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Lentinus edodes heterogalactan: Antinociceptive and anti-inflammatory effects

E.R. Carbonero [a], A.H.P. Gracher [a], D.L. Komura [a], R. Marcon [b], C.S. Freitas [c], C.H. Baggio [c], A.R.S. Santos [d], G. Torri [e], P.A.J. Gorin [a], M. Iacomini [a,*]

[a] *Departamento de Bioquímica e Biologia Molecular, Universidade Federal do Paraná, CP 19046, CEP 81531-980 Curitiba, PR, Brazil*
[b] *Departamento de Farmacologia, Centro de Ciências Biológicas, Universidade Federal de Santa Catarina, Campus Universitário, Trindade, Florianópolis 88049-900, SC, Brazil*
[c] *Departamento de Farmacologia, Universidade Federal do Paraná, CP 19031, CEP 81531-980 Curitiba, PR, Brazil*
[d] *Departamento de Ciências Fisiológicas, Centro de Ciências Biológicas, Universidade Federal de Santa Catarina, Campus Universitário, Trindade, Florianópolis 88040-900, SC, Brazil*
[e] *Istituto di Ricerche Chimiche e Biochimiche "G. Ronzoni", Via Colombo n. 81, 20133 Milan, Italy*

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ABSTRACT

Cold aqueous extraction of basidiocarps (fruiting bodies) of the edible mushroom *Lentinus edodes* (*shiitake*) gave rise to a heteropolysaccharide, whose chemical structure, antinociceptive and anti-inflammatory properties were determined. Its chemical structure was based on monosaccharide composition, methylation analysis, and NMR spectroscopy (^{1}H, ^{13}C, HSQC, HSQC-TOCSY, HSQC-NOESY, and coupled HMQC). It was found to be a fucomannogalactan with a main chain of (1 → 6)-linked α-D-galactopyranosyl units, partially substituted at O-2 by single-unit β-D-Man*p* or α-L-Fuc*p* side chains. The polysaccharide produced a marked and dose-related effect when assessed against acetic acid-induced visceral nociception. Prevention of peritoneal capillary permeability and leukocyte infiltration caused by the acetic acid was similar in potency and effectiveness.

1. Introduction

Mushrooms are rich in dietary fiber, minerals, and vitamins and low in fat (Manzi, Aguzzi, & Pizzoferrato, 2001). They have recently become attractive as food (physiologically functional) and as sources for the development of drugs (Manzi & Pizzoferrato, 2000). Some mushroom components can lower cholesterolemia, modulate the immune system, and inhibit tumor growth (Smith, Rowan, & Sullivan, 2002; Wasser, 2002). Extracts of many mushrooms, including *Lentinus edodes*, *Agaricus blazei*, *Ganoderma lucidum*, and *Grifola frondosa*, suppress tumor growth by controlling the immune system of the host (Schepetkin & Quinn, 2006). In the global market, *L. edodes* (*shiitake*) is the second most popular edible mushroom, its importance being attributed to both its nutritional value and medical application (Hatvani, 2001). Several important components, including biologically active polysaccharides (lentinan), dietary fiber, ergosterol, vitamin B1, B2 and C, and minerals have been isolated from its basidiocarp, mycelium, and culture medium (Choi et al., 2006). Lentinan, a β-glucan, is the most important polysaccharide isolated from *L. edodes,* because of its immunomodulatory and antitumor effects. Related structures that also have antitumor activity have been isolated from *G. frondosa* (grifolan) (Kato et al., 1983) and *Schizophyllum commune* (schizophyllan) (Tabata, Ito, & Kojima, 1981). These polysaccharides are now used in clinics in Japan, Korea, China, and other Asian countries (Zaidman, Yassin, Mahajna, & Wasser, 2005). Other important polysaccharides isolated from mushrooms are heteropolymers, such as heterogalactans, which, usually, have a main chain of (1→6)-linked α-D-galactopyranose that can be substituted at O-2 by L-Fuc*p*, 3-*O*-D-Man*p*-L-Fuc*p* or D-Man*p* groups. These structures occur in several basidiomycetes, namely *Flammulina velutipes*, *Ganoderma applanatum, Laetiporus sulphureus, Coprinus comatus*, among others (Alquini, Carbonero, Rosado, Cosentino, & Iacomini, 2004; Fan et al., 2006; Mukumoto & Yamaguchi, 1977; Usui, Iwasaki, & Mizuno, 1983).

Besides antitumor activity, anti-inflammatory effects have been reported. Glucans of *Trametes gibbosa* and *Dictyophora indusiata* were active *in vivo* against inflammation induced by carrageenan (Hara, Kiho, Tanaka, & Ukai, 1982; Poucheret, Fons, & Rapior, 2006). However, most of the investigations on anti-inflammatory action of mushrooms were carried out with crude polysaccharide extracts (Lindequist, Niedermeyer, & Jülich, 2005; Poucheret et al., 2006). We now determine the detailed chemical structure of a pure fucomannogalactan from basidiocarps of *L. edodes*, and

* Corresponding author. Tel.: +55 (41) 3361 1655; fax: +55 (41) 3266 2042.
E-mail address: iacomini@ufpr.br (M. Iacomini).

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investigate its antinociceptive and potential anti-inflammatory properties, using a model of inflammatory pain in mice.

2. Materials and methods

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 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 or 210 °C (constant temperature) was used for quantitative analysis of alditol acetates and partially *O*-methylated alditol acetates, respectively.

Ultrafiltration was performed on a filter holder (Sartorius – Model 16249), with compressed air at 10 psi as carrier gas.

NMR spectra (^1H, ^{13}C, DEPT, HSQC, HSQC-TOCSY, HSQC-NOESY, and coupled HMQC) were obtained using a 600 MHz Bruker Avance spectrometer incorporating Fourier transform. Analyses were performed at 40 °C on a polysaccharide sample dissolved in D_2O. Chemical shifts are expressed in δ relative to acetone at δ 32.77 (^{13}C) and 2.21 (^1H), referred to DSS (2,2-dimethyl-2-silapentane-3,3,4,4,5,5-d_6-5-sulfonate sodium salt; δ = 0.0 for ^{13}C and ^1H), in accordance with IUPAC recommendations.

2.2. Biological material

Fresh *L. edodes* (2 kg) was furnished by Makoto Yamashita Company (Miriam Harumi Yamashita), located in São José dos Pinhais, State of Paraná, Brazil. The basidiocarps were grown on sweet chestnut (*Castanea sativa*) logs.

2.3. Extraction and purification of the polysaccharide

Fresh basidiocarps of *L. edodes* (2 kg) were freeze-dried, resulting in 162 g of material, which were pulverized and their polysaccharides were extracted with water at 4 °C for 6 h (×5, 2000 ml). Each extract was filtered; the filtrate was collected, and centrifuged at 9000 rpm at 20 °C for 20 min, giving a clear solution. The combined aqueous extracts were evaporated to a small volume, followed by addition of excess EtOH (3:1; v/v). The polysaccharide precipitate was collected by centrifugation at 8500 rpm at 10 °C for 20 min, and was dissolved in H_2O, dialyzed against distilled water for 20 h to remove low-molecular-weight material, and freeze-dried to give fraction CW. This was then dissolved in distilled water and the solution submitted to freezing followed by mild thawing at 4 °C, which furnished cold water-soluble (SCW) and insoluble fractions (ICW), which were separated by centrifugation (8500 rpm at 4 °C for 20 min). The soluble portion (SCW) was treated with Fehling solution and precipitated Cu^{2+} complex was centrifuged off. This was neutralized with aq. HOAc, dialyzed against tap water, deionized with mixed ion exchange resins, and freeze-dried. The product was further purified by ultrafiltration through a 300 kDa M_r cut-off membrane (Millipore; polyethersulfone), giving rise to retained (EFPCW) and eluted material (FMG).

2.4. Monosaccharide composition of polysaccharide fractions

Monosaccharide components of the polysaccharide fractions were identified and their ratios were determined following hydrolysis with 1 M TFA for 8 h at 100 °C. The resulting aldose mixtures were converted to alditol acetates (GC–MS) by successive $NaBH_4$ and/or NaB^2H_4 reduction, and acetylation with Ac_2O-pyridine (1:1, v/v) for 12 h at room temperature.

2.5. Determination of homogeneity of polysaccharide fractions and the molecular weight of FMG

Determination of homogeneity and molar mass (M_w) were 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) were dissolved in the same solvent and filtered through a nitrocellulose membrane (Millipore), 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.

2.6. Methylation analysis

Per-*O*-methylation of the fucomannogalactan (FMG) was carried out by the method of Ciucanu and Kerek (1984). The sample (10 mg) was dissolved in dimethyl sulfoxide (1 ml), and powdered NaOH (20 mg) and iodomethane (CH_3I) (1 ml) were added. After 30 min at 25 °C with vigorous stirring, the mixture was maintained overnight at 25 °C. The reaction was interrupted by addition of water, neutralization with HOAc, dialysis against distilled water and freeze-drying. The products were submitted to one more cycle of methylation, and the products were isolated by partition between $CHCl_3$ and water. The per-*O*-methylated derivatives from the lower layer were hydrolyzed with 45% aqueous formic acid (1 ml) for 6 h at 100 °C, followed by NaB^2H_4 reduction and acetylation as above (item 2.4), to give a mixture of partially *O*-methylated alditol acetates, which was analyzed by GC–MS.

2.7. Absolute configuration of monosaccharides

The enantiomeric configuration of monosaccharides in FMG was determined by reductive amination with chiral 1-amino-2-propanol in the presence of sodium cyanoborohydride, followed by acetylation and GC analysis of the resulting 1-deoxy-1-(2′-hydroxypropylamino)–alditol mixture (Ultra-2 column, Hewlett–Packard) (Cases, Cerezo, & Stortz, 1995).

2.8. Experimental animals

Male Swiss mice (25–35 g) were kept in an automatically controlled temperature room (23 ± 2 °C) in 12 h light–dark cycles, with water and food freely available. Animals were acclimatized to the laboratory for at least 2 h before testing and were used only once. The experiments were performed following the protocol, approved by the Institutional Ethics Committee of the Federal University of Santa Catarina (UFSC), carried out in accordance with current protocols for the care of laboratory animals and ethical guidelines for investigation of experimental pain in conscious animals (Zimmermann, 1983). The numbers of animals and intensities of noxious stimuli were the minimum necessary to demonstrate consistent effects of the drug treatments.

2.9. Abdominal constriction, peritoneal capillary permeability, and leukocyte infiltration caused by intraperitoneal injection of 0.6% acetic acid

Abdominal constrictions in mice were induced according to a previously described procedure (Lucena et al., 2007), which in response to the intraperitoneal injection (i.p.) of acetic acid (0.6%),