Carbohydrate Polymers **6** (1986) 407–421

Structure of Curdlan that is Resistant to (1→3)β-D-Glucanase

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(Received: 27 February 1986)

SUMMARY

A structure that is resistant to (1→3)β-D-glucanase is formed when an aqueous suspension of curdlan ($\overline{DP}_n$ = 455) is heated to high temperatures. The resistance increases with increasing temperature and time of heat treatment. On similar treatment, (1→3)β-D-glucan ($\overline{DP}_n$ = 131) also becomes resistant to (1→3)β-D-glucanase. Electron microscopic examination showed it is a pseudocrystalline form with an electron-dense structure that is resistant to (1→3)β-D-glucanase, but that fine fibrils are disrupted by the enzyme. The resistant structure may be formed by a hydrophobic reaction.

INTRODUCTION

Curdlan, which is composed of (1→3)β-D-glucosidic linkages, forms a firm gel irreversibly when heated in aqueous suspension (Harada *et al.*, 1968; Harada, 1983). This polymer is produced by many strains of *Agrobacterium* and some strains of *Rhizobium*, and attempts have

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Carbohydrate Polymers 0144-8617/86/$03.50 —

been made to use it industrially and commercially as a food additive (Harada, 1983). Curdlan has been studied extensively as a thermogel (Harada, 1983). Its conformation has been shown by X-ray analysis to change from a single helix to a triple helix when it is heated as an aqueous suspension at above 120°C (Takeda *et al.*, 1978). This finding has been confirmed by ^{13}C-NMR studies (Saito *et al.*, 1981). Konno *et al.* (1978) showed that a hydrophobic reaction occurs when curdlan is heated at high temperatures. In this work we examined the change in the conformation of curdlan on heating by comparing the abilities of unheated curdlan and curdlan heated in aqueous suspension to act as substrates for (1→3)β-D-glucanase.

MATERIALS AND METHODS

Substrates

The sample of curdlan used was produced by *Alcaligenes faecalis* var. *myxogenes* 10C3K. Its $\overline{DP}_n$ was 455. A sample of the hydrolytic product of curdlan ($\overline{DP}_n = 131$) was kindly supplied by Takeda Chemical Industries, Japan, through Dr A. Kainuma. The sample was prepared as follows. Curdlan was hydrolyzed at 75°C for 30 min by 85% formic acid and its hydrolyzate was neutralized. The precipitate obtained by centrifugation from the product was fractionated with ethanol (27–70%) in water and the fractions with $\overline{DP}_n = 131$ were obtained. The $\overline{DP}_n$ was measured by the method of Manners *et al.* (1971).

Preparation of samples for enzymatic treatment

An aqueous suspension of curdlan or the polymer ($\overline{DP}_n = 131$) was prepared by neutralization, with 0·1 N HCl to pH 7, of an aqueous solution of the sample (100 mg) in 0·05 N NaOH (10 ml) followed by dialysis to remove the resultant NaCl. Volumes of 20 ml of the suspension in test tubes were heated at various temperatures, then tested as enzyme substrates.

Enzymes

Zymolyase T100 (Kitamura, 1982) and β-D-glucanase from *Trametes sanguinea* (Nara *et al.*, 1965) were obtained by courtesy of Dr Kita-

mura and Mr Nakanishi from Kirin Beer Co., Takasaki, Japan, and Takeda Chemical Industries, Takasago, Japan, respectively.

Enzymatic reaction

Mixtures of 20 ml of aqueous suspensions of samples, prepared as described above, 20 ml of 0·2 M phosphate buffer, pH 7·5, or 0·1 M acetate buffer, pH 4·8, and 10 ml of a solution of 1 mg of Zymolyase T100 or 50 mg of the enzyme preparation from *Trametes sanguinea* were incubated at 32°C for 8 h. Aliquots of 2 ml were taken at appropriate intervals and boiled for 5 min to inactivate the enzyme.

Measurement of hydrolysis of (1→3)β-D-glucosidic linkages

The reducing power of 1 ml samples of digests was measured by the method of Somogyi (1952) and Nelson (1944).

Measurement of remaining polymer after enzymatic hydrolysis

After incubation of the sample with an enzyme for 8 h, the reaction mixture (10 ml) was centrifuged and the resulting precipitate was dried *in vacuo* and weighed.

Preparation of samples for electron microscopy

Samples of suspensions were taken before and after enzyme treatment. In the latter case, after incubation with an enzyme for 8 h, the reaction mixture was not heated to stop the reaction but dialyzed to remove inorganic salts and the oligosaccharides produced. Then it was centrifuged at 50000*g*. In this way remaining insoluble polymer was precipitated and the supernatant containing soluble enzyme protein was removed. Water was added to the insoluble polymer precipitated, and the mixture was centrifuged to remove trace amounts of remaining enzyme protein. The suspension of the precipitate was used for electron microscopy. For negative staining, one drop of a suspension of each preparation was mixed with a small amount of uranyl acetate (2%) on a microscope slide. A micro-drop of the mixture was deposited on a grid covered with carbon-coated collodion film that had been subjected to ion-cleaning to remove oily material and was allowed to dry in air in a dust-free fume cupboard.

Observation of samples by electron microscopy

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

Paper chromatography

Paper chromatography was carried out by the descending method on Toyo-Roshi No. 50 paper with a solvent system of 1-butanol–pyridine–water (6:4:3, v/v). Sugars were detected on paper chromatograms with silver nitrate reagent.

RESULTS AND DISCUSSION

The actions of Zymolyase, a preparation of (1→3)β-D-glucanase from a strain of *Arthrobacter luteus*, on curdlan with and without heat treatment in aqueous suspension were examined at appropriate times, as shown in Fig. 1. The heating temperatures and times used were 60°C for 30 min, 95°C for 30 min, and 120°C for 30 min and 4 h. The temperature of 60°C is the starting temperature for making a 'low-set' gel (Maeda *et al.*, 1967), 95°C is the temperature for making a 'high-set' gel as a result of hydrophobic bond formation (Konno *et al.*, 1978), and 120°C is thought to be the temperature at which the conformation changes from a single to a triple helix (Takeda *et al.*, 1978). Figure 1 shows the initial rates and the final extents of hydrolysis. The rates of hydrolysis of preparations of curdlan in aqueous suspension decreased in the following order: with heat treatment at 60°C for 30 min, without heating, with heat treatment at 95°C for 30 min, and with heat treatment at 120°C for 30 min and 4 h. The final extents of hydrolysis of the preparations without heating and with heating at 60°C for 30 min were almost the same while the extents of hydrolysis of the samples prepared by heating at 95°C and 120°C were considerably lower. The resistance of the preparations to hydrolysis was calculated from the results in Fig. 1 and expressed as a percentage of the resistance of the unheated preparation, because no residual polymer was obtained in the hydrolyzate of the unheated preparation. These results are shown in Table 1 (column a). The amount of residual poly-

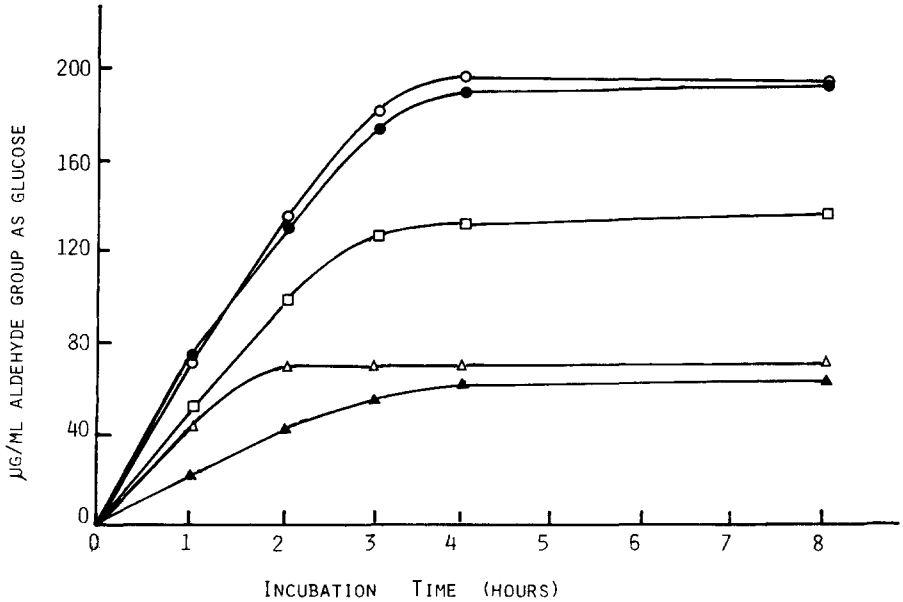


Fig. 1. Hydrolytic action of (1→3)β-D-glucanase (Zymolyase) on curdlan ($\overline{DP}_n$ = 455) preparations that have been subjected to various heat treatments: ○, without heating; ●, with heating at 60°C for 30 min; □, with heating at 95°C for 30 min; △, with heating at 120°C for 30 min; ▲, with heating at 120°C for 4 h.

mer in the hydrolyzates was determined by centrifugating the various enzymatic hydrolyzates and drying and weighing the precipitate (Table 1, column b). Values given in columns a and b in the table, determined from the amount of sugar released and the dry weight of the residual polymer, respectively, were essentially the same. The preparation after heat treatment at 60°C for 30 min (low-set gel) was not resistant to the enzymatic action. This is probably because, as has been previously suggested (Maeda *et al.*, 1967), this structure is maintained by hydrogen bonding. The percentage of enzyme-resistant material formed by heat treatment was about 30–34% in that heated at 95% for 30 min, and 59–65% and 70–73% in samples prepared by heating at 120°C for 30 min and 4 h, respectively. The resistant structure may be formed by a hydrophobic reaction since a hydrophobic reaction occurs when an aqueous suspension of curdlan is heated at above 80°C (Konno *et al.*, 1978). In fact, preparations of curdlan after heat

TABLE 1
Resistance of Curdlan ($\overline{DP}_n = 455$) Gel to Enzymatic Hydrolysis by Zymolyase ((1→3)β-D-glucanase)

Conditions of heat treatment	*Percentage of resistant curdlan gel*[a]	
	a[b]	*b*[c]
None	0	0
60°C, 30 min	1	0
95°C, 30 min	30	34
120°C, 30 min	65	59
120°C, 4 h	70	73

[a] These values were obtained from suspensions that had been incubated for 8 h with the enzyme.
[b] Calculated from the amounts of sugars released by enzyme treatment of curdlan with and without heat treatment.
[c] Determined from the weight of solid matter remaining after enzyme treatment.

treatment at 95°C and 120°C (high-set gel) are insoluble in 0·1 N NaOH but soluble in dimethylsulphoxide.

On paper chromatograms of hydrolyzates after incubation of various preparations for 8 h, the spots corresponding to glucose were strongest and many spots from oligosaccharides were detected. A comparatively intense spot of $R_{glucose}$ 0·40 was identified as laminaribiose, by comparison with moving of the spots of standard laminaribiose obtained with three different developing solvent systems in the paper chromatograms. These results were quite different from those of Kitamura *et al.* (1974). They observed the formation of laminaripentaose only in a hydrolyzate of curdlan with Zymolyase. The difference between their results and the results of this study may be due to differences in the purities of the enzyme preparation used: their preparation may have been much purer. Crude Zymolyase may contain an enzyme(s) producing a large amount of glucose.

Takahashi & Harada (1968) reported that the gel obtained by heat treatment at 120°C shows high syneresis resulting from the formation of hydrophobic bonds. Incorporation of water into this structure may become difficult. Thus, we examined how the structure of curdlan gel caused by heat treatment at high temperatures changes on enzyme treatment. The fine structures of gels formed with and without heating have been previously examined by electron microscopy (Koreeda *et al.*, 1974; Harada *et al.*, 1979). Therefore, in this work, gels formed by heat treatment at 95°C for 30 min or 120°C for 4 h before and after enzyme treatment for 8 h were examined by electron microscopy, and compared with the preparations of curdlan without heating and with heating at 60°C, which have not been treated with enzyme (Fig. 2). As previously mentioned, no polymer remained after enzyme treatment of the unheated preparations and preparations heated to 60°C. The 95°C-set preparation (Fig. 2(C)) showed a complicated arrangement of microfibrils connected to each other by elementary fibrils while the preparation after enzyme treatment (Fig. 2(D)) showed microfibrils without fine elementary fibrils. Thus, the elementary fibrils seemed to be destroyed by enzyme treatment. In curdlan gel prepared by heating at 120°C for 4 h before enzyme treatment (Fig. 2(E)), electron dense materials associated with microfibrils that were bound with fine fibrils were observed. After enzyme treatment (Fig. 2(F)) only leaf-like pseudocrystalline forms were observed without fine fibrils. Thus, a crystalline structure that was resistant to enzyme treatment was formed on heating the aqueous suspension. The crystalline form obtained as a precipitate after enzyme treatment was estimated by the method of Manners *et al.* (1971) to have $\overline{DP}_n$ of about 350. Thus, the end of the curdlan molecule heated at 120°C was hydrolyzed by the enzyme. This suggests that the major part of the curdlan molecule participates in a pseudocrystalline structure and the ends of the molecules not involved in this structure are attacked by the enzyme.

The structures remaining after enzyme treatment of the gels formed by heating at 95°C and 120°C contained different proportions of the original polymer and were also different in their appearance when examined by the electron microscope. The difference in electron microscopic appearance might be due to differences in the amounts of hydrophobic bonds. In gels heated at 120°C, more hydrophobic bonds were formed resulting in triple or multiple helices (Takeda *et*

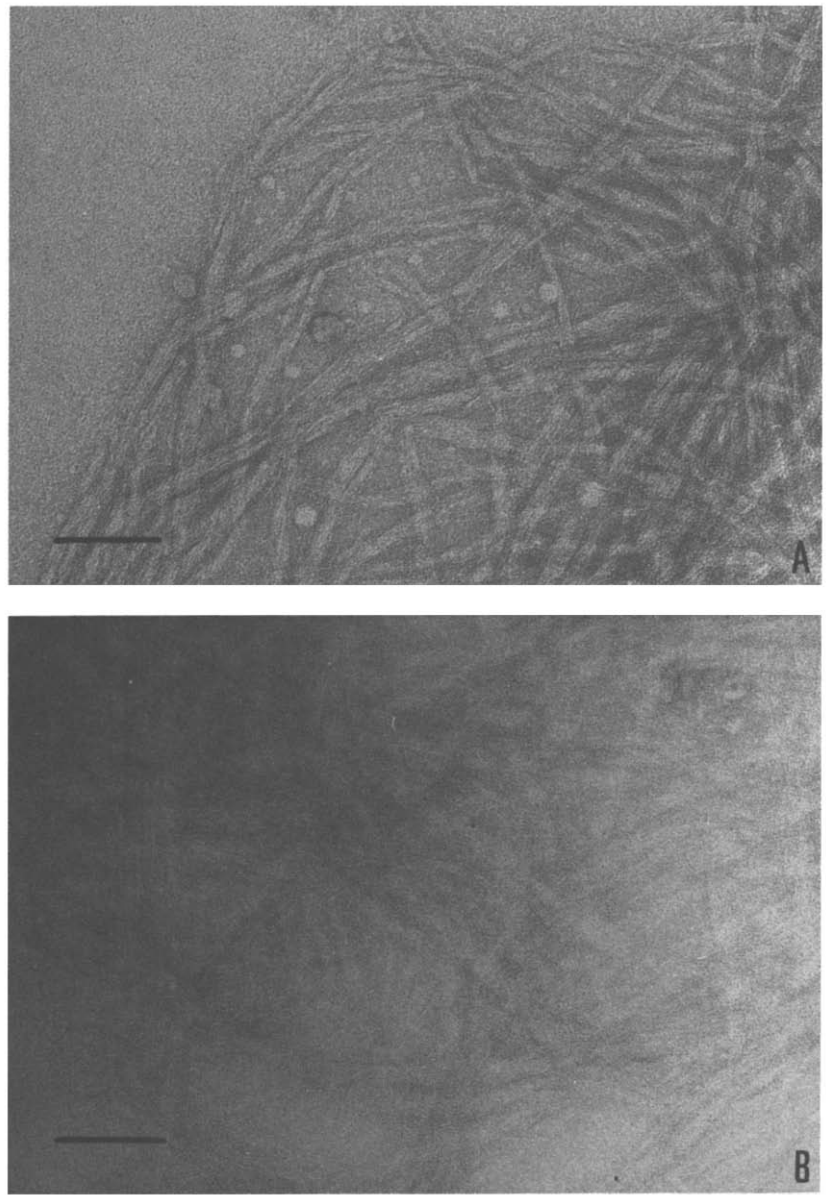


Fig. 2. Electron micrographs of curdlan ($\overline{DP}_n = 455$) before and after enzyme treatment: (A) regeneration from alkaline solution on neutralization with hydrochloric acid; heating at 60°C for 30 min without enzyme treatment (B) at 95°C for 30 min (C and D) and at 120°C for 4 h (E and F) before (C and E) and after (D and F) enzyme treatment. Scale bars represent 0·1 μm.

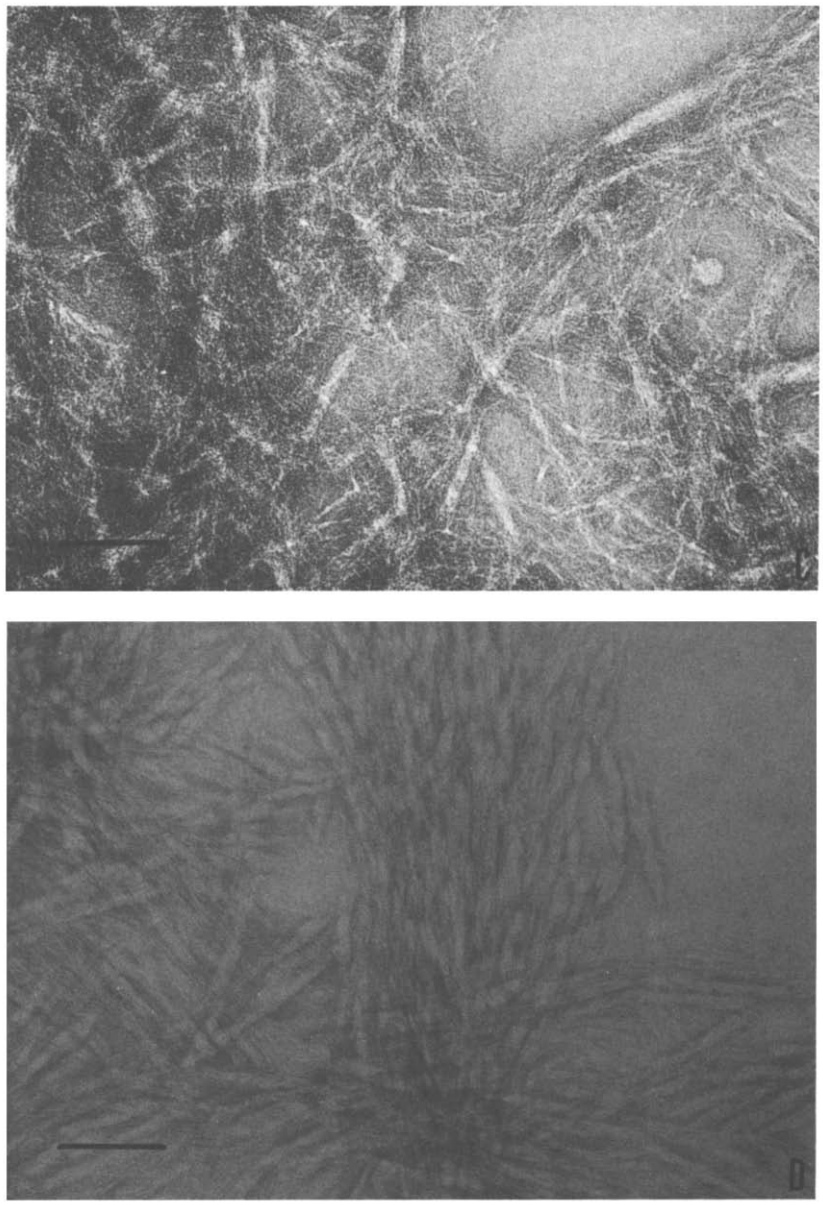
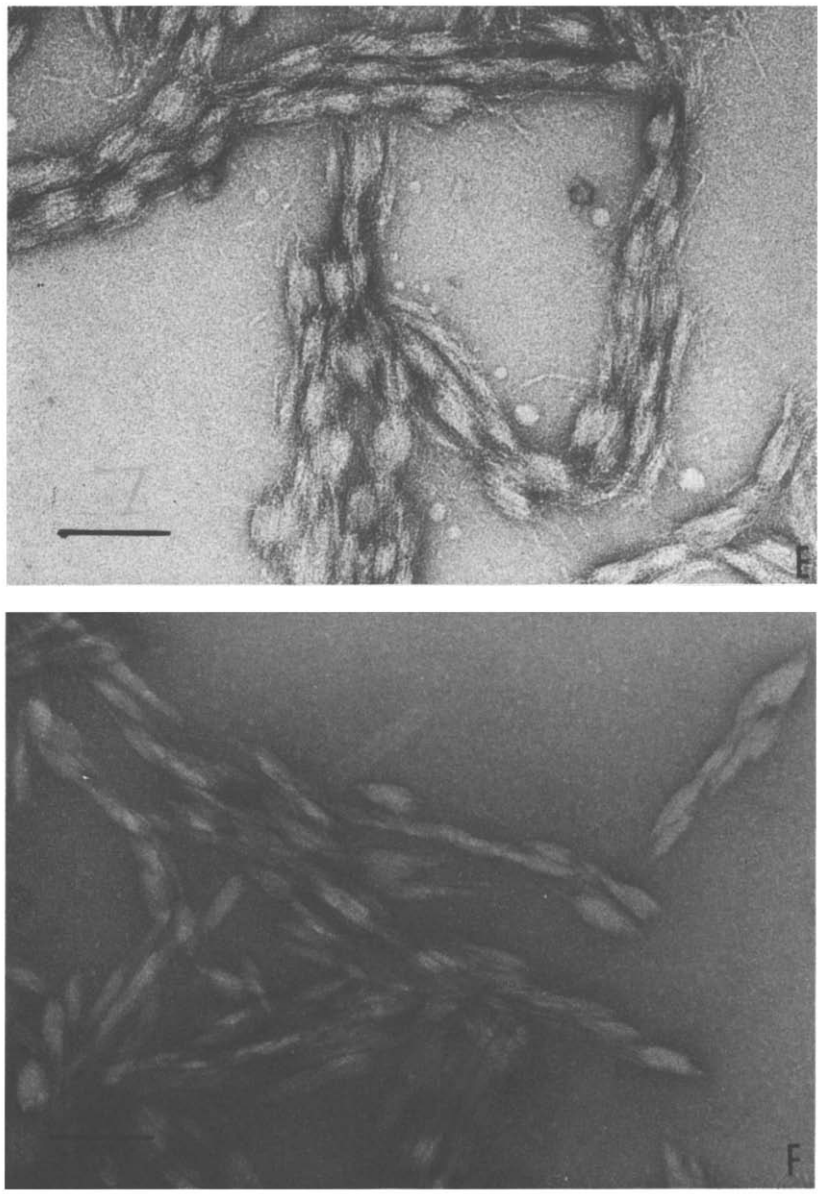


Fig. 2 — *contd.*



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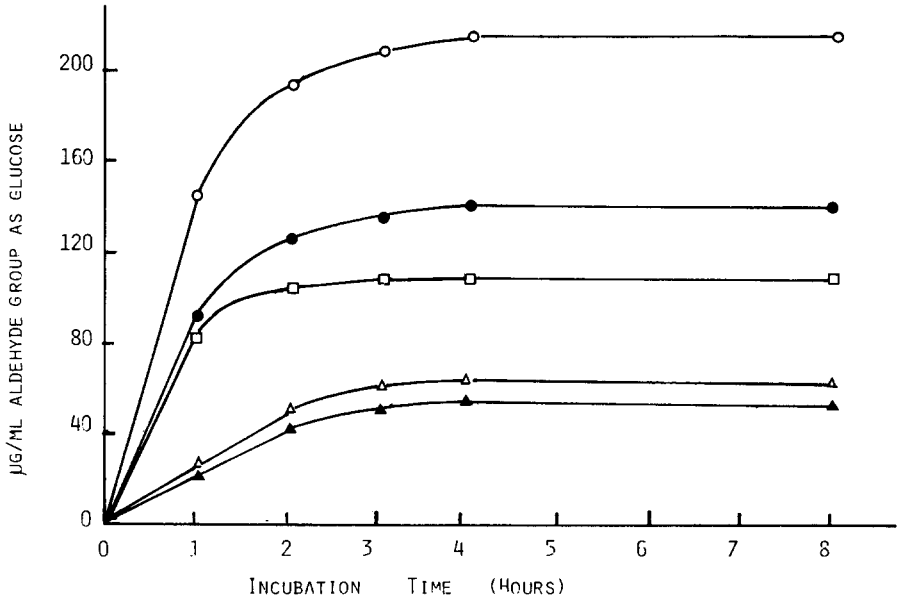

Fig. 3. Hydrolytic action of (1→3)β-D-glucan (Zymolyase) on the polymer ($\overline{DP}_n = 131$) preparations that have been subjected to various heat treatments: ○, without heat-treatment; ●, with heating at 60°C for 30 min; □, with heating at 95°C for 30 min; △, with heating at 120°C for 30 min; ▲, with heating at 120°C for 4 h.

al., 1978) and thus the gels would be expected to have a higher electron density.

Enzymes produced by *Trametes sanguinea* can hydrolyze curdlan. The various preparations of curdlan described above were treated with these enzymes by the same method as that used with Zymolyase. Similar patterns of enzymatic action were obtained. The reducing powers of all the preparations were about twice those obtained with Zymolyase. The enzyme from *Trametes* produced a much larger amount of glucose than that produced by Zymolyase in the final stage of the enzyme treatment.

The action of Zymolyase on (1→3)β-D-glucan of $\overline{DP}_n = 131$, a hydrolysis product of curdlan, was examined (Fig. 3). Preparations of unheated glucan and glucan heated at 60°C for 30 min, at 95°C for 30 min and at 120°C for 30 min or 4 h were used as aqueous suspensions. The preparation without heat treatment was hydrolyzed fastest

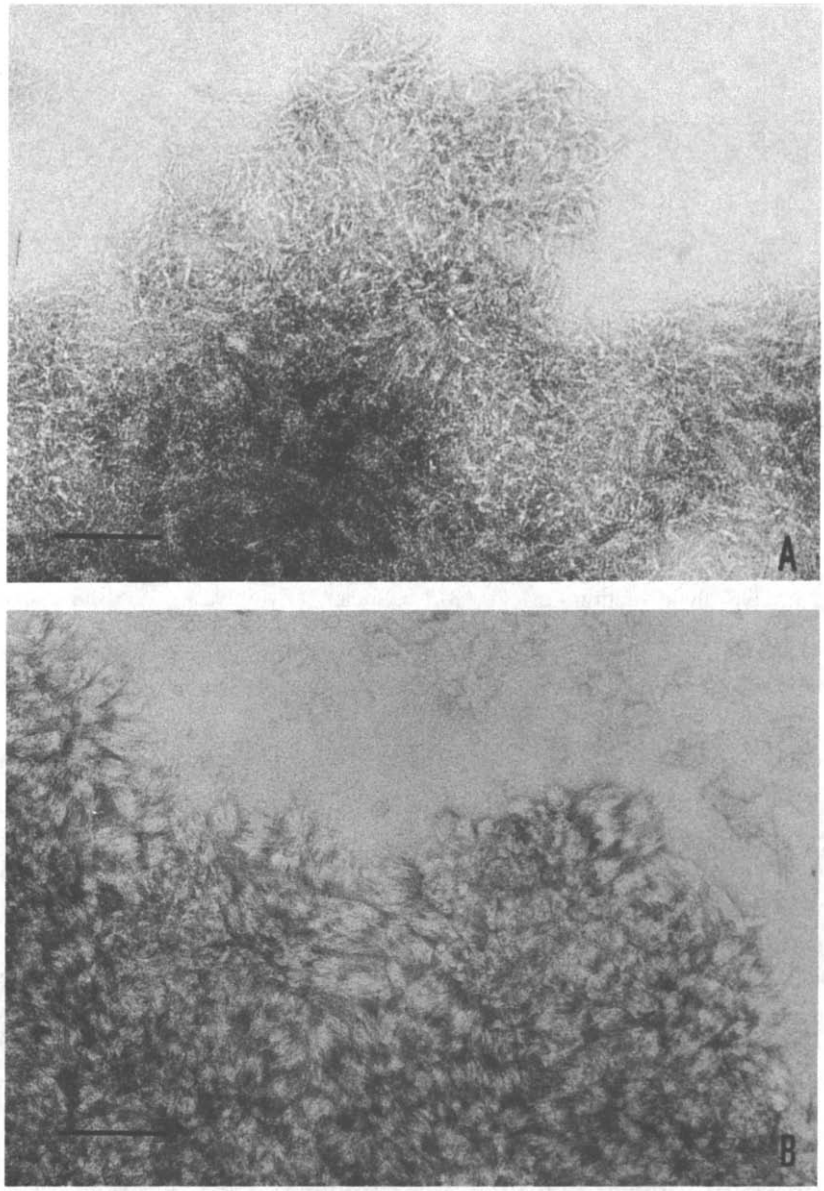

Fig. 4. Electron micrographs of the polymer ($\overline{DP}_n = 131$) after enzyme treatment: (A) regenerated from alkaline solution on neutralization with hydrochloric acid; heating at 60°C for 30 min (B), at 95°C for 30 min (C), and at 120°C for 4 h (D). Scale bars represent 0·1 μm.

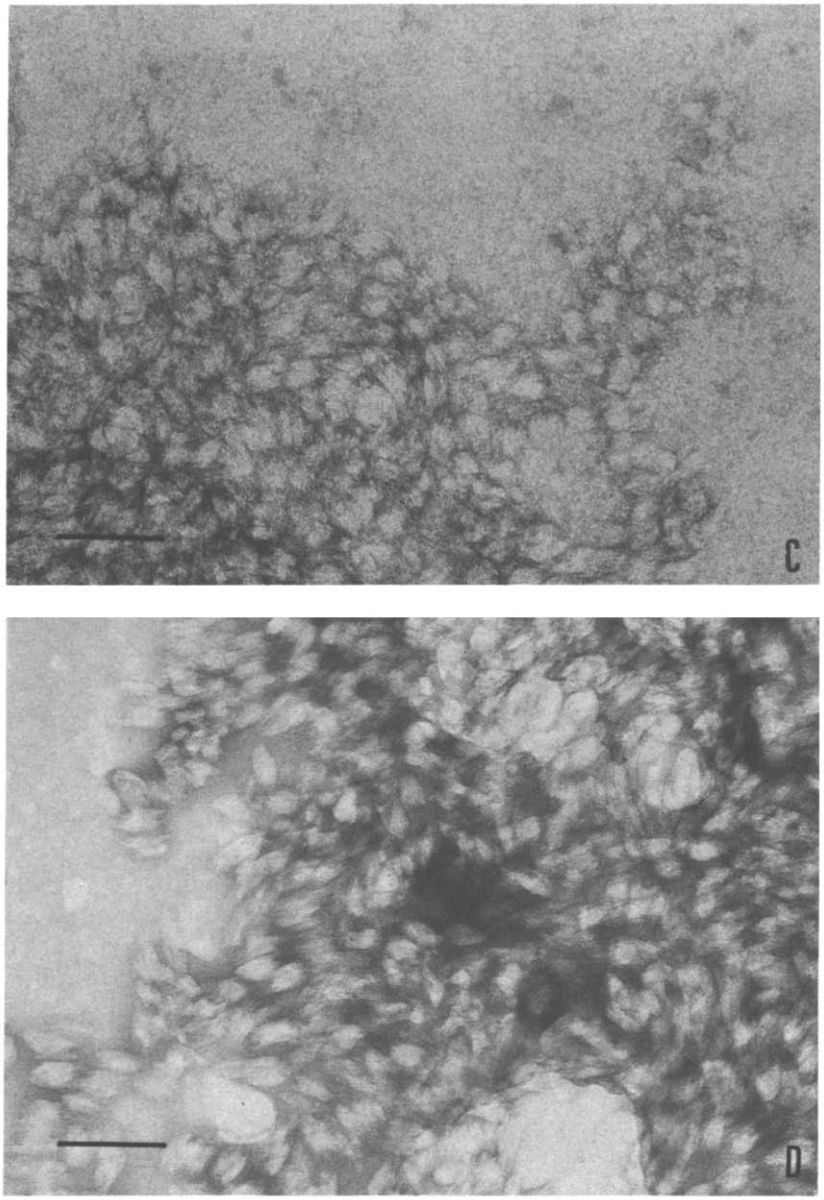

Fig. 4 — *contd.*

and most extensively. The final extents of hydrolysis were about 65% after heat treatment at 60°C, about 50% after heat treatment at 95%, and about 30% and 25% after heat treatment at 120°C for 30 min and 4 h, assuming that the rate of hydrolysis of unheated glucan was 100% because no polymer was precipitated from the unheated sample after enzyme treatment. Electron micrographs of the polymers remaining after enzyme treatment of gels formed by heating at 60°C and 95°C for 30 min and at 120°C for 4 h are shown in Fig. 4. The amount of resistant polymer of $\overline{DP}_n = 131$ was much more than that of curdlan ($\overline{DP}_n = 455$); in particular, the resistance of low-set gels of the two samples, formed by heating at 60°C, were very different. We consider that hydrophobic bonds are formed much more easily on heating at lower temperatures in the polymer ($\overline{DP}_n = 131$) than in curdlan ($\overline{DP}_n = 455$). Electron micrographs of preparations heated at 60°C for 30 min, at 95°C for 30 min and at 120°C for 4 h had almost the same appearance. This finding supported the above conclusion that hydrophobic bonds are formed even on heating at 60°C.

ACKNOWLEDGEMENT

We thank Dr M. Hisamatsu, Mie University, for preliminary studies of this work and Mr T. Ishibashi, Osaka University, for skillful assistance with the electron microscope.

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