STUDIES ON ANTITUMOR POLYSACCHARIDES, ESPECIALLY D-II, FROM MYCELIUM OF *CORIOLUS VERSICOLOR*

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Abstract—A water-soluble polysaccharide, D-II with marked antitumor activity was isolated from the cultured mycelium of *Coriolus versicolor* by extraction with hot-water, fractional precipitation with ethanol and ion-exchange chromatography. D-II strongly inhibited the growth of Sarcoma-180 transplanted subcutaneously in mice by intraperitoneal, intravenous, subcutaneous or intra-muscular administration at a dose of 5 mg/kg. The molecular weight was estimated to be 2,000,000 by gel-filtration or 6,500,000 by light scattering analysis. The chemical structure of D-II was then investigated by periodate oxidation, methylation analysis, Smith degradation, and a combination of controlled Smith degradation and methylation analysis. These studies proposed that D-II is composed of a unit structure of four D-glucose residues, and is a glucan consisting of β-D-1,3-linked main chain in which one for every three D-glucose residues is branched at C-6 with β-D-1,6-linkage.

Immuno-chemotherapy is applied clinically for the treatment of cancer and a number of drugs which accelerate immune functions have been developed. Though there are reports on antitumor polysaccharides, relationships between the structures and activities have not been elucidated. Various studies have been performed on polysaccharides of *Basidiomycetes*, and particularly the structures of lentinan (1), schizophyllan (2, 3) and coriolan (4) have been well identified.

As to *Coriolus versicolor*, the antitumor activity of PSK and ATS, both been reported to contain protein, and the protein component of PSK is reportedly essential to the antitumor property (5). The structure of the polysaccharide component in PSK was reported by Hirase et al. (6), but the chemical bond of the protein component with polysaccharide and its role in the antitumor activity remains unclear. Miyazaki et al. reported the chemical structure of coriolan, an antitumor polysaccharide of the cultured filtrate from *Coriolus versicolor* (4).

We isolated polysaccharide, D-II from the mycelium of *Coriolus versicolor*, the origin of which differs from that of PSK, and the properties and chemical structure were investigated.
MATERIALS AND METHODS

Materials: Amberlite IR-120 was purchased from Orugano Co., Tokyo, Japan. Duolite A-101D and Duolite C-20 were products of Diamond Alkali Co., U.S.A.. DEAE-cellulose (DE52) was a product of Whatman Ltd., England. Bio-Gel A-5m was a product of Bio-Rad Laboratories, U.S.A.. Chemicals were commercially guaranteed reagents and were used without further purification.

Preparation of antitumor polysaccharide: The cultured mycelium of C. versicolor (4) was washed with distilled water, disintegrated in a mixer, and extracted with boiling water for 5 hr. The suspension was filtered to remove the insoluble materials. After concentrating the aqueous extract under reduced pressure, the crude polysaccharide was obtained by precipitation with ethanol (4-fold volume).

In 10 liters of 0.25 M sodium acetate, 100 g of the crude polysaccharide were suspended, and allowed to dissolve at 22°C for 3 hr with stirring; then, the insoluble materials were removed by successive centrifugation (7,500 x g, 2°C). To the slightly turbid supernatant, a quarter volume of cold ethanol was added, and after allowing the preparation to stand for 2 hr at 5°C, the precipitate was collected by centrifugation. This procedure was repeated again. The precipitate obtained was dissolved in 2 liters of distilled water, and applied to a Duolite A-101D (OH-type) column (10 x 12 cm) and to a Duolite C-20 (H-type) column (10 x 12 cm), and subsequently, was rinsed with distilled water to obtain the eluate. DEAE-cellulose (OH-type, 100 g) was added to the eluate, and the mixture was stirred for 1 hr at 5°C. It was passed through a glass filter (Top 26G-2), and washed with distilled water to obtain the non-adsorbed fraction. The fraction was concentrated to about 2.5 liters under reduced pressure. To the concentrated solution, solid sodium acetate was added to obtain the final concentration of 0.25 M, and then, cold ethanol was gradually added to 5% (v/v) over ice water. After centrifugation (7,500 x g, 0°C, 20 min) to remove the precipitate, cold ethanol was added to the supernatant in the final concentration of 20% (v/v). The precipitate was collected by centrifugation and dialyzed against distilled water. The dialysate was lyophilized to obtain a polysaccharide, D-I.

Assay of antitumor activity: Random bred 5 week old male JCL-ICR strain mice (CLEA Apan Inc. Tokyo) were used for the antitumor assay. Sarcoma-180 cells in ascitic form were maintained in JCL-ICR strain mice by weekly i.p. passages. Sarcoma-180 (1 x 10⁷ cells/mouse) was implanted s.c. into groups of mice (8 mice a group), and a sample was given i.p. after 24 hr, the tumor was weighed 21 days after the implantation, and inhibition ratio of the tumor growth was calculated by the following formula:

\[
\text{Inhibition ratio (\%)} = (1 - B/A) \times 100
\]

where A is the average tumor weight of the control group and B is that of the tested group.

Assay of Sugar: Sugar content was determined by the phenol-sulfuric acid method (7). The reduced terminal of polysaccharide was quantified by the method of Somogyi (8). The degree of polymerization of polysaccharide was determined by the method of Misaki et al. (9).
Assay of protein: Protein content was determined by the method of Lowry et al. (10).

Zone electrophoresis: Zone electrophoresis using a glass fiber was performed in 1% sodium borate at 2.6 mA/cm for 2.5 hr. Using Pevikon C-870, this procedure was performed in 1% sodium borate at 4 mA/cm² for 3.5 hr.

Ultracentrifugation: The sedimentation patterns of D-II were measured using a Hitachi 282 Analytical Centrifuge with RA60 HC rotor and a double sector cell in 0.083% aqueous solution, and a Schlieren picture was obtained every 6 min after 60,000 rpm had been reached.

Periodate oxidation of D-II: D-II (25 mg) was oxidized with 17.6 mM sodium periodate (27 ml) at 8°C in the dark. The consumption of periodate was determined by Maraprade’s method (11) and the production of formic acid by the method of Whistler and Hickson (12).

Smith degradation of D-II: Smith degradation was performed by the method of Goldstein et al. (13). After completely oxidizing D-II (50 mg) with 17.6 mM sodium periodate, 2 ml of ethyleneglycol was added, the mixture was stirred for 30 min and was then dialyzed against water for 24 hr. The dialysate was concentrated in vacuo to a small volume, and reduced with sodium borohydride (50 mg) in the dark, with stirring, for 16 hr at room temperature. After decomposing the excess of borohydride in an acidic condition with acetic acid, the reduced polysaccharide was dialyzed against water for 48 hr. The dialyzate was lyophilized and hydrolyzed with 0.5 N sulfuric acid (3 ml) in a sealed tube for 15 at 100°C. The hydrolyzate was neutralized with barium carbonate, and the suspension was filtered. The filtrate was passed through a small Amberlite IR-120 (H-type) column, and evaporated in vacuo to dryness.

Methylation analysis of D-II: D-II was completely methylated by repeating three times the procedure used by Hakomori (14). The complete methylation was confirmed by absorption of IR spectrum at 3500 cm⁻¹. The methylated D-II was hydrolyzed with 90% formic acid for 2 hr at 100°C and then with 0.5 N sulfuric acid for 5 hr at 100°C. The methylated sugar was separated and purified by TLC with a developer of acetone-benzene (1:1, v/v), according to the method of Tscheche and Balle (15). p-Anسانidine hydrochloric acid reagent (16) was used as a color developer.

Controlled Smith degradation and methylation analysis of D-II: Controlled Smith degradation was performed by the method of Goldstein et al. (13). The glucan polyol-I obtained from D-II (glucan-I) by the periodate oxidation and the borohydride reduction was subjected to a mild hydrolysis with 0.1 N sulfuric acid for 24 hr at 28°C. The resulting precipitate was collected by centrifugation, dialyzed against water 24 hr, and lyophilized to obtain glucan-II. The glucan-II was treated similarly as described above and glucan polyol-II and glucan-III were prepared. Glucan-I, -II and -III were completely methylated and then hydrolyzed to obtain the corresponding methylated hydrolyzate. The methylated sugars were separated by TLC then weighed.

Gas chromatography of sugar: Sugar was analyzed as an alditol acetated by the method of Sawardeker et al. (17).

PMR spectrum of methylated sugar: The chemical shift of proton by the methoxyl group of methylated sugar was determined in CDCl₃ or D₂O by XL-NMR Spectrometer (Varian).
RESULTS

_Purification of antitumor polysaccharide_: Antitumor polysaccharide was purified as an index of antitumor activity against Sarcoma-180. The fractionation and purification are shown schematically in Fig. 1. The results of purification are summarized in Table 1. The purified D-II preparation possessed the most potent antitumor activity. The products obtained by hydrolysis of D-II with 2 N sulfuric acid for 5 hr at 100°C were identified as D-glucose alone by TLC or GLC. The D-glucose content was estimated to be between 94.2 and 98.4% and the protein content was not more than 0.05%. Zone electrophoretic analysis of D-II was revealed by the presence of only one band. Purity of D-II was also confirmed by ultracentrifugation in 0.083% aqueous solution which was not viscous, and the sedimentation patterns showed a single peak, Fig. 2.

**Diagram:**

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Mycelium
   extracted with hot water for 5 hr at 100°C
  |
Extract
  4 volumes of ethanol added and centrifugation carried out
  |
Sup.    Ppt.
  suspended in 0.25 M sodium acetate and centrifuged
  |
Ppt.    Sup.
  added one-fourth volume of ethanol and centrifuged
  |
Sup.    Ppt. (A-1)
  treated with Duolite A-101D and C-20
  |
  Passed through soln. (IP-0)
  |
  DEAE-cellulose
  |
  Passed through soln. (D-P)
  |
  fractionated with ethanol and centrifuged
  |
D-I (0–5%)
D-II (5–20%)

Fig. 1. Extraction, fractionation and purification of polysaccharides from cultured mycelium of _Coriolus versicolor_.
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TABLE 1. Fractionation and purification of antitumor polysaccharides from mycelium of *Coriolus versicolor*

| Component                        | Weight (g) | Sugar (g) | Protein (g) | Dose (mg/kg) | Ratio of inhibition (%) |
|----------------------------------|------------|-----------|-------------|--------------|-------------------------|
| Crude polysaccharide             | 100        | 60        | 14          | 30           | 52.0                    |
| Ethanol fractionation: A-I        | 14.9       | 12.6      | 1.2         | 10           | 69.2                    |
| Duolite A-101D and C-20: IP-O    | 11.8       | 10.9      | 0.30        | 2.0          | 63.4                    |
| DEAE-cellulose: D-P               | 7.4        | 7.29      | 0.056       | 0.1          | 32.0                    |
| : D-E                            | 2.7        |           | 0.1         |              | 27.0                    |
| : Ethanol fractionation: D-I      | 2.29       |           | 0.1         |              | 43.7                    |
| : D-II                           | 3.52       | 3.49      | 0.0081      | 0.1          | 34.9                    |

One group included 8 animals. Test samples were given in a one i.p. administration at 24 hr after Sarcoma-180 implantation. Results were expressed by inhibition on the 21st day after inoculation. Sugar was determined by phenol-sulfuric acid method.

**Fig. 2.** Sedimentation patterns of D-II. After reaching full speed of 63,000 rpm, the photographs were taken at a constant intervals of 6 min.

**Physico-chemical properties of D-II:** D-II was insoluble in organic solvents such as alcohol, acetone, chloroform, and pyridine but was more soluble in hot water, aqueous alkali, formic acid, and dimethyl sulfoxide than in water of room temperature. D-II dissolved in water or saline to about 0.6%, but D-II did not dissolve in higher concentrations.
due to the increase of the viscosity. The kinematic viscosity (ν) and the absolute viscosity (γ) were 2.082 (c=0.1, H₂O, 30°C) and 10.1, respectively. D-II showed the specific optical rotation of [α]D (c=0.2, H₂O) = +25° and [α]D (c=0.2, 0.1 N NaOH) = +24.5°.

The elementary analysis of D-II showed C at 38.3%, H at 5.67% and N at 0.14%; a small quantity of nitrogen was present. The infrared spectra showed absorption at 894 cm⁻¹ which shows the presence of β-glucosidic linkage. When D-II was exposed to vapour at 120°C for 30 min, the antitumor activity did not diminish and remained stable.

The remarkable increase of solubility and decrease of viscosity were observed with the change of D-II properties following ultrasonic treatment (19.5 KHz, 18 min). The solubility of D-II increased up to 1%, and simultaneously the kinematic viscosity decreased from 2.082 to 1.195. Suspecting cleavage and other changes in the molecule of D-II, the homogeneity of ultrasonic treated D-II was examined by ultracentrifugal analysis. It gave a single peak as shown in Fig. 3, and there was no apparent change in the homogeneity.

*Estimation of molecular weight of D-II:* The molecular weight of D-II was estimated by the number of reduced terminals and the degree of polymerization, but only small measurements were obtained due to the low solubility of D-II. The molecular weight was estimated to be over 300,000. The molecular weight was estimated to be 2,000,000 by gel-filtration on Bio-Gel A-5 m using blue dextran 2000 as the standard. The molecular weight was also estimated physico-chemically to be 6,500,000 by light scattering analysis with a light scat-
tering photometer (Union Giken), and that of ultrasonic treated D-II was estimated to be 5,170,000.

### TABLE 2. Antitumor activity of D-II against Sarcoma-180

| Administration route | Dose (mg/kg) | Average tumor weight (g ± S.E.) | Ratio of inhibition (%) |
|----------------------|-------------|---------------------------------|-------------------------|
| Control              |             | 1.816 ± 0.668                   |                         |
| D-II                 | i.p.        | 0.5 0.946 ± 0.259                | 47.9                    |
|                      | 5           | 0.149 ± 0.051                   | 91.8                    |
|                      | i.v.        | 0.5 0.801 ± 0.148                | 55.9                    |
|                      | 5           | 0.081 ± 0.031                   | 95.5                    |
|                      | s.c.        | 0.5 1.350 ± 0.377                | 38.9                    |
|                      | 5           | 0.570 ± 0.241                   | 62.9                    |
|                      | i.m.        | 0.5 1.069 ± 0.307                | 25.7                    |
|                      | 5           | 0.150 ± 0.047                   | 68.6                    |

One group included 8 animals. Test samples were given in a one i.p. administration at 24 hr after Sarcoma-180 implantation. Results were expressed by inhibition on the 21st day after inoculation.

**Antitumor activity of D-II:** Table 2 shows the variation of the antitumor activity of D-II with the route of administration. With on dose of D-II given i.p. (5 mg/kg to mice), the inhibition ratio was 91.8%. D-II was also effective when given i.v., s.c., or i.m., and the effects did not differ regarding the antitumor effect seen with the i.p. administration. However, the p.o. administration was ineffective even when given in a dose of 250 mg/kg x 20 every 24 hr.

**Chemical structure of D-II:** D-II finally consumed 0.52 mole of periodate per anhydroglucose unit, with the production of 0.24 mole of formic acid.

GLC of Smith degraded products of D-II as their alditol acetate derivatives showed two peaks, one corresponding to glycerol, and the other to D-glucose in the molar ratio of 1:2.7. An insoluble substance produced on Smith degradation was methylated and then hydrolyzed. The resulting hydrolyzate was analyzed by GLC, to be an alditol acetate derivative, and was estimated as 2,4,6-tri-O-methylglucose.

In PMR spectrum of completely methylated D-II, the anomeric proton signal was

![Fig. 4. 100 MHz PMR spectrum of methylated D-II in CDCl₃.](image-url)
4.75 ppm as shown in Fig. 4, this being the signal value of laminarin shown by Miyazaki et al. (18); thus, D-II is a glucan having β-1,3-glucosidic linkage.

The hydrolyzate of completely methylated D-II was separated by TLC to obtain compounds I, II and III as shown in Fig. 5. The weights were 11.2 mg, 19.4 mg and 10.0 mg, respectively, and the weight ratio was about 1:2:1. The positions of OMe group of the compounds were determined by PMR spectrum, and their chemical shifts are shown in Table 3. According to the values shown by Terui et al. (19), compounds I, II and III were identified as 2,3,4,6-tetra-, 2,4,6-tri- and 2,4-di-O-methylglucose, respectively. These were also confirmed by GLC-MS of the acetylated derivatives of these compounds.

The above-described results showed that D-II was estimated to be a glucan composed of a unit of four glucose residues, which has a main chain consisting of β-1,3-glucosidic linkage containing branched points at C-6, and in which one or two glucose residues were branched with β-1,6-glucosidic linkage.

The glucan polyol-I was subjected to controlled Smith degradation (13), and a considerable amount of precipitate (glucan-II) was obtained. The weight recovery ratio of glucan-II to D-II (glucan-I) was 76.5%, suggesting that one of four D-glucose residues was removed by the controlled Smith degradation. The glucan-II was similarly treated, and the weight recovery ratio of glucan-III to glucan-II was 96%. Glucan-I, II and -III were

### Table 3. Chemical shifts obtained from 100 MHZ PMR spectrum of compounds I, II and III in D₂O

| Compound | C-6 | C-2(α) | C-4 | C-2(β) | C-3 |
|----------|-----|--------|-----|--------|-----|
| I        | 3.38| 3.45   | 3.52| 3.58   | 3.61|
| II       | 3.38| 3.45   | 3.51| 3.58   | —   |
| III      | —   | 3.45   | 3.53| 3.58   | —   |

a, (α): -anomer, b, (β): -anomer
then methylated and hydrolyzed. The methylated hydrolyzates were separated by TLC and collected. The molar ratios are shown in Table 4; they were estimated to be β-1,3-glucan.

Reviewing the results, it is estimated that D-II is a glucan consisting of β-1,3-linked main chain in which one for every three D-glucose residues is branched at C-6 with β-1,6-linkage as shown in Fig. 6.

**DISCUSSION**

Antitumor polysaccharides are obtained from Basidiomycetes, and most are glucan. These polysaccharides consist of β-1,3-, β-1,4- and β-1,6-glucosidic linkages: there is pachymaran (20) as glucan consisting of β-1,3-linked linear chain, PSK fractions (6), schizophyllan (2, 3) and coriolan (4) as glucan consisting of β-1,3-linked linear chain which has a branch of β-1,6-linkage, PSK fractions (6) as glucan consisting of β-1,4-linked linear chain having a branch of β-1,3- or β-1,6-linkage, and lentinan (1) as glucan consisting of β-1,3-linked linear chain, with a few internal β-1,6-linkages, having a branch of β-1,3- or β-1,6-linkage. The first-order structure of D-II estimated in the present experiment is composed of a unit structure of four D-glucose residues, and is a glucan consisting of β-1,3-linked linear chain in which a D-glucose residue is branched at C-6 with β-1,6-linkage. It resembles schizophyllan and coriolan, but physico-chemical properties such as molecular weight, specific optical rotation, solubility and viscosity differ. It was revealed that the first-order structure of PSK fraction with a potent antitumor activity is a glucan consisting of β-1,4-linked linear chain having branches of β-1,3- and β-1,6-linkages and that the fraction contains

| Molar ratio | 2,3,4,6-(OMe)₄ | 2,4,6-(OMe)₃ | 2,4-(OMe)₂ |
|-------------|----------------|--------------|------------|
| Glucan-I    | 0.543          | 1.00         | 0.510      |
| Glucan-I treated by 0.1 N H₂SO₄ at 28° for 24 hr. | 0.538          | 1.00         | 0.506      |
| Glucan-II   | —              | 1.00         | 0.083      |
| Glucan-III  | —              | 1.00         | 0.039      |
protein; thus it is quite different from our refined D-II.

Pachyman which consists of β-1,3-glucosidic linked linear chain having a branch of β-1,6-glucosidic linkage such as is the case with D-II, has no antitumor activity but showed the antitumor activity when subjected to periodate oxidation, reduction and controlled Smith degradation to be composed of only β-1,3-glucosidic linked linear chain (21); it also shows the antitumor activity by subjecting to physical alteration such as urea treatment (22). The antitumor activity of lentinan or pachymaran is lost by denaturation with urea or dimethyl sulfoxide, but is regained by removal of these substances (20). Thus, the structure for development of the antitumor activity in glucan is assumed to relate to the second- or third-order structure as well as the first-order, and in the structure-activity correlation in polysaccharide, its high-order structure or mycel formation will play an important role rather than that of the first-order.

In the present experiment, D-II was found to possess a potent antitumor activity when given i.p., i.v., s.c. and i.m. to JCL-ICR mice Sarcoma-180 system, but p.o. administration of even a large dose showed no antitumor activity, Ito et al. have reported that p.o. administration of coriolan showed antitumor activity (23). The host-depending antitumor action of a substance such as polysaccharide is suggested to be greatly affected by the strain of animal and the administration route.

Coriolan is a glucan derived from Coriolus versicolor cultured filtrate, and D-II is obtained from the mycelium; however, though the degree of antitumor activity and the first-order structure are similar, physical properties such as molecular weight, solubility and viscosity are different. The former is a metabolic product and the latter is a cell constituent.

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