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Research Article

Solid-State ^{13}C CP MAS NMR Spectroscopy as a Tool for Detection of (1 → 3, 1 → 6)-β-D-Glucan in Products Prepared from *Pleurotus ostreatus*

O. Fričová and M. Kovaľaková

Department of Physics, Faculty of Electrical Engineering and Informatics, Technical University of Košice, Park Komenského 2, 042 00 Košice, Slovakia

Correspondence should be addressed to O. Fričová; olga.fricova@tuke.sk

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The ^{13}C CP/MAS NMR solid-state NMR technique was used to detect the presence of β-D-glucan and trace compounds in samples prepared from dried, naturally grown oyster mushroom (*Pleurotus ostreatus*) and commercially available products of dried, specially cultivated oyster mushroom and β-D-glucan isolated from this mushroom. The NMR spectra of all samples displayed signals typical for (1 → 3, 1 → 6)-β-D-glucan; however, signals which could be assigned to other trace compounds—(1 → 3)-α-glucan, chitin, and proteins—were also observed in the spectra. The amount of trace compounds was negligible in the commercially available products.

1. Introduction

Oyster mushroom (*Pleurotus ostreatus*) is highly fancied due to its content of biologically active substances. Among the most notable metabolites extracted from this edible mushroom are polysaccharides belonging mainly to β- and α-D-glucans, which are important regarding their immunomodulating, antitumor, and tissue-healing abilities [1–7]. The content of cholesterol-lowering agents—chitin and chitosan—increases the nutritional value of this medical mushroom [8, 9].

β-D-Glucans are polysaccharides composed of D-glucose monomers linked by β-glycosidic bonds, and they are intensively investigated owing to their anticancer efficacy [1–3, 5, 8, 10–19]. Research has shown that insoluble (1 → 3, 1 → 6)-β-D-glucan (Figure 1) has greater biological activity than its soluble (1 → 3, 1 → 4)-β-D-glucan counterparts. The differences between β-D-glucan linkages and chemical structure are significant in terms of solubility, mode of action, and overall biological activity [1]. The most active forms of (1 → 3)-β-D-glucans are those containing side chains in positions 1 and 6. Activity of β-D-glucans depends on molecular weight, degree of branching, and structure of the β-D-glucans molecule. The highest anti-cancer effect is achieved by β-D-glucans with the degree of branching between 0.20 and 0.33 and high molecular weight [2]. β-D-Glucan isolated from *Pleurotus ostreatus*—pleuran—belongs to this group of β-D-glucans. The content of β-D-glucans in dried *Pleurotus ostreatus* ranges between 24 and 38% wt. [9]. Pleuran has a (1 → 3)-β-D-glucan backbone with (1 → 6)-linked residues. It may contain a small proportion of interior (1 → 6)- and (1 → 4)-linked residues [7, 20].

NMR spectroscopy is considered to be a unique method for the study of structure and molecular dynamics in solids. Modern solid-state NMR techniques such as magic angle spinning (MAS) and cross-polarisation (CP) enable conformation study of solid materials [21]. According to our knowledge, only a few papers [8, 10, 12, 16, 17] deal with NMR study of β-D-glucans in the solid state, despite the fact that solid-state NMR spectroscopy is a nondestructive technique that preserves the 3D structure of the analyzed polymer [16, 21]. Measured ^{13}C CP/MAS NMR spectra of

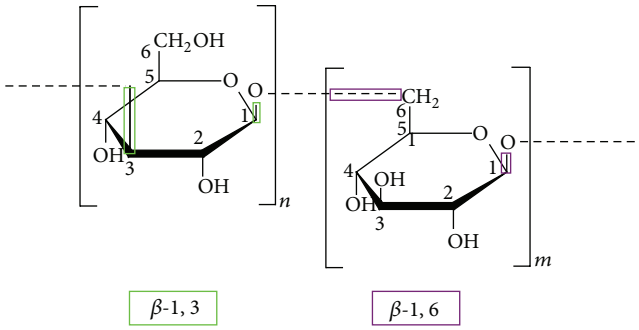

Figure 1: Structure of $(1 \rightarrow 3, 1 \rightarrow 6)$-$\beta$-D-glucan.

powdered $(1 \rightarrow 3, 1 \rightarrow 6)$-$\beta$-D-glucan isolated from *Pleurotus ostreatus* (pleuran) are consistent with those published in [8, 11]. The presence of compounds in the sample of $(1 \rightarrow 3, 1 \rightarrow 6)$-$\beta$-D-glucan can be recognized by resonance signals outside the range of 50–110 ppm. As signals not coming from β-D-glucan are present in the measured ^{13}C CP/MAS NMR spectrum, another two samples of *Pleurotus ostreatus*—a dietary supplement sold in pharmacies and a natural sample—were also studied.

Hydration of polysaccharides gives rise to conformational stabilization which is reflected in spectra by narrowing and splitting of resonance lines [12, 17, 22, 23]. Hence, ^{13}C CP/MAS NMR experiments were also performed on a hydrated sample of $(1 \rightarrow 3, 1 \rightarrow 6)$-$\beta$-D-glucan isolated from *Pleurotus ostreatus*.

2. Materials and Methods

The sample S1 of $(1 \rightarrow 3, 1 \rightarrow 6)$-$\beta$-D-glucan isolated from *Pleurotus ostreatus* and the sample S2 powdered *Pleurotus ostreatus* are commercial products sold in pharmacies as dietary supplements. The cultivation of *Pleurotus ostreatus* for their production is specially controlled. To achieve better resolution of the NMR spectrum the sample S1 was hydrated for a week placed on a glass dish in a desiccator with water. The sample S3 was prepared from dried and ground natural edible mushroom *Pleurotus ostreatus*.

The solid-state ^{13}C CP/MAS NMR measurements were carried out on the 400 MHz Varian solid-state NMR spectrometer in the Centre for Solid-State NMR Spectroscopy of the Slovak National NMR Centre at the Technical University of Košice. The high-resolution ^{13}C CP/MAS NMR spectra were recorded at the resonance frequency of approximately 100 MHz with the use of 4 mm rotors and MAS frequency of 12000 Hz and $\pi/2$ pulse duration of 1.9 μs. In the CP experiments the Hartmann-Hahn condition was achieved with the radio frequency field strength of 58 kHz, a contact time of 1 ms, and relaxation delay of 4 s between two consecutive scans. A high-power proton-decoupling field of 92 kHz was applied during data acquisition. The spectra were obtained at room temperature averaging over 5000–33000 scans.

The chemical shifts were referenced to the TMS using adamantane as an external standard.

3. Results and Discussion

The measured ^{13}C CP/MAS NMR spectrum of sample S1 (β-$(1 \rightarrow 3, 1 \rightarrow 6)$-D-glucan isolated from *Pleurotus ostreatus*) is depicted in Figure 2(a). The peak at 103.6 ppm corresponds to C1 carbon in the β-glycosidic bond, while the broad signal at 86.1 ppm relates to $(1 \rightarrow 3)$-linked residues of C3. The signals of C5, C2, and C4 are superimposed in the range of 65–80 ppm, where signals of $(1 \rightarrow 6)$-linked C3 and C6 carbons are usually observed. C6 carbon in $(1 \rightarrow 3)$- and in $(1 \rightarrow 6)$-linkage gives signals at 62.4 and 69.3 ppm, respectively [8, 11, 18, 19]. The peak position of $(1 \rightarrow 3)$-linked residues of C3 at 86.1 ppm indicates a triple helix conformation of β-D-glucan chains [12, 16]. The relatively large width of this peak may be caused by the presence of different allomorphs in the sample [10, 12, 16].

Besides the signals related to the carbons of $(1 \rightarrow 3, 1 \rightarrow 6)$-$\beta$-D-glucan, additional peaks were observed in the ^{13}C CP/MAS NMR spectrum (Figure 2(a)). The signals near 175 ppm (C=O), 56 ppm (C2), and 23 ppm (CH_3) may indicate the presence of chitin [8, 24–27]. A broad peak of carbonyl group (near 175 ppm) and peaks of aliphatic carbons (near 33 ppm) might indicate the presence of some proteins; however, no signal in the aromatic region (120–140 ppm) was observed [8, 28, 29]. Hence, we assume that these signals came from traces of compounds remaining after isolation of $(1 \rightarrow 3, 1 \rightarrow 6)$-$\beta$-D-glucan from *Pleurotus ostreatus*. A certain asymmetry of the peak of C1 carbon can be explained by the overlapping of two peaks with close chemical shifts [8, 11–17] corresponding to C1 carbon in $(1 \rightarrow 3)$- and $(1 \rightarrow 6)$-linkages, although C1 carbon in $(1 \rightarrow 4)$-linked residues of β-D-glucan produces a signal near 102 ppm [7, 13, 15, 20]. Moreover, the peak of C1 carbon of chitin is also positioned near 104 ppm [24–27]. Another reason for the asymmetry might be the presence of $(1 \rightarrow 3)$-α-glucan [7, 8, 22]. To confirm this assumption the ^{13}C CP/MAS NMR spectrum was measured for hydrated sample S1H, as better resolved spectra were observed for hydrated polysaccharides [12, 17, 23].

The ^{13}C CP/MAS NMR spectrum of hydrated $(1 \rightarrow 3, 1 \rightarrow 6)$-$\beta$-D-glucan isolated from *Pleurotus ostreatus* (sample S1H) is depicted in Figure 2(b). In comparison to the spectrum of dry sample S1, the peaks in the spectrum of S1H are narrower and split, and some of them slightly change their position as a consequence of conformational changes under hydration. The presence of $(1 \rightarrow 3)$-α-glucan is confirmed by the right-hand side shoulder near 101 ppm of the C1 signal of $(1 \rightarrow 3, 1 \rightarrow 6)$-$\beta$-D-glucan coming from C1 carbons of $(1 \rightarrow 3)$-α-glucan [7, 8, 22]. The small signal near 83 ppm may arise from C3 carbons $(1 \rightarrow 3)$-α-glucan [7, 8, 22], although the C4 carbons of chitin also resonate near the same chemical shift [24–27]. The signal near 80 ppm is typical for C4 carbons of $(1 \rightarrow 4)$-linked residues of β-D-glucans [7, 13, 15, 20]. To interpret the part of the ^{13}C CP/MAS NMR spectrum in the range of 65–79 ppm is rather complicated as in this interval of chemical shifts the signals of C5, C2, and C4 and of $(1 \rightarrow 6)$-linked C3 and C6 carbons of β-$(1 \rightarrow 3, 1 \rightarrow 6)$-D-glucan [7, 8, 11, 18–20], the peaks of C3 and C5 carbons of chitin [24–27], and the signals of C2-5 carbons of α-glucan [7, 8, 22] are observed.

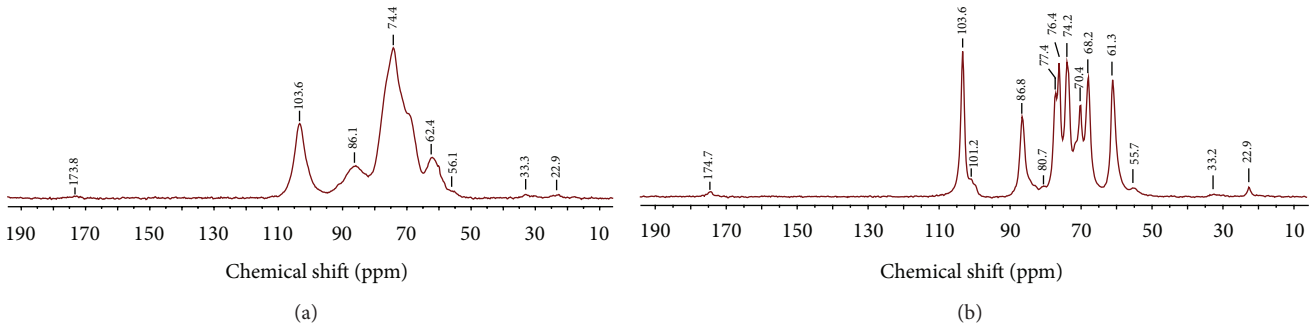

Figure 2: ^{13}C CP/MAS NMR spectrum of β-D-glucan isolated from *Pleurotus ostreatus*: dry sample S1 (a), and hydrated sample S1H (b).

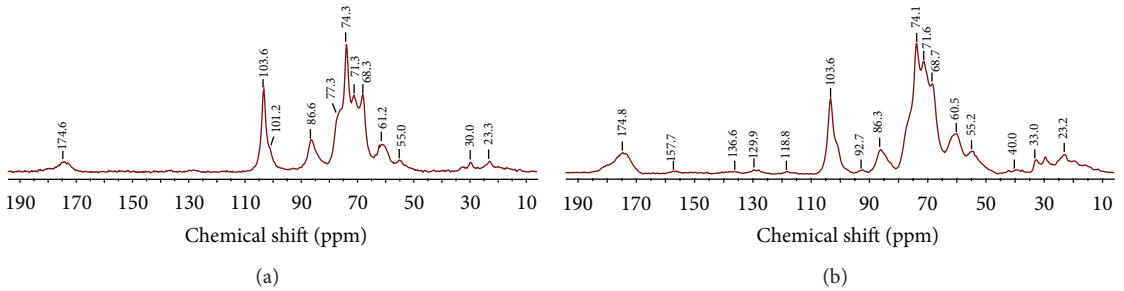

Figure 3: ^{13}C CP/MAS NMR spectrum of *Pleurotus ostreatus*: dietary supplement S2 (a), and natural sample S3 (b).

To prove the purity of sample S1 the ^{13}C CP/MAS NMR spectrum of sample S2 (a dietary supplement sold in pharmacies) was measured (Figure 3(a)). Signals of β-(1 → 3, 1 → 6)-D-glucan (C1 at 103.6 ppm, (1 → 3)-linked C3 at 86 ppm, and (1 → 3)-linked C6 at 62 ppm) [7, 8, 11, 20] and chitin (C=O at 175 ppm, C2 near 55 ppm, and CH_3 at 23 ppm) [24–27] can be clearly identified in this spectrum. The shoulder observed at the right-hand side of the peak at 103.6 ppm is associated with C1 carbons of (1 → 3)-α-glucan [7, 8, 22]. Other carbons of chitin [24–27], (1 → 3)-α-glucan [7, 8, 22], and β-(1 → 3, 1 → 6)-D-glucan [8, 11, 18, 19] produce peaks in a tangle of signals between 65 and 80 ppm. The presence of proteins is confirmed by the broad peak of carbonyl group (near 175 ppm) and peaks of aliphatic carbons (near 30 ppm), and, in contrast to the spectrum of sample S1, by minor signals in the aromatic region (120–140 ppm) [8, 28, 29]. Some protein carbons also resonate in the range of 50–90 ppm [28, 29]. In addition, the weak signal near 93 ppm might be attributed to aldoses or some oligosaccharides [13].

The ^{13}C CP/MAS NMR spectrum of natural *Pleurotus ostreatus* (sample S3) (Figure 3(b)) has similar features to that of sample S2 (Figure 3(a)), except that the signals outside the range of 45–110 ppm, in which signals of β-(1 → 3, 1 → 6)-D-glucan are observed, and the signal near 93 ppm are enhanced. Additional minor broad peaks with chemical shifts near 158, 119, and 40 ppm also appear in the spectrum of sample S3, revealing the presence of other compounds not mentioned previously. This means that in the sample of natural *Pleurotus ostreatus* the content of β-(1 → 3, 1 → 6)-D-glucan is lower than that in the sample of *Pleurotus ostreatus* specially cultivated for the production of food supplements.

4. Conclusions

Powder samples of oyster mushrooms and of (1 → 3, 1 → 6)-β-D-glucan isolated from *Pleurotus ostreatus* were studied using ^{13}C CP/MAS NMR. The NMR spectrum of (1 → 3, 1 → 6)-β-D-glucan revealed the occurrence of some other compounds in the sample; the asymmetry of the C1 peak can be caused by the presence of (1 → 3)-α-glucan. Resonance lines of chitin and other compounds containing carbonyl and aliphatic carbons remaining after isolation were also observed in the spectrum. Traces of some proteins appeared in the sample of *Pleurotus ostreatus* specially cultivated for the production of food supplements. The sample of natural *Pleurotus ostreatus* contained some other compounds in addition to those present in the cultivated sample. Lower content of (1 → 3, 1 → 6)-β-D-glucan in the naturally grown mushroom than in the cultivated one was inferred from the shape of the ^{13}C CP/MAS NMR spectra.

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