Carbohydrate Polymers **10** (1989) 299–313

Difference of Molecular Association in Two Types of Curdlan Gel

Yoshimi Kanzawa, Tokuya Harada*

Kobe Women's University, Suma-ku, Kobe-shi, 654, Japan

Atuo Koreeda, Akira Harada

Institute of Scientific and Industrial Research, Osaka University, Mihogaoka, Ibaraki-shi, 567, Japan

&

Kenji Okuyama

Faculty of Technology, Tokyo University of Agriculture and Technology, Koganei-shi, Tokyo, 184, Japan

(Received 26 October 1987; revised version received 3 February 1988; accepted 10 February 1988)

ABSTRACT

The molecular association in a curdlan gel formed by neutralizing an alkaline solution of curdlan with carbon dioxide was compared with those in gels obtained by heating aqueous suspensions of curdlan at various temperatures.

The neutralized and 60°C-set preparations were soluble in 0·01 M sodium hydroxide, whereas preparations set at above 90°C were soluble only in concentrations of sodium hydroxide above 1 M. The absorption of Aniline blue or Congo red to the preparations decreased with an increase in the temperature of heat treatment and the adsorption to a gel heated at 120°C for 4 h was about 30% of that for the unheated neutralized gel. Seventy-three per cent of the heated preparation was resistant to treatment with 32% sulfuric acid at 32°C for 30 days, whereas none of the neutralized gel was resistant. An electron micrograph of the resistant part of the curdlan showed that it had a pseudocrystalline form. X-ray studies showed a much higher crystalline structure in the resistant part than in the preparation without heat treatment. The X-ray patterns were almost the same for preparations treated with 32% sulfuric acid or (1→3)-β-glucanase.

*To whom correspondence should be addressed.

INTRODUCTION

Curdlan is a bacterial polysaccharide containing predominantly (1→3)-β-D-glucosidic linkages (Harada, 1983; Harada *et al.*, 1968, 1987). It becomes a firm, resilient gel when heated in aqueous suspension at above 80°C. We have shown that gel formation is caused by a hydrophobic interaction (Konno *et al.*, 1978) and that such gels exhibit a high level of syneresis (Takahashi *et al.*, 1986). Gels showing little syneresis can be formed when aqueous alkaline solutions of curdlan are neutralized with carbon dioxide (Kanzawa *et al.*, 1987) in the absence of heating. The two gels differ in that the gel obtained by neutralization is reversible, whereas that formed by heating at higher temperatures is irreversible (Kanzawa *et al.*, 1987). Moreover, the former gel is susceptible to (1→3)-β-D-glucanase, whereas a large part of the latter gel is resistant to this enzyme (Takahashi *et al.*, 1986). This paper reports studies on the difference in molecular associations of curdlan gels obtained with and without heating at various temperatures.

MATERIALS AND METHODS

Substrates

The curdlan used, which was produced by *Alcaligenes faecalis* var. *myxogenes* 10C3K, had a $\overline{DP}n$ of 455. The gel obtained by heating was prepared as follows. Aqueous suspensions of curdlan (1%) were heated at various temperatures for 30 min or 4 h. Then two volumes of acetone were added, and the mixtures were homogenized in a Waring blender. The homogenate was dehydrated with acetone by vacuum filtration at room temperature followed by drying *in vacuo* in the desiccator. Neutralized gels were prepared as follows (Kanzawa *et al.*, 1987). Appropriate amounts of curdlan were solubilized in 0·05 M sodium hydroxide with stirring to give a 1% solution. Beakers of the solution were kept in a carbon dioxide atmosphere in a desiccator and left to stand for 6 h. During this time the alkaline solution was neutralized to about pH 7·0 with carbon dioxide and became a gel. The gel was dehydrated and dried by the same method as mentioned above for the heated gel.

Solubility of curdlan gels in alkaline solutions of sodium hydroxide

To 5 ml of an aqueous suspension of the dehydrated gel, 5 M sodium hydroxide solution was added dropwise until the turbid mixture became clear. The concentration of sodium hydroxide required to solubilize the

preparation completely was calculated from the volume of sodium hydroxide added. The amount of soluble matter was determined as the difference between the amount of insoluble matter and that present initially, and calculated as a percentage of the initial amount of material.

Adsorption of dyes to curdlan preparation

An aqueous solution of Aniline blue (200 μg/ml) or Congo red (200 μg/ml) was added dropwise to 5 ml of aqueous suspension of the curdlan preparation (10 mg) with mixing using a magnetic stirrer until the solution became colored by unadsorbed dye. The amount of dye adsorbed was calculated by subtracting the dye remaining in the supernatant from the total amount of dye added.

Preparation of samples for acid hydrolysis

Neutralized curdlan gel was prepared as described above. Aliquots of the neutralized gel were heated at 60°C, 95°C or 120°C for 30 min or 4 h, and then homogenized in a Potter homogenizer and used in the acid hydrolysis study.

Acid hydrolysis

Mixtures of 50 ml of homogeneous aqueous suspensions of preparations and 50 ml of sulfuric acid at two concentrations were incubated at 32°C for 30 days. Aliquots of 2 ml or 10 ml were taken at intervals. The 2 ml was mixed with calcium hydroxide solution to neutralize the sulfuric acid. The resulting precipitate of calcium sulfate was removed by centrifugation and washed. The supernatant and washing solution were adjusted to a known volume for the measurement of the residual polymer.

Measurement of acid hydrolysis

The reducing power of the supernatant of the acid hydrolysate after treatment with calcium hydroxide was measured by the method of Somogyi (1952) and Nelson (1944).

Measurement of the residual polymer after acid hydrolysis

After incubation of the preparations with 32% sulfuric acid for appropriate times, the acid hydrolysates (10 ml) were centrifuged and the resulting precipitates were washed, dried *in vacuo* and weighed.

Preparation of curdlan with resistance to acid hydrolysis after heating at 120°C for 4 h

A preparation of the neutralized curdlan (5 g) gel was treated with 500 ml of 32% sulfuric acid for 30 days at 32°C and then centrifuged to remove the soluble sugars released and the remaining sulfuric acid. The precipitate was washed twice with 100 ml of water by centrifugation. The material that was resistant to sulfuric acid was mixed with 100 ml of acetone in a Waring blender and the mixture was filtered. The material on the filter was washed with acetone to remove the water and dried *in vacuo*.

Preparation of samples for electron microscopy

Samples of suspensions were taken before and after acid hydrolysis. After incubation for 30 days, the preparations were centrifuged at 50 000 ***g*** and the precipitate was washed, suspended in water and dialyzed against distilled water to remove the remaining sulfuric acid. A suspension of the precipitate was then used for electron microscopy. For negative staining, the method previously reported was used (Takahashi *et al.*, 1986).

Observation of samples by electron microscopy

Negatively stained preparations were examined in a Hitachi H-600FE, 100 kV, electron microscope with a field emission electron gun. Electron micrographs were taken at an original magnification of × 50 000.

X-ray diffraction patterns

The X-ray diffraction patterns of the preparations in the powdered form were obtained using a Rotaflex Ru-200 (Rigaku Denki) X-ray generator under the following conditions: Cu Kα radiation; voltage, 40 kV; current 25 mA; point-focusing camera; irradiation time, 36 h; camera length 34·9 mm.

RESULTS AND DISCUSSION

The solubilities in sodium hydroxide of 1% curdlan preparations with and without heating are shown in Table 1. Preparations obtained from alkaline solution by neutralization and from aqueous suspension by

TABLE 1
Solubilities in Sodium Hydroxide of Curdlan Preparations Obtained with and without Heating

	Heating conditions				
	None	*60°C/30 min*	*95°C/30 min*	*120°C/30 min*	*120°C/4 h*
NaOH concentration (M) for complete solubilization	0·01	0·01	1·0	1·3	2·2
Solubility in 0·01 M NaOH (%)	100	100	—	—	13

heating at 60°C for 30 min were soluble even in 0·01 M NaOH. The neutralized gel is similar to the gel obtained by heating the aqueous suspension at 60°C (Kanzawa *et al.*, 1987). Preparations obtained by heating at 95°C for 30 min and at 120°C for 30 min or 4 h were soluble in 1·0, 1·3 and 2·2 M sodium hydroxide, respectively; only 13% of the preparation obtained by heating at 120°C for 4 h was soluble in 0·01 M sodium hydroxide. The molecules of curdlan in preparations obtained by heating at higher temperatures appear to be associated much more closely.

Aniline blue is known to be absorbed to curdlan to form indigo blue (Nakanishi *et al.*, 1974) and Congo red is a stain for polysaccharides containing contiguous (1→4)-β-D-glucosidic linkage units and for some other polysaccharides, e.g., (1→3)-β-D-glucans including curdlan (Wood, 1980). Therefore, we next examined the ability of curdlan preparations with and without heating to form complexes with Aniline blue and Congo red (Table 2). The adsorptions of these dyes to preparations obtained by heating at 60°C, 95°C for 30 min and 120°C for 30 min or 4 h, were about 100%, 67%, 47% and 30%, respectively, of their adsorptions of the nonheated preparation. Heat treatment at higher temperatures results in closer molecular association and so these dyes which are soluble in aqueous solution may not have access to curdlan molecules. However, cellulose, which is an insoluble fiber, is known to adsorb Congo red strongly (Zevenhuizen *et al.*, 1986), so the mechanisms of adsorption of Congo red to curdlan and cellulose seem to differ.

Previously, we found that curdlan preparations obtained by heating at 120°C for 4 h were very resistant to (1→3)-β-D-glucanase (Takahashi *et al.*, 1986). Here, we examined the resistance of curdlan preparations formed with and without heating, to acid hydrolysis. First, we tested the effect of treatment with 16% sulfuric acid at 32°C, because this is the

TABLE 2
Formation of Complexes of Curdlan with Aniline Blue and Congo Red

		Heating conditions				
		None	*60°C/30 min*	*95°C/30 min*	*120°C/30 min*	*120°C/4 h*
Amounts of dye (mg) adsorbed to 10 mg curdlan	Aniline blue	0·94	0·94	0·61	0·45	0·27
	Congo red	0·57	0·54	0·36	0·23	0·17
Adsorption (%) of dye to curdlan	Aniline blue	100	100	69	48	29
	Congo red	100	100	64	46	30

concentration used to hydrolyze starch granules to obtain their crystalline part as amylodextrin (Kainuma & French, 1971). However, no hydrolysis of curdlan by 16% sulfuric acid was observed even after 30 days' incubation. So we next treated preparation with 32% of sulfuric acid at 32°C for 30 days. The (1→3)-β-D-glucosidic linkage was found to be much more resistant than the (1→4) and (1→6)-α-D-glucosidic linkages present in starch. The time courses of hydrolysis by 32% sulfuric acid of curdlan preparations with and without heating at 120°C for 4 h are shown in Fig. 1. The resultant soluble reducing sugars expressed as glucose and the amounts of remaining insoluble polymer were determined. After 15 days, when the hydrolysis had reached steady levels, the polymer of curdlan was completely solubilized, whereas about 67% of heat-treated polymer remained insoluble. The amount of soluble sugar in the hydrolysate of the treated polymer was about 30% of that in the unheated preparation after incubation for 30 days. Thus, the percentage of insoluble material calculated from the above data was about 70%. Values for the amount of soluble sugar released continued to

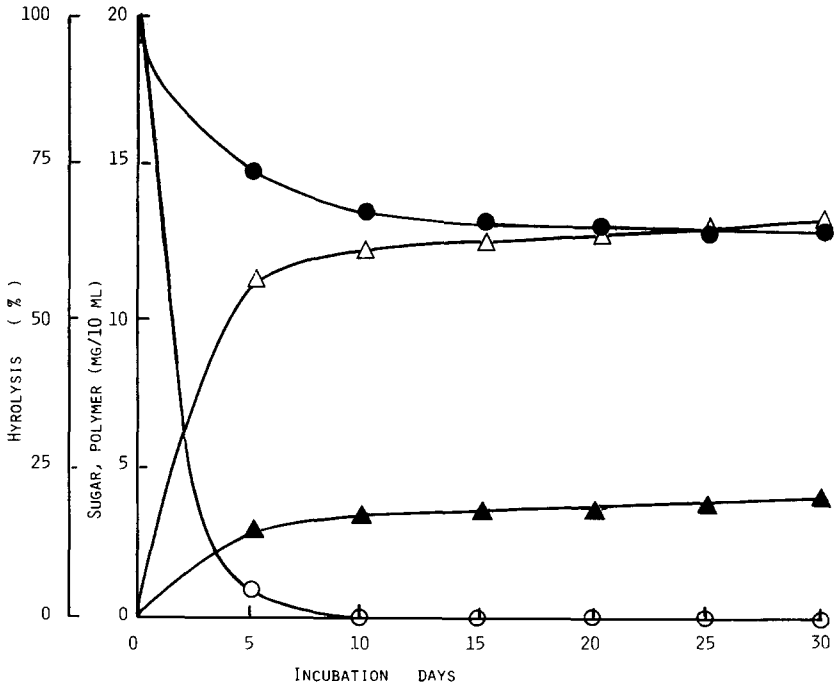

Fig. 1. Hydrolytic action of 32% sulfuric acid on preparations with (●, ▲) and without (○, △) heat treatment at 120°C for 4 h. Soluble sugars released, expressed as glucose (△, ▲); remaining polymer (○, ●).

increase until the end of the 30 days' incubation period, apparently because the released sugars were gradually hydrolyzed to smaller molecules and finally to glucose. The results of paper chromatography of the sugars supported this explanation. Data on the resistance of curdlan with and without heating to treatment with sulfuric acid for 30 days are summarized in Table 3. A small but significant percentage of the preparation obtained by heating at 60°C for 30 min was resistant, although the preparation was previously shown not to be resistant to enzymatic hydrolysis (Takahashi *et al.*, 1986). The reason for this difference is unclear. The percentage of the preparation resistant to acid hydrolysis determined by two methods (A and B) (Table 3), was 34–38%, 54–61% and 66–70%, respectively, for preparations prepared by heating at 95°C for 30 min, 120°C for 30 min and 120°C for 4 h. These values are close to those obtained for the resistance to (1→3)-β-D-glucanase (Takahashi *et al.*, 1986).

Aqueous suspensions of the parts of heated curdlan that were resistant to acid hydrolysis were further tested for resistance to Zymolyase — endo(1→3)-β-D-glucanase from *Arthrobacter* sp. — by the method described previously (Takahashi *et al.*, 1986). Less than 0·5% of the preparations were released as reducing sugars by this enzyme. Thus the resistance to enzymatic and acidic hydrolysis seemed to be due to the same mechanism.

Figure 2 compares the transmission electron micrographs of the acid-resistant parts with the original preparation for the various degrees of heating used. The micrographs of the acid-resistant parts were very similar to those obtained for the enzyme-resistant parts (Takahashi *et al.*, 1986). The regions of higher electron density seemed to be resistant to

TABLE 3
Resistance of Curdlan with and without Heating, to Treatment with 32% Sulfuric Acid for 30 Days at 30°C

	Percentage of resistant material with various heating conditions				
	None	*60°C/30 min*	*95°C/30 min*	*120°C/30 min*	*120°C/4 h*
A[a]	0	6	38	61	70
B[b]	0	5	34	54	66
Enzyme[c]	0	0	34	59	63

[a]Calculated from the amounts of sugars released by the treatment.
[b]Determined from the weight of solid matter remaining after treatment.
[c]Data from Takahashi *et al.* (1986).

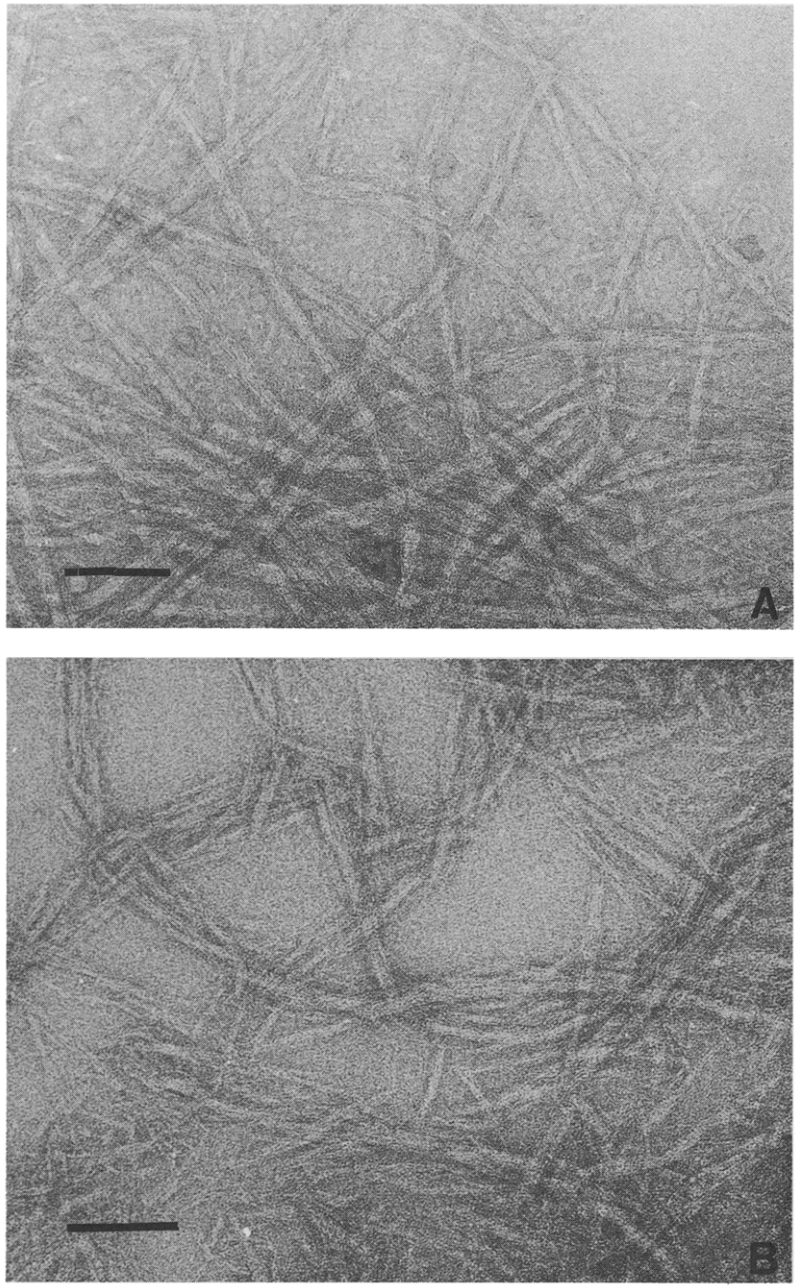

Fig. 2. Electron micrographs of a neutralized preparation (A) and heated preparations before (B, D, F) and after (C, E, G) hydrolysis with 32% sulfuric acid. Heat treatment was at 60°C for 30 min (B, C), at 95°C for 30 min (D, E) and at 120°C for 4 h (F, G). Bars represent 0·1 μm.

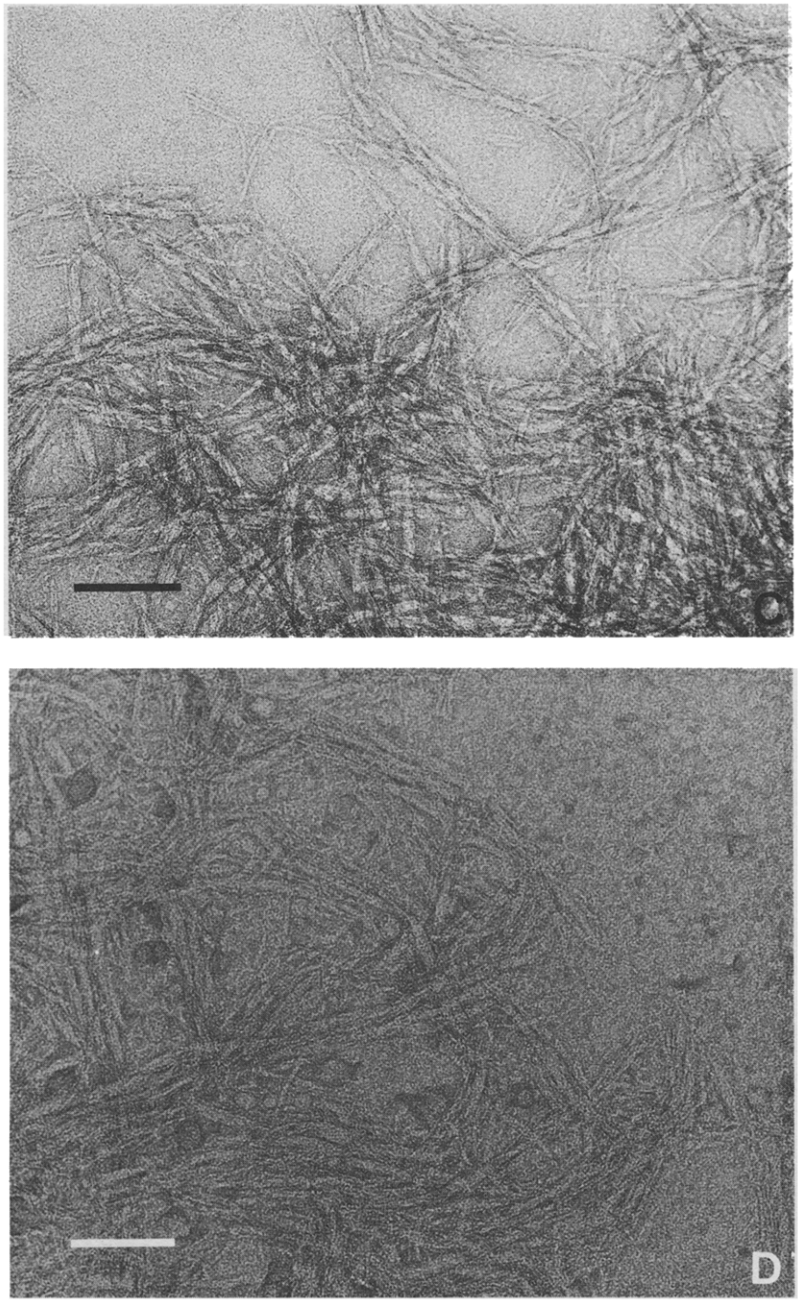

Fig. 2. — *contd.*

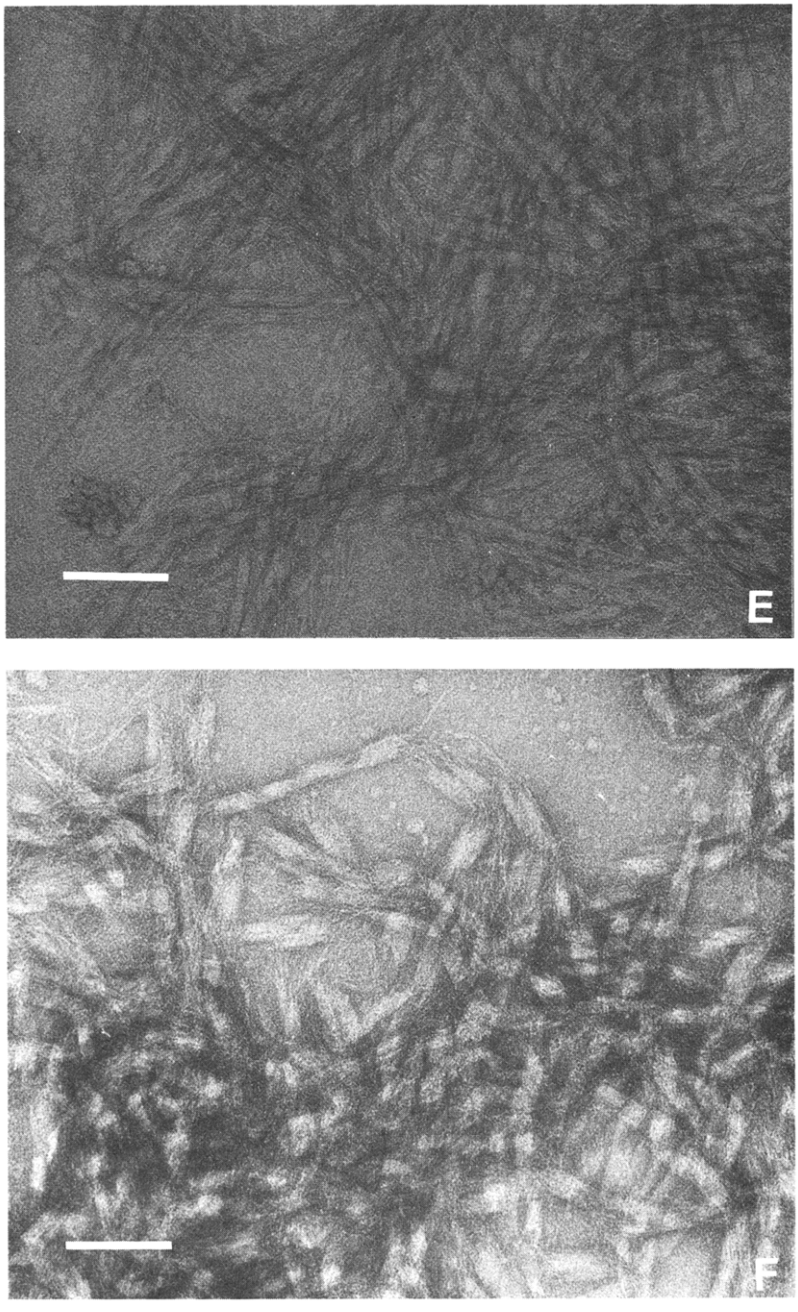

Fig. 2. — *contd.*

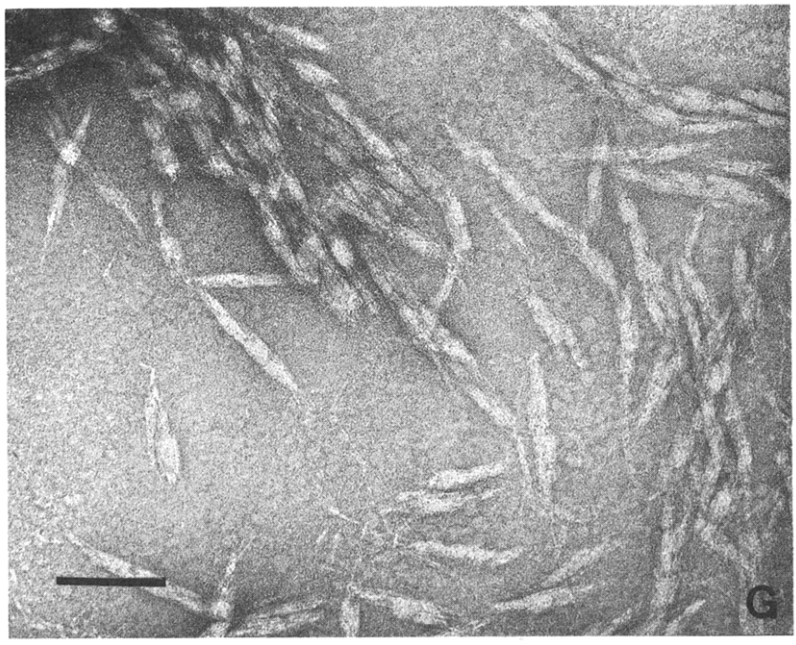

Fig. 2. — *contd.*

both enzymic and acid attack. The curdlan gel obtained without heating is formed by hydrogen bonds involving water, whereas molecules in resistant parts of the gels obtained with heating at higher temperatures may bind to each other with the release of water, causing syneresis. The elementary fibrils released seem to be destroyed by acid treatment. Previously, we reported that hydrophobic bonds are present in gels obtained by heating at higher temperatures (Konno *et al.*, 1978).

The X-ray diffraction patterns of the powdered preparations from the gel developed by neutralization (non-resistant part) and the resistant part of curdlan gels obtained by acid hydrolysis after heating at 120°C for 4 h are shown in Fig. 3 and the data are summarized in Table 4. The X-ray diffraction pattern of the part resistant to enzymatic hydrolysis obtained as described previously (Takahashi *et al.*, 1986) is also shown in Fig. 3. The resistant portions show much more crystallinity than the preparation formed without heating. Enzymic and acid-resistant preparations show very similar patterns. We reported previously (Maeda *et al.*, 1967) that the preparation formed by heating at 100°C for 30 min gave a more crystalline structure than that prepared without heating. Heat treatment at higher temperatures resulted in a pseudocrystalline form, unlike the

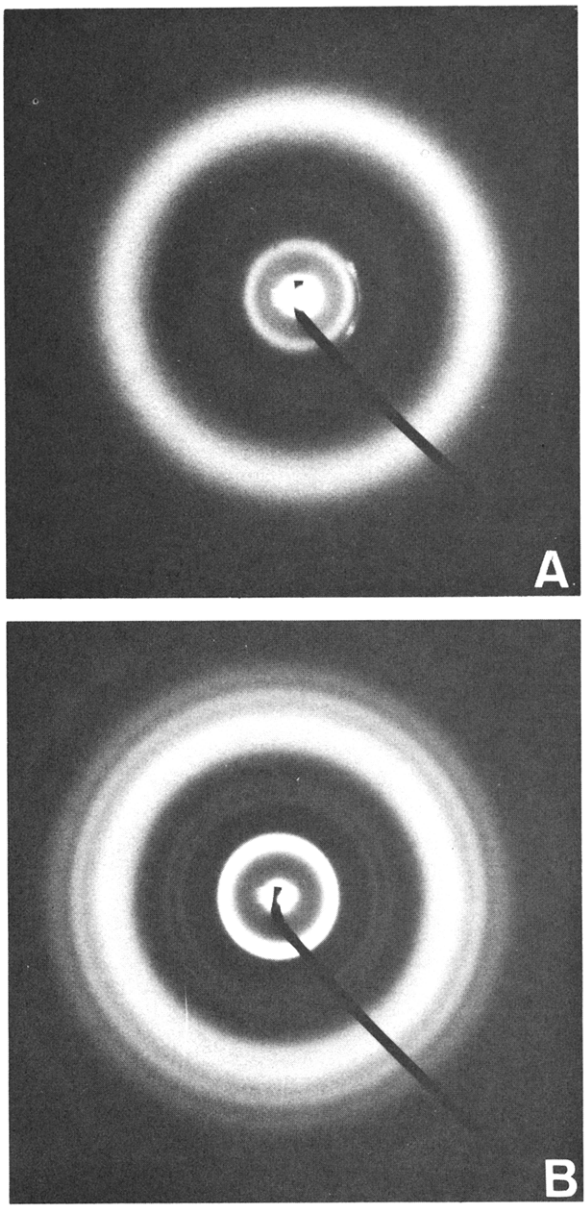

Fig. 3. X-ray diffraction patterns of curdlan powder with and without resistance to acid and enzyme hydrolysis. (A) neutralized preparation; (B) part resistant to acid hydrolysis in the preparation heated at 120°C for 4 h; (c) part resistant to enzyme hydrolysis in the above preparation.

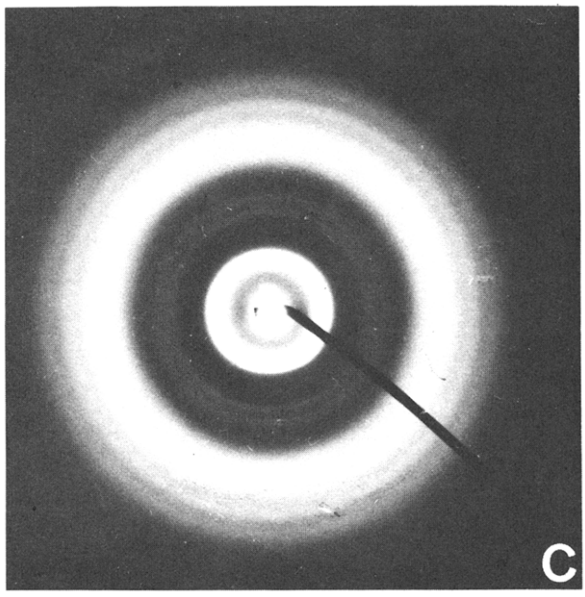

Fig. 3. — *contd.*

TABLE 4
Observed Spacings and Intensities of Powder Patterns of Gels That Were and Were Not Resistant to Acid Hydrolysis

Non-resistant		*Resistant*	
Spacing (Å)	*Intensity*	*Spacing (Å)*	*Intensity*
4·64	Broad	2·96	Very weak
8·25	Weak	3·63	Strong
17·39	Very strong	3·99	Strong
		4·44 broad	Medium
		4·86 broad	Medium
		5·93	Medium
		7·69	Medium
		13·50	Very strong

arrangement in the original or neutralized preparations without heating. Takeda *et al.* (1978) found by X-ray diffraction analysis of an extended preparation of curdlan that heating at 120°C converted curdlan from a single to a triple helix. Fulton and Atkins (1980) proposed a change in conformation of curdlan from a 7:1 triple helix to 6:1 triple helix on

heating in water. In any case, these results showed that, when curdlan in aqueous suspension is heated at higher temperature, it changes to a crystalline form with the release of water.

ACKNOWLEDGEMENTS

The authors thank Mr T. Ishibashi for skillful assistance in the electron microscopy and Mr Y. Ito for help in X-ray diffraction studies.

REFERENCES

Fulton, W. S. & Atkins, E. D. T. (1980). *Fiber Diffraction Method*, ed. A. D. French & K. H. Garner. *ACS Symp. Ser.*, **141**, 363–83.
Harada, T. (1983). *Biochem. Soc. Symp.*, **48**, 97–116.
Harada, T., Misaki, A. & Saito, H. (1968). *Arch. Biochem. Biophys.*, **124**, 292–8.
Harada, T., Sato, T. & Harada, A. (1987). *Bull. Kobe Women's Univ.*, **20**, 143–64.
Kainuma, K. & French, D. (1971). *Biopolymer.*, **10**, 1673.
Kanzawa, Y., Harada, T., Koreeda, A. & Harada, A. (1987). *Agric. Biol. Chem.*, **51**, 1839–43.
Konno, A., Kimura, H., Nakagawa, T. & Harada, T. (1978). *Nippon Nogei Kagaku Kaishi*, **52**, 247–50, (in Japanese).
Maeda, I., Saito, H., Masada, M., Misaki, A. & Harada, T. (1967). *Agric. Biol. Chem.*, **31**, 1184–8.
Nakanishi, I., Kimura, K., Kusui, S. & Yamazaki, E. (1974). *Carbohydr. Res.*, **32**, 47–52.
Nelson, N. (1944). *J. Biol. Chem.*, **132**, 375.
Somogyi, M. J. (1952). *J. Biol. Chem.*, **195**, 19–23.
Takahashi, F. & Harada, T. (1986). *Kasei-gaku Zashi*, **37**, 251–6.
Takahashi, F., Harada, T., Koreeda, A. & Harada, A. (1986). *Carbohydr. Polym.*, **6**, 407–21.
Takeda, T., Yasuoka, N., Kasai, N. & Harada, T. (1978). *Polym. J.*, **10**, 365–8.
Wood, P. J. (1980). *Carbohydr. Res.*, **85**, 271–87.
Zevenhuizen, L. P. T. M., Bertoechi, C. & von Neerven, A. R. W. (1986). *Antonie van Leeuwen Hoek*, **52**, 381.