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Thermostable β-Glycosidase-CBD Fusion Protein for Biochemical Analysis of Cotton Scouring Efficiency

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Multidomain proteins for the biochemical analysis of the scouring efficiency of cotton fabrics were constructed by the fusion of a reporter moiety in the N-terminal and the cellulose binding domain (CBD) in the C-terminal. Based on the specific binding of the CBD of *Cellulomonas fimi* exoglucanase (Cex) to crystalline cellulose (Avicel), the reporter protein is guided to the cellulose fibers that are increasingly exposed as the scouring process proceeds. Among the tested reporter proteins, a thermostable β-glycosidase (BglA) from *Thermus caldophilus* was found to be most appropriate, showing a higher applicability and stability than GFP, DsRed2, or a tetrameric β-glucuronidase (GUS) from *Escherichia coli*, which were precipitated more seriously during the expression and purification steps. When cotton fabrics with different scouring levels were treated with the BglA-CBD and incubated with X-Gal as the chromogenic substrate, an indigo color became visible within 2 h, and the color depth changed according to the conditions and extent of the scouring.

Keywords: Cotton scouring, biochemical analysis, fusion protein, cellulose binding domain, *Thermus caldophilus*, β-glycosidase

Cotton fiber consists of various components, including cellulose, lignin, and cuticle. When manufacturing cotton fabrics, the scouring process to remove the cuticle layer of cotton fiber is one of the most important processes determining the fabric quality, as it affects the water absorption and dyeing efficiency of the cotton fabrics [13, 19]. Although this scouring process is normally performed using a hot alkaline hydrolysis method [11], recent attention has been focused on a biotechnological method using a cutinase and pectin lyase mixture [2, 20, 25]. The effectiveness of scouring is determined by measuring either the time taken for a water drop to be absorbed into the fabric [1] or the chromaticity using a 1% Direct Red 81 dye solution. However, such methods only provide a qualitative result and are unsuitable when the scouring level is low or different between sites. Thus, for a precise measurement, the water-contact angle on cotton fabrics is used, but this requires expensive equipment and special skills [10].

More recently, a biochemical assay of scouring was introduced based on a fusion protein consisting of a cellulose binding domain (CBD) at the N-terminal and the reporter enzyme β-glucuronidase (GUS) at the C-terminal. An increase of CBD-GUS activity on a cotton fabric is then correlated with the extent of the scouring process, since the amount of bound CBD-GUS increases proportionally when more cellulose fibers become available as the binding site for the multidomain protein [6]. The resulting activity on the fabric is visualized using an indigo-blue reaction product with 5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid (X-Gluc) as the substrate. CBDs have already been found in many enzymes related to carbohydrate decomposition, including cellulase, xylanase, pectate lyase, and β-glycosidase [18, 24] in bacterial species [21, 22], such as *Bacillus subtilis*, *Clostridium thermocellum*, *Cellulomonas fimi*, and *Clostridium cellulovorans*, and fungi [3, 26], such as *Trichoderma reesei* and *Trichoderma viride*.

However, when using enzyme activity for the biochemical analysis of a specific substance, the stability of the enzyme is critical in determining the reliability and reproducibility of the analysis results. Thus, various strategies have been explored to stabilize enzyme functions, including immobilization in a porous matrix, protein engineering to

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improve the stability [14, 16, 17], and the isolation of highly stable enzymes from thermophilic bacteria [4].

Accordingly, for a reliable assessment of cotton scouring, this study constructed multidomain functional proteins, consisting of various reporter proteins at the N-terminal, including a β-glycosidase (BglA) from *Thermus caldophilus*, along with a *Cellulomonas fimi* CBD located at the C-terminal. The monomeric BglA-CBD protein demonstrated an improved usability as a scouring reporter when compared with GFP, DsRed2, or a tetrameric β-glucuronidase (GUS) from *Escherichia coli* [7].

Materials and Methods

Materials

The exoglucanase (Cex) gene used as the CBD was derived from *C. fimi* KCTC 9143. pET21a(+) (Novagen, U.S.A.) was used as the expression vector, whereas *E. coli* DH5α (Takara, Japan) was used for all the plasmid constructions and *E. coli* BL21(DE3) (Novagen, U.S.A.) was used as the host for the recombinant protein expression. The 100% cotton knitted fabrics (tricot, medium-weight greige) used were obtained from Woosung Ltd., Ansan, Korea. The color-development substrates used in the staining reactions were 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; Promega, U.S.A.) and 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc; Sigma, U.S.A.) for the BglA and GUS, respectively.

Scouring of Cotton Fabrics

The scouring treatments were performed as described by Kim *et al.* [15]. The alkali scouring was conducted in a bath containing 3.0% (w/v) Na_2CO_3, 0.4% (w/v) scouring agent (Scourol, Dong-A), 0.4% (w/v) chelating agent, and 0.4% (w/v) wetting agent, at 98°C for an hour. Following the incubation, the fabrics were neutralized with 0.4% (w/v) CH_2O_2, and then rinsed twice with hot and cold water. The enzymatic scouring was carried out in a bath with 0.1% (w/v) alkaline pectinase (BioPrep 3000L, Novozymes, Korea) in a 50 mM Tris-HCl buffer (pH 8.2), and 0.4% (w/v) Irgapadol PR was used as the wetting agent. The treatment was conducted at 60°C for 30 min, after which the temperature was raised to 98°C for 2 min.

Gene Manipulation

The β-glycosidase gene (*bgl*A) and β-glucuronidase gene (*gus*A) were amplified from the chromosomes of *T. caldophilus* and *E. coli*, respectively, using a PCR (Fig. 2A). The primers used for the *bgl*A amplification were 5'-GATCCATATGACCGAGAACGCCGAAAAG (primer 1; underlined: NdeI site) and 5'-ACTAGTCGGGGTCGGC-GTCGGAGGGAGCTGGGCCCGCGCGATCCG (primer 2), whereas the primers used for the *gus*A amplification were 5'-GATCCATATG-TTACGTCCTGTAGAAACC (primer 3; underlined: NdeI site) and 5'-ACTAGTCGGGGTCGGCGTCGGAGGTTGTTTGCCTCCCTG-CTG (primer 4).

The CBD gene was amplified from the chromosomal DNA of *C. fimi* using the following primers: 5'-CCTCCGACGCCGACCCCG-ACTAGTGGTCCGGCCGGGTGCCAGG (primer 5) and 5'-CCC-AAGCTTGCCGACCGTGCAGGGCGTG (primer 6; underlined: HindIII site).

Next, the *bglA* and CBD genes were connected by an overlap extension PCR using primers 1 and 6, and the *gusA* and CBD genes were connected using primers 3 and 6, resulting in 1.7 kb and 2.2 kb DNA products, respectively. Each amplified DNA product was then digested with NdeI and HindIII and inserted into pET21a(+), resulting in the expression vectors pET bglA-CBD and pET GUS-CBD (Fig. 2A). In addition, pET vectors to express the CBD fusion proteins with EGFP or DsRed2 as the reporter were also constructed following the strategy in Fig. 2A.

Expression and Purification

E. coli BL21(DE3) was transformed with the expression plasmids and grown in an LB medium (10 g/l tryptone, 5 g/l yeast extract, and 10 g/l NaCl) to produce the fusion proteins. Since isopropyl-β-D-thiogalactoside (IPTG) was found to increase the insoluble expression of the CBD fusion proteins, the cultivation was carried out in the absence of IPTG at 30°C for 20 h. The harvested cells were then suspended in 50 mM sodium phosphate buffer A (pH 7.4) containing 0.5 M NaCl, 5 mM imidazole, 1 mM PMSF, 1 mM DTT, and 0.5% Triton X-100 and disrupted by sonification on ice. The cell debris was removed by centrifugation for 30 min at 12,000 ×*g*, and the supernatant was loaded onto a HisTrap HP 5 column (Amersham Biosciences, Sweden) pre-equilibrated with buffer A using an ACTA system (Amersham Biosciences, Sweden). The elution was conducted with a gradient of 0 to 300 mM imidazole, and the fractions containing enzymatic activity were collected. Thereafter, the purified proteins were dialyzed against 50 mM sodium phosphate buffer B (pH 7.5) containing 0.2 M NaCl, 1 mM EDTA, 0.5 mM PMSF, 1 mM DTT, 0.5% Triton X-100, and 20% glycerol.

Determination of Enzyme Activity

The BglA and GUS enzyme activities were determined by measuring the concentration of *o*-nitrophenol released from 2-nitrophenyl-β-D-galactopyranoside (ONPG). For this, 100 μl of each purified fusion protein in buffer Z (60 mM $Na_2HPO_4 \cdot 7H_2O$, 40 mM $NaH_2PO_4 \cdot H_2O$, 10 mM KCl, 1 mM $MgSO_4 \cdot 7H_2O$, 50 mM β-mercaptoethanol, pH 7.0) was placed in a conical 96-well plate, and 20 μl of ONPG (4 mg/ml) was added. After incubating at 37°C for 10 min in a thermal cycler (iCycler; Bio-Rad, U.S.A.), the reaction was terminated by the addition of 50 μl of 1 M Na_2CO_3. The well plates were centrifuged for 20 min at 3,500 ×*g* on a well-plate centrifuge (Hanil Sci. Ind., Incheon, Korea), 150 μl of the supernatant was then transferred to a flat-bottom 96-well plate, and the absorbance measured at 405 nm using a model 550 microplate reader (Bio-Rad, U.S.A.) [5, 9].

Evaluation of Cotton Scouring Level Using BglA-CBD

The scouring level of the sample fabrics, which had been scoured using an alkaline or enzymatic method, was evaluated using the purified BglA-CBD. First, the protein was added to a PBS buffer (pH 7.4) containing 0.5% Triton X-100, and then the scoured fabrics were immersed in the enzyme solution and incubated at room temperature for 1 h. Next, the reaction solution was decanted, and the cotton fabrics were washed several times with a PBS buffer to remove any unbound BglA-CBD in the reaction solution. The washed cotton fabrics were then immersed in a PBS buffer containing 0.4 g/l X-Gal and allowed to stand at room temperature until an indigo color appeared. The color intensity (K/S value) of the dyed

cotton fabrics was calculated from the reflectance data according to the Kubelka-Munk equation: $K/S=(1-R)^2/2R$ [12], where K is the absorption coefficient, S is the scattering coefficient, and R is the reflectance of the dyed fabric at the wavelength of maximum absorption (λ_{max}). In the present study, the dye absorbance was measured within the visible spectrum range between 400–700 nm.

RESULTS AND DISCUSSION

Preparation of CBD Fusion Proteins

The cellulose binding moiety used for the scouring level assay was the CBD of the Cex from *C. fimi*, because of its high affinity to cellulose and stability once adsorbed on the cellulose surface [21, 22]. For the chromogenic reporter moiety, a monomeric BglA from *T. caldophilus* and tetrameric GUS from *E. coli* were tested for their usability as a reporter domain using two chromogenic substrates, X-Gal and X-Gluc, respectively. Fusion proteins with GFP or DsRed2 as the reporter domain were also tested. All the reporter proteins were located in the N-terminal, and the CBD domain was in the C-terminal (Fig. 2A). The linker moiety between the CBD and the reporter protein consisted of an extended proline-threonine linker (PPTPTPTS), to avoid any possible structural hindrance to each domain function [8, 23] (Fig. 1, Fig. 2B).

The *E. coli* cells cultivated in an LB medium at 30°C without IPTG were analyzed by 12% SDS-PAGE for the expression of the CBD fusion proteins. As shown in Fig. 3A, the BglA-CBD and GUS-CBD were detected at the expected molecular masses, corresponding to 62.6 kDa and 82.4 kDa, respectively. However, when the ONPG-hydrolyzing activities of the soluble fractions were compared, the BglA-CBD activity was at least 10-fold higher than the GUS-CBD activity (data not shown). The low activity of

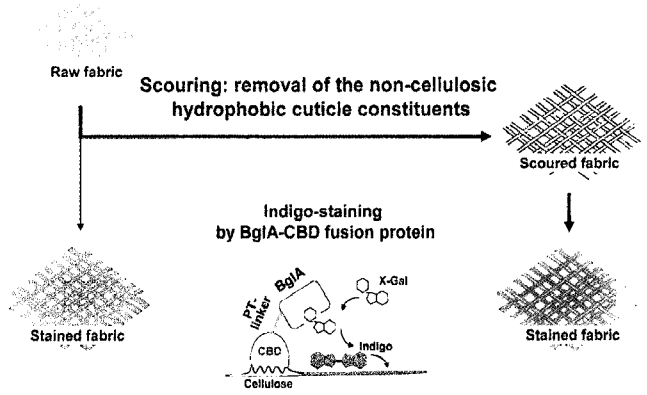

Fig. 1. Enzymatic assessments of cotton scouring level using thermostable β-glycosidase-CBD fusion protein.

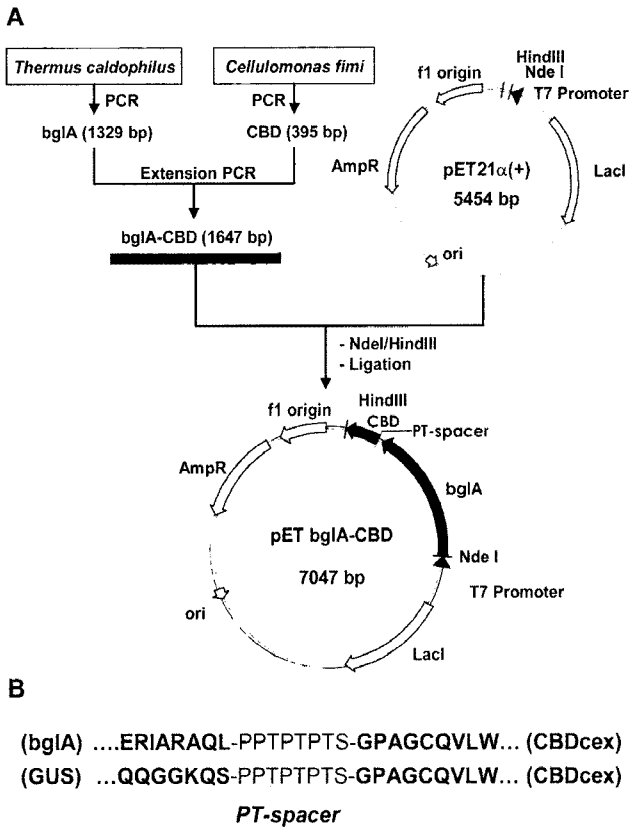

Fig. 2. Construction of CBD fusion proteins.
A. β-Glycosidase (*bglA*) of *Thermus caldophilus* combined with cellulose binding domain of *Cellulomonas fimi* Cex using overlap extension PCR. The cloning of the β-glucuronidase gene (*gusA*) of *Escherichia coli* also followed the same strategy. **B.** Short proline-threonine linker (PT-spacer) between catalytic and cellulose binding domains. All the fusion proteins contained a C-terminal His-tag.

the GUS-CBD in the soluble preparation seemed to be related to its large tetramer size (molecular mass 329.6 kDa), which may have hindered correct folding, resulting in the precipitation of misfolded proteins. Next, soluble preparations of the BglA-CBD and GUS-CBD were loaded onto a Ni-NTA chromatograph for His-affinity purification, yet only the BglA-CBD was successfully purified to a significant purity level (lane 4 in Fig. 3). The purified proteins were then stored at −70°C for several months until use. Meanwhile, since the GFP and DsRed2 with the CBD were found to be extremely insoluble in *E. coli*, soluble preparations of the proteins were determined to be inadequate for the scouring efficiency analysis.

The thermal stability analysis was carried out by exposing the BglA-CBD to a wide temperature range from 25°C to 90°C for 30 min, and then measuring the remaining activity of each protein under standard assay conditions. As a result, the BglA activity was found to remain stable up to 80°C (Fig. 3B), consistent with a previous report on the free enzyme [4]. In contrast, the GUS-CBD showed a significant decrease in activity, even at 45°C, suggesting a considerably low thermal stability.

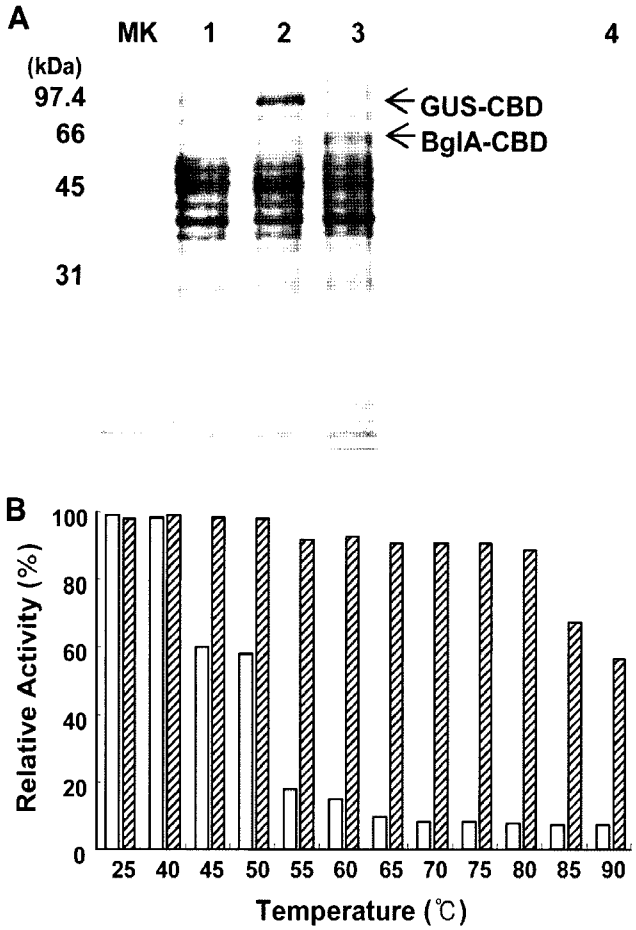

Fig. 3. Purification and characterization of CBD fusion proteins.

A. SDS-PAGE analysis. Abbreviations: M, size markers; lane 1, *E. coli* BL21 (DE3); lane 2, *E. coli* cells expressing GUS-CBD; lane 3, *E. coli* cells expressing BglA-CBD; lane 4, His-tag purified BglA-CBD protein. **B.** Thermal stability of GUS-CBD (□) and BglA-CBD (▨). The fusion proteins were treated at different temperatures for 30 min, and the remaining activity was determined under standard assay conditions.

Indigo Staining of Crystalline Cellulose by CBD Fusion Protein

The CBD fusion proteins were investigated as a catalyst for the indigo staining of crystalline cellulose (Avicel, Fluka). The BglA-CBD and GUS-CBD preparations were each added to a PBS buffer (pH 7.4) containing 1% (w/v) Avicel and left to stand at room temperature for 1 h. The supernatants were then removed and the Avicel was washed two times with a PBS buffer (pH 7.4) to eliminate any unbound CBD fusion proteins. Next, the coloring substrates X-Gal and X-Gluc for BglA and GUS, respectively, were added to the washed Avicel at a concentration of 5 μg/ml, and left to stand at room temperature for 1 h. After a quick centrifugation, color images of the Avicel precipitates were captured using a digital camera. Here, the indigo staining represents the hydrolysis of X-Gal or X-Gluc and the formation of indigo chromophores (**X:** 5-bromo-4-chloro-3-indole), as depicted in Fig. 1.

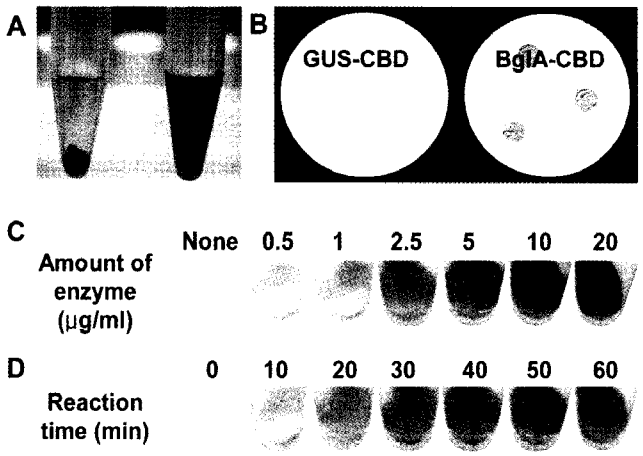

Fig. 4. Enzymatic indigo-colorization of Avicel.

A. Color reaction produced by BglA-CBD in the presence (left) or absence (right) of Avicel. The BglA-CBD used was 5 μg/ml and the reaction was conducted for 1 h at room temperature. **B.** Avicel powders color-developed by GUS-CBD (left) and BglA-CBD (right). **C.** Effect of amounts of BglA-CBD on color reaction. The staining reaction was conducted for 1 h at room temperature. **D.** Effect of time period on color reaction. The BglA-CBD amount was fixed at 5 μg/ml for each reaction.

In the presence of 1% Avicel, most of the indigo color was detected on the surface of the cellulose, with only a trace amount of color in the supernatant (left tube in Fig. 4A). In contrast, when the color reaction was carried out in the absence of Avicel, most of the color was detected in the solution (right tube in Fig. 4A). Based on the clear difference between the indigo blue reactions, the indigo blue on the Avicel surface was considered to be developed just by the enzyme adsorbed to the Avicel. When the GUS-CBD was tested for a color reaction, no significant color reaction was detected on the Avicel powder, as shown in Fig. 4B.

When the color reaction was investigated with varying amounts of the BglA-CBD for 60 min, the indigo chromaticity on the Avicel surface increased depending on the amount enzyme and became approximately saturated after 10 μg/ml (Fig. 4C). The indigo chromaticity also increased depending on the reaction periods, yet became saturated after around 40 min when 5 μg/ml enzyme was included in the reaction solution (Fig. 4D).

Assay of Cotton Scouring Using BglA-CBD

Cotton fabrics that had been scoured using an alkaline or enzymatic method were treated for 1 h with 20 μg/ml of the BglA-CBD and washed 2 times with a PBS buffer (pH 7.4) containing 0.5% Triton X-100 to eliminate any remaining free enzymes. The washed cotton fabrics were then immersed in a PBS buffer (pH 7.4) containing 0.4 g/l X-Gal and allowed to stand at room temperature until developing an indigo color. Although the color development time differed depending on the BglA-CBD and X-Gal concentrations, the color development was visually

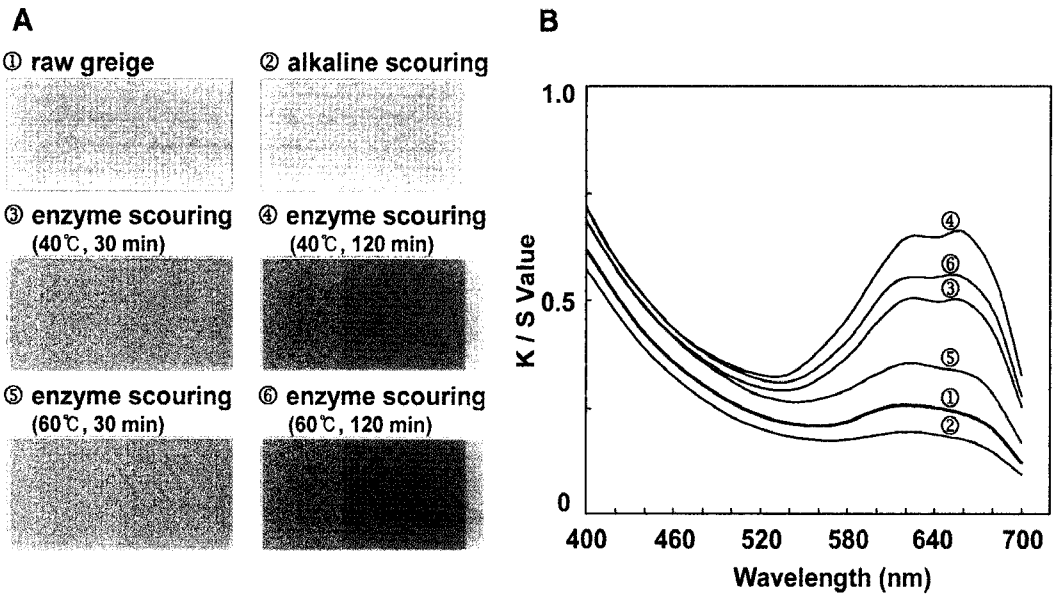

Fig. 5. Analysis of cotton scouring using BglA-CBD.
A. Images of cotton fabrics stained using BglA-CBD. **B.** Spectral analysis of stained cotton fabrics.

distinguishable at around 2 h after the color reaction (Fig. 5A). However, the fabric samples processed by an alkaline scouring method showed no detectible color change during the analysis, presumably due to a limited accessibility of the BglA-CBD to the alkaline scoured fabric surfaces, resulting from an unexpected inhibitory effect of the alkaline scouring agents.

The color intensity of the enzyme-scoured fabrics was clearly dependent on the scouring conditions, with a deeper color representing an extended scouring time (④, ⑥ in Fig. 5A). When the adsorption characteristics of the fabric samples in Fig. 4C were investigated, the K/S-values showed the maximum difference at wavelengths between 600–680 nm, depending on the scouring conditions (Fig. 5B). The K/S values exhibited that the enzymatic scouring was performed effectively at 40°C, yet weakly at 60°C, thereby agreeing with the color depth in Fig. 4C.

Consequently, this study demonstrated that the BglA-CBD, consisting of a *T. caldophilus*-derived thermostable BglA and *C. fimi* Cex-derived CBD, was highly stable and effective for use in a biochemical assay of the enzymatic scouring level of cotton fabrics.

Acknowledgments

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