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Enzymatically produced nano-ordered short elements containing cellulose I_β crystalline domains

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Abstract

This paper reports an attempt to characterize the residues extracted after enzymatic hydrolysis of *Cladophora* microcrystalline cellulose comprising two crystalline allomorphs of cellulose, Iα and I_β. Cellulose I_α is preferentially hydrolyzed by *Trichoderma* cellulase (Hayashi, Ishihara, Sugiyama, & Okano, 1998a). This selective hydrolysis process produced short elements. X-ray diffraction, electron diffraction and FT-IR analyses revealed that these short elements were highly crystalline, similar to the untreated microcrystalline cellulose, and mostly consisted of the I_β phase. The length distribution of the crystalline elements was ca. 350 nm. Furthermore, the average length of 350 nm corresponded to the degree of polymerization of 690 for β-glucan chains obtained by size exclusion chromatographic analysis. The close agreement indicates that individual molecular chains may be extended in the longitudinal direction of the obtained crystalline element. These characteristic short elements have the potential to act as nano-ordered particles and may be useful as fillers to enhance the mechanical properties of various materials.

Keywords: Microcrystalline cellulose; Cellulase; Cellulose Iα; Selective degradation; Short elements

1. Introduction

Cellulose is β-1,4-glucan polymer that self-aggregates to form minimal sized crystalline fibers, designated cellulose microfibrils (CMFs), in the cell wall of plants, algae and tunica of animals. Each CMF is composed of two crystalline allomorphs with parallel arrangements of the molecular chains that are generally accepted as cellulose I_α and I_β (Atalla & VanderHart, 1984; Horii, Hirai, & Kitamaru, 1987; Sugiyama, Persson, & Chanzy, 1991a; VanderHart & Atalla, 1984). Cellulose I_α and I_β have one-chain triclinic and two-chain monoclinic lattices, respectively (Sugiyama, Okano, Yamamoto, & Horii, 1990; Sugiyama Vuong, & Chanzy, 1991b). The ultrastructural localizations of the cellulose I_α and I_β allomorphs in a single microfibril have been investigated and two models have been proposed. In the first, the two crystalline domains are localized laterally (Sugiyama et al., 1991b; Yamamoto, Horii, & Hirai, 1996), while in the second, the two domains coexist alternately longitudinally or laterally (Imai & Sugiyama, 1998).

Recently, we investigated enzymatic hydrolyzates of CMFs secreted from a bacterium, *Acetobacter xylinum*, and two algae, *Cladophora* sp. and *Valonia* sp., that included both cellulose I_α and I_β (Hayashi, Ishihara, Sugiyama, & Okano, 1998a,b). The results indicated that cellulose I_α was more susceptible to enzymatic degradation by *Trichoderma* cellulase than cellulose I_β. Furthermore, short elements rich in cellulose I_β were frequently observed in the residues. Enzymatic hydrolysis by *Trichoderma* cellulase is believed to degrade cellulose crystals in a processive manner from their reducing or non-reducing end (Boisset, Fraschini, Schulein, Henrissat, & Chanzy, 2000; Henrissat, 1998; Imai, Boisset, Samejima, Igarashi, & Sugiyama, 1998). However, we hypothesized that the short elements could not be produced from CMFs as a result of the endo-processive action of cellobiohydrolase (CBH) alone. Assuming the preferable

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hydrolysis of cellulose I_α by cellulase as described above, the supremolecular structure of the crystalline cellulose should also contribute to the production of the short elements in addition to the manner of the enzymatic action.

Therefore, the present study attempted to characterize the larger residues after enzymatic hydrolysis of CMFs and the short elements (designated shortened microcrystalline cellulose: SMCC) obtained from algal cellulose of *Cladophora* sp. using transmission electron microscopy (TEM), atomic force microscopy (AFM) and Fourier transformation-infrared (FT-IR) spectroscopy. Finally, we propose a mechanism for SMCC formation on the basis of the supremolecular structure of *Cladophora* CMFs, which are constructed from cellulose I_α and I_β allomorphs.

2. Experimental

2.1. Enzyme preparation

The crude enzyme used was the commercially available 'Meicelase' (Meiji Seika Co. Ltd, Japan) prepared from *Trichoderma viride*. Exo-1,4-β-glucanase I (CBH I) was isolated and purified by multiple column chromatographies using anion and cation exchangers according to a previously described method (Honda, Takahashi, Nishimura, Kahei, & Ganno, 1981). However, a trace amount of contamination was identified in the purified CBH I preparation after SDS PAGE, and hence it is referred as the CBH I-rich fraction.

2.2. Preparation of cellulose substrates

Cell wall cellulose was collected from *Cladophora* sp. in the sea at Chiba, Japan. To purify the substrate, it was sequentially treated with 0.1 N aqueous NaOH at 100 °C for 2 h and then 0.05 N aqueous HCl at room temperature overnight, before final rinsing with distilled water. Following homogenization and freeze-drying, the residues were collected as CMFs. The CMFs were further treated with a 66% (w/w) aqueous H_2SO_4 solution at room temperature for 3 h with strong stirring and then washed by centrifugation to obtain a non-flocculating aqueous suspension of microcrystalline cellulose (designated MCC) at pH 5.0. The MCC was freeze-dried for further study.

2.3. Enzymatic hydrolysis

The obtained MCC was separately hydrolyzed with the crude cellulase and CBH I-rich fraction as follows. Equal quantities of the substrate suspension (1–5 mg of MCC) and the enzyme in 5 ml of 0.1 M of sodium acetate buffer at pH 4.8 were incubated with shaking for 2–3 days at 48 °C for crude cellulase and 38 °C for the CBH I-rich fraction. In addition, MCC samples hydrolyzed with shaking were compared with samples hydrolyzed without shaking. After the incubation, the precipitated residues (designated the larger residues) were isolated by centrifugation, thoroughly washed with 1% NaOH solution and then distilled water, and finally freeze-dried. Following the addition of an excess of 99.5% ethanol to the supernatants in each washing process mentioned above, short elements (designated SMCCs) were obtained by centrifugation at 10,000–18,000 rpm, and then freeze-dried. The larger residues were repeatedly treated with the enzyme until the total weight loss of the sample reached 80%.

2.4. Acid hydrolysis

To compare the enzymatic hydrolyzates with acid hydrolyzates, the purified *Cladophora* CMFs were treated with 2.5 N HCl at 100 °C for 3 or 5 h. The acid-hydrolyzed MCC samples were also subjected to TEM, FT-IR and size-exclusion chromatographic (SEC) analyses.

2.5. Transmission electron microscopy

The samples were observed with a JEM-2000EX electron microscope (JEOL Co. Ltd) operated at an accelerating voltage of 200 kV. Samples of untreated MCC, acid-hydrolyzed MCC, precipitated larger residues and SMCCs were individually suspended in 50% ethanol. A drop of each suspension was deposited on a carbon-coated Cu grid, and negatively stained with 1.5% uranyl acetate solution. Selected area diffractograms were also obtained without staining. The images and diffractograms were recorded on Mitsubishi electron microscope (MEM) films. The electron diffractograms were traced with a micro-densitometer (3CS; Joyce-Loebl Co. Ltd) to estimate the *d*-spacings of the crystalline structure.

2.6. Infrared spectroscopy

Each sample was mounted on a KBr thin plate of about 1 mm in thickness. FT-IR spectra were obtained from a circular area of 100 μm in diameter using a Nicolet-Magna 550 FT-IR spectrometer equipped with a Nic Plan microscopic accessory. The wave number range scanned was 4000–650 cm^{-1}, and 64 scans of 4 cm^{-1} resolution were signal-averaged and stored. The internal standard band at 2900 cm^{-1} that is assigned to C–H stretching in methyl and methylene groups in the crystalline region was commonly employed. The relative ratios between the cellulose I_α and I_β in the samples were determined by the intensity ratios of their typical FT-IR bands, namely 750 cm^{-1} for cellulose I_α and over 710 cm^{-1} for cellulose I_β, using a calibration curve based on previous results (Yamamoto et al., 1996).

2.7. Determination of molecular weight

Some of the SMCCs were isolated and freeze-dried after 2 or 8 days of enzymatic treatments. The residues were then

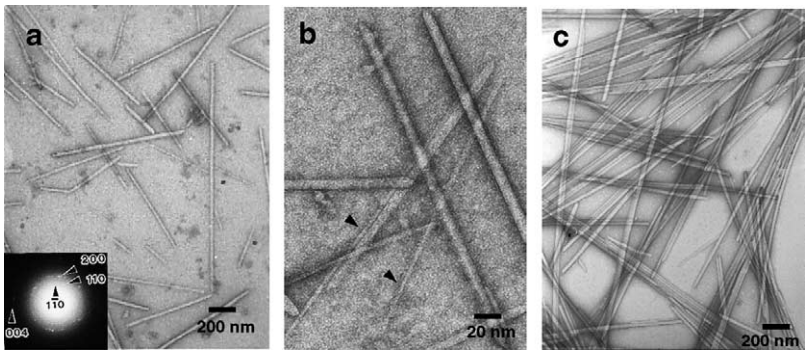

Fig. 1. Electron micrographs of the negatively-stained short elements (SMCCs) obtained after 4 and 8 days of treatment with the CBH I-rich fraction (a, b), and MCC after acid hydrolysis (c). The arrowhead shows fibrillated SMCCs (b).

treated with an ice-cooled mixture of nitric acid and phosphorus pentoxide for 30 min for conversion into cellulose trinitrate for the SEC analysis. The products were filtered and washed thoroughly with water. The SEC was performed with tetrahydrofuran as the eluent to determine the average molar weight (M_w) of the cellulose trinitrate obtained from the enzymatic hydrolyzates (Shibazaki, Kuga, Onabe, & Brown, 1995). The M_w of the acid hydrolyzates of *Cladophora* CMFs was measured in the same manner.

2.8. Atomic force microscopy

A drop of the SMCC suspension or the larger residues was deposited onto a mica plate (8×8 mm), and then air-dried. The samples were observed using a multimode AFM (NanoScope IIIa; Digital Instruments) equipped with a scanner with a 10 μm range (E-scanner). All images (400×400 pixels) were obtained using a tapping mode in air. The cantilever used was 130 μm in length and had an unmodified silicon nitride tip with a spring constant of 40 Nm^{-1}. The scan rate was 1.5 Hz and the scan angle was varied to obtain the optimum contrast.

3. Results and discussion

3.1. Characterization of the residues after enzymatic hydrolysis

Short elements (SMCCs) were produced during the enzymatic degradation processes of both the crude cellulase and the CBH I-rich fraction (Fig. 1a and b). During the 2 days enzymatic hydrolysis, the yield of short elements reached 15–24% of the initial sample, as shown in Table 1. The SMCCs were produced constantly at almost the same rate for the individual enzymes in the residues after the repeated enzymatic degradation processes. Distinct fibrillation was scarcely observed in the samples after 4 days of enzymatic hydrolysis (Fig. 1a). However, the larger residues and SMCCs became thinner and/or fibrillated after 8 days of treatment (Fig. 1b, arrow head), similar to the case for hydrolysis of I_β-rich cotton-ramie type cellulose (Hayashi et al., 1998b). On the other hand, the 5 h acid hydrolysis of *Cladophora* CMFs did not produce such SMCCs (Fig. 1c).

The lengths of the obtained SMCCs did not differ significantly between the crude cellulose and the CBH I-rich fraction. The distribution in length of the SMCCs produced by the degradation with the crude cellulase was evaluated statistically, as shown in the histogram in Fig. 2. The most frequently observed lengths were between 300 and 400 nm.

The SEC results revealed the distributions of the degree of polymerization (DP) based on the M_w for the cellulose molecules in the untreated CMFs, acid-treated CMFs and SMCCs. The results for the SMCCs after treatment with the crude cellulase for 2 and 8 days are shown in Table 2. The DP of the SMCCs was about 690, whereas the acid-hydrolyzed CMFs did not show a significant change in the DP after the treatment was prolonged. More interestingly, the M_w/M_n value of the SMCCs, indicating the distribution of the molecular weight, was much lower than that of the acid-hydrolyzed samples and even smaller than that of the untreated CMFs. These results indicate that only the enzymatically produced SMCCs had a narrower distribution of DP around 690. The value of 690 for the DP of β-glucan molecules coincides with the average length of 300–400 nm of the SMCC rod samples, which were obtained experimentally by TEM observation as described above.

Table 1
Differences in the yields and I_α contents between the enzymatic treatments with and without shaking

		Yield (%)	Icc content (%)
Control		100	55±17
Meicelase shaked	Larger residue	29.3	43±12
	SMCC	21.2	29±7
Meicelase non-shaked	Larger residue	44.3	47±9
	SMCC	15.3	38±2
CBH I rich fraction shaked	Larger residue	47.3	45±6
	SMCC	24.0	39±5
CBH I rich fraction not-shaked	Larger residue	56.4	44±15
	SMCC	21.8	37±7

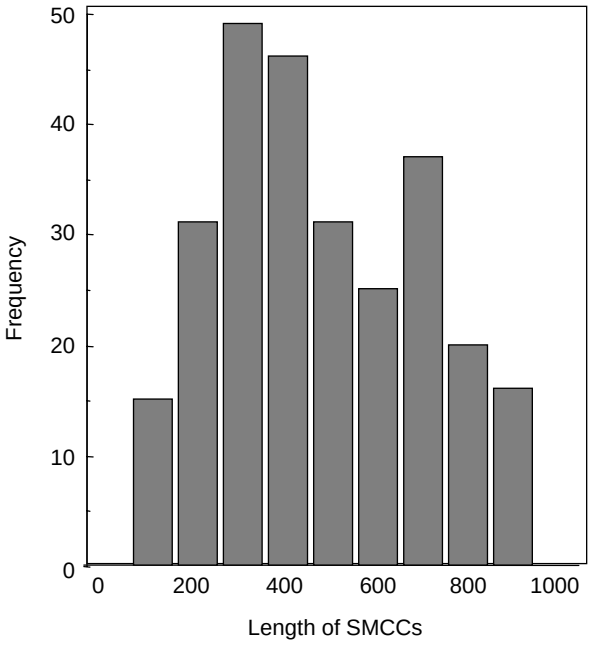

Fig. 2. Statistic frequencies of the lengths of SMCCs treated with the crude cellulase for 4 and 8 days or the CBH I-rich fraction for 2, 4 and 6 days.

This indicates that the length of the SMCC rods may coincide with the extended chain length of the contained β glucan molecules, when it is calculated based on the anhydroglucose unit length of 0.5 nm.

The X-ray diffractograms of the untreated CMFs and the SMCCs are shown in Fig. 3. The SMCCs exhibited a high crystallinity after the enzymatic treatments, similar to the untreated CMFs, indicating that non-crystalline regions may not be present in newly produced SMCCs after enzymatic hydrolysis. The field limiting diffraction pattern of the SMCCs inserted in Fig. 1a exhibited clear reflected spots that also indicate high crystallinity. The *d*-spacings for the SMCCs corresponded to the values for the (1T0) plane (0.60 nm) and the (110) plane (0.54 nm), which are typically identified as cellulose I_β. These results indicate that the major crystalline parts of the SMCCs consist of a highly-ordered cellulose I_β phase.

The cellulose I_α and I_β contents in the obtained samples calculated on the basis of the IR analyses are listed in Table 1. The initial sample contained $55 \pm 17\%$ cellulose I_α. After enzymatic hydrolysis with shaking at 50 wt% of the weight loss, 52% of the initial cellulose I_α content was

Table 2
Changes in the average degree of polymerization (DP) based on SEC chromatograms for the short elements (SMCCs), together with untreated and acid-treated CMFs of *Cladophora* sp.

Treatment	Sample				
	Untreated CMF	Short elements (SMCCs)	Acid treated CMF		
			30 min	60 min	180 min
DP	2870	690	3050	3150	3130
M_w/M_n	9.6	4.7	25.3	21.8	10.0

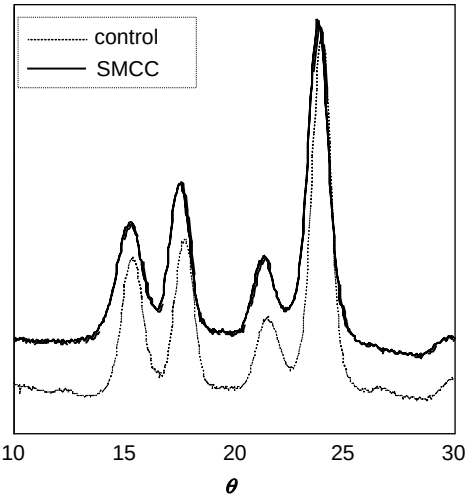

Fig. 3. X-ray diffractograms of untreated CMFs (broken line) and SMCCs (solid line).

enzymatically degraded. This result also indicated that the enzyme did not uniformly attack every MCC during the reaction time course since the I_α content differed between the larger residues and SMCCs. The FT-IR results suggested that the SMCCs were predominantly composed of cellulose I_β, although a trace of cellulose I_α was still remained (Fig. 4), when the bands in the region of 3450–3150 cm^{-1} were compared before and after the enzymatic hydrolysis. However, the absorption bands at 3240 cm^{-1} due to cellulose I_α were drastically decreased after the enzymatic hydrolysis. A trace amount of unhydrolyzed cellulose I_α may still remain at the ends of the SMCCs, after taking into account the preferable enzymatic attack on cellulose I_α. It is generally accepted that certain treatments are able to convert cellulose I_α to cellulose I_β, but they require a high pressure and high temperature (Debzi, Chanzy, Sugiyama, Tekely, & Excoffier, 1991; Horii et al., 1987). In the present study, the enzymatic degradation could not convert the crystalline cellulose to the microcrystalline form, since the hydrolysis was performed at 38 and 48 °C under atmospheric pressure.

Previously, it was reported that short elements were present in I_β-dominant cotton-ramie type cellulose (Halliwell, 1966; Halliwell & Riaz, 1970; King, 1966; Marsh, 1957; Sprey & Bochem, 1993). However, we consider that the SMCC formation from algal-bacterial type cellulose in our case does not represent the same phenomenon. As described above, the two allomorphs cellulose I_α and I_β are distributed in a complex manner in the algal-bacterial cellulose, whereas cotton-ramie type cellulose mainly contains cellulose I_β. The results in the present study appear to confirm that preferential degradation of cellulose I_α accompanied by the SMCC formation occurs in *Cladophora* CMFs, an algal-bacterial type cellulose.

In addition, we investigated the effects of shaking during the enzymatic treatment. It has been considered that shaking

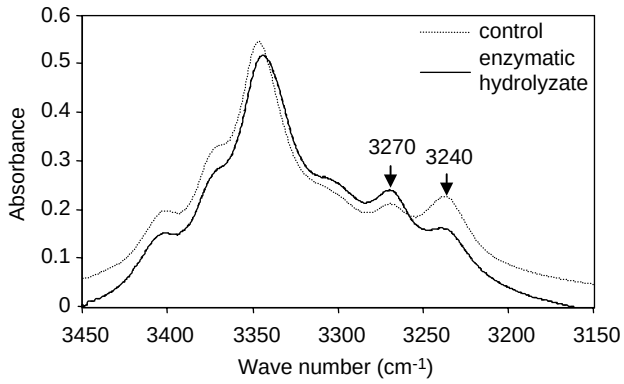

Fig. 4. FT-IR spectra for untreated CMFs (broken line) and SMCCs (solid line) in the characteristic regions for the cellulose I_α and I_β absorption bands The absorption bands near 3240 and 3270 cm^{-1} are assigned to cellulose I_α and I_β, respectively.

samples during the process of degradation may accelerate the action of the enzyme (Lee, Evans, Lane, & Woodward, 1996). The yields and cellulose I_α contents of SMCCs after treatment with the crude cellulase and CBH I-rich fraction with and without shaking are shown in Table 1. The results indicated that shaking during the treatment had an effect on both acceleration of the enzymatic hydrolysis and the preferable degradation of I_α. In particular, the production of SMCCs seemed to be dependent on shaking in the case of the crude cellulase. It is well known that *Trichoderma* crude cellulase contains at least four different enzymes, each of which reacts synergistically with CMFs, which contain complicated distributions of cellulose I_α and I_β, leading to efficient acceleration of the preferable degradation of cellulose I_α to yield SMCCs. It is also considered that shaking may provide the enzymes with more opportunity to interact with new reaction sites. In the case of treatment with the CBH I-rich fraction, however, the production of SMCCs and decrease in cellulose I_α in the residues were independent of shaking during the incubation. This may be caused by the different manners of degradation between the crude cellulase and an isolated enzyme as described above. These results imply that mechanical force may play an important role not only in the enzymatic movement of CBH I to new sites on the substrate, but also in cutting the CMFs after CBH I attack. Furthermore, the fact that the SMCCs obtained from the series of enzymatic hydrolyses in Table 1 showed almost the same values for their cellulose I_α contents indicates that the supremolecular structure of CMFs, such as the distribution of I_α/I_β composites, may contribute to the production of SMCCs during the enzymatic degradation.

3.2. Supremolecular structure of the I_α/I_β composite crystalline phases in microcrystalline cellulose fibers

An atomic force microscope (AFM) was employed in a tapping mode in air to observe the surface morphology of the samples. Fig. 5 shows the AFM height images of

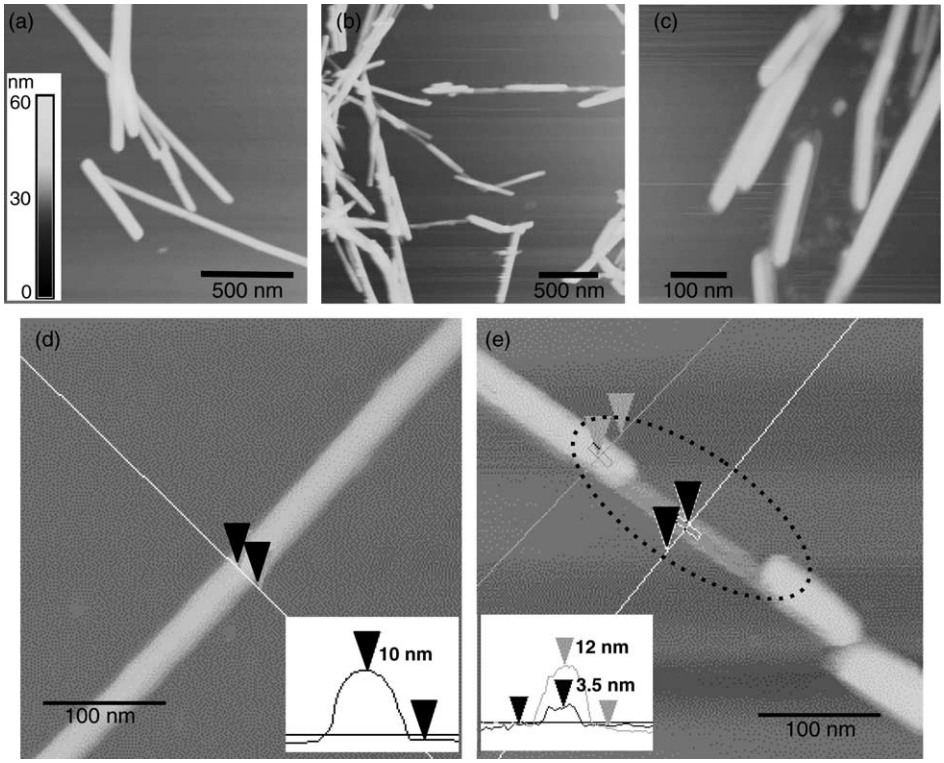

Fig. 5. AFM height images (a, c, and d) and section analyses of an untreated CMF (b), and a longer residue (e). The circled area shows a cut-off area observed in the longer residues.

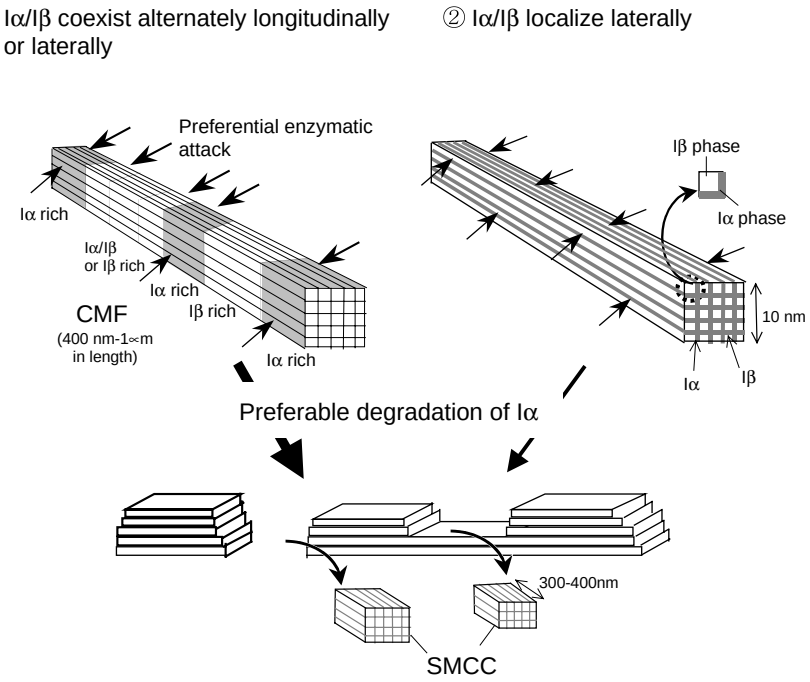

Fig. 6. A hypothesis for the formation of SMCCs by enzymatic hydrolysis of *Cladophora* CMFs, in relation to the possible supremolecular structure of CMFs.

untreated CMF (a), longer residues after the enzymatic degradation (b) and short elements (SMCCs) (c). The enzymatic hydrolysis produced drastic changes to the surface of the MCC. From the AFM observations, the color of the untreated CMFs was uniform, which means that the thickness of the untreated CMF is about 10 nm, and furthermore their surface was considered to be smooth (Fig. 5a and d). After the enzymatic treatment, however, the surface of the longer residues and SMCCs became relatively rough (Fig. 5b, c and e). AFM observations also revealed that the longer residues had areas cut off as blocks from the original surface not only at the ends but also at the middle (circle in Fig. 5e). The lengths of the cut-off areas were within several hundred nanometers, whereas their widths were almost the same as that of the untreated CMFs. Characteristically, the thickness at the cut-off area was 1–4 nm (see the inserted profile of (e); indicating that two or three molecular cellulose chains may be included.

The thicknesses of untreated CMFs, SMCCs and longer residues with and without shaking were 11.7 ± 4.7, 8.06 ± 5.67, 9.65 ± 4.12 and 8.48 ± 5.66 nm, respectively. The thicknesses of the residues after enzymatic hydrolysis were thinner than those of the initial samples. In connection with the length of the SMCCs shown in Fig. 2, it is interesting that the width of the cut-off areas with the enzymatic treatments appeared to be almost identical to the size of the SMCCs.

From these results, the cut-off areas are supposed to be removed by preferential degradation of cellulose I_α-rich blocks or correspond to cellulose I_β-rich domains surrounding I_α-rich parts. The former assumption may agree with the hypothesis for the cellulose I_α/I_β distributions proposed by Imai and Sugiyama (1998), and the latter with that proposed by Sugiyama et al. (1991) and Yamamoto et al. (1996). In other words, our observations indicate that the domains of cellulose I_α may be partly buried as a block or sheet along the longitudinal axis in the CMFs of *Cladophora* sp., in which cellulose I_α/I_β are considered to be distributed in a complex manner as interpreted by Imai and Sugiyama (1998). Thus, we can postulate that when the enzyme attacked CMFs, cellulose I_α domains were preferentially hydrolyzed, and the remaining cellulose I_β-rich domains were consequently extracted as intact crystalline blocks under our reaction conditions. This phenomenon can be attributed to the low susceptibility to the enzymes of the highly-ordered and relatively stable I_β arrangement of β glucan chains for the SMCCs, as illustrated schematically in Fig. 6. The kinks or defects caused by the enzymatic attack may be the initial stage of the cutting of CMFs or peeling off the blocks.

4. Conclusions

Enzymatic hydrolysis of *Cladophora* CMFs yielded short elements, designated SMCCs, with high crystallinity. The SMCCs were mainly composed of a cellulose I_β crystalline phase with a width of 10 nm and a length ca. 350 nm, corresponding to the average DP of 690 for the β glucan molecules. However, a trace of cellulose I_α still remained in the SMCCs. This may be induced by the preferential degradation of I_α crystalline domains by the *Trichoderma* crude cellulase and CBH I-rich fraction.

AFM observation revealed the appearance of some missing blocks in the residual MCC within a few hundred

nanometers at either the ends or the middle. These findings indicate the possibility that crystalline I_α domains of a certain size were buried along the longitudinal axis of the *Cladophora* CMFs. The synergism of the enzymes contained in the crude cellulase and the mechanical force applied by shaking the samples during the enzymatic treatment also assisted the production of SMCCs. The SMCCs have the potential to act as nano-ordered bio-particles. In other words, the SMCCs containing ordered fiber elements on a nano-scale are expected to be useful as fillers for the reinforcement of various impact materials as well as addition to the food and cosmetic industries.

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