[Chem. Pharm. Bull. 19(4) 821—826 (1971)]

UDC 547.458.05.09 : 581.192 : 615.277.4.011.5

Antitumor Polysaccharides from Some Polyporaceae, *Ganoderma applanatum* (Pers.) Pat[1] and *Phellinus linteus* (Berk. et Curt) Aoshima[2]

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(Received November 20, 1970)

Antitumor polysaccharide preparations G-Z and P-Z were fractionated from the water soluble extracts of *Ganoderma applanatum* (Pers.) Pat and *Phellinus linteus* (Berk. et Curt) Aoshima, basidiomycetes of Polyporaceae, respectively, by fractional precipitation with ethanol and cetyltrimethylammonium hydroxide. The structures of G-Z and P-Z consist of β-(1→3), (1→4) linked D-glucose residue and β-(1→3) linked D-glucose residue, respectively. These polysaccharide preparations have marked antitumor activity against transplanted sarcoma 180 in mice, and a complete regression of tumors was observed in more than half of animals with no sign of toxicity. Some derivatives of P-Z were synthesized and their antitumor effects were also examined.

Previously we have reported that water soluble extracts of some basidiomycetes of Polyporaceae, *Ganoderma applanatum* (Pers.) Pat, *Phellinus linteus* (Berk. et Curt) Aoshima and *Coriolus versicolor* (Fr.) Quél. and some edible mushrooms inhibit the growth of sarcoma 180 implanted subcutaneously in mice,[4,5] and that the polysaccharide preparations, lentinan[6,7] isolated from *Lentinus edodes* (Berk.) Sing., and pachymaran[8] derived chemically from pachyman, have a prominent activity in a quite small dosage. Besides, the antitumor effect of the polysaccharide preparations from some other basidiomycetes, such as *Schizophyllum commune*,[9] *Crepidotus*[10] and others[11,12] also have been reported.

The present paper concerns with the fractionation and the purification of the crude water soluble extracts of *Ganoderma applanatum* and *Phellinus linteus*, and with some structural studies and the antitumor effects of these polysaccharide preparations and its derivatives.

Experimental

Basidiomycetes——The dried fruit bodies of wild *Ganoderma applanatum* and *Phellinus linteus* used were commercially available samples in Tokyo, which were collected in various parts of Japan. *G. applanatum* is also known as *Elfvingia applanata* (Pers.) Karst and *Fomes applanatus* (Pers.) Gillet which are synonyms.

Paper Chromatography——Paper chromatograms were run on Toyo Roshi No. 51A paper by descending method with following solvent systems (v/v): (A) *n*-butanol–AcOH–H_2O (4:1:5), (B) *n*-butanol–pyridine–H_2O (6:4:3). Reducing sugars were detected by aniline hydrogen phthalate or *p*-anisidine sulfuric acid in *n*-butanol saturated with water.

1) Japanese name: Kofuki-Sarunokoshikake.
2) Japanese name: Meshimakobu.
3) Location: *Tsukiji 5-1-1, Chuo-ku, Tokyo.*
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Thin-Layer Chromatography——Thin-layer chromatography was performed on Silica gel G (Merck) with solvent system (C) benzene–MeOH (96:4), and products of partial acetoylsis were detected with dil. H_2SO_4 by baking for *ca.* 30 min at 130° on the system C.

Gas Chromatography——Gas chromatograms of complete acid hydrolysates were developed by a model Shimadzu GC-1B gas chromatograph equipped with flame-ionization detector in a glass column (150 cm $\times$ 0.4 in side diameter) which was packed with 0.8% OV-17 (80—100 mesh) on 0.3% Shimalite W at 210°.

Electrophoresis——For the confirmation of purity of each polysaccharide fraction, paper electrophoresis was carried out on Whatman GF-83 glass fiber paper in following system; 0.1M sodium borate buffer pH 9.3, 1600V/40 cm, 1 hr, 0°. *p*-Anisidine sulfuric acid was used as spray reagent and each spot of polysaccharide fraction was detected by baking for *ca.* 30 min at 130°.

Opitcal Rotation——Unless otherwise stated, optical rotations of each sample were measured in 0.1N NaOH aqueous solution in concentration of 1% with 1 cm cell.

Infrared Spectra——Infrared analysis of the each polysaccharide fraction was done as KBr disk.

Extraction of Crude Polysaccharides from Basidiomycetes——The dried fruit bodies of *G. applanatum* (21 kg) were cut into small pieces and immediately extracted with boiling water (150 liters) for 6 hr, and this procedure was repeated thrice. The extracts (G-A) were then filtered and concentrated under the reduced pressure. Crude polysaccharide fraction (G-B) was precipitated with addition of five times volume of EtOH from the concentrated dark brown solution. The precipitates were collected by centrifugation, washed with EtOH and ether, and dried *in vacuo* at room temperature. Yield of crude polysaccharide fraction (G-B) was 375 g. The corresponding fraction (P-B) from *P. linteus* (20 kg) weighed 122 g. The supernatants (G-C and P-C) in EtOH precipitation had no antitumor activity, in both basidiomycetes.

Fractionation and Purification of the Crude Polysaccharides from *G. applanatum* into Polysaccharide Preparation G-Z——Fractionation and purification of crude polysaccharide fraction G-B into polysaccharide preparation G-Z are shown schematically in Chart 1.

Ganoderma applanatum (dried fruit bodies, 21 kg)
↓ cut to small piece, extraction with hot water, 6 hr, 90—100°, filtration
filtrate (G–A)
↓ concentration under the reduced pressure, precipitation by addition of five times volume of ethanol, centrifugation
precipitate (G–B) | supernatant (G–C)
↓ solubilization with water (5.5 liters), precipitation with cetyltrimethylammonium hydroxide (pH 11.3), overnight, centrifugation
precipitate | supernatant (G–E)
↓ solubilization with 5% acetic acid, centrifugation
supernatant
↓ precipitation with ethanol, centrifugation
precipitate 53 gm (G–D)
↓ deproteinization with Sevag method, centrifugation
aqueous phase
↓ precipitation with ethanol
precipitate 18.5 gm (G–F)
↓ decolorization with hydrogen peroxide
cream-coloured powder 3.9 gm (G–Z)

Chart 1. Fractionation and Purification of Water Soluble Extracts from *Ganoderma applanatum* into Polysaccharide Preparations G–F and G–Z

304 g of the fraction G-B was dissolved into 5.5 liters of water, and to the solution, 0.25M aqueous CTA-OH[13] was added dropwise under vigorous stirring until no more precipitate was formed (pH 11.3). All the

13) The abbreviation used are: CTA-OH is cetyltrimethylammonium hydroixde.

precipitate formed was collected by centrifugation at 9000 rpm for 5 min, then suspended in 2.7 liters of 5% AcOH, stirred to solubilize for 5 hr, and the insoluble materials were removed by filtration. The filtrate was added to three times volume of EtOH, and the precipitate formed was collected by centrifugation, washed with EtOH and ether, and dried *in vacuo*. Yield of this precipitate (G-D) was 53 g. The supernatant (G-E) by CTA-OH precipitation had no antitumor effect. Fraction G-D was deproteined by Sevag's method,[14] and yield of deproteined fraction (G-F) was 18.5 g. Fraction G-F, brown powder, was decolored by H_2O_2. G-F was dissolved in water, adjusting to pH 8 with 10% NH_4OH, and then added 10% aqueous H_2O_2. At this step, small amount of precipitate formed was removed by centrifugation, and the supernatant was added to three times volume of EtOH, and precipitate formed was collected, washed with EtOH and ether, and dried *in vacuo* to give a cream-colored powder, polysaccharide preparation G-Z, in a yield of 3.9 g.

Fractionation and Purification of the Crude Polysaccharides from *P. linteus* into Polysaccharide Preparation P-Z——Fractionation and purification of crude polysaccharide fraction P-B into polysaccharide preparation P-Z are shown schematically in Chart 2.

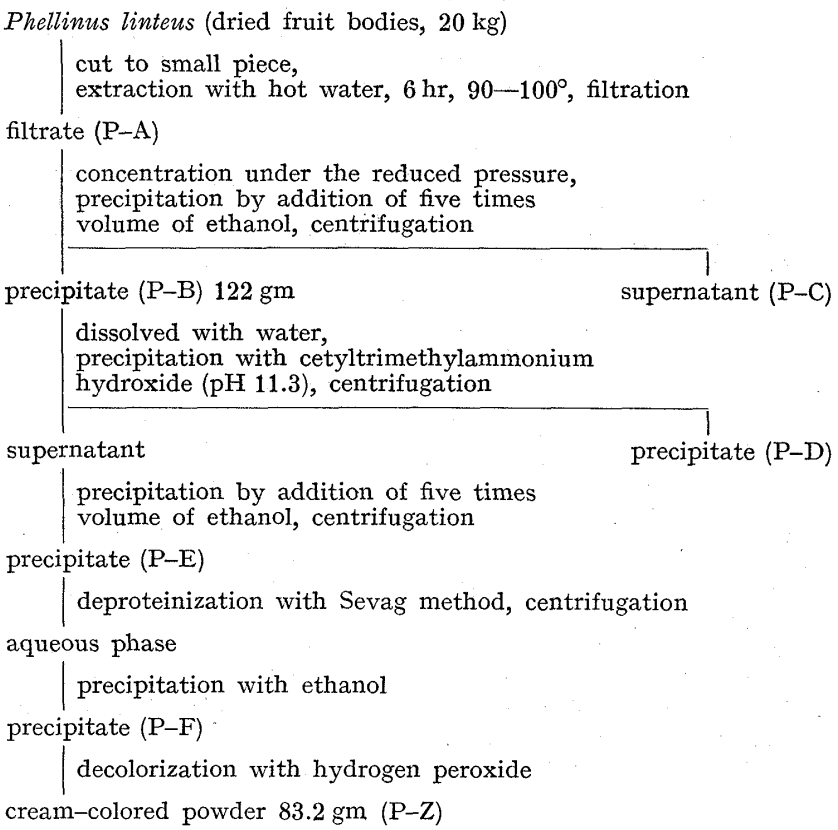

Chart 2. Fractionation and Purification of Water Soluble Extracts from *Phellinus linteus* into Polysaccharide Preparations P–F and P–Z

30 g of fraction P-B was dissolved into 1.35 liter of water, and centrifuged to remove a small amount of insoluble materials, and 0.25M aqueous CTA-OH (53.9 ml) was added dropwise under stirring to the solution until no more precipitate was formed (pH 11.5). All the precipitate formed was removed by centrifugation at 8500 rpm for 10 min, and the supernatant was added to five times volume of EtOH. The precipitate formed was collected, washed by EtOH and ether, and dried *in vacuo*. Thus a brown powder, fraction P-E, was obtained in a yield of 7.2 g. P-E was decolored by H_2O_2, deproteined by Sevag's method, and cream-colored powder, polysaccharide preparation P-Z, was obtained in a yield of 83.2g from 143 g of fraction P-B.

Chemical Properties of Polysaccharide Preparations G-Z and P-Z——Polysaccharide preparations G-Z $[\alpha]_D^{23.5}$ -23° (c=1, in 0.1N NaOH) and P-Z $[\alpha]_D^{23.5}$ -8.5° (c=2, in 0.1N NaOH) are easily soluble in water, slightly soluble in dimethylformamide, and insoluble in almost all of organic solvents. G-Z and P-Z behaved as one spot in high voltage glassfiber paper electrophoresis (G-Z: M_G=1.41; P-Z: M_G=1.66), and did not contain nitrogen and sulphur except carbon, hydrogen, oxygen and minutes amount of ashes. Infrared spectra of G-Z and P-Z exhibited absorption at 890 cm^{-1} assigned to β-glucosidic linkage. G-Z passed scarcely through

14) A.M. Staub, "Method in Carbohydrate Chemistry V," Academic Press, New York, 1965, pp. 5—6.

DIAFLO ultrafiltration membrane XM-50 (for 50000 M.W.) after removal of low molecular weight substances by use of UM-10 membrane (for 10000 M.W).

Complete Acid Hydrolysis and Partial Acetolysis of G-Z and P-Z——G-Z and P-Z were hydrolyzed by boiling in a sealed tube successively with 1NH_2SO_4 at 100° for 6 hr, then the solution was neutralized with $BaCO_3$, filtered and evaporated under reduced pressure at 30° to a syrup. The detection of the monosaccharide constituents was carried out by paperchromatography using solvent system (A) and (B), and gas chromatography after trimethylsilylation of hydrolyzates in dried pyridine. The results showed that the hydrolyzates of these samples, G-Z and P-Z, consisted almost entirely of glucose, although small amount of xylose was matography after trimethylsilylation of hydrolyzates in dried pyridine. The results showed that the hydrolyzates of these samples, G-Z and P-Z, consisted almost entirely of glucose, although small amount of xylose was contained in G-Z. (G-Z: Rf=0.20 (solvent system A), retention time 7.7, 10.45, 14.20 min. Glucose: Rf=0.20, retention time 10.45, 14.20 min. Xylose: 7.7 min; P-Z: Rf=0.24, retention time 10.45, 14.20 min. Glucose: Rf=0.24, retention time 10.45, 14.20 min).

Partial acetolysis of slightly impure polysaccharides G-F and P-F were carried out with following method: A cold 86 ml of the mixture of $(AcO)_2O$, glacial AcOH and conc. H_2SO_4 (48:32:6) were added to 1 g of each sample and the mixture was kept at 30° for 7 days, and then for 30 min at 80°. The solution was cooled and poured into ice water, neutralized with Na_2CO_3 and then extracted with $CHCl_3$. The $CHCl_3$ extract was washed with water and dried with Na_2SO_4, and evaporated *in vacuo* to a syrup. The determination of oligosaccharide acetates was performed by thin-layer chromatography using solvent system (C). Only laminaribiose octaacetate was detected as the product in partial acetolysis of P-F and no other disaccharides could be detected by this method. In partial acetolysis of G-F, on the other hand, cellobiose octaacetate besides laminaribiose acetate as main product was detected (Rf: sample from P-F, 0.62, 0.34,; sample from G-F, 0.62, 0.42, 0.34; glucose acetate, 0.61,; cellobiose acetate, 0.44; laminaribiose acetate, 0.34).

Some Derivatives of P-Z——Sulfonation: *p*-Nitrobenzenesulfonyl chrolide (15 g) was added to an ice-cooled solution of P-Z (6 g) in a mixture of anhydrous pyridine (100 ml) and dimethylformamide (20 ml). After the solution has been stirred at room temperature for 3 days, water (2 ml) was added. 3 Volumes of EtOH were added to the solution, and resulting precipitate was collected, washed with water and EtOH, and dried *in vacuo*. Compound so obtained was pink powder, PZ-Ns, (6 g).

Iodation: PZ-Ns (3 g) was dissolved in a mixture of acetonyl acetone (200 ml) and dimethylformamide (20 m), NaI (3 g) was added, and the solution was refluxed for 5 hr, and then cooled. 7 volumes of EtOH were added to the solution, and the resulting precipitate was collected, washed with water and EtOH, and dried *in vacuo*. Compound obtained was yellow powder, PZ-I, (1 g).

Reduction: P-Z (2 g) was dissolved in water (50 ml) ,$NaBH_4$ (3 g) was added, and the solution was stirred for 24 hr. The resulting solution was dialized for 2 days and then evaporated to concentrated solution. 5 volumes of EtOH were added to the solution. The resulting precipitate was washed with water and acetone. The white powder, PZ-R, (1.2 g) was obtained.

Assay of Antitumor Activity——Antitumor activity was tested by the method described previously.[7] 7-day-old ascites of sarcoma 180 was transplanted subcutaneously in 0.05 ml (about 8×10^6 cells) doses in the right groin of mice. Test samples dissolved in distilled water were injected intraperitoneally once a day for 10 days starting 24 hr after tumor transplantation. At the end of the 5th week, the mice were killed, and the tumors were dissected out and weighed to determine the inhibition ratios, and complete regression of the tumors was recorded.

Result and Discussion

Antitumor polysaccharide preparations G-Z and P-Z were fractionated from the water soluble extracts of *G. applanatum* and *P. linteus* by fractional precipitation with EtOH and CTA-OH, and fractional solubilization with AcOH. Fractionation by precipitation of polysaccharides with CTA-OH was especially effective regarding to the antitumor effect.

The antitumor effects of the polysaccharide preparations and their crude subfractions from these basidiomycetes against subcutaneously implanted sarcoma 180 are shown in Table I and II, which also list the results on some derivatives of P-Z.

The results obtained with G-B and P-B were not so significantly different from those with samples G-A and P-A except a slight improvement in the inhibition ratios, but these results showed that active fractions (G-B, P-B) and non-active fractions (G-C, P-C) were completely devided by alcohol precipitation. Fractionation of G-B and P-B by CTA-OH to G-D, P-E and G-E, P-D also effected a separation in antitumor activity. Although fractions G-D and P-E were toxic, the polysaccharide preparations G-F and P-F after deproteinization by Sevag's method did not show any toxicity, suggesting that some toxic materials were extracted with

TABLE I. Antitumor Effect of Polysaccharide Fractions from *Ganoderma applanatum* against Sarcoma 180 Implanted Subcutaneously in Swiss Albino Mice,; Route: *i.p.*; Vehicle: Aq. dest.

Samples	Doses (mg/kg × day)	Average tumor weight (g)	Inhibition ratio (%)	Complete regression
G-A	200 × 10	2.4	64.9	5/10
	control	6.8		0/10
G-B	200 × 10	3.6	61.9	2/10
	control	9.6		0/10
G-C	200 × 10	7.3	−19.3	0/10
	control	6.0		0/10
G-D	110 × 10	2.1	78.1	1/8
	control	9.6		0/10
G-E	150 × 10	9.4	1.5	0/4
	control	9.6		0/10
G-F	150 × 10	1.3	85.6	6/9
	control	9.0		0/9
G-F	100 × 10	0.4	95.3	5/10
	control	8.5		0/10
G-Z	50 × 10	3.4	54.7	5/9
	control	7.5		0/9

TABLE II. Antitumor Effect of Polysaccharide Fractions and Their Some Derivatives from *Phellinus linteus* against Sarcoma 180 Implanted Subcutaneously in Swiss Albino Mice; Route: *i.p.*; Vehicle: Aq. dest.

Samples	Doses (mg/kg × 10)	Average tumor weight (g)	Inhibition ratio (%)	Complete regression
P–A	200 × 10	0.2	96.7	7/8
	control	6.8		0/8
P-B	115 × 10	0.8	89.2	5/8
	control	7.4		0/10
P-D	150 × 10	2.6	64.9	4/8
	control	7.4		0/10
P-E	150 × 10	0.6	91.9	7/10
	control	7.4		0/10
P-F	150 × 10	0.5	94.0	9/10
	control	8.4		0/9
P-Z	50 × 10	0.2	94.4	7/9
	control	3.6		0/9
PZ-Ns	150 × 10	0.2	90.0	8/9
PZ-I	150 × 10	1.3	65.0	0/10
PZ-R	150 × 10	0.3	85.0	5/8
	control	2.0		1/9

$CHCl_3$. A considerable improvement in the inhibition ratios was observed in the samples, G-F and P-F. A complete regression of tumors was observed in more than half of the mice and inhibition ratios were over 95% in G-F, P-F and P-Z.

G-Z and P-Z were considered to be a glucan, as the hydrolysates of these samples consisted almost entirely of glucose. P-Z was considered to have a β-(1→3)-linked D-glucose residue, based on the result of the partial acetolysis, in which only laminaribiose was detected and no other disaccharides could be observed. On the other hand, G-Z was considered to consist partially of a mixture of β-(1→3) and (1→4) linked D-glucose residues by the same chemical method. Laminaribiose and cellobiose were detected as disaccharides.

Recently, some investigations of antitumor effect of β-(1→3) glucans, for example, lentinan,[6] pachymaran[8] and others,[9,10] from natural sources have been reported, and a significance of higher structure of the polysaccharides was available on the structure-activity relationship.[15] As one of series of trials for the elucidation of this correlation, the chemical modifications of P-Z were carried out. PZ-Ns prepared by sulfonation on P-Z have the same effect as that of P-Z, indicating that this modification should be useful for study on the relationship between antitumor activity and physical properties. This sulfonated PZ-Ns was converted to iodide compound, PZ-I, which decreased the antitumor effect. The significant difference between PZ-Ns and PZ-I may be caused by some structural changes in its secondary structure due to the iodation.[16] For PZ-R prepared by the reduction with $NaBH_4$, its activity was similar to that of P-Z, indicating that reduced end-groups of the polysaccharide did not contribute to the antitumor activity. The work on the metabolism[17] of the labeled polysaccharides prepared by the reduction with NaB_3H_4, and on the action mechanism of the polysaccharides as a immuno-accelerator[18] are published elsewhere.

Of interest may be the fact the basidiomycetes of Polyporaceae have been said to be crude anticancer drugs in Japan and some Asian countries, and that the polysaccharides from the Polyporaceae were now found to be somewhat active against transplanted tumor in mice.

Acknowledgement We thank to Dr. W. Nakahara for his encouragement, to Miss M. Noda her cooperation in antitumor test, and Messrs N. Wada, S. Suzuki, Y. Yamazaki and M. Matsui for their skinful assistances. A part of the expenses for this study was supported by a Grand in Aid of the Japanese Ministry of Health and Welfare.

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