JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. 2010, 58, 10899–10905 10899
DOI:10.1021/jf1004034

Site-Specific Saturation Mutagenesis on Residues 132 and 450 of *Candida rugosa* LIP2 Enhances Catalytic Efficiency and Alters Substrate Specificity in Various Chain Lengths of Triglycerides and Esters

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The catalytic versatility of recombinant *Candida rugosa* LIP2 has been known to have potential applications in industry. In this study, site-specific saturation mutagenesis on residues L132 and G450 of recombinant LIP2 has been employed to investigate the impact of both residues on substrate specificity of LIP2. Point mutations on L132 and G450 were done separately using mutagenic degenerate primer sets containing 32 codons to generate two libraries of mutants in *Pichia pastoris*. Replacements of amino acid on these mutants were identified as L132A, L132I, G450S, and G450A. In lipase activity assay, L132A and L132I mutants showed a shift of preference from short- to medium-chain triglyceride, whereas G450S and G450A mutants retained preferences as compared to wild-type LIP2. Among mutants, G450A has the highest activity on tributyrin. However, hydrolysis of *p*-nitrophenyl (*p*-NP) esters with L132A, L132I, and G450S did not show differences of preferences over medium- to long-chain esters except in G450A, which prefers only medium-chain ester as compared to wild-type LIP2. All mutants showed an enhanced catalytic activity and higher optimal temperature and pH stability as compared to wild-type LIP2.

KEYWORDS: *Candida rugosa* LIP2; *Pichia pastoris*; site-specific saturation mutagenesis; mutants; substrate specificity

INTRODUCTION

For over 150 years, lipases have been extracted, purified, and characterized. Lipases belong to a class of biocatalysts with an unsurpassable role in swiftly growing biotechnology and have remarkable ability to carry out novel reactions in both aqueous and nonaqueous media (*1*). These enzymes are involved in a wide variety of reactions on fatty acids, alcohols, or esters for foods, flavors, fragrances, and pharmaceuticals (*2*). The progress at this stage in producing lipases is largely dependent on the development of molecular biology tools.

Among many lipase-producing types of yeast, *Candida rugosa* (formerly *Candida cylindracea*) is most frequently used as a commercial source of lipase and has been extensively studied for wide biotechnological applications because of its high catalytic activity, stereoselectivity, and regioselectivity in both hydrolysis and synthesis (*3*). *C. rugosa* lipase (CRL) is a mixture of several lipase isoforms. LIP1−LIP7 share 84% homology in amino acid sequences. They differ in their N-glycosylation sites, isoelectric points, and some local features of their hydrophobicity profiles (*4*, *5*). These lipase isoforms consist of 534 amino acids and have an average molecular weight of 60 kDa. Each *C. rugosa* lipase isoform has different substrate specificity, thermal stability during biocatalytic activity, and stereoselectivity (*6*−*9*). These characteristics lead to divergent catalytic performances (*8*) and remarkable variations of catalytic efficiency in most applications (*10*).

Our laboratory is continuing the use of recombinant CRL isoforms to characterize and improve catalytic performance by directed evolution. Among CRL isoforms, LIP2 displayed high preference for short-chain fatty acid, high activity toward cholesteryl esters, and long-chain alcohols in esterification (*11*). Previous study showed that native LIP2 expression in *C. rugosa* was promoted by both olive oil and oleic acid even in the presence of repressing sugar in a culture medium optimization experiment (*12*). In a swap-flap study of CRL, the chimera Trx-LIP4/lid2 increased the hydrolysis of short-chain fatty acid (*13*). Additionally, a successful conversion of the 17 nonuniversal serine codons (CTG) in *C. rugosa* LIP2 gene into universal serine codons (TCT) was done

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Published on Web 09/27/2010

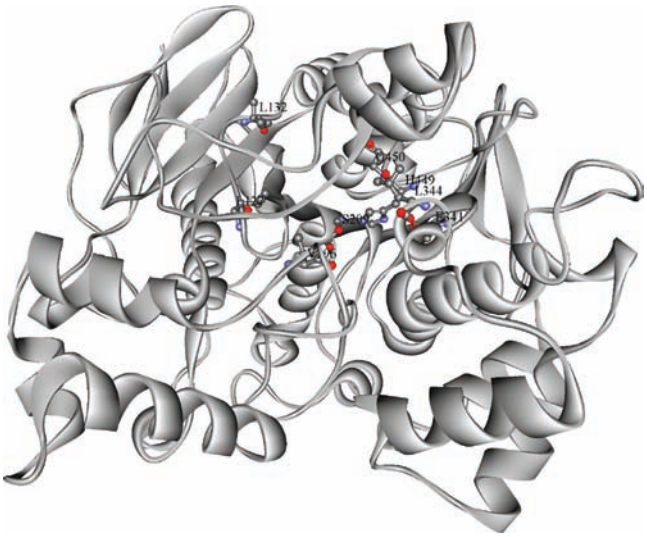

Figure 1. Molecular structure of *Candida rugosa* LIP2. In colored ball and stick form amino acids are L132, L127, G450, S209, V296, L344, E341, and H449, whereas the rest of the protein is in grayscaled solid ribbon.

by overlap extension PCR-based multiple mutagenesis (*14*). The crystal structure of closed confirmation of *C. rugosa* LIP2 revealed several amino acid changes in the flap or lid at residue 69, the substrate-binding site at residues 127, 132, and 450, and the cavity of the hydrophobic tunnel at residues 296 and 344 as compared to LIP1 and LIP3 (*15*). The substrate-binding site is a hydrophobic pocket, and the location of these residues is identified in previous studies to be responsible for the differences of substrate specificity among lipase isoforms. These amino acid residues are important factors in the variation of the catalytic activity of LIP2.

The advent of recombinant DNA biotechnology revolutionizes the study and strategy to alter the activity, specificity, and stability of enzymes for certain applications. One of the techniques uses directed evolution, which accelerates and mimics natural recombination events. Previously, we reported that site-specific saturation mutagenesis at two residues of recombinant LIP4 alters its substrate specificity. The effects of alteration on the substrate specificity of *C. rugosa* LIP4 was observed in mutants with improved hydrolytic activity of triglycerides toward short, medium, and long chains (*16*). LIP2 has great potential for various applications and unique properties. We have sought to perform site-directed mutagenesis at L132 and G450, two important residues on the substrate-binding site (**Figure 1**). We found that the mutants of LIP2 at these residues showed properties remarkably different from those of wild-type LIP2.

EXPERIMENTAL PROCEDURES

Materials and Strains. *Pichia pastoris* SMD1168H, *Escherichia coli* TOP10F, expression vector pGAPZαC, and Zeocin were from Invitrogen (Carlsbad, CA). Plasmid and DNA extraction system kits were supplied by Viogene (Taiwan). The substrates (*p*-nitrophenyl esters and triglycerides) were purchased from Sigma-Aldrich-Fluka (St. Louis, MO). Primer oligonucleotides were produced by GENSET Singapore Biotech Pte Ltd. Enzymes for the recombinant DNA experiments were purchased from Promega Co. (Madison, WI). DNA purification kits were from Qiagen Co. (Madison, WI). Protein molecular mass markers were obtained from Bio-Rad (Richmond, CA). DNA sequencing kit was purchased from Perkin-Elmer Cetus (Wellesley, MA). All common reagents and buffers were obtained from Sigma or Aldrich.

Construction of Randomly Mutated Expression, pGAPZαC-*mlip2*. The construction of the expression vector of recombinant wild-type LIP2 (pGAPαC-*lip2*) was done using the procedure previously described (*17*). This expression vector pGAPZαC-*lip2* was transformed into *E. coli* TOP10F as a host for cloning and grown in low-salt Luria−Bertani (LB) broth with Zeocin (25 mg/mL) for 16 h. The plasmids were extracted and used as template for overlap-extension PCR in saturation mutagenesis of the two important amino acids (L132 and G450) in substrate-binding site as selected (*16*).

Table 1. Mutagenic Primers Used for the Saturation Mutagenesis of the *lip2* Gene[a]

primer	position of mutagenesis	oligonucleotide sequence (5′ to 3′)
2-N		CGGAATTCCGTACCCACCGCCACGCTCGCCAACGGC
2-C		GCTCTAGAAACTACACAAAGAAAGACGGCGGGTT
2-1F	132	GGC GGT GGG TTT GAG CTT GGC GGC TCC AGC **NNK** TTT CCA GGA GAC
2-1R	132	GTC TCC TGG AAA **MNN** GCT GGA GCC GCC TTG CTC AAA CCC ACC GCC
2-2F	450	GGC ACC TTC CAC **NNK** AAC GAC ATC ATC TGG CAG GAC
2-2R	450	GTC CTG CCA GAT GAT GTC GTT **MNN** GTG GAA GGT GCC

[a] The primers 2-N, 2-1F, and 2-2F were used as forward primers, and the primers 2-C, 2-1R, and 2-2R were used as reverse primers. The positions of saturation mutagenesis are in bold. The created restriction sites (*Eco*RI in 2-N and *Xba*I in 2-C) are underlined. The nucleic acid codes are M for A or C residue, K for G or T residue, and N for A, G, C, or T residue.

The construction of randomly mutated LIP2 was performed using the degenerate mutagenic primers set shown in **Table 1** and two rounds of PCR using GeneAmp PCR System 9700 by Applied Biosystem as previously described (*16*). The secondary PCR products were digested with *Eco*RI and *Xba*I and ligated into pGAPZαC to generate the expression vectors of L132 mutants (pGAPZαC-*mlip2*-L132) and the expression vectors of G450 mutants (pGAPZαC-*mlip2*-G450).

Transformation, Expression, and Screening of *P. pastoris*. Ten micrograms of each constructs (pGAPZαC-*lip2*, pGAPZαC-*mlip2*-L132, and pGAPZαC-*mlip2*-G450) quantified by UV absorbance was linearized with *Avr*II and transformed into *P. pastoris* SMD1168H by electroporation at 1.5 kV, 25 μF, and 400 Ω using a Gene Pulser apparatus supplied with Pulse Controller (Bio-Rad). Mutants were incubated on YPDS (1% yeast extract, 2% peptone, 2% dextrose, 1 M sorbitol, and 2% agar, pH 7.2) plates containing Zeocin (100 μg/mL). Individual colonies were picked and patched on emulsion YPD plates containing 1% tributyrin (C_4) or trilaurin (C_{12}). Both substrates were used to monitor the screening because wild-type LIP2 prefers short to medium fatty acyl chain hydrolysis. The clear zone of lipase-secreting *P. pastoris* mutants was compared to SMD1168H with pGAPZαC empty vector as a negative control. The selected mutants were cultured in YPD, and the mutated LIP2 DNAs were sequenced from genomic DNA by *lip2*-specific PCR to identify the replacements.

Purification of Wild-Type and Mutants of LIP2. In a shaking flask culture, the selected *P. pastoris* mutants were grown in a 2000 mL flask containing 1000 mL of YPD medium with Zeocin (500 μg/mL) at 30 °C for 3 days. The extraction and purification steps were performed as previously described (*16*, *18*). The culture medium was collected and concentrated by ultrafiltration on the Labscale TFF System with Pellicon XL devices coupling Biomax-30 membranes (Millipore, Bedford, MA). The filtrate was then applied onto a pre-equilibrated TE buffer (20 mM Tris-HCl, 2 mM EDTA, pH 7.0) HiPrep 16/10 Octyl FF column (Amersham Pharmacia Biotech Inc., Piscataway, NJ). The column was washed with 5 column volumes of TE buffer with 4 mM CHAPS, and the bound proteins were then eluted with 5 column volumes of TE buffer with 30 mM CHAPS. The eluted protein was dialyzed against TE buffer, and the dialyzed protein was applied to a HiPrep 16/10 Q XL column (Amersham Pharmacia Biotech) equilibrated with TE buffer. The proteins were eluted using a linear gradient concentration of $(NH_4)_2SO_4$ (0−300 mM) in 5 column-bed volumes. Fractions were collected, and protein concentration was determined using the Bio- Rad protein assay kit. The enzyme activity was measured using *p*-NP butyrate as a substrate (as described in the next section). The molecular masses of the purified recombinant lipases were determined in denaturing condition by SDS-PAGE. Purified protein was stored in a storage buffer (60 mM KCl, 10 mM Tris-HCl, 1.25 mM EDTA, 1% Triton X-100, and 17% glycerol, pH 7.5) at −20 °C.

Table 2. Substrate Specificities of the Wild-Type (WT) and Mutants of LIP2 (L132A, L132I, G450S, and G450A) with Triglycerides of Various Chain-Length Fatty Acids

		specific activity[a] (units/mg)				
substrate	no. of C atoms in fatty acid chain	WT	L132A	L132I	G450S	G450A
tributyrin	4	1027 ± 46 (100%)	1611 ± 96 (15%)	939 ± 52 (52%)	714 ± 0 (100%)	5000 ± 0 (100%)
tricaprylin	8	487 ± 53 (47%)	1167 ± 235 (11%)	969 ± 104 (53%)	476 ± 41 (67%)	2833 ± 288 (57%)
trilaurin	12	475 ± 86 (46%)	10833 ± 721 (100%)	1818 ± 157 (100%)	369 ± 41 (52%)	1500 ± 433 (30%)
tripalmitin	16	32 ± 2 (3%)	275 ± 43 (3%)	163 ± 23 (9%)	6 ± 1 (1%)	143 ± 11 (3%)

[a] Unit definition: 1 unit of activity is the amount of enzyme necessary to hydrolyze 1.0 μmol of ester bond per minute at 37 °C and pH 7.0. Values are means ± SD from three independent experiments.

Table 3. Substrate Specificities of the Wild-Type (WT) and Mutants of LIP2 (L132A, L132I, G450S, and G450A) with *p*-Nitrophenyl (*p*-NP) Esters of Various Chain-Length Fatty Acids

		specific activity[a] (units/mg)				
substrate	no. of C atoms in fatty acid chain	WT	L132A	L132I	G450S	G450A
p-NP butyrate	4	2118 ± 24 (74%)	8328 ± 466 (43%)	3250 ± 61 (58%)	2148 ± 10 (44%)	25548 ± 2637 (40%)
p-NP caproate	6	109 ± 38 (4%)	368 ± 11 (2%)	99 ± 1 (2%)	89 ± 9 (2%)	1229 ± 79 (2%)
p-NP caprylate	8	992 ± 37 (35%)	743 ± 25 (4%)	3021 ± 34 (54%)	2042 ± 39 (42%)	25052 ± 119 (39%)
p-NP caprate	10	1492 ± 205 (52%)	6141 ± 796 (31%)	1546 ± 16 (28%)	1600 ± 224 (33%)	19185 ± 970 (30%)
p-NP laurate	12	2679 ± 689 (94%)	17952 ± 15 (92%)	4731 ± 873 (84%)	4917 ± 40 (100%)	63481 ± 450 (100%)
p-NP myristate	14	2664 ± 262 (94%)	15717 ± 1351 (80%)	4171 ± 240 (74%)	3667 ± 600 (75%)	24727 ± 819 (39%)
p-NP palmitate	16	2845 ± 51 (100%)	19565 ± 1457 (100%)	5605 ± 88 (100%)	4602 ± 36 (94%)	33775 ± 1130 (53%)
p-NP stearate	18	1588 ± 76 (56%)	8678 ± 632 (44%)	2360 ± 75 (42%)	2346 ± 64 (48%)	18271 ± 32 (29%)

[a] Unit definition: 1 unit of activity is the amount of enzyme necessary to hydrolyze 1.0 μmol of *p*-NP ester per minute at 37 °C and pH 7.0. Values are means ± SD from three independent experiments. Relative activities (percent) of each enzyme are given in parentheses, with the highest activity denoted as 100%.

Substrate Specificity Assay. The lipase activity was assayed by a Hitachi U-2010 spectrophotometer as previously described (*16*). The hydrolysis of *p*-NP esters was carried out at 37 °C in 500 μL of 50 mM sodium phosphate buffer (pH 7.0) containing 0.5% Triton X-100 and 5 mM solution of the corresponding *p*-NP ester. The increase in absorbance was recorded for 10 min at 348 nm (isosbestic point of the *p*-nitrophenol/*p*-nitrophenoxide couple). One unit of activity was defined as the quantity of enzyme necessary to release 1 μmol of *p*-nitrophenol per minute under the above conditions. The lipolytic activity was evaluated titrimetrically using triglycerides with fatty acids of various chain lengths as substrates. The release of nonesterified fatty acids was monitored continuously by a pH-stat (Radiometer Copenhagen, Bagsvaerd, Denmark) during titration with 50 mM NaOH. The emulsification reagent contained NaCl (17.9 g), KH_2PO_4 (0.41 g), glycerol (540 mL), gum arabic (10 g), and distilled water in 1 L. Each lipolytic activity assay was carried out in a 37 °C thermostat reaction vessel containing 2.5 mL of 40 mM triglyceride substrate emulsion, 6.5 mL of distilled water, and 1 mL of the enzyme solution. One unit of lipolytic activity was defined as the amount of lipase necessary to produce 1 μmol of fatty acid per minute under the assay conditions.

Effects of pH and Temperature on Enzyme Activity and Stability. Temperature and pH effects were assessed using *p*-NP butyrate as a substrate. The optimal temperature for the esterase reaction was investigated in the range of 20−90 °C at pH 7.0. The optimal pH was investigated in the pH range of 3.0−9.0 using Good's buffer (50 mM each of Bicine, CAPS, sodium acetate, and BisTris propane). To analyze thermal stability, the lipase was incubated for 10 min at various temperatures in the range of 37−90 °C. Residual activity was determined spectrophotometrically at 37 °C and pH 7.0 using *p*-NP butyrate as a substrate.

Molecular Modeling of Wild-Type and Mutants of LIP2. The structure of *C. rugosa* LIP2 with accession no. 1GZ7 from Protein Data Bank (http://www.ncbi.nlm.nih.gov/protein) was retrieved and used to generate mutants and understand enzyme−substrate interactions in silico. The mutation, superimposition, and docking of substrates were done according to the tools and protocols available in Discovery Studio 2.5.5 (Accelrys).

RESULTS

Screening of the Mutants of LIP2 in *P. pastoris*. Positive clones of *P. pastoris* SMD1168H colonies containing pGAPZαC-*mlip2*-L132 or -G450 were picked from YPDS-Zeocin (100 μg/mL) plates and patched on 1% tributyrin or trilaurin emulsion YPD plates to identify the lipase-secreting mutants. Over 400 colonies from each mutant pool were screened by their clear zone on the opaque emulsion. Four colonies with significant clear zones as compared to pGAPZαC-*lip2* mutants were isolated.

The four colonies revealed four different amino acid replacements as confirmed by the DNA sequence analysis. The mutants were identified as L132A, L132I, G450S, and G450A on the basis of the amino acid replacements.

Substrate Specificities of the Recombinant Wild-Type and Mutants of LIP2. The four mutants were collected and cultured in 1000 mL of YPD-Zeocin (500 μg/mL) medium, and the protein was extracted and purified. Lipase activity was measured, and the preferences for triglycerides containing fatty acids of various chain lengths are shown in **Table 2**. The four mutants displayed a trend in preference for various fatty acid chain lengths as compared to the wild-type LIP2. The wild-type LIP2 preferentially catalyzed hydrolysis of short-chain triglyceride (tributyrin) among various lengths of substrate. Mutants L132A, G450A, and L132I (except in tributyrin, C_4) showed an increase of activity in all substrates as compared to wild-type LIP2. When L132 was replaced with Ala and Ile (L132A and L132I), the mutants displayed a shift of preference from short- to medium-chain triglyceride. Between these two mutants, L132A has the highest activity toward medium-chain triglyceride (trilaurin, C_{12}) and long-chain triglyceride (tripalmitin, C_{16}) by 23- and 9-fold relative to wild-type LIP2, respectively. Mutant lipases G450S and G450A retained a preference for short-chain triglyceride, a characteristic similar to that of wild-type LIP2. In addition, G450A showed the highest activity short-chain triglyceride of any other mutants or the wild-type LIP2 by a 5-fold increase.

Esterase activity was measured through hydrolysis of *p*-NP esters containing fatty acids of various chain lengths, and the result is shown in **Table 3**. Wild-type LIP2 showed a preference toward medium- to long-chain ester substrates (C_{12}−C_{16}). L132A and L132I displayed a similar preference as wild-type LIP2. These

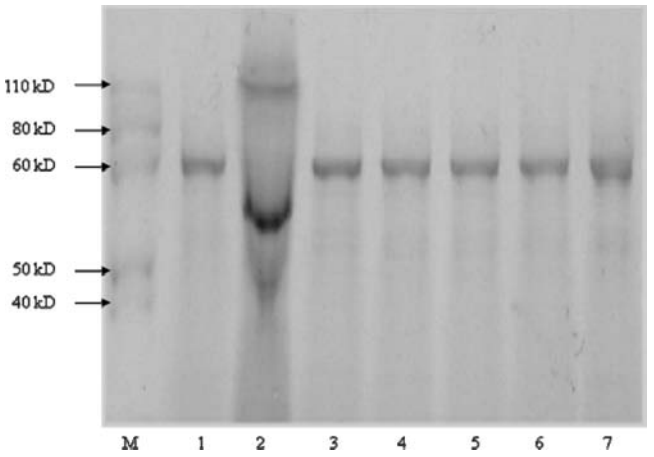

Figure 2. SDS-PAGE (12%) of the wild-type and mutants of LIP2 obtained during purification. Proteins were stained with Coomassie blue. Lane M, marker proteins with relative molecular masses in kDa indicated on the right. Lane 1, commercial lipase from Sigma (L-1745); lane 2, crude enzyme from wild-type LIP2; lane 3, purified wild-type LIP2; lane 4, purified L132A; lane 5, purified L132I; lane 6, purified G450S; lane 7, purified G450A.

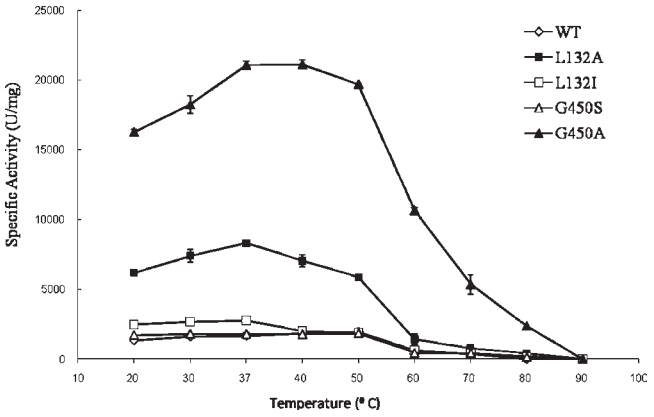

Figure 3. Temperature effect on the wild-type and mutants of LIP2 (L132A, L132I, G450S, and G450A). The activity was measured by spectrophotometric method using *p*-NP butyrate as substrate at pH 7.0. The actual protein concentrations of each sample were 0.10 μg/μL (WT), 0.12 μg/μL (L132A), 0.22 μg/μL (L132I), 0.28 μg/μL (G450S), and 0.04 μg/μL (G450A).

mutants showed an enhanced activity in C_{12}, C_{14}, and C_{16} substrates over wild-type LIP2. A change of preference in G450A was revealed by decrease of activity toward long-chain esters (C_{14} and C_{16}). However, G450A still significantly preferred medium-chain-length ester (C_{12}). Although a shift of preference was observed in G450A, the mutant showed a superior enhanced activity in all ester substrates as compared to wild-type LIP2 and other mutants.

Purification of Wild-Type and Mutants of LIP2. The extracellular wild-type and mutants of LIP2 produced by *P. pastoris* SMD1168H were purified from the supernatant of the cell culture by ultrafiltration, Octyl Sepharose Fast Flow, and Q Sepharose anion exchange column chromatography. The purified wild-type and mutants of LIP2 have an average molecular weight of 60 kDa (**Figure 2**). This result is consistent with CRL isoforms reported in previous studies.

Characterization of the Recombinant Wild-Type and Mutants of LIP2. The effects of temperature on wild-type LIP2, L132A, L132I, G450S, and G450A are depicted in **Figure 3**. The optimal temperatures for L132A, L132I, and G450A while using *p*-NP butyrate as substrate were 37, 37, and 40 °C, respectively. Throughout

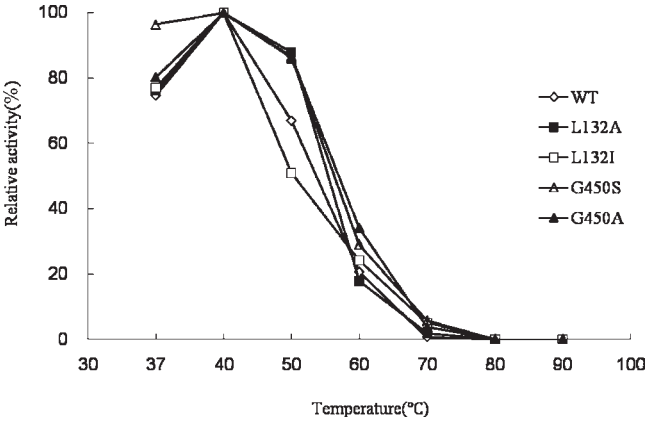

Figure 4. Thermal stability of the wild-type and mutants of LIP2 (L132A, L132I, G450S, and G450A). The residual activities after 10 min of incubation at different temperatures were measured by spectrophotometric method using *p*-NP butyrate as substrate at 37 °C and pH 7.0. The actual protein concentrations of each sample were 0.10 μg/μL (WT), 0.12 μg/μL (L132A), 0.22 μg/μL (L132I), 0.28 μg/μL (G450S), and 0.04 μg/μL (G450A). The 100% activities of the wild-type LIP2 (WT), L132A, L132I, G450S, and G450A were 5506, 14892, 4721, 5207, and 65634 units/mg, respectively.

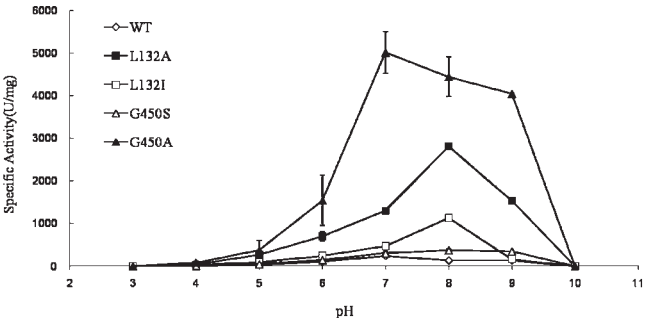

Figure 5. pH effect on the wild-type and mutants of LIP2 (L132A, L132I, G450S, and G450A). The activity was measured by spectrophotometric method using *p*-NP butyrate as substrate at 37 °C. The actual protein concentrations of each sample were 0.10 μg/μL (WT), 0.12 μg/μL (L132A), 0.22 μg/μL (L132I), 0.28 μg/μL (G450S), and 0.04 μg/μL (G450A).

the temperature range, G450A and L132A demonstrated the highest specific activity as compared with other mutants and wild-type LIP2. The two mutants shared a similar curve pattern but differed in the strengths of their specific activities. In addition, G450A displayed a 3- and 20-fold higher activities than L132A and wild-type LIP2, respectively.

The thermal stability in terms of residual activities of wild-type LIP2, L132A, L132I, G450S, and G450A after being subjected at different temperatures for 10 min was assayed (**Figure 4**). The relative activity of all enzymes was highest at 40 °C, but as the temperature increased, the relative activity of the enzymes started to drop. A comparison among enzymes' thermostability at 50 °C showed mutants L132A and G450A to be more stable than wild-type LIP2.

The pH dependences of wild-type LIP2, L132A, L132I, G450S, and G450A were characterized using *p*-NP butyrate substrate at 37 °C as shown in **Figure 5**. The curve pattern of the mutants was similar but differed slightly in the optimum pH. The optimal pH value for L132A and L132I was found at 8.0, whereas that of G450A was at 7.0. In comparison with the wild-type LIP2 and mutants, G450A again showed the highest specific activity among the other mutants at all pH values tested. Significant

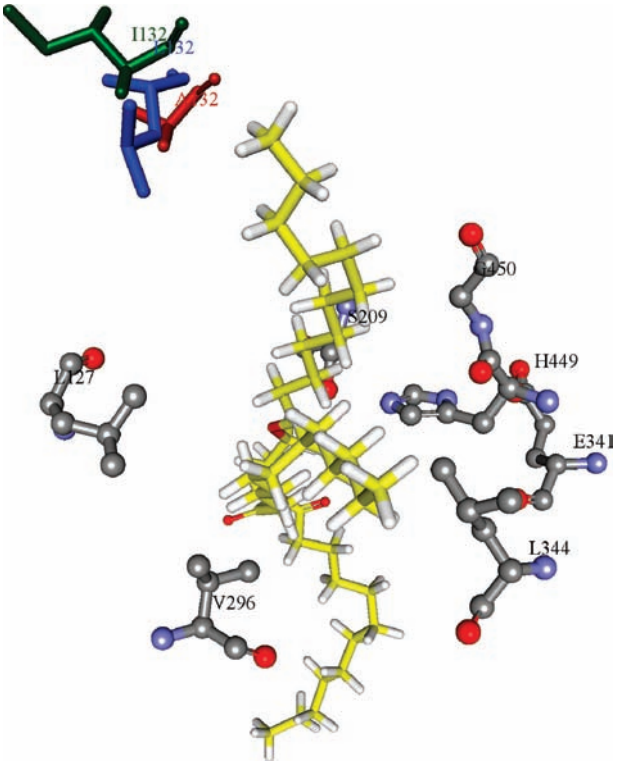

Figure 6. Molecular model of superimposed structures of wild-type and mutants of LIP2 (L132I and L132A) with trilaurin docked. In colored stick form molecules: trilaurin, yellow; L132 (wild-type), blue; L132A, red; and L132I, green.

differences of the specific activity between G450A and wild-type LIP2 were observed at pH 7.0 (16-fold), pH 8.0 (12-fold), and pH 9.0 (11-fold).

DISCUSSION

We have performed site-specific saturation mutagenesis of LIP4 at residues A296 and V344 and observed the mutants have significant change in catalytic activity, thermal stability, and substrate chain-length specificity from those of wild-type LIP4. In this paper, the same experimental methodology was used to examine LIP2. Two important amino acid residues (L132 and G450) located at the substrate-binding site were considered for directed evolution to study their impact on catalytic efficiency and substrate specificity of *C. rugosa* LIP2.

In the previous work, a comparison of pure recombinant LIP4 and LIP2 revealed that recombinant LIP2 holds a unique and remarkable catalytic property over LIP4. LIP2 has a high specific activity toward cholesteryl esters and high activity toward long-chain alcohols in the esterification of myristic acid and shows a preference for short-chain fatty acids such as butyric acid in the esterification of propanol with various acyl chain lengths (*11*). These characteristics are attributed to the key amino acids located at the flap/lid, the mouth of the hydrophobic tunnel, and the substrate-binding site. Residues L132 and G450 are identified as important amino acids in the substrate-binding site that may be responsible for the versatility of the enzyme's activity (*15*). In this study, wild-type LIP2 preferentially catalyzed hydrolysis of short-chain triglyceride (tributyrin) and medium- to long-chain *p*-NP esters (C_{12}, C_{14}, and C_{16}). The mutation of residues L132 and G450 resulted in new properties. In L132A and L132I, the mutants shifted their specificity from short- to medium-chain triglycerides and maintained their preference for *p*-NP esters as compared to

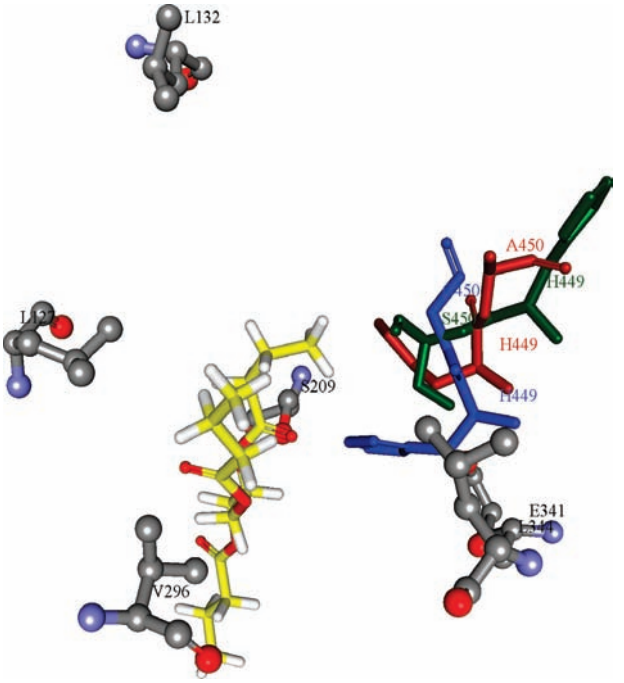

Figure 7. Molecular model of superimposed structures of wild-type and mutants of LIP2 (G450S and G450A) with tributyrin docked. In colored stick form: tributyrin, yellow; G450 (wild-type), blue; G450A, red; and G450S, green.

wild-type LIP2. The other mutants, G450S and G450A, displayed preferences similar to those of wild-type LIP2 in the hydrolysis of triglyceride, but different preferences in *p*-NP esters. In addition, all mutants displayed an enhanced activity in all tested substrates except for G450S (in triglyceride).

The shift of preference observed in mutant L132A can be explained by its side-chain differences of the amino acid. Both Leu and Ala are hydrophobic by nature, but the latter has a smaller side chain compared to the former. The replacement of the small side-chain group at residue 132 may be enhanced to accommodate medium- to long-chain triglycerides in the substrate-binding site region as depicted in **Figure 6**. This preference was also observed in the hydrolysis of esters of L132A. In L132I mutant, a less significant increase in specific activity was observed in all substrates as compared to L132A. Amino acids, Leu and Ile, have the same number of carbons in the side chain but differ in stereochemistry as seen in **Figure 6**. The difference of stereochemistry of Leu and Ile at position 132 may result in a different activity between mutant and wild-type. The presence of the methyl group as substituent in a compound usually increases its lipophilicity and reduces its solubility in water. However, the presence of these ethyl−methyl groups in the side chain of isoleucine could enhance its affinity toward medium- to long-chain fatty acids, although L132A and L132I did not change the chain-length specificity of esterase activity from medium- to long-chain fatty acid esters but showed increase in specific activity as compared to wild-type LIP2. Another interesting finding from superimposed structures of L132A, L132I, and wild-type LIP2 in silico is the same orientation of oxygen (carbonyl structure) of the mutants pointing toward the substrate (**Figure 6**). This electronegative oxygen can react with an electrophile such as unsaturated fatty acid, which may prefer to hydrolyze medium- to long-chain triglycerides, but the mechanism is unclear. The replacement of G450 by hydrophobic Ala, the same residue for other CRL isoforms at the same position (*16*), might increase the hydrophobic interaction with the triglycerides resulting in improved catalytic activity toward triglycerides. However, the replacement of G450 by a hydrophilic

Ser decreases the activity toward all substrates. The nature of Gly residue on 450 may fit a hydrophilic and hydrophobic environment because of its single hydrogen side chain. Aside from that, lipases' catalytic triad is conserved in Ser-209, His-449, and Glu-341 in which the Gly-450 keeps the formation of the triad functional (*15*). Hence, the catalytic activity of G450S on triglyceride is not only affected by hydrophilic Ser but also might be affected by the opposing interaction of side chains from His-449 and Ser-450 as shown in **Figure 7**. However, this observation did not change the original preference of wild-type LIP2 for esters in G450S.

In addition, the optimal temperature and pH of G450A and L132A were remarkably higher compared to those of wild-type LIP2. Temperature and pH are two of several factors that determine enzyme catalytic efficiency. At elevated temperature many enzymes are inactivated or denatured. There are several hypotheses of thermal stability of the enzyme derived from molecular dynamics and pyrolysis studies. One possible explanation is the rigidity of the protein structure,which minimizes the effects of thermal fluctuation. The protein dissipates a large amount of energy without affecting its tertiary structure (*19*). It is also hypothesized that enzyme stability is related to its specific amino acid sequence (*20*), and mutation of the enzyme affects the stability if the affected regions trigger irreversible inactivation process (*21*). Interestingly, we obtained mutants G450A and L132A that have a common amino acid replacement, which is Ala. In thermal decomposition studies, Ala shows the highest temperature range of decomposition as compared to Gly (*22*). The melting point of the amino acids involved in G450A and L132A is as follows: Ala (mp 297 °C), Leu (mp 288 °C), and Gly (mp 262 °C). Aside from that, Ala is also a nonreactive amino acid because of its methyl side chain, whereas Gly and Leu can interact with water. The water−lipid interface also increases the lipolytic activity of lipase. Water acquires and maintains the environment of the enzyme for catalytic activity. However, at different temperature and pH the dynamics of these factors are disturbed. The mechanism for water−amino acid of the enzyme interactions at various temperatures and pH values and the melting point of the studied amino acid related to thermal stability are still unclear. Apparently, there is scarce information to explain the high residual activity after the mutants subjected to various temperatures and pH values, and this could be a good area for further research.

Point mutation is usually adequate to find a change in catalytic performance of an enzyme (*23*, *24*) and, sometimes, combinations of mutations at different sites (*25*, *26*) or focused random mutation of a pair of amino acids in the enzyme's substrate-binding site can provide further improvement (*27*). In this case, the mutants of LIP2 through single-point mutation sufficiently showed an enhanced catalytic activity and substrate specificity as compared to wild-type LIP2. For instance, the G450A mutant showed a preference for short-chain fatty acids such as butyric acid and medium-chain fatty acids such as caprylic acid. This mutant could be used for the hydrolysis of short-chain fatty acid in ripening of cheese for flavor balance (*28*, *29*) and production of food-grade caprylic acid as supplements (*30*). Aside from that, the L132A mutant can also be a potential enzyme for oleochemical industries because it has a high preference for lauric acid (*31*). With these remarkable results, mutants of LIP2 can be a powerful enzyme for future applications in research or industry.

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Received for review February 6, 2010. Revised manuscript received August 6, 2010. Accepted September 13, 2010.