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Hydrolytic resolution of (*R*,*S*)-3-hydroxy-3-phenylpropionates by esterase from *Klebsiella oxytoca*: Effects of leaving alcohol, covalent immobilization and aqueous pH

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ABSTRACT

The kinetic analysis for hydrolytic resolution of (*R*) and (*S*)-ethyl 3-hydroxy-3-phenylpropionate in biphasic media is carried out via a thermally stable esterase (SNSM-87) from *Klebsiella oxytoca*. The resultant kinetic constants are compared with those using (*R*,*S*)-ethyl 2-substituted carboxylic acid ester as the substrate. An optimal enantioselectivity of $V_S/V_R = 16$ for **4** using free SNSM-87 is rationalized via the structure–reactivity correlations in terms of logarithms of specificity constants varied with the inductive parameter of leaving alcohol moiety, and can further increase to an acceptable value of $V_S/V_R = 37$ using SNSM-87 immobilized on Sepabeads@ EC-HA. The pH-reactivity profiles for all enzyme preparations are analyzed in order to elucidate the modest enantioselectivity of $V_S/V_R = 26$ for **2** containing a 3-hydroxy moiety in comparison with $V_S/V_R = 323$ for (*R*,*S*)-ethyl 2-hydroxy-2-phenylacetate containing a 2-hydroxy moiety using SNSM-87 immobilized on Eupergit C 250L.

1. Introduction

Optically active 3-hydroxy carboxylic acids and their derivatives are important chiral synthons used for the synthesis of a number of pharmaceuticals such as fluoxetine, β-lactams, pheromones, β-blockers, lipid A and L-carnitine [1–4]. They can be synthesized by condensation of enolates or iodoacetic acid with carbonyl compounds [5,6], reduction of their corresponding prochiral 3-keto esters using chemical or biocatalytic methods [7,8], or deracemization of racemic esters via various microbial cells [9,10]. As an alternative method, biocatalytic resolution of (*R*,*S*)-3-hydroxy carboxylic esters has attracted the interest of synthetic chemists, and was generally carried out via lipase-catalyzed transesterification at the 3-hydroxy moiety in organic solvents or hydrolysis at the 3-*O*-acyl group in the aqueous solution in order to obtain high enantioselectivity [11–16]. However for the former, the separation of resultant products of 3-*O*-acyl ester from remaining substrates is difficult or tedious. For the latter, an addition synthesis step for the preparation of 3-*O*-acyl ester is needed, when comparing with the simple approach of hydrolysis at the 1-carboxylic moiety of (*R*,*S*)-3-hydroxy carboxylic acid esters although the enantioselectivity is usually low as the catalytic site is remote from the stereocenter. Therefore, a novel approach of using substrate engineering, medium engineering, enzyme immobilization, or their combinations is required in order to improve the biocatalyst performance.

A thermal stable esterase originated from *Klebsiella oxytoca* was recently disclosed to possess an excellent enantioselectivity for the hydrolytic resolution of (*R*,*S*)-2-substituted carboxylic acid esters [17–22]. This discovery has induced our interests for the exploration of the esterase as an enantioselective biocatalyst for the resolution of (*R*,*S*)-3-hydroxy carboxylic acids. With the hydrolytic resolution of (*R*,*S*)-3-hydroxy-3-phenylpropionates in biphasic media as the model system (Scheme 1), it is aimed to obtain the (*S*)-acid product as an important intermediate for the synthesis of antidepressants of tomoxetine and fluoxetine [14,16,23–26]. A detailed kinetic analysis for (*R*)- and (*S*)-ethyl 3-hydroxy-3-phenylpropionate is first carried out. The resultant kinetic constants are compared with those previously reported for (*R*,*S*)-ethyl 2-substituted carboxylic acid esters. By using the substrate engineering approach of changing the leaving alcohol moiety, the enzyme performance is improved and elucidated from the structure–reactivity correlations. The modification of enzyme surface charges by covalently immobilizing SNSM-87 on an epoxy-(oxirane)-activated support of Eupergit C 250L or hexamethylenamino-activated support of Sepabeads@ EC-HA is finally adopted for further enhancing the enzyme enantioselectivity.

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Nomenclature

ee_s	enantiomeric excess for the substrate
E	enantiomeric ratio greater than one, defined as the ratio between k_{2S}/K_{mS} and k_{2R}/K_{mR}
(E_t)	enzyme concentration in aqueous phase (mg/ml)
F	inductive parameter for R group of the leaving alcohol
K_p	partition coefficient defined as the ratio of substrate concentration in aqueous phase to that in organic phase
k^*_{2R}, k^*_{2S}	kinetic constants for (*R*)- and (*S*)-enantiomers, respectively, in aqueous phase (mmol/(g h))
K_{mR}, K_{mS}, K^*_{mR}, K^*_{mS}	kinetic constants for (*R*)- and (*S*)-enantiomers, respectively, in aqueous phase (mM)
k^*_{2R}, k^*_{2S}	kinetic parameters for (*R*)- and (*S*)-enantiomers, respectively, in aqueous phase (mmol/(g h))
(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_t	conversions of (*R*,*S*)-ester

2. Materials and methods

2.1. Materials

A *K. oxytoca* esterase of SNSM-87 was kindly donated from the Research & Development Center, Nagase & Co. Ltd. (Kobe, Japan). Chemicals of analytical grade were commercially available: *N*-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) from Acros (Milwaukee, WI); (*S*)-3-hydroxy-3-phenylpropionic acid and 2,2,2-trifluoroethanol from Aldrich (Milwaukee, WI); (*R*)-3-hydroxy-3-phenylpropionic acid from Alfa Aesar (Ward Hill, MA); (*S*)-ethyl 2-hydroxy-4-phenylbutyrate and Eupergit C 250L from Fluck (Buchs, Switzerland); (*R*)-ethyl 2-hydroxy-4-phenylbutyrate from TCI (Tokyo, Japan); ethyleneglycolmonoethyl ether from Merck (Darmstadt, Germany); ethanol, isooctane, isopropanol, hexane and methanol from Tedia (Fairfield, OH). Highly porous methacrylic polymer beads of Sepabeads@ EC-HA were kindly donated from Resindion S.R.L. (Mitsubishi Chemical Cooperation, Binasco, Italy). Citrate, acetate, phosphate and carbonate were employed for the preparation of pH 3, pH 4–5, pH 6–8 and pH 9–10 buffers, respectively.

2.2. Synthesis of (*R*)- and (*S*)-3-hydroxy-3-phenylpropionates

To 650 mmol alcohol was added 5.35 mmol (*R*)- or (*S*)-3-hydroxy-3-phenylpropionic acid and 5 mmol sulfuric acid with stirring at 55 °C for 8 h. After removing the remaining alcohol under reduced pressure, the residue was dissolved in 20 ml dichloromethane, washed in succession with 30 mM NaOH solution (3× 20 ml) and deionized water (20 ml). The organic phase was separated, dried over anhydrous magnesium sulfate, filtered and concentrated under reduced pressure, giving the desired (*R*)- and (*S*)-3-hydroxy-3-phenylpropionate.

2.3. Analysis

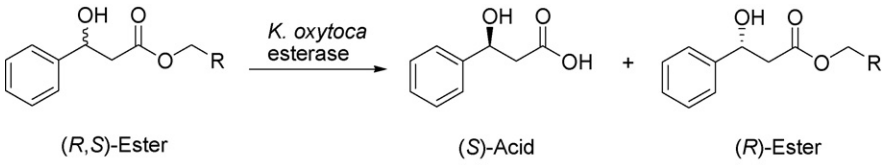
Hydrolysis of (*R*)-, (*S*)- or (*R*,*S*)-3-hydroxy-3-phenylpropionates in biphasic media was monitored by HPLC with UV detection at 220 nm using a Chiralcel OD-H chiral column (Daicel, Tokyo). The mobile phase (hexane:isopropanol, v/v) and retention time for the internal standard of 2-nitrotoluene, (*R*)- and (*S*)-ester were as follows: 80/20 and 3.1/5.2/3.6 min for **1**, 80/20 and 3.1/6.1/4.3 min for **2**, 80:20 and 3.1/4.4/3.6 min for **3**, 90:10 and 3.3/9.9/7.3 min for **4**, and 80:20 and 3.1/6.1/4.1 min for **5**, respectively. Samples were removed from the organic phase and injected onto the HPLC at different time intervals for analysis, from which the time-course conversion and initial rate (V_R or V_S) for each enantiomer, racemate conversion (X_t), and enantiomeric excess for the substrate (ee_s) were determined. The analytical conditions for (*R*)- and (*S*)-ethyl 2-hydroxy-4-phenylbutyrate were previously reported [20].

2.4. Enzyme immobilization

The enzyme immobilization was carried out in 10 ml phosphate buffers (1 M, pH 7) containing 100 mg Eupergit C 250L and 10 mg SNSM-87 at 25 °C and 150 rpm for 4 h. The supports were filtered, washed in succession with the buffer (3× 10 ml) and deionized water (10 ml) each for 0.5 h, lyophilized for 24 h, weighted, and then stored at 4 °C prior to use. Similarly, enzyme immobilization on Sepabeads@ EC-HA was performed by adding 5012 mg supports to 100 ml phosphate buffers (50 mM, pH 7.4) containing 500 mg EDC, 600 mg NHS, and 500 mg SNSM-87 at 25 °C and 150 rpm for 24 h. The supports were filtered, washed in succession with the buffer (3× 100 ml) and deionized water (2× 100 ml) each for 0.5 h, lyophilized for 24 h, weighted, and then stored at 4 °C prior to use. Details for the determination of SNSM-87 immobilized on both supports were demonstrated elsewhere [27–29].

2.5. Kinetic analysis

Unless specified, a biphasic medium consisting of 2.83 ml buffer (300 mM, pH 6) and 20 ml isooctane containing a specific concentration of (*R*)-**2**, (*S*)-**2**, (*R*)- or (*S*)-ethyl 2-hydroxy-4-phenylbutyrate was stirred with a magnetic stirrer at 400 rpm and 55 °C. Reaction



R: (**1**) $(CH_2)_4CH_3$, (**2**) CH_3, (**3**) H, (**4**) $CH_2OC_2H_5$, (**5**) CF_3

Scheme 1. Hydrolytic resolution of (*R*,*S*)-3-hydroxy-3-phenylpropionates in biphasic media.

started when 0.5 ml buffer (pH 6) containing 33.3 mg SNSM-87 was added to the resultant solution. Samples were removed from the organic phase and injected onto the HPLC at different time intervals for analysis. Similar experiments without containing the enzyme were performed for estimating the non-enzymatic initial rate V and partition coefficient K_p. By subtracting V from the initial rates, the enzymatic initial rates V_R and Vs were determined and the kinetic constants estimated by using the rate equations derived from an extended Michaelis–Menten kinetics [20].

In order to investigate effects of leaving alcohol on the enzyme performance, similar experiments except for 1 mM (*R*,*S*)-3-hydroxy-3-phenylpropionates with or without adding the enzyme were carried out. In order to study effects of enzyme surface charges modification, experiments with SNSM-87 immobilized on different supports for various substrates were performed. In order to elucidate the enzyme kinetic behaviors, the aqueous pH varied from 3 to 10 for 1 mM (*R*,*S*)-**2** with or without containing the enzyme was employed for carrying out the hydrolysis.

2.6. Estimation of kinetic constants

On the basis of rate-limiting acylation step of generalized expanded Michaelis–Menten kinetics, one may derive two rate equations for the esterase-catalyzed hydrolysis of (*R*)- and (*S*)-esters as follows [20]:

$$V_R = \frac{k^*_{2R}(S_R)(E_t)}{K^*_{mR} + (S_R)} \quad (1)$$

$$V_S = \frac{k^*_{2S}(S_S)(E_t)}{K^*_{mS} + (S_S)} \quad (2)$$

Notations (E_t), (S_R) and (S_S) denote the initial enzyme, (*R*)- and (*S*)-ester concentration in the aqueous phase, respectively. The latter two concentrations can be related to the substrate concentration in the organic phase $(S_{R0})_{org}$ as $(S_R) = K_p(S_R)^* = K_p(S_{R0})_{org}/(1 + K_p V_{aq}/V_{org})$ or $(S_S) = K_p(S_S)^* = K_p(S_{S0})_{org}/(1 + K_p V_{aq}/V_{org})$. Details for the definitions of kinetic constants, specificity constants $k_{2R}/K_{mR} (= k^*_{2R}/K^*_{mR})$ and $k_{2S}/K_{mS} (= k^*_{2S}/K^*_{mS})$, and enantiomeric ratio E $(= k_{2R}K_{mS}/k_{2S}K_{mR})$ can be found elsewhere [20]. Moreover for the reaction condition leading to $K^*_{mR} \gg (S_R)$ and $K^*_{mS} \gg (S_S)$, k_{2R}/K_{mR} and k_{2S}/K_{mS} can be estimated from $V_R/(E_t)/(S_R)$ and $V_S/(E_t)/(S_S)$, respectively.

3. Results and discussion

3.1. Kinetic analysis

The time-course concentrations in the organic phase for each substrate can be employed for estimating the partition coefficient, initial enzymatic and non-enzymatic rates. Fig. 1 illustrates the initial enzymatic rates varied with substrate concentrations in the aqueous phase for (*R*)-**2**, (*S*)-**2**, (*R*)- or (*S*)-ethyl 2-hydroxy-4-phenylbutyrate, with which the kinetic constants k^*_{2R} and K^*_{mR} as well as k^*_{2S} and K^*_{mS} and hence the E value are estimated by using Eqs. (1) and (2). Almost the same K^*_{mR} and K^*_{mS} are found for a specific substrate (Table 1), implying that (*R*)- and (*S*)-ester have the same affinity on combining the enzyme to form enzyme–substrate complexes or tetrahedral intermediates [20]. Moreover, lower values of K^*_{mR} and K^*_{mS} for the substrate containing a 3- or 4- but not 2-phenyl moiety are demonstrated, implying that a hydrophobic patch composed of amino acid residues around the large group of the active site model may provide π–π interactions for favorable recognizing of 3- or 4-phenyl moiety.

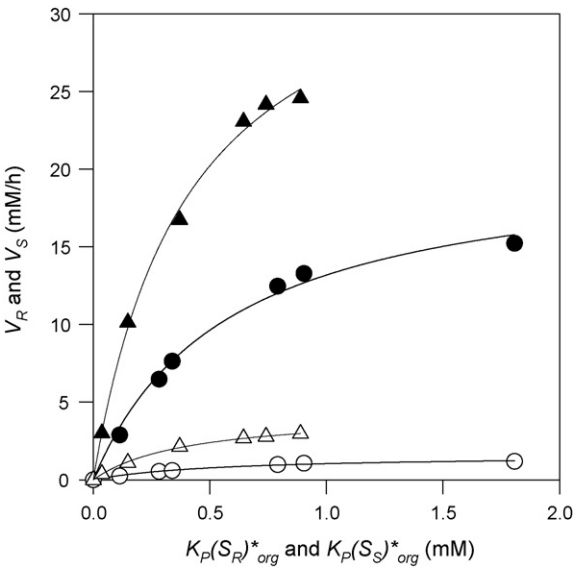


Fig. 1. Variations of the initial rate with substrate concentration in aqueous phase for (*R*)-**2** (○), (*S*)-**2** (●), (*R*)-ethyl 2-hydroxy-4-phenylbutyrate (△), and (*S*)-ethyl 2-hydroxy-4-phenylbutyrate (▲); best-fit results (—). Conditions: 20 ml isooctane and 3.33 ml pH 6 phosphate buffer containing 33.3 mg SNSM-87 at 55 °C and 400 rpm.

Table 1 also demonstrates an order-of-magnitude higher k^*_{2S} (or k^*_{2R}) for the fast-reacting enantiomer containing an electronegative atom attached to the 2-carbon atom, such as for 2-hydroxy, 2-chloro and 2-methoxy but not 2-methyl, 2-hydroxymethyl and 2-hydrogen moiety. This implies that a hydrophilic patch composed of amino acid residues around the medium group to the 2-carbon atom may exert hydrogen bonding or polar attractions to the electronegative atom, leading to stabilization of the transition state for enhancing proton transfer from catalytic imidazolium to the leaving alcohol [20]. Apparently, the enantioselectivity of $E = 12$ for **2** is not high enough for industrial applications.

3.2. Effects of leaving alcohol moiety

Effects of changing the leaving alcohol moiety on K_p, V, specific initial rates, V_R/V_S, X_t, and ee_s for free SNSM-87 are represented in Table 2. With **2** as the reference, an increase of the hydrophobicity

Table 1
Partitioning coefficient, kinetic constants and E value for SNSM-87-catalyzed hydrolysis of (*R*,*S*)-ethyl esters.

(*R*,*S*)-Ethyl ester	K_p	k^*_{2R} (mmol/(h g))	K^*_{mR} (mM)	k_{2R}/K_{mR} (l/(h g))	k^*_{2S} (mmol/(h g))	K^*_{mS} (mM)	k_{2S}/K_{mS} (l/(h g))	E
2-Hydroxy-2-phenylacetate[a]	1.2E−1	8.6E−1	2.7	3.2E−1	4.1E+1	2.5	1.6E+1	51
2-Hydroxy-3-phenylpropionate[a]	7.5E−2	4.3	4.1E−1	1.1E+1	3.7E+1	4.1E−1	9.0E+1	8
2-Hydroxy-4-phenylbutyrate	8.0E−2	9.3E−1	4.1E−1	2.3	1.3E+1	7.7E−1	1.7E+1	7
2-Chloro-2-phenylacetate[a]	9.8E−2	1.9E−1	2.2	8.7E−2	2.7E+1	3.3	8.3	95
2-Methyl-2-phenylacetate[a]	9.5E−2	4.8E−3	2.4	2.0E−3	1.8	2.9	6.4E−1	317
2-Hydroxymethyl-2-phenylacetate[a]	4.3E−1	2.7	8.9	3.0E−1	5.4E−3	8.7	6.2E−4	487
2-Methoxy-2-phenylacetate[a]	2.2E−2	1.7E−1	1.6	1.1E−1	2.3E+1	9.0E−1	2.5E+1	227
3-Hydroxy-3-phenylpropionate	2.4E−1	1.6E−1	5.5E−1	2.9E−1	2.1	5.8E−1	3.6	12

[a] Data from [22]. Notation in values is the exponent, e.g. E−1 = 10^{-1}.

Table 2
Effects of leaving alcohol on inductive parameter F, partition coefficient K_p, non-enzymatic rate V, specific initial rates, V_S/V_R, X_t and ee_s.

Ester	F	K_p	V (mM/h)	SNSM-87	V_R/E_t (mmol/(h g))	$V_S/(E_t)$ (mmol/(h g))	V_S/V_R	E_t (mg/ml)	Time (h)	X_t (%)	ee_s (%)
1	−0.01	7.7E−3	1.83E−4	Free	5.83E−4	6.88E−3	12	20	72	35.1	41.0
				Immobilized[a]	2.25E−4	5.98E−3	27	10.5	72	31.1	39.5
2	0.01	2.4E−1	1.65E−3	Free	2.38E−2	2.88E−1	12	10	5	61.3	100.0
				Immobilized[a]	6.93E−3	1.81E−1	26	2	48	44.4	65.3
				Immobilized[b]	4.03E−3	7.58E−2	19	2	48	29.3	34.6
3	0.03	7.1E−1	5.40E−3	Free	6.92E−2	1.00	14	1	20	61.5	100.0
				Immobilized[a]	2.17E−2	5.56E−1	26	2	20	58.6	95.8
				Immobilized[b]	1.17E−2	2.57E−1	22	1	24	43.2	63.1
4	0.11	5.1E−1	4.50E−3	Free	1.05E−1	1.70	16	5	1.7	62.7	100.0
				Immobilized[a]	2.27E−2	4.91E−1	22	2	20	57.2	96.0
				Immobilized[b]	1.77E−2	6.48E−1	37	1	24	52.6	92.8
5	0.38	2.9E−2	2.25E−3	Free	2.65E−2	2.10E−1	8	5	20	60.5	100.0
				Immobilized[a]	3.93E−3	4.46E−2	11	2	72	27.2	29.1
				Immobilized[b]	1.18E−2	9.55E−2	8	2	48	32.1	31.5

Conditions: 20 ml isooctane containing 1 mM (*R*,*S*)-ester and 3.33 ml pH 6 buffer (300 mM) containing free or immobilized enzyme at 55 °C and 400 rpm.
[a] Eupergit C 250L as the support.
[b] Sepabead® EC-HA as the support.

of leaving alcohol moiety of **1** or **5** may decrease K_p and hence the substrate solubility in the aqueous phase. The inductive parameter F of 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. An enhancement of F results in increasing of $V_R/(E_t)$ and $V_S/(E_t)$, and hence V_R/V_S acting as an index of enzyme enantioselectivity for **4**. As an industrial acceptable E value is at least 20, the optimal enantioselectivity of $V_R/V_S = 16$ is still not good for the practical application.

In order to characterize the enzyme kinetic behavior by eliminating substrate concentration effects, the specificity constants k_{2R}/K_{mR} (i.e. $V_R/(E_t)/(S_R)$) and k_{2S}/K_{mS} (i.e. $V_S/(E_t)/(S_S)$) are estimated, and their logarithms varied with F are represented in Fig. 2. A two-stage Brønsted slope with the breaking point at $F = 0.11$ for (*S*)-**4** is shown, indicating that breakdown of the tetrahedral adduct to the acyl-enzyme intermediate is rate-limiting if the substrate contains a difficult leaving alcohol moiety. It may change to formation of the tetrahedral adduct if the (*S*)-esters contain an easy leaving alcohol moiety. However, only one Brønsted slope for all (*R*)-esters is depicted, indicating that breakdown of the tetrahedral adduct is rate-limiting and may be attributed to the concerted but inefficient proton transfer from catalytic imidazolium to the leaving alcohol. Therefore, a maximum enantioselectivity at $F = 0.11$ for **4** is rationalized. Similar kinetic behaviors for the hydrolysis of (*R*,*S*)-2-substituted carboxylic acid esters are previously reported [20–22,29].

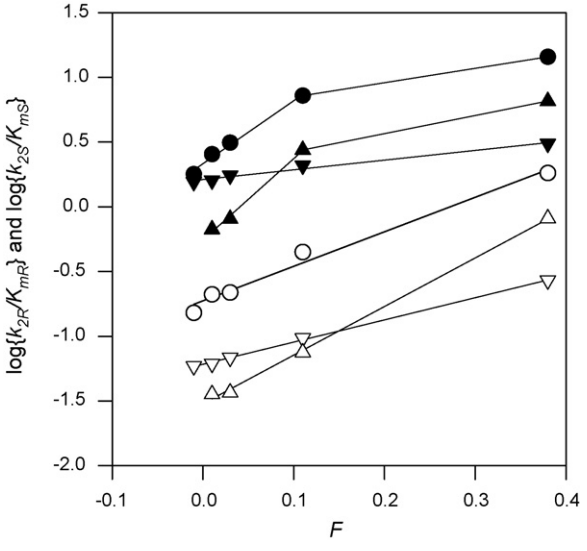


Fig. 2. Variations of $\log(k_{2R}/K_{mR})$ (empty) and $\log(k_{2S}/K_{mS})$ (filled) with the inductive parameter F for free SNSM-87 (● and ○), SNSM-87 immobilized on Eupergit C 250L (▼ and ▽) and Sepabead® EC-HA (▲ and △).

A detailed analysis of the structure–reactivity correlations for (*R*,*S*)-3-hydroxy-3-phenylpropionates and (*R*,*S*)-2-hydroxy-2-phenylacetates [29] indicates that minor changes of the Brønsted slope 2.88 of the latter to 2.67 of the former for all slow-reacting (*R*)-esters and that of 1.01–1.11 for all fast-reacting (*S*)-esters containing a easy leaving alcohol moiety are found. However, the slope greatly decreases from 36.34 of the former to 4.78 of the latter for all (*S*)-esters containing a difficult leaving alcohol moiety, and hence yields an E value decreasing from 51 of (*R*,*S*)-ethyl 2-hydroxy-2-phenylacetate to 12 of (*R*,*S*)-ethyl 3-hydroxy-3-phenylpropionate (Table 1). Similar kinetic behaviors of giving a high Brønsted slope for the fast-reacting enantiomer containing a difficult leaving alcohol moiety of other (*R*,*S*)-2-substituted carboxylic acid esters were obtained [20–22,29]. These indicate that the 2-substituted but not 3-hydroxy moiety must have strong interactions with the hydrophilic patch on causing miniature conformation change of the transition state for the fast-reacting enantiomer, which is more sensitive and favorable for enhancing proton transfer from the catalytic imidazolium by increasing the inductive parameter. Therefore, any technique that can increase the Brønsted slope for the fast-reacting enantiomer containing a difficult leaving alcohol should improve the enzyme enantioselectivity.

3.3. Effects of enzyme immobilization

Chemical modification methods are effective on altering the enzyme surface charges, and hence change the electrostatics and ionization constants of amino acid residues of the active-site [30]. For example, an epoxy-activated support of Eupergit C 250L was previously employed for the SNSM-87 immobilization for decreasing the positive surface charges and hence the ionization constant of catalytic imidazolium [29]. Moreover by immobilizing the enzyme on a hexamethylenamino-activated support of Sepabeads® EC-HA, the enzyme negative surface charges decrease and lead to an enhancement of the ionization constant of catalytic imidazolium [28]. Therefore, the Brønsted slope for the fast-reacting (*S*)-2-hydroxy-2-phenylacetates containing a difficult leaving alcohol has varied from 36.34 of free SNSM-87 to 38.09 and 7.24 of both immo-

bilized enzymes, respectively [28,29]. This approach is adopted for the present substrate for improving the enzyme enantioselectivity.

Table 2 demonstrates effects of changing the leaving alcohol moiety of (*R*,*S*)-3-hydroxy-3-phenylpropionates on K_p, V, specific initial rates, V_R/V_S, X_t, and ee_s for both immobilized enzymes. In general, for a specific substrate the enzyme activity but not enantioselectivity deteriorates after the covalent immobilization. With **2** using free SNSM-87 as the reference, an optimal specific activity $V_S/(E_t) = 6.48 \times 10^{-1}$ mmol/h g with $V_S/V_R = 37$ is obtainable if one selects SNSM-87 immobilized on Sepabeads@ EC-HA for the resolution of **4**. The logarithms of k_{2R}/K_{mR} and k_{2S}/K_{mS} varied with F and hence the resultant structure–reactivity correlations for both immobilized enzymes are further represented in Fig. 2. A two-stage Brønsted slope with the breaking point at $F = 0.11$ for the (*S*)-esters but not their antipodes is shown for SNSM-87 immobilized on Sepabeads@ EC-HA. One may attribute the maximum enantioselectivity of $V_S/V_R = 37$ mainly to the increasing of Brønsted slope from 4.87 to 6.28 for (*S*)-esters containing a difficult leaving alcohol. Moreover, the slope decreases from 4.87 to 0.75 and coincides with that for (*S*)-esters containing an easy leaving alcohol for SNSM-87 immobilized on Eupergit C 250L, and then leads to decreasing of the enantioselectivity by increasing *F*. This behavior is different from that of giving a maximum $V_S/V_R = 867$ at $F = 0.13$ using (*R*,*S*)-2-hydroxy-2-phenylacetates as the substrate [29], and can be elucidated from the variation of ionization constants of catalytic imidazolium as described below.

3.4. Effects of aqueous pH

As the imidazole moiety of catalytic histidine acting as a general acid–base catalyst is directly involved in the reaction, it must be uncharged for catalysis and may play an essential role on creating the chiral discrimination ability for all esters. Therefore, an estimation of the ionization constant of catalytic imidazolium at the presence of substrate is needed in order to evaluate the contribution of uncharged imidazole moiety on the specificity constants. Fig. 3 illustrates the pH effect on changing the relative enzyme activity of (*R*)-**2** and (*S*)-**2** for each enzyme preparation. As an approximate for $K^*_{mR} \gg (S_R)$ and $K^*_{mS} \gg (S_S)$ at the present reaction condition, this figure can be regarded as the pH effect on varying the relative k_{2R}/K_{mR} and k_{2S}/K_{mS}. An empirical equation $k_{2i}/K_{mi} = (k_{2i}/K_{mi})_{int}/[1 + K_{1i}/(H^+) + (H^+)/K_{2i}]$ ($i = R$ and S) with (H^+) as the proton concentration has been employed for estimating the intrinsic specificity constants $(k_{2i}/K_{mi})_{int}$, ionization constants for the catalytic imidazolium moiety K_{2i} and other acidic or basic groups perturbing the activity K_{1i} [28,29]. Unfortunately, the curve fitting for the data of Fig. 3 is not satisfactory for the estimation of K_{1i} and K_{2i} for each enzyme preparation. Yet in comparison with the curves for free SNSM-87, the left-hand-side bell-shape curve for (*R*)- or (*S*)-**2** shifts to the right for SNSM-87 immobilized on Eupergit C 250L, but to the left for the enzyme immobilized on Sepabeads@ EC-HA. This implies that the ionization constant K_{2i} will shift to a lower value for the former and a higher one for the latter, and hence mainly contributes to changing of the Brønsted slope for the fast-reacting (*S*)-esters containing a difficult leaving alcohol. Therefore one may deduce that the left-hand-side bell-shape curve will shift more to the left for increasing K_{2S} and hence the proton transfer and enantioselectivity, if the surface negative charges further decrease or even the surface positive charges increase by using the enzyme engineering approach such as site-directed mutations or chemical modifications.

In a previous report for the resolution of (*R*,*S*)-ethyl 2-hydroxy-2-phenylacetate, $pK_{2S} = 5.16$ and $pK_{2R} = 5.37$ for free SNSM-87 have changed to 5.89 and 7.22 for SNSM-87 immobilized on Eupergit C 250L, respectively [29]. Therefore in pH 6 buffers, more fraction of uncharged catalytic imidazole exists for catalyzing the (*S*)-ester

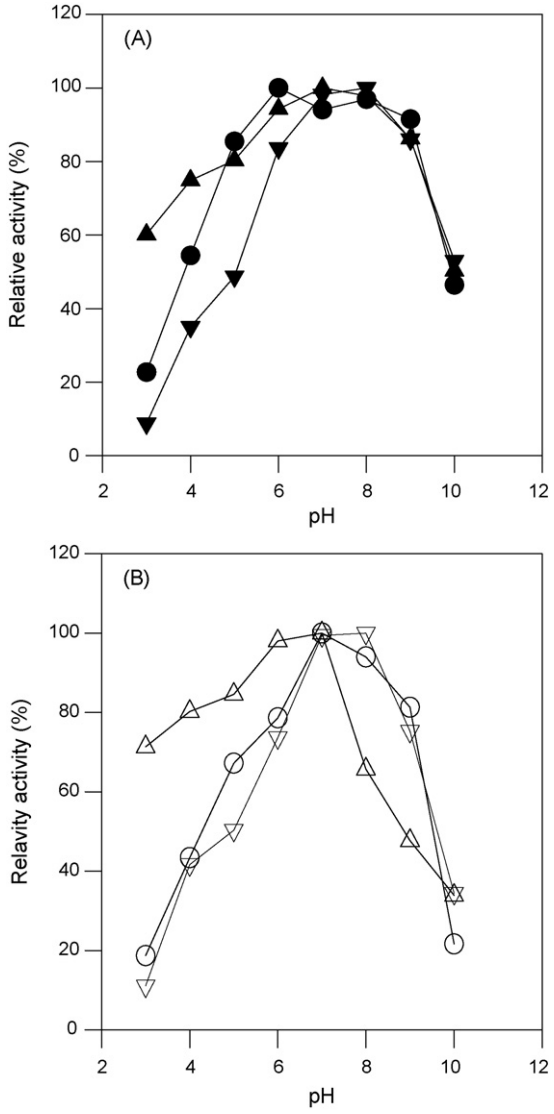


Fig. 3. Relative activity varied with aqueous pH for (A) (*S*)-**2** and (B) (*R*)-**2**; for free enzyme (● and ○), enzyme immobilized on Eupergit C 250L (▼ and ▽), and enzyme immobilized on Sepabead@ EC-HA (▲and □). Conditions: 20 ml isooctane containing 1 mM (*R*,*S*)-**2** and 3.33 ml buffer (300 mM) containing 33.3 mg SNSM-87, 100 mg immobilized enzyme on Eupergit C 250L or 400 mg immobilized enzyme on Sepabead@ EC-HA at 55 °C and 400 rpm.

but not its antipode for the immobilized enzyme. This is advantageous for giving a low k_{2R}/K_{mR} and hence a high enantioselectivity of $V_S/V_R = 323$. As the left-hand-side bell-shape curve of (*R*)-**2** for the immobilized enzyme shifts more to the left in comparison with that of (*R*)-ethyl 2-hydroxy-2-phenylacetate (or those of (*S*)-**2** and (*S*)-ethyl 2-hydroxy-2-phenylacetate), lower values of pK_{2i}, e.g. two units less than pH 6, for (*R*)- and (*S*)-**2** are approximated from Fig. 3. Therefore in pH 6 buffers, all catalytic imidazole moieties are regarded in the uncharged state and lead to $k_{2i}/K_{mi} = (k_{2i}/K_{mi})_{int}$ for (*R*)- and (*S*)-**2**. This implies that the merit of giving lower fraction of uncharged catalytic imidazole for catalyzing (*R*)-ethyl 2-hydroxy-2-phenylacetate leading to a low k_{2R}/K_{mR} for (*R*)-**2** disappears and gives a modest value of $V_S/V_R = 26$ for **2**.

4. Conclusions

A thermally stable *K. oxytoca* esterase is explored for the hydrolytic resolution of (*R*,*S*)-3-hydroxy-3-phenylpropionates in biphasic media. The structure–reactivity correlations in terms of

logarithms of specificity constants varied with the inductive parameter of leaving alcohol moiety are employed for interpreting the reaction mechanism and rationalizing the optimal enantioselectivity for **4**. The enantioselectivity can further improve to $V_S/V_R = 37$ using SNSM-87 immobilized on Sepabeads® EC-HA as the biocatalyst. The pH-reactivity profiles of (*R*)- and (*S*)-**2** are compared with those of (*R*)- and (*S*)-ethyl 2-hydroxy-2-phenylacetate for all enzyme preparations, showing the profound effect of 2- and 3-hydroxy moiety on changing the ionization state of catalytic imidazolium and hence the enzyme enantioselectivity.

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