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Enhancement of enantioselectivity in lipase-catalyzed resolution of *N*-(2-ethyl-6-methylphenyl)alanine by additives

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Abstract

The lipase B from *Candida antarctica* (CAL-B)-catalyzed the enantioselective resolution of *N*-(2-ethyl-6-methylphenyl)alanine from the corresponding racemic methyl ester is investigated. However, the enantioselectivity of CAL-B towards the resolution is not high enough to obtain enantiomerically pure compound. With the aim to improve the enantioselectivity of enzyme, various additives including macrocyclic dioxotetraamines, organic compounds and ionic liquids are tested in the CAL-B-catalyzed hydrolysis. Under the optimized conditions, the highest enantioselectivity of CAL-B ($E > 100$) is achieved in diethyl ether/water (15%, v/v), which is about 9.7-fold more enantioselective than that in pure buffered aqueous solution ($E = 12.1$). When the macrocylic dioxotetraamine (n-C_6H_{13}-MDTA) is added in the reaction medium, both catalytic activity and enantioselectivity of CAL-B are markedly enhanced (*E*-value is up to 87.6 at 45.6% conversion only in 1.5 h). While the *E*-value is improved only up to 44.7 using ionic liquid ([ETOMG]BF_4) as additive, it can establish the green environment of enzymatic catalysis.

Keywords: *Candida antarctica B* lipase; Resolution; (*S*)-*N*-(2-ethyl-6-methylphenyl)alanine; Additives

1. Introduction

(*S*)-(−)-*N*-(2-ethyl-6-methylphenyl)alanine ((*S*)-NEMPA) is an important precursor in the synthesis of most widely used herbicides such as (*S*)-metolachlor [1]. (*S*)-NEMPA is currently produced by chemical synthesis in large quantities [2]. Nevertheless, the chemical method requires drastic reaction conditions that may cause racemization, decomposition or side reactions. Especially for industrial process, long reaction time at higher temperature is unfavorable due to high-energy consumption [3]. An alternative to such processes will be to use a highly active biocatalyst in a very efficient manner. The use of enzymes as chiral catalysts to prepare optically active compounds from either racemic or prochiral substrates has been widely studied [4,5]. Among a variety of enzymes, there has been a growing interest in the use of lipase [6,7] (triacylglycerol ester hydrolase, EC 3.1.1.3) for kinetic resolution of racemic compounds through esterification, hydrolysis and transesterification reactions because of its wide substrate specificity and ability to recognize chirality. However, the use of lipase as chiral catalyst to prepare enantiopure (*S*)-NEMPA from the corresponding racemic ester has not been widely studied.

Of the lipases from different organisms, *Candida antarctica B* lipase (CAL-B) [8,9] is a very efficient catalyst and is widely used in practice. CAL-B has been found to tolerate a great variation in experimental conditions and it has been shown in numerous publications [10–13] to be a particularly efficient enzyme, catalyzing a great number of different organic reactions including many that have been scaled up to commercial scale.

In the previous work (data not published), we had attempted to develop a stereoselective hydrolytic process for studying the feasibility of resolution of NEMPA via CAL-B (Scheme 1). The effect of microenvironment on the activity and enantioselectivity of CAL-B was initially investigated, and found that the lipase CAL-B was very active and nearly 50% of (*R*, *S*)-*N*-(2-ethyl-6-methylphenyl)alanine methyl ester ((*R*, *S*)-NEMPA-ME) was hydrolysis only after 2 h at 37 °C, but displayed poor enantiose-

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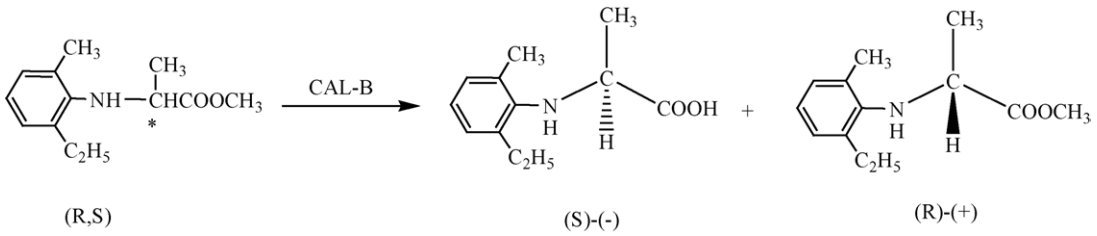

Scheme 1. Enantioselective hydrolysis of methyl ester of NEMPA using CAL-B.

lectivity ($E = 10.6$) towards the (*S*)-enantiomer of the acid. The above results in terms of the *E*-value imply that more studies on improving the enzyme enantioselectivity and hence the optical purity for the desired enantiomer are needed. From an application point of view, among the methods reported to enhance the enantioselectivity of enzyme-mediated reactions, treatment of the reaction mixture with an additive is a very attractive and convenient way to improve the outcome of the reaction because of its simplicity for practical use. An excellent review in this field by Theil [14] has elucidated the advantages associated with the additives based system, and some compounds have so far been reported to enhance the enantioselectivity of lipase-catalyzed reaction [15–31]. However, for the enzymatic hydrolysis of NEMPA ester, achieving an improvement in enantioselectivity by additive method is to our knowledge without precedent in the literatures. Herein, three different types of additives (macrocyclic dioxotetraamines, organic compounds and ionic liquids) are selected to observe their effects on the CAL-B-catalyzed resolution of NEMPA.

2. Materials and methods

2.1. Materials

The lipase B from *Candida antarctica* (CAL-B) was kindly donated by Novo Nordisk Industries (China). Macrocylic dioxotetraamines (MDTA) and derivatives were kindly donated by Department of Chemistry, Sichuan University (Chengdu, China). Ionic liquids were kindly donated by College of Chemistry, Jilin University (Changchun, China). The authenticity of (*R*, *S*)-*N*-(2-ethyl-6-methylphenyl)alanine methyl ester ((*R*, *S*)-NEMPA-ME) prepared during the study was confirmed by spectroscopic analysis including 300 MHz NMR (Mercury-300B, VARIAN, USA) and GC–MS (Saturn 220, VARIAN, USA). Reactions were routinely monitored on silica-gel plates (Qingdao Haiyang Chemical Co., LTD., China) using UV light for detection of the spots. All the organic solvents were reagent grade and used without further purification. Other reagents were all analytical grade or better.

2.2. Determination of conversion and enantiomeric excess ($e.e._p$)

The analysis of the reaction mixtures and the determination of enantiomeric excesses of (*S*)-NEMPA were performed by capillary zone electrophoresis (P/ACE MDQ, Beckman, USA) with a 59 cm (49 cm to detector) × 50 μm i.d. eCAP™ neutral capillary (Beckman, USA). The conversion of reaction was determined by using 100 mmol/L triethylamine/acetic acid buffer (TEAA, pH 5.5) as background electrolyte. The enantiomeric excess of (*S*)-NEMPA was successfully analyzed in 100 mmol/L TEAA, pH 5.5 by using 40 mmol/L 2,6-di-*O*-methyl-β-cyclodextrin (DM-β-CD, Beckman, USA) as a buffer additive. The analysis was performed with applied voltage at −20 kV, and the absorbance was recorded at 200 nm.

Enantiomeric ratio (*E*) of hydrolysis of (*R*, *S*)-NEMPA-ME was calculated from the conversion (*c*) and enantiomeric excess ($e.e._p$) of (*S*)-NEMPA, using the equation [32]: $E = \ln[1 - c\ (1 + e.e._p)]/\ln[1 - c\ (1 - e.e._p)]$, where $e.e._p = (c_S - c_R)/(c_S + c_R)$, while c_S and c_R are the concentrations of (*S*)- and (*R*)-enantiomers, respectively. The absolute configuration of the enantiomers was established by comparison of the measured optical rotation with the literature data [2].

2.3. Preparation of (R, S)-NEMPA-ME

The reaction mixture of 2-ethyl-6-methylaniline (8.4 mL, 60 mmol), $NaHCO_3$ (5.5 g, 65 mmol) and methyl-2-bromopropionate (180 mmol), was stirred under nitrogen atmosphere and slowly heated up to 120–125 °C in 1 h. Then, the dark reaction mixture was continually kept at the temperature for 18 h with stirring. After it was cooled, the reaction mixture was transferred into 30 mL of ice water and extracted with ethyl acetate. The ethyl acetate fractions were dried over sodium sulfate and concentrated in a rotary evaporator at 40 °C. The resulting ester after normal work up was purified by column chromatography on silica gel using ethyl acetate and petroleum ether (1:5) as the eluant to furnish the corresponding methyl ester (8.9 g). *N*-(2-ethyl-6-methylphenyl)alanine methyl ester (NEMPA-ME):GC–MS *m*/*z*: 221(25, M^+), 162 (100, $M^+ - (C{=}O)OCH_3$), 133 (30, $M^+ - (C{=}O)OCH_3\text{-}C_2H_5$), 77 (11, Ph); GC: 98% area. ^{1}H NMR ($CDCl_3$): δ 7.02–6.96 (m, 2H, Ph), 6.88–6.83 (t, 1H, Ph, $J = 7.2$ Hz), 3.96–3.94 (q, 1H, CH, $J = 6.9$ Hz), 3.81 (S, 1H, NH), 3.66 (S, 3H, CH_3), 2.69–2.66 (m, 2H, CH_2), 2.30 (S, 3H, CH_3), 1.38–1.35 (d, 3H, CH_3, $J = 6.9$ Hz), 1.26–1.21 (t, 3H, CH_3, $J = 7.5$ Hz); ^{13}C NMR ($CDCl_3$): δ 176.2, 143.6, 135.3, 129.6, 129.1, 126.9, 122.4, 55.8, 52.2, 24.6, 19.9, 19.2, 14.7.

2.4. Biocatalytic hydrolysis of (R, S)-NEMPA-ME in aqueous buffer

CAL-B (10 mg) was added to a suspension of (*R*, *S*)-NEMPA-ME (0.5 mmol, 110 mg) of phosphate buffer (100 mmol/L,

pH 8.0, 5.0 mL). The resulting mixture was shaken at 25 °C and the pH was maintained using 0.1 mol/L NaOH solution. Aliquots were periodically drawn and analyzed on capillary electrophoresis. When the hydrolysis reached 48% conversion, a saturated solution of $NaHCO_3$ (10 mL) was added to the reaction mixture. The mixture was first extracted with ether (3 × 20 mL) to remove the unchanged ester. The aqueous mixture was then acidified to pH 5.5 with 0.1 mol/L HCl and was again extracted with ether (3 × 20 mL) to remove the acid product. Acid extracts were dried over anhydrous Na_2SO_4 and evaporated to give (*S*)-(−)-NEMPA (48.5 mg, $e.e._p$ = 78.9%).

2.5. Biocatalytic hydrolysis of (R, S)-NEMPA-ME in aqueous buffer with organic compound

To the aqueous phosphate buffer (100 mmol/L, pH 8.0) containing diethyl ether (15%, v/v) 5.0 mL, CAL-B (10 mg) and (*R*, *S*)-NEMPA-ME (0.5 mmol, 110 mg) were added. The mixture was stirred at 25 °C and the pH was maintained using 0.1 mol/L NaOH solution. When the hydrolysis reached 49% conversion, a saturated solution of $NaHCO_3$ (10 mL) was added to the reaction mixture. The mixture was first extracted with ether (3 × 20 mL) to remove the unchanged ester. The aqueous mixture was then acidified to pH 5.5 with 0.1 mol/L HCl and was again extracted with ether (3 × 20 mL) to remove the acid product. Acid extracts were dried over anhydrous Na_2SO_4 and evaporated to give (*S*)-(−)-NEMPA (49.6 mg, $e.e._p$ > 98%).

2.6. Biocatalytic hydrolysis of (R, S)-NEMPA-ME in aqueous buffer with macrocylic dioxotetraamine

To the aqueous phosphate buffer (100 mmol/L, pH 8.0) containing *n*-C_6H_{13}-MDTA (1.0 mg/mL) and Tween-80 (5.0 mg/mL) 5.0 mL, CAL-B (10 mg) and (*R*, *S*)-NEMPA-ME (0.5 mmol, 110 mg) were added. The resulting mixture was shaken at 25 °C and the pH was maintained using 0.1 mol/L NaOH solution. When the hydrolysis reached 48% conversion, a saturated solution of $NaHCO_3$ (10 mL) was added to the reaction mixture. The mixture was first extracted with ether (3 × 20 mL) to remove the unchanged ester. The aqueous mixture was then acidified to pH 5.5 with 0.1 mol/L HCl and was again extracted with ether (3 × 20 mL) to remove the acid product. Acid extracts were dried over anhydrous Na_2SO_4 and evaporated to give (*S*)-(−)-NEMPA (49.0 mg, $e.e._p$ = 94.6%).

2.7. Biocatalytic hydrolysis of (R, S)-NEMPA-ME in aqueous buffer with ionic liquid

To the aqueous phosphate buffer (100 mmol/L, pH 8.0) containing ionic liquid [ETOMG]BF_4 (50 vol.%) 5.0 mL, CAL-B (10 mg) and (*R*, *S*)-NEMPA-ME (0.5 mmol, 110 mg) were added. The resulting mixture was shaken at 25 °C and the pH was maintained using 0.1 mol/L NaOH solution. When the hydrolysis reached 49.5% conversion, a saturated solution of $NaHCO_3$ (10 mL) was added to the reaction mixture. The mixture was first extracted with ether (3 × 20 mL) to remove the unchanged ester. The aqueous mixture was then acidified to pH 5.5 with 0.1 mol/L HCl and was again extracted with ether (3 × 20 mL) to remove the acid product. Acid extracts were dried over anhydrous Na_2SO_4 and evaporated to give (*S*)-(−)-NEMPA (48.8 mg, $e.e._p$ = 92.3%). After separation of the product, the ionic liquid was extracted from the reaction mixture by using dichloromethane for further use.

3. Results and discussion

3.1. Effect of macrocyclic dioxotetraamines on the activity and enantioselectivity of CAL-B

Some researches [33,34] have indicated that the metal complexes of macrocyclic dioxotetraamines show important biological functions as models for metalloproteins and oxygen carriers, but its usage as additive in the enzyme-catalyzed resolution has not been concerned. In this paper, 2,6-dioxo-1,4,7,10-tetraazacyclododecane (MDTA, a) and three different long chain alkyl substituted MDTA bearing an alcohol pendant (b–d) [35] were selected as additives for the first time to investigate their effect on the catalytic properties of CAL-B in the hydrolysis of (*R*, *S*)-NEMPA-ME (Table 1). Herein, it should be pointed out that macrocyclic dioxotetraamines were selected as additives in this work, exactly because their structures are similar to that of crown esters, whereas Itoh et al. [19–21] found crown esters as additives can remarkably enhance the reaction rate and enantioselectivity in lipase-catalyzed hydrolysis of carboxylic esters.

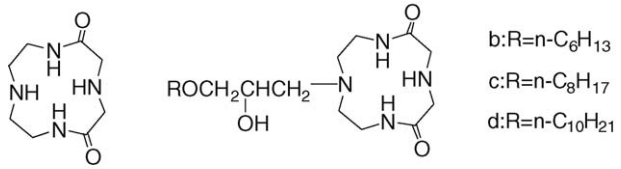

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Table 1
Effect of different MDTA on the activity and enantioselectivity of CAL-B[a]

Amine	Additive amount (mg/mL)	Time (h)	Conversion (%)	$e.e._p$ (%)	*E*-value
None	–	3.0	45.3	78.7	16.3
MDTA	1.0	1.8	46.2	71.0	10.9
n-C_6H_{13}-MDTA	1.0	1.5	45.6	94.6	87.6
n-C_8H_{17}-MDTA	1.0	1.2	47.9	83.2	25.0
n-$C_{10}H_{21}$-MDTA	1.0	0.5	43.7	50.0	4.3

[a] Reaction conditions: substrate: 0.5 mmol, CAL-B: 10 mg, Tween-80: 5 mg/mL, phosphate buffer (100 mmol/L, pH 8.0, 5.0 mL), temperature: 25 °C.

MDTA containing lipophilic long alkyl chain cannot be dissolved enough in pure buffer, but it can be well dissolved in a micellar solution formed by surfactants. The type and additive amount of surfactant were selected according to the previously results about the effect of surfactants on CAL-B (data not shown), thus 5 mg/mL Tween-80 was selected as emulsifier, eliminating the effect of surfactant itself on CAL-B. For further ensuring the exactness of experimental results, blank experiments were performed, and the results show that no appreciable hydrolysis took place in the absence of enzyme.

Under the same conditions, the catalytic activity of CAL-B was drastically improved in the comicellar solution with MDTA, and the effect of MDTA bearing an alcohol pendant was notable than that of free bearing MDTA, especially, the increase in the size of lipophilic long chain of alcohol pendant was favorable for the improvement of the activity of CAL-B. The enantioselectivity of CAL-B was also influenced by MDTA. When the free bearing MDTA was added in the reaction medium, the enantioselectivity of CAL-B decreased; whereas *n*-C_6H_{13}-MDTA and *n*-C_8H_{17}-MDTA drastically enhanced the selectivity of CAL-B ($E = 87.6$ and 25.0, respectively), at the same time, having a preference for (*S*)-enantiomer. *n*-$C_{10}H_{21}$-MDTA appeared to improve the enzyme activity, however, it made the enantioselectivity of CAL-B decrease markedly (*E*-value is only 4.3), which makes it practically worthless.

The results indicate that the large rate enhancement is observed only in the presence of both CAL-B and MDTA, which may be ascribed to the employed MDTA may interact with certain sites of the lipase, modifying the lipase local conformation and increasing fitness of the substrate introduced to the active site of the enzyme, thereby activating the lipase and changing its enantioselectivity. On the other hand, the interaction between MDTA and the product acid may inhibit the reverse reaction, which may explain both accelerations of the hydrolysis rate and enhancement of the enantioselectivity. The activity and enantioselectivity of CAL-B have a close relationship with the types of MDTA. MDTA bearing lipophilic long chain facilitates the interaction between substrate and enzyme molecule, but the increase of alkyl chain length decreases the enzyme selectivity, which may be attributed to the spatial hindrance generated by the longer lipophilic chain. The results are similar to that reported by Itoh et al. [19–21].

Since the hydrolysis in the presence of *n*-C_6H_{13}-MDTA provided a much higher *E*-value, this was confirmed as a useful additive that improves this enzymatic reaction. So, we selected *n*-C_6H_{13}-MDTA as additive to further study the effect of different additive amount on the CAL-B-catalyzed resolution (Fig. 1). The activity of CAL-B increased with the increase of *n*-C_6H_{13}-MDTA concentration, while above 2.0 mg/mL, the catalytic activity of enzyme decreased slightly. However, the changes in the enantioselectivity were different. The results show that the lower additive amount is in favor of the selectivity of CAL-B, and the optimum concentration of *n*-C_6H_{13}-MDTA appears to be at 1.0 mg/mL ($E = 87.6$), but further increase of concentration, thereafter, will make the lipase enantioselectivity decrease.

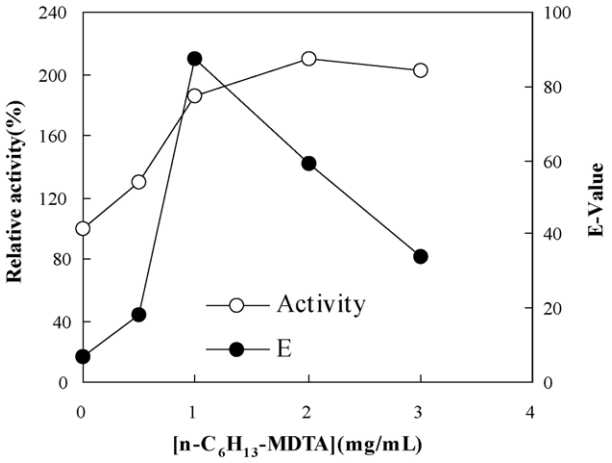


Fig. 1. Effect of *n*-C_6H_{13}-MDTA concentrations on the activity and enantioselectivity of CAL-B.

3.2. Effect of organic compounds on the activity and enantioselectivity of CAL-B

In order to find more effective compounds to improve the enantioselectivity of enzyme, we investigated the effect of organic compounds as additives, which are regarded as solvents in common [36,37], to the reaction system for the lipase-catalyzed hydrolysis. In this study, organic compounds with different hydrophobicities (log *P*) [38,39] were added in the aqueous medium, and the effects of organic compounds on the activity and enantioselectivity of CAL-B were observed (Table 2). In all experiments, the biocatalytic system shows the same stereopreference for (*R*, *S*)-NEMPA-ME giving (*S*)-acid. The addition of triethylamine to the reaction medium drastically improved the enzyme activity with a decrease in the enantioselectivity of enzyme (*E*-value is only 7.1), presumably due to the formation of a soluble ion-pair between the acid generated and triethylamine, thus shifting the equilibrium in the direction of the product [18,27,40]. Whereas the addition of other organic compounds generally enhanced the enantioselectivity of CAL-B compared with that in pure buffered medium, despite of a decrease in enzyme activity. The observations suggest that

Table 2
CAL-B-catalyzed hydrolysis of (*R*, *S*)-NEMPA-ME in different aqueous-organic media[a]

Organic solvent	Log *P*	Time (h)	Conversion (%)	e.e.$_p$(%)	*E*-value
None	–	2	32.5	78.7	12.1
Acetonitrile	−0.33	2	29.7	83.7	15.9
Acetone	−0.23	2	31.7	82.9	15.5
THF	0.49	2	28.2	82.8	14.6
Diethyl ether	0.85	2	19.6	87.8	19.0
Triethylamine	1.60	1	49.8	59.8	7.1
DIPE	1.90	2	28.3	84.8	16.8
Toluene	2.50	2	17.4	84.3	13.9
Cyclohexane	3.20	2	27.1	82.1	13.7
n-Hexane	3.50	2	25.1	87.6	20.1
n-Heptane	4.00	2	23.1	86.5	17.9

[a] Reaction conditions: substrate: 0.5 mmol, CAL-B: 10 mg, organic compound/phosphate buffer (100 mmol/L, pH 8.0, 5.0 mL) 1:9, temperature: 25 °C.

this enhancement of enantioselectivity may be the results of different extent of enantioselective inhibition, and as a result, the (*S*)-enantiomer becomes overwhelmingly the faster reacting enantiomer. The strategy of enantioselective inhibition to enhance the selectivity of lipase-catalyzed reaction has previously been reported by Guo and Sih [17] and Kinoshita and Ohno [41].

The additive amount of organic compound is another important parameter worthy of careful optimization, since it may have deep influences on the enzyme activity and enantioselectivity. According to the above results, it is worthy to note that the use of diethyl ether and *n*-hexane as additives is to be preferred ($E = 19.0$ and 20.1, respectively). So, herein the additive amount effects of diethyl ether and *n*-hexane on the activity and enantioselectivity of CAL-B were studied. When diethyl ether was added in the reaction medium, the maximum *E*-value of CAL-B was obtained at 15% (v/v) ($E > 100$, Table 3), which was about 9.7-fold more enantioselective than that in pure buffered medium. For *n*-hexane, the results were not so similar as diethyl ether, and the maximum enantioselectivity of CAL-B was stabilized at the additive amount ranging from 10 to 15% (v/v) ($E = 20.1$, Table 3). The conversion ratios of reactions in various amounts of organic compounds had also been measured, and found that it decreased with the increase of additive amount of organic solvent. From the results, we presume that diethyl ether may be a stronger and more (*R*)-selective inhibitor than *n*-hexane.

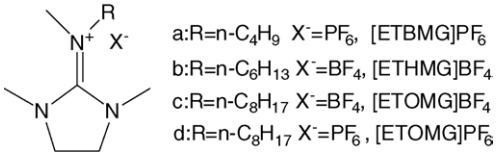
3.3. Effect of ionic liquids on the activity and enantioselectivity of CAL-B

The groundbreaking success of ionic liquids [42–50] has paved the way for convenient, efficient and environmentally friendly methodologies for a wide array of chemical reactions having significant synthetic value. To date, reactions involving lipases have benefited most from the use of ionic liquids; in some cases, remarkable results with respect to enantioselectivity [31,51–53] or enzymatic stability [54–57] was observed. In this paper, we herein report a simple alternative procedure for CAL-B-catalyzed hydrolysis of (*R*, *S*)-NEMPA-ME in pure buffer with alkylguanidinium-based (a–d) ionic liquids as additives. Table 4 summarized the effect results of different ionic liquids on the activity and enantioselectivity of CAL-B.

Table 4
Effect of ionic liquids on the CAL-B-catalyzed enantioselective hydrolysis of (*R*, *S*)-NEMPA-ME[a]

Ionic liquids	Solubility in H_2O	Conversion (%)	e.e.$_p$ (%)	*E*-value
None	–	20.7	70.6	6.9
[ETBMG]PF_6	S	32.1	54.6	4.4
[ETHMG]BF_4	I	47.3	28.1	2.2
[ETOMG]BF_4	I	12.0	73.2	7.1
[ETOMG]PF_6	I	23.9	68.5	6.6

[a] Reaction conditions: substrate: 0.5 mmol, CAL-B: 10 mg, ionic liquid/phosphate buffer (100 mmol/L, pH 8.0, 5.0 mL) 1:3, temperature: 25 °C, reaction time:1 h.



In the alkylguanidinium-based ionic liquids, using ionic liquids [ETBMG]PF_6 and [ETHMG]BF_4 as additives, the enantiomeric excesses of (*S*)-NEMPA were 54.6% e.e.$_p$ at 32.1% conversion and 28.1% e.e.$_p$ at 47.3% conversion, respectively. When the ionic liquids [ETOMG]BF_4 and [ETOMG]PF_6 were added in the reaction medium, the selectivity of CAL-B had the increasing trend ($E = 7.1$ and 6.6, respectively); however, the conversion ratios of reaction were decreased, which may be ascribed to the increasing viscosity of ionic liquid [31] with the increase of chain length of *R* substitute. It needs to be pointed out that the *R* substitute of [ETOMG]BF_4 is the same as that of [ETOMG]PF_6, but the effect on the catalytic properties of CAL-B is different, which may have a close relationship with the ionic liquids bearing the different anion. As evident from the above results, the properties and molecular structure of ionic liquids, and the types of anion contained in ionic liquid influence the activity and enantioselectivity of CAL-B.

In view of improving the enantioselectivity of CAL-B, we selected the ionic liquid [ETOMG]BF_4 as additive, which had the trend to improve the selectivity of CAL-B, to further investigate the effect of additive amount. From the data summarized in Table 5, it was evident that the reaction rate was retarded significantly with the increase of the amount of [ETOMG]BF_4, but the selectivity of CAL-B was enhanced. Similar to the effects of organic compounds as additives, the enhancement of

Table 3
Effect of different volume fractions of organic compounds on the CAL-B-catalyzed resolution of NEMPA[a]

Organic solvent (v/v)	Diethyl ether			*n*-Hexane		
	Conversion (%)	e.e.$_p$ (%)	*E*-value	Conversion (%)	e.e.$_p$ (%)	*E*-value
0	32.5	78.7	12.1	32.5	78.7	12.1
5	28.9	81.8	13.8	27.8	82.0	13.7
10	19.6	87.6	19.0	25.1	87.6	20.1
15	15.3	>98	>100	23.4	88.2	20.7
20	10.8	95.0	43.7	19.7	85.0	15.1

[a] Reaction conditions: substrate: 0.5 mmol, CAL-B: 10 mg, organic compound/phosphate buffer (100 mmol/L, pH 8.0, 5.0 mL), temperature: 25 °C, reaction time: 2 h.

Table 5
Effect of additive amount of ionic liquid [ETOMG] BF_4 on the CAL-B-catalyzed enantioselective hydrolysis of (*R*, *S*)-NEMPA-ME[a]

Ionic liquid (vol.%)	Time (h)	Conversion (%)	e.e.$_p$ (%)	*E*-value
0	3	45.3	78.7	16.3
15	5	42.9	79.4	15.9
25	9	47.3	81.1	20.7
50	12	38.4	92.3	44.7
60	24	20.5	92.0	30.3

[a] Reaction conditions: substrate: 0.5 mmol, CAL-B: 10 mg, phosphate buffer (100 mmol/L, pH 8.0, 5.0 mL), temperature: 25 °C.

Table 6
Recyclability of the ionic liquid [ETOMG] BF_4[a]

Run no.	Conversion (%)	e.e.$_p$ (%)
1	38.4	92.3
2	38.1	92.5
3	37.6	92.4
4	37.2	91.8
5	36.8	91.2

[a] Reaction conditions: substrate: 0.5 mmol, CAL-B: 10 mg, ionic liquid/phosphate buffer (100 mmol/L, pH 8.0, 5.0 mL) 1:1, temperature: 25 °C.

enzyme selectivity may also be the consequence of an inhibitory phenomenon. At the optimum additive condition (1:1 composition of ionic liquid [ETOMG]BF_4 and buffer), the markedly enhanced enantioselectivity towards the (*S*)-enantiomer was observed, and the enantiomeric excess was 92.3% e.e.$_p$ at 38.4% conversion.

Under the optimum additive conditions of ionic liquid [ETOMG]BF_4 (50 vol.%), we had also examined the recyclability of ionic liquid (Table 6). No substantial diminution in conversion and enantioselectivity was observed, even after recycling it for four consecutive runs. The results show that the using stability of ionic liquid [ETOMG]BF_4 is better.

4. Conclusion

A kinetic resolution process for producing (*S*)-*N*-(2-ethyl-6-methylphenyl)alanine from racemic methyl ester using CAL-B-catalyzed hydrolysis was investigated. The present results show that addition of certain organic compound (diethyl ether), macrocylic dioxotetraamine (*n*-C_6H_{13}-MDTA) or ionic liquid ([ETOMG]BF_4) in the reaction system can enhance the enantioselectivity of CAL-B to some extent, making CAL-B become highly enantioselective biocatalyst. With this strategy, a practical process has been successfully developed for the conversion of (*R*, *S*)-NEMPA-ME to (*S*)-NEMPA using CAL-B. Further work in this area is being done in our laboratory, and will be reported in due course.

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