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Thermal Deactivation Kinetics of *Pseudomonas fluorescens* Lipase Entrapped in AOT/Isooctane Reverse Micelles

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ABSTRACT: Thermostability of the lipase (EC 3.1.1.3) was found to be increased by the enzyme-entrapment in 50 mM AOT/isooctane reverse micelles. The half-life (15.75 h) of *Pseudomonas fluorescens* lipase entrapped in reverse micelles at 70 °C was 9.72- and 11.41-fold longer than those solubilized in a glycerol pool or in 10 mM phosphate buffer (pH 8.0), respectively. The enzyme deactivation model considering a two-step series-type was employed, and deactivation constants for the second step (k_2) at all temperatures were drastically decreased after the lipase was entrapped in reverse micelles. In particular, k_2 (0.0354 h^{-1}) at 70 °C in reverse micelles was 12.33- and 13.14-fold lower than in a glycerol pool or in the phosphate buffer, respectively. The deactivation energies (from k_1, k_2) for the lipase entrapped in the reverse micelles, solubilized in a glycerol pool, or in the aqueous buffer were 7.51, 26.35 kcal/mol, 5.93, 21.08 kcal/mol, and 5.53, 17.57 kcal/mol, respectively.

KEYWORDS: *Pseudomonas fluorescens lipase, reverse micelles, glycerolysis, thermostability, deactivation kinetics*

INTRODUCTION

In recent years, considerable scientific and economic interest has been devoted to the potential applications of lipases (triacylglycerol hydrolases, EC 3.1.1.3) in biotechnological processes. Hydrolysis, esterification, and interesterification reactions of fat and oils catalyzed by lipases have been intensively studied and performed in various systems to explore the inherent advantages of using these biocatalysts.[1,2] The major applications of lipases have been in the enantioselective synthesis of precursors for pharmaceutically active compounds[3−5] and the conversion of natural fat and oils into high-value products, e.g., monoacylglycerols (MAG).[6,7]

MAGs are the most widely used emulsifiers in the food, pharmaceutical, and cosmetic industries. Several approaches have been investigated for the enzymatic production of MAG. In addition to the selective hydrolysis of lipids using 1,3-specific lipases and esterification of free fatty acids with glycerol, glycerolysis, the exchange reaction of acylgroups in triglycerides with glycerols, made the high production yields of MAG possible.[8−10] Because many of the enzymatic reactions catalyzed by lipases require biphasic media, a different approach has been the encapsulation of lipases in reverse micelles,[11−14] which display the advantages of one phase systems. Apart from providing considerable interfacial area, this aqueous system is able to retain and often enhance the catalytic activities of enzymes.[13] Furthermore, increased stability has been reported in many cases, despite the fact that a complete understanding of the parameters that affect enzyme deactivation in reverse micelles has not yet been revealed.

The structure of reverse micelles consists of an aqueous microdomain facing the polar heads of the surfactant that surrounds this core and interact with the bulk organic solvent (nonpolar phase) through hydrophobic interactions. The polar cores of the inverted micelles have the ability to solubilize a significant amount of hydrophilic components (e.g., water or glycerol), which depends on the physicochemical properties of the reversed micellar system. When the enzymes are microencapsulated, they are located at the interior aqueous phase of reverse micelles although the degree of interaction with the surfactant interface is variable and of significance in the case of lipases. This location is assumed to protect the enzyme from the adverse effects of organic solvents and facilitate lipase-catalyzed reactions in a dominant-organic solvent phase.[13] To evaluate the feasibility and potentiality of specific lipase/reverse micelles, kinetics and thermostability studies should be carried out.

We have reported the general characteristics of lipase-catalyzed glycerolysis of triolein in AOT (sodium bis-[2-ethylhexyl] sulfosuccinate)/isooctane reverse micelles for the effective production of MAG.[15] In the present work, it has been shown in detail that the thermostability of *Pseudomonas fluorescens* lipase entrapped in a reversed micellar system was significantly increased by the presence of AOT as a shell and a

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glycerol pool as a core phase. To understand the thermal stabilization of *Pseudomonas fluorescens* lipase by AOT and glycerol, a two-step series-type deactivation kinetic equation, and the glycerolysis of triolein in AOT/isooctane reverse micelles were chosen as an enzyme deactivation kinetic model and as a model reaction system, respectively.

MATERIALS AND METHODS

Chemicals. Ten kinds of organic solvents of HPLC grade (purchased from Burdick and Jackson, Muskegon, MI, USA) listed in Table 1 were used as the reaction media. They were stored over a type 4 Å molecular sieve and filtered prior to use. Five kinds of surfactants listed in Table 1 were purchased from Sigma-Aldrich (St. Louis, MO, USA). AOT was purified according to the method of Tamamushi and Watanabe[16] and dried over P_2O_5 under vacuum.

Table 1. Initial Velocity[a] of *Pseudomonas fluorescens* Lipase in Reverse Micelles Formed by AOT in Various Organic Solvents (the Upper Section) and Relative Activity of the Lipase in Reverse Micelles Formed by Various Surfactants in Isooctane (the Lower Section)[b]

organic solvent	initial velocity[c]
n-hexane	1.27 ± 0.09
cyclohexane	1.75 ± 0.09
n-heptane	1.51 ± 0.15
octane	0.97 ± 0.06
isooctane	4.59 ± 0.07
benzene	2.35 ± 0.19
isopropylbenzene	0.73 ± 0.11
acetone	1.79 ± 0.13
toluene	0.58 ± 0.07
isopropylether	1.01 ± 0.17
surfactant	**relative activity (%)[d]**
AOT	100.0 ± 5.1
polyethylene glycol 6000	28.6 ± 4.7
CTAB[e]	7.2 ± 2.1
Triton X-100	30.8 ± 6.2
DPPC[f]	53.4 ± 4.9

[a]Initial velocity is expressed as μmol of triolein decreased/h in 10 mL of reaction mixture. [b]Reaction conditions are as follows: enzyme concentration, 0.41 units/g glycerol; water concentration in glycerol, 4.0%; temperature, 40°C; AOT concentration, 50 mM; [glycerol]/[triolein], 30 (0.184 g glycerol). [c]The values are mean ± standard deviation based on three independent experiments in triplicate (n = 3). [d]The values are mean±standard deviation based on three independent experiments in triplicate (n = 3). [e]CTAB: cetyltrimethylammonium bromide. [f]DPPC, dipalmitoylphosphatidylcholine; surfactant concentration, 50 mM.

Purified *Pseudomonas fluorescence* lipase purchased from Sigma-Aldrich was solubilized in glycerol of molecular biology grade (99.9%, obtained from Sigma-Aldrich). Although *Pseudomonas fluorescence* lipase was highly purified according to its manufacturer's information, its purity was confirmed by electrophoresis. The one clear band without any other detectable bands in the electrophoretic pattern suggested that this lipase could be directly used for the experiments without any additional purification step (data not shown). The specific activity of the enzyme for glycerolysis was 2.14×10^{-2} units/mg protein. The definition of specific activity is the same as described in Analysis of the Lipase-Catalyzed Glycerolysis section.

1-Monoolein and 1,2- and 1,3-diolein were obtained from Sigma-Aldrich (99.9%). Oleic acid, 2-monoolein, and triolein of 99.9% purity from Supelco (Bellefonte, PA, USA) were used as the standard lipids in HPLC work.

Preparation of Reverse Micelles. Reverse micelles containing the lipase were formed by adding appropriate amounts of glycerol with predetermined amounts of water and lipase powder to a solution of 50 mM surfactant in organic solvent. The mixture was then vortex mixed for 15 s to obtain clear micellar solutions. The desired water content was usually defined as the R value, which means the molar ratio of water to surfactant ($[H_2O]/[\text{surfactant}]$), and the glycerol content was defined as the G value, which means the molar ratio of glycerol to surfactant ([glycerol]/[surfactant]). Clear micellar solutions of the enzyme could be made within a restricted range from 0 to 300 mM glycerol under the experimental conditions, and no turbidity was observed after 50 h at 40 °C. The R and G values were calibrated taking the amount of water inherently present in the organic solvent, glycerol, and surfactant into consideration.

Analysis of the Lipase-Catalyzed Glycerolysis. Glycerolysis activity was measured at 40 °C using triolein and glycerol as substrates. A screw-cap culture tube was filled with 10 mL of 50 mM surfactant−organic solvent solution containing triolein (6.67 mM). The desired amount of glycerol with lipase and water was injected into the culture tube, and the reaction was initiated by vortex mixing the mixture until it was clear. After incubation at 40 °C for 5 h, a sample of 0.2 mL was removed from the reaction mixture with a small hypodermic syringe. To the sample in a test tube 3.0 mL of chloroform was added, and the test tube was shaken vigorously for 2 min and then left for at least 1 h to inactivate the enzyme. Then 0.5 mL of water was added, the test tube was again shaken for 1.5 min, and the mixture was centrifuged for 5 min at 2000*g*. The lower chloroform layer was taken out and stored in a round-bottom flask. The upper water layer was re-extracted twice with 3.0 mL of chloroform. A blank was prepared by the same procedure as described above except glycerol pool without enzyme was added.

One unit of enzyme for the glycerolysis was defined as the amount of lipase that reacted with 1 μmol of triolein/min under the assay conditions. All of the data are averages of triplicate samples and are reproducible within ±10%.

The content of triolein, 1,2-diolein, 1,3-diolein, 1-monoolein, 2-monoolein, and oleic acid in the condensed chloroform layer was determined by high performance liquid chromatography, as described in the our previous report.[17]

Measurement of the Lipase Thermostability. After incubation of the enzyme dissolved in 10 mM phosphate buffer (pH 8.0), glycerol, or reverse micelles consisting of 50 mM AOT-isooctane-glycerol pool at 40, 50, 60, and 70 °C, aliquots were taken at the indicated times, and the residual glycerolysis activity was assayed according to the procedure described in the previous section

Determination of the Lipase Deactivation Kinetic Parameters. The data for deactivation in the 10 mM phosphate buffer (pH 8.0), glycerol, or AOT/isooctane reverse micelles were fitted to a two-step series-type deactivation kinetic model,[18] and the kinetic parameters were determined using a nonlinear regression procedure based on the Marquardt−Levenberg method of iterative convergence included in a solver tool of Microsoft Office Excel 2007 software.

Protein Assay and Water Content Determination. Protein in the aqueous phase was assayed by the Bradford method,[19] and the water content of the sample was determined with a Karl Fischer moisture meter (model MKS-1, Kyoto Electronics Manufacturing Co., Japan).

RESULTS AND DISCUSSION

Selection of Organic Reaction Medium and Surfactant. The 10 types of organic solvents tested were *n*-hexane, cyclohexane, heptane, octane, isooctane, benzene, isopropylbenzene, acetone, toluene, and isopropylether. The reason for the choice of these organic solvents was that they have been frequently used in studies of lipase-catalyzed reactions.[13,20,21] Water-miscible solvents, including ethanol and methanol, could not be used because it was observed that reverse micelles were not formed in these solvents. Halogenated hydrocarbons and

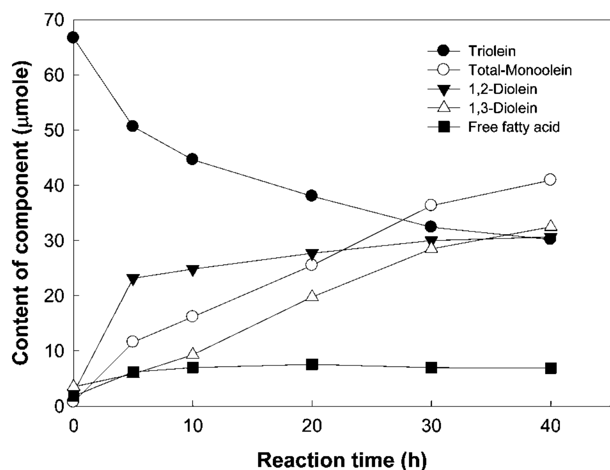

Figure 1. Time course of *Pseudomonas fluorescens* lipase-catalyzed glycerolysis at a fixed concentration of water (4.0% (v/v) in glycerol; *R*, 0.65); lipase, 0.41 units/g glycerol; AOT, 50 mM; [glycerol]/[triolein] = 30 (*G*, 4.00).

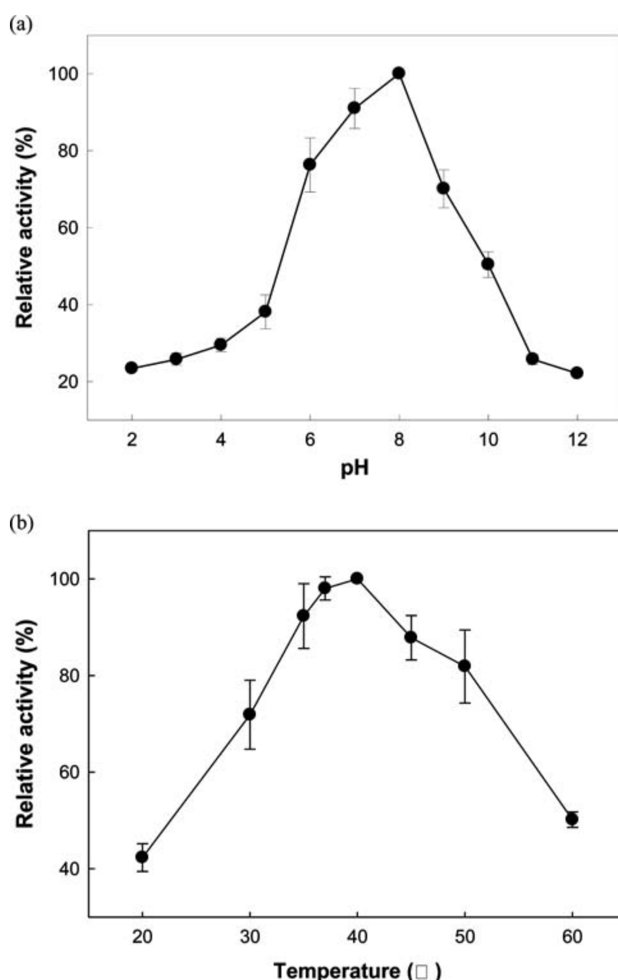

Figure 2. Effect of pH (a) and temperature (b) on *Pseudomonas fluorescens* lipase-catalyzed glycerolysis activity in 50 mM AOT/isooctane reverse micelles at [glycerol]/[triolein] = 30 (*G*, 4.00) and *R* = 0.65.

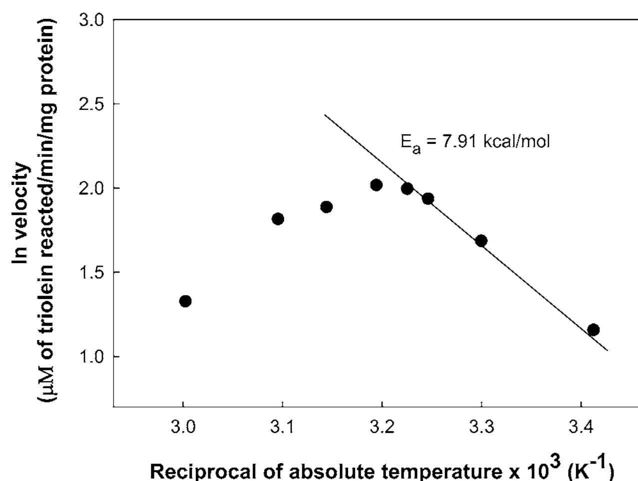

Figure 3. Arrhenius plot for the determination of activation energy of the lipase-catalyzed glycerolysis in AOT/isooctane reversed micellar system and reaction conditions are the same as described in Figure 2.

carbonyls were excluded because of their high solubility in water and toxicity to human beings. As shown in Table 1, isooctane was the most effective for the enzyme-catalyzed glycerolysis of triolein in reverse micelles.

To select the most suitable surfactant, reverse micelles were formed with five kinds of surfactant at a concentration of 50 mM in isooctane. As shown in Table 1, glycerolysis activity was the highest when reverse micelles were formed by AOT, and it decreased in the order, dipalmitoyl phosphatidylcholine, Triton X-100, polyethylene glycol 600, and cetyltrimethyl ammonium bromide.

On the basis of these results, isooctane and AOT were employed throughout this study as the organic reaction medium and surfactant, respectively. These results are consistent with previous reports.[12,20,22]

Time Course of Glycerolysis and Analysis of Glycerolysis Activity. Figure 1 shows a typical time course of the glycerolysis reaction. Oleic acids at both α- and β-positions of a triolein molecule seemed to be transferred to a glycerol molecule because both 1,2- and 1,3-diolein were detected in the product mixture at the start of glycerolysis. In the beginning, the content of 1,2-diolein was higher than that of 1,3-diolein, but later the relationship was reversed. The content of 1,2-diolein even decreased slightly due to the acyl migration from the β-position to the α-position of the glycerol backbone.[23]

Of the two isomers of monoolein, 1-monoolein was observed almost exclusively, whereas very little 2-monoolein was formed. This result indicates that *Pseudomonas fluorescens* lipase used in this study could not attack the β-position of the glycerol backbone in the two kinds of substrates (triolein and glycerol). The decrease of triolein as a function of reaction time at a fixed concentration of enzyme in glycerol (0.41 units/g glycerol, 3.5 mg protein in 10 mL of enzyme reactant) and water (*R*, 0.65) is shown in Figure 1. The initial velocity of the disappearance of triolein was 4.49 μmol/h, and the removal of triolein was linearly proportional to the reaction time up to 5 h. Moreover, the R^2 value, which indicates the relationship between the reaction time and the extent of glycerolysis and is the coefficient for fitting the curve for the experimental data of enzymatic glycerolysis in 50 mM AOT/isooctane reverse micelles, was found to be 99.61% (*R* = 99.81%). Therefore, the specific activity for *Pseudomonas fluorescens* lipase-catalyzed glycerolysis of triolein in 50 mM AOT/isooctane reverse

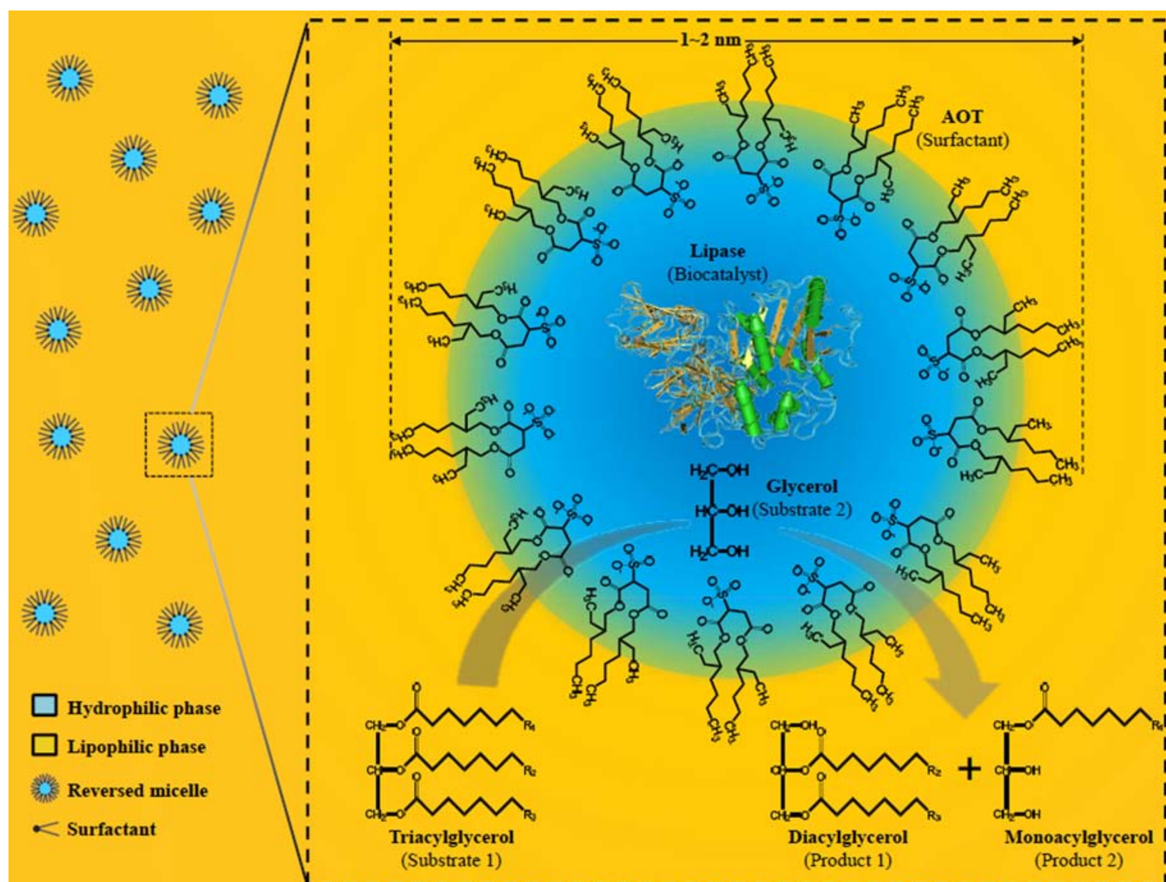

Figure 4. Schematic representation of AOT/isooctane reversed micellar system containing lipase and glycerol at an aqueous core phase and identifying the locations of triacylglycerol at a continuous phase and AOT at shell of a reverse micelle.

micelles was calculated as 0.41 units/g glycerol or 2.14×10^{-2} units/mg protein.

From the results mentioned above, it can be concluded that the assay method for glycerolysis activity used in this study has several advantages over conventional methods; conventional assays have generally utilized an aqueous emulsion of the insoluble substrate (triacylglycerol) to which activators had to be added in addition to the emulsifier. It has been known that the values of kinetic parameters obtained from the emulsion systems are highly dependent on the assay conditions, and they cannot be readily compared because the reaction velocity of glycerolysis depends on the interfacial area between the substrate and enzyme, which is created in the emulsion, and the homogeneity of the reaction system. A reversed micellar system, however, can introduce a single aqueous phase by solubilizing the water-insoluble substrate (triolein) and a small amount of glycerol with little water in the organic solvent (isooctane) that contains the surfactant (AOT). Finally, quantitative analysis by HPLC of glycerolysis in the reverse micelles is convenient, sensitive, and reproducible because (i) the procedure excludes the laborious step of preparing a fresh emulsion for every experimental run, and (ii) an enormous interfacial area between the water-insoluble substrate and the aqueous phase containing glycerol with water provides high degrees of reproducibility and sensitivity, and therefore, many samples can be assayed successively.

General Characteristics. The dependence of the glycerolysis activity on pH in reverse micelles is shown in Figure 2a. Here, the pH values are those of the glycerol pools containing various buffers and the lipase from which the micellar solutions were prepared (pH 2.0, 10 mM KCl−HCl buffer; pH 3.0−5.0, 10 mM citrate-phosphate buffer; pH 6.0−8.0, 10 mM phosphate buffer; pH 9.0−10.0, 10 mM Tris buffer; pH 11.0−12.0, carbonate buffer.). The pH profile was a typical bell-shaped curve, and the *Pseudomonas fluorescens* lipase in this study was found to be most active at pH 8.0. The enzyme was not active in the acidic (lower than pH 4.0) or the extremely alkaline (higher than pH 11.0) ranges of pH.

Figure 2b shows the effect of temperature on the lipase-catalyzed glycerolysis activity. Reverse micellar solutions containing triolein were preincubated for 15 min at each temperature before the addition of glycerol with water and lipase. The optimum temperature was 40 °C. The activation energy (E_a) of the glycerolysis reaction in AOT/isooctane reverse micelles calculated from the Arrhenius equation was relatively low, 7.91 kcal/mol (Figure 3), when compared with 8.40 kcal/mol reported in a two-phase system for a similar temperature range.[24] The difference might be caused by the increased interfacial area between the lipase molecules solubilized in the glycerol pool containing a very small amount of water (core in the reverse micelles) and triolein (substrate) solubilized in isooctane (continuous phase in the reversed micellar system) resulting from the AOT (Figure 4). This result is consistent with the report of another group.[13] Therefore, we reconfirmed that the AOT/isooctane reversed micellar system could be suitable and efficient for the lipase-catalyzed glycerolysis of triacylglycerol because it enhanced the collision factor between the enzyme and substrate molecule.

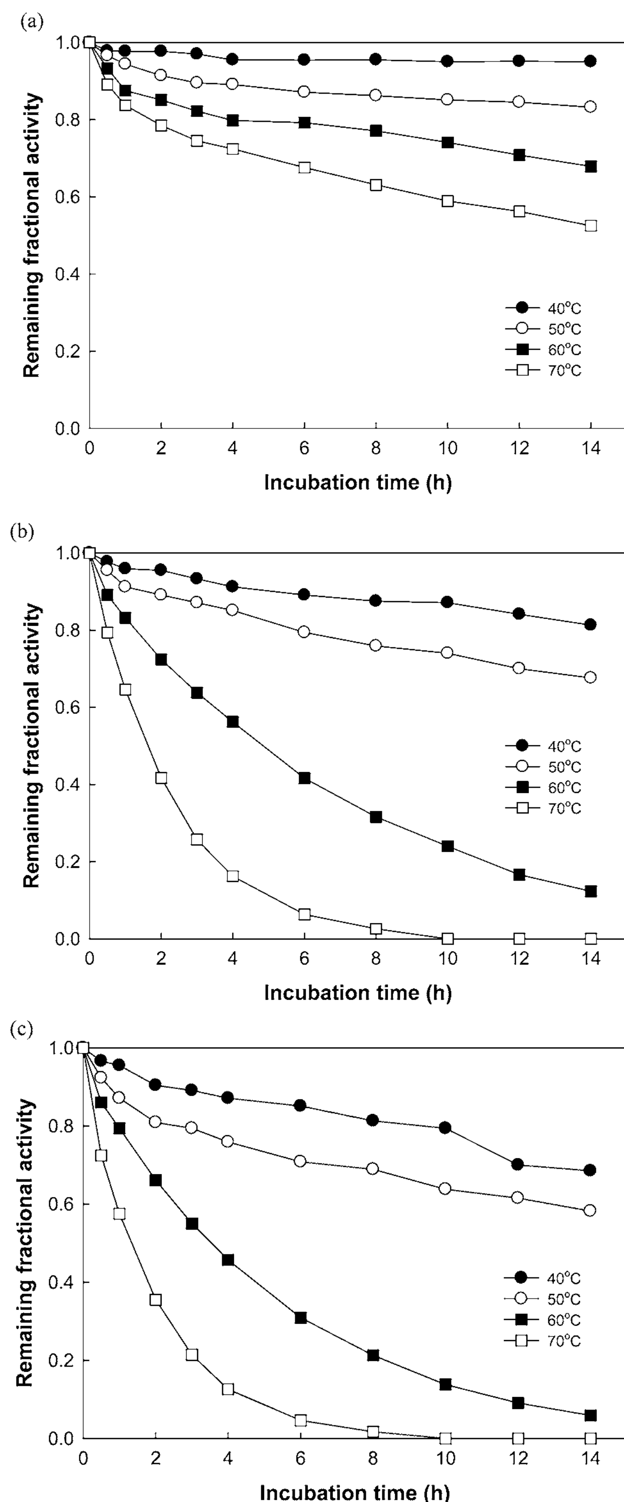

Figure 5. Thermal deactivation profiles of *Pseudomonas fluorescens* lipase in 50 mM AOT/isooctane reverse micelles (a), glycerol pool (b), and 10 mM phosphate buffer (pH 8.0) (c).

Table 2. Deactivation Kinetic Parameters of *Pseudomonas fluorescens* Lipase in 10 mM Phosphate Buffer (pH 8.0), Glycerol Pool, and 50 mM AOT/Isooctane Reverse Micelles

parameter	40 °C	50 °C	60 °C	70 °C
in 10 mM phosphate buffer (pH 8.0)				
k_1 (h^{-1})	1.5138	2.4147	2.7846	4.6405
k_2 (h^{-1})	0.0233	0.0315	0.1882	0.4652
α_1	0.9394	0.8758	0.8562	0.7970
In Glycerol Pool				
k_1 (h^{-1})	0.9983	1.5352	1.9127	2.7985
k_2 (h^{-1})	0.0113	0.0231	0.1402	0.4366
α_1	0.9498	0.9118	0.8695	0.8215
In 50 mM AOT/Isooctane Reverse Micelles				
k_1 (h^{-1})	0.8271	1.0519	1.5963	2.1770
k_2 (h^{-1})	0.0012	0.0057	0.0176	0.0354
α_1	0.9627	0.9164	0.8760	0.8421

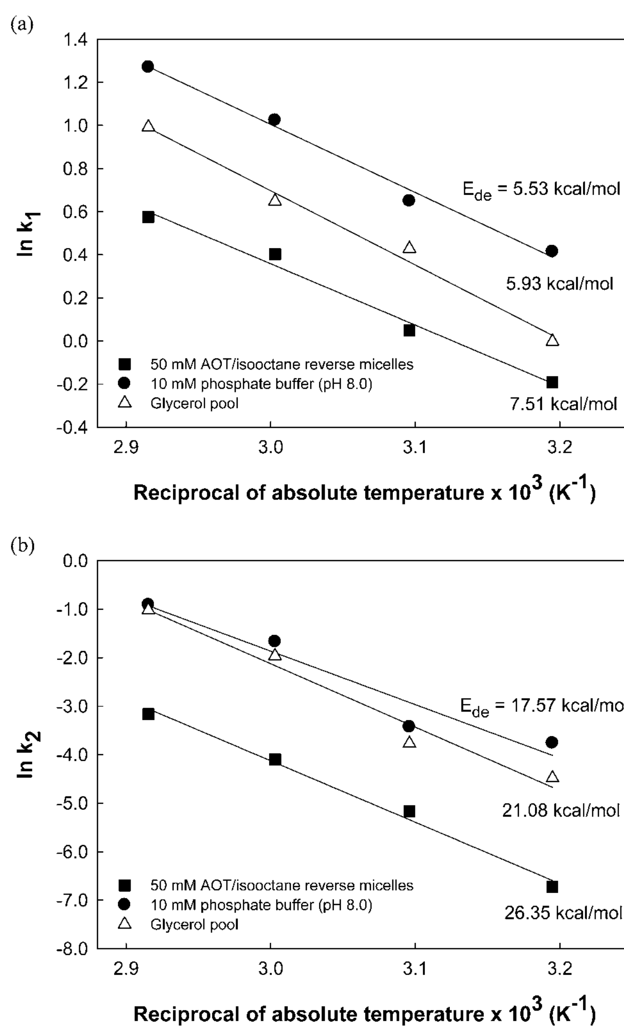

Figure 6. Arrhenius plots of deactivation rate constants ((a) ln k_1 vs K^{-1}; (b) ln k_2 vs K^{-1}) in 50 mM AOT/isooctane reverse micelles, glycerol pool, and 10 mM phosphate buffer (pH 8.0).

Thermostability of Lipase. Figure 5 represents the thermal deactivation profiles of *Pseudomonas fluorescens* lipase entrapped in AOT/isooctane reverse micelles (a), solubilized in a glycerol pool (b), or in buffer (c) and incubated at 40, 50, 60, or 70 °C. The stability of the enzyme was determined by measuring the residual activity of aliquots removed at certain times during the incubation. The rate of glycerolysis in 10 mM phosphate buffer (pH 8.0) dropped rapidly to 50% after 34.38, 19.76, 3.59, and 1.38 h incubation at 40, 50, 60, and 70 °C, respectively. In contrast, the denaturation of the enzyme proceeded more slowly in the glycerol pool, and the activity remained at 90.16, 81.22, 48.62, and 13.04% of the original activity after 5 h incubation at 40, 50, 60, and 70 °C,

respectively. Greater stability was observed in 50 mM AOT/isooctane reverse micelles, and the residual activities were 95.03, 87.85, 78.45, and 69.72% after 5 h incubation at 40, 50, 60, and 70 °C, respectively. In summary, the thermostability of the lipase entrapped in AOT/isooctane reverse micelles at all temperatures was greater than when it was solubilized in glycerol or in 10 mM phosphate buffer (pH 8.0). The half-life (15.75 h) of lipase entrapped in AOT/isooctane reverse micelles at 70 °C was 9.72- and 11.41-fold longer than when the enzyme was solubilized in the glycerol pool or in the aqueous buffer, respectively. The AOT could stabilize the enzyme by suppressing interactions between the lipase and isooctane and by preventing denaturation of the enzyme in the hydrophobic solvent (isooctane, the environmental continuous phase).[25] In addition, the solvation of the lipase in the glycerol pool may reduce the water activity, decreasing the interaction between the surface of the lipase molecules and micellar water, which plays a key role in heat-induced denaturation of the lipase.[26]

To quantify thermal deactivation of the enzyme dissolved in each of the media, a mathematical treatment was undertaken. Because the deactivation curves did not fit single-step first-order kinetics, a model considering two-step series-type enzyme deactivation was employed:

$$E \xrightarrow{K_1 \ \alpha_1} E_1 \xrightarrow{K_2 \ \alpha_2} E_2 \quad (1)$$

in which E, E_1, and E_2 are homogeneous enzymatic states (initial, intermediate, and final, respectively) having different specific activities. This model was reported in the literature[26] and is expressed in eq 2 assuming that α_2 (the ratio of the glycerolysis activity at the E_2 state to that at the E state) = 0, which means that the final form of the enzyme (E_2) is totally deactivated:

$$a = \left(1 + \frac{\alpha_1 k_1}{k_2 - k_1}\right)\exp(-k_1 t) - \left(\frac{\alpha_1 k_1}{k_2 - k_1}\right)\exp(-k_2 t) \quad (2)$$

In the formula, a is the fractional remaining activity, k_1 and k_2 are the first-order deactivation rate constants for the first and second deactivation steps, respectively, and α_1 is the ratio of the glycerolysis activity at the E_1 state to that at the E state. The parameters α_1, k_1, and k_2 were calculated from the experimental data and are shown in Table 2.

As shown, k_1 is significantly larger than k_2. The specific activity of the intermediate is less than that of the initial enzyme state ($\alpha_1 < 1$), and the final state is totally deactivated ($\alpha_2 = 0$). This biphasic behavior has been extensively discussed in various publications[27] and may be exhibited by both soluble and immobilized enzymes. The convexity (toward the origin) exhibited by these curves is a function of the k_1, k_2, and α_1 values. Relatively higher values k_1 together with moderate values of α_1 and k_2 yield a higher degree of convexity.[28,29] A higher degree of convexity implies more sensitivity to the rate of enzyme deactivation. From Figure 5 and Table 2, the significant reductions of convexity (Figure 5) and k_2 values (Table 2) by the entrapment of the lipase molecule in AOT/isooctane reverse micelles were revealed.

More specifically, it can be seen from Table 2 that k_2 at all temperatures drastically decreased after the enzyme was entrapped in AOT/isooctane reverse micelles, whereas k_1 was not significantly reduced. In particular, k_2 (0.0354 h^{-1}) at 70 °C in reverse micelles was 12.33- and 13.14-fold lower than in the glycerol pool (0.4366 h^{-1}) or in phosphate buffer (0.4652 h^{-1}), respectively. The value of α_1 was considerably improved by the formation of AOT/isooctane reverse micelles, indicating that the molecular structure of lipase entrapped in AOT/isooctane reverse micelles was more stable and active than in the glycerol pool or in phosphate buffer.

The values of k_1 and k_2 at a particular temperature were plotted in an Arrhenius diagram in Figure 6. The plot of ln (k_1 or k_2) vs the reciprocal of absolute temperature (K) gave a straight line; the deactivation energy (E_{de}) was determined from the slope ($-E_{de}/R$) of the lines, in which R is the gas constant. The deactivation energies (obtained from k_1, k_2) for the lipase entrapped in 50 mM AOT/isooctane reverse micelles, or solubilized in a glycerol pool, or in 10 mM phosphate buffer (pH 8.0) were 7.51, 26.35 kcal/mol, 5.93, 21.08 kcal/mol, and 5.53, 17.57 kcal/mol, respectively. This result indicates that the AOT/isooctane reversed micellar system increased the stability of the lipase structure and its resistance to heat-induced denaturation.

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Notes

The authors declare no competing financial interest.

REFERENCES

(1) Ognjanovic, N.; Bezbradica, D.; Knezevic-Jugovic, Z. Enzymatic conversion of sunflower oil to biodiesel in a solvent-free system: process optimization and the immobilized system stability. *Bioresour. Technol.* **2009**, *100*, 5146−5154.

(2) Park, K.-M.; Lee, D. E.; Sung, H.; Lee, J.; Chang, P.-S. Lipase-catalysed synthesis of erythorbyl laurate in acetonitrile. *Food Chem.* **2011**, *129*, 59−63.

(3) Zheng, R.-C.; Li, A.-P.; Wu, Z.-M.; Zheng, J.-Y.; Zheng, Y.-G. Enzymatic production of (*S*)-3-cyano-5-methylhexanoic acid ethyl ester with high substrate loading by immobilized *Pseudomonas cepacia* lipase. *Tetrahedron: Asymmetry* **2012**, *23*, 1517−1521.

(4) Brem, J.; Liljeblad, A.; Paizs, C.; Toşa, M. I.; Irimie, F.-D.; Kanerva, L. T. Lipases A and B from *Candida antarctica* in the enantioselective acylation of ethyl 3-heteroaryl-3-hydroxypropanoates: aspects on the preparation and enantiopreference. *Tetrahedron: Asymmetry* **2011**, *22*, 315−322.

(5) Lee, D. E.; Park, K. M.; Choi, S. J.; Chang, P. S. Optimal production and structural characterization of erythorbyl laurate obtained through lipase-catalyzed esterification. *Food Sci. Biotechnol.* **2012**, *21*, 1209−1215.

(6) Pyo, Y. G.; Hong, S. I.; Kim, Y.; Kim, B. H.; Kim, I. H. Synthesis of monoacylglycerol containing pinolenic acid via stepwise esterification using a cold active lipase. *Biotechnol. Prog.* **2012**, *28*, 1218−1224.

(7) Esteban, L.; del Mar Muñío, M.; Robles, A.; Hita, E.; Jiménez, M. J.; González, P. A.; Camacho, B.; Molina, E. Synthesis of 2-monoacylglycerols (2-MAG) by enzymatic alcoholysis of fish oils using different reactor types. *Biochem. Eng. J.* **2009**, *44*, 271−279.

(8) Majid, N.; Cheirsilp, B. Optimal conditions for the production of monoacylglycerol from crude palm oil by an enzymatic glycerolysis

reaction and recovery of carotenoids from the reaction product. *Int. J. Food Sci. Technol.* **2012**, *47*, 793−800.
(9) Damstrup, M. L.; Jensen, T.; Sparsø, F. V.; Kiil, S. Z.; Jensen, A. D.; Xu, X. Solvent optimization for efficient enzymatic monoacylglycerol production based on a glycerolysis reaction. *J. Am. Oil Chem. Soc.* **2005**, *82*, 559−564.
(10) Akoh, C. C.; Sellappan, S.; Fomuso, L. B.; Yankah, V. V. Enzymatic synthesis of structured lipids. In *Lipid Biotechnology*, 1st ed.; Kuo, T. M., Gardner, H. W., Eds.; Marcel Dekker: New York, NY, 2002; 489−519.
(11) Xue, L.; Zhao, Y.; Yu, L.; Sun, Y.; Yan, K.; Li, Y.; Huang, X.; Qu, Y. Choline acetate enhanced the catalytic performance of *Candida rogusa* lipase in AOT reverse micelles. *Colloids Surf., B* **2013**, *105*, 81−86.
(12) Moniruzzaman, M.; Hayashi, Y.; Talukder, M. R.; Kawanishi, T. Lipase-catalyzed esterification of fatty acid in DMSO (dimethyl sulfoxide) modified AOT reverse micellar systems. *Biocatal. Biotransform.* **2007**, *25*, 51−58.
(13) Carvalho, C. M. L.; Cabral, J. M. S. Reverse micelles as reaction media for lipases. *Biochimie* **2000**, *82*, 1063−1085.
(14) Ghosh, M.; Maiti, S.; Brahmachari, S.; Das, P. K. GNP confinement at the interface of cationic reverse micelles: influence in improving the lipase activity. *R. Soc. Ser. Adv. Sci.* **2012**, *2*, 9042−9051.
(15) Chang, P. S.; Rhee, J. S. Characteristics of Lipase-Catalyzed Glycerolysis of Triglyceride in AOT-Isooctane Reversed Micelles. *Biocatal. Biotransform.* **1990**, *3*, 343−355.
(16) Tamamushi, B.; Watanabe, N. The formation of molecular aggregation structures in ternary system: aerosol OT/water/isooctane. *Colloid Polym. Sci.* **1980**, *258*, 174−178.
(17) Chang, P. S.; Rhee, J. S. Simultaneous high-performance liquid chromatographic analysis of oleic acid and simple glycerides of oleic acid. *J. Chromatogr.* **1989**, *465*, 438−441.
(18) Talukder, M. M. R.; Zaman, M. M.; Hayashi, Y.; Wu, J. C.; Kawanishi, T. Thermostability of *Cromobacterium viscosum* lipase in AOT/isooctane reverse micelle. *Appl. Biochem. Biotechnol.* **2007**, *141*, 77−83.
(19) Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein−dye binding. *Anal. Biochem.* **1976**, *72*, 248−254.
(20) Park, K. M.; Kwon, O. T.; Ahn, S. M.; Lee, J.; Chang, P. S. Characterization and optimization of carboxylesterase-catalyzed esterification between capric acid and glycerol for the production of 1-monocaprin in reversed micellar system. *New Biotechnol.* **2010**, *27*, 46−52.
(21) Goswami, D.; Basu, J. K.; De, S. Lipase applications in oil hydrolysis with a case study on castor oil: a review. *Crit. Rev. Biotechnol.* **2013**, *33*, 81−96.
(22) Zaman, M. M.; Hayashi, Y.; Talukder, M. M. R.; Kawanishi, T. Activity of acetone-treated *Chromobacterium viscosum* lipase in AOT reverse micelles in the presence of low molecular weight polyethylene glycol. *Biochem. Eng. J.* **2006**, *29*, 46−54.
(23) Mattson, F. H.; Volpenhein, R. A. Synthesis and properties of glycerides. *J. Lipid Res.* **1962**, *3*, 281−296.
(24) Luna, D.; Posadillo, A.; Caballero, V.; Verdugo, C.; Bautista, F. M.; Romero, A. A.; Sancho, E. D.; Luna, C.; Calero, J. New biofuel integrating glycerol into its composition through the use of covalent immobilized pig pancreatic lipase. *Int. J. Mol. Sci.* **2012**, *13*, 10091−10112.
(25) Talukder, M. M. R.; Hayashi, Y.; Takeyama, T.; Zamam, M. M.; Wu, J. C.; Kawanishi, T.; Shimizu, N. Activity and stability of *Chromobacterium viscosum* lipase in modified AOT reverse micelles. *J. Mol. Catal. B: Enzym.* **2003**, *22*, 203−209.
(26) Longo, M. A.; Combes, D. Thermostability of modified enzymes: a detailed study. *J. Chem. Technol. Biotechnol.* **1999**, *74*, 25−32.
(27) Henley, J. P.; Sadana, A. Categorization of enzyme deactivations using a series-type mechanism. *Enzyme Microb. Technol.* **1985**, *7*, 50−60.
(28) Hennecke, H.; Bock, A. Modification of phenylalanyl-tRNA synthetase from *Escherichia coli* by histidine-specific reagents. Effects on structure and function. *Eur. J. Biochem.* **1974**, *50*, 157−166.
(29) D'Angiuro, L.; Cremonesi, P. Immobilization of glucose oxidase on sepharose by UV-initiated graft copolymerization. *Biotechnol. Bioeng.* **1982**, *24*, 207−216.