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AOT/isooctane reverse micelles with a microaqueous core act as protective shells for enhancing the thermal stability of *Chromobacterium viscosum* lipase

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ABSTRACT

According to the different environmental systems for lipase reactions, changes in thermal stability were investigated by employing the *Chromobacterium viscosum* lipase and a two-step series-type deactivation model. The half-life (6.81 h) of the lipase entrapped in reverse micelles at 70 °C was 9.87- and 14.80-fold longer than that in glycerol pool or in aqueous buffer. The deactivation constants for the first and second step (k_1 and k_2) at all temperatures drastically decreased when the lipase was entrapped in reverse micelles. In particular, k_1 (3.84 h^{-1}) at 70 °C in reverse micelles was 1.57-fold lower than that in aqueous buffer (6.03 h^{-1}). Based on the fluorescence spectrometry, the amount of excited forms of tryptophan and tyrosine increased markedly during the thermal-treatment in aqueous buffer, whereas no significant fluctuation was noted in the reversed micellar system. These results indicated that the encapsulation in reverse micelles could be favorable for preventing the enzyme from heat-induced denaturation.

1. Introduction

Lipase (triacylglycerol hydrolase, E.C. 3.1.1.3) is one of the enzymes that have been widely applied in industrial bioconversions, such as the production of emulsifiers and functional esters, through hydrolysis, glycerolysis and esterification (Houde, Kademi, & Leblanc, 2004; Park, Lee, Sung, Lee, & Chang, 2011). For decades, many studies related to the enzymatic characterization of lipase, immobilization of the lipase and optimization of the enzyme–substrate reaction conditions have been extensively reported to enhance the performance of lipase-catalyzed bioconversions (Lee, Park, Choi, Shim, & Chang, 2013; Moniruzzaman, Hayashi, Talukder, & Kawanishi, 2007; Ognjanovic, Bezbradica, & Knezevic-Jugovic, 2009). Particularly, because of the unique characteristics of lipase-catalyzed reactions between hydrophilic enzymes and hydrophobic substrates, studies focused on the construction of highly efficient reaction systems have been considered to be more important (Chen et al., 2012; Klibanov, 2001).

The structure of reverse micelles consists of an aqueous microdomain (polar phase, core) facing the polar heads of the surfactant that surrounds this core and interacts with the bulk organic solvent (non-polar phase), which is supported by hydrophobic interactions (Carvalho & Cabral, 2000). In a reversed micellar system, lipase is encapsulated in the inner aqueous phase, and enzymatic reactions occur at the interfacial area between the enzymes and the substrates that are solubilized in the external non-polar phase (Correa, Silber, Riter, & Levinger, 2012). Excluding the characteristics that the reactant displays as a homogeneous, monophasic media, the encapsulation of lipases in reverse micelles has been preferred as a novel approach because of its various advantages, including its enormous interfacial area, simple control of the reaction variables and easy monitoring of water content (Park, Kim, Choi, & Chang, 2012; Park, Kwon, Ahn, Lee, & Chang, 2010; Sereti, Zoumpanioti, Papadimitriou, Pispas, & Xenakis, 2014).

Recently, the activation energy of lipase catalyzed reaction in reverse micelles was reported to be lower than that in biphasic

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media, which resulted in a reasonable advantage in the aspect of bioconversion efficiency. Moreover, increased stability (i.e., retention of the catalytic activity for a relatively long term) 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 (Zaman, Hayashi, Talukder, & Kawanishi, 2006). Several notable studies demonstrated that the catalytic activity of lipase was maintained in anhydrous organic solvents under thermal treatment at 100 °C, which was contradictory to the fact that temperatures over 60 °C could cause the denaturation of enzymes (Zaks & Klibanov, 1984). Based on the information presented above, we have conducted an investigation on the changes in thermal-deactivation kinetics according to different reaction systems for the glycerolysis catalyzed by the lipase from *Pseudomonas fluorescens* (Park et al., 2013). As a result, the encapsulation of lipase in reverse micelles decreased the rate constant for the second deactivation-step (k_2) and increased the activation energy for denaturation (E_{de}), leading to the enhancement of resistance to heat-induced denaturation.

The primary purpose of the present study was to confirm the quantitative protection effect experienced after the encapsulation of the lipase in reverse micelles (i.e., the enzyme insulation by the encapsulation in reverse micelles defends its catalytic activity from thermal-deactivation) with a different reaction (hydrolysis catalyzed by *Chromobacterium viscosum* lipase). Additionally, the study aimed to elucidate the shielding effect against the structural denaturation caused by thermal-treatment. A further aim was to reveal that the water content in reverse micelles (referred to as the *R* value, [water]/[surfactant]) influences the thermostability through enzyme-deactivation kinetics by employing a two-step series-type equation model.

2. Materials and methods

2.1. Chemicals and reagents

Purified *C. viscosum* lipase, with a reported catalytic activity of 3400 units/mg solid, was purchased from Millipore (Billerica, MA, USA). Bis-(2-ethylhexyl) sulfosuccinate sodium salt (AOT) was purchased from Sigma–Aldrich (St. Louis, MO, USA) and was purified according to the method of Tamamushi and Watanabe (1980). Isooctane, spectrophotometric grade, was dehydrated over a 4 Å molecular sieve (Sigma–Aldrich) and filtered through a 0.45-μm membrane filter prior to use as the reaction medium in the reverse micelles. Triolein and glycerol (Sigma–Aldrich) were used as substrates in lipase-catalyzed hydrolysis and glycerolysis.

Cupric acetate reagent, used as a coloring reagent, was prepared according to the method of Lowry and Tinsley (1976). The cupric acetate solution was prepared by dissolving 5 g of cupric acetate in 100 ml of distilled water. The prepared cupric acetate solution was then filtered through Whatman No. 1 filter paper, and the pH was adjusted to 6.1 using pyridine. Oleic acid, 1-monoolein, 2-monoolein, 1,2-diolein, 1,3-diolein, and triolein with 99.9% purity were obtained from Sigma–Aldrich and used as the standard lipids in HPLC work.

2.2. Verification of enzyme purity by sodium dodecyl sulfate–polyacrylamide gel electrophoresis

In this study, the *C. viscosum* lipase, which has been widely applied to industrial bioconversions, was selected as a model enzyme. Although *C. viscosum* lipase was highly purified according to the manufacturer's information, the purity of the enzyme was confirmed by electrophoresis. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was conducted using the Laemmli method with a 12% polyacrylamide gel (Laemmli, Beguin, & Gujer-Kellenberger, 1970). Lipase was eluted (1:4, v/v) in sample buffer containing SDS and 2-mercaptoethanol. The eluted sample was boiled for 3 min before loading onto the gels. Electrophoresis was performed at a constant current of 20 mA per gel for 60 min at 25 °C in a Hoefer SE 250 mini-gel system (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom). For protein staining, Coomassie Brilliant Blue R-250 (Sigma–Aldrich) was used.

2.3. Preparation of reverse micelles

Reverse micelles containing lipase were formed by adding appropriate amounts of glycerol, with predetermined amounts of water and lipase powder, to a solution of 50 mM AOT/isooctane. The solution was then mixed vigorously for 1 min to obtain a transparent micellar solution. The desired water content was defined as the *R* value, which indicates the molar ratio of water to surfactant ($[H_2O]$/[surfactant]) (Banerjee, Ghosh, & Datta, 2011), and the glycerol content was defined as the *G* value, which indicates the molar ratio of glycerol to surfactant ([glycerol]/[surfactant]) (Park et al., 2010). The *R* and *G* values were calibrated by considering the amount of water inherently present in the isooctane, glycerol, and AOT. In this study, experiments were conducted under optimized conditions using a *G* value of 4.0 and a *R* value of 10.0 (Park et al., 2013).

2.4. Analysis of glycerolysis activity

Glycerolysis activity was analyzed at 40 °C using triolein and glycerol as substrates. A screw-cap tube, the reactor, was filled with 10 ml of 50 mM AOT/isooctane solution containing triolein (6.67 mM). The desired amount of glycerol and water containing lipase was injected into the tube, and the reaction was initiated by vortex mixing until the mixture was clear. After incubation at 40 °C for a predetermined time, 0.2 ml of the sample was withdrawn from the reaction mixture with a small subcutaneous syringe. In total, 3.0 ml of chloroform was added to the sample, and the test tube was shaken vigorously for 2 min and then put aside for at least 1 h to inactivate the enzyme. Then, 0.5 ml of water was added, and the test tube was again shaken for 1.5 min. The mixture was centrifuged for 5 min at 2000×*g*. The lower chloroform layer was withdrawn 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 identical procedure, as described above, except that a glycerol pool without enzyme was added (Chang, Rhee, & Kim, 1991). 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 (Chang & Rhee, 1990). One unit of enzyme was defined as the amount of lipase that catalyzed the reaction of 1 μmol of triolein/min under the assay conditions. All data are averages of triplicate samples and are reproducible within ±10%.

2.5. Analysis of hydrolysis activity

The hydrolysis activity was analyzed according to the copper-soap colorimetric method. A screw-cap tube was filled with AOT/isooctane reversed-micellar solution that was pre-incubated in a water bath at 37 °C with magnetic stirring at 800 rpm. Lipase-catalyzed hydrolysis was initiated by adding triolein (10%, w/v) as a substrate for the reversed micellar solution. Each aliquot (400 μl) of reactant was collected at predetermined intervals and diluted with 4.6 ml of isooctane. Subsequently, 1 ml of cupric acetate reagent and 1 ml of acetonitrile (used to eliminate the turbidity caused by AOT) was added and mixed vigorously using a vortex

mixer for 30 s. The absorbance of the upper layer (colourized isooctane layer) was measured at 715 nm to quantitatively determine the free fatty acids produced (Prazers, Garcia, & Cabral, 1993). One unit of enzyme was defined as the amount of lipase that released 1 μmol of equivalent fatty acid from triolein in 1 min under the assay conditions. All of the data are the averages of triplicate samples and are reproducible within ±10%. The calibration curve was obtained by calculating the regression equation of the molar concentration of oleic acid and the absorbance at 715 nm.

2.6. Determination of thermal stability

Aliquots were taken at indicated times during incubation of the lipase dissolved in 50 mM Tris–HCl buffer (pH 8.0), glycerol, or reverse micelles consisting of 50 mM AOT/isooctane at various temperatures. The residual glycerolysis activity and hydrolysis activity were analyzed according to the procedure described in the previous section.

2.7. Determination of deactivation kinetic parameters

The data for the deactivation in the 50 mM Tris–HCl buffer (pH 8.0), glycerol (used for glycerolysis), and AOT/isooctane reverse micelles were fitted to a two-step series-type deactivation kinetic model (Knezevic, Siler-Marinkovic, & Mojovic, 1998; Moquin & Temelli, 2008; Talukder, Zaman, Hayashi, Wu, & Kawanishi, 2007). The kinetic parameters were determined using a non-linear regression procedure based on the Marquardt–Levenberg method of iterative convergence included in a solver tool in Microsoft Office Excel 2007 (Microsoft, Redmond, WA, USA) (Valério et al., 2009).

2.8. Structural analysis of thermally deactivated lipase

Circular dichroism (CD) is increasingly recognized as a valuable technique for elucidating the secondary structure of proteins (Greenfield, 2007; Kelly, Jess, & Price, 2005). The analysis was performed on a homogeneous *C. viscosum* lipase at a concentration of 1.0 mg/ml. CD spectrometry (Chirascan™-plus, Applied Photophysics Leatherhead, Surrey, UK) was accomplished in 50 mM Tris–HCl buffer (pH 8.0) or reverse micelles consisting of 50 mM AOT/isooctane in the Far-UV regions (190–260 nm), with a path length of 0.5 mm and bandwidth of 1.0 nm. To study the effect of temperature on the secondary structure of *C. viscosum* lipase, CD spectrometric analysis was performed at 40, 50, 60, and 70 °C. The structural changes in the lipase were analyzed from the CD spectra using the CDNN secondary structure analysis software (Applied Photophysics). In addition, the variance of aromatic amino acids at the active site of the *C. viscosum* lipase was analyzed using a fluorescence spectrometer (Spectra004Dax M2^e, Molecular Devices Corp., CA, USA) (Melo, Costa, & Cabral, 1996). After incubation of the lipase dissolved in 50 mM Tris–HCl buffer (pH 8.0) or entrapped in reverse micelles consisting of 50 mM AOT/isooctane at 40, 50, 60, or 70 °C for 30 min, the emission slit of the substance excited at 280 nm was measured using a fluorescence spectrometer.

3. Results and discussion

3.1. SDS–PAGE

To obtain the thermal-deactivation kinetic parameters with high accuracy and reproducibility, the purity of the lipase was analyzed by electrophoresis. A result of the SDS–PAGE analysis showed a single band with molecular weight of approximately 33 kDa, which confirmed that the lipase from *C. viscosum* was of high purity (Fig. S1, Supplementary data).

3.2. Analysis of glycerolysis activity and thermal stability

Fig. 1 shows the thermal deactivation profiles of glycerolysis by the *C. viscosum* lipase entrapped in AOT/isooctane reverse micelles (a), solubilized in a glycerol pool (b), and in buffer (c) incubated at 40, 50, 60, or 70 °C. The stability of the enzyme was determined by measuring the residual activity during the incubation. It was observed that the half-life of the lipase from *C. viscosum* in

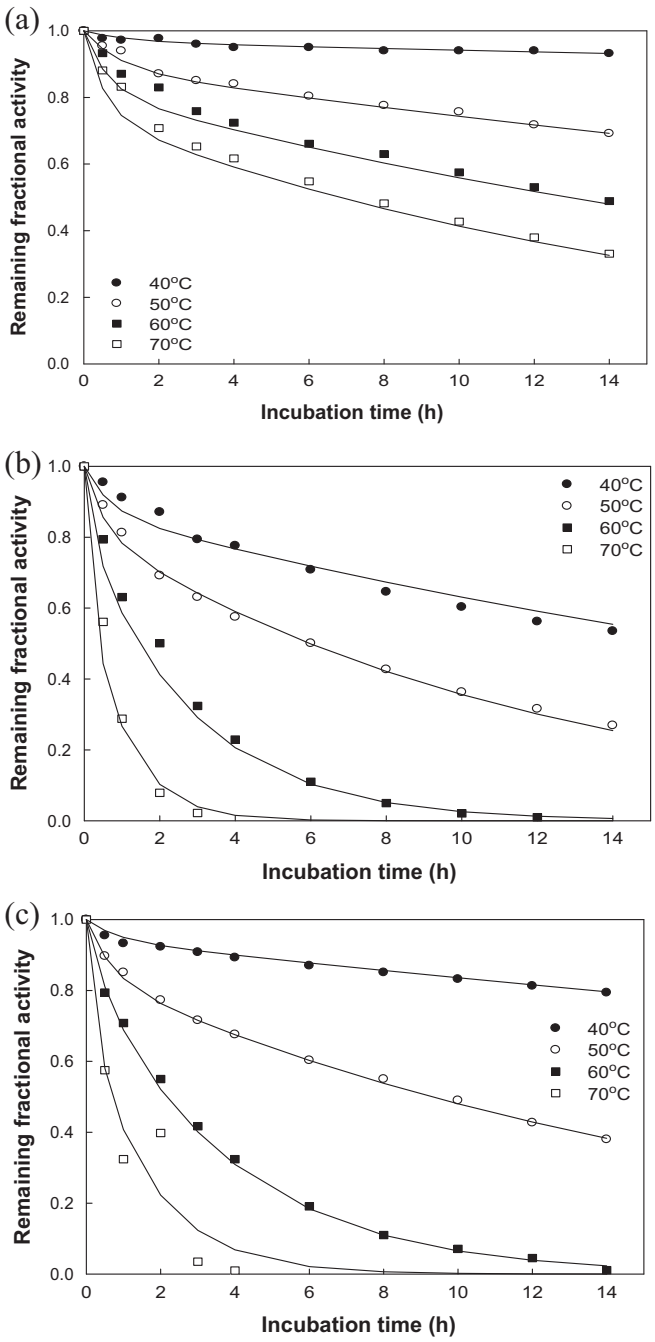

Fig. 1. Thermal deactivation profiles of *Chromobacterium viscosum* lipase-catalyzed glycerolysis in 50 mM AOT/isooctane reverse micelles (a), 50 mM Tris–HCl buffer (pH 8.0) (b), and the glycerol pool (c).

50 mM Tris–HCl buffer (pH 8.0) was 17.83, 5.81, 1.48 and 0.46 h incubation at 40, 50, 60, and 70 °C, respectively. However, the denaturation of the enzyme proceeded more slowly in the glycerol pool, and the activity remained at 89.03%, 63.98%, 24.61%, and 4.17% of the initial activity after 5 h incubation at 40, 50, 60, or 70 °C, respectively. The remaining fractional activities in 50 mM AOT/isooctane reverse micelles were obtained as 95.03%, 87.85%, 78.45% and 69.72% after 5 h incubation at 40, 50, 60, or 70 °C, respectively. In summary, the thermal stability of lipase entrapped in 50 mM AOT/isooctane reverse micelles at all temperatures was more stable than that in the glycerol pool or in 50 mM Tris–HCl buffer (pH 8.0). The stability of an enzyme has been frequently reported in terms of half-life. The half-life (6.81 h) of lipase entrapped in AOT/isooctane reverse micelles at 70 °C was 9.87- and 14.80-fold longer than that in the glycerol pool or in the aqueous medium, respectively. The thermal stability of the enzymes was reported to be maintained by AOT either preventing the denaturation with organic solvents or suppressing interactions between the lipase and isooctane (Delorme et al., 2011).

The thermal deactivation of the enzyme stored in each medium was statistically quantified. The thermal deactivation profiles of *C. viscosum* lipase did not match single-step first order kinetics, indicating that the fractional rate of deactivation was not constant. Therefore, a two-step, series-type enzyme deactivation model was selected in the analysis of enzyme deactivation kinetics.

$$E \xrightarrow{k_1} {}^{\alpha_1}E_1 \xrightarrow{k_2} {}^{\alpha_2}E_2 \quad (1)$$

In the above reaction stage (Eq. (1)), E, E_1, and E_2 can be described as the initial, intermediate, and final lipase status, respectively. For this time, the activity differs in each lipase state. This model of the deactivation mechanism of enzymes was previously reported in the literature (Henley & Sadana, 1985) and is expressed in Eq. (2) by assuming that α_2 is 0 and the final form of the enzyme (E_2) is totally deactivated by heat.

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

In this formula, a refers to the fractional remaining activity; k_1 and k_2 refer to the first-order deactivation rate constants for the first and second deactivation steps, respectively; and α_1 is the ratio of glycerolysis activity in the E_1 state related to the activity in the E state. The deactivation kinetic parameters (α_1, k_1 and k_2) were calculated through experimental data and are expressed in Table 1.

As shown in Table 1, k_1 was greater than k_2. In this case, the specific activity of the initial enzyme state was more than that of the intermediate ($\alpha_1 < 1$), and the final state was completely deactivated ($\alpha_2 = 0$). This biphasic behavior and two-step series deactivation kinetics have been extensively discussed in various studies and could be exhibited by both soluble and immobilized enzymes (Sadana, 1988; Voll et al., 2011). 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 together with moderate values of α_1 and k_2 yield a higher degree of convexity. A higher degree of convexity implies more sensitivity to the rate of enzyme deactivation. Fig. 1 and Table 1 display the significant reduction of convexity (Fig. 1) and k_2 values (Table 1) of the entrapped lipase molecule in AOT/isooctane reverse micelles.

Table 1
Deactivation kinetic parameters of *Chromobacterium viscosum* lipase-catalyzed glycerolysis in 50 mM Tris–HCl buffer (pH 8.0), the glycerol pool, and 50 mM AOT/isooctane reverse micelles.

Parameter	40 °C	50 °C	60 °C	70 °C
In 50 mM Tris–HCl buffer (pH 8.0)				
k_1 (h^{-1})	1.4846	1.9960	3.8856	6.0303
k_2 (h^{-1})	0.0325	0.0842	0.3467	0.9530
α_1	0.8538	0.7916	0.7510	0.5813
In the glycerol pool				
k_1 (h^{-1})	0.8985	1.5119	2.5057	3.7714
k_2 (h^{-1})	0.0122	0.0567	0.2594	0.5894
α_1	0.9348	0.8147	0.7649	0.6102
In 50 mM AOT/isooctane reverse micelles				
k_1 (h^{-1})	0.8235	1.3613	2.2768	3.8381
k_2 (h^{-1})	0.0026	0.0178	0.0382	0.1395
α_1	0.9640	0.8732	0.7986	0.7254

Table 1 shows the differences in the effect of temperature in the enzyme storage conditions. At all temperatures, k_1 did not greatly differ depending on the storage conditions, whereas k_2 drastically decreased after the enzyme was entrapped in AOT/isooctane reverse micelles. In particular, k_2 at 70 °C in aqueous medium was 6.83- and 1.62-fold greater than that in AOT/isooctane reverse micelles or in the glycerol pool. The value of α_1 in AOT/isooctane reverse micelles was greater than that in the glycerol pool and the aqueous medium. The thermal stability in AOT/isooctane reverse micelles was more stable and active than in the glycerol pool or in the aqueous medium.

Logarithmic values of k_1 and k_2 at a specific temperature were plotted as an Arrhenius diagram (Fig. S2, Supplementary data). The plot of $\ln(k_1$ or $k_2)$ versus the reciprocal of the absolute temperature (K^{-1}) produced a straight line. The deactivation energy (E_{de}) was determined from the slope ($-E_{de}/R$) of the line, 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, in a glycerol pool, and in 50 mM Tris–HCl buffer (pH 8.0) were (12.80, 32.98 kcal/mol), (11.93, 32.24 kcal/mol), and (11.65, 28.10 kcal/mol), respectively. From the results mentioned above, the AOT/isooctane reversed micellar system enhanced the stability of the lipase and its resistance to heat-induced denaturation.

3.3. Analysis of hydrolysis activity and thermal stability

The thermal stability of the lipase in an aqueous medium and in AOT/isooctane reverse micelles was identified in hydrolysis. These tests were conducted under the conditions previously described in the 'Section 2'. Fig. 2 displays the thermal deactivation profiles for the hydrolytic activities of *C. viscosum* lipase entrapped in AOT/isooctane reverse micelles (a) and in buffer (b) incubated at 40, 50, 60, and 70 °C. The stability of the enzyme was determined by measuring the residual activity of aliquots from each enzyme sample during the incubation. The half-lives of the lipase from *C. viscosum* in 50 mM Tris–HCl buffer (pH 8.0) at 40, 50, 60, and 70 °C were 12.67, 7.75, 1.68, and 0.15 h, respectively. However, the denaturation of the enzyme proceeded more slowly in the 50 mM AOT/isooctane reverse micelles, and the remaining fractional activities were 89.55%, 83.66%, 40.74%, and 11.07% after a 5 h incubation at 40, 50, 60, and 70 °C, respectively. In summary, the thermal stability of the hydrolytic activity of the lipase entrapped in AOT/isooctane reverse micelles at all temperatures was higher than that in 50 mM Tris–HCl buffer (pH 8.0). The half-life (0.32 h) of the lipase entrapped in AOT/isooctane reverse micelles at 70 °C was 2.13-fold longer than that in the aqueous medium.

The deactivation kinetic parameters α_1, k_1, and k_2 were calculated from the experimental data and are shown in Table 2. As shown in Fig. 2 and Table 2, k_1 was significantly larger than k_2 in hydrolysis. Moreover, the significant reductions of convexity (Fig. 2) and k_2 values (Table 2) occurred when the lipase molecule was entrapped in AOT/isooctane reverse micelles. More specifically, Table 2 shows that k_2 drastically decreased after the enzyme was entrapped in AOT/isooctane reverse micelles at all temperatures. In particular, k_2 (0.1694 h^{-1}) at 70 °C in reverse micelles

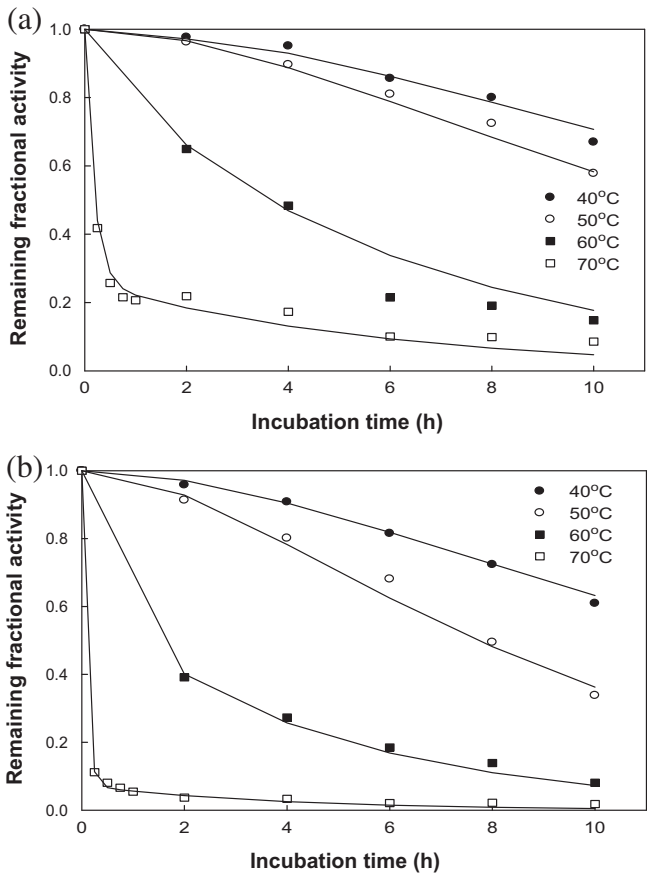

Fig. 2. Thermal deactivation profiles of *Chromobacterium viscosum* lipase-catalyzed hydrolysis in 50 mM AOT/isooctane reverse micelles (a) and 50 mM Tris–HCl buffer (pH 8.0) (b).

Table 2
Deactivation kinetic parameters of *Chromobacterium viscosum* lipase-catalyzed hydrolysis in 50 mM Tris–HCl buffer (pH 8.0) and 50 mM AOT/isooctane reverse micelles.

Parameter	40 °C	50 °C	60 °C	70 °C
In 50 mM Tris–HCl buffer (pH 8.0)				
k_1 (h^{-1})	0.6065	0.6691	1.8540	4.1521
k_2 (h^{-1})	0.0165	0.0920	0.2105	1.3684
α_1	0.9469	0.7984	0.5281	0.1721
In 50 mM AOT/isooctane reverse micelles				
k_1 (h^{-1})	0.1982	0.3584	0.8125	1.3804
k_2 (h^{-1})	0.0037	0.0322	0.0812	0.1694
α_1	0.9903	0.9073	0.7307	0.2507

was 8.08-fold lower than that in Tris–HCl buffer (1.3684 h^{-1}). The value of α_1 and the glycerolysis kinetic parameter considerably improved with the formation of AOT/isooctane reverse micelles. The values of k_1 and k_2 calculated from experimental data at a particular temperature were plotted as an Arrhenius diagram (Fig. S3, Supplementary data). The relationship of $\ln(k_1, k_2)$ to the reciprocal of absolute temperature produced a straight line. The deactivation energies (obtained from k_1, k_2) for the lipase entrapped in 50 mM AOT/isooctane reverse micelles and 50 mM Tris–HCl buffer (pH 8.0) were (12.63, 28.82 kcal/mol) and (10.24, 26.58 kcal/mol), respectively. In summary, these results in glycerolysis and hydrolysis indicated that the AOT/isooctane reversed micellar system enhanced the stability and resistance to heat-induced denaturation of the lipase.

On the other hand, it was revealed that the overall thermal stability in glycerolysis was better than that in hydrolysis, which might result from the protective effect of the glycerol pool against the thermal deactivation of the lipase. In the case of hydrolysis, the deactivation profiles were exhibited solely by the thermal stability of the lipase, excluding the aforementioned protective effect. Additionally, because the water content was a critical factor affecting the catalytic performance in hydrolysis, further experiments were needed to investigate whether water content correlates with thermal deactivation.

3.4. Effect of water content on the thermal stability of the lipase in reverse micelles

Based on the previous experiments that evaluated the thermal stability of the lipase in reverse micelles and in an aqueous buffer, the lipase was shown to be more heat-resistant in the encapsulated form in the reverse micellar system. In this section, changes in enzyme-deactivation energies depending on the *R* value (referred to the molar ratio of water to AOT) were monitored to investigate the effect of water content on the thermal stability of the lipase in reverse micelles. Fig. 3 shows the thermal deactivation energy profiles of the *C. viscosum* lipase entrapped in AOT/isooctane reverse micelles with different *R* values. The stability of the enzyme was determined by measuring the residual activity of aliquots from each enzyme sample during the incubation. As expected, the deactivation energy decreased with increasing *R* values because of the higher denaturation degree exerted by the higher mobility and collision of the enzyme protein molecule.

The deactivation energies derived from k_1 and k_2 drastically decreased at *R* values more than 10.0. The results indicated that water content less than 10.0 for the *R* value in AOT/isooctane reverse micelles could be favorable for resisting heat-induced denaturation of the lipase. However, it has been generally recognized that too low a water content (i.e., less than 10.0 for the *R* value) acts as the lack of essential water (as a substrate) in the hydrolysis, which may lead to suppression of the catalytic performance of the lipase. Therefore, to obtain high thermal stability and activity expression ability for the most efficient bioconversion, the lipase-catalyzed reaction should be performed at an *R* value of 10.0.

3.5. Structural analysis of thermally deactivated lipase

The secondary structure of the *C. viscosum* lipase was analyzed using Far-UV CD spectra in the range of 190–260 nm. In the theory

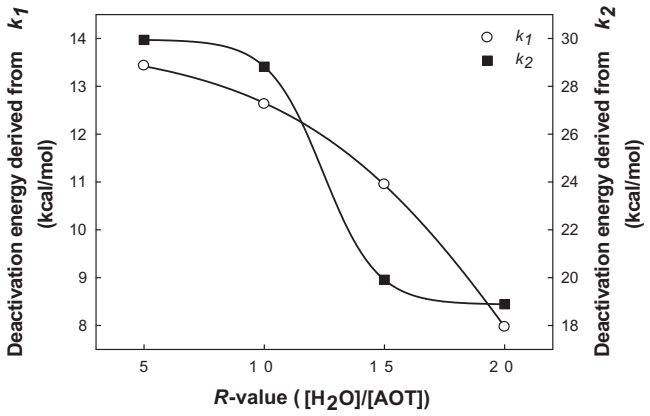

Fig. 3. Deactivation energy profiles of *Chromobacterium viscosum* lipase-catalyzed hydrolysis in 50 mM AOT/isooctane reverse micelles with different *R* values.

of CD spectrometry, α-helix proteins have negative bands at 222 and 208 nm and a positive band at 193 nm, and proteins with β-sheets have a negative band at 218 nm and a positive band at 195 nm, whereas disordered proteins, such as β-turn and random coils, have negative bands at 189 nm and 198 nm and positive bands at 210 nm and 212 nm (Greenfield, 2007). To analyze structural changes resulting from temperature fluctuations, Far-UV CD spectra were measured after incubation at 40, 50, 60, and 70 °C for 30 min. There were no significant changes in far-UV CD spectra at temperature ranging from 40 °C to 70 °C (data not shown). More specifically, the secondary structure was calculated from the CD spectra and amino acids composition (Table S1). The secondary structure of the *C. viscosum* lipase without thermal treatment (negative control) was confirmed to consist of the following: α-helix (17.0%), anti-parallel β-sheet (13.0%), β-turn (20.8%), and random coil (43.1%). Regardless of the lipase deactivation (loss of activity), the composition of the secondary structure of the lipase did not fluctuate significantly. A negligible change (approximately 0.2%) was noted for the anti-parallel β-sheet in both aqueous medium and reversed micellar system.

According to the report of Melo et al., the thermal deactivation of the *C. viscosum* lipase was not because of protein denaturation, such as secondary and tertiary structure changes (Melo, Taipa, Castellar, Costa, & Cabral, 2000; Melo et al., 1996; Walde, Han, & Luisi, 1993). It is well known that aromatic amino acids at the active site of enzymes play a role as an important factor influencing the intrinsic activity. A fluorescence spectrometric method (excited 280 nm and emission at 280–340 nm) has been widely employed to detect excited forms of aromatic amino acids, such as tryptophan and tyrosine. As shown in the fluorescence emission spectra (Fig. 4), the tryptophyl contribution increased in proportion as the temperature increased during the thermal treatment in the aqueous medium. Contrary to that, no significant change was noted in the intensity range of 280–340 nm in the fluorescence spectrum, which indicated that the encapsulation in the reverse micelles could suppress the conversion of aromatic amino acids into excited forms. This phenomenon can be explained by the report that tryptophan residues are usually the main contributors to the emission of globular proteins because of the Tyr-to-Trp energy transfer and quenching by nearby groups on the peptide chain (Lakowicz, 2009). The increase of the tyrosyl contribution likely reflects a less efficient Tyr-to-Trp energy transfer (Saito, Tachibana, Hayashi, & Wada, 1981). From the CD and fluorescence spectrometry, thermal deactivation of the lipase from *C. viscosum* was related to minor changes in the protein conformation.

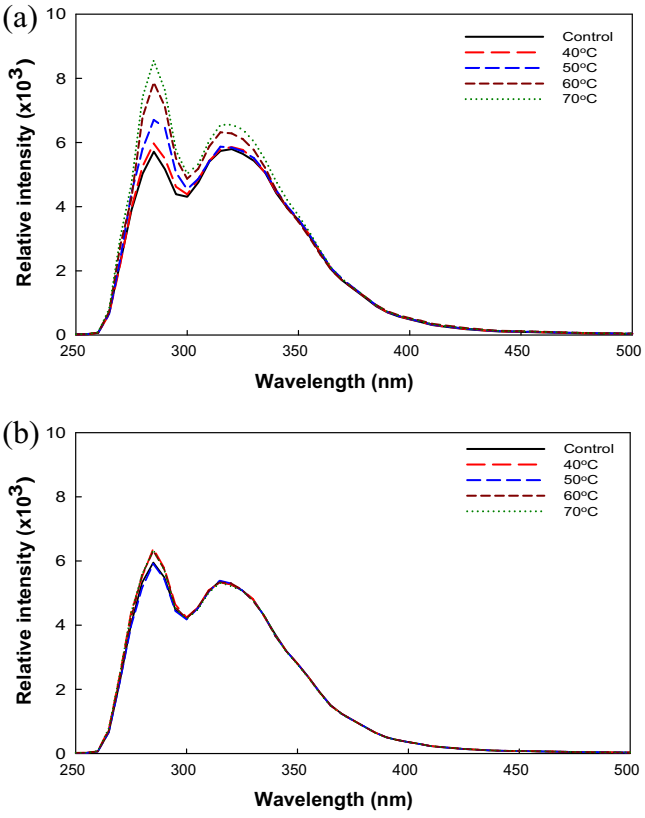

Fig. 4. Fluorescence emission spectra of *Chromobacterium viscosum* lipase after excitation at 280 nm in 50 mM Tris–HCl buffer (a) and in 50 mM AOT/isooctane reverse micelles (b).

4. Conclusion

In this study, the changes in thermal stability and the enzyme-deactivation kinetic according to different reaction systems was investigated by employing glycerolysis and hydrolysis catalyzed by a *C. viscosum* lipase and a two-step series-type deactivation model. To conclude, the deactivation constants for the first and second step (k_1 and k_2) of the lipase in reversed micelles was lower than those in a glycerol pool or aqueous medium system, which led to a longer half-life of the enzyme during the thermal-treatment. Thus, the encapsulation in reverse micelles could be favorable for protecting the enzyme from heat-induced denaturation.

Based on the findings from the CD and fluorescence spectrometry, it was evident that the thermal deactivation was caused by the minor modification of aromatic amino acids found in the active site rather than by changes in secondary structure of the lipase from *C. viscosum*.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2015.01.120.

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