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Analytical Methods

Characterization of a heterogalactan: Some nutritional values of the edible mushroom *Flammulina velutipes*

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Abstract

Production and consume of mushrooms have grown in the world, and beside these, the nutritional properties and biological active components of fungi have received more attention by researchers. Considering these, a mannofucogalactan was isolated from *Flammulina velutipes*, and characterized using ^{13}C and ^{1}H (obs.), ^{13}C HMQC nuclear magnetic resonance spectroscopy and methylation analysis. The monosaccharide composition of this polymer was determined by GC–MS and showed Fuc*p*, Man*p*, and Gal*p* in the molar ratio 20:16:64, respectively. ^{13}C NMR and ^{1}H (obs.), ^{13}C HMQC indicated an anomeric region containing signals (C-1/H-1) at δ 102.9/5.19, 102.0/5.16, and 98.8/5.05 corresponding, sequentially, to non-reducing end of α-D-Man*p*, 3-*O*-substituted α-L-Fuc*p*, and 6-*O*- and 2,6-di-*O*-substituted α-D-Gal*p* units. Along with methylation analysis, these data showed a structure with a main chain composed of 6-*O*-substituted Gal*p* units, partially substituted at O-2 by 3-*O*-D-mannopyranosyl-L-fucopyranosyl, α-D-mannopyranosyl, and in a minor proportion, α-L-fucopyranosyl groups. Furthermore, some nutritional values of this edible mushroom were evaluated, like amino acid and mineral nutrient contents.

Keywords: *Flammulina velutipes*; Mushroom; Heterogalactan

1. Introduction

Fungi have been utilized, for many years, in Asian countries like a very nutritive food and medicine. In 1978, the world mushroom production was about 1.1 million tones, and this production rose to 6.16 million tones, in 1997 (Chang, 2005). Now, consume of mushrooms are growing worldwide due to influence of oriental culture and studies about their nutritional values and pharmacological properties (Guterrez, Mantovani, Eira, Ribeiro, & Jordão, 2004; Yang, Lin, & Mau, 2002). Researchers are considering the edible basidiomycetes like health foods because they are good sources of vitamins, minerals, proteins, carbohydrates, high amounts of fibers and they are poor in fat (Manzi, Gambelli, Marconi, Vivanti, & Pizzoferrato, 1999). Considerable amounts of the essential amino acids and minerals like potassium, calcium and magnesium were observed in various species of *Pleurotus* (Akindahunsi & Oyetayo, 2006; Manzi et al., 1999). From many studies about these basidiomycetes, a number of fungal components have demonstrated biological activities, and among these, polysaccharides have received more attention as therapeutic molecules. An example are β-glucans, commonly extracted from mushrooms, which could present, normally, backbones of β-(1 → 3)- and/or β-(1 → 6)-linked D-glucopyranosyl units. These molecules showed biological effects, like protection against free-radical oxidation (Toklu et al., 2006), increased host responses against tumor or

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infections (Brown & Gordon, 2003; Leung, Fung, & Choy, 1997; Zheng, Jie, Hanchuan, & Moucheng, 2005) among others.

There are few studies about heteropolysaccharides extracted from macrofungi. Some of them, which are described as heterogalactans, heteromannans, and heteroglucans (Schepetkin & Quinn, 2006), have been tested in vitro and/or in vivo, and demonstrated biological activities like proliferation of lymphocytes, antitumor, and/or anticoagulant activities (Cho, Koshino, Yu, & Yoo, 1998; Ikekawa et al., 1982; Yoon et al., 2003).

Flammulina velutipes (Curt. ex Fr.) Sing. is one of the most popular edible mushrooms in Japan, and its morphological features are influenced by the incidence of light and temperature (Sakamoto, Ando, Tamai, & Yajima, 2007; Sakamoto, Tamai, & Yajima, 2004). Glucans and some heteropolysaccharides were isolated from this basidiomycete and some of them presented antitumor activity (Ikekawa et al., 1982; Leung et al., 1997; Mukumoto & Yamaguchi, 1977; Yoshioka, Sano, & Ikekawa, 1973). Smiderle et al. (2006) characterized a xylomannan from *F. velutipes*, that consisted of a main chain of (1 → 3)-linked α-Man*p* units, partially substituted at *O*-4 with single unit side chains of β-Xyl*p*, or of β-Xyl*p*-(1 → 3)-β-Xyl*p* groups. We now have fractionated extracts of *F. velutipes* fruiting bodies, obtained and characterized structurally a heteropolysaccharide described as a mannofucogalactan. Beside these, some nutritional values of this mushroom were analyzed.

2. Experimental

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 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-225 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. The homogeneity and molar mass of 30E fraction were determined by high-performance size-exclusion chromatography (HPSEC–MALLS), using a Waters 510 HPLC pump at 0.6 ml/min, with four gel permeation columns in series with exclusion sizes of 10^6–5×10^3 Da, using a refractive index (RI) detector. Poly (ethylene oxide) of $M_w = 11{,}600$ was used as standard to calibrate the columns. The eluent was 0.1 mol/l aq. $NaNO_2$ with 200 ppm aq. NaN_3. Eluted fraction (30E) was dissolved in the eluent, filtered in membrane (0.22 μm), and injected (100 μl loop) at a 1 mg/ml concentration.

2.2. Fungal material

F. velutipes is recognized by a sticky, orange-brown cap of 1–3 cm broad and dark, finely pubescent stipe of 1.5–7 cm tall and 0.2–0.7 cm thick. The basidiomycete was purchased at the Public, Municipal Market of Curitiba, State of Paraná (PR), Brazil, and was identified by the Prof. Dr. Fábio Rosado from the Centro Universitário de Maringá (CESUMAR), Maringá-PR.

2.3. Polysaccharide extraction and purification

The dried fungus (88 g) was milled and submitted to aqueous extraction (3× at 100 °C, 1000 ml).

The aqueous extract was evaporated to a small volume and polysaccharide precipitated by addition to excess EtOH (3:1, v/v). The product was dialyzed against tap water for 48 h, concentrated under reduced pressure and freeze-dried. The extract was then dissolved in water and the solution submitted to freezing followed by mild thawing at 4 °C. The soluble fraction (SW), following centrifugation, was treated with Fehling solution (100 ml) and the insoluble Cu^{2+} complex formed was isolated by centrifugation. Both complex (FP) and supernatant (FS) were neutralized with HOAc, dialyzed against tap water and deionized with Dowex 50 × 8 (H^+ form) ion-exchange resin (Fig. 1).

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 res-

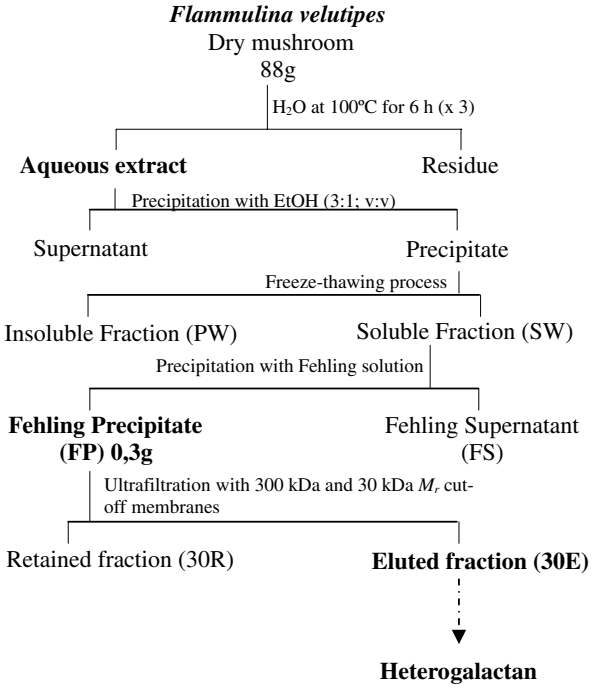

Fig. 1. Extraction and purification of heterogalactan (30E).

idue was successively reduced with excess of $NaBH_4$ and acetylated with Ac_2O–pyridine (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 GC–MS as indicated above and identified by their typical retention times and electron impact profiles.

2.5. Methylation analysis

Per-*O*-methylation of the isolated polysaccharide (1 mg each) was carried out using powdered NaOH in DMSO–MeI (Ciucanu & Kerek, 1984). The product was treated with formic acid 45% at 100 °C for 15 h, followed by evaporation to dryness (Stortz, Cases, & Cerezo, 1997). The residues were converted into partially *O*-methylated alditol acetates, and analyzed by GC–MS as described above.

2.6. NMR analyses

^{13}C 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 D_2O and examined at 70 °C. Chemical shifts are expressed in ppm (δ) relative to resonance of acetone at δ 30.20 (^{13}C) and 2.22 (^{1}H).

2.7. Minerals

The mineral element contents were determined according to the AOAC. (2000) procedures, methods 999.10/999.11/986.15.

2.8. Total amino acids

Amino acid contents of basidiomycete were determined, following hydrolysis, using a Beckman 6300 amino acid analyzer, incorporating System Gold software.

3. Results and discussion

A dry sample (88 g) of *F. velutipes* was submitted to hot aqueous extraction at 100 °C and treated with excess of ethanol (3:1, v/v) and the resulting precipitate was separated for centrifugation, dialyzed against tap water, and freeze-dried (Fig. 1). The soluble fraction obtained from this process was a mixture of polysaccharides and it contained fucose, xylose, manose, galactose and glucose in a molar ratio of 10:9:25:28:28, respectively.

This fraction was purified by treatment with Fehling solution, giving another sample (FP), which was purified by ultrafiltration with membranes of 300 and 30 kDa M_r cut-off, sequentially. This purified fraction (30E) contained fucose, mannose, and galactose (20:16:64, respectively) as monosaccharide components, consistent with a heterogalactan.

In order to elucidate the linkage type of the polymer isolated, it was methylated and converted to mixtures of partially *O*-methylated alditol acetates, which were analyzed by GC–MS. It was characterized as a partially branched polysaccharide by the presence of 2,3,4-Me_3-Fuc*p* (7%), 2,3,4,6-Me_4-Man*p* (14%), and 2,3,4,6-Me_4-Gal*p* (3%). The presence of the 2,4-*O*-Me_2-Fuc*p* (12%) derivative indicates that Fuc*p* was substituted at *O*-3. The 2,3,4-*O*-Me_3-Gal*p* (37%), and 3,4-*O*-Me_2-Gal*p* (27%) derivatives compose the (1 → 6)-linked main chain.

The ^{13}C NMR and HMQC spectra (Fig. 2) contained three principal C-1/H-1 signals that correspond to α-D-mannopyranosyl non-reducing end (δ 102.9/5.19), α-L-fucopyranosyl (δ 102.0/5.16), and 6-*O*- or 2,6-*O*-substituted α-D-galactopyranosyl units (δ 98.8/5.05). The α-configurations of these units could be confirmed by the signals of H-1 in low-field (Table 1). The ^{13}C NMR spectrum showed a signal in δ 16.4 from the methyl group (C-6) of the fucopyranosyl residues. Signals at δ 74.1/3.85, 67.6/3.77, 3.98, and 62.0/3.84 referred to C-5/H-5 of α-D-Man*p* non-reducing end, C-6/H-6 of 6-*O*-substituted α-D-Gal*p*, and C-6/H-6 of α-D-Man*p* non-reducing end units, respectively, (Alquini, Carbonero, Rosado, Cosentino, & Iacomini, 2004; Cho et al., 1998). In according to

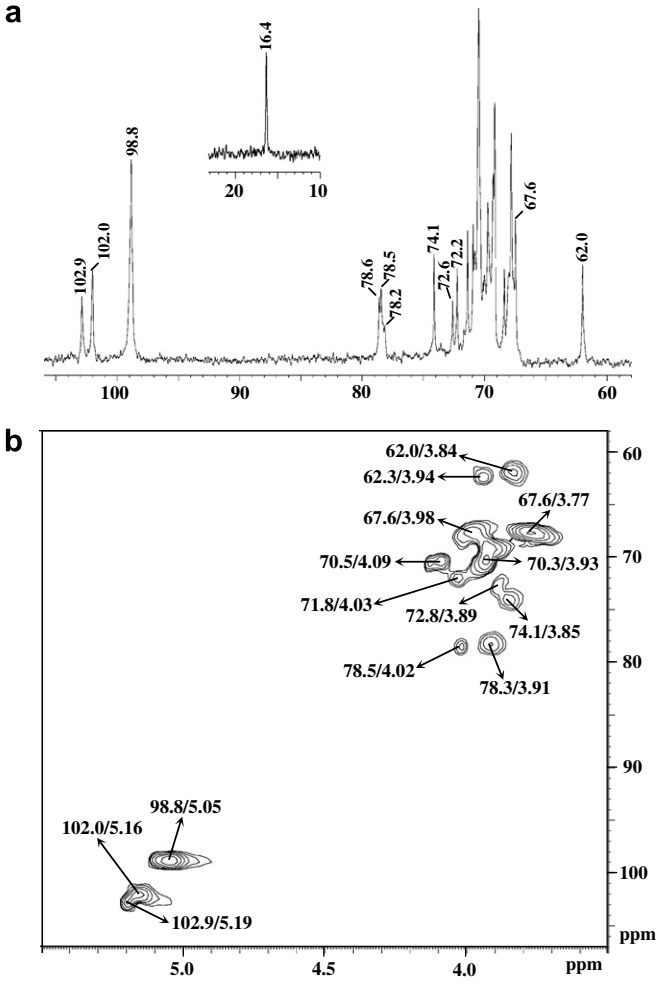

Fig. 2. ^{13}C NMR (a) and HMQC (b) spectra of heterogalactan, in D_2O at 70 °C, chemical shifts are expressed in ppm.

Table 1
Assignments of signals in the NMR analyses of heterogalactan from *F. velutipes*

Signal (C/H)[a]	Assignment
102.9/5.19	C-1/H-1 of D-mannopyranosyl non-reducing end units
102.0/5.16	C-1/H-1 of L-fucopyranosyl units
98.8/5.05	C-1/H-1 of 2-*O*-substituted D-galactopyranosyl units
78.5/4.02	C-2/H-2 of 2-*O*-substituted D-galactopyranosyl units
78.3/3.92	C-3/H-3 of 3-*O*-substituted L-fucopyranosyl units
74.1/3.85	C-5/H-5 of D-mannopyranosyl non-reducing end units
67.6/3.77, 3.98	C-6/H-6 of 6-*O*-substituted D-galactopyranosyl units
62.0/3.84	C-6//H-6a, b of D-mannopyranosyl non-reducing end units
16.4/1.30	CH_3-6/CH_3-6 of L-fucopyranosyl units

[a] The chemical shifts are expressed as ppm (δ).

Table 2
Mineral element contents of the edible mushroom *F. velutipes*

Mineral contents (mg/100 g)	
Ca	117.55
K	2897.59
P	940.33
Na	75.45
Mg	143.04
Mn	0.96
Fe	9.63
Zn	6.77
Se	<0.50[a]
Li	<0.20[a]

[a] Li and Se in mg/kg and the others minerals in mg/100 g.

data, the polysaccharide characterized showed a main chain of (1 → 6)-linked α-D-Gal*p* units, partially substituted at O-2 by 3-*O*-D-mannopyranosyl-L-fucopyranosyl, α-D-mannopyranosyl, and in a minor proportion, α-L-fucopyranosyl groups (Fig. 3).

A polysaccharide with the same main chain but substituted at O-2 only by 3-*O*-D-mannopyranosyl-L-fucopyranosyl groups was extracted from the fruit bodies of *Fomitella fraxinea* (Cho et al., 1998). Alquini and co-workers (2004) described a similar structure, but with different molar ratio of mannose, fucose and galactose, for the basidiomycete *Laetiporus sulphureus*. There are few studies about the structure of these heteropolymers due to difficulty in purified or characterize them. However, it would be encouraged, since there are reports that heteropolysaccharides can present physiological benefits (Cho et al., 1998; Ikekawa et al., 1982; Yoon et al., 2003).

Ko, Liu, Tsang, and Hsieh (2007) valuated the total of carbohydrate (58.0%), protein (27.5%), fat (7.0%), and ash (7.4%) of *F. velutipes*. Since ash account a considerable quantity, the mineral nutrient content was analyzed and is given in Table 2. The high levels of potassium (2897.59 mg/%) and phosphorus (940.33 mg/%) can be associated with wood-decomposing fungi (Sanmee, Dell, Lumyong, Izumori, & Lumyong, 2003). The first one is the most abundant mineral encountered in various species of edible mushrooms (Akindahunsi & Oyetayo, 2006; Manzi et al., 1999). Others macronutrients observed were calcium (117.55 mg/%) and magnesium (143.04 mg/%). The low concentration of sodium (75.45 mg/%) and high amounts of potassium, which can lower blood pressure, is a good reason that mushrooms are recommended for heart patients or those on salt restricted diets (Akindahunsi & Oyetayo, 2006; Manzi et al., 1999). The micronutrients encountered, in minor proportions, were Fe, Zn, Mn, Se and Li, important for a supplementary diet.

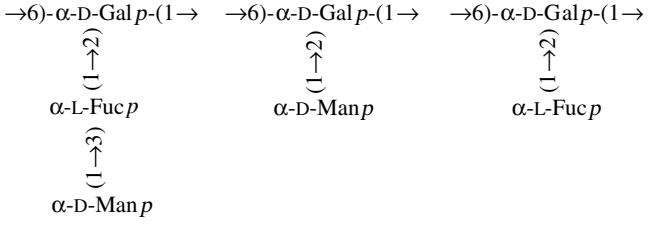

Fig. 3. Fragments of structure of the mannofucogalactan isolated from *F. velutipes*.

The amino acid content was availed too and it is reported as the percentage of the sum of amino acids (Table 3). The most abundant compounds are lysine and glycine. The first one is an essential amino acid limiting in most vegetable proteins. The others components that are present in a considerable quantity are asparagine and glutamine, which are backbones precursors for make the others amino acids, and they are storage forms of nitrogen (Akindahunsi & Oyetayo, 2006). From 17 amino acids encountered in this basidiomycete, six of them, which are essential and have important nutritional value, are present in considerable quantity. According to Chiang, Yen, and Mau (2006), *F. velutipes* is a good source of free amino acids, especially GABA that is a non essential amino acid that functions as a neurotransmitter. The results presented above show that these mushrooms are good sources of carbohydrates, proteins, fibers, essential amino acids and minerals, and the consume of them must be encouraged.

Table 3
Amino acid composition[a] of *Flammulina velutipes*

Amino acid	Molar composition (mg/g)
Glutamine	9.975
Serine	7.686
Glycine	28.482
Histidine	1.456
Arginine	3.880
Threonine	10.047
Alanine	7.591
Proline	4.947
Tyrosine	3.471
Valine	6.539
Methionine	3.108
Cysteine	8.760
Isoleucine	5.090
Leucine	5.404
Phenylalanine	5.654
Lysine	30.896

[a] Determined by amino acid analyzer.

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