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Note

Isolation and characterization of antitumor active β-D-glucans from the fruit bodies of *Ganoderma applanatum*

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There is a strong tradition in Japan from older times that Basidiomycetes belonging to the *Polyporaceae* family are effective against cancer. Ikekawa *et al.* have already reported that the hot-water extracts from the *Polyporaceae* fungus family inhibit the growth of solid-tumor Sarcoma 180 implanted subcutaneously in mice, and the activity is attributable to (1→3)-β-D-glucans[1,2]. Such antitumor glucans have been also obtained from a number of other sources, such as yeast, fungi, bacteria, and plants[3].

Two highly purified β-D-glucans were isolated from the hot-water extracts of fruit bodies of *Ganoderma applanatum* by a combination of fractional procedures, including precipitation with ethanol and with Cetavlon, and sequential chromatography on columns of DEAE-cellulose, Sephadex G-100, and Sepharose CL 4B, and affinity chromatography on concanavalin A–Sepharose CL 4B. Both of the β-D-glucans designated as F-I-1a1-β and F-I-1a2-β gave single, symmetrical peaks on ultracentrifugation, on zone electrophoresis, and by gel filtration on Sepharose CL 4B. The molecular weights of F-I-1a1-β and F-I-1a2-β were estimated by sedimentation analysis to be 1.01×10^6 and 3.02×10^5, respectively. The protein content of these glucan fractions, as determined by the method of Lowry *et al.*[4], was negligibly low (0.12–0.15%). The antitumor effect of the glucans against subcutaneously implanted Sarcoma 180 are shown in Table I. The antitumor active fraction F-I-1a was further divided into the very active fractions F-I-1a1-β and F-I-1a2-β. The highest activity of the material tested was observed in F-I-1a2-β (complete regression 5/5, with a single dose of 1 mg/kg). The ID_{50} indicates noteworthy activity

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TABLE I

ANTITUMOR EFFECT AND OPTIMUM DOSE OF GLUCANS FROM *G. applanatum* AGAINST SARCOMA 180 IN MICE[a]

Samples	*Dose (mg/kg/day) I*	*Average tumor diameter on day 25* *Treated/control*	*Inhibition (%)*	*Complete regression on day 45*	*ID_{50}[b] (mg/kg)*
F-I-1a	3	3.3/12.5	74	4/5	
	1	5.3/12.5	58	3/5	0.36
	0.3	5.8/12.5	54	2/5	
F-I-1a1-β	3	4.8/12.5	62	3/5	
	1	8.4/12.5	33	2/5	1.9
	0.3	9.0/12.5	28	1/5	
F-I-1a2-β	10	0/16.6	100	5/5	
	3	1.5/16.6	91	3/5	0.15
	1	0/16.6	100	5/5	
	0.3	5.0/16.6	70	2/5	
Control	- -			0/5	

[a]Sarcoma 180 cells ($2 \cdot 10^6$) were inoculated subcutaneously into the breast region of mice. [b]Dose level that inhibits tumor growth in 50% of the control.

at the low dose level of 0.15 mg/kg. The present sample is one of the most effective antitumor glucans reported[5-7].

F-I-1a1-β showed $[\alpha]_D$ +8° (0.5M, sodium hydroxide) and F-I-1a2-β +23 (water). Both also showed characteristic i.r. absorption for β-glucosides at 890 cm^{-1}. Methylation analysis employing g.l.c.–m.s. of *O*-methylalditol acetates used characteristic retention-times (*T*-values)[8] and response-factors previously determined for the individual constituents[9]. F-I-1a1-β gave the 2,3,4,6-tetra, 2,3,4- or 2,3,6-tri, and 2,4-di-*O*-methyl derivatives in the molar ratio of 1.0:2.2:1.0, and F-I-1a2-β in the molar ratio of 1.0:2.4:1.1. Treatment of F-I-1a1-β with sodium metaperiodate led to consumption of 0.43 mol of periodate and release of 0.20 mol of formic acid per glucose residue, and for F-I-1a2-β the values were 0.41 mol of periodate and 0.19 mol of formic acid. The results suggest that D-glucose residues are present both as chain residues linked through O-3, and as branch points at O-6. The ^{13}C-n.m.r spectra of these two glucan fractions were characteristic of a (1→6)-branched (1→3)-β-D-glucan as reported for schizophyllan[10,11] and lentinan[11]. The proton n.m.r. spectra of the glucans in D_2O at 90° also showed anomeric signals at δ 4.75 and 4.56 (doublet, $J_{1,2}$ 8.0 Hz) involved in the β-(1→3)- and β-(1→6)-linked glucopyranosyl residues, respectively[12]. From these results, we concluded that the highly purified β-D-glucans have very similar basic structural features

In contrast, the antitumor inactive fractions F-I-1a1-α and F-I-1a2-α were shown by methylation analysis and by ^{1}H- and ^{13}C-n.m.r. spectroscopy[12,13] to be α-D-glucans of the glycogen type having average chain-lengths of ~8 glucose residues. The former had $[\alpha]_D$ +182° and the latter +178° (water). These glycogen-types of

glucans have been detected in numerous Basidiomycetes and in yeast, and is recognized as a fungal component[14].

Additional evidence for the structures of the very active antitumor fractions was provided by enzyme digestion. The enzymic hydrolyzate of F-I-1a1-β and F-I-1a2-β by the exo-(1→3)-β-D-glucanase of *Basidiomycetes sp.* QM 806[15] after 48 h of incubation revealed only two components, corresponding to glucose and gentiobiose, by paper chromatography (p.c.) and by high-performance liquid chromatography (l.c.). F-I-1a1-β gave gentiobiose and glucose in the molar ratio of 1.0:1.8, and F-I-1a2-β gave the ratio 1.0:1.9, as shown in Fig. 1. The elution pattern of the enzymic digestion products on a column of Bio-gel P-2 also showed two peaks for D-glucose and apparent glucose disaccharides, and no trace of the initial polymer was detected. F-I-1a2-β was further subjected to prolonged periodate oxidation, and the resulting polysaccharide polyaldehyde was reduced to the corresponding polyalcohol by conventional treatment with sodium borohydride[16]. The polyalcohol was then analyzed by enzymic hydrolysis as already mentioned. The expected D-glucose and 6-*O*-substituted D-glucose[17] were detected. The latter was separated by l.c. and identified as **1** from its ^{13}C-n.m.r. spectrum, as shown in Fig. 2, using assignments by analogy with earlier data[13,18]. The molar ratio of D-glucose and 6-*O*-substituted D-glucose was 2.1:1.0: this value corresponds closely to the result of enzymatic analysis of F-I-1a2-β. These structural investigations indicate that the two highly

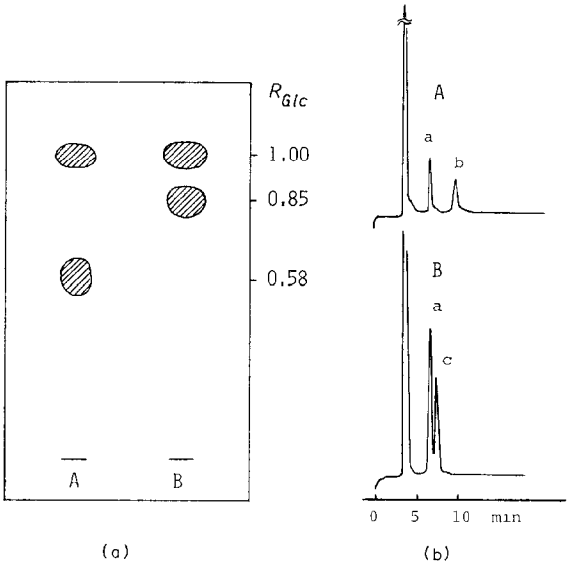

Fig. 1. (*a*) Paper chromatogram of the enzymic digests of F-I-1a2-β (A) and polysaccharide polyol (B). [Solvent, 12:5:4 ethyl acetate–pyridine–water. Gentiobiose; R_{Glc} 0.58, 6-*O*-substituted D-glucose; R_{Glc} 0.85.] (*b*) High-pressure, liquid chromatogram of enzymic digests of F-I-1a2-β (A) and its polysaccharide polyol (B). [0 designates the point of sample injection. The broken, base-line peak denotes the solvent front. Peaks *a*, *b*, and *c* correspond to glucose, gentiobiose, and 6-*O*-substituted glucose, respectively. Solvent; 3:7 water–acetonitrile pumped at 0.8 mL.min^{-1}.]

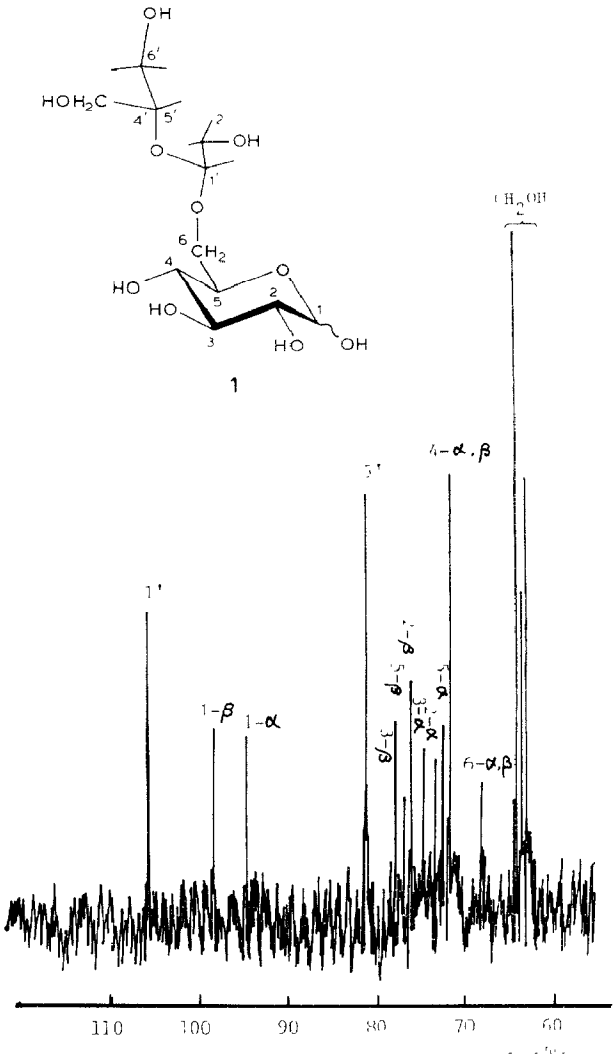

Fig. 2. ^{13}C-N.m.r. spectrum of 6-*O*-substituted D-glucose (2 mg/0.3 mL) in D_2O at room temperature. [The spectrum was measured in a 5-mm tube at 25 MHz with complete proton decoupling. Chemical shifts: in p.p.m. downfield from external Me_4Si. Accumulation: 30,000. Repetition, 0.8 s.]

purified β-D-glucans are highly branched polysaccharides containing a framework of (1→3)-linked β-D-glucopyranosyl residues, substituted at O-6 in every third residue with single branches; (1→6) inter-residue linkages are absent.

Concerning the relationship between polysaccharide structure and antitumor activity, it is interesting that F-I-1a1-β and F-I-1a2-β, although having very similar structures, have different levels of activity, being higher in the latter and lower in the former. The same relationship has also been observed in the antitumor β-D-

glucans from the fruit bodies of *Fomotopsis pinicola*[19], indicating that not only are the primary structures of antitumor β-D-glucans important for activity, but such factors as molecular size and solubility in water are also significant.

EXPERIMENTAL

Polyporaceae *fungi*. — The dried fruit-bodies of wild *G. applanatum* used were collected in Senzu, Shizuoka prefecture of Japan. The material was identified by Dr. K. Aoshima.

General methods. — Sugar composition was determined by g.l.c. of alditol acetate derivatives of the monosaccharides produced by hydrolyzing the corresponding polysaccharide specimens in 0.5M sulfuric acid for 5 h at 105°, using a column[20] of 3% ECNSS-M at 190°. Optical rotations were measured with a Union Automatic Digital Polarimeter PM-101. I.r. spectra were recorded with a Jasco A-102 spectrometer, and ^{1}H- and ^{13}C-n.m.r. spectra with a JEOL FX-100 MHz spectrometer with deuterium oxide as the solvent and sodium 4,4-dimethyl-4-silapentanesulfonate as the internal standard. Zone electrophoresis was conducted on Whatman GF/A glass microfiber paper (5 × 57 cm) in 0.1M sodium tetraborate (pH 9.3) at 2,000–3,000 V for 60 min, and carbohydrates were detected according to Shida *et al.*[21]. Sedimentations were performed in a synthetic boundary-cell at 60,000 r.p.m. at 20° by using a Hitachi model USA-1A ultracentrifuge, equipped with an RA60HC rotor. The concentration of polysaccharide was 0.1% in water or 0.5M sodium hydroxide. P.c. of hydrolyzates of enzymic digestion was performed on Toyo No. 50 filter paper by the multiple-ascending method.

Isolation of antitumor β-D-glucans. — The fungus (3 kg) was cut into small pieces immediately after harvesting and disintegrated in a blender. The resulting slurry was treated with 90% ethanol (30 L) for 2 h. The residues were extracted with hot water (20 L) for 3–4 h, and this procedure was repeated once more. The combined extracts were concentrated and dialyzed. A precipitate that formed during dialysis was removed by centrifugation and discarded, and the crude polysaccharide (12.5 g) was precipitated by an excess of ethanol. The water-extracted fraction was dissolved in water (200 mL) and the solution applied to the top of a column (4 × 65 cm) of DEAE-cellulose (Cl^- form). The column was eluted with water (4 L), and the almost colorless effluent concentrated to low volume (200 mL) and poured into an equal volume of methanol. The precipitate was collected by centrifugation, washed with ethanol, and dried *in vacuo*. This fraction was designated as F-I (2.1 g). Next, 200 mL of ethanol was added to the supernatant solution; the precipitate obtained was termed F-II (0.42 g). The final fraction (1.1 g, termed F-III) was recovered from the supernatant solution by addition of 400 mL of ethanol.

F-I (2.1 g) was applied to a column of Sephadex G-100 and fractionation was conducted as described (Gel filtration) to yield F-I-1 (1.5 g) and F-I-2 (0.3 g). F-I-1 was eluted very near the solvent front (tube numbers 25–34) and gave glucose as the main sugar on acid hydrolysis, whereas F-I-2 (eluted in tube numbers 35–55)

gave a heterogalactan fraction[22]. For F I-1, gradual precipitation was performed by treatment with cetyltrimethylammonium bromide (Cetavlon) in borate buffer at different pH values[21]. Fraction F-I-1 (1.4 g) was dissolved in water and treated with equal volumes of 0.15M Cetavlon and 0.1M borate buffer (pH 8.0). The solution was adjusted to pH 9.0 with 0.5M sodium hydroxide and the precipitate formed was washed, dissolved in 2M acetic acid, and the solution poured into 3 volumes of methanol. The precipitate (F-I-1a) was successively washed with methanol and acetone, and dried *in vacuo*; yield 920 mg. The supernatant solution was treated by the foregoing procedure to give 250 mg of an additional product (F-I-1b). The supernatant solution at pH 11.0 was poured into three volumes of methanol to give 120 mg of precipitate (F-I-1c). F-I-1a (800 mg) was then divided into F-I-1a1 (tube numbers 25–30, 70 mg) and F-I-1a2 (tube numbers 31–46, 640 mg) on a column of Sepharose CL 4B.

Further subfractionation of F-I-1a1 and F-I-1a2 was performed on a column (3.0 × 30 cm) of concanavalin A–Sepharose CL 4B[22]. A solution of F-I-1a2 (50 mg) was applied to the top of the column, which was eluted with 0.1M sodium phosphate buffer at pH 7.0 and 25°. The effluent was collected in 10-mL fractions. The eluate was monitored for neutral carbohydrates by the phenol–sulfuric acid assay. When nonadsorbed carbohydrate had been eluted, the eluant was changed to 5mM methyl α-D-glucoside in sodium chloride at pH 7.0. The eluted polysaccharide, passed through a column, was continuously dialyzed by using a Zemeh Dialyzer (Funakoshi Pharmaceutical Co. Ltd.), in order to remove methyl α-D-glucoside contained in the eluant. The nonadsorbed fraction (tube numbers 9–17, F-I-1a2-β) was concentrated, dialyzed, and freeze-dried to give 30 mg of product, and the adsorbed fraction (tube numbers 44–49, F-I-1a2-α) gave 12 mg. Similarly, subfractionation of F-I-1a1 gave 33 mg of F-I-1a1-β and 8 mg of F-I-1a1-α.

Assay of antitumor activity. — Antitumor activity was tested by the method of Chihara *et al.*[23]. 6 7-Day-old ascites of Sarcoma 180 was transplanted subcutaneously into the breast region of female ICR/JCL mice (7 weeks). The effective dose was determined in two ways. The test samples, dissolved in saline, were injected intraperitoneally at a single dosage, starting 3 days after tumor transplantation. After 25 days, the tumors were dissected out and the dimensions measured to determine the inhibition ratio. At the end of 45 days, the mice were killed, and complete regression of the tumors was recorded.

Gel filtration. — Solutions of the polysaccharide (30–90 mg) in 2–3 mL of water were applied to columns of Sephadex G-100 (2.5 × 115 cm), and Sepharose CL 4B (2.6 × 100 cm). The columns were eluted with 0.05M sodium chloride, collecting 13-mL fractions, in the case of Sephadex G-100 and 8-mL fractions for Sepharose CL 4B. The degree of polymerization (d.p.) of the enzymic hydrolyzate was determined by gel filtration through a column (1.5 × 100 cm) of Bio-gel P-2, followed by elution with water. Fractions of 1 mL were collected. The carbohydrate content of each fraction was determined by the phenol–sulfuric acid method.

Methylation of glucan. — Each glucan was fully methylated by the method of Hakomori[24], as modified by Lindberg[8]. Methylated sugars were converted into their

alditol acetates as already mentioned, and analyzed by g.l.c.–m.s. A Hitachi 063 gas chromatograph coupled to a Hitachi RMU6M mass spectrometer data-system was used, with 3% ECNSS-M on Gas Chrom Q (stainless steel, 0.3 × 200 cm) at 180°.

Periodate oxidation and polysaccharide polyol. — To 15 mg samples of F-I-1a1-β and F-I-1a2-β, 0.01M sodium metaperiodate was added, and the mixture was stirred in the dark for 20 days at 5°. The periodate consumption was measured spectrophotometrically[25]. Formic acid was titrated with 0.01M sodium hydroxide and, following reduction of the excess of periodate, the solution was made neutral with barium carbonate. In the case of F-I-1a2-β, the filtrate was reduced with sodium borohydride (15 mg) for 20 h and then adjusted to pH 5.0 by addition of acetic acid. The solution was dialyzed against running water for 2 days, and freeze dried. The resulting polysaccharide polyol was subjected to enzymic analysis.

Enzymic hydrolysis. — Exo-(1→3)-β-D-glucanase was prepared from a culture of *Basidiomycetes sp.* QM 806 according to the method of Huotari *et al.*[15]. The purified enzyme had 120 units of specific activity per mg, as assayed under the conditions described. Samples (5 mg) were dissolved in 1 mL of 0.05M acetate buffer (pH 4.8), and incubated for 48 h at 40° with the enzyme. After inactivation of the enzyme by heating for 10 min at 100°, each incubation mixture was analyzed by p.c., by l.c., and by gel filtration on a column of Bio-gel P-2. L.c. was performed with a PNH_2-10/s2504 column (4 mm i.d. × 25 cm) in a Shimazu LC-3A liquid chromatograph equipped with an RID-4 differential refractometer.

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