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Hydrolytic effects of scaffolding proteins CbpB and CbpC on crystalline cellulose mediated by the major cellulolytic complex from *Clostridium cellulovorans*

Sang Duck Jeon [a], Su Jung Kim [a], Sung Hyun Park [a], Gi-Wook Choi [b], Sung Ok Han [a,*]

[a] Department of Biotechnology, Korea University, Seoul 136-701, Republic of Korea
[b] Changhae Advanced Institute of Technology, Changhae Ethanol Co., Ltd., Jeonju 561-203, Republic of Korea

HIGHLIGHTS

- The scaffolding protein, CbpB and CbpC bind cell surface of *C. cellulovorans*.
- The CbpB and CbpC complex leads to increase crystalline cellulose hydrolysis.
- The mixture of CbpB and CbpC complex with CbpA complex induces to synergy effect.
- CbpC complex efficient hydrolyze released substrate after CbpA complex degradation.

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ABSTRACT

The role of the scaffolding proteins, cellulose binding protein B and C (CbpB and CbpC, respectively) were identified in cellulolytic complex (cellulosome) of *Clostridium cellulovorans* for efficient degradation of cellulose. Recombinant CbpB and CbpC directly anchored to the cell surface of *C. cellulovorans*. In addition, CbpB and CbpC showed increased hydrolytic activity on crystalline cellulose incubated with exoglucanase S (ExgS) and endoglucanase Z (EngZ) compared with the activity of free enzymes. Moreover, the results showed synergistic effects of crystalline cellulose hydrolytic activity (1.8- to 2.2-fold) when CbpB and CbpC complex with ExgS and EngZ are incubated with cellulolytic complex containing mini-CbpA. The results suggest *C. cellulovorans* critically uses CbpB and CbpC, which can directly anchor cells for the hydrolysis of cellulosic material with the major cellulosome complex.

1. Introduction

Biofuels such as bioethanol, biodiesel and biobutanol from renewable biomass have been investigated to replace fossil fuel-based energies in the near future and are often produced from edible materials. For the production of these second-generation biofuels, lignocellulosic biomass is used as a feedstock in the biorefinery process (Yu et al., 2013). Lignocellulose is composed primarily of three substances, which are all different types of polymers called cellulose (Wiman et al., 2012), hemicellulose and lignin, and they are associated with each other (Blanch et al., 2011). The major component of lignocellulose is cellulose, which can be degraded into the fermentable sugar glucose and consists of clusters of polymeric backbone chains containing β (1 → 4) glucosidic bonds between each glucose monomer and relatively weak hydrogen bonds between each polymer cluster (Laureano-Perez et al., 2005; Sanchez-Segado et al., 2012). Some cellulolytic microorganisms studied extracellularly were shown to secrete lignocellulolytic enzyme, including several types of cellulases and hemicellulases (Blanch et al., 2011; Penttila et al., 1986).

The cellulolytic complex called cellulosome was recently described as one of nature's most elegant and elaborate nanomachines to efficiently deconstruct lignocellulosic biomass (Fontes and Gilbert, 2010). Cellulosomes are active toward various substrates, such as crystalline cellulose, xylan, mannan and pectin, it has been most widely studied in anaerobic clostridia, namely *Clostridium thermocellum* (Stahl et al., 2012), *Clostridium cellulovorans* (Hyeon et al., 2013) and *Clostridium cellulolyticum* (Fendri et al., 2009). Cellulosome assembly is mediated by a highly specific protein:protein interaction between dockerin modules in the cellulosomal catalytic subunits and repeated cohesin modules on the scaffolding protein (Bayer et al., 2004). Cohesins are 150-residue modules that are usually present as tandem and multiple copies

* Corresponding author. Tel.: +82 2 3290 3151.
E-mail address: samhan@korea.ac.kr (S.O. Han).

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in scaffolding proteins (Fontes and Gilbert, 2010). All cellulosomal enzymes contain a twice-repeated sequence called dockerin, which is not found in non-cellulosomal enzymes (Craig et al., 2006). The recognition between cohesins and dockerins is mainly mediated by hydrophobic interactions (Karpol et al., 2009). The cellulosomal enzymes are glycosyl hydrolase enzymatic subunits of various specificities such as exoglucanases, endo-glucanases, xylanases and mannanases that act synergistically to efficiently break down lignocellulose (Craig et al., 2006). The synergistic interaction of multiple enzymes and their substrates helps to overcome the rate-limiting step of converting crystalline forms of cellulose to cellobiose, leading to the efficient degradation of crystalline polysaccharides in plants (Monschein et al., 2013).

In *C. cellulovorans*, Cellulose binding protein A (CbpA) is the main scaffolding protein in cellulosome assembly. CbpA consists of nine cohesin modules, the cellulose binding module (CBM) and the bacterial surface layer homology (SLH) domains that mediate binding between cellulosome and the cell surface (Hyeon et al., 2013). This protein is known to play a critical role in cellulosome assembly and lignocellulose degradation in *C. cellulovorans*. However, the cellulosome system in *C. cellulovorans* was predicted to be mediated not only by CbpA but by other scaffolding proteins. Recently, a genomic study was performed in *C. cellulovorans*, and the researchers found other scaffolding proteins called cellulose binding protein B and C (CbpB and CbpC, respectively) (Tamaru et al., 2011). The molecular weights of CbpB and CbpC are 47.2 and 46.5 kDa, respectively. In genomic sequence analysis of *C. cellulovorans* CbpB and CbpC, consisting of a CBM of family 3, a putative SLH domain and a cohesin module, were newly found and tandemly localized in the *C. cellulovorans* genome; however, no such scaffolding proteins were found in other cellulosomal clostridia (Tamaru et al., 2011).

In recent years, the complexity and diversity of bacterial cellulosomes have become apparent through the molecular and biochemical studies of multiple scaffolding proteins from *Acetivibrio cellulolyticus*, *Bacteroides cellulosolvens*, *Ruminococcus flavefaciens* and *C. thermocellum*. Many cellulosome-producing bacteria, including *B. cellulosolvens*, *C. thermocellum* and *R. flavefaciens*, have remarkable similarity, and these microbes use the type II cohesin–dockerin system to efficiently anchor cellulosome to the cell surface (Xu et al., 2004). In particular, in *C. thermocellum*, several scaffolding proteins (SdbA, OlpB, and Orf2) have been shown to contribute to the assembly of poly-cellulosomes that may contain the cellulosome complex. The main scaffolding protein CipA, derived from cellulosome, may adhere to the bacterium cell surface by binding to the single type II cohesin domains found in OlpA and OlpC because of their SLH domains (Fontes and Gilbert, 2010). The interaction of CipA with the three anchoring secondary scaffoldings tethers the *C. thermocellum* cellulosomes to the cell surface, leading to the hypothesis of the "enzyme-microbe synergy," also referred to here as "cellulosome-cell synergy" (Hong et al., 2014) .

To investigate the unique role of the novel scaffolding proteins in *C. cellulovorans*, the contribution of CbpB and CbpC was revealed by this study for the assembly of cellulosome and deconstruction of the plant cell wall complex. For this purpose, in the present study, recombinant CbpB and CbpC were obtained by overexpression in *Escherichia coli*. To analyze the synergistic effects of cellulosome from *C. cellulovorans* on cellulosic substrates, two different cellulosomes were assembled, including the cellulosomal enzymes and mini-CbpA (mCbpA) containing CBM, SLH and two cohesin modules, because recombinant CbpA is cleaved at the boundary of the nine cohesins. Moreover, the differences in the synergistic reactions against cellulosic substrates between these cellulosomes were measured using analysis of reducing sugar from an enzymatic reaction. A putative domain for cell surface interaction through several analytic methods was also investigated. Taken together, our results demonstrated the importance and contribution of the scaffolding proteins CbpB and CbpC to cellulosome assembly and hydrolysis of cellulosic substrate from *C. cellulovorans*.

2. Methods

2.1. DNA manipulations

The *C. cellulovorans* ATCC35296 strain, which was used to isolate its genomic DNA, was grown under strict anaerobic conditions at 37 °C in 1-L round-bottom flasks in a medium containing 1% (w/v) cellobiose (Sigma–Aldrich) (Doi et al., 1998). *E. coli* DH5α bacteria were used as host cells for DNA manipulation, and the *E. coli* BL21 (DE3) strain (Real Biotech Corporation) was used as a host for the pET22b (+) (Novagen) derivative plasmids pET22b-CbpB and CbpC. The mature CbpB and CbpC DNA sequences were amplified in the reaction conditions (50 mM Tris–HCl, 50 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, pH 7.9) from the genomic DNA of *C. cellulovorans* using a forward primer (5′-ATGCTCGAG-TACTTGTGTATTTATG-3′; the *Xho*I site is underlined) and a reverse primer (5′-TGACGGATCCTATGAAAAAGAACAA-3′; the *BamH*I site is underlined) for CbpB and a forward primer (5′-ATAGCTCGAGTG-CAGTTGCATTTAT-3′, the *Xho*I site underlined) and a reverse primer (5′-GTCGGGATCCTATGAAAAAGAAAAG-3′; the *BamH*I site underlined) for CbpC. The polymerase chain reaction (PCR) product was digested with *EcoR*I and *Xho*I and was ligated into the corresponding restriction sites in the pET22b (+) vector to generate pET22b-CbpB and pET22b-CbpC. To enable the secretion of CbpB and CbpC, we used the commercial secretion pET22b (+) vector that contains a *pelB* signal sequence and poly-histidine (His6) tag at its C-terminus under the control of the T7 promoter (Jeon et al., 2012b).

2.2. Preparation of the scaffolding proteins and cellulosomal enzymes

E. coli BL21 (DE3) cells that harbored the pET22b-CbpB and pET22b-CbpC plasmids were grown at 37 °C in Luria-Bertani broth containing ampicillin (50 μg/ml) to the optical density value of 0.9 when measured at 600 nm (Hazlewood et al., 1993). The expression and purification of recombinant exoglucanase S (ExgS) (Murashima et al., 2002), endoglucanase Z (EngZ) (Jeon et al., 2012b) and endoglucanase E (EngE) (Murashima et al., 2003) were performed as previously described. The culture was then cooled to 18 °C, and isopropyl thio-β-D-galactoside (IPTG) was added to the culture extract at a final concentration of 0.4 mM. After an additional overnight growth period, the cells were collected via centrifugation (4500×*g*, for 10 min at 4 °C) and resuspended in 10 ml of lysis buffer (50 mM Na_2HPO_4, 400 mM NaCl, and 10 mM imidazole; pH 8.0). The cells were collected via centrifugation (4,500×*g*, for 30 min at 4 °C) and lysed using ultrasonication. The supernatant from the crude cell extract was applied to a Ni-NTA column, which bound to the His-tag of the recombinant protein, and the column was washed with wash buffer (50 mM NaH_2PO_4, 400 mM NaCl, and 25 mM imidazole; pH 8.0). The bound recombinant CbpB and CbpC were eluted using an elution buffer (50 mM Na_2HPO_4, 400 mM NaCl, and 250 mM imidazole; pH 8.0). The purified protein was collected and dialyzed against PBS buffer (pH 7.3). The dialyzed protein was concentrated via ultrafiltration (Millipore; molecular weight cut-off of 10 kDa) (Jeon et al., 2012b). The concentration of the purified protein was measured using the Bradford assay from the protein assay kit (Bio-Rad), and bovine serum albumin was used to generate the standard curve (Bradford, 1976).