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Comparison of a Fungal (Family I) and Bacterial (Family II) Cellulose-Binding Domain

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A family II cellulose-binding domain (CBD) of an exoglucanase/xylanase (Cex) from the bacterium *Cellulomonas fimi* was replaced with the family I CBD of cellobiohydrolase I (CbhI) from the fungus *Trichoderma reesei*. Expression of the hybrid gene in *Escherichia coli* yielded up to 50 mg of the hybrid protein, $CexCBD_{CbhI}$, per liter of culture supernatant. The hybrid was purified to homogeneity by affinity chromatography on cellulose. The relative association constants (K_r) for the binding of Cex, $CexCBD_{CbhI}$, the catalytic domain of Cex (p33), and CbhI to bacterial microcrystalline cellulose (BMCC) were 14.9, 7.8, 0.8, and 10.6 liters g^{-1}, respectively. Cex and $CexCBD_{CbhI}$ had similar substrate specificities and similar activities on crystalline and amorphous cellulose. Both released predominantly cellobiose and cellotriose from amorphous cellulose. $Cex-CBD_{CbhI}$ was two to three times less active than Cex on BMCC, but significantly more active than Cex on soluble cellulose and on xylan. Unlike Cex, the hybrid protein neither bound to α-chitin nor released small particles from dewaxed cotton fibers.

Efficient enzymatic degradation of insoluble polysaccharides, such as cellulose, raw starch, and chitin, often requires a tight interaction between the enzymes and their substrates. Many polysaccharidases comprise multiple domains. Typically, bacterial and fungal cellulases consist of at least a cellulose-binding domain (CBD) and a catalytic domain, often separated by a linker rich in proline and hydroxyamino acids or in glycine (20). Additional domains are sometimes present, especially in bacterial cellulases (5, 59). The CBDs endow the proteins with a high affinity for native, ordered cellulose, with hydrolysis of both soluble and insoluble substrates occurring in the catalytic domain. Although the two individual domains can act independently, hydrolysis of insoluble cellulose is greatly enhanced by the presence of the CBD (24, 58), and the nature of the CBD can significantly influence the activity of a given cellulase on various forms and allomorphs of cellulose (10).

Catalytic domains and CBDs are grouped into different families based on sequence similarities (5, 11, 20, 26). At present, there are 12 cellulase and 8 different CBD families (59). The exoglucanase/xylanase Cex from *Cellulomonas fimi* is composed of a family F catalytic domain and a C-terminal, 100-residue-long family II CBD. The cellobiohydrolase CbhI from *Trichoderma reesei* has a family C catalytic domain linked to a 36-residue-long, C-terminal family I CBD (11, 59). The structures of CBD_{CbhI} (30) and CBD_{Cex} (62a) determined by nuclear magnetic resonance are quite different, possibly reflecting functional differences. Both CBDs contain very few charged amino acids and have an unusually large number of conserved aromatic residues (20). Three tryptophans and three tyrosines lie on the surfaces of CBD_{Cex} (62a) and CBD_{CbhI} (30), respectively. These aromatic residues are involved in the interaction of the CBDs with the cellulose surface (9, 14, 43, 44).

This paper describes the construction of a hybrid protein comprising the catalytic domain of Cex and the CBD of CbhI, which has allowed a direct comparison of the properties of a bacterial and a fungal CBD and of their effects on the activity of the catalytic domain of Cex.

MATERIALS AND METHODS

Materials. Microcrystalline cellulose (Avicel PH101) was from FMC International, Little County Island, Cork, Ireland. Bacterial microcrystalline cellulose (BMCC) was prepared from cultures of *Acetobacter xylinum* ATCC 23769 as described before (21). Regenerated cellulose was obtained by phosphoric acid treatment of Avicel PH101 as reported previously (11). *p*-Nitrophenyl-β-D-cellobioside (pNPC), 4-methylumbelliferyl-β-D-cellobioside, cellulose CF1, and α-chitin (purified powder from crab shells) were from Sigma Chemical Company, St. Louis, Mo. Birchwood 4-*O*-methylglucuronoxylan (Roth 7500; molecular weight, ~25,000) was from Carl Roth KG, Karlsruhe, Germany.

Bacterial strains and plasmids. *Escherichia coli* JM101 [*supE thi-1* Δ(*lac-proAB*) (F′ *traD36 proAB lacI*^{q}ZΔM15)] or JM109 e14$^-$ [*mcrA recA1 endA1 gyrA1 thi-1 hsdR17* r_K^- m_K^+ *supE44 relA1* Δ(*lac-proAB*) (F′ *traD36 proAB lacI*^{q}ZΔM15)] (63) was used as the bacterial cloning host. *E. coli* R1360 and TB1 [F$^-$ *ara* Δ(*lac-proAB*) *rpsL* Str^r (φ80*dlac*Δ(*lacZ*)M15) *hsdR* r_K^- m_K^+] (4) were used as hosts for the production of recombinant proteins. Plasmids pTZ18U (36), pUC12, and pUC18 were used as cloning vectors. Plasmid pUC12-1.1*cex*, encoding full-size Cex, was described previously (38). Plasmid pUC18-$cbhI_{tail}$, containing sequence encoding the CBD and linker region of cellobiohydrolase I (CbhI) from *T. reesei*, was kindly provided by T. Teeri (Technical Research Centre of Finland [VTT], Espoo, Finland).

Media and growth conditions. Cultures were grown at 30°C in liquid tryptone-yeast extract-phosphate medium (TYP) (47) or on Luria broth (LB) agar, supplemented with ampicillin (200 μg/ml). Clones expressing Cex and $CexCBD_{CbhI}$ were screened for cellulase activity on plates containing 4-methylumbelliferyl-β-D-cellobioside (20 μg/ml).

General DNA procedures. Plasmid DNA preparation and electrophoresis of DNA fragments were performed by routine procedures (2, 47). Enzymatic treatments of DNA molecules were carried out as recommended by the manufacturers. DNA fragments were recovered after electrophoresis with the GeneClean kit (BIO/CAN Scientific Inc., Mississauga, Ontario, Canada). Bacteria were transformed by the $CaCl_2$ method (25) or by electroporation (2).

PCRs. Oligonucleotide primers were synthesized with an Applied Biosystems 380A automated DNA synthesizer and purified by polyacrylamide gel electrophoresis (PAGE) and reversed-phase chromatography on Sep-Pak columns (Millipore) (1). Primer sequences were designed to reduce mispriming and to minimize primer-dimer production (27). PCRs (100 μl total volume) were carried out in standard buffer (20 mM Tris-HCl [pH 8.3], 25 mM KCl, 0.05% Tween 20, 0.1% gelatin) with 2.0 ng of template DNA, 25 to 50 pmol (300 ng) of primers, 0.5 to 1.5 mM $MgCl_2$, 5 to 10% dimethyl sulfoxide, 0.2 mM (each) the four deoxynucleoside triphosphates, and 2 U of *Taq* DNA polymerase. Thirty successive cycles were performed as follows: denaturation at 96°C for 15 s, annealing at 55°C for 30 s, and primer extension at 72°C for 1.5 min.

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Construction of *cexCBD$_{cbhI}$* gene fusion. PCR was used to fuse the *cex* sequence encoding the catalytic domain with the *cbhI* fragment encoding the binding domain and linker (see Fig. 1). An *Eco*RI restriction site (boldface) was introduced at the 5′ end of *cex* with primer 1 (5′-GCACTA**GAATTC**CCACGT CACAGGGTGCACCCGGCA-3′), and a sequence corresponding to 21 nucleotides at the 5′ end of the *cbhI* PCR fragment (underlined) was added to the 3′ end of the *cex* fragment with oligonucleotide primer 2 (5′-AGGGTTGCCGC CGCTAGGGTTGCTCGCGCCGAAGGCCTCCATCAC-3′). A sequence corresponding to 24 bases at the 3′ end of the *cex* PCR fragment (underlined) was added to the 5′ end of the *cbhI* fragment with oligonucleotide primer 3 (5′-GT GATGGAGGCCTTCGGCGCGAGCAACCCTAGCGGCGGCAACCCT-3′), and a *Hin*dIII restriction site (boldface) was introduced at the 3′ end of *cbhI* with primer 4 (5′-CATGAC**AAGCTT**TCGCACGGAGCTTTACAGGCA-3′). The products of the primary reactions were then combined and fused together in another PCR, with the homologous regions acting as primers. Primers 1 and 4 were also included to allow amplification of the fusion product *cexCBD$_{cbhI}$*. The 1.3-kb fragment thus obtained was purified, digested with *Eco*RI and *Hin*dIII, and ligated into the *Eco*RI and *Hin*dIII sites in the polylinker of pTZ18U to generate pTXI.1. The 0.98-kb *Sty*I-*Xma*I PCR fragment from pTXI.1 was exchanged for the original fragment obtained after digestion of pUC12-1.1*cex* with *Sty*I and *Xma*I. The integrity of the insert encoding CexCBD$_{CbhI}$ in the final construct pDD1 was then verified by sequence analysis of the double-stranded DNA by the dideoxy chain termination method (48) with modified T7 DNA polymerase and [α-^{35}S]dATP (53).

Enzyme purification. Overnight cultures of *E. coli* TB1 or R1360 harboring pUC1.2-1.1*cex* were diluted 500-fold in TYP supplemented with 200 μg of ampicillin per ml and grown to A_{600} of 1.5 to 2.0. Cex production was induced by the addition of 0.1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG), and the cultures were incubated for a further 18 h at 30°C. The cells were harvested by centrifugation at 4°C and 13,000 × *g* for 5 min. The supernatant was set aside, and the cells were resuspended in sucrose buffer (20% sucrose in 30 mM Tris-HCl [pH 8.0], 1 mM EDTA). After incubation at room temperature for 10 min, the cells were collected by centrifugation at 4°C and 13,000 × *g* for 15 min, resuspended in 5 mM ice-cold $MgSO_4$, and incubated on ice for 10 min. The supernatant obtained after centrifugation at 4°C and 16,000 × *g* for 20 min contained the periplasmic proteins. The clarified culture supernatant and the periplasmic fraction were pooled, and the proteins were precipitated with ammonium sulfate (75%, wt/vol) at 4°C overnight. The pellet obtained after centrifugation (16,000 × *g*, 4°C, 30 min) was dissolved in 50 mM potassium phosphate buffer, pH 7.0. The solution was passed through a cellulose CF1 column. The column was washed with 1 M NaCl–50 mM potassium phosphate buffer, pH 7.0, and adsorbed proteins were desorbed with distilled water. Alternatively, proteins were adsorbed on Avicel and eluted with 6 M guanidinium hydrochloride to elute adsorbed proteins, as previously described (24, 42). Desorbed protein was further purified by size exclusion chromatography on Superose 12 (Pharmacia) with 100 mM potassium phosphate, pH 7.0, containing 150 mM NaCl.

Overnight cultures of *E. coli* R1360 harboring pDD1 were diluted 1,000-fold in TYP containing 200 μg of ampicillin per ml and grown for 24 h at 30°C. CexCBD$_{CbhI}$ was recovered and purified as described above for Cex.

The p33 fragment was obtained by limited proteolysis of Cex with partially purified *C. fimi* protease as described before (24). p33 was purified to homogeneity by size exclusion chromatography in 50 mM potassium phosphate buffer, pH 7.0, on Superose 12 (Pharmacia) or by anion-exchange chromatography on an EconoQ cartridge (Bio-Rad) with a linear gradient (60 ml total) of 0 to 300 mM NaCl in 10 mM potassium phosphate buffer, pH 6.0 or 7.0. CBD$_{Cex}$ was obtained and purified by cellulose affinity chromatography on CF1 as described previously (39). Cellobiohydrolase I (CbhI) was purified from the culture supernatant of *T. reesei* (Novoclast 1.5L; a gift from Novo, Denmark) by affinity chromatography on *p*-aminobenzyl-1-thio-β-D-cellobioside as the ligand linked to Sepharose 4B (57) and ion-exchange chromatography on DEAE-Sepharose CL6B (6).

Protein concentration determination. The concentrations of purified proteins were estimated by UV absorbance (49). The estimated extinction coefficient (280 nm, 1 mg ml^{-1}, 1 cm) for CexCBD$_{CbhI}$ (1.34) was in good agreement with that predicted (8) from the tryptophan and tyrosine content of the protein (1.43). Coefficients for Cex (1.61) (21) and CBD$_{Cex}$ (2.31) (39) and the molar extinction coefficient for CbhI (73,000 M^{-1} cm^{-1}) (58) were reported previously.

Enzyme assays. Cellulase activities on Avicel PH101, BMCC, and phosphoric acid swollen cellulose were assayed at pH 6.0 and 37°C by measuring the liberated reducing sugars, as D-glucose equivalents, with the *p*-hydroxybenzoic acid hydrazine reagent (34). Specific activities were expressed as micromoles of reducing sugar released per minute per micromole of enzyme. Release of reducing sugars (D-xylose equivalents) from Roth xylan at pH 6.0 and 50°C was measured by the dinitrosalicylic acid assay (37).

For Avicelase activities, enzyme (final concentration, 0.17 nmol/ml) was incubated for 18 to 24 h at 37°C with 15 mg of substrate in 1.5 ml of 50 mM sodium citrate buffer, pH 6.0, containing 0.02% bovine serum albumin (BSA) (CB buffer). For hydrolysis of BMCC (18 h, 37°C), incubation mixtures contained 1 nmol of enzyme and 2 mg of substrate in 1.5 ml of CB buffer. Activity on regenerated cellulose was assayed after incubation (2 h) of 20 pmol of enzyme with 10 mg of substrate in 2 ml of CB buffer. Samples (250 μl) were withdrawn and centrifuged. From 20 to 100 μl of the supernatant was diluted in 50 mM sodium citrate buffer, pH 6.0 (500 μl, final), and reducing sugars were measured (420 nm) after addition of 1 ml of *p*-hydroxybenzoic acid hydrazine reagent.

Activities on birchwood xylan were determined after incubating (5 min at 37°C) 50 μl of enzyme (0.25 to 0.35 μM) with 450 μl of solubilized Roth xylan (0.1 to 1.0%, wt/vol) in 50 mM potassium phosphate buffer, pH 6.0, containing 0.02% BSA. The reducing sugars released were measured (540 nm) after dilution of the incubation mixtures in 750 μl of dinitrosalicyclic acid and boiling for 5 min. Specific activities were expressed as units per micromole of enzyme, with 1 U being defined as 1 μmol of reducing sugar (D-xylose equivalent) released per min (3).

Release of *p*-nitrophenol upon hydrolysis of pNPC (pH 7.0, 37°C) was monitored continuously at 400 nm. Samples contained 15 μM enzyme and 0.03 to 6.3 mM pNPC in 50 mM potassium phosphate buffer, pH 7.0. The molar extinction coefficient for *p*-nitrophenol, determined from a standard curve, was 9,860 M^{-1} cm^{-1} at pH 7.0 and 37°C. Specific activities were expressed as micromoles of phenol released per minute per micromole of enzyme. Kinetic parameters were derived from a direct fit to the Michaelis-Menten model.

The soluble sugars released from phosphoric acid-swollen cellulose by Cex, CexCBD$_{CbhI}$, and CbhI were analyzed by high-performance liquid chromatography (HPLC) on a Shimadzu system equipped with an SIL6B autoinjector, an RID6A refractive index detector, and a CR501 Chromatopac integrator/plotter. Cellodextrins were separated on a Dextro-Pak column (8 by 100 mm; 4-μm particle size) (Waters) equipped with a resolve C_{18} Guard-Pak column. Water was used as the mobile phase at a flow rate of 1 ml min^{-1}. Reaction mixtures contained 1 μM enzyme and 10 mg of phosphoric acid-swollen Avicel in 1 ml (5 mM) of potassium phosphate buffer, pH 7.0. Samples were withdrawn after 0, 12, and 24 h of incubation at 37°C; cellulose was removed by centrifugation, and the supernatant was boiled for 5 min to inactivate enzyme prior to HPLC analysis.

Small-particle release from dewaxed cotton was determined by light scattering at 600 nm during incubation (37°C for up to 24 h) of 10 μM enzyme with 10 mg of cotton in 2 ml (50 mM) of potassium phosphate buffer, pH 7.0, as described before (15).

Adsorption assays. For quantitative analysis, enzymes (0.2 to 30 μM) were mixed (4°C) end over end with 1 mg of BMCC in a final volume of 1 ml of buffer (50 mM potassium phosphate, pH 7.0). After 1 h, cellulose was removed by centrifugation (13,000 × *g*, 4°C), and unbound enzyme left in the supernatant was measured (280 nm) and used to calculate the amount of enzyme bound to the BMCC. All experiments were done in triplicate. Relative equilibrium constants (K_r) and binding parameters were obtained as described previously (21).

Qualitative adsorption experiments were performed in 1-ml syringes packed with 10 to 50 mg of glycan. The microcolumns were equilibrated in potassium phosphate buffer (50 mM, pH 7.0), and samples were added and allowed to flow freely through the columns. Either culture supernatant (3 to 5 ml) or purified protein (2 to 10 μg) was used. Non-specifically bound protein was removed by five washes with 1 ml of high-salt buffer (50 mM phosphate buffer [pH 7.0], 1 M NaCl), each followed by five washes with 1 ml of low-salt buffer (50 mM phosphate buffer [pH 7.0]). Sodium dodecyl sulfate (SDS) loading buffer (40 μl) was added to the glycans, either immediately or after an additional five washes with 1 ml of distilled water each. After the suspension was boiled for 5 min, 20-μl fractions of supernatant were analyzed by SDS–12.5% PAGE (33).

RESULTS

Construction of the gene fusion and production of the hybrid protein. A gene fusion encoding a protein in which the 128 C-terminal amino acids, comprising the proly-threonyl linker and CBD of Cex (61), were replaced with the 67 amino acids comprising the linker and CBD (block BA) of CbhI (29, 58) (see Fig. 2A) was constructed by PCR (Fig. 1). Three PCRs, two for amplification of the individual fusion partners and one to amplify the final product, were used to construct the gene fragment encoding CexCBD$_{CbhI}$ (Fig. 1). Approximately one mutation, most often a G-to-A substitution, was introduced in every 80 bp during synthesis of *cexCBD$_{cbhI}$* with *Taq* polymerase. Although conditions were chosen to reduce the misincorporation of base pairs (27), the mutation rate was still 2.1×10^{-4} nucleotides per cycle. This is comparable to reported rates of 1.1×10^{-4} to 1.7×10^{-4} (46, 54) but higher than the average expected mutation rate of less than 5×10^{-6} errors per cycle for the conditions used (18). The low fidelity of nucleotide incorporation by *Taq* polymerase is probably the result of the relatively large number of cycles used in the PCR and the high G+C content (73%) of *C. fimi* DNA. Problems with the high mutation rate were easily solved by replacing the majority of the PCR fragment with the wild-type sequence by using two internal restriction sites, *Sty*I and *Xma*I (Fig. 1). Only

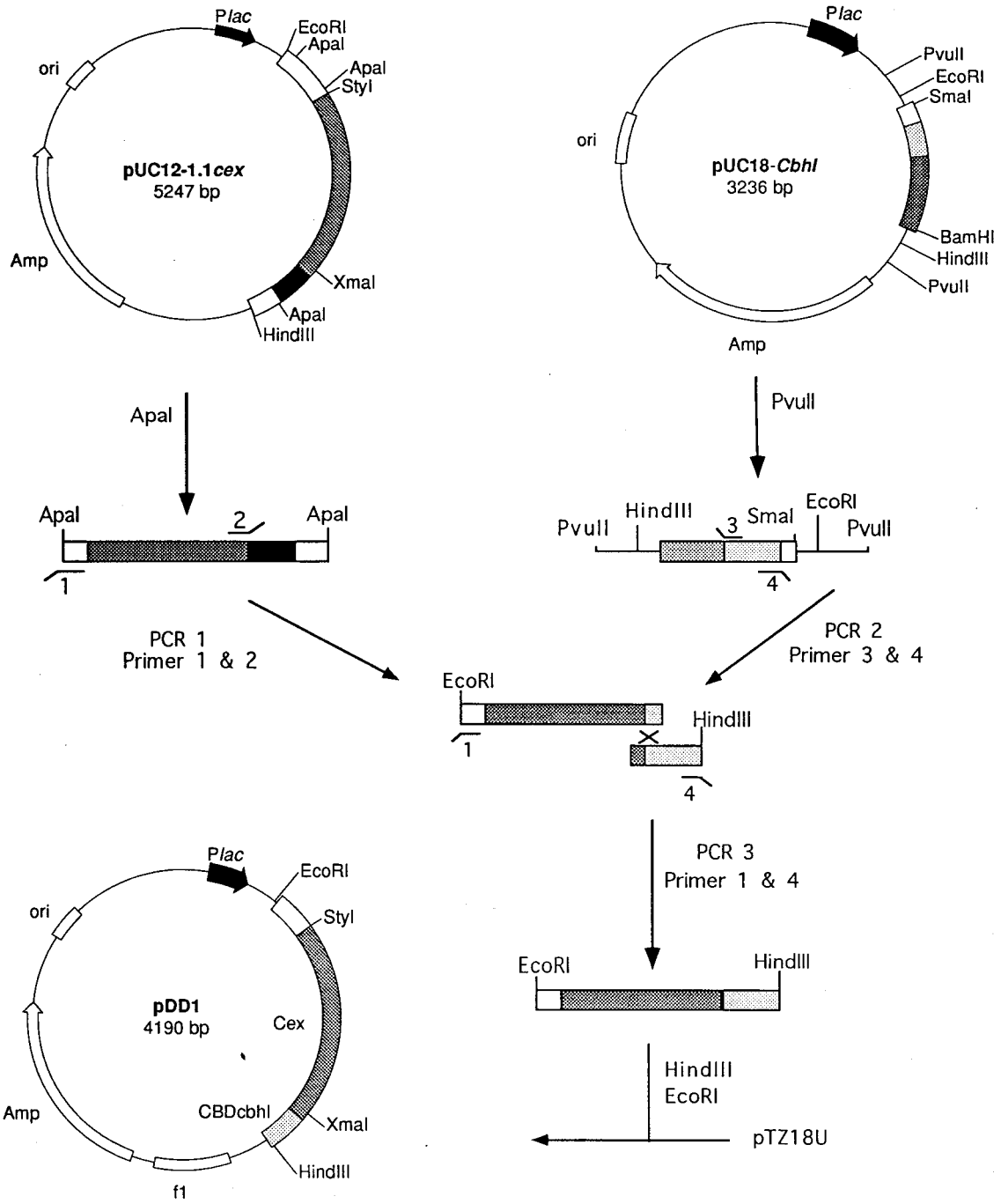
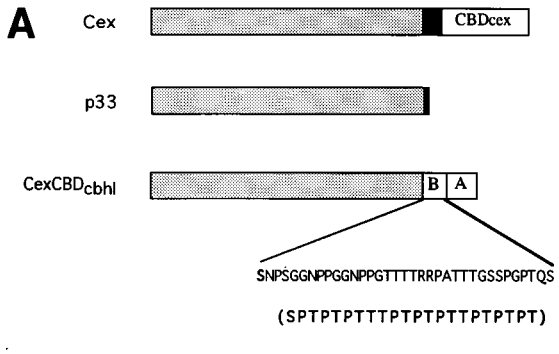


FIG. 1. Construction of the pDD1 expression vector. The gene fusion between the fragment encoding the Cex catalytic domain and the fragment encoding CBD_{CbhI} was accomplished by three separate PCRs as described in Materials and Methods. The *Eco*RI and *Hin*dIII restriction sites introduced at the 5′ and 3′ ends, respectively, of the final PCR product were used to clone $cexCBD_{cbhI}$ into pTZ18U. The majority of the PCR fragment was then replaced with the wild-type sequence by using two internal restriction sites, *Sty*I and *Xma*I, to generate pDD1.

one silent mutation (GCC to GCT) in a codon for alanine was found in the final $cexCBD_{cbhI}$ sequence of pDD1. However, an error was noticed in the DNA sequence encoding the AB region (CBD and linker) of CbhI. The published nucleotide sequence CGT (50) was found to be CCG CCT. This changes the Arg (amino acid residue 443) in the linker (block B) to ProPro, which agrees with the amino acid sequencing of CbhI (16). This error was not the result of a mutation generated during the PCR, since the same CCG CCT sequence was identified by resequencing the $cbhI_{tail}$ fragment in pUC18-$cbhI_{tail}$.

The junction between the bacterial and fungal domains was formed by Ser-315 at the end of the Cex catalytic domain and Asn-432 at the beginning of the B region of CbhI (58) (Fig. 2A). The DNA fragment encoding the hybrid protein was cloned in pDD1 adjacent to the *lac* promoter (Fig. 1). The Cex



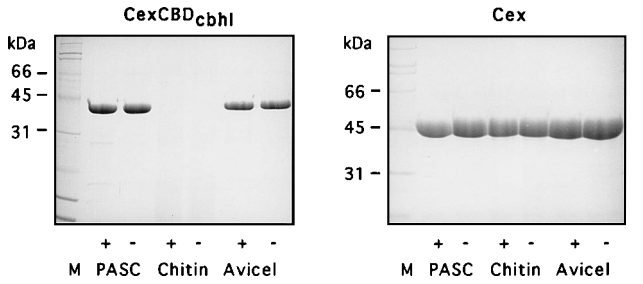


FIG. 3. SDS-PAGE analysis of the binding of $CexCBD_{CbhI}$ and Cex on various β-1,4-glucans. Binding of enzyme (2 to 10 mg) onto phosphoric acid-swollen cellulose (PASC, 10 mg), α-chitin (50 mg), or Avicel (10 mg) was analyzed on gels containing 12.5% acrylamide, before (−) and after (+) a washing step with water, as described in Materials and Methods. Molecular mass standards were run in lane M.

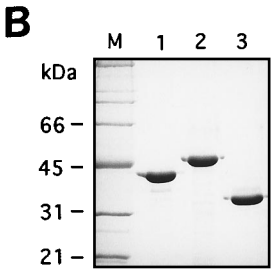


FIG. 2. (A) Schematic representation of the domain structures of Cex, p33, and $CexCBD_{CbhI}$. The catalytic domain of Cex (family F) is represented by the shaded area. A and B denote the CBD and linker, respectively, of CbhI from *T. reesei*. The amino acid sequence of the linker, starting at the boundary between it and the Cex catalytic domain (Ser-315), is shown under B. The original *C. fimi* linker in Cex (solid box) is shown in parentheses for comparison. (B) SDS-PAGE analysis of the various polypeptides after affinity chromatography on cellulose CF1. A 10-μg amount of purified $CexCBD_{CbhI}$ (lane 1), Cex (lane 2), or p33 (lane 3) was analyzed on gels containing 12.5% acrylamide. Molecular mass standards were run in lane M; sizes are shown to the left.

leader peptide was included to direct translocation of the hybrid to the periplasm. After growth of *E. coli* R1360 harboring pDD1 for 18 to 24 h at 30°C without IPTG, the culture supernatant contained 30 to 50 mg of the 41-kDa hybrid protein per liter, together with a degradation product of 33 kDa (p33).

Purification of $CexCBD_{CbhI}$. Cex and $CexCBD_{CbhI}$ were purified to homogeneity from the periplasm and culture supernatant of *E. coli*, respectively, by affinity chromatography on either Avicel or CF1 cellulose. $CexCBD_{CbhI}$ was desorbed from CF1 with water, as previously demonstrated for Cex (24, 42). In water, $CexCBD_{CbhI}$ was rapidly degraded to a 33-kDa fragment, presumably by contaminating proteases. This degradation product was similar to that found in the culture supernatant. It did not adsorb to cellulose but was active on xylan and pNPC. The Cex catalytic domain of 33 kDa was also released by in vitro proteolysis of $CexCBD_{CbhI}$ or Cex with *C. fimi* protease (24) (Fig. 2B, lane 4). Degradation was greatly reduced by adsorption to Avicel and desorption with 6 M guanidinium hydrochloride. Proteins obtained after refolding were indistinguishable from those purified by chromatography on CF1 cellulose. Soluble sugars contaminating the purified enzyme were removed by size exclusion chromatography. Typical yields were 15 to 20 and 35 to 40 mg of purified Cex and $CexCBD_{CbhI}$, respectively, per liter of culture.

Adsorption of $CexCBD_{CbhI}$, Cex, and p33 to insoluble glycans. Cex and $CexCBD_{CbhI}$ bound strongly to Avicel, phosphoric acid-swollen cellulose, and BMCC and could be desorbed only by denaturation with guanidinium hydrochloride or SDS (Fig. 3). Cex but not $CexCBD_{CbhI}$ also bound tightly to α-chitin, a polymer of β-1,4-linked *N*-acetylglucosamine residues. Repeated washes with low-salt buffer, high-salt buffer (1 M NaCl), or water did not desorb the protein from the chitin (Fig. 3). Cex and $CexCBD_{CbhI}$ did not adsorb to insoluble xylan (birchwood, oat spelt, or larchwood xylan), pachyman (insoluble polymer of β 1,3-linked glucopyranosides), or laminarin (results not shown). Purified p33 did not bind to any significant extent to any of these polysaccharides.

Adsorption of Cex, $CexCBD_{CbhI}$, CbhI, and p33 to BMCC. Binding equilibrium was reached almost instantly at lower enzyme concentrations and within 5 min at higher enzyme-to-BMCC ratios (Fig. 4). There was neither increased adsorption nor net desorption of any of the polypeptides during incubation for a further 5 h. Hydrolysis of BMCC, as determined by the release of total sugar, was not detected with the highest concentration of Cex, $CexCBD_{CbhI}$, or CbhI after 2 h of incubation at 4°C.

Relative association constants (K_r), defined as the affinity constant K_a times the total number of initial adsorption sites (N_0), were estimated by using a model of overlapping potential binding sites on the cellulose surface, comprising multiple repeating units of cellobiose residues (21). K_r values were obtained from the initial slopes of $1/[B]$ versus $1/[F]$ plots, where B is the bound protein and F is the concentration of free protein (21). Estimates for the equilibrium association constant (K_a) and the number of lattice sites occupied by a single ligand (a) were calculated by using an estimated value for N_0 of 101 μmol of lattice residues per g of BMCC (21).

The K_r for CBD_{Cex} was about 30% higher than that for Cex, but single molecules of CBD_{Cex} or Cex both occupied approximately the same number of 110 face lattice residues (a = 23 to 25 mol mol^{-1}) (Table 1). The K_r for $CexCBD_{CbhI}$, on the other hand, was about one half that for Cex, and $CexCBD_{CbhI}$ occupied twice as many lattice residues as Cex (Table 1).

Influence of the CBD type on the hydrolytic activity of the Cex catalytic domain. Although low, the activity of Cex on Avicel and phosphoric acid-swollen cellulose was not significantly influenced by replacing CBD_{Cex} with CBD_{CbhI} (Table 1). Furthermore, both Cex and the hybrid enzyme released predominantly cellobiose and cellotriose upon extended incubation with amorphous cellulose (Fig. 5). By contrast, the specific activity of $CexCBD_{CbhI}$ on highly crystalline BMCC was almost 2.5-fold lower than that of Cex. The Cex catalytic domain alone did not hydrolyze this substrate. Hydrolysis of soluble substrates, such as pNPC and birchwood xylan, was mark-

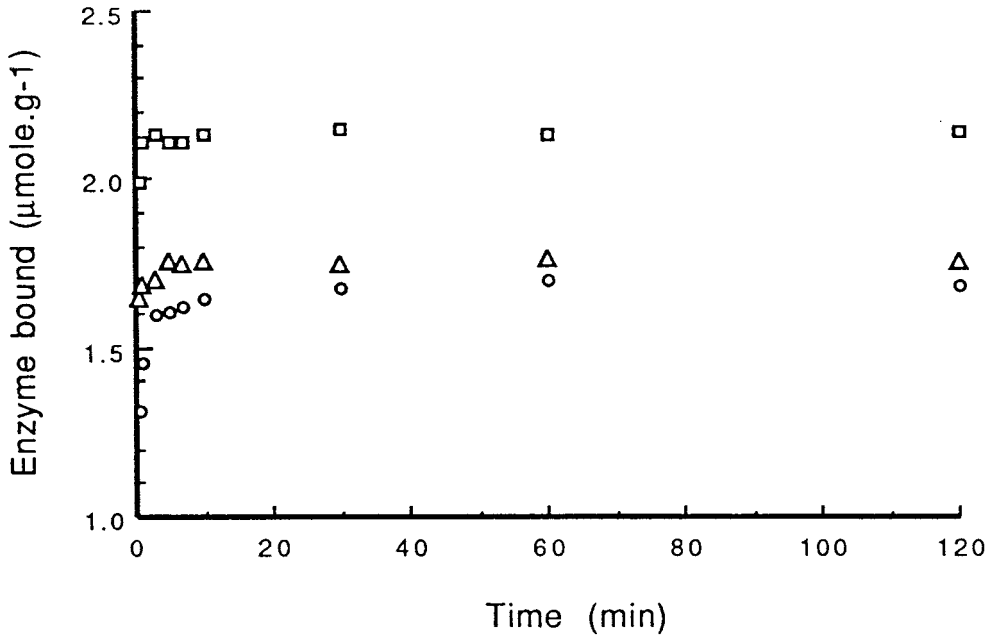


FIG. 4. Kinetics of adsorption of the various proteins to BMCC. Adsorption of Cex ( ), CexCBD$_{CbhI}$ (△), and CbhI ( ) was monitored as described in Materials and Methods.

edly increased in the absence of CBD$_{Cex}$ and when the bacterial CBD was replaced by its fungal counterpart (Tables 2 and 3). This increase was the consequence of an increased k_{cat}, because the K_m of CexCBD$_{CbhI}$ for pNPC was only slightly increased (Table 3). CexCBD$_{CbhI}$ and p33 were 30 and 16% more active on xylan, respectively, than Cex (Table 2).

Both Cex and CBD$_{Cex}$, but neither CexCBD$_{CbhI}$ nor p33, released small particles from cotton fibers (Fig. 6).

DISCUSSION

CbhI from *T. reesei* and Cex from *C. fimi* are both modular proteins consisting of N-terminal catalytic domains and C-terminal CBDs separated by a short linker (24, 60). The role of the CBDs in the activities of the enzymes is not entirely understood, but one function is to target the enzyme to its substrate, thereby increasing the local concentration on the cellulose surface (29). Nevertheless, the two domains retain their functions when separated by proteolytic cleavage or by genetic manipulation. Isolated CBD$_{Cex}$ can be produced easily by expression of the encoding DNA fragment in *E. coli* (39); this is not the case for CBD$_{CbhI}$, which has thus far been obtained only as a fusion protein in this host organism. Alternatively, the individual CBD$_{CbhI}$ can be obtained in vitro by peptide synthesis (30) or by limited proteolysis of CbhI (60). Our previous work with CBD fusion proteins has demonstrated that the functional properties of the CBD in the hybrid proteins are very similar to those of free CBD (21). In this study, fusion of CBD$_{Cex}$ or CBD$_{CbhI}$ to the catalytic domain of Cex allowed us to conveniently produce the polypeptides, to compare their binding properties, and to examine possible effects of the CBD on the catalytic activity.

There are significant differences in the properties of the family I CBD from CbhI and the family II CBD from Cex. The binding affinity of Cex for BMCC is approximately twice that of the hybrid protein CexCBD$_{CbhI}$ (Table 1). Since the catalytic domain of Cex has negligible affinity for cellulose, it is reasonable to conclude that the affinities of the linked domains directly reflect the affinities of the CBDs.

CbhI has a slightly higher affinity than CexCBD$_{CbhI}$ for BMCC. The catalytic domain of CbhI (core I) might contribute to the binding interaction. Indeed, core I has a significant residual affinity for phosphoric acid-swollen cellulose and Avicel (56, 58); however, under the binding conditions used in this study, the affinity of core I for BMCC was too low to accurately estimate K_r. Interaction between glycan moieties in the glycosylated linker of native CbhI (7, 60) and cellulose might also increase the affinity of the enzyme for cellulose, as it does for glycosylated Cex (40). The linker in CexCBD$_{CbhI}$ is not glycosylated. Whether glycosylation and/or core I binding is responsible for the enhanced binding of CbhI remains to be established.

There is believed to be a correlation between the affinity of a cellulase for cellulose and its ability to hydrolyze this substrate (28). Our results support this hypothesis for the hydrolysis of crystalline cellulose. Thus, p33, with very little affinity for BMCC, is incapable of hydrolyzing it. Cex, on the other

TABLE 1. Adsorption parameters for binding of CexCBD$_{CbhI}$, Cex, CbhI, CBD$_{Cex}$, and p33 to BMCC[a]

Enzyme	K_r (liters g^{-1})	a/N_0 (g μmol^{-1})	a (mol mol^{-1})	K_a (liters μmol^{-1})
CexCBD$_{CbhI}$	8.70 ± 0.60	0.40 ± 0.03	40.4 ± 2.8	0.086 ± 0.005
Cex	14.93 ± 0.67	0.23 ± 0.01	22.8 ± 1.3	0.148 ± 0.008
p33	0.77 ± 0.05	0.14 ± 0.01	13.8 ± 1.1	0.008 ± 0.001
CBD$_{Cex}$	21.74 ± 1.42	0.26 ± 0.02	25.3 ± 1.7	0.215 ± 0.017
CbhI	10.64 ± 0.91	0.38 ± 0.03	38.7 ± 2.3	0.105 ± 0.006

[a] The parameters for each polypeptide were derived from adsorption data plotted in double-reciprocal form as described in the text. The values for K_a and a were calculated by using N_0 = 101 μmol of lattice residues per g of cellulose, as described before (21). The values shown are means ± accumulated standard errors.

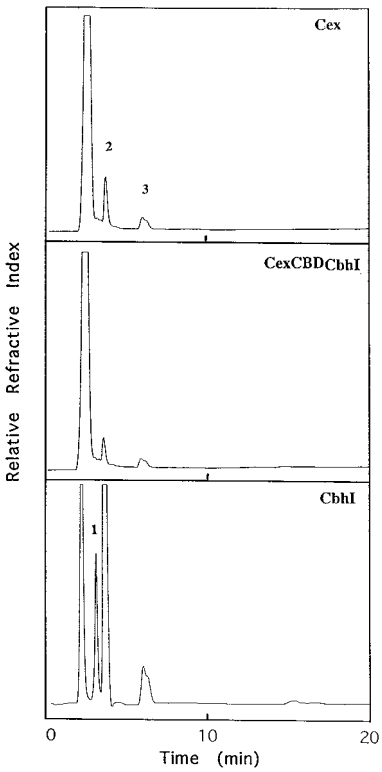


FIG. 5. HPLC analysis of the degradation products released upon hydrolysis of amorphous cellulose with Cex, $CexCBD_{CbhI}$, and CbhI. Soluble sugars released after incubation (37°C, 24 h) of phosphoric acid-swollen Avicel (10 mg/ml) with 1 μM enzyme were analyzed on a Waters Dextropak column as described in Materials and Methods. Peaks: 1, glucose; 2, cellobiose; 3, cellotriose.

hand, has a low but detectable activity on BMCC. There is a reasonably good correlation between the decrease in affinity (approximately 2-fold) and the decrease in activity (~2.5-fold) when CBD_{Cex} is replaced with CBD_{CbhI}. However, it is unlikely that there is a correlation between (CBD-mediated) binding of the cellulase and hydrolysis of phosphoric acid-swollen cellulose. Indeed, the presence of the CBD does not enhance hydrolysis of phosphoric acid-swollen cellulose by the catalytic domain of Cex (Table 2). Similarly, both CbhI and its isolated catalytic domain (core I) have the same specific activity on regenerated cellulose (58). The role of the binding domain thus appears to depend on the nature and accessibility of the cellulosic substrate, as previously demonstrated with the CBD of endoglucanase A (CenA) from *C. fimi* (10).

Both Cex and CBD_{Cex} release small particles from cotton

TABLE 2. Specific activities of $CexCBD_{CbhI}$, Cex, CbhI, and p33 on various soluble and insoluble substrates[a]

Enzyme	Sp act (μmol/min/μmol)				
	BMCC	Avicel	PASC	Xylan	pNPC
$CexCBD_{CbhI}$	0.005	0.10	25.3	4,058	426.4
Cex	0.012	0.11	26.9	3,086	243.4
p33	0.000	0.06	25.1	3,584	311.0
CbhI	4.250	1.37	11.5	ND[b]	ND

[a] Activities were determined as described in Materials and Methods. Specific activities are expressed as micromoles of reducing sugar released per minute per micromole of enzyme. PASC, phosphoric acid-swollen cellulose.
[b] ND, not determined.

TABLE 3. Kinetic parameters for the hydrolysis (37°C, pH 7.0) of pNPC by $CexCBD_{CbhI}$, Cex, and p33

Enzyme	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (M^{-1} min^{-1})
$CexCBD_{CbhI}$	662.6	482.2	7.28×10^5
Cex	603.1	266.1	4.41×10^5
p33	607.2	336.4	5.54×10^5

fibers. The CBDs of CenA and Cex also disrupt the surface of cotton and ramie cellulose fibers (12, 15) and prevent the flocculation of BMCC (22). These family II CBDs disperse the substrate in a nonhydrolytic fashion, presumably by breaking hydrogen bonds. Furthermore, CBD_{CenA} synergizes with the catalytic domain of CenA, either in the intact enzyme or in a mixture of the genetically or proteolytically separated domains, thereby enhancing the hydrolysis of crystalline cellulose (13). Fine-particle release, fiber dispersion, particle disruption, and synergy between CBDs and catalytic domains are likely all manifestations of the same (disruptive) properties of the family II CBDs. This defines an active role for the CBD in substrate utilization in addition to its targeting function. $CexCBD_{CbhI}$ does not release small particles from cotton, and an additional "active role" for the family I CBDs (29, 44) thus seems highly improbable. Perhaps the active penetration of substrate by fungal hyphae obviates the need for a disruptive CBD in fungal enzymes.

Cex but not $CexCBD_{CbhI}$ binds to α-chitin (Fig. 3). CBD_{CenA} also binds to α-chitin (39), and chitin binding may thus be a property common to all family II CBDs. Indeed, some chitinases contain family II CBDs which may function as chitin-binding domains (17, 45). The failure of the family I CBD of CbhI to bind to chitin suggests that the family I and family II CBDs recognize different structures on the cellulose surface.

As yet there is not an adequate model for packing of CBDs or CBD-containing enzymes on the cellulose surface. Both Cex and its isolated CBD on the one hand, and CbhI and Cex CBD_{CbhI} on the other, occupy the same number of ligand sites on BMCC (~25 and 40 mol of CBD per mol of lattice residues, respectively), suggesting that the CBD is the dominant partner in determining packing density. It is intriguing that the family II CBD with triple the number of amino acid residues occupies fewer sites than the smaller family I CBD, apparently allowing denser packing of the polypeptides on the cellulose surface. The overall dimensions of the CBD_{Cex} and CBD_{CbhI} structures determined by nuclear magnetic resonance are 45 by 25 by 25 Å (4.5 by 2.5 by 2.5 nm) and 30 by 18 by 10 Å (3.0 by 1.8 by 1.0 nm), respectively (30, 62a), so CBD_{Cex} should occupy a larger space than CBD_{CbhI}. Further interpretation of the differences requires knowledge of the contact areas of the CBD on the cellulose. Current evidence suggests that the exposed tryptophan residues on the surface of the family II CBDs and exposed tyrosine residues on the surface of family I CBDs are involved in the interaction of the CBD with cellulose (14, 44). Structural evidence for both CBD_{Cex} and CBD_{CbhI} indicates that this contact involves the whole face of the CBD containing these exposed aromatic residues. From this assumption, the footprints (shadows) of CBD_{Cex} and CBD_{CbhI} should be about 0.39 and 0.21 μm^2, respectively. These values are clearly inconsistent with the apparently higher packing density of CBD_{Cex}. The ability of CBD_{Cex} but not CBD_{CbhI} to disrupt cellulose fiber structure, thereby creating additional surface area for CBD binding, could explain this paradox.

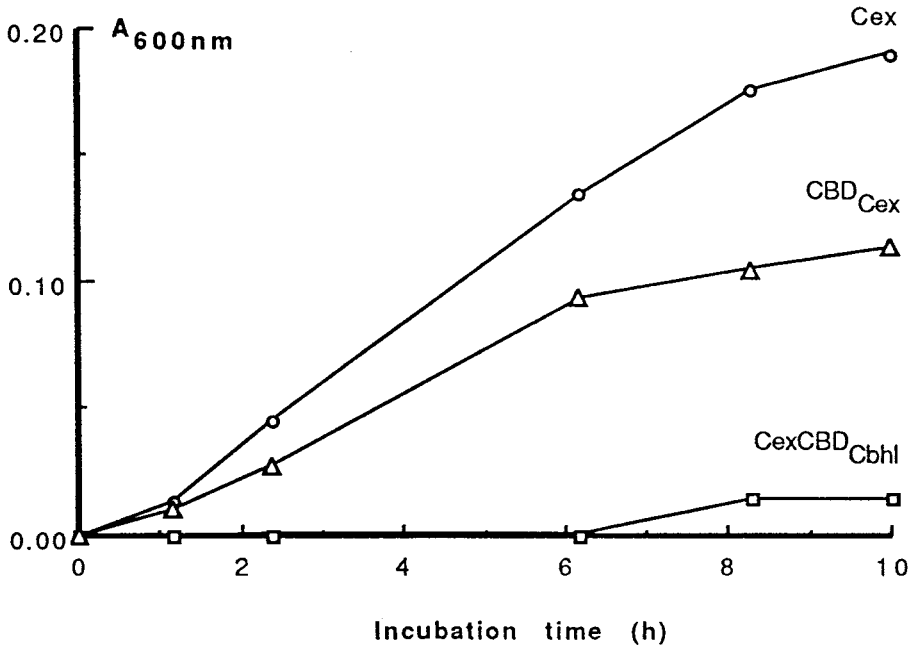


FIG. 6. Kinetics of small-particle release from dewaxed cotton upon incubation with Cex, $CexCBD_{CbhI}$, and CBD_{Cex}. Particle release was estimated from the A_{600} readings during incubation (37°C) of cotton (5 mg/ml) with 10 μM enzyme in 50 mM potassium phosphate buffer, pH 7.0.

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