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Engineering of *Clostridium phytofermentans* Endoglucanase Cel5A for Improved Thermostability

Running title: Engineering of *C. phytofermentans* Endoglucanase

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Abstract

A family 5 glycoside hydrolase from *Clostridium phytofermentans* was cloned and engineered through a cellulase cell-surface display system in *E. coli*. The presence of cell-surface anchoring, a cellulose binding module, or His-tag greatly influenced the activities of wild-type and mutant enzymes on soluble and solid cellulosic substrates, suggesting the high complexity of cellulase engineering. The best mutant had 92%, 36% and 46% longer half-lives at 60°C on carboxymethyl cellulose, regenerated amorphous cellulose, and Avicel, respectively.

The production of biofuels from non-food cellulosic biomass would benefit the economy, environment, and national energy security (17, 32). The largest technological and economical obstacle is release of soluble fermentable sugars at prices competitive with those from sugarcane or corn kernels (17, 31). One of the approaches is discovering new cellulases from cellulolytic microorganisms followed by cellulase engineering for enhanced performance on pretreated solid substrates. But cellulase engineering remains challenging because enzymatic cellulose hydrolysis is complicated, involving heterogeneous substrates (33, 37), different action-mode cellulase components (18), synergy and/or competition among cellulase components (36, 37), and declining substrate reactivity over the course of conversion (11, 26). Directed enzyme evolution, independent of knowledge of the protein structure and the enzyme-substrate interactions (6, 34), has been conducted to generate endoglucanase mutants, such as enhanced activities on soluble substrates (14, 16, 22), prolonged thermostability (20), changed optimum pH (24, 28), or improved expression levels (21). Here we cloned and characterized a family 5

glycoside hydrolase (Cel5A) from a cellulolytic bacterium *Clostridium phytofermentans* ISDg (ATCC 700394) (29, 30) and engineered it for enhanced thermostability.

Characterization of Cel5A. The DNA fragment encoding the mature form of Cel5A (ABX41541) was cloned into vector pET-20b(+) to give plasmid pET20b-Cel5A (Fig. 1). Recombinant Cel5A by *E. coli* BL21 Star (DE3) was highly soluble and purified by Bio-Rad Profinity IMAC Ni-Resins precharged with Ni^{2+} (Hercules, CA). The purified enzyme had an apparent molecular mass of about 45 kDa, and its optimal pH and temperature were 7.0 and 55ºC on carboxymethyl cellulose (CMC), respectively. The recombinant Cel5A drastically decreased the viscosity of a 1% (w/v) CMC solution, suggesting that it is an endoglucanase (35). At pH 7.0, Cel5A had activities of 18.1 ± 0.8, 1.70 ± 0.04 and 0.24 ± 0.06 U/mg on CMC, regenerated amorphous cellulose (RAC) (33), and Avicel but had no detectable activity on bacterial microcrystalline cellulose and xylan at 50ºC.

Improve the thermostability of Cel5A by directed evolution. For potential cellulase recycling (38), Cel5A was engineered for better thermostability. A random *cel5A* mutant library generated by error-prone PCR (4) was expressed by plasmid pGF101-Cel5A (Fig. 1) so that Cel5A was displayed on the cell surface of *E. coli* by fusing with an outer-membrane protein ice nucleation protein (INP) (12). After overnight incubation at 37°C, the colonies on the agar plates without isopropyl-beta-D-thiogalactopyranoside (IPTG) were duplicated onto fresh LB plates containing 50 μM IPTG. The duplicate plates were incubated at room temperature for about 40 hours and then heated at 70°C for 20 min. The plates were subsequently overlaid with soft agar containing 0.5% CMC and incubated at 37°C overnight. After Congo-red staining and washing, the size of the yellow halo zone around a colony reflected endoglucanase activity on CMC (35). Approximately 20 colonies with halo zones larger than the wild-type after the heat treatment

were identified from *ca.* 20,000 colonies (Fig. 2). The selected colonies were retrieved from the original plates and their thermostabilities were further confirmed. After DNA sequencing, five mutants with two or three amino acid substitutions (Table S1) were selected for further characterization.

CBM addition of Cel5A mutants. The five selected mutants that had potentially improved thermostability were evaluated as fusion proteins each with a family 3 cellulose binding module (CBM3) from the *C. thermocellum* scaffoldin (10, 19) added to the N-terminus (Fig. 1), because (i) CBM3 is well-known to enhance hydrolysis of endoglucanase on insoluble cellulosic substrates (2, 7) and (ii) CBM3 helps heterologous protein expression in *E. coli* (10, 19). We first added a family 3 cellulose binding module (CBM3) to the N-terminus (Fig. 1) of each selected mutants and found that only two (m1 and m18) were more thermostable than the wild-type Cel5A. Substrate pre-binding experiments suggested that cellulase thermostability was greatly affected by whether the enzyme was immobilized or free with the substrates (Table S2). We also evaluated the wild-type Cel5A with a His-tag at the C-terminus (WT-His) or a CBM3 at the N-terminus (CBM3-WT) (Fig. 3A). The specific activities of WT-His Cel5A on RAC and Avicel were 66% and 11% of that on CMC, respectively, due to a decreasing cellulose accessibility to cellulase order of CMC, RAC and Avicel (11, 37). Adding an N-terminal CBM3 to Cel5A did not influence its activity on soluble substrate CMC, but decreased its activity on insoluble substrates RAC and Avicel (Fig. 3A). This surprising result does not agree with previous reports that addition of CBM3 enhanced endoglucanase activities on solid cellulosic substrates (2) and removal of CBM3 greatly decreased the enzyme activity on solid substrates (7). We speculated that this negative CBM-addition effect could be attributed to a short linker length (8 AA) between CBM3 and Cel5A. Therefore, we constructed plasmid pET20b-Cel5A-Ig-CBM2 (Fig.

1) for expressing a Cel5A fusion protein (WT-Ig-CBM2) that has a C-terminal sequence (containing a 41-AA linker, an Ig-like module and a CBM2) from another *C. phytofermentans* family 5 glycoside hydrolase (Cel5B, Genbank accession number YP_001560295). WT-Ig-CBM2 did not show any significant improvement of activities on insoluble substrates compared to CBM3-WT (Fig. 3A), implying that the addition of CBM might not always enhance endoglucanase activity on solid substrates. We further investigated the effects of His-tag on Cel5A activities on various substrates (Fig. 3B). The wild-type Cel5A without His-tag was expressed and purified from plasmid pCIG-Cel5A (Fig. 1). Removing the His-tag increased Cel5A specific activities on RAC and Avicel by 48% and 22%, respectively, but had no significant effect on its activity on CMC (Fig. 3B), suggesting that His-tag effects on the Cel5A activities were substrate dependant.

Because of the negative effects of CBMs and His-tag on Cel5A activities, we expressed the tag-free wild-type Cel5A based on plasmid pCIG-Cel5A (Fig. 1) for producing mutants m1 (N144I/N291K), m18 (E158V/V245G), and their combination mutant m1-18 (N144I/N291K/E158V) by site-directed mutagenesis. Table 1 showed that m1, m18, and m1-18 had similar activities to wild-type Cel5A on both soluble and insoluble substrates. On CMC, m1-18 presented a nearly doubled half-life (14.8 min) compared to the wild-type (7.7 min); on RAC and Avicel, m1-18 exhibited increased half-lives by 36% and 46% on RAC and Avicel, respectively.

The complexity of cellulase engineering. Removal of the CBMs from natural cellulases drastically decreased their activities on solid substrates (1, 3, 5, 8, 13, 15, 23), and addition of the *C. thermocellum* CBM to *C. thermocellum* endoglucanase CelD significantly improved its activity on solid cellulosic materials (2). However, our research showed that addition of the *C.*

111 *thermocellum* CBM3 or Ig-CBM2 fragment of *C. phytofermentans* Cel5B to *C. phytofermentans*
112 Cel5A did not enhance its activities on solid substrates (Fig. 3A). One possible reason was that
113 Cel5A might not interact efficiently with the CBM for generating a proper conformation for
114 enhanced activity on insoluble substrates (25, 27). Some of the putative thermostable mutants
115 that were displayed on the cell surface were found to be non-thermostable when purified in a free
116 form (Fig. 2 and Table S2), suggesting that the cell-surface display technology may be more
117 suitable for screening enzyme mutants for whole cell catalysis.
118 Previous directed evolution studies successfully enhanced endoglucanase catalytic efficiency
119 (14, 16, 22) based on the assays on soluble cellulose derivatives. Because there is no clear
120 relationship between the endoglucanase activities on soluble substrates and on solid cellulose
121 substrates (9, 34), the development of high-throughput cellulase assays on solid cellulosic
122 substrates is urgently needed.
123
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231

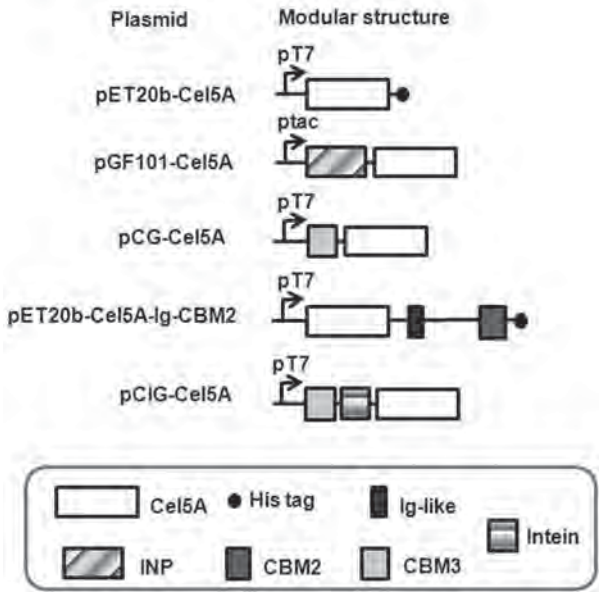
232 ## Figure Legends

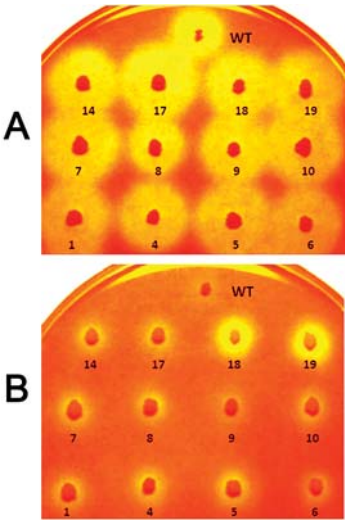
233

234 **FIG. 1.** Schematic representation of the expression plasmids.

235 **FIG. 2.** Congo-red staining of *E. coli* JM 109 colonies surface displaying engineered *C.*
236 *phytofermentans* endoglucanase mutants on LB-Amp-IPTG agar plates overlaid with a CMC soft
237 agar layer. (A) The colonies selected from a library of putatively positive mutants without heat
238 treatment and (B) the same colonies were pretreated at 70ºC for 20 min.

239 **FIG. 3.** Effects of the CBM tags (A) and His-tag (B) on the molar specific activities of wild-
240 type Cel5A on CMC, RAC, and Avicel.





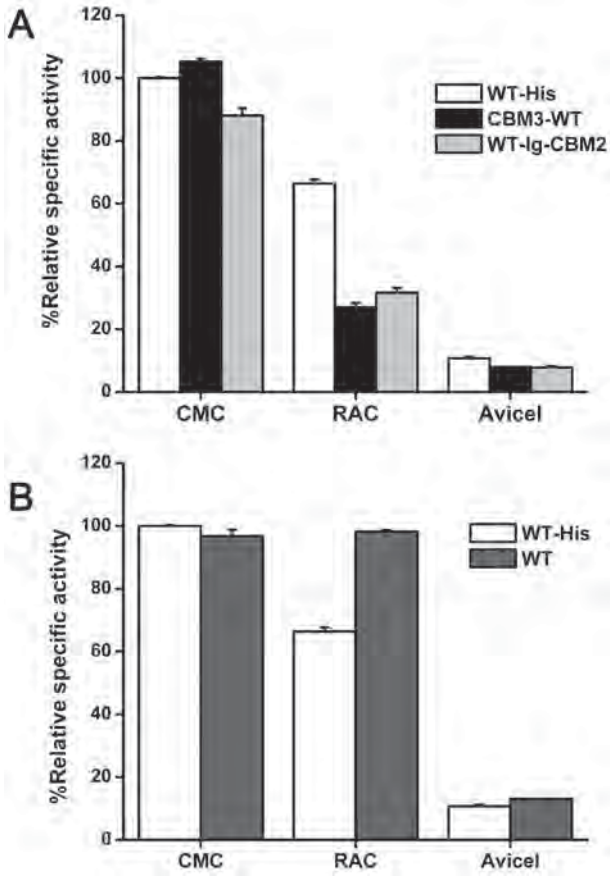


TABLE 1. Reducing end (RE) release and half- life times ($t_{1/2}$) at 60°C of wild-type Cel5A and mutants m1, m18 and m1-18.

	CMC		**RAC**		**Avicel**	
	RE release[a] (µmol/mg protein)	$t_{1/2}$ (min)	RE release[a] (µmol/mg protein)	$t_{1/2}$ (min)	RE release[a] (µmol/mg protein)	$t_{1/2}$ (min)
WT	95 ± 2	7.7 ± 1.1	95.9 ± 0.5	3.6 ± 0.1	12.8 ± 0.1	4.8 ± 0.3
m1 (N144I/N291K)	97 ± 4	9.1 ± 0.7	84.7 ± 0.1	4.6 ± 0.2	13.2 ± 0.3	6.6 ± 0.4
m18 (E158V/V425G)	101 ± 2	7.9 ± 0.7	95.1 ± 4.6	3.2 ± 0.2	13.8 ± 0.4	4.5 ± 0.3
m1-18 (N144I/N291K/E158V)	95 ± 2	14.8 ± 1.7	84.7 ± 0.7	4.9 ± 0.3	12.7 ± 1.6	7.0 ± 0.7

[a] in 30-min reaction.