[Chem. Pharm. Bull. 21(8)1772—1776(1973)]

UDC 547.814.5.02.05 : 581.192 : 615.322.011.5

Studies on Antitumor Polysaccharides of *Flammulina velutipes* (Curt. ex Fr.) Sing. I

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(Received February 13, 1973)

Antitumor polysaccharides were obtained from the aqueous extract of *Flammulina velutipes* (Curt. ex Fr.) Sing., one of popular edible mushrooms. Fractionation was made mainly using ultrafiltration, DEAE Sephadex adsorption and ethanol precipitation.

Two polysaccharides with high tumor inhibition were isolated and they were chemically different. One was a glucan and the other polysaccharide contained mainly glucose, galactose, mannose and arabinose.

It was found by T. Ikekawa, *et al.* that aqueous extracts of some edible mushrooms were very effective for inhibiting growth of transplanted Sarcoma 180 by the host-mediated antitumor bioassay.[2] This paper concerns with fractionation and purification of the antitumor active components of *Flammulina velutipes* (Curt. ex Fr.) Sing. It is one of popular edible mushrooms cultivated in our country and its Japanese name is "enokitake".

On the other hand, we reported in the previous paper that antitumor polysaccharide fractions were isolated from extracts of *P. ostreatus*, and some of the fractions highly inhibited the growth of the experimental tumor.[3] As shown in Chart 1, fractionation of the aqueous extract of *F. velutipes* was made according almost to the same procedure as in the previous paper.[3]

EA_3 fraction was not ultrafiltrated by using a Diaflo membrane PM-30 and not adsorbed to DEAE Sephadex (borate type), and it was obtained by addition of one volume of ethanol and centrifugation. By addition of two more volumes of ethanol to the supernatant, EA_7 fraction was obtained. EA_5 fraction was not ultrafiltrated by the Diaflo membrane as well as EA_3 fraction, but was adsorbed to the DEAE Sephadex. The fraction adsorbed to DEAE Sephadex was eluted by 1% sodium hydroxide solution, and after neutralization and dialysis EA_5 fraction was precipitated with addition of three volumes of ethanol. EA_6 fraction was a fraction ultrafiltrated by the membrane in the acetone precipitate of the aqueous extract and it was dialyzed using cellophane tube and lyophilized. Thus four fractions, EA_3, EA_5, EA_6 and EA_7 were obtained.

By a high voltage electrophoresis using glass fiber paper and borate buffer (pH 9.4), EA_6 fraction gave mainly two spots, which would be substances with molecular weight lower than 30000. EA_5 fraction showed one spot by the high voltage electrophoresis, but EA_3 and EA_7 fractions were not developed by condition of the electrophoresis described in the experimental section.

The fractions except EA_6 had no ultraviolet (UV) absorption other than end absorption. Nuclear magnetic resonance (NMR) spectra showed no other characteristic signal than C-H, and infrared (IR) spectra also showed no characteristic absorption except C-H, O-H and C-O vibrations in EA_5, EA_3 and EA_7 fractions.

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1) Location: *Tsukiji 5-1, Chuo-ku, Tokyo.*
2) *a*) T. Ikekawa, N. Uehara, Y. Maeda, M. Nakanishi, and F. Fukuoka, *Cancer Res.*, **29**, 734 (1969); *b*) T. Ikekawa, M. Nakanishi, N. Uehara, G. Chihara, and F. Fukuoka, *Gann*, **59**, 155 (1968).
3) Y. Yoshioka, T. Ikekawa, M. Noda, and F. Fukuoka, *Chem. Pharm. Bull.* (Tokyo), **20**, 1175 (1972).

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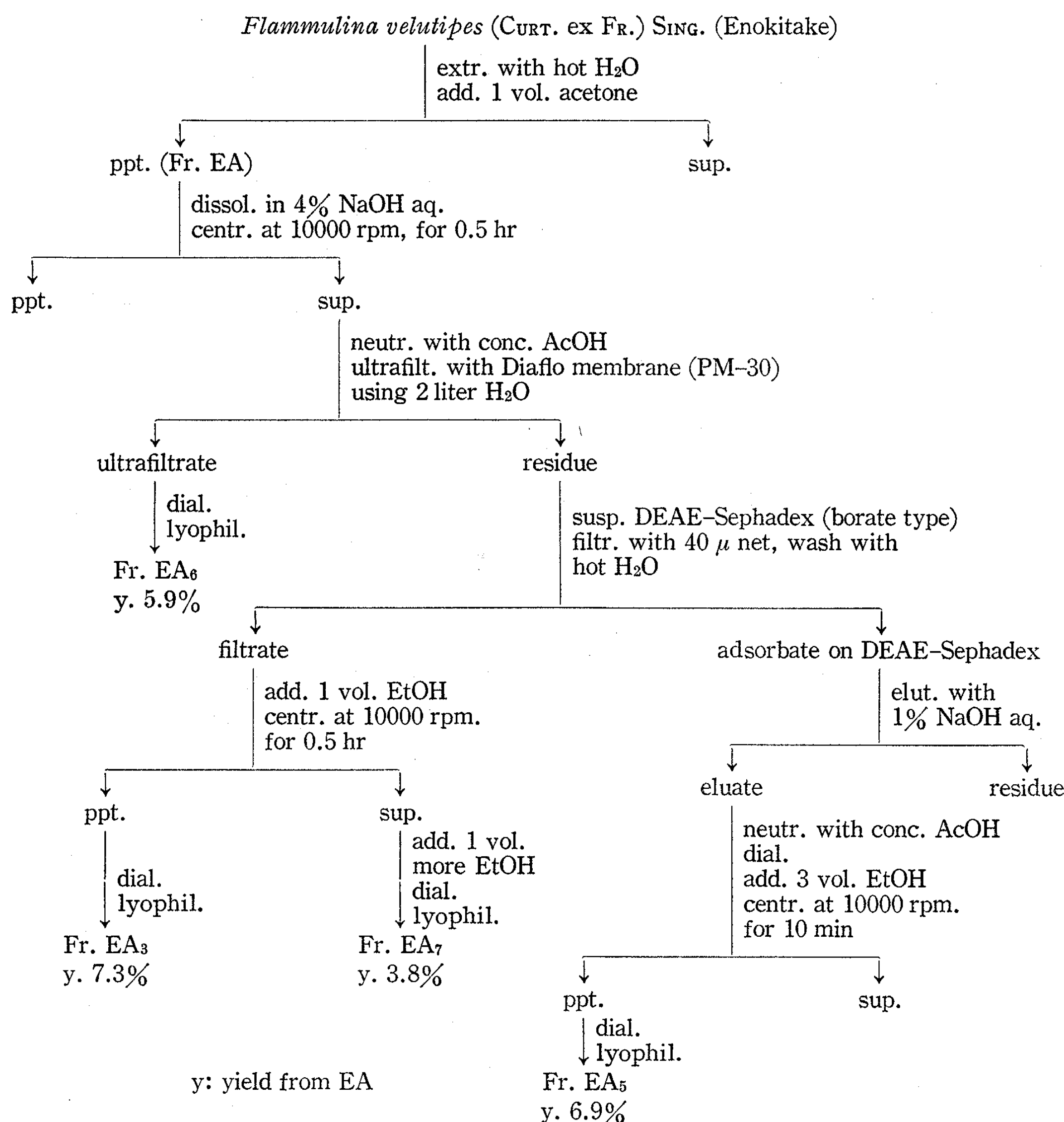

Chart 1. Fractionation of Aqueous Extract

Some basic properties of each fraction were described in Table I. In the Table elemental analysis showed that EA_6 fraction had 3.82% nitrogen. Nitrogen's character of EA_5, EA_3 and EA_7 fractions was not clarified now, but no nitrogen reagent was used during the fractionnation and purification and the fractions had only less than 1% nitrogen.

Total carbohydrate content of each fraction was measured by phenol sulphuric acid method,[4] and EA_3 and EA_7 fractions gave 100% carbohydrate content though EA_5 fraction showed a slightly low value.

Though it was found that the fraction HA_5 corresponding to EA_5 fraction in *P. ostreatus* contained acidic sugars,[3] in EA_5 fraction carbonyl group was not detected by infrared (IR) spectral analysis, and no acidic sugar was found by liquid chromatography. EA_5 fraction gave $[\alpha]_D^{24}$ $+57.4°$ and positive plain curve in an optical dispersion curve, but EA_3 fraction gave $[\alpha]_D^{24}$ $-15.6°$ and did not gave clear plain curve in an optical dispersion curve.

Qualitative and/or quantitative analyses of sugar components were made by thin-layer chromatography (TLC), gas chromatography (GC) and liquid chromatography of acid hydrolyzates of each fraction. EA_6 fraction was completely hydrolyzed with 1 N sulphuric acid,

4) M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, and F. Smith, *Anal. Chem.*, **28**, 350 (1956).

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Table I. Physico-chemical Properties

Fraction		EA_5	EA_3	EA_7	EA_6
Elemental analysis	C	39.17%	39.76%	39.98%	41.39%
	H	6.85	7.08	7.02	6.92
	N	0.90	0.67	0.89	3.82
ORD (in H_2O)		positive plain		positive plain	
Total carbohydrate content[a)]		94%	100%	100%	70%
$[\alpha]_D^{24}$ (c=0.5, H_2O)		+57.4°	−15.6°	+48.2°	−14.2°

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

and other fractions were completely hydrolyzed with 2N sulphuric acid to analyze sugar components. In TLC analyses of the hydrolyzates Silica gel G and microcrystalline cellulose were used, and tetramethylsilyl derivatives and trifluoroacetyl derivatives of the hydrolyzates were applied to gas chromatographic analyses. Quantitative analysis of the sugar components was carried out by mean of liquid chromatography, using strong basic resin (JEOL LC-R-3, borate type).

These analytical results were summarized in Table II. EA_3 fraction contains 92.5% glucose, and this finding in *F. velutipes*, whose main sugar component is glucose, is the same result to that of the fraction (HA_3) corresponding to EA_3 in *P. ostreatus*. But optical rotations showed different results from each other.

Table II. Component Sugar

Sugar	Fraction											
	EA_5			EA_3			EA_7			EA_6		
Method	TLC[a)]	GC[b)]	LC[c)]	TLC	GC	LC	TLC	GC	LC	TLC	GC	LC
Glucose	++	+++	45.0%	+++	+++	92.5%	+++	+++	41.2%	++	++	18.9%
Galactose	++	+++	32.0		+	1.6	+	++	17.9	+++	++	44.9
Mannose	+	++	13.4		+	2.9	+	++	28.3	++	++	23.2
Xylose	+	+	2.4			1.7		+	7.3	+	+	2.4
Arabinose		+	7.2			1.3		+	5.3	+		10.6

a) thin-layer chromatography
plate: Silica gel G impregnated with 5% NaH_2PO_4; solvent: *n*-BuOH: acetone: H_2O (4:5:1)
plate: microcrystalline cellulose; solvent: pyridine: EtoAc: AcOH: H_2O (5:5:1:3), *n*-BuOH: AcOH: H_2O (3:1:1)
b) gas chromatography
column: 0.75% OV-17; temperature: 160° or temp. programming 140°—160°; carrier gas; N_2 gas
c) liquid chromatography
stationary phase: JEOL resin LC-R-3 (borate form); mobil phase: 0.11M pH 7.5→0.25M pH 9.0→0.35M pH 9.6 borate buffer.
coloration: orcinol-H_2SO_4, measured the optical density at 423 mμ and 510 mμ.

In a point of view of antitumor activity these two fractions have very high activity for inhibition of the growth of implanted Sarcoma 180. That is to say, EA_3 showed 82% inhibition ratio in a dose of 1 mg/kg/day, and in *P. ostreatus* HA_3 showed 80% inhibition ratio in the same dose. So this finding showed that optical rotation of the polysaccharides or mode of linkage between sugars might not be concerned with the antitumor activities.

EA_5 fraction was constituted with several kind of sugars, that is glucose, galactose, mannose and arabinose. This suggested that EA_5 might still be a mixture of some kind of polysaccharides in this stage. In liquid chromatography it is difficult to distinguish between arabinose and fructose in a general condition of the borate buffer used for neutral sugars as shown in Fig. 1. But it was determined by gas–liquid chromatography (GLC) analysis of trifluoroacetate using XF-1105 column that EA_5 contained arabinose and not fructose. Fur-

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ther purification of antitumor active component from EA_5 is under progress. It is of interest that glucose percentage of EA_5 is lower than that of the fraction HA_5 of *P. ostreatus* which correspond to EA_5 and mainly consist of glucose, and in the present stage the antitumor activity of EA_5 is very high in spite of low glucose percentage, which is 84% inhibition ratio in a dose of 1 mg/kg/day.

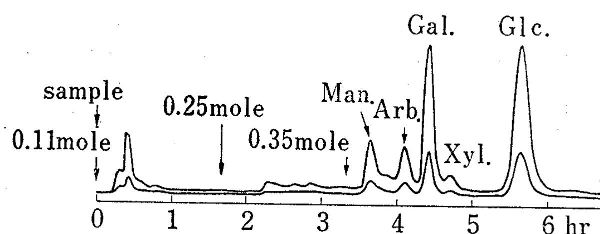

Fig. 1. Liquid Chromatography of the Hydrolyzate of EA_5

stationary phase: JEOL resin LC-R-3 (borate type)
mobile phase: 0.11M borate buffer (pH 7.5)→0.25M borate buffer (pH 9.0)→0.35M borate buffer (pH 9.6)
coloration: orcinol-H_2SO_4, and measured the optical density at 423 mμ.

As shown in Table I nitrogen percentage of EA_6 fraction was 3.82% and it came from peptides. Amino acid analysis of the complete acid hydrolyzate showed that EA_6 had 16 amino acids.

The brief communication on antitumor activities of each fraction is reported in our paper.[5]

Experimental

General Method——UV spectra were obtained by a Cary spectrophotometer Model 14, and IR spectra by a Japan Spectroscopic Co., Model DS-402 G. NMR spectra were performed with a Japan Electron Optics Lab., JNM-3H-60.

Electrophoresis——High voltage paper electrophoresis was made using Whatman glass fiber paper (GF 83) and 0.1 M borate buffer (pH 9.4) at 50 V/cm. It was developed for 15 min. The developed paper was colored by spraying ammonium vanadate sulphuric acid solution and heating.

Isolation and Purification——Fruit bodies of *Flammulina velutipes* (Curt. ex Fr.) Sing. (60 kg) were extracted with H_2O (60 liter) under mechanically stirring and heating overnight. The residual bodies were removed by centrifugation and the extract was condensed to *ca.* 23 liter and one volume of acetone was added.

The precipitate was collected by centrifugation and washed with acetone, and EA fraction (444 g) was obtained after drying up.

EA (10 g) was dissolved in 4% of NaOH aq. solution (450 ml) and after removing the residue with centrifuging at 10000 rpm for 0.5 hr, the supernatant was neutralized with AcOH to pH 6.0. The solution of EA was put into a Diaflo chamber and ultrafiltrated using a Diaflo membrane PM-30 and *ca.* 2 liter of H_2O, and then fraction of molecular weight less than 30000 was filtrated through, and the fraction of molecular weight more than 30000 remained in the chamber. The ultrafiltrate of the Diaflo membrane was dialyzed against tap water and then deionized water for 3 days, respectively. After lyophilization of the non-dialysable solution, EA_6 fraction (586 mg.) was obtained as a white powder.

To the residual solution in the chamber, 60 ml of DEAE Sephadex A-50 (borate type) was suspended with shaking often and stood overnight. The DEAE Sephadex was collected with filtration using 40 μ net. Another fresh DEAE Sephadex (100 ml) was added to the filtrate again and stood overnight and filtrated as the previous procedure. This procedure was repeated two more times. The Sephadex was combined and washed with hot water several times. The washings and filtrate were combined and one volume of EtOH was added after condensation. The precipitate was collected with centrifuging at 10000 rpm for 10 min, and washed with 60% of EtOH aq. solution. It was dialyzed against deionized water for 3 days, and EA_3 (734 mg) was obtained by lyophilization.

Two more volumes of EtOH was added to the supernatant of the one volume EtOH precipitation and the precipitate was collected by centrifuging at 10000 rpm, for 10 min. After dialysis and lyophilization EA_7 (378 mg) was obtained. The adsorbate on the DEAE Sephadex was eluted with 2 liter of 1% of NaOH aq. solution and the eluate was neutralized with AcOH to pH 6.0. After dialysis against tap water and condensation three volumes of EtOH was added and the precipitate was collected. Thus, EA_5 (678 mg) was obtained after dialysis against deionized water and lyophilization.

Acid Hydrolysis——a) To analyze amino acids, EA_6 (20 mg) was dissolved in 6 N HCl aq. solution (20 ml) and heated in a sealed tube at a constant boiling point for 18 hr. After evaporation in a reduced pressure, it was analyzed by an amino acid analyser, a Japan Electron Optics Lab., JLC-5AH. Amino acid components of EA_6 were 16 amino acids, which was almost same to the result on flammulin.[6] The ratio was as

5) T. Ikekawa, Y. Yoshioka, M. Emori, T. Sano, and F. Fukuoka, *Cancer Chemotherapy Reports*, Part 1, **57**, 85 (1973).
6) *a*) N. Komatsu, H. Terakawa, K. Nakanishi, and Y. Watanabe, *J. Antibiotics*, **16A**, 139 (1963); *b*) Y. Watanabe, K. Nakanishi, N. Komatsu, T. Sakabe, and H. Terakawa, *Bull. Chem. Soc.*, (Japan), **37**, 747 (1964).

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follows; alanine, 26.6; glycine, 23.0; leucine, 19.8; valine, 18.7; threonine, 17.8; tyrosine, 17.2; isoleucine, 11.9; serine, 10.7; phenylalanine, 9.7; proline, 7.6; methionine, 1.0; aspartic acid, 30.8; glutamic acid, 27.8; histidine, 14.9; arginine, 12.6 and lysine, 5.6.

b) To analyze sugar components of EA_3, EA_5, and EA_7, 30 mg of each sample was dissolved in 35 ml of 2N H_2SO_4 solution and refluxed for 7 hr. Then it was neutralized with $Ba(OH)_2$ aq. solution. Excess Ba^{2+} ion was removed passing through strong acidic ion exchange resin (Amberlite IR-120, H^+ type). To hydrolyze EA_6 1N H_2SO_4 solution was used instead of 2N H_2SO_4 solution.

Gas-Liquid Chromatography——Gas-liquid chromatograph, Shimadzu Model GC 4A (PF) attached with a hydrogen flame detector was used to determine neutral component sugars.

a) TMS Derivatives: Trimethylsilyl derivatives were made from the hydrolyzates in dried pyridine by addition of trimethylchlorosilazane and bis(trimethylsilyl)-acetaminde and heated at 60° for 15 min. They were chromatographed on a column of 0.75% OV-17 coating supporte.

b) TFA Derivatives: To a solution of the sample 1% $NaBH_4$ aq. solution was added and stood few hr at room temperature. After destruction the excess $NaBH_4$ by Amberlite IR-120 (H^+ type) the solution was dried up and then MeOH was added and evaporated to dryness again. Addition and evaporation was repeated several times to remove borate. The reduction products were treated with trifluoroacetic acid anhydride in AcOEt solution.[7]

Liquid Chromatography——The liquid chromatographic analysis was done using a Japan Electron Optics Lab.'s liquid chromatograph, Model JLC-3BC. Strong basic resin (JEOL LC-R-3) was used as a stationary phase, and measured UV absorbancy at 423 and 510 mμ when colored with orcinol-H_2SO_4 method. To analyze neutral sugars stationary phase was exchanged to borate form and eluted at first with 0.11 M borate buffer (pH 7.5) for 100 min, next 0.25 M borate buffer (pH 9.0) for 100 min., and finally 0.35 M boratc buffer (pH 9.6). In analysis of acidic sugars the resin was used at Cl^- form and eluted with 0.005 N HCl, but no acidic sugar was detected in the hydrolyzates of EA_3, EA_5, and EA_7. In order to analyze quantitatively the sugar components, integration of each peak area should be made in a chromatogram and its value was compared with that of the corresponding peak in the chromatogram of standard samples. Percentage in Table II shows the numerical value of each sugar converting the content of all neutral sugars as 100%.

Thin-Layer Chromatography——In thin-layer chromatography Silica gel G[8]impregnated with 5% NaH_2PO_4 and microcrystalline cellulose[9] were used as plates, and a diphenylamine aniline solution, aniline phthalate solution or alkaline silver nitrate solution as spray reagents. Development solvents were as follows: in Silica gel G, *n*-BuOH: acetone: H_2O; 4: 5: 1, and in microcrystalline cellulose, pyridine: EtOAc: AcOH: H_2O; 5: 5: 1: 3, and *n*-BuOH: AcOH: H_2O; 3: 1: 1. Usually double development was applied.

Acknowledgement The authors thank to Drs. W. Nakahara and F. Fukuoka of this Institute for their kind encouragement and advices throughout this work. They are grateful to Dr. Takeda, the Faculty of Pharmaceutical Sciences, University of Tokyo for his co-operation in amino acid analysis. Thanks are also due to the members of the analytical center in the Faculty of University of Tokyo for elemental analysis and ORD measurement.

7) T. Imanari, Y. Arakawa, and Z. Tamura, *Chem. Pharm. Bull.* (Tokyo), **17**, 1967 (1969).
8) Yu, S. Ovodov, V.E. Vaskovsky, E.V. Evtushenko, R.G. Ovodava, and T.F. Soloveva, *J. Chromatog.*, **26**, 111 (1967).
9) M.L. Wolform, D.L. Patin, and Rosa. M. de Lederkremer, *J. Chromatog.*, **17**, 488 (1965).

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