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Optimization of chemical sulfation, structural characterization and anticoagulant activity of *Agaricus bisporus* fucogalactan

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ABSTRACT

A fucogalactan (E) was isolated from aqueous extract of *Agaricus bisporus*. The monosaccharide composition, methylation, and NMR analyses showed it is constituted by a (1→6)-linked α-D-Gal*p* main-chain, partially methylated at O-3, and partially substituted at O-2 by non-reducing end-units of α-L-Fuc*p* or α-D-Gal*p*. HPSEC analysis showed it had *Mw* of 1.28×10^4 g mol^{-1}. The polysaccharide was sulfated modifying reaction time, molar ratio of sulfation agent to hydroxyl group on the polysaccharide ($\eta ClSO_3H$/OH ratio), and ratio of total reaction volume to weight of sample (V_T/w ratio; μL mg^{-1}). The degree of substitution (DS) was evaluated for all sulfated derivatives. The sulfated fucogalactan with the highest DS value (2.83) had the best anticoagulant activity on Activated Partial Thromboplastin Time (APTT) and Protrombin Time (PT) assays. This sulfated fucogalactan, named E100, was obtained with the optimal conditions of $\eta ClSO_3H$/OH ratio of 18, V_T/w ratio of 100, in 6 h of reaction. The results showed that E100 produces a linear increment of APTT for concentrations of 15–45 μg mL^{-1}, whereas PT was almost constant between 20 and 400 μg mL^{-1}, suggesting an anticoagulant activity via inhibition of the intrinsic pathway of blood coagulation. NMR and methylation analyses showed that α-D-Gal*p* units of the main chain were greatly sulfated on 2-*O*-, 3-*O*-, and 4-*O*-positions.

1. Introduction

Mushrooms have been valued by humankind as an edible and medical resource, containing a number of bioactive molecules, with therapeutic properties that confer a source of powerful new pharmaceutical products. Polysaccharides of water-soluble fractions are the best known and most potent mushroom-derived substances with immunomodulatory and antitumoral activities (Wasser, 2002; Zhang, Cui, Cheung, & Wang, 2007), anti-inflammatory and antinociceptive activities (Guerra Dore et al., 2007; Smiderle et al., 2008), immunostimulatory activity (Smiderle et al., 2013) and antioxidative activity (Samanta et al., 2015). Studies have shown anti-inflammatory and antinociceptive activities for fucogalactans isolates of *Agaricus brasilensis*, *Agaricus bisporus* and *Agaricus bisporus var. hortensts* (Komura et al., 2010; Ruthes, Rattmann, Carbonero, Gorin, & Iacomini, 2012), fucomannogalactans of *Lentinus edodes* (Carbonero et al., 2008) and mannogalactans of *Pleurotus pulmonarius* (Smiderle et al., 2008), attributing a relationship between structure of the polysaccharide and its activity. Despite the biological activities of mushroom fucogalactans, there is no information about their anticoagulant activity.

Although heparin is the most used drug to treat venous and arterial thrombosis, it can cause serious adverse effects such as thrombocytopenia (Menajovsky, 2005), skin rashes, contact dermatitis, urticaria, skin necrosis (Schindewolf, Lindhoff-Last, Ludwing, & Boehncke, 2012; Villanueva, Nájera, Espinosa, & Borbujo, 2012), and contamination problems by animal pathogens due to its origin (de Kort, Buijsman, & van Boeckel, 2005; Konkle et al., 2001). There are many studies focused in evaluating possible anticoagulant and antithrombotic activities of sulfated polysaccharides. Chemically sulfated polysaccharides such as sulfated mannogalactan of *Agaricus brasilensis* (Gracher, Cipriani, Carbonero, Gorin, & Iacomini, 2010), sulfated citrus pectin (Maas et al., 2012), sulfated carrageenans (de Araújo et al., 2013), and sulfated glycoglucuronomannan (de Oliveira Barddal, Gracher, Simas-Tosin, Iacomini, & Cipriani, 2015) have shown anticoagulant and antithrombotic activities.

Sulfated polysaccharides are usually obtained using chlorosulfonic acid (O'Neill, 1955) or SO_3-pyridine (Larm et al., 1979) as sulfation agents. However, the conditions to obtain chemically sulfated polysaccharides are generally different for each study, and there is little information about obtaining optimal sulfation conditions of polysaccharides to produce molecules with improved

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biological activities. The influence of reaction time and amount of reagents on sulfation of ophiopogonpolysaccharide for antiviral activity (Zhang et al., 2012), and the influence of volume ratio of solvents on sulfation of polysaccharides from persimmon fruits for anticoagulant activity (Lu, Mo, Guo, & Zhang, 2012) have been investigated. In addition, both chemical sulfation and biological activity depend on composition, structure and conformation of the polysaccharide.

The anticoagulant and antithrombotic activities of native sulfated galactans (Fonseca et al., 2008; Pereira et al., 2005) and native sulfated fucans (Pomin & Mourão, 2012) are already known. In order to obtain a polysaccharide with anticoagulant activity, a fucogalactan obtained from aqueous extract of *A. bisporus*, structurally characterized through monosaccharide composition, methylation and NMR analysis, was chemically sulfated. The sulfation method was optimized modifying reaction time (t), ratio of reaction total volume to weight of sample (V_T/w), and molar ratio of sulfation agent to hydroxyl group on the polysaccharide ($\eta ClSO_3H$/OH), in order to obtain greater anticoagulant activity. The *O*-methylalditol acetates found on the methylation analysis of the fucogalactan were considered to calculate the molar ratios for chemical sulfation. The optimization was made based on Activated Partial Thromboplastin Time (APTT) and Protrombin Time (PT) assays. The sulfated fucogalactan with the highest anticoagulant activity was structurally characterized and its dose-response effect investigated.

2. Materials and methods

2.1. Biological material

A. bisporus fruiting bodies (Champignon de Paris) were provided by Makoto Yamashi Company (Miriam Harumi Yamashita), São José dos Pinhais, State of Paraná, Brazil. The mushrooms were taken to the laboratory within 12 h of collection, debris were manually removed (without washing), and then the sample was freeze-dried and milled.

2.2. Extraction and purification of the fucogalactan (E)

The extraction and purification of the fucogalactan E was carried out as described by Ruthes et al. (2013). Freeze-dried and milled *A. bisporus* fruiting bodies (720 g) were extracted with H_2O in a weight (g) to volume (L) ratio of 60:1 at 20 °C for 4 h (×6). The aqueous extract was concentrated and polysaccharides were precipitated adding excess of ethanol (EtOH, 3:1 v/v), followed by centrifugation at 8000 rpm, at 5 °C for 25 min. The polysaccharide fraction was then dissolved in H_2O, dialyzed against tap water using a 6–8 kDa cut-off membrane (Spectra/Por®, Regenerated Cellulose Membrane) for 24 h to remove low-molecular-weight compounds. After dialysis, the polysaccharide fraction was frozen and then allowed to thaw slowly resulting in an insoluble fraction, which was separated by centrifugation at 8000 rpm, at 5 °C for 25 min (Gorin & Iacomini, 1984). The supernatant fraction was treated with Fehling solution (Jones & Stoodley, 1965), giving a precipitated Cu^{2+} complex, which was separated by centrifugation. The precipitate was neutralized with acetic acid (HOAc), dialyzed against tap water (48 h), deionized with cation-exchange resin (H^+ form), and then freeze-dried. The fraction was further purified by closed dialysis against distilled water through a membrane with a 100 kDa cut-off (Spectra/Por®, Cellulose Ester Membrane). The eluted material (E) was concentrated and freeze-dried.

2.3. Chemical sulfation

The fucogalactan E was sulfated with $ClSO_3H$ according to the method described by O'Neill (1955), modifying reaction time (h), molar ratio of sulfation agent to hydroxyl group on the polysaccharide ($\eta ClSO_3H$/OH ratio) and ratio of total reaction volume to weight of sample (V_T/w ratio; μL mg^{-1}). To determine the $\eta ClSO_3H$/OH ratio, the total moles of OH in the polysaccharide was calculated from molar contribution of the OH groups of each monomer of the fucogalactan E, taking into account the percentage of *O*-methylalditol acetates found in the methylation analysis. Thus, the total moles of OH in the polysaccharide ($n_{T_{OH}}$), dependent of each monomer of the polysaccharide (i), was calculated according to equation 1; where w_p is the weight of the polysaccharide, %met is the percentage of a specific *O*-methylalditol acetate derivative, *MW* is the molecular weight of the monomer that gave rise to the specific *O*-methylalditol acetate derivative, and n_{OH} is the moles of OH in the monomer.

$$n_{T_{OH}} = \frac{w_p}{100}\sum_{i=1}^{N} \%\text{met}_i \times \frac{n_{OH_i}}{MW_i} \quad (1)$$

2.3.1. Chemical sulfation in different reaction times (t)

$ClSO_3H$, in a $\eta ClSO_3H$/OH ratio of 18, was added to a solution containing formamide:pyridine (1:1 v/v) in a V_T/w ratio of 1000. The addition was made carefully, dropwise, at 4 °C and the reaction was carried out in 1, 3, 6, 12 and 24 h at 8 °C in a closed system.

2.3.2. Chemical sulfation in different $\eta ClSO_3H$/OH ratios

$ClSO_3H$ was added to a solution containing pyridine:formamide (1:1 v/v) in a V_T/w ratio of 1000, in $\eta ClSO_3H$/OH molar ratios of 1, 2, 4, 9, 17, 18 and 22. The addition was made carefully, dropwise, at 4 °C and the reaction was carried out in 6 h at 8 °C in a closed system.

2.3.3. Chemical sulfation in different V_T/w ratios

$ClSO_3H$, in a $\eta ClSO_3H$/OH ratio of 18, was added to a solution containing formamide:pyridine (1:1 v/v) in 700, 200 and 100 V_T/w ratios. The addition was made carefully, dropwise, at 4 °C and the reaction was carried out in 6 h at 8 °C in a closed system.

All sulfation reactions were stopped and neutralized to pH 7.0 with $NaHCO_3$ 10%, and dialysed against distilled water, using a membrane with a 12–14 kDa cut-off (Spectra/Por®, Regenerate Cellulose Membrane). The material retained was concentrated and freeze-dried.

2.4. Structural analysis of the polysaccharides

2.4.1. Monosaccharide composition

1.0 mg of native or sulfated polysaccharide was hydrolyzed with 2.0 M TFA at 100 °C for 8 h, followed by evaporation to dryness. The resulting monosaccharides were solubilized in 1 mL of water and reduced to alditols with $NaBH_4$ (2.0 mg). After 18 h, HOAc was added, the solution evaporated to dryness and the resulting boric acid removed as trimethyl borate by co-evaporation with MeOH. Acetylation was carried out with Ac_2O-pyridine (1:1; v/v; 1.0 mL) at room temperature for 18 h, and the resulting alditol acetates extracted with $CHCl_3$. The samples were then analyzed by GC–MS (Varian Saturn 2000R-3800 gas chromatograph coupled to a Varian Ion-Trap 2000R mass spectrometer), using a DB-225 column (30 m × 0.25 mm i.d.) programmed from 50 to 220 °C at 40 °C/min, with helium as carrier gas. The alditol acetates were identified by their typical retention times and electron ionization spectra.

2.4.2. Methylation analysis of the fucogalactan E

The methylation analysis was carried out using NaOH-Me_2SO-MeI (Ciucanu & Kerek, 1984). 2.0 mg of polysaccharide were solubilized in Me_2SO (500 μL), followed by addition of NaOH (150 mg) and MeI (500 μL). The mixture was vigorously shaken