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Studies on Antitumor Activity of Some Fractions from Basidiomyces. I. An Antitumor Acidic Polysaccharide Fraction of *P. ostreatus* (Fr.) Quél

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An antitumor active aqueous extract was obtained from *P. ostreatus* (Fr.) Quél. The fractionation by ethanol precipitation, ultrafiltration with molecular sieve membrane and adsorption on DEAE-Sephadex were effective to purify an antitumor active fraction A_5 as one spot on a electrophoresis, and to isolate another active fraction A_3 and an inactive fraction A_6. The active fraction A_5 and A_3 were constituted of macromolecular polysaccharide, but molecular size of the inactive fraction A_6 was smaller than those of the active fractions. The main component sugar was glucose in the fraction A_5 and A_3, but it was galactose in the fraction A_6. The fraction A_5 differed from the fraction A_3 in a point of including acidic sugar.

In recent years, many active preparations inhibiting growth of transplanted solid tumor were obtained from microorganisms,[2] fungi,[3] lichens,[4] and plants,[5] *etc.* Important aspects of these studies may be the mechanism of antitumor action of the components and essential chemical structures for the antitumor activity in the components. It has been considered that the inhibitory effect for transplanted solid tumor is not due to simple direct cytotoxicity on tumor cells, but to host-mediated action. These antitumor active components have been realized to be not low molecular toxic substances but macromolecular polysaccharide fractions. However, it is known that some macromolecular polysaccharides have a tendency to enclose other substances. In the studies on antitumor activity of macromolecular polysaccharide preparations obtained from natural sources, one should be careful not to overlook the possible enclosure of substances other than polysaccharides.

This paper describes isolation of antitumor active fractions against transplanted sarcoma 180 from *Pleurotus ostreatus* (Fr.) Quél (an edible mushroom with popular name in Japanese, "Hiratake"), and purification of an antitumor acidic polysaccharide fraction, and elucidation of some chemical structures of the polysaccharide in the fraction.

The tumor inhibition ratio of a hot water extract from *P. ostreatus* was 75% in the doses of 200 mg/kg/day for 10 days, and the tumors in 5 of 10 mice completely regressed within 5 weeks. The antitumor components were concentrated by 50% ethanol precipitation, and the precipitate obtained (fraction HA) showed 91% inhibition ratio in 50 mg/kg doses

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by the same bioassay. The fraction HA was extracted with 4% sodium hydroxide solution, and the extract was neutralized, and components of molecular weight under 30000 and inorganic salts were removed by molecular sieve ultrafiltration using Diaflo PM-30 membrane. The ultrafiltrates were saved and dialyzed, and lyophilized to obtain a fraction A_6. An anion exchanger, DEAE-Sephadex (borate form), was suspended in the residual solution which did not pass through the PM-30 membrane, and some substances were adsorbed on DEAE-Sephadex. The DEAE-Sephadex was collected by filtration using 40 μ nylon mesh, and washed with hot water carefully. The filtrate and washings were precipitated with one volume ethanol to obtain a fraction A_3. The adsorbate on DEAE-Sephadex was recovered by 2% sodium hydroxide elution, and the eluate was neutralized and dialyzed, and precipitated by adding three volumes of ethanol. The precipitate was redialyzed against deionized water, and a fraction A_5 was obtained by lyophilization. The fraction A_5 showed antitumor activity of 95% inhibition ratio in 5 mg/kg doses. The fractionation procedure was summarized in Fig. 1.

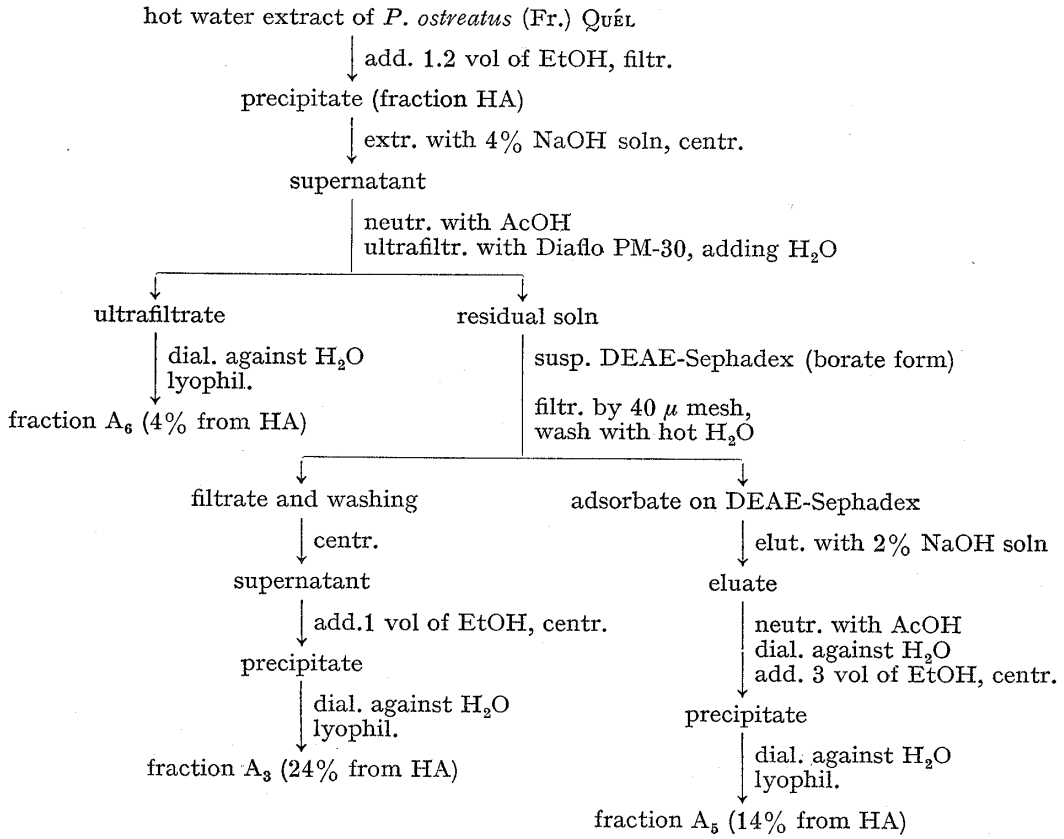

Fig. 1. Fractionation of Aqueous Extract of *P. ostreatus* (Fr.) Quél

The fraction HA, A_5 and A_6 were analyzed by high voltage electrophoresis using glass fibre paper in 0.1M borate buffer. The fraction HA gave three main spots (spots I, II, and III), as shown in Fig. 2. The fraction A_6 showed one spot (spot II), and the residual solution not filtrable by the PM-30 membrane gave two spots corresponding to spots I and III. This showed that the ultrafiltration step was effective to remove the substance giving spot II. Further, materials corresponding to spot III was successfully removed by the treatment with DEAE-Sephadex. Thus the fraction A_5 gave one spot corresponding to spot I, as shown in Fig. 2. By this fractionation procedure, the fraction A_5 was purified as a component showing one spot on the electrophoresis.

Ultraviolet (UV) spectra of each fraction did not show any characteristic absorption except end absorption, and elemental analyses suggested that nitrogen containing com-

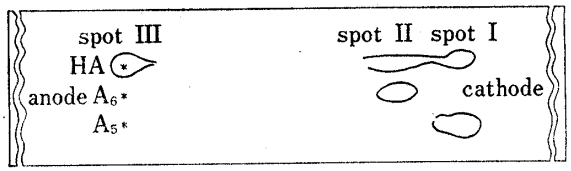

Fig. 2. Paper Electrophoresis of Fraction prepared from *P. ostreatus* (Fr.) Quél

strip: Whatman glass fibre GF 83
solvent: 0.1M borate buffer of pH 9.3
field strength: 50 V/cm, for 45 min
reagent: ammonium vanadate-H_2SO_4

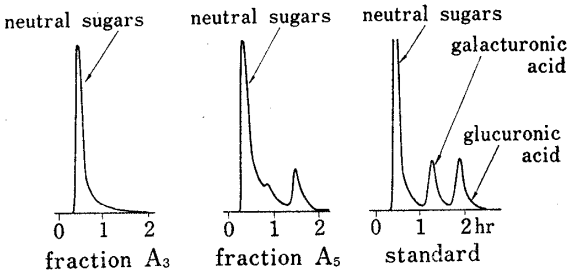

Fig. 3. Automatic Liquid Chromatography of Acidic Sugar in Fraction prepared from *P. ostreatus* (Fr.) Quél

pounds were absent from each fraction, as shown in Table I. Thus contamination of proteins, peptides and nucleic acids in the fractions were excluded. No proton signals of nuclear magnetic resonance (NMR) spectra of each fraction were observed except those of about 5.5 to 3 ppm, that was the common region of polysaccharides. This suggested that the fractions were not contaminated by lipids. All optical rotatory dispersion (ORD) spectra of each fraction drew plain curves, and the curves of the fraction A_3 and the fraction A_6 were positive plain but that of the fraction A_5 was negative plain. Complete acid hydrolyzates of each fraction were chromatographed on thin layer plates, and sugars were detected by anisaldehyde-H_2SO_4 reagent, as shown in Table II. The ammonium vanadate-H_2SO_4 reagent was sprayed on another plate and heated, and by this treatment sugars and other organic substances might be observed. No spot other than that of sugars was detected. Results by other color reactions were consistent with this observation. These results showed that the major constituents of the fractions should be polysaccharides.

Table I. Some Chemical Properties of Fractions prepared from *P. ostreatus* (Fr.) Quél

fraction		A_3	A_5	A_6
Elemental analysis	C	42.7%	38.1%	39.6%
	H	6.9	6.4	6.9
	N	0	0.1	0.8
	S	0	0	0
	P	0.5	0	0.9
	ash	0.5	0	5.4
ORD		posit. plain	negat. plain	posit. plain
Total carbohydrate content[a)]		105%	85%	97%
Shift of IR absorption band				
from salt form			1610	
to acid form		—	→1725	
by methylation		—	→1735	

a) by phenol-H_2SO_4 method using glucose as a standard

However, it was found by quantitative analysis of total carbohydrate content with phenol-H_2SO_4 method that a colorimetric value of the fraction A_5 was only 85%, in contrast to 105% of the value of the fraction A_3, as shown in Table I. It might be assumed that the decrease in the experimental value of A_5 was due to existence of some derived sugars besides neutral sugars, since an experimental value of glucuronic acid by this analysis gave only 35% of that of glucose. The existence of some derived sugar in the fraction A_5 was suggested by an absorption band of infrared (IR) spectrum at 1610 cm^{-1}. The absorption band was shifted to 1725 cm^{-1} with changing from salt form to un-ionized acid form by adding hydrochloric acid or passing through cation exchanger, Dowex 1 (H^+ form). This shifting

is characteristic of carboxylic acids. But the same finding was not observed in the fraction A_3. The carbonyl group was confirmed by IR spectrometry of the methylated A_5. That is to say, an absorption band at 1735 cm^{-1} appeared by the methylation of the fraction A_5, and that at 1610 cm^{-1} disappeared, but that of the methylated A_3 did not observed. It was suggested that carboxylic derivatives of sugars existed in the fraction A_5 in the form of ionized acid. Further, an analysis by adsorption chromatography was employed for determination of acidic sugars in the fraction A_5. The complete acid hydrolyzates of each fraction were applied to a column of anion exchanger, JEOL Resine LC-R-3 (chlorate form), and eluted by 0.005N hydrochloric acid, and sugars were detected by orcinol-H_2SO_4 method. This liquid chromatogram of the hydrolyzate of the fraction A_3 showed one peak at the region of neutral sugars, but that of the fraction A_5 showed other delayed peaks besides the peak of neutral sugars, as shown in Fig. 3. Since elemental analysis gave no P and S in the fraction A_5, these acidic sugars giving the delayed peaks might be the carboxylic sugars as suggested above. But the retention times of these carboxylic sugars in the fraction A_5 did not coincide in position with those of glucuronic acid and galacturonic acid. Further study on the acidic sugars are now in progress.

Composition of neutral sugars was analysed by liquid chromatography of sugar-borate complex. The complete acid hydrolyzates of each fraction in borate solution were applied to a column of basic resin transformed into borate form (JEOL Resin LC-R-3), and eluted with stepwise increase of borate concentration and pH in the eluents. The sugars were detected by orcinol-H_2SO_4 method. In this experimental condition, acidic sugars were strongly adsorbed on the resin and not eluted. It was found by this liquid chromatography that the fraction A_5 was mainly composed of glucose, as well as the fraction A_3, but a main conponent sugar of the fraction A_6 was galactose, as shown in Table II. This finding was comfirmed by gas chromatography of TMS derivatives and thin layer chromatography on the hydrolyzates also. These results demonstrated that the fraction A_6 obtained by the ultrafiltration differed from the fraction A_5 not only in terms of molecular weight, but also in composition of the sugars.

Table II. Chemical Analysis of Complete Acid Hydrolyzate of Fraction prepared from *P. ostreatus* (Fr.) Quél

Sugar	Fraction								
	A_3			A_5			A_6		
Method	TLC[a]	GC[b]	LC[c]	TLC	GC	LC	TLC	GC	LC
Glucose	‖	‖	100	‖	‖	100	‖	+	84
Galactose			3	±		5	‖	‖	100
Mannose			±	±		2	+	+	57
Other neutral sugars			1			±	±		13
Acidic sugars			—			+			

a) thin-layer chromatography
plate: Silica gel G impregnated with 5% NaH_2PO_4; solvent: BuOH–acetone–H_2O (4:5:1); reagent: anisaldehyde-H_2SO_4

b) gas chromatography
column: 1.5% OV 17, and 0.75% SE 30; temperature: 160°, and 200°; carrier gas: N_2 gas, flow rate of 42 ml/min

c) liquid chromatogaphy
stationary phase: JEOL Resin LC-R-3 (borate or chlorate form); mobile phase: 0.11M pH 7.5—0.25M pH 9.0—0.35 M pH 9.6 borate buffer, or 0.005M HCl

Although the antitumor activity of the fraction A_5 showed the remarkable inhibition ratio of 95% in 5 mg/kg doses, the fraction A_6 little inhibited tumor growth in the same doses. This marked contrast in these antitumor activity may be due to the difference of component

sugar and/or molecular weight. Tumor inhibition ratio of the fraction A_3 was almost same as that of fraction A_5 in 5 mg/kg doses. It has been pointed out that the fraction A_5 differed from the fraction A_3 in existence of the acidic sugars though the main component sugar was glucose in both cases. But the fraction A_3 might be similar to the fraction A_5 in terms of molecular size. Of interests are antitumor activity molecular weight relationship and effect of minor component sugars as acidic sugars of the fraction A_5 on the antitumor action. These points will be presented in the series.

Experimental

The UV absorbances were measured with a Cary spectrophotometer Model 14. The IR spectra were obtained with a Japan Spectroscopic Co. Model DS-402 G. The NMR spectra were obtained with a Japan Electron Optics Lab. JNM-3H-60. High voltage paper electrophoreses were performed on Whatman glass fibre GF 83 at 50 V/cm, using 0.1M borate buffer of pH 9.3. The spots were detected by spraying ammonium vanadate-H_2SO_4 reagent and heating at 110°. Thin-layer chromatographic analyses were carried out on a plate of Silica gel G impregnated with 5% NaH_2PO_4, using a solvent system of BuOH–acetone–H_2O (4:5:1). Spraying reagents used for detection were anisaldehyde–H_2SO_4 and ammonium vanadate-H_2SO_4. Total carbohydrate contents were determined by phenol-H_2SO_4 method using glucose as a standard.

Isolation and Purification——The fruit bodies of *P. ostreatus* (Fr.) QUEL were homogenized and extracted with boiling water for 20 hr, and the aqueous extract was filtered. The filtrate was precipitated by adding 1.2 volumes of EtOH, and the precipitate was collected by filtration and dried. A brownish white fibrous substance was obtained (fraction HA). Ten g of HA was extracted with 490 g of 4% NaOH aqueous solution under stirring overnight at room temperature, and the extract was centrifuged at 10000 rpm for 1 hr. The pH of the supernatant was made to 6 by adding AcOH. The solution was placed in a filtration chamber (Diaflo apparatus Model 401) fitted with Daiflo PM-30 membrane, and ultrafiltered under continuous addition of 2 liters of water by applying a pressure. The ultrafiltrate passing through the PM-30 membrane (under molecular weight 30000) was concentrated in vacuum, and dialyzed against tap water and deionized water for 3 days, respectively. The slightly yellowish flake was yielded by lyophilization (fraction A_6). Yield from HA, 4%. Sixty ml of DEAE-Sephadex (borate form) swelled in water was suspended in the residual solution non-filtered through the PM-30 membrane. After the mixture was occasionally stirred with care not to destroy the Sephadex, it stood overnight. The DEAE-Sephadex adsorbed substances was filtered by 40 μ nylon mesh, and washed with hot water several times carefully. The filtrate and washings were combined, and the procedure described above was repeated three times. The filtrate and washings were centrifuged at 10000 rpm for 1 hr to make sure of excluding impurity, and the supernatant was precipitated by addition of 1 volume of EtOH. The precipitate was collected by centrifugation and washed with 60% EtOH a few times, and dialyzed against deionized water for 3 days. Lyophilization yielded the white flake (fraction A_3). Yield from HA, 24%. The DEAE-Sephadex adsorbed substances was put into a column, and the adsorbate was eluted with 2% NaOH solution. The eluate was neutralized with AcOH, and dialyzed against tap water for 3 days, and precipitated by addition of 3 volumes of EtOH. The precipitate was collected by centrifugation, and redialyzed against deionized water for 3 days, and lyophilized to obtain a white flake, fraction A_5. Yield from HA, 14%.

Complete Acid Hydrolysis——Each fraction was heated in 1N H_2SO_4 solution by a boiling water bath for 4.5 hr. The reaction mixture was neutralized with $BaCO_3$, and $BaSO_4$ was filtered off by a Millipore membrane. The filtrate and washings were passed through a column of Amberlite CG 120 (H^+ form), and concentrated at 37° under a reduced pressure, and lyophilized to obtain each hydroyzate.

Gas Chromatography——Gas liquid chromatographic analyses were made with a Shimazu Model GC 4A (PF) Gas Chromatograph attached with a hydrogen flame detector. Samples for gas chromatography were prepared by treatment of the hydrolyzates of each fraction with trimethylchlorosilane, hexamethyldisilazane and bis(trimethylsilyl)acetamide. The TMS-derivatives were chromatographed at 160° with N_2 carrier flow of 42 ml/min, on a 2 m×3 mm of glass column packed with Shimalite W (100—120 mesh) coated with 1.5% OV 17 or at 200° on a column prepared with 0.75% SE 30.

Liquid Chromatography——The liquid chromatographic analyses were performed with a Japan Electron Optics Lab. liquid chromatographic autoanalyzer Model JLC-3BC. For analyses of component acidic sugars, the hydrolyzates of each fraction were applied to a 13 cm×0.8 mm of column of anion exchanger, JEOL Resin LC-R-3 (chlorate form), and eluted with 0.005N HCl. The components were detected with orcinol-H_2SO_4 method, and absorbancies at 510 mμ and 425 mμ were automatically recorded. The sample for component neutral sugars were prepared by dissolving the hydrolyzates of each fraction in 0.15M borate buffer of pH 8.0. These sugar-borate complexes were applied to the column of JEOL Resin LC-R-3 transformed into borate form. A first buffer of 0.11M borate buffer of pH 7.5 was run with flow rate of 0.52 ml/min during 120 min, and thereafter a second buffer of 0.25M borate of pH 9.0 was flowed during 80 min, and the components were finally eluted by 0.35M borate buffer of pH 9.6. Integrated area of the peak

of each sugar was measured in the chromatogram, and quantity of the sugar was calculated by comparing the value with that of the corresponding peak in a standard chromatogram. In Table II, the sugar content was expressed as a relative value to that of a main sugar in the fraction.

Methylation of the Fraction A_5——A mixture of NaH (400 mg) and dimethylsulfoxide (DMSO) (10 ml) was refluxed at 80° for 2.5 hr, and added to the DMSO solution (40 ml) of the fraction A_5 (200 mg), and stirred for 1 hr at room temperature. Then CH_3I (20 ml) was carefully added to the reaction mixture with stirring, and it was further stirred overnight at room temperature. The reaction product was continuously extracted with $CHCl_3$, and $CHCl_3$ was evaporated out. This procedure was repeated three times. The methylated product was finally obtained by washing with petroleum ether, and its absorption near 3400 cm^{-1} in the IR spectrum was negligibly small.

Assay Method of Antitumor Activity——Mice used were males and females of the Swiss albino strain, initially weighing about 20 g. Ascites of sarcoma 180 was subcutaneously injected in 0.05 ml (about 8×10^6 cells) into the right groin of mouse. After 24 hr of tumor implantation, the sample was injected intraperitoneally daily for 10 days. The growth of solid tumor was charted weekly, and the tumor was weighed at the end of 5 weeks. The inhibition ratio was calculated by comparing the tumor weight of the treated mouse with that of the control.

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