Journal of Bioscience and Bioengineering
VOL. xx No. xx, 1–5, 2017

www.elsevier.com/locate/jbiosc

Characterization of the cellulosomal scaffolding protein CbpC from *Clostridium cellulovorans* 743B

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Received 14 March 2017; accepted 20 April 2017
Available online xxx

***Clostridium cellulovorans* 743B, an anaerobic and mesophilic bacterium, produces an extracellular enzyme complex called the cellulosome on the cell surface. Recently, we have reported the whole genome sequence of *C. cellulovorans*, which revealed that a total of 4 cellulosomal scaffolding proteins: CbpA, HbpA, CbpB, and CbpC were encoded in the *C. cellulovorans* genome. In particular, *cbpC* encoded a 429-residue polypeptide that includes a carbohydrate-binding module (CBM), an S-layer homology module, and a cohesin. CbpC was also detected in the culture supernatant of *C. cellulovorans*. Genomic DNA coding for CbpC was subcloned into a pET-22b+ vector in order to express and produce the recombinant protein in *Escherichia coli* BL21(DE3). Measurement of CbpC adsorption to crystalline cellulose indicated a dissociation constant of 0.60 μM, which is a similar to that of CBM from CbpA. We also subcloned the region encoding xylanase B (XynB) with the dockerin from *C. cellulovorans* and analyzed the interaction between XynB and CbpC by GST pull-down assay. It was observed that GST-CbpC assembles with XynB to form a minimal cellulosome. The activity of XynB against rice straw tended to be increased in the presence of CbpC. These results showed a synergistic effect on rice straw as a representative cellulosic biomass through the formation of a minimal cellulosome containing XynB bound to CbpC. Thus, our findings provide a foundation for the development of cellulosic biomass saccharification using a minimal cellulosome.**

[**Key words:** *Clostridium cellulovorans*; Cellulosome; Scaffolding protein; Xylanase; Carbohydrate-binding module]

Lignocellulosic biomass mainly consists of cellulose, hemicellulose, and lignin. To facilitate its degradation, pretreatments incorporating chemical treatments using acid or alkali and physical treatment by blasting or hydrothermal treatment are mainstream (1). However, as these methods are associated with problems in terms of environmental burden and energy balance, saccharification using enzymes produced by microorganisms or the microorganisms themselves is considered as a potential alternative biological pretreatment. It has been revealed that some anaerobic microorganisms produce enzyme complexes termed cellulosomes, which can efficiently degrade cellulose (2). Cellulosomes comprise multi-protein complexes in which enzymes such as cellulase and hemicellulase are bound to scaffolding proteins. *Clostridium cellulovorans* was discovered as a mesophilic, anaerobic bacterium producing cellulosomes (3,4). Subsequently, the cellulose binding protein A (*cbpA*) gene encoding the scaffold protein from *C. cellulovorans* was cloned and a gene cluster containing several cellulase and hemicellulase genes was found downstream of the *cbpA* gene (5,6). CbpA has a number of enzyme-binding sites termed cohesins as well as a carbohydrate-binding module (CBM) (4,7–9). The cohesins play an important role in establishing interactions with a variety of cellulosomal enzymes containing dockerins and binding these enzymes to CbpA. In addition, a small nonenzymatic protein called HbpA, which is a scaffolding protein having only a surface layer homology module (SLH) and a single cohesion, has also been reported in this bacterium (10).

Recently, we have reported the whole genome sequence of *C. cellulovorans* (11), which led to the identification of two novel genes encoding the scaffolding proteins CbpB and CbpC. CbpB and CbpC encode a 439 and 429 residue polypeptide, respectively, that include a CBM, a SLH and a cohesin (12). Accordingly, it was speculated that *C. cellulovorans* produces a cellulosome containing four scaffolding proteins: CbpA, HbpA, CbpB, and CbpC, and is capable of degrading cellulosic biomass (13). CbpB and CbpC were subsequently found to be expressed on the cell surface layer of *C. cellulovorans* and to exhibit increased activity by forming a complex with CbpC and the cellulosomal cellulases exoglucanase S (ExgS) or endoglucanase Z (EngZ) (14).

In addition, genomic analysis further revealed that *C. cellulovorans* contains three cellulosomal xylanase genes, with subsequent proteome analysis demonstrating that this bacterium expresses xylanase A (XynA) and xylanase B (XynB) when it is cultured on xylan as a carbon source. In turn, these proteins form cellulosomes by binding with CbpA, which is considered to constitute an important function in xylan degradation (15–17). Moreover, it has been reported that XynA itself forms a cellulosome by binding to CbpA, which results in

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1389-1723/$ – see front matter
http://dx.doi.org/10.1016/j.jbiosc.2017.04.014

an increase in its activity (18). XynB has also been shown to form cellulosomes, albeit without a concomitant increase in activity (17,19). Additionally, CbpB and CbpC can assemble with cellulosomal cellulases such as ExgS and EngZ; however, it is not known whether these will assemble with a hemicellulase such as xylanase.

Therefore, in this study, we investigated whether (i) *C. cellulovorans* express CbpC when cultured in a medium containing xylan; (ii) whether CbpC binds to crystalline cellulose contained in cellulosic biomass; and (iii) whether cellulosomes assembled by CbpC and XynB through dockerin-cohesin interactions were constructed and increased the activity against rice straw as a representative cellulose biomass.

MATERIALS AND METHODS

Cloning and expression Each gene encoding the CbpC and XynB proteins was amplified by polymerase chain reaction (PCR) from *C. cellulovorans* genomic DNA. The PCR product encoding CbpC was ligated into the Nde1/Xho1-digested pET22b vector (Merck) and the BamH1/EcoR1-digested pGEX-2T GST fusion vector (GE Healthcare), respectively, and the product encoding XynB was ligated into the Sma1/Xho1-digested pET50b NusA fusion vector (Merck). BL21 cells transformed with each plasmid were grown at 37°C to OD_{600} 0.5, induced with isopropyl-β-D-thiogalactopyranoside (0.1 mM final concentration), and harvested by centrifugation after 40 h at 20°C.

Protein purification The CbpC protein was purified using Avicel affinity chromatography (7) and CbpC fused with a GST tag (GST-CbpC) was purified by affinity chromatography using Glutathione Sepharose 4B (GE Healthcare). Each protein was buffer changed into 50 mM Tris-HCl (pH 7.5). A further purification step was carried out by anionic exchange chromatography using Toyopearl DEAE-650M (Tosoh). The purity of each fraction was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The CbpC and the GST-CbpC proteins were subsequently subjected to analysis for adsorption to crystalline cellulose and the GST-pull-down assay, respectively. In addition, XynB was purified via nickel affinity chromatography using Ni Sepharose 6 Fast Flow (GE Healthcare). The NusA tag added to the recombinant was removed by Turbo 3C protease (Accelagen) cleavage. Subsequently, the Turbo 3C protease and the cleaved NusA tag were removed using another round of nickel affinity chromatography. A further purification step by anionic exchange chromatography was performed using the same method as for CbpC.

Preparation of native cellulosomes from medium supernatant and purification of cellulose binding proteins *C. cellulovorans* 743B (ATCC 35296) was grown under strictly anaerobic conditions at 37°C for two weeks in 100 mL of a previously described medium (3,7), which included 1% xylan from birch wood (Sigma). The preparation of native cellulosomes and proteins binding to the crystalline cellulose were purified using Avicel affinity chromatography as described by Shoseyov and Doi (7).

Western blot analysis An anti-CbpC antibody was obtained from a rabbit immunized with purified recombinant CbpC and an anti-CbpA antibody was provided by Professor Roy H. Doi at University of California, Davis. For western blot analysis, native cellulosomes and proteins binding to the crystalline cellulose were separated by SDS-PAGE and blotted onto a polyvinylidene fluoride membrane (ATTO). The membrane was treated with anti-CbpC or anti-CbpA antibody (diluted 1:3000) as the primary antibody. After washing, the secondary antibody reaction was carried out by adding ECL Plus Western blotting reagent pack Anti rabbit lgG (GE Healthcare) in fresh blocking buffer. After incubation at 25°C for 1 h and then four washes with washing buffer, the membrane was dipped in ECL Western Blotting Detection Reagents (GE Healthcare). The secondary antibody was detected using LightCapture II (ATTO).

Determination of the CbpC-Cellulose dissociation constant Samples of CbpC (typically 23–117 mg) were added to 1.5 mL capacity microcentrifuge tubes containing 50 mM Na_2HPO_4, 12 mM citric acid (PC buffer), pH 7.0 and the desired amount of cellulose. The final volume was always 1 mL. Assay tubes were mixed slowly at 37°C for 1 h. The samples were then spun in a microcentrifuge for 3 min to sediment the cellulose and cellulose–protein complexes. After the buffer had been removed, the pellet was washed by resuspension in 1 mL PC buffer. The wash was separated out by centrifugation and discarded. Pellets were then resuspended in a final volume of 1 mL PC buffer. Aliquots (300 μL) of this well-mixed suspension were taken for protein determinations using the MicroBCA kit (Thermo Fisher Scientific). The free CbpC protein concentration, [P], was determined by subtracting the bound protein concentration, [PS], from the total CbpC added to the tube, $[P]_t$ as follows:

$$[P] = [P]_t - [PS]$$

The system was analyzed by assuming a simple equilibrium interaction:

$$P + S \underset{k_{-1}}{\overset{k_1}{\leftrightarrows}} PS \quad (1)$$

where the dissociation constant, K_d, is defined as

$$K_d = \frac{k_{-1}}{k_1} = \frac{[P][S]}{[PS]} \quad (2)$$

The data were analyzed by the Langmuir equation:

$$[PS] = \frac{[PS]_{max}[P]}{K_d + [P]} \quad (3)$$

Interaction analysis between CbpC and XynB by GST pull-down assay GST-CbpC and XynB were mixed in equal ratios in 20 mM Tris-HCl (pH 7.5) containing 1 mM $CaCl_2$ and 100 mM NaCl and kept for 1 h at 4°C. Their assembly was confirmed by GST pull-down assay using MicroSpin GST Columns (GE Healthcare) and SDS-PAGE.

Effect of the addition of CbpC on xylanase activity In this experiment, XynB was used in the form of an Nus-fusion protein as a cellulosomal enzyme and rice straws were prepared as substrates as follows. The rice straws was washed with water and dried at 60°C. After thorough drying, the straws were used as a substrate after grinding into a powder having 100 μm or less particle size. The xylanase activity was assayed in the presence of a 0.5 % (w/v) concentration of rice straw at 37°C in 25 mM acetate buffer (pH 6.0) containing 15 mM $CaCl_2$ by measuring the liberated reducing sugars using a modified Somogyi-Nelson assay method (20).

RESULTS AND DISCUSSION

Protein purification A signal peptide is present at the N-terminus of CbpC and XynB with a consensus sequence of Val-X-Ala-Ser and Val-X-Ala-Glu, respectively, in which the predicted cleavage site is located between positions 28 (Ala) and 29 (Ser) or positions 29 (Ala) and 30 (Glu) (21). In the recombinant proteins, the signal peptide was removed. Each of the purified preparations gave a major band upon SDS-PAGE analysis, with molecular sizes in good agreement with those deduced from the nucleotide sequences (Fig. 1). However, XynB experienced partial degradation over time (date not shown). In comparison, XynB fused with the NusA tag was comparatively more stable than that with the NusA tag removed. Therefore, XynB fused with the NusA tag was used in the activity measurement of the scaffold protein complexed with XynB.

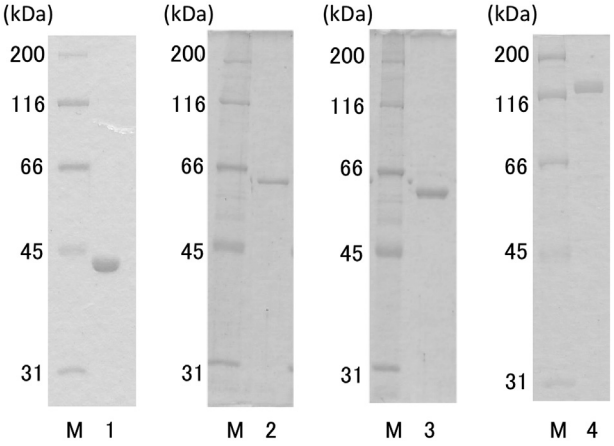

FIG. 1. SDS-PAGE of purified proteins. The gels were stained with Coomassie Brilliant Blue and the samples were loaded in the following order: M, protein maker (molecular masses shown at the left); lane 1, CbpC purified sample; lane 2, GST-CbpC purified sample; lane 3, XynB purified sample; lane 4, NusA-XynB purified sample.

Please cite this article in press as: Nakajima, D., et al., Characterization of the cellulosomal scaffolding protein CbpC from *Clostridium cellulovorans* 743B, J. Biosci. Bioeng., (2017), http://dx.doi.org/10.1016/j.jbiosc.2017.04.014