

Enhancement of lipase activity from *Acinetobacter* species SY-01 by random mutagenesis and the role of lipase-specific chaperone

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Abstract

To achieve improved lipase activity for the hydrolysis of various monoesters, a large number of mutant lipases from *Acinetobacter* species were constructed and analyzed in a high throughput manner by direct evolution. Enzyme libraries were created in *Escherichia coli*, transfected into *Bacillus subtilis* 168 by an electroporation method and expressed. The transfected library was screened using a *Rhodamine B* plate containing olive-oil and a phenol-red pH indicator plate containing glyceryl tricaprlylate as substrates. One lipase variant, M58, which has five amino acid (S21F, I02G, S103F, D299E and N300H) mutations, was selected and characterized. The acyl chain-length selectivity of the in vitro expressed lipases against various *p*-nitrophenyl (*p*-NP) monoesters were compared by their relative hydrolysis rates. The result showed that lipase mutant M58 increased its selectivity for the short-C2 and C4 and the long-chain esters, C10, C12, C14 and C16, but decreased its selectivity for the middle-chain esters, C6 and C8. Wild-type and M58 lipases had been expressed in the presence and absence of its lipase-specific foldase (*lif*) in *B. subtilis* 168 and relative activity was measured. As a result, the activities of the wild-type and M58 lipases in the presence of *lif* increased by 1.8-fold, with enhanced stability.

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Keywords: *Acinetobacter* species; Lipase; Lipase-specific chaperone; Lipase-specific foldase (*lif*); Random mutagenesis; Substrate selectivity; Direct evolution

1. Introduction

Directed evolution is a powerful technique that has been used to improve various properties of enzymes. Genes are subjected to iterative cycles of random mutagenesis and/or recombination, expressed in a suitable host system and subsequently screened high-throughput and selected for improved variants [1,2].

Random mutagenesis combined with high-throughput screening is a useful method for exploring the functional amino acid residues of a protein. This method includes two processes: introduction of mutation into the gene of the target protein, and subsequent screening of objective clones from the given mutational library. It is particularly important to establish a suitable screening system. Simple and effective screening procedures enable the identification of functional residues in the target protein [2,3].

Lipase-specific chaperone or *lif* proteins need to form complexes with their enzyme substrates to exert their biological function. The helper proteins seem to act as molecular chaperones that assist lipase proteins in proper folding to achieve their native conformation. The natural ‘substrate’ for *lif* is not yet clearly defined nor is the location of *lif* action exactly known. Binding of the lipase–*lif* complex to the export machinery could result in a conformational change leading to the release of lipase from the complex and its subsequent translocation extracellularly [4,5].

Lif proteins function in vivo as chaperones that assist in the folding of lipases into their active, protease-resistant conformations [5]. Finally, the correctly folded lipase is secreted through the outer membrane mediated by one or more Xcp Q, T, U, V, or W proteins [6]. Unlike the situation in *Pseudomonas*, no essential lipase helper protein has been identified in *Acinetobacter* during the secretion mechanism process [7]. In our previous report, the extracellular lipase of *Acinetobacter* species SY-01 was found to be presumably exported from the cell via a two-step translocation process, as found in other group I *Pseudomonas* lipases and the active

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lipase was characterized using a *Bacillus* system [8]. However, in the *Pseudomonas* species, *lif* proteins are invariably encoded downstream of the structural lipase gene and this is the case in *Acinetobacter* species SY-01. Genetic characterization of an additional mutant demonstrates that lipase production in *Acinetobacter* species SY-01 also requires a periplasmic *lif* protein that is membrane bound.

In the present study, to improve activity of the lipase from *Acinetobacter* species SY-01 for hydrolysis of various lipid substrates, we utilized the random mutagenesis method by Clontech Diversify PCR Random Mutagenesis Kit [9] and screened the mutant from the *Rhodamine B* plate [10] and the pH indicator plate using the modified phenol red screening method [3]. A mutant was selected and screened for triglyceride selectivity and monoester specificity and compared to the wild-type. The effect of *lif* on the activity and stability of the mutant was also compared to the wild-type using a monoesterase substrate.

2. Material and methods

2.1. Plasmids, bacterial strains, and culture conditions

Escherichia coli was grown in Luria-Bertani (LB) medium (1.0% NaCl, 0.5% yeast extract and 2.5% tryptone, pH 7.5) at 37 °C. *Bacillus subtilis* 168 containing recombinant plasmid was grown in rich medium (20 mM K₃PO₄, 1.0% glucose, 1.0% polypeptone, 0.5% beef extract, 0.2% yeast extract, 1.0% KCl and 0.01% MgSO₄, pH 7.5) on a gyratory shaker with 200 rpm at 30 °C. The *E. coli* strain DH5 α was used as a host for the manipulation of recombinant plasmids. *Bacillus subtilis* 168 was used as a host for the secretion of lipase from recombinant plasmids. Antibiotics were used as the indicated final concentrations, in plates and in liquid media: ampicillin, 100 μ g/ml; kanamycin, 50 μ g/ml.

2.2. Chemicals

Restriction enzymes and T4 DNA ligase were obtained from New England BioLabs (Beverly, MA). Sequence primers were synthesized by COSMO Co. Ltd. (Seoul, South Korea). *Bacillus* shuttle vector, pLIPSM(1), was obtained from Bioleaders Co. (Taejeon, Korea). *Rhodamine B* dye, phenol-red, copper(II)-acetate-1-hydrate aqueous solution, chromogenic reagent containing diethyldithio-carbamic acid, triglycerides and various *p*-NP monoesters were obtained from Sigma (Seelze, Germany). The Diversity PCR Random Mutagenesis Kit was purchased from Clontech (Palo Alto, CA).

2.3. Random mutagenesis and construction

The Diversity PCR Random Mutagenesis Kit was applied for random mutagenesis, and the random mutagenesis conditions were described below.

Two synthetic oligonucleotides, containing each *Bam*HI (forward primer) and *Sal*I (reverse primer) restriction site, were used as primers for mutation and amplification. PCR for the random mutagenesis was carried out using 10 ng of template DNA of the wild-type lipase, 25 pmol of each primer, 1 \times Diversity dNTP mix, 1 \times PCR buffer, MnSO₄ (8 mM) X μ l, dGTP (2 mM) X μ l, and 1.25 U Taq DNA polymerase made upto a total volume of 50 μ l. PCR consists of 25 cycles using 30 s of denaturing at 94 °C, followed by extension at 68 °C for 1 min. The volume of X was controlled depending on the ratio of mutants. PCR reaction was repeated with the mutated PCR product as a template until we get a mutant of enhanced hydrolysis compared with wild-type. To produce a random mutant plasmid library, the randomly mutated PCR product and the pLIPSM(1) vector were digested with *Bam*HI and *Sal*I restriction enzymes and ligated with T4 DNA ligase. Efficiency of random mutant plasmid library was 1 \times 10⁸ cfu/plate in *E. coli*, then the plasmid library stock was transfected in *B. subtilis* 168 by electroporation [11]. Library transfected into *B. subtilis* 168 was screened compared with wild-type on *Rhodamine B* plate containing olive-oil as substrate and on phenol-red pH indicator plate containing glyceryl tricaprlylate as a substrate.

2.4. Plasmid construction and over-expression

The construction of recombinant plasmid pSYLipSM(1), wild-type, is described below. The 1017 bp lipase full gene was cloned into the *Bacillus* shuttle vector, pLIPSM(1). The recombinant plasmid was transfected and amplified into DH5 α (Fig. 1).

The construction of the recombinant plasmid, containing *Acinetobacter* species SY-01 lipase gene (or mutant gene) and *lif*, was performed as follows. The *Bam*HI/*Sal*I (containing end codon at 3'-terminal) lipase full gene (1017 bp) and *Sal*I/*Pst*I (containing ribosome binding site (rbs) before start codon at 5'-terminal) *lif* were cloned into pLIPSM(1). The recombinant plasmid was transfected and amplified into DH5 α (Fig. 1b). Cloned recombinant plasmids were transfected into *B. subtilis* 168 using the electroporation method [11].

The over-expression conditions of the wild-type and mutant were the same and are described below. Cells containing recombinant plasmid were grown at 37 °C overnight in 10 ml LB broth containing 50 μ g/ml kanamycin, inoculated in 1 l of rich broth containing 50 μ g/ml kanamycin, then cultured at 30 °C for 48 h at 200 rpm with saturation aeration. Cultured cells were centrifuged at 4000 rpm for 15 min, and supernatant was lyophilized. The lyophilized powder was re-suspended in 30 ml of 20 mM Tris-HCl buffer, pH 7.5, containing 5 mM CaCl₂, and dialyzed in 4 l of 20 mM Tris-HCl buffer, pH 7.5, at 4 °C for 24 h. The dialyzed solution was purified by FPLC using anion-exchange chromatography on a Fast flow Q-Sepharose column (Amersham Pharmacia Co.,

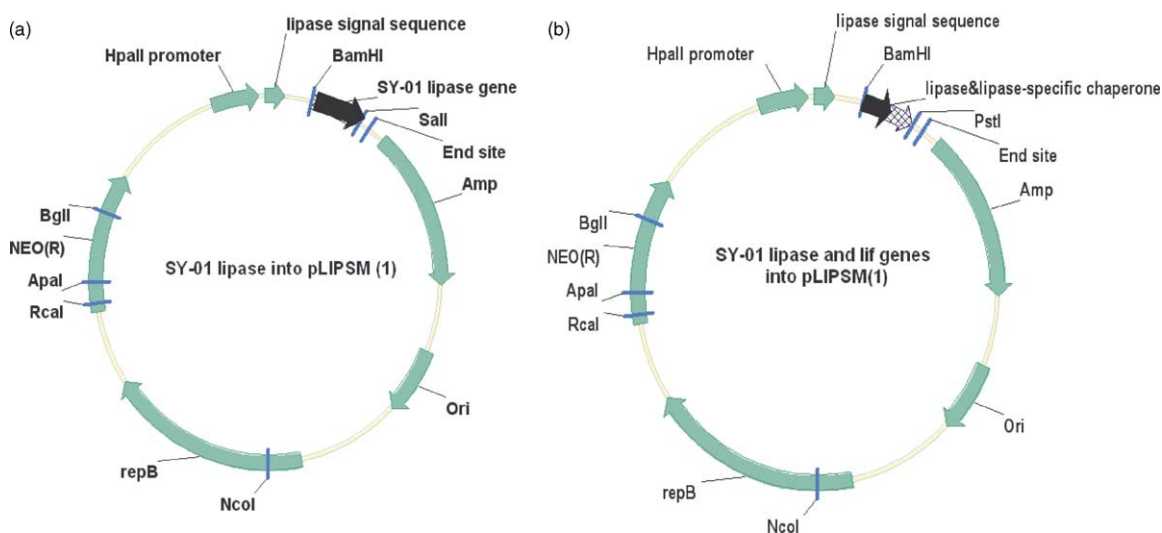


Fig. 1. Construction of recombinant plasmid containing lipase and recombinant plasmid containing lipase and lif from *Acinetobacter* species SY-01. (a) The *Bam*HI/*Sal*I lipase full gene (1017bp) was cloned into the *Bacillus* shuttle vector, pLIPSM(1) containing the *Hpa*II promoter and the lipase signal sequence. (b) The *Bam*HI/*Sal*I (containing end codon at 3'-terminal) lipase full gene and *Sal*I/*Pst*I (containing rbs site before start codon at 5'-terminal) lif gene were cloned into pLIPSM(1) vector.

Piscataway, NJ), a Resource Q column (Amersham Pharmacia Co.) and gel filtration column superdex-200 (Amersham Pharmacia Co.) sequentially. Fast flow Q-Sepharose column was equilibrated with 20 mM Tris-HCl buffer, pH 7.5, containing 5 mM CaCl₂ and eluted with a linear gradient (0.2–0.8 M NaCl) in the same buffer at flow rate of 2 ml/min. The lipase-containing fractions were dialyzed with 2 l of 20 mM Tris-HCl buffer, pH 7.5, containing 5 mM CaCl₂. After the resource Q column was equilibrated with 20 mM Tris-HCl buffer, pH 7.5 containing 5 mM CaCl₂, the dialyzed sample was eluted with a linear gradient 0–1 M NaCl at flow rate of 2 ml/min. Finally, the sample was filtered using gel filtration column, superdex-S200, and the purified lipase was analyzed with SDS-PAGE. The protein concentration was determined according to the Bradford method [12] using bovine serum albumin as a standard.

2.5. Selection of the mutant library

Lipase activity was confirmed in LB agar plates containing olive-oil as a substrate and *Rhodamine B* [10]. *Rhodamine B* (1 mg/ml) was dissolved in distilled water and sterilized by filtration. LB agar medium containing 1% (w/v) olive-oil was autoclaved and cooled to approximately 60 °C and then 0.001% (w/v) of *Rhodamine B* solution (1 mg/ml) was added. Lipase activity was monitored by irradiating the plates with UV light at 350 nm.

For screening mutants using phenol-red pH indicator plate, the reactions were set up in a 96-well microplate [3] and the total volume was 200 µl split as follows: cell supernatant (100 µl) containing the mutated lipase gene and 100 µl of emulsified 20 mM Tris-HCl (pH 8) containing 10 µl tricaprilyn, 5 mM CaCl₂ and 0.001% indicator

phenol-red dye. The reaction mixture was incubated at 37 °C.

2.6. Assay of lipase activity

2.6.1. Method I

Lipase activity was measured by a spectrophotometry assay [13] with the synthetic substrate *p*-NP butyrate, which was dissolved in acetonitrile at a concentration of 10 mM. Subsequently, absolute ethanol, acetonitrile and 50 mM potassium phosphate buffer (pH 7.5) were added to a final composition of 1:4:95 (v/v/v) acetonitrile/ethanol/buffer. The purified enzyme was mixed with the *p*-NP butyrate solution and the final concentration was 3 mM. The reaction mixture was incubated at 50 °C for 30 min and finally the reaction was stopped by addition of 150 µl of 1 M acetone. Absorbance at 405 nm was measured with a spectrophotometer (SpectraMax 340PC³⁸⁴, Molecular Device Co., Sunnyvale, CA) against an enzyme-free blank and one enzyme unit was defined as the release of 1 nmol of *p*-nitrophenol/ml/min. The extinction coefficient for *p*-nitrophenol under these conditions was 14,630 cm⁻¹ M⁻¹ [14]. One unit of lipase was defined as the amount of lipase that caused the release of 1 µmol of *p*-NP/min. Relative activity was calculated by regarding maximum activity at given conditions as 100%.

2.6.2. Method II

The relative hydrolytic activity of the lipase towards different triglycerides was determined by a modified spectrophotometric assay using the formation of copper soaps for the detection of free fatty acids [15]. The substrate solution was consisted of triglycerides (100 mM) emulsified

with homogenizer (Daehan, HOG001) in distilled water with gum arabic (0.2 mM) at maximum speed (15,000 rpm) for 2 min. An aqueous solution of copper(II)-acetate-1-hydrate (90 mM), adjusted to pH 6.1 with pyridine, was used as the copper reagent. The chromogenic reagent contained diethylthio carbamic acid (5.8 mM) dissolved in absolute ethanol. The reaction was started by addition of 10 μ l of enzyme solution in 0.99 ml 20 mM Tris buffer (pH 10) contained 5 mM CaCl₂. The enzyme reaction was carried out for 1 h at 50 °C with slow agitation. Immediately after incubation, 1 ml of the reaction mixture was transferred to a test tube containing 0.5 ml of 5N HCl in order to stop reaction. Fatty acids were subsequently extracted by addition of 3 ml *n*-hexane and mixed vigorously using a vortex for 2 min. The organic phase (2.5 ml) was transferred to a fresh test tube filled with 1 ml copper reagent. The mixture was vortexed for 1.5 min and phase separation was achieved by centrifugation (13,000 rpm, Eppendorf). Then, the organic phase (2 ml) was mixed with 0.4 ml of the chromogenic reagent and the absorption was measured at 430 nm by spectrophotometer (UV-1601PC, Shimadzu Co., Columbia, MD).

2.7. DNA sequencing of the mutant lipase

The fluorescence-based dideoxy DNA cycle sequencing method was used to determine the sequences of the PCR products. DNA sequencing was performed using an ABI 373 automated sequencer (PE Applied Biosystems, CA, USA).

2.8. Stability study

For the stability studies of lipase from *Acinetobacter* species SY-01, the enzyme was incubated in a 50 mM potassium phosphate buffer, pH 7.5, at 30 °C. At time intervals, aliquots were withdrawn and placed into tubes in ice and assayed for their remaining activity using *p*-NP butyrate as a substrate.

3. Results

3.1. Random mutagenesis and screening for direct evolution

Mutant genes were generated according to the method of random mutagenesis using Diversity PCR Random Mutagenesis Kit as described. *Acinetobacter* species SY-01 lipase variants were produced using the Diversity PCR Random Mutagenesis Kit with library sizes of 1×10^8 cfu/plate in *E. coli*. Approximately 8000 clones were transformed into *B. subtilis* 168 and each was screened on *Rhodamine B* plates and pH indicator phenol-red plates leading to the identification of variants with an increased activity for hydrolysis of various triglycerols and monoesters scheme described in Fig. 2.

Table 1

Substituted amino acids codon of M58

Substituted amino acids codon by DNA codon change	
TCT to TTT.....	S21F
GCT to GGT.....	A102G
TCT to TTC.....	S103F
GAT to GAA.....	D299E
AAT to CAT.....	N300H

The first screening was based on the *Rhodamine B* plate assay containing olive-oil as substrate at 37 °C, and about 250 positive clones were identified from 8000 transformants. Finally, one positive clone (M58) was selected from the first 250 positive clones based on the phenol-red pH indicator assay with tricaprylin as a substrate. A positive result was indicated by a change in color from red to yellow caused by the hydrolyzed free fatty acid (Fig. 3). These results suggested that the mutant M58 has higher hydrolysis activity compared to the wild-type towards tricaprylin.

3.2. Sequence analysis and purification of M58

The recombinant plasmid was extracted from randomly mutated clones, and the DNA sequence of the inserted fragment was determined. Five base changes, resulting in the replacement of five amino acid residues, were found in this randomly mutated gene. The amino acid substitutions were located in the N-terminal region (S21F, A102G and S103F) and C-terminal region (D299E, N300H) (Table 1).

The recombinant strains producing the wild-type and M58 lipases were cultured, and purified by sequential treatment with anion exchange fast flow Q-sepharose, resource Q, and Superdex-S200 columns. The procedures to express and purify M58 and the wild-type lipases were identical. M58 lipases had an apparent molecular mass of 43.8 kDa (40 amino acids of signal sequence and multi-cloning site in vector were included) were purified (Fig. 4B).

3.3. Comparison of the activities of the wild-type and M58

Fig. 5 shows the relative activities for hydrolysis of a various triglycerides as determined by assay method II. Activity of M58 was higher than that of wild-type. The rates of increased activity of M58 over the wild-type were >7- and >1.5-fold towards glyceryl tricaprylate (C8) and glyceryl trioleate (C18:1), respectively. M58 and the wild-type hydrolyzed synthetic substrates containing the acyl group with chain length between C2 and C16 (Fig. 6). These relative activities were determined by assay method I. The relative activity of M58 for the short-chain substrates C2 and C4 increased 2- and 2.5-fold compared with that of wild-type. The relative activity of M58 for the middle-chain substrates,

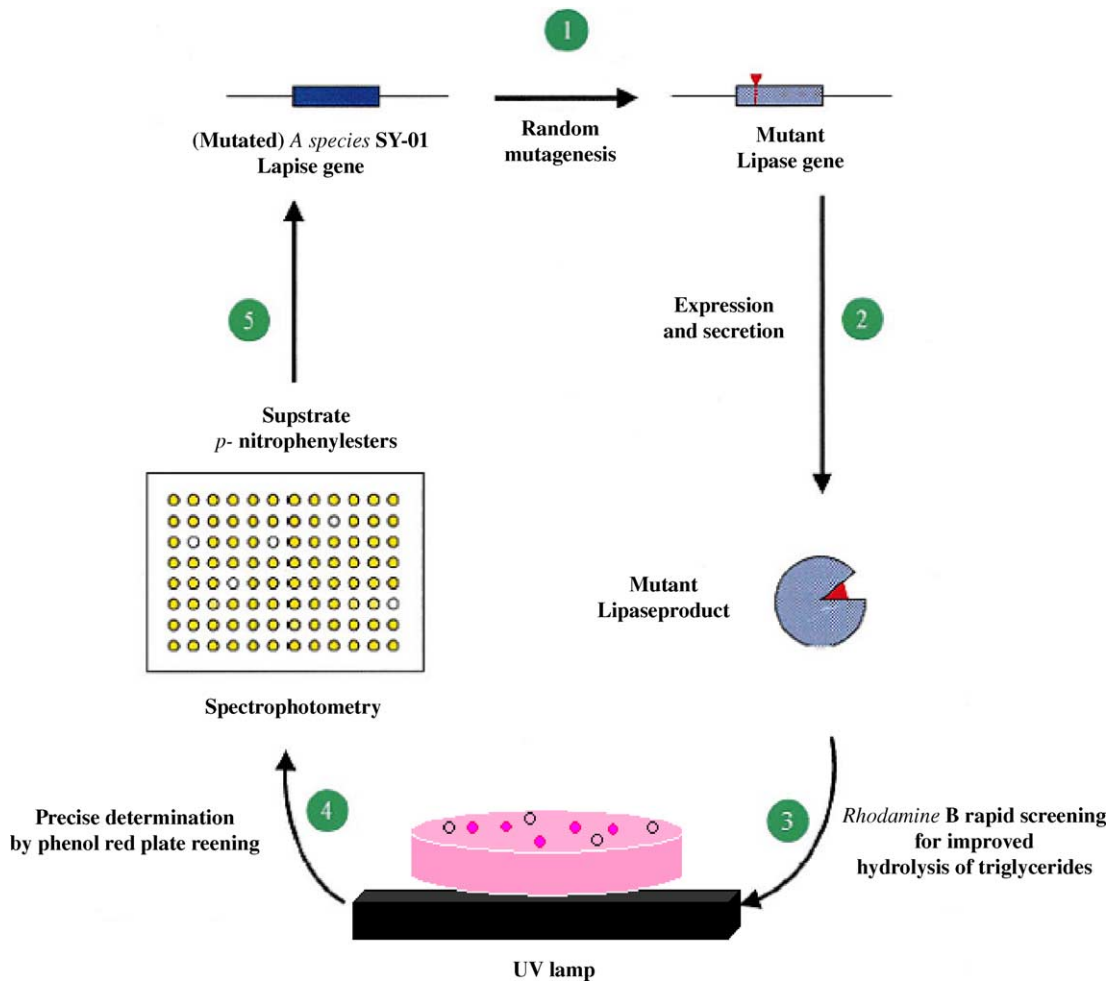


Fig. 2. Scheme of random mutagenesis and screening.

C6 and C8, decreased compared to the wild-type. For the long-chain substrates, C10, C12 and C14, the relative activity of M58 increased more than approximately five-fold over that of the wild-type. In particular, the relative activity of M58 increased by 12-fold over that of the wild-type towards C16 substrate. The substrate specificity of M58 was also altered compared to the wild-type.

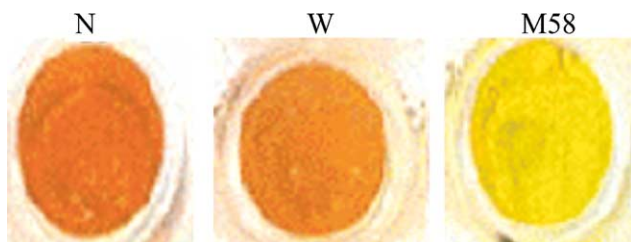


Fig. 3. M58 was screened using the pH indicator plate assay on 96-well plate. N, cell crude from only pLIPSM(1) vector as a negative control; W, cell crude from recombinant plasmid containing wild-type lipase; M58, cell crude from recombinant plasmid containing mutated lipase (M58).

3.4. The effect of lif on the activity and stability of lipase

Lif from *Acinetobacter* species SY-01 were cloned on the same plasmids and over-expressed to investigate the effect of lif on the wild-type and M58 lipase activity (Fig. 1b). The expressed proteins of the wild-type and M58 in the presence and absence of lif were purified and characterized by assay method I using *p*-NP butyrate. In the absence of lif, the specific activity of M58 expressed was 286 U/mg at 50 °C, which is higher than 180 U/mg of the wild-type lipase. After overproduction of lif, specific activity of M58 was increased to 598 U/mg of M58 and 332 U/mg of wild-type (Table 2).

Table 2
Specific activity of purified wild-type and M58 lipases for *p*-NP butyrate with lif or without lif

Lipases	Wild-type		M58	
	+	-	+	-
Lipase-specific chaperone	+	-	+	-
Total protein (mg)	2	2	2	2
Total activity (U)	664	360	1196	572
Specific activity (U/mg)	332	180	598	286

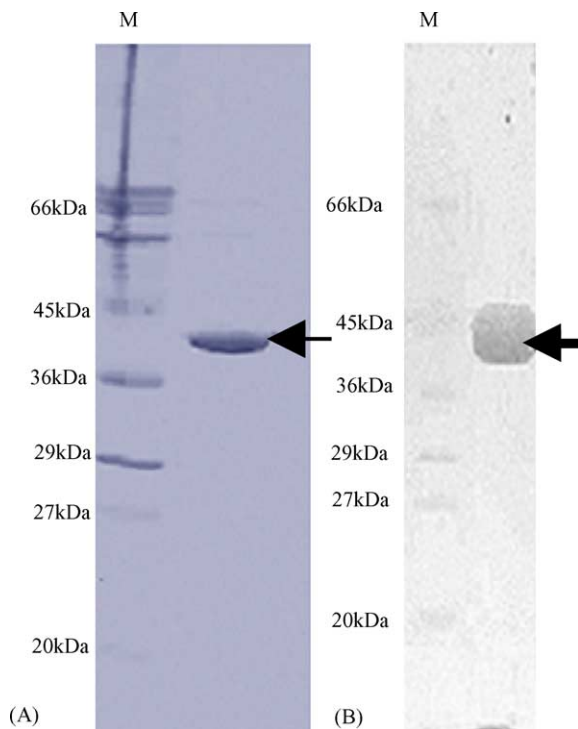


Fig. 4. SDS-PAGE analysis for purification of wild-type and M58 lipases. (A) Purified wild-type lipase, M: molecular weight marker, (B) purified M58 lipase, M: molecular size marker.

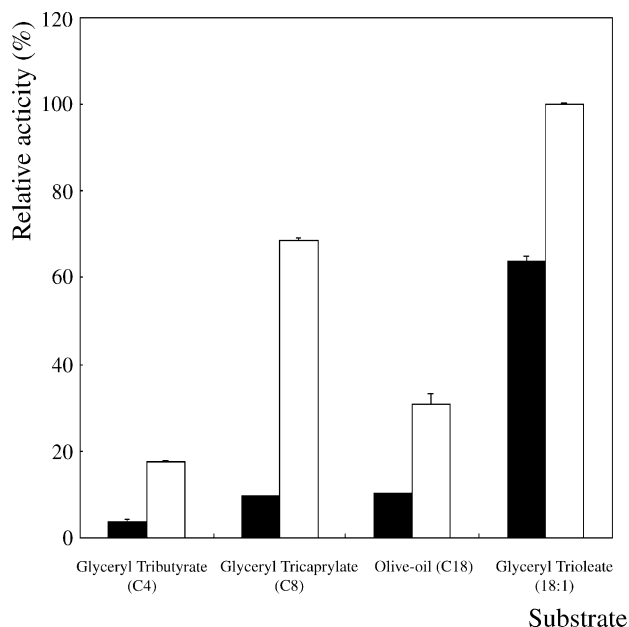


Fig. 5. Relative activities of wild-type and M58 lipases for various triglycerols. Lipase activities on each substrate are expressed as the percentage and activities given are the average values of triplicate measurements by assay method II in Section 2. The solid bar is wild-type lipase and the empty bar is mutant.

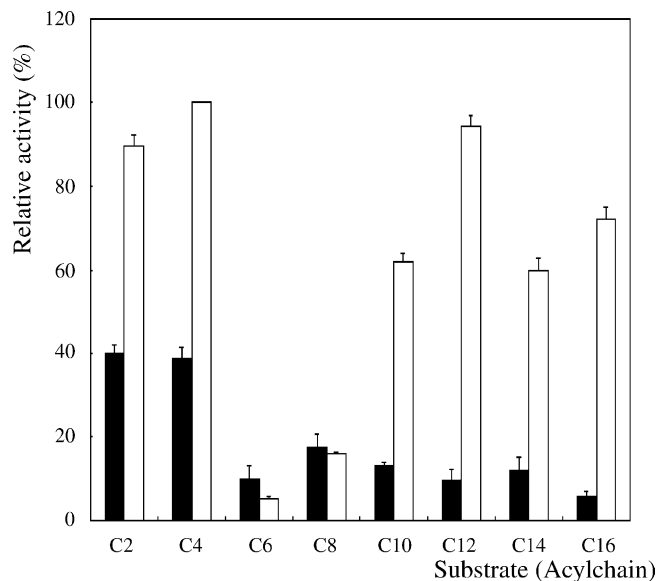


Fig. 6. Chain length selectivity of wild-type and M58 lipases from *Acinetobacter* species SY-01. Activity measurements were performed using *p*-NP monoesters with different chain lengths in acetate:ethanol:phosphate buffer (pH 7.5) (1:4:95). The reaction mixture was incubated at 50 °C for 30 min and finally the reaction was stopped by addition of 150 μ l of 1 M acetone. Absorbance at 405 nm was measured with a spectrophotometer (SpectraMax 340PC³⁸⁴, Molecular Device Co., Sunnyvale, CA) against an enzyme-free blank and one enzyme unit was defined as the release of 1 nmol of *p*-nitrophenol/ml/min. The data presented are based on comparisons to the maximum activity (100%). Activities given are the average values of triplicate measurements. The solid bar represents the wild-type lipase and the empty bar M58 lipase.

The relative activity of wild-type and mutant increased dramatically as did the reaction velocity in the presence of lif (Table 3).

To test the stability of lipases, the enzymes were incubated in a 50 mM potassium phosphate buffer, pH 7.5, at 30 °C. At time intervals, aliquots were assayed for their remaining activity by assay method I. The result showed that the activity of enzymes without lif decreased faster than that with lif. Moreover, the activity of M58 with lif was about 50% whereas that of mutant M58 without lif was 30% lower after 16 h (Fig. 7). These results strongly demonstrate that lif can participate in activity of lipase and contribute to its stability in vivo.

Table 3
Kinetic parameters of wild-type and M58 lipases for *p*-NP butyrate with lif or without lif

Lipases	Wild-type		M58	
	+	-	+	-
Lipase-specific chaperone	+	-	+	-
V_{max} (μ mol/min/mg)	33.16	29.06	44.56	32.24
K_m (mM)	17.5	23.73	7.5	22.5
V_{max}/K_m (μ mol/min/mg/mM)	1.89	1.22	5.94	1.43

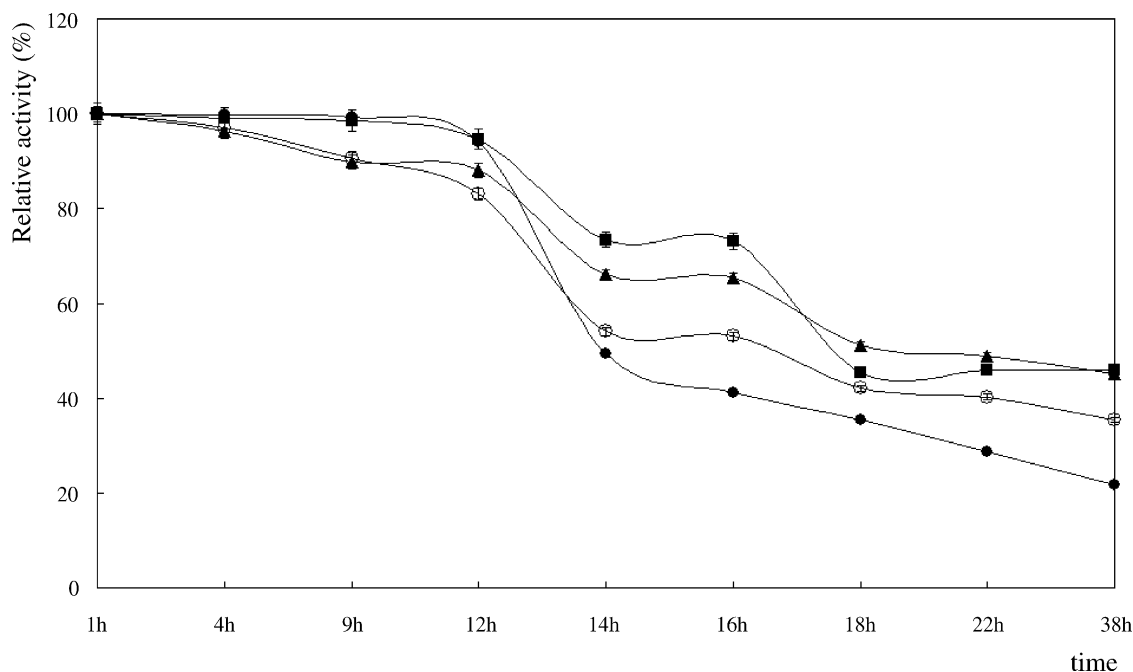


Fig. 7. Stability test of wild-type and mutant lipase from *Acinetobacter* species SY-01 dependent on presence the lip. For the stability, all enzymes were assayed after incubation at 50 °C for 30 min at pH 7.5. The filled square is the expressed mutant in the presence of lip. The filled triangle is the expressed wild-type in the presence of lip. The empty circle is the expressed mutant only in absence of lip. The filled circle is the expressed wild-type only in absence of lip. The data presented are based on comparisons to maximum activity (100%). Activities given are the average values of triplicate measurements.

4. Discussion

In this work, a mutant lipase with altered substrate specificities and higher hydrolysis activity was generated. We have attempted to obtain lipase containing a higher activity for hydrolysis of various monoesters by random mutagenesis than the wild-type lipase and studied the role of the lip. A library of lipase mutants was transfected into *B. subtilis* 168 because recombinant lipases expressed in this system could be obtained in extracellular form [8]. Secretory lipases in the supernatant were used for the screening of mutants containing hydrolysis activity towards triglycerides compared with wild-type lipase of *Acinetobacter* species SY-01. As a result, one clone was selected, grown, and the DNA sequence of the lipase mutant gene was determined. Five amino acids substitution (S21F, A102G, S103F, D299E and N300H) were responsible for the faster hydrolysis and altered substrate specificity of the enzyme activity. Three amino acids, S21F, A102G and S103F, were located in the N-terminal region and two amino acids, D299E and N300H, were in the C-terminal region (D299E, N300H). The amino acid sequences of the lipase from *Acinetobacter* species SY-01 were compared with bacterial lipases from homology family I.1 (*Pseudomonas aeruginosa*, *Vibrio cholerae*, *Pseudomonas fragi*, *Acinetobacter calcoaceticus* BD413, *Pseudomonas wisconsinensis*, *Pseudomonas fluorescens*, and *Pseudomonas vulgaris*). Since the movements of the loop between strand β 4 and helix α 2 (*Acinetobac-*

ter species SY-01 lipase aa residues 99–106), of the oxyanion loop (*Acinetobacter* species SY-01 lipase aa residues 64–70), and of the lid region are correlated [16], the changed Gly residue from the conserved Ala¹⁰² residue might play an important stabilizing role during opening of the lid of these lipases [17]. Moreover, Ala¹⁰² and Ser¹⁰³ residues are the conserved turn residues located between strand β 4 and helix α 4 and highly conserved in structure analysis of the group I *Proteobacterial* lipases, based on comparisons of deduced aa sequences of the mature proteins [17,18]. Tetra peptide motif Gly-Hyd-X-Gly (Hyd = Met, Leu, Val) located between strand β 3 and helix α 4 were highly conserved in group I.1 and I.2 of bacterial lipases and contributed to stabilization of lipase. It was connected to the loop between β 3 and helix α 2 via the hydrogen bond Arg¹¹⁰ NH1-Ala¹⁰² O [17,19].

Ala¹⁰² and N³⁰⁰ residues are similar conserved residues in group I.1 and I.2 of *Proteobacterial* lipases and the function of these residues has not yet been published. Most of the conserved residues had significant function in the acyl portion of the substrate pocket and mutation of these residues resulted in an improvement of the selectivity for the short- and long-chain substrates, and decreasing selectively for middle-chain substrates and an increase in lipase activity [17–19].

The mutant M58 has a higher activity for hydrolysis of triglycerides and monoesters than that of the wild-type. The hydrolysis rate of M58 for the eight esters with different acyl chain lengths had shown the shift of substrate selectiv-

ity compared to wild-type. The relative hydrolysis rates of the medium-chain (C6 and C8) and long-chain (C10–C16) esters to the short-chain (C2 and C4) esters were calculated, and the results are shown in Fig. 6. M58 more rapidly hydrolyzed short- and long-chain length substrates, but had lower activity towards middle chain length substrates than the wild-type. M58 had altered substrate specificity in comparison to the wild-type.

To investigate the effect of *lif* on the activity and stability of lipase from *Acinetobacter* species SY-01, the wild-type and mutant M58 lipase was expressed with and without the *lif* gene. *Lif* aided lipase conformation enhancing resistance of degradation by protease during lipase secretion, but also aided lipase conformation during translation [4,5]. Interestingly, higher activity and stability were shown in the expressed protein of the wild-type and mutant M58 with *lif* than those without *lif*. These results showed that most of the extracellular lipase of *Acinetobacter* species SY-01 required a *lif* in order to fold in the periplasm into its active, protease-resistant conformation prior to their secretion. Co-expression of the lipase with *lif* showed that *lif* plays an important role in the activity and stability of lipases as evidenced by the dramatic increase in the reaction velocity and thermostability of both the wild-type and mutant M58 lipases in the presence of *lif*.

Acknowledgments

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