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Cloning, Expression in *Escherichia coli*, and Enzymatic Properties of a Lipase from *Pseudomonas* sp. SW-3

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The lipase gene (*lipA*) and its activator gene (*lipB*) of *Pseudomonas* sp. SW-3 were cloned and sequenced. The *lipB* was found to be present immediately downstream of *lipA*. The deduced amino acid sequences of *lipA* and *lipB* showed a high level of homology to those of other lipases belonging to the family I.1 of bacterial lipases. When *lipA* was expressed in *Escherichia coli* using T7 promoter, an active lipase was produced in cells carrying both *lipA* and *lipB*, but not in cells harboring only *lipA*. Recombinant lipase (rPSL) overproduced in an insoluble form was solubilized in the presence of 8 M urea, purified in a urea-denatured form and refolded by removing urea in the presence of the Ca^{2+} ion. rPLS had maximum activity at pH 8.0 and 50°C, was stable at pHs from 7.0 to 9.0 and below 50°C, and showed the highest activity toward the *p*-nitrophenyl ester of palmitate (C16).

Key words: DNA cloning, expression, lipase, *Pseudomonas*, refolding

Lipase (EC 3.1.1.3) is a lipolytic enzyme, which catalyzes the hydrolysis of long-chain triacylglycerides and is widely distributed in various animals, plants and microorganisms. Microbial lipases have received much attention because of their potential use in a variety of biotechnological applications (Jaeger *et al.*, 1999). Several bacterial lipase genes have been cloned and their primary structures characterized (Jaeger *et al.*, 1994; 1999). Bacterial lipases are classified into eight different families. Family I is the largest and consists of six subfamilies on the basis of amino acid sequence and some fundamental biological properties (Arpigny and Jaeger, 1999). Among them, lipases from the genus *Pseudomonas* have been well characterized and reviewed in detail (Gilbert, 1993; Rosenau and Jaeger, 2000). *Pseudomonas* lipases are classified into three families, I.1, I.2 and I.3, which correspond to the previously described groups I, II and III, respectively, based on amino acid sequence homology (Jaeger *et al.*, 1994; Arpigny and Jaeger, 1999). Family I.1 includes lipases from *P. aeruginosa*, *P. alcaligenes* and *P. fragi* with a M_r of 30 kDa. Family I.2 includes lipases from *Burkholderia cepacia* (formerly *P. cepacia*), *Burkholderia glumae* (formerly *P. glumae*) and *P. luteola* with a M_r of about 33 kDa. In addition, family I.1 and I.2 lipases show a high degree (more than 40%) of amino acid sequence homology and each contain two cysteine residues, which form a single disulfide bond. These lipases require a chaperone-like protein, called lipase-specific foldase (Lif), to ensure correct folding and secretion (Jaeger *et al.*, 1994). The *lif* genes are located just downstream of the lipase genes and are also classified into two groups according to amino acid sequence homology (Jaeger *et al.*, 1994). In addition, those lipases having the N-terminal signal sequences are secreted via the type II secretion pathway (Rosenau and Jaeger, 2000). However, unlike families I.1 or I.2, family I.3 lipase with a M_r of 50 kDa from *P. fluorescens* do not contain cysteine residues and require any Lif-like protein (Jaeger *et al.*, 1994). This lipase has the secretion signal at the C-terminal region and is secreted via the type I secretion pathway called the ATP-binding cassette transporter system (Rosenau and Jaeger, 2000).

We have isolated a bacterium producing an extracellular lipase from soil and have identified this strain as a member of *Pseudomonas* species. In this paper, we describe the cloning and overexpression of the lipase gene in *Escherichia coli*, and the characterization of the recombinant enzyme produced by *in vitro* refolding.

Materials and Methods

Isolation of lipase-producing microorganisms

A small amount of soil collected from Gyeongnam area, Korea, was suspended in sterile water at an appropriate concentration, and spread on nutrient agar medium containing olive oil (25 g/*l*) and Victoria Blue B (4 g/*l*). Growing colonies with blue color zones were isolated and

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those with large clear zone were selected. The general characteristics of the isolated strain were determined according to *Bergeys Manual of Systematic Bacteriology* (Krieg and Holt, 1984). Other physiological tests including carbon-source utilization were performed using the API 20E system (BioMerieux).

Cloning of the lipase gene

Chromosomal DNA of *Pseudomonas* sp. SW-3 was obtained from cells grown in LB medium at 30°C for 15 h, as previously described (Marmur, 1961). Two degenerate oligonucleotide primers were synthesized to clone the lipase gene from *Pseudomonas* sp. SW-3: 5'-primer, 5'-ACGCG(T/C)TA(C/T)CCGATCATCCT(C/G)-GT(G/C)CACGGC-3 and 3'-primer, 5'-CCGCCCTGGCTGTG(A/G)CCGA(C/T)-(G/C)AG(G/A)TTGAC-3'. These primers were synthesiz ed based on sequences of the conserved regions including the HG sequence part in the N-terminus and active site serine in primary structures of previously cloned lipases of *Pseudomonas* (Gilbert, 1993; Rosenau and Jaeger, 2000). Polymerase chain reaction (PCR) was performed as follow: the first 5 cycles at 94°C for 40 sec, 40°C for 40 sec and 72°C for 1 min, and the following 30 cycles at 94°C for 40 sec, 55°C for 40 sec and 72°C for 1 min. The PCR product of about 240 bp was subcloned into pT7Blue vector (Novagen, USA) and its nucleotide sequence was determined. A 240 bp fragment identified as a part of *lipA* was labeled with digoxigenin using a DIG-labeling kit (Boehringer Mannheim, Germany) and used as a probe for Southern hybridization. In order to clone the complete lipase gene (*lipA* and *lipB*), genomic DNA prepared from *Pseudomonas* sp. SW-3 was completely digested with various restriction enzymes, then size-fractionated electrophoretically on 0.6% agarose gels (w/v) and transferred to a Hybond N^+ nylon membrane (Amersham Pharmacia Biotech, USA). Southern hybridization and detection were performed using the DIG system (Boehringer Mannheim, Germany), according to the manufacturer's instructions. DNA fragments hybridized with the probe were subcloned into the corresponding sites of pUC 119 or pBluescript SK(+) vector and then sequenced. The analysis of sequence data and sequence similarity searches were performed using the BLAST(N) program of the National Center for Biotechnology Information (NCBI). Homology alignment was performed with the CLUSTAL W program (Thompson *et al.*, 1994) using MacVector 6.5 software (Oxford Molecular Group, UK).

Construction of expression plasmids

To construct the expression plasmid pET-LipAB including both *lipA* and *lipB*, lipase gene was amplified by PCR using *Pyrobest* DNA polymerase (Takara Biosci. Japan) with a sense P1 primer that contained a unique *Nde*I restriction site and an ATG initiation codon (boldface): 5'-CCAT**ATG**AGCATCATATATCGATGG-3' and an antisense P3 primer that contained a unique *Bam*HI restriction site: 5'-AGGATCCTTATTGATCAAAGTGAATGCG-3'. The amplified gene fragment (1786 bp) was inserted into the *Sma*I site of pUC119 vector, yielding pULipAB and the nucleotide sequence of the inserted fragment was confirmed by DNA sequencing. The *Nde*I/*Bam*HI fragment of pULipAB was introduced into the corresponding sites of pET29a(+) plasmid, resulting in pET-LipAB. To construct the expression plasmid pET-LipA including only *lipA*, a 948 bp fragment was amplified by PCR with a sense P1 primer and an antisense P3 primer that contained a unique *Hind*III restriction site: 5'-ACAAGCTTTACAGTCCAA GTTGTTGC-3'. This fragment was inserted into the *Sma*I site of pUC119 vector, yielding pULipA. After confirming the nucleotide sequence of the inserted fragment by DNA sequencing, the *Nde*I/*Hind*III fragment of pULipA was inserted into the corresponding sites of pET29a(+) plasmid, resulting in pET-LipA.

Expression of the lipase gene in E. coli

E. coli BL21(DE3) cells transformed with pET-LipAB or pET-LipA were grown in 100 ml of LB medium containing 50 μg/ml of kanamycin at 37°C with shaking until the absorbance at 600 nm was approximately 0.6. Subsequently, expression of the lipase gene was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM and incubating at 37°C for 3 h. After centrifugation at 5,000×*g* for 30 min at 4°C, pellets were suspended in 50 mM Tris-HCl buffer (pH 8.0) and treated with lysozyme (0.1 mg/ml), and then disrupted by sonication for 5 min with a 30 sec pulse. The cell components were separated into soluble and insoluble fractions by centrifugation at 12,000×g for 20 min. The supernatant was recovered as an extracellular fraction after 90% saturation of ammonium sulfate followed by dialysis against the same buffer.

Refolding and purification of recombinant lipase (rPSL)

E. coli BL21(DE3) harboring pET-LipA was cultured in 500 ml of LB medium containing 50 μg/ml of kanamycin at 37°C with shaking. Protein expression and cellular fractionation were carried out as described above. The insoluble fraction containing inclusion bodies was suspended in 50 mM Tris-HCl buffer (pH 8.0) containing 2% Triton X-100 (v/v) and 10 mM EDTA and incubated at room temperature for 10 min with occasional mixing. Inclusion bodies were recovered as a pellet after centrifugation at 25,000×g for 10 min. After washing three times with the same buffer, inclusion bodies were dissolved in 30 ml of buffer A: 50 mM Tris-HCl buffer (pH 8.0) containing 8 M urea, 10 mM dithiothreitol (DTT), 5% glycerol (v/v) and 1 mM EDTA. After incubation for 1 h at room temperature, the suspension was applied to a column (1.6×20 cm) of DEAE-Sepharose CL-6B (Amersham Pharmacia Biotech, USA) equilibrated with buffer A. The column

was then washed with the same buffer to remove unbound proteins, and the bound proteins were eluted by a linear gradient of 0 to 1 M NaCl. After fractions containing rPSL were collected, they were diluted with 2 volumes of 50 mM Tris-HCl buffer (pH 8.0) containing 2 M urea and 5 mM $CaCl_2$ and dialyzed against 50 mM Tris-HCl buffer (pH 8.0) containing 5 mM $CaCl_2$ at 4°C for 3 days by very slow dropping using peristaltic pump. No precipitate was generated during this refolding process. After centrifugation at 30,000×g for 1 h the supernatant was concentrated with Centriprep-10 (Amicon, USA) and then applied to a column (1×30 cm) of Superose™ 12 (Amersham Pharmacia Biotech, USA) equilibrated with 50 mM Tris-HCl buffer (pH 8.0) containing 5 mM $CaCl_2$ and 0.15 M NaCl. The elution was performed at a flow rate of 0.5 ml/min, and the rPSL eluted from the column was used for biochemical characterization.

Measurement of lipase activity and gel electrophoresis

To discriminate between lipase and esterase activities, a more lipase-specific assay was preformed using rhodamine B-olive oil agar plate as previously described (Kouker and Jaeger, 1987). Lipase activity was determined spectrophotometrically using a standard assay toward *p*-nitrophenyl palmitate (pNPP), as previously described (Stuer *et al.*, 1986). The substrate specificity of the lipase was measured spectrophotometrically using *p*-nitrophenyl esters of various acyl chain length as substrates (0.5 mM *p*-nitrophenyl esters), as previously described (Amada *et al.*, 2000). One unit of enzymatic activity was defined as the amount of enzyme that produced 1 μmol of *p*-nitrophenol per min at 30°C. Protein concentration was determined by using the Bio-Rad protein assay kit according to the manufacturer's instructions with bovine serum albumin (Sigma, USA) as the protein standard for the calibration curve. SDS-PAGE was performed on 12% running gel as described by Laemmli (1970), and resolved proteins were visualized by Coomassie staining following standard procedures. A low range protein standard (Bio-Rad, USA) was used as molecular mass marker. Expression levels were estimated as a percentage of total cellular proteins using Alphaimager™ 1220 Documentation and Analysis System (Alpha Innotech, USA).

Determination of pH and temperature optima and stability

Following buffers were used to investigate the effect of pH on lipase activity: 50 mM NaH_2PO_4/Na_2HPO_4 (pH 6.0 to 7.6), 50 mM Tris/HCl (pH 7.5 to 9.0) and 50 mM glycine/NaOH (pH 9.0 to 11.0) containing 5 mM $CaCl_2$. The effect of pH on lipase stability was determined by incubating aliquots of the purified lipase in buffers with different pH values for 30 min at 37°C. The optimum temperature for lipase activity was determined over the range of 20-80°C by the spectrophotometric assay. Assay mixtures were equilibrated at the required temperature before adding the enzyme. The effect of temperature on lipase stability was determined by incubating aliquots of the purified lipase for 30 min in 50 mM Tris-HCl buffer (pH 8.0) containing 5 mM $CaCl_2$ at various temperatures.

Results and discussion

Identification of lipase-producing bacteria

Of the nine lipase-producing strains obtained, one strain with the highest lipase activity was selected for further study. This isolate was a Gram-negative, motile, non-spore-forming, and aerobic rod-shaped bacteria which generated a mucoid colony on nutrient agar. In addition, the isolate was positive for oxidase, catalase, gelatin hydrolysis, arginine hydrolase, and growth at 41°C, but was negative for β-galactosidase, denitrification, glucose acidification, indole production and V-P test. The isolate showed the highest level similarity with *Pseudomonas aeruginosa* on comparing its taxonomical properties with those of class I species of the *Pseudomonas* genus (Krieg and Holt, 1984) (data not shown). On the basis of the above results, the strain was identified as a *Pseudomonas* species and designated as *Pseudomonas* sp. SW-3.

Cloning and sequencing of the lipase (PLS) gene

PCR fragments of the expected size (240 bp) were obtained using degenerated primers, subcloned and sequenced. One of them was found to have the predicted amino acid sequence showing 65% identity to the *P. aeruginosa* lipase (Martinez *et al.*, 1999) and more than 50% identities to the other *Pseudomonas* lipases (Chihara-Siomi *et al.*, 1992; Kumura *et al.*, 1998). Thus, it was used as a hybridization probe to isolate the complete lipase gene from genomic DNA of *Pseudomonas* sp. SW-3. A probe hybridized to an approximately 1.4 kb *Kpn*I and 2.7 kb *Hind*III fragments (data not shown), which were subcloned and sequenced. Nucleotide sequence analysis revealed an open reading frame (ORF) starting at bp 228 with a GTG codon and ending at a TAA at bp 1161, thereby encoding a protein of 311 amino acids. A potential ribosome-binding sequence, AAGGA, similar to those utilized in *E. coli* was found 7 bp upstream of the GTG initiation codon (data not shown). It is noticeable that the ORF of *lipA* has an atypical GTG start codon, unlike other *Pseudomonas* lipases having a typical ATG start codon (Kugimiya *et al.*, 1986; Aoyama *et al.*, 1988; Chihara-Siomi *et al.*, 1992; Tan and Miller, 1992; Kumura *et al.*, 1998).

On the other hand, families I.1 and I.2 lipases have other genes involved in the secretion and/or regulation of lipase production, located immediately downstream of the structural genes (Jaeger *et al.*, 1994). In order to investigate whether another gene is present in the *Pseudomonas* sp. SW-3 genome, genomic DNA was digested with various restriction enzymes and subjected to Southern

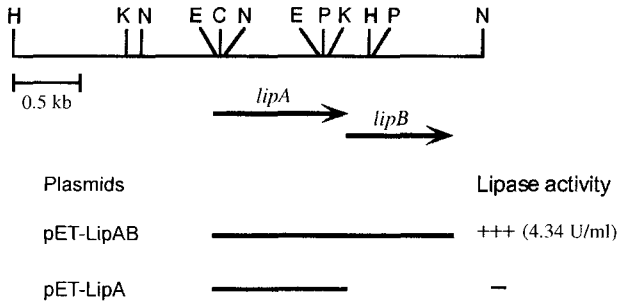

Fig. 1. Restriction map of the 3.6 kb *Hind*III/*Nco*I fragment containing *lipA* and *lipB* of *Pseudomonas* sp. SW-3. *lipA* and *lipB* indicate the structural genes for LipA and LipB, respectively, and arrows indicate the direction of transcription. Production of lipase activity is indicated on the right. Restriction enzymes: H, *Hind*III; K, *Kpn*I; N, *Nco*I; E, *Eco*RV; C, *Cla*I; P, *Pst*I.

hybridization using a DIG-labelled *Kpn*I/*Hind*III fragment (420 bp) as a probe. About 2.0 kb *Nco*I fragment was hybridized with the probe and sequenc- ed. Nucleotide sequence analysis also revealed another ORF starting at bp 1163 with an ATG codon and ending at a TAA at bp 2000, thereby encoding a protein of 279 amino acids (data not shown). Therefore, these two genes were designated *lipA* and *lipB*, respectively (Fig. 1). The sequence data obtained in this study have been deposited in the GeneBank and EMBL Data bank under accession number AF216286.

Sequence comparision of LipA and LipB with other lipases

Comparison of the amino acid sequence of LipA with those of other bacterial lipases showed an high identity of 64% with *Vibrio cholerae* lipase, 54% with *Pseudomonas*

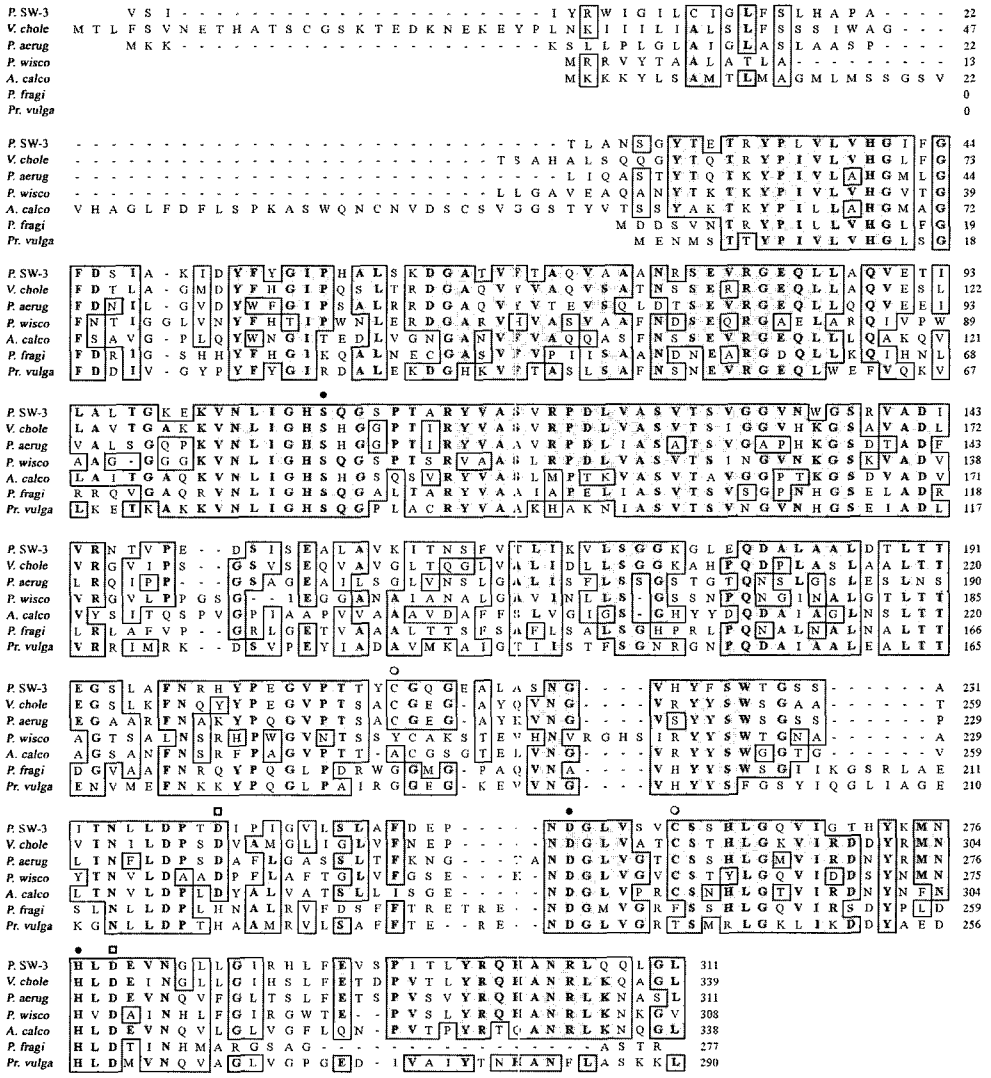

Fig. 2. Multiple alignment of the amino acid sequence of LipA from *Pseudomonas* sp. SW-3 (*P.* SW-3) with other lipases: *Vibrio cholerae* (*V. chole*), NP_232620; *Pseudomonas aeruginosa* (*P. aerug*), BAA23128; *Pseudomonas wisconsinensis* (*P. wiscon*), AAB53647; *Acinetobacter calcoaceticus* (*A. calco*), AAD29441; *Pseudomonas fragi* (*P. fragi*), P08658; *Proteus vulgaris* (*Pr. vulga*), AAB01071. Amino acids that are conserved in at least four of the seven sequences are shown in black boxes. Numbers to the right refer to the last amino acid on the line. Conserved residues belonging to the catalytic triad are indicated by filled circles. The two Asp residues invclved in the Ca^{2+}-binding site are marked by open squares. The two Cys residues forming a disulfide bond are indicated by open circles.

aeruginosa lipase, and 50% with *Pseudomonas wisconsinensis* lipase (Fig. 2). It also revealed more than 45% identity with other lipases belonging to family I.1, whereas no significant similarity was found between LipA and other known lipases, indicating that LipA is a member of the family I.1. Lipases are known to have an active-site consensus sequence Gly-X-Ser-X-Gly and to form a catalytic triad consisting of Ser, Asp and His residues (Arpigny and Jaeger, 1999). LipA had the Gly-X-Ser-X-Gly sequence at positions 106-110, and Ser, Asp and His residues at 108, 255 and 277, respectively. In addition, two Asp residues, known Ca^{+2}-binding sites, were found at positions 240 and 279, and two Cys residues forming a disulfide bond at positions 210 and 261 were also conserved (Arpigny and Jaeger, 1999) (Fig. 2).

Comparison of the amino acid sequence of LipB with those of other bacterial lipases showed an identity of 36% with *Vibrio cholerae* lipase, 25% with *Pseudomonas wisconsinensis* lipase, and 23% with *Acinetobacter calcoaceticus*, whereas no significant similarity was found between LipB and other known lipases. (data not shown). On the other hand, while this work was in progress, the sequence of a lipase gene from *Pseudomonas* sp. strain KFCC 10818 was deposited in GeneBank/EMBL database with accession no. AF125523. This lipase shows very remarkable sequence similarity with that of *Pseudomonas* sp. SW-3, with an identity of 97% over virtually the entire length of the sequence. This lipase gene is composed of *lipK* and *limK* and its genetic organization is almost identical to that of *Pseudomonas* sp. SW-3. However, a comparison of its amino acid sequence with that of *Pseudomonas* sp. SW-3 showed eight residues substituations between LipK and LipA, and between LimK and LipB, respectively.

Overexpression of the lipase gene in E. coli

When *E. coli* BL21(DE3) cells carrying pET-LipAB and pET-LipA were induced for 3 h with 0.5 mM IPTG at 37°C, lipase activity was detected in both the extracellaular and intracellular fractions of cells harboring pET-LipAB, while no activity was observed in cells harboring pET-LipA (Fig. 1). However, a protein band corresponding to LipA was not detected in any of the fractions of pET-LipAB, while a protein with molecular mass of about 31 kDa was observed in the insoluble fraction of pET-LipA (data not shown). These results indicate that LipB is essential for the production of an active lipase like Lif (Jaeger *et al.*, 1994) and that the expression level of the active enzyme is very low in this expression system. It is known that because of the complex gene regulatory and secretion mechanisms, the overproduction of rPLS as active enzymes in heterologous hosts like *E. coli* is difficult (Rosenau and Jaeger, 2000). Our results are similar to those of several other *Pseudomonas* lipases, as only very low lipase activities were detected in the cell lysates

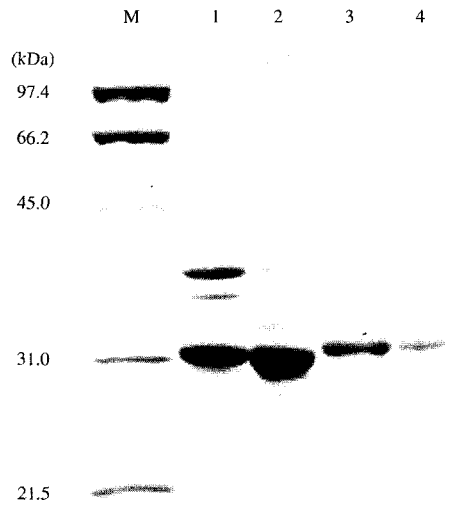

Fig. 3. SDS-PAGE of rPSL produced in *E. coli* cells harboring pET-LipA. Lane M, molecular weight marker; lane 1, insoluble fraction; lane 2, sample after washing with 2% TritonX-100 and 10 mM EDTA; lane 3, sample after DEAE-Sepharose CL-6B chromatography; lane 4, sample after Superose^{TM} 12 gel filtration.

of *E. coli* cells, even though these harbored plasmids containing both lipase and Lif genes (Frenken *et al.*, 1993; Ihara *et al.*, 1995; Iizumi and Fukase, 1994; Jorgensen *et al.*, 1991; Oshima-Hirayama *et al.*, 1993). The present results are similar to those of other *Pseudomonas* lipases, i.e., when only lipase gene, without the Lif gene, was expressed, the recombinant lipase was overproduced in the form of inactive inclusion bodies in *E. coli* (Lee *et al.*, 1993; Amada *et al.*, 2000; Traub *et al.*, 2001).

Refolding, purification and characterization of rPLS

After washing with 2% Triton X-100 (v/v) and 10 mM EDTA, rPLS was solubilized in the buffer containing 8 M urea and then purified by anion exchange column chromatography in the presence of 8 M urea (Fig. 3, lanes 2 and 3). Active enzyme was obtained by successive refolding and finally purified by gel filtration column chromatography. The purified enzyme was identified as a single band with a molecular mass of about 31 kDa on SDS-PAGE (Fig. 3, lane 4). The purification yield was 30% and the specific activity was 36.3 U/mg on *p*-nitrophenyl palmitate (pNPP).

The purified rPLS showed optimal catalytic activity toward pNPP at pH 8.0 and retained more than 80% of its activity at 37°C for 30 min in various buffers over pH ranging from 7.5 to 9.0 (Fig. 4A). The enzyme exhibited maximum activity at 50°C and retained more than 90% of its activity after incubation for 30 min at 50°C (Fig. 4B).

The effect of metal ions on rPLS activity was examined with pNPP as a substrate at a concentration of 2 mM at pH 8.0. Its activity was inhibited by Fe^{2+}, Mg^{2+}, Hg^{2+} and Co^{2+} cations (50-80% inhibition), whereas Na^{+} and Ca^{2+} enhanc-

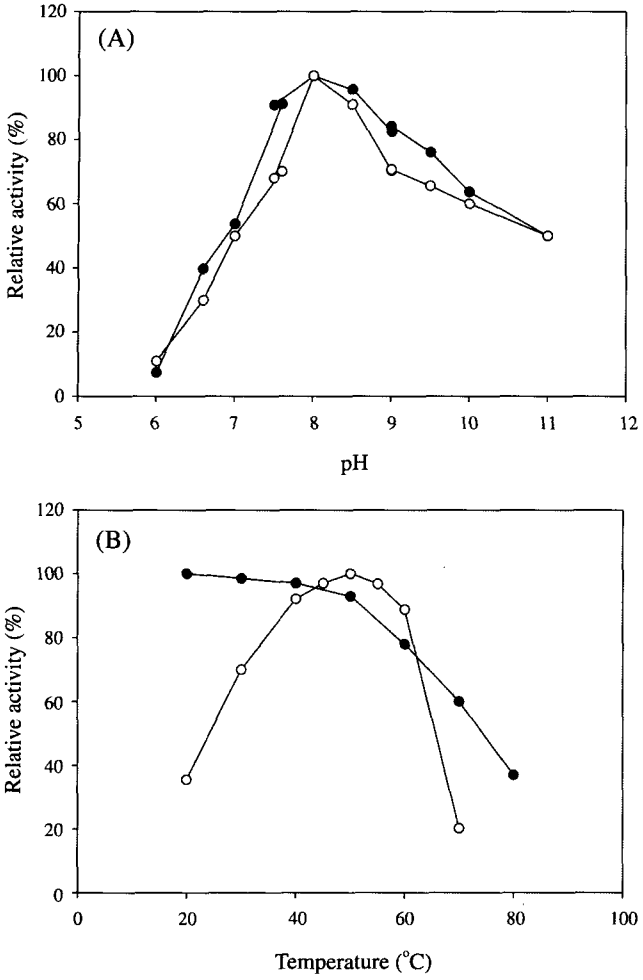

Fig. 4. Effect of pH (A) and temperature (B) on rPSL activity and stability. Effects of pH and temperature on the activity (○) and stability (●) were determined as described in Materials and Methods. The remaining activity was measured under the standard assay condition using *p*-nitrophenyl palmitate as substrate.

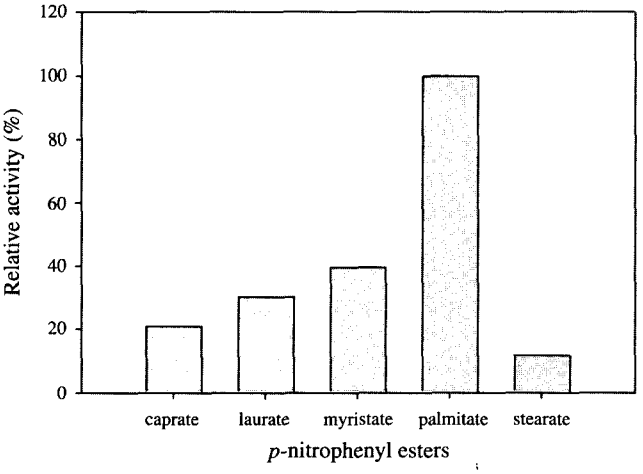

Fig. 5. Acyl-chain length specificity of the purified rPLS. Activity measurements were performed using *p*-nitrophenyl esters of various acyl chain length as substrates [0.5 mM *p*-nitrophenyl ester, 10% acetonitrile (v/v), 10 mM $CaCl_2$, 50 mM Tri-HCl, pH 8.0].

ed its lipase activity (20-45% activation) (data not shown). The substrate specificity of rPLS was investigated with *p*-nitrophenyl monoesters of various acyl chain length. As shown in Fig. 5, the hydrolysis rate was highest for pNPP (C16), and the activities decreased for substrates with shorter and longer chain lengths. This result indicates that this enzyme has a preference for the monoester of palmitic acid. These properties in optimum pH and substrate specificity are very similar to those of the lipase from *Acinetobacter calcoaceticus* BD413 (Kok *et al.*, 1995).

Acknowledgments

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