Journal of Chemical Technology and Biotechnology *J Chem Technol Biotechnol* **74**:751–758 (1999)

Lipase-catalyzed enantioselective esterification of *S*(+)-naproxen ester prodrugs in cyclohexane

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Abstract: A lipase-catalyzed enantioselective esterification process in cyclohexane was developed for the synthesis of *S*(+)-naproxen ester prodrugs containing the moiety of *N,N*-dialkylamino, ethylene glycol or alkyl ether of ethylene glycol. A high enantiomeric ratio of 44 was obtained when di(ethylene glycol) was selected as the best acyl acceptor. A reversible ping-pong Bi Bi mechanism has been employed to elucidate the enzymatic behavior of the initial conversion rate for *S*(+)-naproxen and the time-course conversions for both enantiomers. Improvement of the enzyme activity was demonstrated when alcohol in excess of its cyclohexane solubility limit was used. The application of excess racemic naproxen in the presence of solid substrate suspensions showed enhanced productivity and enantioselectivity for the desired *S*(+)-ester. Studies of the recovery and racemization of the remaining *R*(−)-naproxen are also reported.
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Keywords: (*S*)-naproxen ester prodrugs; lipase; kinetic resolution; esterification

NOTATION

(C)	Alcohol concentration ($\mathrm{mmol\,dm^{-3}}$)
ee_P	Enantiomeric excess for product, $(X_A - X_B)(X_A + X_B)^{-1}$
ee_S	Enantiomeric excess for substrate, $(X_A - X_B)(2 - X_A - X_B)^{-1}$
E^{**}	Enantiomeric ratio defined as $k_2K'_{ml}/k'_2K_{ml}$
(E_t)	Total enzyme concentration ($\mathrm{mg\,cm^{-3}}$)
I	Kinetic parameter defined in Table 1 of a previous report[16]
k_f	Enzyme deactivation constant for *S*(+)-naproxen ($\mathrm{h^{-1}}$)
k_1, k_3, k_{-2}, k_{-4}	Kinetic constants shown in eqns (1) and (2) ($\mathrm{cm^3\,h^{-1}\,mg^{-1}}$)
k_2, k_4, k_{-1}, k_{-3}	Kinetic constants shown in eqns (1) and (2) ($\mathrm{mmol\,cm^3\,dm^{-3}\,h^{-1}\,mg^{-1}}$)
K_{eq}	Equilibrium parameter defined in eqn (6)
K_{ml}	Kinetic parameter defined as $(k_2 + k_{-1})/k_1$ ($\mathrm{mmol\,dm^{-3}}$)
K_{m3}	Kinetic parameter defined as $(k_4 + k_{-3})/k_3$ ($\mathrm{mmol\,dm^{-3}}$)
(P)	Water concentration ($\mathrm{mmol\,dm^{-3}}$)
(Q_A), (Q_B), (Q)	Concentrations of *S*(+)-ester, *R*(−)-ester and racemic ester, respectively ($\mathrm{mmol\,dm^{-3}}$)
(S_A), (S_B), (S)	Concentrations of *S*(+)-naproxen, *R*(−)-naproxen and racemic naproxen, respectively ($\mathrm{mmol\,dm^{-3}}$)
t	Time (h)
V_A	Reaction rate of *S*(+)-naproxen ($\mathrm{mmol\,dm^{-3}\,h^{-1}}$)
X_A, X_B, X	Conversions of *S*(+)-naproxen, *R*(−)-naproxen and racemic naproxen, respectively

Superscript

	In the kinetic constants for *R*(−)-enantiomer

Subscripts

B	*R*(−)-enantiomer
eq	equilibrium
0	Initial state

1 INTRODUCTION

Recently, much effort has centered on the synthesis or resolution of optical pure drugs or agrochemicals by chemical or biochemical methods.[1–3] One may attribute this change of interest to (1) medical or ecological

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Contract/grant sponsor: Chinese National Science Council; contract/grant number: NSC-84-2214-E006-026
(*Received 11 May 1998; revised version received 9 February 1999; accepted 22 February 1999*)

benefits from using optically pure enantiomers, (2) the more severe regulatory climate and (3) advances in the synthesis or resolution of optically pure compounds. Profens (2-arylpropionic acids), an important group of non-steroidal anti-inflammatory drugs (NSAIDs), have their pharmacological activity mainly in the $S(+)$-enantiomer.[4] Therefore, considerable efforts are being made to synthesize optically pure profens, although most NSAIDs are still being used as racemates in therapeutics.

Previous pharmacological studies of the acidic NSAIDs have indicated that gastrointestinal (GI) side effects such as ulceration and hemorrhage constitute the most frequent adverse reactions. Developing NSAID prodrugs which temporarily mask the NSAID acidic group has been proposed.[5–10] An alternative method of avoiding GI side effects is to administer the drug via the transdermal route. However, since the percutaneous absorption of many NSAIDs is low,[11] a prodrug approach presents a very promising method to enhance the skin permeability of the drug without provoking skin damage and irritancy caused by penetration enhancers.[12–14] The esterification of profens is an effective way to prepare the prodrug. However, a careful selection of the alcohol is needed, because a good hydrophilic–lipophilic balance of the prodrug is essential to give an acceptable bioavailbility.[5–10]

In previous reports, we have successfully developed a lipase-catalyzed process in organic solvents to directly synthesize 4-morpholinoethyl $S(+)$-naproxen and $S(+)$-ibuprofen ester prodrugs from the corresponding racemates.[15,16] In the present work, this enzymatic process is extended to the synthesis of other useful $S(+)$-naproxen ester prodrugs in cyclohexane. The best alcohol was selected from various alcohols (containing *N,N*-dialkylamino, ethylene glycol or alkyl ether of ethylene glycol) in terms of their enzyme enantioselectivity and activity. Then, the effect of lipases from different sources and preparations as well as the enzyme kinetics were investigated and applied to time-course analysis. Finally, the feasibility of enhancing the productivity and enantioselectivity of the desired $S(+)$-ester was investigated by employing excess racemic naproxen and/or alcohol as the substrates.

2 MATERIALS AND METHODS

2.1 Materials

Optically pure $S(+)$-naproxen ($S(+)$-2-(6-methoxyl-2-naphthyl)propionic acid) and CR lipase (triacylglycerol ester hydrolases, EC 3.1.1.3) from *Candida rugosa* (type VII, 835 μmol h^{-1} mg^{-1} for the hydrolysis of olive oil at pH 7.2 and 37 °C) were purchased from Sigma (St Louis, MO). Lipase MY (30 μmol h^{-1} mg^{-1} for the hydrolysis of olive oil at pH 7.0 and 37 °C) from the same microorganism was provided by Meito Sangyo Industries Ltd (Japan). Cyclohexane, acetic acid, ethanol and isopropanol of HPLC grade from Tedia (Fairfield, OH) were used without further purification. Other chemicals of analytical grade were commercially available as follows: 2-nitrotoluene from Fluka (Switzerland); ethylene glycol, di(ethylene glycol), tri(ethylene glycol), tetra(ethylene glycol), di(ethylene glycol)methyl ether, tri(ethylene glycol)-butyl ether from Aldrich (Milwaukee, WI); 2-dimethylaminoethanol, 2-(2-dimethylamino-ethoxy)ethanol, 3-dimethylamino-1-propanol, 3-di-ethylamino-1-propanol, 1-diethylamino-2-propanol, 4-dimethylamino-1-butanol and 6-dimethylamino-1-hexanol from Tokyo Kasei (Japan).

2.2 Analysis

HPLC was used to monitor the esterification of racemic naproxen with various alcohols. The mobile phase was composed of *n*-hexane:ethanol:acetic acid (80:20:0.5, v/v) at a flow rate of 1 cm^3 min^{-1}. A UV detector at 270 nm was used for quantification at 25 °C. A chiral column (Regis (S, S)-Whelk-01, Morton Grove, IL) capable of separating the internal standard of 2-nitrotoluene, $R(-)$- and $S(+)$-naproxen, $R(-)$- and $S(+)$-naproxen di(ethylene glycol) esters, $R(-)$- and $S(+)$-naproxen tri(ethylene glycol) esters with the retention times of 4.0, 9.5, 15.8, 19.4, 25.3, 26.5 and 40.3 min, respectively, was employed.

2.3 Alcohol selection

Unless specified, 50 mg CR lipase was added to 10 cm^3 of cyclohexane containing 0.8 mmol dm^{-3} racemic naproxen and 2 mmol dm^{-3} alcohol. The resultant mixture was stirred with a magnetic stirrer at 37 °C, and samples were withdrawn for HPLC analysis at different time intervals. The best alcohol was selected on the basis of enzyme activity and enantioselectivity and applied in the following experiments.

2.4 Selection of lipases from different sources and preparations

Similar experiments to those as in Section 2.3 were carried out to investigate the effect of enzyme sources (i.e. CR lipase and Lipase MY) and preparations on enzyme activity and enantioselectivity. The purified lipase prepared from Lipase MY was kindly supplied by Professor JS Dordick from Rensselaer Polytechnic Institute. It was originally donated by Altus Biologics Inc (Cambridge, MA), dialyzed against 50 mM phosphate buffer (pH 7.0) overnight and then lyophilized for 24 h. The modified lipase was prepared by following the procedure of Colton *et al.*[17] To 80 cm^3 of deionized water at 4 °C was added 8 g of Lipase MY, followed by stirring for 30 min. Isopropanol (80 cm^3, 4 °C) was slowly added to the solution and stirred for 48 h. The resultant solution was centrifuged for 30 min at 3000 rpm to remove the precipitate, dialyzed against 4 °C deionized water for 72 h, and then lyophilized overnight.

2.5 Effects of substrate concentration

Similar experiments to those in Section 2.3 were carried out except that the concentration of the

selected alcohol was changed from 0.09 to $4\,\mathrm{mmol\,dm^{-3}}$. The same experiments were performed at $2\,\mathrm{mmol\,dm^{-3}}$ alcohol and the racemic naproxen concentration varied from 0.17 to $0.8\,\mathrm{mmol\,dm^{-3}}$. In order to increase the $S(+)$-ester productivity while simultaneously maintaining the product optical purity, experiments were also carried out at $2\,\mathrm{mmol\,dm^{-3}}$ alcohol and the racemate concentration of 3 or $5\,\mathrm{mmol\,dm^{-3}}$ higher than the solubility limit in cyclohexane.

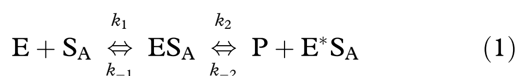
2.6 $R(-)$-Naproxen extraction and racemization

Di(ethylene glycol) solution (pH 13.1) and aqueous solutions (pH 10.7, 11.5 and 12.9) containing sodium hydroxide were prepared. At room temperature, different volumes of the above solution were vigourously mixed with $5\,\mathrm{cm^3}$ of the reaction medium of $(S)=0.42\,\mathrm{mmol\,dm^{-3}}$, $(Q)=0.22\,\mathrm{mmol\,dm^{-3}}$, $ee_P=0.61$ and $ee_S=0.32$ for which the CR lipase had been previously remove by filtration. The resultant solutions were settled for phase separation. The organic phase was sampled for HPLC analysis at different time intervals. After drying the remaining aqueous phase by evaporation, the resultant solid was dissolved in $15\,\mathrm{cm^3}$ of ethylene glycol and racemized at 120°C for 11h. An aqueous solution containing 37.5% hydrogen chloride was added in drops to adjust the pH to about 1. Then, the resultant solution was vigorously mixed with $15\,\mathrm{cm^3}$ of cyclohexane for 20min and the supernatant was sampled for HPLC analysis.

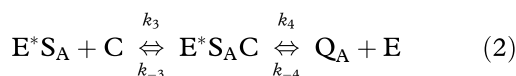
3 THEORETICAL MODEL

3.1 Model description

In a previous work,[16] a reversible ping-pong Bi Bi mechanism with the alcohol as a competitive inhibitor to the enzyme was employed to describe the kinetic behavior of lipase-catalyzed esterification of racemic ibuprofen with 4-morpholine ethanol in cyclohexane. When the alcohol inhibition effect is negligible, the mechanism for the esterification of $S(+)$-naproxen is expressed as:



$$\mathrm{E} + \mathrm{S_A} \underset{k_{-1}}{\overset{k_1}{\Leftrightarrow}} \mathrm{ES_A} \underset{k_{-2}}{\overset{k_2}{\Leftrightarrow}} \mathrm{P} + \mathrm{E^*S_A} \quad (1)$$



$$\mathrm{E^*S_A} + \mathrm{C} \underset{k_{-3}}{\overset{k_3}{\Leftrightarrow}} \mathrm{E^*S_AC} \underset{k_{-4}}{\overset{k_4}{\Leftrightarrow}} \mathrm{Q_A} + \mathrm{E} \quad (2)$$

where C, E, P, Q_A and S_A represent alcohol, enzyme, water, $S(+)$-ester and $S(+)$-naproxen, respectively. For the $R(-)$-enantiomer, the subscript A is changed to B and a superscript of prime is added to the kinetic parameters in eqns (1) and (2).

By assuming all enzyme complexes to be in a pseudo-steady state, the rate equations for both enantiomers can be derived as eqns (1)–(3) of a previous report,[16] in which the terms containing the kinetic parameter for alcohol inhibition are neglected.

One may further assume linear deactivation kinetics for the lipase, with k_f and k'_f as the deactivation constants for $S(+)$-and $R(-)$-naproxen, respectively. Therefore, integration of the rate equations by using the stoichiometric balances for the substrate and product yields the time-course concentrations for both enantiomers. However, since too many kinetic parameters are involved, simplification of the rate equations is needed before parameter fitting from the experimental data.

3.2 Parameters fitting of k_2/K_{m1} and K_{m3}/k_4 at the initial stage

At the initial stage, reaction reversibility and enzyme deactivation can be neglected. If one further assumes $K_{m1} \gg (S_A)$, $K'_{m1} \gg (S_B)$, $k'_4/K'_{m3}=k_4/K_{m3}$ and a high enantiomeric ratio of $E^{**}=k_2K'_{m1}/k'_2K_{m1}$ for the enzyme, the rate equation for $S(+)$-naproxen can be simplified to:

$$V_A \frac{-(S_A)}{\mathrm{d}t} = \frac{k_2(S_A)(E_t)/K_{m1}}{1 + \dfrac{k_2K_{m3}(S_A)}{k_4K_{m1}(C)}} \quad (3)$$

Therefore, from the variation of the initial rate of V_A with the initial alcohol concentration of (C), the parameters of k_2/K_{m1} and K_{m3}/k_4 can be determined.

3.3 Parameters fitting of k'_2/K'_{m1}, k'_f, k_f and K_{eq}

When racemic naproxen is applied as the substrate, one cannot follow the above procedure for $S(+)$-naproxen to estimate the kinetic parameters for $R(-)$-naproxen. This is due to the low reactivity of the latter, and the enzyme deactivation effect should be considered. Therefore by controlling $(C) \gg (S_B)$, neglecting the reversible effect of the product and assuming k'_4/K'_{m3} equal to k_4/K_{m3}, eqn (3) is modified as:

$$-\frac{\mathrm{d}(S_B)}{\mathrm{d}t} = \frac{k'_2(S_B)(E_t)\exp[-k'_ft]/K'_{m1}}{1 + \dfrac{k'_2K_{m3}(S_B)}{k_4K'_{m1}(C)}} \quad (4)$$

Integration of eqn (4) gives:

$$t = -\ln\left\{\frac{k'_f}{(E_t)}\left[\frac{K'_{m1}}{k'_2}\ln(1-X_B) - \frac{K_{m3}}{(C)k_4}(S_B)_0X_B\right]+1\right\}/k'_f \quad (5)$$

Then by using the time-course data of $X_B<0.1$, the parameters of k'_2/K'_{m1} and k'_f can be estimated from the time-course conversion of $R(-)$-naproxen and eqn (5).

The equilibrium constant for both enantiomers can be calculated from:

$$K_{eq} = \left\{\frac{(Q_A)(P)}{(S_A)(C)}\right\}_{eq} = \left\{\frac{(S_A)_0X_A(X_A+X_B)}{(1-X_A)[(C)_0-(S_A)_0(X_A+X_B)]}\right\}_{eq} \quad (6)$$

where the subscript 'eq' indicates an equilibrium state.

It is also reasonable to make the following assumptions:

(1) The kinetic parameters of k_4/K_{m3} and k'_4/K'_{m3} are equal, since they represent the second-order rate constants for the enzyme complexes E^*S_A and E^*S_B for reaction with the achiral alcohol in the deacylation step.
(2) The terms of $I(P)/K_{eq}$ and $I'(P)/K_{eq}$ in eqns (1) and (2) of a previous paper[16] for both enantiomers are negligible by comparing with the alcohol concentration.
(3) The irreversibility of the product can be used to simplify the denominators of the rate equations for both enantiomers. Therefore, the rate equations for both enantiomers can be simplified as follows:

$$-\frac{\mathrm{d}(S_A)}{\mathrm{d}t}=\frac{k_2(E_t)\exp(-k_f t)\left[(S_A)-\dfrac{(2(S_A)_0-(S_A)-(S_B))((S_A)_0-(S_A))}{K_{eq}((C)_0-2(S_A)_0+(S_A)+(S_B))}\right]}{K_{m1}\left\{1+\left[\dfrac{1}{((C)_0-2(S_A)_0+(S_A)+(S_B))}\right]\left[\dfrac{k_2K_{m3}(S_A)}{K_{m1}k_4}+\dfrac{k'_2K_{m3}(S_B)}{K'_{m1}k_4}\right]\right\}} \quad (7)$$

$$-\frac{\mathrm{d}(S_B)}{\mathrm{d}t}=\frac{k'_2(E_t)\exp(-k'_f t)\left[(S_B)-\dfrac{(2(S_A)_0-(S_A)-(S_B))((S_B)_0-(S_B))}{K_{eq}((C)_0-2(S_A)_0+(S_A)+(S_B))}\right]}{K'_{m1}\left\{1+\left[\dfrac{1}{(C)_0-2(S_A)_0+(S_A)+(S_B))}\right]\left[\dfrac{k_2K_{m3}(S_A)}{K_{m1}k_4}+\dfrac{k'_2K_{m3}(S_B)}{K'_{m1}k_4}\right]\right\}} \quad (8)$$

The remaining deactivation constant of k_f can then be determined from parameter fitting between the time-course conversion of X_A and the theoretical results obtained from solving eqns (7) and (8) by employing a fourth-order Runge–Kutta technique.

4 RESULTS AND DISCUSSION

4.1 Alcohol selection

Table 1 demonstrates the effect of *N,N*-dialkylamino-alcohol on the enantioselective synthesis of the $S(+)$-naproxen ester prodrug using lipase-catalyzed esterification in cyclohexane. With the exception of 3-dimethylamino-1-propanol, increasing the chain length of the primary alcohol results in the enhancement of enzyme activity. This is especially true when the alcohol contains a bulky moiety such as diethylamino or dimethylaminoethoxy. A similar tendency for the effect of alcohol chain length on the lipase activity in the esterification of racemic naproxen was also reported.[18] However, the lipase shows very poor enantioselectivity, with the enantiomeric ratio less than 1.5, for all alcohols in the table. When a secondary alcohol of 1-diethylamino-2-propanol was used, a decrease of the enzyme activity without any improvement in the enantiomeric ratio was found. Therefore, based on these results, the *N,N*-dialkylaminoalcohols employed in the present report cannot be applied as the acyl acceptor to the synthesis of $S(+)$-naproxen ester prodrug. However, when 4-morpholine ethanol (an *N,N*-dialkylaminoalcohol and an exception to the observed rule regarding *N,N*-dialkylaminoalcohols) was used in the same reaction system, a high enzyme enantioselectivity and activity was obtained, as previously reported.[15] This is also true when the present esterification reaction is replaced by a transesterification reaction where racemic 2,2,2-trifluoroethyl naproxen ester is employed as an acyl donor.[19]

Table 1. Effect of *N,N*-dialkylaminoalcohol on the enantioselectivity and activity of CR lipase in cyclohexane at 37 °C

Alcohol	*t(h)*	*X*	*ee*$_P$	*E***
2-Dimethylaminoethanol	53.5	0.091	0.11	1.1
3-Dimethylamino-1-propanol	50.5	0.059	0.008	1.0
4-Dimethylamino-1-butanol	53.5	0.14	0.22	1.3
6-Dimethylamino-1-hexanol	58	0.25	0.24	1.5
1-Diethylamino-2-propanol	48	0.13	0.07	1.1
3-Diethylamino-1-propanol	53	0.35	0.14	1.3
2-(2-Dimethylaminoethoxy)-ethanol	50.5	0.40	0.08	1.2

Reaction conditions: $(C)_0 = 2\,\mathrm{mmol\,dm^{-3}}$, $(E_t) = 5\,\mathrm{mg\,cm^{-3}}$, $(S_A)_0 = (S_B)_0 = 0.4\,\mathrm{mmol\,dm^{-3}}$.

When an alcohol containing ethylene glycol or alkyl ether of ethylene glycol was applied as an acyl acceptor, an improvement of the enzyme enantioselectivity was demonstrated as shown in Table 2. A maximum enantiomeric ratio of 44 for di(ethylene glycol) was obtained. However, a maximum initial rate for $S(+)$-naproxen were also found when tri(ethylene glycol) was employed. In general, no rules were found to predict the influence of alcohol chain length on the enantioselectivity and activity of the lipase. Moreover, when an alcohol containing an alkyl ether of ethylene glycol was added, a poor enzyme enantioselectivity and activity was observed (Table 2). Therefore, based on observed enzyme performance, di(ethylene glycol) was chosen as the best alcohol and employed in all subsequent experiments.

4.2 Selection of lipases from different sources and preparations

Several lipases from different sources and preparations were tested for their ability to catalyze the enantio-

Table 2. Effect of alcohol containing ethylene glycol or alkyl ether of ethylene glycol on the enantioselectivity and activity of CR lipase in cyclohexane at 37 °C

Alcohol	t (h)	X	ee_P	E**	$[V_A/(E_t)] \times 10^3$ (mmol cm^3 dm^{-3} h^{-1} mg^{-1})
Ethylene glycol	4.0	0.16	0.65	4.5	5.92
Di(ethylene glycol)	5.0	0.12	1.0	44	10.7
Tri(ethylene glycol)	4.0	0.19	0.87	16	16.7
Tetra(ethylene glycol)	3.0	0.076	0.90	10	3.84
Di(ethylene glycol)methyl ether	3.0	0.13	0.62	5.4	3.44
Tri(ethylene glycol)butyl ether	4.5	0.10	0.39	4.1	1.92

Reaction conditions: $(C)_0 = 2$ mmol dm^{-3}, $(E_t) = 5$ mg cm^{-3}, $(S_A)_0 = (S_B)_0 = 0.4$ mmol dm^{-3}.

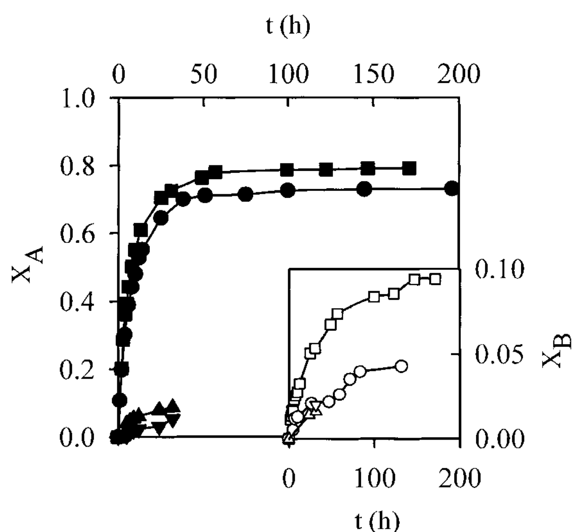


Figure 1. Time-course conversions of X_A and X_B using lipases from different sources and preparations as the biocatalyst in cyclohexane: $(S_A)_0 = (S_B)_0 = 0.4$ mmol dm^{-3}, $(C)_0 = 2$ mmol dm^{-3}, $(E_t) = 5$ mg cm^{-3}, 37 °C. For CR lipase: ●, ○; Lipase MY: ■, □; modified lipase: ▼, ▽; purified lipase: ▲, △.

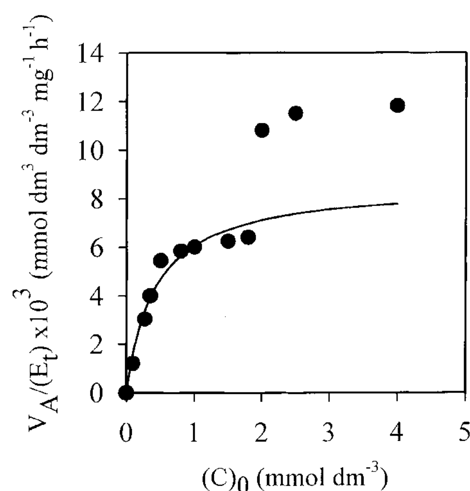


Figure 2. Effects of alcohol concentration on the specific initial rate of $S(+)$-naproxen with CR lipase as biocatalyst: $(S_A)_0 = (S_B)_0 = 0.4$ mmol dm^{-3}, $(E_t) = 5$ mg cm^{-3}, 37 °C. Experimental data, ●; theoretical value,—.

selective esterification of racemic naproxen with di(ethylene glycol) in cyclohexane. As shown in Fig 1, both CR lipase and Lipase MY have a similar performance on the time-course conversion of $S(+)$-naproxen. A slightly higher equilibrium conversion of X_A for the latter was obtained. This may be due to the lower water content in Lipase MY. However, this lipase also possesses a higher time-course conversion for $R(-)$-naproxen, as illustrated in the figure, and results in a lower enantiomeric ratio of 27.

The time-course conversions for $S(+)$-naproxen which result from employing lipases of different preparations as the biocatalyst are also illustrated in Fig 1. A very low enzyme activity for both purified and modified lipases was obtained. This may be due to the large particle size of the enzyme after lyophilization, which gives a poor dispersion of the enzyme molecule in cyclohexane in comparison with the crude lipase. Moreover, the enantiomeric ratios of 6.1 and 3.5 for the purified and the modified lipase, respectively, were determined from the experimental data. Based on the cumulative results of these tests, CR lipase was selected as the best enzyme and applied in the kinetic study.

4.3 Effect of substrate concentration

The variation of the specific initial rate of $S(+)$-naproxen with the alcohol concentration in cyclohexane is represented in Fig 2. The rate curve slows from a rapid start to a plateau region as 1.8 mmol dm^{-3} is approached, showing no sign of inhibition behavior. This is unlike 4-morpholine ethanol in an identical system, where an inhibitory-type maximum was observed at an alcohol concentration of 8 mmol dm^{-3}.[15] This indicates that 4-morpholine ethanol, but not di(ethylene glycol), can be regarded as an enzyme inhibitor in the kinetic analysis.

In Fig 2, an abrupt increase of enzyme activity at around 2 mmol dm^{-3} is shown, rising rapidly to the second and much higher plateau. One may attribute this interesting behavior to the hydrophilic nature of the alcohol. At a di(ethylene glycol) concentration of 2 mmol dm^{-3}, which is higher than the solubility limit of the alcohol in cyclohexane, the excess alcohol disperses in cyclohexane to form the secondary liquid phase. Then, after vigourous shaking, the dispersed alcohol phase, having extracted some naproxen molecules, presumably forms a relatively pure thin film of alcohol adhering to parts of the surface of the enzyme particles. Because naproxen dissolves preferably in alcohol, therefore, the naproxen concentration in the alcohol phase around the enzyme particles is higher

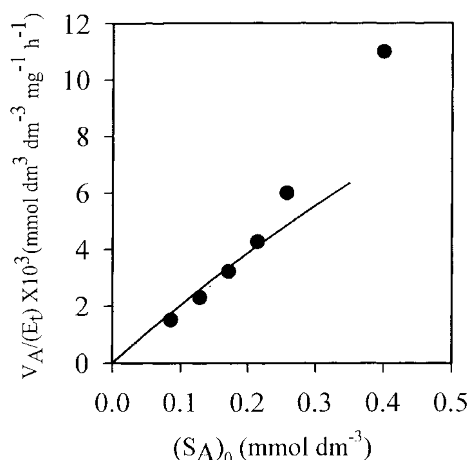


Figure 3. Effects of $S(+)$-naproxen concentration on the specific initial rate of $S(+)$-naproxen with CR lipase as biocatalyst: $(C)_0=2.0\,\text{mmol}\,\text{dm}^{-3}$, $(E_t)=5\,\text{mg}\,\text{cm}^{-3}$, 37 °C. Experimental data, ●; theoretical value, —.

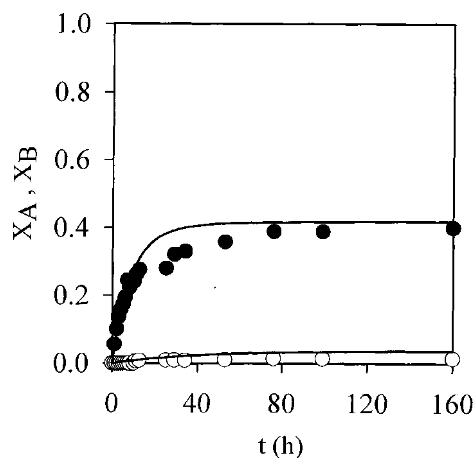


Figure 4. Time-course conversions with CR lipase as biocatalyst: $(S_A)_0=(S_B)_0=0.4\,\text{mmol}\,\text{dm}^{-3}$, $(C)_0=1.0\,\text{mmol}\,\text{dm}^{-3}$, $(E_t)=5\,\text{mg}\,\text{cm}^{-3}$, 37 °C. Experimental data: X_A, ●; X_B, ○; theoretical value,—.

than the naproxen concentration in the cyclohexane phase. This will enhance total enzyme activity. Further increase of the alcohol concentration will produce more alcohol volume to extract enough naproxen from the cyclohexane to cause a reduction in the naproxen/cyclohexane concentration. This in turn will dilute the naproxen concentration in the alcohol phase. Thus, the slightly positive slope of the upper plateau of Fig 2 may be attributed to two conflicting effects, (1) a positive effect resulting from the increase of coverage and depth of the alcohol surface film adhering to the enzyme particles and (2) a negative effect from the decreasing naproxen concentrations in both phases as the alcohol volume increases.

The effect of $S(+)$-naproxen concentration on the specific initial rate of $S(+)$-naproxen at an alcohol concentration of $2\,\text{mmol}\,\text{dm}^{-3}$ is presented in Fig 3, where the enzyme activity is found to be enhanced by increasing the substrate concentration. Normally, such a plot would show a straight line followed by a diminishing slope. The continuing slope increase seen in Fig 3 is notable. However, we can currently provide no quantitative analysis which can elucidate the rapid increase of enzyme activity when $S(+)$-naproxen concentration exceeds approximately $0.22\,\text{mmol}\,\text{dm}^{-3}$. Therefore, further studies which investigate the reaction rate in terms of substrate partitioning between both phases, as well as studies of the possibility of using a cyclohexane-free system to increase the solubility and productivity of racemic naproxen in the alcohol, are needed.

By using eqn (3) and the data for alcohol concentrations less than $2\,\text{mmol}\,\text{dm}^{-3}$ in Fig 2, the kinetic parameters can be determined as $k_2/K_{m1} = 2.14\times10^{-2}\,\text{cm}^3\,\text{h}^{-1}\,\text{mg}^{-1}$ and $K_{m3}/k_4 = 48.3\,\text{h}\,\text{mg}\,\text{cm}^{-3}$. Good agreements were observed between the experimental data and the theoretical results as calculated from the equation. However, kinetic parameters of $k_2/K_{m1} = 4.2\times10^{-2}\,\text{cm}^3\,\text{h}^{-1}\,\text{mg}^{-1}$ and $K_{m3}/k_4 = 1050\,\text{h}\,\text{mg}\,\text{cm}^{-3}$ were reported previously for a system using 4-morpholine ethanol as an acyl acceptor and Lipase MY as the biocatalyst.[15] Since CR lipase and Lipase MY have a similar initial time-course conversions for $S(+)$-naproxen (Fig 1), a comparison of those parameters indicates that changing the alcohol from 4-morpholine ethanol to di(ethylene glycol) resulted in a large effect on the enzyme performance, as reflected on the two-fold decrease of k_2/K_{m1} in the acylation step and 22-fold increase of k_4/K_{m3} in the deacylation step.

4.4 Time-course analysis

Some experimental data for the time-course conversions of X_A and X_B with an alcohol concentration of less than $2\,\text{mmol}\,\text{dm}^{-3}$ are presented in Figs 4 and 5. By using the time-course data of X_B and eqn (5), the kinetic parameters for $R(-)$-naproxen are estimated as $k'_f = 2.5\times10^{-2}\,\text{h}^{-1}$ and $k'_2/K'_{m1} = 2.3\times10^{-4}\,\text{cm}^3\,\text{h}^{-1}\,\text{mg}^{-1}$, respectively. The equilibrium constant is found as 0.17 from the equilibrium conversion of $S(+)$-naproxen and eqn (6).

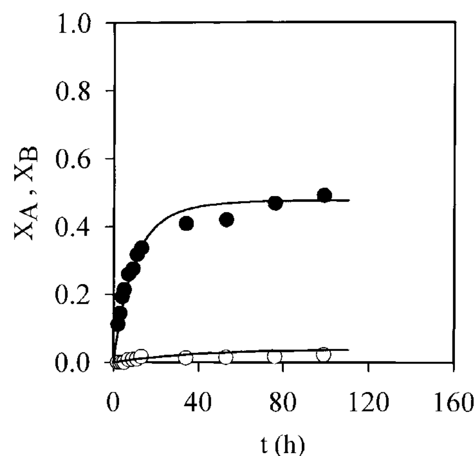


Figure 5. Time-course conversions with CR lipase as biocatalyst: $(S_A)_0=(S_B)_0=0.4\,\text{mmol}\,\text{dm}^{-3}$, $(C)_0=1.5\,\text{mmol}\,\text{dm}^{-3}$, $(E_t)=5\,\text{mg}\,\text{cm}^{-3}$, 37 °C. Experimental data: X_A, ●; X_B, ○; theoretical value,—.

The remaining enzyme deactivation constant, k_f, for $S(+)$-naproxen is estimated as $4.5 \times 10^{-2} h^{-1}$, which was 1.8-fold that for $R(-)$-naproxen. This indicates that the lipase may deactivate more rapidly when $S(+)$-naproxen is the substrate. Similar results were found previously when 4-morpholine ethanol was the acyl acceptor.[15,16] Moreover, it has been proposed that the gradual unfolding of the enzyme molecule due to the formation and adsorption of water in cyclohexane may account for the difference of enzyme deactivation constants for both enantiomers. In general, good agreements between the experimental data and the theoretical results are illustrated in Figs 4 and 5. This implies that the simplification of rate equations to eqns (7) and (8) is a good approximation for predicting the time-course conversion for both enantiomers.

4.5 Reaction with excess naproxen

Since $S(+)$-naproxen is not an inhibitor of the lipase (Fig 3), one may use a high naproxen concentration to enhance the productivity of the desired $S(+)$-ester. When excess racemic naproxen in the presence of a solid substrate suspension is added to cyclohexane, a saturated concentration for each enantiomer is maintained as long as the dissolution rate of the substrate is much higher than the reaction rate. Then, regardless of the product formation, each enantiomer may react with a constant rate to keep $ee_P = (44-1)/(44+1) = 0.96$. Figure 6 illustrates the time-course concentration and ee_P for the products by using naproxen concentration as the parameter. As expected, when $3\,mmol\,dm^{-3}$ naproxen is used, an approximately two-fold increase of $S(+)$-ester concentration is obtained at any specific time. However, the ee_P value does not remain at the theoretical value of 0.96. Similar results were found when a higher naproxen concentration of 5 mM was employed (data not shown). Two possible explanations were suggested for the elucidation of this deviation. First, since the deactivation constant of $S(+)$-naproxen is higher than that of $R(-)$-naproxen, the effective enzyme for the former decreases more rapidly than that for the latter. This will certainly lower the rates of $S(+)$-naproxen and hence the ee_P value as the reaction proceeds. Secondly, when the dissolution rate of $S(+)$-naproxen is not much higher than the reaction rate, (S_A) will decrease with time, as illustrated in Fig 7. However, this is not the case for $R(-)$-naproxen due to the much lower reaction rate. Of course, the formation of water may also enhance the solubility of naproxen, resulting in the increase of $R(-)$-naproxen concentration with time. The distribution of (R)-, (S)-naproxen as well as the solid racemate in the phase diagram of the present system as (S)-naproxen gradually depletes is another possibility to give a higher (R)-naproxen concentration Then, the mass action effect of the (R)-substrate concentration on the reaction rate will obviously decrease the ee_P value. Therefore, a further study of decreasing the reaction rate by applying less enzyme, increasing the dissociation rate by enhancing the stirring rate and using finer naproxen particles, or investigating the phase diagram of the system is needed.

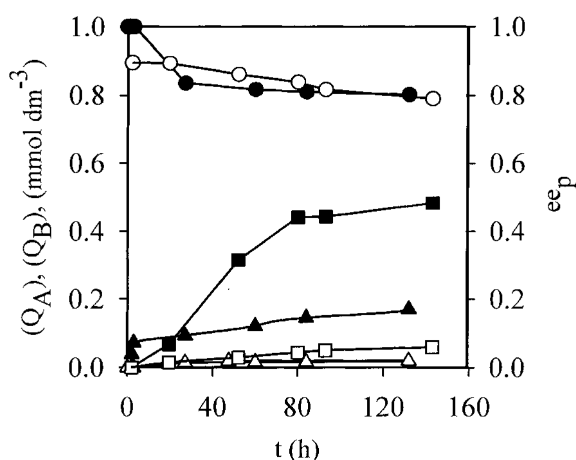


Figure 6. Time-course ee_P and concentrations of $R(-)$- and S(+)-ester with CR lipase as biocatalyst: $(C)_0 = 2\,mmol\,dm^{-3}$, $(E_t) = 5\,mg\,cm^{-3}$, 37 °C. For $(S_A)_0 = (S_B)_0 = 0.4\,mmol\,dm^{-3}$: (Q_A), ▲; (Q_B), △, ee_P, ●. For $(S_A)_0 = (S_B)_0 = 1.5\,mmol\,dm^{-3}$: (Q_A), ■; (Q_B), □; ee_P, ○.

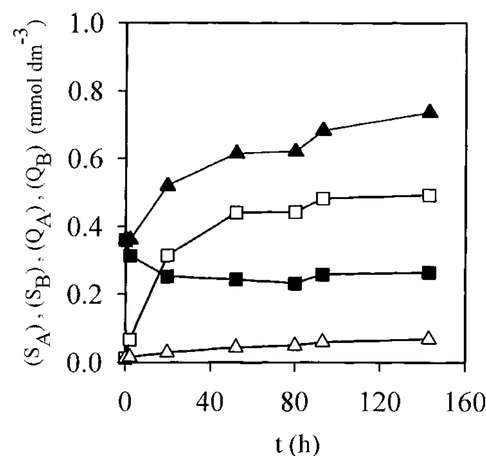


Figure 7. Time-course concentrations of $R(-)$- and $S(+)$-naproxen and their esters with CR lipase as biocatalyst: $(S_A)_0 = (S_B)_0 = 1.5\,mmol\,dm^{-3}$, $(C)_0 = 2\,mmol\,dm^{-3}$, $(E_t) = 5\,mg\,cm^{-3}$, 37 °C; (S_A), ■; (S_B), ▲; (Q_A), □; (Q_B), △.

4.6 R(-)-Naproxen extraction and racemization

The present enzymatic synthesis process will become more competitive if the undesired substrate of $R(-)$-naproxen can be separated and racemized. In a preliminary study, an aqueous solution of pH higher than 10.7 was used to extract the remaining naproxen from a reaction medium of $(S) = 0.42\,mmol\,dm^{-3}$, $(Q) = 0.22\,mmol\,dm^{-3}$, $ee_P = 0.61$ and $ee_S = 0.32$. With the best extraction condition (extraction time of 5 min, organic/aqueous ratio (v/v) of 3 and pH 11.5), the organic phase retains about 94% of the product, with a trace of naproxen. However, one cannot use a di(ethylene glycol) solution to extract the remaining naproxen, since the ester product can also dissolve in it.

After drying the aqueous phase and adding ethylene

glycol, the solution was racemized at 120 °C for 11 h, acidified to pH 1, and re-extracted with cyclohexane. An HPLC analysis of the organic phase indicated that an overall recovery of 59% with $ee_S = 0.12$ for the racemic naproxen was obtained. Of course, a higher recovery is possible if further re-extraction from ethylene glycol solution is performed. Moreover, a higher degree of racemization with a lower ee_S might be obtained if a longer time and/or a higher temperature were applied during racemizing.

5 CONCLUSIONS

A lipase-catalyzed enantioselective esterification process in cyclohexane was developed for the synthesis of useful *S*(+)-naproxen ester prodrugs containing the moiety of *N*,*N*-dialkylamino, ethylene glycol or alkyl ether of ethylene glycol. However, only di(ethylene glycol), with a high enantiomeric ratio of 44, was selected as the suitable acyl acceptor. A reversible ping-pong Bi Bi mechanism has been employed to elucidate the enzymatic behaviors of the initial conversion rate for *S*(+)-naproxen and the time-course conversions for both enantiomers. Improvement of the enzyme activity was demonstrated when alcohol in excess of its cyclohexane solubility limit was used. The application of excess racemic naproxen in the presence of solid substrate suspensions showed enhanced productivity and enantioselectivity for the desired *S*(−)-ester. A preliminary study on finding the extraction conditions indicated that *R*(+)-naproxen could be separated from the organic phase and effectively racemized.

ACKNOWLEDGEMENTS

Financial support from the Chinese National Science Council with Grant No NSC-84-2214-E006-026 is appreciated.

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