Heterologous Production of *Clostridium cellulovorans* engB, Using Protease-Deficient *Bacillus subtilis*, and Preparation of Active Recombinant Cellulosomes

Koichiro Murashima,1 Chyi-Liang Chen,2 Akihiko Kosugi,1 Yutaka Tamaru,1 Roy H. Doi,1* and Sui-Lam Wong2

Section of Molecular and Cellular Biology, University of California, Davis, California 95616,1 and Division of Cellular, Molecular and Microbial Biology, Department of Biological Sciences, University of Calgary, Calgary, Alberta T2N 1N4, Canada2

Received 21 May 2001/Accepted 25 September 2001

In cellulosomes produced by *Clostridium* spp., the high-affinity interaction between the dockerin domain and the cohesin domain is responsible for the assembly of enzymatic subunits into the complex. Thus, heterologous expression of full-length enzymatic subunits containing the dockerin domains and of the scaffolding unit is essential for the in vitro assembly of a “designer” cellulosome, or a recombinant cellulosome with a specific function. We report the preparation of *Clostridium cellulovorans* recombinant cellulosomes containing the enzymatic subunit EngB and the scaffolding unit, mini-CbpA, containing a cellulose binding domain, a putative cell wall binding domain, and two cohesin units. The full-length EngB containing the dockerin domain was expressed by *Bacillus subtilis* WB800, which is deficient in eight extracellular proteases, to prevent the proteolytic cleavage of the enzymatic subunit between the catalytic and dockerin domains that was observed in previous attempts to express EngB with *Escherichia coli*. The assembly of recombinant EngB with the mini-CbpA was confirmed by immunostaining, a cellulose binding experiment, and native polyacrylamide gel electrophoresis analysis.

*Clostridium cellulovorans* produces a cellulase enzyme complex (cellulosome) containing a variety of cellulolytic subunits attached to the nonenzymatic component termed CbpA (2). All cellulosomal enzymatic subunits contain a twice-repeated sequence, usually at their C termini, called the dockerin domain. These dockerin domains are considered to bind to the sequence, usually at their C termini, called the dockerin domain. All cellulosomal enzymatic subunits contain a twice-repeated sequence, which is repeated nine times in CbpA (14). So far, we have cloned and sequenced eight cellulosomal cellulase genes from *C. cellulovorans* (engB [3], engE [16], engH [18], engK [18], engL [15], engM [15], engY [17], and exgS [9]). In addition, the genes for cellulosomal mannanase ([manA]15 and pectate lyase (pelA)17) have also been cloned and sequenced.

In order to understand the hydrolytic mechanism of the cellulosome and to use cellulosomes for industrial purposes, it is very important to make recombinant cellulosomes, or “designer” cellulosomes, with specific functions. To prepare recombinant cellulosomes, it is necessary to express the cellulosomal enzyme subunits that retain their dockerin domains. *Escherichia coli* is a very efficient host for expression of heterologous genes (25, 27; S.-C. Wu, J. C. Yeung, S. J. Szarka, and S.-L. Wong, presented at the 11th International Conference of Antibody Engineering, San Diego, California, 2000). We used *B. subtilis* WB800 as an expression host to produce intact EngB, one of the cellulosomal subunits of *C. cellulovorans*. In addition, a mini-CbpA, which contained a cellulose binding domain (CBD), a putative cell wall binding domain (hydrophilic domain), and two cohesins of CbpA, was constructed and expressed by *E. coli*. The binding between the recombinant EngB (rEngB) and the mini-CbpA was determined to confirm that the rEngB had a dockerin domain and that a designer minicellulosome had been assembled in vitro.

**MATERIALS AND METHODS**

Bacterial strains and media. *B. subtilis* WB800 (Wu et al., presented at the 11th International Conference of Antibody Engineering) was used as an expression host for EngB production with plasmid pWB800-EngB. Cells carrying the expression plasmid were cultivated in superrich medium (6) containing kanamycin at a final concentration of 10 µg/ml. *E. coli* BL21(DE3) (Novagen) was used as an expression host for mini-CbpA and EngB production using pET-22b-mini-CbpA and pET-B2 (4), respectively. Recombinant strains were cultivated in Luria-Bertani medium supplemented with ampicillin (50 µg/ml).
Plasmid construction for mini-CbpA expression by *E. coli*. The mini-CbpA was designed to consist of a CBD, a hydrophilic domain, and two cohesins of scaffoldin protein CbpA (14) (Fig. 1). For expression of mini-CbpA by *E. coli*, pET-22b (Novagen) was used as a vector. The mini-CbpA gene was amplified using genomic DNA from *C. cellulovorans* as a template with a pair of primers, mini-CbpA F (GTATACCATGGCGACCG) and mini-CbpAB (5'-GGCTCGAGTATAGGATCTCCAAT). The PCR primers were designed to allow in-frame fusion at the N-terminal end of mini-CbpA with the PelB signal sequence and at the C-terminal end with the His tag from pET-22b. The amplified 1,680-kb fragment had an NcoI site at the 5' end and an XhoI site at the 3' end (Fig. 1). This fragment was digested with NcoI and XhoI and inserted into pET-22b digested with the same pair of restriction enzymes to generate pET-22b-mini-CbpA.

Expression and purification of mini-CbpA. *E. coli* BL21(DE3) harboring pET-22b-mini-CbpA was grown in 300 ml of medium at 37°C to an optical density at 600 nm of 0.9. Isopropyl-β-D-thiogalactoside was then added to a final concentration of 0.4 mM, and the culture was grown at 30°C for 3 h. After collection of *E. coli* cells by centrifugation, the periplasmic proteins were extracted into 50 ml of extraction buffer (20 mM Tris-HCl [pH 8.0], 10 mM EDTA) by the osmotic shock method (10). A 50-ml aliquot of the supernatant was applied to a CF11 cellulose binding domain, hydrophilic domains, and cohesin domains of CbpA (14) are shown. Numbers are amino acid residues counted from the translation start of CbpA. The amplified mini-CbpA fragment is also indicated.

FIG. 1. Schematic diagram of mini-CbpA. The signal sequence, a cellulose binding domain, hydrophilic domains, and cohesin domains of CbpA are also indicated.

Plasmid construction for *engB* expression by *B. subtilis*. For production of *engB* by *B. subtilis* WB800, the expression vector pWB980-EngB, which is a derivative of pWB980 (24), was constructed. The *engB* gene was amplified using plasmid PC2 (3) as a template with a pair of PCR primers, EngB F (5'-GGAA GCTTTGGACACTACAGCATAAGCAGTGATCC) and EngB B (5'-GAGGAT GCTGTGGTCTGGTAAATGATCAG). The amplified 1.48-kb fragment encoded mature EngB and contained a 247-bp sequence downstream of the *engB* gene. This fragment has a HindIII site at the 5' end and an XhoI site at the 3' end. This design allowed the production of mature EngB with its authentic N-terminal sequence. Since the amplified fragment had an internal HindIII site (861 bp from 5' end), this fragment was inserted into pWB980 by a two-step cloning strategy. The 1.48-kb fragment was first digested with HindIII and XhoI. The resulting 855-bp HindIII-XhoI fragment was inserted into the HindIII-XhoI site of pWB980, and the resulting plasmid was designated pWB980-EngB. To install the 3' end of *engB*, the 1.48-kb fragment was digested with XbaI and SphI, and a 625-bp XbaI-SphI fragment was inserted into pWB980-EngB to generate pWB980-EngB (Fig. 2).

FIG. 2. Construction of pWB980-EngB. Plasmid pWB980 carries both the constitutively expressed P43 promoter (20) and the engineered levansucrase signal sequence (22). The *engB* gene was inserted into pWB980 to generate pWB980-EngB by a two-step cloning strategy as described in Materials and Methods. The sequences coding for the kanamycin resistance marker and levansucrase signal sequence are represented by *kan* and *sacB* SP, respectively. The arrows show the transcription directions for the genes.
by the osmotic shock method (10). The protein from 10 ml of culture broth was concentrated into 1 ml of 20 mM Tris-HCl buffer (pH 8.0).

**Assay of CMCase activity.** Carboxymethylcellulose (medium viscosity; Sigma) degradation activity (CMCase activity) was assayed in 50 mM sodium acetate buffer (pH 6.0) at 37°C by measuring the liberated reducing sugars, as d-glucose equivalents, by the Somogyi-Nelson assay method (23). Activities were expressed as units, with 1 U defined as the amount of enzyme releasing 1 μmol of reducing sugar per min.

**Protein determination.** Protein concentrations were measured with the bicinchoninic acid protein assay system (Pierce) using bovine serum albumin as a standard. The molar amounts of mini-CbpA and EngB were calculated by use of the theoretical molecular masses of mini-CbpA (58.0 KDa) and EngB (48.5 KDa).

**Determination of mini-CbpA binding with rEngB.** To test the binding of the mini-CbpA with the rEngB, the purified mini-CbpA (2 μg per lane) was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted to a polyvinylidene difluoride membrane. The blotted membrane was then immersed in 50 ml of blocking solution (phosphate-buffered saline [137 mM NaCl, 2.7 mM KCl, 8.1 mM Na2HPO4, 1.5 mM KH2PO4], 1% Tween, 1% skim milk) containing 1 ml of the crude EngB expressed by *E. coli* or the EngB found in the periplasmic fraction of *E. coli* at 4 h at room temperature. After being washed with 1% Tween in phosphate-buffered saline, the membrane was immunostained with anti-EngB. To determine the effect of EDTA on the binding of the rEngB expressed by *B. subtilis* WB800 (rEngB-B) with the mini-CbpA, 15 mM EDTA was added to the blocking solution with the crude EngB solution from *B. subtilis*.

**Native PAGE analysis of EngB**–**mini-CbpA complex.** To form the EngB-mini-CbpA complex, 2 pmol of purified rEngB-B was mixed with various amounts of purified mini-CbpA in 50 μl of binding buffer (100 mM sodium acetate buffer [pH 6.0], 15 mM CaCl2) and incubated at 4°C for 1 h, and then this solution was mixed with 50 μl of 2.0% cellulose (Avicel; FMC Corporation) solution and shaken for 1 h at 4°C. After centrifugation, the CMCase activity of the supernatant was assayed as described above. To calculate the percentage of EngB bound to cellulose, the CMCase activity of the supernatant was divided by the activity of the solution without cellulose.

**Native PAGE analysis of EngB**–**mini-CbpA complex.** To form the EngB-mini-CbpA complex, 20 pmol of the purified EngB expressed by *B. subtilis* WB800 and 20 pmol of the purified mini-CbpA were mixed in 5 μl of the binding buffer described above and kept for 1 h at 4°C. This solution was then subjected to native PAGE with a 10% ready-made gel (Bio-Rad). After electrophoresis, the gel was silver stained.

**RESULTS AND DISCUSSION**

**Expression and purification of recombinant mini-CbpA.** The mini-CbpA carried a CBD, a hydrophilic domain, and two cohesins domains (14) with a C-terminal His tag from the vector pET-22b. Since the mini-CbpA was designed to contain the CBD at its N terminus and the His tag at its C terminus, the nondegraded mini-CbpA was expected to be the vector pET-22b. When recombinant mini-CbpA was expressed, most of the mini-CbpA was secreted into the culture broth. We chose *C. cellulovorans* WB800 harboring pWB980-EngB as an expression host (Fig. 2). This vector contained the P43 promoter (20), the sacB gene, pWB980-EngB was constructed (Fig. 2). This vector expressed the signal peptide of *B. subtilis* WB800 (lane 2). The expressed mini-CbpA was secreted to the periplasmic space by means of the signal sequences (22), and the kanamycin resistance marker. The P43 promoter is a strong and constitutively expressed promoter and was used to direct the transcription of engB during growth (Fig. 2). The sacB signal sequence allowed the recombinant protein to be secreted into the culture broth. When recombinant *B. subtilis* WB800 harboring pWB980-EngB was grown at 37°C, most of the rEngB was expressed as inclusion bodies (data not shown). In contrast, when grown at 30°C, the cells secreted most of the rEngB-B into the culture broth. The culture supernatants of *B. subtilis* WB800(pWB980-EngB) collected at different time points were analyzed by Western blotting with anti-EngB. As shown in Fig. 4A, one stained band appeared at the position of 48 kDa. The molecular mass of this band was in good agreement with the calculated molecular mass of the rEngB-B (48.5 KDa) (3). This band accumulated for up to 30 h of the culture period, and no degradation of the rEngB-B was observed up to 48 h. These results indicated that the rEngB-B secreted into the culture broth was very stable during the culture period. The use of *B. subtilis* WB800 was essential for the production of intact EngB. Degraded EngB was observed even when *B. subtilis* WB700, deficient in seven extracellular proteases (27), was used as an expression host (data not shown). This suggests that the cell wall protease WprA (11),...
which is inactivated in B. subtilis WB800, is the protease that directly or indirectly contributes to the degradation of EngB in B. subtilis WB800.

The recombinant EngB expressed by E. coli (rEngB-B) showed two bands smaller than those of the rEngB-B, and no band was detected at the position of 48 kDa, which is the calculated molecular mass of rEngB-E (4) (Fig. 4B). These results suggested that the rEngB-E was degraded by E. coli proteases during the cultivation period and/or the extraction procedure.

**Purification of rEngB-B.** As the first purification step, the rEngB-B in the culture supernatant was precipitated with 40% saturation of ammonium sulfate. The precipitated fraction was then applied to an anti-EngB immunoadfinity column, and the bound rEngB-B was eluted with ethanolamine buffer (pH 11.5). After anti-EngB affinity chromatography, the eluted rEngB-B was almost homogeneous, with only small amounts of contaminants. To remove the contaminants, the eluted rEngB-B was applied to an anion-exchange column. After anion-exchange chromatography, the purified rEngB-B showed a sharp band on SDS-PAGE analysis (Fig. 3, lane 2). The molecular mass of the purified rEngB-B was 48 kDa. The specific activity of the purified rEngB-B was 22.2 U/mg of protein (as measured by the CMCase assay).

**Interaction between mini-CbpA and recombinant EngB.** With purified mini-CbpA and rEngB-B on hand, we tested their binding interaction by showing a cross-reaction between the two with anti-EngB. The anti-EngB was determined to be highly specific for EngB and showed no cross-reactions with the mini-CbpA (data not shown). The purified mini-CbpA blotted onto a membrane was soaked with rEngB-B from the culture supernatant or from the periplasmic fraction and probed with anti-EngB. Only the membrane incubated with the rEngB-B showed one band at the mini-CbpA position (Fig. 5A). No band could be detected with the membrane treated with rEngB-E (Fig. 5B). These results suggested that rEngB-B could bind with mini-CbpA and that rEngB-E could not. The addition of 15 mM EDTA inhibited the interaction between rEngB-B and mini-CbpA (data not shown). Pages et al. (13) showed that calcium ion is necessary for the binding between dockerin domains and cohesin domains of C. cellulolyticum and C. thermocellum. Since the dockerin domain sequence of EngB has a putative calcium binding motif (13, 18) which is homologous to the calcium binding loop in the EF-hand motif (7), rEngB-B is also considered to require calcium ion to bind with the cohesin domain of mini-CbpA.

Since EngB does not contain a CBD, the purified rEngB-B cannot bind to cellulose. To determine whether rEngB-B mixed with mini-CbpA could attach to cellulose via the CBD of mini-CbpA, a mixture of rEngB-B and mini-CbpA was tested in a cellulose binding experiment. The purified rEngB-B (2.0 pmol) was mixed with various amounts of mini-CbpA, and then the CMCase activity of the rEngB-B bound to cellulose was determined. Since addition of mini-CbpA did not have an effect on the CMCase activity of rEngB-B (data not shown), CMCase activity bound to cellulose was considered to indicate the amount of rEngB-B attached to mini-CbpA. As shown in Fig. 6, CMCase activity bound to cellulose was dependent on the amount of added mini-CbpA. These results indicated that the rEngB-B bound to the mini-CbpA could attach to cellulose via the CBD of mini-CbpA. We previously have characterized the recombinant CBD of CbpA and have shown that 50% of the recombinant CBD added to a cellulose suspension can bind to cellulose (5). If all of the purified rEngB-B could bind with mini-CbpA, about 50% of the rEngB-B would be expected to attach to cellulose. As expected, about 50% of the added rEngB-B (2.0 pmol), which was mixed with more than 1.0 pmol of mini-CbpA, did bind to cellulose. These results suggested that all of the rEngB-B was bound to the mini-CbpA. Moreover, although it is very difficult to determine accurate molar amounts of mini-CbpA and rEngB-B, 1.0 pmol...
of mini-CbpA is likely to be enough to bind with 2.0 pmol of rEngB-B.

To confirm that all of the purified rEngB-B could bind to mini-CbpA, the mixture of rEngB-B and mini-CbpA (molar ratio of 1:1) was analyzed with native PAGE. The results are shown in Fig. 7. Compared with the purified rEngB-B (Fig. 7, lane 1) and the purified mini-CbpA (Fig. 7, lane 3), the mixture of rEngB-B and mini-CbpA showed one new band (Fig. 7, lane 2) and no rEngB-B band. This new band was considered to be the rEngB-B–mini-CbpA complex, and all of the rEngB-B appeared to be bound to the mini-CbpA to form minicellulosomes. Since the mini-CbpA was designed to have two cohesin domains, the mixture could form two types of complexes (one or both cohesin domains of mini-CbpA could be occupied by rEngB-B). However, the mixture of rEngB-B and mini-CbpA showed only one new band in spite of the existence of a free mini-CbpA band. Kataeva et al. (8) showed that the mixture of cellulomosomal cellulase CelD and Cip16 containing two cohesin domains from C. thermocellum formed only one type of complex even if an excess amount of Cip16 was present. By use of sedimentation equilibrium analysis, they concluded that both cohesin domains of the complex were occupied by the CelDs. Based on this observation, the mixture of mini-CbpA and rEngB-B may have formed only complexes in which both cohesin domains were occupied.

We have shown that an intact C. cellulovorans EngB endoglucanase can be produced by B. subtilis WB800, that rEngB-B can interact with a mini-CbpA produced by E. coli, and that the rEngB-B–mini-CbpA complex can bind to cellulose. This is a first step that we hope will lead to the production of a variety of designer cellulosomes. In future experiments we hope to express the C. cellulovorans–derived mini-CbpAs and cellulomosomal enzymes in B. subtilis and create designer cellulosomes in vivo.

ACKNOWLEDGMENTS

We thank Helen M. Chan and Judy Wu for their excellent technical support and Wilfred de la Cruz for helpful discussions.

This research was supported by grant DE-DDF03-92ER20069 from the U.S. Department of Energy and by the Natural Sciences and Engineering Research Council of Canada.

REFERENCES

the N terminus of the *Clostridium cellulovorans* major cellulosomal subunit EngE. J. Bacteriol. 181:3270–3276.


