Modifying the chain-length selectivity of the lipase from *Burkholderia cepacia* KWI-56 through *in vitro* combinatorial mutagenesis in the substrate-binding site

Junhao Yang¹, Yuichi Koga², Hideo Nakano¹³ and Tsuneo Yamane¹

¹Laboratory of Molecular Biotechnology, Graduate School of Biological and Agricultural Sciences, Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464-8601 and ²Chubu Science and Technology Center, Sakae 2-17-22, Naka-ku, Nagoya 460-0008, Japan

The mature lipase of *Burkholderia cepacia* KWI-56 was synthesized in an enzymatically active form using an *in vitro* Escherichia coli S30 coupled transcription/translation system by expressing the mature lipase gene (*lip*) in the presence of its specific activator. To investigate the substrate specificity of the lipase comprehensively, a large number of mutant lipases were constructed and analyzed in a high throughput manner by combining overlapping PCR and *in vitro* protein synthesis. In this paper, Phe119 and Leu167, which are located in the acyl portion of the substrate-binding pocket of the lipase of *B. cepacia* KWI-56, were substituted with six hydrophobic amino acid residues by the *in vitro* combinatorial mutagenesis. The wild-type and 35 mutant genes amplified by PCR were directly used as templates for the *in vitro* transcription/translation. The acyl chain-length selectivity of the *in vitro* expressed lipases against p-nitrophenyl butyrate, p-nitrophenyl caprylate and p-nitrophenyl palmitate, was compared by their relative hydrolysis rates. Two mutant lipases, L167V and F119A/L167M, which showed a significant shift in substrate selectivity were further expressed in *vivo* and refolded *in vitro*. It was found that L167V raised its preference for the short-chain ester, whereas F119A/L167M improved its selectivity for the long-chain ester.

Keywords: *Burkholderia cepacia*lin *vivo* protein synthesis/lipase/protein engineering/substrate selectivity

Introduction

Lipases (EC 3.1.1.3) are water-soluble enzymes that catalyze the hydrolysis of triacylglycerols and a large variety of natural and unnatural esters. They differ from one another in their physical properties and biochemical features and are selective in respect to the fatty-acid chains. The hydrolysis of ester bond catalyzed by lipase involves acylation and deacylation steps (Grochulski et al., 1994; Kazlauskas, 1994). The substrate selectivity in the hydrolysis reaction is believed to be determined by the tetrahedral intermediate formed in the acylation step.

Since the beginning of the 1990s, the three-dimensional structures of many lipases of mammalian, yeast and microbial origin have been determined. The structures of lipases resemble those of other hydrolytic enzymes—the α/β fold hydrolases (Ollis et al., 1992; Misset et al., 1994). These hydrolytic enzymes differ widely in origin and catalytic function, however, all of them have a common catalytic triad, including Ser, His and Asp (or Glu). Among all the lipases with known structures, another common feature is that their substrate-binding site is located inside a pocket on top of the central β-sheet. Recently, the lipases were subdivided into three groups according to their shapes of substrate pocket: (i) lipases with a hydrophobic, crevice-like binding site located near the protein surface; (ii) lipases with a funnel-like binding site; and (iii) lipases with a tunnel-like binding site (Pleiss et al., 1998). For example, three closely related lipases, which are from *Burkholderia glumae* (BGL), *Burkholderia cepacia* (BCL) and *Pseudomonas aeruginosa* (PAL), have typical funnel-like substrate-binding sites (Nobel et al., 1993; Kim et al., 1997; Nardin et al., 2000). In the case of BCL, it contains a binding pocket like an elliptical funnel with a length of 17 Å. The width at its base is 4.5 Å and increases to 10.5 Å at the entrance to the binding site. The left- and the right-hand walls viewed along the alcohol–acid axis are 10.5 and 16.5 Å, respectively (Pleiss et al., 1998). This substrate-binding pocket can be divided into two parts for alcohol and fatty acid. In BCL there are seven amino acid residues (Leu17, Pro113, Phe19, Leu164, Leu167, Val266 and Val 267) lining the acyl chain-binding portion of the pocket (Lang et al., 1998). But according to Pleiss, another three amino acid residues, Ser117, Ala120 and Val123 are also included (Pleiss et al., 1998). The acyl chain of ester substrate binds at the hydrophilic bottom of the funnel composing Ser87 and Pro113, and is surrounded by the other amino acid residues.

The shape, size and hydrophobicity of the substrate-binding site may correlate with a fatty-acid chain-length preference of respective lipase. It is also clear from the known crystal structures that only a small number of residues are lining the scissile fatty-acid binding site; therefore, mutation of these residues is expected to change the chain-length selectivity. Rational mutagenesis was applied to the investigation of the chain-length selectivity of the lipase from *Rhizopus delemar*. In the report by Klein et al., a double mutant V209W/F112W showed improved selectivity for the short-chain substrate (Klein et al., 1997). The replacement of Val209 and Phe112 with bulkier tryptophans in the acyl chain-binding groove hinders the docking of fatty acids longer than butyric acid. Similarly, a single F95D and a double F95D/F214R mutation (Joerger and Haas, 1994) resulted in an improvement of the enzyme activity toward tricaprylin in comparison with triolein. But in these studies, the library size to be investigated in *vivo* is relatively limited because the tedious work of cloning and expression of mutant enzymes is necessary.

The *in vitro* protein synthesis system supplies an efficient method to prepare mutant protein products directly from PCR-amplified DNA templates (Nakano and Yamane, 1998; Nakano et al., 1999). Therefore, it has become an alternative method in the investigation of the structure–function relationship. The *in vitro* protein synthesis has been successfully applied in screening novel proteins by ribosome display (Mattheakis et al., 1994; Hanes and Plückthun, 1997; He and Taussig,
1997). In addition, the method of in vitro scanning saturation mutagenesis was used for the investigation of the binding pocket of an antibody (Burks et al., 1997; Chen et al., 1999). High throughput of mutant proteins can be investigated efficiently by the in vitro method because it is not necessary to clone the mutant genes. Compared with the traditional in vivo protein engineering method, the advantage of the in vitro system is obvious.

In this work, we applied the method of in vitro combinatorial mutagenesis in investigating the chain-length selectivity of the lipase of B.cepacia KWI-56. The extracellular lipase from B.cepacia KWI-56 (previously named as Pseudomonas sp. KWI-56) was purified and characterized as a thermostable enzyme with potential industrial uses (Iizumi et al., 1990). Its structural gene (lip) was cloned together with an activator gene (act), which was located just downstream of the lipase gene and necessary for the active production of the lipase in vivo (Iizumi and Fukase, 1991; Iizumi et al., 1994). In our previous report, active lipase was synthesized in an in vitro coupled transcription/translation system using Escherichia coli S30 extract by expressing the mature lipase gene (rilp) in the presence of the presynthesized activator (Yang et al., 2000).

In the present work, two amino acid residues in the acyl pocket of the substrate pocket, Phe119 and Leu167, were targeted for combinatorial substitution by Ala, Val, Ile, Leu, Met and Phe. Thirty-five mutant lipases and the wild-type lipase were prepared by in vitro coupled transcription/translation from PCR-amplified DNA templates. The substrate selectivity of these lipases to p-nitrophenyl butyrate, p-nitrophenyl caprylate and p-nitrophenyl palmitate were investigated comprehensively. Two mutant lipases with an apparently significant selectivity shift were further expressed in E.coli and renatured in vitro, and their improvement in acyl chain selectivity was confirmed.

Materials and methods

Materials
LA Taq DNA polymerase was purchased from TaKaRa Shuzo (Kyoto, Japan). DNA primers for PCR were synthesized by Nippon Flour Mills (Tokyo, Japan). p-Nitrophenyl ester of saturated fatty acids were purchased from Sigma. Dimethyl sulfoxide and 1-propanol were from Wako Pure Chemical Industries (Osaka, Japan). Triton X-100 was purchased from ICN Biomedicals Inc. (Aurora, OH, USA).

Bacterial strains and preparation of plasmids are described previously (Yang et al., 2000).

PCR mutagenesis

Mutations in the recombinant lipase gene, rilp, were generated by the overlapping PCR method as described previously (Yang et al., 2000). To introduce double mutations the whole sequence of T7 promoter, rilp and T7 terminator was first amplified independently using three pairs of primers. The sequences of primers T2, T2–F2 and T2–R2 were described in our previous report. The DNA sequences of the other primers are as follows: F119R, CTGGGACCGCGATGCGCGT; L167R, CGTCTCGACGCGCGCGAG; F119XF, GCCCGATCCGGGCTCGGNNNGCC; L167XF, GCTCGCGCGCCTCGACGNNNNAC; F119XF, GCCCGCGCCTCGACGNNNNAC; L167XF, GCTCGCGCGCCTCGACGNNNNAC. Whereas, X represents the six amino acids in substitution, NNN represents the codon for each amino acid: GCG for Ala, GTG for Val, ATC for Ile, CTG for Leu, ATG for Met and TTC for Phe, respectively. The standard condition for the first-step PCR in 20 µl of reaction mixture was as follows: 25 cycles of 10 s at 98°C, 30 s at 60°C and 30 s at 72°C. The annealing temperatures and extension time varied depending on the sequences of primers and the fragments to be amplified. To connect these three fragments, 1 µl of each amplified fragment was mixed together and followed by 10 cycles of denaturation (10 s at 98°C), annealing (20 s at 60°C), and extension (30 s at 72°C) in 20 µl of the reaction mixture without any primer. Finally, the reconstituted fragment was amplified by 30 cycles of 10 s at 98°C, 30 s at 56°C, 1 min 10 s at 72°C after the addition of 0.5 mM T2 primer. LA Taq DNA polymerase was used throughout this experiment.

In vitro coupled transcription/translation

Escherichia coli S30 extract in acetate buffer [9.9 mM Tris–acetate, pH 7.4, containing 14 mM Mg(OAc)2 and 60 mM KOAc] was prepared according to the procedure of Ellman (Ellman et al., 1991). The in vitro coupled transcription/translation was carried out as described previously (Yang et al., 2000) with some modifications. One percent of Triton X-100 and 0.3 mg/ml of the activator was also included in the reaction mixture. The reagents described above were mixed on ice and incubated at 30°C for 1 h.

Construction of the expression plasmids for the mutant lipases

The mutations L167V and F119A/L167M were introduced into rilp by overlapping PCR as described above. Then, the mutant genes were ligated to NdeI and EcoRI restricted pRSFET, yielding the expression plasmids for the two mutant lipases. The whole sequences of the mutant lipases were confirmed by DNA sequencing.

In vivo expression and preparation of the inclusion bodies of the lipases

Escherichia coli BL21(DE3)pLysS harboring the expression plasmid of the lipase was cultivated in 800 ml of LB medium containing 1% glucose, 50 µg/ml of ampicillin and 25 µg/ml of chloramphenicol at 37°C to achieve an optical density of ~0.6 at 660 nm. IPTG was added into the culture broth to a final concentration of 0.4 mM, and the culture was incubated at 37°C for another 3 h. The cells harvested by centrifugation from the expression cultivation were suspended in 50 mM Tris–HCl buffer (pH 8.0) containing 30 mM NaCl. After ultrasonic disruption, the supernatant obtained by centrifugation at 3000 g for 10 min was collected as the crude cell extract. Then, the soluble and insoluble proteins were separated by centrifugation at 8000 g for 20 min. The inclusion bodies thus collected were resuspended in 1 M sucrose and centrifuged at 8000 g for 20 min. The pellets thus collected were washed twice with the 50 mM Tris–HCl buffer (pH 8.0) containing 30 mM NaCl and the purity of the inclusion bodies was analyzed by SDS–PAGE.

In vitro denaturation and renaturation of the lipases

The refolding of the lipase of B.cepacia KWI-56 was performed by the method as described by Traub et al. (Traub et al., 2001). The purified inclusion bodies were dissolved in buffer B (10 mM Tris–HCl, 8 M urea, 0.1 M sodium phosphate, pH 8.0) at room temperature for 1 h with mild stirring. Following centrifugation at 8000 g for 20 min, the supernatant was subjected to refolding. One hundred microliters of lipase solution obtained was refolded in 100 ml of distilled water in the presence of the same amount of the lipase activator, which was purified as described previously (Yang et al., 2000). After
refolding at 4°C for 20 h, the lipase activity was assayed with p-nitrophenyl butyrate, p-nitrophenyl caprylate and p-nitrophenyl palmitate, respectively.

**Determination of protein concentration**

Protein concentration was determined by the Bradford method with a Bio-RAD kit (Hercules, CA) in accordance with the instructions of the manufacturer.

**Lipase activity assay**

p-Nitrophenyl acetate, p-nitrophenyl propionate, p-nitrophenyl butyrate, p-nitrophenyl caproate, p-nitrophenyl caprylate, p-nitrophenyl caprate and p-nitrophenyl palmitate were first solubilized with dimethyl sulfoxide in one-tenth of the final volume, then diluted to 0.5 mM with 50 mM potassium phosphate buffer (pH 7.0) containing 0.5% Triton X-100. Ten microliters of properly diluted lipase was added to 90 µl of the substrates, respectively, in a 96-well microtiter plate. After hydrolysis at 37°C for 1 h, the reaction was stopped by 100 µl of 1-propanol. The absorbance at 405 nm was measured on a microtiter plate autoreader soon after the reaction was stopped. One unit of lipase activity is defined as the amount of the enzyme required to liberate 1 mmol of p-nitrophenol in 1 min at 37°C. Relative hydrolysis rates of the *in vitro* expressed lipases were calculated by dividing the optical density for p-nitrophenyl acetate, p-nitrophenyl propionate, p-nitrophenyl butyrate, p-nitrophenyl caproate, p-nitrophenyl caprate and p-nitrophenyl palmitate by that for p-nitrophenyl caprylate.

**Results and discussion**

With increasing knowledge about the three-dimensional structures of lipases, it has become possible to explain some of the enzyme properties based on their structures. For example, the interfacial activation of lipase is revealed by the presence of a lid (or flap) structure and the special structure of the alcohol-binding pocket of the lipase B from *Candida antarctica* is used to explain its enantioselectivity for secondary alcohols (Uppenberg et al., 1995). But we are still a long way from the design for a lipase with desired properties by molecular simulation due to the complexity of the protein structure. Therefore, an effective experimental method is required to deal with large libraries of mutant enzymes in the engineering of a lipase.

Burks et al. combined PCR mutagenesis, *in vitro* transcription/translation and ELISA to deal with high throughput mutant antibodies (Burks et al., 1997). This method was proved effective for engineering antibody-binding specificity and for comprehensive structure–function studies and analysis (Chen et al., 1999). In our previous report (Yang et al., 2000), we applied the method of *in vitro* PCR mutagenesis and coupled transcription/translation to study the roles of a disulfide bridge and a calcium-binding site in the activation of the lipase from *B. cepacia* KW1-56. Presently, we are using this system to study a larger library of the mutant lipases, specifically their acyl chain-length selectivity. **Selection of amino acid residues in the substrate-binding site for substitution**

Although the three-dimensional structure of the lipase from *B. cepacia* KW1-56 is not determined yet, it is expected to resemble that of BCL (Kim et al., 1997) with little difference because their amino acid sequence homology is as high as 94%. The accession numbers of BCL and KW1-56 lipase of DDBJ are M58494 and S77842, respectively. Among the 10 acyl-binding amino acid residues the only difference between the *B. cepacia* KW1-56 lipase (Figure 1) and BCL is that the former contains Leu instead of Val at the 266th position. A molecular model of BCL docked with saturated fatty acid methyl esters in the tetrahedral intermediate state was built by Pleiss et al. (Pleiss et al., 1998) describing the acyl chain binding by respective amino acids. In BCL, the fatty-acid chain binds up to C6 to a narrow cleft at the hydrophobic bottom of the funnel, which is formed by Pro113 and catalytic Ser87. It is lined by Val266/Val267 and Leu167/Leu17 on its left- and right-hand side, respectively. At the end of this cleft near Ser117 and Ala120, the fatty acid kinks sharply and binds to the left-hand wall in a cleft up to C9 and then to a hydrophobic-binding site from C10 to C14, which is formed by Phe119, Val123 and Leu164.

By comparing the molecular models of the two lipases, Leu167 of the *B. cepacia* KW1-56 lipase, which is located at the right-hand side of the substrate pocket, may interact with the fatty-acid chain shorter than C6 from the catalytic Ser87 in the intermediate state. In contrast, Phe119 may only interact with the acyl chain from C10 to C14, which is relatively distant from Ser87. Both Leu167 and Phe119 are directly involved in binding the acyl portion of ester substrates and locate on different sides of the pocket. On the other hand, as Phe119 locates near the opening entrance of the binding pocket, it may also affect the docking of the substrate into the active site of the lipase. If the acyl chain-length selectivity of the lipase is determined in the tetrahedral intermediate state, Leu167 may affect the substrate with various chain sizes because its position is closer to the catalytic Ser87. In contrast, Phe119 may only affect substrates with long fatty-acid chains. Since double mutation will reveal more information about the substrate pocket than single mutation, Phe119 and Leu167 were selected for mutagenesis in a combinatorial manner to investigate their effects on substrate selectivity.

Since the protein surface at the border of the substrate
pocket of the lipase is hydrophobic and is supposed to interact with the hydrophobic interface of substrates. Hydrophobic amino acids were favored in mutagenesis to avoid a substantial decrease in lipase activity. As a matter of fact, nine of the 10 amino acid residues except Ser117 included in the acyl portion of the substrate pocket appeared relatively conserved to hydrophobic amino acid residues among the proteobacterial lipases (Sullivan et al., 1999). Leu167 was strictly conserved in this family, whereas Phe119 could be replaced by Thr, Val, Leu or Ile in different species. To avoid the likelihood of a serious damage to lipase activity, Phe119 and Leu167 were substituted independently with Ala, Val, Ile, Leu, Met and Phe forming a library of one wild-type and 35 mutants. It is expected that such mutations would not make a large change to the overall structure of the substrate pocket, but make some delicate modification on the shape and hydrophobicity of the binding pocket.

Overlapping PCR to introduce mutations into the lipase gene

Mutant genes were generated according to the method of overlapping PCR as described in Materials and methods. The same terminal sequence was introduced into the 5’- and 3’-end of the lipase sequence in the first-step PCR. After an overlapping extension, a single primer (T2) corresponding to the attached terminal sequence was used in the final amplification of the whole sequence. Because the primer sequence did not exist in the template, amplification of the original sequence of the wild-type lipase was reduced greatly. The mutant lipase genes, thus prepared by overlapping PCR extension, could be directly used as templates for in vitro expression without interference from the wild-type lipase gene. Since there is a variation in both protein synthesis and folding efficiency, the final activities of in vitro expressed lipases deviated from batch to batch. Thus, the construct prepared with the wild-type sequence was used on each microtiter plate to provide an accurate calibration. The wild-type sequence, which provides the calibration for the mutants in the in vitro protein synthesis, was generated by the same overlapping PCR method. All the overlapping PCR products were confirmed by gel electrophoresis to contain the DNA with the correct size at a proper concentration before in vitro expression.

In vitro expression of the lipases and substrate specificity investigation

Thirty-six lipases were synthesized by E.coli in vitro coupled transcription/translation in 96-well microtiter plates. The wild-type sequence was also used in each expression plate alongside the mutants for the calibration.

The activities of the 36 lipases, including the wild-type, were simultaneously measured towards p-nitrophenyl butyrate, p-nitrophenyl caprylate and p-nitrophenyl palmitate, which contains four, eight and 16 carbons of acyl chain, respectively, in 96-well microtiter plates. In the enzyme activity assay the absorbance at 405 nm was controlled below 0.75 to keep the data in a linear relationship with lipase activity.

As a consequence, all the mutant lipases were expressed in E.coli in vitro coupled transcription/translation in 96-well microtiter plates. Thus, the construct prepared with the wild-type sequence contained the DNA with the correct size at a proper concentration before in vitro expression.

The wild-type sequence is in bold.

### Table I. Relative hydrolysis rates of the mutant and wild-type lipases for \( p \)-nitrophenyl caprylate/\( p \)-nitrophenyl butyrate (C8/C4)

<table>
<thead>
<tr>
<th>C8/C4</th>
<th>119A</th>
<th>119V</th>
<th>119I</th>
<th>119L</th>
<th>119M</th>
<th>119F</th>
</tr>
</thead>
<tbody>
<tr>
<td>167A</td>
<td>3.0</td>
<td>3.2</td>
<td>3.4</td>
<td>3.3</td>
<td>3.0</td>
<td>3.2</td>
</tr>
<tr>
<td>167V</td>
<td>2.8</td>
<td>3.1</td>
<td>3.0</td>
<td>3.2</td>
<td>2.7</td>
<td>3.1</td>
</tr>
<tr>
<td>167I</td>
<td>2.9</td>
<td>2.8</td>
<td>2.9</td>
<td>3.9</td>
<td>3.2</td>
<td>2.8</td>
</tr>
<tr>
<td>167L</td>
<td>3.5</td>
<td>3.3</td>
<td>3.3</td>
<td>3.9</td>
<td>3.0</td>
<td>3.3</td>
</tr>
<tr>
<td>167M</td>
<td>3.7</td>
<td>3.1</td>
<td>3.5</td>
<td>3.8</td>
<td>3.8</td>
<td>3.1</td>
</tr>
<tr>
<td>167F</td>
<td>4.3</td>
<td>4.8</td>
<td>4.7</td>
<td>5.0</td>
<td>4.5</td>
<td>4.8</td>
</tr>
</tbody>
</table>

\(^a\) The relative hydrolysis rates were calculated from the data of three experiments. The SD was <5%.

\(^b\) The wild-type sequence is in bold.

### Table II. Relative hydrolysis rates of the mutant and wild-type lipases for \( p \)-nitrophenyl palmitate/\( p \)-nitrophenyl butyrate (C16/C4)

<table>
<thead>
<tr>
<th>C16/C4</th>
<th>119A</th>
<th>119V</th>
<th>119I</th>
<th>119L</th>
<th>119M</th>
<th>119F</th>
</tr>
</thead>
<tbody>
<tr>
<td>167A</td>
<td>2.9</td>
<td>2.8</td>
<td>3.3</td>
<td>3.0</td>
<td>2.6</td>
<td>3.1</td>
</tr>
<tr>
<td>167V</td>
<td>1.6</td>
<td>1.7</td>
<td>1.7</td>
<td>1.8</td>
<td>1.5</td>
<td>1.3</td>
</tr>
<tr>
<td>167I</td>
<td>1.6</td>
<td>1.8</td>
<td>1.8</td>
<td>1.9</td>
<td>1.7</td>
<td>1.9</td>
</tr>
<tr>
<td>167L</td>
<td>2.4</td>
<td>2.2</td>
<td>2.2</td>
<td>2.6</td>
<td>1.9</td>
<td>2.1</td>
</tr>
<tr>
<td>167M</td>
<td>4.3</td>
<td>3.3</td>
<td>3.5</td>
<td>3.6</td>
<td>3.3</td>
<td>2.9</td>
</tr>
<tr>
<td>167F</td>
<td>4.0</td>
<td>3.6</td>
<td>3.6</td>
<td>3.2</td>
<td>3.1</td>
<td>3.0</td>
</tr>
</tbody>
</table>

\(^a\) The relative hydrolysis rates were calculated from the data of three experiments. The SD was <5%.

\(^b\) The wild-type sequence is in bold.

As evident from Tables I and II, the wild-type lipase is originally more active to the medium- and the long-acyl chain esters than to the short-acyl chain esters. The activity ratio of \( p \)-nitrophenyl caprylate/\( p \)-nitrophenyl butyrate (C8/C4) for the wild-type lipase was 3.3 (Table I), whereas the ratio of \( p \)-nitrophenyl palmitate/\( p \)-nitrophenyl butyrate (C16/C4) was 2.1 (Table II). The results in Tables I and II suggested that most of the mutants showed their relative hydrolysis rates closer to that of the wild-type, but there were some mutants that showed an apparent shift in substrate selectivity. For example, the highest C8/C4 rate was 5.0 (F119L/L167F), and the lowest was 2.7 (F119M/L167V) among the 36 kinds of lipases. On the other hand, the highest C16/C4 rate was 4.3 (F119L/L167M), whereas the lowest was 1.3 (L167V) in this library.

The data in Tables I and II also revealed that substitution of Phe119 with other hydrophobic amino acids did not change the substrate selectivity very much. In contrast, the substitution at 167 caused a larger variation in the relative hydrolysis rate. This result suggested that Leu167 played a more important role than Phe119 for the lipase in substrate selectivity toward esters with different fatty-acid length. According to the molecular model of B.cepacia lipase, Phe119 is located near the entrance of the binding pocket which has weak interaction with acyl chains less than 8C in the intermediate state of hydrolysis. On the other hand, it is relatively wide at the opening entrance; therefore, the substitution of the Phe119 by
other hydrophobic amino acids may affect the binding with long-acyl chain to a lesser extent.

From Tables I and II, it is obvious that substitution of Leu167 by Val and Ile improved the selectivity of the lipase to C4. On the contrary, when Leu167 was substituted by Met and Phe, its selectivity for C8 and C16 was increased. By investigating the shape of the substrate-binding funnel, it is clear that the space is narrow near the bottom of the binding funnel where amino acid 167 is located. Therefore, the change in bulkiness, shape and hydrophobicity of the side chain of this amino acid may directly affect the binding of the short acyl chain of C4 as well as that of the longer acyl chains of C8 and C16. Therefore, the final effect of the mutation on selectivity shift depends on the difference of the affect on the respective substrate.

**Investigation of the mutant lipases after in vivo expression and in vitro refolding**

Because the productivity of target proteins by the in vitro system is very low, there was not enough amount of lipase to be purified. In order to investigate the mutant lipases with significantly improved substrate selectivity in the pure state, L167V and F119A/L167M were expressed in E.coli. L167V and F119A/L167M were selected for further investigation because L167V showed the lowest C16/C4, whereas F119A/L167M showed the highest C16/C4 (Table II). In addition, these two showed the largest shift to higher and lower, respectively, compared with the wild-type in both Tables I and II.

The two mutant genes were inserted into the expression plasmid pRSET and transformed into E.coli BL21(DE3) pLysS. Inclusion bodies of the wild-type lipase and the mutant lipases were synthesized and purified as described in Materials and Methods. The inclusion bodies were denatured by 8 M urea and refolded in vitro, and their specific activities toward p-nitrophenyl butyrate, p-nitrophenyl caprylate and p-nitrophenyl palmitate were assayed (Table III).

Although the relative hydrolysis rates of the wild-type lipase, L167V and F119A/L167M showed some difference between the in vitro expressed and the renatured in vivo expressed enzymes, their selectivity shift for C4 or C16 did not change. The improved selectivity of L167V for the short-acyl substrate (C4) and the improved selectivity of F119A/L167M for the long-acyl substrate (C16) revealed by the in vitro system were confirmed by the in vivo expressed lipases.

Moreover, the relative activities of respective lipases to the seven ester substrates (C2, C3, C4, C6, C8, C10, C16) were measured and are shown in Figure 2, where the activity for C8 is used as standard in each enzyme. It indicates that F119A/L167M has a tendency to prefer the longer chain substrates such as C10 and C16 whereas L167V improved its selectivity for shorter chain substrates. In all the enzymes of Figure 2, the relative activities toward C4 were much lower than toward longer acyl chain substrates. Then, their relative activities decreased gradually, as the acyl chain lengths of substrates became shorter. However, the relative activities of L167V for the short acyl chain substrates were the highest among the three enzymes.

As the results show in Table III, the specific activity of L167V to the short-acyl substrate (C4) is almost the same as that of the wild-type. So, it is possible to speculate that the substitution of Leu167 with Val does not interfere with the binding of p-nitrophenyl butyrate to the catalytic site. But its specific activities to the medium-chain (C8) and the long-chain (C16) substrates decreased. As a consequence, L167V increased its selectivity to the ester with a short fatty-acid chain (Figure 2).

The substitution of Leu167 with Val will decrease the volume of the side chain of the amino acid. This replacement may not interfere with the binding of ester having a short acyl chain such as C4, but affect the binding of C8 and C12. This replacement is likely to decrease the hydrophobic interaction between the amino acid residue and the fatty acid.

It may also cause a modification in the shape of the side chain of amino acid 167. Revealed by the molecular model of BCL (Pleiss et al., 1998), space is very narrow at the bottom of the funnel, and the acyl chains will kink at C6 to the opening mouth of the substrate-binding site. In the mutant lipase of L167V, the two methyl groups of Val 167, which may expose in two directions within the binding pocket, will interfere with the binding of fatty acids with medium chain and long chain such as C8, C10 and C12.

In contrast, the double mutation of F119A and L167M decreased the specific activities of the enzyme to the three
ester substrates, C4, C8 and C16, as shown in Table III. In addition, Figure 2 shows that the activities of F119A/L167M were increased as the acyl chain of substrates became longer, whereas the wild-type enzyme and L167V decreased their activities for such a long acyl chain substrate.

In this double mutant, the substitution of Phe119 with Ala may increase the size of the opening entrance of the substrate pocket, therefore making it easier for the large ester substrate to dock to the active site, which may in part explain the preference for the long ester. On the other hand, Met at 167 may take weaker hydrophobic interaction than Leu with the fatty-acid chains of the ester substrates. But the effect of this substitution would be more serious on the short-chain substrates (less than C4) than on the long-chain substrates (C10, C16), possibly because the long acyl chain of C10 and C16 will be able to contact another hydrophobic amino acid residues due to their high flexibility. As a result, the preference of the wild-type lipase would be shifted from the medium-chain substrate (C8) to the long-chain substrates (C16) by F119A/L167M.

Conclusions

We have demonstrated in this report that in vitro combinatorial mutagenesis enables a rapid screening of a large number of site-directed mutant enzymes, and supplies comprehensive information about some important amino acid residues. The in vitro screening results identify some candidates with improved properties for further characterization after traditional in vivo expression and purification. The in vitro mutagenesis method can greatly increase the speed of finding a promising candidate with the desired property, and it is proven to be highly efficient for the engineering of the enzyme.

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