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An unusual water-soluble β-glucan from the basidiocarp of the fungus *Ganoderma resinaceum*

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Abstract

An unusual water-soluble glucan was isolated from the fruiting bodies of *Ganoderma resinaceum* following alkaline extraction. Its structure was determined using NMR spectroscopy (^{13}C, ^{1}H, and ^{1}H (obs.), ^{13}C HMQC), methylation analysis, and controlled Smith degradation. It was a highly branched β-D-glucan containing a (1→3)-linked β-D-glucopyranosyl main chain, partially substituted at O-6, principally, by side chains of 4-O-substituted β-D-glucopyranosyl groups, on average to every two residues of the backbone.

Keywords: *Ganoderma resinaceum*; Fungus; Polysaccharide; Structure; β-Glucan

1. Introduction

Polypores have been widely considered to be a large group of terrestrial fungi of the phylum Basidiomycota (basidiomycetes), which are a major source of pharmacologically active substances (Zjawiony, 2004). All *Ganoderma* spp. cause tree white-rot, because they degrade woody cell walls, with selective delignification and simultaneous rot, and are one of the most popular medicinal fungi with a long history in oriental countries. Polysaccharides and triterpenes were the most investigated as antitumor and antiviral agents (Paterson, 2006). Aqueous and alkaline extracts of these basidiocarps contain predominantly polysaccharides, which are the principal components of their cell walls. They attracted significant attention in recent years due mainly to their immunomodulatory activity, which results in antitumor effects (Wasser, 2002; Zhang, Cui, Cheung, & Wang, 2007). Differences in activity can be correlated with solubility in water, size of the molecules, degree of branching rate, and conformation (Wasser, 2002).

Mushroom polysaccharides are mainly glucans with various structures. β-D-Glucans are the most common structures of basidiomycetes, having a branched structure, varied degree of substitution, containing β-(1→3) linkages in main chain and additional β-(1→6) branch points (Paterson, 2006; Schmid, Stone, Brownlee, McDougall, & Seviour, 2006; Wasser, 2002) as lentinan (Sasaki & Takasura, 1976), schizophyllan (Tabata, Ito, & Kojima, 1981), among others. Recently, various glucans have been isolated from the basidiocarps of several mushrooms, such as a β-D-glucan from *Agaricus blazei* Murr, which contained a (1→6)-linked β-D-glucopyranosyl backbone substituted at O-3 by single units of β-D-glucopyranose or by side chains of (1→3)-linked β-D-glucopyranose linked to one in every three units (Dong, Yao, Yang, & Fang, 2002). From *Astraeus hygrometricus* was isolated a water-soluble glucan with α-(1→4), β-(1→6) Glc*p* repeating units (Chakraborty, Mondal, Malay, Rout, & Islam, 2004). Rout and co-workers

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(2005) reported the presence of a branched glucan consisting of (1→3)-, (1→6)-linked Glc*p* units with both α- and β-configurations, in *Pleurotus florida*.

Polysaccharides from *Ganoderma resinaceum* have not been previously investigated and we now describe the isolation and chemical characterization of an unusual β-glucan from the basidiocarps of *G. resinaceum*.

2. Materials and methods

2.1. Organism

The fungi were collected from tree trunks growing in the city of Cascavel, State of Paraná, Brazil. It was identified as *G. resinaceum* Boud (Ryvarden, 2004) by Dra. Adriana M. Gugliota, Department of Mycology, Botanic Institute of São Paulo, and deposited in the HUNOP (Herbarium of the Universidade Estadual do Oeste do Paraná-UNIOESTE), Registration No. 2667.

2.2. Polysaccharide extraction and purification

Powdered basidiocarps of the *G. resinaceum* (50 g) were defatted with $CHCl_3$–MeOH (2:1 v/v; 600 mL) and the residual material was submitted to aqueous (100 °C for 3 h, 800 mL) and alkaline (2% and 5% aq. KOH, 800 mL) extractions containing traces of $NaBH_4$ each at 100 °C for 3 h (extracted 3×). The alkaline extracts (K2 and K5, respectively) were neutralized with HOAc, dialyzed against tap water, and after 48 h was freeze dried. Fraction K5 was submitted to a freeze–thawing process, which furnished insoluble (IK5) and soluble (SK5) material, which were separated by centrifugation (15 min, 9000 rpm, 25 °C). The soluble fraction (SK5) was submitted to a second process with addition of HOAc to pH 5.0, giving a precipitate (PSK5) and supernatant (SSK5) which were dialyzed against tap water for 12 h. Fraction SSK5 was further submitted to dialysis with a 16 kDa M_r cut-off membrane, furnishing retained (RSSK5), and eluted (ESSK5) fractions. ESSK5 was then submitted to ultrafiltration through a membrane of 10 kDa M_r cut-off (Millipore-regenerated cellulose), giving rise to retained (DRSSK5) and eluted (DESSK5) fractions (Fig. 1).

2.3. Monosaccharide composition

Total hydrolysis of the fractions was carried out in sealed ampoules with M TFA for 8 h at 100 °C, followed by evaporation to dryness. The residue was successively reduced with sodium borohydride, acetylated with acetic anhydride-pyridine (Wolfrom & Thompson, 1963a,1963b) and the resulting alditol acetates examined by GC–MS with a Saturn ion-trap spectrometer (Varian), using a capillary column of DB-225 (30 m × 0.25 mm i.d.), programmed from 50 to 220 °C at 40 °C min^{-1}, then hold,

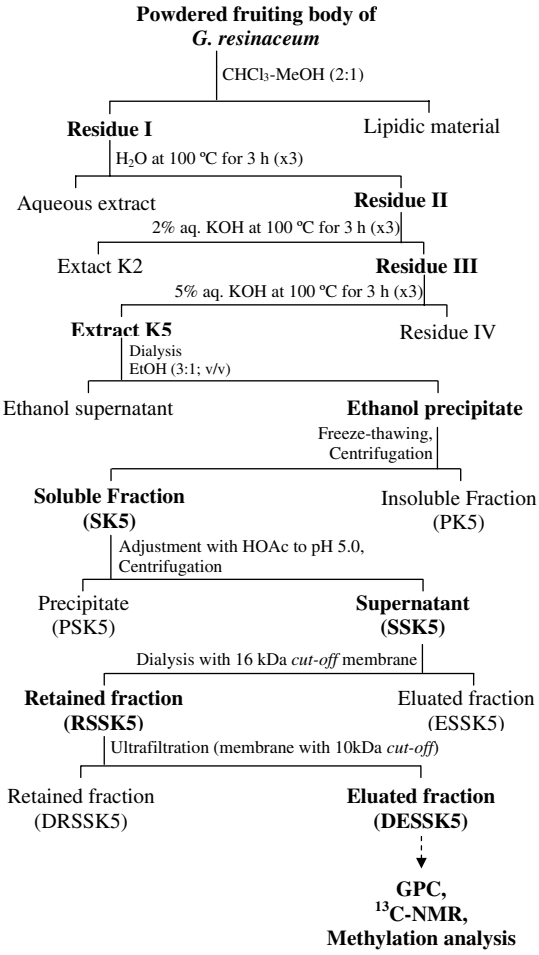

Fig. 1. Extraction and purification of β-glucan from *G. resinaceum*.

the total analysis time being 26 min. The carrier gas was He with a flow rate of 2 mL min^{-1}.

2.4. Methylation analysis

Per-*O*-methylation of DESSK5 was carried out as described by Ciucanu and Kerek (1984) using powdered NaOH in DMSO–MeI. The product was converted into partially O-methylated aldoses and treated with 45% v/v aq. formic acid (1.0 mL, 15 h at 100 °C) followed by evaporation and the product converted to partially *O*-methylated alditol acetates, which were analyzed by GC–MS, using a capillary column of DB-225 (30 m × 0.25 mm i.d.), programmed from 50 to 220 °C (40 °C min^{-1}), then hold. The carrier gas was He with a flow rate of 2 mL min^{-1}.

2.5. NMR analyzes

Nuclear magnetic resonance (NMR) spectra were obtained using a 400 MHz Bruker model DRX Avance spectrometer with a 5 mm inverse probe. ^{13}C NMR (100.6 MHz) analyzes were performed at 70 °C, with the samples being dissolved in D_2O or Me_2SO-d_6. Chemical

shifts of samples are expressed in ppm (δ) relative to acetone or Me_2SO-d_6 at δ 30.2, 39.7 (^{13}C) and 2.22, 2.40 (^{1}H), respectively.

2.6. Controlled Smith degradation

The polysaccharide (100 mg) was oxidized in 0.05 M $NaIO_4$ (100 mL) at room temperature for 72 h in the dark. 1,2-Ethanediol was added, the solution dialyzed, and the resulting polyaldehydes reduced with $NaBH_4$ for 24 h, the solution neutralized with HOAc, dialyzed, and concentrated (Goldstein, Hay, Lewis, & Smith, 1965). The residue was partially hydrolyzed with TFA pH 2.0 (30 min at 100 °C) (Gorin, Horitsu, & Spencer, 1965) and dialyzed against tap water using membranes with a size exclusion of 2 kDa and the solution containing retained material (SD-DESSK5) was freeze-dried.

2.7. Determination of homogeneity and their molar mass (M_w)

A Waters size exclusion chromatography (SEC) apparatus coupled to a differential refractometer (RI), and a Wyatt Technology Dawn-F multi-angle laser light scattering (MALLS) detector was used for examination of the fraction DESSK5. Four Waters Ultrahydrogel columns 2000/500/250/120 were connected in series and coupled with a multi-detection equipment. Sample, previously filtered through a membrane (0.22 μm; Millipore), was injected (100 μL loop) at a 1 mg mL^{-1} concentration. The eluent was 0.1 mol L^{-1} aq. $NaNO_3$ containing 0.5 g L^{-1} NaN_3. The specific refractive index increment (dn/dc) was determined by using a Waters 2410 detector.

2.8. Specific optical rotation

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

3. Results and discussion

As shown in Fig. 1, defatted basidiocarps of *G. resinaceum* were submitted, successively, to aqueous (4.3% yield) and alkaline extractions. Fraction K5 (5.9% yield), obtained by hot aq. 5% KOH extraction, contained mainly glucose (96.5%) and traces of the mannose (1.6%), galactose (0.7%), and xylose (1.2%).

In order to the purification, this fraction was first submitted to several freeze–thawing procedures until no more precipitation occurred. After centrifugation, cold-water soluble (SK5 fraction; 3.5% yield) and insoluble (PK5 fraction; 2.4% yield) subfractions were isolated (Fig. 1). The monosaccharide compositions of the both fractions were similar, having glucose as their main component.

Fraction SK5 was heterogeneous as shown by HPSEC, so its was dialyzed through a 16 kDa M_r cut-off membrane, furnishing two fractions: ESSK5 (eluted) and RSSK5 (retained). Due to the heterogeneity of the RSSK5 fraction, it was submitted to ultrafiltration (10 kDa M_r cut-off membrane), giving rise to retained (DRSSK5) and eluted fractions (DESSK5). Fraction DESSK5 was homogeneous on HPSEC (Fig. 2), and had M_w 2.6×10^4 g/mol (dn/d$c = 0.173$), and when analyzed by GC–MS showed glucose as its main monosaccharide indicating a glucan.

In order to elucidate the glycosidic linkages types of this polymer, fraction DESSK5 was submitted to methylation analysis, which showed a branched structure with a non-reducing end (24.7%), 3-O-(26.8%) and 4-O-substituted (28.5%), and 3,6-di-O-substituted units (20.0%) of glucopyranose (Table 1).

^{13}C NMR and ^{1}H (obs.), ^{13}C HMQC spectra (Figs. 3 and 4a, respectively) had signals corresponding to assign-

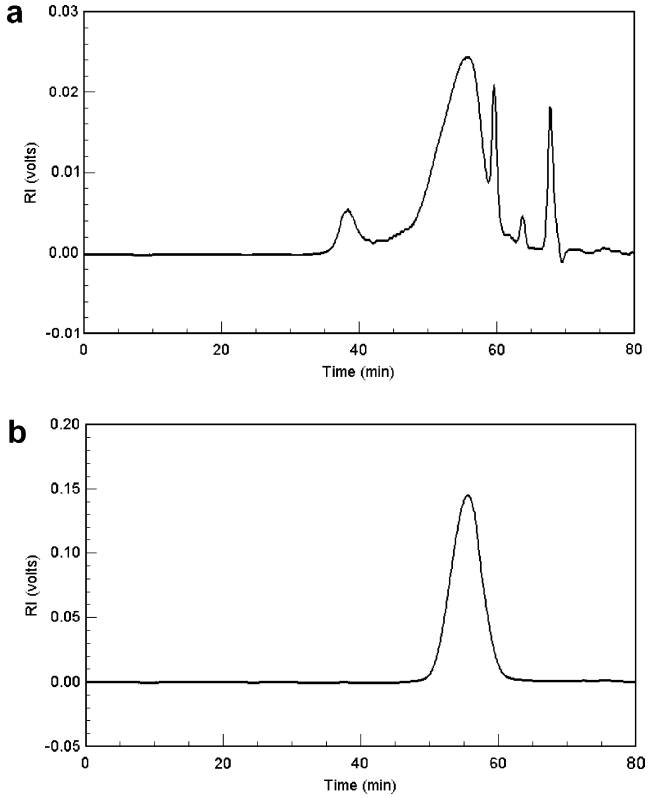

Fig. 2. HPSEC elution profiles of fractions SSK5 (a) and DESSK5 (b).

Table 1
Partially *O*-methylalditol acetates formed on methylation analysis of *G. resinaceum* polysaccharide

Partially O-methylated alditol acetates[a]	T_R[b]	mol %	Linkage type[c]
2,3,4,6-Me_4-Glc	9.31	24.7	Glc*p*-(1→
2,4,6-Me_3-Glc	11.95	26.8	3→)-Glc*p*-(1→
2,3,6-Me_3-Glc	13.25	28.5	4→)-Glc*p*-(1→
2,4-Me_2-Glc	18.72	20.0	3,6→)-Glc*p*-(1→

[a] Analyzed by GC–MS after methylation, total acid hydrolysis, reduction (NaB^2H_4) and acetylation.
[b] Retention time (min).
[c] Based on derived *O*-methylalditol acetates.

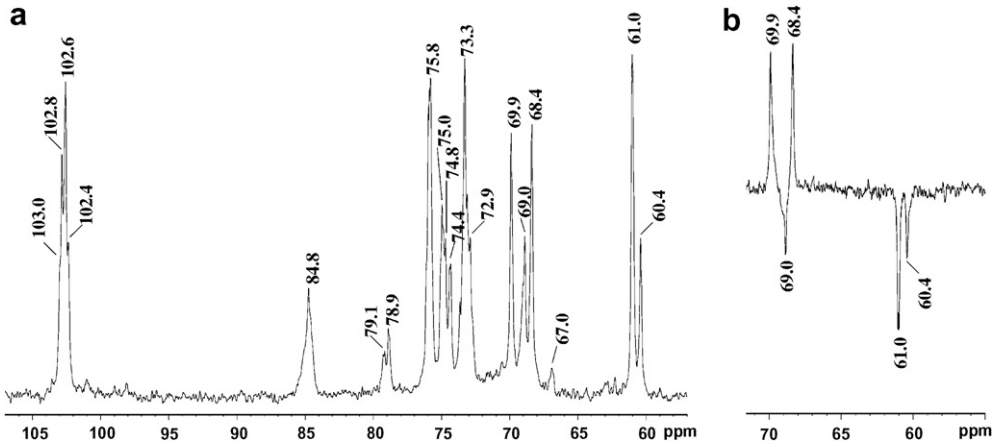

Fig. 3. ^{13}C NMR (a) and -CH_2-OH region in the ^{13}C-DEPT (b) spectra of the *G. resinaceum,* in D_2O at 70 °C (chemical shifts are expressed in ppm).

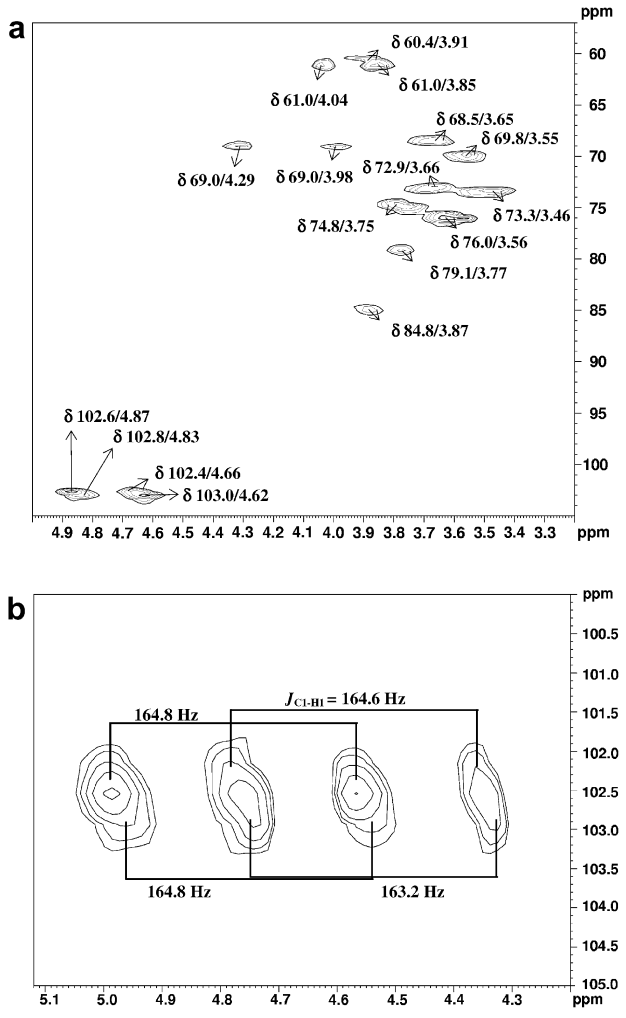

Fig. 4. ^{1}H (obs), ^{13}C HMQC (a) and its coupled (b; anomeric region) spectra of the *G. resinaceum* β-glucan, in D_2O at 70 °C (chemical shifts are expressed in ppm).

ments from the polysaccharide, namely C-1/H-1 at δ 103.0/4.63 corresponding to non-reducing end units of β-Glc*p*, while those at δ 102.8/4.85, 102.4/4.66, and 102.6/4.87 were from 3-O-, 4-O-, and 3,6-di-O-substituted β-Glc*p* units, respectively. The β-configuration was shown by C-1 signals at high (Hall & Johnson, 1969) and H-1 signals at low frequency, which are agreement with the values of coupling constants ($J_{C\text{-}1,\ H\text{-}1}$) of 163.2 (δ 103.0), 164.8 (δ102.8 and 102.6), and 164.6 Hz (δ 102.4) observed in the coupled HMQC spectrum (Fig. 3b) (Perlin & Casu, 1969) and $[\alpha]_D^{20}$–1.6° (H_2O; c 0.5).

The signals at δ 84.8/3.88 and δ 79.1/3.77 arose from O-3 and O-4 substitutions of units of β-Glc*p*, respectively. Non-substituted and O-substituted –CH_2-6 signals are at δ 61.0/4.04/3.85 and 60.4/3.91, and δ 69.0/4.29/3.98, respectively (Figs. 3 and 4a). These C-6 signals were confirmed by an inverted signal in the DEPT spectrum (Fig. 3b). All the signals were assigned by comparing them with literature values for the similar polysaccharides (Barbosa, Steluti, Dekker, Cardoso, & Corradi da Silva, 2003; Carbonero et al., 2005; Saitô, Ohki, Yoshioka, & Fukuoka, 1976).

The structure of the backbone of DESSK5 was identified by a controlled Smith degradation, which gave a product (SD-DESSK5) that was analyzed by ^{13}C NMR (Fig. 5). It was a linear (1→3)-linked β-glucan with six typical signals at δ 102.9; 86.2; 76.4; 72.8; 68.5; and 60.9, arising from C-1, C-3, C-5, C-2, C-4, and C-6, respectively (Gorin, 1981).

These results indicated that DESSK5 had a main chain of (1→3)-linked β-D-glucopyranosyl residues, partially, substituted at O-6 by side chains of 4-O-substituted β-D-glucopyranosyl units, on an average of one to every two residues of the backbone.

β-D-Glucans containing this main chain have been found in diverse basidiomycetes. Polysaccharides found in *Ganoderma lucidum* shown to have the same main chain, mainly substituted in O-6 by non-reducing ends of β-Glc*p* units and side chains of different lengths (Bao, Liu, Fang, & Li, 2001; Sone, Okuda, Noriko, Kishida, & Misaki, 1985). Glucans substituted only by single units of β-Glc*p* were also found in other *Ganoderma* spp., such as *G. applanatum* (Usui, Iwasaki, Mizuno, Tanaka, Shinkai, & Arakawa, 1983), *G. japonicum* (Ukai, Yokoyama, Hara,

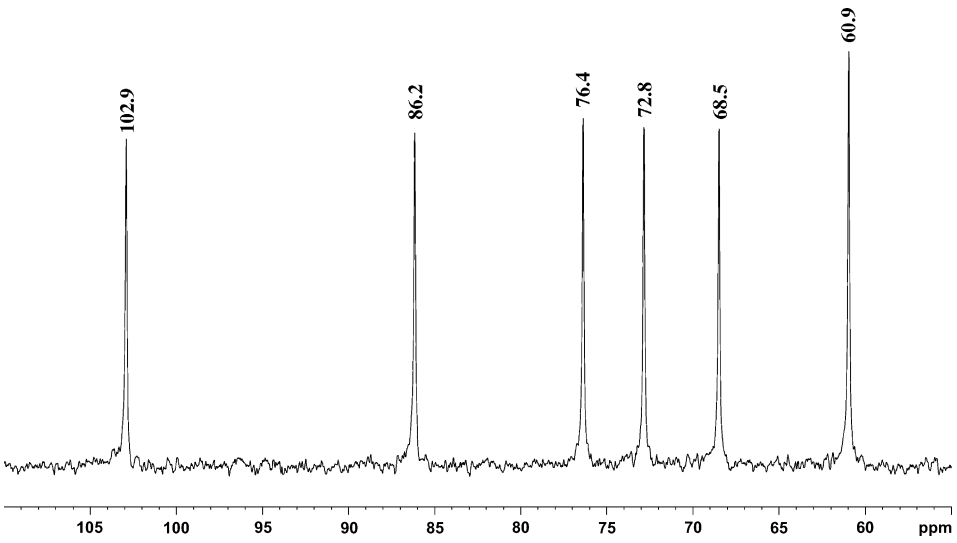

Fig. 5. ^{13}C NMR spectrum of the glucan obtained on controlled Smith degradation (SD-DESSK5) derived from the *G. resinaceum* glucan, in Me_2SO-d_6 at 70 °C (chemical shifts are expressed in ppm).

& Kiho, 1982), and *G. tsugae* (Wang et al., 1993). On the other hand, a glucan having a structure now described has not been previously described in basidiomycetes.

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