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Enhanced activity toward PET by site-directed mutagenesis of *Thermobifida fusca* cutinase–CBM fusion protein

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ABSTRACT

In the present study, cutinase–CBM_{CenA} fusion protein was genetically modified in the carbohydrate-binding module (CBM) binding sites, by site-directed mutagenesis, to enhance its activity toward polyethylene terephthalate (PET) fiber. The effects of tryptophan at particular positions of CBM_{CenA} on the binding and hydrolysis of polyester substrate were investigated by replacing each of Trp14, Trp50 and Trp68 with leucine or tyrosine, respectively. All the mutants were expressed in *Escherichia coli* and purified to homogeneity. Enzyme characterization showed that the mutants displayed similar thermostability and pH stabilities in response to the native enzyme. Furthermore, W68L and W68Y, among all the mutants, exhibited significant improvement in binding and catalytic efficiency (1.4–1.5 fold) toward PET fiber when compared to that of the native enzyme. The enhanced binding and hydrolytic activity might be a result of creating new hydrogen bond or hydrophobic interaction between the enzyme and PET fiber.

1. Introduction

Cutinase (EC 3.1.1.74) is a versatile serine hydrolase that display hydrolytic activity toward not only cutin, the structural polyester of plants cuticle, but also a broad variety of esters including triglycerides and polymers (Arpigny & Jaeger, 1999; Carvalho, Aires-Barros, & Cabral, 1999; Egmond & de Vlieg, 2000). Recently, it has been reported that cutinase has a high potential to degrade cotton cuticle and improve the surface properties of polyethylene terephthalate (PET) fibers in an environmentally friendly way (Araujo, Casal, & Cavaco-Paulo, 2008; Degani, Gepstein, & Dosoretz, 2002; Ronkvist, Xie, Lu, & Gross, 2009). However, due to the fact that the PET fibers with high crystallinity are non-natural substrates of the enzyme, cutinase exhibits low affinity and catalytic efficiency (Araujo et al., 2007; Carla & Cavaco-Paulo, 2008; Guebitz & Cavaco-Paulo, 2008).

Several efforts have been made to improve the hydrolytic activity of cutinases toward PET fibers by site-directed mutagenesis of specific amino acid residues surrounding the active center (Araujo et al., 2007; Herrero Acero et al., 2011; Silva et al., 2011). More recently, we presented a novel approach to increase *Thermobifida fusca* cutinase activity toward cotton fibers by fusing the enzyme with carbohydrate-binding module (CBM) of cellulase CenA from *Cellulomonas fimi* (Zhang et al., 2010), in which the CBM binds specifically to cotton fiber rich in cellulose. However, the polyester structure of PET, polymerizated from terephthalic acid and ethylene glycol, is far different from cellulose, which is a polysaccharide consisting of a linear chain of β(1→4) linked D-glucose units. Thus, the CBM of naturally binding to cellulose does not necessarily fit PET fiber.

Previously, it has been reported that tryptophan residues at particular positions of *C. fimi* CBM_{CenA} played important roles in binding cellulose (Din et al., 1994). To further investigate the effect of tryptophan of CBM on the binding and hydrolysis of polyester substrate and eventually obtain the mutants with higher specific activity toward polyester fibers, in the present work, tryptophans in the CBM_{CenA} from cutinase–CBM_{CenA} fusion protein were mutated to leucine or tyrosine, respectively. Comparative studies were made on the biochemical properties of the mutations toward PET fiber. The mechanisms for the effect of these mutations on binding and hydrolysis of PET fiber were also analyzed. To the limit of our knowledge, this is the first report of improving hydrolytic efficiency of cutinase toward polyesters by mutating the CBM binding sites of cutinase–CBM.

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2. Materials and methods

2.1. Bacterial strains, plasmids and reagents

Escherichia coli JM109 and BL21(DE3) were used as the host strains for plasmid extraction and protein expression. The plasmid pET20b/cutinase–CBM_{CenA} was laboratory stock and constructed previously (Zhang et al., 2010). Enzymes used for DNA manipulations were purchased from TakaRa Biotechnology Co. Ltd. The primers were synthesized by Shanghai Sangon Biological Engineering Technology & Services Co. Ltd. DNA sequencing was performed by Shanghai Generay Biotechnology Co. Ltd.

The PET fibers were supplied by Nantong Fiber Co. Ltd. The denier of the fiber is 65 and the length is 120 mm. All other chemicals were of analytical grade unless indicated.

2.2. Mutagenesis and DNA manipulations

Each of Trp14, Trp50 and Trp68 in cutinase–CBM_{CenA} was mutated by leucine, tyrosine or phenylalanine, respectively. All mutant genes were generated by the standard QuikChange mutagenesis methodology (Stratagene), using plasmid pET20b/cutinase–CBM_{CenA} as template. This approach is based on the PCR amplification of an entire plasmid by mutagentic primers (Table 1). QuikChange PCR was performed using 35 successive cycles as following: denaturation at 94 °C for 10 s, annealing at 60 °C for 5 s, and extension at 72 °C for 5 min. The sample (10 μl) was then incubated with Dpn I at 37 °C for 2 h to remove the methylated template. The PCR product was transformed into *E. coli* JM109, and plasmid mini-preparations were screened by restriction enzyme digestion. All coding regions in the mutant plasmids were verified by DNA sequencing. The resulting mutant plasmids with the correct sequences were transformed chemically into *E. coli* BL21 (DE3) for protein expression.

2.3. Enzyme production and purification

E. coli BL21 (DE3) strains were grown in Luria–Bertani medium at 37 °C for 8 h with 100 μg/ml ampicillin added to the medium. The culture was then transferred into TB medium containing ampicillin (100 μg/ml) on a rotary shaker (200 rpm) at 30 °C. When the culture reached an optical density at 600 nm (OD_{600}) of about 1,5-isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added to a final concentration of 0.4 mM. The culture after induction 24 h was centrifuged (10,000 × *g*, 30 min, 4 °C) and the supernatant was collected. The proteins were purified from the culture supernatants on a nickel affinity column as described in previous work (Zhang et al., 2010). The protein purity was assessed on SDS-PAGE, and protein concentrations were determined by absorbance at 280 nm using the Bio-Rad protein assay kit (Bio-Rad), with purified bovine serum albumin (Promega) as the standard.

Table 1
Primers used for site-directed mutagenesis of cutinase–CBM_{CenA}.

Mutation	Primer (5′ → 3′)
W14L	Forward: GCCGTCACCAACCAG**TTG**CCCGGCGGCTTCGGC Reverse: GCCGAAGCCGCCGGG**CAA**CTGGTTGGTGACGGC
W14Y	Forward: GCCGTCACCAACCAG**TAT**CCCGGCGGCTTCGGC Reverse: GCCGAAGCCGCCGGG**ATA**CTGGTTGGTGACGGC
W50L	Forward: CGGATCCAGCAGCTG**TTG**AACGGCACCGCGTCG Reverse: CGACGCGGTGCCGTT**CAA**CAGCTGCTGGATCCG
W50Y	Forward: CGGATCCAGCAGCTG**TAT**AACGGCACCGCGTCG Reverse: CGACGCGGTGCCGTT**ATA**CAGCTGCTGGATCCG
W68L	Forward: GTCACCAGCCTGCCC**TTG**AACGGCAGCATCCCG Reverse: CGGGATGCTGCCGTT**CAA**GGGCAGGCTGGTGAC
W68Y	Forward: GTCACCAGCCTGCCC**TAT**AACGGCAGCATCCCG Reverse: CGGGATGCTGCCGTT**ATA**GGGCAGGCTGGTGAC
W68F	Forward: GTCACCAGCCTGCCC**TTT**AACGGCAGCATCCCG Reverse: CGGGATGCTGCCGTT**AAA**GGGCAGGCTGGTGAC

The codons corresponding to the specific mutations introduced are indicated in bold.

2.4. Enzyme assays

Enzyme activity was determined as described previously, utilizing *p*-nitrophenyl butyrate (*p*NPB) as the substrate in 20 mM Tris–HCl (pH 8.0) at 20 °C (Chen et al., 2008).

2.5. Model simulation

The theoretical structures of the native and mutant CBM_{CenA} were obtained by homology modeling from the SWISS-MODEL protein-modeling server (http://www. expasy.ch/swissmod/SWISS-MODEL.html). For the comparative modeling, the structure of *C. fimi* exoglucanase (PDB code 1EXG) with a sequence identity of 50% was used as a template.

2.6. Determination of thermostability and pH stability

The thermostability was determined by incubating the enzymes in 50 mM of Tris–HCl buffer (pH 8.0) at 50 °C. At different intervals, samples were taken and assayed for residual activity using *pNPB* as substrate.

To determine the pH stability, 50 mM of potassium phosphate (pH 6.0–7.0), Tris–HCl (pH 7.0–9.0) and glycine–NaOH (pH 9.0–10.0) were used as buffers. The enzymes were pre-incubated in the various buffers at 37 °C for 24 h, followed by the determination of residual activity using *p*NPB as substrate.

2.7. Binding assays

The PET fibers were washed before use to remove impurities. Washing was performed twice at 60 °C on a rotary shaker (200 rpm). The fibers were immerged in distilled water with a certain liquor-to-fiber ratio for 30 min, and then rinsed twice and left to dry at room temperature. The liquor-to-fiber ratio is depending on the type of fibers as well as the treated enzymes. In the present study, initially, three kinds of liquor-to-fiber ratios (25:1, 40:1 and 100:1) were evaluated, and the results showed that the best ratio of hydrolysis effect was 100:1. Thus, a liquor-to-fiber ratio of 100:1 was utilized to perform the binding assays.

The above pretreated samples (1 g) were mixed with 0.5% (v/v) bovine serum albumin (BSA) in 50 mM Tris–HCl buffer (pH 8.0) at 25 °C and incubated for 30 min with a liquor-to-fiber ratio of 100:1. BSA was added in order to avoid potential non-specific binding of the enzyme to substrate according to previous reports (Irwin, Zhang, & Wilson, 2000; Quentin, Ebbelaar, Derksen, Mariani, & van Der Valk, 2002). Subsequently, equal units of purified native and mutant enzymes (10 U/mL toward *p*NPB) were added in the above solution, with shaking at 60 rpm at 25 °C. The samples were withdrawn periodically and centrifuged at 3000 × *g* for 2 min. The amount of enzyme in the supernatant was determined from the measurement of enzymatic activity. The quantity of adsorbed enzyme was calculated by subtracting the amount of the enzyme in the supernatants from the amount of enzyme added initially. The controls were under the same assay conditions without enzyme sample. All values reported in this study are mean values of triplicate experiments.

The filter paper was cut into square swatches and the binding activity was determined following the similar method as described above.

2.8. Determination of PET-hydrolyzing activity

The release of aromatic compounds from PET fibers was estimated by incubating 1 g of PET fiber and 50 μM of purified enzyme preparations in Tris–HCl buffer (50 mM, pH 8.0). The reaction mixtures were incubated with orbital agitation (200 rpm) for 24 h at 50 °C. At time intervals, aliquots were centrifuged and the absorbance at 240 nm was determined. The absorbance values of controls containing deactivated enzyme were subtracted from those of the reaction mixtures containing active enzyme. The deactivated enzyme was obtained by heating the enzyme at 100 °C for 5 min. All experiments were carried out in triplicate.

The above liquid samples was further analyzed by using an Agilent 1200 HPLC system (Agilent Technologies, Palo Alto, CA), with an ASI-100 automated sample injector and a PDA-100 photodiode array detector. A reversed phase column RP-C18 (Showa Denko, Japan, 4.6 mm ID × 150 mm with precolumn) was used. Analysis was carried out with 20% acetonitrile, 20% 10 mM sulphuric acid and 60% (v/v) water as eluent at a wavelength of 240 nm. The flow rate was set to 0.8 mL min^{-1} and the column temperature was maintained at 30 °C. The injection volume was 10 μL.

3. Results

3.1. Expression and purification of mutants

Each of Trp14, Trp50 and Trp68 in cutinase–CBM_{CenA} was mutated by leucine or tyrosine, respectively. All the mutants were successfully constructed by site-directed mutagenesis and verified by DNA sequencing. The mutants were expressed in *E. coli* BL21(DE3) at similar amount of level as the native enzyme. Purified mutant proteins were obtained by ammonium sulfate fraction and Ni-Sepharose affinity chromatography. Purity and molecular weight of mutants were checked by SDS-PAGE. All proteins were purified to apparent homogeneity and displayed a molecular mass of approximately 45 kDa (date not shown). In addition, the purified mutants were active with specific activities using *p*NPB as the substrate ranged from 18,000 to 20,000 U/μmol (Table 2), as similar as the native enzyme.

3.2. Characterization of mutants

The relative thermostability of enzymes at 50 °C is shown in Fig. 1A as the function of incubation time. The native and mutant enzymes retained more than 70% of their activities after incubation at 50 °C for 24 h, indicating high thermostability which is advantageous for application in PET fiber modification processes. The pH stability was determined at pH values between 6 and 10 (Fig. 1B), and similar pH stabilities at a pH range of 6–9 were observed for native and mutant enzymes.

3.3. Binding properties of mutants

The native enzyme and mutants were assayed on binding capability toward PET fiber (Fig. 2A) as well as filter paper (Fig. 2B).

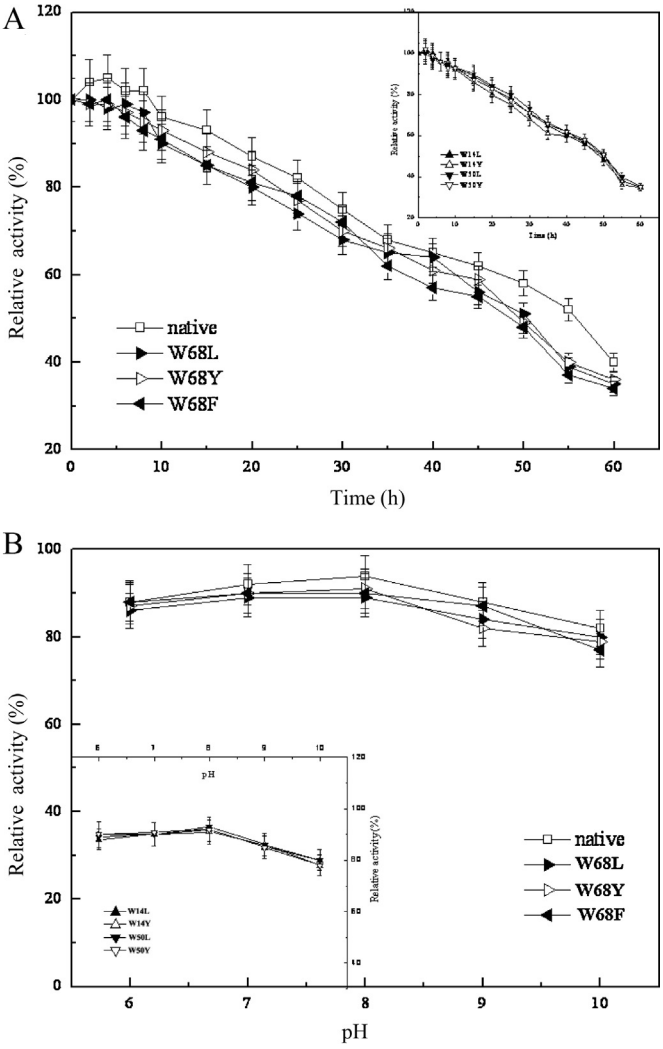

Fig. 1. Comparison of the thermostability and pH stability between the native and mutant enzymes. (A) The stability of the enzymes was performed in phosphate buffer (50 mM, pH 8.0) at 50 °C. (B) The enzyme activity was determined after incubation 24 h at 37 °C in the following buffers-potassium phosphate (pH 6.0–7.0), Tris–HCl (pH 7.0–9.0), and glycine–NaOH (pH 9.0–10.0). All enzyme activities were performed in triplicate.

The time-course results illustrate that the binding activity of the enzymes increased gradually over 1 h and then leveled off. Compared to the native enzyme, all the mutant enzymes have decreased binding on filter paper (Fig. 2B) by more than 40%, which indicated that the modified residues (Trp14, Trp50 and Trp68) were indeed corresponding to cellulose binding. As for PET fiber (Fig. 2A), when compared with the native enzyme, the binding of mutants W68L and W68Y on PET fiber were enhanced by 14% and 17%, while other mutants showed no obvious difference.

3.4. Hydrolytic activity of mutants toward PET fibers

Besides for binding, the hydrolytic efficiency of native and mutant enzymes toward PET fiber was also analyzed and the results were evaluated by spectrophotometry and HPLC (Figs. 3 and 4).

Table 2
Specific activities of native enzymes and mutants.

Enzymes	Native	W14L	W14Y	W50L	W50Y	W68L	W68Y	W68F
Specific activity (U/μmol protein)	20,000	19,400	18,900	19,700	18,300	19,300	18,200	18,500

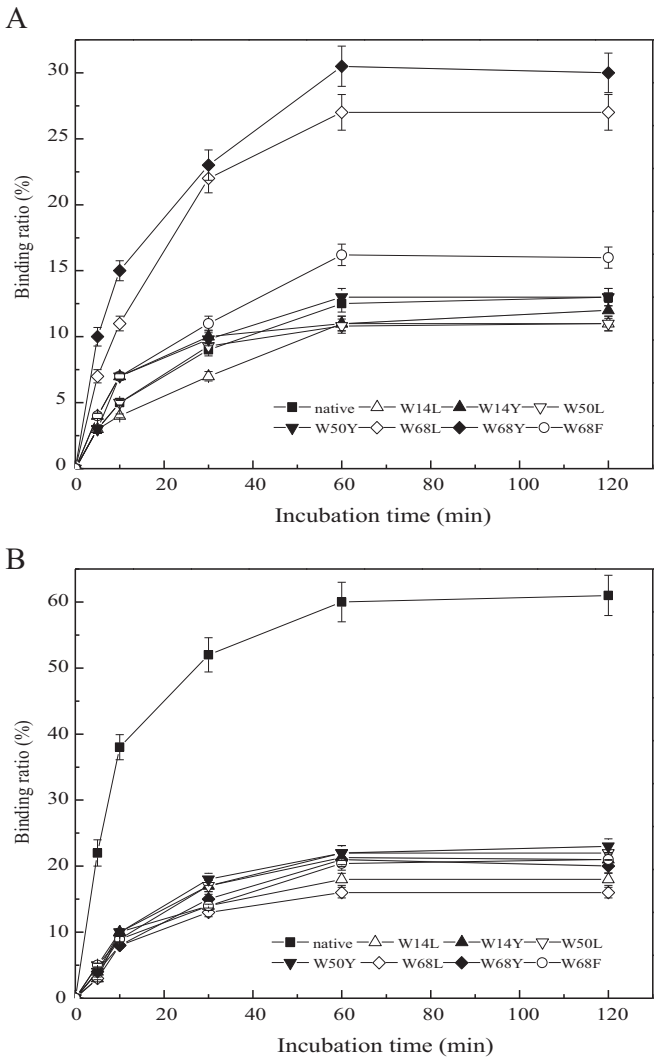

Fig. 2. Time profile of binding activity of enzyme on PET fiber (A) and filer paper (B). Pretreated PET samples or filter paper swatches (1 g) were treated with purified native or mutant enzymes (10 U/mL) and incubated in 50 mM phosphate buffer (pH 8.0) at 25 °C for 2 h.

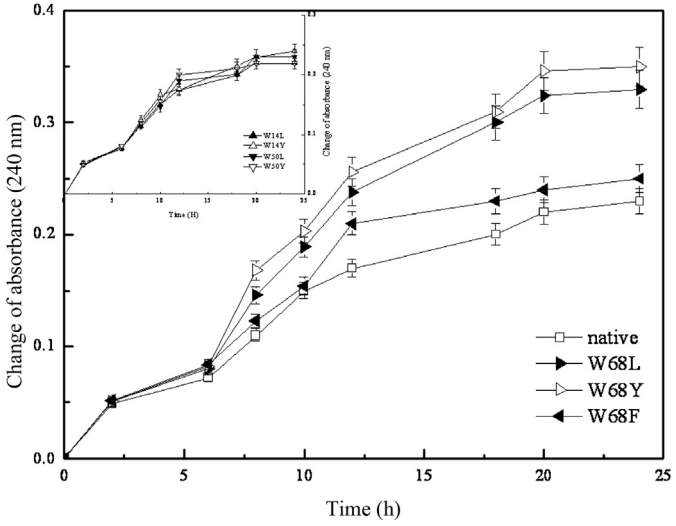

Fig. 3. Increasement of the absorbance at 240 nm for enzymatic treatment. The reaction mixtures containing 50 μM of purified native or mutant enzymes with 1 g of PET fiber in 50 mM phosphate buffer (pH 8.0) at 50 °C for 24 h. The values are average differences between the active enzymatic treatment and a control with deactivated enzyme.

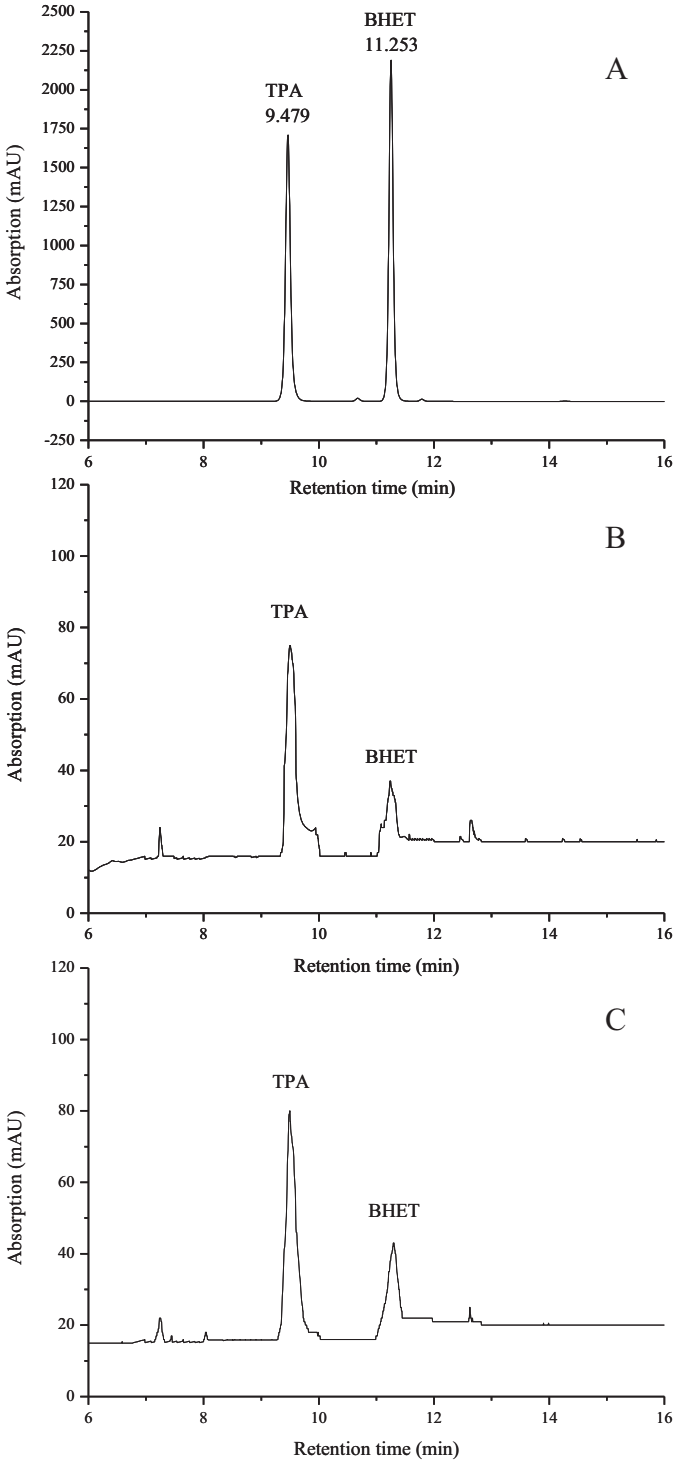

Fig. 4. HPLC analysis of products released from PET fibers. PET fibers (1 g) was treated by same amount of native enzyme or W68Y in 50 mM phosphate buffer (pH 8.0) at 50 °C for 24 h. (A) Calibration standard; (B) hydrolysis of PET fiber with the native enzyme; (C) hydrolysis of PET fiber with W68Y.

Previously, Alisch et al. (2004) and Yoon, Kellis, and Poulose (2002) have shown a biocatalytic modification of PET fiber by measuring an increase at absorbance of 240 nm in the reaction mixtures due to the release of terephthalic acid (TPA) and its esters from the PET substrate. In the present study, Fig. 3 indicates that the UV absorbance increased slightly and gradually with increasing reaction time, which implies that hydrolysis of PET occurred and that products were formed due to the catalytic action of native and

Name	Source	Partial Sequence	Accession No.
		12-16 32-36 48-52 66-70	
CenA	*Cellulomonas fimi*	--NQWPG---SSWKL---QLWNG---LPWNG--	AAA23084.1
Cex	*Cellulomonas fimi*	--NQWNT---DGWTL---QAWSS---APWNG--	AAB34464.1
Cel6A	*Thermobifida fusca*	--NEWND---TGWTV---NAWNA---VGHNG--	AAZ55112.1
Cel5A	*Thermobifida fusca*	--S-WDN---SQWEV---QVWNA---VSWNS--	AAZ54939.1
EngD	*Clostridium cellulovorans*	--NSWGS---NGWTL---NMWSA---AGYNG--	AAA23233.1
XynA	*Pseudomonas fluorescens subsp. cellulosa*	--SEWST---NNWNV---SGWNA---MSWNG--	S13391

Fig. 5. Multiple sequence alignment of family CBM2 from different sources. The positions of tryptophan residues are shown in bold.

mutant enzymes. Compared to the native enzyme, the amounts of released aromatic compounds were increased by 1.4 fold for the mutant W68L and by 1.5 fold for the mutant W68Y, however, other mutants released almost the same amounts of product as to that of the native enzyme (Fig. 3). These results are in agreement with the above binding experiment.

Enzymatic hydrolysis of PET fibers by W68Y as well as the native enzymes was also studied by monitoring the hydrolytic products by HPLC. Terephthalic acid (TPA) (t_r = 9.479) and bis(2-hydroxyethyl) terephthalate (BHET) (t_r = 11.253) were identified as the main hydrolysis products in the samples treated by both the native enzyme and the mutant (Fig. 4).

4. Discussion

This study was aimed at facilitating the hydrolysis of cutinase–CBM_{CenA} fusion protein toward PET fiber. Considering that CBM natively binds to cellulose, site-directed mutagenesis was employed in CBM in order to enhance the binding between cutinase–CBM_{CenA} and PET, which subsequently led to the increasement of enzyme catalytic efficiency.

CBM_{CenA} belongs to the family of CBM2 and sequence comparison of proteins in this family showed that three tryptophan residues, corresponding to Trp14, Trp34 and Trp50 of CBM_{CenA}, are strictly conserved; a fourth tryptophan residue, corresponding to Trp68, is less conserved (Fig. 5). Based on the predicted three-dimensional structure of CBM_{CenA} (Fig. 6), Trp34 was located in the intramolecular, and Trp14, Trp50 and Trp68 distributed at the surface. The stacking of surface tryptophan residues against the multi-sugar rings has been shown to contribute to the specificity of enzyme–carbohydrate interactions (Din et al., 1994; Levy & Shoseyov, 2002). Thus, in the present study, the three tryptophan residues exposed on the surface in CBM_{CenA} were selected for site-directed mutagenesis in an attempt to assess their roles in binding to the substrate of PET.

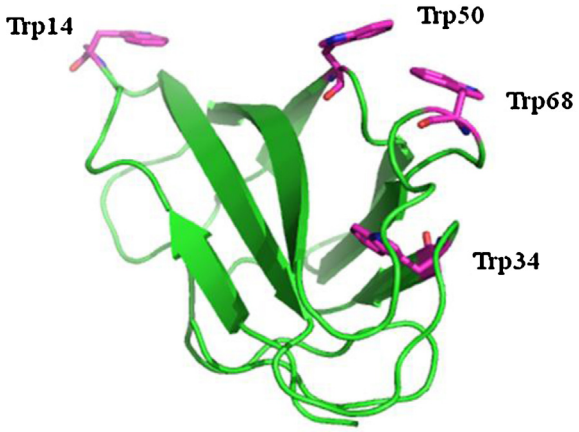

Fig. 6. Structure model of CBM_{CenA} from *C. fimi* with Trp14, Trp34, Trp50 and Trp68 is shown.

Candidate residues to be mutated were chosen through analysis of the structures of PET fibers, the mutation of tryptophan toward leucine or tyrosine were suggested due to that: (i) leucine residue gets an alkyl hydrophobic environment, which might induce higher adsorption on PET fiber rich in alkyl part; (ii) tyrosine residue may form hydrogen bond and has strong aromatic hydroxyl interaction with aromatic ring of PET, which might anchor the CBM to the surface of PET fiber better.

According to above analysis, in the present study, each of Trp14, Trp50 and Trp68 in CBM_{CenA} was mutated to leucine or tyrosine, respectively. The results showed that, when compared to that of the native enzyme, only the mutants of W68L and W68Y had an enhanced binding capacity toward PET fiber, which led to increased catalytic activity as well, other mutants had no significant difference from that of the native enzyme (Figs. 2 and 3).

Previously, Hiraishi, Hirahara, Doi, Maeda, and Taguchi (2006) proposed that the interaction between the residues of substrate-binding domain of poly[(*R*)-3-hydroxybutyrate] (PHB) depolymerase from *Ralstonia pichettii* T1 and the PHB surface was hydrogen bonds and hydrophobic interaction. In addition, Din et al. (1994) and Levy and Shoseyov (2002) have reported that the strictly conserved tryptophan residues of CBM were contributed to the specificity of enzyme–carbohydrate interactions. Based on these reports as well as our above observations, we proposed a hypothesis of the interaction between tryptophan residues of CBM and the substrate of PET. It seemed like that there are not only hydrophobic interactions between the hydrophobic ring of tryptophan and phenyl or methyl groups in PET, but also hydrogen bonds between tryptophan and ester bonds in PET. However, the formation of hydrogen bond between tryptophan and PET is sometimes limited and depending on the location and orientation of tryptophan due to the fact that: (i) the PET molecule has no hydrogen donor, and the hydrogen acceptor, the oxygen atoms, is restricted in the skeleton of the PET molecule; and (ii) tryptophan itself has a certain hydrophobic and rigid property.

According to our model, at position of 14 and 50, the direction of tryptophan may be conducive to the formation of hydrogen bonds with PET fiber, and thus the mutations at these two positions have no positive results. However, the tryptophan at position of 68 was located in the edge of surface and its imino (providing hydrogen atoms) was located on the opposite of the substrate. When Trp68

was mutated to leucine, the flexible hydrophobic long chain in the latter might tend to strengthen the hydrophobic interaction with alkyl chains of PET, and when Trp68 was mutated to tyrosine, hydroxyl group in the latter may face to PET substrate, which led to form a new hydrogen bond between tyrosine and PET. As a matter of fact, this possibility was verified by our further experiment of mutation Trp68 to phenylalanine, in which the hydroxyl group was absent and unable to form additional hydrogen bond, the binding ability of W68F to PET was significantly decreased, as expected (Fig. 2).

In summary, through site-directed mutagenesis of CBM binding sites, we have obtained modified cutinase–CBM_{CenA} fusion protein with enhanced activity toward PET fiber, namely mutants W68L and W68Y. The improved binding and hydrolytic activity of these two mutants might be a result of creating new hydrogen bond or enhancing the hydrophobic interaction with PET fiber at position 68. These mutants may have potential application in the processing of textile fiber.

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