Molecular cloning and expression of a cold-adapted lipase gene from an Antarctic deep sea psychrotrophic bacterium *Pseudomonas* sp. 7323

JIN-WEI ZHANG [1,2,3*], **RUN-YING ZENG** [2,3*]

[1]*Institute for Biomedical Research, Xiamen University, Xiamen, P.R.China*

[2]*Key Laboratory of Marine Biogenetic Resources, State Oceanic Administration, Xiamen, P. R. China*

[3]*Third Institute of Oceanography, State Oceanic Administration, Xiamen, P. R. China*

[*]Corresponding author:

Tel.: +86 592 2195323; fax: +86 592 2085376.

E-mail addresses: Jin-Wei Zhang; jinweizhang@21cn.com

Run-Ying Zeng; runyingzeng@yahoo.com.cn

Running title: **Cold-adapted lipase from psychrotrophic strain 7323**

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 64466 Da. LipA has a GXSXG motif, which is conserved in lipases/esterases and generally contains the active-site serine. The lipase purified from the *E. 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.

Keywords: Deep Sea Sediment—**Cold-Adapted** Lipase—Sequencing and Expression—Characterization

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; Feller et al., 1996). What`s more, 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 also they 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; P¨ortner, 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 J. L. Arpigny **(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 *E. 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 **(74º25'E, 66º55'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 ºC 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. **T**he 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 ºC 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 named 7323, showing highest lipolytic activity, was picked out for further study.**

General DNA Manipulation. **R**estriction 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. **T**he strain 7323 was cultivated at 15 °C for 3 days in fermentation medium containing 10 g tributyrin, 5 g tryptone, 1g 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 by 50 cm). The column was washed with 1

liter 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 liter. 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, Uppsala, Sweden)** (3 by 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 SDS-PAGE **(Model 491 Prep Cell, Bio-Rad, Hercules, CA, U.S.A.)**.

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. 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, Uppsala, Sweden)** 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.

In order to **overproduce** LipA, the *lipA* gene was amplified by PCR using **rTaq DNA polymerase (Takara)** and a combination of forward (5`-ACT ACG GAT CCC TAT TAA TAC

GCA TAC-3`) and reverse (5`- GTA TCG ATC G*TC A*GG CGA TCA CGA TTC CAT-3`) primers, where the underlines represent the *BamH*I 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 *BamH*I and *Nhe*I and ligated to the large *BamH*I-*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 3h, 4h, 5h. Cells were then harvested by centrifugation at 6000×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, U.S.A), 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 by using slab gels, as described by Laemmli (Laemmli, 1970). The proteins on the gels were stained with Coomassie Brilliant Blue R-250.

Characterization of Wild-Type and Recombinant LipA. **L**ipase activity was measured by the spectrometric method, which 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 min at 30 ºC. The substrate solution was consisted of 100 μM *p*-nitrophenyl monoster and 20 mM Tris-HCl buffer (pH9.0) 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 ºC 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 (pH9.0) 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 min under the above conditions. The protein concentration was measured by the method of Bradford (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 ºC) in 20 mM Tris-HCl buffer, pH 9.0. To examine the thermostability, the enzymes were incubated at various temperatures (5-60 ºC) for 30 min in a 50 mM Tris-HCl buffer (pH 9.0) containing 0.05 mg/ml of BSA, and the residual activity was then determined at 30 ºC 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 preincubated 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 ºC in 20 mM Tris-HCl (pH 9.0) buffers containing 0.005% various detergents.

Nucleotide Sequence Accession Numbers. **T**he nucleotide sequences of 16S rRNA gene and lipase gene (*lipA*) have been deposited in EMBL under accession nos. AM111061 and AM229328 respectively.

Results

Identification of Strain 7323. **T**axonomical 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, 1984). 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

Gene Cloning and Analyzing of the Lipase LipA. **S**equence analysis of the amplified DNA revealed an open reading frame (ORF) of 1854 nucleotides, which encodes a protein of 617 amino acids with a predicted molecular mass of 64 466 Da and isoelectric point of 4.5, 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 *Pseudomonas fluorescens* lipase (LipB) (GenBank AAG22559), 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), which 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).

Fig. 2

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

Table 1

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.** And 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 corresponding with the molecular mass of about 67 000 Da appeared 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 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. **W**ith *p*-nitrophenyl caprate as a substrate, both wLipA and rLipA showed most active at pHs between 8.5 and 9.0. And the pH stability of wLipA and rLipA were stable between pH 6 and 12 at the indicated pH range when incubated at 4 ℃ 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 ℃ toward *p*-nitrophenyl caprate (Fig. 4A). The wLipA lipase can exhibit 20% of its highest activity at 0 ℃, and 37% at 10 ℃, where rLipA show 15% and 30% activity respectively at the corresponding temperatures. Heat treatment at 60 ℃ for 5 min on wLipA and rLipA resulted in a 65% and 70% decrease respectively in enzyme activity, whereas their half-life at 30 ℃ 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 ℃ for optimal stability.

Fig. 4

Effects of Metal Ions pH and Chemical Reagents on the Enzyme Activity. **T**he 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 ℃. 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 EDTA(Table 2). The effects of various detergents on 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 to 2 fold 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% (wt/vol) for 1 h did not display significant differences from the activity observed without addition of detergent.

Substrate and Positional Specificity. **T**he 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 *Escherichia* coli Top 10 F` cells harboring a plasmid coding for the *lipA* gene, which was transcripted under the control of the Ipp promoter. And 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 of wLipA and rLipA exhibited optimal temperature at 30 °C towards *p*-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 showed heat-unstable above 25 °C, which mean 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 *Pseudomonas 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 ℃ and 55 ℃ 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 are always exist in most gram-negative bacteria proteins to involve 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 of 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, lost most 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. And 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 U/mg 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 7860 U/mg and 8532 U/mg respectively, while KB-Lip showed 103 U/mg and PML displayed 3600 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`s more, LipA highlighs 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, also in the detergent with cold water washing.

Acknowledgements

This work was supported by Hi-Tech research and development program of China (No. 2007AA091407).

References

Ahn, J.H., Pan, J.G. and Rhee, J.S. (1999). Identification of the tliDEF ABC transporter specific for lipase in *Pseudomonas fluorescens* SIK W1. *J Bacteriol* 181**:** 1847-1852.

Akatsuka, H., Kawai, E., Omori, K., Komatsubara, S., Shibatani, T. and Tosa, T. (1994). The lipA gene of *Serratia marcescens* which encodes an extracellular lipase having no N-terminal signal peptide. *J Bacteriol* 176**:** 1949-1956.

Akatsuka, H., Kawai, E., Omori, K. and Shibatani, T. (1995). The three genes lipB, lipC, and lipD involved in the extracellular secretion of the *Serratia marcescens* lipase which lacks an N-terminal signal peptide. *J Bacteriol* 177**:** 6381-6389.

Alquati, C., De Gioia, L., Santarossa, G., Alberghina, L., Fantucci, P. and Lotti, M. (2002). The cold-active lipase of *Pseudomonas fragi.* Heterologous expression, biochemical characterization and molecular modeling. *Eur J Biochem* 269**:** 3321-3328.

Amada, K., Haruki, M., Imanaka, T., Morikawa, M. and Kanaya, S. (2000). Overproduction in *Escherichia coli*, purification and characterization of a family I.3 lipase from *Pseudomonas* sp. MIS38. *Biochim Biophys Acta* 1478**:** 201-210.

Arpigny, J.L., Feller, G. and Gerday, C. (1995). Corrigendum to "Cloning, sequence and structural features of a lipase from the antarctic facultative psychrophile *Psychrobacter* immobilis B10" [Biochim. Biophys. Acta 1171 (1993) 331-333]. *Biochim Biophys Acta* 1263**:** 103.

Arpigny, J.L. and Jaeger, K.E. (1999). Bacterial lipolytic enzymes: classification and properties. *Biochem J* 343**:** 177-183.

Bradford, M.M. (1976). A rapid and sensitive method for the quatitation of microgram quantities of protein utilizing the pronciple of protein-dye binding. *Anal Biochem* 72**:** 248-254.

Brockerhoff, H. and Jensen, R.G. (1974). Lipolytic Enzymes. *Acad emic Press, New York*.

Choo, D.W., Kurihara, T., Suzuki, T., Soda, K. and Esaki, N. (1998). A cold-adapted lipase of an Alaskan psychrotroph, *Pseudomonas* sp. strain B11-1: gene cloning and enzyme purification and characterization. *Appl Environ Microbiol* 64**:** 486-491.

Clark, M.S., Clarke, A., Cockell, C.S., Convey, P., Dietrich Iii, H.W., Fraser, K.P.P., Johnston, I.A., Methe, B.A., Murray, A.E., Peck, L.S., Roemisch, K. and Rogers, A.D. (2004). Antarctic Genomics. *Comp Func Geno* 5**:** 230-238.

Clarke, A. and Leakey, R. (1996). The seasonal cycle of phytoplankton, macronutrients and the microbial community in a near shore Antarctic marine ecosystem. *Limnol Oceanogr* 41**:** 1281-1294.

Feller, G. and Gerday, C. (2003). Psychrophilic enzymes: hot topics in cold adaptation. *Nat Rev Microbiol* 1**:** 200-208.

Feller , G., Narinx, E., Arpigny, J.L., Aittaleb, M., Baise, E., Genicot, S. and Gerday, C. (1996). Enzymes from psychrophilic organisms. *FEMS Microbiol Rev* 18**:** 189-202.

Feller , G., Narinx, E., Arpigny, J.L., Zekhnini, S.J. and Gerday, C. (1994). Temperature dependence of growth, enzyme secretion and activity of psychrophilic Antarctic bacteria. *Appl Microbiol Biotechnol* 41**:** 477-479.

Feller, G., Thiry, M., Arpigny, J.L. and Gerday, C. (1991). Cloning and expression in *Escherichia coli* of three lipase-encoding genes from the psychrotrophic antarctic strain *Moraxella* TA144. *Gene* 102**:** 111-115.

Gerday, C., Aittaleb, M., Bentahir, M., Chessa, J.P., Claverie, P., Collins, T., D'amico, S., Dumont, J., Garsoux, G., Georlette, D., Hoyoux, A., Lonhienne, T., Meuwis, M.A. and Feller, G. (2000). Cold-adapted enzymes: from fundamentals to biotechnology. *Trends Biotechnol* 18**:** 103-107.

Kawai, E., Idei, A., Kumura, H. and Shimazaki, K. (1999). The ABC-exporter genes involved in the lipase secretion are clustered with the genes for lipase, alkaline protease, and serine protease homologues in *Pseudomonas fluorescens* no. 33. *Biochim Biophys Acta* 1446**:** 377-382.

Kojima, Y., Kobayashi, M. and Shimizu, S. (2003). A novel lipase from *Pseudomonas fluorescens* HU380: gene cloning, overproduction, renaturation-activation, two-step purification, and characterization. *J Biosci Bioeng* 96**:** 242-249.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature,* 227**:** 680-685.

Lee, Y.P., Chung, G.H. and Rhee, J.S. (1993). Purification and characterization of *Pseudomonas fluorescens* SIK W1 lipase expressed in *Escherichia coli. Biochim Biophys Acta* 1169**:** 156-169.

Li, X., Tetling, S., Winkler, U.K., Jaeger, K.E. and Benedik, M.J. (1995). Gene cloning, sequence analysis, purification, and secretion by *Escherichia coli* of an extracellular lipase from *Serratia marcescens*. *Appl Environ Microbiol* 61**:** 2674-2680.

P¨Ortner, H.O. (2002). Climate variations and the physiological basis of temperature dependent biogeography: systematic to molecularhierarchy of thermal tolerance in animals. *Comp Biochem Physiol A* 132**:** 739-761.

Palleroni, N. J. (2005). Genus I. Pseudomonas. In Bergey's Manual of Systematic Bacteriology, 2nd edn, vol. 2, pp. 323–379. Edited by D. J. Brenner, N. R. Krieg, J. T. Staley and G. M. Garrity. New York: Springer.

Peck, L.S. (2002). Ecophysiology of Antarctic marine ectotherms: limits to life. *Polar Biol* 25**:** 31-40.

Rashid, N., Shimada, Y., Ezaki, S., Atomi, H. and Imanaka, T. (2001). Low-temperature lipase from psychrotrophic *Pseudomonas* sp. strain KB700A. *Appl Environ Microbiol* 67**:** 4064-4069.

Salmond, G.P.C. and Reeves, P.J. (1993). Membrane traffic wardens and protein secretion in Gram-negative bacteria. *Trends Biochem Sci* 18**:** 7-12.

Suzuki, T., Nakayama, T., Kurihara, T., Nishino, T. and Esaki, N. (2001). Cold-active lipolytic activity of psychrotrophic *Acinetobacter* sp. strain No. 6. *J Biosci Bioeng* 92**:** 144-148.

Verde, C., De Rosa, M.C., Giordano, D., Mosca, D., De Pascale, D., Raiola, L., Cocca, E., Carratore, V., Giardina, B. and Di Prisco, G. (2005). Structure, function and molecular adaptations of haemoglobins of the polar cartilaginous fish Bathyraja eatonii and Raja hyperborea. *Biochem J* 389**:** 297-306.

Yen, M.R., Peabody, C.R., Partovi, S.M., Zhai, Y., Tseng, Y.H. and Saier, M.H. (2002). Protein-translocating outer membrane porins of Gram-negative bacteria. *Biochim Biophys Acta* 1562**:** 6-31.

Zhang, J.W., Lin, S. and Zeng, R.Y. (2007). Cloning, Expression, and Characterization of a Cold-Adapted Lipase Gene from an Antarctic Deep-Sea Psychrotrophic Bacterium, *Psychrobacter* sp. 7195. *J Microbiol Biotechnol* 17**:** 604-610.

Zhang, J.W. and Zeng, R.Y. (2007). Psychrotrophic amylolytic bacteria from deep sea sediment of Prydz Bay, Antarctic: diversity and characterization of amylases. *W J Microbiol Biotechnol* 23: 1551-1557.

Fig.1 Phylogenetic tree based on 16S rRNA gene sequence of strain 7323. The tree was constructed by neighbor-joining method.

Fig. 2 Alignment of the amino acid sequence of lipases from *Pseudomonas* sp. 7323 (LipA).

Fig. 3. Polyacrylamide gel electrophoresis of rLipA and wLipA.

Fig. 4. Effect of temperature（A）and pH（B）on the lipase activity of wLipA and rLipA.

Table 1. (A) Purification of wLipA from *Pseudomonas* sp. 7323; (B) Purification of rLipA from *E. coli* Top 10F` harboring plasmid pLLP-OmpA-*lipA.*

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

Table 3. Effects of detergents on wLipA and rLipA activity.

Table 4. Substrate specificity of the purified wLipA and rLipA.

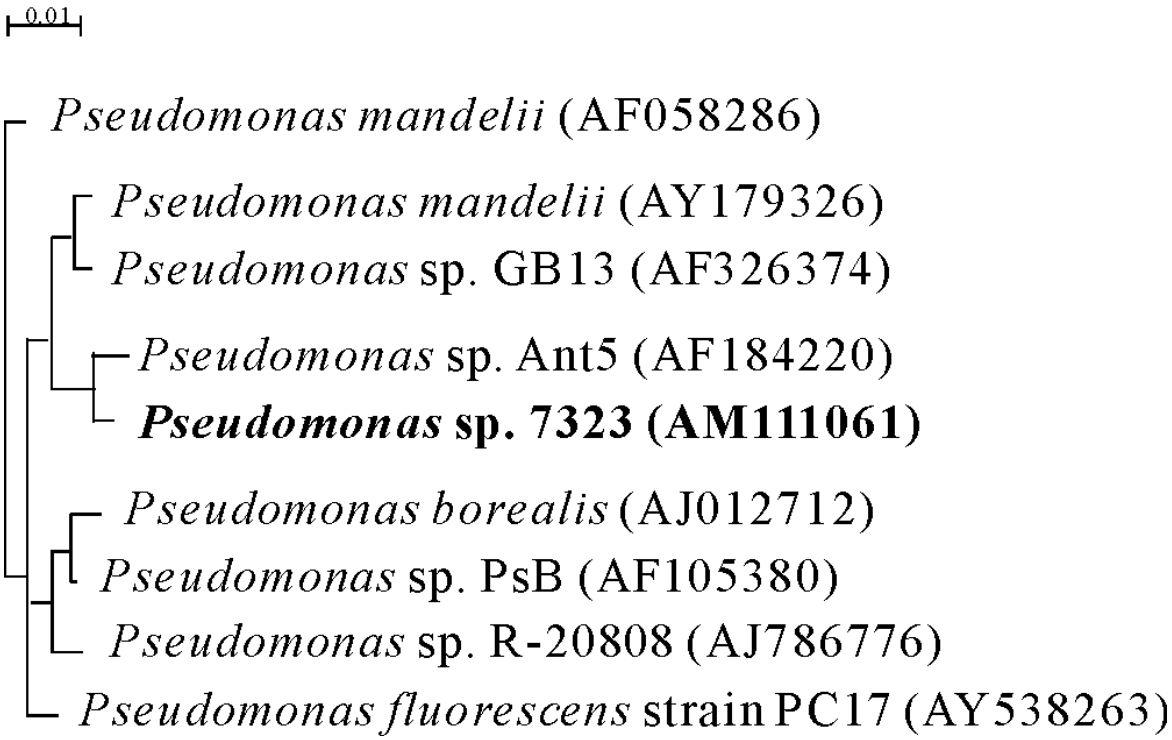

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequence of strain 7323.

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.

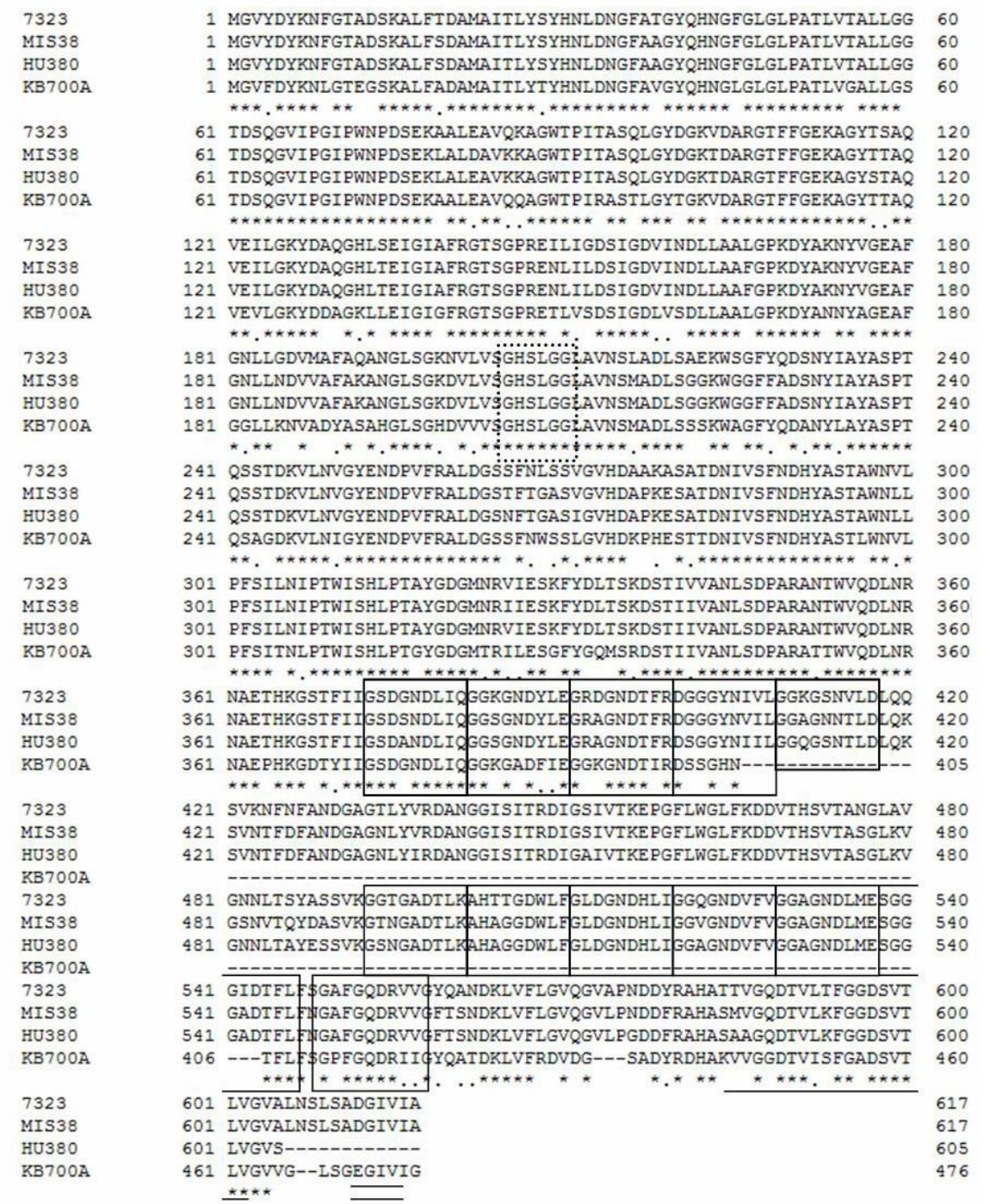

Fig. 2. Alignment of the amino acid sequence of lipases from *Pseudomonas* sp. 7323 (LipA). 7323: *Pseudomonas* sp.7323 lipase; MIS38: *Pseudomonas* sp. MIS38; HU380: *Pseudomonas fluorescens* HU380; KB700A: *Pseudomonas* sp. KB700A; The active-site consensus GXSXG sequence motif is boxed with broken 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. (A) Purification of wLipA from *Pseudomonas* sp. 7323; (B) Purification of rLipA from *E. coli* Top 10F` Harboring Plasmid pLLP-OmpA-*lipA*

Purification method	Total protein (mg)	Total Activity (U[a])	Sp Act (U/mg)	Purifi-cation (fold)	Yield (%)
(A)					
Culture supernatant[b]	28	1221	43.6	1	100
DEAE Sepharose CL-6B	8.5	1001	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
(B)					
Cell extract[c]	76	3936.8	51.8	1	100
Ni-NTA column	2.8	1937.3	691.9	13.4	49.2
Prep. SDS-PAGE	2.1	1588.2	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 concentrated10-fold to 200 ml. [c] Cell extract from two liters of culture.

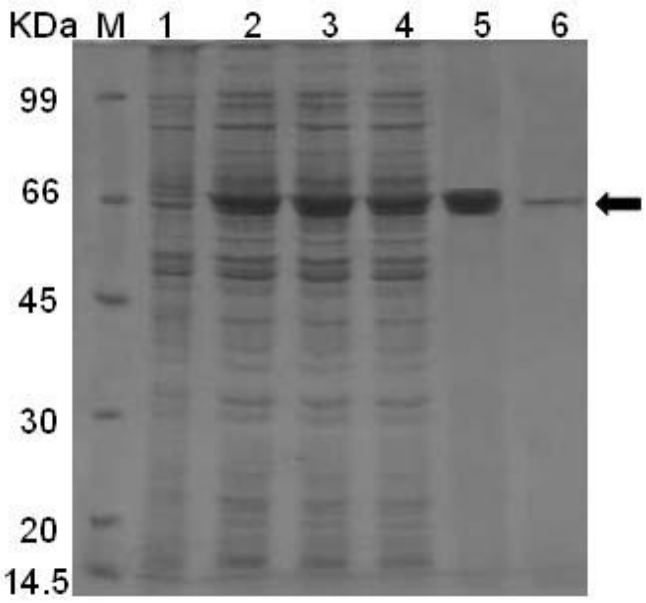

Fig. 3. Polyacrylamide gel electrophoresis of rLipA and wLipA.
Lane M, molecular weight markers; lane 1, *E. coli* Top 10 F` carrying pLLP-OmpA-*lipA* without IPTG induction for 6h at 30 °C; lane 2, 3, 4, *E. coli* Top 10 F` carrying pLLP-OmpA-*lipA* after IPTG induction for 6h, 5h, 4h respectively at 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.

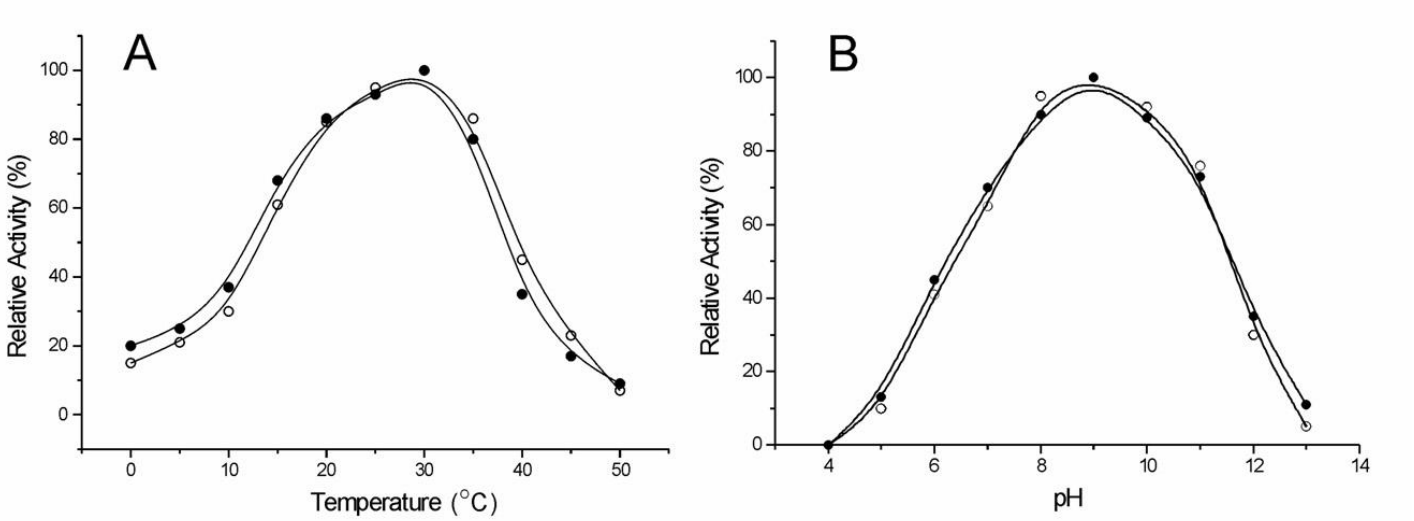

Fig. 4. Effect of temperature（A）and pH（B）on the lipase activity of wLipA (●) and rLipA(○).

（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 fro 10 min, and *p*-nitrophenol formed was measured. wLipA, solid circle; rLipA, hollow circle.

Table 2. Effects of Various Metal Ions[a] on wLipA and rLipA Aactivity

Divalent cation (5 mM) or EDTA (5 mM)	Relative activity (%)[a] wLipA	Relative activity (%)[a] rLipA	Divalent cation (5 mM) or EDTA (5 mM)	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).

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

[a] In the presence of 0.005% detergent.

[b] After 1 h of incubation with 1% detergent.

DFP, diisopropylfluorophoshate; PMSF, phenylmethane sulfonylfluoride;
PCMB, p-chloromercuribenzoic acid;
CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate.

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**
(A) Triglyceride				
Triacetin (C2:0)	525	**7**	570	**6**
Tributyrin (C4:0)	7360	**100**	8532	**100**
Tricaproin (C6:0)	6360	**86**	6900	**80**

Tricaprylin (C8:0)	4850	**65**	5250	**61**
Tricaprin (C10:0)	2670	**36**	2900	**34**
Trimyristin (C14:0)	1585	**21**	1690	**19**
Tripalmitin (16:0)	860	**11**	925	**10**
Olive oil	2530	**34**	2760	**32**
(B) *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.