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Improved thermostability and the optimum temperature of *Rhizopus arrhizus* lipase by directed evolution

Wei-Ning Niu, Zhao-Peng Li, Da-Wei Zhang, Ming-Rui Yu, Tian-Wei Tan *

Beijing Key Lab of Bioprocess, Beijing University of Chemical Technology, Beijing 100029, PR China

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Abstract

To expand the functionality of lipase from *Rhizopus arrhizus* (RAL) we have used error-prone PCR and DNA shuffling methods to create RAL mutants with improved thermostability and the optimum temperature. One desirable mutant with three amino acids substitution was obtained. The mutated lipase was purified and characterized. The optimum temperature of the mutant lipase was higher by 10 °C than that of the wild-type RAL (WT-RAL). In addition, the thermostability characteristic of the mutant was also improved as the result of directed evolution. The half-life ($T_{1/2}$) at 50 °C of the mutant exceeded those of WT-RAL by 12-fold. To confirm which substitution contributed to enhance thermostability and the optimum temperature for lipase activity, three chimeric lipases: chimeric lipase 1(CL-1; A9T), chimeric lipase 2 (CL-2; E190V) and chimeric lipase 3 (CL-3; M225I) from the WT-RAL gene were constructed. Each of the chimeric enzymes was purified and characterized. Amino acid substitution at position 190 was determined to be critical for lipase thermostability and the optimum temperature, while the residue at position 9 and 225 had only marginal effect. The mutational effect is interpreted according to a simulated three-dimensional structure for the mutant lipase.

Keywords: *Rhizopus arrhizus*; Lipase; Stability; Directed evolution; DNA shuffling; Error-prone PCR

1. Introduction

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are enzymes that catalyze reversibly the cleavage of ester bonds of triacylglycerol to yield free fatty acids, diacylglycerols, and monoacylglycerols. They have therefore been widely used in industrial applications, such as in chemical, food, pharmaceutical, and detergent industries [1–3]. However, the mostly reaction must be performed at a high temperature, so lipase that has a high thermostability and a high optimum temperature for lipase activity is required.

The lipases from *Rhizopus* species have been proved useful to produce structured lipids due to their strong 1,3-regiospecificity [1]. Many *Rhizopus* lipases genes were cloned and expressed in *Saccharomyces cerevisiae* and *Pichia pastoris*, such as lipases derived from *Rhizopus oryzae* (ROL) [4], *Rhizopus niveus* (RNL) [5] *Rhizopus delemar* (RDL) [6]. We also cloned RAL gene from *Rhizopus arrhizus* L-03-R-1 [7]. The cloned RAL comprises a prosequence of 97 amino acids and a mature lipase region of 269 amino acids, as deduced from the nucleotide sequence [Genebank: DQ489719]. The amino acids sequences of RAL from *R. arrhizus* L-03-R-1 were 97% identical to ROL from *R. oryzae* DSM 853 [Genebank: AF229435]. RAL and ROL exhibit many similarities in structure and biochemical properties. However, their substrate specificities, such as fatty acid-chain length preference, are different. We constructed an expression system for RAL in *P. pastoris*, with the aim of allowing the production of high amounts of active enzymes [7]. Because of lower thermal stability the wild-type RAL has not been used widely.

Numerous approaches to optimize lipase activity, selectivity and stability have been used with various degrees of success. For example, immobilization of the lipase on solid supports and the use of non-aqueous solvents have often resulted in noticeable changes in the enzymic activity and stability [8]. The combinatorial library of ROL mutated in the lid domain was displayed on yeast cell surface for altered substrate specificity [9]. Several rational protein engineering approaches applied to ROL and RDL have produced mutant proteins with altered substrate selectivity [10–12], but these improvements were relatively modest when compared with the results obtained by directed evolution technologies. Indeed, the application of random mutagenesis

* Corresponding author. Tel.: +86 10 6441 6691; fax: +86 10 6471 5443.
E-mail address: twtan@mail.buct.edu.cn (T.-W. Tan).

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or DNA shuffling, coupled with high-throughout screening, has often resulted in remarkable improvements in numerous characteristics of enzymes. For example, the activity and thermostability of *Candida antarctica* lipase B were improved by directed evolution [13,14]. Error-prone PCR applied to RNL has generated mutants, which the optimum temperature was higher by 15 °C than that of the wild-type lipase [15]. The mutated *N*-carbamyl-D-amino acid amidohydrolase has been industrialized because the thermostability and catalytic activity of enzyme was improved by directed evolution [16,17].

In this paper, we studied the application of directed evolution combined with high temperature screening to identify the mature RAL mutant with improved thermostability and optimum temperature. One amino acid substitution that would contribute to improve function is identified by site-directed mutagenesis. The mutational effect was interpreted using a simulated structural mode of the mutant.

2. Materials and methods

2.1. Strains, plasmids and media

Escherichia coli strain DH5α [F^-, *endA1*, *hsdR17* (r_k^-, m_k^+), *supE44*, *thi-1*, λ^-, *recA1*, *gyrA96*, *ΔlacU196*, *ϕ80dlacZΔM15*] was used as a host for recombinant DNA manipulation. *P. pastoris* GS115 and the pPICZαA vector used for the lipase gene expression and library construction were bought from Invitrogen (Beijing, China). The wild-type strain *R. arrhizus* L-03-R-1 was from our laboratory, and cultivated in PDA medium (200 g l^{-1} potato extract, 20 g l^{-1} glucose). The vector pMD-18T for cloning the lipase gene was supplied from Takara (Dalian, China). The recombinant *E. coli* containing plasmid pMD-18T-RAL was selected using LB medium supplemented with ampicillin, X-Gal and IPTG. The recombinant *E. coli* containing plasmid pPICZαA-RAL was selected using low salt LB medium (yeast extract 0.5%, tryptone 0.5%, NaCl 0.5%) supplemented 25 μg ml^{-1} Zeocin (Invitrogen, Beijing). The recombinant yeasts were selected using YPD–Zeocin medium (1% yeast extract, 2% peptone, 2% glucose) supplemented by 100 μg ml^{-1} Zeocin. For plate medium, 1.5% agar was added.

2.2. Cloning of the mature RAL gene

The mature RAL gene was amplified by PCR methodology using genome DNA from the *R. arrhizus* L-03-R-1 as a template and synthetic oligonucleotides as two primers (primer1: 5′-CTCG*GAATTC*TCTGATGGTGGTAAGGTTGTTGC-3′ and primer2: 5′-CAGT*GCGGCCGC*TTACAAACAGCTTCCTTC-GTT-3′). Restriction enzyme recognition sites, *Eco*RI and *Not*I are shown in italics. Total DNA from *R. arrhizus* L-03-R-1 was isolated by benzyl chloride method [18].

The PCR-amplified fragments were purified by V-gene PCR purification kit (V-Gene, China), and were cloned into the pMD-18T vector. The recombinant plasmid was named pMD-18T-RAL.

The pMD-18T-RAL was digested with *Eco*RI and *Not*I. The lipase gene fragments were purified and ligated into pPICZαA—linearized with the same enzymes to give vector pPICZαA-RAL. The lipase gene was in frame downstream of the α-factor prepro-sequence from *S. cerevisiae* (Fig. 1). The construction was checked by restricted digestion and DNA sequencing.

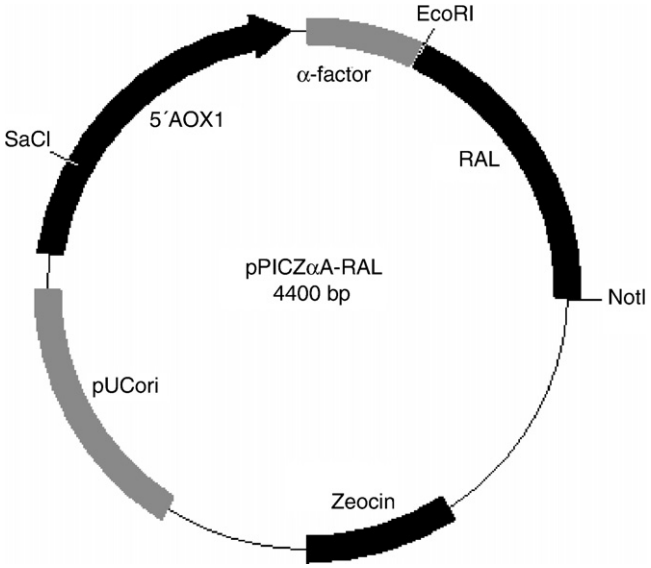

Fig. 1. Structure of plasmid pPICZαA-RAL. 5′AOX1 promoter is the methanol-inducible promoter, α-factor is the secretion signal sequence from *S. cerevisiae*, RAL indicates the protein-coding regions of the *R. arrhizus* mature lipase gene. Zeocin is the Zeocin resistance gene functional in *E. coli* and *P. pastoris*, and pUC ori is the origin of replication. *Sac*I is the restriction sites used to linearize the plasmid before electroporating into *P. pastoris*.

The constructed pPICZαA-RAL vector was linearized with *Sac*I, and then transformed into *P. pastoris* GS115 cells by electroporation using a 0.2 cm cuvette and GenePulser (Bio-Rad, USA). The transformed cells were plated on to YPD–Zeocin agar plates for isolation of the recombinant clones.

2.3. Library construction using error-prone PCR and DNA shuffling

The fragment of the mature RAL gene was obtained from the pMD-18T-RAL vector by PCR with primer1 and primer2. The purified product was then used as template for error-prone PCR test with the same primers. One hundred microlitres reaction mixture composed of 0.2 mM dNTP, 80 pmol primer, 0.1 μg template and 6 mM $MgCl_2$, 0.1 mM $MnCl_2$, 10× PCR buffer and 5 U Taq polymerase (Takara, China), is subjected to PCR under conditions: one cycle: 94 °C, 3 min; 35 cycles: 94 °C, 1 min, 55 °C, 1 min, 72 °C, 1 min; one cycle: 72 °C, 10 min [19]. The purified product of error-prone PCR was digested with bovine pancreas DNase I (Takara, China) by following procedure. Fifty microlitres volume of solution containing 2 μg DNA was mixed with 5 μl of 0.4 M Tris–HCl (pH 7.5) and 5 μl of 0.08 M $MgCl_2$; digestion was initiated with the addition of 1 μl of DNase I (0.02 U μl^{-1}) into the mixture at 25 °C; following incubation for 15 min, the digestion was terminated by heating the reaction solution to 90 °C for 10 min. The digested fragments were

separated by gel electrophoresis. The desired 100–300 bp DNA fragments were isolated and purified using the Omega gel extraction kit (Omega, USA).

Reassembly of the DNaseI digested fragments was conducted in a 50 μl reaction mixture containing 5 μl of 10× Pfu buffer, 6 μl of dNTP (2.5 mM each), 38 μl digested fragment DNA and 1 μl (5 U μl^{-1}) of Pfu polymerase. A progressive hybridization PCR was conducted under the following conditions: 94 °C for 3 min followed by 45 cycles of (94 °C for 30 s, 55–50 °C for 30 s, 72 °C for 60 s), and finally 72 °C for 10 min [20]. The reassembled reaction mixture (1 μl) along with primer1 and primer2 was used to amplify the full-length gene by PCR: 94 °C for 3 min followed by 30 cycles of (94 °C for 30 s, 55 °C for 30 s, 72 °C for 60 s), and finally 72 °C for 10 min. The PCR-amplified fragments were purified, and then digested with *Eco*RI and *Not*I.

Digested pPICZαA vector (300 ng) with *Eco*RI and *Not*I was combined with purified PCR fragments (250 ng) containing the full-length reassembled genes. The resulting DNA mixture was transformed into freshly prepared *E. coli* DH5α (80 μl) using the Bio-Rad GenePulser according to the manufacturer's instructions. The cell suspension was plated on LB–Zeocin plates and incubated at 37 °C for 24 h until the clones appeared. Transformants of *E. coli* were pooled and the plasmid DNA was isolated using a plasmid DNA preparation kit (Omega, USA). The recombinant plasmids (20 μg) were linearized with *Sac*I, and then transformed into *P. pastoris* GS115 cells by electroporation. The transformants were selected by plating and incubating on YPD–Zeocin medium plates at 30 °C for 3 days.

2.4. Enzyme expression and library screening

Fluorescent plate assay was adopted as the preliminary screening method to find mutants having lipase activity at high temperature in the presence of methanol. The positive transformants were replicated on the YPD plates and the originals stored. The duplicated plates were incubated for 2 day at 30 °C by adding 200 μl methanol. These plates were overlaid with fluorescent soft agar (YPD medium plate containing 5 g l^{-1} olive oil and 10 mg l^{-1} rhodamin B with 0.5% methanol) and cultured for 1 day at 50 °C [19], and the colonies forming the clear fluorescent halo were picked up from the original plates as positive clones. The second selection, these positive clones were cultivated in a BMGY medium (10 g l^{-1} yeast extract, 20 g l^{-1} Bacto peptone, 5 g l^{-1} glycerol, 4×10^{-5}% biotin) at 30 °C for 20 h to an optical density (OD_{600}) of 2–6. The culture was centrifuged at 1500 × *g* for 5 min at room temperature. The pellet was resuspended in 50 ml BMMY medium (10 g l^{-1} yeast extract, 20 g l^{-1} Bacto peptone, 4×10^{-5}% biotin). Lipase production was then induced by the addition of 5 g l^{-1} methanol at 30 °C for 48 h. Supernatant of each culture was collected by centrifugation at 1500 × *g* for 5 min. The lipase activity of each solution was measured at 35 and 45 °C, and those mutant strains which had higher activity at 45 °C than at 35 °C were selected for their lipase activity at high temperature.

2.5. DNA sequencing of the mutant lipase

The mutant RAL gene was amplified by PCR methodology using genome DNA from the recombinant GS115 as a template and synthetic oligonucleotides as two primers (5′*AOX1*: 5′-GACTGGTTCCAATTGACAAGC-3′ and 3′*AOX1*: 5′-CAAATGGVATTCTGACATCCT-3′). The purified PCR-amplified fragments were sequenced by using an ABI Prism 373A DNA Sequencer (Applied Biosystems, USA).

2.6. Enzyme purification

The culture supernatant of each mutant was concentrated by ultrafiltration on a 10,000 molecular weight cut-off membrane (Millipore, USA), and then dialyzed against 0.02 M sodium phosphate buffer (PBS, pH 6.0) at 4 °C overnight. The dialyzed solution was centrifuged at 12,000 × *g* for 15 min, and the supernatant was loaded on an SP Sepharose Fast Flow column (1.6 cm × 20 cm) equilibrated with 0.02 M PBS. Elution was performed with a linear gradient of 0–0.5 M NaCl at a rate of 2 ml min^{-1}. The active fractions were collected. The lipase solution was brought to 1.5 M saturation with $(NH_4)_2SO_4$, and applied to a Butyl Sepharose Fast Flow column (5 ml, Amersham, China) equilibrated with 1.5 M $(NH_4)_2SO_4$. Elution was performed with a linear gradient of 1.5–0 M $(NH_4)_2SO_4$ at a rate of 1 ml min^{-1}. The active fractions were pooled, and the purity of lipase was analyzed by SDS-PAGE.

2.7. Protein concentration determination

Protein measurements were carried out by the method of Bradford [21], using bovine serum albumin as standard. In chromatography experiment, the protein in fraction was measured by absorbance at 280 nm.

2.8. Analysis of N-terminal amino acid sequence

The recombinant lipase (wild-type RAL gene) were separated by means of SDS-PAGE, and blotted onto a PVDF membrane. The lipase bands were cut out and analyzed using a ABI proCise 492 cLC protein sequencer (Applied Biosystems, USA).

2.9. Lipase activity assay

Lipase activity was determined according to the olive oil emulsion method [22]. The fatty acids released were determined by titration with 50 mM NaOH solution. Each sample was assayed three times and the average value was taken. One unit of lipase activity was defined as the release of 1 μmol of fatty acid per minute.

The optimum temperature for lipase activity was found by measuring the activity while incubating over a temperature range of 30–60 °C in a water bath. The thermostability of lipase was determined as follows: approximately 1.0 ml of each purified enzyme preparation (0.1 mg ml^{-1} protein in 50 mM PBS, pH 7.0) was placed into a 1.5 ml microcentrifuge tube and incubated at 50 °C in a water bath. Periodically, 0.1 ml aliquots were removed, and then stored at −20 °C for further analysis.

2.10. Construction of the chimeric enzymes

Three chimeric lipases of single site mutation at amino acid positions 9, 190 and 225 of RAL from wild-type plasmid pPICZαA-RAL were conducted by PCR with appropriate primers and Takara MutanBEST Kit (Dalian, China) according to the manufacturer's instructions. Three chimeric lipases were named CL-1 (A9T), CL-2 (E190V) and CL-3 (M225I), respectively. Each chimeric plasmid was transformed to *P. pastoris* GS115 according to the above-mentioned method (Section 2.3), and each chimeric lipase was purified and characterized.

2.11. Prediction of three-dimensional structure

The three-dimensional structure of WT-RAL and mutant RAL was obtained by comparative protein modeling with the program SwissModel (http://swissmodel.expasy.org//SWISS-MODEL.html) using as template lipases with the homologous amino acid sequence of 1LGY (*R. niveus* lipase) and 4TGL (*Rhizomucor miehei* lipase), whose structures had been solved by X-ray diffraction. 1LGY and 4TGL have 98.5 and 59% of homology in amino acid sequence with lipase from *R. arrhizus* L-03-R-1. Both of 1LGY and 4TGL structure were used simultaneously to model RAL molecules.

3. Results

3.1. Sequence analysis and expression of the mature RAL in P. pastoris

S. cerevisiae was successfully used for expressing active ROL having the full-length prosequence (97 amino acids) [23]. Although the mature ROL gene fused with the pre-α-factor-encoding gene was not functionally expressed in *S. cerevisiae*, but the same construction functioned in *P. pastoris* [24]. To circumvent this problem and to develop a high-level expression system suitable for high-throughput screening we cloned the mature RAL gene from strain *R. arrhizus* L-03-R-1 into a *P. pastoris* expression system.

The amino acids sequences of the cloned RAL from *R. arrhizus* L-03-R-1 were 97% identical to ROL from *R. oryzae* DSM 853 [4]. The amino terminal sequence of the recombinant RAL was Glu–Ala–Glu–Ala–Glu–Phe–Ser–Asp–Gly–Gly–Lys–Val–Val, and the last seven residues corresponded to the N-terminal of the original mature RAL from *R. arrhizus* L-03-R-1 [7]. It proved that the α-factor signal sequence was cleaved correctly at the Kex2 cleavage site. No significant differences in the expression levels of the WT-RAL and the mutants were observed.

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1   TCTGATGGTGGTAAGGTTGTTGCTGCTACTACTGCTCAGATCCAAGAGTTCACCAAGTAT
    S  D  G  G  K  V  V  A  A  T  T  A  Q  I  Q  E  F  T  K  Y
61  GCTGGTATCGCTGCCACTGCCTACTGTCGTTCTGTTGTCCCTGGTAACAAGTGGGATTGT
    A  G  I  A  A  T  A  Y  C  R  S  V  V  P  G  N  K  W  D  C
121 GTCCAATGTCAAAAGTGGGTTCCTGATGGCAAGATCATCACTACCTTTACCTCCTTGCTT
    V  Q  C  Q  K  W  V  P  D  G  K  I  I  T  T  F  T  S  L  L
181 TCCGATACAAATGGTTACGTCTTGAGAAGTGATAAACAAAAGACCATTTATCTTGTTTTC
    S  D  T  N  G  Y  V  L  R  S  D  K  Q  K  T  I  Y  L  V  F
241 CGTGGTACCAACTCCTTCAGAAGTGCCATCACTGATATCGTCTTCAACTTTTCTGACTAC
    R  G  T  N  S  F  R  S  A  I  T  D  I  V  F  N  F  S  D  Y
301 AAGCCTGTCAAGGGCGCCAAAGTTCATGCTGGTTTCCTTTCCTCTTATGAGCAAGTTGTC
    K  P  V  K  G  A  K  V  H  A  G  F  L  S  S  Y  E  Q  V  V
361 AATGACTATTTCCCTGTCGTTCAAGAACAATTGACCGCCCACCCTACTTATAAGGTCATC
    N  D  Y  F  P  V  V  Q  E  Q  L  T  A  H  P  T  Y  K  V  I
421 GTTACCGGTCACTCACTCGGTGGTGCACAAGCTTTGCTTGCCGGTATGGATCTCTACCAA
    V  T  G  H  S  L  G  G  A  Q  A  L  L  A  G  M  D  L  Y  Q
481 CGTGAACCAGGGTTGTCTCCCAAGAATTTGAGCATCTTCACTGTCGGTGGTCCTCGTGTT
    R  E  P  G  L  S  P  K  N  L  S  I  F  T  V  G  G  P  R  V
541 GGTAACCCCACCTTTGCTTACTATGTTGAATCCACCGGTATCCCTTTCCAACGTACCGTT
    G  N  P  T  F  A  Y  Y  V  E  S  T  G  I  P  F  Q  R  T  V
601 CACAAGAGAGATATCGTTCCTCACGTTCCTCCTCAATCCTTCGGATTCCTTCATCCCGGT
    H  K  R  D  I  V  P  H  V  P  P  Q  S  F  G  F  L  H  P  G
661 GTTGAATCTTGGATGAAGTCTGGTACTTCCAACGTTCAAATCTGTACTTCTGAAATTGAA
    V  E  S  W  M  K  S  G  T  S  N  V  Q  I  C  T  S  E  I  E
721 ACCAAGGATTGCAGTAACTCTATCGTTCCTTTCACCTCTATCCTTGACCACTTGAGTTAC
    T  K  D  C  S  N  S  I  V  P  F  T  S  I  L  D  H  L  S  Y
781 TTTGATATCAACGAAGGAAGCTGTTTGTAA
    F  D  I  N  E  G  S  C  L  -
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Fig. 2. Sequence of the mature lipase gene from *R. arrhizus*. The mutate nucleotides and the corresponding amino acids were underlined.

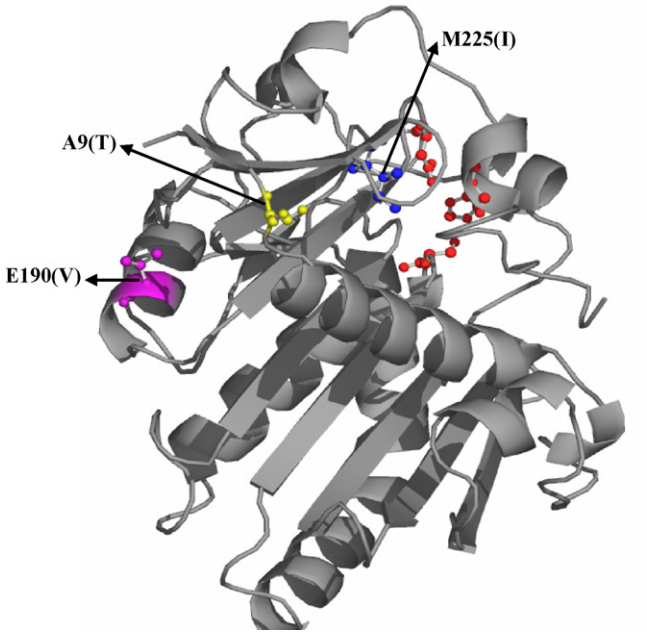

Fig. 3. Schematic illustration of the structure of the mutant RAL. This mutant structure was derived from the structure of the native *R. niveus* lipase. Presented by a ball and stick are the site of mutation (A9T, E190V, M225I) and the catalytic center (Ser145, Asp204, His257).

3.2. Screening of mutant library and isolation of the mutant lipase

Error-prone PCR mutagenesis and DNA shuffling were used for generating libraries of the mature RAL mutants. The mutant library was constructed using the WT-RAL gene as template. The error-prone PCR conditions were selected to achieve a nucleotide mutational rate of ~0.2% (determined by sequencing 10 randomly selected clones), and then DNA shuffling was used for generating library as described in Section 2.

An improvement in stability and the lipase activity at high temperature was achieved by means of fluorescent plate assay from recombinant cells carrying a randomly mutated gene. The prelimimary selection, based on the fluorescent plate assay at 50 °C, identified six positive clones which had clear fluorescent circle from among about 5500 transformants. One positive clone from the first six positive clones was selected for the second selection based on the lipase activity at 45 °C. The ratio of the activity of this mutant at 45 °C against that at 35 °C was 1.16, while that of the WT-RAL was only 0.44.

The genome was extracted from the selected mutant, and the DNA sequence of the inserted fragment was determined. Four base changes (*G25A*, *A569T*, *A570T*, *G675C*), resulting in the replacement of three amino acids (A9T, E190V, M225I), were found in this randomly mutated gene. Sequence of the mature lipase gene from *R. arrhizus* was shown in Fig. 2. The mutate nucleotides and the corresponding amino acids were underlined. The amino acid substitution was located in the N-terminal region (A9T) and C-terminal region (E190V and M225I). Furthermore, the mutated amino acid (M225I) was near the active center of RAL in its tertiary structure (Fig. 3).

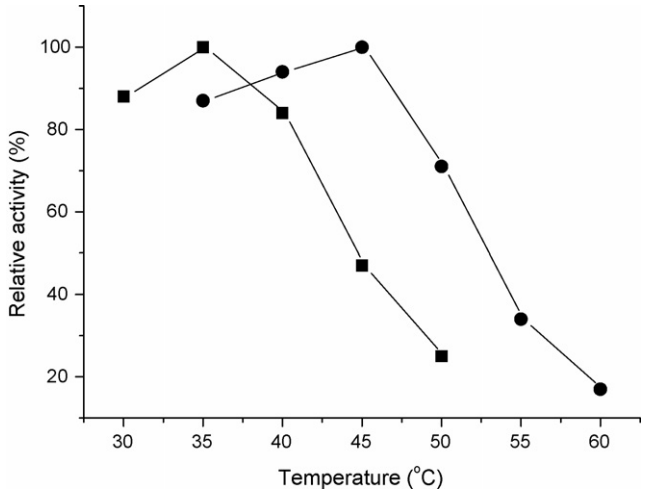

Fig. 4. Temperature dependence of lipase activity. Shown are the WT-RAL (filled squares) and randomly mutant RAL (filled circles). The activity was assayed at the indicated temperature for 10 min. Activity values are each given as a percentage of the most active sample. The most active samples of each lipases were 2500 U mg^{-1} of protein at 35 °C for WT-RAL and 2050 U mg^{-1} of protein at 45 °C for the mutant RAL.

3.3. Purification and characterization of the randomly mutated lipase

The WT-RAL and randomly mutated lipases were purified using SP Sepharose Fast Flow chromatography and Butyl Sepharose Fast Flow chromatography as described in Section 2. The specific activity at 35 °C of the randomly mutated lipase was 1850 U mg^{-1} of protein, this value being 75% that of the wild-type lipase (2500 U mg^{-1}).

The optimum temperature of the randomly mutated lipase was 45 °C, this value being 10 °C higher than that of the WT-RAL (Fig. 4). Irreversible thermal inactivation of WT-RAL and the mutants was characterized by incubating each enzyme in an aqueous buffer at 50 °C. The inactivation profiles of the WT-RAL and mutant RAL were presented in Fig. 5. Stability of the

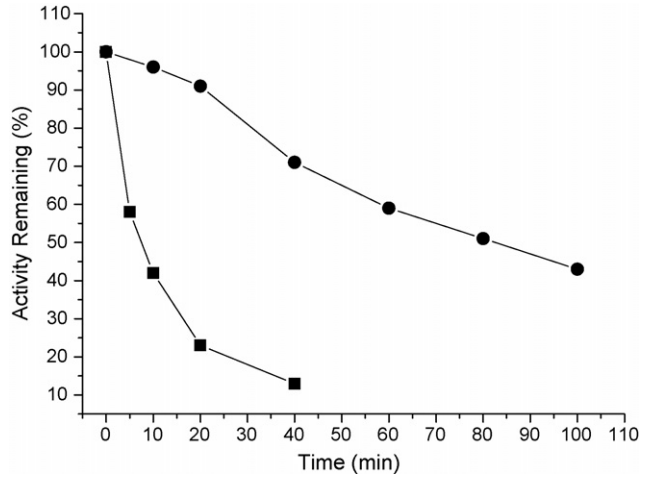

Fig. 5. The thermal inactivation profiles of WT-RAL (filled squares) and the mutant RAL (filled circles) at 50 °C (for experimental details see Section 2). The values are averages of three replicates.

Table 1
The $T_{1/2}$ of WT-RAL and some mutants

Lipase	Amino acid changes	$T_{1/2}$ (min)[a]
WT-RAL	–	7
Mutant	A9T, E190V, M225I	83
CL-1	A9T	7
CL-2	E190V	76
CL-3	M225I	9

[a] $T_{1/2}$ values are based on the inactivation experiment presented in Section 2.9.

mutant RAL against irreversible inactivation was significantly higher than that of the WT-RAL. The approximated $T_{1/2}$ at 50 °C of the WT-RAL, mutant lipase and chimeric lipases were presented in Table 1. The half-life ($T_{1/2}$) at 50 °C of the mutant RAL exceeded those of WT-RAL by 12-fold.

3.4. Construction and characterization of the chimeric enzymes

Three chimeric enzymes were constructed to confirm the position in the randomly mutated lipase that was responsible for the thermostability and the activity at high temperature. The amino acid substitution of one mutative lipase (CL-1) was Ala 9 to Thr in the N-terminal region of the lipase. The position of amino acid substitutions in the other two mutated enzymes (CL-2, CL-3) was Glu190 to Val and Met225 to Ile in the C-terminal region of the lipase, respectively. Each of mutated lipase was purified, and the thermostability and the optimum temperature for lipase activity were measured. The enzymatic characteristics for the thermostability and the optimum temperature of CL-1 and CL-3 were almost same as those of the WT-RAL, while that of CL-2 was almost same as those of the randomly mutated lipase. Consequently, one amino acid substitution E190V was revealed to have enhanced the thermostability and the optimum temperature for lipase activity.

4. Discussion

We have constructed a combinatorial library producing active RAL through a *P. pastoris* expression system, and succeeded in creating a lipase that could maintain its activity at a high temperature by error-prone PCR and DNA shuffling methods. One amino acid substitution (E190V) responsible for the thermostability and the higher optimum temperature of the enzyme activity was identified by the chimeric enzymes.

The lipase from *R. arrhizus* L-03-R-1 has 98.5% of homology with RNL in amino acid sequence. The three-dimensional structure of RNL has already been resolved [25]. Consequently, the 3D structure of WT-RAL and the mutant RAL could be easily predicted by comparing it with the structure of RNL. In this study, the mutate amino acids (A9T and E190V) were on the surface of the protein and the mutate amino acid (M225I) was inside the protein structure (figure not shown). The position (E190V) at which the mutation took place for improving the thermostability and the optimum temperature was far from the catalytic center of the enzyme in its tertiary structure, but the position (M225I) was near of the catalytic center (Fig. 3). The replacement of Met225 with Ile may result in the conformational change of the catalytic center of the enzyme. In fact, the specific activity of the mutant RAL was only 75% that of the WT-RAL.

There are two assumptions that can be made to explain the improved thermostability and the optimum temperature for RAL by only an amino acid substitution. The first assumption is stabilization of the α-helix. The position (E190V) is on the α-helix as shown in Fig. 3. Hydrophobic interaction is crucial for stabilizing of α-helix. Consequently, the replacement of the hydrophilic amino acid (Glu) with the hydrophobic amino acid (Val) would be most likely to enhance the interaction between hydrophobic side chains at intra-helical spacing. In this helix, Ala186 and Tyr187 near the mutated amino acid (Glu190Val) were the hydrophobic amino acids. Therefore, intra-helical hydrophobic interaction was enhanced by the substitution of Glu with Val. Consequently, the α-helix have been stabilized and the thermostability of lipase was improved.

The second assumption to explain the thermal stability of the mutant is strengthening the interaction between secondary structures. We compared the conformational change near the mutated amino acid (E190V) between WT-RAL with the mutant RAL (Fig. 6). The result is that a loop structure (from Ser237 to Thr241) adjacent to the α-helix was stabilized for a conforma-

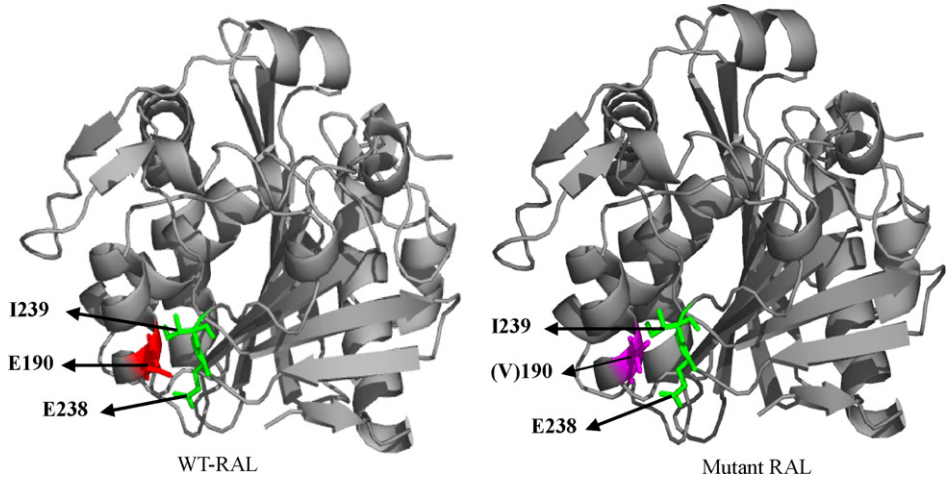

Fig. 6. The comparison of the conformational change near the mutated amino acid (E190V) between WT-RAL with the mutant RAL.

tional change. In WT-RAL, the distance between the ε-oxygen of Glu190 and the ε-oxygen of Glu238 in this loop structure is only 2.91 Å. Since Glu repels in the neutral pH range, the loop structure may become unstable. The distance between the γ-carbon of valine and the β-carbon of Glu238 was likely too short to maintain a stable structure in respect of the unchanged main-chain torsion angles when Glu190 was replaced with Val. To give greater stability the side-chain torsion angle of the replaced valine may be changed. Consequently, hydrophobic interaction between the γ-carbon of valine and the δ-carbon of Ile239 was created by the conformational change of side chain (Fig. 6). So the stabilization of the loop structure was enhanced by the replacement of Glu with Val. From Lys242 to Leu269 is a random structure. His257, which is located in this random structure, is one amino acid residue of the catalytic triad of an enzyme. Therefore, the stabilization of the loop structure (from Ser237 to Thr241) is suggested to be important for the lipase activity at high temperature.

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