Pretreatment of *Chromobacterium viscosum* lipase with acetone increases its activity in sodium bis-(2-ethylhexyl) sulfosuccinate (AOT) reverse micelles

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Abstract: The activity of *Chromobacterium viscosum* lipase for hydrolysis of olive oil in sodium bis-(2-ethylhexyl) sulfosuccinate (AOT) reverse micelles was increased by pretreatment with acetone. In contrast to the untreated lipase, no sharp fall in the activity of the treated lipase at higher *W*0 (water to AOT molar ratio) values was observed. The fluorescence emission intensity of the treated lipase in reverse micelles was higher than that of the untreated lipase but the maximal emission wavelength (λmax) was the same for both lipases. A kinetic model that considers the free substrate in equilibrium with the substrate adsorbed on the micellar surface was successfully used to better understand the activity enhancement. The Michaelis constant (Km) and substrate adsorption equilibrium constant (Kad) were reduced by lipase pretreatment with acetone whereas the maximum reaction rate (vmax) remained unaltered.

Keywords: lipase; pretreatment; acetone; hydrolysis; reverse micelle; olive oil

INTRODUCTION

The hydrolysis, esterification and interesterification reactions of lipids and fats catalyzed by lipases (EC 3.1.1.3) have been intensively studied in various systems to explore the inherent advantages of using these biocatalysts.1–3 As lipase-catalyzed hydrolysis of fats or oils takes place at the interface, reverse micelles have been used for hosting these reactions4,5 due to the large interfacial area promoting contact between enzyme and substrates. The anionic double-tailed surfactant AOT [sodium bis-(2-ethylhexyl) sulfosuccinate] is frequently used in reverse micellar enzymology. The motivation to use the AOT reverse micellar system includes the large amount of published data on the physico-chemical properties of the AOT reverse micelles and the ease of reverse micellar formation.

In order to enhance the activity of lipases entrapped in AOT reverse micelles, several methods have been reported: use of chemically modified AOT,6 and introducing nonionic surfactant such as Tween 85 or small molecular weight polyethylene glycols as additives.7–9 The preparation of chemically-modified AOT is very complicated and the activity enhancement is also limited. The additive complicates the downstream separation.

The pretreatment of enzymes with polar organic solvents has been proved to be very effective in enhancing activity and enantioselectivity of enzymes.10–13 It has the advantages of simple operation and being less time consuming. In most cases, the pretreated enzymes were used in organic solvents in powder form; no report is available on them in reverse micellar systems to improve enzyme performance.

In this work, the activity of acetone-treated *Chromobacterium viscosum* lipase-catalyzed hydrolysis of olive oil in AOT reverse micelles was investigated and compared with that of untreated lipase. The fluorescence properties and kinetics of treated and untreated lipases in reverse micelles and water were also compared.

MATERIALS AND METHODS

Materials

Purified *C viscosum* lipase (glycerol-ester hydrolase, EC 3.1.1.3) was provided by Asahi Chemical Industry Co, Ltd, Tokyo, Japan. Isooctane, acetone, AOT and olive oil were purchased from Wako Pure Chemical Industries Co, Osaka, Japan. The saponification value of olive oil was 195, determined by the method

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Activity of Chromobacterium viscosum lipase in AOT reverse micelles

Acetone treatment of lipase
Five mL of 0.1 M Tris–HCl buffer (pH 7) containing C viscosum lipase (10 mg) was mixed with acetone (5 mL). The mixture was magnetically stirred at 4 °C for 1 h, frozen and then lyophilized to obtain the powdered lipase.

Preparation of reverse micelles
Isooctane containing dissolved AOT was used as the reverse micellar solution. The water to AOT molar ratio ($W_0$) was adjusted by adding the desired amount of buffer and stirring at 25 °C until the mixture became transparent. The powdered lipases (treated and untreated) were directly added to reverse micellar solution an a stirred at 500 rpm for 15 min; the mixture was then centrifuged at 40 × g and the supernatant was used as the lipase-containing micellar solution. The protein content in the lipase-containing reverse micellar solution was determined by the measurement of absorbance at 278 nm using a Shimadzu (Kyoto, Japan) spectrophotometer (BioSpec-1601) following a method previously described$^{15,16}$ and lipase concentration was calculated from the standard curve.

Determination of lipase activity
Lipase activity was defined as the initial reaction rate, $v$ (mol fatty acids dm$^{-3}$ s$^{-1}$). The reaction was initiated by adding olive oil into pre-incubated micellar solution containing lipase and then stirred at 25 °C and 1000 rpm for 20 min as it was found that within this time range free fatty acid production was linearly dependent on time. The produced fatty acids were analyzed by the Lowry and Tinsley method.$^{17}$ All data were the average of at least five batches of experiments under identical conditions, and were reproducible within ±5%. All concentration terms were based on the total volume of the system unless otherwise specified. The $C_{lipase}$ was kept constant at 2 mg dm$^{-3}$.

Fluorescence measurement
Fluorescence emission spectra of the treated and untreated C viscosum lipases entrapped in reverse micelles and water were recorded from 300 to 400 nm using a Hitachi (Tokyo, Japan) F-3010 fluorescence spectrophotometer with an excitation wavelength of 280 nm, a selective wavelength for tryptophan (Trp) residues. The band pass for excitation and emission was 5 nm. All spectra were corrected by subtracting a blank spectrum (without lipases). The concentration of lipases (treated and untreated) in reverse micelles or water for fluorescence study was kept at 10 mg dm$^{-3}$.

RESULTS AND DISCUSSION
Effect of water to AOT molar ratio, $W_0$
Figure 1 shows that the shape of the activity–$W_0$ profile was changed when the lipase was pretreated with acetone, whereas the optimal $W_0$ value (around 10) remained unaltered. It has been recognized that the optimum $W_0$ value is related to a situation where the inner diameter of the micelle corresponds to the size of encapsulated lipase.$^{4,18,19}$ This rule still held true for the case of acetone-treated lipase. The activity of the treated lipase at optimal $W_0$ was about 170% of that of the untreated lipase. The enhanced activity might be attributed to the change in lipase conformation from closed to open form$^{10}$ remaining unaltered. It has been recognized that the optimum $W_0$ value is related to a situation where the inner diameter of the micelle corresponds to the size of encapsulated lipase. $W_0$ value (around 10) remained unaltered. It has been recognized that the optimum $W_0$ value is related to a situation where the inner diameter of the micelle corresponds to the size of encapsulated lipase. $W_0$ value (around 10) remained unaltered. It has been recognized that the optimum $W_0$ value is related to a situation where the inner diameter of the micelle corresponds to the size of encapsulated lipase.
lipase has three tryptophan residues; among them Trp 209 and Trp 283 are closely located to the lipase active site (catalytic triad Ser 87, His 285 and Asp 263). The active site is covered by helix α5, known as lid, and the hydrophobic residue Ser 87 forms the nucleophilic center. The increased exposure of hydrophobic Trp residues to the lipase surface might therefore give rise to more exposure of the binding site of lipase (hydrophobic) and lead to the change in lipase conformation from the closed to open form.

and this conformational change is reversible when dissolved in water. The maximal emission wavelength (λmax) of both the lipases in reverse micelles occurred at 328 nm, which was blue-shifted relative to the emission peak in water (=334 nm). Tryptophan emission of lipases is sensitive to the environment and a blue shift is generally observed with the decrease in polarity of the environment.21 The result thus indicates that the reverse micellar water pool is in the hydrophobic state.9,22 The change in lipase conformation accompanied by the acetone treatment was thus retained in the reverse micelles due to insufficient water and the hydrophobic state of the micellar water pool.

The three-dimensional crystal structure of C viscosum lipase (protein data bank 1 CVL) was analyzed to better understand the lipase conformational change by pretreatment with acetone in response to the tryptophan fluorescence characteristics of lipase. The C viscosum lipase has three tryptophan residues; among them Trp 209 and Trp 283 are closely located to the lipase active site (catalytic triad Ser 87, His 285 and Asp 263). The active site is covered by helix α5, known as lid, and the hydrophobic residue Ser 87 forms the nucleophilic center. The increased exposure of hydrophobic Trp residues to the lipase surface might therefore give rise to more exposure of the binding site of lipase (hydrophobic) and lead to the change in lipase conformation from the closed to open form.

Figure 2. Fluorescence emission spectra of the treated and untreated lipases after excitation at 280 nm. Experimental conditions: Clipase = 10 mg dm\(^{-3}\), C\(_{AOT}\) = 0.05 mol dm\(^{-3}\), W\(_0\) = 10, buffer pH = 8.0, C\(_{NaCl}\) (in buffer) = 0.3 mol dm\(^{-3}\), temperature 25°C.

**Reaction kinetics**

A kinetic model\(^{23}\) (eqn (1)) was used to understand the lipase activation by pretreatment with acetone.

\[
v = \frac{v_{\text{max}} [S_T]}{K_m (1 + K_{ad} [S_S]) + S_T}
\]

where \(v\) is reaction rate, \(v_{\text{max}}\) is maximum reaction rate, \(K_m\) is Michaelis constant, \(K_{ad}\) is adsorption equilibrium constant, \([S_T]\) is substrate (olive oil) concentration and \([S_S]\) is surfactant concentration. The kinetic parameters for treated lipase were obtained from Lineweaver–Burk plots and the plots of the apparent Michaelis constant \(K_m\) against \([S_S]\) as described previously\(^{23}\) and listed in Table 1. For convenience of comparison, the kinetic parameters for the untreated lipase are also listed. Figure 3 shows that the activity values calculated by using the model (eqn (1)) agreed well with the experimental data.

From Table 1, it is seen that the Michaelis constant \(K_m\) and substrate adsorption equilibrium constant \(K_{ad}\) were reduced by pretreatment whereas maximum reaction rate \(v_{\text{max}}\) remained unaltered. As the \(K_m\) value reflects the affinity between enzyme and substrate, it is expected that the pretreatment with acetone enhanced the activity of lipase.

**Table 1. Comparison of the kinetic parameters for the treated and untreated lipases**

<table>
<thead>
<tr>
<th>Kinetic parameter</th>
<th>Treated lipase</th>
<th>Untreated lipase(^{23})</th>
</tr>
</thead>
<tbody>
<tr>
<td>(K_m) (mol dm(^{-3}))</td>
<td>0.037</td>
<td>0.083</td>
</tr>
<tr>
<td>(K_{ad}) (mol(^{-1}) dm(^3))</td>
<td>7.6</td>
<td>16.2</td>
</tr>
<tr>
<td>(v_{\text{max}}) (mol dm(^{-3}) s(^{-1}))</td>
<td>2.68 \times 10(^{-5})</td>
<td>2.70 \times 10(^{-5})</td>
</tr>
</tbody>
</table>

Figure 3. Comparison of the treated lipase activity obtained experimentally with that predicted by model (eqn (1)). Reaction conditions: C\(_{lipase}\) = 2 mg dm\(^{-3}\), W\(_0\) = 10, buffer pH = 8.0, C\(_{NaCl}\) (in buffer) = 0.3 mol dm\(^{-3}\), temperature 25°C.
Activity of *Chromobacterium viscosum* lipase in AOT reverse micelles

and substrate, the decrease in $K_m$ indicates that the combination of substrate with enzyme active site became easier, possibly due to the change in lipase conformation as a consequence of acetone treatment. The $K_{ad}$ value reflects two factors: the adsorption of substrate on the micellar/surfactant surface, and the interactions between lipase and the head groups of AOT molecules. Since the pretreatment of lipase could not affect the substrate adsorption on the surface of the reverse micelles, the significant decrease in $K_{ad}$ value further confirms that the interactions between lipase and the head groups of AOT molecules was decreased.

**CONCLUSIONS**

The activity of *C. viscosum* lipase for hydrolysis of olive oil in AOT reverse micelles was significantly increased by pretreatment of lipase with acetone. The increase in lipase activity was attributed to the change in lipase conformation from the closed to open form through the exposure of hydrophobic residues to the lipase surface. The classical bell-shaped activity–$W_0$ profile observed for untreated lipase was not found for the treated lipase. The kinetic results suggested that acetone treatment enhanced the affinity of the lipase with substrate and reduced the interactions with AOT head groups.

**REFERENCES**