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# Fractionation of Acidic Antitumor β-Glucan of *Grifola frondosa* by Anion-Exchange Chromatography Using Urea Solutions of Low and High Ionic Strengths

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The antitumor β-glucan from the fruit body of *Grifola frondosa* was resolved by DEAE-Sephadex chromatography into neutral and acidic components, both of which were found to be growth-inhibitory against sarcoma 180 solid tumor inplanted in ICR male mice (Ohno *et al.*, *Chem. Pharm. Bull.*, **33**, 1181 (1984)). In the present study, the acidic β-glucan was further fractionated by diethylaminoethyl (DEAE)-Sephadex chromatography followed by ammonium sulfate precipitation, and the resultant β-glucan subfractions were assayed for antitumor effect.

The β-glucan fraction absorbed on DEAE-Sephadex A-25 was eluted with 8 M urea, an inducer of helix-random coil transition of β-1,3-glucan. The eluted glucan fraction (fraction N (8M)) was a β-1,3-glucan branched at the 6 position and possessing almost the same properties as those of a neutral β-1,3-glucan obtained from the neutral fraction (fraction N (0M)). The remaining acidic β-glucan fraction was eluted with 0.45 M ammonium bicarbonate in 8 M urea (fraction A), and was further separated into a β-1,3-glucan branched at the 6 position (fraction AP) and a β-1,6-glucan (fraction AS) fractions by ammonium sulfate precipitation. Fraction AP showed potent antitumor activity, while that of fraction AS was quite weak. These results suggest that *G. frondosa* contains three kinds of acidic glucans, (1) branched β-1,3-glucan non covalently coupled with an acidic component, (2) branched β-1,3-glucan tightly coupled with an acidic component, and (3) acidic β-1,6-glucan, and that the antitumor activity of the crude extracts of *G. frondosa* is due to both the neutral and the acidic β-1,3-glucans.

**Keywords**——antitumor activity; *Grifola frondosa*; β-1,3-glucan; acidic glucan; conformation; urea solution

Fungal β-1,3-glucan is one of the biological response modifiers currently being used clinically for cancer immunotherapy.[1] One of the physico-chemical characteristics of the β-1,3-glucan is the formation of a gel under physiological conditions.[2,3] This property was found to be attributable to triple helix formation of the polysaccharide chains, which can be broken down to random coil form by the action of aqueous NaOH solution at concentrations higher than 0.2 N, dimethyl sulfoxide (DMSO), or other solvents.[2,3] It is assumed that the antitumor activity of β-glucans requires this type of ultrastructure.[4]

Recently, we studied the structure and antitumor activity of polysaccharides obtained from *G. frondosa*,[5] and reported that this fungus contains neutral and acidic glucans possessing antitumor effect,[5b] and a 6-branched β-1,3-glucan was obtained from the neutral fraction. This paper is concerned with the characterization of acidic β-glucan fractions of *G. frondosa*.

## Materials and Methods

**Separation of NMF-5 on a DEAE-Sephadex A 25 Column**——NMF-5 (600 mg) dissolved in $H_2O$ (200 ml) was applied to a column of DEAE-Sephadex A-25 ($HCO_3^-$) (50 ml) and eluted first with water to give "grifolan NMF-

5N"[5d] (fraction N (0M)). Then, the column was eluted with 2 M urea, 8 M urea (fraction N (8M)), 0.45 M $NH_4HCO_3$ containing 8 M urea (fraction A), and finally 2 M NaCl containing 8 M urea (fraction B). Each fraction was dialyzed against water and then lyophilized (yields: N (0M), *ca.* 50%; N (2M), *ca.* 1%; N (8M), *ca.* 8%; A, *ca.*10%).

**Separation of Fraction A by Ammonium Sulfate Fractionation**——Powdered ammonium sulfate was added to an aqueous solution of fraction A (200 mg) in $H_2O$ (200 ml) to give 80% saturation at 4 °C under stirring overnight. The precipitate was recovered by centrifugation (15000 rpm for 10 min), dialyzed against water and then lyophilized. The supernatant was dialyzed against water and lyophilized (fraction AS, yield 67%). The precipitated fraction was further purified on a DEAE Sephadex A 25 ($HCO_3^-$) column equilibrated with 8 M urea, and 0.45 M ammonium bicarbonate containing 8 M urea fraction was collected, dialyzed and then lyophilized (fraction AP, yield 10%).

**Other Methods**——Purification of grifolan, evaluation of antitumor activity, methylation analysis, carbon-13 nuclear magnetic resonance ($^{13}$C-NMR) spectroscopy and other physicochemical analyses were performed as described previously.[5]

## Results

### Separation of the Acidic Glucan Fraction on a DEAE-Sephandex A-25 Column under "Random Coil" Conditions

NMF-5, which is a cold alkali extract obtained from the matted mycelium of *G. frondosa*, [5d] was applied to a DEAE-Sephadex A-25 column with $H_2O$ as solvent (Fig.1). The pass-through fraction (fraction N (0M)) contained a neutral $\beta$-1,3-glucan called grifolan NMF-5N.[5d] The absorbed fraction was eluted first with 2 M urea, but no material was eluted from the column. Then, the column was eluted with 8 M urea, which can induce conformational transition from gel to random coil, and a carbohydrate-rich fraction (fraction N (8M)) was eluted. Next, the column was eluted with 0.45 M ammonium bicarbonate containing 8 M urea (fraction A). Finally, elution was done with 2 M NaCl containing 8 M urea (fraction B), although no carbohydrates were recovered from this eluate.

Because fraction N (8M) was eluted at a high concentration of urea which would induce helix-to-random coil transition, it can be presumed that this fraction consisted of neutral glucan under the "random coil" conditions. These facts suggest that, before DEAE-Sephadex chromatography, fraction N (8M) contained an acidic part (A-part) which was absorbed on the column at low urea concentration. This acidic part would be released from fraction N (8M) in the 8 M urea and retained in the column. Fraction N (8M) was found to be composed of glucose, and the results of methylation analysis (Table I) showed that the molar ratio of 2,3,4,6-$Me_4$-Glc: 2,4,6-$Me_3$-Glc: 2,4-$Me_2$-Glc was 1 : 2.2 : 1.2. The $^{13}$C-NMR spectrum in

Table I. Molar Ratios of *O*-Methyl-*O*-acetyl Glucitols Derived from the Methylated Polysaccharides

| Alditol acetate of | NMF-5[a] | Grifolan NMF-5N | N (8M)[b] | A[c] | AP[d] | AS[e] |
|---|---|---|---|---|---|---|
| 2,3,4,6-$Me_4$-Glc[f] | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| 2,4,6-$Me_3$-Glc | 1.8 | 2.0 | 2.2 | 1.5 | 2.2 | 0.8 |
| 2,3,4-$Me_3$-Glc | 0.3 | 0 | 0.1 | 0.7 | 0.4 | 1.5 |
| 2,3,6-$Me_3$-Glc | 0.1 | 0 | 0.1 | 0 | 0.1 | 0.1 |
| 3,4,6-$Me_3$-Glc | 0 | 0.1 | 0.1 | 0 | 0 | 0 |
| 2,4-$Me_2$-Glc | 1.2 | 1.2 | 1.2 | 1.1 | 1.4 | 0.9 |
| 2,3-$Me_2$-Glc | 0 | 0 | 0 | 0 | 0 | 0.1 |
| 3,6-$Me_2$-Glc | 0 | 0 | 0 | 0 | 0 | 0.1 |

*a*) Crude polysaccharide fraction extracted from the matted mycelium of *Grifola frondosa* with aqueous sodium hydroxide at 4 °C. *b*) 8 M urea-eluted fraction of NMF-5 from the DEAE-Sephadex column. *c*) 0.45 M ammonium bicarbonate-eluted fraction of NMF-5 from the DEAE-Sephadex column. *d*) Ammonium sulfate-precipitated fraction of the 0.45 M ammonium bicarbonate-eluted fraction. *e*) Ammonium sulfate-unprecipitable fraction of the 0.45 M ammonium bicarbonate-eluted fraction. *f*) Based on 2,3,4,6-tetra-*O*-methyl-1,5-di-*O*-acetyl glucitol as unity.

TABLE II. Antitumor Effect of Acidic Fractions[a]

| Sample | Dose × times (μg/mouse) | Tumor weight[c] (g, mean ± S.D.) | Inhibition ratio (%)[b] | CR[b] |
|---|---|---|---|---|
| Grifolan | 20 × 10 | 0.2 ± 0.7[e] | 95 | 9/10 |
| NMF-5N | 100 × 10 | 0.1 ± 0.2[e] | 97 | 1/10 |
| Fraction AP | 20 × 10 | 2.7 ± 1.8 | 33 | 1/8 |
| | 100 × 10 | 0.2 ± 0.4[e] | 95 | 2/9 |
| Fraction AS | 20 × 10 | 3.5 ± 2.8 | 12 | 0/10 |
| | 100 × 10 | 1.7 ± 2.2[d] | 59 | 2/9 |
| Control | 0 | 4.0 ± 2.8 | 0 | 0/20 |
| Grifolan | 20 × 5 | 3.9 ± 3.1[d] | 68 | 2/10 |
| NMF-5N | 100 × 5 | 0.1 ± 0.1[e] | 99 | 5/9 |
| Fraction N (8M) | 20 × 5 | 2.2 ± 3.7[e] | 82 | 4/10 |
| | 100 × 5 | <0.1[e] | >99 | 7/10 |
| Control | 0 | 12.3 ± 7.5 | 0 | 0/20 |

*a*) Sarcoma 180 tumor cells ($5 \times 10^6$) were inoculated subcutaneously into ICR male mice. Each sample was administered as saline solution by intraperitoneal injection at days +1—+10 (10 times) or days +1, +3, +5, +7, +9 (5 times). *b*) Inhibition ratio and complete regression (CR) 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*) $p<0.001$}.

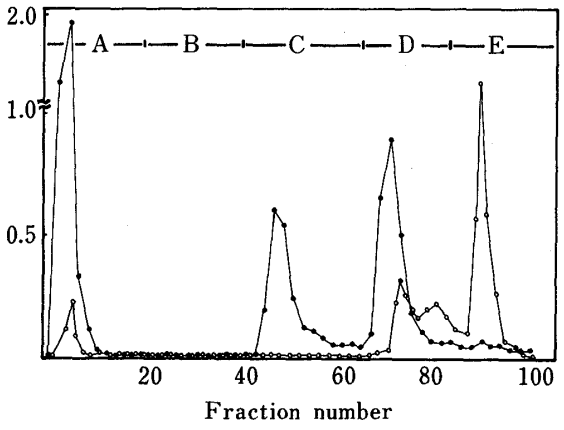


Fig. 1. Elution Profile of NMF-5 on a Column of DEAE-Sephadex A-25

NMF-5 (40 mg) dissolved in $H_2O$ was applied to a column of DEAE-Sephadex A-25 ($HCO_3^-$) (25 ml) equilibrated with water. The column was eluted with (A) water, (B) 2 M urea, (C) 8 M urea, (D) 0.45 M $NH_4HCO_3$ containing 8 M urea, and (E) 2 M NaCl containing 8 M urea. Fractions of 2.5 ml were collected, and carbohydrate and protein were monitored by the phenol–sulfuric acid method (490 nm; —●—) and by ultraviolet absorption measurement (280 nm; —○—), respectively.

DMSO-$d_6$ showed signals quite similar to those of grifolan (Fig. 2 a,b). Further, the $^{13}$C-NMR spectrum in $H_2O$ showed only broad signals (Fig. 2 c, d), and this observation is also consistent with the characteristic features of a β-1,3-glucan branched at the 6 position, such as lentinan and grifolan.[5,6] Fraction N (8M) showed an antitumor effect on the solid form of sarcoma 180 tumor in ICR mice at a dose similar to the effective dose of grifolan (Table II). These results suggest that the structure, physicochemical properties, and antitumor effect of fraction N (8M) are quite similar to those of grifolan NMF-5N, which was purified from fraction N (0M).

**Comparison of Conformations of Grifolan NMF-5N and Fraction N(8M)**

β-1,3-Glucan is known to form so-called "gel structure". This structure is broken down to form a random coiled structure in aqueous NaOH solutions at concentrations higher than 0.2 N[2] or in high concentrations of urea.[5f] The conformational transition can be determined by observing the change of signal strength in the $^{13}$C-NMR spectrum.[3] In order to clarify the gel structure of grifolan that was destroyed at higher urea concentration, the $^{13}$C-NMR spectra of grifolan in various concentration of urea were measured. As shown in Fig. 3, the

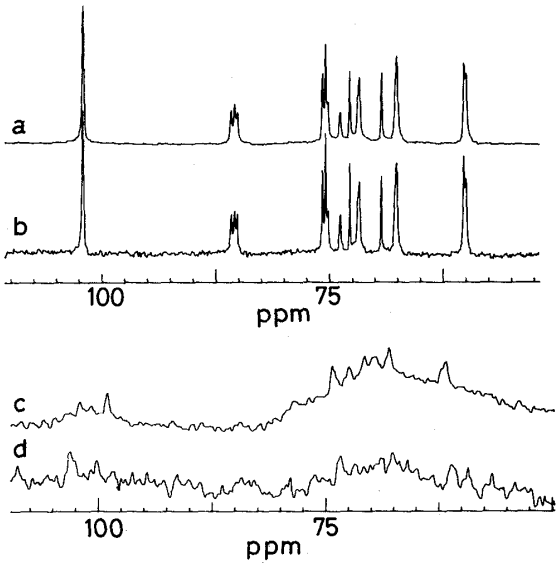


Fig. 2. $^{13}$C-NMR Spectra of Fraction N (8M) in DMSO-$d_6$ or $H_2O$

a, grifolan NMF-5N in DMSO-$d_6$; b, fraction N (8M) in DMSO-$d_6$; c, grifolan NMF-5N in $H_2O$; d, fraction N (8M) in $H_2O$.

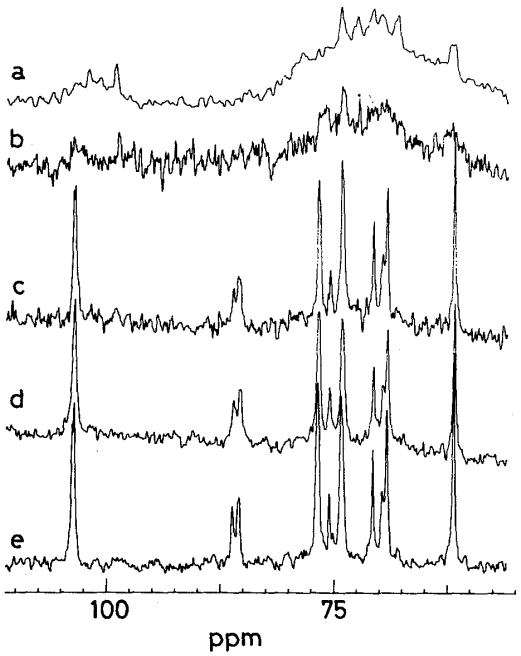


Fig. 3. $^{13}$C-NMR Spectra of Grifolan NMF-5N in the Presence of Various Concentrations of Urea

a, in $H_2O$; b, in 2 M urea; c, in 4 M urea; d, in 6 M urea; e, in 8 M urea.

$^{13}$C-NMR spectrum of grifolan showed only broad signals at 0 to 2 M urea, but showed sharp signals at concentrations of more than 4 M urea. These facts suggest that, before fractionation by DEAE-Sephadex chromatography, fraction N (8M) existed as a complex consisting of certain acidic compounds (A-part) and neutral-$\beta$-1,3-glucan (glucan–A-part complex), and that 8 M urea treatment resulted in the degradation of the gel structure of the glucan part to random coil, yielding neutral glucan.

**Separation of Fraction A by Ammonium Sulfate Fractionation**

Fraction A (carbohydrate, 73%; protein, 7%; uronic acid, 11%) was further separated with ammonium sulfate and yielded a precipitated fraction (fraction AP; carbohydrate, 82%; protein 11%; uronic acid, 2%) and a supernatant fraction (fraction AS; carbohydrate, 75%; protein, 5%; uronic acid, 10%) after dialysis and lyophilization. As shown in Table I, methylation analysis of fractions AP and AS demonstrated that these fractions contained branched $\beta$-1,3-glucan and $\beta$-1,6-linkage-rich glucan ($\beta$-configurations of both fractions were assumed from the results of $^{13}$C-NMR spectroscopy,[5b] respectively. Both fractions were assayed for antitumor activity against the solid form of sarcoma 180 in ICR mice. Fraction AP was nearly as effective as the neutral glucan, but fraction AS showed less activity (Table II). These findings suggest that the $\beta$-1,3-glucan moiety plays a major role in the antitumor activity of both neutral and acidic glucan fractions.

## Discussion

The data presented in this paper show (1) that, under "random coil" conditions, the acidic fraction obtained from the alkaline extract of *G. frondosa* could be separated into neutral (8M) $\beta$-1,3-glucan (fraction N (8M)), acidic $\beta$-1,3-glucan (fraction AP), and acidic $\beta$-1,6-glucan (fraction AS), and (2) that the basic structures of neutral $\beta$-1,3-glucan (grifolan),

neutral (8M) β-1,3-glucan (fraction N (8M)), and acidic β-1,3-glucan (fraction AP) are quite similar in terms of primary structure, conformation and antitumor activity. It is likely that the neutral (8M) β-1,3-glucan, fraction N (8M), before passing through a column of DEAE-Sephadex is acidic (glucan–A-part conjugate), and removal of the acidic component (A-part) from the parent complex occurs on treatment with 8 M urea. It is also suggested that grifolan and neutral (8M) β-1,3-glucan, fraction N (8M), are the same polysaccharide except for the interaction with acidic component(s). Previously, we reported that *G. frondosa* contained both neutral and acidic antitumor glucans.[5b] It was clarified that the acidic antitumor glucan is also a β-1,3-glucan, the bulk of which forms the acidic polymer with the A-part component. The acidic polymer might contain the A-part within the helices of the main chains, because release of the A-part did not take place easily under physiological conditions. The similarity of antitumor activity of the complex and grifolan also suggests that the conformational difference (probably small) of the main chains is not critical for the manifestation of antitumor activity. Although the composition and structure of the A-part are not yet known, the association of β-glucan and the A-part is interesting in relation to the immunomodulating activities. It would also be interesting to know whether these conjugates are contained *in situ* in the fruit body of the fungus.

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