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Exopolysaccharide produced by *Pleurotus sajor-caju*: Its chemical structure and anti-inflammatory activity

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ABSTRACT

Edible mushrooms are high nutritional value foods, which contain proteins, fibers, minerals, vitamins, and carbohydrates. Among their carbohydrates are some polysaccharides with recognized therapeutic effects. It was reported in this manuscript the structural characterization and antinociceptive and anti-inflammatory activities of an exopolysaccharide (EPS) produced by *Pleurotus sajor-caju*. The purified EPS was a mannogalactan (PEIsR), which was composed by mannose (37.0%), galactose (39.7%), and 3-*O*-methyl-galactose (23.3%). The polysaccharide was purified by freeze-thawing and dialysis, and it was characterized by GC-MS analysis and NMR spectroscopy. The mannogalactan is constituted by a main chain of (1→6)-linked α-D-Gal*p* and 3-*O*-methyl-α-D-Gal*p* units. Some of the α-D-Gal*p* units were substituted at O-2 by non-reducing end units of β-D-Man*p*. According to the literature review conducted, this is the first time that a methylated polysaccharide was observed on EPS of *P. sajor-caju*. The mannogalactan was able to reduce the nociception, *in vivo*, in the writhing and formalin tests and also reduced the carrageenan-induced paw edema, which indicates that it could be an effective antinociceptive and anti-inflammatory agent.

1. Introduction

Edible mushrooms have been consumed for centuries and are appreciated not only for their texture and flavor but also for their nutritional value. Mushrooms are rich in proteins, fibers, secondary metabolites, minerals, vitamins, and present low amounts of fat and calories [1,2]. Moreover, some mushroom compounds, such as polysaccharides, have shown therapeutic effects. Among the effects, the most reported are the antitumoral, antinociceptive, anti-inflammatory, and immunoenhancing activities [3–5].

Fungi polysaccharides are usually extracted from the mushroom fruiting bodies [4–7], and the most common are homoglycans, such as (1→3), (1→6)-linked β-D-glucans [5]. Several biological effects have been reported to these glucans [3]. In addition, heteropolysaccharides as heterogalactans, galactomannans, mannoglucans, have also been extracted [6–9]. Mannogalactans were isolated from the fruiting bodies of several *Pleurotus* species (*P. eryngii, P. geesteranus, P. pulmonarius, P. ostreatus 'florida'*, and *P. ostreatoroseus*) [6–8,10]. It was observed that the mannogalactan from *P. pulmonarius* presented a marked antinociceptive effect when tested in mice, which shows that the heteropolysaccharides of mushrooms can also exhibit therapeutic properties [6].

Fungi polysaccharides can also be obtained by submerged liquid fermentation, and be recovered from the culture broth, as exopolysaccharides, or extracted from the mycelium [11]. This is a biotechnological approach that allows a better control of the production of bioactive polysaccharides by fungi [12].

The submerged liquid fermentation has been used to obtain polysaccharides produced by a variety of basidiomycetes of the *Pleurotus* genus. However, information about purification and chemical characterization of these polymers are uncommon [2,13].

In the present study, a mannogalactan was produced by *Pleurotus sajor-caju* using the submerged liquid fermentation technique. This polysaccharide was purified and chemically characterized, and its antinociceptive and anti-inflammatory effects were evaluated.

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2. Materials and methods

2.1. Microorganism and submerged liquid fermentation

Pleurotus sajor-caju (Fr.) Singer was obtained from the Botanical Sciences Center of the Institute of Botany of São Paulo, Brazil (under the code CCB 019) and maintained in Petri dishes containing WDA (1 L of wheat extract, 20 g of dextrose, and 15 g of agar) at 4 °C [14].

The submerged liquid fermentation initiated by preparing the inoculum in 2 L Duran flasks containing 400 mL of medium using the best condition for the EPS production, defined by Assis et al. [15]. The composition of the medium was 2.5 g/L $(NH_4)_2SO_4$, 0.2 g/L $MgSO_4{\cdot}7H_2O$, 1.0 g/L K_2HPO_4, 1.0 g/L peptone, 1.0 g/L $CaCO_3$, with an initial glucose concentration of 20 g/L, without yeast extract. The flasks were inoculated with 7-days-old mycelium (grown in a Petri dish). After inoculation, the flasks were incubated at 30 °C and maintained under reciprocal stirring at 120 rpm for six days. The inoculum (400 mL) was then transferred to the bioreactor to initiate the culture.

The submerged liquid fermentations were conducted as batch processes using a stirred tank bioreactor Biostat B model with a working volume of 4 L, using the same medium as described above. The experiments were performed at 30 °C, at 300 rpm, with an air flow of 0.25 L/min, and an initial K_L a (oxygen transfer coefficient) value of 15 h^{-1}. The pH was maintained at 4.0. The fermentations were interrupted when glucose reached a minimal concentration (determined by glucose oxidase kit, Glucox 400, Doles Reagents), at time 490 h. After that, the fermented liquid and biomass were separated by centrifugation.

2.2. Extraction and purification of the exopolysaccharide (EPS)

The extraction and purification of the EPS from the fermented liquid of *P. sajor-caju* were performed according to Fig. 1. The fermented liquid was concentrated to reach 800 mL, to which four volumes of cold EtOH were added. This procedure yielded a precipitate (PE), which was separated by centrifugation (4.500 × *g*, 20 min, 4 °C), dialyzed (2 kDa cut-off membrane) against distilled water to remove low molecular weight compounds, and freeze-dried to obtain a retentate fraction (PEI). This fraction was dissolved in water (100 mL) and submitted to freeze-thawing process [16] several times, yielding a soluble (PEIs) and an insoluble (PEIi) fractions, which were separated by centrifugation (4.500 × *g*, 20 min, 4 °C). The soluble fraction (PEIs) was subjected to closed dialysis in a 6–8 kDa cut-off membrane against distilled water, to yield a retentate (PEIsR) and an eluted (PEIsE) fractions.

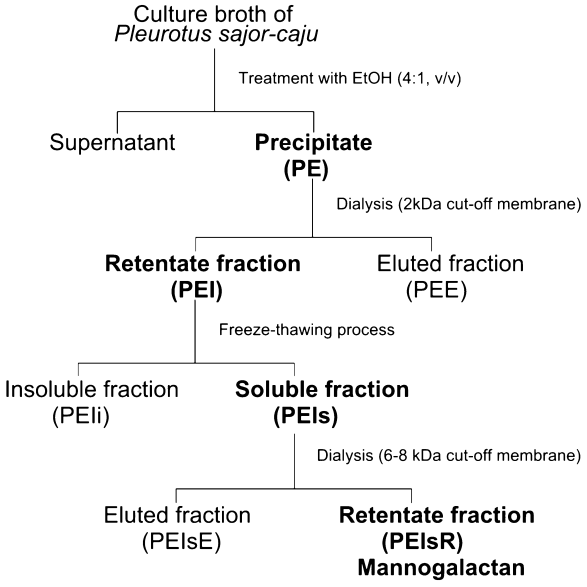

Fig. 1. Extraction and purification of the mannogalactan (PEIsR).

2.3. Monosaccharide analysis

Each EPS fraction (~2 mg) was hydrolyzed with TFA (1 M) at 100 °C for 12 h and then evaporated to dryness. The monosaccharides were successively reduced with $NaBH_4$ or $NaBD_4$ and acetylated with Ac_2O-pyridine (1:1, v/v; 300 μL) for 12 h at room temperature. The resulting alditol acetates were analyzed by gas chromatography-mass spectrometry (GC-MS) using a Varian Saturn 2000R-3800 gas chromatograph coupled to a Varian Ion-Trap 2000R mass spectrometer, with He as the carrier gas. A DB-225 capillary column (30 m × 0.25 mm i.d.), which was maintained at 50 °C during injection and then programmed to increase to 220 °C at a rate of 40 °C/min, was used for the quantitative analysis of the alditol acetates. The products were identified by their typical retention times and electron impact profiles.

2.4. Methylation analysis

The per-*O*-methylation of the mannogalactan (10 mg) was performed by dissolution in DMSO (0.5 mL), followed by addition of iodomethane (0.5 mL) and powdered NaOH (200 mg) [17]. The sample was subjected to vigorous stirring for 30 min at room temperature, and then maintained quiescent overnight. The reaction was interrupted with water and neutralized with HOAc. The per-*O*-methylated derivatives were recovered by partitioning with $CHCl_3$. After drying the $CHCl_3$, the per-*O*-methylated derivatives were subjected to hydrolysis using 45% HCO_2H at 100 °C for 10 h and then evaporated to dryness. The resulting mixture of *O*-methyl monosaccharides was reduced with $NaBD_4$ and acetylated with Ac_2O-pyridine (1:1, v/v; 300 μL) for 12 h at room temperature to obtain a mixture of *O*-methylalditol acetates. These derivatives were analyzed by GC-MS using the same conditions as described for alditol acetates (item 2.3), with the exception that the final temperature was 215 °C. These derivatives were identified by their typical retention times and electron impact spectra [18].

2.5. HPSEC analysis

Homogeneity and average molar mass (M_w) of the mannogalactan were determined by high-performance size-exclusion chromatography (HPSEC) coupled to refractive index (RID) and multi-angle laser light-scattering detectors (MALLS) [19]. Four gel-permeation Ultrahydrogel columns in series with exclusion sizes of 7 × 10^6, 4 × 10^5, 8 × 10^4, and 5 × 10^3 Da, were used. The eluent was 0.1 M aq. $NaNO_2$ containing 200 ppm aq. NaN_3 at 0.6 mL/min. The sample, previously filtered through a membrane (0.22 μm), was injected (100 μL loop) at a concentration of 1 mg/mL. The specific refractive index increment (d*n*/d*c*) was determined, and the results were processed with software provided by the manufacturer (Wyatt Technologies) [20].

2.6. NMR spectroscopy

The NMR spectra were obtained using a 400-MHz Bruker model DRX Avance spectrometer. The ^{13}C-NMR (100.6 MHz) and ^{1}H-NMR (400.13 MHz) analyses were performed at 70 °C, and the samples were dissolved in Me_2SO-d_6. The chemical shifts were expressed in ppm (δ) relative to Me_2SO-d_6 at δ 39.7 (^{13}C) and δ 2.40 (^{1}H). The NMR signals were assigned according to ^{1}H/^{13}C HSQC and 1D selective TOCSY experiments, and literature data.