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Bioactive polysaccharides from *Costus spicatus*

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Abstract

Three polysaccharides, glucans with mean M_r's of 7.50×10^5, 4.25×10^5 and 2.15×10^5, were isolated from fresh stems of *Costus spicatus* by fractionation on Sephacryl S-300 HR and Sephadex G-25. Chemical and spectroscopic studies indicated that they have a highly branched glucan type structure composed of α-(1 → 4) linked D-glucopyranose residues with (1 → 3), (1 → 6) branching points, and a small amount of (1 → 6) branching to α-(1 → 3) linked D-glucopyranose residues. The polysaccharides inhibit capillary permeability and demonstrate phagocytosis stimulating properties. © 2003 Elsevier Science Ltd. All rights reserved.

Keywords: *Costus spicatus*; Costaceae; Polysaccharides; Antiinflammatory activity; Phagocytic activity

1. Introduction

Costus spicatus Swartz (Costaceae), commonly called 'cana-do-brejo' in Brazil, is a medicinal plant found in wet coastal forests. The rhizomes of this plant are used for the treatment of complaints of the bladder and urethra and to expel kidney stones (Manfred, 1947). An infusion of the aerial parts is taken to treat colds, sore throats, dysentery and diarrhea (Cruz, 1965). Recently, steroidal saponins (da Silva, Bernardo, & Parente, 1999a,b) which showed a slight haemolytic effect were isolated from the rhizomes of *C. spicatus*. Flavonol glycosides (da Silva, Bernardo, & Parente, 2000) have been reported from its leaves and demonstrated inhibitory activity of nitric oxide production by activated macrophages. Here, we report the isolation and chemical characterization of three neutral polysaccharides responsible for the antiinflammatory and immunomodulatory activities.

2. Experimental

2.1. Materials

Carbohydrate content was analyzed by colorimetric assays according to the procedure of Dubois, Gilles, Hamilton, Hebers, and Smith (1956), without previous hydrolysis of the sample, and by GC–EIMS of the glucitol acetates (Sawardeker, Sloneker, & Jeanes, 1965). Protein content was analyzed by the method of Bradford (1976) using ovalbumin as a standard. The experimental data were tested for statistical differences using Student's *t* test. The M_r's of Cs1, Cs2 and Cs3 were estimated from the calibration curve of elution of standard dextrans (average M_r's 2,000,000, 413,000, 282,000, 148,000, 68,000, 37,500, 19,500 and 9500; Sigma) on Sephacryl S-300 HR (5 cm × 85 cm; Pharmacia).

Dialysis was carried out using tubing with an M_r cut-off 12,000. Optical rotations were measured on a Perkin Elmer 243B polarimeter. ^{1}H- and ^{13}C-NMR spectra were obtained on a Varian Gemini 200 NMR spectrometer operating at 200 MHz for δ_H and 50 MHz for δ_C, in D_2O containing sodium 2,2-dimethyl-2-silapentane-5-sulfonate as an internal standard. GC analyses were performed using a Shimadzu GC-17A gas chromatograph coupled with a Shimadzu GCMS-QP5050A gas chromatograph mass spectrometer operating at 70 eV. GC was carried out with FID, using a glass capillary column (0.25 m × 25 m, 0.25 micron, J.W. Scientific Inc.) DB-1. TLC of monosaccharides were performed on silica gel coated plates (Merck) in *n*-BuOH–pyridine–H_2O (6:4:3), and sugars were detected by spraying with orcinol–H_2SO_4.

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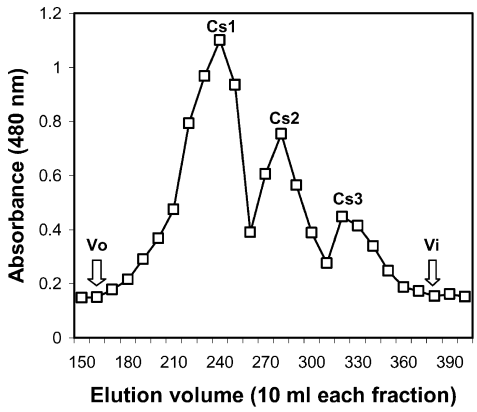

Fig. 1. Elution diagram of Cs1, Cs2 and Cs3 from Sephacryl S-300 HR (0.1 M Tris-HCl). V_o, void volume; V_i, inner volume.

2.1.1. Plant material

Stems of *C. spicatus* Swartz were collected at Ilha do Fundão, Rio de Janeiro in September 1996, and identified by Luci S. Valle. A voucher specimen (no. R192950) is deposited at the herbarium of the National Museum, Rio de Janeiro, Brazil.

2.2. Methods

2.2.1. Extraction

Fresh stems of *C. spicatus* (100 g), previously cut into small pieces, were extracted with hot water (500 ml) at 80 °C under stirring for 1 h. The aqueous extract was filtered through Whatmann filter paper (4 μm) and the filtrate centrifuged. By precipitation with two volumes of EtOH (12 h stirring and 24 h standing at 4 °C), a resulting precipitate was obtained following centrifugation and subsequent lyophilization (yield: 213 mg). The amorphous powder was dissolved in 0.01% sodium sulfate (100 ml) and added to 5% cetyltrimethylammonium bromide (CTAB; 20 ml). After centrifugation, the supernatant was poured into two volumes of EtOH and the precipitate obtained was dissolved in water (100 ml), dialyzed and lyophilized to yield a neutral polysaccharide fraction (153 mg).

2.2.2. Fractionation

A sample of the neutral polysaccharide fraction (100 mg) was dissolved in 0.1 M Tris-HCl buffer (pH 7.0), and applied to a Sephacryl S-300 HR column (5 cm × 85 cm; 1650 cm^3). Fractions of 5 ml corresponding to the peaks Cs1 (1050–1250 ml), Cs2 (1350–1500 ml) and Cs3 (1600–1750 ml) (Fig. 1) were pooled dialyzed and freeze-dried. The powder corresponding to each peak was dissolved in water (2 ml) and applied to a Sephadex G-25 column (1.5 cm × 50 cm; 15 g), and 5 ml fractions were collected (100–150 ml). The eluates obtained were concentrated, and lyophilized to yield Cs1 (38 mg), Cs2 (23 mg) and Cs3 (15 mg). The carbohydrate content of the fractions was measured.

2.2.3. Molar carbohydrate composition and D, L configuration

Monosaccharides were analyzed as their trimethylsilylated methylglucosides obtained after methanolysis (0.5 M HCl in MeOH, 24 h, 80 °C) and trimethylsilylation (Kamerling, Gerwig, Vliegenthart, & Clamp, 1975). The configurations of the glucosides were established by capillary GC and GC–MS of their trimethylsilylated (−)-2-butylglucosides (Gerwig, Kamerling, & Vliegenthart, 1978).

2.2.4. Methylation analysis

Polysaccharides Cs1, Cs2 and Cs3 were methylated with DMSO–lithium methylsulfinyl carbanion–CH_3I (Parente et al., 1985). The methyl ethers were obtained after hydrolysis (4 N TFA, 2 h, 100 °C) and analyzed as alditol acetates by GC–MS (Sawardeker et al., 1965).

2.2.5. Antiinflammatory activity

Antiinflammatory activity was evaluated by measuring acetic acid-induced vascular permeability (Whittle, 1964). Male mice (BALB/c, 2 months old, 15–20 g) in groups of five were dosed orally with Cs1, Cs2 and Cs3 and a positive control, indomethacin (10 mg/kg body weight) before the intravenous injection of 4% Evans blue (10 ml/kg body weight). After injection of the dye, 0.1N acetic acid (10 ml/kg body weight) was injected intraperitoneally. Twenty minutes later, the mice were killed with an overdose of ether and the viscera were exposed after a 1 min period to allow blood to drain away from the abdominal wall. The animal was held by a flap of the abdominal wall and the viscera were irrigated with 10 ml of saline over a petridish. The washing was filtered through glass wool and transferred to a test tube. To each tube was added 0.1 ml of 1N NaOH in order to clear any turbidity due to protein, and the absorbance was read at 590 nm.

2.2.6. Phagocytic activity

Male mice (BALB/c, 2 months old, 15–20 g) were used in groups of five. Cs1, Cs2 and Cs3, and a positive control, zymosan, were each dissolved in physiological saline and dosed intraperitoneally (50 mg/kg body weight) once a day, for 5 days. After 48 h, mice were injected via the tail vein with coloidal carbon (Pelikan drawing ink A.17 black). The ink was diluted eight times with phosphate-buffered saline containing 1% gelatin before use, the amount of the resulting solution used was 10 ml/kg body weight. Blood samples were drawn from the orbital vein at 0, 3, 6, 9, 12 and 15 min. The blood (25 μl) was dissolved in 0.1% sodium carbonate (2 ml) and the absorbance at 660 nm was determined according to Biozzi, Benacerraf, and Halpern (1953).

Table 1
Methylation analysis of Cs1, Cs2 and Cs3 glucans

Methylated alditol acetate derivatives	Relative retention times[a]	Molar ratios[b]			Structural features
		Cs1	Cs2	Cs3	
2,3,4,6-tetra-*O*Me Glc	1.00	3.03	1.35	1.43	Glc 1 →
2,3,6-tri-*O*Me Glc	1.16	81.81	69.69	74.28	→ 4 Glc 1 →
2,4,6-tri-*O*Me Glc	1.18	3.03	1.35	4.28	→ 3 Glc 1 →
2,3,4-tri-*O*Me Glc	1.21	6.06	2.68	1.43	→ 6 Glc 1 →
2,6-di-*O*Me Glc	1.29	3.03	5.36	4.28	→ 4 Glc 1 → 3 ↑
2,3-di-*O*Me Glc	1.36	1.52	8.05	8.58	→ 4 Glc 1 → 6 ↑
2,4-di-*O*Me Glc	1.42	1.52	11.52	5.72	→ 3Glc 1 → 6 ↑

[a] Relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl glucitol.
[b] Calculated from peak areas and molecular weight of derivatives.

3. Results and discussion

The crude neutral polysaccharide fraction extracted from the stems of *C. spicatus* contained 99.5% carbohydrate and 0.5% protein. A sample of this fraction was fractionated by means of Sephacryl S-300 HR and desalted by means of Sephadex G-25 gel permeation chromatography, leading to the isolation of three neutral, protein-free polysaccharide fractions Cs1, Cs2 and Cs3 (Fig. 1).

The fractionation procedures were monitored by carbohydrate content. The sugar molecules Cs1, Cs2 and Cs3 were determined to be only glucose by the identification on TLC of the acid hydrolysates and by GC of the trimethylsilylated methylglucosides derivatives prepared from the monosaccharides. The absolute configuration of the glucose was determined by GC of its trimethylsilylated (−)-2-butylglucosides. D-glucopyranose was identified by GC–EIMS of the pertrimethylsilylated butylglucosides. Cs1, Cs2 and Cs3 glucans showed positive specific rotations, $[\alpha]_D^{20}$ +100° (*c* 0.1, H_2O), $[\alpha]_D^{20}$ +200° (*c* 0.1, H_2O) and $[\alpha]_D^{20}$ +150° (*c* 0.1, H_2O), respectively, and showed characteristic absorption at 840 cm^{-1} in the IR spectra due to an α-configuration. The average M_r's of Cs1, Cs2 and Cs3 glucans were estimated to be 7.50×10^5, 4.25×10^5 and 2.15×10^5, respectively, based on the calibration curve of the elution volume of standard dextrans from gel filtration on Sephacryl S-300 HR.

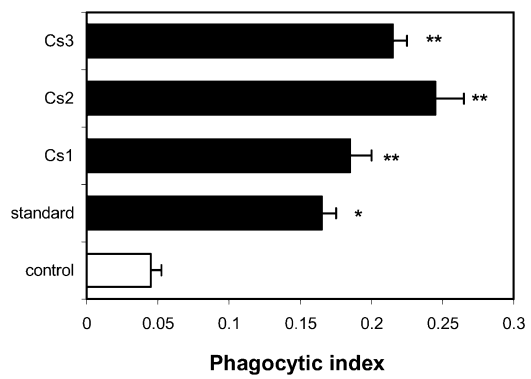

Fig. 2. Effects of Cs1, Cs2 and Cs3 polysaccharides on phagocytic activity. Significantly different from the positive and negative control groups; $*p < 0.01$, $**p < 0.05$.

Cs1, Cs2 and Cs3 glucans were methylated by the method of Parente et al. (1985). The fully methylated products were hydrolyzed with acid, converted into the alditol acetates, and analyzed by GC and GC–MS. Cs1, Cs2 and Cs3 glucans furnished 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl glucitol, 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl glucitol, 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl glucitol, 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl glucitol, 1,3,4,5-tetra-*O*-acetyl-2,6-di-*O*-methyl glucitol, 1,4,5,6-tetra-*O*-acetyl-2,3-di-*O*-methyl glucitol and 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methyl glucitol (Table 1). The results of methylation analyses indicated that Cs1, Cs2 and Cs3 glucans contained mainly (1 → 4) linked glucosyl residues and branching points at *O*-3 and *O*-6 of (1 → 4) linked glucosyl residues. They possess exceptional α-1,3-linked units in addition to the usual α-1,4-linear linkage. On the other hand, the presence of α-1,6-linked units in Cs1, Cs2 and Cs3 is characteristic. In addition to 4,3- and 4,6-branching points, the polysaccharides contain a substantial amount of another 3,6-branching points in different proportions (Table 1). The ^{1}H-NMR spectra of Cs1, Cs2 and Cs3 glucans in D_2O showed anomeric proton signals at δ 5.38 as a broad singlet. Further, the ^{13}C-NMR spectra showed a signal due to an anomeric carbon of α-D-glucopyranose at δ 102.26 ppm (Yamada, Kiyohara, & Otsuka, 1984).

According to the literature, several glucan type polysaccharides were shown to possess antiinflammatory (Czarnecki & Grzybek, 1995) and immunomodulatory (Tomoda, Ohara, Shimizu, & Gonda, 1994) activities. In order to confirm the popular use of *C. spicatus*, the

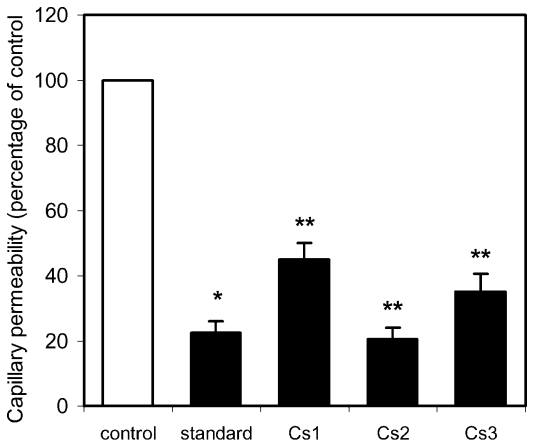

Fig. 3. Antiinflammatory properties Cs1, Cs2 and Cs3 polysaccharides. Significantly different from the positive and negative control groups; $*p < 0.01$, $**p < 0.05$.

pharmacological properties of Cs1, Cs2 and Cs3 glucans were investigated. Their effects on the reticuloendothelial system were demonstrated by the carbon clearance test (Biozzi et al., 1953). As shown in Fig. 2, the phagocytic indices for Cs1, Cs2 and Cs3 glucans were significantly increased, suggesting immunomodulatory properties. In addition to this, Cs1, Cs2 and Cs3 glucans inhibited the increase in vascular permeability caused by acetic acid, which is a typical model of first stage inflammatory reaction (Whittle, 1964). The standard drug indomethacin (10 mg/kg body weight) also reduced the leakage (Fig. 3).

In conclusion, several studies suggested that the mechanisms of action of the glucan type polysaccharides might be due to their action on the reticuloendothelial system, through phagocytosis stimulation, along with an effect on vascular reactions, affecting the capillary permeability (Whistler, Bushway, Singh, Nakahara, & Tokuzen, 1976). Consequently, in spite of the highly branched structures of the polysaccharides, it can be presumed that if humoral amylase can hydrolyze certain (1 → 4) linkages, the residual chains of (1 → 3) and (1 → 6) linked residues may be long enough to contribute to the phagocytic enhancement and vascular protection (Gonda, Takeda, Shimizu, & Tomoda, 1992). The pharmacological results obtained may help explain the use of the stems of *C. spicatus* in the Brazilian traditional medicine.

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