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R-Stereopreference Analysis of Lipase Novozym®435 in Kinetic Resolution of Flurbiprofen

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ABSTRACT Immobilized lipase from *Candida antarctica* (Novozym®435) was employed in the kinetic resolution of racemic flurbiprofen by enantioselective esterification with methanol. It was found that the lipase has the R-stereopreference and the reaction matches Bi Bi Ping Pong mechanism with dead-end inhibition of methanol. Furthermore, the R-stereopreference was analyzed in details from the aspects of enzymatic kinetic mechanism and reaction activation energy of both enantiomers. The R-enantiomer shows lower activation energy and higher maximum reaction rate than the S-enantiomer, which implies the R-stereopreference of the lipase and makes the kinetic resolution of flurbiprofen via enzymatic reaction feasible. *Chirality 19:245–249, 2007.*

KEY WORDS: Novozym®435; flurbiprofen; stereopreference; maximum reaction rate; activation energy

INTRODUCTION

The Profen family (which are chiral acids) are, an important class of non-steroidal anti-inflammatory drugs (NSAIDs), widely used for alleviating pain and inflammation associated with tissue injury. The therapeutic action is mainly due to the S-enantiomers, while the R-enantiomers may have unwanted physiological side effects and toxicity.[1,2] For instance, the S-enantiomer of flurbiprofen (2-fluoro-α-methyl-[1,1′-biphenyl]-4-acetic acid) possesses most of the anti-inflammatory action, nevertheless the presence of the R-enantiomer is reported to enhance gastrointestinal toxicity.[3] Therefore the removal of R-enantiomers from the racemates is desirable.

Among the technologies of enantioseparation of chiral acids, kinetic resolution of profens by lipase-catalyzed enantioselective esterification in nonconventional media have been widely studied in recent years.[4–7] Among the lipases from different resources, the lipase from *Candida antarctica* (Novozym®435), an immobilized preparation of lipase B, exhibits the outstanding characteristic of R-stereopreference in hydrophobic organic media.[7] In a previous study, a simple and practical procedure to experimentally optimize the kinetic resolution of racemic flurbiprofen using Novozym®435 has been developed.[8] The lipase was found to show significant R-stereopreference with an E value of around 6–7 under the optimal conditions. Furthermore, a comprehensive understanding of the enzymatic reaction has been obtained, e.g., the change of various reaction conditions such as substrate type and concentration affects the distribution of water among the components significantly and thus influences the resolution to varying extents. However, few literature reports were found to investigate the R-stereopreference of lipase in detail from the viewpoint of reaction kinetics. Therefore, in this, work kinetic resolution of racemic flurbiprofen was chosen as an example to study the kinetic mechanism of the enantioselective esterification and obtain better understanding of the R-stereopreference of the lipase.

MATERIALS AND METHODS

Enzymatic and Chemical Materials

Novozym®435 (immobilized lipase from *Candida antarctica* with water content of 1–2% (w/w) and activity of 10,000 PLU/g) was purchased from Novo Nordisk A/S (Bagsvaerd, Denmark). Racemic flurbiprofen was obtained from Sigma-Aldrich. HPLC grade *n*-hexane and 2-propanol from Merck-Schuchardt were used as the mobile phase without further purification. All other chemicals used in this work were of analytical grade and became available from various commercial sources.

EXPERIMENTAL PROCEDURES

In a typical experiment, 10 ml of a solution containing varying amounts of racemic flurbiprofen and alcohol were treated in an ultrasonic bath for 2 min to make a homogeneous solution. A sample of the lipase was then introduced into the reaction mixture without ultrasonic treatment.

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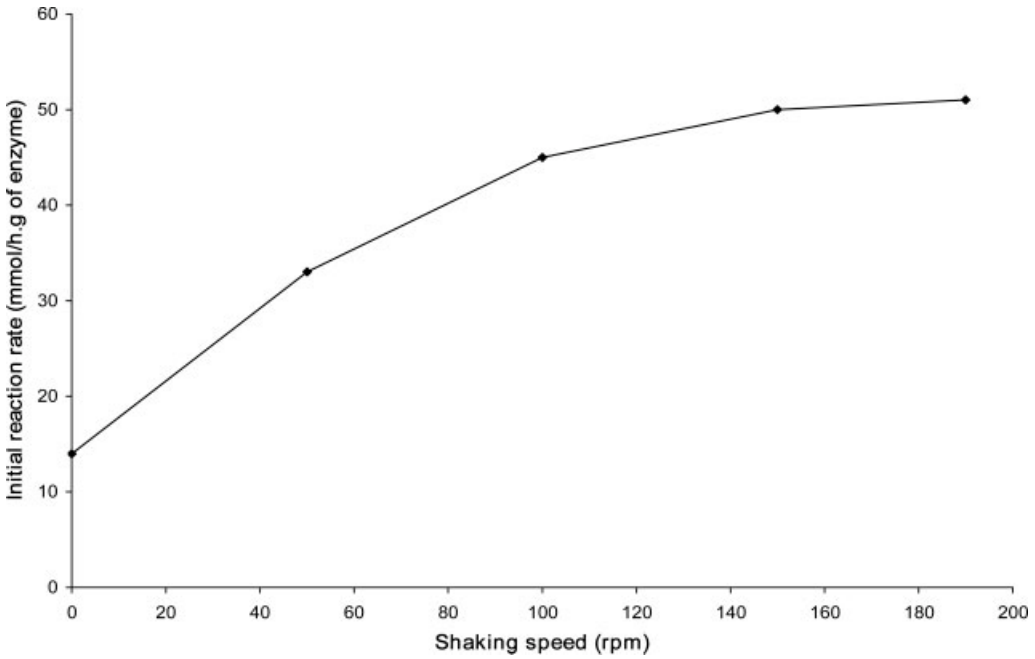

Fig. 1. Effect of shaking speed on the initial reaction rate.

The reaction was performed at 180 rpm and 45°C in a closed flask placed in a water shaker. At certain time intervals, 40 μl of the reaction mixture was withdrawn and diluted in 1 ml of n-hexane, for HPLC analysis.

A supplementary experiment was carried out to verify the validity of using racemic flurbiprofen as substitutes for the chiral substrates *R*- and *S*-flurbiprofen in the kinetic study. In the experiment, the molar ratio of the feed *S*-flurbiprofen and methanol was around 12 (i.e., 15 mM *S*-flurbiprofen and 200 mM methanol), which was in the range of optimal feed ratios obtained previously.[8] It was found that the experimental results were similar to those when 30 mM of racemic flurbiprofen and 200 mM methanol were employed. Based on the experimental results, competition between *R*- and *S*-flurbiprofen is negligable in the optimal reaction conditions and racemic flurbiprofen was used as a substitute for the chiral substrates *R*- and *S*-flurbiprofen in the kinetic study.

Analytical Method

The concentrations of *R*- and *S*-enantiomer of flurbiprofen were determined by HPLC (SHIMADZU SCL-10AVP) with CHIRALPAK AD-H column (Daicel Chemical Ind., Japan) at a wavelength of 254 nm. The mobile phase (the flowrate is 0.8 cm^3/min) consists of *n*-hexane and 2-propanol with the volume ratio of 90:10. The *R*- and *S*-enantiomer were separated well with the retention time of 8.1 and 10.3 min, respectively.

RESULTS AND DISCUSSION

Analysis of Enzymatic Kinetics of R- *and* S-*Flurbiprofen*

Elimination of diffusion limitation. Both external and internal diffusion limitations must be eliminated to obtain accurate kinetic results. The reaction rates at different shaking speeds can be used to evaluate the effect of external mass transfer on reaction. The reaction rate increased with increasing shaking speed in a parabolic relationship to a maximum around 150–190 rpm as shown in Figure 1. Therefore, for shaking speeds higher than 150 rpm, the external diffusion limitation was no longer significant.

To account for the internal diffusion, Thiele's Modulus, which represents the radio of reaction rate to the internal diffusion rate, was calculated. Thiele's modulus, Φ, is expressed as[9]:

$$\Phi = \frac{v}{D_E C}\left(\frac{V_p}{A_p}\right)^2 \quad (1)$$

where, v is the reaction rate (mmol/s g of enzyme); D_E is the effective diffusivity coefficient (cm^2/s); C is the concentration of the reactant (mM); V_p is the volume of the enzyme particle (cm^3/g); A_p is the surface area of the particle (cm^2/g). Since the particle of the immobilized enzyme is spherical, Thiele's modulus can be rearranged in the following form:

$$\Phi = \frac{v}{D_E C}\left(\frac{R}{3}\right)^2 \quad (2)$$

In eqs. 1 and 2, D_E can be expressed as[10]:

$$D_E = D_s \frac{\varepsilon_p}{\tau} H \quad (3)$$

where H is a hindrance factor that accounts for steric interactions between the diffusing solute and the pore wall as well as the enhanced drag on the solute resulting from the presence of the wall. This factor could be neglected in the study because the radius of the pore is much larger than that of the solute molecule in this experiment; ε_p is the po-

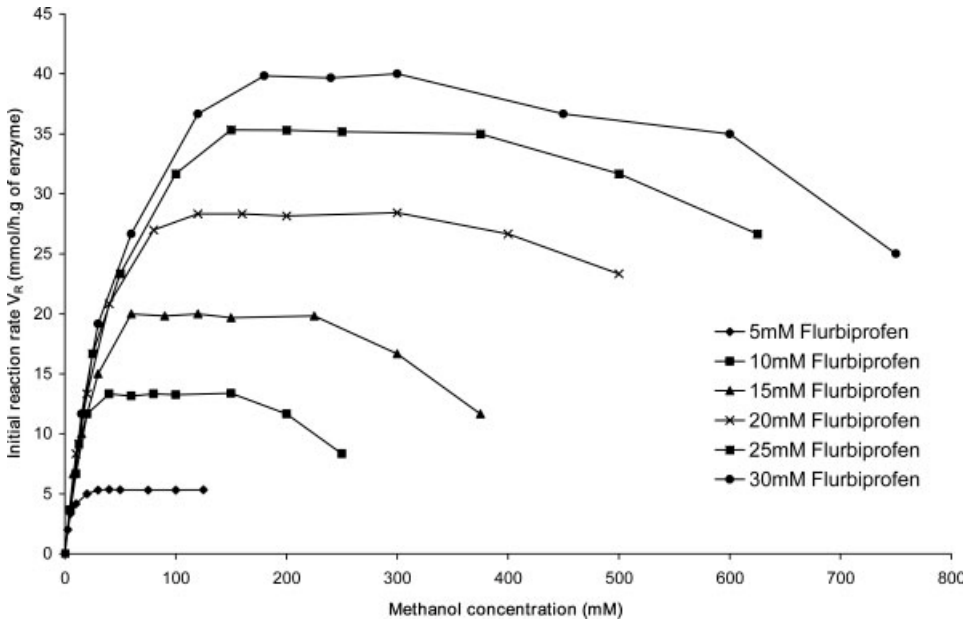

Fig. 2. Effects of the concentrations of *R*-flurbiprofen and methanol on the initial reaction rate.

rosity of the particle; τ is the tortuosity factor of the particle, which is larger than unity by definition; D_s is the substrate diffusivity in the liquid (cm^2/s), which can be estimated from the following correlation[11]:

$$D_s = \frac{\kappa T}{\mu} V_A^{0.33} \quad (4)$$

where, κ is a constant; T is the reaction temperature, K; μ is the viscosity of the reaction medium, cP; V_A is the molar volume of flurbiprofen, cm^3. The values of κ, T, μ, V_A, ε_p, and τ for estimation of DE are 17.5×10^{-8}, 318 K, 0.98 cP, 128.7 cm^3, 0.5, and 6. From eq. 3 and 4, D_E was estimated to be 2.35×10^{-5} cm^2/s. In fact, with high shaking speed in the reaction conditions, D_s is expected to be much higher than estimated and thus a much higher D_E is possible.

The internal diffusion term can be further maximized if all the particles are assumed to be at the maximum size of 0.9 mm. For the reaction rates, we assumed that the maximum reaction rate was achieved in the initial reaction runs. This value was around 0.040 mmol/(s g), which is

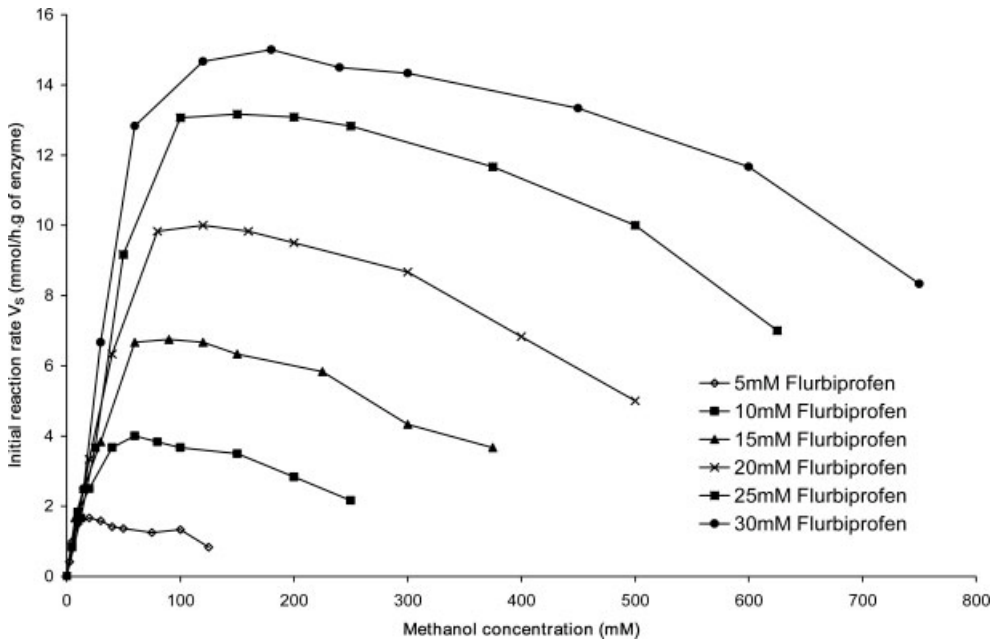

Fig. 3. Effects of the concentrations of *S*-flurbiprofen and methanol on the initial reaction rate.

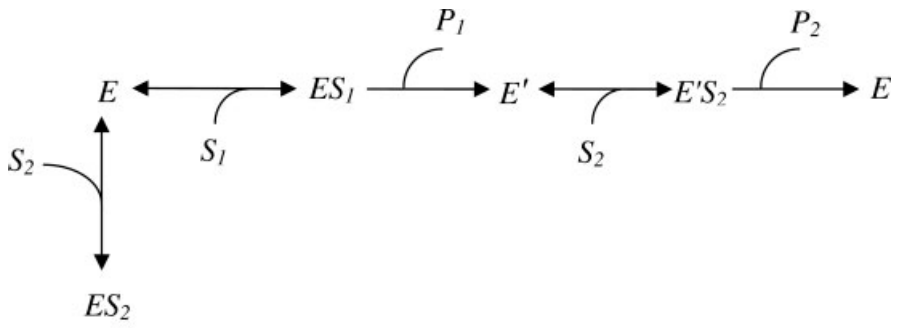

Fig. 4. Reaction mechanistic model: Bi Bi Ping Pong mechanism with dead-end inhibition of methanol.

the maximum reaction rate for *R*-flurbiprofen at a concentration of 30 mM. Substituting all these values into Thiele's modulus, the estimated value is about 0.051. At such a small value of Thiele's modulus, the effect of internal diffusion can be neglected.

Reaction mechanism. A series of kinetic experiments were carried out under the optimal reaction conditions obtained from a previous study.[8] The initial reaction rate increases with the increasing of *R*-flurbiprofen concentration, as shown in Figure 2. Furthermore, when methanol concentration is increased, the effect of substrate inhibition by methanol becomes notable and causes the reaction rate to decrease rapidly. In particular, to examine the irreversibility of inhibition, one reaction with relatively high inhibition (which used 30 mM flurbiprofen and 750 mM methanol as feeds) was studied in detail; after the reaction, the lipase was washed, dried and reused at low feed concentration of methanol (180 mM). As a result, only one-third of the lipase activity remained compared with the value when it was firstly used under the same conditions. Thus, it can be assumed that the inhibition is irreversible, and methanol can be considered as a dead end inhibitor to the lipase. For S-enantiomer, as shown in Figure 3, it has almost the same results as that for R-enantiomer except for the different reaction rates.

For a double substrate reaction catalyzed by the lipase, two mechanisms were proposed. The first one is the ternary complex mechanism or the sequential mechanism,[12] which proposed both substrates must bind to the enzyme before the first product is released. This mechanism assumes that the enzyme can accommodate two substrates at a time. At constant methanol concentrations (constant S_2), the general initial rate equation for this mechanism can be expressed as a linear equation:

$$\frac{1}{v} = \left(\frac{K_3K_1}{K_2V_{\max}} + \frac{K_1K_3}{V_{\max}S_2}\right)\frac{1}{S_1} + \left(\frac{1}{V_{\max}} + \frac{K_3}{V_{\max}S_2}\right) \quad (5)$$

The second mechanism is the substituted enzyme complex mechanism or the more commonly known Ping Pong mechanism.[12] In this mechanism, the enzyme releases a product in between the addition of substrates to the enzyme. More specifically, the mechanism is called Bi Bi Ping Pong mechanism because between the addition of two substrates there occurs alternate release of two products. After flurbiprofen binds to the enzyme, water is released. Following that, methanol binds to the substituted enzyme and the second product, the ester, is then released. The mechanism is shown in Figure 4. The initial reaction rate equation for this mechanism can be expressed as a linear equation:

$$\frac{1}{v} = \frac{C_1S_2\left(1+\frac{S_2}{K_i}\right)}{V_{\max}S_2}\frac{1}{S_1} + \frac{S_2+C_2}{V_{\max}S_2} \quad (6)$$

It is worth noting that in both mechanisms methanol was assumed to be a dead-end inhibitor, i.e., that methanol binds to the enzyme to form another species that can no longer participate in the esterification reaction.

By fitting the experimental data to the above two equations, it was found that the data matched the Bi Bi Ping Pong mechanism. All parameters for the esterification of R- and S-enantiomers are shown in Table 1.

Analysis of Enzymic Kinetic Parameters of R- *and* S-*Flurbiprofen*

In Table 1, difference in the value of $V_{\max}$ was found. It is obvious that the $V_{\max}$ for *R*-flurbiprofen is much higher than that of the S-enantiomer. This means that the resistance to reaction in the *R*-flurbiprofen is smaller than that for *S*-flurbiprofen, and thus leads to the R-stereospecificity of the enzyme. The K_i found for both enantiomers were similar. This was expected as K_i, the dissociation constant of the methanol-enzyme complex, is only determined by

TABLE 1. Parameters of two enantiomers of flurbiprofen for the Bi Bi Ping Pong equation

Kinetic parameters	*R*-flurbiprofen	*S*-flurbiprofen
$V_{\max}$ (mmol/h g of *E*)	125.00	36.36
K_i	0.36	0.41
C_1	0.64	0.13
C_2	54.91	3.52

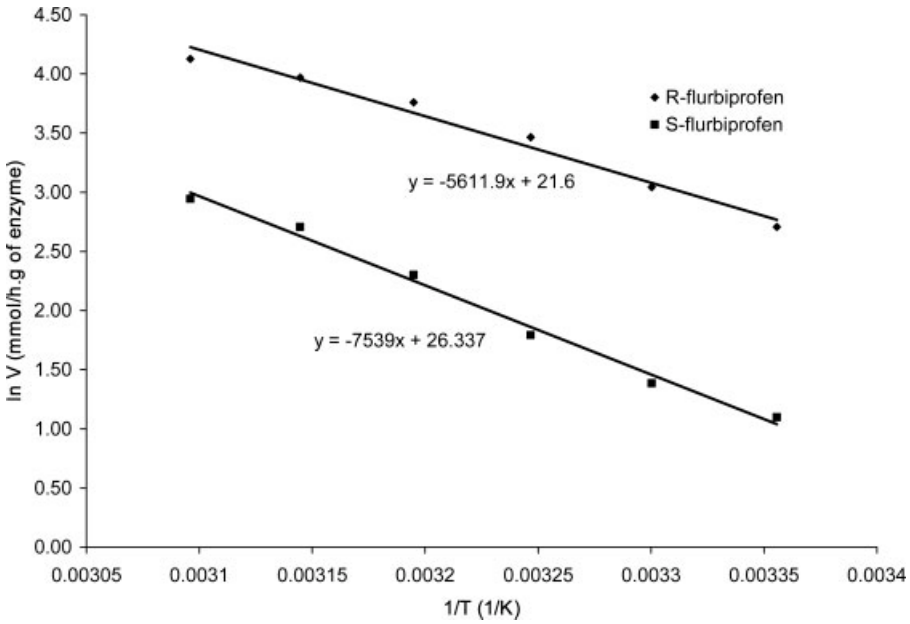

Fig. 5. Effect of temperature on the initial reaction rates of the two enantiomers of flurbiprofen.

the affinity of methanol for the enzyme. Thus there should not be any difference in the values. The values of C_1 and C_2 found cannot be related easily to any observable phenomenon, because they involve the complex relationship of a few rate constants.

Analysis of Reaction Activation Energy of R- *and* S-*Flurbiprofen*

The effect of temperature on initial reaction rate was also studied. The reactions were carried out within the range of 25–50°C at concentrations of 30 mM flurbiprofen and 200 mM methanol. Logarithms of the initial reaction rate were plotted versus reciprocal of the reaction temperature. Linear relationships were found for both enantiomers as shown in Figure 5. This implies that the relationship of initial reaction rate with temperature can be described by the Arrhenius equation. The reaction activation energy for R- and S-enantiomer was found to be 46.7 and 62.7 kJ/mol, respectively. The higher value of the activation energy for S-enantiomer suggests it is more difficult for the esterification of *S*-flurbiprofen to take place. In other words, the energy barrier for R-enantiomer reaction is lower than that for S-enantiomer. Thus it is more favorable for the esterification of R-enantiomer, which results in the R-stereopreference of the enzyme.

In addition, the high values of activation energy are probably due to the fact that for the immobilized enzyme, the activation energy for protein unfolding must be higher than that for free ones; otherwise it was unable to maintain the enzyme in its native structure of unfolding. Besides, the organic solvent used, cyclohexane, may affect the conformation of the enzyme and made it more rigid, leading to higher activation energy.

LITERATURE CITED

1. Hayball PJ. Chirality and nonsteriodal anti-inflammatory drugs. Drugs 1996;52:47–58.
2. Shen TY. Perspectives in nonsteroidal anti-inflammatory agents. Angew Chem Int Ed 1972;11:460–472.
3. Wechter WJ, Bigornia AE, Murray ED, Levine BH, Young JW. Rac-flurbiprofen is more ulcerogenic than its (*S*)-enantiomer. Chirality 1993;5: 492–494.
4. Wu JY, Liu SW. Influence of alcohol concentration on lipase-catalyzed enantioselective esterification of racemic naproxen in isooctane: under controlled water activity. Enzyme Microb Technol 2000;26: 124–130.
5. Wu JY, Liang MT. Enhancement of enantioselectivity by altering alcohol concentration for esterification in supercritical CO_2. J Chem Eng Jpn 1999;32:338–340.
6. Shang CS, Hsu CS. Lipase-catalyzed enantioselective esterification of (S)-naproxen hydroxyalkyl ester in organic media. Biotechnol Lett 2003; 25:413–416.
7. D'Antona N, Lombardi P, Nicolosi G, Salvo G. Large scale preparation of enantiopure S-ketoprofen by biocatalysed kinetic resolution. Process Biochem 2002;38:373–377.
8. Zhang HY, Wang X, Ching CB, Wu JC. Experimental optimization of enzymic kinetic resolution of racemic flurbiprofen. J Biotechnol Appl Biochem 2005;42:67–71.
9. Bailey JE, Ollis DF. Biochemical engineering fundamentals, 2nd ed. Singapore: McGraw-Hill; 1986.
10. Blanch HW, Clark DS. Biochemical Engineering. New York: Marcel Dekker; 1996.
11. Perry RH, Green DW, Maloney JO. Perry's chemical engineering's handbook, 6th ed. Singapore: McGraw-Hill; 1984.
12. Segal IH. Enzyme kinetics. New York: Wiley; 1975.