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Thermostability of *Cromobacterium viscosum* Lipase in AOT/Isooctane Reverse Micelle

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Abstract

The thermostability of *Cromobacterium viscosum* lipase (EC 3.1.1.3) entrapped in AOT (sodium bis-[2-ethylhexyl] sulfosuccinate) reverse micelles was increased by the addition of short-chain polyethylene glycol (PEG 400). Two different approaches were considered: (1) the determination of half-life time and (2) the mechanistic analysis of deactivation kinetics. The half-life of lipase entrapped in AOT/isooctane reverse micelles with PEG 400 at 60°C was 28 h, ninefold higher than that in reverse micelles without PEG 400. The lipase entrapped in both reverse micellar systems followed a series-type deactivation mechanism involving two first-order steps. The deactivation constant for the first step at 60°C in PEG containing reverse micelles was 0.055 h^{-1}, 11-fold lower than that in reverse micelles without PEG, whereas it remained almost constant for the second step. The inactivation energy of the lipase entrapped in reverse micelles with and without PEG 400 was 88.12 and 21.97 kJ/mol, respectively.

Index Entries: *Cromobacterium viscosum*; lipase; polyethylene glycol; reverse micelles; thermostability.

Introduction

Hydrolysis, esterification, and interesterification reactions of lipid and fats catalyzed by lipases (EC 3.1.1.3) have been intensively studied in various systems to explore the inherent advantages of using these biocatalysts

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(1–3). Lipase-catalyzed hydrolysis of fats or oils takes place at the interface, and, therefore, reverse micelles have been used to host these reactions *(4,5)* because they have a large interfacial area that promotes contact between enzyme and substrates. The anionic double-tailed surfactant AOT (sodium bis-[2-ethylhexyl] sulfosuccinate) is frequently used in reverse micellar enzymology. The motivation to use the surfactant AOT includes the large number of published data on the physicochemical properties of AOT reverse micelles and the ease with which reverse micelles are formed. However, the activity of lipase was inhibited by strong interactions with AOT molecules *(6–8)*.

To improve the activity of lipases entrapped in AOT reverse micelles, several methods have been reported: the use of chemically modified AOT *(9)*, pretreatment with acetone *(10)*, and the introduction of nonionic surfactant such as Tween-85 or small molecular weight polyethylene glycols (PEGs) as additives *(11–13)*. In addition to the activity of lipase, stability and thermostability are prerequisites for the industrial application of lipase. Although the stability of lipase in AOT reverse micelles has been intensively studied in the last decade *(11,14–16)*, less attention has been focused on the thermostability of lipase *(17,18)*.

We have reported that the addition of PEGs of a molecular weight of <1000 enhanced the activity and stability of *C. viscosum* lipase entrapped in AOT/isooctane reverse micelles and that PEG of a molecular weight of 400 was the most effective *(11)*. In the present work, we show that the thermostability of *C. viscosum* lipase entrapped in AOT reverse micelles was significantly increased in the presence of PEG 400. A deactivation kinetic model *(19)* was used to better understand the thermal stabilization of lipase by PEG 400.

Materials and Methods

Chemicals

Purified *C. viscosum* lipase (glycerol-ester hydrolase, EC 3.1.1.3) was provided by Ashahi Chemical (Tokyo, Japan). AOT, isooctane, PEG (PEG 400), and olive oil were purchased from Wako Pure Chemical (Osaka Japan). All chemicals were of analytical grade and used as received.

Preparation of Reverse Micelles

Isooctane containing dissolved AOT was used as the reverse micellar solution. Reverse micelles containing the lipase were prepared by injecting lipase solution in 0.1 *M* Tris-HCl buffer (pH 7.0) into reverse micellar solution. The water-AOT molar ratio (W_o) was adjusted by adding the desired amount of buffer. The mixture was then magnetically stirred until an optically transparent solution was observed. The system with PEG 400 was prepared by mixing micellar solution in a glass bottle in which PEG 400 (0.0125 mol/dm^3) was added in advance.

Assay of Enzyme Hydrolytic Activity

Lipase activity was defined as the initial reaction rate, V (mol of fatty acid/[dm^3·s]). The initial reaction rate was measured using hydrolysis of olive oil as a model reaction at optimal conditions *(11)*: C_{lipase} = 2 mg/dm^3, buffer pH = 8.0, C_{AOT} = 0.05 mol/dm^3, $C_{olive\ oil}$ = 0.055 mol/dm^3, C_{NaCl} = 0.3 mol/dm^3 (in buffer), temperature = 25°C, and W_o = equivalent to 10 and 8 for simple AOT and AOT/PEG 400 reverse micelles, respectively. The reaction was initiated by adding olive oil to preincubated micellar solutions containing lipase and then stirred at 25°C and 1000 rpm for 20 min, because it was found that within this time period the free fatty acid produced was linearly dependent on time. The produced fatty acids were analyzed by the Lowry and Tinsely *(20)* method. The remaining fractional activity is the ratio of initial reaction rates measured after and before heat-induced deactivation of lipase. All data were the average of at least five batches of experiments under identical conditions and were reproducible within ±5%. All concentration terms were based on the total volume of the system unless otherwise specified.

Measurement of Enzyme Thermostability

Samples containing lipase without olive oil were placed in a water bath at 45, 50, 55, and 60°C; removed periodically; and cooled down in ice *(19)*. Remaining hydrolytic activity of lipase was assayed according to the procedure described in the previous section.

Determination of Enzyme Deactivation Kinetic Parameters

The deactivation data in reverse micelles with or without PEG 400 were fitted to a two-step series-type deactivation kinetic model *(19)*, and the kinetic parameters were determined using a nonlinear regression procedure based on the Marquardt-Levenberg method of iterative convergence included in Origin 6.1 (2001) software.

Results and Discussion

Effect of Reaction Temperature

The activity-temperature profiles for the lipase in both systems were similar and the optimal activity was observed at about 25°C (Fig. 1). However, lipase activity in reverse micelles with PEG 400 at 60°C was almost the same as that in reverse micelles without PEG 400 at an optimal temperature of 25°C. This result motivated us to investigate the thermostablity of lipase in reverse micelles with and without PEG 400.

Thermostability of Lipase

Figure 2A,B presents the thermal deactivation profiles of *C. viscosum* lipase entrapped in reverse micelles with and without PEG 400 at 45, 50, 55,

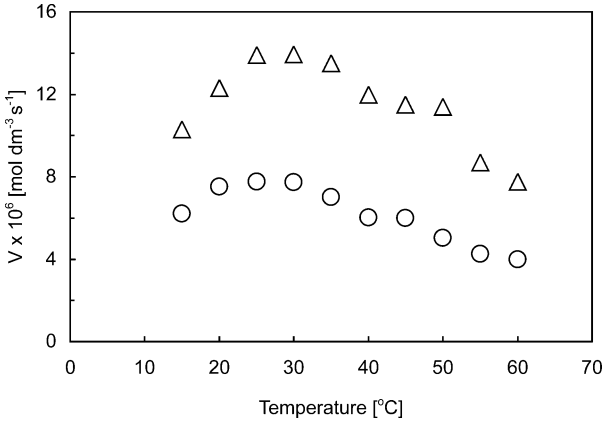

Fig. 1. Effect of reaction temperature on lipase activity in AOT/isooctane reverse micelles with (△) and without (○) PEG 400. Reaction conditions: C_{lipase} = 2 mg/dm^3, C_{AOT} = 0.05 mol/dm^3, $C_{olive\ oil}$ = 0.055 mol/dm^3, buffer pH = 8.0, C_{NaCl} (in buffer) = 0.3 mol/dm^3.

and 60°C. The thermostability of lipase in reverse micelles with PEG 400 at all temperatures was higher than that in reverse micelles without PEG 400. Specifically, the half-life of lipase entrapped in reverse micelles with PEG 400 at 60°C was 28.5 h, ninefold higher than that in reverse micelles without PEG 400 (Fig. 2B). PEG 400 stabilized the enzyme because of the suppression of interactions between lipase and surfactant molecules *(14)*. In addition, the salvation of PEG 400 may reduce the water activity, decreasing the interaction between the lipase surface and micellar water, which plays a key role in heat-induced denaturation of lipase *(19)*. We have reported *(14)* that PEG 400 is located at the interior surface of the micellar interface and participates in redistribution of trapped, bound, and free micellar water.

Figure 2 also shows that not only the half-life but also the deactivation kinetics changed after adding PEG 400. The deactivation curves for lipase entrapped in reverse micelles with PEG 400 were concave, whereas they were convex for reverse micelles without PEG 400. Longo and Combes *(19)* reported that when enzyme bound to additives (e.g., glycoside) in an aqueous system, the nature of the deactivation curves transferred from convex to concave. It is reasonable that terminal hydroxyl groups of PEG molecule form a hydrogen bond with lipase peptide chain, changing the shape of deactivation curves and improving thermostability.

To quantify thermal deactivation in individual micellar systems, mathematical treatment was undertaken. Since the deactivation curves (Fig. 2A,B) did not fit single-step first-order kinetics, a model considering the two-step series-type enzyme deactivation was used:

$$E \xrightarrow{k_1} \overset{\alpha_1}{E_1} \xrightarrow{k_2} \overset{\alpha_2}{E_2} \qquad (1)$$

in which E, E_1, and E_2 are enzymatic homogeneous states (initial, intermediate, and final, respectively) having different specific activities. This model

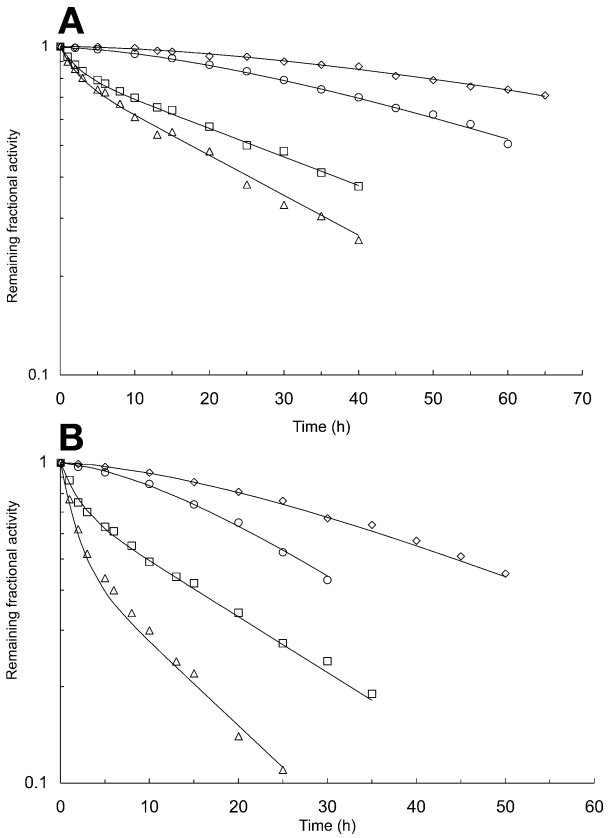

Fig. 2. Thermal deactivation profiles of *C. viscosum* lipase in AOT/isooctane reverse micelles. Symbols represent experimental data and lines represent theoretical profiles obtained from model Eq. 2. **(A)** With PEG 400 at 45°C (◇) and 50°C (○) and without PEG 400 at 45°C (□) and 50°C (△); **(B)** with PEG 400 at 55°C (◇) and 60°C (○) and without PEG 400 at 55°C (□) and 60°C (△).

was reported in the literature *(19)* and is expressed in Eq. 2 assuming that α_2 (ratio of lipase activity at E_2 to E state) = 0, which means that the final form of the enzyme (E_2) is totally deactivated:

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

in which a is the fractional remaining activity; k_1 and k_2 are, respectively, the first-order deactivation rate constant for the first and second deactivation steps; and α_1 is the ratio of lipase activity at E_1 to E state. The parameters α_1, k_1, and k_2 were calculated from the experimental data and are shown in Table 1.

It can be seen from Table 1 that k_1 at all temperatures was significantly decreased after adding PEG 400, whereas k_2 remained almost constant. In particular, k_1 (0.055 h^{-1}) at 60°C in reverse micelles with PEG 400 was 11-fold lower than that (0.6 h^{-1}) in reverse micelles without PEG 400. The value of

Table 1
Deactivation Kinetics Parameters of *C. viscosum* Lipase
in Reverse MicellesWith and Without PEG 400

	Reverse micelle without PEG 400				Reverse micelle with PEG 400			
Parameter	45°C	50°C	55°C	60°C	45°C	50°C	55°C	60°C
k_1 (h^{-1})	0.410	0.468	0.520	0.600	0.012	0.020	0.031	0.055
k_2 (h^{-1})	0.020	0.028	0.040	0.060	0.022	0.031	0.040	0.062
α_1	0.80	0.77	0.67	0.27	0.95	0.90	0.89	0.88

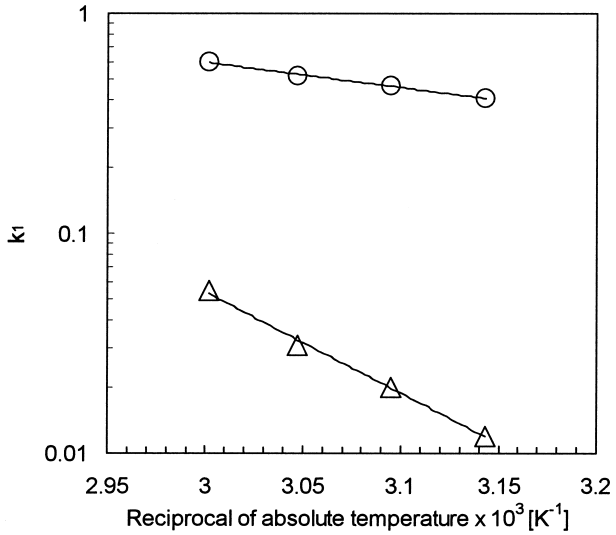

Fig. 3 Arrhenius plot of deactivation rate constant with (△) and without (○) PEG 400.

α_1 was significantly improved by the addition of PEG 400, indicating that the intermediate form (E_1) of lipase entrapped in reverse micelles with PEG 400 was more active than that in reverse micelles without PEG 400.

Since the value of k_2 at a particular temperature was almost the same for the systems with and without PEG 400, only the values of k_1 were plotted in the Arrhenius diagram in Fig. 3. The plots of Ln(k_1) vs the reciprocal of absolute temperature (K) gave a straight line, and the inactivation energy, E_A, was determined from the slope (E_A/R) of the lines, in which R is the gas constant. The inactivation energy for lipase entrapped in reverse micelles with and without PEG 400 was 88.12 and 21.97 kJ/mol, respectively. This result indicates that PEG 400 increased the rigidity of lipase structure, causing high resistance to heat-induced denaturation.

Conclusion

PEG 400 is an excellent additive for improving the thermostability of *C. viscosum* lipase in AOT reverse micelles in addition to its activity *(11)*. The improved thermostability was attributed to the decreased interactions between lipase and AOT molecules and the increased rigidity of the lipase structure.

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