Site-Directed Mutagenesis of a Highly Active *Staphylococcus epidermidis* Lipase Fragment Identifies Residues Essential for Catalysis[1]

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ABSTRACT: A fragment of *Staphylococcus epidermidis* lipase gene (Lys-303 to Lys-688) was inserted into plasmid pET-20b(+). The resulting C-terminal His-tagged recombinant protein (43 kDa) was overexpressed in *Escherichia coli* BL21(DE3) as a highly active lipase and was purified with nickel-coupled resin. Putative catalytic sites were determined by site-directed mutagenesis. Mutant enzymes (S418C and H648K) lost enzyme activities, which strongly suggests that the proposed residues of Ser-418 and His-648 are involved in catalysis. Site-directed mutagenesis showed that in comparison with wild-type enzyme, the M419A and V649I enzymes showed a 2.0- and 4.0-fold increase in the k_{cat}/K_m, respectively, but the M419I, M419Q, V649A, and V649L variants lost enzyme activities. The wild-type enzyme and the V649I mutant favored the hydrolysis of *p*-nitrophenyl esters of butyrate, but the M419A favored decanoate. The results suggested that the amino acid residues (Met-419 and Val-649), following the catalytic triad, could affect the substrate specificity and/or catalytic efficiency.

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KEY WORDS: Site-directed mutagenesis, *Staphylococcus epidermidis* lipase, structure-function.

Lipases (triacylglycerol hydrolase, EC 3.1.1.3) are widely distributed in nature. The principal biological function of lipases is the breakdown of lipids as an initial event in the utilization of fat as an energy source. They have become increasingly important in biotechnology (1,2). The characteristic properties such as substrate specificity, regioselectivity, and enantioselectivity among various lipases allow wide applications such as in the production of emulsifiers (3,4), fatty acid esters (5), fatty acids (6–8), and carbohydrate derivatives (9,10). A Ser-His-Asp catalytic triad occurs in lipases, which are responsible for hydrolyzing triglycerides into diglycerides and subsequently, monoglycerides and free fatty acids (11).

Staphylococcus epidermidis strain 9 lipase gene (*gehC*) has been cloned and expressed in *Escherichia coli* (12). The gene consisted of a single open reading frame of 2064 nucleotides, which encoded a protein of 688 amino acids with a predicted molecular mass of 77 kDa. A lipase with an electrophoretic mobility equivalent to a 97 kDa protein, but corresponding to the 77 kDa lipase, was detected in extracts of *E. coli* harboring *gehC*. Interestingly, a 43 kDa lipase was purified from *S. epidermidis* strain 9 culture supernatant fluid. It was suggested that the *S. epidermidis* lipase of 97 kDa electrophoretic mobility was secreted as a proprotein and subsequently cleaved between Ala-302 and Lys-303 by a proteolytic enzyme to produce the 43 kDa lipase (386 amino acids). A partial *gehSE1* gene from *S. epidermidis* strain RP62A, similar in sequence to the lipase gene of *S. epidermidis* strain 9, and coding a lipase fragment corresonding to virtually the entire mature *S. epidermidis* strain 9 lipase (Asn-7 of the strain 9 lipase is the first amino acid and Lys-386 the last) has been overexpressed as a fusion protein with an N-terminal His-tag in *E. coli* (13) and characterized. However, no structure-function studies have been done.

In the present report, we show that a highly active C-terminal His-tagged recombinant *S. epidermidis* lipase fragment (identical with *S. epidermidis* strain 9 lipase gene, with Lys-1 of the mature lipase as the first amino acid and Lys-386 the last) can be overexpressed in *E. coli.* It was purified by immobilized Ni-resin column chromatography and characterized. The effects on activity and specificity of several amino acids around the active site of the recombinant lipase were investigated by site-directed mutagenesis. The results reveal that the amino acid residues following the catalytic triad residues affect the substrate specificity and catalytic specificity.

MATERIALS AND METHODS

Materials. Enzymes for the recombinant DNA experiments were purchased from either Promega Co. (Madison, WI) or B.M. Biochemicals (Mannheim, Germany). Oligonucleotide primers were made by Bio-Synthesis Co. (Taipei, Taiwan), and a Taq DNA polymerase and DNA sequencing kit was obtained from HT Biotechnology Ltd. (Cambridge, England) and U.S. Biochemicals (Cleveland, OH), respectively. A geneclean

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kit was purchased from Bio101 Co. (Vista, CA) and a plasmid DNA purification kit was purchased from Promega Co. Isopropyl thio-β-D-galactoside (IPTG) was obtained from B.M. Biochemicals, and *p*-nitrophenyl butyrate, Fast blue RR salt, α-naphthyl butyrate, and EDTA were purchased from Sigma Co. (St. Louis, MO). Protein molecular weight markers were obtained from Novel Experimental Technology Co. (San Diego, CA). Other chemicals were reagent grade.

Bacterial strains, plasmid, and bacterial growth conditions. Escherichia coli HB101 (14) was provided by Promega Co. *Escherichia coli* BL21(DE3) and plasmid pET-20b(+) were obtained from Novagene Co. (Madison, WI). The bacteria were grown at 37 or 30°C in L-broth (LB) or on LB/1.5% bacto-agar plates. Ampicillin (50 µg/mL final) was added when needed.

Recloning of a fragment of gehC *gene.* A fragment of the *gehC* gene (designated "Lys-303" gene and encoding the amino acid sequence corresponding to that of the lipase following proteolytic cleavage between Ala-302 and Lys-303) was synthesized using chromosomal DNA as template from a *S. epidermidis* isolated from a patient in Chang-Gung Hospital (Keelung, Taiwan) by the polymerase chain reaction (PCR) method (15). An N-terminal primer (5′-GGGGCCATGGAACAAAAACAATATAAAAAT-3′) and a C-terminal primer (5′-GGGGCTCGAGTTTATTGTTGATGTTAATTG) were used, and chromosomal DNA of *S. epidermidis* was the template for the above PCR. The product of the PCR was digested with *Nco*I and *Xho*I, ligated to a 3.7 kbp *Nco*I/*Xho*I-digested pET-20(+) DNA fragment and transformed *E. coli* strain HB101 (Promega) and into *E. coli* BL21(DE3) (Novagen). The DNA sequence of the Lys-303 gene was confirmed (16) with plasmid isolated from BL21(DE3) and was identical with *S. epidermidis* strain 9 lipase gene. All recombinant DNA experiments followed standard protocols (17) or protocols recommended by manufacturers. The expressed lipase contained six additional His residues attached at the C-terminal.

Site-directed mutagenesis. Mutant genes were synthesized from *S. epidermidis* chromosomal DNA by two-step, three-primer PCR (15) in which the N-terminal and C-terminal oligonucleotides were identical to those used for the synthesis of the "Lys-303" gene (12), the oligonucleotides used for the synthesis of mutant genes are listed in Table 1. The products of the PCR were digested with *Nco*I and *Xho*I, and were ligated to a *Nco*I/*Xho*I-predigested pET-20b(+) DNA fragment. The desired ligated products were cloned, first into HB101, then into BL21(DE3). The DNA sequences surrounding mutant codons were determined (16) from plasmids isolated from BL21(DE3).

Protein work. BL21(DE3) cells, harboring the desired wild-type lipase gene (12) on plasmid pET-20b(+), were grown at 30°C in LB/ampicillin to an optical density (600 nm) of 0.5. IPTG was added to the cultures (100 mL each) for a final concentration of 4 mM, and the cells were harvested 3 h after the IPTG induction. For protein purification of the recombinant lipase, the cells [suspended in a buffer of 20 mM Tris/HCl (pH 7.9), 5 mM imidazole, 0.5 M NaCl, and 0.05% Tween 20] were broken on ice in a Microson (Microsonix Inc., New York, NY) Ultrasonic Cell Disruptor (10 × 30 s at 50 W), and after 10,000 × *g* centrifugation, the proteins in the supernatant fraction were separated by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE) (18). The separated proteins were stained with Coomassie Brilliant Blue (19) and assayed for the esterase activity on gels (20) after the removal of SDS (21). Supernatant fractions with positive gel esterase results were loaded into $NiSO_4$-charged His-bind resins (Novagene Co.). After binding, the resins were washed with a buffer [20 mM Tris/HCl (pH 7.9), 60 mM imidazole, 0.5 M NaCl]. The lipase was eluted with another buffer [20 mM Tris/HCl (pH 7.9), 1 M imidazole, 0.5 M NaCl] and dialyzed against a 25 mM phosphate buffer (pH 6.8). Protein concentrations were determined according to a dye-binding procedure (22), using the Bio-Rad protein assay kit system.

Enzyme assay. The purified enzymes and substrate *p*-nitrophenyl butyrate (2.64 mM predissolved in 2.1% Triton X-100) were mixed in 50 mM phosphate buffer (pH 6.0). The reactions were carried out at 37°C for 15 min and were terminated by the addition of acetone (1:1, vol/vol) (23). The absorption values of the reaction product (*p*-nitrophenol) at 346 nm were determined, and the hydrolase activity (a general indicator for the lipolytic activity) of the purified enzymes was obtained, after converting the absorption values into mmols of *p*-nitrophenol quantity (at pH 6.0) curve. The molar extinction coefficient of *p*-nitrophenol, under this condition, was 2988 $M^{-1}\cdot cm^{-1}$.

TABLE 1
Oligonucleotides Used for the PCR-Site-Directed Mutagenesis of the *Staphylococcus epidermidis* Lys-303 Recombinant Lipase Gene[a]

Primers	Oligonucleotides
Wild type	5′-GACCACCCATACTATGACCAAC-3′
S418*C*	5′-ACCCATAC**A**ATGACCAAC-3′
M419*L*	5′-GACCACC**G**A**G**ACTATGACCAAC-3′
M419*A*	5′-GACCACC**GGC**ACTATGACCAAC-3′
M419*Q*	5′-GACCACC**TTG**ACTATGACCAAC-3′
Wild type	5′-GGGATCATGTAGACTTTGTAGG-3′
H648*K*	5′-TGGGAT**A**A**A**GTAGACTTT-3′
V649*L*	5′-GGGATCAT**C**T**C**GACTTTGTAGG-3′
V649*I*	5′-GGGATCAT**A**TCGACTTTGTAGG-3′
V649*A*	5′-GGGATCATG**CT**GACTTTGTAGG-3′

[a]Capital letters and capital italic letters denote wild type and mutant type amino acid, respectively. The mutated nucleotide is shown in bold. PCR, polymerase chain reaction.

RESULTS AND DISCUSSION

The nucleotide sequence of the *gehC* Lys-303 gene fragment, cloned from a strain of *S. epidermidis,* isolated from a patient in Chang-Gung Hospital (Keelung, Taiwan) was identical to the *S. epidermidis* 9 lipase gene reported previously (12). To form a restriction enzyme cleavage site necessary for the cloning, the Lys-303 was changed to Glu. Six histidine residues were added to the C-terminus of the lipase to facili-

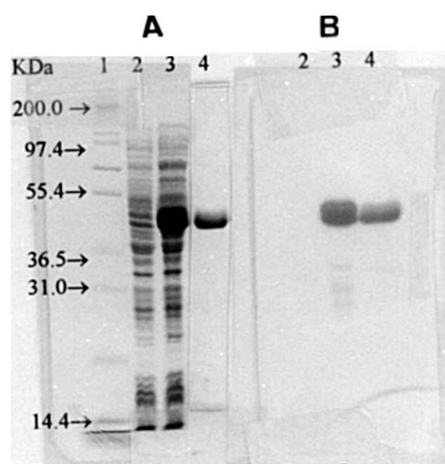

FIG. 1. Expression of the recombinant *Staphylococcus epidermidis* lipase with 6 X His-tail in *Escherichia coli* BL21(DE3). Cells were harvested 4 h after isopropyl thio-β-D-galactoside induction and sonicated. Proteins of the respective cellular extracts were separated by sodium dodecyl sulfate/polyacrylamide gel electrophoresis. Part A, Coomassie Brilliant Blue stained. Part B, esterase activity stained. Lane 1, protein molecular weight markers; lane 2, BL21(DE3) containing pET-20b(+); lane 3, BL21(DE3) containing pET-20b(+) with *S. epidermidis* lipase gene; lane 4, lipase purified with His-Bind resin.

tate enzyme purification. As shown in Figure 1, this slight modification did not retard the overexpression of the lipase gene in *E. coli* BL21(DE3) or destroy the enzyme activity. The purified protein (43 kDa) showed a single major band on SDS/PAGE and on general esterase activity stained gels (Fig. 1). In the previous report, *S. epidermidis* produced a lipase of 97 kDa, as determined by SDS/PAGE, which was degraded by *S. epidermidis* proteolytic activity to a 43 kDa form. Active lipases in the range 97–43 kDa were observed during the purification procedure (12,13). A part of the *S. epidermidis* strain RP62A mature lipase (the first amino acid is Asn-7 and Lys-386 the last) with a N-terminal His-tag has been overexpressed in *E. coli* (13). The amino acid sequence is nearly identical (97.8% identity) to the lipase gene of *S. epidermidis* strain 9 and has Y453→F and E675→D substitutions in the 688 amino acid prolipase (13). In the present work, we showed that a *S. epidermidis* strain 9 full length mature lipase fragment with a C-terminal His-tag could be overexpressed in *E. coli* and purified easily by immobilized Ni-resin column. The purified enzyme was used for the following biochemical studies.

A pH–activity profile for the "Lys-303" enzyme was investigated using *p*-nitrophenyl butyrate as a substrate, and the optimal pH was found to be 6.0 (Table 2). The result is similar to the recombinant lipase of *S. epidermidis* strain RP62A (13). Enzyme activities at various temperatures were analyzed with *p*-nitrophenyl butyrate as substrate, and the lower temperatures were found to be favored, with an optimum at 25°C (Table 3). The ability of the enzyme to hydrolyze *p*-nitrophenyl esters of various chain length was studied. Esters of butyrate and caprate were clearly favored (Table 4). In comparison with the substrate specificity of *S. epidermidis*

TABLE 2
Effect of pH on the Recombinant *Staphylococcus epidermidis* Lipase[a]

pH	Relative activity (%)
3.0	0
4.0	4
5.0	23
6.0	100
7.0	76
8.0	35

[a]The activity was assayed at 37°C using *p*-nitrophenyl butyrate as substrate (2.64 mM) in 50 mM Good's buffer (mixture of BICINE [*N,N*-bis(2-hydroxyethyl)-glycine], CAPS [3-(cyclohexylamino)-1-propane sulfonic acid], sodium acetate and bis-Tris propane, 50 mM each, adjusted to the desired pH by HCl or NaOH). The relative activity at a given pH was calculated by dividing the absolute reaction rate by that at pH 6.0.

strain RP62A lipase, our studies show similar results (13). Finally, kinetic analyses using *p*-nitrophenyl butyrate as substrate showed K_m, k_{cat} and k_{cat}/K_m were 0.90 mM, 25.1 s^{-1} and 28.2 $s^{-1}mM^{-1}$ (Table 5), respectively. In comparison, the K_m, k_{cat} and k_{cat}/K_m of *S. hyicus* lipase were 2.07 mM, 0.53 s^{-1} and 0.257 $s^{-1}mM^{-1}$, respectively (25). The *S. epidermidis* lipase had much higher substrate-binding affinity and catalytic efficiency than the *S. hyicus* lipase.

The putative catalytic triad of *S. epidermidis* lipase was predicted to be Ser-418, Asp-609, and His-648 from sequence comparison with *S. aureus* and *S. hyicus* (12,24). The amino acid residues (Met-419 and Val-649), following catalytic triad, could presumably affect the substrate specificity and catalytic efficiency (26). Site-directed mutagenesis experiments were performed to study the role of these amino acids.

For mutant S418C, no general lipolytic activity was detected on gels following electrophoresis although a predominant overexpressed 43 kDa protein was detected by Coomassie Brilliant Blue staining . Furthermore, no significant levels of esterase activity were detected from S418C ex-

TABLE 3
Effect of Temperature on the Activity of Recombinant *Staphylococcus epidermidis* Lipase[a]

Temperature (°C)	Relative activity (%)
5	31
10	47
15	66
20	87
25	100
30	98
35	77
40	23
45	3
50	2

[a]Lipase activity was monitored by the formation of *p*-nitrophenol from *p*-nitrophenyl butyrate (23). The relative activity at a given temperature was calculated by dividing the absolute rate by 25°C.

TABLE 4
Substrate Specificity of the Recombinant *Staphylococcus epidermidis* Lipase and Mutant Type Lipases[a]

Substrates	Relative activity(%)		
	Wild type	M419A	V649I
p-Nitrophenyl acetate	19	141	322
p-Nitrophenyl butyrate	100	190	550
p-Nitrophenyl caproate	37	145	199
p-Nitrophenyl caprylate	43	155	265
p-Nitrophenyl caprate	74	227	338
p-Nitrophenyl laurate	38	128	114
p-Nitrophenyl myristate	13	40	19
p-Nitrophenyl palmitate	5	11	2
p-Nitrophenyl stearate	8	1	0

[a]Lipase activity was monitored by the formation of *p*-nitrophenol from *p*-nitrophenyl esters (23). Relative activities were calculated by dividing the absolute activities by that of the *p*-nitrophenyl butyrate for wild type (9440 μmol/μg/min). Each data point is the average value of three independent measurements.

tracts, when compared to those of wild type extracts. The results strongly suggested that Ser-418 was a member of the catalytic triad since simply changing the hydroxyl group to a thiol group caused the loss of enzyme activity.

His-648 was predicted to be a component of the catalytic triad. Mutant (H648K) protein expressed in *E. coli* exhibited very low lipolytic activity on gels when compared to that of wild type cells. In comparison with wild type enzyme, kinetic analysis showed little change in the K_m for substrate *p*-nitrophenyl butyrate, but the k_{cat} greatly decreased to 10.4% (Table 5). The results suggested that H648 is critical for catalysis but not for substrate binding and could be a component of the catalytic triad.

The amide NH group of the residue following the catalytic serine has been suggested to be associated with stabilization of the oxyanion that forms the tetrahedral intermediate in the reaction through hydrogen bond formation (27). To study the role of the residue following the catalytic serine, site-directed mutants were constructed. For mutant enzymes M419L and M419Q, no general lipolytic activity was detected on gels. Conversely, the M419A mutant enzyme showed increased lipolytic activity on gels compared with wild-type enzyme. The mutation apparently affected the enzyme's ability to stabilize the transition state more than it affected the substrate binding ability of the enzyme. Kinetic analysis using *p*-nitrophenyl butyrate as substrate (Table 5) showed that in comparison with wild-type enzyme, the M419A increased the catalytic efficiency (k_{cat}/K_m) by 2.0-fold, which was dominated by the k_{cat} effect. The kinetic result suggested that the amino acid residue following the catalytic serine not only was involved in stabilization of the oxyanion hole (as seen by alteration of k_{cat}) but also was an important determinant for substrate binding and specificity (as indicated by the alteration of k_m in the mutants), presumably defined by the side chain. The mutation of Met-419 to Ala significantly broadened the specificity of the enzyme and increased the activity toward larger substrates. As shown in Table 4, the best substrates for wild-type and M419A enzymes were *p*-nitrophenyl butyrate and *p*-nitrophenyl caprate, respectively. Among the *p*-nitrophenyl esters tested, the M419A mutant enzyme showed increased activity against all substrates except *p*-nitrophenyl stearate.

TABLE 5
Kinetic Parameters for Wild Type and Mutants of *Staphylococcus epidermidis* Lipase[a]

Enzyme	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}\cdot mM^{-1}$)
Wild type	0.90 ± 0.08	25.1 ± 0.6	28.2 ± 2.0
M419A	3.97 ± 0.12	217.6 ± 14.0	55.0 ± 2.9
H648K	0.95 ± 0.35	2.6 ± 0.9	3.6 ± 1.3
V649I	3.02 ± 0.21	335.8 ± 15.7	112.1 ± 6.8

[a]Lipase activity was monitored by the formation of *p*-nitrophenol from *p*-nitrophenyl butyrate (23). Data are means ± standard error and were obtained from three experiments.

The amino acid residues following His-648 could presumably affect the substrate specificity and/or catalytic efficiency. To study the role of Val-649, site-directed mutants were constructed. Mutants V649A and V649L lost enzyme activity, while a V649I mutant enzyme showed improvement in the specific activity for the hydrolysis of *p*-nitrophenyl butyrate over that of wild type enzyme (Table 5). In comparison with wild-type enzyme, the V649I enzyme showed a 4.0-fold improvement in the k_{cat}/K_m, and a 13.4-fold increase in k_{cat}, for substrate *p*-nitrophenyl butyrate. These suggest that Val-649 affects enzyme catalysis.

The above facts suggest that the amino acid residues following catalytic Ser-418 significantly influence the substrate specificity and/or catalytic efficiency of the enzyme. In comparison with wild-type enzyme, the M419A mutant enzyme prefer *p*-nitrophenyl caprate (Table 4). The substitution of small side chain in M419A might enlarge the pocket of the catalytic site, enabling the binding and hydrolysis of substrate with longer carbon chain. The active site became more plastic. Furthermore, V649I is better suited for *p*-nitrophenyl caprate, and this phenomenon is likely to be due to a slight increase in the hydrophobicity around the substrate binding site, which may influence certain kinetic behaviors of the lipase. Therefore, Val-649 can play an important role in substrate specificity and can serve as a good candidate for the engineering of enzyme specificity.

Comparison with the amino acid sequences of the other four characterized staphylococcal lipases could further identify amino acids that can play roles in substrate-binding. The amino acid sequence of the *S. epidermidis* lipase is similar to the *S. hyicus* lipase. The C-terminal regions are closely related, with 43% of the 386 residues being identical and 35% being conservative changes (12) The significant difference in biochemical properties between these two enzymes suggests that dissimilar amino acid residues, particularly those around the active site, are important for substrate specificity and enzyme catalytic efficiency. Further protein engineering work is needed to improve enzyme activity and biochemical properties. Our studies also showed that the recombinant 43 kDa lipase could be expressed and purified easily. This success will

overcome a time-consuming, multi-step purification problem.

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