Available online at www.sciencedirect.com
ScienceDirect
Carbohydrate Research 343 (2008) 1456–1462
Carbohydrate RESEARCH

A gel-forming β-glucan isolated from the fruit bodies of the edible mushroom *Pleurotus florida*

Juliana C. Santos-Neves, Maria Izabel Pereira, Elaine R. Carbonero, Ana Helena P. Gracher, Philip A. J. Gorin, Guilherme L. Sassaki and Marcello Iacomini*

Departamento de Bioquímica e Biologia Molecular, Universidade Federal do Paraná, CP 19046, CEP 81531-980 Curitiba, PR, Brazil

Received 26 October 2007; received in revised form 19 February 2008; accepted 3 March 2008
Available online 10 March 2008

Abstract—Glucans of basidiomycetes are considered to be an important class of polysaccharides, as they can act as biological response modifiers. We now isolate a gel-forming, water-soluble β-glucan, with a molecular mass of 1.2×10^6 g/mol (HPSEC), from the fruit bodies of the edible mushroom *Pleurotus florida*, via alkaline extraction, followed by fractionation by freeze–thawing. Structural assignments were carried out using mono- and bi-dimensional nuclear magnetic resonance spectroscopy, monosaccharide composition, methylation analyses, and a controlled Smith degradation. It was a branched β-glucan, with a main chain of (1→3)-linked-Glc*p* residues, substituted at O-6 by single-unit Glc*p* side chains, on average to every fourth residue of the backbone.

Keywords: Polysaccharides; Edible mushrooms; *Pleurotus florida*; β-Glucans

1. Introduction

Mushrooms are known for their nutritional and medicinal value and also for the diversity of bioactive compounds that they contain.[1] Some species of mushrooms, such as those of the genera *Lentinus, Grifola*, *Ganoderma*, *Agaricus*, and *Pleurotus*, have medicinal or functional properties.[2,3]

Pleurotus spp. is a basidiomycete distributed worldwide throughout hardwood forests, having a greater number of species in temperate climates. *Pleurotus* spp. are commonly called oyster mushrooms, all known species of *Pleurotus* being edible, and several being commercially cultivated.[4] The popularity of the genus is on the increase because they are a good source of non-starchy carbohydrates, have a high content of dietary fiber, and contain moderate quantities of good quality proteins with most of the essential amino acids, minerals and vitamins.[5] Among the different species of oyster mushrooms, the most known are *P. citrinopileatus*, *P. ostreatus*, *P. ostreatoroseus*, *P. pulmonarius*, *P. eryngii*, and *P. florida* (=*P. ostreatus* var. *florida*).[6]

P. florida is an excellent edible and highly nutritious mushroom, which is a common species in tropical countries.[5] As well as the nutritional value and the easy cultivation of these mushrooms, they are a good source of compounds such as polysaccharides that can act as biological response modifiers (BRMs). They have been shown to modulate the immune system, have hypoglycemic activity, lower blood pressure and lipid concentrations, and inhibit tumor growth, inflammation, and microbial action.[8,9]

Several polysaccharides have been isolated from different species of *Pleurotus*. A (1→3), (1→6) glucan containing both α and β linkages was isolated from *P. florida*.[7] Branched β-glucans, with a main chain of (1→3)-linked-β-Glc*p* residues, substituted at O-6 by single-unit β-Glc*p* side chains, on the average to one in every third residue of the backbone, were obtained from *P. ostreatoroseus* and *P. eryngii*.[10] A similar β-glucan with antitumor activity was isolated from *P. ostreatus*[11] and such an action of these glucans can be due to the (1→3)-β-linkages in the main chain, (1→6)-β-substitution, and their solubility in water.[12]

* Corresponding author. Tel.: +55 41 33611655; fax: +55 41 32662042; e-mail: iacomini@ufpr.br

0008-6215/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carres.2008.03.001

Now we describe the structural features of a water-soluble glucan present in the alkaline extract of fruit bodies of *P. florida*.

2. Experimental

2.1. General experimental procedures

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 the 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. Partially O-methylated alditol acetate mixtures were similarly analyzed, but with a program from 50 °C to 215 °C at 40 °C/min, then hold.

^{13}C NMR spectra were obtained using a 400 MHz Bruker model DRX Avance spectrometer incorporating Fourier transform. Analyses were performed at 70 °C on samples dissolved in Me_2SO-d_6. Chemical shifts of samples are expressed in δ (ppm) relative to Me_2SO-d_6 at δ 39.7 and 2.40 for ^{13}C and ^{1}H signals, respectively.

The determination of the homogeneity and molar mass (M_w) of the purified fraction (ISK2) was performed on a Waters high-performance size-exclusion chromatography (HPSEC) apparatus coupled to a differential refractometer (RI) and a Wyatt Technology Dawn-F Multi-Angle Laser Light Scattering detector (MALLS). Waters Ultrahydrogel columns (2000, 500, 250, and 120) were connected in series and coupled with multidetection equipment, using a $NaNO_2$ solution (0.1 M) as eluent, containing 0.5 g/L NaN_3. The polysaccharide solutions (1 mg mL^{-1}) were dissolved in the same solvent and filtered through a Millipore nitrocellulose membrane, with pores of 0.22 or 0.45 μm. HPSEC data were collected and analyzed by the Wyatt Technology ASTRA program. The specific refractive index increment (d*n*/d*c*) was determined using a Waters 2410 detector. All experiments were carried out at 25 °C.

2.2. Biological material

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

2.3. Extraction and purification of polysaccharide

Extraction and purification of the polysaccharide from the fruit bodies of *P. florida* was carried out according to Figure 1. Dried fruit bodies (196 g) were successively extracted with water (25 and 100 °C) and 2% aq KOH (100 °C for 6 h, each). The combined alkaline extracts were neutralized with HOAc, dialyzed against tap water for ~48 h, concentrated under reduced pressure to small volumes, and freeze-dried, giving rise to fraction K2. This fraction was then dissolved in H_2O, and the solution was frozen and then allowed to thaw slowly. The resulting insoluble material (IK2) was centrifuged off (9000 rpm for 15 min at 25 °C). The soluble fraction (SK2) was submitted to a second process of freezing, followed by mild thawing at 4 °C, which furnished cold water-soluble (SSK2) and insoluble gel-like fractions (ISK2), which were separated by centrifugation (9000 rpm at 4 °C for 30 min).

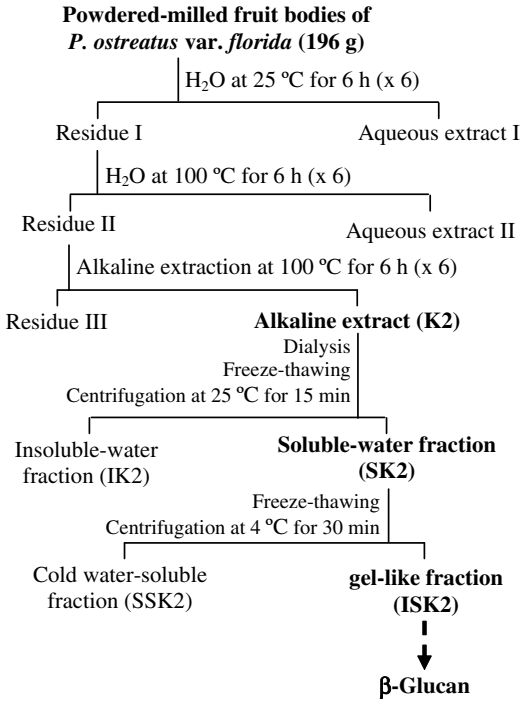

Figure 1. Scheme of extraction and purification of the β-glucan from the fruit bodies of *P. florida*.

2.4. 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 acetylated with 1:1 Ac_2O–pyridine (2 mL) at room temperature for 12 h.[13,14] The resulting alditol acetates were analyzed by GC–MS as indicated above and identified by their typical retention times and electron impact profiles.

2.5. Methylation analysis

O-Permethylation of the isolated polysaccharide (10 mg) was carried out using 40% aq NaOH (3 mL) and

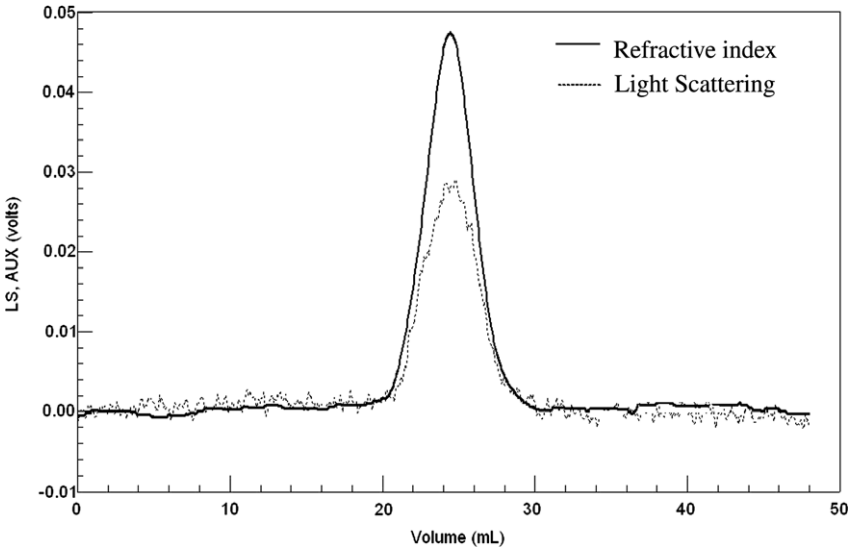

Figure 2. HPSEC elution profile of ISK2 fraction.

Me_2SO_4 (3 mL), added dropwise.[15] This process, after isolation of the products by neutralization, dialysis, and evaporation, was repeated. Complete O-permethylation of the polysaccharide was carried out using NaOH–Me_2SO–MeI.[16] The O-permethylated derivatives (2 mg) were hydrolyzed with 45% v/v formic acid (1 mL) at 100 °C for 15 h, followed by evaporation to dryness. The resulting mixture of O-methylaldoses was reduced with NaB^2H_4 and acetylated as above (item 2.4) to give a mixture of partially O-methylated alditol acetates, which was analyzed by GC–MS.

2.6. Controlled Smith degradation

ISK2 (100 mg) was submitted to oxidation with 0.05 M aq $NaIO_4$ (20 mL) for 72 h at 25 °C in the dark. The sample was then dialyzed against tap water for 48 h and treated with $NaBH_4$ (pH 9–10) for ~20 h.[17] The solution was dialyzed and freeze-dried, and the product was successively partially hydrolyzed (TFA pH 2.0, 30 min, 100 °C),[18] and dialyzed against tap water using membranes with a size exclusion of 2 kDa, retained material being freeze-dried to give SM-ISK2 (72 mg).

Table 1. Partially *O*-methylalditol acetates formed on methylation analysis of the β-glucan (ISK2) and those obtained on controlled Smith degradation (SM-ISK2)

Partially O-methylated alditol acetates	t_R[b]	% Area of fragments		Linkage type[a]
		ISK2	SM-ISK2	
2,3,4,6-Me_4-Glc	9.38	20.4	Tr.[c]	Glc*p*-(1→
2,4,6-Me_3-Glc	12.23	59.5	99.8	3→)-Glc*p*-(1→
2,4-Me_2-Glc	19.05	20.1	—	3,6→)-Glc*p*-(1→

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

3. Results and discussion

P. florida was shown to contain 90% moisture, removed in a freeze dryer, and the product was then successively extracted with water and aqueous alkali. Fractionation of the alkaline extract (K2, 15.3% yield) by a freezing/thawing process furnished water-soluble (SK2; 9.2% yield) and insoluble (IK2; 6.1% yield) polysaccharide fractions, which were separated by centrifugation. In order to separate a gel-forming fraction (ISK2; 3.6%

Table 2. ^1H and ^{13}C NMR chemical shifts of native β-glucan (ISK2)[a]

Units		1	2	3	4	5	6	
							6a	6b
→3)-β-Glc*p*-(1→	^{13}C	102.9	72.9/72.8	86.6/86.3/86.2	68.6	76.5/76.4	61.0/60.9	61.0/60.9
	^1H	4.51	3.21	3.48	3.26	3.26	3.68	3.48
→3,6)-β-Glc*p*(1→	^{13}C	103.0	72.7	86.0	68.7	74.9	68.5	68.5
	^1H	4.51	3.21	3.48	3.26	3.51	4.05	3.54
β-Glc*p*-(1→	^{13}C	103.1	73.7	76.6	70.3	76.2	61.2	61.2
	^1H	4.21	3.01	3.11	3.10	3.26	3.68	3.48

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

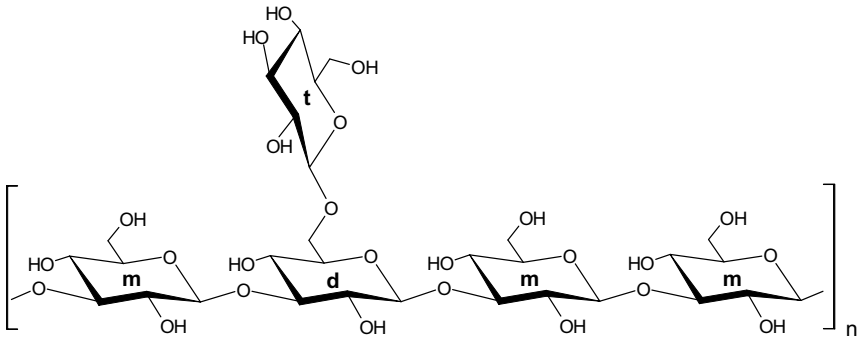

Figure 3. Structure of the β-glucan obtained from *P. florida*.

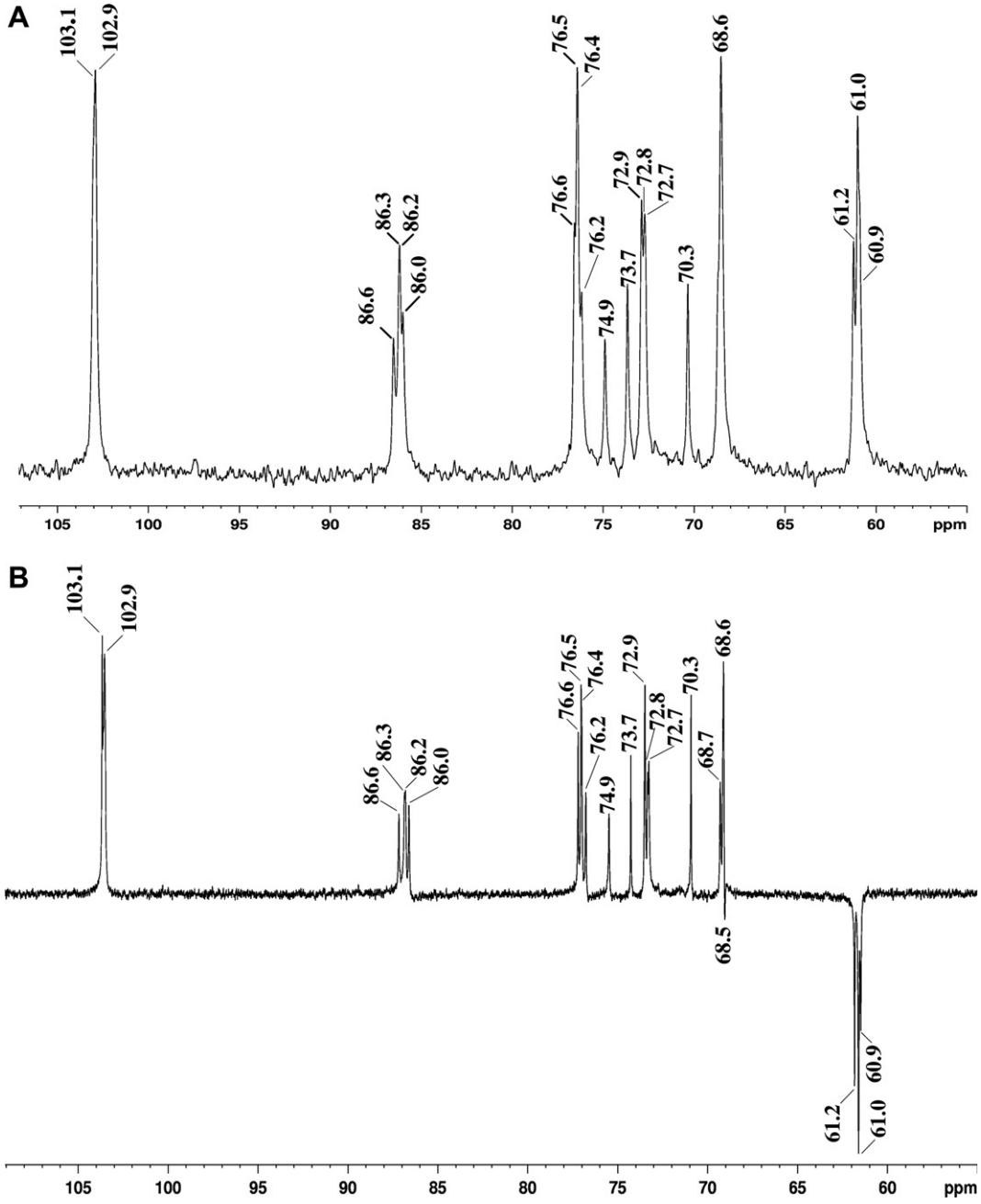

Figure 4. ^{13}C NMR (A) and DEPT (B) spectra of the β-glucan in Me_2SO-d_6 at 70 °C (chemical shifts are expressed in ppm).

yield), SK2 was submitted to a second process of freezing followed by mild thawing at 4 °C, then centrifugation (Fig. 1). This process gave pure ISK2 fraction, which showed a homogeneous elution profile on HPSEC, with M_w 1.2×10^6 g/mol ($dn/dc = 0.159$) (Fig. 2).

The purified gel-like fraction (ISK2) was a glucan giving only glucose on hydrolysis (GC–MS). Analysis by GC–MS of its O-permethylated homopolymer suggested the presence of a branched (1→3), (1→6)-linked β-glucan due to the presence of alditol acetates of 2,3,4,6-Me_4Glc, 2,4,6-Me_3Glc, and 2,4-Me_2Glc, in a molar ratio of nearly 1:3:1, respectively (Table 1).

ISK2 was examined using NMR spectroscopy, and its signals were assigned using 1D (^{1}H, ^{13}C, and DEPT) and 2D NMR spectra (^{1}H (obsd) ^{13}C HMQC, COSY, and TOCSY) (see in Table 2). All the signals were assigned using the literature values for similar polysaccharides.[19–21] These data suggest the structure of the polysaccharide isolated as shown in Figure 3.

^{13}C NMR (Fig. 4A) and ^{1}H (obsd), ^{13}C HMQC spectra (Fig. 5A) contained signals (C-1/H-1) in the

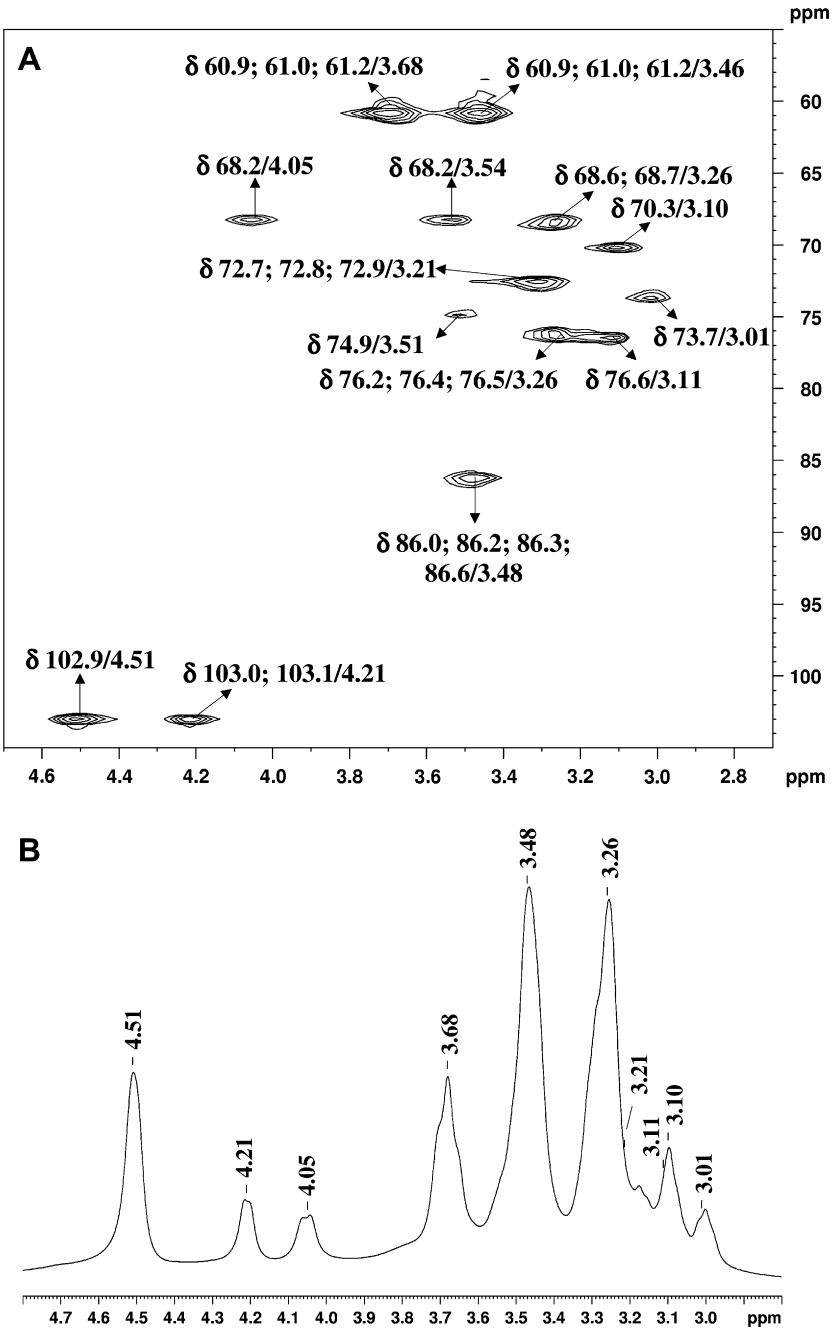

Figure 5. ^{1}H (obsd),^{13}C HMQC (A), and ^{1}H NMR (B) spectra of the β-glucan in Me_2SO-d_6 at 70 °C (chemical shifts are expressed in ppm).

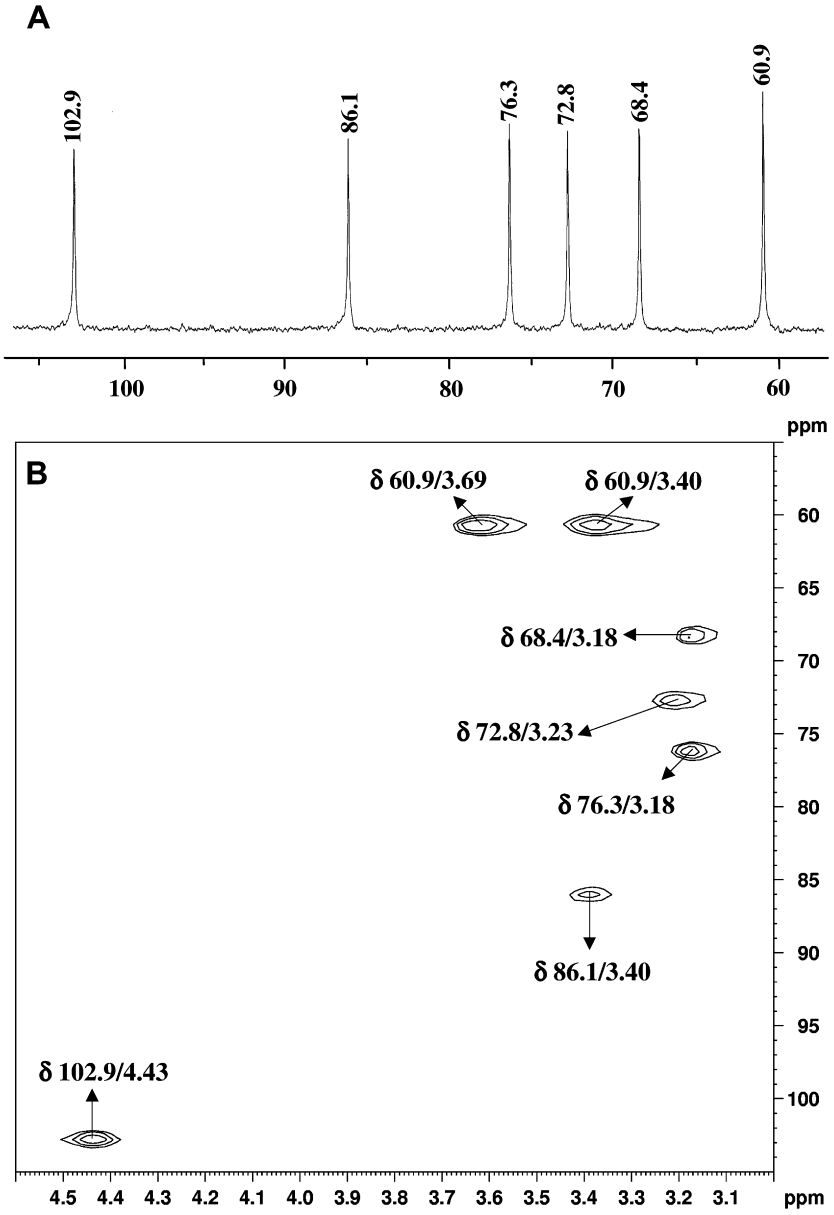

Figure 6. ^{13}C NMR (A) and ^{1}H (obsd), ^{13}C HMQC (B) spectra of β-glucan obtained on controlled Smith degradation (SM-ISK2), in Me_2SO-d_6 at 70 °C (chemical shifts are expressed in ppm).

anomeric region at δ 103.1/4.21 corresponding to non-reducing end units (t) (Fig. 3), while those at δ 102.9/4.51 are from 3-O-substituted (m) and 3,6-di-O-substituted residues (d). The β-configuration was shown by low-frequency H-1 (δ 4.51 and 4.21) and high-frequency C-1 signals (δ 103.1 and 102.9) (Figs. 4A and 5A, B).[22]

The glycosidic linkages of the glucan were shown by the presence of 3-O-substituted signals at δ 86.6, 86.3, and 86.0, and O-substituted-CH_2-6 signal at δ 68.2 (in the form of an HMQC doublet at δ 68.2; 4.05/3.54, Fig. 5A). O-6 substitution was confirmed from the respective reversed peak in the DEPT spectrum (Fig. 4B).

The structure of the backbone of this glucan was characterized by a controlled Smith degradation, which gave a product (SM-ISK2) that was analyzed by ^{13}C NMR spectroscopy and ^{1}H (obsd) ^{13}C HMQC (Fig. 6A and B, respectively). It proved to be a linear (1→3)-linked β-glucan with typical C/H signals at δ 102.9/4.43; 86.1/3.40; 76.3/3.18; 72.8/3.23; 68.4/3.18, and 60.9/3.69;3.40, arising from C-1/H-1, C-3/H-3, C-5/H-5, C-2/H-2, C-4/H-4, and C-6/H-6a;b, respectively.[23] It was methylated, and the alditol acetates on GC–MS analysis revealed almost exclusively a 2,4,6-Me_3Glc derivative (99.8%). This result further confirms that the non-reducing ends units of β-Glc*p* attached to the branch point at O-6 of glucan were completely oxidized by periodate.

In summary, the results of monosaccharide composition, methylation data, NMR spectroscopic analysis,

and controlled Smith degradation of ISK2 showed it to be a glucan containing a (1→3)-linked, β-D-glucopyranosyl main chain, partially substituted at O-6 by single units of β-D-glucopyranosyl groups, on the average, to every fourth residue of the backbone.

Glucans are common components of basidiomycetes, and the degree of substitution varies with the organism.[23] Usually, these polymers have the same main chain and substitution and the degree of branching is 1 in 3, such as scleroglucan,[19] lentinan,[24] grifolan,[25] among others, and these glucans have a strong antitumor effect.[12] However, the present glucan, having the same structure and degree of branching as that from *P. ostreatus*, had a stronger antitumor activity at doses of 0.1 mg/kg ($74 \pm 10\%$ of inhibition) and 0.2 mg/kg ($95 \pm 3\%$ of inhibition) against Sarcoma 180.[20] In addition, other studies indicated that the distribution of the single glycosyl units along the main chain confers their immunomodulating activity, their solubility in water also being important for biological applications.[26] These studies suggest that the presently investigated glucan could act as a biological response modifier (BRM). We propose that part of the medicinal value of the edible *Pleurotus* spp. can be attributed to their glucans.

Acknowledgments

The authors would like to thank the Brazilian funding agencies: Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), and Fundação Araucária.

References

1. Breene, W. M. *J. Food Protect.* **1990**, *53*, 883–894.
2. Smith, J. E.; Rowan, N. J.; Sullivan, R. *Biotechnol. Lett.* **2002**, *24*, 1839–1845.
3. Wasser, S. P. *Appl. Microbiol. Biotechnol.* **2002**, *60*, 258–274.
4. Ragunathan, R.; Gurusamy, R.; Palaniswamy, M.; Swaminathan, K. *Food Chem.* **1996**, *55*, 139–144.
5. Gunde-Cimerman, N. *Int. J. Med. Mushrooms* **1999**, *1*, 69–80.
6. Kües, U.; Liu, Y. *Appl. Microbiol. Biotechnol.* **2000**, *54*, 141–152.
7. Rout, D.; Mondal, S.; Chakraborty, I.; Pramanik, M.; Islam, S. S. *Carbohydr. Res.* **2005**, *340*, 2533–2539.
8. Guzman, G. *Int. J. Med. Mushrooms* **2000**, *2*, 95–123.
9. Chang, R. *Nutr. Rev.* **1996**, *54*, 91–93.
10. Carbonero, E. R.; Gracher, A. H. P.; Smiderle, F. R.; Rosado, F. R.; Sassaki, G. L.; Gorin, P. A. J.; Iacomini, M. *Carbohydr. Polym.* **2006**, *66*, 252–257.
11. Yoshioka, Y.; Tabeta, R.; Saitô, H.; Uehara, N.; Fukuoka, F. *Carbohydr. Res.* **1985**, *140*, 93–100.
12. Zhang, M.; Cui, S. W.; Cheung, P. C. K.; Wang, Q. *Trends Food Sci. Technol.* **2007**, *18*, 4–19.
13. Wolfrom, M. L.; Thompson, A. *Methods Carbohydr. Chem.* **1963**, *2*, 65–68.
14. Wolfrom, M. L.; Thompson, A. *Methods Carbohydr. Chem.* **1963**, *2*, 211–215.
15. Haworth, W. N. *J. Chem. Soc.* **1915**, *107*, 8–16.
16. Ciucanu, I.; Kerek, F. *Carbohydr. Res.* **1984**, *131*, 209–217.
17. Goldstein, I. J.; Hay, G. W.; Lewis, B. A.; Smith, F. *Methods Carbohydr. Chem.* **2005**, *5*, 361–369.
18. Gorin, P. A. J.; Horitsu, K.; Spencer, J. F. T. *Can. J. Chem.* **1965**, *43*, 950–954.
19. Tabata, K.; Ito, W.; Kojima, T.; Kawabata, S.; Misaki, A. *Carbohydr. Res.* **1981**, *89*, 121–135.
20. Yoshioka, Y.; Tabeta, R.; Saito, H.; Uehara, N.; Fukuoka, F. *Carbohydr. Res.* **1985**, *140*, 93–100.
21. Chauveau, C.; Talaga, P.; Wieruszeski, J. M.; Strecker, G.; Chavant, L. *Phytochemistry* **1996**, *43*, 413–415.
22. Hall, L. D.; Johnson, L. F. *J. Chem. Soc., Chem. Commun.* **1969**, 509–510.
23. Gorin, P. A. *J. Adv. Carbohydr. Chem. Biochem.* **1981**, *38*, 13–104.
24. Sasaki, T.; Takasuka, N. *Carbohydr. Res.* **1976**, *47*, 99–104.
25. Kato, K.; Mutoh, K.; Egashira, T.; Hiura, M.; Ueno, Y. *Agric. Biol. Chem.* **1978**, *42*, 1073–1074.
26. Bohn, J. A.; BeMiller, J. N. *Carbohydr. Polym.* **1995**, *28*, 3–14.