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Formolysis of a Potent Antitumor (1→6)-β-D-Glucan–Protein Complex from *Agaricus blazei* Fruiting Bodies and Antitumor Activity of the Resulting Products

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ABSTRACT

*A potent antitumor (1→6)-β-D-glucan–protein complex, isolated from the water-insoluble residue of [*Agaricus blazei*] fruiting bodies, was formolyzed in order to separate the complex into its polysaccharide and protein components and assay the antitumor activity of each component. A pure glucan obtained did not exhibit strong activity. The results suggest that the protein component is necessary for the potent activity of the complex.*

INTRODUCTION

Agaricus blazei (Japanese name; Himematsutake or Kawariharatake) is known as a home remedy having many physiological activities. From this

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fungus we isolated cytotoxic steroids (Kawagishi *et al.*, 1988*a*) and a lectin (Kawagishi *et al.*, 1988*b*). And also we have reported fractionation and antitumor activity of the water-insoluble residue of the fruiting bodies of the fungus. Among those fractions, the most active fraction, FIII-2-b, was a (1→6)-β-D-glucan–protein complex consisting of 43·3% of protein and 50·2% of carbohydrate (Kawagishi *et al.*, 1989). Because we were interested in determining whether the polysaccharide or the protein, or both, is essential to the potent antitumor activity of FIII-2-b, we tried to separate the material into its polysaccharide and protein components. This report describes formolysis of FIII-2-b in order to separate the complex into its two components and antitumor activity of the resulting products.

MATERIALS AND METHODS

Materials

Fruiting bodies of cultured *A. blazei* were kindly provided by the Iwade Institute of Mushroom (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 and fractionation

The extraction and fractionation were performed according to our method previously reported (Mizuno *et al.*, 1986; Kawagishi *et al.*, 1989).

Formolysis

FIII-2-b (100 mg) was dissolved in 80% formic acid (5 ml) and heated at 85°C for 45 min. After cooling, the reaction mixture was partitioned between $CHCl_3$-butanol (5/1 = v/v, 1 ml) and aqueous layers. The organic layer (FIII-2-b_1) was concentrated *in vacuo* and lyophilized. To the aqueous layer was added ethanol (3 vols), and the precipitate (FIII-2-b_3) was collected by centrifugation (10 000 × *g*). The supernatant (FIII-2-b_2) was concentrated *in vacuo* and lyophilized. FIII-2-b_3 was treated with water at 100°C for 2 h. Ethanol was added to the reaction mixture after cooling, and the precipitate was collected by centrifugation (10 000 × *g*) and lyophilized, giving FIII-2-b'_3.

Composition of constituent sugars

The polysaccharides were completely hydrolyzed by heating in 0·5–1·0 M H_2SO_4 at 100°C for 3–6 h. The resulting monosaccharides were reduced with $NaBH_4$, and then acetylated with acetic anhydride and pyridine. The constituent sugars were determined by standard GLC–MS of their alditol acetates (Mizuno *et al.*, 1986). The total sugars were measured by the phenol–sulfuric acid method with reference to glucose (Dubois *et al.*, 1956).

Composition of amino acids

Amino acids were analyzed with a Hitachi 835 amino acid analyzer after hydrolysis of samples in 6 M HCl at 110°C for 20 h in sealed, evacuated tubes.

NMR spectra

The NMR spectra were recorded at 25°C in 0·3 M NaOD (FIII-2-b) or D_2O (FIII-2-b_3 and FIII-2-b'_3) with a JEOL GSX-400 spectrometer. The chemical shifts were referenced to internal sodium 2,2,3,3-tetradeuterio-4,4-dimethyl-4-silapentanoate (TSP). All the ^{1}H-NMR experiments were performed with suppression of the HOD resonances by homo-gated decoupling.

Molecular weight

Molecular weights were estimated by gel filtration using Toyopearl HW55S standardized with standard dextrans (Pharmacia).

Methylation analysis

The polymer was completely methylated by the Hakomori method (Hakomori, 1964), and hydrolyzed first with 87% formic acid and then 0·5 M 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 GLC–MS method (Mizuno *et al.*, 1986).

Assay for antitumor activity

Assay for antitumor activity was performed according to a method previously reported (Shimura *et al.*, 1983) as follows. Antitumor activity was measured by intraperitoneal (i.p.) injection of mice bearing Sarcoma 180. Five-week-old male ICR/Slc mice (25 ± 2 g) were housed in cages in an air-conditioned room and supplied with commercial diet (Oriental Yeast Co., Japan) and water *ad libitum*. Sarcoma 180 solid tumor was initially supplied by the National Cancer Research Institute of Japan and maintained in the laboratory of Mie University School of Medicine. A fragment, about 3 mm in diameter, of the 14-day-old tumor was implanted subcutaneously into the right groin of mice by a trocar. The samples dissolved or suspended in saline were injected intraperitoneally at various intervals after the tumor implantation. In the control group, mice were injected with saline only in the same dose and were maintained for the same time period as those in the treated groups. Tumor growth was indicated by measuring the tumor size 3 weeks after implantation. The inhibition ratios were calculated by the following formula: inhibition ratio (percent) = {1 − (average of tumor size in treated group/average of tumor size in control group)} × 100. Complete regression of tumors and mortality were compared with those of the control mice 6 weeks after tumor implantation.

RESULTS AND DISCUSSION

The residue after boiling water extraction of the fruiting bodies of *A. blazei* was further extracted with boiling 1% ammonium oxalate, then 5% sodium hydroxide at 30°C. The sodium hydroxide solution was acidified with acetic acid, and ethanol was added to the supernatant after removing the resulting precipitate. The most antitumor active fraction (Table 1), FIII-2-b, was obtained by gel filtration of the ethanol precipitate. This fraction consisted of 50·2% of carbohydrate and 43·3% of protein. Although the carbohydrate and the protein were eluted as a single symmetrical peak on gel filtration, there is no direct evidence of a covalent linkage between the sugar and the protein. The molecular weight of the fraction estimated by gel filtration is $1-5 \times 10^4$. Mainly on the basis of analyses of ^{1}H-NMR (Fig. 2(a)) and ^{13}C-NMR (Fig. 4(a)) spectra, it was suggested that the polysaccharide part is an almost pure (1→6)β-D-glucopyranan. That was the first report of the association of antitumor activity with a glucan containing only β-(1→6) linked residues (Kawagishi *et al.*, 1989).

TABLE 1
Antitumor Activity of FIII-2-b and the Formolyzed Products against Sarcoma 180 in Mice[a]

Experimental group	*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
FIII-2-b	10	0·4	99·1	8/10	0/10
Control	5	31·4	0	0/5	1/5
FIII-2-b_1	5	15·6	50·3	0/5	0/5
FIII-2-b_2	5	14·9	52·5	0/5	0/5
FIII-2-b_3	5	22·0	29·9	0/5	0/5
FIII-2-b'_3	5	24·8	21·0	0/5	0/5

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

In order to determine whether the polysaccharide or the protein, or both of them, are indispensable to its very strong antitumor activity, we tried to separate the complex into the sugar and the protein part enzymatically. However the polysaccharide–protein was insoluble in all buffers tested and only soluble in strong alkali solutions such as 0·3 M NaOH. It was therefore unaffected by trypsine, pronase P and chymotrypsin. Therefore we tried the separation chemically. The sugar-free protein component could not be obtained by any method attempted. However, among various conditions or reagents tested, formolysis with 80% formic acid at 85°C for 45 min has given an almost pure carbohydrate. The procedure is summarized in Fig. 1. The reaction mixture was partitioned between $CHCl_3$–butanol and aqueous layers. The organic layer contained a protein-rich fraction (FIII-2-b_1). Precipitate (FIII-2-b_3) was obtained by adding ethanol to the aqueous layer and removing supernatant (FIII-2-b_2) by centrifugation. FIII-2-b_3 was then deformylized by heating in water, giving FIII-2-b'_3.

The ^{1}H-NMR spectrum of FIII-2-b_3 (Fig. 2(b)) is very similar to that of FIII-2-b (Fig. 2(a)) except for the signals at 8·0–8·2 ppm. Since the peaks could be assigned to formyl protons, it is indicated that FIII-2-b_3 is a partially formylated (1→6)-β-D-glucopyranan.

The ^{1}H-NMR (Fig. 2(c)), COSY (Fig. 3) and ^{13}C-NMR (Fig. 4(b)) spectra of FIII-2-b'_3 suggest that this product is a simple (1→6)-β-D-glucopyranan (Usui *et al.*, 1973, 1974; Bassieux *et al.*, 1977; Saito *et al.*, 1977) and completely deformylated. Methylation analysis confirmed its structure. The analysis of the glucan gave 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-

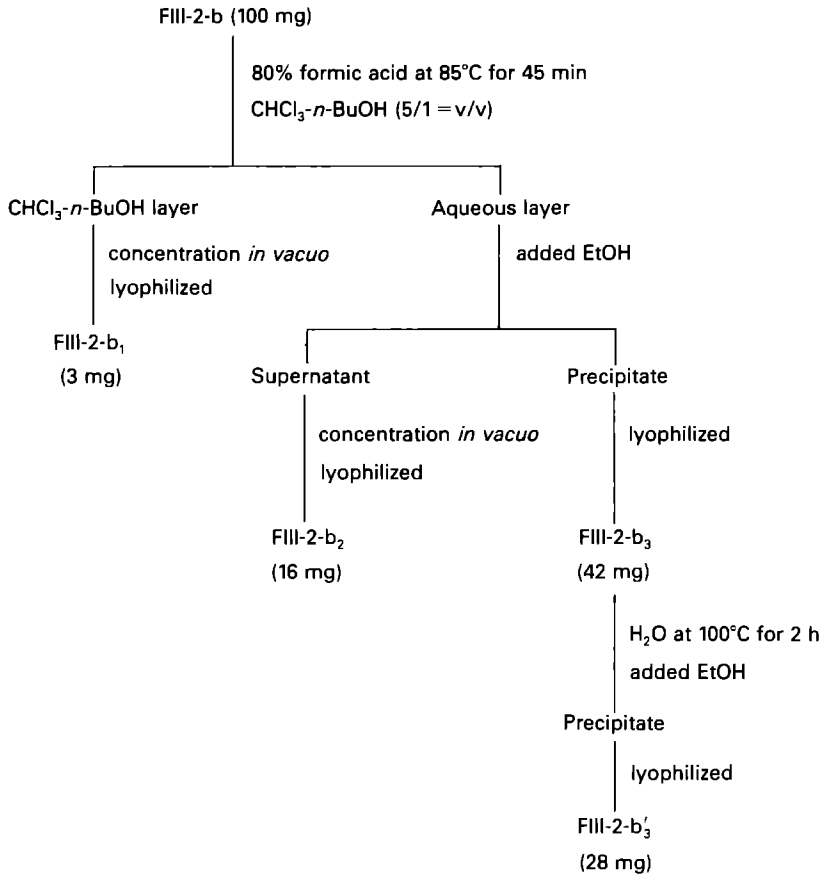

Fig. 1. Formolysis of FIII-2-b and fractionation of the formolyzed products.

methyl-D-glucitol (91%) and 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol. The value for carbohydrate of the fraction was more than 98%. Furthermore, the molecular weight of FIII-2-b'_3 estimated by gel filtration using dextran standards is $1 \sim 5 \times 10^4$ (data not shown) and this value was very similar to that of FIII-2-b. All the results mentioned above suggested that the carbohydrate component of FIII-2-b could be obtained purely by the formolysis, without significant degradation of the component.

Antitumor activity of each fraction is listed in Table 1. Formylated glucan FIII-2-b_3 and complete-deformylated glucan FIII-2-b'_3 did not exhibit potent activity. On the other hand, FIII-2-b_1, whose carbohydrate content is 23% and amino acid composition is similar to that of FIII-2-b (Table 2), and FIII-2-b_2 (the carbohydrate-content is 50%) have

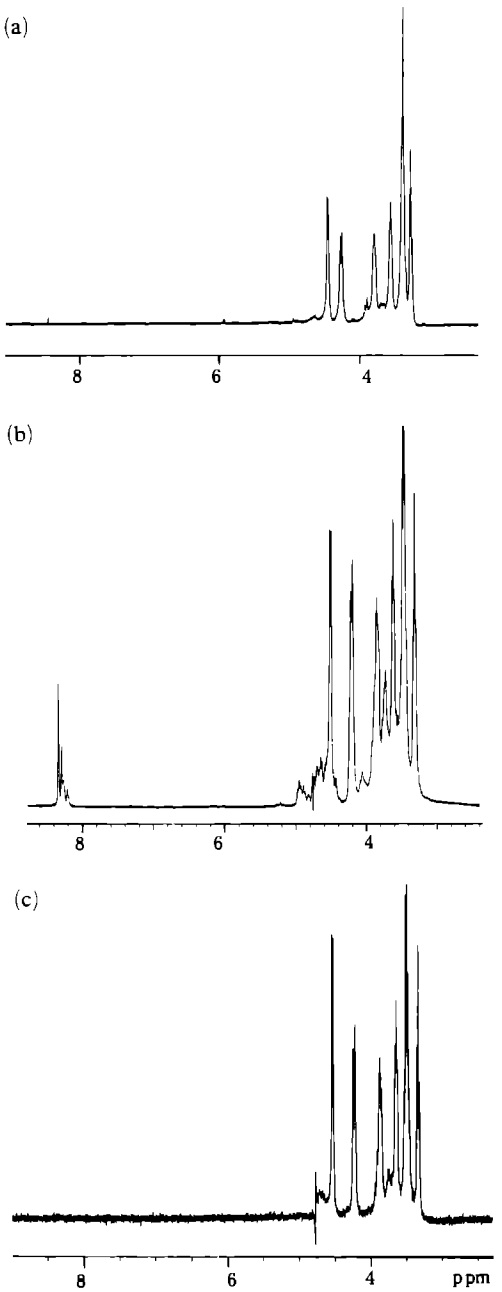

Fig. 2. ^{1}H-NMR spectra of FIII-2-b, FIII-2-b_3 and FIII-2-b'_3. (a) FIII-2-b; (b) FIII-2-b_3; (c) FIII-2-b'_3.

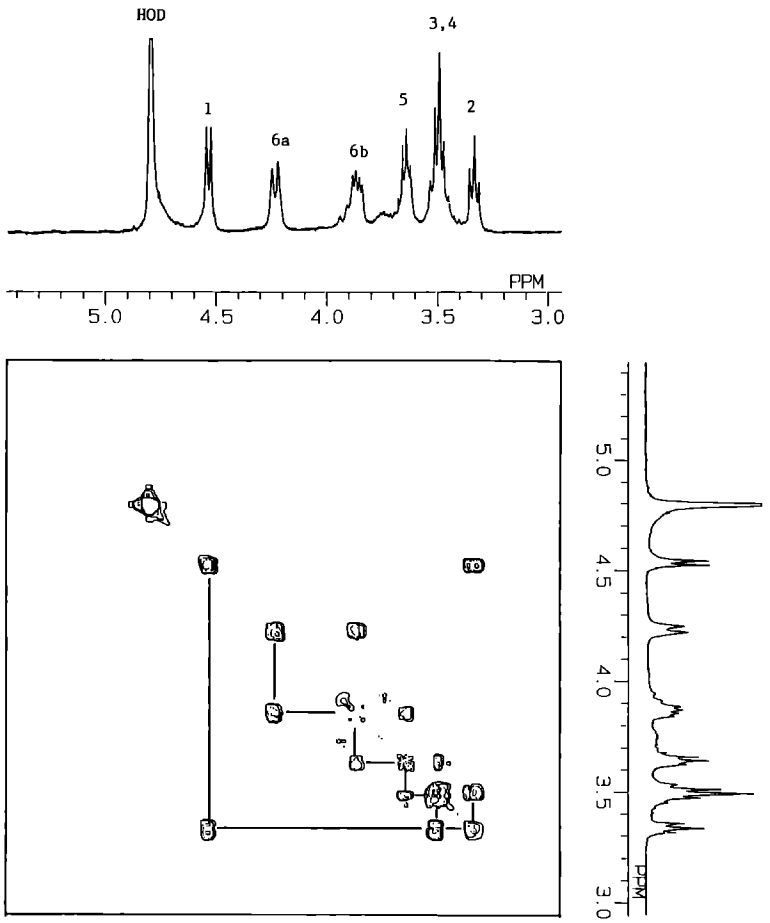

Fig. 3. COSY spectrum of FIII-2-b$'_3$. Lines connect signals from the glucosyl residue. A conventional 1D proton spectrum with signal assignments added is given above.

stronger activity than the protein-free glucans. Since we failed to obtain sugar-free protein, it is uncertain whether or not the protein alone will exhibit potent antitumor activity. However, at least these results allow us to conclude that the protein component is essential for antitumor activity of the complex and the glucan alone cannot exhibit strong activity like the glucan–protein complex, FIII-2-b.

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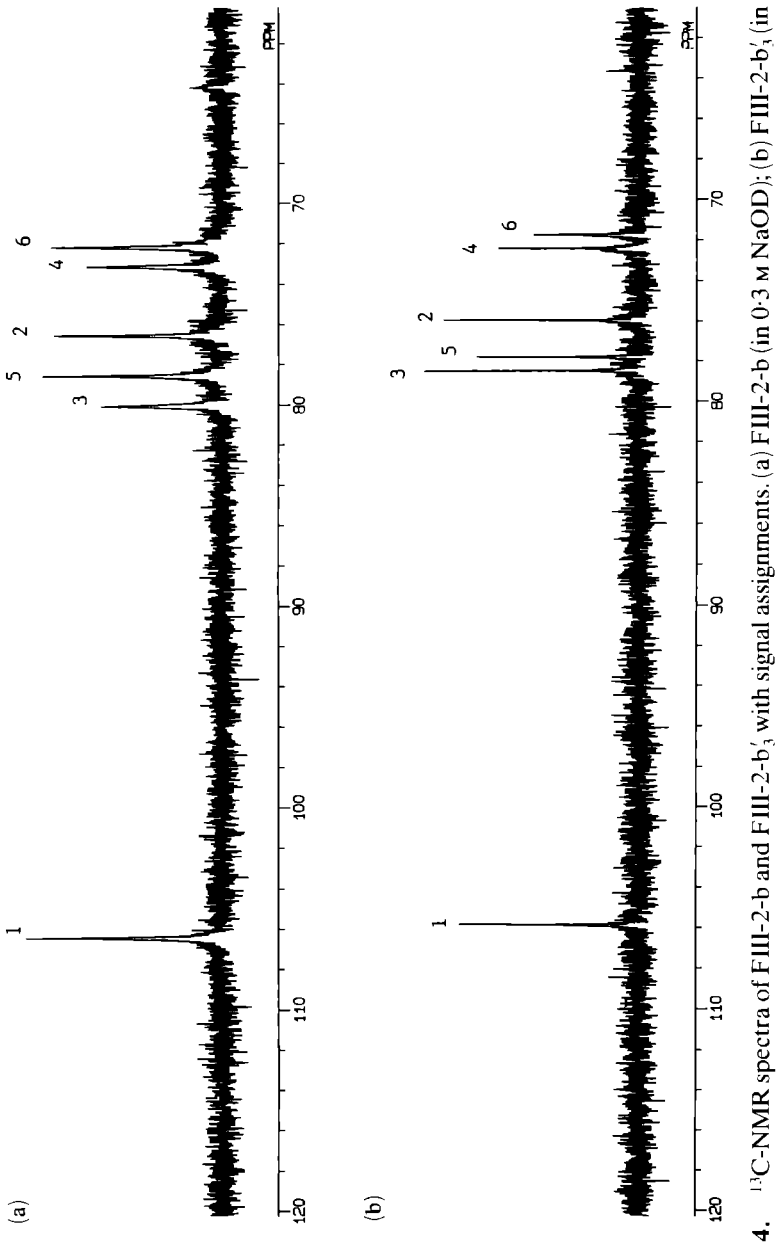

Fig. 4. ^{13}C-NMR spectra of FIII-2-b and FIII-2-b$'_3$ with signal assignments. (a) FIII-2-b (in 0·3 M NaOD); (b) FIII-2-b$'_3$ (in D_2O).

TABLE 2
Amino Acid Composition of FIII-2-b and FIII-2-b_1

Amino acid	*FIII-2-b*	*FIII-2-b_1*
	(mol.%)	
Asx	10·7	9·9
Thr	5·2	5·9
Ser	5·3	6·8
Glx	11·1	9·8
Gly	9·3	12·1
Ala	11·9	12·0
Val	4·9	4·7
Met	1·1	0·5
Ile	3·3	3·5
Leu	10·8	12·3
Tyr	2·4	2·6
Phe	4·5	5·5
Lys	5·3	3·9
His	2·1	1·4
Arg	5·2	3·8
Pro	6·9	5·3
	100·0	100·0

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