ACCELERATED COMMUNICATION

Cleavage of cellulose by a CBM33 protein

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Abstract: Bacterial proteins categorized as family 33 carbohydrate-binding modules (CBM33) were recently shown to cleave crystalline chitin, using a mechanism that involves hydrolysis and oxidation. We show here that some members of the CBM33 family cleave crystalline cellulose as demonstrated by chromatographic and mass spectrometric analyses of soluble products released from Avicel or filter paper on incubation with CelS2, a CBM33-containing protein from *Streptomyces coelicolor* A3(2). These enzymes act synergistically with cellulases and may thus become important tools for efficient conversion of lignocellulosic biomass. Fungal proteins classified as glycoside hydrolase family 61 that are known to act synergistically with cellulases are likely to use a similar mechanism.

Keywords: CBM33; cellulose oxidation; GH61; cellulose degradation

Introduction

For long the biochemically challenging and economically important process of enzymatic cellulose degradation was thought to be achieved by the synergistic action of endo- and exo-acting cellulases.[1,2] Still there have been speculations that other factors may be involved, in particular factors that would make the crystalline and recalcitrant polysaccharide more accessible to the hydrolytic enzymes.[1,3–5] In order for cellulases to act on cellulose chains organized in a crystalline matrix, the enzyme will need to "extract" several consecutive sugars from their crystalline context and bind them in its active site. This is energetically demanding,[6,7] except perhaps for chain ends that may be present in limiting amounts in substrates with high crystallinity.

Studies on the enzymatic depolymerization of chitin, a cellulose analog used as a structural component in, for example, crustaceans, insects and fungi, have shown that, indeed, the concept of synergistically acting endo- and exo-enzymes may be incomplete. Some years ago, it was shown that chitinolytic bacteria produce proteins classified as family 33 carbohydrate-binding modules (CBM33; see Refs. 8,9) and most importantly, these proteins act synergistically with chitinases.[10,11] In a very recent study, it was shown that these proteins in fact are enzymes that cleave chitin chains while still being in their crystalline context, using an unprecedented mechanism that involves a hydrolytic and an oxidative step.[12] Thus, the CBM33 generates two new chain ends on the crystalline surface, one normal

Abbreviations: CBM, carbohydrate-binding module; DP, degree of polymerization; Glc, glucose; GlcA, gluconic acid; GlcLA, gluconolactone; HPAEC, high pressure anion exchange chromatography; MALDI-TOF MS, matrix-assisted laser desorption/ionization time of flight mass spectrometry.

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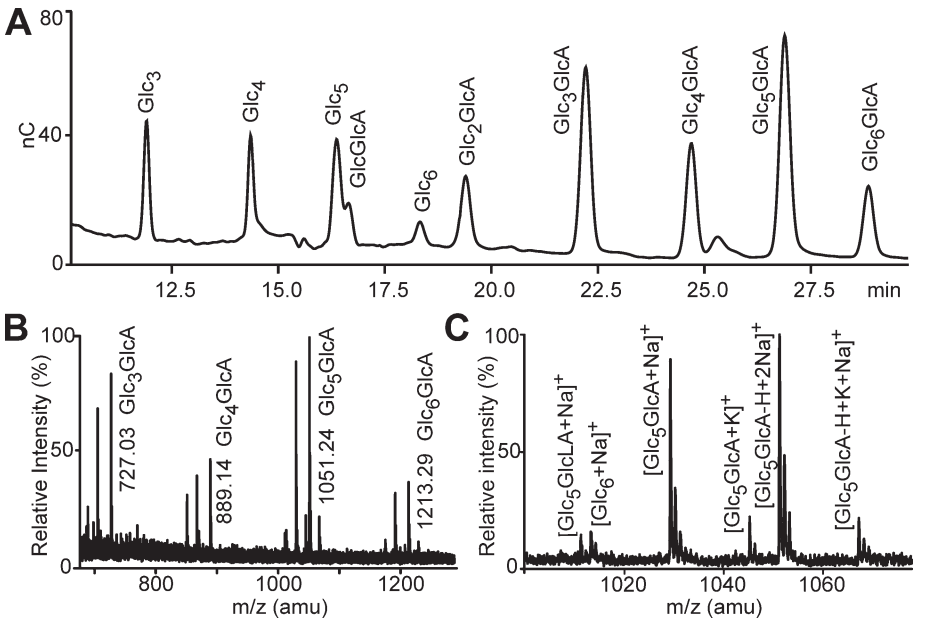

Figure 1. The action of CelS2. Panels A and B show soluble native ($Glc_{3–6}$) and oxidized ($Glc_{2–6}GlcA$) cello-oligosaccharides generated by CelS2 activity on Avicel, as detected by HPAEC (A) and MALDI-TOF MS (B). In panel B, only the major peaks in each oligosaccharide cluster are labeled. Panel (C) shows details of the mass spectrum for Glc_5GlcA. The oxidized oligosaccharide is observed as sodium and potassium adducts, and as sodium and potassium adducts of the oligosaccharide sodium/potassium salts, as is commonly seen for carbohydrates containing carboxylic groups.[19] Observed masses (*m/z*) in the Glc_5GlcA cluster are 1011.22 (Glc_5GlcLA+Na), 1013.25 (Glc_6+Na), 1029.24 (Glc_5GlcA+Na), 1045.21 (Glc_5GlcA+K), 1051.24 (Glc_5GlcA-H+2Na), and 1067.23 (Glc_5GlcA-H+K+Na). GlcLA indicates the lactone form of the oxidized cello-oligosaccharide.

nonreducing and an "oxidized reducing end," that is, an aldonic acid. It was also shown that the activity of CBM33 proteins could be boosted by adding external electron donors such as ascorbic acid.

These observations raise the question whether there exist proteins that act in a similar way on cellulose. Indeed, several bacteria that are able to degrade crystalline cellulose contain multiple CBM33 proteins[13,14] and some of these are known to be coregulated with cellulases.[15,16] Proteins classified as glycoside hydrolase family 61 (GH61) are structurally similar to CBM33[17,18] and are known to act synergistically with cellulases. However, the potentiating mechanism of these proteins remains enigmatic and activity has so far only been shown for rather complex substrates, that is, not for pure cellulose.[17] In this study, we demonstrate that CelS2 from *Streptomyces coelicolor* A3(2), a two-domain protein consisting of a 194-residue CBM33 supplemented with a well-known 99-residue cellulose-binding domain (CBM2), indeed is capable of cellulose cleavage, generating oxidized chain ends and boosting cellulase activity. This is the first time such an activity is experimentally shown.

Results and Discussion

We have cloned and analyzed the functionality of CelS2 (Uniprot ID: Q9RJY2) from *S. coelicolor* A3(2) consisting of a CBM33 domain and a C-terminal cellulose-binding domain classified as a CBM2. Figure 1 shows high pressure anion exchange chromatography (HPAEC) and matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) analyses of soluble oligosaccharides released from Avicel by CelS2 and demonstrates that this protein cleaves crystalline cellulose by a mechanism that leads to the formation of oxidized products. The observed masses [Fig. 1(B,C)] correspond to those for oxidized cello-oligosaccharides and the detection of the lactone form of the oxidized cello-oligosaccharides [two atomic mass units smaller than the native cello-oligosaccharide; Fig. 1(C)] confirms that the CelS2-derived products are aldonic acids (i.e., analogous to the products detected for CBM33 enzymes acting on chitin). The MS data and the chromatogram [Fig. 1(A)] of the oxidized CelS2-generated products also correlated with what was observed for in-house generated cello-oligosaccharide aldonic acids (Glc_nGlcA; Supporting Information Fig. S1). Figure 1(A and C) reveals the presence of both native and oxidized cello-oligosaccharides in the product mixtures. This is likely due to the low degree of polymerization (DP) of Avicel (~100; See Ref. 20), which implies that a soluble native cello-oligosaccharide is generated each time CelS2 cleaves a chain near the reducing end. As the CBM33 enzymatic mechanism includes both a hydrolytic and oxidative step,[12] it is also possible

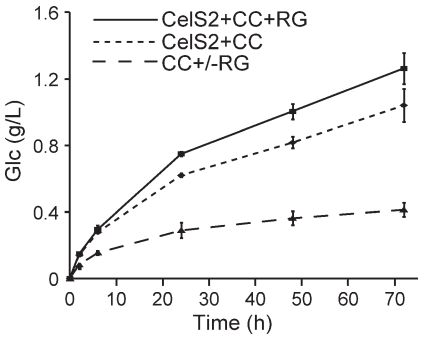

Figure 2. Degradation of high-molecular weight filter paper cellulose by Celluclast (CC), in the presence or absence of CelS2 and reduced glutathione (RG) as shown by the increase of soluble cello-oligosaccharides (Glc and Glc_2; converted to total Glc) over time. Under these conditions, reactions with only CelS2 did not yield detectable amounts of Glc or Glc_2 (not shown). The chitin-active CBM33, CBP21[12] did not affect CC efficiency (not shown). RG had no effect in reactions with only CC; only one of the two overlapping curves is shown. Data are mean ± SD ($N = 3$); error bars indicate SD. See the Materials and Methods section for experimental details.

that CelS2 occasionally generates normal reducing ends, by-passing the oxidative step. This would imply that the hydrolytic step must be the first in the order of events. Clarification of this issue awaits further studies on the catalytic mechanism of CBM33s.

As the solubility of cello-oligosaccharides is low and as these oligosaccharides tend to remain bound to the crystalline substrate, it is difficult to show soluble CelS2-generated products when using substrates with high DP. Indeed, we detected only very minor amounts of soluble products on incubation of filter paper (estimated DP ~2000; see Ref. 21) with CelS2 under the conditions used for producing Figure 1. However, by addition of a "catalytic amount" of cellulase activity, the action of CelS2 on filter paper could be visualized as a "boosting effect" on cellulose degradation, as shown in Figure 2. The data show that the hydrolytic enzymes become more active as they are presented to a more amenable substrate resulting from the action of CelS2. Figure 2 also shows that this boosting effect is increased in the presence of reductants, albeit less vigorously than previously observed for CBM33 proteins acting on chitin.

Interestingly, oxidized soluble products generated by CelS2 [Fig. 1(A and B)] show a dominance of products with an even number of sugars. This may indicate that CelS2 cleavage happens on a well-ordered chain, as would be the case when the chain is in a crystalline context. CBM33s acting on chitin show similar product patterns.[12]

These results reveal a new paradigm for enzymatic cellulose conversion and perhaps for enzymatic conversion of polysaccharides in general. Proteins classified as GH61 seem to be the fungal counterpart of the bacterial CBM33 proteins, as they are structurally similar and have a potentiating effect on cellulases,[17] although their mechanism remains unknown. The fact that such a "cellulase-boosting activity" not only occurs in fungi (GH61) but also in bacteria (CBM33) indicates the importance of this activity in nature. The occurrence of this activity in bacteria also has practical consequences as it is easier to produce the bacterial CBM33s recombinantly, with a cellulase-free background.

One important conclusion from our results is that CBM33 proteins vary with respect to their substrate specificities. In addition, we note that many microorganisms contain multiple CBM33 or GH61 encoding genes. Thus, it seems possible that additional substrate specificities may be discovered in the future. Another important issue for future studies is the role of the divalent metal ion which remains somewhat enigmatic for both CBM33 and GH61 enzymes.[11,17,18] We have used Mg^{2+} in this study because an initial screening showed high activity when this metal was added. However, several metals worked well, confirming the apparent promiscuity that has been observed in other studies of both CBM33s[11] and GH61s.[17] Purified CelS2 retained considerable activity without the addition of metals and it is likely that this activity is due to high affinity binding of another as yet unidentified metal. Addition of ethylenediaminetetraacetic acid (EDTA) inhibited the enzyme. Clearly, more work needs to be done on the role of metals in CelS2, as well as in other CBM33s and in GH61s.

Finally, and most importantly, the present findings have major implications for further development of an efficient cellulose-based biorefinery. CBM33 and GH61 enzymes may turn out to be important tools for achieving more efficient enzymatic conversion of recalcitrant lignocellulosic biomass. Further process optimization work is needed, as it is currently not immediately obvious what enzyme ratios (i.e., hydrolases versus CBM33/GH61) lead to optimal conversion rates and how these ratios depend on the substrate and the presence of reducing power (in the substrate or externally added; Forsberg *et al*., unpublished observations and Refs. 14,17). Still, there is no doubt that this novel class of enzymes will play a major role in the current quest for efficient enzymatic processes for biomass conversion.

Materials and Methods

Cloning, expression, and purification

The gene encoding the mature form of CelS2 (Uniprot ID: Q9RJY2; residues 35-364) from *S. coelicolor* A3(2) was cloned into the pET-32 LIC vector following the instructions provided by the supplier (Novagen). Successful constructs were sequenced for verification and transformed into *E. coli* Rosetta DE(3)

cells that were cultured at 37°C, and induced by 0.1 m*M* isopropyl β-D-1-thiogalactopyranoside (IPTG) at O.D. = 0.6, followed by 20 h culturing at 20°C and finally harvesting by centrifugation. Cell pellets were resuspended in 20 m*M* Tris-HCl pH 8.0, 100 μ*M* phenylmethylsulfonyl fluoride (PMSF), 0.1 mg/mL lysozyme (Sigma), and 1U/mL DNAse (Fluka) and lysed by sonication. Cell debris was removed by centrifugation and CelS2 was purified by standard immobilized metal affinity chromatography (IMAC) purification protocols using the Nickel-NTA IMAC resin (Qiagen). Purified protein was concentrated using Sartorius Vivaspin protein concentration devices with a 10 kDa cutoff. To obtain a native N-terminus, which is crucial for enzyme activity,[12] the pure protein was digested with Factor Xa according to the instructions supplied by the manufacturer (Novagen). The free His-Tag and Factor Xa were removed using IMAC chromatography and Xarrest agarose beads (Novagen), respectively. The buffer of the pure protein was finally changed to 20 m*M* Tris pH 8.0. Processing of the His-Tag and protein purity were verified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis. Protein concentrations were quantified using the Bio-Rad Bradford micro assay (Bio-Rad).

Chemical oxidation of cello-oligosaccharides to aldonic acids/lactones

Cello-oligosaccharides were obtained by trifluoroacetic acid (TFA) hydrolysis according to the method described by Wing and Freer[22] and lyophilized. The material was oxidized using a mild oxidation method that has been shown to selectively oxidize the hemiacetal carbon of carbohydrates to generate aldonic acids.[23,24] The oligosaccharide (2.90 g which included an unspecified amount of salt) was suspended in a minimum amount of water (10 mL) and mixed with an iodine solution (7.3 mmol iodine in 15 mL methanol). While stirring, a 4% (w/w) solution of KOH in methanol (48 mL) was added dropwise for ≈15 min. The solution was heated to 40°C for 1 h until the color disappeared. Cooling in the refrigerator overnight yielded a precipitate of white crystals that was filtered and washed with cold methanol. Drying in a desiccator gave 0.96 g of off-white crystals. ^{13}C NMR spectra showed the carboxylic carbon resulting from the oxidation at about 178 ppm (internal), anomeric carbons at 100–105 ppm, and carbinol carbons at 60 – 80 ppm. Signals for the hemiacetal region (91–97 ppm) were low relative to the signal for the internal C1 carbons at 100–105 ppm (i.e., much lower than in the nonoxidized material), confirming that C1 had been oxidized. For assignment of NMR signals, see Ref. 25; for an example of a study showing that the hemiacetal C1 still would show chemical shifts in the 91–97 ppm region if another carbon, such as C6, had been oxidized, see Ref. 26.

Separation and fractionation of cello-oligosaccharide aldonic acids/lactones

Oligosaccharides and their lactones/aldonic acids were separated by porous graphitic carbon chromatography run in reverse phase mode on an Ultimate 3000RSLC (Dionex corp.) with a Hypercarb 10 × 150 mm^2 (5 μm) column (Thermo Scientific), operated at 70° with 5 mL/min flow rate, a 693 μL sample loop, and charged aerosol detection (Corona Ultra, ESA). The following eluents were used: 0.05% (v/v) trifluoroacetic acid (A), acetonitrile with 0.05% (v/v) trifluoroacetic acid (B). Oligosaccharides were eluted using the following gradient; 100% A for 1.8 min, then a linear gradient running for 25.6 min to reach 27.5% B, and finally running 24.4 min to reach 60% B. 60%B was kept for 13.4 min, followed by a rapid change back to initial conditions, which was kept for 16.5 min (column reconditioning). Fractions were collected using a 1:20 custom-made post-column split directing the flow to the detector and the collecting tubes, respectively. The identities of the solutes were verified by MALDI-TOF analysis (using an identical method as described in Ref. 12).

Qualitative analysis of native and oxidized cello-oligosaccharides

Soluble products generated by CelS2 activity on cellulosic substrates were identified by MALDI-TOF MS, using previously published methods,[12] and by HPAEC using a Dionex Bio-LC equipped with a CarboPack PA1 column operated with a flow rate of 0.25 mL/min 0.1*M* NaOH and column temperature of 30°C. Cello-oligosaccharides were eluted by applying a stepwise linear gradient with increasing amounts of NaOAc, going from 0.1*M* NaOH to 0.1*M* NaOH/0.1*M* NaOAc in 10 min, then to 0.1*M* NaOH/0.3*M* NaOAc in 25 min and then to 0.1*M* NaOH/1.0*M* NaOAc in 5 min. Column reconditioning was achieved running initial conditions for 9 min. Eluted oligosaccharides were monitored by PAD detection. Chromatograms were recorded and analyzed using Chromeleon 7.0.

Cellulose degradation experiments

Quantitative assays were performed using 10 mg/mL filter paper (Whatman no.1) in 20 m*M* sodium acetate buffer pH 5.5 in the presence or absence of 40 μg/mL CelS2 and/or 0.8 μg/mL Celluclast (Novozymes). Celluclast is an enzyme cocktail produced by *T. reesei* (Rut C-30) where the dominant enzymes are Cel7A (40–60%), Cel6A (12–20%), Cel7B (5–10%), and Cel5A (1–10%); see Ref. 27. In reactions where no CelS2 was present, 40 μg/mL purified bovine serum albumin (BSA) (NEB) was added to maintain an identical protein load. Qualitative analysis of soluble products generated by CelS2 alone was performed by MALDI-TOF MS or HPAEC using

10 mg/mL Avicel PH-101 (Sigma) as substrate in 25 m*M* Bis-Tris pH 6.5. Reduced glutathione (0.5 m*M*) and ascorbic acid (1.0 m*M*) were used as external electron donors in the quantitative and qualitative experiments, respectively. All reactions contained 1.0 m*M* $MgCl_2$ and were incubated with shaking at 900 rpm at 50°C. Peak assignments in HPAEC were based on the use of purified external standards of native or chemically oxidized cello-oligosaccharides.

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