Structure and Antitumor Activity of the Less-Branched Derivatives of an Alkali-Soluble Glucan Isolated from *Omphalia lapidescens*. (Studies on Fungal Polysaccharide. XXXVIII[1])

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The structure and antitumor activity of Smith-type degradation products (OL-2-I, OL-2-II and OL-2-III) of an alkali-soluble glucan, OL-2, isolated from a crude fungal drug "Leiwan" (*Omphalia lapidescens*) were investigated. Methylation analysis suggested that OL-2-I was a (1→3)-β-D-glucan with approximately one branch at every three main chain glucosyl units at each C-6 position; OL-2-II was a (1→3)-β-D-glucan with approximately one branch at two main chain glucosyl units at each C-6 position, and OL-2-III was a (1→3)-β-D-glucan with approximately one branch at twenty four main chain glucosyl units at each C-6 position (number of all main chain glucosyl units is on average). OL-2-I, OL-2-II and OL-2-III which were Smith-type degradation products of OL-2, showed potent antitumor activity against the solid form of sarcoma 180 in ICR mice. These results indicated that the degree of β-linked branching at position 6 was remarkably related to the antitumor activity.

Keywords Leiwan (*Omphalia lapidescens*); 6-O-branched (1→3)-β-D-glucan; Smith-type degradation; methylation analysis; branching degree; antitumor activity

Introduction

Previously we reported that OL-2, isolated from a fungal crude drug "Leiwan" (*Omphalia lapidescens*, a member of the Agaricales species in Trichlomataceae), is a (1→3)-β-D-glucan possessing approximately two branches at every three main chain glucosyl units at each C-6 position, and that its antitumor activity against the solid form of sarcoma 180 in ICR mice was negative.[2] The inactivity of OL-2 seemed to arise from its highly branched structure.[2–5] If OL-2 is changed to a less branched structure by Smith-type degradation, the products (less-branched derivatives: OL-2-I, OL-2-II and OL-2-III) are expected to have antitumor activity. This paper is concerned with the structure and antitumor activity of OL-2 and its smith-type degradation products.

Results and Discussion

In order to obtain a (1→3)-β-D-glucan possessing approximately one branch at every two, three or twenty four main chain glucosyl units, on average, at each C-6 position, Smith-type degradation of OL-2 was performed with equivalent periodate. OL-2 (100 mg) was oxidized with 0.22, 0.11 or 0.42 mmol of periodate. Oxidized OL-2 was reduced with borohydride, and the products were treated with 0.1 M trifluoroacetic acid (TFA) for 72 h at 37 °C (OL-2-I, Smith-type degradation products oxidized with 0.22 mmol of periodate; OL-2-II, with 0.11 mmol of periodate; and OL-2-III, with 0.42 mmol of periodate, Chart 1). OL-2 and its Smith-type degradation products were analyzed by methylation.[6] The molar ratios of alditol acetates derived from methylated OL-2, OL-2-I, OL-2-II and OL-2-III are shown in Table I. The foregoing data indicated that OL-2-I is a (1→3)-β-D-glucan with approximately one branch at every three main chain glucosyl units, that OL-2-II is a (1→3)-β-D-glucan with approximately one branch at every two main chain glucosyl units and that OL-2-III is a (1→3)-β-D-glucan with approximately one branch at every twenty four main chain glucosyl units. All unit numbers are average and all at each C-6 position.

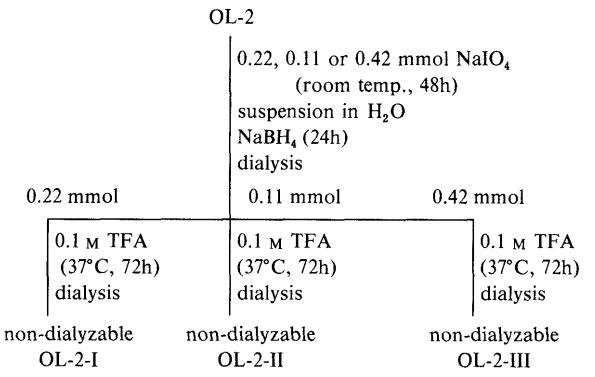

Chart 1. Smith-Type Degradation of OL-2

Branched (1→3)-β-D-glucan(lentinan,[7] grifolan,[8] schizophyllan (SPG),[9] and SSG obtained from *Sclerotinia sclerotiorum* IFO 9395[10]) with side chains of a single D-glucosyl group at C-6 exhibit antitumor activity against sarcoma 180 implanted in mice. Therefore, the antitumor activities of OL-2, OL-2-I, OL-2-II, OL-2-III, OL-2 derivatives with less-branch, and OR-OL-2c,[2] an OL-2 derivative without a side chain, were assayed by comparing the growth of solid form of sarcoma 180 tumor cells in ICR mice. As shown in Table II, OL-2-I and OL-2-II showed potent antitumor activity at a dose of about 100 and 500 μg × 5/mouse. OL-2-III was lower active than OL-2-I and OL-2-II at the same dose. OL-2 and OR-OL-2c did not inhibit the growth of solid form of sarcoma 180.

TABLE I. Relative Molar Ratios of Alditol Acetates Derived from Methylated OL-2 and Derived Glycans

Component	Molar ratio			
	OL-2	OL-2-I	OL-2-II	OL-2-III
1,5-Di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl glucitol	0.99	0.85	0.79	0.47
1,3,5-Tri-*O*-acetyl-2,4,6-tri-*O*-methyl glucitol	0.58	2.20	1.04	23.7
1,3,5,6-Tetra-*O*-acetyl-2,4-di-*O*-methyl glucitol	1.00	1.00	1.00	1.00

TABLE II. Antitumor Activity of OL-2 and Derived Glycans against Sarcoma 180 Solid Tumor[a]

Sample	Dose ×5 (μg/mouse)	Tumor weight[c] (g, mean±S.D.)	Inhibition (%)[b]	Complete regression[b,g]
Experiment-1				
OL-2	100	4.29±4.00[f]	−4.1	0/7
	500	5.04±3.46[f]	−22.3	0/8
OL-2-I	100	0.04±0.07[d]	99.0	6/9
	500	0.03±0.09[d]	99.3	5/7
OL-2-III	100	0.67±1.43[e]	83.7	5/7
	500	0.68±1.49[e]	83.5	4/7
SPG	100	1.01±1.21[e]	74.0	2/7
	500	0.14±0.18[d]	96.6	4/7
Control		4.12±2.07		0/7
Experiment-2				
OL-2-II	100	0.49±1.28[d]	92.6	5/7
	500	0.05±0.12[d]	99.2	6/9
Control		6.71±3.28		0/8
Experiment-3				
OL-2	20	5.51±1.02[f]	−28.5	0/7
	100	5.55±1.00[f]	−29.6	0/8
OR-OL-2c	20	5.18±1.47[f]	−21.0	0/9
	100	2.40±2.71[f]	36.6	2/8
Control		4.28±2.80		0/7

a) Sarcoma 180 tumor cells (5×10^6) were inoculated subcutaneously (day 0). Each fraction was administered as a saline solution by intraperitoneal injection on days 7, 9, 11, 13 and 15. *b*) Inhibition and complete regression were determined 35 d after tumor inoculation. *c*) The significance of differences was evaluated according to Student's *t*-test. Significant difference from control [*d*) $p<0.001$, *e*) $p<0.01$, *f*) not significant]. *g*) Number of complete regressions/mice used.

We previously described that the degree of branching was an important factor in the antitumor activity.[2,3] These experimental results indicated that the degree of branching in 6-O-branched (1→3)-β-D-glucan was strongly related to the activity.

Results confirmed that the highly branched structure of (1→3)-β-D-glucan had no the antitumor activity, while (1→3)-β-D-glucan possessing one branch at two or three main chain glucosyl units had strong antitumor activity. Also, the antitumor activity of the less-branched glucan, such as that with one branch at twenty four main chain glucosyl units, was still positive, but that of OR-OL-2c with no side chain was negative.

Available experimental evidence suggests that there are several physical and chemical parameters such as molecular weight, degree of branching and conformation which affect the antitumor and immunopharmacological activities of (1→3)-β-D-glucans.[11] However, it is relatively difficult to distinguish among those parameters; for example, reduction of the molecular weight changed the conformation from a gel to a sol; solubility of the glucan depends on the degree of branching. Reduction of the OL-2 side chain slightly increased its solubility. The physicochemical change in OL-2 might influence, at least in part, the antitumor activity. However, solubility would not be a critically important change because, in carboxymethylated or hydroxyethylated SSG and curdlan, significantly high substitution of these residues results in a loss of antitumor activity without affecting the solubility.[12] Considering these facts, substitution of more than half the residues of the main chain in OL-2 would be the most important contributing factor in a significantly low level of antitumor activity.

It was previously demonstrated that (1→3)-β-D-glucans have antitumor and various immunopharmacological activities.[7-11] However, the initial and/or very early events between the host and the glucan have not been examined in detail. Some reports have suggested the critical importance of receptors on macrophages, such as the complement, fibronectin, and glucan receptors on the phagocytosis and immunopharmacological activities of glucans.[13] Nevertheless, no detailed investigation of the structure of these receptor molecules, especially of the glucan receptor, has been made. Knowledge of the structure–activity relationships of these receptors might help to clarify why higher substitutions on the main chain reduce antitumor activity.

Experimental

Materials SPG was provided by Kaken Pharmaceutical Co.

Preparation of OL-2 OL-2 was prepared as previously described.[14]

Smith-Type Degradation of OL-2 OL-2 (100 mg) was dissolved in 5 ml of 0.5 M sodium hydroxide solution, and the pH was adjusted to 5.5 with 0.5 M hydrochloric acid. The volume was increased to 100 ml with water, and sodium metaperiodate at 0.22, 0.11 or 0.42 mmol was added. The mixtures were stirred in the dark at room temperature for 48 h. Sodium borohydride (*ca.* 200 mg) was added, and the mixtures were stirred overnight. Excess sodium borohydride was decomposed by acidification with acetic acid. Mixtures were dialyzed against water for 48 h and concentrated to about 45 ml. Thereafter, 5 ml of 1 M TFA was added to the concentrates, which were then hydrolyzed for 72 h at 37 °C. Hydrolysates were dialyzed against running water for 24 h and the non-dialyzable fraction was further dialyzed against distilled water for 24 h. The non-dialyzable fractions were concentrated *in vacuo*, then precipitates formed upon the addition of ethanol were collected by centrifugation, washed with ethanol, acetone and ether, and dried *in vacuo* (OL-2-I, products oxidized with 0.22 mmol of periodate; OL-2-II, with 0.11 mmol of periodate; OL-2-III, with 0.42 mmol of periodate). The yields of OL-2-I, OL-2-II and OL-2-III were 73, 83 and 52 mg, respectively.

Methylation OL-2 and its Smith-type degradation products (OL-2-I, OL-2-II and OL-2-III) were methylated by the method of Hakomori (three times)[6] until they showed no significant infrared (IR) absorption due to hydroxyl groups at 3500 cm^{-1}. Each methylated polysaccharide was heated with 90% formic acid at 100 °C for 4 h. Formic acid was distilled off, and the residue was hydrolyzed with 1 M TFA at 100 °C for 8 h followed by evaporation to dryness. The resulting partially *O*-methylated sugars were reduced with sodium borohydride at room temperature for 8 h to the corresponding alditols, then acetylated.[15] The results are given in Table I.

Gas Liquid Chromatography (GLC) and GLC–Mass Spectroscopy (GLC-MS) of Partially Methylated Alditol Acetates GLC of the partially *O*-methylated alditol acetates was performed using a glass column (0.3 × 200 cm) packed with 5% (w/w) ECNSS-M on Chromosorb W (AW-DMCS, 80—100 mesh), and analyzed at 180 °C under N_2 at a flow rate of 50 ml/min.

GLC-MS of the partially *O*-methylated alditol acetates was performed using a glass column (0.3 × 200 cm) packed with 3% (w/w) silicon OV-225 on Gas chrom Q, and analyzed at 170 °C under He at a flow rate of 50 ml/min. The electron impact mass spectra were recorded by a JEOL JMS-D 300. The following results were obtained: 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl glucitol, *m/z*: 43, 45, 71, 87, 101, 117, 129, 145, 161 and 205, 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl glucitol, *m/z*: 43, 45, 87, 101, 117, 129 and 161, 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methyl glucitol, *m/z*: 43, 87, 117, 129, and 189.

Evaluation of Antitumor Activity The antitumor activity was evaluated against the solid form of sarcoma 180 tumor cells. Tumor cells (5×10^6) were inoculated subcutaneously into the right groin of mice. Each fraction was administered as a saline solution by intraperitoneal injection on days 7, 9, 11, 13 and 15 d (dose: 20, 100 or 500 μg/mouse/d). Five weeks after tumor inoculation, the mice were sacrified. The inhibition was calculated as follows:

(1 − average tumor weight of the treated group/ average tumor weight of the control group) × 100 (%) [16]

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