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Purification and Structural Characterization of an Antitumor β-1,3-Glucan Isolated from Hot Water Extract of the Fruit Body of Cultured *Grifola frondosa*

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Polysaccharides in the hot water extract of the fruit bodies of *G. frondosa* contain α-1,4-, α-1,6-, β-1,6- and β-1,3-glucosidic linkages, and include an antitumor-active substance assumed to be a β-1,3-glucan (N. Ohno *et al.*, *Chem. Pharm. Bull.*, **32**, 1142 (1984)). In this paper, the purification and structural characterization of this glucan are described. Prior to the hot water extraction, the fruit bodies were digested with amylases, in order to decrease the content of α-1,4-glucan in the fruit body. After the treatment, the fruit bodies were extracted with hot water, cold sodium hydroxide, and then hot sodium hydroxide, successively. Antitumor β-1,3-glucan in the hot water extract was purified by using diethylaminoethyl (DEAE)-Sephadex A-25 column chromatography and stepwise precipitation with ammonium sulfate. The purified glucan had $[\alpha]_D$ +6.3 (H_2O), and an average molecular weight of 5.6×10^6 (8 M urea–0.2 N NaOH), and shifted λ_{max} of Congo Red to 500 nm (0.1 N NaOH). From the results of methylation analysis and carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectroscopy, this glucan was concluded to be a branched β-1,3-glucan possessing a branch at position 6 of every third main-chain glucosyl unit.

Keywords——*Grifola frondosa*; antitumor polysaccharide; 1,3-β-D-glucan; amylase digestion; fruit body; hot water extract

Introduction

The crude polysaccharide fraction (GF-1) obtained from the hot water extract of the fruit body of *Grifola frondosa* showed potent antitumor activity on murine allogeneic and syngeneic tumors.[1] The fraction was composed mainly of α-1,4-, α-1,6-, β-1,3-, and β-1,6-linkages, and the antitumor activity was due to the β-1,3-glucan, based on the results of amylase digestion and periodate oxidation.[1] Because this fraction contained only a small amount of active glucan, purification of the active substance was quite difficult. In this study, purification and structural characterization of the active glucan in this fraction were carried out, after amylase digestion of the fruit body.

Materials and Methods

Enzymic Treatment of the Fruit Body——The pulverized fruit bodies (100 g) suspended in distilled water (1600 ml) were boiled for 5 min to sterilize and degas the preparation. Amyloglucosidase (Sigma, A-7255, 400 mg) and NaN_3 (1.6 g) were added to the suspension and the reaction mixture was incubated at 37 °C for 24 h with constant shaking, then centrifuged. The treated fruit bodies were resuspended in 0.1 M Tris–HCl buffer pH 6.9 containing 0.1% NaN_3 to make 1600 ml. α-Amylase (Sigma, A-6380, 40 mg) was added to the suspension and the reaction mixture was incubated at 37 °C for 24 h with constant shaking. The resulting suspension was centrifuged and the treated fruit body was washed with distilled water. The treated fruit bodies were frozen and lyophilized (yield 35%).

Partial Purification of Antitumor Glucan in the Hot Water Extract from the Fruit Body——The cultured fruit bodies were extracted 7 times with hot water by autoclaving (121 °C, 1 h). The combined extract was concentrated and the crude polysaccharide fraction (HW) was precipitated by adding 1 vol of EtOH. Solutions of HW fraction (0.3 g) in 8 M urea (15 ml) were applied to columns of diethylaminoethyl (DEAE)-Sephadex A-25 (HCO_3^-) (3 × 10 cm). Each column was eluted with 8 M urea, the eluate was dialyzed, and the non-dialyzable fraction was collected (HW-1). HW-1 (1 g) was dissolved in 50 mM Tris–HCl buffer (pH 6.9, 500 ml) containing 0.1% NaN_3 and digested with α-amylase (Sigma, A-6380, 20 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 (HW-1a). The non-dialyzable fraction was concentrated and the polysaccharide fraction was precipitated by adding 1 vol of EtOH (HW-1a1). HW-1a1 (40 mg) was dissolved in 0.02 M Tris–HCl buffer (pH 7.4) and applied to a column of concanavalin A-Sepharose (3 × 10 cm). The column was eluted with the same buffer, and the fraction was dialyzed and lyophilized (HW-1a1p).

Purification of Grifolan 1N——Amylase-digested fruit bodies were extracted 7 times with hot water by autoclaving (121 °C, 1 h). The combined extract was concentrated and the crude polysaccharide fraction (AHW) was precipitated by adding 1 vol of EtOH. Solutions of AHW fraction (100 mg) in 8 M urea were applied to columns of DEAE-Sephadex A-25 (HCO_3^-) (3 × 10 cm). Each column was eluted with 8 M urea, the eluate was dialyzed, and the non-dialyzable fraction was collected (AHW-1). AHW-1 (100 mg) dissolved in H_2O (100 ml) was sequentially precipitated by 30, 60, 80, 100% saturation with $(NH_4)_2SO_4$ (AHW-1b, -1c, -1d, -1e). The final supernatant was named AHW-1f. AHW-1b, -1c were named grifolan 1N.

General Methods——Evaluation of antitumor activity, quantitative analysis, and other physicochemical procedures were performed as described previously.[2]

Results

Partial Purification of Antitumor Glucan from the Hot Water Extract

For purifying the antitumor glucan, separation of the glucan fraction from the hot water extract was investigated. The yield and methylation data for each fraction are shown in Table I. The hot water extract (HW) was applied to DEAE-Sephadex A-25 and the flow-through fraction was obtained (HW-1). HW-1 was digested with α-amylase (HW-1a), and then precipitated with 1 volume of ethanol (HW-1a1). HW-1a1 was applied to a concanavalin A-

Table I. Yield and Methylation Data of Fractions Obtained from the Hot Water Extract of *G. frondosa* Fruit Body

Fraction	Yield[a]	Me_4	Me_3			Me_2		X-1	X-2	X-3
			2,4,6	2,3,4	2,3,6	2,4	2,3			
HW-1	31	1.0	0.1	0.1	8.8	0	0.9	0.2	0.1	
HW-1a	7.8	1.0	0.2	0.2	0.1	0.1	0.5	0.4	0.3	
HW-1a1	2.4	1.0	0.7	0.1	0.3	0.3	0.1	0.8	0.3	
HW-1a1p	1.5	1.0	2.3	0	0	1.1	0	0	0	1.0

a) From the hot water extract (%).

Table II. Antitumor Effect of HW-1a1p on Solid Form of Sarcoma 180[a]

Sample	Dose × 10 (μg/mouse)	Tumor weight[c] (g, mean ± S.D.)	Inhibition ratio (%)[b]	Complete regression[b]
HW-1a1p	20	1.6 ± 2.7	58	3/8
	100	< 0.01[e]	> 99	6/10
	250	0.4 ± 0.1[d]	96	2/8
Nil (control)		3.7 ± 2.6		0/12

a) Sarcoma 180 tumor cells (5×10^6) were inoculated subcutaneously. Each sample was administered as saline solution by intraperitoneal injection from day 1 to day 10. *b*) Inhibition and complete regression were determined at 35 d after tumor inoculation. *c*) The significance of differences was evaluated according to Student's *t*-test. Significant difference from the control (*d*) $p < 0.01$, *e*) $p < 0.001$).

Sepharose column and the flow-through fraction was obtained (HW-1a1p) (yield 1.5%). This glucan showed potent antitumor activity (Table II), and the optimum dose was about 50 times less than that of the crude extract. HW-1a1p contained 2,4,6-Me_3, 2,4-Me_2 as major components in a molar ratio of about 2 : 1 as determined by methylation analysis. This result suggests that the major component was a β-1,3-glucan, but contaminants were still present; for example, peak X-3 (Table I) was also a major component. Peaks X-1, -2, -3 were unknown components that did not correspond to glucosidic linkage derivatives. Further purification of this glucan was not performed because of the complexity of the purification step and lower yield.

Amylase Digestion of the Fruit Body of *G. frondosa*

To make purification of the antitumor glucan easier, digestion of the fruit bodies with amylases was investigated. The fruit bodies were pulverized to a powder and digested with amyloglucosidase and then α-amylase. The residual fraction was obtained by centrifugation (yield 35%). For monitoring the amylase digestion, nuclear magnetic resonance (NMR) spectra were taken periodically. As shown in Fig. 1, the content of α-1,4-linkage was decreased after these treatments. Signal A in Fig. 1 corresponds to the anomeric carbon of the α-1,4-linkage. After the enzyme digestion, this signal was no longer apparent.

Chemical Properties of Hot Water, Cold Alkali, and Hot Alkali Extracts from the Enzyme-Digested Fruit Body

Hot water extract (AHW), cold alkali extract (ACA), and hot alkali extract (AHA) were obtained from the enzyme-treated fruit bodies by the methods previously used. As compared with the enzyme-untreated fruit bodies, the yield of the hot water extract was decreased (*cf.*, HW; 10.7%). However, methylation analysis indicated a large amount of 2,4,6-Me_3 derivatives. The contents of 2,4,6-Me_3 derivatives in the ACA and AHA fractions were also larger than those from untreated fruit bodies. In the ^{13}C-NMR spectra of these fractions, almost all of the α-anomeric carbon signals had disappeared (signal A in Fig. 2). These results suggest that the use of enzyme digestion prior to extraction does make it easier to obtain purified β-1,3-glucan.

Purification of the Antitumor β-1,3-Glucan from AHW Fraction

AHW fraction was applied to a DEAE-Sephadex A-25 column, and the flow-through fraction (AHW-1) was obtained. Methylation analysis, showed that this fraction contained large amounts of 2,4,6-Me_3, and 2,4-Me_2 derivatives and a trace amount of 2,3,4-Me_3 derivative. This result is quite different from the ratio obtained in HW fraction (see HW-1 in Table I). This further supports the usefulness of the enzyme treatment of the fruit bodies. For further purification, AHW-1 was precipitated with 30, 60, 80, and 100% ammonium sulfate.

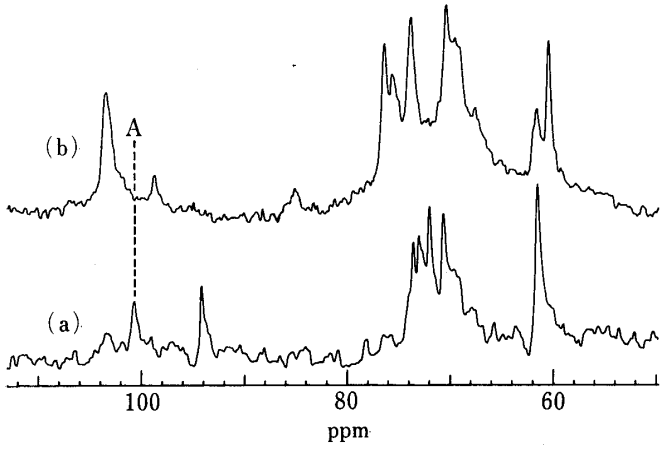

Fig. 1. ^{13}C-NMR Spectra of Aqueous Suspensions of Native (a) and Amylase-Digested (b) Fruit Bodies of *G. frondosa*

Arrow A indicates the anomeric carbon signal of α-glucans.

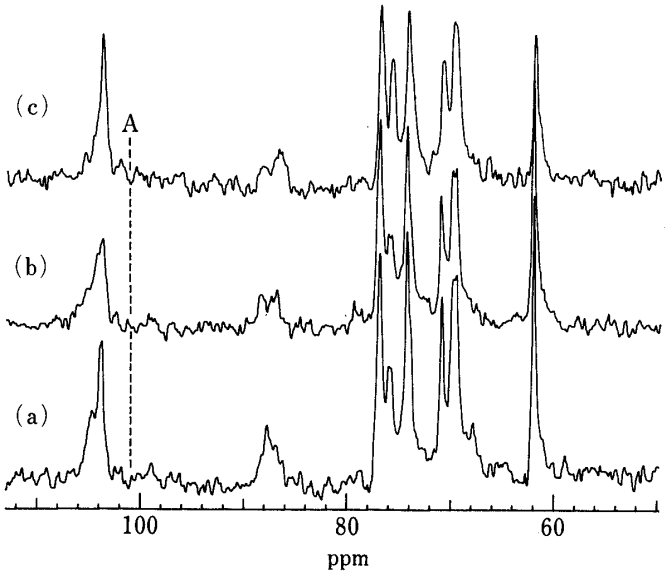

Fig. 2. ^{13}C-NMR Spectra in 0.2 N NaOH of AHW (a), ACA (b), and AHA (c) Fractions

Arrow A indicates the anomeric carbon signal of α-glucan.

TABLE III. Chemical Properties of Hot Water, Cold Alkali, and Hot Alkali Extracts from Amylase-Digested Fruit Body

	AHW[a)]	ACA[a)]	AHA[a)]
Yield (%)	3.6	8.3	2.6
Sugar content	74.4	88.7	63.0
Protein content	23.7	5.5	11.3
Methylation analysis			
2,3,4,6-Me_4	1.0	1.0	1.0
2,4,6-Me_3	1.2	2.0	1.5
2,3,4-Me_3	0.6	0.4	0.5
2,4-Me_2	0.9	1.2	1.0
Component sugar (Glc/Gal/Man)	1.0/0.03/0.04	1.0/0.01/0.01	1.0/0.02/0.01

a) Hot water (AHW), cold alkali (ACA), or hot alkali (AHA) extract of the amylase-digested fruit bodies. Experimental details are given in Materials and Methods.

TABLE IV. Yield and Methylation Analysis of Fractions Obtained from AHW Fraction

	AHW-1[a)]	AHW-2[a)]	AHW-1b[b)]	AHW-1c[b)]	AHW-1f[b)]
Yield (%)[c)]	57	33	22	9	17
Sugar content	95	78	97	nd	nd
Protein content	1.4	5.9	0.8	nd	nd
Methylation data					
2,3,4,6-Me_4	1.0	1.0	1.0	1.0	1.0
2,4,6-Me_3	2.2	0.7	1.9	1.8	1.3
2,3,4-Me_3	0.2	1.1	0	0	0.9
2,4-Me_2	1.1	0.8	1.1	1.0	0.8
3,4,6-Me_3	0	0	0.1	0.1	0

a) Flow-through (AHW-1) and absorbed (AHW-2) fractions on DEAE-Sephadex A-25. *b*) -1b, 30% ppt; -1c, 60% ppt; -1f, 100% sup. *c*) From AHW fraction (%).

Methylation analysis revealed only 2,3,4-Me_3 derivative in the supernatant fraction (AHW-1f) (Table IV). The 30 and 60% precipitated fractions (AHW-1b, -1c) contained only Me_4, 2,4,6-Me_3, and 2,4-Me_2 derivatives in a molar ratio of about 1 : 2 : 1. The molar ratio of this

TABLE V. Antitumor Activity of Grifolan 1N on Solid Form of Sarcoma 180[a]

Sample	Dose ×5 (μg/mouse)	Tumor weight[c] (g, mean±S.D.)	Inhibition ratio (%)[b]	Complete regression[b]
Grifolan-1N	20	0.2±0.6[d]	98	7/10
	100	0.1±0.1[d]	>99	7/10
Nil (control)	—	9.3±6.8	0	0/12

a) Sarcoma 180 tumor cells (5×10^6) were inoculated subcutaneously. Each sample was administered as saline solution by intraperitoneal injection at days 1, 3, 5, 7, 9. *b*) Inhibition ratio and complete regression were determined at 35 d after tumor inoculation. *c*) The significance of differences was evaluated according to Student's *t*-test. Significant difference from the control (*d*) $p<0.001$).

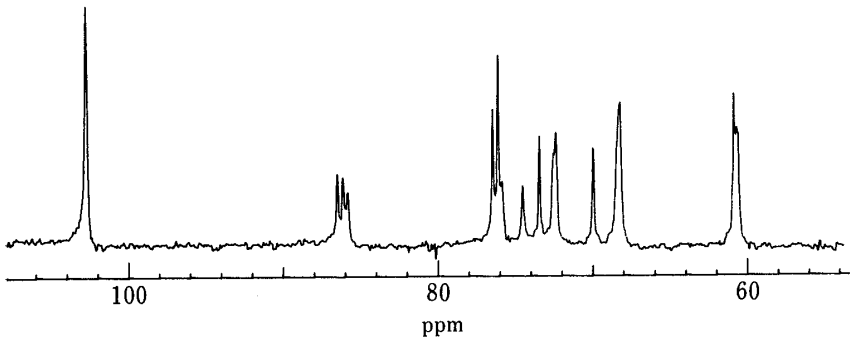

Fig. 3. ^{13}C-NMR Spectrum of Grifolan 1N in DMSO-d_6 at 60 °C

glucan is consistent with that of HW-1a1p fraction as shown in Table I. This glucan showed potent antitumor activity (Table V). The optimum dose was similar to those of the glucans obtained from alkali extracts. This glucan was named grifolan 1N.

Physicochemical Properties of the Purified Antitumor Glucan

In Sepharose CL-4B chromatography with 0.2 M NaOH–8 M urea, most of the glucan was eluted at $K_{av}=0.136$. By comparing this with the values for standard dextrans, the average molecular weight of the glucan was estimated to be 5.6×10^6. The glucan showed $[\alpha]_D$ +6.3. As shown in Fig. 3, the ^{13}C-NMR spectrum of the glucan in dimethyl sulfoxide (DMSO)-d_6 showed signals quite similar to those of scleroglucan and the β-1,3-glucans obtained from the alkali extract of the fruit bodies and the matted mycelium. The results of methylation analysis and ^{13}C-NMR spectroscopy suggest that the glucan is a β-1,3-glucan branched at position 6 of every third main-chain glucosyl unit.

Congo Red is known to form complexes with several β-1,3-glucans, and the absorption maximum of Congo Red is shifted markedly to longer wavelength.[3] In the presence of grifolan 1N, λ_{max} of Congo Red (480 nm) was shifted to 500 nm (0.1 N NaOH), 502 nm (0.2 N NaOH); 502 nm (0.3 N NaOH), or 504 nm (0.4 N NaOH). These shifts return to the shortest wavelength (*ca.* 480 nm) at NaOH concentrations higher than 0.2 N in the cases of curdlan,[3] grifolan 7N[2b] and grifolan NMF-5N,[2c] but not in the case of grifolan 1N. This may be due to its higher molecular weight (M_r 5.6×10^6).

Discussion

In this paper, the purification and characterization of the antitumor β-1,3-glucan obtained by hot water extraction from the enzyme-digested fruit bodies of *G. frondosa* are described. It was shown that the antitumor activity of the hot water extract was due to a branched β-1,3-glucan. As regards structure, cold alkali-extracted β-1,3-glucan, and hot

alkali-extracted β-1,3-glucan were quite similar. However, (1) the molecular weight of this glucan was the greatest among the above glucans (this glucan, 5.6×10^6; cold alkali-extracted, 7.5×10^5; hot alkali-extracted, 1.2×10^6) and (2) complexes formed between glucan and Congo Red were the most stable in the case of this glucan (the complex did not disappear at concentrations higher than 0.3 N NaOH).

Glucans possessing similar structural units have been obtained from *Lentinus edodes* (lentinan),[4] *Schizophylum commune* (schizophyllan),[5] and *Sclerotinia glucanicum* (sclero-glucan).[6] Some of them are expected to be used clinically in the near future for cancer therapy. Recently, we have obtained a polysaccharide fraction, GF-1, from HW fraction.[1] GF-1 is composed of α-1,4-, α-1,6-, β-1,6-, and β-1,3-linkages and the antitumor activity appears to be due to β-1,3-glucan. GF-1 showed characteristic features compared with other antitumor glucans.[1] Further studies are in progress.

Based on HW fraction as a starting material, partially purified glucan was obtained in less than 2% yield (Table I). However, by using the pretreatment with amylases, purified glucan was obtained in about 30% yield (Table IV, AHW-1b, AHW-1c). Many kind of fungi are known to possess antitumor polysaccharides, and this method should be generally useful for purifying antitumor glucans.

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