A Thesis for the Degree of Master of Science

Effect of Water Content on the Thermal Stability of *Chromobacterium viscosum* Lipase in AOT/isooctane Reverse Micelles

역미셀계 내에서의 수분 함량이 *Chromobacterium viscosum* lipase의 열 안정성에 미치는 영향

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Hong, Sung Chul

Department of Agricultural Biotechnology

Seoul National University

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지도교수 장 판 식

이 논문을 석사학위 논문으로 제출함

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서울대학교 대학원 농생명공학부

홍 성 철

홍성철의 석사 학위 논문을 인준함

위 원 장 서 진 호 (인)

부위원장 장 판 식 (인)

위 원 최 영 진 (인)

Abstract

According to the different reaction systems such as aqueous media, glycerol pool, and reverse micelles, changes in thermal stability and enzyme-deactivation kinetic parameters were investigated in this study, employing glycerolysis and hydrolysis catalyzed by *Chromobacterium viscosum* lipase and two-step series-type deactivation model. Thermal stability of the lipase (triacylglycerol hydrolase, EC 3.1.1.3) was found to be increased by the enzyme-entrapment in AOT/isooctane reverse micelles. The half-life (6.81 hr) of *Chromobacterium viscosum* lipase entrapped in reverse micelles at 70°C was 9.87- and 14.80-fold longer than those solubilized in a glycerol pool and in 50 mM Tris-HCl buffer (pH 8.0), respectively. The enzyme deactivation model considering a two-step series-type was employed and deactivation constants for the first and second step (k_1 and k_2) at all temperatures were drastically decreased after the lipase was entrapped in reverse micelles. In particular, k_1 (3.84 hr^{-1}) at 70°C in reverse micelles was 1.57-fold lower comparing to that in the aqueous buffer (6.03 hr^{-1}). The deactivation energies (from k_1, k_2) for the lipase entrapped in the reverse micelles, solubilized in a glycerol pool, and in the aqueous buffer were (12.80 kcal/mol, 32.98 kcal/mol), (11.93 kcal/mol, 32.24 kcal/mol), and (11.65 kcal/mol, 28.10 kcal/mol), respectively. Based on the experiments in hydrolysis, it was also

found to be consistent with these phenomena indicate the lipases in reverse micelles were more stable against thermal treatment than those in aqueous media. From the structural analysis of thermally-deactivated lipase through fluorescence spectrometry to detect the conversion of aromatic residues, the amount of excited forms of tryptophan and tyrosine increased markedly in the case of thermal-treatment in the aqueous buffer, whereas there was no significant fluctuation in the reversed micellar system.

Keywords: reverse micelles; deactivation kinetics; thermal stability; *Chromobacterium viscosum* lipase; water content; circular dichroism

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1. Introduction

Lipase (triacylglycerol hydrolase, E.C. 3.1.1.3) is one of the enzymes that have been widely applied to the industrial bioconversions such as synthesizing emulsifiers and functional esters through hydrolysis, glycerolysis, and esterification (Houde, Kademi, & Leblanc, 2004; K.-M. Park, Lee, Sung, Lee, & Chang, 2011). For decades, many studies related to enzymatic characteristics, immobilization of lipase, and optimization for reaction conditions have been extensively investigated to enhance the yield of the lipase-catalyzed bioconversion (Lee, Park, Choi, Shim, & Chang, 2013; Moniruzzaman, Hayashi, Talukder, & Kawanishi, 2007; Ognjanovic, Bezbradica, & Knezevic-Jugovic, 2009). Particularly, due to the unique characteristics of lipase-catalyzed reaction between hydrophilic enzymes and hydrophobic substrates, the researches on efficient reaction systems have been considered to be more important (Klibanov, 2001; Stamatis, Xenakis, Menge, & Kolisis, 1993).

The structure of reverse micelles consists of an aqueous micro-domain 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 reversed micellar

system, lipase exists as a form encapsulated on the inner aqueous phase and an enzymatic reaction occurs at the interfacial area between the enzymes and the substrates solubilized in the external non-polar phase (De & Maitra, 1995). Excluding even the characteristic that reactant displays the homogeneity as monophasic media, the encapsulation of lipases in reverse micelles has been preferred as a novel approach method due to its various advantages such as enormous interfacial area, simple control of the reaction variables, and easy monitoring of water content (K. M. Park, Kim, Choi, & Chang, 2012; K. M. Park, Kwon, Choi, Son, Lim, Yoo, et al., 2013; K. M. Park, Kwon, Ahn, Lee, & Chang, 2010).

Recently, there was a report that the activation energy of lipase catalyzed reaction in reverse micelles was lower than that in biphasic media, which results in a reasonable advantage in the aspect of bioconversion efficiency. Moreover, increased stability (i.e. retain the catalytic activity for 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 (Talukder, Takeyama, Hayashi, Wu, Kawanishi, Shimizu, et al., 2003; Zaman, Hayashi, Talukder, & Kawanishi, 2006). On the other hand, Some interesting studies demonstrated

that the catalytic activity of lipase was maintained in anhydrous organic solvent under the thermal treatment at 100^{o}C, which is contradictory to the fact that high temperature over 60^{o}C could cause the denaturation of enzymes in general conditions (Zaks & Klibanov, 1984; Zaks & Klibanov, 1985).

Based on the knowledge above, the primary purpose of this study is to investigate whether the encapsulation of lipases in reverse micelles protects the catalytic activity from thermal-deactivation. A further aim of this study is to elucidate such phenomena through enzyme-deactivation kinetics employing the two-step series-type equation model.

2. Materials and Methods

2.1. Chemicals and reagents

Purified *Chromobacterium viscosum* lipase, with a reported catalytic activity of 3,400 units/mg solid, was purchased from Millipore (Billerica, MA, USA). Bis (2-ethylhexyl) sulfosuccinate sodium salt (Aerosol-OT or AOT) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and purified according to the method of Tamamushi and Watanabe (Tamamushi & 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 a reaction medium in the reversed micellar system. Olive oil, triolein, and glycerol (Sigma-Aldrich) were used as substrates in lipase-catalyzed hydrolysis and glycerolysis.

Cupric acetate reagent, used as a color reagent, was prepared according to the method of Lowry and Tinsley (Lowry & 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.

1-Monoolein, 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.

2.2. Determination of enzyme purity using sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Although *Chromobacterium viscosum* lipase was highly purified according to its manufacturer's information, its purity was confirmed by electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis was conducted with Laemmli method using 12% polyacrylamide (Laemmli, Beguin, & Gujer-Kellenberger, 1970). The lipase was eluted (1:4) 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 constant current of 20 mA per gel for 60 min at 25°C and Hoefer SE 250 mini-gel system (GE Healthcare). For protein staining, Coomassie Brilliant Blue R-250 was used (Cleveland, Fischer, Kirschner, & Laemmli, 1977).

2.3. 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 AOT/isooctane. The mixture was then mixed vigorously for 1 min to obtain transparent micellar solutions. The desired water content was usually defined as the *R* value, which means 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 means the molar ratio of glycerol to surfactant ([glycerol]/[surfactant]) (K. M. Park, Kwon, Ahn, Lee, & Chang, 2010). The *R* and *G* values were calibrated taking the amount of water inherently present in the isooctane, glycerol, and AOT into consideration. In this study, experiments were conducted with the most optimized condition using the *G* value at 4.0 and the *R* value at 10.0 (Chang, Rhee, & Kim, 1991; Prazeres, Garcia, & Cabral, 1992).

2.4. Determination of lipase glycerolysis activity

Glycerolysis activity was measured at 40^{o}C using triolein and glycerol as substrates. A screw-cap tube, used as our 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 the mixture until it was clear. After incubation at 40^{o}C for a predetermined time, 0.2 mL of sample was

taken out from the reaction mixture with a small subcutaneous syringe. To the sample in a test tube was added 3.0 mL of chloroform, and the test tube was shaken vigorously for 2 min and then put aside for at least 1 hr 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 2,000 xg. 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 (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 1μmol of triolein/min under the assay conditions. All of data are averages of triplicate samples and are reproducible within ± 10%.

2.5. Determination of the lipase hydrolysis activity

Hydrolysis activity was determined according to copper-soap

colorimetric method. A screw-cap tube was filled with AOT/isooctane reversed micellar solution that was pre-incubation in a water bath at 37^{o}C with magnetic stirring at 800 rpm. Lipase-catalzyed hydrolysis was initiated by adding olive oil (10%, v/v) as a substrate to 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 turbidity caused by AOT) was added and mixed vigorously using a vortex mixer for 30 s. The absorbance of the upper layer (colorized 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 hydrolyzed with 1 μmol of fatty acids/min under the assay condition.

All of the data are 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. Measurement of the lipase thermal stability

After incubation of the enzyme dissolved in 50 mM Tris-HCl buffer (pH 8.0), glycerol, and reverse micelles consisting of 50 mM AOT/isooctane at predetermined temperature interval, aliquots were taken at the indicated times, and the residual glycerolysis activity and hydrolysis activity were assayed according to the procedure described in the previous section.

2.7. Determination of the lipase deactivation kinetic parameters

The data for 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), and 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 of Microsoft Office Excel 2007 software (Aymard & Belarbi, 2000; Press, Teukolsky, Vetterling, & Flannery, 1992; Valério, Krüger, Ninow, Corazza, de Oliveira, Oliveira, et al., 2009).

2.8. Structural analysis of thermally deactivated lipase

Circular dichroism (CD) is being increasingly recognized as a valuable technique for analyzing the secondary structure of proteins (Greenfield, 2007; Kelly, Jess, & Price, 2005). Measurements were performed on homogeneous *Chromobacterium viscosum* lipase at a concentration of 1.0 mg/mL. CD spectrometer (Chirascan™-plus, Applied Photophysics, Ltd., Leatherhead, Surrey, UK) was performed 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), path length (0.5 mm), and bandwidth (1.0 nm). To study the effects of temperature on the second structure of *Chromobacterium viscosum* lipase, CD was measured at 40, 50, 60, and 70^{o}C. The structural changes of lipase were analyzed from the CD spectra using the CDNN secondary structure analysis software. In addition, the variance of aromatic amino acid at the active site of the *Chromobacterium viscosum* lipase was analyzed using a fluorescence spectrometer (Spectra004Dax M2^{e}, Molecular Devices Corp., CA, USA) (E Pinho 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, and 70^{o}C for 30 min, the emission slit of the substance excited to 280 nm was measured using a fluorescence spectrometer.

3. Results and discussion

3.1. SDS-PAGE

In this study, *Chromobacterium viscosum* lipase which has been widely applied to industrial bioconversions was chosen as a model enzyme. To obtain the thermal-deactivation kinetics with accuracy and reproducibility, purity of the lipase was analyzed by electrophoresis. As a result of SDS-PAGE analysis showing a single band with molecular weight of approximately 33 kDa, it was confirmed that the lipase from *Chromobacterium viscosum* has high purity.

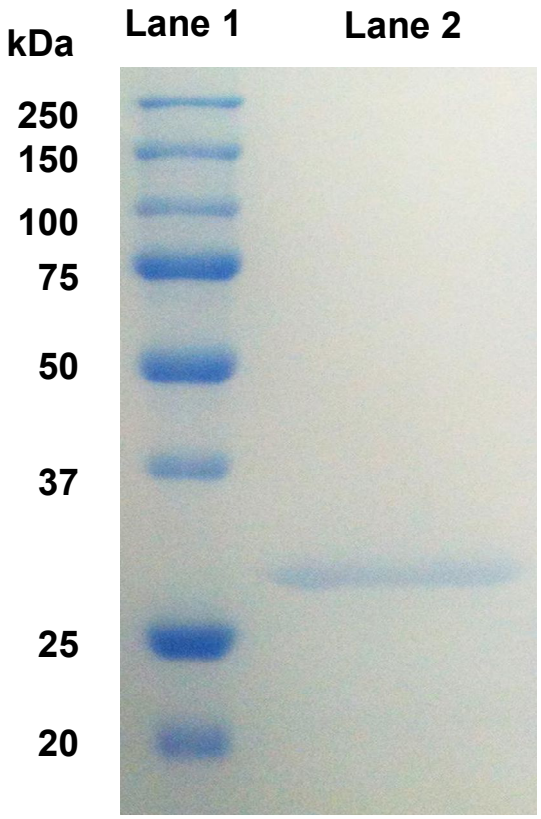


Figure 1. SDS-PAGE of the *Chromobacterium viscosum* lipase. Lane 1, Marker; Lane 2, *Chromobacterium viscosum* lipase.

3.2. Analysis of glycerolysis activity and thermal stability

Figure 2 shows the glycerolysis thermal deactivation profiles of *Chromobacterium viscosum* lipase entrapped in AOT/isooctane reverse micelles (a), solubilized in a glycerol pool (b), and in buffer (c) incubated at 40, 50, 60, and 70^{o}C. The stability of enzyme was determined by measuring the residual activity of aliquots removed at certain times during the incubation. It is observed that the half-life of lipase from *chromobacterium viscosum* in 50 mM Tris-HCl buffer (pH 8.0) is 17.83, 5.81, 1.48 and 0.46 hr incubation at 40, 50, 60, and 70^{o}C, respectively. While, 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 original activity after 5 hr incubation at 40, 50, 60, and 70^{o}C, respectively. Remaining fractional activities in 50 mM AOT/isooctane reverse micelles were obtained as 95.03, 87.85, 78.45 and 69.72% after 5 hr incubation at 40, 50, 60, and 70^{o}C, respectively. In summary, the thermal stability of lipase entrapped in 50 mM AOT/isooctane reverse micelles at all temperatures was more stable than when it was solubilized in the glycerol pool or in 50 mM Tris-HCl buffer (pH 8.0). Stability of enzyme frequently reported in terms of half-life. The half-life (6.81 hr) of lipase entrapped in AOT/isooctane reverse micelles at

70°C was 9.87- and 14.80-fold longer than when the enzyme stored in the glycerol pool or in the aqueous media system, respectively. It was reported that thermal stability of the enzymes is maintained by AOT preventing denaturation with organic solvents or suppressing interactions between the lipase and isooctane (Delorme, Dhouib, Canaan, Fotiadu, Carrière, & Cavalier, 2011).

To quantify thermal deactivation of the enzyme stored in each of the media, a mathematical technique of statistics was used. In thermal deactivation profiles did not appropriate for single-step first order kinetics, so a two-step series-type enzyme deactivation model was selected for analysis of enzyme deactivation kinetics.

$$E \xrightarrow{k_1} E_1 \overset{\alpha_1}{\xrightarrow{k_2}} E_2 \overset{\alpha_2}{} \qquad [1]$$

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

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

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. α_1 is the ratio of glycerolysis activity in the E_1 state to the activity in the E state. Deactivation kinetics parameters which were 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 has a greater than k_2. In this case, the specific activity of the initial enzyme state is more than that of intermediate ($\alpha_1<1$), and the final state is totally deactivated ($\alpha_2=0$). This two-step series deactivation kinetics has been extensively discussed in various literatures and may be exhibited by both soluble and immobilized enzymes (Sadana, 1988; Voll, Krüger, de Castilhos, Cabral, Ninow, & Corazza, 2011). This biphasic behavior has been extensively discussed in various publications 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 together with moderate values of α_1 and k_2

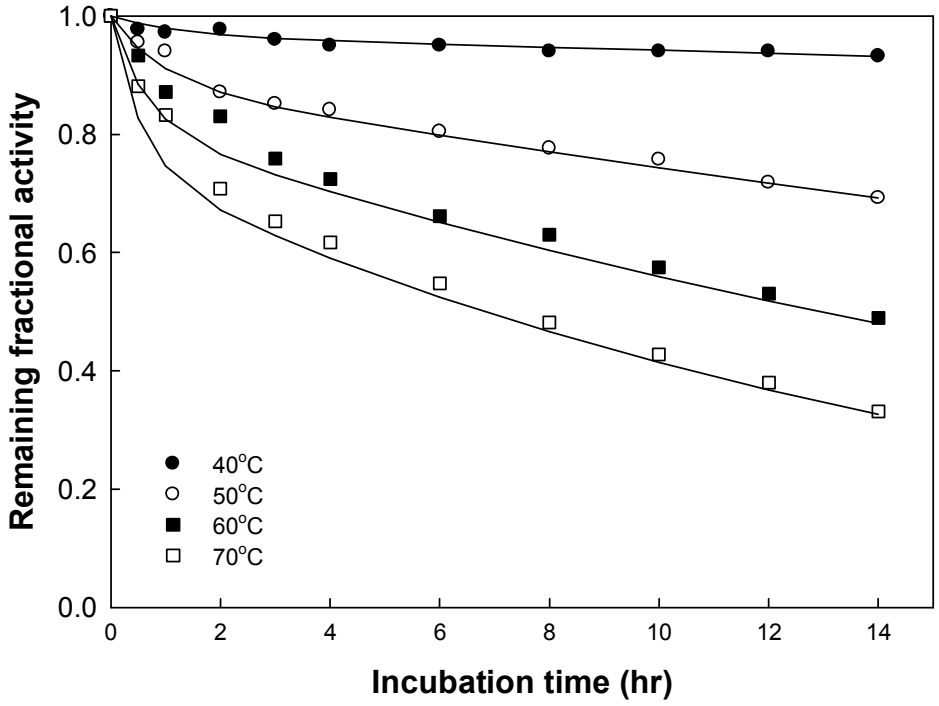
yield a higher degree of convexity. A higher degree of convexity implies more sensitivity to the rate of enzyme deactivation. From Figure 2 and Table 1, the significant reduction of convexity (Figure 2) and k_2 values (Table 1) by the entrapment of the lipase molecule in AOT/isooctane reverse micelles were revealed.

Table 1 clearly shows the differences in the effect of temperature in the enzyme storage conditions. At all temperature, k_1 did not greatly differ depending on the storage conditions, while k_2 drastically decreased after the enzyme was entrapped in AOT/isooctane reverse micelles. In particular, k_2 at 70^{o}C in aqueous media was 6.83- and 1.62-fold greater than that in AOT/isooctane reverse micelles or in glycerol pool, respectively. The value of α_1 in AOT/isooctane reverse micelles was greater than that in the glycerol pool and the aqueous media system, respectively. It was indicated that thermal stability in AOT/isooctane reverse micelles more stable and active than in glycerol pool or in the aqueous media system.

The values of k_1 and k_2 calculated through experimental data were used as Arrhenius equation by temperature to express like Figure 3. Through ln (k_1, k_2) according to reciprocal of absolute temperature, the equation of a straight line can be found. At this time, the slope ($-E_{de}/R$) of the lines, in which R is

the gas constant, are index to determine deactivation energy. Figure 3 shows the results of calculation of the dependence of storage media system. The deactivation energies 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.

(a)


Remaining fractional activity
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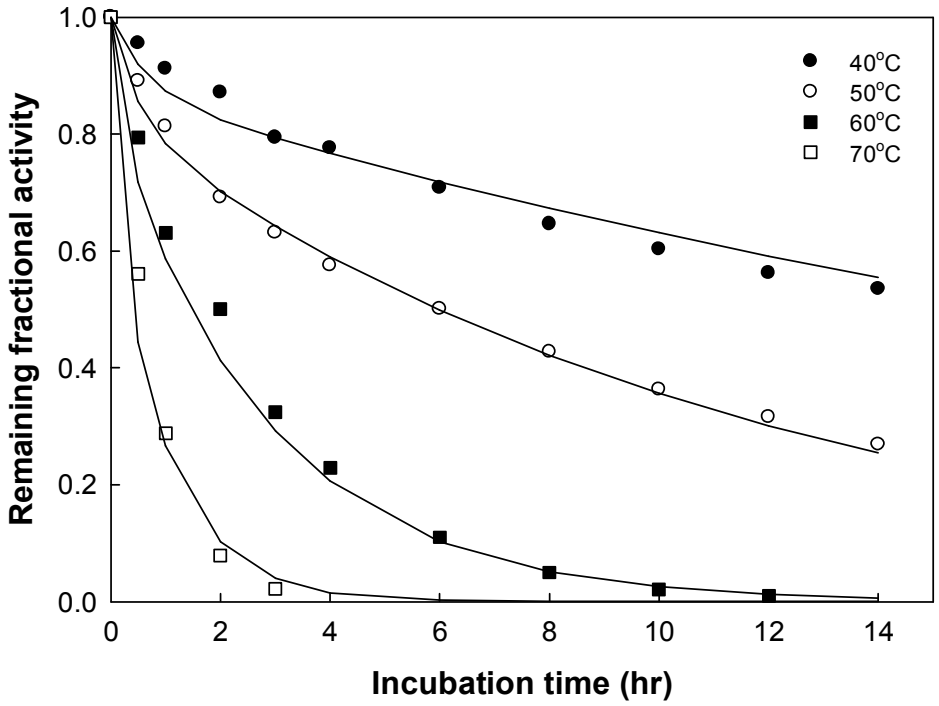
0.8
0.6
0.4
0.2
0.0
0
2
4
6
8
10
12
14
Incubation time (hr)
40°C
50°C
60°C
70°C

(b)


Remaining fractional activity
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0.6
0.4
0.2
0.0
0
2
4
6
8
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12
14
Incubation time (hr)
40°C
50°C
60°C
70°C

(c)

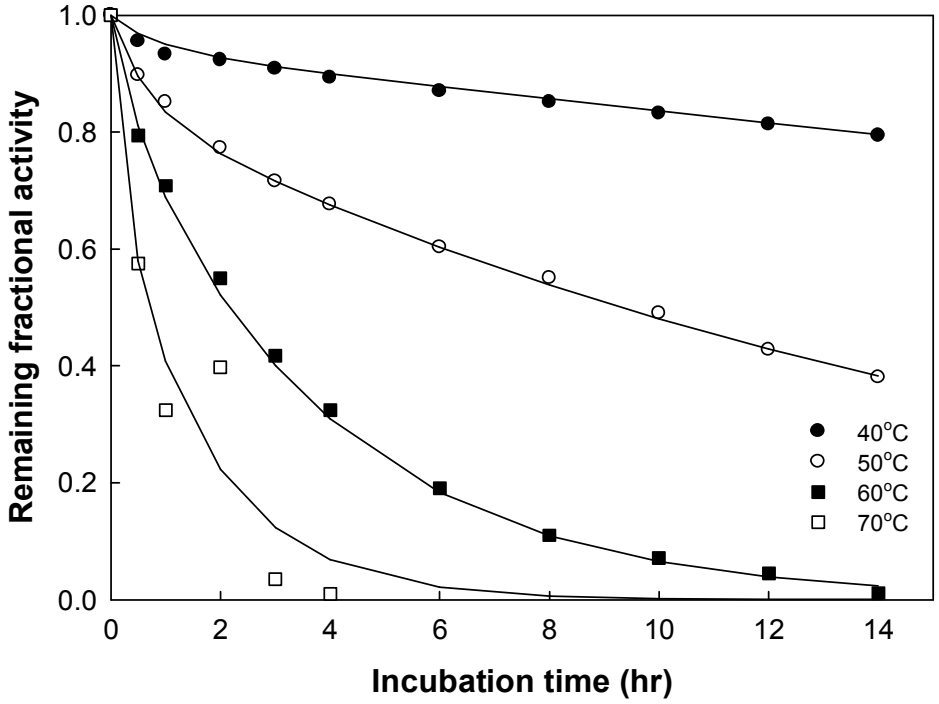
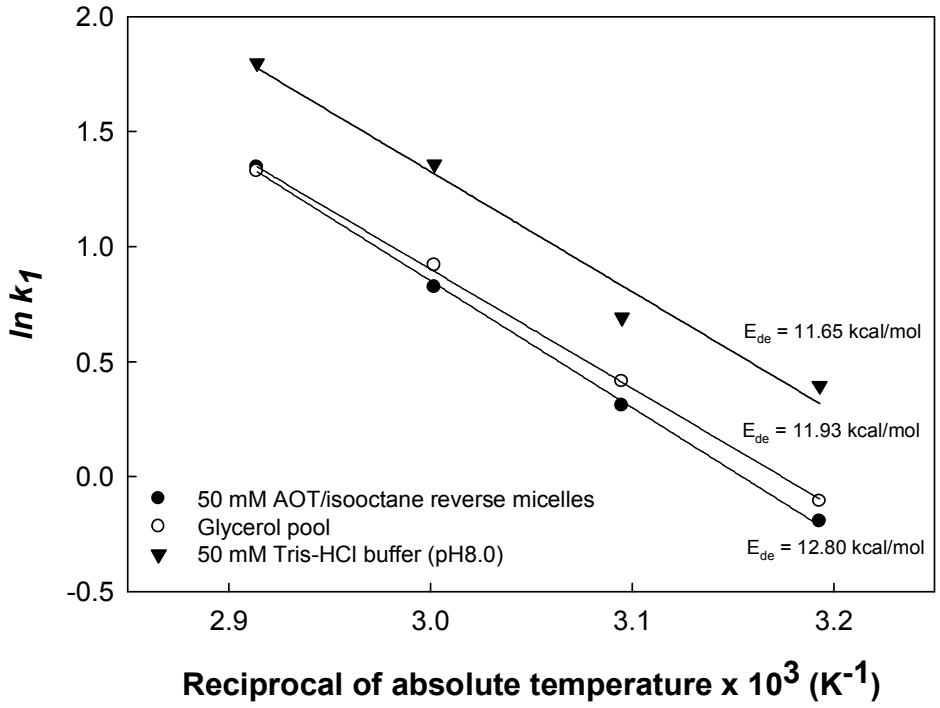


Figure 2. Thermal deactivation profiles of *Chromobacterium viscosum* lipase-catalyzed glycerolysis in 50 mM AOT/isooctane reverse micelles (a), 50 mM Tris-HCl buffer (pH 8.0) (b), glycerol pool (c).

Table 1. Deactivation kinetic parameters of *Chromobacterium viscosum* lipase-catalyzed glycerolysis in 50 mM Tris-HCl buffer (pH 8.0), 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 (hr^{-1})	1.4846	1.9960	3.8856	6.0303
k_2 (hr^{-1})	0.0325	0.0842	0.3467	0.9530
α_1	0.8538	0.7916	0.7510	0.5813
In Glycerol Pool				
k_1 (hr^{-1})	0.8985	1.5119	2.5057	3.7714
k_2 (hr^{-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 (hr^{-1})	0.8235	1.3613	2.2768	3.8381
k_2 (hr^{-1})	0.0026	0.0178	0.0382	0.1395
α_1	0.9640	0.8732	0.7986	0.7254

(a)


2.0
1.5
1.0
0.5
0.0
-0.5
ln k1
2.9
3.0
3.1
3.2
Reciprocal of absolute temperature x 10^3 (K^-1)
50 mM AOT/isooctane reverse micelles
Glycerol pool
50 mM Tris-HCl buffer (pH8.0)
Ede = 11.65 kcal/mol
Ede = 11.93 kcal/mol
Ede = 12.80 kcal/mol

(b)

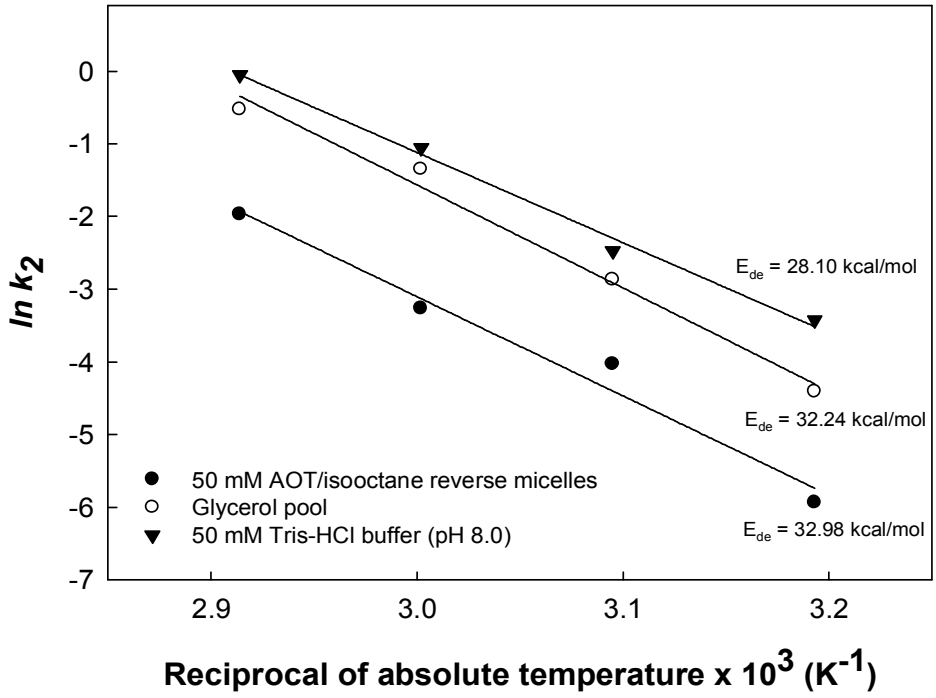


Figure 3. Arrhenius plots of deactivation rate constants ((a) *ln* k_1 vs K^{-1}; (b) *ln* k_2 vs K^{-1}) in *Chromobacterium viscosum* lipase-catalyzed glycerolysis.

3.3. Analysis of hydrolysis activity and thermal stability

Thermal stability of the lipase in an aqueous media and in AOT/isooctane reverse micelles was identified in hydrolysis, which is conducted under the same condition as in glycerolysis. Figure 4 represents the hydrolysis thermal deactivation profiles of *Chrmobacterium viscosum* lipase entrapped in AOT/isooctane reverse micelles (a) and in buffer (b) and incubated at 40, 50, 60, and 70^{o}C, respectively. The stability of the enzyme was determined by measuring the residual activity of aliquots from each enzyme sample during the incubation. It is observed that the half-life of lipase from *Chrmobacterium viscosum* in 50 mM Tris-HCl buffer (pH 8.0) is 12.67, 7.75, 1.68 and 0.15 hr incubation at 40, 50, 60, and 70^{o}C, respectively. While, the denaturation of the enzyme proceeded more slowly in 50 mM AOT/isooctane reverse micelles, and the remaining fractional activities were 89.55, 83.66, 40.74 and 11.07% after 5 hr incubation at 40, 50, 60, and 70^{o}C, respectively. In summary, the hydrolysis thermal stability of lipase entrapped in AOT/isooctane reverse micelles at all temperatures was more stable than when it was solubilized in 50 mM Tris-HCl buffer (pH 8.0). The half-life (0.32 hr) of lipase entrapped in AOT/isooctane reverse micelles at 70^{o}C was 2.13-fold longer that when the enzyme stored in the aqueous media system.

To quantify thermal deactivation of the enzyme stored in each of the media, a mathematical techniques of statistics was used. The deactivation kinetic parameters α_1, k_1, and k_2 were calculated from the experimental data and are shown in Table 2.

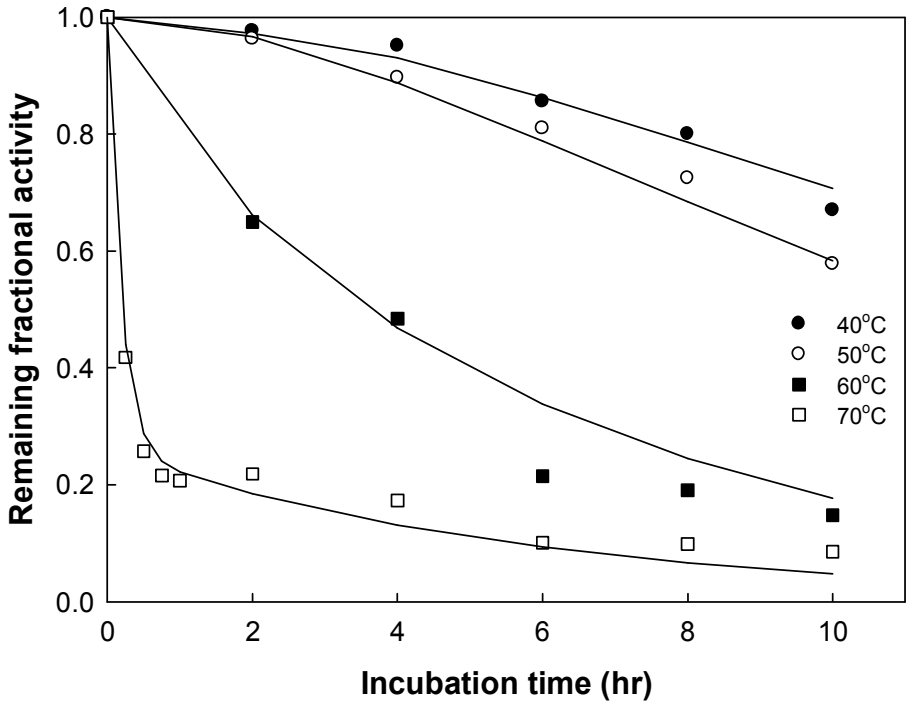
As shown, k_1 is also significantly larger than k_2 in hydrolysis. From Figure 4 and Table 2, the significant reductions of convexity (Figure 4) and k_2 values (Table 2) by the entrapment of the lipase molecule in AOT/isooctane reverse micelles were revealed.

More specifically, it can be also seen from Table 2 that k_2 at all temperatures drastically decreased after the enzyme was entrapped in AOT/isooctane reverse micelles. In particular k_2 (0.1694 hr^{-1}) at 70°C in reverse micelles was 8.08-fold lower than in Tris-HCl buffer (1.3684 hr^{-1}). The value of α_1 was considerably improved by the formation of AOT/isooctane reverse micelles, along with that of glycerolysis kinetic parameter.

The values of k_1 and k_2 at a particular temperature calculated through experimental data were plotted in an Arrhenius equation in Figure 5. Through ln (k_1, k_2) according to reciprocal of absolute temperature gave 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 Tirs-HCl buffer (pH 8.0) were 28.74, 42.05 kcal/mol and 27.13, 40.22 kcal/mol. In summary, these results in glycerolysis and hydrolysis indicate that the AOT/isooctane reversed micellar system enhanced the stability of the lipase and resistance to heat-induced denaturation.

(a)


Remaining fractional activity
1.0
0.8
0.6
0.4
0.2
0.0
0
2
4
6
8
10
Incubation time (hr)
40°C
50°C
60°C
70°C

(b)

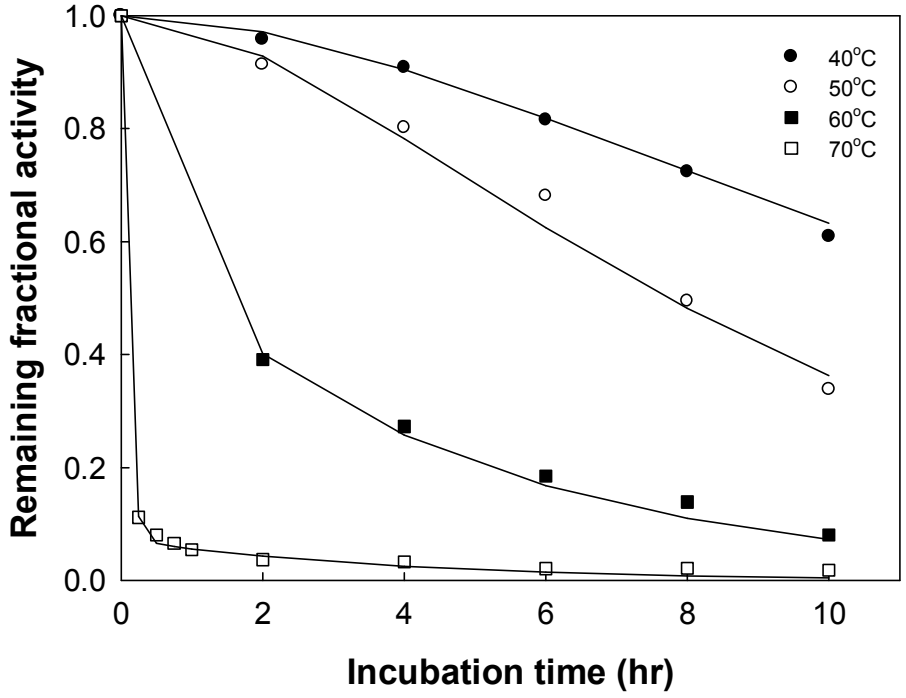
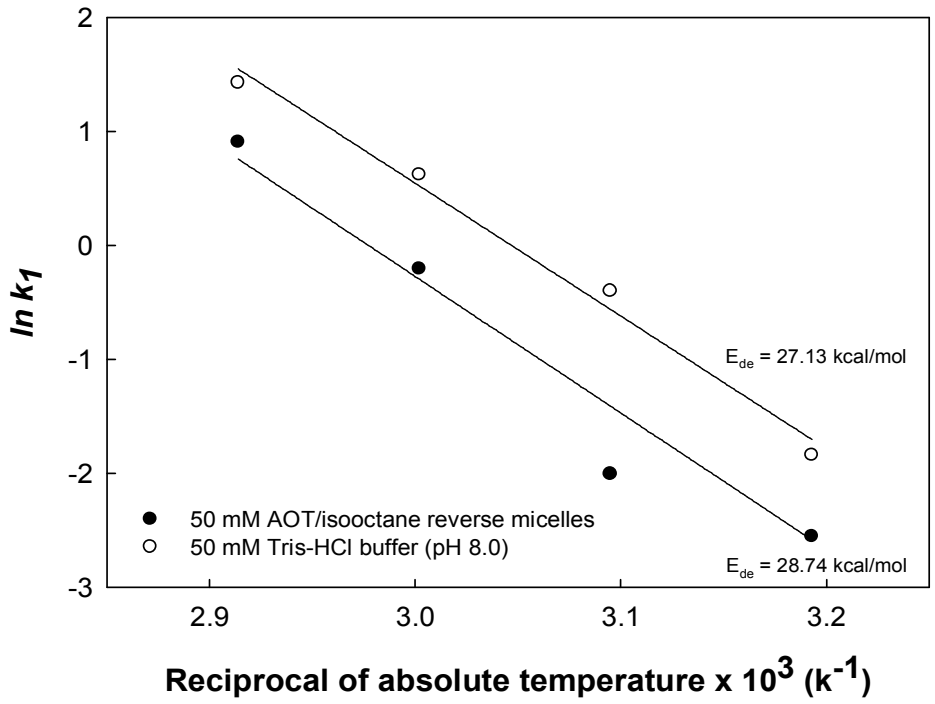


Figure 4. Thermal deactivation profiles of *Chromobacterium viscosum* lipase-catalyzed hydrolysis in 50 mM AOT/isooctane reverse micelles (a), and 50 mM Tris-HCl buffer (pH 8.0).

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 (hr^{-1})	0.1589	0.6691	1.8540	4.1521
k_2 (hr^{-1})	0.0109	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 (hr^{-1})	0.0778	0.1345	0.8125	2.4680
k_2 (hr^{-1})	0.0011	0.0322	0.0812	0.1694
α_1	0.9903	0.9073	0.7307	0.2507

(a)


2
1
0
-1
-2
-3
ln k1
2.9
3.0
3.1
3.2
Reciprocal of absolute temperature x 10^3 (k^-1)
50 mM AOT/isooctane reverse micelles
50 mM Tris-HCl buffer (pH 8.0)
Ede = 27.13 kcal/mol
Ede = 28.74 kcal/mol

(b)

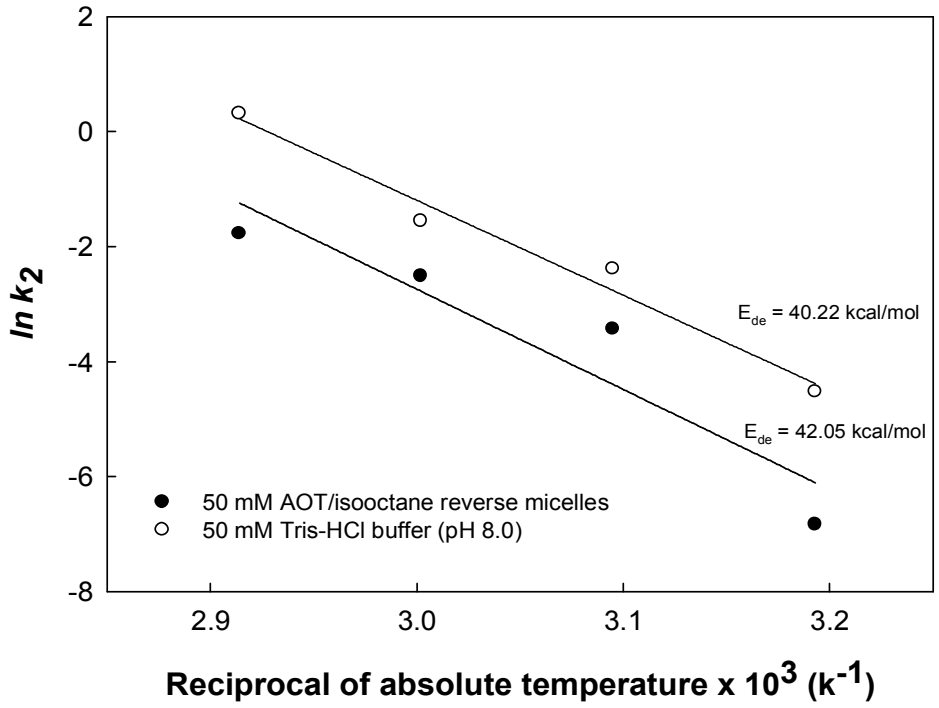


Figure 5. Arrhenius plots of deactivation rate constants ((a) *ln* k_1 vs K^{-1}; (b) *ln* k_2 vs K^{-1}) in *Chromobacterium viscosum* lipase-catalyzed hydrolysis.

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

Based on the previous experiments evaluating the thermal stability of lipase in reverse micelles and in an aqueous buffer, it was revealed that the lipase was more heat-resistant in the case of the encapsulated form in reverse micellar system. In this section, changes in enzyme-deactivation parameters depending on *R* value (referred to the molar ratio of water to surfactant) were monitored to investigate the effect of water content on the thermal stability of lipase in reverse micelles.

Figure 6 shows the thermal deactivation profiles of *Chrmobacterium 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. The half-life of the lipase in the reversed micellar system with *R* value 20 is 0.55, 0.35, and 0.15 hr under the incubation at 50, 60, and 70^{o}C, respectively. In comparison to that of *R* value 20, the denaturation of the enzymes proceeded more slowly in the *R* value 10 and 15. The lipase in reverse micelles with *R* value 5 which means relatively low water content was found to have the greatest stability than the others, and the residual activity dropped

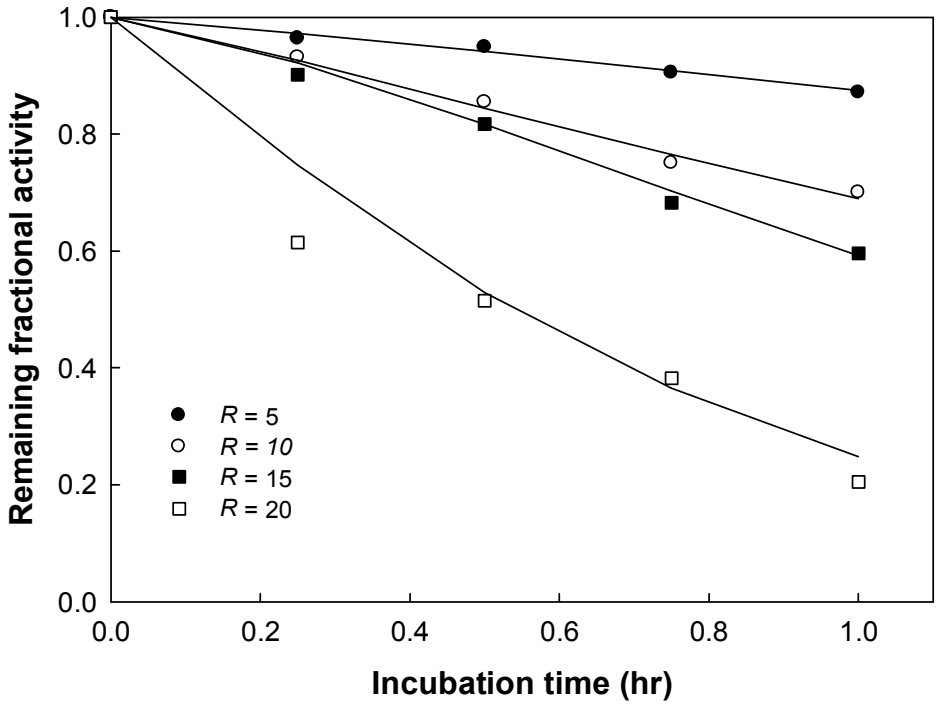
by a half after 3.81, 2.33, and 0.77 hr under the incubation at 50, 60, and 70°C, respectively. The half-life (0.77 hr) of lipase in *R* value 5 AOT/isooctane reverse micelles at 70°C was 3.67-, 4.81-, and 5.13-fold longer than those in the *R* value 10, 15, and 20, respectively.

To quantify thermal deactivation of the enzyme entrapped in each *R* value AOT/isooctane reverse micelles, the parameters α_1, k_1, and k_2 were calculated from the experimental data and are shown in Table 3.

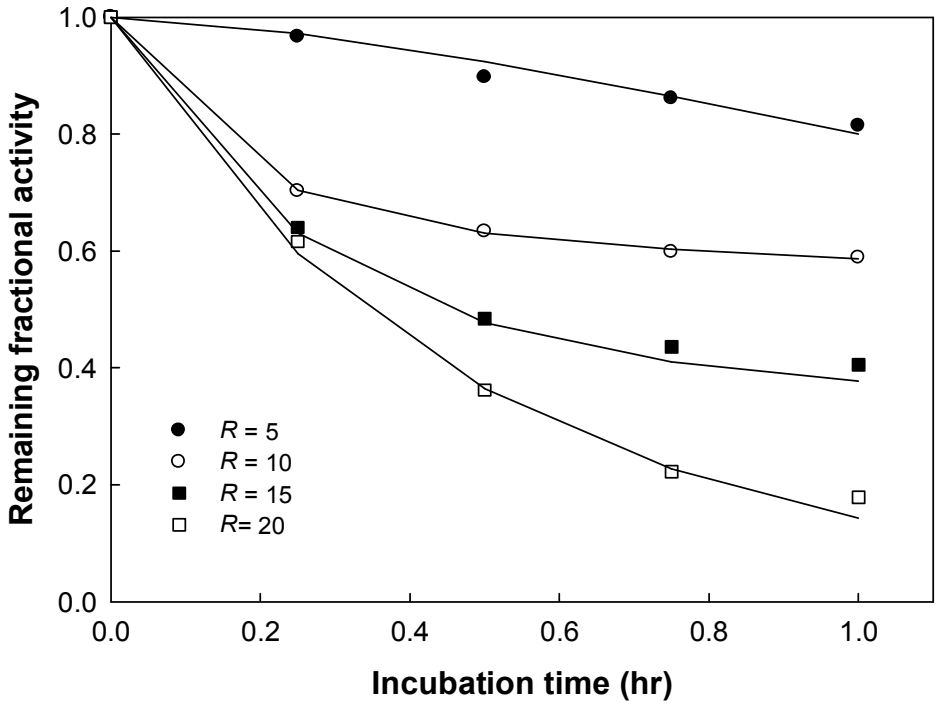
k_1 is significantly larger than k_2, and k_2 at all temperatures drastically decreased after the enzyme was entrapped in *R* value 5 AOT/isooctane reverse micelles. In particular, k_2 (0.1012 hr^{-1}) at 70°C in *R* value 5 was 3.44-, 3.45-, and 8.28-fold lower than k_2 in *R* value 10 (0.3484 hr^{-1}), *R* value 15 (0.3492 hr^{-1}), and *R* value 20 (0.8381 hr^{-1}), respectively. The values of k_1 and k_2 at a particular temperature calculated through experimental data were plotted in an Arrhenius equation in Figure 7. Through ln (k_1, k_2) according to reciprocal of absolute temperature gave a straight line. The deactivation energies obtained from k_1 for the lipase entrapped in reverse micelles with *R* value 5, 10, 15, and 20 were 16.00, 13.65, 11.97, and 8.99 kcal/mol, respectively. The pattern of those obtained from k_2 was consistent with the result from k_1 (27.94, 22.78, 19.91, and 18.89 kcal/mol, respectively). In

summary, the results indicate that relative lower water content in AOT/isooctane reversed micellar system could be favorable for resistance to heat-induced denaturation of the lipase.

(a)


Remaining fractional activity
Incubation time (hr)
R = 5
R = 10
R = 15
R = 20
0.0
0.2
0.4
0.6
0.8
1.0

(b)


Remaining fractional activity
Incubation time (hr)
0.0
0.2
0.4
0.6
0.8
1.0
R = 5
R = 10
R = 15
R= 20

(c)

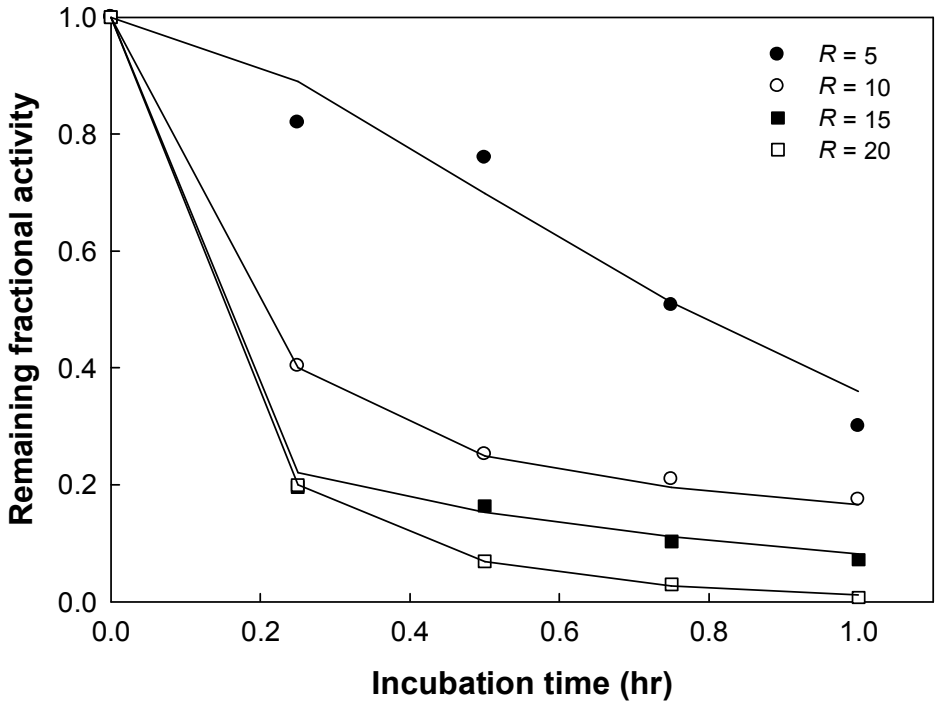
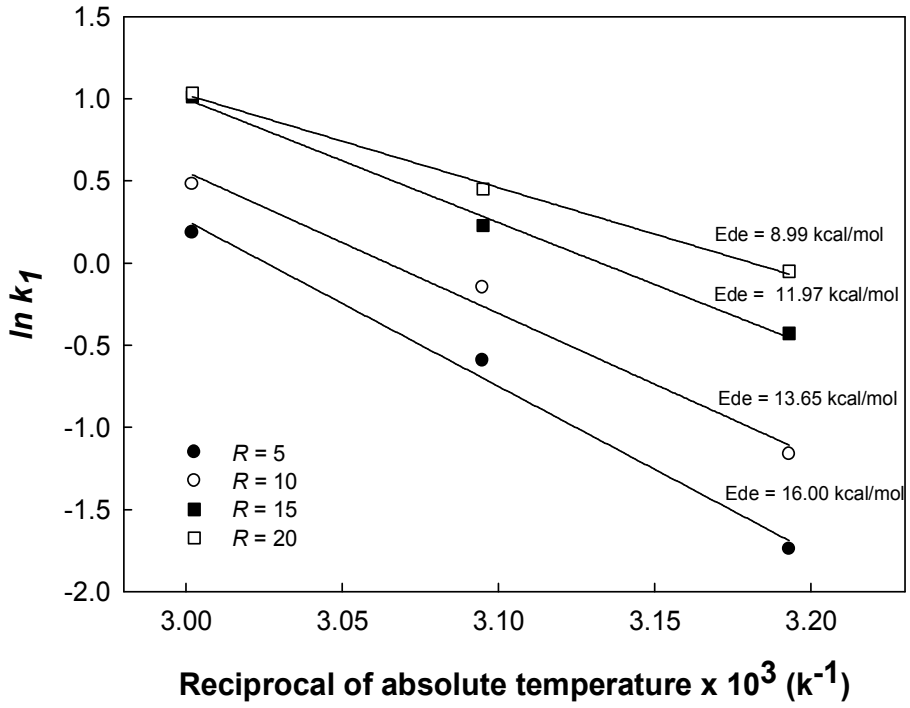


Figure 6. Thermal deactivation profiles of lipase form *Chromobacterium viscosum* in 50 mM AOT/isooctane reverse micelles. (a) R = 5 (●), R = 10 (○), R = 15 (■), and R = 20 (□) at 50 °C; (b) at 60 °C; (c) at 70 °C.

Table 3. Deactivation kinetic parameters of *Chromobacterium viscosum* lipase in 50 mM AOT/isooctane reverse micelles

Parameter	50°C	60 °C	70 °C
***R* value 5**			
k_1 (hr^{-1})	0.1753	0.5520	1.2012
k_2 (hr^{-1})	0.0035	0.0325	0.1012
α_1	0.9773	0.8493	0.5731
***R* value 10**			
k_1 (hr^{-1})	0.3123	0.8614	1.6135
k_2 (hr^{-1})	0.0225	0.1021	0.3484
α_1	0.9154	0.6345	0.2640
***R* value 15**			
k_1 (hr^{-1})	0.6521	1.2554	2.7512
k_2 (hr^{-1})	0.0319	0.1021	0.3492
α_1	0.8270	0.3902	0.2637
***R* value 20**			
k_1 (hr^{-1})	0.9512	1.5687	2.8042
k_2 (hr^{-1})	0.0864	0.1751	0.8381
α_1	0.7106	0.3186	0.2590

(a)


1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.0
ln k1
3.00
3.05
3.10
3.15
3.20
Reciprocal of absolute temperature x 10^3 (k^-1)
Ede = 8.99 kcal/mol
Ede = 11.97 kcal/mol
Ede = 13.65 kcal/mol
Ede = 16.00 kcal/mol
R = 5
R = 10
R = 15
R = 20

(b)

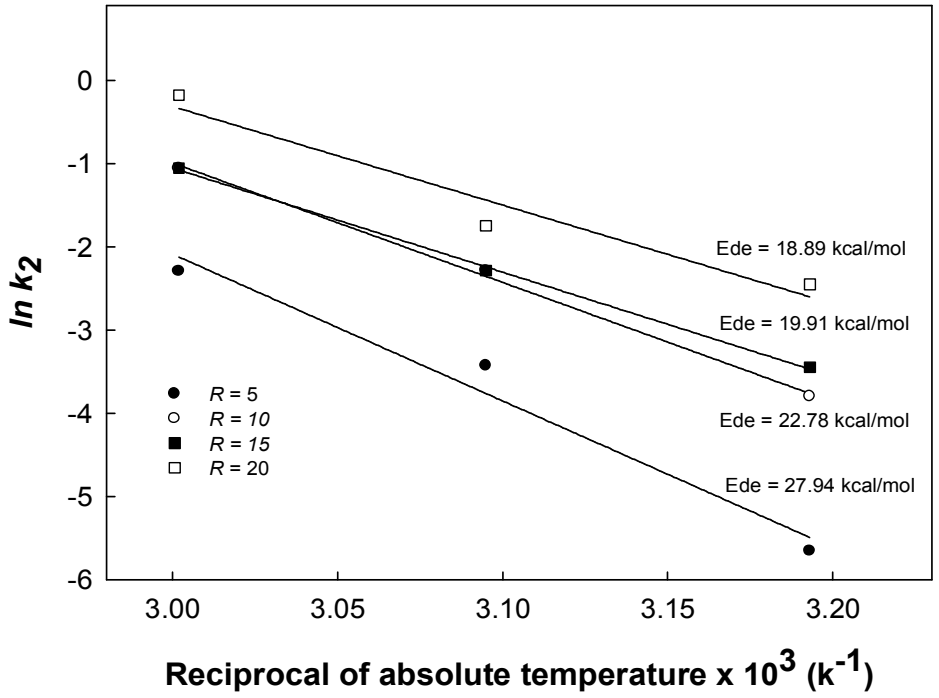


Figure 7. Arrhenius plots of deactivation rate constants ((a) *ln* k_1 vs K^{-1}; (b) *ln* k_2 vs K^{-1}) in 50 mM AOT/isooctane reverse micelles.

3.5. Structural analysis of thermally-deactivated lipase

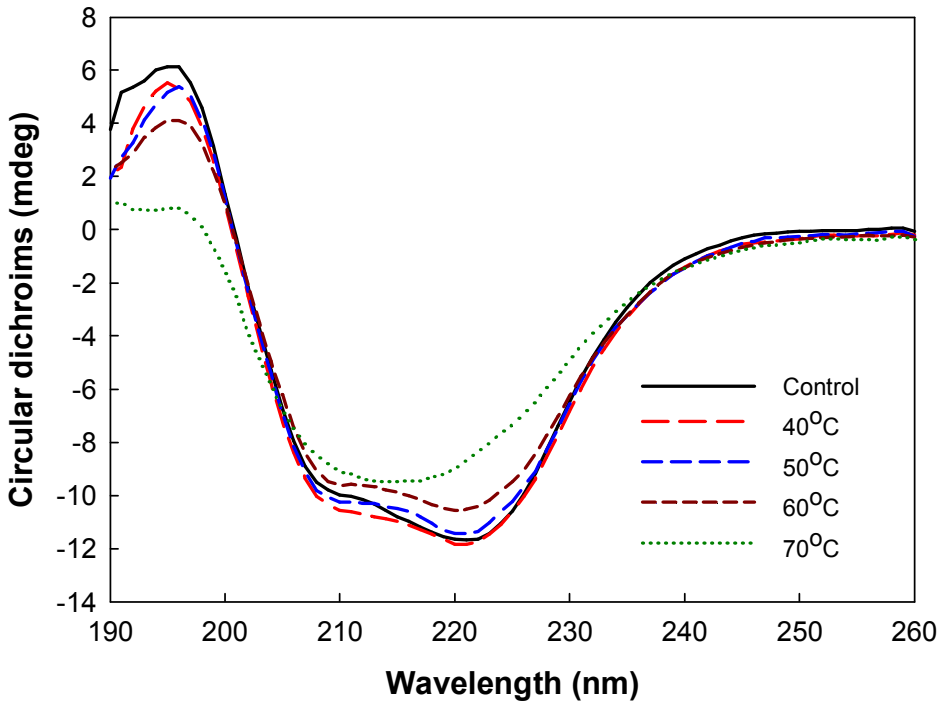
The secondary structure of the *Chromobacterium viscosum* lipase was analyzed using Far-UV CD spectra in the range of 190 to 260 nm. In theory of CD spectrometry, α-helix proteins have negative bands at 222 and 208 nm and a positive band at 193 nm and proteins with β-sheet have a negative band at 218 nm and a positive band at 195 nm, whereas disordered proteins such as β-turn and random coil have negative bands at 189 nm and 198 nm and positive bands at 210 nm and 212 nm, respectively. (Greenfield, 2007). For analysis of structural changes by temperature, Far-UV CD spectra were measured after incubation at 40, 50, 60 and 70^{o}C for 30 min. The CD spectra of the *Chromobacterium viscosum* lipase with thermal treatment in Tris-HCl buffer (pH 8.0) and in 50 mM AOT/isooctane reverse micelles were shown in Figure 8. CD spectra show no significant changes after incubation at 40, 50, 60 and 70^{o}C on far-UV range. More specifically, the secondary structure was calculated from CD spectra and amino acids composition (Table 4). The secondary structure of the *Chromobacterium viscosum* lipase without thermal treatment (negative control) was confirmed to consist of α-helix (17.0%), anti-parallel β-sheet (13.0%), β-turn (20.8%), and random coil (43.1%), respectively. Regardless of lipase deactivation (loss of activity), the

composition of secondary structure of the lipase did not fluctuate significantly with negligible change (approximately 0.2%) in anti-parallel β-sheet in both aqueous media system and reversed micellar system.

According to the report of Melo et al., thermal deactivation of *Chromobacterium viscosum* lipase is not due to protein denaturation such as secondary and tertiary structures changed (E Pinho Melo, Costa, & Cabral, 1996; E. P. Melo, Taipa, Castellar, Costa, & Cabral, 2000; 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 on the intrinsic activity. Fluorescence spectrometric method (excited 280 nm and emission at 280-340 nm) has been widely employed for the detecting excited forms of aromatic amino acids such as tryptophan and tyrosine. As shown in the fluorescence emission spectra (Figure 9), the tryptophyl contribution increased in proportion as the rise in temperature of thermal treatment in the aqueous media system. This phenomenon is explained those theory. tryptophan residues are usually the main contributors to the emission of globular proteins due to Tyr-to-Trp energy transfer and quenching by nearby groups on the peptide chain (Lakowicz, 2009), with a few exceptions (E Pinho Melo, Costa, & Cabral, 1996). The increase on the tyrosyl contribution

probably reflects a less efficient Tyr-to-Trp energy transfer (Saito, Tachibana, Hayashi, & Wada, 1981). Tyrosyl contribution increases for other proteins upon denaturation (Brand & Cagan, 1977). The CD data indicate that secondary structures remain native during deactivation observed after incubation. Therefore, deactivation is not due to protein denaturation, but subtle conformational rearrangements caused from tryptophyl. Contrary to that, there was no significant change in the intensity at 280-340 nm of fluorescence spectrum, which indicates that the encapsulation in reverse micelles could suppress the conversion of aromatic amino acids into excited forms. Based on the finding from the CD and fluorescence spectrometry, it was shown that thermal deactivation is not due to the changes in secondary structure, but is related to the minor changes in protein conformation of lipase from *Chromobacterium viscosum*.

(a)


Circular dichroims (mdeg)
8
6
4
2
0
-2
-4
-6
-8
-10
-12
-14
190
200
210
220
230
240
250
260
Wavelength (nm)
Control
40°C
50°C
60°C
70°C

(b)

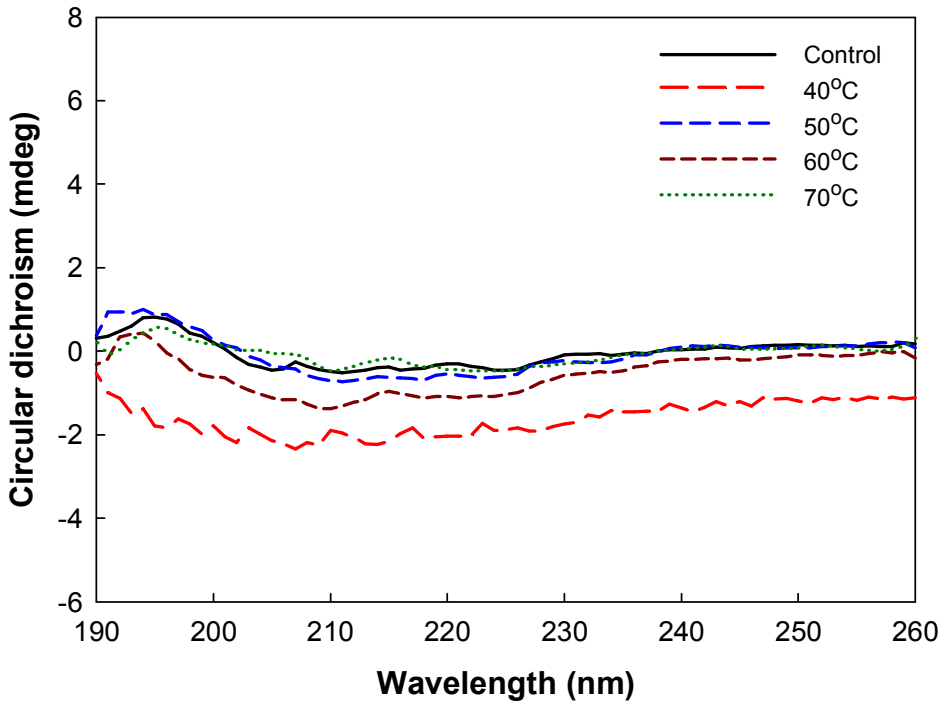
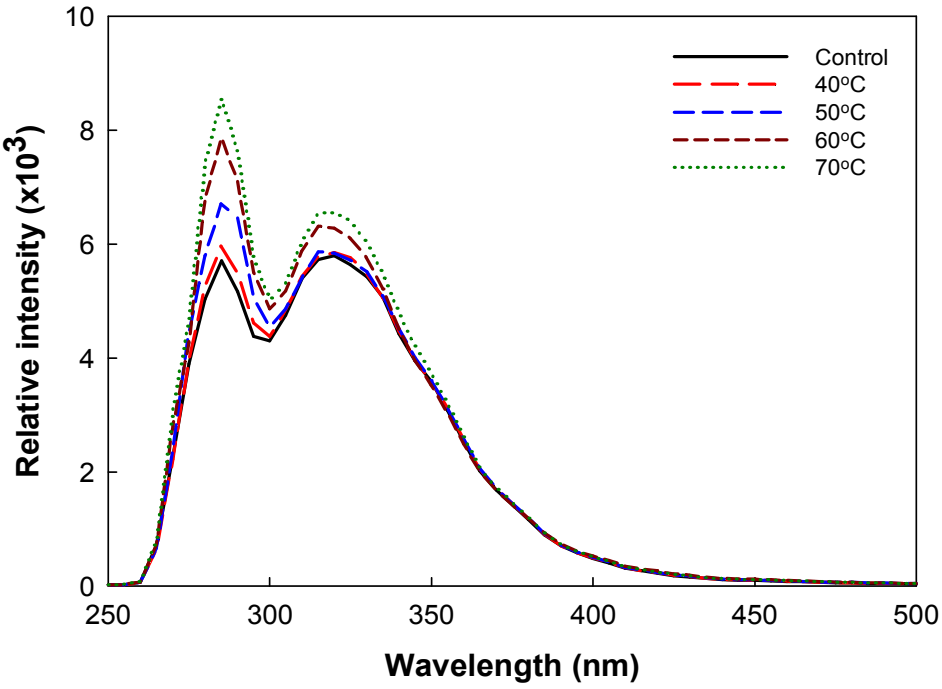


Figure 8. Far-UV CD spectra of *Chromobacterium viscosum* lipase in 50 mM Tris-HCl buffer (pH 8.0) (a), and in 50 mM AOT/isooctane reverse micelles (b).

Table 4. Content (%) of structure elements for *Chromobacterium viscosum* lipase in 50 mM Tris-HCl buffer (pH 8.0) and in 50 mM AOT/isooctane reverse micelles at different temperature.

Temperature (°C)	α-Helix (%)	Antiparallel β -sheet (%)	Parallel β -sheet (%)	β -Turn (%)	Random coil (%)
In 50 mM Tris-HCl buffer (pH 8.0)					
37	14.9	29.6	16.4	21.3	48.8
40	14.9	29.6	16.4	21.3	48.8
50	14.8	29.6	16.4	21.3	48.8
60	14.8	29.7	16.4	21.3	48.8
70	14.8	29.8	16.4	21.3	48.8
In 50 mM AOT/isooctane reverse micelles					
37	17.1	41.8	13.0	20.8	43.1
40	17.2	41.8	12.8	20.8	42.3
50	17.1	41.5	13.0	20.7	42.1
60	17.2	41.7	12.9	20.8	42.7
70	17.0	42.0	13.1	20.8	43.3

(a)


Relative intensity (x10^3)
Wavelength (nm)
Control
40°C
50°C
60°C
70°C
0
2
4
6
8
10
250
300
350
400
450
500

(b)

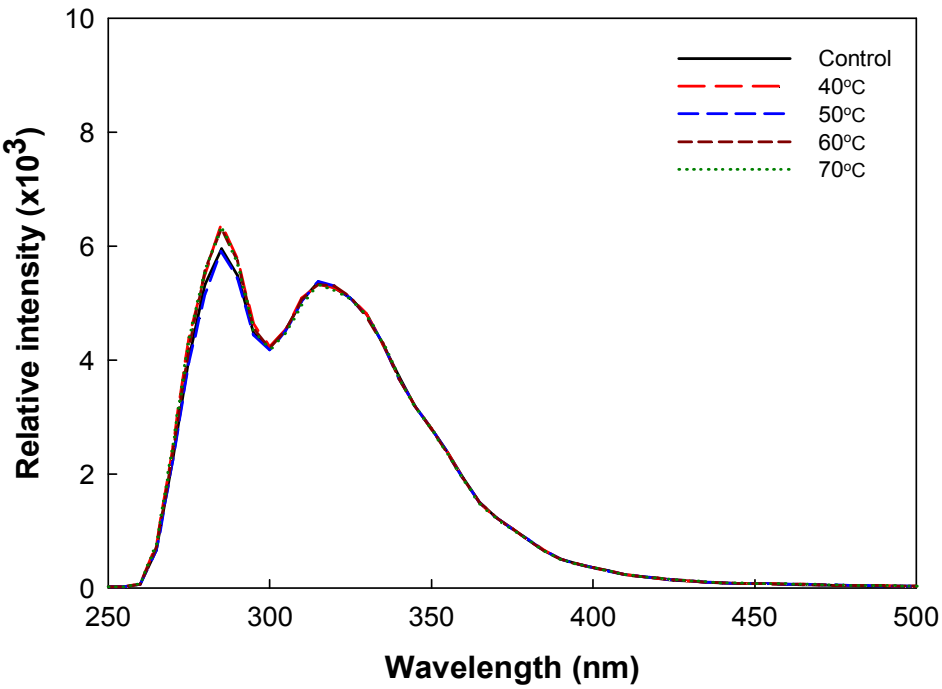


Figure 9. Fluorescence emission spectra of *Chromobacterium viscosum* lipase after excitation at 280 nm in 50 mM Tris-HCl buffer (pH 8.0) (a), and in 50 mM AOT/isooctane reverse micelles (b).

4. Conclusion

Due to the unique characteristics of lipase-catalyzed reaction between hydrophilic enzymes and hydrophobic substrates, efficient reaction systems to enhance conversion yield and thermal stability, have been considered to be significant factor for enzymatic bioconversions. In this study, changes in thermal stability and enzyme-deactivation kinetics according to different reaction systems including aqueous media, glycerol pool, and reverse micelles were investigated, employing glycerolysis and hydrolysis catalyzed by *Chromobacterium viscosum* lipase and two-step series-type deactivation kinetic model.

In glycerolysis model, the deactivation energy in reversed micellar system was approximately 1.10 (k_1), 1.17 (k_2) times greater, so that the lipase was more heat-resistant and also retained its catalytic activity rather than in aqueous buffer. Also, in hydrolysis, the residual activity of the lipase was less changed in reversed micellar system than in aqueous media system, showing the deactivation energy of the enzyme in reverse micelles was to be 1.06 (k_1), 1.05 (k_2) times and more stable than in aqueous media system. From these results, it is plausible to assume that the enhanced stability of the enzyme in reverse micelles might be acquired from the encapsulated form to

suppress not only the interaction with organic solvent but also external heat transfer.

Based on the findings that the lipase was more heat-resistant in reversed micellar system than that in a aqueous buffer, the changes in enzyme-deactivation parameters depending on *R* value were monitored to investigate the effect of water content on the thermal stability of the lipase in reverse micelles. The half-life (0.77 hr) of lipase in reverse micelles with *R* value 5 at 70^{o}C was 3.67-, 4.81-, and 5.13-fold longer than those in the *R* value 10, 15, and 20, respectively, which indicates that relatively lower water content in AOT/isooctane reversed micellar system could be favorable for resistance against heat-induced denaturation of the lipase.

From the structural analysis employing CD and fluorescence spectrometry, it was evident that thermal deactivation is not caused by the changes in secondary structure, but is related to the minor modification of amino acids consist in active site of the lipase from *Chromobacterium viscosum*. Furthermore, the encapsulation of lipase in reverse micelles displays an advantage in prevention of the enzyme-protein rearrangement from thermal treatment.

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국문초록

본 연구에서는 *Chromobacterium viscosum* lipase의 glycerolysis 및 hydrolysis 모델에서 효소반응계에 따른 열 안정성 변화 양상 및 enzyme-deactivation kinetics 연구를 수행하였다. Enzyme-deactivation kinetics로부터 효소동력학적 상수를 결정하기 위해서 two-step series-type deactivation kinetic 모델을 선정하였다. Glycerolysis에서 Tris-HCl buffer (pH 8.0), glycerol pool 및 역미셀계 내 효소의 잔존 활성을 살펴본 결과, 70°C에서 효소의 반감기는 역미셀계 환경이 Tris-HCl buffer (pH 8.0)와 glycerol pool에 대비하여 각각 14.80배와 9.87배 더 긴 반감기를 나타냈다. Two-step series-type deactivation kinetic 모델의 수식에 의해, 불활성화 속도상수(k_1,

k_2)를 구한 결과, 실험한 모든 온도조건에서 역미셀계의 불활성화 상수가 다른 효소반응계에 비해 낮은 것으로 확인하였다. 특히 70°C에서 k_1 (3.84 hr^{-1})은 aqueous media system에 비해 1.57배 낮게 나타났다. 속도상수 k_1, k_2로부터 효소불활성화 에너지를 환산한 결과, 역미셀계에서는 각각 12.80과 32.98 kcal/mol로 나타났으며, glycerol pool의 경우 11.93 및 32.24 kcal/mol로 계산되었다. Aqueous media system의 경우, 11.65 및 28.10 kcal/mol로 세가지 효소반응계 중 효소불활성화 에너지가 가장 작은것으로 확인하였다. 이러한 현상은 hydrolysis에서도 유사한 패턴을 나타냈으며, 그 결과, glycerolysis와 hydrolysis 반응 모두 효소안정성 측면에서 역미셀계가 aqueous media system 보다 유리한 것으로 판단하였다. 역미셀계가 aqueous media system에 비해 효소의 열 안정성이 높은 것을 토대로, 역미셀계 내의 수분 함량에 따른 효소의 열 안정성을 살펴본 결과, 수분 함량이 적을수록 효소의 열 안정성은 높은 것으로 나타났다. 효소의 열 불활성화에 의한 구조적 분석을 위해, circular dichroism (CD)과 fluorescence spectrometry를 이용하여 단백질의 2차구조 및 방향족 아미노산의 변화를 관찰하였다. 구조분

석 결과, 효소단백질의 2차구조는 보관조건 및 온도와 무관하게 유의적인 변화를 나타내지 않았다. 반면, 효소의 구성하는 방향족 아미노산인 tryptophan과 tyrosine의 변화를 살펴본 결과, 역미셀계와 달리 aqueous media system에서는 보관온도가 높을수록 tryptophyl과 tyrosyl과 같이 excited 형태로 전환되는 현상이 촉진되었다.