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Kinetics of Lipase Reactions in Two Phase System

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이상계내에서 리파제의 반응동력학

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Two phase reaction system was used to hydrolyze the olive oil for fat splitting. Kinetics of lipases in two phase system were investigated by determining the hydrolysis rate of triglycerides at various olive oil concentrations in isooctane using the microbial lipases from *Candida rugosa* and *Rhizopus arrhizus*. The rate equation in lipid hydrolysis for various olive oil concentrations in two phase system was deviated from the Michaelis-Menten kinetics. The results suggested that the olive oil concentration in isooctane affects the interfacial area. The dependency of the interfacial area on olive oil concentration is greater at the lower olive oil concentration than at the higher substrate concentration. We modified the rate equation by considering the interfacial area between two phases depending on the olive oil concentration in solvent phase.

Enzymatic hydrolysis of lipids in two phase system using organic solvents were advantageous with respects to products separation or enzyme recovery, and with respect to application to the solid lipids (1-3).

In our previous report (4), the characteristics of lipases from *Candida rugosa* and *Rhizopus arrhizus* were studied for fat splitting and interesterification, respectively, in two phase system for their specificity (5). The result showed that the reaction curve did not follow the Michaelis-Menten kinetics. Compared to the ordinary enzyme, especially at lower olive oil concentration, the reaction rates for each lipase were much lower and did not also follow the first order reaction either. In the ordinary enzyme reaction, the reaction velocity follows the first order reaction, i.e., reaction rate of enzyme increased in proportion to the substrate concentration at lower substrate concentration. Although Mukataka *et al.* (6) suggested that the rate equation derived by considering the interfacial area between the two phases of organic and aqueous solutions was well represented by the Michaelis-Menten kinetics, any effort to draw the Lineweaver-Burk plot of these lipases reactions of our data in two phase system came to naught. For interpreting this problem it was necessary to modify the rate equation of the lipase reaction in two phase system in order to clarify the mechanism of this lipase reaction by analyzing the rate data of the lipase reaction for the various olive oil concentrations in isooctane.

Objective of this paper is to study the rate equation of lipase reactions in two phase system by considering the interfacial area which is dependent on substrate concentration and proportional ratio (hold up portion), and the properties of the equation for the lipase reaction systems for the comparative study.

Kinetic Model

In two phase lipase reaction system such as aqueous-organic solvents system where the lipid is

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dissolved in the organic solvent while the lipase is dissolved in the aqueous buffer solution, the reaction occurs mostly at the interface between the two phases of organic-aqueous buffer solution. The rate equation for these reaction systems can be derived as follows:

It is assumed that the enzyme reaction at the interface proceeds according to the scheme of the Michaelis-Menten model (7).

$$E+S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES \xrightarrow{k_{+2}} E+P \qquad [1]$$

In this model, the concentration of the enzyme-substrate complex is also assumed to be constant. Then, if $[E]_i$, $[S]_i$, and $[ES]_i$ represent the concentration of the enzyme, substrate, and the enzyme-substrate complex at the interface, respectively, the rate of change, $d[ES]_i/dt$, will be:

$$\frac{d(ES)_i}{dt}=k_{+1}[E]_i[S]_i-(k_{-1}+k_{+2})[ES]_i=0 \qquad [2]$$

Here, to simplify the mechanism of the reaction, we shall assume that each concentration of the enzyme and the substrate at the interface is proportional to that in the aqueous phase and in the organic solvent phase, respectively:

$$[E]_i=C_1\times E/V(1-\phi) \qquad [3]$$

$$[S]_i=C_2\times S/V\phi \qquad [4]$$

where E and S are the respective number of molecules of enzyme and substrate in the reaction system, V is the total volume of the reaction mixture, and ϕ is the volume fraction of lipid (or solvent phase containing the substrate) in the reaction mixture. C_1 and C_2 are proportionality constants.

By indicating the interfacial area per unit volume of the reaction mixture as a and the concentration of total enzyme and substrate in the mixture as $[E]_0$ and [S], respectively, equation [3] and [4] become:

$$[E]_i=\frac{C_1}{1-\phi}([E]_0-a[ES]_i) \qquad [5]$$

$$[S]_i=C_2[S]/\phi \qquad [6]$$

Substituting equation [5] and [6] to equation [2] and assuming quasi-steady state, $[ES]_i$ is given by

$$[ES]_i=\frac{(1/a)[E]_0[S]}{\frac{k_{-1}+k_{+2}}{k_{+1}}\frac{\phi(1-\phi)}{C_1C_2a}+[S]}=\frac{(1/a)[E]_0[S]}{Km\frac{\phi(1-\phi)}{C_1C_2a}+[S]}$$

where

$$Km=\frac{k_{-1}+k_{+2}}{k_{+1}}$$

Consequently, the rate of product formation per unit volume, v, in two phase system reaction is represented by

$$v=k_{+2}a[ES]_i=\frac{k_{+2}[E]_0[S]}{Km\frac{\phi(1-\phi)}{aC_1C_2}+[S]}=\frac{Vmax[S]}{Km'+[S]} \qquad [8]$$

where

$$Vmax=k_{+2}[E]_0 \qquad [9]$$

$$Km'=Km\frac{\phi(1-\phi)}{aC_1C_2} \qquad [10]$$

and Km and Km′ are Michaelis-Menten constants of homogeneous enzyme reaction and two phase reaction, respectively. Supposing that the interfacial area per unit volume, a, remains unaltered by the change of substrate concentration in organic solvent, the value of apparent Michaelis-Menten constant, Km′, does not change at given ϕ. At constant Km, equation [8] becomes similar to the rate equation derived by Michaelis-Menten for the homogeneous enzyme reaction.

In the standard organic-water system, that is to say, in substrate free organic-water system, interfacial area per unit volume, a, is defined as

$$a=\frac{6}{dm} \qquad [11]$$

where dm is average particle size (diameter) of dispersed drops. Equation [11] shows that interfacial area is determined by solvent fraction (ϕ), and therefore, Km′ changes with ϕ only.

In the lipase reaction in two phase system, where

the substrate is dissolved in the organic solvent while the lipase is in aqueous solution, the change in the substrate (olive oil) concentration will alter the properties of the solvent phase (organic solvent-substrate mixture) such as interfacial tension, γ; viscosity, η; and density, d. Therefore, we can assert Km' is changed, since a is changed with the substrate concentration in solvent.

In two phase system, according to coalescence and break up theory (8), i.e., equilibrium theory, the formation of drops by agitation was equilibrated as follows;

$$B_0 \underset{k_b}{\overset{k_c}{\rightleftharpoons}} B_d \qquad [12]$$

where k_c and k_b are rate constants, B_0 is undropped two phase, and B_d is dropped dispersed phase broken to n-particles.

At equilibrium ($k_c = k_b$) in the scheme of equation [12], drop size distribution in agitated two phase systems was determined experimentally (9-12).

$$dm = k\gamma^{3/5}\eta^{-3/5}N^{-6/5} \qquad [13]$$

where k is proportional constant,
γ is interfacial tension,
η is viscosity of continuous phase and
N is agitation speed

Interfacial tension (γ) and viscosity (η) in equation [13] are changed with the change of substrate concentration, whereas other parameters can be controlled constantly regardless of the change of substrate concentration. According to the experimental data (12), γ and η are given by

$$\gamma \propto [S]^{\alpha} \qquad [14]$$

$$\eta \propto [S]^{\beta} \qquad [15]$$

In equation [13], dm will be

$$dm = K[S]^{(3/5)\alpha-\beta} \qquad [16]$$

where K is overall proportional constant. From the equation [11] interfacial area becomes;

$$a = \frac{6\phi}{K[S]^{(3/5)\alpha-\beta}} = \Omega[S]^{(3/5)\delta} \qquad [17]$$

where Ω is proportionality constant including ϕ, N, K, and $\delta = \beta - \alpha$.

Equation [17] shows that change of substrate concentration in organic solvents affects the interfacial area, which is formed by agitating the solvent-water phase in lipase catalyzed reaction in two phase system. In the lipase reaction, olive oil concentration in solvent (isooctane) was changed, while enzyme concentration in aqueous phase is controlled constantly. Reaction velocity, v, is defined by substituting equation [17] to [8]

$$v = \frac{Vmax[S]}{Km\frac{\phi(1-\phi)}{C_1C_2[S]^{3\sigma/5}} + [S]} \qquad [18]$$

$$= \frac{Vmax[S]}{Km'' + [S]} \qquad [19]$$

where

$$Km'' = Km\frac{\phi(1-\phi)}{C_1C_2\Omega[S]^{3\sigma/5}} \qquad [20]$$

Equation [20] is a formula which deviates from the Michaelis-Menten equation, especially at lower substrate concentration.

To find out value, δ, we can construct the plot between v and [S] at lower substrate concentration ([S] $\ll$ Km). v is defined

$$v = \frac{Vmax[S]}{Km\frac{\phi(1-\phi)}{C_1C_2\Omega[S]^{3\sigma/5}}} = \frac{Vmax}{Km\frac{\phi(1-\phi)}{C_1C_2\Omega}} \times [S]^{1+3\sigma/5} \qquad [21]$$

The logarithmic form of equation [21] becomes

$$\ln v = \tau \ln[S] + \ln\frac{Vmax}{Km\frac{\phi(1-\phi)}{C_1C_2\Omega}} \qquad [22]$$

where τ is $1 + (3/5)\delta$.

From the plot of ln v vs. ln [S] of equation [22], the slope represents τ, the degree of contribution to the change of lipase activity by the change of olive oil concentration. Thus τ is defined as "contribution coefficient" in two phase reaction system.

Materials and Methods

Materials

Lipases from *C. rugosa* and *R. arrhizus*, olive oil and tripalmitin were purchased from Sigma

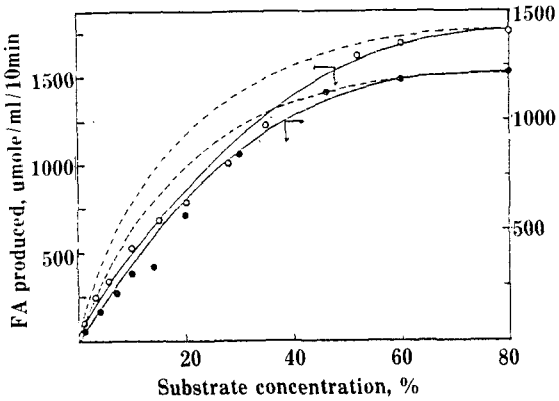

Fig. 1. Reaction velocity of lipase from *C. rugosa* and *R. arrhizus* to the olive oil in two phase system.

Reaction conditions: pH of enzyme solution, 6.0; temperature, 30°C; ratio of solvent phase to aqueous phase, 8:2; agitation speed, 800 rpm; reaction time, 10 min; reaction volume, 5 ml. The data are the mean values of three determinations. Symbols: ○, lipase from *C. rugosa*; ●, lipase from *R. arrhizus*. Dotted curves are constructed for Michaelis-Menten kinetics, in which the values of apparent Km and Vmax were determined from the Lineweaver-Burk plot for the data of initial velocities only to the higher olive oil concentration.

Chemical Co. (St. Louis, MO. USA). Isooctane were purchased from Tokyo Kasei Chemical Co., Ltd. (Tokyo, Japan). All other reagents and chemicals used were of analytical grade.

Methods

Reactor system, the methods of lipase assay, and determination methods of kinetics were the same as described in previous paper (4). Lipase activity in two phase system were determined by the method of Kwon and Rhee (13). One unit of lipase activity was defined as one micromole of fatty acid produced per 1 hr under the analytical conditions.

Results and Discussion

To analyze the rate equation of lipases in two phase system, lipid hydrolysis rate of olive oil were measured in two phase system with various substrate concentrations. Fig. 1 shows the effects of olive oil concentration on the lipid hydrolysis rate by lipases from *C. rugosa* and *R. arrhizus* in two phase system.

Mukataka *et al.*(6) proposed a kinetic model for two phase enzyme reaction by considering the interfacial area between the two phases of organic and aqueous solutions and the volume ratio of the two phases. They assumed that the interfacial area per unit volume, *a*, remains unaltered by the change of substrate concentration in organic solvent. Consequently at a given interfacial area (*a*), the value of apparent Michaelis-Menten constant, Km′ does not change. For this reason, they suggested the derived rate equation of lipase reaction represents the Michaelis-Menten kinetics well. However, they neglected the fact that the interfacial area is determined not only by the proportional ratio of the two phases but also by other physical parameters of solvent such as density, viscosity, interfacial tension, etc. (9, 14).

In the lipase reaction with two phase system, where the substrate is dissolved in the organic solvent while the lipase is in aqueous solution, the change of the substrate concentration must alter the properties of the solvent phase such as interfacial tension, viscosity and density. Therefore, the interfacial area is a variable depending on the substrate concentration. Based on this fact, we think that

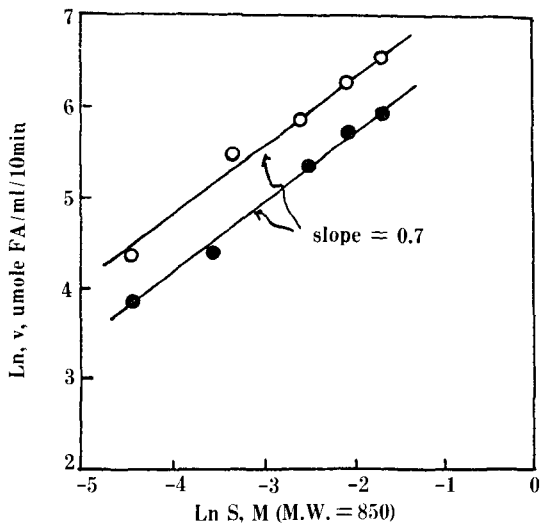

Fig. 2. Plot of ln v vs. ln S in the hydrolysis of olive oil in two phase system.

Symbols: ○, lipase from *R. arrhizus*; ●, lipase from *C. rugosa*.

Km′ is changed with the change of substrate concentration in solvent.

Assumming that olive oil concentration affects the viscosity and interfacial tension of the solvent phase (equation [14], and [15]), we modified interfacial area per unit volume (a) and apparent Michaelis-Menten constant (Km″) which were changed by the substrate concentration (equation [17], [20]). The value of Km″ depends not only upon the volume ratio of two phases, ϕ, but also upon the olive oil concentration, [S]. Therefore, the rate equation of olive oil hydrolysis deviated from the Michaelis-Menten kinetics. According to the proposed kinetic model (equation [19], [20]), when the substrate concentration is increased up to about Km″ or over Km″ ([S] $\gg$ Km″), the value of Km″ becomes Km′. Consequently reaction rate follows the Michaelis-Menten kinetics. However, at lower olive oil concentration ([S] $\ll$ Km″), substrate concentration affects the value of Km″ greatly to decrease the lipase activity in two phase system.

We notice that the reaction velocity of lipase from *C. rugosa* and *R. arrhizus* in two phase system were much lower than the ordinary enzyme reaction, especially at low olive oil concentration, and that the rate equation of lipid hydrolysis by the lipases deviated from the Michaelis-Menten kinetics (Fig. 1). Therefore, we concluded that this result agrees with the kinetic model suggested above.

To determine the contribution coefficient in equation [22], τ, at lower olive oil concentration ([S] $\ll$ Km″), equation [22] was plotted against substrate concentration (Fig. 2). The slope of ln vs. ln [S] plot represented the value of Tan. The slope of plot for both lipases are almost the same ($\tau = 0.7$), thus the value of δ is -0.5 for each lipase. The result showed that the slope (degree of contribution to the change of lipase activity) is not affected by the kinds of lipases but affected by the such reaction parameters as γ, η, d and Ω only.

Substituting in equation [17] by -0.5 the interfacial area is given as $a = \Omega[S]^{-0.3}$. From this formula we assert that the dependency of the interfacial area on olive oil concentration is greater at the lower olive oil concentration than at the higher substrate concentration. This is the reason why the rate equation of olive oil hydrolysis deviated from the Michaelis-Menten kinetics at lower concentration.

In conclusion, the interfacial area of dispersed water phase (containing lipase) in two phase-lipase reaction system was affected by the olive oil concentration in isooctane. Based on this fact, we derived the rate equation of lipase in two phase system. This modified equation is adequate to explain the reason why the rate equation of two phase system for both lipases do not follow the Michaelis-Menten kinetics, especially at the lower olive oil concentration. However, further researches are required to clarify the suggested model above by determining the drop size and constant variables such as C_1, C_2, a, Ω, and so on. If the value of C_1, C_2, a, Ω is obtainable, we can simulate the lipase reaction mechanism of two phase system and expect the reaction rate of lipase in two phase system or reaction mechanisms of other enzymes in two phase system.

요 약

이상계(二相界)를 이용해서 올리브유를 가수분해하는 데 있어서, 리파제의 반응동력학을 검토했다. 반응계는 *Candida rugosa*와 *Rhizopus arrhizus* 리파제를 수용액상에 녹이고 기질인 올리브유를 유기용매인 이소—옥탄에 녹여서 서로의 계면에서 효소반응이 일어나는 이상계 반응이다. 각각의 리파제에 대해서 이소—옥탄에 올리브유를 여러 농도로 녹여서 유지 분해속도를 측정함으로써 리파제의 반응동역학을 보았다. 이상계 내에서 반응 속도 방정식은 일반 효소의 속도 방정식의 Michaelis-Menten 식을 따르지 않았다. 이 결과는 이상계에서 유기용매내의 올리브유의 농도가 계면면적의 변화에 영향을 주기 때문에 비롯되는 것으로 생각된다. 그래서 올리브유의 농도가 계면면적에 영향을 주지 않는다고 가정한 식에 변형을 가해서 새로운 식을 유도했다. 이 식은 특히 올리브유가 낮은 농도에서 심하게 Michaelis-Menten 식에서 벗어나는 현상을 설명할 수 있다.

Nomenclatures

[E] Enzyme concentration
[S] Substrate concentration
[ES] Enzyme-substrate concentration
k_{+1} Rate constant for forward reaction in equation [1]
k_{-1} Rate constant for backward reaction in equation [1]
k_{+2} Rate constant for the product formation in equation [1]
a Interfacial area per unit volume
[P] Product concentration
i Subscript denotes interfacial value
C_1 Proportionality constant for enzyme
C_2 Proportionality constant for substrate
V Total volume of reaction mixture
ϕ Fraction of solvent phase in the reaction mixture
Km Michaelis-Menten constant
Km′ Apparent Michaelis-Menten constant
Km″ Apparent Michaelis-Menten constant in two phase system
dm Average particle diameter of dispersed drops
γ Interfacial tension
η Viscosity
d Density
v Rate of product formation per unit volume, velocity
V_{max} Maximum velocity
B_o Undropped 2-phase in equation [12]
B_d Dropped dispersed phase broken to n-particles in equation [12]
k_c Rate constant in equation [12]
k_b Rate constant in equation [12]
α Exponential constant in equation [14]
β Exponential constant in equation [15]
δ Exponential constant, $\delta = \beta - \alpha$
k Proportionality constant in equation [13]
K Proportionality constant in equation [16]
Ω Proportionality constant in equation [17]
τ Contribution coefficient in equation [22]

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