

Purification and Characterization of Two Highly Thermophilic Alkaline Lipases from *Thermosyntropha lipolytica*[∇]

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Two thermostable lipases were isolated and characterized from *Thermosyntropha lipolytica* DSM 11003, an anaerobic, thermophilic, alkali-tolerant bacterium which grows syntrophically with methanogens on lipids such as olive oil, utilizing only the liberated fatty acid moieties but not the glycerol. Lipases LipA and LipB were purified from culture supernatants to gel electrophoretic homogeneity by ammonium sulfate precipitation and hydrophobic interaction column chromatography. The apparent molecular masses of LipA and LipB determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis were 50 and 57 kDa, respectively. The temperature for maximal activity of LipA and LipB was around 96°C, which is, so far as is known, the highest temperature for maximal activity among lipases, and the pH optima for growth determined at 25°C (pH^{25°C} optima) were 9.4 and 9.6, respectively. LipA and LipB at 100°C and pH^{25°C} 8.0 retained 50% activity after 6 and 2 h of incubation, respectively. Both enzymes exhibited high activity with long-chain fatty acid glycerides, yielding maximum activity with trioleate (C_{18:1}) and, among the *p*-nitrophenyl esters, with *p*-nitrophenyl laurate. Hydrolysis of glycerol ester bonds occurred at positions 1 and 3. The activities of both lipases were totally inhibited by 10 mM phenylmethylsulfonyl fluoride and 10 mM EDTA. Metal analysis indicated that both LipA and LipB contain 1 Ca²⁺ and one Mn²⁺ ion per monomeric enzyme unit. The addition of 1 mM MnCl₂ to dialyzed enzyme preparations enhanced the activities at 96°C of both LipA and LipB by threefold and increased the durations of their thermal stability at 60°C and 75°C, respectively, by 4 h.

Thermosyntropha lipolytica is an anaerobic, thermophilic, organotrophic, lipolytic, alkali-tolerant, gram-positive-type bacterium. It was isolated from an alkaline hot spring of Lake Bogoria, Kenya, by using a mineral medium supplemented with food-grade olive oil (53), with the specific intent of finding bacterial lipases exhibiting high stability and activity at elevated temperatures and alkaline pH values.

Lipases (carboxyl ester hydrolases; E.C. 3.1.1.3) are enzymes that generally catalyze the synthesis and hydrolysis of long-chain fatty acid esters (14, 45). They are ubiquitous in nature, produced by animals, plants, and fungi, as well as bacteria, but have not yet been reported in archaea. Lipases are activated at the water-lipid interface; they show little activity when the substrate is in the monomeric form, and the activity increases dramatically above the solubility limit, i.e., when the substrate starts to form emulsions. This fact has led to the emergence of a phenomenon known as interfacial activation, which describes substrate emulsions as a necessity for maximum lipolytic activity (8, 14, 45, 56) for the majority of lipases. However, there are a few exceptions that are not interfacially activated despite homology to other known lipases (19, 31, 33).

Lipases have a conserved three-dimensional structure (2), share the α/β hydrolase fold, and have the same catalytic mechanism. The catalytic center contains the catalytic triad serine-histidine-aspartic or glutamic acid. On the other hand, lipases usually have little if any similarity in their primary

sequences, molecular masses, pH and temperature optima, substrate and positional specificities, cofactors, and cellular locations (2, 17).

Lipases are among the most versatile of the studied enzyme classes and are used in a number of applications in various industries, including the pharmaceutical, dairy, detergent, cosmetic, oleochemical, fat-processing, leather, textile, cosmetic, and paper industries (4, 7, 21, 22, 46, 57).

Many lipases, including those from mesophilic organisms, have been found to be fairly thermo- and alkali stable. However, it is likely that enzymes produced by thermophilic, alkali-tolerant bacteria will be more thermostable at alkaline pHs while exhibiting high specific activities at elevated temperatures (>70°C) and alkaline pHs (pH of >9). While a few lipases have been reported from aerobic thermoalkaliphilic bacteria (5, 27, 26, 30, 41), here we report on the purification and characterization of the first lipases from an anaerobic thermophilic bacterium, *Thermosyntropha lipolytica*.

MATERIALS AND METHODS

Culture and growth conditions. *Thermosyntropha lipolytica* DSM 11003^T was grown in 20- and 100-liter fermentors in a basal medium containing 0.75% yeast extract as the carbon and energy sources under nitrogen gas phase. The basal medium contained (per liter) 0.3 g of K₂HPO₄, 0.3 g of KCl, 0.5 g of NaCl, 1.0 g of NH₄Cl, 0.1 g of MgCl₂ · 6H₂O, 0.02 g of CaCl₂ · 2H₂O, 3.0 g of NaHCO₃, 3.0 g of Na₂CO₃, 0.5 g of Na₂S · 9H₂O, 0.15 g of cysteine, 2 ml of vitamin solution, and 2.5 ml of trace element solution (15). The pH of the medium for growth determined at 25°C (pH^{25°C}) (57) was adjusted to 8.2, and the growth temperature was 60°C.

Lipase assays. Lipase was routinely assayed spectrophotometrically using *p*-nitrophenyl laurate (p-NPL) (Sigma) and *p*-nitrophenyl palmitate (p-NPP) (Sigma) as substrates (59). The reaction mixture contained 0.3 μ M monomeric enzyme, 25 μ M MnCl₂, 1.088 ml of freshly prepared mixed buffer of 100 mM HEPES (Sigma), 100 mM TAPS (Sigma), and 100 mM CAPS buffer (Sigma), and 12 μ l of 300 mM p-NPL or p-NPP in acetonitrile (final concentration in the

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assay, 3 mM). The assay was typically run for 20 min at 96°C (the assay was linear for up to 45 min). The reaction mixture was cleared by centrifugation before the amount of liberated *p*-nitrophenol was determined at A_{405} .

To verify the activity obtained with the chromogenic substrate, assays were performed using triglycerides as substrates and measuring the liberated fatty acids by using an NEFA C kit (Waco) as instructed by the vendor. One unit is defined as the amount of the enzyme catalyzing the release of 1 μ mol of *p*-nitrophenol ($\epsilon = 1.82 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$) from *p*-NPL/*p*-NPP or 1 μ mol of free fatty acids from triglycerides per min.

Purification of lipases. All purification steps were performed at room temperature. Twenty liters of medium was inoculated with 2 liters of an exponentially growing preculture. The pH was adjusted to 8.2^{25°C} (7.6^{60°C}), and the growth temperature was 60°C. After 18 h, the cells were separated by using an Amicon hollow-fiber filter with a 1-million Da cutoff, and the supernatant was recovered. The extracellular lipase was concentrated from the culture supernatant by filtration with a 10-kDa Amicon hollow-fiber filter (Millipore) and subsequent step-wise saturation to 60% and 75% ammonium sulfate. The precipitate was collected by centrifugation, dissolved in 10 mM sodium phosphate buffer, pH 8.0, and applied to a 30-ml octyl Sepharose fast-flow column (Amersham Biosciences). The column was pre-equilibrated with 20 mM Tris buffer, pH 8.0, containing 2 M (NH₄)₂SO₄ (buffer A). The bound protein was eluted with a linearly decreasing gradient from 450 ml of buffer A to 450 ml of 20 mM Tris buffer, pH 8.0. Fractions containing high specific lipase activity [around 1.8 M and 0.5 M (NH₄)₂SO₄] were pooled, desalted, and concentrated by using a filter membrane (Millipore). The lipases were tested for purity on native and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels as described by Sambrook et al. and Ausubel et al. (3, 44). The gels were stained with GelCode blue stain reagent (Pierce). The molecular masses of the enzymes were estimated graphically by interpolation from a logarithmic graph of molecular mass versus relative migrations using standard proteins (Sigma).

Protein concentration. The protein concentrations were determined by using a bicinchoninic acid protein assay kit (Pierce) following the manufacturer's instructions.

The effect of temperature on activity and stability. A temperature gradient incubator (Scientific Industries, Inc., Bohemia, NY) was used to determine the temperature for maximal activity of both lipases. The enzyme assay was performed at pH^{25°C} 9.4 and 9.6, which were equal (58) to pH^{60°C} 8.5 and 8.8 for LipA and LipB, respectively. The enzyme assays were performed as described above, using a combination buffer containing 100 mM HEPES, 100 mM TAPS, and 100 mM CAPS.

Thermostability was analyzed in triplicate by measuring the residual activity after incubating the enzyme in Tris buffer, pH^{25°C} 8.0, at 60°C, 75°C, and 100°C for various times in series of sealed 2-ml serum bottles from which three bottles were sacrificed for every time point. Each sample contained 15 μ g of protein in 0.6 ml of 100 mM Tris buffer, pH 8.0. After the incubation, the samples were concentrated and desalted when necessary by using 10-kDa-cutoff Centricon filter tubes (Millipore); they were then assayed in triplicate for lipase activity and protein concentration.

Determination of substrate specificity. The substrate specificity and chain length selectivity were determined spectrophotometrically using a variety of *p*-NP esters (Sigma) and triglycerides. The levels of fatty acids liberated from triglycerides (all from Sigma) were determined by using a NEFA C kit. Long-chain glycerides ($\geq C_{12}$) were dissolved in acetonitrile.

Determination of positional specificity. The positional specificities of LipA and LipB were analyzed by thin-layer chromatography using a modified version of the procedure reported by Lesuisse et al. (31). The enzyme assays were carried out at the temperature and pH optima of both lipases for 8 h. The assay solution contained 5 mM triolein and 1 mM MnCl₂ in a 100 mM TAPS buffer. The assay mixture was sonicated for 30 s before the addition of 50 μ g of the purified enzyme and was terminated by rapid cooling in an ice bath. The reaction products were extracted with 2 ml of cold diethylether. The extract was concentrated by evaporation and applied on reversed-phase (multi-K) thin-layer chromatography plates (Whatman). Monolein, 1,3-diolein, 1,2-diolein, and triolein (Sigma) were used as the reference standards. The plates were developed with a mixture of chloroform-acetone-acetic acid (96:4:1). The separated esters were visualized by spraying the plates with 0.1% iodine in chloroform.

Effects of metal ions, EDTA, and PMSF. To determine the effects of metal ions, EDTA, and phenylmethylsulfonyl fluoride (PMSF) on enzyme activity, different concentrations of up to 10 mM were added directly to the standard *p*-NPL assay mixture. In addition to the standard assay, the effects of calcium, manganese, and iron, along with EDTA and PMSF, were tested with triolein as the substrate, and the liberated fatty acids quantified using a NEFA C kit.

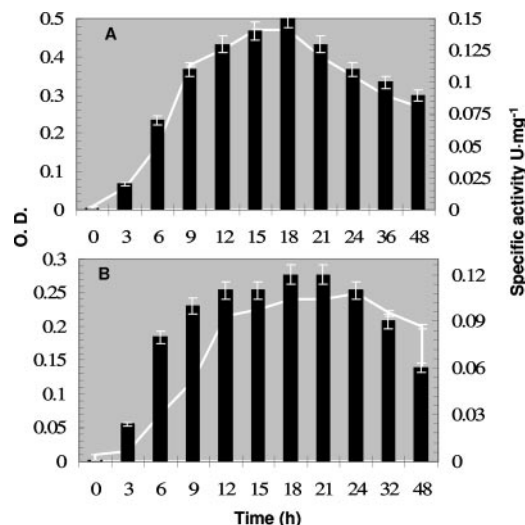


FIG. 1. Extracellular lipase activity during growth at pH^{60°C} 7.6 (A) and pH^{60°C} 9.0 (B). Optical density (O.D.; curve) and specific activity (bars) in units of activity per mg protein in the supernatant which contained 0.75% yeast extract (wt/vol), leading to a high protein value and thus low specific activity. Error bars show standard deviations. A 500-ml amount of anaerobic medium was inoculated with 5% (vol/vol) of a 12-h-old culture of *T. lipolytica*, the pH of the medium was adjusted by using 0.2 M HCl and 0.2 N NaOH, and the culture was incubated at 60°C. Every time a sample was withdrawn, the pH of the culture was adjusted. After the optical density was measured, the sample was centrifuged and used for protein quantification and lipase assays.

RESULTS

Lipase level in the culture. Svetlitsnyi et al. (53) reported that *T. lipolytica* constitutively produces lipase activity. Here, we report that the maximum formation of lipase activity occurred when the cell biomass reached its maximum (Fig. 1). At pH^{60°C} 7.6, the optimum pH for growth, the maximum specific activity using *p*-NPL was 0.15 U mg⁻¹ supernatant protein after 15 to 18 h of growth. At pH^{60°C} 9.0, the maximum specific activity was 0.12 U mg⁻¹ supernatant protein after 21 h of growth (Fig. 1). The supernatant contained 0.75% yeast extract from the medium, rendering the specific activity relatively low.

Purification of lipases LipA and LipB. The lipases were purified 140-fold from the culture supernatant using ammonium sulfate precipitation and hydrophobic interaction chromatography (Table 1). Two different lipases were eluted from the octyl Sepharose column using a descending ammonium sulfate gradient. LipA, weakly bound, was eluted at 1.8 M ammonium sulfate, and the second lipase (LipB), more strongly bound, was eluted with 0.5 M ammonium sulfate. SDS-PAGE analysis yielded molecular masses of 50 (LipA) and 57 (LipB) kDa (Fig. 2). Their N-terminal sequences were NGGGATLPLQTSGLTAGFAP and VKVMATLPADYVAQVIENVKRR, respectively, suggesting that they are two distinct enzymes.

LipA and LipB were cold labile; i.e., both irreversibly lost activity when stored at 0 to 4°C for 24 h or when frozen in various buffers, including phosphate (pH^{25°C} 8.0) buffer. The effect could be minimized by freezing in solutions containing 40% glycerol (vol/vol) and 2 mg/ml bovine serum albumin,

TABLE 1. Purification of extracellular LipA and LipB from *Thermosyntropha lipolytica*

Step, enzyme	Total vol (ml)	Total activity (units)	Total protein (mg)	Sp act (U mg ⁻¹)	% Yield	Purification factor
Supernatant	22,000	2,900	28,600	0.1	100	1
Holofiber with 10-kDa cutoff	5,000	2,640	3,250	0.81	91	8
(NH ₄) ₂ SO ₄ precipitation	800	1,580	392	4.0	54.5	40
Octyl Sepharose column, LipA	300	660	47	14	23	140
Octyl Sepharose column, LipB	210	390	29	13.4	13.4	134

leading to residual catalytic activities of 75% and 90% for LipA and LipB, respectively.

Effects of pH and temperature on enzyme activity and stability. The maximal observed activities for LipA and LipB were at 96°C with the 20-min p-NPL assays (Fig. 3). Because p-NPL is relatively unstable at 96°C and a pH of ≥ 10.0 , the true substrate, trioleate, was used for determining the pH ranges and optima at the optimum temperature of 96°C. A broad pH^{25°C} range for activity was observed for both enzymes. They exhibited a biphasic curve with intermediate maxima of around 8.7 (LipA) and 8.9 (LipB) besides the pH optima of around pH^{25°C} 9.4 (LipA) and 9.6 (LipB) (Fig. 4A). At room temperature, both enzymes were unstable when incubated at acidic pHs (below 7.0) but were stable for 36 h at alkaline pHs, retaining 100% activity at pH^{25°C} 7.0 to 12.0 (Fig. 4B).

LipA and LipB showed maximum thermostability at temperature and pH optima in the presence of 0.5 to 2 M (0 to 2 M were tested) ammonium sulfate. They retained 50% activity after incubation at 100°C for 6 h (LipA) and 2 h (LipB). The temperatures for a 24-h half life were 74.1°C (LipA) and 76.5°C (LipB) (graphs not shown).

Substrate specificity and positional specificity. With the use of triglycerides as substrates, both LipA and LipB exhibited no significant activity with tributyrin (C₄) and tricaproin (C₆) but intermediate high activity toward glycerides with long-chain fatty acids (C₁₂ to C₁₈) and an activity that was about fivefold higher with triolein, i.e., a triglyceride containing unsaturated C₁₈ fatty acids, than with glycerides containing saturated fatty acids (Fig. 5). Among the p-NP esters, similar specificities were observed. The highest activity was observed with the saturated C₁₂ ester; unfortunately, the p-NP oleic acid ester was not

available. This substrate specificity identifies LipA and LipB as true lipases. The hydrolytic products of triolein after up to 8 h of incubation with LipA or LipB were increasing amounts of 2-monoolein, indicating that the enzymes specifically hydrolyze the *sn*-1,3 position of triolein (detailed data not shown).

Effects of metal ions, PMSF, and EDTA on the enzymes' activities. The catalytic activities of LipA and LipB of *T. lipolytica* were neither enhanced nor inhibited by the presence of 1 mM to 10 mM CaCl₂ (Table 2), but a 15% decrease in activity occurred at 25 mM CaCl₂. LipA (pH^{25°C} 9.4) and LipB (pH^{25°C} 9.6) showed various degrees of inhibition by the metal ions tested (Table 2), and the inhibition was greater as the concentration of the ion increased. Manganese (in the form of MnCl₂) at concentrations between 0.1 and 2 mM increased the activities of LipA and LipB by threefold in comparison to the activities found in assays conducted in the absence of metal ions or in the presence of calcium (0.1 to 10 mM). The effect of manganese was confirmed by carrying out lipase assays using triolein as the substrate and quantifying the liberated fatty acids. In the presence of 5 mM EDTA, nearly half of the activity of both LipA and LipB was lost, and the activity was completely diminished in the presence of 10 mM EDTA. These results indicate that LipA and LipB might require a specific metal for activity.

Subsequent metal analyses (by inductively coupled plasma emission spectrometry) yielded data showing that purified LipA and LipB contained Mn²⁺ ions instead of the Zn²⁺ ions

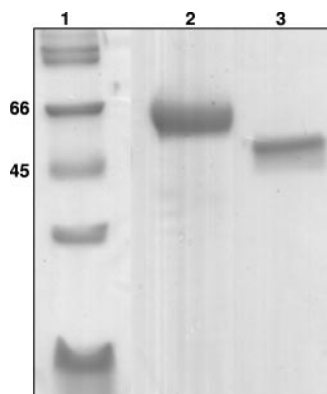


FIG. 2. Purified LipA and LipB proteins were analyzed by SDS-PAGE. Lanes: 1, molecular mass marker (kDa); 2, purified LipB from octyl Sepharose column; 3, purified LipA after Q-Sepharose fast-flow chromatography. Gels were stained with GelCode blue stain reagent.

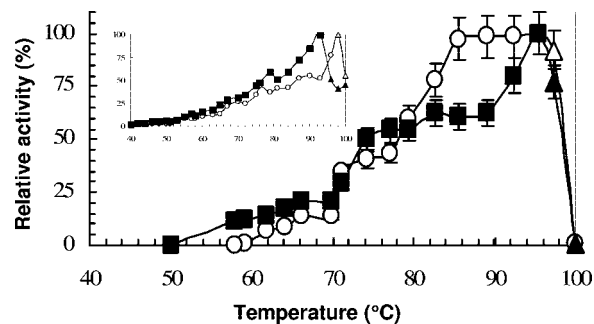


FIG. 3. Effect of temperature on LipA (○) and LipB (■) activities. The lipase assay was conducted as mentioned in Materials and Methods. The substrate used for the assay was p-NPL. A similar temperature profile but lower specific activity was also obtained when p-NPL was replaced with p-NPP. The insert is the temperature profile when only TAPS buffer was used at pH 9.0. A true substrate, trioleate, was used at temperatures of $\geq 98^\circ\text{C}$, and the liberated fatty acids were quantified by using a NEFA C kit. The 100% relative activities were $11.8 \pm 0.5 \text{ U mg}^{-1}$ and $13.0 \pm 0.6 \text{ U mg}^{-1}$ for LipA and LipB, respectively. The activity values were corrected for control values obtained without enzyme addition. Error bars show standard deviations.

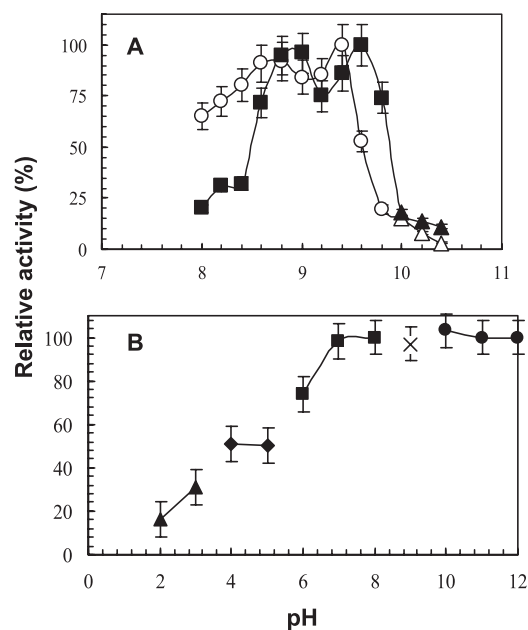


FIG. 4. Effect of pH^{25°C} on activity of LipA (○) and LipB (■) (A) and on stability of LipA (B). The 100% relative activities were 12.4 ± 0.5 U mg⁻¹ and 12.8 U mg⁻¹ ± 0.3 U mg⁻¹ for LipA and LipB, respectively. Because the p-NPL substrate was not stable at pH^{25°C} 10 or above, triolein was used at the high pH (panel A, ▲); the liberated fatty acids were measured by using an NEFA C kit. (B) LipA and LipB showed nearly identical profiles for stability; thus, LipB was omitted for clarity. For the stability experiments, 100 mM of the following buffers were used: glycine-HCl (▲), sodium acetate buffer (◆ and ◇), MES buffer (■), TAPS buffer (×), and CAPS buffer (●). Results are expressed as percentage of maximal activity. The lipase assays were conducted as described in Materials and Methods.

found in thermophilic lipases from *Geobacillus stearothermophilus*. After correction against the values of the dialysis buffer, the enzymes contained 0.92 ± 0.1 (mean ± standard deviation) mol Ca²⁺ and 0.6 ± 0.06 mol Mn²⁺ ions per mol of monomeric enzyme. Since the addition of manganese ions resulted in a threefold-greater activation of the enzyme, it is assumed that the native enzyme contains 1 mol Mn²⁺ ions per mol lipase and that approximately 40% of the Mn²⁺ ions are removed from the protein during the purification procedure or during dialysis.

Lipases are generally members of the serine hydrolases, with serine as an essential residue for catalytic activity. The addition of 10 mM of the serine hydrolase inhibitor PMSF inhibited the activity of LipA and LipB (Table 2), indicating that LipA and LipB are also serine hydrolases.

DISCUSSION

Two highly thermostable enzymes are present. Very few lipases from thermophiles and none from an anaerobic thermophile have been characterized so far (43). The anaerobic thermophile *T. lipolytica* produced two extracellular lipases which appear to be encoded by two distinct genes, as indicated by their different biochemical properties and different N-terminal sequences. However, this needs to be verified in the future by sequence analysis of the encoding genes. Attempts to

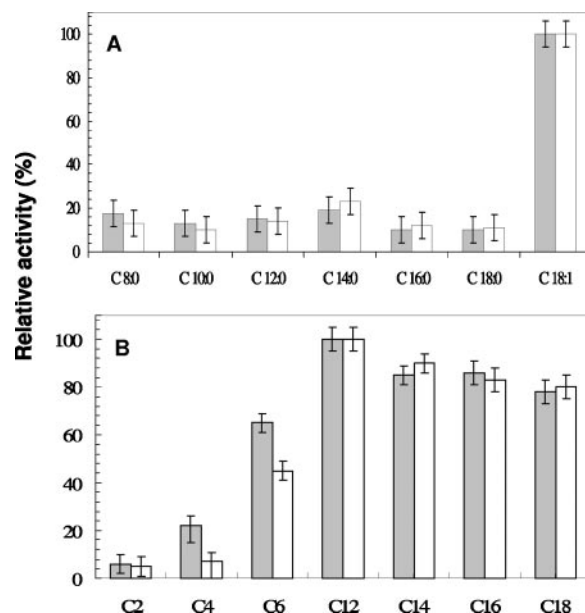


FIG. 5. The activities of purified LipA (open bars) and LipB (grey bars) toward substrates with different acyl chain lengths were determined under standard conditions. (A) Activities with 5 mM triglycerides. The 100% relative activities represent 0.64 ± 0.03 U mg⁻¹ (LipA) and 0.79 ± 0.04 U mg⁻¹ (LipB) using trioleic acid as the substrate. (B) Activities with 3 mM *p*-nitrophenyl esters. The 100% activities represent 12.4 ± 0.5 U mg⁻¹ (LipA) and 13.0 ± 0.6 U mg⁻¹ (LipB) with *p*-NPL as the substrate. Error bars show standard deviations.

clone the two lipases were unsuccessful. A maximum temperature of 96°C (while using the true substrate triolein instead of the usually used *p*-NP esters) indicates that these two lipases are the most thermophilic ones so far reported. The only other lipase with a similar high thermal activity and stability was found, surprisingly, in the mesophilic *Burkholderia cepacia* (39). Its lipase retains 50% activity after 13 h of incubation at

TABLE 2. Effects of various metal ions and inhibitors on lipase activity

Metal ion or inhibitor	Relative remaining activity (%) of enzyme at indicated concn of ion or inhibitor			
	LipA		LipB	
	1 mM	10 mM	1 mM	10 mM
Ca ^a	100 ± 5	100 ± 5	100 ± 5	100 ± 5
Mg	95 ± 5	65 ± 5	97 ± 5	73 ± 5
Fe ^a	12 ± 5	0	20 ± 5	0
Mn ^{a,b}	340 ± 5	270 ± 15	315 ± 15	250 ± 15
K	65 ± 5	30 ± 10	85 ± 5	70 ± 10
Zn	50 ± 15	44 ± 15	62 ± 15	36 ± 15
Na	78 ± 5	70 ± 5	83 ± 5	80 ± 5
Cs	77 ± 5	37 ± 10	72 ± 5	44 ± 5
Cu	45 ± 10	0	56 ± 10	0
Al	28 ± 5	0	30 ± 5	0
Ni	80 ± 5	22 ± 5	50 ± 5	15 ± 5
Co	60 ± 5	60 ± 5	68 ± 5	49 ± 5
PMSF	70 ± 15	6 ± 5	62 ± 15	0
EDTA	60 ± 15	11 ± 5	81 ± 5	0

^a Assays were conducted with triolein and lipase activity quantified by using an NEFA C kit.

^b The addition of Mn or Mn and Ca together gave the same value.

90°C. However, comparing the physical properties of these lipases is quite difficult due to the differences in the assay substrates and procedures. None of the lipases from aerobic thermophiles exhibit the high thermostability of LipA and LipB (27, 36, 50).

Role of metal content in activity and stability of LipA and LipB. Many bacterial lipases for which structures are available contain a Ca^{2+} -binding site. These include the enzymes from *Chromobacterium viscosum* (28), *Pseudomonas aeruginosa* (35), and *Burkholderia glumae* (basonym *Pseudomonas glumae*) (BGL) (37). Many lipases have been found to require certain metals for activity and/or to enhance activity and (thermo)stability. The two thermophilic lipases from *Geobacillus stearothermophilus* (basonym *Bacillus stearothermophilus*), P1 (55) and L1 (24), contain, besides Ca^{2+} ions, Zn^{2+} ions, which is unique among the lipases that have been characterized so far. Zinc is believed to be involved in enhancing the thermostability of these enzymes. In addition, the activity of the thermophilic lipase of *Geobacillus thermoleovorans* strain ID-1 (30) is enhanced by the addition of calcium and zinc ions. The activities of other lipases, from *Pseudomonas* sp. (13, 38), *Acinetobacter* sp. (51), *Bacillus licheniformis* (25), and *Bacillus subtilis* 168 (31), are enhanced by calcium. Staphylococcal lipases require calcium for activity and stability (40, 16). Kim et al. showed by means of fluorescence emission kinetics that calcium ions enhance the thermostability of the *G. stearothermophilus* lipase (27).

The importance of calcium ions and their involvement in stabilizing the tertiary structures of lipases was observed in the crystal structure of the *B. glumae* lipase (37). Calcium ions function as ligands to a number of adjacent residues at the active site. The loss of the calcium ion through either pH change or mutation to a residue that affects the calcium interactions has been proposed to disrupt the enzyme structure and decrease its thermal stability, as observed in *Staphylococcus hyicus* (49). However, the addition of calcium ions neither enhanced nor reduced the activity and/or stability of LipA or LipB. Based on the observed stoichiometry, the Ca^{2+} ion appears to be tightly bound. In contrast, manganese ions specifically enhanced the activities of both LipA and LipB by threefold (Table 2). Based on the metal analysis, the Mn^{2+} ion does not bind as tightly as the Ca^{2+} ion. Moreover, the effect of manganese on the thermostability of LipA and LipB; i.e., manganese extended the half-lives of both enzymes by 4 h at 60°C and 75°C, respectively, suggests that LipA and LipB contain a manganese-coordinated domain similar to the Zn-coordinated domain found in *G. stearothermophilus* lipases, with a similar function of stabilizing the protein structure. The crystal structure of *G. stearothermophilus* lipase indicated that the zinc-coordinated extra domain makes tight interactions with the loop extended from the C terminus of the lid helix, thus making strong, hydrophobic interactions with its neighboring domains, including the core domain. It has been proposed that these interactions lead to rigid packing of the active site, and they appeared to play an important role in the enzyme catalytic activity and thermostability of the enzyme. This is in agreement with the observed maximal activity at 65°C. The lid apparently requires high temperatures in order to move away and to expose the active site (24). The Mn^{2+} ion, a slightly larger metal ion than the Zn^{2+} ion, might have the same effect in

LipA and LipB as zinc in other lipases, contributing to the enzyme activity and stability at the elevated temperatures. Other structural features, such as high hydrophobic interactions, higher hydrogen bonding, amino acid substitutions, and high salt bridge content, among others (10, 11, 18, 23, 32, 42, 54), which were observed and studied in thermophilic proteins, could contribute to the thermostability of LipA and LipB, and have not been investigated here due to lack of the encoding gene sequence and crystal structure. However, the most profound effect on thermostability was noticed with the addition of 0.5 to 2 M ammonium sulfate to the enzyme solution prior to assaying activity, suggesting that hydrophobic interactions are important for the integrity of the LipA and LipB protein structures. This conclusion is in agreement with the results of Benjwal and coworkers, who showed that molar concentrations of NaCl or Na_2SO_4 increased the apparent melting temperature of a lipoprotein by up to 20°C and that these salts decelerated protein unfolding (6). Similar effects have also been reported for other enzymes (1, 20, 52). Ammonium sulfate is a chaotropic agent. It increases the chaos (entropy) in water and thereby increases hydrophobic interactions; the proteins become less flexible, which stabilizes the tertiary structure and lowers domain unfolding.

Substrate specificity and true lipases. The substrate specificity preferences of LipA and LipB are different from those of other described thermophilic lipases. Whereas LipA and LipB both had similar preferences for long-chain fatty acid esters (C_{12} to C_{18} p-NP esters) and, especially, toward the unsaturated ones present in triolein ($\text{C}_{18:1}$ as glycerol ester), other described thermophilic lipases have maximal activity with shorter, acryl esters, and several could be called lipase esterases and esterases instead of true lipases. For example, the thermophilic enzyme from the aerobic strain *G. thermoleovorans* ID-1 showed maximum activity toward tricaproin (C_6) and *p*-nitrophenyl caproate (C_6) (9), that from *G. stearothermophilus* P1 toward *p*-nitrophenyl caprate (C_{10}) and tricaprylin (C_8) (50), that from *G. stearothermophilus* L1 towards *p*-nitrophenyl caprylate (C_8) and tripropionin (C_3) (26), and that from *Geobacillus thermocatenulatus* toward *p*-nitrophenyl butyrate and tributyrin (C_4) (48).

LipA and LipB hydrolyze specifically the ester bonds at positions 1 and 3 of triglycerides. This specificity is similar to that exhibited by lipases from, e.g., *Bacillus* sp. strain THL027 (12), *G. thermocatenulatus* (47), *B. subtilis* 168 (31), and some gram-negative bacteria (29, 34).

Most of the lipases that are utilized commercially are from *Pseudomonas* species, presumably because these lipases were the first to be isolated, cloned, and characterized. Their three-dimensional structures were also among the earliest to be revealed. Moreover, most of these lipases have undergone a variety of molecular modifications yielding effective and efficient enzymes with desirable characteristics (17, 22). However, the properties of LipA and LipB should make these enzymes of great interest for specific industrial applications. The described properties at high temperatures and activities at alkaline pHs extend the diversity of lipases and indicate that extremophilic bacteria are a rich source of biotechnologically interesting enzymes, including lipases in particular.

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