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Note

Gel-to-sol transition of ^{13}C-labeled (1 → 3)-β-D-glucan, ^{13}C-SSG, assessed by ^{13}C nuclear magnetic resonance (^{13}C-NMR) spectroscopy

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It has been known that several (1 → 3)-β-D-glucans possess immunomodulating activities including antitumor effects [1]. These glucans have been included as members of a group of biological response modifiers (BRM) and two of them, lentinan (LTN; from *Lentinus edodes*) [2] and schizophyllan (SPG; from *Schizophyllum commune*) [3], have been applied clinically in cancer patients in Japan.

It is known that ^{13}C-NMR spectroscopy is useful for analyzing the primary- and ultra-structure of (1 → 3)-β-D-glucans [4–6]. However, natural abundance spectra required high concentrations of sample and time for measurement. In addition, ^{13}C-NMR spectra of these glucans were measured in Me_2SO solutions in which glucans form a sol conformer resulting in disappearance of signals under physiological conditions [7,8]. The (1 → 3)-β-D-glucosyl residues produce a stable triple helical structure which strongly contributes to the gel formation. A part of the main chain moiety can also exist as a single helical structure, the ratio being dependent on the various solvent conditions [9–11].

SSG is a (1 → 6)-β-D-monoglucosyl branched (1 → 3)-β-D-glucan obtained from the liquid-cultured broth of a fungus, *Sclerotinia sclerotiorum* IFO 9395 [8,12], belonging to the *Ascomycotina*, and possessing immunomodulating and antitumor activities [13–16].

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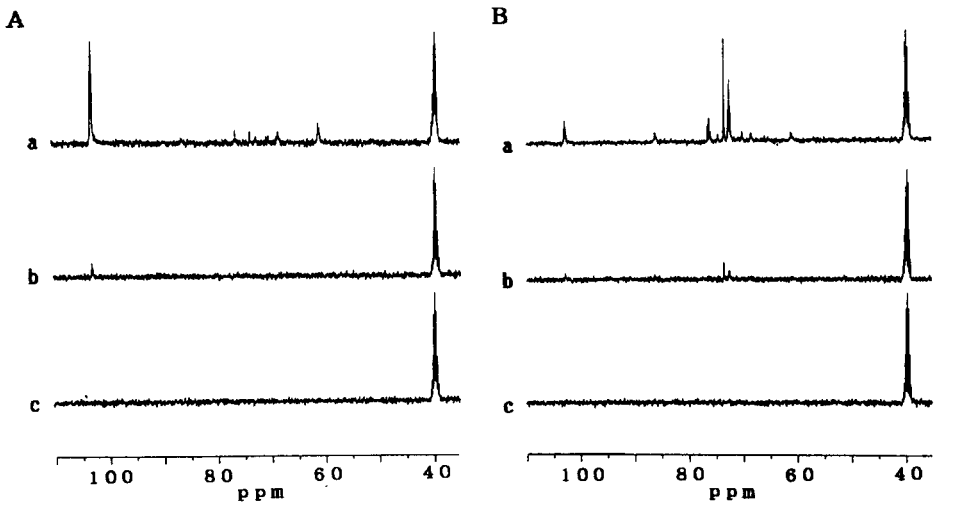

Fig. 1. Relationships between ^{13}C-signal intensities and concentration of ^{13}C-SSGs in Me_2SO-d_6. Each concentration of ^{13}C-SSG was dissolved in Me_2SO-d_6, and ^{13}C-NMR spectra were measured at 60°C. All spectra were obtained from 2000 scans. (A) [1-^{13}C]-SSG, (B) [2-^{13}C]-SSG. a, 10 mg mL^{-1}; b, 1 mg mL^{-1}; c, 0.1 mg mL^{-1}.

The number of branching points in SSG is greater than that in LTN and SPG (the ratios of branching points to main chain glucosyl residues in SSG, LTN and SPG are 1:2, 2:5, and 1:3, respectively). From the view point of structure-activity relationships, too high or too low a ratio of branching points abrogates BRM activities. Thus, physicochemical examination of both main chain to side chain ratios would be of value.

Previously, we reported the preparation of metabolically ^{13}C-labeled SSG (^{13}C-SSG) [17]. In the report, we show that the ^{13}C-label was not equally distributed in all the positions of carbon using D-[1-^{13}C]- or D-[2-^{13}C]-glucose as ^{13}C source. However, the ^{13}C-NMR spectra of ^{13}C-SSGs had sufficient signal intensities obtained within 30 min accumulation time to enable examination of the ^{13}C-NMR spectroscopic properties, and sol-to-gel transitions of ^{13}C-SSG at low concentration.

1. Results and discussion

Relationship between ^{13}C-signal intensity and concentration of ^{13}C-SSG.—In the case of natural abundance ^{13}C-NMR spectra of (1 → 3)-β-D-glucan, a high concentration of sample and much time are required to obtain well resolved spectra, but these are substantially reduced by metabolic enrichment of ^{13}C-nuclei [17]. The relationship between ^{13}C-signal intensity and concentration of ^{13}C-SSG was examined, as shown in Fig. 1, in which all spectra were obtained from 2000 scans. ^{13}C-signal intensities of both [1-^{13}C]-SSG (**1A**) and [2-^{13}C]-SSG (**1B**) were decreased in a concentration dependent manner. At 1 mg mL^{-1} (physiological concentration) well-resolved spectra could be obtained when measured with at least 10 000 scans.

Sol-to-gel transition.—One of the characteristic properties of antitumor (1 → 3)-β-D-glucan is the formation of a gel structure under physiological conditions. Since this gel-to-sol transition is reversible, neutralization of alkali solution reconstruct the gel from the sol structure. To examine whether this phenomenon could be observed by using ^{13}C-SSGs, ^{13}C-NMR spectra of ^{13}C-SSGs in various concentrations of alkali (sodium hydroxide) solution were measured. As shown in Fig. 2A ([1-^{13}C]-SSG) and 2B ([2-^{13}C]-SSG), the deteriorations of S/N ratio (signal/noise ratio) were observed at 0.15 M in both spectra, and at 0.05 M, it became difficult to distinguish between signal and noise. Under neutral conditions, no signals were observed in either spectra.

Secondly, ^{13}C-NMR spectra of ^{13}C-SSGs in Me_2SO-d_6 solution containing various concentrations of water were measured. As shown in Fig. 3A ([1-^{13}C]-SSG) and 3B ([2-^{13}C]-SSG), at about 17% of water both signals of ^{13}C-SSGs disappeared. These

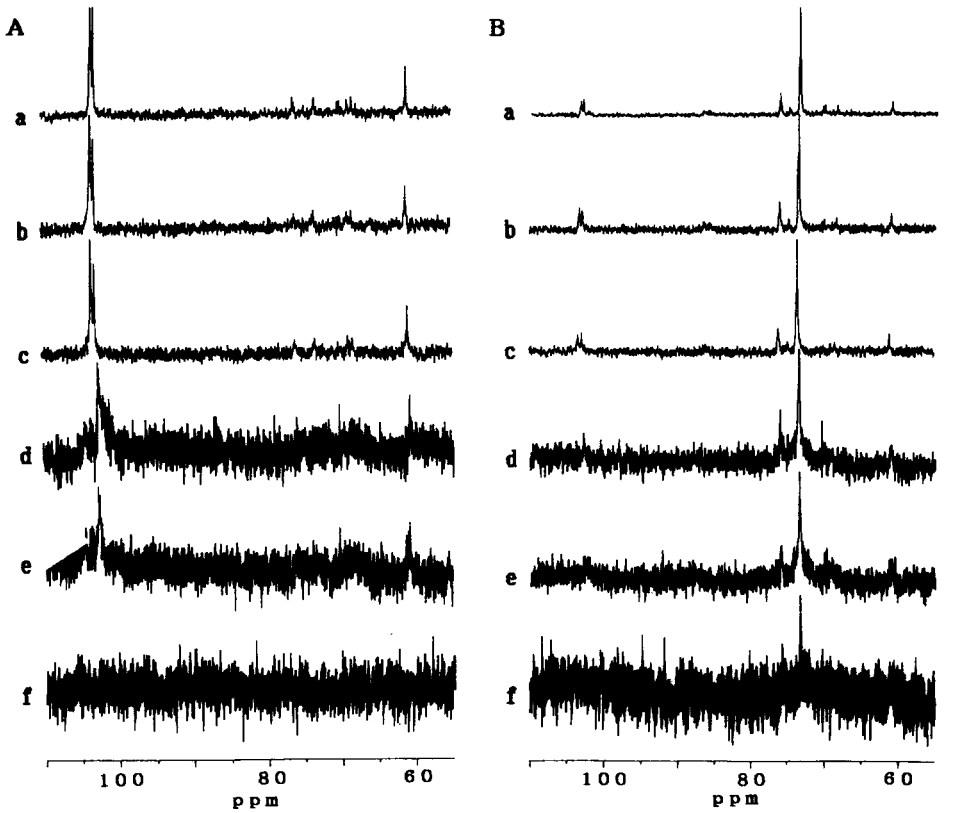

Fig. 2. Sol-to-gel transitions of ^{13}C-SSGs in sodium hydroxide solution measured by ^{13}C-NMR spectroscopy. To the solution of [1-^{13}C] and [2-^{13}C]-SSGs (10 mg) dissolved in 0.3 N of sodium hydroxide (1 mL) appropriate amounts of hydrochrolic acid solution (6 N) was added to give the indicated normality, and ^{13}C-NMR spectra were measured at room temperature. All spectra were obtained from 2000 scans. (A) [1-^{13}C]-SSG, (B) [2-^{13}C]-SSG. a, 0.30 M of sodium hydroxide; b, 0.25 M; c, 0.20 M; d, 0.15 M; e, 0.10 M; f, 0.05 M.

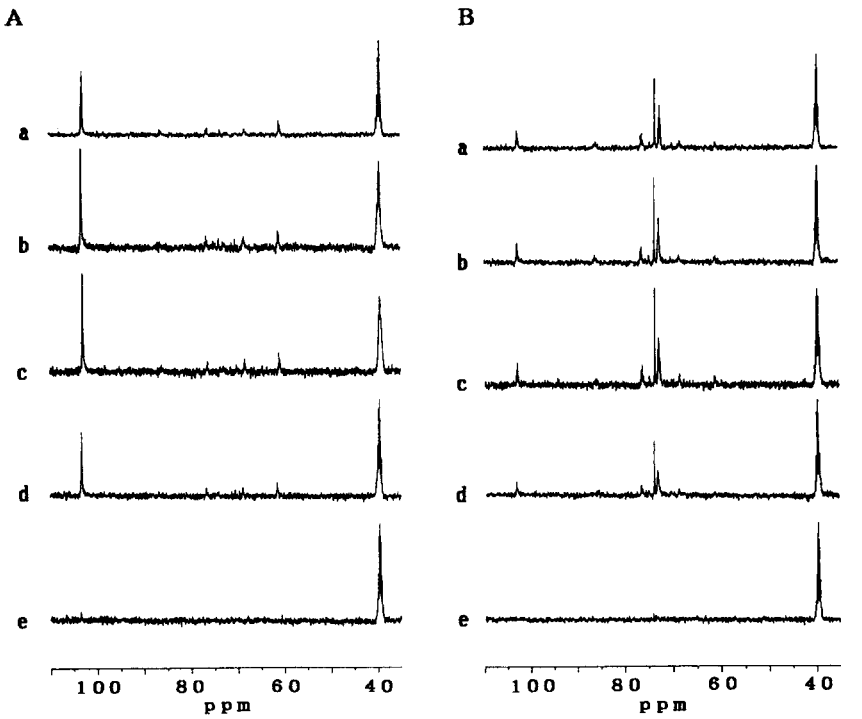

Fig. 3. Sol-to-gel transitions of ^{13}C-SSGs in Me_2SO-d_6 by adding water assessed by ^{13}C-NMR spectroscopy. To the solution of [1-^{13}C] and [2-^{13}C]-SSGs (10 mg) dissolved in Me_2SO-d_6 (1 mL) distilled water was added to give the indicated normality, and ^{13}C-NMR spectra were measured at 60°C. All spectra were obtained from 2000 scans. The solvent signal appears at 39.5 ppm as a multiplet. (A) [1-^{13}C]-SSG, (B) [2-^{13}C]-SSG, a, 0% of water; b, 5%; c, 9%; d, 13%; e, 17%.

results are consistent with previously reported natural abundance ^{13}C-NMR [7,8,18], and suggested that ^{13}C-SSGs have the identical property to unlabeled SSG. This is thought to be important to apply to the biochemical analyses.

Sol-to-gel transition at low concentrations.—The formation of intra- and inter-molecular hydrogen bonds play important roles in gel structure which is reduced at low concentrations of SSG. Figure 4 shows spectra of ^{13}C-SSGs in Me_2SO-d_6 obtained on adding various concentrations of water. Most of the signals became difficult to distinguish from noise at around 20% of water which suggested that most of the glucan segment was changed from sol to gel, independent of the concentration of glucans. In the spectrum of [1-^{13}C]-SSG, shown in Fig. 4A, however, a weak signal at 103 ppm (corresponding to C-1s of both the main chain and branch), was still observed at 23% of water (Fig. 4Af). To precisely compare the disappearance of signals in high and low concentration of SSG, signal intensities of major carbons were plotted (Fig. 5). Figure 5A and 5B show the signal intensities in high or low concentration of samples, respectively, which shows a slower loss of signals in low concentrations which might

reflect inter-molecular hydrogen bonding interactions.

Signals for C-2 of main chain and branch glycosyl units showed different ^{13}C-chemical shifts, which disappeared at the same concentration (Fig. 5). This result suggested that even though the side chain glucosyl unit does not directly contribute to the formation of triple helical conformation, loss of the mobility of the main chain significantly influences the physicochemical state of the side chain glucose.

2. Experimental

Materials.—D-[1-^{13}C] and D-[2-^{13}C]-Glucose were purchased from Cambridge Isotope Laboratories, Woburn, Massachusetts.

Preparation of ^{13}C-SSG.—Sclerotinia sclerotiorum IFO 9395 was grown in the medium (25 mL) containing yeast extract (0.3%), polypepton (1.0%), unlabeled glucose (1.6%) and ^{13}C-glucose (0.4%) with shaking at 25°C for 4 days. After the mycelia and broth were separated by centrifugation, the broth was mixed with 1 vol. of EtOH, and fibrous products collected. The product was washed with 50% EtOH, and dissolved in 8 M urea. The resulting solution was applied to the columns of DEAE Sephadex A-25(Cl^-) and SP Sephadex C-25(Na^+). The eluate was dialyzed against tap and distilled water, and precipitated by EtOH.

^{13}C-NMR studies.—^{13}C-NMR spectra (100.6 MHz) of ^{13}C-SSG were recorded at 60°C for solutions in Me_2SO-d_6, or at room temperature for alkali solutions, with a BRUKER AM-400 spectrometer. The spectra were obtained in the pulsed FT mode with complete proton decoupling. The spectra were obtained from 2000 to 10000 scans with a 655-ms pulse interval.

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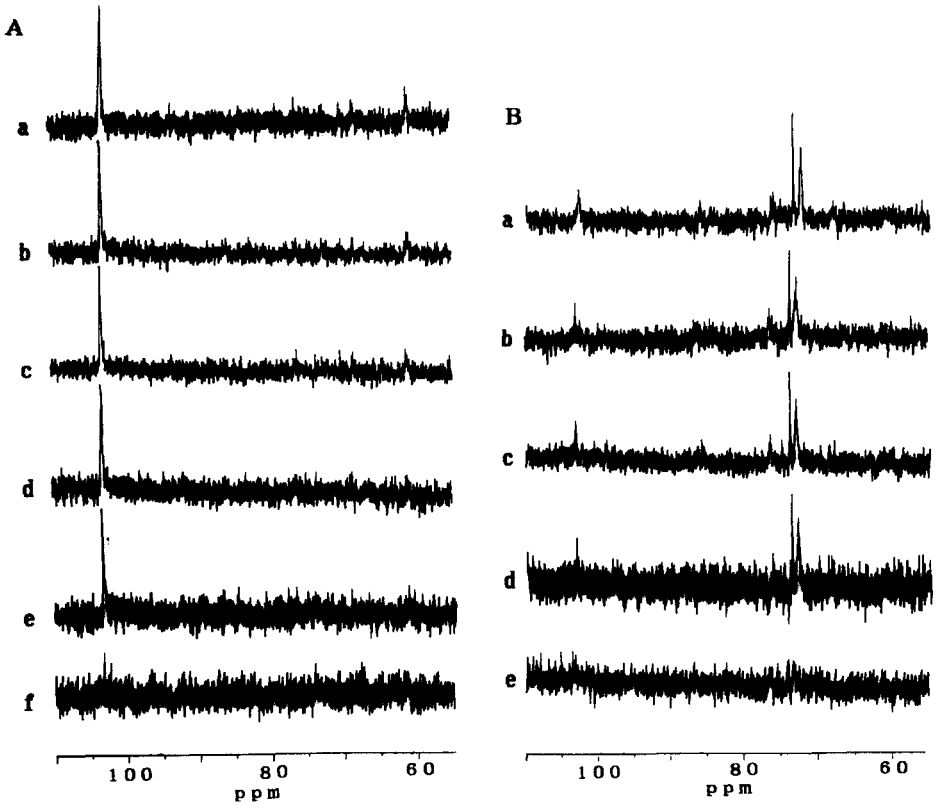

Fig. 4. Sol-to-gel transitions of low concentration of ^{13}C-SSGs in Me_2SO-d_6 by adding water assessed by ^{13}C-NMR spectroscopy. To the solution of [1-^{13}C] and [2-^{13}C]-SSGs (1 mg) dissolved in Me_2SO-d_6 (1 mL) distilled water was added to give the indicated normality. Following protocols were similar to Fig. 4 except for that all spectra were obtained from 10 000 scans. (A) [1-^{13}C]-SSG, (B) [2-^{13}C]-SSG. a, 0% of water; b, 9%; c, 13%; d, 16%; e, 20%; f, 23%.

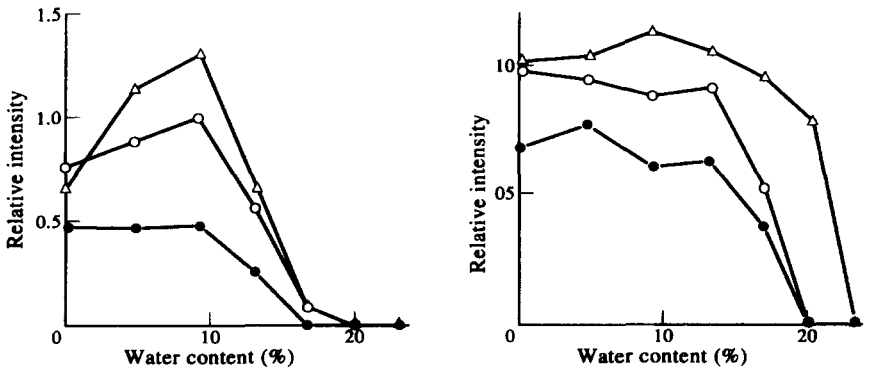

Fig. 5. Changes of relative signal intensities with water content. Each value is shown as the intensities of ^{13}C-signal of ^{13}C-SSGs relative to the signal of solvent, Me_2SO-d_6. (A) 10 mg mL^{-1} of ^{13}C-SSGs as shown in Fig. 3, (B) 1 mg mL^{-1} of ^{13}C-SSGs as shown in Fig. 4. △, C-1s of [1-^{13}C]-SSG (103ppm); ○, C-2 of [2-^{13}C]-SSG (73.5 ppm, branch); ●; C-2s of [2-^{13}C]-SSG (72.5 ppm, main chain).

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