Carbohydrate Research, 189 (1989) 273–279
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

(1→3)-α-D-GLUCAN FROM AN ALKALINE EXTRACT OF *Agrocybe cylindracea*, AND ANTITUMOR ACTIVITY OF ITS *O*-(CARBOXYMETHYL)ATED DERIVATIVES*

TADASHI KIHO, ISAO YOSHIDA, KATSUYUKI NAGAI, SHIGEO UKAI†,
Gifu Pharmaceutical University, Mitahora-higashi, Gifu 502 (Japan)

AND CHIHIRO HARA
Shotoku Gakuen Women's Junior College, Nakauzura, Gifu 500 (Japan)

(Received June 1st, 1988; accepted for publication, November 15th, 1988)

ABSTRACT

The structure of an alkali-soluble D-glucan (AG-AL) from the fruit body of *Agrocybe cylindracea* was investigated by a combination of chemical and spectroscopic methods indicating that it was a linear (1→3)-α-D-glucan (molecular weight, ~560,000), $[\alpha]_D^{20}$ +195° (*c* 0.5, M sodium hydroxide). Both water-soluble and gelatinous products obtained by *O*-(carboxymethyl)ation of AG-AL showed potent antitumor activity against the solid form of Sarcoma 180 in mice, although the native D-glucan had little effect on the tumor.

INTRODUCTION

Agrocybe cylindracea (Fr.) Maire (*Bolbitiaceae*) is an edible mushroom, and the fruiting body can be obtained by cultivation of the fungus. We have isolated a water-insoluble α-D-glucan (AG-AL) from an alkaline extract of the fruiting body, and prepared water-soluble AG-AL-CMS and gelatinous AG-AL-CMI as *O*-(carboxymethyl)ated products from AG-AL. (1→3)-β-D-Glucans have been chemically modified, *e.g.* (carboxymethyl)ated[2], to raise antitumor activity of the glucans, but, to the best of our knowledge, carboxymethylation of (1→3)-α-D-glucan has not been reported. We now describe the structural characterization of AG-AL, and the antitumor activity of AG-AL, AG-AL-CMS, and AG-AL-CMI, where CM denotes carboxymethyl, S means "water-soluble", and I, "water-insoluble or gelatinous".

*Polysaccharides in Fungi, Part XXIII. For Part XXII, see ref. 1.
†To whom inquiries should be addressed.

0008-6215/89/$ 03.50

RESULTS AND DISCUSSION

A water-insoluble glucan (AG-AL) obtained from an alkaline extract of the fruiting bodies of *Agrocybe cylindracea* showed a symmetrical peak on gel filtration in M sodium hydroxide. AG-AL contained no nitrogen (by elementary analysis), and the total hexose content was found to be 98% (by the phenol–sulfuric acid method[3]). On hydrolysis, AG-AL gave glucose, as shown by paper chromatography (p.c.) of the hydrolyzate, and gas–liquid chromatography (g.l.c.) of the alditol acetate prepared from the hydrolyzate. The glucan was insoluble in water, and soluble in M sodium hydroxide, and had a high, positive specific rotation, $[\alpha]_D^{20}$ +195° (*c* 0.5, M sodium hydroxide). The glucan showed characteristic absorbance at 925 and 850 cm^{-1} in the infrared (i.r.) spectrum, indicating the presence of the α-D-configuration[4]. The calibration curve shown in Fig. 1 was made by gel filtration of standard dextrans on Toyopearl HW-65 with M sodium hydroxide; the molecular weight of AG-AL was estimated to be ~560,000.

Methylation analysis of AG-AL by the Hakomori method[5] gave 2,4,6-tri-*O*-methyl-D-glucose and a trace of 2,3,4,6-tetra-*O*-methyl-D-glucose (molar percent, 98.3:1.7), as confirmed by g.l.c. and g.l.c.–mass spectrometry of their alditol acetates. The results indicated that the D-glucopyranosyl residues in AG-AL are

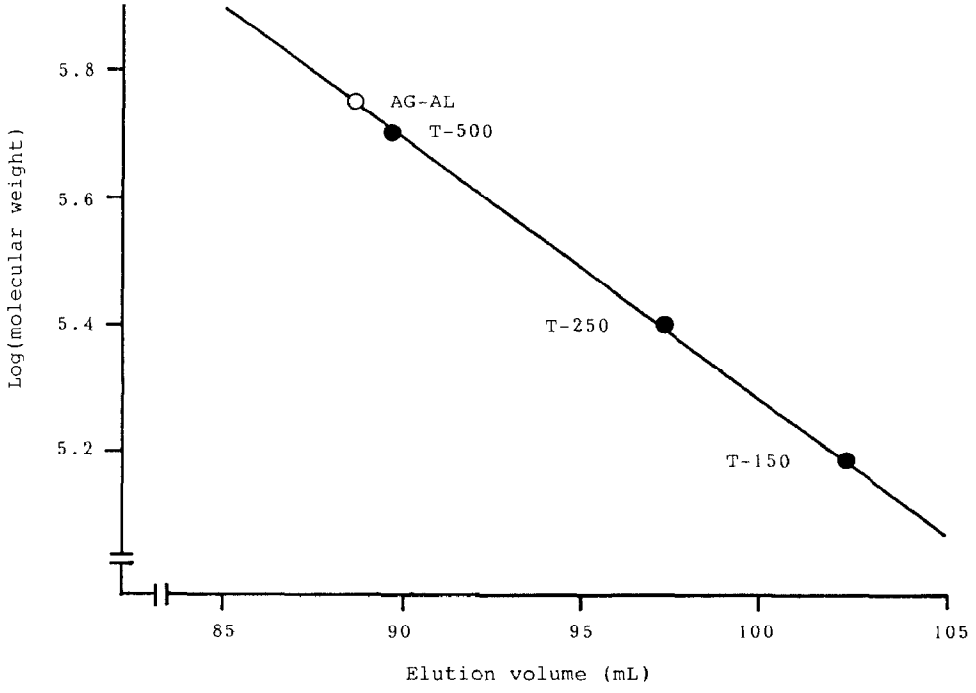

Fig. 1. Determination of the molecular weight of AG-AL by gel filtration on Toyopearl HW-65 with standard dextrans (T-500, T-250, and T-150).

entirely (1→3)-linked. On periodate oxidation, AG-AL consumed very little periodate, which is consistent with a (1→3)-linked structure.

On mild hydrolysis with acid, AG-AL gave glucose, a disaccharide,and a number of oligosaccharides, as shown by p.c. A linear relationship existed between the presumed degree of polymerization of the oligosaccharides and their $\log[R_F/(1 - R_F)]$ values[6], indicating that a homologous series of oligosaccharides was released.

The ^{13}C-nuclear magnetic resonance (n.m.r.) spectrum of AG-AL in M sodium deuteroxide is shown in Fig. 2. The resonance at 103.9 p.p.m. is assigned to the anomeric carbon atoms of (1→3)-linked α-D-glucopyranosyl residues. The assignments were based on data[7] for α-(1→3) linkages in alkaline solution at 85° and our data for (1→3)-β-D-glucan (curdlan), as follows: signals at 106.19 (C-1), 89.99 (C-3), 79.60 (C-5), 76.47 (C-2), 71.59 (C-4), and 64.05 p.p.m. (C-6) under the same conditions as for the AG-AL.

The foregoing data indicate that the water-insoluble, alkali-soluble glucan AG-AL is a linear (1→3)-α-D-glucan. Alkali-soluble, (1→3)-linked α-D-glucans have been isolated from other fungi[8,9], but these glucans contain the (1→4) linkages of glycogen, and the data on their molecular weights were not given, although S-glucan (containing a trace of D-xylose) without (1→4) linkages, from *Schizophyllum commune,* was characterized[10].

Many (1→3)-β-D-glucans have antitumor activity against Sarcoma 180 implanted in mice[11]. We attempted to assay any antitumor effect of the insoluble (1→3)-α-D-glucan (AG-AL). AG-AL was ineffective on this tumor in mice when administered daily by the intraperitoneal (*i.p.*) route at does of 1–20 mg/kg for 10 days (see Table I). An insoluble curdlan type of (1→3)-β-D-glucan (PS)[12] was used

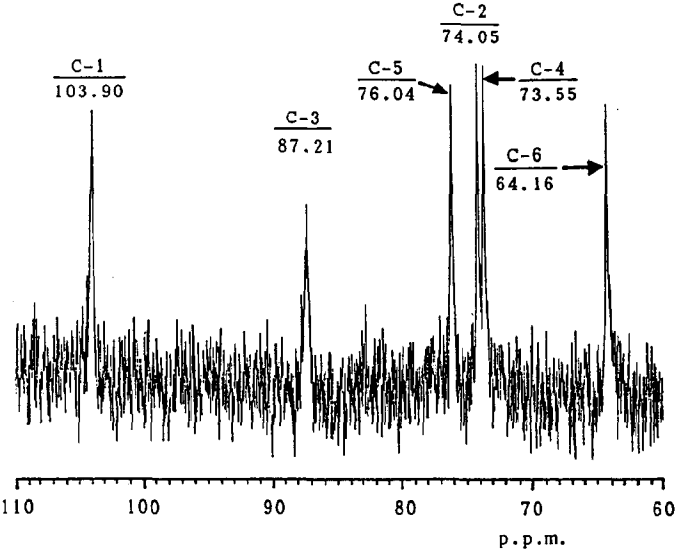

Fig. 2. ^{13}C-N.m.r. spectrum of AG-AL in M NaOD.

TABLE I

ANTITUMOR ACTIVITY OF AG-AL, AG-AL-CMS, AND AG-AL-CMI AGAINST SARCOMA 180

Sample	*Dose (mg/kg/day)*	*Mean tumor weight ±s.d. (g)*	*Inhibition ratio (%)*	*Complete regression*
Experimental-1				
AG-AL	1	4.99 ±2.13	6.0	0/6
	10	3.66 ±0.21	31.1	0/6
	20	6.25 ±4.04	−17.7	0/6
PS[a]	10	1.11 ±1.26[b]	79.1	2/6
Control		5.31 ±3.55		0/6
Experimental-2				
AG-AL-CMS	1	1.98 ±1.73[c]	78.3	0/6
	10	2.38 ±2.95[c]	74.0	0/6
	20	1.08 ±1.05[d]	88.2	0/6
Control		9.14 ±4.39		0/6
Experimental-3				
AG-AL-CMI	1	1.49 ±1.35[b]	62.8	1/6
	10	1.29 ±0.99[b]	67.8	1/6
	20	0.95 ±0.11[b]	76.3	0/6
CMPS[e]	10	0.33 ±0.50	91.8	4/6
Control		4.00 ±2.36		0/6

[a]Curdlan-type (1→3)-β-D-glucan from *Alcaligenes faecalis* var. *myxogenes* IFO 13140. [b]Significant difference from control, $p < 0.05$. [c]$p < 0.01$. [d]$p < 0.005$. [e]Carboxymethylation product of PS.

as a positive control. Sasaki *et al.*[2] reported that carboxymethylation of (1→3)-β-D-glucans increases the solubility and antitumor potency. Therefore, we applied carboxymethylation to (1→3)-α-D-glucan. Water-soluble (AG-AL-CMS) and gelatinous (AG-AL-CMI) products were obtained by carboxymethylation of the water-insoluble (1→3)-α-D-glucan (AG-AL). The degree of substitution by carboxymethyl groups per D-glucosyl residue was determined by the method previously reported[13], and found to be 0.80 for AG-AL-CMS and 0.82 for AG-AL-CMI. As shown in Table I, both *O*-(carboxymethyl)-D-glucans were found to exhibit high antitumor activity at doses of 1–20 mg/kg, *i.p.*, daily for 10 days, and the activity of AG-AL-CMS to be slightly higher than that of AG-AL-CMI.

It is of interest that the antitumor effect of (1→3)-α-D-glucan was activated by carboxymethylation. The antitumor activity has been discussed in connection with the conformational behavior of linear and branched (1→3)-β-D-glucans[14,15].

EXPERIMENTAL

Materials. — The fruiting bodies of *Agrocybe cylindracea* (Fr.) Maire were kindly provided by Aichi Prefectural Forest Experimental Station, Japan. Toyopearl HW-65, standard dextrans (dextran T-500, T-250, and T-150), and curdlan were respectively purchased from Toyo Soda Co., Ltd, Pharmacia Fine Chemicals, and Wako Pure Chemical Ind. Co. (1→3)-β-D-Glucan (PS) and

carboxymethylated PS (CMPS) were kindly donated by Takeda Chemical Ind., and *O*-(carboxymethyl)cellulose, sodium salt, was obtained from Daicel Chemical Ind.

General. — All evaporations were conducted under diminished pressure at a bath temperature not exceeding 40°. Specific rotations were measured with a JASCO DIP-4 automatic polarimeter. P.c. was performed by the double-ascending method on Toyo No. 51 filter paper with the following solvent systems: (*A*) 6:4:3 (v/v/v) 1-butanol–pyridine–water, and (*B*) 6:1:3 (v/v/v) 1-propanol–ethyl-acetate–water. Sugars were detected with an alkaline silver nitrate reagent[16]. I.r. spectra were recorded with a JASCO A-120 spectrometer. G.l.c. was performed in a Shimadzu GC-4CM apparatus equipped with a flame-ionization detector, and (*1*) a glass column (1.5 m × 0.3 cm) packed with 3% of ECNSS-M on Gaschrom Q (100–120 mesh) at 185° for unmethylated sugars, or (*2*) a glass column (2 m × 0.3 cm) packed with 3% of OV-225 on Chromosorb W (80–100 mesh) at 185° for methylated sugars. All g.l.c. analyses were conducted by first converting the sugars into their alditol acetates[17], and the peak areas were measured with a Shimadzu C-R3A Chromatopac. G.l.c.–m.s. was performed with a JEOL JMS-D 300 apparatus equipped with a glass column (1 m × 0.2 cm) packed with 3% of OV-225 and operated at 185°. The mass spectra were recorded at an ionizing potential of 70 eV, an ionizing current of 50 μA, and a temperature of the ion source of 220°.

Isolation of the polysaccharide. — The fresh fruiting bodies (450 g) were homogenized with 0.9% sodium chloride (1 L). The residue was extracted twice with hot water (1 L) for 6 h, and then twice with 5% sodium carbonate (1 L) for 24 h at 4°. The residue was extracted twice with M sodium hydroxide (1 L) containing a small proportion of sodium borohydride for 24 h at room temperature under a nitrogen atmosphere, and the extracts were collected by centrifugation, made neutral with 2M hydrochloric acid, and dialyzed against distilled water. The insoluble material in the non-dialyzable fraction was collected by centrifugation, and lyophilized, to afford the polysaccharide (AG-AL) in 0.37% yield.

Gel filtration and estimation of molecular weight. — Gel filtration of AG-AL (2 mg/0.5 mL) and standard dextrans on a column (92.5 × 1.5 cm) of Toyopearl HW-65 with M sodium hydroxide as the eluant, was performed as described previously[15]. A calibration curve was constructed by use of dextran T-500 (mol. wt. 495,000), dextran T-250 (253,000), and dextran T-150 (154,000) as shown in Fig. 1, and therefrom the molecular weight was estimated.

Analysis of component sugars. — The polysaccharide was hydrolyzed with 90% formic acid for 3 h at 100°, and then with 2M trifluoroacetic acid for 4 h at 100°. The hydrolyzate was evaporated to remove the acid, and analyzed by p.c. and g.l.c. (column *1*) as alditol acetates.

Methylation analysis. — AG-AL (10 mg) was methylated 6 times by the Hakomori method[5], as previously described[15]. The final methylation product showed no hydroxyl absorption band in its i.r. spectrum. The fully methylated glucan was heated successively with 90% formic acid for 4 h at 100°, and with 2M

trifluoroacetic acid for 5 h at 100°. The resulting partially methylated alditol acetates were analyzed[1] by g.l.c. (column *2*) and g.l.c.–m.s.

Periodate oxidation. — AG-AL (10 mg) was oxidized with 10mM sodium metaperiodate (40 mL) as previously described[15].

Partial hydrolysis with acid. — AG-AL (9.8 mg) was suspended in 50% (v/v) sulfuric acid (5 mL), and the suspension was kept for 42 h at 4° and for 1.5 h at room temperature, with stirring. The acid was neutralized with barium carbonate, and the mixture centrifuged. The supernatant liquor was applied to a small column of Amberlite CG-120 (H^+) resin, the eluate evaporated, and the residue analyzed by p.c. (solvent *B*).

^{13}C-N.m.r. spectroscopy. — The ^{13}C-n.m.r. spectra were recorded with a JEOL-GX 270 spectrometer, operated at 24° in the Fourier-transform mode with complete proton-decoupling, for solutions in M NaOD (23 mg/0.5 mL). Sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS, Aldrich Chemical Co.) was used as an external standard.

O-(Carboxymethyl)ation. — The D-glucan was carboxymethylated according to the method of Sasaki *et al.*[2]. A suspension of AG-AL (106 mg) in 2-propanol (2.8 mL) was stirred for 30 min at room temperature. A solution (30%) of sodium hydroxide (0.28 mL) was added, with stirring, during 60 min, and the mixture stirred vigorously for 90 min. Monochloroacetic acid (127 mg) was then added, and the mixture was stirred for 5 h at 60–70°. The suspended product was recovered by filtration, and successively washed with 7:3 methanol–acetic acid, 4:1 methanol–water, methanol, and acetone. The product was suspended in water (50 mL), and dialyzed against distilled water at 4°. The non-dialyzable fraction was separated by centrifugation, to give a water-soluble product, $[\alpha]_D$ +176° (*c* 0.135, M sodium hydroxide) (AG-AL-CMS, 118.4 mg) and a gelatinous product, $[\alpha]_D$ +156° (*c* 0.139, M sodium hydroxide) (AG-AL-CMI, 43.2 mg), which showed a characteristic absorption band at 1730 cm^{-1} in their i.r. spectra.

Antitumor activity. — Antitumor activity of the D-glucan and the *O*-(carboxymethyl)-D-glucans was assayed by a described procedure[15]. Sarcoma 180 ascites cells (2×10^6 cells) were transplanted subcutaneously into the right groin of male ddY mice. The saline suspensions or solutions were sterilized for 20 min at 120°, and then injected *i.p.* daily for 10 days, starting 24 h after the transplantation. After 30 days, the mice were sacrificed, and the tumors were extirpated and weighed. The inhibition ratios (%) were given by $[(A - B)/A] \times 100$, where A is the average tumor weight of the control group, and B is that of the test group.

REFERENCES

1 T. KIHO, M. ITO, K. NAGAI, C. HARA, AND S. UKAI, *Chem. Pharm. Bull.*, 36 (1988) 3032–3037.
2 T. SASAKI, N. ABIKO, K. NITTA, N. TAKASUKA, AND Y. SUGINO, *Eur. J. Cancer*, 15 (1979) 211–215.
3 M. DUBOIS, K. A. GILLES, J. K. HAMILTON, P. A. REBERS, AND F. SMITH, *Anal. Chem.*, 28 (1956) 350–356.
4 S. A. BARKER, E. J. BOURNE, AND D. H. WHIFFEN, *Methods Biochem. Anal.*, 3 (1956) 213–245.
5 S. HAKOMORI, *J. Biochem. (Tokyo)*, 55 (1964) 205–208.

6 D. FRENCH AND G. M. WILD, *J. Am. Chem. Soc.*, 75 (1953) 2612–2616.
7 H. M. DAVIS, W. J. BOYKO, AND J. R. EDWARDS, *Carbohydr. Res.*, 152 (1986) 279–282.
8 E. BARRETO-BERGTER AND P. A. J. GORIN, *Adv. Carbohydr. Chem. Biochem.*, 41 (1983) 68–103.
9 J. G. H. WESSELS AND J. H. SIETSMA, in W. TANNER AND F. A. LOEWUS (Eds.), *Fungal Cell Walls: A Survey*, in *Encyclopaedia of Plant Physiology* (New Series), Vol. 13B, Springer-Verlag, Berlin, 1981, pp. 360–361.
10 J. G. H. WESSELS, D. R. KREGER, R. MARCHANT, B. A. REGENSBURG, AND O. M. H. DEVRIES, *Biochim. Biophys. Acta*, 273 (1972) 346–358.
11 R. L. WHISTLER, A. A. BUSHWAY, P. P. SINGH, W. NAKAHARA, AND R. TOKUZEN, *Adv. Carbohydr. Chem. Biochem.*, 32 (1976) 235–275.
12 S. HONDA, H. SUGINO, T. ASANO, AND A. KAKINUMA, *Immunopharmacology*, 11 (1986) 29–37.
13 S. UKAI, A. HONDA, K. NAGAI, T. KIHO, AND T. TSUCHIYA, *Abstr. Annu. Meet. Pharm. Soc. Jpn.*, 108th, Hiroshima, 1988, p. 662.
14 K. OGAWA, T. WATANABE, J. TSURUJI, AND S. ONO, *Carbohydr. Res.*, 23 (1972) 399–405.
15 T. KIHO, M. ITO, K. NAGAI, C. HARA, AND S. UKAI, *Chem. Pharm. Bull.*, 35 (1987) 4286–4293.
16 W. E. TREVELYAN, D. P. PROCTER, AND J. S. HARRISON, *Nature (London)*, 166 (1950) 444–445.
17 J. H. SLONEKER, *Methods Carbohydr. Chem.*, 6 (1972) 20–24.