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Fractionation and Purification of the Polysaccharides with Marked Antitumor Activity, Especially Lentinan, from *Lentinus edodes* (Berk.) Sing. (an Edible Mushroom)[1]

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SUMMARY

Six polysaccharide preparations, lentinan, LC-11, LC-12, LC-13, EC-11, and EC-14, were isolated from the water-soluble extracts of *Lentinus edodes* (Berk.) Sing., an edible mushroom, by fractional precipitation with ethanol and cetyltrimethylammonium hydroxide, fractional solubilization with acetic acid, and diethylaminoethyl cellulose column chromatography.

The first two polysaccharide preparations have a strong antitumor effect; lentinan, especially, markedly inhibited the growth of Sarcoma 180 implanted s.c. in mice, inducing almost complete regression of tumors at doses of 1 mg/kg X 10 with no sign of toxicity. LC-11 was also effective in 5 to 25 mg/kg X 10 doses.

The purity and properties of these antitumor polysaccharides are discussed.

INTRODUCTION

The antitumor activity of polysaccharide preparations from various natural sources, such as higher plants (1, 12, 21, 22, 27, 29, 30), fungi (3–6, 8, 9, 14–17, 20, 23, 24, 31, 36, 41), lichen (11, 25, 37, 38), and bacteria (7, 26, 33, 35, 39), have been reported of late, in addition to the well-known yeast cell-wall polysaccharide, zymosan (3, 4).

However, most of these polysaccharides have not been adequately confirmed as to their purities and chemical and physical properties.

In previous papers (6, 14, 15), we reported that water-soluble extracts of some basidiomycetes inhibited the growth of Sarcoma 180 implanted s.c. in mice and that the polysaccharide preparations from water extracts of *L. edodes*, an edible mushroom popular in Japan, had a prominent activity in very small dosages. The present paper concerns the details of fractionation, purification, and antitumor activity of these polysaccharide preparations.

MATERIALS AND METHODS

Mushroom. *L. edodes* (Berk.) Sing. [synonyms: *Cortinellus edodes* (Berk.) S. Ito and Imai, *Armillaria edodes* (Berk.) Sacc., and *Cortinellus shiitake* (Takeda) Henn.] used was on sale in Tokyo markets. It was washed with cold water to eliminate adherent extraneous matter.

Reagents. CTA-OH,[2] ethanol, methanol, sodium hydroxide, chloroform, 1-butanol, boric acid, and other reagents used were all of commercial purities.

Electrophoresis. For confirmation of the purity of each polysaccharide preparation, paper electrophoresis was carried out on Whatman GF-83 glass fiber paper in the following 2 systems: System A, borate buffer, pH 9.3, 2 kV, 45 min, 0°; System B, 2% aqueous sodium hydroxide, 700 V, 14 hr, 0°. *p*-Anisidine-sulfuric acid was used as spray reagent in both cases, and each spot of polysaccharides was generally detected by baking at 130° for about 30 min.

Column Chromatography. Crude LC-11 and LC-13 fractions were placed on a DEAE-cellulose column (phosphate type, 3.5 x 30 cm), and the column was eluted stepwise with water, borate buffer, and aqueous sodium hydroxide, with anthrone as a color reagent. Colorimetry was carried out on a Coleman Model 6D junior spectrophotometer at 620 mμ.

pH Measurement. pH measurements were usually carried out at room temperature under stirring by pH meter.

Optical Rotation. Optical rotations of each sample were measured in aqueous solution or in 1% 2.5 N aqueous sodium hydroxide with a 1-cm cell, except for some samples.

Ultracentrifugation. The sedimentation patterns of lentinan were measured in a Hitachi Model UCA-1A analytical centrifuge at 21.2° in 1% aqueous sodium hydroxide, and photographs were taken 30, 50, 70, 90, and 160 min after 40,610 rpm had been reached.

Assay of Antitumor Activity. Swiss albino and SWM/Ms mice weighing about 20 g were used for the antitumor assay. Seven-day-old Sarcoma 180 ascites, 0.05 ml (about 8 X 10^6 cells), were transplanted s.c. into the right groins of mice. The test samples, dissolved or suspended in distilled water, were injected i.p., after sterilization at 120° for 15 min, daily for 10 days starting 24 hr after tumor implantation. The growth of tumors was charted weekly for 5 weeks. At the end of the 5th week, the mice were killed, and the tumors were extirpated

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[2] The abbreviations used are: CTA-OH, cetyltrimethylammonium hydroxide; DEAE-cellulose, dimethylaminoethyl cellulose.

and weighed. The inhibition ratios were calculated by the following formula:

$$\text{Inhibition ratio } (\%) = [(A - B)/A] \times 100$$

where A is the average tumor weight of the control group, and B is that of the treated group.

RESULTS

Extraction of the Crude Polysaccharides from the Mushroom

The fresh fruit bodies of *L. edodes* (200 kg) were washed with water, disintegrated in a mixer or Waring Blendor, and extracted with boiling water (1000 liters) for 8 to 15 hr. The suspension obtained was filtered or centrifuged to remove the insoluble matter; the aqueous extract was concentrated under the reduced pressure to the point where a slight turbidity was observed. The crude polysaccharide fraction was obtained by precipitation with ethanol in 2 steps from the concentrated extract. First, addition of an equal volume of ethanol to the concentrated solution gave the fibrous substance, Fraction L (320 g) and, after removal of this fraction and addition of a further 3 volumes of ethanol, a brown powder precipitation, Fraction E (200 g), was obtained and collected by filtration or centrifugation. The supernatant from the ethanol precipitation had no antitumor effect.

Fractionation and Purification of the Crude Fractions into Polysaccharide Preparations, LC-11, LC-12, LC-13, LC-33 (Lentinan), EC-11, and EC-14

Fractionation of L into LC-1 and LC-3 Fractions. Fraction L, 50 g, was suspended in 2 liters of water and homogenized to a viscous, brown solution with a Waring Blendor at room temperature. Then 20 liters of water were added, and the mixture was stirred for 1 to 2 hr to obtain a clear homogeneous solution. To the solution, 0.2 M aqueous CTA-OH, pH 13.2, was added dropwise with vigorous stirring. After the formation of a small quantity of fibrous precipitate at pH 7 to 8, a large quantity of white precipitate appeared at a pH 10.5 to 11.5. Aqueous CTA-OH was added until no more precipitate was formed (pH 12.8). All the precipitates were collected by centrifugation at 9000 rpm for 5 min, washed with ethanol, suspended in 1.2 liters of 20% acetic acid, and stirred for 5 min at 0°, fractionating the precipitates into an insoluble part, LC-3, and a soluble part, LC-1. Four volumes of ethanol were added to the soluble part, and the precipitate formed was collected, washed twice with ethanol and once with ether, and dried under vacuum at room temperature.

Fractionation and Purification of LC-3 Fraction into LC-33 (Lentinan). Further purification of LC-3 gave 2 spots with high-voltage electrophoresis (System A). LC-3 was washed with 1 liter of 50% acetic acid for 3 min at 0° by stirring in Waring Blendor, centrifuged, and fractionated into insoluble and soluble parts. The insoluble part was dissolved in 2 liters of 6% aqueous sodium hydroxide, contaminants were removed by centrifugation, 4 liters of ethanol were added to the supernatant, and the precipitate formed was collected, washed twice with ethanol and then once with ether, and dried under vacuum to give powdered LC-33. The fraction was deproteined by the Sevag method (40) with chloroform and 1-butanol, followed by precipitation with 3 volumes of ethanol, and was washed twice with methanol and once with ether and dried under vacuum at room temperature in a desiccator over calcium chloride.

Fractionation and Purification of LC-1 Fraction into LC-11, LC-12, and LC-13 Fractions. LC-1, 23 g, was dissolved in 1.2 liters of water, and contaminants were removed by centrifugation. Aqueous 0.2 M CTA-OH was added dropwise to the supernatant under stirring with careful measurement of rise of pH, and LC-1 was fractionated into 5 fractions, LC-1A, LC-1B, LC-1C, LC-1D, and LC-1E. By these procedures, 3 polysaccharide fractions, pure LC-12 (LC-1D), crude LC-11 (LC-1B), and crude LC-13 (LC-1E), were obtained. Each preparation gave 1 spot in high-voltage paper electrophoresis in both System A and System B.

For completely pure polysaccharide preparations, purification of crude LC-11 and LC-13 fractions were undertaken with the use of DEAE-cellulose column chromatography. One g of LC-1B was dissolved in 10 ml of water and chromatographed on a column (3.5 x 32 cm) packed with DEAE-cellulose (phosphate type). At the first stage, water was eluted and fractionated into a 25-ml volume of eluent, then eluted with 0.25 M phosphate buffer and 0.25 N aqueous sodium hydroxide. Column chromatography of LC-1E was similarly carried out on a 3.0- x 35-cm column. Thus, the pure polysaccharide preparations, LC-11 (450 mg) and LC-13 (650 mg), were obtained from the fractionation of LC-1B and LC-1E, respectively.

Fractionation and Purification of Crude Fraction E into Polysaccharide Preparations EC-11 and EC-14. Fraction E, 310 g, was dissolved in 6 liters of water and centrifuged to remove the insoluble contaminants; then 3.6 liters of aqueous CTA-OH were added until no more precipitate was formed (pH 12.6). To the supernatant, after centrifugation, 3 volumes of ethanol were added; the precipitate formed was then collected and decolorized by eluting with water through Duolite A-7 (acetate form) ion-exchange resin to obtain a white powder of EC-1 by precipitation with 4 volumes of ethanol. EC-1, 24.9 g, was dissolved in 1 liter of 0.1 M aqueous boric acid, and 0.2 M aqueous CTA-OH was added dropwise during careful pH measurements. The precipitate formed was treated with 10% acetic acid; after centrifugation 3 volumes of ethanol were added to the supernatant, and 14.2 g of pure EC-11 preparation were recovered by evaporation of the ethanol under reduced pressure to remove boric ester.

To the supernatant after CTA-OH precipitation, 5 volumes of ethanol were added and 2.91 g of white powdered EC-14 preparation were obtained.

Fractionation and purification of the crude fractions, L and E, into 6 polysaccharide preparations are shown schematically in Chart 1.

Confirmation of Purity of Six Polysaccharide Preparations

In Table 1, the optical rotations and solubility of each polysaccharide are summarized. It is evident that 6 polysaccharides, *i.e.*, LC-11, LC-12, LC-13, EC-11, EC-14, and lentinan,

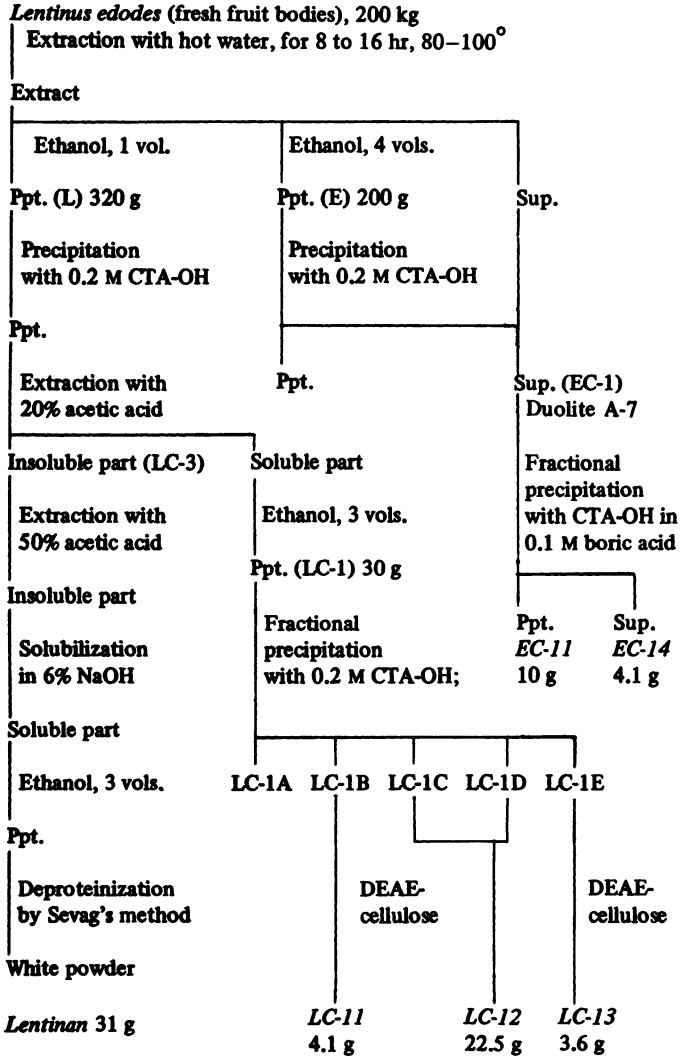

Chart 1. Extraction of polysaccharides from *L. edodes* and fractionation of water-soluble extract into 6 polysaccharide preparations, lentinan, LC-11, LC-12, LC-13, EC-11, and EC-14.

are fractionated from the water-soluble extracts of *L. edodes*. Each polysaccharide behaves as 1 spot on high-voltage glass fiber electrophoresis with Systems A and B. Purity of lentinan was also confirmed by ultracentrifugation in 1% aqueous sodium hydroxide solution, and sedimentation patterns of lentinan showed a single peak, as shown in Fig. 1.

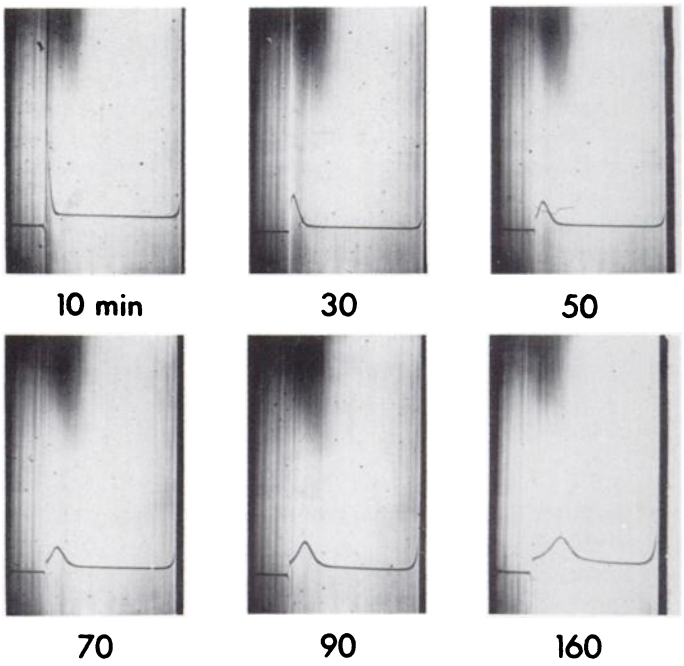

Fig. 1. Sedimentation patterns of lentinan showed a single peak, in 1% aqueous sodium hydroxide at 212°. Photographic exposures were made 30, 50, 70, 90, and 160 min after 40,610 rpm had been reached.

Deproteinization was carried out by Sevag's method with an appropriate purification process for removal of the contaminating protein or glycoprotein. These polysaccharides do not contain nitrogen, sulfur, phosphorus, and ashes.

Table 1

Optical rotation and solubility of each polysaccharide preparation from L. edodes

Fractions	$[\alpha]_D^{20}(c = 1)$	Solubility
Lentinan	+19.5 to +21.5° (10% NaOH)	Water insoluble Alkali soluble
LC-11	−16.3° (H_2O)	Water soluble
LC-12	+181.3° (H_2O)	Water soluble
LC-13	−15.7° (1% NaOH)	Water difficult to solve
EC-11	+92.8° (H_2O)	Water easily soluble
EC-14	−13.2° (H_2O)	Water soluble

Chemical Properties of Lentinan

Lentinan is insoluble in cold water, acid, and almost all of organic solvents such as alcohol, ether, chloroform, pyridine, and hexamethylphosphoramide; slightly soluble in hot water and dimethyl sulfoxide; and soluble in aqueous alkali and formic acid. These solubility behaviors are quite similar to that of the β-(1→3) glucan analogous polysaccharides known so far. It is quite stable against strong acid such as sulfuric acid and hydrochloric acid but is easily decomposed with the action of alkali at a raised temperature.

Elementary analysis of lentinan shows C at 40.54% and H at 7.02%. No nitrogen, phosphorus, sulfur, and any other element, as well as ashes, are detected. Lentinan begins to decompose at 150° and is completely decomposed at 250°.

Infrared spectra and nuclear magnetic resonance spectra show absorptions at 890 cm^{-1} and τ 5.4, respectively, which show the presence of β-glycoside linkage.

Antitumor Activity

The results of the antitumor testing of 6 polysaccharides with Swiss albino mice are shown in Table 2. The tumor-inhibitory effect of lentinan was highly striking. In 1 to 5 mg/kg X 10 doses, the inhibition ratio was 95 to 97.5%, and in dosages of 0.2 mg/kg X 10, the tumors underwent complete regression in 6 out of 10 mice. The results of the assay with SWM/Ms mice are shown in Table 3. Four lots of lentinan (α, β, γ, δ) showed the same antitumor activity, and inhibition ratio was almost 100% in 1 mg/kg X 10 doses. With 0.5 to 1 mg/kg X 10 doses, the inhibition ratio of lentinan was 95 to 100%, but with doses of 5 mg/kg X 10 and of 0.1 mg/kg X 10, the antitumor activity decreased and complete regression of tumor did not occur.

Our preliminary report (6) showed that the tumor had regressed in all the mice given 15 to 30 mg/kg X 10 of LC-1. From the results reported here, it was clarified that LC-11 was the active principle of LC-1 fraction, other components of LC-1 (namely, LC-12 and LC-13) showing no antitumor activity. Lentinan and LC-11 were nontoxic as far as could be detected during antitumor tests.

DISCUSSION

Characteristic methods for the preparation of purified polysaccharides in our experiments were extraction by hot water, not with alkali, fractional precipitation of polysaccharides with a quaternary ammonium salt such as CTA-OH, and fractional solubilization of the salts of polysaccharides with a dilute acid such as acetic acid. Application of precipitation of polysaccharides with CTA-OH (34) was useful, because the inactive EC-11 and EC-14 was not precipitated by the reagents while the active LC-11 and lentinan were precipitated. Fractional solubilization with acetic acid was used to free the polysaccharides from the salts and to recover the polysaccharides by precipitation with addition of ethanol.

Table 2

Antitumor effect of 6 polysaccharide preparations from L. edodes against Sarcoma 180 implanted s.c. in Swiss albino mice

The polysaccharides were injected i.p. with distilled water as the vehicle.

Sample	Doses (mg/kg X 10)	Body weight change (g)	Average tumor weight (g)	Inhibition ratio (%)	Complete regression
Lentinan	25	+1.8	1.7	73.0	2/9
	5	+1.2	0.15	97.5	7/10
	1	+2.7	0.31	95.1	6/10
	Control	+6.0	6.3		0/10
	5	+2.2	0.2	97.3	7/10
	0.2	+4.1	1.6	78.1	6/10
	Control	+6.7	7.3		0/10
LC-11	25	+3.3	0.7	91.0	5/8
	5	+3.3	0.5	93.6	6/10
	1	+4.5	3.5	55.1	1/9
	Control	+4.6	7.8		0/10
LC-12	25	+2.7	7.0	−37.3	0/10
	5	+3.5	6.0	−17.6	0/10
	1	+3.0	5.1	0	0/10
	Control	+3.5	5.1		0/10
LC-13	25	+2.5	4.4	6.4	0/10
	5	+4.8	5.6	−19.1	0/10
	Control	+1.0	4.7		0/10
EC-11	50	+2.1	6.2	−1.6	0/10
	Control	+5.9	6.1		0/10
EC-14	50	+3.0	4.4	42.1	0/10
	Control	-1.0	7.6		0/10

Table 3

Antitumor activity of lentinan against sarcoma 180 implanted s.c. in SWM/Ms mice
α, β, γ, and δ are lots of lentinan.

Sample	Doses (mg/kg X 10)	Body weight change (g)	Average tumor weight (g)	Inhibition ratio (%)	Complete regression
Lentinan (α)	1	+4.8	0	100	7/7
Lentinan (β)	1	+5.4	0.2	98.8	6/8
Lentinan (γ)	1	+3.7	0.05	100	7/8
Lentinan (δ)	1	+5.6	0.1	99.4	5/8
	Control	+1.7	9.5		0/10
Lentinan (α)	5	+5.0	6.2	44.0	0/9
	0.5	+1.9	0.6	94.7	8/10
	0.1	+4.6	8.9	19.1	0/9
	Control	+4.5	11.0		0/10

Antitumor polysaccharides have been reported to occur in some higher plants, fungi, basidiomycetes, lichens, and bacteria. These antitumor polysaccharides varied from glucan (5, 6, 8, 17, 23, 25, 31, 38), mannan (41), and hemicellulose (22) to heteroglycan (28) and lipopolysaccharides (7, 26, 39), but most of them were of uncertain chemical purity.

We believe that the active polysaccharides reported in this paper are more reliable in their purity than any other antitumor polysaccharide reported so far, which would make them promising tools for investigation of the mechanism of antitumor action.

The mechanism of the antitumor effect of these polysaccharides has not been determined, but effectiveness of pretreatment and ineffectiveness on tumor cell cultures suggested that the action may be host mediated and not directly cytocidal. Unlike bacterial lipopolysaccharides, which caused cell necrosis, lentinan and LC-11 left no scar at the site of a complete tumor regression.

The presence of optimum dosage suggests that the action mechanism of lentinan may have some relation to immunological response of host. Recently, immunological research on tumor has been conducted by many researchers, especially from the therapeutic and prophylactic viewpoint (10, 13, 18, 19, 28, 32). In the common action mechanism of host defense against tumors and viruses, interferon or interferon inducer behave similarly to, but not the same as, polysaccharides. It may be that polysaccharides play a role of interferon or interferon inducer (2). The details of action mechanism of polysaccharides in host are now under investigation, especially to clarify the entity intervening between polysaccharides and tumors. Discussions on correlation between structure and antitumor activity of polysaccharides will be described in succeeding papers.

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