Further Examination on the Structure of an Alkali-Soluble Glucan Isolated from *Omphalia lapidescens*[1] (Studies on Fungal Polysaccharide. XXXVI[2])

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Analytical data of Smith-type degradation products and carbon-13 nuclear magnetic resonance spectroscopy of an alkali-soluble glucan, OL-2, which was isolated from a crude fungal drug "Leiwan" (*Omphalia lapidescens*), suggested that OL-2 was a β-1,3-D-glucan possessing approximately two branches at every three main chain glucosyl unit at each C-6 position. Antitumor activities of OL-2 and the degradation product, OR-OL-2c, against the solid form of sarcoma 180 in ICR mice were negative.

Keywords Leiwan (*Omphalia lapidescens*); 6-*O*-branched β-1,3-D-glucan; Smith-type degradation; methylation analysis

Introduction

Previously we reported that a β-D-glucan, designated as OL-2, which was isolated from a fungal crude drug "Leiwan" (*Omphalia lapidescens*, belonging to Agaricales in Trichlomataceae) should be a 6-*O*-branched 1,3-linked glucan. Results of the structural analysis of OL-2 suggested that it might be shown as formula A or B (Fig. 1).[3]

Antitumor activity of OL-2 against the solid form of sarcoma 180 in ICR mice was almost negative, and it seemed to arise from its highly branching structure.[4] If OL-2 has an A type structure, it will be changed to a less branched structure by mild Smith-type degradation, and the product can be expected to have antitumor activity. However, in the case of a B type structure it can be changed to β-1,3-linked linear structure, and it must remain inactive.[5]

Results and Discussion

For the determination of the structure of OL-2, Smith-type degradation was carried out. After periodate-oxidation repeated and borohydride-reduction, the product (OR-OL-2) was treated with mild acid hydrolysis using 0.1 M trifluoroacetic acid (TFA) for 24 and 72 h (Chart 1). Each hydrolyzate was analyzed by the methylation procedure.[6] The molar ratios of alditol acetates derived from methylated OL-2, OR-OL-2, non-dialyzable fractions of 24 h-hydrolyzate (OR-OL-2a) and of 72 h-hydrolyzate (OR-OL-2c) are shown in Table I. The treatments of periodate-oxidation repeated and borohydride-reduction caused partial de-

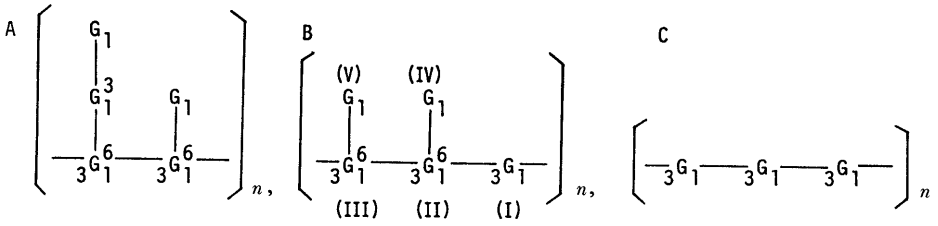

Fig. 1. Possible Structures of OL-2 and OR-OL-2c
G = D-glucopyranosyl residue.

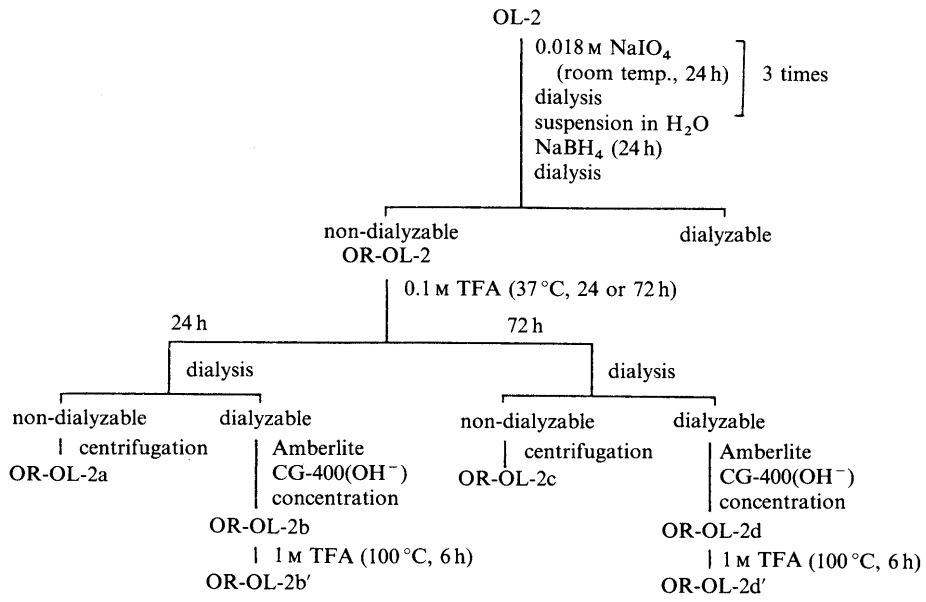

Chart 1. Smith-Type Degradation of OL-2

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

Component	Molar ratio			
	OL-2	OR-OL-2	OR-OL-2a	OR-OL-2c
1,5-Di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl glucitol	0.86	ND	ND	ND
1,3,5-Tri-*O*-acetyl-2,4,6-tri-*O*-methyl glucitol	0.52	1.52	5.12	+++
1,3,5,6-Tetra-*O*-acetyl-2,4-di-*O*-methyl glucitol	1.00	1.00	1.00	ND

ND = not detected.

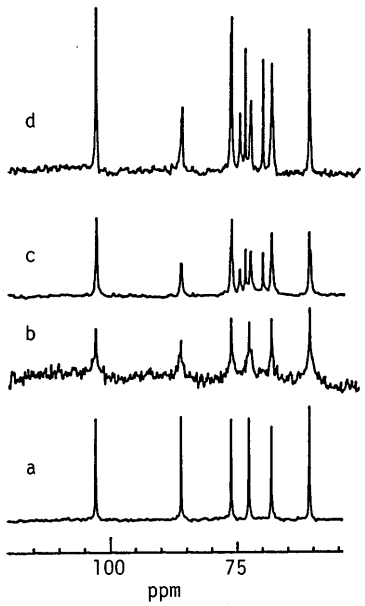

Fig. 2. ^{13}C-NMR Spectra of OL-2 and OR-OL-2c in DMSO-d_6 at 60 °C

a, curdlan; b, OR-OL-2c; c, SSG; d, OL-2.

branching. Therefore the molar ratio of 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl glucitol derived from OR-OL-2 increased. The molar ratio of tri-*O*-methyl derivative of the 24 h hydrolyzate increased, and 72 h hydrolysis gave a completely de-branched product, OR-OL-2c, which gave only 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl glucitol.

The carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectra of OL-2 and OR-OL-2c are shown in Fig. 2, and the signal assignments are listed in Table II. The ^{13}C-NMR spectrum of OL-2 in dimethyl sulfoxide-d_6 (DMSO-d_6) was quite similar to that of lentinan,[7] grifolan obtained from *Grifola frondosa*[8] and the glucan (SSG) obtained from *Sclerotinia sclerotiorum* IFO 9395,[9] therefore, OL-2 had to contain a large amount of C-6 branched β-1,3-glucosidic linkage. Further, the ^{13}C-NMR spectra of OR-OL-2c and curdlan[8] were quite similar to each other.

From these results, it was concluded that OR-OL-2c consisted of a β-1,3-linked straight chain (Fig. 1c). The molecular weight was estimated to be approximately 50000 (OL-2: *ca.* 250000) by Toyopearl HW-55F gel filtration (Fig. 3). The discrepancy of molecular weights between OL-2 and OR-OL-2c suggested that fragmentation might occur during Smith-type degradation. The dialyzable fractions of 24 h-hydrolyzate (OR-OL-2b) and of 72 h-hydrolyzate

Table II. ^{13}C-NMR Chemical Shifts of OL-2, OR-OL-2c, SSG and Curdlan in DMSO-d_6 Solution at 60 °C[a]

Glucans[b,c]		C-1	C-2	C-3	C-4	C-5	C-6
OL-1	I	102.92	72.38	86.10	68.29	76.23	60.76
	II, III	102.92	72.38	85.93	68.29	74.54	68.29
	IV, V	102.92	73.49	76.41	69.98	76.23	60.93
SSG	I	102.80	72.38	86.10	68.23	76.12	60.64
	II	102.80	72.38	85.93	68.23	74.48	68.23
	III	102.80	73.43	76.41	69.98	76.12	60.87
OR-OL-2c		102.86	72.61	86.04	68.29	76.17	60.76
Curdlan		102.86	72.73	86.10	68.29	76.23	60.76

a) Assignments were performed with reference to those in ref. 12. *b*) I, II, III of OL-2 represents main chain glucosyl units and IV, V of OL-2 represents a glucosyl unit at a branching point. IV, V of OL-2 is present at C-6 of residue II, III of OL-2. *c*) I, II of SSG represents main chain glucosyl units and III of SSG represents a glucosyl unit at a branching point. III of SSG is present at C-6 of residue II of SSG.

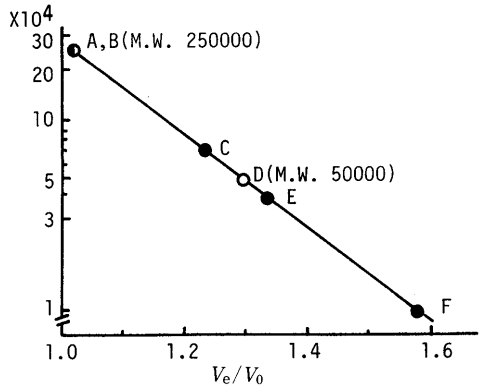

Fig. 3. Estimation of Molecular Weight of OL-2 and OR-OL-2c on Toyopearl HW-55

Solvent: 0.5 M NaOH. A, dextran T-250; B, OL-2; C, dextran T-70; D, OR-OL-2c; E, dextran T-40; F, dextran T-10.

(OR-OL-2d) derived from OR-OL-2 were analyzed by thin layer chromatography (TLC) and gas liquid chromatography (GLC) after conversion into alditol acetates. Only glycerol was detected from OR-OL-2b and OR-OL-2d, and glucitol was not detected from them. Further acid hydrolyzates (OR-OL-2b′ and OR-OL-2d′) of OR-OL-2b and OR-OL-2d with 1 M TFA for 6 h also gave the same results. Under physiological conditions, β-1,3-D-glucans adopt a herical conformation. They induce metachromasy with Congo red, as do helix-forming glucans in a diluted alkali-solution.[10] The absorption maximum of Congo red shifted largely to a longer wavelength in the presence of OL-2 (0.1 M NaOH 530 nm), however, it did not shift in the presence of OR-OL-2c. OL-2 showed metachromasy coupled with Congo red, and formed a complex with Congo red in a diluted alkali-solution.

In view of these results, it was reasonable to conclude that OL-2 consisted of a type B structural unit. Antitumor activities of OL-2 and OR-OL-2c were assayed by comparing the growth of the solid form of sarcoma 180 tumor cells in ICR mice. Both fractions did not show growth inhibition. It was known that curdlan itself did not show significant antitumor activity,[5] and OR-OL-2c showed the same result.

Experimental

Preparation of OL-2 The preparation of OL-2 was carried out

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according to the previous paper.[3]

Smith-Type Degradation of OL-2 OL-2 (50 mg) was dissolved in 2 ml of 0.5 M sodium hydroxide solution, and the pH of the solution was adjusted to 5.5 with 0.5 M hydrochloric acid. After the addition of 0.22 M sodium metaperiodate (4 ml), the volume was increased to 50 ml with water. The mixture was allowed to stand with stirring in the dark at room temperature. After 24 h, 4 ml of ethylene glycol were added, then the solution was dialyzed against running water for 24 h (the oxidation procedure was repeated three times). The internal solution was concentrated to about 20 ml, sodium borohydride (*ca.* 100 mg) was added to the concentrate, and then the mixture was stirred overnight. The excess sodium borohydride was decomposed by acidification with acetic acid. The mixture was dialyzed against water for 2 d, concentrated to a syrup (OR-OL-2), and hydrolyzed with 3 ml of 0.1 M trifluoroacetic acid (TFA) for 24 h or 72 h at 37 °C. Each hydrolyzate was dialyzed and the non-dialyzable fraction was further dialyzed against running water for 24 h. Non-dialyzable fractions thus obtained were concentrated *in vacuo*, respectively, then precipitates formed upon the addition of ethanol were collected by centrifugation, washed with ethanol, acetone and ether, and dried *in vacuo* (hydrolyzate for 24 h: OR-OL-2a, hydrolyzate for 72 h: OR-OL-2c). The dialyzable fractions were passed through an Amberlite CG-400 (OH^- formed) column, and the eluates were concentrated to dryness *in vacuo* (hydrolyzate for 24 h: OR-OL-2b, hydrolyzate for 72 h: OR-OL-2d). Further, those were hydrolyzed with 1 M TFA for 6 h, respectively (hydrolyzate for 24 h: OR-OL-2b′, hydrolyzate for 72 h: OR-OL-2d′). The hydrolyzates were examined by TLC using ethylacetate–pyridine–water (10 : 4 : 3, v/v) and cellulose plate (Merck). The hydrolyzates were also derived to alditol acetates by the usual way,[7] and analyzed by a Hitachi 163 instrument using a glass column (0.3 × 200 cm) of 5% (w/w) ECNSS-M on Chromosorb W (AW-DMCS, 80—100 mesh), at 150 °C under N_2 at a flow rate of 50 ml/min.

Methylation OL-2 and its products (OR-OL-2, OR-OL-2a and OR-OL-2c) were methylated by the method of Hakomori (three times), respectively,[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, and then acetylated by the usual way.[11] The results are given in Table I.

GLC and GLC-Mass Spectroscopy (GLC-MS) of Partially Methylated Alditol Acetates GLC of the partially *O*-methylated alditol acetates was carried out by 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 carried out by 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. In GLC-MS, the following results were observed: 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl glucitol, *m/z*: 43, 45, 71, 87, 101, 117, 129, 145, 161, 205; 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl glucitol, *m/z*: 43, 45, 87, 101, 117, 129, 161; 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methyl glucitol, *m/z*: 43, 87, 117, 129, 189.

^{13}C-NMR Spectral Analysis ^{13}C-NMR spectra were recorded at 60 °C for solutions in DMSO-d_6 with a JEOL-FX 200 (for carbon-13 at 50.1 MHz) spectrometer. The spectra were obtained in the pulsed Fourier-transform mode with complete proton decoupling.[8]

Interaction with Congo Red The complex-formation of OL-2 or OR-OL-2c (1 mg/ml) with Congo red (38 μM) was evaluated from the shift in the visible absorption of Congo red induced by the presence of OL-2 or OR-OL-2c in 0.1 M sodium hydroxide at 20 °C.[10]

Evaluation of Antitumor Activity Evaluations of antitumor activity were carried out according to the previous paper.[8,9]

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References and Notes

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