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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

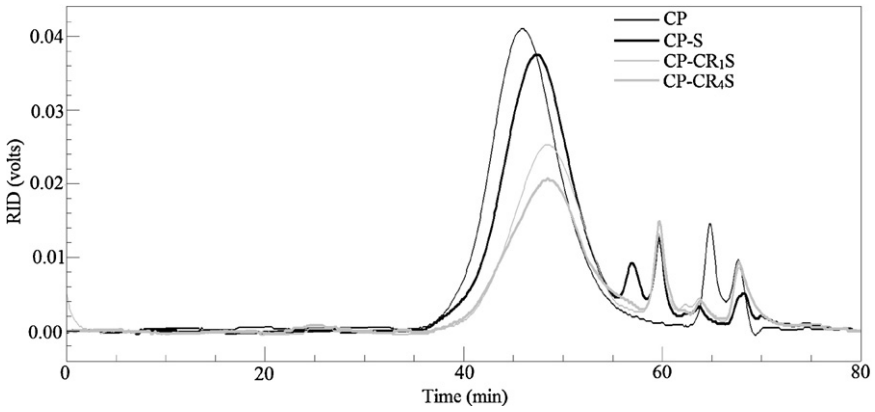

Fig. 1. Elution profiles obtained on HPSEC analysis of CP, CP-S, CP-CR_1S and CP-CR_4S.

Ethics Committee of Federal University of Paraná approved all the procedures adopted in this study (authorization number 428).

2.2.6. Venous thrombosis

Thrombus formation was induced by promoting a combination of stasis and hypercoagulability (Berry, Girard, Lochot, & Lecoffre, 1994; Vogel, Meleuman, Bourgondiën, & Hobbelen, 1989). Rats were anesthetized and their right carotid artery was cannulated for injection of vehicle (saline), sulfated polysaccharides or heparin, and thromboplastin. The abdominal vena cava was dissected, and loose sutures were placed between the right renal vena and femoral veins, and in the left renal vena. Vehicle, sulfated polysaccharides, or heparin were infused into the right carotid artery and allowed to circulate for 5 min. Thrombus formation was then induced by injection of thromboplastin (5 mg/kg body weight), followed 20 s later by stasis of a 0.7 cm segment of the abdominal vena cava, for 20 min. The thrombus formed inside the occluded segment was then pulled out, washed with saline, dried for 1 h at 60 °C, and weighed. For each group ($n = 6$), the mean thrombus weight ± SEM was determined and expressed as percentages of thrombus weight, 100% representing absence of any inhibition of thrombosis formation (thrombus weight with vehicle administration).

2.2.7. Inhibition of α-thrombin or factor Xa in the presence of sulfated polysaccharides

The assays were performed in 96-well plates. The final concentrations of the reactants included 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) and 0–100 μg/ml sulfated polysaccharide in 75 μl of TS/PEG buffer (0.02 M Tris/HCl, 0.15 M NaCl, and 1.0 mg/ml polyethylene glycol 8000, pH 7.4). The α-thrombin or factor Xa was finally added to initiate the reaction. After 1 min of incubation at 37 °C, 25 μl of chromogenic substrate S-2238 for α-thrombin or S-2222 for factor Xa (Chromogenix AB, Molndal, Sweden) were added (100 μM final concentration), and absorbance at 405 nm recorded over 15 min (Infinite M200, Tecan Group, Switzerland). The change of absorbance was proportional to the α-thrombin or factor Xa activity. In the absence of sulfated polysaccharides, the thrombin activity was considered 100%. Assays without AT and HCII were also performed.

2.2.8. Hemorrhagic effect

Rats were anesthetized, and their right carotid artery was cannulated for administration of vehicle (PBS, Phosphate Buffered Saline), heparin or polysaccharides. After circulation for 5 min, the tails were cut 5 mm from their tip, and immersed in 40 ml of distilled water for 1 h. Determination of blood loss was established by measuring spectrophotometrically the amount of hemoglobin dissolved in water, compared to a standard curve obtained at 540 nm (Mendes-Silva et al., 2003). For each treatment group ($n = 6$), the mean of blood loss volume ± SEM was determined.

2.2.9. Statistical analysis

Results are expressed as the mean ± standard error of the mean (SEM) and the statistical significance of the results was determined using one-way analysis of variance (ANOVA), followed by Tukey's test. Data were considered different at a significance level of $p < 0.05$.

3. Results

3.1. Preparation and structural analysis of the sulfated polysaccharides

Citrus pectin (CP) is a polysaccharide formed mainly by →4)-α-D-Gal*p*A-(1→ repeating units. CP, extracted from mesocarp of *C. sinensis*, contained essentially galacturonic acid (95.5%), which was identified by TLC analysis and quantified colorimetrically. CP also contained traces of neutral sugars: galactose (1.8%), glucose (1.5%), arabinose (0.8%), and rhamnose (0.4%). It was subjected to one or four carboxy-reduction cycles, resulting in fractions CP-CR_1 and CP-CR_4, which contained 40% and 2% of Gal*p*A units, respectively. Each polysaccharide (100 mg) was then chemically sulfated, giving CP-S (145 mg), CP-CR_1S (156 mg) and CP-CR_4S (195 mg).

In order to verify the integrity of the polysaccharide chains after chemical sulfation, HPSEC analysis of CP, CP-S, CP-CR_1S, and CP-CR_4S were performed, and their molecular weights determined. Their elution profiles presented a main peak between 40 and 55 min (Fig. 1). Smaller peaks visualized after 55 min may represent fragments of the polysaccharides. The molecular weights of CP, CP-S, CP-CR_1S, and CP-CR_4S were 120 kg/mol ($dn/dc = 0.142$), 148 kg/mol ($dn/dc = 0.133$), 132 kg/mol ($dn/dc = 0.142$), and 91 kg/mol ($dn/dc = 0.107$), respectively.

According to methylation analysis (Table 1), CP-CR_4S was 2-*O*- (53.2%), 3-*O*- (55.5%) and 6-*O*-sulfated (91.1%), with more than one third (36.6%) of the galactose units being tri-sulfated. CP-CR_1S was 2-*O*- (22.9%), 3-*O*- (24.3%) and 6-*O*-sulfated (50.2%).

As fraction CP-CR_1S contained 40% of Gal*p*A units, it was carboxy-reduced prior to methylation analysis, to determine the sulfation pattern of its Gal*p*A units. The proportion of alditol acetates of resulting 3,6-Me_2-Gal and 2,6-Me_2-Gal increased after carboxy-reduction, indicating that Gal*p*A units of CP-CR_1S were 2-*O*- (11.1%) and 3-*O*-sulfated (9.3%). However, according to the

Table 1
Structural components of CP-CR_4S, CP-CR_1S and CP-CR_1SCR, based on methylation analysis.

O-Methylalditol acetate[a]	Mol (%)[b]			Substitution position
	CP-CR_4S	CP-CR_1S	CP-CR_1SCR	
2,3,6-Me_3-Gal	3.5	6.5	34.6	–
2,6-Me_2-Gal	1.9	1.7	11.0	3-*O*-
3,6-Me_2-Gal	1.5	1.6	12.7	2-*O*-
2,3-Me_2-Gal	22.4	20.4	27.2	6-*O*-
2-Me-Gal	17.0	8.5	6.5	3-*O*- and 6-*O*-
3-Me-Gal	15.1	7.2	5.7	2-*O*- and 6-*O*-
Gal	36.6	14.1	2.3	2-*O*-, 3-*O*- and 6-*O*-
GalA[c]	2.0	40.0	–	–

[a] *O*-Methylalditol acetate, obtained by successive per-*O*-methylation, hydrolysis, reduction with $NaBD_4$, and acetylation, then analysis by GC–MS (DB-225 column).
[b] Percentage of peak area relative to total peak area.
[c] Determined colorimetrically according to Filisetti-Cozzi and Carpita (1991) in a separate experiment.

relative increase of a 2,3,6-Me_3-Gal derivative, 28.1% of Gal*p*A units of CP-CR_1S had no sulfate group.

3.2. In vitro anticoagulant activity of polysaccharides

The sulfated polysaccharides CP-S, CP-CR_1S and CP-CR_4S had anticoagulant activity, being able to extend APTT in a dose-dependent manner (Fig. 2), and doubling APTT at concentrations of 28.7, 13.2, and 4.9 μg/ml, respectively (APTT control = 35.8 s). Non-sulfated polysaccharides (CP, CP-CR_1 and CP-CR_4) did not affect APTT.

3.3. In vivo antithrombotic activity of sulfated polysaccharides

The antithrombotic effect of CP-S, CP-CR_1S and CP-CR_4S was investigated by a venous thrombosis model, in rats. In the control group (vehicle), the average dried thrombus weight was 5.2 ± 0.3 mg (mean ± SEM), corresponding to 100% thrombosis. CP-CR_1S and CP-CR_4S inhibited thrombus formation in a dose-dependent manner. CP-CR_4S was more potent, with ED_{50} of 1.7 mg/kg, while CP-CR_1S had an ED_{50} of 3.01 mg/kg. Heparin, the positive control in the test, showed an ED_{50} of 0.06 mg/kg (Fig. 3). However, in the group treated with CP-S at dose of 5 mg/kg, the average dried thrombus weight was 5.45 ± 1.2 mg (mean ± SEM), that was not statistically different from control group (vehicle). Since CP-S has no antithrombotic effect in this high dose, other doses were not tested.

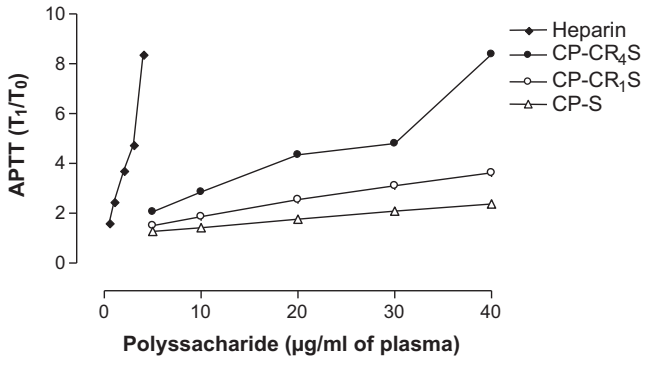

Fig. 2. Anticoagulant activities measured by activated partial thromboplastin time (APTT). Normal human plasma was incubated with different concentrations of CP-CR_4S, CP-CR_1S, CP-S or heparin. Results are expressed as T_1/T_0, which is the ratio between the clotting time in the presence (T_1) and absence of polysaccharide (T_0) in the incubation mixture ± standard error of the mean (SEM) ($n = 2$). $T_1/T_0 = 8.38$ indicate complete inhibition of plasma coagulation. All doses of polysaccharide CP-CR_1S, CP-CR_4S and heparin, as well as the CP-S doses of 20, 30 and 40 μg/ml of plasma, showed significant differences with $p < 0.001$ compared with the negative control group (saline, 35.8 ± 0.0 s). The dose of 10 μg/ml of CP-S showed $p < 0.01$.

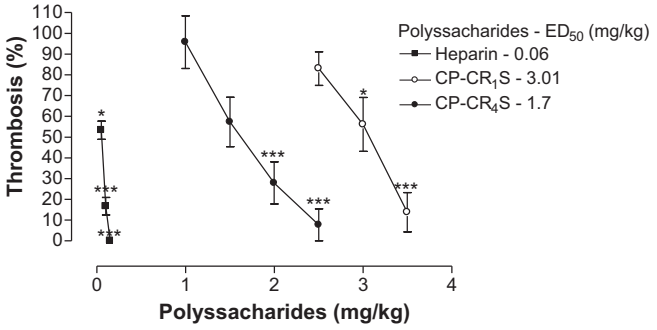

Fig. 3. Venous antithrombotic effect after intravascular administration of sulfated polysaccharides in rats. Thrombus formation was induced by promoting a combination of stasis and hypercoagulability. Different doses of CP-CR_4S (●), CP-CR_1S (○), or heparin (■) were administered in the right carotid artery and allowed to circulate for 5 min. Thromboplastin (5 mg/kg body weight) was then injected and 20 s later, 0.7 cm of an isolated segment of the abdominal vena cava was tied off. After stasis for 20 min, the thrombus formed on the interior was pulled out, dried and weighed. Results are expressed as % thrombus weight (mean ± SEM, $n \geq 6$, $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. saline), 100% representing absence of any thrombosis inhibition (thrombus weight in the absence of polysaccharide administration).

3.4. Effect of sulfated polysaccharides on inhibition of α-thrombin and factor Xa

Like heparin, CP-CR_1S and CP-CR_4S are able to inhibit α-thrombin and factor Xa in a serpin-dependent mechanism (Fig. 4 and Table 2). CP-CR_1S inhibited α-thrombin, in the presence of AT or HCII, at IC_{50} of 2.9×10^{-3} μg/ml and 0.46 μg/ml, respectively. However, CP-CR_4S was more effective, at IC_{50} of 1.0×10^{-3} μg/ml with AT and 0.35 μg/ml with HCII. Concerning factor Xa, CP-CR_1S and CP-CR_4S had an IC_{50} of 0.46 μg/ml and 0.11 μg/ml, respectively, in the presence of AT. However, the inhibitory effect on factor Xa was slight, when compared with heparin with $IC_{50} = 2.6 \times 10^{-3}$ μg/ml.

3.5. Hemorrhagic effect with sulfated polysaccharides

The hemorrhagic effect was evaluated using four times the dose of polysaccharide required to inhibit 50% of thrombus formation.

Table 2
Concentrations of sulfated polysaccharides required to inhibit 50% of α-thrombin and factor Xa in the presence of AT and HCII.

Polysaccharide	IC_{50} (μg/ml)		
	α-Thrombin		Factor Xa
	With AT	With HCII	With AT
Heparin	9.5×10^{-4}	0.53	2.6×10^{-3}
CP-CR_4S	1.0×10^{-3}	0.35	0.11
CP-CR_1S	2.9×10^{-3}	0.46	0.46

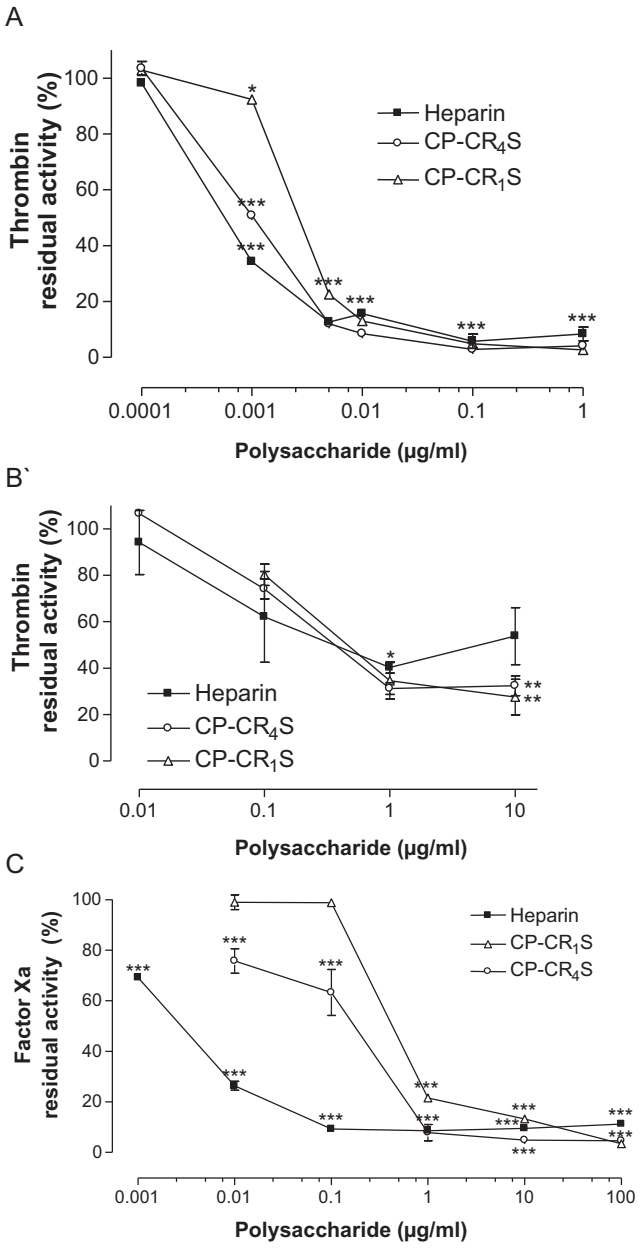

Fig. 4. Effect of sulfated polysaccharides on inhibition of α-thrombin by AT (A), α-thrombin by HCII (B), and factor Xa by AT (C), 6 nM of α-thrombin or 8 nM factor Xa and 100 nM AT or 15 nM HCII were incubated with different concentrations of CP-CR4S (●), CP-CR_1S (○), or heparin (■). After 1 min at 37 °C, 100 μM of specific chromogenic substrate were added, and α-thrombin or factor Xa activity, expressed as a proportion of absorbance at 405 nm (means, $n = 3$), with 100% of activity considered to be that occurring without addition of polysaccharide.

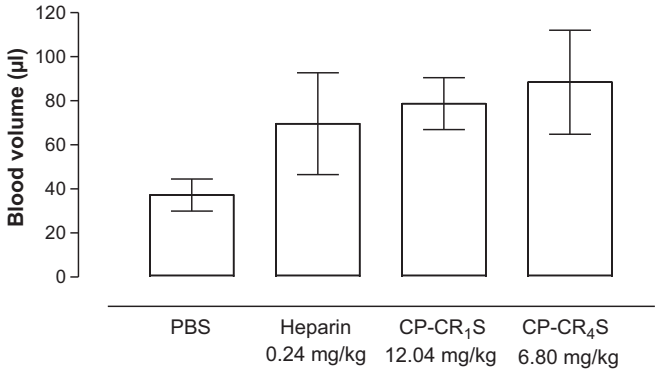

Fig. 5. Hemorrhagic effect after intravascular administration of sulfated polysaccharides in rats. CP-CR_4S, CP-CR_1S or heparin was administered in the right carotid artery. The polysaccharide dose used was four times that of ED_{50} obtained by the antithrombotic test. After circulation for 5 min, the tails were cut 5 mm from their tip, and immersed in 40 ml of distilled water for 1 h. The determination of the blood loss was established by measuring the amount of hemoglobin dissolved in water, by spectrophotometric method, compared to a standard curve obtained at 540 nm.

Although a visual analysis of the graph may indicate an increase in hemorrhagic process when polysaccharides were administered (Fig. 5), we found no statistically significant difference on comparison with the control group (PBS). PBS was used as vehicle, because with saline there was copious bleeding. It is unknown why saline caused this effect.

4. Discussion

Heparin is largely effective for prevention and treatment of thromboembolic problems. However, its anticoagulant effect is unpredictable, with risk of bleeding, requiring close laboratory monitoring for its safe use (Mousa, 2007). In addition, due to its animal origin, biological contamination by animal pathogens is a serious concern (Mourão & Pereira, 1999). Consequently, attempts have been made to obtain alternatives to heparin. It has been demonstrated that chemical sulfation of polysaccharides can provide anticoagulant and antithrombotic agents (Cipriani et al., 2009; Gracher et al., 2010; Martinichen-Herrero et al., 2005).

Studies have demonstrated that chemical sulfation of pectins, including citrus pectin, give products with anticoagulant properties (Bae et al., 2009; Cipriani et al., 2009), with the activity depending on the quantity of sulfate groups (Fan et al., 2011; Vityazev et al., 2010).

Citrus pectin is formed almost entirely by →4)-α-D-Gal*p*A-(1→ repeating groups, and its chemical sulfation can lead to structures 2-*O*- and 3-*O*-sulfated. When carboxy-reduced, galacturonic acid residues are converted to galactose, which can also be sulfated at O-6. Thus, citrus pectin was submitted to one or four carboxy-reduction cycles, resulting in two polysaccharides with different Gal*p*A to Gal*p* ratios, which were chemically sulfated and biologically tested as anticoagulants and antithrombotics.

Using different reagents for sulfation, Vityazev et al. (2010) demonstrated that chlorosulfonic acid is the optimal reagent for chemical sulfation of pectins, considering sulfation degree, destruction degree, and yield of sulfated derivatives. However, they demonstrated that chemical sulfation with chlorosulfonic acid can lead to partial destruction of the polysaccharide. So, since chlorosulfonic acid was employed for chemical sulfation, acid hydrolysis of the polysaccharides may have occurred.

In order to verify the integrity of the polysaccharides, their molecular weights were determined. The native polysaccharide CP had a M_w of 120 kg/mol, whereas its sulfated derivative CP-S had a M_w of 148 kg/mol. Glycosidic linkages including anomeric carbon of uronic acids units are more resistant to acid hydrolysis. Thus, the high content of Gal*p*A in CP is responsible for its acid hydrolysis resistance, and the increase of molecular weight is due to the addition of sulfate groups. Moreover, according to the protocol adopted, chemical sulfation was developed at cold temperature, avoiding a high cleavage degree of the polysaccharides. To prepare CP-CR_1S and CP-CR_4S, carboxy-reduction method was employed on CP and its galacturonic acid units were converted to galactose units. CP-CR_1S still had a high Gal*p*A content (40%) and apparently did not present cleavage during the chemical sulfation procedure, since its M_w was 132 kg/mol. Nevertheless, Gal*p*A content of CP-CR_4S was only 2%, and its M_w was 91 kg/mol. It shows that reduction of Gal*p*A to Gal*p* makes the polysaccharide more susceptible to cleavage.

The sulfation patterns of the polysaccharides were determined by methylation analysis, where *O*-methyl substitutes the free hydroxyl groups. Sulfated hydroxyl groups are not methylated, allowing the determination of their position. To determine the sulfation pattern of Gal*p*A units of CP-CR_1S, it was carboxy-reduced, generating CP-CR_1SCR. This is necessary because the partially *O*-methylated alditol acetates derivatives of acid monosaccharides are less volatile and resistant to analysis by GC–MS. Therefore, according to the analysis of CP-CR_1SCR, the sulfation pattern of Gal*p*A units in CP-CR_1S was indicated. As observed, chemical sulfation occurred preferably at 6-*O*-position over 3-*O*- or 2-*O*-positions, probably due to stereochemistry of CP-CR_1 and CP-CR_4. Since CP was constituted basically by Gal*p*A units (95.5%), its 6-*O*-positions are unavailable for sulfation. Then, although not quantified by methylation analysis, sulfation of CP occurs at 2-*O*- and/or 3-*O*-position.

In order to determine if the polysaccharides had an anticoagulant effect, the APTT test was employed, since it is used to monitor patients undergoing anticoagulation therapy. All the chemically sulfated polysaccharides extended APTT, suggesting that they can act as antithrombotics. The presence of sulfate groups is essential to the activity of CP-S, CP-CR_1S and CP-CR_4S, since non-sulfated polysaccharides (CP, CP-CR_1 and CP-CR_4) did not affect APTT. The anticoagulant activity was dependent on the sulfation pattern of the polysaccharides. With more sulfation at O-6, the polysaccharide became more potent. The absence of 6-*O*-sulfate on CP-S may explain its low anticoagulant activity. In the thrombosis experiment, CP-CR_4S proved to be a more potent inhibitor of venous thrombosis than CP-CR_1S, whereas CP-S had no antithrombotic effect, showing again the positive effect of the 6-*O*-sulfate group.

The anticoagulant and antithrombotic effects of heparin are mainly related to α-thrombin and factor Xa inhibition by a mechanism dependent on AT and HCII (Bourin & Lindahl, 1993; Casu, 1985). In order to determine if CP-CR_1S and CP-CR_4S act the same way as heparin, their effects on α-thrombin and factor Xa activities were evaluated. Like heparin, they were able to inhibit α-thrombin and factor Xa in a serpin-dependent mechanism. However, the effect on factor Xa was slight, indicating that CP-CR_1S and CP-CR_4S act preferentially inhibiting α-thrombin (Table 2).

An ideal antithrombotic drug should inhibit thrombus formation, without increasing the risk of bleeding. Visual analysis of Fig. 5 may indicate an increase in the hemorrhagic process when polysaccharides were administered, but we found no statistically significant differences on comparison with the control group (PBS). Although heparin has been related to bleeding risk (Mousa, 2007), this effect was not observed at the dose employed. The polysaccharide dose used to evaluate the effect of bleeding was four times that of ED_{50} obtained by the antithrombotic test. Thus, the results indicate that administration of these polysaccharides does not cause a hemorrhagic process, at these doses, and that they are potentially safer with respect to their hemorrhagic potential. Although the antithrombotic effect of CP-CR_1S and CP-CR_4S are slight when compared with heparin, there is a lower risk of a super dose, with lower risk of bleeding.

Our results indicate that replacement of a carboxyl group with a sulfate group in citrus pectin increases anticoagulant and antithrombotic effects, without affecting the hemorrhagic effect. It is known that the sulfation pattern of polysaccharides influence their anticoagulant and antithrombotic activities (Pomin, 2009). Sulfated galactans from red seaweeds have been studied as anticoagulant and antithrombotic agents (Fonseca et al., 2008; Pereira et al., 2005). Those from *Botryocladia occidentalis* and *Gelidium crinale* have a very similar structure, composed of repetitive disaccharide →4-α-D-Gal*p*-(1 → 3)-β-D-Gal*p*-(1→, with the same size chain, but they differ slightly in the sulfation pattern and in the proportions of 2,3-di-*O*-sulfated and 3-*O*-sulfated α-Gal*p* residues, with the galactan from *G. crinale* being less sulfated. As a consequence of these differences, the two sulfated galactans differ in their anticoagulant and antithrombotic activities (Fonseca et al., 2008; Pereira et al., 2005). The sulfated galactan from *G. crinale* has a lower anticoagulant activity when compared with the polysaccharide from *B. occidentalis* (Pereira et al., 2005). Also, sulfated galactan from *B. occidentalis* is a potent antithrombotic compound in low doses, whereas that from *G. crinale* exhibits procoagulant effect. In high doses, these effects are inverse. Carboxy-reduction employed on this study converted citrus pectin in a (1 → 4)-linked α-D-galactan. Here, proportion and/or distribution of 6-*O*-sulfated units may be crucial for the anticoagulant and antithrombotic effects of CP-CR_1S and CP-CR_4S. Moreover, CP-S, which is devoid of 6-*O*-sulfated units, had low anticoagulant activity and has not antithrombotic effect. Since galactans from seaweeds are structurally different from those derived from citrus pectin, considering glycosidic linkages and galactose anomers, a direct comparison of their activities is not possible. However, as demonstrated for seaweeds sulfated galactans, sulfation patterns affect the activity of CP-CR_1S and CP-CR_4S. Since CP-CR_4S was more effective as an anticoagulant and antithrombotic, stereospecific chemical sulfation protocols can be further employed to investigate the critical structural motif responsible for promoting stronger effects.

Possible unintended pharmacologic consequences, like allergenic side-effects, can be associated with these chemically sulfated polysaccharides. In this way, caution and new biological tests are still necessary before they are employed. Even so, CP-CR_4S appears to be a promising antithrombotic agent, since it does not involve bleeding risk and is obtainable from a widely available source. Moreover, chemically sulfated polysaccharides can also be an alternative to provide *in vitro* anticoagulation for use in clinical laboratories.

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