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Kinetic characterization of a glycoside hydrolase family 44 xyloglucanase/endoglucanase from *Ruminococcus flavefaciens* FD-1

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ABSTRACT

Two forms of *Ruminococcus flavefaciens* FD-1 endoglucanase B, a member of glycoside hydrolase family 44, one with only a catalytic domain and the other with a catalytic domain and a carbohydrate binding domain (CBM), were produced. Both forms hydrolyzed cellotetraose, cellopentaose, cellohexaose, carboxymethylcellulose (CMC), birchwood and larchwood xylan, xyloglucan, lichenan, and Avicel but not cellobiose, cellotriose, mannan, or pullulan. Addition of the CBM increased catalytic efficiencies on both CMC and birchwood xylan but not on xyloglucan, and it decreased rates of cellopentaose and cellohexaose hydrolysis. Catalytic efficiencies were much higher on xyloglucan than on other polysaccharides. Hydrolysis rates increased with increasing cellooligosaccharide chain length. Cellotetraose hydrolysis yielded only cellotriose and glucose. Hydrolysis of cellopentaose gave large amounts of cellotetraose and glucose, somewhat more of the former than of the latter, and much smaller amounts of cellobiose and cellotriose. Cellohexaose hydrolysis yielded much more cellotetraose than cellobiose and small amounts of glucose and cellotriose, along with a low and transient amount of cellopentaose.

1. Introduction

Ruminococcus flavefaciens is a ruminant bacterium highly active on plant cell walls. Two of its strains, *R. flavefaciens* 17 and *R. flavefaciens* FD-1, have been extensively studied [1–4]. Both *R. flavefaciens* strains produce cellulosomes, extracellular multi-protein complexes anchored to their cell membranes that break down cellulose and hemicelluloses. *R. flavefaciens* FD-1 has the largest number of hemicellulases and cellulases found in a cellulosome [5]. Two common features of the cellulosomes produced by each strain are the large number of different proteins, including hydrolases, cohesins, dockerins, and scaffoldin, incorporated into them and, unlike many cellulosome structures, the lack of carbohydrate binding modules (CBMs) attached to their scaffoldin domains. The latter factor makes the association of CBMs with catalytic domains (CDs) functionally necessary so that the cellulosomes can digest insoluble polysaccharides [6–11].

R. flavefaciens FD-1 endoglucanase B (CelB) and *R. flavefaciens* 17 cellulase (EndB) are enzymatic components of cellulosomes. The CDs of these two endoglucanases (EGs) are members of glycoside hydrolase family 44 (GH44), found in the CAZy database [12]. Both EGs have CDs that, like all GH44 CDs, are composed of a large number of subsites, each holding one carbohydrate residue. The subsites are numbered from negative to positive, starting with the nonreducing end of the bound substrate, with subsites −1 and +1 being on either side of the cleavage point. Both CDs are attached to CBMs that form a novel family [13].

With one exception, GH44 enzymes are produced by prokaryotes, both aerobic and anaerobic. GH44 is currently composed of 34 amino acid sequences. These sequences are often combined with other GHs or CBMs to form modular proteins.

Before this work, only a few GH44 members had been kinetically explored, yielding a large range of activities and optimal reaction temperatures. They include EGs from *Caldocellulosiruptor saccharolyticus* [14], *Clostridium acetobutylicum* [15], *Clostridium thermocellum* [16], some *Paenibacillus* strains [17,18], and an uncultured bacterium (CelM2) [19], along with EndB [13] and CelB [2], the enzyme featured in this paper, which is active on carboxymethylcellulose (CMC) (10 U/mg), oat spelt xylan (3.5 U/mg), and [^{14}C]

Abbreviations: CBM, carbohydrate binding module; CelB, *Ruminococcus flavefaciens* FD-1 endoglucanase B; CD, catalytic domain; CEC, capillary electrophoresis chromatography; CMC, carboxymethylcellulose; EG, endoglucanase; EndB, *Ruminococcus flavefaciens* 17 endoglucanase B; GH44, glycoside hydrolase family 44; HEPES, N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid); MWCO, molecular weight cutoff; Ni-NTA, nickel-nitrilotriacetic acid; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TLC, thin layer chromatography.

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cellulose (0.0036 U/mg). Temperature and pH optima for CelB had not been published.

These experimental results indicate that GH44 enzymes exclusively cleave β-1,4 glycosidic bonds between glucosyl and xylosyl residues, and that they have varying abilities to attack lichenan, xylan, xyloglucan, and different cellulose forms.

The CBM attached to CelB is unstudied, and its amino acid sequence is not found in the CAZy database [12], suggesting that it is unique. The other member of this novel CBM family, the 150-residue CBM on the EndB C-terminus, binds Avicel but is otherwise relatively unstudied [13]. Some activity data on CMC and lichenan have been reported for a single 87 kDa EndB band, and some activities have been estimated for CelB components (five bands of different molecular weights) on CMC and oat spelt xylan [2]. However, no Michaelis–Menten parameters are known for a single CelB band, and no studies have been conducted on the effect of the CBM on activities of either CelB or EndB.

This article will present both the impact of the CBM on Michaelis–Menten kinetic parameters of the CelB CD and its effect on product formation.

2. Experimental procedures

2.1. Gene sequencing and construction

The *R. flavefaciens* FD-1 gene, *celB*, encoding CelB was cloned into the pBluescript plasmid, pBAW101, by Vercoe et al. [4]. Dr. Bryan A. White of the University of Illinois at Urbana-Champaign kindly donated an *Escherichia coli* XL1-Blue clone with the plasmid to us. Sequencing of the vector at the Iowa State University DNA Facility to confirm the *celB* sequence uncovered a frameshift error, giving a sequence that conflicted with that previously published for *celB* [4]. The recently published correct sequence codes for a protein containing the CelB CD, a CBM similar to that in *R. flavefaciens* 17 EndB, and a dockerin domain [5].

The pBAW101 plasmid was used to amplify a gene fragment using the polymerase chain reaction with *Taq* polymerase encoding the CelB CD and CBM (forward primer 5′-AATACATATGGCAGGAGGTTTTGATATG-3′, reverse primer 5′-TCTCCTCGAGCTACTGCGGCTCATCAC-3′). The product was cloned into pGEM-T Easy vectors (Promega, Madison, WI) and sequenced at the Iowa State University DNA Facility. The gene fragment was then ligated into the NdeI site of pET-14b (Novagen, San Diego, CA) and transformed into *E. coli* BL21 (DE3) (Novagen). Strategene's (San Diego, CA) QuikChange II site-directed mutagenesis kit was used to insert a stop codon to remove the CBM from the CelB CD (forward primer 5′-GGTAACCGAGAAGACTGAGTAATTCAAGGATCCTTCTTC-3′, reverse primer 5′-GAAGAAGGATCCTTGAATTACTCAGTCTTCTCGGTTACC-3′). The mutated plasmids were transformed into *E. coli* XL1-Blue and sequenced to confirm the mutated DNA sequence. The plasmid was then transformed into *E. coli* BL21 (DE3) using electroporation. This resulted in two clones, one coding for the isolated CD (61.6 kDa) and the other coding for the CD and CBM (CD/CBM, 78.0 kDa).

2.2. Protein production and purification

An overnight culture was grown in 50 mL LB medium [20] supplemented with 0.132 mM carbenicillin at 37 °C with 250 rpm shaking. It was added to an auto-induction medium (2.78 mM glucose, 54.3 mM glycerol, 5.85 mM lactose, 1.2% tryptone, 2.4% yeast extract, 25 mM succinate, 5 μM $Fe_2(SO_4)_3$, 19 mM KH_2PO_4, 45 mM K_2HPO_4, 2 mM $MgSO_4$, and 45 mM NaH_2PO_4) [21], supplemented with 0.132 mM carbenicillin. A concentrated, sterilized phosphate buffer solution listed in the auto-induction recipe was added to the other media components to bring the pH to 7. The resulting suspension was cultured at 25 °C with 250 rpm shaking for approximately 36 h.

The cells were harvested by centrifugation at 8000 × *g* for 10 min and resuspended in 5–20 mL nickel-nitrilotriacetic acid (Ni-NTA) binding buffer (25 mM HEPES, pH 7.0, 300 mM NaCl, and 10 mM imidazole). They were lysed four successive times in an SLM Aminco (Rochester, NY) French press at 125 MPa. A 15 mL Ni-NTA His·Bind Superflow™ (Novagen) column resin was used to purify His-tagged proteins. The column was washed with Ni-NTA wash buffer (25 mM HEPES, pH 7.0, 300 mM NaCl, and 20 mM imidazole), and the CD or CD/CBM was eluted with Ni-NTA elution buffer (25 mM HEPES, pH 7.0, 300 mM NaCl, and 250 mM imidazole) (Novagen). A 50 mL Sephadex G-25 (GE Healthcare, Piscataway, NJ) column was used to desalt the protein into 50 mM HEPES buffer, pH 6.8. The protein was concentrated using an Amicon (Millipore, Billerica, MA) stirred-cell ultrafilter with a Millipore Biomax polyethersulfone membrane (5 kDa MWCO) and a Vivaspin6 (Sartorius, Elk Grove, IL) polyethersulfone 5 kDa MWCO spin filter at 8000 × *g*. All procedures were conducted at 4 °C.

Protein concentrations were determined using the Pierce (Rockford, IL) bicinchoninic acid assay [22] and bovine serum albumin standards. SDS-PAGE gels produced by standard procedures recommended by Pierce showed Coomassie Blue bands other than the desired ones only when they were strongly overloaded.

2.3. Substrates

The following substrates were incubated with the CD or CD/CBM: the cellooligosaccharides [(β-D-glucopyranosyl-(1→4))$_n$-β-D-glucose, n = 1–5] cellobiose (Sigma, St. Louis, MO, catalog C-7252), cellotriose (Seikagaku, Tokyo, Japan, 400400-1), cellotetraose (Seikagaku, 400402-1), cellopentaose (Seikagaku 400404-1), and cellohexaose (Seikagaku 400406-1), Avicel (microcrystalline cellulose) (Fluka, Buchs, Switzerland, 11363, lot 430118/1), low-viscosity CMC (cellulose derivatized mainly with 2-O- and 6-O-linked carboxyl groups, averaging 0.6–0.95 groups per glucopyranosyl residue) (Sigma C-5678, lot 065K0111), lichenan [(β-D-glucopyranosyl-(1→3,1→4))$_n$-D-glucose, n = high] from *Cetraria islandica* (Fisher, Pittsburgh, PA, 155231, lot 9964F), mannan [(β-D-mannopyranosyl-(1→4))$_n$-D-mannose, n = high] (Sigma M-7504, lot 44C-1764), pullulan [(α-maltotriosyl-(1→6)-α-maltotriosyl)$_n$-D-glucose, n = high] (TCI America, Portland, OR, P0978, lot GA01), xylan (β-D-xylopyranosyl-(1→4))$_n$-β-D-xylose, n = high, with significant branching initiated and terminated by other sugar residues) from birchwood (Sigma X-0502, lot 129H0901) or larchwood (Sigma X-3875, lot 125C-00582), and xyloglucan ((β-D-glucopyranosyl-(1→4))$_n$-β-D-glucose, n = high, with significant branching initiated by (1→)-linked α-xylopyranosyl residues) from tamarind gum (V-Labs, Covington, LA, PS136, lot DX060731J)

2.4. Thin-layer chromatography of carbohydrate hydrolysis products

The CD (10.55 μM) and CD/CBM (8.33 μM) were incubated separately at 25 °C in 0.1 M sodium acetate (NaOAc) buffer, pH 5.0, for 16 h with 750 mg/L cellobiose, cellotriose, cellotetraose, cellopentaose, and cellohexaose, along with 20 g/L Avicel, 20 g/L CMC, 10 g/L lichenan, 10 g/L mannan, 20 g/L pullulan, and 20 g/L xylan from birchwood or larchwood. The hydrolysis products were analyzed by thin-layer chromatography (TLC). A 60 Å silica gel plate (Whatman, Florham Park, NJ) was spotted with hydrolyzate and developed using a single ascent of acetonitrile/ethyl acetate/1-propanol/water (1.7:0.4:1:1) mobile phase [23]. The plate was dipped into a 5% (w/v) H_2SO_4, 0.5% (w/v) naphthol solution in ethanol and incubated at 95 °C until the carbohydrate spots developed color.

2.5. Capillary electrophoresis chromatography of carbohydrate hydrolysis products

The CD or CD/CBM (2.24 μM) was incubated with 3.2 g/L solutions of cellotetraose (4.80 mM), cellopentaose (3.86 mM), or cellohexaose (3.23 mM) in 0.1 M NaOAc buffer, pH 5.0, at 25 °C while mixing. Samples of 5–10 μL were taken at varying intervals for up to about 10 h and subjected to capillary electrophoresis chromatography (CEC). Each sample was denatured for 2 min at >90 °C and then lyophilized. The lyophilized samples were resuspended in 2 μL of 1 M sodium cyanoborohydride in tetrahydrofuran and 2 μL of 20 mM 8-aminopyrene-1,3,6-trisulfonic acid trisodium salt (APTS) in 15% v/v acetic acid. The derivatization mixtures were incubated for 2 h at 55 °C and then resuspended in 96 μL of distilled, deionized water. Samples were further diluted so their peak areas were in the linear range established by the calibration curves of glucose, cellobiose, cellotriose, cellotetraose, cellopentaose, and cellohexaose. Aliquots of 100 μL of diluted samples were placed in the trays of a Beckman (Beckman Coulter, Fullerton, CA) P/ACE capillary electrophoresis instrument in the Iowa State University W.M. Keck Metabolomics Research Laboratory. Three to five microliters was taken from a sample and injected for 3–5 s at 0.034 bar on an uncoated capillary. Separation took place at 23.5 kV using Beckman Carbohydrate Gel Buffer N [24]. Peaks on the resulting chromatographs were assigned to the appropriate cellooligosaccharide based on retention time and then integrated to determine the area under the curve. This area was used to quantify cellooligosaccharide concentration in the initial sample based upon the generated calibration curves and dilution factors.

2.6. Assays for enzyme activity

Values of k_{cat} and K_M for hydrolysis of CMC, birchwood xylan, xyloglucan, and Avicel were determined by using the tetrazolium blue assay [25] to measure reducing sugar production. Typical reaction conditions consisted of incubating CD (0.0276 μM) or CD/CBM (0.0218 μM) with 0.025–10 g/L substrate in 0.1 M NaOAc buffer, pH 5.0, at 25 °C. At least six samples of 100–300 μL were taken at 2–5 min intervals for 20–30 min, except for those from the Avicel incubation, which were taken every 30 min for 4–5 h. Each sample was placed in a boiling water bath to quench the reaction. Then 4 mL of reagent (0.1% (w/v) tetrazolium blue, 0.05 M NaOH, and 0.5 M sodium potassium tartrate) was added to each sample, and all samples, standards, and controls were placed in a 90 °C water bath for 5 min to develop reagent color [25]. A glucose standard curve was used to determine reducing sugar. Specific activity for each substrate concentration was determined by a linear regression of the reducing sugar concentration liberated versus incubation time and dividing the slope by the mass of protein in the sample. Enzyme units are defined as μmol glucose liberated/min under the assay conditions. A plot of spe-