Antitumor Activity of *Hypsizigus marmoreus*. I. Antitumor Activity of Extracts and Polysaccharides

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Antitumor activity of *Hypsizigus marmoreus*, one of the most popular Japanese edible mushrooms, was investigated. The aqueous and methanol extracts were tested against allogeneic tumor, solid sarcoma 180 and syngeneic tumor, Meth A fibrosarcoma. The aqueous extract was highly active in inhibiting growth of solid sarcoma 180, but not as much for Meth A fibrosarcoma. Fractionation of antitumor substances of the aqueous extract isolated four polysaccharides. Chemical analysis revealed one of them to be β-(1-3)-glucan with a remarkable inhibitory effect against tumor-growth of sarcoma 180.

Keywords antitumor activity; mushroom; *Basidiomycetes*; *Hypsizigus marmoreus*; sarcoma 180; Meth A fibrosarcoma; polysaccharide

Introduction

It has long been believed in Japan that certain mushrooms are effective in halting cancer, and we had studied the antitumor activity of *Basidiomycetes*, reporting for the first time that *Coriolus versicolor* (FR.) QUÉL. showed this activity[1]; thereafter, so-called PSK was isolated from the same mushroom.[1,2] Our study also illustrated that Japanese edible mushrooms were highly active in inhibiting tumor-growth and an antitumor glucan was isolated from *Lentinus edodes* (BERK.) SING.[3] Polysaccharides and protein-binding polysaccharides were isolated from *Flammulina velutipes* (CURT. *ex.* FR.) SING. and other edible mushrooms, and their antitumor activity reported.[4-10]

In this study we describe the antitumor activity of a fruit-body of another Japanese edible mushroom, *Hypsizigus marmoreus* (bunashimeji in Japanese), which has recently becomes one of the most popular edible mushrooms in Japan.

Materials and Methods

Isolation Procedure Aqueous Extraction: Fruit-body (10 kg) of *Hypsizigus marmoreus* T-2 strain cultivated and supplied by the Mushroom Spawn Center of Iiyama Agricultural Co-operatives, Iiyama City, Nagano Prefecture, Japan was chopped and 40 l of deionized water was added; 4 h extraction under refluxing, the aqueous extract was filtered through by 60-mesh filter. A further 20 l of deionized water was added to the residual fruit-body and heated for another hour. The extracts were combined and condensed in vacuum, and after lyophilization the aqueous extract (YH, 146 g) was obtained.

Methanol Extraction: To a chopped fruit body (270 g), 600 ml of MeOH was added and the material was extracted for 5 h under refluxing. After extraction and removal of the solvent, MeOH extract (YM, 6.76 g) was obtained.

Fractionation Procedure To the condensed aqueous extract of the fruit body, one volume of EtOH was added and centrifuged, and the precipitate was collected. The precipitate was dissolved in 4% NaOH solution and after removing precipitates by centrifugation, the supernatant was neutralized by dil. AcOH and ultrafiltrated by Diaflo membrane (PM-30). The inner fraction (mol. wt. more than 30000) was lyophilized and a grayish white powder YH-1 fraction (21 g) was obtained. YH-1 fraction (10 g) was dissolved in deionized water (300 ml), and diethylaminoethyl (DEAE)-Sephadex (borate type, 60 ml) was added and allowed to stand overnight with stirring. After removing the DEAE-Sephadex by filtration, aqueous solution of non-adsorbated substances was obtained. One volume of EtOH was added to this solution and a precipitate (YH-2 fraction, yield: 0.4%) was isolated by centrifugation. A supernatant from which YH-2 fraction was removed was concentrated and freeze dried to obtain YH-3 fraction (yield: 94.1%). Substances adsorbed into DEAE-Sephadex, on the other hand, were eluted by 1% NaOH solution and the eluate solution was neutralized by dil. AcOH and three volume of EtOH was added. The precipitate thus obtained was isolated by centrifugation to be YH-4 fraction (yield: 0.8%) as shown in Fig. 1. The supernatant was evaporated in vacuum to remove the solvents, and YH-5 fraction (yield: 4.8%) was obtained after lyophilization.

Chemical Analysis Protein content was determined by Folin–Lowry method using bovine serum albumin as a standard and sugar content using D-glucose as a standard by phenol–sulfuric acid method. The sugar components were determined by follows; 50 mg of YH or YH-5 was dissolved in 100 ml of 1 N H_2SO_4 solution and heated for 4 h. After neutralization of the acid hydrolysate, the sugar components were determined by high performance liquid chromatography (HPLC). An instrument of HPLC (LC-5A, Shimadzu) with a column of TSK gel sugar AXI (Toyo Soda) attached was used. As a mobile phase, 0.45 M borate buffer (pH 8.8, flow rate: 0.4 ml/min) and as a reaction phase, 0.45 M borate buffer containing 1% cyanoacetamide (pH 8.8, flow rate: 0.4 ml/min) were used; and column temp. was 60 °C and reaction temp. 100 °C, and monitoring was done intensity at 280 nm. In amino acid analysis, after YH was hydrolyzed in 5.7 N HCl at 110 °C for 24 h, the solvent was evaporated in vacuum and analysis of the hydrolysate was performed by an amino acid analyzer (Hitachi, L-8500).

Infrared (IR) spectra were run using a KBr tablet by JASCO IRA-1.

Animal Female ICR mice supplied by Japan Crea Co., Ltd. (Tokyo) weighing 24±3 g and female BALB/c mice supplied by Charles River, Japan (Kanagawa) weighing 17±3 g were used.

Tumor Sarcoma 180 and Meth A fibrosarcoma maintained in this research institute by a weekly passage were used. In sarcoma 180, 1.0×10^6 cells/mouse was subcutaneously transplanted into ICR mice, and in Meth A fibrosarcoma, 1.0×10^6 cells/mouse was also subcutaneously transplanted into BALB/c mice to test the antitumor activity.

Antitumor Test *in Vivo* The test-sample was dissolved in physiological saline when intraperitoneally administratered, or in deionized water with per-oral administration. Treatment was started from 24 h after the tumor transplantation, and the test-sample was administered once daily for 10 d. Solid tumors of sarcoma 180 were dissected out 35 d after the tumor transplantation and the tumor growth inhibition ratio was calculated as reported in our previous papers.[7,9,10] In Meth A fibrosarcoma, the solid tumor weight was measured 21 d after the tumor transplantation, and the tumor growth inhibition ratio was determined by the same way.

Cytotoxic Activity Test *in Vitro* A mouse leukemia cultured cell line, L-5178Y (5.0×10^4 cells/ml) maintained in this research institute was cultured in RPMI-1640 medium containing 10% fetal calf serum in a CO_2 incubator for 48 h with or without test-samples. Cell counting was done using a micro cell-counter (Sysmex Co., Ltd.), and IC_{50} (50% inhibition concentration) was determined as described earlier.[9,10]

Statistical Analysis The difference in the mean tumor weight between the treated and control groups was calculated statistically by Student's *t* test. *P* values below 0.05 were considered significant.

Results and Discussion

In our studies on antitumor activity of the popular edible mushroom, *Hypsizigus marmoreus* the aqueous extract (YH) showed remarkably high activity against solid sarcoma 180 (Table I). Particularly at a dose of 30 or 100 mg/

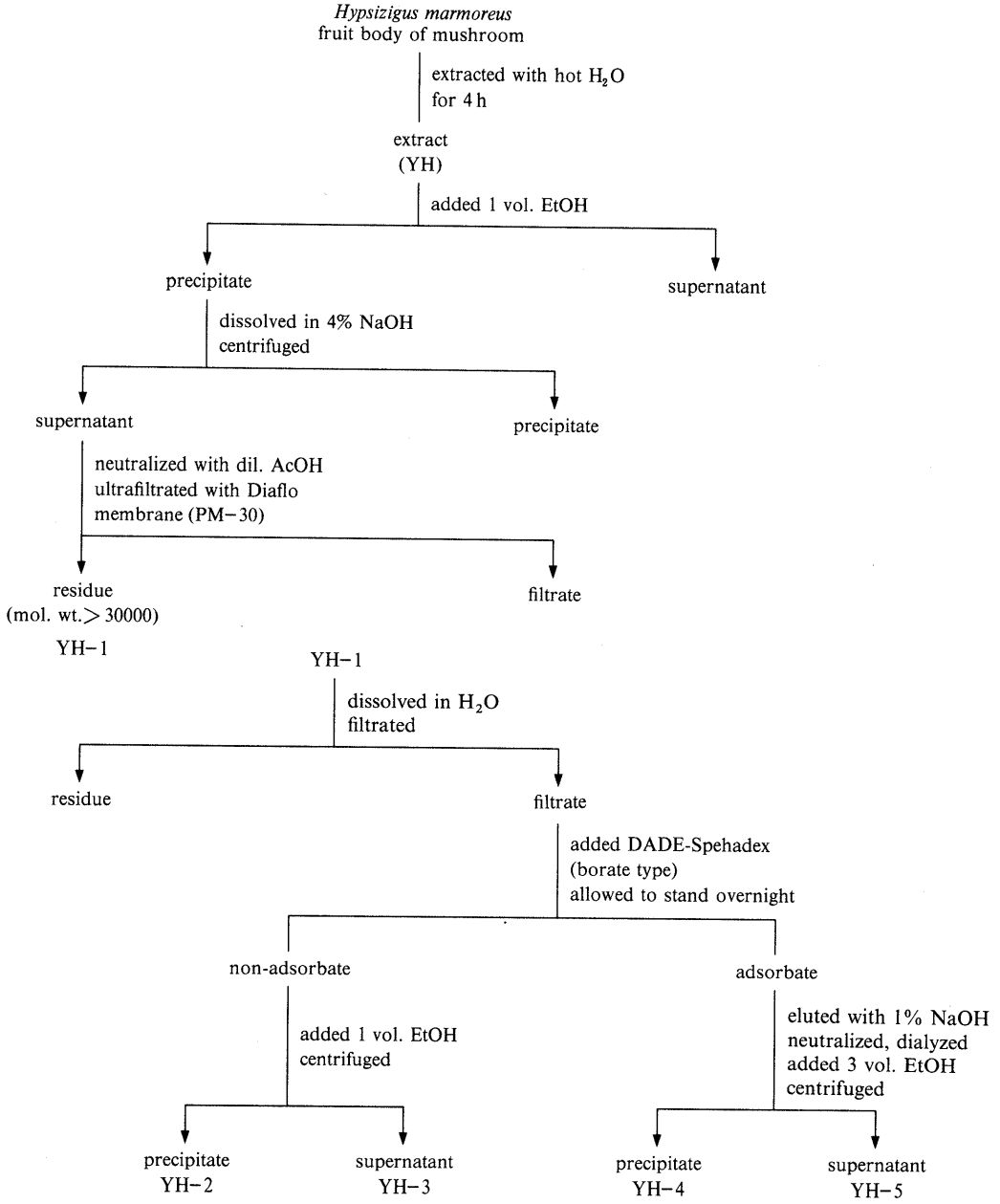

Fig. 1. Isolation of the Antitumor Polysaccharides from *Hypsizigus marmoreus*

TABLE I. Antitumor Activity of Extracts of *Hypsizigus marmoreus* against Solid Sarcoma 180 by Intraperitoneal Administration

Sample	Dose (mg/kg)	Tumor weight (g) mean±S.E.	Inhibition ratio (%)	Complete regression[b)]
	Control	9.4±2.3		0/6
YH	100	0.0	100	6/6
YH	30	0.0	100	6/6
YH	10	2.7±1.6[a)]	71	3/6
	Control	3.4±0.7		
YM	100	3.0±0.7	12	0/6
YM	30	3.3±0.8	3	0/6

a) $p<0.05$. *b*) No. of regressed mice/No. of total mice. Tumor: sarcoma 180, 1.0×10^6 cells/mouse s.c. transplantation. Animal: female ICR mouse. Administration: i.p. day 1 to 10. Vehicle: saline.

kg/d ×10, the tumors of all mice completely regressed, and at a dose of 10 mg/kg/d×10, 72% tumor growth inhibition was shown, though a methanol extract of this mushroom did not show any inhibitory activity by this assay. Oral administration of the aqueous extract showed approximately 20% tumor growth inhibition, though this was not significant (Table II). Table III shows the experimental result of antitumor activity against syngenetic tumor of Meth A fibrosarcoma, and the aqueous extract had a certain activity although it was not significant.

TABLE II. Antitumor Activity of Aqueous Extract of *Hypsizigus marmoreus* against Solid Sarcoma 180 by Oral Administration

Sample	Dose (mg/kg)	Tumor weight (g) mean±S.E.	Inhibition ratio (%)
	Control	5.9±1.4	
YH	1000	4.6±1.3	22
YH	500	4.4±0.4	25

Tumor: sarcoma 180, 1.0×10^6 cells/mouse s.c. transplantation. Animal: female ICR mouse. Administration: *p.o.* day 1 to 10. Vehicle: distilled water.

TABLE III. Antitumor Activity of Aqueous Extract of *Hypsizigus marmoreus* against Solid Meth A Fibrosarcoma by Intraperitoneal Administration

Sample	Dose (mg/kg)	Tumor weight (g) mean±S.E.	Inhibition ratio (%)
	Control	6.9±0.5	
YH	200	5.4±0.7	22
YH	100	5.1±0.5	26
YH	50	6.4±0.5	7

Tumor: Meth A fibrosarcoma, 1.0×10^6 cells/mouse s.c. transplantation. Animal: female BALB/c mouse. Administration: i.p. day 1 to 10. Vehicle: saline.

TABLE IV. Chemical Components of Fruit Body of *Hypsizigus marmoreus*

Component	Result
Water	91.0%
Protein	3.0%
Fat	0.8%
Fiber	0.9%
Ash	0.7%
Sugar	3.6%
P	105 mg/100 g
Fe	0.68 mg/100 g
Ca	1.4 mg/100 g
Na	2.56 mg/100 g
K	356 mg/100 g
Thiamine (Vitamin B_1)	0.24 mg/100 g
Riboflavin (Vitamin B_2)	0.20 mg/100 g
Niacin	8.38 mg/100 g

TABLE V. Sugar and Amino Acid Components of the Aqueous Extract of *Hypsizigus marmoreus*

Sugar	(%)	Amino acid	(%)
Glucose	88.0	Glutamic acid	29.9
		Arginine	10.1
Xylose	3.6	Aspartic acid	9.3
		Alanine	7.3
Galactose	2.6	Lysine	5.2
		Serine	4.8
Mannose	2.3	Glycine	4.3
		Valine	4.3
Rhamnose	1.8	Leucine	4.0
		Phenylalanine	3.8
Arabinose	1.6	Proline	3.8
		Threonine	3.7
		Histidine	3.1
		Isoleucine	2.6
		Tyrosine	2.2
		Cystine	1.2
		Methionine	0.4

Chemical components of the *H. marmoreus* T-2 strain mushroom itself are shown in Table IV. Here, protein and sugar contents are almost the same as those of *Pleurotus ostreatus* QUÉL. (hiratake in Japanese) and niacin content is higher than those of *Lentinus edodes* (BERK.) SING. (shiitake in Japanese) and *Flammulina velutipes* (CURT. *ex.* FR.) SING. (enokitake in Japanese) according to the Japan Standard Table of Food Components. In the aqueous extract glucose was the main component in sugar and in amino acid, glutamic acid, arginine and aspartic acid were dominant as shown in Table V. Purification of antitumor components in this study was done by the same procedure as that of antitumor polysaccharides of *F. velutipes*.[5] The aqueous extract was constituted mainly of high molecular weight substances.

TABLE VI. Antitumor Activity of Polysaccharides Isolated from *Hypsizigus marmoreus* against Solid Sarcoma 180 by Intraperitoneal Administration

Sample	Dose (mg/kg)	Tumor weight (g) mean±S.E.	Inhibition ratio (%)	Complete regression[c]
Control		4.5±1.1		0/11
YH-2	1	2.3±1.1	49	1/6
	3	1.0±0.9[a]	78	4/6
	10	0.0	100	6/6
YH-3	3	2.7±1.2	40	0/6
YH-4	1	0.0	100	6/6
	3	0.4±0.4[a]	91	5/6
YH-5	1	0.0	100	6/6
	3	0.03±0.03[b]	99	5/6
	10	0.0	100	6/6

a) $p<0.05$. *b*) $p<0.01$. *c*) No. of regressed mice/No. of total mice. Tumor: sarcoma 180 solid, 2.0×10^6 cells/mouse s.c. transplantation. Animal: female ICR mouse. Administration: i.p. day 1 to 10. Vehicle: saline.

The antitumor activity of the polysaccharides isolated as a nitrogen free substance in this study was tested against solid sarcoma 180 as shown in Table VI. Among them, YH-2 and YH-3 fractions showed 78 and 40% tumor growth inhibition at a dose of 3 mg/kg/d × 10. However, YH-4 and YH-5 fractions showed 100% tumor growth inhibition at a dose of 1 mg/kg/d × 10, though at a dose of 3 mg/kg/d × 10 the tumor of one mouse remained as described here. The inhibitory effect of each polysaccharide fraction against proliferation of L-5178Y cells *in vitro* was also investigated. IC_{50} values of YH-4, YH-5 fractions and the aqueous extract (YH) were more than 1000 μg/ml, indicating that they had no cytotoxic effect on cultured cell lines. The antitumor polysaccharides isolated from *H. marmoreus* may thus be biological response modifiers (BRM) accompanying with the antitumor activity.[3-10]

The active polysaccharide, YH-5 fraction contains 82% glucose and 18% galactose. IR absorption and carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectra of the polysaccharides were analyzed and compared the spectrum with that of a β-(1-3)-gluco-oligomer, laminariheptaose, which had an absorption band at 890 cm^{-1} based on β-(1-3)-linkage. The polysaccharides isolated from mushrooms are clinically used in Japan, but their efficacy in clinical application is not recognized in the rest of world so that usage is limited. The β-(1-3)-glucan, YH-5 fraction was somewhat water-soluble and its biological activity as a BRM may be of interest.

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