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Crystallization of the catalytic domain of *Clostridium cellulolyticum* CelF cellulase in the presence of a newly synthesized cellulase inhibitor

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Abstract

The catalytic domain of the CelF processive endocellulase, a family 48 glycosyl hydrolase from *Clostridium cellulolyticum* has been crystallized in the presence of a newly synthesized inhibitor (methyl 4-*S*-β-cellobiosyl-4-thio-β-cellobioside), by vapour diffusion, using PEG as a precipitant. The protein crystallizes in the orthorhombic $P2_12_12_1$ space group and diffracts to a resolution of 2.0 Å. The unit-cell parameters are $a = 61.4$, $b = 84.5$, $c = 121.9$ Å.

1. Introduction

Clostridium cellulolyticum, a cellulolytic, mesophilic and anaerobic bacterium, produces extracellular cellulolytic macromolecular complexes called cellulosomes (Madarro *et al.*, 1991; Gal *et al.*, 1998): these complexes allow bacteria to grow on crystalline cellulose as sole source of carbon and energy (Giallo, Gaudin, Bélaich, Petitdemange & Caillet, 1983). Cellulosomes from various *Clostridium* species have been studied for several years (Lamed & Bayer, 1988; Doi, Goldstein, Hashida, Park & Takagi, 1994; Gal *et al.*, 1998). They are composed of a scaffolding protein, which is able to bind strongly to cellulose, around which are organized various enzymatic subunits (Salamitou, Tokatlidis, Béguin & Aubert, 1992; Salamitou *et al.*, 1994; Pagès *et al.*, 1996).

CelA and CelC, two components of the *C. cellulolyticum* cellulosome, have been extensively characterized, and the three-dimensional structure of the catalytic core of CelA, a family 5-glycosyl hydrolase (Henrissat, 1991), has been solved (Ducros *et al.*, 1995). CelA and CelC are endocellulases (β-1,4-hydrolyzing enzymes showing a random attack on cellulose polymers) which are composed of two domains: a catalytic core and a dockerin domain at their C terminus (Fierobe *et al.*, 1991, 1993). The presence of the CelA dockerin domain is essential for a high-affinity interaction between CelA and a cohesin domain of the *C. cellulolyticum* scaffolding protein CipC (Pagès *et al.*, 1996).

Cellulolytic systems of fungi and bacteria contain several endocellulases and at least one processive cellulase (Davies & Henrissat, 1995). Processive cellulases only weakly degrade cellulosic substrates but they have been reported to be essential components for the efficient degradation of crystalline cellulose (Davies & Henrissat, 1995), together with endocellulases and β-glucosidases (Fägerstam & Pettersson, 1980; Henrissat, Driguez, Viet & Schülein, 1985; Barnett, Berka & Fowler, 1991).

CelF has recently been characterized to be a processive cellulase which also performs some random attacks on cellulose (Reverbel-Leroy *et al.*, 1996; Reverbel-Leroy, Pagès, Bélaich, Bélaich & Tardif, 1997). Moreover, this protein is believed to play a key role in *C. cellulolyticum* cellulosomes, as one of the three major components of these complexes. On the basis of amino-acid sequence similarities, CelF is composed of two domains: an N-terminal catalytic domain which was classified to be a member of glycosyl hydrolase family 48, and a C-terminal dockerin domain which was shown to be necessary for the interaction with a cohesin domain of CipC. Many cellulases have been described as members of family 48 of glycosyl hydrolases (formerly family L) (Henrissat & Bairoch, 1996): CelS from *C. thermocellum* strain ATCC27405 (Wang, Kruus & Wu, 1993), CbhB from *Cellulomonas fimi* (Shen, Gilkes, Kilburn, Miller & Warren, 1995), CelA from *Caldocellum saccharolyticum* (Té'o, Saul & Berquist, 1995), CelY from *C. stercorarium* (Bronnenmeier, Rucknagel & Staudenbauer, 1991; GenBank accession number P50900) (classified on the basis of the entire sequence comparison), and P70 from *C. cellulovorans* (Doi *et al.*, 1993), ORF1p from *C. josui* (Fujino, Karita & Ohmiya, 1993), E6 from *Thermomonospora fusca* (Barr, Hsieh, Ganem & Wilson, 1996), S8 from *C. thermocellum* strain YS (Morag, Halevy, Bayer & Lamed, 1991) (classified upon a partial sequence comparison). No structure has been solved so far for any member of this family. Here, we describe the cocrystallization of the catalytic core of CelF with a thiooligosaccharide inhibitor. The first structure of EG1 cellulase from *Humicola insolens* complexed to a non-hydrolyzable tetrathiocellopentaose has been determined by X-ray crystallography recently (Sulzenbacher, Driguez, Henrissat, Shülein & Davies, 1996). The use of an analogue of substrate in which all the interglycosidic O atoms were substituted with S atoms was necessary since this enzyme EG1 (family 7) hydrolyzes various glycosidic bonds of cellotetraose and cellopentaose molecules (Claeyssens & Henrissat, 1992). For the present study, the CelF cleavage pattern of cellotetraose into two cellobiose units (Reverbel-Leroy *et al.*, 1997) led us to design and synthesized a molecule in which only the scissile O atom was replaced by an S atom.

2. Materials and methods

2.1. *Protein expression and purification*

CelF was obtained from BL21(DE3)[pETFc] culture as previously described (Reverbel-Leroy *et al.*, 1997). In short, the

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culture was incubated at 310 K until OD_{600} = 2 was reached. Then, the culture was stabilized at 289 K for 30 min before adding 40 μM isopropyl β-D-thiogalactopyranoside (IPTG), and incubated for 16 h at the same temperature. Harvested cells were broken using a French press. CelF was purified by Ni^{2+} affinity chromatography from the crude extract supernatant. The apparent molecular mass of the purified protein was found to be 79 kDa by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis. After 1 week of storage at 277 K, a truncated form of CelF (71 kDa) appeared. The truncated CelF was separated from the intact form by chromatography on an Ni–NTA column as previously described (Reverbel-Leroy *et al.*, 1997). The purified protein (truncated form) was dialysed against water and concentrated on 24 mg ml^{-1} using a PM 30 membrane (Amicon) prior to performing crystallization experiments. Protein concentration was estimated by the method of Lowry *et al.* using bovine serum albumin as a standard (Lowry, Rosebrough, Farr & Randall, 1951). Homogeneity of purified truncated protein was checked by SDS–PAGE and isoelectric focusing (IEF) analysis.

2.2. *Synthesis of IG4 inhibitor*

General methods and materials are as previously described (Moreau, Viladot, Samain, Planas & Driguez, 1996). The synthesis is illustrated in Fig. 2.

2.2.1. *2,3,6-Tri-O-acetyl-1-S-acetyl-4-O-(2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl)-β-D-glucopyranose (compound 2).* Compound 2 (Fig. 2) (2.4 g, 58%) was synthesized from the peracetylated cellobiose (4 g, 5.76 mmol) *via* the bromide (Fig. 2, compound 1) as previously described (Schou, Rasmussen, Schülein, Henrissat & Driguez, 1993; Moreau *et al.*, 1996). M.p. 466 K (from ether); $[\alpha]_D$-19° (*c* 0.56, $CHCl_3$); rate percent of the theoretical composition for $C_{28}H_{38}O_{18}S$: C, 48.41; H, 5.47; S, 4.61%; rate percent found: C, 48.40; H, 5.52, S, 4.70%; FABMS(+): *m/z* 718 $(M^+Na)^+$, 618 $(M\text{-SAc})^+$; ^{13}C-NMR ($CDCl_3$) δ = 191.72 ($S\underline{C}OCH_3$); 170.3, 170.1, 170.0, 169.5, 169.4, 169.2, 168.9 ($O\underline{C}OCH_3$); 100.5 (C^{II}-1); 80.0 (C-1); 77.1, 75.9, 73.4, 72.8, 71.9, 71.5, 69.2, 67.8 (C-2,3,4,5; C^{II}2,3,4,5); 61.8, 61.5 (C-6; C^{II}-6); 30.7 ($SCO\underline{C}H_3$); 20.7, 20.5, 20.5, 20.4 ($OCO\underline{C}H_3$).

2.2.2. *Methyl O-β-D-glucopyranosyl-(1→4)-S-β-D-glucopyranosyl-(1→4)-O-(4-thio-β-D-glucopyranosyl)-(1→4)-β-D glucopyranoside [methyl 4^{II}-S-β-cellobiosyl-4-thio-β-cellobioside]; (IG4) (compound 6).* Methyl 4^{II}-*O*-trifyl-lactoside (compound 4), obtained from methyl 2,3,6-tri-*O*-benzoyl-4-*O*-(2,3,6-tri-*O*-benzoyl-β-D-galactopyranosyl)-β-D-glucopyranoside (Cox, Metzner & Reist, 1978) (compound 3) (590 mg, 0.53 mmol) as described before for the corresponding galactoside (Schou *et al.*, 1993) was added to a solution of fully acetylated thiocellobiose (compound 2) (370 mg, 0.53 mmol) in dry *N,N*-dimethylformamide (5 ml) containing diethylamine (1.2 ml) (Bennet, von Itzstein & Kiefel, 1994). The resulting mixture was stirred under argon at room temperature for 15 h, then diluted with ethyl acetate (60 ml), and extracted with water (2 × 50 ml). The extract was dried, concentrated, and partially purified by flash chromatography (acetone–cyclohexane 1:2). Impure compound 5 was isolated (470 mg, 53%). A stirred solution of impure compound 5 (155 mg) in dry methanol (10 ml) was treated with 1 *N* methanolic sodium methoxide (690 µl). The solution was stirred at room temperature for 15 h, then neutralized (Amberlite IRN 77H^+), filtered, and the filtrate was concentrated to dryness. The product was dissolved in water, freeze dried, and subjected to high-pressure liquid chromatography (µ-Bondapak NH2 column using acetonitrile–water 75:25 as an eluent). The so-obtained expected compound 6 (also called IG4) was pure (53 mg, 42% from compound 3). $[\alpha]_D$-34° (*c* 0.44, H_2O); rate percent of the theoretical composition for $C_{25}H_{44}O_{20}S$. $4H_2O$: C, 39.06; H, 6.81; S, 4.17%; rate percent found: C, 38.91; H, 6.39; S, 4.24%; FABMS(+): *m/z* 697 $(M)^+$, ^{1}H-NMR (D_2O) δ = 4.56 (d, 1H, H^{III}-1, $J_{1,2}$ = 9.8 Hz), 4.39, 4.38 (d, 2H, H^{II}-1, H^{IV}-1 $J_{1,2}$ = 7.9 Hz), 4.28 (d, 1H, H-1, $J_{1,2}$ = 7.9 Hz), 3.46 (s, 3H, OCH_3), 2.80 (dd, 1H, H^{II}-4, $J_{4,3} = J_{4,5}$ = 10.9 Hz); ^{13}C-NMR (D_2O) δ = 104.5, 104.0, 103.8 (C-1, C^{II}-1, C^{IV}-1), 85, 1 (C^{III}-1), 80.1, 80.0, 79.7, 77.9, 77.5, 77.0, 76.2, 75.8, 74.6, 74.3, 74.1, 73.7, 70.9 (C-2,3,4,5, C^{II}-2,3,5, C^{III}-2,3,4,5, C^{IV}-2,3,4,5), 62.7, 62.0, 61.5, (C-6, C^{II}-6, C^{III}-6, C^{IV}-6), 58.7 (OCH_3), 48.4 (C^{II}-4).

2.3. *Enzyme assays*

Cellulase activity was assayed by mixing 1 ml of enzyme (50 µg) with 4 ml of phosphoric acid-swollen cellulose (PAS-cellulose) in a 20 m*M* Tris–maleate buffer, pH 6. Final concentrations of PAS–cellulose in the assays were from 1 to 8 g l^{-1}. PAS–cellulose was prepared from Avicel (Fluka) as described by Wood (Wood, 1988). PAS-cellulase activity was also determined in the presence of the methyl 4-*S*-β-cellobiosyl-4-thio-β-cellobioside potential inhibitor (IG4) (0.6 m*M*) in each assay. In the $1/V = f(1/[S])$ curve, each *V* was calculated from a 20 min kinetics; [*S*] was PAS-cellulose concentration. The amounts of reducing sugars released in 1 ml were measured at various incubation times at 318 K, using the Park and Johnson method (Park & Johnson, 1949) after removing the solids by centrifugation. One unit of cellulase activity (international unit) corresponds to 1 µmol of D-glucose equivalent released per minute.

2.4. *Crystallization and preliminary crystallographic analysis*

Crystallization trials were carried out using the hanging-drop vapour-diffusion method (McPherson, 1982). Crystals of the truncated CelF were obtained at 293 K using polyethylene glycol (PEG) 4000 as a precipitant. The hanging drops containing 4 µl of 71 kDa CelF solution (20 mg ml^{-1}) in water, 2 m*M* IG4 (methyl 4-*S*-β-cellobiosyl-4-thio-β-cellobioside), and 4 µl of screening buffer, were laid down on siliconized microscope slides. Drops were equilibrated in Linbro tissue-culture plates against a 500 µl of reservoir solution containing only 0.5 or 0.3 *M* NaCl. First small crystal plates were enhanced to a size of 500 × 500 × 50 µm in a week, using macroseeding (Stura & Wilson, 1991).

A crystal was mounted in a 0.7 mm diameter glass capillary containing a small volume of solution from the well and sealed by wax. They were characterized on a MAR Research image-plate scanner mounted on a Rigaku RU-200BH rotating-anode generator working at 40 kV and 80 mA. Oscillation range and time per frame were 1° and 500 s, respectively. The data were processed using the *DENZO* program (Otwinowski, 1993) and the *CCP4* suite of crystallographic programs (Collaborative Computational Project, Number 4, 1994).

3. Results and discussion

CelF cellulase was overproduced in *Escherichia coli* BL21 (DE3) [pETFc]. The recombinant protein, including a C-

terminal hexahistidine tag, was purified by affinity chromatography on Ni–NTA column. During low-temperature (277 K) storage of the purified protein, a C-terminal truncated form of CelF appeared. The C-terminal duplicated segment may be wholly or partly lacking in the truncated CelF, as previously observed with CelA and CelC (Fierobe *et al.*, 1991, 1993). This type of spontaneous proteolytic phenomenon, independent of any external protease action, has been described with several other proteins (Stephenson & Clarke, 1989; Tomizawa, Yamada, Hashimoto & Imoto, 1995). Truncated and intact CelF were separated by performing chromatography on an Ni–NTA column, since the truncated form was not retained by the resin while the intact form is adsorbed. The concentrated purified truncated protein, showing a single IEF and SDS–PAGE band, was stored in water at 277 K.

CelF degrades three soluble cellodextrins: cellohexaose, cellopentaose and cellotetraose. The latter is the only oligomeric substrate which presents a unique scissible bond (Reverbel-Leroy *et al.*, 1997) (Fig. 1). An inhibitor was synthesized in which the unique scissible O atom of cellotetraose was replaced by an S atom. The resulting compound, the methyl 4-*S*-β-cellobiosyl-4-thio-β-cellobioside (called IG4) was obtained as described in §2 (Fig. 2) and was shown to be resistant to CelF hydrolysis.

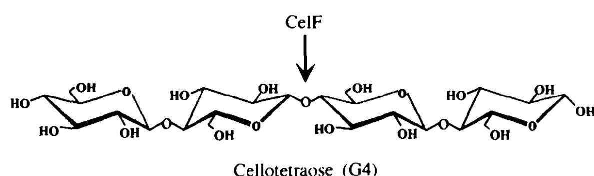

Fig. 1. Pattern of cellotetraose cleavage by CelF. Black arrow indicates the cleavage site of the cellotetraose molecule (G4) by CelF.

Some activities of truncated CelF with or without 0.6 m*M* of the methyl 4-*S*-β-cellobiosyl-4-thio-β-cellobioside potential inhibitor (IG4) were assayed using PAS–cellulose as a substrate. Fig. 3 shows Lineweaver–Burk plots $1/V = f(1/[S])$. K_m and K'_m were rated at 2.4 and 6.3 g l^{-1}, respectively. The curves revealed that IG4 is a competitive inhibitor. K_i was found to be 0.4 m*M*, according to the $K'_m = K_m(1 + [I]/K_i)$ Michaëlis formula. This high value is not very surprising. On the one hand, cellotetraose is not a very good substrate for CelF compared to PAS-cellulose (data not shown). On the other hand, the thiosaccharide analogue may bind in different sites than the natural sugar because of the presence of the thio-linkage. Studies performed by Schou *et al.*, 1993) on the inhibition of *H. insolens* CBHII by various methylthiocellooligosaccharides yielded similar K_i values, especially with the methylthiocellotetraoside (K_i = 0.27 m*M*).

Crystallization was performed by screening a series of PEG and ammonium sulfate conditions at various pH values and at two incubation temperatures (277 and 293 K). Various buffer systems were used (Acetate, Tris–HCl, Tris–maleate and HEPES) to generate pH values between 4 and 10. Two sets of screening were used, one without and the other with addition of 1 m*M* of the IG4 inhibitor in the droplet. Crystals were only obtained at 293 K in a drop containing 10 mg ml^{-1} protein,

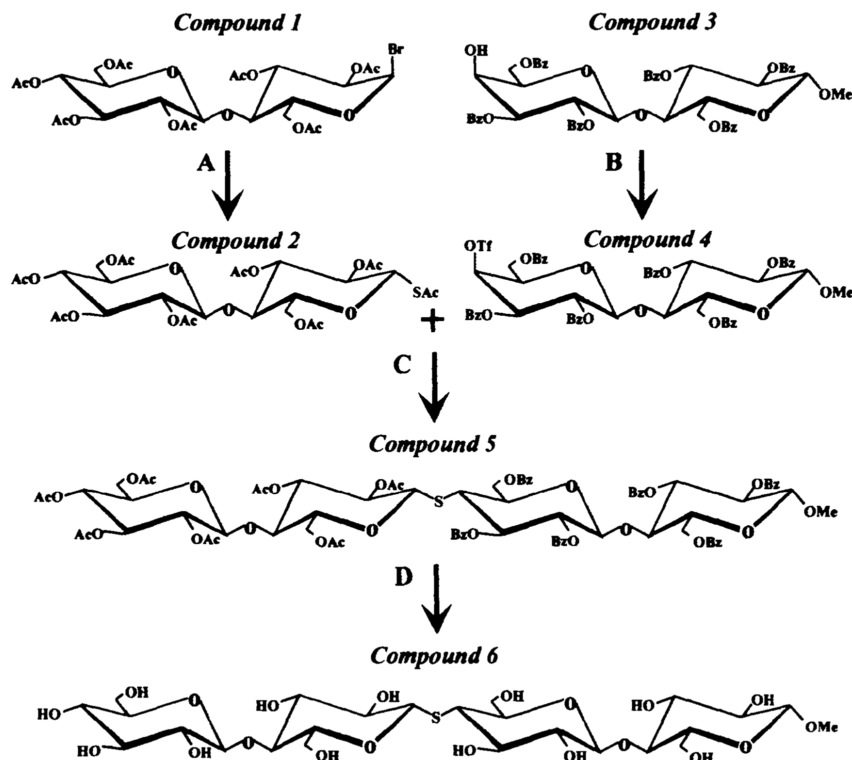

Fig. 2. Synthesis of the methyl 4-*S*-β-cellobiosyl-4-thio-β-cellobioside inhibitor (IG4). The different steps of the synthesis are *A*, $(tBu)_4N$-SAc, toluene; *B*, $(CF_3SO_2)_2O$, CH_2Cl_2, pyridine; *C*, DMF, Et_2NH; *D*, NaMeO, MeOH. The generic names of compounds are given in §2.

1 mM IG4 and 8%(w/v) PEG 4000 in 50 mM sodium HEPES pH 7.5. Since nucleation appeared to be the limiting factor, additional crystals were grown in the same buffer by macroseeding techniques. In this way, crystals of truncated CelF grew to a final size of 500 × 500 × 50 µm over a period of a week with 0.3 M NaCl in the reservoir.

Crystals of the CelF–IG4 complex obtained belonged to the orthorhombic $P2_12_12_1$ space group with cell dimensions a = 61.4, b = 84.5, c = 121.9 Å. According to the methods of Matthews (Matthews, 1968), the unit cell has a single molecule per asymmetric unit, corresponding to a solvent content of about 45%. A preliminary data set was collected up to 2.0 Å, but weak reflections could be observed to at least 1.9 Å. The data were reduced from 181 313 measurements to 43 643 unique reflections with an overall completeness of 99.9% (100% in final bin) and an overall multiplicity of 4.2 (3.4 in final bin). A final R_{merge} on intensities of 73% (24.8% in final bin) and an average $I/\sigma(I)$ of 8.0 were obtained (3.0 in final bin).

CelF, a family 48 glycosyl hydrolase, hydrolyses cellulose by an initial endo cut, followed by a processive mode of action along the cellulose chain (Reverbel-Leroy *et al.*, 1997). The structure solution of CelF should provide interesting data to understand the catalytic mechanism leading to the observed cleavage pattern. Family 48 contains several key enzymes, *e.g.* CelS from the cellulosome of *C. thermocellum* (Kruus, Wang, Ching & Wu, 1995) or CelF from *C. cellulolyticum* (Reverbel-Leroy *et al.*, 1997). No three-dimensional structure information is yet available for these important enzymes. The crystals of truncated CelF obtained in the present study are suitable for a crystallographic structure determination and the search for heavy-atom derivatives is in progress. This three-dimensional structure should provide information for a better understanding of the catalytic mechanism of cellulases belonging to family 48.

Only crystals of CelF–thiooligosaccharide inhibitor complex were obtained, which is another example of the interesting use of thiooligosaccharides as tools in glycobiology.

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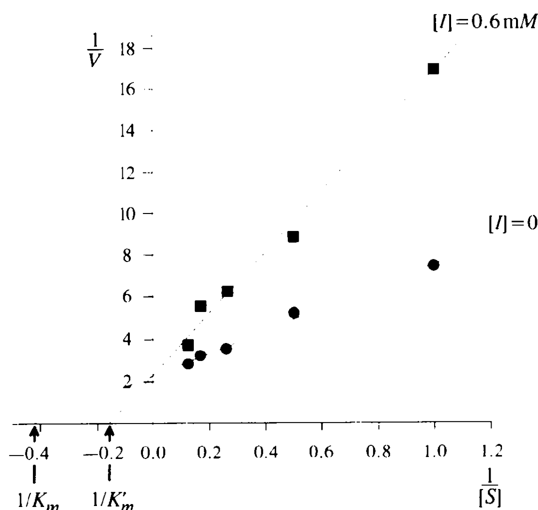

Fig. 3. Determination of the IG4 K_i by reciprocal inversed plot with PAS-cellulose as substrate. $1/V$ is expressed in mg IU^{-1} and $1/[S]$ in l g^{-1}. Black arrows indicate the positions of $1/K_m$ and $1/K'_m$.

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