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FURTHER STUDY OF THE STRUCTURE OF LENTINAN, AN ANTI-TUMOR POLYSACCHARIDE FROM *Lentinus edodes*

TAKUMA SASAKI AND NOBUO TAKASUKA
National Cancer Center Research Institute, Tsukiji 5-1-1, Chuo-ku, Tokyo (Japan)
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ABSTRACT

The structure of lentinan, an anti-tumor polysaccharide from *Lentinus edodes*, has been further investigated. Periodate oxidation, Smith degradation, methylation analysis, and bioassay were the principal methods used. These studies showed that a branched molecule having a backbone of (1→3)-β-D-glucan and side chains of both β-D-(1→3)- and β-D-(1→6)-linked D-glucose residues, together with a few internal β-D-(1→6)-linkages, is present.

INTRODUCTION

In very small dosages against certain allogeneic tumors, particularly Sarcoma 180 in mice, lentinan isolated from *Lentinus edodes*, an edible mushroom popular in Japan, has a definite growth-inhibitory activity which seems to be elicited by the stimulation of cell-mediated responses. The fine structure of lentinan has not been completely elucidated, but it was reported from this laboratory that lentinan is a (1→3)-β-D-glucan with a molecular weight of about a million; it was studied by total hydrolysis, partial hydrolysis, and digestion by exo-(1→3)-β-D-glucanase from *Sclerotium*[1]. If this structure is correct, lentinan should be closely related chemically to the bacterial (1→3)-β-D-glucan from *Alcaligenes faecalis* var. *myxogenes* strain 10C3K, given the trivial name curdlan[2]. However, recent investigations[3] showed that the shift in the absorption maximum of Congo Red complexed with the fractions from acid-degraded lentinan is somewhat different from that reported[4] for the complex with the fractions from degraded curdlan. It was considered that this difference might be due to the presence of other glycosidic linkages in the lentinan molecule. Accordingly, we re-examined the structure of lentinan and its partially hydrolyzed fragment, the latter being designated "small lentinan".

RESULTS AND DISCUSSION

"Small lentinan" prepared by hydrolysis with formic acid had $[\alpha]_D^{25}$ +29.0° in water, and showed an i.r. spectrum entirely identical to that of lentinan. Probably

due to the difference in the molecular weight, lentinan (mol. wt. 1,000,000) was insoluble in water, but "small lentinan" (mol. wt. 16,200) was soluble. The solubilities of (1→3)-β-D-glucans are known to be related to their molecular weight[4].

Oxidation of lentinan and "small lentinan" with sodium metaperiodate at 22° resulted in consumption by each of 0.57 mole of periodate per D-glucose residue, with the production of 0.22 and 0.21 mole of formic acid, respectively. The same values were obtained on oxidation for 20 days at 5°. This result indicates that the chemical structure of "small lentinan" is similar to that of lentinan, which contains "triol" groups [*i.e.*, nonreducing ends, or internal (1→6)-glycosidic linkages, or both]. The production of formic acid was somewhat lower than that calculated on the basis of the consumption of metaperiodate.

More information on the structure of lentinan was obtained from examination by Smith degradation[5]. Lentinan was subjected to periodate oxidation at 22°, and the resulting, oxidized glucan was reduced with sodium borohydride to the corresponding polyalcohol. Complete hydrolysis with 95% formic acid and then M sulfuric acid yielded glycerol and glucose in the ratio of 2:5.

On mild hydrolysis with 0.05M sulfuric acid at 22°, the lentinan polyalcohol gave the degraded glucan in 66% yield, with simultaneous liberation of glycerol only. In addition to this, the fact that, after retreatment by Smith degradation, the degraded glucan released a negligible proportion of material that consumed periodate indicated that no cleavage of β-D-(1→3)-linkages occurred during the mild hydrolysis. From these studies, lentinan has branch points, two for every five D-glucose residues.

Analysis of the ^{13}C n.m.r. spectra of "small lentinan" and lentinan had indicated that they contain branch points, one for every three D-glucose residues[6]. This branched structure is consistent with the results of methylation analysis. Direct methylation of "small lentinan" and of lentinan by the Hakomori method[7] afforded *O*-methyl-D-glucans. Methanolysis of these fully methylated polysaccharides, and

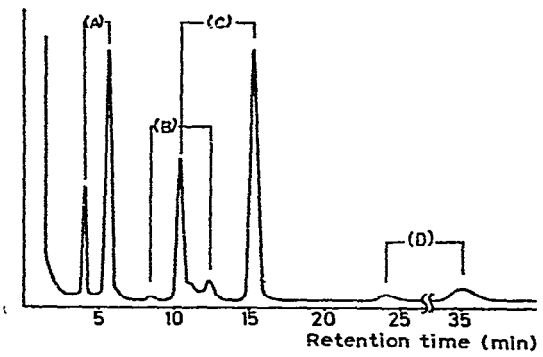

Fig. 1. Gas–liquid chromatogram of the methanolysis products of permethylated lentinan and permethylated "small lentinan": peaks A, methyl 2,3,4,6-tetra-*O*-methylglucosides; peaks B, methyl 2,3,4-tri-*O*-methylglucosides; peaks C, methyl 2,4,6-tri-*O*-methylglucosides; peaks D, methyl 2,4-di-*O*-methylglucosides.

examination of the methyl *O*-methyl-D-glucosides by gas–liquid chromatography, indicated the presence of residues of 2,3,4,6-tetra-, 2,4,6-tri-, 2,3,4-tri-, and 2,4-di-*O*-methylglucose in the ratios of 5:17:1:5, as shown in Fig. 1.

The presence of the 2,4-dimethyl derivative suggested that branching occurs at C-6. The presence could be confirmed of branch points at C-6 only. The occurrence of 2,3,4-tri-*O*-methylglucose indicated that lentinan has internal, (1→6)-glycosidic linkages as branches, one for every 28 D-glucose residues. If lentinan contained internal (1→6)-glycosidic linkages in the β-D-(1→3)-linked main-chain, Smith degradation should cause hydrolysis of the main chain of lentinan, and it would yield small fragments; but its Smith-degradation product was still insoluble in water. In addition to its low solubility, the fact that, after treatment by Smith degradation, the degraded lentinan maintained a marked anti-tumor activity indicated that lentinan has a relatively long sequence of β-D-(1→3)-glycosidic linkages in its main chain.

Recently, Whistler and co-workers[8] reported that scleroglucan, an anti-tumor polysaccharide (from *Sclerotium glucanicum*) that has a main chain of β-D-(1→3)-glucopyranosyl residues, with every third or fourth residue carrying a β-D-(1→6)-glucopyranosyl group, showed an extreme decrease in activity after removal of branches from the scleroglucan by Smith degradation. However, even when lentinan was debranched and then hydrolyzed, no loss of its activity occurred, as shown in Table I. Both the debranched and debranched-hydrolyzed lentinan preparations were still effective against Sarcoma 180 at a level of 2.0 mg/kg for five days, and they

TABLE I

ANTITUMOR EFFECT OF LENTINANS AND SMITH-DEGRADED LENTINAN

Sample	*Dose (mg/kg × day)*	*Average tumor-weight (g)*	*Inhibition ratio (%)*	*Complete regression*[a]
Lentinan	2.0×5	0.5	83.0	6/7
Debranched lentinan	2.0×5	0.01	99.7	5/6
Debranched and hydrolyzed lentinan	2.0×5	0.29	90.0	3/5
"Small lentinan"	2.0×5	0.07	97.6	5/6
Control	—	2.91	—	0/6
Lentinan	1.0×10	0.06	99.2	6/8
"Small lentinan"	1.0×10	0.15	98.0	6/8
Control	—	7.61	—	0/8

[a]Ratio of number of mice showing complete regression to number of mice tested.

showed 99.7 and 90.0% inhibition, respectively. The significant difference between scleroglucan and lentinan may be attributed to their different branched structures; the former is a highly branched D-glucan with a (1→3)-β-D-linked main-chain in which every third or fourth residue carries, at C-6, a single D-glucosyl group as a branch, and, therefore, it is converted into a linear polysaccharide by Smith degradation. In contrast, the latter has β-D-(1→3)-linked side-chains, and it exhibits anti-

tumor activity after treatment by Smith degradation; in addition, it contains β-D-(1→6)-linked side-chains at C-6 which are attached to the β-D-(1→3)-linked main-chain. From the data obtained concerning negligible consumption of periodate after retreatment by Smith degradation, the β-D-(1→3)-linked side-chains seem to be relatively long, or to consist of a few chains, or both.

In this connection, it is interesting that the analysis[6] of the ^{13}C n.m.r. spectra of "small lentinan" and lentinan in deuterium oxide suggests that the β-D-(1→3)-linked side-chains exist, and supports the foregoing conclusions.

From the combined evidence now presented, it is proposed that lentinan has a main chain consisting of β-D-(1→3)-linked D-glucopyranosyl residues containing branch points (two for every five D-glucose residues) at C-6, and that the side chains of lentinan consist of β-D-(1→6)-linked and β-D-(1→3)-linked D-glucose residues attached to the (1→3)-β-D-glucan constituting the main chain. A few, internal β-D-(1→6)-linkages might also be present as β-D-(1→6)-linked side-chains.

EXPERIMENTAL

General methods. — The specific rotations and i.r. spectra were respectively measured at 25° with a Japan Spectroscopic Co., Model DIP-S, automatic polarimeter and a JASCO IRA-I infrared spectrometer. Gas–liquid chromatography (g.l.c.) was conducted with a Shimadzu Seisakusho Ltd., Model GC-4APF instrument.

Preparation of "small lentinan". — Lentinan was kindly supplied by Dr. Goro Chihara of this Institute. "Small lentinan" was prepared with 80% formic acid by the method of Ogawa *et al.*[4]. Lentinan (1 g) was heated with 80% formic acid for 20 min at 85°. The product, freed from formic acid and water by evaporation *in vacuo* at room temperature, was a D-glucan formate, and the i.r. spectrum showed a band at 1720 cm^{-1}, indicating the presence of formyl groups. The formyl groups were hydrolyzed off by suspending the D-glucan formate in water, and keeping the suspension in a boiling-water bath for 3 h. The i.r. spectrum of the product showed no band at 1720 cm^{-1}. The degraded D-glucan was extracted with water (50 ml), and the extract filtered. The product was precipitated from the extract by addition of ethanol (650 ml), and the precipitate was collected by centrifugation; the yield of "small lentinan" was 89 mg (8.9%). Its average molecular weight, as determined by the Somogyi–Nelson method[9], was 16,200; $[\alpha]_D^{25}$ +29.0° (*c* 1, water).

Periodate oxidation of lentinan and "small lentinan". — Lentinan (50.17 mg) and "small lentinan" (25.50 mg) were oxidized with 15mM sodium metaperiodate (100 ml for lentinan, and 50 ml for "small lentinan") at 22° in the dark. The consumption of periodate and production of formic acid were respectively determined by titration with 0.01M sodium hydroxide (with Bromocresol Purple as the indicator), and by the Fleury–Lange method[10] using 0.01N iodine solution. After 72 h, lentinan reduced 0.57 mole of periodate per D-glucose residue, with the production of 0.22 mole of formic acid; whereas, after 27 h, "small lentinan" reduced 0.57 mole of periodate per D-glucose residue, with the production of 0.21 mole of formic acid.

Smith degradation of lentinan. — Lentinan (250.23 mg) was subjected to periodate oxidation with 15mM sodium metaperiodate (500 ml) at 22° in the dark. After complete oxidation (72 h), ethylene glycol (20 ml) was added, and the mixture was stirred for 30 min (to decompose the excess of the reagent) and then dialyzed in water for 24 h. The dialyzate was concentrated *in vacuo* to a small volume, and the material was reduced with sodium borohydride (400 mg) in the dark, with stirring, for 18 h at room temperature. The excess of borohydride was decomposed by addition of 0.1M acetic acid to pH 5.5, and the reduced polysaccharide was dialyzed in water for 48 h. After lyophilization of the recovered material, the glucan polyalcohol was obtained as a white powder (216 mg). A portion (18.4 mg, dry weight) of the lentinan polyalcohol was heated with 95% formic acid (1 ml) in a sealed tube for 10 h at 100°, and then with M sulfuric acid in a sealed tube for 5 h at 100°. The hydrolyzate was made neutral with barium carbonate, the suspension filtered, and the filtrate evaporated *in vacuo* to dryness. G.l.c. of the resulting sugars as their per(trimethylsilyl)ated derivatives on a column of silicone GE SE-52 showed two peaks, one corresponding to glycerol, and the other to glucose in the ratio of 2:5.

In another experiment, lentinan polyalcohol (100 mg) was subjected to mild hydrolysis with 0.05M sulfuric acid (10 ml) for 20 h at 22°, and the degraded glucan was collected by centrifugation; water-insoluble material (yield, 66 mg). Only glycerol could be detected by g.l.c. of the per(trimethylsilyl)ated derivatives of the products obtained from the supernatant liquor of the degraded glucan.

Methylation analysis of "small lentinan" and lentinan. — A mixture of sodium hydride (200 mg) and dimethyl sulfoxide (5 ml) was heated for 3 h at 70°, and then added to a solution of "small lentinan" (100 mg) in dimethyl sulfoxide (20 ml). The mixture was stirred for 5 h at room temperature, and methyl iodide (1.2 g) was carefully added, with further stirring for 12 h at room temperature. All operations were conducted under dry nitrogen in an apparatus that had been oven-dried and assembled hot under nitrogen. The reaction mixture was diluted with water, and extracted with chloroform; the extract was well washed with water, dried (sodium sulfate), and concentrated *in vacuo* to a small volume, to which petroleum ether was added dropwise, to give a fully substituted (by i.r. spectrum) *O*-methylglucan (41 mg), $[\alpha]_D^{22}$ −13.0° (*c* 1.0, chloroform). For lentinan, three such methylations were required.

Methanolysis of methylated "small lentinan" and of methylated lentinan. — The methylated "small lentinan" and methylated lentinan were each heated with methanolic chloride in a sealed tube for 10 h at 100°, and the resulting methanolysis products were subjected to g.l.c. on a column of 1% of neopentyl glycol succinate on Chromosorb W at 145°. The peaks obtained for "small lentinan" and lentinan were the same and indicated the presence of 2,3,4,6-tetra-, 2,4,6-tri-, 2,3,4-tri-, and 2,4-di-*O*-methylglucose in the ratios of 5:17:1:5; the peaks were identified by comparison with those given by authentic specimens.

Assay of anti-tumor activity. — ICR-JCL mice weighing about 20 g were used for the anti-tumor assay. Seven-day-old Sarcoma-180 ascites (0.05 ml, $\sim 6 \times 10^6$ cells) was transplanted subcutaneously into the right groins of mice. The test samples,

dissolved or suspended in distilled water, after sterilization for 20 min at 120°, were intraperitoneally injected daily for 5, or 10, days, starting 24 h 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 and weighed. The inhibition ratios were calculated by use of the formula: 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.

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