



Formation of disordered regions is caused by drying in cellulose microfibrils of *Egeria densa*

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Abstract Periodically disordered regions can be induced by drying in cellulose microfibrils of several plant species. In this study, cellulose nanocrystals were prepared from never-dried and oven-dried cellulose samples from *Egeria densa* by sulfuric acid hydrolysis and observed with transmission electron microscopy. Cellulose nanocrystals from never-dried samples were long ($> 1 \mu\text{m}$ in length), curved smoothly, and had very few kinks. In contrast, cellulose nanocrystals from oven-dried samples were shorter (50–600 nm in length) and were almost entirely straight. These results indicated that never-dried cellulose microfibrils lacked the disordered regions along the microfibril and exhibited a continuous, low bending rigidity. Never-dried and oven-dried cellulose samples from *E. densa* were subjected to acid hydrolysis, dissolution in 1% LiCl/dimethylacetamide, and size-exclusion

chromatography–multi-angle laser-light scattering analysis. After acid hydrolysis, oven-dried samples showed a significantly lower degree of polymerization than never-dried samples. In addition, oven-dried samples showed a unimodal and narrow molar mass distribution, whereas never-dried samples showed a bimodal distribution after acid hydrolysis. These results indicated that oven-drying induces the disordered regions along the cellulose microfibril and that the disordered regions are cleaved by acid hydrolysis.

Keywords Cellulose microfibril · *Egeria densa* · SEC–MALLS · Acid hydrolysis

Abbreviations

CNC	Cellulose nanocrystal
DP	Degree of polymerization
Dpp	Degree of polymerization of the peak maximum
Dpw	Weight-average degree of polymerization
DMAc	Dimethylacetamide
LiCl	Lithium chloride
LODP	Leveling-off degree of polymerization
Mp	Molar mass of the peak maximum
Mw	Weight-average molar mass
SEC	Size-exclusion chromatography
MALLS	Multi-angle laser-light scattering
TEM	Transmission electron microscopy
TEMPO	2,2,6,6-Tetramethylpiperidine-1-oxyl

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Introduction

Cellulose is the most abundant biopolymer on Earth. In addition to its utilization in clothes and paper, cellulose is a potential nanomaterial generated through chemical and mechanical treatments (Isogai et al. 2011). In a plant cell wall, cellulose molecules are oriented in a single direction and constitute crystalline cellulose microfibrils. The cellulose microfibrils of terrestrial plants are approximately 3 nm in width. During acid hydrolysis of cellulose microfibrils, the degree of polymerization (DP) becomes constant. The reported DP value after acid hydrolysis is 200–400, which is the leveling-off degree of polymerization (LODP) (Battista et al. 1956; Hayashi et al. 1974; Helmy and Abd El-Motagali 1993). This phenomenon has been explained by the fringed micellar model consisting of a crystalline region of 100–150 nm in length interconnected by a periodically disordered region in the longitudinal direction (Scallan 1971). The periodically disordered regions along the cellulose microfibril purified from ramie (*Boehmeria nivea*) bast fibers were detected by small-angle neutron scattering with deuterium substitution (Nishiyama et al. 2003).

However, several reports indicate that the presence of periodically disordered regions is not the native structure of a cellulose microfibril. Lai-Kee-Him et al. (2002) reported that cellulose synthesized in cultured cells of *Rubus fruticosus* maintained long fibrils after acid hydrolysis, although those synthesized in vivo were short. In addition, cellulose microfibrils isolated from never-dried *Chara australis*, which were of similar size and crystal structure to those of terrestrial plants (Koyama et al. 1997; Wada et al. 1995), maintained long fibrils after acid hydrolysis, whereas oven-dried cellulose samples were shorter in length (Horikawa et al. 2018). Funahashi et al. (2018) reported that never-dried cedar cellulose had a higher DP value after acid hydrolysis than oven-dried cellulose and softwood bleached kraft pulp. These results indicate that the periodically disordered regions could be formed by the mode of cellulose synthesis or by artificial means, such as dehydration and pulping. However, the mechanism of the formation of the disordered region remains unclear.

Shinoda et al. (2012) discussed that the disordered regions were the cause of depolymerization when cellulose was subjected to

2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO)-mediated oxidation. In addition, Fukuzumi et al. (2013) reported that longer TEMPO-oxidized cellulose nanofibrils had higher values for mechanical properties, such as elastic modulus, tensile strength, and extensibility, than shorter nanofibrils. Therefore, it is important to elucidate the mechanism of the formation of the disordered regions for industrial use of cellulose.

In this study, never-dried and oven-dried cellulose samples purified from *Egeria densa*, which is a submerged aquatic angiosperm, were subjected to acid hydrolysis and size-exclusion chromatography–multi-angle laser-light scattering (SEC–MALLS) analysis. The molar mass distribution in the samples was analyzed by peak deconvolution with non-linear least square minimization. In addition, cellulose nanocrystals (CNC) were prepared and observed by transmission electron microscopy (TEM). The aim was to clarify the effect of oven-drying on cellulose microfibrils of *E. densa*.

Materials and methods

Plant materials and cellulose purification

Egeria densa plants were purchased from Kagaku Club (Shiga, Japan). Specimens were cut into small pieces with scissors and purified to holocellulose via sodium chlorite oxidation in water at 70 °C for 1 h to remove lignin (Wise et al. 1946). After the delignification process, the products were boiled in 5% NaOH for 2 h to remove hemicellulose and other non-cellulosic components. After the removal of hemicellulose, specimens were again subjected to sodium chlorite oxidation in water at 70 °C for 1 h for decolorization. A portion of the sample was then oven-dried at 120 °C with an infrared moisture analyzer (FD-720, Kett Electric Laboratory, Tokyo, Japan) until no decrease in mass was observed. After oven-drying, the sample was soaked in deionized water and degassed for uniformity in the acid hydrolysis.

X-ray diffraction

Freeze-dried sample was pressed into disk pellets using a hand press. XRD pattern was collected in reflection mode with Cu-K α radiation (0.1542 nm),

which was generated from an Ultra X-18HF (Rigaku, Tokyo, Japan) at 40 kV and 300 mA. Peak deconvolution of the cellulose was performed by least-squares fitting. Peaks from the lattice planes and the amorphous peak were assigned by gaussian functions, while background scattering was assigned by a linear function. The deconvolution of the peaks from diffractograms was performed at the range of 10–30 degrees of 2θ with Python 3.7.0 software and Imfit 0.9.13, which is a module for curve-fitting by non-linear least square minimization. The full width at half maximum (FWHM) value of the peak from (200) was measured to understand the order of cellulose microfibril.

Neutral sugar analysis with gas chromatography

Oven-dried sample was cut into small pieces and was treated with 72% (w/w) H_2SO_4 (0.3 mL) in a glass tube fitted with a teflon-lined screw cap. After 4 h at 30 °C, the acid was diluted with deionized water (8.4 mL) and heated for 1 h at 120 °C with an autoclave machine. After cooling, myo-inositol (0.5 mL of a 20 mg/mL solution) was added as an internal standard. Then, pH of the solution was adjusted to 5.6 by adding saturated aqueous solution of $\text{Ba}(\text{OH})_2$. The solution was centrifuged at 3000 rpm for 15 min. 2 mL of the supernatant was reduced with 20 mg of sodium borohydride for 1 night at room temperature. Then, the excess reagent was destroyed by treatment with acetic acid until gas evolution ceased. The solution was evaporated to dryness and dehydrated by adding 20 mL of ethanol until acetic acid odor disappeared. The dehydrated residue was acetylated by adding 2 mL acetic anhydride and 0.1 mL H_2SO_4 for 1 h at 55 °C in a water bath. The acetylated sample was extracted with ethyl acetate to remove the acetylation reagents and then, evaporated with a rotary evaporator. The resulting acetates were dissolved in acetone (1 mL) and 1 μL of this solution was injected into the gas chromatograph. The analysis was carried out on a GC-2025 gas chromatograph (Shimadzu, Kyoto, Japan) fitted with AOC-20i autosampler (Shimadzu) and SP-2330 fused silica capillary column (15 m $\times$ 0.25 mm, 0.2 μm film thickness; Supelco Inc., Pennsylvania, USA). Helium was used as a carrier gas. On-column injection and FID detection was used. The column, the injection, and the

detector temperature were ramped 210 °C, 240 °C, and 250 °C, respectively.

Preparation of cellulose nanocrystals

The cellulose samples were hydrolyzed with 50% (w/w) H_2SO_4 at 70 °C for 1 h. The products were washed repeatedly with deionized water by centrifugation until reaching a neutral pH. The samples were suspended in deionized water and agitated at 1000 rpm and 4 °C for 1 night. After centrifugation, the supernatant was observed with a transmission electron microscope.

Transmission electron microscopy

An 8 μL aliquot of the CNC dispersion was mounted on a copper grid (Oken-shoji, Tokyo, Japan), which was supported by a carbon film. The excess liquid was absorbed with filter paper, and 3 μL of 2% uranyl acetate negative stain was added before drying. Excess solution was blotted with filter paper, and then allowed to stand and dry by natural evaporation. The sample grid was observed at 120 kV using a JEM 1400 transmission electron microscope (JEOL, Tokyo, Japan).

Acid hydrolysis and dissolution of cellulose samples in 8% (w/v) lithium chloride/dimethylacetamide

Cellulose samples were hydrolyzed with 2.5 N HCl at 100 °C for 30, 60, 120, or 240 min. The acid hydrolysates were washed repeatedly by centrifugation in distilled water until a neutral pH was attained. Dissolution of cellulose in lithium chloride (LiCl)/dimethylacetamide (DMAc) was conducted according to a method previously reported (Ono et al. 2016) with slight modification. The hydrolyzed cellulose samples (5 mg) were suspended in 100% ethylenediamine (1 mL) and centrifuged. This washing was repeated twice, and then the samples were shaken at room temperature for 4 days. The resulting mixtures were centrifuged to separate excess ethylenediamine from the mixture. The samples were soaked in methanol (1 mL) and centrifuged. This washing was repeated twice with fresh methanol. The samples were soaked in fresh methanol (1 mL) and shaken at room temperature for 3 h. The mixtures were centrifuged to separate excess methanol. The samples were then soaked

in DMAc (1 mL) and centrifuged. This DMAc washing was repeated twice. The samples were then soaked in fresh DMAc (1 mL) and the mixtures were shaken overnight at room temperature. After centrifugation to remove excess DMAc, the samples were suspended in 8% (w/v) LiCl/DMAc (1 g) and the mixtures were shaken at room temperature for 3 months. The 8% (w/v) LiCl/DMAc in which the cellulose samples were dissolved was diluted with fresh DMAc to 1% (w/v) LiCl/DMAc for SEC–MALLS analysis.

SEC–MALLS analysis

A SEC column and Guard column (KD-806 M and KD-G, respectively, Shodex, Tokyo, Japan), a refractive index detector (RI-71S, Shodex), and a MALLS detector (DAWN HELEOS-II, $k=658$ nm, Wyatt Technologies, Goleta, USA) were used. The SEC eluent was 1% (w/v) LiCl/DMAc. The value 0.131 was used as the specific refractive index increment (dn/dc) of cellulose in 1% LiCl/DMAc at 40 °C (Ono et al. 2016). All sample solutions were filtered through 0.45 μm polytetrafluoroethylene disposable membranes (Advantec, Tokyo, Japan) prior to injection. Data acquisition and processing were performed using ASTRA V software (Wyatt Technologies) and molar mass distribution, the weight-average molar mass (M_w), and the molar mass of the peak maximum (M_p) were determined. The DP of cellulose was calculated as the molar mass divided by 162. To investigate the depolymerization behavior during acid hydrolysis, peak fitting was performed with Python 3.7.0 software and lmfit 0.9.13, which is a module for curve-fitting by non-linear least square minimization. Monteiro (2015) reported that a log-normal distribution can be used to fit the molar mass distribution for characterization of polymers. Therefore, to simplify the fitting calculation, molar mass (M) was converted to a common logarithm and one fraction of the molar mass distribution was defined by a Gaussian function (Eq. 1).

$$F(a, b, c) = a \exp\{-4\ln 2(\log M - b)^2/c^2\} \quad (1)$$

This function contains three fitting parameters: peak height (a), position of the peak maximum (b), and full width at half maximum (c). The differential weight fraction, $dw/d(\log M)$, was fitted as the sum of the multiple functions (Eq. 2).

$$dw/d(\log M) = \sum_i F(a_i, b_i, c_i) \quad (2)$$

From the b value obtained from the fitting calculation, M_p was calculated (Eq. 3).

$$M_p = 10^b \quad (3)$$

Results and discussion

Characterization of cellulose prepared from *Egeria densa*

X-ray diffraction patterns of cellulose obtained from *E. densa* is shown in Fig. 1. The data showed that *E. densa* cellulose has the cellulose I crystal structure and that the crystallinity is similar to those of celluloses obtained from terrestrial plants and *Chara australis* (Horikawa et al. 2018).

The relative composition of the neutral monosaccharides of the cellulose obtained from *E. densa* is shown in Table 1. Total proportion of the monosaccharides other than glucose was > 17%. It indicates that a few hemicelluloses remained in the cellulose sample even after alkaline treatment.

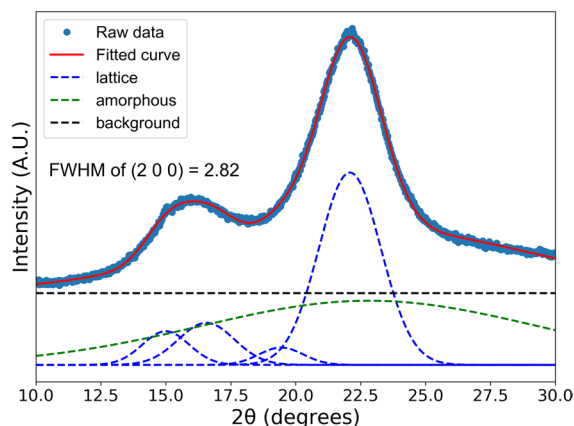


Fig. 1 X-Ray diffraction pattern of the purified celluloses prepared from *Egeria densa*

Table 1 Relative monosaccharide composition (%) of cellulose prepared from *Egeria densa*

Glucose	Mannose	Xylose	Rhamnose	Galactose
82.2	7.43	7.01	1.73	1.64

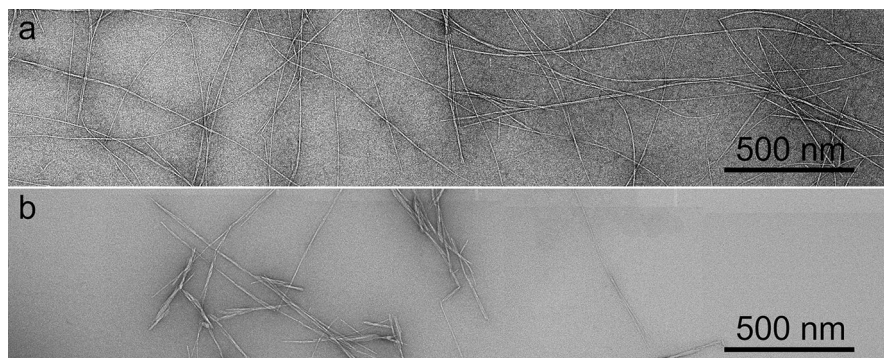
TEM observation of cellulose nanocrystals obtained from *E. densa*.

Transmission electron micrographs of never-dried and oven-dried CNCs are shown in Fig. 2. The CNCs showed about 3 nm width, which is similar to those obtained from terrestrial plants. Most never-dried CNCs were too long to allow recognition of the ends of fibrils in the TEM images, despite observation at a wide field of view, but a small number of CNCs were of 50–600 nm in length (Fig. 2a). In contrast, most oven-dried CNCs were of 50–600 nm in length and barely curved (Fig. 2b). Accurate evaluation of the CNC length was difficult because the never-dried CNCs were too long and the oven-dried CNCs were too aggregated. However, oven-drying treatment apparently shortened the CNCs prepared by acid hydrolysis. Furthermore, never-dried CNCs curved smoothly and had very few kinks. These results indicated that never-dried cellulose microfibrils from *E. densa* had neither a fringed micellar structure consisting of a crystalline region and periodically disordered regions nor a perfectly rigid rod structure; instead, the microfibrils showed low bending rigidity. The morphological features of the CNCs obtained from *E. densa* are in contrast to those of the CNCs obtained from *Valonia macrophysa*, which have about 20 nm width and showed a number of kinks (Chanzy and Henrissat 1985).

Change in molar mass distribution of cellulose by acid hydrolysis.

The molar mass distributions of the cellulose samples are shown in Fig. 3. The cellulose before acid hydrolysis showed a bimodal pattern in which the low-molar-mass fraction was very small. The bimodal molar mass distribution or SEC elution pattern were previously reported for hardwood pulp and holocellulose (Ono et al. 2015; 2016; Sjöholm et al. 2000; Yamamoto et al. 2011). Sjöholm et al. (2000) reported that the high-molar-mass fraction could be assigned to cellulose, whereas the low-molar-mass fraction could be assigned to xylan in SEC analysis of hardwood pulp. After acid hydrolysis, the molar mass distributions of all samples had a similar small shoulder peak at a lower molar mass region than cellulose LODP values in the previous reports. The molar mass distribution patterns are similar to those of acid-hydrolyzed birch kraft pulp, which contains a high proportion of xylose (Håkansson et al. 2005). Holocellulose from *E. densa* contains xylose (Rabemanolontsoa and Saka 2012). As shown with the neutral sugar analysis, in addition, a few hemicellulose remained even after alkaline treatment in the cellulose sample used in this research. Therefore, it is likely that the low-molar-mass fraction can be assigned to hemicellulose, such as xylan. In addition to the hemicellulose peak, never-dried cellulose hydrolysates showed a bimodal pattern consisting of high- and low-molar-mass fractions. In contrast, oven-dried cellulose hydrolysates showed an almost unimodal molar mass distribution near to the low-molar-mass fraction of never-dried cellulose hydrolysates. In addition, oven-dried cellulose showed a significantly lower molar mass distribution than never-dried cellulose hydrolysates, even before hydrolysis. This finding

Fig. 2 Transmission electron micrographs of cellulose nanocrystals prepared from **a** never-dried and **b** oven-dried cellulose from *Egeria densa*. The samples were negatively stained with uranyl acetate



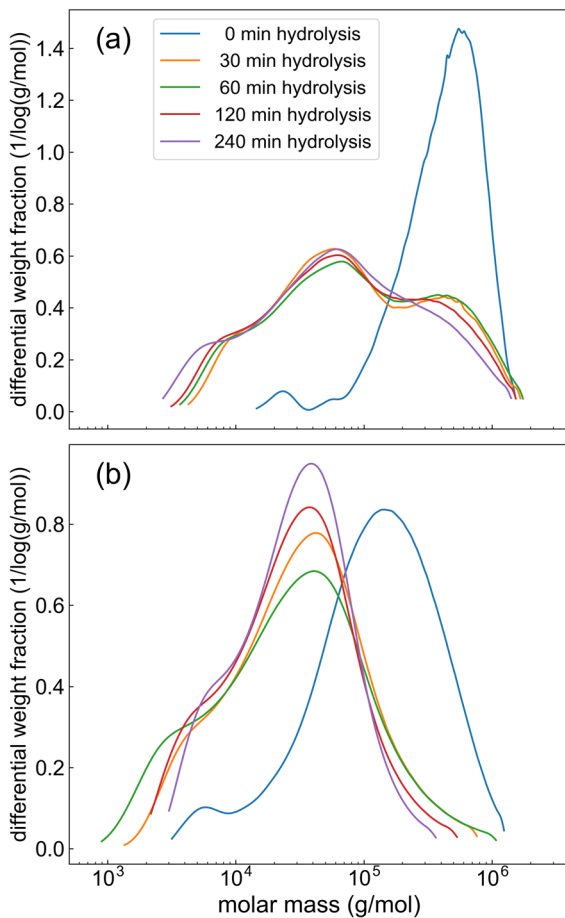


Fig. 3 Molar mass distributions of cellulose from *Egeria densa* that was acid hydrolyzed in the never-dried state (a) and the oven-dried state (b)

indicated that oven-drying caused cleavage along the cellulose microfibrils. Depolymerization resulting from oven-drying treatment has been observed previously (Funahashi et al. 2018). However, the depolymerization caused by oven-drying was more pronounced in the present study. This may be because of the harsh oven-drying process with infrared radiation at 120 °C.

The results of peak deconvolution of the molar mass distributions by non-linear least square minimization of the never-dried and oven-dried cellulose hydrolysates are shown in Figs. 4 and 5, respectively. Fitting calculations for the never-dried cellulose were performed with three Gaussian distributions: cellulose of high- and low-molar-mass fractions and hemicellulose. In contrast, calculations for the

oven-dried cellulose were performed with two Gaussian distributions: cellulose and hemicellulose. From 60 to 240 min hydrolysis, the weight-average degree of polymerization (DP_w) of the never-dried and oven-dried samples gradually decreased (Fig. 6a). In contrast, the degree of polymerization of the peak maximum (DP_p) of the high-molar-mass fraction of the never-dried samples was > 2900, even after 240 min hydrolysis (Fig. 6b). In addition, the DP_p of the oven-dried samples and low-molar-mass fraction of the never-dried samples remained almost constant and was < 400, which is a so-called LODP pattern. However, from 60 to 240 min hydrolysis, the peak height of the high-molar-mass fraction of never-dried cellulose apparently decreased and the peak tail of the high-molar-mass region of oven-dried cellulose decreased (Figs. 4 and 5). The DP_w similarly decreased during the corresponding hydrolysis period (Fig. 6a). These results led to the prediction that the high-molar-mass fraction will disappear and the molar mass distribution will become unimodal, similar to those of oven-dried hydrolysates, with further hydrolysis, as observed in a previous report (Funahashi et al. 2018).

A contradiction between the TEM observations and SEC–MALLS analysis is apparent. Most of the never-dried CNCs prepared by acid hydrolysis were > 1 μm in length (Fig. 2), although a large proportion of the cellulose molecules in the never-dried hydrolyzed samples had DP_p < 400, which corresponded to a cellulose length of 200 nm (Figs. 5 and 6b). Therefore, we speculate that in the SEC–MALLS analysis of never-dried hydrolysates, cellulose molecules in the high- and low-molar-mass fractions corresponded to the microfibril core and surface, respectively. It is reasonable to expect that depolymerization in never-dried cellulose occurs mainly on the microfibril surface because of the accessibility to H⁺. Based on this assumption, the unimodal molar mass distribution of the oven-dried hydrolysates indicated that oven-drying treatment induced disordered regions along the microfibril. Decrease in the peak height of the high-molar-mass fraction during acid hydrolysis in never-dried cellulose indicated that cellulose molecules in the core of the never-dried cellulose microfibril were not resistant to hydrolysis and were depolymerized during prolonged acid hydrolysis.

Based on the present results, a schematic model of the changes in *E. densa* cellulose microfibrils during

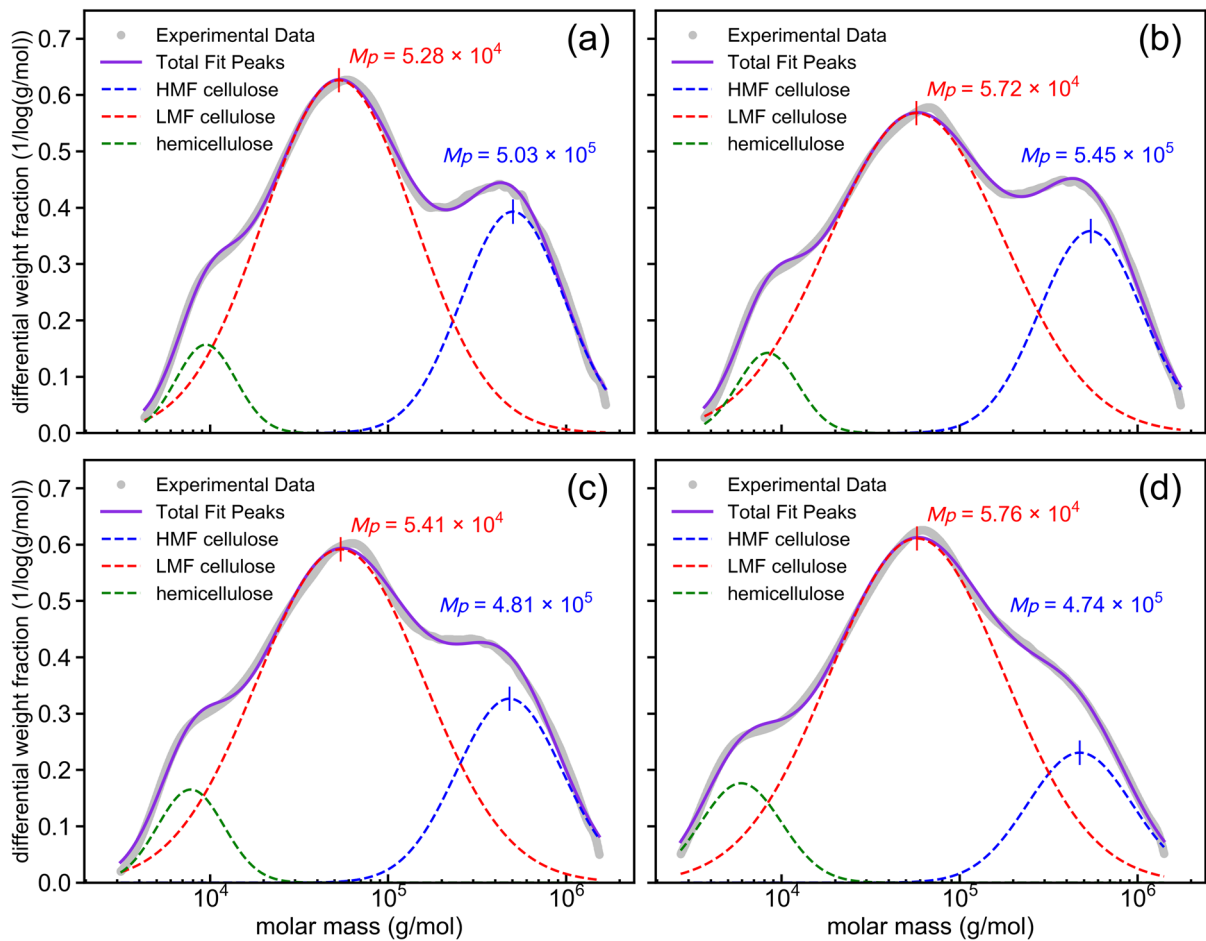


Fig. 4 Molar mass distributions and their peak fitting results of never-dried cellulose that was acid hydrolyzed for 30 min (a), 60 min (b), 120 min (c), and 240 min (d). The gray line indicates experimental data. The violet line indicates the sum of the fitting function. Blue, red, and green dashed lines indi-

cate the high-molar-mass fraction of cellulose, low-molar-mass fraction of cellulose, and hemicellulose, respectively. HMF, High-molar-mass fraction; LMF, Low-molar-mass fraction; M_p , Molar mass of the peak maximum

oven-drying or remaining in the never-dried state, and with acid hydrolysis is illustrated in Fig. 7. In never-dried cellulose microfibrils, cellulose molecules in the microfibril surface are cleaved because of the high accessibility to H^+ , whereas molecules in the microfibril core remain almost intact. As a result, the molar mass distribution shows a bimodal pattern in which the high-molar-mass fraction corresponds to cellulose molecules in the microfibril core and the low-molar-mass fraction corresponds to molecules in the microfibril surface. Oven-drying induces disordered regions along the microfibrils and cleavage with a reduced frequency. Depolymerization by acid hydrolysis occurs in the disordered regions and, therefore,

the molar mass distribution shows a unimodal pattern. Note that never-dried cellulose microfibrils will be degraded into short fibrils with prolonged acid hydrolysis.

This research supports the insight that the periodically disordered regions depolymerized by acid hydrolysis are not a native structure of cellulose microfibrils, at least in *E. densa*. However, many questions concerning the disordered regions remain unanswered. What is the mechanism whereby oven-drying induces periodically disordered regions in cellulose microfibril? What causes the disordered

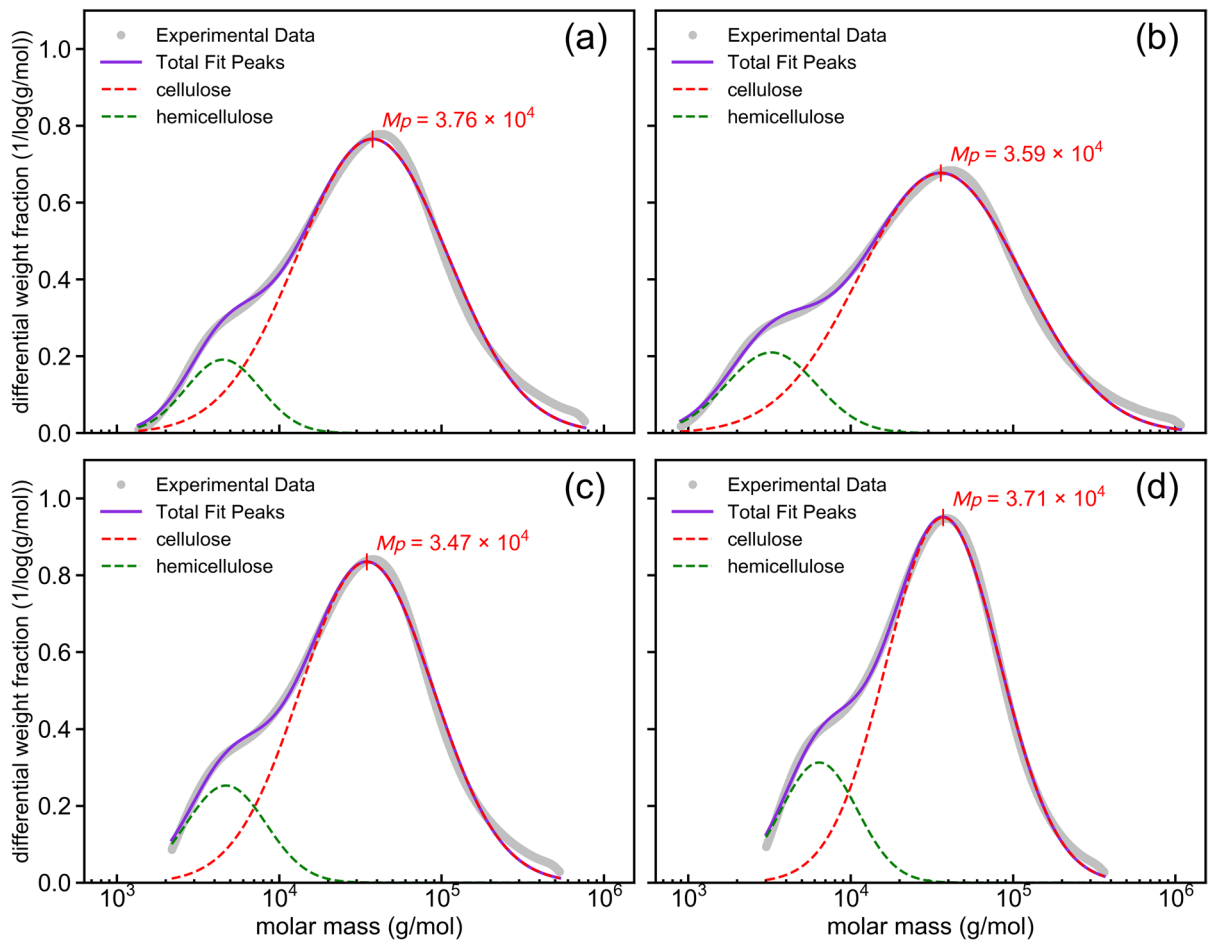
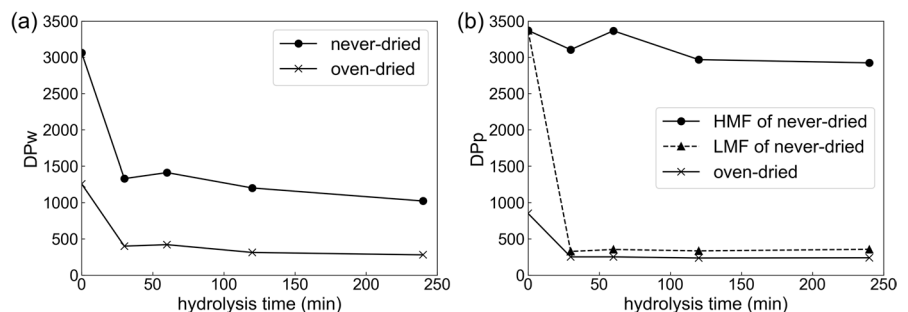


Fig. 5 Molar mass distributions and their peak fitting results of oven-dried cellulose that was acid hydrolyzed for 30 min (a), 60 min (b), 120 min (c), and 240 min (d). The gray line indicates experimental data. The violet line indicates the sum

of the fitting function. Red and green dashed line indicate cellulose and hemicellulose, respectively. Mp, Molar mass of the peak maximum

Fig. 6 Changes in weight-average degree of polymerization (DPw) (a) and degree of polymerization of the peak maximum (DPp) (b) of cellulose from *Egeria densa* with various durations of hydrolysis. HMF, High-molar-mass fraction; LMF, Low-molar-mass fraction



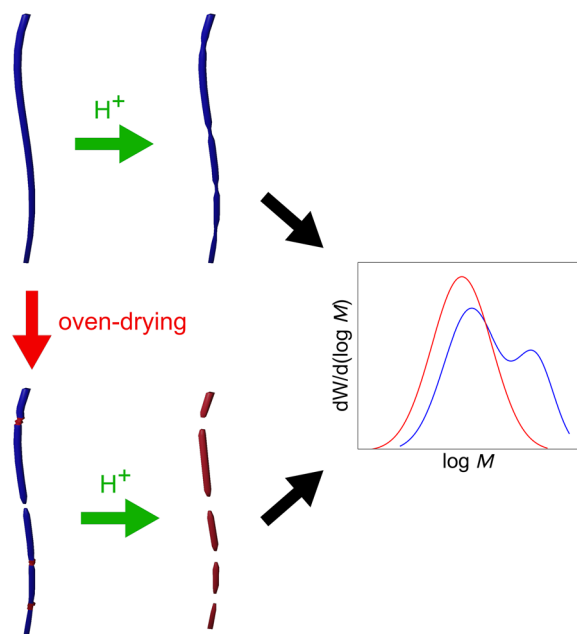


Fig. 7 Schematic model of *Egeria densa* cellulose microfibrils subjected to acid hydrolysis with or without oven-drying treatment and their molar mass distribution. $dW/d(\log M)$, Differential weight fraction; $\log M$, Common logarithm of molar mass

regions other than oven-drying? Plant species, the purification process, and drying conditions may affect the presence of the disordered regions and require investigation in future research.

Conclusion

This study showed that never-dried cellulose from *E. densa* maintains long microfibrils and has a bimodal molar mass distribution with a small high-molar-mass fraction after acid hydrolysis. In contrast, oven-dried cellulose comprises shorter microfibrils and has a lower molar mass distribution with a unimodal distribution after acid hydrolysis. Peak deconvolution indicated that the high-molar-mass fraction of never-dried cellulose hydrolysates was less degraded during acid hydrolysis. These results indicate that depolymerization of cellulose molecules by acid hydrolysis mainly occurs on the microfibril surface in never-dried cellulose and in the disordered regions along the microfibril in the oven-dried samples. We hypothesize that

oven-drying induces disordered regions along the microfibrils.

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Author contributions J.S. and Y.H. conceived and designed the research. S.K. and Y.H. conducted the experiments and wrote the manuscript. All authors read and approved the manuscript.

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Data availability No datasets were generated or analysed during the current study.

Declarations

Competing interests The authors declare no competing interests.

Ethical approval Not applicable.

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