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Kinetics of lipase-catalyzed hydrolysis of olive oil in AOT/isooctane reversed micelles

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Abstract

The kinetics of lipase-catalyzed hydrolysis of olive oil in AOT/isooctane reversed micellar media was studied. It was shown that the deactivation of lipase had a great influence on the reaction kinetics. Based on whether the enzyme deactivation and influences of both product and substrate on enzyme stability were included or not, four different kinetic models were established. The simulating results demonstrated that the kinetic model, which including product inhibition, enzyme deactivation and the improvements of lipase stability by both product and substrate, fit the experimental data best with an overall relative error of 4.68%.

Keywords: Kinetics; Lipase; Hydrolysis of olive oil; AOT; Reversed micelles

1. Introduction

The use of lipase for production of fatty acids from hydrolysis of triacylglyceride has been attempted as an energy-saving method [1]. The *Candida rugosa* lipase is most frequently used because of its random specificity and fairly low price. The lipase, as a kind of surface-active enzyme, can reveal its activity only at the interface. The reversed micellar system provides a large interface. So, the lipase-catalyzed hydrolysis, synthesis and transesterification of glyceride in reversed micelles were fully investigated in the past decades [2–11].

An important problem for application of the lipase in reversed micelles is the deactivation of the enzyme [3,9–11]. Our previous work [12] showed that the activity of lipase in AOT/isooctane reversed micellar media decreased rapidly to about 15% of the initial value after 4 h [12]. A first-order deactivation mechanism from active lipase (E_a) to partially inactive lipase (E_i) was proposed. The model equation as below describing the residual activity with time was found to fit the experimental data well:

$$R_a = \alpha + (1 - \alpha)\exp(-k_d t) \quad (1)$$

where α is the activity ratio of E_i to E_a and k_d is the rate constant of enzyme deactivation. The existence of oleic acid, the product of olive oil hydrolysis, can improve the stability of lipase in AOT reversed micelles. It was shown that the apparent rate constant of lipase deactivation while oleic acid existed was decreased by a factor of $1/(1 + [P]/K_P)$ [12], in which K_P is the dissociation constant of lipase-product complex. The deactivation of enzyme greatly influences the kinetics of reaction in reversed micelles. So, the kinetics of deactivation should be included while studying the reaction kinetics catalyzed by enzyme in reversed micellar systems.

In this study, four different kinetic models of lipase-catalyzed hydrolysis of olive oil were established and used to simulate the experimental data. It was found the kinetic

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model which including product inhibition, enzyme deactivation and influences of both product and substrate on lipase stability was in most coincidence with the experimental data.

2. Theory

2.1. Kinetic model of competitive product inhibition (Model I)

The lipase in AOT/isooctane reversed micelles is inhibited by oleic acid [9,10], a product of olive oil hydrolysis. It is assumed that the reaction mechanisms are as below:

$$E + S \rightleftharpoons ES \xrightarrow{k_{cat}} E + P \tag{2a}$$

$$E + P \overset{K_P}{\rightleftharpoons} EP \tag{2b}$$

Then the reaction rate can be derived as:

$$v = -\frac{d[S]}{dt} = \frac{k_{cat}[E]_0[S]}{K_m\left(1 + \frac{[P]}{K_P}\right) + [S]} \tag{3}$$

Integrating Eq. (3) and rearranging one can obtain the relationship of x (conversion) versus t:

$$k_{cat}[E]_0 t = [S]_0 x - K_m\left\{\ln(1-x) + \frac{[\ln(1-x) + x][S]_0}{K_P}\right\} \tag{4}$$

The catalytic constant (k_{cat}), Michaelis constant (K_m) and dissociation constant of EP (K_P) had been determined [12] to be 0.366 mol h^{-1} g^{-1}, 0.19 and 0.015 mol L^{-1} separately at pH 7.0, 30 °C and W_0 of 8.0. So, the conversion at any time can be calculated with Eq. (4).

2.2. Kinetic model including product inhibition and enzyme deactivation (Model II)

In Model I, the deactivation of lipase was omitted. Our previous study [12] had shown that lipase deactivation in AOT/isooctane reversed micelles was serious. Based on the relationship of R_a versus t, i.e. Eq. (1), if all the active enzyme (containing free lipase E_a, lipase-product complex E_aP and lipase-substrate complex E_aS) decay at the same rate, the reaction rate including enzyme deactivation is:

$$v = \frac{k_{cat}[E]_0[\alpha + (1-\alpha)\exp(-k_d t)][S]}{K_m\left(1 + \frac{[P]}{K_P}\right) + [S]} \tag{5}$$

Rearranging Eq. (5) one can get the relationship of x versus t:

$$\frac{dx}{dt} = \frac{k_{cat}[E]_0(1-x)[\alpha + (1-\alpha)\exp(-k_d t)]}{K_m\left(1 + \frac{[S]_0 x}{K_P}\right) + [S]_0(1-x)} \tag{6a}$$

The initial value of conversion is:

$$t = 0, \quad x = 0 \tag{6b}$$

The Eq. (6) can be numerically solved with the fourth-order Runge–Kutta method.

2.3. Kinetic model including product inhibition, enzyme deactivation and influence of product on lipase stability (Model III)

The product, oleic acid, can improve the stability of lipase in reversed micelles [9,12]. It is assumed the active lipase-oleic acid complex does not decay. Certainly, the substrate may also influence lipase stability. In other words, the active lipase-substrate complex may decay at a different rate comparing with free lipase. But this is difficult to verify individually. Here, we assume E_aS decays at the same rate with E_a. For simplification, it is assumed that the partially inactive lipase is also competitively inhibited by the product, and the dissociation constant of E_iP is identical with that of E_aP. Then the reaction mechanisms are:

$$E_a + S \rightleftharpoons E_aS \xrightarrow{k_{cat}} E_a + P \tag{7a}$$

$$E_a + P \overset{K_P}{\rightleftharpoons} E_aP \tag{7b}$$

$$E_i + S \rightleftharpoons E_iS \xrightarrow{\alpha k_{cat}} E_i + P \tag{7c}$$

$$E_i + P \overset{K_P}{\rightleftharpoons} E_iP \tag{7d}$$

$$E_a \xrightarrow{k_d} E_i \tag{7e}$$

$$E_aS \xrightarrow{k_d} E_iS \tag{7f}$$

The concentration of all the active lipase is:

$$[E_{a,tot}] = [E_a] + [E_aS] + [E_aP] \tag{8}$$

where the concentrations of E_aS and E_aP are given by:

$$[E_aS] = \frac{[E_a][S]}{K_m} \tag{9}$$

$$[E_aP] = \frac{[E_a][P]}{K_P} \tag{10}$$

Then the deactivation rate of the active lipase is:

$$-\frac{d[E_{a,tot}]}{dt} = k_d([E_a] + [E_aS]) \tag{11}$$

Combining Eqs. (8)–(11), one can obtain:

$$-\frac{d[E_{a,tot}]}{dt} = \frac{k_d[E_{a,tot}]\left(1 + \frac{[S]}{K_m}\right)}{1 + \frac{[S]}{K_m} + \frac{[P]}{K_P}} \tag{12}$$

The concentration of partially inactive lipase is:

$$[E_{i,tot}] = [E]_0 - [E_{a,tot}] \tag{13}$$

The reaction rate contains the contribution of active lipase and partially inactive lipase:

$$v = \frac{k_{cat}[E_{a,tot}][S]}{K_m\left(1+\frac{[P]}{K_P}\right)+[S]} + \frac{\alpha k_{cat}[E_{i,tot}][S]}{K_m\left(1+\frac{[P]}{K_P}\right)+[S]}$$
$$= \frac{\{\alpha[E]_0 + (1-\alpha)[E_{a,tot}]\}\, k_{cat}[S]}{K_m\left(1+\frac{[P]}{K_P}\right)+[S]} \quad (14)$$

Rearraging Eqs. (12) and (14), one can obtain original differential equations describing the relationships of $[E_{a,tot}]$ and x with t as below:

$$\frac{d[E_{a,tot}]}{dt} = \frac{k_d[E_{a,tot}]\left\{1+\frac{[S]_0(1-x)}{K_m}\right\}}{1+\frac{[S]_0(1-x)}{K_m}+\frac{[S]_0 x}{K_P}} \quad (15a)$$

$$\frac{dx}{dt} = \frac{k_{cat}(1-x)\{\alpha[E]_0+(1-\alpha)[E_{a,tot}]\}}{K_m\left(1+\frac{[S]_0 x}{K_P}\right)+[S]_0(1-x)} \quad (15b)$$

The initial conditions are:

$$t = 0, [E_{a,tot}] = [E]_0 \quad (15c)$$

$$t = 0, \quad x = 0 \quad (15d)$$

2.4. Kinetic model including product inhibition, enzyme deactivation and influences of both product and substrate on lipase stability (Model IV)

While assuming the substrate, just like the product, can also improve the stability of lipase, in other words, the active lipase-substrate complex does not decay, the reaction mechanisms are:

$$E_a + S \rightleftharpoons E_aS \xrightarrow{k_{cat}} E_a + P \quad (16a)$$

$$E_a + P \overset{K_P}{\rightleftharpoons} E_aP \quad (16b)$$

$$E_i + S \rightleftharpoons E_iS \xrightarrow{\alpha k_{cat}} E_i + P \quad (16c)$$

$$E_i + P \overset{K_P}{\rightleftharpoons} E_iP \quad (16d)$$

$$E_a \xrightarrow{k_d} E_i \quad (16e)$$

Similarly to that in Section 2.3, one can obtain the original differential equations as below:

$$\frac{d[E_{a,tot}]}{dt} = \frac{k_d[E_{a,tot}]}{1+\frac{[S]_0(1-x)}{K_m}+\frac{[S]_0 x}{K_P}} \quad (17a)$$

$$\frac{dx}{dt} = \frac{k_{cat}(1-x)\{\alpha[E]_0+(1-\alpha)[E_{a,tot}]\}}{K_m\left(1+\frac{[S]_0 x}{K_P}\right)+[S]_0(1-x)} \quad (17b)$$

The initial conditions are the same as Eqs. (15c) and (15d).

3. Experimental

3.1. Materials

Candida rugosa lipase (Type VII, EC 3.1.1.3) purchased from Sigma was used without further purification for the experiments. The specific activity of lipase was marked as 880 U/mg solid (one unit of enzyme activity (U) was defined as the amount of lipase that liberated 10^{-6} mol fatty acids per hour in aqueous solution at pH 7.7, 37 °C). Bis(2-ethylhexyl) sodium sulfosuccinate (AOT) and highly refined olive oil were obtained from Sigma. All other chemicals were of analytical reagent grade.

3.2. Hydrolysis reaction

A stock solution of lipase of 6 g L^{-1} in 0.05 mol L^{-1} phosphate buffer solution (pH 7.0) was prepared daily and was stored before use at 4 °C in the refrigerator.

Bottles of 100 mL were filled with 25 mL AOT/isooctane reversed micellar solution containing 0.05 mol L^{-1} AOT and substrate at various concentrations in the range of 0.175–0.695 mol L^{-1} based on the ester bond of olive oil [12]. The hydrolysis reaction was initiated by adding the desired volume of lipase solution (the concentration of lipase was maintained at 0.0432 g solid per liter for all the experiments in this study) and then vortex mixing until the solution became clear (ca. 10 s). Reactions were carried out at 30 °C in a shaking water bath at 150 rpm. At predetermined time intervals, aliquots of the reaction mixture were withdrawn and the liberated free acid was determined by the Lowry method [13]. The value of W_0 was fixed at 8.0 for all experiments. It was adjusted through adding the required amount of buffer solution without enzyme.

4. Results and discussion

Fig. 1 showed time dependence of conversion at the initial substrate concentration of 0.348 mol L^{-1}. The reaction rate was moderate while $t < 10$ h, but thereafter gradually decreased with time. With the kinetic parameters of $k_{cat} = 0.366$ mol h^{-1} g^{-1}, $K_m = 0.19$ mol L^{-1}, $k_d = 0.75$ h^{-1}, $\alpha = 0.15$ and $K_P = 0.015$ mol L^{-1} [12], the theoretical curves calculated by model equations were also shown in Fig. 1. The Eqs. (6), (15) and (17) were all numerically solved by the fourth-order Runge–Kutta method. While $t < 5$ h, the results predicted by Model I (curve 1) fit with the experimental data well. But the deviation increased greatly with time when $t > 5$ h. This was in coincidence with the results of Chiang and co-workers [9]. One of the main reasons for the deviation was the deactivation of lipase [12]. But as shown in Fig. 1, while the lipase deactivation was included in the kinetic model, the conversions calculated by Model II were lower than the experimental data. So, the operational stability of lipase in actual reaction mixture was higher than free lipase

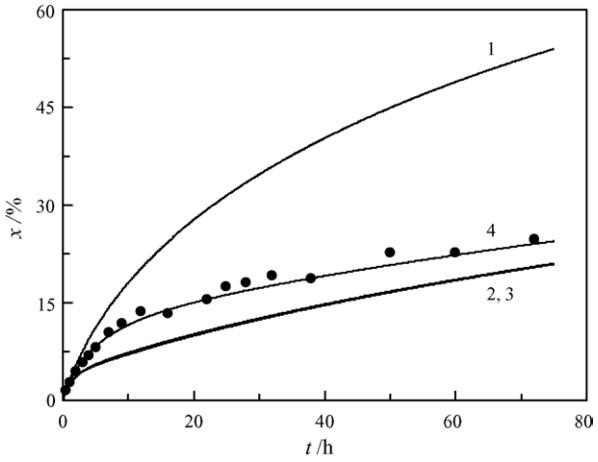

Fig. 1. Time dependence of conversion of olive oil hydrolysis catalyzed by lipase in AOT/isooctane reversed micelles at 30 °C, pH 7.0, $W_0 = 8.0$, taking initial substrate concentration of 0.348 mol L^{-1} as example. Solid lines are calculated with Model I (curve 1), Model II (curve 2), Model III (curve 3) and Model IV (curve 4). Dots (●) are experimental data.

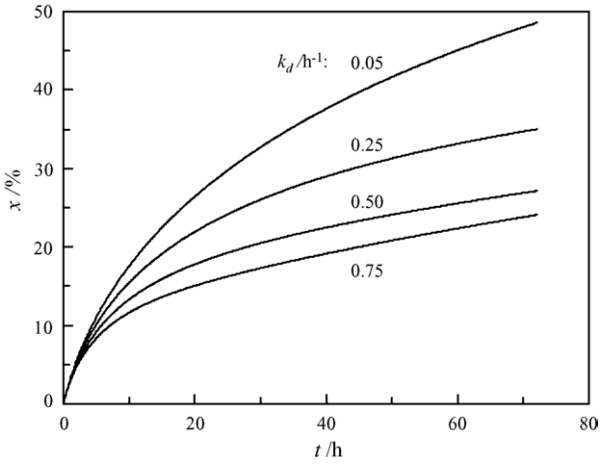

Fig. 3. Time courses of lipase-catalyzed hydrolysis of olive oil in AOT/isooctane reversed micelles predicted by Model IV with different values of k_d. Other kinetic parameters are the same as in Fig. 1.

in reversed micelles. As we know, the product, oleic acid, can improve the stability of lipase [12]. In Model III, the improvement of lipase stability by product was included. But the conversion at any time calculated by Model III (curve 3) was nearly the same with that calculated by Model II. As shown in Fig. 1, curve 3 was nearly superposed to curve 2. This could be explained by the low product concentration at the beginning of the reaction ($t < 10$ h), just in this period, the lipase decayed rapidly. So, the enhancement of product to lipase stability did not significantly influence the time course of olive oil hydrolysis. While the influence of substrate was also included, Model IV gave a theoretical curve (curve 4), which fit well with the experimental data for the whole stage of reaction. So, the existence of substrate can improve lipase stability too, and the improvement mechanism can be explained by that the lipase-substrate complex does not decay. It should be noted that the enhancement of substrate to lipase stability influences the reaction more significantly than that of product for its higher concentration at the beginning of reaction.

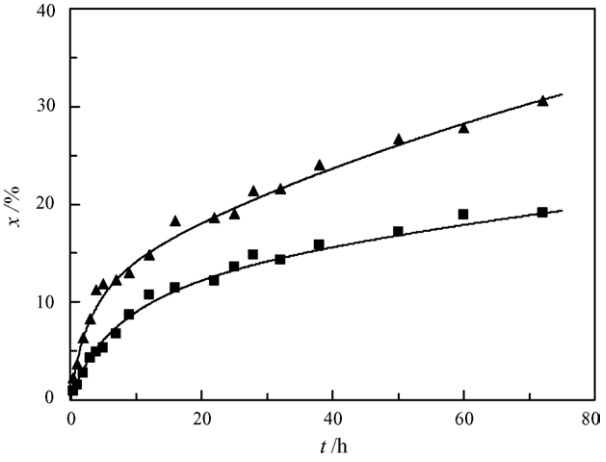

Fig. 2. Time courses of lipase-catalyzed hydrolysis of olive oil in AOT/isooctane reversed micelles with initial substrate concentrations of 0.175 mol L^{-1} (▲) and 0.695 mol L^{-1} (■) at the same conditions as in Fig. 1. Solid lines are calculated with Model IV.

Fig. 2 showed time courses of olive oil hydrolysis at different initial substrate concentrations. The theoretical curves calculated by Model IV were in good agreement with the experimental data with an overall relative error of 4.68%.

With conversions calculated by Eq. (17) plots of conversion versus t at different k_d were shown in Fig. 3. The conversion at any time increased with the decrease of k_d. Comparing with that at $k_d = 0.75\ h^{-1}$, decreasing k_d to $0.05\ h^{-1}$ will lead to a two-fold increase in conversion at 72 h. So, the deactivation of enzyme is a momentous obstacle that limiting the application of enzymatic reactions in reversed micelles. How to improve enzyme stability, such as through developing new reversed micellar systems and/or optimization of reaction conditions, should be fully investigated in the near future.

5. Conclusions

The deactivation of lipase greatly influences the kinetics of olive oil hydrolysis in AOT/isooctane reversed micelles. The product, oleic acid and the substrate can both improve the lipase stability in reaction mixture. The improvement can be explained by the mechanisms of both enzyme-product and enzyme-substrate complexes do not decay. The kinetic model, which including product inhibition, enzyme deactivation and influences of both product and substrate on enzyme stability, fits the experimental data well with an overall relative error of 4.68%.

References

[1] Z.D. Knezevic, S.S. Siler-Marinkovic, L.V. Mojovic, Appl. Microbiol. Biotechnol. 49 (1998) 267.
[2] C.M.L. Carvalho, J.M.S. Cabral, Biochimie 82 (2000) 1063.
[3] F. Jing, X. An, W. Shen, J. Mol. Catal. B: Enzym. 24/25 (2003) 53.
[4] K.S. Freeman, T.T. Tang, R.D.E. Shah, D.J. Kiserow, L.B. McGown, J. Phys. Chem. B 104 (2000) 9312.
[5] G.W. Zhou, G.Z. Li, J. Xu, Q. Sheng, Colloid Surf. A-Physicochem. Eng. Asp. 194 (2001) 41.
[6] S. Fadiloglu, Z. Soylemez, J. Agric. Food Chem. 46 (1998) 3411.
[7] N. Krieger, M.A. Taipa, E.H.M. Melo, J.L. Lima, M.R. Aires-Barros, J.M.S. Cabral, Appl. Biochem. Biotechnol. 67 (1997) 87.
[8] M. Svensson, G.D. Rees, B.H. Robinson, G.R. Stephenson, Colloid Surf. B-Biointerfaces 8 (1996) 101.
[9] S.W. Tsai, C.L. Chiang, Biotechnol. Bioeng. 38 (1991) 206.
[10] D.G. Hayes, E. Gulari, Biotechnol. Bioeng. 35 (1990) 793.
[11] F.C. Huang, Y.H. Ju, Appl. Biochem. Biotechnol. 50 (1995) 323.
[12] C. Yao, S. Tang, J. Zhang, Y. Yu, J. Mol. Catal. B: Enzym. 18 (2002) 279.
[13] D. Han, J.S. Rhee, Biotechnol. Bioeng. 28 (1986) 1250.