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Lipase-catalyzed enantioselective hydrolysis of methyl 2-fluoro-2-arylpropionates in water-saturated isooctane

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Abstract

Lipases from *Candida rugosa*, *Candida antartica* B and *Carica papaya* are employed as the biocatalyst for the hydrolytic resolution of methyl 2-fluoro-2-arylpropionates in water-saturated isooctane, in which excellent to good enantioselectivity without the formation of byproducts is obtained for the papaya lipase when using (*R*,*S*)-2-fluoronaproxen methyl ester (**1**) and methyl (*R*,*S*)-2-fluoro-2-(4-methoxyphenyl)propionate (**2**), but not methyl (*R*,*S*)-2-fluoro-2-(naphth-1-yl)propionate (**3**) as the substrates. The thermodynamic analysis indicates that the enantiomer discrimination for the papaya lipase is driven by the difference in activation enthalpy for compound **1**, **2** or (*R*,*S*)-naproxen methyl ester (**4**). The kinetic analysis also demonstrates that in comparison with (*S*)-**4**, the insertion of the 2-fluorine moiety in (*R*)-**1** has increased k_2, but not K_m, and consequently the lipase activity.

Keywords: Lipases; Enantioselective hydrolysis; Methyl 2-fluoro-2-arylpropionates; Naproxen methyl ester

1. Introduction

Organic molecules containing fluorine atoms may act as hydrogen-bonding receptors on inducing remarkable conformational changes in biologically active compounds such that their pharmacological activity and selectivity are greatly improved. Their high electronegativity causes dramatic electronic changes that can significantly affect the reactivity and stability of the neighboring groups [1,2]. Fluorinated non-steroidal anti-inflammatory drugs (NSAIDs), especially the profen (2-arylpropionic acids) family, have received particular attention [3–8]. The replacement of 2-hydrogen of a profen with the quasi-isosteric fluorine can not only mimic the parent with respect to biological behavior but also convey a greater configuration stability to the chiral carbon, thus providing tools for studying *in vivo* behavior of the individual enantiomers of these NSAIDs.

The optically pure 2-fluoro-2-arylpropionic acids have been synthesized by either hydrolyzing optical active cyanohydrins or resolving the racemates *via* diastereomeric crystallization [8,9]. Nevertheless, the enzymatic resolution method represents another attractive choice because satisfactory yields and optical purity for the desired (*R*)-2-fluoroprofens have been reported by employing lipase-catalyzed hydrolysis of (*R*,*S*)-2-fluoroibuprofen (2-fluoro-2-(isobutylphenyl)propionic acid) methyl ester [3]. Unfortunately, recent attempts to hydrolyze (*R*,*S*)-2-fluoronaproxen ((*R*,*S*)-2-fluoro-2-(6-methoxynaphth-2-yl)propionic acid) methyl ester *via* various lipases in buffers were unsuccessful. The by-products 2-(6-methoxynaphth-2-yl)acrylic acid and (*R*,*S*)-2-hydroxynaproxen (2-hydroxy-2-(6-methoxynaphth-2-yl)propionic acid) were mainly formed in addition to the expected 2-fluoronaproxen (2-fluoro-2-(6-methoxynaphth-2-yl)propionic acid) [10]. A mechanistic hypothesis has been proposed to elucidate the byproduct formation, according to which the enzyme facilitates the elimination of the fluoride ion from the hydrolyzed acid, forming

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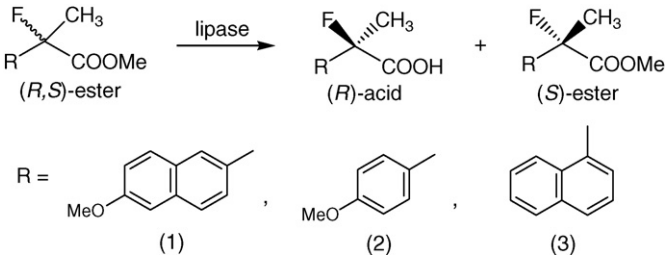

Scheme 1.

an α-carboxy-stabilized carbocation followed by a nucleophilic attack of water as well as β-elimination of the hydrogen.

We believed that this intriguing process could be improved if the reaction conditions and lipase sources were carefully selected. Therefore, as a continuation of exploring *Carica papaya* lipases as the versatile biocatalyst for resolving racemic acids, the hydrolysis of methyl (*R*,*S*)-2-fluoro-2-arylpropionates in water-saturated isooctane (Scheme 1) was first investigated [11–13]. Comparisons of the enzyme performances with those using (*R*,*S*)-naproxen methyl ester as the substrate *via* different lipases and temperatures were then reported.

2. Materials and methods

2.1. Materials

Lipase MY (CRL) from *Candida rugosa*, and Novozym 435 (NOVO 435) from *Candida antartica* B were provided by Meito Sangyo (Tokyo, Japan) and Novo Nordisk (Bagsvaerd, Denmark), respectively. A partially purified papaya lipase (pCPL) stored in the aqueous-insoluble aggregate of crude papain was kindly donated by Challenge Bioproducts (Yun-Lin Hsien, Taiwan). Other chemicals of analytical grade were commercially available. (*R*,*S*)-2-Fluoronaproxen methyl ester (**1**), methyl (*R*,*S*)-2-fluoro-2-(4-methoxyphenyl)propionate (**2**) and methyl (*R*,*S*)-2-fluoro-2-(naphth-1-yl)propionate (**3**) were synthesized and identified as previously described [10].

(*R*,*S*)-Naproxen (2-(6-methoxynaphth-2-yl)propionic acid) methyl ester (**4**) was synthesized by first adding 5.35 mmol (*R*,*S*)-naproxen and 5 mmol sulfuric acid to 650 mmol methanol and stirring at 65 °C for 18 h. After removing the remaining methanol by vacuum, the residue was extracted with a mixture composed of 20 ml NaOH (1 M) and 20 ml ethyl acetate. The organic phase was separated, dried over $MgSO_4$, filtered and evaporated under reduced pressure, giving the desired product. ^{1}H NMR spectra were recorded at 400 MHz on a Bruker AC-400 spectrometer in deuteriochloroform solutions with tetramethylsilane as an internal standard. Chemical shifts are reported in ppm from tetramethylsilane as follows: 51.58 (3H, d), 3.50–3.85 (3H, m), 3.90 (3H, s), 4.10 (1H, s), 7.12–7.26 (2H, q), 7.38–7.41 (1H, d), 7.70–7.73 (3H, m).

2.2. General remarks for analytical procedure

The hydrolysis of methyl esters of (*R*,*S*)-2-fluoro-2-arylpropionic acid and (*R*,*S*)-naproxen were monitored by HPLC using a chiral column ((*R*,*R*)-WHELK-01, Regis) capable of separating the internal standard of 2-nitrotoluene, (*R*)- and (*S*)-esters with the following retention times as: 2-nitrotoluene (3.5 min), (*R*)-**1** (4.52 min), (*S*)-**1** (6.43 min), (*R*)-**2** (3.10 min), (*S*)-**2** (3.53 min), (*R*)-**3** (3.07 min), (*S*)-**3** (4.04 min), (*R*)-**4** (7.70 min) and (*S*)-**4** (10.0 min). The composition of *n*-hexane:isopropanol = 9:1 (v/v) and flow rate of 1.5 ml/min for the mobile phase and UV detection at 270 nm were employed for quantification at a column temperature of 25 °C.

2.3. General procedure for enzymatic hydrolysis in water-saturated isooctane

Twenty-five milligrams of lipase was added to 5 ml water-saturated isooctane containing 1 mM ester with magnetic stirring at 55 °C. Samples were removed at different time intervals for HPLC analysis. From the time-course conversions, the initial rate for each enantiomer and hence the *E* value (defined as the ratio of initial rates) was estimated. Similar experiments were carried out for pCPL and CRL except that the temperature was changed to 35 or 65 °C. More experiments were performed at 45 °C for **1** and **4** with concentrations that varied from 1 to 10 mM. The kinetic constants for the fast-reacting enantiomer (i.e., (*R*)-**1** or (*S*)-**4**) were estimated based on the initial rate varied with the substrate concentration.

3. Results and discussion

3.1. Effects of acyl donor and lipase source

Fig. 1 illustrates the time-course conversions of (*R*)- and (*S*)-ester (i.e., X_R and X_S) for pCPL-catalyzed hydrolysis of methyl (*R*,*S*)-2-fluoro-2-arylpropionates in water-saturated isooctane at 55 °C, from which the specific initial rates for both substrates (i.e., V_R and V_S) and hence the enantiomeric ratio (i.e., *E* value)

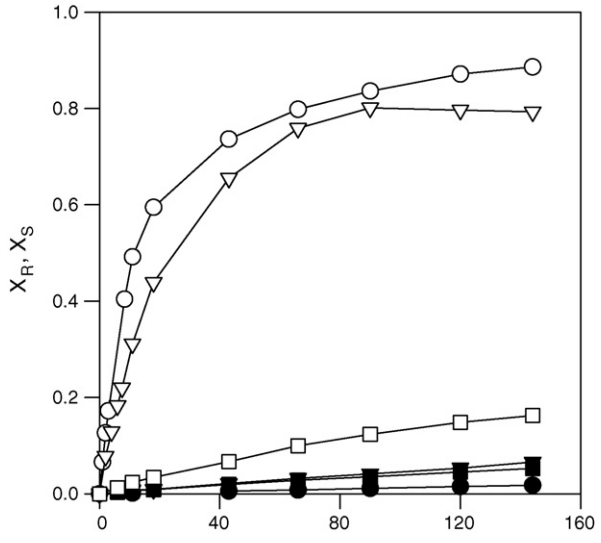

Fig. 1. Time-course conversions of (*R*)-ester (empty) and (*S*)-ester (solid): (●, ○) for substrate **1**, (▼, ▽) for substrate **2** and (■, □) for substrate **3**. *Conditions*: 1 mM ester, 5 mg/ml pCPL in water-saturated isooctane at 55 °C.

Table 1
Effects of acyl donor and lipase source on specific initial rates (with unit as mmol/h g) and *E* value

	pCPL			CRL			NOVO 435		
	V_R	V_S	E	V_R	V_S	E	V_R	V_S	E
1	6.1(E−3)	1.2(E−5)	493	1.5(E−3)	1.7(E−5)	85	1.5(E−3)	7.9(E−2)	52
2	3.2(E−3)	5.6(E−5)	76	2.6(E−3)	5.2(E−4)	5	7.1(E−3)	3.8(E−2)	6
3	1.5(E−4)	4.0(E−5)	4	2.9(E−4)	1.7(E−4)	2	1.4(E−3)	9.4(E−3)	5
4	1.5(E−5)	1.2(E−3)	80	3.0(E−5)	1.2(E−3)	40	4.2(E−2)	2.2(E−2)	2

Conditions: 1 mM methyl ester and 5 mg/ml lipase in 5 ml water-saturated isooctane at 55 °C. Values in parentheses as exponents, e.g., (E−5) = 10^{-5}.

were estimated. The values are reported in Table 1, where excellent enantioselectivity ($E = 493$) was obtained for compound **1** containing a hydrophobic 6-methoxynaphth-2-yl moiety at the chiral carbon. When the 2-aryl substituent is replaced with a less bulky 4-methoxyphenyl moiety, the enzyme activity decreases for the fast-reacting (*R*)-**2** but increases for the slow-reacting (*S*)-**2**, resulting in 6.5-fold decrease in the *E* value. The *Xs* conversions for both **1** and **2** approach asymptotic values at reaction times longer than 90 h, implying that (*R*)-2-fluoro-2-(4-methoxyphenyl)propionic acid and (*R*)-2-fluoronaproxen may act as lipase inhibitors [11,12]. A greater reduction of the enantioselectivity ($E = 4$) was shown when compound **3** containing a 2-naphth-1-yl moiety was replaced. In comparison with the reactivity of **1** or **2**, the V_R but not V_S for **3** were more than an order-of-magnitude lower. This may be attributed to the spatial hindrance of the 2-naphth-1-yl group that impeded the formation or breakage of the tetrahedron intermediate of the fast-reacting enantiomer in the acylation step.

The chemical shifts in ppm from tetramethylsilane for 3H of the 2-methyl group have been reported as follows: δ2.02 for **1**, δ1.96 for 2-fluoronaproxen and δ1.70 for 2-hydroxynaproxen [3,10]. In order to check if the side reactions previously reported for the hydrolysis of (*R*,*S*)-2-fluoronaproxen methyl ester in buffers also occurred in the present system, samples taken from the pCPL-catalyzed hydrolysis of **1** after 144 h were subjected to ^{1}H NMR analysis. The chemical shifts of δ2.04 and δ1.97 with a trace at δ1.76 were observed, implying that the formation of 2-hydroxynaproxen in water-saturated isooctane was negligible.

Similar behaviors for the CRL-catalyzed hydrolysis are depicted in Fig. 2, where the great decrease in the enantioselectivity for **1** ($E = 85$), **2** ($E = 5$) and **3** ($E = 2$) is shown (Table 1). Interestingly, the reduction of the *E* value was mainly due to the decreased V_R for **1** and the increased V_S for **2** or **3**. CRL but not pCPL may have a stringent active site to locate the 2-aryl substituent for slow-reacting enantiomers, and the replacement of the 2-substituent with a less bulky 4-methoxyphenyl or naphth-1-yl moiety can enhance the enzyme activity. Moreover, CRL was less stable at 55 °C; the X_S for each substrate approaches a plateau at 24 h (Fig. 2), as has been reported for the hydrolysis of (*R*,*S*)-naproxen 2,2,2-trifluoroethyl ester in organic solvents [14].

Similar results for the NOVO 435-catalyzed hydrolysis are reported in Fig. 3 and Table 1. The specific initial rate for each enantiomer was more than an order-of-magnitude higher when comparing with pCPL and CRL performances. The reduction of the enantioselectivity for **1** ($E = 52$), **2** ($E = 6$) and **3** ($E = 5$) was observed along with an opposite stereo-selectivity due to

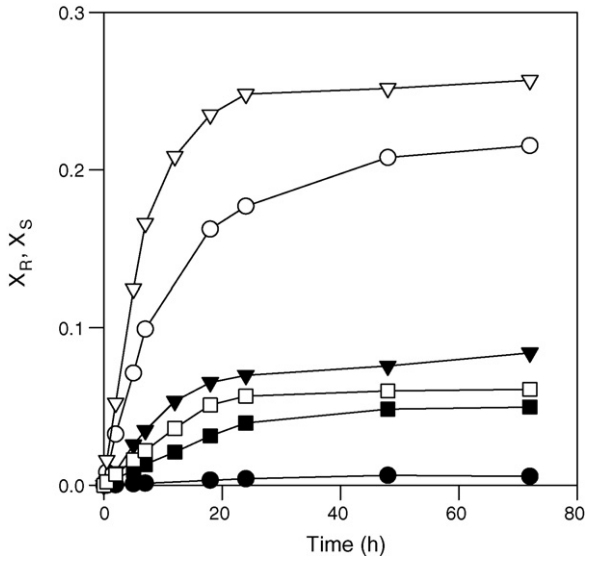

Fig. 2. Time-course conversions of (*R*)-ester (empty) and (*S*)-ester (solid): (●, ○) for substrate **1**, (▼, ▽) for substrate **2** and (■, □) for substrate **3**. *Conditions*: 1 mM ester, 5 mg/ml CRL in water-saturated isooctane at 55 °C.

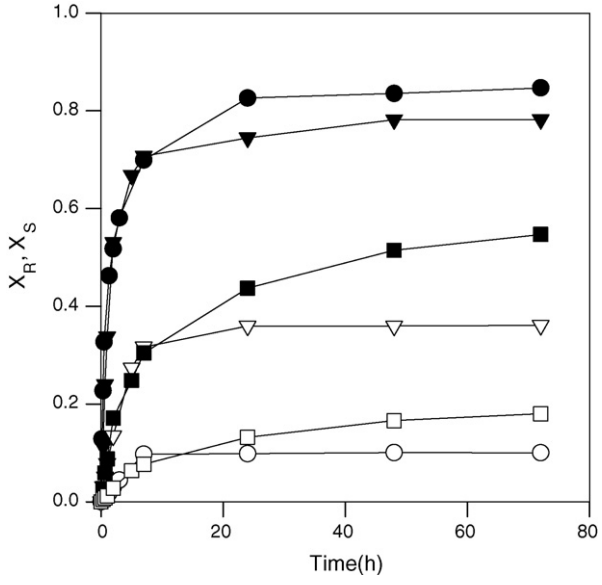

Fig. 3. Time-course conversions of (*R*)-ester (empty) and (*S*)-ester (solid): (●, ○) for substrate **1**, (▼, ▽) for substrate **2** and (■, □) for substrate **3**. *Conditions*: 1 mM ester, 5 mg/ml NOVO 435 in water-saturated isooctane at 55 °C.

the increased enzyme activity of the slow-reacting enantiomer. Based on the highest enantioselectivity for all the substrates, pCPL was selected as the best lipase for the kinetic analysis.

In order to study the effect of inserting a 2-flurorine atom on the kinetic behaviors of pCPL, the lipase-catalyzed hydrolysis of **4** in water-saturated isooctane at 45 °C was performed. From the time-course variations of X_R and X_S (data not shown), V_R, V_S and E value were estimated and are reported in Table 1. Good enantioselectivity for **4** ($E = 80$), although not as good as $E = 493$ for **1**, was obtained for pCPL, which was attributed to the decreasing lipase activity of the fast-reacting enantiomer. Similarly, fair enantioselectivity for **4** ($E = 40$) was found for CRL, although not as good as $E = 85$ for **1**. These results were mainly due to the increasing enzyme activity for the slow-reacting enantiomer. Moreover, bad enantioselectivity was found for NOVO 435 for **4** ($E = 2$) in comparison with $E = 52$ for **1**. Again, this was due to the increasing lipase activity for the slow-reacting enantiomer.

3.2. Thermodynamic analysis

Table 2 demonstrates the effect of temperature on the specific initial rate and E value for pCPL and CRL. In general, increasing the temperature enhances V_R and V_S slightly followed by a decrease in the E value. However, this behavior was not observed for CRL if (*R*)-**2** was used instead of (*R*)-**1** as the substrate, because V_R slightly decreased from 45 to 55 °C. Therefore, the bulky 6-methoxynaphth-2-yl moiety might provide more hydrophobic interactions with the enzyme active site that would avoid a conformation change of CRL at the higher temperature. Unfortunately a reduction of the temperature from 55 to 35 °C for **3** only increases the E value from 4 to 11 for pCPL. Moreover, the E value at any specific temperature greatly decreases when compound **1** is replaced with **4** for pCPL.

A thermodynamic analysis was used to investigate the effect of solvent type, acyl donor and acceptor and lipase type on the temperature dependence of the E value in lipase-catalyzed kinetic resolutions [11,12,15,16]. The difference in activation free energy of the transition states of both enantiomers can be separated into the differences between the activation enthalpy ($\Delta\Delta H$) and the activation entropy ($\Delta\Delta S$). Therefore, it is possible to determine whether the enantiomer discrimination is enthalpy-driven or entropy-driven. From the liner relationships between ln(E) and the inverse of the absolute temperature for pCPL (Table 2), the thermodynamic parameters $-\Delta\Delta H$ and $-\Delta\Delta S$ for **1**, **2** and **4** were estimated and are reported in Table 3. Based on the comparison of $-\Delta\Delta H$ and $-T\Delta\Delta S$ at 45 °C for each substrate, the enantiomer discrimination is found to be driven by the difference in activation enthalpy. The same result has been reported when employing lipases from different sources and (*R*,*S*)-naproxen 2,2,2-trifluoroethyl ester as the biocatalyst and substrate, respectively [14].

Table 3
Comparisons of $-\Delta\Delta H$ (with unit as kJ/mol), $-\Delta\Delta S$ (with unit as J/mol K) and $-T\Delta\Delta S$ (with unit as kJ/mol) for pCPL at 45 °C

Compounds	$-\Delta\Delta H$	$-\Delta\Delta S$	$-T\Delta\Delta S$	Driven by
1	9.1	−23.7	−7.5	$-\Delta\Delta H$
2	26.2	43.3	13.8	$-\Delta\Delta H$
4	11.4	−1.5	−0.48	$-\Delta\Delta H$

A detailed analysis of $-\Delta\Delta H$ and $-\Delta\Delta S$ (Table 3) between **4** and **1** indicates that the insertion of 2-flurorine atom may have changed the enthalpy and entropy of the transition state for the enantiomer of compound **1**. An active site model with three enantioselective pockets (i.e., large, medium and small pockets for aryl, methyl and hydrogen moieties to the chiral carbon, respectively) and a hydrophilic amino acid side chain between the medium and small pockets has been proposed to elucidate the kinetic and thermodynamic behaviors in pCPL-catalyzed hydrolysis of (*R*,*S*)-2-profen thioesters [11]. In comparison with the size of the 2-methyl substituent, the 2-fluorine or 2-hydrogen moiety of the fast-reacting enantiomer must favorably reside in

Table 2
Effects of temperature and lipase source on specific initial rates (with unit as mmol/h g) and E value

	1			**2**			**3**			**4**		
	V_R	V_S	E	V_R	V_S	E	V_R	V_S	E	V_R	V_S	E
35 °C												
pCPL	4.0(E−3)	6.6(E−6)	607	2.6(E−3)	1.8(E−5)	151	7.0(E−5)	6.5(E−6)	11	n.d.	n.d.	n.d.
CRL	6.4(E−4)	5.2(E−6)	123	1.7(E−3)	1.5(E−5)	111	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
45 °C												
pCPL	5.2(E−3)	9.3(E−6)	559	2.9(E−3)	2.7(E−5)	107	8.0(E−5)	8.0(E−6)	10	1.2(E−5)	1.0(E−3)	90
CRL	9.4(E−4)	9.7(E−6)	97	3.2(E−3)	2.5(E−5)	121	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
55 °C												
pCPL	6.1(E−3)	1.2(E−5)	493	3.2(E−3)	4.2(E−5)	76	1.5(E−4)	4.0(E−5)	4	1.5(E−5)	1.2(E−3)	80
CRL	1.5(E−3)	1.7(E−5)	85	2.6(E−3)	5.2(E−4)	5	2.9(E−4)	1.7(E−4)	2	3.0(E−5)	1.2(E−3)	40
65 °C												
pCPL	8.0(E−3)	1.7(E−5)	445	3.5(E−3)	5.8(E−5)	62	n.d.	n.d.	n.d.	1.9(E−5)	1.4(E−3)	71
75 °C												
pCPL	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	2.5(E−5)	1.5(E−3)	62

Conditions: 1 mM methyl ester and 5 mg/ml lipase in 5 ml water-saturated isooctane. Values in parentheses as exponents, e.g., (E−5) = 10^{-5}. Notation "n.d." as not determined.

Table 4
Initial rates (with unit as mmol/h) varied with substrate concentrations (with unit as mM) for (*R*)-**1** and (*S*)-**4**

Conc.	0.5	1.0	2.0	3.0	4.0	5.0
(*R*)-**1**	2.58(E−2)	5.12(E−2)	1.00(E−1)	1.22(E−1)	1.33(E−1)	1.36(E−1)
(*S*)-**4**	5.20(E−3)	1.00(E−2)	1.75(E−2)	2.15(E−2)	2.20(E−2)	2.40(E−2)

Conditions: 5 mg/ml pCPL in 5 ml water-saturated isooctane at 45 °C. Values in parentheses as exponents, e.g., (E−5) = 10^{-5}.

the small pocket, giving the same stereo-preference for (*R*)-**1** or (*S*)-**4**. Yet, the high electronegativity of the 2-fluorine atom might form a non-covalent bond, e.g., a hydrogen bond, with the hydrophilic side chain, which would decrease the enthalpy and entropy of the transition state. It is postulated that if the side chain locates at the position near the medium pocket, the non-covalent bonding of the transition state of (*S*)-**1** would be stronger than that of (*R*)-**1**, which would lead to the great reduction of $-\Delta\Delta H$ and hence $-\Delta\Delta S$ in comparison with those for **4**. Yet, more studies on verifying this hypothesis are needed if the 3D structure of the papaya lipase can be resolved.

3.3. Kinetic analysis

By using pCPL as the biocatalyst, the initial rates varied with the substrate concentrations in water-saturated isooctane at 45 °C for (*R*)-**1** and (*S*)-**4** are shown in Table 4. The kinetic constants $k_2 = 4.5 \times 10^{-2}$ mmol/h g and $K_m = 2.9$ mM for (*R*)-**1** as well as $k_2 = 7.3 \times 10^{-3}$ mmol/h g and $K_m = 2.4$ mM for (*S*)-**4** were estimated by assuming a Michaelis–Menten kinetics for each substrate. The similar K_m values imply that the 2-flurorine atom has negligible effect on changing the (*R*)-**1** affinity to the enzyme active site. Moreover, a 6.2-fold enhancement of k_2 could be attributed to the electron-withdrawing ability of the 2-flurorine atom to stabilize the transition state leading to the formation of tetrahedral intermediate in the acylation step.

4. Conclusions

In comparison with the lipases from *Candida rugosa* and *Candida antarctica* B, *Carica papaya* lipase was found to possess excellent to good enantioselectivity for the hydrolytic resolution of **1** and **2** at 55 °C. Negligible side reactions that formed (*R*,*S*)-2-hydroxynaproxen byproduct were verified from the ^{1}H NMR analysis. The enzyme activity for (*R*)-**3** was greatly reduced and hence the enantioselectivity was related to the spatial hindrance of the 2-naphth-1-yl group which might prevent the formation of the tetrahedral intermediate in the acylation step. The thermodynamic analysis indicates that the enantiomer discrimination for pCPL is driven by the difference in activation enthalpy for the transition states of **1**, **2** or **4**. Moreover, the kinetic analysis using pCPL as the biocatalyst for **1** and **4** indicates that the insertion of the 2-fluorine atom increases k_2 but not K_m, and hence the lipase activity for (*R*)-**1** is increased.

Acknowledgments

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