J. Biochem. 120, 915-921 (1996)

Substitutions of Ser for Asn-163 and Pro for Leu-264 Are Important for Stabilization of Lipase from *Pseudomonas aeruginosa*[1]

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Received for publication, May 1, 1996

The lipase gene from *Pseudomonas aeruginosa* was randomly mutated by error-prone PCR to obtain thermostable mutants, followed by screening for thermostable mutant lipases. Out of about 2,600 transformants, four thermostable clones were obtained. Their nucleotide sequences showed that they had two or three amino acid substitutions. Analysis of the thermal stabilization of these mutant lipases indicated that Asn-163 to Ser and Leu-264 to Pro mutations were essential for the increased stability of the lipase. We expressed a mutant lipase (StLipA-5) having only the Asn-163 to Ser mutation and another (StLipA-6) having only the Leu-264 to Pro mutation in *P. aeruginosa* PAO 1161, purified them, and then confirmed that the temperature which causes a 50% decrease in the activity of the non-treated enzyme on treatment for 30 min was increased by 1.5 and 3°C, respectively, compared to the wild-type enzyme. However, the thermal stability of the mutant lipase (StLipA-7) having both mutations was increased only by 2.5°C. These mutant lipases were stabilized through a decrease in activation entropy. Kinetic studies showed that the k_{cat}/K_m values of StLipA-5, StLipA-6, and StLipA-7 were decreased by 14.4, 52.9, and 26.0%, respectively. Interestingly, the pH-stabilities of StLipA-6 and StLipA-7 were also increased, especially at alkaline pH. Based on these results, the tertiary structure and mechanism of stabilization of the lipase were discussed.

Key words: lipase, pH-stabilization, *Pseudomonas aeruginosa*, random mutagenesis, thermal stabilization.

Lipases [EC 3.1.1.3] have been found in many species of animals, plants, and microorganisms. They can hydrolyze various kinds of esters and, in organic solvents, they can also catalyze the reverse reactions, *e.g.*, the synthesis of esters, and *trans*-esterifications. Therefore, lipases are industrially important enzymes for food refinement, as additives in washing detergents, as diagnostic enzymes, and for paper manufacturing (*1*). Among various kinds of lipases, microbial extracellular lipases are industrially useful because of ease of mass production. In particular, the highly homologous lipases from *Pseudomonas aeruginosa* and *Pseudomonas* sp. are valuable because they are able to catalyze interesting reactions such as hydrolysis of polycaprolactone, synthesis of macrolactone, and so on (*2-4*). One important enzymatic characteristic for the application of enzymes is thermal stability. Therefore, the stability-enhanced mutants of the lipase from *P. aeruginosa* will be useful for various industrial fields.

The primary structure of the lipase from *P. aeruginosa*, deduced from the nucleotide sequence of its gene, consists of a mature protein of 285 amino acids preceded by a signal sequence of 26 amino acids (*5, 6*). It was also found that an additional protein, a so-called chaperonin of the lipase, whose gene was located immediately downstream of the lipase gene, was necessary for expression of the active lipase (*6-8*). Furthermore, a three-dimensional model of the lipase was proposed by comparison of its amino acid sequence with those of other hydrolases with known three-dimensional structures (*9*). However, it is difficult to determine the positions and residues for specific mutations to increase the thermal stability of the enzyme. On the other hand, random mutagenesis is practical when coupled with efficient screening procedures to identify clones expressing variant enzymes with the properties of interest (*10-12*).

In this work, in order to determine the amino acid changes that are essential for stabilization of the lipase from *P. aeruginosa* and to widen the applicability of this lipase to industrial fields, we tried to increase its thermal stability by the random mutagenesis technique. We obtained four thermostable mutant lipases and found that two mutations are important for thermal stabilization of the lipase. From the results of analysis of enzymatic properties of the thermostable mutant lipases, the tertiary structure and mechanism of stabilization of the lipase are also discussed in terms of the three-dimensional model proposed by Jaeger *et al.* (*9*).

MATERIALS AND METHODS

Isolation of the Lipase Gene—P. aeruginosa ATCC 31156 was used as the DNA donor for the lipase gene.

[1] The nucleotide sequence data reported here appear in the GSDB, DDJB, EMBL, and NCBI nucleotide sequence databases, with accession numbers D50587, for *lipA*, and D50588, for *lipM*.

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Abbreviation: LB, Luria broth.

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Chromosomal DNA of *P. aeruginosa* ATCC 31156, obtained by the method of Marmur (*13*), was digested with *Bam*HI. Then 8.3-kbp fragment containing the lipase gene (*lipA*) and lipase modulator gene (*lipM*) (*7, 8*) were isolated by colony hybridization with the nucleotide probe corresponding to N-terminal 19 amino acid residues of the mature form of the enzyme, using pBluescriptII KS(+) (Toyobo, Osaka) as a vector, and *Escherichia coli* XLIBlue (*14*) as a host strain. The isolated plasmid containing *lipA* and *lipM* was named pWtLipA-1 (Fig. 1).

Construction of a Randomly Mutated DNA Library—(a) PCR mutagenesis: Plasmid pLipA-T carrying *lipA*, constructed as described below, was linearized by digestion with *Sca*I, and the linearized plasmid was used as a template for PCR. Two oligonucleotides, 5′-CTCGAGGTCGACGGTATCGA-3′ and 5′-CAGGAAACAGCTATGACCATG-3′, corresponding to the sequences on pBluescriptII SK(−), were used as 5′ and 3′ PCR primers, respectively. PCR was carried out as described by Cadwell and Joyce (*15*). (b) Library construction: pWtLipA-3 constructed as described below and PCR-mutagenized lipase gene fragments were digested with *Hin*dIII and *Bam*HI. The large fragment of pWtLipA-3 and mutagenized lipase genes were ligated, and *E. coli* XLIBlue was transformed with the hybrid plasmids by electroporation (*16*).

Screening of Thermostable Lipase by a Filter Assay—Colonies harboring the plasmids carrying the randomly mutated lipase genes on LB-plates were transferred to nylon membranes (Hybond-N, Amersham, Buckingamshire, UK), and the cells were incubated on LB-plates supplemented with 50 μg/ml ampicillin and 1 mM IPTG for 6 h. The cells on membranes were treated with a solution comprising 20 mM Tris-HCl (pH 7.5), 1 mg/ml lysozyme, and 1 mM Na-EDTA (pH 8.0) at 37°C for 5 min. The membranes were transferred onto paper filters wetted with a solution comprising 10 mM Tris-HCl (pH 7.5) and 1% (w/v) Triton X-100, and incubated at 37°C for 5 min. Then the membranes were incubated at 70°C for 45 min on a paper filter wetted with 10 mM Tris-HCl (pH 7.5). The colonies, colored yellow, were selected by incubating the filters at 37°C for 30 min on 0.8% agarose plates containing 10 mM Tris-HCl (pH 7.5) and 1 mM *p*-nitrophenyl palmitate. The plasmids prepared from the four colonies were named pStLipA-1, pStLipA-2, pStLipA-3, and pStLipA-4, respectively.

Plasmid Construction—The scheme for construction of plasmids carrying the wild-type and mutant lipase genes is summarized in Fig. 1.

pWtLipA-2: pWtLipA-1 was digested with *Nru*I, then with *Bal*31. After treatment with Klenow fragment, the plasmid was ligated with *Hin*dIII linker. Then the plasmid was digested with *Sma*I, followed by digestion with *Bal*31, treatment with Klenow fragment and ligation with *Sma*I linker. The 2.4-kbp *Hin*dIII-*Sma*I fragment containing *lipA* and *lipM* was inserted into pUC19-EN, which was constructed by digestion of pUC19 with *Eco*RI followed by Klenow treatment and ligation with *Nco*I linker, and the plasmid was named pWtLipA-2.

pWtLipA-3: To construct pWtLipA-3, which has a *Bam*HI site immediately downstream of the stop codon of *lipA* on pWtLipA-2, site-directed mutagenesis was performed. The 685-bp *Eco*RI-*Sal*I fragment containing the downstream of *lipA* was inserted into M13mp19. The single-stranded DNA of the hybrid plasmid was prepared and site-directed mutagenesis was performed using Mutan-K (Takara Shuzo, Kyoto) with a synthetic oligonucleotide, 5′-AACGCCAGCCTGTAGGATCCCGG-3′. The 685-bp *Eco*RI-*Sal*I fragment was again isolated and ligated with the 4.4-kbp *Eco*RI-*Sal*I fragment of pWtLipA-2.

pLipA-T: The *Hin*dIII-*Bam*HI 980-bp fragment coding *lipA* on pWtLipA-3 was inserted into pBluescriptII SK(−), and the resulting plasmid was named pLipA-T.

pStLipA-5, pStLipA-6, and pStLipA-7: The 394-bp *Eco*RI-*Bam*HI fragment of pStLipA-1 was ligated with the 4.7-kbp *Eco*RI-*Bam*HI fragment of pWtLipA-3 to construct pStLipA-5 containing the StLipA-5 gene. The 580-bp *Hin*dIII-*Eco*RI fragment of pWtLipA-3 was ligated with the 4.5-kbp *Hin*dIII-*Eco*RI fragment of pStLipA-4 to construct pStLipA-6 containing the StLipA-6 gene. The 713-bp *Hin*dIII-*Pst*I fragment of pStLipA-5, the 262-bp *Pst*I-*Bam*HI fragment of pStLipA-6, and the 4.1-kbp *Hin*dIII-*Bam*HI fragment of pWtLipA-3 were ligated to construct pStLipA-7 containing the StLipA-7 gene.

pWtLipA-3P, pStLipA-5P, pStLipA-6P, and pStLipA-7P: The 2.4-kbp *Hin*dIII-*Sma*I fragment containing pWtLipA-3, pStLipA-5, pStLipA-6, and pStLipA-7 was ligated with pEG400 (*17*), a broad host range plasmid for Gram-negative bacteria, to construct pWtLipA-3P, pStLipA-5P, pStLipA-6P, and pStLipA-7P, respectively.

Other DNA Manipulations—Plasmid DNA was prepared as described by Birnboim and Doly (*18*). To introduce a plasmid into *P. aeruginosa* PAO 1161 (*trp, leu, r*$^-$, *m*$^-$) (*19*), we performed triparental matings (*20*) as described below. Matings were performed by mixing about 10^9 cells each of the donor (*E. coli* XLIBlue containing the plasmid carrying the wild-type or a mutant lipase gene), recipient (*P. aeruginosa* PAO 1161), and *E. coli* HB101 containing pRK2013 (*20*) after washing the cells with 10 mM potassium phosphate (pH 7.3). The suspensions were filtered on 0.45 μm Millipore filters and the filters were incubated at 30°C on nonselected LB-agar plates for 6 h. *P. aeruginosa* PAO 1161 carrying the wild-type or a mutant lipase gene was isolated by plating the cells on *Pseudomonas* isolation agar (Difco, Detroit, MI) plates containing 650 μg/ml of streptomycin followed by incubation at 30°C for 20 h. Nucleotide sequences were determined by the dideoxynucleotide chain termination method (*21*) using [α-^{35}S]dCTP (>1,000 Ci/mmol, Amersham) and M13 phage single-stranded DNAs as templates with a Sequenase Version 2.0 DNA Sequencing Kit (Toyobo). All other recombinant DNA techniques were performed as described by Sambrook *et al.* (*22*).

Purification of the Recombinant Lipases—The recombinant wild-type or thermostable mutant lipase was purified from the culture medium of recombinant *P. aeruginosa* PAO 1161, basically as described by Chihara-Siomi *et al.* (*5*) with some modifications. Proteins in the culture supernatant were precipitated with 80% (w/v) saturation of $(NH_4)_2SO_4$ and resuspended in 1 mM sodium phosphate (pH 7.5). The suspension was chromatographed on a butyl-Sepharose FF FPLC column (2.6×10 cm) (Pharmacia, Uppsala, Sweden) and a MonoQ FF FPLC column (0.5×5 cm) (Pharmacia), instead of an octyl-Sepharose CL-6B column and a DEAE-Sepharose column. The buffers used were 1 mM sodium phosphate (pH 7.5) for the former and 50 mM sodium phosphate (pH 7.0) for the latter. The

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active fractions collected were concentrated by ultrafiltration, then dialyzed against 50 mM sodium phosphate (pH 7.0).

The specific activities of the purified lipases, measured with cholesterol linoleate as a substrate as described below, are shown in Table II.

Enzyme Assay—The tributyrin diffusion-agar method (*23*) was used to analyze the thermal stabilization of the wild-type and mutant lipases expressed in *E. coli*. Cells were grown in 40 ml of LB medium supplemented with 50 μg/ml ampicillin. At the exponential growth phase (A_{660} = 0.5-0.6), the lipases were induced with 5 mM IPTG. After further cultivation for 2.5 h, the cells were collected by centrifugation, washed with 10 mM Tris-HCl (pH 7.5), and resuspended in 2 ml of the same buffer, then disrupted by sonication. A 2-μl portion of each cell lysate was spotted on a tributyrin diffusion-agar plate and incubated at 37°C for 6 h.

Lipase activity was measured with cholesterol linoleate as a substrate according to the method of Uwajima and Terada (*24*).

Measurement of the Thermal Stablity of the Purified Lipase—Thermal stability was measured by incubation at different temperatures in 50 mM sodium phosphate (pH 7.0). The protein concentration was 100 μg/ml. Lipase activity remaining after treatment was measured as described above.

The heat inactivation rate constants at 66, 68, 70, and 72°C were calculated, and the activation enthalpy (ΔH^*) and activation entropy (ΔS^*) were estimated from an Arrhenius plot.

Measurement of the Kinetic Parameters of the Lipase—The enzyme activity was measured as described above except that the concentration of the substrate was 200, 100, 67, 50, or 40 μM. The K_m and k_{cat} values were estimated from Lineweaver-Burk plots, Eadie-Hofstee plots, and Hanes-Woolf plots, the average values being calculated.

Measurement of the pH-Stability of the Lipase—The purified lipase (2 μg) was incubated at 40°C for 30 min in 750 μl of Johnson-Lindsay buffer (pH 2-12). After the solution had been adjusted to pH 7.0 with NaOH or HCl, the remaining lipase activity was measured as described above.

Other Methods—Protein concentrations were determined by the method of Lowry *et al.* (*25*) using bovine serum albumin as a standard.

RESULTS

Isolation of Thermostable Mutant Lipases—The 980-bp *Hin*dIII-*Bam*HI fragment containing *lipA* was inserted into pBluescriptII SK(−), and this plasmid (pLipA-T) (Fig. 1) was used as a template for random mutagenesis by error-prone PCR. PCR-amplified fragments containing randomly mutated *lipA* were ligated into the upstream of *lipM* on pWtLipA-3, and the hybrid plasmids were used to transform *E. coli* XLIBlue.

The mutant *lipA*-containing transformants (about 2,600 colonies) were analyzed for heat-resistant enzyme activity under conditions under which the native enzyme was completely inactivated, as described under "MATERIALS AND METHODS." We obtained four clones, and from these clones we prepared plasmids (pStLipA-1, -2, -3, and -4), and determined the nucleotide sequences of the mutagenized regions (980-bp *Hin*dIII-*Bam*HI fragments) of these plasmids. Table I summarizes the base substitutions caused by the error-prone PCR and the amino acid substitutions deduced from them. These mutant lipases (StLipA-1, -2, -3, and -4) exhibited two or three amino acid substitutions. Their notable mutational characteristics were as follows: StLipA-1 and StLipA-2 had an identical Asn-163 to Ser mutation, and StLipA-3 and StLipA-4 had an identical Leu-264 to Pro mutation. These results suggest that the two mutations are important for increased thermal stability of the lipase.

To confirm the importance of the two mutations for thermal stabilization of the lipase, we constructed plasmid pStLipA-5 carrying the StLipA-5 gene containing only the Asn-163 to Ser mutation, and pStLipA-6 carrying the StLipA-6 gene containing only the Leu-264 to Pro mutation, and expressed them in *E. coli* to examine their thermal stability. As the lipase activity was detected in the cell fraction in *E. coli*, we prepared cell lysates and examined the thermal stability of the lipases by detection of the formation of a clear zone on tributyrin plates (Fig. 2). The clear zone formed by StLipA-5 after treatment at 70 or 75°C was the same as that formed by StLipA-1, and clearer than that formed by StLipA-2. The clear zone formed by StLipA-6 was clearer than those formed by StLipA-3 and StLipA-4. These results indicate that the two mutations are important for increased thermal stability of the lipase, and suggest that the other mutations detected in StLipA-1, StLipA-2, StLipA-3, and StLipA-4 do not contribute to the increase in thermal stabilization. Therefore, we investigated precisely the effects of the Asn-163 to Ser and Leu-264 to Pro mutations on the enzymatic properties of the lipase.

Expression of the Lipase in P. aeruginosa PAO 1161—In *E. coli*, the lipases were not expressed sufficiently for quantitative analysis of their enzymatic properties even when the genes were inserted into a high expression vector such as pKK233-2 (data not shown). Therefore, we chose *P. aeruginosa* PAO 1161 as a host strain for expression of the wild-type and thermostable mutant lipases because it has been improved for gene engineering, and little lipase activity is detected in both its culture medium and cell fraction (data not shown).

The recombinant *P. aeruginosa* PAO 1161, with the introduced plasmid pWtLipA-3P containing *lipA* and *lipM*, produced the lipase, and its activity was found in the culture medium. The maximal activity was 2.0±0.4 units/ml of broth with cholesterol linoleate as a substrate, which was almost 500 times higher than that produced by *E. coli*. When the DNA fragment containing *lipA* and *lipM* was ligated in reverse relative to the lactose promoter in the vector, lipase activity was not found (data not shown). These results indicate that the lipase is expressed through the function of the lactose promoter in pEG400 in *P. aeruginisa* PAO 1161. We constructed plasmids pStLipA-5P and pStLipA-6P, and introduced them into *P. aeruginosa* PAO 1161 to express StLipA-5 and StLipA-6, respectively.

Thermal Stability of the Mutant Lipases—The wild-type and mutant lipases were purified from the culture medium of recombinant strains, and their heat resistance was investigated. Figure 3 shows that the heat resistance of the wild-type enzyme was increased by the Asn-163 to Ser or Leu-264 to Pro mutation. The T_m values of StLipA-5 and

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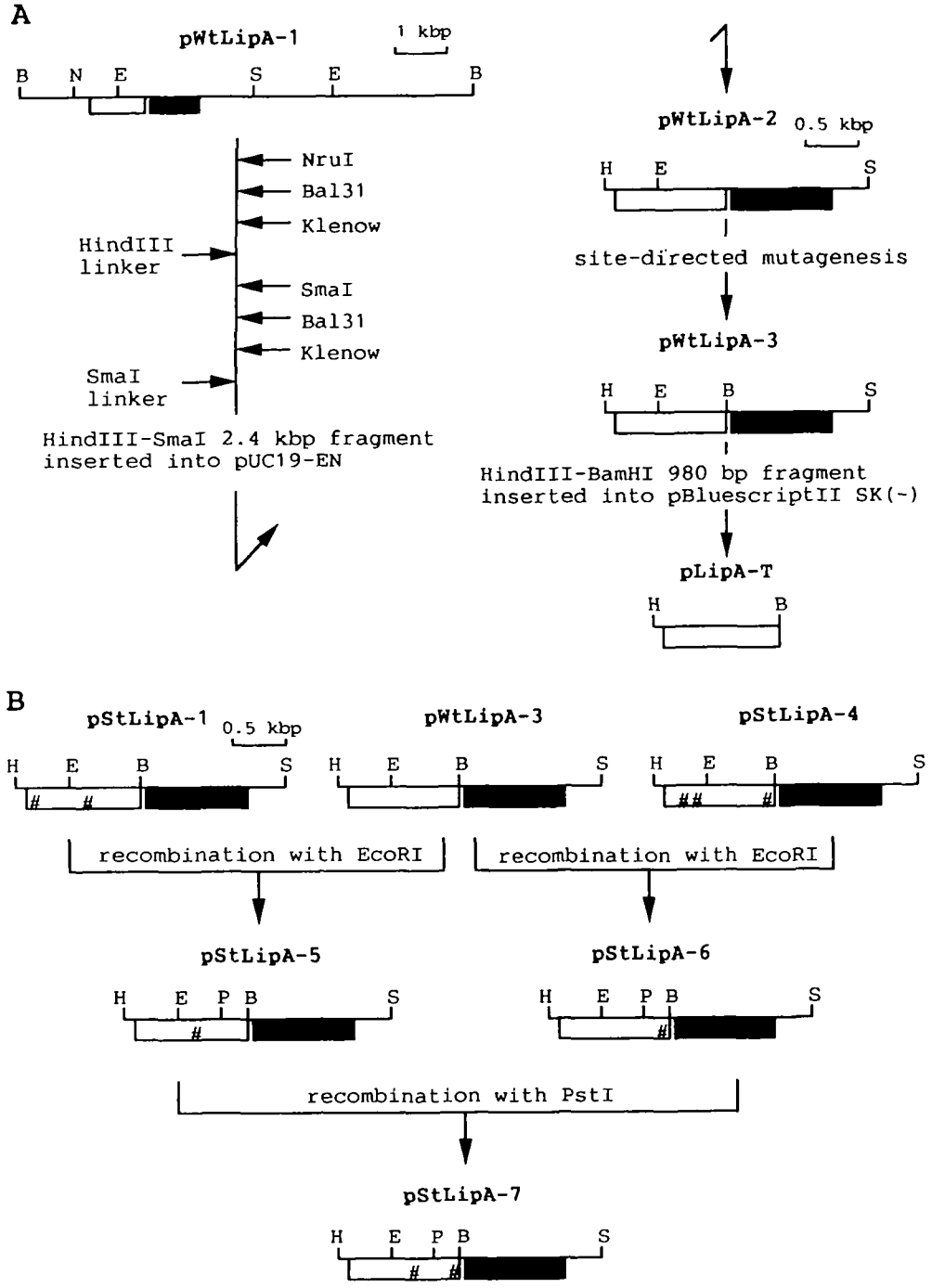

Fig. 1. **The scheme for construction of plasmids carrying the wild-type and mutant lipase genes.** The inserted fragment of each plasmid is shown. (A) Subcloning of the *lipA* and *lipM* genes. (B) Construction of pStLipA-5, pStLipA-6, and pStLipA-7. ▭ and ■ indicate the *lipA* and *lipM* genes, respectively. H, *Hin*dIII; E, *Eco*RI; B, *Bam*HI; S, *Sma*I; and P, *Pst*I. # indicates the location of a nucleotide change observed in the mutant lipase gene.

StLipA-6, that is, the temperature which causes a 50% decrease in the activity of the non-treated enzyme on treatment for 30 min, were 1.5 and 3°C higher than that of the wild-type enzyme, respectively (Table II).

To study the relationship between the two mutations, we constructed plasmid pStLipA-7P carrying the StLipA-7 gene, which has both the Asn-163 to Ser and Leu-264 to Pro mutations, and expressed it in *P. aeruginosa* PAO 1161. The enzyme was purified and its thermal stability was investigated (Fig. 3). The T_m value of StLipA-7 was only 2.5°C higher than that of the wild-type enzyme, demonstrating that, the thermal stabilization effects of the two mutations were not additive (Table II).

Thermodynamic Parameters of the Mutant Lipases—To investigate the mechanism of stabilization of the mutant lipases, the thermal stabilities of the wild-type and mutant lipases were investigated at various temperatures ranging from 66 to 72°C, and the results at 70°C are shown in Fig. 4A assuming first-order kinetics. The inactivation rate constant (k) at each temperature was calculated and the activation parameters for the heat inactivation were estimated from Arrhenius plots (Fig. 4B). The activation enthalpy (ΔH^*) and activation entropy (ΔS^*) values for the mutants are listed in Table II. These results show that the

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TABLE I. **Base changes on random mutagenesis and deduced amino acid substitutions in the mutant genes coding thermostable lipases.**

Mutant[a]	Nucleotide change	Location of nucleotide change[b]	Amino acid substitution[b]
StLipA-1	(A/T) to (C/G)	7	Lys-(−24) to Glu
	(A/T) to (G/C)	566	Asn-163 to Ser
StLipA-2	(T/A) to (C/G)	13	Ser-(−22) to Pro
	(A/T) to (G/C)	272	Glu-65 to Gly
	(A/T) to (G/C)	566	Asn-163 to Ser
StLipA-3	(G/C) to (A/T)	821	Arg-248 to Gln
	(T/A) to (C/G)	869	Leu-264 to Pro
StLipA-4	(A/T) to (T/A)	437	Gln-120 to Leu
	(A/T) to (T/A)	523	Ser-149 to Cys
	(T/A) to (C/G)	869	Leu-264 to Pro
StLipA-5	(A/T) to (G/C)	566	Asn-163 to Ser
StLipA-6	(T/A) to (C/G)	869	Leu-264 to Pro
StLipA-7	(A/T) to (G/C)	566	Asn-163 to Ser
	(T/A) to (C/G)	869	Leu-264 to Pro

[a]The mutants under the broken line are recombinants constructed as described under "MATERIALS AND METHODS." [b]The locations of nucleotides are numbered from the first nucleotide of the open reading frame of *lipA*. The locations of amino acids are numbered from the N-terminal amino acid of the mature enzyme, and amino acids with negative numbers are in the signal sequence.

TABLE II. **T_m values, activation parameters for heat inactivation, kinetic parameters, and specific activities of the wild-type and mutant lipases.** The T_m values and the activation parameters were estimated from Fig. 3 and Fig. 4B, respectively. The kinetic parameters and specific activities were calculated from triplicate experiments, respectively.

Enzyme	T_m (°C)	ΔH^* (kJ/mol)	ΔS^* (J/mol/K)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}\cdot\mu M^{-1}$)	Specific activity (U/mg)
Wild-type	66.0	332	665	84.0	8.70	0.104	44.0
StLipA-5	67.5	212	311	83.0	7.35	0.089	40.0
StLipA-6	69.0	275	491	102	5.00	0.049	20.0
StLipA-7	68.5	301	569	88.7	6.80	0.077	38.0

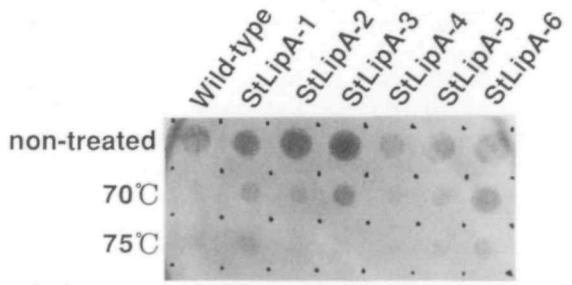

Fig. 2. **Clear zone formation by the wild-type and mutant lipases in *E. coli*.** An *E. coli* cell lysate containing the wild-type or a mutant lipase was plated on a tributyrin diffusion-agar plate, directly (non-treated) or after treatment at 70 or 75°C.

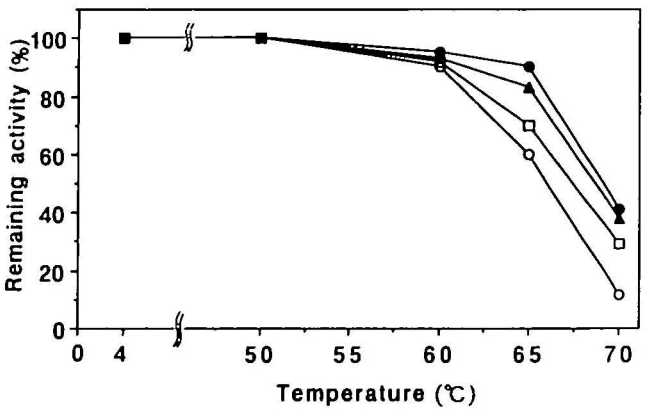

Fig. 3. **Thermal stability of the wild-type and mutant lipases.** The enzymes were incubated at the indicated temperatures for 30 min in 50 mM sodium phosphate (pH 7.0), then the remaining activity was measured as described under "MATERIALS AND METHODS." Duplicate experiments were performed, and the values whose error ranges were within 5% were averaged. (○) Wild-type, (□) StLipA-5, (●) StLipA-6, and (▲), StLipA-7.

introduction of the Asn-163 to Ser or Leu-264 to Pro mutation decreased both ΔH^* and ΔS^*, and that these mutants are, therefore, stabilized by a decrease in ΔS^*.

Kinetic Parameters of the Mutant Lipases—The effects of these amino acid substitutions on the catalytic activity of the lipase were then studied. The wild-type and mutant lipases showed Michaelis-Menten saturation kinetics when the initial velocity was plotted against the concentration of the substrate (data not shown). Table II summarizes the average values of K_m and k_{cat} calculated from Lineweaver-Burk plots, Eadie-Hofstee plots, and Hanes-Woolf plots. For StLipA-5, the k_{cat} value was mainly affected (15.5% decrease), while for StLipA-6, both the K_m and k_{cat} values were affected (21.4% increase and 42.5% decrease, respectively). StLipA-7 has the same (Leu-264 to Pro) mutation as StLipA-6, but the K_m value was smaller than that of StLipA-6, and the k_{cat} value was larger than that of StLipA-6. These results suggest that the introduction of the Asn-163 to Ser mutation into StLipA-6 led to structural complementation, with which the activity was improved.

pH-Stability of the Mutant Lipases—Figure 5 shows the pH-stabilities of the wild-type and mutant lipases. The mutation of Leu-264 to Pro did not change the pH-stability of the enzyme, but the mutation of Asn-163 to Ser in-

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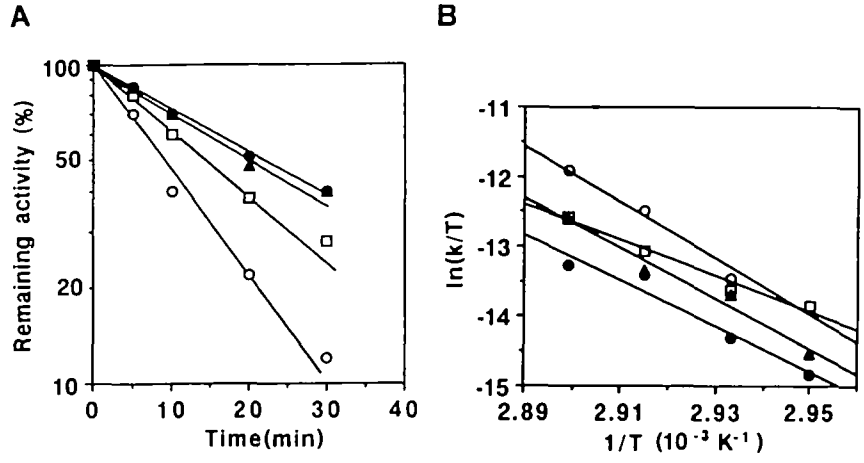

Fig. 4. **(A) Time course of heat inactivation of the wild-type and mutant lipases.** The enzymes were heated at 70°C in 50 mM sodium phosphate (pH 7.0). The remaining activity was measured as described under "MATERIALS AND METHODS." **(B) Arrhenius plots for heat inactivation of the wild-type and mutant lipases at 66 to 72°C.** The method of least squares was used for these graphs. (○) Wild-type, (□) StLipA-5, (●) StLipA-6, and (▲) StLipA-7.

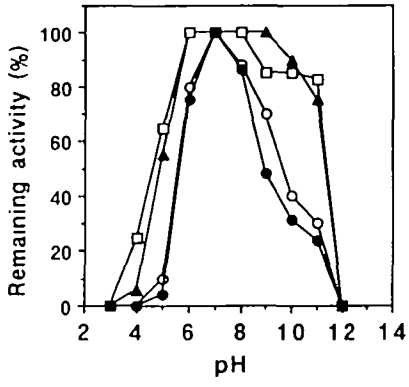

Fig. 5. **Effects of pH on the stability of the wild-type and mutant lipases.** The enzymes were incubated at 40°C for 30 min in Johnson-Lindsay buffer at the indicated pH values, and then the remaining activity was measured as described under "MATERIALS AND METHODS." Duplicate experiments were performed, and values whose error ranges were within 5% were averaged. (○) Wild-type, (□) StLipA-5, (●) StLipA-6, and (▲) StLipA-7.

creased the pH-stability at pH 4 to 11, especially at pH values higher than 8.

DISCUSSION

By the combination of random mutation and filter assaying, we isolated four thermostable lipases and concluded that Asn-163 to Ser and Leu-264 to Pro mutations were important for increased thermal stability of the lipase. However, we could not isolate a thermostable mutant lipase having only one mutation, like StLipA-5 or StLipA-6, from random mutant library. The efficiency of introduction of random mutation by error prone PCR performed in this work was about 3 bp per 1 kbp, and 7 clones out of 8 clones isolated randomly from the library had several mutations (data not shown). If the efficiency could be lowered, mutant lipases like StLipA-5 and StLipA-6 might be isolated. And if such conditions could be used, other mutant lipases having only one mutation could be isolated that would show decreased thermal stability when combined with another mutation. We tried to use *P. aeruginosa* directly as a host strain for the construction of a random mutant library of the lipase gene, but the efficiency of transformation of *P. aeruginosa* was about 100 times lower than that of *E. coli* (data not shown). Therefore, we used *E. coli* as a host strain for the construction of a random mutant library of the lipase gene, and used *P. aeruginosa* as a host strain for expression of the mutant genes obtained. It has been shown that the lipase from *P. aeruginosa* was activated through the function of a lipase modulator protein considered to be a chaperonin, whose gene is located immediately downstream of the lipase gene (*7, 8*). Therefore, with this system, a mutant lipase that could not interact with the chaperonin (in this case, LipM) was not selected as a thermostable mutant lipase, even if the mutant lipase has important mutations that increase the thermal stability.

Both the Asn-163 to Ser and Leu-264 to Pro mutations caused a decrease in the activation entropy of unfolding (Table II). Matthews *et al.* showed that by replacing glycine with alanine, which has less backbone conformational flexibility, or by replacing alanine with proline, whose pyrrolidine ring restricts the number of possible conformations, the stability of T4 lysozyme increased (*26*). These mutant T4 lysozymes were stabilized due to decreases in the backbone entropy of unfolding. The mutant lipases might also be stabilized thermodynamically by the same mechanism as the mutant T4 lysozymes as discussed by Arase *et al.* (*12*).

The amino acid sequence of the lipase from *P. aeruginosa* ATCC 31156 was identical with that from *P. aeruginosa* PAO1, whose three-dimensional model was constructed by Jaeger *et al.* (*9*), except for two amino acid residues, Val-130 for Ile and Ile-178 for Val (*6*). Therefore, the three-dimensional model of the lipase from *P. aeruginosa* ATCC 31156 must be basically the same as that constructed by Jaeger *et al.*, and the mechanism of stabilization and the tertiary structure of the mutant lipases obtained can be discussed in terms of that model. According to the model, Leu-264 exists in the same loop as His-251, one of the presumed catalytic amino acids (Ser-82, Asp-229, and His-251). Therefore, introduction of the Leu-264 to Pro mutation, might cause structural changes around His-251, resulting in decrease of the catalytic efficiency to about 50% of the value of the wild-type lipase (Table II). However, the catalytic efficiency of StLipA-6 containing only the Leu-264 to Pro mutation seemed to be partially complemented by the introduction of the Asn-163 to Ser mutation (Table II). According to the model, Asn-163 also exists in a loop

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structure, which is close to the three loops in which presumed catalytic amino acids exist. Therefore, Asn-163 might be close to the catalytic amino acids, and the introduction of the Asn-163 to Ser mutation might cause a structural change that results in the recovery of the catalytic efficiency. The thermal stability caused by the Asn-163 to Ser and Leu-264 to Pro mutations was not additive, unlike in the cases of other enzymes reported (*12, 27, 28*); therefore, the structural changes caused by the two mutations may not be independent.

The pH-stability profiles of the mutant lipases indicate that Asn-163 is also one of the critical residues that decide the alkaline stability of the enzyme (Fig. 5), and it might be that the hydroxyl group of Ser-163 contributes to the increased alkaline stability.

To verify the mechanism of stabilization and the tertiary structure of the lipase described above, we need to study other mutant lipases with other amino acid substitutions at position 163 or 264, or the same substitutions around position 163 or 264, and to perform X-ray crystallographic analysis of these mutant lipases. Through such studies we might obtain or design more thermostable mutants than those obtained in this study.

We wish to thank Kazuo Yamaguchi, Kazuko Yamaguchi, Keiichi Yano, and Mika Yoshida for amino acid sequence analysis of the lipases. We also thank Akiko Saito and Mayumi Enoki for the synthesis of DNA, and Hideharu Anazawa for providing us with plasmid pEG400.

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