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Cloning, Expression, and Characterization of a Cold-Adapted Lipase Gene from an Antarctic Deep-Sea Psychrotrophic Bacterium, *Psychrobacter* sp. 7195

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Abstract A psychrotrophic strain 7195 showing extracellular lipolytic activity towards tributyrin was isolated from deep-sea sediment of Prydz Bay and identified as a *Psychrobacter* species. By screening a genomic DNA library of *Psychrobacter* sp. 7195, an open reading frame of 954 bp coding for a lipase gene, *lipA1*, was identified, cloned, and sequenced. The deduced LipA1 consisted of 317 amino acids with a molecular mass of 35,210 kDa. It had one consensus motif, G-N-S-M-G (GXSXG), containing the putative active-site serine, which was conserved in other cold-adapted lipolytic enzymes. The recombinant LipA1 was purified by column chromatography with DEAE Sepharose CL-4B, and Sephadex G-75, and preparative polyacrylamide gel electrophoresis, in sequence. The purified enzyme showed highest activity at 30°C, and was unstable at temperatures higher than 30°C, indicating that it was a typical cold-adapted enzyme. The optimal pH for activity was 9.0, and the enzyme was stable between pH 7.0–10.0 after 24 h incubation at 4°C. The addition of Ca^{2+} and Mg^{2+} enhanced the enzyme activity of LipA1, whereas the Cd^{2+}, Zn^{2+}, Co^{2+}, Fe^{3+}, Hg^{2+}, Fe^{2+}, Rb^{2+}, and EDTA strongly inhibited the activity. The LipA1 was activated by various detergents, such as Triton X-100, Tween 80, Tween 40, Span 60, Span 40, CHAPS, and SDS, and showed better resistance towards them. Substrate specificity analysis showed that there was a preference for trimyristin and *p*-nitrophenyl myristate (C_{14} acyl groups).

Keywords: Deep-sea sediment, cold-adapted lipase, *Psychrobacter*, recombinant protein

Enzymes from psychrotrophic and psychrophilic microorganisms have recently received increasing attention, owing to their relevance for both basic and applied research [1, 8]. This effort has been stimulated by the recognition that cold-adapted enzymes might offer novel opportunities for biotechnological exploitation based on their high catalytic activity at low temperatures, low thermostability, and unusual specificities. These properties offer potential economic benefits in different fields such as detergents, textile and food industries, and bioremediation of polluted soils and wastewaters at low and moderate temperatures, through substantial energy savings in large-scale processes that would not require the expensive heating of reactors [7, 12].

Lipases (triacylglycerol acyl hydrolases) (EC 3.1.1.3) are characterized by their activity towards hydrolyzing water-insoluble emulsified triacylglycerols to liberate fatty acids and glycerols [16, 17, 23, 30]. These enzymes are excreted by many bacteria and fungi. According to Arpigny and Jaeger [3], 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-adapted or cold-active lipolytic enzymes isolated from psychrophilic bacteria have been studied, such as lipase (PFL) produced by *Pseudomonas fragi* (X14033) [1]; cold-adapted lipase (KB-Lip) produced by a psychrotrophic *Pseudomonas* sp. strain KB700A [23]; cold-adapted lipase (LipP) excreted by an Alaskan psychrotrophic *Pseudomonas* sp. strain B11-1 [7]; extracellular esterase (X67712) from *Psychrobacter immobilis* B10 [5]; and *Moraxella* sp. TA144 [10].

We have isolated several psychrotrophic bacteria producing cold-adapted lipase from an Antarctic deep-sea sediment. One such strain, which showed sigmoidal growth even at −3°C and had high activity and better thermostability, was further studied, including the gene cloning of its lipase

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gene (*lipA1*) and the purification and characterization of the recombinant enzyme LipA1.

MATERIALS AND METHODS

Sample Collection

The deep-sea sediment was collected by a multicore sampler at the depth of 900 m of site PN5-6 (740°25'E, 66°55'S) during the 18th cruise of the Chinese Antarctic Research (Nov. 2001-Mar. 2002). The sediment was transferred to sterile falcon tubes in a clean bench and kept at 4°C until use.

Isolation of Psychrotrophic 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 supernatant was grown at 10°C on marine 2216E medium containing 0.5% tryptone, 0.1% yeast extract, 0.01% $FePO_4$, and ASW. The strains recovered from marine 2216E agar plates were tested for their lipolytic activity by inoculating them on the screen medium plates containing 0.1% yeast extract, 0.01% $FePO_4$, 3.4%NaCl, and 1% tributyrin. A strain showing the highest lipolytic activity, 7195, was picked out for further study.

Construction of Genomic DNA Library and Screening for Lipase Gene

The chromosome DNA was prepared by standard methods [24] and partially digested with Sau3AI. The digested DNA fragments with sizes between 1 to 5 kb were recovered from the agarose gel by electroelution and then ligated with pUC118, which had been previously digested with BamHI. The genomic DNA library was constructed by transforming the recombined plasmids into competent *E. coli* BL21(DE3) [24]. A total of 28,000 clones were re-inoculated on the Luria-Bertani (LB) agar plates supplemented with ampicillin (50 μg/ml), 50 μM isopropyl-β-D-thiogalactopyranoside (IPTG), 1% tributyrin, and 1% gum arabic. The clones showing lipase activity were identified by the clear zone on the plates after being incubated at 30°C for 24 h. Then, they were preserved in glycerol stock by adding 300 μl of glycerol 100% in 600 μl of fresh LB culture, vortex, and keep at −80°C.

Lipase Activity Assay

Lipase activity was determined by measuring the absorbance at 405 nm of the *p*-nitrophenol released from *p*-nitrophenyl palmitate [21, 23]. One unit of the activity (U) was defined as the amount of enzyme releasing 1 μmol *p*-nitrophenol per min at 30°C. The substrate solution consisted of 100 μM *p*-nitrophenyl palmitate 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 purified LipA1 was incubated at 30°C for 10 min. The reaction was terminated by adding 2 ml of 4 M NaOH, and then the amount of *p*-nitrophenol liberated was measured spectrometrically at 405 nm.

As for the triglyceride substrate, the lipase activity was examined by titrating free fatty acids liberated from substrate with alkali [26]. The reaction mixture, consisting of 5 ml of substrate (25% triglyceride emulsified with a 1.5% polyvinylalcohol solution), 4 ml of 20 mM Tris-HCl buffer (pH 9.0), 100 μl of 0.5 M $CaCl_2$, and 900 μl of enzyme solution, was incubated at 30°C for 30 min with magnetic stirring at 500 rpm. The reaction was stopped by the addition of 20 ml ethanol, and the liberated fatty acid was titrated with 10 mM NaOH. One unit of lipase activity was defined as the amount of enzyme required to release 1 μmol of fatty acids per min under these conditions.

Purification of LipA1

The purification of LipA1 was achieved at 4°C as follows, where 20 mM Tris-HCl (pH 9.0) was used as the standard buffer unless otherwise stated. The recombinant *E. coli* BL21 (DE3) harboring pUC-*lipA1* was cultured in LB medium containing ampicillin (0.2 mg/ml) and 50 μM IPTG for 12 h at 30°C. Then, 20 ml of the culture was inoculated into 2 l of the same medium and incubated for 1 day at 25°C. The harvested cells were disrupted by sonication. The supernatant was subjected to a two-step ammonium sulfate precipitation, and the protein precipitation at a salt saturation between 25% and 75% was resuspended in buffer. After dialysis, the pre-purified protein was applied to chromatography on a DEAE Sepharose CL-4B (Pharmacia) column and a Sephadex G-75 (Pharmacia) column in sequence. For the ion-exchange chromatography, the protein was eluted in a linear gradient of 0.2 to 2.0 M NaCl with standard buffer. For the gel-filtration chromatography, the enzyme was eluted with a linear gradient of 0.005 to 1.0 M Tris-HCl buffer (pH 9.0). Between these two chromatography steps, the active fraction was concentrated by ammonium sulfate precipitation at 75% saturation and dialyzed against the standard buffer. Finally, the enzyme preparation eluted from the Sephadex G-75 column was desalted and concentrated by dialysis and lyophilization, and then was applied to preparative SDS-polyacrylamide gel electrophoresis (PAGE) (Model 491 Prep Cell, Bio-Rad).

Characterization of Lipase

The optimal activity temperature of the purified LipA1 was determined by measuring the activity at temperatures ranging from 0°C to 50°C with the interval of 5°C. The thermostability of the purified LipA1 was examined by

measuring the residual activities of enzyme fractions, which were pre-incubated at 10°C, 30°C, and 60°C in 20 mM Tris-HCl (pH 9.0) for different periods of time.

The activities of LipA1 in buffers with different pH values at 30°C were detected to analyze the effect of pH on the lipase activity. The buffers included Glycine-HCl (pH 2.2, 3.0, and 3.6); sodium acetate (pH 3.6, 4.5, and 5.6); Tris-malate (pH 5.6, 6.6, and 7.4); Tris-HCl (pH 7.4, 8.0, 8.6, and 9.0); and Glycine-NaOH (pH 8.6, 9.0, 9.5, 10.6, 11, and 12). The pH stability of LipA1 was determined by measuring the residue activity after 24-h incubation in 20 mM Tris-HCl buffer with different pH values at 4°C.

The effects of metal ions and detergents on LipA1 were analyzed by measuring the activities in the presence of 5 mM various metal ions and 0.005% detergents, respectively. The resistances of LipA1 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% of various detergents.

Nucleotide Sequence Accession Numbers

The nucleotide sequences of the 16S rRNA gene, *lipA1*, and pAL1-3 have been deposited in the EMBL under the accession numbers AM111050, AM229326, and AM229327, respectively.

RESULTS AND DISCUSSION

Identification of Strain 7195

The optimal and highest temperatures for growth of strain 7195 were 15°C and 35°C, respectively, exhibiting sigmoidal growth even at −3°C. Because it was not able to grow at 37°C or more, we could not obtain all the data of the substrate utilization by the microorganism identification system (MicroStation, BIOLOG), which made it unidentifiable. As determined by the 16S rRNA gene, it was most closely related to the genus *Psychrobacter* (Fig. 1), showing the highest similarity (99.18%) to *Psychrobacter* sp. CK1. Thus, we placed the strain in the genus *Psychrobacter*, as *Psychrobacter* sp. 7195.

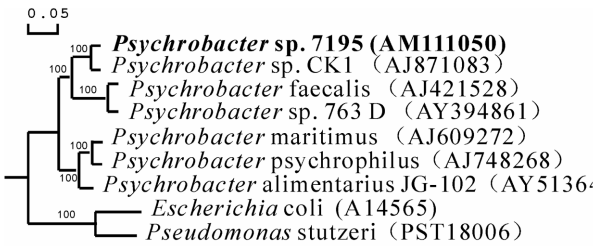

Fig. 1. Phylogenetic tree based on the 16S rRNA gene sequence of strain 7195.

The phylogenetic tree was constructed from a matrix of pairwise genetic distances by the maximum-parsimony algorithm and the neighbor-joining method using the DNAMAN program. The scale bar shows 0.05 substitutions per base position. Numbers refer to the bootstrap values for each node out of a total of 100 replicate resamplings. The strain 7195 is indicated by bold letters.

The generation times of *Psychrobacter* sp. 7195 in LB at 4, 8, 15, and 25°C were 6, 3, 1.5, and 1 h, respectively. The lipase activity in culture supernatant of the culture run at 25°C was 8% less than what was produced by the cells grown at 10°C. It decreased slightly with increasing temperature.

Cloning of the *lipA1* Gene

A *Psychrobacter* sp. 7195 genomic library with a total of 28,000 recombinants with a mean insert size of 1–5 kb was constructed and screened for lipase activity. Five recombinant colonies producing clear zones were isolated and the size of DNA insert verified. One clone with the insert size of about 1,200 bp was selected and the plasmid designated pAL1-3. It produced a relatively clearer and larger clear zone compared with other recombinants at 25°C. The nucleotide sequence of the genomic DNA insert of pAL1-3 was determined to be 1,208 bp. Since the recombinant colony harboring pAL1-3 formed a clear zone in the presence of IPTG, LipA1 was probably expressed under the control of its inherent promoter in *E. coli*. Analysis of the insert DNA sequence with DNAMAN (Version 5.2) software revealed a single open reading frame with the size of 954 bp, which encoded a putative protein of 317 amino acids with a predicted molecular weight of 35,210. A putative ribosome binding site (5'-CAAGGA-3') was found seven bases upstream from the initial codon ATG. BLAST analysis indicated that this gene was homologous to bacterial lipase genes, and it was designated *lipA1*.

Comparison of Amino Acid Sequences

LipA1 showed a high sequence similarity to Lip1 from *Psychrobacter immobilis* B10 [4] and Lip3 from *Moraxella* sp. TA144 [9], with amino acid sequence identity of 93% and 85%, respectively. They all belonged to Family V lipolytic enzymes [3]. The alpha/beta hydrolase fold from *Psychrobacter cryohalolentis* K5 and the esterase from *Pseudomonas aeruginosa* PAO1 [25] also showed some sequence identity to those of LipA1, with homologies of 79% and 35%, respectively. The most probable cleavage site for the signal peptide of LipA1 would be between Ala-27 and Val-28 in the prolipase sequence [4, 5, 29]. Numerous lipases present a wide diversity of amino acid sequences but all contain a consensus motif Gly-X1-Ser-X2-Gly, except for Family II lipase [3], which was also present in LipA1 starting at residue 140. Furthermore, another consensus peptide, His-Gly, present in the N-terminal part of all known lipases except for the fungal lipases [5], was also present in LipA1 (Fig. 2). Therefore, Ser142 probably acted as the nucleophile to form an acyl intermediate with the substrate, as many hydrolytic enzymes do. The active-site serine was encoded by AGC in the same

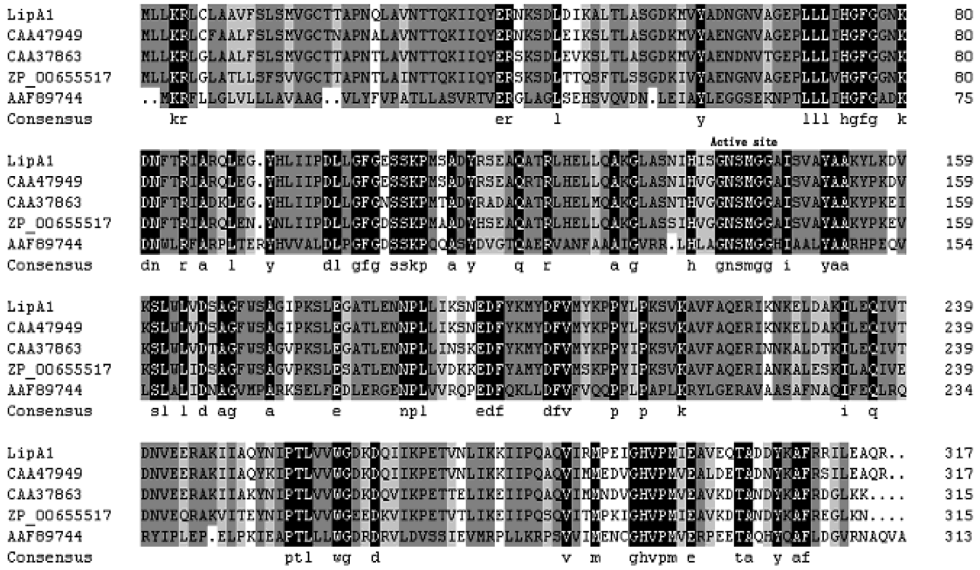

Fig. 2. Amino acid sequence alignment between the LipA1 and other four lipases with high identity.
LipA1: lipase from *Psychrobacter* sp. 7195; CAA47949: lipase (Lip1) from *Psychrobacter immobilis* B10; CAA37863: lipase (Lip3) from *Moraxella* sp. TA144; ZP_00655517: Alpha/beta hydrolase fold from *Psychrobacter cryohalolentis* K5; AAF89744: esterase from *Pseudomonas aeruginosa* PAO1.

manner as many other lipases: AGY (Y, a pyrimidine) is known as the common code for the active-site serine residue of most lipases [22].

Purification of LipA1

LipA1 was purified with a 13.7% yield by 35-fold purification (Table 1). The purified LipA1 showed a single band equal to the molecular mass of about 35 kDa on SDS-PAGE, which was consistent with the subunit molecular weight (35,210) of LipA1 deduced from the nucleotide sequence of *lipA1* (Fig. 3). The molecular masses of reported cold-adapted lipases varied from 30 to 50 kDa, mostly around 30–40 kDa [15, 27]. For example, the PFL from *Pseudomonas fragi* was 32,500 kDa [1]; LipP form *Pseudomonas* sp. Strain B11-1 was 33,714 kDa [7]; Lip1 from *Psychrobacter immobilis* B10 was 35,288 kDa [4]; KB-lip from *Pseudomonas* sp. Strain KB700 was 49,924 kDa [23]; and Lip3 and Lip2 from *Moraxella* TA144 were 34,662 kDa and 47,222 kDa, respectively [10]. By contrast, the molecular mass of mesophilic lipases was bigger than that of cold-adapted lipases; for instance, the PUFA (with optimal temperature at 45°C) from *Pseudomonas fluorescent* HU380 was 64,000 kDa [18], and PML (with optimal temperature at 55°C) from *Pseudomonas* sp. MIS38 was 64,510 Da [2].

Effects of Temperature on the Enzyme Activity

The LipA1 showed maximum activity at 30°C toward *p*-nitrophenyl palmitate (Fig. 4A) and exhibited 18% and 35% of its highest activity at 0°C and 10°C, respectively. To our knowledge, the optimal activity temperature of LipA1 was lower than that of other cold-active lipases reported, except for PFL from *Pseudomonas fragi* [1], which displayed the highest activity at 29°C.

The thermostability study of LipA1 revealed that it was stable at 10°C, whereas it retained half of its activity after 5 h and 15 min incubation at 30°C and 60°C, respectively (Fig. 4B). The rapid inactivation at high temperature of LipA1 was a typical property of cold-adapted enzymes. LipA1 was a little more stable than lipases from other psychrotrophs at high temperature; *e.g.*, *Pseudomonas* sp. Strain KB700 [23], *Pseudomonas fragi* [1], *Pseudomonas* sp. Strain B11-1 [7], and *Acinetobacter* O_{16} [6]. Heat treatment at 60°C for 5 min resulted in a 70% decrease in the activity of KB-lip [23]. The activity of PFL [1] and LipP [7] decreased rapidly after a few minutes of incubation

Table 1. Purification of LipA1 from *E. coli* BL21(DE3) harboring pAL1-3.

Purification method	Total protein (mg)	Total activity (U[a])	Special activity (U/mg)	Purification (fold)	Yield (%)
Cell extract	168	5,150	31	1	100
DEAE Sepharose CL-4B	5.7	1,400	246	7.9	27
Sephadex G-75	0.9	950	1,055	34	18
Prep. SDS-PAGE	0.65	705	1,084	35	13.7

[a]Activity was measured by determination of *p*-nitrophenol formed from *p*-nitrophenyl palmitate as a substrate.

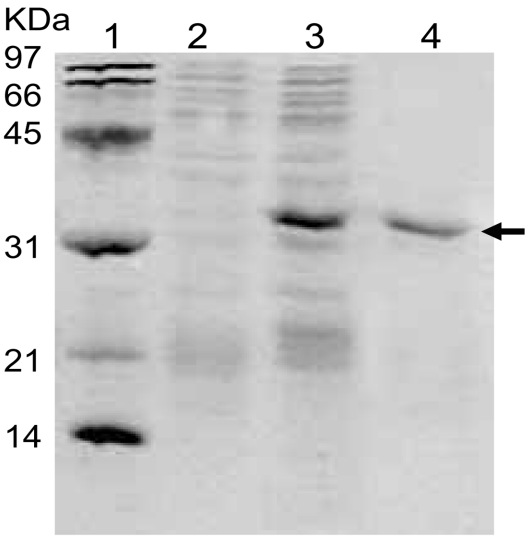

Fig. 3. SDS-PAGE of LipA1.
Lane 1, molecular weight markers; lane 2, *E. coli* BL21(DE3) carrying pUC118 after IPTG induction for 24 h at 25°C; lane 3, *E. coli* BL21(DE3) carrying pUC118 harboring pAL1-3 after IPTG induction for 24 h at 25°C; and lane 4, the purified LipA1 after DEAE Sepharose CL-4B and Sephadex G-75 chromatographies (amount of protein loaded, 10 μg). The position of LipA1 is indicated by an arrow.

at 50°C and 45°C, respectively. One exceptional case was the lipase of *Moraxella* TA144 [11] isolated from the Antarctic, which also kept nearly 15% of its activity after 5 h incubation at 60°C.

Effects of pH on the Enzyme Activity

LipA1 was most active at pH 9.0 (Fig. 4C), which was similar to other alkaline lipases isolated from psychrotrophs. The pH optima for the reactions catalyzed by *Pseudomonas fragi* (PFL) [1], *Pseudomonas* sp. strain B11-1 (LipP) [7], *Pseudomonas* sp. strain KB700 (KB-lip) [23], *Pseudomonas cepacia*, *Pseudomonas pseudoalcaligenes*, and *Pseudomonas mendocina* [28] were reported to be between 8 and 9. The LipA1 was stable at the pH range from 7 to 10 when incubated at 0°C for 24 h using *p*-nitrophenyl palmitate as substrate, retaining more than 90% residue activity (Fig. 4D). Among other cold-adapted lipases isolated from psychrotrophs, the recombinant lipase (LipP) from *Pseudomonas* sp. strain B11-1 was recorded to be stable between pH 6 and 9 [7], and the recombinant lipases from *Pseudomonas pseudoalcaligenes* and *Pseudomonas mendocina* were reported to be stable between pH 5 and 10, whereas the enzyme from *Pseudomonas cepacia* DSM 50181 was

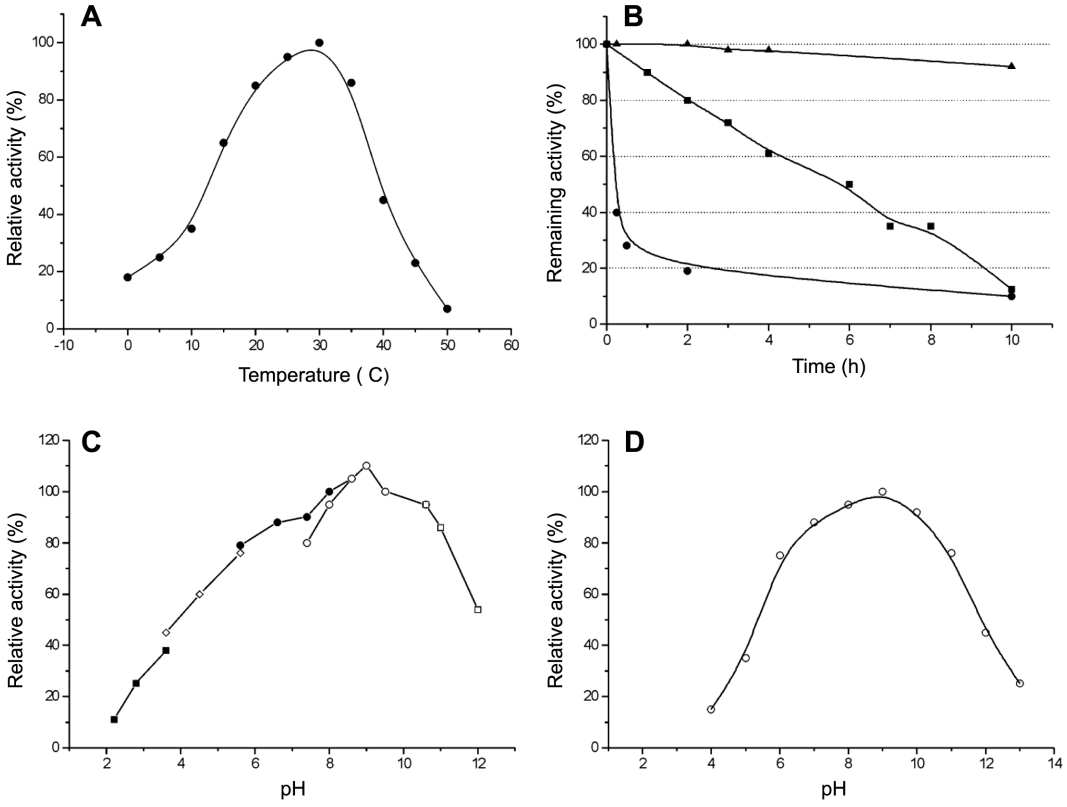

Fig. 4. Effect of temperature and pH on the lipase activity of the LipA1.
A. Activity at different temperatures. **B**. Thermostability (-▲- 10°C, -■- 30°C, -●- 60°C). **C**. Activity at different pHs (-■- glycine-HCl, -◇- sodium acetate, -●- Tris-malate, -○- Tris-HCl, -□- glycine-NaOH). **D**. pH stability (enzyme was incubated with 20 mM Tris-HCl buffer at various pH values at 4°C for 24 h).

reported to be stable under acidic (pH<2.0) and alkaline (pH>12.0) conditions [28].

Effects of Metal Ions on the Enzyme Activity

The activity of LipA1 increased by 17% and 23% in the presence of Ca^{2+} and Mg^{2+}, respectively, and was strongly inhibited by Cd^{2+}, Zn^{2+}, Co^{2+}, Fe^{3+}, Hg^{2+}, Fe^{2+}, Rb^{2+}, and EDTA, especially Co^{2+} (75% inhibited) and Cd^{2+} (65% inhibited). The ions Na^{+}, K^{+}, Li^{+}, Mn^{2+}, and Cu^{2+} had no effect on the catalytic activity of LipA1. The Ca^{2+} ion was generally found to stimulate the lipase activity [3, 28] by facilitating the removal of free fatty acids formed in the reaction at the water-oil interface [14, 20], in the same way as the lipases from *Pseudomonas* sp. strain KB700 [23], *Pseudomonas fluorescens* HU380 [18], *Aspergillus niger* [13], *Humicola lanuginosa* [20], and many other lipases of Family I [3]. The effect of Cu^{2+} on LipA1 was distinct from other lipases reported. The enzymes from *Pseudomonas* sp. strain KB700A (KB-Lip) [23], *Pseudomonas* sp. strain B11-1 (LipP) [7], *Pseudomonas* sp. MIS38 [2], and *Pseudomonas fluorescens* sp. HU380 [18] were strongly inhibited by Cu^{2+}, whereas LipA1 was not influenced. Only the lipase of *Pseudomonas fluorescens* sp. SIK W1 [19] displayed a tendency similar to that of the LipA1, being not inhibited by Cu^{2+}.

Effects of Detergents

The effects of various detergents on LipA1 are summarized in Table 2. In general, an increase in LipA1 activity with the addition of detergents was observed. The presence of SDS or 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) slightly increased the activity by 21% and 35%, respectively. Approximately 1.5- to 2-fold, increase in activity were observed by adding Triton X-100, Tween 80, Tween 40, Span 60, and Span 40. However, we found that the 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 adding the detergent, indicating that the LipA1 had better resistance to detergents. For example, LipA1 displayed 18% decrease, whereas 95% of the activity of the lipase from *Pseudomonas fluorescens* strain SIK W1 was abolished in the presence of SDS [19]. The stability against detergents will make LipA1 avail in the detergents industry.

Table 2. Effects of detergents on LipA1 activity.

Detergent	Relative activity (%)	Residual activity (%)
–	100	100
CHAPS	121	79
SDS	135	82
Triton X-100	176	85
Tween 80	196	86
Tween 40	167	71
Span 60	212	104
Span 40	149	91

Table 3. Substrate specificity of the purified LipA1.

Substrate	Special activity (U/mg)	Relative activity (%)
(A) Triglyceride		
Triacetin ($C_{2:0}$)	215	9
Tributyrin ($C_{4:0}$)	623.8	27
Tricaprin ($C_{10:0}$)	1,685	72
Trimyristin ($C_{14:0}$)	3,826.6	164
Tripalmitin ($C_{16:0}$)	3,218	138
Tristearin ($C_{18:0}$)	2,269	97
Triolein ($C_{18:1}$)	2,054	88
Olive oil	2,331.5	100
(B) *p*-Nitrophenyl esters		
p-Nitrophenyl acetate (C_2)	87.6	8
p-Nitrophenyl butyrate (C_4)	165.5	16
p-Nitrophenyl caprate (C_{10})	753	71
p-Nitrophenyl myristate (C_{14})	1,206	114
p-Nitrophenyl palmitate (C_{16})	1,055	100
p-Nitrophenyl stearin (C_{18})	576.5	55

Olive oil and *p*-nitrophenyl palmitate were used as the standard in (A) and (B), respectively.

Substrate Specificity

The enzymatic activity of LipA1 was determined by using various triglycerides and *p*-nitrophenyl monoesters as a substrate at pH 9.0 and 30°C. The LipA1 had the ability to hydrolyze triglycerides, indicating that it was a lipase rather than esterase. The middle- to long-chain fatty acids of *p*-nitrophenyl monoesters and triglycerides were good substrates for LipA1 (Table 3). The LipA1 hydrolyzed *p*-nitrophenyl myristate (C_{14}) and trimyristin (C_{14}) most preferably among the substrates examined (Table 3).

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