[Chem. Pharm. Bull. 36(3)1198—1204(1988)]

Conformations of Fungal β-D-Glucans in the Fruit Body of Edible Fungi Assessed by Cross Polarization-Magic Angle Spinning Carbon-13 Nuclear Magnetic Resonance Spectroscopy

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(Received July 22, 1987)

Fungal (1→3)-β-D-glucans possess two kinds of conformations in the solid state, *i.e.*, helix (curdlan type) and native (laminaran type) (Ohno *et al.*, *Chem. Pharm. Bull.*, **34**, 2555 (1986); Saito *et al.*, *Bull. Chem. Soc. Jpn.*, **59**, 2093 (1986)). In this paper, the glucan conformations in the fruit bodies of several edible fungi were examined by using carbon-13 cross polarization-magic angle spinning (CP/MAS) nuclear magnetic resonance (NMR) spectroscopy. The fruit bodies were washed extensively with water, defatted by refluxing with ethanol, and/or treated with mata-periodate then borohydride. Most of the fruit bodies showed C-3 signals at about 86 ppm, resembling those of the native form. These findings suggested that the (1→3)-β-D-glucan conformation in the fruit body is the "native form."

Keywords——cross-polarization-magic angle spinning-NMR; edible fungus; (1→3)-β-D-glucan; antitumor glucan; conformation

(1→3)-β-D-Glucan is one of the major constituents in the cell wall of fungi. Many of these glucans have been found to show antitumor activity and some of them, lentinan and schizophyllan, are now used clinically. The ultrastructure of (1→3)-β-D-glucan is thought to be important for their antitumor activity. Recently, antitumor activity was also observed on feeding of mushroom powder. Thus, it would be interesting to know the conformation of glucans in the fruit body.

We have studied the carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectra of β-glucan in the fruit body of edible mushrooms and yeasts as aqueous suspensions.[1] Most of these glucans were sensitive to periodate-oxidation. The same method was also applicable to yeast cells, and signals from mannan and glucan were detected.

Saito *et al.* have applied cross-polarization–magic angle spinning (CP/MAS NMR) spectroscopy to determine the conformations of these glucans, and suggested that the chemical shift of C-3 is strongly affected by the torsion angles.[2] It was indicated that (1→3)-β-D-glucans possess at least 4 types of solid-state conformations: form I (curdlan type), form II (laminaran type), form III (laminarapentaose type), and form IV (dimethyl sulfoxide (DMSO)-adduct). Recently, we have studied the conformations of (1→3)-β-D-glucan fractions obtained by various preparation procedures from the fungi *Grifola frondosa* and *Sclerotinia sclerotiorum*,[3] and suggested that two kinds of conformations, native and helix, were present.[3b,c] It is also suggested that, in the liquid culture, the glucans (LLFD, LELFD) were synthesized in the native form and transformed into the helix form during the purification by treatment with urea and/or sodium hydroxide. By comparison with the spectra reported by Saito *et al.*, it appears that the helix form corresponds to form I (curdlan type) and the native form to form II (laminaran type).

Further, we applied CP/MAS ^{13}C-NMR spectroscopy to the fruit body of *Grifola frondosa* and the sclerotia of *Sclerotinia sclerotiorum*, and suggested that the conformation of (1→3)-β-D-glucan in the fruit body and in the sclerotia is the "native form."[4] In this paper, to

compare the conformation of (1→3)-β-D-glucans in various fungi, CP/MAS spectra of several fruit bodies were measured with or without treatment with periodate-borohydride.

Materials and Methods

Tested Fungi——*Grifola frondosa* (maitake) was a gift from Nippon Beet Sugar Mfg. Co., Ltd., *Peziza vesiculosa* (o-chawantake), *Scutellinia scutellata* (kuriiro-chawantake), *Morhella esculenta* (amigasatake), *Geliella javanica* (o-gomutake), *Rhizina undulata* (tsuchikurage), *Hypoxylon fragiforme* (akakobutake), *Grifola umbellate* (chyorei) were obtained from local fields. *Lentinus edodes* (shiitake), *Flammulina velutipes* (enokidake), *Pholiota nameko* (nameko), *Agaricus bisporus* (mushroom), *Pleurotus ostreatus* (hiratake) and *Lyophyllum ulmarium* (shirotamogitake) were obtained from a vegetable store.

Other Methods——Preparation of the defatted and periodate-oxidized, borohydride-reduced fruit body[1] and ^{13}C-NMR spectroscopy[3,4] were performed by the methods discribed previously.

Results and Discussion

^{13}C-CP/MAS spectra of several fungi belonging to Basidiomycotina and Ascomycotina, and a yeast, are shown in Figs. 1—3. Figures 1 and 3 show the defatted mushroom and Fig. 2 shows the spectra of some of the mushrooms after periodate oxidation and borohydride reduction. As described in the previous paper, many kinds of edible mushrooms contained β-glucans having both high and low segmental mobility, and the former was suggested to correspond to (1→6)-β-D-glucan and the latter to (1→3)-β-D-glucan, because several mushrooms showed quite similar ^{13}C-NMR spectra and the signals of *G. frondosa* disappeared on treatment with sodium metaperiodate.[1] Figure 4 shows the spectra of periodate-oxidized, borohydride-reduced (I/B) mushrooms as aqueous suspensions. All the spectra (Fig. 4a—g) were similar to the spectrum of I/B-treated *G. frondosa*,[1] suggesting the removal of highly mobile β-glucan, (1→6)-β-D-glucan, from these mushrooms.

Figure 5f and g show the CP/MAS spectra of LELFD and grifolan, respectively, as reference spectra.[3] Signals at around 86 ppm (in LELFD) suggest the "native conformation" and those at around 90 ppm (in grifolan) the "helix conformation." In this study, CP/MAS spectra of 16 species of fungi were measured and the spectral data are summarized in Table I in groups based on the C-3 shifts and the facts described below. Groups A, B, and C have the chemical shift of C-3 at around 86 ppm, 90 ppm, and no representative peak, respectively. Thirteen of the 16 species gave signals at around 86 ppm. Only yeast gave signals at around 90 ppm.

Figures 1g and 2g show the spectra of *L. edodes*. The spectrum of the I/B-treated mushroom showed a clearer C-3 signal than the parent mushroom, and the signal appears at 85 ppm, as in *G. frondosa*. *L. edodes* is known to contain lentinan and other skeletal (1→3)(1→6)-β-D-glucans.[5] To examine the conformation more precisely, the fruit body of *L.*

TABLE I. Classification of Edible Fungi by the Chemical Shift of Carbon-3 in CP/MAS ^{13}C-NMR Spectroscopy

Group	Name
A	*Flammulina velutipes*, *Lyophyllum ulmarium*, *Pholiota nameko*, *Pleurotus ostreatus*, *Lentinus edodes*, *Grifola umbellata*, *Grifola frondosa*, *Galiella javanica*, *Hypoxylon fragiforme*, *Morchella esculenta*, *Peziza vesiculosa*, zymosan, *Saccharomyces cerevisiae*
B	Zymosan, *Saccharomyces cerevisiae*
C	*Agaricus bisporus*, *Rhizina undulata*, *Scutellinia scutellata*

A, Carbon-3 signal near 86 ppm; B, carbon-3 signal near 90 ppm; C, carbon-3 signal was not clearly identified.

edodes was extracted successively with hot water, cold alkali, and hot alkali as done in the case of *G. frondosa*,[3a] and polysaccharide fractions, L-HWE, L-CAE, and L-HAE, were obtained. The results of methylation (data not shown) and ^{13}C-NMR spectroscopy (Fig. 6), indicated that each fraction contained (1→3)-β-D-glucan, because every spectrum in 0.2 N sodium hydroxide showed signals at 86 ppm, attributable to substituted C-3 (Fig. 6a—c). Further, the ^{13}C-NMR spectrum of L-CAE in DMSO-d_6 (Fig. 6d) suggests that the primary structure of CAE is similar to that of grifolan (Fig. 6e).[6] Figure 5c—e shows the CP/MAS spectra of these glucans. Carbon-3 of HWE showed a broad signal and those of CAE and HAE appeared at 90 ppm, suggesting the conformations of CAE and HAE to be helix form. Further, the CP/MAS spectrum of lentinan was reported to be that of a curdlan form.[2] These results suggested that (1→3)-β-D-glucan in the fruit body of *L. edodes* possesses native conformation, and the conformation was transformed to helix form during the extraction and purification. Lentinan is a glucan purified from a hot water extract, but it also showed helix conformation, whereas the fungus possessed native conformation. These differences probably resulted from the use of alkaline conditions during purification of lentinan, which might induce the gel-to-sol transition of (1→3)-β-D-glucans. A similar observation was reported in the case of *G. frondosa*.[3]

P. ostreatus,[7] *F. velutipes*,[8] *P. nameko*,[9] *G. umbellata*,[10] and *L. ulmarium*,[11] are mushrooms showing antitumor activity and are known to contain (1→3)-β-D-glucans. HA-glucan (*P. ostreatus*) was reported to have native form.[7] Figure 1 (a, c—f) shows the CP/MAS spectra of the parent mushrooms. *P. ostreatus*, *F. velutipes*, *P. nameko*, *G. umbellata*, and *L. ulmarium* showed the C-3 signal at 86 ppm, suggesting the conformation of the glucans in these mushrooms to be native form. In the case of I/B-treated mushrooms (Fig. 2(c—f)), peak signals appeared below 86 ppm. Previously, we suggested that the signal at 54 ppm was attributable to the C-2 carbon of chitin,[4] and the C-4 carbon of chitin appeared at 82 ppm. Considering the intensity of signal at 54 ppm, all of the mushroom contained chitin to some extent. The resolution of signals in CP/MAS spectra is not high, and the C-3 signal of glucan and the C-4 signal of chitin were not clearly resolved. Chitin also showed signals at *ca.* 175 ppm (carbonyl) and at *ca.* 25 ppm (acetylmethyl). These signals appear in all the spectra shown in Figs. 1 and 2. However, they may not reflect the exact content of chitin, because these two regions would contain signals from other macromolecules, such as proteins and nucleic acids.

Figures 1b and 2b show the CP/MAS spectra of the defatted and the I/B-treated *A. bisporus*. In this fungus, the signal at about 86 ppm was quite weak compared with the signal at about 82 ppm, which is attributable to chitin, as described above. CP/MAS spectra of this fungus also show a stronger signal at 54 ppm than those of other mushrooms, suggesting a high chitin content in the mushroom; Michalenko *et al.* examined the composition of mycelial wall of *A bisporus* and suggested that the walll consisted of 43%(w/w) chitin, 14% KOH soluble glucan and 27% β-glucan which contained (1→3)-β-D-glucanase digestable linkages.[12a] Their chitin content is consistent with the result presented in this paper. The antitumor activity of this mushroom has been to be low,[12b] supporting the low (1→3)-β-D-glucan content.

Comparing the spectra in Figs. 1 and 2, the sharpest C-3 signals were observed in the case of *G. frondosa*. Some of the spectra show only broad signals and some show a signal overlapping with the C-4 carbon of chitin. The signal width in the CP/MAS spectrum would indicate the variation of the torsion angles: small variation results in a narrow signal. Thus, it is suggested that the torsion angle of (1→3)-β-D-glucan in *G. frondosa* is strictly regulated.

Fungi of Ascomycotina contain many antitumor (1→3)-β-D-glucans, *e.g.* scleroglucan,[13] PS-1426,[14] SSG,[3c] PVG,[15] and CO-1.[16] In the previous papers, we have reported that the conformation of SSG, which is secreted into the culture medium of *Sclerotinia sclerotiorum*

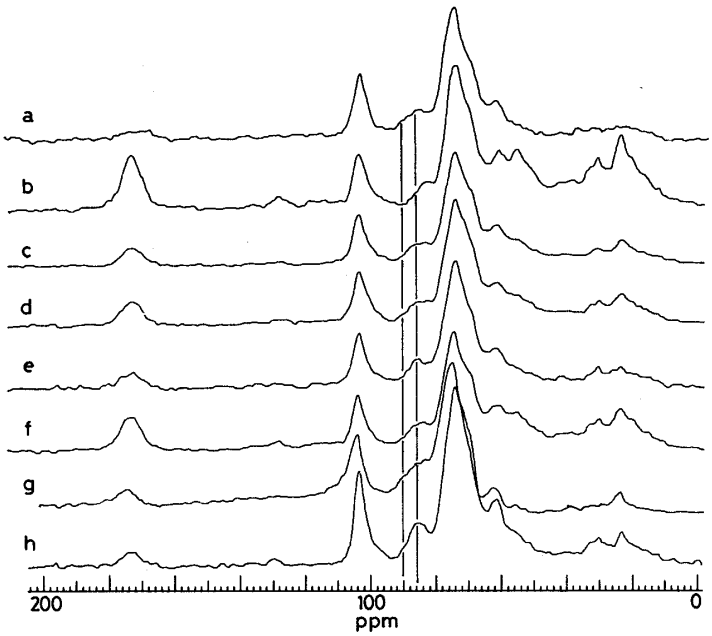

Fig. 1. CP/MAS ^{13}C-NMR Spectra of the Defatted Fungi

a, *G. umbellata* (sclerotia); b, *A. bisporus* (fruit body); c, *F. velutipes* (fruit body); d, *L. ulmarium* (fruit body); e, *P. nameko* (fruit body); f, *P. ostreatus* (fruit body); g, *L. edodes* (fruit body); h, *G. frondosa* (amylase treated fruit body).

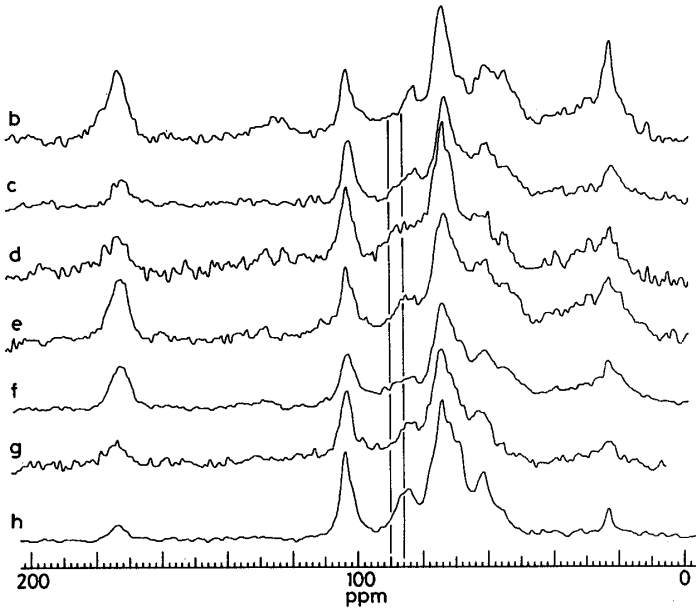

Fig. 2. CP/MAS ^{13}C-NMR Spectra of the Periodate-Oxidized, Borohydride-Reduced Fruit Body

b—h, these letters indicate the same kind of fungi as in Fig. 1.

IFO 9395, may be native, and was transformed to helix by various drastic treatments. Further, the sclerotia of *S. sclerotiorum* contained several (1→3)-β-D-glucans and these glucans showed native conformations.[17] It is speculated that the (1→3)-β-D-glucans in Ascomycotina are also present in the native form, similar to the glucans from Basidiomycotina. Figure 3 shows the spectra of *G. javanica*, *R. undulata*, *H. fragiforme*, *M. esculenta*, *S. scutellata*, and *P. vesiculosa*, as well as the skeletal components of yeast (zymosan) and a yeast cell (*S. cerevisiae*), all of which belong to Ascomycotina. Except for Fig. 3b and e, all spectra show signals attributable to the native form of (1→3)-β-D-glucan. The C-3 signal of *G. javanica* (Fig. 3a) was the narrowest.

Previously, it was found that the cold alkaline extracts of *P. vesiculosa* contained two kinds of antitumor (1→3)-β-D-glucans, PVP and PVG, which have a branch at C-6 of every eight and every five main chain glucosyl units, respectively.[15] PVG was purified from PVS. The CP/MAS spectra of PVP and PVS are shown in Fig. 5a and b. PVP shows the C-3 signal

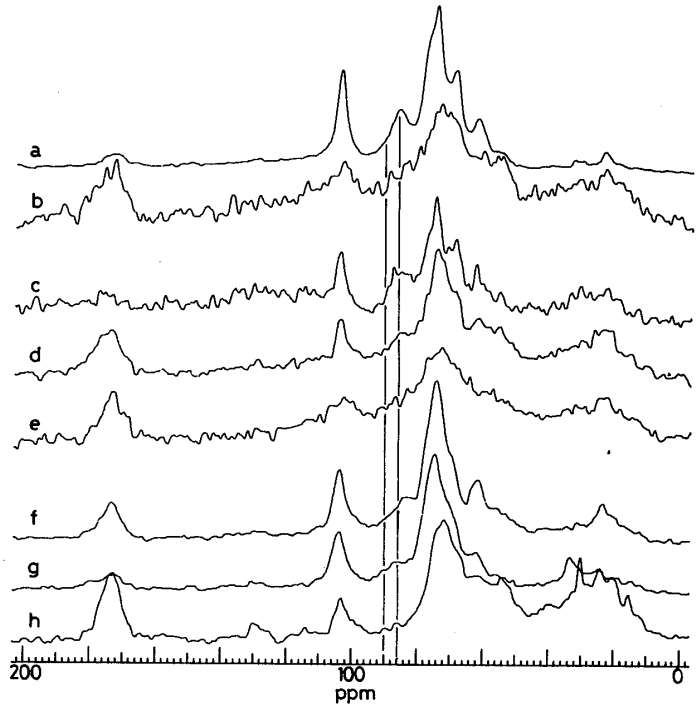

Fig. 3. CP/MAS ^{13}C-NMR Spectra of the Defatted Fungi

a, *G. javanica* (fruit body); b, *R. undulata* (fruit body); c, *H. fragiforme* (fruit body); d, *M. esculenta* (fruit body); e, *S. scutellata* (fruit body); f, *P. vesiculosa* (fruit body); g, zymosan; h, *S. cerevisiae*.

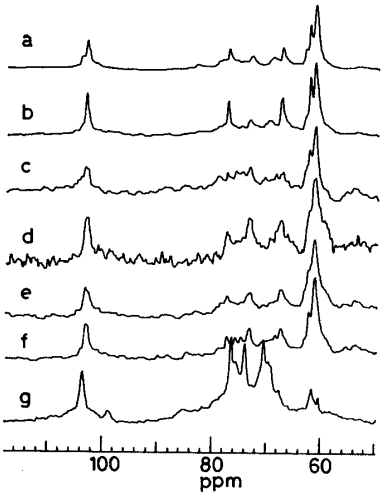

Fig. 4. ^{13}C-NMR Spectra of Periodate-Oxidized, Borohydride-Reduced Mushrooms as Aqueous Suspensions

a, *F. velutipes*; b, *L. edodes*; c, *L. ulmarium*; d, *A. bisporus*; e, *P. nameko*; f, *P. ostreatus*; g, amylase-treated *G. frondosa* (before I/B treatment, as a control).

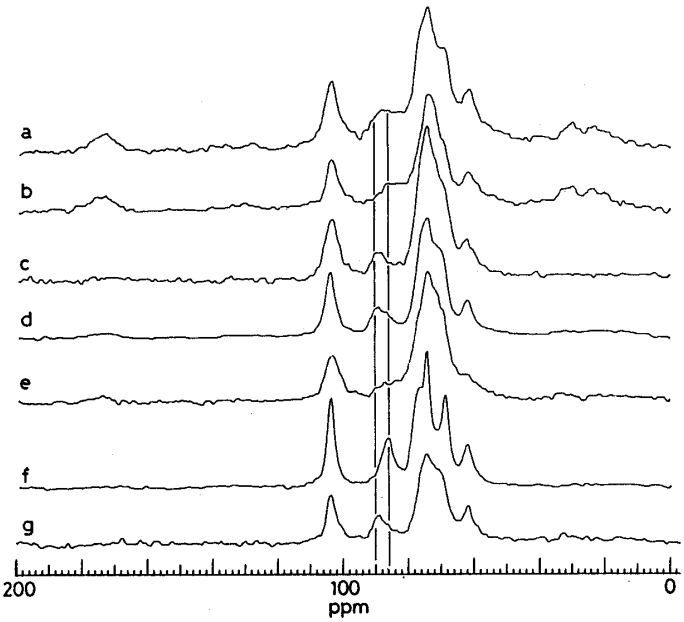

Fig. 5. CP/MAS ^{13}C-NMR Spectra of (1→3)-β-D-Glucan Fractions

a, PVP from *P. vesiculosa*; b, PVS from *P. vesiculosa*; c, L-HAE from *L. edodes*; d, L-CAE from *L. edodes*; e, L-HWE from *L. edodes*; f, LELFD from *G. frondosa*; g, grifolan NMF-5N from *G. frondosa*. Chemical shifts of the carbon signals of the reference compounds were as follows. LELFD: C-1, 103.4 ppm; C-2, 74.2 ppm; C-3, 86.3 ppm; C-4, 69.0 ppm; C-5, 76.4 ppm; C-6, 62.4 ppm. Grifolan NMF-5N: C-1, 103.9 ppm; C-2, 74.5 ppm; C-3, 89.2 ppm; C-4, 70.1 ppm; C-5, 74.5 ppm; C-6, 61.6 ppm.

at 88 ppm and PVS shows it at 86 ppm. It is suggested that PVP possesses more helical conformation than PVS. The result for the defatted fruit body suggests that these glucans are also synthesized as the native form (peak at 84 ppm). Chemical characterization of other fungi belonging to Ascomycotina has not been done, but the spectra shown in this paper strongly suggested the presence of the native form of (1→3)-β-D-glucan. The spectra of Fig. 3b and e

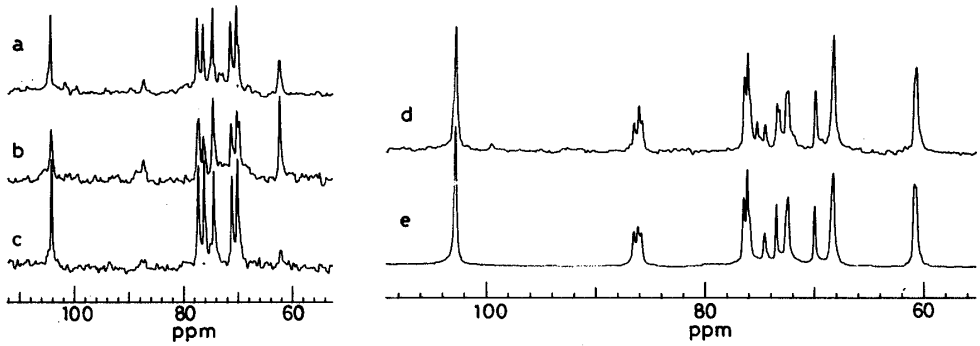

Fig. 6. ^{13}C-NMR Spectra of Glucan Fractions in 0.2 N Sodium Hydroxide or DMSO-d_6

a, L-HWE in 0.2 N NaOH; b, L-CAE in 0.2 N NaOH; c, L-HAE in 0.2 N NaOH; d, L-CAE in DMSO-d_6; e, grifolan NMF-5N in DMSO-d_6.

have quite low resolution, so the conformation of glucans could not be defined.

Figure 3 g and h shows the spectra of *Saccharomyces cerevisiae* and zymosan, which is a skeletal component of *S. cerevisiae*. It is known that the yeast cell contains a complicated framework of (1→3)-β-D-glucans.[18] In contrast to the other fungi, both showed signals at 86 and 90 ppm in the C-3 region. It is assumed that the yeast cell contains both native and helix conformers of (1→3)-β-D-glucans and the conformation and the primary structure of the yeast (1→3)-β-D-glucan are not similar to those of other (1→3)-β-D-glucans obtained from the culture broth, mushroom and sclerotia of mushrooms.

Conclusion

This paper deals with the solid state conformation of (1→3)-β-D-glucans in various mushroom, sclerotia, or a yeast. It was found that: (1) most of the (1→3)-β-D-glucans (13 in 16 species) possess native conformations, and (2) the (1→3)-β-D-glucan in *S. cerevisiae* possesses both native and helix conformations.

Recently, it was found that antitumor (1→3)-β-D-glucans possess more than two conformers in the solid state.[2,3] For example, lentinan possesses curdlan form and HA-glucan possesses laminaran form. Further, LELFD, obtained from *G. frondosa*, and SSG from *S. sclerotiorum*, possess both conformers in ratios that depend on the purification processess used. We speculate that all fungal (1→3)-β-D-glucans, except for those from yeasts, possess "native" conformation *in situ*. Because the helix form of (1→3)-β-D-glucans such as LELFD and SSG is induced after denaturation of the native form, in which the gel network of (1→3)-β-D-glucan strands is once degraded to sol and then regenerated to the gel,[19] it seems probable that the glucan strands of the native form are synthesized in parallel, possibly directly as a triple helix, since such a conformation could not easily be formed after the completion of the chain synthesis. On the other hand, in the case of the skeletal glucans of *L. edodes*, there are at least three types of the (1→3)-β-D-glucans, whose differences include the ratio of branching points.[5] Further, each species of fungus possesses (1→3)-β-D-glucans having characteristic properties; *e. g.* the branching of SSG,[20] grifolan,[6] and PVG[15] amounts to 1/2, 1/3 and 1/5, respectively. It is reasonable to think that the biosynthesis of the main chain moiety of fungal (1→3)-β-D-glucans proceeds through quite similar mechanisms while that of the branching points proceeds under different conditions in each fungus. In the case of yeast, the biosynthesis of the cell wall proceeds in parallel with degradation of the cell wall. Thus, as in the bacterial cell wall, the structure of the (1→3)-β-D-glucan might be quite

complicated, and might include different conformers.[18] It would be worth investigating the regulation of (1→3)-β-D-glucan biosynthesis and the control mechanism of (1→3)-β-D-glucan conformation.

Acknowledgments The authors thank Mr. K. Nunomura for collecting fungi and Miss M. Yamanokuchi for technical assistance.

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