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Sulfation pattern of citrus pectin and its carboxy-reduced derivatives: Influence on anticoagulant and antithrombotic effects

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ABSTRACT

Citrus pectin (CP), a polysaccharide composed of [→4)-α-D-Gal*p*A-(1→]$_n$, was submitted to one or four carboxy-reduction cycles, resulting in CP-CR$_1$ and CP-CR$_4$, which had 40% and 2% of Gal*p*A units, respectively. The polysaccharides were chemically sulfated and their anticoagulant and antithrombotic effects determined. Sulfated polysaccharides (CP-S, CP-CR$_1$S and CP-CR$_4$S) had different anticoagulant activities, doubling APTT at concentrations of 28.7, 13.2, and 4.9 μg/ml respectively. CP-CR$_1$S and CP-CR$_4$S also showed antithrombotic activity *in vivo* with ED$_{50}$ of 3.01 and 1.70 mg/kg, respectively. Like heparin, they inhibited thrombin by a mechanism dependent on AT and HCII. Their hemorrhagic potential was also similar to that of heparin. According to methylation analysis, 91.1% and 50.2% of 6-*O*-position in CP-CR$_4$S and CP-CR$_1$S were sulfated, respectively. Therefore, substitution of carboxyl groups by sulfate esters in these polysaccharides increases the anticoagulant and antithrombotic effects.

1. Introduction

Venous thrombosis is a multifactorial disease characterized by excessive formation of clots in veins, without occurrence of any type of vascular injury. It affects 1 in every 20,000 people per year, the risk increasing exponentially with age. The disease can present itself in different ways, such as pulmonary embolism or deep vein thrombosis. Risk factors for the development of thrombosis are divided into genetic factors and acquired, like immobility, pregnancy, and surgery (Fowkes, Price, & Fowkes, 2003; Rosendaal, 1999). One of the most popular treatments of venous thrombosis is based on heparin, a highly sulfated glycosaminoglycan. Its structure is mainly formed by repeating disaccharide groups of →4)-α-D-glucosamine-(1→4)-α-L-iduronic acid(1→ (Mourão & Pereira, 1999; Petitou, Casu, & Lindahl, 2003).

The anticoagulant action of heparins takes place indirectly, depending on the interaction of a specific pentasaccharide segment with antithrombin (AT). Thus, heparin facilitates the interaction of AT with thrombin and factor Xa (Mourão & Pereira, 1999). In addition to this mechanism, heparin also enhances thrombin inhibition by heparin cofactor II (HCII), at higher concentrations, apparently independently of a specific sequence of monosaccharides (Siè et al., 1989).

Despite its popularity, the use of heparin as an anticoagulant drug has limitations due to serious adverse effects that it may entail, such as thrombocytopenia, bleeding, osteoporosis, skin rashes, contact dermatitis, urticaria and skin necrosis, eosinophilia, among others. In addition, due to its animal origin, biological contamination by animal pathogens is a major concern (Longhi, Laks, & Kalil, 2001; Mourão & Pereira, 1999; Perrinaud et al., 2006). Consequently, attempts have been made to develop alternatives to heparin, including studies with naturally or chemically sulfated polysaccharides (Cipriani et al., 2009; Gracher, Cipriani, Carbonero, Gorin, & Iacomini, 2010; Martinichen-Herrero, Carbonero, Gorin, & Iacomini, 2005; Mourão & Pereira, 1999; Mourão, 2004; Pomin, 2009). Both anticoagulant and antithrombotic actions are related to the presence of sulfate esters, their position and distribution along the sugar-chain. Moreover, these properties are influenced by the sugar type, glycosidic linkage of the sugar-chain and its stereochemistry (Pomin, 2009).

Galactans from seaweeds and fucans from marine invertebrates are the most common naturally sulfated polysaccharides that have shown anticoagulant and antithrombotic activities (Fonseca, Oliveira, Melo, Benevides, & Mourão, 2008; Melo, Pereira, Foguel, & Mourão, 2004; Mourão & Pereira, 1999; Pereira, Mulloy, & Mourão, 1999; Yoon, Pyun, Hwang, & Mourão, 2007). Concerning chemical sulfation, polysaccharides from different sources have been studied, including those from lichens, mushrooms, and plants (Cipriani et al., 2009; Gracher et al., 2010; Martinichen-Herrero et al., 2005).

Recently, anticoagulant and antithrombotic effects of chemically sulfated citrus pectin were demonstrated (Cipriani et al., 2009). Citrus pectin (CP) is a widely available polysaccharide in

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nature, consisting almost entirely of →4)-α-D-Gal*p*A-(1→ repeating units. CP has now been submitted to carboxy-reduction steps, followed by chemical sulfation, and the influence of the sulfation pattern on anticoagulant and antithrombotic effects was investigated.

2. Experimental

2.1. Materials

Citrus pectin was extracted from fresh mesocarp of *Citrus sinensis* (L.) Osbeck. Powdered material (100 g) was submitted to extraction with 0.01 M HCl (500 ml) under reflux for 1 h, followed by filtration, the filtrate then being adjusted to pH 7.0 with aq. NaOH, dialyzed, and freeze-dried. Unfractionated heparin sodium salt from porcine intestinal mucosa (202 IU/mg) was obtained from Sigma (St. Louis, MO, USA).

2.2. Methods

2.2.1. Carboxy-reduction

Carboxy-reductions were performed by the carbodiimide method (Taylor & Conrad, 1972), using $NaBH_4$ as reducing agent. Citrus pectin (500 mg) was dissolved in 20 ml of MES buffer [2-(*N*-morpholine)-ethanesulfonic acid] (0.2 M, pH 4.75), to which carbodiimide [cyclohexyl-3-(2-morpholinoethyl) carbodiimide] was added slowly with stirring, in a ratio of 24 mg of carbodiimide for each 1 mg of uronic acid in the sample. The reaction mixture was stirred for a total of 2 h. 2 M Tris buffer (pH 7.0) was added until pH 7.0 (10 ml) and then $NaBH_4$ to a final concentration of 2 M. The reduction occurred over 14 h and was stopped by adding acetic acid to pH 5. The solution was finally dialyzed with an 8 kDa cut-off membrane for 48 h, and the retained solution was concentrated and lyophilized. The citrus pectin was subjected to one or four carboxy-reduction cycles, resulting in fractions CP-CR_1 and CP-CR_4, respectively.

2.2.2. Chemical sulfation

Citrus pectin (CP) and its carboxy-reduced derivatives (CP-CR_1 and CP-CR_4) were chemically sulfated, resulting in samples CP-S, CP-CR_1S and CP-CR_4S, according to the method described by O'Neill (1955), which was slightly modified. The polysaccharides (100 mg) were then solubilized in formamide (10 ml), pyridine (10 ml) was then added, followed by chlorosulfonic acid (in a proportion of 10 mol of chlorosulfonic acid per mol of free hydroxyl), which was added dropwise over 1 h at 0 °C. The mixture was maintained at 4 °C for 12 h, and 10% (w/v) aq. $NaHCO_3$ was added until effervescence ceased. The solution was then dialyzed and freeze dried.

2.2.3. Structural analysis of polysaccharides

The average molar mass (M_w) of the polysaccharides was 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 0.1 M aq. $NaNO_2$ containing 200 ppm aq. NaN_3 at 0.6 ml/min. Each sample, previously filtered through a membrane (0.22 μm), was injected (100 μl loop) at a concentration of 1 mg/ml. The specific refractive index increment (d*n*/d*c*) of the polysaccharides was determined and the results were processed with software provided by the manufacturer (ASTRA 4.70.07, Wyatt Technologies).

Sugar composition was determined by GC–MS analysis of alditol acetates. The polysaccharide (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 $NaBH_4$ (2 mg), and, 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 ml) at room temperature for 12 h, and the resulting alditol acetate extracted with $CHCl_3$. This was 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 present on citrus pectin was identified by silica-gel 60 thin layer chromatography (TLC; Merck). CP was hydrolyzed as described above and analyzed by TLC. The plates were developed by ethyl acetate:*n*-propanol:acetic acid:water (4:2:2:1, v/v/v/v) and stained with orcinol–sulfuric acid. Authentic standards of glucuronic and galacturonic acids were used. The uronic acid contents of the polysaccharides were quantified using the *m*-hydroxybiphenyl colorimetric method (Filisetti-Cozzi & Carpita, 1991).

The sulfation pattern was determined by methylation analysis. The polysaccharides were solubilized in water, followed by addition of cation-exchange resin (H^+ form), stirring for 30 min. The solution pH was 1.0–2.0. The resin was filtered off, the filtrate neutralized with pyridine, and freeze-dried (Nagasawa, Inoue, & Tokuyasu, 1979). The pyridinium salts were solubilized in Me_2SO (1 ml), followed by addition of powdered NaOH (300 mg) and MeI (1 ml) (Ciucanu & Kerek, 1984). 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 an 8 kDa cut-off membrane and then freeze-dried. The per-*O*-methylated polysaccharides were hydrolyzed with 0.5 ml of 72% H_2SO_4 (w/v) for 1 h at 0 °C, followed by addition of water (4.0 ml) and heating at 100 °C for 17 h (Saeman, Moore, Mitchell, & Millet, 1954). The hydrolyzates were neutralized with $BaCO_3$, filtered, reduced with $NaBD_4$ and acetylated with acetic anhydride–pyridine (1:1, v/v; 1 ml) for 14 h at room temperature. The reactions were stopped by addition of ice, and the partially *O*-methylated alditol acetates extracted with chloroform, which was washed several times with 5% aq. $CuSO_4$ (w/v) for elimination of residual pyridine. The partially *O*-methylated alditol acetate mixtures 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 (Sassaki, Gorin, Souza, Czelusniak, & Iacomini, 2005).

2.2.4. Clotting assay (APTT)

The activated partial thromboplastin time (APTT) test was determined with a Dade Actin kit (Dade Behring, Marburg, DE), in a COAG-A-MATE XM coagulometer (OrganonTeknika Corporation, Durhan, NC), using a pool of normal human plasma.

Plasma (90 μl) was incubated at 37 °C with saline, heparin, or polysaccharides (10 μl) and rabbit cephalin (100 μl). After 2 min, 0.025 M $CaCl_2$ (100 μl) was added, and the clotting time measured. Results were 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 ± standard error of the mean (SEM) ($n = 2$). $T_1/T_0 = 8.38$ indicate complete inhibition of plasma coagulation.

2.2.5. Animals

Experiments were conducted on male or female Wistar rats (170–250 g) from the colony of 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