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Characterization of CenC, an Enzyme from *Cellulomonas fimi* with Both Endo- and Exoglucanase Activities

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The *cenC* gene, encoding β-1,4-glucanase C (CenC) from *Cellulomonas fimi*, was overexpressed in *Escherichia coli* with a *tac*-based expression vector. The resulting polypeptide, with an apparent molecular mass of 130 kDa, was purified from the cell extracts by affinity chromatography on cellulose followed by anion-exchange chromatography. N-terminal sequence analysis showed the enzyme to be properly processed. Mature CenC was optimally active at pH 5.0 and 45°C. The enzyme was extremely active on soluble, fluorophoric, and chromophoric glycosides (4-methylumbelliferyl β-glycosides, 2′-chloro-4′-nitrophenyl-β-D-cellobioside, and 2′-chloro-4′-nitrophenyl-lactoside) and efficiently hydrolyzed carboxymethyl cellulose, barley β-glucan, lichenan, and, to a lesser extent, glucomannan. CenC also hydrolyzed acid-swollen cellulose, Avicel, and bacterial microcrystalline cellulose. However, degradation of the latter was slow compared with its degradation by CenB, another *C. fimi* cellulase belonging to the same enzyme family. CenC acted with inversion of configuration at the anomeric carbon, in accordance with its classification as a family 9 member. The enzyme released mainly cellobiose from soluble cellodextrins and insoluble cellulose. Attack appeared to be from the reducing chain ends. Analysis of carboxymethyl cellulose hydrolysis suggests that CenC is a semiprocessive enzyme with both endo- and exoglucanase activities.

Cellulose is an abundant natural polymer with a repeating unit of β-1,4-linked cellobiosyl residues. Plants and other organisms synthesize cellulose in the form of microfibrils consisting of both crystalline and noncrystalline regions (9). Cellulolytic organisms typically produce endo-β-1,4-glucanases (EC 3.2.1.4), cellobiohydrolases (EC 3.2.1.91), and β-glucosidases (EC 3.2.1.21) that interact synergistically to degrade cellulose microfibrils. It is thought that endoglucanases randomly attack bonds in noncrystalline regions on the microfibril surface to produce new chain ends for attack by exo-acting cellobiohydrolases. In turn, the erosion of the microfibril surface by cellobiohydrolases reveals further noncrystalline regions for endoglucanase attack. β-Glucosidases prevent the accumulation of cellobiose to inhibitory levels (50).

Although this general model is widely accepted, several aspects of cellulose digestion remain unexplained. For example, some studies indicate a substantial difference in the ability of individual endoglucanases to interact synergistically with cellobiohydrolases. This finding is difficult to explain if all endoglucanases attack noncrystalline regions in the same random fashion. There are other aspects of cellulose digestion that are not explained by the general model, and it is evident that a more complete understanding of the process requires further information about the action of individual enzymes and their interactions in whole systems (50).

Previous studies have demonstrated that the cellulase system of *Cellulomonas fimi*, a gram-positive saprophytic bacterium, includes four enzymes classified as endoglucanases (CenA, -B, -C and -D), two cellobiohydrolases (CbhA and CbhB), and one enzyme (Cex) with both β-1,4-glucanase and β-1,4-xylanase activity. All of these enzymes contain a distinct catalytic domain and at least one cellulose-binding domain (CBD); in some cases, additional domains are present (31, 32, 40). The catalytic domains belong to one of five families of β-1,4-glucanases and β-1,4-xylanases in the established classification of glycosyl hydrolases based on amino acid sequence similarity (22). Most of their CBDs belong to a large group of related domains (family II) in a similar sequence-based classification (50, 51). While most of the *C. fimi* β-1,4-glucanases have recognizable counterparts in other cellulase systems from bacteria and fungi, one of the enzymes, CenC, has structural and functional features that distinguish it from related enzymes. These include two CBDs (N1 and N2) in tandem at the N terminus with distinct structures and specificities and the occurrence of two additional tandem domains (C1 and C2) of unknown functions at the C terminus (13, 14, 50). These features suggest the possibility that CenC has unusual enzymatic properties. Previous studies on CenC have been limited by the rather poor expression of its recombinant gene in *Escherichia coli*; as a result, very little is known about its activity. These considerations prompted us to develop an improved expression system for CenC and to examine its hydrolytic properties in greater detail. The results suggest that the attack of cellulosic substrates by CenC is significantly different from that by other *C. fimi* β-1,4-glucanases.

MATERIALS AND METHODS

Materials. Microcrystalline cellulose (Avicel PH101) was from FMC International, Little Island, County Cork, Ireland. Bacterial microcrystalline cellulose (BMCC) was prepared from cultures of *Acetobacter xylinum* (ATCC 23769) as described previously (18). Acid-swollen cellulose (PASC) was obtained by phosphoric acid treatment of Avicel PH101, as reported previously (13). Carboxymethyl cellulose (CMC; Na salt, low-viscosity grade, nominal degree of polymerization of 400, nominal degree of substitution of 0.7), lichenan, *p*-nitrophenyl β-glycosides, and 4′-methylumbelliferyl β-glycosides were from Sigma Chemical Company, St. Louis, Mo. Barley β-glucan (viscosity, 20 to 30 centiStokes) was from MegaZyme Ltd., North Rocks, New South Wales, Australia. Cellooligo-

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saccharides (>99% pure) were from Seikagaku America Inc., Rockville, Md. 2′-Chloro-4′-nitrophenyl-β-D-cellobioside ($CNPGlc_2$) and 2′-chloro-4′-nitrophenyl-lactoside (CNPLac) were synthesized as described previously (12).

Bacterial strains, plasmids, and growth conditions. *E. coli* JM101 {*supE thi-1* Δ(*lac-proAB*) [F′ *traD36 proAB lacI*^{q}*ZΔM15*]} (54) and TB1 {F$^-$ *ara* Δ(*lac-proAB*) *rpsL* (Str^r) [φ80*dlac*Δ(*lacZ*)*M15*] *hsdR* (r_K^- m_K^+)} (4) were used as host strains for maintenance of the plasmids and for production of recombinant protein. Cultures were grown at 30°C in liquid tryptone-yeast extract-phosphate medium (TYP) (37) or on Luria broth (LB) agar, supplemented with kanamycin (100 μg/ml). Cellulase activity was detected on 0.5% (wt/vol) CMC plates by Congo red (1%, wt/vol) staining (47).

General DNA procedures. Plasmid DNA preparation and electrophoresis of DNA fragments were performed by routine procedures (2, 37). 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 (21) 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). Each PCR mixture (total volume, 50 μl) contained 10 to 100 ng of template DNA, 25 to 50 pmol (300 ng) of primers, 2 mM $MgCl_2$, 6% dimethyl sulfoxide, 0.2 mM 2′-deoxynucleotide 5′-triphosphates, and 1 U of *Taq* DNA polymerase in 50 mM Tris-HCl buffer (pH 8.3). Twenty-eight successive cycles were performed as follows: denaturation at 94°C for 15 s, annealing at 57°C for 1.4 min, and primer extension at 72°C for 1.5 min.

Construction of the expression vector for *cenC*. The original vector pTZ-JC2 (13) did not contain useful restriction sites for subcloning the *cenC* gene in pTugEO7K3. Appropriate restriction sites were introduced by PCR (see Fig. 1). A *Nhe*I site coinciding with the N-terminal end (Ala-Ser) of the mature protein was introduced as a silent mutation at the 5′ end of *cenC*, with the oligonucleotide 5′TTACCTCAT**ATG**GCTAGCCCGATCGGGGAGGGAACG-3′ as primer (*Nhe*I site underlined). An ATG start codon (boldfaced in sequence above) previously shown to enhance the expression of CenC in *E. coli* over the original *C. fimi* translational start codon GTG (14) was also included as part of a *Nde*I site. A *Hin*dIII restriction site (underlined) and an *Eco*RI restriction site (boldfaced) were introduced at the 3′ end of *cenC* with the oligonucleotide 5′-AGAAT**GAATTC**AAGCTTAGCTGCGCGGACGCTGCAC-3′ as primer. The resulting 3.24-kb PCR fragment was digested with *Nde*I and *Eco*RI and subcloned at the *Nde*I-*Eco*RI restriction sites in the polylinker of pAED4 (see Fig. 1) to give pDC1. The 2.46-kb *Apa*I-*Ppu*MI PCR fragment encoding all of CenC with the exception of the C-terminal repeats C1 and C2 (14) was removed by digestion of pDC1 with *Apa*I, *Ppu*MI, and *Sma*I and replaced with the *Apa*I-*Ppu*MI fragment from pTZ-JC2 to give pDC1n. pDC1n and pTμgO7K3 were cut with *Nhe*I and *Hin*dIII, and the whole *cenC* fragment obtained from pDC1n was recloned in frame with the Cex leader in pTμgO7K3 to give the final construct pTC1n. This construct was verified by sequencing of the double-stranded DNA by the dideoxy chain termination method (38) with modified T7 DNA polymerase and [α-^{35}S]dATP (46).

Enzyme purification. Overnight cultures of *E. coli* TB1, harboring pTC1n, were diluted 500-fold in TYP supplemented with 100 μg of kanamycin per ml and grown at 30°C to an A_{600} of 4.0 to 5.0. CenC production was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.1 to 0.5 mM. The cultures were incubated for an additional 3 to 4 h at 30°C. The cells were harvested by centrifugation at 4°C for 10 min at 13,000 × *g*. All subsequent steps were performed on ice or at 4°C. The cells were washed twice with 5 mM potassium phosphate buffer (pH 7.0) containing 0.02% NaN_3 and resuspended in 50 mM potassium phosphate buffer (pH 7.0) containing 5 mM EDTA and 1 mM phenylmethylsulfonyl fluoride. The cells were ruptured by two passages through a French pressure cell (21,000 lb/in^2). Fresh phenylmethylsulfonyl fluoride was immediately added to a final concentration of 1 mM, and residual cell debris was removed by centrifugation for 30 min at 16,000 × *g*. DNA in the clarified supernatant was removed by overnight precipitation with streptomycin sulfate (final concentration, 1.5% [wt/vol]) followed by centrifugation for 30 min at 16,000 × *g*. Clarified cell extract containing CenC was passed through a CF1 (Sigma) cellulose column (5 by 30 cm; 50 mM potassium phosphate buffer [pH 7.0]) at 4°C and a flow rate of 1 ml/min. The column was washed with 1 M NaCl–50 mM potassium phosphate buffer (pH 7.0), and adsorbed proteins were desorbed with distilled water. Fractions (10 ml) of the eluate were assayed for protein and activity against CMC and $CNPGlc_2$. The active fractions were pooled, adjusted to pH 6.0 with potassium phosphate buffer (20 mM final), and further purified by ion-exchange chromatography on an EconoQ cartridge or a MacroQ column (Bio-Rad) previously equilibrated with 20 mM potassium phosphate buffer (pH 6.0). Bound protein was desorbed (1 ml/min) with a linear gradient of 0 to 1 M NaCl (pH 6.0). The purity of the protein fractions with $CNPGlc_2$ activity was determined by sodium dodecyl sulfate (SDS)–10% PAGE. Peak fractions containing pure protein were pooled, dialyzed against 50 mM potassium phosphate buffer (pH 7.0)–0.02% NaN_3, and concentrated by ultrafiltration through an Amicon PM-30 membrane. Protein was stored at 4°C. CenA, CenB, CenD, and Cex were purified by affinity chromatography on cellulose CF1 by published methods (27, 41).

Protein determination. Protein concentrations were determined with a dye-binding assay (6) and bovine serum albumin (BSA) as a standard. The concentrations of purified proteins were estimated by UV absorbance (39). The estimated extinction coefficient (280 nm, 1 mg/ml, 1 cm) for CenC (1.80) was in good agreement with that predicted (8) from the tryptophan and tyrosine content of the protein (i.e., 1.86). Extinction coefficients for CenA (2.64), CenB (2.13), CenD (2.48), and Cex (1.61) were as reported previously (18).

Enzyme assays. Cellulase activities on Avicel PH101, BMCC, CMC, and PASC were assayed at pH 6.0 and 37°C by measuring the liberated reducing sugars, as D-glucose equivalents, with the hydroxybenzoic acid hydrazide reagent (26). Release of reducing sugars (D-glucose equivalents) from barley β-glucan, lichenan, or glucomannan was measured by the dinitrosalicylic acid assay (33). Activities were expressed as units per micromole of enzyme, with 1 U defined as the amount of enzyme releasing 1 μmol of reducing sugar per min.

Avicelase activities were assayed after incubation of 0.17 nmol of enzyme with 10 mg of Avicel in 1.0 ml of 50 mM sodium citrate buffer (pH 6.0) containing 0.02% BSA and 0.02% NaN_3 (CB buffer) for 18 h at 37°C. 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. Hydrolysis of CMC was determined after incubation (30 min at 37°C) of 1 pmol of CenA, -B, or -C, or 20 pmol of CenD or Cex, plus 0.5% (wt/vol) CMC in 0.5 ml of CB buffer. Activity on PASC was assayed after incubation (2 h at 37°C) of 20 pmol of enzyme with 10 mg of phosphoric acid-swollen Avicel in 1 ml of CB buffer. Two-hundred-fifty-microliter aliquots of the incubation mixtures were withdrawn at regular intervals. After centrifugation (twice, 5 min each time; 16,000 × *g*), 20 to 100 μl of supernatant was diluted in 50 mM sodium citrate buffer (pH 6.0; final volume, 500 μl) and reducing sugars were measured (at 420 nm) after the addition of 1 ml of hydroxybenzoic acid hydrazide reagent.

Hydrolysis of β-glucan and lichenan was determined after incubation of 0.5 pmol of enzyme with 0.5% (wt/vol) substrate in 50 mM potassium phosphate buffer (pH 7.0) containing 0.02% BSA and 0.02% NaN_3 at 30°C for 2 h. Hydrolysis of glucomannan (0.5%, wt/vol) was done essentially as described for β-glucan after 8 h of incubation at 30°C. The reducing sugars released upon hydrolysis were measured (at 540 nm) after addition of 1 ml of dinitrosalicylic acid.

Release of 2-chloro-4-nitrophenol upon hydrolysis of $CNPGlc_2$ and CNPLac (pH 7.0, 37°C) was monitored continuously at 405 nm. Samples contained 1.7 pmol of CenC and 0.125 to 2.5 mM substrate in 50 mM potassium phosphate buffer (pH 7.0)–0.02% NaN_3. 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 equation with the *GraFit* program (25).

Hydrolysis of 4-methylumbelliferyl glycosides was monitored in microtiter plates after incubation (37°C) of 0.1 to 0.5 pmol of enzyme with 200 to 500 μM fluorophoric substrate in 50 mM potassium phosphate buffer (pH 7.0)–0.02% NaN_3 for up to 24 h. Hydrolysis was qualitatively monitored by UV illumination.

Enzymatic hydrolysis of cellodextrins (cellotriose to cellohexaose) was monitored by high-performance liquid chromatography (HPLC) on a Shimadzu system equipped with an SIL6B autoinjector, a 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. Reactions contained 125 to 200 μg of cellodextrin (cellotriose-cellohexaose) and 10 to 50 pmol of enzyme in 10 mM potassium phosphate buffer (pH 7.0). Aliquots were analyzed after 30 s of incubation at 25°C or after 30 to 60 min at 37°C to determine anomeric products before and after mutarotation, respectively.

Soluble products released by CenB or CenC from BMCC and PASC were also monitored by HPLC. Reaction mixtures (1 ml) contained 1 nmol of enzyme and 1 mg of BMCC or PASC in 5 mM potassium phosphate buffer (pH 7.0). Aliquots were withdrawn after 0, 12, and 24 h of incubation at 37°C; the cellulose was removed by centrifugation, and the supernatant was boiled for 5 min to inactivate enzyme prior to HPLC analysis.

Viscometric assay. Increases in specific fluidity (ϕ_{sp}) and reducing sugars during CMC hydrolysis were determined as described previously (19).

N-terminal amino acid sequence analysis. For N-terminal sequence determinations, proteins were resolved by SDS-PAGE (10 to 12% polyacrylamide) and electroblotted (0.5 A, 25 min) onto polyvinylidene difluoride membranes (Immobilon; Millipore) (28). Coomassie blue-stained bands were excised and sequenced directly by automated Edman degradation analysis in an Applied Biosystems 470 gas-phase sequenator.

RESULTS

Production and purification of CenC. CenC is extremely protease sensitive, and expression of *cenC* under the control of the *lac* promoter in the original pTZ-JC2 vector resulted in low yields of protein (14). Therefore, the *cenC* gene was recloned into the high expression vector pTugEO7K3, a derivative of pTugA (20), under the tight control of the *tac* promoter (Fig. 1). The original CenC leader peptide was replaced with the one

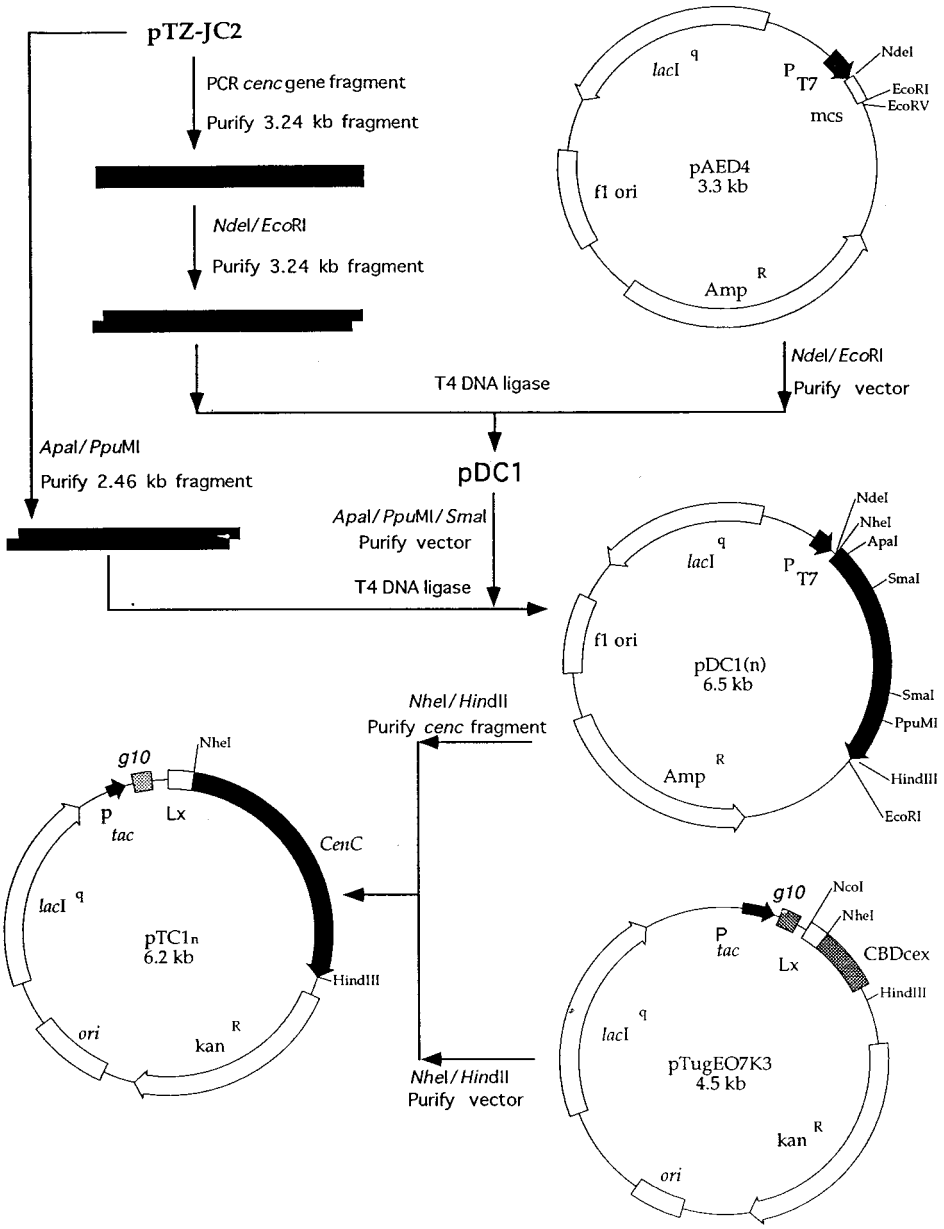

FIG. 1. Construction of the *cenC* expression vector. *Nde*I-*Nhe*I and *Hin*dIII-*Eco*RI restriction sites were introduced at the 5′ and 3′ ends of *cenC*, respectively, by PCR. The resulting PCR fragment was cloned at the *Nde*I and *Eco*RI sites in the polylinker of the pAED4 vector to give pDC1. The 2.5-kb *Apa*I-*Ppu*MI PCR fragment in pDC1 was replaced with the corresponding native fragment obtained from pTZ-JC2 to give pDC1n. pDC1n was then cut with *Nhe*I and *Hin*dIII, and the corresponding *cenC* fragment was subcloned at the *Nhe*I-*Hin*dIII sites of pTugEO7K3 behind the Cex leader peptide to yield the final construct pTC1n.

from *C. fimi* Cex. This peptide efficiently directs translocation of several proteins to the periplasm of *E. coli*, and upon extended incubation, the proteins leak into the culture medium (34). Furthermore, the processing site located between the two alanines in the Ala-Ala-Ser sequence at the boundary of the leader peptide and the CBD of Cex was correctly recognized by the *E. coli* leader peptidase (34).

After 4 h of induction, 87% of the carboxymethyl cellulase activity was present in the cytoplasm of *E. coli*, with the remainder in the culture supernatant. Further incubation resulted in considerable cell lysis and release of CenC into the culture medium. After 18 h of induction, 55% of the activity was found in the culture medium, 40% was found in the cytoplasm, and only 5% of the total activity was detected in the periplasm. However, longer incubation periods also resulted in substantially more degradation of CenC.

Purification of CenC on Avicel was unsuccessful because the enzyme could not be desorbed under mild conditions even though the polypeptide corresponding to the CBDs N1 and N2 is readily desorbed with distilled water (13). Affinity chromatography of the cell extracts on CF1 cellulose, on the other hand, gave a partially purified fraction (Table 1) containing two major polypeptides with apparent molecular masses of 130 and 40 kDa. A previously observed degradation product with a molecular mass of 120 kDa (14) was absent. The 130-kDa (Fig. 2, peak 1) and 40-kDa (Fig. 2, peak 2) polypeptides were separated by anion-exchange chromatography on MacroQ. The 130-kDa polypeptide was identified as CenC by its activity

TABLE 1. Purification of CenC from cell extracts of *E. coli*

Step	Vol (ml)	Total protein (mg)	Total activity (U)[a]	Yield (%)	Sp act (U · mg)	Purification factor (fold)
Cell extracts	75.0	1,897.5	233,889	100.0	123.3	1.0
Streptomycin sulfate step	75.0	1,380.0	188,700	80.7	136.7	1.1
Cellulose affinity	7.0	13.8	62,160	26.6	4,504.3	36.5
MacroQ	25.0	4.3	32,200	13.8	7,576.5	61.5

[a] Activities were measured at 37°C and 405 nm with 2′-chloro-4′-nitrophenyl-β-D-cellobioside as the substrate. Units were defined as the amount of enzyme needed to release 1 μmol of 2-chloro-4-nitrophenol per min.

on $CNPGlc_2$ (at 405 nm). CenC was purified approximately 62-fold with a final yield of 14% (Table 1). Purified CenC had a very high specific activity (37°C, pH 7.0) of ~7,600 U/mg on $CNPGlc_2$.

The sequence Ala-Ser-Pro-Ile-Gly-Glu-Gly-Thr-Phe-Asp obtained by Edman degradation analysis of the 130-kDa polypeptide corresponded with that of native CenC and confirmed that the protein was properly processed. However, little activity was detected in the periplasm, suggesting that the leader peptide was functional but that the enzyme was trapped in the inner membrane. Edman degradation analysis and binding to cellulose identified the 40-kDa fragment as N1N2.

Properties of CenC. The apparent molecular mass of CenC determined by SDS-PAGE was 130 kDa (Fig. 2), slightly larger than the molecular mass of 112.2 kDa deduced from the amino acid sequence. This difference may be the result of a slightly aberrant behavior of the highly acidic CenC (calculated pI, 4.2) during electrophoresis. The optimum pH and temperature for hydrolysis of $CNPGlc_2$ or CNPLac were 5.0 and 45°C, respectively.

Activities on soluble and insoluble polymeric substrates. CenC hydrolyzed a variety of β-1,4-glucans (Table 2), and the activity decreased roughly with increasing crystallinity of the substrate (CMC > PASC > BMCC ≥ Avicel). CenA and CenB showed a similar trend (Table 2). The activities of CenC on these substrates resembled most closely those of CenA, with a slightly lower activity on Avicel but a significantly higher activity on BMCC. However, the specific activities of CenC (and CenA) on these crystalline substrates, especially BMCC, were significantly lower than those of CenB and CenD (Table 2). CenB and CenD were eight times more active than CenC and 50 times more active than CenA on BMCC.

On the other hand, with specific activities of ~1,000 mol of glucose residues released per mol of enzyme per min, CenC and CenB were the most active *C. fimi* carboxymethyl cellulases (Table 2); CenC was originally classified as an endoglucanase on this basis (14). Endoglucanase D (CenD) had a surprisingly low activity (47 $\mu mol \cdot min^{-1} \cdot \mu mol^{-1}$) on CMC. Although viscometric analysis of CMC hydrolysis suggests that CenD is a true endoglucanase (Fig. 3), its activity on this substrate was about 20-fold lower than the activity of the other *C. fimi* endoglucanases (Table 2).

CenC was also highly active on mixed β-(1,3-1,4) glucans such as barley β-glucan and lichenan and to a lesser extent on glucomannan (Table 2). This property was shared only by CenB (Table 2). Hydrolysis of glucomannan was probably restricted to the β-1,4-glucosidic linkages since galactomannan and mannan were not hydrolyzed by either CenC or CenB. The high activity of CenC on barley β-glucan is believed to be a property of endoglucanases, although several cellobiohydrolases, including CBH II from *Trichoderma reesei*, also slowly hydrolyze this substrate (3). As observed with CMC, CenD had a three- to fivefold-lower activity than CenA, CenB, or CenC on β-glucan.

Viscometric analysis of CMC hydrolysis. Hydrolysis of CMC by CenA, CenB, and CenD resulted in a rapid increase of the specific fluidity (ϕ_{sp}) relative to the amount of reducing sugars released, indicative of a random action of these endoglucanases (Fig. 3). The slopes were 95.9, 72.2, and 88.1 $\phi \cdot ml \cdot mmol^{-1}$ for CenA, CenB, and CenD, respectively. In contrast, the exoglucanase CbhA, acting from the chain ends, resulted in a much lower increase in specific fluidity relative to the sugars released (17.1 $\phi \cdot ml \cdot mmol^{-1}$). A similar value was previously obtained for CbhB, another *C. fimi* exoglucanase (40). The slope for CenC was 33.4 $\phi \cdot ml \cdot mmol^{-1}$, in between those obtained for the endo- and exoglucanases (Fig. 3).

Activities on small chromophoric and fluorophoric compounds. Several *C. fimi* cellulases hydrolyzed 4-methylumbelliferyl glycosides with release of the fluorophore (Fig. 4). CenC hydrolyzed both the 4-methylumbelliferyl cellobioside and the 4-methylumbelliferyl lactoside (Fig. 4, lane 4). In contrast,

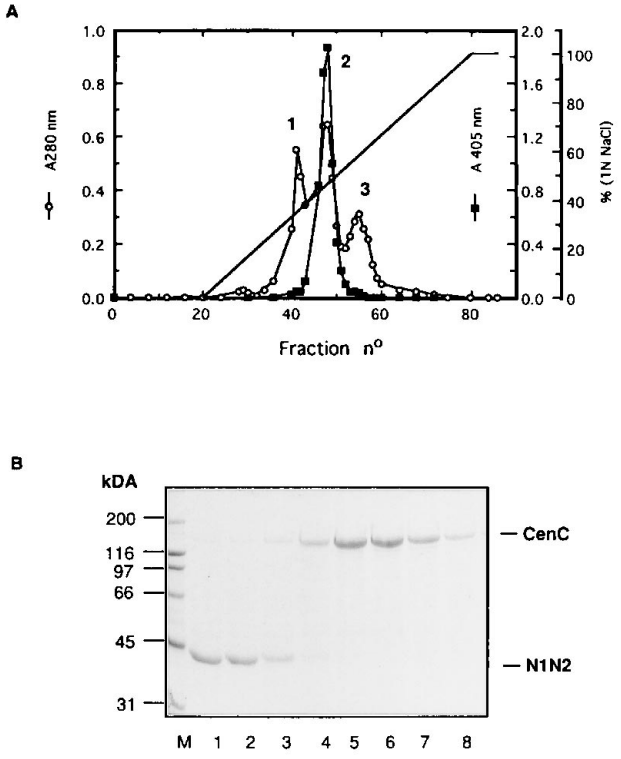

FIG. 2. Anion-exchange chromatography of CenC after affinity chromatography on cellulose. (A) Purification of CenC on MacroQ. Fractions were assayed for protein (○) and activity against $CNPGlc_2$ (■). (B) SDS-PAGE analysis of the MacroQ fractions corresponding to peak 1 and peak 2 in panel A. Aliquots (5 to 20 μl) of each fraction were analyzed on gels containing 10% acrylamide. Lanes: M, size markers; 1 to 3, fractions 41 to 43, respectively; 4 to 8, fractions 46 to 50, respectively.

TABLE 2. Activities of *C. fimi* enzymes on soluble and insoluble glucans

Enzyme	Sp act[a] on:						
	Avicel[b]	BMCC[b]	CMC[b]	PASC[b]	β-Glucan[c]	Lichenan[c]	Glucomannan[c]
CenA	2.18[c]	0.21	760	244	2,180	3,940	ND[d]
CenB	2.22	10.87	928	66	5,700	3,240	912.5
CenC	0.99	1.55	1,016	114	3,900	3,220	722.0
CenD	2.42	9.66	47	81	940	1,220	ND
Cex	0.16	ND	10	46	ND	ND	ND

[a] Specific activities are expressed as micromoles of reducing glucose per micromole of enzyme per minute. Carboxymethyl cellulase activities were monitored for 30 min; PASC, β-glucan, and lichenase activities were done over 2 h; Avicel and BMCC activities were measured over 18 h.
[b] Activity was determined with the hydroxybenzoic acid hydrazide reagent after incubation of the enzyme with substrate at 37°C.
[c] Activity was determined with the dinitrosalicylic acid reagent after incubation of the enzyme with substrate at 30°C.
[d] ND, not detected.

neither CenB, another family 9 enzyme, nor CenA cleaved these substrates at the agluconic bond, even after prolonged incubation (Fig. 4, lanes 2 and 3). CenD, a retaining enzyme, was most active on the 4-methylumbelliferyl cellotrioside but also hydrolyzed the cellobioside (Fig. 4, lane 5). Cex was active on a range of substrates: both the cellobioside and the cellotrioside were rapidly hydrolyzed, but the lactoside and the xyloside were hydrolyzed more slowly (Fig. 4, lanes 6). None of the *C. fimi* enzymes hydrolyzed the 4-methylumbelliferyl glucoside, arabinoside (Fig. 4), galactoside, or mannoside (data not shown).

Both $CNPGlc_2$ and CNPLac were hydrolyzed by CenC with similar kinetics (Table 3).

Stereochemistry and mode of action on cellodextrins. CenC was extremely active on cellodextrins with a degree of polymerization of >3. Cellotriose was hydrolyzed slowly. Cellobiose was the major product from cellotriose, cellotetraose, cellopentaose, and cellohexaose. In contrast, CenB was much less active on the cellodextrins and produced significantly more glucose (Fig. 5C). Cellotriose, which was only very slowly hydrolyzed by CenB, also accumulated in the reaction mixtures of cellotetraose, cellopentaose, or cellohexaose.

Cellohexaose was cleaved by CenC into cellobiose and cellotriose with a trace of cellotetraose (Fig. 5). Cellotetraose was completely converted to cellobiose upon further incubation (30 min). Chromatographic resolution of the α- and β-anomers showed a ratio of α-cellotriose/β-cellotriose of approximately 2.1:1 after 30 s of incubation at 25°C (Fig. 5A). The equilibrium ratio of 1:1.8 was almost completely attained (1:1.6) after a further incubation for 30 min (Fig. 5B). Therefore, the reaction clearly proceeded with inversion of anomeric configuration, characteristic of a single-displacement mechanism (43). Moreover, cellopentaose was cleaved to cellobiose and cellotriose, with essentially the same ratio of α-cellotriose to β-cellotriose as that observed for cellohexaose (not shown). Production of α-cellotriose indicates that cellobiose is produced from the reducing end of cellopentaose during hydrolysis by CenC.

Degradation products of cellulose hydrolysis. More than 98% of the total sugar produced from PASC following incubation with 1 nmol of CenC for 24 h was cellobiose, with only trace amounts of glucose (~1.6%) (data not shown). A similar pattern was obtained with BMCC. There was no accumulation of cellotriose or higher-molecular-weight products from either substrate. In contrast, CenB released 20 to 25% glucose and 9 to 12% cellotriose upon incubation with both substrates for 24 h (data not shown).

DISCUSSION

Biochemical and kinetic characterizations of CenC reveal several properties which are inconsistent with its classification as a randomly acting endoglucanase. The data obtained in this study also suggest that the classification of β-1,4-glucanases in two mutually exclusive groups, endoglucanases and exoglucanases, is inappropriate.

The most commonly used criterion to identify endoglucanases is activity on CMC (47, 53). True exoglucanases would have no activity in the highly sensitive CMC-Congo red plate assay (19, 53). However, at high enzyme concentrations, some cellobiohydrolases do give a positive response in this assay (40). The positive result probably reflects a low intrinsic endoglucanase activity (40), which can result in erroneous identification of exocellulases or xylanases as endoglucanases (42). Indeed, it has been suggested previously that there are no strictly exohydrolytic β-1,4-glucanases (16, 45). Because detection of exohydrolytic activity requires the use of large amounts of enzyme and long incubation times, slight contamination with endoglucanases, especially for enzymes purified from the culture media of cellulolytic organisms, could account for the apparent endoglucanase activities of the enzymes. However, it appears that CenC has both endoglucanase and exoglucanase activity because it is very active on CMC but it effects a relatively small increase in specific fluidity of CMC per unit of reducing sugar released. This suggests that CenC hydrolyzes CMC by a semiprocessive mechanism; i.e., the enzyme appears

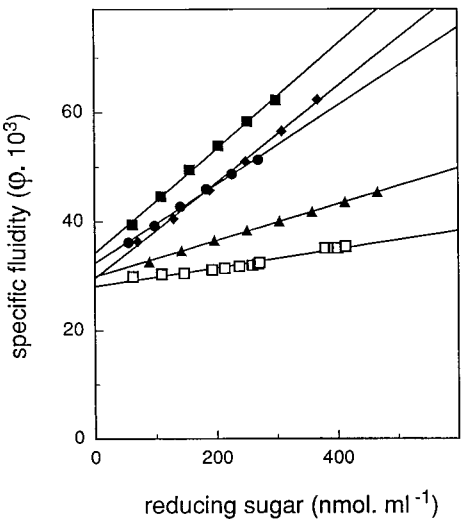

FIG. 3. Hydrolysis of CMC by *C. fimi* cellulases. Specific fluidity (ϕ) is plotted versus the release of reducing sugar upon hydrolysis of CMC by CenA, CenB, CenC, CenD, and CbhA. Reaction mixtures contained 3.6 nM CenA (■), 24 nM CenB (●), 2.5 nM CenC (▲), 20 nM CenD (◆), or 500 nM CbhA (□) and 3.2% (wt/vol) CMC in 50 mM sodium citrate (pH 7.0).

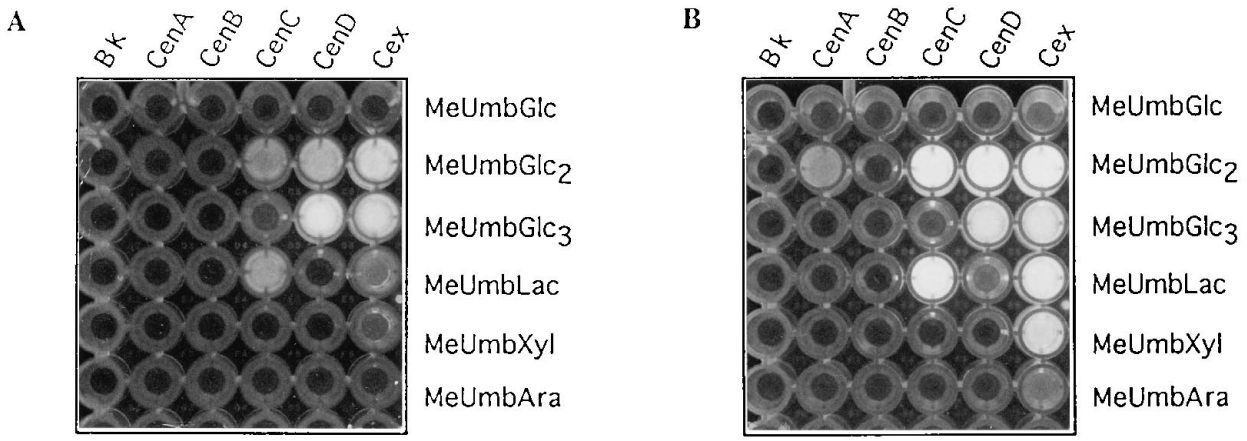

FIG. 4. Analysis of the hydrolysis of 4-methylumbelliferyl glycosides by CenA, CenB, CenC, CenD, and Cex. Hydrolysis of the fluorophoric glycosides was assessed qualitatively by UV illumination after incubation of 0.1 to 0.5 pmol of enzyme with 200 to 500 μM glucoside (MeUmbGlc), cellobioside ($MeUmbGlc_2$), cellotrioside ($MeUmbGlc_3$), lactoside (MeUmbLac), xyloside (MeUmbXyl), or arabinoside (MeUmbAra) for 30 min (A) or 2 h (B) at 37°C.

to initiate attack on CMC by random attack of internal β-1,4-glucosidic bonds, presumably adjacent to unsubstituted residues, but then proceeds processively from the site of hydrolysis along the β-1,4-glucan chain in an exoglucanolytic manner (Fig. 6B) until one or more substituted glucose residues are encountered. It is not yet clear whether attack of unsubstituted cellulose occurs by a similar mechanism, but such processive activity would explain the almost exclusive release of cellobiose from BMCC. An endo-1,4-β-D-glucanase with a similar semiprocessive activity was recently purified from the culture filtrate of *Fusarium oxysporum* (11). In contrast, the more random action of CenB on cellulose results in the formation of substantial amounts of glucose and cellotriose (Fig. 6C).

The original classification of CenC as a family 9 (formerly family E) cellulase (14) was based entirely on sequence similarities between its catalytic domain and those of various other β-1,4-glucanases (22). Underlying this classification is the concept that the members of a particular family share a common three-dimensional structure (22) and active-site topology (17). As a result, conservation of the catalytic machinery, dictated by the spatial arrangement of catalytic residues (see below), and of the reaction mechanism, as indicated by the stereochemistry of the product, is expected. The finding that hydrolysis by CenC, like several other family 9 enzymes (17, 30) such as CenB from *C. fimi*, proceeds with inversion of anomeric configuration (Fig. 5C) is consistent with this concept.

Glycosyl hydrolase family 6 contains both endoglucanases and exocellobiohydrolases. The endoglucanases have a groove-shaped active site, whereas in the cellobiohydrolases, the active site is enclosed by two large surface loops resulting in a tunnel-shaped structure (36, 44). Regions that contribute to the formation of these loops are evident as insertions when the amino acid sequences of the family 6 endoglucanases and exocellobiohydrolases are compared (31, 36, 44). The tunnel-shaped active site of the cellobiohydrolases is consistent with a processive mechanism in which the enzymes continually release cellobiose from the ends of cellulose molecules while remaining bound to the substrate (Fig. 6A). Family 9 (subfamily 1) currently contains nine enzymes. In contrast to family 6, sequence comparison of the family 9 enzymes does not reveal the presence of cellobiohydrolases and most enzymes were identified as endoglucanases. CelD from *Clostridium thermocellum* is the only family 9 subfamily 1 enzyme for which structural information is available (24). Its active site forms an extended cleft composed of six substrate-binding sites, A to F (24). Comparison of CenC and the other family 9 enzymes does not reveal any obvious insertions that might correspond to extended surface loops enclosing the CenC active site. It therefore seems reasonable to assume that the CenC active site has a cleft-like structure similar to that of CelD. The active site of CenC appears to be readily accessible to the cellulose chains, and such a structure is consistent with the ability of CenC to attack CMC randomly. Presumably, binding is sufficiently tight to prevent rapid dissociation of the substrate after the initial attack, allowing the enzyme to continue processively (Fig. 6B).

Until recently, it was thought that all cellobiohydrolases hydrolyzed cellulose from the nonreducing ends. However, there is now evidence that hydrolysis by CbhB from *C. fimi* (40), CBH I from *T. reesei* (52), and Avicelase II from *Clostridium stercorarium* (7) proceeds from the reducing end. Although the determination of the exact bond cleavage frequencies requires the introduction of a radioisotope label, HPLC analysis of the hydrolysis patterns for cleavage of cellopentaose and cellohexaose indicates that CenC also liberates cellobiose from the reducing ends. Assuming similar active-site topologies for CenC and CelD, CenC would bind the reducing chain end in subsite F and cleave the glucosidic bond between subsites D and E (24), resulting in release of cellobiose from the reducing chain end. This assumption is supported by the finding that most functionally important residues identified in the six substrate-binding sites of CelD are conserved in CenC. In this context, it is interesting to note that both CenC (this study) and CelD (10, 23, 48) are highly active on chromophoric and fluorophoric glycosides derived from β-D-1,4-cellobiose and lactose. The absence of significant activity of CenB on these substrates supports the classification of CenC and CenB in different subfamilies (50).

Hydrolysis by CenC of the agluconic bond of chromophoric glycosides (especially cellobiosides) is not inconsistent with the

TABLE 3. Kinetic parameters for hydrolysis (37°C, pH 7.0) of CNPLac and $CNPGlc_2$ by CenC

Substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)
CNPLac	166	30.7	1.85×10^5
$CNPGlc_2$	297	27.3	0.92×10^5

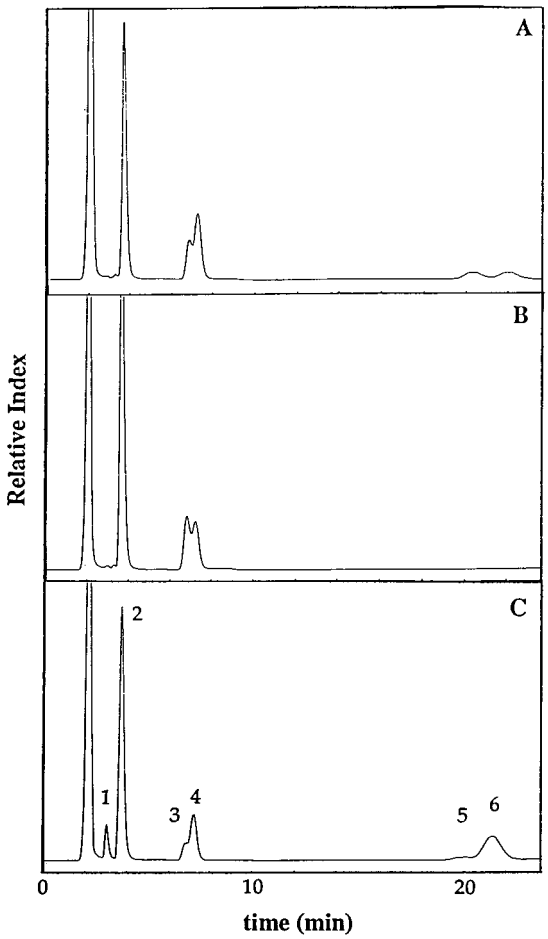

FIG. 5. Products of the hydrolysis of cellohexaose by CenC. The products were analyzed by HPLC. (A) Hydrolysis products from cellohexaose after 30 s of incubation with CenC at 25°C; (B) hydrolysis products after incubation at 37°C for 30 min, conditions which allow mutarotation at the anomeric carbon; (C) hydrolysis products from cellohexaose after 30 s of incubation with CenB at 25°C. The β- and α-anomers of cellotriose (peak 3 and 4) and cellotetraose (peak 5 and 6) are resolved; those of glucose (peak 1) and cellobiose (peak 2) are not.

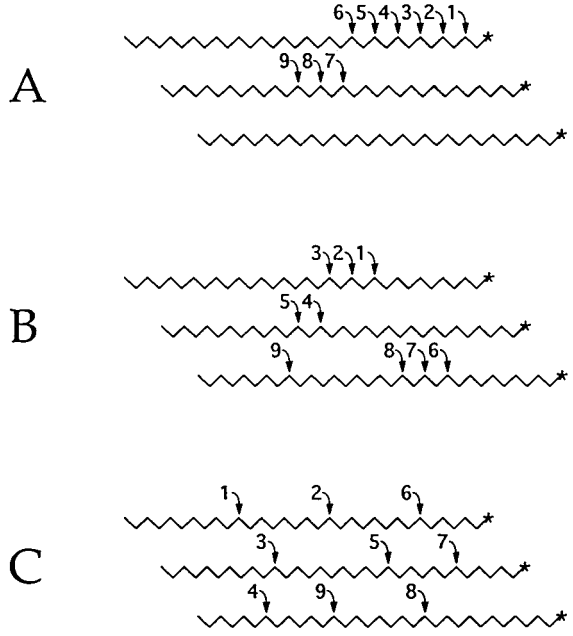

FIG. 6. Attack by processive (A), semiprocessive (B), and nonprocessive (C) cellulases. In each case, the cellulose substrate is represented by three adjacent β-1,4-glucan chains. Each chain consists of 16 cellobiosyl residues with the reducing end denoted by an asterisk. Numbered arrows show the sequence of nine successive hydrolytic events. (A) A processive enzyme (exoglucanase [arrows 1 to 6] or endoglucanase [arrows 7 to 9]) that initiates attack at a reducing end and continues by removing many successive cellobiosyl residues from the same chain before jumping to a new site nearby; (B) a semiprocessive enzyme that initiates attack at an internal β-1,4-glucosidic bond and removes only a few successive cellobiosyl residues from the new reducing end before jumping to another site; (C) a nonprocessive enzyme that jumps to a new site after each attack. The mechanisms shown are similar to those proposed by Robyt and French (35) to describe the attack of starch by α-amylases.

preferential release of cellobiose from the reducing end of natural substrates because the hydrophobicity of the aglucons is known to affect the mode of binding of the glycosides in the active site (5, 29). For example, CBH I from *T. reesei* readily hydrolyzes $CNPGlc_2$ and CNPLac with release of the phenolate (49), even though kinetic analysis using ^3H-labeled cellooligosaccharides (52) and structural evidence obtained from cocrystallization of CBH I with substrate analogs (15) indicate that hydrolysis proceeds by removal of cellobiose from the reducing ends.

In summary, the data presented here suggest that the classification of cellulases into endoglucanases and exoglucanases is artificial. It is now apparent that at least some exoglucanases have measurable, albeit low, endoglucanase activity. Similarly, some endoglucanases, such as CenC, appear to act processively at the ends of cellulose molecules following an initial, random attack. Furthermore, the long-held view that all exoglucanases attack cellulose from the nonreducing end is clearly untenable. The topology of the active site probably determines the direction of attack, although a cellulose-binding domain, if present, may also be influential.

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