–MS of derived alditol acetates (Table 1). The position of the *O*-methylgroup was confirmed by the presence of the ions at *m*/*z* 130 and 190, after reduction (NaB^2H_4) and acetylation.

Fractionation and purification of EPW-PE and EPW-PO was carried out by a freeze-thawing procedure, resulting in a cold-water insoluble (IEPW-PE, 2.5% yield; IEPW-PO, 2.7% yield) and soluble fractions (SEPW-PE, 5.3% yield; SEPW-PO, 5.0% yield). The insoluble fractions consisted of a β-glucan with (1 → 3)-linked main chains, partially substituted at O-6, as previously described (Carbonero et al., 2006).

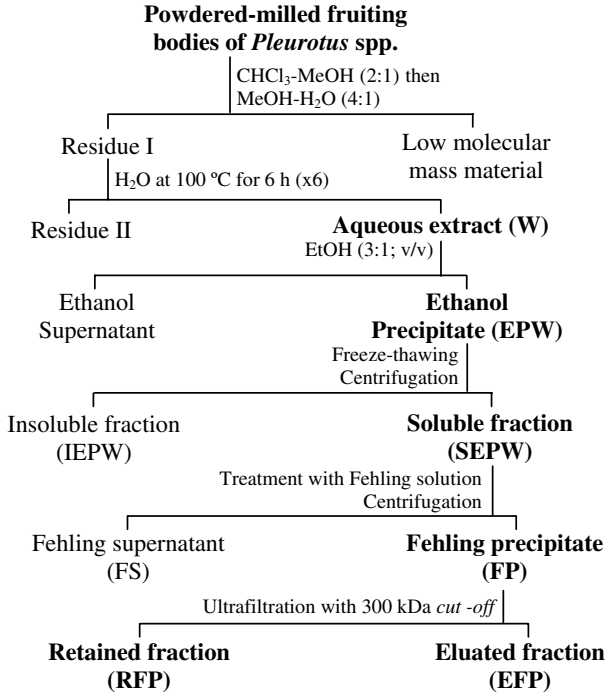

Fig. 1. Scheme of extraction and purification of the galactan from the *P. eryngii* (PE) and *P. ostreatoroseus* (PO).

Table 1
Monosaccharide composition and yields of polysaccharide fractions obtained from *P. eryngii* and *P. ostreatoroseus*

Fractions	Yields (%)[a]		Monosaccharides (%)[b]			
			Man	3-*O*-Me-Gal	Gal	Glc
P. eryngii	EPW-PE	7.8	2	3	5	90
	SEPW-PE	5.3	12	13	28	47
	IEPW-PE	2.5	Tr.	Tr.	0.5	99
	FP-PE	3.3	11	18	69	2
	RFP-PE	2.1	–	25	75	–
P. ostreatoroseus	EPW-PO	7.7	4	Tr.	4	91
	SEPW-PO	5.0	23	7	23	47
	IEPW-PO	2.7	Tr.	Tr.	Tr.	99
	FP-PO	2.2	31	8	55	6
	RFP-PO	0.8	–	25	75	–

Tr. ⩽ 0.5%
[a] Yields based on dry fungi.
[b] Alditol acetates obtained on successive hydrolysis, $NaBH_4$ and/or NaB^2H_4 reduction, and acetylation, analyzed by GC–MS.

The cold-water soluble fractions were then treated with Fehling solution, giving rise to supernatants (FS-PE and FS-PO, 2.0% and 2.8% overall yields, respectively) and precipitates (FP-PE and FP-PO, 3.3% and 2.2% overall yields, respectively). The precipitates (FP-PE and FP-PO) contained mannose, galactose, glucose, and 3-*O*-methylgalactose (Table 1). These gave heterogeneous elution profiles by HPSEC-MALLS, so they were purified by ultrafiltration (300 kDa M_r cut-off membrane), giving rise to retained (RFP-PE and RFP-PO) and eluted (EFP-PE and EFP-PO) fractions. The RFP fractions were homogeneous on HPSEC, and had M_w 17.9×10^4 g/mol ($dn/dc = 0.168$) for *P. eryngii*, and M_w 16.5×10^4 g/mol ($dn/dc = 0.168$) for *P. ostreatoroseus*. The monosaccharide composition of this fraction was similar for both species, being 3-*O*-Me-Gal and Gal in a 1:3 molar ratio (Table 1).

As the ^{13}C NMR spectra of the galactans from the two species of the *Pleurotus* were indistinguishable (Fig. 2a and b), further analyses were carried out only on that of *P. eryngii*. Signals corresponding to all carbons from the polysaccharide (RFP) were assigned using 2D NMR spectra (^1H (obs.) ^{13}C HMQC, COSY, HETEROTOCSY and, HMBC) (Table 2).

The ^{13}C NMR spectra of the galactans had signals of C-1 at δ 100.6 corresponding to predominant Gal*p* units, while that at δ 100.5 was from 3-*O* Me-Gal*p* residues. The signals at δ 71.0, 72.3, 72.2, and 71.6 arose from C-2, C-3, C-4, and C-5, respectively, of Gal*p* units, while those at δ 70.0, 81.6, 68.1, and 71.6 were from similar carbons of 3-*O*-Me-Gal*p* residues. An HMQC signal at δ 59.0/3.43 corresponded to $O\underline{CH_3}$. The linkage of this polymer was shown by the presence of an *O*-substituted $-\underline{C}H_2$-6 sig-

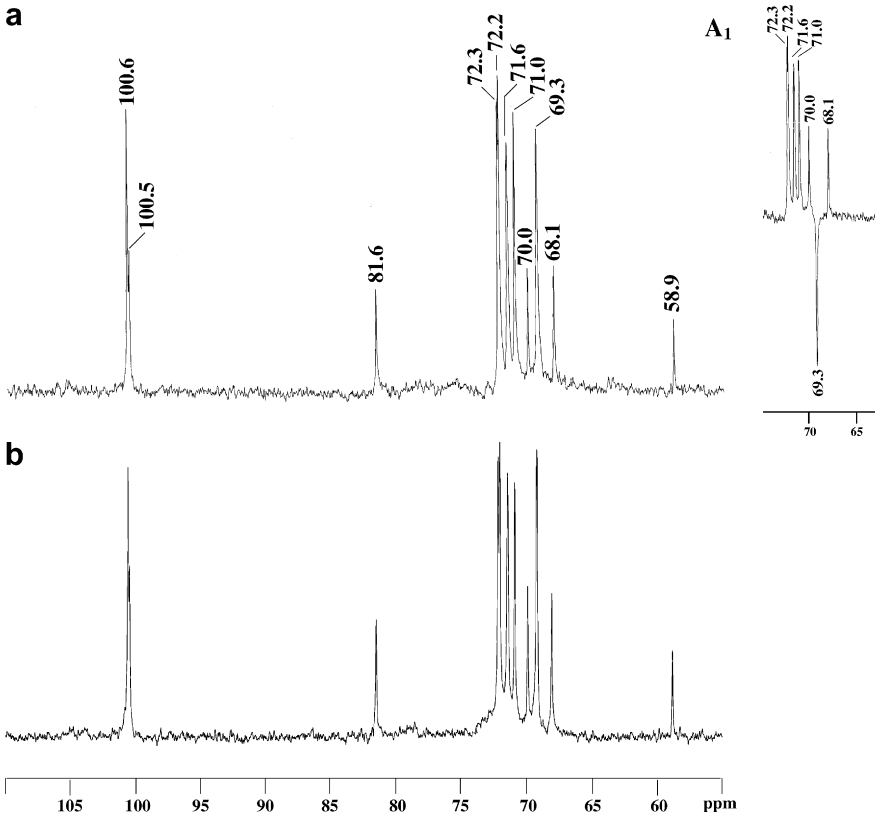

Fig. 2. ^{13}C NMR spectra, obtained at 70 °C, of galactan from *P. eryngii* (a) and *P. ostreatoroseus* (b) in D_2O. Inset of DEPT (A1).

Table 2
^{1}H and ^{13}C NMR data [δ (ppm)][a] of the galactan from *P. eryngii*

Unit		1	2	3	4	5	6		*O*-Me
							6a	**6b**	
α-Gal*p*	^{13}C	100.6	71.0	72.3	72.2	71.6	69.3	69.3	
	^{1}H	4.98	3.84	3.87	4.04	4.21	3.92	3.69	
3-*O*-Me-α-Gal*p*	^{13}C	100.5	70.0	81.6	68.1	71.6	69.3	69.3	59.0
	^{1}H	4.98	3.88	3.55	4.3	4.21	3.92	3.69	3.43

[a] Assignments were based on ^{13}C, DEPT, COSY, and HMQC analysis.

nal from Gal*p* and 3-*O*-Me-Gal*p* residues at δ 69.3 (in the form of an HMQC doublet at δ 3.69; 3.92/69.3, Fig. 3). This was confirmed by an inverted DEPT signal (Fig. 2A$_1$). HMBC correlations were obtained from H-1 (δ 4.98) to C-6 (δ 69.3) for both units. The α-configuration was shown by high-frequency signals H-1 (δ 4.98 for both units) and low-frequency C-1 signals (δ 100.6 and 100.5) (Fig. 3), confirmed by the coupling constant $J_{C\text{-}1,H\text{-}1} = 173$ Hz observed in a coupled HMQC spectrum (Perlin and Casu, 1969) and by the high specific rotation $[\alpha]_D^{20} + 83$ (H_2O; *c* 1.0).

COSY (Fig. 4) and HETEROTOCSY analysis (Fig. 5) was also helpful to elucidate the structure of RFP, since the coupling of all protons of the each unit made possible assignments of their respective carbons using HMQC (Fig. 3).

In order to confirm of the linkage type of this polymer, RFP fractions were submitted to methylation analysis, which showed only the alditol acetates of 2,3,4-Me_3Gal, and traces of 2,3,4,6-Me_4Gal (GC–MS), consistent with a linear 3-*O*-methylated and non-methylated (1 → 6)-linked Gal*p* units.

A Smith degradation incorporating mild hydrolytic conditions on RFP-PE was carried out to determine the sequence of 3-*O*-Me-Gal*p* and Gal*p* units. ESI-MS (+ve mode) (not shown) of the product gave rise to molecular ions (Na^+ adduct) at *m/z* 291, 463, and 643 corresponding to 3-Me-α-D-Gal*p*-(1 → 1)-L-glycerol, 3-Me-α-D-Gal*p*-(1 → 6)-3-Me-α-D-Gal*p*-(1 → 1)-L-glycerol, and 3-Me-α-D-Gal*p*-[(1 → 6)-3-Me-α-D-Gal*p*]$_2$(1 → 1)-L-glycerol, respectively. These data show that the Gal*p* and 3-*O*-methyl Gal*p* units are arranged irregularly.

The above results show that the polysaccharides consisted of linear partially 3-*O*-methylated (1 → 6)-linked α-galactopyranans. Vinogradov and Wasser (2005) have also shown very similar structure formed in a submerged

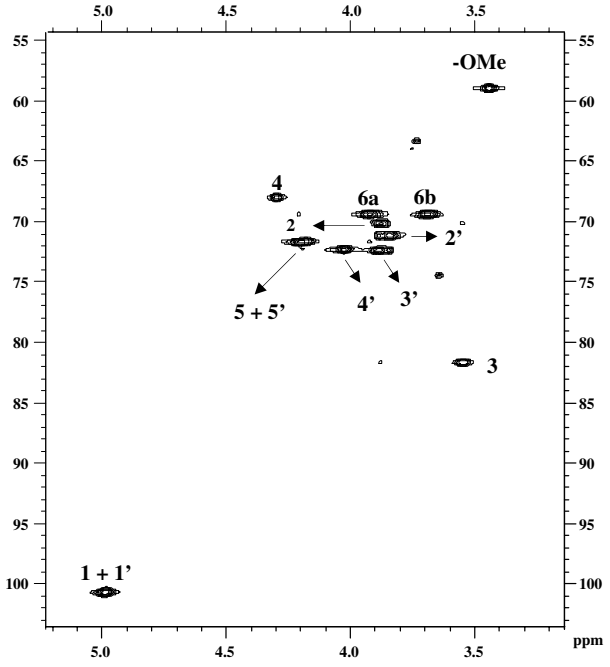

Fig. 3. ^{1}H (obs.), ^{13}C HMQC spectrum of galactan from *P. eryngii* in D_2O at 40 °C.

culture of the mushroom *Inonotus levis*, and in which *O*-methyl substitution occurred in about half of the galactosyl units. Non-reducing end units of glucuronic acid were also present. Rosado et al. (2003) also characterized a partially *O*-methylated galactan from extracellular polysaccharide produced by *P. ostreatoroseus*, although it had (1 → 4)-linkages. The partially 3-*O*-methylated (1 → 6)-linked α-galactan structure from *P. eryngii* and *P. ostreatoroseus* has not been previously described.

3. Experimental

3.1. General experimental procedure

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 600 MHz Bruker Avance spectrometer incorporating Fourier transform. Analyses were performed at 40 °C or 70 °C on samples dissolved in D_2O. 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. 1D (^{1}H, ^{13}C, and DEPT) and 2D NMR spectra [^{1}H (obs.) ^{13}C HMQC,

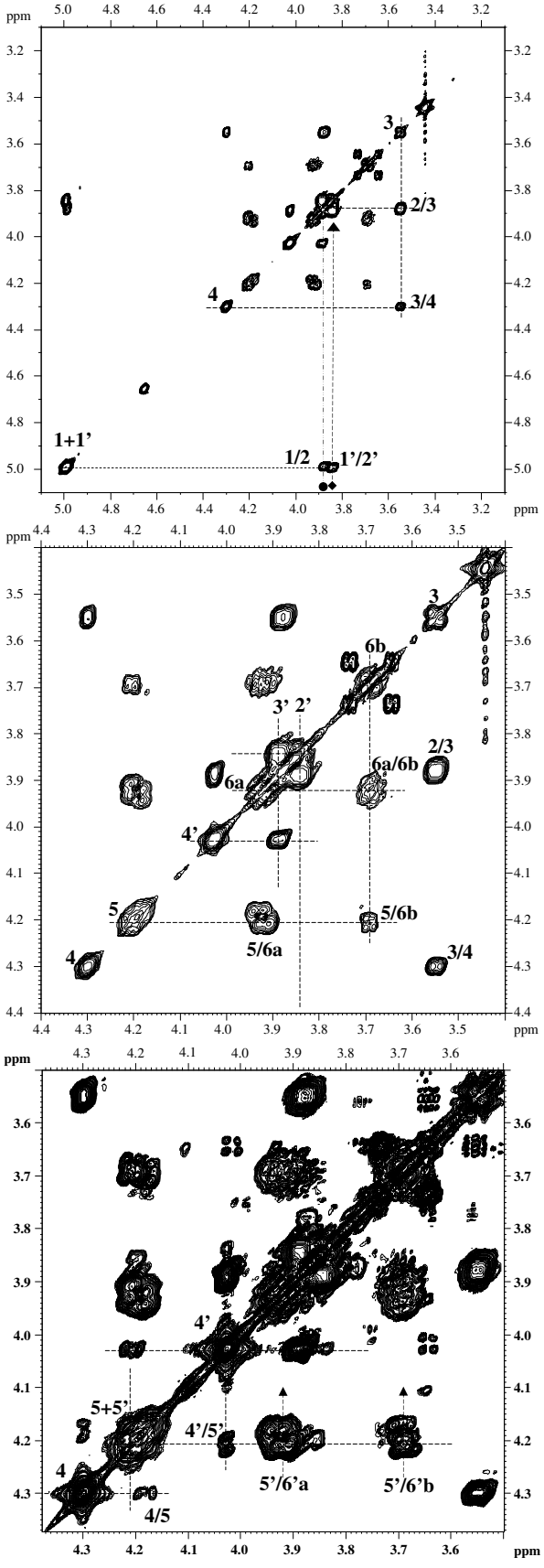

Fig. 4. COSY spectrum of galactan from *P. eryngii* in D_2O at 40 °C.

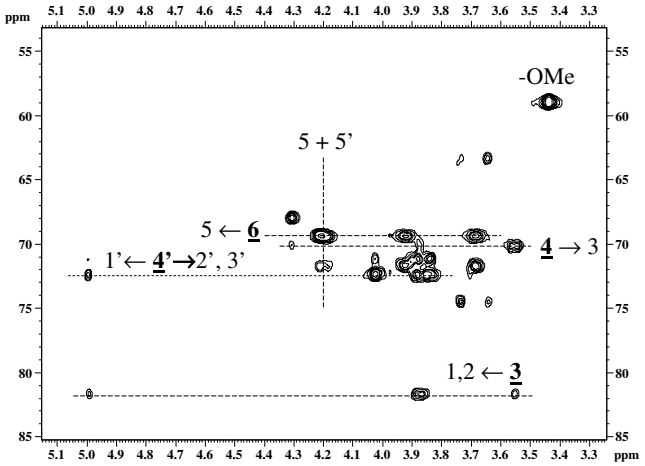

Fig. 5. HETEROTOCSY spectrum of galactan from *P. eryngii* in D_2O at 40 °C.

COSY, HMBC, coupled HMQC, and HETEROTOCSY] were obtained by following the Bruker manual.

The specific rotation of RFP-PE was determined at 20 °C, using a 10 cm cell and sodium D line (589.3 nm) on a Rudolph Autopol III automatic polarimeter.

ESI-MS analysis was carried out using Quattro Ultima equipment in the positive-ion mode on a sample from RFP-PE previously dissolved in MilliQ H_2O. It was applied using a manual loop injector (10 μL volume) on to a flow rate of 20 μL/min.

3.2. Extraction and purification of polysaccharides

Extraction and purification of the polysaccharides from the fruiting bodies of the two species of *Pleurotus* spp. was carried out according to Fig. 1. Powdered-milled bodies (*P. eryngii*, 64 g; *P. ostreatoroseus*, 66 g) were extracted with 2:1 (v/v) $CHCl_3$-MeOH at 60 °C for 3 h (3×, 350 mL each) and then with 4:1 (v/v) MeOH-H_2O at 60 °C for 3 h (×3, 350 mL each), to remove low-molecular-weight material. The residues were each submitted to extraction with H_2O at 100 °C for 6 h (6×, 800 mL). The combined aq. extracts were evaporated to a small volume with the polysaccharide precipitated by addition to excess EtOH (3:1; v/v); the resulting polysaccharide precipitates were dissolved in H_2O, and dialyzed against tap water, giving rise to fractions EPW-PE and EPW-PO, respectively. These fractions were frozen and then allowed to thaw slowly and resulting insoluble material (IEPW-PE and IEPW-PO fractions) were removed by centrifugation at 9000 rpm for 15 min, at 25 °C. The supernatants (SEPW-PE and SEPW-PO fractions) were treated with Fehling solution (Jones and Stoodley, 1965) and precipitated Cu^{++} complexes (FP-PE and FP-PO) were removed by centrifugation at 9000 rpm for 15 min, at 25 °C. The supernatants (FP-PE and FP-PO) were neutralized with HOAc, dialyzed against tap water, deionized with mixed ion exchange resins, and then freeze-dried.

Fraction FP was further purified by ultrafiltration through a membrane of 300 kDa M_r cut-off (Millipore-regenerated cellulose), giving rise to eluted (EFP) and retained (RFP) material.

3.3. Monosaccharide composition

Monosaccharide components of the polysaccharides were identified and their ratios were determined following hydrolysis with 2 M TFA for 8 h at 100 °C, and conversion to alditol acetates (GC–MS) by successive $NaBH_4$ and/or NaB^2H_4 reduction, and acetylation with Ac_2O-pyridine (1:1, v/v) for 12 h at room temperature (Wolfrom and Thompson, 1963a,b).

3.4. Determination of homogeneity of polysaccharides and their molecular weight

The homogeneity and molar mass (M_w) of the purified fractions (RFP-PE and RFP-PO) 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 polysaccharides solutions were filtered through a membrane, with pores of 0.22 μm diameter (Millipore). The specific refractive index increment (d*n*/d*c*) was determined by Waters 2410 detector.

3.5. Methylation analysis of polysaccharides

Per-*O*-methylation of the polysaccharides (10 mg each) was carried out using NaOH–Me_2SO–MeI (Ciucanu and Kerek, 1984). The per-*O*-methylated derivatives were hydrolyzed with 5c. H_2SO_4–H_2O (1:1, v/v) (1 h, 0 °C), followed by dilution to 5.5% v/v (10 h, 100 °C), neutralization ($BaCO_3$), and filtration (Saeman et al., 1954). The resulting mixture of *O*-methylaldoses was reduced with NaB^2H_4 and acetylated as above (item 3.3) to give a mixture of partially *O*-methylated alditol acetates, which was analyzed by GC–MS.

3.6. Controlled Smith degradation

RFP-PE (10 mg) was submitted to oxidation with 0.05 M aq. $NaIO_4$ (10 mL) for 72 h at 25 °C in the dark. Samples was then dialyzed against tap water for 48 h and treated with $NaBH_4$ (pH 9–10) for ~20 h (Goldstein et al., 2005). The solutions were dialyzed and freeze-dried, and the product was successively partially hydrolyzed (TFA pH 2.0, 30 min, 100 °C) (Gorin et al., 1965), evaporated to dryness, and the residue analyzed by ESI-MS.

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