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# Lipase-catalyzed production of optically active (*S*)-flurbiprofen in aqueous phase reaction system containing chiral succinyl β-cyclodextrin

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**Abstract**

The lipase-catalyzed production of optically active (*S*)-flurbiprofen was carried out in a dispersion reaction-system induced by chiral succinyl β-cyclodextrin (suβ-CD). The optimal reaction conditions were 500 mM (*R*,*S*)-flurbiprofen ethyl ester ((*R*,*S*)-FEE), 600 units of *Candida rugosa* lipase per 1 mmol of (*R*,*S*)-FEE, and 1000 mM suβ-CD at 37 °C for 72 h. An extremely high enantiomeric excess of 0.98 and conversion yield of 0.48 were achieved in the dispersed aqueous phase reaction system containing chiral suβ-CD added as a dispenser and chiral selector. The inclusion complex formability of the immiscible substrate (*S*)- and (*R*)-form of FEE with suβ-CD was compared using a phase-solubility diagram, DSC, and $^1$H NMR. (*S*)-Isomer formed a more stable and selective inclusion complex with chiral suβ-CD. It was hydrolyzed much more selectively by lipase from *C. rugosa*, due to the selective structural modification through inclusion complexation with chiral suβ-CD.


*Keywords:* (*S*)-Flurbiprofen; (*R*,*S*)-Flurbiprofen ethyl ester; Lipase; Enantioselective hydrolysis; Succinyl β-CD; Chiral selector

## 1. Introduction

(*R*,*S*)-2-(3-Fluoro-4̃-biphenyl) phenyl-propionic acid [(*R*,*S*)-flurbiprofen], which belongs to a family of 2-arypropionic acid, is one of the well-known non-steroidal anti-inflammatory drug (NSAID) that exhibits anti-inflammatory, analgesic, and antipyretic properties [1,2]. The enantiopure (*S*)-enantiomer exhibits a stronger anti-inflammatory activity as high as 30-fold higher compared to *rac*-flurbiprofen; however, flurbiprofen is still currently produced in large quantities as a racemic mixture [3].

Recently, the enzymatic resolution of *rac*-2-arypropionic acid into the optically pure (*S*)-2-arypropionic acid using lipase (triacylglycerols ester hydrolases, EC 3.1.1.3) or esterase as the chiral catalyst has drawn much attention [4–7]. To increase the resolution ability of lipase and esterase, various methods have been attempted, such as the modification of lipase using organic solvent [8,9], immobilization of lipase [10], and utilization of surfactant [11] and crown ether [12] to overcome the low solubility of immiscible substrate *rac*-2-arypropionic acid.

Cyclodextrin (CD) is a doughnut-shaped molecule with a hydrophilic surface and hydrophobic inside, and it can form inclusion complexes with various immiscible hydrophobic guest molecules, including (*R*,*S*)-FEE [13]. The physical–chemical properties of guest molecules can be significantly modified through inclusion complexation. In our previous works, the inclusion complexation of CDs was applied for the lipase-catalyzed enzyme reaction in aqueous reaction systems, such as the hydrolysis of immiscible triolein [14] and esterification between insoluble oleic acid and *n*-butanol to form oleic acid butyl ester [15]. Furthermore, the selective inclusion complexation of CDs and their derivatives with optical isomer-pairs has also been ap-

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plied to the enantioselective hydrolysis of immiscible (*R*,*S*)-ketoprofen ethyl ester into optically active (*S*)-ketoprofen [16].

In this work, the chiral CDs and their derivatives were applied in the lipase-catalyzed enantioselective hydrolysis of (*R*,*S*)-FEE to the optically active (*S*)-flurbiprofen. The selective inclusion complexation of immiscible (*S*)- and (*R*)-FEE enantiomer with the chiral succinyl β-CD (suβ-CD) used as a dispenser or chiral selector was evaluated by a phase-solubility diagram, DSC, and $^1$H NMR. The kinetic parameters of the lipase-catalyzed hydrolysis reaction of (*S*)- and (*R*)-FEE were compared to clarify the characteristic of the enantioselective hydrolysis of (*R*,*S*)-FEE in the dispersed aqueous phase reaction system induced by suβ-CD.

## 2. Materials and methods

### 2.1. Lipase

Lipase from *Candida rugosa* (Sigma Co., St. Louis, USA) was used as the catalyst for the enantioselective hydrolysis of (*R*,*S*)-FEE. The activity was measured according to the method of Abramic et al. using triolein after a minor modification [16,17]. A 100 mM of triolein was emulsified by ultrasonication for 1 min in 50 mM phosphate buffer (pH 7.0) containing 0.3% gum Arabic, and then reacted with a certain amount of lipase of at 37 °C, 200 rpm for 30 min. The oleic acid produced was measured based on the calorimetric method of Lowry and Tinsley [18], and one unit of lipase was defined as the amount of lipase liberating 1 μmol of oleic acid per minute.

### 2.2. Synthesis of (R,S)-flurbiprofen ethyl ester and separation of (R)-enantiomer from rac-mixture

The (*R*,*S*)-FEE was synthesized from commercially available (*R*,*S*)-flurbiprofen (Kolon Co., Seoul, Korea) using an esterification reaction [19], where 30% (w/v) of (*R*,*S*)-flurbiprofen was dissolved in ethanol and esterified at 25 °C for 10 h in the presence of 3% (w/v) sulfuric acid. The resulting (*R*,*S*)-FEE was vacuum evaporated, then freeze-dried after washing with 1 M sodium bicarbonate solution.

The (*S*)-form isomer FEE was similarly synthesized from commercially available (*S*)-flurbiprofen (Sigma Co., St. Louis, USA). Meanwhile, the commercially unavailable (*R*)-isomer was separated before esterification as follows from the residual reaction mixture after the enantioselective hydrolysis [20]. The (*R*)-FEE and (*S*)-flurbiprofen extracted using two-fold ethyl acetate were washed again three times using 1 M sodium bicarbonate to remove any contaminated (*S*)-flurbiprofen up to a purity as high as 98%, as confirmed by HPLC.

### 2.3. CDs and their derivatives

The CDs and their derivatives used as the dispenser and chiral selectors were α-, β-, γ-CDs, glucose-α-CD, maltose-α-CD, methylated-β-CD, TRIMEB-CD, DIMEB-CD HP-β-CD, succinylated-β-CD, carboxylethylated-β-CD, acetylated-β-CD, 2-hydroxyl ethylated-β-CD, peracetylated-β-CD, octenylsuccinylated-β-CD, and phosphated-β-CD (Cyclolab Ltd., Budapest, Hungary).

### 2.4. Hydrolysis of (R,S)-flurbiprofen ethyl ester into (S)-flurbiprofen in aqueous phase reaction system containing chiral suβ-CD

A 50 mM of (*R*,*S*)-FEE dissolved in a sodium phosphate buffer (pH 7.0) was mixed with 100 mM suβ-CD, then sonicated for 1 min to form the inclusion complex. After adding 600 units of lipase from *C. rugosa* per 1 mmol (*R*,*S*)-FEE, it was hydrolyzed at 37 °C, 300 rpm for 72 h. The amount of lipase, the mixing ratio between (*R*,*S*)-FEE and suβ-CD, and concentration of (*R*,*S*)-FEE inclusion complex were changed accordingly.

### 2.5. Phase-solubility diagram for (S)- and (R)-FEE with suβ-CD

An excess amount of immiscible (*S*)- or (*R*)-FEE was dissolved in 1 ml of a phosphate buffer (pH 7.0) containing different amounts of suβ-CD for complexation at 25 °C, 200 rpm for 12 h. The (*S*)- or (*R*)-enantiomer dissolved in the aqueous phase after complexation was separated using a 0.45 μm membrane, then the (*S*)- and (*R*)-FEE were extracted from the inclusion complexes using a five-fold amount of chloroform. The dissolved (*S*)- and (*R*)-FEE were analyzed by HPLC to construct a phases-solubility diagram, and also the stability constant ($K_c$) was calculated according to the method of Higuchi–Connors [21].

### 2.6. Inclusion complexation, DSC thermodiagram, and $^1$H NMR

The inclusion complexation of (*S*)- or (*R*)-FEE with suβ-CD was carried out according to the freeze-drying method of Masashi et al. [22] as follows. The (*S*)- or (*R*)-FEE dissolved in 35 ml of a 50 mM phosphate buffer (pH 7.0) containing the same molar volume of suβ-CD was mixed with 100 μl of a 28% aqueous ammonium solution at 200 rpm for 24 h. The resulting inclusion complexes were freeze-dried, then dried at room temperature after being washed three times with ethyl ether to remove any residual impurities.

The thermodynamic stability of the inclusion complexes was evaluated by DSC (Seiko Co. Ltd., Tokyo, Japan) scanning at a speed of 10 °C/min from 25 to 250 °C. The structural features of the inclusion complexes were also analyzed by 500 MHz $^1$H NMR (Varian Inc., California, USA) at 25 °C

after being dissolved in 0.5 ml $D_2O$, then compared with the structural features of the native (*S*)- and (*R*)-FEE.

### 2.7. Kinetic evaluation of lipase-catalyzed hydrolysis of (S)- or (R)-FEE in dispersed aqueous phase reaction system containing suβ-CD

The (*S*)- or (*R*)-FEE was hydrolyzed separately in a dispersed aqueous phase reaction system induced by suβ-CD after mixing 50 mM of (*S*)- or (*R*)-FEE, 100 mM of suβ-CD, and 600 units of lipase/mmol substrates in 50 mM sodium phosphate buffer (pH 7.0) at 37 °C, 300 rpm for 48 h to evaluate the kinetic parameters.

### 2.8. Analytical methods

The (*R*,*S*)-FEE, (*S*)-flurbiprofen, and (*R*)-flurbiprofen were measured by HPLC (Gilson Inc., France) under the following conditions: column; RS-Tech TBB chiral column (0.46 cm × 25 cm), detection; UV (250 nm) spectrometer, mobile phase; (*n*-hexane/methyl *tert*-butyl ether/acetate: 6/4/0.01), and flow rate 2.0 ml/min. The retention time for (*R*,*S*)-FEE, (*S*)-flurbiprofen, and (*R*)-flurbiprofen was found at 1.65, 3.49, and 4.77 min, respectively. The enantiomeric excess (ee) representing the content of (*S*)-flurbiprofen in the reaction mixture and conversion yield (*C*) from (*R*,*S*)-FEE to (*S*)-flurbiprofen were calculated as follows.

$$\text{Enantiomeric excess (ee)} = [(S)\text{-profen} - (R)\text{-profen}]/[(S)\text{-profen} + (R)\text{-profen}]$$

$$\text{Conversion yield } (C) = [(S)\text{-profen} + (R)\text{-profen}]/[\text{initial}(R,S)\text{-profenethylester}]$$

## 3. Results and discussion

### 3.1. Selection of chiral CD derivatives for enantioselective hydrolysis of (R,S)-flurbiprofen ethyl ester

Different kinds of CD and their derivatives were applied for the lipase-catalyzed enantioselective hydrolysis of immiscible (*R*,*S*)-FEE using an aqueous phase reaction system. As shown in Table 1, the conversion yield and enantiomeric excess of enantiopure (*S*)-flurbiprofen were influenced either positively or negatively, mainly depending on the molecular structures of the CD and its derivative used as the disperser and chiral selector. The conversion yield of optically pure (*S*)-flurbiprofen increased after supplementing with α-CD, β-CD, glucose-α-CD, carboxylmethylated-β-CD and succinylated-β-CD, yet most preferably with suβ-CD from 0.041 to 0.362.

Table 1
Effect of CD and CD derivatives on conversion yield (*C*) and enantiomeric excess (ee)

| Kind of CDs | Conversion yield (*C*) | Enantiomeric excess (ee) |
|---|---|---|
| None | 0.041 | 0.613 |
| α-CD | 0.104 | 0.776 |
| β-CD | 0.043 | 0.733 |
| γ-CD | N.D. | N.D. |
| Glucose-α-CD | 0.106 | 0.905 |
| Maltose-α-CD | N.D. | N.D. |
| Methylated-β-CD | 0.005 | 0.500 |
| HP-β-CD | 0.004 | 0.714 |
| Succinylated-β-CD | 0.362 | 0.943 |
| Carboxylmethylated-β-CD | 0.086 | 0.861 |
| Acetylated-β-CD | N.D. | N.D. |
| 2-Hydroxylethylated-β-CD | N.D. | N.D. |
| TRIMEB-CD | 0.006 | 0.212 |
| DIMEB-CD | 0.004 | 0.327 |
| Peracetylated-β-CD | 0.006 | 0.710 |
| Octenylsuccinylated-β-CD | 0.007 | 0.745 |
| Phosphated-β-CD | N.D. | N.D. |

Reaction was carried out at 200 units of lipase/mmol of (*R*,*S*)-FEE, 50 mM of (*R*,*S*)-FEE, 50 mM sodium phosphate buffer (pH 7.0), 300 rpm, 37 °C, and for 72 h, but adding 50 mM of CD and CD derivatives.

The enantiomeric excess representing the content of (*S*)-flurbiprofen in the reaction mixture also increased significantly from 0.613 without any CD to 0.943 after supplementing with suβ-CD. These increments may be influenced by two factors: the size of the hydrophobic cavity in the native CDs and the functional groups in the CD derivatives, such as glucose, carboxylmethyl, or succinyl, as described in our previous work [16].

### 3.2. Optimization of aqueous phase reaction system containing chiral succinyl β-cyclodextrin

The lipase-catalyzed production of optically active (*S*)-flurbiprofen in an aqueous phase reaction system containing chiral suβ-CD was carried out under different reaction conditions: the amount of lipase, the molar mixing ratio of immiscible (*R*,*S*)-FEE to suβ-CD, and the amount of inclusion complex of immiscible (*R*,*S*)-FEE to suβ-CD. As illustrated in Fig. 1, as the amount of lipase from *C. rugosa* increased, the conversion yield and enantiomeric excess increased proportionally up to 600 units lipase per 1 mmol of (*R*,*S*)-FEE. The optimal molar mixing ratio for inclusion complexation between immiscible (*R*,*S*)-FEE and chiral selector suβ-CD was 1.0:2.0, respectively. Even at the extremely high immiscible substrate concentration 500 mM of (*R*,*S*)-FEE, a conversion yield as high as 0.48 could be achieved.

Fig. 2 compares the progress of the lipase-catalyzed enantioselective hydrolysis of (*R*,*S*)-FEE into (*S*)-flurbiprofen carried out with or without the chiral suβ-CD. The maximum conversion of 0.48, approaching the maximum yield of 0.50, was achieved after 96 h. In the aqueous reaction system

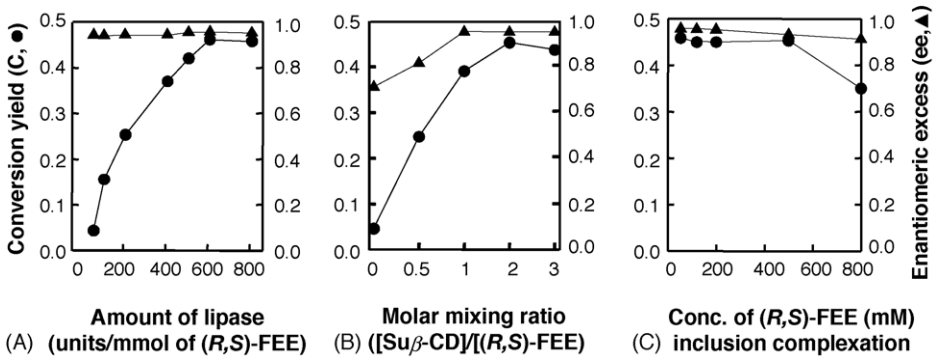


Fig. 1. Effect of reaction conditions on conversion yield (*C*) and enantiomeric excess (ee) in lipase-catalyzed enantioselective hydrolysis of (*R*,*S*)-flurbiprofen ethyl ester using succinyl β-CD as chiral selector. Reaction was carried out at 50 mM sodium phosphate buffer (pH 7.0), 37 °C, and 300 rpm for 72 h. (A) Amount of lipase, (B) molar mixing ratio between suβ-CD and 50 mM (*R*,*S*)-FEE, (C) concentration of (*R*,*S*)-FEE inclusion complexation.

dispersed by the chiral suβ-CD, a high enantiomeric excess of up to 0.90 was also maintained from the initial reaction stage, indicating a strong selectivity for the (*S*)-form of FEE rather than the (*R*)-form, and it increased steadily up to 0.98.

### 3.3. Phase-solubility of immiscible (S)- and (R)-flurbiprofen ethyl ester in aqueous phase reaction system containing chiral suβ-CD

The phase-solubility of the immiscible (*S*)- and (*R*)-FEE was compared using different amounts of the chiral suβ-CD, as shown in Fig. 3. The phase-solubility increased linearly according to the $A_L$ type of Higuchi–Connors [21], although the (*S*)-type FEE dissolved twice as well. The stability constants for (*S*)- and (*R*)-FEE, defined as the slope/intercept [1 − slope] in the phase-solubility diagrams, were also calculated as 265 and 246 $M^{-1}$, respectively, indicating a higher solubility and more stable inclusion complexation with the (*S*)-enantiomer [23,24]. As such, the higher solubility of (*S*)-FEE in the aliquot phase seemed to provide more opportunity for the lipase-catalyzed resolution to interact with (*S*)-FEE than with the less soluble (*R*)-FEE.

### 3.4. DSC and $^1$H NMR analysis of inclusion complexes of (S)- and (R)-flurbiprofen ethyl ester with suβ-CD

In DSC thermodiagram of the inclusion complex of (*S*)-FEE, the endothermic peak of raw guest molecule (*S*)-FEE (a) disappeared, and one endothermic peak of suβ-CD at position of 65.9 °C (d) was observed. Meanwhile, in the inclusion complex of (*R*)-FEE (e), two peaks were observed, the original (*R*)-FEE peak at the position of 188.6 °C (b) and that of suβ-CD at 65.9 °C (c) as shown in Fig. 4, providing a distinct evidence of a stronger and more stable inclusion complexation with (*S*)-FEE compared to (*R*)-FEE [25]. A stronger and more stable inclusion complexation can also be confirmed

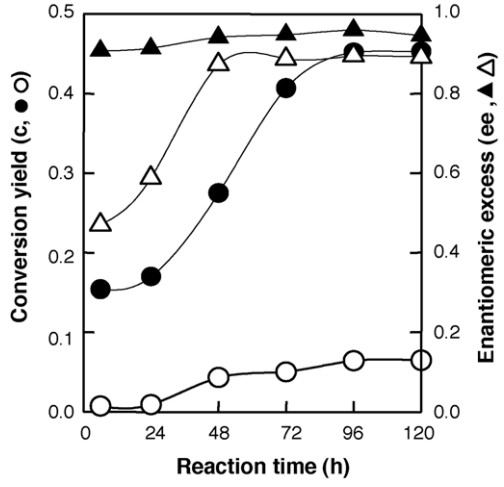


Fig. 2. Production profile of (*S*)-flurbiprofen in the aqueous phase reaction system containing chiral succinyl β-CD. Reaction was carried out at 600 units of lipase/mmol (*R*,*S*)-FEE, 500 mM (*R*,*S*)-FEE, 1000 mM of suβ-CD, 50 mM sodium phosphate buffer (pH 7.0), 37 °C, and 300 rpm for 120 h. Closed symbols (●, ▲): dispersed reaction system induced by chiral suβ-CD; open symbols (◯,△): aqueous reaction system.

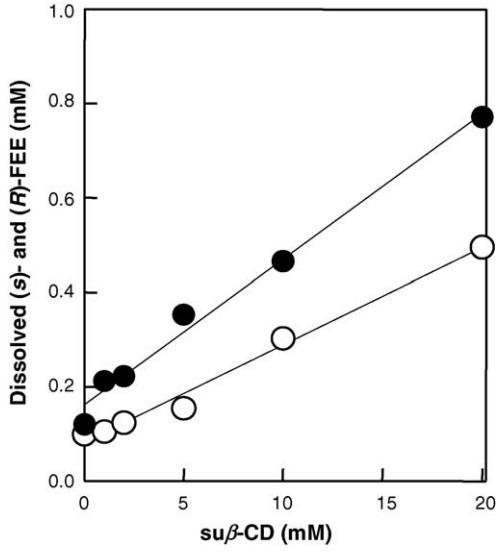


Fig. 3. Phase-solubility diagram of (*S*)-flurbiprofen ethyl ester and (*R*)-flurbiprofen ethyl ester according to the concentration of succinyl β-CD. (*S*)-FEE (●), (*R*)-FEE (◯).

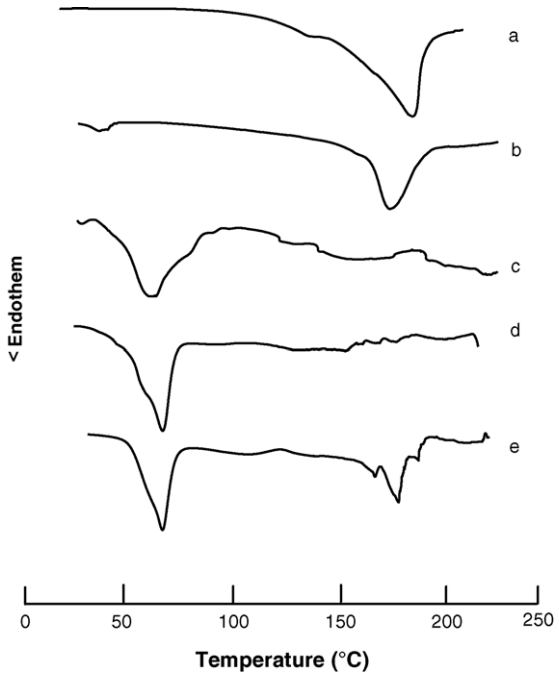


Fig. 4. DSC thermodiagrams of inclusion complexes of (*S*)- and (*R*)-flurbiprofen ethyl ester with succinyl β-CD. (a) (*S*)-FEE, (b) (*R*)- FEE, (c) suβ-CD, (d) inclusion complexes of (*S*)-FEE and (e) (*R*)-FEE.

by the fusion enthalpies ($\Delta H$), which were calculated to be 80.61 and 72.17 J/g, respectively.

It is known that chemical shift changes ($\Delta\delta$) in the inner protons H3 and H5 of a CD molecule can reflect the insertion of an aromatic group in hydrophobic guest molecules inside the macrocycle cavity, and can also be used as proof of inclusion complexation [26,27]. As shown in Table 2, the most remarkable $\Delta\delta$ values were absorbed by the internal protons H3 and H5 of the enantiomer-pairs, while the external protons H1, H2, and H4 presented lower $\Delta\delta$ values, providing again clear evidence of an inclusion complex interaction between the enantiomer pairs and the suβ-CD.

Table 2
$^1$H NMR chemical shifts and their changes of inclusion complexes of (*S*)- and (*R*)-flurbiprofen ethyl ester with succinyl β-CD

| | H1 | H2 | H3 | H4 | H5 | H6 |
|---|---|---|---|---|---|---|
| suβ-CD | 5.089 | 3.685 | 3.985 | 3.592 | 3.756 | 3.882 |
| suβ-CD/(*R*)-FEE | 5.088 | 3.677 | 3.965 | 3.592 | 3.728 | 3.881 |
| suβ-CD/(*S*)-FEE | 5.085 | 3.674 | 3.949 | 3.588 | 3.711 | 3.880 |
| $\Delta\delta$ (A) | 0.004 | 0.011 | 0.036 | 0.004 | 0.045 | 0.002 |
| $\Delta\delta$ (B) | 0.001 | 0.008 | 0.020 | 0.000 | 0.028 | 0.001 |

suβ-CD; free succinyl β-CD, (A); (suβ-CD) – (suβ-CD/(*S*)-FEE), (B); (suβ-CD) – (suβ-CD/(*R*)-FEE).

Additionally, $\Delta\delta$ values for H3 forming a ring near the larger opening of the cavity are related to the stability of inclusion complex, while those for H5 forming a ring near the smaller opening of the cavity indicate the penetration depth of aromatic group [28]. (*S*)-FEE showed larger $\Delta\delta$ values for H3 and H5, suggesting that it forms a more stable inclusion complex with an aromatic group of (*S*)-FEE, which penetrates more deeply into the cavity.

### *3.5. Kinetic analysis of enantioselective hydrolysis of (S)- or (R)-flurbiprofen ethyl ester in aqueous phase reaction system containing chiral suβ-CD*

The immiscible substrates of (*S*)- and (*R*)-FEE were hydrolyzed separately in both an aqueous phase reaction system and aqueous phase reaction system dispersed by the chiral suβ-CD (A), then the kinetic parameters were evaluated using an integrated form of the simple Michaelis–Menten equation (B), as depicted in Fig. 5. The lipase from *C. rugosa* cannot hydrolyze either (*S*)- or (*R*)-form FEE well when chiral suβ-CD was not added as a dispenser and chiral selector. However, the enantioselectivity to (*S*)-form FEE was enhanced significantly in the dispersed aqueous phase reaction system containing suβ-CD, but not to (*R*)-FEE.

The $V_m$ values for (*S*)-FEE increased remarkably from 0.046 to 0.785 mM/h after the addition of suβ-CD, whereas those for (*R*)-FEE only increased slightly from 0.033 to

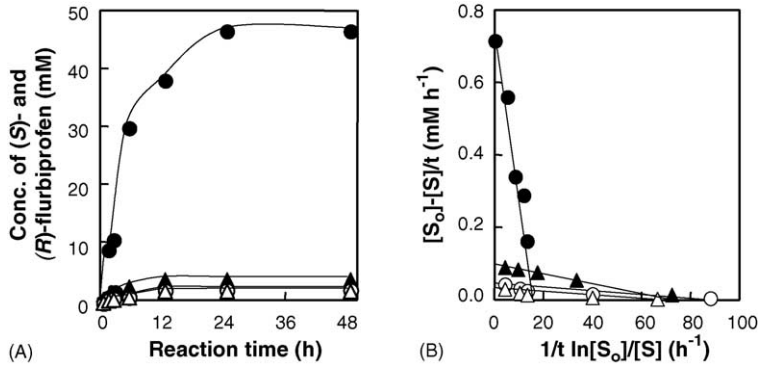


Fig. 5. The kinetics of lipase-catalyzed hydrolysis on immiscible substrate (*S*)- and (*R*)-FEE in the aqueous phase reaction system without and with chiral suβ-CD. Reaction was carried out at 600 units of lipase/mmol substrates, 50 mM (*S*)-, (*R*)-FEE and, 100 mM of suβ-CD, 50 mM sodium phosphate buffer (pH 7.0), 37 °C, and 300 rpm for 1–48 h. Lipase-catalyzed enantioselective hydrolysis reaction: (*S*)-FEE + suβ-CD (●); (*S*)-FEE (○); (*R*)-FEE + suβ-CD (▲); (*R*)-FEE (△).

0.098 mM/h. The apparent $K_m$ values for (*S*)-FEE also increased from $5.2 \times 10^{-4}$ to $4.7 \times 10^{-2}$ mM with suβ-CD, more significantly that those for (*R*)-FEE from $4.8 \times 10^{-4}$ to $1.4 \times 10^{-3}$ mM, indirectly indicating a steric hindrance to accepting lipase from *C. rugosa* after inclusion complexation.

The enhanced enantioselectivity caused by the chiral suβ-CD may have been partially the result of two effects: the facilitated dispersion of the immiscible substrate in the aliquot phase, and the selective inclusion complexation of (*S*)- or (*R*)-FEE with the chiral suβ-CD. Nonetheless, the enhancing effect cannot be elucidated fully only by above reasons.

This may be more directly related to the different stereoscopic conformational changes of the inclusion complex of each (*S*)- and (*R*)-FEE with suβ-CD, such as the different tilt angle induced by steric repulsion and the attraction of the succinyl group around the chiral center of substrates. The active site of lipase from *C. rugosa* seems to have preferential accessibility to the geometric structure in the ester bonding site in (*S*)-FEE, even though further research needs to be conducted, including geometric structure analysis using computer-aided molecular modeling.

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