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SHORT COMMUNICATION

Enhanced enantioselectivity of immobilized *Candida antarctica* lipase for hydrolysis of ketoprofen ethyl ester at pH 1

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Abstract−The immobilized *Candida antarctica* lipase, a commercially available and one of the most commonly used enzymes, showed significantly improved enantioselectivity (twice) when used in a very acidic environment (pH 1.0) than in the normal pH 7.0 for the hydrolysis of ketoprofen ethyl ester at 45 °C. The enzyme was still 60% active at pH 1.0 compared to the activity at pH 7.0 and its stabilities at the two pH values were almost the same. The improved enantioselectivity was ascribed to the conformational change of the enzyme in the very acidic environment.

Key words: *Candida antarctica*, Hydrolysis, Ketoprofen, Lipase, Resolution

INTRODUCTION

Enzymatic resolution of racemic compounds has been extensively studied as a promising method for producing optically pure enantiomers [1,2]. In many cases, however, the enantioselectivity of commercial enzymes is not as high as expected to make the enzymatic resolution processes commercially acceptable. Many methods have been proposed to improve enzyme enantioselectivity, such as pretreatment of enzymes with polar solvents [3-5], chemical modification of enzymes [6], medium engineering [7-9], substrate engineering [10,11] and protein engineering [12] etc.; among them the pretreatment and medium engineering methods are relatively simpler.

Ketoprofen, a non-steroid anti-inflammatory drug, is clinically used in a racemic mixture. Its biological activity, however, is mainly contributed by the S-enantiomer [3] and the R-enantiomer can be used as a toothpaste additive to prevent periodontal disease [13]. The commercial immobilized *Candida antarctica* lipase shows good activity but low enantioselectivity when used as a biocatalyst for ketoprofen resolution reactions. We tried to improve the enantioselectivity of this enzyme preparation by pretreatment with some polar organic solvents such as 2-propanol and acetone but failed, although this method worked very well for other enzymes [3-5]. During our experiments on enzymatic hydrolysis of ketoprofen ethyl ester, we occasionally found that this enzyme preparation showed a much higher enantioselectivity in a very acidic environment than in the normal pH solution. Here we report the results in detail.

MATERIALS AND METHODS

1. Chemicals

Immobilized *Candida antarctica* lipase (EC 3.1.1.3, adsorbed on macroporous acrylic resin) and ketoprofen were purchased from Sigma. Organic solvents (HPLC grade) were from Merck. All other chemicals used were of a reagent grade and obtained commercially.

2. Preparation of Ketoprofen Ethyl Ester

Ketoprofen ethyl ester was prepared following the method of Kim et al. [14]. Thirty grams of ketoprofen was dissolved in 100 ml ethanol followed by addition of 3 g of sulfuric acid. The mixture was stirred overnight at room temperature, followed by evaporation of ethanol and addition of 1 M $NaHCO_3$ to adjust the pH to 9-10. Then equal volume of ethyl acetate was added to extract the ketoprofen ethyl ester which was collected after evaporation of ethyl acetate.

3. Enzymatic Hydrolysis of Ketoprofen Ethyl Ester

Ketoprofen ethyl ester (200 mg) was dissolved in 10 ml 0.1 M phosphate buffer of a predetermined pH and 100 mg immobilized *Candida antarctica* lipase was added. The mixture was kept in a shaking water bath (180 rpm) at 45 °C for 2 h followed by filtration to remove the lipase. The filtrate was acidified to pH 1-2 by addition of 1 M H_2SO_4. The ketoprofen produced and the remaining ester were extracted with ethyl acetate (2×10 ml) and subjected to HPLC analysis.

4. Assay of Time Stability of *Candida antarctica* Lipase at Different pHs

The immobilized lipase was incubated in buffer of predetermined pH at 45 °C under shaking (180 rpm) and the lipase stability was analyzed by detecting the residual activity at different incubation times. As the dry immobilized lipase could not be well dispersed in buffer without the presence of ester substrate, the dry lipase was first wetted with drops of isooctane prior to use.

5. Analytical Methods

Ketoprofen was analyzed by HPLC with a Chiracel OJ-H column (4.6×250 mm). Samples (5 μl) were eluted by a mixture of n-hexane: 2-propanol: acetic acid (90 : 10 : 0.5, v/v/v) at 1.0 ml min^{-1} and detected at 254 nm.

RESULTS AND DISCUSSION

1. Effect of Buffer pH on Activity and Enantioselectivity of Immobilized *Candida antarctica* Lipase

Fig. 1 shows that the substrate conversion tended to increase with increasing buffer pH while the enzyme enantioselectivity sharply decreased when the pH was below 2 and the variation became less

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significant afterwards. At pH 1.0, the enantioselectivity (E=6.6) was twice that (E=3.0) at pH 7.0. The substrate conversion reached 36.1% at pH 1.0 and 50.2% at pH 7.0 after 2 h; the former was about 72% of the latter. The higher enantioselectivity might be attributed to the conformational change of the enzyme at the very acidic environment. Liu et al. [15] reported a similar phenomenon that the lipase from *Candida rugosa* (Lipase OF) showed much higher activity at lower pH environment for hydrolysis of ketoprofen ester. The higher substrate conversion at higher pH might be partially ascribed to the better solubilization of the produced ketoprofen in a more alkaline environment, favoring the hydrolysis reaction. In addition, the conformational change might also negatively affect the enzyme activity.

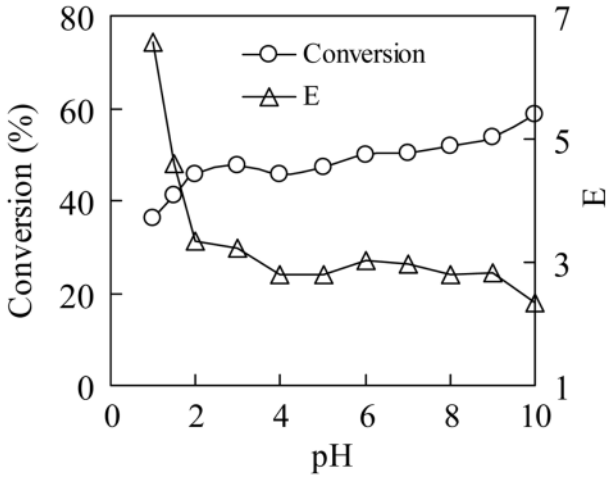

Fig. 1. Effect of pH on enzymatic hydrolysis of ketoprofen ethyl ester. Ketoprofen ester: 200 mg; *Candida antarctica* lipase 100 mg; buffer 10 ml; temperature 45 °C; shaking speed 180 rpm; reaction time 2 h. The pH was varied from 1.0, 1.5, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 to 10.0.

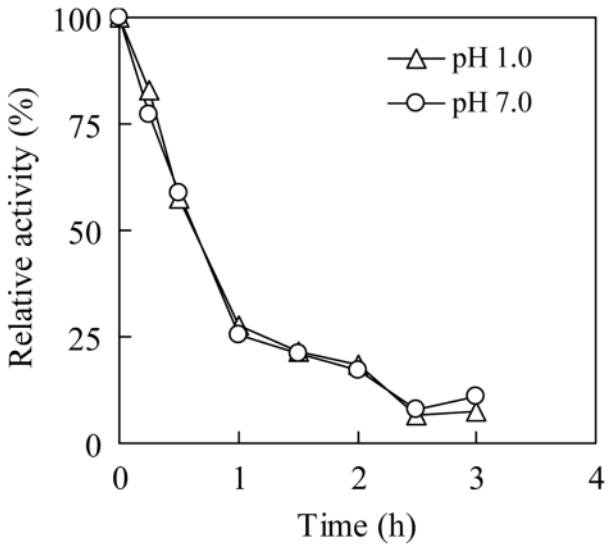

Fig. 2. Stability of immobilized *Candida antarctica* lipase at different pHs as a function of time. The lipase was incubated in buffer of predetermined pH at 45 °C and the residual activity (R) was assayed at different incubation times. Assay conditions: ketoprofen ester 200 mg; Candida antarctica lipase 100 mg; buffer 10 ml; temperature 45 °C; shaking speed 180 rpm.

2. Time Stability of Immobilized *Candida antarctica* Lipase at pH 1.0 and 7.0

Fig. 2 shows that the lipase stabilities at pH 1.0 and 7.0 were almost the same. After incubation for about 40 min, half of the lipase activity was lost in both cases. The final relative activity of the lipase at pH 7.0 (9.3%) was slightly higher than that at pH 1.0 (6.9%). The initial activities of the lipase at pH 1.0 and 7.0 were calculated as 6.4 and 10.9 mg ketoprofen min^{-1} g^{-1} lipase, the former was about 60% of the latter.

Assuming that the lipase follows the first-order deactivation model [16]:

$$E \xrightarrow{k_d} E_d \tag{1}$$

Here E and E_d represent active and partially deactivated enzymes, respectively. k_d is the deactivation rate constant.

The concentrations of active [E] and partially deactivated [E_d] enzymes can be expressed as:

$$[E] = E_0 \exp(-k_d t) \tag{2}$$

$$[E_d] = E_0[1 - \exp(-k_d t)] \tag{3}$$

Here E_0 represents the initial enzyme concentration and t is time.

Assuming that the active and partially deactivated enzymes have the same Michaelis constant (K_m), the relative activity (R) can be calculated as:

$$R = \frac{k_{cat}[E] + \alpha k_{cat}[E_d]}{k_{cat}E_0} = \alpha + (1-\alpha)\exp(-k_d t) \tag{4}$$

Here k_{cat} is the catalytic activity constant, α represents the ratio of the specific enzyme activity at the final state (E_d) to that at the initial state (E_0).

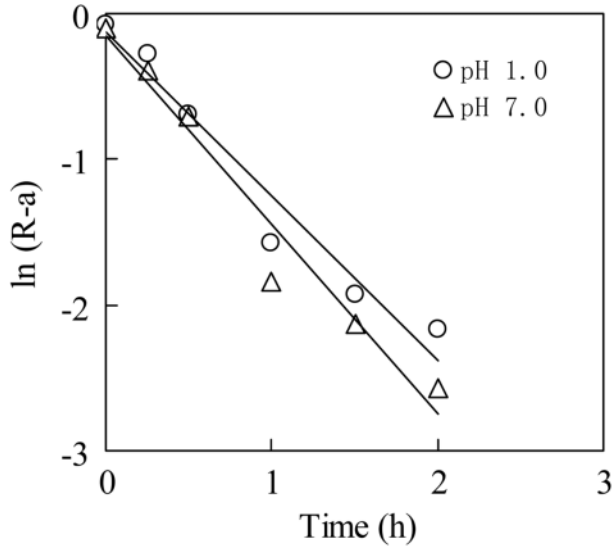

Fig. 3. Plot of ln(R−α) against time according to Eq. (5) for determination of deactivation rate constants at different pH. 100% activity corresponds to 6.4 and 10.9 mg ketoprofen min^{-1} mg^{-1} lipase at pH 1.0 and 7.0, respectively.

For determination of the deactivation rate constant (k_d), Eq. (4) is transformed to the following form:

$$\ln(R-\alpha)=\ln(1-\alpha)-k_d t \quad (5)$$

The plots of $\ln(R-\alpha)$ against t (Fig. 3) show good linear correlations ($r^2>0.95$), indicating that the first-order deactivation model is applicable. The calculated k_d values at pH 1.0 and 7.0 are 1.1/h and 1.3/h, respectively, almost the same.

In summary, the commercially available immobilized *Candida antarctica* lipase showed significantly improved enantioselectivity without significant loss of activity when used in a very acidic environment compared to the normal pH environment for hydrolysis of ketoprofen ethyl ester. The enzyme stability was hardly changed with the variation of pH environment. The improved enantioselectivity was ascribed to the conformational change of the enzyme in the very acidic environment.

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REFERENCES

1. N. Kamiya, H. Kasagi, M. Inoue, K. Kusunoki and M. Goto, *Biotechnol. Bioeng*., **65**, 227 (1999).
2. K. Watanabe, T. Koshiba, Y. Yasufuku, T. Miyazawa and S. Ueji, *Bioorg. Chem*., **29**, 65 (2001).
3. M. G. Kim, E. G. Lee and B. H. Chung, *Process Biochem*., **35**, 977 (2000).
4. I. J. Colton, S. N. Ahmed and R. J. Kazlauskas, *J. Org. Chem*., **60**, 212 (1995).
5. C. S. Chang and C. S. Hsu, *J. Chem. Technol. Biotechnol*., **80**, 537 (2005).
6. Y. Yano, K. Shimada, J. Okai, K. Goto, Y. Matsumoto and R. Ueoka, *J. Org. Chem*., **68**, 1314 (2003).
7. K. W. Kim, B. Song, M. Y. Choi and M. J. Kim, *Org. Lett*., **3**, 1507 (2001).
8. H. J. Park, W. J. Choi, E. C. Huh, E. Y. Lee and C. Y. Choi, *J. Biosci. Bioeng*., **87**, 545 (1999).
9. T. Itoh, S. Han, Y. Matsushita and S. Hayase, *Green Chem*., **6**, 437 (2004).
10. H. Yang, E. Henke and U. T. Bornscheuer, *J. Org. Chem*., **64**, 1709 (1999).
11. J. S. Shin, S. Luque and A. M. Klibanov, *Biotechnol. Bioeng*., **69**, 577 (2000).
12. U. T. Bornscheuer, *Curr. Opin. Biotechnol*., **13**, 543 (2002).
13. P. F. Gong, H. Y. Wu, J. H. Xu, D. Shen and Y. Y. Liu, *Appl. Microbiol. Biotechnol*., **58**, 728 (2002).
14. S. H. Kim, T. K. Kim, G. S. Shin, K. W. Lee, H. D. Shin and Y. H. Lee, *Biotechnol. Lett*., **26**, 965 (2004).
15. Y. Y. Liu, J. H. Xu, Q. G. Xu and Y. Hu, *Biotechnol. Lett*., **21**, 143 (1999).
16. A. Sadana and J. P. Henley, *Biotechnol. Bioeng*., **30**, 717 (1987).