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BIOTECHNOLOGY and BIOENGINEERING

Improvements of Enzyme Activity and Enantioselectivity Via Combined Substrate Engineering and Covalent Immobilization

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ABSTRACT: Esterases, lipases, and serine proteases have been applied as versatile biocatalysts for preparing a variety of chiral compounds in industry via the kinetic resolution of their racemates. In order to meet this requirement, three approaches of enzyme engineering, medium engineering, and substrate engineering are exploited to improve the enzyme activity and enantioselectivity. With the hydrolysis of (*R*,*S*)-mandelates in biphasic media consisting of isooctane and pH 6 buffer at 55°C as the model system, the strategy of combined substrate engineering and covalent immobilization leads to an increase of enzyme activity and enantioselectivity from $V_S/(E_t) = 1.62$ mmol/h g and $V_S/V_R = 43.6$ of (*R*,*S*)-ethyl mandelate (**1**) for a *Klebsiella oxytoca* esterase (named as SNSM-87 from the producer) to 16.7 mmol/h g and 867 of (*R*,*S*)-2-methoxyethyl mandelate (**4**) for the enzyme immobilized on Eupergit C 250L. The analysis is then extended to other (*R*,*S*)-2-hydroxycarboxylic acid esters, giving improvements of the enzyme performance from $V_S/(E_t) = 1.56$ mmol/h g and $V_S/V_R = 41.9$ of (*R*,*S*)-ethyl 3-chloromandelate (**9**) for the free esterase to 39.4 mmol/h g and 401 of (*R*,*S*)-2-methoxyethyl 3-chloromandelate (**16**) for the immobilized enzyme, $V_S/(E_t) = 5.46$ mmol/h g and $V_S/V_R = 8.27$ of (*R*,*S*)-ethyl 4-chloromandelate (**10**) for free SNSM-87 to 33.5 mmol/h g and 123 of (*R*,*S*)-methyl 4-chloromandelate (**14**) for the immobilized enzyme, as well as $V_S/(E_t) = 3.0$ mmol/h g and $V_S/V_R = 7.94$ of (*R*,*S*)-ethyl 3-phenyllactate (**11**) for the free esterase to 40.7 mmol/h g and 158 of (*R*,*S*)-2-methoxyethyl 3-phenyllactate (**18**) for the immobilized enzyme. The great enantioselectivty enhancement is rationalized from the alteration of ionization constants of imidazolium moiety of catalytic histidine for both enantiomers and conformation distortion of active site after the covalent immobilization, as well as the selection of leaving alcohol moiety via substrate engineering approach.

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Introduction

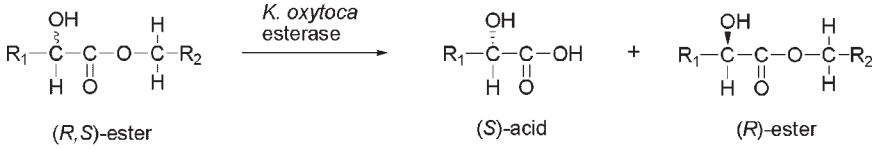
Esterases, lipases, and serine proteases are widely applied as versatile biocatalysts for preparing a variety of pharmaceuticals and fine chemicals with or without containing a chiral center (Kazlauskas and Bornscheuer, 1998). Their active site consisting of a Ser-His-Asp/Glu catalytic triad catalyzes the hydrolysis or synthesis by following an acylation-deacylation displacement mechanism (Bornscheuer and Kazlauskas, 2006; Dodson and Wlodawer, 1998; Hedstrom, 2002). On the basis of absence of pre-steady-state burst or comparison of kinetic constants, the acylation step for the formation of acyl-enzyme intermediate is frequently determined as the rate-limiting step in the hydrolase-catalyzed hydrolysis of esters (Bocola et al., 2003; Botta et al., 2002; Fujii et al., 2005; Heffner and Norin, 1999; Sutton and Quinn, 1990; Wang et al., 2007a,b) or amides (Bott et al., 2003; Case and Stein, 2003; Ishida and Kato, 2003). Since the catalytic histidine is highlighted as a general acid-base catalyst in the whole acylation step, an expanded Michaelis–Menten mechanism is further employed to interpret the solvent isotope effects or structure-reactivity correlations in terms of rate-limiting formation or breakdown of the tetrahedral adduct (Bott et al., 2003; Ema et al., 1998; Hirohara and Nishizawa, 1998; Nishizawa et al., 1997; Sutton and Quinn, 1990; Wang et al., 2007a,b).

Improvements of enzyme enantioselectivity via substrate engineering approach are well recognized in bioorganic syntheses (Ema et al., 1996, 1998; Faber, 2004; Magnusson et al., 2001; Miyazawa et al., 1999, 2006; Tsai et al., 2006; Yang et al., 1999; Zheng et al., 2006). Recently in the hydrolytic resolution of (*R*,*S*)-2-chloromandelates or (*R*,*S*)-2-chlorophenyl acetates in biphasic media, a two-stage Brønsted slope for the fast-reacting esters, but not for their antipodes, was determined from the structure-reactivity correlations (Wang and Tsai, 2005; Wang et al., 2006). This indicates that by altering the leaving alcohol moiety the rate-limiting acylation step for the fast-reacting esters may shift from breakdown to formation of the tetrahedral adduct, and leads to an optimal enantioselectivity for the methyl esters. If the rationale of obtaining the optimal enantioselectivity is valid for the serine-type hydrolase that catalyzes the hydrolytic resolution of esters containing a chiral center on the acyl part, the question is then how one can shift the breaking point of two-stage Brønsted slope to the right-hand side such that the optimal enantioselectivity can further enhance. Apparently, an increase of pK_a of imidazole moiety of catalytic histidine (K_a as the ionization constant of imidazolium group) is a possible solution, as it may effectively decrease proton transfer from the imidazolium to leaving alcohol moiety, and hence the Brønsted slope, for the fast-reacting reacting enantiomer.

The enzyme engineering approach of using site-directed mutation, evolution, chemical modification, or a combination of these techniques has been applied for improving enzyme activity, enantioselectivity, or stability (DeSantis and Jones, 1998; Penning, 2001; Reetz, 2001, 2006 and references herein; Berglund and Park, 2005; Davis, 2003; de Kreij et al., 2002; Ivarsson et al., 2007; Toscano et al., 2007 and references herein; Yasukawa and Inouye, 2007). In comparison with other techniques where only 20 primary proteinogenic amino acids or their closely resemble non-coded counterparts can be selected for incorporation into the biocatalyst, chemical modification of amino acid sidechain with or without site-specificity allows an almost unlimited variety of groups to be introduced. Although most of the currently employed methods for enzyme chemical modification are based on reactions of limited selectivity and efficiency, non-specific modifications of multiple sites to create heterogeneous mixtures are still commonplace and hence strongly require novel strategies such as combination of chemical modification with molecular modeling technique (and site-directed mutation) for the amino acids in the active site or on the enzyme surface, leading to promising biocatalysts.

A literature survey indicates that the alteration of enzyme surface charge via site-directed mutagenesis or chemical modification is an effective way to alter the active-site electrostatics, and hence the ionization constants of side-chain of catalytic amino acids in the active-site (Afzal et al., 2007; de Kreij et al., 2002; Yasukawa and Inouye, 2007). Indeed, increasing of positive surface charge of the serine proteases will destabilize the protonated imidazolium group of catalytic histidine and thus lowers its pK_a, while increasing of negative surface charge can stabilize the imidazolium, thereby raising its pK_a (DeSantis and Jones, 1998). Moreover, the additive effect of multiple modifications is frequently observed (Loewenthal et al., 1993). These findings have induced our hypothesis that the pK_a of imidazolium of catalytic histidine may increase when decreasing the positive surface charge of serine-type hydrolases via the multipoint attachment of amino group in the enzyme surface with chemical reagents (de Segura et al., 2004; Knezevic et al., 2006; Li et al., 2007; Othman et al., 2008). Therefore, this study is aimed to the combination of structure-reactivity correlations in substrate engineering and enzyme covalent immobilization on rationalizing the improvement of enzyme activity and enantioselectivity.

The hydrolytic resolution of (*R*,*S*)-mandelates in biphasic media via a thermally stable *Klebsiella oxytoca* esterase is first applied as the model system (Scheme 1) for studying effects of substrate engineering on enhancing the enzyme activity and enantioselectivity. Instead of using chemical reagents for the chemical modification, an epoxy-(oxirane)-activated support of Eupergit C 250L that can form multipoint attachment with amino group (or thiol and phenolic ones) in the enzyme surface is then employed for preparing the



(*R*,*S*)-ester (*S*)-acid (*R*)-ester

R_1: Ph; R_2: (1) CH_3, (2) H, (3) $CH_2OC_2H_5$, (4) CH_2OCH_3, (5) 3-pyridine, (6) CF_3, (7) 2-pyridine

R_2: CH_2; R_1: (8) 2-Cl-Ph, (9) 3-Cl-Ph, (10) 4-Cl-Ph, (11) CH_2Ph

R_2: H; R_1: (12) 2-Cl-Ph, (13) 3-Cl-Ph, (14) 4-Cl-Ph, (15) CH_2Ph

R_2: CH_2OCH_3; R_1: (16) 3-Cl-Ph, (17) 4-Cl-Ph, (18) CH_2Ph

Scheme 1. Hydrolytic resolution of (*R*,*S*)-2-hydroxycarboxylic acid esters in biphasic media.

immobilized enzyme, when considering the benefits of easy recovery from reaction media, long-term stability, and controlled modulation for altering the activity and selectivity in industrial applications. The Brønsted slopes estimated from the structure-reactivity correlations for the free and immobilized enzymes are compared and interpreted by considering the pK_a alternation of imidazolium of catalytic histidine after the immobilization. The study is finally extended to other (*R*,*S*)-2-hydroxycarboxylic acid esters (Scheme 1), showing the prospects of using the immobilized enzyme for preparing optically pure 2-hydroxycarboxylic acids as important building blocks for the synthesis of chiral pharmaceuticals and resolving agents in the resolution process (Groeger, 2001).

Materials and Methods

Materials

A *Klebsiella oxytoca* esterase (named as SNSM-87 by the producer) in the crude preparation was kindly donated from the Research & Development Center, Nagase & Co. Ltd. (Kobe, Japan). Other chemicals of analytical grade were commercially available: 2-hydroxymethyl pyridine from Acros (Geel, Belgium); acetophenone, (*R*,*S*)-ethyl mandelate ((*R*,*S*)-ethyl 2-hydroxyphenylacetate), (*R*,*S*)-mandelic acid and 2,2,2-trifluoroethanol from Aldrich (Milwaukee, WI); (*R*,*S*)-2-chloromandelic acid ((*R*,*S*)-2-(2-chlorophenyl)-hydroxyacetic acid), (*R*,*S*)-3-chloromandelic acid ((*R*,*S*)-3-chloro-2-hydroxyphenylacetic acid) and (*R*,*S*)-4-chloromandelic acid ((*R*,*S*)-4-chloro-2-hydroxyphenylacetic acid) from Alfa Aesar (Ward Hill, MA); Eupergit C 250L from Fluck (Buchs, Switzerland); 3-hydroxymethyl pyridine from TCI (Tokyo, Japan); 2-ethoxyethanol and 2-methoxyethanol from Merck (Darmstadt, Germany); (*R*,*S*)-3-phenyllactic acid ((*R*,*S*)-2-hydroxy 3-phenylpropanoic acid) from Sigma (St. Louis, MO); ethanol, isooctane, isopropanol, hexane and methanol from Tedia (Fairfield, OH). Phosphate was employed for the preparation of the pH 6 buffer.

Preparation of 2-Hydroxycarboxylic Acid Esters

To 650 mmol alcohol was added 5.35 mmol (*R*,*S*)-2-hydroxycarboxylic acid and 5 mmol sulfuric acid and stirred at 65°C for 18 h. After removing the remaining alcohol 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 (*R*,*S*)-2-hydroxycarboxylic acid ester of purity more than 98% based on the HPLC area (Wang et al. 2007a).

Analysis

The hydrolysis of (*R*,*S*)-2-hydroxy acid esters in biphasic media is monitored by HPLC using a chiral column from Daicel (Chiralcel OD-H, OJ-H or OD; Tokyo, Japan) that is capable of separating the internal standard of acetophenone, (*R*)- and (*S*)-ester. Detailed analytical conditions are represented in Table SI. Samples from the organic phase were removed and injected onto the HPLC at different time intervals for analysis, from which the time-course conversions and hence the initial rate for each enantiomer was determined.

Enzyme Immobilization

Unless specified, immobilizations were carried out in 10 mL phosphate buffers (1 M, pH 7) containing 100 mg of Eupergit C 250 L 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. The enzyme bound in terms of free SNSM-87 was determined as 76.4 mg per g of the immobilized support (Method 2 in Wang et al., 2008). This is confirmed from the weight increase of 36.4 mg per g of the immobilized support and the decrease of band absorption at about 907 cm^{-1} for the asymmetric stretching of epoxide ring in FTIR spectra (Fig. S1). Similar results were reported from the reduction of band absorption at 4,520 cm^{-1} in FT-NIR spectra (Fig. 3 in Li et al., 2007).

Kinetic Analysis

Unless specified, biphasic media consisting of 2.83 mL pH 6 buffer (300 mM) and 20 mL isooctane containing 1 mM (*R*,*S*)-2-hydroxycarboxylic acid ester were stirred at 55°C with a magnetic stirrer at 400 rpm. Reaction started when 0.5 mL pH 6 buffer containing a specific amount of free SNSM-87 or immobilized supports was added to the resultant solution. 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 the initial rate for each substrate were determined. The non-enzymatic hydrolysis and substrate partitioning in the biphasic media by using the HPLC analysis were also performed for estimating the non-enzymatic initial rate V and partition coefficient K_P for each substrate. Then, the enzymatic initial rate V_R or V_S was obtainable by subtracting V from the initial rate for each substrate. Similar experiments of varying (*R*,*S*)-ethyl mandelate concentration in the biphasic media were carried out, from which the kinetic constants were estimated.

Model Development

Estimation of Kinetic Constants

A generalized expanded Michaelis–Menten mechanism including a two-step process for the acylation step for the

hydrolase-catalyzed hydrolysis is employed to describe the enzymatic kinetics. By using the pseudo-steady-state approximation for all Michaelis complexes and tetrahedral intermediates and assuming an equilibrium partitioning for each substrate between the two phases, the enzymatic initial rates V_R and V_S based on the aqueous phase can be expressed as follows (Wang et al., 2007a):

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

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

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 in the organic phase $(S_{R0})_{org}$ (i.e., $(S_{S0})_{org}$) as $(S_R) = (S_S) = K_P(S_R)^*_{org}$, where K_P, $(S_R)^*_{org}$ $(= (S_{R0})_{org}/(1 + K_P V_{aq}/V_{org}))$, 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. Moreover, the kinetic parameters in the above equations are defined in Supporting Information Section.

When the enzyme has high enantioselectivity for the (S)-enantiomer, the concentration of (S)-ester is negligible in comparison with that of its antipode in determining the initial rate V_R. Therefore, one can at first employ Equation (1) to estimate $K_{mR}^{**} = K_{mR}^{*}$ and $k_{2R}^{**} = k_{2R}^{*}$, and then K_{mS}^{**} and k_{2S}^{**} (and hence K_{mS}^{*} and k_{2S}^{*}) from Equation (2). One can then further investigate variations of $k_{2S}^{**}/K_{mS}^{**} (= k_{2S}^{*}/K_{mS}^{*} = k_{2S}/K_{mS})$ and $k_{2R}^{**}/K_{mR}^{**} (= k_{2R}^{*}/K_{mR}^{*} = k_{2R}/K_{mR})$, and then the enantiomeric ratio E $(= k_{2S}K_{mR}/k_{2R}K_{mS})$ with the acyl or leaving alcohol moiety of the substrate. Moreover in the case of $K_{mR}^{**} \gg (S_R)$ and $K_{mS}^{**} \gg (S_S)$ for both substrates, the specificity constants 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. Detailed derivations may refer to Wang et al. (2007a).

Estimation of Intrinsic Kinetic Constants

The enzyme reactivity for each substrate can be evaluated by specificity constants k_{2R}/K_{mR} and k_{2S}/K_{mS}. Thus, the intrinsic specificity constants $(k_{2R}/K_{mR})_{int}$ and $(k_{2S}/K_{mS})_{int}$, and hence intrinsic enantiomeric ratio E_{int} $(= (k_{2S}/K_{mS})_{int}/(k_{2R}/K_{mR})_{int})$, related to the ionization constants K_{1i} and K_{2i} $(i = R$ and $S)$ in pH-activity profiles can be determined by using a non-linear least-squares regression method to the following empirical equation, where (H^+) means the proton concentration at the specified pH.

$$k_{2i}/K_{mi} = \frac{(k_{2i}/K_{mi})_{int}}{1 + K_{1i}/(H^+) + (H^+)/K_{2i}}, \quad i = R, S \quad (3)$$

Since the imidazole moiety of catalytic histidine as a general acid-base catalyst is directly involved in the reaction, it must be uncharged for catalysis and has ionization constants K_{2i}. It can be assumed that protonation of other acidic or basic groups may perturb the activity, and is considered by ionization constants K_{1i}.

Results and Discussion

Effects of Leaving Alcohol Moiety of (*R,S*)-Mandelates

Table I demonstrates variations of leaving alcohol moiety on changing K_P, $V_R/(E_t)$, $V_S/(E_t)$, V_S/V_R (or E-value), X_t, and

Table I. Effects of leaving group on inductive parameter F, partition coefficient K_P, specific initial rates, V_S/V_R, conversion X_t and ee_S for (R,S)-mandelate esters.

Ester	R_1	R_2	F	K_P	V (mM/h)	Enzyme	$V_R/(E_t)$ (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**)[a]	Ph	CH_3	0.01	1.70E−1	1.65E−3	Free[a]	3.72E−2	1.62	4.36E+1	0.750	24	58.5	100.0
						Immobilized	1.44E−3	4.65E−1	3.23E+2	0.457	24	17.2	20.6
(**2**)	Ph	H	0.03	6.81E−1	1.80E−3	Free[a]	2.01E−1	3.19E+1	1.59E+2	0.125	2	44.6	77.8
						Immobilized	8.36E−3	3.69	4.41E+2	0.230	7	38.6	62.3
(**3**)	Ph	$CH_2OC_2H_5$	0.11	7.40E−1	9.80E−3	Free	4.13E−1	3.91E+1	9.47E+1	0.125	3	49.7	91.5
						Immobilized	1.54E−2	1.19E+1	7.68E+2	0.230	7	43.8	77.4
(**4**)	Ph	CH_2OCH_3	0.13	7.70E−1	1.28E−2	Free	4.56E−1	4.25E+1	9.32E+1	0.125	3	51.0	95.8
						Immobilized	1.93E−2	1.67E+1	8.67E+2	0.230	5	45.6	83.2
(**5**)	Ph	3-pyridyl	0.24	1.01	6.20E−2	Free	1.44	8.27E+1	5.74E+1	0.125	1.5	51.2	93.4
						Immobilized	4.13E−2	3.43E+1	8.29E+2	0.230	2	42.5	73.5
(**6**)	Ph	CF_3	0.38	8.09E−2	5.04E−2	Free[a]	2.48E−1	7.92	3.19E+1	0.250	7	48.5	81.0
						Immobilized	5.69E−3	3.32	5.83E+2	0.230	7	38.1	61.0
(**7**)	Ph	2-pyridyl	0.40	9.42E−1	1.26E−1	Free	2.71	1.08E+2	3.99E+1	0.125	1.5	52.2	90.7
						Immobilized	9.74E−2	4.49E+1	4.60E+2	0.230	2	43.0	74.4

Conditions: 20 mL isooctane containing 1 mM (R,S)-mandelate ester and 3.33 mL pH 6 buffer (300 mM) containing free or immobilized enzyme for all esters at 55°C and 400 rpm.

[a]Data from Wang et al. (2007a).

ee_S, when free SNSM-87 is employed for the hydrolysis of (*R*,*S*)-mandelates in biphasic media consisting of isooctane and pH 6 buffer at 55°C. With (*R*,*S*)-methyl mandelate (**2**) as the reference, increasing the carbon-chain of leaving alcohol except for those containing a polar methoxy, ethoxy, 3-pyridyl or 2-pyridyl group results in the decease of K_P. This will reduce the substrate solubility and generally the specific enzymatic rate in the aqueous phase. As V_S/V_R acts as an index of enzyme enantioselectivity, free SNSM-87 is regarded as an excellent biocatalyst for resolving **2**, **3**, and **4** but not **1** and **5–7**.

The inductive parameter F of R_2 moiety 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. Therefore, it has been included in appropriate linear free energy relationships in a wide variety of reaction series (references in Hansch et al., 1991). In order to examine the enzymatic kinetic behavior, the specificity constants k_{2R}/K_{mR} and k_{2S}/K_{mS} are calculated and their logarithms are illustrated in Figure 1. A two-stage Brønsted slope with the breaking point at 0.03 for the fast-reacting esters is shown, implying that the rate-limiting step for (*S*)-esters containing a difficult leaving alcohol is breakdown of tetrahedral adduct to the acyl-enzyme intermediate. However, it changes to formation of tetrahedral adduct when (*S*)-esters contain an easy leaving alcohol. Moreover, only one Brønsted slope for all (*R*)-esters in the whole range of inductive parameters is shown, indicating that breakdown of tetrahedral adduct is rate-limiting. This can be attributed a concerted, but not effective, proton transfer from the imidazolium to the leaving alcohol moiety (Wang et al., 2007a). Therefore, it is rational to obtain maximum enantioselectivity at $F = 0.03$ for the methyl ester (**2**).

Similar kinetic behaviors for the resolution of (*R*,*S*)-2-chloromandelates and (*R*,*S*)-2-chlorophenyl acetates have been reported by varying the leaving alcohol moiety (Wang et al., 2007a,b). The structure-reactivity correlations are further represented in Table II, where the specificity constant with a steep ascent slope is observed for the fast-reacting esters containing a difficult leaving alcohol. The Brønsted slope varied from 25.73 of (*S*)-2-chloromandelates to 36.34 (*S*)-mandelates and then 57.97 of (*R*)-2-chlorophenyl acetates implies strong influence of the 2-substituent of acyl part on the rate-limiting breakdown of tetrahedral adduct. Therefore, the current approach and interpretation of obtaining an optimal *E*-value by varying the leaving alcohol moiety may generally apply to all serine-type hydrolases that obey the rate-limiting acylation step in hydrolytic resolution. Moreover, any technique, such as enhancing of the nucleophilic power of catalytic serine oxygen atom that can stabilize the transition state for formation of tetrahedral adduct for the fast-reacting esters, may shift the breaking point to a higher *F*-value, and hence increases the enzyme activity and enantioselectivity (Anderson et al., 2006). Similarly, any method that can increase the pK_a of imidazolium of catalytic histidine may effectively decrease the Brønsted slope of rate-limiting breakdown of tetrahedral adduct for fast-reacting esters, and thus increases the enzyme enantioselectivity.

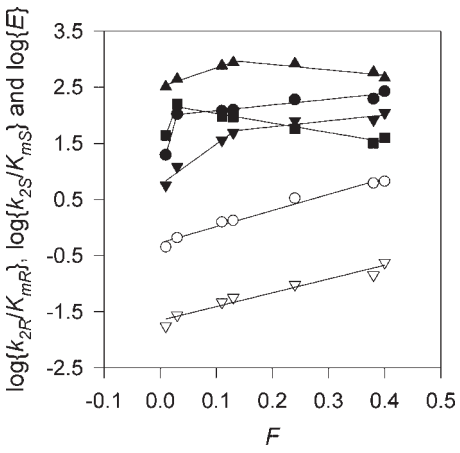


Figure 1. Variations of (○, ▽) log(k_{2R}/K_{mR}), (●, ▼) log(k_{2S}/K_{mS}), and (■, ▲) log(*E*) with the inductive parameters of leaving alcohol group of (*R*,*S*)-mandelates; (○, ●, ■) for free enzyme and (▽, ▼, ▲) for immobilized enzyme. (—) Best-fit results.

Effects of Covalent Immobilization

Effects of varying the leaving alcohol moiety of (*R*,*S*)-mandelates on changing $V_R/(E_t)$, $V_S/(E_t)$, V_S/V_R, X_t, and ee_S are demonstrated in Table I when the immobilized enzyme is employed for catalysis. In comparison with free SNSM-87, the immobilized enzyme generally shows lower catalytic efficiency, for example, $V_S/(E_t)$ dropping in a range from 11.6% of (*S*)-**2** to 41.9% of (*S*)-**6**, and $V_R/(E_t)$ from 2.3% of (*R*)-**6** to 4.2% of (*R*)-**4**. However, the enzyme enantioselectivity (i.e., V_S/V_R) greatly increases in a range from 2.8-fold of **2** to 18.3-fold of **6** after the immobilization. Obviously, the enantioselectivity enhancement can be attributed to the multipoint attachment of SNSM-87 to the support, but not to the diffusion limitation inside the support, as the latter will decrease the apparent enantioselectivity (Tsai and Dordick, 1996).

The logarithms of k_{2R}/K_{mR} and k_{2S}/K_{mS}, and hence log(*E*), for the immobilized enzyme were estimated and illustrated in Figure 1. A two-stage Brønsted slope for the fast-reacting (*S*)-esters is obtained, yet, with the breaking point shifting to $F = 0.13$. This implies that the electro-withdrawing effect of R_2 moiety on the rate-limiting breakdown of tetrahedral adduct decreases, owing to less efficient proton transfer from the imidazolium moiety. Similarly, only one Brønsted slope for all (*R*)-esters in the whole range of inductive parameters is demonstrated, indicating that the breakdown of tetrahedral adduct is rate-limiting and can be attributed a concerted, but not effective, proton transfer from the imidazolium to the leaving alcohol group. Therefore, it is

Table II. Structure-reactivity correlations for SNSM-87-catalyzed hydrolysis of various esters in biphasic media.

Substrates	Structure-reactivity correlations	Constrains
(*R*,*S*)-2-chloromandelates (free enzyme)[a]		
(*R*)	$\log(k_{2R}/K_{mR}) = -2.30 + 4.14F$, $r^2 = 0.99$	$-0.09 \le F \le 0.38$
(*S*)	$\log(k_{2S}/K_{mS}) = 1.07 + 25.73F$, $r^2 = 1.0$	$-0.09 \le F \le 0.03$
	$\log(k_{2S}/K_{mS}) = 1.85 + 1.53F$, $r^2 = 0.98$	$0.03 \le F \le 0.38$
(*R*,*S*)-2-chlorophenyl acetates (free enzyme)[b]		
(*R*)	$\log(k_{2R}/K_{mR}) = 0.27 + 57.97F$, $r^2 = 1.0$	$-0.01 \le F \le 0.01$
	$\log(k_{2R}/K_{mR}) = 0.87 + 2.28F$, $r^2 = 0.99$	$0.01 \le F \le 0.38$
(*S*)	$\log(k_{2S}/K_{mS}) = -1.02 + 4.15F$, $r^2 = 0.96$	$-0.01 \le F \le 0.38$
(*R*,*S*)-mandelates (free enzyme)		
(*R*)	$\log(k_{2R}/K_{mR}) = -0.27 + 2.88F$, $r^2 = 0.98$	$0.01 \le F \le 0.40$
(*S*)	$\log(k_{2S}/K_{mS}) = 0.93 + 36.34F$, $r^2 = 1.0$	$0.01 \le F \le 0.03$
	$\log(k_{2S}/K_{mS}) = 1.98 + 1.01F$, $r^2 = 0.92$	$0.03 \le F \le 0.40$
(*R*,*S*)-mandelates (immobilized enzyme)		
(*R*)	$\log(k_{2R}/K_{mR}) = -1.65 + 2.45F$, $r^2 = 0.95$	$0.01 \le F \le 0.40$
(*S*)	$\log(k_{2S}/K_{mS}) = 0.76 + 7.24F$, $r^2 = 0.97$	$0.01 \le F \le 0.13$
	$\log(k_{2S}/K_{mS}) = 1.59 + 1.05F$, $r^2 = 0.84$	$0.13 \le F \le 0.40$

[a]Data from Wang et al. (2007a).
[b]Data from Wang et al. (2007b).

rational to obtain maximum enantioselectivity at the inductive parameter of 0.13 for **4** (Fig. 1 or Table I).

The structure-reactivity correlations for the immobilized enzyme are also represented in Table II, where the Brønsted slope for the rate-limiting breakdown of tetrahedral adduct of (*S*)-mandelates greatly decreases to 7.24 in comparison with 36.34 of free SNSM-87. On the contrary, very similar slopes of 1.01 and 1.05 for the rate-limiting formation of tetrahedral adduct of (*S*)-mandelates, as well as those of 2.88 and 2.45 for the rate-limiting breakdown of tetrahedral adduct of (*R*)-mandelates, are found for both enzyme preparations. In order to rationalize the different structure-reactivity correlations after the immobilization, a kinetic analysis by considering the pH perturbation on amino acid sidechain of catalytic groups is needed. Nevertheless, the enzyme activity and enantioselectivity greatly improves from $V_S/(E_t) = 1.62$ mmol/h g and $V_S/V_R = 43.6$ of **1** for free SNSM-87 to 16.7 mmol/h g and 867 of **4** for the immobilized enzyme.

Kinetic Analysis for (*R,S*)-Mandelates

The kinetic constants (Table III) of using **1** as the substrate for both enzyme preparations are estimated by coupling Equations (1) and (2) and the initial rates varied with substrate concentrations (Fig. S2). Regardless of the enzyme preparation employed, the same order-of-magnitude of all kinetic parameters K^*_{mR} (i.e., $(E)(S_R)/[(ES_R) + (E^*S_R)]$) and K^*_{mS} (i.e., $(E)(S_S)/[(ES_S) + (E^*S_S)]$) implies that either (*R*)-**1** or (*S*)-**1** has similar affinity on combining the enzyme preparation to enzyme-substrate complex and tetrahedral adduct. However on the basis of k^*_{2R}, about 48-fold enhancements of k^*_{2S} for free SNSM-87 are obtained and lead to $E = 50$ (or 158-fold enhancements of k^*_{2S} and $E = 400$ for the immobilized enzyme). These results indicate that proton transfer from the imidazolium for breakdown of tetrahedral adduct must play an essential role on creating the chiral discrimination ability for both enzyme preparations. Therefore, an estimation of the ionization constants for the imidazolium of catalytic histidine at the presence of substrates is needed.

Figure S3 illustrates effects of changing pH on relative enzyme activity of (*R*)- or (*S*)-ethyl mandelate at 55°C (Wang et al., 2008). Since $K^{**}_{mR} \gg (S_R)$ and $K^{**}_{mS} \gg (S_S)$ for both enzyme preparations at the present reaction conditions (Tables I and III), this figure can be regarded as effects of varying pH on the relative specificity constants k_{2R}/K_{mR} and k_{2S}/K_{mS}. From a non-linear least-squares regression of the experimental data to Equation (3), the ionization constants in terms of pK_{1i} and pK_{2i} for each substrate and enzyme preparation are tabulated (Table IV). Slight changes of pK_{1R} from 9.87 to 9.96 and pK_{2S} from 5.16 to 5.37 are obtained for the enzyme after immobilization. However, more changes

Table III. Effects of enzyme immobilization on kinetic constants and *E* values.

	Free enzyme[a]	Immobilization enzyme
k^*_{2R} (mmol/h g)	8.6E−1	3.1E−2
K^*_{mR} (mM)	2.7	2.8
k_{2R}/K_{mR} (L/h g)	3.2E−1	1.1E−2
$(k_{2R}/K_{mR})_{int}$ (L/h g)	6.1E−1	1.9E−1
k^*_{2S} (mmol/h g)	4.1E+1	4.9
K^*_{mS} (mM)	2.5	1.2
k_{2S}/K_{mS} (L/h g)	1.6E+1	4.4
$(k_{2S}/K_{mS})_{int}$ (L/h g)	1.8E+1	5.4
E	5.0E+1	4.0E+2
E_{int}	3.0E+1	2.8E+1

Conditions: hydrolysis of (*R*,*S*)-ethyl mandelate in biphasic medium containing 20 mL isooctane and 3.33 mL pH 6 buffer at 55°C. Values in the parentheses are exponents, for example, $E-1 = 10^{-1}$.
[a]Data from Wang et al. (2007a).

Table IV. Ionization constants pK_{1i} and pK_{2i} estimated from Equation (3) for each substrate and enzyme preparation.

	i	pK_{1i}	pK_{2i}
Free enzyme	*R*	9.87	5.96
	S	9.89	5.16
Immobilized enzyme	*R*	9.96	7.22
	S	9.33	5.37

Conditions: hydrolysis of 1 mM (*R*,*S*)-ethyl mandelate in biphasic medium containing 20 mL isooctane and 3.33 mL pH 6 buffer at 55°C.

of pK_{1S} from 9.89 to 9.33 and even from 5.89 to 7.22 for pK_{2R} occur. There is still no explanation of giving the different pK_{1R} and pK_{1S}, as well as pK_{2R} and pK_{2S}, for each enzyme preparation, although a literature survey indicates that pK_a values of catalytic groups in the active site are perturbed differently when the (*R*)- or (*S*)-enantiomer of a substrate is bound and leads to a pH-dependence of enantioselectivity (Guncheva et al., 2004; Lummer et al., 1999; Secundo and Phillips, 1996). Since K_{2i} ($i = R, S$) may refer to the ionization constants of imidazolium of catalytic histidine, an enhancement of pK_{2S}, especially pK_{2R}, is apparent after the immobilization. Unfortunately, we can not distinguish the formation of C–N bond between oxirane moiety of the support and amino group in the enzyme surface from C=O and C–O–C stretching of ester and amide backbone of the support in FTIR spectra (Fig. S1). Yet, this bond formation has been confirmed between certain primary or secondary amine with oxirane group of polymeric microsphere on forming secondary or tertiary β-hydroxy amine (Choi et al., 2003). The presence of β-hydroxy moiety will certainly weaken the basicity of resulting amine in comparison with the original primary or secondary amine (Perrin et al., 1981). Therefore, the decrease of positive surface charge in the enzyme surface at pH 6 buffer is plausible, and may confirm the hypothesis of pK_{2i} enhancement after the immobilization.

By substituting pK_{1i} and pK_{2i} ($i = R, S$) into Equation (3), the intrinsic specificity constants $(k_{2i}/K_{mi})_{int}$ for both enzyme preparations are determined and represented in Table III. A ratio of 3.3 defined as $(k_{2S}/K_{mS})_{int}$ of free SNSM-87 to that of the immobilized enzyme gives a difference of 3.28 kJ/mol between the free energy difference of transition and ground states of (*S*)-**1** for both enzyme preparations. Similarly, a ratio of 3.2 based on $(k_{2R}/K_{mR})_{int}$ and hence a difference of 3.17 kJ/mol for (*R*)-**1** are obtainable. Therefore, the covalent immobilization might cause minute conformation distortion such that the strength of hydrogen bonding in the oxyanion hole of active site for the transition state of each enantiomer may decrease, and leads to nearly the same E_{int} for both enzyme preparations. By assuming all pK values (Table IV) to be valid for other (*R*,*S*)-mandelates, the ratios in a range from 2.4 of (*S*)-**4** to 2.2 of (*S*)-**7** based on $(k_{2S}/K_{mS})_{int}$, as well as those from 3.2 of (*R*)-**1** to 3.7 of (*R*)-**7** based on $(k_{2R}/K_{mR})_{int}$, are determined from Table I. These indicate that the conformation distortion indeed has exerted nearly the same power on the rate-limiting formation of tetrahedral adduct of (*S*)-mandelates and breakdown of tetrahedral adduct of (*R*)-mandelates, regardless of the inductive parameter employed. However for the rate-limiting breakdown of tetrahedral adduct of (*S*)-mandelates, an increase of $(k_{2S}/K_{mS})_{int}$ ratio from 3.3 of (*S*)-**1** to 9.6 of (*S*)-**2** implies a more strong cooperated interaction between the imidazolium group (in terms of pK_{2S}) for proton transfer and R_2 moiety of leaving alcohol (in terms of *F*) for breakdown of tetrahedral adduct. Therefore, a 3.5-fold reduction of E_{int} for **1** is expected for the immobilized enzyme. Fortunately, this reduction is compensated from the more increase of pK_{2R} in comparison with pK_{2S} for the immobilized enzyme, and leads to a higher *E* owing to the more uncharged imidazole moiety of catalytic histidine for catalyzing (*S*)-mandelates.

Effects of Acyl Donor

Other (*R*,*S*)-2-hydroxycarboxylic acid esters (**8**–**18**) containing an aryl or arylalphatic moiety at the 2-carbon position are selected as the substrates for exploiting the immobilized enzyme as an efficient biocatalyst. Table V demonstrates variations of *V*, $V_R/(E_t)$, $V_S/(E_t)$, V_S/V_R, X_t, and ee_S with the acyl moiety and leaving alcohol. Very similar kinetic behaviors of changing the leaving alcohol moiety are found when (*R*,*S*)-mandelates are replaced by other esters. For example in the hydrolysis of (*R*,*S*)-3-chloromandelates via free SNSM-87, the specific initial rate $V_S/(E_t)$ rapidly increases from 1.56 mmol/h g of (*S*)-**9** to 26.3 mmol/h g of (*S*)-**13**, and then gradually to 39.4 mmol/h g of (*S*)-**16**. However, the specific initial rate $V_R/(E_t)$ smoothly changes from 0.0372 mmol/h g of (*R*)-**9** to 0.180 mmol/h g of (*R*)-**13**, and then 0.640 mmol/h g of (*S*)-**16**, leading to an optimal V_S/V_R of 143 for the methyl ester **13**. Therefore, a two-stage Brønsted slope with the breaking point at $F = 0.03$ for (*S*)-3-chloromandelates but only one Brønsted slope for all (*R*)-3-chloromandelates are expected, if the logarithms of k_{2R}/K_{mR} and k_{2S}/K_{mS} are further estimated and plotted.

An enhancement of enantioselectivity V_S/V_R from 126 of **9** to 365 of **13**, and then 401 of **16**, is demonstrated in Table V when the immobilized enzyme is employed for catalysis. However, the catalytic efficiency for (*R*)- or (*S*)-3-chloromandelates in general decreases after the immobilization, indicating that the breaking point of two-stage Brønsted slope will shift to an inductive parameter higher than 0.03. Similar arguments as described for (*R*,*S*)-mandelates and (*R*,*S*)-3-chloromandelates are also valid when using (*R*,*S*)-2-chloromandelates, (*R*,*S*)-4-chloromandelates, or (*R*,*S*)-3-phenyllactic acid esters as the substrate (Table V). Therefore by following the strategy of combined substrate engineering and covalent immobilization, the enzyme activity and enantioselectivity has greatly improved from $V_S/(E_t) = 1.56$ mmol/h g and $V_S/V_R = 41.9$ of **9**, 5.46 mmol/h g and 8.27 of **10**, as well as 3.0 mmol/h g

Table V. Effects of immobilization on hydrolytic resolution of (*R*,*S*)-2-hydroxycarboxylic acid esters.

Ester	R_1	R_2	K_P	V (mM/h)	Enzyme	$V_R/(E_t)$ (mmol/h g)	$V_S/(E_t)$ (mmol/h g)	V_S/V_R	(E_t) (mg/mL)	Time (h)	X_t (%)	ee_S (%)
(**8**)	2-Cl-Ph	CH_3	7.01E−2	7.20E−4	Free[a]	2.07E−4	6.30E−1	3.04E+3	2.000	7	50.3	100.0
					Immobilized	ND	1.62E−1	≫1	1.142	24	15.4	18.2
(**9**)	3-Cl-Ph	CH_3	7.97E−2	1.20E−3	Free[a]	3.72E−2	1.56	4.19E+1	0.750	24	58.0	100.0
					Immobilized	2.88E−3	3.63E−1	1.26E+2	0.457	24	13.9	15.8
(**10**)	4-Cl-Ph	CH_3	7.10E−2	1.56E−3	Free[a]	6.60E−1	5.46	8.27	0.500	5	66.6	100.0
					Immobilized	4.29E−2	1.36	3.20E+1	0.457	24	35.8	50.5
(**11**)	CH_2Ph	CH_3	7.50E−2	1.42E−3	Free[a]	3.78E−1	3.00	7.94	2.000	2	70.4	100.0
					Immobilized	6.65E−2	1.26	1.90E+1	0.457	24	34.9	44.8
(**12**)	2-Cl-Ph	H	1.59E−1	9.60E−4	Free	4.80E−4	5.31	1.11E+4	2.000	1	51.0	100.0
					Immobilized	ND	1.73	≫1	0.457	7	37.1	58.7
(**13**)	3-Cl-Ph	H	1.45E−1	1.38E−3	Free[a]	1.80E−1	2.63E+1	1.46E+2	0.125	7	48.7	90.7
					Immobilized	9.37E−3	3.44	3.65E+2	0.230	7	38.4	61.3
(**14**)	4-Cl-Ph	H	1.56E−1	2.12E−3	Free	2.88	9.15E+1	3.30E+1	0.125	2	59.2	100.0
					Immobilized	2.72E−1	3.35E+1	1.23E+2	0.230	3	54.0	100.0
(**15**)	CH_2Ph	H	1.46E−1	1.53E−2	Free[a]	2.27	7.90E+1	3.50E+1	0.125	2	59.6	100.0
					Immobilized	1.48E−1	1.12E+1	7.60E+1	0.230	5	42.6	70.3
(**16**)	3-Cl-Ph	CH_2OCH_3	1.51E−1	3.11E−3	Free	6.40E−1	3.94E+1	6.16E+1	0.125	3	49.6	89.2
					Immobilized	5.28E−2	2.11E+1	4.01E+2	0.457	3	50.3	98.2
(**17**)	4-Cl-Ph	CH_2OCH_3	1.43E−1	5.65E−3	Free	9.84	9.77E+1	9.93	0.125	2	59.6	83.3
					Immobilized	8.96E−1	4.21E+1	4.69E+1	0.230	5	49.1	85.7
(**18**)	CH_2Ph	CH_2OCH_3	1.60E−1	2.88E−2	Free	1.15E+1	1.03E+2	8.94	0.250	2	60.1	82.6
					Immobilized	2.36E−1	3.73E+1	1.58E+2	0.230	5	48.2	89.7

Conditions: 1 mM (*R*,*S*)-2-hydroxycarboxylic acid esters in biphasic media containing 20 mL isooctane and 3.33 mL pH 6 buffer at 55°C. ND as "not determine."

[a]Data from Wang et al. (2007a).

and 7.94 of **11** for free SNSM-87 to $V_S/(E_t) = 39.4$ mmol/h g and $V_S/V_R = 401$ of **16**, 33.5 mmol/h g and 123 of **14**, as well as 40.7 mmol/h g and 158 of **18** for the immobilized enzyme, respectively.

Conclusions

With the hydrolysis of (*R*,*S*)-mandelates in biphasic media consisting of isooctane and pH 6 buffer at 55°C as the model system, the enzyme activity and enantioselectivity for free SNSM-87 is enhanced via the substrate engineering approach of using a variety of leaving alcohols with different inductive parameters. A two-stage Brønsted slope for the fast-reacting (*S*)-mandelates, but only one-stage Brønsted slope for all (*R*)-esters, is rationalized to give an optimal enantioselectivity of $V_S/V_R = 159$ at $F = 0.03$ of **1**. The enantioselectivity is further increased to $V_S/V_R = 867$ at $F = 0.13$ of **4** when SNSM-87 immobilized on Eupergit C 250L is employed for catalysis. A thorough kinetic analysis for both enzyme preparations indicates that the covalent immobilization might yield minute conformation distortion of the active site and an enhancement of ionization constants for imidazolium moiety of catalytic histidine, for example, pK_{2R} from 5.89 to 7.22 for (*R*)-**1** and pK_{2S} from 5.16 to 5.37 for (*S*)-**1**. These will decrease the catalytic efficiency $V_S/(E_t)$ and $V_R/(E_t)$ of the immobilized enzyme, but can still lead to an enantioselectivity improvement if pH 6 buffer of the aqueous phase and an inductive parameter of 0.13 for the leaving alcohol moiety are selected. The analysis is also extended to other (*R*,*S*)-2-hydroxycarboxylic acid esters of (*R*,*S*)-2-chloromandelates, (*R*,*S*)-3-chloromandelates, (*R*,*S*)-4-chloromandelates and (*R*,*S*)-3-phenyllactic acid esters, showing the prospects of using the strategy of combined substrate engineering and covalent immobilization for improving the enzyme performances.

Nomenclature

ee_S	enantiomeric excess for remaining (*R*)-esters
E	enantiomeric ratio, defined as $k_{2S}K_{mR}/k_{2R}K_{mS}$ or V_S/V_R
E_{int}	intrinsic enantiomeric ratio, defined as $(k_{2S}/K_{mS})_{int}/(k_{2R}/K_{mR})_{int}$
(E_t)	enzyme concentration in terms of free SNSM-87 in aqueous phase (g/L)
F	inductive parameter for leaving alcohol
(H^+)	proton concentration of aqueous phase (M)
k^*_{2i}, k^{**}_{2i}	kinetic parameters, $i = R$ and S for (*R*)- and (*S*)-enantiomer 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_{1i}, K_{2i}	ionization constants defined in Equation (3), $i = R$ and S for (*R*)- and (*S*)-ester, respectively (M)
K_{mi}, K^*_{mi}, K^{**}_{mi}	kinetic constants, $i = R$ and S for (*R*)- and (*S*)-ester, respectively, in aqueous phase (mM)
$(k_{2i}/K_{mi})_{int}$	intrinsic specificity constants, $i = R$ and S for (*R*)- and (*S*)-ester, respectively (L/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, without the presence of aqueous phase (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*)-ester based on aqueous phase, respectively (mM/h)
V_{aq}, V_{org}	volumes of aqueous and organic phases, respectively (mL)
X_t	racemate conversion defined as $\{1-[(S_R)_{org}+(S_S)_{org}]\times[1+K_pV_{aq}/V_{org})]/[(S_{R0})_{org}+(S_{S0})_{org}]\}$ or $\{1-[(S_R)_{org}+(S_S)_{org}]/[(S_R)^*_{org}+(S_S)^*_{org}]\}$

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