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A novel branched αβ-glucan isolated from the basidiocarps of the edible mushroom *Pleurotus florida*

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Abstract

As polysaccharides of the mushroom genus *Pleurotus* can act as biological response modifiers, we now describe the chemical characterization of a new water-soluble αβ-glucan from the fruiting bodies of the edible mushroom *Pleurotus florida.* It was obtained via successive aq. extraction, freeze-thawing, precipitation with Fehling solution, and dialysis through a membrane with M_r 1000 kDa cut-off. It was analyzed using NMR spectroscopy, HPSEC, monosaccharide composition, methylation analysis, and a controlled Smith degradation. It was a branched glucan (M_w 1.1×10^6 g/mol), with a main-chain of (1→3)-linked-α-D-Glc*p* residues, partially substituted at *O*-6 by single-unit β-D-Glc*p,* and a minor proportion by side-chains with 3-*O*-substituted β-D-Glc*p* groups.

Keywords: Polysaccharides; Edible mushroom; *Pleurotus florida*; αβ-glucan

1. Introduction

Edible mushrooms are excellent foods that can be incorporated into well-balanced diets due to their low content of fat and energy, and high content of dietary fiber and functional compounds (Breene, 1990; Manzi, Aguzzi, & Pizzoferrato, 2001). Their benefit to health include immunomodulatory, anti-tumoral, and hypocholesterolemic effects (Lavi, Friesem, Geresh, Hadar, & Schwartz, 2006). In numerous molecules synthesized by macrofungi are known to be bioactive, such as polysaccharides, glycoproteins, terpenoids, lectins, among others (Moradali, Mostafavi, Ghods, & Hedjaroude, 2007).

Pleurotus is an important genus of edible basidiomycetes which are commonly called oyster mushroom: all known species are edible, with several being commercially cultivated (Ragunathan, Gurusamy, Palaniswamy, & Swaminathan, 1996). The popularity of this genus is on the increase, especially because of its flavor and texture (Kim et al., 2007). The species are also rich in minerals, are good sources of protein, and have short life cycle (Yildiz, Yildiz, Gezer, & Temiz, 2002). Among oyster mushrooms, the best known *Pleurotus* spp. are *Pleurotus citrinopileatus*, *Pleurotus ostreatus*, *Pleurotus ostreatoroseus*, *Pleurotus pulmonarius*, *Pleurotus eryngii*, and *Pleurotus florida* (=*P. ostreatus* var. *florida*) (Kües & Liu, 2000).

A great number of polysaccharides have been isolated from basidiomycetes and their chemical structures determined. These are homo- and heteropolymers, the most common of the former being glucans; most with the β-configuration. Glucans containing α- and β-configurations are few found in basidiomycetes. A polysaccharide composed of -(1→4)-α-D-Glc*p*- and -(1→6)-β-D-Glc*p*-units occurs in *Astraeus hygrometricus* (Chakraborty, Modal, Pramanik, Rout, & Islam, 2004). Glucans with both α- and β-configurations were isolated from *P. florida* and *P. sajor-caju* (Rout, Mondal, Chakraborty, Pramanik, & Islam, 2005; Pramanik, Chakraborty, Modal, & Islam, 2007). We describe the structural features of an unusual water-soluble

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glucan with both α- and β-configurations present in an aqueous extract of basidiocarps of *P. florida*.

2. Materials and methods

2.1. Organism

Fresh basidiocarps of *P. florida* (2 kg) were furnished by Makoto Yamashita Company (Miriam Harumi Yamashita), located in São José dos Pinhais, State of Paraná, Brazil. The basidiomycete was grown on wheat straw supplemented with wheat–corn powder at a temperature of, ~20 °C.

2.2. Extraction and purification of glucan

The fresh basidiocarps of *P. florida* (2 kg) were desiccated in a freeze dryer giving a product (196 g) which was extracted successively with water at 4 °C (3000 mL, for 6 h; 6×) and 100 °C (3000 mL, for 6 h; 6×). Each extract was filtered, and the filtrate was collected, and centrifuged at 9000 rpm at 25 °C for 15 min to give a clear supernatant. The aq. extracts were evaporated to small volume, and added to excess EtOH (3:1; v/v), and the resulting polysaccharidic precipitate were obtained by centrifugation at 9000 rpm for 15 min at 25 °C, which were then dissolved in H_2O, dialyzed against distilled H_2O to remove low-molecular-weight material, and freeze-dried, giving rise to fractions CW and HW, respectively (Fig. 1).

CW and HW fractions were dissolved in H_2O and the solutions were frozen and then allowed to thaw slowly. Cold water-insoluble materials (ICW and IHW, respectively) were centrifuged-off (9000 rpm for 15 min at 25 °C). The supernatants (SCW and SHW, respectively) were treated with Fehling solution (Jones & Stoodley, 1965) and precipitated polysaccharides (FP–CW and FP–HW, respectively) were fractionated using their soluble Cu^{2+} complexes (FS–CW and FS–HW, respectively) by centrifugation. Aq. solutions of FSCW and FSHW were neutralized with HOAc, dialyzed against tap water, deionized with mixed ion exchange resins and then freeze-dried (Fig. 1).

FS–CW and FS–HW were further fractionated by dialysis through a membrane of 1000 kDa M_r cut-off [Spectra-Por® Biotech Polyvinilidene Difluoride (PVDF)], giving rise to eluted (MEFS–CW and MEFS–HW, respectively) and retained (MRFS–CW and MRFS–HW, respectively) materials (Fig. 1).

2.3. Determination of homogeneity of polysaccharide and their molar mass

The homogeneity and molar mass (M_w) of the fractions 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 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 using a Waters 2410 detector.

2.4. Monosaccharide composition of polysaccharide fractions

Total hydrolysis of the fractions (1 mg) was carried out with 2 M TFA at 100 °C for 8 h, followed by evaporation to dryness. The residue was successively reduced with excess $NaBH_4$ and acetylated with Ac_2O-pyridine

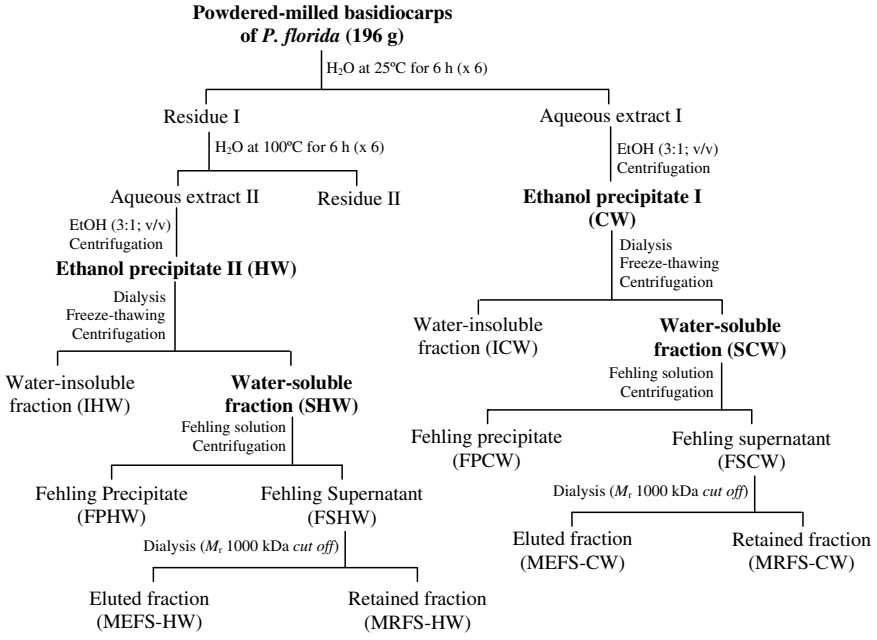

Fig. 1. Scheme of extraction and purification of the polysaccharides from the fruit bodies of *P. florida*.

(1:1, v/v; 2 mL) at room temperature for 12 h (Wolfrom & Thompson, 1963a; Wolfrom & Thompson, 1963b). The resulting alditol acetates were analyzed by gas liquid chromatography–mass spectrometry (GC–MS) with 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, programmed from 50 to 220 °C at 40 °C min^{-1}, then hold, the total analysis time being 26 min.

2.5. Methylation analysis of polysaccharide fractions

Per-*O*-methylation of fractions (10 mg) was carried out using NaOH—Me_2SO—MeI (Ciucanu & Kerek, 1984). This process, after isolation of the products by neutralization, dialysis and evaporation was repeated. The per-*O*-methylated derivatives (2 mg) were hydrolyzed with 45% v/v formic acid (1 mL) at 100 °C for 15 h, followed by evaporation to dryness and the product converted to partially *O*-methylated alditol acetates (see Section 2.4). The mixtures were analyzed by GC–MS, using a capillary column of DB-225 (30 m × 0.25 mm i.d.), programmed from 50 to 210 °C (40 °C min^{-1}), then hold, the total analysis time being 36 min.

2.6. NMR analyses of polysaccharide fractions

NMR spectra (^1H, ^{13}C and DEPT) were obtained using a 400 MHz Bruker model DRX Advance spectrometer incorporating Fourier transform. Analyses were performed at 70 °C on sample dissolved in D_2O or Me_2SO-d_6. Chemical shifts are expressed in δ relative of acetone at δ 30.2 (^{13}C) and 2.22 (^1H) for samples soluble in D_2O, and relative of Me_2SO-d_6 at δ 39.70 (^{13}C) and 2.40 (^1H) for samples insoluble in D_2O.

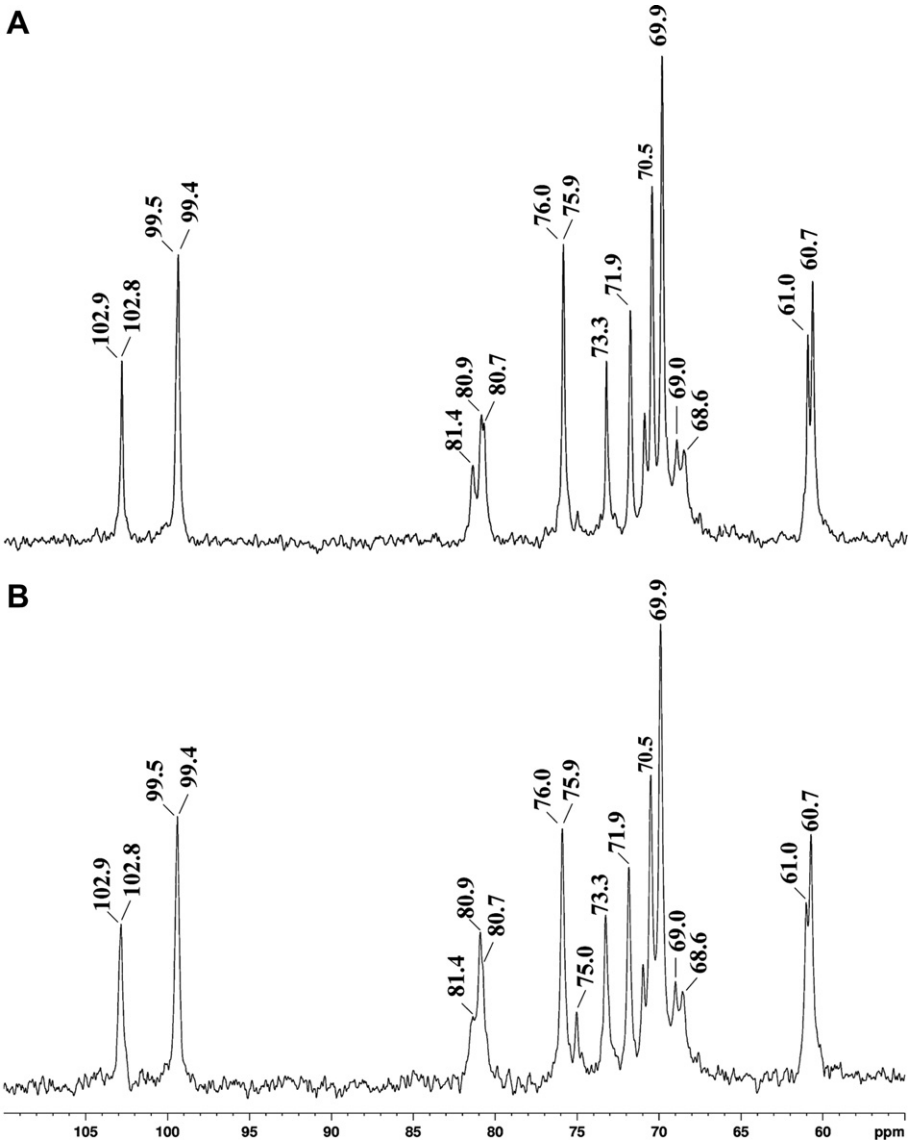

Fig. 2. ^{13}C NMR spectra of the MRFS–CW (A) and MRFS–HW fractions (B), in D_2O at 70 °C (chemical shifts are expressed in ppm).

Table 1
Partially *O*-methylalditol acetates formed on methylation analysis of a αβ-glucan and those obtained on three successive controlled Smith degradation (RSm1, RSm2, and RSm3)

Partially *O*-methylated alditol acetates	R_t[b]	% Area of fragments				Linkage type[a]
		MRFS–HW	RSm1	RSm2	RSm3	
2,3,4,6-Me_4-Glc	9.38	22.0	7.0	5.0	1.0	Glc*p*-(1→
2,4,6-Me_3-Glc	12.23	55.0	86.5	92.0	99.0	3→)-Glc*p*-(1→
2,4-Me_2-Glc	19.05	23.0	6.5	3.0	–	3,6→)-Glc*p*-(1→

[a] Based on derived *O*-methylalditol acetates.
[b] Retention time (min).

2.7. Controlled Smith degradations of native glucan

The native glucan (MRFS–HW, 100 mg) was submitted to oxidation with 0.05 M aq. $NaIO_4$ (20 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, Hay, Lewis, & Smith, 1965). The solution was dialyzed and freeze-dried, and the product was successively partially hydrolyzed (TFA pH 2.0, 30 min, 100 °C) (Gorin, Horitsu, & Spencer, 1965), and dialyzed against tap water using membranes with a size exclusion of 2 kDa, giving rise to retained (RSm1, 41 mg) and eluted material (ESm1). The residual product (RSm1) was submitted to more two cycles of oxidation, resulting in resistant fractions RSm2 (33 mg), RSm3 (22 mg) and eluted material ESm2, ESm3, respectively, which were obtained after dialyses.

3. Results and discussion

The basidiocarps (fruiting bodies) of the "white oyster" mushroom, *P. florida,* after desiccation in a freeze dryer gave 10% of the original weight which was then submitted to successive extractions with water at 4 °C and 100 °C, and the extracted polysaccharides were recovered as ethanol precipitates, giving the fractions CW (9.0 g) and HW (2.9 g), respectively. Fractionation of the aq. extracts by freezing/thawing furnished cold water-soluble (SCW, 4.1 g; SHW, 2.0 g, respectively) and insoluble polysaccharides, which were separated by centrifugation. The water-soluble fractions were then treated with Fehling solution, giving rise to soluble (FSCW, 3.6 g; FSHW, 1.2 g, respectively) and insoluble Cu^{+2} complexes, previously reported to be a partially 3-*O*-methylated mannogalactan (Rosado et al., 2003).

FSCW and FSHW both contained Glc (~97%) as their main monosaccharide, and traces of mannose and galactose, and gave rise to heterogeneous elution profiles on HPSEC. They were then purified by dialysis (1000 kDa M_r cut-off membrane). The retained respective fractions (MRFS–CW, 57 mg; MRFS–HW, 398 mg) were homogeneous by HPSEC, and had M_w 1.1×10^6 g/mol ($d_n/d_c = 0.157$ mL/g) giving only glucose on hydrolysis (GC–MS). Comparison of their ^{13}C NMR spectra (Fig. 2A and B), showed that they contained the same structural components, and as MRFS–HW was obtained in a greater yield, it was chosen for further investigation.

MRFS–HW was submitted to methylation analysis and GC–MS of resulting *O*-methylalditol acetates showed a branched structure with non-reducing end, 3-*O*-, and 3,6-di-*O*-substituted units of glucopyranose, in a molar ratio of ~1:2:1 (Table 1).

^{13}C NMR spectra of MRFS–HW (Figs. 2A, B and 3) showed C-1 signals of non-reducing end (δ 102.8) and 3-*O*-substituted β-D-Glc*p* units (δ 102.9), as well as those C-1 signals corresponding to 3-*O*- (δ 99.4) and 3,6-di-*O*-substituted α-D-Glc*p* units (δ 99.5) (Gorin, 1981). The

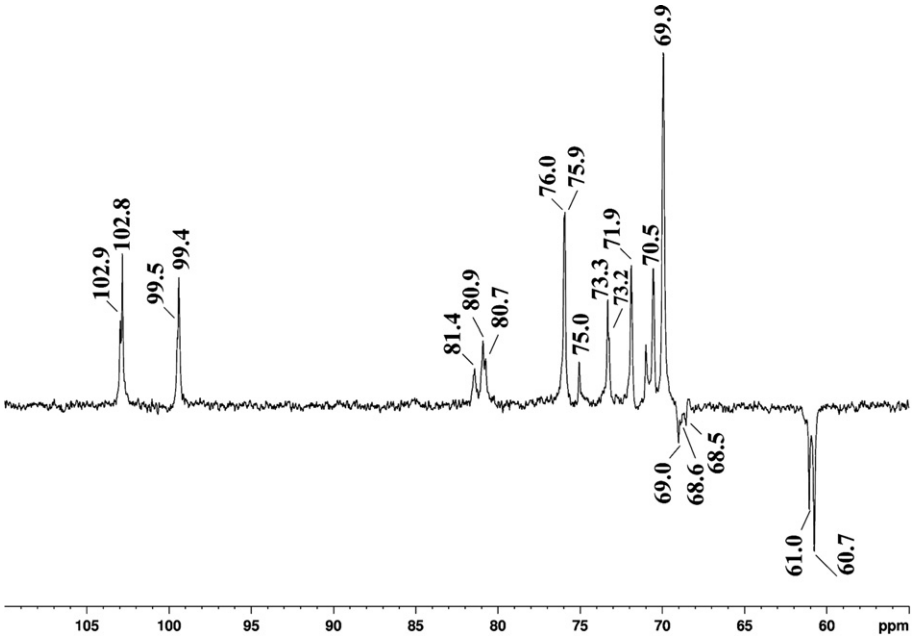

Fig. 3. DEPT spectrum of the water-soluble glucan (MRFS–HW fraction), in D_2O at 70 °C (chemical shifts are expressed in ppm).

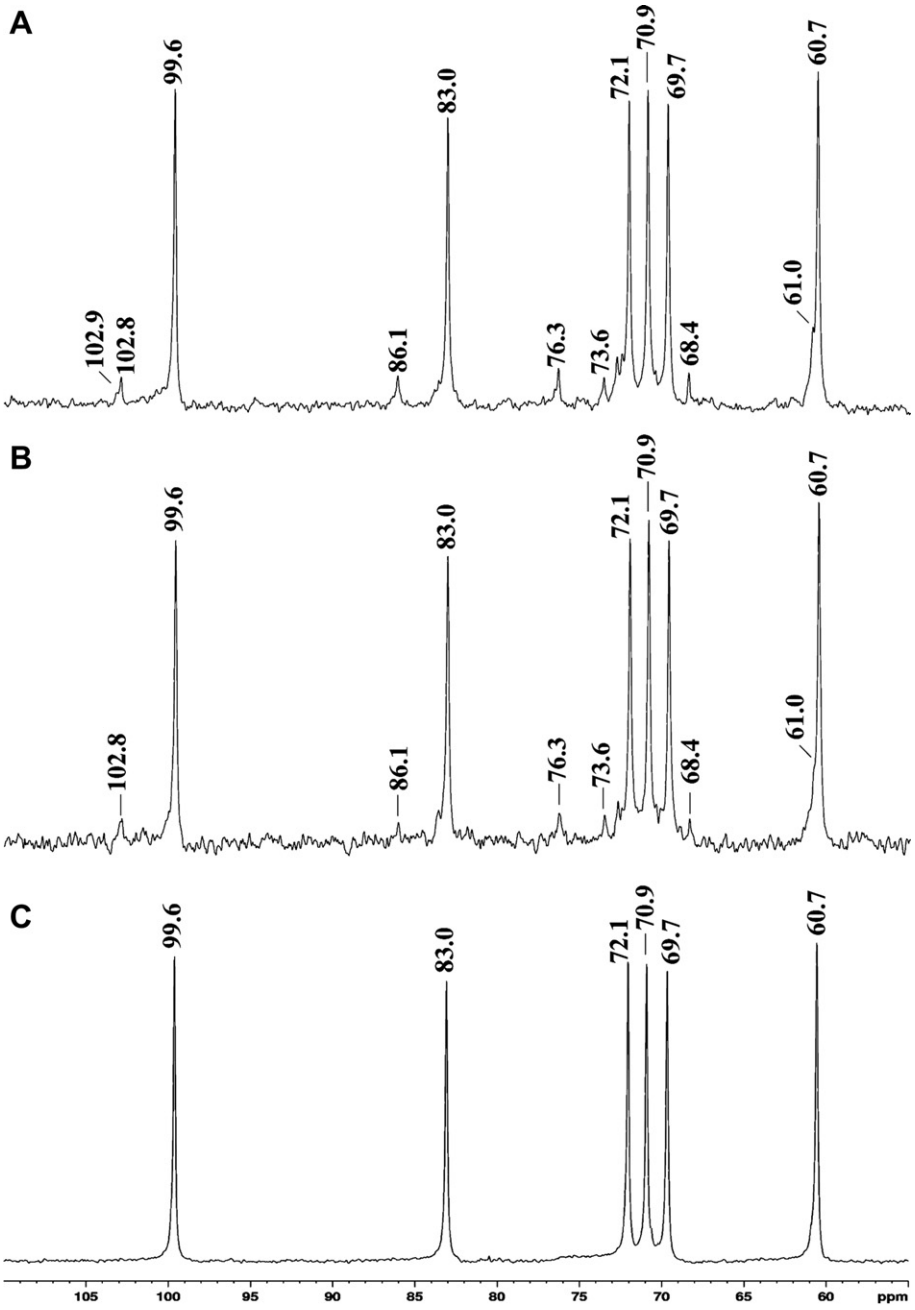

Fig. 4. ^{13}C NMR spectra of the resistance products obtained by three successive controlled Smith degradation [RSm1 (A), RSm2 (B), and RSm3 (C), respectively], in Me_2SO-d_6 at 70 °C (chemical shifts are expressed in ppm).

α-configuration was shown by low frequency resonances of C-1 at δ 99.5 and δ 99.5 and H-1 high field ones at δ 5.43. β-Configurations were shown by δ 102.9 and δ 102.8 (C-1) and δ 4.59 (H-1).

The glycosidic linkages of the glucan were shown by the presence of 3-*O*-substituted signals at δ 81.4, 80.9 and 80.7, and *O*-substituted-CH_2-6 signals at δ 69.0, 68.6, and 68.5 (Fig. 2A and B), confirmed from the inverted DEPT signals (Fig. 3).

The main-chain structure of the glucan was determined via three successive controlled Smith degradations, giving rise to polysaccharides RSm1, RSm2 and RSm3, respectively. The ^{13}C NMR spectrum of the RSm1 (Fig. 4A) was considerably simplified, with almost complete removal of β-D-Glc*p* side-chain units (C-1 at δ 102.9 and 102.8; C-2, 73.6; substituted C-3, 86.1; C-4, 68.4; C-5, 76.3; C-6, 60.9).

The spectrum of RSm2 (Fig. 4B) showed a continuation of side-chain removal, which was complete in RSm3 (Fig. 4C). This was a linear (1→3)-linked α-glucan, due to presence of six signals at δ 99.6, 70.9, 83.0, 69.7, 72.1 and 60.7, arising, respectively, from C-1 to C-6 in ^{13}C NMR spectrum (Fig. 4C) (Gorin, 1981).

These data agree with methylation analysis (Table 1), which showed a marked decrease of non-reducing and

3,6-di-*O*-substituted Glc*p* units, with concomitant increase of 3-*O*-substituted Glc*p* units.

The eluted materials (ESm1, ESm2 and ESm3) obtained by Smith degradation incorporating mild hydrolytic conditions were analyzed by GC–MS as alditol acetates and showed only glycerol, suggesting that each cycle of periodate oxidation removed the most external (1→3)-linked β-D-Glc*p* units. Thus, the maximum length of the (1→3)-linked β-D-Glc*p* side-chains units is three, although they consisted mainly as single-units, since after the first step of Smith degradation the most of them was removed.

The glucan is thus a branched glucan (M_w 1.1×10^6 g/mol), with a main-chain of (1→3)-linked α-D-Glc*p* residues, partially substituted at *O*-6 mostly by single-units of β-D-glucopyranose and a minor proportion by 3-*O*-substituted β-D-glucopyranosyl groups of three units. To own knowledge, basidiomycetes glucans with this structure have not been previously described.

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