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ORIGINAL PAPER

A thermoalkaliphilic lipase of *Geobacillus* sp. T1

Thean Chor Leow · Raja Noor Zaliha Raja Abd Rahman · Mahiran Basri · Abu Bakar Salleh

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Abstract A thermoalkaliphilic T1 lipase gene of *Geobacillus* sp. strain T1 was overexpressed in pGEX vector in the prokaryotic system. Removal of the signal peptide improved protein solubility and promoted the binding of GST moiety to the glutathione-Sepharose column. High-yield purification of T1 lipase was achieved through two-step affinity chromatography with a final specific activity and yield of 958.2 U/mg and 51.5%, respectively. The molecular mass of T1 lipase was determined to be approximately 43 kDa by gel filtration chromatography. T1 lipase had an optimum temperature and pH of 70°C and pH 9, respectively. It was stable up to 65°C with a half-life of 5 h 15 min at pH 9. It was stable in the presence of 1 mM metal ions Na^+, Ca^{2+}, Mn^{2+}, K^+ and Mg^{2+} , but inhibited by Cu^{2+}, Fe^{3+} and Zn^{2+}. Tween 80 significantly enhanced T1 lipase activity. T1 lipase was active towards medium to long chain triacylglycerols (C10–C14) and various natural oils with a marked preference for trilaurin (C12) (triacylglycerol) and sunflower oil (natural oil). Serine and aspartate residues were involved in catalysis, as its activity was strongly inhibited by 5 mM PMSF and 1 mM Pepstatin. The T_m for T1 lipase was around 72.2°C, as revealed by denatured protein analysis of CD spectra.

Keywords *Geobacillus* sp. · Thermoalkaliphilic · Overexpression · Purification · Thermostable lipase

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T. C. Leow · R. N. Z. R. A. Rahman (✉) · M. Basri · A. B. Salleh
Enzyme and Microbial Technology Research Group, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia
e-mail: rnzaliha@biotech.upm.edu.my

Introduction

Lipases or acylglycerol hydrolases (E.C. 3.1.1.3) are enzymes that catalyze the hydrolysis of long chain triglycerides with the formation of diacylglyceride, monoglyceride, glycerol and free fatty acids at the interface between the insoluble substrate and water through interfacial activation (Brockman et al. 1988; Verger 1997). Apart from their natural substrates, lipases catalyze the enantio- and regio-selective-hydrolysis and synthesis of a broad range of natural and non-natural esters (Schmidt-Dannert 1999).

Most biocatalysts are inherently labile; therefore, their operational stability is of paramount importance for any bioprocess (Lllanes 1999). Enzymes derived from thermophilic bacteria are more resistant to denaturation than their mesophilic counterparts (Coolbear et al. 1992); thermostable lipases of thermophiles with their inherent stability therefore received much more attention for their potential application in the detergent, oil and fat, dairy and pharmaceutical industries (Gao et al. 2000). Furthermore, running bioprocesses at elevated temperatures lead to higher diffusion rate, increased solubility of lipids and other hydrophobic substrates in water and reduced risk of contamination (Becker et al. 1997).

Although thermophilic bacteria could be a good candidate for thermostable lipase producers, only a minute amount of enzyme can be obtained and may not be suitable for industrial application. Moreover, cloning such thermophilic genes into more suitable mesophilic hosts is now at hand to produce stable proteins (Lllanes 1999). Therefore, the prokaryotic system remains the most widely used approach towards overexpression for fundamental studies and commercial purposes. However, the successful production of heterologous protein requires the thoughtful integration of information from bacterial genetics, physi-

ology, nucleic acid and protein chemistry and biochemical engineering (Georgiou 1996). The fusion of a protein of interest to a large-affinity tag, such as maltose-binding protein (MBP), thioredoxin (TRX) or glutathione-*S*-transferase (GST), can be advantageous in terms of increased expression, enhanced solubility, protection from proteolysis, improved folding and purification via affinity chromatography, but with a minimal effect on enzyme tertiary structure and biological activity (Smyth et al. 2003).

A *Geobacillus* sp. strain T1 was shown to have optimum growth condition at a temperature of 65°C and pH 6.5 in nutrient broth. This made the lipase isolated from such strain a good candidate for a thermostable enzyme; however, the production of lipase was low. The production was markedly increased by expressing T1 lipase (with signal peptide) as fusion protein in prokaryotic systems (Leow et al. 2004). However, the expressed fusion protein was not able to bind to the immobilized ligands of support matrix for purification. Here, we report on the manipulation of gene expression (without signal peptide) for ease of T1 lipase purification and the study on its biochemical and structural characteristics for potential use in the industrial applications. This enzyme might represent the most thermostable recombinant lipase from the same group of lipases (family I.5) so far reported.

Materials and methods

Bacterial strains and plasmids

Recombinant plasmid (pGEX/T1) (Leow et al. 2004) carrying open reading frame of thermostable T1 lipase gene (GenBank accession no. AY260764) from *Geobacillus* sp. strain T1 [deposited in DSMZ (DSM 18318) and NITE (NBRC 101842)] was used as a source for subcloning. *Escherichia coli* TOP10 [F′ *mcr*A, Δ(*mrr-hsd*RMS-*mcr*BC),Φ80*lac*ZΔM15, Δ*lac*X74, *deo*R, *rec*A1, *ara*D139, Δ(*ara-leu*)7697, *gal*U, *gal*K, *rps*L(StrR), *end*A1, *nup*G] and BL21(De3)plysS [F- *ompT hsdSB(rB-mB-) gal dcm* (DE3) pLysS(CmR)] were grown in LB medium at 37°C. Vector pGEX-4T1 (Amersham Bioscience, England, UK) was used for subcloning and expression.

DNA manipulation

The plasmid DNA was isolated with a QIAGEN miniprep spin kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The PCR product was purified with a GeneClean Kit (Qbiogene, Carlsbad, USA), as described by the supplier. *Escherichia coli* competent cells were prepared using a conventional $CaCl_2$ method (Sambrook et al. 1989).

Expression of the thermostable T1 lipase gene

The T1 lipase (without signal peptide) gene was amplified from recombinant plasmid pGEX/T1 (with signal peptide) (Leow et al. 2004) by designing a set of primers with incorporated restriction enzyme sites *Bam*H1/*Eco*R1. The primers EH2-F: 5′-GAC GGG ATC CGC ATC CCT ACG CGC CAA TGA T-3′ and pGEX-Rev: 5′-AAT AGA ATT CTT AAG GCT GCA AGC TCG CCA A-3′ were used to remove signal peptide. The amplified gene and plasmid pGEX-4T1 were digested with the same restriction enzymes, *Bam*H1 and *Eco*R1. The ligated plasmid was used to transform *E. coli* TOP10 and screened with tributyrin LB agar plates with appropriate antibiotics. The extracted recombinant plasmid pGEX/T1S (without signal peptide) was used to transform BL21 strain. *Escherichia coli* BL21(De3)pLysS harboring recombinant plasmids were grown in 1 l blue cap bottles containing 200 ml of LB medium supplemented with 100 μg/ml ampicillin and 35 μg/ml chloramphenicol on a rotary shaker (200 rpm) at 37°C. The recombinant clones were induced with 1 mM of isopropyl-β-D-thiogalactopyranoside (IPTG) at A_{600nm} ~ 0.5 for different induction periods (0, 4, 8, 12, 20, 28, 36 and 44 h). It was further optimized by varying the concentration of IPTG (w/o, 0.025, 0.05, 0.1, 0.5, 1.0, 1.5 and 2.0 mM) and induction at A_{600nm} (0.25, 0.50, 0.75 and 1.00). The cultures (10 ml) were harvested by centrifugation and resuspended with an equal volume of 50 mM of potassium phosphate buffer (pH 7.0) prior to sonication (Branson 250 sonifier; output 2, duty cycle 30 and min 2) and cleared by centrifugation (12,000 rpm, 20 min). The clear crude lysate was used for lipase assay.

Assay of lipase activity

The lipase activity was assayed colorimetrically as described by Kwon and Rhee (1986). The samples were assayed at 70°C under shaking conditions (200 rpm) for 30 min. Lipase activity was determined by measuring the amount of free fatty acid released based on the standard curve of free fatty acids. One unit of lipase activity was defined as 1 μmole of fatty acid released per minute under the assay condition. Assays were carried out in triplicate and results are the mean of the obtained experimental reading.

Electrophoresis

SDS-PAGE was carried out on 12% running gel by using the method of Laemmli (1970). A broad range of protein

standard (MBI Fermentas, St Leon-Rot, Germany) was used as a molecular mass marker.

Purification of thermostable T1 lipase

A volume of 400 ml of recombinant culture was harvested by centrifugation and resuspended with 40 ml of PBS (pH 7.4) containing 5 mM of DTT prior to sonication. The sonicated cell lysate was cleared through centrifugation at 12,000*g* for 30 min. Glutathione-Sepharose HP (10 ml) was packed into XK 16/20 column. The glutathione-Sepharose HP column was equilibrated with ten-column volume (CV) of PBS (pH 7.4). The cleared cell lysate was then loaded on a glutathione-Sepharose HP column at a flow rate of 0.25 ml/min. The column was washed with the same buffer until no protein was detected. The bound lipase was eluted with thrombin cleavage buffer (50 mM Tris–HCl, 100 mM NaCl and 0.33 mM $CaCl_2$), pH 8.0 supplemented with 10 mM reduced glutathione. The fusion protein was subjected to thrombin cleavage at 16°C for 20 h and buffer exchanged using Sephadex G-25 at a flow rate of 1 ml/min. The GST tag and thrombin were further removed by using Glutahione-Sepharose HP, HiTrap Glutathione-Sepharose 4FF and HiTrap Benzamidine in series after dialyzing the digested mixture with 2 l of PBS (pH 7.4) at 4°C.

Characterization of T1 lipase

The effect of temperature on T1 lipase was measured at temperatures ranging from 40 to 80°C at 5°C intervals for 30 min. Enzyme stability test was conducted by pre-incubating T1 lipase at various temperatures ranging from 60 to 70°C at 5°C intervals up to 14 h prior to lipase assay. The effect of pH on T1 lipase activity was measured at various pHs (pH 4–12) under agitation rate of 200 rpm for 30 min. The buffer system involved various 50 mM acetate buffer (pH 4–6), potassium phosphate buffer (pH 6–8), Tris–HCl buffer (pH 8–9), glycine–NaOH buffer (pH 9–11) and Na_2HPO_3/NaOH buffer (pH 11–12). pH stability test was performed by pre-incubating the T1 lipase at various pHs (1:1) for 30 min, at 200 rpm. T1 lipase was tested with various metal ions (Na^+, Mg^{2+}, Ca^{2+}, Fe^{2+}, Mn^{2+}, K^+, Zn^{2+}, Cu^{2+}) and surfactants (Tween 20, Tween 40, Tween 60, Tween 80, Triton X-100, SLS, SDS) at a concentration of 1 mM at 50°C for 30 min. Triglycerides (triacetin, tributyrin, tricaproin, tricaprylin, tricaprin, trilaurin, trimyristin, tripalmitin, tristearin and triolein) and natural oils (olive oil, soybean oil, corn oil, sunflower oil, rice brain oil, coconut and palm oil) were also tested for T1 lipase activity. Various inhibitors were tested at a final concentration of 5 mM (PMSF, DTT, β-mercaptoethanol, EDTA) and 1 mM (pepstatin) at 50°C for 30 min. The samples were subjected to lipase assay as described earlier.

CD spectral analysis of T1 lipase

The circular dichroism (CD) spectra analysis of T1 lipase was conducted using a J-810 spectropolarimeter (JASCO, Tokyo, Japan). The thermal dentaturation was monitored in duplicate by following the ellipticity at 220 nm at a 1°C/min heating rate from 55 to 85°C. The concentration of T1 lipase was 1 mg/ml and the top of the 10 mm cell was completely closed using a cap. Data pitch, bandwidth, response and scanning speed were set to be 0.1°C, 1 nm, 8 s, and 1°C per min, respectively. The fitting lines were extrapolated from the stable region to the range of denaturation (61.20–83.80°C) to obtain the T_m value. The measurement of CD spectra from 195 to 250 nm was performed at each temperature (60–75°C at 5°C interval) using the same cell. The concentration was set at 0.33 mg/ml. The top of the cell was completely closed using a cap to minimize evaporation. Data pitch, bandwidth, response, scanning speed and accumulation were set to be 0.1 nm, 1 nm, 8 s, 50 nm per min, and four times, respectively.

Results

Expression of the T1 lipase

Thermophiles including *Geobacillus* sp. strain T1 are generally low in their lipase production. Molecular cloning offers an alternative route of high-level expression economically in a shorter time. However, expression of the T1 lipase gene (with signal peptide) under the control of strong *tac* promoter (Leow et al. 2004) interfered with the binding of GST fusion lipase to the support matrix of affinity glutathione-Sepharose column hindering the purification process. Therefore, expression of T1 lipase gene was carried out by omitting the signal peptide to allow rigid fusion of GST tag to T1 lipase, thereby exposing the GST binding domain to facilitate protein purification. The strategy was that the restriction enzyme sites, *Bam*H1 and *Eco*R1, were incorporated into the primers, EH2-For and pGEX-Rev, for subcloning of T1 lipase gene (without signal peptide) to the pGEX system. Parameters such as induction time, inducer concentration and induction at A_{600nm} were taken into consideration during the optimization of T1 lipase expression. Lipase expression levels of 23,915 U/l (induction time), 31,609 U/l (inducer concentration) and 41,902 U/l (A_{600nm} induction) were obtained when the recombinant cultures were induced for 12 h with 0.025 mM of IPTG at $A_{600nm} \sim 0.75$. Removal of signal peptide

increased the solubility of protein, as indicated qualitatively (SDS-PAGE) and quantitatively (lipase assay). The expression level was around 3.8 times higher than T1 fusion lipase (with signal peptide) [11,041 U/l] expressed under the same condition. The evidence for improper folded T1 fusion lipase (with signal peptide) is demonstrated by lesser expression level of T1 fusion lipase (with signal peptide) compared to T1 fusion lipase (without signal peptide), which was seen in soluble fractions of Fig. 1. However, the expression obtained under this condition was about 279-folds higher as compared to thermostable lipase of wild-type *Geobacillus* sp. strain T1 (150 U/l). In fact, the T1 lipase gene of wild-type *Geobacillus* sp. strain T1 might be regulated by weaker promoter regions as compared to its strongly regulated *tac* promoter in the recombinant plasmid. Low copy numbers might be an additional cause of the low production level.

Purification of T1 lipase

The absence of signal peptide rigidly fused the T1 lipase to the GST tag, since the absence of 28 amino acid residues limited the movement of T1 lipase in covering the GST moiety. Therefore, high-binding affinity to immobilized glutathione on the support matrix was observed with the removal of the signal peptide. Table 1 shows that the recovery for fusion lipase was around 71.3% with a purification fold of 2.3. Purification of GST fusion lipase increased the specific activity from 208.6 (crude cell lysate) to 489.5 U/mg (purified fusion lipase). The fusion lipase

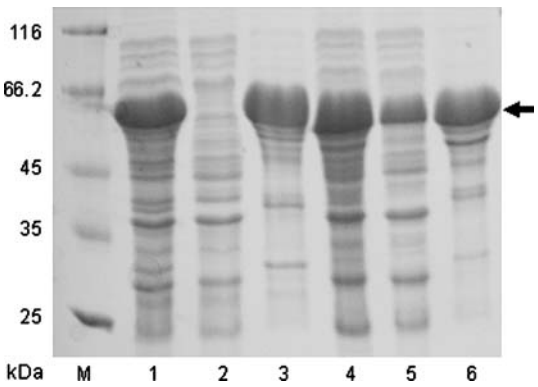

Fig. 1 Expression of T1 fusion lipase with and without signal peptide. Equivalent fractional volumes were visualized by SDS-PAGE (12%). *Lane 1* molecular weight marker; *lane 1–3* recombinant cultures harboring pGEX/T1 (with signal peptide); *lane 4–6* recombinant cultures harboring pGEX/T1S (without signal peptide). Whole cell extract after induction (*lane 1*, *4*); soluble protein (*lane 2*, *5*); pellet (*lane 3*, *6*). Standard protein markers were β-galactosidase (116 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), lactate dehydrogenase (35 kDa), restriction endonuclease Bsp 981 (25 kDa) and β-lactoglobulin (18.4 kDa). *Arrow* indicates fusion T1 lipase

was eluted out with thrombin cleavage buffer, pH 8.0 supplemented with 10 mM of reduced glutathione, instead of performing on-column cleavage, because the concentrated fusion lipase easily aggregated at pH close to the pI of the fusion T1 lipase.

Meanwhile, T1 lipase was obtained through subsequent purification steps. The fusion lipase was cleaved with thrombin protease at 16°C for 20 h. It was buffer exchanged using Sephadex G-25 column into PBS (pH 7.4), while removing the free glutathione. Thrombin cleavage buffer at high pH not only prevented the formation of aggregation of highly concentrated fusion lipase, but also reduced the formation of precipitate upon incubation with thrombin protease. The cleaved fusion protein was loaded onto affinity chromatography glutathione-Sepharose HP, HiTrap glutathione-Sepharose 4FF and Benzamidine FF (high sub) in series to remove the GST tag and thrombin after being dialyzed against PBS (pH 7.4) overnight. A specific activity of 958.2 U/mg was obtained after thrombin cleavage and removal of GST tag to yield a mature lipase. This corresponded to a purification fold of 4.6 and final recovery of 51.5%.

Samples from different purification steps were analyzed by SDS-PAGE (Fig. 2). The fusion lipase (without signal peptide) was purified through a single step procedure (lane 3). SDS-PAGE analysis showed that the purified T1 lipase (lane 5) appeared as a 39 kDa homogeneous subunit after removal of 26 kDa GST tag from the digested fusion lipase (lane 4). The native molecular weight of T1 lipase was estimated to be 43,000 Da by comparing its partition coefficient value (K_{av}) with the values obtained using ribonuclease A (MW 13,700), chymotrypsinogen A (MW 25,000), ovalbumin (MW 43,000) and albumin (MW 67,000) as calibration standards. The chromatography revealed that the T1 lipase was in monomeric form under running condition. The size was similar to its predicted molecular weight, but slightly bigger than its denatured form obtained through SDS-PAGE.

Characterization of T1 lipase

Expression of the target protein as GST fusion protein helps in facilitating protein purification in bulk quantity and higher purity. However, the native entity of T1 lipase needs to be characterized to explore its potential in industrial applications. The temperature profile of T1 lipase is of great significance for high temperature operational bioprocesses, as T1 lipase is most active in the temperature range of 65–75°C, with maximal activity at 70°C (Fig. 3a). T1 lipase was intrinsically stable at 60 and 65°C, as revealed in Fig. 3b, with half-lives of 12 and 5 h 15 min, respectively. Further increase in treatment temperature made a big impact on enzyme stability. At 70°C, the

Table 1 Summary of the purification procedure for the T1 lipase

Purification steps	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Recovery (%)	Purification fold
Cell lysate	28,848.1	138.3	208.6	100.0	1.0
Affinity 1	20,557.1	42.0	489.5	71.3	2.3
Affinity 2	14,852.7	15.5	958.2	51.5	4.6

The GST fusion lipase was purified under native conditions through affinity chromatography. Affinity 1 represents glutathione sepharose HP, whereas affinity 2 represents glutathione sepharose HP, glutathione sepharose 4FF and benzamidine FF (high sub) attached in series

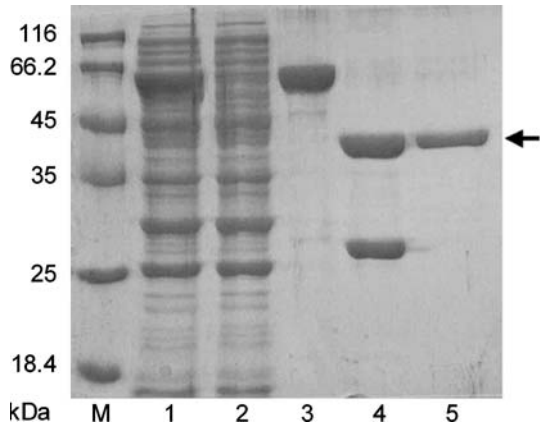

Fig. 2 SDS-PAGE (12%) of T1 lipase at different purification steps. *M* standard protein markers were *β*-galactosidase (116 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), lactate dehydrogenase (35 kDa), restriction endonuclease Bsp 981 (25 kDa) and *β*-lactoglobulin (18.4 kDa); crude cell lysate (*lane 1*); flow through (*lane 2*); purified fusion lipase (*lane 3*); fusion lipase after thrombin cleavage at 16°C (*lane 4*); and purified T1 lipase (*lane 5*). *Arrow* indicates purified T1 lipase

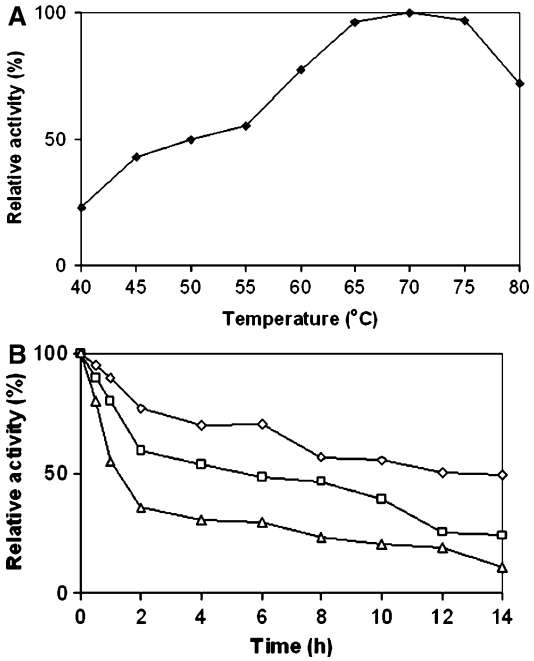

Fig. 3 Effect of temperature on T1 lipase activity (**a**) and stability (**b**). The purified T1 lipase was assayed at different temperatures (40–80°C). For the stability test, the purified T1 lipase was incubated at different temperatures: 60°C (*open diamond*); 65°C (*open square*); 70°C (*open triangle*) under shaking conditions for different durations. The residual activity was measured colorimetrically with olive oil as substrate (1:1, v/v) under shaking conditions

optimum temperature for activity, its half-life was 1 h 10 min. However, it was able to retain >20% of its lipase activity at the same temperature even up to 10 h of treatment time.

The purified T1 lipase displayed a broad pH activity of pH 6–11 with an optimum pH of 9 in 50 mM glycine-NaOH buffer, when olive oil (70% oleate residue) was used as the substrate. Despite the high activities within a broad pH range, almost none or low T1 lipase activity was observed at pH below 6.0 and above 11.0. Its enzyme-catalyzed reactions are influenced by the pH of the reaction because active sites are composed of ionizable groups. Therefore, proper ionic form is needed in order to maintain the conformation of active site and binding of the substrate as well as catalyzing the reaction. pH treatment showed that T1 lipase was relatively stable at alkaline pH condition. T1 lipase was fairly stable (>60% of lipase activity) at pH 9–11 upon treatment at 50°C for 30 min. Treatment of the enzyme at pH below six strongly destabilized T1 lipase. Full lipase activity was not regained at that pH range, where the enzyme was not stable due to the formation of improper ionic forms of enzyme, and eventually resulted in irreversible enzyme inactivation.

The effect of tested metal chloride (1 mM) on recombinant T1 lipase activity differs significantly (Table 2). The metal ions Na^+ and K^+ from Group I of the periodic table gave slight enhancement effect of 6.5 and 8.0%, respectively, after 15 min of treatment (data not shown). Nevertheless, both ions had a negligible effect on the activity after prolonged treatments of 30 min. Metal ions from Group II of the periodic table were also tested. Metal ion Mg^{2+} gave slight inhibition on T1 lipase activity, but Ca^{2+} only gave a stabilizing effect to T1 lipase. Heavy metals (Cu^{2+}, Fe^{3+} and Zn^{2+}) gave more impact on T1 lipases activity. Cu^{2+}, Fe^{3+} and Zn^{2+} influenced the T1 lipase activity by exhibiting more than 50% of inhibition

Table 2 Effect of effectors and substrates on T1 lipase activity

Effect	Relative activity (%)
Metal ions	
w/o	100
Na	99
K	81
Mg	81
Ca	100
Mn	87
Fe	39
Cu	49
Zn	22
Surfactants	
w/o	100
Tween 20	122
Tween 40	101
Tween 60	126
Tween 80	188
Triton X-100	72
SLS	48
SDS	5
Triacylglycerols	
Triacetin (C2)	5
Tributyrin (C4)	20
Tricaproin (C6)	70
Tricaprylin (C8)	77
Tricaprin (C10)	93
Trilaurin (C12)	100
Trimyristin (C14)	98
Tripalmitin (C16)	83
Tristearin (C18)	29
Triolein (C18:1)	75
Natural oils	
Olive oil	100
Sun flower oil	151
Corn oil	146
Palm oil	118
Soy bean oil	116
Rice bran oil	87
Coconut oil	75

Effectors such as metal ions (1 mM) and surfactants (1 mM) were used for treatment prior to assay at 70°C, pH 9 for 30 min, whereas tricylglycerols (100 mM) and natural oils (1:1, v/v) were used with substrate specificities

after 30 min of treatment. Among them, Zn^{2+} greatly inhibited T1 lipase activity by forming a white precipitate upon treatment even with half of the tested Zn^{2+} ion concentration. In contrast, Mn^{2+} slightly inhibited (13%) the lipase activity after 30 min of treatment. In general, T1 lipase was stable with most of the surfactants tested, particularly non-ionic detergents (Table 2). Among the tested surfactants, enhancement effect was seen in T1 lipase with the presence of Tween 20-80. Astonishingly, significant enhancement of 88% activation was seen with Tween 80. Triton X-100 gave 38% inhibition to T1 lipase. SDS on the other hand almost completely inhibited T1 lipase, while SLS greatly inhibited its lipase activity by 52%.

T1 lipase hydrolyzed triacylglycerols with acyl-group chain lengths between C10 and C16, with an optimal activity with C12 (trilaurin) (Table 2). The hydrolytic rate on medium chain triacylglycerols (C8–C12) was between 77 and 100% of optimal activity. Whereas, there were 5–70% and 29–98% of optimal lipase activity encountered with short chain (C2–C6) and long chain (C14–C18) of triacylglycerols, respectively. In addition, T1 lipase hydrolyzed triolein (C18:1) more than tripalmitin (C18:0). The preference of T1 lipase for natural oils was studied by comparing the hydrolytic rate of natural oils (Table 2). In general, T1 lipase hydrolyzed natural oils such as sunflower oil, corn oil, palm oil and soybean oil more rapidly than olive oil; however, it had a low hydrolytic rate for coconut oil, which contains more than 80% of total saturated fatty acids.

Several metal chelating agents, reducing agents, serine and aspartate inhibitors were used to study the inhibition effect and to verify the amino acids that play a significant role in enzyme catalysis. T1 lipase encountered less inhibition effect with reducing agents (DTT and β-mercaptoethanol) and metal chelating agent EDTA with less than 22% inhibition at 5 mM final concentration. In contrast, a complete inhibition effect was observed with PMSF (5 mM) and pepstatin (1 mM) with remaining activity of 11 and 14% after 30 min treatment at 50°C, respectively. The inhibition studies showed that serine and aspartate residues participated in enzyme catalysis as revealed by amino acid sequences alignment among thermostable lipases from family I.5 (Fig. 4).

CD spectral analysis

The CD spectra (molecular ellipticity) of T1 lipase were analyzed as a function of temperature at 220 nm (Fig. 5). Wavelength at 220 nm was set to monitor the transition of α-helical to unordered structures, as they exhibited characteristic signals at this wavelength. The high-tension voltage (HT) value gradually increased until T_m of T1 lipase was reached and then decreased as the lipase was unfolded above that melting temperature point (data not shown). It indicated loss of protein secondary structure, followed by an increase in unordered conformations. The sigmoidal shape of the resulting denaturation curve indicated a monophasic helix-coil transition of T1 lipase within

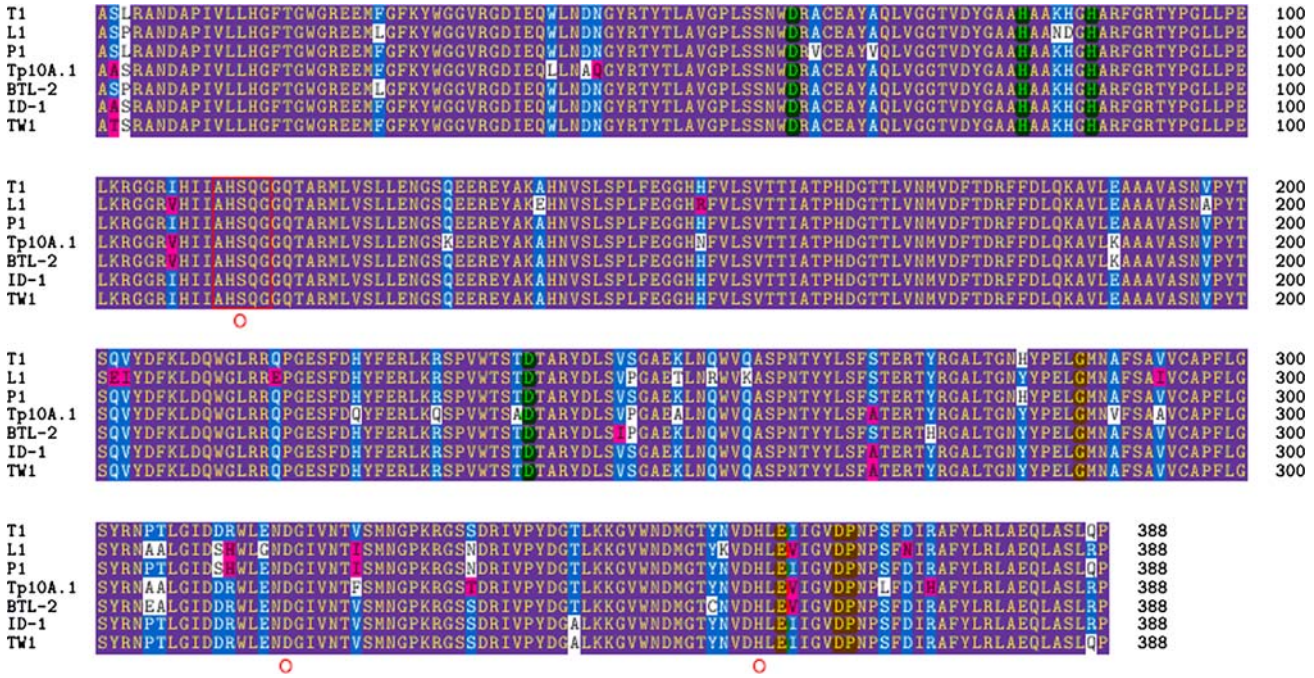

Fig. 4 Sequence alignment of family I.5 lipases. The sequences alignment was generated using CLUSTALW and TEXSHADE in Biology Workbench 3.2 (http://www.workbench.sdsc.edu/). The alignment was generated using T1 lipase (AY260764), L1 lipase (U78785; 1KU0), P1 lipase (AF237623; 1JI3), Top10A.1 lipase (AF141874), BTL2 lipase (X95309), ID-1 lipase (AF134840) and TW1 lipase (AY786185). The conserved pentapeptide and catalytic triad (S113, D317, and H358) are shown in *box* and marked with *open circle*, respectively. The Ca-binding sites (G286, E360, D365 and P366) and Zn-binding sites (D61, H81, H87 and D238) were colored *green* and *yellow*, respectively

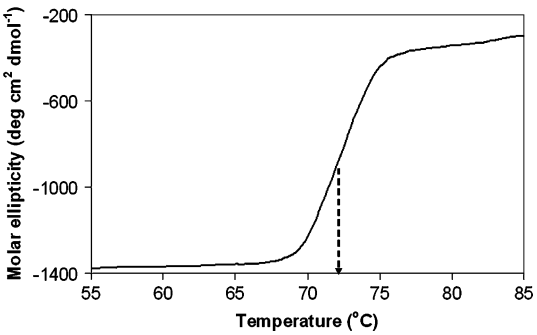

Fig. 5 Denatured protein analysis of T1 lipase. The thermal denaturation of T1 lipase was monitored by following the ellipticity at 220 nm from temperatures 55 to 85°C. The *arrow* indicates the T_m value of T1 lipase

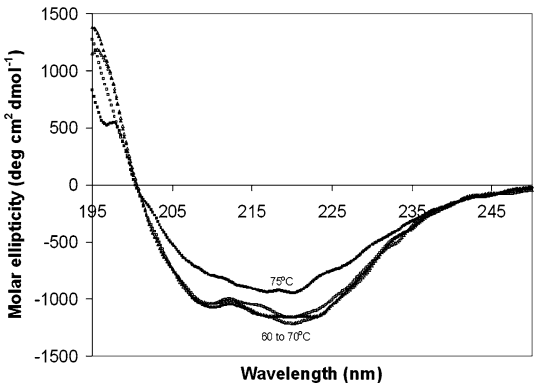

Fig. 6 CD spectra of T1 lipase at various temperatures. The CD spectra of T1 lipase between wavelengths 195 and 250 nm was measured at temperatures 60°C (*open triangle*), 65°C (*filled triangle*), 70°C (*open square*) and 75°C (*filled square*)

the analyzed temperature range. The fitting lines were extrapolated from the stable region to the range of denaturation (61.20–83.80°C) to obtain the T_m value. The T_m value for T1 lipase for this transition was 72.2°C, while the unfolding enthalpy ΔH and entropy ΔS were –158.67 kcal/mol and –0.46 kcal/mol K, respectively. The remarkable sensitivity of the far-UV to the backbone conformation of proteins allowed measuring the CD spectra of T1 lipase at various temperatures (Fig. 6). As an asymmetrical packing of intrinsically achiral peptide groups of T1 lipase was determined by the CD spectra, changes in temperatures caused the conformational changes. Small changes of CD spectra were encountered at temperatures between 60 and 70°C. At that range, a transition of weight (%) α-helices to β-sheets was observed. Further increase in temperature had a big impact on protein structure, whereby the random coil

was significantly increased and β-sheet was completely lost.

Discussion

Enzyme stability is dictated by its three-dimensional structure, through inherited genetic and environmental evolution. These characters confer high thermal stability to biocatalysts derived from thermophiles prevailing in various industrial processes. Reports on family I.5 lipases expression in prokaryotic system are available (Cho et al. 2000; Kim et al. 1998; Li and Zhang 2005; Schmidt-Dannert et al. 1996; Sinchaikul et al. 2001; Sunna et al. 2002). Nevertheless, the conserved pentapeptide (Ala-Xaa-Ser-Xaa-Gly) and catalytic triad (Ser113, Asp317 and His358) are identical among those lipases, as revealed by amino acid sequences alignment (Fig. 4). Besides, alignment with crystal structures of P1 lipase (1JI3) and L1 lipase (1KU0) accurately annotated Ca-binding sites and Zn-binding sites (Tyndal et al. 2002; Jeong et al. 2002). Metal ions can act as electrophiles that seek the possibility of sharing electron pairs with other atoms, so that a bond or a charge–charge interaction could be formed (Glusker et al. 1999). The binding of Ca^{2+} ensured structural stabilization by holding the catalytic residues at their actual position and thus ensuring that T1 lipase does not easily lose its conformation at elevated temperatures. The severe inhibition effect of Zn^{2+} on T1 lipase might be due to the toxic effect generated by free Zn^{2+}. This is because tightly bound Zn^{2+} appears to control the lipase activation and stabilization of L1 lipase (Choi et al. 2005).

In some cases, recombinant protein might alter some of the properties of enzymes. Expression of T1 lipase as GST fusion protein in prokaryotic systems was expected to allow rapid purification of recombinant T1 lipase through affinity chromatography. On the contrary, it was not true for T1 fusion lipase (with signal peptide), which might be due to the high hydrophobic region of GST and signal peptide, leading to conformational changes and improper folding of the GST binding domain and subsequently hindering it from binding to the affinity column. Physicochemical properties of T1 lipase are important in seeking any possibility for use in industrial applications. From this study, the optimal temperature of recombinant T1 lipase was the same compared to its crude wild-type enzyme (70°C). On the other hand, the recombinant LipA lipase of *Bacillus* sp. Tp10A.1 (Sunna et al. 2002), L1 lipase (Kim et al. 1998) and P1 lipase (Sinchaikul et al. 2001) of *Bacillus stearothermophilus* and BTL2 lipase of *Bacillus thermocatenulatus* (Schmidt-Dannert et al. 1996) had reported optimum temperatures of 55–60°C. However, the optimal temperatures of those lipases were far higher than that of recombinant *Bacillus licheniformis* lipase (Nthangeni et al. 2001) in this case whereby no adaptation or directed evolution for enzyme derived from mesophile.

From the structural point of view, high temperatures might have induced a temperature switch of the lid and exposed the active site for catalysis. However, longer exposure may trigger nonspecific aggregation due to hydrophobic interaction at areas around the active site, especially at high temperature. Therefore, prolonged treatment time significantly decreased the lipase activity, as T1 lipase tended to lose its native conformation as a result of breaking of the intrinsic interaction. The denatured protein analysis and weights of secondary structure of T1 lipase well supported the thermal stability test. A shift of CD spectra to the *X*-axis indicated a decrease in the stability of T1 lipase. A total loss of T1 lipase activity at 75°C resulted from a big change in secondary structure beyond its T_m, in which a total loss of β-sheets' structure and instantaneous increase in random coil were observed. Nevertheless, T1 lipase (predicted p*I* ~ 6.12) with a half-life of 12 h was much more stable than the reported P1 lipase (p*I* ~ 6.17) with a half-life of 5 h in pH 8.5, and recombinant lipases from ID-1 lipase (p*I* ~ 6.19) and TW1 lipase (p*I* ~ 6.07) had a half-life of less than 1 h in pH 7.5 when the enzymes were treated at 60°C under the tested condition (Sinchaikul et al. 2001; Cho et al. 2000; Li and Chang 2005). These thermostable lipases are highly similar, whereby pH may dominate the protein stability besides sequence variation among those lipases. Treatment of enzymes at a pH near its p*I* may probably cause decreased stability, as the enzymes easily form aggregates or undergo denaturation during treatment.

The activation of T1 lipase by surfactants Tween 20–80 may have triggered lid opening by behaving like an insoluble water–oil interface through micelle activation or substrate emulsion stabilization. However, BTL2 lipase was completely inhibited by Tween 80, while P1 lipase showed a moderate inhibition in the presence of Tween 20 (Schmidt-Dannert et al. 1996; Sinchaikul et al. 2001). On the other hand, SDS (0.1%) showed moderate inhibition on P1 lipase (Sinchaikul et al. 2001), because of a more rigid structure and closed lid conformation at lower treatment temperatures (37°C). The inhibition effect caused by SDS and SLS might be due to binding of the ionic surfactant to hydrophobic and hydrophilic residues of T1 lipase, thus initiating unfolding of the tertiary structure and resulting in losing catalytic efficacy.

In general, the activity of lipases is enhanced with insoluble substrates (such as emulsions) through interfacial activation as compared with the same substrates in true monomeric solutions (Verger 1997). Variation in the affinity of T1 lipase to medium to long carbon chain-length triacylglycerols, with trilaurin (C12) being the best tri-

acylglycerol, may be due to the triggering of lid opening during catalysis. In agreement, point mutation on lid region affected chain length specificity (Santarossa et al. 2005). In contrast, BTL2 lipase (Schmidt-Dannert et al. 1996) showed high activity towards tributyrin (C4), whereas the P1 lipase (Sinchaikul et al. 2001) preferred tricaprylin (C8) as substrate. The result implied that there are some structural and functional differences between T1 lipase and other thermostable lipases in spite of the significant similarity of the amino acid sequences.

T1 lipase shows preference to natural oils such as sunflower oil and corn oil with high unsaturated/saturated ratio. This provides a good potential for the use of T1 lipase as additive in detergent formulation and as biocatalyst for natural oils-based industries, due to its broad substrate specificity and selectivity. However, coconut oil (mainly C12:0, 47%; C14:0, 18%) with a low content of unsaturated fatty acids was less preferable, even though saturated lauric acid (C12) was in abundance (47%). In contrast, the recombinant L1 lipase showed preference for solid lipids, such as palm oil and beef tallow (Kim et al. 1998). T1 lipase can be effectively used at high temperatures for producing fatty acids in the food industry by virtue of its thermostability, as some of the lipids such as palm oil and beef tallow are partially or totally solid at room temperature. Hydrolysis at such high temperatures was almost impossible for other lipases from mesophilic and psychrophilic sources.

T1 lipase of *Geobacillus* sp. strain T1 is highly similar to other reported thermostable BTL2 lipase, L1 lipase and P1 lipase of *Bacillus* spp., but it shows some differences in properties with respect to temperature activity and stability, metal ions, surfactant stability and substrate specificity.

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