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A β-glucan from the fruit bodies of edible mushrooms *Pleurotus eryngii* and *Pleurotus ostreatoroseus*

Elaine R. Carbonero, Ana Helena P. Gracher, Fhernanda R. Smiderle, Fábio R. Rosado, Guilherme L. Sassaki, Philip A.J. Gorin, Marcello Iacomini *

Departamento de Bioquímica e Biologia Molecular, Universidade Federal do Paraná, CP 19046, CEP 81531-990, Curitiba, PR, Brazil

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Abstract

The glucans of basidiomycetes are an important class of polysaccharides with potential biological activities. In this work, the β-glucans were isolated from the fruiting bodies of edible mushrooms, *Pleurotus eryngii* and *Pleurotus ostreatoroseus*, via extraction with hot water, and then fractionation by freeze-thawing. The insoluble glucans gave similar ^{13}C NMR spectra, monosaccharide composition and methylation analyses, and *P. eryngii* was selected for further controlled Smith degradation, and DEPT and ^{1}H (obs.), ^{13}C HMQC spectroscopy. It was a branched β-glucan, with a main chain of (1 → 3)-linked-Glc*p* residues, substituted at O-6 by single-unit β-Glc*p* side-chains, on average to every third residue of the backbone, as in scleroglucan.

Keywords: Polysaccharides; Edible mushrooms; *Pleurotus* spp.; β-Glucans

1. Introduction

Mushrooms are known for their nutritional and medicinal value and the diversity of their bioactive components (Ng, 1998). These organisms have long been valued as highly tasty and nutritional foods by many societies throughout the world. Many worldwide cultures, especially in the Orient, recognize that extracts from certain mushrooms can have profound health promoting benefits. Edible mushrooms, which demonstrate medicinal or functional properties, include species of the genera *Lentinus*, *Hericium*, *Grifola*, *Flammulina*, *Pleurotus*, and *Tremella* (Kües & Liu, 2000).

Pleurotus spp. occurs throughout the hardwood forests of the world that include the most diverse climates (Gunde-Cimerman, 1999; Rosado, Carbonero, Kemmelmeier, Tischer, & Iacomini, 2002). The production of *Pleurotus* has been increasing at a rapid rate. These mushrooms have attracted much attention owing to them being a good source of non-starchy carbohydrates, with a high content of dietary fiber, moderate quantities of proteins with most of the essential amino acids, minerals, and vitamins (Croan, 2004). They have been shown to modulate the immune system, have hypoglycemic activity and to inhibit tumor growth (Gunde-Cimerman, 1999; Wasser, 2002).

Polysaccharides represent a structurally diverse class of macromolecules of widespread occurrence in nature and offer the highest capacity for carrying biological information because they have the greatest potential for structural variability. The monosaccharide units in oligosaccharides and polysaccharides can interconnect at several points to form a wide variety of branched or linear structures (Ooi & Liu, 2000). This enormous potential variability gives the necessary flexibility for precise regulatory mechanisms of various interactions in higher organisms.

Recent advances in chemical technology have allowed the isolation and purification of some compounds, especially polysaccharides which posses strong immunomodulation and anti-cancer activities. They are used as biological response modifiers (Rout, Mondal, Chakraborty, Pramanik,

* Corresponding author. Tel.: +55 41 33611655; fax: +55 41 32662042.
E-mail address: iacomini@ufpr.br (M. Iacomini).

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& Islam, 2005). The polysaccharides isolated from mushroom fruiting bodies are either water soluble or/and insoluble glucans and heteropolysaccharides with different main- and side-chains. There is great interest on these molecules because they can act as biological response modifiers (Gonzaga, Ricardo, Heatly, & Soares, 2005; Lavi, Friesem, Geresh, Hadar, & Schwartz, 2006; Smith, Rowan, & Sullivan, 2002).

We now describe the isolation and chemical characterization of a β-glucan from the fruiting bodies of *Pleurotus eryngii* and *Pleurotus ostreatoroseus*.

2. Materials and methods

2.1. General experimental procedures

All solutions were evaporated at <40 °C under reduced pressure. Centrifugation was carried out at 9000 rpm for 15 min, at 25 °C. Alditol acetate mixtures formed from polysaccharides were analyzed by GC–MS using a Varian model 3300 gas chromatograph linked to a Finnigan Ion-Trap, model 810-R12 mass spectrometer, using a DB-23 capillary column (30 m × 0.25 mm i.d.) programmed from 50 to 220 °C at 40 °C/min, then hold. Partially O-methylated alditol acetate mixtures were similarly analyzed, but with a program from 50 to 215 °C at 40 °C/min, then hold.

2.2. Polysaccharide extraction and purification

Extraction and purification of the β-glucans from the fruiting bodies of the two species of *Pleurotus* were processed according to Fig. 1. Powdered-milled fruiting 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 residue was submitted to extraction with water at 100 °C for 6 h (6×, 800 mL each). The combined aq. extracts were evaporated to a small volume and polysaccharide precipitated by addition to excess EtOH (3:1). The precipitates from *P. eryngii* (EPW-PE) and *P. ostreatoroseus* (EPW-PO) were dialyzed against tap water for 48 h, concentrated under reduced pressure to small volumes, which were freeze-dried. EPW-PE and EPW-PO were then dissolved in water and the solutions submitted to freezing followed by mild thawing at 4 °C, which furnished soluble (SEPW-PE and SEPW-PO) and insoluble gel-like fractions (IEPW-PE and IEPW-PO), which were separated by centrifugation.

2.3. Monosaccharide composition

Each fraction (1 mg) was hydrolyzed with 2 M TFA at 100 °C for 8 h, followed by evaporation to dryness. The residue was successively reduced with excess of $NaBH_4$ and/or NaB^2H_4 and acetylated with Ac_2O–pyridine (1:1, v/v; 2 mL) at room temperature for 12 h (Wolfrom & Thompson, 1963a, 1963b). The resulting alditol acetates were analyzed by GC–MS as indicated above and identified by their typical retention times and electron impact profiles.

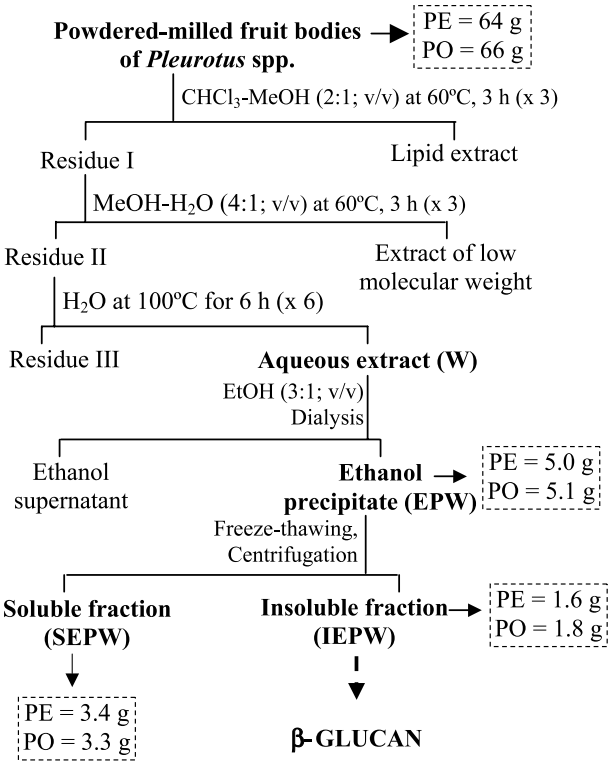

Fig. 1. Scheme of extraction and purification of the β-glucan from the *Pleurotus eryngii* (PE) and *P. ostreatoroseus* (PO).

2.4. Methylation analysis

Per-O-methylation of the isolated polysaccharides (10 mg each) was carried out using 40% aq. NaOH (3 mL) and Me_2SO_4 (2 mL), added dropwise (Haworth, 1915). The process, after isolation of the products by neutralization, dialysis, and evaporation was repeated, and the methylation was found to be complete. The products were treated with 50% v/v aq. H_2SO_4 (0.5 mL v/v, 1 h, 0 °C), followed by a dilution until it reached 5.5% (addition of 4.0 mL of distilled water). The solution was kept at 100 °C for 18 h (Saeman, Moore, Mitchell, & Millet, 1954), and was neutralized with $BaCO_3$, filtered, and the filtrate evaporated to dryness. The residues were converted into partially O-methylated alditol acetates, and analyzed by GC–MS (as described above).

2.5. NMR analyses

^{13}C DEPT and ^{1}H(obs.), ^{13}C HMQC determinations were carried out using a 400 MHz Bruker model DRX Avance spectrometer incorporating Fourier Transform. Samples were dissolved in Me_2SO-d_6 and examined at 50 or 70 °C. Chemical shifts are expressed in ppm (δ) relative

to resonance of Me_2SO-d_6 at δ 39.70 (^{13}C) and 2.40 (^{1}H) for samples examined in this solvent.

2.6. Controlled Smith degradation

IEPW-PE (300 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, 2005). The solutions were dialyzed and freeze-dried.

The products were then submitted to partial acid hydrolysis (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 and retained material (SM-PE, 97 mg) was freeze-dried.

3. Results and discussion

As shown in Fig. 1, *P. eryngii* and *P. ostreatoroseus* were extracted with $CHCl_3$–MeOH and then MeOH–H_2O to remove low-molecular weight compounds. Each resulting residue was submitted to aqueous extractions at 100 °C, and the extracted polysaccharides were recovered by ethanol precipitation (fractions EPW-PE and EPW-PO for *P. eryngii* and *P. ostreatoroseus*, respectively) were dialyzed against tap water, and the solution freeze-dried (EPW-PE, 7.8% yield; EPW-PO, 7.7% yield).

EPW-PE and EPW-PO both contained glucose as their main component, besides mannose, galactose and 3-*O*-methyl-galactose (Table 1). The presence of 3-*O*-methyl galactose was confirmed by GC–MS ions at *m*/*z* 130 and 190 after $NaBD_4$ reduction and acetylation.

For purification, EPW-PE and EPW-PO were submitted to several freeze-thawing procedures until no more precipitation occurred (Fig. 1). After centrifugation of the fractions, cold-water soluble SEPW-PE (5.3% yield) and SEPW-PO (5.0% yield) and insoluble PEPW-PE (2.5% yield) and PEPW-PO (2.7% yield) subfractions were isolated (Fig. 1). Table 1 shows both cold water-soluble fractions (SEPW-PE and SEPW-PO) to contain glucose, mannose, galactose and 3-*O*-methyl-galactose, while the insoluble fractions (IEPW-PE and IEPW-PO) show glucose as main monosaccharide components, consistent with a predominant glucan.

In order to elucidate the linkage type of glucans, IEPW-PE and IEPW-PO were submitted to methylation analysis, which showed, for both, mainly alditol acetates of 2,3,4,6-Me_4Glc (~26%), 2,4,6-Me_3Glc (~49%), and 2,4-Me_2Glc (~25%), showing the presence of a branched (1 → 3), (1 → 6)-linked β-glucan (Table 2; Fig. 2). These data agree with those of a ^{13}C NMR analysis (Figs. 3A and B for *P. eryngii* and *P. ostreatoroseus*, respectively), which showed similar β-glucans, structurally similar to those of some basidiomycetes (Tabata, Ito, & Kojima, 1981; Yoshioka, Tabeta, Saito, Uehara, & Fukuoka, 1985). Due to the similarity shown in the ^{13}C NMR spectra and methylation analysis, further analyses were carried out on the β-glucan of *P. eryngii*.

^{13}C NMR (Figs. 3A and B) and ^{1}H (obs.), ^{13}C HMQC spectra, obtained using Me_2SO-d_6 as solvent (Fig. 4), had signals corresponding to all carbons from the polysaccharide: C-1/H-1 at δ 103.1/4.21 corresponding to non-reducing ends units (C) (Fig. 2), while those at δ 102.9/4.51 are from 3-O-substituted (A) and 3,6-di-O-substituted units (B). The β-configuration was shown by C-1 signals at low field (Hall & Johnson, 1969) and H-1 signals at high field. The signals at δ 86.6 and 86.3 arose from substitutions at O-3 in units A, while those at δ 86.0 and 76.6 are from similar substitutions in units B and free O-3 from non-reducing end units of β-Glc*p* (C), respectively. Signals at δ 76.4; 76.1

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

Fractions	Yields[a] (%)	Monosaccharides[b] (%)			
		Man	3-*O*-MeGal	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
P. ostreatoroseus					
EPW-PO	7.7	4	Tr.	4	91
SEPW-PO	5.0	23	6.5	23.5	47
IEPW-PO	2.7	Tr.	Tr.	Tr.	99

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.

Table 2
Partially *O*-methylalditol acetates formed on methylation analysis of *P. eryngii* and *P. ostreatoroseus* polysaccharides (IEPW-PE and IEPW-PO, respectively) and that obtained on controlled Smith degradation (SM-PE)

Partially O-methylated alditol acetates	% Area of fragments			Linkage type[a]
	IEPW-PE	IEPW-PO	SM-PE	
2,3,4,6-Me_4-Glc	26	26	2	Glc*p* – (1→
2,4,6-Me_3-Glc	49	49	98	3→)-Glc*p* – (1→
2,4-Me_2-Glc	25	25	–	3,6→)-Glc*p* – (1→

[a] Based on derived *O*-methylalditol acetates.

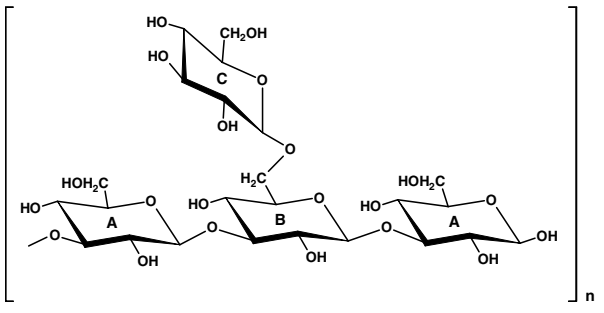

Fig. 2. Structure of the β-glucan obtained from *Pleurotus* spp.

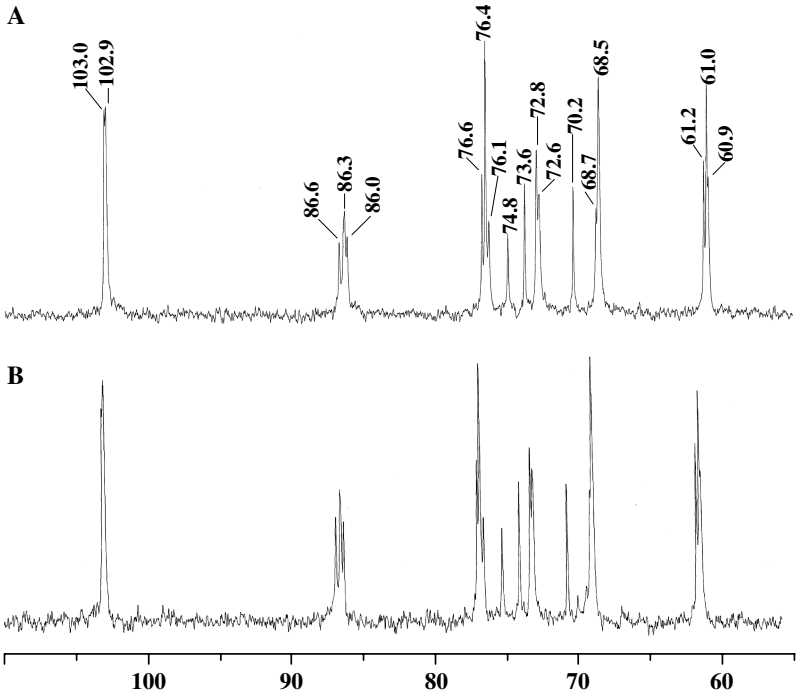

Fig. 3. ^{13}C NMR spectra of the β-glucans obtained from *P. eryngii* (A) and *P. ostreatoroseus* (B), in Me_2SO-d_6 at 70 °C (chemical shifts are expressed in ppm).

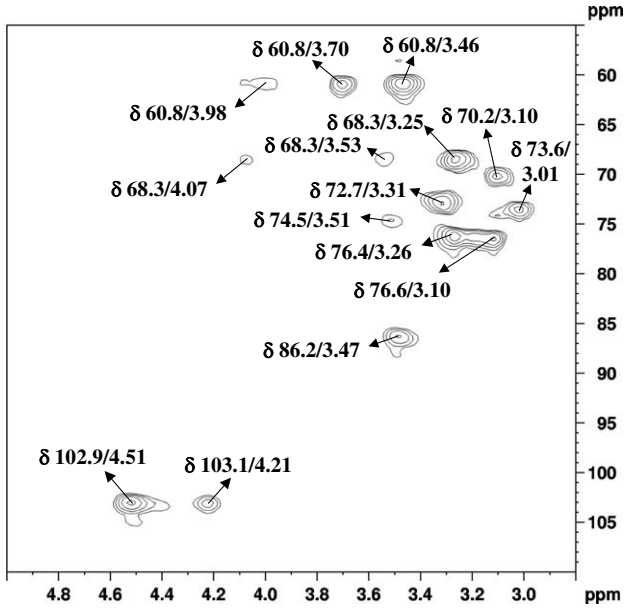

Fig. 4. ^{1}H (obs.), ^{13}C HMQC spectrum of the β-glucan obtained from *P. eryngii*, in Me_2SO-d_6 at 70 °C (chemical shifts are expressed in ppm).

and 74.8 arise from C-5 of units A, A and/or C and B, respectively. Non-substituted and O-substituted $-CH_2$ signals are at δ 61.2; 61.0; 60.9 and 68.4, respectively. The C-6 signals were confirmed from the inverted signal of the DEPT spectrum (Fig. 5). All the signals were assigned by comparing them to those found in the literature for the corresponding polysaccharides (Tabata et al., 1981; Yoshioka et al., 1985).

The structure of the main chain of IEPW-PE was identified by a controlled Smith degradation, which gave a product (SM-PE) that was analyzed by ^{13}C NMR. It proved to be a linear (1 → 3)-linked β-glucan with six typical signals at δ 102.9; 86.1; 76.3; 72.8; 68.4 and 60.9, arising from C-1, C-3, C-5, C-2, C-4, and C-6, respectively (Fig. 6) (Gorin, 1981).

Thus, according to the monosaccharide composition, methylation data, NMR spectroscopic analysis, and controlled Smith degradation for cold water-insoluble fractions (IEPW-PE and IEPW-PO), that are consisted of glucans to contain a (1 → 3)-linked, β-D-glucopyranosyl main chain, partially substituted at O-6 by (single units) β-D-glucopyranosyl groups, on average, to every third residue of the backbone.

This type of polysaccharide has been found in diverse basidiomycetes and the degree of substitution varies with the fungus. In 1963, it was found to be 1 in 3 for the scleroglucan of *Sclerotium glucanicum* (Johnson et al., 1963). With *Auricularia auricula-judae* (Misaki, Kakuta, Sasaki, Tanaka, & Miyaji, 1981), *Boletus erythropus* (Chauveau, Talaga, Wieruszeski, Strecker, & Chavant, 1996), *Ganoderma applanatum* (Usui et al., 1983), *Grifora frondosa* (Mizuno, Ohsawa, Hagiwara, & Kuboyama, 1986), *G. umbrellata* (Kato, Mutoh, Egashira, Hiura, & Ueno, 1978), *Pleurotus ostreatus* (Yoshioka et al., 1985), and *Schizophyllum commune* (schizophyllan; Tabata et al., 1981) the glucan from these fruiting body has the same degree of branching that the structures isolated in this work. There as a great interest in these molecules, since such glucans have antitumor (Jong & Donovick, 1989;

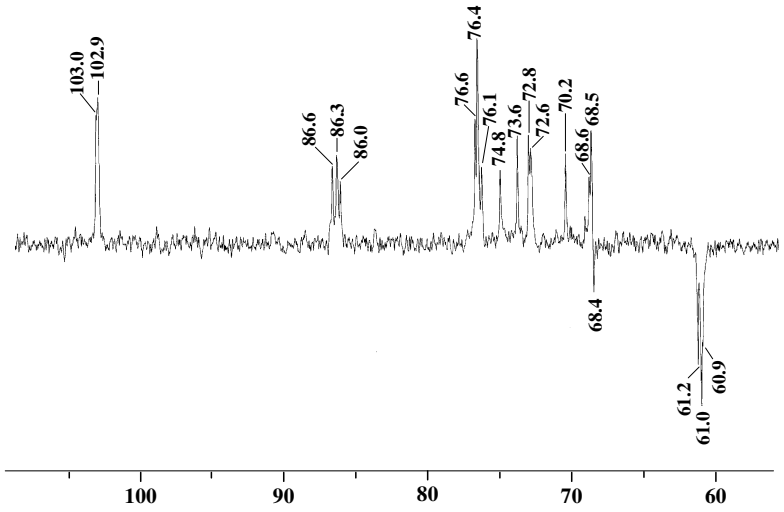

Fig. 5. DEPT spectrum of the β-glucan obtained from *P. eryngii*, in Me_2SO-d_6 at 70 °C (chemical shifts are expressed in ppm).

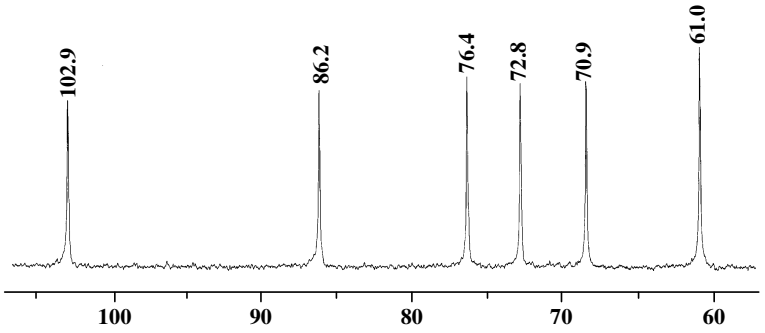

Fig. 6. ^{13}C NMR spectrum of Smith degraded glucan obtained from *P. eryngii* (SM-PE), in Me_2SO-d_6 at 70 °C (chemical shifts are expressed in ppm).

Sasaki & Takasura, 1976; Ukawa, Ito, & Hisamatsu, 2000), and anti-inflammatory (Hara, Kiho, Tanaka, & Ukai, 1982) activity, suggesting that part of the medicinal value of *Pleurotus* spp. can be attributed to them.

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