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Kinetic characterization of esterification catalyzed by *Rhizopus delemar* lipase in lecithin–AOT microemulsion systems

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Abstract

The esterification of oleic acid with octyl alcohol catalyzed by *Rhizopus delemar* lipase in microemulsion systems was investigated in terms of the kinetic parameters. The amphiphiles used were AOT and lecithin, both singly and as dual components. The enzyme activity in the lecithin system was high under hydrophobic conditions, i.e., at a W_L ($\equiv [H_2O]/[\text{lecithin}]$) of 4.5, whereas in the AOT system it was high at W ($\equiv [H_2O]/[\text{AOT}]$) = 7. The reaction kinetics were recognized to follow the ping–pong bi–bi mechanism. A dual lecithin–AOT amphiphile system was effective in lipase catalyzed esterification, the maximum initial reaction rate being larger than those obtained in either of the single systems. The optimal molar fraction of lecithin for the maximum initial reaction rate differed with the water concentration in the microemulsion phase. Under relatively hydrophobic conditions, i.e., in a system with a lower W_D ($\equiv [H_2O]/[\text{lecithin} + \text{AOT}]$), the initial reaction rate had a higher maximum value. © 1998 Elsevier Science B.V.

Keywords: Lecithin; AOT; Microemulsion; Esterification; Lipase; Kinetic parameters; Dual amphiphiles

1. Introduction

Microemulsion systems formed by amphiphilic molecules have been studied as enzyme reaction media for hydrophobic substrates [1–4]. The catalytic activity of an enzyme hosted in a microemulsion is stabilized by solubilized water molecules and amphiphiles. The anionic amphiphile AOT is frequently employed because of its nature of spontaneous assemblage, thermodynamic stability and the fact that it does not require an additional cosurfactant. Lipase is known to catalyze hydrolysis and esterification for hydrophobic substrates. Microemulsion systems formed by amphiphiles are therefore considered to offer an attractive means of obtaining a high level of enzyme activity in a hydrophobic solvent [1–4].

Recently, microemulsions formed by phospholipids and lecithins have become of interest as biomimetic reaction systems in hydrophobic media [5–9]. Because lecithins could be found in some natural products, e.g., soybean and egg yolk, it is anticipated they can be employed as biocompatible amphiphilic components to stabilize enzymes in microemulsion systems. The

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structure of the lecithin assemblage in an organic solvent takes a peculiar form, which changes with the amount of water solubilized in the system [11,20–22].

Enzyme activity in microemulsion systems depends to a marked extent on the solubilized water concentration. This dependence has been reported not only in reaction systems but also in protein extraction systems [1–4,18]. The main factors affecting enzyme activity have been found to occur in structural changes of the amphiphile aggregates and in the hydrophilic surroundings of the enzyme molecules [7,13].

Kinetic studies are very important in exploring reaction mechanisms and evaluating catalytic activity. Although there have been a few reports on the kinetics of lecithin microemulsion systems [5–9], in these studies the addition of a cosurfactant was needed in order to investigate the kinetics. Furthermore, the kinetic parameters differ with the media components, especially water, the species of lecithin and their respective concentrations. In this respect, the kinetics and kinetic parameters for lipid transformation in systems using lecithin have not been sufficiently investigated.

Kinetic parameters are especially important in order to evaluate and design a practical esterification process under most suitable conditions, which give a high level enzymatic activity. Here authors present kinetic results using *Rhizopus delemar* lipase in AOT, lecithin and dual amphiphilic systems. The kinetic results in both systems are compared and discussed with a difference in amphiphile characteristics. Optimal conditions for obtaining a higher lipase activity was determined and the findings are discussed with respect to kinetic parameters and its specificity ratio.

2. Experimental

2.1. Materials

Lecithin from soybean (assay 95% as phosphatidylcholine) was supplied by Nihon Siber Hegner (Tokyo) and Lucas Meyer (Hamburg), and di-2-ethylhexyl sodium sulfosuccinate (abbreviated as AOT; assay 97%) was purchased from Nacalai Tesque (Kyoto). They were used without further purification. Lipase (*Rhizopus delemar*) was purchased from Seikagaku Kogyo (Tokyo). This enzyme has a specific activity of 830 units/mg solid for the hydrolysis of olive oil at 303 K according to the literature [10]. Iso-octane was used as an organic solvent and was purchased from Nacalai Tesque. Oleic acid was purchased from Wako Pure Chemical Industries (Osaka) and octyl alcohol from Tokyo Kasei (Tokyo). All other chemicals used were of analytical reagent grade.

2.2. Methods

2.2.1. Preparation of microemulsion

Iso-octane solution (0.04 dm^3) containing the desired concentrations of amphiphile and substrates was incubated in screw-capped vials with magnetic stirring (ca. 10 s^{-1}) at 303 K. The water content in the microemulsion system was adjusted by adding lipase solution and buffer solution (50 mM KH_2PO_4–50 mM NaOH, pH 6.0). In every experimental run, 6 mg solid lipase was solubilized. The mixtures were sonicated for 60 s to solubilize them. The water content was determined by a Karl–Fischer titrator (MKS-1s; Kyoto Electronics, Kyoto).

2.2.2. Enzyme assay

The enzyme activity of lipase for esterification was determined by measuring the decreasing profile of oleic acid according to the methods previously described [17,19]. The procedures were as follows. Samples (0.2 cm^3) were taken at a desired running time and put into 4.8 cm^3 benzene. Cupric acetate aqueous solution (1 cm^3) containing pyridine (5 w/v%, pH 6.0) was then added into the benzene solution and the solutions were mixed for 30 s. After centrifugation at 2500 rpm for 120 s, the upper organic phase contained oleic acid was monitored by a UV spectrophotometer (U-3210; Shi-

madzu, Kyoto) at 715 nm. All experiments were performed at 303 K.

3. Results and discussion

3.1. Effect of water concentration on lipase activity

Determining the amounts of fatty acid and alcohol to be added is important not only for the control of phase viscosity but also to enable the enzyme reaction to proceed effectively [9,25]. Water concentration in microemulsion system also have a large effect on the reaction activity of lipase. In this paper water concentrations are indicated by the following definition, i.e., $W \equiv [H_2O]/[AOT]$ and $W_L \equiv [H_2O]/[lecithin]$.

Fig. 1 shows the effects of the substrate concentrations on the apparent viscosity for a lecithin concentration of 25 mM and W_L of 4.5. The apparent viscosity decreased with increasing concentration of oleic acid or octyl alcohol. It is known that the viscosity of a lecithin microemulsion system increases with the water concentration [11,20–22]. In this study, the kinetics was investigated at the substrate concentration where the apparent viscosity corresponded to the level of iso-octane.

Fig. 2 shows the effect of the W and W_L value on the initial reaction rate in the lecithin and AOT microemulsion systems for the esterification of oleic acid (40 mM) with octyl alcohol (50 mM). The initial reaction rate in this study was defined as the decrease in the slope of the oleic acid concentration for 15 min after the start of the reaction.

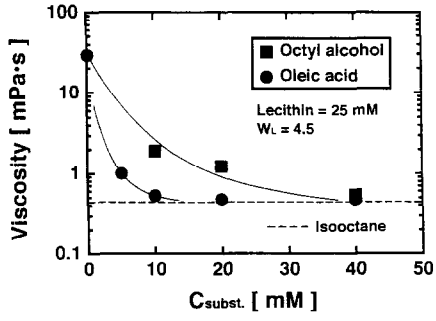

Fig. 1. Effects of substrate concentrations on apparent viscosity of lecithin microemulsion: lecithin = 25 mM, W_L = 4.5.

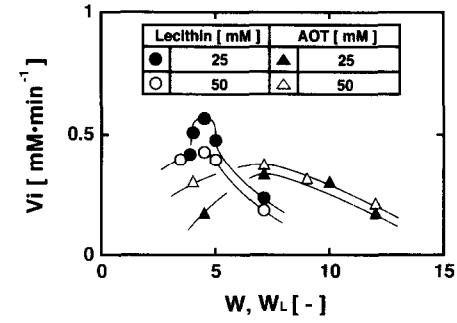

Fig. 2. Effect of W and W_L value on initial reaction rate in lecithin and AOT microemulsion systems for oleic acid (40 mM) and octyl alcohol (50 mM).

In the lecithin microemulsion system, the maximal reaction rate was obtained at a W_L of 4.5. The optimal W value for the reaction rate of the lecithin system was lower than that of the AOT system ($W = 7$). A similar tendency for the reaction rate to be maximal at a lower amount of water has been noted in the hydrolysis reactions of triolein [9] and *p*-nitrophenylpalmitate [7].

The difference in the optimal W values for the maximal reaction rates can be attributed to the hydrophilic environment around the lipase molecule. The water bound with the head group of an amphiphile differs according to the amphiphile species. It has been reported that for the lecithin molecule it is about one water molecule [12], whereas for the AOT molecule it is nine water molecules [3]. As a result of the difference in the amount of bound water, in the lecithin system the hydrophilic environment within the microemulsion required for maximum activity is achieved with less water than the amount needed in the AOT system.

When $W_L > 4.5$ for the lecithin system and $W > 7$ for the AOT system, the activity of the lipase, which acts as a catalyst of esterification, decreased due to a shift in the chemical equilibrium in favor of hydrolysis in these more hy-

drophilic ranges. The lipase activity decreased more markedly in the lecithin microemulsion system than in the AOT system. The assemblages formed by lecithin molecules change their shape from spherical to cylindrical or rod-like, depending on the amount of solubilized water [11,20–22]. Such sterical changes in the lecithin assemblage seem to have an affect on the activity of lipase.

3.2. Effect of amphiphile concentration on lipase activity

Fig. 3 shows the effect of the amphiphile concentration on the initial reaction rate. Based on the results shown in Fig. 2, the molar ratios of water to amphiphile (W_L and W) in each system were set at the optimal condition, i.e., $W_L = 4.5$ for the lecithin system and $W = 7$ for the AOT system. The optimal concentration in the lecithin system was found to be 25 mM. This lecithin concentration gave the maximal reaction rate at $W_L = 4.5$ in our experimental system. In the AOT system, on the other hand, the optimal concentration appeared to be around 50 mM, but no clearly defined value could be discerned.

3.3. Effect of water concentration on ester yield

Fig. 4 shows the effect of the W and W_L value on the ester yield after 24 h in the lecithin and AOT systems. The ester yield was defined as the ratio of the ester concentration after 24 h to the stoichiometrical ester concentration. The amphiphile concentration in each experimental system was set at 25 mM for the lecithin system and 50 mM for the AOT system, respectively. The maximal ester yield was obtained at $W_L = 4.5$ in the lecithin system and it was obtained in the range of $W = 4$ to 7 in the AOT system. Under these water conditions, the conversion yield was obtained to be ca. 90%.

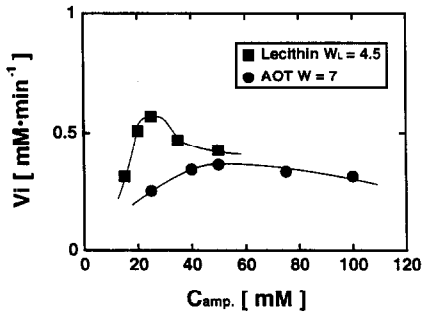

Fig. 3. Effect of amphiphile concentration on initial reaction rate for oleic acid (40 mM) and octyl alcohol (50 mM): $W_L = 4.5$ in the lecithin microemulsion system; $W = 7$ in the AOT microemulsion system.

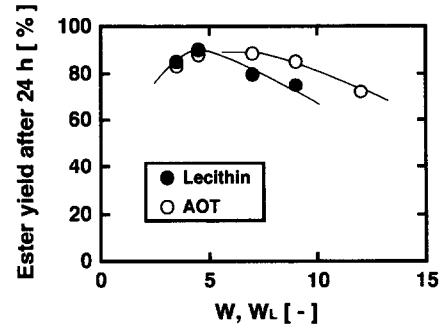

Fig. 4. Effect of W and W_L value on ester yield after 24 in lecithin and AOT microemulsion systems for oleic acid (40 mM) and octyl alcohol (50 mM): $C_{amp} = 25$ mM in the lecithin microemulsion system; $C_{amp} = 50$ mM in the AOT microemulsion system.

In the lecithin microemulsion system, the shape of assemblage reported was changed from a spherical globule to a rodlike one with increasing water and lecithin concentration and its formed network construction became to be complicate. In the AOT system, the stable microemulsion globule was not formed under a water enriched conditions [26,27]. These structural changes in microemulsion systems resulted a negative effect on lipase activity. On the other side, in the lower water condition, lipase molecules solubilized in restrictive aqueous environment and could not preserve its activity.

3.4. Kinetic study

To elucidate the mechanism of esterification in the microemulsion system formed by lecithin molecules, a kinetic study was performed as a

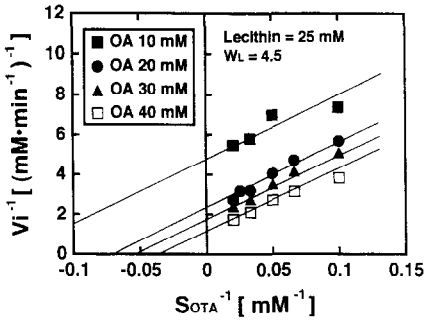

Fig. 5. Lineweaver–Burk plots for octyl alcohol concentration at various oleic acid concentrations: lecithin = 25 mM, $W_L = 4.5$.

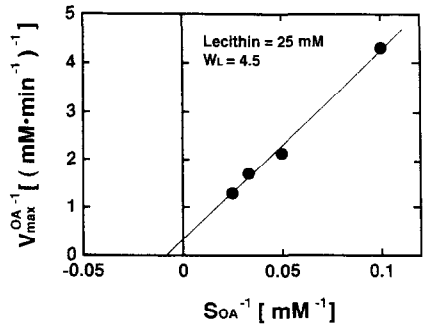

Fig. 6. Double reciprocal plot of maximum velocity obtained from Fig. 5.

function of the concentration of each substrate. The kinetics of the AOT microemulsion system was also investigated using the same substrates.

Fig. 5 shows Lineweaver–Burk plots for the octyl alcohol concentration at different concentrations of oleic acid, using a lecithin concentration of 25 mM and a W_L of 4.5. Data obtained were linearly correlated in double reciprocal plots, so the reaction followed Michaelis–Menten kinetics. The data correlations at the different concentrations of oleic acid ran parallel with each other. This profile shows that the esterification proceeded according to the ping–pong bi–bi mechanism. This mechanism has been reported for esterifications catalyzed by various lipase species in AOT microemulsions and in other reaction systems [14–16]. Lipase is considered to be an acyl-enzyme composed of catalytic active triads, i.e., Ser–His–Asp (or -Glu) [23,24]. Lipase forms a complex with oleic acid (carboxylic-lipase intermediate) and this complex then reacts with octyl alcohol [14].

The kinetic parameters for oleic acid were determined by the double reciprocal plotting of the apparent maximum velocity, obtained from Fig. 5, versus the concentrations of oleic acid (Fig. 6). The reaction parameters for octyl alcohol could be determined from Fig. 5. Table 1 shows the kinetic parameters obtained in the experimental results for both the lecithin and AOT systems. The turnover number of the lecithin system was about 3-fold higher than that of the AOT system. As concerns on the turnover number, the lecithin microemulsion system was found to be superior to the AOT system with respect to the lipase activity as a catalyst of the esterification. The Michaelis constants in the lecithin system, obtained for both oleic acid and octyl alcohol, were higher than those in the AOT system. This suggested that, in the lecithin system, the higher concentration of substrates is necessary to obtain the maximal activity of lipase.

The catalytic behavior of enzyme was also determined by chemicophysical surroundings active site and a substrate solubilized in reaction media. The specificity ratio, i.e., the ratio of turnover number to Michaelis constant, is often used to evaluate apparently enzyme activity in a practical system. There are very few papers about the specificity ratio in amphiphilic mi-

Table 1
Comparison of kinetic parameters in lecithin and AOT microemulsion systems for esterification of oleic acid with octyl alcohol

	K_{cat} (s^{-1})	K_m^{OA} (mM)	K_m^{OTA} (mM)	K_{cat}/K_m^{OA} ($M^{-1} \cdot s^{-1}$)	K_{cat}/K_m^{OTA} ($M^{-1} \cdot s^{-1}$)
Lecithin	13.3	128.2	76.9	103.7	173.0
AOT	4.7	12.5	27.0	376.0	174.1

Lecithin = 25 mM, $W_L = 4.5$.
AOT = 50 mM, $W = 7$.

croemulsion systems [15,16]. Esterifications resemble with our paper, e.g., lauric acid/octanol or (-)menthol, in AOT-iso-octane system has investigated using *Pseudomonas cepacia* or *Penicillium simplicissimum* lipase, respectively. The specificity ratio obtained for oleic acid in the lecithin system, 103.7 M^{-1} s^{-1}, was less than one third of the AOT system. On the other hand, the ratio of octyl alcohol was almost same in both amphiphile systems. The effect of used amphiphile systems on the specificity ratio depended on the substrates.

When the esterification reaction occurs, the substrates have to diffuse and encounter with an active site of lipase. Electrostatic interaction and steric inhibition have been discussed as primary factors for the encountering of enzyme and substrates. In a lecithin used system, although the electrostatic interaction was lower than that of the AOT system owing to its moderate electric charge, the steric hindrance of the lecithin molecule affecting the encounter of substrate and enzyme was great due to its large molecular size. The fluidity of the interface layer of microemulsions formed by the lecithin molecule was lower than that formed by other amphiphilic components [25]. It also made a barrier of encountering substrate and enzyme.

3.5. *Lipase activity in lecithin–AOT dual-component microemulsion system*

With the aim of developing optimal conditions for esterification catalyzed by lipase using amphiphiles, a lecithin–AOT dual-component system was applied to the reaction. It was anticipated that this dual amphiphile system would possess the benefits of the two individual amphiphile components. For lecithin, these are its biocompatibility and moderate electrostatic interaction, and for AOT, its ability to act as a well-forming reagent of microemulsion globules.

Fig. 7 shows the initial reaction rates in the lecithin–AOT microemulsion system for a total concentration of 25 mM amphiphiles, in which

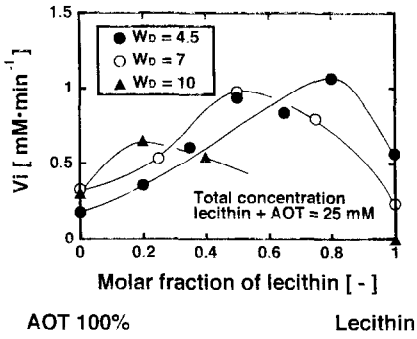

Fig. 7. Effects of molar fraction of lecithin and W values on initial reaction rate. The total concentration of the amphiphile components was set up at 25 mM.

the initial reaction rate is plotted as a function of the molar fraction of lecithin. At each W_D value, the initial reaction rate increased with the molar fraction of lecithin. The W_D indicated the water concentration in dual lecithin–AOT amphiphile microemulsion system, $W_D \equiv [H_2O]/[\text{lecithin} + \text{AOT}]$. This can be attributed to the electrostatic field around the lipase in the microemulsion being moderated by the addition of lecithin, thus enhancing the enzyme activity [13].

The maximum values of the initial reaction rates under the W_D conditions examined were larger than those obtained in either of the single amphiphile systems composed of only AOT or lecithin. For lipase-catalyzed esterification, the dual system is therefore preferable to a single amphiphile system.

The optimal molar fraction for the maximum initial reaction rate differed with the water concentration, expressed as the W_D value. A higher maximum initial reaction rate was obtained under relatively hydrophobic conditions, i.e., in a lower W_D system, suggesting that this is the most favorable condition for esterification by lipase.

In the higher molar fractions of lecithin of each W_D system, the initial reaction rate was decreased because of the structural change of the assemblage due to the excess lecithin molecule.

4. Conclusions

The esterification of oleic acid with octyl alcohol catalyzed by *Rhizopus delemar* lipase was investigated in microemulsion systems formed by single amphiphilic components, i.e., lecithin or AOT, as well as in a dual system containing both amphiphiles.

In the single systems, the maximum initial reaction rates were obtained at $W_L = 4.5$ with lecithin and $W = 7$ with AOT, while the optimal amphiphile concentrations were 25 mM for lecithin and about 50 mM for AOT. A higher level of enzyme activity was obtained in the lecithin microemulsion, which had lower concentrations of both amphiphile and water than the AOT system.

The reaction kinetics accorded to the ping–pong bi–bi mechanism. The kinetic parameters could be obtained from a Michaelis–Menten analysis. The parameters of the lecithin system were larger than those of the AOT system.

A dual lecithin–AOT amphiphile system was examined to take advantage of the property of lecithin as a biocompatible component and of AOT as a good reagent for the formation of microemulsions. The maximum values of the initial reaction rates were larger than those obtained in either of the single systems. The optimal molar fraction of lecithin for the maximum initial reaction rate differed with the water concentration expressed as the W_D value. A relatively hydrophobic condition, i.e., a system with a lower W_D value, gave the highest initial reaction rate.

5. Nomenclature

C_{amp}: concentration of amphiphile (mM)
C_{subst}: concentration of substrate (mM)
K_m: Michaelis constant (mM)
k_{cat}: turnover number (s^{-1})
V_i: initial reaction rate ($mM \cdot min^{-1}$)
V_{max}: maximum velocity ($mM \cdot min^{-1}$)
W: molar ratio of water to AOT (mol-H_2O/mol-AOT)
W_D: molar ratio of water to amphiphiles, lecithin + AOT (mol-H_2O/mol-(lecithin + AOT))
W_L: molar ratio of water to lecithin (mol-H_2O/mol-lecithin)
OA: oleic acid
OTA: octyl alcohol

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