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ORIGINAL ARTICLE

Molecular Cloning and Expression of a Cold-Adapted Lipase Gene from an Antarctic Deep Sea Psychrotrophic Bacterium *Pseudomonas* sp. 7323

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Abstract A psychrotrophic bacterium producing a cold-adapted lipase was isolated from the deep-sea sediment of Prydz Bay, Antarctic and identified as a *Pseudomonas* strain. Determination of the nucleotide sequence of the gene encoding a lipase from *Pseudomonas* sp. 7323 (*lipA*) revealed that LipA is composed of 617 amino acid residues with a calculated molecular weight of 64,466 Da. LipA has a GXSXG motif, which is conserved in lipases/esterases and generally contains the active-site serine. The lipase purified from the *Escherichia coli* transformant (rLipA) by metal-chelating chromatography exhibited the same electrophoretic mobility as did the wild-type lipase (wLipA) purified from strain 7323, and both enzymes were quite similar in physicochemical properties. The optimal temperature and pH value for the lipases activity were 30°C and 9.0, respectively. They were unstable at temperatures above 25°C and only retained half of their highest activity after incubation at 60°C for 5 min. These results indicated that the enzymes were typical alkaline cold-adapted enzymes. Both enzymes were particularly activated by Ca^{2+}. Additionally, the enzymes hydrolyzed *p*-nitrophenyl caprate and tributyrin at the highest velocity among the other *p*-nitrophenyl esters and triglycerides.

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Introduction

The Antarctic is regarded as an extreme environment with predominantly low temperatures, and bacteria living there must grow and carry out all their metabolic processes at temperatures near 0°C in which the broadly studied mesophilic microorganisms are unable to do (Feller et al. 1994, 1996). What is more is that the evolutionary history and geographical isolation of the Antarctic have produced a unique environment, rich in species adapted to the extreme conditions (Clark et al. 2004), and make the Antarctic marine organisms highly stenothermal in response to stable water temperatures (Verde et al. 2005). Therefore, deep-sea bacteria and their metabolic enzymes have attracted more and more research interests, as they are not only essential in some fundamental scientific study areas but they also provide potential for commercial development (Peck 2002) due to their high catalytic activity at low temperatures, low thermo stability, and unusual specificities. Recent microbial studies of the deep ocean have led to significant new discoveries of unusual microbial diversity, metabolic activity, and natural products of interest to biotechnology and bioremediation (Clarke and Leakey 1996; Peck 2002; Pörtner 2002; Gerday et al. 2000; Zhang and Zeng 2007). However, partly due to the great difficulties in collecting deep-sea samples, it remains relatively untapped.

Lipases are glycerol ester hydrolases (EC 3.1.1.3) that are characterized by their ability to hydrolysis of triacylglycerols to free fatty acids and glycerol (Lee et al. 1993; Brockerhoff and Jensen 1974). They resemble

esterases in catalytic activity but differ in that their substrates are water-insoluble fats containing medium to long fatty acyl chains (Brockerhoff and Jensen 1974). They are versatile enzymes excreted by many most living organisms, which demonstrate stereo- and region-selectivity in both hydrolysis and synthesis reactions, making them good candidates for production of optically active compounds used in the pharmaceutical and agricultural industries. This is particularly the case for cold-active or cold-adapted lipases which have great potential in the fields of waster water treatment, bioremediation in fat-contaminated cold environment, active compounds synthesis in cold conditions, and so on (Suzuki et al. 2001).

According to Arpigny and Jaeger (1999), bacterial lipolytic enzymes can be classified into eight families (I to VIII), and lipases in family I can be classified into six subfamilies, subfamilies I.1 to I.6. Most of the bacterial lipases fall in families I and II. At present, many cold-active or cold-adapted lipolytic enzymes isolated from psychrophilic bacteria have been studied, such as lipase (PFL) produced by *Pseudomonas fragi* (Alquati et al. 2002); cold-adapted lipase (KB-Lip) produced by a psychrotrophic *Pseudomonas* sp. strain KB700A (Rashid et al. 2001); cold-adaptive lipase (LipP) excreted by *Pseudomonas* sp. strain B11-1 (Choo et al. 1998); cold-adapted lipase (LipA1) excreted by *Psychrobacter* sp. 7195 (Zhang et al. 2007); extracellular esterase from *Psychrobacter* immobilis B10 (Arpigny et al. 1995), and *Moraxella* sp. TA144 (Feller et al. 1991). In this study, we report a cold-adapted lipase LipA which belong to subfamily I.3 secreted by *Pseudomonas* sp. 7323 and was also expressed in *Escherichia coli* with alkaline-soluble protein (rLipA), which retained similar physicochemical properties to the wild-type lipase (wLipA).

Materials and Methods

Sample Collection

The deep-sea sediment was collected by multi-core sampler at the depth of 900 m of site PN5-6 (74°25′E, 66°55′S) during the 21st cruise of Chinese Antarctic Research (Nov. 2004–Mar. 2005). The collected sample was sectioned into 3-cm slices in clean bench immediately and kept in −20°C on board. Subsampling was carried out in laboratory by discarding the surface sample in clean bench, and only the central section was subjected to further analysis.

Isolation of Cold-Adapted Lipolytic Bacteria

The sediment was diluted with artificial sea water (ASW) containing 0.3% NaCl, 0.07% KCl, 0.53% $MgSO_4 \cdot 7H_2O$, 1.08% $MgCl_2 \cdot 6H_2O$, and 0.1% $CaSO_4 \cdot 7H_2O$, and the supernatants were grown at 10°C on marine 2216E medium containing 0.5% tryptone, 0.1% yeast extract, 0.01% $FePO_4$ and ASW, pH 7.2. The different strains recovered from marine 2216E agar plates were tested for their enzymatic activity. The distinctive strains recovered from marine 2216E agar plates were tested for their lipolytic activities by inoculating on minimal medium agar plates containing 0.1% yeast extract, 0.01% $FePO_4$, 3.4% NaCl, supplemented with 1% tributyrin, pH 8.0. A strain with clear zone, which is named 7323, showing highest lipolytic activity, was picked out for further study.

General DNA Manipulation

Restriction enzymes and DNA polymerase were purchased from Toyobo (Osaka, Japan) as well as Takara Shuzo (Kyoto, Japan). Each enzyme was used according to the recommendations of the manufacturer. DNA ligations were performed using DNA ligation kit (Toyobo). Genomic DNA and plasmid DNA were isolated using genomic and plasmid DNA isolation kits, respectively (Qiagen, Hilden, Germany). A DNA purification kit (Toyobo) was used to recover DNA fragments from agarose gels.

Purification of Wild-Type Lipase

The strain 7323 was cultivated at 15°C for 3 days in fermentation medium containing 10 g tributyrin, 5 g tryptone, 1 g yeast extract, 0.01 g $FePO_4$ per liter sea water, pH 7.0. All of the following steps were performed at 4°C. After centrifugation, the supernatant solution was fractionated with ammonium sulfate, and a fraction of 25% to 75% saturation was collected. After dialysis, the enzyme solution was applied to a DEAE Sepharose CL-6B column (Pharmacia, Uppsala, Sweden; 3×50 cm). The column was washed with 1 l of the buffer supplemented with 0.2 M NaCl, and the enzyme was eluted with a linear gradient of 0.2 to 2.0 M NaCl with a total volume of 1.0 l. The active fractions were concentrated with 75% saturation of ammonium sulfate. The enzyme solution, dialyzed against 20 mM Tris–HCl buffer (pH 9.0), was applied to a Sephadex G-75 column (Pharmacia; 3×30 cm) equilibrated with the same buffer. The enzyme was eluted with a linear gradient of 0.005 to 1.0 M Tris–HCl buffer (pH 9.0) with a total volume of 500 ml. Subsequently, fractions containing lipolytic activity were desalted and concentrated by dialysis in the same buffer and lyophilization. Finally, the resulting enzyme preparation was eluted with 20 mM Tris–HCl buffer (pH 9.0) and applied to preparative sodium dodecyl sualfate–polyacrylamide gel electrophoresis (SDS-PAGE; Model 491 Prep Cell, Bio-Rad, Hercules, CA, USA).

Gene Cloning, Expression, and Purification of the Cold-Adapted Lipase LipA

The primers based on known pseudomonas lipase sequences in Genbank database were designed as follows: (*lipA* F) 5′-ATG GYT GTR TAS GAC AWA AGA AC-3′ (nucleotides 0 to + 23) and (*lipA* R) 5′-TCA GGC SAT YAC WAT RCC ATC AGC-3′ (+1854 to + 1830). The genomic DNA of strain 7323 was used as template. Polymerase chain reaction (PCR) amplification was performed with an initial denaturation step of 2 min at 95°C and then 30 cycles of 30 s denaturation at 94°C, 45 s at 54°C for primer annealing, and 2 min at 72°C for primer extension. The PCR product was cloned into pGEM-T vector (Pharmacia) and transform *E. coli* DH 5α. The plasmid pT-*lipA* DNA was purified from the positive transformant for sequencing by Shanghai Shenggong Biological Engineering Technology & Service Company.

To overproduce LipA, the *lipA* gene was amplified by PCR using rTaq DNA polymerase (Takara) and a combination of forward (5′-ACT A*CG GAT CC*C TAT TAA TAC GCA TAC-3′) and reverse (5′-GTA T*CG ATC GTC A*GG CGA TCA CGA TTC CAT-3′) primers where the italicized represent the *Bam*HI and *Nhe*I sites, respectively, and both ATG codon for the initiation of the translation and the sequence complementary to the termination codon TGA were deleted. The plasmid pT-*lipA* derivative which contains the *lipA* gene was used as a template in this experiment. The resultant 1.8-k bp DNA fragment was digested with *Bam*HI and *Nhe*I and ligated to the large *Bam*HI–*Nhe*I fragment of plasmid pLLP-OmpA to create an overexpression plasmid pLLP-OmpA-*lipA* for the *lipA* gene. Recombinant proteins are produced as fusion with a His_6 tag at the N terminus. For the overproduction of LipA, an overproducing strain, which was constructed by transforming *E. coli* Top 10 F′ with the plasmid, was grown at 37°C. When the absorbance at 660 nm of the culture reached around 0.6, 1 mM isopropyl-β-D-thiogalactopyranoside as added to the culture medium, and cultivation was continued for an additional 3, 4, and 5 h. Cells were then harvested by centrifugation at 6,000×*g* for 10 min and subjected to the purification procedures.

The following purification steps were all carried out at 4°C. Cell extract of *E. coli* cells harboring the *lipA* gene was dialyzed with a binding buffer (50 mM Tris pH 9.0, 300 mM NaCl, 5 mM imidazole). The enzyme suspension was loaded onto a Ni-NTA column (Qiagen, Valencia, CA, USA), and unbound protein was washed out with 50 mM imidazole. The His-tagged LipA bound to Ni-NTA resin was then eluted with 300 mM imidazole solution. To check the purity of LipA, SDS-PAGE (10%) was performed using slab gels as described by Laemmli (1970). The proteins on the gels were stained with Coomassie Brilliant Blue R-250.

Characterization of Wild-Type and Recombinant LipA

Lipase activity was measured by the spectrometric method which was described previously (Rashid et al. 2001), with a slight modification, using various *p*-nitrophenyl monoesters of fatty acids with acyl chain length from 2 to 16 as a substrate. One unit of the activity (U) was defined as the amount of enzyme releasing 1 μmol *p*-nitrophenol per minute at 30°C. The substrate solution was consisted of 100 μM *p*-nitrophenyl monoster and 20 mM Tris–HCl buffer (pH 9.0) containing 1% acetonitrile and 4% isopropanol (*v*/*v*). The reaction mixture containing 985 μl substrate solution, 10 μl 0.5 M $CaCl_2$ and 5 μl enzyme solution was incubated at 30°C for 10 min. The reaction was terminated by adding 2 ml 4 M NaOH, and then the amount of *p*-nitrophenol liberated was measured by the spectrometric at 405 nm. The enzyme solution contained purified lipase in 20 mM Tris–HCl buffer (pH 9.0) at a concentration of 0.2 μg/μl.

Lipase activity was also examined by titrating free fatty acids liberated from triglycerides with alkali (Amada et al. 2000). The reaction mixture, consisting of 55 μl of triglyceride and appropriate amount of enzyme in 1.5 ml of 20 mM Tris–HCl buffer (pH 9.0) containing 5 mM $CaCl_2$ was incubated at 30°C for 30 min with magnetic stirring at 500 rpm. The enzyme reaction was stopped by the addition of 5 ml of acetone–ethanol (1:1, *v*/*v*), and the liberated fatty acid was titrated with 10 mM NaOH. One unit of lipase activity was defined as the activity required to release 1 μmol of fatty acids per minute under the above conditions. The protein concentration was measured by the method of Bradford (1976) with bovine serum albumin as a standard.

The optimum temperature for lipase activity of LipA was determined by assaying their hydrolytic activity on *p*-nitrophenyl caprate at various temperatures (0–50°C) in 20 mM Tris–HCl buffer, pH 9.0. To examine the thermostability, the enzymes were incubated at various temperatures (5–60°C) for 30 min in a 50 mM Tris–HCl buffer (pH 9.0) containing 0.05 mg/ml of bovine serum albumin (BSA), and the residual activity was then determined at 30°C and pH 9.0.

The optimum pH on the activity of LipA was determined over the range between 4 and 11 at 30°C. GTA buffer (100 mM 3,3-dimethylglutaric acid, 100 mM Tris, 100 mM 2-amino-2-methyl-1,3-propanediol), a universal buffer, was used in these experiments. To determine the pH stability of the enzyme, it was pre-incubated at various pHs (pH 4–11) for 30 min in the presence of 0.05 mg/ml BSA, and the residual lipase activity was then determined at 30°C and pH 9.0. In this case, GTA buffer was also used.

The effects of metal ions and detergents on LipA were analyzed by measuring the activities in the presence of

5 mM various metal ions and 0.005% detergents, respectively. The resistances of LipA towards detergents were surveyed by determining the residual activity after 1-h incubation at 4°C in 20 mM Tris–HCl (pH 9.0) buffers containing 0.005% various detergents.

Nucleotide Sequence Accession Numbers

The nucleotide sequences of 16S ribosomal RNA (rRNA) gene and lipase gene (*lipA*) have been deposited in EMBL under accession nos. AM111061 and AM229328, respectively.

Results

Identification of Strain 7323

Taxonomical studies on lipase-producing microorganism strain 7323 were performed. The strain is Gram-negative, rod-shaped, aerobic, motile with polar multitrichous flagella, catalase-positive, oxidase-positive, and produced acid from glucose. From these results, this strain was classified into the genus of *Pseudomonas* according to Bergey's manual (Palleroni 2005). The optimum and highest temperatures for its growth were 15°C and 35°C, respectively, exhibiting sigmoidal growth even at 0°C. Identification by bacterial 16S rRNA gene sequence analysis showed that strain 7323 was most closely related to the genus *Pseudomonas* (Fig. 1), with the highest levels of similarity (99.443%) to *Pseudomonas* sp. Ant5 (AF184220). Thus, we placed this strain in the genus *Pseudomonas* as *Pseudomonas* sp. 7323. Data obtained from RDP (Ribosomal Database Project) also suggested that strain 7323 is a member of the *Pseudomonas* genus.

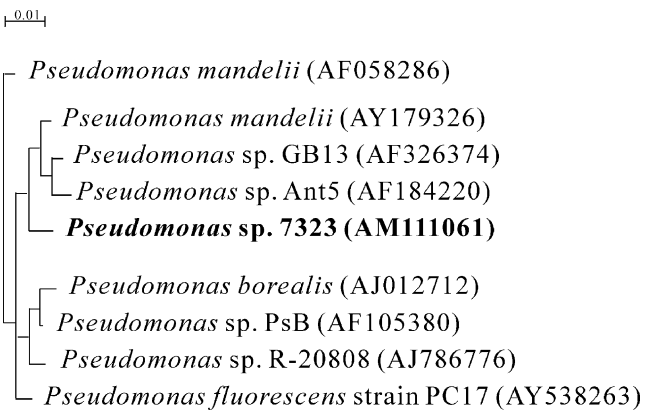


Fig. 1 Phylogenetic tree based on 16S rRNA gene sequence of strain 7323. The phylogenetic tree was constructed from a matrix of pairwise genetic distances by the maximum-parsimony algorithm and the neighbour-joining method using the DNAMAN program, and 1,000 trials of bootstrap analysis were undertaken. The lipase-producing strain 7323 is indicated by bold letters

Gene Cloning and Analyzing of the Lipase LipA

Sequence analysis of the amplified DNA revealed an open reading frame of 1,854 nucleotides, which encodes a protein of 617 amino acids with a predicted molecular mass of 64,466 Da and isoelectric point of 4.5 and showed a G + C content of 46%. Homology searched indicated that LipA showed amino acid sequence identities of 89% to *Pseudomonas* sp. UB48 lipase (LipUB48; GenBank AF202538), 89% to *Pseudomonas* sp. MIS38 lipase (PML; GenBank BAA84997), 89% to *Pseudomonas fluorescens* HU380 lipase (LipA; GenBank BAC98500), 86% to *uncultured bacterium* lipase (LipB; GenBank AAP76489), 86% to *P. fluorescens* lipase (LipB; GenBank AAG22559), and 79% to *Pseudomonas* sp. KB700A (KB-lip; GenBank BAB64913; Fig. 2). A GHSLG sequence which has been suggested to contain the active-site serine residue for PML (Amada et al. 2000) is fully conserved in the LipA sequence (Fig. 2). The C-terminal secretion signals, such as a repetitive GGXGXDXUX sequence motif (where X represents any amino acid residue and U represents a large hydrophobic residue), are generally observed in a signal peptide-independent mechanism of the protein secretion in Gram-negative bacteria (Yen et al. 2002). In addition, an 18-residue amphipathic α-helix and an extreme C-terminal motif consisting of a negatively charged residue followed by four hydrophobic residues, which have been suggested to be involved in an ABC-transporter system for PML (Amada et al. 2000), PFL (Ahn et al. 1999), and SML (Li et al. 1995; Akatsuka et al. 1994), are well conserved in the LipA sequence (Fig. 2).

Purification of LipA from Strain 7323 and *E. coli* Top 10 F′ Transformant with pLLP-OmpA-lipA

The wild-type LipA lipase (wLipA) was purified from cell free supernatant through anion exchange (DEAE Sepharose CL-6B) and gel filtration (Sephadex G-75). The final preparation of lipase was purified 13.7-fold, with a yield of 23% and a specific activity of 596.6 U/mg protein (Table 1). The purified enzyme was judged to be homogeneous on SDS-PAGE (Fig. 3).

The LipA protein was expressed as a 6× His-tagged fusion protein (recombinant LipA, rLipA) in *E. coli* containing pLLP-OmpA-*lipA* under the control of the Ipp promoter and induced by IPTG, and the expressed protein was secreted to extracellular by OmpA secretion signals at the N-terminal regions. It was purified with a 40.3% yield by 14.6-fold purification by metal-chelating chromatography and subjected to SDS-PAGE. An apparent protein band

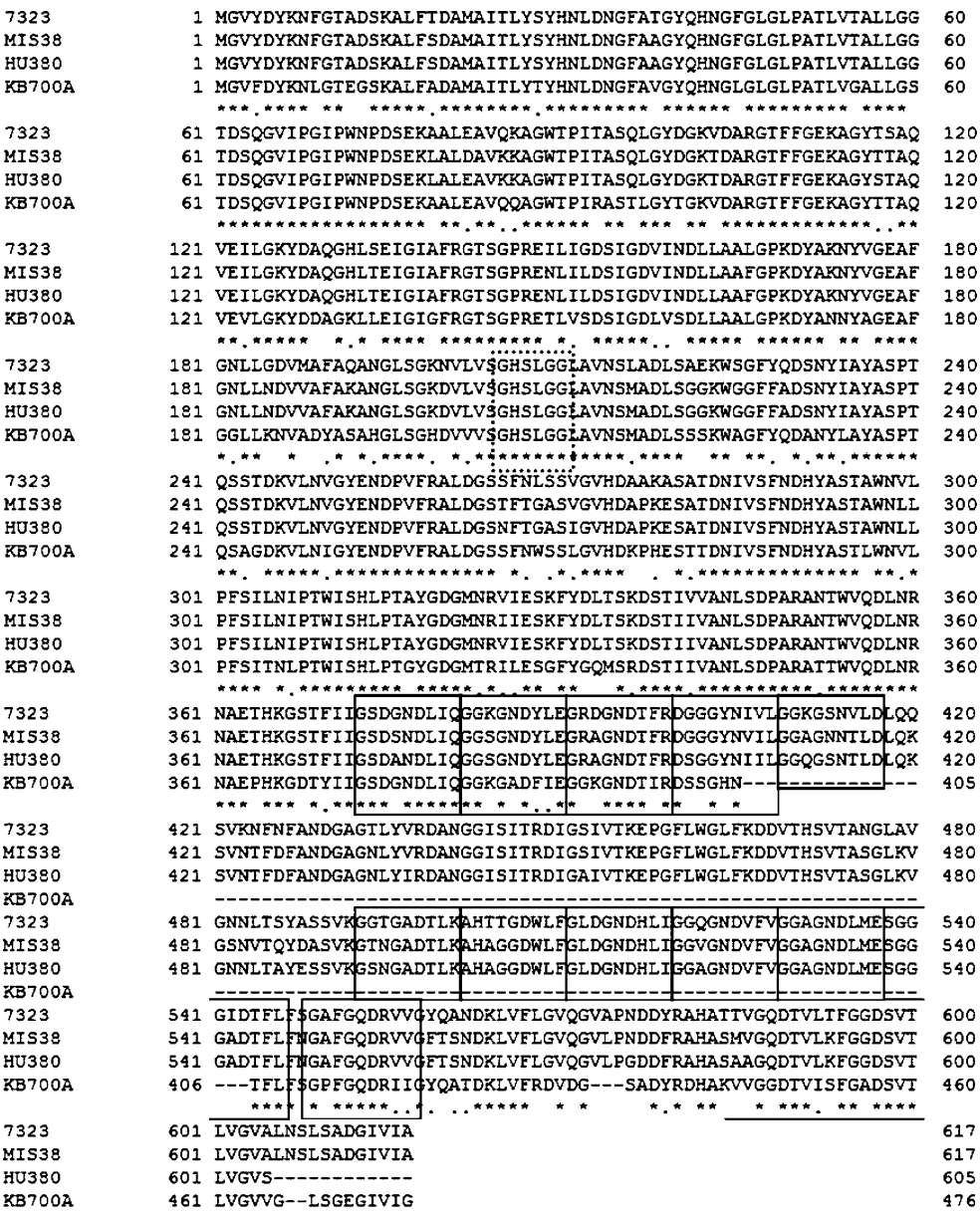


Fig. 2 Alignment of the amino acid sequence of lipases from *Pseudomonas* sp. 7323 (LipA). 7323: *Pseudomonas* sp.7323 lipase; MIS38: *Pseudomonas* sp. MIS38; HU380: *Pseudomonas fluorescens* HU380; KB700A: *Pseudomonas* sp. KB700A; The active-site consensus GXSXG sequence motif is boxed with broken line. A nine-residue GGXGXDXUX sequence motif is also boxed following by the first box. A putative 18-residue amphipathic sequence motif and an extreme C-terminal five-residue sequence motif in lipase from 7323 are underlined and double underlined respectively

Table 1 Purification of wLipA from *Pseudomonas* sp. 7323 and of rLipA from *E. coli* Top 10 F′ harboring plasmid pLLP-OmpA-*lipA*

Purification method	Total protein (mg)	Total Activity (U)[a]	Sp Act (U/mg)	Purification (fold)	Yield (%)
wLipA from *Pseudomonas* sp. 7323					
Culture supernatant[b]	28	1,221	43.6	1	100
DEAE Sepharose CL-6B	8.5	1,001	117.8	2.7	82
Sephadex G-75	0.6	315.6	526	12.1	25.8
Prep. SDS-PAGE	0.47	280.4	596.6	13.7	23
rLipA from *E. coli* Top 10 F′					
Cell extract[c]	76	3,936.8	51.8	1	100
Ni-NTA column	2.8	1,937.3	691.9	13.4	49.2
Prep. SDS-PAGE	2.1	1,588.2	756.28	14.6	40.3

[a] Activity was measured by determination of *p*-nitrophenol formed from *p*-nitrophenyl caprate as a substrate. The definition of unit is given in the text
[b] Two liters of filtered supernatant were concentrated tenfold to 200 ml
[c] Cell extract from two liters of culture

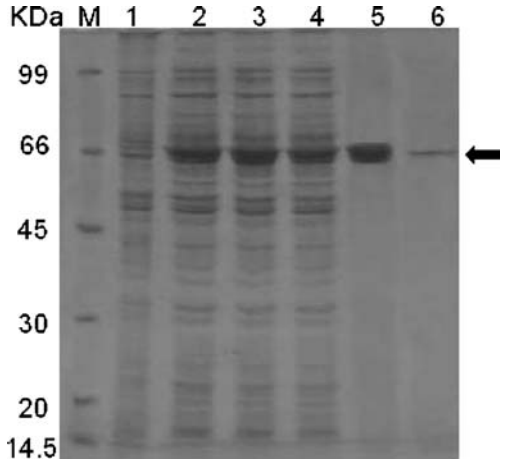


Fig. 3 Polyacrylamide gel electrophoresis of rLipA and wLipA. Lane M, molecular weight markers; lane 1, *E. coli* Top 10 F` carrying pLLP-OmpA-*lipA* without IPTG induction for 6h at 30 °C; lane 2, 3, 4, *E. coli* Top 10 F` carrying pLLP-OmpA-*lipA* after IPTG induction for 6h, 5h, 4h respectively at 30 °C; lane 5, the purified rLipA from *E. coli* Top 10 F` transformant with pLLP-OmpA-*lipA* (amount of protein loaded, 50 μg); and 6, the purified wLipA from strain 7323 after DEAE Sepharose CL-6B and Sephadex G-75 chromatography (amount of protein loaded, 10 μg). The position of LipA is indicated by an arrow

corresponding with the molecular mass of about 67,000 Da appearing in the culture supernatant that was induced by IPTG, which is consistent with the subunit molecular weight (67,210 Da) of rLipA deduced from the nucleotide sequence of rLipA and the wLipA, was observed (Fig. 3). It indicated that the *lipA* gene was successfully expressed in the heterologous host *E. coli*. The expressed rLipA containing 6× His was an alkaline-soluble (0.5 M Tris–HCl, pH 9.0) protein.

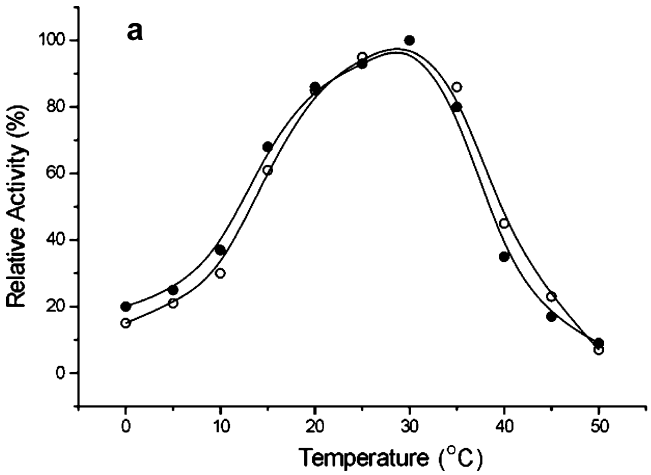
Characterization of wLipA and rLipA Lipase

Effects of pH and Temperature on the Enzyme Activity

With *p*-nitrophenyl caprate as a substrate, both wLipA and rLipA showed most active at pHs between 8.5 and 9.0. The pH stability of wLipA and rLipA were stable between pH 6 and 12 at the indicated pH range when incubated at 4°C for 24 h. We then examined the effects of temperature on the activity of the enzymes at pH 9.0. They both showed maximum activity at 30°C toward *p*-nitrophenyl caprate (Fig. 4A). The wLipA lipase can exhibit 20% of its highest activity at 0°C and 37% at 10°C where rLipA show 15% and 30% activity, respectively, at the corresponding temperatures. Heat treatment at 60°C for 5 min on wLipA and rLipA resulted in a 65% and 70% decrease, respectively, in enzyme activity, whereas their half-life at 30°C is about 4.5 h (data not shown). Both the wild-type lipase and the soluble expressed recombinant proteins are cold-adapted enzymes, which have to be maintained below 10°C for optimal stability.

Effects of Metal Ions pH and Chemical Reagents on the Enzyme Activity

The purified wLipA and rLipA, respectively, were incubated with various metal ions, and the remaining activity was measured with *p*-nitrophenyl caprate as the substrate at 30°C. wLipA and rLipA were both activated by Ca^{2+} (1.5-fold), Mg^{2+}, Mn^{2+} (1.1- to 1.3-fold) and was not affected by Na^{+}, K^{+}, Li^{+}, but was strongly inhibited by Cd^{2+}, Zn^{2+}, Co^{2+}, Fe^{3+}, Hg^{2+}, Fe^{2+}, Rb^{2+}, Cu^{2+} and ethylenediaminetetraacetic acid (EDTA; Table 2). The effects of various detergents on



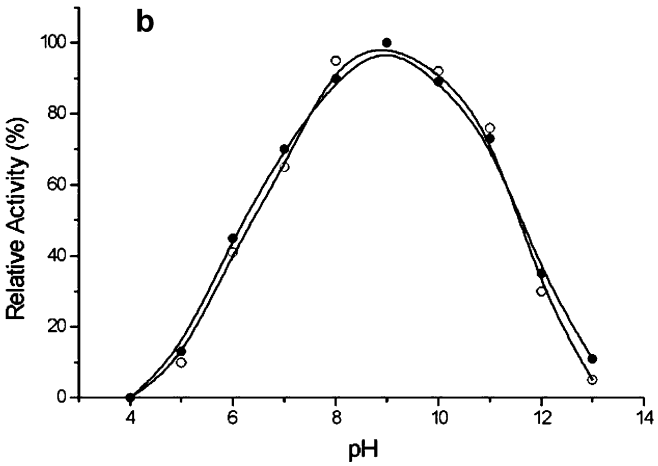


Fig. 4 Effect of temperature (**A**) and pH (**B**) on the lipase activity of wLipA and rLipA. **A**: The enzymes was incubated with a mixture containing 20 mM Tris-HCl(pH 9.0). 1% acetonitrile, 4% isopropanol and 0.1 mM *p*-nitrophenyl caprate at various temperatures for 10 min, and *p*-nitrophenol formed was measured. The value obtained at 30°C was taken as 100% wLipA, solid circle, rLipA, hollow circle. **B**: The enzymes was incubated with 0.1 mM *p*-nitrophenyl caprate in 20 mM Tris-HCl buffer at various pH values at 25°C for 10 min and *p*-nitrophenol formed was measured wLipA, solid circle, rLipA, hollow circle

Table 2 Effects of various metal ions on wLipA and rLipA activity

Divalent cation (5mM) or EDTA (5mM)	Relative activity (%)[a] wLipA	Relative activity (%)[a] rLipA	Divalent cation (5mM) or EDTA (5mM)	Relative activity (%)[a] wLipA	Relative activity (%)[a] rLipA
–	100	100	Mn^{2+}	129	136
EDTA	65	58	Cd^{2+}	40	35
Na^{+}	97	102	Zn^{2+}	40	41
K^{+}	100	103	Cu^{2+}	100	104
Li^{+}	105	101	Co^{2+}	20	25
Ca^{2+}	156	147	Fe^{3+}	58	56
Mg^{2+}	115	123	Fe^{2+}	46	48
Rb^{2+}	55	56	Hg^{2+}	26	31

[a] Metal chlorides were used in the assay
[b] Both LipA (0.05 mg/ml) were incubated in 20 mM Tris–HCl (pH 9.0) containing each compound at 4°C for 1 h. Remaining activity was determined with 0.1 mM *p*-nitrophenyl caprate at 30°C for 10 min and expressed as the percent of the control value (with no addition)

wLipA and rLipA are summarized in Table 3. In general, we observed an increase in both enzymes' activity with the addition of detergents. The presence of SDS, DFP, PMSF, CHAPS, and PCMB with 0.005% concentration slightly increased the activity (15 to 30%), and approximately 1.5-fold to twofold increases in activity were observed with Triton X-100, Tween 80, Tween 40, Span 60, and Span 40. However, we found that levels of activity of the enzyme after incubation with these detergents at 1% (*w*/*v*) for 1 h did not display significant differences from the activity observed without addition of detergent.

Substrate and Positional Specificity

The hydrolytic activity of the wLipA and rLipA lipases towards various simple *p*-nitrophenyl esters and triglycerides was examined at 30°C and pH 9.0. As shown in Table 4, the enzymes hydrolyzed *p*-nitrophenyl caprate (C_{10} acyl group) and tributyrin (C_4 acyl group) at the highest velocity among the other *p*-nitrophenyl esters and triglycerides, respectively. The enzyme LipA hydrolyzed short to middle-chain fatty acids rather than long-chain fatty acids of simple *p*-nitrophenyl esters and triglycerides.

Discussion

pLLP-OmpA vector was constructed by adding histidine residues to the LipA enzyme, and purification was conducted by metal-chelating chromatography from the extract of recombinant *E. coli* Top 10 F′ cells harboring a plasmid coding for the *lipA* gene, which was transcripted under the control of the Ipp promoter. The wild-type lipase (wLipA) from *Pseudomonas* sp. strain 7323 was also purified for comparison. The His tag additions did not reduce the specific activities of rLipA and retained similar physicochemical properties to wLipA. Both wLipA and rLipA exhibited optimal temperature at 30°C towards *p*-

Table 3 Effects of detergents on wLipA and rLipA activity

Detergent	Relative activity (%)[a] wLipA1	Relative activity (%)[a] rLipA1	Residual activity (%)[b] wLipA1	Residual activity (%)[b] rLipA1
None	100	100	100	100
DFP	118	121	92	90
PMSF	124	119	80	82
CHAPS	134	126	80	79
PCMB	115	117	85	90
SDS	130	128	82	82
Triton X-100	165	160	82	85
Tween 80	186	190	83	86
Tween 40	202	200	87	85
Span 60	215	218	100	104
Span 40	172	180	95	91

DFP diisopropylfluorophoshate, *PMSF* phenylmethane sulfonylfluoride, *PCMB*, *p*-chloromercuribenzoic acid, *CHAPS* 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate
[a] In the presence of 0.005% detergent
[b] After 1 h of incubation with 1% detergent

Table 4 Substrate specificity of the purified wLipA and rLipA

Substrate	Sp Act of wLipA (U/mg)	Relative activity (%) wLipA1	Sp Act of rLipA (U/mg)	Relative activity (%) rLipA1
Triglyceride				
Triacetin (C2:0)	525	7	570	6
Tributyrin (C4:0)	7,360	100	8,532	100
Tricaproin (C6:0)	6,360	86	6,900	80
Tricaprylin (C8:0)	4,850	65	5,250	61
Tricaprin (C10:0)	2,670	36	2,900	34
Trimyristin (C14:0)	1,585	21	1,690	19
Tripalmitin (16:0)	860	11	925	10
Olive oil	2,530	34	2,760	32
p-Nitrophenyl esters				
p-Nitrophenyl acetate (C2)	12	2	16	2
p-Nitrophenyl butyrate (C4)	38	7	50	7
p-Nitrophenyl caproate (C6)	110	21	137	19
p-Nitrophenyl caprylate (C8)	252	49	326	47
p-Nitrophenyl caprate (C10)	520	100	690	100
p-Nitrophenyl laurate (C12)	286	15	380	54
p-Nitrophenyl myristate (C14)	87	17	115	16
p-Nitrophenyl palmitate (C16)	24	4	32	4
p-Nitrophenyl stearin (C18)	4	0.8	5	0.7

The enzyme reaction was carried out as described in "Materials and Methods"

nitrophenyl caprate under the optimal pH 9.0 and behaved about 20% of the highest activity at 0°C toward the substrate, but they both were heat-unstable above 25°C, which means that both of the enzymes bear the typical property of cold-adapted enzyme (Feller and Gerday 2003). The subunit molecular mass of the purified wLipA or rLipA (67 kDa) was larger than those of typical cold-active or cold-adapted *Pseudomonas* lipase (approximately 30–40 kDa). For example, the PFL from *Pseudomonas fragi* was 32,500 kDa (Alquati et al. 2002); LipP form *Pseudomonas* sp. Strain B11–1 was 33,714 kDa (Choo et al. 1998), and KB-lip from *Pseudomonas* sp. Strain KB700 was 49,924 kDa (Rashid et al. 2001). Although the LipA from strain 7323 showed highest amino acid sequence with PUFA from *P. fluorescent* HU380 (Kojima et al. 2003) and PML from *Pseudomonas* sp. MIS38 (Amada et al. 2000), PUFA and PML manifested the optimal temperature at 45°C and 55°C, respectively.

The primary structure of LipA indicated that it was a member of subfamily I.3 of bacterial lipolytic enzymes according to the classification and properties of bacterial lipolytic enzymes reported previously (Arpigny and Jaeger 1999). The deduced amino acid sequence of strain 7323 lipase is very similar (81–89%) to those of group III or subfamily I.3 lipase. Sequence comparison suggested that the active-site serine residue GHSLG, the N-terminal amino acid sequence, and the C-terminal secretion signals of LipA in strain 7323 are consistent with the case of lipase from *P. fluorescent* HU380 (Kojima et al. 2003), *Pseudomonas* sp. MIS38 (Amada et al. 2000), *P. fluorescens* SIK W1 (Ahn et al. 1999), *P. fluorescens* no. 33 (Kawai et al. 1999), *Pseudomonas* sp. Strain KB700 (Rashid et al. 2001). The glycine-rich repeats of the consensus GGXGXDXUX near the C-terminal always exist in most Gram-negative bacteria proteins to be involved in the protein secretion by a signal peptide-independent mechanism (Salmond and Reeves 1993; Akatsuka et al. 1995; Kojima et al. 2003). These proteins are also secreted through both inner and outer membranes into the growth medium without the aid of an N-terminal signal peptide (Akatsuka et al. 1994).

Both the wLipA and rLipA were dependent on divalent cations, like Mg^{2+}, Mn^{2+}, particularly Ca^{2+}, to improve their activity. It has been reported that the presence of the Ca^{2+} ion greatly enhanced the enzymatic activities of PFL (Alquati et al. 2002), SML (Li et al. 1995), PML (Amada et al. 2000), and KB-Lip (Rashid et al. 2001) due to the Ca^{2+}-binding motifs GXXGXD in the C terminus of the enzymes. KB-Lip displayed a tendency similar to that of LipAs and lost most of its activity in the presence of Zn^{2+}, Co^{2+}, Cu^{2+}, while lipase from strain WIK W1 was not inhibited by them (Lee et al. 1993). Enzyme stability against SDS also differed; LipAs displayed little decrease in activity when incubated with 1% SDS for 1 h as in the case of KB-Lip (Rashid et al. 2001), while 95% of the activity of the enzyme from strain SIK W1 was abolished in the presence of SDS. DFP, PMSF, CHAPS, and PCMB did not significantly affect the lipase activity. In additional, LipAs' activities were greatly improved in the presence of Triton X-100, Tween 80, Tween 40, Span 60, and

Span 40. wLipA and rLipA both hydrolyzed glyceryl tributyrate (C4) most preferably among various triglyceride substrates examined. However, it hydrolyzed triglycerides with longer acyl chain length, including olive oil, with comparable efficiencies. Thus, both of them showed rather wide preference of triglycerides, while they hydrolyzed *p*-nitrophenyl esters as well. wLipA and rLipA both hydrolyzed *p*-nitrophenyl caprate (C_{10}) most preferably and showed the specific activities of 520 and 690 U/mg, respectively. Under the same conditions and with their optimal substrate *p*-nitrophenyl caprate, KB-Lip displayed 543 U/mg (Rashid et al. 2001), while PML showed only 110 U/mg (Amada et al. 2000). Among the triglycerides, tributyrin (C_4) was the preferable substrate to wLipA and rLipA, showing the specific activities of 7,860 and 8,532 U/mg, respectively, while KB-Lip showed 103 U/mg and PML displayed 3,600 U/mg.

In conclusion, the final specific activity of the purified rLipA protein reconstituted agreed with the purified wLipA from strain 7323, demonstrating the full catalytic function of the native lipase. What is more is that LipA highlights very active and stable in several detergents and metal ions mentioned above at low and moderate temperatures. These properties may offer potential economic benefits and are expected to be useful in the treatment of domestic and industrial wastes during the winter in temperate countries and also in the detergent with cold water washing.

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