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Construction of engineered yeast with the ability of binding to cellulose

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Abstract

The genes encoding cellulose binding domain (CBD) from cellobiohydrolase I (CBHI) and cellobiohydrolase II (CBHII) of the filamentous fungus *Trichoderma reesei* were expressed on the cell surface of the yeast *Saccharomyces cerevisiae* by cell surface engineering. The CBD genes were fused to the gene encoding the *Rhizopus oryzae* glucoamylase secretion signal sequence, and expressed under the control of the glyceraldehydes-3-phosphate dehydrogenase (GAPDH) promoter. Each of CBDs was successfully displayed on the yeast cell surface by fusing their genes to the gene encoding the 3′-half of α-agglutinin of *S. cerevisiae* having a glycosylphosphatidylinositol anchor attachment signal. Tandemly aligned CBHI (CBD1) and CBHII (CBD2) fusion gene was also constructed to display simultaneously both CBDs on the cell surface of *S. cerevisiae*. Binding affinity of the CBD-displaying yeast cells to a cellulose substrate was similar between the CBD1- and CBD2-displaying yeast cells. However, the cells displaying the fusion protein of CBD1 and CBD2 showed much higher binding affinity to cellulose than either of the single CBD-displaying yeast cells. The binding affinity of the cells was increased by treating the cellulose with phosphoric acid.

Keywords: Cellulose binding domain; Cellobiohydrolase; Cellulose; Cell surface engineering; *Saccharomyces cerevisiae*; *Trichoderma reesei*

1. Introduction

Cellulose, the major polysaccharide compound of plant cell wall consisting of 100–10,000 glucose units linked by β-1,4-glycosidic bonds, is the most abundant organic material on earth. Cellulose is hydrolyzed by cellulolytic enzyme system: endoglucanase (EC 3.2.1.4, endo-1,4-β-D-glucan 4-glucanohydrolase), cellobiohydrolase (EC 3.2.1.91, 1,4-β-D-glucan cellobiohydrolase, exoglucanase) and β-glucosidase (EC 3.2.1.21, β-D-glucoside glucohydrolase). These three types of cellulases act synergistically to degrade crystalline cellulose [1,2]. Like many other cellulases, two cellobiohydrolases (CBHI and CBHII) of the filamentous fungus *Trichoderma reesei* have a cellulose-binding domain (CBD) linked by the linker peptide to the catalytic core domain. The CBDs, the domains of CBHI and CBHII, display two distinct faces, one being a flat form and the other a rough form [3]. The flat face is the binding surface to

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cellulose and contains several aromatic amino acids. CBD of CBHI (CBD1) and CBD of CBHII (CBD2) have similar binding properties, but CBD1 binds reversibly and CBD2 binds irreversibly [4].

For the display of each CBD on the yeast cell wall, the cell surface engineering technique in *Saccharomyces cerevisiae* [5] was used. We have already reported the cell surface-engineered yeast cells with cellulases [6,7] and the possibility of the conversion of cellulosic materials to useful ones including ethanol has been indicated. For this purpose, the display of only CBD was carried out on the yeast cell surface. The basic analysis of the binding ability of CBD to cellulose might contribute not only to understanding of the binding mechanism of CBD but also to wide development of the application—whole-cell biosensors and bioadsorbents etc.—by immobilization of yeasts.

In this study, binding property of each CBD-displaying yeast cells to cellulose was investigated. By using Avicel or phosphoric acid-swollen Avicel, the binding affinity of CBD1-, CBD2-, and CBD1/CBD2-displaying cells was compared.

2. Experimental

2.1. Strains and media

Escherichia coli strain DH5α [F^- *endA1 hsdR17* (rK^-/mK^+) *supE44 thi-1* λ^- *recA1 gyr96*Δ*lacU169* (ϕ*80lacZ*Δ*M15*)] was used as a host for recombinant DNA manipulation. *E. coli* was cultivated in LB medium (1% tryptone (Difco, MI, USA), 0.5% yeast extract (Difco), and 0.5% sodium chloride) containing 100 μg/ml ampicillin. *S. cerevisiae* MT8-1 (*MATa*, *ade*, *his3*, *leu2*, *trp1*, *ura3*) was cultivated either in YPD medium (1% yeast extract, 2% peptone (Difco), and 2% glucose) or SD-W medium (0.67% yeast nitrogen base without amino acids (Difco), 2% glucose, 0.003% L-leucine, 0.002% L-histidine HCl, 0.002% adenine sulfate, and 0.002% uracil) containing 2% casamino acids (Difco) [8].

2.2. Construction of plasmids and transformation of yeast

The plasmids pMCBD1 and pMCBD2 were constructed as follows: a fragment of CBD1 (CBD of CBHI) from *T. reesei* was amplified from the total cDNA prepared from mRNA of *T. reesei* as a template by the polymerase chain reaction (PCR), with the primers 5′-AATAATTCCCCGCGGGAACCCAGTCTC ACTACGGCCAGTGCGG-3′ and 5′-ATTATTCCGC TCGAGCCCAGGCAC TGAGAG TAG TAAGGGT T CA-3′. A DNA fragment of CBD2 (CBD of CBHII) was also amplified by PCR, with the primers 5′-A ATAATTCCCCGCGGGACAAGCTTGCTCAAGCG TCTGGGGCCAA-3′ and 5′-ATTATTCCGCTCGA GCCAAGACACTGGGAGTAATAGTCGTTGGA-3′. Each amplified PCR products was digested with *Sac*II and *Xho*I, and inserted into the *Sac*II–*Xho*I site of the multicopy-type yeast cell–surface displaying cassette vector pCAS1 [9]. The DNA fragment encoding $RGS(H)_4$ [Arg-Gly-Ser-His-His-His-His] was inserted into the *Sac*II site of the 5′-end of the CBD encoding DNA for the immunofluorescent labeling and a DNA encoding six repeated amino acid sequences of GGGS (linker-1) was inserted into the *Xho*I site of the 3′-end of the CBD encoding DNA to insert a linker between the regions encoding the C-terminal 320 amino acids of α-agglutinin and each CBD. The plasmid pMCBD3, which was tandemly fused to the CBD1 encoding DNA and CBD2 encoding DNA [10], was constructed as follows: the gene encoding CBD2 and the following three amino acid residues, the original linker of CBHII, was amplified from the cDNA prepared from total mRNA of *T. reesei* as a template by PCR with the primers 5′-AATAAT TCCCCGCGGGACAAGCT TGCTCAA GCGTCTGGGGCCAA-3′ and 5′- GCTTGAAGAT CTAGCGCCGGGAAGACACTGGGAGTAATAG-3′. The amplified fragment was digested by *Sac*II and *Bgl*II, and inserted into the *Sac*II–*Bgl*II site of pCAS1. The gene encoding 19 amino acid residues, the original linker of CBHI, and the following CBD1 was prepared similarly by PCR with the primers 5′-GGCG GAAGA TCT CC TGGCACCACCACCACCCGCCG CCCAGCC-3′ and 5′-ATTATTCCGCTCGAGCCCA GGCACTGAGAGTAGTAAGGGTTCA-3′. The amplified linker-CBD1 fragment was cut with *Bgl*II and *Xho*I, and ligated with *Bgl*II–*Xho*I site of the pCAS1 containing the CBD2 linker gene. The linker gene between the CBD2 and CBD1 genes was named the linker-2 gene, encoding the amino acid sequence, PGARSPGTTTTRRPATTTGSSPGP. In this case, the linker-2 had the original linker sequences from CBD1

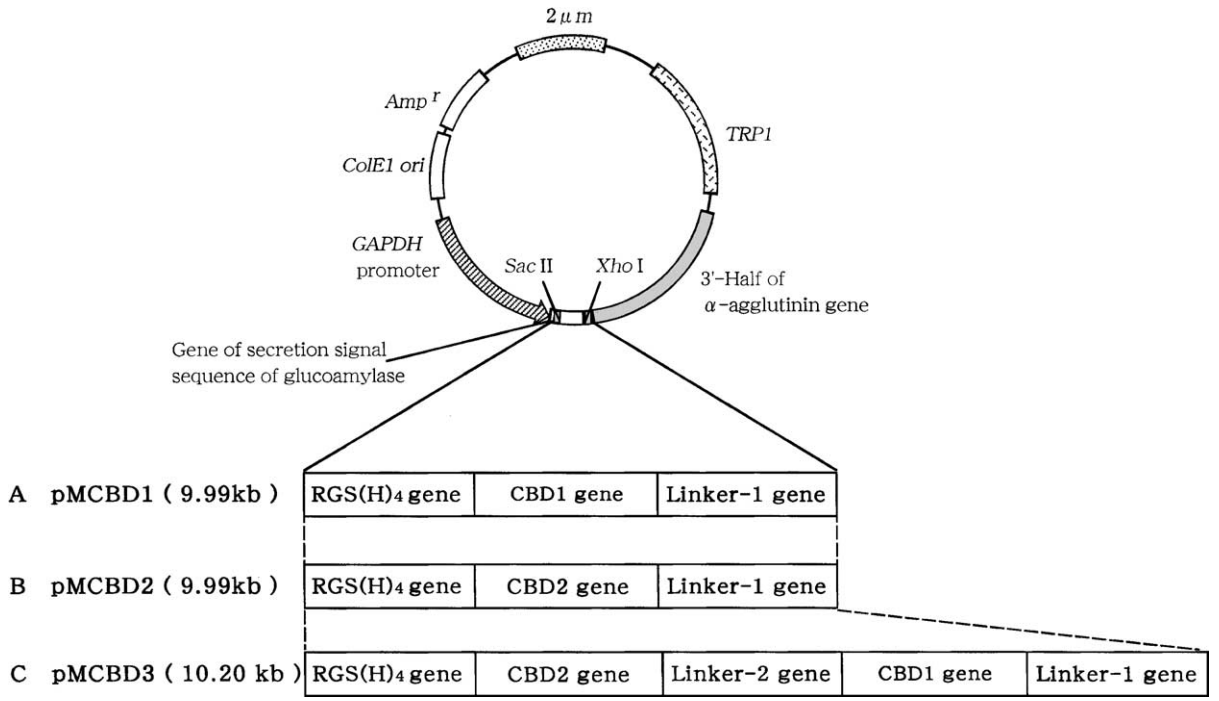

Fig. 1. Construction of multicopy plasmids for displaying CBDs on the yeast cell surface. (A) pMCBD1, the plasmid for displaying CBD1, (B) pMCBD2, the plasmid for displaying CBD2, (C) pMCBD3, the plasmid for displaying CBD1–CBD2.

(19 amino acid residues) and CBD2 (three amino acid residues) and the extra sequences (two amino acid residues). The genes of $RGS(H)_4$ and the linker fragment (the linker-1 gene) were inserted into the fusion plasmid of CBD2 and CBD1. The resulting plasmid was named pMCBD3. The plasmids pMCBD1, pMCBD2 and pMCBD3 (Fig. 1) were transformed into *S. cerevisiae* by the lithium acetate method [11].

2.3. Immunofluorescence microscopy

Immunofluorescent labeling of the cells was performed as follows: $RGS(H)_4$ antibody (Qiagen, Hilden, Germany) used as a primary antibody at a dilution rate of 1:1000 was mixed with the cells at room temperature for 1.5 h. The cells were washed with 10 mM PBS (phosphate-buffered saline, pH 7.2) and exposed to the second antibody, Alexa Fluor™ 488 goat anti-mouse IgG (H + L) conjugated antibody (Molecular Probes, OR, USA), diluted 1:300, at room temperature for 1 h. After washing the antibody with 10 mM PBS, the fluorescence of the cells was observed by a fluorescent microscopy.

2.4. Binding assay of yeast cells to Avicel

Avicel (Fluka Chemie GmbH, Buchs, Switzerland) was washed five times with Milli-Q water to remove small particles. Cultivated yeast cells (6.7×10^7 cells) were collected and washed twice with 3 ml of 50 mM potassium phosphate buffer (KPB, pH 7.2) containing 50 mM NaCl. Yeast cells were incubated with 0.05 g Avicel in a test tube at room temperature for 18 h over agitating with a rotary shaker at 14 rpm. After incubation, test tubes were stood statically for 5 min and 3 ml of the supernatant were taken to measure the absorbance at 600 nm for evaluation of the non-adsorbed cells.

2.5. Binding assay of yeast cells to phosphoric acid-swollen Avicel

Yeast cells were washed twice with 50 mM KPB containing 50 mM NaCl before reacting with 200 μl

phosphoric acid-swollen Avicel [11,12]. After incubating for 18 h, the mixture was filtrated through an 11 μm nylon net filter (Millipore, MA, USA) twice. The A_{600} of the filtrate was measured to evaluate the non-adsorbed cells.

3. Results

3.1. Evidence of CBD display on the yeast cell surface

For display of CBDs on the surface of yeast *S. cerevisiae*, the genes encoding CBDs fused to the gene encoding *Rhizopus oryzae* glucoamylase secretion signal sequence and the 3′-half of α-agglutinin including the glycosylphosphatidylinositol (GPI)-anchor attachment signal were expressed under the control of the glyceraldehydes 3-phosphate dehydrogenese (GAPDH) promoter. To confirm the localization of each CBD on the yeast cell surface, the immunofluorescent labeling of the yeast cells was performed with the RGS(H)$_4$ antibody as the primary antibody and Alexa Fluor™ 488 goat anti-mouse IgG (H+L) conjugated antibody as the second antibody. The yeast cells harboring pMCBD2 were clearly labeled by green color of fluorescein isothiocyanate (FITC) (Fig. 2B), although cells harboring the control plasmid pCAS1 (Fig. 2D) were not labeled. The yeast cells harboring pMCBD1 and pMCBD3 were also clearly labeled (data not shown). These results demonstrated that each CBD was displayed on the surface of yeast cells.

3.2. Binding property of CBD-displaying cells

The binding ability of the CBD-displaying cells to cellulose was quantified by using Avicel and the phosphoric acid-swollen Avicel as substrates. Binding of the each yeast cells were determined by measuring the A_{600} of the non-bound yeast cells. Binding ratio (%) was calculated as:

$$\text{Binding ratio}(\%) = \frac{(A_{\text{control}} - A_{\text{sample}})}{A_{\text{control}} \times 100}$$

As shown in Fig. 3, the CBD-displaying yeast cells could bind to both types of cellulose, while the non-displaying yeast cells could not. All CBD-displaying yeast cells have higher binding affinity to the phosphoric acid-swollen Avicel than that to Avicel. Especially, binding affinity of yeast cells displaying the fusion protein of CBD1 and CBD2 to the phosphoric acid-swollen Avicel (Fig. 3B) was twice higher than

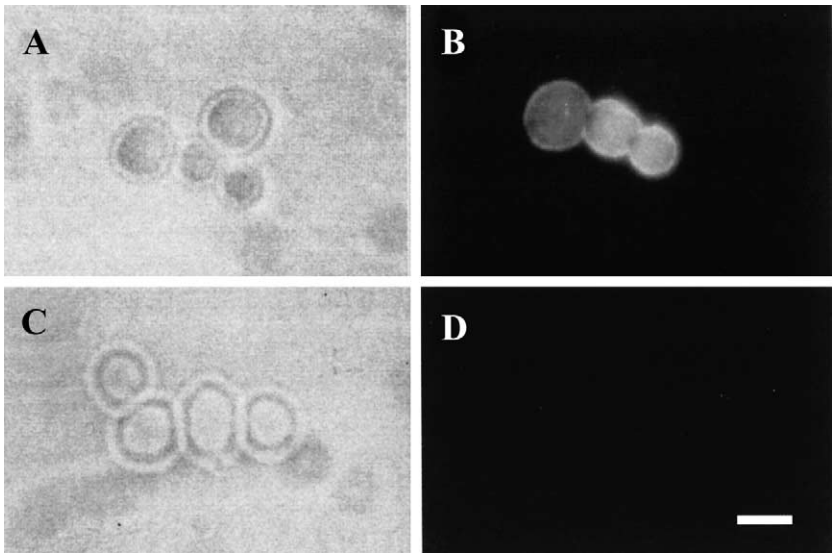

Fig. 2. Immunofluorescent labeling of the transformed yeast cells using the RGS(H)$_4$ antibody and Alexa Fluor™ 488 goat anti-mouse IgG (H+L) conjugated antibody. Phase micrographs (A, C), and immunofluorescence micrographs (B, D). A, B cells harboring pMCBD2; C, D cells MT8-1 harboring pCAS1 (control). Bar 5 μm.

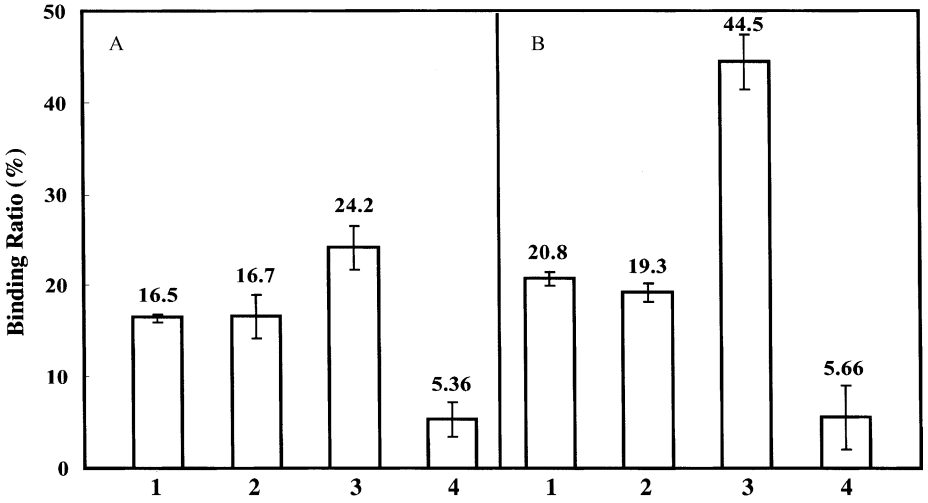

Fig. 3. Binding ratio of the yeast cells to cellulose. Avicel (A) and the phosphoric acid-swollen Avicel (B) were used as the cellulose substrates. The data shown are mean (+/−) S.D, $n = 3$. 1, Cells harboring pMCBD1; 2, cells harboring pMCBD2; 3, cells harboring pMCBD3; 4, cells harboring pCAS1 (control).

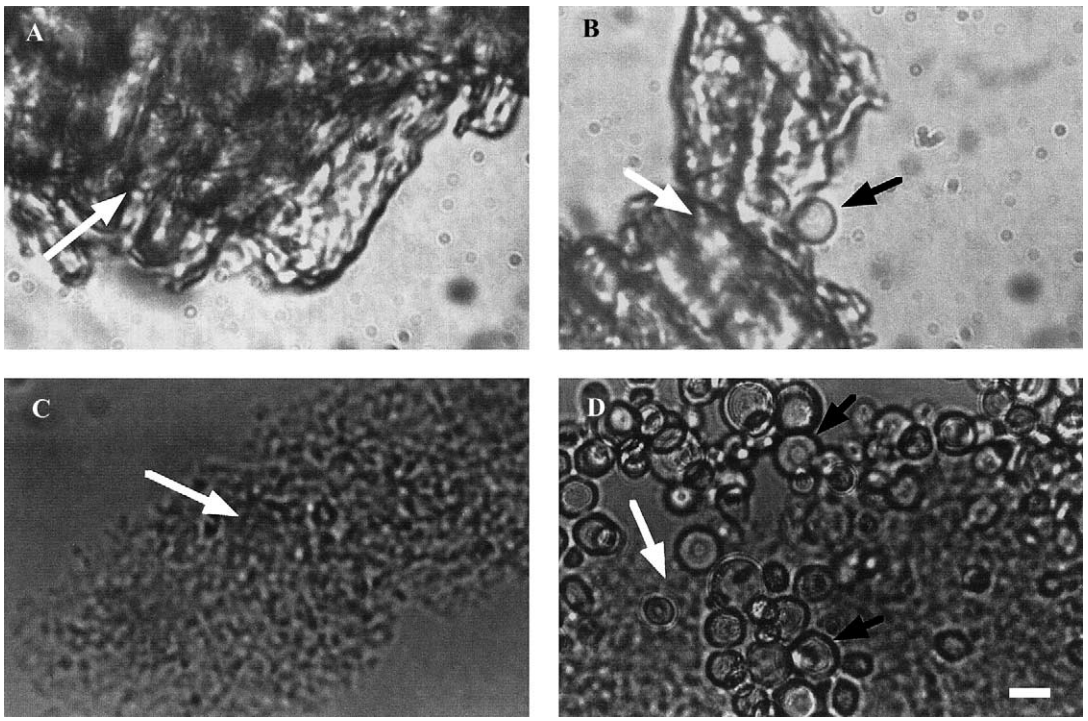

Fig. 4. Microscopical observation of cellulose after incubation with cells harboring pMCBD3. Avicel (A, B), and the phosphoric acid-swollen Avicel (C, D), were used as the cellulose substrates. A, C cells harboring pCAS1 (control); B, D cells harboring pMCBD3. White arrows, Avicel or the phosphoric acid-swollen Avicel; black arrows, yeast cells. Bar 5 μm.

that to Avicel (Fig. 3A). Binding ratio of the yeast cells harboring pMCBD1 and pMCBD2 were similar, but that of the yeast cells harboring pMCBD3 was higher, showing 24.2% on Avicel (Fig. 3A), and 44.5% on the phosphoric acid-swollen Avicel (Fig. 3B).

3.3. Microscopical observation of CBD-displaying yeast binding to cellulose

Binding of CBD-displaying yeast cells to Avicel (Fig. 4A and B) or the phosphoric acid-swollen Avicel (Fig. 4C and D) was observed by a microscopy. To compare the binding of CBD-displaying yeast cells and control cells to cellulose, binding reaction was performed with a surplus amount of cells and small amount of cellulose. The yeast cells displaying the fusion protein of CBD1 and CBD2 on the cell surface exhibited the binding ability to either cellulose substrates (Fig. 4B and D), while the yeast cell with no CBD on the cell surface did not (Fig. 4A and C). Clearly, the phosphoric acid-swollen Avicel was the better substrate for binding than Avicel.

4. Discussion

We successfully displayed three different types of CBDs on the cell surface of yeast *S. cerevisiae* MT8-1, and confirmed that each CBD-displaying yeast cells could bind to the cellulose surface. No studies have ever tried to bind yeast cells to cellulose by yeast cell surface displaying of a fungal CBD, although the binding of recombinant *Staphylococcus carnosus* or *E. coli* onto cellulose by CBD-display on the cell surface was reported in a recent paper [13,14]. The binding ratio of the yeast cells harboring pMCBD3, which is displaying two tandemly aligned CBDs (fusion protein of CBD1 and CBD2), was almost twice higher than the yeast cells harboring pMCBD1 or pMCBD2. In the case of the yeast cells harboring the genome-integrated gene for displaying CBD1 or CBD2, the cells almost could not bind to Avicel (data not shown). From these results, it can be concluded that the binding ratio of yeast cells is dependent on the number of displayed CBD molecules on the cell surface. The difference in the binding properties between Avicel and the phosphoric acid-swollen Avicel was not clear since the mechanism of the binding reaction of CBD and cellulose is unknown. Phosphate ions may activate the binding face of Avicel.

This study is the first report of the construction of engineered yeast which can bind to cellulose through CBDs. By using CBD- and mutated CBD-displaying yeast cells, the binding mechanism of CBDs to cellulose will be analyzed. Furthermore, the construction of a bioreactor system with sequential and/or multistep enzyme reactions on the cellulose filter will be possible by arranging of the engineered yeast cells which co-display CBDs and target enzymes.

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