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Selective degradation of the cellulose I_α component in *Cladophora* cellulose with *Trichoderma viride* cellulase

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Abstract

We previously reported that the algal-bacterial type cellulose microfibril was more susceptible to enzymatic attack than the cotton–ramie type cellulose. In cellulose crystallite (CC) of the algal–bacterial type cellulose, the cellulose I_α crystal component was more selectively degraded than the cellulose I_β crystal component. The shortened CC was observed frequently in the residue of *Cladophora* CC. Fibrillation was observed in the residual *Halocynthia* CC and repeatedly hydrolyzed *Cladophora* CC that richly contained cellulose I_β. These results may suggest the supermolecular structure of CCs. © 1998 Elsevier Science Ltd.

Keywords: Cellulose crystallite; *Cladophora* cellulose; *Halocynthia* cellulose; *Trichoderma* cellulase; Fibrillation; Cellulose I_α

1. Introduction

The structural investigation of cellulose crystallite by solid-state CP/MAS ^{13}C NMR revealed spectral differences among samples of several cellulose origins [1–4]. A crystalline model composed of cellulose I_α and cellulose I_β components was proposed, and it was reported that the algal–bacterial type celluloses are rich in cellulose I_α (triclinic), while cellulose I_β (monoclinic) is predominant in the cotton–ramie type celluloses. Atalla and VanderHart [1] and VanderHart and Atalla [2] suggested that these differences were caused by the difference of the hydrogen-bonding pattern between cellulose I_α and I_β [5–8]. It has been determined that the cellulose I_α is metastable and can be converted into I_β by hydrothermal treatment in the presence of NaOH [9]. Sugiyama et al. characterized these two crystalline structures: the triclinic structure with dimensions of $a = 0.674$ nm, $b = 0.593$ nm, $c = 1.036$ nm, $\alpha = 117°$, $\beta = 114°$, $\gamma = 81°$, cell volume = 0.3395 nm^3, and calculated density = 1.582; the monoclinic structure with dimensions of $a = 0.801$ nm, $b = 0.817$ nm, $c = 1.036$ nm, $\alpha = \beta = 90°$, γ(monoclinic angle) = 97.3°, cell volume = 0.6725 nm^3, and calculated density = 1.599 [10].

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The mechanism of the enzymatic degradation of crystalline cellulose is still poorly understood because of the complexity of the substrate: cellulose in nature is insoluble, fibrous and composed of both crystalline and amorphous regions. Wardrop and Jutte [11] and Chanzy and Henrissat [12], Henrissat and Chanzy [13], Chanzy et al. [14,15] have observed that the hydrolyzed residue of the *Valonia* cellulose microcrystal becomes fibrillated or narrow after the treatment with *Trichoderma reesei* cellulase, and it was shortened by action of the cellulase from *Humicola insolens* [12]. White and Brown [16,17] also observed the fibrillation in the bacterial cellulose microfibril treated with cellulase derived from *T. viride*. No other detailed study has been reported on the enzymatic degradation of the crystals of the algal–bacterial type cellulose composed of two crystalline components.

We previously reported that the algal–bacterial type cellulose microfibril was more susceptible to cellulase attack than the cotton–ramie type cellulose, and the cellulose I_α in the algal–bacterial type cellulose microfibril was more selectively degraded than the cellulose I_β [18]. The substrate used in that work was a homogenized sample, which contained both crystalline and amorphous regions, and their cellulose microfibrils aggregated tightly. Recently, Revol et al. [19] reported that the chiral nematic order structure was arranged by 'highly crystalline microfibrils' [19]. They collected the highly-crystalline samples from the crystalline region of cellulose and suspended them in water, whereby the highly-crystalline cellulose dispersed one by one. If this sample is treated with cellulase, it is not necessary to consider the influences of the amorphous region and of the aggregation of cellulose crystallites during enzymatic treatment. Therefore, we used a suspension of cellulose crystallite (CC) from *Cladophora* sp., which is rich in cellulose I_α, as the substrate in this report. Another aqueous suspension of CC of *Halocynthia* sp. that is exclusively the cellulose I_β component [20] was also used as a control substrate. The hydrolysis residues were examined by FTIR spectroscopy and by electron-diffraction analyses to characterize them.

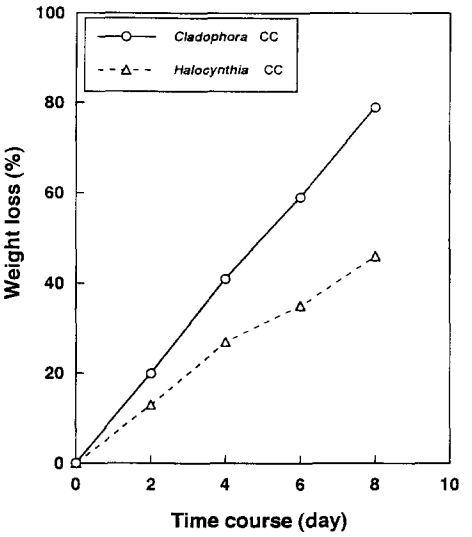

Fig. 1. Time course of the enzymatic hydrolysis of *Cladophora* and *Halocynthia* CCs.

2. Results

The weight losses in the time course of each sample are shown in Table 1. Fig. 1 shows a linear relationship of the weight loss and the hydrolysis time; the slope of the *Cladophora* CC was steeper than the *Halocynthia* CC.

Table 1
The changes in weight loss, *d*-spacings of *Cladophora* and *Halocynthia* CCs and the change in the composition of I_α and I_β of *Cladophora* CC after enzymatic hydrolysis

Sample	Time course (day)	Weight loss (%)	*d*-Spacings (nm)		Composition of I_α/I_β	
			A1	A2	I_α (%)	I_β (%)
Cladophora	0	0	0.622	0.530	53	47
	2	20	–	–	50	50
	4	41	0.611	0.535	49	51
	6	59	0.590	0.535	39	61
	8	79	0.612	0.538	32	68
Halocynthia	0	0	0.605	0.537		
	2	13	–	–		
	4	27	0.606	0.537		
	6	35	0.592	0.537		
	8	46	0.605	0.536		

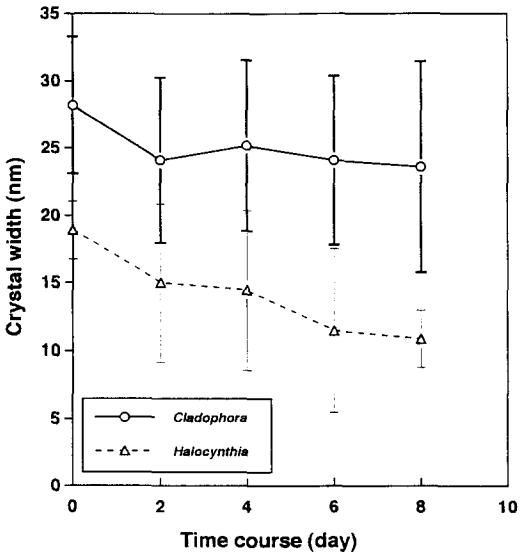

Fig. 2. The changes of the widths of the residues of *Cladophora* and *Halocynthia* CCs which were measured from electron micrographs.

From the measurement of the negatively stained crystallites in electron micrographs, the original *Cladophora* CC was 24–34 nm in width and the *Halocynthia* CC was 17–22 nm, and their lengths were variable (500 nm to several mm). Fig. 2 shows the changes in the crystallite width of the residues of *Cladophora* and *Halocynthia* CCs that were measured from electron micrographs. In the residual *Halocynthia* CC, the width became significantly smaller by enzymatic treatment, while that of the *Cladophora* CC decreased slightly.

Fig. 3 shows the *Cladophora* CC before (a) and after (b) enzymatic treatment. The number of CC with both ends to CC without one end and both ends was 86 to 449, while the numbers changed to 132 to 99 after two days' treatment. The length of CC after the treatment was in a wide range and short CC of about 500 nm in length were frequently observed as shown in Fig. 3b. On the contrary, *Halocynthia* CC clearly did not change in length but became narrower in width during the enzymatic hydrolysis. Some fibrillation was observed in the residues of *Halocynthia* CC treated with cellulase (Fig. 4).

Fig. 5 shows changes of the IR spectra of *Cladophora* (a) and *Halocynthia* (b) in the duration of enzymatic treatment. Bands near 3240 cm^{-1} and near 750 cm^{-1} are characteristic of the cellulose I_α component, whereas those near 3270 cm^{-1} and near 710 cm^{-1} denote the cellulose I_β component [21–24]. In the OH stretching region of the *Cladophora* CC, the absorption of the band near 3240 cm^{-1} decreased with increasing time of cellulase treatment (Fig. 5a). The original *Cladophora* CC is comprised of both the cellulose I_α and cellulose I_β crystal component [22], while the original *Halocynthia* CC has only cellulose I_β [23], as confirmed by the FTIR data. The results of IR absorption indicate that the residue of *Cladophora* CC became rich in the cellulose I_β component. The second-derivative spectrum of the residues of *Cladophora* CC (Fig. 5c) gets close to that of the

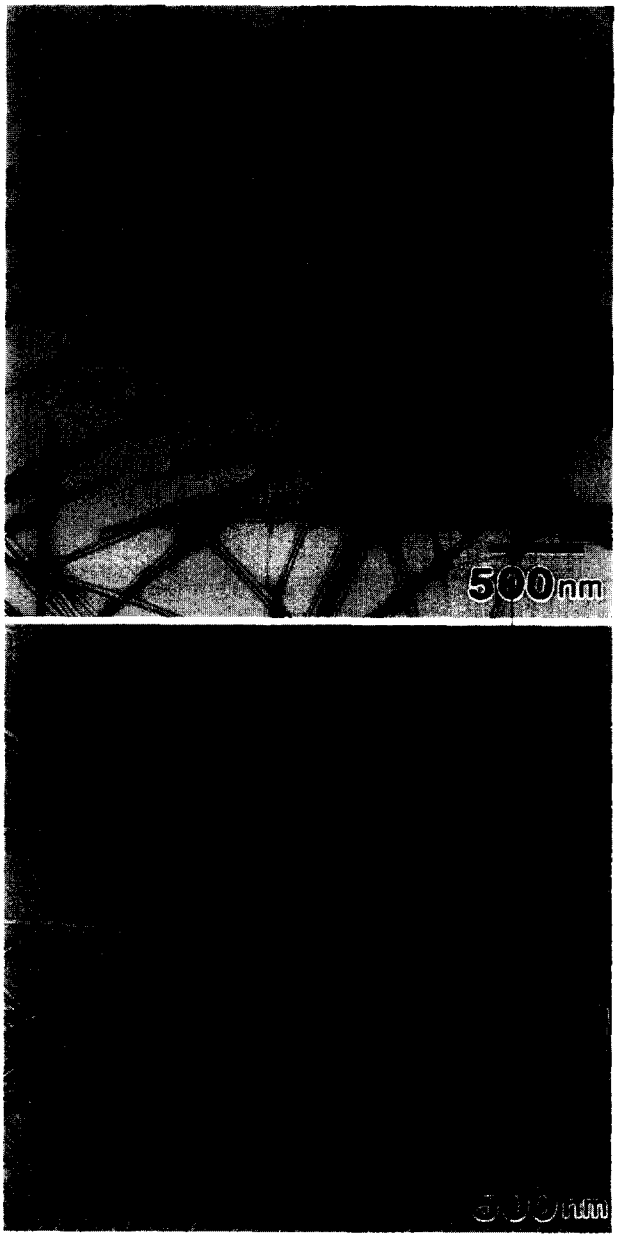

Fig. 3. The electron micrographs of the residual *Cladophora* CC (a) before and (b) after 2 days of enzymatic treatment.

Halocynthia residues (Fig. 5d) near the 3350 cm^{-1} region. In the spectra resulting from heavy-atom bending, both C–O and ring mode [21,25] at the region 800–650 cm^{-1}, the absorption at 750 cm^{-1} in the residual *Cladophora* CC also decreased. The change in the composition of cellulose I_α and cellulose I_β as calculated from the peak height of 750 cm^{-1} and 710 cm^{-1} according to the method of Yamamoto et al. [26] is shown in Table 1. The original *Cladophora* CC consisted of 53% of the cellulose I_α and 47% of the cellulose I_β. After enzymatic hydrolysis, the composition of the cellulose I_α changed to nearly half that of cellulose I_β. In response to the compositional change, the absorption bands at about 3270 cm^{-1} and 710 cm^{-1} in *Halocynthia* CC were rendered observable by enzymatic treatment (Fig. 5b).

Fig. 6 shows the electron-diffraction diagrams of *Cladophora* and *Halocynthia* CCs before and after enzymatic treatment. In the residues of *Cladophora* CC, a triclinic diffraction spot of $(\bar{1}03)$ plane disappeared, and the spot of the second-layer meridian reflection of (002) plane was observed clearer than that of the untreated sample.

From the geometry of the two-chain monoclinic unit cell and the one-chain triclinic unit cell, it is considered that in the algal–bacterial type cellulose whose cellulose crystal contains both a triclinic and monoclinic system, the relative intensities of the meridional diffraction spots at the fourth layer line (004) should be more intense than those of the second layer (002) because the (002) in the triclinic system do not coincide with those in the monoclinic system as meridional lattice points [10,27]. For simplicity, we refer to the fourth- and second-layer meridional spots as (004) and (002) on the basis of the monoclinic system. According to the studies on the intensity ratio of (002)/(004) [10,28], the intensity ratio of the algal–bacterial type cellulose is below 0.1, and that of the cotton–ramie type cellulose is above 0.1. Fig. 7 shows the changes of the intensity ratio of (002)/(004) of *Cladophora* and *Halocynthia* CCs during enzyme treatment. The ratio of *Cladophora* CC was 0.06, whereas enzyme treatment made the value larger than 0.1. The value of *Halocynthia* CC, however, changed little. The *d*-spacing of the A1 $(1\bar{1}0)$ and A2 (110) spots of *Cladophora* CC changed into those of typical cellulose I_β, whereas that of the *Halocynthia* crystallite did not change, even in the advanced stages of enzymatic hydrolysis (Table 1).

Fig. 8 shows the change of the infrared ratio, a_{1429} cm^{-1}/a_{893} cm^{-1}, which was a measure of crystallinity index by IR. The crystallinity index shows gradual decrease in the residual *Cladophora* CC; on the other hand, the crystallinity index of residual *Halocynthia* CC increased remarkably.

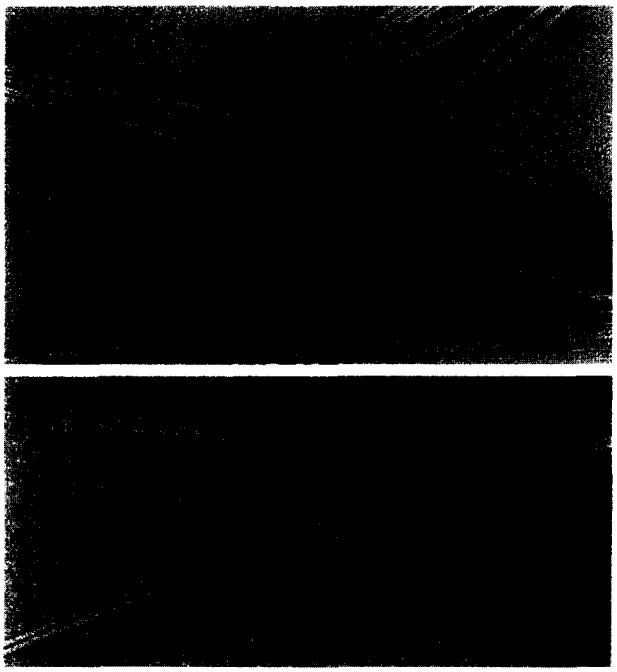

Fig. 4. The electron micrograph of *Halocynthia* CC of (a) untreated and (b) the residue treated with cellulase for 2 days.

3. Discussion

The CCs were less accessible to the enzyme than the cellulose sample from cell walls described in the previous paper [18]. These results did not agree with those of Chanzy and Henrissat [12] who dealt with the enzymatic susceptibility of *Valonia* cellulose pellicles and crystallites. The disagreement should be explained because our samples contained exclusively highly ordered cellulose chain molecules from the crystalline region of the cell walls. Nevertheless the *Valonia* sp. produces larger crystallites than either the *Cladophora* sp. and *Halocynthia* sp.

Two phenomena observed during the enzymatic hydrolysis of the *Cladophora* and *Halocynthia* CCs are (1) a constant rate of the weight loss and (2) a steeper slope for *Cladophora* than for *Halocynthia*. These observations indicate two facts: one is that both cellulose crystallites are completely free from amorphous cellulose, and the other is that the *Cladophora* CC is more susceptible than the *Halo-*

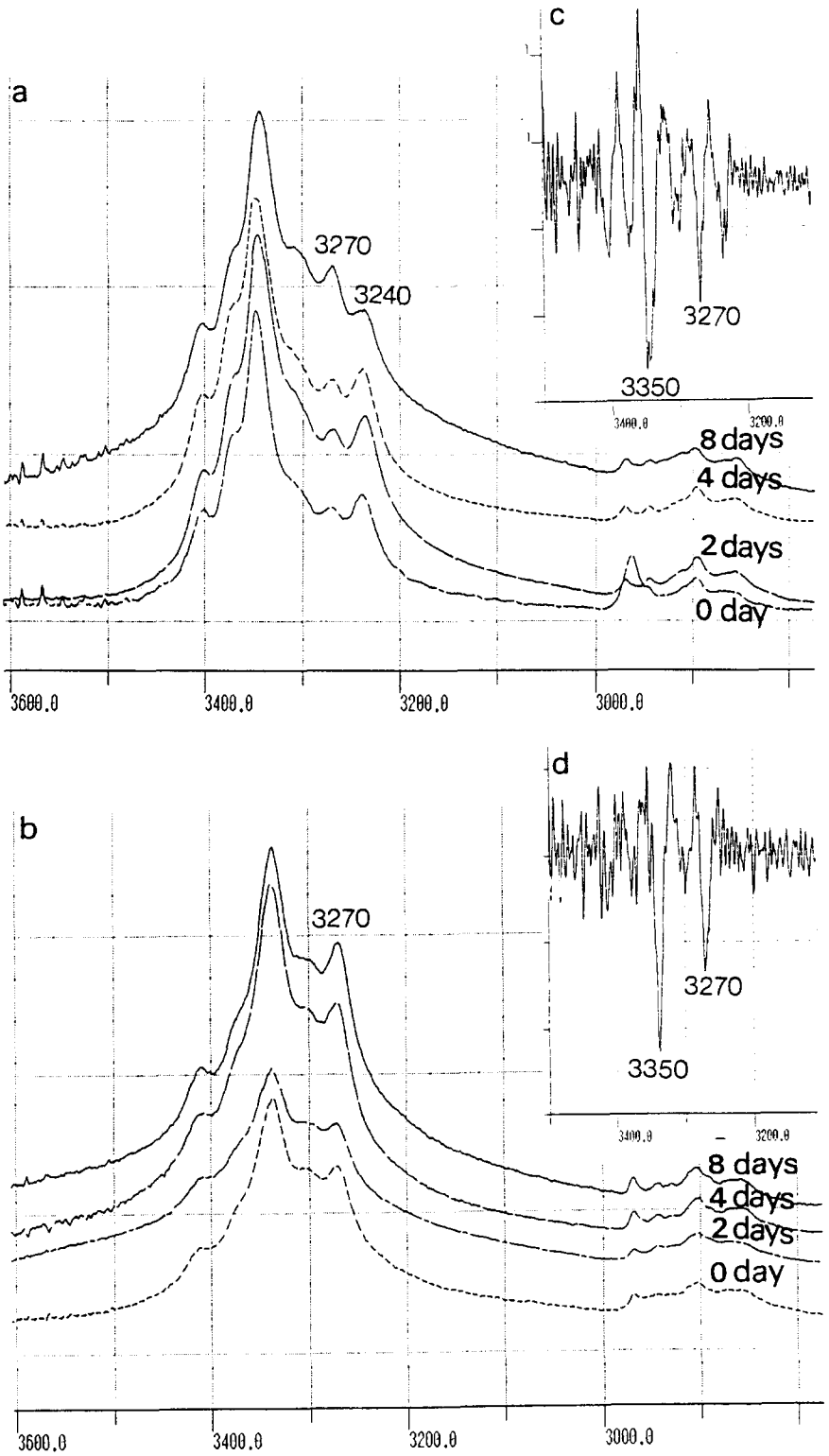

Fig. 5. FTIR spectra in the region from (a) 3600 cm^{-1} to 2600 cm^{-1} of *Cladophora* CC and (b) *Halocynthia* CC treated with cellulase for 0, 2, 4, and 8 days, and (c,d) the second-derivative spectra of the residues treated for 8 days.

cynthia CC to the enzyme. The two CCs, even though with quite similar features, are different in their crystal structure. Consequently, cellulose I_α is more susceptible to enzyme action compared with cellulose I_β.

The results of IR absorption and the *d*-spacings of the residual CCs indicate that the characteristics in the hydrolyzed residues might be explained from the difference of the crystal component. The results of the ratio of cellulose I_α to cellulose I_β as calculated

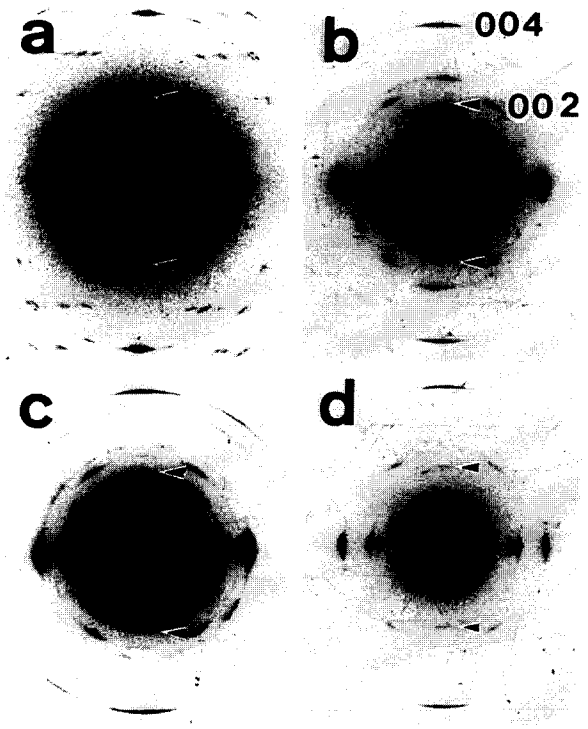

Fig. 6. The electron-diffraction diagrams of *Cladophora* CC (a) untreated and (b) treated with cellulase for 8 days and of *Halocynthia* CC (c) before and (d) after 8 days of enzymatic treatment.

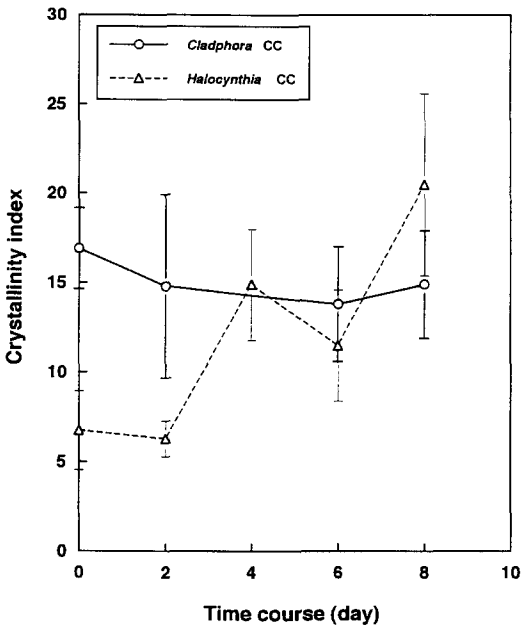

Fig. 8. The changes of the infrared ratio, a_{1429} cm^{-1} / a_{893} cm^{-1}, of the *Cladophora* and *Halocynthia* CCs after enzymatic treatment.

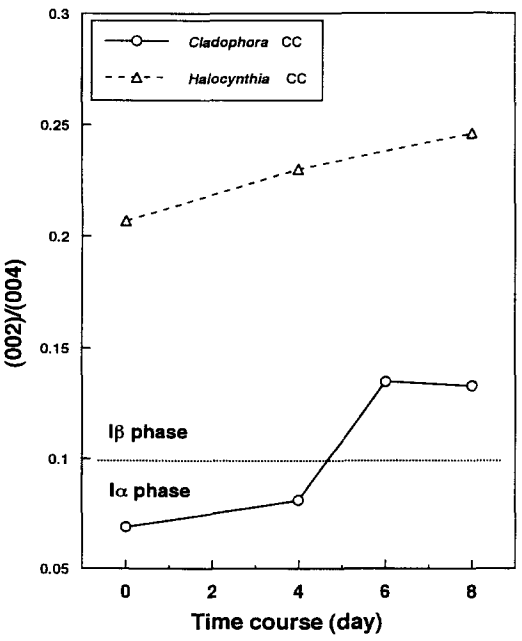

Fig. 7. The changes of the intensity ratio of (002)/(004) of *Cladophora* and *Halocynthia* CCs during enzymatic treatment.

from the IR absorption confirmed that the cellulose I_α in the *Cladophora* CC was hydrolyzed more selectively with cellulase than was the cellulose I_β. The electron-diffraction diagrams also supported the contention that the enzymatic action on *Cladophora* CC gave rise to the selective removal of the cellulose I_α component with respect to the cellulose I_β component. The disappearance of the diffraction spot $(\bar{1}03)$(T) from the cellulose sample composed of cellulose I_β was already confirmed by Sugiyama et al. [10]. This result was the same as that in the previous report [18].

The measurements of the sizes in the residual CCs suggested that the enzyme tended to shorten the length of the *Cladophora* CC or to narrow the width of *Halocynthia* CC. Some fibrillation was observed in the residues of *Halocynthia* CC. This fibrillation was also observed in the residues of *Cladophora* CC that were treated repeatedly with cellulase. We have already reported the thinned and fibrillated microfibrils that occur in the cotton–ramie type cellulose where cellulose I_β is dominant, when treated with *T. viride* cellulase [29]. Therefore, the fibrillation seems to be associated with the region of the cellulose I_β crystal component of the CCs.

The results of the FTIR analyses indicate a great deal about the supermolecular structure of the CCs. The untreated CCs have high crystallinity; however, the ratio of absorption at 1429 cm^{-1} and at 893 cm^{-1} shows the difference of minute changes in those highly crystalline regions. The behavior of the crystallinity index differs markedly between the residual *Cladophora* and *Halocynthia* CCs. The modest reduction in the crystallinity index in the *Cladophora* CC might reflect the selective degradation of the I_α component. On the other hand, the increase of the crystallinity of the *Halocynthia* CC suggests that the CC contains a highly crystallized region, so that the structure in a CC should be not homogeneous. The increase of the peak height in the FTIR spectra of the residual *Halocynthia* CC may also indicate the presence of disordered chains of cellulose on the surface or within the crystallite. Partly disordered chains were proposed by Rowland and Howley [30]. Verlhac et al. [31] compared the availability of the surface hydroxyl groups in *Valonia*, bacterial cellulose and cotton linter, and found the high surface-perfection of the *Valonia* and bacterial cellulose crystals and the fully disorganized cellulose in the cotton samples [31]. Our results will support their findings. Further investigation is needed to clarify the causes of these phenomena concerning the morphological changes of the CCs.

It was concluded that the CC of algal–bacterial type cellulose is more susceptible than that of the cotton–ramie type cellulose to enzymatic attack, and cellulose I_α component in the algal–bacterial type cellulose is much more selectively hydrolyzed than cellulose I_β. In addition, shortened CCs are observed frequently in the residual *Cladophora* CC, whereas some fibrillation is observed in the residue of *Halocynthia* crystallite and repeatedly hydrolyzed *Cladophora* CC which contains large amounts of cellulose I_β. The fibrillation seems to be characteristic of the cellulose I_β crystallite. FTIR analysis suggests the differences in supermolecular structure between the *Cladophora* and *Halocynthia* CCs, as well as the differences in their crystal component.

4. Experimental

Substrates.—The substrates used were the suspensions of cellulose crystallites (CCs) of *Cladophora* sp. and *Halocynthia* sp. The purified sample of *Cladophora* sp. and bleached tunic of *Halocynthia* sp. were treated with 100 mL of 65% sulfuric acid (w/w). The treatment conditions were 25 °C for 30 min for the *Cladophora* sp. and 70 °C for 30 h for the *Halocynthia* sp. The CCs thus obtained were then washed with distilled water successively by centrifugation until a pH range of from 1 to 5 was achieved.

The crystallinity index (CrI) calculated from X-ray profiles of the purified samples before acid hydrolysis were 82% (*Cladophora* sp.) and 86% (*Halocynthia* sp.), respectively [18], and the average crystallite sizes decided from $(1\bar{1}0)$ and (110) [1] planes by Scherrer's equation [32] were as follows: 11 nm × 9 nm for *Cladophora* sp. and 8 nm × 9 nm for *Halocynthia* sp. Therefore, the sizes of the two substrates were not appreciably different.

Enzymatic treatment.—The above samples were hydrolyzed with a commercial cellulase 'Meicelase' (Meiji Seika Kaisha, Tokyo, Japan) derived from *Trichoderma viride* as previously described [18]. The cellulase preparation was fractionated by ultrafiltration membranes. The fraction with molecular mass higher than 10 kDa (2.5–5 mg) was added to 5 mg of substrate in 5 mL of 0.1 M sodium acetate buffer and incubated at 48 °C for 2 days. After centrifugal separation of the reaction mixture into the residues and products, the residues were successively and thoroughly washed with 0.1 N NaOH and distilled water, and then freeze-dried. The enzymatically hydrolyzed residues were repeatedly treated four times with a new cellulase solution under the same conditions to remove the susceptible portion. The enzymatic susceptibility was determined by ratio of the weight loss to the initial sample weight.

Observation by TEM.—The residues from two-day enzymatic treatments were examined under the transmission electron microscope (TEM; JEM-2000EX, JEOL). Some of the freeze-dried residues were suspended in water. Drops of the suspension were deposited on a carbon-coated grid. These were used without further treatment for electron diffraction, and negatively stained with 1.5% uranyl acetate for imaging. TEM was operated at an accelerating voltage of 100 kV and 200 kV for imaging and of 200 kV for electron diffraction. The width of the CCs were measured from the electron micrographs of nega-

[1] Throughout this study, indexing for major equational and meridional crystallographic planes are based on the monoclinic model [21]. However, the specific reflections are given by triclinic indexing by indicating (T) after Miller indices.

tively stained samples. In the micrographs their lengths were also compared with the number of CCs with both ends to the number of the CCs with one or no end. The diffraction diagrams were measured with a microdensitometer equipped with a microscopic accessory (3CS, Joice Loeble). From the profiles the half-width of the three major equational diffractions illustrated as A_1, A_2 and A_3 were measured. The *d*-spacing was calculated from the diffraction profiles obtained by the densitometer.

FTIR spectral analysis.—Fourier-transform infrared (FTIR) analyses were carried out using KBr discs containing hydrolyzed residues with an FTIR-8100 M spectrometer equipped with a microscopic accessory (Shimadzu). All the FTIR spectra were recorded in the transmission mode with a resolution of 2 cm^{-1} in the range 4000–650 cm^{-1}. The crystallinity indexes were compared with the infrared ratios by the method of Nelson and O'Connor [33]. The ratio of absorption at 1429 cm^{-1} (CH_2-scissoring motion) and absorption at 893 cm^{-1} (vibrational mode involving C-1 of β-linked glucose) was used [24].

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