An Organic Solvent-Tolerant Alkaline Lipase from Cold-Adapted *Pseudomonas mandelii*: Cloning, Expression, and Characterization

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A gene encoding a novel organic solvent-tolerant alkaline lipase, *lipS* (GenBank ID JQ071496), was cloned from cold-adapted *Pseudomonas mandelii*. Re-combinant LipS was expressed in *Escherichia coli* as a 32-kDa soluble protein and was purified by standard procedures. It catalyzes the hydrolysis of triacylglycerols into glycerol and free fatty acids. It demonstrates more than 80% of its activity under alkaline conditions, pH 8–10.5, with an apparent optimum temperature range of 40–50°C. It maintained thermal stability from 4 to 50°C. After 1 h of incubation at 60°C, approximately 50% of its activity remained. It retained its activity in organic solvents, and activity increased in the presence of ethanol and of DMSO. Our data indicate that LipS is an alkaline lipase with relatively high thermal stability and notable tolerance of organic solvents.

**Key words:** alkaline lipase; organic solvent; *Pseudomonas mandelii*; psychrotrophic bacterium

Gene cloning of *lipS*. The *lipS* gene was cloned from *Pseudomonas mandelii* JR-1 by polymerase chain reaction (PCR) in two steps. At the first step, primers were designed based on non-coding sequences surrounding the lipase coding sequence (UniProt ID Q3KIU1) in the *Pseudomonas fluorescens* PFL-1 genome. The forward primer (GS4F) was 5′-GTGTTTCTCGGTTTCGCTCT-3′, approximately 40 bp downstream. The reverse primer (GS5R) was 5′-GAGAGACATATGAGTGCGTTTCTGCT-3′, approximately 170 bp upstream of Q3KIU1, and the reverse primer (GS5R) was 5′-GTGATTCATTTCCGAGGCCTG-3′, approximately 40 bp downstream. The resulting PCR product from *P. mandelii* JR-1 was subcloned into a TA vector and sequenced. At the second step, the *lipS* gene was amplified from the TA vector and subcloned into a pET28a vector. The forward primer used (GS56F) was 5′-GAGAGACATATGAGTGCGTTTCTGCTGCAACG-3′ (Nde I site underlined and the N-terminal part of *lipS* in bold-face type). The reverse primer used (GS57R) was 5′-GAGAGACATATGAGTGCGTTTCTGCTGCAACG-3′ (Nde I site underlined and the C-terminal part of *lipS* in bold-face type). A His6 tag and an 11 amino acid linker sequence (HHHHHHSSGLVPRGSH), which came from a pET28 vector, was located on the N-terminus of LipS. The construct was confirmed by DNA sequencing.

**Materials and Methods**

Materials. Bacterium *Pseudomonas mandelii* JR-1 was deposited in the Korean Collection for Type Culture (Daejeon, Korea) as KCTC 12151BP. TA cloning vector was purchased from InTiRON Biotechnology (Daejeon, Korea). pET28a expression vector was from Novagen (Boston, MA), Ex Taq DNA polymerase was from Takara Biotechnology (Seoul, Korea). HisTrap FF column and HiTrap Q-Sepharose FF column were from GE Healthcare (Piscataway, NJ), esters for p-nitrophenyl were from Sigma (St. Louis, MO), and all other reagents were from Sigma unless noted otherwise.

Gene cloning of LipS. The *lipS* gene was cloned from *Pseudomonas mandelii* JR-1 by polymerase chain reaction (PCR) in two steps. At the first step, primers were designed based on non-coding sequences surrounding the lipase coding sequence (UniProt ID Q3KIU1) in the *Pseudomonas fluorescens* PFL-1 genome. The forward primer (GS4F) was 5′-GTGTTTCTCGGTTTCGCTCT-3′, approximately 170 bp upstream of Q3KIU1, and the reverse primer (GS5R) was 5′-GTGATTCATTTCCGAGGCCTG-3′, approximately 40 bp downstream. The resulting PCR product from *P. mandelii* JR-1 was subcloned into a TA vector and sequenced. At the second step, the *lipS* gene was amplified from the TA vector and subcloned into a pET28a vector. The forward primer used (GS56F) was 5′-GAGAGACATATGAGTGCGTTTCTGCTGCAACG-3′ (Nde I site underlined and the N-terminal part of *lipS* in bold-face type). The reverse primer used (GS57R) was 5′-GAGAGACATATGAGTGCGTTTCTGCTGCAACG-3′ (Nde I site underlined and the C-terminal part of *lipS* in bold-face type). A His6 tag and an 11 amino acid linker sequence (HHHHHHSSGLVPRGSH), which came from a pET28 vector, was located on the N-terminus of LipS. The construct was confirmed by DNA sequencing.

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Sequence analysis. A homology search was done by BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). Multiple sequence alignments were by ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

Expression and purification of LipS. The pET28a vector containing the entire open reading frame of LipS was transformed into Escherichia coli BL21 (DE3). A single colony grown on an LB/kanamycin plate was selected for additional overnight growth at 37°C, followed by inoculation into a 250-mL LB/kanamycin broth. At the mid-log phase (OD600nm = 0.6–0.8) the incubation temperature was lowered to 15°C. After the addition of 0.1 mM of IPTG, the cells were grown for an additional 16 h. They were harvested at 10,000 x g for 5 min. The pellet was resuspended in binding buffer (20 mM Tris, 100 mM NaCl, 5% glycerol, pH 8.0), followed by sonication at 4°C. After centrifugation at 13,000 x g for 15 min, the supernatant was collected and the imidazole concentration was adjusted to 5 mM for nickel-chelated affinity chromatography. LipS was purified manually using a 1-mL HisTrap column. The bound LipS was eluted using a step-gradient (100–500 mM imidazole in binding buffer). Imidazole was removed with dialysis buffer (20 mM CAPS, 25 mM KCl, 5% glycerol, pH 10.5). Anion-exchange chromatography was carried out by the AKTA Explorer system (GE Healthcare) equipped with a 1-mL HiTrap Q-Sepharose FF column equilibrated with dialysis buffer and using a linear gradient of KCl (25–1,000 mM). Fractions showing lipase activity were collected. All purification steps were carried out at 4°C. The purified enzymes were frozen in N2, and were stored at −80°C.

Enzyme assay. Lipase activity was measured on a routine basis using 0.1 mM p-nitrophenyl octanoate (PNPO, C8) in reaction buffer (100 mM Tris-Cl, 100 mM NaCl, 0.3% Triton X-100, pH 8.5). The accumulation of p-nitrophenol was measured with a Shimadzu UV-160 spectrophotometer at 400 nm for 5 min. One enzyme unit was defined as 1 μmol of p-nitrophenol per minute from the p-nitrophenyl ester substrate at 25°C.

MALDI-TOF MS. Mass spectrometric analysis of the two bands seen in an SDS gel (Fig. 2B) was done using an Applied Biosystems 4700 proteomics analyzer at Genomine, Inc. (Pohang, Korea). Both MS and MS/MS data were acquired, and the Mascot program (http://www.matrixscience.com) was used for sequence tag searches.

Results and Discussion

Gene cloning and sequence analysis. To clone the lipS gene, we used the genome information of Pseudomonas fluorescens PFO-1, which is closely related to P. mandelii, and designed primers based on the non-coding region sequences surrounding the gene encoding P. fluorescens PFO-1 lipase (Uniprot ID Q3KIU1). The PCR product was amplified and subcloned into a TA vector (data not shown). Then an 891-bp lipS gene was amplified from the TA vector by PCR and subcloned into a pET28 vector (data not shown). The LipS contained a Gly-His-Ser-Gln-Gly sequence (Fig. 1, yellow box), a motif characteristic of the serine lipase family (Gly-X-Ser-X-Gly). LipS has a calculated molecular weight of 32,263 Da and a pl of 9.2. Multiple sequence alignment revealed that it had 80% sequence identity at the amino acid level to an organic solvent-tolerant lipase, rPFL, from P. fluorescens JCM5963 (Fig. 1). LipS, Q3KIU1, and rPFL had 296 identical amino acid residues. Another solvent-tolerant lipase, Lip9 from P. aeruginosa LST-03, showed 44% sequence identity but exhibited a conserved serine lipase motif (Fig. 1). Two Asp residues were identified as calcium-binding motifs (Fig. 1, filled circle).

Expression and purification of LipS. An entire open reading frame of lipS was constructed on a pET28a vector with N-terminal His6 residues. The recombinant LipS protein was successfully expressed as a soluble protein in E. coli BL21 (DE3) and purified by nickel chelate affinity chromatography followed by Q-Sepharose column chromatography (Table 1). However, a large fraction of the LipS did not bind to the nickel resins (Fig. 2, lane FT), leading to a relatively low yield of 5%. A method to improve the yield of LipS from protein purification is being sought. Elution profiles from both nickel chelate and Q-Sepharose columns kept pace with the lipase activity (data not shown). LipS appeared as a 32-kDa protein on a Coomassie Blue-stained SDS gel (Fig. 2, lane Q). The peptide sequence (VNLIGHSQGSLTAR) determined by MALDI-TOF MS from the 32-kDa band also matched the corresponding LipS sequence (underlined in Fig. 1). A minor approximately 35 kDa band appeared above the 32 kDa band, but MALDI-TOF MS resulted in no match.

Substrate specificity of LipS. LipS showed broad substrate preferences for p-nitrophenyl esters C4 to C16 in length, and maximal.

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Table 1. Purification of LipS from E. coli BL21 (DE3)

<table>
<thead>
<tr>
<th>Total protein (mg)</th>
<th>Total activity (units)</th>
<th>Yield (%)</th>
<th>Purity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell lysate</td>
<td>97.8</td>
<td>6,480</td>
<td>66.3</td>
</tr>
<tr>
<td>Nickel-chelate column</td>
<td>1.95</td>
<td>325</td>
<td>167</td>
</tr>
<tr>
<td>Q-Sepharose column</td>
<td>0.5</td>
<td>115</td>
<td>230</td>
</tr>
</tbody>
</table>

*Protein concentration was determined by a BioRad protein staining assay with bovine serum albumin as standard protein.

* Lipase activity was determined using PNPO as substrate.

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Fig. 1. Multiple Sequence Alignment for LipS (P. mandelii JR-1, Q3KIU1 (P. fluorescens PFO-1), rPFL (P. fluorescens JCM5963), and Lip9 (P. aeruginosa LST-03).

GXXG (yellow). LipS possesses a catalytic triad consisting of Ser99 (yellow), Asp263 (cyan), and His267 (cyan) residues. Calcium-binding motif ( ). The peptide sequence determined by MALDI-TOF MS is underlined.
Hydrolysis was observed for C8 (100%), followed by C12 (59%), C4 (49%), and C16 (31%) (Fig. 3). LipS had 3% activity for \(p\)-nitrophenyl acetate (PNPA, C2). In our previous study, an extracellular esterase, EstK, purified from \(P.\) mandelii, showed substrate specificity to short-chain fatty acids, particularly PNPA.11) These results collectively indicate that multiple, differentially expressed lipase genes are produced by \(P.\) mandelii, reflecting its broad lipolytic activity.

**Kinetic analysis**

The \(K_M\) of LipS with PNPO as substrate, determined from a Lineweaver-Burk plot, was 279 \(\mu\)M. The \(k_{cat}\) of LipS was 138 s\(^{-1}\).

**Optimum pH and temperature**

Cold-adapted lipases typically have an optimum pH of 7–8 and an optimum temperature in a range of 20–40\(^\circ\)C. More than 80% of LipS activity occurred at pH 8–10.5 (Fig. 4A), suggesting that it is an alkaline lipase. The apparent optimum temperature was determined for 0.1 mM PNPO in reaction buffer (100 mM Tris-Cl, 100 mM NaCl, 0.3% Triton X-100, pH 8.5) at 4–80\(^\circ\)C. The buffer pH was adjusted to 8.5 for each temperature.

**Thermal stability**

Thermal stability analysis revealed LipS maintained more than 80% of its activity in a range of 4–50\(^\circ\)C (Fig. 5). After incubation at 60 \(^\circ\)C for 1 h, it had retained 50% of its activity (Fig. 5). To maintain activity at low temperatures, cold-adapted enzymes generally exhibit structural flexibility at the expense of stability with a decreased number of intramolecular non-covalent bonds (e.g., hydrogen bonds, ionic interactions, and hydrophobic interactions) relative to their warmer-temperature counterparts. Thus LipS is unique in possessing relatively high thermal stability as an enzyme derived from a cold-adapted bacterium. Similarly, a cold-adapted esterase from \(P.\) cryohalolentis K\(5^T\) was found to maintain about 60% of its activity upon incubation at 80 \(^\circ\)C for 1 h.17)

**Effects of organic solvents, detergents, and metal ions**

The hydrolytic activity of LipS was measured in the presence of various organic solvents (Fig. 6A). LipS maintained its activity in acetone, acetonitrile,
Organic solvent-tolerant lipase rPFL from **P. fluorescens** JCM596313) in terms of relative tolerance and elevated catalytic activity in the presence of organic solvents.18)

As shown in Fig. 1, highest enzymatic activity for LipS was observed in the presence of Ca\(^{2+}\) with a 55% increase (Fig. 6C). The effects of K\(^{+}\), Mg\(^{2+}\), Na\(^{+}\), and Ni\(^{2+}\) were minimal, but the presence of Cu\(^{2+}\), Zn\(^{2+}\), and EDTA decreased enzymatic activity significantly.

In conclusion, we cloned, expressed, and characterized an organic solvent-tolerant lipase from **Pseudomonas mandelii** JR-1. This lipase, LipS, is attractive for potential application in a wide variety of commercial uses requiring alkaline conditions, reactions and/or extraction using organic solvents, and it has a relatively high thermal stability range of 4–50°C.

### Acknowledgment

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### References


### Fig. 6. Effects of Organic Solvents, Detergents, and Metal Ions.

**A.** The effects of organic solvents was measured after incubation of LipS for 1 h (white bar) and 24 h (gray bar) in a reaction buffer (100 mM Tris-Cl, 100 mM NaCl, 0.3% Triton X-100, pH 8.5) containing 30% (w/v) of each organic solvent at 25°C for 5 min with 0.1 mM PNPO. **B.** The effect of detergents were measured in the same reaction buffer containing 0.1% (w/v) (white bar) or 1.0% (w/v) (gray bar) of detergent at 25°C for 5 min with 0.1 mM PNPO. The effects of metal ions were measured in the same reaction buffer containing 5 mM of each metal ion at 25°C for 5 min with 0.1 mM PNPO. Data correspond to mean ± SD from three experiments.

Isopropanol. To our surprise, it was activated by ethanol (1.85-fold) and DMSO (1.95-fold) after 1 h of incubation. Furthermore, the catalytic activity of LipS was maintained at an elevated level for 24 h in ethanol (1.22-fold) and DMSO (1.67-fold). In contrast, LipS activity in aqueous buffer solution decreased 50%. LipS activity in methanol and acetic acid were about 20% as compared to control.

In this regard, LipS was similar to the organic solvent-tolerant lipase rPFL from **P. fluorescens** JCM596313) in terms of relative tolerance and elevated catalytic activity in the presence of organic solvents.

The effects of detergents on the catalytic activity of LipS were examined, as shown in Fig. 6B. Overall a 0.1% (w/v) concentration of detergents was more effective than 1.0% (w/v). At a 0.1% (w/v) detergent concentration, LipS activity increased by 77% in Tween 80, 50% in Tween 20, and 25% in Triton X-100, but it was inhibited 75% by SDS. In a previous study, some surface-active substances were found to stabilize the interfacial area, facilitating the access of the substrate to the enzyme.18)