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ISOLATION AND CHARACTERIZATION OF AN ANTITUMOR ACTIVE AGAR-TYPE POLYSACCHARIDE OF *Gracilaria dominguensis*

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ABSTRACT

Cold water extraction of the red alga *Gracilaria dominguensis*, followed by cetyltrimethylammonium bromide fractionation, gave a highly sulfated, agar-type polysaccharide which inhibited the transplantation of Ehrlich ascites carcinoma in mice. The structure of the polysaccharide has been investigated by methylation analysis, and ^{1}H- and ^{13}C-n.m.r. spectroscopy, and was shown to be mainly composed of alternating (1→3)-linked β-D-galactopyranosyl 6-sulfate and (1→4)-linked 3,6-anhydro-α-L-galactopyranosyl residues.

INTRODUCTION

Antitumor activity has been demonstrated for a large variety of polysaccharides derived from natural sources[1]. Certain seaweeds have long been used in traditional Chinese herbal medicine in the treatment of cancer.

The brown algae, *Sargassum fulvellum*[2,3], *Sargassum kjellmanianun, Laminaria augustata, Laminaria japonica, Eklonia cava, Eisenia bicyclis*[4], and *Laminaria religiosa*[5], afforded polysaccharide fractions containing sodium alginate or sulfated fucoidans which showed antitumor activity either against sarcoma 180 or against L-1210 leukemia in mice. However, the antitumor activity of red seaweed galactans has been studied to a lesser extent. Carrageenan from *Gloiopeltis furcata* was effective against sarcoma 180 solid tumor[6]. Such polysaccharides were observed to enhance the host's defense mechanism against neoplasia in certain cases by increase of natural killer-cell activity and of cellular immunity associated with T cells[4].

We report herein the structural characterization and antitumor activity of CT-1, a cold, water-soluble agar isolated from *Gracilaria dominguensis*.

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RESULTS AND DISCUSSION

Three successive cold-water extractions of air-dried *Gracilaria dominguensis* gave a polysaccharide fraction in a total yield of 11.6%. Further fractionation with cetyltrimethylammonium bromide yielded acidic polysaccharide CT-1 (5.1%). CT-1 was examined for homogeneity by gel-filtration chromatography in Ultrogel AcA-22 and ultracentrifugation. The elution profile showed only one peak, and the polysaccharide gave a single and symmetrical peak on ultracentrifugation, from which it was concluded that CT-1 is an essentially homogeneous polymer. The molecular weight of CT-1 was estimated by sedimentation analysis to be 1.25×10^5.

Acid hydrolysis of the polysaccharide and paper chromatography of the hydrolyzate revealed the presence of galactose and 6-*O*-methylgalactose or xylose (or a mixture of both). 5-Hydroxymethyl-2-furaldehyde was also detected by ^{1}H- and ^{13}C-n.m.r. spectroscopy, and it is presumed to be a decomposition product of 3,6-anhydrogalactosyl residues.

Chemical data obtained on the polysaccharide CT-1 are given in Table I. Constituent sugars were quantitatively determined by g.l.c.–m.s. of their alditol acetates. 3,6-Anhydrogalactosyl residues were estimated by the method of Matsuhiro and Zanlungo[7]. The values for the monosaccharide residues in Table I are expressed as molar percentages of total carbohydrate present. Protein and sulfate contents are given as mass percentages. Comparison of the molar percentages of D-galactose and 6-*O*-methyl-D-galactose with those of 3,6-anhydro-L-galactose and 4-*O*-methyl-L-galactose[8] showed that the molar ratio of D to L residues is ~1:1, which is in the range found for galactan sulfates.

The i.r. spectrum of the sulfated galactan exhibited the characteristic, broad band absorption for sulfate groups at 1240 cm^{-1}, and a moderate band at 820 cm^{-1}, indicating the presence of sulfated primary hydroxyl groups. In addition, the i.r. spectrum showed a weak band at 940 cm^{-1} which has been previously assigned to the presence of 3,6-anhydrogalactosyl residues[9].

Combination of chemical degradation techniques with ^{13}C-n.m.r. spectros-

TABLE I

CHEMICAL COMPOSITION OF CT-1[a]

D-*Galactose*	*6-O-Methyl-galactose*	*4-O-Methyl-galactose*	*Xylose*
41.4	5.6	1.6	3.0
Mannose	*3,6-Anhydro-galactose*	*% Protein*	*%SO_3Na*
0.8	47.6	3.0	15.4

[a]All monosaccharide residues are expressed as molar percentages.

TABLE II

^{13}C-N.M.R. CHEMICAL SHIFTS OF AGAROSE AND CT-1

Polysaccharide	*Residue*	*C-1*	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*
Agarose[a]	D-Gal	102.4	70.2	82.2	68.4	75.3	61.4
	3,6-Anh-L-Gal	98.3	69.8	80.1	77.3	75.6	69.4
CT-1	D-Gal-6*S*	102.5	70.1	82.1	68.4	72.9	67.4
	D-Gal	102.4	70.1	82.1	68.4	75.6	61.5
	3,6-Anh-L-Gal	98.3	69.8	80.1	77.8	75.6	69.4

[a]Signals assigned according to refs. 10, 11, and 12.

copy gave the main information concerning the sulfated galactan structure. ^{13}C-N.m.r. chemical shifts of CT-1 and agarose are shown in Table II. In the ^{13}C-n.m.r. spectrum of native CT-1, two prominent anomeric signals were observed at δ 102.5 and 98.3, corresponding to both anomeric carbon atoms in the disaccharide repeating units of the polysaccharide in which 3-*O*-substituted D-galactopyranosyl 6-sulfate residues alternate with 4-*O*-substituted 3,6-anhydro-L-galactopyranosyl residues[10]. The high degree of sulfation at C-6 of 3-*O*-substituted β-D-galactopyranosyl residues is indicated not only by the very low intensity of the signal at δ 61.4, but also by the signals at δ 67.4 (C-6) and 72.9 (C-5), the former being assigned through an APT experiment[13]. Another C-1 signal was observed at δ 102.4, which together with that at δ 98.3 was indicative of unsulfated (1→3)-linked β-D-galactopyranose residues alternating with (1→4)-linked 3,6-anhydro-α-L-galactopyranose residues[14] (see Table II).

The ^{13}C-n.m.r. spectrum of the polysaccharide isolated after hot, aqueous sodium hydroxide–sodium borohydride treatment of CT-1 was identical with that of the native biopolymer. This result was expected as sulfate groups at C-6 of (1→3)-linked β-D-galactopyranosyl residues are stable under alkaline conditions[15]. Furthermore, the ^{13}C-n.m.r. spectrum of the polysaccharide isolated after solvolytic desulfation[16] of CT-1 was identical with that of agarose. From the ^{13}C-n.m.r. study of CT-1 and its derivatives, it could be concluded that the sulfated galactan belongs to the "agar" type and also that the main disaccharide component is composed of a (1→3)-linked β-D-galactopyranose 6-sulfate alternating with a (1→4)-linked 3,6-anhydro-α-L-galactopyranose residue.

Additional valuable information about the structure of CT-1 was obtained by ^{1}H-n.m.r. spectroscopy. The spectrum of the partial hydrolyzate of native CT-1 was similar to that of agarose[17]. The presence of galactosyl 6-sulfate residues was confirmed by the intense doublet at δ 4.2, and the lack of a pyruvic acid ketal substituent was also confirmed by the absence of the corresponding singlet signal at δ 1.56. The weak methoxy signal observed at δ 3.4 indicated that the content of 6-*O*-methylgalactosyl residues in CT-1 was very low, confirming the g.l.c.–m.s. results (see Table I).

CT-1 was acetylated and then methylated by the Hakomori procedure[18],

TABLE III

G.L.C.–M.S. DATA FOR THE METHYLATED ALDITOL ACETATES

Component (as alditol acetate)	*Mol-percent*
2,3,4-Me_3Xyl	2.8
2,3,4,6-Me_4Gal	0.2
3,6-Anhydro-2-MeGal	47.6
2,4,6-Me_3Gal	16.1
2,4-Me_2Gal	26.7
2,6-Me_2Gal	4.7
3,6-Me_2Gal	0.8
4,6-Me_2Gal	0.9

followed by a modified Haworth procedure[19]. Complete methylation was assumed[20] to be achieved because the hydrolyzate of the permethylated polysaccharide was free from galactose and mono-*O*-methylgalactoses. Permethylated CT-1 was hydrolyzed by heating at 100° in 90% formic acid, followed by 0.1M sulfuric acid. The results of g.l.c.–m.s. analysis of the alditol acetates of the sugars in the hydrolyzate are given in Table III. Because 3,6-anhydro-2-*O*-methyl-L-galactose is degraded during acid hydrolysis, the mol-per cent value in Table III was corrected by use of the mol-per cent value of 3,6-anhydro-L-galactose in the native, unmethylated polysaccharide. 1,4,5-Tri-*O*-acetyl-3,6-anhydro-2-*O*-methylgalactitol was detected in the hydrolyzate of methylated CT-1. All of the mol-per cent values were then corrected accordingly.

The large proportion of 2,4-di-*O*-methylgalactose in the hydrolyzate of the methylated polysaccharide confirmed that an important part of the D-galactopyranosyl residues are linked through O-3 and are sulfated at C-6 in the structure of CT-1. The presence of 2,4,6-tri-*O*-methylgalactose in the hydrolyzate proved that D-galactosyl, 6-*O*-methylgalactosyl, and 4-*O*-methylgalactosyl residues are linked at O-3 in the native polysaccharide. The unexpected large proportion of this derivative may be attributed to desulfation under the methylation conditions employed.

Another component of the hydrolyzate was identified as 2,6-di-*O*-methylgalactose. A possible source might be a (1→3)-linked D-galactose residue having a sulfate group or a monosaccharide type subsituent at C-4, or both. 2,3,4-Tri-*O*-methylxylose was a minor component of the hydrolyzate, showing that xylopyranosyl residues are present as nonreducing end-groups, probably linked to the backbone of the polysaccharide as xylosyl substituents. The 1,5-diacetate of 2,3,4,6-tetra-*O*-methylgalactose was another component found in the hydrolyzate, and it originated from terminal nonreducing galactosyl groups. Its corrected molar percentage was 0.2, suggesting that if all the nonreducing end-groups in the methylated polysaccharide were 2,3,4,6-tetra-*O*-methylgalactose and assuming that little branching occurs, then the number-average degree of polymerization may be ~600, which agrees with the mol.wt. of CT-1.

TABLE IV

ANTITUMOR ACTIVITY OF SULFATED GALACTAN FROM *Gracilaria dominguensis*[a]

Sample	*Dose (mg/kg)*	*Tumor free animals*	*Survival (%)*
CT-1	15	3/10	30
	30	3/10	30
	60	9/10	90
Control		0/20	0

[a]See Experimental section for details of the assay.

Two other minor components of the hydrolyzate were 3,6- and 4,6-di-*O*-methylgalactose. A possible source of the former could be (1→4)-linked L-galactosyl residues with a sulfate group, a monosaccharide-type substituent, or both at C-2. The latter might be associated with the presence of (1→3)-linked D-galactosyl residues having the same substitution pattern as the former.

These methylation results showed that the polysaccharide is composed of approximately equal amounts of (1→3)- and (1→4)-linked units. Also methylation data were in good agreement with the ^{1}H- and ^{13}C-n.m.r. structural study of CT-1.

The antitumor activity of CT-1 against the Ehrlich ascites carcinoma in mice is shown in Table IV. In the untreated group, the mean-survival time of the animals was 20 days, whereas the tumor-free animals belonging to the group treated with a dose of 60 mg/Kg of CT-1 were alive over 30 days. Experiments showed that CT-1 has no cytocidal effect on the *in vitro* growth of Ehrlich ascites carcinoma cells. These results suggested that the effect of CT-1 might be mainly host-mediated.

EXPERIMENTAL

Isolation and purification of CT-1. — The alga *Gracilaria dominguensis* was collected on the north coast of Cuba, close to Havana. It was carefully hand-sorted prior to air drying at 35°. The seaweed was then milled, exhaustively washed with ethanol at room temperature, and the remaining powder dried at 40°. This (300 g) was extracted with cold distilled water (3 L) for 6 h, the insoluble material was removed on a cloth filter, and the filtrate centrifuged at 3000 r.p.m. for 20 min. The supernatant solution was poured into ethanol (15 L), and the precipitate removed by centrifugation, redissolved in water (1 L), and then dialyzed against distilled water (6 × 20 L). Lyophilization of the dialyzate yielded the cold-water polysaccharide fraction. The insoluble material was successively reextracted twice with cold water in the same way to yield the total polysaccharide fraction (34.8 g, 11.6%). It was fractionated with cetyltrimethylammonium bromide by the procedure described by Scott[20]. Yield of acidic polysaccharide CT-1 was 15.3 g (5.1%).

Materials and methods. — Standard agarose was purchased from Koch–

Light Lab. I.r. spectra were recorded with a Pye Unicam PU 9512 spectrophotometer for KBr disks. ^{13}C-N.m.r. spectra were recorded with a Jeol FX 90Q spectrometer operating at 22.53 MHz on solutions (100 mg/mL) in D_2O at 80° and with dimethyl sulfoxide as internal reference (δ 39.45). ^{1}H-N.m.r. spectra were recorded with the same instrument at 90 MHz on samples previously hydrolyzed according to Izumi[17], and with sodium 2,2-dimethylpentane-5-sulfonate as internal reference. G.l.c.–mass spectra were recorded with a Jeol DX-300 mass spectrometer, equipped with a bonded-phase OV-17 capillary column (0.21 mm × 25 m); the carrier gas was He with a flow of 0.8 mL/min and the column temperature was programmed from 100 to 300° at 8°/min for alditol acetates and at 4°/min for partly *O*-methylated alditol acetates. The method of Matsuhiro and Zanlungo[7] was used to determine 3,6-anhydrogalactose. Protein content was determined by the method of Lowry *et al.*[21], and that of sulfate by the procedure of Dodgson and Price[22]. Whatman No. 1 paper and the solvent system 6:3:3 butanol–pyridine–water were used for paper chromatography by the descending method. Sugars were detected with the $AgNO_3$–NaOH reagent[23] and the aniline phthalate reagent spray (Merck).

Gel-filtration chromatography and sedimentation analysis of CT-1. — A solution of CT-1 (5 mg) in phosphate saline buffer (pH 7, 0.5 mL) was applied to a column (2.5 × 40 cm) of Ultrogel AcA-22 (LKB) and eluted with the same buffer at 48 mL/h. Fractions (2 mL) were monitored on the basis of the phenol–H_2SO_4 reaction[24]. Sedimentation analysis was performed according to procedures described earlier[25,26].

Treatment of CT-1 with alkali. — The polysaccharide (0.5 g) in water (25 mL) was treated with hot aqueous $NaBH_4$ and NaOH as previously described[27]. The contents of sulfate and 3,6-anhydrogalactose in the alkali-treated polysaccharide (0.38 g) were identical with that of the native CT-1.

Acetylation of CT-1. — The polysaccharide (0.5 g) was dissolved in formamide (50 mL), dry pyridine (37 mL), and acetic anhydride (75 mL), and isolated as previously described[27] (yield 0.65 g).

Desulfation of the polysaccharide. — The polysaccharide (1 g) was dissolved in water (200 mL) and Amberlite IR-120 (H^+) cation-exchange resin was added until pH 2. The resin was removed by filtration, the acidity of the filtrate neutralized with pyridine, and the pyridinium salt of the polysaccharide isolated by freeze-drying (0.9 g). This was dissolved in dry dimethyl sulfoxide (50 mL) and pyridine (1 mL), and desulfated according to the procedure of Usov *et al.*[16]. The sulfate content was 0%.

Methylation analysis of acetate of CT-1. — The polysaccharide acetate (0.6 g) was methylated by the Hakomori procedure[18]. The material was further remethylated by a modification of the Haworth procedure[19] to give the fully *O*-methylated polysaccharide (0.2 g). The methylated material was hydrolyzed with 90% formic acid for 1 h at 100°, followed by 0.1M H_2SO_4 for 16 h at 100°. The identity of the hydrolysis products was determined by g.l.c.–m.s.

Assay of CT-1 for antitumor activity. — The activity of CT-1 was assayed by

the following procedure: A solution of CT-1 in normal saline solution was injected intraperitoneally (15–60 mg/kg daily for 5 days) into IBF, male mice (20–23 g). After 48 h, seven-day-old Ehrlich ascites carcinoma cells (2×10^6 cells) were implanted intraperitoneally into the mice. After 4 weeks, the control and treated groups were compared for survival and tumor-free animals.

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REFERENCES

1 R. L. WHISTLER, A. A. BUSHWAY, P. P. SINGH, W. NAKAHARA, AND R. TOKUZEN, *Adv. Carbohydr. Chem. Biochem.*, 32 (1976) 235–275.
2 I. YAMAMOTO, T. NAGUNO, M. FUJIHARA, M. TAKANASHI, Y. ANDO, M. OKADA, AND K. KAWAI, *Jpn. J. Exp. Med.*, 47 (1977) 137–140.
3 M. FUJIHARA, N. IIZIMA, I. YAMAMOTO, AND T. NAGUNO, *Carbohydr. Res.*, 125 (1984) 97–106.
4 I. YAMAMOTO, M. TAKAHASHI, E. TAMURA, H. MARUYAMA, AND H. MORI, *Hydrobiologia*, 116/117 (1984) 145–149.
5 H. MARUYAMA AND I. YAMAMOTO, *Hydrobiologia*, 116/117 (1984) 534–536.
6 T. NISHIZAWA, *Marine Algae in Pharmaceutical Sciences*, Vol. 3, Walter de Gruyter, Berlin, 1979, pp. 243–264.
7 B. MATSUHIRO AND A. ZANLUNGO, *Carbohydr. Res.*, 118 (1983) 276–279.
8 A. J. R. ALLSOBROOK, J. R. NUNN, AND H. PAROLIS, *Carbohydr. Res.*, 36 (1974) 139–145.
9 D. J. STANCIOFF AND N. P. STANLEY, *Proc. Int. Seaweed Symp., 6th*, 1969, 545–609.
10 A. I. USOV, E. G. IVANOVA, AND A. S. SHASHKOV, *Bot. Mar.*, 26 (1983) 285–294.
11 F. M. NICOLAISEN, I. MEYLAND, AND K. SCHAUMBURG, *Acta Chem. Scand., Ser. B*, 34 (1980) 103–107.
12 A. I. USOV, S. V. YAROTSKY, AND A. S. SHASHKOV, *Biopolymers*, 19 (1980) 977–990.
13 S. L. PATT AND J. N. SHOOLERY, *J. Magn. Reson.*, 46 (1982) 535–539.
14 A. I. USOV, *Bot. Mar.*, 27 (1984) 189–202.
15 E. PERCIVAL, *Methods Carbohydr. Chem.*, ((1980) 281–285.
16 A. I. USOV, K. ADAMYANTS, AND K. KOCHETKOV, *Carbohydr. Res.*, 18 (1971) 336–338.
17 K. IZUMI, *Biochim. Biophys. Acta*, 320 (1973) 311–317.
18 S.-I. HAKOMORI, *J. Biochem. (Tokyo)*, 55 (1964) 205–208.
19 T. C. S. DOLAN AND D. A. REES, *J. Chem. Soc.*, (1963) 3534–3539.
20 J. E. SCOTT, *Methods Carbohydr. Chem.*, 5 (1965) 38.
21 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR, AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265–275.
22 K. S. DODGSON AND R. G. PRICE, *Biochem. J.*, 84 (1962) 106–110.
23 W. E. TREVELYAN, D. P. PROCTER, AND J. S. HARRISON, *Nature (London)*, 166 (1950) 444–445.
24 J. E. HODGE AND B. T. HOFREITER, *Methods Carbohydr. Chem.*, 1 (1962) 380–394.
25 W. J. ARCHIBALD, *J. Phys. Coll. Chem.*, 51 (1947) 1205.
26 M. S. SCHACHMAN, *Ultracentrifugation in Biochemistry*, Academic Press, New York, 1959.
27 D. J. BRASCH, H. M. CHANG, C. T. CHUAH, AND L. D. MELTON, *Carbohydr. Res.*, 97 (1981) 113–125.