Digestion of crystalline cellulose substrates by the *Clostridium thermocellum* cellulosome: structural and morphological aspects

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The action of cellulosomes from *Clostridium thermocellum* on model cellulose microfibrils from *Acetobacter xylinum* and cellulose microcrystals from *Valonia ventricosa* was investigated. The biodegradation of these substrates was followed by transmission electron microscopy, Fourier-transform IR spectroscopy and X-ray diffraction analysis, as a function of the extent of degradation. The cellulosomes were very effective in catalysing the complete digestion of bacterial cellulose, but the total degradation of *Valonia* microcrystals was achieved more slowly. Ultrastructural observations during the digestion process suggested that the rapid degradation of bacterial cellulose was the result of a very efficient synergistic action of the various enzymic components that are attached to the scaffolding protein of the cellulosomes. The degraded *Valonia* sample assumed various shapes, ranging from thinned-down microcrystals to crystals where one end was pointed and the other intact. This complexity may be correlated with the multi-enzyme content of the cellulosomes and possibly to a diversity of the cellulosome composition within a given batch. Another aspect of the digestion of model celluloses by cellulosomes is the relative invariability of their crystallinity, together with their I_α/I_β composition throughout the degradation process. Comparison of the action of cellulosomes with that of fungal enzymes indicated that the degradation of cellulose crystals by cellulosomes occurred with only limited levels of processivity, in contrast with the observations reported for fungal enzymes. The findings were consistent with a mechanism whereby initial attack by a cellulosome of an individual cellulose crystal results in its 'commitment' towards complete degradation.

Key words: bacterial cellulose, enzymic digestion, processivity, synergism, *Valonia* cellulose.

INTRODUCTION

A number of studies have been devoted to furthering the understanding of the enzymic degradation of cellulose [1–8]. In order to solubilize crystalline cellulose substrates, bacteria and fungi produce either a collection of free cellulases and/or multicellulase complexes known as cellulosomes [9,10]. Despite these studies, the exact mechanism by which either isolated enzymes or multi-component enzymic systems are able to attack and digest crystalline biopolymers, such as cellulose, remains a mystery.

Cellulose-based materials are usually complex substrates consisting of a hierarchy of structures. In Nature, the polymer chains of native cellulose (or cellulose I) are organized into slender crystalline microfibrils. The microfibrils themselves are further assembled into plant cell walls, the tunica of some sea animals, pellicles of bacterial origin, etc. The microstructure of the crystalline microfibrils also adds a degree of complexity to the picture; in addition to variable amounts of amorphous components, the cellulose microfibril also consists of crystalline nanodomains of two distinct allomorphs: cellulose I_α and I_β [11,12]. Within a given cellulose sample, the fraction of the I_α and I_β phase depends on its source. It has been established that these two phases exhibit different susceptibilities towards chemical as well as enzymic reagents [13–15]. Thus, with a complex solid such as cellulose, the kinetics of enzymic digestion depends on both the macro- as well as the micro-structure of the substrate. Establishing the relative influence of parameters such as accessibility, crystallinity and nanostructure of the substrate is one of the major hurdles that has to be cleared for the full understanding of the enzymic degradation of cellulose.

The availability of models of cellulose microfibrils and different crystalline forms has advanced our understanding of cellulose biodegradation. In this context, the model substrates have been subjected to the action of either individual enzymes or controlled mixtures of enzymic components. Using this approach, each microfibril or crystal has an equivalent accessibility, and thus only the contributions of crystallinity and the perfection of the desired substrate have to be taken into account. To date, structural and morphological studies on the digestion of model cellulose crystals have been achieved, essentially with fungal enzymes from *Trichoderma reesei* and *Humicola insolens* [14–20]. In these studies, ultrastructural observations revealed the processivity and/or synergism of these enzyme systems. In some cases, spectroscopic evidence has also shown that the I_α phase of cellulose was more susceptible toward enzyme degradation than the I_β counterpart [14–15].

The present work is devoted to the study of the digestion of model cellulose crystals by cellulosomes from *Clostridium thermocellum*. These bacterial cellulases differ markedly from those of most fungal systems. In aerobic fungi, the various enzymes that constitute the cellulases occur as separate entities, each of them having a specific action. In contrast, cellulosomes consist of discrete multi-functional, multi-enzyme complexes [21–24], wherein all the enzymic factors leading to cellulose degradation are physically attached to a central scaffold component. Despite a number of studies on the molecular and ultrastructural

Abbreviations used: TEM, transmission electron microscopy; FTIR, Fourier-transform IR spectroscopy.

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definition of the cellulosomes [25–28], the details of their action on crystalline cellulose are still unknown.

In this report, we present some structural and ultrastructural details on the digestion of model cellulose systems by cellulosomes from *C. thermocellum*. Two types of substrate were investigated: isolated ribbons of bacterial cellulose and *Valonia* cellulose microcrystals. The action of the cellulosomes was visualized directly by transmission electron microscopy (TEM). Modifications of the crystallinity and of the hydrogen bonding of the substrate were monitored by Fourier-transform IR spectroscopy (FTIR) as well as by X-ray diffraction analysis.

MATERIALS AND METHODS

Isolation and purification of the cellulosome from *C. thermocellum*

Cellulosome samples were prepared from cellulose-grown cells as described earlier [29]. *C. thermocellum* was cultured at 60 °C on 0.5 % (w/v) microcrystalline cellulose (Avicel; Merck). After 30 h near-complete digestion of the substrate was observed and the cells and residual cellulose were removed by centrifugation. The cell-free broth was cooled on ice and phosphoric-acid-swollen cellulose (0.12 g/l culture), prepared by the method of Lamed et al. [30], was added. After 2 h at 4 °C, the suspension was centrifuged at 10000 ***g*** and stabilizing buffer (12 mM $CaCl_2$/2 mM EDTA/50 mM Tris/HCl buffer, pH 7.5) (30 ml/g of cellulose adsorbent) was added. The suspension was dialysed at 50 °C in a cellulose acetate dialysis bag (Fisher Scientific) until the suspension was clear (3–5 h). The sample was centrifuged at 10000 ***g***, and the supernatant was concentrated and subjected to Sepharose 4B chromatography using 50 mM Tris/HCl buffer, pH 7.5, as the equilibration and elution buffer. The major peak was collected and the fractions were pooled; NaN_3 [0.05 % (w/v) final concentration] was added and the preparation was stored at a concentration of 0.9 mg/ml.

Cellulose samples

Bacterial cellulose

Cubes of bacterial cellulose from Nata de coco, a food-grade commercial cellulose (Fujicco Co., Kobe, Japan), were used throughout. The cubes were extensively washed with tap water in order to remove the sweet syrup. They were then soaked for one week in 1 % (w/v) aqueous NaOH. At this concentration of alkali most of the proteins remaining are solubilized but native cellulose is unmodified. After neutralization with a few drops of concentrated HCl, the cubes were rinsed several times with distilled water. The purified cubes were then homogenized for 10 min in a Waring blender, operated at full speed. The suspensions were stored at 4 °C with 0.01 % (w/v) NaN_3.

Valonia ventricosa

V. ventricosa vesicles were harvested from the seabed off the Lower Keys, Florida. They were slit open, emptied and temporarily dried for transportation. In the laboratory, the dried vesicles were rinsed with tap water to remove sand particles and other contaminants and were then washed with 2 % aqueous NaOH at room temperature. After neutralization with distilled water, they were treated with 0.5 % (w/v) HCl for 30 min. The vesicles were boiled in 2 % aqueous NaOH for 6 h; the solution was changed for fresh 2 % aqueous NaOH every 2 h. This treatment removed most of the encrusting hemicelluloses, but left intact the cellulose. The purified vesicles were neutralized with dilute HCl and washed with distilled water. They were stored at 4 °C in distilled water containing 0.01 % (w/v) NaN_3.

For the preparation of *V. ventricosa* microcrystals, purified cell wall fragments were first homogenized for 10 min at 10000 rev./min. The resulting suspensions were then hydrolysed into microcrystals by refluxing for 5 h in 3.5 M HCl, with stirring. The resulting microcrystals of cellulose were neutralized with a few drops of 10 % aqueous NaOH and washed several times with distilled water. The suspensions of *Valonia* cellulose were stored at 4 °C in distilled water containing 0.01 % (w/v) NaN_3.

Digestion of *Valonia* and bacterial cellulose by cellulosomes

Samples (1 mg) of *Valonia* and bacterial cellulose were incubated without agitation at 55 °C in 50 mM Tris/HCl, pH 7, containing 10 mM $CaCl_2$. A range of cellulosome concentrations was added to these suspensions such that the total reaction volume was kept at 1 ml and the final ratio of cellulosome to cellulose ranged from 2 to 8 % (w/w). The hydrolysis was terminated at various time periods from 0 to 24 h for bacterial cellulose and 0 to 16 days for *Valonia* cellulose. In order to prevent any biological contamination, 0.001 % NaN_3 (w/v) was added to the *Valonia* suspensions. The hydrolysed cellulose suspensions were first centrifuged and the supernatant recovered for further sugar analysis. The pellet was washed at room temperature for 20 min with 1 % NaOH followed by several washes with distilled water. The suspensions were stored at 4 °C in distilled water containing 0.01 % (w/v) NaN_3.

Measurement of the extent of enzymic degradation

The amount of reducing sugars released during enzymic degradation was estimated using a ferricyanide method adapted from [31]. The ferricyanide reagent was prepared as follows: 300 mg of potassium hexacyanoferrate III and 28 g of hydrated Na_2CO_3 were dissolved in 1 litre of distilled water, 1 ml of aqueous 5 M NaOH was then added to make the reagent alkaline. Following enzymic degradation, 1 ml of ferricyanide reagent was added to 100 μl of the supernatants and the absorbance was measured at 420 nm. A linear calibration was obtained from averaging a series of measurements which corresponded to blanks and totally solubilized samples, assumed to be 100 % degraded.

TEM

Drops of bacterial or *Valonia* cellulose suspensions were deposited on carbon-coated copper grids, which had been pretreated by glow discharge. Images were recorded with a Philips CM 200 CRYO electron microscope operated at an accelerating voltage of 80 kV under low-dose mode. Specimens of bacterial cellulose were viewed without staining, whereas *Valonia* microcrystals were negatively stained with 2 % (w/v) uranyl acetate.

FTIR spectral analysis

Suspensions of hydrolysed as well as initial samples were evaporated into thin films. FTIR analyses were performed on these films with a Perkin Elmer 1720 X spectrophotometer. All FTIR spectra were recorded in the absorbance mode with a resolution of 2 cm^{-1} in the range 4000–400 cm^{-1}. Line-shape analyses of the spectra were carried out in the range 800–400 cm^{-1}, according to the method of Yamamoto et al. [32], in order to evaluate the relative fraction of cellulose I_α in each cellulose sample.

X-ray diffraction analysis

Dried, digested cellulose samples were inserted into thin-walled X-ray capillaries, mounted on a goniometer head and X-rayed

on a 5-circle diffractometer at the ID2 beam line (European Synchrotron Radiation Facility, Grenoble, France). The X-ray patterns were recorded for 4 min (with a Mo attenuator), during which time the samples were rotated by 360° about the capillary axis. A wavelength of 0.07000 nm was used and the patterns were recorded with a 345 mm MAR-Research image plate positioned 170 mm from the sample. In all cases, the apparent lateral width of the crystals was calculated for the three equatorial diffraction lines at $2\theta = 10.2°$, 7.4° and 6.7°, using the Scherrer formula: $t = 0.9\,\lambda/B\cos\theta$, where t is the crystal width, λ the wavelength, B the diffraction line-width at half peak size and θ the Bragg angle.

RESULTS

Solubilization of cellulose by cellulosomes

The solubilizing activity of cellulosomes on cellulose was deduced from the formation of reducing sugars. With bacterial cellulose, cellulosome concentrations ranging from 2 to 8 % (w/w, cellulosome to cellulose) were tested (Figure 1). It was found that within

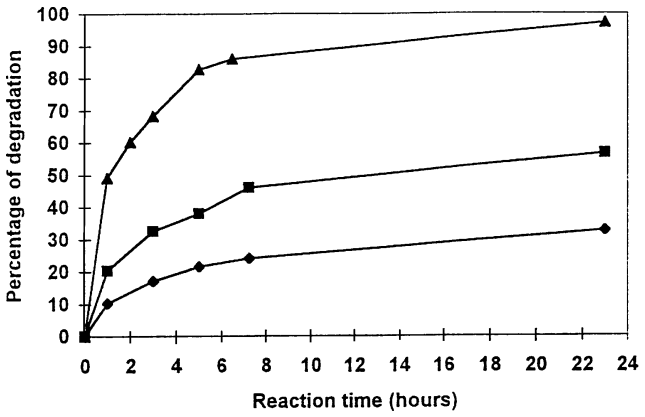

Figure 1 Degradation of bacterial cellulose by cellulosome samples from *C. thermocellum*

Aliquots (100 μg) of bacterial cellulose ribbons were treated with cellulosome preparations at a protein/substrate ratio (w/w) of 2% (◆), 4% (■) or 8% (▲) and the percentage of degradation was plotted against the reaction time.

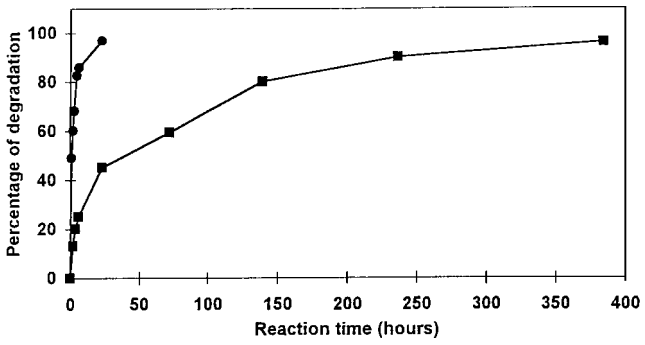

Figure 2 Comparative degradation of *Valonia* and bacterial cellulose by cellulosome samples from *C. thermocellum*

The experiment was carried out as described in the legend to Figure 1, using samples (100 μg) of *Valonia* cellulose microcrystals (■) or bacterial cellulose ribbons (●) treated with cellulosome preparations [8% (w/w) protein/substrate ratio].

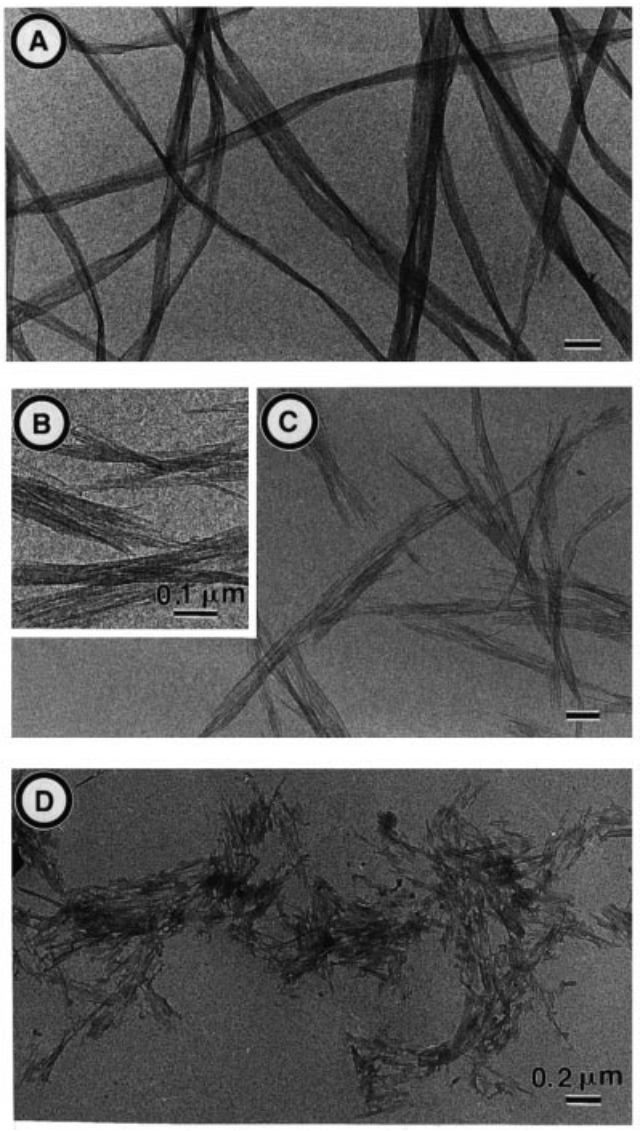

Figure 3 TEM of cellulosome-induced degradation of bacterial cellulose ribbons

(**A**) Untreated cellulose ribbons. (**B**) and (**C**) Cellulose ribbons after 3 h of digestion with a cellulosome preparation from *C. thermocellum* [8% (w/w) protein/substrate ratio]. (**D**) As in (**B**) but after 6.5 h of digestion. (**A**), (**C**) and (**D**) are at the same magnification; the magnification in (**B**) is twice that of the other micrographs.

24 h a concentration of 8 % was sufficient to completely solubilize dispersed bacterial cellulose ribbons. On the other hand, two weeks were necessary to achieve the same effect with the 2 % concentration. Similar treatment of *Valonia* microcrystals (Figure 2) required 16 days to reach 95 % solubilization. Thus under the experimental conditions used, *Valonia* cellulose microcrystals were hydrolysed at least 16 times more slowly than bacterial cellulose ribbons.

Morphological observations of the action of cellulosomes on cellulose

The progress of biodegradation by cellulosomes was followed by TEM, using either cellulose ribbons from the bacterium *A. xylinum*, or the larger cellulose microcrystals from the alga

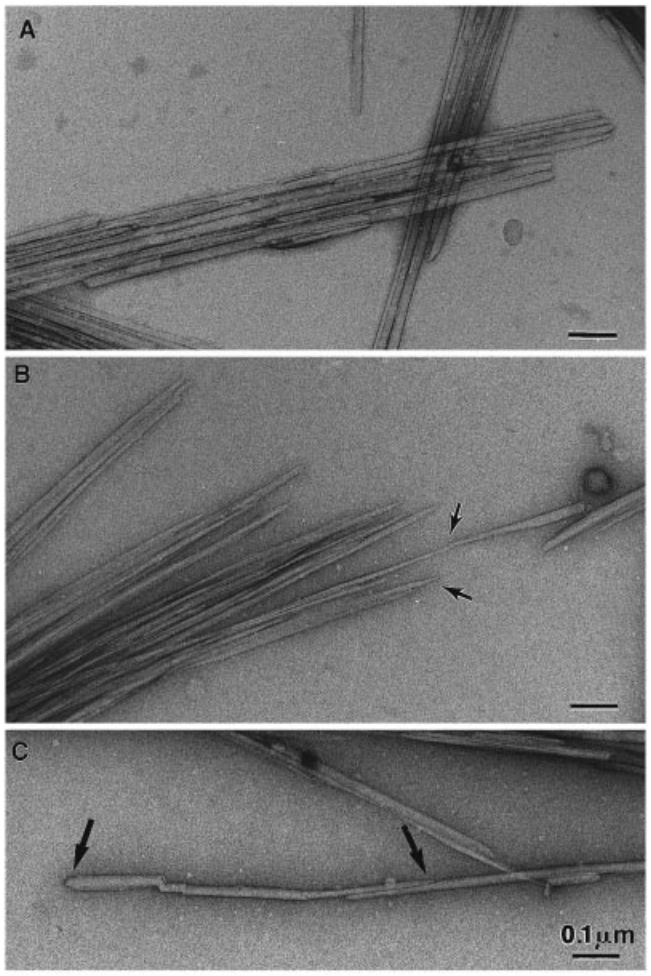

Figure 4 TEM of cellulosome-induced degradation of *V. ventricosa* cellulose microcrystals

(**A**) Untreated microcrystals. (**B**) and (**C**) Microcrystals after 16 days of digestion with a cellulosome preparation from *C. thermocellum* [8% (w/w) protein/substrate ratio]. The arrows in (**B**) indicate pointed microcrystals, typical of the unidirectional action of cellulase samples. The arrows in (**C**) indicate the two tips of a unidirectionally eroded microcrystal.

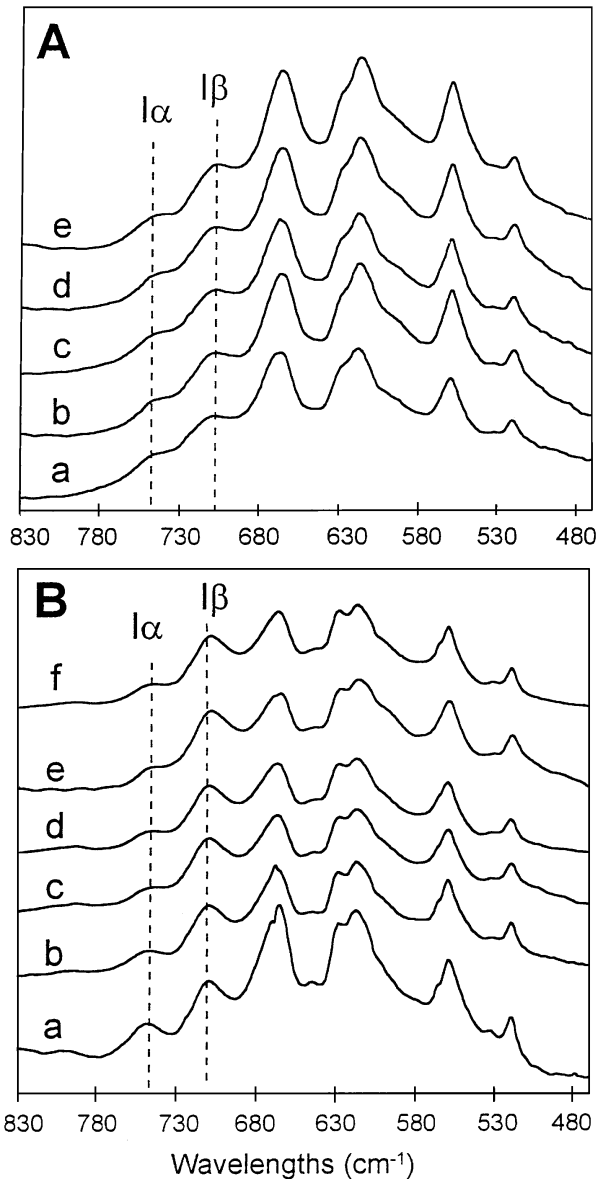

Figure 5 IR spectra of cellulosome-digested cellulose samples

(**A**) Bacterial cellulose ribbons were treated with cellulosome samples from *C. thermocellum* [8% (w/w) protein/substrate ratio]. The IR spectra of samples were recorded after the following reaction times: (a) 0, (b) 1 h (c) 2 h (d) 3 h and (e) 6.5 h. (**B**) A similar experiment performed on *Valonia* cellulose microcrystals. The IR spectra were recorded after the following reaction times: (a) 0, (b) 23 h (c) 72 h (d) 139 h (e) 236 h and (f) 384 h.

Valonia. The results for *A. xylinum* cellulose are presented in Figure 3. Figure 3(A) shows an untreated sample consisting of almost endless, twisted ribbons, measuring 0.1–0.2 μm in width and approx. 10 nm thick. After 3 h of degradation (a time period corresponding to roughly 60% digestion), the morphology was dramatically altered. The ribbons were no longer continuous but became spliced into shorter elements of only 1–2 μm in length (Figures 3B and 3C). As evident from these micrographs, the shorter elements display substantial sub-fibrillation and each appears to comprise a number of smaller needle-like crystals 2–5 nm in width. Thus the digestion of the samples appeared to reflect both sectioning of the ribbons and longitudinal erosion of the sub-elements which constitute bacterial cellulose ribbons. Figure 3(D) shows the result of a sample that was digested for 6.5 h; approx. 85% of the sample was solubilized. At this level of digestion, the residual cellulose consisted of aggregates of very small, short and narrow crystals.

The effect of cellulosome action on *Valonia* microcrystals appeared less striking compared with that on the bacterial cellulose ribbons (Figure 4). Figure 4(A) shows an untreated sample, where the microcrystals exhibit classical characteristics consisting of isolated or clustered monocrystalline elements of around 20 nm in width and variable lengths, ranging from 500 nm to several micrometres. After 16 days of degradation, which resulted in solubilization of 95% of the samples (Figures 4B and 4C), the hydrolysed *Valonia* sample appeared very heterogeneous and intact microcrystals could still be found together with damaged crystals. Some of the crystals appeared to have a tapered tip, a feature indicative of a co-ordinated enzymic mode of action. In some cases, the erosion of the crystals was clearly unidirectional. This phenomenon is illustrated in Figure

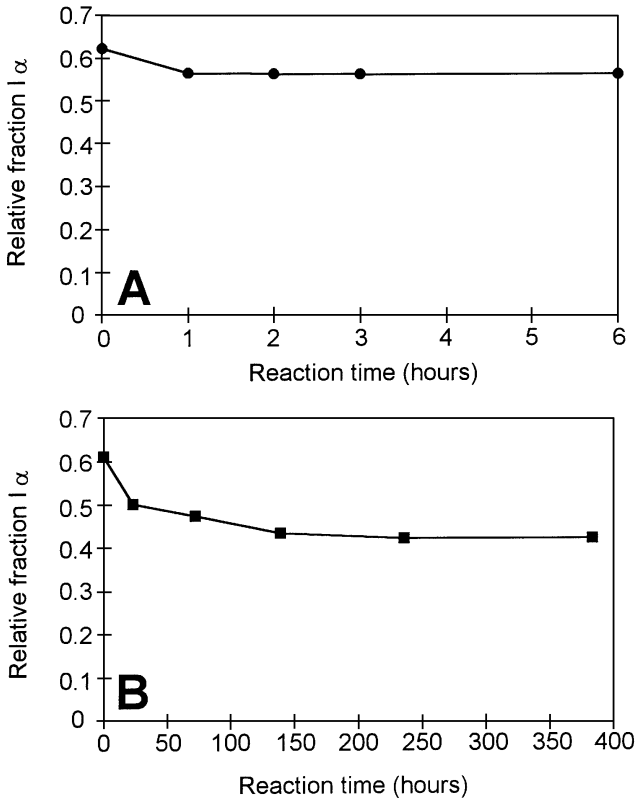

Figure 6 Relative fraction of I_α phase following cellulosome-induced hydrolysis of crystalline cellulose samples

The ratio of the I_α/I_β phases in the desired samples was calculated from the IR spectra in Figure 5 and plotted against time. (**A**) Bacterial cellulose ribbons. (**B**) *Valonia* cellulose microcrystals.

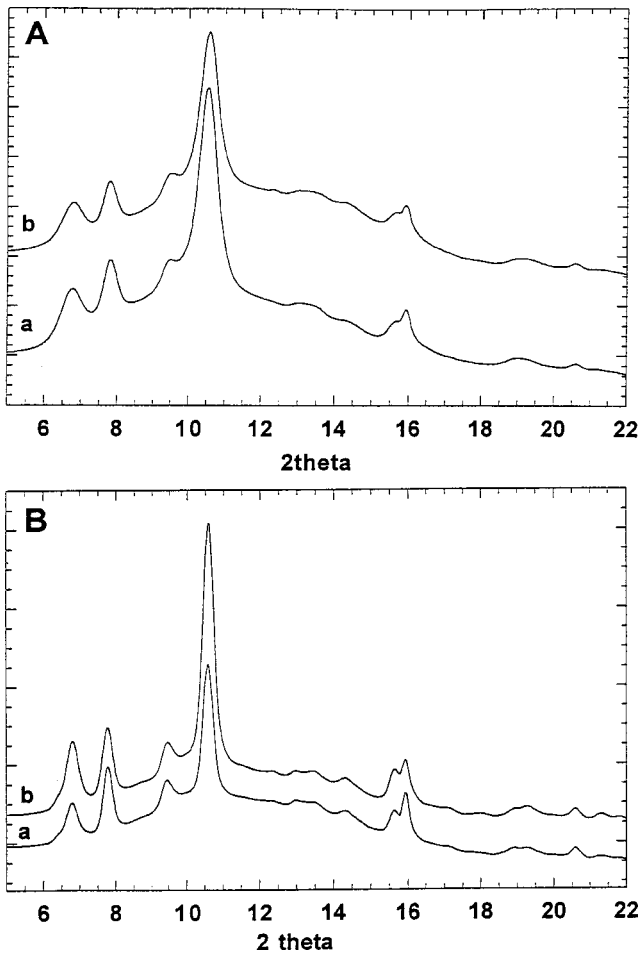

Figure 7 X-ray diffraction of cellulosome-degraded crystalline cellulose samples

(**A**) Bacterial cellulose ribbons; (a) untreated sample and (b) after 6.5 h of digestion with cellulosome preparation from *C. thermocellum* [8% (w/w) protein/substrate ratio]. (**B**) *Valonia* cellulose microcrystals; (a) untreated sample and (b) after 16 days of digestion.

4C, where the left-most end of the indicated microcrystal appeared essentially intact compared with the other end, which was much thinner and formed a pointed tip.

FTIR analysis of the I_α/I_β composition of the cellulose samples during degradation by cellulosomes

Following the work of Sugiyama et al. [33], the IR absorption bands near 3240 cm^{-1} and 750 cm^{-1} in the FTIR spectra of native cellulose can be assigned to the I_α phase of cellulose, whereas the bands near 3270 cm^{-1} and 710 cm^{-1} correspond to the contributions from cellulose I_β. The identification of these bands enabled the recent development of an IR method, devised by Yamamoto et al. [32], which allowed quantification of the I_α/I_β ratio within a given sample. The analysis was based on the deconvolution of the 800–500 cm^{-1} region of the spectra. A sample of this region of the FTIR spectra, during the course of cellulosome treatment, is shown in Figure 5(A) for bacterial cellulose and Figure 5(B) for *Valonia* specimens. For both samples, very little difference was observed between the untreated and degraded material, even when extensive digestion had occurred. Upon deconvoluting the spectra and determining the I_α/I_β ratio for each sample, it was found that the I_α content of bacterial cellulose decreased only slightly from 62% in the untreated sample to 56% after 6.5 h of degradation (Figure 6A). Similarly, the I_α content of *Valonia* cellulose decreased from 65% in untreated samples to 45% after 16 days of degradation, corresponding to 95% solubilization (Figure 6B).

Effect of cellulosomes on the apparent crystal size of cellulose samples

The results of X-ray analysis of the cellulose samples were similar to those of the FTIR studies. The X-ray spectra of the degraded samples differed only slightly from those of the untreated material. This phenomenon is illustrated in the powder diffraction traces presented in Figure 7. Figure 7(A) shows the traces of bacterial cellulose before (a) and after (b) biodegradation. Both traces indicate about the same amount of crystallinity. Quantitative analysis, using the Scherrer equation applied to the three strong equatorial diffraction peaks located at $2\theta = 10.2°$, 7.4° and 6.7°, revealed that the lateral width of the bacterial cellulose crystals increased slightly during the degradation process. An average width of 6 nm was found for the untreated sample, compared with 7 nm after a 6.5-h digestion period. In the case of *Valonia*, a 16-day treatment of the microcrystals had little effect on the crystallinity of the remaining fraction of the sample. Nevertheless, measurement of the width of the initial and

degraded crystals indicated a slight decrease of 11 nm for the initial sample and 10 nm for the sample treated for 16 days.

DISCUSSION

The results of the present study confirm the earlier observations of Johnson et al. [34], who were among the first to report the efficiency of a crude cell-free cellulase system from *C. thermocellum* for the biodegradation of crystalline cellulose. These authors showed that the activity of this cellulase system toward cotton was at least 50 times higher than that of the extracellular cellulase system from *T. reesei* [8,35]. In the present work, the degradation of bacterial and *Valonia* cellulose was investigated, both substrates being of much higher crystallinity than cotton. Cotton has a crystallinity of the order 45%, that of bacterial cellulose is given as 75% and that of hydrolysed *Valonia* is close to 100% [36]. Nevertheless, despite its high crystallinity, bacterial cellulose can be totally solubilized by a concentration of 8% cellulosomes within a period as short as 24 h. Using the same cellulosome concentration, the solubilization of the acid-etched *Valonia* microcrystals took more time, but a value of 95% solubilization was reached within two weeks.

The modifications imparted to the morphology of the substrates during hydrolysis by cellulosomes were informative. The bacterial cellulose images (Figure 3) were indicative of a digestion mode, where all the various cellulase factors acted more or less in unison. Indeed the splicing of the cellulose ribbons into shorter elements is the signature of an 'endo' mode of action, whereas that of sub-fibrillation corresponds to a processive action, often associated with an 'exo' mechanism [16,18]. In relation to these ultrastructural observations, the high rate of degradation of the bacterial cellulose ribbons indicates that a very efficient synergism must take place among the enzymic components that are physically associated within the cellulosomes.

The images of the partially degraded *Valonia* microcrystals (Figure 4) also provide additional details concerning the action of cellulosomes. As opposed to the digestion of bacterial cellulose, the hydrolysis of *Valonia* microcrystals is characterized by specific digestion features, such as crystal thinning, pointed tips, etc. These features are comparable with those observed previously during the digestion of *Valonia* microcrystals by fungal cellulases [16–19]. With the fungal enzymes, thinning was attributed to the high processivity of cellobiohydrolase I (Cel7A) from either *T. reesei* or *H. insolens* [16,18,19]. The pointed-tip feature was attributed to the somewhat less processive unidirectional degradation by cellobiohydrolase II (Cel6A) from the same fungal sources [17,18,20]. In this context, directionality refers to the direction in which the chains are degraded (e.g. non-reducing versus reducing, or vice-versa) while processivity refers to the iterative degradation of the chains, irrespective of the directionality of the attack.

The micrographs in Figures 4(B) and 4(C) show crystals with either or both features, and some appear totally unchanged thus suggesting that individual cellulosomes from the same batch appear to possess a substantial diversity of action. This diversity may be related to heterogeneity in the composition of the individual cellulosomes, even within a given batch. The pointed-tip feature suggests that some or many of the cellulosomes contain enzyme activities mainly capable of degrading cellulose processively in one direction. In this context, the major cellulosomal enzyme is the family-48 cellulase, CelS (Cel48), which represents about 40% of the total enzymic component of the cellulosome. It was shown previously that the properties and overall cellulolytic activity of the cellulosome appear to be directly dependent on this processive enzyme [37]. The direction of processive cleavage by a related family-48 enzyme has been suggested recently to occur from the reducing to the non-reducing end [38]. At least two of the other major enzymic components of the cellulosome also have been classified as cellobiohydrolases, namely CbhA and CelK, each of which comprises about 10% of the total enzyme content. The directionality of chain degradation by these enzymes, however, is not known.

The digestion of the cellulose substrates described above by cellulosomes from *C. thermocellum* appears to have almost no effect on the I_α/I_β composition of the substrates. This is particularly true for bacterial cellulose, where this ratio appears to remain constant throughout the entire course of degradation. This is less true for *Valonia* cellulose, for which a slow decrease in I_α content was observed. Thus unlike fungal systems which seem to attack the I_α phase of cellulose preferentially [14,15], the cellulosomes appear to hydrolyse both the crystalline I_α and I_β phases of cellulose at an almost equivalent rate.

The limited influence of digestion time on the crystallinity of the degraded samples is another feature of cellulosome action. Indeed, throughout the progress of digestion, the crystallinity as well as the apparent crystal size of the sample appears to remain constant. For bacterial cellulose, there was even a slight increase in the apparent crystalline width, which might be due to selected erosion of either crystal defects or of the narrowest crystals. For *Valonia* cellulose, there is almost no change in the crystallinity of the sample, even when 95% of the sample had been digested.

From the results of the present study, a general pattern seems to emerge for the biodegradation of crystalline cellulose substrates by cellulosomes from *C. thermocellum*. One of the major organizational roles of the scaffolding protein of the cellulosome is to bring several catalytic subunits into close proximity [9,35]. In the case of bacterial cellulose degradation, the resulting co-ordinated multi-enzymic attack is so efficient and rapid that the basic features of cellulose crystal degradation (splicing, thinning and crystal-tip sharpening) all appear to occur at the same time. As a result, the substrate is degraded in a near-uniform manner, along both its length and width.

Valonia cellulose microcrystals appear significantly less reactive to *C. thermocellum* cellulosomes than bacterial cellulose. This could be due to the larger lateral dimension of the microcrystals and/or to a paucity in amorphous regions or defects. Only moderate crystal thinning and crystal-tip sharpening were observed with this substrate. The thinning of *Valonia* cellulose microcrystals was the dominant factor of their biodegradation with crude fungal cellulase complexes from *T. reesei*, *H. insolens* or *Schizophyllum commune* [16,39]. This feature, which was associated with a high processivity of the enzymes, was not only observed in the TEM images but was also reflected in X-ray and spectroscopic observations. Indeed, the thinning of the crystals gave X-ray diffractograms where all the equatorial diffraction intensities had substantially decreased, whereas the meridional ones were left unchanged [16,39]. In correlation with the X-ray observations, the $I\alpha$ component of *Valonia* cellulose was attacked preferentially [14] in processive fungal degradation. As these features are only moderately observed in the action of cellulosomes on *Valonia* microcrystals, we conclude that cellulosome action exhibits a relatively limited processive character.

A final aspect of the degradation of *Valonia* microcrystals by cellulosomes from *C. thermocellum* is the heterogeneity of the degradation, a phenomenon which had already been observed to some extent in the digestion of the same substrate by fungal cellulases [16,39]. It is indeed quite striking to observe that intact crystals are still found, even after 15 days of degradation, when 95% of the sample has been digested. Thus it seems that the rate-

determining step for degradation of an individual *Valonia* cellulose crystal is the initial attack; once the attack has been successful, the crystal undergoes rapid and total digestion. Such a mechanism explains why intact crystals are still observed even when near-complete solubilization is achieved, and no significant overall loss of crystallinity can be detected in the remaining crystals. An alternative explanation may be that the residual intact *Valonia* microfibrils simply represent a defect-free subpopulation of crystals that are particularly recalcitrant to attack, even by cellulosomes.

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