Thrombosis Research 126 (2010) e180–e187

Contents lists available at ScienceDirect

Thrombosis Research

journal homepage: www.elsevier.com/locate/thromres

Regular Article

Antithrombin and heparin cofactor II-mediated inactivation of α-thrombin by a synthetic, sulfated mannogalactan

Ana Helena P. Gracher [a], Thales R. Cipriani [a], Elaine R. Carbonero [b], Philip A.J. Gorin [a], Marcello Iacomini [a,*]

[a] Laboratório de Química de Carboidratos, Departamento de Bioquímica e Biologia Molecular, Universidade Federal do Paraná, Curitiba, Paraná, Brazil
[b] Departamento de Química, Universidade Federal de Goiás, Catalão, Goiás, Brazil

ARTICLE INFO

Article history:
Received 26 October 2009
Received in revised form 14 April 2010
Accepted 20 April 2010
Available online 31 May 2010

Keywords:
Sulfated polysaccharide
Mannogalactan
Pleurotus ostreatoroseus
Antithrombotic
Antithrombin
Heparin cofactor II

ABSTRACT

Introduction: A mannogalactan from *Pleurotus ostreatoroseus* (MgPr) was chemically sulfated to give MgPr-S1, which was evaluated for its anticoagulant and antithrombotic activities, bleeding tendency, and platelet aggregation.
Materials and methods: MgPr-S1 was partially characterized by HPSEC-MALLS, methylation analysis, and ^{13}C NMR spectroscopy. Its anticoagulant activity was determined by assays of aPTT, TT, α-thrombin and factor Xa residual activity, heparin cofactor II (HCII)-, or antithrombin (AT)-mediated inhibition. The antithrombotic effect was evaluated in rats using a venous thrombosis model and the bleeding tendency was also tested *in vivo.* Platelet aggregation was investigated by an adaptation of the method of Born [1].
Results: The hydroxyl groups of β-D-Man*p* units and OH-2 and OH-4 of the (1→6)-linked α-D-Gal*p* units were preferentially substituted. The anticoagulant activity of MgPr-S1 was mainly by thrombin inhibition with antithrombin and HCII, and had an effect on platelet aggregation induced by ADP and α-thrombin. It almost completely inhibited thrombus formation *in vivo* at a dose of 6 mg/kg and heparin inhibited thrombus formation at a dose of 0.200 mg/kg.
Conclusions: These results suggested that the chemically sulfated mannogalactan could act as an alternative to heparin as anticoagulant.

Thrombotic disorders are one of the major causes of morbidity and mortality, with venous thrombosis being a disease of aging in both males and females [2] and is also related to immobilization, trauma, malignant disease, family history, obesity, estrogen therapy, and other factors [3]. Blood coagulation is a highly regulated process, having thrombin as a central point of regulation and amplification of clot formation [4]. Its contribution to venous thrombosis is evidenced by extensive investigations on the efficacy of thrombin inhibitors in venous thromboembolic prophylaxis and treatment. Both anticoagulant and antiplatelet therapies are effective to treat and prevent arterial thrombosis, although platelet inhibitors are more often used since platelets play an important role in the development of this pathology [5]. Heparin is the most used drug to treat venous and arterial thrombosis, which although effective, heparin has limitations. It must be administered, mainly, parenterally or subcutaneously, requires careful laboratory monitoring, because its anticoagulant response is variable and is obtained from an animal source, which increases concern on contamination by pathogens [6,7].

In an attempt to obtain antithrombotic agents as alternatives to heparin, innumerable polyanionic macromolecules have been examined. Sulfated polysaccharides can be obtained from different sources, such as marine algae, invertebrates [8], or by chemical sulfation [9–11]. Chemically sulfated polysaccharides from mushrooms could be an alternative, because the original macromolecules are readily available, chemically defined, and have several biological activities, such as antiviral and antitumoral [12–14].

Pleurotus ostreatoroseus is an edible mushroom and a ready source of molecules such as polysaccharides with antitumoral and immunomodulatory effects. Polysaccharides isolated from this basidiomycete are homopolymers, such as glucans [15], and heteropolymers, namely mannogalactans [16]. Usually, galactans and heterogalactans from *Pleurotus* spp. are partially *O*-methylated.

We have been seeking anticoagulants from non-animal sources, as alternatives to heparin, with lower health risks. For this purpose, we now chemically sulfate a mannogalactan isolated from the edible mushroom *Pleurotus ostreatoroseus*, which is composed of a main chain of (1→6)-linked α-D-galactopyranosyl units, some of which are partially *O*-methylated at O-3. These were partially substituted at O-2 by β-D-mannopyranose residues [16]. Synthetic polysaccharides derived from naturally occurring ones are of great interest, as therapeutic substitutes of natural products and to increase understanding of mechanisms of their biological reactions [17].

* Corresponding author. Laboratório de Química de Carboidratos, Departamento de Bioquímica e Biologia Molecular, Universidade Federal do Paraná. CP 19046, CEP 81.531–980, Curitiba, PR, Brazil. Tel.: +55 41 3361 1655; fax: +55 41 3266 2042.
E-mail address: iacomini@ufpr.br (M. Iacomini).

0049-3848/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.thromres.2010.04.008

Materials and Methods

Materials

The mannogalactan was isolated from the fruiting bodies of edible basidiomycete *Pleurotus ostreatoroseus* Sing, via successive aqueous extraction, freeze thawing, and precipitation of the supernatant with Fehling solution [16]. Unfractionated heparin (UFH-202 IU/mg), adenosine diphosphate (ADP), and factor Xa chromogenic substrate were obtained from Sigma (St. Louis, MO, USA), rabbit cephalin and 0.025 M $CaCl_2$ solution from Dade Behring (Marburg, DE), and α-thrombin, factor Xa, heparin cofactor II (HCII) and antithrombin (AT) from Haematologic Technologies Inc. (Essex Junction, VT, USA). α-Thrombin chromogenic substrate S-2238 and rabbit brain thromboplastin were from Instrumentation Laboratory (Lexington, MA, USA), and pyridine, formamide and chlorosulfonic acid, were from Merck (Darmstadt, Germany). Normal human plasma was obtained by centrifugation (2000 g for 15 min at 22 °C) of a pool of citrated normal human plasma (nine parts of blood to one of 3.8% trisodium citrate) from healthy volunteer donors. Platelet rich plasma (PRP) was obtained from peripheral blood from drug-free human volunteers as described above. The blood was centrifuged at 110 g for 15 min at room temperature. For aggregation assay with α-thrombin as agonist, blood was obtained from drug-free human volunteers and was collected in 5 mM EDTA. Platelets were obtained by centrifugation of the PRP at 800 g for 15 min followed by washing twice with calcium-free Tyrode's buffer, pH 6.5, containing 0.1% glucose, 0.2% gelatin, 0.14 M NaCl, 0.3 M $NaHCO_3$, 0.4 mM NaH_2PO_4, 0.4 mM $MgCl_2$, 2.7 mM KCl, and 0.2 mM EGTA. All other chemicals and reagents were of analytical grade.

Methods

Chemical sulfation

Polysaccharide was sulfated according to the method described by O'Neill [18] with slight modifications: the mannogalactan (360 mg, 3.57×10^{-4} mmol), was solubilized in formamide (26 mL), pyridine (26 mL) then added, and the mixture vigorously stirred for 24 h, followed by dropwise addition of chlorosulfonic acid (6.5 mL, 97.5 mmol) over 1 h at 0 °C. The mixture was maintained at 4 °C for 12 h, ice-water then added, followed by 10% (w/v) aqueous $NaHCO_3$ until effervescence ceased. The solution was dialyzed against running water using 14 kDa M_w cut-off membrane and then freeze dried, providing the sulfated mannogalactan (720 mg), named MgPr-S1.

The degree of substitution (DS) of sulfated derivative was determined after hydrolysis with 1 M HCl for 5 h at 100 °C, the resulting $BaSO_4$ being measured turbidimetrically [19].

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 DB-225 capillary column (30 m × 0.25 mm i.d.), held at 50 °C during injection and then programmed at 40 °C min^{-1} to 220 °C (constant temperature) was used for qualitative and quantitative analysis of alditol acetates. Partially *O*-methylalditol acetate mixtures were similarly analyzed, but with a program from 50 °C to 215 °C at 40 °C/min, then hold.

^{13}C NMR spectra were obtained using a 400 MHz Bruker model DRX Avance spectrometer incorporating Fourier transform. Analyses were performed at 70 °C at a frequency of 100.6185 MHz, on samples dissolved in D_2O. Chemical shifts of samples are expressed in δ (ppm) relative to acetone at δ 30.2.

Determination of the homogeneity and molar mass (M_w) of the sulfated polysaccharide was performed using 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, corresponding to 7×10^6, 4×10^5, 8×10^4, and 5×10^3 size exclusion limit respectively) 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 diluted in the same solvent and filtered through a Millipore nitrocellulose membrane, 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 (*dn/dc*) was determined using a Waters 2410 detector. All experiments were carried out at 25 °C.

Methylation analysis

Partial *O*-methylation of sulfated polysaccharide (10 mg) was carried out using 40% aq. NaOH (1 mL) with dropwise addition of Me_2SO_4 (1 mL) [20]. The reaction medium was neutralizated, dialyzed, and evaporated. Complete *O*-permethylation was carried out using NaOH–Me_2SO–MeI [21]. Per-*O*-methylated derivatives (5 mg) were hydrolyzed with 45% v/v formic acid (1 mL) at 100 °C for 15 h, and then evaporated to dryness. The resulting mixture of *O*-methylaldoses was successively reduced with $NaBD_4$ and acetylated with 1:1 Ac_2O–pyridine (200 μL) overnight at room temperature to give a mixture of partially *O*-methylated alditol acetates, which was analyzed by GC–MS [22].

In vitro anticoagulant activity

The assay was carried out using sulfated polysaccharides, dissolved in saline at various concentrations. Activated partial thromboplastin time (aPTT) was performed using the kit Dade Actin® (Dade Behring, Marburg, DE). Normal human plasma (90 μL) was incubated with 10 μL of saline, native polysaccharide (5, 10, 25, 50, and 100 μg/mL, final concentration), sulfated polysaccharide (5, 10, 25, 50 and 100 μg/mL, final concentration) or heparin (2, 5 and 10 μg/mL, final concentration) at 37 °C for 2 min. Rabbit cephalin (100 μL) was then added and incubated at 37 °C for 3 min. Finally, 0.025 M $CaCl_2$ solution (100 μL) pre-warmed to 37 °C, was added to the mixtures, and the clotting time was measured up to 300 s in triplicate using a COAG-A-MATE® XM coagulometer (Organon Teknika Corporation, Durhan, NC).

For determination of thrombin time (TT), normal human plasma (90 μL) was incubated with 10 μL of saline, native polysaccharide (5, 10, 25, 50 and 100 μg/mL, final concentration), sulfated polysaccharide (5, 10, 25, 50 and 100 μg/mL, final concentration), or heparin (2, 5 and 10 μg/mL, final concentration) at 37 °C for 2 min, at which the thrombin time reagent (Behring, Marburg, Germany), 200 μL, was added. The time for the appearance of a fibrin clot (s) was measured up to 300 s in triplicate using a COAG-A-MATE® XM coagulometer (Organon Teknika Corporation, Durhan, NC).

Results are expressed as T_1/T_0, which is the ratio between the clotting time in the presence (T_1) and absence of polysaccharide (T_0) in the incubation mixture ± SD (n = 3), whose $T_1/T_0 = 13.39286$ for aPTT and $T_1/T_0 = 26.90583$ for TT assays were considered to indicate complete inhibition of plasma coagulation.

Animals

Venous thrombosis experiments were conducted on female and male Wistar rats (180–220 g) and tail transection bleeding time experiments were on female Wistar rats (180–220 g) from the colony of the Federal University of Paraná, Curitiba, Brazil. They were maintained under standard laboratory conditions (12 h light/dark cycle, temperature 22 ± 2 °C), with standard pellet food and water *ad libitum*. The animals were anesthetized with an intramuscular injection of a mixture of ketamine (100 mg/kg body weight) and xylazine (16 mg/kg). The Institutional Ethics Committee of Federal