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Sulfonation and anticoagulant activity of fungal exocellular β-(1→6)-D-glucan (lasiodiplodan)

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ABSTRACT

An exocellular β-(1→6)-D-glucan (lasiodiplodan) produced by a strain of *Lasiodiplodia theobromae* (MMLR) grown on sucrose was derivatized by sulfonation to promote anticoagulant activity. The structural features of the sulfonated β-(1→6)-D-glucan were investigated by UV–vis, FT-IR and ^{13}C NMR spectroscopy, and the anticoagulant activity was investigated by the classical coagulation assays APTT, PT and TT using heparin as standard. The content of sulfur and degree of substitution of the sulfonated glucan was 11.73% and 0.95, respectively. UV spectroscopy showed a band at 261 nm due to the unsaturated bond formed in the sulfonation reaction. Results of FT-IR and ^{13}C NMR indicated that sulfonyl groups were inserted on the polysaccharide. The sulfonated β-(1→6)-D-glucan presented anticoagulant activity as demonstrated by the increase in dose dependence of APTT and TT, and these actions most likely occurred because of the inserted sulfonate groups on the polysaccharide. The lasiodiplodan did not inhibit the coagulation tests.

1. Introduction

β-Glucans are found mainly in the cell wall of yeasts and filamentous fungi, as minority constituents in the cytosol, and can also be secreted as exo-biopolymers to the environment (Williams, 1997). They have emerged as an important class of bioactive products with biological response modifying (BRM) activities (Bohn & BeMiller, 1995). Besides immunoprotective activity, exopolysaccharides of the β-glucan type have been examined in relation to the antithrombotic, antioxidant, antiviral, anti-inflammatory, anticoagulant actions, and antiproliferative activity on breast cancer cells (Brandi et al., 2011; Cunha et al., 2012; Kato et al., 2010; Martinichen-Herrero, Carbonero, Gorin, & Iacomini, 2005; Wang et al., 2010).

The biological activities can be presented by the molecules *in natura*, or through chemical modification. The chemical derivatization of glycans offers an opportunity to enhance their action, and even develop activity in non-bioactive molecules, which can be used as new pharmacological agents (Mantovani et al., 2008; Vetvicka, Vetvickova, Frank, & Yvin, 2008). Furthermore, the inclusion of sulfonate groups on glycans enables the generation of water-soluble molecules, and this is important for anticoagulant and other biological activities (Mendes et al., 2009).

Glucans of the β-(1→3)- and β-(1→3;1→6)-types are the most described in scientific articles and patents. They have linear, branched or cyclic (Laroche & Michaud, 2007) structures, and are targets for research for their pharmacological and immunological effects (Vetvicka et al., 2008). β-(1→6)-D-Glucans are widely known as pustulan produced by lichens of Umbilicariaceae species (Narui, Sawada, Culberson, Culberson, & Shibata, 1999), and are commonly present as constituents of the fungal and yeast cell wall (Klis, Mol, Hellingwerf, & Brul, 2002). As exocellular biopolymers, β-(1→6)-D-glucans are uncommon, if not rare, and are known to be produced by only some fungi (Cunha et al., 2012; Vasconcelos et al., 2008).

The biological activity of the β-glucans from fungi including mushrooms that comprise both β-(1→3)- and (1→6)-D-glucans are considered the most effective immune stimulatory agents (Rop, Mlcek & Jurikova, 2009), and the presence of branches at C-6 on the β-(1→3)-glucan chain as well as a triple helix conformation are important structural features determining BRM activity of these polysaccharides (Bohn & BeMiller, 1995; Leung, Liu, Koon, & Fung, 2006).

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Substances with anticoagulant properties have been used both in therapeutic processes and *in vitro* to treat medical conditions, and natural heparin is most widely used (Wang, Li, Zheng, Normakhamatov, & Guo, 2007). Unfortunately, heparin shows some contra-indications such as bleeding, and moreover, because heparin is extracted from animal tissues, it tends to cause a risk of contamination by animal-derived pathogens. Development of alternatives to heparin therefore is an important field of research. No mammalian source of heparin or its derivatives are considered to be ideal choices, and consequently, polysaccharide sulfonates (natural or chemically derivatized) are of special interest (Glauser et al., 2009; Pomin, 2012).

An abundant source of anticoagulant polysaccharides is marine algae that contain a variety of natural sulfated galactans and sulfated fucans (Melo, Pereira, Foguel, & Mourão, 2004). Other sulfated polysaccharides with anticoagulant activity are found in marine invertebrates (Pomin, 2012). Chemically sulfonated polysaccharides, which present anticoagulant and antithrombotic activity, have been obtained from various polysaccharide types such as microbial β-glucans (Brandi et al., 2011; Mendes et al., 2009) and dextrans, and plant-derived galactoglucomannans and galactomannans (Martinichen-Herrero et al., 2005; Yang, Du, Wen, Li, & Hu, 2003).

In this work we describe for the first time the sulfonation of an exocellular β-(1→6)-D-glucan (lasiodiplodan) from *Lasiodiplodia theobromae* MMLR grown on sucrose as carbon source (Vasconcelos et al., 2008), and the effect of sulfonation on promoting the solubility of this polysaccharide in aqueous solutions, and the physiological activity as an anticoagulant as alternative to heparin.

2. Experimental

2.1. Materials

Sodium heparin (5000 UI/mL) was purchased from Akzo Organon (São Paulo, Brazil). Human plasma was obtained by centrifugation (450 × *g*/15 min at 25 °C) of citrated blood. Blood coagulation time reagents: activated partial thromboplastin time (APTT), thrombin time (TT) and prothrombin time (PT), were acquired from In-Vitro Diagnóstica S/A (Itabira, MG, Brazil). Thrombin and antithrombin were obtained from Haematologic Technologies, USA, and chromogenic substrate S-2238 from Chromogenix, Sweden. Chlorosulfonic acid was obtained from Vetec (Rio de Janeiro, RJ, Brazil).

2.2. Production and preparation of β-(1→6)-D-glucan (lasiodiplodan)

β-(1→6)-D-Glucan (lasiodiplodan) was produced by *L. theobromae* MMLR and grown on nutrient medium containing sucrose as previously described (Vasconcelos et al., 2008). Cell-free culture fluid was obtained after removal of the mycelium by centrifugation (5500 × *g*/20 min) at 4 °C. The supernatant was treated with 3 volumes of absolute ethanol, the precipitated material recovered and dissolved in distilled water, followed by extensive dialysis against frequent changes of distilled water over 48 h, and then freeze-dried.

2.3. Analytical techniques

Carbohydrate was determined by the phenol–sulfuric acid method (Dubois, Gilles, Hamilton, & Rebers, 1956) with D-glucose as standard. Protein was measured by the Bradford method (Bradford, 1976) using bovine serum albumin as standard.

2.4. Sulfonation

Sulfonation of β-(1→6)-D-glucan was performed according to O'Neill (1955) with some modifications: lasiodiplodan powder (50.0 mg) was solubilized in dry formamide (10.0 mL) with vigorous stirring for 24 h at room temperature. Then 10.0 mL of pyridine was added to the mixture followed by continuation of vigorous stirring for another 30 h at room temperature. Chlorosulfonic acid (4.0 mL) was next added drop-wise to the mixture in an ice-bath over an interval of 2 h, and then left at 4 °C for 12 h. The reaction was terminated by adding ice-water, and neutralized by adding a solution of 10% (w/v) $NaHCO_3$ until all CO_2 evolution ceased. The reaction mixture was then dialyzed exhaustively against distilled water for 6 days with several changes of water, and the dialysate concentrated under reduced pressure (<39 °C) and lyophilized. The product obtained was referred to as sulfonated β-(1→6)-D-glucan. The sulfonation reaction was repeated two further times until a degree of substitution (DS) of greater than 0.80 was obtained.

2.5. Determination of the degree of substitution (DS)

Samples of sulfonated β-(1→6)-D-glucan (1.0 mg) were hydrolyzed using 1.0 M HCl (1.0 mL) for 5 h at 100 °C. To determine the DS, 0.2 mL of hydrolyzed the sulfonated β-(1→6)-D-glucan sample was reacted with 3.8 mL of 3% (w/v) trichloroacetic acid (TCA) in a glass tube, and then 1.0 mL of protector solution (6.0 g NaCl, 0.5 mL c.HCl, 2.5 mL of 0.1% (w/v) gelatin and 47.0 mL distilled water) and 0.03 g $BaCl_2$ were added. The contents were stirred for 1 min and left for 15 min. The resulting $BaSO_4$ formed was measured turbidimetrically at 360 nm. The DS, which designates the average number of sulfonyl groups on each sugar-residue, was established from the sulfur content according to Whistler and Spencer (1964), in which *S* = % sulfur:

$$S(\%) = \frac{(BaSO_4, \mu g) \times 0.1374 \times 100}{\text{Sample}, \mu g}$$

$$\text{DS} = \frac{162 \times S}{3200 - 102 \times S}$$

2.6. Homogeneity of the β-(1→6)-D-glucan and sulfonated β-(1→6)-D-glucan

The homogeneity of the β-(1→6)-D-glucans (natural and sulfonated) was determined by gel permeation chromatography. One milligram of each of the 2 polysaccharide samples was dissolved in water (1.5 mL) and applied to a Sepharose CL-4B column (1.5 cm × 30 cm), and eluted with distilled water at a flow rate of 0.5 mL/min. Fractions (1.5 mL) were collected and analyzed for carbohydrate (490 nm). The void volume (19.5 mL) was determined using blue dextran.

2.7. Spectroscopy analysis

Fourier-transform infra-red (FT-IR) spectra of the exopolysaccharides samples (β-(1→6)-D-glucan and sulfonated β-(1→6)-D-glucan, 1 mg) were recorded using KBr pellets (250.0 mg) on a Bruker Vector 22 Model spectrometer. The ultraviolet–visible (UV–vis) absorption spectra for dilute aqueous solutions (1.0 mg/mL) of β-(1→6)-D-glucan and sulfonated β-(1→6)-D-glucan were determined using a Shimadzu 1601 UV-Vis spectrophotometer. Nuclear magnetic resonance spectroscopy of carbon thirteen (^{13}C NMR) analysis of β-(1→6)-D-glucan and sulfonated β-(1→6)-D-glucan were carried out using a 400 MHz Bruker model DRX Avance spectrometer incorporating Fourier transform. Samples (~20.0 mg) were dissolved in Me_2SO-d_6 and

examined at 50 or 70 °C. Chemical shifts are expressed in ppm (δ) relative to resonance of Me_2SO-d_6 at 39.70 for samples examined in this solvent.

2.8. Blood clotting assays

The anticoagulant activity of the samples (β-(1→6)-D-glucan and sulfonated β-(1→6)-D-glucan; 0–200 μg/mL) was determined by measuring the clotting times (in seconds) of human plasma using the reagents prothrombin time (PT), thrombin time (TT) and activated partial thromboplastin time (APTT) according to the manufacturer's instructions. Each assay was performed at 37 °C. Heparin (0–30 μg/mL) was used as standard. Normal human plasma (900 μL) was mixed with 100 μL of exopolysaccharides solution (β-(1→6)-D-glucan and sulfonated β-(1→6)-D-glucan), or heparin dissolved in isotonic saline, within the concentration ranges indicated above. Isotonic saline (100 μL) was used in the control group. In each of the blood clotting assays, the reagents (PT, TT and APTT) and all glass tubes were pre-heated at 37 °C before to use. The assays were performed in triplicate and the results represent the means ± SD.

2.9. Inhibition of thrombin by antithrombin in the presence of β-(1→6)-D-glucan, sulfonated β-(1→6)-D-glucan and heparin

The inhibition of thrombin by antithrombin was performed according to Melo et al. (2004) with the following modifications: incubations were performed in disposable microcuvettes. The reaction mixture contained 25 μL of human plasma or purified antithrombin (40 nM), and 25 μL of exopolymer solution (β-(1→6)-D-glucan or sulfonated β-(1→6)-D-glucan; 0–400 μg/mL), or heparin (0–1 μg/mL) in TS/PEG buffer (0.02 M Tris/HCl, 0.15 M NaCl, and 1.0 mg/mL polyethylene glycol, pH 7.4). Thrombin (10 μL, 20 nM) was next added to the mixture to initiate the reaction, and after 60 s incubation (room temperature), 500 μL of TS/PEG buffer containing 25 μM chromogenic substrate S-2238 for thrombin was added, and the absorbance at 405 nm recorded for 300 s. No inhibition occurred in the control experiment in which thrombin was incubated with antithrombin in the absence of sulfated polysaccharides.

3. Results and discussion

3.1. Sulfonation and structural characterization

The exocellular β-(1→6)-D-glucan was selected for this work as it presents important characteristics for biological activity with regards to purity, uniformity, homogeneity (Fig. 1) and triple helix conformational structure at previously described (Vasconcelos et al., 2008). As hydrated the biopolymer formed a viscous solution, and the derivatization by sulfonation was a way to improve its solubility in aqueous solutions, and to promote anticoagulant activity. It is well known that the introduction of charged groups on a neutral polysaccharide chain improves water solubility and can enhance its biological activities such as anticoagulation (Brandi et al., 2011; Jung, Bae, Lee, & Lee, 2011).

In this work, sulfonation (three cycles) was performed using formamide as solvent, pyridine as catalytic reagent and chlorosulfonic acid as the hydroxyl group donor. The effectiveness of each cycle of the sulfonation reaction was monitored by UV–vis and FT-IR analysis. The integrity of sulfonated material was performed by gel permeation chromatography on Sepharose CL-4B (Fig. 1), and presented a single carbohydrate peak. Following sulfonation, UV–vis spectroscopy showed a new band at 261 nm (Fig. 2) that can be attributable to the $n \rightarrow \pi^*$ transition of sulfonate or the unsaturated bond formed in the sulfonation process (Brandi et al., 2011; Yang et al., 2003).

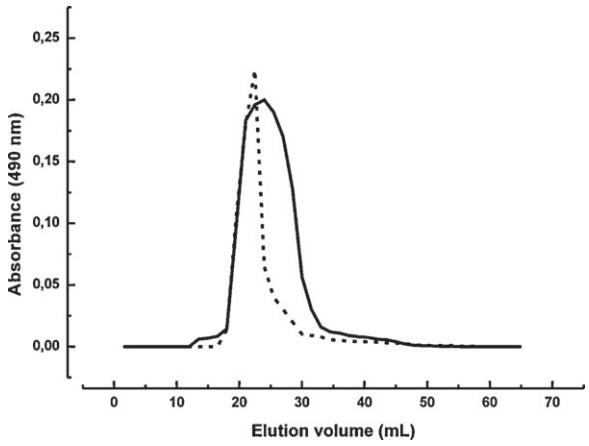

Fig. 1. Gel permeation chromatography profile of the β-(1→6)-D-glucan (- - -) and sulfonated β-(1→6)-D-glucan (—) on a column of Sepharose CL-4B. The column (1.5 cm × 30 cm) was eluted with water at a flow rate of 0.5 mL/min.

The success of the reaction was accompanied by the appearance of two characteristics absorption bands on FT-IR spectra of the sulfonated β-(1→6)-D-glucan (Fig. 2): one at 1258 cm^{-1} describing an asymmetrical S=O stretching vibration (Yang, Du, Huang, Wan, & Li, 2002; Zhang, Zhang, Zhou, Chen, & Zeng, 2000), whereas the band at 810 cm^{-1} representing a symmetrical C—O—S vibration associated with a C—O—SO_3 group (Brandi et al., 2011; Nie, Shi, Ding, & Tao, 2006) demonstrated that lasiodiplodan was successfully sulfonated. Additionally, a new band at 1631 cm^{-1} could be related to the unsaturated bond formed due to the sulfonation process (Yang et al., 2003).

The sulfur content of the sulfonated polysaccharide was determined by calculating the DS, and in this case was 0.95. In studies related to anticoagulant activities of chemically modified β-glucans, the DS was around 1.95 (Martinichen-Herrero et al., 2005), 1.74 (Nie et al., 2006) and 1.54 (Brandi et al., 2011). According to published reports, DS values equal or higher than 0.80 are required for anticoagulant activity (Han, Yao, Yang, Liu, & Gao, 2005). The content of sulfur obtained for the sulfonated β-(1→6)-D-glucan was 11.73%, and within the range of values necessary for anticoagulant activity.

The positions of the sulfonyl groups in polysaccharides can be determined by ^{13}C NMR (Han et al., 2005; Zhang et al., 2008), and the literature shows that the main position for the entry of sulfonyl groups in β-(1→3)-glucans is the primary carbon, i.e. at C-6, followed by C-2 and C-4 (Telles et al., 2011; Zhang et al., 2000).

The ^{13}C NMR spectra of native β-(1→6)-D-glucan and its sulfonated derivative are presented in Fig. 3. The chemical shift at 103.0 ppm of β-(1→6)-D-glucan was attributed to the anomeric carbon and that at 69.0 ppm to substituted C-6. The signals at 75.9, 75.0, 73.2 and 69.9 ppm were attributed to C-3, C-5, C-2 and C-4, respectively (Narui et al., 1999; Sassaki et al., 2002). The ^{13}C NMR spectrum of sulfonated glucans was more complicated with broader signals, resulting from the sulfonation of the hydroxyl groups (Brandi et al., 2011). Usually following sulfonation, the spectra became more complicated because the carbons directly attached to the electronegative sulfonated ester groups shift down field, while the carbons indirectly attached (neighborhood) to the sulfonyl group shift to an upfield position (Perlin & Casu, 1982; Telles et al., 2011).

The chemical shifts in the ^{13}C NMR spectrum of the sulfonated β-(1→6)-D-glucan presented a small variation (+0.3 ppm), in relation to the original biopolymer. The new signal at 96.3 ppm was

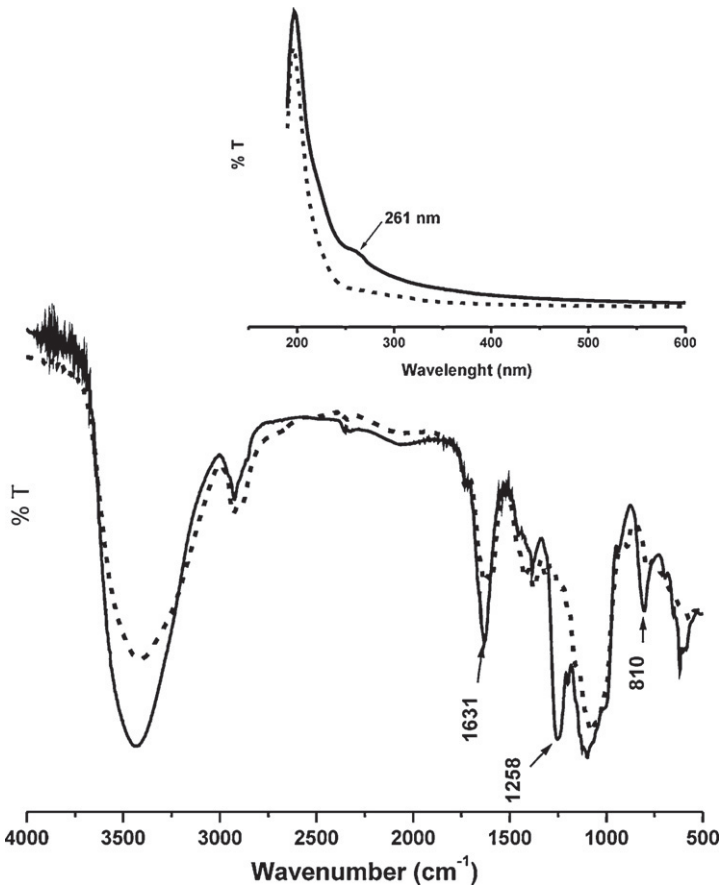

Fig. 2. FT-IR and UV–vis (inset) spectra of β-(1→6)-D-glucan before (- - -) and after sulfonation (—).

attributed to C-1 as the signal of C-1 splits when an —OH group on C-2 is substituted with a sulfonyl group. In addition, the chemical shift at 73.2 ppm assigned as C-2 also split and moved downfield (73.6 ppm), suggesting that part of C-2 was sulfonated. The intense signal at 70.2 ppm corresponded probably to the substituted C-6 and free C-4, while that at 71.9 ppm could be assigned to C-4 substituted by sulfonyl groups (α shift). The small signal at 68.6 ppm (β shift) can be related to carbon indirectly attached to sulfonyl groups. Probably part of C-3 was also substituted, however, the attribution is difficult because the chemical shifts may correspond to more than one carbon. From the results we concluded that non-selective sulfonation of β-(1→6)-D-glucan has occurred, as C-2 and C-4 were split indicating partial substitution.

After the sulfonation reaction, the solutions of sulfonated β-(1→6)-D-glucan became less viscous and more water soluble, which facilitates the bioassays to determine anticoagulant activity through traditional tests of blood coagulation with heparin as the reference.

3.2. Anticoagulant activity

3.2.1. APTT, TT and PT clotting times

To investigate the anticoagulant property of β-(1→6)-D-glucan and sulfonated β-(1→6)-D-glucan, APTT, PT and TT assays were conducted using normal human plasma and the effects of the β-glucans on the clotting times measured. The results were compared with those for heparin as the reference standard.

The APTT test is related to the phase of intrinsic coagulation in plasma and measures the function of blood coagulation factors XII, XI, IX and VIII. PT is related to the extrinsic phase, which depends upon the tissue factor of the activation process and measures the integrity of the common phase of coagulation. The TT assay evaluates the conversion of plasmatic fibrinogen to fibrin in the presence of exogenous thrombin. The coagulation time in the last stage of the coagulation cascade of events is the conversion of fibrinogen to fibrin by thrombin (Beutler, Coller, Lichtman, & Kipps, 2001; Melo et al., 2004; Mendes et al., 2009).

The results for the APPT, TT and PT assays are shown in Table 1. The sulfonated polysaccharide was able to prolong the APPT and TT times in a concentration-dependent manner. With regard to the APTT and TT results, the anticoagulant effect of sulfonated β-(1→6)-D-glucan at 30 and 40 μg/mL concentration was ∼5 and ∼7 times greater, respectively, than the control. These results demonstrated an important *in vitro* anticoagulant activity for the sulfonated β-(1→6)-D-glucan as demonstrated by the increases in the dose-dependence of APTT and TT, and this action could be attributable to the degree of sulfonation (DS 0.95).

The APTT prolongation time suggests inhibition of the intrinsic coagulation pathway, whereas prolongation of TT time indicates inhibition of thrombin-mediated fibrin formation (Wang et al., 2007). No prolongation of PT demonstrated there was no inhibition of the extrinsic pathway of coagulation (Mao et al., 2009). Since the anticoagulant effect of heparin is not mediated by modulation of the extrinsic system, the sulfonated β-(1→6)-D-glucan is a poor inhibitor of the extrinsic pathway. The APTT and TT values were compared with heparin, and a concentration ∼10 times greater of the sulfonated β-(1→6)-D-glucan was necessary to achieve the same effect as exhibited by heparin. The sulfonated β-(1→3;1→6)-D-glucan (named botryosphaeran) from *Botryosphaeria rhodina* MAMB-05 grown on fructose (Mendes et al., 2009), and glucose

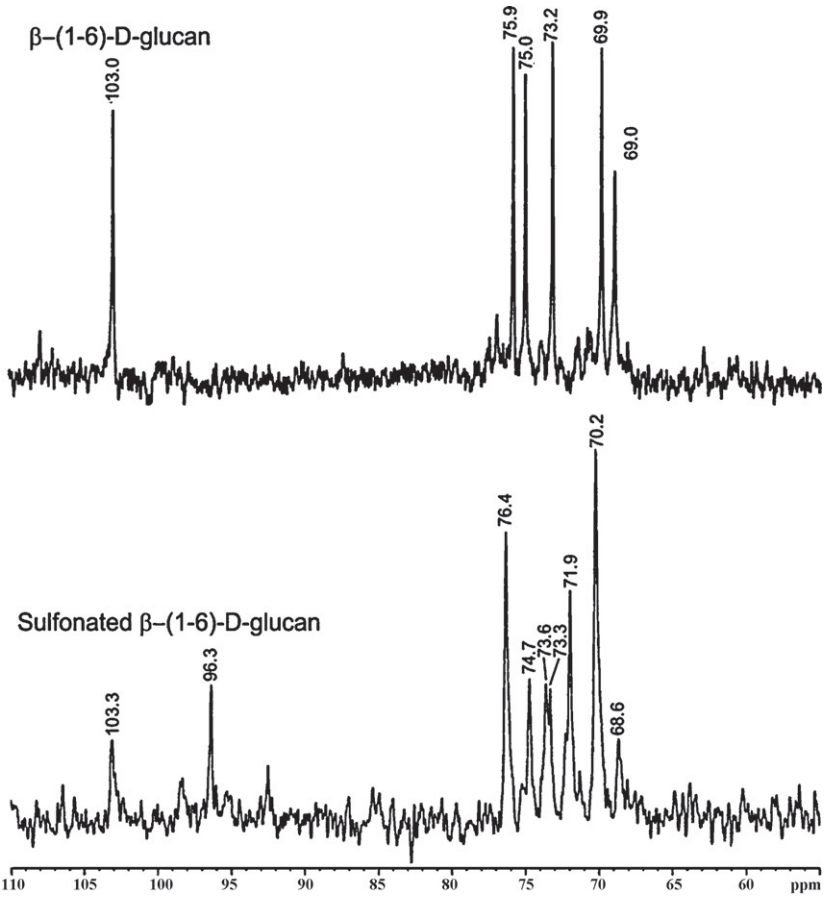

Fig. 3. ^{13}C NMR spectra of exocellular β-(1→6)-D-glucan and sulfonated β-(1→6)-D-glucan.

(Brandi et al., 2011), showed similar results as anticoagulants. According to Martinichen-Herrero et al. (2005), sulfated polysaccharides with a lower anticoagulant activity than heparin could exhibit a potent antithrombotic effect with less hemorrhagic risk.

As with heparin, the weakest effect was observed in the PT assay for the sulfonated β-(1→6)-D-glucan. The relative lack of effect of sulfonated β-(1→6)-D-glucan on the PT is consistent with the observation that this test is also not sensitive to heparin, and several other sulfated polysaccharides (Martinichen-Herrero et al.,

Table 1
Anticoagulant activity of normal human plasma in the presence of β-(1→6)-D-glucan, sulfonated β-(1→6)-D-glucan and heparin as measured by the activated partial thromboplastin time (APTT), thrombin time (TT) and prothrombin time (PT).

Polysaccharide	Amount (μg/mL)	Clotting times (s)		
		APTT	TT	PT
β-(1→6)-D-Glucan	Control (0)	46.65 ± 0.8	19.45 ± 0.4	12.87 ± 0.8
	1	39.45 ± 0.2	15.92 ± 0.4	16.04 ± 0.1
	5	39.94 ± 0.6	16.56 ± 0.5	14.87 ± 0.1
	10	42.30 ± 0.1	14.24 ± 0.3	15.77 ± 0.1
	15	41.07 ± 0.7	14.53 ± 0.2	14.27 ± 0.2
	20	41,09 ± 0.2	14.43 ± 0.4	16.87 ± 0.1
Sulfonated β-(1→6)-D-glucan	Control (0)	46.65 ± 0.8	19.45 ± 0.4	12.87 ± 0.8
	1	46.79 ± 0.2	15.67 ± 0.3	15.12 ± 0.0
	5	58.27 ± 0.1	26.75 ± 1.6	17.56 ± 0.1
	10	70.55 ± 3.6	50.87 ± 1.1	17.52 ± 0.4
	15	96.97 ± 1.3	70.06 ± 0.6	18.65 ± 0.2
	20	105.69 ± 0.1	187.54 ± 1.5	18.61 ± 0.4
	30	228.49 ± 3.7	227.06 ± 2.2	17.45 ± 0.4
	40	298.13 ± 1.0	249.45 ± 0.6	17.17 ± 0.7
Heparin[a]	Control (0)	46.65 ± 0.8	19.45 ± 0.4	12.87 ± 0.8
	1	55.60 ± 0.6	95.00 ± 0.7	16.56 ± 0.2
	2	76.19 ± 1.0	275.98 ± 1.3	17.93 ± 0.6
	3	106.08 ± 0.3	592.70 ± 2.9	19.20 ± 0.2

[a] Sodium heparin, 5000 IU/mL. The results represent mean times ± SD.

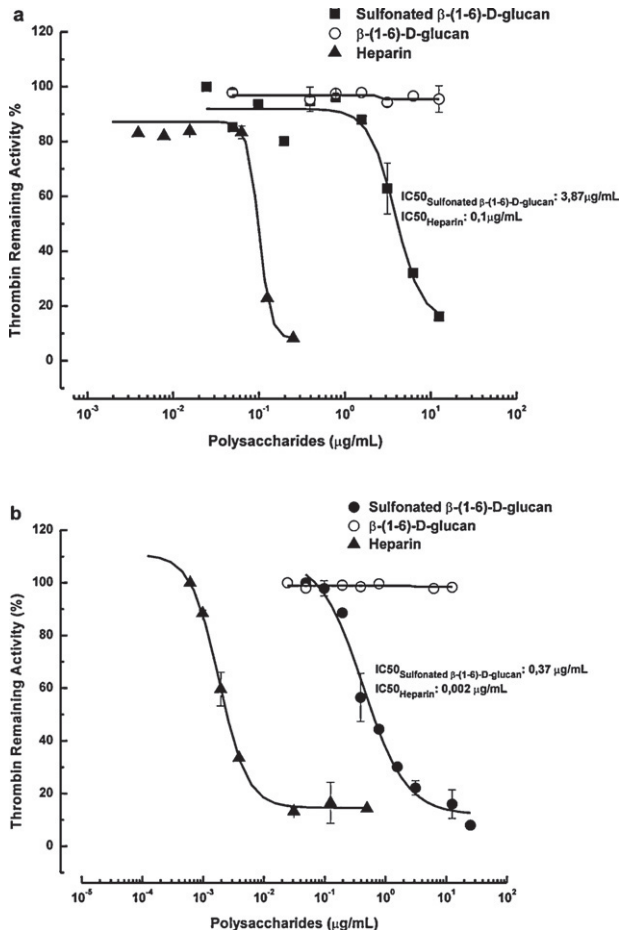

Fig. 4. Concentration dependence of β-(1→6)-D-glucan, sulfonated β-(1→6)-D-glucan and heparin on the inactivation of thrombin by (a) antithrombin of human plasma and (b) a purified antithrombin preparation.

2005). The β-(1→6)-D-glucan (lasiodiplodan) did not inhibit the APTT, TT and PT assays, and demonstrated that the presence of sulfonyl groups was an essential requirement for these anticoagulant activities.

3.2.2. Inhibition of thrombin by antithrombin in the presence of β-(1→6)-D-glucan, sulfonated β-(1→6)-D-glucan and heparin

The biological mechanism of sulfonated polysaccharides occurs by the potentiation of plasmatic cofactors, which are physiological inhibitors of the coagulation cascade as antithrombin that acts by inhibiting thrombin and factors Xa, XIIa, XIa and IX, and may have its action strengthened by the presence of heparin (Melo et al., 2004; Mendes et al., 2009). To elucidate the inhibitory mechanism of the anticoagulant activity of sulfonated β-(1→6)-D-glucan, the effects on thrombin activity were studied using chromogenic substrates in the presence of plasma as a source of physiological inhibitors (antithrombin and heparin cofactor II), and also purified antithrombin.

Fig. 4(a) and (b) shows that the sulfonated β-(1→6)-D-glucan was able to potentiate thrombin inhibition in a manner similar to that of heparin. However, comparing the values of IC_{50} (concentration of the sulfonated β-(1→6)-D-glucan necessary to obtain 50% inhibition of thrombin activity) obtained for both sets of assays, the activity of the sulfonated β-(1→6)-D-glucan was approximately 180-fold lower than that of heparin in experiments using the purified antithrombin, and ∼38-fold lower with human plasma. These results suggest that besides the activation of antithrombin, the sulfonated β-(1→6)-D-glucan could possibly contribute to an increase in the action of another physiological inhibitor of thrombin (heparin cofactor II) absent in the assays with purified antithrombin. Heparin cofactor II is an inhibitor of serine protease and thrombin. Antithrombin inhibits all intrinsic pathway coagulation enzymes (Mao et al., 2009).

Therefore, the results of the anticoagulant tests described in this work demonstrated that the sulfonated β-(1→6)-D-glucan exhibited anticoagulant activity, and was most likely involved with the intrinsic pathway. The lasiodiplodan did not present inhibiting activity at any of the concentrations examined, demonstrating that the presence of sulfonyl groups in this β-(1→6)-D-glucan was an important characteristic of anticoagulant action.

4. Conclusions

An exocellular β-(1-6)-D-glucan (lasiodiplodan) was sulfonated (three cycles) using formamide as solvent, pyridine as catalytic reagent and chlorosulfonic acid as the hydroxyl group donor. The effectiveness of each sulfonation reaction cycle was monitored by UV–vis and FT-IR analysis, and only the sulfonated β-(1-6)-D-glucan showed sulfonate or the unsaturated bond formed in the sulfonation process. The content of sulfur and DS obtained for the sulfonated β-(1→6)-D-glucan was 11.73% and 0.95, respectively, which indicated that there was approximately one sulfonyl group per residue of glucose. The positions of the sulfonyl groups introduced in lasiodiplodan were determined by ^{13}C NMR, and from the results it was concluded that non-selective sulfonation of the β-(1→6)-D-glucan had occurred, with C-2 and C-4 being partially substituted. It is possible that C-3 also received a sulfonyl group. The prolongation of APTT in the presence of the sulfonated β-(1→6)-D-glucan suggested inhibition of the intrinsic pathway of coagulation, while an extension of the TT time probably indicated inhibition of the reaction resulting in the conversion of fibrinogen into fibrin. Thus, this work demonstrated that the chemical derivatization of exocellular β-(1→6)-D-glucan by sulfonation produced a modified polysaccharide resulting in anticoagulation activity.

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