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Sulfation of fucogalactan from *Agaricus bisporus*: Different patterns in the chemical structure and their effects on anticoagulant activity

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ABSTRACT

A fucogalactan from *Agaricus bisporus* was sulfated by two methodologies based on an optimized sulfation method. The direct action of chlorosulfonic acid and SO_3-pyridine complex over the sulfation reaction and its effects on anticoagulant activity were evaluated. The products of chemical sulfations were two sulfated fucogalactans named E100 and ESL respectively. Clotting assays (APTT, PT and TT) showed that both sulfated polysaccharides have anticoagulant activity, and that ESL was more potent compared to E100. The FXa, T and FXIIa activities in the presence of the sulfated polysaccharides were determined. The better anticoagulant activity of ESL could be related to anti-FXIIa activity and also probably to its higher bioavailability. The HPSEC analysis showed similar *Mw* of 1.08×10^4 g mol^{-1} and 1.00×10^4 g mol^{-1} for E100 and ESL respectively. NMR and methylation analyses indicated a heterogeneous sulfation pattern for E100, whereas ESL showed conserved unsulfated (1→6)-linked α-D-Gal*p* residues in the main-chain and a more homogeneous sulfation pattern. The DS values of ESL and E100 were 1.0 and 2.8 respectively, indicating that the sulfation pattern is more important for the anticoagulant activity than the amount of sulfate.

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1. Introduction

Biological activity of mushroom polysaccharides has been evaluated aiming pharmaceutical products. The proper cultivation and high production of these organisms have drawn attention in the search for molecules with biological interest. Studies of polysaccharides derived from mushrooms have increased due to the functional properties triggered by biological interactions. The antioxidative [1–3], immunomodulatory [4], immunostimulatory [5], antitumoral [6,7], anti-inflammatory [8] and antinociceptive activities [9,10] are the most known biological properties of polysaccharides derived from mushrooms. However, the anticoagulant and antithrombotic activities of polysaccharides from mushrooms are little known. An acidic polysaccharide of *Auricularia auricula* [11] and a chemically sulfated glucogalactomannan of *Pleurotus sajor-caju* [12] with anticoagulant activities have been investigated.

The sulfated group is related to various biological activities, mainly those involving blood clotting effects [13]. Studies focused in the evaluation of native sulfated polysaccharides that regulate the clotting system have been performed [14–16]. In addition, chemically sulfated polysaccharides have been synthesized to obtain molecules with anticoagulant and antithrombotic properties [17,18].

Strategies for obtaining chemically sulfated polysaccharides vary according to the type of structure and the preference for the type of sulfation process. These biomolecules are usually obtained using chlorosulfonic acid ($ClSO_3H$) [19] or SO_3-pyridine complex [20] as sulfation agents. A glycoglucuronomannan [21], β-(1→6)-D-Glucans [18,22], citrus pectin [23] and a mannogalactan [24] have been chemically sulfated with excess of $ClSO_3H$. On the other hand, polysaccharides from persimmon fruits [25], an ophiopogonpolysaccharide [26], glucans [27] and fucans [28] have been chemically sulfated through excess of SO_3-pyridine complex. Nevertheless, indicating a sulfation agent as the most appropriate is still difficult. For example, citrus pectin has been chemically sulfated through $ClSO_3H$ [23,29], SO_3-pyridine complex [30] or $N(SO_3Na)_3$ synthetic reagent [31] to obtain an anticoagulant and antithrombotic agent. Reports indicate that $ClSO_3H$ generates harsh conditions that produce severe changes in the pectin structure and alters the bioactivity of the sulfated derivatives [30]. However, even though the sulfation with excess of $ClSO_3H$ has led to partial hydrolysis of galacturonans, it proved to be an optimal reagent for sulfation of pectins [32].

In addition, it is important to consider that both the reaction conditions (sulfation agent, temperature, solvents and time) and the characteristics of the polysaccharide (composition, structure,

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spatial arrangement, degree of substitution – DS and molecular weight – *Mw*) are significant factors that could alter the anticoagulant and antithrombotic activities. Moreover, the same types of polysaccharides vary structurally according to their origin. Thus, chemical sulfation of the same types of polysaccharides from different sources can generate sulfated polysaccharides with different anticoagulant activities. In these conditions, comparisons between sulfation methods could be something inadequate. Besides that, there are no reports that compare the use of sulfation methods on a well-characterized molecule, properly describing the structural differences of the sulfated polysaccharides obtained and the implications on their anticoagulant activity.

In this study, a fucogalactan from *A. bisporus* was chemically sulfated through two sulfation processes usual for polysaccharides. The sulfations were made through $ClSO_3H$ and SO_3-pyridine complex methods. The methodologies considered sulfation conditions based on an optimized sulfation method. The influence of the sulfated fucogalactans on activated partial thromboplastin time (APTT), prothrombin time (PT), thrombin time (TT), and their effects on the factor Xa (FXa), α-thrombin (T) and factor XIIa (FXIIa) activities were analyzed. Moreover, the sulfated fucogalactans were structurally compared in relation to monosaccharide composition, methylation and nuclear magnetic resonance (NMR) analyses.

2. Experimental

2.1. Material

Fruiting bodies of *A. bisporus* (champignon de Paris) were provided by Makoto Yamashita Company (Miriam Harumi Yamashita), São José dos Pinhais, State of Paraná, Brazil.

2.2. Methods

2.2.1. Extraction and purification of fucogalactan E

The fucogalactan was extracted and purified according to described by Román et al. [33], and conventionally described as fucogalactan E.

2.2.2. Chemical sulfation

In order to obtain the sulfated fucogalactans through both $ClSO_3H$ and SO_3-pyridine complex methodologies, fucogalactan E was chemically sulfated considering the optimal sulfation conditions based on an optimized sulfation method [33]. Thus, a molar ratio of sulfation agent to hydroxyl group on the polysaccharide ($\eta ClSO_3H$/OH or ηSO_3-pyridine/OH ratios) of 18, a ratio of total reaction volume to weight of sample (V_T/w ratio; $\mu L\,mg^{-1}$) of 100 in 6 h of reaction were considered. Likewise, the *O*-methylalditol acetates found on the methylation analysis of the fucogalactan E were considered to calculate the $\eta ClSO_3H$/OH and ηSO_3-pyridine/OH ratios in the chemical sulfation.

2.2.2.1. Chemical sulfation of fucogalactan E with $ClSO_3H$ as sulfation agent. Fucogalactan E was sulfated according to the method described by O'Neill [19] with some modifications. 100.0 mg of the polysaccharide were dissolved in 9,8 mL of a solution containing formamide:pyridine (1:1 v/v), in a closed system with constant stirring. Later, 2050 μL of $ClSO_3H$ were carefully added, dropwise, at 4 °C ($\eta ClSO_3H$/OH ratio = 18 and V_T/w ratio = 100). The sulfation reaction was carried out in 6 h at 8 °C in a closed system. The reaction was stopped and neutralized to pH 7.0 with 10% $NaHCO_3$, and dialyzed against distilled water, using a 12–14 kDa cut-off membrane (Spectra/Por®, Regenerate Cellulose Membrane). The retained material (E100) was concentrated and freeze-dried.

2.2.2.2. Chemical sulfation of fucogalactan E with SO_3-pyridine complex as sulfation agent. Fucogalactan E was sulfated according to the method described by Larm et al. [20] with some modifications. A first solution was prepared dissolving 100.0 mg of the polysaccharide in 5.0 mL of *N,N*-dimethylformamide and then stirred for 12 h. A second solution was prepared dissolving 4.92 g of SO_3-pyridine complex in 5.0 mL of *N,N*-dimethylformamide and then stirred for 1 h. The first solution was then added to the second solution and the sulfation reaction was carried out in 6 h at 25 °C in a closed system (ηSO_3-pyridine/OH ratio = 18 and V_T/w ratio = 100). The reaction was stopped and neutralized to pH 7.0 with 10% $NaHCO_3$, and dialyzed against distilled water, using a 12–14 kDa cut-off membrane (Spectra/Por®, Regenerate Cellulose Membrane). The retained material (ESL) was concentrated and freeze-dried.

2.2.3. Structural analysis of the polysaccharides

2.2.3.1. NMR spectroscopy. 1D-NMR spectra (^{13}C, ^{1}H and DEPT) were prepared using a 400 MHz Avance III spectrometer with 5 mm direct probehead (BBO). Analyses were carried out at 70 °C on samples dissolved in D_2O. Chemical shifts are expressed in δ relative to acetone at δ 2.21 (^{1}H) and 32.77 (^{13}C), based on DSS (2,2-dimethyl-2-silapentane-5-sulfonate-d6 sodium salt; δ = 0.0 for ^{1}H and ^{13}C). 2D-NMR Edited-HSQC (COSY, TOCSY and heteronuclear) spectra were obtained with a Bruker 600 MHz AVANCE III NMR spectrometer with a 5 mm inverse gradient probehead (QXI). HSQC, HSQC-COSY and HSQC-TOCSY experiments, with spectral widths of 6393 Hz (^{1}H) and 1200 Hz (^{13}C), were recorded for quadrature detection in the indirect dimension, using 4 scans per series of 2 K × 512 and a width data points with zero filling in F1 (2 K) prior to Fourier transformation.

2.2.3.2. Homogeneity and Mw. The *Mw* of the polysaccharides were determined by High Performance Size Exclusion Chromatography (HPSEC) coupled to refractive index and multi-angle laser light scattering detectors. Four 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 a solution containing $NaNO_2$ (0.20 M) and NaN_3 (200 ppm) at 0.6 mL min^{-1}. Each polysaccharide was dissolved and filtered through a 0.22 μm pore size filter (Millipore). The injection (100 μL) was made at a concentration of 1 mg mL^{-1}. The specific refractive index increment (*dn/dc*) of the polysaccharides was determined and the results were processed with software provided by the manufacturer (ASTRA 4.70.07, Wyatt Technologies).

2.2.3.3. DS analysis of the sulfated polysaccharides. The sulfate released resulting of hydrolysis (1 M HCl at 100 °C for 5 h) of the sulfated polysaccharides was quantified by the $BaCl_2$-gelatin turbidimetric method [34]. Inorganic Na_2SO_4 was used as standard and the sulfur percentage (S%) in the sulfated polysaccharides was calculated from the inorganic sulfate content. The DS was calculated according to the equation: DS = (Mm × S%)/(3200–102 × S%) [35], where Mm is the weighted mean of molecular weight of the monosaccharides in the polysaccharide according to the percentages of *O*-methylalditol acetates.

2.2.3.4. Monosaccharide composition. 2.0 mg of the sulfated polysaccharide were hydrolyzed with 2.0 M TFA at 100 °C for 8 h, followed by evaporation to dryness. The resulting monosaccharides were solubilized in 1.0 mL of distilled water and reduced to alditols with 2.0 mg of $NaBH_4$ in 8 h. The reaction was neutralized with HOAc, freeze-dried 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. The resulting Alditol Acetates (AAs) were extracted with $CHCl_3$, washed with 2% $CuSO_4$ and the organic

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