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Effect of heating on formation of curdlan gels

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The characteristics of curdlan preparations that were dehydrated after heating at various temperatures in water were examined by X-ray diffraction, differential scanning calorimetry and electron microscopy. X-Ray diffraction patterns showed that crystallinity was higher in preparations heated to above 80°C, with heated preparations at 170°C having the highest crystallinity. Differential scanning calorimetry showed that the temperature of the endothermic peak caused by swelling decreased and that the peak area became smaller with increasing heating temperatures. Moreover, preparations of (1 → 3)-β-D-glucans of low molecular mass, $\overline{DP}$n 131 and 49, with and without heating at 120°C gave similar X-ray diffraction patterns and differential scanning calorimetry curves to those of the original curdlan. The crystallinity was particularly high without heating in the preparation of $\overline{DP}$n 49 though the endotherm associated with the breakage of hydrogen bonds was lower and the enthalpy smaller than found for the original curdlan. Electron microscopic studies showed that longer and wider microfibrils were formed in curdlan gels, while shorter microfibrils or crystals were formed with the lower molecular mass samples of (1 → 3)-β-D-glucan, which are incapable of forming gels.

INTRODUCTION

Harada *et al.* (1992, 1993) found that curdlan, a bacterial polysaccharide produced commercially for food and industrial areas, and composed entirely of (1 → 3)-β-D-glucosidic linkages, forms two types of gel. One type is formed by neutralization of its alkaline solution or by heating an aqueous suspension at about 60°C and then cooling (Kanzawa *et al.*, 1989*b*). This gel has much lower gel strength and syneresis than the other type obtained by heating an aqueous suspension of curdlan to above 80°C. A hydrophobic reaction occurs during formation of the latter gel (Kanzawa *et al.*, 1989*a*). The group of Kasai, Okuyama and Harada (Takeda *et al.*, 1978; Okuyama *et al.*, 1991) demonstrated by X-ray diffraction analysis that the single stranded helices in curdlan are converted to triple stranded helices by heating at the higher temperature and then cooling. Recently, Konno and Harada (1991) studied the thermal properties of curdlan in aqueous suspension and curdlan gel by differential scanning calorimetry (DSC). DSC curves of curdlan in aqueous

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suspension showed a sharp endothermic peak at 50–64°C caused by swelling followed by a broad endothermic peak and two exothermic peaks. Harada *et al.* (1979, 1991) and Konno *et al.* (1994) also examined the ultra-structure of curdlan by transmission electron microscopy and found that heating at 120°C in water resulted in a pseudo-crystalline form with an electron dense structure.

This paper deals with the properties of powdered preparations of both curdlan and low molecular mass (1 → 3)-β-D-glucans obtained by dehydration after heating aqueous suspensions at various temperatures. X-Ray diffraction analysis and DSC has been carried out on these samples. The significance of electron micrographs of these systems is also discussed.

MATERIALS AND METHODS

Preparation of curdlan powders dehydrated after heating in water

A solution of 1% curdlan in 0·3 N alkaline solution was dialyzed against distilled water and the resultant gel was homogenized in a Waring blender. Samples of the solution were heated at various temperatures for 20 min. For heating at temperatures above 100°C, a stainless reactor tube (Taiatsu, Scientific Glass Co.) was used. The heated preparations were cooled, homogenized with a Waring blender and mixed with an equal volume of acetone. The mixtures were centrifuged at 20 000×*g* for 10 min and the precipitates were dehydrated with acetone to form powders.

Preparation of low molecular-mass (1 → 3)-β-D-glucans

Low molecular mass (1 → 3)-β-D-glucans of $\overline{DP}n$ 131 and 49 were kindly supplied through Dr Kakinuma of the Institute of Fermentation Production of Takeda Chemical Industries. These preparations were obtained from curdlan by partial hydrolysis with 85% formic acid for 30 min at 81–90°C ($\overline{DP}n = 49$) or 76–83°C ($\overline{DP}n = 131$), followed by fractionation (Asano, T. & Kakinuma, A., personal communication).

X-Ray diffraction patterns

X-Ray diffraction patterns of preparations in the powdered form were recorded on an imaging plate (DIP 100, MAC Science) using a graphite-monochromatized CuKα radiation (1·5418 Å) from an X-ray generator (ROTA FIEX RU-200, Rigaku).

Differential scanning calorimetry

Differential scanning calorimetry was carried out in a Super High Sensitive DSC 120 (Seiko Instruments Inc.). The dispersed curdlan obtained in a Waring blender was sealed in a silver pan of 70 μl. Distilled water was used as reference material. In order to obtain a hot baseline the weights of the water and curdlan suspension samples were the same. Calibration for temperature and enthalpy was done using indium as a standard. The temperature was raised from 30–170°C at a rate of 1°C min^{-1}.

Preparation of samples for electron microscopy

Samples of 0·1% neutralized gels and heated gels were examined by transmission electron microscopy. For negative staining, one drop of a suspension or solution of each preparation was mixed with a small amount of 2% uranyl acetate on a microscope slide. A microdrop of the mixture was deposited on a grid covered with carbon-coated film that had been subjected to ion-cleaning to remove contaminating oily material. The drop was dried in a dust-free fume cupboard.

Observation of samples by electron microscopy

Negatively stained preparations were examined under a Hitachi H-600FE electron microscope at an accelerating voltage of 100 kV. Electron micrographs were taken at an original magnification of 50 000.

RESULTS AND DISCUSSION

Firstly the authors, examined the X-ray diffraction patterns of powders of curdlan dehydrated with acetone after heating at various temperatures (Fig. 1). Fine powders obtained by freeze-drying from the wet centrifuge pellet without use of acetone gave almost the same pattern as the acetone powders with X-ray analysis, although the wet centrifuge pellet did not give a clear pattern. The peak at about $2\theta = 6°$ became sharper with increasing temperature, especially at above 120°C. Two peaks between 10 and 15° gradually appeared on heating at 120°C and three or five peaks between 15 and 25° also appeared on heating at 80°C and more. These peaks may be due to the formation of the triple stranded helix followed by hydrophobic interactions as judged by comparison with previously reported X-ray results (Takeda *et al.*, 1978; Fulton & Atkins, 1980; Chuah *et al.*, 1985; Okuyama *et al.*, 1991), and ^{13}C NMR (Saito *et al.*, 1991) studies. Referring to the report of Chuah *et al.* (1985), the curdlan peak at $2\theta = 6°$ corresponds to the (100) reflection with a spacing of 13·6 Å and two peaks between 10 and 15° correspond respectively to (110) reflection with a 7·82 Å spacing and (200) with a 6·78 Å spacing. Those between 15 and 25° correspond to the strong (113) reflection with a spacing of 4·98 Å,

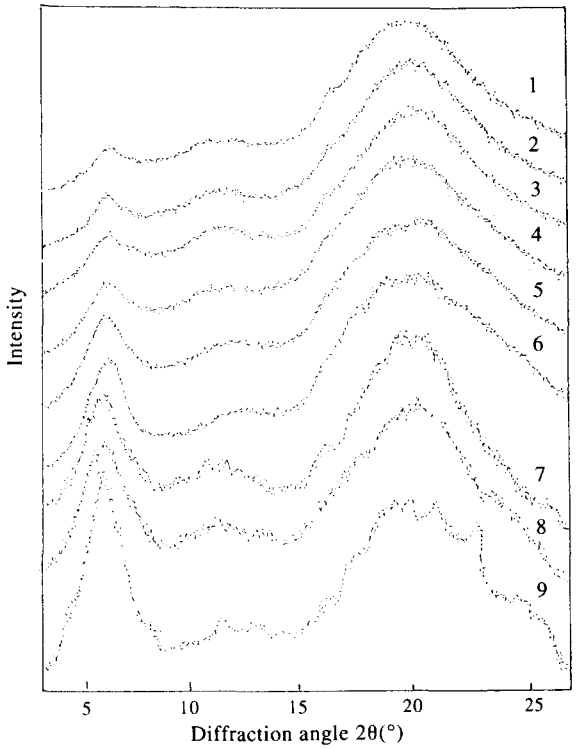

Fig. 1. X-Ray diffraction patterns of curdlan powders obtained after heating at various temperatures in water. Heating temperatures were: 1, without heating; 2, 55°C; 3, 60°C; 4, 70°C; 5, 80°C; 6, 100°C; 7, 120°C; 8, 145°C; 9, 170°C.

(203) with a spacing of 4·54 Å, (123) with a spacing of 3·93 Å, (303) with a spacing of 3·64 Å and (222) with a spacing of 3·61 Å.

Previously, an endothermic peak was observed at around 60°C, due to swelling on heating of both curdlan dispersion and the cooled curdlan gel (Konno *et al.*, 1994). The preparation obtained by freeze-drying without use of acetone gave a similar endothermic peak, which was independent of the method of dehydration. Figure 2 shows that this swelling temperature decreases with an increase in the heating temperature. The peaks of the samples heated at 120–170°C were almost the same. The difference between the swelling temperatures of the neutralized preparation and those heated at 170°C was about 8°C. The enthalpy decreased with an increase in the heating temperature, being about 0·5 mj mg^{-1} for the original curdlan and about 0·1 mj mg^{-1} for, preparation heated at 120–170°C. This decrease may be because the proportion of molecules capable of forming hydrogen bonds decreased as a result of hydrophobic interactions caused by heating at above 80°C. Heating preparations at 70–120°C causes irreversible interactions and the residual material may be susceptible to breakage of hydrogen bonds. At the temperature giving an exothermic peak between 140 and 160°C, the pseudo-crystalline form appeared, although the peak was reported to be due to the formation of microfibrils with an electron dense structure (Konno *et al.*, 1994). At 170°C, the pseudo-crystalline form was broken, as shown in previous studies (Konno *et al.*, 1994). When the preparation heated at 170°C was cooled to 20°C and then re-heated to 170°C without dehydration, the heating curve did not show an exothermic peak (Konno *et al.*, 1994). The structure might change to some extent during the process of preparation of powders. The X-ray diffraction patterns of the preparation heated at 170°C is noteworthy. The structure of curdlan molecules in the 170°C-set preparation showed the highest crystalline forms although the microfibrils did not have a pseudo-crystalline form (Konno *et al.*, 1994). Thus, the fine structure of neutralized powders differs significantly from those of curdlan obtained after heating at above 120°C, and especially above 160°C in water and cooling. Kuge *et al.* (1977) reported that curdlan gel melted at about 160°C but that the powders obtained from the melted matter did not form gel on re-heating to a higher temperature. On heating from 160–170°C, the ultra-structure changed from a pseudo-crystalline

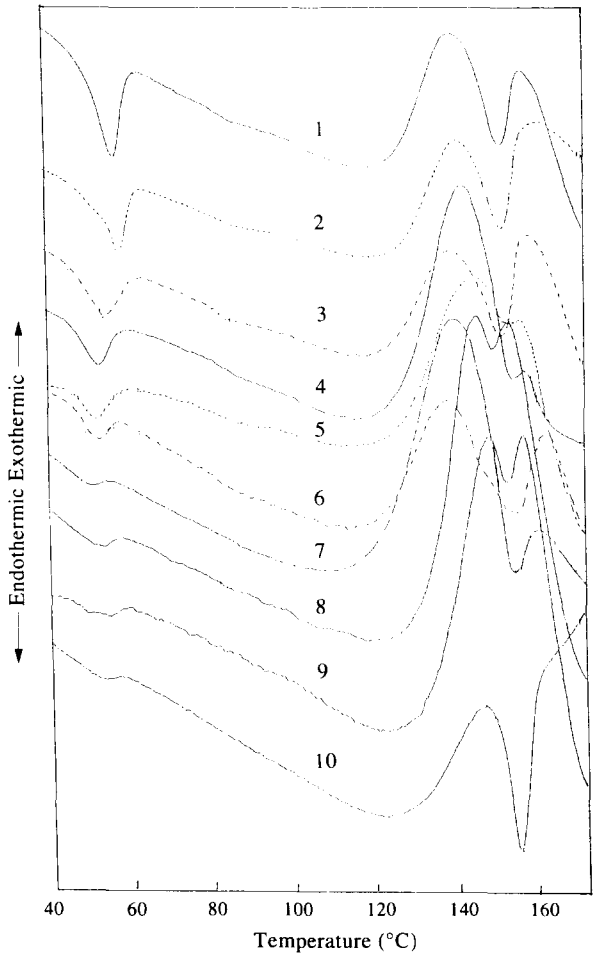

Fig. 2. Thermograms of curdlan powders obtained after heating at various temperatures in water. Heating temperatures were; 1, without heating; 2, 55°C; 3, 60°C; 4, 70°C; 5, 80°C; 6, 100°C; 7, 120°C; 8, 145°C; 9, 160°C; 10, 170°C.

form to microfibrils (Konno *et al.*, 1994), but the latter had a much higher crystallinity than the former, as shown in Fig. 1.

We examined the X-ray diffraction patterns of preparations of (1 → 3)-β-D-glucan of DPn 131 and 49 obtained with and without heating at 120°C, and found that they showed a higher crystallinity than the original curdlan (Fig. 3). The unheated preparations of DPn 49 showed a sharp peak around 6°, but preparations heated at 120°C showed much higher crystallinity than those without heating. Compared with the sharp diffraction around $2\theta = 6°$ for heated specimens A2 and B2, the corresponding diffraction for unheated preparation is broad and shifted to the lower angle (preparation A1), or has a shoulder at the low angle side (preparation B1). These features may come from the other modification of curdlan, the so called Form I (Okuyama *et al.*, 1991), which has a fairly strong diffraction at 15·4 Å ($2\theta = 5·7°$). These patterns of heated preparations were similar to that of the original curdlan heated at 120°C. By X-ray diffraction analysis, it is not possible to differentiate between the structures of gels in the original curdlan from those of sols of

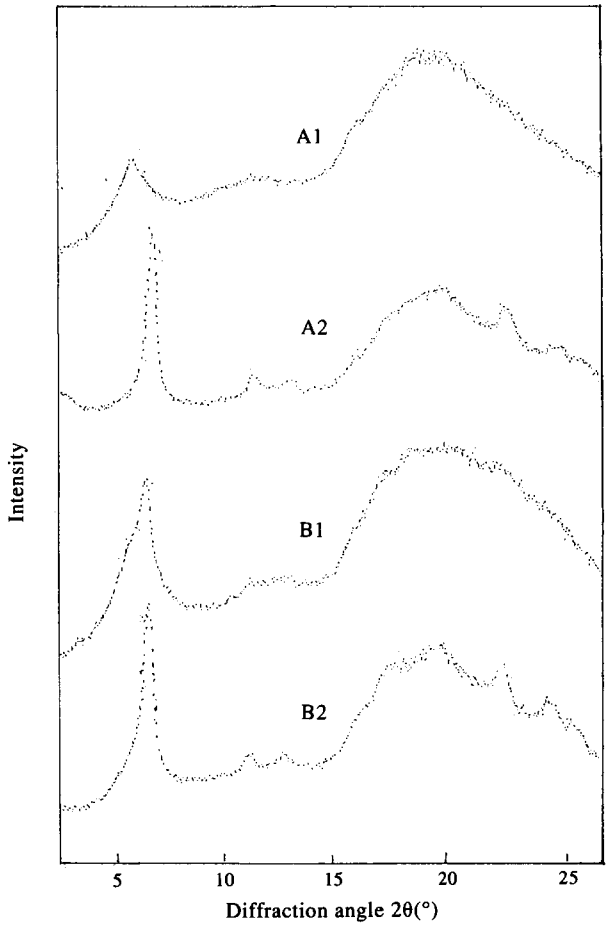

Fig. 3. X-Ray diffraction patterns of powders of low molecular mass (1 → 3)-β-D-glucan; $\overline{DP}$n 131 (A) and 49 (B) before (1) and after (2) heating at 120°C.

$\overline{DP}$n 131 and 49 (Nakanishi *et al.*, 1974; Ogawa *et al.*, 1973) in aqueous suspensions. Thermograms of low molecular mass (1 → 3)-β-D-glucan are shown in Fig. 4. The temperatures of endothermic peaks of the preparations of $\overline{DP}$n 131 and 49 without heating were about 45 and 27°C, respectively, which were lower than that of 55°C for the original curdlan and their enthalpies were 0·1 and 0·2 mj mg^{-1}, respectively, which were also less than that of the original curdlan. These results indicate that with a decrease in the size of the molecules, the breakage of their hydrogen bonds becomes easier. An exothermic peak above 130°C was observed as in the original preparation of curdlan. On heating at 120°C, the peak at 25°C of $\overline{DP}$n 49 disappeared, whereas the broad peak at about 40°C $\overline{DP}$n 131 remained almost unchanged.

Electron micrographs of the original curdlan and low molecular mass (1 → 3)-β-D-glucan have been reported (Harada *et al.*, 1979, 1991). To observe much clearer differences between images of the micrographs of curdlan and its low molecular mass form, we re-examined the micrographs of these compounds with and without heating at 120°C in water (Fig. 5). For this purpose, we used an improved Hitachi H-600FE electron microscope that gave a much more stable electron beam. A powder preparation is unsuitable for electron microscopy because micrographs of dispersions are required to determine the detailed structure. Therefore, we observed preparations prepared by dialysis of a 0·05% sample in 0·3 N NaOH and used the resultant neutralized dilute gels with and without heating at 120°C for 20 min. The microfibrils of the curdlan with-

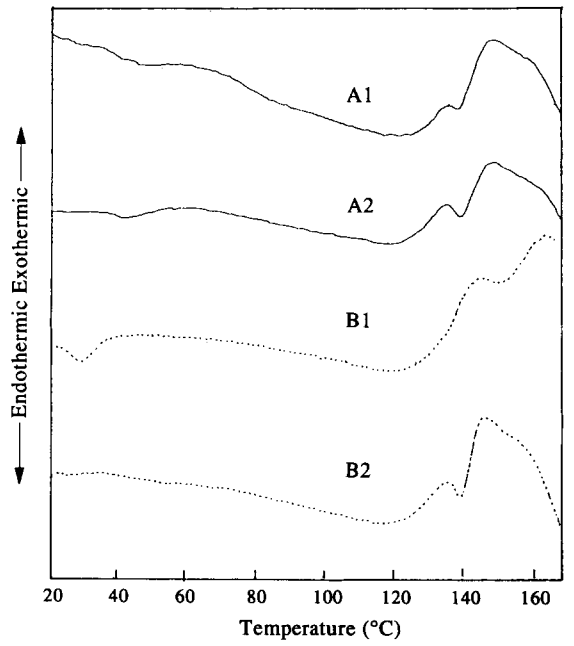

Fig. 4. Thermograms of powders of low molecular mass (1 → 3)-β-D-glucan, $\overline{DP}$n 131 (A) and 49 (B) before (1) and after (2) heating at 120°C.

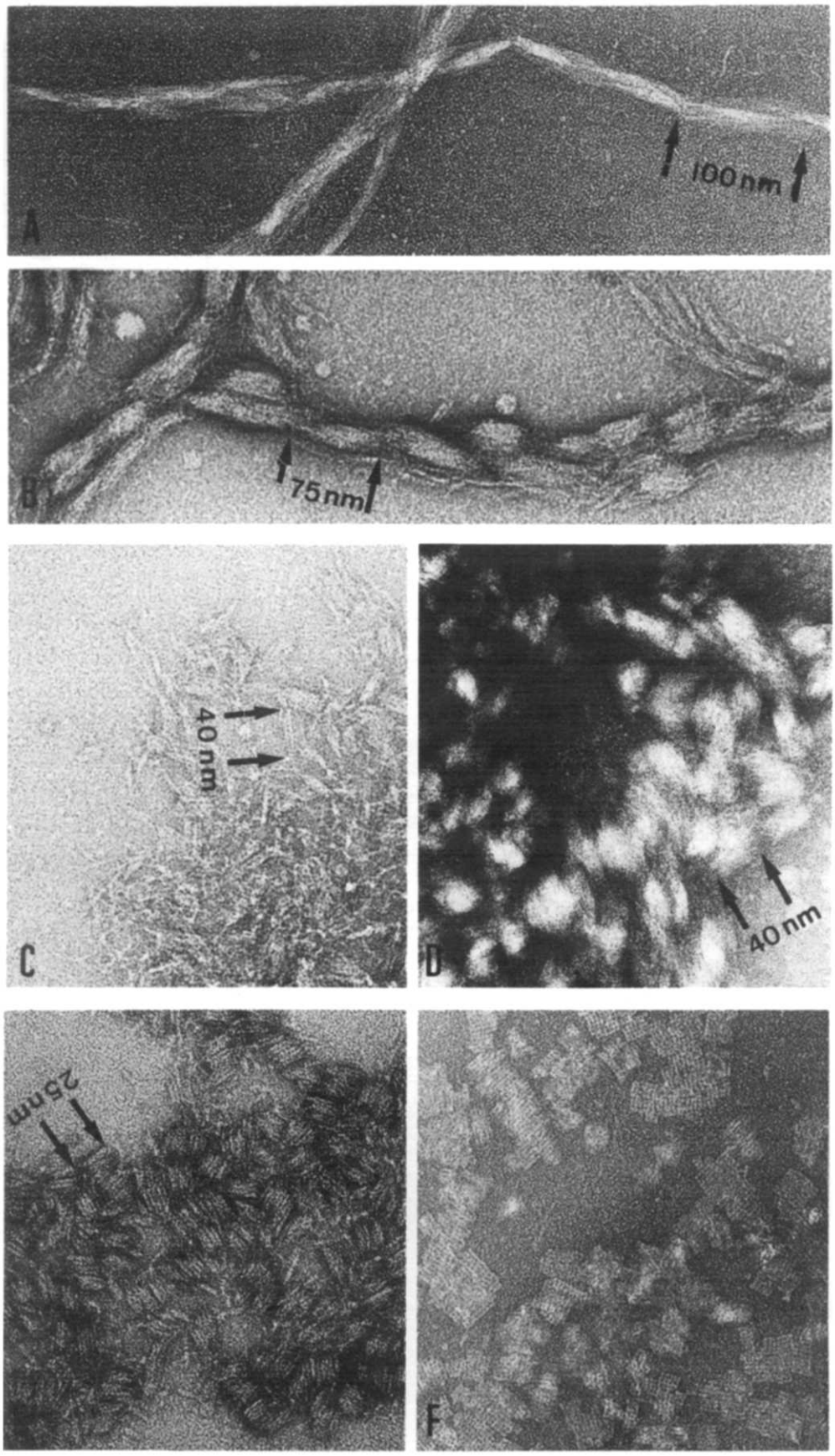

Fig. 5. Electron micrographs of the original curdlan ($\overline{DP}n$ 450) (A, B) and low molecular mass (1 → 3)-β-D-glucans, $\overline{DP}n$ 131 (C, D) and 49 (E, F) before (A, C, E) and after (B, D, F) heating at 120°C for 30 min.

out heating were endless forms composed of fibril units of about 100 nm length and 20–25 nm width, but those of the 120°C heated preparation were loosely combined or separated fibril units of about 75 nm length and about 30 nm width. The micrographs of $\overline{DP}$n 131 and 49 without heating differed, the former showing elementary fibrils of about 40 nm length and the latter showing elementary fibril units combined in parallel of about 25 nm length. The micrographs of heated preparations of $\overline{DP}$n 131 and 49 showed elementary fibrils each combined in parallel of about 40–50 nm length and elementary fibril units each combined in parallel to form crystals of different sizes, respectively. The preparations without heating with $\overline{DP}$n 450, 131 and 49 gave the structures of microfibrils with a length of about 100 nm, 40 nm and 25 nm, respectively. The ratio of calculated lengths in chains to average degrees of polymerization was 91:26:10 whereas the ratio of the length of microfibrils observed by electron microscopy was 40:16:10. The differences between the ratios may be caused by the behavior of the extending chains. The chains of fibrils in the preparations of $\overline{DP}$n 49 stretched in parallel with each other as shown in Fig. 5(E). However, the chains of fibrils in the samples of $\overline{DP}$ns 131 and 450 entangled, resulting in a reduction in their lengths as shown in Fig. 5(A) and (C). Fibril units in the original curdlan connect with each other in the axial direction to form long microfibrils on neutralization of an alkaline solution or on heating at 120°C, whereas microfibrils in preparations of $\overline{DP}$n 131 and 49, which are incapable of forming gel, are too short to connect with each other in the axial direction with or without heating. From these electron microscopic results, long, wide microfibrils may be required to form gels. Molecules forming such microfibrils tend to be immobile and involve water, thus forming a gel.

By X-ray diffraction analysis and DSC, behaviors of association of curdlan molecules in parallel on heating were recognized. The preparations were obtained after the samples observed by electron microscopy were precipitated with acetone, and gave similar X-ray patterns and DSC thermograms to the usual acetone powders. Thus, no information was obtained with X-ray analysis and DSC on formation of longer microfibrils by connection of fibrils with each other in the axial direction.

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