CelAB, a Multifunctional Cellulase Encoded by *Teredinibacter turnerae* T7902^T, a Culturable Symbiont Isolated from the Wood-Boring Marine Bivalve *Lyrodus pedicellatus*[∇]

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We characterized a multifunctional cellulase (CelAB) encoded by the endosymbiont *Teredinibacter turnerae* T7902^T. CelAB contains two catalytic and two carbohydrate-binding domains, each separated by polyserine linker regions. CelAB binds cellulose and chitin, degrades multiple complex polysaccharides, and displays two catalytic activities, cellobiohydrolase (EC 3.2.1.91) and β -1,4(3) endoglucanase (EC 3.2.1.4).

Increasing demand for renewable energy sources has sparked growing interest in enzymes capable of degrading cellulose, the primary constituent of plant matter, to sugars that can be used for the production of ethanol. In nature, cellulose is degraded by the combined activities of multiple enzymes containing various glycoside hydrolase (GH) domains and carbohydrate binding modules (CBM) (1). While the great majority of glycosidases reported to date contain one GH domain, a small number contain multiple GH domains (2, 4, 5, 7, 9, 14, 16, 18, 20, 21). An example is CelA (8), from *Teredinibacter turnerae* T8201, an endosymbiont of the wood-boring bivalve *Psiloteredo healdi*. Although CelA is largely uncharacterized, amino acid sequence homology of its two GH domains suggests both endoglucanase and cellobiohydrolase activities. Here we describe CelAB, a homolog of CelA, produced by *T. turnerae* T7902^T, an endosymbiont of the shipworm *Lyrodus pedicellatus*. Substrate specificity, binding properties, and cleavage products of purified CelAB were examined to evaluate its potential multiple enzymatic activities and carbohydrate binding affinities.

A recombinant plasmid encoding a protein with hydrolytic activity against pNP-cellobioside and carboxymethylcellulose (CMC), when expressed in *Escherichia coli*, was identified in a Lambda Zap II genomic library (Stratagene) of *T. turnerae* T7902^T. Using EZ:Tn <Kan-2> transposon mutagenesis (Epicenter), an open reading frame was found encoding a putative cellulase, designated CelAB. CelAB is composed of 1,010 amino acids with a predicted molecular mass of 108 kDa (GenBank accession no. EF562510). It displays an unusual domain composition of GH5, CBM5, CBM10, and GH6 and is 98.2% identical in amino acid sequence to CelA (Fig. 1A). Similar domains have been identified in a variety of functionally diverse proteins from phylogenetically diverse organisms (Table 1). Each domain of CelAB is separated by polyserine linker regions (15) of 38, 55,

and 54 residues in length, containing 32, 40, and 43 serine residues, respectively. It is interesting to note that while CelAB differs from CelA by 1.8% in amino acid sequence identity, no changes are observed in the linker regions, suggesting an unexpectedly high degree of functional conservation for regions with no identified sequence-dependent function.

Sequence homology of CelAB to domains of known function suggests it may be capable of both endoglucanase and cellobiohydrolase activities and may bind cellulose and, perhaps, other polysaccharides. Most known representatives of the GH5 family display endoglucanase activity (though this family also includes proteins with at least 11 other activities), while only cellobiohydrolase and β -1,4 glucanase activities have been demonstrated in family GH6 domains to date (12). Both CBM5 and CBM10 families are further classified as type A surface binding modules, all of which are thought to bind highly crystalline substrates (3), including cellulose and chitin.

CelAB was cloned and expressed in a *Kluyveromyces lactis* Δ cts1 strain (6) (a gift from P. Colussi and C. Taron, New England Biolabs [NEB]) because of its attractive scale-up potential for protein production. Cloning was performed according to the manufacturer's guidelines using the following PCR primers: forward, TATATATAGATCTACCTCTGCAGCTTTCGCGG; and reverse, containing a hemagglutinin (HA) epitope, ATATATAGCGGCCGCTTATGCATAATCTGGAACATCATATGGATACGTCAGGTCGGAGGCGG. Culture supernatants of this strain rapidly depolymerized 5% CMC, suggestive of endoglucanase activity (Fig. 1B).

To test the carbohydrate binding characteristics of CelAB, various substrates were incubated with culture supernatant of *K. lactis* Δ cts1 celAB cells, washed with 3 M urea, and assayed by immunoblots with anti-HA (Cell Signaling Technologies) for the presence of CelAB. CelAB bound to cellulose and chitin, while it did not bind to xylan or curdlan (Fig. 1C). The observed binding was resistant to 8 M urea, 5 M NaCl, and exposure to pH 3 or 10 (data not shown) but was reversible in 20 mM NaOH (pH 12). The capability of CelAB to bind two structurally distinct polysaccharides may reflect differential

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FIG. 1. (A) Structural features of CelAB. Domains are noted. Black box, type II secretion signal; striped boxes, polyserine linker regions. (B) Endoglucanase activity of CelAB. Rapid reduction in viscosity of a 5% CMC gel by *K. lactis* Δ cts-1 *celAB* (solid line) culture supernatant compared to the *K. lactis* Δ cts-1 background strain (dashed line) suggests endoglucanase activity. (C) Carbohydrate binding characteristics of CelAB. Immunoblot detection of CelAB indicates binding to Avicel, cellulose, and chitin but not xylan or curdlan. Lanes, 1, Avicel; 2, cellulose; 3, chitin; 4, xylan; 5, curdlan. Molecular mass markers (kDa) are indicated to the left.

specificities of its two distinct CBM domains, although this remains to be confirmed experimentally.

CelAB was purified from *K. lactis* Δ cts1 *celAB* cells by applying culture supernatants to a chitin column (NEB) following the manufacturer's guidelines, except the proteins were eluted with 20 mM NaOH and then neutralized with 1/10 volume of 1 M Tris (pH 8.0). Active fractions were applied to a High Trap DEAE column and then fractionated on a Superdex 75 preparative column (5 cm by 90.2 cm) to isolate the full-length product. CelAB was purified as a variably glycosylated protein that produced a broad high-molecular-weight smear observed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Incubation of the glycosylated protein with peptide:N glycosidase F (PNGase F [NEB]), which cleaves N-linked-glycan chains from proteins, produced a single sharp protein band of approximately the expected molecular mass for CelAB (Fig. 2A). The identical purification procedure performed on the *K. lactis* Δ cts1 background expression strain did not yield any detectable protein in immunoblots, Coomassie-stained gels, or activity assays. CelAB produced in *E. coli* appeared to be enzymatically identical to that produced by the *K. lactis* Δ cts-1 *celAB* strain, suggesting that neither host glycosyla-

tion of CelAB nor contaminating proteins derived from the *K. lactis* Δ cts-1 strain affected activity (data not shown).

Temperature and pH optima of 42°C and pH 6.0 were determined for purified CelAB based on the cleavage of pNP-cellobioside (Fig. 2B) (18). Substrate specificity of CelAB was determined by detecting the production of reducing sugar ends (10) after incubation with various substrates in 50- μ l reaction mixtures containing 1% (wt/vol) substrate, 0.15 μ g of CelAB, and 50 mM sodium citrate (pH 6.0). Reaction mixtures were incubated for 2 h at 42°C. The production of reducing sugars was detected with barley β -glucan, CMC, laminarin, and lichenan (Table 2). These activities along with lack of activity against Avicel suggested that the activity of CelAB is directed primarily towards amorphous substrates.

Endoglucanase and cellobiohydrolase activities for CelAB were assayed with coumarin-labeled cello-oligosaccharides synthesized and assayed as previously described (19). CelAB cleaved cellotetraose to produce cellobiose as the sole product, by definition a cellobiohydrolase activity. Hydrolysis of cello-pentaose produced cellotriose and cellobiose, consistent with both cellobiohydrolase and β -1,4 endoglucanase activities (Fig. 2C). Cellobiose concentrations up to 30 mM did not inhibit cellobiohydrolase activity (data not shown).

TABLE 1. Properties of functional domains in CelAB

CelAB domain	Most similar characterized protein ^a	GenBank accession no.	% Identity ^b	% Similarity ^b	Reference
GH5	GH domain of cellulase from <i>Cellvibrio japonicus</i>	CAA60493	64	81	11
CBM5	CBM of cellulase from marine bacterium DY3	AAP04424	58	69	22
CBM10	CBM of mannanase from <i>C. japonicus</i>	AAO31761	67	75	13
GH6	GH domain of cellobiohydrolase from <i>Cellulomonas fimi</i>	P50401	49	64	17

^a As determined by a BLASTP search of the nonredundant database on 5 April 2007.

^b Percent identity and percent similarity were calculated by pairwise BLASTP alignment.

FIG. 2. Enzymatic characterization of CelAB. (A) Glycosylation of CelAB by the *K. lactis* Δ cts-1 strain. Treatment of CelAB purified from the *K. lactis* Δ cts-1 celAB strain with PNGase F resulted in a single protein band at the expected molecular mass. Lane 1, PNGase F-treated CelAB; lane 2, untreated CelAB. Molecular mass markers (kDa) are given to the left. (B) Temperature and pH optima for CelAB based on the hydrolysis of pNP-cellobioside. (C) Endoglucanase and cellobiohydrolase activity of CelAB demonstrated with coumarin-linked substrates. Lanes: 1 D-glucose-coumarin; 2, cellobiose-coumarin; 3, cellobiose-coumarin; 4, cellobiose-coumarin; 5, cellobiose-coumarin; 6, CelAB plus cellobiose-coumarin; 7, CelAB plus cellobiose-coumarin. (D) Cleavage site of coumarin-linked cellobiose and cellopentaose produced by CelAB. CelAB produced cellobiose-coumarin from cellobiose-coumarin (below left) indicative of cellobiohydrolase (CBH) activity and released cellobiose-coumarin and cellobiose-coumarin from cellopentaose-coumarin (below right), indicative of cellobiohydrolase and β -1,4 endoglucanase (ENG) activities. Open stars, coumarin; solid circles, glucose.

It remains to be determined whether the two catalytic activities observed in CelAB reflect the distinct properties of its two GH domains. If so, the potential exists for synergistic interaction between these domains, possibly explaining its 25-fold

increase in specific activity compared to Cel5A from *Saccharophagus degradans*, a similar enzyme with two predicted endoglucanase domains (18). Further domain analysis of CelAB may provide insights into the potential for coordinated activity within multifunctional cellulases and may be useful in the discovery and optimization of industrial cellulases.

TABLE 2. Activity of CelAB

Substrate ^a	Sp act in U/ mg (SD) ^b
β -Glucan.....	51.11 (9.48)
CMC	24.88 (1.59)
Laminarin	2.66 (0.47)
Lichenan	0.50 (0.13)
PASC	ND

^a PASC, phosphoric acid-swollen cellulose. Production of reducing sugars was not detected with Avicel, chitin, curdlan, pachyman, starch, or xylan after 72 h of incubation.

^b One unit is equal to the amount of protein that liberates 1 μ mol of glucose equivalents of reducing ends from a substrate per hour in a 2-h reaction at pH 6.0 and 42°C as determined by the Nelson-Somogyi reducing sugar microassay (10). All reactions were run in triplicate, with the standard deviation (SD) reported in parentheses. ND, not determined: reducing sugars were detected after 24 h but in quantities too small to allow accurate determination of specific activity.

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