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Acid hydrolysis behaviour of microbial cellulose II

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A mutant strain of *Acetobacter xylinum* produces cellulose of anomalous band-like form ('native band'), and this material has been found to be cellulose II, presumably having a folded-chain structure (according to recent work by Kuga *et al.*). In addition to the previous results of electron diffraction, X-ray analysis showed that this band material was composed of virtually pure cellulose II. We have studied the acid hydrolysis behaviour of this material to obtain additional evidence for the proposed structure. When hydrolysed with 1 N hydrochloric acid at 100°C, the degree of polymerization (*DP*) of the material decreased rapidly from 322 ($\overline{DP_w}/\overline{DP_n} = 3.83$) to 18.3 ($\overline{DP_w}/\overline{DP_n} = 1.19$). The latter value (levelling-off *DP*) corresponds to the observed width (10 nm) of strand-like constituents of the band material. The sample dissolved in and regenerated from 8.75% aqueous sodium hydroxide lost its original characteristic morphology and became irregular-shaped agglomerates. The levelling-off *DP* of this regenerated sample was 55.2 ($\overline{DP_w}/\overline{DP_n} = 2.53$), a typical value for common regenerated celluloses. These findings as a whole strongly suggest that the cellulose molecules in the native band are selectively cleaved at sharply folded parts by acid, producing fragments of the length of folding periodicity.

(Keywords: bacterial cellulose; acid hydrolysis; chain folding)

INTRODUCTION

Acetobacter xylinum produces a ribbon of crystalline cellulose I microfibrils, which is extruded into the medium. The ribbons are extruded parallel to the longitudinal axis of the cell. With this bacterium, a mutant strain is known to produce an anomalous form of cellulose called 'native band' cellulose[1–3]. This material has a broad, band-like shape, and is extruded perpendicularly from the cell. As examined by transmission electron microscopy, native band cellulose is composed of strand-like structures with a lateral dimension of 10 nm. Electron diffraction analysis revealed that these strands are composed of well defined cellulose II crystallites, in which glucan chains are oriented perpendicular to the strand axis. On the other hand, the degree of polymerization (*DP*) of this material was determined to be ca. 200 by size exclusion chromatography[2]. These facts strongly suggested that the band material is composed of cellulose II molecules with a chain-folded structure.

The occurrence of regular chain folding is an established phenomenon for certain synthetic linear polymers. For instance, the crystalline lamella of polyethylene has a chain-folding structure, and the chain length at which folding occurs varies with the crystallization temperature. To prove this, Keller showed that the molecular-weight distribution of polyethylene treated with nitric acid did not decrease continuously but through development of discrete peaks in size exclusion chromatography, finally resulting in one peak corresponding to the thickness of a lamella[4,5]. As for polysaccharides, mannan was confirmed to form a chain-folding structure under certain artificial conditions[6,7]. On the other hand, the possibility of a folded-chain crystal of underivatized cellulose has been studied extensively[8–11], but conclusive evidence has been lacking.

In this paper, the acid hydrolysis behaviour of native band cellulose was studied in detail, to obtain further evidence for the occurrence of regular chain folding in this material.

EXPERIMENTAL

Cellulose samples

The following cellulose materials were prepared for acid hydrolysis:

(a) wild-type normal bacterial cellulose,
(b) mutant native band cellulose,
(c) regenerated native band cellulose, and
(d) refined cotton linter

The wild-type strain of *Acetobacter xylinum* (ATCC23769) produces a tough pellicle consisting of

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strongly entangled ribbon-like microfibrils at the surface of Schramm–Hestrin (SH) culture medium[12]. The culture was statically incubated at 28°C for 6–9 days until the pellicle was about 8 mm thick. The harvested pellicle was rinsed with distilled water and boiled in aqueous 1% NaOH for 30 min to remove the incorporated cells and culture medium components. Then the pellicle was thoroughly washed with distilled water to neutrality. The defibrated bacterial cellulose from the wild-type strain was used as a control for experiment.

A mutant native band-producing strain was derived from ATCC23769 as reported[2] and statically incubated in SH medium. The product was formed at the surface of liquid culture as a fragile thin layer. This material was purified in the same way as above.

Native band cellulose shows a relatively low average degree of polymerization (DP)[2], and could be dissolved in aqueous NaOH solutions of higher than 8% (w/w). Never-dried native band cellulose was dissolved in 8.75% NaOH solution. The dissolved cellulose was precipitated by diluting the solution with 10 times volume of distilled water and neutralized with dilute hydrochloric acid. In this procedure the cellulose sample was recovered quantitatively.

Acid hydrolysis

Approximately 50 mg of cellulose was suspended in 10 ml of 1 N hydrochloric acid in a test tube and heated in a water bath at 100°C for various periods (5–180 min). After treatment, the test tube was cooled immediately and the sample suspension was diluted with a large amount of water. The insoluble residue was collected by centrifugation, and washed with distilled water to neutrality.

Electron microscopy

The sample was negatively stained with 2% uranyl acetate solution on an ultrathin carbon film and examined with a transmission electron microscope (JEOL 2000EX) at 100 kV.

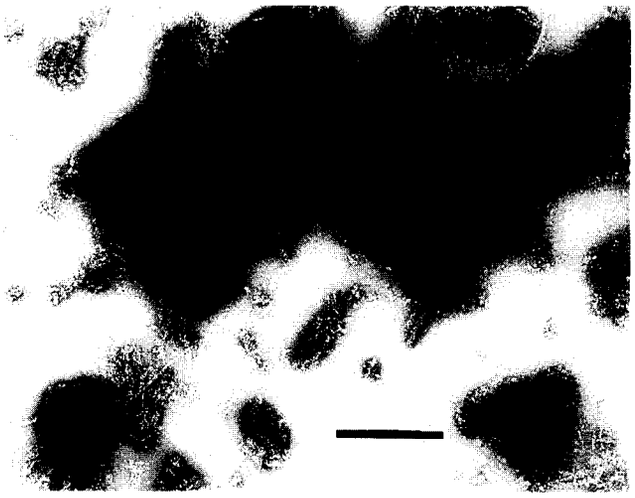
X-ray diffraction

The freeze-dried native band cellulose and normal bacterial cellulose were pressed into a pellet of 6 mm diameter (ca. 3.5 mg). X-ray diffraction patterns were recorded with a Rint 2000 (Rigaku) diffractometer with Ni-filtered Cu Kα radiation generated at 40 kV and 30 mA. The scanning rate was 0.5 deg min^{-1} of the diffraction angle 2θ.

Determination of molecular weight

The hydrolysate was freeze-dried and converted to cellulose trinitrate by treating with an ice-cooled mixture of nitric acid and phosphorus pentoxide for 30 min[13]. The product was collected on a filter paper and thoroughly washed with cold distilled water. The cellulose nitrate was dissolved in tetrahydrofuran and analysed by size exclusion chromatography: columns, TSK GMHXL and TSK G2500H6, each 300 × 7.5 mm i.d., connected in this order; Tosoh pump (Shimadzu, LC6A); u.v. detector (S310AM2, Soma Optics). The solvent, tetrahydrofuran, was eluted at 0.5 ml min^{-1}. The elution peak was converted into a molecular weight of polystyrene ($MW_{\text{polystyrene}}$) by the calibration obtained from a series of monodisperse polystyrene standards

(a)



(b)

(c)

Figure 1 Transmission electron micrographs of (a) native band cellulose, (b) native band cellulose hydrolysed with 1N HCl for 60 min and (c) sonicated hydrolysate of native band cellulose. Scale bars are 1 μm for all micrographs

($MW = 8.42 \times 10^6$, 6.77×10^6, 2.89×10^6, 1.30×10^6, 7.91×10^5, 4.46×10^4, 9.7×10^3, 2.8×10^3 9.5×10^2, 5.5×10^2, 1.0×10^2; Tosoh). The DP of the sample was given as:

$$DP_{\text{cellulose}} = MW_{\text{polystyrene}}/C$$

where C is the experimentally determined correction factor[14]. Reference 14 shows a relation between polystyrene and cellulose trinitrate in ångström lengths on size exclusion chromatography study. We obtained:

$C = 0.0015$ at $3\,300\,000 - 450\,000$ $MW_{\text{polystyrene}}$ range

$C = 0.00167$ at $450\,000 - 160\,000$ $MW_{\text{polystyrene}}$ range

$C = 0.00195$ at $160\,000 - 50\,000$ $MW_{\text{polystyrene}}$ range

The DP determined by this method for standard cellulose (Filter Pulp, Advantec; $\overline{DP_w} = 216$, $\overline{DP_n} = 69$) agreed well with its viscosity-average DP ($DP_v = 200$).

RESULTS AND DISCUSSION

Figure 1a is a transmission electron micrograph of the mutant native band cellulose, showing the broad, band-like shape different from the ribbon-like microfibrils produced by the normal strain. The acid hydrolysate of the native band cellulose treated for 60 min largely retained its original morphology, but small broken pieces were frequently observed (*Figure 1b*). By sonication (Fischer Sonic Dismembrator model 300) for 30 s, the hydrolysate was disintegrated into strand-like components (*Figure 1c*). The morphology of the original native band was scarcely changed by the same sonication treatment. These differences before and after hydrolysis indicate that the material was severely degraded by acid.

Figure 2 shows X-ray diffraction patterns of the native band and normal bacterial cellulose by the reflection method. The normal cellulose gave a typical X-ray pattern for cellulose I (data not shown). The native band cellulose is well defined cellulose II. For each sample, the X-ray diffractogram for never-dried wet samples before purification was the same as this pattern (data not shown). Thus, the purification by using alkali does not affect the morphology and crystal structure of the native band cellulose.

Polystyrene standards gave a calibration curve as shown in *Figure 3*. The change in slope at $MW \simeq 1.0 \times 10^4$ is caused by the use of a series of two columns. $MW_{\text{polystyrene}}$ was calibrated to the cellulose samples as determined from this graph and converted into $DP_{\text{cellulose}}$. *Figure 4* shows the change in the size exclusion chromatogram of cotton linter by acid hydrolysis. While the cotton linter before acid hydrolysis had a broad and complicated distribution pattern, as hydrolysis proceeded the DP shifted to a lower range and the distribution narrowed. The normal ribbon bacterial cellulose had a high average DP and a narrow distribution. Upon acid hydrolysis, the chromatograms shifted to a lower DP range with a broader distribution (*Figure 5*). On the other hand, the native band cellulose had a symmetrical pattern, and the distribution became sharper by acid hydrolysis (*Figure 6*).

The changes in average DP_w by acid hydrolysis are plotted in *Figure 7*. The DP was decreased by acid hydrolysis, and the existence of a so-called levelling-off

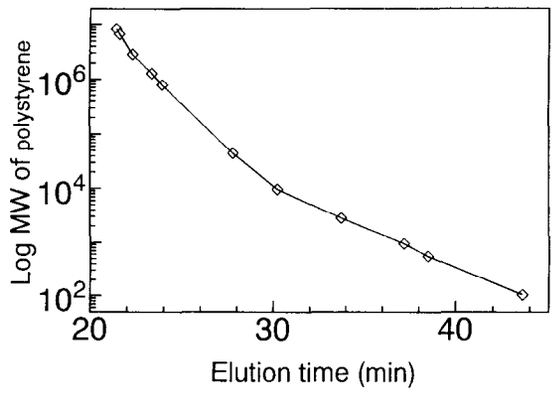


Figure 3 Calibration curve for monodisperse polystyrene standards

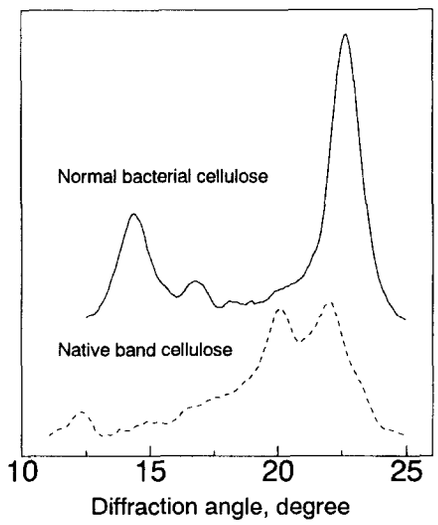


Figure 2 X-ray diffraction diagrams of normal ribbon cellulose and native band cellulose

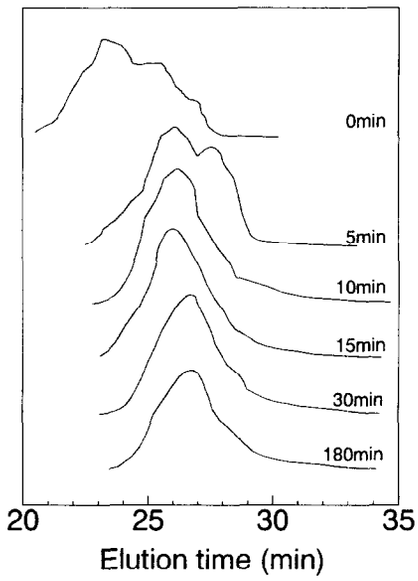


Figure 4 Change in size exclusion chromatogram of cotton linter cellulose by hydrolysis with 1 N HCl at 100°C

DP (*LODP*) was observed for each sample. Cotton linter had a *LODP* of around 260. The *LODP* of normal wild-type ribbon bacterial cellulose was about 650. The *LODP* of the mutant native band cellulose was 18.3, and the *DP* distribution of the hydrolysate was noticeably sharp ($\overline{DP_w}/\overline{DP_n} = 1.19$,) being virtually monodisperse.

As an independent evaluation of *DP*, the acid hydrolysate was examined with ^{1}H n.m.r. The number-average *DP* was calculated from the ratio of the number of the C1 proton to that of the proton at the reducing end. The calculated DP_n was about 17, agreeing closely with the chromatography results.

For normal ribbon bacterial cellulose and refined cotton linter, the *LODP* is considered to correspond to certain periodicity in the higher-order structure of cellulose, such as the length of microcrystallite[15,16]. As for the native band cellulose, the *LODP* seems to correspond to its folding period of cellulose chains in the strand. A typical width of the strand composing the band material as seen by TEM was 10 nm (ref. 2). Thus the *LODP* obtained from acid hydrolysis is in excellent agreement with the proposed folded-chain structure. The native band cellulose probably is hydrolysed selectively at the edges of the folded sheets, where parts of cellulose molecules are exposed to the medium. The uniformity of hydrolysate's *DP* indicates a high regularity in the period of chain folding. Thus the acid hydrolysis behaviour of the native band cellulose strongly supports the occurrence of cellulose chain folding.

To confirm further the relation between the band morphology and the periodicity of *DP* 20, regenerated native band cellulose was hydrolysed by acid. By regeneration from alkali solution, the native band cellulose completely lost its characteristic band-like morphology as in *Figure 8*. *Figure 9* shows the change in size exclusion chromatograms of regenerated native band cellulose by acid hydrolysis. The rapid decrease in average *DP* and levelling-off behaviour were the same as those of original native band cellulose, but the *DP* distribution of hydrolysate of regenerated native band cellulose was broader than the original material. Also, its

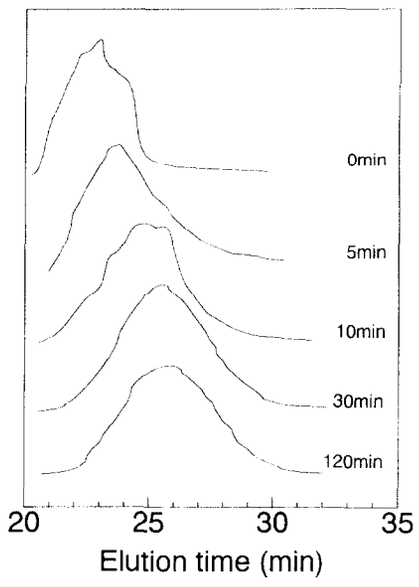


Figure 5 Change in size exclusion chromatogram of normal bacterial cellulose by hydrolysis with 1 N HCl at 100°C

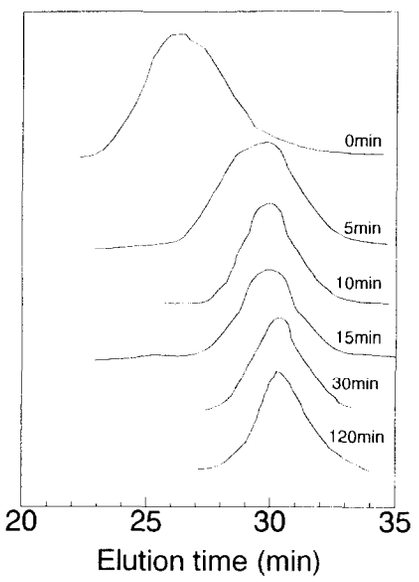


Figure 6 Change in size exclusion chromatogram of native band cellulose by hydrolysis with 1 N HCl at 100°C

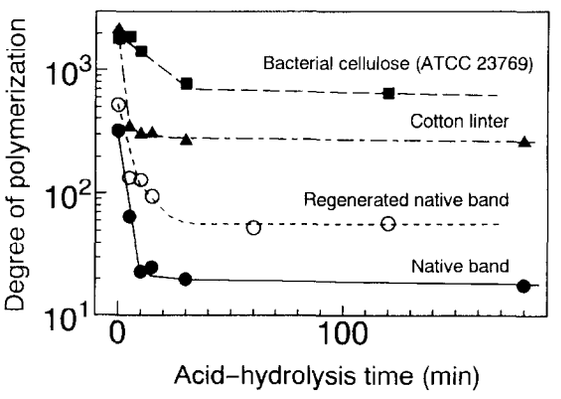


Figure 7 Change in average DP_w of cellulose samples by hydrolysis with 1 N HCl at 100°C

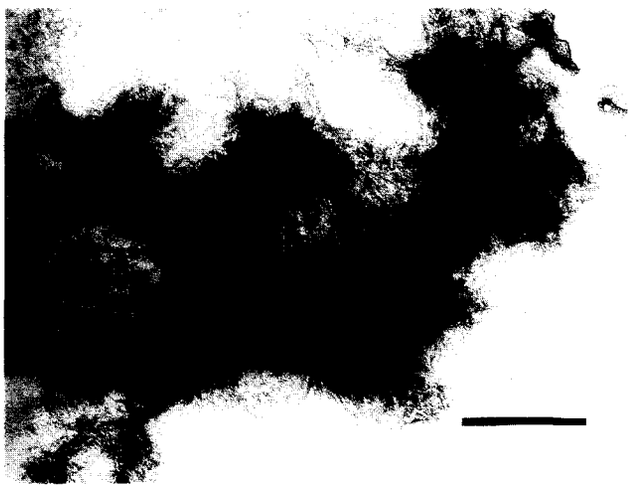


Figure 8 Transmission electron micrograph of native band cellulose regenerated from 8.75% sodium hydroxide solution. Scale bar is 200 nm

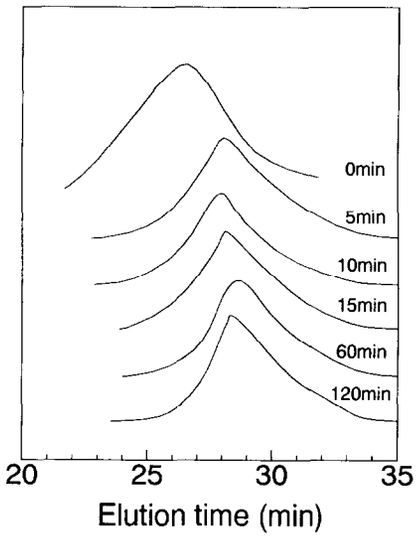


Figure 9 Change in size exclusion chromatogram of regenerated native band cellulose by hydrolysis with 1N HCl at 100°C

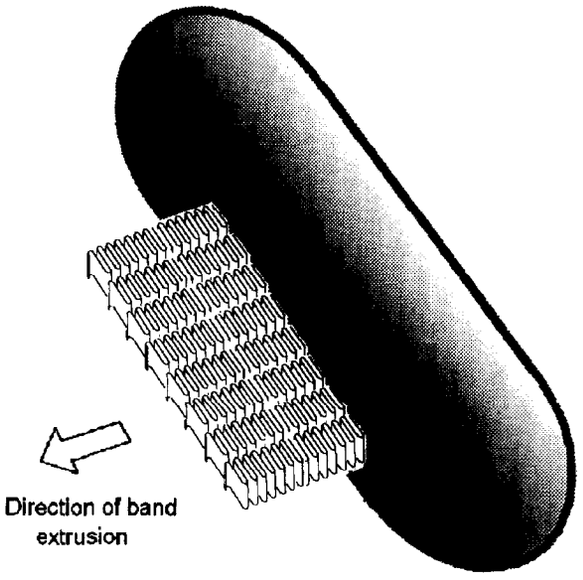


Figure 10 Model for native band cellulose production by mutant *Acetobacter xylinum*. Note the folded glucan chain mini-sheets originating from the subunits of the linear synthesizing complex

LODP was about 50 ($\overline{DP_w}/\overline{DP_n} = 2.53$), which is a typical value for regenerated cellulose materials such as rayon fibres (*Figure 7*). This indicates that the *LODP* of 20 for native band cellulose is substantially related to its unusual morphology.

Besides synthetic linear polymers, some cellulose derivatives have been known to form folded-chain crystals[9–11]. Some models of chain folding in the cellulose molecule have been proposed[8] and supported by conformational analysis computed in terms of stereochemistry. Both Rees[17] and Yathindra[18] showed the presence of a comparatively stable conformation different from the normal glycosidic linkage, causing a cellulose chain to loop back within three or four successive glucan units. The present finding of a characteristic hydrolysis behaviour of native band cellulose indicates that such a conformation can be actually formed in this anomalous form of cellulose.

In the previous study[2], it was observed that the number of folded sheets in the native band corresponds well with the number of subunits of the cellulose-synthesizing complex in *A. xylinum*. Also, the number of glucan chains synthesized by a single subunit has been estimated as around 10–16[19,20]. A recent proposal on the multistep crystallization of cellulose is of relevance[21]. The crystallization of cellulose I normally proceeds with two steps, the first involving van der Waals forces to generate a mini-sheet of glucan chains, and the second step involving the association of the mini-sheets into the microfibril by H bonding. Based on this evidence and the results of this study, we propose a possible mechanism for construction of the native band cellulose as follows: (a) a monomolecular glucan chain mini-sheet of approximately 16 glucan chains is formed as a vertical plane immediately after extrusion from the subunit; (b) this entire sheet then folds upon itself in accordion-like fashion to generate the single cellulose II strand directed away from the cell and perpendicular to the cell's longitudinal axis (*Figure 10*). Takagi[3] speculates that the polymerization reaction itself may be suppressed by the conditions of nutrient stress, or that some of the export pores may become plugged or the diameters of the export pores could undergo conformational changes, thus effecting the unique export and glucan chain sheet folding to produce cellulose II *in vivo*. How such an unusual molecular arrangement can take place and which genetic and cellular transformations in the bacterium lead to this mode of cellulose biosynthesis remain to be elucidated.

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