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Altering the Substrate Specificity of *Candida rugosa* LIP4 by Engineering the Substrate-Binding Sites

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Candida rugosa (formerly *Candida cylindracea*) lipase (CRL) is an important industrial enzyme that is widely used in biotechnological applications such as the production of fatty acids and the synthesis of various esters. CRL comprises at least seven isozymes (LIP1−LIP7), which share a similar amino acid sequence but with different specificities for substrates. Previously, LIP4 was reported to have higher esterase activity toward long acyl-chain ester and lower lipase activity toward triglycerides. A296 and V344 of LIP4 were predicted to play decisive roles in its substrate specificity. In this study, site-specific saturation mutagenesis has been employed to study the substrate specificity of LIP4. Point mutations were separately introduced into A296 and V344 positions using degenerate primer sets containing 32 codons to generate two libraries of variants. LIP4 variants were heterologously expressed in the yeast *Pichia pastoris*. A specific plate assay was used to identify lipase-producing *P. pastoris* clones in a medium containing tributyrin. LIP4 variants with high activity toward short fatty acyl-chain triglyceride (tributyrin) were screened. Specificity analysis and biochemical characterization indicated that the recombinant variants A296I, V344Q, and V344H had properties remarkably different from those of wild-type LIP4. All three variant enzymes had significantly higher specific activities toward tributyrin than LIP4. In addition to short-chain triglyceride, A296I and V344Q also improved hydrolytic activities of triglycerides toward medium- and long-chain triglycerides tested. The results suggested that A296 played an important role in lipase activity and high-temperature dependence of LIP4, whereas it had no effect on the chain-length specificity in lipolytic reaction. The V344 residue had a significant effect on the substrate chain-length specificity of LIP4.

KEYWORDS: ***Candida rugosa*; lipase; saturation mutagenesis; substrate specificity; *Pichia pastoris***

INTRODUCTION

Candida rugosa (formerly *Candida cylindracea*) lipase (CRL) is an important industrial enzyme that is widely used in biotechnological applications such as the production of fatty acids and the synthesis of various esters (*1*, *2*). It possesses stereoselectivity and regioselectivity in the synthesis of pharmaceuticals, glycoderivatives, and carbohydrate esters used in the manufacture of many foods and fragrances (*2*). Because the individual lipase isoforms are not isolated and purified before most applications, the native or wild-type enzyme exhibits a wide range of substrate specificities, acyl chain-length specificities, and stereoselectivities. However, crude enzyme preparations obtained from the various commercial suppliers exhibit remarkable variation in their catalytic efficiency and stereospecificity (*3*). We have observed variations in the protein composition of three commercial preparations of CRL that caused the essential differences in catalytic efficiency, substrate specificity, and thermostability (*4*). The purified isoenzymes have been reported

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to display different substrate specificities and thermal stabilities (*5*−*7*). Several CRL genes have been cloned (*8*−*10*). Proteins deduced from the DNA sequences showed high similarity (84%) in amino acid sequence across the entire protein family (*11*).

Separation of CRL isoenzymes is highly desirable to allow their use under well-defined conditions. However, high identity in their protein sequences causes similarities in the physical properties of the lipases that create technical difficulties in the isolation of individual isoenzymes from cultures of *C. rugosa* on a preparative scale for industrial applications. In addition, the purification procedure may affect the properties of different isoforms (*12*, *13*), and the differential expression level of the five lipase genes makes it difficult to purify each isoenzyme directly from cultures of *C. rugosa* on a preparative scale for industrial applications (*14*, *15*). Therefore, gene cloning and heterologous expression can be suggested as the most suitable approach for the production, characterization, and optimization of biocatalytic properties of pure isoforms. We have changed the nonuniversal CTG codons in *LIP1*, *LIP2*, *LIP3*, and *LIP4* to universal serine codons using site-directed mutagenesis. The functional expressions of these genes were successfully accomplished in heterologous hosts including bacteria *Escherichia coli* and yeast *Pichia pastoris* (*16*−*22*). According to the expression of LIP mRNA, the amount of native LIP4 in CRLs is extremely low (*14*). Moreover, the recombinant LIP4 had higher esterase activities toward long-chain ester and lower lipase activities than the commercial CRL (a mixture of various isoforms) (*21*). This substrate specificity is different from that of recombinant LIP1, which is the major isoform present in the commercial CRL preparation and showed higher lipolytic activity toward medium (C8−C10) acyl-chain esters (*22*).

The 3-D structural analysis is very important in our understanding of lipase functions. The 3-D structure of three CRL isoforms (LIP1, LIP2, and LIP3) purified from the commercial crude enzyme mixtures have been elucidated (*23*−*26*). The structure of *C. rugosa* lipase and conformational flexibility were reviewed (*27*). All three lipases have essentially two binding sites, one for the substrate or acyl group and the other for alcohol binding. To understand the structural basis of acyl chain-length specificity of LIP4, we compared the amino acid sequences of the substrate (acyl group)-binding sites of the CRL isoforms on the basis of the high similarity in sequence and revealed variations that may affect substrate specificity and catalytic properties.

Directed evolution methods mimic natural evolution in the test tube by using modern molecular biology methods of random mutation and recombination and provide a powerful tool in protein engineering without requiring knowledge of enzyme structures or catalytic mechanisms. It has been demonstrated that directed evolution can produce enzymes with altered substrate specificity (*28*, *29*). In addition, site-specific saturation mutagenesis is one of the directed protein evolution methods, which have been used for the development of biocatalysts with novel properties (*30*).

We found the residues 296 and 344 of the substrate-binding residues in LIP4 displaying significant differences from other LIP isoforms. Both residues are located at the mouth of the hydrophobic acyl group-binding tunnel and may be responsible for the different substrate specificity of LIP4 compared with other isoforms. Site-specific saturation mutagenesis is a powerful technique to introduce all possible amino acid substitutions into a specific position within a target gene. In this study we used this technique to analyze the roles that these two substrate-binding sites may play in the specificity of LIP4.

Table 1. Amino Acid Residue Difference of the Fatty Acyl-Binding Sites in the Various CRL Isoforms

residue no.[a]	LIP1	LIP2	LIP3	LIP4	LIP5
216	M	F	L	M	L
296	**F**	**V**	**F**	**A**	**F**
300	S	P	S	P	T
344	**F**	**L**	**I**	**V**	**L**
414	G	A	A	A	T

[a] The residue positions at which the amino acids are unique to LIP4 are shown in bold.

MATERIALS AND METHODS

Materials and Strains. The substrates (*p*-nitrophenyl esters and triacylglycerols) were purchased from Sigma-Aldrich-Fluka (St. Louis, MO). The *P. pastoris* expression vector pGAPZαC (Invitrogen, Carlsbad, CA) was manipulated in *E. coli* strain TOP10F (Invitrogen), which was used as a host for cloning and grown in low-salt Luria−Bertani (LB) broth supplemented with zeocin (25 mg/mL) (Invitrogen). The *P. pastoris* strain KM 71 (Invitrogen), harboring the recombinant plasmids, was used for expressing recombinant wild-type LIP4 and mutated LIP4. All *P. pastoris* transformants were cultured in YPD (1% yeast extract, 2% peptone, and 2% dextrose; pH 6.3) broth containing 100 μg/mL of zeocin at 30 °C. 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). A DNA sequencing kit was purchased from Perkin-Elmer Cetus (Wellesley, MA). Other chemicals used were of the purest grades available.

Construction of Randomly Mutated LIP4s. Among the predicted substrate-binding associated residues, LIP4 showed significant difference from other LIP isoforms at residues A296 and V344 (**Table 1**). These substrate-binding sites were selected as the targets for saturation mutagenesis to change the fatty-acid chain-length preference in this study.

Construction of the *P. pastoris* expression vector of wild-type LIP4 (pGAPZαC-LIP4) has been described previously (*20*). This plasmid vector was used as a template for overlap-extension PCR in saturation mutagenesis of the two substrate-binding sites selected (**Figure 1**). The degenerate mutagenic primers sets containing 32 codons are shown in **Table 2**. To create the A296 mutant library, the two pairs of primers, 4-N and 4-1R and 4-1F and 4-C, were first used to amplify a 5′-end 880 bp fragment and a 3′-end 790 bp fragment. After the first PCR, these two fragments were mixed by overlapping as the template for the second PCR. The second PCR used primers 4-N and 4-C, which contain *Kpn*I and *Xba*I restriction sites, respectively, to amplify the full length of mutated *lip4* open reading frame. The secondary PCR product (1670 bp) was digested with *Kpn*I and *Xba*I and ligated into pGAPZαC to generate the expression vectors of A296 mutants (pGAPZαC-mLIP4-A296). In the same way, the V344 mutants were created by using the two pairs of primers, 4-N and 4-2R and 4-2F and 4-C, to amplify 5′-1060 bp and 3′-330 bp fragments in the first PCR. The secondary PCR product (1670 bp) was digested with *Kpn*I and *Xba*I and ligated into pGAPZαC to generate the expression vectors of V344 mutants (pGAPZαC-mLIP4-V344).

Expression and Screening of Mutated LIP4s. The plasmids (10 μg) harboring the engineered wild-type *lip4* and mutated *lip4* were linearized with *Avr*II and transformed into *P. pastoris* KM 71 by electroporation. High-voltage pulses (1.5 kV) were delivered to 100 μL samples in 0.2 cm electrode gap cuvettes using a Gene Pulser apparatus supplied with the Pulse Controller (Bio-Rad). Transformants were plated on YPDS (1% yeast extract, 2% peptone, 2% dextrose, 1 M sorbitol, and 2% agar, pH 7.2) plates containing 100 μg/mL zeocin (Invitrogen) to isolate zeocin-resistant clones. Individual colonies containing lipase-secreting transformants were picked and patched on emulsion YPD plates containing 1% tributyrin. The clear zone on the opaque emulsion identified the lipase-secreting *P. pastoris* transfor-

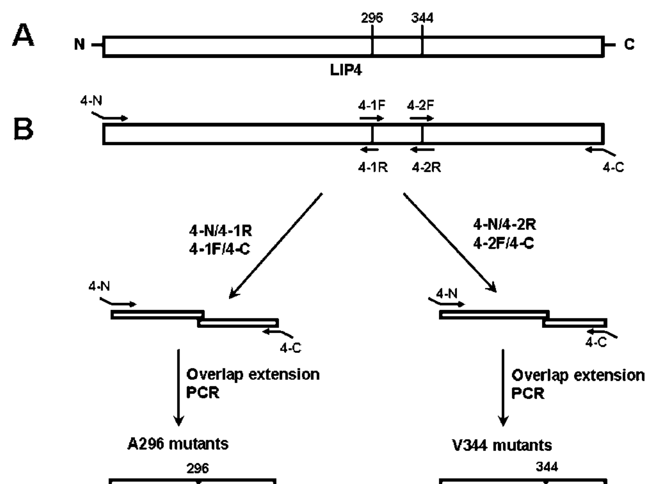

Figure 1. Mutagenesis of *lip4* gene introduced by overlap extension PCR. (**A**) The targets of mutated amino acids are indicated with their residue numbers along the polypeptide chain of LIP4. (**B**) The arrows indicate the mutagenic primers that were used to alter the codons of the amino acid targets in the *lip4* gene. For each mutant pool, the first-step PCR generated two DNA fragments that were overlapped and amplified in the second-step PCR. The oligonucleotide sequences of the mutagenic primers are shown in **Table 2**.

mants. Tributyrin is a triglyceride substrate for lipase containing short fatty acyl chain. The tributyrin emulsion plate let us conveniently screen the mutant LIP4 with changed (short) fatty acyl chain preference. *P. pastoris* transformed with pGAPZαC, free of any target gene, was used as a negative control. The sequences of mutated LIP4 DNAs, which were isolated from the *P. pastoris* transformants' genomic DNA by *lip4*-specific PCR, were confirmed by full-length DNA sequencing to identify the replacements.

Purification of Wild-Type and Mutated LIP4 Enzymes. Selected *P. pastoris* transformants were grown in 500 mL flasks containing 200 mL of YPD medium with 100 μg/mL zeocin at 30 °C for 3 days. The culture medium was concentrated by ultrafiltration on the Labscale TFF System with Pellicon XL devices coupling Biomax-30 membranes (Millipore, Bedford, MA) and then applied onto a HiPrep 16/10 Octyl FF column (Amersham Pharmacia Biotech Inc., Piscataway, NJ) equilibrated with TE buffer (20 mM Tris-HCl, 2 mM EDTA, pH 7.0). The column was washed with 5 column volumes of TE buffer plus 4 mM CHAPS. Bound proteins were then eluted with 5 column volumes of TE buffer containing 30 mM CHAPS. The eluted protein was dialyzed against TE buffer.

The dialyzed proteins were then applied to a HiPrep 16/10 Q XL column (Amersham Pharmacia Biotech) equilibrated with TE buffer, and 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*-nitrophenyl (*p*-NP) butyrate as a substrate (as described under Enzyme Characterization). The molecular masses of the purified recombinant lipases were determined in denaturing conditions 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.

Enzyme Characterization. The initial rates of *p*-nitrophenol esterase activities were measured by a Hitachi U-2010 spectrophotometer (*31*). 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 a 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 triacylglycerols with fatty acids of various chain lengths as substrates. The release of nonesterified fatty acids was monitored continuously by titration using 50 mM NaOH with a pH-stat (Radiometer Copenhagen, Bagsvaerd, Denmark), adjusted to a constant end-point value. The substrate emulsion was prepared by a modification of the Tiss method (*32*). The emulsification reagent contains, in 1 L, NaCl (17.9 g), KH_2PO_4 (0.41 g), glycerol (540 mL), gum arabic (6 g), and distilled water. Each lipolytic activity assay was carried out in a 37 °C thermostatted reaction vessel containing 2.5 mL of 40 mM triacylglycerol 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.

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 20−90 °C at pH 7.0. The optimal pH was investigated in the pH range of 3.0−10.0 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 caprylrate as a substrate.

RESULTS

Screening the Randomly Mutated LIP4s. To screen the large libraries of mutant lipases, an easy and effective experimental method was used in this study. All of the individual transformed *P. pastoris* KM 71 colonies containing pGAPZαC-mLIP4-A296 or -V344 were picked and patched on 1% tributyrin emulsion YPD plates to identify the lipase-secreting transformants. We screened over 200 colonies from each mutant pool and isolated three colonies with significant clearer zones than wild-type LIP4 transformant. After DNA sequence analysis, the amino acid replacements of these three mutants were identified as A296I, V344Q, and V344H. They were suggested to show changed substrate specificity from long to short acyl chain preference.

Substrate Specificities of the Recombinant Wild-Type and Mutant LIP4s. For lipase activity assay, the preferences for triacylglycerols containing fatty acids of various chain lengths

Table 2. Mutagenic Primers Used for the Saturation Mutagenesis of the *lip4* Gene

primer[a]	position of mutagenesis	oligonucletide sequence (5′ to 3′)[b]
4-N		CTCGGATCGGTACCCACTGCCACGCTCGCC
4-C		CCCTCTAGACTAAACAAAAAACGACGACGG
4-1F	296	GCCACCAGCGACACTCCGGGG**NNK**TTGGCGTACCCCTCGTTGCGG
4-1R	296	CCGCAACGAGGGGTACGCCAA**MNN**CCCCGGAGTGTCGCTGGTGGC
4-2F	344	GACGAGGGCACA**NNK**TTTGCGTTGCTGCTGTTGAACGTGACTACG
4-2R	344	CGTAGTCACGTTCAACAGCAGCAACGCAAA**MNN**TGTGCCCTCGTC

[a] The primers 4-N, 4-1F, and 4-2F were used as forward primers, and the primers 4-C, 4-1R, and 4-2R were used as reverse primers. [b] The positions of saturation mutagenesis are in bold. The created restriction sites (*Kpn*I in 4-N and *Xba*I in 4-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.

Table 3. Substrate Specificities of the Wild-Type (WT) and Mutant LIP4s (A296I, V344Q, and V344H) with Triglycerides of Various Chain-Length Fatty Acids

substrate	specific activity[a] (units/mg)			
	WT	A296I	V344Q	V344H
tributyrin (C4)	40.3 ± 0.3 (57)[b]	1112.2 ± 36.8 (67)	820.8 ± 3.6 (100)	142.5 ± 0.0 (100)
tricaprylin (C8)	70.0 ± 0.0 (100)	1666.7 ± 0.0 (100)	124.0 ± 12.6 (15)	20.0 ± 3.0 (14)
trilaurin (C12)	46.5 ± 0.3 (67)	107.5 ± 0.6 (6)	97.9 ± 7.2 (12)	3.0 ± 0.1 (2)
tripalmitin (C16)	3.1 ± 0.1 (4)	45.7 ± 2.1 (3)	107.3 ± 2.5 (13)	4.0 ± 0.9 (3)

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

Table 4. Substrate Specificities of the Wild-Type (WT) and Mutant LIP4s (A296I, V344Q, and V344H) with *p*-Nitrophenyl (*p*-NP) Esters of Various Chain-Length Fatty Acids

substrate	10^{-3} × specific activity[a] (units/mg)			
	WT	A296I	V344Q	V344H
p-NP butyrate (C4)	2.9 ± 0.2 (47)[b]	41.3 ± 3.8 (100)	5.3 ± 0.4 (61)	1.7 ± 0.2 (55)
p-NP caproate (C6)	0.4 ± 0.1 (7)	10.6 ± 1.1 (26)	0.8 ± 0.1 (10)	0.3 ± 0.0 (10)
p-NP caprylate (C8)	4.3 ± 0.2 (70)	28.3 ± 0.2 (69)	6.3 ± 0.4 (74)	2.3 ± 0.2 (74)
p-NP caprate (C10)	4.6 ± 0.5 (75)	9.3 ± 0.4 (22)	6.5 ± 0.5 (76)	2.3 ± 0.3 (73)
p-NP laurate (C12)	4.4 ± 0.2 (73)	6.5 ± 1.4 (16)	6.3 ± 0.6 (74)	2.4 ± 0.1 (78)
p-NP myristate (C14)	3.3 ± 0.5 (54)	2.1 ± 0.5 (5)	7.0 ± 1.2 (82)	2.1 ± 0.1 (67)
p-NP palmitate (C16)	6.1 ± 0.1 (100)	3.3 ± 1.1 (8)	8.6 ± 1.3 (100)	3.1 ± 0.2 (100)

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

are shown in **Table 3**. In the tributyrin-plate screening three mutants (A296I, V344Q, and V344H) displayed higher activities than the wild type toward short-chain triacylglycerol (tributyrin). In addition, A296I and V344Q also showed higher activities than the wild type toward medium- and long-chain triacylglycerols. Actually, A296I favored medium-chain triacylglycerol and had a similar pattern of chain-length preference as wild-type LIP4. However, V344Q and V344H favored short-chain triacylglycerol and displayed different chain-length selectivity from the wild-type LIP4.

Hydrolysis of *p*-NP esters containing fatty acids of various chain lengths by LIP4, A296I, V344Q, and V344H is demonstrated in **Table 4**. V344Q and V344H had the same preference as the wild-type LIP4 for the ester substrate with long fatty acyl chain (C16). However, A296I significantly showed preference opposite to that of the wild-type LIP4 and was highly active toward short-chain fatty acid esters (C4−C6). A296I showed 14- and 26-fold the activity of LIP4 toward *p*-NP butyrate (C4) and *p*-NP caproate (C6), respectively.

Characterization of the Recombinant Wild-Type and Mutant LIP4s. The effects of temperature on wild-type LIP4, A296I, V344Q, and V344H are depicted in **Figure 2**. The optimal temperatures for LIP4, A296I, V344Q, and V344H were 37, 50, 37, and 37 °C, respectively, using *p*-NP butyrate as substrate. Obviously, A296I demonstrated an improved temperature optimum (50 °C) and much higher activity when compared with other mutants and wild-type LIP4 throughout all of the temperature range. As compared with the wild-type LIP4, A296I showed about 10−20-fold higher activity at all temperatures tested. It was also advantageous that A296I retained about 57% of the highest enzyme activity at 20 °C.

The pH dependences of LIP4, A296I, V344Q, and V344H were studied using *p*-NP butyrate substrate at 37 °C (**Figure 3**). The optimal pH values for the three mutant LIP4s in this study were all 8.0, which was obviously higher than that of wild-type LIP4 (pH 7.0). In comparison with the wild-type LIP4, A296I again showed the highest specific activity among the

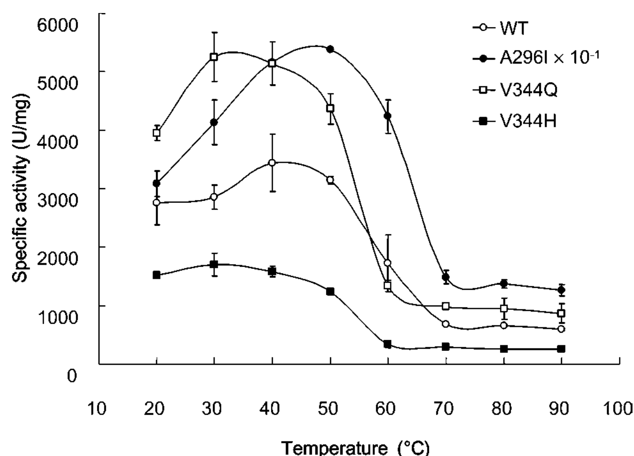

Figure 2. Temperature effect on the wild-type and mutant LIP4s (A296I, V344Q, and V344H). The activity was measured by spectrophotometric method using *p*-NP butyrate as substrate at pH 7.0.

other mutants at all pH values tested. Significant differences of the specific activity between A296I and wild-type LIP4 were observed at pH 8.0 (48-fold) and pH 9.0 (79-fold).

The residual activities of LIP4, A296I, V344Q, and V344H after heating for 10 min at various temperatures were assayed (**Figure 4**). Wild-type LIP4 and A296I showed similar thermostabilities and retained 80% of the highest activity after incubation at 60 °C. V344H was stable at 37−50 °C. However, its thermostability drastically decreased as the temperature was raised and retained only 23% of the highest activity after incubation at 60 °C.

DISCUSSION

In this paper, we used an easy and effective experimental method to generate LIP4 mutants and screened them functionally. By using saturation mutagenesis and functional screening we have generated three mutants with altered substrate specifici-

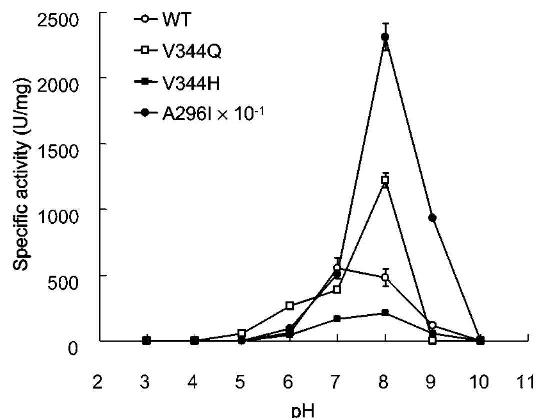

Figure 3. pH effect on the wild-type and mutant LIP4s (A296I, V344Q, and V344H). The activity was measured by spectrophotometeric method using *p*-NP butyrate as substrate at 37 °C.

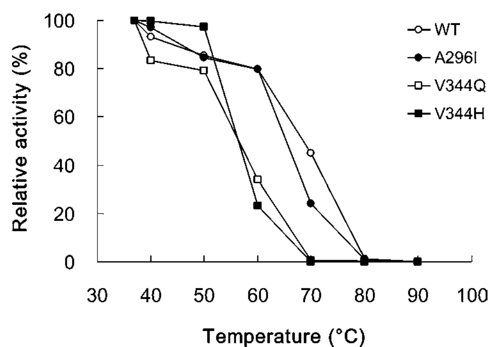

Figure 4. Thermal stability of the wild-type and mutant LIP4s (A296I, V344Q, and V344H). The residual activities after 10 min of incubation at different temperatures were measured by spectrophotometric method using *p*-NP caprylate as substrate at 37 °C and pH 7.0. The 100% activities of the wild-type LIP4 (WT), A296I, V344Q, and V344H were 4723, 69019, 7368, and 2307 units/mg, respectively.

ties and activities. Two amino acid residues (A296 and V344) located at the substrate-binding sites had been shown to have different effects on the catalytic properties of LIP4.

According to the 3-D structure of CRLs, there is a long, narrow, hydrophobic tunnel, characterized as the binding site of the acyl group of the lipid substrate. Residues 296 and 344 are located at a hydrophobic pocket near the entrance of the hydrophobic tunnel, where it may be involved in the initial recognition and transport of hydrophobic substrates into the binding site (*33*, *34*). The lower hydrolytic activity of LIP4 toward triglycerides may be due to the small and less hydrophobic Ala residue at the 296 position. The replacement of A296 by hydrophobic Ile, which more resembles those residues of other CRL isoforms at the same position (**Table 1**), might increase the hydrophobic interaction with the triglycerides and, therefore, showed highly improved activity toward triglycerides (**Table 3**). Although A296I did not change the chain-length specificity of lipase activity, it somehow shifted the chain-length specificity of esterase activity to short-chain fatty acid esters (**Table 4**). Perhaps the larger side chain of isoleucine interfered with the binding of long-chain fatty acid of *p*-NP esters more significantly than triglycerides. Previously, mutation of amino acids of recombinant LIP1 at different locations inside the tunnel resulted in mutants with different chain-length specificities (*35*). Here we showed that the residue 296 outside the tunnel could also affect the chain-length specificity.

Single amino acid substitution of V344Q or V344H actually changed the chain-length specificity of LIP4 lipase activity (**Table 3**). These results suggested that a hydrophobic residue at position 344 is necessary for the binding of the medium- or long-chain triglycerides. Once the V344 is replaced by a hydrophilic residue such as glutamine (Q) or histidine (H), the mutant LIP4 began to favor only short-chain triglycerides. A tailor-made enzyme to modify the molecular recognition of 2-arylpropionic esters by recombinant LIP1 using site-directed mutagenesis of Phe344 and Phe345 was reported (*34*). Phe345, but not Phe344, was shown to play an important role in the *S*-enantiomer preference. Therefore, residue 344 of CRL would be crucial for the chain-length specificity instead of enantiospecificity.

Usually, a single amino acid substitution is sufficient to obtain the necessary change in performance of enzyme during directed evolution (*36*, *37*); sometimes, combinations of mutations at different sites can provide further improvement (*38*, *39*). It has been proved that focused random mutation of a pair of amino acids in the enzyme's substrate-binding site (active site) can create variants with an increased range of substrates (*40*). More research effort is definitely needed to understand the chemistry and molecular biology of CRL isoforms.

CRLs have been widely used in biotechnological applications. To obtain a stable and reproducible result in a particular application, a purified preparation of recombinant CRL is a better choice than the crude commercial enzyme. The present study demonstrated an efficient method to engineer CRL and obtain recombinant CRL mutants with new catalytic properties. For example, A296I showed high specific activity to hydrolyze *p*-NP esters with short-chain fatty acids, such as butyric acid (**Table 4**). It might be useful in the industrial production of flavor esters. Furthermore, the optimal temperature and pH of A296I were higher than those of the wild-type LIP4. Esterification activity assays of the A296I, V344Q, and V344H mutants are underway. Catalysis of ester synthesis of these mutants might be different from that of LIP4 in terms of substrate specificity. In general, the present work not only expanded the industrial application of LIP4 but also provided a useful hint for modifying the other *C. rugosa* lipase isoforms.

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