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Three Native Cellulose-Depolymerizing Endoglucanases from Solid-Substrate Cultures of the Brown Rot Fungus *Meruliporia (Serpula) incrassata*

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Three extracellular cellulose-depolymerizing enzymes from cotton undergoing decay by the brown rot fungus *Meruliporia (Serpula) incrassata* were isolated by anion-exchange and hydrophobic interaction chromatographies. Depolymerization was detected by analyzing the changes in the molecular size distribution of cotton cellulose by high-performance size-exclusion chromatography. The average degree of polymerization (DP; number of glucosyl residues per cellulose chain) was calculated from the size-exclusion chromatography data. The very acidic purified endoglucanases, Cel 25, Cel 49, and Cel 57, were glycosylated and had molecular weights of 25,200, 48,500, and 57,100, respectively. Two, Cel 25 and Cel 49, depolymerized cotton cellulose and were also very active on carboxymethyl cellulose (CMC). Cel 57, by contrast, significantly depolymerized cotton cellulose but did not release reducing sugars from CMC and only very slightly reduced the viscosity of CMC solutions. Molecular size distributions of cotton cellulose attacked by the three endoglucanases revealed single major peaks that shifted to lower DP positions. A second smaller peak (DP, 10 to 20) was also observed in the size-exclusion chromatograms of cotton attacked by Cel 49 and Cel 57. Under the reaction conditions used, Cel 25, the most active of the cellulases, reduced the weight average DP from 3,438 to 315, solubilizing approximately 20% of the cellulose. The weight average DP values of cotton attacked under the same conditions by Cel 49 and Cel 57 were 814 and 534; weight losses were 9 and 11%, respectively.

Brown rot basidiomycetes cause the most destructive form of wood decay by rapidly depolymerizing the cellulose component, often before significant weight loss occurs (9). The average degree of polymerization (DP; number of glucosyl residues per cellulose molecule) of cellulose in wood is rapidly reduced from about 10,000 (18) to approximately 250. These residual fragments apparently correspond to the size of cellulose crystallites, which are formed by cleavages within the amorphous regions of cellulose microfibrils (2). As a result of the initial attack on cellulose, the wood becomes friable and brittle. Brown rot fungi are unique in that they utilize the hemicellulose and cellulose components of wood without removing the lignin (9, 27, 32, 34). The characteristic brown color of the decayed wood is probably due to oxidative changes in the remaining lignin (31).

The enzymatic cellulolytic system of brown rot fungi has not been extensively investigated, but it appears that it differs from that of other well-characterized cellulose-degrading microorganisms. Such fungi as *Phanerochaete chrysosporium* and *Trichoderma reesei* effectively degrade crystalline cellulose via a synergistic mechanism between endoglucanases and cellobiohydrolases (14). Endoglucanases cleave within cellulose molecules, generating nonreducing ends upon which cellobiohydrolases act. In contrast, most brown rot fungi cannot degrade pure crystalline cellulose and do not have detectable cellobiohydrolase activity (28, 45, 46). Exceptions to this generalization are a few brown rot fungi belonging to the *Coniophoraceae* family (45, 46). Two cellobiohydrolases were recently isolated from *Coniophora puteana*, which degrades crystalline cellulose (48). Endoglucanases, which are produced by both types of fungi, have been isolated from at least three brown rot fungi, including *Polyporous schweinitzii* (30), *Lenzites trabea* (synonym; *Gloeophyllum trabeum*) (23), and *Tyromyces palustris* (29). These acidic cellulases range in molecular mass from 16 to 45 kDa. In general, it appears that most endoglucanases of brown rot fungi are constitutively expressed and are not catabolically repressed by glucose (8, 24).

Endoglucanase activity is commonly determined by measuring the release of reducing sugars from insoluble modified cellulose substrates or water-soluble cellulose derivatives and, less commonly, by determining the reduction in viscosity of carboxymethyl cellulose (CMC) solutions. These methods are limited in that they do not directly detect internal cleavages of glycosidic bonds of natural insoluble cellulose substrates. Recently, we reported the use of high-performance size-exclusion chromatography (SEC) to determine changes in the molecular size distribution of cotton cellulose attacked by the brown rot fungus *Postia placenta* (36). In that investigation, we concluded that *P. placenta* degrades cotton cellulose by cleaving completely through the cellulose microfibrils at the amorphous sites. The aim of this study was to isolate and characterize cellulose-depolymerizing enzymes from cotton being degraded by *M. incrassata* in solid substrate cultures by using the SEC method of analysis.

MATERIALS AND METHODS

Fungal strain and culture conditions. Because the complete cellulose-depolymerizing system of brown rot fungi has not been demonstrable in liquid cultures, solid-substrate cultures were used in this study. *Meruliporia (Serpula) incrassata* was the brown rot fungus of choice, because we (37) and others (28) have found that it depolymerizes cotton cellulose more rapidly

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and consistently than the more commonly studied brown rot fungus *P. placenta*.

M. incrassata (Berk. & Curt.) Murr. (MAD-563; ATCC 11236) was grown in solid-substrate cotton cultures in 2.8-liter Fernbach flasks (12 total). Each flask contained 150 ml of a chemically defined medium into which was placed 100 g of dewaxed cotton (a gift from The Procter and Gamble Cellulose Company, Memphis, Tenn.). The chemically defined medium consisted of 0.5% glucose and BIII medium (33), except that 25 mM NH_4NO_3 replaced ammonium tartrate as the nitrogen source. The flasks and contents were sterilized by autoclaving, inoculated with six mycelium-covered malt agar plugs (diameter, 1.5 cm), and incubated at 27°C under 70% relative humidity for 4.5 weeks.

Extracellular crude extract. After incubation, the degraded cotton cellulose (~1.2 kg total dry weight) was removed from the flasks and immersed in 10 liters of water (4°C) for 1 h to release extracellular proteins. The extract was squeezed from the cellulose with a hydraulic press, filtered by vacuum (glass fiber filter; model SP-10; Baxter), and concentrated on a Minitan tangenital-flow ultrafiltration system equipped with a polysulfone membrane (M_r cutoff, 10,000) (Millipore Corp., Bedford, Mass.). The extract (100 ml) was then centrifuged to remove fine cellulose particulates and concentrated to 10 ml on an Amicon Diaflo Ultrafiltration PM10 membrane (M_r cutoff, 10,000) in a stirred-cell ultrafiltration unit (Pharmacia LKB Biotechnology Inc., Uppsala, Sweden). Total protein was approximately 2 mg/ml.

pH profile study of crude extract. The concentrated crude extract (0.5-ml aliquots) was incubated with 10 mg of cotton cellulose in a total volume of 1 ml at different pH values at 27°C for 48 h with magnetic stirring. The buffers used were HCl-KCl for pH 2.0 and McIlvaine buffer (citrate-phosphate) (52) for the pH range of 2.6 to 7.0; final buffer concentrations were 50 mM. The crude extract was assayed at the various pH values for both carboxymethyl cellulase (CMCase) and cotton cellulose-depolymerizing activities; assays are described below.

Purification of enzymes. Concentrated crude extract (2 ml) was chromatographed on a MonoQ HR5/5 anion-exchange column interfaced with a fast protein liquid chromatography (FPLC) system (Pharmacia). The column was previously equilibrated with 10 mM bis-Tris-HCl (pH 6.5). The proteins were eluted with an NaCl gradient in the buffer described above (0 to 0.5 M in 50 ml) at a flow rate of 1 ml/min. The eluent was monitored at 280 nm by using a UV-VIS detector (Pharmacia). Fractions (1 ml) having CMCase and/or cotton cellulose-depolymerizing activity were pooled and combined with other fractions from five separate MonoQ trials before the next purification step. Alternatively, the entire 10 ml of concentrated crude extract was applied to a MonoQ HR10/10 anion-exchange column and eluted with the same buffer and salt gradient (0 to 0.5 M NaCl in 400 ml) at a flow rate of 4 ml/min.

Protein fractions having cellulase activity were further purified by hydrophobic interaction chromatography on a phenyl-Superose HR5/5 column (Pharmacia) equilibrated with 50 mM potassium phosphate buffer (pH 7.0) containing 1.7 M $(NH_4)_2SO_4$. Two of the cellulases were eluted with a 5-ml descending salt gradient (1.7 to 0.85 M) and then with a 15-ml descending salt gradient (0.85 to 0 M) with the same phosphate buffer devoid of $(NH_4)_2SO_4$. The flow rate was 0.5 ml/min. The third cellulase was eluted with the same buffer, but the series of the two gradients was reversed. Fractions (1 ml) having CMCase and/or cotton cellulose-depolymerizing activity were pooled.

CMCase assays. Measurement of endo-1,4-β-glucanase activity was patterned after the Commission on Biotechnology recommended method (56), based on release of reducing sugars from CMC. The activities in the crude extract and in the FPLC fractions were determined. The reaction mixtures consisted of 0.5 ml of 2% CMC (Hercules 7LF with a degree of substitution of 0.7; degree of substitution = substituent per glucosyl residue; Hercules, Wilmington, Del.) in 50 mM Na citrate (pH 3.5) containing 0.02% NaN_3 (citrate-azide buffer) and 0.5 ml of diluted enzyme solution; incubation was at 27°C for 30 min. Reducing sugars were determined with the dinitrosalicylic acid method with glucose as the standard (56). The enzyme solutions were diluted to give values of less than 0.5 mg of reducing sugar. One unit of activity is defined as the amount of enzyme required to release 1 μmol of reducing sugar min^{-1} as glucose.

Endoglucanase activity was also determined with CMC (Hercules 7MF; degree of substitution, 0.7) and a modified viscosity method of analysis (56). Six milliliters of a 0.5% CMC solution (in the citrate-azide buffer described above) were placed in a size 100 viscometer (Fisher Scientific Co., Pittsburgh, Pa.) and equilibrated at 30°C. The reaction was started by adding 1 ml of diluted enzyme to the CMC solution. Flow rates were determined at 5-min intervals after the addition of enzyme. One unit of activity is defined as the amount of enzyme required to change the inverse specific viscosity by $0.01 \cdot min^{-1}$ under the conditions described.

Depolymerization of cotton cellulose. Cotton cellulose-depolymerizing activity in the chromatography fractions was determined by incubating aliquots of the fractions with 0.5 to 1.0 mg of dewaxed cotton cellulose (Procter and Gamble Cellulose) in a total volume of 1 ml of citrate-azide buffer. Samples were magnetically stirred and incubated at 27°C for 2 or 7 days. After the incubation period, the samples were centrifuged for approximately 10 min at 16,000 × *g*. Total soluble sugars released in the supernatants were determined by the phenol-sulfuric acid method (7). These values, corrected for water of hydrolysis, were used to calculate the percent weight losses of the cellulose. The residual cellulose pellets were washed three times with 1.5 ml of water and oven dried overnight at 60°C prior to the determination of the molecular size distributions.

Molecular size distribution of cellulose. In the initial pH profile experiment, the average DP of cellulose was estimated viscosimetrically after cellulose solubilization in cupriethylene diamine (GFS Chemicals, Columbus, Ohio) (1). In subsequent experiments, the molecular size distribution of the cellulose samples was determined by SEC of the tricarbanilate derivatives dissolved in tetrahydrofuran (55). In derivatizing 1 mg of dried cotton, 3.0 ml of anhydrous pyridine and 0.5 ml of phenylisocyanate were used. We have found this method to give reproducible results (data not shown). Four high-performance liquid chromatography tandem SEC columns, Shodex KF803, KF805, and KF807 (Waters Co., Milford, Mass., and Millipore Corp.) and Waters Co. 10-nm μStyragel, were used for the analysis. The weight average and number average DPs (DP_w and DP_n, respectively) of the cellulose samples were calculated as described previously (57).

Other analytical methods. Protein concentrations were determined by the bicinchoninic acid method after precipitation with trichloroacetic acid (5), with bovine serum albumin (Bio-Rad, Richmond, Calif.) as the standard. Isolated proteins were analyzed on a precast sodium dodecyl sulfate (SDS)-polyacrylamide gel (gradient, 10 to 15%; Pharmacia) by using a Pharmacia PhastSystem. Standards (Bio-Rad) had molecular weights of 97,400 (rabbit muscle phosphorylase), 66,200 (bovine serum albumin), 45,000 (hen egg white ovalbumin), 31,000 (bovine carbonic anhydrase), 21,500 (soybean trypsin inhibi-

tor), and 14,400 (hen egg white lysozyme). Isoelectric focusing of the isolated enzymes was performed on a precoat (pH range, 3 to 6; Serva, Heidelberg, Germany) with a Bromma 2117 Multiphor apparatus (LKB, Bromma, Sweden). Standards (Sigma) had pI values of 3.6 (amyloglucosidase), 4.6 (soybean trypsin inhibitor), 5.1 (β-lactoglobulin A), and 5.9 (carbonic anhydrase B). Proteins were stained with Coomassie blue after all of the above analytical separations were performed. Glycoproteins were detected on nitrocellulose blots by a glycan detection kit, which is based on an enzyme immunoassay (Boehringer-Mannheim Corporation, Indianapolis, Ind.).

RESULTS

By using anion-exchange and hydrophobic interaction chromatographies, we isolated three endoglucanases from cotton cellulose that was being degraded by *M. incrassata*. In addition to the more conventional assays for detecting endoglucanase activity, we used depolymerization of insoluble cotton cellulose, which in fact was the only method that detected one of the enzymes. The activities of the isolated cellulases on CMC and cotton were compared, and selected physical properties of the enzymes were determined.

Fungal growth and enzyme extraction. *M. incrassata* grew exceptionally well in the solid-substrate cultures, in which approximately one-third of the cotton cellulose absorbed the liquid medium. Most of the fungal biomass was observed on the drier portions of the cellulose, where long hyphal strands, emanating from the agar inoculation plugs, penetrated through the cellulose fibers into the nutrients at the bottom of the flask. The average DP of the drier, decayed cellulose was around 300. Enzyme production and purification were most successful when cultures were harvested before 5 weeks, i.e., before the production of a yellow pigment, presumably a secondary metabolite. The use of the hydraulic press was successful in facilitating the recovery of extracellular enzymes. Approximately 20 mg of total protein per kg of cotton was recovered in the concentrated crude extract, which had a pH of approximately 4.

pH activity profile of enzymes in the crude extract. Both the cotton-depolymerizing activity and the sugar release activity in the concentrated crude extract were maximum in the acidic pH range (Fig. 1), with maximum activity at pH 2.5 to 4.0. The enzymes were still quite active at pH 2.0, and a sharp decrease in activity was observed between pH 4.0 and 5.0.

Anion-exchange chromatography. Fractionation of the proteins in the concentrated crude extract on a MonoQ anion-exchange chromatography column resulted in the resolution of at least three peaks containing CMCase (Fig. 2A) and/or cotton cellulose-depolymerizing activity (Fig. 2B). The most active proteins, which eluted from the column with 0.20, 0.35, and 0.37 M NaCl, were eventually designated Cel 49, Cel 25, and Cel 57 on the basis of their molecular weights (see below). Pooled chromatography fractions containing Cel 49 and Cel 25 had significant CMCase activity and also depolymerized cotton cellulose. Pooled fractions containing Cel 57, by contrast, did not have significant CMCase activity but did depolymerize cotton cellulose. Although moderate CMCase activity was detected in fractions 26 to 30 (Fig. 2A), we did not pursue the identity of this enzyme(s) because these fractions did not significantly depolymerize cotton cellulose (data not shown). The only fractions that contained discernible cotton cellulose-depolymerizing activity are shown in Fig. 2B.

Hydrophobic interaction chromatography. Final purification of the three cellulases was achieved by hydrophobic interaction chromatography. To maintain good peak resolution, MonoQ fractions containing Cel 25 were separated in multiple runs on the HR5/5 phenyl-Superose column. Fractions containing the same activity were pooled. During the purification, Cel 49 and Cel 25 (Fig. 3A and B) were detected with the CMC-based assay for reducing sugar, whereas Cel 57 was detected with the cotton depolymerization assay (Fig. 3C, inset). Cel 49 and Cel 57 are quite hydrophobic, eluting from the column with potassium phosphate buffer containing 0.34 and 0.48 M ammonium sulfate, respectively, whereas Cel 25 eluted from the column earlier with potassium phosphate containing 1.16 M ammonium sulfate. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) revealed single bands for each of the three cellulases (Fig. 4A). The purifications of Cel 25 and Cel 49 are described in Table 1.

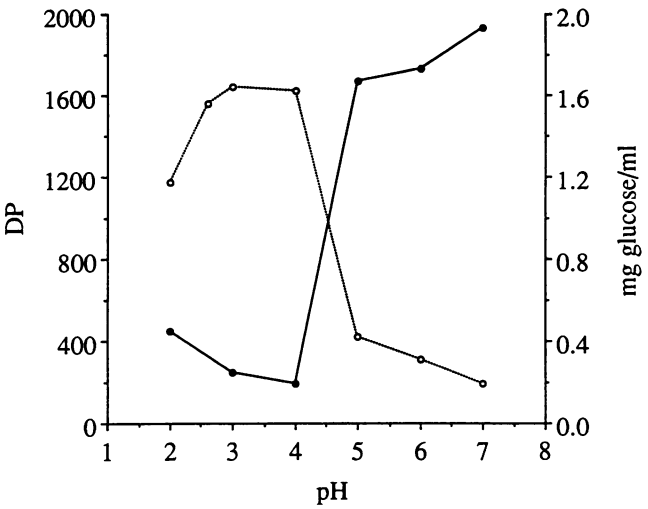

FIG. 1. pH activity profile of cellulases in crude extract. The pH profile of the cellulases in the crude extract was determined with cotton cellulose as the substrate. The average DP of the cellulose (●) and the amount of reducing sugars released as glucose (○) were determined. One-milliliter samples were incubated with 10 mg of cotton cellulose for 48 h at 27°C. The average DP was determined viscosimetrically.

Physical properties of the three cellulases. The molecular weights of the respective cellulases, determined by SDS-PAGE (Fig. 4A), are as follows: Cel 49, 48,500; Cel 25, 25,200; and Cel 57, 57,100. Isoelectric focusing on a gradient gel (pH 3 to 6) revealed that all of the cellulases are more acidic than the lowest pI standard marker (pI 3.6) (Fig. 4B). Cel 57 appeared to be more acidic than the other two cellulases. All three cellulases were indicated to be glycoproteins by the enzyme immunoassay technique (data not shown).

CMCase activities and cotton cellulose-depolymerizing activities of the isolated *M. incrassata* cellulases. The isolated cellulases of *M. incrassata* had markedly different activities on CMC and cotton cellulose. Cel 25 and Cel 49 released significant amounts of reducing sugar from CMC, whereas Cel 57 did not (Table 2). The specific activity of Cel 25 was 376 U/mg, approximately twice that of Cel 49. The temperature optimum of Cel 25 was 50°C (data not shown). To determine if the cellulases randomly hydrolyze internal bonds of CMC, a viscosimetric analysis was also performed. Again, Cel 25 and Cel 49 had significant activities, (2,201 and 1,313 U/mg, respectively), whereas that of Cel 57 was negligible (51 U/mg).

Cotton cellulose depolymerization by the individual enzymes was investigated in detail by examining the changes in the

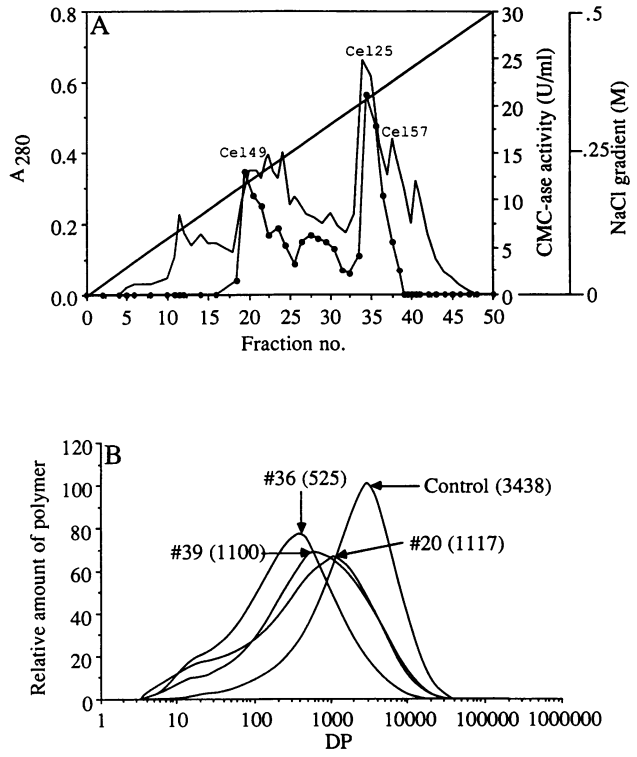

FIG. 2. MonoQ anion-exchange chromatogram of the crude extract. (A) Anion-exchange chromatography of concentrated crude extract (2 ml) on an HR5/5 MonoQ column. Fractions (1 ml) were assayed for CMCase activity (●) and cotton cellulose-depolymerizing activity (see panel B). The diagonal line indicates the NaCl gradient. (B) Molecular size distributions of cotton cellulose incubated with various MonoQ fractions. Approximately 0.5 mg of cotton was incubated with 400 μl of each fraction in a total of 1 ml of 50 mM sodium citrate buffer (pH 3.5) containing 0.02% NaN_3. The samples were magnetically stirred for 48 h at 27°C. Numbers next to arrows designate MonoQ fractions. The numbers in parentheses are the DP_w values of the sample.

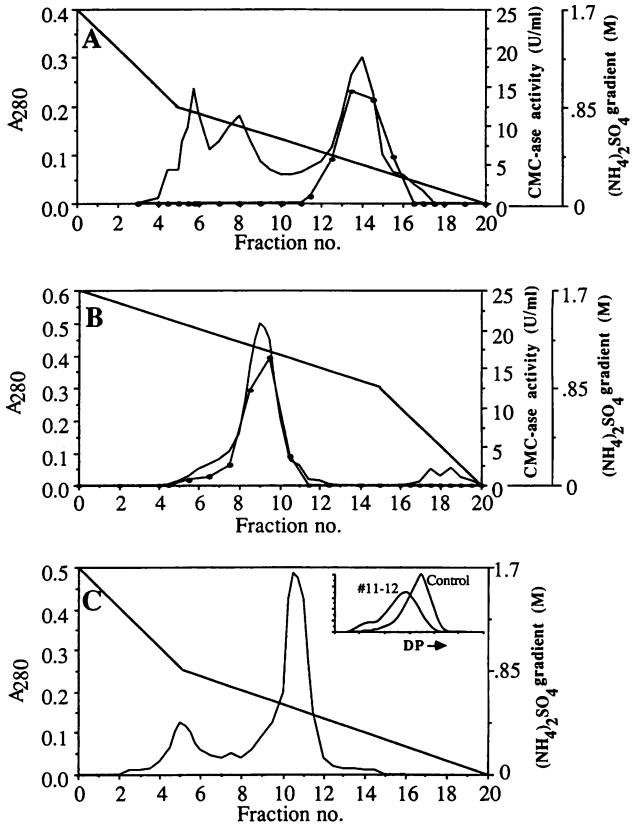

FIG. 3. Hydrophobic interaction chromatography of MonoQ fractions. Hydrophobic interaction chromatography of MonoQ fractions 19 to 21 (Cel 49) (A), 34 to 37 (Cel 25) (B), and 38 to 40 (Cel 57) (C) on a phenyl-Superose HR5/5 column. Fractions (1 ml) were assayed for either CMCase (●) or cellulose-depolymerizing activity (C, inset). Diagonal lines indicate $(NH_4)_2SO_4$ gradients.

molecular size distributions after 7-day incubations. The size-exclusion chromatograms of cellulose attacked by the cellulases revealed single major peaks that shifted to lower DP positions, indicating that extensive depolymerization had occurred (Fig. 5). A smaller, less pronounced peak at a DP of ≅20 also appeared when cellulose was attacked by Cel 49 or Cel 57. The slight narrowing of the major peak in the molecular size distribution of cellulose attacked by Cel 25 indicates a decrease in polydispersity (DP_w/DP_n; an indication of the range of molecular sizes) (Table 2). With Cel 49 and Cel 57, however, the major peak broadened, resulting in increased polydispersities. Cel 25 solubilized 20% of the cellulose, reducing the DP_w from 3,438 to 328 (Table 2). The DP_n was reduced from 656 to 70. The DP_w and DP_n of cotton attacked by Cel 49 were 814 and 94, and those for Cel 57 were 534 and 71, respectively (Table 2). Approximately 9% (Cel 49) and 11% (Cel 57) of the cellulose were solubilized by these two enzymes.

DISCUSSION

Cellulose is composed of highly ordered crystalline regions that are connected by less-ordered amorphous regions. Because of this complexity and insolubility, most cellulolytic microorganisms employ a consortium of enzymes in converting cellulose to soluble oligomers and monomeric sugars. Endoglucanases, which are thought to initiate the degradation process by cleaving internal glycosidic bonds, can be specifically assayed by determining the changes in the molecular size distribution of insoluble celluloses. In this study, we used high-performance SEC to demonstrate in vitro depolymerization of cotton cellulose by three cellulases of the brown rot fungus *M. incrassata*.

The cellulose-depolymerizing system of *M. incrassata* was expressed in solid-substrate cultures as evidenced by a sevenfold reduction in the viscosity average DP of the cotton. Extraction of the degrading cellulose resulted in the recovery of approximately 20 mg of protein per kg of cellulose. This value probably underestimates the total protein because cellulases bind tightly to their substrates (17, 47). Datta (11), who used a similar extraction procedure in isolating fungal enzymes from solid-substrate cultures, recovered 4 mg of total protein per kg of wood pulp. In that study, the nutrient-amended pulp was incubated with the white rot fungus *P. chrysosporium* for 3 days.

The average DP of cotton cellulose incubated with crude extracts of cotton undergoing decay by *M. incrassata* was reduced from about 2,000 to approximately 300. This is analogous to the leveling-off DP that is observed on acid hydrolysis in which cellulose crystallites are released (2, 44), suggesting that enzymes in the crude extract preferentially

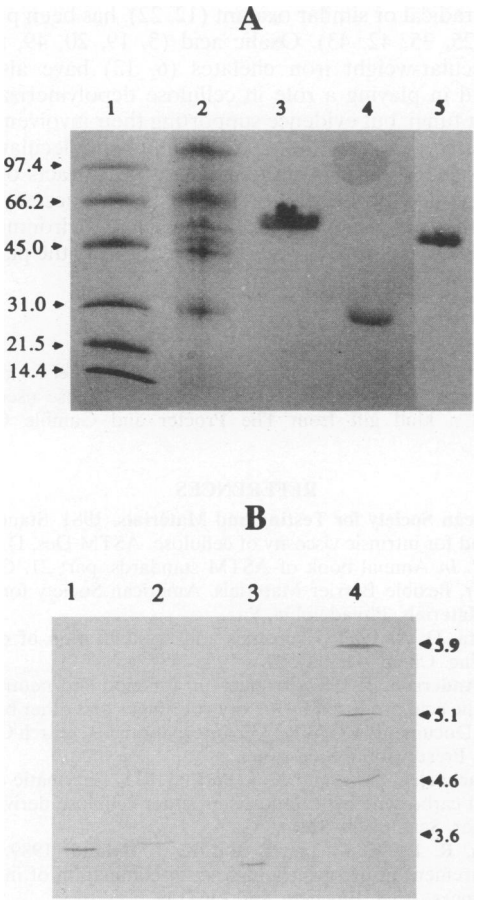

FIG. 4. SDS-PAGE and isoelectric focusing of cellulases. (A) SDS-PAGE of cellulases on a 10 to 15% gradient gel. Lanes: 1, molecular weight standards; 2, crude extract; 3, Cel 57; 4, Cel 25; 5, Cel 49. Numbers next to arrows indicate molecular weights of standards in kilodaltons. (B) Isoelectric focusing of cellulases on a pH 3 to 6 gel. Lanes: 1, Cel 25; 2, Cel 49; 3, Cel 57; 4, pI standards. Numbers next to arrows indicate the pIs of the standards. All proteins were stained with Coomassie blue.

cleaved through the cellulose microfibrils at the amorphous sites. This is the first report of in vitro cellulose depolymerization to the leveling-off DP by crude extracts of brown-rotted material. The previous minimum reported DP, obtained by incubating cotton cellulose with extracts of degraded wood, was 1,100 (26). We and others (54) have found that cellulose degradation by enzyme preparations is greatly enhanced by stirring the reaction mixtures. Stirring might mechanically increase the surface area of the cellulose microfibrils, creating new accessible sites for the cellulases. Depolymerization of cotton cellulose to the minimum DP (obtained by acid and enzymatic hydrolysis) by crude extracts seen here mimicked in vivo cellulose depolymerization by the brown rot fungi *P. placenta* (36) and *M. incrassata* (37) in soil block cultures.

Two of the three enzymes isolated here, Cel 25 and Cel 49, were also active on CMC and were obtained in near homogeneity in a two-step purification scheme with final yields of 3 and 18%. These yields are not necessarily due to inefficiency of the methods of purification. They may instead reflect the presence of additional CMCases in the crude extract. Such interfering activities may also account for the apparent decrease in the calculated specific activity of Cel 49 after the MonoQ anion-exchange chromatography step. The third isolated cellulase, Cel 57, is unique in that it depolymerized and degraded cotton cellulose but did not release reducing sugars from CMC nor did it significantly reduce the viscosity of CMC solutions. CMC might not be a substrate for Cel 57 because the substituted carboxymethyl groups sterically hinder its binding to the cellulose; the extent of enzymatic hydrolysis of CMC decreases as the degree of carboxymethyl substitutions increases (4, 16, 41). To our knowledge, this is the first report of a cellulase that depolymerizes and solubilizes cotton cellulose by cleaving the internal glycosidic bonds and yet does not degrade the water-soluble derivative CMC.

The isolated *M. incrassata* cellulases are most likely β-1,4-endoglucanases because they cleave the internal bonds of the cellulose molecules. In addition, isolated cellobiohydrolases, such as CBHI and CBHII of *T. reesei* (40) and Cex (38) and CbhA (39) of *Cellulomonas fimi*, do not affect the molecular size distribution of cotton nor do they release significant amounts of reducing sugars from cellulose. Like other brown rot endoglucanases (23, 29, 30), the cellulases isolated here are acidic, exhibiting combined optimum activity in the pH 3 to 4 range. It seems reasonable that these enzymes should be active at low pHs, because brown rot fungi secrete significant amounts of organic acids (53) that lower the pH of their environments.

Our data suggest that the isolated endoglucanases of *M. incrassata* cleaved within the amorphous regions of the cotton cellulose microfibrils, as evidenced by peak shifts to lower DP positions in the size-exclusion chromatograms, accompanied by relatively little solubilization of the cellulose. Cel 25 was the most active depolymerizer; it reduced the DP_w of cotton cellulose to 328, near the leveling-off DP, while solubilizing approximately 20% of the cellulose. This suggests that Cel 25 cleaved completely through the cellulose microfibrils at the amorphous sites, presumably leaving behind a relatively crystalline cellulose residue. We observed a similar mode of attack with endoglucanase CenA of the bacterium *C. fimi* (38) and endoglucanase EGI of the filamentous fungus *T. reesei* (40). In those studies, CenA and EGI solubilized approximately 30% of the cellulose in 8 days and reduced the DP_w of cotton cellulose to 150 and 212, respectively. The molecular size distribution of cotton cellulose attacked by CenA or EGI was

TABLE 1. Purification of endoglucanase Cel 25 and Cel 49 from *M. incrassata*[a]

Purification step	Protein (mg)		Total activity (U)[b]		Sp act (U/mg)		Yield (%)	
	Cel 25	Cel 49	Cel 25	Cel 49	Cel 25	Cel 49	Cel 25	Cel 49
MonoQ	1.5	1.4	251	64	167	46	21	5
Phenyl-Superose	0.59	0.16	220	32	376	201	18	3

[a] The concentrated crude extract (before purification) contained 23.8 mg of protein and had a total activity of 1,200 U, a specific activity of 50 U/mg, and a 100% yield.

[b] One unit of activity is defined as the amount of enzyme required to release 1 μmol of reducing sugar from CMC per min.

TABLE 2. *M. incrassata* cellulase activities on cotton cellulose and CMC[a]

Cellulase	Activities on:					
	Cotton cellulose				CMC	
	Wt loss (%)	DP_w	DP_n	Polydispersity (DP_w/DP_n)	CMCase (U/mg)[b]	Viscosity (U/mg)[c]
None	0	3,438	656	5.2	0	0
Cel 25	20	328	70	4.7	376	2,201
Cel 49	9	814	94	8.7	201	1,313
Cel 57	11	534	71	7.5	0	51

[a] Activities of cotton cellulose were determined by analyzing the molecular size distributions of the cellulose after incubation with the individual cellulases as described in the legend to Fig. 5.

[b] One unit of activity is defined as the amount of enzyme required to release 1 μmol of reducing sugars as glucose per min.

[c] One unit of activity is defined as the amount of enzyme required to change the inverse specific viscosity by 0.01/min.

slightly different from that of cellulose attacked by Cel 25 in that, in addition to the major peak centered over a DP of ≅200, a second smaller peak emerged at a DP of ≅11.

Cel 49 and Cel 57 also appeared to attack the more-accessible, amorphous regions of the cellulose microfibril as suggested by reduced DP_ws (814 and 534) at low weight losses (about 10%). From these results, in addition to the appearance of a smaller second peak (DP, ≅20) in the chromatograms, we speculate that these enzymes also utilize a mechanism of cotton cellulose degradation similar to that of CenA and EGI. This is in contrast to the pattern seen when cotton cellulose is attacked by endoglucanases CenB and CenD of *C. fimi*, in which the DP_ws (1,280 and 950, respectively) remained relatively high even though more than 20% of the cellulose was solubilized (38).

The role of the endoglucanases of brown rot fungi in the extensive and rapid depolymerization of cellulose in wood is not yet clear. Flournoy et al. (15) and Srebotnik et al. (51) have shown that the pores in sound wood cell walls are probably too small for enzymes (of the size described here) to penetrate and are not enlarged significantly early in the fungal attack. This enigma was realized early on by Cowling and Brown (10). As a result, an oxidative mechanism of cellulose depolymerization, involving Fenton's reagent (Fe^{2+} + H_2O_2), which generates hydroxyl radical or similar oxidant (12, 22), has been proposed (10, 21, 25, 35, 42, 43). Oxalic acid (3, 19, 20, 49, 50) and low-molecular-weight iron chelates (6, 13) have also been implicated in playing a role in cellulose depolymerization by brown rot fungi, but evidence supporting their involvement has been limited. Our attempts to find a low-molecular-weight oxidative cellulose-depolymerizing agent in extracts of cotton being degraded by brown rot fungi were unsuccessful. It is possible that brown rot fungi create a microenvironment that transiently opens up the wood cell wall, allowing the passage of the endoglucanases.

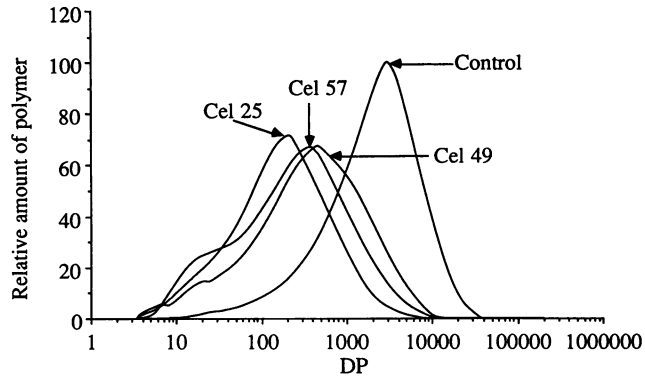

FIG. 5. Molecular SEC of cotton cellulose incubated with purified *M. incrassata* endoglucanses. One milligram of cotton cellulose was incubated with 10 nmol of Cel 25, 5 nmol of Cel 49, or 20 nmol of Cel 57 at 27°C for 7 days. Areas under the curves have been corrected to reflect weight losses. See Materials and Methods for details.

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