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Chemical Structure of Antitumor Polysaccharide, Coriolan, produced by *Coriolus versicolor*

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Coriolan, an extracellular polysaccharide of *Coriolus versicolor* is a glucan, $[\alpha]_D^{22}$ −35.0°, slightly soluble in water and has an antitumor activity. Results of periodate oxidation and methylation studies showed that the glucan has a highly branched structure possessing (1-3)- and (1-6)-linkages.

Recently some antitumor polysaccharides obtained from the culture filtrates of Basidiomycetes have been reported.[2] In 1971, the authors reported[3] that an antitumor polysaccharide, obtained from the mycelium of *Coriolus versicolor* (*Polysticus versicolor*; Japanese name "Kawaratake") by hot water extraction, inhibited the growth of sarcoma-180 implanted subcutaneously in mice. Hirase, *et al.*[4] also reported that protein-rich polysaccharide fractions from the mycelium of *Coriolus versicolor* showed an antitumor activity.

We have now obtained a polysaccharide from the culture filtrate of *Coriolus versicolor*. This polysaccharide does not contain nitrogen from elementary analysis, and it differed evidently from the intracellular polysaccharide fraction. In this paper, chemical structure of the extracellular polysaccharide will be discussed.

The polysaccharide obtained by the procedure described in the experimental part showed $[\alpha]_D^{22}$ −35.0 (c=0.2, 0.1 M NaOH), and was revealed homogeneous by paper electrophoresis with borate buffer and column chromatography on Sepharose 2B, slightly soluble in water, and any significant absorption band was not observed in its infrared (IR) spectrum. This polysaccharide consisted of D-glucose and the glucose content was estimated as 97.2%. Estimation of the reducing power of this polysaccharide by the method of Somogyi-Nelson[5] showed 0.004 calculated as glucose. This polysaccharide is designated as coriolan for the sake of convenience.

Structure determination of coriolan was carried out by the Smith type degradation and methylation analysis. The Smith type degradation gave unoxidized glucose and a remarkable quantity of glycerol. From this result, it is apparent that coriolan is composed of 1—2 or 1—6 linked or terminal glucopyranose residue which produces glycerol by periodate oxidation followed by borohydride reduction and 1—3 linked or branching glucopyranose residue which is not oxidized. Coriolan was methylated by the method of Hakomori[6] and the acid hydrolyzate of the methylated compound was analyzed by paper chromatography. Tetra-O-methyl, tri-O-methyl, and di-O-methyl-D-glucose were detected in the relative molar ratio of 0.91:

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2) H. Nakayoshi, *Nippon Saikingaku Zasshi*, **22**, 645 (1967); *idem, ibid.*, **23**, 7 (1968); N, Komatsu, S. Okubo, and S. Kikumoto, *Gann*, **60**, 134 (1969).
3) S. Naruse, K. Fujii, and H. Ito, *Proc. Japan Cancer Assoc. 30th Annu. Meet.*, 171 (1971); H. Ito, K. Fujii, S. Naruse, M. Sugiura, and T. Miyazaki, *Mie Med. J.*, **22**, 103 (1972).
4) S. Hirase, S. Nakai, and S. Otsuka, *Proc. Japan Cancer Assoc. 29th Annu. Meet.*, 227 (1970).
5) M. Somogyi, *J. Biol. Chem.*, **195**, 19 (1952).
6) S. Hakomori, *J. Biochem.* (Tokyo), **55**, 205 (1964).

2.03: 1.0. These methylated glucose fractions separated from the hydrolyzate by paper chromatography showed a single spot in thin-layer chromatography (TLC) and paper electrophoresis. Tetra-O-methyl and tri-O-methyl-D-glucose fractions were identified with authentic samples of 2,3,4,6-tetra-O-methyl-D-glucose and 2,4,6-tri-O-methyl-D-glucose, respectively, by gas chromatography, thin-layer chromatography and paper electrophoresis. Di-O-methyl-D-glucose fraction seemed to be 2,4-di-O-methyl-D-glucose from M_G value of paper electrophoresis, and further investigations by proton magnetic resonance (PMR) and mass spectrometry were carried out. The PMR spectrum of di-O-methyl-D-glucose fraction 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 affected by the anomeric proton of the α and β configration, respectively.[7] The signal at 3.54 ppm was ascribed to the methoxyl group at C-4. Thus, the di-O-methylglucose fraction was considered as 2,4-di-O-methyl-D-glucose.

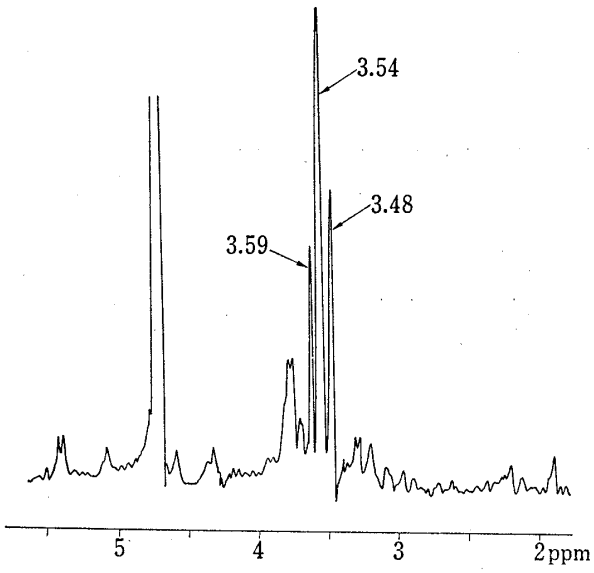


Fig. 1. PMR (100 MHz) in D_2O of Di-O-methyl Fraction separated from the Hydrolyzate of the fully methylated Extracellular Polysaccharide

The fully methylated polysaccharide was hydrolyzed and the hydrolyzate was converted into alditol acetate and analyzed by gas chromatography (Fig. 2) and mass spectrometry. Two of the three peaks, retention time 5.4 and 10.2 min, were found to be identical with authentic samples of 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-glucitol and 1,3,5-tri-O-acetyl-2,4,6-tri-O-methyl-D-glucitol by gas chromatography and mass spectrometry. The mass spectrum of the peak with retention time of 24.9 min contained fragments having *m/e* 43, 87, 117, 129, 189, and 233 (Fig. 3). This fragmentation pattern showed that the di-O-methyl fraction is 2,4-di-O-methyl-D-glucose.[8]

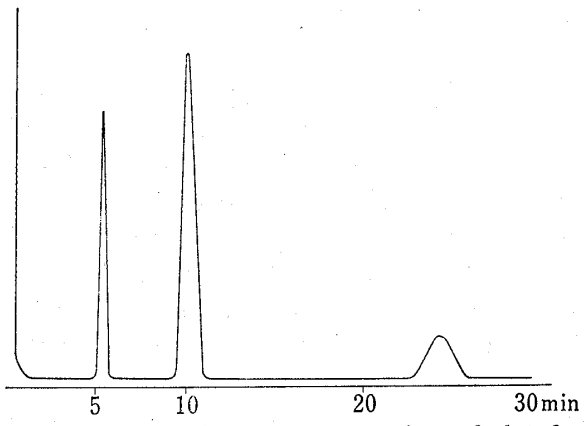


Fig. 2. Gas Chromatogram of methylated Sugars, as Their Alditol Acetate, obtained from the Hydrolyzate of the fully methylated Extracellular Polysaccharide
condition: 3% ECNSS-M, at 180°, N_2 50/min

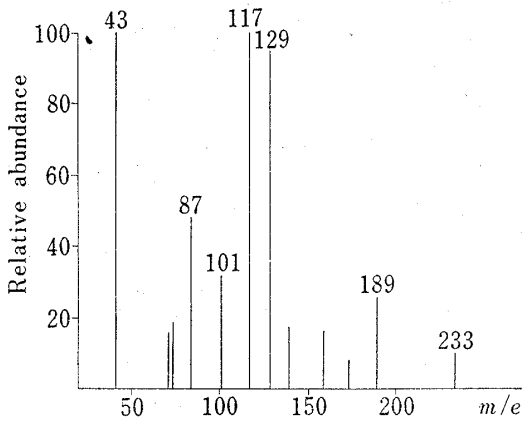
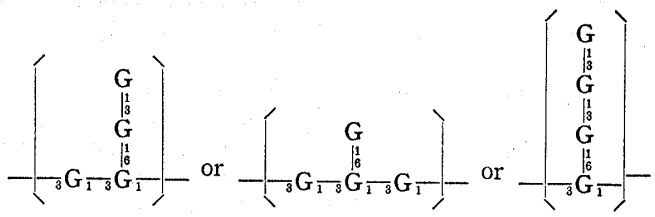


Fig. 3. Mass Spectrum of Alditol Acetate of Di-O-methyl Glucose Fraction (the Peak with Retention Time of 24.9 min in Fig. 2) determined with Hitachi RMU-7L at 70 eV Ionization Voltage

7) T. Terui, T. Yadomae, H. Yamada, O. Hayashi, and T. Miyazaki, *Chem. Pharm. Bull.* (Tokyo), in press.
8) H. Björndal, B. Lindberg, and S. Svensson, *Carbohyd. Res.*, 5, 433 (1967).

From these results it is concluded that coriolan contains one or more of the following units.



A B C

Antitumor activity of coriolan has been reported in a separate paper.[9]

Experimental

Isolation and Purification of Coriolan——The culture medium was composed of 5% sucrose, 25% onion extract, 5% soy sauce (Yamasa Shoyu), 0.1% KH_2PO_4, and 0.05% $MgSO_4$ in tap water. *C. versicolor* hyphae separated purely from its fruit body, were inoculated in this medium and cultivated at 30° for 3 days.

The mycelial growth from submerged cultures was filtered off, an equal volume of EtOH was added to the filtrate, and the precipitated polysaccharide was collected by centrifugation and washed successively with EtOH, acetone, and ether, and dried *in vacuo*. One liter of culture medium gave 0.5 g of crude polysaccharide. The crude polysaccharide was dissolved in water (0.1%) and purified by fractional precipitation with EtOH. This procedure was repeated four times, the final polysaccharide fraction was dissolved in water, and dialyzed against running water and distilled water. Finally, white powder was obtained by lyophilization, and its yield was 0.25 g/1000 ml of culture medium. Paper electrophoresis using 0.026M borate buffer (pH 10) showed a single spot (detected with periodate Schiff reagent.[10] A solution of the coriolan (1.1 mg) in 0.2M NaCl (2 ml) was applied to a column (1.2×37 cm) of Sepharose 2B and elution was effected with 0.2M NaCl. The rate of flow through the column was 8.8 ml/1 hr, 2.2 ml fraction were collected, each fraction was mixed with 0.2% anthrone reagent, and the optical density was read at 625 mμ on a colorimeter. A single peak was obtained.

Component and Quantitative Estimation of Coriolan——Coriolan (10 mg) in 2 ml of 1M H_2SO_4 sealed in a tube was heated at 100° for 6 hr. After neutralization and filtration, the filtrate was concentrated to a small volume and the concentrate was examined by paper chromatography using the solvent systems of (1) AcOEt–pyradine–H_2O (10: 4: 3) and (2) BuOH–AcOH–H_2O (4: 1: 5). One spot corresponding to glucose was detected with the spray reagents of *p*-anisidine-HCl[11] and $AgNO_3$–NaOH.[12] The glucose area on the chromatogram was sprayed with D-glucose oxidase (glucostat) solution. The paper was kept in moist air in a closed chamber for 30 min and then dried. When it was sprayed with 0.04% Bromphenol Blue in EtOH, a yellow spot appeared on the chromatogram showing the sugar to be D-glucose. Nitrogen content in 1515.5 μg coriolan was estimated by elementary analysis and nitrogen was not detected. Total sugar content of coriolan was estimated to be 97.2% by phenol-H_2SO_4 method[13] using D-glucose as standard.

Properties of Coriolan——Coriolan, $[\alpha]_D^{22}$ −35.0° (c=0.2, 0.1M NaOH), was slightly soluble in water, and any significant absorption band was not observed in its IR spectrum (KBr). Determination of the reducing power by the method of Somogyi–Nelson[5] gave the ratio of glucose to the coriolan as 1: 0.004.

Smith-type Degradation of Coriolan——Coriolan (53.0 mg) was dissolved in 90 ml of H_2O and its total volume was made up to 100 ml with 8 ml of 0.22M $NaIO_4$ and H_2O. The mixture was allowed to stand in the dark at room temperature for 108 hr. After oxidation, ethylene glycol (0.6 g) was added to destroy the excess periodate and the reaction mixture was dialyzed against running water. Non-dialyzable substance was reduced by stirring with 50 mg of $NaBH_4$ overnight. The excess $NaBH_4$ was destroyed by acidification with AcOH, the reaction mixture was evaporated to dryness, and heated with 0.5M H_2SO_4 at 100° for 14 hr. The hydrolyzate was neutralized with $BaCO_3$ and filtered, and the filtrate was concentrated to a small volume. The concentrate was examined by paper chromatography and TLC. Two spots corresponding to glucose and glycerol were detected.

Methylation Analysis of Coriolan——Coriolan (155 mg), in Me_2SO, was methylated by treatment[6] with methylsulfinyl sodium and MeI. The fully methylated coriolan showed no significant OH band in

9) H. Ito, K. Fujii, Y. Terada, S. Naruse, S. Takeda, M. Sugiura, and T. Miyazaki, *Mie Med. J.*, 23, 67 (1973).
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11) L. Hough, J.K.N. Jones, and W.H. Wadman, *J. Chem. Soc.*, **1702** (1950).
12) W.E. Trevelyan, D.P. Proctor, and J.S. Harrison, *Nature*, **166**, 444 (1950).
13) M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, and F. Smith, *Anal. Chem.*, **28**, 350 (1956).

3500 cm^{-1} region in its IR spectrum. A portion of methylated coriolan was heated with 90% HCOOH at 100° for 5 hr. HCOOH was distilled off and the residue was further hydrolyzed with 0.5M H_2SO_4 at 100° for 5 hr. The hydrolyzate was neutralized with $BaCO_3$ and filtered, and the filtrate was concentrated to a syrup.

Paper chromatogram of the hydrolyzate using the solvent system AcOEt–AcOH–H_2O (9: 2: 2), showed three spots corresponding to tetra-O-methyl, tri-O-methyl, and di-O-methyl-D-glucose. In order to estimate the relative molar ratio of tetra-, tri-, and di-O-methyl-D-glucose, the hydrolyzate was spotted on a filter paper and separated with the above solvent system. After air drying, the corresponding area on the chromatogram was quantitatively extracted with hot MeOH. The extracts were evaporated to dryness and residues were dried in a vacuum desiccator. Weights of tetra-, tri-, and di-O-methyl-D-glucose fractions were 7.0, 14.7, and 6.6 mg, respectively, their molar ratio being 0.91: 2.03: 1.0.

These methyl-D-glucose fractions were examined by paper electrophoresis using 0.026M borate buffer (pH 10). The M_G values of tetra-, tri-, and di-O-methyl-D-glucose fractions were 0, 0, and 0.03, respectively. Reference di-O-methyl-D-glucose[14] had $M_G < 0.05$ (2,4-di-O-methyl-D-glucose), 0.135 (2,3-), 0.28 (3,4-), 0.55 (3,6-), and 0.19 (4,6-).

These methyl-D-glucose fractions were dissolved in D_2O and examined by PMR. The PMR spectra were obtained with a JNM-4H 100 spectrometer at 100 MHz, and ppm value are relative to sodium 2,2,3,3-tetradeutero-3-(trimethylsilyl)propionate.

A portion of methylated coriolan was converted into methyl glucoside heating with 1M HCl–MeOH in a sealed tube for 10 hr at 100°. MeOH was evaporated and HCl was removed by evaporation in a vacuum desiccator over NaOH. The resulting methyl glucosides were dissolved in a minimum amount of MeOH and the solution was used for GLC. After removal of MeOH and HCl, the methanolyzate was trimethylsilylated according to Sweeley, *et al.*[15] Gas liquid chromatography (GLC) of methyl glucosides and trimethylsilylated derivatives of methyl glucosides was made with a Shimadzu DC-1C unit, equipped with a flame ionization detector, using a 175 × 0.5 cm glass column packed with 15% poly(butane-1,4-diol) succinate on Chromsorbe W-80 (100 mesh); column temperature 165° and 170°.

The hydrolyzate of the methylated coriolan, obtained by treatment as described above, was reduced with $NaBH_4$. The resulting, partially methylated alditols[16] were treated with mixed solution of Ac_2O and pyridine (1: 1) and the product was chromatographed by GLC on a column (200 × 0.3 cm) containing 3% ECNSS-M (100—120 mesh) at 180° (Fig. 2). These alditol acetates derived from the hydrolyzate of the fully methylated coriolan were subjected to GC–MS. The mass spectra were obtained with a Hitachi RMU-7L mass spectrometer.

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15) C.C. Sweeley, R. Bentley, M. Makita, and W.W. Welles, *J. Am. Chem. Soc.*, **85**, 2497 (1963).
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