*J. Microbiol. Biotechnol.* (2007), **17**(11), 1782–1788

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY



# Effect of Multiple Copies of Cohesins on Cellulase and Hemicellulase Activities of *Clostridium cellulovorans* Mini-cellulosomes

**CHA, JAEHO[1,†]*, SATOSHI MATSUOKA[1], HELEN CHAN[1], HIDEAKI YUKAWA[2], MASAYUKI INUI[2], AND ROY H. DOI[1]**

[1]*Section of Molecular and Cellular Biology, University of California, Davis, California 95616, U.S.A.*
[2]*Research Institute of Innovative Technology for the Earth, Kyoto 619-0292, Japan*

Received: March 15, 2007 Accepted: May 1, 2007

**Abstract** Cellulosomes in *Clostridium cellulovorans* are assembled by the interaction between the repeated cohesin domains of a scaffolding protein (CbpA) and the dockerin domain of enzyme components. In this study, we determined the synergistic effects on cellulosic and hemicellulosic substrates by three different recombinant mini-cellulosomes containing either endoglucanase EngB or endoxylanase XynA bound to mini-CbpA with one cohesin domain (mini-CbpA1), two cohesins (mini-CbpA12), or four cohesins (mini-CbpA1234). The assembly of EngB or XynA with mini-CbpA increased the activity against carboxymethyl cellulose, acid-swollen cellulose, Avicel, xylan, and corn fiber 1.1–1.8-fold compared with that for the corresponding enzyme alone. A most distinct improvement was shown with corn fiber, a natural substrate containing xylan, arabinan, and cellulose. However, there was little difference in activity between the three different mini-cellulosomes when the cellulosomal enzyme concentration was held constant regardless of the copy number of cohesins in the cellulosome. A synergistic effect was observed when the enzyme concentration was increased to be proportional to the number of cohesins in the mini-cellulosome. The highest degree of synergy was observed with mini-CbpA1234 (1.8-fold) and then mini-CbpA12 (1.3-fold), and the lowest synergy was observed with mini-CbpA1 (1.2-fold) when Avicel was used as the substrate. As the copy number of cohesin was increased, there was more synergy. These results indicate that the clustering effect (physical enzyme proximity) of the enzyme within the mini-cellulosome is one of the important factors for efficient degradation of plant cell walls.

**Keywords:** Cellulosome, *Clostridium cellulovorans*, cellulase, hemicellulase

*Corresponding author
Phone: 82-51-510-2196; Fax: 82-51-514-1778;
E-mail: jhcha@pusan.ac.kr

Mailing address: San 30, Jangjun-dong, Geumjeong-ku, Busan 609-735, Korea
†Present address: Department of Microbiology, College of Natural Sciences, Pusan National University, Busan 609-735, Korea

Many cellulolytic microorganisms have been studied extensively for the degradation of naturally abundant lignocelluloses to valuable products such as fermentable sugars, chemicals, and liquid fuels. Efficient enzymatic degradation of insoluble polysaccharides requires a tight interaction between the enzymes and their substrates and the cooperation of multiple enzymes to enhance the hydrolysis, owing to the complex structure [14, 16, 22, 26]. Cellulosomes, which have been identified and characterized in cellulolytic clostridia such as *Clostridium thermocellum*, *C. cellulolyticum*, and *C. cellulovorans* and ruminal bacteria, are defined as multienzyme complexes having high activity against crystalline cellulose and related plant cell wall polysaccharides [5]. A common feature of the cellulosomes is that they consist of a large number of catalytic components arranged around noncatalytic scaffolding proteins. CbpA, the scaffolding protein of the *Clostridium cellulovorans* cellulosome, possesses one family 3 cellulose binding domain (CBD), nine cohesin domains, and four hydrophilic domains or surface layer homology domains (HLD or SLH) [24]. Binding of the cellulosome to cellulose is mediated by CBD [2, 7, 18].

Each cohesin domain is a subunit-binding domain that interacts with a docking domain, called dockerin, of each catalytic component of cellulosomal enzymes [1, 23]. The amino acid sequences of nine repeated cohesins in CbpA are highly similar to each other [24]. The cohesin-dockerin interaction is crucial for cellulosome assembly. It is well known that the integrity of the cellulosome is critical for the hydrolysis of crystalline cellulose, and dissociated components have weak activity against the substrate [10, 15]. Recently, we assembled recombinant cellulosomes containing only two cohesin domains of CbpA *in vitro*, and the mini-cellulosomes enhanced their activities against crystalline cellulose compared with free cellulosomal enzymes, although the activity of recombinant mini-cellulosomes was much lower than the activity of the native cellulosome [19].

The stimulation of cellulolytic activity may be explained by several factors. Previous data have shown that the CBD of CbpA appears to play a major role in binding the cellulosome to its substrate. This brings the CbpA-bound enzymes in close proximity to the substrate, and the complex is much more efficient in degrading cellulose than the individual free enzymes [8]. The HLD also increases cellulose degradation activity by binding the cellulosome complex to the substrate as well as to the *C. cellulovorans* cell surface [11]. The function of cohesins of CbpA is to interact with the dockerin domains present in all cellulosomal enzymes and to bind these enzymes to CbpA to form the large enzyme complex. A 1:1 stoichiometry has been assumed for the interaction between individual cohesin and dockerin domains [1, 23].

In spite of many publications devoted to the cellulosome and its components, it is still a question as to how the cellulosomes degrade plant cell walls efficiently. In order to assess the relative contribution of the cohesin domains to plant cell wall degradation, we compared the synergistic effects on the hydrolysis of cellulose and hemicellulose by endoglucanase EngB and endoxylanase XynA mini-cellulosomes with a different number of cohesin domains.

In this paper, we describe how the multiple copy number of cohesins affects cellulase and hemicellulase activities. Synergistic effect was observed when the cellulosomal enzyme concentration was increased to be proportional to the copy number of cohesins in mini-cellulosomes. As the number of cohesins was increased, the synergy effect was more distinct. The synergy degree observed by three different mini-cellulosomes is discussed.

## MATERIALS AND METHODS

### Materials

The carboxymethyl cellulose (CMC, medium viscosity) and xylan from oat spelts were purchased from Sigma. Avicel (crystalline cellulose) was purchased from FMC Corporation. Acid-swollen cellulose was prepared from Avicel as described previously [9]. The corn fiber was kindly provided by David J. Johnston of the U.S. Department of Agriculture.

### Bacterial Strains and Media

*Escherichia coli* BL21(DE3) (Novagen) was used as an expression host for mini-CbpA1 and mini-CbpA12 production with pET-22b-mini-CbpA [19, 28]. *Bacillus subtilis* WB800, which is a strain deficient in eight extracellular proteases, was used as an expression host [29, 30] for mini-CbpA1234, EngB, and XynA production with pDG148-mini-CbpA1234, pWB980-EngB [20], and pDG148-XynA. Recombinant strains were cultivated in super-rich medium [4] supplemented with ampicillin (50 μg/ml) or kanamycin (30 μg/ml).

### Construction of Recombinant Plasmids Encoding Mini-CbpA1234 and XynA

Mini-CbpA1234 was designed to consist of a CBD domain, two HLD domains, and four cohesins of scaffolding protein CbpA and was expressed from the inducible pDG148 vector [25] in *B. subtilis*. The gene was designed to allow an in-frame fusion at the C-terminal end with a His tag to add its sequence in a primer. A defined part of CbpA containing its SD sequence, signal peptide sequence, and CBD-HLD1-Coh1-Coh2-HLD2-Coh3-Coh4 was amplified from genomic DNA of *C. cellulovorans* by using primers 5'-GTCGTCGACATGAGGGGGAGCAAT-TATGCA-3' and 5'-GCAGCATGCTCAGTGGTGGTGG-TGGTGGTGGTTAAATGTTAATGTAAGATCTCCA-3'. The amplified 2.8-kbp fragment digested with SalI and SphI was introduced between the SalI and SphI sites of the pDG148 to generate pDG148-mini-CbpA1234. The *xynA* gene containing its SD sequence and signal peptide sequence was amplified from genomic DNA from *C. cellulovorans* by using primers 5'-GTCGTCGACCTTGAATAAAGTA-GTTATAATGTA-3' and 5'-GCAGCATGCTCAGTGGTGG-TGGTGGTGGTGGAATGCACCATTTAACATTGTACC-3', and the amplified 1.6-kbp fragment digested with XhoI and SphI was inserted into pDG148 as a mini-CbpA1234 construction to generate pDG148-XynA.

### Expression and Purification of the Recombinant Mini-CbpAs and Cellulosomal Enzymes

For production of recombinant mini-CbpA1 and mini-CbpA12, *E. coli* BL21(DE3) cells harboring pET-mini-CbpA1 and pET-mini-CbpA12 were grown, and recombinant proteins were induced by adding IPTG as an inducer. *E. coli* cells were grown in 1 l of LB medium at 30°C to a density of 0.6 at 600 nm, and IPTG was added to a final concentration of 0.5 mM. Then, the culture was grown for an additional 4 h. For the production of the recombinant EngB, *B. subtilis* WB800 cells harboring pWB980-EngB was grown in 1 l of super-rich medium for 12 h at 30°C. For the production of recombinant XynA and mini-CbpA1234, *B. subtilis* WB800 cells harboring pDG148-mini-CbpA1234 and pDG148-XynA were grown, and recombinant proteins were induced by adding IPTG as an inducer. *B. subtilis* cells were grown in 1 l of super-rich medium at 30°C to a density of 0.6 at 600 nm, and IPTG was added to a final concentration of 0.5 mM. Then, the culture was grown for an additional 4 h. The recombinant proteins mini-CbpA1, mini-CbpA12, and EngB were purified as described previously [19, 20]. Mini-CbpA1234 and XynA were purified in the same manner, as follows. After the *B. subtilis* cells grown as described above were removed by centrifugation, the culture supernatant was applied to 2 ml of nickel-nitrilotriacetic acid agarose resin (QIAGEN), and the proteins bound to the resin were purified and pooled as previously described for EngB [20]. The pooled solution was desalted and

concentrated into 50 mM Tris-HCl buffer (pH 8.0) by use of the Ultrafree 10-kDa membrane (Millipore). The purity of the concentrated solution was analyzed by SDS-PAGE.

**Protein Determination**

Protein was measured by using the method of Bradford [3] with a protein assay kit from Bio-Rad with bovine serum albumin as a standard. The molar amount of each recombinant protein was calculated by use of the estimated molecular mass of each protein by SDS-PAGE.

**Assembly of Recombinant Cellulosomes**

The purified mini-CbpA and the recombinant cellulosomal subunits were mixed in various ratios (0.2–1 nmol of each protein) in 100 μl of binding buffer (25 mM sodium acetate buffer [pH 6.0], 0.1 mM $CaCl_2$) and kept for 1 h at 4°C. The assembly of mini-CbpA's and cellulosomal subunits was confirmed by native PAGE analysis as described previously [20].

**Determination of Cellulase and Hemicellulase Activities**

The enzymatic activities were assayed in the presence of a 0.5% (w/v) concentration of each polysaccharide at 37°C in 50 mM acetate buffer (pH 6.0). The enzyme concentration used was related to the type of substrate. For the soluble substrates, samples were collected at appropriate times and immediately mixed with 0.38 M sodium carbonate containing 1.8 mM cupric sulfate. For the insoluble substrates, the experiments were done by slowly shaking the reaction mixture. Activities were expressed in units, with 1 U defined as the amount of enzyme releasing 1 μmol of reducing sugar per min. The released reducing sugar, as D-glucose equivalent, was determined by reductometry with the Dygert *et al.* [6] method. All experiments were repeated at least three times.

## RESULTS AND DISCUSSION

**Preparation of Recombinant Mini-CbpA Proteins, rEngB and rXynA**

To investigate whether the copy number of cohesin domains affects cellulase and hemicellulase activities, three recombinant mini-CbpA proteins containing one, two, and four cohesins were constructed and expressed by *E. coli* or *B. subtilis*. In addition, rEngB and rXynA were constructed to serve as enzymatic cellulosomal subunits. Among the recombinant mini-CbpA proteins, mini-CbpA1, mini-CbpA12, and mini-CbpA1234 were composed of a CBD and HLD, and of one, two, and four cohesin domains, respectively. The recombinant proteins mini-CbpA1 and mini-CbpA12 were expressed successfully by *E. coli* as soluble proteins and purified almost to homogeneity by nickel affinity chromatography. The rEngB and rXynA, which consist of a glycosyl hydrolase family 5 catalytic domain and a dockerin domain, and a glycosyl hydrolase family 11 catalytic domain, nodB-like domain, and a dockerin domain, respectively, and mini-CbpA1234 were expressed and secreted in *B. subtilis*. In the case of *B. subtilis*, the enzymes in the culture supernatant were directly purified to homogeneity by nickel affinity chromatography. The apparent molecular mass of each purified protein was determined by SDS-PAGE analysis (Fig. 1).

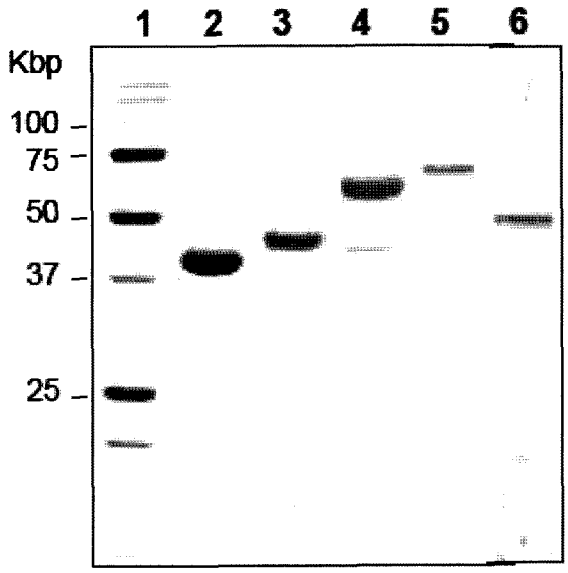


**Fig. 1.** SDS-PAGE of the purified cellulosomal enzymes and mini-CbpAs.
Lane 1, molecular mass standard; lane 2, EngB; lane 3, mini-CbpA1; lane 4, mini-CbpA12; lane 5, mini-CbpA1234; lane 6, XynA.

**Contribution of Mini-CbpA to Cellulase and Hemicellulase Activity**

Previously, it was shown that individual cellulosomal enzymes from *C. cellulovorans* retain the activity against cellulose, but the greater hydrolysis of native cellulose is affected by cellulosomal enzymes associated with CbpA, implying a pivotal role for CbpA [11, 21]. The impact of mini-CbpA's on cellulase and hemicellulase activities also proved that mini-CbpAs enhanced the cellulase activity on celluloses containing a greater crystalline structure [13].

In this study, the impact of three different mini-CbpAs that have different number of cohesins within mini-CbpA was examined. The mini-cellulosomes were assembled by mixing the cellulosomal enzyme. The mini-CbpA and the formation of mini-cellulosomes was confirmed by nondenaturing PAGE. Upon titration of EngB with increasing amounts of mini-CbpA12 (Fig. 2A) or mini-CbpA1234 (Fig. 2B), a new band corresponding to the complex appeared, whereas the intensity of the band corresponding to free EngB decreased. Similar results were obtained using XynA. A new band was considered to

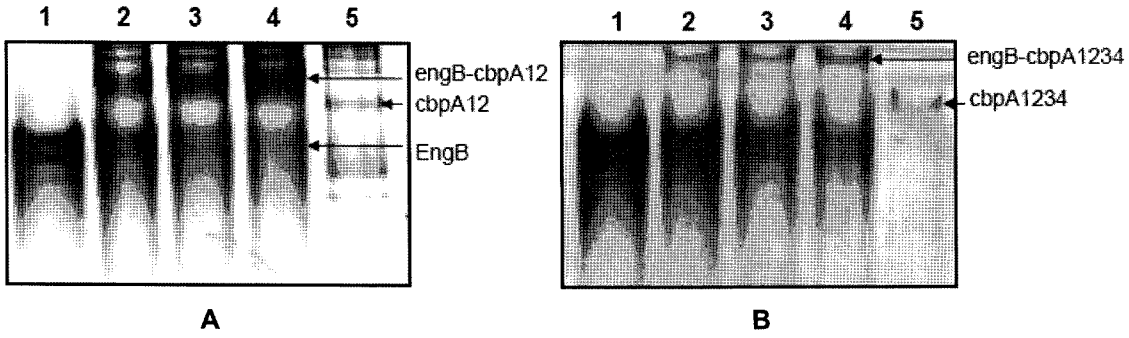


**Fig. 2.** Nondenaturing PAGE of the purified EngB and the purified mini-CbpA complex.
**A.** Mini-CbpA12 complex. Lane 1, EngB alone; Molar ratios of EngB/mini-CbpA12 were lane 2, 1:0.4; lane 3, 1:0.5; lane 4, 1:0.7; lane 5, mini-CbpA12 alone. **B.** Mini-CbpA1234. Lane 1, EngB alone; Molar ratios of EngB/mini-CbpA1234 were lane 2, 1:0.2; lane 3, 1:0.3; lane 4, 1:0.45; lane 5, mini-CbpA1234 alone.

be the band for the "EngB mini-cellulosome or XynA mini-cellulosome" as described previously [21]. The results are in good agreement with the previous studies about *C. thermocellum* cellulosome complex formation. The binding of cellulosomal enzymes EngB and XynA to mini-CbpA12 and mini-CbpA1234 was demonstrated by the interaction Western blotting technique developed in our laboratory [27]. These results indicated that mini-CbpA's could bind both EngB and XynA.

The hydrolytic activity of recombinant mini-cellulosomes was assayed on various celluloses and hemicellulloses. Addition of mini-CbpA12 increased the activity of EngB towards CMC by up to 2-fold (Fig. 3). The increase of the activity was dependent on the increase of EngB/mini-CbpA12 molar ratio, with little increase past the equivalent point. To investigate whether mini-CbpA enhances the cellulase and hemicellulase activities of catalytic subunits of the cellulosome, we compared the hydrolytic activity of free EngB and XynA against different forms of celluloses and hemicelluloses, with an equimolar mixture of each EngB and XynA with mini-CbpA12. As shown in Table 1,

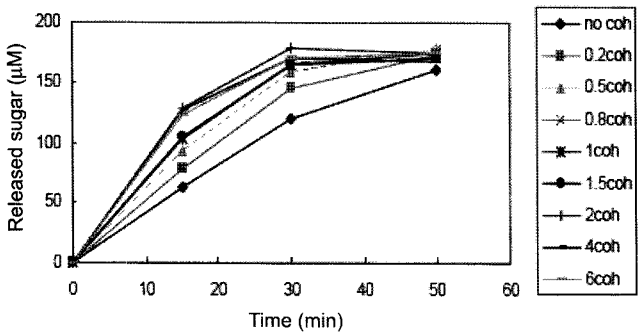


**Fig. 3.** Time course of hydrolysis of CMC by EngB mini-cellulosome.
Hydrolysis was performed in 1 ml of 0.5% CMC suspended in 50 mM sodium acetate buffer (pH 6.0). EngB (1 nM) was mixed with mini-CbpA12 in the various molar ratios indicated.

the large increases of the EngB and XynA activities were obtained by the addition of mini-CbpA12. The most distinct increase of the enzyme activity was observed on corn fiber. The result showed that whatever the type of cellulose or hemicellulose, the complex of EngB mini-cellulosome or XynA mini-cellulosome exhibited higher activity than observed for free enzyme. The enhancement of the cellulase and hemicellulase activities by addition of mini-CbpA was also observed when mini-CbpA1 and mini-CbpA1234 were added (data not shown). Such an increase in activity was also observed previously, where EngL or XynA bound to the mini-CbpA's (mini-CbpA12 or mini-CbpA56) over

**Table 1.** Effects of mini-CbpA12 on the activity of EngB and XynA against celluloses and hemicelluloses.

| Substrate | Enzyme | Free enzyme | Mini-CbpA12 | |
|---|---|---|---|---|
| | | Amt[a] (U/μmol) | Amt (U/μmol) | SF[b] |
| CMC | EngB | 4,027 (52) | 4,824 (770) | 1.2 |
| | XynA | 22 (3.2) | 25 (5.2) | 1.1 |
| AS-cellulose | EngB | 5.1 (0.27) | 5.7 (0.01) | 1.1 |
| | XynA | ND[c] | ND | |
| Avicel | EngB | 1.2 (0.04) | 1.6 (0.28) | 1.3 |
| | XynA | ND | ND | |
| Xylan | EngB | 62 (8.7) | 74 (7.3) | 1.2 |
| | XynA | 621 (141) | 722 (173) | 1.2 |
| Corn fiber | EngB | 0.074 (0.010) | 0.115 (0.020) | 1.6 |
| | XynA | 0.052 (0.003) | 0.073 (0.012) | 1.4 |

[a]The amount of released reducing sugars from each substrate (0.5%) at 37°C is given. 1 U was defined as the amount of enzyme releasing 1 μmol of reducing sugar per min. Each enzyme and mini-scaffolding protein were in equimolar amounts. CMC, As-cellulose, Avicel, xylan, and corn fiber were degraded by 1 nM for 30 min (EngB), 17 nM for 2 h (XynA), 40 nM for 12 h, 40 nM for 24 h, 20 nM for 30 min (EngB), 3.4 nM for 1 h (XynA), and 250 nM for 2 h, respectively. The number in parenthesis indicates standard deviation.
[b]SF, ratio of reducing sugar released by mini-cellulosome to reducing sugars released by the corresponding free enzyme.
[c]ND, not detected.

**Table 2.** Combined effects of EngB mini-cellulosome and XynA mini-cellulosome on xylan and corn fiber degradation.

| Mini-cellulosome | Xylan[b] | | | | Corn fiber[c] | | | |
|---|---|---|---|---|---|---|---|---|
| | EngB Amt[a] (U/μmol) | XynA Amt (U/μmol) | EngB+XynA Amt (U/μmol) | SF[d] | EngB Amt (U/μmol) | XynA Amt (U/μmol) | EngB+XynA Amt (U/μmol) | SF |
| No CbpA | 52.6 (4.9) | 605 (96) | 840 (149) | 1.3 | 0.074 (0.012) | 0.052 (0.003) | 0.098 (0.017) | 0.8 |
| CbpA1 | 65.0 (9.9) | 900 (199) | 990 (37.3) | 1.0 | 0.096 (0.020) | 0.073 (0.020) | 0.140 (0.066) | 0.8 |
| CbpA12 | 66.8 (2.7) | 913 (190) | 1016 (15.0) | 1.0 | 0.115 (0.019) | 0.073 (0.012) | 0.170 (0.018) | 0.9 |
| CbpA1234 | 58.9 (1.2) | 723 (80) | 970 (29.7) | 1.2 | 0.115 (0.007) | 0.096 (0.057) | 0.187 (0.003) | 0.9 |

[a]The amount of released reducing sugars from each substrate (0.5%) at 37°C is given.
[b]Xylan was degraded by 5 nM EngB mini-cellulosome and 5 nM XynA mini-cellulosome for 30 min.
[c]Corn fiber was degraded by 250 nM XynA mini-cellulosome and 250 nM EngB mini-cellulosome for 24 h.
[d]SF, ratio of reducing sugar released by EngB plus XynA mini-cellulosome by the sum of each corresponding mini-cellulosome.

enzyme alone increased the activity by 1.4–2.0-fold against cellulose and hemicellulose [13]. The increase of the activity after the binding of free enzyme to the mini-CbpA is thought to be due to the conformational changes of the participating components, thereby allowing the enzyme more freedom to distribute on the preferred substrate sites.

### Combined Effect of EngB- and XynA-Mini-Cellulosomes to Hemicellulase Activity

Previously, it was shown that simultaneous reactions with two different hemicellulases (ArfA and XynA) or cellulases (EngE, EngH, and ExgS) and hemicellulase (XynA) exhibit synergistic effects on plant cell wall substrates [13, 21]. It was explained that degradation of xylan networks between cellulose microfibrils by xylanases might allow cellulases to access and degrade cellulose microfibrils embedded in the deeper structure [21]. In this experiment, the synergistic effects between EngB and XynA were investigated by using two different hemicelluloses, oat spelts xylan containing over 70% xylose residues, 10% glucose, and 15% arabinose residues; and corn fiber containing 15% cellulose and 40% arabinoxylan. EngB and XynA were simultaneously incubated with different hemicelluloses for 30 min (xylan) and 24 h (corn fiber) at 37°C. No significant synergy was observed between EngB mini-cellulosome and XynA mini-cellulosome against xylan and corn fiber substrates. The amount of reducing sugar released from the simultaneous reaction with EngB and XynA was not significantly increased compared with that released from the sum of EngB or XynA alone (Table 2). In the case of the xylan substrate, XynA and EngB activity was over 90% versus less than 10%, which indicates xylan attack is dominant, thus synergy was not found. Although EngB and XynA showed similar activities against corn fiber, the basic endocellulase and xylanase activities were too low to find synergy. Therefore, XynA and EngB might degrade different regions of the substrates (arabinoxylan component by XynA and cellulose component by EngB). As at least eight different kinds of cellulases and four kinds of hemicellulases are known to bind to scaffolding protein CbpA, the construction of the mini-cellulosome complexes composed of several kinds of cellulases and hemicellulases with mini-CbpAs will be necessary to observe the high synergy shown by purified cellulosomes from *C. cellulovorans* culture broths.

### Synergistic Effect of Mini-Cellulosomes with Different Number of Cohesins on Cellulase Activity

Experiments were designed to test whether the EngB activity enhancement by mini-CbpA is due to the clustering effect that allowed simultaneous attack by more than one enzyme acting at the same site of the substrate. The synergy effects of mini-cellulosomes with one, two, and four cohesins were determined by using three different cellulosic substrates: CMC, AS-cellulose, and Avicel. Since one cohesin domain binds one cellulosomal enzyme, EngB was mixed with each mini-CbpA at a molar ratio of 1:1, 2:1, and 4:1 to assemble the recombinant mini-cellulosomes. The amount

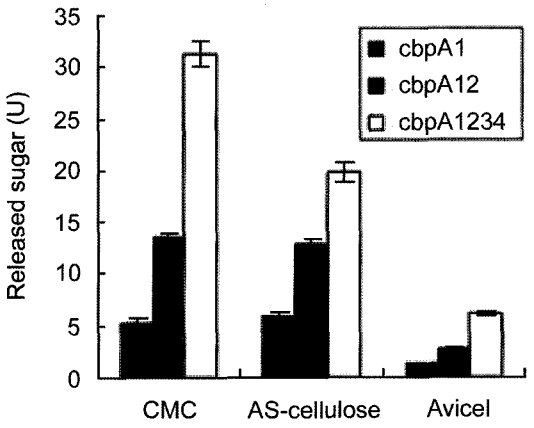


**Fig. 4.** Synergistic effects between EngB and mini-CbpAs on cellulose substrates.
EngB was mixed with mini-CbpA1, mini-CbpA12, and mini-CbpA1234 at a molar ratio of 1:1, 2:1, and 4:1, respectively. The amount of released sugar (units) for CMC substrate should be multiplied by 1,000.

**Table 3.** Synergistic effect of mini-CbpAs on EngB activity against cellulose substrates.

| Substrate | w/CbpA1 | | w/CbpA12 | | w/CbpA1234 | |
|---|---|---|---|---|---|---|
| | Amt[a] (U) | SF[b] | Amt (U) | SF | Amt (U) | SF |
| CMC | 5,257 (646) | 1.2 | 13,400 (1898) | 1.4 | 31,199 (3475) | 1.5 |
| AS-cellulose | 5.93 (0.18) | 1.1 | 12.79 (1.56) | 1.2 | 19.74 (0.56) | 1.3 |
| Avicel | 1.25 (0.12) | 1.2 | 2.74 (0.57) | 1.3 | 6.01 (1.77) | 1.8 |

[a]The amount of released reducing sugars from each substrate (0.5%) at 37°C is given. EngB was mixed with mini-CbpA1, mini-CbpA12, and mini-CbpA1234 at a molar ratio of 1:1, 2:1, and 4:1, respectively. The number in parenthesis indicates standard deviation. CMC was degraded by 0.25 nM enzyme for mini-CbpA1, 0.5 nM for mini-CbpA12, and 1 nM for mini-CbpA1234 for 7 min. As-cellulose and Avicel were degraded by 10 nM enzyme for mini-CbpA1, 20 nM for mini-CbpA12, and 40 nM for mini-CbpA1234 for 30 h.

[b]SF, ratio of reducing sugar released by mini-cellulosome to reducing sugars released by the corresponding free enzyme.

of reducing sugar released from each substrate was examined and is shown in Fig. 4 and Table 3.

As we discussed above, the amount of reducing sugar released was increased when any mini-CbpA was combined with free EngB compared with that released with EngB alone. The synergy effect was increased in the order of mini-CbpA1234>mini-CbpA12>mini-CbpA1 with all three substrates. The highest degree of synergy was observed with mini-CbpA1234 (1.8-fold) and then mini-CbpA12 (1.3-fold), and the lowest synergy was observed with mini-CbpA1 (1.2-fold) when Avicel was used as a substrate. The result of the synergy effect indicates that the increase of the degree of synergy is proportional to the increase of the copy number of cohesins in mini-cellulosome. This result implies that the physical proximity of the catalytic subunits of EngB in the mini-cellulosome complex might help each other to attack the cellulosic substrates more efficiently. However, the physical association of two or more molecules of EngB did not significantly enhance the degradation of Avicel.

Mini-cellulosomes constructed from *C. cellulovorans* components have shown synergy between cellulases [19], between cellulases and hemicellulases [21], and between cellulosomal and non-cellulosomal enzymes [12, 17]. In all cases, synergy was observed, indicating that the synergy between enzymes in cellulosomes makes the cellulosome structure more effective in attacking the substrate. As combined with our present results and the previous studies about the role of the cellulose binding domain and hydrophilic domain, the synergism observed by mini-cellulosome may be due to the combinatorial effect of the cellulose-binding domain, the hydrophilic domain, and the clustering effect of the enzyme (physical enzyme proximity).

## Acknowledgments

The research was supported in part by the Department of Energy grant DE-FG02-04ER15553 and the RITE Institute to R.H.D. J. Cha was supported by the Overseas Research grant from Pusan National University.

## REFERENCES

1. Bayer, E. A., E. Morag, and R. Lamed. 1994. The cellulosome -- a treasure-trove for biotechnology. *Trends Biotechnol.* **12:** 379–386.
2. Boraston, A. B., B. W. McLean, J. M. Kormos, M. Alam, N. R. Gilkes, C.A. Haynes, P. Tomme, D. G. Kilburn, and R. A. Warren. 1999. Carbohydrate-binding modules: Diversity of structure and function, pp. 202–211. *In* H. J. Gilbert, G. J. Davies, B. Henrissat, and B. Svensson (eds.), *Recent Advances in Carbohydrate Bioengineering.* The Royal Society of Chemistry, Cambridge, United Kingdom.
3. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:** 248–254.
4. Cho, H.-Y., H. Yukawa, M. Inui, R. H. Doi, and S.-L. Wong. 2004. Production of minicellulosomes from *Clostridium cellulovorans* in *Bacillus subtilis* WB800. *Appl. Environ. Microbiol.* **70:** 5704–5707.
5. Doi, R. H. and A. Kosugi. 2004. Cellulosomes: Plant-cell-wall-degrading enzyme complexes. *Nat. Rev. Microbiol.* **2:** 541–551.
6. Dygert, S., L. H. Li, D. Florida, and J. A. Thoma. 1965. Determination of reducing sugar with improved precision. *Anal. Biochem.* **13:** 367–374.
7. Gerngross, U. T., M. P. Romaniec, T. Kobayashi, N. S. Huskisson, and A. L. Demain. 1993. Sequencing of a *Clostridium thermocellum* gene (*cipA*) encoding the cellulosomal SL-protein reveals an unusual degree of internal homology. *Mol. Microbiol.* **8:** 325–334.
8. Goldstein, M. A., M. Takagi, S. Hashida, O. Shoseyov, R. H. Doi, and I. H. Segel. 1993. Characterization of the cellulose-binding domain of the *Clostridium cellulovorans* cellulose-binding protein A. *J. Bacteriol.* **175:** 5762–5768.
9. Ichiishi, A., S. Sheweita, and R. H. Doi. 1998. Characterization of EngF from *Clostridium cellulovorans* and identification of a novel cellulose binding domain. *Appl. Environ. Microbiol.* **64:** 1086–1090.
10. Kataeva, I., G. Guglielmi, and P. Béguin. 1997. Interaction between *Clostridium thermocellum* endoglucanase CelD and polypeptides derived from the cellulosome-integrating protein CipA: Stoichiometry and cellulolytic activity of the complexes. *Biochem. J.* **326:** 617–624.

11. Kosugi, A., Y. Amano, K. Murashima, and R. H. Doi. 2004. Hydrophilic domains of scaffolding protein CbpA promote glycosyl hydrolase activity and localization of cellulosomes to the cell surface of *Clostridium cellulovorans*. *J. Bacteriol.* **186:** 6351–6359.
12. Kosugi, A., K. Murashima, and R. H. Doi. 2002. Characterization of non-cellulosomal subunits, AfrA and BgaA from *Clostridium cellulovorans*, that cooperate with the cellulosome in plant cell wall degradation. *J. Bacteriol.* **184:** 6859–6865.
13. Koukiekolo, R., H.-Y. Cho, A. Kosugi, M. Inui, H. Yukawa, and R. H. Doi. 2005. Degradation of corn fiber by *Clostridium cellulovorans* cellulases and hemicellulases and contribution of scaffolding protein CbpA. *Appl. Environ. Microbiol.* **71:** 3504–3511.
14. Kusuma, K., G. H. Chon, J.-S. Lee, J. Kongkiattikajorn, K. Ratanakhanokchai, K. L. Kyu, J. H. Lee, M. S. Roh, Y. Y. Choi, H. Park and Y. S. Lee. 2006. Hydrolysis of agricultural residues and kraft pulps by xylanolytic enzymes from alkaliphilic *Bacillus* sp. strain BK. *J. Microbiol. Biotechnol.* **16:** 1255–1261.
15. Lamed, R., E. Setter, and E. A. Bayer. 1983. Characterization of a cellulose-binding, cellulase-containing complex in *Clostridium thermocellum*. *J. Bacteriol.* **156:** 828–836.
16. Lee, Y. E. and P. O. Lim. 2004. Purification and characterization of two thermostable xylanases from *Paenibacillus* sp. DG-22. *J. Microbiol. Biotechnol.* **14:** 1014–1021.
17. Morag, E., E. A. Bayer, and R. Lamed. 1990. Relationship of cellulosomal and noncellulosomal xylanases of *Clostridium thermocellum* to cellulose-degrading enzymes. *J. Bacteriol.* **172:** 6098–6105.
18. Morag, E., A. Lapidot, D. Govorko, R. Lamed, M. Wilchek, E. A. Bayer, and Y. Shoham. 1995. Expression, purification, and characterization of the cellulose-binding domain of the scaffoldin subunit from the cellulosome of *Clostridium thermocellum*. *Appl. Environ. Microbiol.* **61:** 1980–1986.
19. Murashima, K., A. Kosugi, and R. H. Doi. 2002. Synergistic effects on crystalline cellulose degradation between cellulosomal cellulases from *Clostridium cellulovorans*. *J. Bacteriol.* **184:** 5088–5095.
20. Murashima, K., C. L. Chen, A. Kosugi, Y. Tamaru, R. H. Doi, and S.-L. Wong. 2002. Heterologous production of *Clostridium cellulovorans engB*, using protease-deficient *Bacillus subtilis*, and preparation of active recombinant cellulosomes. *J. Bacteriol.* **184:** 76–81.
21. Murashima, K., A. Kosugi, and R. H. Doi. 2003. Synergistic effects of cellulosomal xylanase and cellulases from *Clostridium cellulovorans* on plant cell wall degradation. *J. Bacteriol.* **185:** 1518–1524.
22. Patthra, P., G. H. Chon, K. Ratanakhanokchai, K. L. Kyu, O.-H. Jhee, J. Kang, W. H. Kim, K.-M. Choi, G.-S. Park, J.-S. Lee, H. Park, M. S. Rho, and Y.-S. Lee. 2006. Selection of multienzyme complex-producing bacteria under aerobic cultivation. *J. Microbiol. Biotechnol.* **16:** 1269–1275.
23. Salamitou, S., O. Raynaud, M. Coughlan, P. Béguin, and J.-P. Aubert. 1994. Recognition specificity of the duplicated segments present in *Clostridium thermocellum* endoglucanase CelD and in the cellulosome-integrating protein CipA. *J. Bacteriol.* **176:** 2822–2827.
24. Shoseyov, O., M. Takagi, M. A. Goldstein, and R. H. Doi. 1992. Primary sequence analysis of *Clostridium cellulovorans* cellulose binding protein A (CbpA). *Proc. Natl. Acad. Sci. USA* **89:** 3483–3487.
25. Stragier, P., C. Bonamy, and C. Karmazyn-Campelli. 1988. Processing of a sporulation sigma factor in *Bacillus subtilis*: How morphological structure could control gene expression. *Cell* **52:** 697–704.
26. Takagi, M., S. Hashida, M. A. Goldstein, and R. H. Doi. 1993. The hydrophobic repeated domain of the *Clostridium cellulovorans* cellulose-binding protein (CbpA) has specific interactions with endoglucanases. *J. Bacteriol.* **175:** 7119–7122.
27. Tachaapaikoon, C., Y. S. Lee, K. Rantanakhanokchai, S. Pinitglang, K. L. Kyu, M. S. Rho, and S.-K. Lee. 2006. Purification and characterization of two endoxylanases from an alkaliphilic *Bacillus halodurans* C-1. *J. Microbiol. Biotechnol.* **16:** 613–618.
28. Tamaru, Y. and R. H. Doi. 2001. Pectate lyase A, an enzymatic subunit of the *Clostridium cellulovorans* cellulosome. *Proc. Natl. Acad. Sci. USA* **98:** 4125–4129.
29. Wu, X.-C., W. Lee, L. Tran, and S.-L. Wong. 1991. Engineering a *Bacillus subtilis* expression-secretion system with a strain deficient in six extracellular proteases. *J. Bacteriol.* **173:** 4952–4958.
30. Wu, S.-C., J. C. Yeung, Y. Duan, R. Ye, S. J. Szarka, H. R. Habibi, and S.-L. Wong. 2002. Functional production and characterization of a fibrin-specific single-chain antibody fragment from *Bacillus subtilis*: Effects of molecular chaperones and a wall-bound protease on antibody fragment production. *Appl. Environ. Microbiol.* **68:** 3261–3269.