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Intein을 이용한 대장균에서의 *Trichoderma reesei* 유래의 Cellobiohydrolase I 섬유소 결합 도메인의 발현

Intein-mediated expression of *Trichoderma reesei* Cellobiohydrolase I Cellulose Binding Domain in *E. coli*

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Abstract

Cellulose binding domains (CBDs) of cellulases are thought to assist in the hydrolysis of insoluble crystalline cellulose. To gain sufficient amount of CBDs, the self-cleavable intein tag was used for expression and purification of *Trichoderma reesei* cellobiohydrolase I CBD in *E. coli*. Synthetic CBD genes, CBD or linker-CBD were cloned into expression vector pTYB11. Recombinant CBDs were successfully purified by intein mediated purification with an affinity chitin-binding domain. The final yields of recombinant CBD and linker-CBD were 3.2 mg/L and 1.4 mg/L, respectively. The functional bindings of recombinant CBDs were confirmed by Avicel binding experiments. The simple and easy purification method using self-cleavable intein tag can be further used in pretreatment of crystalline cellulose or characterization of engineered CBDs.

키워드 : *Trichoderma reesei, cellulose binding domain, intein, 재조합발현*
Keywords : *Trichoderma reesei, cellulose binding domain, intein, recombinant expression*

1. INTRODUCTION

The use of lignocellulosic biomass to produce biofuels is currently limited by various shortcomings in the enzymatic hydrolysis reaction [4]. Reaction rates, especially at high conversions, are low and cellulose intrinsic properties such as crystallinity, surface area, porosity or its insoluble nature mainly account for the slow enzymatic reaction [4]. Cellobiohydrolase I (Cel7A), the exocellulase cleaving off reducing ends, is the rate-limiting step of cellulose hydrolysis. Cellobiohydrolase I consist of catalytic domain with cellulose binding domain [5].

Cellulose Binding Domains (CBDs) are found as functional domains of many cellulolytic enzymes [5][12]. The primary function of the CBD is to bind the enzyme or enzyme complex to cellulose and facilitate the interaction of the catalytic site with the substrate [8][13]. CBDs are found in a wide variety of bacterial and fungal cellulases, and to date have been classified into about 13 different families based on their amino acid sequence homologies [9].

Cellulase CBDs are thought to assist in the hydrolysis of insoluble crystalline cellulose

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[4][14][16]. Previously, CBDs of cellulases, such as from *Trichoderma reesei*, are used in a pretreatment step and to reduce the crystallinity of cellulose, with the expectation of a higher cellulose rate in subsequent hydrolysis of cellulose using a mixture of cellulases and without the need for any intermediate purification step [4].

Because CBDs may be a valuable tool in pretreating cellulose and eventually afford faster enzymatic conversion of cellulose to glucose, thus contributing to more affordable processes in the production of biofuels, previous efforts have mainly focused into the production of CBDs via a site-specific cleavage of CBDs from intact cellobiohydrolase I by papain and its subsequent purification step using ion exchange column chromatography [4][11].

To study the effect of CBDs of *T. reesei* on enzymatic hydrolysis of lignocellulose, it is required to an efficient production of CBD with large amount. Currently, most CBD are prepared by proteolysis of intact cellobiohydrolase I. But its entire protocols depend on tedious protease cleavage between cellobiohydrolase catalytic domain and CBD [11].

Recently, some self-cleaving tags including intein tag have been proposed as a simple method for recombinant proteins or peptides via fusion purification, cleavage and target protein separation in a minimal step [15]. Especially, intein-mediated expression has been successfully used for purification of recombinant antimicrobial peptides like cathelicidin [10], b-defensin [2], cecropin [3], and dermcidin [6]. In this study, recombinant CBDs were expressed in intein-mediated manner. The simple purification method for recombinant CBD preparation was described.

2. MATERIALS AND METHODS

2.1. Bacterial strains, plasmids, and growth conditions

E. coli (XL10-gold) was used as the host strain for the plasmid preparation and grown at 37℃with shaking at 180 rpm in LB broth containing 100 μg/ml ampicillin or LB ampicillin agar plate. Plasmid pGEM T-easy vector and pTYB11 vector were used for the cloning and expression of recombinant.

2.2. Synthesis of synthetic cel7A gene and PCR amplification

Firstly, the synthetic *cel7*A gene of *T. reesei* (1,542 bp) was chemically synthesized by Bioneer company (Daejeon, Korea) on the basis of *P. pastoris* codon-optimized scheme for the purpose of dual protein expressions in both *E. coli and P. pastoris*. The recombinant pGEM T-Easy vector harboring synthetic *cel7*A gene was employed as a PCR template for CBDs cloning. The sequence of PCR primers (5′-3′) to amplify CBD and linker-CBD DNAs are as follows: CBD-F (GGT GGT TGC TCT TCC AAC ATG ACC CAA TCT CAT TAC GGG), LKRCBD-F (GGT GGT TGC TCT TCC AAC ATG GGA AAT CGT GGC ACC ACC), CBDHis-R (GTA GGC GGC GGC CGC TTA ATG ATG ATG ATG ATG ATG CAG GCA CTG ACT GTA). PCR products of CBD (129bp, CBD-F/CBDHis-R) and linker-CBD (192bp, LKRCBD-F/CBDHis-R) were obtained by combination of two forward PCR primers with a *Sap*I restriction site (underlined) and one reverse primer with a *Not*I restriction site (underlined). The PCRs were done by Ex-taq polymerase (Takara Korea Biomedical Inc., Seoul, Korea) using the following conditions: Incubation at 94 ℃ for 3 min, 28 cycles at 94 ℃ for 30 sec, 55 ℃ for 30 sec, 72 ℃ for 1 min and a final incubation at 72 ℃ for 10 min.

2.3. Construction of recombinant Intein-C BD fusion

The PCR products were digested with the restriction enzymes *Sap*I and *Not*I, and subsequently ligated to pTYB11 previously digested with *Sap*I and *Not*I. Ligation mixtures were used to transform competent *E. coli* XL10-gold cells. Recombinant plasmid DNAs were isolated and confirmed by DNA sequencings, and then transformed into competent BL21(DE3)pLysS cells for the expression of CBDs

2.4. Expression and purification of CBDs in *E.coli*

E. coli BL21(DE3)pLysS harboring recombinant pTYB11 plasmids were cultivated in 4 ml LB broth with 100 μg/ml of ampicillin at 37 ℃ for 3 hrs, then 0.4 mM IPTG was addedto each culture to induce gene expression

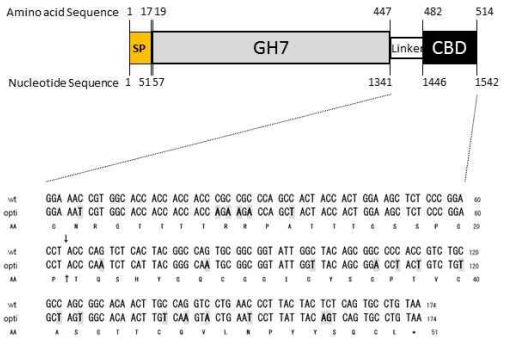

Figure 1. Sequence comparison between wild type and optimized linker-CBD region of Cel7A. Modified nucleotides are denoted as bold faces. Arrows indicate the junction between linker and CBD region. wt: wild type, opti: codon-optimized, AA: amino acid.

and further incubated at 25 ℃ for 12 hrs. The cellpellets were resuspended with 0.5 ml of lysis buffer (500 mM NaCl, 20 mM Tris, pH8.0), and then lysised by sonication (Sonics Vibra Cell Model CV18, Sonics & Materials, Inc., Newtown, CT). Soluble protein fraction of the cell extracts were harvested by centrifugation at 15,000 rpm x 15 min at 4 ℃, and then intein-CBDs fusion proteins were purified by chitin affinity chromatography according to the manufacture's manual (New England Biolabs, Ipswich, MA). Soluble protein fraction and purified proteins were analyzed in 10% Gradient acrylamide SDS-PAGE (Elpis-Biotech, Daejeon, Korea) or Mini-Proteans TGX Any kD gels (Bio-Rad, Hercules, CA), respectively to verify intein-fused CBDs expression.

3. RESULTS AND DISCUSSION

3.1. Design of synthetic CBDs for recombinant expression

Initially, the synthetic *cel7*A gene of *T. reesei* (1,542 bp) was synthesized on the basis of *P. pastoris* codon-optimized scheme for the purpose of dual protein expressions in both *E. coli and P. pastoris*. The gene was designed for both removal of restriction enzymes sites and maximizing translation efficiency. The DNA sequence and deduced amino acid of synthetic and wild type CBD gene was compared (Figure 1).

3.2. Intein-mediated expression of CBDs in recombinant *E.coli*

The intein-CBDs fusion proteins were successfully expressed in *E. coli* as soluble fractions and purified into single proteins (Figure 2).

To release CBDs from intein fusion proteins, cells were harvested from 150 ml each culture by centrifugation and the pellets were re-suspended in 2 ml of the lysis buffer to subject to sonication. Soluble proteins fractions were applied to 1 ml of 20% (w/v) chitin column, and then washed column with wash buffer (500 mM NaCl, 20 mM Tris HCl, pH8.0) to remove non-specific bound proteins. For the intein cleavage, 0.5 ml of cleavage buffer (500 mM NaCl, 20 mM Tris HCl, pH 8.0, 50 mM -mercaptoethanol) was added into chitin column and incubated at room temperature for overnight. A sample of the chitin column after cleavage was analyzed by gradient SDS-PAGE analysis and stained by coomasie blue. The purified CBDs were subsequently dialyzed using Thermo Scientific Slide-A-Lyzer Dialysis Cassettes (3.5KMWCO, Rockford, IL) against 0.1M Na-acetate buffer (pH5.0). The protein concentrations were determined through mini-

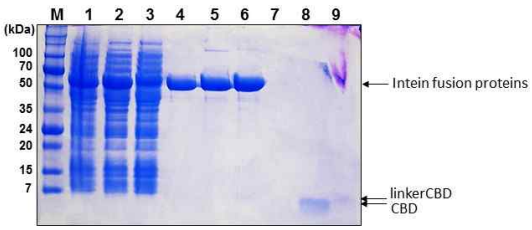

Fig. 2. Expression of pTYB11-CBDs fusion proteins in BL21(DE3)plysS cells. 10% Gradient SDS-PAGE stained with coomassie blue. M: Molecular weight marker, lane 1-3: crude extracts from IPTG induced cultures of pTYB11, pTYB11-CBD, pTYB11-linkerCBD, lane 4-6: chitin-column purified fusion proteins of pTYB11, pTYB11-CBD, pTYB11-linkerCBD, lane 7-9: eluted proteins of pTYB11, pTYB11-CBD, pTYB11-linkerCBD by DTT incubation

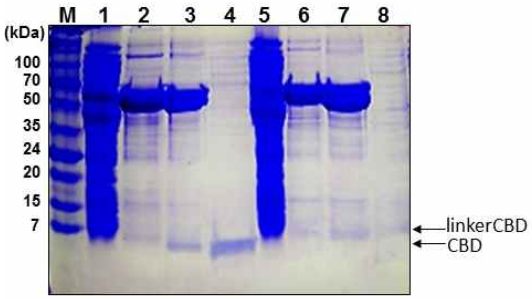

Fig. 3. Purification of CBDs from chitin beads. 10% Gradient SDS-PAGE stained with coomassie blue. M: molecular weight marker, lane 1: CBD crude extract, lane 2: purified intein-CBD protein, lane 3: proteins bound to chitin beads after b-mercaptoethanol cleavage, lane 4: eluted CBD, lane 5: linker-CBD crude extract, lane 6: purified intein-linker-CBD protein, lane 7: proteins bound to chitin beads after b-mercaptoethanol cleavage, lane 8: eluted linker-CBD.

bradford protein assay kit (Bio-Rad). CBD and linker-CBD were efficiently released from intein fusion proteins as shown in Figure 3. The size of eluted CBD and linker-CBD were matched with expected molecular weights of recombinants CBD (Mw=4567.99) and linker-CBD (Mw=6623.18), respectively. Approximately, more than 50% of CBD and linker-CBD were cleaved from 12 hr incubation with b-mercaptoethanol (Figure 3) and 3.2 mg/L of CBD and 1.37 mg/L of linker-CBD were obtained from 150 ml scale experiments.

3.3. Verification of recombinant CBD by mass spectrophotometer analyses

The amino acid sequences of purified CBD and linker-CBD were further analyzed by nanoLC-ESI-MS/MS analysis [6]. Mass data measured for one peptide fragment was 1743.7626 (mass=0.0550) which corresponds to the expected mass for the peptide (RPATTTGSSPGPTQSHY) from linker-CBD (GNRGTTTTRRPATTTGSSPGPTQSHYGQCGGIGYSGPTVCASGTTCQVLNPYYSQCLHHHHHH) peptide. This result suggests that recombinant linker-CBD band was an authentic linker-CBD originated from *T. reesei* cellobiohydrolase I.

3.4. Functional study of recombinant CBD

To test functional binding of purified CBDs with crystalline cellulose, 500 µl of 2% (w/v) Avicel samples in 100 mM sodium acetate buffer (pH 5.0) were mixed with 50 µl of cleaved CBD and linker-CBD, then incubated at room temp for 1 hr by rotation. After centrifugation, the pellet was washed three times with 1 mL of 50 mM sodium acetate buffer (pH 5.0). The pellet was resuspended by 1x SDS-PAGE loading buffer, and analyzed by SDS-gel analysis. Both CBD and linker-CBD peptides were absorbed with avicel in spite of several washing steps as shown in Figure 4. This result suggests that the recombinant CBDs retain the functional binding affinity of *T. reesei* cellulose binding domains.

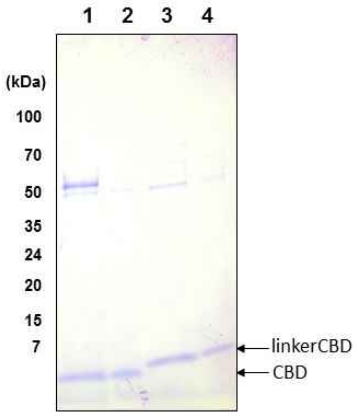

Fig. 4. Functional test of CBDs by Avicel binding experiment. Mini-Proteans TGX Any kD gels (Bio-rad) stained with coomassie blue. Lane 1: CBD, lane 2: CBD after avicel binding, lane 3: linker-CBD, lane 4: linker-CBD after avicel binding.

4. CONCLUSION

I reported here a new intein-fused expression and purification strategy for the production of CBD or linker-CBD peptides. The self-cleavage property of intein avoided using chemical reagents or proteases for CBD cleavage. So far, most CBD purification strategy depends on tedious serial purification steps after protease cleavages of intact cellobiohydrolase I or fusion

proteins. If intein cleavage step is optimized, the simplified intein-fusion expression method could contribute to large scale production of CBDs. Therefore, the purified CBDs can be further used in pretreatment of crystalline cellulose or characterization of other engineered CBDs.

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