# Surfactant Enhancement of (*S*)-Naproxen Ester Productivity from Racemic Naproxen by Lipase in Isooctane

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In the enantioselective esterification of racemic Naproxen with trimethylsilyl methanol in isooctane by *Candida cylindracea* lipase, improvements in (*S*)-naproxen ester productivity and enzyme selectivity were demonstrated by adding bis(2-ethylhexyl) sodium sulfosuccinate (AOT) as the best surfactant. The effect of water content on the enhancement of enzyme activity was elucidated from the reduced adsorption of surfactant molecules on the lipase. A competitive inhibition by the alcohol and a noncompetitive inhibition by the surfactant to the enzyme were found from the kinetic analysis. By using a two-phase extraction, a complete separation of the surfactant from the organic solution was obtained. © 1996 John Wiley & Sons, Inc.
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## INTRODUCTION

Recently, much effort has centered on the synthesis of optically pure drugs and agrochemicals as they are more effective and have fewer side effects compared with their racemates.[10,18] Profens (2-arylpropionic acids), an important group of nonsteroidal anti-inflammatory drugs, have an asymmetric center at the 2-position, and the (*S*)-enantiomer often shows higher activity than the antipode. Naproxen [2-(6-methoxy-2-naphthyl) propionic acid] is currently the only member marketed as a single enantiomer and has been the subject of intense study.[4,17] Various strategies have been proposed to resolve the racemic naproxen by using selectively diastereoisomeric crystallization or chromatographic separation techniques. Recently, enzymatically enantioselective hydrolysis of naproxen esters by lipases has been addressed, in which high enantioselectivity for the (*S*)-enantiomer was obtained by using *Candida cylindracea* lipase.[2,3,5,7,15,16]

Lipase is able to catalyze esterification or transesterification reaction if water is replaced by a microaqueous organic medium.[1,8,12] In a previous report, an enzymatic resolution process was developed which produced naproxen enantiomers via esterification in isooctane.[20] The lipase from *C. cylindracea* was found to preferentially esterify (*S*)-naproxen and showed a high enantioselectivity and reactivity by using trimethylsilyl methanol as an acyl acceptor. Since the solubility of naproxen in isooctane is very low at ambient temperature, other organic solvents with lower hydrophobicity were tested to enhance naproxen solubility and productivity.[21,23] Results indicated that the enzyme activity and selectivity rapidly decreased as the solvent hydrophobicity decreased. Therefore, further studies on the reaction conditions, leading to a higher productivity, enzyme reactivity, and enantioselectivity, are still necessary if this enzymatic resolution method is to compete with the conventional diastereomeric crystallization process.

Here, increases in the productivity of (*S*)-naproxen ester from enantioselective esterification of racemic naproxen in isooctane by lipase were studied. The strategy was to find a suitable surfactant added to the reaction medium to yield a reaction with improved productivity. Effects of the surfactant concentration and water content on the enzyme activity were first presented. Then, the improved productivity of (*S*)-naproxen ester was demonstrated by comparing the results without adding the surfactant. The kinetic model that was modified from the previous report[22] was also investigated.

## MATERIALS AND METHODS

### Materials

Optical pure (*S*)-naproxen [(*S*)-2-(6-methoxy-2-naphthyl) propionic acid] and lipase (triacylglycerol ester hydrolases, EC 3.1.1.3) from *Candida cylindracea* (type VII, 860 units $mg^{-1}$ solid) were purchased from Sigma (St. Louis, MO). High-performance liquid chromatography (HPLC) grade organic solvents were dried over 4-Å molecular sieves and used without further purification. Anionic surfactant of bis(2-ethylhexyl) sodium sulfosuccinate (AOT), nonionic surfactant of tetraethylene glycol dodecyl ether ($C_{14}F_4$), cationic surfactant of cetyltrimethylammonium bromide (CTAB), and other chemicals were the highest purity commercially available.

### Analytical Procedure

The esterification was monitored by HPLC using a chiral column [Regis (S, S) Whelk-01, Morton Grove,

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IL] capable of separating the surfactant, (*R*)- and (*S*)-naproxen, and their esters without derivatization. The mobile phase (0.4 mL $min^{-1}$) was composed of *n*-hexane:ethanol:acetic acid (80:20:0.5). Ultraviolet (UV) detection at 254 nm was used for quantification at ambient temperature.[19]

Using a Shimadazu UV-160 spectrophotometer at 220 nm and 37°C, a calibration curve related the absorptivity to the concentration of trimethylsilyl methanol in isooctane was prepared. Thus the partition coefficient of the alcohol between isooctane and a 67 m*M* phosphate buffer (pH 7) was determined as 1.6.

### Reaction in Isooctane

Racemic naproxen was prepared by racemizing (*S*)-naproxen at 140°C in ethylene glycol with sodium hydroxide.[14] Unless specified, to 3.0 m*M* of the racemic naproxen and 100 m*M* of trimethylsilyl methanol dissolved in 20 mL of isooctane with or without AOT was added 140 mg of the crude lipase. The resultant mixture was stirred with a magnetic stirrer at 37°C. Samples were removed for HPLC analysis at different time intervals, from which conversions for the racemate and each enantiomer were calculated. Similar experiments were carried out except that different volumes of the above phosphate buffer were added.

More experiments were performed at the water content $W_o$ (molar ratio of water to the surfactant) of 5.56, where AOT and substrate concentrations were varied as parameters. From the specific initial rates, the kinetic parameters and their combinations for each enantiomer were determined. Similar experiments by using $C_{14}F_4$ as the surfactant, with or without adding the above phosphate buffer, were also carried out.

### Reaction in Reversed Micelles

A 0.6-mL portion of the above buffer ($w_o$ = 5.56) that contained lipase (20 mg $mL^{-1}$) was injected (i.e., injection method) into 20 mL AOT/isooctane reversed micelles, where the acid, alcohol, and AOT concentrations were 3, 100, and 300 m*M*, respectively. Stirring was taken in a vortex mixer until the solution became clear (ca. 30 s). Samples were withdrawn from the solution at 37°C for HPLC analysis at different time intervals.

Similar experiments were carried out except that the same enzyme mass (12 mg) was directly added to the reversed micelles (i.e., solid-state solubilization method) that contained the same amounts of the above surfactant, substrates, and water content. Therefore, the lipase would gradually solubilize into the inner water pool of the reversed micelles. Using the injection method, we also carried out the experiment at various AOT concentrations, where the enzyme mass and the water content were fixed at 4.32 mg and $W_o$ = 8, respectively.

### Adsorption of Water and AOT on Enzyme Mass

Using a Karl Fischer titrator, we have measured the water contents in 219 mg lipase, 20 mL isooctane containing 300 m*M* AOT, and the latter solution with 0.6 mL of the above buffer ($W_o$ = 5.56). Moreover, to 20 mL isooctane that contained 300 m*M* AOT and 0.6 mL of the above buffer was added 219 mg of the lipase. The solution was then mixed by a magnetic stirrer for an hour at 37°C. After centrifuging, the water content in the superstratum was measured.

Four milliliters of isooctane that contained 20 m*M* AOT and 200 mg lipase was mixed by a magnetic stirrer for an hour at 37°C. After centrifuging, the AOT concentration in the superstratum was measured by HPLC analysis. A similar experiment was carried out in the above solution in which the water content $W_o$ was 5.56.

### AOT Separation by Two-Phase Extraction

Aqueous solutions of different pH and ionic strength were prepared by adding suitable amounts of sodium hydroxide and sodium chloride into the distilled water. After filtrating the lipase from the reaction medium, equal amounts of the aqueous solution and the filtrate (e.g., 10 mL) were mixed overnight at different temperatures. Then, AOT and naproxen concentrations in the supertratum were measured by HPLC analysis.

## MODEL DEVELOPMENT

According to the previous analysis, a Ping-Pong Bi–Bi mechanism with the alcohol as a competitive inhibitor could be applied to describe the kinetic behavior of lipase-catalyzed esterification reaction.[22] By further assuming that AOT acted as a noncompetitive inhibitor, the mechanism for the (*S*)-enantiomer might be expressed as

$$E + S_A \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES_A \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} H_2O + E^*S_A \qquad (1)$$

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

$$E + C \underset{k_{-5}}{\overset{k_5}{\rightleftharpoons}} EC \qquad (3)$$

$$E(ES_A,\ E^*S_A,\ or\ E^*S_AC) + I \underset{k_{-6}}{\overset{k_6}{\rightleftharpoons}} EI\ (ES_AI,\ E^*S_AI,\ or\ E^*S_ACI) \qquad (4)$$

For the (*R*)-enantiomer, the subscript *A* is changed to *B* in Eqs. (1), (2), and (4) and a superscript "′" (prime) is added to the kinetic parameters in Eqs. (1) and (2).

In the enantioselective resolution of a racemate in an organic solvent where an excess of the acyl acceptor is presented to obtain a high enantiomeric excess for the product, one might assume irreversible kinetics as the conversion is not high. Thus the rate equation for each enantiomer was derived as follows:

$$V_A = \frac{[k_2/K_{m1}]^*(S_A)\,(E)}{D} \quad V_B = \frac{[k_2'/K_{m1}']^*(S_B)\,(E)}{D} \tag{5}$$

where the denominator and the kinetic constants and their combinations were defined as

$$D = 1 + \frac{(C)}{K_c^*} + \left\{\frac{(S_A)\,[1 + k_2/k_4]}{K_{m1}} + \frac{(S_B)\,[1 + k_2'/k_4']}{K_{m1}'} + \frac{1}{(C)}\left[\frac{[k_2/K_{m1}]^*(S_A)}{[k_4/K_{m3}]^*} + \frac{[k_2'/K_{m1}']^*(S_B)}{[k_4'/K_{m3}']^*}\right]\right\} \tag{6}$$

$$K_{m1} = [k_{-1} + k_2]/k_1 \qquad K_{m1}' = [k_{-1}' + k_2']/k_1'$$

$$K_{m3} = [k_{-3} + k_4]/k_3 \qquad K_{m3}' = [k_{-3}' + k_4']/k_3'$$

$$K_c = k_{-5}/k_5 \qquad K_I = k_{-6}/K_6 \qquad K_c^* = K_c\left[1 + \frac{(I)}{K_I}\right]$$

$$[k_2/K_{m1}]^* = \frac{k_2[1 + (I)/K_I]}{K_{m1}} \qquad [k_2'/K_{m1}']^* = \frac{k_2'[1 + (I)/K_I]}{K_{m1}'}$$

$$[K_{m3}/k_4]^* = \frac{Km_3[1 + (I)/K_I]}{k_4} \qquad [K_{m3}'/k_4']^* = \frac{K_{m3}'[1 + (I)/K_I]}{k_4'} \tag{7}$$

Then the enantiomeric ratio, defined as the ratio of $k_2/K_{m1}$ to $k_2'/K_{m1}'$, can be found once the kinetic constants are determined.

Equation (6) can be simplified if we assume that $K_{m1}$ and $K_{m1}'$ for esterification of organic acids by this lipase are much greater than $(S_A)$ and $(S_B)$, respectively. Indeed, this has been shown to be the case in numerous organic solvents including hydrocarbons.[13,22] Moreover, if the lipase has a high selectivity to the $(S)$-enantiomer, Eq. (6) reduces to

$$D = 1 + \frac{(C)}{K_c^*} + \frac{[k_2/K_{m1}]^*(S_A)}{[k_4/K_{m3}]^*(C)} \tag{8}$$

Therefore from Eqs. (5) and (8), the time course of $(S)$-naproxen conversion was derived as

$$t = -\ln\left\{\frac{k_f}{(E)}\left[\frac{1 + (C)/K_c^*}{[k_2/K_{m1}]^*}\ln(1 - X_A) - \frac{[K_{m3}/k_4]^*(S_A)_o X_A}{(C)}\right] + 1\right\} k_f^{-1} \tag{9}$$

where a linear deactivation kinetics for the enzyme with $k_f$ as the deactivation constant and a negligible consumption of the alcohol has been assumed.

If the experimental conditions are controlled such that $(C) \gg (S_A)$, Eq. (8) may be further simplified as

$$D = 1 + (C)/K_c^* \tag{10}$$

Therefore a linear relationship between the initial rate and the enantiomer concentration was observed from Eqs. (5) and (10), and Eq. (9) was reduced to

$$X_A = 1 - \exp\left\{\frac{-[k_2/K_{m1}]^*(E)\,[1 - \exp(-k_f t)]}{k_f[1 + (C)/K_c^*]}\right\} \tag{11}$$

Under these assumptions, Eq. (11) was also valid for $(R)$-naproxen, with the subscript $A$ replaced by $B$ and the superscript "′" (prime) added to $k_2$ and $K_{m1}$. By furthermore neglecting the enzyme deactivation in the organic solvent, Eq. (11) was simplified as

$$X_A = 1 - \exp\left\{\frac{-[k_2/K_{m1}]^*(E)t}{1 + (C)/K_c^*}\right\} \tag{12}$$

## RESULTS AND DISCUSSION

### Effect of AOT Concentration

Using pure isooctane as the reaction medium, the time course of naproxen conversion and the variation of enantiomeric excesses for the substrate and the product with the conversion are illustrated in Figure 1. Following the previous analysis[22] by using Eq. (12) for $X_A$ and the same form for $X_B$ with $(I) = 0$, the kinetic parameter combinations of $[k_2/K_{m1}]/[1 + 100K_c^{-1}]$ and $[k_2'/K_{m1}']/[1 + 100K_c^{-1}]$ were determined as $3.57 \times 10^{-2}$ mL mg$^{-1}$ h$^{-1}$ and $8.00 \times 10^{-5}$ mL mg$^{-1}$ h$^{-1}$, respectively. Then,

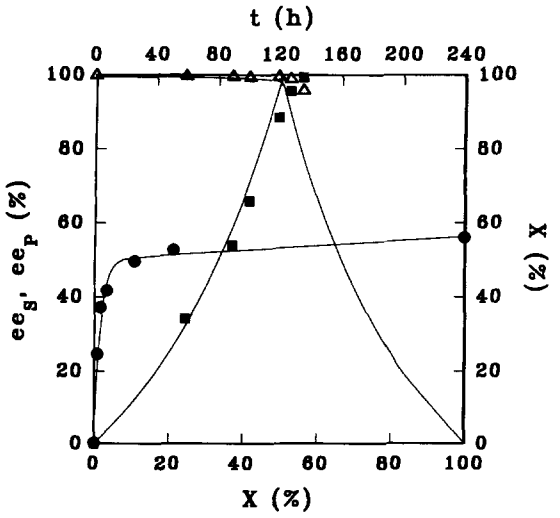


**Figure 1.** Time course of naproxen conversion and enantiomeric excesses for the substrate and the product in pure isooctane at 37°C; $(S)_0 = 0.2$ m$M$, $(C) = 100$ m$M$, $(E) = 7.0$ mg mL$^{-1}$. (●) For $X$ vs. $t$; (△) for ee$_p$ vs. $X$; (■) for ee$_s$ vs. $X$; solid line for theoretical results by using Eq. (12) for $X_A$, the same form for $X_B$, and the definitions of $X$, ee$_p$, and ee$_s$.

a high enantiomeric ratio of 425 for the enzyme was calculated. Good agreement between experimental and theoretical results is illustrated in the figure, where no enzyme deactivation ($k_f$ = 0) was found.

By adding AOT to isooctane to form dry reversed micelles, a two-order-of-magnitude enhancement of the saturated naproxen solubility at the surfactant concentration of 500 m*M* was obtained (Table I). When 100 m*M* $C_{14}F_4$ was applied in isooctane where no reversed micelles were formed, this solubility was only reduced from 4.79 m*M* (for 100 m*M* AOT) to 2.14 m*M*. Consequently, one may attribute the increase of naproxen solubility to the change of microenvironment around the naproxen molecule by the surfactant that presented either in reversed micelles or free molecules. The dissolved naproxen molecules seemed to exist among the reversed micelles, but not in the inner polar core or on the micellar membrane of the reversed micelles. However, there was still no evidence to prove it.

With the addition of AOT in the reaction medium, very low conversions for (*S*)-naproxen at various surfactant concentrations were observed (Table I). For examples, compared with the result of $X_A$ = 99% (calculated from $X$ = 49.5% and $ee_p$ = 99.6%) at 26 h in Figure 1, only 5% of the (*S*)-naproxen conversion at 23 h for (*I*) = 100 m*M* in Table I was found. An inhibitory behavior of the surfactant to the enzyme was also shown in the table, in which $X_A$ decreased as AOT concentration increased.

When a small amount of the above buffer ($W_o$ = 5.56) was added to the reaction medium, $X_A$ (and hence the enzyme activity) was greatly improved after comparing the time-course results at any AOT concentration in Table I and Figure 2. Possible explanations were as follows:

1. Lipase might absorb some water that was added to the solution and increased the enzyme activity.
2. Water would solubilize into the inner core of the dry reversed micelles to form the inner water pool. This could provide an environment to solubilize some alcohol that acted as a competitive inhibitor to the enzyme. Thus the effective alcohol concentration around the lipase was reduced. This would certainly increase the enzyme activity.

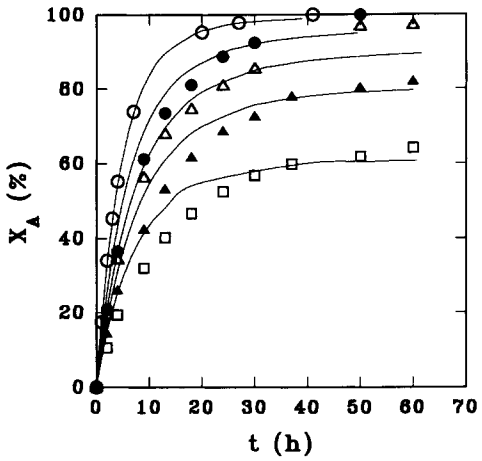


**Figure 2.** Time course of (*S*)-naproxen conversion in isooctane at 37°C with (*I*) as parameter; $(S)_0$ = 3 m*M*, (*C*) = 100 m*M*, (*E*) = 7 mg $mL^{-1}$, $W_0$ = 5.56. (○) For (*I*) = 100 m*M*; (●) for (*I*) = 200 m*M*; (△) for (*I*) = 300 m*M*; (▲) for (*I*) = 400 m*M*; (□) for (*I*) = 500 m*M*; solid lines for theoretical results by using Eq. (9).

3. Some enzyme would solubilize into the water pool of the reversed micelles. Thus the resultant lipase in the reversed micelles may possess the so-called "superactivity" to the substrate to enhance the enzyme activity.[11]
4. The AOT in isooctane may exist in the form of dry reversed micelles, free molecules, and those adsorbed on the enzyme mass. When water was added, the surfactant molecules might redistribute and reduce those adsorbed on the lipase. This certainly reduced the surfactant inhibition to the enzyme and increased the enzyme activity.

## How Enzyme Activity Was Improved by Adding Water

Since the crude lipase contains some hydrophilic additives such as lactose to absorb water, a water content of 8.34% (w/w) in the enzyme was found. This water was certainly enough to keep the enzyme in the active form to obtain the results of Figure 1. When 0.6 mL buffer ($W_o$ = 5.56) was added to isooctane with or without 219 mg lipase, the water content in the superstratum

**Table I.** Effect of AOT concentration on saturated naproxen solubility and time course of (*S*)-naproxen conversion[a] at 37°C.

| | (*I*) = 0 | (*I*) = 100 m*M* | (*I*) = 200 m*M* | (*I*) = 300 m*M* | (*I*) = 400 m*M* | (*I*) = 500 m*M* |
|---|---|---|---|---|---|---|
| Saturated solubility, m*M* | 0.20 | 4.79 | 8.44 | 14.9 | 19.1 | 21.1 |
| $X_A$, % | | | | | | |
| 23 h | | 5.0 | 3.8 | 3.0 | 2.4 | 2.2 |
| 92 h | | 13.7 | 10.9 | 9.8 | 7.7 | 7.2 |
| 188 h | | 18.3 | 15.8 | 14.4 | 11.9 | 10.6 |

[a] $(S)_0$ = 3 m*M*, (*C*) = 100 m*M*, and (*E*) = 7 mg $mL^{-1}$ in 20 mL isooctane.

**Table II.** Water and AOT contents in the organic solution at 37°C.

| Water content | 8.34% (w/w)[a] | 0.73% (w/v)[b] | 3.58% (w/v)[c] | 3.66% (w/v)[d] |
|---|---|---|---|---|
| AOT concentration, m*M* | 20.0[e] | 17.9[f] | 19.0[g] | |

[a]In 219 mg lipase.
[b]$(I)$ = 300 m*M* in 20 mL isooctane.
[c]$(I)$ = 300 m*M* and $W_0$ = 5.56 (0.6 mL buffer) in 20 mL isooctane.
[d]In 219 mg lipase, $(I)$ = 300 m*M* and $W_0$ = 5.56 in 20 mL isooctane.
[e]$(I)$ = 20 m*M* in 4 mL isooctane.
[f]200 mg lipase and $(I)$ = 20 m*M* in 4 mL isooctane.
[g]200 mg lipase, $(I)$ = 20 m*M* and $W_0$ = 5.56 ($8.0 \times 10^{-3}$ mL buffer) in 4 mL isooctane.

hardly changed (Table II). Since the water solubility in pure isooctane is very low, this result indicated that all the added water was solubilized into the reversed micelles. Then the explanation of water absorption by the lipase to increase the enzyme activity was abandoned.

The equilibrium concentrations of trimethylsilyl methanol in isooctane and the above buffer at 37°C have been measured, with which the partition coefficient of 1.6 was calculated. As an example in the case of 100 m*M* AOT in Figure 2, only 1.8 molar percent of the alcohol was extracted into the water pool of the reversed micelles. Thus, the second explanation of reducing the effective alcohol concentration around the lipase to enhance the enzyme activity could be ruled out.

With reversed micelles as the reaction media, the results in Table III obviously indicated that, compared to the injection method, a 27-fold decrease of the specific initial rate was obtained by using the solid-state solubilization method. This was due to the gradual solubilization of the lipase from the organic solvent into the reversed micelles and deactivated the enzyme. Moreover, comparing the specific initial rate of $5.80 \times 10^{-4}$ m*M* mL $h^{-1}$ $mg^{-1}$ in Table III for the solid-solubilization method, with that of $2.42 \times 10^{-2}$ m*M* mL $h^{-1}$ $mg^{-1}$ calculated for the case of $(I)$ = 300 m*M* in Figure 2, one may neglect the activity contribution of the enzyme that solubilized into the reversed micelles in the latter. Then, the third explanation of enzyme solubilization into the reversed micelles to increase the conversion in Figure 2 was ruled out.

Table III also demonstrated the effect of AOT concentration on the time-course conversion and specific initial rate for (*S*)-naproxen by using the injection method. No (*R*)-naproxen ester was found during the experiment, which implied a good enantioselectivity of the enzyme to the (*S*)-isomer. However, a rapid enzyme deactivation was observed, resulting in nearly no improvement of $X_A$ at the time of more than 91 h in each case. Moreover, an inhibitory behavior by the surfactant was found as the specific initial rate decreased on increasing the AOT concentration. Therefore, this lipase

**Table III.** Effects of enzyme solubilization method on the time course of conversion and specific initial rate for (*S*)-naproxen in reversed micelles at 37°C.

| Method | $X_A$, % (time, h) | | | | $V_A(E)^{-1} \times 10^2$, mM mL $h^{-1}$ $mg^{-1}$ |
|---|---|---|---|---|---|
| Solid-state solubilization | 0.56[a] (24) | 1.15 (49) | 2.59 (74) | 2.45 (99) | 0.058 |
| Injection | 6.44[a] (10) | 9.81 (25) | 12.3 (50) | 14.1 (100) | 1.61 |
| | 6.81[b] (24) | 7.93 (51) | 8.41 (91) | 8.54 (120) | 1.97 |
| | 2.03[c] (24) | 2.75 (52) | 3.21 (88) | 3.62 (120) | 0.59 |
| | 0.81[d] (24) | 1.06 (52) | 1.27 (88) | 1.58 (120) | 0.23 |
| | 0.56[e] (24) | 0.73 (52) | 0.87 (88) | 1.03 (120) | 0.16 |

[a]$(S)_0$ = 3 mM, $(C)$ = 100 m*M*, $(E)$ = 0.6 mg $mL^{-1}$, $(I)$ = 300 m*M*, $W_0$ = 5.56 (0.6 mL buffer) in 20 mL isooctane.
[b]Conditions as in footnote a except for $(E)$ = 0.216 mg $mL^{-1}$, $(I)$ = 100 m*M*, $W_0$ = 8 (0.288 mL buffer).
[c]Conditions as in footnote b except for $(I)$ = 200 m*M*, $W_0$ = 8 (0.576 mL buffer).
[d]Conditions as in footnote b except for $(I)$ = 300 m*M*, $W_0$ = 8 (0.864 mL buffer).
[e]Conditions as in footnote b except for $(I)$ = 400 m*M*, $W_0$ = 8 (1.15 mL buffer).

can be classified as a membrane active enzyme, as previously reported for the lipase from other sources.[9,14] After considering the applicable enzyme concentration, the enzyme specific activity, stability, and ease of recovery, we concluded that lipase solubilized in the present reversed micelles has an inferior performance to that suspended in isooctane with or without adding AOT.

Table II demonstrates the decrease of surfactant concentration in the superstratum from 20 to 17.9 m$M$ after adding 200 mg lipase into the solution that contained 20 m$M$ AOT. This indicated that some AOT molecules have adsorbed on the enzyme mass. However, by furthermore adding $8.00 \times 10^{-3}$ mL ($W_o$ = 5.56) of the buffer into the above solution, the surfactant concentration readjusted to 19 m$M$. This implied that 1.1 m$M$ of AOT molecules originally adsorbed on the enzyme mass dissolved back to the solution. Therefore, adding water to the reaction medium might redistribute AOT molecules. This obviously reduced the adsorption, and hence the inhibition, of the surfactant to the lipase and resulted in an increase of the enzyme activity.

## Effects of Water Content

Table IV demonstrates the effect of water content on the enzyme selectivity and activity. A high enantiomeric excess for the product ($ee_p > 99\%$) was found regardless of the amount of water in the solution. The strong activating effect of water was intriguing. The addition of as little as 0.1 mL buffer ($W_o$ = 2.78) was sufficient to reduce the amount of surfactant adsorbed on the lipase and increased about eightfold enzyme activity. In general, the increase of $W_o$ at a fixed AOT concentration might reduce more surfactant molecules that originally adsorbed on the enzyme and enhanced the enzyme activity. However, this also provided more water in the reversed micelles to solubilize enzyme and decreased the enzyme activity. Consequently, there might exist an optimal water content, such as $W_o$ = 5.56 in Table IV, leading to a maximum enzyme activity.

**Table IV.** Effect of water content on the activity and selectivity of the lipase at 37°C.[a]

| $W_0$ | Buffer, mL | $V_A(E)^{-1} \times 10^2$, mM mL $h^{-1}$ $mg^{-1}$ | $X_A$, % | $ee_S$, % | $ee_p$, % |
|---|---|---|---|---|---|
| 0 | 0 | 0.47 | 5.1[b] | 6.1 | 99.9 |
| 1.39 | 0.05 | 0.58 | 28.7 | 18.8 | 99.8 |
| 2.78 | 0.1 | 3.60 | 67.2 | 50.3 | 99.8 |
| 4.17 | 0.15 | 3.86 | 71.3 | 55.2 | 99.8 |
| 5.56 | 0.2 | 4.06 | 73.0 | 56.8 | 99.7 |
| 6.94 | 0.25 | 3.86 | 56.3 | 39.3 | 99.8 |
| 8.33 | 0.3 | 3.72 | 52.8 | 36.4 | 99.8 |

[a] $(S)_0$ = 3 m$M$, $(C)$ = 100 m$M$, $(E)$ = 7 mg $mL^{-1}$, and $(I)$ = 100 m$M$ in 20 mL isooctane.
[b] At 23 h; others at 7 h.

The same behaviors were observed in Figure 3 as AOT concentration was changed to 300 m$M$. Since more AOT molecules might adsorb on the lipase in this case, a higher water content of $W_o$ = 4.17 was required to give an improved enzyme activity. Thus one might deduce that at the lower AOT concentration of 20 m$M$, the water content with $W_o$ less than 2.78 was required to give the similar behavior. However, when more enzyme such as $(E)$ = 50 mg $mL^{-1}$ was applied at $(I)$ = 20 m$M$, a higher water content of $W_o$ = 12.5 was necessary, as illustrated in Figure 3. This implied that the amounts of solubilized enzyme in the reversed micelles and the adsorbed surfactant on the suspended lipase were greatly affected by the water content, AOT, and enzyme concentrations. Therefore, more research is necessary in order to quantitatively predict their influence on the enzyme activity.

Some examples were given to show the benefit of adding AOT to enhance the productivity. Since the saturated naproxen solubility in isooctane at 37°C was very low, only $8.29 \times 10^{-2}$ and $9.90 \times 10^{-2}$ m$M$ of $(S)$-naproxen ester at 8 and 26 h, respectively, were calculated (Fig. 1). However, more than a 13-fold increase of the productivity (1.11 and 1.47 m$M$ at 7 and 27 h, respectively) was obtained for the case of $(I)$ = 100 m$M$ in Figure 2. Of course, more productivity was obtainable when using a higher naproxen concentration at high AOT concentrations.

## Kinetic Study

Figure 4 illustrates the variation of specific initial rate of $(S)$-naproxen with the alcohol (or naproxen) concentration. Increasing the acid concentration resulted in a nearly linear enhancement of the activity. However, an asymmetric curve with a maximum at the alcohol concentration between 100 and 200 m$M$ was found, which

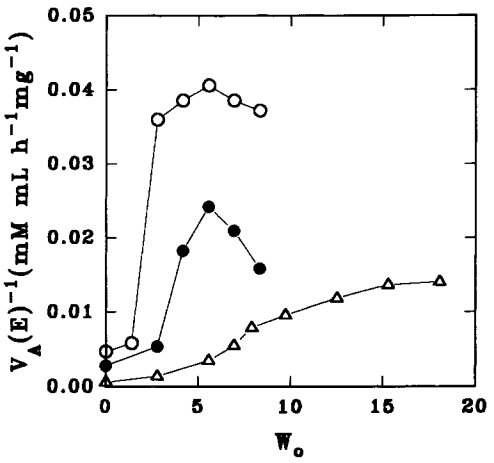


**Figure 3.** Variation of $V_A(E)^{-1}$ with $W_0$ in isooctane at 37°C; $(C)$ = 100 m$M$. (○) For $(S)_0$ = 3 m$M$, $(I)$ = 100 m$M$, $(E)$ = 7 mg $mL^{-1}$; (●) for $(S)_0$ = 3 m$M$, $(I)$ = 300 m$M$, $(E)$ = 7 mg $mL^{-1}$; (△) for $(S)_0$ = 0.5 m$M$, $(I)$ = 20 m$M$, $(E)$ = 50 mg $mL^{-1}$.

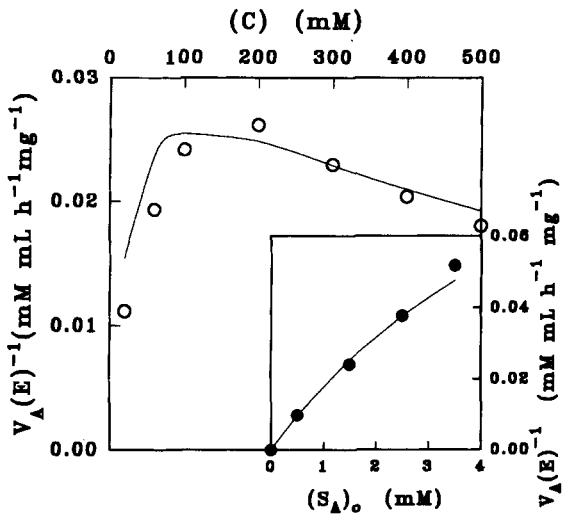


**Figure 4.** Variation of specific initial rate with substrate concentration in isooctane at 37°C; ($I$) = 300 m$M$, ($E$) = 7 mg $mL^{-1}$, $W_0$ = 5.56. (●) For $V_A(E)^{-1}$ vs. $(S_A)_0$ at ($C$) = 100 m$M$; (○) for $V_A(E)^{-1}$ vs. ($C$) at $(S_A)_0$ = 1.5 m$M$; solid lines for theoretical results by using Eqs. (5) and (8).

implied that the alcohol might have acted as an enzyme inhibitor. Thus using a nonlinear regression technique coupled with Eqs. (5) and (8) for ($S$)-naproxen, the kinetic parameter combinations of $[k_2/K_{m1}]^*$ = 2.50 × $10^{-2}$ mL $h^{-1}$ $mg^{-1}$, $K_c^*$ = 582 m$M$, and $[K_{m3}/k_4]^*$ = 736 mg h $mL^{-1}$ were determined. Agreements between the theoretical and the experimental results were obtained, as illustrated in Figure 4.

Figure 5 represents the variation of specific initial rate of ($S$)-naproxen with AOT concentration at various substrate concentrations. By using the above constants coupled with Eqs. (5), (7), and (8), the kinetic parameters and their combinations of $[k_2/K_{m1}]$ = 7.60 × $10^{-2}$ mL $h^{-1}$ $mg^{-1}$, $K_c$ = 193 m$M$, $K_I$ = 149 m$M$, and $[K_{m3}/k_4]$ = 244 mg h $mL^{-1}$ were found. Good agreements between the theoretical and experimental results are demonstrated in the figure. Therefore, the noncompetitive inhibition by AOT on the enzyme was confirmed.

Using the above kinetic constants and the data in Figure 2 with Eq. (9), we have determined the deactivation constants of 3.9 × $10^{-2}$, 5.3 × $10^{-2}$, 5.9 × $10^{-2}$, 7.1 × $10^{-2}$, and 9.8 × $10^{-2}$ $h^{-1}$ for ($I$) values of 100, 200, 300, 400, and 500 m$M$, respectively. Then the time course of the theoretical conversion for ($S$)-naproxen is represented in Figure 2, in which some deviation to the experimental data in high surfactant concentrations was observed. Since no enzyme deactivation was found in pure isooctane,[22] one might speculate that the present deactivation behavior was caused by the low activity of enzyme that gradually solubilized into the reversed micelles. Therefore, a higher deactivation constant might imply a high enzyme solubilization rate. Indeed, at a fixed water content of $W_o$ = 5.56, a high surfactant concentration would provide more reversed micelles to collide and solubilize the enzyme. This implied that the enzyme solubilization rate (and hence the enzyme deactivation rate constant) should increase with the surfactant concentration, as observed. More research is required in investigating the effect of enzyme mass, surfactant concentration, and water content on the rate and the equilibrium amount of enzyme that solubilized into the reversed micelles. After that, one could accurately monitor the time courses of enzyme solubilization and substrate conversion.

Figure 6 illustrates the time courses of both enantiomers with 50 mg $mL^{-1}$ enzyme (with a different lot number) concentration. For simplicity, Eq. (10) was applied to determine the kinetic parameter combinations

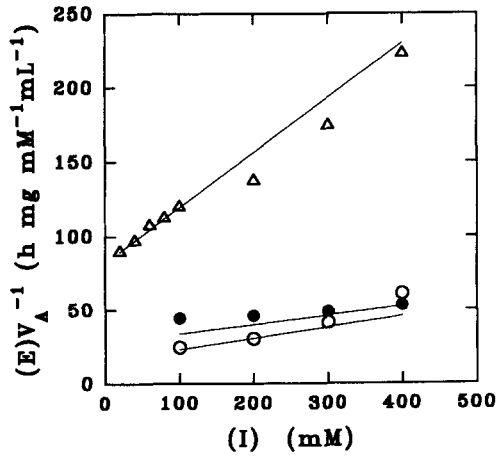


**Figure 5.** Variation of $(E)V_A^{-1}$ with ($I$) in isooctane at 37°C; ($E$) = 7 mg $mL^{-1}$, $W_0$ = 5.56. (○) For $(S)_0$ = 3 m$M$, ($C$) = 100 m$M$; (●) for $(S)_0$ = 3 m$M$, ($C$) = 400 m$M$; (△) for $(S)_0$ = 0.5 m$M$, ($C$) = 100 m$M$; solid lines for theoretical results by using Eqs. (5), (7), and (8).

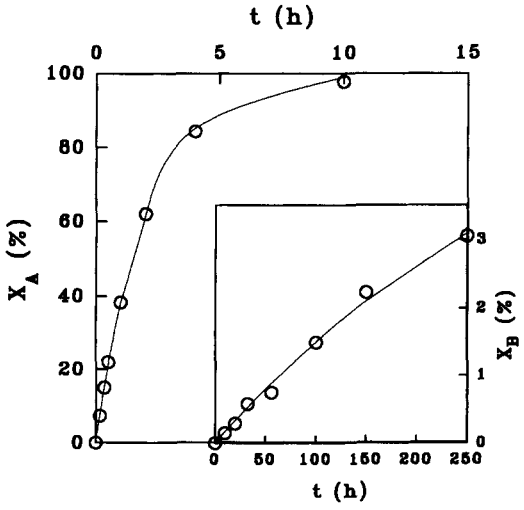


**Figure 6.** Time courses of ($R$)- and ($S$)-naproxen conversion in isooctane at 37°C; $(S)_0$ = 3 m$M$, ($C$) = 100 m$M$, ($I$) = 300 m$M$, ($E$) = 50 mg $mL^{-1}$, $W_0$ = 5.56. (○) For experiments; solid lines for theoretical results by using Eq. (11) for $X_A$ and the same form for $X_B$.

of $[k_2/K_{m1}]^*/[1 + 100/K_c^*]$ and $[k_2'/K_{m1}']^*/[1 + 100/K_c^*]$ as $9.62 \times 10^{-3}$ and $3.40 \times 10^{-6}$ mL mg$^{-1}$ h$^{-1}$, respectively. Then, a high enantiomeric ratio of 2830 was calculated. Moreover, the deactivation constant of $2.6 \times 10^{-3}$ h$^{-1}$, instead of $5.9 \times 10^{-2}$ h$^{-1}$ for $(I) = 300$ m$M$ in Figure 2, was determined. This indeed supported the above deduction that $k_f$ was not a deactivation constant; otherwise they should be equal. Therefore more studies on the real mechanism of enzyme solubilization into the reversed micelles was necessary.

We have tried adding other surfactants such as CTAB and $C_{12}F_4$. The former has a low solubility, and hence little improvement in naproxen solubility, in isooctane and was abandoned. A comparison of the time courses of (*S*)-naproxen conversion with AOT and $C_{12}F_4$ as the surfactants is illustrated in Figure 7. There was still no explanation for the lower $X_A$ in the latter, when isooctane saturated with the buffer (but not the anhydrous isooctane) was applied as the reaction medium. However, after considering the effect of surfactant on improving the saturated naproxen solubility and enzyme activity, we have chosen AOT as the best surfactant.

### AOT Separation by Two-Phase Extraction

The benefit of using AOT to enhance the (*S*)-naproxen productivity has been described. Therefore, the present synthesis process will be more competitive if AOT and the unreacted (*R*)-naproxen can be easily separated and racemized. A preliminary study indicated that an aqueous solution with 100 m$M$ of sodium chloride and at pH 9 or 10, or without adding the salt at pH 9, could be applied to completely extract AOT from the organic phase at 40°C. For example, by using the latter, AOT was completely separated and only 18% of (*R*)-naproxen remained in the organic phase. Further research on finding an optimal condition (i.e., suitable aqueous pH, temperature, and salt concentration) to completely extract the unreacted acid and AOT and that to racemize (*R*)-naproxen in the raffinate is necessary.

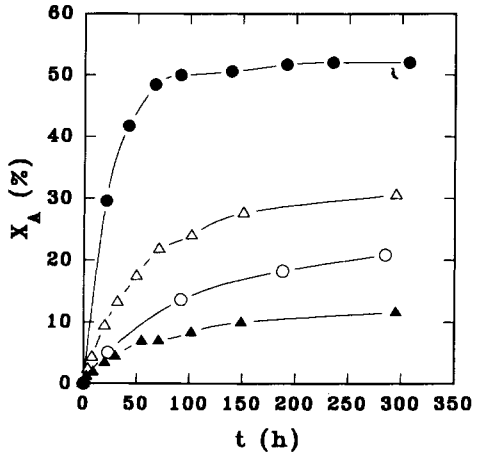


**Figure 7.** Time courses of (*S*)-naproxen conversion in isooctane at 37°C; $(C) = 100$ m$M$, $(I) = 100$ m$M$, $(E) = 7$ mg mL$^{-1}$. With AOT as the surfactant and $(S)_0 = 3$ m$M$: (●) for $W_0 = 2.78$; (○) for $W_0 = 0$. With $C_{14}F_4$ as the surfactant and $(S)_0 = 2$ m$M$: (▲) for isooctane saturated with the buffer solution; (△) for $W_0 = 0$.

## CONCLUSIONS

The surfactant effect on the lipase-catalyzed enantioselective esterification of racemic naproxen with trimethylsilyl methanol at 37°C in isooctane was investigated. Naproxen solubility in the solvent has been improved by adding AOT as the best surfactant. The low enzyme activity by the strong surfactant inhibition was relaxed by the addition of a phosphate buffer of pH 7. Investigation of the possible mechanism for this improvement indicated that, on adding the buffer, AOT in the solution might redistribute, resulting in the decrease of the surfactant adsorbed on the enzyme to decrease the inhibition.

The apparent fit of the specific initial rate and the time course for (*S*)-naproxen supported the proposed Ping-Pong Bi–Bi reaction mechanism with a competitive inhibition for the alcohol and a noncompetitive inhibition for AOT. Moreover, deactivation of the lipase in the organic solution was observed, which was attributed to the enzyme solubilization into the reversed micelles. The unfavorable surfactant inhibition on the enzyme activity could be compensated by the enhancement of naproxen solubility and resulted in an increase of (*S*)-naproxen ester productivity. A higher enantioselectivity for the enzyme has also been found when AOT was added. Moreover, a preliminary study showed that AOT could be completely removed from the organic solution by using a two-phase extraction.

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## NOMENCLATURE

| | |
|---|---|
| $(C)$ | alcohol concentration (m$M$) |
| $D$ | parameter defined in Eq. (6) or (8) |
| $(E)$ | enzyme concentration (mg mL$^{-1}$) |
| $ee_P$ | enantiomeric excess for product, defined as $[X_B - X_A]/[2 - X_A + X_B]$ |
| $ee_S$ | enantiomeric excess for substrate, defined as $[X_A - X_B]/[X_A + X_B]$ |
| $(I)$ | surfactant concentration (m$M$) |
| $K_{m1}$, $K_{m3}$, $K_c$, $K_I$ | kinetic constants defined in Eq. (7) (m$M$) |
| $k_f$ | deactivation constant (h$^{-1}$) |
| $k_2$, $k_4$, $k_{-1}$, $k_{-3}$, $k_{-5}$, $k_{-6}$ | kinetic parameters (mM mL h$^{-1}$ mg$^{-1}$) |
| $k_1$, $k_3$, $k_5$, $k_6$, $k_{-2}$, $k_{-4}$ | kinetic parameters (mL h$^{-1}$ mg$^{-1}$) |
| $(S)$, $(S_A)$, $(S_B)$ | concentrations of racemic naproxen, (*S*)-naproxen, and (*R*)-naproxen, respectively (m$M$) |
| $(S)_o$, $(S_A)_o$, $(S_B)_o$ | initial concentrations of $(S)$, $(S_A)$, and $(S_B)$, respectively (m$M$) |
| $t$ | time (h) |

| | |
|---|---|
| $V_A$, $V_B$ | reaction rates of (*S*)- and (*R*)-naproxen, respectively ($mM\ h^{-1}$) |
| $X$ | conversion of racemic Naproxen, $=[X_A + X_B]/2$ |
| $X_A$, $X_B$ | conversion of (*S*)- and (*R*)-naproxen, respectively |

*Superscripts*

| | |
|---|---|
| ' | kinetic parameters applied for (*R*)-naproxen |
| * | kinetic parameter combinations in Eq. (7) |

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