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Journal of Molecular Catalysis B: Enzymatic 48 (2007) 16–22
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
www.elsevier.com/locate/molcatb

Enzymatic hydrolytic resolution of (*R*,*S*)-α-chlorophenyl acetates in biphasic media

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Received 2 March 2007; received in revised form 19 May 2007; accepted 31 May 2007
Available online 3 June 2007

Abstract

A thermally stable carboxylesterase (SNSM-87) from *Klebsiella oxytoca* is explored as an enantioselective biocatalyst for the hydrolytic resolution of (*R*,*S*)-α-chlorophenyl acetates in biphasic media, where the ethyl ester possessing the highest enantioselectivity ($E^* = 95$) is selected as the best substrate and rationalized from the linear free energy relationships in terms of the logarithms of kinetic constants and enantiomeric ratio varied with the inductive parameter of leaving alcohol. An expanded Michaelis-Menten mechanism for the rate-limiting acylation step is adopted for the kinetic analysis. Two-stage Brønsted slopes of 58.0 and 2.28 for the fast-reacting (*R*)-esters, as well as only one-stage slope of 4.15 for the slow-reacting (*S*)-esters, were found and elucidated from the change of rate-limiting step. The replacement of α-chloro substituent to α-methyl or α-hydroxy moiety indicates that the α-substituent has profound effects on varying the enzyme activity, enantioselectivity, and optical-preference for SNSM-87 and lipases of pCPL, Lipase MY, NOVO 435 and Lipase PS.
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Keywords: (*R*,*S*)-α-Chlorophenyl acetates; Enantioselective hydrolysis; SNSM-87 carboxylesterase; Lipases

1. Introduction

α-Haloarylacetic acids and their derivatives, e.g. methyl and ethyl esters of α-bromo-*o*-tolylacetic acid, are known as important intermediates for synthesizing many drugs such as prostaglandin, prostacyclin, semi-synthetic penicillin and thiazolium salts [1–8]. Although lipases have been widely employed for preparing optically pure carboxylic acids [9,10], they are scarcely reported for resolving α-chloroarylacetic acids. In the esterification of (*R*,*S*)-α-chlorophenylacetic acid with butyl alcohol in hexane via a *Candida cylindracea* lipase, very low 8% conversion at 168 h was achieved [1]. Although the lipase activity was improved (58% conversion at 5 h) by using (*R*,*S*)-2,2,2-trifluoroethyl α-chlorophenyl acetate as the substrate in phosphate buffers, very poor enantioselectivity ($E^* = 2$) coupled with the formation of mandelic acid side product was found. These shortcomings have been overcome by changing the reaction medium to hexane containing aniline [2]. A dynamic kinetic resolution process in biphasic media by using cross-linked enzyme crystals from a *Candida rugosa* lipase and resin-bound phosphonium chloride as the racemization catalyst was furthermore developed, giving 90% yield and $ee_P = 90\%$ of (*R*)-α-chlorophenylacetic acid from the corresponding (*R*,*S*)-methyl ester [4]. Similarly, the hydrolysis of (*R*,*S*)-2,2,2-trifluoroethyl α-chlorophenyl acetate in water-saturated isooctane containing trioctylamine and a partially purified *Candida rugosa* lipase resulted in 93.0% yield and $ee_P = 89.5\%$ for the (*R*)-acid product, where the enantiomeric ratio $E^* = 33.6$ was estimated [11]. These results indicate that in order to obtain the desired product of high yield and optical purity, an improvement on enzyme activity and enantioselectivity is needed.

Recently, a thermally stable carboxylesterase (SNSM-87) from *Klebsiella oxytoca* was disclosed, leading to excellent enantioselectivity for the hydrolytic resolution of many carboxylic acid esters, e.g. ethyl mandelate, ethyl 2-chloromandelate, methyl esters of 3-tert-butoxycarbonyl-5,5-dimethyl-1,3-thiazoline-4-carboxylic acid, 2-arylpropionic acids, and 2-aryloxypropionic acids [12–15]. As a continuation

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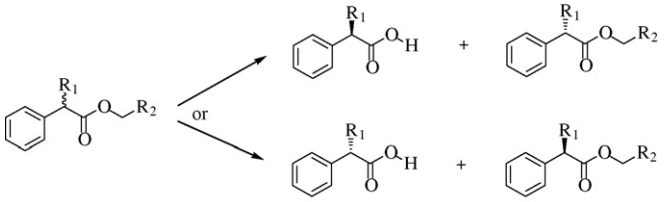
doi:10.1016/j.molcatb.2007.05.008

Nomenclature

aee_s enantiomeric excess for the substrate
E^* enantiomeric ratio, defined as the ratio between k_{2R}/K_{mR} and k_{2S}/K_{mS}
(E_t) enzyme concentration in aqueous phase (mg/ml)
k_{1R}, k_{1S} kinetic constants for (*R*)- and (*S*)-enantiomers, respectively, in aqueous phase (l/g h)
k_{2R}, k_{3R}, k_{-1R}, k_{-3R} kinetic constants for (*R*)-enantiomer in aqueous phase (mmol/g h)
k_{2S}, k_{3S}, k_{-1S}, k_{-3S} kinetic constants (*S*)-enantiomer in aqueous phase (mmol/g h)
k^*_{2R}, k^*_{2S}, k^{**}_{2R}, k^{**}_{2S} kinetic parameters for (*R*)- and (*S*)-enantiomers, respectively, in aqueous phase (mmol/g h)
K_p partition coefficient, defined as the ratio of substrate concentration in the aqueous phase to that in the organic phase
K_{mR}, K_{mS}, K^*_{mR}, K^*_{mS}, K^{**}_{mR}, K^{**}_{mS} kinetic constants for (*R*)- and (*S*)-enantiomers, respectively, in aqueous phase (mM)
K_{3R}, K_{3S} kinetic constants defined as $(k_{2R}+k_{-3R})/k_{3R}$ and $(k_{2S}+k_{-3S})/k_{3S}$, respectively
(S_R), (S_S) (*R*)- and (*S*)-ester concentrations in aqueous phase, respectively (mM)
$(S_R)_{org}$, $(S_S)_{org}$ (*R*)- and (*S*)-ester concentrations in organic phase, respectively (mM)
$(S_R)^*_{org}$, $(S_S)^*_{org}$ initial (*R*)- and (*S*)-ester concentrations in organic phase in equilibrium with those in aqueous phase, respectively (mM)
$(S_{R0})_{org}$, $(S_{S0})_{org}$ initial $(S_R)_{org}$ and $(S_R)_{org}$, respectively (mM)
V non-enzymatic initial rates for (*R*)- or (*S*)-ester based on aqueous phase (mM/h)
V_R, V_S enzymatic initial rates for (*R*)- and (*S*)-esters based on aqueous phase, respectively (mM/h)
V_{aq}, V_{org} volumes of aqueous and organic phases, respectively (ml)
X_R, X_S, X_t conversions of (*R*)-, (*S*)- and (*R*,*S*)-ester, respectively

of previous researches, we aim to explore the hydrolase as an efficient biocatalyst for the hydrolytic resolution of (*R*,*S*)-α-chlorophenyl acetate in biphasic media (Scheme 1), in which the desired (*S*)-ester from the organic phase or (*R*)-acid from the aqueous phase can be recovered. The effect of leaving alcohol moiety on the enzyme performance is first examined, leading to the rate-limiting acylation step in the overall reaction. An extended Michaelis-Menten kinetics is then employed for estimating the kinetic parameters varied with the inductive parameter of leaving alcohol, with which the obtained Brønsted slopes are employed for rationalizing the optimal enzyme enantioselectivity for the ethyl ester substrate. Comparisons of the enzyme performance are also made when the α-chloro substituent is replaced by α-methyl or α-hydroxy moiety, as well as the carboxylesterase changed to lipases suspending in water-saturated isooctane.



R_1: (**1**) Cl, (**2**) CH_3, (**3**) OH

R_2: (**a**) $(CH_2)_4CH_3$, (**b**) CH_3, (**c**) H, (**d**) $CH_2OC_2H_5$, (**e**) CF_3

Scheme 1.

2. Material and Methods

2.1. Materials

(*R*,*S*)-Ethyl mandelate from Aldrich (Milwaukee, WI), (*R*,*S*)-α-chlorophenylacetyl chloride and (*R*,*S*)-α-methylphenylacetic acid from Fluka (Buchs, Switzerland) were purchased. Lipases (triacylglycerol ester hydrolases, EC 3.1.1.3) from different sources were obtained as follows: Lipase MY from *Candida rugosa* (Meito Sangyo, Nagoya, Japan), pCPL as a partially purified *Carica papaya* lipase (Challenge Bioproducts, Yun-Lin Hsien, Taiwan), Lipase PS from *Pseudomonas cepacia* (Amano, Nagoya, Japan), and NOVO 435 from *Candida antarctica* B (Novo Nordisk, Bagsvaerd, Denmark). A *Klebsiella oxytoca* carboxylesterase (SNSM-87) was kindly donated from the Research & Development Center, Nagase & Co. Ltd. (Kobe, Japan). Other chemicals of analytical grade were commercially available.

2.2. Synthesis of (R,S)-α-substituted-phenyl acetates

(*R*,*S*)-α-Chlorophenylacetyl chloride (2.8 g, 13.3 mmol) was added slowly to 15 ml alcohol containing triethylamine (1.63 g, 16 mmol) at room temperature. After 3 h, the solution was poured into deionized water (3 × 15 ml) and extracted with ethyl acetate (15 ml). The organic phase was separated, dried over $MgSO_4$, filtered and evaporated under reduced pressure, giving the desired (*R*,*S*)-α-chlorophenyl acetate [11].

To 15 ml ethanol was added 5.35 mmol (*R*,*S*)-α-methylphenylacetic acid and 5 mmol sulfuric acid and stirred at 65 °C for 18 h. After removing the remaining ethanol by vacuum, the residue was extracted by using the solution 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 (*R*,*S*)-ethyl α-methylphenyl acetate.

2.3. Analysis

Hydrolysis of (*R*,*S*)-α-substituted-phenyl acetates in biphasic media is monitored by HPLC using a chiral column from Daicel (Chiralcel OD or Chiralcel OJ-H; Tokyo, Japan) that is capable

Table 1
HPLC analytical conditions

Ester	Chiral column	Mobile phase (hexane/IPA, v/v)	Flow rate (ml/min)	Retention time (IS/(*S*)-ester/(*R*)-ester, min)
1a	OJ-H	97/3	1.5	4.6/12.3/11.3
1b	OJ-H	97/3	1.5	4.6/13.9/15.9
1c	OJ-H	97/3	1.5	4.6/13.8/15.0
1d	OJ-H	97/3	1.5	4.6/12.8/14.1
1e	OJ-H	97/3	1.5	4.6/9.9/11.8
2a	OJ-H	90/10	1.0	5.8/7.7/8.5
3a	OD	80/20	1.2	3.6/4.1/5.8

Conditions: acetophenone as internal standard (IS); UV detection at 230 nm for quantification at room temperature.

of separating the internal standard acetophenone, (*R*)- and (*S*)-ester. Detailed analytical conditions are represented in Table 1. Samples were removed from the organic phase and injected onto the HPLC at different time intervals for analysis. Then, the conversion for each enantiomer (X_R or X_S), racemate conversion ($X_t = 0.5(X_R + X_S)$), and enantiomeric excesses for the substrate (ee_S) were calculated.

2.4. Kinetic resolution of (R,S)-ethyl α-substituted-phenyl acetates

Unless specified, a biphasic medium consisting of pH 6 phosphate buffers (300 mM, 2.83 ml) and 20 ml isooctane containing 1 mM (*R*,*S*)-α-substituted-phenyl acetate was stirred at 55 °C with a magnetic stirrer (400 rpm). Reaction started when 3.33 mg SNSM-87 dissolving in 0.5 ml buffers was added to the resultant solution. The initial rate for each substrate was determined from the time-course conversion. Similar experiments without adding the enzyme in the biphasic media for estimating the non-enzymatic initial rate (V) and partition coefficient (K_P) were also carried out. The initial enzymatic rate V_R or Vs was then calculated by subtracting V from the initial rate for each substrate. More experiments of varying the substrate concentration were carried out, from which the kinetic constants were estimated by using an extended Michaelis-Menten kinetics. Finally, lipase-catalyzed hydrolysis in 20 ml water-saturated isooctane containing 1 mM (*R*,*S*)-ethyl α-substituted-phenyl acetate was performed at 55 °C for studying the α-substituent effect on enzyme activity and enantioselectivity.

3. Model Development

As SNSM-87 can be regarded a serine-type carboxylesterase that follows the acylation-deacylation displacement mechanism [9,10,15,16]. A generalized expanded Michaelis-Menten mechanism including a two-step process for the acylation step is shown in Scheme 2. Each substrate (S_R or S_S) combines the enzyme (E) to form Michaelis complexes ES_R and ES_S. The imidazole of the catalytic histidine acting as a general base assists the nucleophilic attack of the serine oxygen, while the resultant imidazolium acts as a general acid to facilitate alcohol expulsion from the tetrahedral intermediates E^*S_R and E^*S_S, and gives the alcohol product P and acyl-enzyme intermediates $E^{**}S_R$ and $E^{**}S_S$.

By using pseudo-steady-state approximations for all Michaelis complexes and tetrahedral intermediates and assuming equilibrium partitioning for each substrate between both phases, the enzymatic initial rates based on the aqueous phase can be derived as follows [15]:

$$V_R = \frac{k_{2R}^{**}(S_R)(E_t)}{K_{MR}^{**} + (S_R)} \quad (1)$$

$$V_S = \frac{k_{2S}^{**}(S_S)(E_t)}{K_{mS}^{**} + (S_S)} \quad (2)$$

Notations (E_t), (S_R) and (S_S) denote the initial concentrations of enzyme, (*R*)- and (*S*)-ester in the aqueous phase, respectively. The latter two can be related to the initial substrate concentration $(S_{R0})_{org}$ or $(S_{S0})_{org}$ in the organic phase as $K_P(SR)^*_{org}$ $(=K_P(S_{R0})_{org}/(1 + K_P V_{aq}/V_{org}))$, where K_P, $(SR)^*_{org}$, V_{aq}, and V_{org} are the partition coefficient, initial substrate concentration in the organic phase of biphasic solution, volumes of aqueous and organic phases, respectively.

The apparent kinetic constants k_{2R}^{**}, k_{2S}^{**} and K_{mR}^{**} (or K_{mS}^{**}) in terms of the kinetic parameters k_{2R}^{*}, k_{2S}^{*} and K_{mR}^{*} $(=K_{mR}/(1 + K_{3R}))$ and K_{mS}^{*} $(=K_{mS}/(1 + K_{3S}))$ are expressed as

$$k_{2R}^{**} = \frac{k_{2R}^{*} K_{mS}^{*}}{K_{mR}^{*} + K_{mS}^{*}} \quad (3)$$

$$K_{mR}^{**} = K_{mS}^{**} = \frac{K_{mR}^{*} K_{mS}^{*}}{K_{mR}^{*} + K_{mS}^{*}} \quad (4)$$

$$k_{2S}^{*} = \frac{k_{2S}^{*} K_{mR}^{*}}{K_{mR}^{*} + K_{mS}^{*}} \quad (5)$$

Moreover, the kinetic parameters are related to the kinetic constants k_{2R}, k_{2S}, K_{3R} $(=(k_{2R} + k_{-3R})/k_{3R})$, K_{3S} $(=(k_{2S} + k_{-3S})/k_{3S})$, K_{mR} $(=(k_{-1R}K_{3R} + k_{2R})/k_{3R})$, and K_{mS} $(=(k_{-1S}K_{3S} + k_{2S})/k_{1S})$ as

$$E + S_R \underset{k_{-1R}}{\overset{k_{1R}}{\rightleftharpoons}} ES_R \underset{k_{-3R}}{\overset{k_{3R}}{\rightleftharpoons}} E^*S_R \xrightarrow{k_{2R}} E^{**}S_R + P$$

$$E + S_S \underset{k_{-1S}}{\overset{k_{1S}}{\rightleftharpoons}} ES_S \underset{k_{-3S}}{\overset{k_{3S}}{\rightleftharpoons}} E^*S_S \xrightarrow{k_{2S}} E^{**}S_S + P$$

Scheme 2.

follows:

$$k^*_{2R} = \frac{k_{2R}}{1 + K_{3R}} \quad (6)$$

$$K^*_{mR} = \frac{K_{mR}}{1 + K_{3S}} \quad (7)$$

$$k^*_{2S} = \frac{k_{2S}}{1 + K_{3S}} \quad (8)$$

$$K^*_{mS} = \frac{K_{mS}}{1 + K_{3S}} \quad (9)$$

If the enzyme has high (*R*)-enantioselectivity, the (*R*)-ester concentration in the organic phase is negligible in comparison with (*S*)-ester concentration when estimating the initial rate V_S. Therefore, one can at first employ Eq. (2) for estimating K^{**}_{mS} and k^{**}_{2S} (i.e. K^*_{mS} and k^*_{2S}), then K^{**}_{mR} and k^{**}_{2R} from Eq. (1), and finally K^*_{mR} and k^*_{2R} from Eqs. (3) and (4). The enantiomeric ratio E^* is defined as $k^{**}_{2R}K^{**}_{mS}/k^{**}_{2S}K^{**}_{mR}$ ($= k^*_{2R}K^*_{mS}/k^*_{2S}K^*_{mR} = k_{2R}K_{mS}/k_{2S}K_{mR}$). As an approximation, one may estimate k^{**}_{2R}/K^{**}_{mR} from $V_R/[K_P(E_t)(S_R)^*_{org}]$ and k^{**}_{2S}/K^{**}_{mS} from $V_S/[K_P(E_t)(S_S)^*_{org}]$, if $K^{**}_{mR} \gg (S_R)$ and $K^{**}_{mS} \gg (S_S)$ in Eqs. (1) and (2) are valid.

4. Results and Discussion

4.1. Effects of leaving alcohol

Table 2 demonstrates effects of changing the leaving alcohol of (*R*,*S*)-α-chlorophenyl acetate on K_P, optical preference, specific enzymatic initial rates $V_R/(E_t)$ and $V_S/(E_t)$, initial rates ratio V_R/V_S, non-enzymatic initial rate V, X_t, and ee_S. With (*R*,*S*)-methyl α-chlorophenyl acetate (**1c**) as the reference, increasing the carbon chain of leaving alcohol except for **1d** containing a polar ethoxy group results in reducing the K_P value. This will decrease the substrate solubility, and hence generally the enzymatic rate, in the aqueous phase. A comparison of all non-enzymatic initial rates indicates that an electro-withdrawing group of 2,2,2-trifluoro or ethoxy moiety in the leaving alcohol is advantageous for the non-enzymatic hydrolysis. However, this will deteriorate the optical purity of product or remaining substrate and should be suppressed.

About 190-fold difference of $V_R/(E_t)$ for the fast-reacting (*R*)-ester is demonstrated when the leaving 2-ethoxyethanol (**1d**) is replaced by 1-hexanol (**1a**). This implies that the acylation step must be rate-limiting as all (*R*)-α-chlorophenyl acetates have the same acyl-enzyme intermediate for proceeding the deacylation step. Similar behaviors of more than 40-fold difference of $V_S/(E_t)$ between (*S*)-**1a** and (*S*)-**1d**, and hence the rate-limiting acylation step for the slow-reacting (*S*)-ester, are perceived. As the initial rates ratio V_R/V_S acts as an index of enzyme enantioselectivity, SNSM-87 is regarded as good for resolving **1b**, modest for **1c** and **1d**, and fair for **1a** and **1e**.

The inductive parameter of R_2 moiety to the leaving alcohol can provide a measure of the relative effect of chain substituent on the electron density and nucleophilic ability of the hydroxyl group [17,18]. The values of this parameter have been established on the basis of the dissociations of bicyclooctane carboxylic acids and quinuclidines, and lead many appropriate linear free energy relationships in a wide variety of reaction series including the dissociation of alcohols [17–20]. In order to examine the intrinsic enzymatic kinetic behavior, the kinetic parameters k^{**}_{2R}/K^{**}_{mR} ($=k_{2R}/K_{mR}$) and k^{**}_{2S}/K^{**}_{mS} ($=k_{2S}/K_{mS}$) have been estimated from $V_R/[K_P(E_t)(S_R)^*_{org}]$ and $V_S/[K_P(E_t)(S_S)^*_{org}]$, respectively, by assuming $K^{**}_{mR} \gg (S_R)$ and $K^{**}_{mS} \gg (S_S)$ for both substrates. Fig. 1 illustrates the LFER in terms of logarithms of k_{2R}/K_{mR} and k_{2S}/K_{mS} varied with the inductive parameter of leaving alcohol. For all (*R*)-esters, a steep ascent of the kinetic parameter with a Brønsted slope 58.0 is shown when increasing the parameter from −0.01 (**1a**) to 0.01 (**1b**). This implies that for **1a** containing a difficult leaving 1-hexanol, the breakdown of tetrahedral intermediate to the acyl-enzyme intermediate is rate-limiting, corresponding to a limiting case of $k^*_{2R} = k_{2R}$ and K_{3R} [$= (ER)/(E^*R)$] $\ll 1$. However, this slope (i.e. 2.28, $r^2 = 0.989$) levels off if the parameter furthermore increases from 0.01 to 0.38 (**1e**), indicating that the formation, or concerted formation and breakdown, of the tetrahedral intermediate is rate-limiting. Only one Brønsted slope (i.e. 4.15, $r^2 = 0.964$) for all (*S*)-esters in the whole range of inductive parameter is shown, implying that the breakdown of tetrahedral intermediate to the acyl-enzyme intermediate is rate-limiting. This corresponds to a limiting case of $k^*_{2S} = k_{2S}$ and K_{3S} [$= (ES)/(E^*S)$] $\ll 1$. Similar kinetic behaviors for the hydrolase-

Table 2
Effects of leaving alcohol or α-substituent of acyl part on SNSM-87-catalyzed hydrolysis in biphasic media

Ester	K_P	Inductive parameter[b]	(E_t) (mg/ml)	$V_R/(E_t)$ (mmol/h g)	$V_S/(E_t)$ (mmol/h g)	Optical-preference	Initial rates ratio	V (mM/h)	Time (h)	X_t (%)	ee_S (%)
1a	5.4(E-2)	−0.01	5.0	1.3(E-2)	1.7(E-3)	*R*	7.4	1.1(E-4)	24	15.3	9.6
1b	9.8(E-2)	0.01	2.0	3.4(E-1)	4.2(E-3)	*R*	82.7	6.6(E-4)	24	53.4	100.0
1c	3.1(E-1)	0.03	1.0	1.3	2.3(E-2)	*R*	55.3	9.6(E-4)	19	56.2	100.0
1d	3.6(E-1)	0.11	1.0	2.5	6.9(E-2)	*R*	37.0	2.1(E-3)	5	56.6	100.0
1e	4.2(E-2)	0.38	1.0	1.1	6.5(E-2)	*R*	16.6	3.4(E-3)	7	49.6	72.7
2b	9.5(E-2)	0.01	4.0	8.3(E-5)	3.6(E-2)	*S*	435.4	3.3(E-4)	96	45.8	80.9
3b[a]	1.2(E-1)	0.01	1.0	3.7(E-2)	1.6	*S*	43.5	1.7(E-3)	7	59.2	100.0

Conditions: 20 ml isooctane containing 1 mM (*R*,*S*)-ester and 3.33 ml pH 6 buffers (300 mM) containing SNSM-87 at 55 °C and 400 rpm.

[a] From [15].

[b] For the R_2 moiety of leaving alcohol [18,19].

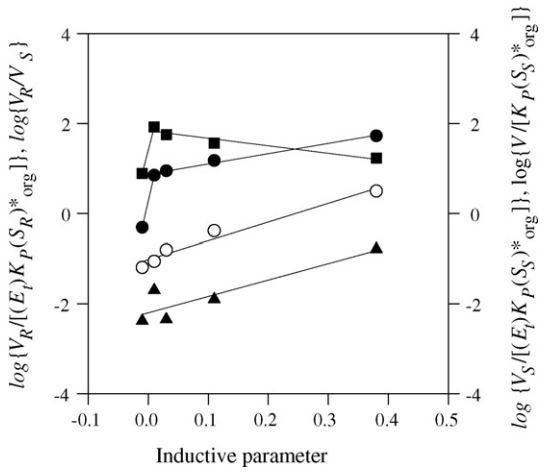


Fig. 1. Variations of (●) $\log\{V_R/[K_p(E_t)(S_R)^*_{org}]\}$, (○) $\log\{V_S/[K_p(E_t)(S_S)^*_{org}]\}$, (■) $log\{V_R/V_S\}$, and (▲) $\log\{V/[Kp(S_S)^*_{org}]\}$with inductive parameter of R_2 moiety of leaving alcohol.

catalyzed hydrolysis of (*R*,*S*)-2-chloromandelates in biphasic media have been reported [15].

Unlike the non-enzymatic general-base esters hydrolysis in the aqueous phase, the leaving alcohol moiety must leave the tetrahedral intermediate with a concerted proton transfer from the imidazolium of the active site. The proton transfer for the slow-reacting (*S*)-esters is evidently not efficient, owing to the partial or complete disruption of the hydrogen bond between the imidazolium and leaving alcohol oxygen at the transition state [21], or even due to the stereoelectronic effect or steric repulsion of the leaving alcohol moiety [22]. Therefore, $k_{3S} \gg k_{2S}$ for (*S*)-**1e** containing a 2,2,2-trifluoro moiety having a high inductive parameter. Fig. 1 also demonstrates the logarithm of an apparent first-order rate constant $V/[K_P(S_R)^*_{org}]$ or $V/[K_P(S_S)^*_{org}]$ varied with the inductive parameter. The Brønsted slope 3.64 ($r^2 = 0.813$) implies that the non-enzymatic reaction and the enzymatic hydrolysis for slow-reacting (*S*)-esters follow the rate-limiting breakdown of tetrahedral intermediates.

The relationships relating the enzyme enantioselectivity in terms of $log(V_R/V_S)$ to the inductive parameter is also represented in Fig. 1, where a maximum value occurs at the inductive parameter 0.01 for **1b**. Obviously, this is mainly due to the change of rate-limiting step for (*R*)-esters. However, the merit of maximum enantioselectivity for **1b** is offset by 7.5-fold reduction of k_{2R}/K_{mR} when comparing with **1e**. In order that all (*R*)-esters can obey the rate-limiting breakdown of tetrahedral intermediate such that the enzyme activity and enantioselectivity simultaneously improves when increasing the inductive parameter, enhancing of the nucleophilic power of serine oxygen atom by altering the coordination and/or bonding of the nucleophile is needed [23]. Similar interpretations as described in Fig. 1 have been reported for the hydrolysis of (*R*,*S*)-2-chloromandelates, where the best methyl ester was found [15]. Then, the current approach and rationalization for obtaining maximum enantioselectivity by varying the leaving alcohol moiety may generally apply to all serine-type hydrolases that obey the rate-limiting acylation step when carrying out hydrolysis, transesterification or aminolysis in the aqueous or organic phase.

4.2. Effects of changing α-substituent

Effects of changing the α-substituent on the partition coefficient, non-enzymatic initial rate, and enzyme performances are represented in Table 2. Similar partition coefficients or non-enzymatic initial rates are found, regardless of the α-substituent employed. Very high enantioselectivity for (*R*,*S*)-ethyl α-methylphenyl acetate (**2b**) is observed, as the initial rates ratio is greater than 100. This has been reported when employing methyl ester as the substrate in an aqueous solution [12]. Yet, the advantage of high enantioselectivity is offset by an order-of-magnitude lower specific initial rate $V_S/(E_t)$ for the fast-reacting substrate, when comparing with $V_R/(E_t)$ of **1b**. On the contrary, when using (*R*,*S*)-α-hydroxyphenyl acetate (**3b**) as the substrate, 4.8 folds higher of $V_S/(E_t)$ for the fast-reacting substrate but 1.9 folds lower of the initial rates ratio were found. To our surprise, the enzyme optical-preference for **3b** is opposite to that of **1b**, regardless of the α-chloro and α-hydroxy moieties having similar group sizes, electronegativities, or inductive parameters. No explanation is found to elucidate this interesting behavior, although it seems plausible that the α-hydroxy group may provide an additional hydrogen bond to the amino acid residuals around the active site and then inverses the enantioselectivity.

In order to get rid of the effect of partition coefficient when studying the enzyme performance, the kinetic constants and E^* value (Table 3) were estimated from Eqs. (1) and (2) coupled with the initial rate varied with the substrate concentration (Fig. 2). Almost the same K^*_{mR} ($= (E)(S_R)/[(ES_R) + (E^*S_R)]$) and K^*_{mS} ($= (E)(S_S)/[(ES_S) + (E^*S_S)]$) were obtained, regardless of the α-substituent employed. This implies that all (*R*)- or (*S*)-esters have the same affinity on converting the free enzyme into either the enzyme-substrate complex or tetrahedral intermediate. The values $K^{**}_{mR} = K^{**}_{mS} = 1.32$ mM and $K_P(S_R)^*_{org} = K_P(S_R)^*_{org} = 0.048$ mM for **1b** were estimated from Eq. (4),

Table 3
Kinetic constants for SNSM-87-catalyzed hydrolysis of (*R*,*S*)-ethyl α-substituted-phenyl acetates

Ester	k^*_{2S} (mmol/h g)	K^*_{mS} (mM)	k_{2S}/K_{Ms} (l/h g)	k^*_{2R} (mmol/h g)	K^*_{mR} (mM)	k_{2R}/K_{mR} (l/h g)	E^*
1b	1.9(E-1)	2.2	8.7(E-2)	2.7(E+1)	3.3	8.3	95
2b	1.8	2.9	6.4(E-1)	4.8(E-3)	2.4	2.0(E-3)	317
3b[a]	4.1(E+1)	2.5	1.6(E+1)	8.6(E-1)	2.7	3.2(E-1)	51

Conditions: 20 ml isooctane containing (*R*,*S*)-ethyl ester and 3.33 ml pH 6 buffer (300 mM) containing 3.33 mg SNSM-87 for all esters except for 13.32 mg for **3b** at 55 °C and 400 rpm.

[a] Data from [15].

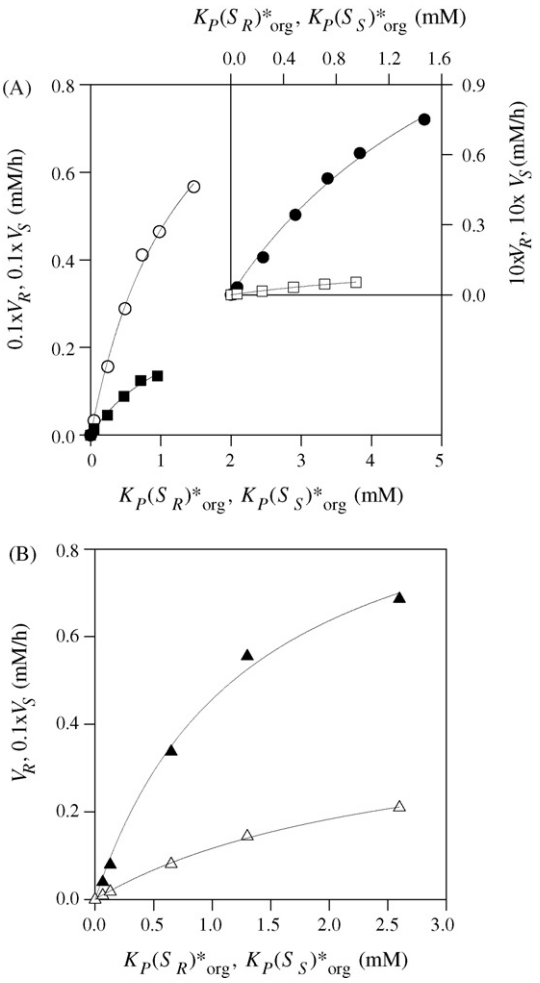


Fig. 2. Variations of initial rate with substrate concentration of the aqueous phase ($K_P(S_R)^*_{org}$ or $K_P(S_S)^*_{org}$); empty and filled symbols for (*R*)- and (*S*)-ester, respectively. (A) (●) and (◯) for ester (**1b**), (■) and (□) for ester (**2b**); (B) (▲) and (□) ester (**3b**); best-fit results (—).

implying that $K^{**}_{mR} \gg (S_R)$ and $K^{**}_{mS} \gg (S_S)$ are the reasonable assumption when preparing Fig. 1.

About 13-fold decrease of k_{2S}/K_{mS} for the fast-reacting substrate is found when (*R*)-**1b** is replaced by (*S*)-**2b**. Similarly, about 43-fold reduction of k_{2R}/K_{mR} for the slow-reacting enantiomer and hence 3.3-fold increase of E^* value is estimated by changing (*S*)-**1b** to (*R*)-**2b**. When (*R*)-**1b** is furthermore substituted by (*S*)-**3b**, only 1.9-fold enhancement of k_{2S}/K_{mS} for the fast-reacting substrate is obtained. Similarly by replacing (*S*)-**1b** with (*R*)-**3b**, about 3.7-fold increase of k_{2R}/K_{mR} for the slow-reacting enantiomer, and then 3.3-fold decrease of E^* value, is perceived. These results indicate that the amino acid residues around the active site do exert different non-covalent bonding to attract or expel the α-substituent. This will lead a miniature change of the transition states for **1b** and **2b**, but a major change of those for **1b** and **3b**, for breaking the tetrahedral intermediate.

In order to investigate if lipases also follow similar kinetic behaviors as described in Table 2 for SNSM-87, the hydrolysis of **1b**-**3b** in water-saturated isooctane was carried out. For **1b**, the specific activity of SNSM-87 for the fast- or slow-reacting substrate is two order-of-magnitudes greater than those of lipases except for NOVO 435 (Table 4). When (*R*,*S*)-2,2,2-trifluoroethyl α-chlorophenyl acetate was employed as the substrate, the same enzyme optical-preference, but with very poor enantioselectivity, for all lipases has been reported [11]. The modest initial rates ratio of 46 for pCPL, 23 for Lipase MY, but only 1.4 for NOVO 435 and 4.7 for Lipase PS, indicate that an improvement on lipase enantioselectivity is possible if the leaving 2,2,2-trifluoroethanol is replaced by ethanol. When changing the α-chloro substituent (1**b**) to α-methyl (**2b**) or α-hydroxy moiety (**3b**), similar enzyme performances, but with much lower enantioselectivity, for all lipases are demonstrated (Table 4). In comparison with **1b**, the inversion of optical preference for **3b** was obtained for all lipases except for Lipase PS. This implies that a complicated interaction among the α-hydroxy moiety and amino acid residuals of the active site may prevail for the lipases.

Table 4
Effects of α-substituent of (*R*,*S*)-ethyl esters on lipase-catalyzed hydrolysis in water-saturated isooctane

Ester	(E_t) (mg/ml)	$V_S/(E_t)$ (mmol/h g)	$V_R/(E_t)$ (mmol/h g)	Optical- preference	Initial rates ratio	Time (h)	X_t (%)	ee_S (%)
pCPL								
1b	20.0	3.75(-5)	1.74(-3)	*R*	46	48.0	44.8	63.8
2b	20.0	8.75(-5)	3.00(-5)	*S*	2.9	72.0	10.8	3.0
3b	20.0	3.25(-4)	1.63(-4)	*S*	2.0	72.0	30.0	3.7
Lipase MY								
1b	5.0	1.40(-4)	3.22(-3)	*R*	23	48.0	16.7	18.4
2b	5.0	5.00(-5)	2.00(-5)	*S*	2.5	96.0	3.0	6.0
3b	5.0	2.06(-3)	5.00(-4)	*S*	4.2	16.0	14.2	4.7
NOVO 435								
1b	1.0	4.62(-1)	3.26(-1)	*S*	1.4	0.5	26.5	4.7
2b	1.0	9.50(-2)	2.18(-1)	*R*	2.3	0.5	14.4	6.0
3b	1.0	3.81(-1)	1.64	*R*	4.3	0.5	28.9	12.7
Lipase PS								
1b	5.0	4.20(-4)	9.00(-5)	*S*	4.7	96.0	16.4	11.7
2b	5.0	3.00(-5)	2.60(-4)	*R*	8.6	96.0	10.2	7.4
3b	5.0	7.00(-4)	3.60(-4)	*S*	1.8	96.0	18.2	27.2

Conditions: 20 ml water-saturated isooctane containing 1 mM (*R*,*S*)-ethyl ester at 55 °C and 400 rpm.

5. Conclusions

With SNSM-87-catalyzed hydrolysis of (*R*,*S*)-α-chlorophenyl acetates in biphasic media as the model system, a maximum enzyme enantioselectivity for the ethyl ester is obtained when varying the leaving alcohol with chain substituent of different electro-withdrawing strength. An expanded Michaelis-Menten mechanism for the rate-limiting acylation step is adopted to derive the rate equations for kinetic analysis. For the fast-reacting (*R*)-esters, the Brønsted slope estimated from $log(k_{2R}/K_{mR})$ varied with the inductive parameter indicates that the breakdown of tetrahedral intermediate is the rate-limiting step for **1a** and **1b**. It shifts to the formation, or concerted formation and breakdown, of tetrahedral intermediate if the parameter increases furthermore to 0.38 for **1e**. However, only one Brønsted slope and hence the rate-limiting breakdown of tetrahedral intermediate is found for all (*S*)-esters, and consequently the optimal enzyme enantioselectivity for ethyl ester substrate is obtained and rationalized. The analysis has been extended to (*R*,*S*)-ethyl α-methylphenyl acetate (**2b**) and (*R*,*S*)-ethyl α-hydroxyphenyl acetate (**3b**), where the α-substituent was shown to have profound effects on the enzyme activity, enantioselectivity, and optical-preference for SNSM-87 and lipases. Based on the enzyme enantioselectivity and activity, SNSM-87 is selected as the best biocatalyst for resolving **1b**-**3b**.

Acknowledgements

Financial supports from National Science Council (Grant No. NSC 95-2221-E-182-075) are appreciated.

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