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An active heparinoid obtained by sulphation of a galactomannan extracted from the endosperm of *Senna macranthera* seeds

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Abstract

A galactomannan was isolated from the endosperm of seeds of *Senna macranthera*. It contained mannose and galactose in a 3.0:1 ratio and consisted mainly of a (1 → 4)-linked β-D-Man*p* main-chain partially substituted at O-6 with α-D-Gal*p* side-chains. Sulphation with SO_3-pyridine gave a product with DS 0.40. According to ^{13}C NMR analysis, the OH-6 groups of galactopyranosyl and mannopyranosyl units were preferentially substituted. The product had 45 IU/mg of anticoagulant activity, as shown by the in vitro activated partial thromboplastin time (APTT), compared with 183 IU/mg for a porcine intestinal mucosa heparin. Two fractions of the sulphated derivative were obtained differing in their affinity to AT III in gel. Only that with a high affinity (DS 1.4) showed a strong anticoagulant activity, as measured by APTT (321 IU/mg) and Heptest. By analogy with heparin, the anticoagulant activity of the derivative could be expressed through binding of the polysaccharide to AT III.

Keywords: *Senna macranthera*; Leguminosae; Endosperm; Sulphated galactomannan; Heparinoid; Anticoagulant activity

1. Introduction

Heparin is a complex sulphated glycosaminoglycan produced by connective-tissue-type mast cells. They have several biological effects, although the best studied is their ability to minimise blood coagulation. Such activity is due to the occurrence of a specific pentasaccharide sequence that mediates an interaction with antithrombin III (AT III) (Atha, Lormeau, Petitou, Rosemberg & Choay, 1985; Casu et al., 1981; Lindahl, Baeckstroem, Thunberg & Leder, 1980; Lindahl, Thunberg, Bäckström, Riesenfeld, Nordling & Björk, 1984). Heparin has been the drug of choice in clinical pre-surgical and post-surgical prophylaxis of thrombotic events. However, because of their side effects, such as bleeding, contamination with highly infectious bovine spongiform encephalopathy, immunosupression (Gorski, Wasik, Nowaczyk & Korczak-Kowalska, 1991), and other disadvantages such as lack of homogeneity and variability in physiological activity, intense investigations have been carried out in order to find a replacement. As the anticoagulant activity of heparins depends on their sulphate content, many heparinoids, prepared by sulphation of polysaccharides from plant or obtained directly from animal tissue, have been studied (Doctor, Lewis, Coleman, Kemp, Marbley & Sauls, 1991; Pavão, Mourão, Mulloy & Tollefsen, 1995; Razi, Feyzi, Björk, Naggi, Casu & Lindahl, 1995). However, such products showed weak anticoagulant action and were often devoid of anti-platelet activity, while others are too toxic for application in humans. Thus, in order to obtain another heparinoid candidate, we have now sulphated a galactomannan extracted from the endosperm of *Senna macranthera* (common Brazilian name, sena) seeds and have submitted the product to appropriate tests.

2. Experimental

2.1. Polysaccharide source

Seeds of *S. macranthera* were collected in the metropolitan region of Curitiba, State of Paraná, Brazil.

2.2. Polysaccharide isolation and purification

Endosperms (100 g) of *S. macranthera* seeds were crushed and extracted with 2:1 (v/v) toluene:EtOH (750 ml) in a Soxhlet apparatus for 100 h and the residual mass refluxed with 2:1 (v/v) MeOH:H_2O (600 ml) for 48 h.

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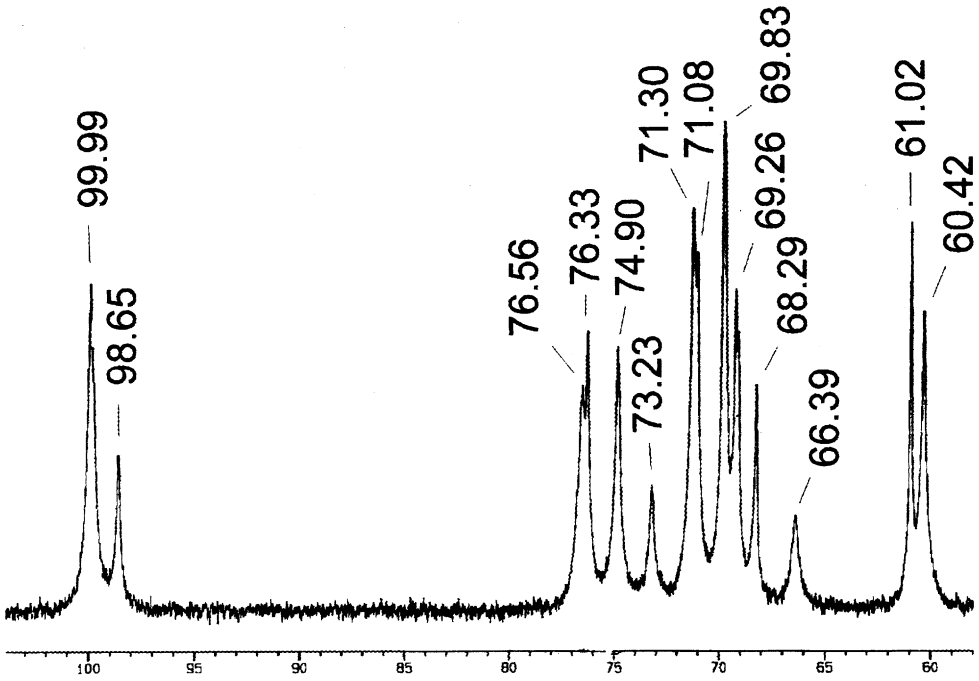

Fig. 1. ^{13}C NMR spectrum of galactomannan from *S. macranthera* endosperms (D_2O at 50°C, numerical values expressed in δ, ppm).

The residue was extracted $\times 5$ with H_2O at room temperature. The extract was submitted to centrifugation at 2000 *g* (15 min), and sufficient NaCl then added to the supernatant (3.0 l) to give a concentration of 0.1 mol/l. The polysaccharide was precipitated from the solution with 2 vol. EtOH. To purify this sample (1.0 g in 300 ml H_2O), aliquots were applied in two separate equal portions to a DEAE-cellulose column (Cl^- form, 33×3.0 cm i.d.), which was eluted with water to give the polysaccharide (yield 10%).

2.3. Monosaccharide composition of native polysaccharide

The polysaccharide was hydrolysed with TFA (1 mol/l) at 100°C for 5 h and liberated monosaccharides converted into alditol acetates by successive $NaBH_4$ reduction and acetylation with Ac_2O-pyridine. GC–MS was performed using a Finnigan Mat ion trap (model 410) mass spectrometer, incorporating a DB-225 capillary column ($30 \text{ m} \times 0.25$ mm i.d.), with helium as carrier gas. Injections were carried out at 50°C and the column was then programmed to 230°C at a rate of 4°/min, and held until the end of the run. Scans were carried out for *m/z* 40–420 every 2 s at 70 eV.

2.4. Sulphation of galactomannan

Following the method of O'Neill (1955), the purified galactomannan (500 mg) was suspended in pyridine-formamide (10:37.5; 47.5 ml) and stirred overnight at 8°C. Sulphation was carried out at the same temperature by continuous addition of chlorosulphonic acid (6.0 ml) for 6 h. The resulting homogeneous solution was neutralised with aqueous $NaHCO_3$ and in order to remove low molecular weight compounds, it was dialysed against tap water. Finally, sufficient NaCl was added to give a concentration of 0.1 mol/l and the sulphated polysaccharide was precipitated from the solution (500 ml) with 2 vol. of EtOH (yield 70%).

2.5. Sulphate content of sulphated galactomannan

The total sulphate content was determined by the method of Dodgson and Price (1962) using $BaCl_2$/gelatin.

2.6. Infrared spectroscopy

IR spectra on KBr pellets were recorded using a BOMEM, MB-100 (Hartman and Baum) spectrometer.

2.7. NMR spectroscopy

^{13}C NMR spectra were obtained using a BRUKER, AVANCE DRX-400 model spectrometer (100 MHz, at 50°C for native and 27°C for the sulphated polymer in D_2O), equipped with a Fourier-Transform System. Chemical shifts are expressed as δ (ppm) relative to the resonance of dimethyl sulphoxide as internal standard ($\delta = 39.7$).

2.8. Molecular weights of purified and sulphated galactomannan

Each sample (10 mg) was dissolved in 1 ml 0.15 mol/l NaCl, filtered successively through Millipore membranes of 3.0 and 1.2 μm pore diameter. The filtrate was applied to a Sepharose CL-4B column (62.5×1.1 cm i.d.), calibrated with standard dextrans (M_r: 77.8×10^3 to 2.0×10^6 Da), and eluted with 0.15 mol/l NaCl. Fractions were collected and their absorption monitored with

phenol-H_2SO_4 at 490 nm to determine total sugar (Dubois, Gilles, Hamilton, Rebers & Smith, 1956).

2.9. Chromatography on agarose-AT III column

Twelve samples (2.0 mg) were successively applied to a column (5.0 × 1.5 cm i.d.) containing the affinity material of AT III bound to agarose (Sigma), and equilibrated with 0.05 M tris/HCl buffer, pH 7.2. Washing with this buffer eluted a polysaccharide fraction and the retained active fraction was then eluted with 2.0 mol/l NaCl. The hexose contents of these fractions were determined (Dubois et al., 1956).

2.10. Biological assays

APTT (activated partial thromboplastin time) values were determined using 80 μl of normal plasma (Biomérieux) and 20 μl of sample or standard Sigma heparin from porcine intestinal mucosa [183 IU/mg), with 100 μl cephalin (Biolab-Biomérieux S.A., Brazil) for 240 s at 37°C. After addition of 100 μl of aqueous $CaCl_2$ (0.025 mol/l), the coagulation time was determined with a OPTION fibrometer.

The inhibition of the Xa factor was measured by the Heptest-method (Brown, 1984) using 80 μl of normal plasma (Biomérieux), 20 μl of sample (3.5 μg/ml) or standard Sigma heparin (4.3 μg/ml) from porcine intestinal mucosa (183 IU/mg) and 100 μl factor Xa (Sigma). The mixture was incubated for 120 s at 37°C. After addition of 100 μl of a 1:1 aqueous mixture of $CaCl_2$:cephalin, the coagulation time was measured.

3. Results and discussion

The galactomannan was prepared by cold aqueous extraction of the seed endosperm followed by fractionation on a column of DEAE-cellulose. It had a mannose to galactose ratio of 3.0:1 (GC–MS of derived alditol acetates) and as expected, gave a ^{13}C NMR spectrum (Fig. 1) typical of a main chain of (1 → 4)-linked β-Man*p* units partially substituted at O-6 with those of α-Gal*p*. Signals were present at δ 99.99 (C-1: β-Man*p*), δ 98.65 (C-1: α-Gal*p*), δ 66.39 (*O*-substituted C-6: Man*p*), δ 61.02 (C-6: Gal*p*), and δ 60.42 (C-6: Man*p*). In the internal region of the spectrum (see Fig. 2), the signal at δ 76.33 corresponded to C-4 of adjacent Man*p* units not substituted at O-6. This had a shoulder at slightly lower field of δ 76.81 (C-4 of 2 adjacent *O*-substituted Man*p*), so that the intermediate signal (δ 76.56) could be from overlapping resonances of diads. These would arise from a group in which only one of the two Man*p* units is substituted (Grasdalen & Painter, 1980; Gupta & Grasdalen, 1989; Manzi, Cerezo & Shoolery, 1986; Petkowicz, Sierakowski, Ganter & Reicher, 1998).

Sulphatation of the galactomannan with SO_3-pyridine gave a product containing 19% sulphate, corresponding to a DS of 0.40. The presence of sulphate was confirmed by a strong band in 1227 cm^{-1}, which corresponds to an S=O stretching vibration (Lloyd, Tudball & Dodgson, 1961), which was absent when the starting material was examined. Sulphation without degradation was shown by gel permeation chromatography (GPC), which gave M_r values of 2.2 and 2.4×10^6 Da for native and sulphated galactomannan, respectively. The derivative was examined using ^{13}C NMR spectroscopy in order to determine its substitution pattern.

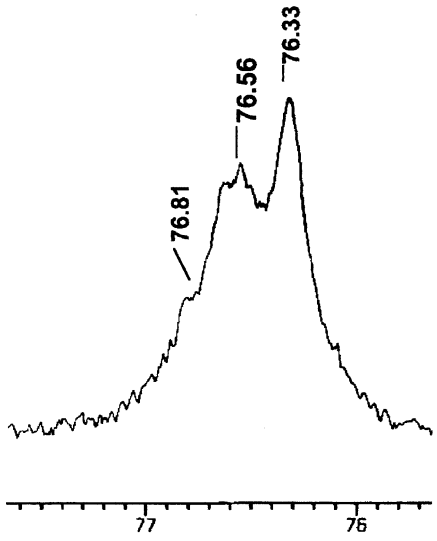

Fig. 2. C-4 region of mannopyranosyl units in the ^{13}C NMR spectrum of galactomannan from *Senna macranthera* endosperms (D_2O at 50°C, numerical values expressed in δ, ppm).

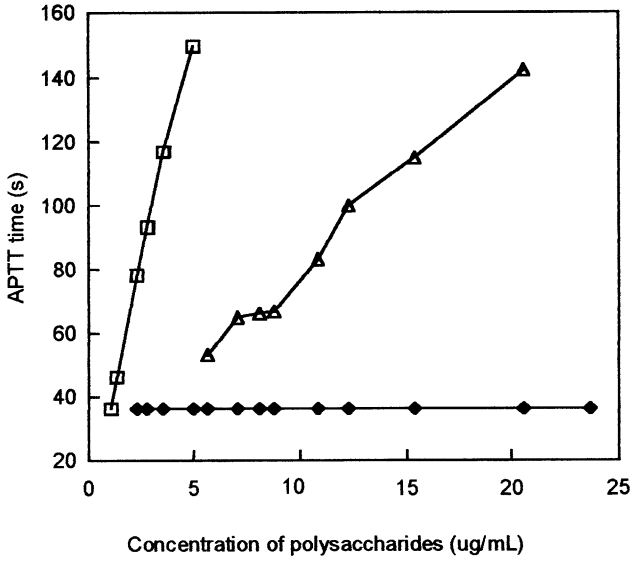

Fig. 3. Effect of sulphated galactomannan using the APTT test. Porcine intestinal mucosa heparin (-□-); native galactomannan (-◆-) and product obtained by sulphation (DS: 0.40; -△-). APTT was measured using an OPTION fibrometer, with 80 μl of normal plasma, 20 μl of sample and 100 μl cephalin for 240 s at 37°C. After addition of 100 μl of aqueous $CaCl_2$ (0.025 mol/l) the coagulation time was determined. Time control: 36 s.

Table 1
Anticoagulant activity of sulphated galactomannan

Sulphated material	APTT[a] (IU/mg)
Active fraction[b]	321
Porcine heparin[c]	183

[a] Activated partial thromboplastin time (APTT).
[b] Sulphated galactomannan was applied to an agarose-AT III column and eluted with 2 mol/l NaCl,, giving a product with DS 1.40.
[c] DS 0.60.

The most notable effect was a diminution of the C-6 signals of non-substituted mannopyranosyl (δ 60.54) and galactopyranosyl (δ 61.12) units, (Grasdalen & Painter, 1980; Gupta & Grasdalen, 1989; Manzi et al., 1986; Sierakowski, Milas, Desbrières & Rinaudo, 2000), which arose from preferential sulphation of the more reactive CH_2OH-6 groups. These were shifted downfield into a crowded region of the spectrum, which prevented their detection as inverted signals using distortionless enhanced polarisation transfer (DEPT).

The sulphated derivative was tested for its APTT, which is the most widely used clinical laboratory test for monitoring anticoagulant activity: by its measurement, factors XII–VIII are mainly indicated (Triplett, 1993). The sulphated galactomannan inhibited APTT at concentrations greater than 5.7 μg/ml (see Fig. 3). No significant difference was found if the heparinoid was successively filtered through Millipore membranes of 3.0 and 1.2 μm compared with unfiltered samples.

The anticoagulant activity of this polysaccharide was low when compared with that of standard heparin from porcine intestinal mucosa (DS sulphate, 0.60). Thus, in order to increase activity and to evaluate if the mechanism of anticoagulant activity is involved with its interaction with AT III, the sulphated galactomannan was fractionated by chromatography on an agarose-antithrombin column. The AT III-bound fraction (DS 1.40), named the active fraction, corresponded to 30% of the applied polysaccharide and was responsible for all of its activity, as the unbound AT III fraction (DS 0.12) did not inhibit APTT. The active fraction showed 321 IU/mg of human plasma by APTT, in comparison to porcine heparin, which had 183 IU/mg (Table 1).

The active bound fraction was also tested by the Heptest, which measures inhibition of the exogenous factor Xa using the plasma factor AT III. The coagulation time was significantly prolonged by the active fraction at 3.5 μg/ml in comparison with porcine heparin at 4.3 μg/ml, confirming the specificity of the AT III interaction.

4. Conclusions

The results suggest that, by analogy to heparin, the anticoagulant activity of the sulphated derivative may be also expressed through binding to AT III, as the only fraction which presented a strong anticoagulant activity was that which bound to AT III. Other investigations, using animal thrombosis models, must be carried out as questions remain as to whether sulphated galactomannan, which has a good in vitro anticoagulant activity, could operate in vivo as a potent antithrombotic agent, useful for clinical purposes. In particular, it is necessary to determine whether it would give rise to a reduced risk, in terms of acute and long-term toxicity.

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