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Molecular engineering of *Rhizopus oryzae* lipase using a combinatorial protein library constructed on the yeast cell surface

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Abstract

We constructed a combinatorial yeast library through cell-surface display of the pro- and mature region of lipase from *Rhizopus oryzae* (ProROL) and obtained clones retaining lipase activity in fluorescent plate assay. The initial reaction rates of hydrolysis and methanolysis could be measured directly as whole-cell biocatalyst without complex treatments such as concentration, purification, and immobilization. The selected clones showed wide-ranging variation of reaction specificity. The K138R mutant showed a 1.3-fold shift of reaction specificity toward methanolysis compared to the wild type, while the V-95D, I53V, P-96S/F196Y, and Q128H/Q197L mutants showed shifts toward hydrolysis of 1.6–5.9-fold. Predictions of the mutants' three-dimensional structure suggested that the hydrogen-bond distance between threonine 83 and aspartic acid 92 may influence reaction specificity, which shifted toward hydrolysis in mutants where this distance was shorter than in the wild type, but toward methanolysis where it was longer. The positions of amino acid residues (aa) 53, 138 and 196 in ProROL are considered the sites that influence hydrogen-bond distance and change reaction specificity. Construction of a surface-displayed combinatorial library in yeast cells is thus a powerful tool in accelerating the combinatorial approach to enzyme engineering and novel whole-cell biocatalyst development.

Keywords: *Saccharomyces cerevisiae*; Lipase; *Rhizopus oryzae*; Whole-cell biocatalyst; Combinatorial library

1. Introduction

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are enzymes that catalyze a variety of reactions, such as hydrolysis of ester bonds, transesterification, and ester synthesis at the interface between substrate and water or in non-aqueous organic solvent [1]. They have therefore been used in a wide range of processes in the chemical, pharmaceutical, detergent, and other industries [2–5], but since their wide-ranging catalytic reactions make the formation of by-products unavoidable, there have been attempts to create lipases that specifically catalyze the target reaction.

Tailor-made enzymes with optimized performance in particular industrial processes can be obtained by various methods such as chemical mutagenesis, ultraviolet irradiation, and creation of mutator strains. In recent years, it has become popular to create mutant libraries by molecular biological methods and to screen for target clones with improved reactivity or reaction specificity [6]. Widely used methods of introducing nucleic mutations into the template sequences encoding the target proteins include DNA shuffling, which recombines blocks of parent-sequence fragments by self-priming polymerase chain reaction (PCR) [7,8]; staggered extension PCR (StEP), which creates template sequences through template switching by brief polymerase-catalyzed primer extension [9]; and error-prone PCR, which uses low-fidelity polymerization conditions [10]. In many cases, however, the screening operation is rate-limiting. A procedure to purify and concentrate the proteins produced is necessary in secretory expression systems and permeabilization in intracellular expression systems [11], additional steps which are barriers to high-throughput screening using conventional methods.

In our previous study, the pro- and mature region of lipase from *Rhizopus oryzae* (ProROL) was successfully

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surface-displayed on yeast cells by fusing the flocculation functional region of Flo1 protein to the N-terminus of ProROL [12], so that the ProROL immobilized on the yeast cell surface could be selected efficiently with no need for purification, concentration or permeabilization, and the yeast cells displaying ProROL utilized as whole-cell biocatalyst. Since the cells with novel catalytic function harbor the gene of the novel ProROL, screening and analysis of the mutated genes can be carried out efficiently. In the present paper, we report a screening method for novel mutant lipase based on a *Saccharomyces cerevisiae* cell-surface displayed mutant library.

2. Materials and methods

2.1. Strains and media

A *ProROL* gene consisting of the genes encoding the pro region (amino acid residues (aa) −97 to −1), which supports folding, and the mature ROL region (aa 1–269) was cloned from *R. oryzae* IFO4697. *S. cerevisiae* MT8-1 (*MATa ade his3 leu2 trp1 ura3*) was used for cell-surface display of mutagenized Flo1-ProROL fusion proteins. Yeasts were grown in complete medium (YPD: 1% yeast extract, 2% peptone, 2% glucose), or minimal medium (SD: 0.67% yeast nitrogen base supplemented with appropriate amino acids and nucleotides, 2% glucose unless otherwise noted). For plate medium, 2% agar was added.

2.2. Construction of expression vector

Error-prone PCR was carried out to introduce approximately three base mutations (corresponding to 1–2-amino acid mutations per protein) in the *ProROL* gene of pWGP3ProROL [11]. Two synthesized oligonucleotides, pGAP (5′-CACCAAGAACTTAGTTTCG) and tGAP (5′-GTAAATTCACTTAAGCC), were used as the primers. Fifty picomoles of each primer was added together with 10 ng of the pWGP3ProROL template gene to a 100-μl PCR reaction mixture. The reaction conditions were: 5.5 mM $MgCl_2$, 0.1 mM $MnCl_2$, 0.2 mM dATP, 1.0 mM dCTP, 1.0 mM dTTP, 1.0 mM dGTP, 10 × buffer, 5 U Taq polymerase (Takara Shuzo Co. Ltd., Shiga, Japan). The reaction was thermocycled for 30 cycles of: 94 °C, 1 min; 40 °C, 2 min; and 72 °C, 2 min [10,13]. The amplified *ProROL* gene fragments containing nucleic replacement mutants were digested with *Bam*HI and *Sal*I and inserted into the *Bgl*II and *Xho*I sites of pWIFS [12] (Fig. 1).

2.3. Construction of combinatorial yeast library

The expression plasmids prepared as described above were transformed into *S. cerevisiae* MT8-1 cells using YEAST MAKER™ (Clonetech Laboratories Inc, Palo Alto, CA) according to the protocol specified by the supplier. The transformants were selected by plating and incubated on SD medium plates for 2 days, after which the plates were duplicated and the originals stored. The duplicated cultures were then grown on SD plates for 3 days and screened as described below.

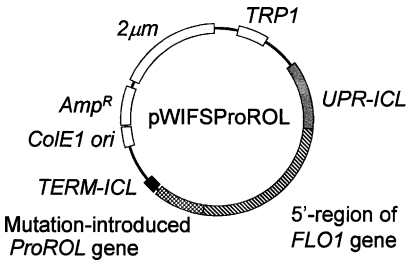


Fig. 1. Expression plasmid pWIFSProROL for surface display of mutagenized ProROL via flocculation functional domain of Flo1 protein anchor.

2.4. Fluorescent plate assay

Fluorescent plate assay was adopted as the first screening to find mutants active in the presence of methanol. The duplicated plates were overlaid with fluorescent soft agar (SD medium plate containing 2.5% soybean oil and 0.001% rhodamin B with or without 50% methanol) and exposed to ultraviolet light of wavelength 350 nm, whereupon active clones fluoresced a light orange color [14].

2.5. Cultivation

Clones selected by fluorescent plate assay were precultured in SD medium at 30 °C for 30 h (OD_{600} > 1.5) and used as starter to incubate 100 ml of SDC medium (SD medium containing 2% casamino acids) in a 500-ml shake flask to give initial OD_{600} of 0.03. Initial concentration of glucose was 0.5%. After cultivation for 8 days, the clones were collected, washed with distilled water, and lyophilized.

2.6. Measurement of lipase activity

For the measurement of methanolysis activity, 16.5 mg-dry cell weight of lyophilized cells displaying mutagenized ProROLs was suspended in 0.5 ml of acetate buffer (0.1 M, pH 7.0) and used as a catalyst. The yeast-cell suspension was added to a mixture of soybean oil and methanol (4.825/0.175 g/g = 1/1, mol/mol) and reaction carried out in a 30-ml screw vial at 37 °C and 140 rpm. Hydrolysis activity of ProROL was measured in the same way but with 0.098 g distilled water instead of methanol.

The methanolysis and hydrolysis products were analyzed by capillary gas chromatography as described below [15]. Aliquots of 150 μl were taken from the reaction mixture at the specified times and centrifuged to obtain the upper layer; 120 μl of this and 20 μl of tricaprylin were then precisely measured into a 10-ml bottle, to which were added a specified amount of anhydrous sodium sulfate as a dehydrating

agent and 3.0 ml of hexane. Tricaprylin served as the internal standard for the capillary gas chromatography, in which 1 μl of treated sample was injected into a GC-18A gas chromatograph (Shimadzu Co., Kyoto, Japan) connected to a DB-5 capillary column (∅ 0.25 mm, 10 m; J&W Scientific, Folsom, CA) for determination of methylester and free fatty acid content in the reaction mixture. The column temperature was held at 150 °C for 0.5 min, raised to 300 °C at 10 °C/min, and maintained at this temperature for 3 min. The temperatures of the injector and detector were set at 245 and 320 °C, respectively.

2.7. Prediction of three-dimensional structure

The three-dimensional structure of the mutants was obtained by comparative protein modeling with the program MODELLER6 (Andrej Šali, The Rockefeller University, http://guitar.rockefeller.edu/modeller/modeller.html) using as template lipases with the homologous amino acid sequences of 1LGY (*R. niveus* lipase) and 4TGL (a complex with *Rhizomucor miehei* lipase and diethylphosphate, a substrate analog), whose structures had been solved by X-ray diffraction. 1LGY and 4TGL have 99 and 55% of homology with ROL from IFO4697 in amino acid sequence. 4TGL was used to predict the ROL–diethylphosphate complex structure. Both of 1LGY and 4TGL were used concurrently to model mutated ROL molecules. The models were used to estimate the hydrogen-bond distances around the active site.

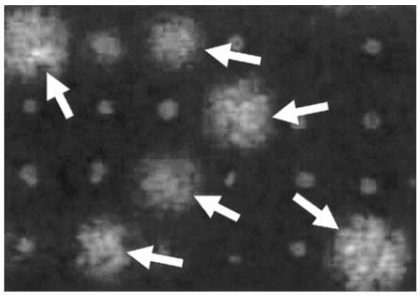
3. Results

3.1. Construction of combinatorial yeast library

A combinatorial yeast library with cell-surface display of mutagenized ProROL was constructed to select ProROL mutants with improved substrate specificity in catalytic reactions. To investigate the diversity of the constructed library, a replica library was covered with fluorescent soft agar without methanol and the mutants exposed to ultraviolet light, whereupon clones maintaining lipase activity fluoresced a light orange color. 45.6% of all mutants showed a detectable lipase activity on the screening plate. When the sequence of the ProROL mutants was determined, the average mutation number in the DNA molecule encoding ProROL (1101 bp) was approximately 3 bp and the maximum 6 bp.

3.2. Fluorescent plate assay

Enzyme molecules generally have both hydrophilic portions, which contact with water, and hydrophobic portions, which fold inside the molecule in aqueous solutions [16]. In the presence of organic solvents or in organic solvent, however, the hydrophobic portion may be exposed and the proteins inactivated by unfolding. ROL is inactivated in methanolysis reaction by methanol, an amphipathic solvent [15]. For the efficient selection of clones maintaining lipase activity in the presence of methanol, a screening assay plate containing 50% methanol was used for the first screening (Fig. 2). Of a total of 713 clones, 11.2% or 80 were positive, that is showed lipase activity in the presence of 50% methanol, and these were used for further investigation.



Total	Positive
713	80 (11.2%)

Fig. 2. Screening of mutants using fluorescent plate assay. In clones maintaining lipase activity, a fluorescent halo formed around the colony (arrowheads). The proportion of positive mutants among all clones is shown in the lower table.

3.3. Change of reaction specificity

The reaction profiles of the 80 ProROL mutants obtained in the first fluorescent plate assay were examined for initial reaction rate of methanolysis and hydrolysis. Table 1 shows the initial reaction rates of 11 ProROL mutants which show the obviously different ratio of metanolysis and hydrolysis activity from that of wild type. For instance, compared with the wild type, a ProROL mutant with two replacements,

Table 1
Variation of hydrolysis and methanolysis activity of mutagenized ProROL displayed on yeast cell surface

Mutant	Initial reaction rate (μM/min)		Methanolysis/ hydrolysis ratio
	Methanolysis	Hydrolysis	
WT	2.27	5.48	0.41
V-95D	0.86	3.42	0.25
S-88P	3.71	10.5	0.35
K-62E	1.39	4.38	0.32
E-44G	2.08	6.78	0.31
K-30E	1.62	5.47	0.30
153V	0.79	10.7	0.07
K138R	1.84	3.43	0.54
S237T	1.46	4.16	0.35
P-96S/F196Y	2.19	10.6	0.21
W-43R/VI26A	2.25	6.55	0.34
Q128H/Q197L	1.13	6.98	0.16

The data represent the averages of two independent experiments.

namely prorine-96 replaced by serine and phenylalanine 196 by tyrosine (P-96S/F196Y), showed 1.9-fold higher initial reaction rate in hydrolysis, but nearly the same initial reaction rate in methanolysis.

The variation in initial reaction rates, however, can be affected by the amount of displayed lipase molecule. Since it seems difficult to measure an accurate amount of displayed ROL on yeast cell surface, so specific reaction specificity was calculated. The ratio of methanolysis to hydrolysis activity of the selected clones is also shown in Table 1. The variation in the ratios indicates the shifts in the reaction specificity of the mutants. Compared with the wild type, K138R showed a 1.3-fold shift in reaction specificity toward methanolysis, while V-95D, I53V, P-96S/F196Y, and Q128H/Q197L exhibited 1.6–5.9-fold shifts toward hydrolysis reaction.

3.4. Prediction of three-dimensional structure

A prediction was made of the three-dimensional structure of the mutants, which greatly influences reaction specificity. In cell-surface display of FSProROL, ROL was produced as a fusion protein with the FS (amino acids 1 to 1099 of Flo1p) and pro-sequence. However, of the proteins used as template, only mature lipase has a registered three-dimensional structure. Modeling was therefore undertaken with clones having a mutation in the mature ROL region. Therefore V-95D, replacement only in pro-region, could not be modeled and P-96S/F196Y, former replacement of two is in pro-region, was predicted as F196Y.

The hydrogen-bond distances around the catalytic triad of ROL (serine 145, aspartic acid 204, and histidine 257) were estimated by prediction of the three-dimensional structure (Table 2). The distance between threonine 83 Oγ and aspartic acid 92 Oδ1 was markedly altered, even though the other hydrogen-bond distances were hardly changed at all. The distance between threonine 83 Oγ and aspartic acid 92 Oδ1 in the hydrolysis-shifted clones I53V and F196Y was 3.66 Å and 4.27 Å, respectively, shorter than in the wild type (4.41 Å). On the other hand, the methanolysis-shifted clone K138R had the distance changed to 4.49 Å, longer than in the wild type.

Table 2
Comparison of important hydrogen-bond distances in structural models of the ROL–diethylphosphate (DEP) complex

Donor–acceptor	Calculated hydrogen-bond distance (Å)				
	WT	I53V	K138R	F196Y	Q128H/Q197L
① Thr 83 Oγ–DEP[a] O2	4.28	4.27	4.27	4.24	4.24
② Thr 83 N–DEP O2	2.84	2.81	2.85	2.85	2.85
③ Leu146 N–DEP O2	3.44	3.46	3.43	3.44	3.44
④ Thr 83 Oγ–Asp 92 Oδ1	4.41	3.66	4.49	4.27	4.42
⑤ His 257 Nε–DEP O1	3.35	3.35	3.34	3.35	3.35
⑥ His 257 Nε–Ser 145 Oγ	3.17	3.10	3.17	3.18	3.15
Reaction specificity shift		H[a]	M[b]	H	H

[a] Hydrolysis.
[b] Methanolysis.

4. Discussion

We have constructed a combinatorial yeast library producing active lipase through a cell-surface display system. The frequency of inactive clones is generally correlated to point mutagenesis rate [6]. The introduction of approximately three base pairs promotes 1–2-amino acid replacement, a rate effective in maintaining lipase activity and changing function. 45.6% of all mutants showed detectable lipase activity on screening plate, and 11.8% of all mutants retained lipase activity in the presence of methanol (Fig. 2). Mutants with 1–2-amino acid replacement also showed variation in initial reaction rate and reaction specificity of lipase (Table 1). We confirmed that directed molecular evolution using a cell-surface display system is reliable for optimization of enzymes. This system, in which the enzyme molecule is immobilized spontaneously and can be easily prepared without separation, concentration, or immobilization, is advantageous for rapid screening of cell-surface displayed lipase with novel catalytic function.

In industrial production processes, cost-effectiveness is improved if the amount of by-product is minimized. In esterification and ester synthesis, hydrolysis reaction proceeds simultaneously in the presence of water, which leads to by-production of acylcarboxylate. It has been previously reported that a 4% water content is necessary for a sufficient reaction rate in methanolysis [15]. Water content also affects the equilibrium position of these reactions as well as the distribution of the product in the media [17], and a shift in reaction specificity is therefore commercially important.

Lipases have lid domains that border the catalytic pocket. At the interface between the hydrophobic substrate and water, the lid domains are opened and the substrates attack the catalytic pocket. The catalytic mechanism of ROL is accounted for as follows [18]: in ROL, when substrate combines with the substrate binding region, a tetrahedral oxyanion intermediate is formed by the action of histidine 257 and stabilized by the threonine 83γ OH (giving up a hydrogen bond to aspartic acid 92) together with the backbone amides of threonine 83 and leucine 146; threonine 83γ reforms its hydrogen bond to aspartic acid 92 and releases the alcohol component; a second tetrahedral transition state is then formed by attack on a water or methanol molecule of the acyl-enzyme and fatty acid released in the same manner (Fig. 3). As shown in Table 2, in the 153V and F196Y mutants, the hydrogen-bond distance between threonine 83 and aspartic acid 92 is shortened and the reaction specificity shifted toward hydrolysis. In the K138R mutation, on the other hand, the distance is increased and the specificity shifted toward methanolysis. This result suggested to us

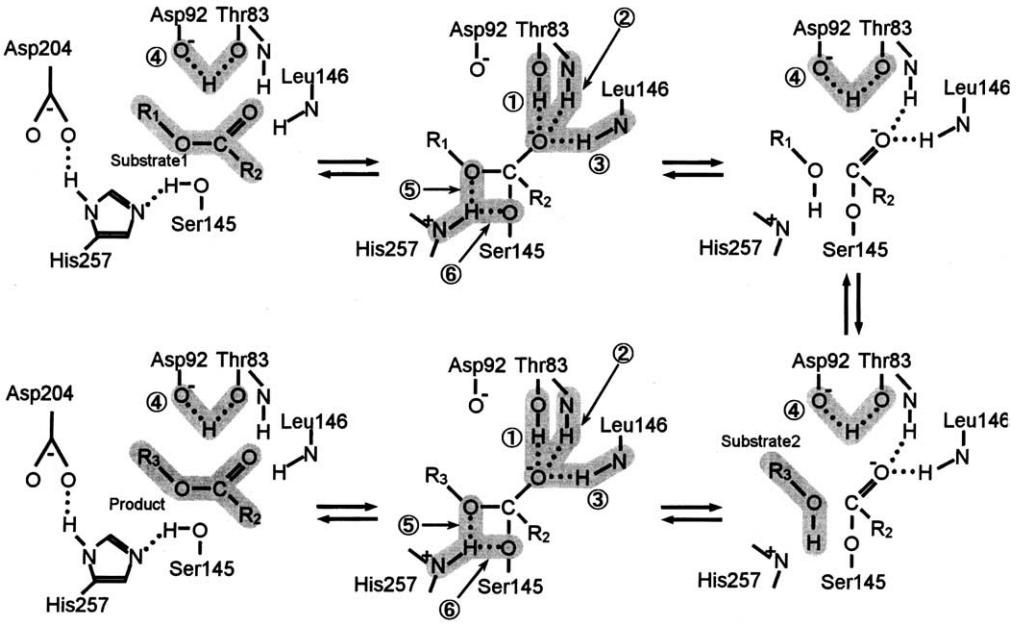


Fig. 3. Schematic representation of ROL ester-cleavage reaction pathway. Hydrogen bonds between Thr83 Oγ and diethylphosphate (DEP) O2 ①, Thr83 N and DEP O2 ②, Leu146 N and DEP O2 ③, Thr83 Oγ and Asp92 Oδ1 ④, His257 Nε and DEP O1 ⑤, His257 Nε and Ser145 Oγ ⑥.

that an important factor in shifting reaction specificity is the hydrogen-bond distance between threonine 83 and aspartic acid 92. The cleavage of this hydrogen bond affects the formation of oxyanion hole that stabilizes first tetrahedral intermediate [18]. A change in this hydrogen-bond distance may influence the stability of tetrahedral intermediate and the attack by a water or methanol molecule on this intermediate. The amino acid residue positions 53, 138, and 196 in ProROL are considered the hot spots that change the hydrogen-bond distance between threonine 83 and aspartic acid 92, which in turn affects reaction specificity. These residues seemed to be laid outside of catalytic pocket in predicted models. Replacements of these positions may cause a distortion in catalytic pocket. By rational design on the basis of hot spot analysis, we should be able to obtain more effective lipases with altered reaction specificity.

In the present study, we efficiently obtained reaction specificity-modified lipase from a combinatorial enzyme library displayed on the yeast cell-surface, in which the cell displaying an engineered enzyme on its surface harbors the gene encoding the displayed enzyme. This makes possible structural and functional analyses and directed selection of engineered proteins. It is also advantageous for industrial processes that the clones obtained are applicable as whole-cell biocatalyst.

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