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Isolation and characterization of polysaccharides from *Agaricus blazei* Murill

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Abstract

An efficient route has been developed to isolate water soluble polysaccharides from *Agaricus blazei* Murill mushroom of Brazilian origin. Using different temperatures: 10, 60 and 100 °C, and precipitation with ethanol, a number of precipitates were extracted which were isolated, purified and fractionated for characterization. The molar mass distribution for the precipitates and fractions from the extraction at 100 °C were determined by GPC. Analysis of the FTIR, ^{1}H NMR and ^{13}C NMR spectra showed a pattern characteristic of glucan–protein complex.

Keywords: *Agaricus blazei* Murill; Polysaccharides; FTIR; NMR

1. Introduction

Mushrooms have been used for many years in oriental culture as tea and nutritional food and because of their special fragrance and texture (Manzi, Gambelli, Marconi, Vivante, & Pizzoferrato, 1999). Their biochemical composition, with significant content of protein, carbohydrate, lipids, enzymes, minerals, vitamins and water, has attracted attention also as a functional health food (Muchove & Muchove, 1989).

The scientific community, in searching for new therapeutic alternatives, has studied many kinds of mushrooms and has found variable therapeutic activity such as anti-carcinogenic, anti-inflammatory, immuno-suppressor and antibiotic, among others (Asfors & Ley, 1993; Longvah & Deosthale, 1998). The *Basidiomycete* and *Ascomycete* class are the most studied because of their food properties, and their low or even non-toxicity to man (Bach et al., 1998; Ebina & Fujimiya, 1998; Wade & Mizuno, 1997).

Numerous mushrooms (Yoshioka, Tabeta, Saitó, Uehara, & Fukuoka, 1985) have been studied for their anti-tumor activity. Extracts of these mushrooms have been clinically used as drugs for therapy (Liu, Ooi, Liu, & Chang, 1996; Ohno, Suzuki, & Yadomae, 1986). Despite their origin, this effect is generally attributed to polysaccharides with 1,3-β-glucan structures (Yoshioka et al., 1985) or a β-glucan–protein complex (Kawagishi et al., 1990). However, the precise structure has not been fully characterized.

The mushroom *Agaricus blazei* Murill, popularly known as 'Cogumelo do Sol' is originally native from Brazil in Piedade, state of S. Paulo. Its anti-tumor and anti-carcinogenic activities have been considered and extensive research has been carried out to elucidate the polysaccharide structure and its relationship to the anti-tumor activity (Grosev, Bozac, & Puppels, 2001; Mizuno et al., 1990; Wannet, Hermans, Drift, & Huub, 2000).

The aim of the present study is to find an efficient route to extraction, isolation and fractionation of the polysaccharides present in the *Agaricus* mushroom and also its structural characterization, as a contribution to better understanding of the chemical nature of these substances as well as other active components. Analysis of chemical

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properties and FTIR and NMR spectra were used to characterize these substances.

2. Experimental

2.1. Materials and methods

The raw material was donated by Guinish Commerce of Importation and Exportation of Nourishing Products Ltd, Susano (S. Paulo). The dried material was cut in slices and packed in plastic bags under vacuum. The mushrooms were pulverized for the experiments.

2.2. Extraction and purification

The dried mushrooms were dissolved in distilled water at 5% (w/w) using different temperatures (10, 60 and 100 °C) and time (1–5 h). The same experiment at 60 °C was performed in a basic medium. The suspensions were centrifuged and clear colorless orange extracts were obtained. The filtrates were neutralized to pH 7.0 by addition of diluted NaOH solution. To the filtrate was added NaCl—1.0% w/v (where w is the weight of NaCl and v the volume of the extract) to induce subsequent precipitation of the polysaccharides in ethanol. It was added ethanol/solution of the extract, 5:1, to obtain the precipitates, which were isolated from the ethanolic medium by centrifugation, washed with acetone and dried. Hydrogen peroxide (30%) was used as a 1:1 ethanol/peroxide mixture for approximately 15 h at ambient temperature for the clarification of the extracted material. Due to its partial solubilization the materials were submitted to a second extraction using ethanol/volume of the extract (4:1). All the precipitates (from initial solubilization at 10, 60 and 100 °C) were isolated by centrifugation, washed with ethanol then acetone, and dried in a sand bath at 40 °C.

To purify the samples from possible insoluble substance, the isolated materials were dissolved in distilled water with stirring at 40 °C for 48 h. After this procedure the suspensions were centrifuged and the soluble phase was removed and submitted to lyophilization.

2.3. Fractionation

The dried sample extracted at 100 °C was dissolved in distilled water with stirring at ambient temperature. The temperature was then increased to 40 °C with stirring for 24 h and then cooled down to ambient temperature. Ethanol was slowly added with moderate stirring until the solution became turbid and stable. The resulting suspension was then heated at 50 °C, in a sand bath for 15 h, to promote solubilization of the fractions. After this procedure, the system was cooled down to ambient temperature. The fractionated portion was isolated by centrifugation (6000 rpm for 20 min) and dried with acetone in sand bath at 40 °C. The remained liquid phase was then submitted to the same procedure to isolate further fractions. Nine fractions were isolated using the procedure previously described, of which the first four, designated f1, f2, f3 and f4, fractionated from the precipitate extracted at 100 °C were analyzed (Paula, Heatley, & Budd, 1998).

2.4. Molar mass determination by gel permeation chromatography (GPC)

Solutions from the isolated precipitates extracted at 100 °C and the fractions were analyzed by GPC. A Shimadzu LC-10AD-GPC instrument with a differential refractometer used as detector and UV–vis SPD-10A to detect the presence of protein (280 nm) was used. Ultragel linear columns (7.8 × 300 mm) were employed with 0.1 M $NaNO_3$ solution as eluent and a flow rate of 0.5 ml/min. The calibration curve was constructed using pullulan standards (molar mass from 10^3 to 10^6 g/mol).

2.5. Structural characterization by FTIR, ^{13}C NMR and ^{1}H NMR spectroscopy

Raw mushroom and the isolated and fractionated samples were analyzed by transmittance IR spectroscopy in the form of KBr discs using a Perkin Elmer Spectrum 1000 FTIR spectrometer. ^{1}H and ^{13}C NMR spectra of solutions in D_2O were recorded at ambient temperature using a Varian Associates Unity 500 spectrometer operating at 500 MHz for ^{1}H and 125.8 MHz for ^{13}C.

2.6. Others constituents

The total carbohydrate content was determined for the precipitate which had been extracted, and for the f1, f2, f3 and f4 fractions isolated at 100 °C using the phenol–sulphuric acid spectrophotometric method (Dubois, Gilles, Hamilton, & Rebers, 1956) with glucose as standard. Absorbance measurements were made using a HP 8453 UV–vis spectrophotometer with a diode array detector. The protein content was determined using the Kjeldahl method (Marks, Buchsbaum, & Swain, 1985). The conversion factor utilized was 4.38, which has been established for some fungi species (Chang, Chao, Chen, & Mau, 2001; Manzi et al., 1999). The elemental analysis for C, N, H, and S was made with a Carlo Erba Model 1110 CHNS/O micro-analyzer equipped with a thermal conductivity detector.

3. Results and discussion

3.1. Isolation and purification

It was believed, from the experimental conditions used, that the best extraction results were restricted to temperature

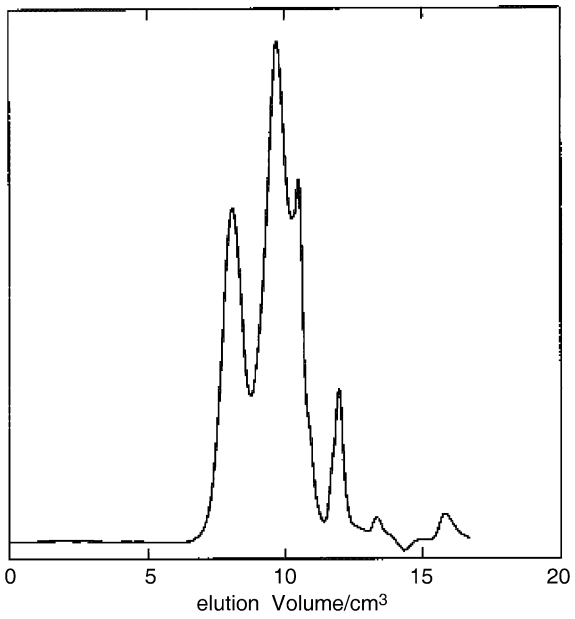

Fig. 1. GPC chromatogram of the polysaccharide extracted at 100 °C.

conditions of 10 and 100 °C for 5 h, which resulted in 6.3 and 11.6% yields, respectively. The insoluble material remaining after purification corresponded to 10% of the precipitate.

3.2. Molar mass distribution by GPC

Fig. 1 shows the GPC chromatogram of the polysaccharide extracted at 100 °C. According to a calibration curve with pullulan standards, the peaks with elution volume of 8.09 and 9.70 cm^3 corresponded to molar masses of 4.55×10^5 and 7.99×10^3 g/mol, respectively. These two peaks also showed UV absorption (280 nm) characteristic of the presence of protein.

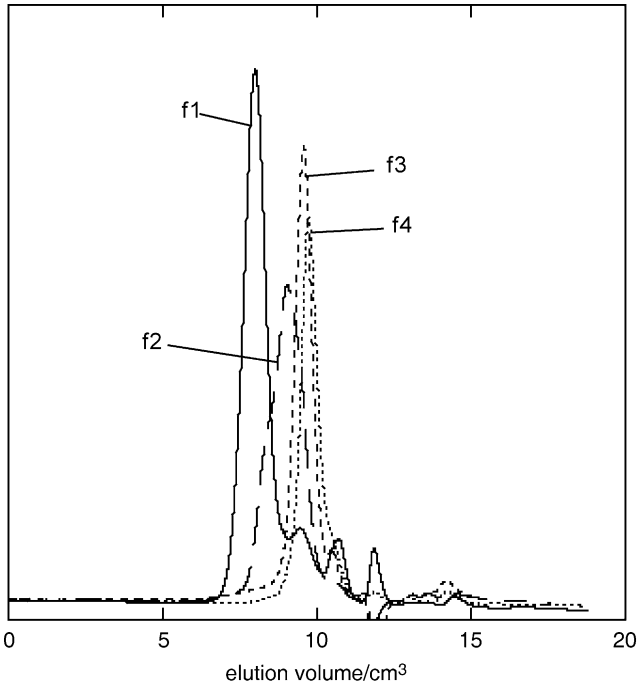

Fig. 2. GPC chromatogram of the fractions of the polysaccharide extracted at 100 °C.

Table 1
GPC elution volumes and corresponding molar masses for f1, f2, f3 and f4 fractions of the polysaccharide extracted at 100 °C

Fraction	Elution volume (cm^3)		Molar mass (g/mol)
	RID detector	UV detector	
f1	7.99	7.78	5.75×10^5
f2	9.05	9.15	4.08×10^4
f3	9.58	9.61	1.07×10^4
f4	9.73	9.71	7.74×10^3

The molar mass distribution was also analyzed for the samples (f1, f2, f3 and f4) fractionated from the precipitate extracted at 100 °C. The GPC chromatograms are presented in Fig. 2. More than one peak was observed for the four fractions, the first one being more representative. Due to limitations of the calibration curve it was possible to determine the molar mass distribution only for the main peak. A response from UV detector (280 nm) was also observed, probably indicating the presence of glucan–protein complex. Table 1 shows the representative values obtained from the analysis of the GPC chromatograms of the fractions with their respective molar mass.

3.3. FTIR spectroscopy

Infrared spectra of precipitates isolated at 10, 60 and 100 °C showed the same pattern and were similar to those of carbohydrates and proteins. Therefore, the IR spectra of the precipitate extracted at only 100 °C is presented (Fig. 3). The attributions of the main absorptions are characteristic of glycosidic structures and are related to OH stretching (3000–3500 cm^{-1}); CO stretching (1028 cm^{-1}); C–O–C stretching (1165 cm^{-1}) and anomeric C_1H group vibration (1074 cm^{-1}). Likewise protein patterns were also observed with characteristic absorptions at 1654, 1544 and 1409 cm^{-1}

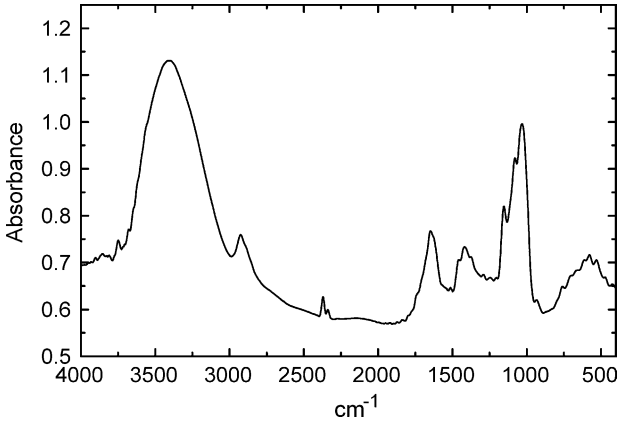

Fig. 3. FTIR spectra of the isolated precipitate extracted at 100 °C.

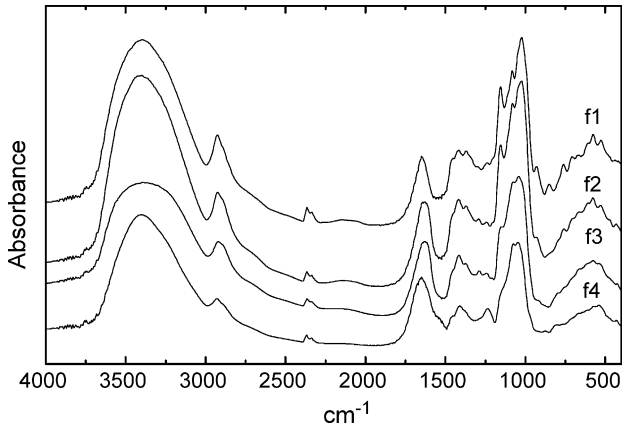

Fig. 4. FTIR spectra of the isolated fractions from the precipitate extracted at 100 °C. (a) f1; (b) f2; (c) f3; and (d) f4.

(Carey, 1992). On the other hand, N–H vibration generally expected at 3400 cm^{-1} could be overlapped by OH stretch vibration at 3410 cm^{-1}. Protein structures could also be represented by the absorption at 1242 cm^{-1}. These attributions may suggest the presence of glucan–protein complex in both samples. It can also be observed that the difference of the relative intensity of the glycosidic and protein groups may reflect the predominance of the protein content at extraction at 10 °C.

The FTIR spectra of the four fractions obtained from the extract at 100 °C (Fig. 4) retain the same characteristic features of the original precipitate, despite the difference on the relative intensity of glycosidic and protein groups absorptions. It seems that carbohydrate absorption bands at 1155 cm^{-1} (νC–O–C), 1080 cm^{-1} (νC_1H), and 931 cm^{-1} (δC_1H) decreases progressively and the characteristic absorption bands related to anion carboxylate (1647 and 1409 cm^{-1}) increase from f1 to f4 fractions. It was also

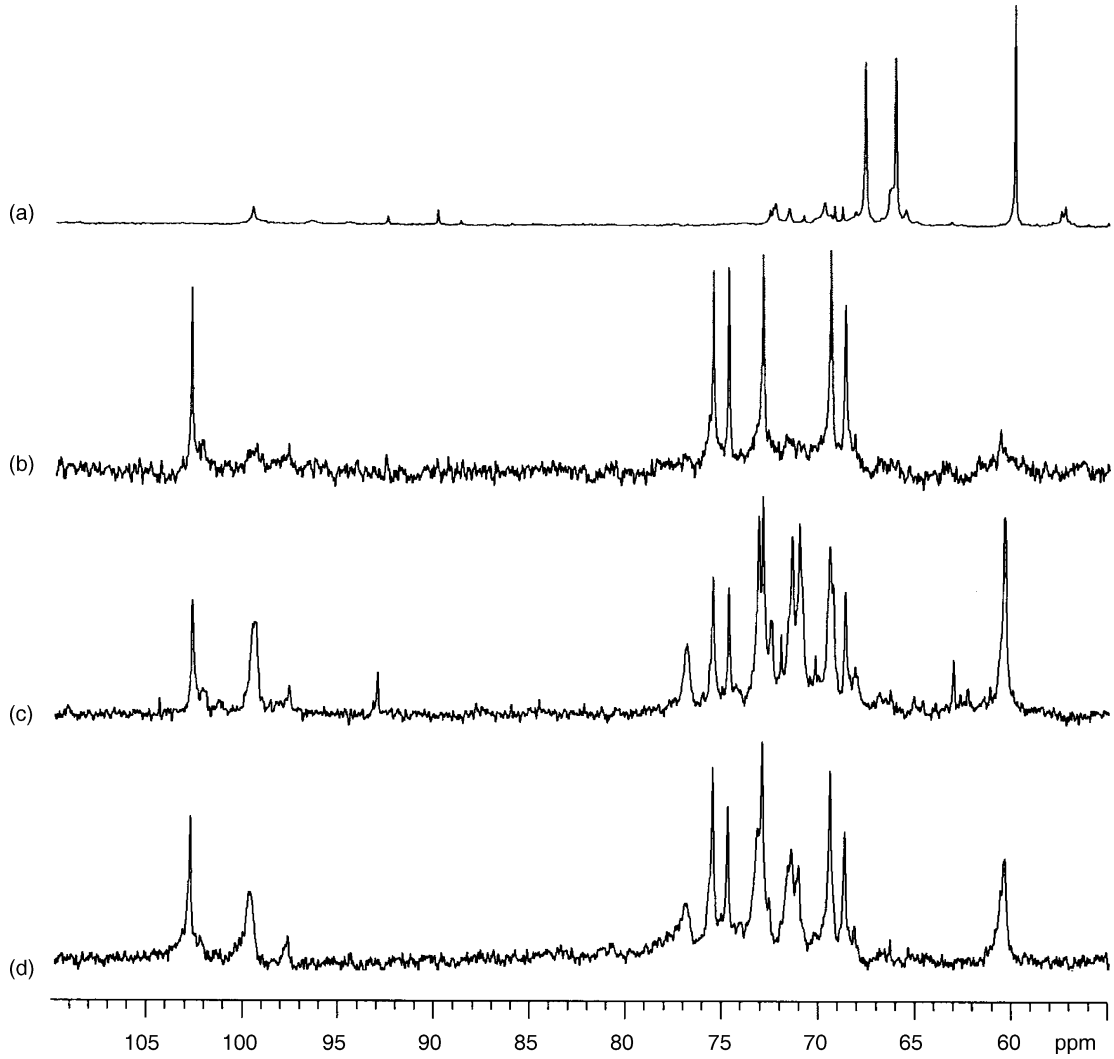

Fig. 5. ^{13}C NMR spectra. (a) Raw mushroom; (b) purified polysaccharide isolated at 10 °C; (c) extract isolated in basic medium; (d) purified polysaccharide isolated at 100 °C.

observed that the band at 1242 cm^{-1} related to protein functionalities increases progressively.

3.4. ^{13}C NMR spectroscopy

^{13}C NMR spectra of raw mushroom and the precipitate which had been isolated from the experiments were recorded for aqueous solutions in D_2O. The spectra are shown in Fig. 5.

The raw material is composed of many diverse constituents, so the assignment of the peaks observed in Fig. 5(a) is difficult. From the spectra of the extracted precipitate shown in Fig. 5(b)–(d), the pattern of carbohydrate structure is clearly more defined. The general representative peaks of carbohydrate (Kawagishi et al., 1990) were assigned as follows: anomeric carbon (C1) signals of glycosides assigned to 100–104 ppm and C2, C3, C4, C5 and C6 from glycosidic ring to 60–80 ppm.

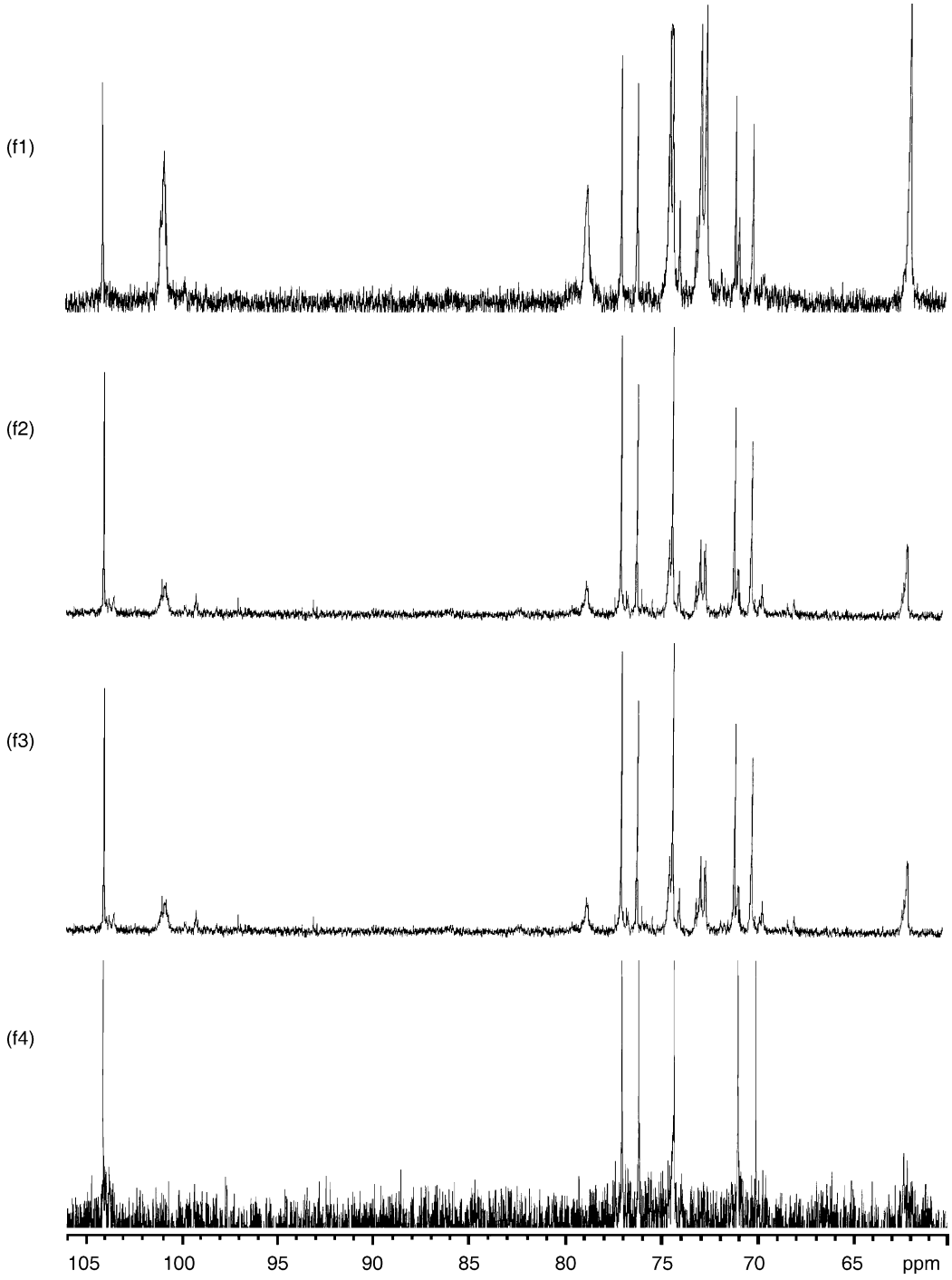

Fig. 6. ^{13}C NMR spectra of fractions. (a) f1; (b) f2; (c) f3; and (d) f4.

Table 2
^{13}C NMR assignments for β(1→6) and α(1→4)-glucan configurations observed in the purified polysaccharides extracted at 10 and 100 °C for 5 h

Configuration	Chemical shift (ppm)					
	C1	C2	C3	C4	C5	C6
β(1→6)[a]	103.0	73.1	75.6	69.5	74.9	68.8
α(1→4)[a]	99.8	71.5	75.3	78.7	71.2	60.1
β(1→6)[b]	103.0	73.1	75.6	69.5	74.9	68.8
α(1→4)[b]	100.5	71.5	75.3	76.7	–[c]	60.7

[a] Extraction at 100 °C.
[b] Extraction at 10 °C.
[c] Not clearly visible.

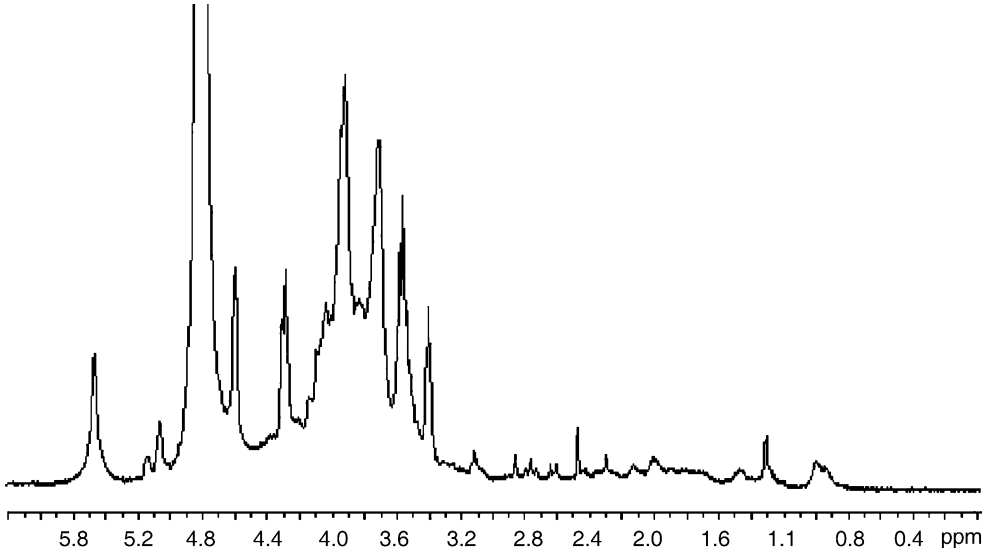

Fig. 7. ^{1}H NMR spectrum of precipitate extracted at 100 °C. The intense peak at 4.8 ppm is from residual HDO in the solvent.

The presence of additional peaks in the range 20–40 ppm (not shown) may suggest the consideration of the presence of glucan–protein structure. The anomeric carbon sign of both α and β configurations were detected at 100 and 104 ppm, respectively (Ohno et al., 1986). Comparison of the spectra, except for the raw material, showed α and β-glucan signals, the β-glucan (which is considered to have anti-tumor activity) being the more abundant. It can also be observed that extraction at 100 °C (Fig. 5(d)) was efficient in isolating β-glucans. The α-glucan signal in this spectra had almost disappeared. Also for the extraction carried out at basic pH, the α-glucan signal was less well resolved.

Fig. 6 shows ^{13}C NMR spectra of the fractions f1, f2, f3 and f4; the peak assignment is given in Table 2. Polysaccharides in β(1→6) and α(1→4) configurations were observed in f1, the α(1→4) configuration showing a significant reduction on the fractionation proceeded.

3.5. ^{1}H NMR spectroscopy

The ^{1}H NMR spectra of the precipitates extracted at 10, 60 and 100 °C have the same pattern and therefore the spectrum of the precipitate extracted at 100 °C only is shown in Fig. 7. The spectrum is well representative of protons from glycosidic groups of carbohydrates. ^{1}H chemical shifts in the anomeric region are observed at 4–6 ppm. A doublet at 4.1 and 4.5 ppm was also observed corresponding to the β configuration and in the region 4.9–5.6 ppm corresponding to α configuration (Kawagishi et al., 1990). The chemical shifts from 3.3 to 4.0 ppm were assigned to protons of carbons C2 to C6 of glycosidic ring (Chauveau, Talaga, Wieruszeski, Strecker, & Chavant, 1996). It seems that at 2.78 ppm it is possible to detect a chemical shift which could be assigned to protein groups related to the glucan–protein structure. These attributions were considered once the groups N–CH_3 and N–H are generally observed at 0.5–3.0 ppm, respectively (Silvestein, Bassler, & Morril, 1994).

Table 3
Carbohydrate content in the isolated precipitate and its fractions extracted at 100 °C

Sample	Carbohydrate (wt%)
Isolated precipitate	37.0
f1	41.7
f2	39.6
f3	33.8
f4	15.6

3.6. Others constituents

The carbohydrate content in the precipitate and its fractions are shown in Table 3. The results observed for the carbohydrate content in the fractions reinforces the analysis suggested by the infrared analysis, in which the concentration decreases with increasing collection time. The determination of proteins by the Kjeldahl method gave a 28.5 wt% content for the raw mushroom. From the nitrogen percentage given by the elemental analysis, protein contents of 24.6 and 25.3 wt% were obtained for the raw mushroom and isolated precipitated, respectively. The protein content of the samples that have been analyzed were similar and comparable with the published values for various species of mushrooms (Longvah & Deosthale, 1998; Manzi et al., 1999).

4. Conclusions

A polysaccharide-complex was isolated from the extraction conditions used. The best extraction results were those realized at 100 °C. The isolated precipitates were identified by FTIR, ^{13}C and ^{1}H NMR as a glucan–protein complex, presenting α and β glycosidic linkage. β-Glucans being predominant than α-glucans, to which the anti-tumor activity may be attributed. The protein content from the glucan–protein fractionation increased as the fractions (f1–f4) were extracted.

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