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The Polysaccharide from *Lampteromyces Japonicus*

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From *Lampteromyces japonicus*, a polysaccharide was extracted with hot water and purified by deproteinization, removal of nucleic acid and DEAE cellulose column chromatography. The purified neutral polysaccharide, $[\alpha]_D^{24}$ +218.3° (c=0.93, H_2O), was white powder and constituted of mannose, glucose and galactose in the molar ratio of 1 : 0.66 : 0.54.

The tumor inhibition ratio of the polysaccharide against the implanted sarcoma 180 in mice was 55.1% in the doses of 40 mg/kg/day for 5 days, and the tumors in 3 of 8 mice completely regressed.

In recent years, many studies on fungal polysaccharides were reported in relation to their antitumor activity and chemical structure.[2]

In regard to *Lampteromyces japonicus*, a toxic mushroom, it was reported by Nakanishi, *et al.*[3] in 1963 that the toxic substance "lampterol," a sesquiterpene, was isolated from methanol extract and shown to have antitumor activity.

This paper describes the extraction of polysaccharide from *L. japonicus*, its purification and some of its chemical characters.

Experimental

Collection of the Fungi, *L. japonicus*——The fungi, (*L. japonicus*), were collected at Mt. Fuji, dried at room temperature and fragmented.

Qualitative and Quantitative Analysis——Sugar was determined by the anthrone method. Protein was measured by Lowry's method[4] or a difference in optical density between 215nm and 225nm (OD_{215}—OD_{225}).[5] The measurement of phosphorus was carried out by Bruce's method.[6] The detection of sulfur was carried out by sodium fusion and addition of 1% sodium nitroprussid solution.

Extraction of Crude Polysaccharide——The dried fruit bodies of *L. japonicus* (dry weight: 200 g) were cut into small pieces and immediately extracted with boiling water (1 liter) for eight hr. This procedure was repeated twice. The extract was then centrifuged at 8000 rpm for 30 min and the supernatant concentrated

1) Location: *Hatanodai, 1-5-8, Shinagawa-ku, Tokyo.*

2) G. Chihara, Y. Maeda, J. Hamuro, T. Sasaki, and F. Fukuoka, *Nature*, **222**, 687 (1969); G. Chihara, J. Hamuro, Y. Maeda, S. Arai, and F. Fukuoka, *ibid.*, **225**, 943 (1970); T. Ikekawa, N. Uehara, Y. Maeda, M. Nakanishi, and F. Fukuoka, *Cancer Res.*, **29**, 734 (1969); G. Chihara, J. Hamuro, Y. Maeda, Y. Arai, and F. Fukuoka, *ibid.*, **30**, 2776 (1970); N. Komatsu, S. Okubo, S. Kikumoto, K. Kimura, G. Saito, and S. Sakai, *Gann*, **60**, 137 (1969); S. Shibata, Y. Nishikawa, C.F. Mei, F. Fukuoka, and M. Nakanishi, *ibid.*, **59**, 159 (1968); T. Ikekawa, M. Nakanishi, N. Uehara, G. Chihara, and F. Fukuoka, *ibid.*, **59**, 155 (1968); T. Sasaki, Y. Arai, T. Ikekawa, G. Chihara, and F. Fukuoka, *Chem. Pharm. Bulll.* (Tokyo), **19**, 821 (1971); Y. Yoshioka, T. Ikekawa, M. Noda, and F. Fukuoka, *ibid.*, **20**, 1175 (1972); T. Miyazaki and N. Oikawa, *ibid.*, **21**, 2545 (1973).

3) K. Nakanishi, M. Ohashi, N. Suzuki, M. Tada, Y. Yamada, and S. Inagaki, *Yakugaku Zasshi*, **83**, 377 (1963); K. Nakanishi, M. Tada, Y. Yamada, M. Ohashi, N. Komatsu, and H. Terakawa, *Nature*, **197**, 292 (1963); H. Shirahama, Y. Fukuoka, and T. Matsumoto, *Nippon Kagaku Zasshi*, **83**, 1289 (1962); N. Harada and K. Nakanishi, *Chem. Comm.*, **1970**, 310.

4) O.H. Lowry, N.J. Rosebrough, A.L. Farr, and R.J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

5) J.B. Murphy and M.W. Kies, *Biochim. Biophys. Acta*, **45**, 382 (1960).

6) N.A. Bruce, "Methods in Enzymology," Vol. VIII, ed. by E.F. Neufeld and V. Ginsberg, Academic Press, New York and London, 1966, p. 115.

under the reduced pressure. Crude polysaccharide fraction [I] was precipitated with addition of five volumes of ethanol from the concentrated dark brown solution and the dark brown precipitate [I] was collected by centrifugation, then dried *in vacuo* at room temperature. The yield of crude fraction [I] was 20 g.

Deproteinization with Pronase E Digestion——Crude polysaccharide fraction [I] was dissolved in 1 liter of water and digested with Pronase E (Kaken Kagaku, 100 mg) overnight at 37° after adjusting to pH 7.8 with 1 N NaOH. The reaction mixture was adjusted to pH 7.8 and Pronase E (50 mg) was again added to the reaction mixture. Afterwards, the reaction mixture was boiled for 5 min, and concentrated to 300 ml. One hundred millilitre of 40% trichloroacetic acid (10% in final concentration) was added and allowed to stand overnight in a refrigerator (about 4°). The deproteinized supernatant was obtained by centrifugation at 8000 rpm for 30 min and dialysed against water for two days, concentrated and precipitated by addition of 3 volumes of ethanol. The deproteinized precipitate [II] obtained was brown. The yield of deproteinized polysaccharide [II] was 3.3 g.

Removal of Nucleic Acid from the Deproteinized Polysaccharide [II]——The removal of nucleic acid was carried out by Kirby's method.[7] The deproteinized polysaccharide fraction [II] (3.3 g) was dissolved in 250 ml of water, and sample solution was mixed with an equal volume of 2.5 M K_2HPO_4, 0.05 volume of 33.3% H_3PO_4 and 1 volume of 2-methoxy ethanol. The mixture was shaken vigorously in a separating funnel. The lower layer, together with any insoluble substance was separated by standing for several hours. The upper layer was discarded. To the lower layer was added a fresh upper layer of the mixture (water–2.5 M K_2HPO_4–2-methoxy ethanol–33.3% H_3PO_4; 250 ml: 250 ml: 250 ml: 12.5 ml; 1: 1: 1: 0.05, by vol.). The newly seperated lower layer containing polysaccharide was dialysed against water for 3 days and precipitated by adding 3 volumes of ethanol. The precipitate [III] obtained was a cream-colored powder. The yield of precipitate [III] was 2.5 g.

DEAE Cellulose Column Chromatography——DEAE cellulose (capacity; 0.95 meq/g) was obtained from Seikagaku Kogyo Ltd. It was used in the chloride form under the following conditions: column size; 3×35 cm, flow rate; 30 ml/hr. The precipitate [III] was dissolved in 50 ml of water and applied to a column of DEAE cellulose, then eluted with water. Each 10 ml of effluent was collected into fractions. The positive fraction (from 200 ml to 400 ml) as shown by the anthrone method was collected, concentrated and precipitated by addition of 3 volumes of ethanol. The purified polysaccharide [IV] obtained was a white powder, yield, 0.7 g, 0.35% (calculated from the weight of the starting material). The remaining materials on DEAE cellulose were eluted by a linear gradient (0—0.5 M NaCl). The positive fraction (from 200 ml to 380 ml) as shown by the anthrone method was collected, dialysed, concentrated and precipitated by addition of 3 volumes of ethanol. The precipitate [V] obtained was a somewhat cream-colored powder. Yield, 0.1 g.

Gel Filtration——The dissolved sample [IV] (15 mg) was applied to a column (3×42 cm) of Sephadex G-200, to estimate the molecular weight by gel filtration method, and eluted with 0.1 M NaCl. Flow rate was 35 ml/hr. The void volume (110—115 ml) was determined by using dextran 2000. Each 5 ml of effluent was fractionally collected and the sugar in each fraction was measured by the anthrone method.

Paper Chromatography——Paper chromatography was carried out on Whatman No. 3 (20×57 cm) by descending method with the following solvent: *n*-BuOH–pyridine–H_2O (6: 4: 3) for 30 hr. The sample [IV] (*ca.* 5 mg) was hydrolysed in 1 N HCl at 100° for 3 hr, neutralized with 1 N NaOH, passed through a column of Amberliet MB-3, concentrated to a syrup and spotted on the paper. Sugars were detected by spraying bentidine trichloroacetic acid and heating at 100°.

Gas Chromatography——Gas chromatography was carried out using a HITACHI K-53 gas chromatograph according to Sawardeker's method.[8] The sample [IV] (ca. 5 mg) was hydrolysed, neutralized, passed through a column of Amberlite MB-3, concentrated and reduced in water (1 ml) with $NaBH_4$ (*ca.* 1—2 mg) for 1 hr at room temperature. After addition of 10% AcOH, treatment with Amberlite MB-3 and concentration, boric acid was removed by codistillation with methanol. The product was treated with acetic anhydride–pyridine, 1: 1, (0.5 ml) at 100° for 2 hr. The acetylation mixture was first diluted with water, concentrated to dryness, dissolved in chloroform and injected into the column. Separations were made at a gas flow rate of 60 ml nitrogen per min on a column (2 m×3 mm) containing 3% of ECNSS-M on Gas Chrom Q (100—120 mesh). Conditions: column temperature; 210°, H_2; 1 kg/cm², air pressure; 1.5 kg/cm². Retention times of xylose as internal standard, mannose, galactose and glucose were 21.2, 37.2, 42.0, and 47.0 min (1.00 1.76: 1.98: 2.22) respectively. Response factors of neutral sugars (Man: Gal: Glc) were 1.00: 0.85; 1.00.

Infrared Spectra and Optical Rotation——Infrared analysis of the purified polysaccharide fraction [IV] was done as KBr disk on a Jasco IRA-1 model. Optical rotation of the purified fraction [IV] was measured with 1 cm cell on a Yanagimoto OR-50 model. The ORD spectrum was recorded on a Jasco model ORD/UV-5 polarimeter using H_2O as a solvent.

Assay of Antitumor Activity——Mice used were males of the ddY strain, initially weighing about 25 g. Fresh ascites fluid, containing approximately 2×10^6 cells of sarcoma 180 in 0.2 ml, was implanted subcutane-

7) K.S. Kirby, *Biochem. J.*, **64**, 405 (1956).
8) J.S. Sawardeker, J.H. Slonecker, and A. Jeanes, *Anal. Chem.*, **37**, 1602 (1965).

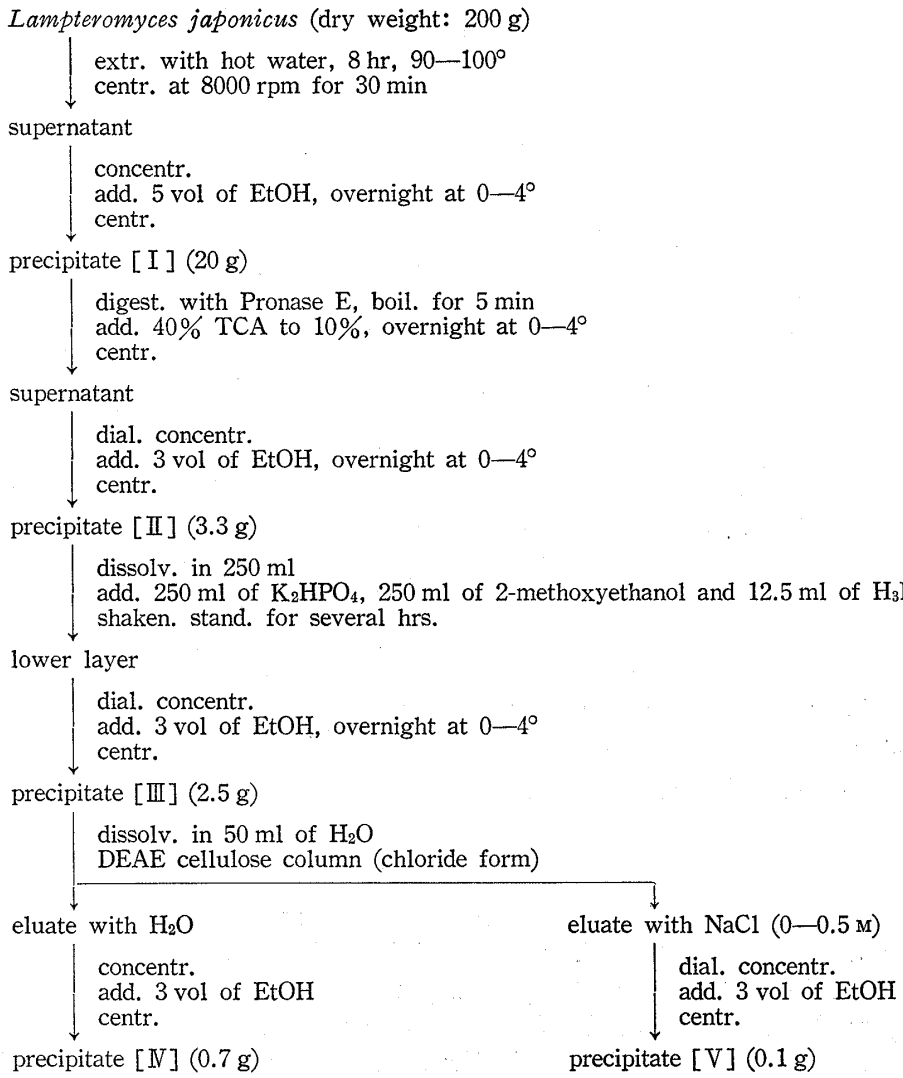

Fig. 1. Extraction and Purification of Polysaccharide from *Lampteromyces japonicus*

ously at left groin of mouse. The aqueous solution of sample [IV] was administered by intraperitoneal injection beginning 24 hr after the implantation, once daily for 5 days. The mice were autopsied 21 days after the implantation to measure tumor weight. Tumor inhibition ratio and number of mice showing complete regression were recorded.

Result and Discussion

The extraction and purification of the polysaccharide from *L. japonicus* were shown in Fig. 1. The precipitate [I], which was extracted with hot water and precipitated by adding 5 volumes of ethanol, was dark brown.

The deproteinized precipitate [II] by Pronase E digestion was brown. The yield decreased to 1/6. Although the protein content of this fraction [II] was 23% by Lowry's method, it was only 4.0% according to the optical density (215—225 nm) method. The abnormal result as measured by Lowry's method may be caused by brown pigment. Organic phosphorus content in the fraction [II] was determined to give 0.3% as phosphorus. Sulfur was not detected.

Contamination of organic phosphorus by nucleic acid was expected and nucleic acid was removed by Kirby's method.[7] That is, the dissolved sample [II] solution was mixed with an equal volume of 2.5M K_2HPO_4, 0.05 volume of 33.3% H_3PO_4 and 1 volume of 2-methoxy

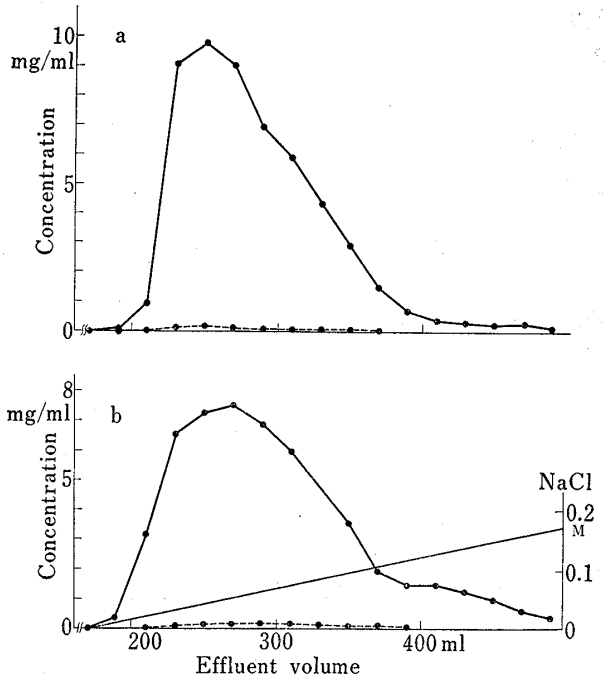

Fig. 2. DEAE Cellulose Column Chromatography

a: elution curves with water,
b: elution curves with NaCl (0—0.5 M)
Sugar (—●—) was measured by anthrone method as galactose.
Protein (---●---) was measured by Lowry's method as albumin.

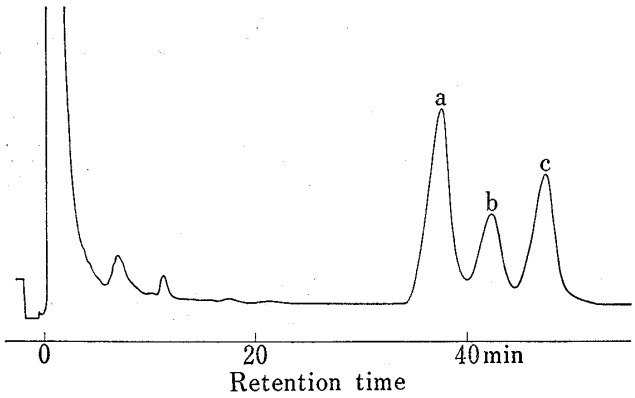

Fig. 3. Gas Chromatography of the Purified Polysaccharide [IV]

column (2 m × 3 mm); 3% ECNSS-M on Gas Chrom Q (100—120 mesh), column temperature; 210°, carrier gas; N_2 60 ml/min
a: mannose, b: galactose, c: glucose

ethanol. The lower layer containing polysaccharide was separated, dialysed and precipitated by addition of 3 volume of ethanol. The precipitate [III] obtained was a cream-colored powder. The protein content was 4% by Lowry's method and 3.3% by the optical density (215—225 nm) method. The organic phosphorus content was negligible. That is, nucleic acid was almost all removed. Fortunately the brown color was also removed in the upper layer. On the other hand, preliminary experiments revealed that the brown pigment was adsorbed on DEAE cellulose, eluted with 0.2N NaOH and shown to promoted tumor activity. The sugar content of this polysaccharide fraction [III] was 120% as galactose and 62% as glucose.

Further, the fraction [III] dissolved was applied to a column (3 × 35 cm) of DEAE cellulose (chloride form), and eluted with water. The contents of sugar and protein in each fraction were measured, and the elution curves are shown in Fig. 2 (a). Thus, the white powder of the purified polysaccharide [IV] was obtained. This fraction [IV] did not contain nitrogen, sulfur and phosphorus. The sugar content of the purified polysaccharide [IV] was 136% as galactose and 66% as glucose.

The remaining material on DEAE cellulose was eluted by a linear gradient (0—0.5M NaCl). The result is shown in Fig. 2(b). The precipitate [V] was a somewhat cream-colored powder.

In order to investigate the neutral sugar components in the purified polysaccharide [IV], paper chromatography was carried out according to the method described under Experimental. Three neutral sugars, mannose, glucose and galactose (R_{Glc}; respectively 1.14, 1.00, 0.87) were detected. The molar ratios of three neutral sugars were analysed by gas chromatography. The conditions were described under Experimental. The sample [IV] was hydrolysed, neutralized, reduced and acetylated, and the alditol acetates were separated on 3% ECNSS-M column. The molar ratios of the neutral sugars in the sample [IV] were 1.00 (mannose): 0.54 (galactose): 0.66 (glucose). The results are shown in Fig. 3. From the above result it was found that mannose was a major component.

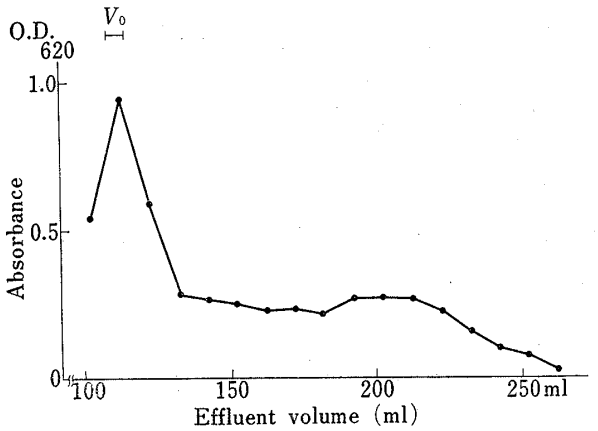

Fig. 4. Gel Filtration of the Purified Polysaccharide [IV] by Sephadex G-200

Sugar was measured by Anthrone method.

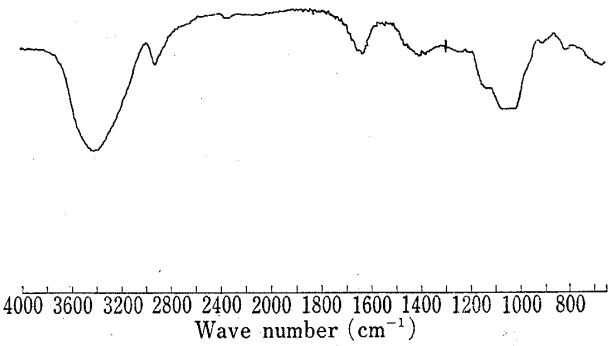

Fig. 5. Infrared Spectrum of the Purified Polysaccharide [IV]

To estimate the molecular weight of the purified polysaccharide [IV], gel filtration was performed by Sephadex G-200 (3×42 cm). The sample [IV] was dissolved, applied to the column, and eluted with 0.1M NaCl. The elution curve is shown in Fig. 4. Since the curve showed a major peak at the void volume and had smooth tailing, it was found that the molecular weight in most of the sample [IV] was greater than 200000, and that the tailing part had a molecular weight less than 200000.

Infrared spectrum of the purified polysaccharide [IV] is shown in Fig. 5. Two small absorption bands at 810 cm^{-1} and 910 cm^{-1} were observed. The absorption near 940 and 970 cm^{-1}, which were valuable for recognition of the main glycosidic linkage of the mannan,[9] was not observed. Consequently, it was not concluded about α- or β-glycosidic bond from IR spectrum. The purified polysaccharide [IV] showed a positive high specific rotation $[\alpha]_D^{24}$ +218.3° (c=0.93, H_2O) and a positive plane curve in ORD.

The antitumor activity of the neutral purified polysaccharide [IV] showed the inhibition ratio of 55.1% against the implanted sarcoma 180 in mice at doses of 40 mg/kg/day for 5 days, and the tumors in 3 of 8 mice completely regressed. Dose responses of this fraction [IV] were shown in Table I.

Table I. Antitumor Effect of the Neutral Purified Polysaccharide [IV] against the Implanted Sarcoma 180 in Mice

Doses (mg/kg×day)	Average tumor weight (g)	Inhibition ratio (%)	Complete regression
40 × 5	0.98	55.1	3/8
Control	2.23		0/8
20 × 5	1.8	35.7	2/10
8 × 5	2.3	17.9	1/10
4 × 5	2.0	28.6	0/10
2 × 5	2.6	7.1	1/10
Control	2.8		0/10

Further studies are required to elucidate the chemical structure and linkage form of this polysaccharide.

9) K. Kato, M. Nitta, and T. Mizuno, *Agr. Biol. Chem.*, **37**, 433 (1973).