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Carbon-13 Nuclear Magnetic Resonance Spectroscopy of Some Edible Mushrooms

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In this paper, carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectra of some edible mushrooms, *Grifola frondosa, Lentinus edodes, Flammulina velutipes, Pholiota nameko, Agaricus bisporus, Lyophyllum cinerascens, Lyophyllum aggregatum,* and *Peziza vesiculosa,* as aqueous suspensions were measured and the glucan compositions in these mushrooms were compared. The ^{13}C-NMR spectrum of each native mushroom gave signals attributable to both low and high molecular weight carbohydrates. Low molecular weight carbohydrates were different in each mushroom. As high molecular weight carbohydrates, all the mushrooms belonging to Basidiomycotina (*G. frondosa, L. edodes, F. velutipes, P. nameko, A. bisporus, L. cinerascens,* and *L. aggregatum*) showed β-linked glucan signals. *G. frondosa,* and *P. nameko* also showed α-glucan signals. After removal of low molecular weight substances and α-glucans by refluxing with 80% ethanol, dialysis or amylase digestion, all of the spectra were quite similar and showed representative signals at 104, 85, 77, 76, 74, 70, 69, 62 ppm. On the other hand, though *P. vesiculosa,* a fungus belonging to Ascomycotina, contains antitumor β-glucans, this mushroom showed strong signals attributable to α-glucans and only weak signals attributable to β-glucans. After treatment of *G. frondosa* with periodate and borohydride, most of the β-glucan signals disappeared. These results suggest that (1) ^{13}C-NMR spectroscopy is applicable to chemotaxonomical examination of fungi, (2) all of the mushrooms belonging to Basidiomycotina contain quite similar glucans, (3) β-signals in these spectra are attributable to periodate-sensitive linkages.

Keywords——^{13}C-NMR; antitumor glucan; glucan; edible mushroom; *Peziza vesiculosa*; *Grifola frondosa*; *Lentinus edodes*

Introduction

Many kinds of mushrooms contain antitumor glucans. Extracts of these mushrooms have been used as traditional drugs for cancer therapy. It is thought that the mechanism of the activity involves activation of host immune systems.[1)] Some of these polysaccharides obtained from edible fungi are now clinically used (PSK,[1b)] lentinan,[1c)] schizophyllan[1d)]). It is considered that not only primary structure but also ultrastructure is important for the antitumor activity. It is known that many of these glucans possess oligosaccharide units consisting of partially branched β-1,3-linked glucose residues. However, the precise structures of these polysaccharides are not yet fully elucidated.

Nuclear magnetic resonance (NMR) spectroscopy is presently making significant contributions in physiology, biochemistry, and clinical diagnosis due to its ability to measure noninvasively the concentrations and the dynamics of metabolites in living tissue and to provide information on the conformations, noncovalent bonding interactions, and molecular motions of polymeric molecules. Recently, we have studied the structure and antitumor activity of a branched β-1,3-glucan obtained from the mushroom and the matted mycelium of *Grifola frondosa.*[2)] During these studies, it was found that the polysaccharides contained in the fungus could be investigated by ^{13}C-NMR spectroscopy of aqueous suspensions of the powdered fruit body.[2b)] A part of these polysaccharides appeared to show motional freedom in the mushroom. On the other hand, we obtained antitumor glucans possessing a small

degree of branching from *Peziza vesiculosa*,[3] a fungus belonging to Ascomycotina. In this paper, we deal with the application of this NMR method to some edible mushrooms belonging to Basidiomycotina and Ascomycotina.

Materials and Methods

Tested Fungi——*Grifola frondosa* (Maitake) were gifted from Nippon Beet Sugar Mfg., Co., Ltd., *Peziza vesiculosa* (O-chawantake) was obtained from local fields. *Lentinus edodes* (Shiitake), *Flammulina velutipes* (Enokidake), *Pholiota nameko* (Nameko), *Agaricus bisporus* (Mashuruum), *Lyophyllum cinerascens* (Shimeji), and *Lyophyllum aggregatum* (Honshimeji) were obtained from a vegetable store.

Preparation of the Defatted Fruit Body——Each fruit body (5 g) was suspended in 80% ethanol and the suspension was refluxed for 30 min. This procedure was repeated several times. After air-drying, the fruit body was washed with water and then lyophilized.

^{13}C-NMR Spectral Analysis——^{13}C-NMR spectra were measured in a 10 mm i.d. tube and recorded at room temperature for aqueous suspensions (several drops of D_2O were added to provide a lock signal) 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. About 300 to 500 mg of each mushroom was suspended in 3 ml of H_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; irradiation frequency (IRFRQ) 199.5 MHz.

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

Preparation of Periodate-Oxidized and Borohydride-Reduced *G. frondosa*——Amylase-digested fruit body (1.5 g) suspended in 50 mM acetate buffer pH 4.5 (200 ml) was treated with sodium metaperiodate (final conc. of 20 mM) at 4 °C in the dark for 2 d with gentle stirring. After the reaction, remaining periodate was destroyed by incubation with ethylene glycol. The resulting suspension was centrifuged and the treated fruit body was washed with water. Then, the fruit body was treated with sodium borohydride at 4 °C for 1 d and the resulting fruit body was washed with diluted acetic acid and water. The fruit body was then lyophilized.

Results

^{13}C-NMR Spectra of Native Mushrooms as Aqueous Suspensions

^{13}C-NMR spectra of native mushrooms which had been lyophilized and powdered prior to use were measured as aqueous suspensions. About 300 mg of these powders was suspended in H_2O (3 ml) using a 10 mm i.d. tube. Several drops of D_2O were added to the sampling tube to provide a lock signal. ^{13}C-NMR spectra were measured in the pulsed Fourier-transform mode with complete proton decoupling. Measurement conditions are described in Materials and Methods. Mushrooms belonging to Basidiomycotina were purchased from a vegetable store and Ascomycotina were collected from local fields. After about 5000 scans each, the spectra shown in Fig. 1 were obtained. Each spectrum gave signals attributable to both lower molecular weight carbohydrates (sharp signals; *a*) in Fig. 1) and polysaccharides (broad signals). Several signals of low molecular weight carbohydrates overlapped with those of polysaccharides. From the spectrum of *G. frondosa*, α,α-trehalose has been identified.[2b] *P. nameko*, *L. cinerascens* and *L. aggregatum* also contained α,α-treharose as a major carbohydrate. On the other hand, except for *P. nameko*, all the mushrooms belonging to Basidiomycotina contained alditols as a major carbohydrate. Further analysis to elucidate the exact structures of these alditols was not performed. However, the above assignments are consistent with the data obtained by chemical analysis.[4] The broader signals were further analyzed by using dialyzed materials as described in the next section.

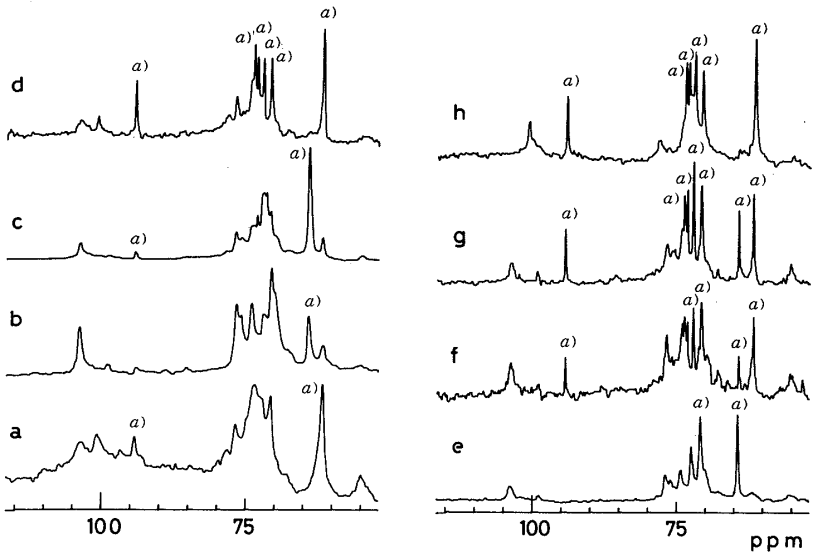

Fig. 1. ^{13}C-NMR Spectra of Lyophilized Mushrooms

a, *G. frondosa*; b, *L. edodes*; c, *F. velutipes*; d, *P. nameko*; e, *A. bisporus*; f, *L. cinerascens*; g, *L. aggregatum*; h, *P. vesiculosa*.
a) indicate signals attributable to low molecular weight carbohydrates.

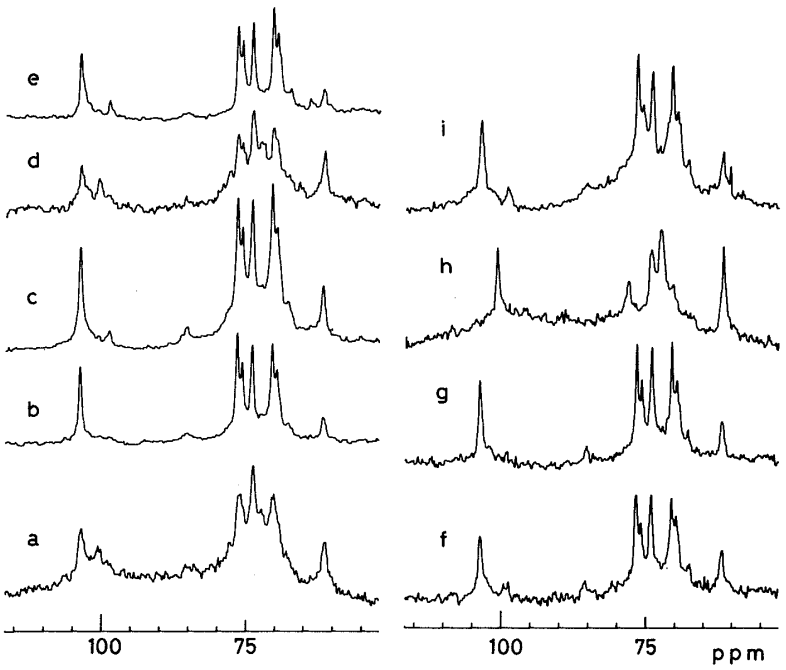

Fig. 2. ^{13}C-NMR Spectra of Defatted Mushrooms

a, *G. frondosa*; b, *L. edodes*; c, *F. velutipes*; d, *P. nameko*; e, *A. bisporus*; f, *L. cinerascens*; g, *L. aggregatum*; h, *P. vesiculosa*; i, amylases-digested *G. frondosa*.

^{13}C-NMR Spectra of Defatted and Dialyzed Mushrooms as Aqueous Suspensions

Mushrooms were defatted by refluxing with 80% ethanol and then lower molecular weight carbohydrates were removed by washing with H_2O. The ^{13}C-NMR spectra of these treated mushrooms were measured by essentially the same procedure as above (Fig. 2). In the previous study [2c] on the fruit body of *G. frondosa*, anomeric carbon signals of α- and β-glucans were assigned to 101 and 104 ppm by concomitant use of chemical analysis. Comparison of the spectra of Basidiomycotina with that of *G. frondosa* showed that all mushrooms, except for *P. nameko* (Fig. 2d), gave only β-glucan signals. *P. nameko* gave both α- and β-

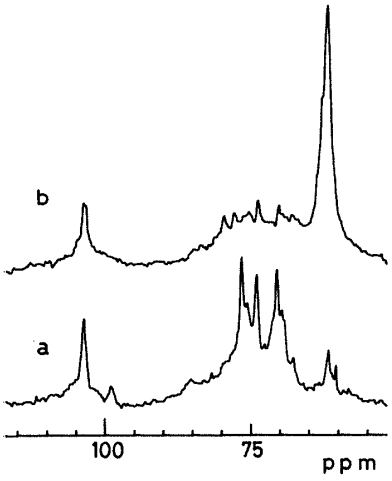

Fig. 3. ^{13}C-NMR Spectra of Periodate- and Borohydride-Treated *G. frondosa*

a, defatted *G. frondosa*; b, periodate-oxidized and borohydride-reduced *G. frondosa*.

glucan signals. After removal of α-glucan by digestion of *G. frondosa* with amylases (Fig. 2i),[2c] the spectra of all Basidiomycotina were quite similar. Representative signals were observed at 104, 85, 77, 76, 74, 70, 69, and 62 ppm. On the other hand, the fruit body of *P. vesiculosa* (which belongs to Ascomycotina) showed no β-glucan signals. Other mushrooms (*Morchella esculenta* (Amigasatake), *Rhizina undulata* (Tsuchikurage), *Galiella javanica* (O-gomutake), *Hypoxylon fragiforme* (Akakobutake)) belonging to Ascomycotina gave poorly resolved signals and also showed weak signals attributable to β-glucans (data not shown). These observations suggest that, in Basidiomycotina, β-glucan structures are quite similar to each other and that, in Ascomycotina, observable β-glucans were minor constituents.

Characterization of β-Glucans Showing NMR Signals

Recently, we reported that *P. vesiculosa* (which belongs to Ascomycotina) contains antitumor β-glucans, PVG and PVP,[3] extractable by aqueous alkali. PVG is a water-soluble β-1,3-glucan possessing one branch at C-6 of every five main chain glucosyl units.[3b] PVP is a water-insoluble β-1,3-glucan that shows little branching.[3a] Moreover, as mentioned above, this mushroom showed only weak β-glucan signals. On the other hand, *G. frondosa* contains a β-1,3-glucan possessing a branch at C-6 of every three main chain glucosyl units and a β-1,6-glucan possessing some branching (the precise structure has not been determined yet).[2d] In order to identify the origin of β-signals, periodate oxidation-borohydride reduction was performed on the fruit body of *G. frondosa*, and the treated mushrooms were analyzed by ^{13}C-NMR spectroscopy (Fig. 3). The anomeric carbon signal was not changed by the treatment (104 ppm). Most of the ring carbon signals (65 to 80 ppm) had disappeared, while signals attributable to polyol (*ca.* 60 ppm) appeared. This suggests that the β-signals at 65 to 80 ppm were attributable to periodate-sensitive residues, *i.e.*, β-1,6-glucan and branched residues of β-1,3-glucans. As shown in Fig. 2h, *P. vesiculosa* showed no β-glucan signals, though it does contain β-glucan.[3] The glucan is known to possess quite few branching points. It is assumed that the lower branching ratio accounts for the undetectability of β-glucan signals in the spectrum of *P. vesiculosa*.

Discussion

In previous papers, we described the polysaccharide composition of *G. frondosa*.[2a] *G. frondosa* contains water-soluble neutral polysaccharide (α-1,4 and α-1,6-linked glucan and branched β-1,3-glucan), and water-soluble acidic polysaccharide (β-1,6-linked glucan possessing some branching and branched β-1,3-glucan). Further, it contained water-insoluble

polysaccharide, a heteropolysaccharide composed of Glc, Xyl, Man. On the other hand, by ^{13}C-NMR spectroscopy of the aqueous suspension, it was found that a part of the polysaccharides contained in the fruit body possesses motional freedom and, therefore, showed signals in the spectrum. The spectrum of periodate-oxidized, borohydride-reduced fruit body (this paper), compared with the previous spectra, suggests that β-1,6-linked glucan and the branching points of the branched β-1,3-glucan show sharp signals, while the main chain of the branched β-1,3-glucan moiety shows weak signals. Therefore, it is suggested that β-1,6-linked glucan and the branching points of branched β-1,3-glucan have motional freedom in the fruit body.

Many antitumor glucans obtained from fungi belonging to Basidiomycotina have been investigated by many laboratories.[1] It is assumed that the primary structures of these antitumor glucans are similar,[1] though precise comparisons of the structures and the antitumor activity have not been performed. The data presented in this paper suggest that β-glucans contained in the fruit body of fungi belonging to Basidiomycotina show quite similar motional freedom in the fruit body and that the polysaccharide components of these fungi are similar. On the other hand, we showed that the antitumor β-1,3-glucan from *Peziza vesiculosa*,[3] a fungus belonging to Ascomycotina, possesses branches at every five main chain glucosyl units. The ^{13}C-NMR spectrum of this fungus is quite different from those of fungi of Basidiomycotina. The spectrum showed only weak β-glucan signals. Further, several fungi belonging to Ascomycotina also showed weak β-glucan signals (data not shown). These observations suggest that the main chain of the β-1,3-glucan has limited mobility in the fruit body of fungi of both Ascomycotina and Basidiomycotina. Furthermore, it is suggested that measurement of the ^{13}C-NMR spectra of fungi as aqueous suspensions is a useful tool to evaluate the presence of glucans with motional freedom. This method should also be useful for examining the chemotaxonomy of fungi.

It is known that the antitumor β-1,3-glucans form helical conformations and produce gel by noncovalent bonding interactions in the physiological pH range. Although polysaccharides generally show well-resolved signals in the neutral pH range, lentinan,[5] grifolan, and curdlan,[6] for example, are known to show less-resolved signals at neutral pH. This suggests that the mobility of glucans in the mushroom might be similar to that of the extracted glucans. More precise characterization of these antitumor glucans by physicochemical and biochemical methods is required, however.

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