[Chem. Pharm. Bull. 28(10)3118—3121(1980)]

Studies on Fungal Polysaccharides. XXVI.[1] An Alkali-soluble Glucan of *Omphalia lepidescence*[2]

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(Received March 5, 1980)

A water-insoluble β-D-glucan designated as OL-2, $[\alpha]_D^{20}$: −0.1° (0.5 N NaOH), was isolated from a fungal crude drug, "Raigan" (dried fruiting body of *Omphalia lepidescence*).

The results of periodate oxidation and methylation studies indicated that OL-2 possessed a highly branched structure consisting of 1,3- and 1,6-linked β-D-glucopyranosyl residues.

Keywords——*Omphalia lepidescence*; methylation analysis; exo-(1→3)-β-D-glucanase treatment; water-insoluble glucan; branched β-glucan

A fungus, *Omphalia lepidescence*, which belongs to Agaricales in Trichlomataceae, is the basis for a well-known crude drug "Raigan" (Leiwan), which is used as a vermifuge. In this paper, we report the results of structural analysis of the major polysaccharide component, a water-insoluble β-glucan, which was isolated by extraction of the crude drug with 0.5 N NaOH in *ca.* 31% yield. This β-glucan, designated as OL-2, $[\alpha]_D^{20}$ −0.1° (c=1.0, 0.5 N NaOH), gave a single spot on glass-fiber paper electrophoresis, and was homogeneous as determined by ultracentrifugation. Upon acid hydrolysis, this polysaccharide gave glucose as the only component sugar. The hexose content of OL-2 was 98% (as glucose) according to the procedure of Dubois *et al.*[4] The proton magnetic resonance (^{1}H-NMR) spectrum of the methylated OL-2 in $CDCl_3$ showed an anomeric proton signal at δ 5.33 (d, J=7.5 Hz) due to H-1 of the β-D-linked residues in the glucan. The infrared (IR) spectrum of OL-2 has an absorption band at 890 cm^{-1}, suggesting a CH bonding vibration in the β-glycosidic linkage. On periodate oxidation, OL-2 consumed 0.79 mol of periodate per anhydrohexose unit, with 0.28 mol of formic acid liberation after 72 hr. The periodate-oxidized OL-2 was treated by the Smith procedure. Paper chromatographic analysis of the hydrolysate revealed the presence of glycerol and unoxidized component sugar unit.

OL-2 was methylated by the methods of Haworth,[5] Hakomori[6] and Purdie,[7] sequentially, and after acid hydrolysis, the resulting permethylated compound was analyzed by paper and thin layer chromatographies (PC and TLC). The relative molar ratios of the tetra-*O*-methyl, tri-*O*-methyl and di-*O*-methyl-D-glucose fractions were found to be 2.0 : 1.0 : 2.0 by the method of Hirst *et al.*[8] These *O*-methyl glucoses were separated by TLC. Tetra-*O*-methyl and tri-*O*-methyl-D-glucoses were identified as the 2,3,4,6- and 2,4,6-*O*-methyl derivatives, respectively, by gas–liquid chromatography(GLC), GLC-mass spectrometry (GC-MS), ^{1}H-NMR spectroscopy

1) Part XXV: H. Tsuchihashi, T. Yadomae, and T. Miyazaki, *Carbohydr. Res.*, **84**, 365 (1980).
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4) M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, and F. Smith, *Anal. Chem.*, **28**, 350 (1956).
5) W.N. Haworth, E.L. Hirst, and H.A. Thomas, *J. Chem. Soc.*, **1931**, 821.
6) S. Hakomori, *J. Biochem.* (Tokyo), **55**, 205 (1964).
7) T. Purdie and J.C. Irvine, *J. Chem. Soc.*, **83**, 1021 (1903).
8) E.L. Hirst, L. Hough, and J.K.N. Jones, *J. Chem. Soc.*, **1948**, 928.

and paper electrophoresis (PE). The di-*O*-methyl-D-glucose fraction was identified as 2,4-di-*O*-methyl-D-glucose by PE, ^{1}H-NMR and GC-MS analyses. The ^{1}H-NMR spectrum of di-*O*-methyl-D-glucose in D_2O is illustrated in Fig. 1. The signals at 3.48 and 3.59 ppm were assigned to the methoxyl group at C-2, and that at 3.54 ppm was ascribed to the methoxyl group at C-4.[9] The fully methylated polysaccharide was hydrolyzed and the hydrolysate was converted into alditol acetates and analyzed by GLC and GC-MS. The mass spectrum of the di-*O*-methyl fraction contained fragments having *m/e* 43, 87, 129 and 189. This fragmentation pattern showed that the di-*O*-methyl fraction corresponded to 2,4-di-*O*-methyl-D-glucose.[10]

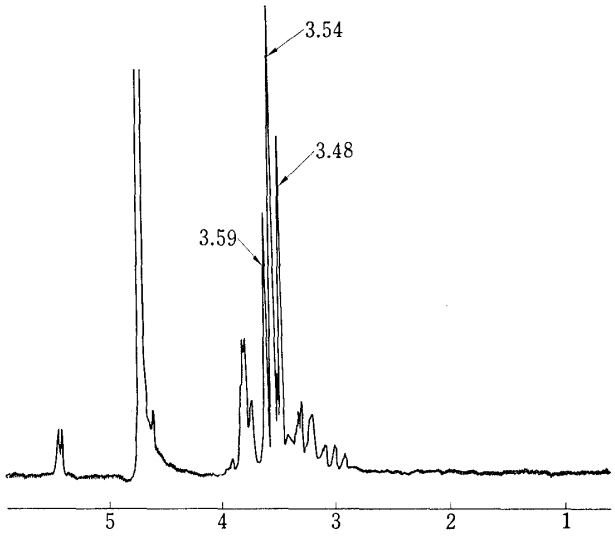
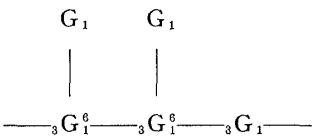


Fig. 1. ^{1}H-NMR Spectrum of the Di-*O*-methyl Fraction derived from OL-2 (100 MHz; D_2O)

Treatment with exo-(1→3)-*β*-D-glucanase, which was prepared from Alternaria solani,[11] had no effect on OL-2. Therefore, the existence of consecutive *β*-1→3 linked D-glucopyranosyl residues can be ruled out.[12]

In view of these results, it is reasonable to conclude that OL-2 consists of structural units of the following type.



G = *β*-D-glucopyranosyl residue

Experimental

Isolation of Crude Polysaccharide——The dried fruiting body of *Omphalia lepidescence*, "Raigan" (100 g), was extracted with distilled water (1 l) for 8 hr at 100°, and this extraction was repeated until the extract was negative to the anthrone reagent. The hot-water insoluble material was extracted exhaustively with 0.1 N NaOH (1 l, 7 times) at room temperature. The residue was further extracted with 0.5 N NaOH (1 l) at room temperature, and the supernatant was neutralized with AcOH, then dialyzed in Visking Cellophane tubing against running water for 2 days. The extraction was repeated exhaustively. The yields of hot water-extractable, 0.1 N NaOH-extractable and 0.5 N NaOH-extractable substances were 1.7 g, 1.5 g and 31.5 g, respectively.

The 0.5 N NaOH-soluble material was washed with water and 0.1 N NaOH, then dissolved in 0.5 N NaOH, and acidified with AcOH. The resulting precipitate was dialyzed in Visking Cellophane tubing against running water for 3 days. The internal water-insoluble substance (OL-2) was collected by centrifugation, washed with water, and dried *in vacuo*. Yield, 31.0 g.

Component Sugar of OL-2——A suspension of OL-2 (10 mg) in 1 N H_2SO_4 (2 ml) in a sealed tube was heated in a boiling water bath for 8 hr. After neutralization ($BaCO_3$) and filtration, a portion of the hydrolysate was concentrated to a syrup and applied to Toyo Roshi No. 50 filter paper for detection of the component sugar. PC was carried out by the ascending method, using AcOEt–pyridine–H_2O (10: 4: 3, solvent system A) and AcOEt–pyridine–AcOH–H_2O (5: 5: 1: 3, solvent system B). Sugar was visualized with alkaline $AgNO_3$[13] and *p*-anisidine–HCl.[14] Only glucose was detected.

Properties of OL-2——The sugar content of OL-2 was 98% (phenol–sulfuric acid method[4]), and no phosphorus[15] was detected in this polysaccharide specimen. It showed $[\alpha]_D^{20}$ −0.1° (*c*=1.0, 1 N NaOH) [IR] ν_{max}^{KBr} cm^{-1}: 890 (*β*-glycosidic linkage).

9) T. Terui, T. Yadomae, H. Yamada, O. Hayashi, and T. Miyazaki, *Chem. Pharm. Bull.*, **22**, 2476 (1974).
10) H. Björndal, B. Lindberg, and S. Svensson, *Carbohydr. Res.*, **5**, 433 (1967).
11) S. Yamato, T. Yadomae, and T. Miyazaki, *J. Ferment. Technol.*, **52**, 706 (1974).
12) T. Miyazaki, N. Oikawa, H. Yamada, and T. Yadomae, *Carbohydr. Res.*, **65**, 235 (1978).
13) W.E. Trevelyan, D.P. Proctor, and F.S. Harrison, *Nature* (London), **166**, 444 (1950).
14) L. Hough, J.K.N. Jones, and W.H. Wadman, *J. Chem. Soc.*, **1950**, 1702.
15) C.H. Fiske and Y. Subbarow, *J. Biol. Chem.*, **81**, 629 (1929).

Periodate Oxidation of OL-2——OL-2 (52 mg) was dissolved in 0.5 N NaOH (2 ml), and the pH of the solution was adjusted to 5.5 with 0.5 N HCl. After addition of 0.22 M $NaIO_4$ (4 ml), the volume was made up to 50 ml with water. The mixture was allowed to stand with stirring in the dark at room temperature. The consumption of $NaIO_4$ and the amounts of HCOOH and HCHO formed were determined by the procedures of Malaprade,[16] Whistler,[17] and O'Dea,[18] respectively. A blank solution without the glucan was processed simultaneously. The numbers of moles of $NaIO_4$ consumed and of HCOOH liberated per anhydrohexose unit of the glucan after 72 hr were 0.79 and 0.28, respectively.

Smith-type Degradation of OL-2——OL-2 (52 mg) was oxidized with $NaIO_4$ as described above. After 72 hr, ethylene glycol (4 ml) was added, then the solution was dialyzed against running water for 24 hr. The internal solution was concentrated to about 20 ml, and $NaBH_4$ (*ca.* 100 mg) was added to the concentrate, then the mixture was stirred overnight. The excess $NaBH_4$ was decomposed by acidification with AcOH. The mixture was dialyzed against water for 2 days, concentrated to a syrup, and hydrolyzed with 1 N H_2SO_4 (3 ml) in a boiling water bath for 6 hr. After neutralization ($BaCO_3$) and filtration, the filtrate was concentrated to a small volume *in vacuo*, and was examined by PC using solvent system A. Two spots corresponding to glucose and glycerol were detected by spraying a solution of alkaline-$AgNO_3$ (*Rf* values: 0.24 and 0.48, respectively).

Methylation of OL-2——OL-2 (2.0 g) was dissolved in the minimum volume of 30% NaOH, then dimethyl sulfate (40 ml) and 30% sodium hydroxide (80 ml) were introduced drop by drop under nitrogen from a separatory funnel, keeping the temperature below 40°. After heating at 60—70° for 6 hr, the reaction mixture was neutralized with HCl and dialyzed against running water for 2 days. The non-dialyzable fraction was concentrated under reduced pressure and dried *in vacuo*. This partial methylation product was then repeatedly methylated by the methods of Hakomori[6] and Purdie,[7] sequentially, in order to obtain the permethylated product. The final product showed no OH absorption near the 3400 cm^{-1} region in its IR spectrum.

Preparation of Alditol Acetate Derivatives from Methylated OL-2——Partially methylated alditol acetates were prepared from methylated OL-2 by the procedure described in a previous paper.[19]

Methanolysis of OL-2——Methylated OL-2 (50 mg) was converted into methyl glucosides by heating with 2 N MeOH–HCl (10 ml) in a sealed tube for 3 hr in a boiling water bath. MeOH was evaporated off, and HCl was removed by evaporation in a vacuum desiccator over $CaCl_2$ and KOH pellets.

GLC of the Methanolysate of Methylated OL-2 and partially Methylated Alditol Acetates——GLC of the methyl glucosides was carried out with a Shimadzu GC-5A unit, equipped with a flame ionization detector, using a glass column (200 × 0.3 cm) packed with 15% polybutane-1,4-diol succinate on Celite 545 (60—80 mesh); column temperature 175°; N_2 flow rate 50 ml/min. GLC of partially methylated alditol acetates was carried out at an N_2 flow rate of 50 ml/min on a glass column (200 × 0.3 cm) containing 5% (w/w) ECNSS-M on Chromosorb W (aw-dmcs, 60—80 mesh), at 180°.

GC-MS of partially Methylated Alditol Acetates from OL-2——Partially methylated alditol acetates from OL-2 were dissolved in acetone, and subjected to GC-MS spectrometry. Determination of the derivatives was carried out with a Shimadzu LKB-9000 mass spectrometer, equipped with a glass column packed with 3% Silicone OV-225 on Chromosorb W (aw-dmcs, 80—100 mesh), operated at 170°; electron energy 70 eV; trap current 60 μA; temperature of the ion source, 310°. Values of *m/e*: tetra-*O*-methyl fraction; 43, 45, 71, 87, 101, 117, 129, 145, 161 and 205, tri-*O*-methyl fraction; 43, 45, 87, 101, 117, 129 and 161, di-*O*-methyl fraction; 43, 87, 117, 129 and 189. Relative retention times of tetra, tri and di compounds were 1: 1.95: 5.10.

^{1}H-NMR Spectrum of Methylated OL-2——The ^{1}H-NMR spectrum of the methylated OL-2 was recorded at 100 MHz with a Varian NV-14 spectrometer at normal operating temperature. Tetramethylsilane was used as an internal standard. The sample concentration was about 5%.

TLC and PE of the Hydrolysate of Methylated OL-2——PC of the hydrolysate of methylated OL-2, which was prepared by heating with 90% HCOOH at 100° for 5 hr, and then with 1 N H_2SO_4 at 100° for 6 hr, was examined using AcOEt–AcOH–H_2O (9: 2: 2), and three spots (*Rf* values: 0.85, 0.66 and 0.39) were detected corresponding to tetra-*O*-methyl-, tri-*O*-methyl- and di-*O*-methyl monosaccharides. TLC of the hydrolysate of methylated OL-2 was carried out using benzene–acetone (1: 1) on a precoated silica-gel plate (Wako-gel C-200). Three fractions (*Rf* values: 0.54, 0.31 and 0.16) were detected. The acetone extract of each fraction was concentrated and dried *in vacuo*. The di-*O*-methyl monosaccharide was identified as 2,4-di-*O*-methyl-D-glucose (MG value: 0.05) by PE (1 mA/cm^2, 2.5 hr) using 1% NaB_4O_7 solution and Whatman GF83.

Attempted Degradation of OL-2 with Exo-(1→3)-β-D-glucanase——Exo-(1→3)-β-D-glucanase from Alternaria solani was used for this purpose. A ratio of 10 units of the glucanase per 10 mg of the glucan

16) L. Malaprade, *Bull. Soc. Chim. France*, **15**, 833 (1934).
17) R.L. Whistler and J.L. Hickson, *J. Am. Chem. Soc.*, **76**, 1671 (1954).
18) L.F. O'Dea and R.A. Gibbons, *Biochem. J.*, **55**, 580 (1953).
19) T. Miyazaki and Y. Naoi, *Chem. Pharm. Bull.*, **22**, 2058 (1974).

was suspended in 3 mM acetate buffer (pH 4.5), and the mixture was allowed to stand with stirring in the dark for 72 hr at 55°.[12] The reducing power was determined at intervals by the Somogyi-Nelson method.[20,21]

20) M. Somogyi, *J. Biol. Chem.*, **160**, 69 (1952).
21) N. Nelson, *J. Biol. Chem.*, **153**, 375 (1944).

[Chem. Pharm. Bull. 28(10)3121—3123(1980)]

Effect of Chondroitin Sulfate A in Combination Therapy with Mitomycin C on Sarcoma 180 Ascites Tumor

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(Received April 1, 1980)

Chondroitin sulfate A (CSA) was found to show a strong synergistic effect with mitomycin C in combination therapy of sarcoma 180 ascites tumor implanted in dd mice. Namely, 50% of mice each implanted with 1×10^6 tumor cells of ascitic form were found to survive on combination treatment with mitomycin C (0.3 mg/kg/day, 5 times) and CSA (1 and 10 mg/kg/day, 5 times). Enhancement of acid phosphatase and β-D-glucuronidase activities of the peritoneal cells in the mice given the combination therapy was evident as compared with those of mice of the control group.

Keywords——acid phosphatase; antitumor agent; chondroitin sulfate A; combination therapy; β-D-glucuronidase; peritoneal cells

Only a few papers have been published on tumor therapy by means of combinations of immunopotentiators with antitumor agents in order to minimize the side reaction and to increase the effect of the latter agents.[2] Previous studies have indicated that modified polysaccharides prepared by the introduction of fatty acid and phosphate groups, *i.e.*, palmitoyldextran phosphate[3] and stearoylmannan phosphate,[4] showed synergistic action with several antitumor agents against transplantable mouse tumors. It has recently been reported by Niitani *et al.*[5] that dextran sulfate showed a synergistic effect with mitomycin C against tumor. These findings seem to indicate that a polyanionic nature is one of the essential factors for the synergistic effect.

Here, we report that chondroitin sulfate A (CSA), a normal component of connective tissue, showed a synergistic effect with mitomycin C against sarcoma 180 ascites tumor. The mechanism was assumed to involve enhancement of the lysosomal enzyme activities in the peritoneal cells treated with CSA.

1) Location: *Komatsushima 4-4-1, Sendai, 983, Japan.*
2) I. Azuma, T. Taniyama, F. Hirao, and Y. Yamamura, *Gann*, **65**, 493 (1974); T. Hattori, and S. Yamagata, *ibid.*, **68**, 115 (1977); L. Chedid, A. Lamensans, F. Parent, M. Parent, A. Adam, J.F. Petit, and E. Lederer, *Cancer Res.*, **33**, 2187 (1973).
3) M. Suzuki, T. Mikami, T. Matsumoto, and S. Suzuki, *Carbohydr. Res.*, **53**, 223 (1977); M. Suzuki, T. Mikami, M. Kadowaki, T. Matsumoto, and S. Suzuki, *Cancer Res.*, **37**, 3448 (1977).
4) M. Suzuki, T. Matsumoto, T. Mikami, and S. Suzuki, *Chem. Pharm. Bull.*, **24**, 1100 (1976); *idem*, *Gann*, **67**, 607 (1976).
5) H. Niitani, A. Suzuki, M. Shimoyama, and K. Kimura, *Gann*, **57**, 193 (1966); M. Shimoyama, H. Niitani, T. Taniguchi, J. Inagaki, and K. Kimura, *ibid*, **60**, 33 (1969); H. Niitani, A. Suzuki, T. Taniguchi, N. Saijo, I. Kawase, and K. Kimura, *ibid*, **65**, 403 (1974).