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Changes in the molecular-size distribution of insoluble cellulose by the action of recombinant Cellulomonas fimi cellulases

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Specific patterns of attacks of cotton, bacterial cellulose and bacterial microcrystalline cellulose (BMCC) by recombinant cellulases of *Cellulomonas fimi* were investigated. Molecular-size distributions of the cellulose were determined by high-performance size-exclusion chromatography. Chromatography of cotton and bacterial cellulose revealed single major peaks centred over progressively lower molecular-mass positions during attack by endoglucanase CenA. In advanced stages, a second peak appeared at very low average size (approx. 11 glucosyl units); ultimate weight losses were approximately 30 %):. The isolated catalytic domain of CenA, p30, gave results very similar to those with complete CenA. CenA did not effectively depolymerize or solubilize BMCC significantly. Molecular-size distributions of cotton and bacterial cellulose incubated with endoglucanases CenB or CenD exhibited one major peak regardless of incubation time; low-molecular-mass fragments did not accumulate. Weight losses were 40 and 35 % respectively. The single peak shifted to lower-molecular-mass positions as incubation continued, but high-molecular-mass material persisted. CenB and CenD readily attacked and solubilized BMCC (approx. 70 %). We conclude that CenA attacks cellulose by preferentially cleaving completely through the cellulose microfibrils at the amorphous sites, and much more slowly by degrading the crystalline surfaces. Conversely, CenB and CenD cleave the amorphous regions much less efficiently while vigorously degrading the surfaces of the crystalline regions of the microfibrils.

INTRODUCTION

Cellulose, the major component of plant biomass, is a linear polymer of anhydroglucose units linked by β- 1,4-glycosidic bonds. Hydrogen-bonding within and between multiple layers of closely packed cellulose molecules results in the formation of cellulose microfibrils [1]. In natural tissues, these fibrils exist in a highly ordered state consisting of crystalline regions that are connected by less-ordered amorphous regions. A variety of fungi and bacteria produce an assortment of extracellular hydrolytic enzymes, including endo- and exo-β-glucanases, which convert this insoluble substrate into soluble cello-oligomers, and ultimately to cellobiose and glucose.

Many studies of enzymic attack of cellulose have focused primarily on the release of reducing sugars from insoluble modified cellulose or water-soluble cellulose derivatives. Very few investigations have examined the effect of enzymes on the macromolecular structure of insoluble cellulose substrates. One approach to such research is to analyse the molecular-size distribution, and in effect the average degree of polymerization (DP, number of glucosyl residues per cellulose chain) of the residual cellulose after different microbial or enzymic treatments. We used this method to demonstrate that the mechanism of *in vivo* cotton cellulose degradation differs fundamentally between wood-degrading fungi causing different types of decay [2].

Molecular-size distributions of cellulose incubated with culture filtrates from cellulolytic micro-organisms have been examined by others. Puls and Wood [3] reported that culture filtrates of *Neocallimastix frontalis, Trichoderma koningii* and *Penicillium pinophilum,* but not *Clostridium thermocellum,* reduced the chain length of birchwood cellulose to various extents. Puls [4] had earlier demonstrated that the average chain length of carbohydrates in steam-pretreated birchwood was somewhat reduced by culture filtrates of *Trichoderma reesei.* Additionally, dramatic shifts in the molecular-size-distribution peaks of partially mercerized cotton by culture filtrates of *Sporotrichum dimorphosporum* and of mechanically modified cotton fibres by cultures of *Trichoderma reesie* were reported by Lauriol et al. [5] and Chang et al. [6] respectively.

Specific modes of attack of insoluble cellulose substrates by individual cellulases, however, have received little attention and are not understood. Part of the reason for this is the difficulty encountered in preparing purified cellulases from the usually complex mixture of enzymes present in cellulolytic systems. This obstacle, however, has been circumvented by the introduction of recombinant gene technology. Genes encoding different cellulases have been individually cloned and expressed in non-cellulolytic hosts, thereby ensuring that the cellulase preparations are devoid of other contaminating glycanases. This approach has been used to isolate and characterize five cellulases from the Gram-positive bacterium *Cellulomonas fimi:* endoglucanases CenA, CenB, CenC and CenD and the exoglycanase Cex [7–13]. All these enzymes are bifunctional proteins consisting of an active-site domain and a cellulose-binding domain (CBD) [13–19]. Unique domains, which are related to the repeats of fibronectin type III, also exist in CenB [18,19] and CenD [13].

The purpose of the present study was to determine the effects of isolated CenA, CenB, CenD and Cex on the molecular-size distribution of cotton cellulose, bacterial cellulose and bacterial microcrystalline cellulose (BMCC). We report the first description of the changes in the molecular-size distribution of insoluble cellulosic substrates by purified cellulases.

Abbreviations used: DP, degree of polymerization; DP_w, weight-averaged degree of polymerization; DP_n, number-averaged degree of polymerization; BMCC, bacterial microcrystalline cellulose; s.e.c., size-exclusion chromatography; CBD, cellulose-binding domain; p30, isolated catalytic domain of CenA.

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EXPERIMENTAL

Celluloses and chemicals

Purified dewaxed cotton cellulose (1AY) was from Procter and Gamble Cellulose. Memphis, TN, U.S.A. Bacterial cellulose [20] and BMCC[21] were prepared with *Acetobacter xylinum* (ATCC 53524 and ATCC 23769) and purified as described previously. Anhydrous pyridine, phenyl isocyanate and h.p.l.c.-grade tetrahydrofuran were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A.

Bacterial cellulases

C. fimi (ATCC 484) CenA [16], CenB [22], CenD [13,22] and Cex [16] were purified from *Escherichia coli* transformed with recombinant plasmids containing the designated gene, as described in the cited papers. As a result. the recombinant enzymes were not glycosylated, whereas CenA and Cex are normally glycosylated in *C., fimi.* Isolated CenA catalytic domain, p.30, was prepared by incubating intact CenA with crude C. *fimi* proteases and subsequently purified as described previously [23]. The enzymes and p30 were maintained at 4°C in 50 mM potassium phosphate bufler (pH 7.0) containing 0.02 % NaN_3 ($P0_4$/azide buffer).

Reactions

Time-course studies of cellulose depolymerization by isolated enzymes were performed at 39°C with continuous magnetic stirring. We and others [24] have found that the cellulases attack cellulose much more efficiently when the reaction mixtures are stirred. In studies of cotton cellulose depolymerization, samples were removed periodically over a 48- or 192-h period. Bacterial cellulose was incubated for 192 h and BMCC for 24 h, At the designated time points, samples were removed and spun for 6–8 min in a microcentrifuge (16000 g). The supernatants were analysed for total sugars. The residual cellulose pellet was washed three times with 1.5 ml of water and oven-dried at 60°C before determination of molecular-size distribution.

Preparation of 'small particles' from cellulose

In previous work [25], it was observed that fine particulate material ('small particles') is released from dewaxed cotton fibres on treatment with CenA; this is visible to the naked eye as a haziness in the supernatant after most of the cellulose mass has settled. Eight samples, each containing 12.5 mg of dewaxed cotton fibre and 10 μM CenA in 2.5 ml of the PO_4/azide buffer, were incubated at 37°C (300 rev./min) for 26 h. The identity of the 'small particles', which were recovered as suspensions in the combined supernatants, removed by aspiration and separated from ' short fibres' by filtration through a fritted plastic disc, was confirmed by phase-contrast microscopy. Approx. 1.2 mg of cellulose 'small particles' was recovered. A haziness in the supernatant also resulted when cotton linters were incubated with CenA, but the suspended particles were not characterized.

Analysis of cellulose and sugar

The molecular-size distributions of the residual cellulose samples (pellets from centrifugation) were determined by s.e.c. of the tricarbanilate derivatives in tetrahydrofuran after evaporation of the reaction mixture [26]. We found this method to give reproducible results (not shown). Four h.p.l.c. s.e.c. columns, Shodex KF807, KF805 and KF803 and 10 nm μ Styragel (Waters and Millipore Co.), were connected in series and used for the analyses. DP_w and DP_n were calculated as previously described [27]. Total sugars in the reaction supernatants were determined by the phenol/H_2SO_4 assay [28]. These values, corrected for water hydrolysis, were used to calculate the percentage weight losses of the cellulose.

RESULTS

Recombinant cellulases from *C.,fimi* were individually incubated with cotton, bacterial cellulose or BMCC for various times to determine their effects on the molecular-size distribution of the cellulose. Weight losses of the cellulose (percentage solubilization) were calculated by determining the weight of soluble sugars and oligosaccharides released from the cellulose. In a subsequent experiment, the molecular-size distribution of 'small particles' generated from dewaxed cotton by CenA was determined.

Changes in the molecular-size distribution of cotton cellulose by *C. fimi* cellulases

Determination of the molecular-size distribution of cotton cellulose attacked by CenA over time revealed single major peaks that progressively shifted to lower DP positions. indicating that extensive cellulose depolymerization occurred (Figure 1a). After

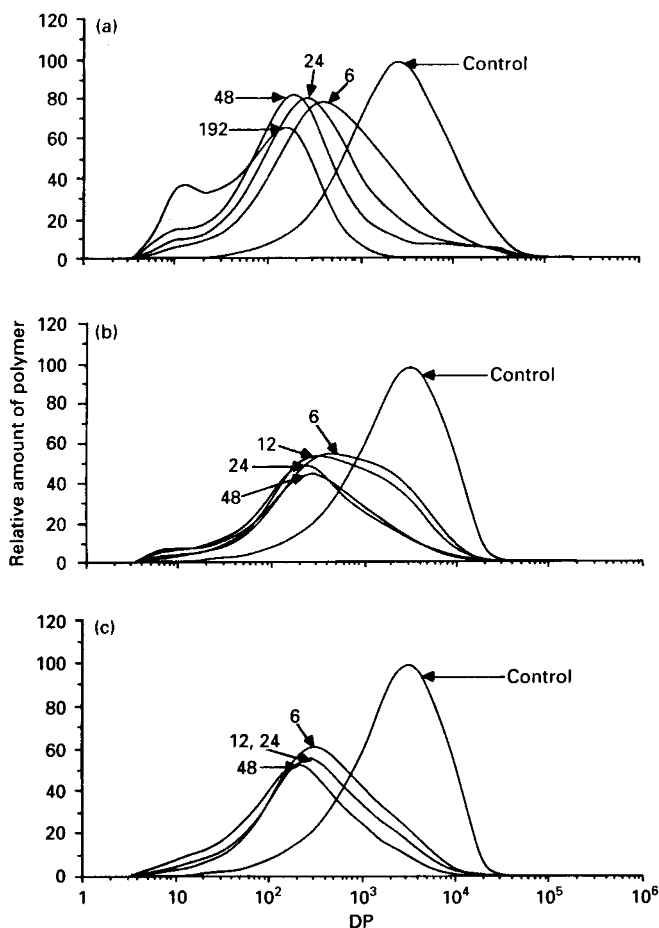

Figure 1 Changes hr the molecular-size distributions of cotton cellulose during attack by the *C. fimi* endoglucanases (a) CenA, (b) CenB and (c) CenD

The assay mixtures contained either 10 mg of cotton cellulose and 2 nmol of CenA or 1 mg of cotton cellulose and 4 nmol of CenB or CenD in 1 ml of P_i/azide buffer Numbers next to the arrows represent length of incubation (h). Areas under the curves have been adjusted to reflect weight loss.

Table 1 Depolymerization of cotton cellulose by CenA, CenB and CenD

For CenA, the reaction mixture contained 2 nmol of enzyme/10 mg of cotton cellulose and for CenB and CenD the mixture was 4 nmol of enzyme/mg of cotton cellulose.

Incubation time (h)	Weight loss (%)	DP_w	DP_n	Polydispersity (DP_w/DP_n)
CenA				
0	0	3499	861	4.1
6	4	1224	157	7.8
24	12	949	101	9.4
48	16	863	68	12.7
192	29	154	34	4.5
CenB				
0	0	3717	800	4.7
6	18	1423	163	8.7
12	22	1277	156	8.2
24	37	750	80	9.4
48	40	762	78	9.8
CenD				
0	0	3717	800	4.7
6	24	952	149	6.4
12	29	759	131	5.8
24	31	854	110	7.8
48	35	519	75	6.9

48 h, a second peak with a maximum at DP ≅ 11 began to appear and became a major component of the sample after 192 h of incubation. The average DP_w values of the cotton cellulose decreased from 3499 to 154 in 192 h, and the average DP_n values decreased from 861 to 34 (Table 1). The initial broadening of the major peak in the molecular-size distribution profiles was reflected in a gradual increase in the polydispersity of the samples (DP_w/DP_n; an indication of the range of molecular sizes). A small amount of high-molecular-mass cellulose remained after 48 h, but had disappeared by 192 h. At 192 h, the major peak narrowed and the low-molecular-mass secondary peak appeared, giving, overall, a reduction of the corresponding polydispersity value. Soluble sugars and oligodextrans were continuously released from the cotton by CenA. Solubilization after 192 h was 29 %

The molecular-size distribution of cotton cellulose incubated with the isolated CenA catalytic domain, p30, for 192 h changed in a pattern very similar to that of cotton incubated with complete CenA. The same bimodal distribution curve was resolved. The DP_w, DP_n and polydispersity values were 242, 45 and 4.8. The final weight loss, however, was less than half that seen with the complete enzyme (results not shown).

Changes in the molecular-size distribution of cotton cellulose on incubation with CenB or CenD were different from those observed with CenA (Figures 1b and lc). All cotton samples, incubated with either CenB or CenD, were resolved as single major peaks in the s.e.c. profile regardless of incubation time; no low-molecular-mass peak appeared. With time, the major peaks broadened and shifted to lower DP positions. The minimum DP_w values of cellulose attacked by CenB (DP, = 762) and CenD (DP_w = 519) remained relatively high compared with those observed with CenA (DP_w = 154), even though CenB and CenD solubilized more cellulose than did CenA. A significant proportion of the enzymically treated cellulose still consisted of high-molecular-mass material, which skewed the average DP_w values towards higher values. This was especially evident for cellulose degraded by CenB for 6 (DP_w = 1423) and 12 h (DP_w = 1277). The average DP_n values for cellulose attacked by both CenB and CenD ultimately decreased from 800 to approximately 80 (Table 1).

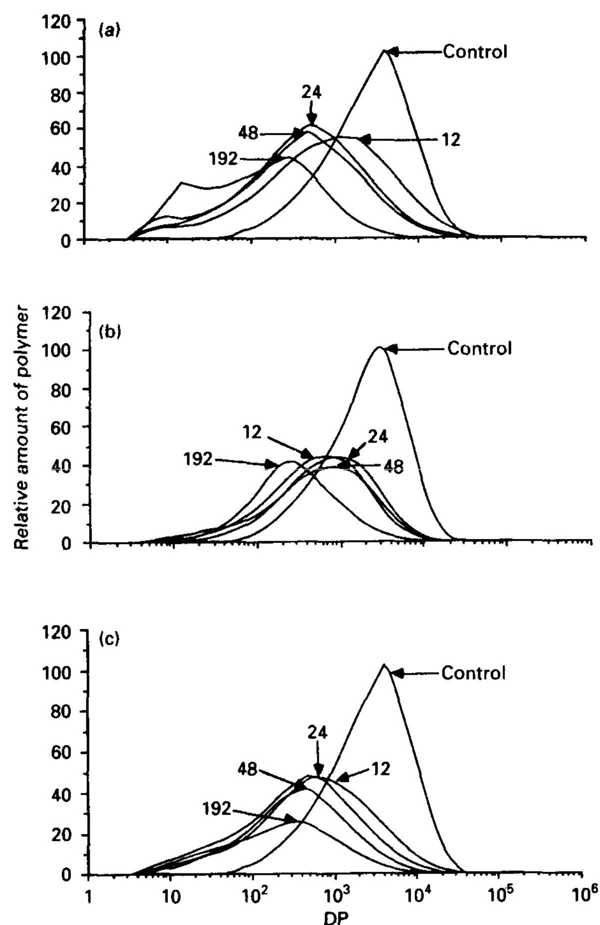

Figure 2 Changes in the molecular-size distributions of bacterial cellulose during attack by the *C. fimi* endoglucanases (a) CenA (b) CenB or (c) CenD

All assay mixtures contained 1 mg of bacterial cellulose and 1 nmol of enzyme in 1 ml of PO_4/azide buffer. Numbers next to the arrows represent length of incubation (h). Areas under the curves have been adjusted to reflect weight loss.

The molecular-size distribution of cotton cellulose incubated with the exoglucanase Cex for 192 h was similar to that of the unattached control, and only insignificant amounts of soluble sugars and oligosaccharides were released form the cotton (5 nmol of Cex was incubated with 0.5 mg of cellulose in 1.0 ml of PO_4/azide buffer at 39°C with stirring). Changes in the molecular-size distribution of cotton cellulose incubated with Cex and CenA (1: 1) over time were similar to those of cotton cellulose incubated with CenA alone (results not shown).

Changes in the molecular-size distribution of bacterial cellulose by *C. fimi* endoglucanases

The general pattern of bacterial cellulose depolymerization by CenA mimicked that of cotton cellulose depolymerization in that the major peak in the s.e.c. profile shifted to progressively lower DPs, and a second low-DP peak (DP ≅ 11) appeared in the most depolymerized sample (Figure 2a). With bacterial cellulose, the average DP_w decreased from 4244 to 332, and the average DP_n decreased from 1229 to 42 in 192 h (Table 2). The major peaks in the s.e.c. profiles representing bacterial cellulose attacked by

Table 2 Depolymerization of bacterial cellulose by CenA, CenB and CenD

The reaction mixtures contained 1 nmol of enzyme/mg of bacterial cellulose,

Incubation time (h)	Weight loss (%)	DP_w	DP_n	Polydispersity (DP_w/DP_n)
CenA				
0	0	4244	1229	3.5
12	9	2362	144	16.4
24	11	1269	107	11.9
48	14	1128	77	14.7
192	30	332	42	7.9
CenB				
0	0	4244	1229	3.5
12	41	1013	208	4.9
24	43	1313	322	4.1
48	45	1147	157	7.3
192	48	514	154	3.3
CenD				
0	0	4244	1229	3.5
12	28	1479	149	9.9
24	28	971	92	10.6
48	41	750	101	7.5
192	57	559	68	8.2

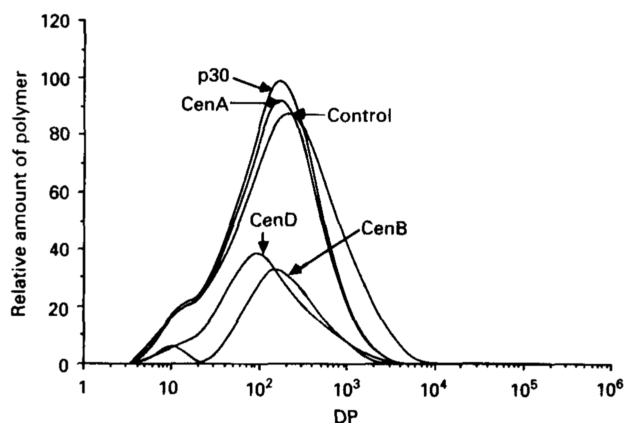

Figure 3 Molecular-size distributions of BMCC on incubation with *C. fimi* CenA, p30, CenB and CenD

BMCC (1 mg) was incubated with 1 nmol of enzyme in 1.0 ml of P/azide buffer for 24 h, Areas under the curves have been adjusted to reflect weight loss.

Table 3 Degradation of BMCC by CenA, p30, CenB and CenD after 24 h

The reaction mixtures contained 1 nmol of enzyme/mg of BMCC.

Incubation time (h)	Weight loss (%)	DP_w	DP_n	Polydispersity (DP_w/DP_n)
None	0	425	68	6.3
CenA	12	250	63	4.0
p30	6	268	63	4.3
CenB	73	256	68	3.8
CenD	65	216	59	3.7

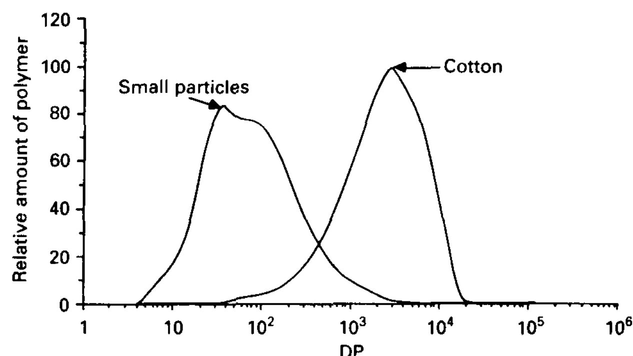

Figure 4 Molecular-size distribution of ' small particles' generated from dewaxed cotton cellulose by CenA in 26 h

Areas under the curves have been normalized, although the 'small particles' represented only approx. 1 % of the total cellulose.

CenA were somewhat broader than those of cotton attacked by CenA. However, the polydispersities of the two types of cellulose followed a similar trend: increasing rapidiy and then decreasing, Total weight loss from bacterial cellulose, as from cotton, increased gradually, resulting in a maximum weight loss of approx. 30 %.

When bacterial cellulose was incubated with p30 for 192 h, a bimodal curve, simllar to that observed for CenA, was again resolved (results not shown). The corresponding weight loss, DP_w, DP_n and polydispersity values were 12 %, 509, 51 and 10.0 respectively.

Changes in the molecular-size distribution of bacterial cellulose on incubation with CenB or CenD again differed from those observed with CenA. The molecular-size distributions of bacterial cellulose attached by CenB or CenD were generally similar to those observed with cotton. Only one peak was resolved for all samples, and the'DP ≅ 11' peak did not appear (Figures 2b and 2c). Bacterial cellulose attacked by CenB for 12, 24 or 48 h had similar molecular-size distributions: single peaks centred over a DP of approx. 1000. After 192 h, however, the major peak had shifted to a lower DP centred over approx. 300. With CenD, the major peak progressively shifted to lower DP positions, eventually also centring over a DP of about 300. As observed with cotton, the single major peak representing bacterial cellulose attacked by CenB and CenD was broadened with time. However, the peak representing cellulose attacked by CenB for 192 h had narrowed, resulting in a subsequent decrease in polydispersity. The DP_w of bacterial cellulose attacked by CenB or CenD was approximately three to four times greater than that attacked by CenA at comparable weight losses of approx. 30-40 % (Table 2). Minimum DP_w and DP_n values of bacterial cellulose incubated with either CenB or CenD were greater than those observed with CenA-degraded bacterial cellulose (Table 2). The polydispersity of cellulose incubated with CenD increased dramatically in the first 24 h and thereafter decreased slightly. CenB and CenD caused substantially greater weight loss (48 % and 57 %) than did CenA.

Degradation of BMCC by *C. fimi* endoglucanases

Changes in the molecular-size distribution of BMCC attacked by CenA or p30 for 24 h were similar, but CenA solubilized twice as much of the cellulose (12 compared with 6 %) (Figure 3; Table 3). In both enzymic treatments, the highest-molecular-mass material

was attacked, resulting in the formation of a single peak with a slight shoulder at DP < 20. The DP_w of the BMCC incubated with either CenA or p30 decreased from 425 to approximately 260, whereas the DP_n remained relatively unchanged. As a result, the polydispersity decreased from 6.3 to 4.0. Degradation of BMCC in 24 h by CenB and CenD was much more extensive than by CenA (Table 3); 73 % and 65 % of the cellulose were solubilized. The molecular-size distribution of BMCC attacked by CenB revealed a major peak centred over a DP of approx. 200 and a much smaller peak centred over a DP of approx. 10. The DP_w and DP_n values ($DP_w = 256$ and $DP_n = 68$) did not differ much from those observed with CenA. The molecular-size distribution of BMCC attacked by CenD was somewhat different from those observed with the other cellulase treatments. Thus the major single peak shifted to a lower DP position centred over a DP of approx. 90. The DP_w, DP_n and the polydispersity values of the BMCC were 216, 59 and 3.7 respectively.

Molecular-size distribution of ‘small particles’ generated from dewaxed cotton fibres by CenA

The s.e.c. profile of ‘small particles’ produced from dewaxed cotton by CenA after 26 h revealed an irregularity shaped peak that was centred over a DP of approx. 60 (Figure 4). A protrusion from the major peak at a DP position of approx. 30 suggested a bimodal distribution of products. The DP_w and DP_n of the cotton ‘small particles’, which made up only approx. 1 % of the total cellulose, were significantly less than for the unattached control: $DP_w = 160$ and $DP_n = 44$ compared with $DP_w = 3391$ and $DP_n = 1171$. The polydispersities were 3.6 and 2.9 respectively.

Discussion

Cellulose in its native form is an unusual substrate for enzymes. Unlike most enzymes that convert soluble substrates into products, cellulases must diffuse to the surfaces of the cellulose microfibrils before contact and catalysis can occur. We chose cotton and bacterial cellulose as substrates for our enzymic depolymerization studies because they are easily purified and chemically identical with the cellulose found in lignocellulosic materials. Cotton, found in the seed hairs of the cotton plant, is the purest form of natural cellulose and consists of elementary microfibrils, which in turn combine to form microfibrils [1]. Bacterial cellulose is synthesized by *Acetobacter xylinum* as long ribbons composed of hydrogen-bonded microfibrils that are smaller in diameter and more crystalline than cotton ceilulose [29]. Relative to the highly crystalline acid-treated *Valonia* cellulose, the degree of crystallinity of cotton cellulose lies in the range 35-45 %, whereas the degree of crystallinity of BMCC is approx. 76 % [30]. Our studies revealed differences in the specific modes of attack of individual *C. fimi* cellulases on these naturally occurring cellulose and differences in the two celluloses with respect to enzymic hydrolysis. The carbanilate derivative of cellulose was employed for these molecular-size distribution studies, because this derivatization procedure has been shown to be non-degradative [31].

Significant peak shifts to lower DP positions in the molecular-size distributions of cotton and bacterial cellulose during attack by the endoglucanase CenA indicates that both types of celluloses were extensively depolymerized. The DP_w of cotton was reduced from 3499 to 154. This is near the ‘levelling off’ DP of cotton cellulose that is also observed on acid hydrolysis; the residual cellulose has been referred to as’ crystallite’ as first observed by Battista [32]. An analogous pattern of attack of bacterial cellulose by CenA was observed, but the apparent limit DP appeared to be higher (300-350). Indeed, the DP of acid-hydrolyses bacterial cellulose, BMCC, was determined to be approx. 400. These results suggest that CenA preferentially cleaved completely through the cellulose microfibrils at the amorphous sites, leaving behind predominantly crystalline cellulose. (Although we did not determine crystailinity here, increases in crystallinity of cellulose on enzymic attack have been reported [33–35].)

Initial peak broadening of both cotton and bacterial cellulose during enzymic attack suggests that CenA cleaved the most accessible amorphous regions of the cellulose first, producing a range of low-molecular-mass fragments. Further hydrolysis of these fragments, in addition to the slower but continuous cleavage of the intact microfibril, resulted in the subsequent narrowing of the major peak. Reduction of the major peak, presumably representing crystallite (DP ≅ 200-250), concurrent with the appearance of a second peak, representing low-molecular-mass material (DP ≅ 11), suggests that CenA did not cut through the remaining crystallites, but slowly removed fragments from their surfaces. The near inability of CenA to depolymerize or solubilize BMCC further supports this notion.

The low-molecular-mass peak (DP ≅ 11) that appeared when cotton and bacterial cellulose were extensively depolymerized by CenA also appeared when these celluloses were attacked by p30, the isolated catalytic domain of CenA. This indicates that the active site, and not the linker sequence or the CBD of the protein, was responsible for the generation and accumulation of these fragments. We presume that oligomers of DP approx. 15 and smaller were formed by the action of CenA and p30, and that those soluble in water (DP approx. 7 and smaller) were washed out in our work-up procedures; the remaining insoluble oligomers averaged DP 11. Why oligomers smaller than 15 were resistant to further attack by CenA and p30 is unknown. One possibility is that the enzyme simply requires longer fragments for activity. Another possibility is that the generated fragments crystallized on to the cellulose crystallite, where they escaped further hydrolysis.

Our work also shows that the ‘small particles’ generated from cotton cellulose with CenA (see ref. [25]) are smaller than the majority of the material generated in the same time period (see Figures la and 4). We suspect that these ‘small particles’, which were separated by the total product mixture by filtration and only represented approx. 1 % of the total cellulose, depicted the smaller portion of the product mixture.

Although removal of the CBD from CenA did not affect the ability of the endoglucanase to cleave cellulose at the amorphous sites, it did affect its ability to solubilize crystalline cellulose. Apparently, the CBD of cellulases plays a significant role in the efficient solubilization of crystalline cellulose [16,36].

Changes in the molecular-size distributions of cotton or bacterial cellulose during attack by CenB or CenD differed from those observed with CenA. Single peaks, shifted to progressively lower DPs, were seen with both CenB and CenD; low-DP material, including the ‘DP ≅ 11’ peak, did not accumulate. These observations suggest that attack of cotton or bacterial cellulose microfibrils by CenB and CenD at amorphous sites was less extensive than with CenA. This was particularly evident during the initial attack of cotton by CenB in which a relatively large proportion of material of DP > 1000 persisted after approx. 30 % solubilization. In contrast, attack by both CenB and CenD at crystalline surfaces appeared to be much more extensive than for CenA. This mode of attack led to comparatively high solubilization rates coupled with high residual DP_w values. Unlike CenA, CenB and CenD readily solubilized 60-70 % of the

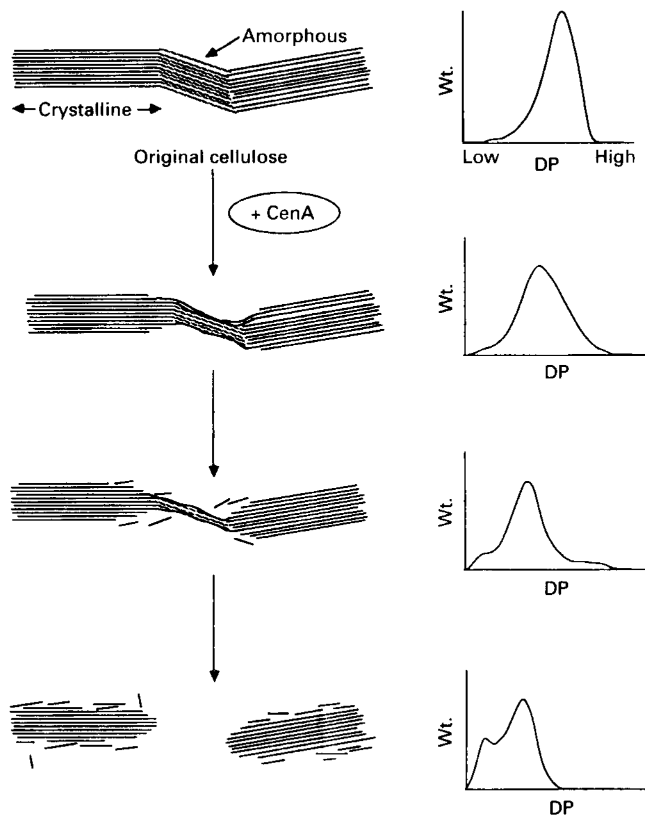

Figure 5 Schematic representation of the proposed mode of cellulose attack by *C. fimi* endoglucanase CenA

It is envisaged that CenA preferentially cleaves cellulose at the less-ordered amorphous sites, slowly degrading the residual highly ordered crystalline regions. The experimental s.e.c. profiles are shown to the right of the corresponding cellulose diagrams. Wt., weight. See the Discussion for details.

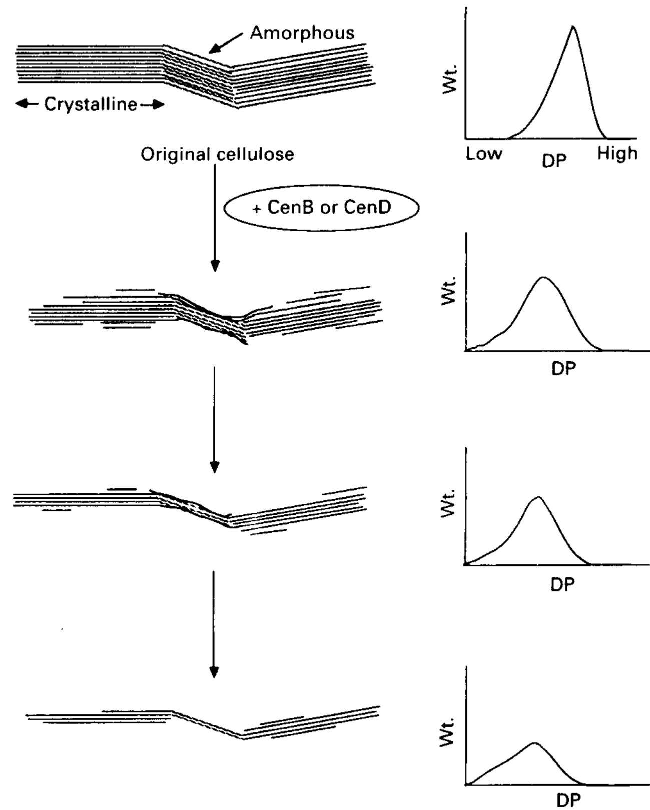

Figure 6 Schematic representation of the proposed mode of cellulose attack by *C. fimi* endoglucanases CenB and CenD

It is suggested that CenB and CenD not only attack cellulose microfibrils at the less-ordered amorphous sites, but also extensively remove cellulose fragments from the surfaces of the highly ordered crystalline regions The experimental s.e.c. profiles are shown to the right of the corresponding cellulose diagrams Wt., weight See the Discussion for details.

BMCC. This is in agreement with a previous report of high weight losses of crystalline cellulose observed on incubation with CenB and CenD, but not with CenA [13].

It is at present unclear why the attack of cellulose microfibrils by CenB and CenD differs from that of CenA. All three enzymes are endoglucanases, but they belong to different families in the classification of β- 1,4-glucanases based on amino acid sequence similarities [37]. In addition, there appears to be no correlation between our results and the stereoselectivities of the endoglucanases; CenA and CenB catalyse hydrolysis with inversion of the anomeric carbon [38], whereas CenD catalyses with retention of the anomeric carbon [13]. Apparent specificities of attack towards the different celluloses by the different *C. fimi* endoglucanases may be attributed to certain domains within the proteins. For instance, sequences related to the repeated domains of fibronectin II exist in CenB (three copies) [19] and CenD (two copies) [13], but not in CenA. Apparently, removal of these sequences in CenB does not affect hydrolysis of CM-cellulose [19]. Similar experiments with crystalline cellulose substrates have not yet been performed. Another domain, already suspected of playing a significant role in the enzymic hydrolysis of crystalline cellulose, is the CBD [16,36]. Indeed, our results demonstrate that removal of the binding domain from CenA affected its ability to solubilize crystalline cellulose, but did not affect its ability to cleave cellulose at the amorphous sites. It is of interest that CenB, which readily solubilizes crystalline cellulose, contains two CBDs [19]. Neither binding domain is necessary for CM-cellulose hydrolysis, but removal of one of them, a *Bacillus*- type CBD at the C-terminus of the catalytic domain, reduces activity. Again, similar structure-function experiments using crystalline cellulose have not yet been carried out. Lastly, the tilted configuration of the catalytic domain of CenA, which is at a constrained angle of 135° to the CBD [23], may function in the efficient cleavage of cellulose microfibrils at the amorphous sites. When this conformation is altered by removing the hinge region that connects the catalytic domain and the CBD, activities on CM-cellulose are reduced [39].

The inability of the *C. fimi* exoglucanase Cex to depolymerize cotton cellulose may be explained by the fact that it is more properly classified as a xylanase than a cellulase [40]. Although it hydrolyses CM-cellulose, it has 1500 times more xylanase activity than any of the other *C. ,fimi* endoglucanases [13,41].

In summary, the recombinant endoglucanase CenA of *C. fimi* attacked cellulose by preferentially cleaving completely through the cellulose microfibrils at the amorphous sites and only slowly degrading the crystalline surfaces (Figure 5). The recombinant endoglucanases CenB and CenD, on the other hand, attacked cellulose not only by slowly cleaving the amorphous regions, but by vigorously attacking the crystalline surfaces of the microfibrils (Figure 6).

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REFERENCES

1 Blackwell, J. (1982) in Cellulose and Other Natural Polymer Systems: Biogenesis, Structure and Degradation (Brown, R. M., Jr., ed.), pp. 403–428, Plenum, New York
2 Kleman-Leyer, K., Agosin, E., Conner, A. H. and Kirk, T. K. (1992) Appl. Environ. Microbiol. **58**, 1266–1270
3 Puls, J. and Wood, T. M. (1991) Bioresource Technol. **36**, 15–19
4 Puls, J. (1987) in Energy from Biomass 1 (Grassi, G. and Zibetta, H., eds.), pp. 224–229, Elsevier Applied Science, London and New York
5 Lauriol, J.-M., Comtat, J., Froment, P., Pla, F. and Robert, A. (1987) Holzforschung **41**, 165–169
6 Chang, M. M., Chang, T. Y. C. and Tsao, G. T. (1981) Adv. Biochem. Eng. **20**, 15–42
7 Gilkes, N. R., Kilburn, D. G., Langsford, M. L., Miller, R. C. Jr., Wakarchuk, W. W., Warren, R. A. J., Whittle, D. J. and Wong, W. K. R. (1984) J. Gen. Microbiol. **130**, 1377–1384
8 O'Neill, G. P., Goh, S. H., Warren, R. A. J., Kilburn, D. G. and Miller, R. C. Jr. (1986) Gene **44**, 325–330
9 Owalabi, J. B., Beguin, P., Kilburn, D. G., Miller, R. C. Jr. and Warren, R. A. J. (1988) Appl. Environ. Microbiol. **54**, 518–523
10 Whittle, D. J., Kilburn, D. G., Warren, R. A. J. and Miller, R. C. Jr. (1982) Gene **17**, 139–145
11 Wong, W. K. R., Gerhard, B., Guo, Z. M., Kilburn, D. G., Warren, R. A. J. and Miller, R. C. Jr. (1986) Gene **44**, 315–324
12 Moser, B., Gilkes, N. R., Kilburn, D. G., Warren, R. A. J. and Miller, R. C. Jr. (1989) Appl. Environ. Microbiol. **55**, 2480–2487
13 Meinke, A., Gilkes, N. R., Kilburn, D. G., Miller, R. C. Jr. and Warren, R. A. J. (1993) J. Bacteriol. **175**, 1910–1918
14 Coutinho, J. B., Gilkes, N. R., Warren, R. A. J., Kilburn, D. G. and Miller, R. C. Jr. (1992) Mol. Microbiol. **6**, 1243–1252
15 Coutinho, J. B., Moser, B., Kilburn, D. G., Warren, R. A. J. and Miller, R. C. Jr. (1991) Mol. Microbiol. **5**, 1221–1233
16 Gilkes, N. R., Warren, R. A. J., Miller, R. C. Jr and Kilburn, D. G. (1988) J. Biol. Chem. **263**, 10401–10407
17 Langsford, M. L., Gilkes, N. R., Singh, B., Moser, B., Miller, R. C. Jr., Warren, R. A. J. and Kilburn, D. G. (1987) FEBS Lett. **225**, 163–167
18 Meinke, A., Braun, C., Gilkes, N. R., Kilburn, D. G., Miller, R. C. Jr. and Warren, R. A. J. (1991) J. Bacteriol. **173**, 308–314
19 Meinke, A., Gilkes, N. R., Kilburn, D. G., Miller, R. C. Jr. and Warren, R. A. J. (1991) J. Bacteriol. **173**, 7126–7135
20 Hackney, J. M., Kramer, G. P., Atalla, R. H., VanderHart, D. L. and Chapman, D. J. (1993) Planta **192**, 461–472
21 Gilkes, N. R., Jervis, E., Henrissat, B., Tekant, B., Miller, R. C. Jr., Warren, R. A. J. and Kilburn, D. G. (1992) J. Biol. Chem. **267**, 6743–6749
22 Meinke, A. M., Schmuck, M., Gilkes, N. R., Kilburn, D. G., Miller, R. C. Jr. and Warren, R. A. J. (1992) Glycobiology **2**, 321–326
23 Pilz, I., Schwarz, E., Kilburn, D. G., Miller, R. C. Jr., Warren, A. J. and Gilkes, N. R. (1990) Biochem. J. **271**, 277–280
24 Vaheri, M. P. (1982) J. Appl. Biochem. **4**, 356–363
25 Din, N., Gilkes, N. R., Tekant, B., Miller, R. C. Jr., Warren, R. A. J. and Kilburn, D. G. (1991) Bio/Technology **9**, 1096–1099
26 Wood, B. F., Conner, A. H. and Hill, C. G., Jr. (1986) J. Appl. Polymer Sci. **32**, 3703–3712
27 Yau, W. W., Kirkland, J. J. and Bly, D. D. (1980) in Modern Size-Exclusion Liquid Chromatography, pp. 315–341, John Wiley & Sons, New York
28 Chaplin, M. F. (1986) in Carbohydrate Analysis: A Practical Approach (Chaplin, M. F. and Kennedy, J. F., eds.), pp. 1–36, IRL Press, Oxford
29 White, R. A. and Brown, M. R. Jr. (1981) Proc. Natl. Acad. Sci. U.S.A. **78**, 1047–1051
30 Kulshreshtha, A. K. and Dweltz, N. E. (1973) J. Polym. Sci. Polym. Phys. Sci. **11**, 487–497
31 Dañhelka, J., Kössler, I. and Bohácková, B. (1976) J. Polym. Sci. Polym. Chem. Ed. **14**, 287–298
32 Battista, O. A. (1950) Ind. Eng. Chem. **42**, 502–507
33 Highley, T. L., Kirk, T. K. and Ibach, B. (1989) Document no. IRG/WP1350, International Research Group on Wood Preservation, Stockholm
34 Norkans, B. (1950) Physiol. Plant **3**, 75–87
35 Paralikar, K. M. and Bhatawdekar, S. P. (1984) J. Appl. Polym. Sci. **29**, 2573–2580
36 Van Tilbeurgh, H., Tomme, P., Claeyssens, M., Bhikhabhai, R. and Petersson, G. (1986) FEBS Lett. **204**, 223–227
37 Gilkes, N. R., Henrissat, B., Kilburn, D. G., Miller, R. C. Jr. and Warren, R. A. J. (1991) Microbiol. Rev. **55**, 303–315
38 Gebler, J., Gilkes, N. R., Claeyssens, M., Wilson, R. A. J. and Withers, S. G. (1992) J. Biol. Chem. **267**, 12559–12561
39 Shen, H., Schumuck, M., Pilz, I., Gilkes, N. R., Kilburn, D. G., Miller, R. C. Jr and Warren, R. A. J. (1991) J. Biol. Chem. **266**, 11335–11340
40 Gilkes, N. R., Claessens, M., Aebersold, R., Henrissat, B., Meinke, A., Morrison, H. D., Kilburn, D. G., Warren, R. A. J. and Miller, R. C. Jr. (1991) Eur. J. Biochem. **202**, 367–377
41 Gilkes, N. R., Langsford, M. L., Kilburn, D. G., Miller, R. C. Jr. and Warren, R. A. J. (1984) J. Biol. Chem. **259**, 10455–10459

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