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TWO DIFFERENT CONFORMATIONS OF THE ANTITUMOUR β-D-GLUCAN PRODUCED BY *Sclerotinia sclerotiorum* IFO 9395

NAOHITO OHNO AND TOSHIRO YADOMAE

Laboratory of Immunopharmacology of Microbial Products, Tokyo College of Pharmacy, Horinouchi 1432-1, Hachioji, Tokyo 192-03 (Japan)

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ABSTRACT

The antitumour β-D-glucan [SSG, (1→3)-linked with a D-glucosyl group attached to position 6 of alternate units] produced by *Sclerotinia sclerotiorum* IFO 9395 afforded a gel in neutral aqueous solution which gave no ^{13}C-n.m.r. signals. Signals appeared on the addition to the gel of sodium hydroxide to 0.15M and the line-width was narrowest near 0.25M base (gel-to-sol transition). SSG bound to Congo Red and shifted the λ_{max} in a manner similar to that of curdlan. Neutralisation of the sol regenerated the gel. SSG and regenerated SSG showed different c.p.–m.a.s. ^{13}C-n.m.r. spectra, especially the signals for C-3 which appeared at 86 and 89 p.p.m., respectively. The c.p.–m.a.s. spectrum of regenerated SSG was similar to that of curdlan, suggesting a helical structure. Thus, SSG and regenerated SSG appear to have different conformations.

INTRODUCTION

Many kinds of antitumour glucans have been studied and lentinan (from *Lentinus edodes*) and schizophyllan (from *Schizophyllum commune*), both of which are branched (1→3)-β-D-glucans, have been applied clinically in Japan. It has been suggested[1] that a triple-helix structure is required for antitumour activity. The antitumour activity of pachyman can be induced by treatment with urea which would alter the conformation of the glucan[2]. Thus, conformation is thought to be important for the antitumour activity of glucans, but elucidation of the conformation by X-ray crystallography has been successful only for a few polysaccharides because they are difficult to crystallise[3,4].

Recently, the conformations of lentinan, curdlan, cellulose, chitin, and cyclomalto-oligosaccharides have been investigated by solid-state cross-polarisation–magic angle spinning (c.p.–m.a.s.) ^{13}C-n.m.r. spectroscopy[5,12], and a good correlation was found with the results of X-ray crystallography. Saito *et al.*[9,10] examined the conformations of lentinan and curdlan by c.p.–m.a.s. n.m.r. spectroscopy and concluded that the chemical shifts of the signals of several carbons in the solid were different from those in solution. These differences were thought to be due to differ-

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ences in fine structure, such as the formation of helices, since the random-coil structure of the oligosaccharides showed chemical shifts similar to those seen in solution[10].

Sclerotinia sclerotiorum IFO 9395 produces an antitumour β-D-glucan (SSG) which is (1→3)-linked with a D-glucosyl group attached to position 6 of alternate units[13]. We now report on an investigation of the structure of SSG in the gel and the solid state, using c.p.–m.a.s. ^{13}C-n.m.r. spectroscopy, binding of Congo Red, and viscosity.

EXPERIMENTAL

Native and regenerated SSG. — Sclerotinia sclerotiorum IFO 9395 was cultured in a medium containing D-glucose (2%), yeast extract (0.3%), and polypeptone (1%) kept in a 15-L jar fermentor at 27° for 2 days. When the viscosity of the culture had reached a maximum, the mycelium was removed by a glass filter, ethanol (1 vol.) was added to the filtrate, and the fibrous product which rose to the surface was collected. This material was reprecipitated from aqueous solution by the addition of ethanol (1 vol.). After three re-precipitations, the product was dried with acetone and ether to give SSG (3 g).

A solution of a portion (1 g) of SSG in 8M urea (700 mL) was applied to a column (10 × 3 cm) of DEAE-Sephadex A-25 (Cl^- form) and eluted with 8M urea (350 mL). The eluate was dialysed against tap water and then distilled water, and concentrated. The addition of ethanol (1 vol.) gave regenerated SSG (70%).

Complex formation with Congo Red. — The change of λ_{max} of Congo Red in the presence of glucans was recorded using a Hitachi 557 spectrophotometer.

Viscosity. — An Ostwald-type viscometer was used. All measurements were performed after equilibration of the solutions at 25°.

^{13}C-N.m.r. spectra. — A JEOL FX 200 instrument was used and spectra were recorded at room temperature (5–100 × 10^3 scans). C.p.–m.a.s. ^{13}C-n.m.r. spectra were recorded with a c.p.–m.a.s. unit together with the appropriate software and with the use of a Dyflon rotor. Contact time, pulse interval, and number of pulses were 1 ms, 1–2 s, and 500–2000 scans, respectively. Chemical shifts relative to that of Me_4Si were determined by using the signal of adamantane (29.5 p.p.m.).

RESULTS AND DISCUSSION

Gel structure of SSG. — Antitumour glucans, such as curdlan, schizophyllan, scleroglucan, and lentinan, form gels[1,14–18] that are degraded to sols by the addition of sodium hydroxide or urea[14,15].

Fig. 1 shows that the viscosity of an aqueous gel of SSG was decreased by the addition of sodium hydroxide and reached a constant value at 0.25M consistent with a change to a random-coil conformation (sol) caused by removal of cross-links. Sodium hydroxide induces similar changes in the conformation of other (1→3)-β-D-

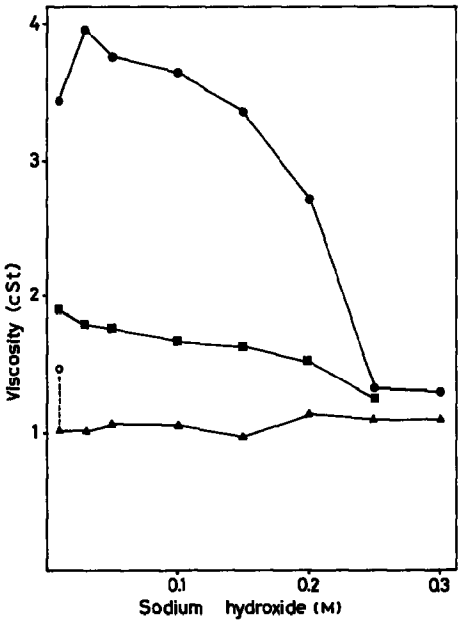

Fig. 1. Dependence of the viscosity of glucans (1 mg/mL) on the concentration of sodium hydroxide: SSG (—●—) or curdlan (—■—) was dissolved in 0.01M sodium hydroxide, and 6M base was added gradually; SSG (—▲—) was dissolved in 0.3M sodium hydroxide, and 6M hydrochloric acid was added gradually. The neutralised SSG was dialysed against water and then measured (--○--).

glucans[14]. When hydrochloric acid was added gradually to the sol, the viscosity was not increased greatly, but, as described below, the segmental motion was lowered. Thus, the cross-links, once degraded, were not re-formed in the same manner. Similarly, high concentrations of urea are known to degrade gels to sols and this was also true of SSG (Fig. 2).

The λ_{max} of Congo Red in the visible region can be shifted markedly to longer wavelength by the addition of a (1→3)-β-D-glucan to a solution of the dye in aqueous sodium hydroxide when the concentration of base is <0.25M, suggesting that the ordered structure of the glucan forms a complex with Congo Red[14,15]. The effect of SSG on the λ_{max} of Congo Red, shown in Fig. 3, was much weaker than that of curdlan. However, regenerated SSG, obtained by dissolution of SSG in 0.3M sodium hydroxide and dilution to the indicated molarity, showed a higher binding potency than the native form (Fig. 4). According to Saito *et al.*[18], the (1→3)-β-D-glucan curdlan forms single and multiple helical chains in both neutral solution and dilute sodium hydroxide. Marchessault *et al.*[4] reported clear X-ray diffraction patterns of the (1→3)-β-D-glucan fibre, which was annealed at 140° in water in a closed vessel, and suggested that the conformation in the crystal was a triple-stranded helix. Ogawa *et al.*[14] showed that the annealed film was considerably less stained with Congo Red than the original one and suggested that the single helical portion of the D-glucan chain forms a complex with Congo Red. Thus, the proportion of single helices in SSG could be lower than in regenerated SSG.

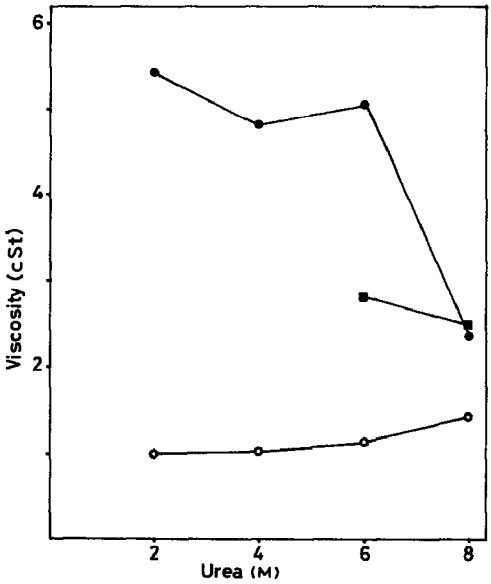

Fig. 2. Dependence of the viscosity of glucans (1 mg/mL) on the concentrations of urea: SSG (—●—) or curdlan (—■—) was dissolved in 2, 4, 6, or 8M urea (—○—). Curdlan was hardly solubilised in 2 and 4M urea.

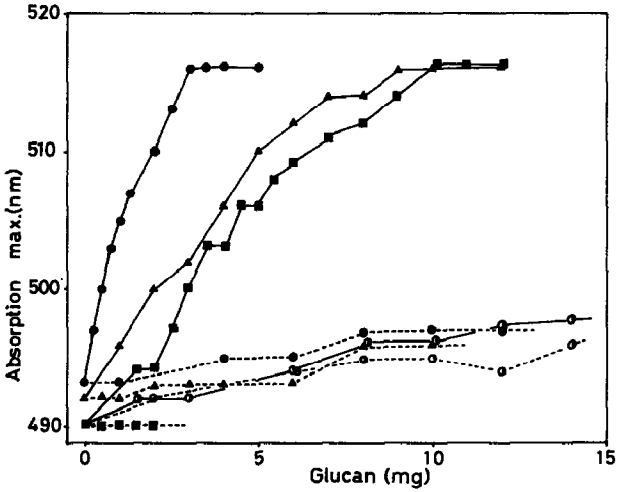

Fig. 3. Absorption maxima of glucan–Congo Red complexes. SSG or curdlan was dissolved (10 mg/mL) in the indicated molarity of sodium hydroxide and added to 10^{-5}M Congo Red (10 mL): —●—, curdlan (0.05M); —■—, curdlan (0.1M); —▲—, curdlan (0.15M); —◑—, curdlan (0.2M); --●--, SSG (0.05M); --■--, SSG (0.1M); --▲--, SSG (0.15M); --◑--, SSG (0.2M).

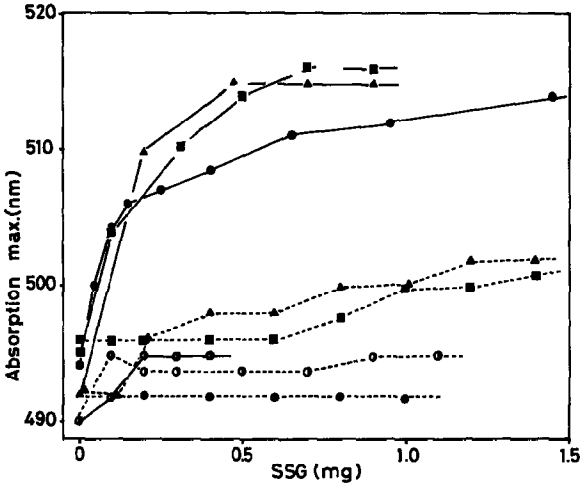

Fig. 4. Absorption maxima of the complexes of Congo Red with SSG and regenerated SSG. Regenerated SSG was prepared by dissolution of SSG in 0.3M sodium hydroxide and then dilution. SSG or regenerated SSG in various concentrations of sodium hydroxide (1 mg/mL) was added to 10^{-6}M Congo Red (10 mL); SSG: --●--, 0.05M NaOH; --■--, 0.1M; --▲--, 0.15M; --◑--, 0.2M; regenerated SSG: —●—, 0.05M NaOH; —■—, 0.1M; —▲—, 0.15M; —◑—, 0.2M.

In order to obtain more information on the architecture of the gel network of SSG, ^{13}C-n.m.r. spectroscopy was applied to study the sodium hydroxide- or urea-induced conformational changes. (1→3)-β-D-Glucan gives few or no ^{13}C signals in the gel state[1,17,18] because of the lowered mobility of glucan chains by cross-links. Degradation of the gel to the sol state by the addition of sodium hydroxide resulted in the appearance of ^{13}C signals. The ^{13}C-n.m.r. spectra of SSG in various concentrations of sodium hydroxide are shown in Fig. 5. No signals were observed in 0.05–0.1M base, but they appeared in >0.15M base. The line-widths of the signals were narrowest in 0.25M base. On the basis of the viscosity and Congo-Red-binding data, the gel structure would be expected to be degraded into the sol near 0.25M sodium hydroxide. The chemical shifts of the signals for C-1 and C-3 were slightly different, depending on the concentration of sodium hydroxide [C-1, 105 and 103 p.p.m. (0.15M), 104 p.p.m. (0.25M); C-3, 88 (0.15M) and 86 p.p.m. (0.25M)]. The peak intensity and line-width gradually changed with change in the concentration of sodium hydroxide. Similar results were observed for lentinan and curdlan (Fig. 5)[1,17,18]. Saito *et al.*[18] assigned the ^{13}C signals observed for a linear (1→3)-β-D-glucan in neutral and dilute alkaline solution to a region of single helical conformation. Thus, it is likely that (*a*) the segmental motion of SSG was highly restricted in 0–0.1M sodium hydroxide, (*b*) in 0.15–0.2M base, some of the cross-links were degraded with the formation of some single helical regions, and (*c*) in >0.25M base, SSG formed a random-coil sol.

The sol-to-gel transition was measured by stepwise addition of hydrochloric acid to the sol of SSG (Fig. 6). The signals were observed until the sodium hydrox-

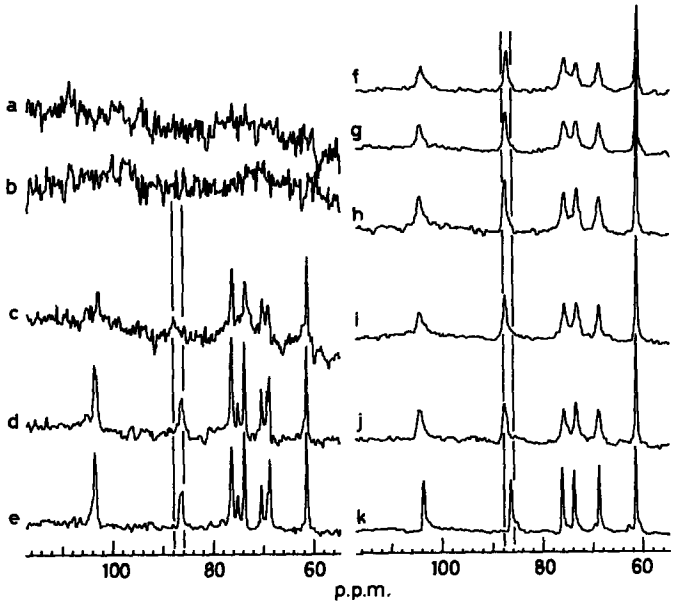

Fig. 5. ^{13}C-N.m.r. spectra of samples (30 mg) of SSG and curdlan in various concentrations of sodium hydroxide (4 mL): a–e, SSG; f–k, curdlan; f, 0.01M NaOH; a,g, 0.05M; b,h, 0.1M; c,i, 0.15M; d,j, 0.2M; e,k, 0.25M.

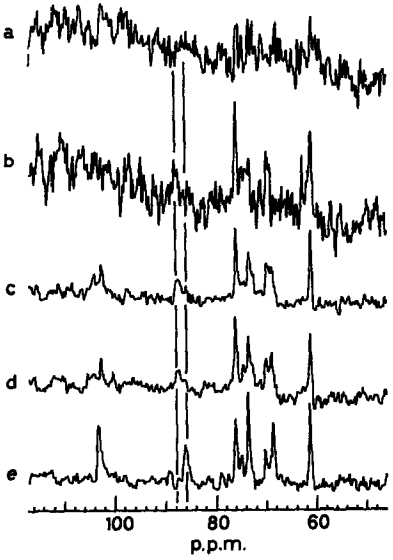

Fig. 6. ^{13}C-N.m.r. spectra of the sol-to-gel transition of SSG. To a solution of SSG (30 mg) in 0.3M sodium hydroxide (4 mL) was gradually added 6M hydrochloric acid to the following molarities: a, 0M; b, 0.05M; c, 0.1M; d, 0.15M; e, 0.2M.

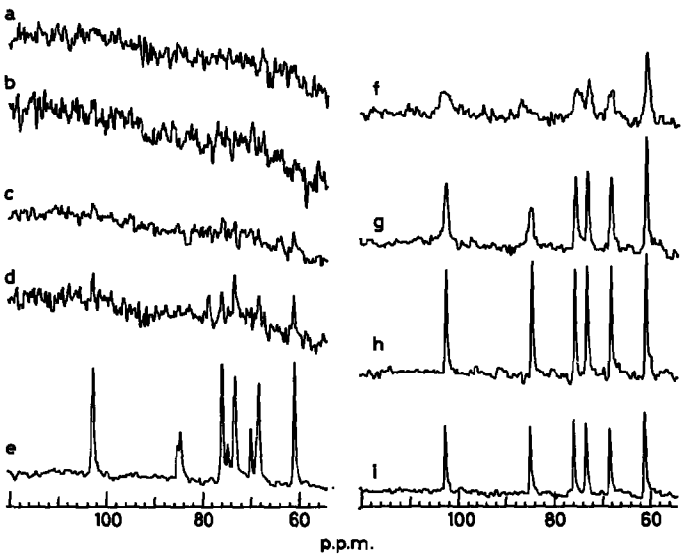

Fig. 7. ^{13}C-N.m.r. spectra of solutions (4 mL) of samples (30 mg) of SSG and curdlan in various concentrations of urea. SSG: a, 8M urea (not heated); b, 2M; c, 4M; d, 6M; e, 8M; curdlan: f, 2M urea; g, 4M; h, 6M; i, 8M.

ide concentration reached 0.05M; in the gel-to-sol transition, signals were observed at an upper concentration of 0.15M. These facts suggest that the cross-links once degraded were not fully restored at the same concentration of sodium hydroxide. During the addition of hydrochloric acid, the signal for C-3 appeared at 88 p.p.m. when the concentration of sodium hydroxide was lower than 0.15M (Fig. 6). It is suggested that, below this concentration of base, some cross-links are formed and the majority of glucan chains are transformed into single- or multiple-helix forms which limit their segmental motion. Further, it is suggested that the single-to-multiple helix transition, which would induce the disappearance of ^{13}C-signals, did not occur easily. The Congo Red-binding and ^{13}C-n.m.r. data indicate that the regenerated SSG contains a higher proportion of single-helix segments than native SSG. This increased proportion of single-helix portion and the limited segmental motion suggest the formation of a "gel" by the addition of hydrochloric acid. These facts and the viscosity data indicate that SSG and regenerated SSG are different.

The stability of the SSG gel in the presence of urea was further studied by ^{13}C-n.m.r. spectroscopy (Fig. 7). The ^{13}C signals of curdlan were seen at >2M urea, and the line-width was smallest at 6–8M urea, suggesting a gel-to-sol transition at ~4M urea. SSG gave a ^{13}C signal only when treated with 8M urea at 100°, in contrast to curdlan, which suggested that the gel was quite stable.

Structure of SSG in the solid state. — The ultrastructure of SSG was compared with that of other (1→3)-β-D-glucans, such as curdlan, laminaran, and pachyman, by c.p.–m.a.s. ^{13}C-n.m.r. spectroscopy. Under physiological conditions, curdlan

possesses a helical structure and laminaran possesses a random-coil structure[10]. C.p.–m.a.s. ^{13}C-n.m.r. spectra obtained from these glucans are shown in Fig. 8. The signal for C-3 in curdlan showed at 90 p.p.m. In the random-coil form (in sodium hydroxide), the signal of C-3 of 3-substituted β-glucosyl residues is observed at 84–86 p.p.m. (Figs. 5–7). Saito *et al.*[12] suggested that the difference in chemical shift of the signal for C-3 in the solid state and in solution reflects conformational differences. The structure of laminaran in the solid state has not been examined, but the data presented here suggest that there is a large proportion of helical regions. The signal of C-3 of SSG appeared at 86 p.p.m. (Fig. 8d). The c.p.–m.a.s. ^{13}C-n.m.r. spectrum of SSG suggested conformational differences between curdlan and SSG in the solid state. Considering the chemical shift of the signal of C-3 of SSG in urea or 0.25M sodium hydroxide (84–86 p.p.m.), the conformation of SSG in the solid state is similar to that in the random-coil conformation.

Fig. 8c also shows the c.p.–m.a.s. ^{13}C-n.m.r. spectrum of regenerated SSG (see Fig. 6), which is quite different to that of SSG (Fig. 8d), especially the chemical shift of the signal of C-3, and similar to those of curdlan (Fig. 8h) and pachyman

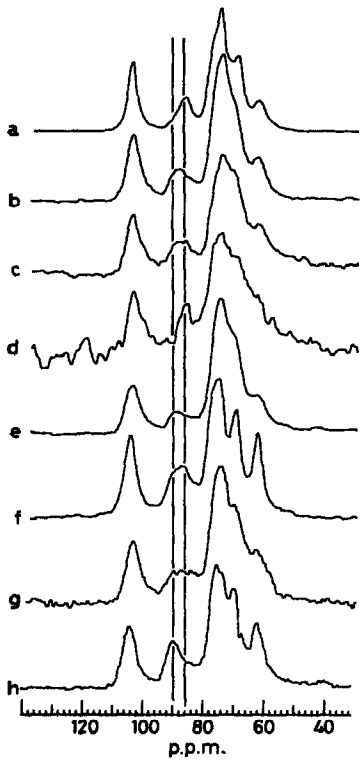

Fig. 8. C.p.–m.a.s. ^{13}C-n.m.r. spectra of a, SSG treated with 8M urea at 100° for 30 min; b, SSG treated with 8M urea at 100° for 6 h; c, SSG treated with 0.3M sodium hydroxide; d, SSG; e, laminarin; f, pachyman; g, curdlan (d.p. 24); h, curdlan.

(Fig. 8f). These data suggest that regenerated SSG could contain a larger proportion of helical regions and that SSG forms two different conformations in the solid state. Figs. 8a and 8b show the spectra of SSG regenerated by treatment with 8M urea. If the heat treatment was insufficient, SSG treated with 8M urea could not form a helical conformation (Fig. 8a), indicating that the stability of the SSG gel is quite high.

Horii *et al.*[7] examined the c.p.–m.a.s. ^{13}C-n.m.r. spectra of regenerated cellulose having different crystallinities, as well as cotton, β-D-glucose, β-cellobiose, and cellopentaose. The spectra of the regenerated celluloses exhibited broad multiplicities of the C-4 and C-6 resonances in a manner similar to those of native cellulose such as cotton and ramie, and also broad tailing of the C-1 resonance. Since these multiplicities changed linearly with crystallinity, they were ascribed to the contributions from the crystalline and non-crystalline components, possibly due to different conformations and torsion angles. Chemical shifts in the c.p.–m.a.s. ^{13}C-n.m.r. spectra may also be affected by packing in the solid state. For cellulose, packing is known to alter the chemical shift by 1–2 p.p.m. However, the difference in chemical shifts between the native and regenerated form is >2 p.p.m. These results suggest that the difference between the native and regenerated forms of SSG is due mainly to differences in torsion angles.

In contrast to cellulose, the c.p.–m.a.s. ^{13}C-n.m.r. spectra of chitin (native) and *N*-acetylchitosan (regenerated chitin) are similar[11]. The results obtained with SSG are similar to those for cellulose. The line widths in the spectra of native SSG are much narrower than those for regenerated SSG, suggesting that the torsion angles in SSG are relatively regular in value. Further, the chemical shift of the C-3 signal is similar to that in solution under denatured conditions. It may be concluded that the conformation of the native SSG is an ordered one.

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