International Journal of Biological Macromolecules 80 (2015) 328–333

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

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

Anticoagulant activity of native and partially degraded glycoglucuronomannan after chemical sulfation

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ARTICLE INFO

Article history:
Received 24 April 2015
Received in revised form 10 June 2015
Accepted 26 June 2015
Available online 29 June 2015

Keywords:
Polysaccharide
Chemical sulfation
Anticoagulant activity

ABSTRACT

Heparin has great clinical importance as anticoagulant and antithrombotic agent. However, because of its risks of causing bleeding and contamination by animal pathogens, several studies aim to obtain alternatives to heparin. In the search for anticoagulant and antithrombotic agents from a non-animal source, a glycoglucuronomannan from the gum exudate of the plant *Vochysia thyrsoidea* was partially hydrolyzed, and both native and partially degraded polysaccharides were chemically sulfated, yielding VThS and Ph-VThS respectively. Methylation analysis indicated that sulfation occurred preferentially at the O-5 position of arabinose units in the VThS and at the O-6 position of mannose units in Ph-VThS. *In vitro* aPTT assay showed that VThS and Ph-VThS have anticoagulant activity, which could be controlled by protamine, and *ex vivo* aPTT assay demonstrated that Ph-VThS is absorbed by subcutaneous route. Like heparin, they were able to inhibit α-thrombin and factor Xa by a serpin-dependent mechanism. *In vivo*, VThS and Ph-VThS reduced thrombus formation by approximately 50% at a dose of 40 IU/kg, similarly to heparin. The results demonstrated that the chemically sulfated polysaccharides are promising anticoagulant and antithrombotic agents.

1. Introduction

The physiological process involving blood coagulation and its interruption is known as hemostasis, and the imbalance in this process is the origin of many ischemic and hemorrhagic diseases.

Heparin is widely used as an anticoagulant and antithrombotic agent, mainly by binding to antithrombin (AT) and heparin cofactor II (HCII), accelerating the rate at which these protease inhibitors form complexes with enzymes involved in blood clotting, particularly α-thrombin and factor Xa, inactivating them [1,2]. It is a glycosaminoglycan primarily consisting of glucosamine (N-acetylated or N-sulfated) and uronic acid units (iduronic or glucuronic) joined by 1→4 glycosidic linkages, with a complex pattern of substitution of sulfate groups [1,3].

Although effective, the use of heparin has limitations since its anticoagulant effect is unpredictable, resulting in risk of bleeding, requiring laboratory monitoring of the patient for a safe use. Furthermore, since it is extracted from animal tissues, especially from the porcine gut and bovine lung, there is a concern for heparin contamination with animal pathogens [4,5]. For these reasons, many studies aim to obtain anticoagulant and antithrombotic agents, as alternatives to heparin, especially those involving naturally or chemically sulfated polysaccharides [3,5–10]. Both anticoagulant and antithrombotic effects are related to the presence of sulfate groups, and their distribution along the polysaccharide chain. Furthermore, these effects are influenced by the type and stereochemistry of the monosaccharides constituting the polysaccharide, and by the kind of the glycosidic linkage that connects them [7]. In relation to that, our intention was to study a polysaccharide containing some structural characteristics similar to heparin, as the alternation of neutral and acidic monosaccharide units presented by the main chain of the polysaccharide from gum exudate of the plant *Vochysia thyrsoidea*.

V. thyrsoidea is a common tree of the Brazilian Cerrado, in the central region of Brazil. Its trunk produces a gum exudate with physicochemical properties similar to gum arabic [11]. The gum exudate of *V. thyrsoidea* is constituted by a glycoglucuronomannan formed by a main chain of →4)-β-D-Glc*p*A-(1→2)-α-D-Man*p*-(1→ repeating groups, with most Man*p* units substituted at O-3 by side chains of a single-unit of Ara*f* [12].

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http://dx.doi.org/10.1016/j.ijbiomac.2015.06.051
0141-8130/

In order to obtain anticoagulant and antithrombotic agents, the glycoglucuronomannan from the gum exudate of the plant *V. thyrsoidea* was partially hydrolyzed, and both native and partially degraded polysaccharides were chemically sulfated. The sulfated polysaccharides showed different biological properties.

2. Experimental

2.1. Material

The polysaccharide used was a glycoglucuronomannan, named VTh, obtained from the gum exudate of *Vochysia thyrsoidea* according to Wagner et al. [12].

2.2. Methods

2.2.1. Partial hydrolysis of VTh

VTh (4 g) was partially hydrolyzed with 0.5 M TFA (600 ml) at 100 °C for 4 h and the solution then evaporated to 200 ml, which was added to EtOH (x4 volumes) to give a precipitate of partially hydrolyzed VTh (Ph-VTh).

2.2.2. Chemical sulfation

VTh and Ph-VTh were chemically sulfated according to the method described by O'Neill [13], with slight modifications. Each polysaccharide (400 mg) was solubilized in formamide (40 ml) and pyridine (40 ml). Chlorosulfonic acid was added dropwise over 1 h at 0 °C (in a proportion of 10 mol of chlorosulfonic acid per mol of free hydroxyl). The mixture was maintained at 4 °C for 12 h, then 10% (w/v) aq. $NaHCO_3$ was added until effervescence ceased. The solution was dialyzed against water with 6–8 kDa cut-off membranes and freeze-dried, resulting in the sulfated polysaccharides VThS and Ph-VThS.

2.2.3. Structural analysis of polysaccharides

The molecular weight (M_w) of the polysaccharides was determined by high performance size exclusion chromatography (HPSEC). The peak elution time of each polysaccharide was compared with a calibration curve obtained using standard dextrans (670 kDa, 487 kDa, 266 kDa, 124 kDa, 72.2 kDa, 40.2 kDa, 17.2 kDa, 9.4 kDa and 5.2 kDa, from Sigma). HPSEC analyses were performed using four ultrahydrogel columns in series, with exclusion sizes of 7×10^6, 4×10^5, 8×10^4, and 5×10^3 Da, and a refractive index detector. The eluent was 0.1 M aq. $NaNO_2$ containing 200 ppm aq. NaN_3 at 0.6 ml/min. Samples and dextrans at a concentration of 1 mg/ml were previously filtered through a membrane (0.22 μm) and injected (100 μl loop).

Sugar composition was determined by GC–MS analysis of alditol acetates. Ph-VTh (2 mg) was hydrolyzed in 2 M TFA (1 ml) at 100 °C for 8 h, the solution then evaporated, and the residue dissolved in water (1 ml). The hydrolyzate was treated with ammonia solution to convert glucuronolactone in glucuronate. Then $NaBH_4$ (2 mg) was added and after 18 h, the solution was neutralized with HOAc, evaporated to dryness and the resulting boric acid removed as trimethyl borate by co-evaporation with MeOH [14]. Acetylation was carried out with Ac_2O-pyridine (1:1, v/v; 1 ml) overnight at room temperature [15], and the resulting alditol acetates extracted with $CHCl_3$, which was washed several times with 5% aq. $CuSO_4$ (w/v) for elimination of residual pyridine. They were 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. Components were identified by their typical retention times and electron ionization spectra.

The uronic acid contents of the polysaccharides were quantified using the m-hydroxybiphenyl colorimetric method [16].

Carboxy-reductions of polysaccharides were performed by the carbodiimide method [17], using $NaBH_4$ as reducing agent.

The inorganic sulfate liberated from acid hydrolysis (1 M HCl at 110 °C for 5 h) of the sulfated polysaccharides was quantified by the $BaCl_2$-gelatin turbidimetric method, using Na_2SO_4 as standard [18]. The sulfur percentage in the sulfated polysaccharides was calculated from the inorganic sulfate content. Then, the degree of substitution (DS) was obtained according to the equation described by Whistler and Spencer [19], with $DS = (155.5 \times S\%)/(3200 - 102 \times S\%)$ for VThS and $DS = (168.5 \times S\%)/(3200 - 102 \times S\%)$ for Ph-VThS.

The sulfation pattern was determined by methylation analysis. Before methylation, sulfated polysaccharides were converted in their pyridinium salts, making them soluble in Me_2SO. They were solubilized in water, followed by addition of cation exchange resin (H^+ form), and stirring for 30 min. The resulting pH was 1.0–2.0. The material was filtered, and the filtrate neutralized with pyridine, and freeze-dried [20]. Polysaccharides (2 mg) were solubilized in Me_2SO (0.5 ml), followed by addition of powdered NaOH (150 mg) and CH_3I (0.5 ml) [21]. The mixtures were vigorously shaken for 30 min and then left for 24 h at rest. After neutralization with HOAc, the samples were dialyzed with a 6–8 kDa cut-off membrane and freeze-dried. The per-*O*-methylated polysaccharides were hydrolyzed with 5.5% H_2SO_4 (v/v) for 16 h at 100 °C. The hydrolyzates were neutralized with $BaCO_3$, filtered, reduced with $NaBD_4$ and acetylated as described above. The partially *O*-methylated alditol acetates were analyzed by GC–MS using the same conditions as described above for alditol acetates, except the final temperature was 215 °C. They were identified by their typical retention times and electron impact spectra [22].

2.2.4. Clotting assay (aPTT)

The anticoagulant activity of polysaccharides was investigated using a standard curve of aPTT log *versus* International Units (IU) of heparin. The assay was conducted with a HemosIL® kit (Instrumentation Laboratory), a COAG-A-MATE® XM coagulometer, and unfractionated heparin (200.47 IU/mg; 6th International Standard). Citrated sheep plasma (100 μl) was incubated at 37 °C with saline or polysaccharides solubilized in saline (100 μl) for 1 min and then aPTT reagent was added (100 μl). After 2.5 min, 100 μl of 25 mM $CaCl_2$ was added and the clotting time measured. For each test ($n = 2$), the aPTT mean ± standard error of the mean (SEM) was determined. aPTT was also employed to evaluate the effect of protamine (Sigma) on the inhibition of the anticoagulant activity of the polysaccharides. In this case, polysaccharides plus 1 or 2 μg of protamine were used in the test.

2.2.5. α-Thrombin and factor Xa activities

The assays were performed in 96-well plates. The final concentrations of the reactants were 100 nM antithrombin (AT) or 15 nM heparin cofactor II (HCII), 6 nM α-thrombin or 8 nM factor Xa (Haematologic Technologies Inc., Essex Junction, VT, USA), 100 μM chromogenic substrate S-2238 for α-thrombin and S-2222 for factor Xa (Chromogenix AB, Molndal, Sweden), and 1 to 1×10^{-3} IU/ml of sulfated polysaccharide in TS/PEG buffer (0.02 M Tris/HCl, 0.15 M NaCl, and 1.0 mg/ml polyethylene glycol 8000, pH 7.4). Polysaccharide, AT or HCII, and α-thrombin or factor Xa were incubated for 1 min at 37 °C, then specific chromogenic substrate was added, and absorbance at 405 nm recorded over 15 min (Infinite M200, Tecan Group, Switzerland). In the absence of sulfated polysaccharides, the α-thrombin and fator Xa activities were considered 100%. Assays without AT and HCII were also performed. For each test ($n = 3$), the α-thrombin or fator Xa residual activity mean ± standard error of the mean (SEM) was determined.