

Characterization of *Thermobifida fusca* Cutinase–Carbohydrate-Binding Module Fusion Proteins and Their Potential Application in Bioscouring^{∇†}

Yao Zhang,^{1,2} Sheng Chen,^{1,2} Meng Xu,^{1,2} Artur Cavoco-Paulo,³
Jing Wu,^{1,2*} and Jian Chen^{1,2*}

State Key Laboratory of Food Science and Technology¹ and School of Biotechnology and Key Laboratory of Industrial Biotechnology,² Ministry of Education, Jiangnan University, Wuxi 214122, China, and Department of Textile Engineering, University of Minho, 4800 Guimaraes, Portugal³

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Cutinase from *Thermobifida fusca* is thermally stable and has potential application in the bioscouring of cotton in the textile industry. In the present study, the carbohydrate-binding modules (CBMs) from *T. fusca* cellulase Cel6A (CBM_{Cel6A}) and *Cellulomonas fimi* cellulase CenA (CBM_{CenA}) were fused, separately, to the carboxyl terminus of *T. fusca* cutinase. Both fusion enzymes, cutinase-CBM_{Cel6A} and cutinase-CBM_{CenA}, were expressed in *Escherichia coli* and purified to homogeneity. Enzyme characterization showed that both displayed similar catalytic properties and pH stabilities in response to *T. fusca* cutinase. In addition, both fusion proteins displayed an activity half-life of 53 h at their optimal temperature of 50°C. Compared to *T. fusca* cutinase, in the absence of pectinase, the binding activity on cotton fiber was enhanced by 2% for cutinase-CBM_{Cel6A} and by 28% for cutinase-CBM_{CenA}, whereas in the presence of pectinase, the binding activity was enhanced by 40% for the former and 45% for the latter. Notably, a dramatic increase of up to 3-fold was observed in the amount of released fatty acids from cotton fiber by both cutinase-CBM fusion proteins when acting in concert with pectinase. This is the first report of improving the scouring efficiency of cutinase by fusing it with CBM. The improvement in activity and the strong synergistic effect between the fusion proteins and pectinase suggest that they may have better applications in textile bioscouring than the native cutinase.

Cotton fiber has a multilayered structure, with its outermost surface being the cuticle that is cross-linked to the primary cell wall of cotton fiber by esterified pectin substances. The major component of the cuticle is cutin, an insoluble polyester composed mainly of saturated C₁₆ and C₁₈ hydroxy and epoxy fatty acids (14, 16, 27, 38). During the process of scouring in the textile industry, the cuticle layer has to be removed in order to improve the wettability of cotton fiber, which then facilitates uniform dyeing and finishing. Traditionally, this process is performed by hot hydrolysis in alkaline medium, which not only consumes large quantities of water and energy but also causes severe pollution and fiber damage (20, 21, 33). Therefore, environment-friendly scouring methods based on biocatalysts have been actively sought (2, 30, 36).

Cutinase is a multifunctional esterase capable of degrading the cutin component of the cuticle. Earlier reports showed that the fungal cutinase from *Fusarium solani pisi* has potential use for cotton cuticle degradation and exhibits a good synergistic effect with pectinase, an enzyme utilized to degrade pectin, in the scouring of cotton fiber (1, 7, 8, 14). Moreover, site-directed mutagenesis has been performed to replace the specific amino acid residues near the active site of cutinase (3) to

improve its hydrolytic activity toward polyesters. More recently, a cutinase from the thermophilic bacterium *Thermobifida fusca* has been identified and overexpressed in *Escherichia coli* in our laboratory (10). The good thermal stability and alkali resistance of this recombinant *T. fusca* cutinase make it potentially more amenable to textile bioscouring (10).

To further improve the applicability and/or catalytic efficiency of *T. fusca* cutinase, the present study attempts to engineer a novel cutin-degrading enzyme, based on analysis of the surface structure of cotton fiber. It has been observed that, in addition to cutin, pectin, proteins and other components, there is also a large amount of cellulose on the surface layer of cotton fiber (23). Thus, it is tempting to hypothesize that if the enzyme can be engineered to specifically bind to cellulose through a “gain of function” modification, its concentration on the surface of cotton fiber could increase significantly. Subsequently, its catalytic efficiency for cutin breakdown could be improved due to a proximity effect. In order to design such an enzyme, a fusion protein strategy in which a cellulose-binding protein/module will be attached to cutinase is considered.

It is well known that cellulase is capable of binding specifically to cellulose (25, 31). This enzyme has two separate modules: a catalytic module and a carbohydrate-binding module (CBM) (11). The two modules are discrete structural and functional units usually connected by a flexible linker (5, 17, 28). CBM has high specific capacities for cellulose binding. Previously, it has been reported that CBM is able to be fused to a chosen target protein by genetic manipulation (36), resulting in enhanced binding of this fusion protein to cellulose (6, 29). For example, fusion proteins were constructed by fusing CBM to

* Corresponding author. Mailing address: State Key Laboratory of Food Science and Technology, Jiangnan University, Wuxi 214122, China. Phone for Jing Wu: 86-510-85327802. Fax: 86-510-85327802. E-mail: jingwu@jiangnan.edu.cn. Phone for Jian Chen: 86-510-85329031. Fax: 86-510-85918309. E-mail: jchen@jiangnan.edu.cn.

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TABLE 1. Primers used in the construction of the fusion genes

Primer	Sequence (5'–3') ^a	Restriction enzyme
P1	GGAATACCATATGTCATGGCCAACCCCTA CGAGCGCGG	NcoI
P2	CGCGGCATCGCCATGAACGGGCAGG TGGA	
P3	TCCACCTGCCCCGTTTCATGGCGATCGCCGCGG	
P4	CATCTCGAGAGAATTCGGGCAGGTAAGGG TCGGAAACAG	EcoRI
P2'	GCGGCAGCCGGGAGCGGGAGGCGGGCG TGGG	
P3'	CCCACGCCGCTCCCGCTCCCGGCTGCCCGC	
P4'	CATCTCGAGAGAATTCGGGGTGCCCGTGCA GGTGGTGC	EcoRI

^a Restriction enzyme sites are indicated in boldface. Portions (15 bp) of the overlapping regions are underlined.

β -glucose nucleotide enzyme (GUS) (13) or β -glycosidase (BglA) (19), which facilitates biochemical analysis of scouring efficiency for cotton fabrics.

In the present study, the CBM from *T. fusca* cellulase Cel6A (CBM_{Cel6A}) and the CBM from *Cellulomonas fimi* cellulase CenA (CBM_{CenA}) were fused, separately, to the carboxyl terminus of *T. fusca* cutinase. The resulting fusion enzymes were compared to the native cutinase in terms of their biochemical properties, as well as the catalytic efficiency in cutin breakdown on cotton fiber. This is the first report of improving the scouring efficiency of cutinase by fusing it with CBM.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The *T. fusca* strain and plasmids pET20b(+)-Tfu_0883 and pBSK-CBM_{CenA} were lab stocks (4, 10). Plasmid pET20b(+)-Tfu_0883 was used as the gene source of *T. fusca* cutinase. The *T. fusca* strain and plasmid pBSK-CBM_{CenA} were used as the gene sources of CBM_{Cel6A} and CBM_{CenA}, respectively. *E. coli* strain BL21(DE3) was used as the expression host, and pET20b(+) was used as the cloning and expression vector. Cells were grown in Luria-Bertani medium at 37°C and, if necessary, ampicillin in a final concentration of 100 μ g/ml was added to the medium.

Construction of cutinase-CBM fusion protein expression vectors. The genes encoding cutinase (NCBI accession number YP_288944) and CBM_{CenA} (NCBI accession number AAA23084.1) were amplified by using plasmids pET20b-Tfu_0883 and pBSK-CBM_{CenA} as the templates, respectively. The gene encoding CBM_{Cel6A} and its linker (NCBI accession number YP_289135) was amplified from *T. fusca* genomic DNA. Overlapping PCR was used to fuse the *T. fusca* CBM_{Cel6A} or *C. fimi* CBM_{CenA} to the C terminus of *T. fusca* cutinase. The sequences of the primers are given in Table 1. The fusion procedure is shown in Fig. 1. An NcoI restriction site (in boldface) was introduced at the 5' end of P1, while an EcoRI restriction site (in boldface) was introduced at the 3' end of P4/P4'. The overlapping area is underlined. The primers were synthesized by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd.

The PCR was performed using 35 successive cycles as follows: denaturation at 94°C for 0.5 min, annealing at 56°C for 0.5 min, and primer extension at 72°C for 1.5 min. PrimeSTAR HS DNA polymerase was utilized during the process. The amplification product was isolated and ligated into the vector pMD18T-simple. The ligation mixture was used to transform chemically competent *E. coli* JM109. The plasmid isolated from these transformants was verified by restriction analysis, and the gene sequence was confirmed by DNA sequencing. The plasmids with the correct sequences for cutinase-CBM_{Cel6A} and cutinase-CBM_{CenA} were named pMD18T/cutinase-CBM_{Cel6A} and pMD18T/cutinase-CBM_{CenA}, respectively. The resulting plasmids were digested with NcoI and EcoRI and ligated into the similarly digested expression vector pET20b(+). The ligation mixture was used to transform chemically competent *E. coli* JM109 cells. The plasmid isolated from these transformants was verified by restriction analysis, and the gene sequence was confirmed again by DNA sequencing. The plasmids with the correct sequences for cutinase-CBM_{Cel6A} and cutinase-CBM_{CenA} were named pET20b/cutinase-CBM_{Cel6A} and pET20b/cutinase-CBM_{CenA}, respectively.

The enzymes used for DNA manipulations were purchased from TaKaRa Biotechnology Co., Ltd. DNA sequencing was performed by Shanghai Generey Biotechnology Co., Ltd. Genomic DNA extraction was performed according to the method of Sambrook et al. (32). Plasmid DNA was extracted by using the Sangon EZ-10 Spin Column Plasmid Mini-Preps kit. Plasmid and PCR products were recovered from agarose gel by using the Sangon purification kit.

Expression and purification of the cutinase-CBM fusion proteins. *E. coli* BL21(DE3) cells harboring pET20b/cutinase-CBM_{Cel6A} or pET20b/cutinase-CBM_{CenA} were grown in TB medium containing ampicillin (100 μ g/ml) at 37°C. When the culture reached an A_{600} of 1.5 to 2.0, IPTG (isopropyl- β -D-thiogalactopyranoside) was added to a final concentration of 0.5 mM. The culture after 18 h of induction was centrifuged (10,000 \times g, 30 min, 4°C), and the supernatant was collected.

The supernatant described above was treated with 70% (wt/vol) saturated ammonium sulfate solution, and the solution was kept at 4°C overnight. Precipitates were collected by centrifugation and dissolved in 50 ml of buffer A (20 mM sodium phosphate, 0.5 M NaCl, and 20 mM imidazole [pH 7.4]). The solutions were subsequently dialyzed against 2 liters of buffer A overnight and applied to a nickel affinity column pre-equilibrated with buffer A. The samples were allowed to bind with Ni-nitrilotriacetic acid agarose at a flow rate of 1 ml/min, followed by washing with buffer A until the UV baseline was reached. Elution was performed using a linear gradient from 0 to 500 mM imidazole in buffer A over 100 min. The fractions containing *p*-nitrophenyl butyrate (pNPB) hydrolase activity were pooled and dialyzed against 2 liters of buffer B (20 mM Tris-HCl [pH 8.0]) at 4°C overnight. The purified enzyme was concentrated by ultrafiltration and stored at -80°C.

Enzyme characterization of cutinase-CBMs. For enzyme characterization, all of the values presented in graphs and tables are the means of three replications. Esterase activity, cutinase activity, and lipase activity were assayed as described previously (10), with pNPB, cutin, and triolein as the substrates, respectively.

Temperature optima of the fusion and native enzymes were measured at temperatures ranging between 20 and 70°C. The reaction was performed in a buffer containing 20 mM Tris-HCl, 10 mM NaCl, and 50 mM sodium taurodeoxycholate at pH 8 using pNPB as the substrate. Since the pH of the Tris buffer is temperature dependent, the buffers were adjusted to pH 8 at the desired temperature. The enzyme activity was measured by preincubating the buffer at the desired temperature for 2 min to allow it to reach the final pH of 8. The thermostability of the enzymes was determined by incubating the enzymes in 20 mM Tris-HCl (pH 8) at 50°C. At different intervals, samples were taken and assayed for residual activity using pNPB as the substrate.

pH optima of the fusion and native enzymes were investigated between pH 6 and 9 using either potassium phosphate buffer (pH 6 to 7) or Tris-HCl buffer (pH 7 to 9). To determine the pH stability, 20 mM concentrations of the following buffers were used: sodium acetate (pH 4 to 6), potassium phosphate (pH 6 to 7), Tris-HCl (pH 7 to 9), and glycine-NaOH (pH 9 to 11). The enzymes were preincubated in the various buffers at 37°C for 24 h, followed by the determination of residual activity using pNPB as the substrate.

The Michaelis-Menten parameters, V_{max} and K_m , were determined from Michaelis-Menten plots of specific activities at various substrate concentrations. Rates were measured in triplicate using pNPB (100 to 2,000 μ M) as the substrate by continuous spectrophotometric analysis. Initial reaction velocities were calculated from the linear region (~60 s) of the reaction progress curve and measured in triplicate by varying the concentration of the substrate. The apparent kinetic constant K_m was calculated by using the GraphPad Prism program.

Protein quantification and SDS-PAGE analysis. Protein concentrations were determined by using the Bio-Rad protein assay kit (Bio-Rad), with purified bovine serum albumin (Promega) as the standard. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 10% acrylamide gels, and the proteins were visualized by staining with Coomassie brilliant blue R-250.

Binding and catalytic activity of cutinase-CBMs toward cotton fiber. The adsorption property of the enzymes was tested with cotton fiber as described previously with minor modifications (4). Prior to treatment, raw cotton fiber was boiled for 1 min and then dried at 40°C overnight. Then, 1 g of pretreated cotton fiber was mixed with 0.1% penetrant and 0.5% bovine serum albumin in Tris-HCl buffer (20 mM, pH 8.0) at 25°C. The mixture was incubated for 30 min to avoid nonspecific binding. Equal units of native cutinase or fusion enzyme (10 U/ml toward pNPB) were then added to this solution, followed by incubation for another 1 h with shaking at 60 rpm. The reaction mixtures were centrifuged at 3,000 \times g for 2 min, and the amount of unbound enzyme was estimated from the residual activity in the supernatant. The amount of cotton fiber-bound enzyme was calculated from the difference between the initial enzyme activity and unbound enzyme activity. Control assays were performed under the same condi-

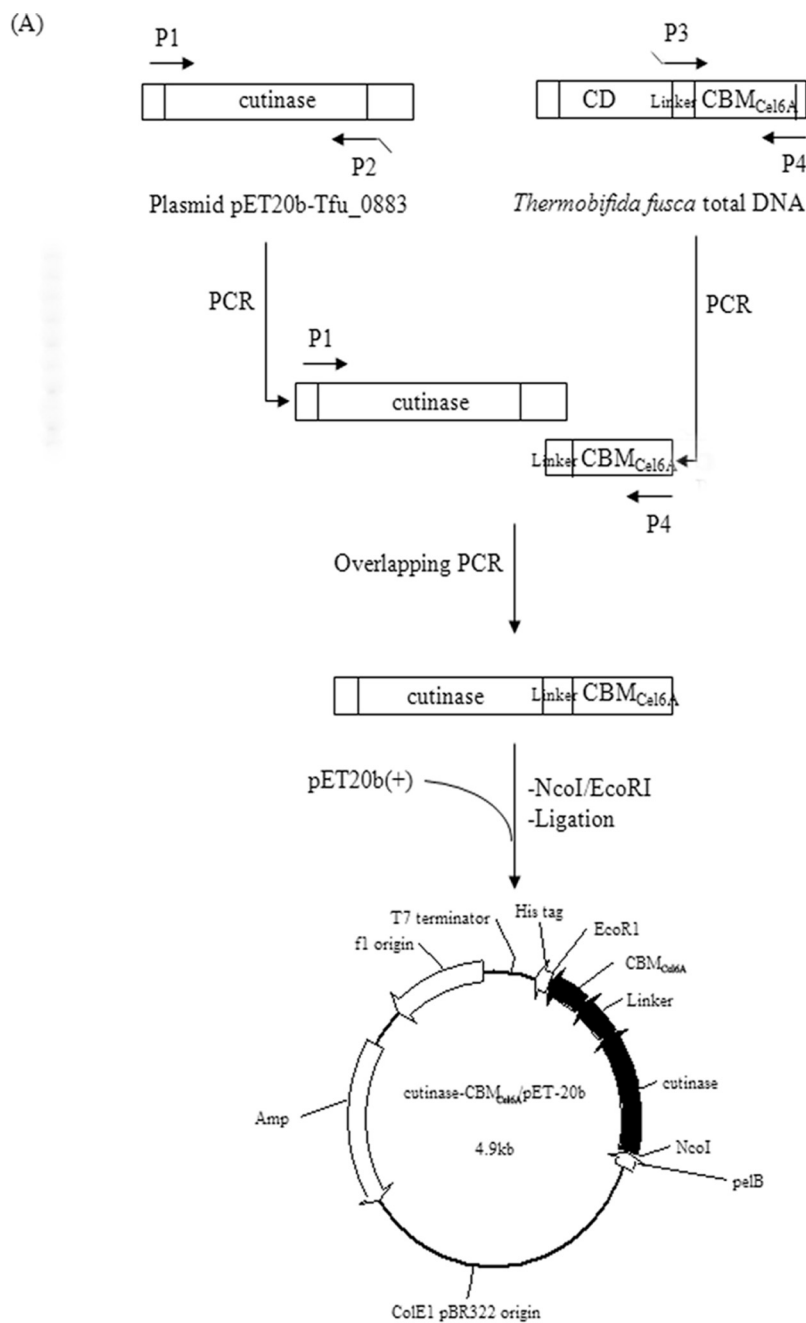


FIG. 1. Construction of cutinase-CBM fusion proteins. (A) *T. fusca* cutinase was joined with the CBM from *T. fusca* Cel6A using overlapping extension PCR. The fusion of *T. fusca* cutinase with CBM from *C. fimi* CenA followed a similar strategy. (B) Amino acid composition of the fusion proteins.

tions, except in the absence of raw cotton fiber. Pectinase was added when needed.

The desorption property of the enzymes was determined according to a method described previously (4). After 2 h of incubation, the reaction solution

described above was diluted (1:20) with Tris-HCl buffer, and the mixture was incubated for another 60 min with shaking at 200 rpm at 25°C. The amount of desorbed enzyme was estimated from the enzymatic activity in the supernatant. All assays were performed in triplicate.

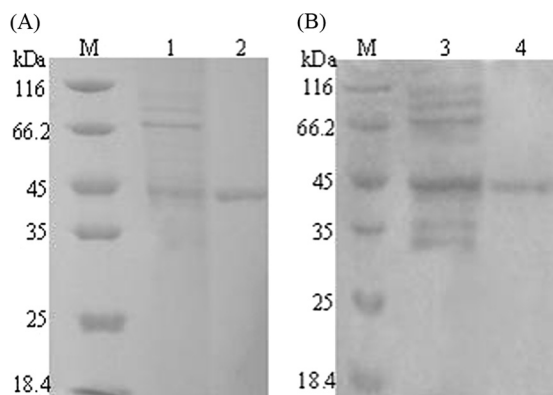


FIG. 2. SDS-PAGE analysis of the expression and purification of cutinase-CBMs. (A) Cutinase-CBM_{Cel6A}. Lanes: M, molecular mass markers; 1, culture supernatant of cutinase-CBM_{Cel6A}; 2, purified cutinase-CBM_{Cel6A}. (B) Cutinase-CBM_{CenA}. Lanes: M, molecular mass markers; 1, culture supernatant of cutinase-CBM_{CenA}; 2, purified cutinase-CBM_{CenA}.

For the determination of released fatty acids catalyzed by cutinase or cutinase-CBM, a 10-ml reaction mixture containing 1 g of raw cotton fiber and 50 μ M native or fusion cutinase was incubated in 20 mM Tris-HCl buffer with or without pectinase. The reaction mixture was shaken at 200 rpm for 18 h at 50°C. At various times, samples were removed and subjected to titration using 20 mM NaOH.

RESULTS

Construction and purification of cutinase-CBM fusion proteins. Cutinase-CBM fusion proteins were generated by fusing either the CBM of cellulase Cel6A from *T. fusca* (CBM_{Cel6A}) or the CBM of cellulase CenA from *C. fimi* (CBM_{CenA}) to the C terminus of *T. fusca* cutinase through overlapping PCR amplification. The fused genes were subsequently inserted into the expression vector pET-20b(+), which encodes a C-terminal His₆ tag and an N-terminal signal peptide PelB to allow the expressed proteins to be secreted. The resulting constructs pET20b/cutinase-CBM_{Cel6A} and pET20b/cutinase-CBM_{CenA} were used for protein expression in *E. coli* BL21(DE3). The *p*NPB hydrolyzing activities in the culture supernatant were 94 U/ml for cutinase-CBM_{Cel6A} and 76 U/ml for cutinase-CBM_{CenA}, values that were 230- and 190-fold greater, respectively, than that of the control culture [in which *E. coli* cells carried the vector pET-20b(+)]. Both fusion enzymes were purified by ammonium sulfate fraction and Ni-Sepharose affinity chromatography (see Tables S1 and S2 in the supplemental material). SDS-PAGE results demonstrated that they were purified to homogeneity with the same molecular mass of ~45 kDa (Fig. 2). In addition, the purified enzymes were active with specific activities of 20,000 U/ μ mol of protein (see Table S1 in the supplemental material) for cutinase-CBM_{Cel6A} and 15,000 U/ μ mol of protein (see Table S2 in the supplemental material) for cutinase-CBM_{CenA}.

Enzymatic properties of cutinase-CBM fusion proteins. The optimal temperature and pH of the fusion enzymes were determined at a temperature range of 20 to 70°C (see Fig. S1A in the supplemental material) and a pH range of 6 to 9 (see Fig. S1C in the supplemental material). For comparative purposes, *T. fusca* cutinase was also subjected to a similar analysis. The

TABLE 2. Monomeric products released from cutin hydrolysis by cutinase-CBMs and cutinase^a

Cutin hydrolysis product	Hydrolysis area (%)		
	Cutinase	Cutinase-CBM _{Cel6A}	Cutinase-CBM _{CenA}
Hexadecanoic acid	27.6	30.6	31.4
Octadecenoic acid	26.7	25.2	27.5
9-Octadecenoic acid	0.50	0.43	0.36
9,12-Octadecadienoic acid	1.08	1.02	1.01
16-Hydroxyhexadecanoic acid	0.54	0.54	0.58
18-Hydroxyoctadeca-9-enoic acid	1.01	0.87	0.91
18-Hydroxyoctadeca-9,12-dienoic acid	1.12	1.76	1.70
9,10,18-Trihydroxyoctadecanoic acid	0.47	0.47	0.51

^a Cutin hydrolysis by cutinase-CBM and cutinase was carried out in 25 mM potassium phosphate buffer (pH 8.0) at 50°C, for 18 h. Each value represents the mean of three independent measurements, and the variation about the mean is below 5%.

results showed that both cutinase-CBM_{Cel6A} and cutinase-CBM_{CenA} exhibited an optimal temperature at 50°C, whereas the native cutinase displayed an optimal temperature at 60°C. Not surprisingly, all native and fusion enzymes exhibited the same optimal pH of 8. Subsequently, the thermostability was determined at the temperature of 50°C (see Fig. S1B in the supplemental material), whereas the pH stability was determined at pH values between 4 and 11 (see Fig. S1D in the supplemental material). The half-life of the native cutinase was 70 h at 50°C, while those of cutinase-CBM fusion enzymes were both 53 h at 50°C. Similar pH stabilities at a pH range of 6 to 9 were observed for all three enzymes.

Previously, it has been shown that *T. fusca* cutinase has broad substrate specificity against cutin, soluble esters, and insoluble triglycerides (10). The activity of cutinase-CBMs toward soluble ester had been confirmed in the purification and characterization analysis described above using *p*NPB as a substrate. For the insoluble triglycerides, both cutinase-CBM_{Cel6A} and cutinase-CBM_{CenA} were found to be capable of hydrolyzing triolein with specific activities corresponding to 128 and 111% of the native cutinase, respectively. Furthermore, their cutin hydrolyzing activities were evaluated under their individual optimal temperature and pH. As shown in Table 2, the C₁₆ and C₁₈ family fatty acid monomers released after enzymatic reaction were 61% for cutinase-CBM_{Cel6A}, 64% for cutinase-CBM_{CenA}, and 59% for the native cutinase. The hydroxy fatty acids, which are specific in cutin, were 3.6% for cutinase-CBM_{Cel6A}, 3.7% for cutinase-CBM_{CenA}, and 3.1% for the native cutinase. These results demonstrated that both cutinase-CBM_{Cel6A} and cutinase-CBM_{CenA} can hydrolyze cutin as efficiently as the native cutinase.

TABLE 3. Kinetic parameters of cutinase-CBMs and cutinase

Cutinase or cutinase-CBM	Mean \pm SD		k_{cat}/K_m (s ⁻¹)
	K_m^{pNPB} (μ M)	k_{cat} (s ⁻¹)	
Cutinase	640 \pm 40	220 \pm 10	0.34
Cutinase-CBM _{Cel6A}	620 \pm 40	200 \pm 10	0.32
Cutinase-CBM _{CenA}	620 \pm 30	180 \pm 10	0.29

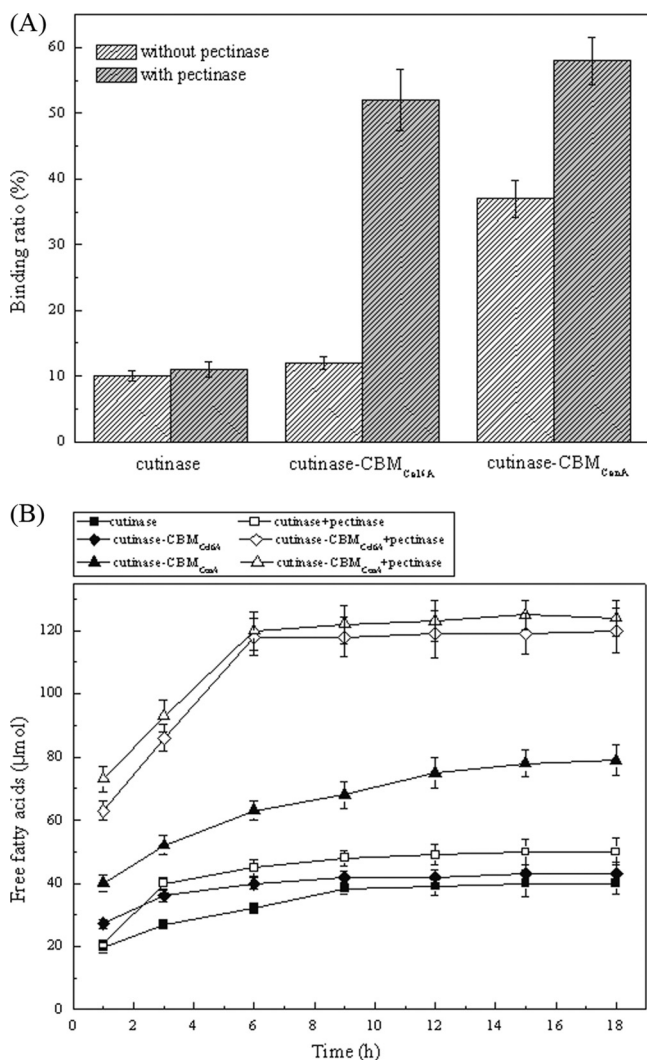


FIG. 3. Scouring effects of cutinase-CBMs and cutinase on cotton fiber. (A) Adsorption on cotton fiber. Purified cutinase or cutinase-CBM fusion protein was incubated in 20 mM Tris-HCl buffer (pH 8.0, 25°C). (B) Analysis of products released from cotton fiber. A 50 μ M concentration of purified cutinase or cutinase-CBM fusion protein was incubated in 20 mM Tris-HCl buffer (pH 8.0, 50°C) with or without pectinase, and the amount of released fatty acids was measured by titration with 0.02 N NaOH.

The kinetics of the fusion enzymes was analyzed using *p*NPB as the substrate (Table 3). Their K_m values were similar to that of the native cutinase, while their catalytic efficiencies (k_{cat}/K_m) were ca. 94% (cutinase-CBM_{Cel16A}) and 85% (cutinase-CBM_{CenA}) of that of the native cutinase.

Binding and hydrolytic activity of cutinase-CBMs toward cotton fiber. Adsorption of the enzyme on the surface of cotton fiber is the first step for cutinase to perform its hydrolysis toward cutin (37). The binding experiments (Fig. 3A) were performed under the conditions with or without the presence of pectinase, an enzyme utilized to remove pectin in bioscouring. The results showed that, compared to *T. fusca* cutinase, the binding of cutinase-CBM_{Cel16A} was enhanced by 2% in the absence of pectinase and 40% in the presence of pectinase, whereas the binding of cutinase-CBM_{CenA} was enhanced by

28% in the absence of pectinase and 45% in the presence of pectinase. After dilution and reequilibration, almost all of the bound enzymes were desorbed. Thus, the binding of both cutinase and cutinase-CBMs with cotton fiber appeared to be reversible.

In addition to binding, their hydrolytic efficiencies toward cotton fiber were also compared (Fig. 3B). In the absence of pectinase, the amount of released fatty acids was similar to that of the native cutinase for cutinase-CBM_{Cel16A} but was 1.8-fold higher for cutinase-CBM_{CenA}. In the presence of pectinase, however, both fusion enzymes released almost the same amount of fatty acids and exhibited a catalytic efficiency 3-fold higher than that of the native cutinase. This result is consistent with that of the above binding experiment. The increased binding capability resulted in enhanced cutin hydrolytic efficiency.

DISCUSSION

Previously, site-directed mutagenesis of the amino acid residues surrounding the active site has been performed in order to obtain higher catalytic efficiency for cutinase (3). In the present study, a fusion protein approach in which cutinase was fused with CBM for improved affinity to cotton fiber was developed. By taking advantage of the CBM's specific binding to cellulose on the surface of cotton fiber where cutin also exists, the concentration of fusion cutinase around cutin would be increased, which may then result in enhanced enzyme catalytic efficiency due to the proximity effect. Considering the conditions of the textile scouring process, the cutinase-CBM fusion protein has to meet the following requirements: (i) no significantly decreased cutin hydrolyzing activity compared with the native cutinase, (ii) good thermal stability and alkali resistance, (iii) good binding affinity to cotton fiber, and (iv) improved scouring effect for cotton fiber.

In order to meet the above requirements, the choice of a suitable CBM is critical. To date, characteristics of CBMs have been explored extensively (22, 35). They are found mainly in carbohydrate degrading enzymes from fungi (e.g., *Trichoderma reesei*) and bacteria (e.g., *C. fimi*), including cellulase, xylanase, mannanase, and a number of nonhydrolytic proteins (24). Considering the source of the cutinase used in the present study, naturally a CBM was first selected from the genome of *T. fusca*.

As identified by the CAZY ModO database (<http://www.afmb.cnrs-mrs.fr/CAZY/>), the genome of *T. fusca* encodes a total of 17 hydrolytic enzymes that possess a CBM (see Table S3 in the supplemental material), with 6 of them experimentally characterized (underlined in Table S3 in the supplemental material) (26). In addition, CBMs are divided into 59 different families (<http://www.afmb.cnrs-mrs.fr/CAZY/>), and those from the second family were found to be able to loosen crystalline cellulose without significant fiber damage (9). Among the six characterized CBMs from *T. fusca*, except for the CBM3 of Cel9A and the CBM4 of Cel9B, all of the others were identified to belong to the second family and thus were candidates for fusing with cutinase.

As for the directionality of the fusion, the homology structural model of cutinase showed that both the N and the C termini of the enzyme were exposed to the solvent side (10). Considering the presence of the N-terminal PeIB signal sequence for transmembrane localization, it appears that the

CBM is better to be fused to the C terminus of cutinase. Another consideration is a possible linker sequence between the cutinase and the CBM. For example, it has been reported that the linker between the CBM and catalytic domain of a bacterial cellulase is composed entirely of the Pro-Thr repetitive sequence (18). Such a linker in cellulases would possess certain flexibility and avoid possible structural hindrance, which ensures the uniform movement of the two domains on the fiber surface (18, 28, 34). Therefore, an appropriate linker between the cutinase and the CBM is desired.

Putting together the above considerations, the CBM from *T. fusca* cellulase Cel6A, which belongs to the second family of CBMs and has a 28-residue linker region, was chosen to be fused to the C terminus of cutinase.

Cutinase-CBM_{Cel6A} was well expressed and purified and was able to hydrolyze not only cutin but also insoluble triglycerides (triolein) and soluble esters (*p*NPB). In addition, it shares similar pH stability as cutinase and displayed an optimal temperature at 50°C and half-life of 53 h at 50°C. When their binding and catalytic efficiency toward cotton fiber were compared, cutinase-CBM_{Cel6A} did not appear to perform significantly better than the native cutinase. However, in the presence of pectinase, cutinase-CBM_{Cel6A} exhibited significant improvement in binding and a dramatic 3-fold increase in catalytic efficiency. This sharp contrast is likely because most of the cellulose on the surface of cotton fiber is not well exposed to the solvent and is embedded in the epidermis full of pectins, proteins, and other components, thus limiting the binding of CBM to cotton fiber. When pectinase was added in the reaction mixture, removal of pectin by this enzyme may have led to the exposure of cellulose, resulting in increased adsorption of cutinase-CBM_{Cel6A}, which eventually led to higher scouring efficiency of cotton fiber.

In addition to CBM_{Cel6A}, we also examined the possibility of using other CBMs that have been experimentally characterized. The CBM of endoglucanase A (CenA) from *C. fimi*, which also belongs to the second family of CBMs, was shown to have high affinity to cellulose (12, 15, 39) and appears to be a suitable candidate. Subsequently, CBM_{CenA} was fused to the C terminus of cutinase using the same linker from *T. fusca* Cel6A. As expected, cutinase-CBM_{CenA} displayed a substrate specificity and catalytic properties similar to those of the native cutinase. Interestingly, although the CBM_{CenA} is from a mesophilic bacterium, this fusion enzyme still retained decent thermostability, which may be due to the presence of a disulfide bond in CBM_{CenA} (15). Notably, significant improvement in the binding and catalytic efficiency toward cotton fiber was observed for cutinase-CBM_{CenA} compared to the native cutinase and cutinase-CBM_{Cel6A}. In addition, similar to cutinase-CBM_{Cel6A}, a strong synergistic effect with pectinase was also observed with cutinase-CBM_{CenA}. Thus, it appears that the scouring effect of cutinase-CBM_{CenA} is better than that of cutinase-CBM_{Cel6A}.

In conclusion, cutinase-CBM fusion proteins were successfully created by fusing a CBM to the C terminus of *T. fusca* cutinase. Compared to the native cutinase, both fusion proteins, cutinase-CBM_{Cel6A} and cutinase-CBM_{CenA}, share similar stabilities and catalytic properties but showed greatly enhanced binding and hydrolytic activity toward cotton fiber. These improvements, as well as the synergistic effect between

the fusion proteins and the pectinase, suggest that the cutinase-CBM fusion proteins may have better application potential in textile bioscouring.

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