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Carbon-13 Nuclear Magnetic Resonance Spectral Analysis of the Fruit Bodies of *Grifola frondosa*

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Carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectral analyses of the cultured fruit body of *Grifola frondosa* and the extracted glucan fractions were performed. NMR spectra of the fruit body were measured by suspending the powdered material in D_2O. The spectrum of the lyophilized fruit body showed signals of mono-, oligo-, and polysaccharides and fatty acids. The spectrum of the fruit body defatted with 80% ethanol showed signals attributable to polysaccharides. By comparison with the spectra of extracts from the fruit body, these signals were assigned to α- and β-glucans. Well-resolved signals were also obtained in the spectra of residues extracted with hot water, and cold and hot alkali. These results suggest that (1) the majority of glucans presented in the fruit body has high motional freedom, (2) this method is suitable for studies of the assembly of biopolymers and the ultrastructure of fungal fruit bodies, (3) this method could be applicable for the standardization of commercially grown mushrooms.

Keywords——^{13}C-NMR; *Grifola frondosa*; β-1,3-glucan; motional freedom; amorphous powder; polysaccharide; fruit body

Various immunomodulators have been obtained from fungi, *e.g.* lentinan, PS-K (Krestin, manufactured by Sankyo), schizophyllan, and TAK (Curdlan, manufactured by Takeda),[1,2] and in addition, immunomodulators from bacterial components, *e.g.* lipid A derivatives and muramyl peptide derivatives, have been studied in detail. The latter developments resulted from an adequate characterization of the chemical composition and ultrastructural characterization of bacterial cell wall, which in turn provided a basis for a synthetic approach. On the other hand, except for the yeast cell wall, the components and cell surface structure of fungi are not well clarified. Precise examination of fungal cell components might result in the discovery of new kinds of immunomodulators.

Grifola frondosa, like *Lentinus edodes*, is an edible mushroom in Japan. We and other groups identified a branched β-1,3-glucan from the fruit body.[3] Like lentinan, the glucan shows a potent antitumor effect on allogeneic and syngeneic tumors on mice by a host-mediated mechanism. NMR spectral analysis is suitable for further studies of the mushroom due to its ability to measure noninvasively the concentrations and dynamics of metabolites in living tissue and to elucidate the conformations, noncovalent bonding interactions, and molecular motions of polymeric molecules.[4] Solid-state and cross polarization magic angle spinning (CP/MAS) NMR spectroscopy is particularly useful in polymer research.[5] In this paper, we will describe the results of ^{13}C-NMR spectroscopy of the glucans in the fruit bodies of *G. frondosa*.

Materials and Methods

Preparation of Each Polysaccharide Fraction and Residues——Hot water-, cold alkali- and hot alkali-extracted

polysaccharide fractions were successively prepared from powdered fruit bodies of *G. frondosa* as described in the previous paper.[3a] The residual fractions were prepared by extensive washing of the residues with water.

^{13}C-NMR Spectral Analysis——^{13}C-NMR spectra were measured in a 10ϕ tube and recorded at 37 °C for solutions in D_2O with a JEOL FX-200 (for carbon-13 at 50.1 MHz) spectrometer. The spectra were obtained in the pulsed Fourier-transform mode with complete proton decoupling. For measuring water-soluble fractions, 100 mg of the fraction was dissolved in 3 ml of D_2O. For measuring residual fractions, 300 mg of the fraction was suspended in 3 ml of D_2O. Representative measurement conditions were as follows: pulse mode (PUMOD) 1; pulse width (PW 1) 15 μs; pulse delay (PD) 100 ms; data points (POINT) 8192; observation frequency width (FREQU) 12004 Hz; sampling time (ACQTM) 341.1 ms; observation frequency (OBFRQ) 50.1 MHz; center frequency offset (OBSET) 83.8 kHz; irradiation center frequency offset (IRSET) 57.7 kHz; temperature (TEMP) 37 °C; irradiation frequency (IRFRQ) 199.5 MHz.

Results

^{13}C-NMR Spectrum of Lyophilized (LY) or Defatted (DR) Fruit Body

The ^{13}C-NMR spectrum of lyophilized or defatted fruit body was measured as a suspension in D_2O. About 300 mg of powder was suspended in 3 ml of D_2O and the spectrum was taken by using a 10ϕ sampling tube. Measurement conditions are given in the figure legends and in the experimental section. ^{13}C-NMR spectra were measured in the pulsed Fourier-transform mode with complete proton decoupling.

About 5000 scans, were run to obtain each spectrum shown in Fig. 1. The spectra of the lyophilized powder (LY), defatted fruit body (DR) and 80% EtOH extract (DE) are shown in Fig. 1a, b, and c, respectively. Relatively sharp signals (*e.g.* signals C, D, G) in the spectrum of LY (Fig. 1a) were not observed in DR (Fig. 1b) but were in DE (Fig. 1c). The major signals observed in DE (Fig. 1c) were identified as being due to α,α-trehalose by comparison with those of the standard material (Fig. 1d). This compound was previously identified by chemical analysis of the fruit body.[6] The broader signals in the spectrum (*e.g.* signals A, B, E, F, G) of LY were also seen in that of DR (Fig. 1b), so these signals were attributable to the polymer fraction. The spectrum of DR was quite similar to that of the cold alkali extracts (HWE, Fig. 2b), confirming that the signals were attributable to polysaccharides. The signals at 101 and

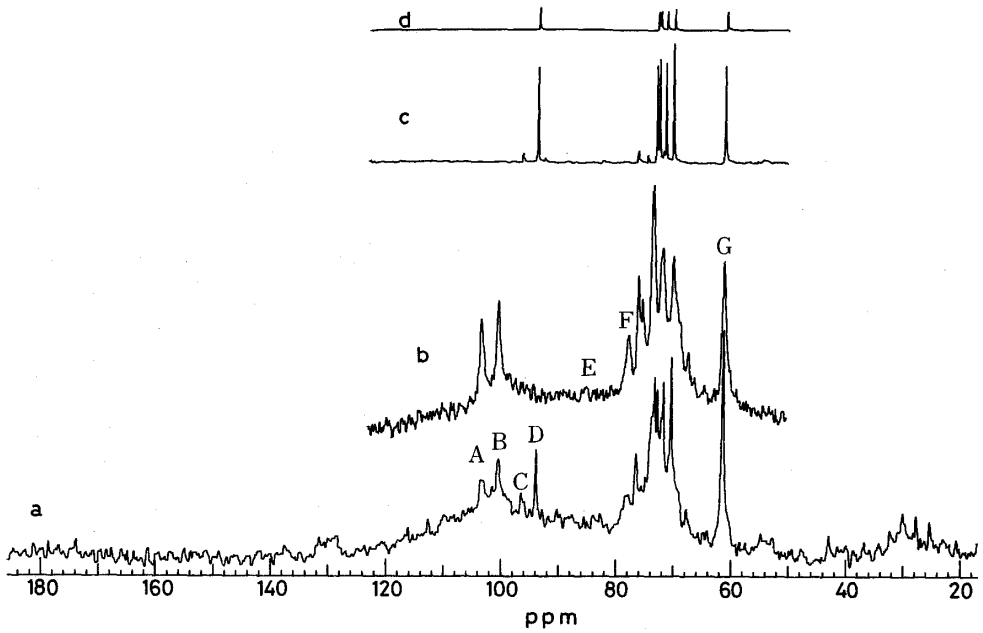

Fig. 1. ^{13}C-NMR Spectra of Lyophilized or Defatted Fruit Bodies

a, lyophilized fruit body (LY); b, defatted fruit body (DR); c, 80% EtOH extract (DE); d, α,α-trehalose.

TABLE I. Chemical Shifts of Some Characteristic Signals in ^{13}C-NMR Spectra Taken in D_2O

Signal	Chemical shift (ppm)	Assignment
A	104	C-1 of β-glucan
B	101	C-1 of α-glucan
C	96	C-1 of β-glucose
D	94	C-1 of α, α-trehalose
E	86	C-3 of 3-substituted β-glucosyl unit
F	78	C-4 of 4-substituted α-glucosyl unit
G	61	C-6 of glucose, α, α-trehalose, and glucans

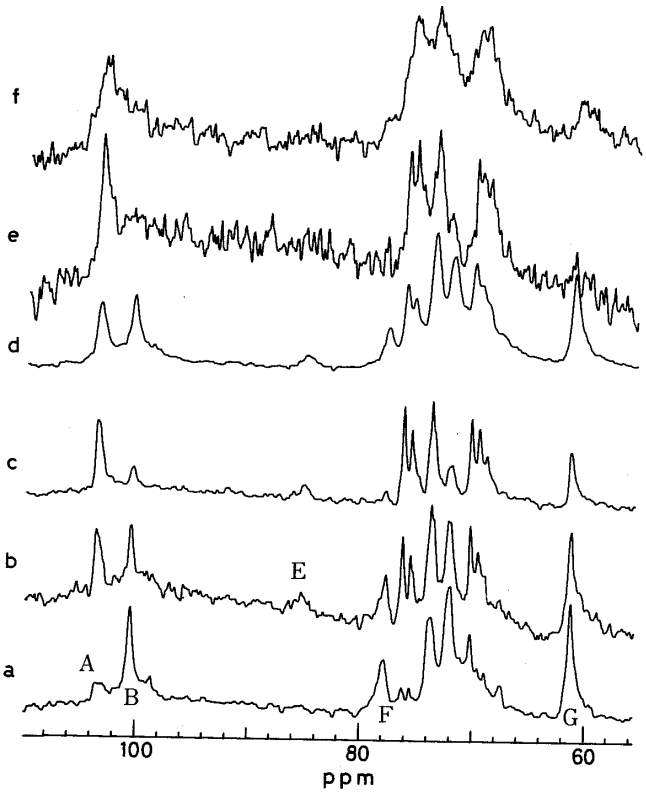

Fig. 2. ^{13}C-NMR Spectra of Various Extracts and Residues

a, hot water extract (HWE); b, cold alkali extract (CAE); c, hot alkali extract (HAE); d, hot water-extracted residue (HWR); e, cold alkali-extracted residue (CAR); f, hot alkali-extracted residues (HAR).

104 can be assigned as C-1 signals of α- and β-glucans, respectively. These results suggest that the polysaccharides contained in the fruit body show a certain motional freedom, and that the NMR method is a useful tool in biochemical research on fungal antitumor polysaccharides.

^{13}C-NMR Spectra of Hot Water-Extracted Residue (HWR), Cold Alkali-Extracted Residue (CAR) or Hot Alkali-Extracted Residue (HAR)

The spectrum of the defatted residue (DR) should contain signals of not only covalently linked but also ionically bound polysaccharides. In order to obtain the signals of covalently linked polysaccharide fraction, the spectra of HWR, CAR, and HAR fractions were measured and compared with those of extracted polysaccharide fractions under similar conditions.

As reported in the previous paper,[3a] hot water extract (HWE) contains mainly α-1,4-glucan (Fig. 2a) (anomeric carbon at 101 ppm and C-4 carbon at 78 ppm), and cold and hot alkali extracts contain mainly β-glucans (Fig. 2b, c) (anomeric carbon at 104 ppm). As shown in Fig. 2 (d, e, f), the HWR, CAR and HAR fractions showed characteristic resonances. The

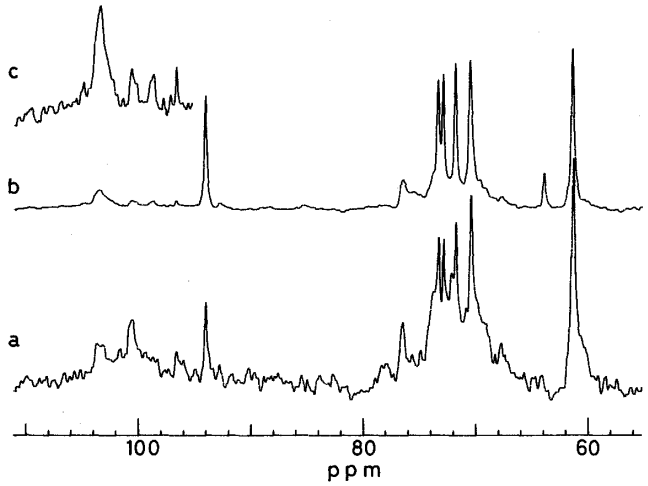

Fig. 3. ^{13}C-NMR Spectra of Fresh and Stored Fruit Bodies

a, fresh fruit body; b, after 2 d at 37 °C; c, expansion of b.

α-signals were the strongest in HWR and the weakest in HAR. The ratio of α and β glucans in these spectra is consistent with that of the extracts. Line width was the broadest in HAR. These results suggest that the covalently bound glucans also possess motional freedom in the fruit body. Such mobility should be positively correlated with the extractability of these glucans. Although more drastic extraction was not performed on HAR, the above observations suggest that the majority of glucans, even if they could not be extracted by hot alkali, possesses high motional freedom in the fruit body.

Application of ^{13}C-NMR Spectroscopy

The results described above indicate that the contents of mono-, oligo-, and polysaccharides in the mushroom can be measured within a few hours by the NMR method. We applied this method for the quality control of lots. Figure 3 shows the ^{13}C-NMR spectra of the fruit body at two different stages after harvest, immediately (a) and after 2 d at 37 °C (b). As shown in Fig. 3b, α-glucan was lowered by maintaining the mushroom at room temperature for 2 d.

Discussion

Recently, fungal immunomodulators, such as lentinan, PS-K, schizophyllan, and TAK have become important in immunopharmacology. Development of bacterial immunomodulators, such as muramylpeptides and lipid A derivatives is more advanced than in the case of fungi, mainly because less is known about the chemical-cell surface structure of fungi.

The data described in this paper indicate that natural abundance ^{13}C-NMR spectral analysis is a useful method for studying the ultrastructure of the fungal cell and cell wall. Recently, Saito *et al.* examined the ultrastructure of glucan without using any solvent by means of solid-state CP/MAS ^{13}C-NMR spectroscopy.[5] Their method should also be applicable to fungal cells and cell wall, and might be complementary with the present approach.

In 1979, it was reported that ^{15}N-NMR is useful for studying the primary structure of peptidoglycan and the amounts of teichoic acid and teichuronic acid in the bacterial cell wall, as well as the dynamic properties of the cell wall polymers.[4a] It was found that all the peptidoglycan ^{15}N peptide resonances obtained in the intact cells and isolated cell walls could be accounted for by residues in the bridge or crossbar regions of the peptide chains, and that only the cross-linking groups had a high degree of motional freedom.

On the other hand, in the case of proteoglycans in cartilage, only the glycosaminoglycan part shows NMR signals.[4b] This result suggests that the glycosaminoglycan is mobile but the

protein part has highly restricted motion possibly arising from a well-defined backbone structure. It seems that the linkage of multiple chondroitin sulfate chains to the proteoglycan core protein and the association of proteoglycan with collagen and other constituents of cartilage matrix do not significantly alter the structure and motion of these chains.

In the polysaccharide system of higher Basidiomycetes, a microfibrillar network of chitin is embedded in a matrix of amorphous glucan. In the case of the fruit body of *Lentinus edodes*, it has been reported that the majority of glucans are extractable by alkali and the alkali-unextractable core matrix is composed of three skeletal glucan parts.[7] The outer part skeletal glucan seems to be composed of mainly β-1,3- and β-1,6-glucoside linkages and has a close structural similarity to lentinan. The middle part of the skeletal glucan appears to be composed mainly of β-1,6-glucosidic linkages. The innermost part of the skeletal glucan is a highly branched glucan with β-1,6- and β-1,3-linkages. Furthermore, in the case of *Schizophyllum commune*, the lateral wall is composed of 2 layers, outer and inner.[8]

In this paper, the spectra of residues obtained by several treatments of the fruit body were compared. The resolution of the spectra of DR and HWR were better than those of CAR and HAR, indicating that the hot water- and cold alkali-extractable glucans are relatively mobile, and suggesting that the glucans in the fruit body can be classified into two groups on this basis. This type of ultrastructure is similar to those of *L. edodes* and *S. commune*. The fact that the α-1,4- and β-1,6-glucosidic linkages were extracted easily from the fuit body suggests that these types of glucans are present in the outer part of the filament.

In the case of chromatin, it is known that the mobility of desoxyribonucleic acid (DNA) is restricted by a protein, histone Hl, and the spectrum of DNA in the chromatin is scarcely observable.[4c] The fungal fruit body is known to contain about 10% of protein, whose function is uncertain. However, the result that DR and HWR show NMR signals suggests that the proteins in the fruit body are not tightly bound to the polysaccharides. Nevertheless, extraction with alkali or heat treatment would be accompanied by the breakage of peptide bonds, so the residues may not have retained the native conformation completely.

On the other hand, other fungi possess water-insoluble β-glucans, and it is known that in them, chitin (one of the water-insoluble polysaccharides, and a fungal cell wall component) shows no NMR signals. Thus there is a qualitative difference between glucans in *G. frondosa* and the other polymers described above. It appears that the extent of linkage between the cell wall backbone and the β-glucan in *G. frondosa* is relatively weak. We may speculate that the antitumor glucan is liberated from the fruit body by enzymes in the digestive organs.

Many kinds of mushrooms are cultured in Japan for food. Quality control of these mushrooms is now based on the color, *etc.* As shown in this paper, NMR analysis might provide a rapid and convenient method for quality control of edible mushrooms.

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