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Biochemical characterization of the cutinases from *Thermobifida fusca*

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ABSTRACT

Thermobifida fusca produces two cutinases which share 93% identity in amino acid sequence. In the present study, we investigated the detailed biochemical properties of *T. fusca* cutinases for the first time. For a better comparison between bacterial and fungal cutinases, recombinant *Fusarium solani pisi* cutinase was subjected to the similar analysis. The results showed that both bacterial and fungal cutinases are monomeric proteins in solution. The bacterial cutinases exhibited a broad substrate specificity against plant cutin, synthetic polyesters, insoluble triglycerides, and soluble esters. In addition, the two isoenzymes of *T. fusca* and the *F. solani pisi* cutinase are similar in substrate kinetics, the lack of interfacial activation, and metal ion requirements. However, the *T. fusca* cutinases showed higher stability in the presence of surfactants and organic solvents. Considering the versatile hydrolytic activity, good tolerance to surfactants, superior stability in organic solvents, and thermostability demonstrated by *T. fusca* cutinases, they may have promising applications in related industries.

1. Introduction

Cutin is a major component of the plant cuticle which constitutes an efficient barrier against desiccation and pathogens [1]. Cutinases are inducible extracellular enzymes secreted by microorganisms capable of catalyzing the cleavage of ester bonds in cutin [2]. They display hydrolytic activity not only towards cutin but also a variety of soluble synthetic esters, insoluble triglycerides, synthetic fibers (polyethylene terephthalate fibers), plastics (polycaprolactone) and others [3,4]. Therefore, cutinases have been recognized as versatile lipolytic enzymes in laundry and dishwashing detergent formulations, and for other applications in the textile, food and chemical industries [5–7]. In addition to their hydrolytic activity, cutinases also show synthetic activity and have potential use for the synthesis of structured triglycerides, polymers, pharmaceuticals and agrochemicals [8].

Cutinases have been found in both fungi and bacteria [9]. Fungal cutinases, such as from *Fusarium solani pisi*, *Monilinia fructicola* [10], *Botrytis cinerea* [11], and *Aspergillus oryzae* [4] have been studied comprehensively. Especially, the cutinase from *F. solani pisi* has been extensively investigated, including gene identification, cloning, expression, characterization, structure elucidation, and applications [12]. In contrast, limited studies have been performed on cutinases from bacterial sources until recently when the genes encoding cutinase from the thermophilic actinomycete *Thermobifida fusca* were identified in our laboratory [13].

T. fusca has two cutinase-encoding open reading frames, *Tfu_0882* and *Tfu_0883*. The two isoenzymes are 93% identical in their amino acid sequence. Initial characterization showed that Tfu_0882 and Tfu_0883 share similar temperature dependence profiles and thermostability, but display higher temperature optimum and greater thermostability compared to fungal cutinases. Although both bacterial and fungal cutinases belong to the a/β hydrolase fold superfamily, the bacterial sequences are significantly longer and demonstrate no similarity to the fungal sequences. In this respect, it has been suggested that the bacterial and fungal enzymes should be classified into prokaryotic and eukaryotic cutinase subfamilies, respectively [13]. A more in-depth analysis of the enzymatic properties of bacterial cutinases will further our understanding on cutinase subfamilies. In addition, such studies will further explore their potential applications in biotechnology.

In the present study, we investigated the detailed biochemical properties of *T. fusca* cutinases. For a better comparison between the two subfamilies of cutinases, recombinant *F. solani pisi*

Abbreviations: FspC, *Fusarium solani pisi* cutinase; TDOC, sodium taurodeoxycholate; *p*NPB, *p*-nitrophenyl butyrate; *p*NPP, *p*-nitrophenyl palmitate; TPA, terephthalic acid; MHET, mono(2-hydroxyethyl terephthalate); BHET, bis(2-hydroxyethyl terephthalate); EMT, 1,2-ethylene-mono-terephthalate-mono(2-hydroxyethyl terephthalate).

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cutinase (FspC) was subjected to the same analysis. The results showed that these enzymes are similar in substrate specificity, substrate kinetics, interfacial activation, and metal ion requirement. However, they differed significantly in their stability in the presence of surfactants and organic solvents.

2. Materials and methods

2.1. Chemicals

Sodium taurodeoxycholate (TDOC), *p*-nitrophenyl butyrate (*p*NPB), *p*-nitrophenyl palmitate (*p*NPP), triolein, tributyrin, terephthalic acid (TPA), mono(2-hydroxyethyl terephthalate) (MHET), bis(2-hydroxyethyl terephthalate) (BHET) and 1,2-ethylene-monoterephthalate-mono(2-hydroxyethyl terephthalate) (EMT) were obtained from Sigma. Other chemicals were obtained from Sinopharm Chemical Reagent Co. Ltd.

2.2. Enzyme preparation

Recombinant cutinases Tfu_0882 and Tfu_0883 were purified from *E. coli* BL21 (DE3) cells harboring the plasmids pET20b(+)-Tfu_0882 or pET20b(+)-Tfu_0883. Recombinant FspC was purified from *Bacillus subtilis* harboring the plasmid pBSMuL3 as previously reported [13].

2.3. Molecular mass determination

The molecular masses of Tfu_0882 and Tfu_0883 were determined under denaturing conditions by SDS-PAGE. The native molecular weight of the protein was determined by analytical gel filtration chromatography using a Superose 12 column (HR 10/30; Pharmacia). Chromatography was performed at ambient temperature on a Pharmacia FPLC system monitoring the eluent at 280 nm. The running buffer was 0.15 M sodium chloride, 50 mM Tris (pH 7.5) and a flow rate of 0.5 ml/min was used. A standard curve was generated using proteins from a molecular weight marker kit (MWGF-200, Sigma). Standards and samples were used at concentrations of 5–10 mg/ml and were loaded onto the column using a 50 μl sample loop. The elution volume, V_e, was determined in triplicate for all samples and standards.

2.4. Esterase assay

Esterase activity was determined by a continuous spectrophotometric assay using *p*-nitrophenyl butyrate (*p*NPB) as the substrate [14]. The standard assay contained in a final volume of 1 ml *p*NPB (1 mM, stock in acetonitrile), enzyme solution, and buffer (20 mM Tris–HCl containing 10 mM NaCl, pH 8.0) at 37 °C. The reaction was initiated by the addition of *p*NPB.

The activity against *p*-nitrophenyl palmitate (*p*NPP) was determined as previously reported [15].

The hydrolysis of *p*NPB and *p*NPP was spectrophotometrically monitored for the formation of *p*-nitrophenol at 405 nm. One unit of enzyme activity was defined as the production of 1 μmol *p*-nitrophenol per minute [16].

2.5. Lipase assay

Lipase activity was measured as previously reported [17] with the following modifications. Triolein or tributyrin was used as the substrate. Triolein or tributyrin emulsion was prepared by emulsifying Triolein/tributyrin in 25 mM potassium phosphate buffer (pH 8) and 0.5% (w/v) gum arabic for 2 min at maximum speed in a Waring blender. The reaction solution contained 2.5 ml of 25 mM potassium phosphate buffer (pH 8) and 2 ml of emulsion and enzyme. The reaction was initiated by adding the enzyme to the reaction solution, incubated for 15 min and quenched by adding 7.5 ml of ethanol. The released fatty acids were quantified by titration with 0.05N NaOH. One unit of lipase activity was defined as the release of 1 μmol of fatty acid per minute.

2.6. Degradation of cutin by the cutinases

1 mg of Tfu_0882, Tfu_0883, and FspC were incubated with 1% (w/v) apple cutin in 25 mM potassium phosphate buffer (pH 8.0). The incubation temperature was 60 °C for Tfu_0882 and Tfu_0883, and 40 °C for FspC. At various time intervals, aliquots were removed and assayed for released fatty acids by titration with 0.02N NaOH.

2.7. Degradation of cyclic PET trimer by the cutinases

0.3 g of a cyclic PET trimer preparation and 10 ml 25 mM phosphate buffer (pH 8) were added to a Erlenmeyer flask. The reaction was initiated by the addition of 40 U *p*NPB activity corresponding to 37 μg, 280 μg, and 94 μg protein of FspC, Tfu_0882 and Tfu_0883, respectively. The incubation was performed at 60 °C for Tfu_0882 and Tfu_0883, and 40 °C for FspC in an incubator (150 rpm) over 72 h. During the incubation, aliquots were removed and prepared for the analysis by RP-HPLC as previously described [6].

The amounts of released soluble hydrolysis products were calculated based on the enzyme concentrations employed (mM/mg protein). The hydrolysis products were detected as previously reported by Hooker et al. [18] for terephthalic acid (TPA), mono(2-hydroxyethyl terephthalate) (MHET) and bis(2-hydroxyethyl terephthalate) (BHET). 1,2-Ethylene-monoterephthalate-mono(2-hydroxyethyl terephthalate) (EMT) was detected and confirmed by LC–MS analysis. The hydrolysis products were calculated as the sum of the amounts of TPA, MHET, BHET and EMT produced.

2.8. Triglyceride position specificity of the cutinases

The reaction solution contained 2.5 ml of 25 mM potassium phosphate buffer (pH 8), 2 ml of triolein emulsion and enzyme. Analysis of the regioselectivity of triolein hydrolysis by cutinase was carried out by incubating the reaction solution at 60 °C (Tfu_0882 and Tfu_0883) or 40 °C (FspC) for 15 min. The reaction mixture was extracted by isopropanol and n-hexane and analyzed by HPLC [19].

2.9. Kinetic analysis of the cutinases

Kinetic studies were performed with *p*NPB (100–2000 μM) as substrate using the continuous spectrophotometric assay as described above. 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. Apparent kinetic constants K_m were calculated from a double-reciprocal plot ($1/v$ vs. $1/[p\text{NPB}]$) of the initial rate data. Results are the average of triplicate assays [20].

2.10. Interfacial activation of the cutinases

Tributyrin emulsion (3–35 mM) was prepared by mixing a given amount of tributyrin in 30 ml of 0.5% gum arabic and 0.15 M NaCl in a Warring blender. The reaction solution contained 2.5 ml of 25 mM potassium phosphate buffer (pH 8) and 2 ml of emulsion and enzyme. The reaction was initiated by adding the enzyme to the reaction solution, incubated in 37 °C for 15 min and quenched

by adding 7.5 ml of ethanol. The released fatty acids were quantified via titration by 0.05N NaOH.

2.11. Effect of metal ions on enzyme activity

Various metal ions ($CaCl_2$, $CuSO_4$, $MgCl_2$, $MnCl_2$, $ZnSO_4$, $CoCl_2$, $NiCl_2$, $BaCl_2$, $PbCl_2$, $CrCl_2$, $HgCl_2$ and $FeSO_4$) at a concentration of 1 mM were added to 5 nM cutinase (20 mM Tris–HCl buffer, pH 8), and the solution was preincubated at 37 °C for 5 min and then assayed for esterase activity against *p*NPB. Esterase activity of the enzyme without added metal ion was defined as 100% [21].

2.12. Effect of surfactants on enzyme activity

Various surfactants (SDS, Triton X-100, Tween 20 and TDOC) at different concentrations were added to 5 nM cutinase (20 mM Tris–HCl buffer, pH 8), and the solution was preincubated at 37 °C for 5 min and then assayed for esterase activity against *p*NPB. Esterase activity of the enzyme without added surfactants was defined as 100% [22]. The SDS inhibition constant was determined by a double-reciprocal plot ($1/v$ vs. $1/[p\text{NPB}]$) of the initial rate data at three concentrations of SDS [23]. The inhibition data were analyzed using a Lineweaver-Burk plot.

2.13. Stability in organic solvent

The stability in organic solvents was tested in a buffer (20 mM Tris–HCl, pH 8) containing 75% (v/v) of various solvents and 5 nM cutinase. After 18 h of incubation at 20 °C, aliquots were removed for determination of residual esterase activity against *p*NPB. Esterase activity of the enzyme without solvents was defined as the 100% level [24].

3. Results and discussion

3.1. Molecular mass determination

The subunit molecular masses of the purified Tfu_0882 and Tfu_0883 were both 29 kDa as determined by SDS-PAGE, which is consistent with their calculated molecular masses of 29.220 kDa and 28.997 kDa, respectively. Their native molecular masses were both 32 kDa as determined by analytical gel filtration chromatography. Therefore, both Tfu_0882 and Tfu_0883 were predicted to be monomeric proteins in solution. The FspC also determined as a monomeric protein in solution (data not shown) which confirmed the previous results [25].

3.2. Substrate specificity

The catalytic efficiency of Tfu_0882, Tfu_0883, and FspC toward cutin was evaluated by measuring the released fatty acid products. As shown in Fig. 1, all three enzymes hydrolyze cutin in a similarly effective way, which is consistent with their physiological function as a cutin hydrolase.

Given the broad substrate specificity of fungal cutinases, we further evaluated the hydrolytic activity of the *T. fusca* cutinases towards additional esters. Previously, it has been shown that Tfu_0882, Tfu_0883 and FspC hydrolyze the insoluble triglyceride triolein [13]. To gain more insight into the position specificity for triolein, the reaction products were analyzed by HPLC. As shown in Fig. 2, the molar concentration of 1,2(2,3)-diolein obtained was not very different from 1,3-diolein for the three enzymes. The cutinases not only hydrolyzed the ester bond of triacylglycerids in the sn-1,3-position, but also in the sn-2 position. None of the three cutinases showed 1,3-position specificity.

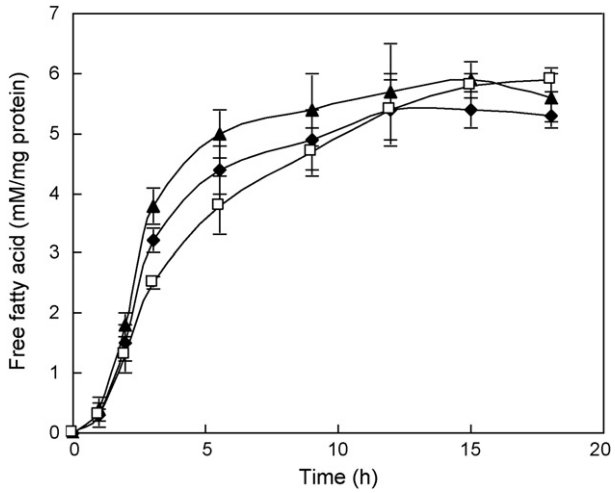

Fig. 1. Degradation of cutin by the cutinases. The enzymes were incubated with 1% (w/v) apple cutin in 25 mM potassium phosphate buffer (pH 8.0) and the amount of released fatty acids was measured by titration with 0.02N NaOH. (♦) Tfu_0883; (▲) Tfu_0882; (□) FspC. Error bars correspond to the standard deviation of three determinations.

Subsequently, the chain length specificity of esters was investigated using *p*-nitrophenyl-fatty acyl esters and triglycerides (Table 1). For all three enzymes, their activities against the C4 *p*-nitrophenyl-fatty acid ester *p*NPB were significantly higher than the corresponding C16 ester *p*NPP. Similarly, their activities against the C4 triglyceride tributyrin were significantly higher than the corresponding C18 ester triolein. Therefore, all three enzymes appeared to have a preference for a shorter carbon chain of the acyl moiety. It is notable that FspC exhibited highest specific activity toward all these four substrates. When comparing the *T. fusca* cutinases, both Tfu_0883 and Tfu_0882 showed similar activities toward triglyceride but differential activities toward *p*-nitrophenyl-fatty acid ester which was hydrolyzed at a significantly higher rate by Tfu_0883.

Recently, it has been reported that FspC can hydrolyze synthetic polyesters such as PET and improve the surface properties of PET fibers in an environmentally friendly way [6,26]. The ability of cutinases to hydrolyze cyclic PET trimers has also been evaluated [18]. As shown in Fig. 3, FspC exhibited the highest activity toward this polyester, while Tfu_0883 exhibited less activity. Surprisingly, Tfu_0882 was virtually inactive towards this polyester despite its high sequence similarity with Tfu_0883, suggesting that the minor sequence differences may be mainly located at the substrate binding site. Further studies, including crystallographic studies may shed light on this dramatic difference between the two *T. fusca* enzymes.

The above results revealed that the *T. fusca* cutinases have a broad substrate specificity against cutin and other polyesters, insoluble triglycerides, and soluble esters. They could therefore be described as intermediate enzymes between lipases and esterases as has been suggested for their fungal counterparts [12]. Such broad specificity is consistent with their open active sites previously predicted [13].

Table 1
Specificity of the cutinases towards the acyl chain length of different esters. Values are means ± SD ($n = 3$).

Substrate	Tfu_0882 cutinase	Tfu_0883 cutinase	FspC
*p*NPB	499 ± 10	1016 ± 15	2083 ± 23
*p*NPP	269 ± 5	383 ± 6	1041 ± 9
tributyrin	237 ± 11	217 ± 12	643 ± 15
triolein	137 ± 7	125 ± 8	321 ± 10

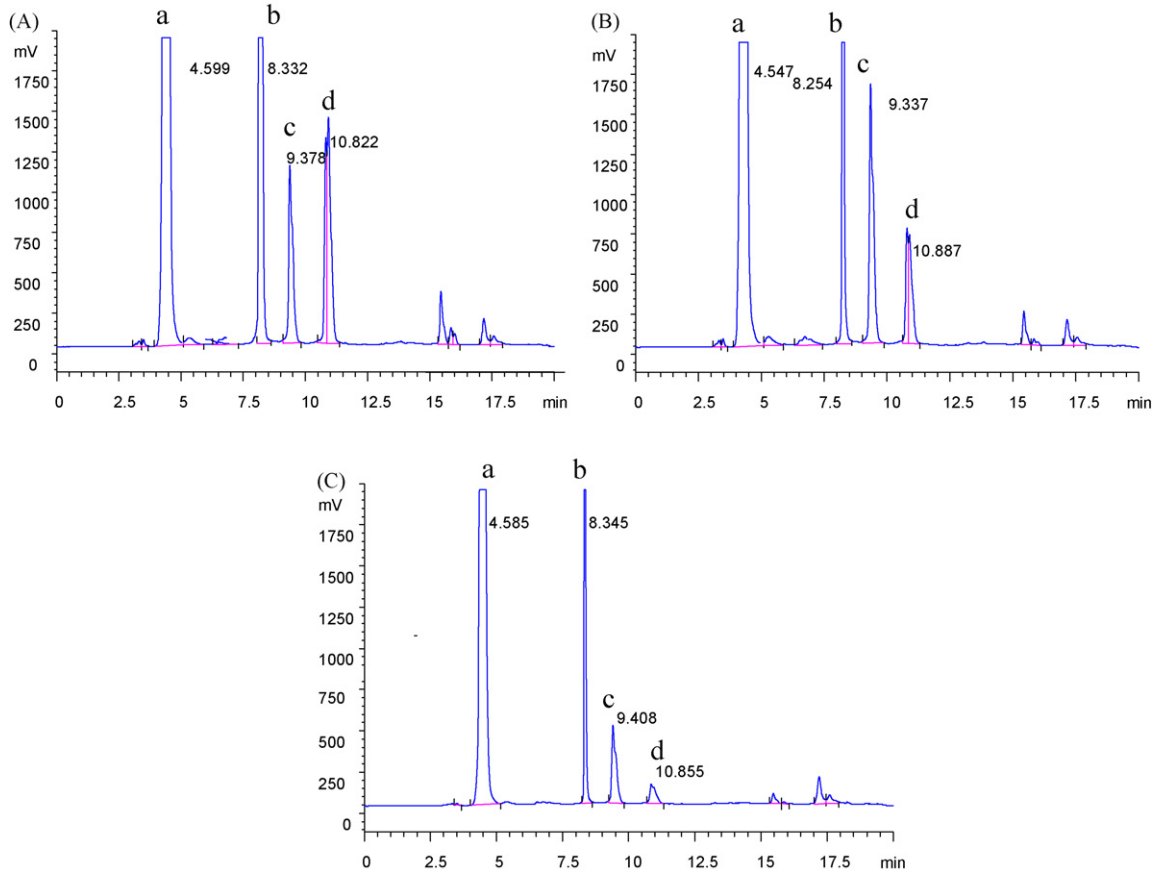

Fig. 2. HPLC chromatogram of triolein hydrolyzed by the cutinases. The enzymes were incubated with triolein emulsion in 25 mM potassium phosphate buffer (pH 8) for 15 min. The reaction mixture was extracted by isopropanol and n-hexane and analyzed by HPLC. (A) Tfu_0882; (B) Tfu_0883; (C) FspC. (a) Triolein; (b) oleic acid; (c) 1,3-diolein; (d) 1,2(2,3)-diolein. The molar ratio of c/d for A, B and C were 0.55, 0.68 and 1.33, respectively.

3.3. Kinetic analysis

The kinetic constants of Tfu_0882, Tfu_0883 and FspC were determined for the commonly used esterase substrate *p*NPB. The results showed that all three cutinases showed Michaelis–Menten kinetics with FspC exhibiting the highest affinity (lowest K_m value) for the substrate (Table 2). In addition, among the three enzyme, FspC exhibited the highest catalytic efficiency with a k_{cat}/K_m of 3.214 s^{-1} μM^{-1}. Interestingly, the catalytic efficiency (k_{cat}/K_m) of Tfu_0883 is twice as much as that of Tfu_0882 even though they showed a 93% identity in amino acid sequences and an almost identical structure of their active sites (data not shown).

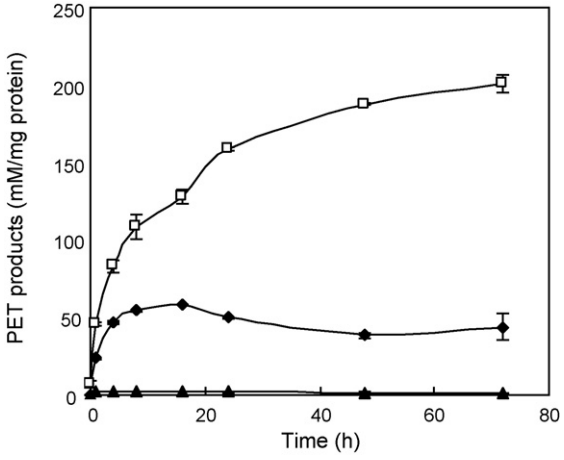

Fig. 3. Degradation of cyclic PET trimers by the cutinases. The enzymes were incubated with cyclic PET trimers in 10 ml 25 mM phosphate buffer (pH 8) and the hydrolysis products were analyzed by RP-HPLC and LC–MS. (♦) Tfu_0883; (▲) Tfu_0882; (□) FspC. Error bars correspond to the standard deviation of three determinations.

3.4. Evaluation of an interfacial activation of the cutinases

Interfacial activation has been observed with most lipases [27]. Tributyrin, a suitable substrate to test the interfacial activation phenomenon of lipases [28] was selected to evaluate the a possible interfacial activation of the cutinases. As shown in Fig. 4, the specific activity of the three cutinases as a function of tributyrin concentration followed normal Michaelis–Menten kinetics. The activity did not sharply increase when the solubility limit of tributyrin (12 mM) was reached. Therefore, Tfu_0882, Tfu_0883 and FspC did not possess interfacial activation phenomenon.

FspC has been previously reported not to exhibit an interfacial activation, which sets it apart from most of the true lipases. The

Table 2
Kinetic parameters of the cutinases. Kinetics parameters of Tfu_0882 and Tfu_0883 were determined at their optimal temperature of 60 °C and FspC at its optimal temperature of 40 °C. Values are means ± SD ($n = 3$).

Kinetic parameter	Tfu_0882 cutinase	Tfu_0883 cutinase	FspC
K_m^{pNPB} (μm)	673 ± 32	505 ± 29	272 ± 10
k_{cat} (s^{-1})	483 ± 19	742 ± 24	837 ± 25
k_{cat}/K_m (s^{-1} μM^{-1})	0.7	1.5	3.2

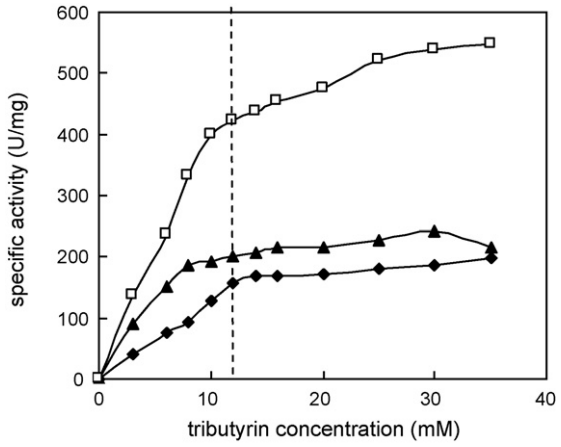

Fig. 4. Evaluation of interfacial activation of the cutinases. The enzymes were incubated with 3–35 mM of tributyrin emulsion at 37 °C for 15 min. The released fatty acids were quantified by titration with 0.05N NaOH. (♦) Tfu_0883; (▲) Tfu_0882; (□) FspC; vertical dot, the critical micelle concentration (CMC) of tributyrin (12 mM).

absence of a lid structure and an exposure of the nucleophilic serine in this enzyme has been described as the main reason for this behaviour [29]. In many true lipases, a lid structure burying the nucleophilic serine has been reported to be involved in interfacial activation. It undergoes a conformational change in response to an adsorption of the enzyme at the oil–water interface [30]. A lid insertion was also absent in the previously developed homology models of Tfu_0882 and Tfu_0883 [13], supporting the results obtained. The absence of interfacial activation in these cutinases further confirms that they are different from true lipases although all the enzymes belong to the α/β hydrolase fold superfamily.

3.5. Metal ion requirement

To determine whether the cutinases requires a metal cofactor for activity, they were incubated with the metal chelator EDTA or metal ions and then assayed for esterase activity against *p*NPB. EDTA (1 mM or 10 mM) did not affect their activities (Table 3), suggesting that the cutinases did not require divalent cations for their activity. When they were incubated with 1 mM of divalent metal ions, Mn, Co, Ni, Mg, Ba, Cu, or Ca did not exhibit a significant effect on the enzyme activity, whereas Zn, Fe, or Pb showed a medium inhibitory effect. Cr, however, inhibited most enzyme activity, whereas Hg completely inactivated the cutinases.

Table 3
Effect of metal ions and metal chelator on cutinase activity. The cutinase were preincubated with metal ions (1 mM) or metal chelator at 37 °C for 5 min, and then assayed for esterase activity against *p*NPB. Values are means ± SD ($n = 3$).

Metal ion or chelator	Tfu_0882 cutinase Relative activity (%)	Tfu_0883 cutinase Relative activity (%)	FspC Relative activity (%)
Control	100 ± 1	100 ± 2	100 ± 2
$MnCl_2$	109 ± 4	124 ± 5	136 ± 5
$CoCl_2$	104 ± 2	124 ± 4	107 ± 3
$NiCl_2$	105 ± 3	107 ± 2	93 ± 3
$BaCl_2$	93 ± 2	107 ± 2	101 ± 2
$CuSO_4$	72 ± 2	94 ± 3	83 ± 2
$CaCl_2$	72 ± 1	82 ± 3	94 ± 4
$MgCl_2$	66 ± 2	111 ± 5	89 ± 2
$ZnSO_4$	50 ± 1	44 ± 2	82 ± 3
$FeSO_4$	55 ± 3	64 ± 1	46 ± 2
$PbCl_2$	52 ± 2	45 ± 3	61 ± 3
$CrCl_2$	11 ± 1	10 ± 1	28 ± 1
$HgCl_2$	N.D.	N.D.	N.D.
EDTA (1 mM)	101 ± 2	101 ± 1	102 ± 3
EDTA (10 mM)	101 ± 2	102 ± 2	103 ± 2

N.D.: no detectable activity.

Table 4
Effect of surfactants on cutinase activity. Cutinase was preincubated with the surfactant at 37 °C for 5 min, and then assayed for esterase activity against *p*NPB. Values are means ± SD ($n = 3$).

Surfactant	Tfu_0882 cutinase Relative activity (%)	Tfu_0883 cutinase Relative activity (%)	FspC Relative activity (%)
Triton X-100			
1 mM	42 ± 3	72 ± 3	102 ± 4
10 mM	42 ± 1	77 ± 3	100 ± 4
Tween 20			
1 mM	34 ± 1	73 ± 4	91 ± 3
10 mM	33 ± 1	84 ± 4	94 ± 3
SDS			
1 mM	87 ± 4	69 ± 2	22 ± 1
10 mM	75 ± 4	43 ± 1	24 ± 1
TDOC			
1 mM	107 ± 3	97 ± 4	123 ± 4
10 mM	89 ± 2	111 ± 3	174 ± 5

3.6. Effect of surfactants on cutinase activity

Application of industrial enzymes often involves relatively harsh conditions such as the presence of surfactants and organic solvents. The activity of the cutinases was tested in the presence of the nonionic surfactants Triton X-100, Tween 20 and the anionic surfactants SDS and TDOC. At concentrations of 1 mM and 10 mM, Triton X-100 and Tween 20 inhibited both cutinases from *T. fusca* but did not significantly reduce FspC activity. SDS inhibited all three enzymes. TDOC, on the other hand, stimulated FspC activity with a 23.11% increase at 1 mM and a 73.65% increase at 10 mM. It did not have a significant effect on the *T. fusca* cutinase activity (Table 4).

The effects of TDOC on cutinase activity were further tested at concentrations up to 100 mM (Fig. 5). Tfu_0882 appeared to be fairly stable in the presence of this surfactant with 71.43% activity remaining at 100 mM. Tfu_0883 was slightly stimulated at TDOC concentrations between 1 mM and 50 mM and fully retained its activity at 100 mM. FspC was significantly stimulated by TDOC with the highest stimulatory effect at 10 mM and almost full activity remaining at 100 mM. TDOC is an anionic surfactant with a bulky side chain which may bind to the hydrophobic sites of proteins

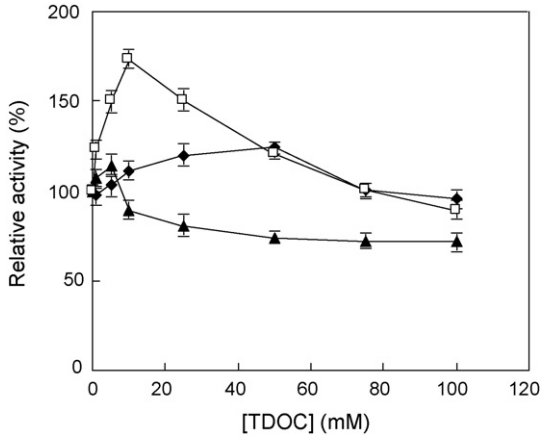

Fig. 5. Effect of TDOC on cutinase activity. The enzyme was incubated with TDOC at 37 °C for 5 min and then assayed for esterase activity against *p*NPB. (♦) Tfu_0883; (▲) Tfu_0882; (□) FspC. Error bars correspond to the standard deviation of three determinations.

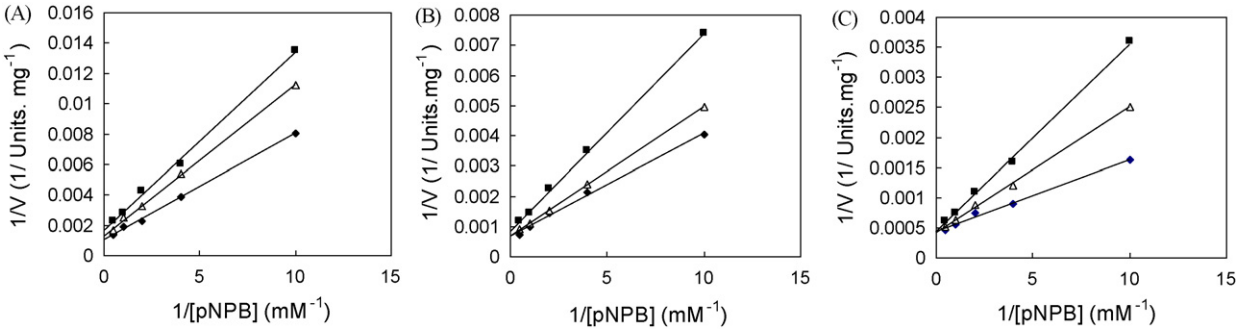

Fig. 6. Inhibition kinetics of the cutinase by SDS. (A) Tfu_0882; (B) Tfu_0883; (C) FspC. Double-reciprocal plots ($1/v$ vs. $1/[p\text{NPB}]$) of the initial rate data were determined at three concentrations of SDS. (■) SDS 1 mM; (△) SDS 0.5 mM; (♦) SDS 0 mM.

Table 5
Stability of the cutinase in organic solvents. The cutinase were incubated with 75% (v/v) of organic solvents in assay buffer at 20 °C for 18 h. Aliquots were removed for determination of residual activity. Values are means ± SD (n = 3).

Organic solvent (75%)	Tfu_0882 cutinase Relative activity (%)	Tfu_0883 cutinase Relative activity (%)	FspC Relative activity (%)
Control	100 ± 2	100 ± 2	100 ± 2
Methanol	87 ± 1	86 ± 3	6 ± 1
Ethanol	100 ± 4	98 ± 2	4 ± 1
Isopropanol	27 ± 3	65 ± 3	1 ± 1
Butanol	44 ± 5	20 ± 3	9 ± 1
Acetone	97 ± 3	99 ± 3	31 ± 2
n-Hexane	96 ± 4	84 ± 3	70 ± 4
Dimethyl sulfoxide	94 ± 4	92 ± 4	32 ± 1

preventing their aggregation and rendering them more stable [31]. FspC is less stable than Tfu_0882 and Tfu_0883 in solution, so that TDOC showed more effective in stimulating FspC.

In addition, inhibition kinetics was performed for SDS to evaluate its inhibitory efficiency on the three cutinases. The inhibition constants were determined by a double-reciprocal plot ($1/v$ vs. $1/[p\text{NPB}]$) of the initial reaction rate at varying concentrations (Fig. 6). SDS appeared to be a competitive inhibitor for all three enzymes with a K_i^{SDS} of 1385.6 μM for Tfu_0882, 944.4 μM for Tfu_0883 and 657.5 μM for FspC. Compared with the other two enzymes, Tfu_0882 appeared to be more resistant to SDS and may be more favorable for applications in detergent formulations.

3.7. Stability of the cutinase in organic solvents

Cutinases have been reported to show esterification and transesterification activity and have potential for use in the production of biodiesel [8,32]. Such applications often involve the use of organic solvents, therefore it is important to evaluate their stability in organic solvents. As shown in Table 5, Tfu_0882 and Tfu_0883 exhibited excellent tolerance to methanol, ethanol, acetone, n-hexane, and dimethyl sulfoxide, but were less stable in isopropanol and butanol. In contrast, FspC was very unstable in these solvents except in n-hexane in which nearly 70% activity remained. These results demonstrated that, compared with fungal cutinase, the bacterial cutinases from *T. fusca* are far more suitable for applications in organic solvents.

4. Conclusions

In summary, this is the first report on detailed biochemical characterization of bacterial cutinases from *T. fusca*. The results demonstrated that bacterial and fungal cutinases are similar in their substrate specificity, kinetics, lack of interfacial activation, and metal ion requirement. However, they differ significantly in their sensitivity to surfactants and dramatically in their sensitivity to organic solvents. Considering their versatile hydrolytic activity, good tolerance to surfactants, superior stability in organic solvents, and superior thermostability the *T. fusca* cutinases may have promising applications in related industries.

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