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THERMAL DENATURATION OF (1→3)-β-D-GLUCANS IN NEUTRAL AQUEOUS SOLUTION ABOVE 130°: EFFECT ON PHYSICOCHEMICAL PROPERTIES

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ABSTRACT

The gel-to-sol transition and degradation of aqueous solutions of (1→3)-β-D-glucans above 100° has been investigated. (1→6)-Branched (1→3)-β-D-glucans (LELFD and SSG) changed from gel to sol at temperatures >130°, as assessed by ^{13}C-n.m.r. spectroscopy. Curdlan, a linear (1→3)-β-D-glucan, remained a gel after heat treatment at 150°. Decreases in molecular weight dependent on the heating time were observed in the case of branched glucans or chemically modified, soluble curdlan (HE-curdlan). The soluble fractions obtained from linear glucans heated for 12 h at 150° were 13.7 and 49.2% for curdlan and debranched LELFD (SD-LE-I/B), respectively. HE-curdlan was denatured by heating in a similar fashion to the branched glucan. The LELFD helix, which has a similar conformation to curdlan in the solid state, was altered to the native form, which is the same as that observed for schizophyllan as assessed by c.p.–m.a.s. (cross-polarization, magic-angle spinning) ^{13}C-n.m.r. These results suggested that side chains or substituted groups of the (1→3)-β-D-glucan are important in gel-to-sol transition above 130° and that long-term heating gradually degraded these glucans into small fragments.

INTRODUCTION

Such immunomodulating activities as antitumor effects of fungal (1→3)-β-D-glucans are well documented[1]. From the results of several physicochemical and pharmacological investigations, it has been suggested that such antitumor activities are closely related to the (1→3)-β-D-glucosyl chain and the molecular weight[2]. A common property of these glucans is low solubility in water because of gel formation. However, low-viscosity glucans are required for clinical use.

Recent studies by Saito *et al.*[3] classified the ultrastructure of (1→3)-β-D-glucans into four types of solid conformations: form I (curdlan type), form II (schizophyllan type), form III (laminarapentaose type), and form IV (dimethyl

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sulfoxide adduct). From a combined study using solid state, c.p.–m.a.s., ^{13}C-n.m.r., and X-ray analysis, it was proposed that forms I and II possess mainly a single-helix and triple-helix, respectively. Lentinan, paramylon, and curdlan are form I, and schizophyllan and scleroglucan are form II, in view of the characteristic ^{13}C-n.m.r. peak-positions. We found that the (1→6)-branched (1→3)-β-D-glucan, grifolan LE, which had been purified from an extracellular polysaccharide mixture (LELFD) produced from liquid-cultured mycelium of *Grifola frondosa*, has potent antitumor activity[4]. Examination of its physicochemical properties using c.p.–m.a.s. ^{13}C-n.m.r. spectroscopy showed that LELFD had native (form II) and helix (form I) conformations, and grifolan LE was form I. It was also demonstrated that the transition from native to helix is induced by dissolution in 8M urea followed by dialysis and lyophilization[5].

It has been shown previously that the conformation of the glucan affected the outcome of formolysis, and the native conformer was more resistant than the helical form[6]. Thus, formolysis of the native conformer was not applicable as a method for the production of soluble glucans. Yanaki *et al.* showed[7] that triple-helical (1→3)-β-D-glucan (SPG) changed conformation in water to single chains when the temperature was increased to >135°. A preliminary examination revealed alteration of the physicochemical properties of the LELFD-helix by heat treatment, and it was observed that the molecular weights of heat-treated products were lower than those of the starting material. These findings suggested the possibility that heat treatment might be a useful method for solubilization and depolymerization of branched (1→3)-β-D-glucans. We report here on the structural factors which affect the depolymerization by heat treatment and on physicochemical changes in the products.

EXPERIMENTAL

Materials. — Curdlan, sodium metaperiodate, sodium borohydride, and the glucose B test kit were purchased from Wako (Osaka, Japan).

Heat treatment of glucans. — Samples were suspended in distilled water (2.5 mg/mL, 10 mL) and heated at temperatures from 100–150° using a glass tube with a screw cap in an aluminum-block heater.

Preparation of SD-LE-I/B. — The polysaccharide LELFD[4] (1 mg/mL, 500 mL) was oxidized with 10mM sodium metaperiodate at 4° in the dark, and periodate consumption was monitored by the method of Avigad[8]. To terminate the reaction, an excess of ethylene glycol was added, and the mixture was dialyzed against tap water for 2 days and then against distilled water for 1 day. The non-dialyzable fraction was reduced with sodium borohydride for 48 h at 4°. After acidification with acetic acid, the mixture was dialyzed and lyophilized to give LELFD-I/B (yield: 85.0%). A portion of LELFD-I/B was partially hydrolyzed with 0.05M sulfuric acid for 72 h at 37°. The mixture, after neutralization, was dialyzed against distilled water for 2 days. The non-dialyzable fraction was concentrated and lyophilized (SD-LE-I/B, yield: 66.4%).

Preparation of boiled curdlan. — Curdlan was suspended in distilled water (100 mg/mL) and boiled for 30 min. After this treatment, an elastic gel was formed and this was lyophilized.

Preparation of HE-curdlan. — Curdlan was dissolved in 100 mL of M sodium hydroxide (20 mg/mL) at 4°. The solution was kept at −10° in Dry Ice bath. Ethylene oxide (30 mL) was then added. The mixture was stirred for 2 h and then overnight at 4°, and made neutral with acetic acid. The non-dialyzable fraction was concentrated and lyophilized to afford HE-curdlan, (yield: 116.0%).

^{13}C-N.m.r. studies. — ^{13}C-N.m.r. spectra were measured with a JEOL FX-200 instrument. Pressure-proof 10-mm (i.d.) sample-tubes with screw caps were used at various temperatures for the aqueous solution. All spectra were obtained from 15 000 to 10 000 scans.

Other methods. — Preparation of LELFD[4] and SSG[9], purification of grifolan LE[4], methylation analysis[10], determination of molecular weight by gel filtration[6], formation of the complex with Aniline Blue[6], and the c.p.–m.a.s. ^{13}C-n.m.r. studies on the solid state[11] were performed as described previously.

RESULTS

Solubilization of gel-forming glucan by heat treatment. — Solubilization of (1→3)-β-D-glucan into an aqueous solution is quite difficult and time-consuming, and the addition of sodium hydroxide or urea followed by dialysis is usually required. At the outset, we used 150° treatment to achieve solubilization of (1→3)-β-D-glucans. Table I shows the carbohydrate contents in the supernatants separated from the treated solution. The supernatant obtained immediately after mixing with water gave soluble fractions of only 6.8% for helix and 29.7% for native LELFD, respectively, as assessed by the phenol–sulfuric acid method. After 30 min at 150°, the soluble fractions increased to the extent that clear solutions were obtained and the sugar contents reached a maximum value after 0.5–1.0 h (89.6% after 1.3 h for helix and 90.7% after 0.5 h for LELFD-native). The effect of temperature on solubilization of LELFD-helix was examined by heating for 0.5 and 3 h (Fig. 1). The sugar contents in the supernatants were significantly increased after heating above 130°, and the maximum value was observed at 150°. These observations suggested the possibility that heat treatment at 150° is advantageous for dissolving (1→3)-β-D-glucans.

Effect of heating time on change of molecular weight. — The molecular weights of the products derived from the heat-treated LELFD-helix were determined by gel filtration. As shown in Fig. 2, the elution profiles of the LELFD-helix heat treated at 150° for 0, 0.5, 3, and 6 h, shifted with a single peak with respect to the total volume of the column, indicating molecular weights of 800 000, 250 000, 21 000, and 6200, respectively. As the 12 h-treated fraction was excluded because of the limitations of the column, we could not determine its molecular weight.

Absence of changes in the primary structure of heat-denatured LELFD. — To

TABLE I

SOLUBILITY OF HEATED LELFD-HELIX[a]

Sample	*Temperature (°)*	*Time (h)*	*Sugar content in supernatant (%)*
LELFD (helix)	150	0	6.8
		0.5	82.9
		1.3	89.6
		2.0	85.5
		3.0	81.6
		6.0	83.4
		9.0	77.0
		12.0	75.9
LELFD (native)	150	0	29.7
		0.5	90.7
		4.0	90.0
		6.0	73.2
		12.0	90.7

[a]Heat-treated glucan suspensions (2.5 mg/mL, 10 mL) were centrifuged (2500 r.p.m. for 5 min), and sugar contents in supernatants were determined by the phenol–sulfuric acid method.

confirm the absence of changes in the primary structure, the heat-denatured LELFD-helix was subjected to methylation analysis. The non-dialyzable fractions of the products were methylated by Hakomori's method[12], and partially methylated alditol acetates were produced and analyzed by gas chromatography. The molar ratios of alditol acetate derivatives of 2,3,4,6-tetra-, 2,4,6-tri-, and 2,4-di-*O*-methyl-D-glucose were 1.00, 2.18 ±0.42, and 1.23 ±0.06, and were thus similar to those from parent LELFD-helix[4]. The ^{13}C-n.m.r. spectra of the LELFD-helix heat treated for 3 and 6 h at 150° showed characteristic triplet C-3 signals in Me_2SO-d_6

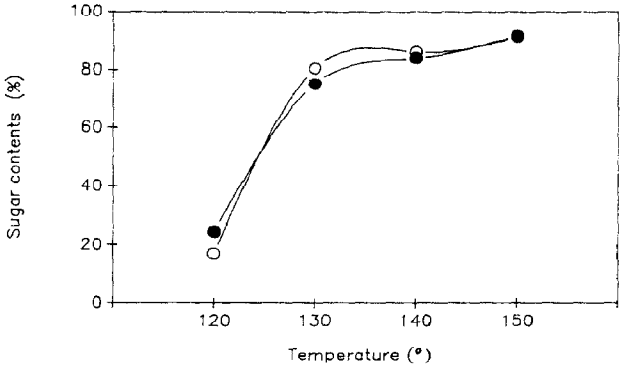

Fig. 1. Effect of temperature on solubility of LELFD-helix: LELFD-helix (2.5 mg/mL in distilled water of 10 mL) was heated for 0.5 (○) and 3 h (●) at various temperatures and the products were centrifuged (2500 r.p.m. for 5 min), and then sugar contents in the supernatants were determined by the phenol–sulfuric acid method.

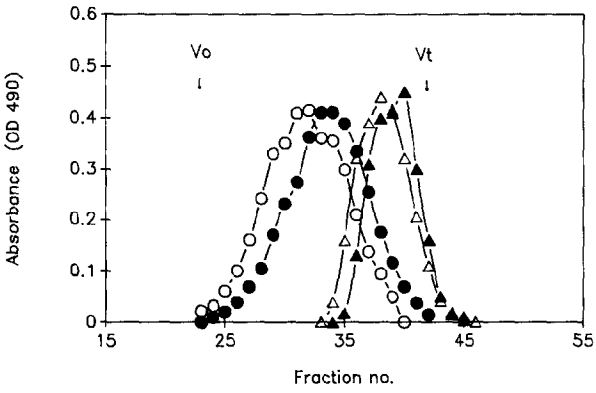

Fig. 2. Elution profiles of heat-treated LELFD-helix from a column of TSK-GEL HW-65F equilibrated with 0.3M sodium hydroxide: 150° ○ 0 h; ● 0.5 h; △ 3 h; and ▲ 6 h; V_o, void volume; V_t, total volume.

(Fig. 3)[13]. These results suggested that the products were (1→3)-β-D-glucan possessing a glucosyl branch at position 6 of every third unit.

Study of the physicochemical properties of products of heat-treated LELFD-helix using ^{13}C-n.m.r. in the solid state. — In order to assess the gel-forming ability of heat-treated LELFD-helix, ^{13}C-n.m.r. spectra of the glucans in water were recorded. The spectra of those heat treated for 0, 0.5, and 1.3 h were broad, indicating gel formation (Fig. 4). However, those heat treated for 3, 6, and 12 h showed relatively sharp signals and gave C-3 signals at 85 p.p.m., suggesting the presence of a significant proportion of random-coil segments[14]. These spectral patterns are in good agreement with the molecular weights of these products.

Examination of glucan–dye complex-forming ability. — The fluorescence intensity of the glucan–Aniline Blue complex in dilute alkaline solution should be closely related to the existence of the single helical conformation in (1→3)-β-D-glucans[15,16]. Table II shows the fluorescence intensity of the glucan–dye complex. Heat-treated LELFD-helix was admixed with Aniline Blue solution and the fluores-

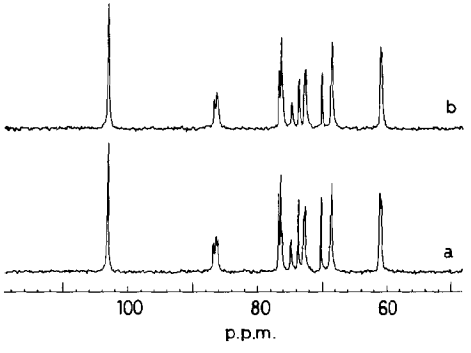

Fig. 3. ^{13}C-N.m.r. spectra (Me_2SO-d_6) of the products of heat-denatured LELFD-helix at 60°: a, 150°, 3 h; b, 6 h.

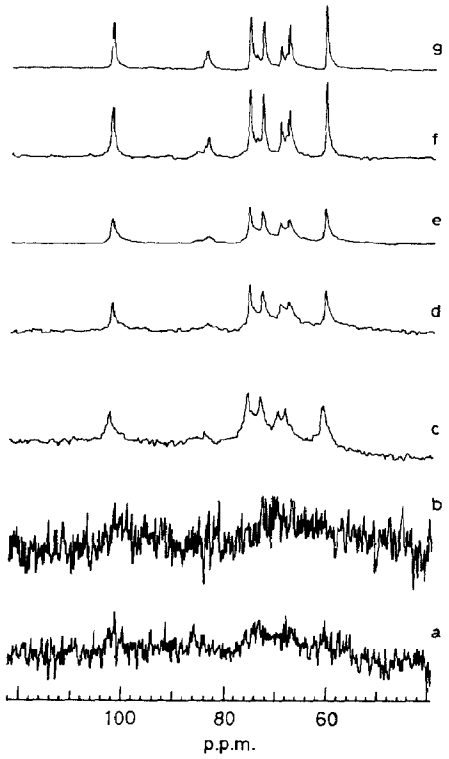

Fig. 4. ^{13}C-N.m.r. spectra (H_2O) of the products of heat-denatured LELFD-helix at 25°: a, 150°, 0 h; b, 0.5 h; c, 1.3 h; d, 2.0 h; e, 3.0 h; f, 6.0 h; and g, 12 h.

TABLE II

RELATIVE FLUORESCENCE INTENSITY OF ANILINE BLUE–GLUCAN COMPLEX[a]

Sample	*Temperature (°)*	*Time (h)*	*Relative intensity (%)*
LELFD-helix	150	0	101.6
		0.5	82.1
		1.3	77.2
		2.0	71.4
		3.0	50.0
		6.0	32.5
		9.0	23.0
		12.0	33.2
Grifolan LE			100.0

[a]Aniline Blue solution (10 μg/mL 0.1M NaOH, 3 mL) was mixed with several samples (100 μg/mL) and the fluorescence intensity was recorded. Each value was calculated from the intensity of grifolan LE taken as 100%.

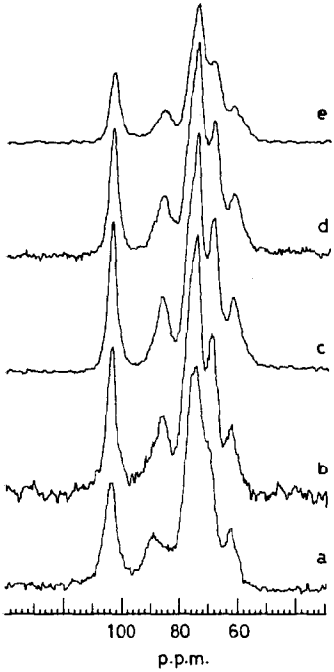

Fig. 5. C.p.–m.a.s. ^{13}C-n.m.r. spectra of a, LELFD-helix; b, LELFD-helix 150°, 0.5 h; c, 3 h; d, 6 h; and e, 12 h.

cence intensity was compared with those of grifolan LE used as a standard. Untreated LELFD showed high fluorescence intensity, comparable to that of grifolan LE. The intensity of treated LELFD decreased in relation with the heating time. These results suggested that the amount of helix in the glucan solution was also diminished gradually by heat treatment.

Conformational change in the solid state of the glucans by heat denaturation. — LELFD-helix has a large number of single-helical conformers, as assessed by a c.p.–m.a.s. ^{13}C-n.m.r. study in the solid state[5]. We examined the conformational change of the LELFD-helix due to heat treatment. As shown in Fig. 5, the C-3 signal at 89 p.p.m. of untreated LELFD was shifted to ~86 p.p.m., indicating an increment in the triple-helix moiety after treatment. This result showed that heat treatment changed the conformation from helix to native. It is also suggested that a molecular weight of about 10 000 can produce the triple-helical conformation in the solid state. In spite of this, these low-molecular-weight products showed little antitumor activity[6].

Molecular-weight change of several (1→3)-β-D-glucans by heat treatment. — To confirm whether alteration of molecular weight is generally observed in other (1→3)-β-D-glucans, several glucans were tested. The branched glucans, grifolan LE and SSG, were degraded in a similar manner to that of LELFD (Fig. 6). A soluble derivative from curdlan. HE-curdlan, showed a decrease in molecular

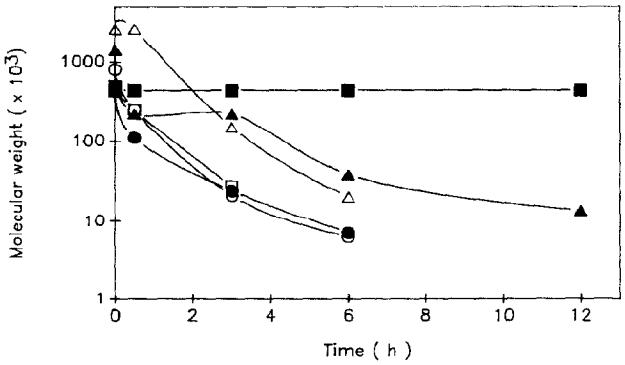

Fig. 6. Alteration of molecular weight of the products by heat treatment. Heat-treated glucans were dissolved in 0.3M sodium hydroxide (2 mg/mL), and eluted from a TSK GEL HW-55 or HW-65 gel-filtration column (2.5 × 70 cm or 1.5 × 73 cm) equilibrated with the same solvent. Fractions (2.9 or 2.4 mL) were assayed by the phenol–sulfuric acid method. Molecular weight was estimated from the elution volume of dextran standards: ○, LELFD-helix; □, LELFD-native; ●, grifolan LE; △, SSG; ▲, HE-curdlan; and ■, dextran T-500.

weight in relation to the heating time (Fig. 6). However, such linear glucans as curdlan and SD-LE-I/B remained insoluble in water (Table III). As denatured curdlan and SD-LE-I/B was not solubilized in 0.3M sodium hydroxide, the molecular weights could not be determined. Monomeric D-glucose was found in 6- and 12-h treated products of LELFD-helix, and was estimated by D-glucose oxidase as 8.0 and 61.8%, respectively.

Observation of gel-to-sol transition during heat treatment. — To determine why solubility of glucans was increased by heat treatment, gel-to-sol transition of glucans during heat treatment was examined by ^{13}C-n.m.r. analysis measured at

TABLE III

SOLUBILITY OF HEATED DENATURED LINEAR (1→3)-β-D-GLUCANS[a]

Sample	*Temperature (°)*	*Time (h)*	*Sugar content in supernatant (%)*
Curdlan	150	0	0
		0.5	0
		3	2.3
		6	2.1
		12	13.7
SD-LE-I/B	150	0	4.5
		0.5	12.5
		3	8.5
		6	17.6
		12	49.2

[a]See footnote of Table I.

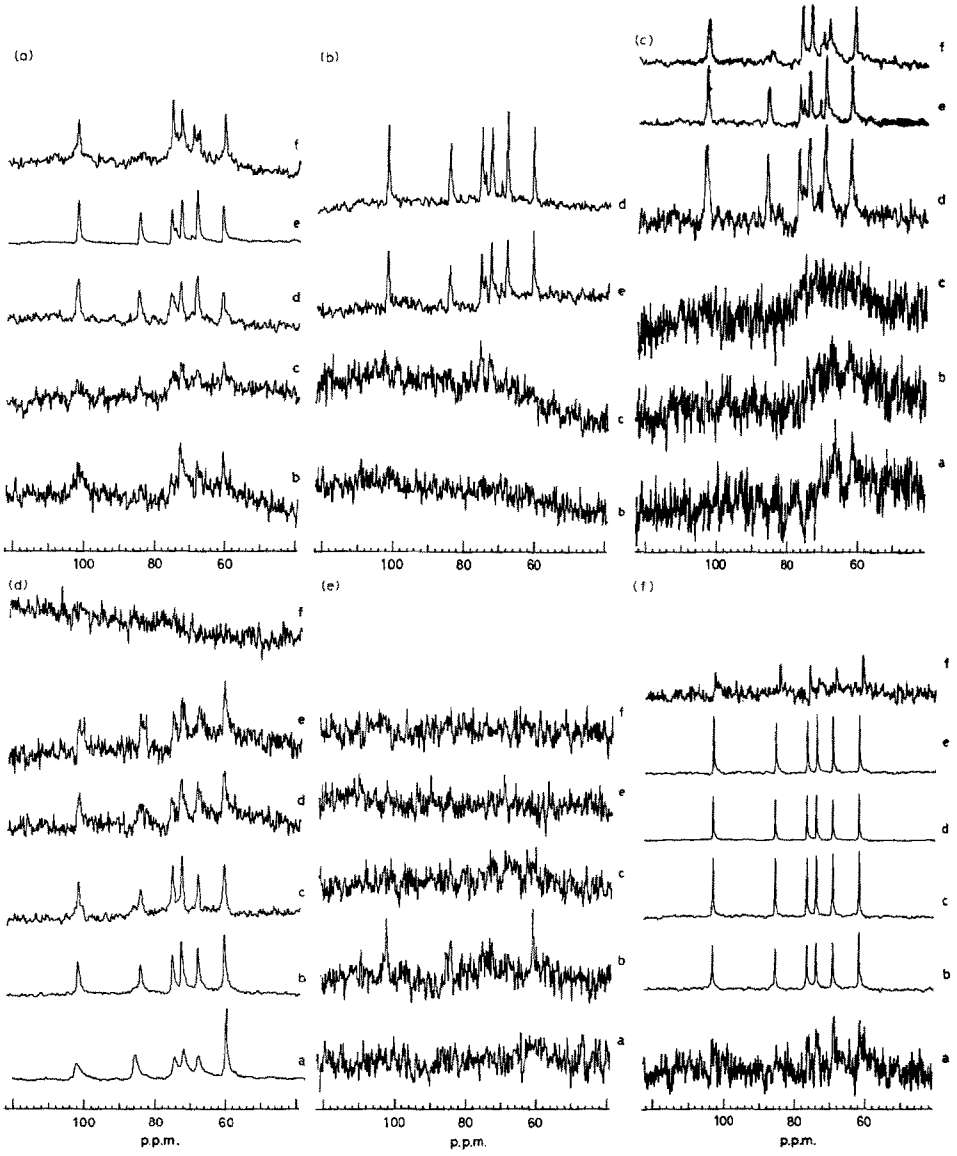

Fig. 7. Gel-to-sol transition of LELFD-helix (a), LELFD-native (b), SSG (c), curdlan (d), boiled curdlan (e), and SD-LE-I/B (f) in water measured by ^{13}C-n.m.r. spectroscopy at various temperatures. Each glucan (40 mg) was suspended in 50% D_2O (1 mL) and then ^{13}C-n.m.r. spectra were recorded at various temperatures: a, 40°; b, 100°; c, 120°; d, 130°; e, 140°; and f, 40° after treatment at 140°.

>100°. Glucans suspended or dissolved in water in pressure-proof glass sample-tubes were used for the analyses. Both the LELFD-helix and LELFD-native [Fig. 7(a) and (b)] showed gel-to-sol transition at ~130°, as assessed by the appearance of signal peaks, and the C-3 signal peak at 85 p.p.m., suggesting the random-coil conformation, was higher than that at lower temperatures. When the temperature was lowered to 40° after 140° treatment, signal broadening was observed, and C-3 signals almost disappeared [Fig. 7(a)]. These gel-to-sol transitions were similar to the other results observed by alkali-neutralization, although the C-3 signal peak at about 89 p.p.m. attributable to the single-helical conformation was not observed[17]. The highly branched glucan, SSG, showed similar results to those of LELFDs [Fig. 7(c)]. Although curdlan [Fig. 7(d)] showed clear signal peaks for C-3 at 89 p.p.m. (suggesting the single-helical conformation) at 40° and at 85 p.p.m. at 100°, broad signals were observed at 130°. The spectra of boiled curdlan [Fig. 7(e)] at 40–140° were distinct from those of untreated curdlan, and it was clear that the gel architecture of boiled curdlan was more rigid than that of untreated curdlan. Sharp signals of the debranched LELFD, SD-LE-I/B, appeared at 100° and disappeared on cooling to 40° [Fig. 7(f)]. Because SD-LE-I/B had a lower molecular weight (38 000) than curdlan, this difference between curdlan and SD-LE-I/B at 140° might result from the difference in their molecular weight. Comparison of the spectra at 40° after treatment at 140° suggested that linear glucans became stiff gels, while branched glucans showed relative increases in the flexibility of the molecule.

DISCUSSION

The present study clarifies the effect of heat treatment on several (1→3)-β-D-glucans. LELFD is a high-molecular-weight (1→6)-β-branched (1→3)-β-D-glucan which has gel-forming ability. When the glucan was suspended in water and heated to 150°, its solubility increased. The increased solubility is thought to be due to gel-to-sol transition, which induces destruction of the gel network, with transformation of the helix structure to a random-coiled one. Further treatment over longer periods of time showed that there is a decrease in molecular weight, and it is suggested that the molecular-weight change affects the ultrastructural change. Another branched (1→3)-β-D-glucan, SSG, also shows similar physicochemical alterations. A linear (1→3)-β-D-glucan, curdlan, remained insoluble and showed less molecular movement than did the parent glucan as the temperature was increased. The HE-curdlan provided a low-molecular-weight fragment similar to that of the branched glucans. The foregoing results indicate that branched glucans showed gel-to-sol transition and increased solubility as a result of heat treatment, whereas linear glucans showed no increase in solubility.

Yanaki *et al.* have already observed by using viscosity measurements[7] that conformational change, from a triple helix to a random coil, occurs on heat treatment. It was previously noted that helical (1→3)-β-D-glucans showed characteristic chemical shifts of C-3 from 88 p.p.m. to 85 p.p.m. in 0.15M and 0.25M sodium

hydroxide solution, respectively, suggesting that the difference in the chemical shifts reflected a conformational change such as from a single helix to a random coil[14]. Our ^{13}C-n.m.r. study also demonstrated that treatment at 130° caused a gel-to-sol transition not seen at 120°, and the C-3 signal appeared at 85 p.p.m., the same as with the alkaline solution. This result strongly suggests that heat treatment has potent destructive effects on the gelation capability of branched glucans. Comparing the peak height of C-3 signals of LELFD between the spectrum at 40° after treatment at 140° and that at 140°, the former gave a broad signal and the C-3 signal almost disappeared, whereas the latter showed relatively sharp and higher signals. The difference suggested that the well-resolved spectra of LELFD and SSG at 140° did not result from a decrease in molecular weight through heat treatment, and that some physical cross-links had been degraded to form the random-coiled sol. Takahashi *et al.*[18] reported that heat-denatured curdlan gel was more resistant to degradation by (1→3)-β-D-glucanase than alkali-neutralized gel, and that heat treatment affected the density of the intermolecular cross-links that reinforced the gel network. It appears that our result could also be explained in a way similar to that of Takahashi *et al.*The SD-LE-I/B also showed no increase in solubility by heat treatment. The foregoing results suggest that the linear glucans have a rigid gel-structure and also suggest the possibility that the glucosyl branches and substituted groups prevent complete reconstruction of the gel by cooling. The gel-to-sol transition of SD-LE-I/B in the ^{13}C-n.m.r. study was not completely compatible with that of curdlan. The sharp signals observed at 40° after treatment at 140° were compatible with the fact that the supernatant of SD-LE-I/B, heat treated at 150° for 12 h, had a sugar content of 49.2%. This phenomenon was thought to result from the difference in molecular weight, because untreated SD-LE-I/B had a low molecular weight (38 000, data not shown).

We have previously reported that the solid-state conformation of LELFD could be classified into two different types: the curdlan type and the schizophyllan type[5]. Recently, the former was defined as a single-helical conformation and the latter as a triple-helical conformation in a study combining high-resolution solid-state ^{13}C-n.m.r. and X-ray diffraction[3]. Both conformers of LELFD showed similar gel-to-sol transition on heat treatment, suggesting that the difference in solid state conformation does not affect the gel-to-sol transition of these forms. We have previously reported that the formolysis of branched (1→3)-β-D-glucans was influenced by the difference in solid state conformation[6]. However, heat degradation was significantly affected by the difference in primary structure rather than solid-state conformation.

From an examination of heat-treated LELFD, it was shown that the tendency to form a glucan–Aniline Blue complex was decreased, and the solution ^{13}C-n.m.r. spectra of the products were altered from broad to sharp, as a function of the heating time. A significant decrease of the helix component is suggested. Similar results were observed with formolized LELFD in our previous report[6]. These and previous results show that a certain molecular weight is apparently required to re-

tain an ordered conformation. The mechanisms of molecular-weight change are not yet clear, but contaminating protein in LELFD might not be related to these phenomena because such purified glucans as grifolan LE, SSG, and HE-curdlan changed to lower molecular weights with a similar pattern to that of LELFDs. Molecular-weight reduction might be correlated with solubility of the glucans, although a soluble (1→6)-α-D-glucan, dextran, showed no alteration of its molecular weight. Therefore, this degradation seemed to be specific to glucosyl linkages or to be affected by other factors. Examination by ^{13}C-n.m.r. in the solid state suggested that heat-treated products contain the native conformer. These results demonstrate that high-temperature treatment of single-helical glucan can easily induce the triple-helical conformation. This phenomenon might be understood from the annealing that was applied to crystallize curdlan[19]. Although high-molecular-weight gel-forming glucan shows poor solubility, this heat treatment has the advantage that even triple-helical glucans provided small fragments. Moreover methylation analysis of the products from LELFD clearly eliminated the possibility of primary-structural change, and it is suggested that this method might be useful for obtaining soluble, branched (1→3)-β-D-glucans. LELFD has been reported to possess potent host-mediated antitumor activity. The influence of heat denaturation on the biological effects is being examined.

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