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Influence of positioning of carbohydrate binding module on the activity of endoglucanase CelA of *Clostridium thermocellum*

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ABSTRACT

This study reports characteristics of different derivatives produced between CelA, a major endoglucanase of *Clostridium thermocellum* and carbohydrate binding domain of family 3a (CBM3a). In addition to the native form of the endoglucanase containing catalytic and dockerin domains (CelA-CD), its derivatives consisting of catalytic domain without dockerin domain (CelA-C), catalytic domain linked with the binding domain at N-, C- and both termini (CelA-BC, CelA-CB and CelA-BCB, respectively), two catalytic domains cloned in tandem (CelA-CC) and two catalytic domains intervened by a binding domain (CelA-CBC) were expressed in *Escherichia coli* at levels of 40, 43, 28, 30, 20, 20 and 10%, respectively of the total cell proteins. Specific activities of CelA-CD, CelA-C, CelA-BC, CelA-CB, CelA-CC, CelA-BCB and CelA-CBC against carboxymethyl cellulose (CMC) were 8.1, 7.0, 12.1, 8.5, 11.8, 10.2 and 23.5 U mg^{-1} enzyme while activities against pre-treated bagasse were 490, 250, 1400, 600, 810, 710 and 2270 μmoles reducing sugars released per μmole of the enzyme, respectively, under the assay conditions used. Thus the activities of CelA-BC and CelA-CBC showed nearly 3- and 5-fold increase against pre-treated bagasse as compared to that of the native form of the enzyme, CelA-CD. Molecular modeling studies using MODELLER show that the binding residues of CBM3a and the active site residues of the catalytic domain are more favorably oriented for binding and hydrolysis of the polysaccharide in the case of CelA-BC as compared to those in CelA-CB, which corresponds with higher activity of the former.

1. Introduction

Clostridium thermocellum is a gram-positive, anaerobic cellulolytic bacterium, which produces cellulases organized in a large cellulolytic complex called cellulosome. The cellulosome of *C. thermocellum* consists of a number of cellulolytic and xylanolytic enzymes bound to a large non-catalytic scaffoldin protein (CipA) of 197 kDa, which in turn is anchored to the cell wall via type II cohesin domains (Bayer et al., 1994). This binding of the complex to the cell surface is mediated by three anchoring proteins; OlpB, Orf2p and SdbA, which interact with type II dockerin domain of scaffoldin, CipA (Dror et al., 2003). A total of 28 catalytic domains, which include at least twelve endoglucanases, four cellobiohydrolases, five xylanases, one mannanase, one chitinase and one lichenase, are present in the cellulosome (Demain et al., 2005; Zverlov et al., 2005).

Generally, cellulolytic enzymes have one or more carbohydrate binding domains that facilitate the binding of the enzyme to insoluble substrate but some of the cellulosomal enzymes lack their own binding domain as they use the binding domain of the scaffoldin protein (Wilson, 2008). However, binding domains have been shown to have varying effects on the xylanase activities. In the case of xylanase XynC of *C. thermocellum* deletion of the binding domain resulted in reduced thermostability as well as reduced activity on insoluble substrates. Further, addition of a second binding domain to the XynC catalytic domain resulted in increased binding to insoluble substrate as well as increased activity. However, in the case of xylanase XynZ deletion of the binding domain showed increased activity (Khan et al., 2010; Sajjad et al., 2010). Kim et al. (2010) developed a cell free expression system and expressed a library of chimeric endoglucanases from glycoside hydrolase family 5 and 12 with CBM family 1 or 2 at either N- or C-terminus. Addition of CBM resulted in an overall increase in activity against insoluble substrates e.g. Avicel and pre-treated corn stover. Similarly, fusion of CBM22 to xylanase of *Bacillus halodurans* S7 also resulted in increased hydrolysis of insoluble oat spelt xylan due to increased

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Table 1
Primers used for PCR amplification of the various sequences of *celA* and the binding domain.

Primer	Sequence[a] (5′–3′)
P1	CGCATATGGCAGGTGTGCCTTTTAAC
P2	GACTCGAGATAAGGTAGGTGGGGTATG
P3	CTCGAGGTCACTCAAAGGATTCGGGAA
P4	GAGCTCGCAGGTGTGCCTTTTAACACA
P5	CACATATGGTATCAGGCAATTTGAAGG
P6	GAGCTCGGCTGACGGCGGTATT
P7	CTCCATGGCAAGGTCACTCAAAGGATT
P8	CTCCATGGCAACACCGACCAAG
P9	CTCGAGACCGGGTTCTTTACCCCA
P10	CCATGGGTGGCAGTGTAGTACCATCA
P11	GTCGACACCGGGTTCTTTACCCCATA
P12	GTCGACGGCAGTGTAGTACCATCAACA

[a] Restriction sites of *Nde*I (CATATG), *Nco*I (CCATGG) and *Sac*I (GAGCTC), *Sal*I (GTCGAC) and *Xho*I (CTCGAG) are underlined.

binding of the chimeric enzyme to the insoluble substrate (Mamo et al., 2007).

Endoglucanase A (CelA) is the only member of glycoside hydrolase (GH) family 8 from *C. thermocellum*. Unlike most other cellulases of the cellulosome, CelA does not possess a binding domain. This report describes the effect of introducing a carbohydrate binding domain of family 3a, which is a component of the scaffoldin (CipA) of the cellulosome, in various positions on expression, activity and other characteristics of the variants produced.

2. Materials and methods

2.1. Bacterial strains and vectors

Bacterial strains used were *Escherichia coli* DH5α for cloning and vector propagation and *E. coli* BL21 CodonPlus (RIPL) for expression of recombinant proteins. These strains were either grown in LB or M9NG medium (Sadaf et al., 2007). Plasmids used were pTZ57R/T (Fermentas Inc, Ontario, Canada) for TA cloning and pET22b(+) as expression plasmid (Novagen, Madison, USA). Chromosomal DNA of *C. thermocellum* ATCC 27405 (kindly provided by Prof. D. B. Wilson, Cornell University, Ithaca, NY) was used as a source of cellulase gene and the binding domain sequences.

2.2. PCR amplification

Based on the published sequences of *celA* and *cipA* (GenBank accession No. K03088 and L08665, respectively), primers were designed using NEBcutter (Vincze et al., 2003), Primer 3.0 (Rozen and Skaletsky, 2000) and OligoCalc (Kibbe, 2007), and these are given in Table 1. Possibility of any secondary structure formation was checked by determining free energy values for the fragment between the ribosomal binding site and the +10 codon using Mfold web-server (Zuker, 2003). Using the respective primers and the genomic DNA of *C. thermocellum* DNA fragments encoding CelA-CD, CelA-C, and CBM3a were amplified. PCR reactions were run for 30 cycles with denaturation at 94 °C for 45 s, annealing at 58 °C for 45 s and extension at 72 °C for 1 min 30 s. Final extension was done at 72 °C for 20 min. PCR products were purified using gel DNA recovery kit (Vivantis Technologies, Malaysia) according to the manufacturer's instructions.

2.3. Cloning into expression plasmid

PCR products were purified and cloned in pTZ57R/T using InsT/Aclone™ PCR product cloning kit (Fermentas Inc, Ontario, Canada). *E. coli* DH5α cells were transformed using standard protocol (Sambrook and Russell, 2001). Positive clones containing recombinant plasmids were screened on LB-agar plates containing ampicillin, isopropyl-β-D-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) with final concentrations of 50 μg ml^{-1}, 0.1 mM and 20 μg ml^{-1}, respectively. The clones were checked by colony PCR as described for the initial PCR, except that amplification was done for 25 cycles and the final extension was carried out at 72 °C for 10 min. In order to check correctness of the constructs, sequencing reactions were done with GenomeLab DTCS – Quick start kit (Beckman Coulter Inc.) according to the manufacturer's instructions using each of the sequence as template. The reaction mixtures were then loaded onto Beckman Coulter CEQ8000 Genetic Analyzer. Sequencing was performed in both directions.

The recombinant plasmids and pET22b(+) were isolated by alkaline lysis method (Sambrook and Russell, 2001), and these were double digested with specified restriction enzymes. Purified restricted fragments were then ligated into linearized pET22b(+) using T4 DNA ligase. To obtain chimeric proteins, the DNA fragments were cloned sequentially in pET22b(+) according to the scheme given in Fig. 1a, using compatible restriction sites and ensuring that the fragments were in-frame. Recombinant plasmids pCelA-CD, pCelA-C, pCelA-BC, pCelA-CB, pCelA-CC, pCelA-BCB and pCelA-CBC, thus prepared were used to transform *E. coli* BL21 CodonPlus (RIPL) cells.

2.4. Expression of cellulase variants

LB broth containing 100 μg ml^{-1} ampicillin was inoculated with overnight culture of *E. coli* BL21 CodonPlus (RIPL) harboring recombinant plasmids and grown at 37 °C to an OD_{600} of 0.5–0.6. Expression of recombinant proteins was then induced with IPTG at a concentration of 0.5 mM. Cells were harvested after 6 h of induction. In a parallel experiment, cells were also induced with 10 mM lactose in the auto-inducing M9NG medium (Sadaf et al., 2007). The induced cells were harvested and resuspended in 0.05 M phosphate buffer, pH 6.0. Cells were then sonicated at 50 amplitude for 0.5 second cycle for 10 min using a UP400S ultraschallprozessor (Dr. Hielscher GmbH, Teltow, Germany). Soluble cytoplasmic and insoluble fractions were separated by centrifugation at 6500 rpm for 15 min. For partial purification of recombinant cellulase variants soluble cytoplasmic fraction was heated at 65 °C for 1 h to precipitate heat labile host cell proteins, which were then removed by centrifugation at 6500 rpm for 15 min. Supernatant was then used for activity determination and other experiments.

2.5. SDS-PAGE and zymographic analysis

Total cell protein, soluble and insoluble fractions were analyzed for expression and sub-cellular localization of recombinant cellulase variants by SDS-PAGE by the method of Laemmli (1970), while zymography was performed as described by Geib et al. (2010) with some modifications. Briefly, two 12% SDS-PAGE gels were prepared one with 0.2% CMC and other without CMC. Samples after mixing with equal volume of 2× sample loading dye (100 mM Tris–Cl, pH 6.8; 20% glycerol; 4% SDS; 1.5% β-mercaptoethanol and 0.1% bromophenol blue) and heating at 85 °C for 10 min, were loaded onto gels and run at a constant voltage of 100 V. After electrophoresis, the gel without CMC was stained with commassie brilliant blue R250 while the other containing CMC was incubated in 0.05 M phosphate buffer (pH 6.0) containing 2.5% Triton X-100 (v/v) for 1 h with slow agitation. After washing with 0.05 M phosphate buffer (pH 6.0) for 1 h, it was incubated at 60 °C for 5 min. The gel was then stained with 0.1% Congo red solution and destained with 1 M NaCl.