The Cellulases Endoglucanase I and Cellobiohydrolases II of *Trichoderma reesei* Act Synergistically To Solubilize Native Cotton Cellulose but not To Decrease Its Molecular Size

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Degradation of cotton cellulose by *Trichoderma reesei* endoglucanase I (EGI) and cellobiohydrolases II (CBHII) was investigated by analyzing the insoluble cellulose fragments remaining after enzymatic hydrolysis. Changes in the molecular-size distribution of cellulose after attack by EGI, alone and in combination with CBHII, were determined by size exclusion chromatography of the tricarbanilate derivatives. Cotton cellulose incubated with EGI exhibited a single major peak, which with time shifted to progressively lower degrees of polymerization (DP; number of glucosyl residues per cellulose chain). In the later stages of degradation (8 days), this peak was eventually centered over a DP of 200 to 300 and was accompanied by a second peak (DP, ≈15); a final weight loss of 34% was observed. Although CBHII solubilized approximately 40% of bacterial microcrystalline cellulose, the cellobiohydrolases did not depolymerize or significantly hydrolyze native cotton cellulose. Furthermore, molecular-size distributions of cellulose incubated with EGI together with CBHII did not differ from those attacked solely by EGI. However, a synergistic effect was observed in the reducing-sugar production by the cellulase mixture. From these results we conclude that EGI of *T. reesei* degrades cotton cellulose by selectively cleaving through the microfibrils at the amorphous sites, whereas CBHII releases soluble sugars from the EGI-degraded cotton cellulose and from the more crystalline bacterial microcrystalline cellulose.

The cellulolytic system of the filamentous fungus *Trichoderma reesei* has been studied in great detail because of its efficiency in degrading native cellulose substrates (4, 7, 17, 18, 20, 29). When degrading cellulose ultimately to glucose, this fungus utilizes an assortment of extracellular hydrolytic enzymes, including at least three endoglucanases (EC 3.2.1.4), two cellobiohydrolases (EC 3.2.1.91), and a β-glucosidase (EC 3.2.1.21). Apparent problems in the conventional separation of these enzyme components, along with poorly characterized cellulose substrates, have made it difficult to ascertain definitively the biochemical functions of the individual proteins within and between the different cellulase enzyme groups. Improved protein isolation techniques, including affinity chromatography and cellulase gene expression in noncellulolytic hosts, have made it possible to study essentially pure enzymes (see reference 29 for a review).

Mechanistic studies of the individual cellulases of *T. reesei* have focused primarily on the production of soluble products from insoluble modified celluloses, water-soluble cellulose derivatives, cello-oligosaccharides, and chromogenic or fluorescent model compounds. Endoglucanase I (EGI), for instance, has been shown to be nonspecific, releasing reducing sugars from amorphous phosphoric acid-swollen cellulose, hydroxyethyl cellulose, and carboxymethyl cellulose, as well as xylans (1, 29). Cellobiohydrolases H (CBHII), on the other hand, releases cellobiose from microcrystalline cellulose (Avicel) and amorphous cellulose and shows low activity toward carboxymethyl cellulose (1, 29). Characterization of the insoluble products of cellulose hydrolysis by the individual *T. reesei* cellulolytic enzymes has received little attention.

We recently used size exclusion chromatography (SEC) to analyze the insoluble cellulose residues that remain after different fungal or enzymatic treatments. The study revealed various mechanisms of cellulose degradation between two wood-degrading fungi (15) and also between different isolated endoglucanases of the bacterium *Cellulomonas fimis* (16). The present study applied the same SEC technique in determining the effects of EGI and CBHII, alone and in combination, on the molecular-size distribution of cotton cellulose and bacterial microcrystalline cellulose (BMCC). We also assessed solubilization of the cellulose during enzyme attack.

**MATERIALS AND METHODS**

**Celluloses and chemicals.** Purified dewaxed cotton cellulose (1AY) was from Procter and Gamble Cellulose, Memphis, Tenn. BMCC was prepared from *Acetobacter xylinum* (ATCC 12733) grown on peptone-yeast extract-glucose medium (12); it was extracted and purified as described previously (10). Anhydrous pyridine, phenyl isocyanate, and high-pressure liquid chromatography (HPLC)-grade tetrahydrofuran were purchased from Sigma Chemical Co., St. Louis, Mo.

**Enzyme preparations.** CBHII of *T. reesei* RUT C-30 was purified by anion-exchange and affinity chromatography as described previously (24).

EGI was isolated from a strain of *T. reesei* that does not produce CBHII or CBHIII (kindly supplied by Oy Alko Ab, Helsinki, Finland). The culture was grown at 29°C in a 15-dm fermenter on a medium containing 3% (wt/vol) whey solids, 1.5% (wt/vol) distillers' spent grain, 0.5% KH$_2$PO$_4$, and 0.5% (NH$_4$)$_2$SO$_4$. The pH was maintained between 4.0 and 5.0. The culture fluid was clarified (38), concentrated by ultrafiltration (PM10 membrane; Amicon), and equilibrated in 12 mM sodium acetate (pH 5.8) before being applied to an anion-exchange DEAE Sepharose FF column (Pharmacia), equilibrated with the same buffer. Proteins were eluted from the column with a sodium chloride gradient (0 to 150 mM). Fractions containing EGI were combined, buffered with 7 mM sodium acetate (pH 3.8), and applied to a cation-exchange CM Sepharose FF column...
(Pharmacia). The bound proteins were eluted with a combined pH and ionic-strength gradient from 7 mM sodium acetate (pH 3.8) to 20 mM sodium acetate (pH 4.3). Endoglucanase activity was assayed by measuring the liberation of reducing sugar from hydroxyethyl cellulose (13). In addition, EGI was identified in certain fractions by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (molecular weight, 55,000) and/or isoelectric focusing (pI = 4.5). Purified Agrobacterium β-glucosidase was a gift from Stephen Withers, University of British Columbia, Vancouver, Canada.

Reactions. In time course studies of cotton cellulose depolymerization, 10 mg of cellulose was incubated with 2 nmol of EGI alone or in combination with 2 nmol of CBHII in 1 ml of 50 mM sodium acetate buffer (pH 5.0) containing 0.02% NaN₃ (acetate-azide buffer). The reaction mixtures were stirred magnetically at 39°C over a 192-h period; stirring greatly enhances enzymatic cellulose hydrolysis (16, 30). At designated time points, samples were removed and spun for 5 min in a microcentrifuge (16,000 x g). The supernatants were analyzed for reducing sugars and total sugars. The residual cellulose pellet was washed three times with 1.5 ml of water and oven-dried at 60°C prior to determination of molecular-size distributions.

In additional experiments with cotton, cellulobiose (1 mg) or β-glucosidase (1 U; amount of enzyme required to release 1 µmol of p-nitrophenol·min from p-Nitrophenyl-β-D-glucopyranoside) was added to the 1-ml reaction mixtures containing both EGI and CBHII. EGI controls also included these additives. The β-glucosidase was added to the reaction mixtures 12 h after EGI and CBHII, i.e., when synergy began to decline. Cellulobiose was added to the reaction mixtures at the same time as the two cellulases. The amount of reducing sugar contributed by the exogenous cellulobiose was subtracted from the total amount of reducing sugars in the reaction mixture supernatants.

To determine the activity of CBHII on a highly crystalline cellulose substrate, 1 nmol of enzyme was incubated with 1 mg of BMCC as described above. Weight losses and molecular-size distributions of the cellulose were determined after 12, 24, 48, and 192 h. In a separate experiment, the stability of the cellulobiose attack was determined by preincubating CBHII at 39°C in acetate-azide buffer for 48 and 96 h before assaying with 1 mg of BMCC for 24 h.

Cellulose and sugar analyses. Molecular-size distributions of the residual cellulose (pellets from centrifugation) were determined by SEC of the tricarballylate derivatives dissolved in tetrahydrofuran (16, 34). Four HPLC SEC columns, Shodex KF803, KF805, and KF807 and 100 Å (10 nm) µStyragel (Waters Co.), were connected in series and used for the analyses. The SEC columns were calibrated with narrow polystyrene standards by using the universal calibration technique (34, 37). The weight- and number-average degrees of polymerization (DPn and DPw) were then calculated (37). Levels of reducing sugars were determined by the dinitrosalicylic acid method, with glucose as the standard (35). Total levels of sugars in the reaction supernatants were determined by the phenol-sulfuric acid assay (3). These values, corrected for water of hydrolysis, were used to calculate the percent weight losses of the cellulose. Analysis of the variation in multiple determinations indicated a maximum 3% standard deviation.

Synergy between enzymes in cotton cellulose degradation was assessed by determining the ratio of the reducing-sugar level produced by the combination of EGI and CBHII to that produced by EGI alone (CBHII alone did not produce detectable levels of reducing sugars).

RESULTS

Isolated T. reesei EGI and CBHII, alone and in combination, were incubated with cotton cellulose for various times. Changes in molecular-size distribution and weight losses (percent solubilization) were determined. The degree of synergy between EGI and CBHII in the production of reducing sugars from the cellulose was investigated in the presence and absence of added β-glucosidase and cellulobiose. Degradation of BMCC by isolated CBHII was also investigated.

Molecular-size distribution of cellulose incubated with EGI, CBHII, and EGI-CBHII. HPLC of cotton cellulose after incubation with EGI revealed a single major peak that shifted to progressively lower DP positions with time. Eventually, a second peak, centered over a DP of approximately 15, also appeared (Fig. 1). After 12 h, the major peak had become broad and was centered over a DP of approximately 700. This resulted in a twofold increase in the polydispersity of the cellulose (DPw/DPn; an indication of the range of molecular sizes in the sample) (Table 1). This major peak was subsequently shifted to a DP of about 300 after 24 h, and the second, less pronounced peak (DP = 15) began to appear. As the incubation continued, the major peak was reduced in size and shifted to a slightly lower DP (~250), while the peak at a DP of 15 increased. A small portion of high-molecular-weight material remained in the sample after 96 h but was absent after 192 h. Ultimately, the DPw and DPn of the cellulose had decreased from 3,718 to 212 and from 803 to 42, respectively (Table 1). The major peak became narrower after 48 h, a change reflected in decreased polydispersity values. During the incubation, EGI gradually solubilized the cellulose, resulting in a maximum weight loss of 34% after 192 h. Reducing-sugar production increased rapidly for the first 48 h and then more slowly thereafter; 3.0 mg of reducing sugars, estimated as glucose, had been released after 192 h from 10 mg of cellulose. In contrast to EGI, CBHII did not affect the molecular-size distribution of cotton cellulose; the single peak centered over a DP of around 4,000 was seen at all incubation times (data not shown). Production of soluble sugars from cotton by CBHII was minimal, i.e., the amount of glucose was below the detection limit of the dinitrosalicylic acid assay (<100 µg·ml⁻¹).

Changes in the molecular-size distribution of cotton cellulose attacked by EGI in combination with CBHII (in experiments otherwise done exactly as described for Fig. 1) were very similar to those observed when cotton was attacked by EGI alone (data not shown). The peak centered at a DP of approximately 15 was also present. A maximum weight loss of approximately 35% was observed after 192 h. Somewhat more reducing sugars were released from cotton by the action of the combined enzymes than by EGI alone; this was especially evident after 12 h (Fig. 2). The degree of synergy increased from 1.4 to approximately 1.8 but then decreased thereafter.

| Table 1. Depolymerization of cotton cellulose by EGI of T. reesei* |
|---|---|---|---|---|
| Incubation time (h) | Weight loss (%) | DPw | DPn | Polydispersity (DPw/DPn) |
| 0 | 0 | 3,718 | 803 | 4.6 |
| 12 | 11 | 1,737 | 196 | 8.9 |
| 24 | 15 | 1,004 | 144 | 7.0 |
| 48 | 21 | 775 | 97 | 8.0 |
| 96 | 31 | 547 | 82 | 6.7 |
| 192 | 34 | 212 | 42 | 5.0 |

* 2 nmol of enzyme per 10 mg of cellulose.
Approximately 3.4 mg of reducing sugars was released from 10 mg of cellulose in 192 h by the combined enzymes.

**Effects of added β-glucosidase and cellobiose on reducing-sugar production by EGI-CBHII.** To determine if end-product inhibition of CBHII contributed to the observed reduction over time in the degree of synergy between EGI and CBHII (Fig. 2), β-glucosidase and cellobiose were separately added to the reaction mixtures. A much greater degree of cooperativity, in terms of reducing-sugar production, was observed between the cellulases in the presence of β-glucosidase than in its absence. This effect was most pronounced 12 h after the addition of the β-glucosidase; the degree of synergy was 2.18, compared with 1.30 in the absence of the β-glucosidase (Fig. 2). After 12 h the degree of synergy decreased. Addition of cellobiose to the reaction mixture containing both EGI and CBHII resulted in a decrease in reducing-sugar production by the cellulases and essentially in the abolition of synergy (Fig. 2).

**Degradation of BMCC by CBHII.** CBHII readily attacked BMCC, solubilizing approximately 40% of the crystalline cellulose in 24 h (Table 2). Extended incubations (48 and 192 h) did not result in further degradation of the cellulose as indicated by total sugar production.

The molecular-size distribution of BMCC degraded by CBHII changed only slightly compared with that of the unattacked control (Fig. 3). Thus, the single peak in the SEC chromatogram became narrower and smaller but remained centered over a DP of 250. However, initial degradation of the longest cellulose molecules by CBHII resulted in a reduction in the DP and polydispersity of the cellulose (Table 2). These values remained essentially constant after 12 h, despite an increase in weight loss. The DP of the cellulose remained relatively unchanged.

CBHII preincubated at 39°C for 48 h solubilized nearly 50% less BMCC in 24 h than fresh enzyme. The cellobiohydrolases was inactive after a 96-h preincubation period.

**DISCUSSION**

Cellulose is a polymer of anhydroglucose (or anhydrocellobiose) units linked by β-1,4-glycosidic bonds. van der Waals forces and hydrogen bonding interactions between and within cellulose molecules, however, make natural cellulose structurally complex; highly crystalline regions are connected by less organized amorphous regions. The resulting bundles, known as cellulose microfibrils, require that cellulolytic microorganisms such as *T. reesei* employ an array of specialized enzymes to convert cellulose to soluble oligomers and ultimately to glucose. It has long been of interest to researchers to understand precisely the contribution of each enzyme component of the cellulolytic system. This study provides insight into the action of two isolated enzymes, EGI and CBHII from *T. reesei*, alone and in combination, on native cotton cellulose.

EGI alone continuously depolymerized cotton cellulose over 192 h, ultimately causing an 18-fold reduction in the DP and a 34% weight loss. In advanced stages of degradation, the majority of the residual cellulose had a DP of 200 to 300, presumably the size of the crystalline regions (2). The pattern of changes in molecular size distribution suggests that EGI degraded the cellulose by selectively attacking the amorphous regions, as does acid (2), generating cellulose crystallites. These were further attacked at their surfaces; cellulose chains of DP ≤ 20 accumulated.

A very similar mode of attack on cotton was observed earlier with the bacterial endoglucanase CenA of *C. fimi* (16). In spite of this, detailed amino acid sequence comparisons (23, 33) place the two endoglucanases in different cellulase families, indicating differences in molecular structure (9). Also, CenA hydrolyzes the β-1,4-glycosidic bond with inversion (32) but EGI hydrolyzes with retention (5) of the anomeric configuration, indicating basic differences in the catalytic mechanism.

Accumulation of small fragments (DP, < 20) in advanced
stages of cellulose degradation by both EGI and CenA is intriguing. EGI and CenA might inherently be unable to attack, or be able to attack only slowly, these small fragments. Another possibility is that newly generated insoluble fragments recrystallized onto the remaining bundles, retarding their further degradation. That the peak was centered at a DP of \( \approx 15 \) rather than at a lower DP might be an artifact, because the workup procedure removed any soluble fragments from the reaction mixtures prior to the molecular-size determinations, thereby eliminating fragments with DPs of \( \leq 7 \) from the analysis. The smaller peak, therefore, might represent only the insoluble portion of fragments ranging up to a DP of \( \approx 20 \).

Although both cellobiohydrolases of \( T. \) reesei liberate almost exclusively cellobiose from crystalline cellulose and do not decrease the viscosity of substituted cellulases such as carboxymethyl cellulose, their exoglucanase character has frequently been questioned in the literature (1, 11, 14, 19, 28). Two recent papers indicate that both CBHI and CBHII produce new reducing end groups on filter paper cellulose (14, 28). However, the efficiency is clearly much lower than that observed with the endoglucanases, and it is extremely difficult to exclude the possibility that low amounts of endoglucanases remain, especially in CBHII preparations after purification (25). The end-product analysis of CBHII cellulose degradation described here is indicative of an exoglucanase mode of action. The activity of our CBHII sample was nondetectable on cotton. Cotton is less crystalline than BMCC and has only about 15% of the number of chain ends. The resistance of the cotton to CBHII evidently is due to a combination of these, and perhaps other, factors. Despite efficient hydrolysis, the chain length distribution in the hydrolyzed BMCC sample was not significantly different from that of the unattached substrate. After solubilization of 40% of the BMCC, the reduction in DP, was only about 100. This suggests that the substrate was not cleaved within the molecules but was attacked from the chain ends, largely at the crystallite surfaces. Initial DP, and polydispersity decreases at 12 h may be attributed to degradation of the longer cellulose chains by CBHII. Since the DP, thereafter remained constant, we speculate that CBHII sequentially removed the outermost layers of the microfibrils, leaving behind relatively intact cellulose crystallite (DP, \( \approx 360 \)).

Our results with both EGI and CBHII can be explained by examining some of the structures of fungal and bacterial cellulases (6, 26, 27). The structures of both \( T. \) reesei exoglucanases reveal active sites buried in long tunnels which are generated by distinct loops on the enzyme surface (6, 26). Unless these loops are capable of major structural adjustments, the shape of the active site prevents the binding of the enzyme to the middle of cellulose chains for attack of chains buried in the crystallite matrix. Endoglucanases homologous to CBHI and CBHII (6, 26, 27) apparently lack the loops and molecular folds and catalytic mechanisms.

Because the pattern of change in the molecular-size distribution of cotton cellulose attacked by EGI-CBHI was essentially the same as that of cotton attacked solely by EGI, our data show that CBHII does not enhance or inhibit cellulose depolymerization by EGI. However, increased amounts of soluble reducing sugars were released by the cellulase combination, especially early in the incubation. Because CBHII alone did not release detectable amounts of reducing sugars from cotton, synergism between EGI and CBHII is documented. Cooperativity between EGI and CBHII of \( T. \) reesei has previously been reported with different cellulose substrates and conditions (1, 8, 11, 21). In particular, it has been shown that pretreatment of filter paper by CBHII does not promote its hydrolysis, although pretreatment by EGI clearly facilitates hydrolysis by CBHII (22). Our data are consistent with the view that such an "endo-exo" type of synergy is based on the action of CBHII on new chain ends generated by EGI (36).

The observed decrease in the degree of synergy between EGI and CBHII after the 12-h incubation was apparently due to end-product inhibition of CBHII by cellobiose (31). This was evidenced by the increase in the degree of synergy when \( \beta \)-glucosidase, which hydrolyzes cellobiose to glucose, was added to the reaction mixture. Additionally, the synergistic effects of EGI and CBHII were essentially eliminated when exogenous cellobiose was added at the same time as the cellulases. Reductions in cooperativity between the cellulases after extended incubations may also be attributed to the instability, and therefore decreased activity, of CBHII. This enabled the EGI-only reaction to catch up in production of reducing sugar.

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REFERENCES
