

Consecutive Reaction Model For Triglyceride Hydrolysis Using Lipase

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Abstrak

Penelitian mengenai hidrolisis trigliserida menggunakan lipase sudah banyak dilakukan. Namun, kinetika perilaku pembentukan produk antara seperti digliserida dan monogliserida masih belum jelas. Triolein dihidrolisis menggunakan *Candida rugosa* lipase dalam suatu sistem minyak-air yang memiliki luas permukaan yang terdefinisi. Pengaruh kondisi operasi seperti luas permukaan minyak-air dan konsentrasi awal enzim terhadap perilaku reaksi hidrolisis bertingkat diselidiki. Kinetik model yang diusulkan dengan mempertimbangkan Langmuir adsorption isotherm lipase dari fasa bulk ke interface minyak dan air dan mekanisme reaksi bertingkat orde satu yang irreversible. Model ini berhasil menggambarkan pengaruh kondisi luas permukaan dan konsentrasi enzim terhadap reaksi hidrolisis triolein secara bertingkat bukan hanya untuk produk akhir tetapi juga untuk produk antara.

Kata kunci : Lipase, perilaku reaksi, dihidrolisis dan triolein

Abstract

A large number of studies have been made on the triglyceride hydrolysis using lipase. However, the kinetics of the formation behavior of the intermediates, such as the diglyceride and monoglyceride, is still not clear. Triolein was hydrolyzed by *Candida rugosa* lipase in the biphasic oil-water system having a definite interfacial area. The effects of the operating factor, such as the oil-water interfacial area and the initial enzyme concentration, on the consecutive hydrolysis behavior were investigated. The kinetic model was proposed by considering a Langmuir adsorption isotherm of lipase in the bulk of the water phase on the oil-water interface and an irreversible pseudo first order consecutive reaction mechanism. The model well described the effects of the initial enzyme concentration and the interfacial area on the consecutive triolein hydrolysis for not only the end product but also the intermediate products.

Keywords : Lipase, consecutive reaction, hydrolysis and triolein

1. Introduction

Triglyceride, a main component of natural oil or fat, is hydrolyzed to consecutively form diacylglycerol, monoacylglycerol and glycerol as well as a fatty acid. Glycerol and fatty acid are widely used as raw materials, and monoacylglycerol is used as an emulsifying agent in the food, cosmetic and pharmaceutical industries[1]. Recently, diacylglycerol has received much attention as a healthy cooking oil because it has a biological activity that prevents the accumulation of body fat and lowers the level of cholesterol in the blood[2].

The Colgate-Emery method has been used for the industrial hydrolysis of triglyceride[3]. This method requires high temperature and pressure (563 K, 50 atm), and has the disadvantages of high energy consumption as well as thermally damaging the products[3]-[5]. Another hydrolysis method using lipase has been proposed to overcome the problems caused by high temperature and pressure. Lipase is a water-soluble enzyme, but the triglyceride is an oil-soluble substrate. The enzymatic reaction is forced to take place at the oil-water interface.

A large number of studies have been made on the triglyceride hydrolysis using

lipase. Most researchers have studied the activities of various lipases and the effects of temperature and pH on the formation rate of the end product, the fatty acid[6]-[11]. A few kinetic models have been proposed in which the effects of the droplet diameter or the oil-water interfacial area on the hydrolysis rate were incorporated for the emulsion system[12]-[14] or the biphasic system[15],[16]. However, the kinetics of the formation behavior of the intermediates, such as the diglyceride and monoglyceride, is still not clear.

In this study, triolein, as a model triglyceride, was hydrolyzed using *Candida rugosa* lipase in a biphasic oil-water system having a definite interfacial area. The effects of the operating factors, such as the initial enzyme concentration and oil-water interfacial area, on the consecutive hydrolysis behavior were investigated. The kinetic model incorporating the intermediate products was proposed by considering the Langmuir adsorption isotherm of lipase in the bulk of the water phase on the oil-water interface and an irreversible pseudo first order consecutive reaction mechanism.

2. Materials and Methods

2.1. Materials

Triolein was purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan, and its purity was 99 %. Lipase from *Candida rugosa* was purchased from Sigma Chemical Co., St. Louis, MO, USA. The enzyme catalyzes all three ester bonds of the triglyceride and has an activity of 8.87×10^5 units per gram. Here, one unit is defined as the amount of enzyme required to hydrolyze 1.0 microequivalent of fatty acid from a triglyceride in one hour. All other chemicals were purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan, and were of reagent grade.

2.2. Triolein hydrolysis

The oil phase was hexane containing triolein. The water phase was phosphate buffer (0.50×10^{-4} mol cm^{-3} , pH 6.7) containing lipase. A cylindrical glass bottle

was used as the reaction vessel. The oil and water solutions were separately preheated. The reaction was started by pouring the oil phase (100 cm^3) on the top of the water phase (25 cm^3). The vessel was shaken in a reciprocal shaker bath at 310 K. The initial concentration of triolein was held constant at 0.767×10^{-4} mol cm^{-3} -oil phase. The shaking rate, the initial enzyme concentration and the oil-water interfacial area were varied in the range of 60-80 spm, 0.02-0.16 g cm^{-3} -water phase and 36.3-66.5 cm^2 , respectively. The interfacial area was changed by using the bottles with different inner diameters. The shaking rate of 80 spm, the initial enzyme concentration of 0.04 g cm^{-3} -water phase and the interfacial area of 50.3 cm^2 were set as the control condition.

2.3. Analysis

The sample solution was withdrawn from the oil phase at specific time intervals and diluted tenfold with isopropanol. The concentrations of substrate and products in the solution were measured using an HPLC system (L-7100, Hitachi, Ltd., Tokyo, Japan) equipped with an Inertsil ODS column (particle size 5.0×10^{-4} cm, i.d. 0.46 cm, length 25 cm, GL Science, Inc., Tokyo, Japan) and a UV detector (L-7400, Hitachi, Ltd., Tokyo, Japan) at 210 nm. The temperature of the column oven was 313 K. The mobile phase composition was methanol : acetone = 100 : 0 (v/v) for 20 min and then the acetone ratio increased up to 20 % (v/v) and the value was maintained from 21 min to 35 min. The flow rate of the mobile phase was 1.0 $\text{cm}^3 \text{min}^{-1}$.

3. Kinetic Model

Figure 1 shows the conceptual model of the triolein hydrolysis by *Candida rugosa* lipase in the biphasic oil-water system. The lipase in the bulk of the water phase is adsorbed on the oil-water interface. The ester bonds of triolein are catalyzed by the adsorbed lipase to consecutively produce diolein, monoolein, and glycerol. Oleic acid is formed at each reaction stage. Only glycerol is hydrophilic and dissolved into the water phase.

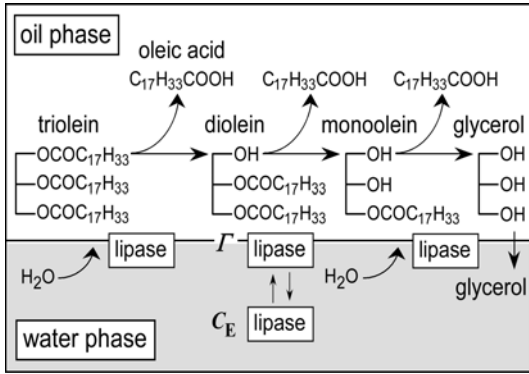
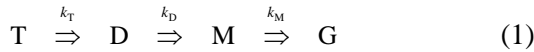


Figure 1.

Conceptual Diagram of Triolein Hydrolysis by *Candida Rugosa* Lipase in Biphasic Oil-water System.

In the kinetic model, the triolein hydrolysis is considered to proceed via an irreversible consecutive reaction mechanism[17]-[18] as



and each reaction step obeys a pseudo first order kinetics. Here, k_T , k_D and k_M are the reaction rate constants for the hydrolysis of triolein (**T**), diolein (**D**) and monoolein (**M**), respectively. Each hydrolysis rate is assumed to be proportional to the amount of lipase that existed at the oil-water interface. The time derivatives of the concentrations of the respective components are expressed as

$$\frac{dC_T}{dt} = -k_T \Gamma A C_T \quad (2)$$

$$\frac{dC_D}{dt} = k_T \Gamma A C_T - k_D \Gamma A C_D \quad (3)$$

$$\frac{dC_M}{dt} = k_D \Gamma A C_D - k_M \Gamma A C_M \quad (4)$$

where Γ is the enzyme concentration at the oil-water interface (g cm^{-2}), A is the oil-water interfacial area of the reaction vessel (cm^2) and C_i is the concentration of the component i (mol cm^{-3}). The initial condition is given as

$$t = 0, C_T = C_{T0}, C_D = 0, C_M = 0 \quad (5)$$

The Langmuir adsorption isotherm is assumed to provide the relationship for the enzyme concentrations between the liquid-

liquid interface and the bulk of the water phase as[19]-[23].

$$\Gamma = \frac{\Gamma_{\max} K_a C_E}{1 + K_a C_E} \quad (6)$$

where Γ_{\max} is the maximum enzyme concentration at the oil-water interface (g cm^{-2}), K_a is the equilibrium constant for adsorption of the enzyme ($\text{cm}^3 \text{g}^{-1}$) and C_E is the enzyme concentration in the bulk of the water phase (g cm^{-3}).

The analytical solutions of Eqs.(2)-(4) are expressed as

$$C_T = C_{T0} \exp(-k_T \Gamma A t) \quad (7)$$

$$C_D = \frac{k_T C_{T0}}{k_D - k_T} \times [\exp(-k_T \Gamma A t) - \exp(-k_D \Gamma A t)] \quad (8)$$

$$C_M = \frac{k_T k_D C_{T0}}{(k_T - k_D)(k_D - k_M)(k_M - k_T)} \times [(k_M - k_D) \exp(-k_T \Gamma A t) + (k_T - k_M) \exp(-k_D \Gamma A t) + (k_D - k_T) \exp(-k_M \Gamma A t)] \quad (9)$$

The concentration of oleic acid, C_O , can be given by the mass balance on the basis of the oleyl group as

$$C_O = (3C_{T0}) - (3C_T + 2C_D + C_M) \quad (10)$$

The mass balance for the enzyme is given as

$$A \Gamma = V_w (C_{E,0} - C_E) \quad (11)$$

Here, V_w is the volume of the water phase (cm^3) and $C_{E,0}$ is the initial enzyme concentration (g cm^{-3}). To eliminate C_E , substituting of Eq.(11) into Eq.(6) yields:

$$\Gamma = \left(b - \sqrt{b^2 - 4 \frac{A}{V_w} \Gamma_{\max} C_{E,0}} \right) / \left(2 \frac{A}{V_w} \right) \quad (12)$$

Here, b in Eq.(12) is given as

$$b = \frac{A}{V_w} \Gamma_{\max} + C_{E,0} + \frac{1}{K_a} \quad (13)$$

Thus, there are five unknown constants, k_T , k_D , k_M , Γ_{\max} and K_a in the model.

The unknown constants were estimated by fitting the analytical solution, Eqs.(7)-(10), with six sets of experimental data obtained under various conditions. A

flowchart for the calculation procedure of the constants is shown in Fig.2. Using an arbitrary set of constants, the analytical solutions were calculated at each reaction time. The best fitted values of the constants were determined using the Simplex method[24] by minimizing the squared-sum of the relative error between the calculated values and the experimental data for the concentrations of triolein, diolein and oleic acid.

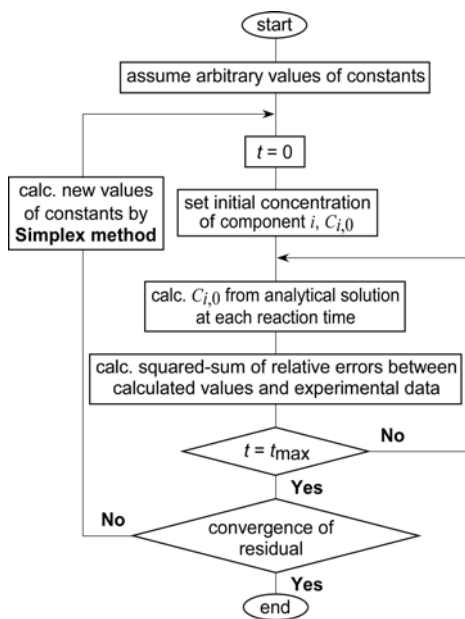


Figure 2.

Flowchart for Calculation Procedure of Model Constants.

4. Results and Discussion

Figure 3 (a) shows the time courses of the concentrations of triolein and oleic acid at the shaking rates of 60 and 80 spm while Figure 1 (b) is those of diolein. Under any condition, the triolein concentration, the square symbol, gradually decreased with the reaction time, while the oleic acid concentration, the circle symbol, increased. The conversion of triolein reached about 85 % at 150 h. The concentration of diolein, the triangle symbol, was low compared with those of triolein and oleic acid, and monoolein was not detected. Therefore, diolein and monoolein were considered to be quickly hydrolyzed. The concentrations of the respective components obtained under

the two agitated conditions almost overlapped each other. This result suggested that the effect of the mass transfer on the hydrolysis was negligible at the shaking rate of more than 60 spm.

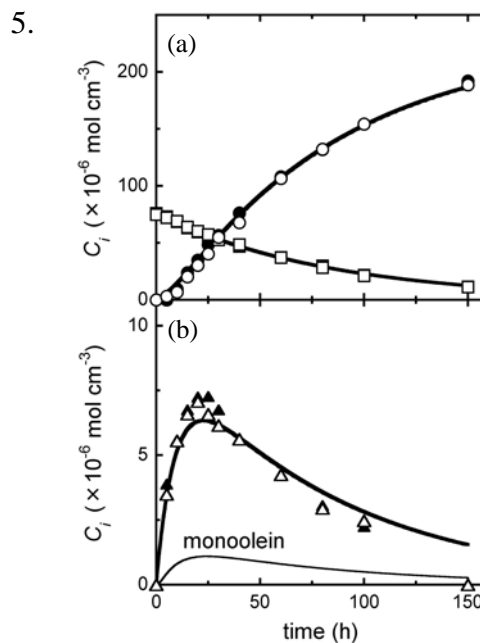


Figure 3.

Effect of Shaking Rate on Triolein Hydrolysis Behavior ($C_{E,0} = 0.04 \text{ g cm}^{-3}$ -water phase, $A = 50.3 \text{ cm}^2$). (a) Concentrations of Triolein (\square) and Oleic Acid (\circ), (b) Diolein Concentration (\triangle) at 60 (open symbols) and 80 (closed ones) spm and Calculated Results For Each Component Concentration by The Model (lines).

The effects of the initial enzyme concentration and the oil-water interfacial area on the consecutive hydrolysis are shown in Figs. 4 and 5, respectively. As the initial enzyme concentration increased from 0.02 to 0.04 g cm^{-3} -water phase in Fig.4, the consumption or formation rate of the respective species increased. However, the results for the 0.04 and 0.16 g cm^{-3} -water phase almost overlapped. Thus, the amount of lipase that existed at the oil-water interface became larger with the increasing enzyme concentration in the water phase, and then tended to become saturated. Other researchers reported similar results in the emulsion system^{8, 14}. Furthermore, as the

interfacial area increased by keeping the volume of water phase constant, the reaction rates increased as shown in Figure 5.

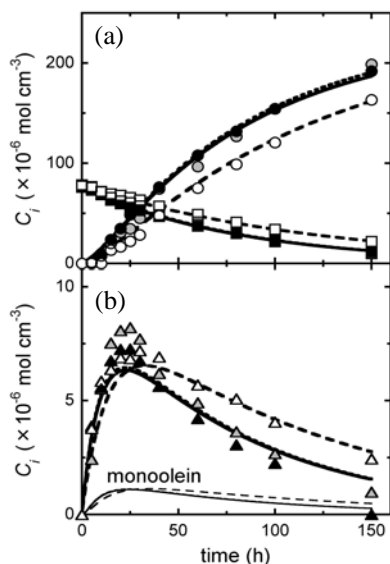


Figure 4.

Effect of Initial Concentration of Enzyme on Triolein Hydrolysis Behavior (Shaking rate = 80 spm, $A = 50.3 \text{ cm}^2$). (a) Concentrations of Triolein (\square) and Oleic Acid (\circ), (b) diolein concentration (\triangle) at 0.02 (open symbols), 0.04 (closed ones) and 0.16 (gray ones) g cm^{-3} -water Phase and Calculated Results For Each Component Concentration by the Model (lines).

The fitted results are shown in Figure 3-5 by the thick lines. Under all conditions, the calculated lines were in good agreement with the experimental results. The model well described the effects of the initial enzyme concentration and the interfacial area on the consecutive triolein hydrolysis. The monoolein concentration was simulated using the model equation, Eq.(9). The results are also shown in Figure.3-5 by the thin lines. The monoolein concentration increased for about 30 h, and then decreased similar to the diolein concentration.

The estimated values of each constant are listed in Table 1. The value of k_T was the smallest among the three reaction rate constants, and hence, the hydrolysis of triolein was very slow compared with the hydrolysis of diolein and monoolein. The value of k_M was higher than k_D , so that the monoolein was hydrolyzed faster than the diolein. The active sites of *Candida rugosa*

lipase are Ser-209, His-449 and Glu-341 in the amino acid sequence structure, and these are hydrophilic amino acid residues[25]-[27]. The hydrophilic substrate is easily accessible to the active site of the *Candida rugosa* lipase. The level of hydrophilicity for the substrate molecules depends on the number of OH groups. Thus, the values of the kinetic constants for the hydrolysis of triolein, diolein and monoolein became larger in order according to the number of OH groups. The maximum enzyme concentration at the oil-water interface, max , was estimated to be $1.47 \times 10^{-2} \text{ g cm}^{-2}$. No literature values were available for this constant. However, the equilibrium constant of the enzyme adsorption at the liquid-liquid interface, K_a , was reported to be in the range of 10^3 - $10^4 \text{ cm}^3 \text{ g}^{-1}$ [19]-[21]. The value was estimated to be $2.64 \times 10^4 \text{ cm}^3 \text{ g}^{-1}$ and was within the range of the literature values.

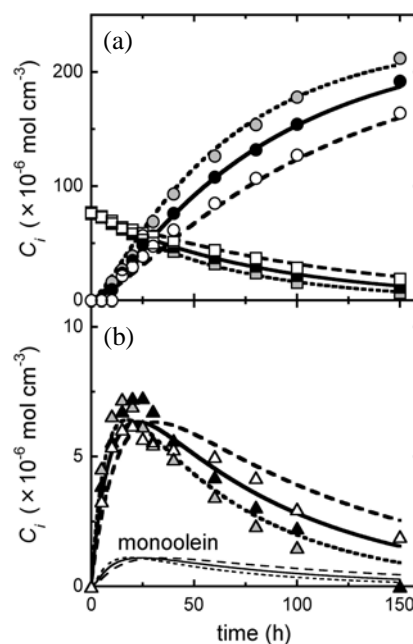


Figure 5.

Effect of Oil-water Interfacial Area on Triolein Hydrolysis Behavior (Shaking rate = 80 spm, $C_{E,0} = 0.04 \text{ g cm}^{-3}$ -water phase). (a) Concentrations of Triolein (\square) and Oleic Acid (\circ), (b) Diolein Concentration (\triangle) at 36.3 (Open symbols), 50.3 (closed ones) and 66.5 (gray ones) cm^2 and Calculated Results For Each Component Concentration by The Model (lines).

Table 1.
Estimated Values of Each Kinetic Constant in
The Model

constants	estimated value
k_T [$\text{g}^{-1} \text{min}^{-1}$]	2.71×10^{-4}
k_D [$\text{g}^{-1} \text{min}^{-1}$]	2.48×10^{-3}
k_M [$\text{g}^{-1} \text{min}^{-1}$]	1.42×10^{-2}
Γ_{\max} [g cm^{-2}]	1.47×10^{-2}
K_a [$\text{cm}^3 \text{g}^{-1}$]	2.64×10^4

6. Conclusions

The hydrolysis experiments of the triglyceride were conducted in the biphasic oil-water system under various operating factors. The concentrations of diolein and monoolein were low compared with those of triolein and oleic acid. Diolein and monoolein were quickly hydrolyzed. The effect of mass transfer on the hydrolysis behavior was negligible at the shaking rate of more than 60 rpm. Each hydrolysis rate was proportional to the amount of enzyme that existed at the oil-water interface depending on the initial enzyme concentration in the water phase and the oil-water interfacial area. The kinetic model was constructed by considering the Langmuir adsorption isotherm of lipase in the bulk of the water phase on the oil-water interface and the irreversible pseudo first order consecutive reaction mechanism. The model well described the effects of the initial enzyme concentration and the interfacial area on the consecutive triolein hydrolysis.

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