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Enzymatic hydrolysis of bacterial cellulose

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Abstract

Native cellulose from the bacterium *Acetobacter xylinum* as well as acid-treated bacterial cellulose prepared from partial hydrolysis of the native bacterial cellulose with 2.5 N HCl were subjected to enzymatic hydrolysis by *Trichoderma viride* cellobiohydrolase I (CBH I) and endoglucanase II (EG II). The activities of the two enzymes were continuously monitored with an oxidation–reduction potential electrode based on the cellobiose dehydrogenase-ferricyanide redox system. The individual CBH I and EG II hydrolyzed both native and acid-treated bacterial celluloses in a similar way. While CBH I rapidly hydrolyzed both cellulose samples, the ability of EG II to hydrolyze these samples was very limited. However, the hydrolytic behavior of the two cellulose samples by the combination of the two enzymes was significantly different. The rate of hydrolysis of the native bacterial cellulose increased drastically with the combination of the two enzymes, while no synergistic increase in hydrolysis rate was observed with the acid-treated cellulose. Electron microscopy demonstrated that the synergistic action of CBH I and EG II for the native bacterial cellulose involved drastic disintegration of the twisted and bent ribbon-like structure of microfibril bundles and gave rise to the formation of linear, needle-like microcrystallites. Thus, the ribbon-like structure of microfibril bundles in the native bacterial cellulose seems to have a high susceptibility for the combined action of the two enzymes. In contrast, the microfibril aggregates of the acid-treated bacterial cellulose were not disintegrated by the combination of the two enzymes. From these observations, it seems reasonable to assume that differences in the assembling pattern of the microfibrils must be one of the major reasons for the significant

Abbreviations: CDH, cellobiose dehydrogenase; CBH I, cellobiohydrolase I; EG II, endoglucanase II; ORP, oxidation-reduction potential; BC, bacterial cellulose

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differences in the synergism of the two enzymes for the two bacterial cellulose samples.

Keywords: Bacterial cellulose; Endoglucanase; Cellobiohydrolase; Cellobiose dehydrogenase; Cellulase; Cellulose degradation

1. Introduction

The primary structure of cellulose is a linear β-(1 → 4)-glucan polymer. However, the secondary and tertiary structures of cellulose are complicated and vary significantly depending on source and preparation. These structural differences of cellulose are very important with regard to their susceptibility to enzymatic hydrolysis [1,2]. Crystalline celluloses from plant cell walls have been widely used as substrates for studies of the function of cellulases. However, such samples are relatively heterogeneous, and their natural structures are considerably altered in the preparation procedures [3]. Moreover, most commercial celluloses are available only in dried forms, and it has been recognized that drying of cellulose gives rise to a significant decrease in its susceptibility to enzymatic hydrolysis, even after a long time of soaking in water [4,5]. Therefore, the use of a never-dried cellulose sample is a better choice of substrate for enzymatic studies.

Never-dried crystalline cellulose from cultures of the Gram-negative bacterium, *Acetobacter xylinum*, can be prepared using a relatively simple protocol [6]. Moreover, cellulose produced by *A. xylinum* has a more homogeneous structure and higher crystallinity than those of plant origin. For these reasons, never-dried bacterial cellulose has recently been the substrate of choice for cellulase studies [7–10]. Although bacterial cellulose is more susceptible to cellulase attack than other crystalline celluloses [2], the reasons for this phenomenon have not been elucidated.

The native bacterial cellulose produced in static cultures of *A. xylinum* possesses a ribbon-like structure which is composed of microfibril bundles. In the biosynthesis of the bacterial cellulose, β-(1 → 4)-glucan chains are first spun into microfibrils with a width of 3.0–3.5 nm. These microfibrils are assembled stepwise to form the ribbon-like structure [11]. Acid-treatment of the native bacterial cellulose with 2.5 N HCl under refluxing conditions results in transformation of the ribbon-like structure into an aggregation of microcrystalline cellulose through disruption and cutting of microfibrils [12].

In this study, the behavior of both native and acid-treated bacterial celluloses when hydrolyzed by purified *Trichoderma* CBH I and EG II individually and in combination, was compared. A new detection system of cellulase activity with the oxidation–reduction potential (ORP) electrode using the CDH–ferricyanide redox system was applied in these studies [13,14]. Furthermore, the morphological changes of the two bacterial cellulose samples that were exposed to enzymatic hydrolysis were observed by transmission electron microscopy. We could, in these experiments, demonstrate significant differences between the native and the acid-treated bacterial celluloses in their susceptibility to hydrolysis by the combination of CBH I and EG II. The possible reasons for these differences are discussed.

2. Experimental

Enzymes.—CBH I and EG II were purified from Meicelase, a commercially available *Trichoderma viride* cellulase mixture (Meiji Seika, Tokyo, Japan). The purification procedure was the following: Meicelase (10 g of dried powder) was dissolved in 100 mL of deionized water and centrifuged to remove insoluble materials. The supernatant obtained was subjected to a QAE-Toyopearl 550 C column (44 × 170 mm) equilibrated with 20 mM sodium acetate buffer, pH 6.0. EG II was eluted with the starting buffer, whereas CBH I was eluted with a 1 M sodium acetate buffer pH 6.0. The fractions containing CBH I were pooled and further purified on a DEAE-Toyopearl 650 M column (44 × 145 mm) equilibrated with 20 mM potassium phosphate buffer, pH 7.0, with a linear gradient of potassium chloride up to 0.5 M. CBH I was then purified by gel filtration on a Toyopearl HW-55 column equilibrated with 20 mM sodium acetate buffer, pH 5.0, and finally purified on a Phenyl-Toyopearl 650 M column (32 × 130 mm) equilibrated with 20 mM potassium phosphate buffer, pH 7.0, in each 0.2 M reverse-stepwise gradient of ammonium sulfate from 0.8 M down to 0.0 M.

The crude EG II, after the QAE-Toyopearl column, was further purified on a SP-Toyopearl 550 C column (44 × 170 mm) equilibrated with 20 mM sodium acetate buffer, pH 4.0, in a linear gradient of sodium chloride up to 0.4 M. The final purification of

EG II was done on a Phenyl-Toyopearl 650 M column (32 × 130 mm) equilibrated with 20 mM potassium phosphate buffer, pH 7.0, in each 0.1 M reverse-stepwise gradient with ammonium sulfate from 0.5 M to 0.0 M.

Purity and identity of the purified CBH I and EG II were confirmed on both SDS- and IEF-gels by comparison with the authentic samples [15,16], kindly provided by Drs. M. Tenkanen and M. Siika-aho of VTT Biotechnology and Food Research, Helsinki, Finland. The concentrations of the purified CBH I and EG II were evaluated from the absorbence at 280 nm ($E = 10.0$). CDH was purified from *Phanerochaete chrysosporium* as previously described [14]. The concentration of CDH was evaluated from the absorbence at 421 nm ($E = 8.4$).

Cellulose samples.—Bacterial cellulose was obtained from static cultures of *A. xylinum* BPR 1071, grown on Hestrin and Schramm medium [6] supplemented with mannitol instead of glucose as a carbon source. The cellulose pellicle thus produced was harvested, washed several times with deionized water, and then dispersed with a homogenizer at 15 K rpm for 5 min on a cooling bath with slush ice. The cellulose sample was then soaked in 0.1% sodium hydroxide at 70 °C for 2 h to remove bacterial cell debris and washed several times with deionized water to get a neutral pH level. The sample obtained by this protocol is here defined as native bacterial cellulose. The sample, provided with a drop of $CHCl_3$, was stored in a refrigerator until used.

The acid-treated cellulose was prepared from the native cellulose by refluxing in 2.5 N hydrochloric acid for 1 h under constant stirring. The sample obtained was washed several times with deionized water and then dialyzed against deionized water. The amorphous cellulose was obtained from the SO_2–amine solution of Avicel by regeneration according to Isogai and Atalla [17]. Celloheptaitol was prepared by the reduction of celloheptaose with sodium borohydride as described in previous reports [18,19].

Enzymatic hydrolysis of cellulose.—Enzymatic hydrolysis of the cellulose samples was performed as follows: 1.5 mg of the cellulose sample was incubated in 1.8 mL of a solution containing 300 μmol of Na-Ac (pH 4.2) and 180 μg of CDH at 30 °C for 2 h. Then, 0.6 mL of a solution containing 1.5 μmol of potassium ferrocyanide and 1.5 μmol of potassium ferricyanide was added. To start the reaction in a final volume of 3 mL, 0.6 mL of a solution containing 300 μg of CBH I or EG II was added for the experiments with a single enzyme, while 0.6 mL of a solution containing 300 μg of both CBH I and EG II was added for the experiments with the combined enzymes. The hydrolysis rate of cellulose by cellulases was continuously monitored by following the changes of ORP [13,14]. With this technique, not only reducing end-groups in cellobiose but also those in cellodextrin and even in cellulose can be detected [20].

X-ray diffraction and FTIR spectra.—The suspensions of cellulose samples were freeze-dried and 100 mg of the sample was pressed into a pellet at 200 kg/cm^2. X-ray diffraction of the sample was estimated at a symmetrical reflection mode with a Rigaku RAD-1. The data were obtained between a diffraction angle of 5° and 40° by Cu K α radiation generated at 35 kV, 20 mA ($\gamma = 0.15406$ nm). The diffraction angle was calibrated using a diffraction line for sodium fluoride. Profile analysis was made by a least-squares peak fitting program assuming a Gaussian diffraction function, and crystallographic features such as *d*-spacing and crystalline width were estimated using the conventional Scherrer equation.

The cellulose suspension was deposited on the surface of a glass slide and allowed to air-dry. The cellulose membrane obtained was easily detached from the glass surface after 15 s of immersion in a

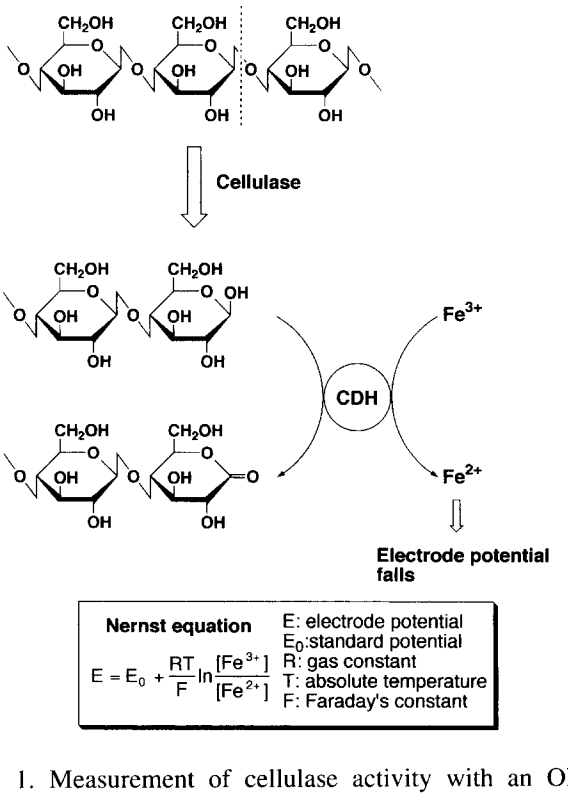

Fig. 1. Measurement of cellulase activity with an ORP electrode based on CDH-ferricyanide redox system.

solution of 0.1% hydrofluoric acid and floated on water. The membrane, after air-drying, was then mounted on a 3-mm hole of the sample holder. The spectra were recorded in the absorbance mode between the range of 4600 and 400 cm^{-1} with a JASCO FTIR 7000 spectrometer.

Determination of maximum adsorption of CDH for cellulose.—Maximum adsorption of CDH for a cellulose sample was estimated according to a previous report [14].

Transmission electron microscopy.—A drop of dispersed cellulose suspension was mounted on a carbon-coated grid, and after squeezing out excess water, the sample was allowed to air-dry. It was investigated with a JEOL JEM-2000EX transmission electron microscope operated at 100 kV. The images were taken by diffraction contrast in the bright field mode, recorded on Mitsubishi electron microscopic film (MEM) and developed by Microfine™ (Fuji Film, Tokyo) at 20 °C for 5 min.

3. Results

Hydrolysis of the cellulose samples by CBH I and EG II.—Hydrolysis of the cellulose samples by CBH I and EG II was monitored with an ORP electrode employing the CDH-ferricyanide redox system as shown in Fig. 1. In this system, the reducing end-groups created in the cellulose by the action of CBH I and EG II are immediately oxidized to the corresponding lactones by excess amounts of CDH. Electrons captured by CDH transfers ferricyanide (Fe^{3+}) to ferrocyanide (Fe^{2+}) and shifts the equilibrium of Fe^{3+}/Fe^{2+}. The change of 1 mV electrode potential corresponds to the formation of approximately 4.8 μM of reducing end groups by the action of cellulases at the working conditions.

The hydrolysis rates of four cellulose samples by CBH I and EG II are shown in Fig. 2. CBH I was able to hydrolyze both native and acid-treated bacterial celluloses at reaction rates comparable to those

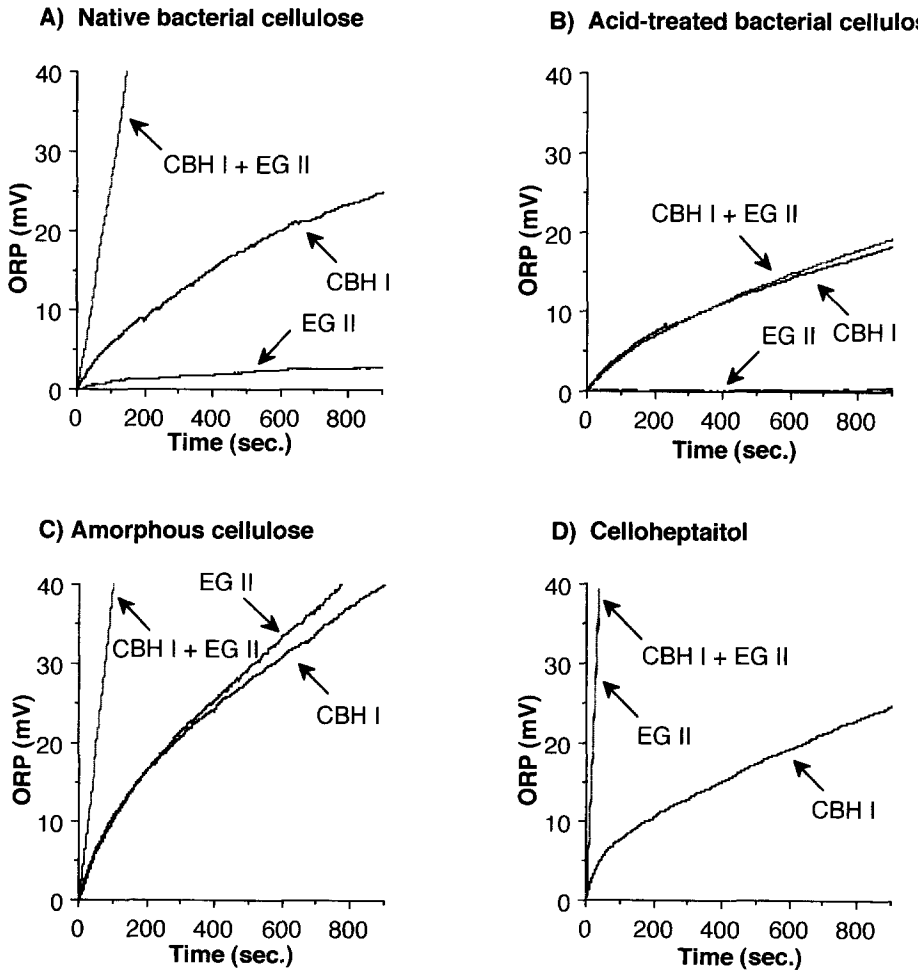

Fig. 2. Time-course changes of ORP for hydrolysis of cellulose samples by CBH I and EG II. (A) Native bacterial cellulose, (B) acid-treated bacterial cellulose, (C) amorphous cellulose, (D) celloheptaitol.

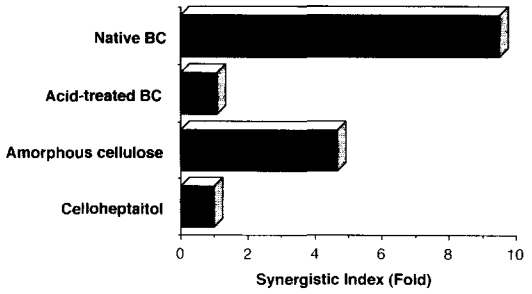

Fig. 3. Synergistic effects on hydrolysis of the cellulose samples by the combination of CBH I and EG II.

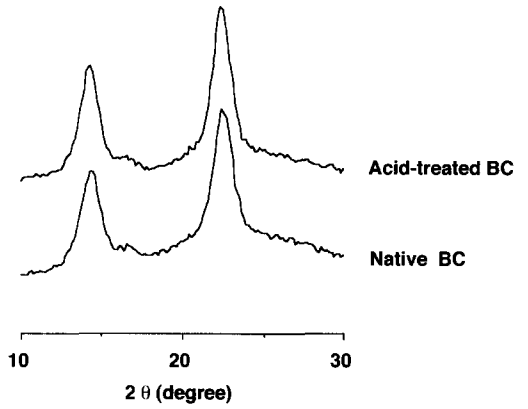

Fig. 4. X-ray diffraction patterns of bacterial cellulose samples.

for the amorphous cellulose and celloheptaitol. However, both native and acid-treated bacterial celluloses showed high resistance to hydrolysis by EG II. These results indicate that amorphous structures, susceptible to hydrolysis by EG II, are very limited in both native and acid-treated bacterial celluloses. In contrast, both the amorphous cellulose and celloheptaitol were hydrolyzed rapidly by EG II. However, the hydrolysis rate of celloheptaitol by EG II was much faster than that of the amorphous cellulose, probably demonstrating the difference in accessibility between a soluble and an insoluble substrate. It is also worth mentioning that EG II hydrolyzed celloheptaitol 36 times faster than CBH I at the same concentration of the enzymes.

Table 1
Crystallographic data for the bacterial cellulose samples

		100	010	110
Native BC	*d*-spacing (nm)	0.615	0.536	0.393
	thickness (nm)	7.8		7.9
Acid-treated BC	*d*-spacing (nm)	0.618	0.540	0.394
	thickness (nm)	8.1		8.3

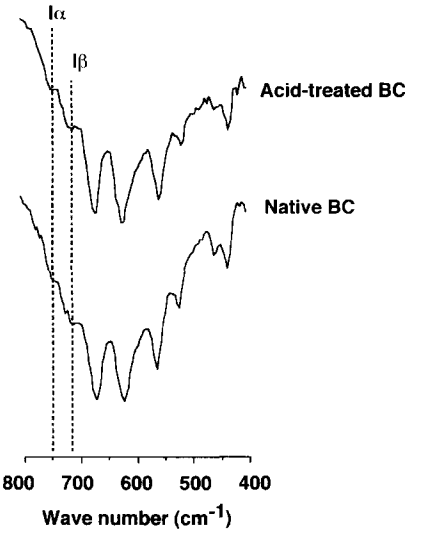

Fig. 5. FTIR spectra of bacterial cellulose samples.

While the individual CBH I and EG II hydrolyzed both native and acid-treated bacterial celluloses in a similar way, the hydrolysis rates for the two celluloses were significantly different with the combination of the two enzymes. The hydrolysis rate of the native bacterial cellulose increased dramatically by the combination of the two enzymes, actually to a level comparable to that for the amorphous cellulose and celloheptaitol. However, the hydrolysis rate of the acid-treated cellulose by the combination of the two enzymes was almost the same as for CBH I alone. In Fig. 3, the synergistic effect obtained by the combination of CBH I and EG II is compared to the non-synergistic sum of the individual action by CBH I and EG II. The combination of the two enzymes gave rise to an almost 10-fold increase in hydrolysis of the native bacterial cellulose, while no synergy

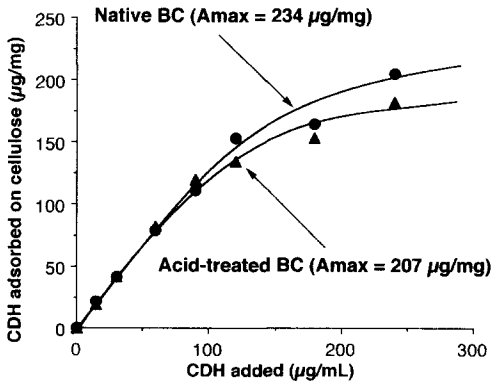

Fig. 6. Adsorption isotherms of CDH on bacterial cellulose samples. A_{max}: Maximum adsorption of CDH (μg) per 1 mg of cellulose.

was observed for the acid-treated bacterial cellulose. A synergistic effect was also detected for the amorphous cellulose, but not for celloheptaitol.

Physical properties of bacterial celluloses.—To investigate the reasons for the significant differences in hydrolysis by the combination of the two enzymes between native and acid-treated bacterial celluloses, several physical properties of the cellulose samples were investigated. As shown in Fig. 4, X-ray analysis demonstrated that both native and acid-treated bacterial celluloses gave very similar diffraction patterns. Moreover, the crystal width and the *d*-spacing of crystal units calculated from diffraction data were almost identical between the two bacterial celluloses (Table 1). In addition, FTIR spectra (Fig. 5) were also similar, and no significant differences were observed in the absorption intensity at 750 and 710 cm^{-1} relating to the $I\alpha/I\beta$ ratio between the two bacterial celluloses [21].

The accessible surface area of the two bacterial celluloses for enzymes was elucidated by their capacity for CDH adsorption. It was found that CDH

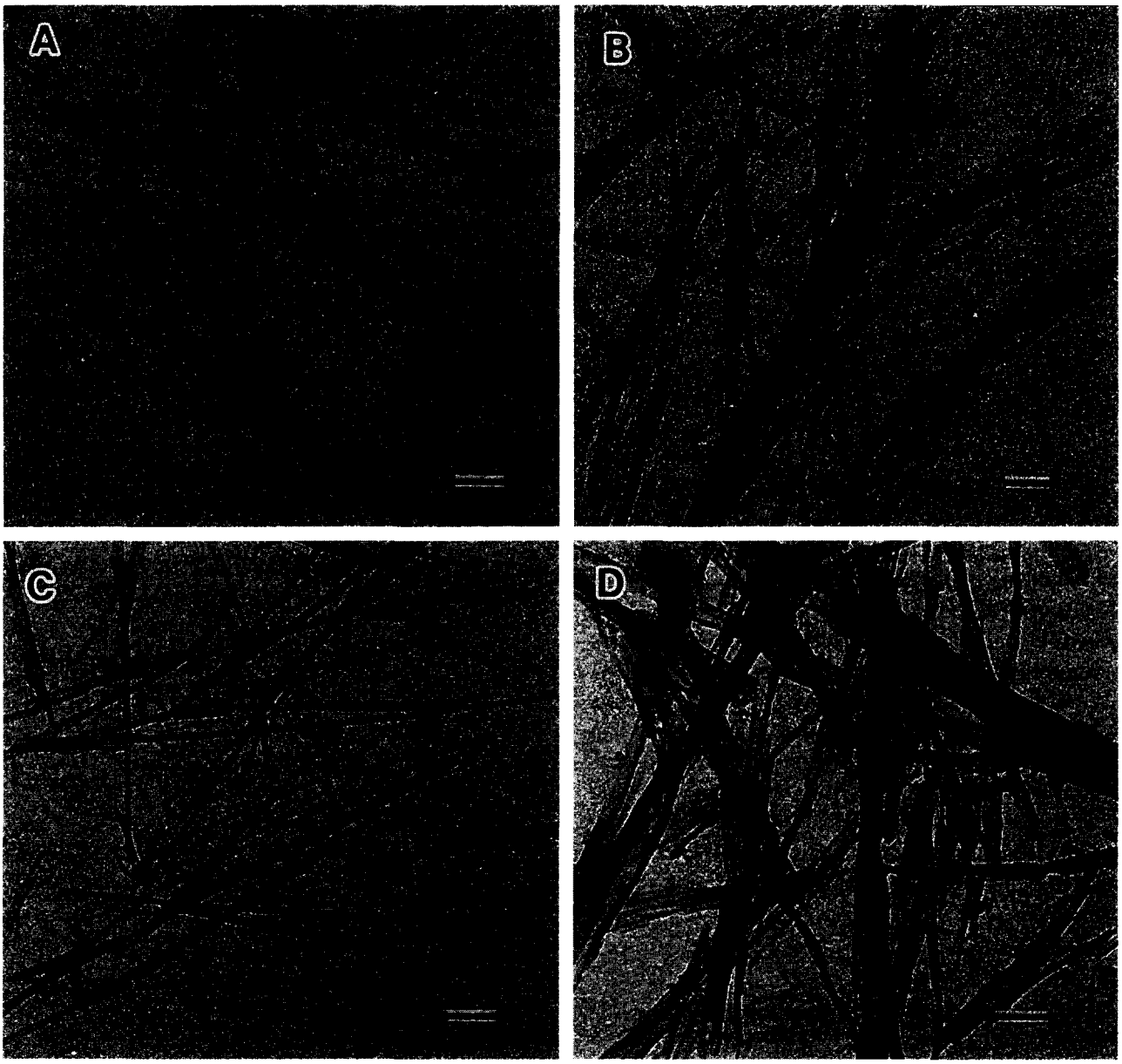

Fig. 7. Morphological changes of bacterial cellulose samples by the combined action of CBH I and EG II (scale bar indicates 200 nm). (A) Native bacterial cellulose (before enzymatic treatment), (B) acid-treated bacterial cellulose (before enzymatic treatment), (C) native bacterial cellulose (after enzymatic treatment for 5 min), (D) acid-treated bacterial cellulose (after enzymatic treatment for 5 min).

adsorbed to both native and acid-treated bacterial celluloses at a similar level and that more than 200 μg of CDH could be adsorbed per mg of both cellulose samples (Fig. 6).

Morphological changes of bacterial celluloses during enzymatic hydrolysis.—Morphological changes of native and acid-treated celluloses in enzymatic hydrolysis by the combination of CBH I and EG II were investigated with transmission electron microscopy. The native bacterial cellulose has a ribbon-like structure composed of microfibril bundles (Fig. 7A). However, this type of structure cannot be seen in the acid-treated cellulose. In the preparation of the acid-treated bacterial cellulose, the ribbon-like structure is disrupted and is converted to re-aggregated linear microfibril bundles with larger width and shorter length (Fig. 7B).

While no morphological changes could be detected in any of the two bacterial celluloses by the individual action of CBH I or EG II in the working condition (data not shown), a combined action of the two enzymes caused drastic morphological changes in the native cellulose (Fig. 7C) but not in the acid-treated cellulose (Fig. 7D). By the combined action of CBH I and EG II, formation of linear, needle-like microcrystallites with various lengths and widths was detected in the native bacterial cellulose.

4. Discussion

The individual enzymes, CBH I and EG II, hydrolyzed both native and acid-treated bacterial celluloses in a similar way. While CBH I rapidly hydrolyzed both cellulose samples, the ability of EG II to hydrolyze these samples was very limited. In addition to the bacterial celluloses, amorphous cellulose and celloheptaitol were also used as substrates. This means that the substrates examined differed both in variation of crystallinity and in degree of polymerization. Surprisingly, the rate of hydrolysis by CBH I for all these cellulose samples was very comparable. Considering that CBH I is an exo-glucanase according to the conventional concept, the behavior of CBH I observed here may be somewhat strange. Several recent reports on the 3D molecular structure of CBH I clearly suggest that CBH I reacts as a glucanase with a processive mode of hydrolyzing for the glucan chain [22–24]. However, the idea of processivity must be distinguished from the concept of the exo-glucanases, which concerns the site for attack on the glucan chain. A recent report on the function of CBH I suggests that this enzyme can attack the glucan chain in a way that is normally ascribed to endo-glucanases [25]. Therefore, more consideration is needed for the site of the initial attack on the glucan chain by CBH I. The results presented here are easy to understand only if it is acknowledged that an initial endo-type attack on the glucan chain can be accomplished by CBH I.

In contrast to the behavior of CBH I, the ability of EG II to hydrolyze both of the bacterial cellulose samples was very limited, while the latter enzyme hydrolyzed the amorphous cellulose and celloheptaitol well. Thus, both of the bacterial celluloses possess, with regard to hydrolysis by EG II, the typical characteristics of a crystalline cellulose, even if both native and acid-treated bacterial celluloses allowed for large amounts of CDH adsorption. We have, in our earlier work, found that CDH adsorbs almost exclusively to the amorphous structure of cellulose [14]. The contradiction as observed between CDH adsorption and EG II hydrolysis may point to ambiguous definitions of what is the amorphous structure of cellulose since the surface of both of the bacterial cellulose samples seems to be disordered or flexible enough to allow for adsorption of CDH, but not for hydrolysis by EG II.

When the combination of CBH I and EG II was used for the hydrolysis of native and acid-treated bacterial celluloses, only the hydrolysis rate for the native bacterial cellulose was drastically increased, and the rate reached a level almost comparable to those for the amorphous cellulose and for celloheptaitol. In contrast, this synergistic effect was not observed for the acid-treated cellulose. Neither X-ray nor FTIR analysis showed differences in crystallinity and crystal form of microfibrils between the two bacterial cellulose samples that were large enough to explain significant differences in hydrolysis by the combination of CBH I and EG II.

Although the major part of microfibrils in both native and acid-treated bacterial celluloses consists of Iα type crystalline units with linear assembling of glucan-chains [21], this ribbon-like structure of microfibril bundles in the native bacterial cellulose is twisted and bent [26]. Therefore, it seems likely that some internal stress must be present to keep this type of structure together [27]. It also seems plausible that if enzymes are able to damage this native structure, the internal stress becomes high enough to disintegrate the ribbon-like structure. Thus, the native bacterial cellulose is fibrillated and disintegrated by the combined action of CBH I and EG II, resulting in a

drastic expansion of the surface area and formation of amorphous structures susceptible to hydrolysis by EG II. This may give rise to the significant increase of the hydrolysis rate observed by the combination of CBH I and EG II. White and Brown have earlier reported that morphological changes of the native bacterial cellulose are caused by cellulases and suggested that the combination of CBH I with EG IV gives rise to drastic structural disruptions [26]. These authors suggested that EG IV must initiate the hydrolytic attack. However, our results demonstrate that CBH I can attack the native bacterial cellulose without any help from EG II. Thus, CBH I is a more likely candidate for accomplishing this initial attack.

Treatment of the native bacterial cellulose with 2.5 N hydrochloric acid under refluxing conditions gives rise to a disruption of the ribbon-like structures and causes a re-aggregation of microfibrils. In this process, the ribbon-like structures of the native bacterial cellulose must be converted to a more stabilized aggregations of microfibril. This might be a possible explanation for the absence of a synergistic effect in the hydrolysis of the acid-treated bacterial cellulose by the combination of CBH I and EG II.

Acknowledgements

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