Carbohydrate Research, 114 (1983) 164–168
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

Note

An antitumor polysaccharide produced by *Microellobosporia grisea*: preparation, general characterization, and antitumor activity

KAZUHIRO INOUE, HIROTO NAKAJIMA, MORIHIRO KOHNO, MASAYUKI OHSHIMA, SHIZUO KADOYA,
Research Institute of Daiichi Seiyaku Co., Ltd., Kitakasai, Edogawa-ku, Tokyo 134 (Japan)

KUNIO TAKAHASHI,

Department of Pharmacognosy and Phytochemistry, Meiji College of Pharmacy, Nozawa, Setagaya-ku, Tokyo 154 (Japan)

AND SHIGERU ABE

Faculty of Pharmaceutical Sciences, Teikyo University, Sagamiko-cho, Tsukui-gun, Kanagawa 199-01 (Japan)

(Received August 9th, 1982; accepted for publication, August 30th, 1982)

Antitumor polysaccharides have been obtained from various sources[1], such as bacteria, fungi, yeasts, lichens, and higher plants. In addition, an extract from the mycelia of an actinomycete, *Microellobosporia grisea*, has been shown[2] to exhibit potent antitumor activity by some routes, including oral administration, and the material was proved to be a polysaccharide composed of glucose and mannose in the approximate molar ratio of 2:1, and containing a cell-wall peptidoglycan, but there is no report that actinomycetes produce extracellular polysaccharides having antitumor activity.

We now describe the preparation, general characterization, and antitumor activity of an extracellular polysaccharide from *Microellobosporia grisea*.

The actinomycete was cultured in a jar fermentor, and the polysaccharide was isolated from the culture filtrate and purified successfully with cetylpyridinium borate[3]. The purified polysaccharide had $[\alpha]_D$ +65° (*c* 0.5, water), and its elemental analysis indicated $C_{10}H_{18}O_9$. Its carbohydrate and protein contents were 115% as glucose, and 0.4% as bovine serum albumin, respectively, and it showed no characteristic absorption in its u.v. spectrum. It also contained no appreciable amount of phosphorus, and no detectable amount of cell-wall peptidoglycan. After acid hydrolysis of the polysaccharide, the sugar composition was analyzed by t.l.c. and g.l.c. T.l.c. revealed the presence of glucose and mannose, and the molar ratio of glucose to mannose was determined to be 1.14:1.00 by g.l.c. analysis. The glucose content of the polysaccharide was also determined, with the glucostat reagent, to be 52%, which is in good agreement with the value (53%) obtained by g.l.c. analysis, and it indicated that the

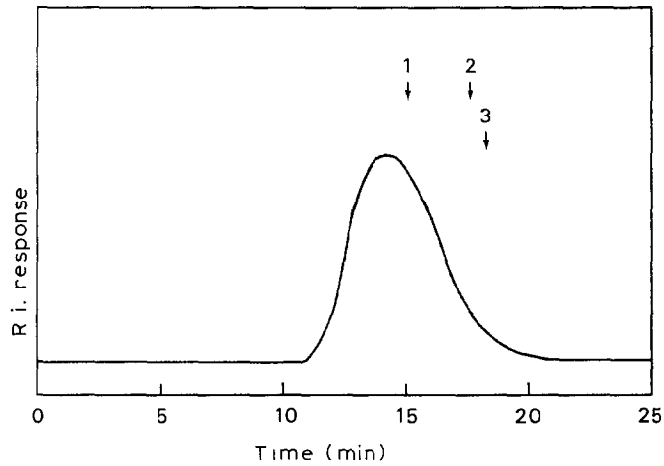

Fig. 1. Gel-permeation chromatography of the polysaccharide on G5000 PW, with 0.1M potassium acetate buffer (pH 6.5) as the carrier (1.0 mL/min). [Arrows indicate the elution positions of the following standard dextrans; 1 (T-500), 2 (T-70), and 3 (T-40).]

glucose was the D enantiomer. Mannose was also identified as the D enantiomer because, after complete hydrolysis of the polysaccharide with acid, the hydrolyzate showed $[\alpha]_D$ +32°, the theoretical value being +35° for a 1.14:1.00 molar ratio of D-glucose to D-mannose.

The molecular weight of the polysaccharide was estimated to be $\sim 1 \times 10^6$ by gel-permeation chromatography on G5000 PW with dextran as the standard (see Fig. 1). The polysaccharide gave an i.r. spectrum typical of neutral polysaccharides, the spectrum showing absorption bands at 3500–3300, 2920, 1100–1000, 880, and 810 cm^{-1}. The unequivocal bands at 880 and 810 cm^{-1} suggested the presence of

TABLE I

ANTITUMOR EFFECT OF THE POLYSACCHARIDE ON EHRLICH CARCINOMA SOLID TUMOR[a]

Group	*Route*	*Dose (mg/kg × days)*	*Tumor weight (mean ±SE) (g)*	*T/C[b] (%)*	*Complete regression[c]*
Treated	i.p.[d]	10 × 10	0.17 ±0.16**[e]	3.2	3/5
		30 × 10	0.60 ±0.31**	11.2	2/5
Control			5.35 ±0.70	100	0/5
Treated	p.o.[f]	50 × 10	3.74 ±0.67	85.0	0/7
Control			4.40 ±0.46	100	0/12

[a]ICR mice were inoculated subcutaneously with Ehrlich carcinoma cells on day 0. [b]Average tumor-weight of treated group/that of the control group. [c]No. of tumor-free mice/no. of mice tested. [d]Intraperitoneally administered daily from day 1. [e]Significant difference from control group (*$p < 0.01$, **$p < 0.001$). [f]Orally administered every other day from day 1.

TABLE II

ANTITUMOR EFFECT OF THE POLYSACCHARIDE ON MM46 ADENOCARCINOMA SOLID TUMOR[a]

Group	*Route*	*Dose (mg/kg days)*		*Tumor weight (mean ± SE) (g)*		*T/C[b] (%)*	*Complete regression[c]*
Treated	i.p.[d]	10	10	0.07	0.05*[e]	7.0	4/8
		30	10	0.06	0.02*	6.0	3/8
Control				1.00	0.18	100	0/7
Treated	p.o.[f]	100	16	0.80	0.17	79.2	0/8
Control				1.01	0.09	100	0/11

[a]C3H/He mice were inoculated subcutaneously with MM46 tumor cells on day 0. [b,c,d,e]See footnotes to Table I. [f]Orally administered daily from day 5.

β-glucosidic and α-mannosidic linkages, respectively, based on the literature[4]. Broad absorption observed at 1640 cm^{-1} was due to bound water[5], which persisted even after the most extensive drying over phosphorus pentaoxide *in vacuo*.

These results indicate that the polysaccharide is a mannoglucan probably having α-mannosidic and β-glucosidic linkages.

The polysaccharide was tested for antitumor activity against mouse allogeneic and syngeneic tumors, solid form, by intraperitoneal administration of 10 and 30 mg/kg for 10 days. As shown in Tables I and II, the polysaccharide exhibited potent antitumor activity against both Ehrlich carcinoma and MM46 syngeneic adenocarcinoma, with high rates of complete regression (3/5 and 4/8 at 10 mg/kg dose, respectively). There was no significant difference between the antitumor effects on these tumors at doses of 10 and 30 mg/kg. In contrast to intraperitoneal administration, however, its oral administration showed no effect on these tumors. The polysaccharide also significantly inhibited the growth of other syngeneic tumors, such as intradermally transplanted Meth A in BALB/c mice (unpublished result).

The mechanism of the antitumor effect exhibited by this polysaccharide has not yet been elucidated, but the results of preliminary studies suggest that the polysaccharide shows the antitumor activity through stimulation of host-mediated response.

EXPERIMENTAL

General methods. — Thin-layer chromatography (t.l.c.) was performed on Kieselgel $60F_{254}$ by the ascending method, using 5:3:1 (v/v) 1-butanol–2-propanol–water. Sugars on thin-layer chromatograms were detected with ammoniacal silver nitrate, or *p*-anisaldehyde–sulfuric acid[6]. Carbohydrate was determined by the phenol–sulfuric acid method[7], protein by the method of Lowry *et al.*[8], and phosphorus by the method of Chen *et al.*[9]. A glucostat reagent (Worthington Biochemical

Co.) was used for assay of D-glucose. Optical rotations were measured with a Perkin–Elmer model 141 polarimeter, and infrared (i.r.) spectra were recorded for KBr disks with a Hitachi model 285 infrared spectrophotometer, and ultraviolet (u.v.) spectra with a Hitachi model 124 spectrophotometer.

Micro-organism. — *Microellobosporia grisea* IFO 12518 was obtained from the Institute for Fermentation, Osaka. The strain was maintained for 3–5 days at 28° on slants of GC agar (D-glucose 2%, peptone 0.5%, corn-steep-liquor 0.5%, yeast extract 0.3%, NaCl 0.5%, $CaCO_3$ 0.3%, and agar 1.5%; pH 7.0), and subcultured every 3–4 weeks.

Media, and cultivation. — Seed culture was prepared as follows. One piece of agar from the GC slant was inoculated into a 500-mL flask containing 100 mL of the GC medium, and incubated for 5 days at 28° on a rotary shaker. This culture (2 mL) was again inoculated and incubated for 3 days, as just described.

The cultivation for production of polysaccharide was conducted in a 30-L, jar fermentor. The seed culture (200 mL) was transferred to the jar fermentor containing 20 L of a production medium (glucose 3%, and corn steep-liquor 2%; pH 7.2), and cultured for 92 h at 28° under aeration (10 L/min) and agitation (250 r.p.m.). During the cultivation, Adecanol LG 805 (Asahi Electro-Chemical Co., Ltd.) was used as an antifoaming agent.

Preparation of polysaccharide. — The culture broth was heated for 20 min at 80°, cooled to room temperature, and filtered to remove the cells and other insoluble materials. The filtrate (~20 L) was passed through a column of Diaion PA306 (Cl^-), in order to remove such polyanionic compounds as nucleic acids and phosphorylated polysaccharides. To the effluent were added 10% cetylpyridinium chloride (0.5 L) and 0.5M sodium borate buffer, pH 10 (1.0 L). The resulting cetylpyridinium borate complex was collected, washed with water, and then dissolved in 2% acetic acid (2.0 L). The polysaccharide, which was precipitated from the acidic solution with ethanol (6.0 L), was dissolved in 0.02% sodium acetate (3.0 L), centrifuged at 13,200*g* for 5 min, and reprecipitated with ethanol (9.0 L) from the supernatant liquor. After washing successively with 75% ethanol, ethanol, and acetone, the purified polysaccharide was dried over phosphorus pentaoxide *in vacuo* for 8 h at 50° (yield, 36 g).

Polysaccharide hydrolysis, and analysis. — The polysaccharide was hydrolyzed in 0.5M sulfuric acid for 6 h at 100° in a sealed ampoule. The hydrolyzate was made neutral with Dowex 1 (CO_3^{2-}) resin, and examined by t.l.c. and g.l.c. G.l.c. was performed with a Hitachi model 163 gas chromatograph equipped with a flame-ionization detector. For quantitative analysis, D-xylose was used as the internal standard. Sugars were separated as the alditol acetates[10] in a glass column (0.3 × 200 cm) packed with 3% of OV-225 on Gas Chrom Q. Nitrogen was used as the carrier gas at a flow rate of 60 mL/min, and the column temperature was kept at 190°.

Estimation of molecular weight. — The molecular weight of the polysaccharide was estimated by gel-permeation chromatography (g.p.c.), with dextran T-500,

T-70, and T-40 (Pharmacia Fine Chem. Co.) as standards. G.p.c. was conducted in a Toyo Soda model 803D liquid chromatograph equipped with a refractive index detector. A G5000 PW column (0.75 × 60 cm) was eluted with 0.1M potassium acetate buffer, pH 6.5, at a flow rate of 1.0 mL/min at 40°.

Assay of antitumor activity. – Ehrlich carcinoma and MM46 adenocarcinoma[11] were maintained as an ascites form by weekly transplantation in ICR-JCL and C3H/He mice, respectively. Seven-day-old Ehrlich carcinoma (3×10^6 cells) was implanted subcutaneously into the right groin of female, 7-week-old, ICR-JCL mice. The polysaccharide sample, dissolved in physiological saline, was injected intraperitoneally, daily for 10 days, starting 24 h after the tumor implantation. On day 30 after the implantation, the mice were killed, and the tumors were extirpated and weighed. The antitumor effect was evaluated by comparing the average tumor-weight of the treated group with that of the control group. The significance of differences in tumor weights was estimated by Student's t-test.

In another antitumor experiment, MM46 adenocarcinoma (4×10^6 cells) was implanted subcutaneously into the right groin of male, 7-week-old, C3H/He mice. The sample was administered intraperitoneally, and the antitumor effect was evaluated as already described.

ACKNOWLEDGMENTS

We are grateful to Profs. S. Shibata and D. Mizuno for their valuable advice on chemical and biological aspects of this work. We also thank Mr. K. Nakatsuka, Mr. N. Higashihashi, Mr. K. Kawamoto, and Mr. S. Hashimoto for their valuable assistance.

REFERENCES

1 R. L. Whistler, A. A. Bushway, P. P. Singh, W. Nakahara, and R. Tokuzen, *Adv. Carbohydr. Chem. Biochem.*, 32 (1976) 235–294.
2 M. Kohno, S. Abe, M. Yamazaki, and D. Mizuno, *Gann*, 73 (1982) 488–494.
3 J. E. Scott, *Methods Carbohydr. Chem.*, 5 (1965) 38–44.
4 H Spedding, *Adv. Carbohydr. Chem.*, 19 (1964) 23–49; W. B. Neely, *ibid.*, 12 (1957) 13–33; S. A. Barker, E. J. Bourne, and D. H. Whiffen, *Methods Biochem. Anal.*, 3 (1956) 213–245.
5 F. S. Parker, *Applications of I.R. Spectroscopy in Biochemistry, Biology and Medicine*, Plenum Press, New York, 1971, pp. 100–140.
6 E. Stahl and U. Kaltenbach, *J. Chromatogr.*, 5 (1961) 351–355.
7 M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith, *Anal. Chem.*, 28 (1956) 350–356.
8 O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, 193 (1951) 265–275.
9 P. S. Chen, T. Y. Toribara, and H. Warner, *Anal. Chem.*, 28 (1956) 1756–1758.
10 J. S. Sawardeker, J. H. Sloneker, and A. Jeanes, *Anal. Chem.*, 37 (1965) 1602–1604.
11 R. F. Irie, *Cancer Res.*, 31 (1971) 1682–1689.