Extremophiles (2007) 11:809–817
DOI 10.1007/s00792-007-0101-2

ORIGINAL PAPER

Cloning and expression of gene, and activation of an organic solvent-stable lipase from *Pseudomonas aeruginosa* LST-03

Hiroyasu Ogino · Yoshikazu Katou · Rieko Akagi · Takashi Mimitsuka · Shinichi Hiroshima · Yuichi Gemba · Noriyuki Doukyu · Masahiro Yasuda · Kosaku Ishimi · Haruo Ishikawa

Received: 3 May 2007 / Accepted: 18 June 2007 / Published online: 27 July 2007

Abstract Organic solvent-tolerant *Pseudomonas aeruginosa* LST-03 secretes an organic solvent-stable lipase, LST-03 lipase. The gene of the LST-03 lipase (Lip9) and the gene of the lipase-specific foldase (Lif9) were cloned and expressed in *Escherichia coli*. In the cloned 2.6 kbps DNA fragment, two open reading frames, Lip9 consisting of 933 nucleotides which encoded 311 amino acids and Lif9 consisting of 1,020 nucleotides which encoded 340 amino acids, were found. The overexpression of the lipase gene (*lip9*) was achieved when T7 promoter was used and the signal peptide of the lipase was deleted. The expressed amount of the lipase was greatly increased and overexpressed lipase formed inclusion body in *E. coli* cell. The collected inclusion body of the lipase from the cell was easily solubilized by urea and activated by using lipase-specific foldase of which 52 or 58 amino acids of N-terminal were deleted. Especially, the N-terminal methionine of the lipase of which the signal peptide was deleted was released in *E. coli* and the amino acid sequence was in agreement with that of the originally-produced lipase by *P. aeruginosa* LST-03. Furthermore, the overexpressed and solubilized lipase of which the signal peptide was deleted was more effectively activated by lipase-specific foldase.

Keywords Lipase · Lipase-specific foldase · Molecular chaperone · Organic solvent-stable · *Pseudomonas aeruginosa*

Communicated by K. Horikoshi.

H. Ogino (✉) · Y. Katou · R. Akagi · T. Mimitsuka · S. Hiroshima · Y. Gemba · M. Yasuda · K. Ishimi · H. Ishikawa
Department of Chemical Engineering,
Osaka Prefecture University, 1-1 Gakuen-cho,
Naka-ku, Sakai, Osaka 599-8531, Japan
e-mail: ogino@chemeng.osakafu-u.ac.jp

N. Doukyu
Department of Life Sciences, Toyo University,
Oura-gun, Gunma 374-0193, Japan

Introduction

Reactions catalyzed by enzymes are carried out in the presence of organic solvents, when the substrates are water-insoluble and when the thermodynamical equilibria are desired to shift from hydrolysis to synthesis. However, most enzymes including the lipase were not stable in the presence of the organic solvents. Therefore, organic solvent-stable enzymes are required for industrial applications of enzymes (Ogino and Ishikawa 2001). A *Pseudomonas* strain, *Pseudomonas aeruginosa* LST-03 was isolated from natural sources (Ogino et al. 1994). This strain is organic solvent-tolerant and secretes an organic solvent-stable lipase, LST-03 lipase (Ogino et al. 1999, 2000).

Several *Pseudomonas* and related strains were found and isolated as lipase producers (Gupta et al. 2004). Lipases from these strains are characterized and cloned. Some of these lipases require a molecular chaperone, a chaperonin, for activation. These molecular chaperonins were lipase-specific and generally called as lipase-specific foldases (Rosenau et al. 2004). The activation mechanisms of the lipases from *Burkholderia cepacia* DSM3959 (Jorgensen et al. 1991; Hobson et al. 1993, 1995; Aamand et al. 1994), *Pseudomonas* sp. 109 (Ihara et al. 1992, 1995; Tanaka et al. 1999, 2000), and *P. aeruginosa* TE3285 (Chihara-Siomi et al. 1992; Oshima-Hirayama et al. 1993; Shibata et al. 1998a, 1998b, 1998c) were relatively well

investigated and discussed. The lipase and foldase formed a 1:1 complex (Hobson et al. 1993, 1995; Aamand et al. 1994). Glutathione (Tanaka et al. 1999, 2000) and calcium ion (Shibata et al. 1998a) were also important factors for activation. Recently, the crystal structure of the complex of the lipase and lipase-specific foldase from *Burkholderia glumae* was determined (Pauwels et al. 2006). However, the mechanism was still not fully clarified.

In this paper, the genes of the organic solvent-stable LST-03 lipase and lipase-specific foldase were cloned and sequenced. Overexpression of the lipase genes was attempted. Furthermore, activation of the expressed LST-03 lipase by recombinant *Escherichia coli* using lipase-specific foldase was performed.

Materials and methods

Organisms

Pseudomonas aeruginosa LST-03, an organic solvent-tolerant microorganism that was isolated from soil and produced a lipolytic enzyme (Ogino et al. 1994), was used as a donor of the LST-03 lipase and its gene. *Escherichia coli* JM109 [*recA1*, *supE44*, *endA1*, *hsdR17*, *gyrA96*, *relA1*, *thi*, λ^-, Δ(*lac-proAB*), F′ [*traD36*, *proAB*$^+$, *lacI*^{q}ZΔ*M15*]] (Yanisch-Perron et al. 1985) was used as a host cell of cloning, subcloning, and expression. *E. coli* BL21(DE3) [F$^-$, *ompT*, *hsdS*$_B$ (r_B^-, m_B^-), *gal*, *dcm*, (DE3)] was used as a host cell of overexpression.

Preparation of Genomic DNA of *P. aeruginosa* LST-03

Pseudomonas aeruginosa LST-03 was grown in an L medium containing 1.0% (w/v) Bacto-tryptone (Difco Laboratories Ltd, Detroit, USA), 0.5% (w/v) yeast extract (Dried Yeast Extract-S, Nihon Pharmaceutical Co. Ltd, Tokyo, Japan), 0.5% (w/v) NaCl, and 1.0% (w/v) glucose, which was adjusted to pH 7.2 with 1 M NaOH. The genomic DNA of *P. aeruginosa* LST-03 was isolated by modifying the procedure of Murray and Thompson (1980). One milliliter of preculture of *P. aeruginosa* LST-03 incubated at 30°C overnight was inoculated into a 500-ml baffled Erlenmeyer flask containing 100 ml of the L broth. The cultivation was performed at 30°C with rotary shaking (150 rpm, 7-cm-diameter shaking) until the late logarithmic phase for 8 h. The cells of the late logarithmic phase were harvested by centrifugation at 5,100*g* for 10 min at room temperature and washed with 50 ml of TE buffer (pH 7.5) containing 10 mM Tris–HCl and 1 mM ethylenediaminetetraacetic acid (EDTA). The cells were collected by centrifugation at 11,000*g* for 10 min at room temperature, then resuspended in 9 ml of TE buffer containing 0.1 g/l lysozyme (from egg white, Wako Pure Chemical Industries, Ltd, Osaka, Japan) and incubated at 37°C with shaking at 60 strokes per min for 1 h. After addition of 0.5 ml of 10% (w/v) sodium dodecyl sulfate (SDS) and 0.1 ml of 10 g/l protease K (E. Merck, Darmstadt, Germany), the suspension was incubated at 50°C with shaking at 160 strokes per min for 30 min, and then at 60 strokes per min overnight for completing lysis. The lysate added with 1.8 ml of 5 M NaCl and 1.5 ml of 10% (w/v) cetyltrimethylammonium bromide (CTAB) in 0.7 M NaCl was incubated at 65°C for 20 min. To the solution an equal volume of a chloroform/isoamyl alcohol (24:1) mixture was added and the mixture was mixed gently by inversing the tube at room temperature for 10 min. Phase separation was achieved by centrifugation at 11,000*g* for 20 min at room temperature. After twice extractions with the chloroform/isoamyl alcohol mixture, extraction using an equal volume of phenol saturated with 1 M Tris–HCl (pH 7.5) was performed. Following an additional extraction with the chloroform/isoamyl alcohol mixture, a twofold volume of cold ethanol (–20°C) was added to the mixture. The precipitated genomic DNA was collected by applying it onto a glass rod and then rinsed with 70% (v/v) ethanol. After evaporating the ethanol in vacuo, the nucleotide was suspended in 3 ml of TE buffer and its concentration was determined by measuring the absorbance of its 20-fold dilution with TE buffer at 260 and 280 nm. Two hundred microliters of a solution containing 1 g/l genomic DNA and 0.01 g/l ribonuclease A (RNase A, Sigma Chemical Company, St Louis, USA) in a microtube were incubated at 37°C overnight. Impurities were removed by extraction with an equal volume of a phenol/chloroform/isoamyl alcohol (25:24:1) mixture and precipitation of the genomic DNA of *P. aeruginosa* by ethanol was performed before the DNA was dissolved in TE buffer. The purity and the concentration of the genomic DNA were determined by measuring the absorbance at 260 and 280 nm. The solution containing the DNA was kept at 4°C until utilization.

Construction of plasmids

For cloning of the genes of the LST-03 lipase and the lipase-specific foldase, amplification of DNA by the polymerase chain reaction (PCR) was performed using genomic DNA from *P. aeruginosa* LST-03 as a template, 5′-GCA T*C*T A*GA CCA TTT CAG CCT GTT TTG CTC-3′ (asterisk and underlined sequence indicate mismatch and *Xba* I site, respectively) and 5′-CTT CC*C CG*G* GAT CGT GGC GAT CTT CAG C-3′ (underlined sequence indicates *Sma* I site) as primers, and LA-Taq (Takara Shuzo Co., Ltd, Kyoto, Japan). Thirty repeated cycles of thermal denaturation at 94°C for 30 s, annealing at 62°C for 30 s, and extension/termination 72°C for 2 min were

performed using a GeneAmp PCR System 2400 (The Perkin-Elmer Corp., Norwalk, CT, USA). The PCR product purified using a PCR purification kit and pUC19 were cleaved with restriction endonucleases, *Xba* I and *Sma* I. After purification of cleaved PCR product and cloning vector, they were ligated using T4 DNA ligase to construct pLC9. *E. coli* JM109 was transformed with the constructed plasmid by a chemical method (Hanahan 1983).

To construct expression plasmids, PCR was performed using pLC9 as a template. The purified PCR products and pET-21c(+) (EMD Bioscience, Inc., Madison, USA.) were cleaved with *Nde* I and *Xho* I and ligated. To construct pE_L9_F9 containing genes of Lip9 and Lif9, 5′-ATG AGA ACC* AT*A TGA AGA AGA AGT CTC TGC-3′ (underlined sequence indicates *Nde* I site) and 5′-TTT CCG C*T*C G*A*G TCA GCG CTG CTC GGC C-3′ (primer A, underlined sequence indicates *Xho* I site) were used as primers. To construct pE_d25L9_F9 containing genes of d25Lip9, Lip9 of which 25 amino acid residues of N-terminal were removed, and Lif9, 5′-CGC TGA TCC AT*A*T*G* A GCA CCT ACA CCC AGA CC-3′ (underlined sequence indicates *Nde* I site) and primer A were used as primers. To construct pE_d52F9 containing a gene of d52Lif9, Lif9 of which 52 amino acid residues of N-terminal were removed, 5′-CGG GAG AAC* A*T*A* TGC CGG CCC CCC AGG-3′ (underlined sequence indicates *Nde* I site) and primer A were used as primers. To construct pE_d58F9 containing a gene of d58Lif9, Lif9 of which 58 amino acid residues of N-terminal were removed, 5′-CCC CCC AGC* A*T*A TGC CGG CCA AGG TCG-3′ (underlined sequence indicates *Nde* I site) and primer A were used as primers. Sequencing of the cloned DNA in all constructed plasmids was performed.

Cultivation of Transformants

The transformed *E. coli* was cultivated using a Luria–Bertani (LB) medium (1.0% (w/v) Bacto-tryptone, 0.5% (w/v) yeast extract, and 1.0% (w/v) NaCl) (Sambrook et al. 1989). For cultivation of *E. coli* JM109 and BL21(DE3), a filtered ampicillin solution was added to be 50 and 100 mg/l ampicillin sodium salt, respectively. For preparation of agar plates, 1.5% (w/v) agar was added. To check the lipolytic activity of transformants on the plate, 0.5% (v/v) tri-*n*-butyrin was added and emulsified.

For expression of the gene following T7 promoter on pET-21c(+) vector, the transformed *E. coli* BL21(DE3) was cultivated in a 500-ml baffled Erlenmeyer flask containing 100 ml of the LB medium containing 100 mg/l ampicillin sodium salt at 37°C with shaking at 150 rpm. When the optical density reached about 1.0 by cultivation for about 4 h, 0.2 mM isopropyl-β-D(–)-thiogalactopyranoside (IPTG) was added. After 3 h-additional incubation at 37°C with shaking, the cells were harvested by centrifugation at 10,000*g* and 4°C for 5 min.

Preparation of cell extract

Collected cells from 950 ml of the culture were suspended in 95 ml of 20 mM Tris–HCl buffer (pH 8.0) and disrupted by ultrasonic disintegration using an ultrasonic disruptor UD-200 (Tomy Seiko Co., Ltd, Tokyo, Japan) at 60 W intermittently in an ice bath. Disrupted cells were centrifuged at 10,000*g* and 4°C for 5 min. The supernatant and the precipitation were collected as soluble and insoluble fractions, respectively.

Solubilization and activation of inclusion bodies

The insoluble fraction was taken into the 20 mM Tris–HCl buffer (pH 8.0) containing 5.3 M urea and the suspension was mixed well. After storage of the suspension at 4°C for over night, 100-fold dilution was made using 20 mM Tris–HCl buffer (pH 8.0). Diluted solution was mixed with soluble fractions prepared from transformants with pE_d52F9 and pE_d58F9, which contained d52Lif9 and d58Lif9, respectively, and then stored at 4°C for 24 h.

Measurement of lipolytic activity

Lipolytic activity was assayed by the BALB-DTNB method, which is a modification of the method of Kurooka et al. (1977). 2,3-Dimercaptopropan-1-ol tributyrate (BALB) was used as a substrate and the thiol groups liberated by hydrolysis were coupled with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB). Then, 1.0 ml of 0.3 mM DTNB in 100 mM Tris–HCl buffer (pH 8.0) and 10 µl of an enzyme sample were mixed. After preincubation at 30°C for 5 min, 0.1 ml of 20 mM BALB in ethanol was added to the reaction mixture and the reaction was allowed to proceed at 30°C for 10 min. The reaction was then stopped by adding 2.0 ml of acetone. The absorbance of the resulting 5-thio-2-nitrobenzoate anion was measured at 412 nm. One unit (I.U.) of lipolytic activity was defined as the amount of enzyme that produced 1 µmol of 5-thio-2-nitrobenzoate anion per min at 30°C.

SDS-polyacrylamide gel electrophoresis

The purified enzyme was dissolved in an SDS treatment solution consisting of 1% (w/v) SDS, 1% (w/v) 2-mercaptoethanol, 10% (w/v) glycerol and 31.25 mM Tris–HCl buffer (pH 6.8), and the resulting solution was heated in a water bath at 100°C for 3 min. The sample was run in a stacking gel consisting of 4.35% (w/v) polyacrylamide, 0.3% (w/v) *N*,*N*'-methylene-bis(acrylamide), and 0.1% (w/v)

SDS and a separating gel containing 14.8% (w/v) polyacrylamide, 0.2% (w/v) *N*, *N'*-methylene-bis(acrylamide), and 0.1% (w/v) SDS under the conditions developed by Laemmli (1970).

Sequencing of N-terminal amino acids

The protein separated by SDS-polyacrylamide gel electrophoresis was electrotransferred on a polyvinylidene difluoride (PVDF) membrane (Immobilon-P, Millipore, Bedford, USA) using 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) solution (pH 11) containing 10% (v/v) methanol as a electroblotting buffer at 50 V for 30 min. The blotting membrane was stained with a solution containing 0.1% (w/v) coomassie brilliant blue R-250, 50% (v/v) methanol, and 1% (v/v) acetic acid. The stained single protein band was cut out and destained in 50% (v/v) methanol. The sequence of the N-terminal amino acids was determined by the Edman degradation method using a Protein Sequencing System (Model 491 Procise, Applied Biosystems).

Results and discussion

N-terminal sequence of LST-03 lipase

Pseudomonas aeruginosa LST-03 was isolated as an organic solvent-stable lipolytic enzyme producer which had organic solvent-tolerance (Ogino et al. 1994). The LST-03 lipase was purified by fractionation using ammonium sulfate, ion exchange column chromatography using DEAE-Sepharose, hydrophobic interaction column chromatography using Phenyl-Sepharose, and desalting column chromatography using HiPrep 26/10 from culture supernatant of *P. aeruginosa* LST-03, as described in a previous paper (Ogino et al. 2000). The purified LST-03 lipase was analyzed by SDS-PAGE, and then the protein band was transferred on a PVDF membrane electrophoreticaly. The transferred protein was analyzed to determine its N-terminal sequence by the protein sequencer. The 20 N-terminal sequence of the LST-03 lipase was Ser–Thr–Tyr–Thr–Gln–Thr–Lys–Tyr–Pro–Ile–Val–Leu–Ala–His–Gly–Met–Leu–Gly–Phe–Asp-.

Cloning and sequencing of the gene of LST-03 lipase

Sequence similarity search with N-terminal sequence of the LST-03 lipase was performed by using the BLAST program. The 20 amino acid sequence of N-terminal of the LST-03 lipase completely coincided with those of several lipases from *Pseudomonas* sp. 109 (Ihara et al. 1991) and *P. aeruginosa* TE3285 (Chihara-Siomi et al. 1992), ATCC 31156 (Shinkai et al. 1996), PAO1 (Wohlfarth et al. 1992), and IGB83 (Martinez and Soberon-Chavez 2001). It had been known that they need a lipase-specific foldase for activation of the lipase, which was called as molecular chaperone, modulator protein, activator protein, or helper protein (Rosenau et al. 2004). Genes of lipase-specific foldase locate in the downstream region of these lipase genes. According to the previous findings (Ihara et al. 1992; Chihara-Siomi et al. 1992; Wohlfarth et al. 1992; Shinkai et al. 1996), one pair of primers were constructed to obtain the genes of the LST-03 lipase and lipase-specific foldase, and polymerase chain reaction (PCR) was performed using genomic DNA from *P. aeruginosa* LST-03 as a template. A 2.6 kbps-DNA fragment was obtained. After digestion of the PCR products and pUC19 with *Xba* I and *Sma* I, they were ligated. *Escherichia coli* JM109 cells were transformed by the ligated plasmids. Some plasmids of 5.3 kbps were extracted from *E. coli* cells and the cloned fragments were confirmed by nucleotide sequencing. The plasmid containing the genes of LST-03 lipase and its foldase was named as pLC9.

Nucleotide sequence of the cloned DNA fragment of 2,629 bps which was shown in Fig. 1 was submitted to DNA Data Bank of Japan (DDBJ) and the accession number was assigned as AB290342. The sequenced DNA contained 66.95% G + C base pairs and two open reading frames. The open reading frame of upstream (*lip9*) consisted of 933 nucleotides and encoded 311 amino acids of the precursor of LST-03 lipase. The open reading frame of downstream (*lif9*) consisted of 1,020 nucleotides and encoded 340 amino acids of the foldase of the LST-03 lipase. Although the primary structures of the LST-03 lipase and lipase-specific foldase from *P. aeruginosa* LST-03 were very similar to some lipases and foldases from *Pseudomonas* sp. 109 (Ihara et al. 1991, 1992) and *P. aerugiosa* PAO1 (Wohlfarth et al. 1992), ATCC31156 (Martinez and Soberon-Chavez 2001), TE3285 (Chihara-Siomi et al. 1992), and PA7 (NCBI Microbial Genomes Annotation Project 2006; Accession No. ZP_01293163), respectively, there were some differences between the putative amino acid sequences of the lipase and the foldase from *P aeruginosa* LST-03 and those from other strains.

The putative amino acid sequence of the LST-03 lipase had high homology with those from other bacteria such as *Vibrio choletae* N16961 (Heidelberg et al. 2000, similarity is 58.8%), *V. parahaemolyticus* RIMD 2210633 (Makino et al. 2003, similarity is 54.7%), *Chromobacterium violaceum* ATCC12472 (Vasconcelos et al. 2003, similarity is 55.7%), *Acinetobacter* sp. SY-01 (Han et al. 2003, similarity is 50.0%), *Burkholderia cepacia* ATCC 21808 (Jorgensen et al. 1991, similarity is 38.6%), and *B. glumae* PG1 (Frenken et al. 1992, 1993, similarity is 38.1%). Although the N-terminal sequence analyzed using the

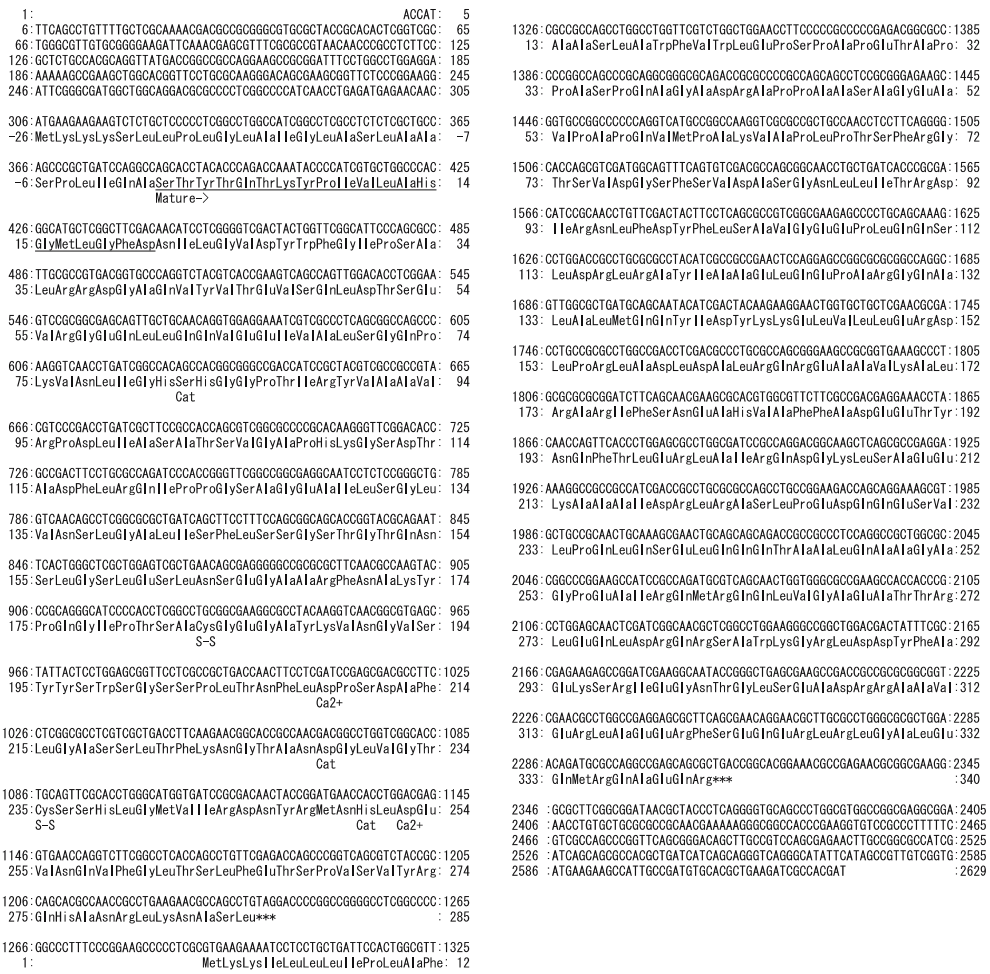

Fig. 1 Sequenced nucleotide and deduced amino acid sequences of the Lip9 (LST-03 lipase) and Lif9 (lipase-specific foldase). The reduced amino acid sequences of the Lip9 and Lif9 are given below the nucleotide sequence. The *numbers* written on both sides of the lines of nucleotides and amino acid sequences indicate the nucleotide and amino acid positions, respectively. "***" indicates the terminal codon of translation. The *underlined* amino acid sequences are determined by the Edman degradation of the purified LST-03 lipase. "Cat", "S–S", and "Ca2+" indicate catalytic site, site of disulfide bond, and calcium binding site, respectively

purified LST-03 lipase was not agreement with that of the putative primary structure from the open reading frame in the cloned DNA fragment, it was found within the putative primary structure. It was suggested that the LST-03 lipase was synthesized as a 32.7 kDa-precursor consisting of 311 amino acids and N-terminal peptide consisting 26 amino acids which was removed as a signal peptide during translocation across the cytoplasmic membrane. This was supported by the evidence that the LST-03 lipase was secreted by *P. aeruginosa* LST-03 (Ogino et al. 1994, 1999, 2000) and by the algorithm for predicting signal sequence cleavage sites (von Heijne 1986). The consensus sequence found in lipases, -Gly–Xaa–Ser–Xaa–Gly-, was found in the LST-03 lipase at amino acid position 80–84. Three-dimensional structures of lipases from *P. aeruginosa* PAO1 (Nardini et al. 2000), *B. cepacia* ATCC21808 (Lang et al. 1998), and *B. glumae* (Noble et al. 1993) were clarified. It was suggested that the active center of the LST-03 lipase was constructed by Ser-82, Asp-229, and His-251. It has a disulfide bond between Cys-183 and Cys-235 and a calcium ion which is bound to Asp-209 and Asp-253. These amino acids residues which constructed the consensus sequence, the active center, the disulfide bond, and the calcium ion binding site were well conserved in all lipases from *Vibrio choletae* N16961, *V. parahaemolyticus* RIMD 2210633, *Chromobacterium violaceum* ATCC12472, *Acinetobacter* sp. SY-01, *Burkholderia cepacia* ATCC 21808, and *B. glumae* PG1.

Lipases which are similar to the LST-03 lipase need foldases for activation. However, the similarities between the foldase from *P. aeruginosa* LST-03 and those from *Acinetobacter* sp. SY-01, *C. violaceum* ATCC12472, *B. glumae* PG1, *B. cepacia* ATCC 21808 (Quyen et al. 1999), *V. parahaemolyticus* RIMD 2210633, and *V. choletae* N16961 were between 20.1 and 32.7%. Amino acid sequence of the foldase is variable compared with that of lipase. N-terminal region of the foldase was hydrophobic (data not shown) and suggested to be assembled in the cytoplasmic membrane.

Expression of *lip9* and *lif9*

The transformant harboring pLC9 (*E. coli* JM109/pLC9) showed lipolytic activity on a plate media containing tri-*n*-

butyrin in the absence of isopropyl-β-D(–)-thiogalactopyranoside (IPTG). When *E. coli* JM109/pLC9 was cultured in the LB broth containing ampicillin in the absence of IPTG, the lipolytic activity was not detected in its supernatant, but in the cell extract. The lipolytic activity of *E. coli* increased by transforming with pLC9, however it was not so high. The amount of the expression of the LST-03 lipase, Lip9, without promoter of *E. coli* was negligible, and the protein was not detected on SDS-PAGE (data not shown).

Overexpression was attempted by using pET system. The DNA fragment containing two open reading frames was amplified by PCR using the forward and reverse primers containing *Nde* I site and *Xho* I site, respectively. pE_L9_F9 encoding Lip9 (LST-03 lipase) and Lif9 (lipase-specific foldase), pE_d25L9_F9 encoding Lip9 of which the signal peptide consisting 26 amino acids of N-terminal was deleted and into which methionine was added as start codon, pE_d52F9 encoding Lif9 of which 52 amino acids of N-terminal were deleted, and pE_d58F9 encoding Lif9 of which 58 amino acids of N-terminal were deleted, were constructed by ligating the digested PCR products and pET-21c(+) with *Nde* I and *Xho* I.

The transformants of *E. coli* BL21(DE3) by pE_L9_F9, pE_d25L9_F9, pE_d52F9, and pE_d58F9 were cultivated in the presence of ampicillin until the optical density at 660 nm of the culture attained about 1.0 for about 4 h. After addition of IPTG to be 0.2 mM, the cultivation was continued for more 3 h for induction of protein expression. The cells were harvested by centrifugation and disrupted by sonication. Supernatant and precipitation of centrifugation of the disrupted cells were separately collected as the soluble and insoluble fractions, respectively. The proteins contained in the soluble and insoluble fractions were analyzed by SDS-PAGE as shown in Fig. 2. When *E. coli* BL21(DE3) transformed by pE_L9_F9 and pE_d25L9_F9 were cultivated, expressed proteins, Lip9 and d25Lip9 were not only found in the soluble fractions, but also in the insoluble fractions. The large amounts of Lip9 and d25Lip9 were found in insoluble fractions. The amount of the expressed Lip9 was increased by the overexpression using pET system with T7 promoter. However, the expressed Lip9 formed insoluble inclusion bodies. Furthermore, the expressed amount of d25Lip9 in insoluble fractions was larger than that of Lip9. By removing signal peptide of Lip9, the expressed amount of the LST-03 lipase, d25Lip9, dramatically increased. Although the accurate concentration in cells is not clear, the expressed amount by using T7 promoter and deleting the signal sequence of the LST-03 lipase increased more than 100-fold according to the estimation using the result of SDS-PAGE.

Lip9 and d25Lip9 were expressed using pE_L9_F9 and pE_d25L9_F9, respectively, with the gene of the foldase which was located in the downstream region of gene of the

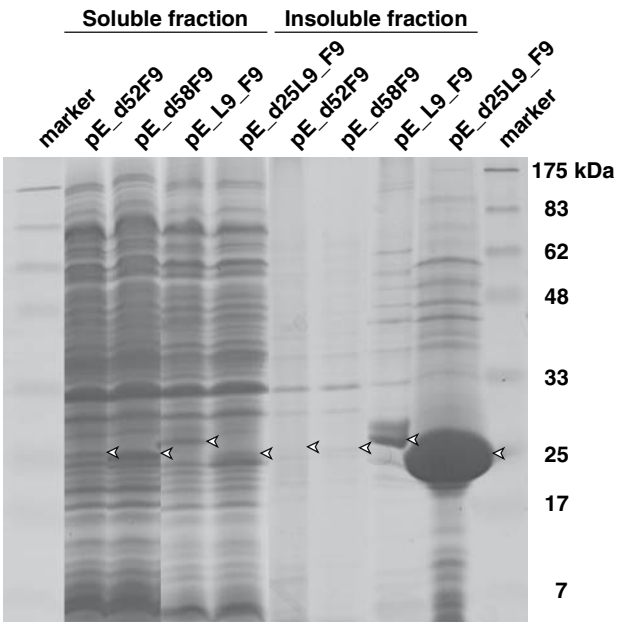

Fig. 2 SDS-PAGE analysis of soluble and insoluble fractions of the disrupted recombinant *E. coli* transformed with expression plasmids. Recombinant *E. coli* transformed with different expression plasmids were cultivated for about 4 h at 37°C. After addition of IPTG for induction of gene expression, 3 h-additional incubation was performed at 37°C. The harvested recombinant cells were disrupted by sonication. The supernatant and precipitation of disrupted cells were collected by centrifugation as soluble and insoluble fractions, respectively. Soluble and insoluble fractions prepared from 0.12 and 0.24 ml of culture, respectively, were analyzed by SDS-PAGE

lipase. However, the amounts of foldases were negligible and they were not detected by SDS-PAGE. The gene of lipase-specific foldase was expressed without the gene of lipase. Some N-terminal amino acid residues of the foldase were deleted, because membrane-associating motif was found at the N-terminal of the Lif9. When *E. coli* BL21(DE3) transformed by pE_d52F9 and pE_d58F9 were cultivated, expressed lipase-specific foldases, d52Lif9 and d58Lif9, respectively, were mainly found in the soluble fractions. The expressed amounts of the foldases, d52Lif9 and d58Lif9, also increased by locating T7 promoter in just upstream region of the genes of the foldases and by expressing solely. However, the amounts of the expressed foldases were not so much in comparison with those of Lip9 and d25Lip9 using T7 promoter.

Lip9 and d25Lip9 contained in insoluble fractions prepared from the transformants harboring pE_L9_F9 and pE_d25L9_F9, respectively, were transferred on PVDF membrane from the gel of SDS-PAGE. The N-terminal sequences of the expressed proteins, Lip9 and d25Lip9, were Met–Lys–Lys–Lys–Ser- and Ser–Thr–Tyr–Thr–Gln-, respectively. Although the N-terminal amino acid of the Lip9, methionine, was not released, the N-terminal methinine of the d25Lip9 was released in *E. coli*. The

methionine of N-terminal of the d25Lip9 may be removed by methionyl aminopeptidase of *E. coli*. The amino acid sequence of the expressed d25Lip9 was in agreement with that of the LST-03 lipase secreted by the original stain of *P. aeruginosa*.

Activation of lipase

It was expected that the expressed Lip9 and d25Lip9 by *E. coli* using T7 promoter form inclusion bodies in the cells. The lipolytic activities of the inclusion bodies of Lip9 and d25Lip9 were negligible. Inclusion bodies of Lip9 and d25Lip9 in the insoluble fractions prepared from the transformants with pE_L9_F9 and pE_d25L9_F9 were solubilzed by using urea. After 100-fold dilution, the soluble fractions prepared from the transformant of *E. coli* BL21(DE3) by pE_d52F9 containing *d52lif9* and pE_d58F9 containing *d58lif9*, were added. The lipolytic activities of Lip9 and d25Lip9 before and after incubation with and without d52Lif9 and d58Lif9 are shown in Fig. 3. The Lip9 and d25Lip9 were co-expressed with Lif9 using pE_L9_F9 and pE_d25L9_F9, respectively, both of which contained *lif9*. Although the lipolytic activities of the solubilized Lip9 and d25Lip9 before incubation and after incubations without lipase-specific foldase were found, they were both very low. It was estimated that the Lip9 and d25Lip9 were activated by small amounts of the foldases contained in insoluble fractions of the transformants of *E. coli* BL21(DE3) by pE_L9_F9 and pE_d25L9_F9. The activities of Lip9 and d25Lip9 before incubation were 0.13 and 23.3 I.U./ml-culture, respectively. However, by the addition of d52Lif9 or d58Lif9, and incubation with one of them, the lipolytic activities of solubilized Lip9 and d25Lip9 were greatly enhanced. The activities of Lip9 and d25Lip9 after incubation with d52Lif9 were 0.35 and 117 I.U./ml-culture, respectively. The activities of Lip9 of which the signal peptide was deleted (d25Lip9) by the incubation with the lipase-specific foldase were more enhanced compared with those of Lip9. However, the difference of the foldases of which 52 or 58 amino acids of N-terminal was deleted was not found. By the incubation with the foldase, the activities of the Lip9 and d25Lip9 were about 2.7-fold and 5.0-fold increased, respectively. Although only the rough specific activities of the d25Lip9 which were solubilized and incubated with d52Lif9 and d58Lif9 were measured, they were almost the same level with that of the LST-03 lipase purified from the culture supernatant of *P. aeruginosa* LST-03 according to the estimated concentration of d25Lip9 using the result of SDS-PAGE (Ogino et al. 2000).

In our previous papers, two lipolytic enzymes, Lip3 (Ogino et al. 2004a) and Lip8 (Ogino et al. 2004b) were cloned from genomic DNA of *P. aeruginosa* LST-03 that was the same strain used in this study. These genes were expressed in *E. coli* and recombinant enzymes were purified and characterized. However, Lip3 and Lip8 were quite different from Lip9, the LST-03 lipase, by both the structure and characteristic properties, and did not require any foldases for their activation.

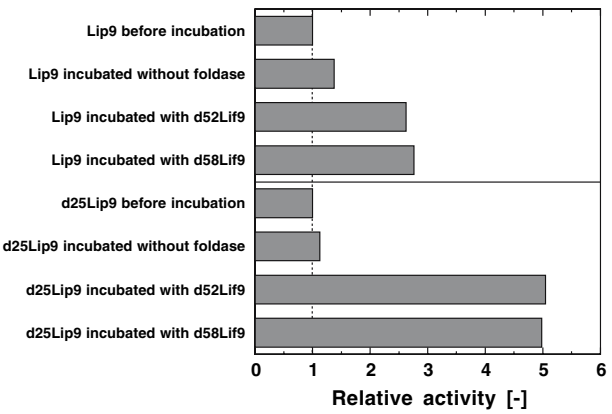

Fig. 3 The lipolytic activities of lipases before and after incubation with and without lipase-specific foldases. Inclusion bodies of Lip9 and d25Lip9 were solubilized using urea and diluted. Before and after incubation of the solutions containing Lip9 and d25Lip9 with and without d52Lif9 and d58Lif9 at 30°C, the lipolytic activities were measured by the BALB-DTNB method

Acknowledgments This work was supported by Industrial Technology Research Grant Program in '05 from New Energy and Industrial Technology Development Organization (NEDO) of Japan and a Grant-in-Aid for Scientific Research (B) (17310131) from the Japan Society for the Promotion of Science. The authors would like to express their appreciation for these supports

References

Aamand JL, Hobson AH, Buckley CM, Jorgensen ST, Diderichsen B, McConnell DJ (1994) Chaperone-mediated activation in vivo of a *Pseudomonas cepacia* lipase. Mol Gen Genet 245:556–564

Chihara-Siomi M, Yoshikawa K, Oshima-Hirayama N, Yamamoto K, Sogabe Y, Nakatani T, Nishioka T, Oda J (1992) Purification, molecular cloning, and expression of lipase from *Pseudomonas aeruginosa*. Arch Biochem Biophys 296:505–513

Frenken LG, Egmond MR, Batenburg AM, Bos JW, Visser C, Verrips CT (1992) Cloning of the *Pseudomonas glumae* lipase gene and determination of the active site residues. Appl Environ Microbiol 58:3787–3791

Frenken LG, Bos JW, Visser C, Muller W, Tommassen J, Verrips CT (1993) An accessory gene, *lipB*, required for the production of active *Pseudomonas glumae* lipase. Mol Microbiol 9:579–589

Gupta R, Gupta N, Rathi P (2004) Bacterial lipases: an overview of production, purification and biochemical properties. Appl Microbiol Biotechnol 64:763–781

Hanahan D (1983) Studies on transformation of *Escherichia coli* with plasmids. J Mol Biol 166:557–580

Han SJ, Back JH, Yoon MY, Shin PK, Cheong CS, Sung MH, Hong SP, Chung IY, Han YS (2003) Expression and characterization

of a novel enantioselective lipase from *Acinetobacter* species SY-01. Biochimie 85:501–510

Heidelberg JF, Eisen JA, Nelson WC, Clayton RA, Gwinn ML, Dodson RJ, Haft DH, Hickey EK, Peterson JD, Umayam L, Gill SR, Nelson KE, Read TD, Tettelin H, Richardson D, Ermolaeva MD, Vamathevan J, Bass S, Qin H, Dragoi I, Sellers P, McDonald L, Utterback T, Fleishmann RD, Nierman WC, White O, Salzberg SL, Smith HO, Colwell RR, Mekalanos JJ, Venter JC, Fraser CM (2000) DNA sequence of both chromosomes of the cholera pathogen *Vibrio cholerae*. Nature 406:477–483

von Heijne G (1986) A new method for predicting signal sequence cleavage sites. Nucleic Acids Res 14:4683–4690

Hobson AH, Buckley CM, Aamand JL, Jorgensen ST, Diderichsen B, McConnell DJ (1993) Activation of a bacterial lipase by its chaperone. Proc Natl Acad Sci USA 90:5682–5686

Hobson AH, Buckley CM, Jorgensen ST, Diderichsen B, McConnell DJ (1995) Interaction of the *Pseudomonas cepacia* DSM3959 lipase with its chaperone, LimA. J Biochem (Tokyo) 118:575–581

Ihara F, Kageyama Y, Hirata M, Nihira T, Yamada Y (1991) Purification, characterization, and molecular cloning of lactonizing lipase from *Pseudomonas* species. J Biol Chem 266:18135–18140

Ihara F, Okamoto I, Nihira T, Yamada Y (1992) Requirement in *trans* of the downstream *limL* gene for activation of lactonizing lipase from *Pseudomonas* sp. 109. J Ferment Bioeng 73:337–342

Ihara F, Okamoto I, Akao K, Nihira T, Yamada Y (1995) Lipase modulator protein (LimL) of *Pseudomonas* sp. strain 109. J Bacteriol 177:1254–1258

Jorgensen S, Skov KW, Diderichsen B (1991) Cloning, sequence, and expression of a lipase gene from *Pseudomonas cepacia*: lipase production in heterologous hosts requires two *Pseudomonas* genes. J Bacteriol 173:559–567

Kurooka S, Okamoto S, Hashimoto M (1977) A novel and simple colorimetric assay for human serum lipase. J Biochem 81:361–369

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685

Lang DA, Mannesse ML, de Haas GH, Verheij HM, Dijkstra BW (1998) Structural basis of the chiral selectivity of *Pseudomonas cepacia* lipase. Eur J Biochem 254:333–340

Makino K, Oshima K, Kurokawa K, Yokoyama K, Uda T, Tagomori K, Iijima Y, Najima M, Nakano M, Yamashita A, Kubota Y, Kimura S, Yasunaga T, Honda T, Shinagawa H, Hattori M, Iida T (2003) Genome sequence of *Vibrio parahaemolyticus*: a pathogenic mechanism distinct from that of *V. cholerae*. Lancet 361:743–749

Martinez A, Soberon-Chavez G (2001) Characterization of the *lipA* gene encoding the major lipase from *Pseudomonas aeruginosa* strain IGB83. Appl Microbiol Biotechnol 56:731–735

Murray MG, Thompson WF (1980) Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res 8:4321–4325

Nardini M, Lang DA, Liebeton K, Jaeger KE, Dijkstra BW (2000) Crystal structure of *Pseudomonas aeruginosa* lipase in the open conformation. the prototype for family I.1 of bacterial lipases. J Biol Chem 275:31219–31225

NCBI Microbial Genomes Annotation Project (2006) EMBL/GenBank/DDBJ databases, Accession No. ZP_01293163

Noble ME, Cleasby A, Johnson LN, Egmond MR, Frenken LG (1993) The crystal structure of triacylglycerol lipase from *Pseudomonas glumae* reveals a partially redundant catalytic aspartate. FEBS Lett 331:123–128

Ogino H, Ishikawa H (2001) Enzymes which are stable in the presence of organic solvents. J Biosci Bioeng 91:109–116

Ogino H, Miyamoto K, Ishikawa H (1994) Organic-solvent-tolerant bacterium which secretes organic-solvent-stable lipolytic enzyme. Appl Environ Microbiol 60:3884–3886

Ogino H, Miyamoto K, Yasuda M, Ishimi K, Ishikawa H (1999) Growth of organic solvent-tolerant *Pseudomonas aeruginosa* LST-03 in the presence of various organic solvents and production of lipolytic enzyme in the presence of cyclohexane. Biochem Eng J 4:1–6

Ogino H, Nakagawa S, Shinya K, Muto T, Fujimura N, Yasuda M, Ishikawa H (2000) Purification and characterization of organic solvent-stable lipase from organic solvent-tolerant *Pseudomonas aeruginosa* LST-03. J Biosci Bioeng 89:451–457

Ogino H, Hiroshima S, Hirose S, Yasuda M, Ishimi K, Ishikawa H (2004a) Cloning, expression and characterization of a lipase gene (*lip3*) from *Pseudomonas aeruginosa* LST-03. Mol Genet Genomics 271:189–196

Ogino H, Mimitsuka T, Muto T, Matsumura M, Yasuda M, Ishimi K, Ishikawa H (2004b) Cloning, expression, and characterization of a lipolytic enzyme gene (*lip8*) from *Pseudomonas aeruginosa* LST-03. J Mol Microbiol Biotechnol 7:212–223

Oshima-Hirayama N, Yoshikawa K, Nishioka T, Oda J (1993) Lipase from *Pseudomonas aeruginosa*. Production in *Escherichia coli* and activation in vitro with a protein from the downstream gene. Eur J Biochem 215:239–246

Pauwels K, Lustig A, Wyns L, Tommassen J, Savvides SN, Van Gelder P (2006) Structure of a membrane-based steric chaperone in complex with its lipase substrate. Nat Struct Mol Biol 13:374–375

Quyen DT, Schmidt-Dannert C, Schmid RD (1999) High-level formation of active *Pseudomonas cepacia* lipase after heterologous expression of the encoding gene and its modified chaperone in *Escherichia coli* and rapid in vitro refolding. Appl Environ Microbiol 65:787–794

Rosenau F, Tommassen J, Jaeger KE (2004) Lipase-specific foldases. Chembiochem 5:152–161

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor

Shibata H, Kato H, Oda J (1998a) Calcium ion-dependent reactivation of a *Pseudomonas* lipase by its specific modulating protein, LipB. J Biochem (Tokyo) 123:136–141

Shibata H, Kato H, Oda J (1998b) Molecular properties and activity of amino-terminal truncated forms of lipase activator protein. Biosci Biotechnol Biochem 62:354–357

Shibata H, Kato H, Oda J (1998c) Random mutagenesis on the *Pseudomonas* lipase activator protein, LipB: exploring amino acid residues required for its function. Protein Eng 11:467–472

Shinkai A, Hirano A, Aisaka K (1996) Substitutions of Ser for Asn-163 and Pro for Leu-264 are important for stabilization of lipase from *Pseudomonas aeruginosa*. J Biochem 120:915–921

Tanaka J, Ihara F, Nihira T, Yamada Y (1999) A low-Mr lipase activation factor cooperating with lipase modulator protein LimL in *Pseudomonas* sp. strain 109. Microbiology 145:2875–2880

Tanaka J, Nihira T, Yamada Y (2000) Glutathione as an essential factor for chaperon-mediated activation of lactonizing lipase (LipL) from *Pseudomonas* sp. 109. J Biochem (Tokyo) 127:597–601

Vasconcelos ATR, de Almeida DF, Almeida FC, de Almeida LGP, de Almeida R, Goncalves JAA, Andrade EM Antonio RV, Araripe J, de Araujo MFF, Filho SA, Azevedo V, Batista AJ, Bataus LAM, Batista JS, Belo A, vander Berg C, Blamey J, Bogo M, Bonato S, Bordignon J, Brito CA, Brocchi M, Burity HA, Camargo AA, Cardoso DDP, Carneiro NP, Carraro DM, Carvalho CMB, Cascardo JCM, Cavada BS, Chueire LMO, Pasa TBC, Duran N, Fagundes N, Falcao CL, Fantinatti F, Farias IP, Felipe MSS, Ferrari LP, Ferro JA, Ferro MIT, Franco GR, Freitas NSA, Furlan LR, Gazzinelli RT, Gomes EA, Goncalves PR, Grangeiro TB, Grattapaglia D, Grisard EC, Guimaraes CT, Hanna ES, Hungria M, Jardim SN, Laurino J, Leoi LCT,

Fassarella L, Lima A, Loureiro MF, Lyra MCP, Macedo M, Madeira HMF, Manfio GP, Maranhao AQ, Martins WS, di Mauro SMZ, de Medeiros SRB, Meissner RDV, Menck CFM, Moreira MAM, Nascimento FF, Nicolas MF, Oliveira JG, Oliveira SC, Paixao RFC, Parente JA, Pedrosa FO, Pena SJD, Perreira JO, Perreira M, Pinto LSRC, Pinto LS, Porto JIR, Potrich DP, Neto CER, Reis AMM, Rigo LU, Rondinelli E, dos Santos EBP, Santos FR, Schneider MPC, Seuanez HN, Silva AMR, da Silva ALC, Silva DW, Silva R, Simoes IC, Simon D, Soares CMA, Soares RBA, Souza EM, Souza KRL, Souza RC, Steffens MBR, Steindel M, Teixeira SR, Urmenyi T, Vettore A, Wassem R, Zaha A, Simpson AJG (2003) The complete genome sequence of *Chromobacterium violaceum* reveals remarkable and exploitable bacterial adaptability. Proc Natl Acad Sci USA 100:11660–11665

Wohlfarth S, Hoesche C, Strunk C, Winkler UK (1992) Molecular genetics of the extracellular lipase of *Pseudomonas aeruginosa* PAO1. J Gen Microbiol 138:1325–1335

Yanisch-Perron C, Vieira J, Messing J (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene 33:103–119