Experimental

General. All the DNA manipulations and bacterial transformation were carried out according to the standard protocols[1] or manufacturers' instructions, unless otherwise stated. *E. coli* JM109 (Toyobo) and pGEM-T (Promega) were routinely used as a host and a cloning vector, respectively, and *E. coli* BL21(DE3) (Stratagene) was used as a host for pET-11a, pET-11d (Stratagene) and their derivatives. *TaKaRa Ex Taq* DNA Polymerase was purchased from Takara Bio. Restriction enzymes were purchased from Toyobo, Stratagene, New England Biolabs, or Takara Bio, and T4 DNA ligase was purchased from Toyobo. Agarose for electrophoresis was purchased from Nacalai Tesque. QIAprep Spin Miniprep Kit, QIAquick PCR Purification Kit, and QIAquick Gel Extraction Kit (Qiagen) were routinely used for DNA isolation and purification. Synthetic oligonucleotides were obtained from Sigma Genosys Japan. DNA sequencing was performed on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems) at Advanced Science Research Center of Okayama University, and then analyzed with MacVector 7.0 (Oxford Molecular). All the genes cloned into the plasmids were fully sequenced in both directions. The molecular-weight marker for SDS-PAGE (lysozyme (14 kDa), β-lactoglobulin (18 kDa), trypsinogen (24 kDa), ovalbumin (45 kDa), albumin (66 kDa)) was purchased from Sigma. The amount of proteins was determined by the method of Bradford using BSA as the standard.[2] TLC was performed on Merck silica gel 60 F_{254}, and silica gel column chromatography was performed using Fuji Silysia BW-127 ZH (100–270 mesh). ^{1}H NMR spectra were measured in $CDCl_3$ at 300 or 200 MHz.

Construction of plasmids, pGBCL and pEBCLΔ48Act. Genomic DNA of *Burkholderia cepacia* NBRC (formerly IFO) 14595 was isolated according to the literature.[3] The DNA encompassing two genes coding for lipase and activator was amplified by PCR with the primers BC-LIP-1F (5'-GAGTTCATTCGTACCGGCAGC-3') and BC-LIP-2R (5'-CACATCACGCGCGCGGCATAAGGC-3'). The conditions for the 100 μL PCR mixture were as follows: 0.5 μM each primer, 0.2 mM each dNTP, genomic DNA (1 ng), 5 units of *TaKaRa Ex Taq* DNA Polymerase, 1.5 mM $MgCl_2$, and 10 μL of PCR buffer. PCR was done for 30 cycles of 98 ˚C for 10 s and 60 ˚C for 1 min followed by a final extension at 72 ˚C for 1 min. The amplified DNA fragment was directly cloned into pGEM-T to yield pGBCL from transformed *E. coli* JM109 cells. The genes coding for lipase and activator were subcloned into pET-11d. The primers used for PCR are as follows: BC-LIP-3F (5'-GAGAACATCCATGGCCAGATCG-3') and BC-LIP-5R (5'-CCGGATCCCATCACGCGCGCGGCATAAGGC-3'), where the restriction sites for *Nco* I

and *Bam* HI are underlined. The conditions for the 100 μL PCR mixture were as follows: 0.5 μM each primer, 0.2 mM each dNTP, pGBCL (1 ng), 2.5 units of *TaKaRa Ex Taq* DNA Polymerase, 1.5 mM $MgCl_2$, and 10 μL of PCR buffer. PCR was done for 30 cycles of (98 ˚C for 30 s, 57 ˚C for 30 s, and 72 ˚C for 1 min) followed by a final extension at 72 ˚C for 5 min. The amplified DNA fragment was digested with *Nco* I and *Bam* HI, and then ligated into pET-11d that had been treated with the same restriction enzymes. *E. coli* BL21(DE3) harboring pEBCLΔ48Act, which lacks 144 nucleotides in frame, was accidentally obtained by transformation of the competent cells.

Construction of an Expression Plasmid, pELIP. The gene encoding the mature lipase without an N-terminal signal sequence was subcloned into an expression vector, pET-11a. The primers used for PCR are as follows: BC-LIP-18F (5'-GGAATTCCATATGGCCGACGACTACGCGAC-3') and BC-LIP-19R (5'-CGGGATCCTCATCGATTACACGCC-3'), where the restriction sites for *Nde* I and *Bam* HI are underlined. The conditions for the 100 μL PCR mixture were as follows: 0.5 μM each primer, 0.2 mM each dNTP, pGBCL (1 ng), 2.5 units of *TaKaRa Ex Taq* DNA Polymerase, 1.5 mM $MgCl_2$, and 10 μL of PCR buffer. PCR was done for 30 cycles of 98 ˚C for 10 s and 55 ˚C for 30 s followed by a final extension at 72 ˚C for 1 min. The amplified DNA fragment was digested with *Nde* I and *Bam* HI, and then ligated into pET-11a that had been treated with the same restriction enzymes. *E. coli* BL21(DE3) harboring pELIP was obtained by transformation of the competent cells.

Construction of an Expression Plasmid, pEACTΔ48. The gene encoding the activator was subcloned into pET-11a. To construct pEACTΔ48, pEBCLΔ48Act was used as the template, and BC-ACT-1F (5'-GGAATTCCATATGACGGCACGTGAAGG-3') and BC-LIP-5R were used as the primers. The PCR conditions were the same as those for pELIP except for the thermal cycling conditions: 98 ˚C for 1 min, 30 cycles of (98 ˚C for 1 min, 57 ˚C for 1 min, and 72 ˚C for 3 min) and a final extension at 72 ˚C for 10 min. The amplified DNA fragment was digested with *Nde* I and *Bam* HI, and then ligated into pET-11a that had been treated with the same restriction enzymes. *E. coli* BL21(DE3) strain harboring pEACTΔ48 was obtained by transformation.

Site-directed Mutagenesis. Mutations were introduced by the overlap-extension PCR method.[4] Mutagenic oligonucleotides used as primers are shown in Table 1.

Table 1. Primers used for site-directed mutagenesis.

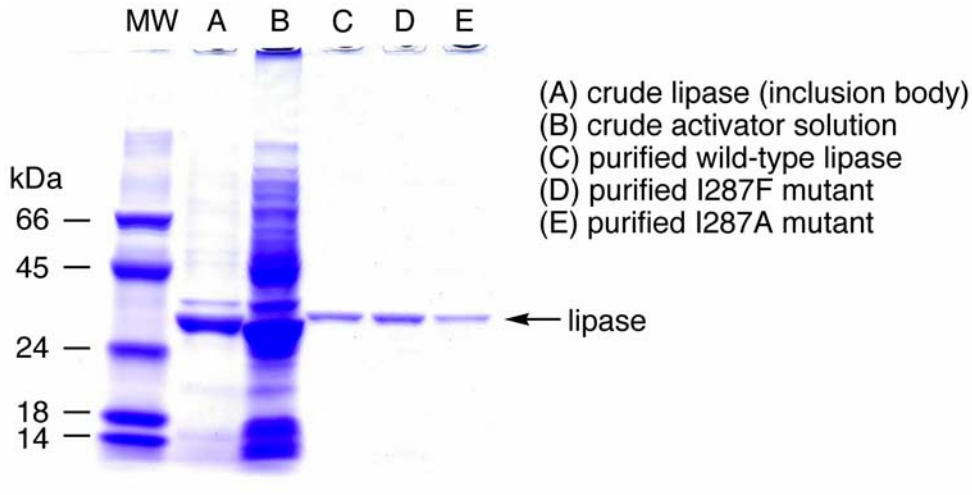
mutant	primer	sequence
I287F	BC-I287F-1F	5'-CTACAAGTGGAACCATTTCGACGAG-3'
	BC-I287F-2R	5'-CTCGTCGAAATGGTTCCACTTGTAG-3'
I287A	BC-I287A-1F	5'-CTACAAGTGGAACCATGCCGACGAG-3'
	BC-I287A-2R	5'-CTCGTCGGCATGGTTCCACTTGTAG-3'
I287L	BC-I287L-1F	5'-AAGTGGAACCATCTCGACG-3'
	BC-I287L-2R	5'-CGTCGAGATGGTTCCACTT-3'
I287M	BC-I287M-1F	5'-CTACAAGTGGAACCATATGGACGAG-3'
	BC-I287M-2R	5'-CTCGTCCATATGGTTCCACTTGTAG-3'

Together with the above mutagenic primers, in the first PCRs, BC-LIP-9F (5'-CCGCCACGTACAACCAGAACTATC-3') and PET-2R (5'-GTTATTGCTCAGCGGTGG-3') were also used, and in the second PCR, BC-LIP-9F and PET-2R were used. The conditions for the 100 μL PCR mixture were as follows: 0.5 μM each primer, 0.2 mM each dNTP, pELIP (1 ng) (first PCR) or the DNA fragment (1 ng) amplified in the first PCR (second PCR), 2.5 units of *TaKaRa Ex Taq* DNA Polymerase, 1.5 mM $MgCl_2$, and 10 μL of PCR buffer. PCR was done for 30 cycles of 98 °C for 10 s and 57 °C for 30 s followed by a final extension at 72 °C for 1 min. The DNA fragments were separated by 1.2% agarose gel electrophoresis and purified with QIAquick Gel Extraction Kit. After the second PCR, the amplified DNA fragment was digested with *Asc* I and *Bam* HI. The DNA fragment was purified as described above, and then ligated into pELIP that had been treated with the same restriction enzymes. *E. coli* BL21(DE3) strain harboring a mutated plasmid DNA was obtained by transformation. Introduction of the mutation was confirmed by DNA sequencing.

Overexpression of Lipase and Activator Genes and Refolding of Lipase. *E. coli* BL21(DE3) harboring pELIP (or its mutated derivative) was used to overproduce the wild-type (or mutant) lipase, while *E. coli* BL21(DE3) harboring pEACTΔ48 was used to overproduce activator. After each of the transformed cells was grown in LB medium (3 mL × 4) containing ampicillin (100 μg/1 mL) at 37 ˚C for 12 h with shaking at 230 rpm, 3 mL of the culture was transfered to the same medium (300 mL × 4) in a 1-L Erlenmeyer flask. The culture was shaken at 230 rpm at 37 ˚C, and IPTG (1 mM) was added when OD_{600} reached 0.8. The cells were further incubated at 37 ˚C for 4 h with shaking at 230 rpm, and then harvested by centrifugation (7000 rpm, 4 ˚C, 5 min). Each of the cell pellet (ca. 6 g) was suspended in 50 mM Tris buffer containing 1 mM EDTA (pH 8.0, 30 mL), and disrupted by

sonication with an Ultrasonic Disruptor UD-200 (Tomy) in an ice bath (sonication at output 3 for 5 min and cooling for 2 min, × 2). After centrifugation (20000 rpm, 4 ˚C, 30 min), activator was contained in the supernatant (activator solution), whereas lipase was contained in the pellet (inclusion body). The insoluble inclusion body was washed with 50 mM Tris buffer containing 1 mM EDTA (pH 8.0, 30 mL), and then dissolved in 0.1 M phosphate buffer containing 8 M urea (pH 8.0, 30 mL) at room temperature for 1 h with mild stirring (90 rpm). Centrifugation (20000 rpm, 4 ˚C, 30 min) gave a denatured lipase solubilized in the supernatant. The activator solution (15 mL) was added to the lipase solution (15 mL), and the mixture was incubated at 4 ˚C for 24 h to refold the lipase.

Purification of Recombinant Lipase. All the purification procedures were carried out at 4 ˚C. The crude solution (30 mL) containing the refolded (wild-type or mutant) lipase was dialyzed against 10 mM phosphate buffer (pH 7.0, 300 mL × 4, 1 L × 2), and then brought to 0.2 M $(NH_4)_2SO_4$ by the addition of solid $(NH_4)_2SO_4$. The solution was centrifuged at 20000 rpm at 4 ˚C for 30 min, and applied to a Butyl-Toyopearl column (ϕ6 × 7 cm) equilibrated with 10 mM phosphate buffer (pH 7.0) containing 0.2 M $(NH_4)_2SO_4$. After the column was washed with 10 mM phosphate buffer (pH 7.0, 100 mL), the lipase was eluted with 10 mM phosphate buffer (pH 7.0, 400 mL) containing 50% ethanol. Fractions with lipase activity (150 mL) were combined, and then immediately applied to a DEAE-Toyopearl column (ϕ4 × 8 cm) equilibrated with 10 mM phosphate buffer (pH 7.0). The proteins were eluted with the same buffer (200 mL), and then with 10 mM phosphate buffer (pH 7.0, 200 mL) containing 0.5 M NaCl. Active fractions eluted at 0.5 M NaCl (70 mL) were combined and concentrated to 20–30 mL by ultrafiltration. Only a single band was detected around ca. 33 kDa by SDS-PAGE (12.5%).



Immobilization of Lipase. The enzyme solution (20–30 mL) and Toyonite-200M were mixed in the ratio of 1 mg (lipase) to 100 mg (Toyonite-200M), and the mixture in a flask was shaken at 125 rpm for 12 h. The immobilized lipase was collected by filtration. After the immobilized lipase was dried in vacuo (4 mmHg) for 5 h, it was stored in an electric desiccator (humidity 20–25%) with a lid of the vial open.

Lipase-Catalyzed Kinetic Resolution of 1. A mixture of alcohol **1** (0.50 mmol), immobilized lipase (typically 100 mg), and three pieces of molecular sieves 3A in dry *i*-Pr_2O (5.0 mL) in a test tube with a rubber septum was stirred at 450 rpm in a thermostat at 30 ˚C. The reaction was started by adding vinyl acetate (85 mg, 1.0 mmol) via a syringe. The progress of the reaction was monitored by GC. The reaction was stopped by filtration at an appropriate conversion (typically 40–50%), and the filtrate was concentrated under reduced pressure. Compounds **1** and **2** were characterized as reported previously.[5] A mixture of **1a** and **2a** was analyzed directly by capillary GC using a CP-cyclodextrin-β-2,3,6-M-19 column (Chrompack, ϕ 0.25 mm × 25 m): Inj. 250 ˚C, Col. 100 ˚C, Det. 220 ˚C, (*S*)-**2a** 17.8 min, (*R*)-**2a** 19.7 min, (*R*)-**1a** 21.9 min, (*S*)-**1a** 23.7 min. Alcohol **1b** and ester **2b** were separated by silica gel column chromatography (hexane/EtOAc = 50:1–5:1). Alcohol **1b** was converted to the corresponding acetate **2b**, and the enantiomeric purity of **2b** was determined by capillary GC using the same column: Inj. 250 ˚C, Col. 100 ˚C, Det. 220 ˚C, (*S*)-**2b** 16.2 min, (*R*)-**2b** 18.4 min.

References

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