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Note

An antitumor activity of the alkali-soluble polysaccharide (and its derivatives) obtained from the sclerotia of *Grifora umbellata* (Fr.) PILÁT

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Antitumor polysaccharides have been obtained from various sources. The highest activity against Sarcoma 180 has been demonstrated[1] for a (1→3)-β-D-glucan having β-D-glucopyranosyl groups (1→6)-linked to every third or fourth residue of the main chain. A water-soluble glucan, from *Grifora umbellata*, having such a structure has been reported by Miyazaki and his coworkers[2] to possess the activity. However, the yield of the polysaccharide is very low. As the water-insoluble residue was found to contain a polysaccharide similar to the water-soluble one, we attempted to extract the polysaccharide effectively from the residue, and reported on its structure[3]. This Note is concerned with the antitumor activity of the alkali-soluble polysaccharide from the water-insoluble residue of the sclerotia of *G. umbellata*, and some of its derivatives.

The water-insoluble residue of the sclerotia was extracted with 2% sodium hydroxide containing 2% of urea, and then with 2% sodium hydroxide alone. After separation of polysaccharides **AP-7** and **AP-9**, which were precipitated by neutralization of the base, **AP-8** and **AP-10** were isolated from the supernatant liquor by addition of methanol. Similar extractions were conducted after treatment of the water-insoluble residue with 10% aqueous zinc chloride, and polysaccharides **AP-1** to **AP-6** were obtained. A polysaccharide (**ZP**) was also obtained from the zinc chloride extract.

Results of bioassay of these polysaccharides are given in Tables I and II. It is evident that some of the alkali-soluble polysaccharides are more effective against Sarcoma 180 than the water-soluble polysaccharide (**WP**). Although there is no difference in activity between **AP-8** and **AP-10**, which were not precipitated by neutralization, there is a big difference in activity between **AP-7** and **AP-9**, which were precipitated by neutralization. **AP-7** and **AP-8** are the polysaccharides extracted with urea-containing sodium hydroxide, and **AP-9** and **AP-10** are obtained with sodium hydroxide alone. Similar phenomena are observed between the polysaccharides **AP-1** and **AP-4**, and **AP-3** and **AP-6**, which were extracted from the residue after

TABLE I

ANTITUMOR ACTIVITY (5TH WEEK) OF THE POLYSACCHARIDES IN THE SCLEROTIA OF *G. umbellata* (PART I)

Sample	*Dose (mg/kg × days)*	*Average tumor size (mm^3)*	*Inhibition ratio (%)*	*Survival*	*Complete regression*
Control		12,466	—	3/7	1/7
WP	10 × 10	0	100	3/8	3/8
ZP	10 × 10	77	99	7/8	5/8
AP-7	10 × 10	0	100	4/8	4/8
AP-8	10 × 10	38	99	7/8	6/8
AP-9	10 × 10	5,406	57	4/7	1/7
AP-10	10 × 10	0	100	6/8	6/8

TABLE II

ANTITUMOR ACTIVITY (5TH WEEK) OF THE POLYSACCHARIDES IN THE SCLEROTIA OF *G. umbellata* (PART II)

Sample	*Dose (mg/kg × days)*	*Average tumor size (mm^3)*	*Inhibition ratio (%)*	*Survival*	*Complete regression*
Control		11,969	—	3/8	0/8
AP-1	10 × 10	0	100	2/7	2/7
AP-2	10 × 10	157	99	4/7	3/7
AP-3	10 × 10	158	99	6/8	4/8
AP-4	10 × 10	—	—	0/8	0/8
AP-6	10 × 10	2,116	82	5/8	2/8

zinc chloride treatment. Treatment of the water-insoluble residue with the salt increased the yield of alkali-soluble polysaccharide, but was accompanied by diminution of the activity of the polysaccharide extracted. However, the polysaccharide regained its activity when it was extracted with urea-containing sodium hydroxide.

It is interesting that the presence of urea in the alkaline solution increased the activity, as well as the yield, of the alkali-soluble polysaccharide. Therefore, the distribution of the molecular weight of these polysaccharides was determined by gel filtration in a column of Sepharose CL-2B. The results are shown in Fig. 1. It is apparent, with some exceptions, that the polysaccharide extracted by urea-containing sodium hydroxide contains a fraction having a molecular weight relatively higher than that of those extracted by alkali alone. Therefore, the activity of the polysaccharide extracted with the urea-containing alkali might be ascribed to the fraction having the higher molecular weight. Another possibility is that the polysaccharide itself gained the activity by being treated with urea.

The chemical structure and the average molecular weight of AP-3 have already been reported[3]. AP-3 can be digested by exo-(1→3)-β-D-glucanase to 74% degree of hydrolysis, with release of D-glucose and gentiobiose in the ratio of 2:1. Therefore,

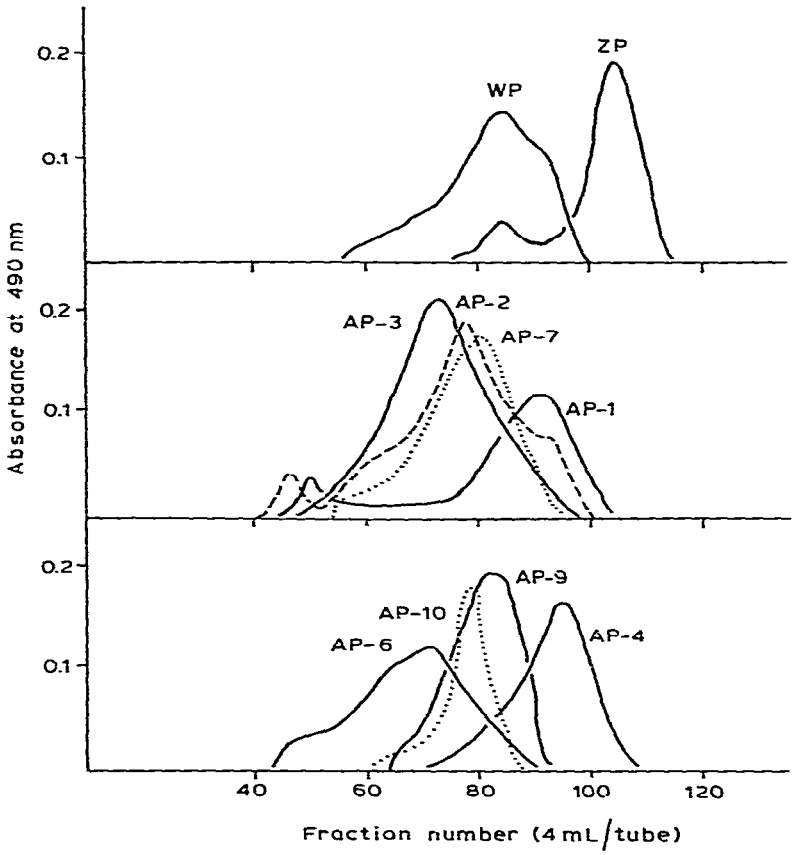

Fig. 1. Molecular-weight distribution of the polysaccharide extracted in different ways from the sclerotia of *G. umbellata*. [A solution of each polysaccharide (2 mg) in 0.2M sodium hydroxide was applied to a column (2.8 × 90 cm) of Sepharose CL-2B, and eluted with 0.2M sodium hydroxide (0.4 mL/min). The carbohydrate content of each fraction was determined by the phenol–sulfuric acid method.]

TABLE III

ANTITUMOR ACTIVITY (5TH WEEK) OF ENZYMICALLY HYDROLYZED AP-3 AND AP-3 DERIVATIVES

Sample	*Dose (mg/kg × days)*	*Average tumor size (mm^3)*	*Inhibition ratio (%)*	*Survival*	*Complete regression*
Control		12,286	—	4/9	1/9
EH-11%	10 × 10	393	97	6/7	5/7
EH-38%	10 × 10	253	98	3/7	2/7
AP-3 polyaldehyde	10 × 10	3,115	75	3/7	2/7
AP-3 Polyalcohol	10 × 10	2,626	79	6/7	4/7

TABLE IV

ANTITUMOR ACTIVITY (4TH WEEK) OF DEBRANCHED AP-3 (LAP) AND SOME OF ITS DERIVATIVES

Sample	*Dose (mg/kg × days)*	*Average tumor size (mm^3)*	*Inhibition ratio (%)*	*Survival*	*Complete regression*
Control		9938	—	1/9	0/9
LAP	10 × 10	5179	48	4/9	3/9
Ac-LAP[a]	10 × 10	7919	20	2/9	0/9
CM-LAP[b]	10 × 10	7486	25	7/9	2/9
HE-LAP[c]	10 × 10	4703	53	6/9	3/9
Me-LAP[d]	10 × 10	3942	60	5/9	4/9

[a]Ac = *O*-acetylated (d.s. 0.40). [b]CM = *O*-(carboxymethyl)ated (d.s. 0.10). [c]HE = *O*-(2-hydroxyethyl)ated (d.s. 0.40). [d]Me = *O*-methylated (d.s. 0.85).

AP-3 was partially hydrolyzed with the enzyme, and the product was subjected to bioassay to learn the relationship between the activity and the molecular weight. As shown in Table III, 38%-degraded AP-3 (EH-38%, mol. wt. 600,000 ±100,000) showed a high inhibition-ratio, but a low complete-regression, whereas 11%-degraded material (EH-11%) did not lose the activity. A molecular weight of >1,000,000 might be indispensable for the polysaccharide to have the activity.

AP-3 is classified as a (1→3)-β-D-glucan whose D-glucosyl residues are partially linked to single β-D-glucopyranosyl groups through (1→6) bonds. The β-D-glucosyl substituents at O-6 of the D-glucosyl residues in the main chain were modified, in order to learn the relationship between the structure of the substituent and the activity; the first modification was achieved by periodate oxidation, and the second by reduction of the product with borohydride. The activity of the products thus obtained is shown in Table III. It was found that the activity of the polysaccharide was lost by the oxidation, but regained by the reduction. From this observation, substitution with such groups as acetyl, carboxymethyl, 2-hydroxyethyl, and methyl was attempted, to introduce various degrees of substitution into linear (1→3)-β-D-glucan (LAP). The derivatives having different degrees of substitution were obtained by changing the proportions of the reagents. LAP was prepared from AP-3 by periodate oxidation followed by Smith degradation. All of the derivatives tested lost the activity. The results of bioassay of each derivative, given in Table IV, indicate that the structure of the substituent at O-6 is very important for activity against Sarcoma 180.

EXPERIMENTAL

General. — Paper chromatography was performed on Toyo No. 50 filter paper by the multiple, ascending method, with 4:1:5 (v/v) 1-butanol–ethanol–water as the solvent. The compounds on a chromatogram were located with alkaline silver

nitrate. ^{1}H-N.m.r. spectra were recorded at 90 MHz with a Hitachi R-22 spectrometer, for solutions in D_2O. Gas–liquid chromatography (g.l.c.) was performed in a Shimadzu GC-4APF apparatus fitted with a flame-ionization detector. The glass column (0.4 × 200 cm) was packed with 3% of OV-1 on Chromosorb W (80–100 mesh), and operated at 220° (for partially methylated, alditol acetates), 230° (for carboxymethyl ethers), and 240° (for 2-hydroxyethyl ethers), with a gas flow-rate of 50 mL of nitrogen per min. Peak areas were measured with a Shimadzu ITG-4A digital integrator. G.l.c.–mass spectrometry was conducted with a Hitachi Model M-52 apparatus equipped with the same column mentioned, and mass spectra were recorded at an ionizing potential of 20 eV.

Preparation of the polysaccharide. — The defatted sclerotia of *Grifora umbellata* (Fr.) were extracted with water in an autoclave (1 kg/cm^2) for 1 h. The extraction was repeated until the supernatant liquor showed a negative reaction with 1-naphthol (Molisch test). Water-soluble polysaccharide (**WP**) was obtained from the supernatant liquor by adding four volumes of methanol; yield 3.9% (based on the defatted sclerotia).

The residual material was divided into three parts. The first part of the residue was similarly treated in an autoclave with 10% aqueous zinc chloride. The Molisch-positive supernatant liquors were combined, and dialyzed against water for 4 days. A greyish precipitate that formed during the dialysis was filtered off. The polysaccharide (**ZP**) was obtained from the dialyzed solution by adding four volumes of methanol; yield 0.15%, based on the water-insoluble residue.

A part of the residual material after treatment with 10% zinc chloride was thoroughly washed with water (to remove the remaining salt), and then extracted by stirring with 2% sodium hydroxide containing 2% of urea for 24 h at 4°. The Molisch-positive, supernatant liquors were combined, and the base was neutralized with hydrochloric acid, with cooling. The polysaccharide (**AP-1**) deposited was de-ionized by dialysis, and then the solution was lyophilized; yield 6.4%, based on the 10% zinc chloride-insoluble residue.

The polysaccharide (**AP-2**) not precipitated by neutralization was obtained by adding four volumes of methanol; yield 1.5% based on the zinc chloride-insoluble residue.

The residue from extraction with 2% sodium hydroxide containing 2% of urea was then similarly extracted with 10% sodium hydroxide containing 5% of urea. The polysaccharide (**AP-3**) precipitated by neutralization was de-ionized by dialysis, and the solution lyophilized; yield 33.9%, based on the zinc chloride-insoluble residue.

Another part of the 10% zinc chloride-insoluble residue was similarly extracted with 2% sodium hydroxide containing no urea. The polysaccharide (**AP-4**) was precipitated when the supernatant liquors were combined, and made neutral with hydrochloric acid, with cooling; the solution was de-ionized by dialysis, and lyophilized; yield 1.8% based on the zinc chloride-insoluble residue. The amount of polysaccharide (**AP-5**) not precipitated by neutralization was very small.

The residue from the extraction with 2% sodium hydroxide was then extracted with 10% sodium hydroxide as already described. The polysaccharide (**AP-6**) was obtained from the supernatant liquor by neutralization with hydrochloric acid, with cooling; yield 23.8%, based on the zinc chloride-insoluble residue.

The second part of the water-insoluble residue was repeatedly extracted by stirring with 2% sodium hydroxide containing 2% of urea for 24 h at 4°. When the supernatant liquor was made neutral with hydrochloric acid, with cooling, the polysaccharide (**AP-7**) was precipitated. After de-ionization by dialysis, the solution was lyophilized; yield 2.1%, based on the water-insoluble residue. The polysaccharide (**AP-8**) not precipitated by neutralization was obtained by adding four volumes of methanol; yield 5.0%, based on the water-insoluble residue.

The third part of the water-insoluble residue was extracted with 2% sodium hydroxide containing no urea, exactly as already described. The polysaccharides (**AP-9** and **AP-10**), corresponding to **AP-7** and **AP-8** in the foregoing procedure, were obtained in a yield of 1.7 and 3.3%, respectively, based on the water-insoluble residue.

Each polysaccharide was completely hydrolyzed with 0.5M sulfuric acid at 100°, and the hydrolyzate was examined by paper chromatography. Besides a large proportion of D-glucose, traces of D-mannose and D-glucuronic acid were detected in **WP**, and D-mannose in **ZP**, but only D-glucose was obtained from the alkali-soluble polysaccharides (**AP-1**–**AP-10**).

Distribution of molecular weight. — A solution (1 mL) of the polysaccharide (2 mg) in 0.2M sodium hydroxide was applied to a column (2.8 × 90 cm) of Sepharose CL-2B. The column was equilibrated, and eluted, with 0.2M sodium hydroxide (0.4 mL/min). The effluent was collected in 4-mL fractions. The carbohydrate content of each fraction was determined by the phenol–sulfuric acid method. The results are given in Fig. 1.

Periodate oxidation and Smith degradation. — A suspension of **AP-3** (1 part) in 0.03M sodium metaperiodate (300 parts) was kept in the dark at 4°, with continuous stirring. At intervals, the periodate consumption was determined by the Fleury–Lange method[4]; when it became constant (0.49 mol per mol of D-glucosyl residue), ethylene glycol was added, and the solution was dialyzed against running water for 2 days. The oxidized product (**AP-3** polyaldehyde) was collected by centrifugation, and then lyophilized. **AP-3** polyaldehyde (1 part) was dissolved in 0.1M sodium hydroxide (100 parts), and reduced with sodium borohydride (0.33 part) for 48 h at room temperature. After decomposition of the excess of borohydride by addition of M acetic acid, the solution was dialyzed against running water. The reduction product (**AP-3** polyalcohol) was collected by centrifugation, and lyophilized. **AP-3** polyalcohol (1 part) was hydrolyzed with 125mM sulfuric acid (100 parts) for 40 h at room temperature. The solution was made neutral with sodium hydroxide, dialyzed against running water for 2 days, and the nondialyzable fraction lyophilized. This product was designated Smith-degraded **AP-3** (**LAP**). **LAP** gave only D-glucose when completely hydrolyzed with 0.25M sulfuric acid at 100°.

Partial hydrolysis by exo-(1→3)-β-D-glucanase. — **AP-3** (1 part) was incubated

with exo-(1→3)-β-D-glucanase in 0.05M acetate buffer, pH 4.7 (500 parts) at 37° as already described[3]. At suitable intervals of time, part of the mixture was heated for 10 min at 100°, and then examined for reducing power (to estimate the degree of hydrolysis). When **AP-3** was completely digested with the enzyme, the degree of hydrolysis was 74%, that is, almost in accordance with the theoretical value deduced from the repeating unit of the polysaccharide. By changing the proportion of the enzyme and the incubation time, 11, 38, and 62% degraded **AP-3** (EH-11%, and the like) were respectively prepared.

O-*Acetylation of* **LAP**. — **LAP** (1 part) was acetylated in dimethyl sulfoxide (80 parts) with acetic anhydride (0.5 part) and pyridine (6 parts) for 2 h at 4°. The mixture was then diluted with water, and dialyzed against running water for 4 days. Partially acetylated **LAP** was obtained from the gelatinous dialyzate by lyophilization. From the ratio of acetyl protons to ring protons in the ^{1}H-n.m.r. spectrum, the degree of substitution was determined to be 0.40.

O-(*Carboxymethyl*)*ation of* **LAP**. — **LAP** (1 part) was *O*-(carboxymethyl)ated, in 2-propanol containing 3% of sodium hydroxide (100 parts), with monochloroacetic acid (1 part) for 5 h at 50°. The mixture was cooled, made neutral with M hydrochloric acid, and de-ionized by dialysis for 4 days. The product was obtained from the dialyzate by lyophilization. Part of the *O*-(carboxymethyl)ated polysaccharide was hydrolyzed with 0.25M sulfuric acid (400 parts) for 6 h at 100°, and the hydrolyzate was reduced with sodium borohydride in the usual way. The reduction product was treated with 2% methanolic hydrogen chloride for 30 min in a sealed tube at 80°, and then the solution was evaporated to dryness. The residue was acetylated with acetic anhydride–pyridine, and the acetates were analyzed by g.l.c. and g.l.c.–m.s. Only two peaks were detected and identified; these corresponded to the alditol acetates from D-glucose and 6-*O*-(carboxymethyl)-D-glucose. From the molar ratio of the compounds, the degree of substitution was determined to be 0.10.

O-(*2-Hydroxyethyl*)*ation of* **LAP**. — **LAP** (1 part) was (2-hydroxyethyl)ated in 1% sodium hydroxide containing 3% of sodium chloride (200 parts) with ethylene oxide (30 parts) for 24 h at 36°. The mixture was made neutral with M hydrochloric acid, and dialyzed against running water for 4 days. The product was obtained from the dialyzate by lyophilization. Part of the (2-hydroxyethyl)ated polysaccharide was hydrolyzed with 0.25M sulfuric acid (400 parts) for 6 h at 100°, and, after the hydrolyzate had been converted into the corresponding alditol acetates, these were analyzed by g.l.c. and g.l.c.–m.s. Only two peaks, corresponding to D-glucose and 6-*O*-(2-hydroxyethyl)-D-glucose, were detected. From the molar ratio of the compounds, the degree of substitution was determined to be 0.40.

O-*Methylation of* **LAP**. — **LAP** (1 part) was methylated with dimethyl sulfate (1.2 parts) by the Haworth procedure. After completion of the reaction, the mixture was made neutral with hydrochloric acid, and dialyzed against running water for 4 days. Partially methylated polysaccharide was obtained from the dialyzate by lyophilization. Part of the methylated polysaccharide was hydrolyzed with 90% formic acid for 3 h at 100°, and then with 0.25M sulfuric acid overnight at 100°.

After the hydrolyzate had been converted into the corresponding alditol acetates, the mixture was analyzed by g.l.c. and g.l.c.–m.s. Seven peaks, corresponding to 2,4,6-tri- (3.8%), 2,6- and 4,6-di- (16.6%), 2- and 6-*O*-methyl-D-glucose (42.5%), and D-glucose (37.1%) were detected. From the molar ratios of the compounds, the degree of substitution by the methyl group was calculated to be 0.85.

Assay of antitumor activity. — A piece of Sarcoma 180 tumor fragment was transplanted subcutaneously into the right groin of 5-week-old, STD-ddY female mice. Test samples, dissolved, or suspended, in saline solution were intraperitoneally injected daily for 10 days, starting 24 h after tumor implantation. The major and minor axes of the tumor were measured by use of a slide caliper, and converted into tumor size (mm^3), on each 7th day. The inhibition ratios were calculated by use of the formula: inhibition ratio $(\%) = (1 - T/C) \times 100$, where C is the average tumor size of the control group, and T is that of the group tested.

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