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Cloning, expression and characterization of an organic solvent tolerant lipase from *Pseudomonas fluorescens* JCM5963

Aijun Zhang, Renjun Gao*, Naibing Diao, Guiqiu Xie, Gui Gao, Shugui Cao**

Key Laboratory for Molecular Enzymology and Engineering of Ministry of Education, Jilin University, Changchun 130021, PR China

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ABSTRACT

A novel lipase gene from an organic solvent degradable strain *Pseudomonas fluorescens* JCM5963 was cloned, sequenced, and overexpressed as an N-terminus His-tag fusion protein in *E. coli*. The alignment of amino acid sequences revealed that the protein contained a lipase motif and shared a medium or high similarity with lipases from other *Pseudomonas* strains. It could be defined as a member of subfamily I.1 lipase. Most of the recombinant proteins expressed as enzymatically active aggregates soluble in 20 mM Tris–HCl buffer (pH 8.0) containing sodium deoxycholate are remarkably different from most subfamily I.1 and I.2 members of *Pseudomonas* lipases expressed as inactive inclusion body formerly described in *E. coli*. The recombinant lipase (rPFL) was purified to homogeneity by Ni-NTA affinity chromatography and Sephacryl S-200 gel filtration chromatography. The purified lipase was stable in broad ranges of temperatures and pH values, with the optimal temperature and pH value being 55 °C and 9.0, respectively. Its activity was found to increase in the presence of metal ions such as Ca^{2+}, Sn^{2+} and some non-ionic surfactants. In addition, rPFL was activated by and remained stable in a series of water-miscible organic solvents solutions and highly tolerant to some water-immiscible organic solvents. These features render this novel lipase attraction for biotechnological applications in the field of organic synthesis and detergent additives.

1. Introduction

Lipases (E.C 3.1.1.3) catalyze the hydrolysis of medium- and long-chain triglycerides to fat acids and glycerol, and they are further distinguished from esterases in their sharply activation at the interface generated by a water-insoluble lipid substrate in aqueous solution [1]. Lipases can also catalyze ester synthesis and transesterification reactions with high regional and stereo selectivity in nonaqueous solvent systems. These features make lipases play an important role in the function of detergent additives, fine chemicals synthesis, pharmaceutical and agrochemical productions [2–5]. Among various bacterial lipases, those from *Pseudomonas* (*Ps.*) genus and *Burkholderia* (*B.*, formerly *Pseudomonas*) genus are the most widely used members in biotechnological applications [6]. According to molecular properties and amino acid sequence homology, *Ps.* lipases are generally classified into three groups designated as subfamily I.1, I.2 and I.3 lipases [7]. To date, some of *Ps.* lipases have been commercially available, such as Amano P, P-30 and Lipase AH (from *B. cepacia*), as well as Lipase AK and Lipase 56P (from *Ps. fluorescens*), they all exhibit tremendous potential in organic synthesis for high-value chemicals. At the same time, the crystal structure determinations of some lipases have also been reported including lipases from *Ps. aeruginosa* (subfamily I.1), *B. cepacia* (subfamily I.2) and *Ps.* sp. MIS38 (subfamily I.3) [8–10].

In recent years, a variety of lipase-encoding genes from different *Ps.* species have been cloned and sequenced, and the corresponding proteins have been expressed in homologous or heterogeneous hosts [11–14]. Unfortunately, the heterogeneous expressions of subfamily I.1 and I.2 *Ps.* lipases are hampered by the fact that a lipase chaperone is necessary for correctly folding to an enzymatically active form [15]. On the other hand, lipase is strongly hydrophobic in nature. When continuously accumulated as foreign protein in cytoplasm, lipase produces toxicity to host cells, leading to their death, or forming inactive inclusion bodies [16]. Therefore, to increase lipase productivity in a biochemically safe and economic expression system, *E. coli* is always causing a tremendous interest among scientists and industrialists. Several previous researches have reported the overexpression of *Ps.* lipases and their chaperon proteins in *E. coli* as inactive inclusion bodies, and subsequently denaturing and refolding are necessary in order to obtain enzymatically active recombinant lipases [13,14]. To our knowledge, there have been only limited researches on the expression

* Corresponding author. Tel.: +86 431 88498972x602; fax: +86 431 88980440.
** Corresponding author. Tel.: +86 431 88924281; fax: +86 431 88499912.
E-mail addresses: gaorj@jlu.edu.cn (R. Gao), sgcao@jlu.edu.cn (S. Cao).

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of *Ps*. 1ipases as enzymatically active form in *E. coli* [11,12], and the expression level is low and the recovery is poor without the coexpression of chaperone protein and the subsequently denaturing and refolding. However, the high-level expression of active *Ps*. lipases in *E. coli* has not yet been reported up to now.

Reactions catalyzed by lipases are often carried out in the presence of organic solvents because there are many advantages when organic solvents are utilized instead of water in some reaction systems, such as the increased solubility of substrates, the relatively easy recovery of products in organic phase and the ability to shift the reaction equilibrium to the synthetic direction [17]. However, most enzymes including lipases are not always stable in organic solvents. Therefore, a lot of efforts have been made in recent years for finding new lipases with excellent activity and stability in organic solvents [18–20]. *Pseudomonas fluorescens* JCM5963 (*Ps. fluorescens* 5963) was isolated from pre-filter tanks in England, and it was reported to be able to survive by utilizing methanol, isopropanol, ethyl acetate, and so on as nutrition source [21]. The present paper describes the cloning, heterogeneous expression, purification and characterization of an organic solvent tolerant lipase of this strain. The recombinant lipase possessed the various advantages existed in many other *Ps*. lipases, and high expression level and satisfactory recovery were achieved through designed well-connected experiments. This work may be of great significance to the increased biotechnological application in organic synthesis by supplying efficient and stable biocatalyst and to the improved production of family I bacterial lipases.

2. Materials and methods

2.1. Strains and materials

The host strain *E. coli* BL21 (DE3) CodonPlus (*E. coli* BLP), the cloning and expression vector pET28b were obtained from Novagen, and the organic solvent degradable strain of *Ps. fluorescens* JCM 5963 was purchased from Japan Collection of Microorganisms. Ex-Taq DNA polymerase, T_4DNA ligase, restriction endonucleases and DNA Marker were purchased from TaKaRa Biotechnology (Dalian, China). Sodium deoxycholate (DOC), isopryl-β-D-thiogalactopyranoside (IPTG), *p*-nitrophenyl fatty acid esters with varying acyl chain lengths, Fast Blue RR, and α-naphtyl caprylate were purchased from Sigma (St. Louis, USA). All the other chemicals were of the highest reagent grade and commercially available.

2.2. Cloning, sequencing and overexpression of the lipase gene

The lipase-encoding gene was amplified from chromosomal DNA by PCR using a pair of degenerate oligonucleotide primers. Forward primer was 5′-CACAGAATTC TCGCCGCATGTCVCAA-3′, reverse primer was 5′-TCCGAAGCTTAACRSYKAG ATCASARKC-3′ (EcoRI and HindIII cutting sites are underlined, respectively). The amplified fragments were inserted in pET28b vector according to the manufacturer's instructions, and the nucleotide sequence was completely sequenced on both the strands. Gene analysis and homology comparison of the sequence with GenBank sequences were performed with ClustalX 1.8 software package and BioEdit version 7.0.9.0 [22,23]. The recombinant plasmid was transformed into *E. coli* BLP for protein expression. The transformed strains were grown in a modified 2YT medium (adding 1% glucose) containing kanamycin (50 μg/ml) at 37 °C until the OD_{600} reached 1.0. Protein expression was induced by adding IPTG to a final concentration of 0.05 mM, and then the culture was shaken for 8 h at 20 °C and the cells were harvested by centrifugation.

2.3. Purification of recombinant lipase

Saturated ammonium sulfate fractionation, Ni-NTA agarose affinity chromatography and Sephacryl S-200 gel filtration chromatography were used in the purification process. Firstly, the frozen cell pellet from 1 l-culture (about 6.5 g wet cells) was thawed and suspended in 30 ml of 20 mM phosphate-buffer (pH 7.0), ultrasonicated and centrifuged. Then solid ammonium sulfate was added into the supernatant to a saturation of 40% (w/v), and the precipitate was recovered by centrifugation. The precipitate was re-suspended in 2 ml of 20 mM phosphate-buffer (pH 6.0) and centrifuged, and the supernatant was removed. This washing process was repeated twice. In the end, the remained precipitate and the cell debris were incorporated and dissolved in 190 ml of 20 mM Tris–HCl buffer (pH 8.0) containing 0.2% DOC (w/v) by mild shaking at 37 °C for 1 h and centrifuged again. Secondly, the supernatant was applied to a Ni-NTA agarose affinity column (5 ml, Amersham, Sweden). Target protein was finally eluted with the elution buffer containing 20 mM Tris–HCl (pH 8.0), 0.1 M NaCl, and 100 mM imidazole. All the solutions in purification course contained 0.2% DOC (w/v). Lastly, the collected fraction was concentrated to 6 ml by a Centricon 10 filter (Millipore, USA), and 1 ml of it was applied on a Sephacryl S-200 gel filtration chromatography. The column (1.0 cm × 100 cm) was equilibrated with 20 mM Tris–HCl (pH 8.0) containing 0.1 M NaCl at a flow rate of 30 ml/h, and the sample was loaded and eluted with the same buffer. The active fraction was collected and concentrated. The purity of rPFL was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), while native polyacrylamide gel electrophoresis (N-PAGE) and activity staining using α-naphtyl caprylate as substrate were performed in order to determinate the exact molecular mass of purified rPFL.

2.4. Enzyme and protein assays

Lipase activity was routinely determined using the *p*-nitrophenyl fatty acid esters as substrates [24]. The standard assay reaction mixture contained 50 mM Tris–HCl buffer (960 μl, pH 8.0), 10 mM *p*NPC_8 dissolved in acetonitrile (20 μl) and undiluted enzyme solution (20 μl). One unit (U) of enzymatic activity is defined as the amount of enzyme required to produce 1 μmol of *p*-nitrophenyl per minute ($\varepsilon_{420\,nm} = 0.016\ \mu M^{-1}$). Unless specially mentioned, all the measurements of lipase activity were conducted under those standard conditions at 55 °C. Lipase activity on olive oil emulsified with gum arabic was determined at pH 8.0 as described previously [25]. Protein concentration was determined by Bradford's method using bovine serum albumin as a standard. All the assays were performed in triplicate, and the average values were taken.

2.5. Enzyme characterization

The effect of temperature on the hydrolytic activity was determined by measuring the hydrolytic activity at different temperatures ranging from 10 to 85 °C. The optimum pH was determined by measuring the hydrolytic activity in buffers with various pH values. The stability of rPFL to temperature was investigated by detecting the residual activity after incubating the purified lipase (0.05 mg/ml) at a temperature range of 10–80 °C for 1 h. The effect of pH on lipase stability was determined by detecting the residual activity after incubating the purified lipase (0.05 mg/ml) in the buffers with different pH values at 25 °C for 20 h. The substrate specificity of rPFL was studied using *p*-nitrophenyl fatty acid esters with various acyl chain lengths under standard conditions. The effects of EDTA, various metal ions and surfactants on the hydrolytic activity were determined by detecting the residual

activity after incubating the purified lipase (0.05 mg/ml) containing EDTA (1 mM), each of various metal ions (1 mM) and each of surfactants (0.1%, w/v) in a 20 mM Tris–HCl buffer (pH 8.0) at 37 °C for 1 h, respectively.

2.6. Effects of organic solvents on rPFL

Activity or stability of rPFL to organic solvents was assayed by measuring the residual activity after incubating the enzyme in the presence of each of various organic solvents (30%, v/v) in 20 mM Tris–HCl buffer (pH 8.0). The sample was prepared by diluting the purified lipase to 0.1 mg/ml with the buffer containing each of a variety of organic solvents. After vortex, the mixture was incubated at 30 °C with constant shaking at 150 rpm for 24 h in screw-capped tubes. Samples were withdrawn periodically from the aqueous phase and residual lipase activity was assayed. The effects of organic solvents on rPFL were expressed as relative activities, and distilled water was added instead of organic solvent as a reference when water-miscible organic solvents were studied.

3. Results and discussion

3.1. Cloning and gene analysis

A pair of degenerated oligonucleotide primers was designed based on the nucleotide sequences immediately upstream and downstream the known coding sequences of lipase. Using these primers, a band of about 900 bp length was amplified from the chromosomal DNA of *Ps. fluorescens* 5963. Sequencing and gene analysis indicated an ORF of 891 bp, a full-length lipase gene motif encoding

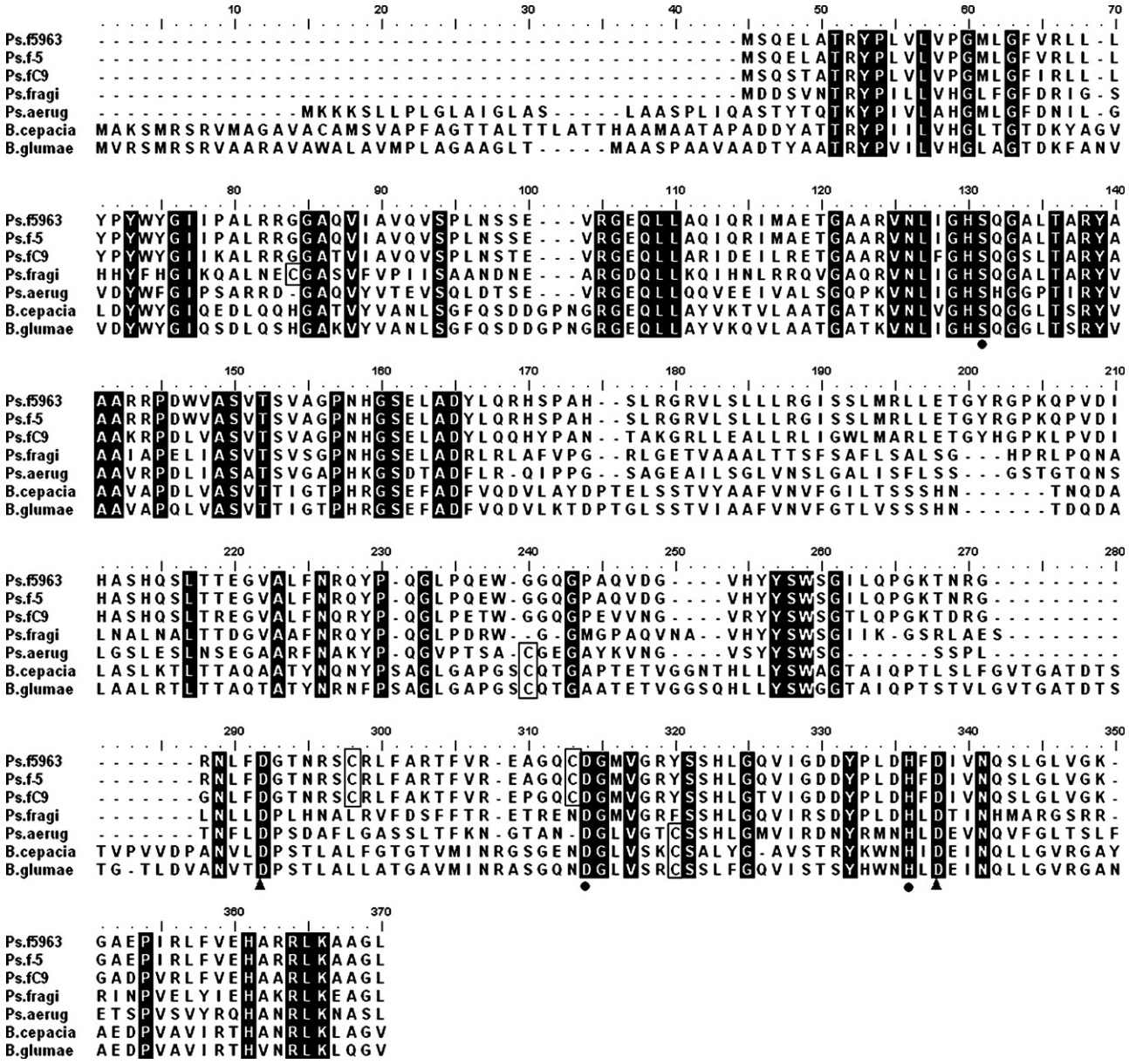

Fig. 1. Alignment of the rPFL sequence with several reported *Pseudomonas* lipases of subfamily I.1 and I.2. The comparison is performed using the ClustalX 1.8 software package and BioEdit version 7.0.9.0. Shaded regions indicate the identical residues. Cys residues are highlighted in rectangles. Symbols: (●) catalytic triad residues; (▲) Asp residues involved in calcium binding. Abbreviations and accession numbers in GenBank of homogeneous lipases: *Pseudomonas fluorescens* JCM5963 (*Ps. fluo*5963, EU310372); *Pseudomonas fluorescens* pf-5 (*Ps. fluo*f-5, AAY96024); *Pseudomonas fluorescens* C9 (*Ps. fluo*C9, AAC15585); *Pseudomonas fragi* (*Ps. fragi*, CAC07191); *Pseudomonas aeruginosa* (*Ps. aerug*, BAA09135); *Burkholderia cepacia* (*B. cepacia*, CAA01276); *Burkholderia glumae* (*B. glumae*, CAA02073).

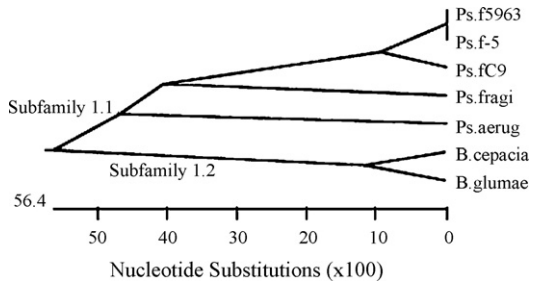

Fig. 2. Phylogenetic tree predicted from sequence alignment of lipases from subfamily I.1 and I.2. The rooted tree is derived by MEGALIGN from the Lasergene sequence analysis software (DNASTAR, Madison). Abbreviations and accession numbers in GenBank are same as that in Fig. 1.

a polypeptide of 296aa residues. The nucleotide sequence data reported here were submitted to GenBank with the accession number EU310372. The translated amino acid sequence was submitted to BLASTP on Esther database and NCBI. As shown in Fig. 1, the protein was absolutely identical with the lipase of *Ps. fluorescens* pf-5 PFL0617 (genome prediction), and it shared 83%, 50% and 43% identities with the subfamily I.1 lipases of *Ps. fluorescens* C9 (over 247 aa), *Ps. fragi* (over 149 aa) and *Ps. aeruginosa* (over 126 aa), respectively, as well as shared 36% identity with both the subfamily I.2 lipases of *B. cepacia* (over 118 aa) and *B. glumae* (over 118 aa). Sequence alignment revealed that the residues forming the catalytic triad and two aspartate residues involved in the Ca^{2+}-binding site described in the known crystal structures were found at homologous positions in all these sequences. However, the two cysteine residues forming a disulfide bridge that were conserved in subfamily I.1 and subfamily I.2 lipases with known three-dimensional structures did not locate at the equivalent positions in the lipases of *Ps. fluorescens* pf-5, *Ps. fluorescens* 5963 and *Ps. fluorescens* C9, which could not form a functional disulfide bridge because they were not at appropriate positions, and there was only one cysteine residue in *Ps. fragi* lipase located close to N-terminus compared with those in the other lipases. In addition, hydrophobicity analysis indicated that there existed a transmembrane region at N-terminal end in the lipases of *Ps. fluorescens* 5963, *Ps. fluorescens* C9 and *Ps. fragi*, respectively, which could not be identified unambiguously as leader sequences for secretion as the signal peptides of *Ps. aeruginosa*, *B. cepacia* and *B. glumae* lipases. The phylogenetic tree predicted from multiple sequence alignment of these homologous lipases indicated that the lipase of *Ps. fluorescens*5963 belongs to subfamily I.1. It was also observed that even though sharing a considerable similarity with well characterized subfamily I.1 and subfamily I.2 lipases, *Ps. fluorescens*5963, *Ps. fluorescens* C9 and *Ps. fragi* lipases were in relatively distant relation to *Ps. aeruginosa*, *B. cepacia* and *B. glumae* lipases and formed an independent clade (Fig. 2). These results suggested a possible and reasonable explanation to the realization of active expression of *Ps. fluorescens* 5963, *Ps. fluorescens* C9 and *Ps. fragi* lipases in *E. coli*, i.e., the lack of disulfide bridge and the secretion signal peptide.

3.2. Expression and purification of rPFL

Overexpression of rPFL as enzymatically active form was obtained under properly controlled culturing conditions as described in the section of materials and methods. The recombinant protein reached to over 20% of the total protein in the cells. The addition of 1% glucose in 2YT medium increased the amount of active expression by 30%. During the course of purification, an important phenomenon was observed, that was, rPFL was unstable in solution and easy to form aggregates like other family I lipases [5,13]. The aggregates were only partially dissolved in Tris–HCl buffer (pH 8.0), but almost completely dissolved in Tris–HCl buffer (pH 8.0) containing 0.2% DOC and showed a high enzymatic activity. The SDS-PAGE results also indicated that less than 30% of the total recombinant protein was dissolved in the supernatant of crude cell lysate, while most of the total recombinant protein existed as aggregates and precipitated with cell debris. The easily soluble fractions existed in the precipitate derived from saturated ammonium sulfate fractionations were removed after washed twice. As shown in Fig. 3A and Table 1, a distinct purification was achieved

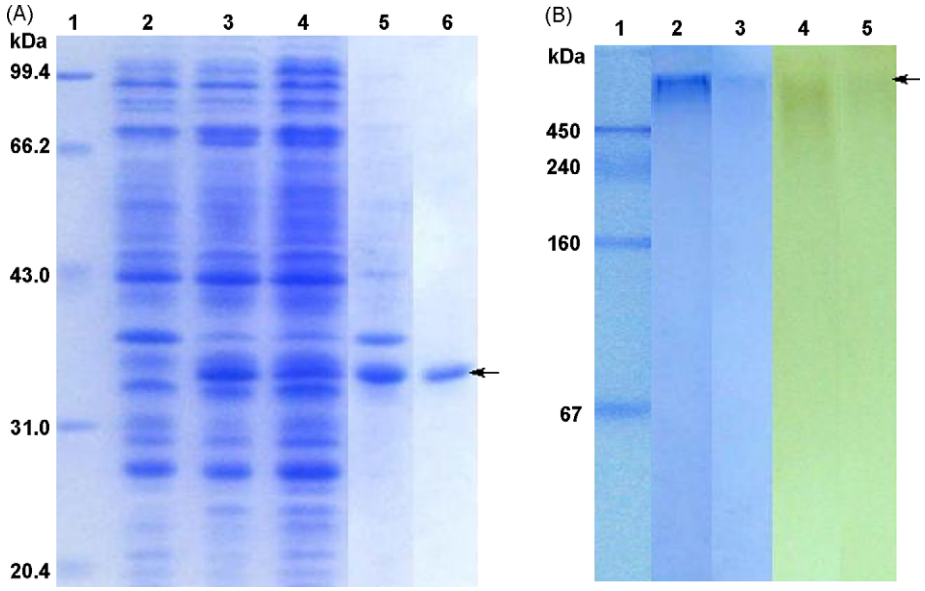

Fig. 3. SDS-PAGE (A) and native gel electrophoresis (B) analysis. The molecular masses of markers are shown in the left. The arrow indicated the target protein rPFL. (A) SDS-PAGE analysis of proteins in each purification step. Lane 1, molecular mass marker proteins; lane 2, total proteins of *E. coli* BLP harboring blank pET28b; lane 3, total proteins of *E. coli* BLP harboring recombinant pET28b; lane 4, supernatant of cell extract; lane 5, cell debris dissolved in buffer containing DOC; lane 6, rPFL purified by Ni-NTA affinity chromatography. (B) Native gel electrophoresis and activity staining of the purified rPFL. Lane 1, native protein marker; lanes 2 and 3, Coomassie brilliant blue R-250 staining at protein concentration of 2 and 0.05 mg/ml; lanes 4 and 5, activity staining at protein concentration of 2 and 0.05 mg/ml.

Table 1
Summary of purification procedures of rPFL expressed in *E. coli*

	Step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification fold[a]	Yield (%)[b]
A	Supernatant of cell lysate	1687.3	51.6	32.7	1	
B	Saturation ammonium sulfate precipitation and cell debris	6115.1	25.7	242.0	7.4	100
C	Ni-NTA agarose affinity	3486.4	8.0	435.8	13.3	57.0
D	Sephacryl S-200 gel filtration	3271.5	7.4	442.1	13.5	53.5

[a] The specific activity of Step A was used as initial value.
[b] The total activity of Step B was used as initial value when the yield of Step C and Step D was calculated because the total activity generated from cell debris in Step B could not be included in the total activity of Step A.

with a 7.4-fold increase of purity compared to cell lysate in Step A and about 60% of the target protein in total proteins estimated on SDS-PAGE gel, and the Ni-NTA affinity chromatography gave a production of 8.0 mg rPFL per g of wet cells, with a purity of over 95% and a specific activity of 435 U/mg. There was only one active peak with a molecular mass over 240kD eluted in the void volume in gel filtration chromatography (data not shown). The molecular mass of the recombinant rPFL was estimated to be 34 kD by SDS-PAGE gel, which agreed well with the theoretically calculated molecular mass. Furthermore, as shown in Fig. 3B, the apparent molecular mass estimated by N-PAGE also indicates that there is no monomer found in the enzyme solution even at a low concentration (0.05 mg/ml), and the purified rPFL is active and exists as aggregates with a high molecular mass at the concentrations of 2 or 0.05 mg/ml.

It has been reported that the folding and secretion of subfamily I.1 and I.2 lipases from *Ps*. species are highly specific processes that normally do not function properly in heterogonous hosts, and the efficient secretion and correct folding to enzymatically active state require the functional assistance of the chaperone proteins [15]. Up to now, there have been only two *Ps*. lipases from psychotropic strains including *Ps. fluorescens* C9 and *Ps. fragi* IFO 34584 belonging to subfamily I.1 reported with enzymatically active expression in *E. coli* to our knowledge. However, no detailed data about production amount and purification were described for *Ps. fluorescens* C9 lipase. As for *Ps. fragi* IFO 3458 lipase, the expression level was relatively low and no more than 2 mg of pure recombinant target protein was recovered per g of wet weight cells. Considering all the results above, we concluded that these lipases exhibit an intrinsic folding capability in vitro without chaperon protein, which support the hypothesis put forward by I. Beacham et al., that is, these subfamily I.1 lipases might be secreted via a signal peptide-independent pathway [11]. Moreover, the lack of the disulfide bridge conserved in homologous *Ps.* lipases may also contribute to the formation of active conformation for these lipases in *E. coli*. In addition, aggregation tendency observed in this work is a nearly universal property for family I lipases because of the strong hydrophobicity in nature, as can be found from previous descriptions of other family I members. For example, *Ps. fluorescens* SIK W 1(subfamily I.3) is readily precipitated in an aggregated form when it is concentrated to ≥5 mg/ml; the *Ps. aeruginosa* lipase exhibits a strong tendency to form high-Mr. aggregates when isolated from the cell-free growth medium or purified and biochemically characterized [26,27].

3.3. Effects of temperature and pH on activity and stability

The temperature-activity curve (Fig. 4) shows the optimal reaction temperature is 55 °C. The purified rPFL keeps more than 80% of the maximum activity at 55 °C in a temperature range of 45–70 °C. After incubated at temperatures of 50 and 60 °C for 1 h, the enzyme remains over 90% and 75% residual activity compared with the initial activity before incubation, respectively. However, the residual activity decreases sharply when the temperature is above 60 °C. The purified rPFL shows an optimum hydrolytic activity at pH 9.0 (Fig. 5A), and is distinguished from most lipases whose optimum pHs are in a range of pH 7.0–8.0 [5]. It exhibits a more than 80% of the maximum activity at pH 9.0 in a range of pH 7.6–10.0, while no activity is detected at pH 4.0–5.8. These results are consistent with the other alkaline lipases from the strains of *Pseudomonas, Acinetobacter and Bacillus subtilis* [5,20,22]. Furthermore, the pH stability curve (Fig. 5B) of rPFL shows that the enzyme is stable over a broad pH range of 5.0–9.5 with a more than 80% residual activity after incubated at 25 °C for 20 h.

3.4. Substrate specificity of rPFL

As shown in Table 2, rPFL exhibits activity toward *p*-nitrophenyl fatty acid esters with a broad range of acyl chain lengths, and the highest hydrolytic activity of rPFL is obtained to *p*NPC$_8$. The activities of rPFL to *p*NPC$_2$, *p*NPC$_4$, *p*NPC$_{10}$ and *p*NPC$_{12}$ are moderate, and only 21.7% and 18.6% of the maximum activities to *p*NPC$_8$ are detected to *p*NPC$_{16}$ and *p*NPC$_{18}$, respectively. These results demonstrate that the rPFL prefers medium-chain length fatty acid esters of *p*-nitrophenyl. Activity detection using olive oil emulsion as substrate demonstrates rPFL exhibits hydrolysis activity of 156 U/mg, which suggests that rPFL can be defined as a true lipase.

3.5. Effects of metal ions and surfactants on rPFL

Metal cations, particularly Ca^{2+}, play important roles in influencing the structure and function of enzymes, and many calcium-stimulated lipases have been reported [13]. Results of

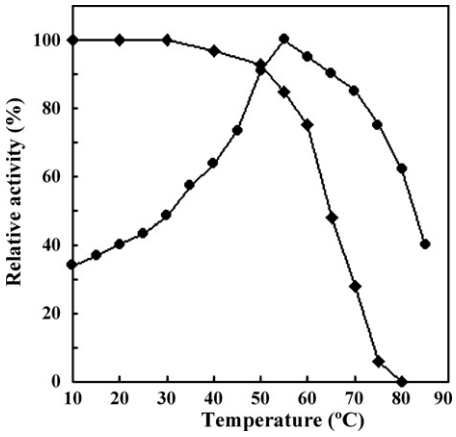

Fig. 4. Effects of temperature on activity (●) and stability (♦) of rPFL. The curve of temperature-activity was determined by measuring the relative activity at different temperatures. The curve of temperature-stability was determined by measuring the residual activity at 55 °C after incubating the purified lipase (0.05 mg/ml) at a temperature range of 10–80 °C for 1 h.

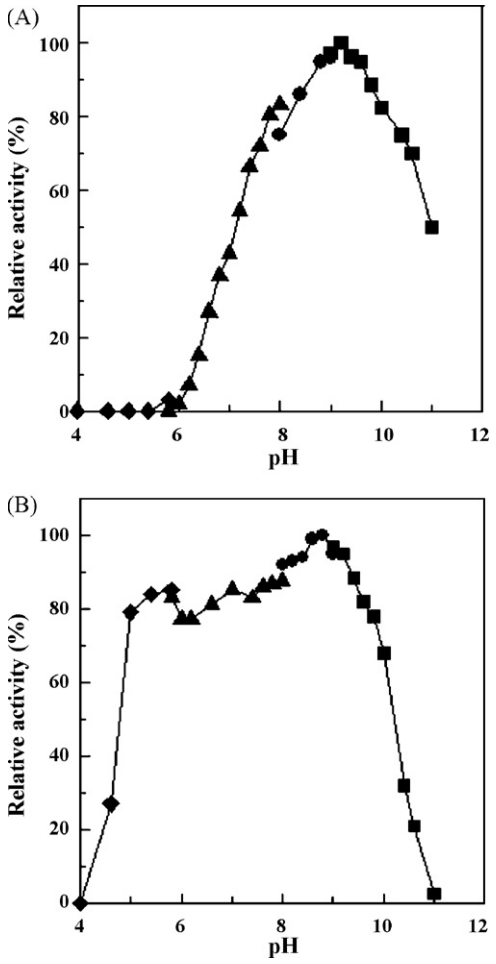

Fig. 5. Effects of pH on activity (A) and stability (B) of rPFL. Buffers: HAc–NaAc ((♦) pH 5.0–5.8); Na_2HPO_4–NaH_2PO_4 ((▲) pH 5.8–8.0); Tris–HCl ((●) pH 8.0–9.0); glycine–NaOH ((■) pH 9.0–11.0). (A) Purified lipase samples were diluted in 20 mM Tris–HCl (pH 8.0) to 0.05 mg/ml. Lipase activity was assayed in various buffer from pH 5.0 to pH 11.0 using $pNPC_8$ (0.2 mM) as substrate at 55 °C. (B) Purified lipase samples were diluted in buffer with different pH values to 0.05 mg/ml, pH adjusted, and incubated for 20 h at 25 °C.

sequence analysis also suggest the presence of a Ca^{2+}-binding site in rPFL. Table 3 indicates that 1 mM Ca^{2+} or Sn^{2+} increases the activity of rPFL by 10% or 5% respectively with respect to the blank control. On the contrary, Fe^{2+}, Cu^{2+}, and Zn^{2+} significantly inhibit the activity of rPFL and Zn^{2+} inhabits nearly 70% activity especially. EDTA inhibits the activity of rPFL by 40%, while non-ionic surfactants Triton X-100 and Tween 20 at 0.1% (v/v) increase its activity by 8% and 9%, respectively. Anionic surfactant SDS completely inhibits the hydrolytic activity of rPFL. On the basis of the results above, we can optimize the activity of rPFL by selectively adding metal ions and proper surfactants in the reaction system.

Table 2
Substrate specificity of rPFL towards *p*-nitrophenyl fatty acid esters with various acyl chain lengths

Substrate	Specific activity (U/mg)	Relative activity (%)
p-Nitrophenyl acetate (C_2)	256.3	58.8
p-Nitrophenyl butyrate (C_4)	298.1	68.4
p-Nitrophenyl caprylate (C_8)	435.8	100
p-Nitrophenyl decanoate (C_{10})	373.0	85.6
p-Nitrophenyl laurate (C_{12})	211.8	48.6
p-Nitrophenyl palmitate (C_{16})	94.6	21.7
p-Nitrophenyl stearate (C_{18})	81.1	18.6

Table 3
Effects of various metal ions, EDTA and various surfactants on the activity of rPFL

Metal ions/surfactants	Concentration (mM)	Relative activity (%)
Blank	–	100
Ca^{2+}	1	110.07
Sn^{2+}	1	105.78
Ba^{2+}	1	100
Mg^{2+}	1	100
Mn^{2+}	1	95.92
Fe^{3+}	1	79.9
Cu^{2+}	1	75.17
Zn^{2+}	1	32.29
EDTA	1	60.82
Tween 20	0.1% (w/v)	108.21
Triton X-100	0.1% (w/v)	109.33
SDS	0.1% (w/v)	0

3.6. Stability of rPFL in organic solvents

Stability and activity in organic solvents are important characteristics of lipases used in organic synthesis. Fig. 6 shows the relative hydrolytic activity of rPFL in the presence of different organic solvents (30% v/v) after incubation for 1 and 24 h. The rPFL was activated in the presence of almost all the water-miscible organic solvents investigated after it was incubated for 1 h, and remained stable after it was incubated for 24 h in the presence of short carbon chain alcohols including isopropanol, methanol and ethanol, as well as acetone and glycerol. About 20% activity of rPFL was lost in the presence of dimethylsulfoxide and tetrahydrofuran after it was incubated for 24 h. The observed highest activities relative to that of the control were 142% in isoamyl alcohol after incubation for 1 h and 120% in isopropanol after incubation for 24 h. In contrast, the rPFL was inactivated to some extent in the presence of water-immiscible organic solvents such as cyclohexane, chloroform and benzene, but its activity was partly recovered with the increased incubation time. The relative activities were 80%, 65% and 33% after incubation for 1 h, but were recovered to 87%, 91% and 56% after incubation for 24 h in the presence of each of these three solvents, respectively. Significant inactivation was observed in the presence of acetic acid. This may be due to the acidity of the solvent that may have inactivated the lipase [18]. The similar results have been reported for lipases from other two organic solvent tolerant *Ps. aeruginosa* strains [19,20]. However, different experiment results were also reported for *Galactomyces geogrichum* Y05 lipase [14]. It showed more than 80% residual activity after incubation for 6 h in 50% (v/v) methanol or cyclohexane, but its activity decreased to 13% or 0 when ethanol or chloroform was involved. Therefore, different

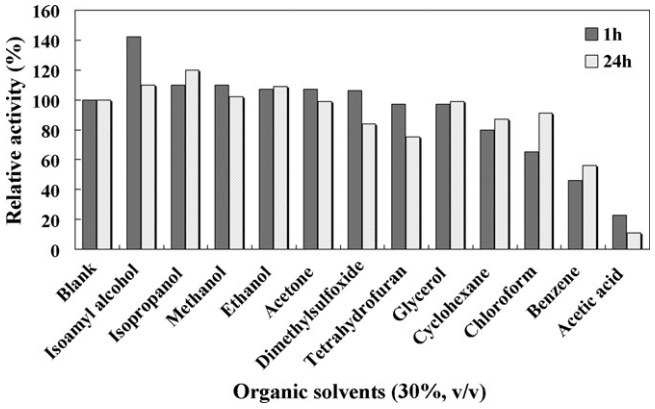

Fig. 6. Effects of various organic solvents on the activity and stability of rPFL. Lipase activity was assayed by measuring the residual activity after incubating the purified enzyme (0.1 mg/ml) in the presence of various organic solvents (30%, v/v) in 20 mM Tris–HCl buffer (pH 8.0) for 1 h and 24 h, respectively.

lipases show distinct stabilities against various organic solvents, and further mechanism investigations are needed.

4. Conclusions

In this study, we have successfully cloned a lipase gene from the organic solvent degradable strain *Ps. fluorescens* 5963, and firstly expressed an organic solvent tolerant lipase of subfamily I.1 as active aggregates at a high level in *E. coli* without coexpressing the helper foldase and the subsequently denaturing and refolding. The purified recombinant lipase not only exhibits high activity in wide ranges of temperatures and pH values, but also behaves quite stable under alkaline, moderate temperature conditions and even in the presence of some surfactants. The most important feature of rPFL is the high activity and extreme stability against a variety of organic solvents. All these results provide sufficient advantages to make rPFL a promising candidate for application in nonaqueous biocatalysis and in the field of detergent additives. In addition, this work will be of great value to both the efficient expression and the large-scale production of *Ps*. lipases widely used in organic synthesis with *E. coli* as a host cell.

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