[Chem. Pharm. Bull. 33(8)3395—3401(1985)]

Structural Characterization and Antitumor Activity of the Extracts from Matted Mycelium of Cultured *Grifola frondosa*

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(Received November 27, 1984)

The fruit body of *Grifola frondosa*, an edible mushroom, is used as a food in Japan. However, the matted mycelium of the fruit body is not utilized. In this paper, structural characterization and antitumor activity of the extracts from the matted mycelium of this fungus were examined. Hot water, cold alkali, and hot alkali extracts of the mycelium all contained polysaccharides; that in the cold alkali extract was composed of glucose, but those in the hot water and hot alkali extracts contained glucose, mannose and/or xylose. Each fraction showed potent antitumor activity against murine solid tumor, Sarcoma-180. The antitumor activities of the alkali extracts were more potent than that of the hot water extract. By means of DEAE-Sephadex chromatography, α-amylase treatment, and SP-Sephadex chromatography, a neutral β-1,3-glucan was purified from the cold alkali extract. From the results of nuclear magnetic resonance spectroscopy, methylation, periodate oxidation, enzymic degradation, and physicochemical characterizations, the purified antitumor glucan was concluded to be a β-1,3-glucan branched at C-6 of every third main-chain glucosyl unit. The structure is quite similar to that of the antitumor glucan obtained from the fruit body of this fungus. It is concluded that the matted mycelium of *G. frondosa* may be useful as a source of antitumor β-1,3-glucan.

Keywords——*Grifola frondosa*; antitumor activity; β-1,3-glucan; matted mycelium

Beta-1,3-glucans show potent antitumor action, which is believed to result from the activation of certain host immune systems. Many kinds of edible mushroom contain glucan of this type. *Grifola frondosa* is a commercially available, edible mushroom belonging to the Basidiomycetes, Aphyllophorales, Polyporaceae. Recently, we reported that the fruit body of *G. frondosa* contains an antitumor active β-1,3-glucan.[1] In this paper, the structure and antitumor activity of the extracts from the matted mycelium (which is normally discarded) of *G. frondosa* are reported.

Materials and Methods

Isolation of Each Polysaccharide Fraction——Polysaccharide fractions were prepared from powdered matted mycelium of *G. frondosa* (cultured at Obihiro, Hokkaido, Japan, by Nippon Beet Sugar Mfg. Co. Ltd.). After being washed with water, the mycelium was lyophilized, and then pulverized to a powder. The powder (65 g) was extracted with water (600 ml) in an autoclave (1 kg/cm^2, 121 °C) for 1 h. The extract was concentrated to a small volume (100 ml) and the polysaccharide fraction was precipitated by adding 4 vol of ethanol (NMF-1). The precipitate was dried by washing it with acetone and then ether. The extraction was repeated 5 to 7 times. The residue after water extraction was further extracted by stirring it with 10% sodium hydroxide containing 5% urea for 24 h at 4 °C. This extract was neutralized with acetic acid and dialyzed extensively against tap water (4 d) and distilled water (4 d). The precipitates produced during dialysis were discarded. The supernatant was concentrated and treated as described for the hot water extract (NMF-5). The extraction was repeated 3 times. The residue was extracted at 65 °C for 1 h with buffer of the same composition and by the same procedures as used for cold alkali extraction. The supernatant fraction was designated as NMF-7.

Purification of Grifolan NMF-5N——NMF-5 (0.3 g) dissolved in H_2O (15 ml) was applied to a column (3 × 10 cm) of DEAE-Sephadex A-25 (HCO_3^-). The column was eluted with water, the eluate was dialyzed and the non-dialyzable fraction was collected. The fraction was dissolved in 50 mM Tris–HCl buffer pH 6.9 (60 ml) and digested with α-amylase (0.3 mg) at 37 °C for 24 h. The reaction was terminated by heating at 100 °C for 5 min, and the mixture was dialyzed against water. The non-dialyzable fraction was concentrated and passed through a column of SP-Sephadex C-25 (H^+). The resulting solution was precipitated with 1 vol of ethanol, and then dried with acetone and ether (yield; about 20%).

Periodate Oxidation and Smith Degradation——Grifolan NMF-5N (11.8 mg) was oxidized with 10 mM sodium metaperiodate (40 ml) at 4 °C in the dark. At appropriate intervals, the periodate consumption was determined by the method of Avigad.[2] When the reaction was completed (171 h) the excess periodate was destroyed by adding ethylene glycol. The reaction mixture was dialyzed against tap water for 2 d, and distilled water for 1 d. The non-dialyzable fraction was reduced with sodium borohydride (50 mg) at 4 °C for 48 h. After acidification with acetic acid, the mixture was dialyzed and lyophilized.

A portion (1.4 mg) of polyol was hydrolyzed with 1 M trifluoroacetic acid (1 ml) at 100 °C for 5 h. The resulting monosaccharide mixture was analyzed by gas liquid chromatography (GLC) of the alditol acetates. Another portion (1.4 mg) of polyol was partially hydrolyzed with 0.1 N sulfuric acid (1.6 ml) at 25 °C for 72 h. The reaction mixture, after neutralization, was dialyzed against distilled water for 2 d. The dialyzable fraction was concentrated and then deionized on columns of Amberlite CG-4B (OH^-) resin and Dowex 50 (H^+) resin. The resulting fraction was derivatized to alditol acetates and analyzed by GLC. The non-dialyzable fraction was analyzed by methylation.

Enzyme Hydrolysis[3]——Grifolan NMF-5N (20 mg) was dissolved in 0.03 M McIlvaine buffer, pH 4.9 (20 ml), and *exo*-β-1,3-glucanase (11.4 mg, 10 ml) was added to the solution. The reaction mixture was incubated at 37 °C for 25 h with constant shaking, and then the reaction was terminated by heating at 100 °C for 15 min. The resulting solution was dialyzed against distilled water for 2 d, and the dialyzable and non-dialyzable fractions were collected. The dialyzable fraction was analyzed by thin-layer chromatography (TLC), high performance liquid chromatography (HPLC) and gel filtration.

General Methods——Evaluation of antitumor activity, quantitative analysis, methylation analysis, and other physicochemical procedures were performed as described previously.[1]

Results

Comparison of the Matted Mycelium and the Fruit Body by Carbon-13 NMR Spectroscopy

In the previous paper, carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectral analysis of the fungi suspended in H_2O was shown to be useful for characterizing the polysaccharide moiety in the fruit body of *G. frondosa*.[1c] In the present work, this method was applied to compare the fruit body and the matted mycelium. Figures 1A, B shows the ^{13}C-NMR spectra. The fruit body showed trehalose as the major oligosaccharide, whereas the

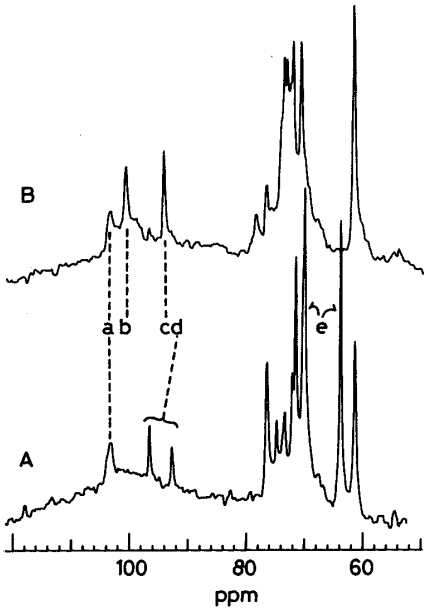

Fig. 1. Carbon-13 NMR Spectra of the Matted Mycelium and Fruit Body in D20

A, lyophilized matted mycelium. B, lyophilized fruit body.

a, anomeric carbon of β-glucan; b, anomeric carbon of α-glucan; c, anomeric carbon of trehalose; d, anomeric carbons of α- and β-glucose; e, mannitol.

matted mycelium showed glucose and mannitol. Both spectra showed anomeric carbons of β-glucan. However, the intensity of the α-glucan signal (101 ppm) in the matted mycelium is quite small. The α-glucan content of matted mycelium varied from lot to lot. It appears that the chemical compositions of the mycelium and the fruit body are different and that the matted mycelium contains a lesser amount of α-glucan than the fruit body.

Characterization of Hot Water, Cold Alkali, and Hot Alkali Extracts

Polysaccharide fractions were extracted successively with hot water, cold alkali, and hot alkali in the same way as done for the fruit body. The extracts were dialyzed extensively against water and the water-soluble fraction was collected. The non-dialyzable fractions were precipitated with four volumes of ethanol. The precipitates were named NMF-1, NMF-5, and NMF-7, respectively. Table I shows the yield and some chemical properties of each extract. When these values are compared with those of the fruit body, the component sugars of NMF-7 are clearly different. The product in the alkali extract of the fruit body (CF-7) was composed of only glucose.[1a)]

Linkages and configurations of each fraction were characterized by methylation and ^{13}C-NMR spectroscopy. As shown in Fig. 2, the ^{13}C-NMR spectrum of NMF-1 showed signals similar to those of CF-1,[1a)] especially as regards the anomeric carbon signals (α, 102 ppm,[a)] in the figure; β, 104 ppm,[b)]) and the C-4 signal (80 ppm,[c)] in the figure) of α-glucan. From these results, NMF-1 is suggested to contain α-1,4-, β-1,3-, and β-1,6-linkages. In the case of the fruit body, CF-5 and -7 contained significant amounts of α-1,4-glucan. However, from the results of methylation analysis, NMF-5 and -7 contained only trace amounts of α-1,4-glucan and the most abundant linkages of these fractions were β-1,3 and β-1,6 linkages.

Table III showed the antitumor activity of each extract on the solid tumor Sarcoma-180

Table I. Some Properties of Polysaccharide Fractions Extracted from the Matted Mycelium of Cultured *G. frondosa*

Fraction	Yield (g)[a)]	Sugar (%)	Protein (%)	Component sugars[b)]
NMF-1	10	74	10	Glc/Man (1.0:0.1)
NMF-5	4.7	66	17	Glc
NMF-7	2.9	72	19	Glc/Man/Xyl (1.0:0.2:0.3)

a) From 65 g of the matted mycelium. *b*) Determined as alditol acetate derivatives by GLC.

Table II. GLC of Alditol Acetates Derived from the Methylated Polysaccharides

Alditol acetate of Glc residue	NMF-5	NMF-7[a)]	DEAE-1[b)]	DEAE-2[c)]	Grifolan NMF-5N	I/B[d)]	I/B (H^+)[e)]
2,3,4,6-Me_4	1.00	1.00	1.00	1.00	1.00	0	0
2,4,6-Me_3	1.82	1.18	1.67	1.53	1.97	1.00	1.00
2,3,4-Me_3	0.29	0.54	0.09	0.65	0.01	0	0
2,3,6-Me_3	0.05	0.51	0.14	0.04	0.02	0	0
2,6-Me_2	0.01	0	0	0.03	0	0	0
3,4,6-Me_3	0.04	0.04	0.04	0.04	0.05	0	0
2,4-Me_2	1.16	1.07	1.05	1.09	1.22	0.55	0.09
2,3-Me_2	0	0.04	0	0	0	0	0
3,6-Me_2	0	0	0	0.04	0	0	0

a) Only glucosyl moieties are listed. *b*) Unabsorbed fraction on DEAE-Sephadex A-25 of NMF-5. *c*) NH_4HCO_3-eluted fraction of NMF-5 from DEAE-Sephadex A-25. *d*) Periodate-oxidized and borohydride-reduced grifolan NMF-5N. *e*) Periodate-oxidized, borohydride-reduced and mild acid-hydrolyzed grifolan NMF-5N.

TABLE III. Antitumor Effect of Polysaccharide Fractions from the Matted Mycelium of Cultured *G. frondosa*[a)]

Sample	Dose ($\mu g \times 10$)	Tumor weight (g, mean ± S.D.)	Inhibition[b)] (%)	Complete regression[b)]	Significance[c)] $p <$
NMF-1	20	5.8 ± 3.4	−55	0/3	n.s.[d)]
	100	2.1 ± 1.9	45	2/6	n.s.
	500	< 0.1	99	3/6	0.01
NMF-5	20	0.5 ± 0.4	87	1/8	0.01
	100	0.1 ± 0.3	97	4/8	0.01
	500	0.5 ± 0.2	86	0/9	0.01
NMF-7	20	0.4 ± 0.9	90	4/9	0.01
	100	0.2 ± 0.3	96	3/10	0.001
	500	0.4 ± 0.7	89	1/6	0.01
Grifolan NMF-5N					
	4	3.3 ± 2.1	12	0/10	n.s.
	20	0.2 ± 0.3	94	2/8	0.01
	100	0.1 ± 0.2	97	2/9	0.001
Control		3.7 ± 2.6	0	0/12	

a) Sarcoma 180 tumor cells (5×10^6) were inoculated subcutaneously. Each sample was administered as saline solution by intraperitoneal injection. *b*) Inhibition and complete regression were determined at 35 d after tumor inoculation. *c*) The significance was evaluated according to Student's *t*-test. $p < 0.05$ was taken as the criterion of a significant difference. *d*) Not significant.

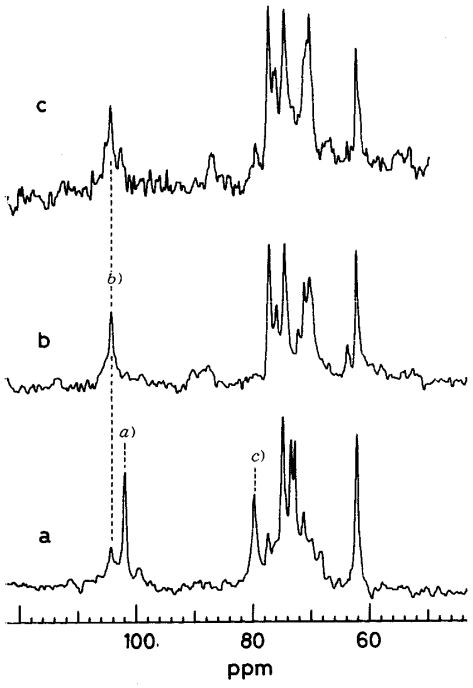

Fig. 2. Carbon-13 NMR Spectra of Polysaccharide Fractions in 0.2 M NaOD

a, NMF-1; b, NMF-5; c, NMF-7.

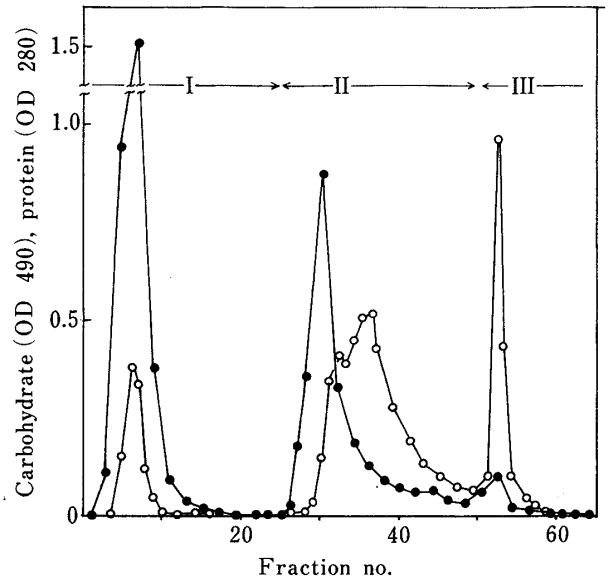

Fig. 3. Elution Profiles of Polysaccharide Fraction NMF-5 from a Column of DEAE-Sephadex A-25 (HCO_3^-)

The column (1.5 × 6 cm) was equilibrated with 8 M urea, and 37 mg of NMF-5 was applied. The column was washed successively with 8 M urea (I), 0.45 M NH_4HCO_3 containing 8 M urea (II), and 2 M NaCl containing 8 M urea (III). Fractions of 2.4 ml were collected, and carbohydrate and protein were assayed by the phenol–H_2SO_4 method (—●—) and by ultraviolet (UV) absorption measurement (—○—), respectively.

in ICR mice. All of the extracts showed potent antitumor activity and the alkali extracts were more potent than the hot water extract.

DEAE-Sephadex Chromatography of Cold Alkali Extract

For further characterization, we used NMF-5 because of its potent antitumor activity and well-defined chemical composition. In the case of the fruit body, glucans were separated into neutral and acidic glucans by DEAE-Sephadex A-25 column chromatography. As the first step of purification, we compared the elution profiles of NMF-5 and CF-5. As shown in Fig. 3, the carbohydrate pattern of NMF-5 was quite similar to that of CF-5.[1b] In Table II, the results of methylation analysis of each fraction are listed. The values for NMF-5 are similar to those in the case of CF-5.[1b]

Characterization of a Purified β-Glucan Obtained from Cold Alkali Extract

During the purification and chemical characterization of the β-glucan obtained from the fruit body,[1d] it was found that α-amylase treatment, DEAE-Sephadex chromatography and ethanol precipitation are useful for purifying the β-1,3-glucan. In this paper, therefore, we applied these methods for purification of the β-glucan from the matted mycelium. As described in Materials and Methods, β-1,3-glucan was purified from the cold alkali extract by sequential use of DEAE-Sephadex chromatography, α-amylase treatment, SP-Sephadex chromatography and ethanol precipitation. The purified glucan, named grifolan NMF-5N, showed $[\alpha]_D$ +3.6 and gave a broad but symmetrical peak on Sepharose CL-4B column chromatography (data not shown). Its average molecular weight was estimated to be 750000. On methylation analysis tetra-*O*-methylglucose, 2,4,6-tri-*O*-methylglucose, and 2,4-di-*O*-methylglucose were detected in a molar ratio of 1 : 1.97 : 1.22 (Table II). As shown in Fig. 4, the ^{13}C-NMR spectrum of the glucan in dimethylsulfoxide-d_6 (DMSO-d_6) showed signals quite similar to those of scleroglucan[4] and the β-1,3-glucan obtained from the fruit body.[1d] These results suggest that the glucan is a β-1,3-glucan branched at C-6 of every third main chain glucosyl unit. When grifolan NMF-5N was digested with *exo*-β-1,3-glucanase, about 50% was digested and recovered from the dialyzable fraction. From the results of TLC, HPLC and gel filtration, the dialyzable fraction contained only gentiobiose and glucose. For further characterization, grifolan NMF-5N was oxidized with sodium metaperiodate and reduced with sodium borohydride, then the reaction products were analyzed. The glucan consumed 0.59 mol of periodate per glucosyl unit. The polyol gave two peaks corresponding to 2,4,6-tri-

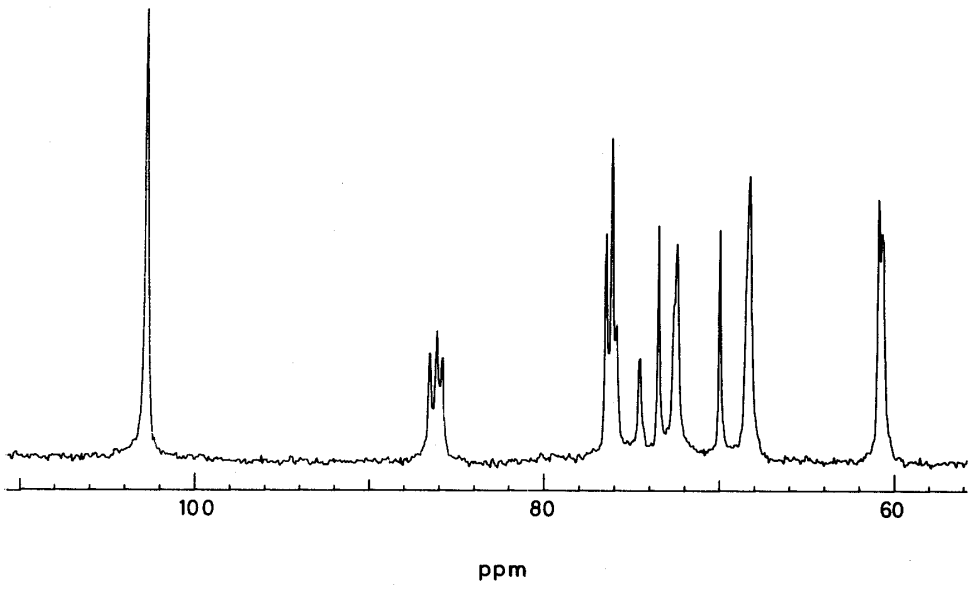

Fig. 4. Carbon-13 NMR Spectrum of Grifolan NMF-5N in DMSO-d_6 at 60 °C

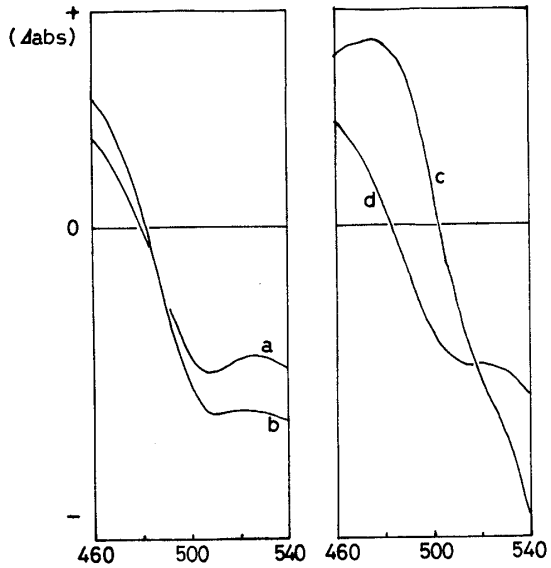

Fig. 5. Differential Spectra of Congo Red in Alkaline Solution in the Presence and Absence of Grifolan NMF-5N

a, Congo Red (0.1 N); b, Congo Red (0.2 N); c, Congo Red + grifolan NMF-5N (0.1 N); d, Congo Red + grifolan NMF-5N (0.2 N).

O-methylglucose and 2,4-di-*O*-methylglucose in a molar ratio of 1 : 0.55. This ratio is consistent with that of the native glucan (Table II). Complete hydrolysis of the glucan gave glucose and glycerol. Mild acid hydrolysis of the polyol gave only glycerol as a dialyzable product and, on methylation analysis of the non-dialyzable fraction, 2,4,6-tri-*O*-methylglucose and 2,4-di-*O*-methylglucose in the ratio of 1 : 0.09 were detected.

Congo Red is known to form complexes with several β-1,3-glucans,[5] and the absorption maximum of Congo Red is shifted markedly to longer wave length. Figure 5 shows the visible absorption spectra of Congo Red in 0.1 and 0.2 N sodium hydroxide solutions in the presence or absence of grifolan NMF-5N. At low alkali concentration (0.1 N), the absorption maximum of Congo Red (480 nm) is markedly shifted to longer wavelength (502 nm) in the presence of glucan, but at higher alkali concentration (0.2 N), the absorption maximum is little shifted.

The antitumor activity of the purified glucan was examined. As shown in Table III, the glucan showed potent antitumor activity against the solid tumor Sarcoma 180 in ICR mice. The optimum dose was similar to that of the glucan obtained from the fruit body.

Discussion

In this paper, we have characterized the matted mycelium of *G. frondosa* and purified an antitumor active β-1,3-glucan. Compared with the fruit body, the characteristic features of the matted mycelium are as follows. (1) The matted mycelium contained less α-glucan. (2) The chemical compositions of the extracts were similar, but the component sugars of NMF-7 were different from those of the other extracts; NMF-7 was composed of Glc, Xyl, Man. (3) The optimum doses for antitumor activity of the extracts were less than those of the corresponding extracts of the fruit body.[1,6] These results suggest that the content of the antitumor-active glucan is higher in the matted mycelium. The neutral antitumor glucan in the mycelium was concluded to be a branched β-1,3-glucan. This structure is similar to that of the active glucan from the fruit body (determined by methylation, NMR spectroscopy, dye binding, *etc.*). Further characterization of the glucan obtained from the matted mycelium should be of interest.

Acknowledgments The authors thank Miss M. Akahane, Mr. Y. Adachi, Mr. J. Saito, and Mr. M. Matsumura for their technical assistance.

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