

The Processive Endocellulase CelF, a Major Component of the *Clostridium cellulolyticum* Cellulosome: Purification and Characterization of the Recombinant Form

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The recombinant form of the cellulase CelF of *Clostridium cellulolyticum*, tagged by a C-terminal histine tail, was overproduced in *Escherichia coli*. The fusion protein was purified by affinity chromatography on a Ni-nitrilotriacetic acid column. The intact form of CelF (M_r , 79,000) was rapidly degraded at the C terminus, giving a shorter stable form, called truncated CelF (M_r , 71,000). Both the entire and the truncated purified forms degraded amorphous cellulose ($k_{cat} = 42$ and 30 min^{-1} , respectively) and microcrystalline cellulose ($k_{cat} = 13$ and 10 min^{-1} , respectively). The high ratio of soluble reducing ends to insoluble reducing ends released by truncated CelF from amorphous cellulose showed that CelF is a processive enzyme. Nevertheless, the diversity of the cellobioses released by truncated CelF from phosphoric acid-swollen cellulose at the beginning of the reaction indicated that the enzyme might randomly hydrolyze β -1,4 bonds. This hypothesis was supported by viscosimetric measurements and by the finding that CelF and the endoglucanase CelA are able to degrade some of the same cellulose sites. CelF was therefore called a processive endocellulase. The results of immunoblotting analysis showed that CelF was associated with the cellulosome of *C. cellulolyticum*. It was identified as one of the three major components of cellulosomes. The ability of the entire form of CelF to interact with CipC, the cellulosome integrating protein, or mini-CipC₁, a recombinant truncated form of CipC, was monitored by interaction Western blotting (immunoblotting) and by binding assays using a BIAcore biosensor-based analytical system.

The biological degradation of cellulose, a recalcitrant substrate, has been studied for several years (25). Many hydrolytic enzymes, especially from fungi and bacteria, have been purified and studied. These enzymes have been classified into two categories, the endoglucanases, which randomly hydrolyze the β -1,4 bonds in the cellulose molecule, and the exoglucanases, which in contrast show a recurrent mode of action from one chain extremity and in most cases release cellobiose units. The latter category was therefore called cellobiohydrolases. Synergistic crystalline cellulose degradation processes have been found to occur between exo- and endoglucanases (6, 15, 16) and between exoglucanases (2, 15). Many endoglucanases have been purified from different organisms and characterized. These enzymes are easily identifiable because they have a high specific activity on carboxymethyl cellulose (CM-cellulose), a soluble substituted substrate. On the other hand, only a few exoglucanases have been identified and studied (34). Cellulolytic systems contain many endoglucanases but only a few exoglucanases, which are more difficult to detect because they only slightly degrade the available cellulosic substrates. They might, however, be key components of cellulolytic systems (7).

Clostridium cellulolyticum belongs to the group of bacteria which synthesize cellulosomes (20). These very stable proteic complexes are composed of a scaffolding protein, CipC (23, 26), and enzymatic proteins. Genes coding for six of these catalytic subunits have been cloned in *Escherichia coli* (1, 11,

26, 29). The corresponding proteins were found to carry a C-terminal reiterated sequence (4), called dockerin domain (3), which mediates interactions with the cohesin domains of the scaffolding protein CipC (23; this study). Recombinant CelA and CelC have been purified and characterized as typical endoglucanases (12, 13).

CelF has been overproduced in *E. coli* by using a T7 expression system (26). Its apparent molecular mass (79 kDa) is similar to that of one of the three major cellulosome components of *C. cellulolyticum* (20). In this study, this interesting protein was purified and characterized.

MATERIALS AND METHODS

Bacterial strains, vectors, and growth conditions. *E. coli* BL21(DE3) (Novagen) was used as the host for recombinant plasmids pETFc and pETCip1 (23, 26). Recombinant strains were grown in Luria-Bertani medium supplemented with ampicillin (200 $\mu\text{g/ml}$) as previously described. *C. cellulolyticum* ATCC 35319 was grown at 32°C in Hungate tubes on basal medium (14) supplemented with 5 g of MN300 cellulose (Serva-Feinbiochemica) per liter.

Purification of CelF. Strain BL21(DE3)[pETFc] was used to obtain large quantities of CelF. The culture was incubated at 37°C until an optical density at 600 nm of 2 was reached. Expression of the heterologous protein was induced by adding 40 μM IPTG (isopropyl- β -D-thiogalactopyranoside) and incubating the culture at 16°C for 16 h. Cells from 2.7 liters of culture were suspended in 70 ml of ice-cold 50 mM Tris-HCl buffer (pH 8) and broken twice in a French press. The crude extract was centrifuged at 8,000 $\times g$ for 20 min. The resulting 60-ml crude extract supernatant was loaded at 1 ml/min on a 20-ml Ni-nitrilotriacetic acid (NTA) column (Novagen) previously equilibrated with 50 mM Tris-HCl buffer (pH 8). The column was then washed with the same buffer before His-tagged CelF was eluted with Tris-HCl (100 mM; pH 4.8). The 300-ml eluate was concentrated to 11.2 ml by ultrafiltration in an Amicon concentrator with a 30K PTK Millipore membrane. The entire purification procedure was carried out at 4°C.

Production and purification of the mini-CipC₁ protein (CBD-HD₁-C₁) from *E. coli* BL21(DE3)[pETCip1] were performed as previously described (23).

Enzyme assays. Cellulase activity was assayed by mixing 1 ml of the appropriate enzyme dilution with 4 ml of either 1% (wt/vol) bacterial microcrystalline

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TABLE 1. Purification of CelF

Purification step	Vol (ml)	Protein (mg)	Activity (IU)	Sp act ^a (mIU/mg)	Yield (%)	Purification (fold)
Crude extract supernatant	60	2,772	32.1	11.6		
Ni-NTA column	11.2	53	17.5	330	54	28

^a Activity was estimated by mixing 4 ml of 1% PAS-cellulose with 1 ml of diluted proteins.

cellulose (BMC-cellulose) (15) or 10% Avicel-cellulose (Fluka) or 2% phosphoric acid-swollen cellulose (PAS-cellulose) in 20 mM Tris-maleate buffer (pH 6). PAS-cellulose was prepared from Avicel (Fluka) as described by Wood (35). The amounts of reducing sugars released in 1 ml were measured at various incubation times at 45°C by the Park and Johnson (24) method after the solids were removed by centrifugation. One unit of cellulase activity (international unit) corresponds to 1 μ mol of D-glucose equivalent released per min. The degradation of 1% CM-cellulose (medium viscosity; Sigma-Aldrich), oat spelt xylan (Sigma-Aldrich), lichenan (Sigma-Aldrich), and laminarin (Sigma-Aldrich) was tested in the same way. The carboxymethyl cellulase activity was also tested by measuring the increase in the fluidity of the CM-cellulose solution during the reaction (13).

Phosphoric acid-swollen cellulase (PAS-cellulase) activity was determined in the presence of potential inhibitor or activator (1 mM EDTA alone and with either 10 mM MgCl₂ or 10 mM CaCl₂ or 10 mM LiCl₂). These components were preincubated with CelF for 10 min before the substrate suspension containing the component(s) at the same concentration was added.

The protein concentration was determined by the method described by Lowry et al. (19) with bovine serum albumin as the standard.

Analysis of degradation products. The soluble products (cellobiose [G2] to celohexaose [G6]) of cellodextrin hydrolysis or PAS-cellulose hydrolysis were analyzed as follows. A 30- μ l volume of 0.2% cellodextrin (Merck) solution was mixed with 190 μ l of diluted enzyme (truncated CelF). To study PAS-cellulose degradation, 500 μ l of 3% substrate solution was mixed with 30 μ l of diluted enzyme (truncated CelF). Samples (100 μ l) were taken at specific incubation times at 45°C, centrifuged if necessary, heated at 100°C for 10 min, and filtered. A 50- μ l sample was loaded for analysis by high-pressure liquid chromatography (HPLC) (Varian) onto a Resex RSO-oligosaccharide column (20 by 1 cm; Interchim) heated at 40°C. The sugars were detected with a refractive-index detector (Varian). Chromatographic data were recorded and analyzed on a computer by means of the LC Star Workstation from Varian. The retention time of each cellodextrin was determined by loading 25 μ l of 10% cellodextrin mixture II (Merck) solution.

Extraction of cellulosome. A 4-ml volume of a 7-day-old culture of *C. cellulolyticum* on cellulose was centrifuged at 14,000 \times g for 20 min at 4°C. A 2-ml sample of supernatant was mixed with 4 mg of Avicel-cellulose. The mixture was adjusted to 50 mM phosphate buffer (pH 7) in a final volume of 4 ml. After 15 min of incubation at 4°C, cellulose was collected by centrifugation and washed twice with phosphate buffer. Proteins adsorbed onto the cellulose pellet (cellulosome) were eluted by boiling the pellet for 10 min in 100 μ l of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer (18).

Immunoblotting. Proteins (3 μ g of intact CelF or truncated CelF, 20 μ l of cellulosome solution) were separated by SDS-PAGE (9% acrylamide) and transferred to a polyvinylidene difluoride (PVDF) Western blotting (immunoblotting) membrane (Boehringer Mannheim). The membrane was incubated overnight in TBS-milk buffer (4% nonfat milk from Régilait, 50 mM Tris-HCl, 150 mM NaCl; pH 7.5) and for 30 min in 10 ml of TBS-milk buffer containing anti-CelF immunoglobulin G1 (IgG1). IgG1 was obtained from anti-CelF rabbit serum saturated with crude extracts of *E. coli* BL21(DE3) by the method described by Ey et al. (10). The blot was washed twice for 10 min with TBS-milk buffer and twice for 10 min with TBS-0.2% Tween before incubation for 30 min in 10 ml of 1:2,000 (vol/vol) goat anti-rabbit IgG conjugated with horseradish peroxidase in TBS-milk buffer (chemiluminescence Western blotting kit; Boehringer Mannheim).

Biotin labeling of protein and biotin-labeled-protein detection. The biotinylation of mini-CipC₁ and the incubation of the PVDF blot with the biotin-labeled protein were performed as described previously (23). Labeled proteins were detected with a Western blotting kit (biotin/streptavidin; Boehringer Mannheim).

Biosensor-based protein binding assay. Real-time protein-protein interaction analysis was performed at 25°C with a BIAcore system (Pharmacia). Biotinylated mini-CipC₁ was coupled to a streptavidin-dextran layer on a sensor chip surface, and intact CelF was used in kinetic runs under the conditions described by Pagès et al. (23). The resulting sensorgram data were analyzed with BIAevaluation software (Pharmacia).

RESULTS

Purification. In a previous study, we constructed two expression phasmids using pET22b+ in order to overproduce CelF in

E. coli BL21(DE3) (26). One of these, pETFc, was designed with a view to obtaining a mature cytoplasmic CelF protein carrying a C-terminal hexahistidine tail. When the induction was performed at 37°C, cytoplasmic inclusion bodies were formed. Under these conditions, we also observed that the protein was subject to some degradation. We optimized the induction of the expression of *celF* from pETFc in order to produce a soluble entire cytoplasmic protein. The optimized conditions are described in Materials and Methods. In short, the culture was incubated at 37°C to an optical density at 600 nm of 2. Induction was performed by adding 40 μ M IPTG and incubating the culture for 16 h at 16°C. The cells collected were broken with a French pressure cell. CelF was purified from the crude extract supernatant by Ni²⁺ affinity chromatography. Fifty-three milligrams of pure CelF was obtained from 2.7 liters of culture (Table 1). The apparent molecular mass of the purified protein was estimated to be 79 kDa by SDS-PAGE analysis (Fig. 1A, lane F_L). This value matched the molecular mass calculated from the sequence (78,821 Da). After 1 week of storage at 4°C, a smaller form of CelF appeared. It was called truncated CelF (Fig. 1A, lane F_S). The whole and the truncated (71-kDa) forms showed the same N-terminal sequence. Truncated CelF lacked part or all of the C-terminal duplicated segment, as previously observed with CelA and CelC (12, 13). Although this type of spontaneous proteolytic phenomenon has not yet been elucidated, it has been described for numerous other proteins (31, 33). Truncated CelF and

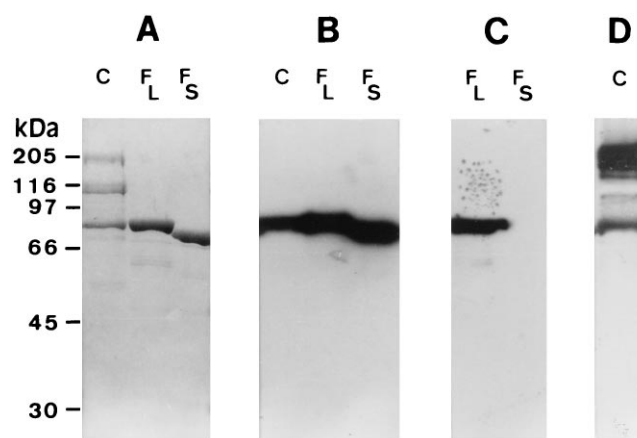


FIG. 1. Identification of CelF as part of the cellulosome. (A) SDS-PAGE (9% acrylamide) of the cellulosome (lane C, 20 μ l), the purified intact CelF (lane F_L, 3 μ g), and the purified truncated CelF (lane F_S, 3 μ g). The gel was stained with Coomassie brilliant blue. Molecular masses are indicated on the left. (B) Western blotting analysis of the same fractions using anti-CelF rabbit antibodies. Lanes are the same as in panel A. (C and D) Interaction of CelF with CipC, the scaffolding protein of the cellulosome. In panel C, intact CelF (lane F_L) and truncated CelF (lane F_S) were blotted onto a PVDF membrane and probed with 7 μ g of biotin-labeled mini-CipC₁ (23). The blot was revealed with streptavidin-peroxidase complexes. (D) Separated cellulosomal proteins were transferred onto a PVDF membrane. The blot was incubated for 30 min in 10 ml of TBS-milk buffer containing intact CelF (8 μ g) and washed twice for 15 min in TBS-milk buffer and twice for 15 min in TBS-Tween before being incubated with antiserum raised against CelF.

TABLE 2. Activities of intact CelF and truncated CelF on various substrates

Substrate	Concn (%, wt/vol)	Sp act (IU/ μ mol) ^a	
		F _L	F _S
Polymeric substrate			
PAS-cellulose	1.6	42.5	30.5
Avicel-cellulose	8	13.4	10.6
BMC-cellulose	0.8	3.9	2.8
CM-cellulose	0.8	ND	1.4
Lichenan	0.8	—	ND
Laminarin	0.8	—	ND
Oat spelts xylan	0.8	—	ND
Oligomeric substrate			
G6	—	—	+++
G5	—	—	++
G4	—	—	+
G3	—	—	ND
G2	—	—	ND

^a F_L, intact CelF; F_S, truncated CelF; ND, not detected; —, not determined.

intact CelF were separated by performing chromatography on a Ni-NTA column, with the truncated form passing through the column and the intact form adsorbed. The pIs of intact CelF and truncated CelF were 5.1 and 4.7, respectively.

Substrate specificity. CelF is able to degrade PAS-cellulose, Avicel-cellulose, and BMC-cellulose (Table 2). The rate of hydrolysis of amorphous cellulose is greater than those of crystalline celluloses. On the other hand, only truncated CelF was able to weakly degrade CM-cellulose, the substituted soluble cellulose. G6, G5, and G4 are degraded, but not G3 and G2. No activity could be detected on xylan, laminarin, or lichenan. The two forms, truncated CelF and intact CelF, exhibit noticeable differences in their activity, as previously observed in the case of endoglucanases CelA and CelC (12, 13).

Nature of reducing ends released. The distribution of reducing sugars released by a cellulase from a cellulosic substrate between insoluble and soluble fractions was determined to distinguish the endocellulases which released a large quantity of insoluble reducing ends (30 to 50%) from the processive enzymes (also called exocellulases), where the insoluble reducing sugar released amounted to <10% (16, 17). The products of PAS-cellulose degradation by truncated CelF and truncated CelA (one endocellulase) were examined and compared as described by Irwin et al. (16). As shown in Fig. 2, the sugars produced by truncated CelF were about 95% soluble and 5% insoluble, whereas those produced by truncated CelA were only 42% soluble and 58% insoluble. These results indicate that CelF is a processive enzyme.

Catalytic properties of CelF. The pH and temperature optima as well as thermostability were studied with truncated CelF, using PAS-cellulose as a substrate. The degradation kinetics were recorded for 30 min. The enzyme was most active between pH 5.5 and 6. The optimum temperature was found to be 55°C. Truncated CelF is stable up to 45°C but is rapidly denatured at 50°C. Addition of either 1 mM EDTA, 10 mM CaCl₂, 10 mM MgCl₂, or 10 mM LiCl₂ had no effect on the specific activity of the enzyme. The apparent K_m and k_{cat} of truncated CelF towards PAS-cellulose were 2.4 g/liter and 30.5 min⁻¹, respectively.

HPLC analysis of reaction products. First, the degradation products of cellodextrins were analyzed. A total of 10% of the G6 was found to be cleaved into 2 G3, and 90% was cleaved into G4 plus G2. G3 and G2 were the only hydrolytic products

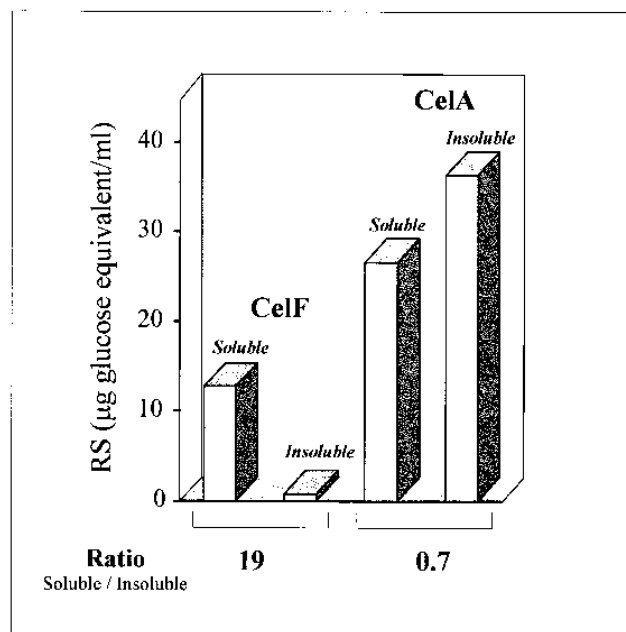


FIG. 2. Distribution of reducing ends between cellulose (insoluble fraction) and supernatant (soluble fraction) after hydrolysis of PAS-cellulose by CelF or CelA. Reducing sugars (RS) were measured at time zero and after incubation of 23 μ g of truncated CelF or 4 μ g of truncated CelA for 30 min with 5 ml of 1.6% (wt/vol) PAS-cellulose in 20 mM Tris-maleate buffer (pH 6) in the supernatant and in the twice-washed insoluble fraction. After 30 min of hydrolysis, <1% of the cellulose had been digested by CelF or CelA (% of digestion = amount of soluble and insoluble reducing sugar released [glucose equivalent] \times 100/cellulose concentration at time zero [glucose equivalent]).

of G5, and G4 yielded only 2 G2. Neither G3 nor G2 was found to be degraded by CelF.

The pattern of the degradation products of PAS-cellulose exhibited a great variety of molecules at the beginning of the reaction (Fig. 3). All the soluble cellodextrins (G6 to G2) were present in the reaction mixture after 10 min of incubation at 45°C. After 1 h, cellobiose was the major product (70%) and G3 and G4 amounted to 20 and 10%, respectively. No G5 and G6 were detected by this stage.

Functional type of CelF. When studying the action of truncated CelF on CM-cellulose, we observed first that the enzyme exhibited a quite limited action on this substrate (26) (Fig. 4a) and second that this degradation process looked like a random β -1,4-linkage hydrolysis process along the whole chain (end-mode of action): a significant increase in fluidity of the CM-cellulose solution was observed during the limited degradation effected by truncated CelF (Fig. 4b). The slopes of curves $F_{sp} = f$ (reducing sugars), where F_{sp} is specific fluidity, obtained with truncated CelF and the endoglucanase CelC were almost identical. We hypothesized that CelF might initially hydrolyze cellulose molecules like an endocellulase and might subsequently hydrolyze the substrate in a recurrent manner. The hypothetical recurrent mode of action might be inhibited by the presence of carboxymethyl groups. In order to check this second point, we tested the capacity of CM-cellulose to trap the enzyme. Thirty micrograms of CelF (truncated CelF) was incubated for 10 min with 2% CM-cellulose before the hydrolysis of PAS-cellulose was evaluated (Fig. 5). A significant decrease in the PAS-cellulase activity was observed when truncated CelF was preincubated with CM-cellulose. Thirty-four percent of the enzyme was not available in that case. Simultaneous incubation of the enzyme with both substrates had only

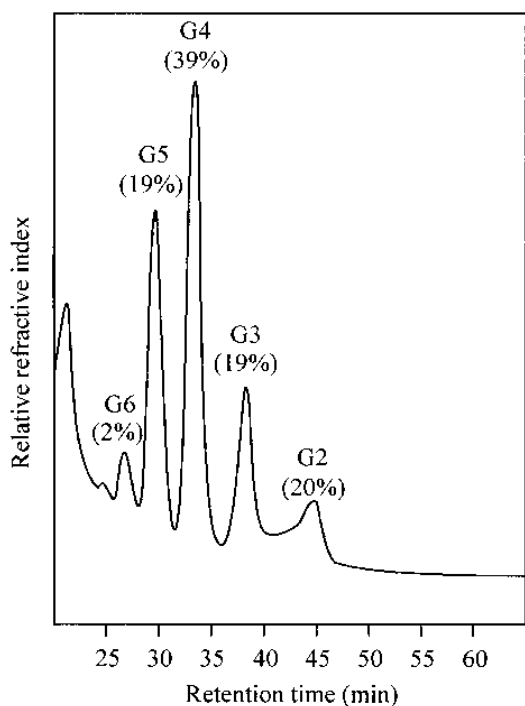


FIG. 3. HPLC analysis of PAS-cellulose degradation products. A 500- μ l volume of 3% PAS-cellulose was incubated with 30 μ l of enzyme solution (19 μ g) for 10 min. A 50- μ l centrifuged and boiled sample was analyzed. The percentages of nanomoles of products are shown in parentheses.

weak effects on the cellulase activity due to the great difference between the K_m values (the apparent K_m towards CM-cellulose was found to be 35 g/liter).

To evaluate the influence of the number of extremities on the PAS-cellulase activity, PAS-cellulose was pretreated with truncated endoglucanase CelA and washed twice with 20 mM Tris-maleate buffer (pH 6) (Fig. 6). A decrease in the cellulase activity of truncated CelF was observed with increasing pre-

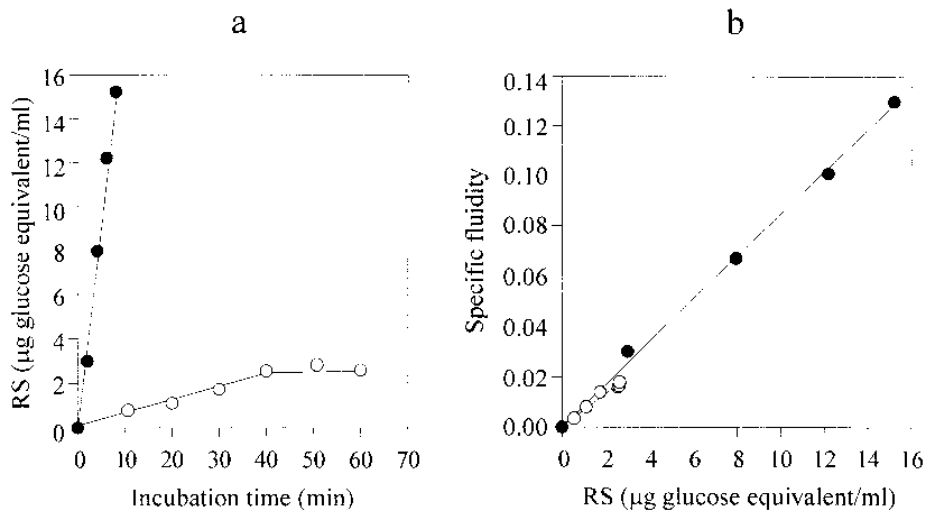


FIG. 4. CM-cellulose hydrolysis by CelF (○) and by the endoglucanase CelC (●). (a) Release of reducing sugars (RS) versus time. (b) Increase in specific fluidity versus release of RS. The specific fluidity (F_{sp}) was calculated by using the formula $F_{sp} = t^0/(t - t^0)$, where t^0 is the flow time of water and t is the flow time of the CM-cellulose solution. Enzymatic reactions were performed at 37°C using medium-viscosity CM-cellulose (Sigma) as a substrate at a final concentration of 0.8% (wt/vol) in 20 mM phosphate buffer (pH 7) and 300 μ g (4,200 pmol) of truncated CelF or 1 μ g (24 pmol) of truncated CelC (12).

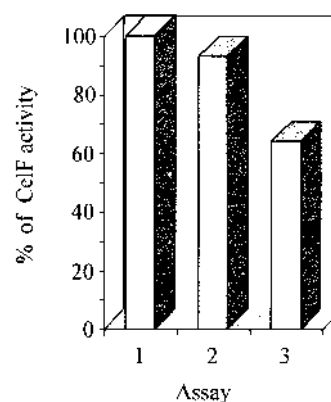


FIG. 5. Trapping of CelF by CM-cellulose. (Assay 1) A 30- μ g sample of truncated CelF was preincubated for 10 min at 45°C in 1 ml of Tris-maleate buffer (pH 6) before 4 ml of 1% PAS-cellulose was added and the hydrolytic activity was evaluated as described in Materials and Methods. (Assay 2) A 30- μ g amount of truncated CelF was preincubated for 10 min at 45°C in 250 μ l of buffer before 4 ml of 1% PAS-cellulose and 750 μ l of buffer containing 15 mg of CM-cellulose were added. (Assay 3) A 30- μ g sample of truncated CelF was preincubated with 15 mg of CM-cellulose in 1 ml of buffer for 10 min before 4 ml of 1% PAS-cellulose was added.

treatment times. Neither CelA nor CelF contained any cellulose-binding domain (9, 26). The results obtained therefore could not be interpreted as reflecting the removal by CelA of the CelF cellulose binding site. They show, rather, that CelA hydrolyzed potential CelF sites.

Identification of CelF as one of the three major components of the cellulosome. The cellulosome was extracted from the supernatant of a culture of *C. cellulolyticum* on cellulose MN300 by adsorption on Avicel. The adsorbed complexes were eluted by being incubated at 100°C in electrophoresis sample buffer before SDS-PAGE analysis. As previously mentioned by Madarro et al. (20), three major components were observed, one of which had the same M_r as intact CelF (Fig. 1A) and was recognized by anti-CelF antibodies (Fig. 1B). When the N-terminal sequence of the corresponding protein

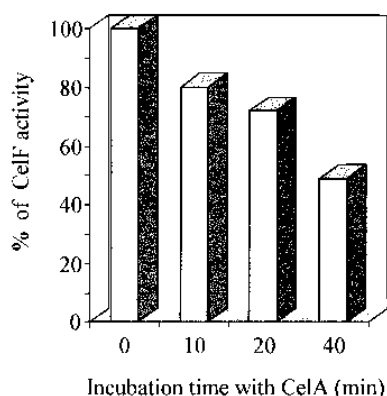


FIG. 6. Degradation of CelA-pretreated PAS-cellulose by CelF. In each case, 4 ml of twice-washed 1% PAS-cellulose pretreated with 4 μ g of truncated CelA was added to 1 ml of diluted truncated CelF (48 μ g). After 40 min of incubation with CelA, <1% of the substrate had been digested (calculated as described for Fig. 2).

isolated from the cellulosome was determined, this major component turned out to be CelF (13a). The analyzed sequence matched the predicted sequence (26).

Interaction between CelF and the scaffolding protein CipC.

(i) Blotting analysis. Intact CelF and truncated CelF were blotted onto a PVDF membrane. The membrane was incubated with biotinylated mini-CipC₁ protein. This truncated form of CipC contains the cellulose-binding domain, hydrophilic domain 1, and cohesin domain 1 (23). The mini-CipC₁ was found to bind to only the entire form (Fig. 1C). Truncated CelF was not recognized. As with the endoglucanase CelA of *C. cellulolyticum*, the C-terminal dockerin domain was necessary for CelF to be recognized by mini-CipC₁ (23). In another interaction blotting analysis, the blotted cellulosome proteins were incubated with intact CelF. Interactions of intact CelF with cellulosomal proteins were detected with anti-CelF antibodies. An intense signal was observed slightly below 205 kDa, corresponding to the binding of intact CelF to the largest major cellulosome component, thought to be CipC (Fig. 1D). As expected, the antibodies also recognized the intact blotted CelF component.

(ii) Biomolecular interaction analysis. The interaction between intact CelF and mini-CipC₁ was studied by using a BIAcore biosensor-based analytical system. Biotinylated mini-CipC₁ was immobilized on a streptavidin sensor chip. Three sensorgrams were obtained with three different intact CelF concentrations (210, 420, and 840 nM) injected for 3 min on immobilized mini-CipC₁. BIAevaluation software was used to analyze each sensorgram and to calculate the association and the dissociation rate constants (k_{on} and k_{off}). The apparent equilibrium dissociation constant was calculated from mean values of k_{on} and k_{off} ($K_d = k_{on}/k_{off} = 10^{-8}$ M). The histidine tail did not seem to prevent the interaction between intact CelF and mini-CipC₁. A K_d of 7×10^{-9} was estimated by studying the formation of the complex mini-CipC₁-CelA (23). This indicates that the two cellulases bind mini-CipC₁ with the same high affinity.

DISCUSSION

As previously described by Fierobe et al. in the case of *celA* and *celC* (12, 13), expression of the *celF* gene in *E. coli* in the present study resulted in the purification of two forms of the recombinant protein, a large form termed intact CelF (the

estimated M_r of which corresponds to the entire mature protein) and a smaller form termed truncated CelF (a C-terminal truncated form with an altered dockerin domain). Truncated CelF is a spontaneous degradation product of the purified intact form and was purified thanks to its inability to bind to Ni-NTA resin. The dockerin domain of *C. cellulolyticum* and *Clostridium thermocellum* cellulases was found to be necessary to anchor them to the cellulosome scaffolding proteins (23, 32). The presence or the absence of the C-terminal dockerin domain affects the activity of CelF, as previously found to occur in the case of endoglucanases CelA and CelC, which indicates that the catalytic domain and the dockerin domain are not independent, maybe because of the very short linker domain in *C. cellulolyticum* cellulases.

CelF is one of the three major components of the cellulosome of *C. cellulolyticum*. CelS, another family 48 glycosyl hydrolase (family L of β -1,4-glycanases) which is very homologous to CelF, is one of the two major components of the cellulosome of *C. thermocellum* (21). Likewise, P70, one of the three major components of the cellulosome of *Clostridium cellulovorans*, has been classified in the same structural family as CelF and CelS (8, 28). These enzymes seem to occupy a predominant position in the clostridial cellulolytic system and probably all play similar roles.

Studies of the degradation of various substrates have indicated that CelF is more active on swollen Avicel than on microcrystalline Avicel or on substituted soluble CM-cellulose. This pattern of substrate specificity, differing completely from that of endoglucanases, which are most active on CM-cellulose (12, 13), along with the ability of CelF to produce a high proportion of soluble reducing ends, indicates that it is a processive enzyme. All the other family 48 enzymes, namely, CelS from *C. thermocellum* ATCC 27405 (17), S8 from *C. thermocellum* YS (22), Avicelase II from *Clostridium stercoararium* (5), and CbhB from *Cellulomonas fimi* (27), have been characterized as exoglucanases. Like CelF, they act preferentially on amorphous cellulose rather than Avicel- or CM-cellulose and release cellobiose and celotriose as final cellulose degradation products. Three-dimensional-structure studies have shown that the active sites of endocellulases (7, 9) are located in a groove where random binding with one long chain of cellulose occurs. Processive enzymes exhibit a tunnel-shape enzymatic site. This topology may explain why these enzymes were found to have little or no activity on CM-cellulose. It was proposed that the recurrent mode of action was blocked by carboxymethyl substituents. Glycosyl hydrolases with different substrate specificities can be found in the same structural family, as is the case for CBHII, a processive enzyme from *Trichoderma reesei*, and endoglucanase E2 from *Thermomonospora fusca*, in the former of which two loops close the groove into a tunnel (7). Davies and Henrissat (7) have suggested that the initial mode of introduction of the substrate into the processive enzyme active site was not at all clear-cut; the possibility that an occasional opening of the tunnel might occur could not be ruled out. Stahlberg et al. (30), upon observing that the *T. reesei* purified CBHII protein was able to release insoluble reducing ends from cellulose, concluded that this enzyme was not a true exocellulase. A similar phenomenon was also observed, although to a lesser extent, with CBHI. Shen et al. (27), upon studying the exocellobiohydrolase CbhB, have observed that this enzyme was able to produce clearance zones on CM-cellulose plates, although the slope of the plot of Fsp versus reducing sugar production was very low compared to that of the endoglucanase CenA. They concluded that CbhB was predominantly but not exclusively exohydrolytic. Morag et al. (22) observed that a truncated form of the cellobiohydrolase S8

purified from the cellulosome of *C. thermocellum* exhibited a low but distinct endoglucanase activity, as revealed by Congo red zymograms on native polyacrylamide gels. Studies of CelF, a recombinant protein which could not be contaminated by any other cellulase, have shown that this processive enzyme also exhibits an endo-mode of action. Indeed, the increase in the fluidity of the CM-cellulose solution observed as a function of the amount of reducing sugars released during hydrolysis on the one hand and the diversity of the PAS-cellulose degradation products at the beginning of the reaction on the other hand show that CelF initially has an endo-mode of hydrolysis. This hypothesis is supported by the fact that PAS-cellulose pretreated with the endoglucanase CelA was less markedly degraded by CelF than the original substrate. CelA and CelF are able to hydrolyze the same sites. Interestingly, the CM-cellulose degradation by truncated CelF is stopped after a short period of incubation. It seems highly probable that under our experimental conditions, the enzyme, which showed a recurrent mode of action on the cellulose molecules, may have been rapidly blocked by carboxymethyl substituents. This was corroborated by the present finding that CM-cellulose is able to trap enzyme molecules, which means that they will then no longer be available for PAS-cellulose hydrolysis. The iterative gliding movement of the enzyme was stopped by steric overcrowding, and the enzyme wedged into the substrate. We therefore concluded that CelF is a processive endocellulase.

The last important point worth discussing is the low catalytic efficiency of CelF and homologous proteins. First, this low efficiency is surprising in a major component of the cellulolytic system. Many explanations can be proposed: (i) we may have lost some essential cofactors during the purification process; (ii) we possibly did not find or we were not able to obtain the best substrate for this enzyme (e.g., cellodextrins with a degree of polymerization of >6); and (iii) this protein, in spite of its low efficiency when acting alone, is of great importance to the synergistic hydrolysis of cellulose by all the enzymes together. As suggested by Davies and Henrissat (7), processivity is probably a key factor contributing to the efficient degradation of crystalline cellulose by cellulolytic systems. If one compares enzymatic parameters of CelF acting on its most efficient substrate, PAS-cellulose ($K_m = 2.4$ g/liter; $k_{cat} = 30$ min $^{-1}$), with those of endoglucanases CelA ($K_m = 4$ g/liter; $k_{cat} = 6,644$ min $^{-1}$) and CelC ($K_m = 2.5$ g/liter; $k_{cat} = 3,900$ min $^{-1}$) acting on their most efficient substrate, CM-cellulose, it can be seen that the K_m values are equivalent but that the k_{cat} of CelF is about 2 orders of magnitude lower than those of endoglucanases. It might be speculated that the recurrent walking of the enzyme along the cellulose molecule is a slow process.

It would now be very interesting to test the potential synergisms by associating CelF with the scaffolding protein CipC and various other *C. cellulolyticum* cellulases in crystalline cellulose degradation studies.

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