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BIOENERGY/BIOFUELS/BIOCHEMICALS

A low molecular mass cutinase of *Thielavia terrestris* efficiently hydrolyzes poly(esters)

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Abstract A low molecular mass cutinase (designated TtcutA) from *Thielavia terrestris* was purified and biochemically characterized. The thermophilic fungus *T. terrestris* CAU709 secreted a highly active cutinase (90.4 U ml^{-1}) in fermentation broth containing wheat bran as the carbon source. The cutinase was purified 19-fold with a recovery yield of 4.8 %. The molecular mass of the purified TtcutA was determined as 25.3 and 22.8 kDa using SDS-PAGE and gel filtration, respectively. TtcutA displayed optimal activity at pH 4.0 and 50 °C. It was highly stable up to 65 °C and in the broad pH range 2.5–10.5. Extreme stability in high concentrations (80 %, v/v) of solvents such as methanol, ethanol, acetone, acetonitrile, isopropanol, and dimethyl sulfoxide was observed for the enzyme. The K_m values for this enzyme towards *p*-nitrophenyl (*p*NP) acetate, *p*NP butyrate, and *p*NP caproate were 7.7, 1.0, and 0.52 mM, respectively. TtcutA was able to efficiently degrade various ester polymers, including cutin, polyethylene terephthalate (PET), polycaprolactone (PCL), and poly(butylene succinate) (PBS) at hydrolytic rates of 3 μmol h^{-1} mg^{-1} protein, 1.1 mg h^{-1} mg^{-1} protein, 203.6 mg h^{-1} mg^{-1} protein, and 56.4 mg h^{-1} mg^{-1} protein, respectively. Because of these unique biochemical properties, TtcutA of *T. terrestris* may be useful in various industrial applications in the future.

Keywords Cutinase · *Thielavia terrestris* · Characterization · Organic solvent · Ester polymer degradation

Abbreviations

MES	2-(*N*-morpholino)ethane sulfonic acid
PBS	Poly(butylene succinate)
PCL	Polycaprolactone
PET	Polyethylene terephthalate
*p*NP	*p*-Nitrophenol
*p*NPA	*p*-Nitrophenyl acetate
*p*NPB	*p*-Nitrophenyl butyrate
*p*NPC	*p*-Nitrophenyl caproate
*p*NPD	*p*-Nitrophenyl dodecanoate
*p*NPH	*p*-Nitrophenyl hexacaprate
*p*NPM	*p*-Nitrophenyl myristate
*p*NPP	*p*-Nitrophenyl palmitate
SDS-PAGE	Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
TtCutA	A low molecular mass cutinase of *Thielavia terrestris* CAU709

Introduction

Alternate environmentally friendly strategies for polymer synthesis and their degradation after use have been in great demand in recent years due to increasing public concern over environmental pollution and the rapid depletion of crude petroleum reserves for synthetic polymers [1, 2, 32]. The incineration of synthetic petroleum-based plastic wastes generates large amounts of carbon dioxide and

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dioxin [27]. To mitigate these problems, biodegradable plastics such as poly(butylene succinate) (PBS), polycaprolactone (PCL), polyethylene terephthalate (PET), and polyurethane have been developed [27, 29].

However, despite their various excellent properties, biodegradable plastics are yet to find widespread use. This is because the cost of producing biodegradable plastic industrially is higher than that of synthetic plastic. Furthermore, current disposal systems cannot recover the cost of producting biodegradable plastics, since most of these plastics are incinerated after use. Recycling processes for biodegradable plastics have not been developed, even though several studies on biodegradable plastic degradation have been carried out [27]. Enzyme-catalyzed degradation offers a unique solution to these challenges, as several enzymes have shown the ability to hydrolyze degradable plastics [1, 4, 27, 46]. Cutinase (EC 3.1.1.74) is a type of hydrolytic enzyme that is capable of catalyzing the cleavage of ester bonds in ester polymers and triglycerides [44]. Recently, cutinases have received much attention due to their potential applications in the pharmaceutical and food industries, in peptide synthesis, and in fine chemical production [1, 36, 46]. A cutinase from *Aspergillus oryzae* has been reported to efficiently hydrolyze the degradable plastic PBS [27]. Similarly, PBS was degraded by cutinases from *Alternaria brassicicola*, *A. fumigates*, *A. oryzae*, *Humicola insolens*, and *Fusarium solani* [1], while a cutinase derived from *Thermobifida fusca* KW3 displayed strong hydrolytic activity toward PET [4]. To date, reported cutinases have shown relatively narrow substrate specificities; each acts on only one or two plastic substrates.

Cutinases are present in a vast species of bacteria [5, 19, 46] and fungi [6, 36, 40]. The biochemical properties of cutinases from mesophilic fungi such as *Monilinia fructicola* [48], *Aspergillus oryzae* [27], *Trichoderma harzianum* [41], *Coprinopsis cinerea* [30], *Fusarium solani* [22], *Alternaria brassicicola* [20], and *F. oxysporum* [12] have been studied. The first report on a cutinase from a thermophilic fungus related to the cutinase from *Humicola insolens* [34]. *Thielavia terrestris* is a thermophilic fungus that grows well at or above 50 °C [38]. This fungus plays an important role in the global carbon cycle, as it secretes biomass-hydrolyzing enzymes with superior biochemical properties [13, 14, 21]. The genome sequence of *T. terrestris* has been elucidated, and the presence of putative esterases was reported [3]. However, there is no report in the literature describing the production and characterization of any cutinase from *Thielavia* sp. We recently isolated a strain of *T. terrestris*, CAU709, which produces cutinases in the presence of wheat bran as the sole carbon source. The present report describes our work to optimize the fermentation conditions for maximal cutinase production, to purify the enzyme, and to characterize its biochemical properties.

Materials and methods

Chemicals

p-Nitrophenol (*p*NP) monomer esters such as *p*-nitrophenyl acetate (*p*NPA), *p*-nitrophenyl butyrate (*p*NPB), *p*-nitrophenyl dodecanoate (*p*NPD), *p*-nitrophenyl hexacaprate (*p*NPH), *p*-nitrophenyl myristate (*p*NPM), *p*-nitrophenyl palmitate (*p*NPP), 4-methylumbelliferyl butyrate (MUF butyrate) and Fast Red TR Salt were purchased from Sigma Chemical Co. (St. Louis, MO, USA). *p*-Nitrophenyl caprylate (*p*NPC) was obtained from HEOWNS Co. (Tianjing, China). Chromatographic resins such as Sephacryl^{TM} S-100 High Resolution, Q Sepharose^{TM} Fast Flow, and CM Sepharose^{TM} Fast Flow were obtained from GE Healthcare (Piscataway, NJ, USA). Triacetin, tributyrin, tricaproin, tricaprylin, and tricaprin were from TCI Co. (Tokyo, Japan). All other chemicals used were of analytical grade unless otherwise stated.

Strain and enzyme production

Thielavia terrestris CAU709 was isolated from a soil sample collected at Beijing, China. It was deposited at the China General Microbiological Culture Collection Center with the accession number CGMSS 6233. The fungus was grown and maintained on potato dextrose agar (PDA) at 4 °C.

For cutinase production, *T. terrestris* CAU709 was grown on a PDA plate at 45 °C for two days. A portion of agar medium (1 cm^2) covered with mycelia was scooped up and transferred to the culture medium. The medium contained 20 g l^{-1} carbon source, 10 g l^{-1} tryptone, 1 g l^{-1} K_2HPO_4, and 0.5 g l^{-1} $MgSO_4$ in distilled water. The fungus was allowed to grow at 50 °C for six days on an orbital shaker. The supernatant of the culture broth was collected by centrifugation (10,000×*g*) and used as the crude enzyme.

The fermentation conditions for cutinase production were optimized by single-factor experiments. The effects of different carbon sources (glucose, sucrose, oleic acid, lauric acid, olive oil, wheat bran, corn stalk, straw, bagasse, and peanut hull) at 1–6 % (w/v), surfactants (Tween 20, 40, 60, 80, Triton X-100, and Triton X-114) at 0.5–2 % (w/v), and incubation times (1–6 days) on the cutinase production were investigated.

Cutinase assay and protein determination

The cutinase activity was measured according to the method of Sumby et al. [45] using *p*-nitrophenyl butyrate (*p*NPB) as

the substrate. Exactly 50 μL of suitably diluted enzyme in 400 μL of 50 mM citrate buffer (pH 4.0) were kept for 2 min at 50 °C for temperature equilibration, and then 50 μL of 20 mM *p*NPB (prepared in pure isopropanol) were added. The mixture was further incubated at 50 °C for 10 min and the reaction was stopped by the addition of 500 μL of chilled 300 mM phosphate buffer (pH 7.0). The absorbance was then measured at 410 nm. One unit of enzyme activity was defined as the amount of enzyme required to release 1 μmol of *p*NP per minute under the above assay conditions.

Protein concentration was measured according to the method of Lowry et al. [26] using bovine serum albumin as the standard. The specific activity of the enzyme was expressed in units per mg of protein.

SDS-PAGE, activity staining, and molecular mass determination

The purity of the enzyme was checked by SDS-PAGE. Electrophoresis was carried out using 4.5 % stacking gel and 12.5 % separating gel, as described by Laemmli [23]. Low molecular weight proteins from GE Co., including phosphorylase b (97.0 kDa), bovine serum albumin (66.0 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (30.0 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa), were used as ladder proteins. Cutinase activity staining was performed according to the methods of Prim et al. [37] and Karpushova et al. [18].

The native molecular mass of purified cutinase was determined by gel filtration using a Sephacryl S-100 column (1.0 cm × 100 cm) with a flow rate of 0.3 mL min^{-1} of 50 mM citrate buffer (pH 4.0). The standard proteins used for column calibration were cytochrome *c* (12.4 kDa), α-chymotrypsinogen A (type II from bovine pancreas, 25.6 kDa), albumin egg (45.0 kDa), bovine serum albumin fraction V (66.2 kDa), and phosphorylase b (from rabbit muscle, 97.2 kDa).

Purification of a low molecular mass cutinase (TtcutA) of *Thielavia terrestris*

The crude enzyme solution was subjected to 80 % ammonium sulfate precipitation. The precipitated proteins were collected and dialyzed against 20 mM acetate buffer (pH 4.5, buffer A) for 12 h. The dialyzed sample was then applied onto a CM Sepharose Fast Flow column (10 cm × 1.0 cm) which was pre-equilibrated with buffer A. The unbound protein fractions were collected and dialyzed against 20 mM Tris–HCl buffer (pH 8.5, buffer B) for 12 h. The dialyzed sample was loaded onto a Q Sepharose Fast Flow column (10 cm × 1.0 cm) which had previously been equilibrated with buffer B. The bound proteins were eluted with a linear gradient of 0–50 mM sodium chloride, followed by a constant elution of 50 mM sodium chloride. The active fractions were concentrated by ultrafiltration, and then loaded onto a Sephadex G-75 gel filtration column (100 cm × 1.0 cm) pre-equilibrated with buffer B containing 150 mM sodium chloride. Proteins were eluted at a flow rate of 0.2 mL min^{-1}. The fractions were analyzed for enzyme activity and purity by SDS-PAGE.

Protein identification by internal peptide sequences

The purified TtcutA was submitted to the National Center of Biomedical Analysis (China) for amino acid sequencing using high-performance liquid chromatography–electrospray ionization tandem mass spectrometry (HPLC–ESI–MS/MS). Mass spectral sequencing was performed using a Q-TOF II mass analyzer (Q-TOF2, Micromass Ltd., Manchester, UK). Peptide sequencing was performed using a palladium-coated borosilicate electrospray needle (Protana, Denmark). The mass spectrometer was used in positive ion mode with a source temperature of 80 °C, and a potential of 800 V was applied to the nanospray probe. MS/MS spectra were transformed using MaxEnt3 software (MassLynx, Micromass), and amino acid sequences were interpreted manually using PepSeq software (BioLynx, Micromass).

Effects of pH and temperature on TtcutA

The effect of pH and temperature on the enzyme activity was determined according to the method of Karpushova et al. [18]. To determine its pH stability, the enzyme activity was measured at 50 °C in 50 mM citrate buffer (pH 4.0). To determine its optimal temperature, the enzyme activity was determined in 50 mM citrate buffer (pH 4.0).

Effects of organic solvents and metal ions on TtcutA

The stability of TtcutA in organic solvents was evaluated according to the method of Hotta et al. [15]. The purified enzyme (40 μg mL^{-1}) was incubated with various organic solvents at 30 °C for 1 h in 50 mM citrate buffer (pH 4.0).

The effects of metal ions and other reagents on the enzyme activity was estimated by incubating the enzyme (40 μg mL^{-1}) in 50 mM citrate buffer (pH 4.0) at 30 °C for 1 h in the presence of 10 mM concentrations of various metal ions, or various detergents such as Tween 20, Tween 80, Triton X-100, and SDS at 5 % (w/v). Aliquots were taken, and the residual activities were measured.

Substrate specificity and the kinetic parameters of TtcutA

The substrate specificity was determined by incubating the cutinase with 2 mM of various *p*-nitrophenyl

esters—*p*NPA, *p*NPB, *p*NPC, *p*NP-caprylate, *p*NPH, *p*NPD, *p*NP-myristate, and *p*NPP—prepared in 50 mM citrate buffer (pH 4.0) containing 0.1 % (v/v) Triton X-100 and 0.1 % (w/v) arabic gum at 50 °C for 10 min. The *p*NPs released in the reaction mixture were quantified as described by Janssen et al. [16]. For olive oil and other synthetic triacylglycerol substrates, including triacetin, tributyrin, tricaproin, tricaprylin, and tricaprin, the enzyme activity was determined by analyzing the fatty acid released by titration using 10 mM NaOH after the mixture had been incubated in 50 mM citrate buffer (pH 4.0) containing 0.1 % (v/v) Triton X-100 and 0.1 % (w/v) arabic gum at 50 °C for 10 min, as described by Eggert et al. [10]. The substrate concentrations were all adjusted to 10 mM except for the olive oil, which was adjusted to 8.72 g l^{-1}. One unit of enzyme activity was defined as the amount of enzyme that required the release 1 µmol of fatty acid per minute under the above assay conditions.

The kinetic parameters of TtcutA towards *p*NPA, *p*NPB, and *p*NPC were determined by measuring the enzyme activities in 50 mM citrate buffer (pH 4.0) at 50 °C. The kinetic parameters K_m, V_{max}, and k_{cat} were calculated using the nonlinear regression analysis program Grafit [24].

Degradation of cutin and other ester polymers by the purified TtcutA

The cutins used were prepared from apple peels, as described by Walton and Kolattukudy [47]. The degradation of cutin was examined by incubating 40 µg of purified TtcutA with 1 mL cutin solution (1 %) dissolved in 25 mM citrate buffer (pH 4.0) at 50 °C for 18 h under gentle shaking conditions. The fatty acids released were quantified by titration with 10 mM NaOH, as mentioned previously. The activity was expressed as the amount of fatty acid (µmol) released per hour per milligram of protein.

The PBS, PCL, and PET-degrading activities of TtcutA were evaluated by measuring the weight losses of the ester polymers after incubating with TtcutA in 25 mM citrate buffer (pH 4.0) at 50 °C for 24 h with gentle shaking, according to the method of Sulaiman et al. [44]. The activity was expressed as the weight loss (mg) per hour per milligram of protein.

The activity of TtcutA towards the linalyl acetate was investigated by incubating 1 mg of purified TtcutA with 1 mL of 1.5 % linalyl acetate dissolved in 50 mM citrate buffer (pH 4.0) at 37 °C for 6 h [17]. The reaction was stopped by the addition of four volumes of ethanol. Samples withdrawn at different time intervals were centrifuged, and the supernatants were analyzed by TLC on Merck silica gel 60 plates with petroleum ether:ethyl acetate (7:1) as developing solution. The compounds separated on the TLC plate were visualized by dipping the plate in a solution of vanillin (5 g l^{-1}) in 30 % (v/v) H_2SO_4 and then heating it at 130 °C.

The ability of TtcutA to degrade polyurethane was tested in glass tubes. Ten micrograms of TtcutA were added to glass tubes containing 1 mL of emulsified polyurethane (0.6 %) and poly(diethylene glycol adipate) (polyDEGA) solution (0.4 %) in 50 mM citrate buffer (pH 4.0), and then incubated at 37 °C for 1 h. Samples withdrawn at different time intervals were observed and the turbidities were measured at 600 nm. The ability of TtcutA to hydrolyze olive oil was investigated according to the methods of Kang et al. [17] and Messias et al. [31] with slight modification. The substrate agar plate was prepared in 50 mM citrate buffer (pH 4.0), and different amounts of protein samples of TtcutA (0–20 µg) were added to the wells and incubated at 50 °C for 24 h. Hydrolysis of the olive oil results in a clear ring around the enzyme hole on the plate under UV light (365 nm).

Results

Cutinase production by *T. terrestris* CAU709

Thielavia terrestris CAU709 secreted cutinases in all tested culture media containing 2 % (w/v) different carbon sources. Higher enzyme activity was observed when wheat bran was used as the sole carbon source. A significant increase in cutinase activity (20 U mL^{-1}) was observed upon the addition of olive oil in wheat bran media (data not shown). Addition of 0.1 % (w/v) Tween 80 significantly increased (84.8 U mL^{-1}) the cutinase activity. The time course of cutinase production under the optimized fermentation conditions presented the highest enzyme activity (90.4 U mL^{-1}) after 96 h of cultivation and a decrease in activity later (Fig. 1). Zymographic analysis showed the presence of at least two cutinases in the fermentation broth with molecular masses of approximately 25 and 27 kDa (Fig. 2). The low molecular mass cutinase (TtcutA) was studied further in detail.

Purification and biochemical characterization of TtcutA from *T. terrestris*

The extracellular low molecular weight cutinase, namely TtcutA, was purified 19-fold with a recovery yield of 4.8 % from the culture supernatant of *T. terrestris* CAU709 (Table 1). The specific activity of the purified enzyme was 983 U mg^{-1} of protein. The molecular mass of TtcutA was found to be approximately 25.3 kDa (Fig. 2) by SDS-PAGE, while the gel filtration experiment gave a molecular mass of 22.8 kDa, suggesting the monomeric form of the enzyme.

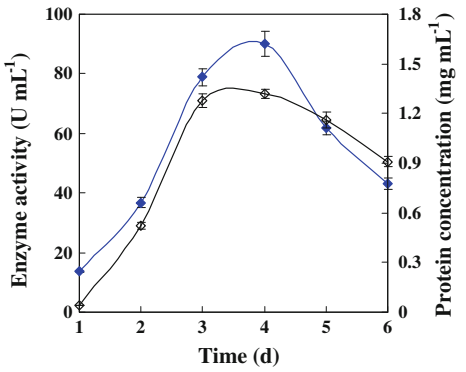

Fig. 1 Time course of cutinase production by *T. terrestris* under submerged cultivation conditions. *Filled diamonds* enzyme activity, *empty diamonds* protein concentration. Values shown are the means of three independent experiments, and the standard errors are indicated

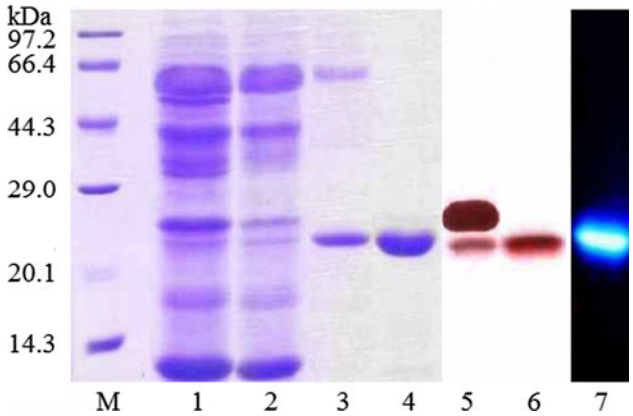

Fig. 2 SDS-PAGE and zymography of the purified TtcutA from *T. terrestris*. *Lane M* low molecular weight protein markers, *lane 1* ammonium sulfate precipitation, *lane 2* after the CM Sepharose Fast Flow column, *lane 3* after the Q Sepharose Fast Flow column, *lane 4* purified TtcutA after the Sephadex G-75, *lane 5* zymogram of crude TtcutA on SDS-PAGE indicated by α-naphthyl acetate, *lanes 6* and *7* zymograms of purified TtcutA on SDS-PAGE indicated by α-naphthyl acetate and MUF butyrate, respectively

The purified TtcutA exhibited maximal activity in the acidic pH range, since the highest enzyme activity was achieved at pH 4.0 in 50 mM citrate buffer (Fig. 3a). The enzyme was stable at 50 °C over a wide pH range (pH 2.5–10.5), as more than 90 % of the enzyme activity was retained after 30 min of incubation (Fig. 3b). The optimum temperature for maximal enzyme activity was 50 °C (Fig. 4a), and TtcutA was stable up to 65 °C for 30 min (Fig. 4b). The mass spectral analysis revealed that TtcutA shares sequence similarity with a putative esterase (XP003656017.1) from *T. terrestris* NRRL 8126.

Stability of TtcutA in the presence of organic solvents, detergents, and metal ions

Excellent stability of TtcutA was observed in the presence of the organic solvents tested (Table 2). The enzyme activity was not affected by methanol and ethanol, but a marginal increase (109 %) and decrease (93 %) in the activity was observed in the presence of isopropanol and acetonitrile, respectively, when the enzyme was present in 50 % (v/v) of the organic solvent. About a 1.4-fold increase in activity was noticed in 80 % DMSO solution. Activation of TtcutA in the presence of 5 % of Tween 20 (194 %), Tween 80 (120 %), and Triton X-100 (192 %) was also seen for the enzyme (data not shown). The effects of different metal ions on cutinase activity were studied. Metal ions like Ca^{2+}, Mn^{2+}, Fe^{3+}, Co^{2+}, Hg^{2+}, and Zn^{2+} inhibited the enzyme activity by 3–20 %.

Determination of substrate specificity and kinetic parameters

The specific activities of TtcutA towards different substrates are presented in Table 3. The enzyme catalyzed a wide range of the substrates tested. Short-chain *p*-nitrophenyl substrates and glycerol esters were found to be good substrates, and the highest specific activities of 1,200 and 416 U mg^{-1} were recorded for *p*NP butyrate (C_4) and

Table 1 Purification of a low molecular mass cutinase (TtcutA) from *T. terrestris*

Purification step	Total activity (U)[a]	Specific activity (U mg^{-1})	Purification factor (-fold)	Yield (%)[b]
Cell-free extract	23,900	62	1	100
$(NH_4)_2SO_4$ fractionation	18,900	118	1.9	79
CM Sepharose Fast Flow	15,400	143	2.3	64
Q Sepharose Fast Flow	3,000	800	13	12
Sephadex G-75	1,160	1,200	19	4.8

[a] The enzyme activity was determined at 50 °C in 50 mM citrate buffer (pH 4.0)

[b] The yield means the ratio of the residual enzyme activity to the initial total enzyme activity as a percentage

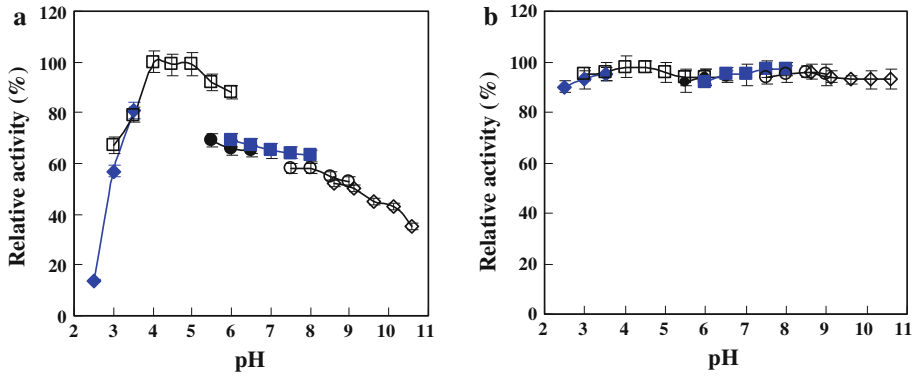

Fig. 3 Effects of pH on the activity (**a**) and stability (**b**) of the purified TtcutA from *T. terrestris*. The optimal pH of the enzyme was determined at 50 °C using *p*NPB as the substrate in 50 mM different buffers with pH values ranging from 2.5 to 10.5. For pH stability, the residual activities were measured at 50 °C in 50 mM citrate buffer (pH 4.0) after the enzyme was incubated at 50 °C at different pH values for 30 min. The buffers used were: *filled diamonds* glycine–HCl buffer (pH 2.5–3.5), *squares* citrate buffer (pH 3.0–6.0), *filled circles* MES buffer (pH 5.5–6.5), *filled squares* phosphate buffer (pH 6.0–8.0), and *empty circles* Tris–HCl buffer (pH 7.5–9.0)

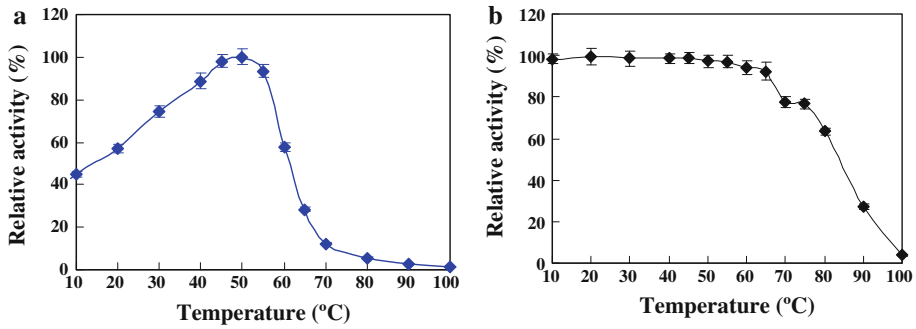

Fig. 4 Effects of temperature on the activity (**a**) and stability (**b**) of TtcutA from *T. terrestris*. The optimal temperature was determined by measuring the enzyme activity at different temperatures (10–100 °C) in 50 mM citrate buffer (pH 4.0). To determine the thermostability, the residual activity of TtcutA was measured each time after incubating the enzyme at various different temperatures (10–100 °C) for 30 min

Table 2 Stability of the purified TtcutA from *T. terrestris* in various organic solvents

Solvent	Relative activity (50 %, v/v) (%)	Relative activity (80 %, v/v) (%)
Control	100 ± 5	100 ± 2
Methanol	99 ± 4	80 ± 3
Ethanol	97 ± 3	92 ± 1
Isopronanol	109 ± 3	111 ± 2
Acetone	95 ± 3	92 ± 1
Acetonitrile	93 ± 4	100 ± 5
DMSO	100 ± 3	141 ± 3

Enzyme samples were mixed with different organic solvents and incubated at 30 °C for 1 h prior to determining the residual activity at 50 °C. Results are presented as the mean ± standard deviation ($n = 3$)

tributyrin (C_4), respectively. As the carbon chain length increased above 4, a drastic reduction in the enzyme activity was observed.

The kinetic parameters of the purified TtcutA are shown in Table 4. Among the three tested substrates, TtcutA exhibited the highest affinity for *p*NPC, followed by *p*NPB and *p*NPA. However, the highest catalytic efficiency of the enzyme was observed for *p*NPA, followed by *p*NPC and *p*NPB.

Hydrolytic properties of the purified TtcutA

TtcutA efficiently degraded apple peel cutin, PBS, PCL, and PET (Table 5), with the hydrolytic rates decreasing in the order PCL > PBS > apple peel cutin > PET. The degradation products of linalyl acetate were analyzed by thin-layer chromatography (TLC). Almost all of the linalyl acetate was converted to linalool (Fig. 5a) after 6 h of enzymatic reaction. The polyurethane-degrading ability of TtcutA was estimated in glass tubes containing 1 % emulsified polyDEGA. After a 1 h incubation period, the turbid emulsion changed to a clear solution (Fig. 5b). The OD_{600} of the turbid solution reduced from 2.193 to 0.001,

Table 3 Substrate specificity of the purified TtcutA from *T. terrestris*

Substrate	Specific activity (U mg^{-1})
pNP esters[a]	
*p*NP acetate (C_2)	169 ± 5
*p*NP butyrate (C_4)	1,200 ± 10
*p*NP caproate (C_6)	482 ± 5
*p*NP caprylate (C_8)	184 ± 4
*p*NP hexacaprate (C_{10})	92 ± 1
*p*NP dodecanoate (C_{12})	71 ± 0.6
*p*NP myristate (C_{14})	31 ± 0.1
*p*NP palmitate (C_{16})	4.0 ± 0.01
Triglycerides[b]	
Triacetin (C_2)	69 ± 3.2
Tributyrin (C_4)	416 ± 12
Tricaproin (C_6)	208 ± 5.6
Tricaprylin (C_8)	208 ± 7
Tricaprin (C_{10})	208 ± 8
Olive oil	122 ± 4

[a] Assays with *p*NP esters as the substrates were performed at 50 °C in 50 mM pH 4.0 citrate buffer containing 0.1 % (v/v) Triton X-100 and 0.1 % (w/v) arabic gum

[b] Activities obtained with olive oil and synthetic triglycerides as substrates were measured in 2.5 mM citrate buffer (pH 4.0) with 0.1 % (v/v) substrate and 0.1 % (w/v) arabic gum

indicating that the enzyme showed the highest hydrolytic efficiency towards polyurethane. A similar effect was also seen for olive oil, since a clear ring around the hole with the enzyme was observed under UV light (Fig. 5c).

Discussion

Recently, Berka et al. [3] reported the secretion of different kinds of enzymes that hydrolyze lignocellulosic substrates by *Thielavia terrestris*. However, the production, purification, and characterization of any cutinase from *T. terrestris* have not yet been reported. The cutinase produced by *T. terrestris* CAU709 was hyperinduced by wheat bran, in contrast to the cutinases produced by *Fusarium oxysporum* [36], *F. oxysporum* [12], and *Aspergillus nidulans* [6],

Table 4 Kinetic parameters for the purified TtcutA from *T. terrestris*

Substrate	V_{max} (μmol min^{-1} mg^{-1})	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})
pNPA	782	7.7	0.33	0.04
pNPB	1,464	1	0.62	0.62
pNPC	517	0.5	0.22	0.42

Enzyme activity was determined at 50 °C in 50 mM pH 4.0 citrate buffer; the enzyme concentration was about 0.2 mg mL^{-1}

Table 5 Degradation of ester polymers by TtcutA from *T. terrestris*

Substrate	Degradation rate (mg h^{-1} mg^{-1} protein)
Cutin	3.0
PBS	56.4
PET	1.11
PCL	203.6

The reaction was carried out at 50 °C for 24 h in 25 mM citrate buffer (pH 4.0). The weight loss after incubation was measured

which were maximally induced by flaxseed oil, soybean rind, and olive oil, respectively. Wheat bran not only serves as a source of carbon and energy but also provides the necessary inducing compounds for microorganisms. It forces different fungi to adapt to the given nutrients for growth [43]. *T. terrestris* can secrete multiple forms of cellulose-degrading enzymes, which aid the utilization of the substrate by microbial cells [13]. Thus, wheat bran induced a relative high level of cutinase production for *T. terrestris* CAU709. In particular, about a fourfold increase in cutinase activity was observed upon the addition of Tween 80. The enhanced enzyme production prompted by the addition of Tween 80 to the fermentation broth may be due to prevention of cell clumping, or enhanced availability of the carbon source, or increased cell growth [28, 35].

Production of cutinases by some fungi—*Fusarium oxysporum* (22.7 U mL^{-1}) [36], *F. oxysporum* (15.5 U mL^{-1}) [12], *F. solani* f. sp. *pisi* (21 U mL^{-1}) [42], and *Aspergillus nidulans* (26.3 U mL^{-1}) [5]—have been reported. Recently, Chen and co-workers reported a thermotolerant cutinase from *Thermobifida fusca* with enzyme activity of 149.2 U mL^{-1} [7]. In this study, *T. terrestris* CAU709 secreted a high level of cutinase (90 U mL^{-1}) when the cheap raw material wheat bran was used as the substrate, and exhibited the potential for industrial production of cutinase. On the SDS-PAGE zymogram of the crude enzyme, two protein bands with molecular masses between 20 and 30 kDa showed cutinase activity. Similarly, the secretion of three cutinases (22, 29, and 37 kDa) by *Aspergillus nidulans* has been reported [6]. The molecular mass of the purified TtcutA was approximately 25.3 kDa in SDS-PAGE (Fig. 2), which is similar to those of many reported microbial cutinases [6, 11, 30, 41]. Its molecular mass is slightly higher than that of the cutinase (20.2 kDa) from *Monilinia fructicola* [48] and lower than those of the cutinases from *Colletotrichum kahawae* (40 kDa, [9]) and *Thermobifida fusca* KW3 (50 kDa, [4]).

The optimal pH of purified TtcutA is obviously lower than those of all other reported cutinases, ranging from pH 6.0–10.0 [4, 8, 11, 20, 22], indicating that TtcutA is an acidic cutinase. Most reported cutinases show pH stability

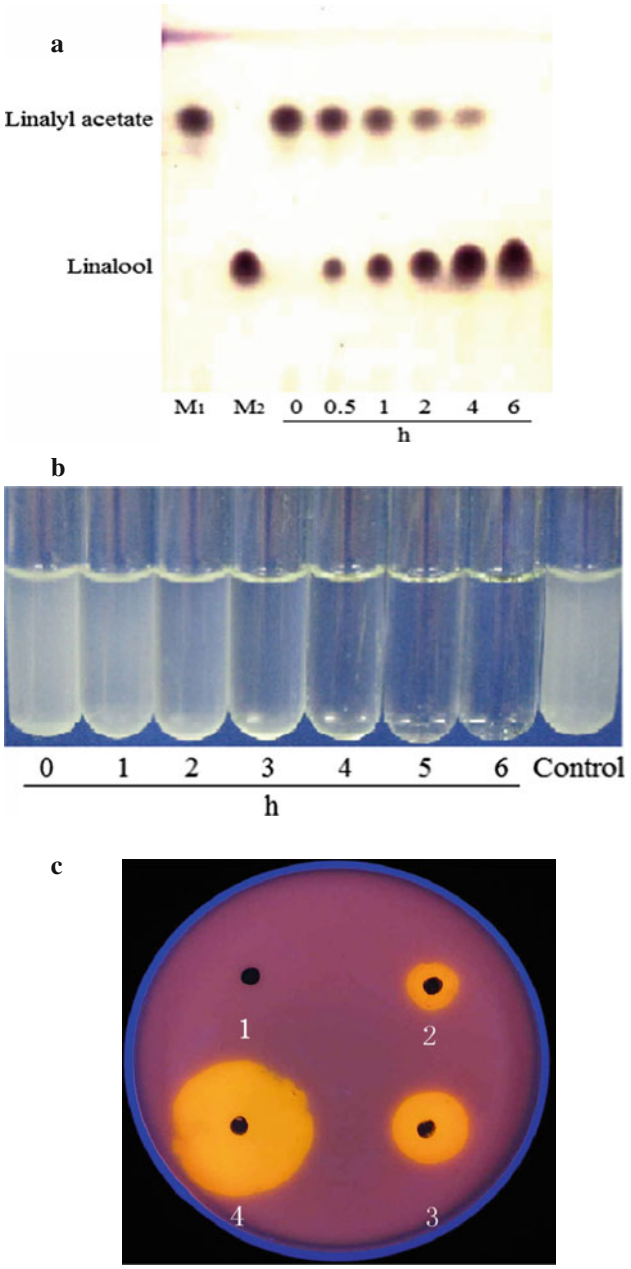

Fig. 5 Degradation of linalyl acetate (**a**), polyurethane (**b**), and olive oil (**c**) by the purified TtcutA from *T. terrestris*. For linalyl acetate, the reaction was carried out at 37 °C in 50 mM citrate buffer (pH 4.0), and the hydrolytic products were analyzed by TLC; *lane* M_1 linalyl acetate, *lane* M_2 linalool. Incubation times (h) are indicated. For polyurethane, the reaction was performed at 37 °C in 50 mM citrate buffer (pH 4.0), and samples withdrawn at different time intervals were observed. *Control* means the sample treated for 1 h without the addition of TtcutA. The hydrolysis of olive oil was investigated on an agar plate: different amounts of TtcutA were added in the holes on the plate and incubated with substrate at 50 °C for 4 h, before the plate was observed under UV light with a wavelength of 365 nm. The enzyme dosages in holes 1, 2, 3, and 4 were 0, 5, 10, and 20 μg, respectively

in near-neutral or alkaline conditions [11, 20, 22], while TtcutA displayed excellent pH stability in the pH range of 2.5–10.5 (Fig. 3b). The optimal temperature for TtcutA is 50 °C (Fig. 4a), which is similar to those for most of the cutinases from *Alternaria brassicicola* [20], *Fusarium solani* [22], and *Thermobifida fusca* [4, 8]. The fact that TtcutA shows activity across a broad pH range and temperature range is noteworthy from the perspective of industrial applications (Fig. 4a). The present cutinase showed excellent thermostability, with a thermal denaturing half-life of 294 min at 55 °C (data not shown). Thermostability is an important property for enzymes; it is absolutely essential when they are used in some special industrial processes employing high temperatures. In addition, enzymes with high thermostability have many advantages over their mesophilic counterparts [11].

The specific activities of TtcutA towards different substrates were studied. The enzyme displayed a broad range of substrate specificity (Table 3). It efficiently hydrolyzed *p*NP ester derivatives in addition to triglycerides. Among all of the tested substrates, the enzyme preferred *p*NP butyrate (C_4); this substrate yielded an activity of 1,200 U mg^{-1}, which is coincident with those for other reported cutinases [20, 41, 44]. The specific activity increased with the carbon chain length up to 4 but later decreased (Table 3). TtcutA also exhibited high activities towards various triglycerides, with the highest activity of 416 U mg^{-1} observed when tributyrin (C_4) was used as the substrate. This is similar to the cutinase from *Alternaria brassicicola* [20]. However, the specific activities of TtcutA towards various triglycerides with carbon chain lengths ranging from six to ten were almost the same (208 U mg^{-1}), which is very different to the behavior seen for other reported cutinases [17, 20]. The kinetic parameters of TtcutA towards *p*NPA, *p*NPB, and *p*NPC were also determined (Table 4). The results indicated that its affinity for the substrate increases as the carbon chain length of the carboxylic ester in the substrate increases. The stability of cutinases in organic solvents is very important, as several reactions of cutinases (such as esterification and transesterification) are carried out in the presence of organic solvents [7]. Excellent stability of TtcutA was seen in all tested organic solvents (Table 2). A similar characteristic has been reported for a cutinase from *Thermobifida fusca* [7]. However, such organic solvent stability was not seen for a cutinase from *Aspergillus nidulans* [6]. It is worth mentioning here that the enzyme activity was enhanced by up to 41 % in the presence of 80 % DMSO. This unique property may enable the enzyme to be used in a processing environment containing organic solvents.

In order to test its ability to degrade cutin, TtcutA was used to hydrolyze apple peel cutin. The results confirmed the nature of the enzyme as a cutinase, as it hydrolyzed cutin at a rate of 3 μmol h^{-1} mg^{-1} of protein (Table 5). This degradation rate is a little lower than those of several cutinases (6 μmol h^{-1} mg^{-1} [44]; and *T. fusca*, 4 μmol h^{-1} mg^{-1} [8]). Biodegradable plastics such as PBS, PCL, and PET are "green" materials, and degrade in the natural environment without polluting it. The degradation of such plastics by cutinases is more effective and environmentally friendly in recycling process [39]. TtcutA degraded PBS, PCL, and PET. It is reported that the cutinase from *A. oryzae* exhibited PBS-degrading ability [27]. Similar to the cutinases from *A. oryzae* [25], *Alternaria brassicicola* [1], and *F. solani* [1], the present enzyme could also efficiently hydrolyze the degradable plastic PCL. The rate of hydrolysis of PET film by TtcutA is much higher (1.1 mg h^{-1} mg^{-1}) than that of a cutinase from *Thermobifida fusca* (0.05 mg h^{-1} mg^{-1} protein, [33]), but lower than that of a recombinant cutinase (12 mg h^{-1} mg^{-1}, [44]). The addition of TtcutA to an emulsion containing polyDEGA (one of the components of polyester polyurethane) clarified the turbid solution (Fig. 5b), suggesting that TtcutA possesses the ability to hydrolyze polyester polyurethane. A cutinase from a compost metagenomic library exhibited a similar ability to degrade polyurethane and linalyl acetate [17]. TtcutA could also hydrolyze esters of a tertiary alcohol; it completely converted linalyl acetate to linalool in 6 h (Fig. 5a). Tertiary alcohols are useful building blocks in chemistry and are regarded as important natural products in pharmaceuticals [17]. It is interesting that TtcutA also efficiently hydrolyzes olive oil with a specific activity of 122 U mg^{-1}, and hydrolysis was exhibited in a dose-dependent manner (Fig. 5c). However, most other reported cutinases show no such catalytic activity [7, 41, 44, 46]. TtcutA may therefore be of potential use in the recycling of biodegradable plastics due to its broad range of substrate specificity.

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