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FRACTIONATION AND ANTITUMOR ACTIVITY OF THE WATER-INSOLUBLE RESIDUE OF *Agaricus blazei* FRUITING BODIES

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ABSTRACT

Some polysaccharide-containing materials were successively extracted from the fruiting bodies of *Agaricus blazei* with aqueous ammonium oxalate and sodium hydroxide, fractionated, and assayed for antitumor activity. From chemical analyses and n.m.r. data, it was concluded that the most active fraction, FIII-2-b, was comprised of protein and a (1→6)-β-D-glucan.

INTRODUCTION

Agaricus blazei Murill (Himematsutake or Kawariharatake in Japanese) is well known in Japan as a home remedy having many physiological activities. From the fruiting bodies of this mushroom, we have already isolated cytotoxic steroids[1], and a lectin having hemagglutinating activity that was strongly inhibited by bovine submaxillary asialomucin[2].

The residue remaining after ethanol extraction of the cytotoxic steroids was further extracted sequentially with boiling water, boiling 1% ammonium oxalate, and 5% sodium hydroxide at room temperature. The polymers in the fractions were then characterized structurally and tested for antitumor activity.

Herein are reported the results for the polymers contained in the last two extracts.

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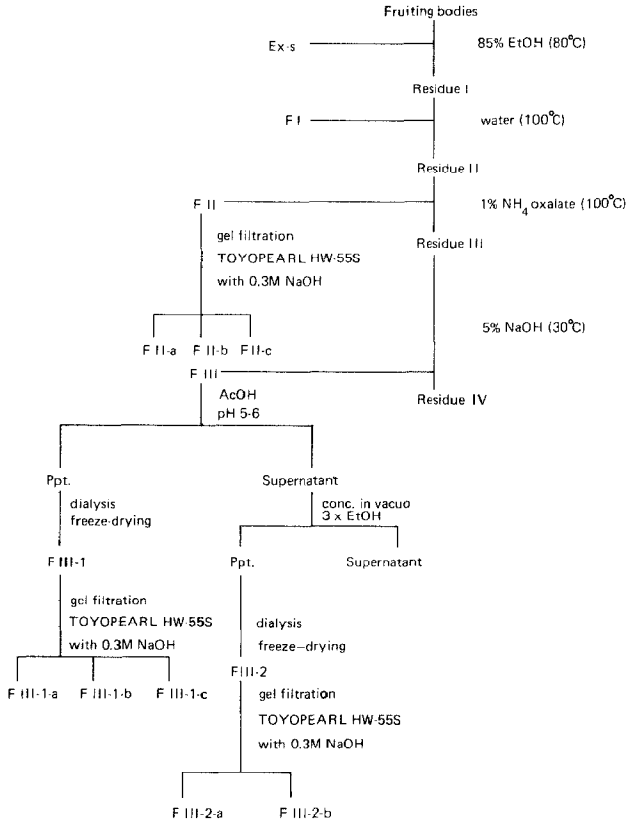
EXPERIMENTAL

Material. — Fruiting bodies of cultured *A. blazei* were a gift from the Iwade Institute of Mushrooms (Japan). Sepharose CL-6B was obtained from Pharmacia (Sweden), and Toyopearl HW55S was from Tosoh (Japan). Other chemicals and reagents were ordinary commercial products.

Extraction. — The extraction and fractionation were performed according to a method previously reported[3]. The steps are diagrammed in Scheme 1.



Scheme 1.

Monosaccharide composition. — The polysaccharides were completely hydrolyzed by heating in 0.5–1M H_2SO_4 for 3–6 h at 100°. The monosaccharides produced were reduced with $NaBH_4$, and the alditols acetylated with acetic anhydride and pyridine. The constituent sugars were determined by standard g.l.c. of their alditol acetates[3]. The total sugars were measured by the phenol–sulfuric acid method with reference to D-glucose[4]. Uronic acid was assayed by the carbazole–sulfuric acid method[5].

Amino acid composition. — Amino acids were analyzed with a Hitachi 835

amino acid analyzer after hydrolysis of samples (1 mg) in 6M HCl (1 mL) for 20 h at 110° in sealed, evacuated tubes.

Protein content. — The protein contents were determined by the method of Lowry *et al.*[6], with reference to bovine serum albumin.

I.r. spectra. — Potassium bromide pellets and a JASCO grating infrared spectrophotometer A 102 were used to record the i.r. spectra.

N.m.r. spectra. — All n.m.r. experiments were conducted with a JEOL GSX-400 spectrometer. Samples (15 mg) were dissolved in 0.5 mL of 0.3M NaOD. The chemical shifts were referenced to internal sodium 2,2,3,3-tetradeuterio-4,4-dimethyl-4-silapentanoate (TSP).

Other physical properties. — The values of $[\alpha]_D$ were determined with a PM 101 Union Giken automatic polarimeter. Molecular weights were estimated by gel filtration using Toyopearl HW55S standardized with standard dextrans (Pharmacia).

Methylation analysis. — Each polymer was completely methylated by the Hakomori method[7], and the product hydrolyzed first with 87% formic acid and then with 0.5M sulfuric acid. The resulting monosaccharides were reduced with $NaBH_4$, followed by acetylation with acetic anhydride and pyridine to give alditol acetates. The partially methylated alditol acetates were identified by the g.l.c. and g.l.c.–m.s. methods[3].

Assay for antitumor activity. — Assay for antitumor activity was performed according to a method previously reported[8].

RESULTS AND DISCUSSION

The antitumor activity of each fraction (see Scheme 1) is listed in Table I. We

TABLE I

ANTITUMOR EFFECTS OF WATER-INSOLUBLE FRACTIONS FROM THE FRUITING BODIES OF *Agaricus blazei* AGAINST SARCOMA 180 IN MICE

Fraction[a]	*No. of mice*	*Average tumor size* (cm^3)[b]	*Inhibition (%)*[b]	*Complete regression*[c]	*Mortality*[c]
Control	14	43.0	0	0/14	11/14
FII-a	5	20.3	52.8	0/5	3/5
FII-b	10	25.6	40.5	0/10	4/10
FII-c	10	8.9	79.3	5/10	2/10
FIII-1-a	5	0.3	99.4	3/5	1/5
FIII-1-b	10	23.2	46.2	1/10	3/10
FIII-1-c	10	17.9	58.3	2/10	4/10
FIII-2-a	5	6.7	84.3	2/5	0/5
FIII-2-b	10	0.4	99.1	**8/10**	**0/10**

[a]Dose, 10 mg/kg per day for 10 days. [b]Three weeks after the tumor implantation. [c]Six weeks after the tumor implantation.

TABLE II

PROPERTIES OF WATER-INSOLUBLE FRACTIONS FROM THE FRUITING BODIES OF *Agaricus blazei*

Fraction	*Yield (%, from Res. II)*	*Hexuronic acid (%, as GlcA)*	*Protein (%)*	*Sugar (%, as Glc)*	*Component sugar*[a]				$[\alpha]_D^{25}$ *(in 5% NaOH; degrees)*
					Glc	*Xyl*	*Man*	*Gal*	
FII	11.9								
FII-a		2.3	3.6	16.0	100	11	4	5	+36.4
FII-b		9.7	17.0	67.1	100	6	0	6	−23.7
FII-c		2.4	67.1	31.5	100	27	16	26	−25.0
FIII-1	4.7								
FIII-1-a		1.6	28.0	17.5		n.d.[b]			n.d.
FIII-1-b		1.3	50.5	47.3	100	1124	0	0	+62.8
FIII-1-c		0.7	84.4	7.7	100	719	0	107	−18.3
FIII-2	7.5								
FIII-2-a		3.3	16.7	61.3		n.d.			n.d.
FIII-2-b		6.7	43.3	50.2	100	4	1	5	−23.0

[a]By g.l.c. (see Experimental). [b]Not determined.

decided to analyze FIII-2-b further, because this fraction could be obtained in the highest yield (7.0% from Residues II) and, along with fraction FIII-1-a, had the highest activity.

Some properties of the fractions obtained are given in Table II. Fraction FIII-2-b showed $[\alpha]_D$ −23° (*c* 1.00, 0.3M NaOH), and gave D-glucose together with small proportions of galactose, mannose, and xylose after complete hydrolysis with acid. The i.r. spectrum showed an absorption band at 913 cm^{-1}. These data suggested that the glycan component was a β-glucan. No signals other than those expected from a (1→6)-β-D-glucan were observed in either the ^{1}H-n.m.r. [δ 4.47 (d, *J* 7.48 Hz, H-1), 4.28 (d, *J* 10.07 Hz, H-6a), 3.82 (m, H-6b), 3.57 (m, H-5), 3.40 (m, H-3,4), 3.29 (dd, *J* 7.48 and 7.48 Hz, H-2)] or COSY (see Fig. 1) or ^{13}C-n.m.r. (see Fig. 2) spectra[9–12]. These data indicated that FIII-2-b mainly contained simple (1→6)-β-D-glucopyranosyl chains as the glucan part. This conclusion ws confirmed by methylation analysis of the glucan. The analysis gave 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methylglucitol as the major peak (89%; *m/z* 233, 189, 161, 117, and 43), together with 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol (*m/z* 205, 161, 117, and 43) and some minor peaks. The value for the protein content of FIII-2-b was 43.3%. Amino acid analysis revealed a high content of acidic amino acids, alanine, and leucine, and a low content of methionine, histidine, and tyrosine (see Table III). The molecular weight of FIII-2-b was estimated by gel filtration using dextran standards to be 1–5 × 10^4. Thus, the data allow the conclusion that FIII-2-b contains a protein and a (1→6)-β-D-glucopyranan.

However, neither the ^{1}H-n.m.r. (see Fig. 1) nor the ^{13}C-n.m.r. spectrum (see Fig. 2) shows any peaks for the protons of the amino acid residues of the protein component. This observation can be explained by the following two suggestions:

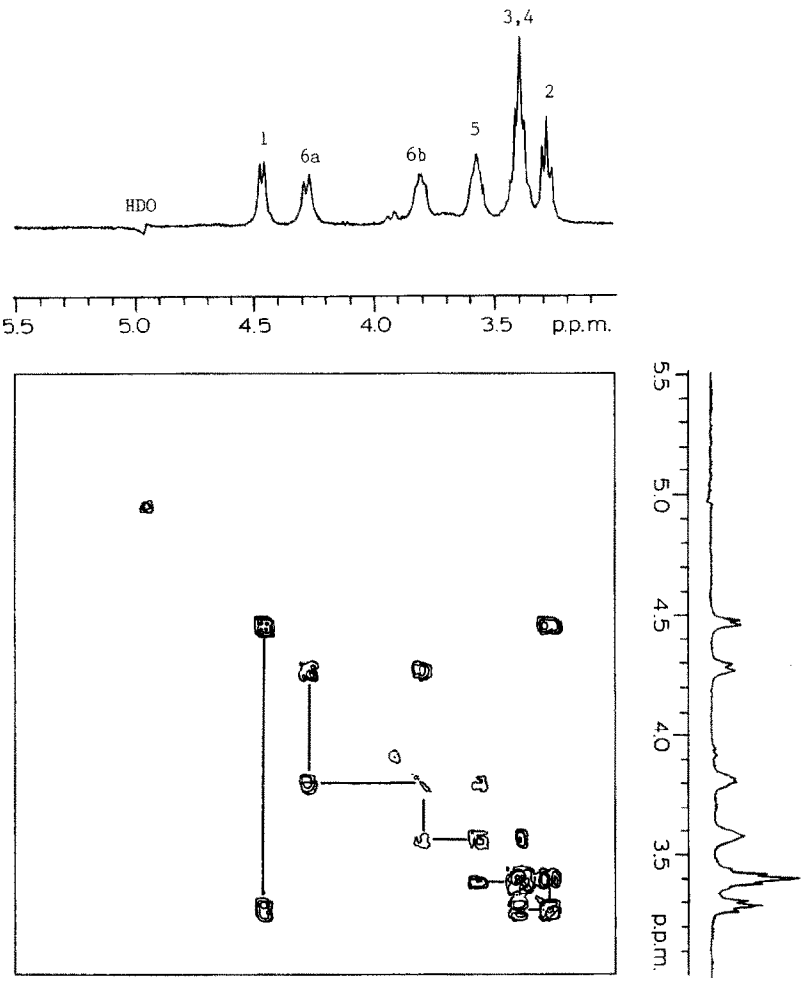


Fig. 1. Two-dimensional COSY spectrum of FIII-2-b. Lines connect signals from the D-glucosyl residues. A conventional 1D proton spectrum with signal assignments added is given.

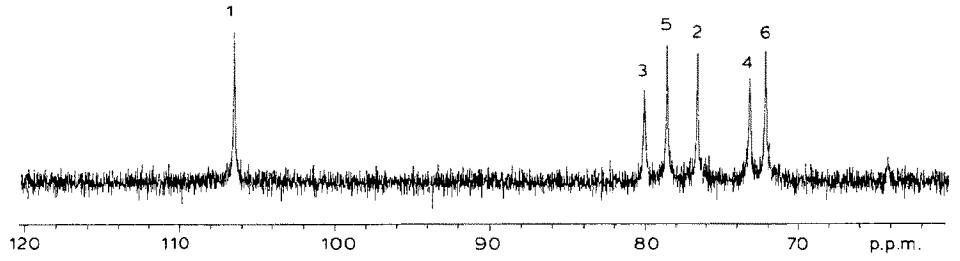


Fig. 2. ^{13}C-N.m.r. spectrum of FIII-2-b, with signal assignments.

TABLE III

AMINO ACID COMPOSITION OF FIII-2-b

Amino acid	*Mol%*	*Amino acid*	*Mol%*
Asx	10.7	Ile	3.3
Thr	5.2	Leu	10.8
Ser	5.3	Tyr	2.4
Glx	11.1	Phe	4.5
Gly	9.3	Lys	5.3
Ala	11.9	His	2.1
Val	4.9	Arg	5.2
Met	1.1	Pro	6.9
			Total 100.0

the molar ratio of the (1→6)-β-glucopyranosyl residues is considerably more than that of each amino acid residue, and peak-broadening occurred because of gel formation in the solvent used. Although the protein and the glucan of FIII-2-b could not be disassociated from each other by precipitation with trichloroacetic acid, ammonium sulfate, or ethanol at various concentrations, and were eluted as a single, synmetrical peak on gel filtration using Toyopearl 55S (data not shown), there is no conclusive evidence of a covalent linkage between the protein and the polysaccharide.

Because we were interested in determining whether the polysaccharide or the protein, or both, is essential to the potent antitumor activity of the material, we tried to separate FIII-2-b into its polysaccharide and protein components enzymically. However, the protein–polysaccharide was insoluble in all buffers tested, and only soluble in such alkaline solutions as 0.3M NaOH. It was, therefore, unaffected by trypsin and pronase P.

Many antitumor polysaccharides from basidiomycetous fungi are known[3–13]. Most of them have (1→3)-β-D-glucan chains as backbones. Among (1→6)-β-glucans, it is known that a (1→6)-β-D-glucan from *Gyrophora esculenta* which is partially acetylated at O-3 of the D-glucosyl residues shows quite marked activity. However its *O*-deacetylated derivative was less active[14]. To the best of our knowledge, this is the first report of the association of antitumor activity with a D-glucan consisting solely of unsubstituted β-D-(1→6)-linked residues.

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