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Functional Analysis of a Hybrid Endoglucanase of Bacterial Origin Having a Cellulose Binding Domain from a Fungal Exoglucanase

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ABSTRACT

A cellulose binding domain (CBD) of an endo-β-1,4-glucanase (Ben) from the bacterium *Bacillus subtilis* BSE616 was replaced with the CBD of exoglucanase I (TexI) from the fungus *Trichoderma viride* HK-75. The resultant hybrid enzyme Ben'-CBD_{TexI}, comprising the catalytic domain (Ben') of Ben and the CBD (CBD_{TexI}) of TexI, was highly expressed at 20% of the total protein in *Escherichia coli*. The molecular mass of the hybrid enzyme was estimated to be ca. 38 kDa by SDS-PAGE, which was in good agreement with that calculated from 305 amino acids of Ben and 42 amino acids of CBD_{TexI}. The hybrid enzyme exhibited almost the same activity as that of the original Ben toward soluble substrates, such as cellooligosaccharides. The hybrid enzyme showed higher binding ability and hydrolysis activity toward microcrystalline cellulose (Avicel), even though the length of the CBD of TexI was four times smaller than that of Ben. The hybrid enzyme was more resistant to tryptic digestion than the original Ben. The efficient binding ability of the hybrid enzyme to Avicel permitted purification of the enzyme using an Avicel-affinity column to the extent of ca. 90% purity.

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Index Entries: *Bacillus subtilis* endoglucanase; *Trichoderma viride* exoglucanase; cellulose binding domain; fusion protein; resistance to tryptic digestion; affinity purification.

INTRODUCTION

Cellulolytic enzymes are classified into two types according to the mode of hydrolysis substrates: endo-β-1,4-glucanase (EC 3.2.1.4) and exo-cellobiohydrolase (EC 3.2.1.91). Both types of cellulase usually possess multiple functional domains. These domains act synergistically for efficient degradation of insoluble substrates, which have a microcrystalline structure.

Almost all bacterial and fungal cellulases studied to date have catalytic domains and cellulose binding domains (CBDs). These two domains are separated by a linker region *(1)*. The CBDs seem to play an important role in the hydrolysis of insoluble substrates by accelerating the interaction between enzyme and substrate. However, which domain is correlated to the specificity of native cellulose hydrolysis is still unclear. The binding ability to Avicel of these cellulolytic enzymes was used for purification and immobilization of the enzymes *(2)*. According to the sequence similarities of catalytic domains and CBDs the cellulolytic enzymes were classified into 12 groups, and 8 different CBD families were proposed *(3)*.

The Ben gene expressed in *Escherichia coli* was characterized previously *(4–7)*. The catalytic domain (Ben') was classified into family A2, and the putative CBD, comprising 133 amino acid residues, was grouped to family III *(3,6–9)*. The binding ability of CBD could be used for immobilization of β-glucosidase of *Cellulomonas fimi (10)*. On the other hand, exoglucanase I (ExoI) from *Trichoderma viride* HK-75 is a major cellulase and exhibits high activity toward Avicel, producing cellobiose predominantly *(11)*.

To compare a fungal and bacterial CBD, a CBD of an exoglucanase/xylanase from *C. fimi* was replaced with the CBD of cellobiohydrolase I from *T. reesei (12)*.

This article describes the construction and expression of a hybrid protein, composed of a catalytic domain of the endo-type bacterial cellulase and a relatively small CBD comprising 36 amino acid residues of the exo-type fungal cellulase (TexI) from *T. viride* HK-75, in order to elucidate the catalytic and binding functions of these cellulases. Also, we report on the resistance of the hybrid protein to tryptic digestion.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

E. coli JM109 was used as a bacterial cloning host. The plasmid pUC19 was used as a cloning vector. An *E. coli* transformant harboring plasmid

pBS1 *(4)* was used for expression of the original Ben as a positive control. Plasmid pENDO *(13)* and pHSG-GPexo carrying a cellulose binding region of the exoglucanase gene from *T. viride* HK-75 (unpublished data) were used as the gene source of Ben and the CBD region of TexI, respectively. Cells were grown in Luria-Burtani (LB) medium supplemented with ampicillin (100 μg/mL) at 37°C. The positive clones were selected on plates containing 0.5% carboxymethyl-cellulose (CMC) or by pouring 2 m*M* 4-methylumbelliferyl-β-D-cellobioside (MUC) solution onto the plates.

Transformation and Recombinant DNA Techniques

Competent cells of *E. coli* were prepared by the method of Hanahan *(14)*. Standard procedures of Sambrook et al. *(15)* were followed for construction of the recombinant plasmids. DNA fragments were recovered with the Gene Clean II kit (Bio 101 Inc., La Jolla, CA).

Polymerase Chain Reactions (PCRs)

Primer sequences were designed to minimize primer-dimer production and to reduce mispriming. Oligonucleotide primers were synthesized by DNA synthesizer (ABI392, Applied Biosystems Inc., Foster City, CA) and purified by OPC columns (Applied Biosystems Inc.). PCR was carried out with a thermal cycler (Program Temp Control System PC-700, Astec Co. Ltd., Tokyo, Japan) in 50 μL of reaction volume. PCRs were performed in the buffer containing 2 m*M* $MgCl_2$, 0.1 m*M* (each) of the four deoxynucleoside triphosphates, 50 pmol forward primer, 50 pmol reverse primer, 5–200 ng template DNA, and 0.5 U *Taq* polymerase. The conditions for 20 successive cycles were as follows: denaturation at 93°C for 1 min, annealing at 56°C for 1.5 min, and primer extension at 72°C for 1.5 min. One additional cycle was performed as follows: denaturation at 93°C for 1 min, primer extension at 72°C for 10 min, and cooling at 4°C.

Construction of the Ben′-CBD_{TexI} Hybrid Gene

By three rounds of PCR, the catalytic domain sequence of Ben (Ben′) was fused with the CBD sequence of TexI, which contained a part of linker sequence (Fig. 1). Following four primers were used in this study.

Primer 1: 5′-CTATGACCATGATTACGCC-3′

Primer 2: 5′-GGCCGGGAGAGCTTCCATCTTTGGTGCCGAGAATG-3′

Primer 3: 5′-CATTCTCGGCACCAAAGATGGAAGCTCTCCCGGCC-3′

Primer 4: 5′-*GTCGTT***AAGCTT**TTACAAGCACTGAGAGTAGT-3′

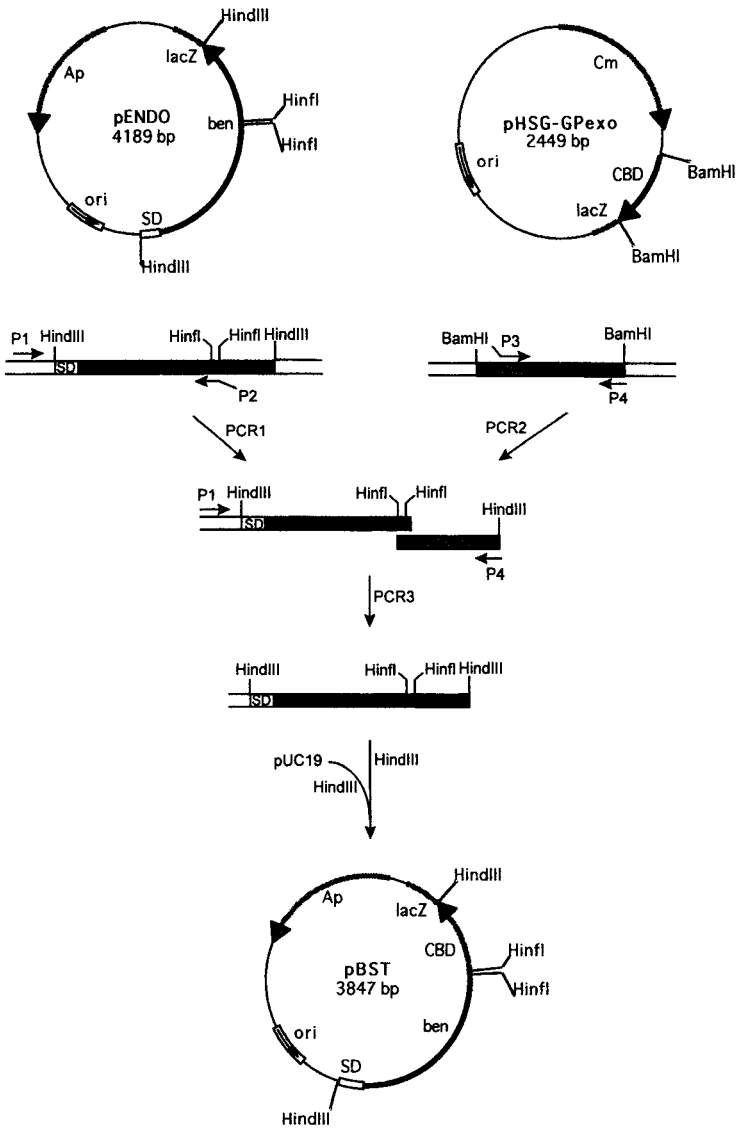

Fig. 1. Construction of pBST expressing Ben'-CBD_{Text}.

A newly introduced *Hin*dIII restriction site (boldface) and extra bases (italic) were introduced at the 5'-end of primer 4. Primers 1 and 3 were designed corresponding to the 5'-sequences of Ben' and CBD regions, respectively. Primers 2 and 4 were complementary to the 3'-sequences of CBD and Ben' regions, respectively. Primers 2 and 3 were designed such that their 3'-ends hybridize to template sequence on one side, and the 5'-ends were complementary to template sequence on the other side. The PCR product 1 coding for Ben', using primers 1 and 2, and the PCR prod-

uct 2 coding for the CBD region, using primers 3 and 4, were individually amplified. The PCR products 1 and 2 thus obtained share overlapping sequences at their respective 3'- and 5'-ends. The second PCR was performed using the PCR products 1 and 2 and primers 1 and 4 to generate the PCR product 3 containing the *ben'-cbd* hybrid gene. The PCR product 3 was purified, digested with *Hin*dIII, and then ligated into the *Hin*dIII-digested pUC19 to generate pBST. The junction sequence of the hybrid gene was verified by sequence analysis of the double-stranded DNA by the dideoxy chain-termination method.

Preparation of Native, Truncated, and Fusion Proteins

After full stationary phase was reached, *E. coli* JM109 (pBST) transformant was harvested by centrifugation at 12,000 *g* for 5 min and washed twice in 50 m*M* sodium citrate buffer (pH 5.5) containing 1 m*M* EDTA and 1 m*M* phenylmethylsulfonyl fluoride (PMSF). The cells were broken by ultrasonication (Branson, Model 350), and the supernatant containing Ben'-CBD_{Cex} fusion protein was collected. For purification of the Ben'-CBD_{Cex} fusion protein, 6 *M* guanidine/HCl were applied to the column after adsorption of the protein to Avicel as in the procedure described previously *(9)*. Desorbed protein was dialyzed against the same buffer and was further purified by Poros HQ (PerSeptive Biosystems Co., Framingham, MA).

The native Ben was prepared from *E. coli* (pBS1) as above and truncated Ben' was prepared as described previously *(8)*.

Enzyme Assay

Endo-β-1,4-glucanase activity was determined by measuring the release of reducing sugar from CMC at pH 5.5 and 50°C according to the procedure of Miller *(16)* as in the method described previously *(9)*. Avicelase activity was assayed in a reaction mixture containing 0.5 or 20% Avicel and the enzyme (typically 5–7 μ*M*) in 1.0 mL of 50 m*M* sodium citrate buffer (pH 5.5). After appropriate time of incubation at 40 or 50°C, cellulose was removed and reducing sugar remaining in the supernatant was measured. One unit of enzyme activity was defined as the amount of enzyme releasing 1 μmol of glucose Eq/min under the assay condition. When *p*-nitrophenyl-β-D-cellobioside (PNPC) was used as a substrate, the amount of liberated *p*-nitrophenol was quantified by its absorbance at 400 nm.

Adsorption Assay

Adsorption property of the fusion protein was tested with Avicel PH-101 as described previously *(9)* with minor modifications. A 50-mg cellulose sample was suspended in 50 m*M* sodium citrate buffer (pH 5.5). After 1 h of incubation of the purified enzymes (typically 0–3.5 μ*M*) in 1 mL of the buffer

on ice, cellulose was removed or collected by centrifugation, and the protein remaining in the supernatant was measured. Nonspecifically adsorbed protein was removed by washing four times with 1 mL of 1 *M* NaCl. Gel-loading buffer containing 0.4% SDS was added to the mixture, and then desorbed protein was analyzed by SDS-PAGE and activity staining.

Activity Staining

After denaturating PAGE, the gel was washed twice in 50 m*M* sodium citrate buffer (pH 5.5) containing 25% (v/v) isopropanol for 1 h at room temperature. The gel was soaked in 50 m*M* sodium citrate buffer (pH 5.5) for 1 h at room temperature, transferred to a fresh buffer containing 1 m*M* MUC, and further soaked for 30 min at 4°C. The gel was transferred onto a glass plate and incubated at 55°C for about 30 min. The MUC-hydrolyzing activity was photographed as fluorescent bands under UV light (254 nm). Alternatively, the gel was transferred to an agar sheet containing 0.5% CMC, incubated at 55°C for about 30 min, and visualized with 0.1% Congo red and 1 *M* NaCl solution.

Tryptic Digestion

Enzyme samples (0.067 units) were digested with 100 μg trypsin in 15 μL of 50 m*M* Tris/HCl (pH 8.0) at 37°C for 1 h. After mixing and boiling with SDS-loading buffer, the cleaved enzymes were analyzed by SDS-PAGE and activity staining.

Reaction Product and Protein Analysis

The reaction products of the fusion protein for cellotriose (G3), cellotetraose (G4), cellopentaose (G5), PNPC, CMC, and Avicel were analyzed by a μBondapak-NH_2 HPLC column (10 μm) and a differential refractometer (Waters) with 73% (v/v) acetonitrile. Protein analyses, such as PAGE, and protein concentration were done as in the methods cited previously *(10)*.

Chemicals

Avicel PH101 was purchased from Fluka (Buchs, Switzerland). CMC, Cellooligosaccharides, PNPC, MUC, PMSF, and trypsin were obtained from Sigma (St. Louis, MO). All other chemicals were standard reagent grades.

RESULTS AND DISCUSSION

Expression of the Hybrid Gene in *E. coli*

The 1.16-kb hybrid gene encoding a protein composed of 305 amino acid residues of Ben and 42 amino acid residues of CBD_{Cex} was successfully constructed by three rounds of PCR, two for amplification of the individ-

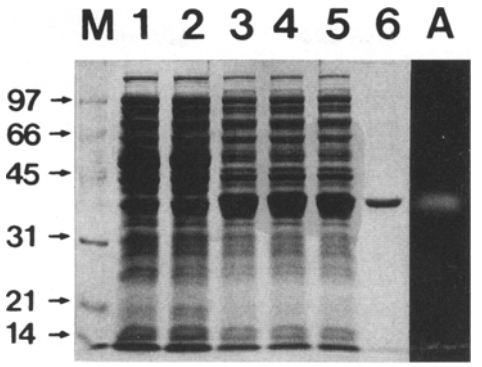

Fig. 2. SDS-PAGE analysis of the expression, binding to Avicel, and activity staining with MUC after renaturation of Ben′-CBD$_{TexI}$. Lane M, standard size markers; lanes 1 and 2, transformants containing a fused gene oriented in reverse; lanes 3, 4, and 5, positive transformants; lane 6, desorbed hybrid sample of lane 5; lane A, activity stained sample of lane 6 *in situ* as described in the text. Each sample lane was loaded with 95 μg of protein, except lane A (8 μg). Numbers on the left side are expressed in kilodaltons.

ual fusion sources, and one to amplify the entire fusion gene (Fig. 1). pBST containing the hybrid gene was transformed into *E. coli* JM109. From the 50 primary candidates, three positive clones were obtained. These clones possessed the same insert and produced active enzyme at the level of 88–93%, compared with *E. coli* cell (pENDO) producing native Ben. The cell extracts of these clones contained large amounts of hybrid protein at the level as high as ca. 20% of the total cell protein (Fig. 2). The sequence at the junction between the bacterial catalytic domain and fungal CBD region was not altered at all during PCR extension (data not shown).

Adsorption of the Hybrid Enzyme to Avicel

It was reported previously that the 52-kDa original native Ben bound to Avicel, whereas the 33-kDa truncated Ben (Ben′), which lacks the CBD, failed to bind to Avicel *(9)*. The CBD$_{TexI}$, originating from a strain of the fungus *T. viride*, retained the native binding ability to cellulosic substrates *(11)*. The hybrid Ben′-CBD$_{TexI}$ produced from *E. coli* (pBST) exhibited significant adsorbing ability to Avicel compared with the Ben′. In order to purify the hybrid Ben′-CBD$_{TexI}$ protein, 10 g of Avicel was added to 40 mL of the cell extracts. After 1 h of adsorption, Ben′-CBD$_{TexI}$ adsorbed to Avicel was washed with 1 *M* NaCl and then desorbed by 6 *M* guanidine/HCl. By this method, finally 37.5% of the hybrid protein was recovered. For analysis of the partially purified Ben′-CBD$_{TexI}$ thus obtained, SDS-PAGE was carried out. After renaturation of the Ben′-CBD$_{TexI}$ on the SDS-PAGE gel, the activ-

ity staining was performed using MUC as a chromogenic substrate (Fig. 2). The molecular mass of the expressed hybrid protein was the same as the expected value of ca. 38 kDa on SDS-PAGE (Fig. 2). The purity of the hybrid protein was more than 90%. The purified Ben'-CBD_{TexI} protein exhibited about 10% higher adsorbing ability toward Avicel than the native Ben, even though the size of the CBD of TexI is about four times smaller than that of Ben. More than 90% of enzyme used was tightly adsorbed to Avicel (Fig. 3). The adsorption capacity of Ben was determined to be 20 mg/g of Avicel *(9)*. The value corresponds to approx 0.38 μmol of Ben bound/g of Avicel. This value is 5.5 times lower than the value, 2.1 μmol, of CBD from *Clostridium cellulovorans (17)*. Purified Ben', which lacks the original CBD, showed no adsorption toward Avicel, like the CenA catalytic domain from *C. fimi (18)*. These results demonstrate that the CBD can be used to construct fusion proteins of interest without a significant loss of enzyme activity, and that these polypeptides can be purified easily by the adsorption to Avicel and subsequent desorption from the Avicel.

Sensitivity to Protease

By *in situ* activity staining, the intact 52-kDa Ben expressed in *E. coli* was found to be almost completely cleaved to the 34-kDa protein by endogenous *E. coli* proteases (Fig. 4). However, the hybrid Ben'-CBD_{TexI} protein was resistant to the hydrolysis, probably because of the lack of cleavage sites for *E. coli* proteases. Thus, the fusion protein retained the predicted size of 38 kDa (Fig. 4). When the cell extracts were treated with trypsin, most of the *E. coli* endogenous proteins were digested to smaller sizes (Fig. 4). The native Ben and truncated Ben' produced from *E. coli* cells harboring pBS1 and pENDO, respectively, were almost completely cleaved to the core catalytic domain of 33 kDa. However, when the hybrid enzyme was treated with trypsin, a predominant band (38 kDa) of the intact enzyme was still detected by the activity staining along with the digested band of 34 kDa. This result indicates that the hybrid Ben'-CBD_{TexI} is much more resistant to tryptic digestion than the native Ben and truncated Ben'. However, longer incubation of the same fusion enzyme with trypsin (6 h) resulted in complete digestion into the 34-kDa protein, indicating that a cleavage site for trypsin is present in the CBD_{TexI} domain. Such cleaved protein failed to bind to Avicel (data not shown). The core catalytic domain (33 kDa) was not cleaved any further and was still active against soluble cellulosic substrates.

Hydrolysis of Cellulosic Substrates

To investigate the enzymic properties of the Ben'-CBD_{TexI} hybrid enzyme, several substrates were digested and the products were analyzed

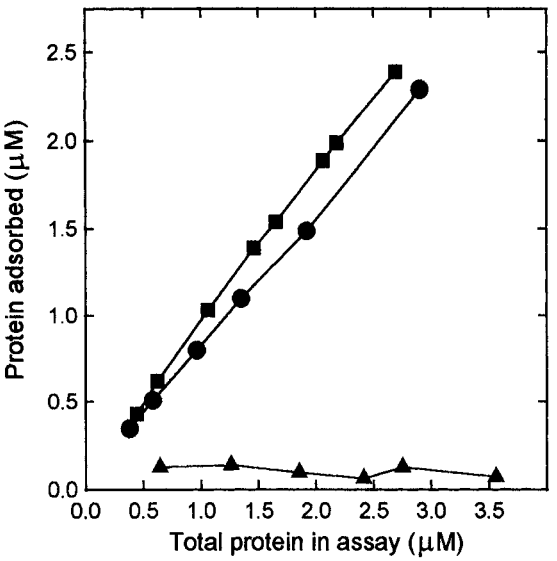

Fig. 3. Adsorption of Ben, Ben′, and Ben′-CBD_{TexI} to Avicel. Purified enzymes were mixed with 50 mg Avicel in a final volume of 1.0 mL of 50 m*M* sodium citrate (pH 5.5) on ice. The initial protein concentration range was 0.38–2.90 μ*M* for Ben, 0.64–3.51 μ*M* for Ben′, and 0.44–2.66 μ*M* for Ben′-CBD_{TexI}. After 1 h, enzyme activity remaining in the supernatant was measured as described in the text. Symbols: ●, Ben; ▲, Ben′; ■, Ben′-CBD_{TexI}.

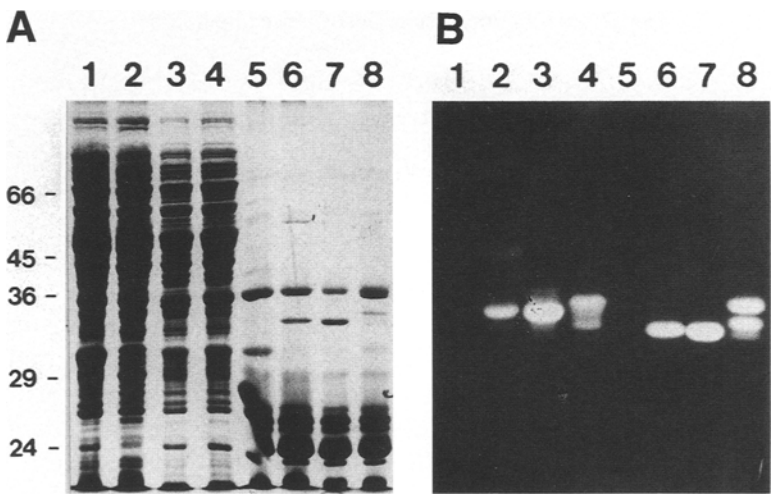

Fig. 4. SDS-PAGE analysis **(A)** and zymogram **(B)** of the native Ben and hybrid Ben′-CBD_{TexI} after limited hydrolysis with trypsin. Lane 1, negative control, extract of *E. coli* JM109 (pUC19); lane 2, extract of *E. coli* JM109 (pBS1); lane 3, *E. coli* JM109 (pENDO); lane 4, *E. coli* JM109 (pBST); lanes 5–8, reacted samples of lanes 1–4, respectively, with trypsin at 37°C for 1 h. Each sample lane, 1–4, was loaded with 250 μg of protein. Agar replica for the zymogram contained 0.5% CMC. Numbers on the left side are expressed in kDa.

Table 1
Comparison of Endo-β-1,4-glucanase Activity of Native Ben and Fusion Protein Ben'-CBD_{TexI} on Various Substrates[a]

Substrate	Concentration of sugars released, m*M*		
	Product	Ben	Ben'-CBD_{TexI}
CMC	G1	0.014	0.021
	G2	0.011	0.011
Avicel	G1	0.011	0.050
	G2	0.021	0.035
	G3	0.009	0.014
G5	G2	7.10	6.77
	G3	1.98	1.50
G4	G2	2.57	2.82
	G3	0.72	0.54
G3	G2	0.90	0.85
PNPC	PNP	0.47[b]	0.42[b]

[a]Reactions were carried out at 50°C with 0.60 μ*M* enzymes as follows: 0.5% CMC and 0.5% Avicel for 12 h; 10 m*M* oligosaccharides, G5, G4, and G3 for 1, 1.5, and 4 h, respectively; 2 m*M* PNPC for 0.5 h.

[b]Represents the increase in absorbance at 400 nm.

by HPLC. When soluble CMC and insoluble Avicel were treated with the native Ben and the hybrid enzyme, small end products, such as glucose (G1), cellobiose (G2), and cellotriose (G3), were detected together with trace amounts of other hydrolysis products (Table 1). The total amount of sugars produced from soluble substrates such as CMC, cellooligosaccharides, and PNPC were almost the same for Ben and the hybrid Ben'-CBD_{TexI}. When cellopentaose (G5) was used as a substrate, the sum of final products, G2 and G3, hydrolyzed by the hybrid enzyme was slightly less than that of Ben. However, similarly to the pattern of Ben activity, the amount of G2 was not equal to that of G3 produced by the hybrid enzyme with a substrate G5 (Table 1). It might suggest that the enzyme has an extra activity in addition to the direct internal cleavage of the substrate G5, similarly to the Ben. This result also indicates that the domain exchange of the C-terminal region between endoglucanase and exoglucanase did not affect hydrolysis of soluble cellulosic substrates. Interestingly, the total amount of sugars produced by the hybrid enzyme from Avicel was 2.4 times higher than that of the Ben (Table 1). This is coincident with the fact that the bind-

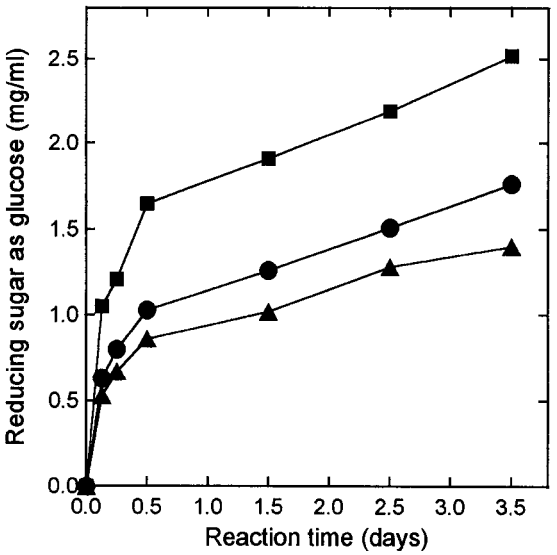

Fig. 5. Digestion of Avicel by Ben, Ben', and Ben'-CBD_{TexI}. Purified enzymes were incubated with 200 mg Avicel in 1.0 mL of 50 m*M* sodium citrate (pH 5.5) at 40°C. The enzyme concentration used was 6.0 μ*M* for Ben, 7.0 μ*M* Ben', and 5.1 μ*M* for Ben'-CBD_{TexI}. Symbols: ●, Ben; ▲, Ben'; ■, Ben'-CBD_{TexI}.

ing ability of the hybrid enzyme was higher than that of Ben (Fig. 3). Although both enzymes digested Avicel, digestion rate by hybrid enzyme was 1.5 times higher than that of the Ben enzyme (Fig. 5).

In this study, we demonstrated that the modes of cellulose hydrolysis, such as endo-type and exo-type, are largely dependent on the catalytic domain, but not dependent on the CBD. We also concluded that the CBD_{TexI} could be widely used in the construction of hybrid proteins without any loss of original enzyme activities. These fusion proteins can be purified easily by adsorption to Avicel and following desorption from Avicel. The Ben'-CBD may be a useful model for construction of fusion protein because of its protease tolerance and higher activity toward microcrystalline cellulose.

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