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Two Different Conformations of Antitumor Glucans Obtained from *Grifola frondosa*

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Several antitumor glucan fractions obtained by various extraction procedures from *Grifola frondosa* were compared by solution and solid (cross polarization, magic angle spinning (CP/MAS)) carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectroscopy. The fractions prepared under milder conditions, *i.e.*, hot water extract (AHW), liquid culture filtrate (LLFD), and extracellular polysaccharide (LELFD), showed a C-3 signal at the same chemical shift as that in DMSO-d_6 solution (native form) in ^{13}C CP/MAS NMR spectroscopy. However, the fractions prepared under drastic conditions, *i.e.*, cold alkali extract (NMF-5, grifolan), showed a C-3 signal at 89 ppm, similar to those of curdlan and attributable to helix structure (helix form). After dissolution of LELFD in 8 M urea followed by dialysis, LELFD also showed a C-3 signal at 89 ppm. These results suggest that the polysaccharides obtained from *G. frondosa* possess two kinds of conformations.

Keywords——CP/MAS; conformation; polysaccharide; antitumor glucan; *Grifola frondosa*; grifolan; ^{13}C-NMR spectroscopy; helix

Many kinds of antitumor glucans have been studied, and lentinan from *Lentinus edodes* and schizophyllan from *Schizophyllum commune* have been applied clinically.[1,2] It has been suggested that the triple helix structure is required for antitumor activity of these polysaccharides. It is well known that, in the case of pachyman, antitumor activity can be induced by treatment with urea, which would alter the conformation of the glucan.[3] Conformation is thought to be important for antitumor activity of glucans, but elucidation of polysaccharide conformation by X-ray crystallography has been successful only for a limited number of polysaccharides because polysaccharides are difficult to crystallize.

We have studied the structure and antitumor activity of grifolan obtained by various extraction procedures from *G. frondosa*.[4] Grifolan is a 6-branched β-1,3-glucan, and the primary structure is similar to that of schizophyllan or scleroglucan. The main chain moiety of grifolan is considered to be important for antitumor activity, because periodate oxidation and borohydride reduction of grifolan did not affect the antitumor activity. However, it is not fully elucidated what kind of conformation is required for the antitumor activity of grifolan.

Recently, conformations of poly- and oligosaccharides, such as lentinan, curdlan, cellulose, chitin, and cyclodextrin, have been investigated by solid state cross polarization, magic angle spinning (CP/MAS) carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectroscopy.[5] These results showed a good correlation between the results of X-ray crystallography and CP/MAS NMR spectroscopy. Saito *et al.* examined the polysaccharide conformations of lentinan and curdlan by CP/MAS NMR spectroscopy.[5] They concluded that the chemical shifts of several carbons of these polysaccharides in the solid are different from those in solution. These differences are thought to result from ultrastructural differences. The above differences presumably result from the formation of helical conformation, because the

random coiled structure of the oligosaccharide showed a chemical shift similar to that seen in solution.

In this paper, we have studied the conformation of glucan fractions prepared from *G. frondosa* in solution and in the solid state by (CP/MAS) ^{13}C-NMR spectroscopy.

Materials and Methods

Preparation of Polysaccharide Fractions——Curdlan oligo was prepared by partial hydrolysis with formic acid and trifluoroacetic acid. Oligosaccharides (DP 24) were partially purified on the basis of solubility in ice-cold water. The degree of polymerization (DP) was determined by Somogyi–Nelson's method.[6] Fraction AHW was prepared from amylase-digested fruit body of *G. frondosa* by hot water extraction.[4f] Fraction NMF-5 was prepared from the matted mycelium of *G. frondosa* by cold alkali extraction.[4e] Grifolan NMF-5N was purified from NMF-5 by diethylaminoethyl (DEAE)-Sephadex A-25 chromatography and ethanol precipitation.[4e] Grifolan NMF-5N was also conventionally prepared from the amylase-digested matted mycelium of *G. frondosa*. Grifolan 7N was purified from F-7, which was obtained from the fruit body of *G. frondosa* by hot alkaline extraction, followed by DEAE-Sephadex A-25 and Sepharose CL-4B chromatographies.[4b] Fraction LLFD was prepared from the liquid culture broth of *G. frondosa* by ethanol precipitation.[4h] Fraction LELFD was prepared by incubation of the mycelium with a buffer composed of glucose and citric acid.[4h]

^{13}C-NMR Spectra——^{13}C-NMR spectra were measured with JEOL-FX 200 instruments. Solution NMR spectra were measured by using 10ϕ sampling tubes at room temperature for aqueous solution and at 60 °C for DMSO-d_6 solution. All spectra were obtained from 5000 to 100000 scans. CP/MAS spectra were recorded with a CP/MAS unit operating with JEOL CP/MAS software. The spectra were measured with the use of a Dyflon rotor. Contact time, pulse interval, and number of pulses were 1 ms, 1—2 s, and 500—2000, scans respectively. Chemical shifts relative to tetramethylsilane (TMS) were determined by using adamantane signals (29.5 ppm).

Results

CP/MAS Spectra of Polysaccharide Fractions Obtained from *Grifola frondosa*

The ultrastructures of polysaccharide fractions obtained from *G. frondosa* were compared by means of CP/MAS NMR spectroscopy. The polysaccharide fractions used in these experiments are listed in Table I. Three polysaccharides were used as references (laminarin, curdlan, curdlan oligo). Under physiological conditions, it is known that curdlan possesses a helix structure and laminarin possesses a random coil structure.

CP/MAS NMR spectra obtained from these polysaccharide fractions are shown in Fig. 1. The polysaccharides could be classified into two groups. One group (group N) showed the C-3 signal at 86 ppm (Fig. 1d, h, i) and the other group (group H) showed the C-3 signal at 89 ppm (Fig. 1e, f, g) (Table II). Assignment of each carbon signal is shown in Table II. The C-3 signal in the former group showed a similar chemical shift to that seen in the random coiled state (in DMSO-d_6) (Fig. 2, Table II). On the other hand, the latter group showed a C-3 signal similar to that of curdlan powder.

Curdlan is known to form a helix structure in the solid or under physiological conditions. However, it is transformed into a random coil structure in 0.2 N or stronger NaOH or in dimethyl sulfoxide (DMSO) solution. The C-3 signal appeared at 90 ppm (Fig. 1b) in the case of helix and 87 ppm (Table II) in the case of random coil. The preparation methods used to prepare the glucan fractions of group N from *G. frondosa* were milder than those in the case of group H. The former fractions were prepared under neutral conditions and the latter under alkaline conditions. Alkaline conditions are known to shift the glucan conformation from random coil to helix.[7] These considerations suggest that the conformation of group H is helical, like that of curdlan. However, the conformation of group N is different, and may reflect the native form because of the mild preparation conditions. The native conformation is presumably coil because the chemical shift of C-3 is similar to that in DMSO solution. Laminarin and curdlan oligo form random coil structure under physiological conditions, but the solid state structure has not been fully elucidated. Saito *et al.* suggested that the signal at

Table I. Polysaccharides and Fractions Used for NMR Spectroscopy

Name	Notes	Ref.
Laminarin	Low molecular weight β-1,3-glucan. Used as a reference compound for random coil structure	
Curdlan	Linear β-1,3-glucan. Used as a reference compound for helix structure	
Curdlan oligo (DP24)	Oligosaccharide (DP24) prepared from curdlan by formic acid and trifluoroacetic acid hydrolysis	
AHW	Hot water extract obtained from amylase-digested fruit body of *G. frondosa*. AHW was composed mainly of 6-branched β-1,3-glucan and β-1,6-glucans (group N)	4*f*
NMF-5	Cold alkali extract obtained from the matted mycelium of *G. frondosa*. NMF-5 was composed mainly of 6-branched β-1,3-glucan and β-1,6-glucans (group H)	4*e*
Grifolan NMF-5N	6-Branched β-1,3-glucan purified from NMF-5 (group H)	4*e*
Grifolan 7N	6-Branched β-1,3-glucan purified from the hot alkali extract of the fruit body of *G. frondosa* (group H)	4*b*
LLFD	6-Branched β-1,3-glucan fraction obtained from the culture filtrate of *G. frondosa* (group N)	4*h*
LELFD	6-Branched β-1,3-glucan fraction obtained from the liquid-cultured mycelium of *G. frondosa* (group N)	4*h*
LELFD (8 M)	8 M urea-treated LELFD (group H)	

Table II. Chemical Shifts of Glucans in Solution and in the Solid State

	C-1	C-2	C-3	C-4	C-5	C-6
(in DMSO: random coil)						
Curdlan[a]	103.7	73.6	86.9	69.2	77.1	61.7
Grifolan NMF-5N[b]	102.8	72.4	86.6	68.3	76.2	60.7
		73.5	86.2	70.0	74.5	68.3
			85.8			60.9
			76.5			
(CP/MAS)						
Laminarin[c]	103.8	74.8[e]	89.3	70.8	74.8[e]	62.2
Curdlan[c]	104.6	73.9	90.1	70.2	76.1	62.5
Curdlan (oligo)[c]	104.0	74.6	90.3	69.7	75.8	62.0
			87.8			
			84.0			
AHW	103.4	74.2[e]	86.5	69.1	74.2[e]	62.0
NMF-5	103.1	72.6[e]	89.3	72.6[e]	72.6[e]	61.9
Grifolan	103.9	74.5[e]	89.2	70.1	74.5[e]	61.6
LELFD (8 M)	103.1	74.0[e]	88.7	69.4	74.0[e]	62.0
LELFD	103.4	74.2	86.3	69.0	76.4	62.4
LLFD	103.9	74.6	86.3	69.0	77.0	61.9
Lentinan[d]	104.3	—	90.3	69.5	76.0	62.2
	105.2					
	103.3					

a) Values from ref. 8. *b*) Values from ref. 4*e*. *c*) Assigned with reference to ref. 5*c*. *d*) Values from ref. 5*c*. *e*) Overlapping signals.

86.4 ppm originates from the random coil structure[5] (this signal was observed only as a shoulder in this paper). This fact also suggests the above assumption that the conformation of group N is coil.

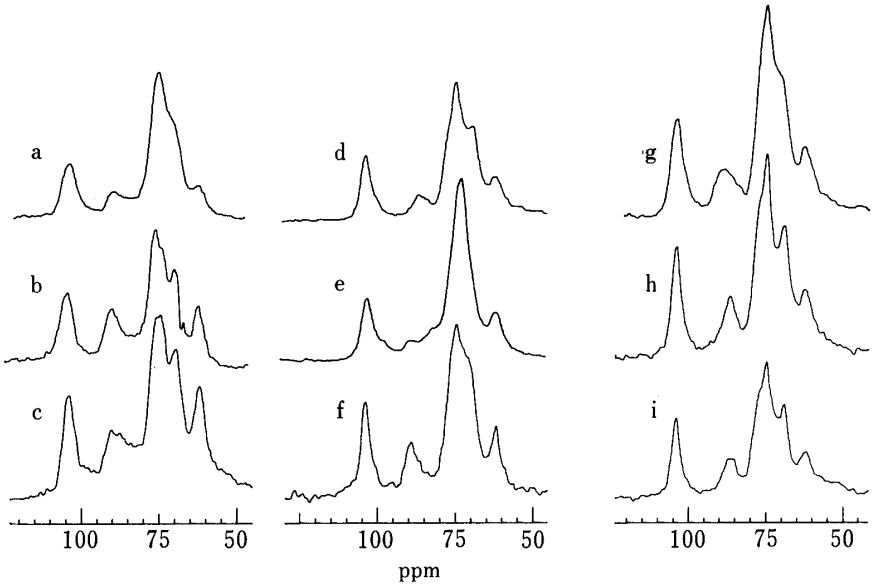

Fig. 1. CP/MAS ^{13}C-NMR Spectra of Polysaccharide Fractions Obtained from *Grifola frondosa*

a, laminaran; b, curdlan; c, curdlan oligo; d, AHW; e, NMF-5; f, grifolan NMF-5N; g, LELFD (8 M); h, LELFD; i, LLFD.

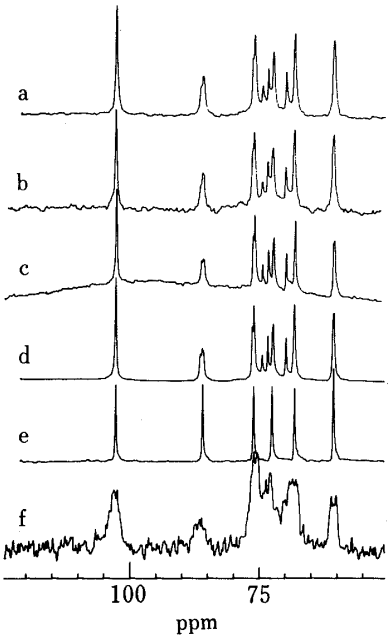

Fig. 2. ^{13}C-NMR Spectra of Polysaccharide Fractions Obtained from *Grifola frondosa* in DMSO-d_6 (Random Coil)

a, LELFD; b, LLFD; c, grifolan 7N; d, grifolan NMF-5N; e, curdlan, f, laminaran.

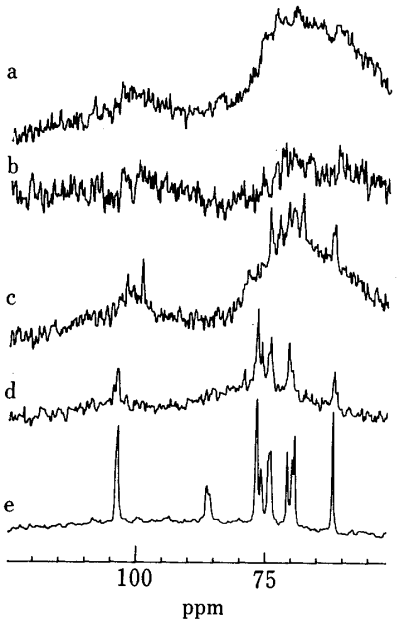

Fig. 3. ^{13}C-NMR Spectra of Polysaccharide Fractions Obtained from *Grifola frondosa* in H_2O (Gel)

a, LELFD; b, LLFD; c, grifolan NMF-5N; d, grifolan 7N; e, laminaran.

Conformational Transition from Native Form to Helix on Treatment with Urea

To clarify the possibility of conformational transition during the extraction and purification procedures, the native form fraction, LELFD, was treated with 8 M urea and then dialyzed. As shown in Fig. 1h, g, the CP/MAS spectra before and after the treatment are quite different. The latter spectrum is similar to that of grifolan (group H; Fig. 1f). This result strongly suggests that polysaccharide conformation was altered, at least in part, during the extraction and purification steps.

Comparison of Native Conformation and Helix under Physiological Conditions

To elucidate the conformations of the polysaccharides under physiological conditions, where the polysaccharides show antitumor activity, gel structures were compared by aqueous ^{13}C-NMR spectroscopy. Previously, it was found that the signals from the main chain region of lentinan could not be observed because helix formation reduced the mobility of the chains, and only the signals attributable to side chains were observable.[1a] As shown in Fig. 3, to compare the mobilities of the conformations, ^{13}C-NMR spectra in H_2O of polysaccharides possessing both conformers were measured. In contrast to the ^{13}C-NMR spectra of these polysaccharides in DMSO-d_6 (Fig. 2, random coil), and laminaran in H_2O (Fig. 3, random coil), polysaccharides in both native and helical conformations showed quite broad signals. Grifolan 7N, which was prepared by hot alkali extraction from the fruit body, showed a quite similar spectrum to lentinan and showed only signals attributable to the side chain moieties. Grifolan NMF-5N, which was prepared by cold alkali extraction from the matted mycelium, showed signals attributable to contaminating α-glucans and showed no β-glucan signals (this conclusion was supported by the ^{13}C-NMR spectrum in DMSO-d_6 (Fig. 2)). LELFD and LLFD showed no detectable signals. These results suggest that both conformations showed reduced mobility of main chain units.

Discussion

In this paper, we have identified two different conformations, native and helix, of polysaccharides obtained from *G. frondosa* by various preparation procedures. Further, the native conformation was found to switch to helical conformation on treatment of polysaccharides with 8 M urea. The helical conformation shown by the polysaccharides obtained from *G. frondosa* is considered to be similar to those of lentinan and curdlan. The polysaccharides purified from the extracts of *G. frondosa* are presumably obtained in helical conformation because their purification steps include 8 M urea treatment. However, the conformation of polysaccharides in the mushroom *in situ* has not yet been examined. The data that the native conformer switched the conformation to helix by denaturing treatment (Fig. 1g, h), suggest the possibility that (if not all of) polysaccharides showing helix conformation after extraction and purification possessed native conformation in the mushroom.

Chemical shift in CP/MAS spectra is suggested to be affected by packing of polysaccharides in the solid state and by the torsion angle of the sugar unit. In the case of cellulose, packing is known to alter the chemical shift by about 1 to 2 ppm. However, the chemical shift difference between the helix and native form is more than 3 ppm. These results suggest that the difference between the present preparations is due not to differences in the packing of carbohydrate chains but to differences of torsion angles. On the other hand, the CP/MAS NMR spectra of chitin (native) and N-acetyl chitosan (regenerated form) are known to be quite similar. However, in the case of cellulose, native and recrystallized preparations showed different conformations. The present results on the conformational change are similar to those in the case of cellulose.

The line width of the CP/MAS spectra of the native form is relatively narrow and similar to those of cellulose and chitin.[5a,b] On the other hand, the line width of the helix form is broad and similar to that of curdlan. These results suggest that the torsion angles of the native form are relatively regular value. Further, the chemical shift of the C-3 signal is similar to that in solution under denatured conditions. Further, Saito *et al.* suggested that the signal of C-3b (86.4 ppm) is due to the random coil conformation.[5c] It may be concluded that the conformation of the native form is an ordered one.

It is thought that the antitumor activity of polysaccharides requires a certain ultrastructure. Hamuro *et al.* converted pachyman, which showed no antitumor activity, into U-

pachyman, which showed antitumor activity, simply by 8 M urea treatment at 70 °C for 4 h.[3] This treatment is similar to that used by us. Therefore, U-pachyman should have helical conformation. However, the polysaccharides possessing native conformation described in this paper showed antitumor activity,[4f,h] whereas pachyman showed no antitumor activity. These considerations suggest that the conformations of pachyman and the native form of grifolan are different. Further, the previous conclusion that helical conformation is required for antitumor activity is not applicable to the case of polysaccharides obtained from *G. frondosa*. These facts also suggest that the halix formation is not always required for the appearance of antitumor activity. Further work on the conformational differences between pachyman and native form grifolan should cast more light on this important problem.

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