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Anticoagulant and antithrombotic activities of a chemically sulfated galactoglucomannan obtained from the lichen *Cladonia ibitipocae*

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Abstract

A galactoglucomannan (GGM), isolated from the lichen *Cladonia ibitipocae*, consisted of a (1 → 6)-linked main chain of α-mannopyranose units, substituted by α- and β-D-galacto (α- and β-D-Gal*p*)-, β-D-gluco (β-D-Glc*p*) - and α-D-mannopyranosyl (α-D-Man*p*) groups, and was sulfated giving a sulfated polysaccharide (GGM-SO_4) with 42.2% sulfate corresponding to a degree of substitution of 1.29. NMR studies indicated that after sulfation, the OH-6 groups of galactopyranosyl and mannopyranosyl units were preferentially substituted. GGM-SO_4 was investigated in terms of its in vitro anticoagulant and in vivo antithrombotic properties. Those of the former were evaluated by its activated partial thromboplastin (APTT) and thrombin time (TT), using pooled normal human plasma, and compared with that of 140 USP units mg^{-1} for a porcine intestinal mucosa heparin. Anticoagulant activity was detected in GGM-SO_4, but not in GGM. The in vivo antithrombotic properties of GGM-SO_4 were evaluated using a stasis thrombosis model in Wistar rats, intravenous administration of 2 mg kg^{-1} body weight totally inhibiting thrombus formation. It caused dose-dependent increases in tail transection bleeding time. The results obtained showed that this sulfated polysaccharides is a promising anticoagulant and antithrombotic agent.

Keywords: Anticoagulant activity; Sulfated polysaccharide; Galactoglucomannan

1. Introduction

Polysaccharides are increasingly used for medical applications, taking advantage of some intrinsic functional properties elicited by biological interactions. Biological and pharmacological properties can be induced or modified in such a polymer by chemical derivatization [1].

Chemical derivatization of polysaccharides, such as sulfation, is important because the presence of this group is related to anticoagulant and antithrombotic effects [2,3] and other biological activities, such as inhibition of HIV-1 infectivity [4–7] and the development of Kaposi's sarcoma-related lesions [8,9].

Anticoagulant and antithrombotic activities are among the most widely studied properties of sulfated polysaccharides [10]. The anticoagulant heparin is an important therapeutic agent for prophylaxis and treatment of thrombosis. This anionic polymer acts by accelerating the inhibitory effects of antithrombin and heparin co-factor II on activated clotting factors [11].

Commercial processing of heparin results in a mixture of glycosaminoglycan chains with diverse structure and varying lengths with an average M_r of 9000–15000 Da, making it polydisperse and heterogeneous [12].

The anticoagulant activity of heparin is due to the presence of a specific pentasaccharide sequence that mediates interaction with antithrombin [13]. The distinguishing structural feature of the antithrombin-binding region is the 3-*O*-sulfated glucosamine residue, which is present in commercial heparin in about one-third of the heparin chains [14].

In an attempt to overcome some of the problems associated with heparin, such as bleeding, thrombocytopenia,

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problems with animal pathogen contamination due to its animal origin, and poor bioavailability [15–18], a number of alternative anticoagulants have been developed. Studies on a number of other sulfated polysaccharides, obtained by sulfation of natural polysaccharide and those occurring naturally, have been carried out related to in vivo and in vitro anticoagulant and antithrombotic properties with the aim of finding substitutes for heparin [3,19–30].

We have now prepared a sulfated galactoglucomannan (GGM-SO_4) by chemical modification from the native polysaccharide (GGM) extracted from the lichenized fungus *Cladonia ibitipocae*. GGM-SO_4 was then examined for its anticoagulant and antithrombotic properties.

2. Experimental

2.1. Materials

Heparin from porcine intestinal mucosa (140 USP units mg^{-1}) was obtained from Sigma (St. Louis, MO, USA) and rabbit brain thromboplastin from Instrumentation Laboratory (Lexington, MA, USA). Normal human plasma was obtained by centrifugation (2000 × *g* for 15 min at 22 °C) of citrated normal human plasma (1/10 vol. of 3.8% trisodium citrate) from a pool of healthy volunteer donors and frozen at −70 °C in aliquots of 0.5 ml until further use. Activated partial thromboplastin (APTT) was determined with reagents from Instrumentation Laboratory (Lexington, MA, USA) and thrombin time (TT) with reagents from Behring (Marburg, Germany). Pyridine, formamide, chlorosulfonic acid, methanol, ethanol, potassium hydroxide, trifluoroacetic acid and phenol red were from Merck (Darmstadt, Germany). Dowex 50 × 8 (H^+ form) resin was from Sigma (St. Louis, MO, USA). All other chemicals and reagents used were of analytical grade.

2.2. Isolation of galactoglucomannan and its sulfation

The galactoglucomannan (GGM) from the lichen *C. ibitipocae* was extracted, purified and characterized as previous described by Woranovicz et al. [31]. It was sulfated according to the method described by O'Neill [32] as follows. The native polysaccharide (1.0 g) was solubilized in formamide (15 ml) and pyridine (10 ml) by vigorous stirring for 24 h, followed by dropwise addition of chlorosulfonic acid (1.6 ml) over 1 h at 0 °C, the mixture being maintained at 4 °C for 12 h. Ice-water was added, followed by 10% (w/v) aqueous $NaHCO_3$ until effervescence ceased. The solution was then dialyzed against water to remove pyridine, salts, and potential degradation products, and then freeze-dried, providing the sodium salt of the sulfated galactoglucomannan. This process was repeated five times, resulting in the sulfated derivative (GGM-SO_4).

The degree of substitution (DS) of sulfated derivatives was determinated by hydrolysis with 1 M HCl for 5 h at 100 °C, the resulting $BaSO_4$ being measured turbidimetrically [33].

2.3. NMR spectroscopy

^{13}C NMR spectra were obtained using a 400 MHz Bruker model DRX Avance spectrometer with a 5 mm inverse probe. The ^{13}C NMR (100.6 MHz) analyses were performed at 50 °C with samples dissolved in D_2O. Chemical shifts of these are expressed in ppm (δ) relative to acetone at δ 30.20 for ^{13}C signals.

2.4. Methylation analysis

GGM-SO_4 was per-*O*-methylated according to the method of Ciucanu and Kerek, using powdered NaOH in Me_2SO–MeI [34]. The per-*O*-methylated derivatives were treated with refluxing 3% HCl–MeOH for 2 h at 80 °C, then 0.5 M H_2SO_4 at 100 °C for 14 h. The resulting mixture of *O*-methylaldoses were reduced with $NaBH_4$ and acetylated with Ac_2O–pyridine (1:1 (v/v); 2 ml) at room temperature for 12 h. The resulting partially *O*-methylated alditol acetates obtained were analyzed by GC–MS using a Varian model 3300 gas chromatograph linked to a Finnigan Ion-Trap, model 810 R-12 mass spectrometer using a DB-225 capillary column (30 m × 0.25 mm i.d.) with He as carrier gas. The analysis was carried out from 50 to 215 °C at 40 °C min^{-1}, the final temperature being maintained constant until the end of its analysis (31 min).

2.5. Clotting assay

The assay was carried out using heparin, GGM and GGM-SO_4, dissolved in saline at various concentrations. Normal human plasma (90 μl) was mixed with 10 μl of a solution of GGM-SO_4 (0–2 mg), GGM (0–2 mg) or heparin (0–100 μg). For controls, saline was added to plasma in a ratio of 1:10. APTT measurements were performed using a kit obtained from Instrumentation Laboratory (IL test™, Lexington, MA, USA). Plasma (100 μl) containing various concentrations of GGM-SO_4, GGM or heparin, was incubated at 37 °C for 1 min. Hundred microliters of bovine cephalin was then added and incubated at 37 °C. After 3 min of incubation, 100 μl of pre-warmed 0.25 M $CaCl_2$ solution were added to the mixtures, and the clotting time was measured in quadruplicate using a COAG-A-MATE® XM coagulometer (Organon Teknika Corporation, Durhan, NC, USA) up to 120 s. For determination of TT, normal human plasma (100 μl) containing various concentrations of heparin, GGM and GGM-SO_4 was incubated at 37 °C for 2 min, at which the thrombin time reagent, 200 μl (Behring, Marburg, Germany), was added. The time for the appearance of a fibrin clot (seconds) was measured up to 100 s using a COAG-A-MATE® XM coagulometer (Organon Teknika Corporation, Durhan, NC, USA). All assays were performed in duplicate and repeated at least three times on different days ($n = 6$).