

## Chemical Modification of Botryosphaeran: Structural Characterization and Anticoagulant Activity of a Water-Soluble Sulfonated (1→3)(1→6)-β-D-Glucan

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The exopolysaccharide botryosphaeran (EPS<sub>GLC</sub>; a (1→3)(1→6)-β-D-glucan from *Botryosphaeria rhodina* MAMB-05) was sulfonated to produce a water-soluble fraction (EPS<sub>GLC</sub>-S) using pyridine and chlorosulfonic acid in formamid. This procedure was then repeated twice to produce another fraction (EPS<sub>GLC</sub>-RS) with a higher degree of substitution (DS, 1.64). The purity of each botryosphaeran sample (unsulfonated and sulfonated) was assessed by gel filtration chromatography (Sephacose CL-4B), where each polysaccharide was eluted as a single symmetrical peak. The structures of the sulfonated and re-sulfonated botryosphaerans were investigated using ultraviolet–visible (UV–Vis), Fourier-transform infrared (FT-IR), and <sup>13</sup>C nuclear magnetic resonance (<sup>13</sup>C NMR) spectroscopies. EPS<sub>GLC</sub> and EPS<sub>GLC</sub>-RS were also assayed for anticoagulation activity, and EPS<sub>GLC</sub>-RS was identified as an anticoagulant.

**Keywords:** Botryosphaeran, sulfonation, anticoagulant activity, *Botryosphaeria rhodina* MAMB-05

Glucans of the (1→3)(1→6)-β-D-glucan type are an important class of fungal polysaccharides that possess interesting biological-response-modifying activities, such as anticancer, anti-inflammatory, and immunomodulation effects [6, 7, 16, 29]. The action mechanisms of these

biopolymers, however, are still not clearly understood, in spite of the plethora of knowledge on their activities [6, 29]. Structurally, the biological activities of these carbohydrate macromolecules are specifically related to their monosaccharide composition, degree of branching, degree of polymerization, the conformation of their constituent chains, and conformation in solution [7, 16, 27]. In addition, their high molecular weight, triple helix conformational structure, and β-(1→6) branching are all favorable structural features [7, 31]. Yet, when combined, these features can lead to solubility problems, imposing obstacles to their application [32]. Attempts to overcome this problem have included fragmentation of the polysaccharides into short chain lengths [15] through chemical or enzymatic hydrolyses [11].

The chemical modification of polysaccharides by sulfonation has been widely studied owing to the advantages of the products so obtained. Thus, sulfonation methods have already been developed for different polysaccharides, including curdlan [2], fucoidan [28], dextran [19], and the botryosphaeran produced on fructose (EPS<sub>FRU</sub>) as a carbon source [23]. It is well known that the introduction of a charged group to a polysaccharide chain improves its water solubility and can lead to enhanced biological activities, such as anticoagulation and antithrombotic effects [3, 13, 23], as well as anti-HIV [18] and antitumor activities [17]. However, these activities are also affected by certain parameters, such as the degree of substitution, degree of branching, molecular weight, and type of sugar constituents [12, 22, 36] comprising the derivatized polysaccharides. Therefore, sulfonation is a rational choice [1].

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*Botryosphaeria rhodina* MAMB-05, an endophytic fungus, produces a family of exopolysaccharides (EPS), named botryosphaerans, structurally characterized as (1→3)(1→6)-β-D-glucans [4] and responsible for increasing the medium viscosity in growing cultures [9] depending on the type of carbon source (carbohydrate) used for growth of the fungus [8, 30]. The botryosphaeran obtained from *B. rhodina* MAMB-05 grown on fructose (EPS<sub>FRU</sub>) was found to show a greater degree of branching with (1→6)-β-linked substituents along the (1→3)-β-glucan chain and formed a less viscous aqueous solution than botryosphaerans produced using other carbohydrate sources [8]. When EPS<sub>FRU</sub> was sulfonated, anticoagulation activity was induced [23]. Botryosphaeran has presented some interesting biological effects at low concentrations that include non-mutagenic and strong anticlastogenic activities [24], and has also been effective in lowering blood glucose levels in diabetes-induced rats and lowering LDL-cholesterol levels in hyperlipidemia-induced rats fed a high-fat diet [25].

Accordingly, the aim of the present work was to overcome the lower solubility and higher viscosity in aqueous solutions exhibited by the botryosphaeran from *B. rhodina* MAMB-05 grown on glucose (EPS<sub>GLC</sub>) through chemical modification by sulfonation. The anticoagulation activity of the sulfonated exopolysaccharide was also evaluated.

## MATERIALS AND METHODS

### Materials

The chlorosulfonic acid was purchased from Vetec (Rio de Janeiro-RJ, Brazil). The thrombin and antithrombin were obtained from Haematologic Technologies USA, and the chromogenic substrate S-2238 from Chromogenix-Sweden. The sodium heparin (5,000 UI/ml) was purchased from Akzo Organon (São Paulo, Brazil). The human plasma was obtained by centrifugation (450 ×g/15 min at 24°C) of citrated blood. The blood coagulation time reagents, such as the activated partial thromboplastin time (APTT), thrombin time (TT), and prothrombin time (PT), were all purchased from In-Vitro Diagnóstica S/A (Itabira-MG, Brazil).

### Production and Preparation of Botryosphaeran

The botryosphaeran (EPS<sub>GLC</sub>) was produced by *Botryosphaeria rhodina* MAMB-05 grown on a Vogel minimal salts medium with glucose as the carbon source, and prepared according to the procedure previously outlined by Steluti *et al.* [30] and Barbosa *et al.* [4].

### Analytical Techniques

The carbohydrate was determined using the phenol-sulfuric acid method according to Dubois *et al.* [10] with D-glucose as the standard sugar. The protein was measured using the Bradford method [5] with bovine serum albumin as the standard.

### Sulfonation of Botryosphaeran

The sulfonation of the botryosphaeran (EPS<sub>GLC</sub>) was performed according to the method of O'Neill [26], as modified by Mendes *et*

*al.* [23]. The resulting sulfonated product was referred to as EPS<sub>GLC</sub>-S. The sulfonation reaction was repeated two more times using a portion of EPS<sub>GLC</sub>-S to obtain an oversulfonated product, designated EPS<sub>GLC</sub>-RS.

### Gel Filtration Chromatography

Samples of the botryosphaerans (EPS<sub>GLC</sub>, EPS<sub>GLC</sub>-S, and EPS<sub>GLC</sub>-RS; 1 mg) were each dissolved in 1 ml of distilled water, fractionated using a column of Sepharose CL-4B (28.8 × 1.5 cm), and eluted with water (0.5 ml/min). Fractions of 1.5 ml were then collected and analyzed for carbohydrate using the phenol-sulfuric acid method. The column was calibrated using blue dextran (Sigma) and the absorbance measured at 625 nm.

### Determination of Degree of Substitution (DS)

Samples of EPS<sub>GLC</sub>-S and EPS<sub>GLC</sub>-RS (1 mg/ml) were each hydrolyzed using 1.0 M HCl for 5 h at 100°C. The DS was then determined as previously described by Mendes *et al.* [23]. The DS, which designates the average number of sulfonyl groups in each sugar residue, was established on the basis of the sulfur content determined from the formulae below [34], where S = % sulfur:

$$S(\%) = \frac{(\text{BaSO}_4, \mu\text{g}) \times 0.1374 \times 100}{(\text{Sample}, \mu\text{g})}$$

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

### Structural Analysis

The UV-Vis absorption spectra for dilute aqueous solutions (1 mg/ml) of the sulfonated (EPS<sub>GLC</sub>-S and EPS<sub>GLC</sub>-RS) and non-sulfonated (EPS<sub>GLC</sub>) botryosphaerans were determined using a Shimadzu 1601 UV-Vis spectrophotometer. The Fourier-transform infrared (FT-IR) spectra for the botryosphaeran samples (EPS<sub>GLC</sub>, EPS<sub>GLC</sub>-S, and EPS<sub>GLC</sub>-RS; 1 mg of each) were recorded on KBr disks (250 mg) using a Bruker Vector 22 Model spectrometer. The <sup>13</sup>C nuclear magnetic resonance (<sup>13</sup>C NMR) spectra were obtained using a 400 MHz Bruker model DRX Avance spectrometer with a 5 mm inverse probe. The <sup>13</sup>C NMR (100.6 MHz) analyses were then performed at 50°C using samples dissolved in D<sub>2</sub>O. The chemical shifts are expressed in ppm (δ) relative to acetone at δ 30.20 for the <sup>13</sup>C signals.

### Assays for Blood Clotting

The anticoagulant activity of the botryosphaerans (EPS<sub>GLC</sub>; and its sulfonated derivative, EPS<sub>GLC</sub>-RS; 0 – 200 μg/ml) was determined by measuring the clotting times (s) of human plasma at 37°C when employing the reagents PT, TT, and APTT according to the manufacturer's instructions, as described elsewhere [23]. The assays were performed in triplicate and the results represent the mean ± SD.

### Thrombin Inhibition by Antithrombin in the Presence of EPS<sub>GLC</sub>, EPS<sub>GLC</sub>-RS, and Heparin

The inhibition assays of thrombin by antithrombin were performed according to Melo *et al.* [21] with modifications, as previously described [23], over a 300 s period. No inhibition occurred in the control experiments, in which thrombin was incubated with antithrombin in the absence of the sulfonated polysaccharides [21].

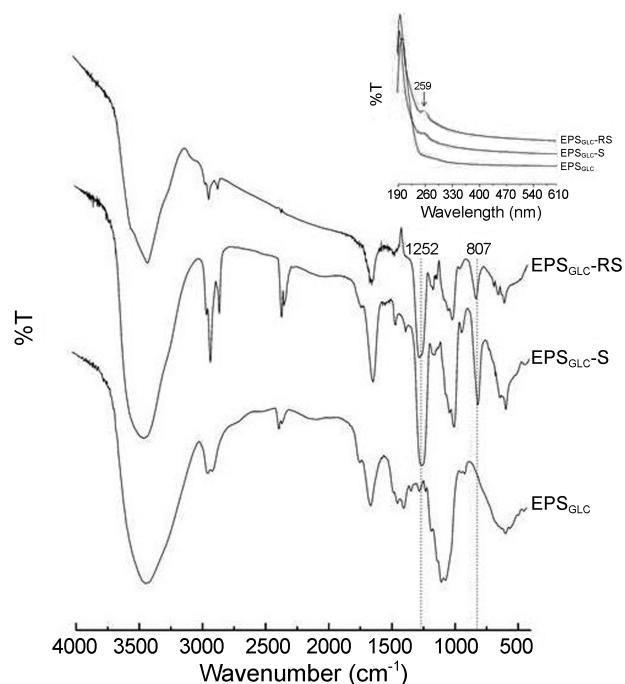
## RESULTS AND DISCUSSION

### Sulfonation and Structural Characterisation

The solvent systems in which polysaccharides are immersed play an important role in their chemical reactions, including derivatization by sulfonation [35]. The solvents *N,N*-dimethyl formamide (DMF) and formamide (FA), commonly used in sulfonation reactions, were tested to solubilize the botryosphaeran. FA showed the best capacity to dissolve the botryosphaeran, but the polysaccharide was almost insoluble in DMF, an aprotic polar solvent. The water solubility of the sulfonated and re-sulfonated botryosphaeran depended on the molar ratio of chlorosulfonic acid-to-hydroxyl groups in the polysaccharide molecule [27]. The reaction time and temperature were chosen to present mild conditions in order to minimize any degradation of the polysaccharide chain. Pyridine is an important part of the sulfonation reaction, acting as a catalytic reagent. As a strong organic base, pyridine nucleophilically attacks polysaccharides and weakens their O-H bonds, facilitating the entrance of the sulfonation reagent.

Gel permeation chromatography of the botryosphaeran prior to the sulfonation reaction revealed only one eluted fraction after the void volume (18.1 ml), indicating that the polysaccharide was homogeneous. The sulfonated botryosphaerans, EPS<sub>GLC</sub>-S and EPS<sub>GLC</sub>-RS, were also eluted as single peaks with similar elution volumes to that of the unsulfonated botryosphaeran, indicating that the sulfonation reaction had not degraded these polysaccharides. The sulfur content and DS values were 15.4% and 1.54, respectively, for the once-sulfonated botryosphaeran (EPS<sub>GLC</sub>-S), and 15.9% and 1.64, respectively, for the re-sulfonated polysaccharide (EPS<sub>GLC</sub>-RS). The chemically modified botryosphaerans were all water-soluble, indicating that the introduction of an anionic sulfonyl group with an appropriate DS enhanced the polysaccharide's water-solubility. The presence of sulfonyl groups is responsible for increasing their solubility in water, as the anionic groups interact better with water molecules. In  $\beta(1\rightarrow3)$ -linked polysaccharides, the primary OH groups at C-6 are about 10 times more accessible to sulfonation than the secondary OH groups at positions C-2 and C-4 [12, 35]. In general, the bioactivities of sulfonated polysaccharides are improved when increasing the DS, and for this reason, the blood clotting assays (see below) were only performed using the higher sulfur-substituted EPS<sub>GLC</sub>-RS.

The UV-Vis spectroscopy of the sulfonated and re-sulfonated botryosphaerans revealed a new broad absorption peak at 259 nm (Fig. 1), which could have been due to the  $n\rightarrow\pi^*$  transition of sulfonate or the formation of an unsaturated bond during the sulfonation process [1]. This peak was missing from the spectrum of the unsulfonated botryosphaeran. The FT-IR spectra (Fig. 1) indicated that sulfonyl groups were inserted into the botryosphaeran



**Fig. 1.** FT-IR spectra of botryosphaeran (EPS<sub>GLC</sub>), plus sulfonated (EPS<sub>GLC</sub>-S) and re-sulfonated (EPS<sub>GLC</sub>-RS) botryosphaerans. The UV-Vis absorption spectrum is presented on a different scale to show the botryosphaeran before and after sulfonation.

molecule. Some characteristic bands at 1,252  $\text{cm}^{-1}$  indicated an asymmetrical stretching vibration, whereas the band at 807  $\text{cm}^{-1}$  was typical of a symmetrical C-O-S vibration associated with a C-O-SO<sub>3</sub> group [1].

The <sup>13</sup>C NMR chemical shift assignments for the botryosphaeran samples EPS<sub>GLC</sub>-S and EPS<sub>GLC</sub>-RS are shown in Table 1. When compared with the original botryosphaeran, the <sup>13</sup>C NMR spectra of the sulfonated polysaccharides [12] were more complicated with broader signals, resulting from the sulfonation of the hydroxyl groups. The anomeric carbon signals were also split into more peaks, another result of the sulfonation. The region at 60.7 – 61.1 ppm, attributable to the chemical shift of free C-6, for the unsulfonated botryosphaeran (EPS<sub>GLC</sub>) was almost absent from the <sup>13</sup>C NMR spectra for the sulfonated botryosphaeran, while new peaks appeared close to 68.0 ppm in the spectra for EPS<sub>GLC</sub>-S and EPS<sub>GLC</sub>-RS. As the downfield shift ( $\alpha$  shift) of a carbon atom linked by a sulfonyl group is nearly 7 to 10 ppm [12, 35], the results indicated that most of the free C-6 was substituted by sulfonyl groups and the C-6<sub>s</sub> signal was shifted to 68.0 ppm. When considering that the botryosphaeran is a  $\beta(1\rightarrow3)$ -D-glucan with 22% side branching at C-6 by glucosyl and gentiobiosyl residues [4], substitution at C-2 and C-4 by sulfonyl groups should have been more difficult owing to steric hindrance. However, the appearance of new peaks upfield ( $\beta$  shift) at 101.4, 100.9, and 100.6

**Table 1.**  $^{13}\text{C}$  NMR chemical shift data for botryosphaeran and sulfonated botryosphaerans (EPS<sub>GLC</sub>-S and EPS<sub>GLC</sub>-RS) measured at 30°C.

Linked-glucose residue	Chemical shifts ( $\delta$ , ppm)							
	C-1	C-2	C-3	C-4	C-5	C-6	C-1	C-6 <sub>s</sub>
Glc <sub>p</sub> -(1→	103.3	73.8	75.5	68.8	76.7	61.1		
→6)-Glc <sub>p</sub> -(1→	102.9	73.0	74.8	68.7	76.3	70.1		
→3,)-Glc <sub>p</sub> -(1→	103.1	73.0	86.3	68.7	76.7	61.1		
						60.9		
						60.7		
→3,6)-GLc <sub>p</sub> -(1→	102.9	73.0	86.0	68.7	76.3	70.1		
			85.5					
EPS <sub>GLC</sub> -S, EPS <sub>GLC</sub> -RS <sup>a</sup>							101.3	~68.0
							100.9	
							100.6	

<sup>a</sup>Chemical shifts found at *O*-sulfonated botryosphaeran.

ppm in the  $^{13}\text{C}$  NMR spectra of EPS<sub>GLC</sub>-S and EPS<sub>GLC</sub>-RS was ascribed to the signal of C-1, probably because the anomeric region was influenced by the adjacent C-2 substitution [12].

#### Anticoagulation Activity of Sulfonated Botryosphaeran, EPS<sub>GLC</sub>-RS

**APTT, TT, and PT clotting times.** Blood clotting is the result of a complex process initiated by an intrinsic or extrinsic system and/or a common pathway. The various coagulation assays indicate the interaction with the different stages of the coagulation process and provide basic information on the mode of action of anticoagulants [33]. Table 2 shows the anticoagulant activities of EPS<sub>GLC</sub>-RS

and EPS<sub>GLC</sub> as measured by the APTT, TT, and PT clotting times, and compares these results with those for heparin. EPS<sub>GLC</sub>-RS was able to prolong the APTT and TT in a concentration-dependent manner. As regards the APTT and TT, the anticoagulant effect of EPS<sub>GLC</sub>-RS with 30 and 40  $\mu\text{g}/\text{ml}$  of plasma was ~6.8 and ~10 times greater, respectively, than that of the control. Therefore, these results demonstrate an important *in vitro* anticoagulant activity related to EPS<sub>GLC</sub>-RS, as evidenced by the increase in the dose-dependence of the APTT and TT, and this activity could be attributable to the high degree of sulfonation of the botryosphaeran (DS 1.64). Previous literature has stated that anticoagulant activity always increases with the DS, and a DS of >0.8 is necessary [13]. Prolongation of

**Table 2.** Anticoagulant activity of normal human plasma in the presence of botryosphaeran (EPS<sub>GLC</sub>), re-sulfonated botryosphaeran (EPS<sub>GLC</sub>-RS), and heparin, measured according to activated partial thromboplastin time (APTT), thrombin time (TT), and prothrombin time (PT).

Polysaccharide	Amount ( $\mu\text{g}/\text{ml}$ )	Clotting times (s)		
		APTT	TT	PT
EPS <sub>GLC</sub>	Control (0)	30.4 ± 0.2	14.9 ± 0.1	15.5 ± 0.25
	10	27.7 ± 0.1	14.2 ± 0.3	15.5 ± 0.4
	15	29.7 ± 0.9	15.9 ± 1.2	14.1 ± 0.4
	20	32.0 ± 0.8	15.7 ± 0.4	15.5 ± 0.7
EPS <sub>GLC</sub> -RS	Control (0)	30.4 ± 0.2	14.9 ± 0.1	15.5 ± 0.2
	10	59.6 ± 0.8	24.4 ± 0.8	15.5 ± 0.5
	15	96.7 ± 3.4	29.8 ± 0.2	16.3 ± 0.4
	20	117.5 ± 2.8	41.2 ± 1.3	16.1 ± 0.7
	30	205.1 ± 2.9	101.7 ± 0.9	16.6 ± 0.6
	40	304.5 ± 2.3	157.7 ± 0.01	16.1 ± 0.2
Heparin <sup>a</sup>	Control (0)	30.4 ± 0.2	14.9 ± 0.1	15.5 ± 0.25
	1	44.0 ± 1.0	27.9 ± 1.4	15.8 ± 0.4
	2	67.7 ± 2.9	50.0 ± 0.7	16.1 ± 0.9
	3	100.1 ± 3.6	88.2 ± 8.3	16.6 ± 0.1

<sup>a</sup>Sodium heparin, 5,000 UI/ml. The results represent mean times ± SD.

the APTT suggests inhibition of the intrinsic coagulation pathway, whereas prolongation of the TT indicates the inhibition of thrombin-mediated fibrin formation [20]. Since the anticoagulant effect of heparin is not mediated by modulation of the extrinsic system, it would appear that  $\text{EPS}_{\text{GLC}}\text{-RS}$  is a poor inhibitor of the extrinsic pathway. When the APTT and TT values were compared with those for heparin, an  $\sim 10$  times greater concentration of  $\text{EPS}_{\text{GLC}}\text{-RS}$  was necessary to obtain the same degree of effect as heparin. In this respect, polysaccharides with a lower anticoagulant activity than that of heparin could exhibit a potent antithrombotic effect with fewer hemorrhagic risks [14]. As with heparin, the weakest effect of the sulfonated botryosphaeran was observed in the PT assay. The relative lack of effect of  $\text{EPS}_{\text{GLC}}\text{-RS}$  on the PT is consistent with the observation that this test is also not sensitive to heparin and several other sulfated polysaccharides [37]. The unsulfonated botryosphaeran did not inhibit the APTT, TT, and PT assays, as the presence of sulfonyl groups is an essential requirement for these anticoagulant activities.

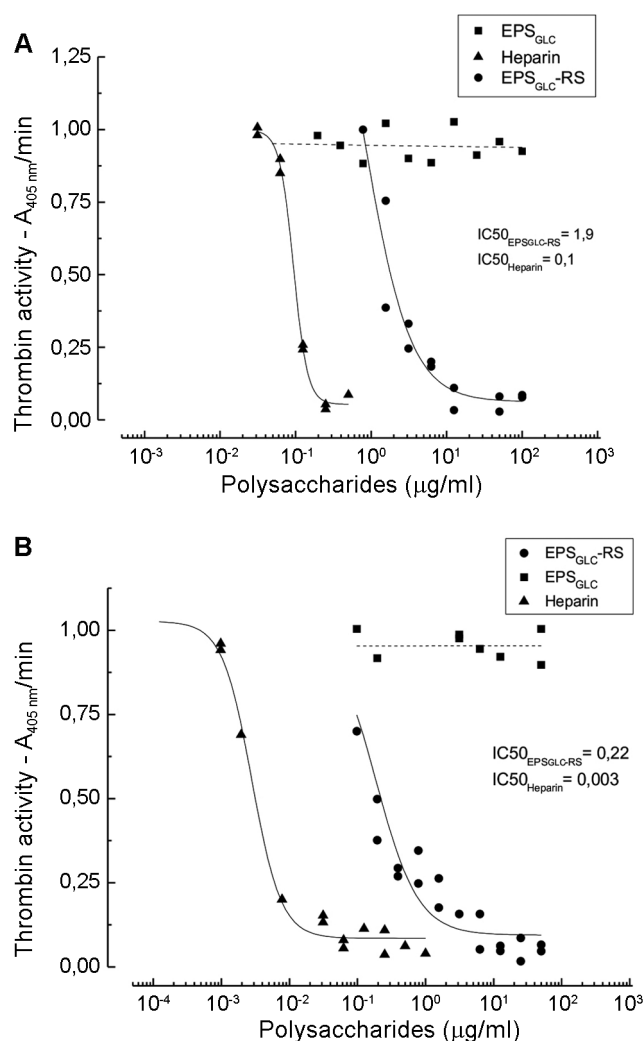
**Inhibition of thrombin by antithrombin in the presence of  $\text{EPS}_{\text{GLC}}\text{-RS}$  and  $\text{EPS}_{\text{GLC}}$ .** To elucidate the inhibitory mechanism of the anticoagulant activity of  $\text{EPS}_{\text{GLC}}\text{-RS}$ , its effect on thrombin activity was measured using chromogenic substrates in the presence of plasma as a source of physiological inhibitors (antithrombin and heparin cofactor II) and also purified antithrombin.

According to the results,  $\text{EPS}_{\text{GLC}}\text{-RS}$  was able to potentiate thrombin inhibition in a manner similar to that of heparin (Fig. 2). However, the sulfonated polysaccharide concentrations required to obtain a 50% inhibition of thrombin activity were higher than the required concentrations of heparin. According to the  $\text{IC}_{50}$  values (Fig. 2) from the tests using human plasma, the activity of  $\text{EPS}_{\text{GLC}}\text{-RS}$  was 19 times lower than that of heparin, and 73 times lower when using purified antithrombin. The difference between these assays was probably due to  $\text{EPS}_{\text{GLC}}\text{-RS}$ , which was necessary to increase the action of heparin cofactor II, another physiological inhibitor of thrombin present in plasma and absent from the tests using purified antithrombin.

Therefore, the results obtained from this study demonstrated that  $\text{EPS}_{\text{GLC}}\text{-RS}$  had a higher anticoagulant activity than the re-sulfonated  $\text{EPS}_{\text{FRU}}$  [23]. One explanation for this difference is that the re-sulfonated  $\text{EPS}_{\text{FRU}}$  had more branches at  $\text{C}_6$ , hindering the entry of sulfonyl groups at the primary hydroxyl.

## Concluding Remarks

Difficulties were encountered in solubilizing the examined botryosphaeran in appropriate solvents for sulfonation. Among the solvents tested, formamide showed the best capacity to dissolve the botryosphaeran, and was subsequently



**Fig. 2.** Effects of nonsulfonated ( $\text{EPS}_{\text{GLC}}$ ) and re-sulfonated ( $\text{EPS}_{\text{GLC}}\text{-RS}$ ) botryosphaerans and heparin on the inhibition of thrombin by (A) antithrombin in human plasma and (B) purified antithrombin preparation.

used in the sulfonation reaction. The sulfonation was performed under mild conditions (low temperature) to prevent sample degradation. The  $\text{EPS}_{\text{GLC}}\text{-S}$  and  $\text{EPS}_{\text{GLC}}\text{-RS}$  preparations were not degraded, as demonstrated by gel filtration chromatography. Finally, this study demonstrated that sulfonation of the botryosphaeran (as  $\text{EPS}_{\text{GLC}}\text{-RS}$ ) produced anticoagulant activity *in vitro*.

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