ELSEVIER

Journal of Molecular Catalysis B: Enzymatic 18 (2002) 279–284

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

www.elsevier.com/locate/molcatb

Kinetics of lipase deactivation in AOT/isooctane reversed micelles

Chuanyi Yao [a,*], Shaokun Tang [b], Jinhong Zhang [c], Yaoting Yu [c]

[a] *Unimicro (Shanghai) Technologies, Co. Ltd., C01 489# Songtao Road, Zhangjiang High-Tech Park, Shanghai 201203, China*
[b] *School of Chemical Engineering and Technologies, Tianjin University, Tianjin 300072, China*
[c] *Institute for Molecular Biology, Nankai University, Tianjin 300071, China*

Received 7 January 2002; received in revised form 30 April 2002; accepted 30 April 2002

Abstract

The stability of lipase in AOT/isooctane reversed micellar solution was investigated. It was found that the lipase deactivated to a stable state that was not completely inactivated. The lipase residual activity after achieving the stable state in AOT/isooctane reversed micelles at 30 °C, pH 7.0, $W_0 = 8.0$ was found to be 0.15, and the first-order deactivation rate coefficient of lipase at the same conditions was regressed to be 0.75 h^{-1}. The stability of lipase was increased while oleic acid was added. Assuming the protection of oleic acid to lipase stability is due to the lipase–oleic acid complex does not decay, the kinetic model of lipase deactivation in AOT/isooctane reversed micellar solution including the influence of oleic acid was established. It was shown with the model equation that the increase in stability of the enzyme by oleic acid could be quantitatively estimated by the dissociation constant of lipase–oleic acid complex which was determined by product inhibition experiments. The model equation fit the experimental data well with an average relative deviation of 3.40%.

Keywords: Kinetics; Lipase; Deactivation; Reversed micelles; Oleic acid

1. Introduction

Enzymatic reactions in reversed micelles have received much more attention for its combining the advantages of aqueous- and organic-phase enzyme systems [1]. Especially the lipase-catalyzed hydrolysis, synthesis and transesterification of glyceride were fully investigated in the past decades. The lipase is a kind of surface-active enzyme, so it can reveal its activity only at the interface. The reversed micellar system provides a large interface. Tsai and Chiang [2] studied the kinetics of lipase-catalyzed hydrolysis of olive oil in the reversed micelles. A kinetic model of competitive inhibition by the product, oleic acid, was proposed and used to simulate the experimental data. They found that the model was in agreement with experimental data only for lower substrate concentrations. The deviation increased with time while the initial substrate concentration was >0.341 M. One of the main reasons for the deviation is the deactivation of lipase. Tsai and Chiang [2] also found that the existence of oleic acid would increase the stability of lipase in reversed micelles. Similar behaviors have also been found by Hayes and Gulari [3] and Huang and Ju [4]. So the effect of lipase deactivation must be included when the dynamic of lipase-catalyzed reactions in reversed micelles is studied.

In this paper, the kinetics of lipase deactivation in AOT/isooctane reversed micellar media was

* Corresponding author. Tel.: +86-21-38953588; fax: +86-21-38953636.
E-mail address: yaochuanyi@hotmail.com (C. Yao).

1381-1177/02/$ – see front matter © 2002 Elsevier Science B.V. All rights reserved.
PII: S1381-1177(02)00107-8

investigated. A mathematical model was established including the influence of oleic acid.

2. Theory

2.1. Kinetics of lipase deactivation in reversed micelles

It was found from the experimental data that the lipase decayed rapidly with time for a period of time. When its activity decreased to a certain value, lipase would not further decay obviously with time. It is assumed the lipase follows the single-step deactivation model as below:

$$E_a \xrightarrow[\alpha]{k_d} E_i \tag{1}$$

where E_a is active lipase, E_i the partially inactive lipase, α the activity ratio of E_i to E_a, k_d the rate constant of lipase deactivation. One can obtain the residual activity of lipase in reversed micelles as [5]:

$$\mathrm{Ra} = \alpha + (1-\alpha)\exp(-k_d t) \tag{2}$$

By rearranging and then taking the natural logarithm of Eq. (2), one can show that

$$\ln(\mathrm{Ra}-\alpha) = \ln(1-\alpha) - k_d t \tag{3}$$

A plot of $\ln(\mathrm{Ra}-\alpha)$ versus t yields a straight line which has a slope of $-k_d$.

2.2. Effect of oleic acid to kinetics of lipase deactivation in reversed micelles

The existence of oleic acid (P) can increase the stability of lipase in reversed micellar media [2]. Assuming that E_aP complex does not decay, the deactivation mechanism of lipase in reversed micelles will be

$$E_a \xrightarrow[\alpha]{k_d} E_i \tag{4}$$

$$E_a + P \overset{K_P}{\rightleftharpoons} E_a P \tag{5}$$

where K_P is the dissociation constant of E_aP, which is given by

$$K_P = \frac{[E_a][P]}{[E_a P]} \tag{6}$$

The total active lipase ($E_{a,tot}$) contains E_a and E_aP:

$$[E_{a,tot}] = [E_a] + [E_a P] \tag{7}$$

Combining Eqs. (6) and (7), one can obtain

$$[E_a] = \frac{[E_{a,tot}]}{1 + ([P]/K_P)} \tag{8}$$

The rate of active lipase deactivation depends on the concentration of E_a:

$$\frac{\mathrm{d}[E_{a,tot}]}{\mathrm{d}t} = -k_d[E_a] \tag{9}$$

The concentration of E_a as given in Eq. (8) may now be substituted into Eq. (9). Then one can obtain

$$\frac{\mathrm{d}[E_{a,tot}]}{\mathrm{d}t} = -K_d[E_{a,tot}] \tag{10}$$

where K_d is defined as

$$K_d = \frac{k_d}{1 + ([P]/K_P)} \tag{11}$$

The concentration of $E_{a,tot}$ is given by integrating Eq. (10):

$$[E_{a,tot}] = E_0 \exp(-K_d t) \tag{12}$$

Then the concentration of partially inactive lipase is given by

$$[E_i] = E_0[1 - \exp(-K_d t)] \tag{13}$$

The residual activity of lipase is

$$\mathrm{Ra} = \frac{k_{cat}[E_{a,tot}] + \alpha k_{cat}[E_i]}{k_{cat} E_0} \tag{14}$$

Substituting $[E_{a,tot}]$ in Eq. (12) and $[E_i]$ in Eq. (13) into Eq. (14), one can obtain the residual activity of lipase in reversed micelles with the influence of oleic acid as

$$\mathrm{Ra} = \alpha + (1-\alpha)\exp(-K_d t) \tag{15}$$

Rearranging Eq. (15) and then taking the natural logarithm one can show that

$$\ln(\mathrm{Ra}-\alpha) = \ln(1-\alpha) - K_d t \tag{16}$$

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. 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. Preparation of reversed micellar solution

A stock solution of lipase of 6 mg/ml in 50 mM phosphate buffer solution (pH 7.0) was prepared daily and was stored before use at 4 °C in the refrigerator. An appropriate amount of aqueous buffer solution containing lipase was injected into a solution of 50 mM AOT in isooctane solution.

Stirring was carried out in vortex mixer until the solution became clear (ca. 10 s). The value of W_0, defined as the molar ratio of water to surfactant, was fixed at 8.0 for all experiments. It was adjusted through adding the required amount of buffer solution without enzyme.

3.3. Determination of lipase activity

Lipase activity was determined by using olive oil as the substrate. A screw-cap culture tube was filled with 5 ml of AOT/isooctane solution containing the substrate of concentration of 0.14 M based on the ester bond of olive oil defined below [2]:

$$S_0(\mathrm{M}) = \frac{191.5 S_0(\mathrm{g\,ml^{-1}})}{56.1} \quad (17)$$

After thermostatting the tube at 30 °C for 20 min in a water bath, the hydrolysis reaction was initiated by adding the desired amount of buffer solution containing lipase into the tube, followed by vortex mixing. The solution was then kept at 30 °C without stirring. The liberated free acids were determined by the Lowry method [6]. One unit of enzyme activity (U) was defined as the amount of lipase that liberated 1 μmol fatty acids per minute under the assay conditions.

3.4. Determination of lipase stability with or without oleic acid

The reversed micellar solutions with oleic acid concentration ranging from 0 to 50 mM at $W_0 = 8.0$ and pH 7.0 was prepared and kept in 30 °C water bath for 20 min. Then a certain amount of lipase solution was added. Aliquots were taken at predetermined time intervals to determine the lipase activity. The residual activity is the ratio of lipase activity at any time to initial activity ($t = 0$).

4. Results and discussion

4.1. Stability of lipase in AOT/isooctane reversed micelles

The reversed micellar solution containing lipase was incubated at 30 °C, pH 7.0 and $W_0 = 8.0$. Aliquots were taken at predetermined time intervals to measure the residual activities. The results were shown in Fig. 1. The activity of lipase in AOT/isooctane reversed micelles decreased rapidly to about 15% of the initial value after 4 h and did not further decay obviously with time. The residual activity after 20 h was regarded as α ($\alpha = 0.15$). The plot of $\ln(\mathrm{Ra} - \alpha)$ versus t yielded a straight line (inner figure in Fig. 1) with a very high correlation coefficient of 0.996. From the

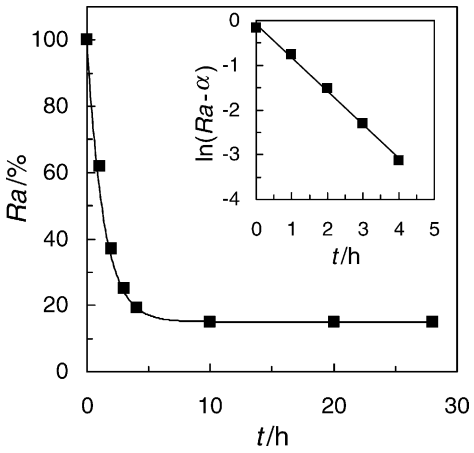

Fig. 1. Stability of lipase in AOT/isooctane reversed micellar solution at 30 °C, pH 7.0, $W_0 = 8.0$. (■) Experimental data, (—) calculated by using Eq. (2).

slope of the straight line the deactivation rate coefficient was found to be 0.75 h^{-1}. Then the values of k_d and α were substituted into Eq. (2). The calculated results from Eq. (2) were presented in Fig. 1 (the solid line). The experimental data were in good agreement with those obtained from Eq. (2) with an average relative deviation of 3.14%.

4.2. Product inhibition

Oleic acid is a product of the hydrolysis of olive oil. Eq. (5) indicates the lipase to be competitively inhibited by the product of oleic acid. This had been verified by former studies [2,3]. That means the value of K_P could be determined by the traditional method. For this, with initial concentrations of oleic acid ranging from 0 to 50 mM, the initial reaction rates (V_0) of lipase-catalyzed hydrolysis of olive oil were measured at substrate concentrations ranging from 0.14 to 0.7 M. The Lineweaver–Burk plots were shown in Fig. 2. The vertical intercepts of straight lines of $1/V_0$ versus $1/S_0$ with concentration of oleic acid as parameter were nearly identical. This further validated the lipase to be competitively inhibited by oleic acid. From Fig. 2, the turnover number (k_{cat}) was obtained to be 0.366 mol h^{-1} g^{-1}. Plotting the apparent Michaelis constants (K_m) obtained from Fig. 2 versus [P] yielded a straight line (Fig. 3). The value of K_P was obtained to be 0.015 M.

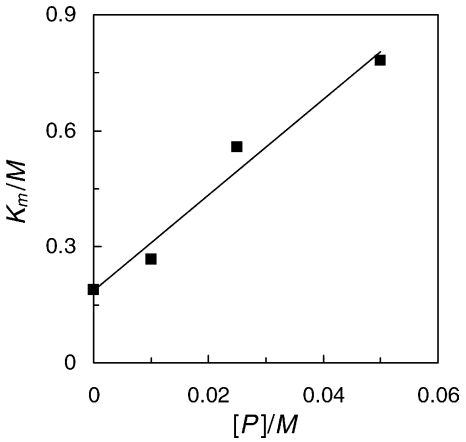

Fig. 3. The apparent Michalis constants of lipase in AOT/isooctane reversed micelles at 30 °C, pH 7.0, $W_0 = 8.0$ while with different concentrations of oleic acid.

4.3. Kinetics of lipase deactivation with oleic acid

The reversed micellar solution, containing lipase and different concentrations of oleic acid, was incubated at 30 °C. The residual activities were measured at predetermined time. The results shown in Fig. 4 indicated the stability of lipase was increased when oleic acid was added. This was in agreement with the results obtained by Tsai and Chiang [2] and Hayes and Gulari [3]. According to Tsai and Chiang [2], oleic acid

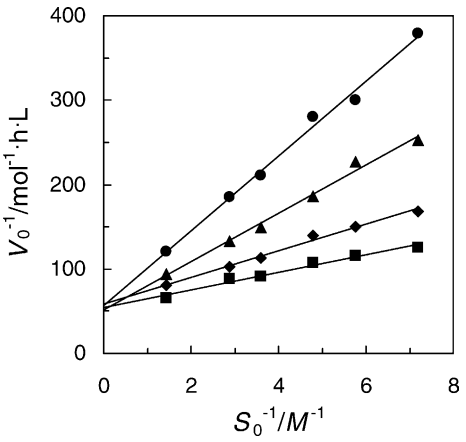

Fig. 2. Lineweaver–Burk plot of initial velocity for olive oil hydrolysis in AOT/isooctane reversed micelles at 30 °C, pH 7.0, $W = 8.0$ with concentration of oleic acid as parameter. [P] (M): (■) 0; (◆) 0.01; (▲) 0.025; (●) 0.05.

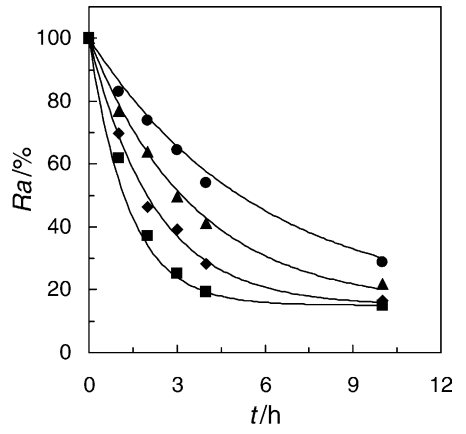

Fig. 4. The influence of oleic acid to the stability of lipase in AOT/isooctane reversed micelles at 30 °C, pH 7.0, $W_0 = 8.0$. (■, ◆, ▲, ●) Experimental data, (—) calculated by using Eq. (15). [P] (M): (■) 0; (◆) 0.01; (▲) 0.025; (●) 0.05.

competes with substrate for the same binding site of the enzyme. When oleic acid occupies the active site of the enzyme, it can be regarded as a protecting agent for the enzyme, which slows down the denaturation rate of the lipase.

Substituting the values of k_d, α and K_P obtained above into Eq. (15), one can calculated the residual activities at any time. The calculated results were also shown in Fig. 4 with solid lines. The results calculated by the model equation were in good agreement with the experimental data with an average relative deviation of 3.40%. This verifies that the model proposed above is reasonable.

In addition, the experimental data shown in Fig. 4 can be used to get the values of k_d and K_P in reverse order. According to Eq. (16), plotting $\ln(Ra-\alpha)$ versus t at each concentration of oleic acid yielded a straight line with a slope of $-K_d$ (Fig. 5). Taking the reciprocal of Eq. (11), one can obtain

$$\frac{1}{K_d} = \frac{1}{k_d} + \frac{[P]}{K_P k_d} \tag{18}$$

With the values of K_d at different $[P]$, plotting $1/K_d$ vs. $[P]$ yielded a straight line which had a vertical intercept of $1/k_d$ and a slope of $(1/K_P)/k_d$ (Fig. 6). Then the values of k_d and K_P were obtained to be $0.63\,h^{-1}$ and 0.019 M. These values were very close to what obtained above. This further validated the model proposed in this paper.

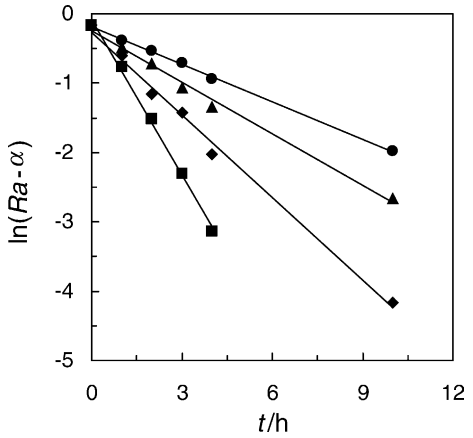

Fig. 5. Relationship of $\ln(Ra-\alpha)$ vs. t of lipase in AOT/isooctane reversed micelles with different concentrations of oleic acid. $[P]$ (M): (■) 0; (◆) 0.01; (▲) 0.025; (●) 0.05.

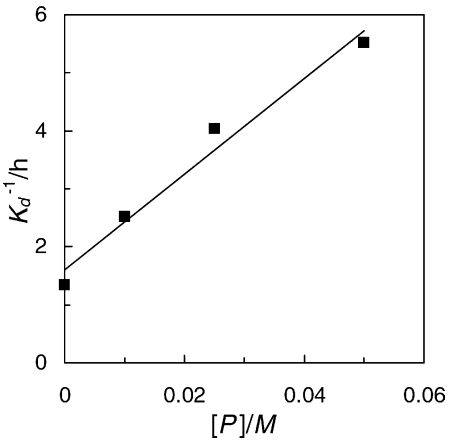

Fig. 6. Relationship of $1/K_d$ vs. $[P]$ (M).

4.4. Discussion

Melo et al. [7] investigated the deactivation of recombinat cutinase in AOT/isooctane reversed micelles, a series deactivation scheme [8] as below was used to simulate the experimental data:

$$\underset{\alpha_1}{E} \xrightarrow{k_1} \underset{\alpha_2}{E_1} \xrightarrow{k_2} E_2 \tag{19}$$

where E is the active enzyme, E_1 and E_2 the two forms of inactive enzyme, α_1 and α_2 the activity ratios of E_1 and E_2 to E. Obviously, while k_2 is equal to 0, Eq. (19) will be the same as Eq. (1). Melo et al. [7] simulated the activity of recombinat cutinase in AOT/isooctane reversed micelles using this model and found the regressive values of k_2 at 30 °C, $W_0 = 10$ and 20 were both equal to 0.

In this paper, it is assumed that the protection of oleic acid to enzyme stability is due to the enzyme–oleic acid complex does not decay. The model of lipase deactivation in reversed micellar solution with oleic acid is established. Comparing Eq. (2) (without oleic acid) and Eq. (15) (with oleic acid), one can show that the deactivation rate coefficient (k_d) is replaced by K_d while oleic acid exists. That means the presence of oleic acid causes k_d to be multiplied by the factor $1/(1+([P]/K_P))$. Obviously, K_d decreases and the stability of lipase increases with the increase of $[P]$. So the occupation of active site of lipase by oleic acid causes not only product inhibition but also the increase of enzyme stability.

5. Conclusions

The kinetic models of lipase deactivation in reversed micellar solution with and without oleic acid are established. They are in good agreement with the experimental data. The presence of oleic acid causes the deactivation rate coefficient to be multiplied by the factor $1/(1+([P]/K_P))$. The occupation of active site of lipase by oleic acid leads to product inhibition of olive oil-hydrolysis and also the increase of lipase stability. The values of deactivation rate coefficient and dissociation constant for the complex E_aP obtained through two different ways are in good agreement with each other. This validated the kinetic models of lipase deactivation in reversed micelles proposed in this paper. It was expected that one should be able to simulate the time course of lipase-catalyzed hydrolysis of olive oil in reversed micelles with the enzyme deactivation kinetics. This work is still in progress.

References

[1] M.P. Pileni, J. Phys. Chem. 97 (1993) 6961–6973.
[2] S.W. Tsai, C.L. Chiang, Biotechnol. Bioeng. 38 (1991) 206–211.
[3] D.G. Hayes, E. Gulari, Biotechnol. Bioeng. 35 (1990) 793–801.
[4] F.C. Huang, Y.H. Ju, Appl. Biochem. Biotechnol. 50 (1995) 323–331.
[5] A. Sadana, Biocatalysis: Fundamentals of Enzyme Deactivation Kinetics, Prentice-Hall, NJ, 1991, pp. 68–131.
[6] D. Han, J.S. Rhee, Biotechnol. Bioeng. 28 (1986) 1250–1255.
[7] E.P. Melo, M.R. Aires-Barros, J.M.S. Cabral, Appl. Biochem. Biotechnol. 50 (1995) 45–50.
[8] J.P. Henley, A. Sadana, Enzyme Microb. Technol. 7 (1985) 50–60.