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Cellulose affinity purification of fusion proteins tagged with fungal family 1 cellulose-binding domain

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ABSTRACT

N- or C-terminal fusions of red-fluorescent protein (RFP) with various fungal cellulose-binding domains (CBDs) belonging to carbohydrate binding module (CBM) family 1 were expressed in a *Pichia pastoris* expression system, and the resulting fusion proteins were used to examine the feasibility of large-scale affinity purification of CBD-tagged proteins on cellulose columns. We found that RFP fused with CBD from *Trichoderma reesei* CBHI (CBD_{TrCBHI}) was expressed at up to 1.2 g/l in the culture filtrate, which could be directly injected into the cellulose column. The fusion protein was tightly adsorbed on the cellulose column in the presence of a sufficient amount of ammonium sulfate and was efficiently eluted with pure water. Bovine serum albumin (BSA) was not captured under these conditions, whereas both BSA and the fusion protein were adsorbed on a phenyl column, indicating that the cellulose column can be used for the purification of not only hydrophilic proteins but also for hydrophobic proteins. Recovery of various fusion proteins exceeded 80%. Our results indicate that protein purification by expression of a target protein as a fusion with a fungal family 1 CBD tag in a yeast expression system, followed by affinity purification on a cellulose column, is simple, effective and easily scalable.

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Introduction

Cellulose-binding domains (CBDs)[1] were initially discovered as a component of carbohydrate-active enzymes (CAZymes), such as cellulases; the CBD serves to promote enzymatic hydrolysis of crystalline cellulose by increasing the concentration of bacterial and fungal cellulases on cellulose surfaces [1,2]. Recently, CBDs were subdivided into carbohydrate-binding module (CBM) families by means of hydrophobic-cluster analysis, and several thousand CBM sequences have been classified into more than 60 families [3,4]. Family 1 CBMs (CBM1) are generally found in fungal cellulolytic enzymes, while family 2 and 3 CBMs (CBM2 and CBM3) are mostly found in bacterial enzymes. These CBDs have in common a hydrophobic flat surface composed of several aromatic residues, such as tyrosine or tryptophan, that interact with the hydrophobic surface of crystalline cellulose [5–7].

CBDs have potential value for various applications in biotechnology, and one interesting possibility is their use in affinity chromatography. Although oligohistidine, glutathione S-transferase (GST), and maltose-binding protein (MBP) have been used as affinity tags for the purification of fusion proteins [8–10], these affinity purification systems are all relatively expensive. On the other hand, CBDs can be genetically fused to a protein of interest to mediate specific binding to cheap and versatile cellulose matrices, suggesting that CBD tags could be effective for low-cost affinity purification of proteins [11–15]. Bacterial CBDs have often been used as affinity tags in *Escherichia coli* protein expression systems, and CBD_{Cex} and CBD_{CenA} (CBM2) from *Cellulomonas fimi* and CipA CBD (CBM3) from *Clostridium* spp. are commercially available [16–21]. In contrast, fungal CBDs belonging to CBM family 1 have not been widely used as tags for fusion proteins, in part because of the difficulties of heterologous expression in *E. coli* systems. Although the CBD of cellobiohydrolase I from *Trichoderma reesei* (CBD_{TrCBHI}) has been used for cellulose affinity chromatography [22,23], the applicability of this system is quite limited.

CBDs belonging to CBM family 1 share the common feature of rather small molecular weight (approximately 3 kDa; less than 40 amino acid residues), with two dithiol bonds, and they contain three hydrophobic amino acids, which form a flat hydrophobic surface through which the CBDs bind to the hydrophobic surface of crystalline cellulose substrates [24,25]. Various reports describe apparently reversible adsorption of fungal CBDs [26–28]. Therefore, fungal CBDs could be suitable for affinity purification of fusion proteins, if the fusion proteins are correctly folded during protein production.

In this study, we examined the feasibility of protein purification by means of expression of a target protein as an N- or C-terminal

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[1] *Abbreviations used:* CBD, cellulose-binding domain; CBM, carbohydrate-binding module; RFP, red-fluorescent protein; CBH, cellobiohydrolase; BSA, bovine serum albumin; DEAE, diethylaminoethyl; CV, column volume.

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fusion with CBM family 1 CBDs from fungal cellobiohydrolases (CBHs) in the methylotropic yeast *Pichia pastoris*, followed by affinity separation of the fusion protein on a cellulose column. The affinity of the fusion protein for the cellulose column was enhanced by the addition of kosmotropic salt, ammonium sulfate, and the fusion protein could be easily eluted with water. Expression level, extent of purification (fold) and purification yield were compared among CBD-tagged fusion proteins. Our results indicate that fungal CBDs are promising tools for simple, effective and easily scalable affinity purification of recombinant proteins.

Materials and methods

Chemicals and strains

All chemicals used in this study were laboratory-grade products from WAKO Pure Chemical Industries, Ltd. (Osaka, Japan) or Sigma–Aldrich (St. Louis, USA). Restriction enzymes, DNA ligase, pDsRed-Monomer vector coding monomeric red-fluorescent protein (RFP), and *E. coli* strain JM109 were obtained from TaKaRa Bio Inc. (Japan). pCR4Blunt-TOPO vector for sequencing, oligonucleotides, *P. pastoris* KM71H for protein production and pPICZαA transforming vector were purchased from Invitrogen (Carlsbad, CA). KOD plus DNA polymerase from Toyobo Co., Ltd. (Japan) was used for polymerase chain reaction. Cellulose powders, CF11 (fibrous), CC31 and CC41 (microgranular) were from Whatman Ltd. (England) and Avicel (Funacel II, average grain size 80 μm) was from Funakoshi Ltd. (Tokyo, Japan).

Plasmid construction and expression of fusion proteins

All fusion proteins used in this study were constructed by replacement of the catalytic core domain of cellobiohydrolases with RFP. Four types of CBD containing native linker regions were PCR-amplified with plasmid vectors harboring four types of fungal cellobiohydrolases, which had previously been cloned in our laboratory. The genes coding C-terminal CBDs from *T. reesei* CBHI (*Tr*Cel7A) and CBH58 from *Phanerochaete chrysosporium* (*Pc*Cel7D), and genes coding N-terminal CBDs from *T. reesei* CBHII (*Tr*Cel6A) and CBH50 from *P. chrysosporium* (*Pc*Cel6A) were amplified by PCR with primers 5–6 for *Tr*Cel7A and 9–10 for *Pc*Cel7D or with primers 7–8 for *Tr*Cel6A and 11–12 for *Pc*Cel6A, as listed in Table 1. The PCR products were ligated into Blunt-TOPO vector according to the manufacturer's instructions. RFP genes designed to be tagged at the N- or C-terminus were similarly amplified from pDsRed-Monomer with primer sets 1–2 and 3–4 and sub-cloned into Blunt-TOPO vector. The inserts ligated into Blunt-TOPO vector were subjected to DNA sequencing after mini-preparation of the plasmids from isolated ampicillin-resistant *E. coli* colonies. These CBD and RFP genes were simultaneously ligated into expression vector pPICZαA using restriction enzyme sites *Eco*RI–*Kpn*I or *Kpn*I–*Not*I, as shown in Table 1. Transformants were prepared by using the constructed plasmids, and the fusion proteins were produced as described previously [29].

The fluorescence intensity and protein concentration of the supernatant of each culture medium were monitored for 6 days after induction with methanol. The fluorescence intensity of the medium was measured with a Fluoroskan Ascent Microplate Fluorometer (Thermo Fisher Scientific Inc., Japan) with excitation at 544 nm and emission at 590 nm. A 180 μl aliquot of 100 mM sodium phosphate buffer (pH 8.0) was added to 20 μl of the supernatant of each culture medium in a 96-well black-wall microplate (Costar, Product Code 3925), and fluorescence measurement was performed after incubation for 5 min at room temperature. Protein concentrations were assayed with the Bio-Rad Protein Assay (Bio-Rad Laboratories, Inc.). SDS–PAGE analysis of each culture medium was performed as described previously [30].

Cellulose affinity purification of RFP-CBD$_{TrCBHI}$ with a CC31 column

The crude extracellular proteins obtained by centrifugation (6000*g*, 30 min) were loaded onto a manually packed CC31 cellulose column (∅10 × 20 mm) and eluted with distilled water at 25 °C. The protein sample containing 1 M ammonium sulfate was also loaded on the column, which had been pre-equilibrated with the same concentration of ammonium sulfate, and the column was washed with 1 M ammonium sulfate solution then eluted with distilled water.

The crude fusion protein sample (1.0 ml, approximately 0.5 mg/ml) was applied to a CC31 column (∅10 × 50 mm) and eluted with water at the flow rate of 0.5, 1.0 or 1.5 ml/min after a 5 min wash with 1 M ammonium sulfate at 4 °C, in order to examine the effect of flow rate. The effect of column volume (CV) was examined with the same protein sample, using ∅10 × 20, ∅10 × 55 and ∅26 × 113 mm columns (CV = 1.6, 4.3 and 60 ml, respectively), which were washed with 3 CV of 1 M ammonium sulfate and eluted with distilled water at 1.5 ml/min. Purity of the product and purification yield were determined by measurement of fluorescence intensity and protein concentration of the elute fractions and loading samples, as described above.

Characterization of celluloses as affinity column materials

The performance of crystalline celluloses (CF11, CC31, CC41 and Avicel) as cellulose affinity column chromatography materials was tested with a manually packed open column (∅10 × 5 mm) using

Table 1
Primers used in this study to amplify genes coding CBD-tagged RFP fusion proteins.

Primer name	Primer (5′ → 3′)	Restriction enzyme
DsRed-C terminal type-forward (1)	TTTGAATTC***AAAAGA***ATGGACAACACCGAGGACGTCATC	*Eco*RI
DsRed-C terminal type-reverse (2)	TTTGGTACCCTGGGAGCCGGAGTGGCGG	*Kpn*I
DsRed-N terminal type-forward (3)	TTTGGTACCATGGACAACACCGAGGACGTCATC	*Kpn*I
DsRed-N terminal type-reverse (4)	TTTGCGGCCGC***CTA***CTGGGAGCCGGAGTGG	*Not*I
TrCBHI-C terminal type-forward (5)	TTTGGTACCGGCAACCCTAGCGGCGGC	*Kpn*I
TrCBHI-C terminal type-reverse (6)	TTTGCGGCCGC***TTA***CAGGCACTGAGAGTAGTAAGGG	*Not*I
TrCBHII-N terminal type-forward (7)	TTTGAATTC***AAAAGA***CAAGCTTGCTCAAGCGTCTGGG	*Eco*RI
TrCBHII-N terminal type-reverse (8)	TTTGGTACCCGATCCGACTGGAGGTACTCTG	*Kpn*I
PcCBHI-C terminal type-forward (9)	TTTGGTACCTTCAGCGGCACCTCCTCCC	*Kpn*I
PcCBHI-C terminal type-reverse (10)	TTTGCGGCCGC***TTA***GTAGCACTGCGAGTAGTAAGG	*Not*I
PcCBHII-N terminal type-forward (11)	TTTGAATTC***AAAAGA***CAGGCGTCGGAGTGGGGAC	*Eco*RI
PcCBHII-N terminal type-reverse (12)	TTTGGTACCCGACGGAGGAGGAGGAGGG	*Kpn*I

The restriction enzymes sites are underlined. Kex2 protease cleavage site (*AAAAGA*) or the terminal codon site are shown in bold italic.

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