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Improvement in Enzyme Activity and Stability by Addition of Low Molecular Weight Polyethylene Glycol to Sodium *bis*(2-ethyl-L-hexyl)sulfosuccinate/ Isooctane Reverse Micellar System

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Abstract

The activity and stability of *Chromobacterium viscosum* lipase (glycerol-ester hydrolase, EC 3.1.1.3)–catalyzed olive oil hydrolysis in sodium *bis* (2-ethyl-l-hexyl)sulfosuccinate (AOT)/isooctane reverse micelles is increased appreciably when low molecular weight polyethylene glycol (PEG 400) is added to the reverse micelles. To understand the effect of PEG 400 on the phase behavior of the reverse micellar system, the phase diagram of AOT/PEG 400/water/isooctane system was studied. The influences of relevant parameters on the catalytic activity in AOT/PEG 400 reverse micelles were investigated and compared with the results in the simple AOT reverse micelles. In the presence of PEG 400, the linear decreasing trend of the lipase activity with AOT concentration, which is observed in the simple AOT reverse micelles, disappeared. Enzyme entrapped in AOT/PEG reverse micelles was very stable, retaining >75% of its initial activity after 60 d, whereas the half-life in simple AOT reverse micelles was 38 d. The kinetics parameter maximum velocity (V_{max}) exhibiting the temperature dependence and the activation energy obtained by Arrhenius plot was suppressed significantly by the addition of PEG 400.

Index Entries: Enzyme stability; reverse micelles; polyethylene glycol; phase diagram; activation energy.

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Introduction

Catalysis by lipases has been investigated thoroughly in recent years for various applications in pharmaceutics, oleochemicals, and foods. Reverse micellar systems have received a great deal of interest in enzymatic studies, one reason being that they possess a large amount of interfacial area, which would promote contact between enzyme and substrate *(1)*. Although feasibility has been demonstrated for various reactions *(2–5)*, disappointments have been encountered for the interaction of surfactant molecules with the enzyme entrapped in reverse micelles. Most of the studies on reverse micellar systems have been performed with the system using an anionic surfactant, sodium *bis*(2-ethyl-l-hexyl)sulfosuccinate (AOT), despite the generally accepted notion that nonionic surfactants are less likely to promote enzyme deactivation owing to the absence of charges at the micellar interface *(2)*. The motivation to use AOT in these studies has been the large quantity of published data on the physicochemical properties of AOT reverse micelles and the ease of reverse micelle formation. However, reports have shown that in many cases AOT causes deactivation of the enzyme *(3–6)* by the electrostatic and hydrophobic interaction with solubilized enzyme.

To overcome this problem, one approach is to chemically modify the enzyme surface. However, it is a laborious and time-consuming modification. It has been shown that the specific hydrolytic activity is significantly reduced by the chemical modification of lipases *(7)*. Another alternative to enhance the enzyme activity is to modify the reverse micellar interface to decrease the surface charge density. Modification of the reverse micellar interface has been reported by using a nonionic surfactant such as Span 85 or Tween-85 *(2,8–10)*. However, Span 85 and Tween-85 are slowly hydrolyzed by lipases *(9)*. Improvement of enzyme activity has also been reported using newly synthesized surfactant *(11)* or modified AOT *(12)*.

Since protein surface characteristics, hydrophile-lipophile balance, and charge distribution appear to play a major role in the catalytic activity, it should be possible to alter enzyme activity by modifying the biocatalyst microenvironment. Several researchers have modified the enzyme surface by creating noncovalent enzyme-additive complex for the enzymatic catalysis in pure organic solvent *(13,14)*. Less attention has been paid to the enzyme entrapped in reverse micelles.

In the previous study *(15)*, the addition of methoxy PEG to the reverse micelles increased the activity of *Chromobacterium viscosum* lipase to some extent *(15)*. The objective of the present study is to increase the enzyme activity and stability by other additives. From this viewpoint, we modified AOT reverse micelles and the enzyme surface by polyethylene glycol (PEG) 400, which is soluble in the micellar water pool. By investigating hydrolysis of olive oil as a model reaction, comparative studies between simple AOT and AOT/PEG systems were conducted and the catalytic properties of the AOT/PEG reverse micelles are discussed.

Attractive interactions between anionic surfactant and the polymer lead to polymer adsorption at the interface *(16)*. Linkage of PEG thus alters the electrical nature of the enzyme surface and micellar interface since charges on the surface become buried beneath the neutral PEG *(17)*. The presence of PEG molecules reduces the attractive interaction between microemulsion droplets *(18)*. There is a high probability that ethylene oxide (EO) groups of PEG molecule decrease the hydrophobicity of reverse micelles like polyoxyethylene groups of nonionic additives such as Tritons *(9)*. More information is desired on how PEG 400 affects the phase behavior of the AOT reverse micellar system, especially the activity, stability, as well as the conformation of lipase.

Materials and Methods

Chemicals

The purified *C. viscosum* lipase (glycerol-ester hydrolase, EC 3.1.1.3) was provided from Ashahi. It is a mixture of lipase A with a mol wt of 120 kDa (p*I* 3.7) and lipase B with a mol wt of 30 kDa (p*I* 7.3). Lipase B is the main portion of the mixture, and its content by weight is >80% *(19)*. AOT, isooctane, and olive oil were purchased from Wako Pure Chemical. PEGs of nominal mol wts of 200, 300, 400, 600, and 1000, whose number of EO units is 4, 6, 9, 13, and 22, respectively, were obtained from Sigma. All chemicals were used without further purification.

Preparation of Reverse Micelles

Isooctane solution in which AOT was dissolved was used as the reverse micellar solution. Reverse micelles containing the lipase were prepared by injecting appropriate amounts of a concentrated buffer solution of lipase into the reverse micellar solution. W_o was adjusted by adding the desired amount of buffer solution: 10.2 *M* NaOH, 0.04 *M* (H_3PO_4 + CH_3COOH + H_3BO_3). The mixture was then stirred with a magnetic bar until a clear and optically transparent solution was obtained. The systems with PEG were prepared by mixing the reverse micellar solutions in a glass bottle in which an appropriate amount of PEG was added in advance. Total lipase concentration was maintained at 2 mg/dm^3 throughout the whole range of w_o values.

Determination of Lipase Activity

Lipase activity was defined as the initial reaction rate, *V*. Reaction was initiated by adding an appropriate amount of olive oil to a preincubated micellar solution containing lipase. The reaction mixture was agitated by a magnetic stirring bar at 500 rpm in a constant-temperature box at 25°C for exactly 20 min. It was found that within this time range, free fatty acid production was linearly dependent on time *(20)*. The fatty acid produced was analyzed by the Lowry technique *(21)*. The details of activity determination have been described elsewhere *(20)*. All the data were the average

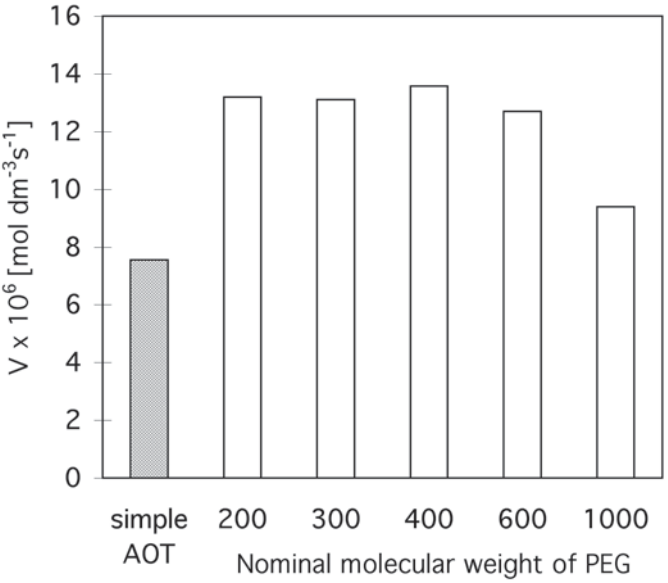

Fig. 1. Effect of PEG molecular weights on lipase activity in reverse micelles. Experimental conditions were as follows: C_{lipase} = 2 mg/dm^3; $C_{PEG\,400}$ = 5 g/dm^3; $C_{olive\,oil}$ = 0.055 *M*; C_{AOT} = 0.05 *M*; pH = 8.0; w_o = 10; C_{NaCl} = 0.3 *M*; reaction temperature = 25°C.

of five replicate experiments and were reproducible within ±5%. The effect of each operational parameter was investigated by varying only the parameter considered, the others being kept unchanged. All of the concentration terms herein are based on the total volume of the reverse micellar system unless otherwise specified.

Determination of Phase Diagram

PEG 400 was added to optically clear, monophasic AOT/water/isooctane reverse micellar solution in a stepwise fashion with a microsyringe. A short period of agitation was required to incorporate PEG into the reverse micelles. The phase diagram was determined by water titration based on the visual observation of the cloudiness-clarity transition as described elsewhere *(22,23)*.

Results and Discussion

Selection of PEG Molecular Weight

Because the dispersed phase (PEG + water) affects micellar size, which dictates lipase activity, the effect of PEG molecular weight was investigated at constant-weight concentration. Figure 1 shows that the lipase activity was increased appreciably when PEGs 200–600 were used, and a further increase in molecular weight decreased the enhancement of activity. The decrease in lipase activity at higher molecular weight can be attributed to

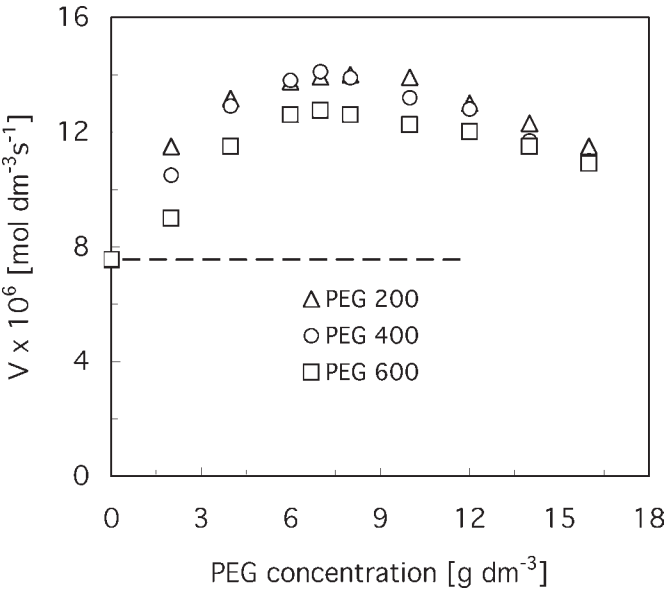

Fig. 2. Effect of PEG concentration on lipase activity in reverse micelles. The dotted line represents the activity in simple AOT reverse micelles. Experimental conditions are the same as those in Fig. 1.

the steric hindrance caused by large PEG molecules, which probably hinder the effective combination of substrate molecules with enzyme-active site. Moreover, as the molecular weight increases, the chain of PEG molecule begins to fold by itself, making it difficult to interact with enzyme *(24)*. As a consequence, the lipase activity decreases when larger molecular weights of PEG are used.

Figure 2 shows the effect of PEG concentration on lipase activity for PEGs 200, 400, and 600. In general, with the increase in PEG concentrations, the lipase activity increased to a maximum value and then began to decrease as the reverse micellar solution became turbid. The maximum value in AOT/PEG reverse micelle was about 200% higher than that in simple AOT reverse micelle. At lower PEG concentrations, the linkage of PEG to the surfactant head groups and the enzyme surface may not develop sufficiently. From Fig. 2 it can be seen that the maximum activity in reverse micelles with PEG 200 or PEG 400 was higher than that in the system with PEG 600. Therefore, PEGs of nominal molecular weights of 200–400 are selected as the most effective to increase lipase activity. In the subsequent studies, PEG 400 was used throughout.

Phase Diagram of AOT/PEG 400/Water/Isooctane System

Figure 3 shows the phase diagram for AOT/PEG 400/water/isooctane system at a fixed temperature of 25°C, containing substrate (olive oil). According to Gibb's phase rule, at the boundary between two phases under

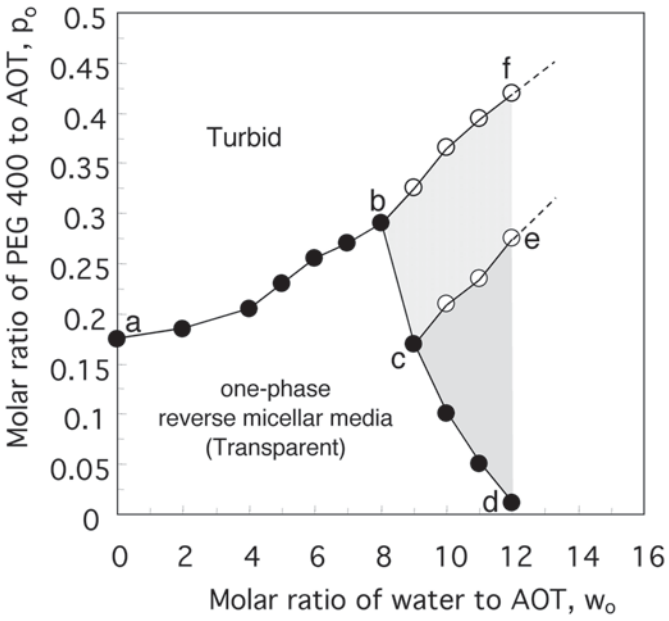

Fig. 3. Phase boundaries for reverse micellar phase of AOT/PEG 400/water/isooctane system: $C_{AOT} = 0.05\,M$; pH = 8.0; $C_{NaCl} = 0.3\,M$; $C_{olive\ oil} = 0.055\,M$; temperature = 25°C. The area below the boundary, abcd, is one-phase reverse micellar media; above it the corresponding area (dotted, shaded, and turbid) is two phase; see text for more details.

constant temperature, the number of degrees of freedom (f) is equal to the number of components (n), which is five for this system. To reduce the value of f to 3, the ratio of olive oil-AOT-isooctane is kept constant, and then a triangular phase diagram with [H_2O], [PEG 400], and [AOT] at the vertices could be formed. When the concentration of AOT in reverse micellar systems used for the enzymatic hydrolysis of olive oil is low, such as 30–100 mM, the phase boundary does not change significantly with the variation of [AOT]. This fact convinces us to transform the triangular phase diagram to one in terms of two variables: water-AOT molar ratio (w_o) and PEG 400-AOT molar ratio (p_o), since the dispersed phase consisting of water and PEG 400 dictates micellar size.

In Fig. 3 the area below the phase boundary abcd represents the region where one–phase reverse micellar medium exists; above it, two–phase and nonmicellar media are formed. The phase boundary abcd illustrates that the solubilization of PEG 400 into micellar inner core increases with water content until $w_o = 8$ and then decreases. This phenomenon is comparable with the phase boundary of the AOT/glycerol/water/isooctane system *(22)*. The increase in PEG solubilization with w_o may be a consequence of the decreased interfacial rigidity of reverse micelles. It has been reported that the solubility of water increases with a decrease in the interfacial rigidity of reverse micelles *(25)*. As the size of micelles increases with w_o, the attractive interaction between colliding micelles increases *(26)*. Therefore, the decrease

in the solubility of PEG 400 at $w_o > 8$ may be attributed to the increase in attractive interaction between colliding micelles and the decrease in interfacial rigidity that leads to the clustering of reverse micelles, which exist at conditions near phase inversion *(22)*. The large increase in micellar collision also causes the phase inversion at higher p_o.

Above the boundary cd, the micellar solution is found to be translucent by the addition of PEG (shaded area in Fig. 3), and when p_o crosses the boundary ce, it becomes transparent (dotted area in Fig. 3). The phase boundary bcd suggests that when a large amount of water is encapsulated in reverse micelles ($w_o > 8$), the media can incorporate less PEG and vice versa. Therefore, the water, which is excluded from the micellar inner core, makes the solution translucent (shaded area in Fig. 3), and when all the excluded water has disappeared by the hydration of PEG, the solution becomes transparent (dotted area in Fig. 3), where hydrated PEG and reverse micellar phases are in equilibrium. It should be emphasized that above the boundary abf further addition of PEG makes the solution turbid.

Effect of Water-AOT Molar Ratio, w_o

The dependence of enzyme activity on w_o has been investigated at constant lipase concentration throughout the whole range of w_o values, as is usual for determination of such a profile *(27)*. Figure 4 (curves a and b) shows that on addition of PEG, the shape of the activity-w_o profile changed significantly from the classic bell shape dependence observed in simple AOT reverse micelles (curve c), and that the optimum w_o shifted toward a low value (7 to 8) according to the concentration of PEG 400. The optimum value is related to a situation in which the inner diameter of the micelles corresponds to the size of encapsulated lipase *(5,28)*. It seems that this conclusion still holds true in the systems to which PEG has been added, in which the size of PEG-lipase complex is almost equal to the size of AOT/PEG 400 reverse micelles. The increased immobility thus reduces the fluctuation of enzyme structure that usually destroys catalytic activity *(28)*.

Fourier transform infrared result *(29)* suggest that at higher w_o values properties of water inside the micellar core are similar to those in the bulk. Therefore, the decrease in lipase activity at higher w_o seems to be owing to the conformational rearrangements in the micellar phase induced by the change in water structure in the hydration shell *(30,31)*. In AOT/PEG 400 reverse micelles, PEG 400 participates in redistribution of water and binds to the enzyme surface by noncovalent linkage such as hydrogen bonding or van der Waals interaction. As a consequence, enzyme is protected from an unfavorable environment by PEG molecules on its surface *(32)*. In addition, solvation of PEG chains by water hinders the molecular mobility necessary for protein conformational rearrangement *(33)*. Hence, activity at higher w_o values has been improved.

The improvement in lipase activity at lower w_o values can be attributed to the increase in micellar size caused by the presence of PEG 400

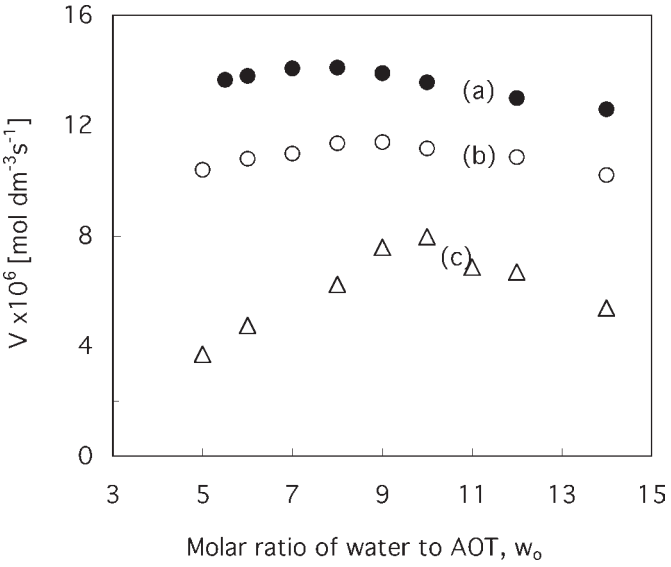

Fig. 4. Dependence of lipase activity on w_o in reverse micelles. (curves a and b) AOT/PEG 400 reverse micelles: (●) C_{AOT} = 0.05 *M*, $C_{PEG\ 400}$ = 0.0125 *M*, (○) C_{AOT} = 0.05 *M*, $C_{PEG\ 400}$ = 0.005 *M*; (curve c) simple AOT reverse micelles: (△) C_{AOT} = 0.05 *M*. Other experimental conditions are the same as those in Fig. 1.

molecules, promoting encapsulation of the lipase into micellar inner core. Increase in the interfacial area with PEG may also be responsible for the faster reaction rate, since it means an increase in substrate availability for the enzyme acting on interfaces. The shifting of optimum w_o toward a low value and improvement in lipase activity at lower w_o reveal that the presence of PEG 400 increases the size of micelles.

General Characteristics

For the lipase entrapped in AOT/PEG 400 reverse micelles, the activity-pH profile is similar to that in simple AOT reverse micelles *(10)* with the optimum pH of 8.0, which is near the isoelectric pH of *C. viscosum* lipase. Here, the pH values are those of the aqueous buffer from which the micelle solution was prepared.

Figure 5 shows the dependence of lipase activity on ionic strength at pH 8.0. In contrast to simple AOT reverse micelles, enzyme activity in AOT/PEG 400 reverse micelles is independent of ionic strength (i.e., concentration of NaCl in buffer) until $C_{NaCl} \approx 0.6$ *M*, after which the activity decreases slightly with the increase in ionic strength. The linkage of PEG owing to the strong interactions with surfactant monolayer inhibits the counterion (Na^+) to approach the SO_3^- head groups of AOT molecules, and the effect of ionic strength on micellar size becomes less effective. As a result, the activity seems to be independent of ionic strength until $C_{NaCl} \approx 0.6\,M$.

The activity-temperature profiles for the enzyme entrapped in AOT/PEG reverse micelles is similar to that in simple AOT reverse micelles *(15)*,

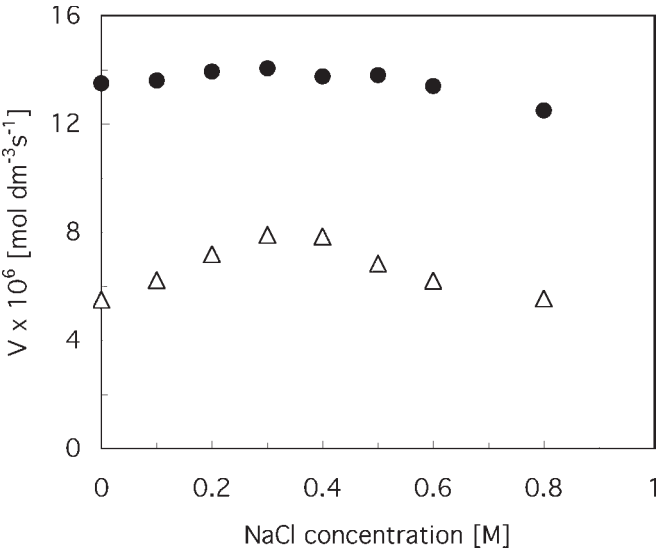

Fig. 5. Dependence of lipase activity on ionic strength (concentration of NaCl in buffer) in reverse micelles. (●) AOT/PEG 400 reverse micelles: C_{AOT} = 0.05 *M*, $C_{PEG\,400}$ = 0.0125 *M*, w_o = 8; (△) simple AOT reverse micelles: C_{AOT} = 0.05 *M*, w_o = 10. Other experimental conditions are the same as those in Fig. 1.

with the optimum activity at a temperature between 25 and 30°C. The activation energy estimated from the Arrhenius equation as described elsewhere *(34)* was found to be 7.60 and 4.8 kcal/mol for the simple AOT and AOT/PEG 400 reverse micellar systems, respectively. The activation energy for the simple AOT reverse micelles is comparable with that reported by others *(3,35)*. The observed decrease in activation energy would tend to indicate that there has been a conformational change in the enzyme *(34)* that may account for the observed increased activity.

Effect of PEG on AOT Concentration Dependence of Activity

The most striking result in the present study is the improvement in lipase activity at higher AOT concentration. It is well established that in simple AOT reverse micellar systems, the enzyme activity decreases with an increase in surfactant concentration *(36,37)*. However, Fig. 6 shows that in the AOT/PEG 400 reverse micellar system, enzyme activity goes through a maximum value with an increase in AOT concentration. The reason for the highest activity at $C_{AOT} \approx 0.05\,M$ is twofold: first, at a constant molar ratio of PEG to AOT, when the AOT concentration increases, the overall PEG concentration also increases, causing the increase in lipase activity; second, with the increase in the concentration of AOT, micelle number increases, and the total amount of substrate adsorbed on the micellar surfactant tails also increases *(20)*, leading to the decrease in free substrate concentration that is responsible for the hydrolysis reaction.

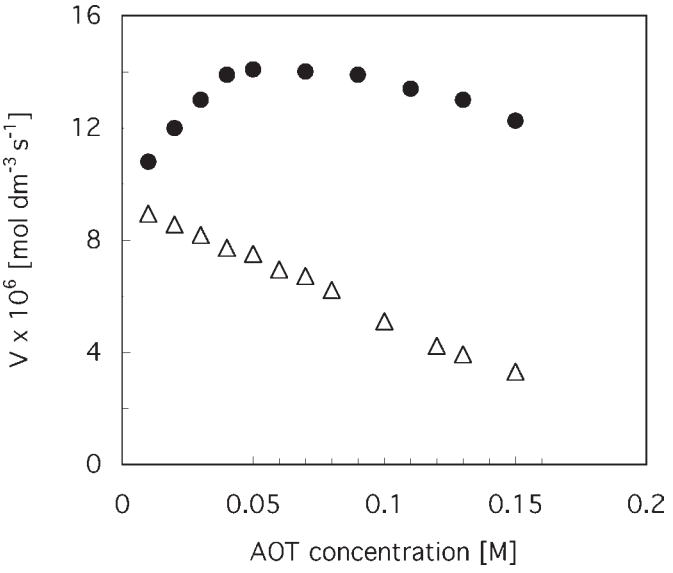

Fig. 6. Effect of AOT concentration on lipase activity in reverse micelles. (●) AOT/PEG 400 reverse micelles: $C_{PEG\,400} / C_{AOT} = 0.25$, $w_o = 8$; (△) simple AOT reverse micelles: $w_o = 10$. Other experimental conditions are the same as those in Fig. 1.

Influence of PEG 400 on Stability of Lipase

Several investigators *(38–40)* have reported that the presence of polymers significantly changes the behavior of the micellar system containing the ionic surfactant AOT. The effect of PEG 400 on the enzyme stability in the micelles is thus a very important factor in bioprocessing using reverse micelles and in recycling of the enzyme. The reverse micellar solution containing lipase without substrate was incubated at 25°C, and the residual activity after incubation was measured. Although several investigators *(3,7,41)* have reported that low w_o is favorable for enzyme stability, we investigated the stability of lipase at optimum w_o values, since the reaction rate at optimum w_o is maximum. We selected 0.1 *M* Tris-HCl solution as a buffer because the half-life of *C. viscosum* lipase in this buffer (about 49 d) is longer than that in other buffers (e.g., phosphate, borate, NaOH-H_3BO_3). Using Tris-HCl buffer, the optimum w_o was found to be 8 and 7 for the simple AOT and AOT/PEG 400 reverse micelles, respectively (data not shown).

From Fig. 7, it is evident that *C. viscosum* lipase entrapped in AOT/PEG reverse micelles is very stable, retaining >75% of its initial activity after 60 d. By contrast, the half-life of the native lipase in simple AOT reverse micelles is about 38 d. The stability of lipase in simple AOT reverse micelles is comparable with that reported elsewhere *(6,42)*. The observed increase in the enzyme stability might be owing to the suppressed interaction of enzyme with anionic surfactant, since charges on the micellar interface and enzyme surface are buried by neutral PEG. In addition, the

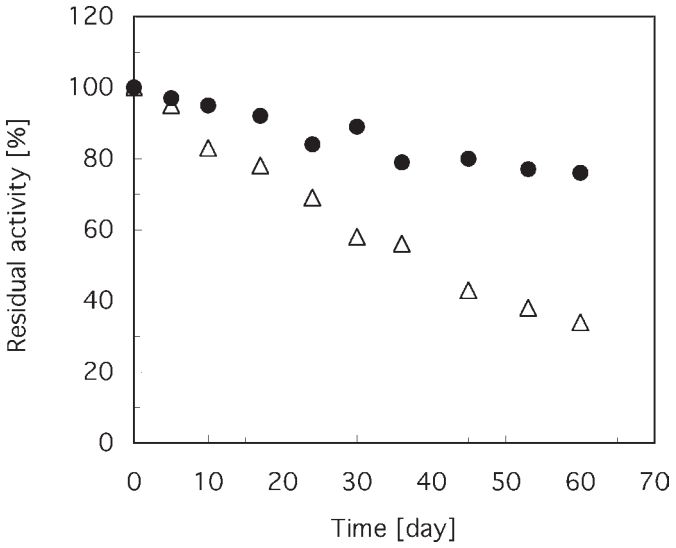

Fig. 7. Stability of lipase in individual reverse micelles at 25°C. (●) AOT/PEG 400 reverse micelles: C_{AOT} = 0.05 *M*, $C_{PEG\ 400}$ = 0.0125 *M*, w_o = 7; (△) simple AOT reverse micelles: C_{AOT} = 0.05 *M*, w_o = 8. Overall enzyme concentration is 2 mg/dm^3 for both reverse micellar systems.

surrounding PEG molecules, which are bound to the enzyme surface by noncovalent linkage, stabilize the enzyme in AOT/PEG reverse micelles. This observation agrees with the results reported by others *(32)* in which lipases are modified by long-chain PEG.

Conclusion

The activity of *C. viscosum* lipase–catalyzed olive oil hydrolysis in AOT/PEG reverse micelles was investigated and found to increase up to two times by the addition of PEGs of nominal mol wts of 200–400. Higher enzyme stability, improvement in lipase activity at high AOT concentration, as well as no sharp decrease in lipase activity at lower and higher w_o reveal that the inner core of the AOT/PEG 400 reverse micelles provides a better environment for enzymatic reaction than that of the simple AOT reverse micelles. Decreased activation energy suggests to some extent the conformational change in lipase at which combination of lipase with olive oil becomes easier *(34)*.

Nomenclature

C_{AOT} = AOT concentration (mol/dm^3)
C_{lipase} = *C. viscosum* lipase concentration (mg/dm^3)
C_{NaCl} = NaCl concentration (mol/dm^3)
$C_{olive\ oil}$ = olive oil concentration (mol/dm^3)
$C_{PEG\ 400}$ = PEG 400 concentration (mol/dm^3)
M = molarity (mol/dm^3)

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