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Note

Circular dichroism of the complex of a (1→3)-β-D-glucan with Congo Red

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A bacterial (1→3)-β-D-glucan, Curdlan type polysaccharide 13140, which consists entirely of β-(1→3)-linked D-glucose residues and contains no other linkages[1], is soluble in aqueous alkaline but not in neutral or acidic solution, and it forms a firm resilient gel when its aqueous suspension (at pH <12) is heated above 54° or its alkaline solution is gently neutralized[2,3] to pH below 12. Ogawa *et al.*[3,4] studied solutions of this polysaccharide and of a partially degraded derivative. They suggested that the (1→3)-β-D-glucan having a number average degree of polymerization (DPn) above ~200 shows an ordered conformation (probably helix) in dilute sodium hydroxide solutions, whereas it practically behaves as a random coil at high-alkaline concentrations, a conformational transition occuring in the region between 0.19 and 0.24M. This helix-coil transition was also found by Saito *et al.*[5] in the study of the ^{13}C-n.m.r. spectrum. X-Ray diffraction analysis of this polysaccharide was performed by Harada[2] and Marchessault *et al.*[6]. The latter authors reported that the chain conformation of (1→3)-β-D-glucan was a triple-stranded 6_1 helix.

Ogawa *et al.*[7] found that the absorption maximum of Congo Red in the visible region was largely shifted to a longer wavelength by the addition of the (1→3)-β-D-glucan into the dye–sodium hydroxide solution with an alkaline concentration below 0.25M, and they suggested that the ordered structure of the D-glucan forms a complex with Congo Red. Nakanishi *et al.*[8] also found a similar complex-formation in neutral media where the D-glucan was in a gel state, and proposed[9] that one molecule of Congo Red is bound to six D-glucose residues of the D-glucan chain. Visible absorption maxima of other dyes having a chemical structure closely related to that of Congo Red, *i.e.*, Color Index Direct Red 2, Red 7, Violet 39, Blue 6, Blue 14, and Blue 15, were also shifted to longer wavelengths by the addition of the D-glucan to solution of the dyes having a low (0.1M) sodium hydroxide concentration[10]. In order to understand the structure of the complex, circular dichroism measurements of the D-glucan–Congo Red complex in an alkaline or neutral aqueous medium were performed.

EXPERIMENTAL

Materials. — Curdlan-type polysaccharide 13140 (original D-glucan) was supplied by Takeda Chemical Industries Ltd. The number average degree of polymerization of the original D-glucan was determined to be 462 by Nakanishi *et al.*[1], by use of a modification of the enzymatic method of Manners *et al.*[11] A (1→3)-β-D-glucan having a lower degree of polymerization (DPn 13.9; determined by the modified Somogyi–Nelson method[4]) was prepared from the original D-glucan by partial degradation and subsequent fractional precipitation. This D-glucan was soluble in water and was named "soluble fraction"[4]. The (1→3)-β-D-glucanitol was prepared by reduction of the aldehyde terminal residue of the soluble fraction with sodium borohydride.

A gel film of the original D-glucan having a thickness of ~2 mm was prepared as follows. The 1% D-glucan–10mM sodium hydroxide solution was spread upon a glass plate at a thickness of ~2 mm. After 24 h a gel film of the D-glucan was obtained by spontaneous lowering of the pH of the D-glucan solution due to atmospheric carbon dioxide and it was thoroughly washed with water until no coloration with phenolphthalein was observed. The gel was stained with Congo Red by immersing it in an aqueous solution of the dye.

Methods. — The circular dichroism spectra were measured with Jasco Model J-15 and J-20A dichrographs equipped with a cuvette having a 10- or 50-mm path length. The absorption spectra were recorded on a Hitachi Model 323 recording spectrophotometer. The molar absorption coefficient (ε) and the molar ellipticity ($[\theta]$) were obtained on the basis of the total dye concentration. The rotation angles at the wavelength of 439 nm were measured with a Union Giken Model PM-70 high sensitivity polarimeter. To calculate the residue rotation ($[m']$) of the D-glucans, the refractive indices of the solvents were determined with a differential refractometer type OR-1 of Shimadzu Seisakusho Co. Ltd. The extinction angle of flow birefringence was measured with a flow birefringence viscometer Model No. B-23 of Rao Instrument Co.

RESULTS

As reported by Ogawa *et al.*[7], at sodium hydroxide concentrations below 0.25M, the absorption maximum of the Congo Red solution in the visible region was largely shifted to a longer wavelength by the addition of the original D-glucan (Fig. 1A). The c.d. of the D-glucan–Congo Red complex in the ratio of 6500 D-glucan units to 1 mole of Congo Red was measured in the presence of 0.05, 0.1 0.2, 0.3, and 0.5M sodium hydroxide, respectively. Similar c.d. spectra were observed at alkali concentration <0.2M, but no c.d. band was observed at a sodium hydroxide concentration >0.3M (Fig. 1B). These results support the previous conclusion[7] that the original D-glucan forms a complex with Congo Red in dilute alkaline media where the D-glucan takes an ordered conformation.

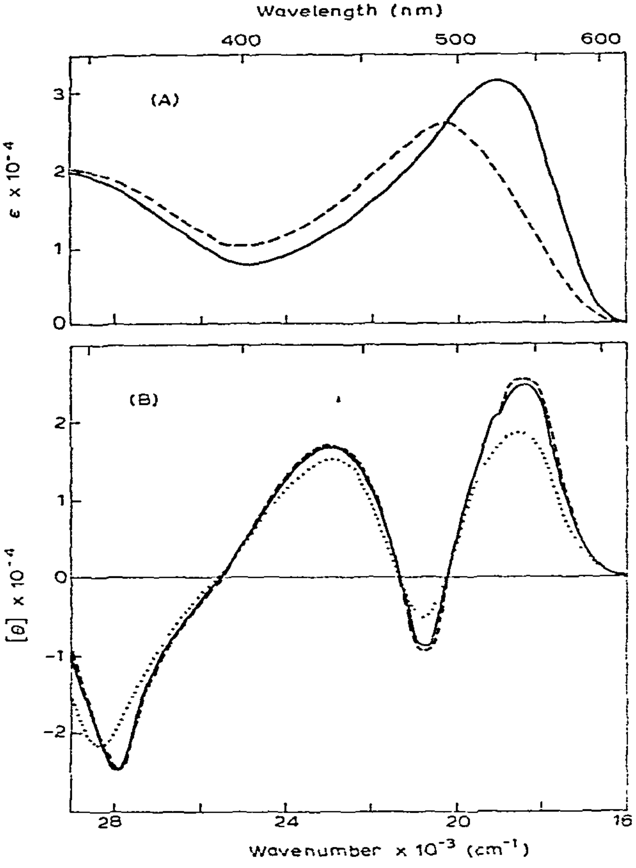

Fig. 1. Absorption and circular dichroism spectra of the original D-glucan–Congo Red system in sodium hydroxide solution. (A) Absorption spectra of Congo Red in 0.10M sodium hydroxide solution in the presence (———) and the absence (– – – –) of D-glucan. (B) Circular dichroism spectra of the D-glucan–Congo Red system at 0.05 (– – – –), 0.10 (———), and 0.20 (·····) M sodium hydroxide concentrations. The total concentration of Congo Red, was 8.0μM and that of D-glucan units (in residue concentration) 52mM. The molar absorption coefficient (ε) and the molar ellipticity ($[\theta]$) were obtained on the basis of the total dye concentration.

When the ratio of D-glucan to Congo Red was decreased, the absorption spectrum of the solution became close to that of free Congo Red solution [7].As shown in Figs. 1B and 2, the original D-glucan–Congo Red–alkaline solution exhibited, in the visible region, four positive c.d. peaks at 18.5×10^3, near 19×10^3, 23.0×10^3, and near 26×10^3 cm^{-1}, and a negative peak near 28×10^3 cm^{-1}. Another negative band in the wave number range of $16-20 \times 10^3$ cm^{-1} may be present, though it is not well-defined as a single c.d. band. Table I shows the variations of the molar absorption coefficient at the wave number of 18.5×10^3 cm^{-1}, and the molar ellipticities at 18.5×10^3 and 23.0×10^3 cm^{-1} with change of the D-glucan to Congo Red ratio. The value of the molar ellipticity of the peak at 18.5×10^3 cm^{-1} increased with increasing ratio value, except where the value of the ratio was low, whereas the molar

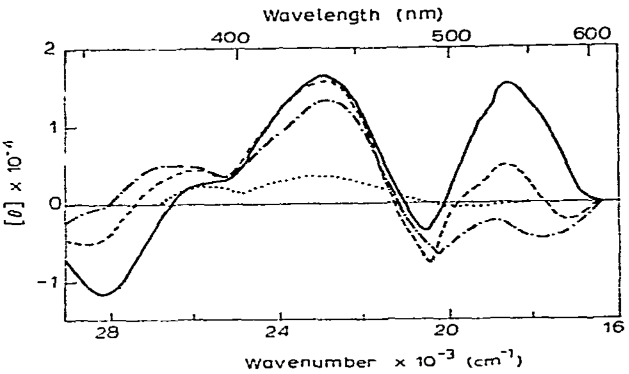

Fig. 2. Circular dichroism spectra of the original D-glucan–Congo Red system at various concentration ratio (R/D) in 0.10M sodium hydroxide solution. Values of R/D are 1290 (———), 390 (– – – – –), 100 (—·—·), and 10 (......), respectively. The concentration of Congo Red was 80μM. The molar ellipticity ([θ]) was obtained on the basis of the total dye concentration.

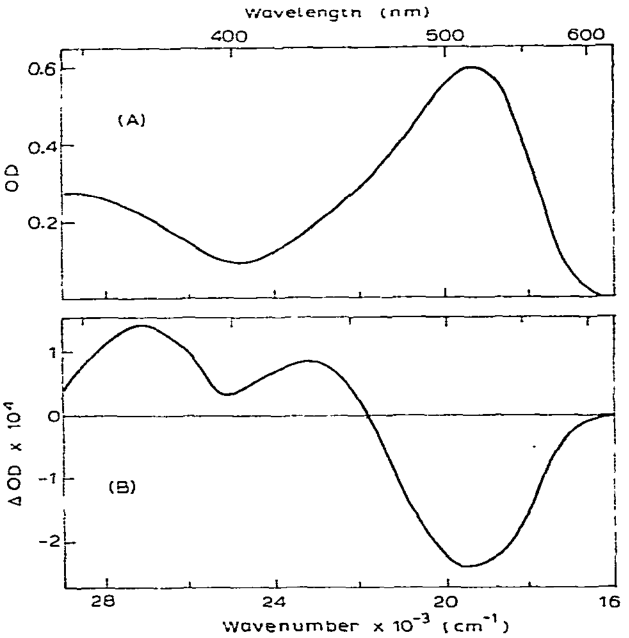

Fig. 3. Absorption (A) and circular-dichroism (B) spectra of a gel film of original D-glucan stained with Congo Red. In this experiment, the molar absorption coefficient and the molar ellipticity could not be obtained, as the total concentration of Congo Red could not be determined.

TABLE I

THE MOLAR ABSORPTION COEFFICIENT AND THE MOAR ELLIPTICITY OF THE ORIGINAL D-GLUCAN–CONGO RED SYSTEM IN VARIOUS PROPORTIONS, IN 0.10M SODIUM HYDROXIDE SOLUTION[a]

Molar proportion of D-*glucan to Congo Red*[a]	*Abs. coefficient* (ε)[b] *at* 18.5×10^3 *cm*$^{-1}$	*Ellipticity* ($[\theta]$)[b]	
		at 18.5×10^3 *cm*$^{-1}$	*at* 23.0×10^3 *cm*$^{-1}$
10:1	1.49	0.00	0.38
46:1	1.86	−0.10	0.63
90:1	2.20	−0.20	1.13
100:1	2.25	−0.33	1.33
390:1	2.56	0.50	1.62
650:1	2.66	0.84	1.61
1290:1	2.72	1.50	1.65
1590:1	2.80	1.74	1.63

[a] Molar residue concentration of the D-glucan to molar concentration of Congo Red (in 80μM solution). [b] The molar absorption coefficient (ε) and the molar ellipticity ($[\theta]$) were obtained on the basis of the total dye concentration.

ellipticity of the peak at 23.0×10^3 cm^{-1} attained a maximum at high values of the ratio. The increase of the molar absorption coefficient at 18.5×10^3 cm^{-1} with increasing ratio value is similar to the increase of the molar ellipticity at 23.0×10^3 cm^{-1} in the low-value range of the ratio. At a high concentration ratio, however, the molar absorption coefficient still increased with the increase of the ratio.

As shown in Fig. 3, the absorption spectrum of the original D-glucan gel stained with Congo Red was similar to that of the D-glucan–Congo Red complet in dilute alkaline solution (Fig. 1A). But the c.d. spectra were different, particularly in the range of $16\text{–}22 \times 10^3$ cm^{-1}.

Fig. 4 shows the effect of additives on the c.d. bands of the original D-glucan–Congo Red complex in 0.1M sodium hydroxide solution. With increasing sodium chloride concentration, little change in the absorption spectrum and molar ellipticity at 23.0×10^3 cm^{-1} was observed, whereas at 18.5×10^3 cm^{-1}, $[\theta]$ decreased considerably. Similar changes were also observed with the addition of urea. On the other hand, with increasing ethyl alcohol concentration, the absorption maximum of the D-glucan–Congo Red complex shifted to a shorter wavelength, and both values of molar ellipticity at 18.5×10^3 and 23.0×10^3 cm^{-1} decreased.

DISCUSSION

The present results suggest that the ordered structure of the D-glucan present in dilute alkaline solution is retained in neutral medium (in a gel state). In dilute alkaline solution, two types of binding of Congo Red with the ordered structure of the D-glucan are present. One (Complex I) is the same binding system as that in the neutral medium (in a gel state of the D-glucan), and it exhibits a negative c.d. band at 19.5×10^3 cm^{-1} and a positive one at 23.0×10^3 cm^{-1}. The other (Complex II) is formed only in the alkaline media, and it shows a positive band at ~18.5 × 10^3 and a negative band at ~28 × 10^3 cm^{-1}.

To clarify the nature of Complexes I and II in the D-glucan–Congo Red system, we examined the effect of additives on the complex formation (Fig. 4): No change in the original D-glucan conformation after the addition of sodium chloride, urea, or ethyl alcohol was observed, when the D-glucan takes the ordered structure (in 0.1M sodium hydroxide solution). Therefore, it may be assumed that these additives affect only the formation of the complex between D-glucan and Congo Red. Sodium chloride and urea may break polar bonds, and ethyl alcohol may interfere with the hydrophobic bond between the biopolymer and the dye. This suggests that Complex I is stabilized by strong hydrogen bonds and/or hydrophobic interactions. The induced

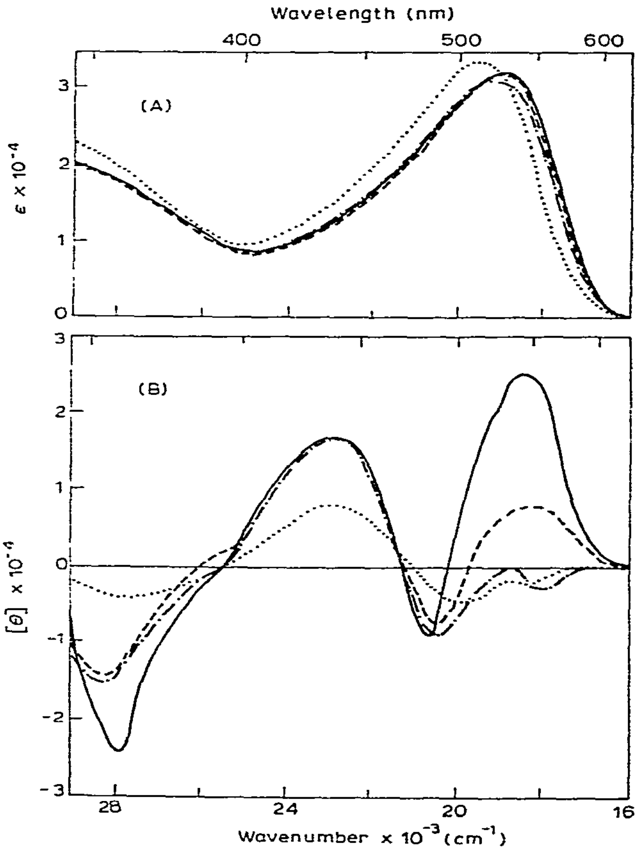

Fig. 4. Change of absorption (A) and circular dichroism (B) spectra of the original D-glucan–Congo Red system (———) in 0.10M sodium hydroxide solution with addition of 1.5M sodium chloride (– – – –), 3.5M urea (—·—·), or 5M ethyl alcohol (......). The total concentration of Congo Red was 8.0μM, and that of D-glucan (in residue concentration) 52mM. The molar absorption coefficient (ε) and the molar ellipticity ([θ]) were obtained on the basis of the total dye concentration.

circular dichroism of Complex II is less stable and dependent upon weak nonpolar interactions, as well as hydrogen bonding and electrostatic interactions, because the molar ellipticity decreased with the increase of sodium chloride, urea, or ethyl alcohol concentration.

With increasing sodium chloride or urea concentration, the absorption spectrum of the D-glucan–Congo Red complex changed little in spite of decreasing the proportion of Complex II (Fig. 4). These results indicate that the contribution of Complex I to the absorbance is larger than that of the Complex II.

In neutral medium, the addition of the soluble fraction, which takes a disordered conformation in the absence of Congo Red[4], shifted the absorption maximum of Congo Red in the visible region to a longer wavelength. This D-glucan–Congo Red complex exhibited a c.d. spectrum similar to that observed with the original D-glucan–Congo Red complex in neutral medium (Fig. 3). These results suggest the presence of an ordered structure in the soluble fraction, when combined with Congo Red.

According to Saito *et al.*[5], the original D-glucan shows single and multiple helical chains in both neutral, water gel, and dilute sodium hydroxide solution (<0.22M). At higher alkaline concentration, the chain conformation of the D-glucan is a random coil. Marchessault *et al.*[6] reported clear X-ray diffraction patterns of the original D-glucan fiber, which was annealed at 140° in water in a closed bomb, and they suggested that the chain conformation of the D-glucan crystal was a triple-stranded helix. We found that the original D-glucan film (which was well stained with Congo Red without annealing) annealed under the same conditions as described by Marchessault *et al.*[6]; its X-ray diffraction pattern corresponded to that obtained by Marchessault *et al.*[6] for the annealed fiber[12]. The annealed film was considerably less stained with Congo Red than the original one. These results suggest that the single helical portion of the D-glucan chain forms a complex with Congo Red.

Effect of urea or ethyl alcohol on the conformation of (1→3)-β-D-glucan in sodium hydroxide solutions. — Ogawa *et al.*[13] studied the effect of sodium chloride on the conformation of the original D-glucan in sodium hydroxide solution by measuring the viscosity, rotation angle, and extinction angle of flow birefringence. They suggested that no change in the D-glucan conformation resulted from the addition of sodium chloride when the original D-glucan took the ordered structure, whereas an increase of the salt concentration caused a conformational transition of the D-glucan to the ordered structure when the D-glucan took a disordered structure in the absence of the salt.

As shown in Fig. 5, at 0.1M sodium hydroxide concentration where the original D-glucan takes an ordered structure, the rotation angle of the D-glucan decreased monotonously with increasing urea or ethyl alcohol concentration, and the change in the extinction angle was small. Whereas, at the alkaline concentration of 0.25M where the original D-glucan takes a disordered structure, the rotation angle changed greatly with increasing urea or ethyl alcohol concentration. The extinction angle of flow birefringence could also be observed. At high concentration of these additives, both values approached those obtained with the solution in 0.1M sodium hydroxide.

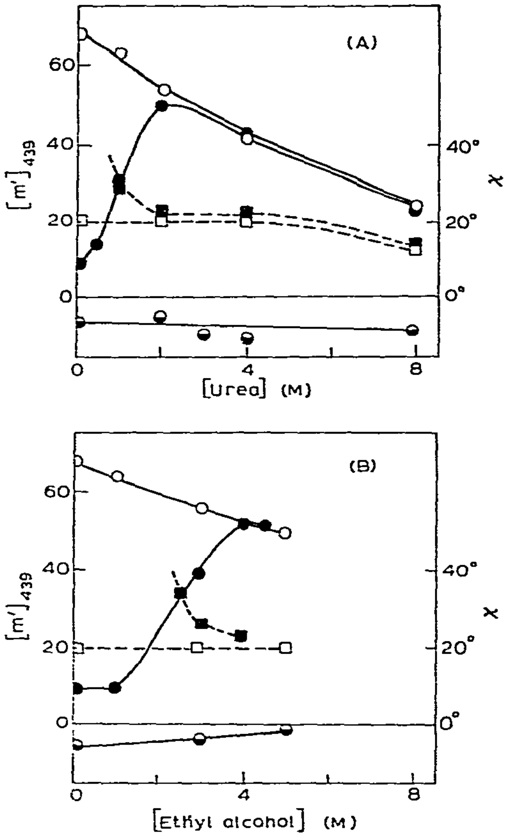

Fig. 5. Dependence of the extinction angle (χ) and the reduced, mean residue-rotation at 439 nm ([m']$_{439}$) of the (1→3)-β-D-glucan–sodium hydroxide solution on the urea (A) or ethyl alcohol (B) concentration. Values of χ of the original D-glucan at 0.10 (☐) and 0.25M (■) sodium hydroxide concentrations, respectively. Values of [m']$_{439}$ of the original D-glucan at 0.10 (○) and 0.25M (●) sodium hydroxide concentration, respectively, and of the soluble fraction at 0.25M (◒). The value of χ was observed for a 0.85% D-glucan solution at the rate of shear of 6000 sec^{-1}.

The change in rotation angle of the soluble fraction* was minimal when the concentrations of these additives were increased.

These changes are similar to those observed on the same D-glucan solutions with decreasing alkaline concentration[3,4], and also to those obtained with increasing sodium chloride concentration[13]. They suggest that no change in the original D-glucan conformation results from the addition of urea or ethyl alcohol when the D-glucan takes the ordered structure (in 0.1M sodium hydroxide solution); whereas at the

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*As soluble fraction, we used a (1→3)-β-D-glucanitol prepared from the soluble fraction, as the low molecular weight (1→3)-β-D-glucan itself was unstable in alkaline solution[4] and because we could assume that no change in the chain conformation occurred by the reduction of the reducing end of the D-glucan. In the neutral water solution, the rotation angle of the D-glucanitol was the same as that of the soluble fraction.

alkaline concentration of 0.25M, the D-glucan conformation changes from a random coil to the ordered structure with increasing concentration of urea or ethyl alcohol.

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