A ^{13}C NUCLEAR MAGNETIC RESONANCE STUDY OF A GEL-FORMING BRANCHED (1→3)-β-D-GLUCAN, A_3, FROM *PLEUROTUS OSTREATUS* (Fr.) QUÉL: DETERMINATION OF SIDE-CHAINS AND CONFORMATION OF THE POLYMER-CHAIN IN RELATION TO GEL-STRUCTURE

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1. Introduction

In recent years, a number of gel-forming branched (1→3)-β-D-glucans with anti-tumor activity were isolated from various natural sources [1–3]. Evidence was presented that conformation of polysaccharide is closely related to its biological activity [4]. Further, conformational studies of polysaccharides in relation to gel-structure are of considerable importance in view of their biological functions [5,6]. However, conformational behaviour of neutral polysaccharides, especially of (1→3)-β-D-glucans, has not been fully investigated in comparison with that of polypeptides and polynucleotides.

In this paper, we demonstrate that ^{13}C n.m.r. measurements of polysaccharide A_3 from *P. ostreatus* in the gel state are able to provide insight into structure and conformation.

2. Experimental

The macromolecular polysaccharide fraction A_3 was obtained from the fruit bodies of *P. ostreatus* (an edible mushroom) by hot-water extraction, ethanol precipitation and ultrafiltration with a Diaflo pM-30 membrane and purified with exclusion of acidic fractions by adsorption on DEAE-Sephadex [2].

^{13}C n.m.r. spectra were obtained on a JEOL PFT-100/EC-100 pulsed Fourier-transform spectrometer. ^{13}C chemical shifts were expressed in parts per million downfield from external tetramethylsilane. Samples were contained in 10 mm o.d. tubes (100 mg/ml). Spin-lattice relaxation times (T_1's) were obtained using the pulse-sequence of 180°–t-90° [7]. Nuclear Overhauser effects [NOE's] were obtained from the ratio of the intensity of fully decoupled spectra to the intensity of spectra in which the proton noise-decoupler was gated off to remove the NOE [8].

Results and discussion

3.1. *Determination of the side-chains and branch points*

The assignments of ^{13}C spectra of A_3 taken in DMSO-d_6 (fig.1) and in alkaline solution, pD 13.7, (fig.2c) are straightforward if account is taken of its β-D-(1→3)- and α-D-(1→4)- linked glucose residues [9], except for the two additional peaks marked as C-5′ and C-6′. The presence of the latter glucose residues is obvious in the ^{13}C spectra of the gel state at neutral pD (fig.2a), though in DMSO and alkaline solutions most of the signals due to α-D-(1→4)- linkages are buried in the intense β-D-(1→3)- linkage signals. The complete loss of the β-D-(1→3)- signals in the gel state suggests that these linkages are the backbone taking on an ordered conformation which immobilizes the segmental motion such that the dipolar field from the neighbouring proton is not averaged out. These results indicate that A_3 is a (1→3)-β-D-glucan branched with α-D-(1→4)- linked glucose residues as the side-chains in disordered conformation. A g.l.c. analysis

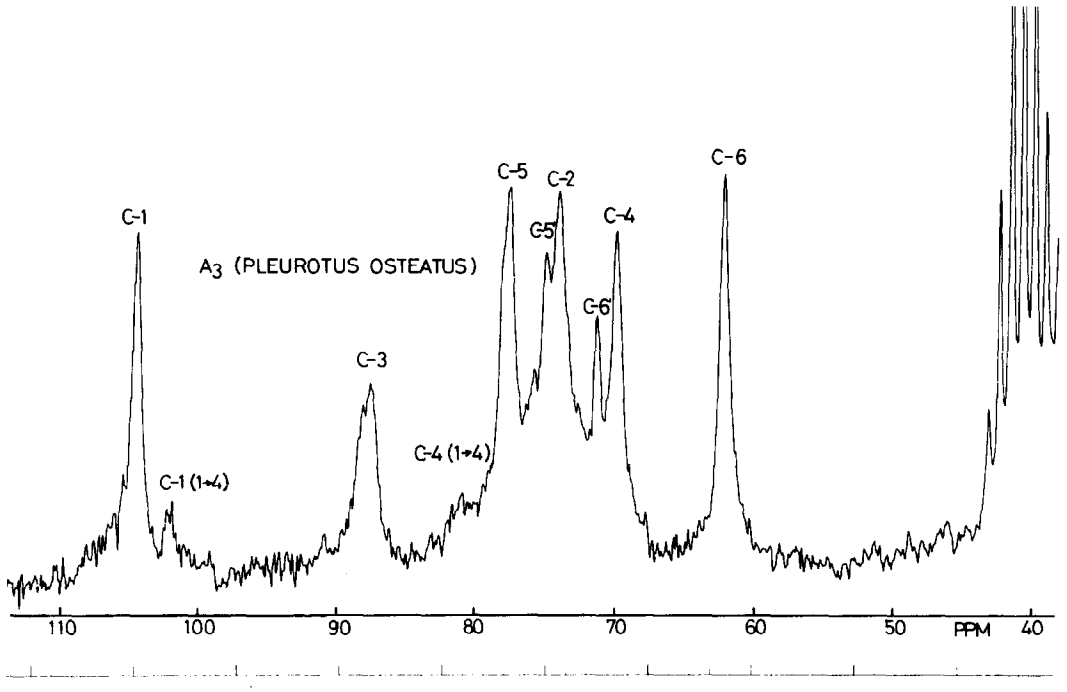

Fig.1. ^{13}C n.m.r. spectrum of A_3 in DMSO-d_6 solution (90° pulse, repetition time 0.6 sec, 40 000 accumulations).

of the methanolysis product of permethylated A_3 is consistent with this conclusion owing to the presence of methyl 2,3,4,6-tetra-*O*-methyl glucoside, methyl 2,3,6-tri-*O*-methyl glucoside, methyl 2,4,6-tri-*O*-methyl glucoside, and methyl 2,4-di-*O*-methyl glucoside (1:3:3:0.6) [10]. The observation of the dimethylate shows that the side-chains are branched at C-6 (hereafter described as C-6′) of the backbone β-D-(1→3)-linkages. The distance between the branch points of the backbone chain is estimated as 3–5 units. However, the peak-intensities of the side-chains are unexpectedly low (30% of the total) with respect to the backbone, in contrast to the g.l.c. result and ^{13}C n.m.r. spectrum of permethylated A_3 (50% of the total).

It is expected that ^{13}C signals of C-6′ and C-5′ (in the branch points) are much influenced by glucosidic-bond formation at C-6′, whereas other signals, C-1′–C-4′, are not. These chemical-shift displacements, C-6′ and C-5′ with respect to C-6 and C-5, respectively, might be estimated from ^{13}C chemical-shift data of 6-*O*-methylglucopyranose [11] and *O*-methylated inositols [12]. Thus, two peaks of fig.1, C-6′ (9.2 ppm downfield from C-6) and C-5′ (2.5 ppm upfield from C-5), are clearly assigned to C-6′ and C-5′ carbons at the branch points. The NOE value of C-6′, 1.4 in DMSO, is much smaller than that of C-6, 1.9 (table 1). The smaller NOE value at C-6′ is explained by the reduced mobility due to glucosidic bond formation. The change of NT_1 values, 196 msec (C-6′) to 100 msec (C-6), is similarly explained, since the T_1 values are shown in the low-temperature side as proved by the decreases of those at 15 MHz (table 1) [13].

3.2. *Conformation and gel-structure*

In view of the widest line-width detectable by high-resolution n.m.r. as approx. 1000 Hz, the correlation time of the backbone motion in the gel is roughly estimated to be much longer than 10^{-6} sec [13]. A multiple-stranded helix, proposed by computer model building [14] as well as in the solid-state conformation of xylan [15], is the most likely conformation to account for such immobility of the backbone. From the aspect of gelation mechanism, the multiple-stranded helix is capable of forming the junction-zone for interchain connection to make an 'infinite' net-

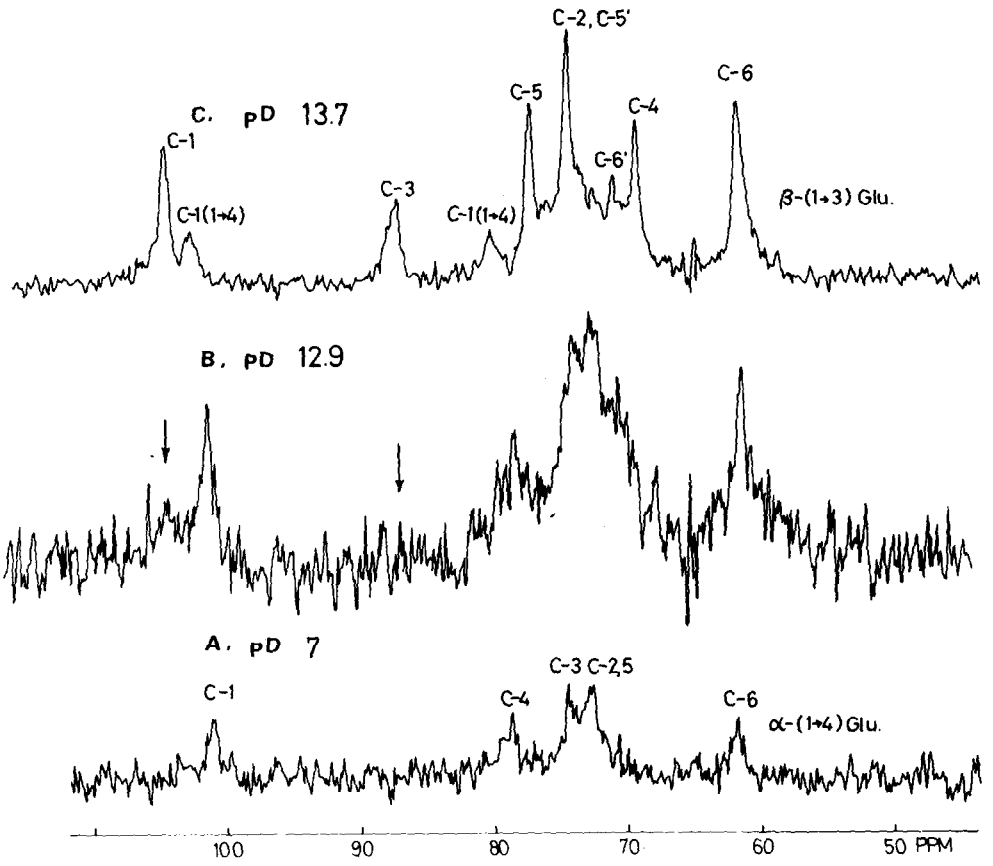

Fig.2. ^{13}C n.m.r. spectra of A_3 in the gel states (A and B) and in homogeneous alkaline solution (C). (A) pD 7, 34 000 accumulations, (B) pD 12.9, 43 000 accumulations, (C) pD 13.7, 36 500 accumulations.

Table 1
Spin-lattice relaxation times (NT_1 values in msec), nuclear Overhauser effects (NOE values) and line-widths (in Hz) of A_3 in the gel state and DMSO solution

	Gel state[a] Side-chain (α-D-(1→4))			DMSO-d_6 solution Backbone (β-D-(1→3))			Side-chain (α-D-(1→4)
	NT_1	NOE	Line-width	NT_1	NOE	Line-width	Line-width
C-1	76	1.4	18	95 (63)[b]	1.5	22	28
C-2	78	1.4	34	91 (49)	1.4		
C-3	78	1.3	29	71 (44)	1.6	42	
C-4	78	1.2	34	84 (44)	1.4	31	∿ 94
C-5	78	1.4	34	97 (55)	1.5	33	
C-6	112	1.6	20	100 (68)	1.9	22	
C-5'[c]				89	1.3		
C-6'[c]				196	1.4		

[a] ^{13}C peaks due to the backbone are completely lost (line-width>1000 Hz).
[b] T_1's measured at 15 MHz (by a JEOL FX-60 spectrometer).
[c] Peaks due to the branching unit.

work. Similar loss of the peak-areas was also reported to occur in the double-helical polynucleotides [16] and ι-carageenan [17], and triple-helical collagen [18]. At the intermediate of the helix-coil transition induced by addition of NaOH (at pD 12.9), very broad C-1 and C-3 signals of the backbones are partially seen, as marked by the arrows (fig.2b). Those signals could be ascribed to the single-helical backbone partially 'melted'. It is found that this transition is reversible.

The line-width, T_1 and NOE values of the side-chain signals in the gel state are very similar to those of the disordered conformation of the backbone in DMSO solution (table 1). The correlation time of the side-chain motion is estimated as 2×10^{-8} sec from the NT_1 values (low-temperature side) and the line widths, whereas 5×10^{-9} sec is obtained from the NOE values. These values imply that the side-chains in the gel reorient at least a hundred times as rapidly as does the backbone. Nevertheless, the possibility of inter-side-chain connection may not be ruled out. Instead, it is reasonable to assume that the side-chains participate partially in the connection, as anticipated from the sizable peak-loss of the side-chains in DMSO and alkaline solution (fig.1 and fig.2c).

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