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Received 7 December 2004/Accepted 28 February 2005

Immobilization of enzymes on some solid supports has been used to stabilize enzymes in organic solvents. In this study, we evaluated applications of genetically immobilized *Rhizopus oryzae* lipase displayed on the cell surface of *Saccharomyces cerevisiae* in organic solvents and measured the catalytic activity of the displayed enzyme as a fusion protein with α-agglutinin. Compared to the activity of a commercial preparation of this lipase, the activity of the new preparation was 4.4 × 104-fold higher in a hydrolysis reaction using p-nitrophenyl palmitate and 3.8 × 104-fold higher in an esterification reaction with palmitic acid and n-pentanol (0.2% H2O). Increased enzyme activity may occur because the lipase displayed on the yeast cell surface is stabilized by the cell wall. We used a combination of error-prone PCR and cell surface display to increase lipase activity. Of 7,000 colonies in a library of mutated lipases, 13 formed a clear halo on plates containing 0.2% methyl palmitate. In organic solvents, the catalytic activity of 5/13 mutants was three- to sixfold higher than that of the original construct. Thus, yeast cells displaying the lipase can be used in organic solvents, and the lipase activity may be increased by a combination of protein engineering and display techniques. Thus, this immobilized lipase, which is more easily prepared and has higher activity than commercially available free and immobilized lipases, may be a practical alternative for the production of esters derived from fatty acids.

Lipases (EC 3.1.1.3) catalyze the hydrolysis of acylglycerides and other fatty acid esters under aqueous conditions and the synthesis of esters in organic solvents (5, 25). Lipase-catalyzed reactions have high substrate specificity or regioselectivity, so lipase-catalyzed ester synthesis in organic solvents is potentially an industrially feasible alternative to conventional chemical methods (1, 4). However, organic solvents may alter the structure and activity of enzymes that usually function in an aqueous environment.

One alternative is to immobilize the enzymes on solid supports in order to increase their function and stability in response to organic solvents or increased temperatures. Enzymes may be stabilized by chemical and physical processes (6, 15, 20, 24). With chemical methods enzymes are immobilized by strong covalent bonding, but changes in protein structure often result. In physical stabilization processes, the interactions between enzymes and solids usually are weaker, resulting in fewer changes in the enzyme’s structure.

Yeast cell surface engineering (12, 13) is an alternative approach that immobilizes an enzyme on the yeast cell surface. Proteins are immobilized by using an outer shell wall cell protein, the C-terminal half of α-agglutinin (10, 26). The gene encoding *Rhizopus oryzae* lipase (ROL), which has been used to produce diesel fuels (8), was fused to a DNA sequence encoding the C-terminal half of yeast α-agglutinin and expressed in *Saccharomyces cerevisiae*. The transformants have hydrolytic activity toward triolein, which indicates that the ROL is immobilized on the yeast cell surface in an active form (27).

Our objectives in this study were (i) to describe an application of an immobilized ROL for ester synthesis in organic solvents and (ii) to identify mutant displayed ROLs with higher esterification activities in organic solvents. We hypothesized that displayed ROLs would be stabilized by the cell wall components and that ROL activity could be easily improved by display techniques. The ROL-displaying yeast could be used as an immobilized lipase in ester synthesis.

**Materials and Methods**

Strains and media. *Escherichia coli* DH5α (F− endA1 hsdR17 (rK− mK−) supE44 thi-1 λ− recA1 gyrA96 deoA1 lacU169 (80lacZΔM15)) was used as a host for DNA manipulation. *S. cerevisiae* strain MT8-1 (MATa ade2 his3 leu2 trp1 ura3) (22) was used as the host for protein expression. *E. coli* was grown in LB medium (10 g/liter tryptone, 5 g/liter yeast extract, 5 g/liter sodium chloride) containing 50 μg/ml ampicillin. YPD medium (10 g/liter yeast extract, 20 g/liter peptone, 20 g/liter glucose) was used to cultivate yeast host cells. Yeast transformants were cultivated in SD-W medium (20 g/liter glucose, 7 g/liter yeast nitrogen base without amino acids [Difco, Detroit, MI], 20 mg/ml adenine sulfate, 20 mg/ml l-histidine-HCl, 30 mg/ml l-leucine, 20 mg/ml uracil) containing 20 g/liter Casamino Acids.

Plasmid construction. Plasmid pWRL17 for expression of ROL on the cell surface of *S. cerevisiae* and control plasmid pMW1 have been described previously (27). In pWRL17, the fusion gene, a pre-α-factor sequence, a pro-ROL-encoding gene and linker sequence, and the DNA coding sequence for the C-terminal 320 amino acids of α-agglutinin are downstream of a GAPDH promoter (27). The plasmid used for expression of the ROL mutants was con-
structed by amplifying mature ROL-encoding fragments by error-prone PCR (3) with primers 5'-TTAGGTCATGTTGCAAACCTGMCATCATCGGAAAA GC-3' and 5'-TTGGCGCTCCAGACCAACCCGAACCCAGAACCCGAAGGAGCAC ACCAGACACCACTGATCCTC-3' (NcoI and XhoI sites are underlined) and with pWRS17S as the template. The error-prone PCR mixture contained 0.1 mM MnCl₂, 5.5 mM MgCl₂, 2.5 mM dATP, 2.5 mM dGTP, 25 mM dCTP, and 5 U Tag polymerase (Takara, Tokyo, Japan)/100 μl. The reaction conditions were 30 cycles of 60° at 94°C, 60° at 70°C, and 120 s at 72°C. These conditions resulted in an average of two or three nucleotide substitutions per coding sequence for each reaction. The amplified fragments were digested with NcoI and XhoI and ligated into the 8.9-kb fragment of pWRSL17 that resulted from digestion with NcoI and XhoI. The resulting plasmid was first amplified in E. coli and then used to transform the yeast using a yeast transformation kit (YEAST MAKER; Clontech Laboratories, Inc., Palo Alto, CA).

Preparation of lipases. ROL-displaying yeast cells were cultivated in SD-W medium containing 2% Casamino Acids for 24 h (stationary phase) and collected by centrifugation at 3,000 × g for 5 min at 25°C. After the cells were washed, the optical density at 600 nm was adjusted to 1.0, and the cells were lyophilized overnight. After lyophilization, 0.7 mg (dry weight) of cells was obtained and used as a whole-cell catalyst. The amount of ROL displayed on the yeast cell surface was estimated to be about 0.2 mg/g (dry weight) of cells. Amano F-AP15 was purchased from Amano Enzyme (Nagoya, Japan) (65% [wt/wt] lipase and 35% dextran) and used as a free ROL.

Assay of lipase activity. Hydrolytic activity in aqueous and organic media was measured with p-nitrophenyl palmitate (pNPP) as the substrate (14). In aqueous solvents, the commercial lipases or ROL-displaying yeast cells (0.5, 1.0, or 2.0 mg) were added to 2 ml 0.1 M Tris-HCl (pH 8.0) containing 1.5 mM pNPP, 0.4% (wt/vol) Triton-X, and 0.1% (wt/vol) arabic gum. In organic solvents, the commercial lipases or ROL-displaying yeast cells (0.5, 1.0, or 2.0 mg) were added to 2 ml of heptane containing 50 mM pNPP. The reaction mixtures were incubated at 30°C, and the A₄₀₀ of the supernatant was measured at 5-min intervals. In heptane, the pNPP of the supernatant was extracted with 1 M Tris-HCl (pH 8.0) before measurement. A reaction rate was calculated using a molar extinction coefficient of 12.75 × 10⁵ cm²/moll (14). One unit of enzyme activity was defined as the amount of enzyme required to hydrolyze 1 μmol of pNPP per min.

Esterification activity in heptane was measured using 100 mM n-pentanol and 100 mM palmitic acid as the substrates (28). The esterification activity was not determined by using saturated alkyl carboxylic acids (C10-0, C14-0, C18:0, and C22:0; 25 mM each) and 100 mM n-pentanol in heptane containing 0.2% (wt/vol) H₂O (Table 2). Five of the 13 mutants had three- to sixfold-higher activities than the wild-type ROL-displaying yeast, and the selectivity for chain length was not changed. The other mutants had the same activities as the wild-type ROL-displaying yeast, or the activities were slightly lower (data not shown).

Analysis of DNA sequences from the ROL-displaying mutants. The mature ROL was 269 amino acids long (2). The three-dimensional structure of ROL is presumed to be similar to that of the Rhizopus niveus lipase as the two proteins exhibit

<table>
<thead>
<tr>
<th>ROL</th>
<th>Conversion (m/mol)¹</th>
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<tbody>
<tr>
<td></td>
<td>No water</td>
</tr>
<tr>
<td>Free ROL</td>
<td>46 ± 4</td>
</tr>
<tr>
<td>Displayed ROL</td>
<td>1.4 × 10⁵</td>
</tr>
</tbody>
</table>

| ¹ The reaction volumes were 1 ml, and the reaction time was 3 h. The data are means ± standard errors based on three independent measurements.  
| ² Water contents were determined by addition of water to dry heptane.  
| ³ Either the enzyme preparation (0.3 mg) or cells (0.3 mg [dry-weight]) were added.
TABLE 2. Esterification activities of mutated ROLs displayed on the yeast cell surface in heptane containing 0.2% H2O

<table>
<thead>
<tr>
<th>Clone</th>
<th>Conversion rates (%) with the following fatty acidsa,b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C10:0</td>
</tr>
<tr>
<td>Wild type</td>
<td>11 ± 1</td>
</tr>
<tr>
<td>E5 (K51E)</td>
<td>38 ± 3</td>
</tr>
<tr>
<td>E8 (G3A, K51E)</td>
<td>47 ± 4</td>
</tr>
<tr>
<td>E10 (S267N)</td>
<td>41 ± 4</td>
</tr>
<tr>
<td>E17 (A24AR)</td>
<td>59 ± 6</td>
</tr>
<tr>
<td>E31 (T241S)</td>
<td>46 ± 4</td>
</tr>
</tbody>
</table>

a C10:0, capric acid; C12:0, myristic acid; C18:0, stearic acid; C22:0, behenic acid. b Cells (3.0 mg [dry weight]) were added to 1-mI reaction mixtures. The wild-type enzyme was the wild-type ROL-displaying yeast.

DISCUSSION

We used a displayed ROL and mutants of this enzyme immobilized on the yeast cell surface in organic solvents as whole-cell biocatalysts. The hydrolytic activity of the displayed ROL in n-heptane was 33 times higher than it was in aqueous solvents. Although the substrate concentration in n-heptane (50 mM pNPP) was 33 times higher than it was in water (1.5 mM pNPP), in heptane the activity of the free enzyme decreased significantly. Since the displayed ROL had higher activity in heptane than in water without any loss of activation, the cell wall-immobilized, displayed lipase appears to be stabilized by an as-yet-unidentified component of the cell wall. The amount of water required to obtain sufficient enzyme activity for hydrolysis in organic solvents depends on the reaction conditions, including the water content of the substrate, the solvents, and the biocatalyst (14). In this reaction system, the solvents and the biocatalyst together appear to contain enough water for pNPP hydrolysis to proceed at at least a minimal level.

The esterification activity of the displayed ROL was much higher than that of the free ROL, and addition of 0.2% (vol/vol) H2O increased its activity ~20-fold. After 3 h ~10 to 20% of the alcohols was converted to esters by 3 mg whole-cell biocatalyst (Table 2). These figures are comparable to those obtained for other immobilized lipases (20) and suggest that the displayed ROL could be used as a whole-cell lipase.

The number of proteins displayed on a yeast cell surface has been estimated to be ~10^7 molecules (17). If this estimate applies to ROL-displaying yeast cells, then they contain ~0.2 mg displayed ROL/g (dry weight) of cells. This amount of enzyme is similar to the amount of available lipase immobilized in other ways (6). The activity of the displayed ROL was estimated to be 1.4 U/mg. Takahashi et al. (23) reported that ROL expressed in S. cerevisiae displayed a hydrolytic activity of 0.5 U/mg toward pNPP, which is similar to the activity of the displayed ROL in the fused form (1.4 U/mg). Display of enzymes on the yeast cell surface has at least two advantages compared to other physical immobilization methods. First, the displayed enzymes can be readily produced in a standard fermentation. No further work is required to either purify or immobilize the enzymes. Second, enzymes displayed on the yeast cell surface can be modified directly by conventional genetic engineering, which enables error-prone PCR, DNA shuffling, and combinatorial mutagenesis to be used quickly and efficiently to create strains with enhanced enzyme activity (19).

Error-prone PCR has been used frequently to improve enzyme activities, including the activities of other lipases (11, 21). The best mutants which we obtained had three- to sixfold-higher esterification activities than the wild type in heptane-0.2% (vol/vol) H2O. None of the mutants had altered glycosylation patterns. Each of the five mutants had one or two mutations, and a K51E mutation occurred in two different mutants. From the three-dimensional structure of ROL based on R. niveus lipase generated with ViewerLite (Accelrys, San Diego, CA), the mutations all altered the surface of the protein and probably increased protein stability by permitting additional hydrogen bonding between altered amino acids and adjacent residues (7, 23). Combinations of these mutations to make new mutants might result in strains with even higher levels of lipase activity.

As described here, lipases immobilized on the cell surface through genetic engineering are more easily prepared and have higher activity than commercialized lipases. Accordingly, ROL-displaying yeasts such as the one described here may be useful as whole-cell biocatalysts in organic media; for example, they may be used in the production of methyl esters of long-chain fatty acids, which are used as biodiesel fuel, and in the production of various esters from waste edible oils, such as tuna oils and sardine oils, which are used as flavors compounds in the cosmetic, food, and beverage industries. The immobilization of the lipase described here and the improvement of its activity through error-prone PCR provide a concrete example of how other immobilized lipases could be constructed and how their activities could be increased.

ACKNOWLEDGMENTS

We thank H. Fukami and M. Nakao of Suntory Institute for Fundamental Research for their comments on the manuscript.

REFERENCES