Functional display of fungal cellulases from *Trichoderma reesei* on phage M13

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Abstract To test whether the phage display technology could be applied in cellulase engineering, phagemids harboring the genes encoding the mature forms of cellobiohydrolase I (CBH I) and endoglucanase I (EG I) from filamentous fungus *Trichoderma reesei* were constructed, respectively. CBH I and EG I fused to the phage coat protein encoded by the *g3* gene were expressed and displayed on phage M13. The phage-bound cellulases retained their activities as determined by hydrolysis of the corresponding substrates. Also, their binding abilities to insoluble cellulose substrate were confirmed by an ELISA method. Overall, these results demonstrate that cellulases can be displayed on phage surface while maintaining their biological function, thus providing an alternative for directed evolution and high-throughput screening for improved cellulases.

Keywords Cellobiohydrolase I · Endoglucanase I · Phage-cellulase · Phage display · *Trichoderma reesei*

Abbreviations

ABTS, 2	2'-Azino-bis-3-ethylbenzthiazoline-6-sulfonic acid
CBD	Cellulose-binding domain
CBH I	Cellobiohydrolase I

CD	Catalytic domain
EG I	Endoglucanase I
epPCR	Error-prone PCR
IPTG	Isopropyl- β -D-thiogalactopyranoside
P III	Gene-3-protein of filamentous phage
PBS(T)	Phosphate-buffered saline (with 0.05% Tween 20)
PEG	Polyethylene glycol
MUC	4-Methylumbelliferyl- β -D-cellobiose
2 $\times$ YT-A(K)(G)	2 $\times$ YT Medium supplied with 100 μ g ampicillin/ml (70 μ g kanamycin/ml) (2% glucose)

Introduction

Cellulose, a linear homopolymer of β -1, 4 linked anhydro glucose units, is the most abundant and renewable biopolymer in the earth (Jarvis 2003; Mansfield et al. 1999). Converting cellulosic biomass to biofuels such as ethanol is considered to be an important alternative to the exhaustion of fossil energies (Demain et al. 2005; Kamm and Kamm 2004; Ragauskas et al. 2006). Multi-component cellulase systems from filamentous fungi such as *Trichoderma* are usually the main architects of cellulose degradation in industrial application (Kirk et al. 2002; Lynd et al. 2002). However, characteristics of cellulases including the relatively low hydrolysis efficiency and the rapidly decreased hydrolytic rate during the hydrolysis process usually result in decreased sugar yields, high enzyme input, and long reaction time, which has been always considered to be the major impediment to the commercial conversion of cellulose. Although the cost of cellulases for the biomass-to-biorefineries has been reduced by combined strategies, cellulases are still relatively costly which limit their

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applicability as biocatalysts in processing (Moreira 2005; Zhang et al. 2006). So it is necessary to improve the enzymatic properties such as activity and stability till it is practical.

Directed evolution has been a powerful tool in enzyme engineering where no detailed structural information of a given protein is required (Cherry and Fidantsef 2003; Otten and Quax 2005; Wong et al. 2006). In combination with various combinatorial mutagenesis methods, directed evolution has been successful in engineering quite a few industrially important enzymes (Chirumamilla et al. 2001; McCarthy et al. 2004). Among others, a well-designed selection or screening strategy typically constitutes the critical step in successfully exploiting the generated molecular diversity. By displaying libraries of mutated proteins on microbial cell surface has thus proved to be a very effective method in this regard. Proteins displayed on cell surface usually adopt the proper conformation and the gene encoding the desired mutant can be simultaneously obtained. Recently, bacterial cell surface display of an carboxymethyl cellulase library for selective screening of improved cellulase variants has been reported (Kim et al. 2000). However, cell-based display has its inherent limitation which prevents it being used under harsh conditions (Wittrup 2001).

As an alternative, phage display has made a tremendous impact on the discovery of peptides binding to a variety of receptors and high-affinity antibodies, and it has been widely used to select and engineer peptides and proteins (Fernandez-Gacio et al. 2003; Kehoe and Kay 2005; Smith 1985). Recently, this technology has also been used in the directed evolution of enzymes taking advantage of the larger libraries it could accommodate and the direct linkage between the phenotype and genotype (Atwell and Wells 1999; Jarvis 2003). Enzymes displayed on phage have also been successfully screened for desired variants with improved biofunction (Ponsard et al. 2001; Verhaert et al. 2002). In this study, we tested the feasibility of applying the phage display technology to screen an enzyme library of desired cellulase variants. Importantly, we showed that either endoglucanase or cellobiohydrolase from *Trichoderma reesei* can be functionally displayed on phage M13, which offers a potentially useful platform for future directed evolution and high-throughput screening of improved enzymes.

Materials and methods

Bacterial strains, plasmids and media

Escherichia coli TG1 (Lyophilized; K12 Δ (lac-pro), sup E, thi, hsd D5/F', traD36, pro AB, lacIq, lac Z Δ -M15) was

used as a suppressor strain for displaying the target protein fused to P III. *E. coli* HB2151 (Lyophilized; K12 Δ (lac-pro), ara, nal^r, thi/F', pro AB, lacIq, lac Z Δ -M15) was used for expression of soluble recombinant protein. pCANTAB 5E (Amersham Bioscience) was used in phage display experiments. Media used for cultivation were described in the manual of expressing module/recombinant phage antibody system (Amersham Bioscience). *E. coli* was grown in Luria-Bertani (LB) medium containing 5 g yeast extract/l; 10 g tryptone/l; and 5 g NaCl/l. For selection, ampicillin was added to a final concentration of 100 μ g/ml. Cell growth was determined by the absorbance value at 600 nm with a spectrometer.

Phagemid construction

The cellulase genes were amplified from pAJ401-EG I and pUCmT-CBH I harboring *eg I* and *cbh I*, respectively. PCR was performed by use of *vent* DNA polymerase (New England Biolabs) with primers with additional *Sfi* I and *Not* I restriction sites shown in Fig. 1. For amplification of *cbh I*, PCR conditions were 1 cycle of 95°C for 3 min followed by 30 cycles of 95°C for 2 min, 60.5°C for 1 min and 72°C for 2 min. Thermol cycling parameters of amplification of *eg I* were the same as those of *cbh I* except for the annealing temperature at 65°C. For both CBH I and EG I, the amplified cellulase genes only encoded their mature forms without the signal sequence and the stop codon. The PCR products were purified and concentrated using cycle-pure kit (Omega Bio-tech, USA). The target cellulase genes were digested with *Sfi* I and *Not* I (New England Biolabs) before being inserted into the pCANTAB 5E digested with the same enzymes with quick ligation kit (New England Biolabs). The two recombinant phagemids were termed pEG I and pCBH I, respectively.

Transformation and phage preparation

E. coli TG1 competent cells were transformed by electroporation by use of a MicroPulser (Bio-Rad laboratories) (Sheng et al. 1995). The ligation mixture of the appropriate phagemid was desalted and concentrated with cycle-pure kit before being used for electroporation. Transformants were plated on SOBAG (5 g yeast extract, 20 g tryptone, and 0.5 g NaCl/l and 0.01 M MgCl₂) agar plates containing 100 μ g ampicillin/ml and 20 mM glucose, and were incubated at 30°C overnight. The separated colonies (TG1/pCBH I, TG1/pEG I) on the selective plates were picked to 2 $\times$ YT-AG (10 g yeast extract, 17 g tryptone, and 5 g NaCl/l plus 100 μ g ampicillin/ml and 2% (w/v) glucose) medium and cultured at 37°C to an OD₆₀₀ of 0.8. M13KO7 (4×10^{10} p.f.u.) was then added in the culture with constantly shaking at 37°C for an hour. Superinfected

A

CD Linker region CBD

cbh I

Sfi I *Not I*

Sfi I *Not I*

g3 signal

fd gene 3

ColE1 ori

pCANTAB 5E
4522 bp

M13 ori

AMP^r

Linker region

CD CBD

eg I

Sfi I *Not I*

Ligation

Sfi I *Not I*

g3 signal

fd gene 3

ColE1 ori

pCBH I

M13 ori

AMP^r

Ligation

Sfi I *Not I*

g3 signal

fd gene 3

ColE1 ori

pEG I

M13 ori

AMP^r

B

CBH I For: CATCTCGGCCCAGCCGCCACAGC
Sfi I

CBH I Rev: ATGAACGCGGCCGCCAGGCACTGAGAGTAGT
Not I

EG I For: TTGACCACGGCCCAGCCGCCAT
Sfi I

EG I Rev: GTCAGCGGCCGCAAGGCATTGCGAGT
Not I

was induced by isopropyl- β -D-thiogalactopyranoside (IPTG) according to the provider's protocol with minor modifications (Amersham Bioscience). Briefly, *E. coli* HB2151 colony harboring the appropriate phagemid was picked to 5 ml $2 \times$ YT-AG and cultured overnight at 30°C. The overnight culture was inoculated into 50 ml of $2 \times$ YT-AG followed by incubation for another hour under the same conditions. Cells were collected by centrifugation and resuspended in 50 ml of $2 \times$ YT-AI with 1 mM of IPTG. The culture continued at 30°C for 24 h before the supernatant containing the soluble recombinant cellulases was obtained by centrifugation. The culture supernatant was further concentrated before being loaded onto a sepharose G100 (pharmacia) column. Fractions containing respective cellulase activities toward either CMC (sigma) for endoglucanase or 4-methylumbelliferyl- β -D-cellobiose (MUC) (sigma) for exoglucanase were collected and exchanged to 20 mM sodium acetate (NaAc) buffer, pH 5.8. The samples were loaded onto a DEAE sepharose FF (pharmacia) anion exchange column and the column was eluted with a linear gradient of 0–0.5 M NaCl. Fractions containing CBH I and EG I were resolved by

sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 10%) (Laemmli 1970). Zymogram analysis of EG I and CBH I were carried out according to the method described previously (Medve et al. 1998).

Activity assays

The endoglucanase activity of EG I was determined by analyzing the reducing sugars by the Smogyi method (Smogyi 1952). EG I was incubated with sodium carboxymethyl cellulose (0.1% CMC) at 50°C. One unit of EG I activity was defined as the amount of enzyme that produced 1 μ mol reducing sugars/min. *p*-Nitrophenyl- β -D-cellobiose (*p*-NPC) was the substrate for assay of the enzymatic activity of CBH I (Deshpande et al. 1984). *p*-NPC (1 mg/ml in 50 mM NaAc pH 5.0) was incubated with CBH I at 50°C. The reaction was terminated by the addition of 1% Na₂CO₃. The released *p*-nitrophenol was measured by the absorbance at 410 nm. One unit of CBH I activity was defined as the amount of enzyme that released of 1 μ mol *p*-nitrophenol/min.

Cellulose-binding assays

Cellulose binding abilities of the phage-displayed EG I and CBH I were assayed by an ELISA method with filter paper (Whatman No. 1) as the immobilized substrate coated in a 96-well plastic microtiter plate (Costar). The 96-well plates were blocked with 10% (w/v) of non-fat milk in PBS for an hour. Phage solution in PBS was preincubated with the blocking buffer for 30 min before being transferred to the pre-coated microtitre plate and incubated for an hour. The plates were then washed sequentially three times with PBST (Tween 20, 0.05%) and three times with PBS. The diluted horseradish peroxidase-conjugated anti-phage antibody (Amersham Bioscience) solution was added and incubated for an hour. After extensive washing, ABTS reagent containing 0.05% H₂O₂ was added to each well. The supernatant was quantified by measuring the absorbance at 410 nm.

Results and discussion

Phagemid construction to display cellulases from *T. reesei*

Two recombinant phagemids, pEG I and pCBH I, to express and display the endoglucanase EG I and cellobiohydrolase CBH I, respectively, were constructed (Fig. 1). The amplified DNA fragments of *cbh I* and *eg I* encoded their corresponding mature forms of enzyme without the signal sequence and the stop codon. The recombinant phagemids were isolated from the culture supernatant of

respective recombinant colonies and were verified by enzymatic digestion and DNA sequencing.

Production of EG I and CBH I in *E. coli*

To investigate whether *E. coli* transfected with the constructed phagemids could express the cellulases in their active forms, protein expression was carried out in *E. coli* HB2151 because of its inability to suppress the amber codon introduced between the target gene and gene III. Distinctive bands of CBH I and EG I were detected from the culture supernatant of *E. coli* transfected with the appropriate phagemids by SDS-PAGE after induction with IPTG as compared to the secreted proteins of transfected *E. coli* HB2151 without induction (Fig. 2). The apparent mobility of CBH I and EG I corresponded to molecular masses which, however, were slightly larger than the theoretically calculated molecular masses. The reason for this discrepancy might lie in the fact that the recombinant cellulases contain an in-frame-fused E-tag. Activities of the recombinant cellulases were further confirmed by active staining of the native-PAGE-resolved expressed proteins of *E. coli* HB2151 transfected with recombinant phagemids (Fig. 3). Taken together, recombinant cellulases as mediated by incorporating in the phagemid vectors were successfully expressed in *E. coli* HB2151.

Phage display of EG I and CBH I

To test whether the relative cellulases displayed on the surface of phage M13 also kept their activities, recombinant phagemids were transfected into *E. coli* TG1. The phage particles were prepared and carefully purified by PEG precipitation to preclude any contaminating soluble proteins. Significant enzymatic activities for both phage-exposed EG I and phage-exposed CBH I were observed for phage particles of transfected *E. coli* as demonstrated by their activities toward CMC and *p*-NPC, respectively (Table 1). In contrast, no activities were detected for control particles where only empty phagemid vectors used to transfect *E. coli*. Interestingly, compared with phage-displayed counterparts, enzyme activities of soluble EG I or CBH I expressed by *E. coli* HB2151 were around 3 fold higher. Nonetheless, considering that wild-type P III would compete with P III fused to cellulases in the phage rescue process resulting in no more than 30% of the phage displaying the recombinant enzyme (Kehoe and Kay 2005; Verhaert et al. 1999), the specific activity/phage-exposed cellulase was in reasonable agreement with the measured enzymatic activity of its corresponding soluble recombinant cellulase. Overall, the results indicated that, as expected, recombinant cellulases in their active forms were

Fig. 2 Recombinant expression and purification of CBH I (◇) (a); and EG I (▽) (b). SDS-PAGE analysis of crude extracts of uninduced cell (lane 1), induced cell (lane 2), purified recombinant CBH I and EG I (lane 3 and 4), and low molecular weight marker with the corresponding molecular mass (lane 5)

Fig. 3 Native PAGE (left) and zymogram (right) analysis of recombinant CBH I (a) and EG I (b) Crude extracts of HB 2151 (lane 1), expressed protein of induced transfected recombinant cells (lane 2, 3)

properly displayed on the phage tip through fusing to P III via its C-terminal cellulose binding domain. Potential structural constraints caused by fusing cellulases to an exogenous P III, however, did not significantly compromise the display of functional cellulases.

To further evaluate whether the phage-displayed cellulases retained the ability to bind to insoluble cellulose substrate, recombinant phage particles were incubated with filter paper discs precoated in the microtiter plate and the bound phage mediated by cellulases were analyzed by ELISA as described in materials and methods. While wild-type phages, M13KO7, were easily washed away from the filter paper, recombinant phages with surface displayed CBH I and EG I bound tightly to cellulose filter paper (Fig. 4). This observation, together with above activity assays, demonstrated that the phage-displayed cellulases by fusing with P III protein not only kept their catalytic activity, but also retained the ability to bind insoluble

Table 1 Activity assay of phage-exposed enzyme and the corresponding soluble enzyme

Enzyme type	Activity of phage suspension ($\times 10^{-3}$ U/ml) ^a	$\times 10^{13}$ pfu/ml ^b	$\times 10^{-18}$ U/phage particle ^c
Phage-exposed EG I	1.6	2.1	77
Phage-exposed CBH I	0.007	5.0	0.14
Soluble CBH I	48.2		0.44
Soluble EG I	0.272		244

^a The catalytic activity was assayed as described in materials and methods

^b Numbers of phage particles were determined by titration (plaque-forming units)

^c The activity/phage particle was calculated from the activity of phage solution and its numbers of phage particles. For calculation of the activity/soluble enzyme (the corresponding soluble enzyme) molecular, the protein concentration was analyzed routinely

Fig. 4 The cellulose binding ability of phage-displayed cellulases. The binding abilities of wild-type M13K07 (bar 1), phage-displayed endoglucanase I (bar 2) and phage-displayed cellobiohydrolase I (bar 3) were indirectly analyzed as determined by absorbance at OD 410 nm with the ELISA method

cellulose substrates, both of which are key determinants for the full biological function of cellulases.

Conclusion

Both endoglucanase I and cellobiohydrolase I (CBH I) from *Trichoderma reesei* were functionally expressed and displayed on the phage surface in their enzymatically active forms. The phage-bound cellulases not only retained their catalytic activities as determined by hydrolysis of the corresponding soluble substrates, but also maintained their binding abilities to insoluble cellulose substrates as confirmed by an ELISA method. Our results presented here indicated that the successful display of these key enzymes in cellulose hydrolysis would provide a technology platform for screening or selection of enzymes with improved catalytic properties produced by directed evolution considering the easy manipulation and the huge library capacity provided by phage display. Compared with systems involving cytoplasmic or secreted enzymes, screening phage-displayed enzymes could avoid the requirement of cell breakage as well as expression-normalization before the evaluation of changes in specific enzyme activities. Besides, DNA sequences encoding the desired enzyme variant could also simultaneously be retrieved and determined.

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