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Activity and stability of *Chromobacterium viscosum* lipase in modified AOT reverse micelles

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Abstract

The modification of AOT/water/isooctane reverse micellar system by various additives was undertaken to enhance the activity and stability of *Chromobacterium viscosum* lipase. Polypropylene glycols (PPG, MW < 700), polypropylene glycols triol (PPGT, MW = 725), glycerol propoxylate (GP, MW = 260) and polyethylene glycol (PEG, MW < 1000) appreciably increased lipase activity. Low molecular weight polyethylene glycol, PEG 400, was found to be the most effective among them. Fourier transformed infrared spectroscopy (FTIR) analysis suggested that PEG 400 molecules participated in the redistribution of water inside the reverse micelles and strongly interacted with the head groups (SO_3^-) of anionic surfactant AOT. Deactivation kinetic at 25 °C indicated that the *C. viscosum* lipase entrapped in AOT/PEG 400 reverse micelles was very stable, retaining over 75% of its initial activity after 60 days, whereas half-life time in simple AOT reverse micelles was 38 days.

Keywords: Hydrolysis; Lipase activity; PEG 400; Reverse micelles and lipase deactivation

1. Introduction

Enzyme lipase (EC 3.1.1.3) has proven to be useful in a myriad of industrial applications. Reverse micelles have been used for hosting various enzymatic reactions [1–3], one reason being their possession of a large interfacial area that promotes contact between enzyme and substrate [4]. This technology allows lipase to act upon hydrophobic substrate, which is not soluble in aqueous media, and to catalyze the hydrolysis of triglycerides since water and lipase exist inside reverse micelles. The anionic double-tailed surfactant AOT [sodium bis-(2-ethyl 1-hexyl) sulfosuccinate] is frequently used for the study of reverse micelles. The motivation to use AOT in micellar studies has been the large amount of published data on the physico-chemical properties of the AOT reverse micelle and the ease of reverse micellar formation. However, activities of enzymes hosted in ionic AOT reverse micellar system are negatively affected by the strong interactions with surfactant molecules [5–7].

To overcome this problem, a modified AOT surfactant or a newly synthesized surfactant can be used to prepare reverse micelles [8,9]. But such modification and synthesis of surfactants are laborious and

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time consuming. In addition, purification of modified AOT in a good yield is very difficult. Although chemical modification of the lipase surface is also reported to overcome this problem, specific hydrolytic activities are significantly reduced by the chemical modification of lipase [10]. Another comparatively simple and easy alternative to enhance the enzyme activity is to use some additives to modify the reverse micelles. Modifications of reverse micelle have been reported by several researchers [11–15]. However, most studies concentrated on the general characteristics of the initial activity of lipase in modified reverse micelles. Less attention was paid to investigation of the effects of additives on the stability of lipase and the properties of water inside the reverse micelles that directly affect lipase activity.

In this study, we modified AOT reverse micelles with various additives, such as polypropylene glycols (PPG), polypropylene glycols triol (PPGT), glycerol propoxylate (GP), polyethylene glycol (PEG) and *n*-alcohols, to increase the activity of *Chromobacterium viscosum* lipase catalyzed hydrolysis of olive oil. When compared with other additives, the short chain polyethylene glycol, PEG 400, is found to be the most effective. The initial activity of *C. viscosum* lipase entrapped in AOT/PEG 400 reverse micelles is characterized first by optimizing the various parameters, and then the properties of the reverse micelle modified by PEG 400 molecules are investigated by Fourier transformed infrared spectroscopy (FTIR). A deactivation kinetic of *C. viscosum* lipase entrapped in AOT/PEG 400 reverse micelle is also studied.

2. Experimental

2.1. Materials

Purified *C. viscosum* lipase (glycerol-ester hydrolase, EC 3.1.1.3) was provided by Ashahi Chemical Industry Co. Ltd., isooctane (AOT), polyethylene glycols, *n*-alcohols and olive oil were purchased from Wako Pure Chemical Industries Co. Polypropylene glycols, polypropylene glycols triol and glycerol propoxylate were purchased from Aldrich Chem. Co. All of the chemicals were used without further purification.

2.2. Preparation of reverse micelles

Isooctane solution in which AOT had been dissolved was used as the reverse micellar solution. Reverse micelles containing the lipase were prepared by injecting appropriate amounts of a concentrated buffer solution containing the lipase into the reverse micellar solution. Water–AOT molar ratio, w_o was adjusted by adding the desired amount of buffer solution. The mixture was then stirred by a magnetic stirrer until clear and an optically transparent solution was obtained. Systems with additives were prepared by mixing reverse micellar solutions in a glass bottle in which an appropriate amount of additive had been added in advance.

2.3. Determination of lipase activity

The lipase activity was defined as the initial reaction rate, V (mol dm^{-3} s^{-1}). Reaction was initiated by adding an appropriate amount of olive oil into a pre-incubated micellar solution containing lipase. The reaction mixture was agitated by a magnetic stirrer at 500 rpm in an incubator at constant temperature 25 °C for exactly 20 min. It was found that within this time range, the free fatty acid production was linearly dependent on time [16]. The produced fatty acid was analyzed by the Lowry and Tinsley technique [17]. All data are the average of five replicated experiments and are reproducible within ±5%. In this paper, all of the concentration terms are based on the total volume of the reverse micellar system unless otherwise specified.

2.4. Lipase stability

Samples containing lipase without substrate were incubated at 25 °C and taken out at the time intervals; and the residual lipase activity was measured at the incubation temperature. Enzyme half-life times were calculated directly from residual activity profiles.

2.5. FTIR spectra

FTIR spectra were taken using Horiba FT-210 Fourier transformed infrared spectrometer using a base line horizontal attenuated total reflectance (HATR) cell. The data were recorded by a Horiba Spectradesk. FTIR spectra were recorded in a 400–4000 cm^{-1} range. Fifty scans were taken for each sample at a

resolution of 4 cm^{-1}. Individual peak positions and intensities were determined by curve fitting of Gaussian bands applying a Marquardt–Levenberg algorithm. Fourier self-deconvolution was used to resolve intrinsically overlapped spectra bands of the O–H stretching region. The Jandel Scientific software was employed for Gaussian deconvolution.

3. Results and discussion

3.1. Effect of additives on the activity of lipase

Effects of the various additives on lipase activity are shown in Fig. 1. Lipase activity is increased by the addition of PPG 425, PPGT 725, GP 260 and PEG 400. PEG 400 was found to be most effective among them. However, normal alcohols (methanol, butanol and tetradecanol) decrease lipase activity. It should be noted that the addition of PPGs of molecular weights above 700, and PEGs above 1000 decrease lipase activity under the same conditions (data not shown). In order to compare the effect of PEG 400 with Tween 85 (polyoxyethylene sorbitan trioleate), the activity of lipase entrapped in AOT/Tween 85 reverse micelles was adopted from our previous paper [12].

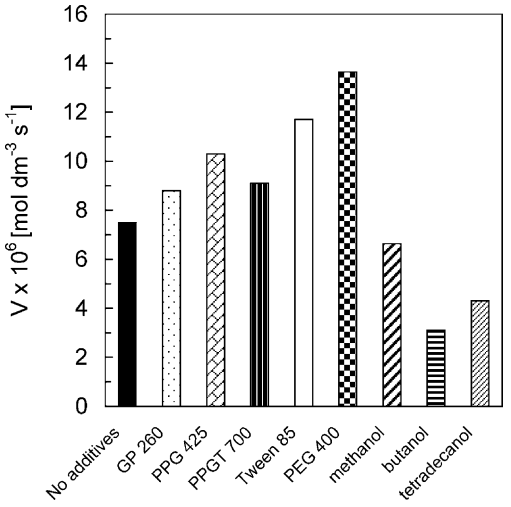

Fig. 1. The effect of additives on lipase activity in AOT/isooctane reverse micelles. Experimental conditions: C_{lipase} = 2 mg dm^{-3}; $C_{additive}/C_{AOT}$ = 0.25; C_{AOT} = 0.05 mol dm^{-3}; $C_{olive\ oil}$ = 0.055 mol dm^{-3}, pH = 8.0, w_o = 10; C_{NaCl} = 0.3 mol dm^{-3}, temperature 25 °C.

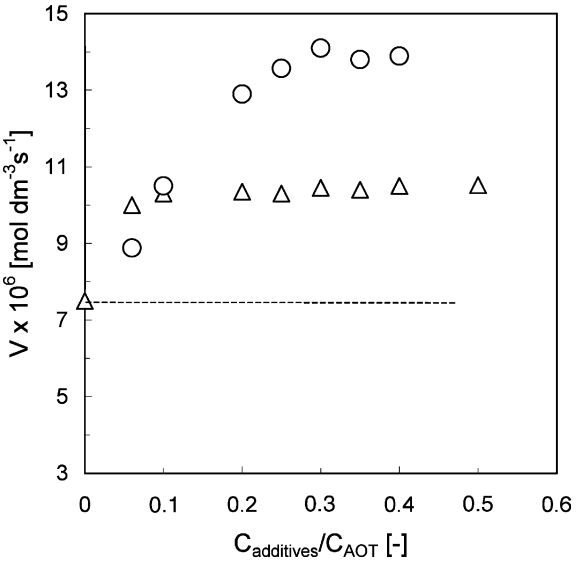

Fig. 2. The effect of additive concentrations on lipase activity in reverse micelles. Symbols (○) and (△) represent the activity of lipase in AOT reverse micelles with polyethylene glycol (PEG 400) and polypropylene glycol (PPG 425), respectively. The dotted line represents the activity in AOT reverse micelles without additive.

To understand the enhancement of lipase activity by PPG 425 and PEG 400, the effect of their concentration was investigated and is shown in Fig. 2. The maximum enhancement of lipase activity is obtained at PEG 400–AOT molar ratio, p_o = 0.3. At higher p_o values, the reverse micellar solution became turbid and the activity at $p_o > 0.4$ was not investigated to avoid errors in spectroscopic measurements of fatty acid. Since the PEG 400 molecule is highly polar and poorly soluble in organic solvent it remains inside the water pool, and influences the reverse micelle in several ways: changes the properties of water inside the reverse micelles, and modifies the micellar interface by interacting with surfactant head groups. In addition the linkage of the PEG molecule to the lipase surface protects the lipase from an unfavorable environment [18]. These influences can thereby enhance lipase activity.

3.2. General characteristics

The initial activity of *C. viscosum* lipase is characterized by the optimizing system parameters listed in Table 1. The effect of each operational parameter is

Table 1
Characterization of the initial activity of *C. viscosum* lipase by optimizing the system parameters at a PEG 400–water molar ratio, $p_o = 0.25$ and $C_{olive\,oil} = 0.055\,mol\,dm^{-3}$

Parameters	Optimal value in AOT reverse micelles	Optimal value in AOT/PEG 400 reverse micelles
w_o	10	8
Buffer pH	8	8
C_{NaCl} in buffer ($mol\,dm^{-3}$)	0.3	No effect until 0.6
Temperature (°C)	25	25
C_{AOT} ($mol\,dm^{-3}$)	Activity decreases with the increase in C_{AOT}	0.05

investigated by varying only the parameter considered, the others being kept constant. As can be seen from Table 1, the optimal pH and temperature in AOT/PEG 400 reverse micelles are the same as those in simple AOT reverse micelles. The value of the optimal pH in both reverse micelles is near to the isoelectric pH of *C. viscosum* lipase (pH 7.3) at which the electrostatic interaction between the lipase and the micellar interface is at a minimum. However, in the presence of PEG 400, the optimum water–AOT molar ratio, w_o is shifted toward a low value (7–8), at which the size of the PEG–lipase complex and the reverse micelles are assumed to be equal because the optimum w_o is related to a situation where the inner diameter of the micelles corresponds to the size of the encapsulated lipase [1,19]. Since the size of reverse micelles increases with w_o value [20] and PEG 400 molecules are solubilized inside the micellar water pool, the shifting of optimum w_o toward a low value suggests an increase in micellar size from the presence of the PEG 400 molecule. It has been reported that ethylene glycol solubilized inside the micellar water pool increased the size of AOT/isooctane reverse micelles [21].

In contrast to simple AOT reverse micelles, the lipase activity in AOT/PEG 400 reverse micelles is independent of ionic strength (i.e. concentration of NaCl in buffer) until $C_{NaCl} = 0.6\,mol\,dm^{-3}$ (Table 1). The linkage of the PEG 400 molecule caused by the strong interaction with the AOT head group inhibits the counter-ion (Na^+) from approaching the interface, and the effect of ionic strength on micellar size become less effective. As a result the lipase activity is not influenced by ionic strength until $C_{NaCl} = 0.6\,mol\,dm^{-3}$, and then it decreases gradually with the increase in NaCl concentration because of a salting out effect. The result suggests that the presence of PEG 400 molecules at the interior surface of the micellar interface protects the lipase from interactions with AOT molecules.

3.3. Lipase stability

The influence of PEG 400 on the stability of *C. viscosum* lipase was investigated at the optimum system parameters listed in Table 1. Tris–HCl solution of $0.1\,mol\,dm^{-3}$ was chosen as the buffer because the half-life of *C. viscosum* lipase in this buffer is longer than that in other buffers (phosphate, borate, NaOH–H_3BO_3, etc). From Fig. 3, it is evident that *C. viscosum* lipase entrapped in AOT/PEG 400 reverse micelles is very stable, retaining over 75% of its initial activity after 60 days. In contrast, the half-life of lipase in simple AOT reverse micelles is about 38 days, which is comparable with that reported elsewhere [22]. It should be noted that stability of lipase at $p_o < 0.25$, might be lower than that at $p_o = 0.25$, as the shifting of the optimum w_o value toward a low value depends on the increase in p_o value: 10 for $p_o = 0$, 9

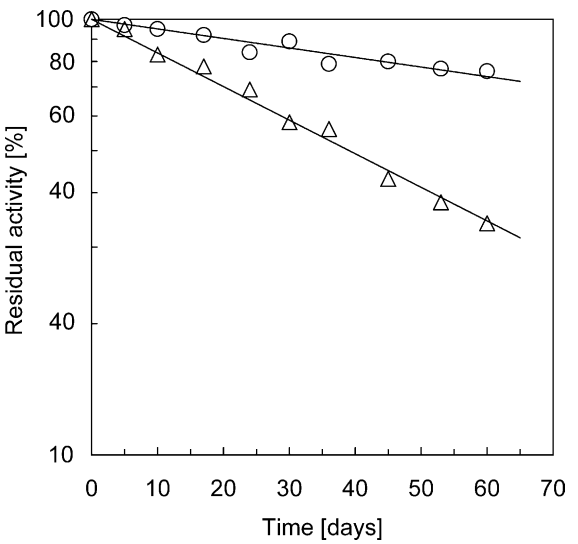

Fig. 3. Stability of lipase in individual reverse micelles at the optimum system parameters listed in Table 1 and $C_{lipase} = 2\,mg\,dm^{-3}$. (○) AOT/PEG 400 reverse micelles for $p_o = 0.25$ and $C_{AOT} = 0.05\,mol\,dm^{-3}$; (△) simple AOT reverse micelles for $C_{AOT} = 0.05\,mol\,dm^{-3}$. Symbols are experimental data and lines are theoretical profiles obtained from the application of the model Eq. (2).

for $p_o = 0.1$, 8 for $p_o = 0.25$. The lipase at lower w_o value is more stable than that at higher w_o value [23].

To quantify lipase deactivation in individual reverse micelles (with or without PEG 400), a mathematical treatment is undertaken. The lipase entrapped in both reverse micellar systems follows a first-order deactivation kinetic (Fig. 3). This means that deactivation might occur in a unique step from active (E) to totally denatured (E_d) state as follows:

$$E \overset{k_d}{\rightarrow} E_d \quad (1)$$

Enzyme fractional residual activity (a) can be expressed as a function of time by the following equation

$$a = \exp(-k_d t) \quad (2)$$

The first-order deactivation constant, k_d per day is calculated in both reverse micelles. Its value is found to decrease after addition of PEG 400: k_d was 0.0176 per day for the lipase in simple AOT reverse micelles (without PEG 400) and it decrease to 0.0051 per day in AOT/PEG 400 reverse micelles.

The thermodynamic parameter standard free energy (ΔG°) of the deactivation process can also be calculated from

$$-\Delta G^\circ = RT \ln\left[\frac{k_B T}{k_d h}\right] \quad (3)$$

where k_B is the Boltzmann's constant ($J\,K^{-1}$), h the Planck's constant (J per day), R the gas constant ($J\,mol^{-1}\,K^{-1}$) and T the temperature (K). The value of ΔG° ($111.1\,kJ\,mol^{-1}$) in simple AOT reverse micelles was increased to $114.3\,kJ\,mol^{-1}$ by the addition of PEG 400. This free energy increment is significant, since the net free energy for stabilization of an enzyme is, in general, small [24]. A significant decrease in the deactivation constant correlates with the increase in free energy and, therefore, in lipase stability. The observed increase in lipase stability is attributed to suppression of the interaction of the lipase with surfactant molecules and the protection of lipase by the non-covalent linkage of PEG molecules to the lipase surface [18].

3.4. FTIR investigation of an individual reverse micellar system

The S=O stretching region of the FTIR spectrum in AOT reverse micelles is shown in Fig. 4. The peak

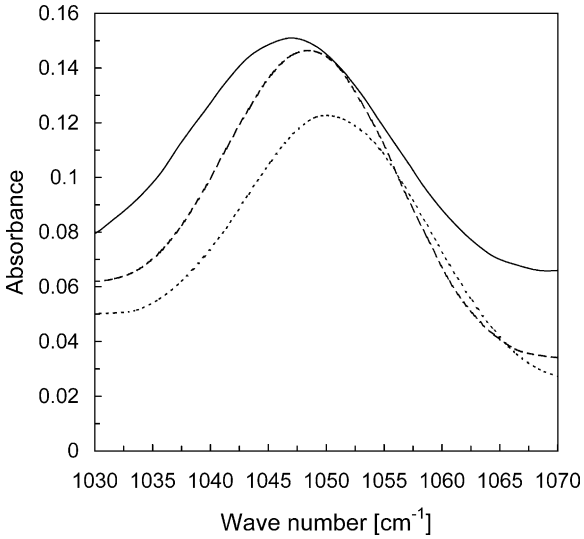

Fig. 4. The effect of PEG 400 on the S=O stretching region of the FTIR spectrum of AOT/isooctane reverse micelles at $C_{AOT} = 0.1\,mol\,dm^{-3}$ and C_{NaCl} (in buffer) $= 0.3\,mol\,dm^{-3}$. (- - -) $w_o = 0$; (– – –) $w_o = 3$; (—) $w_o = 0$, $p_o = 0.2$.

position is shifted toward a low frequency by the addition of PEG 400 into the reverse micelle, which suggests the direct interaction of PEG 400 molecule with head groups (SO_3^-) of AOT molecules. As the peak for $p_o = 0.2$ without water ($w_o = 0$) is shifted to a lower frequency than that for $w_o = 3$, the PEG 400 molecule interacts with the micellar interface more strongly than does water, or not just the terminal hydroxyl (–OH) group of PEG 400 is responsible for the interaction, but also the oxygen of ethylene oxide ($–CH_2CH_2O–$) unit. The result indicates the presence of PEG 400 molecules at the interior surface of the micellar interface.

The O–H stretching region of the FTIR spectrums ($3100–3700\,cm^{-1}$) for simple AOT/water/isooctane and AOT/PEG 400/water/isooctane reverse micelles at $w_o = 8$ are shown in Fig. 5. The addition of PEG 400 increases the absorbance and alters the shape of the spectrum in the O–H stretching region. To analyze further, the O–H stretching region of PEG 400 and water-containing reverse micelles are deconvoluted into three Gaussian peaks centered at 3580 ± 10, 3445 ± 10 and $3240 \pm 20\,cm^{-1}$ with half widths at half heights of 40 ± 5, 90 ± 10 and $110 \pm 20\,cm^{-1}$, respectively. These values are in good agreement with

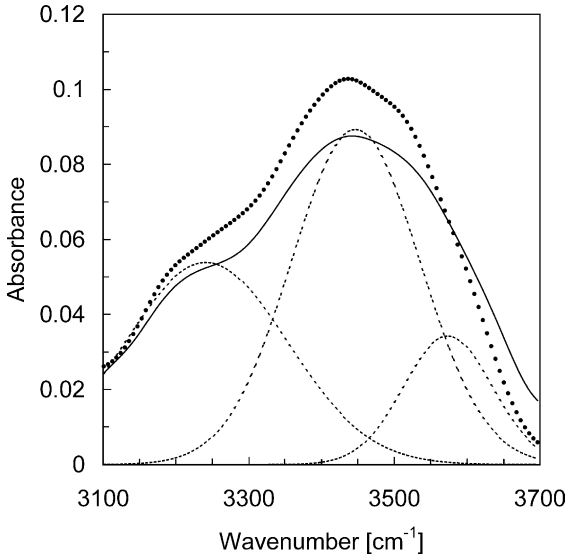

Fig. 5. O–H stretching region of the FTIR spectrum of an individual reverse micelle. (···) AOT/PEG 400 reverse micelles: $C_{AOT} = 0.1\,mol\,dm^{-3}$, $p_o = 0.2$, $w_o = 8$; C_{NaCl} (in buffer) $= 0.3\,mol\,dm^{-3}$; (—) simple AOT reverse micelles: $C_{AOT} = 0.1\,mol\,dm^{-3}$, $w_o = 8$; C_{NaCl} (in buffer) $= 0.3\,mol\,dm^{-3}$; (- - -) peaks indicted by deconvolution for free, bound and trapped water in AOT/PEG 400 reverse micelles.

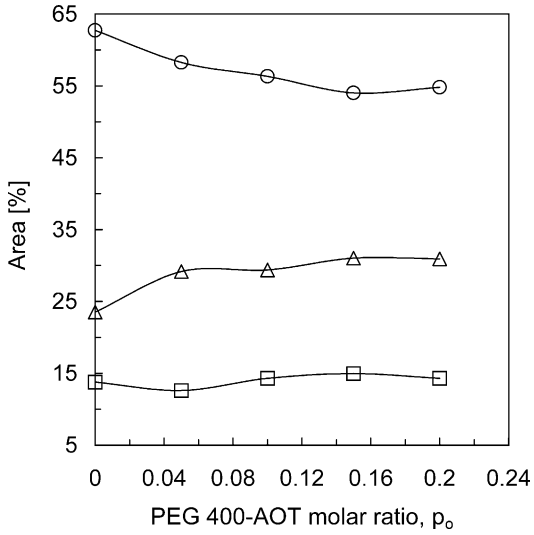

Fig. 6. The effect of PEG 400 concentration on the percentage area of free water, bound water and trapped water in AOT/isooctane reverse micelles at $C_{AOT} = 0.1\,mol\,dm^{-3}$, $w_o = 8$ and C_{NaCl} (in buffer) $= 0.3\,mol\,dm^{-3}$. (○) Bound water; (△) free water; and (□) trapped water.

those reported elsewhere [25,26]. The average standard deviation between the sum of three Gaussian peaks and the raw data is less then 0.035.

On the basis of reported FTIR studies [25], the peak at $3240 \pm 20\,cm^{-1}$ is attributed to free water, which undergoes a similar extent of hydrogen bonding as in bulk states. The peak at $3445 \pm 10\,cm^{-1}$ is assigned to the solubilized dimers, which have distorted hydrogen bonding due to contact with the micellar interface, here called bound water. The peak at $3580 \pm 10\,cm^{-1}$ refers to weakly hydrogen-bonded or monomeric water; called trapped water.

The area percentages of deconvoluted peaks for free, bound and trapped water at $w_o = 8$ for different PEG 400 concentrations are plotted in Fig. 6. The result shows that compared with simple AOT reverse micelles ($p_o = 0$), the percentage of free water is increased and the amount of bound water is decreased by the addition of PEG 400. However, the percentage of trapped water remains unchanged. The decrease of bound water is attributed to the lowering of the interactions between the micellar water and the polar head group in AOT molecules by the presence of PEG 400. As a result, the percentage of free water increases, which suggests that the hydrophobicity of the reverse micelle is decreased [20]. Compared with normal bulk water, the bound water is motionally restricted in the micelles, and has an ice like structure that inhibits the orientation of the active site of lipase toward the micellar interface. The decrease in the percentage of bound water then suggests that the active site of lipase is oriented more easily toward the micellar interface.

4. Conclusions

Polyethylene glycol (PEG 400) increased lipase activity more significantly than other additives. *C. viscosum* lipase entrapped in AOT/PEG 400 reverse micelles exhibited higher stability than that encountered in simple AOT reverse micelles. Increased half-life correlated with a decrease in the deactivation constant and an increase in free energy. FTIR results showed that the addition of PEG 400 molecules weakened the interaction between the head groups

(SO_3^-) of AOT molecules and micellar water that then led to a decreased of hydrophobicity in the system. The increase of free water and the decrease of bound water inside the reverse micelle suggested that PEG 400 participated in the redistribution of water, and makes the orientation of the active site of lipase toward the micellar interface easy. As a result, the combination of the active site of lipase with the substrate (olive oil) becomes more effective. These influences account for the observed increases of lipase activity.

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