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Note

Purification of a new polysaccharide from *Cyttaria johowii* (Esp.) and studies on its antitumor activity

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Cyttariaceae (Discomycetes) is a family of fungi confined to the Southern hemisphere, where the different species grow as parasites of *Nothofagus*, on which they produce characteristic tumors[1].

We have previously studied the polysaccharides from *Cyttaria harioti*[2–5], and have initiated chemical studies on *Cyttaria johowii*, a species that has fruit bodies of different characteristics. On this basis, Gamundi[1] suggested that the species could be included together with *C. hookeri* in a subgenus. These chemical studies could help to establish the relationship between the species.

RESULTS AND DISCUSSION

Three polysaccharide fractions: CJ 1, $[\alpha]_D$ +5.1° (dimethyl sulfoxide); CJ 2, $[\alpha]_D$ +45.5° (dimethyl sulfoxide); and CJ 3, $[\alpha]_D$ −14.7° (dimethyl sulfoxide) were separated from an aqueous extract of the fruit bodies of *C. johowii* by fractional precipitation with ethanol. The last fraction, the most abundant, was chosen for this study.

The purified polysaccharide was precipitated in 92% yield at an ethanol concentration of 29–30% and was eluted as a single peak from Sephadex G-100. Because of its low solubility in water, dimethyl sulfoxide was used as solvent. By comparison with the elution volume of known dextrans, an average molecular weight of 40,000 was estimated. This value is in agreement with the degree of polymerization of 235 determined by the method of Unrau and Smith[6], considering the terminal, reducing monosaccharide to be linked through O-3.

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Acid hydrolysis was performed by dissolving the polysaccharide in 90% formic acid and then hydrolyzing it in 0.5M sulfuric acid for various time-intervals. Total hydrolysis gave glucose and a small amount of fructose, as detected by paper chromatography. The same monosaccharides, in 92:3 molar ratio, were identified by g.l.c. of the trimethylsilyl ethers (columns *A* and *B*). Analysis by the phenol sulfuric acid method[7] gave 100% of total sugar. A D-glucose content of 89% was determined with D-glucose oxidase[8]. The difference could be attributed to fructose that had undergone partial decomposition under conditions for total hydrolysis. Treatment with hot formic acid caused partial degradation. After removal of the formyl groups the degraded product (75% of the original) became insoluble. Analysis of the soluble sugars by paper chromatography showed spots corresponding to fructose, glucose, laminarabiose, gentiobiose, and two unidentified, higher oligosaccharides. A disaccharide that could not be resolved from laminarabiose in solvent *A* was detected with the reagent for ketoses (*c*). In solvent *B*, the keto-disaccharide having R_{Glc} 0.73 could be separated from laminarabiose (R_{Glc} 0.59) and gentiobiose (R_{Glc} 0.37). G.l.c. (column *B*) showed, besides peaks corresponding to trimethylsilyl ethers of the known sugars, another compound having a retention time similar to that of the turanose [*O*-α-D-glucopyranosyl-(1→3)-D-fructose] derivative. Preliminary studies indicate that it is laminarabiulose [*O*-β-D-glucopyranosyl-(1→3)-D-fructose]. Synthesis of this disaccharide, which is not described in the literature, will be presented elsewhere. The formation of a glycosylfructose by partial hydrolysis would indicate that at least some fructose residues are not terminal. Gentiobiose could be differentiated from isomaltose only by g.l.c. with a capillary column. Gel filtration of the degraded polysaccharide revealed that its molecular size was not significantly different from the original. This finding indicates that the ketose is not incorporated in the main chain, but is most probably the starting point for some branches that are hydrolyzed under mild conditions.

TABLE I

METHYLATION ANALYSIS OF *Cyttaria johowii* POLYSACCHARIDE

O-Methylalditol acetate	*Relative retention times*		*Percent of total*[a]
	3% ECNSS-M	*8% NPGS*	
	180°	*215°*	
2,3,4,6-Me$_4$Glc	1.00	1.00	31.3
Me$_3$X	1.69	1.42	0.4
2,4,6-Me$_3$Glc	1.95	1.68	23.5
2,3,4-Me$_3$Glc	2.55	1.90	6.2
2,3,6-Me$_3$Glc	2.55	1.98	6.0
2,4-Me$_2$Glc	5.20	3.48	32.6

[a]Peak areas on 8% NPGS. Similar values were obtained with the 3% ECNSS-M column.

The polysaccharide was methylated by the Hakomori method[9], and analysis of the methylated sugars liberated by hydrolysis, gave the results presented in Table I. These indicate a highly branched structure having 3-O- and 6-O-substituted D-glucopyranosyl residues. A minor, unidentified component might correspond to a methylated glucitol or mannitol acetate derived from the fructose-linked residues.

The low specific rotation, $[\alpha]_D$ −34° (dimethyl sulfoxide), supports the β-configuration for the polysaccharide. Fungal D-glucans having β-(1→3) and (1→6) linkages have been reported to have antitumor activity[10–12]. It was of interest to investigate whether the polysaccharide from *C. johowii* was effective against Sarcoma 180 in mice. These studies show that tumor growth was significantly inhibited during the initial days (59% on day 8) and that the inhibitory effect became weaker by day 30 (25%) with respect to the control animals. However, whereas all of the controls died after 75 days, 14 out of 15 treated animals showed complete tumor regression. The fructoglucan had no toxic effects in the test animals, although some dehydration was observed in the initial days.

EXPERIMENTAL

Material. — Fruit bodies of *Cyttaria johowii* Espinosa collected in Villa Angostura (Neuquén, Argentina) in January were used in this study. The host was *Nothofagus dombeyi*.

Analytical methods. — Paper chromatography was performed by the descending method on Whatman No. 1 paper with: *A*, 1-butanol–pyridine–water (6:4:3, v/v) or *B*, (5:2:2) 1-butanol–ethanol–water. Detection was effected with (*a*) silver nitrate–sodium hydroxide[13], (*b*) *p*-anisidine hydrochloride[14], or (*c*) resorcinol–hydrochloric acid[14]. Optical rotations were recorded with a Perkin–Elmer 421 spectrophotometer. Total carbohydrate was determined by the phenol–sulfuric acid method[7] and glucose in the hydrolyzate was analyzed[8] with D-glucose oxidase (Type VII), peroxidase (Type II), and *o*-dianisidine (Sigma). G.l.c. was performed with a Hewlett–Packard 5830 A gas chromatograph equipped with glass columns packed with (*A*) 3% SE-30 on Chromosorb WAW-DMCS (80–100 mesh) with nitrogen at a flow rate of 32 mL.min^{-1}; T_i 250°; T_d 225°; T_c programmed (1°/min) from 160–210°; (*B*) 2% OV-101 on Chromosorb WAW-DMCS (60–80 mesh), nitrogen 25 mL.min^{-1}; T_i 280°; T_d 280°; T_c was kept for 5 min at 165° and then programmed (4°/min) to 280°; (*C*) 3% ECNSS-M on Gas Chrom Q (100–120 mesh), nitrogen 30 mL.min^{-1}; T_i 210°; T_d 215°; T_c 180°; and (*D*) 8% NPGS on Chromosorb WAW-DMCS, nitrogen 26 mL.min^{-1}; T_i 280°; T_d 250°; T_c 215°. A glass capillary column (0.2 mm × 12 m) with SP 2100 was used for the identification of gentiobiose: T_i 280°; T_d 280°; T_c 235°. G.l.c.–m.s. was performed with a Varian 1440 gas chromatograph coupled to a Varian-MAT CH7A spectrometer. Standards prepared with authentic samples and cochromatography was used for identification. The trimethylsilyl ethers were prepared by dissolving the dried sample in pyridine and heating the solution with Tri-Sil (Pierce) for 10 min at 60°. Gel filtration was performed on Sephadex G-100

(200–400 mesh). A sample (5 mg) of the polysaccharide dissolved in 1 mL of dimethyl sulfoxide was applied to a column (72 × 2 cm) pre-equilibrated and then eluted with the same solvent. Fractions (3 mL) were collected and monitored for total carbohydrate. Blue Dextran 2000 (Pharmacia Fine Chemicals, Uppsala, Sweden) was used for determination of the void volume. The average molecular weight was determined by using dextrans of known molecular weight (Sigma).

Isolation and purification of the polysaccharide. — Powdered, dried fruit-bodies of *C. johowii* (50 g) were successively extracted with chloroform and methanol in a modified Soxhlet apparatus. The remaining material (45 g) was extracted with water (2 L) in a Waring blender at room temperature and then centrifuged. The extraction was repeated twice, and the combined supernatant solutions were concentrated to 2 L under diminished pressure below 40 ° and then fractionated with ethanol. Three fractions were separated: CJ_1, 0.2 g, $[\alpha]_D^{20}$ +5.1 ° (dimethyl sulfoxide) precipitated with 16 % ethanol; CJ_2, 0.31 g, $[\alpha]_D^{20}$ +45.5 (dimethyl sulfoxide) was obtained with 25 % ethanol, and CJ_3 1.3 g, $[\alpha]_D^{20}$ −14.7 (dimethyl sulfoxide) precipitated when the ethanol concentration was increased to 28 %. Polysaccharide CJ_3 was purified by redissolution in water and reprecipitation with ethanol (to 30 %) until the specific rotation remained constant. The purified polysaccharide dissolved very slowly in water, but was soluble in dimethyl sulfoxide; it had $[\alpha]_D^{20}$ 34 (*c* 0.3, dimethyl sulfoxide). The degree of polymerization was determined as described[4].

Acid hydrolysis. — A solution of the polysaccharide (10.5 mg) in 90 % formic acid (2 mL) was heated in a sealed tube for 3 h at 100°. The formyl groups were hydrolyzed by diluting to 50 % with water and heating for 3 h at 100 °. The degraded product was evaporated under diminished pressure with the addition of water to aid in the removal of the acid, and further hydrolyzed with 0.5M sulfuric acid (1 mL) for 24 h at 105°. The hydrolyzate was made neutral with barium carbonate, filtered, and the filtrate decationized by stirring with Dowex 50 (H^+) resin, and evaporated. In one experiment, the product after hydrolysis of the formyl groups was suspended in water, centrifuged, and the precipitate hydrolyzed by heating with 0.5M sulfuric acid for 3 h at 100°. After this time, most of the product (77 %) remained insoluble and was separated from the soluble sugars by centrifugation.

Methylation analysis. — The polysaccharide (12 mg) was methylated by the Hakomori method[9] as described[15]. The methylated product was soaked in 72 % sulfuric acid (1 mL) with external cooling. The solution was kept for 2 h at room temperature, the acid was diluted to 12 %, and the solution was then heated for 4 h at 100 °.

The solution was made neutral with barium carbonate and the methylated sugars in the hydrolyzate were converted into their alditol acetates as already described[4]. Analysis was performed by g.l.c. with columns *C* and *D*. The identity of the alditol acetates was confirmed by g.l.c.–m.s.

Assay of antitumor activity. — Two-month-old, BALB/C mice of both sexes were used for the antitumor assay. Sarcoma 180 ascites (2 × 10^6 cells), maintained by serial passage in mice, were transplanted subcutaneously into the right groin

of the mice. The test polysaccharide dissolved in distilled water (1 mg/mL) and sterilized, for 20 min at 120° was injected intraperitoneally at a dose of 5 mg/kg daily for 5 days, starting 24 h after tumor implantation. Tumor growth was measured by use of a caliper applied to the surface of the animal. Fifteen mice and the same number of control animals were used for the experiment.

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