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Efficient resolution of (*R,S*)-1-(1-naphthyl)ethylamine by *Candida antarctica* lipase B in ionic liquids

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ABSTRACT

The resolution of (*R,S*)-1-(1-naphthyl)ethylamine ((*R,S*)-NEA) by *Candida antarctica* lipase B (CALB) in ionic liquids (ILs) containing 1-alkyl-3-methylimidazolium cations ($[C_n mim]^+$) and $[Tf_2N]^-$, $[BF_4]^-$, and $[PF_6]^-$ anions was investigated. When the alkyl chain on the cation contained less than six carbons, the lipase activity corresponded with the hydrophobicity of the ILs, but further increase in the chain length suppressed the enzyme activity. The enzyme activity decreased depending on the anion, where $[Tf_2N]^-$ > $[PF_6]^-$ > $[BF_4]^-$. The effects of acyl donors, pH, temperature, water activity, and substrate concentration on the resolution were determined. Under the optimal conditions, the conversion of (*R,S*)-NEA and enantiomer excess of (*R*)-*n*-octyl acyl-NEA was 49.3% and 99.2%, respectively. The resolution kinetics of (*R,S*)-NEA by CALB in $[C_6mim][Tf_2N]$ were studied and a ping-pong mechanism with a two substrate inhibition model was selected. The kinetic parameters of the fitting results were as follows: Michaelis constant of (*R,S*)-NEA K_{ma}, 461.8 mmol/L; Michaelis constant of vinyl *n*-octanoateK_{mb}, 262.1 mmol/L; inhibition constant of (*R,S*)-NEA K_{ia}, 8737.2 mmol/L; inhibition constant of vinyl *n*-octanoateK_{ib}, 62336.8 mmol/L; maximum reaction rate r_{max}, 0.352 mmol/(mg min). Moreover, circular dichroism revealed that incubation of CALB in $[C_6mim][Tf_2N]$ resulted in increased β-sheet content; its secondary structure was stable.

1. Introduction

(*R*)-1-(1-naphthyl)ethylamine ((*R*)-NEA) is an important pharmaceutical intermediate for the preparation of calcimimetic Cinacalcet Hydrochloride [1,2], and is used as a chiral intermediate for the resolution of Pregabalin intermediates [3]. It is also an important raw material for the preparation of heterogeneous chiral hydrogenation catalysts [4,5]. Therefore, considerable research has been carried out on preparation methods for chiral NEA, which can be divided into three categories. The first is chemical resolution methods; for example, (*D*)-tartaric acid was used as a chemical separation agent for the resolution of (*R,S*)-NEA [6]. The process was long and the optical purity of the product was low, and a large amount of chemical waste was generated. The second category is asymmetric synthesis methods; (*R,S*)-1-(1-naphthyl)acetophenone was used for the synthesis of (*R*)-NEA in the presence of a chiral catalyst [7]. This process was simple, but the chiral catalyst was structurally complex, expensive, and its large-scale production was difficult.The third category is biological methods; an enzyme catalyst transaminase was applied for the preparation of chiral NEA in the presence of 1-(1-naphthyl)ethanone [8] and *Candida antarctica* lipase B (CALB) was used for the resolution of (*R,S*)-NEA [9,10]. Compared to transaminase, CALB does not require a coenzyme, is inexpensive, and has other advantages [11].

Notably, organic solvents were used as reaction media for the resolution of (*R,S*)-NEA by CALB; environmental and safety concerns of the volatile organic solvents directly affected the enzymatic resolution of (*R,S*)-NEA. Ionic liquids (ILs), new reaction media with low saturated vapor pressures, exhibit incombustibility, hydrophobicity, and excellent thermal stability [12,13]. Recently, ILs have been reported to show favorable characteristics as reaction media in enzyme catalysis to improve enzyme enantioselectivity and thermal stability [14,15]. Therefore, in this study, CALB was selected as the catalyst and ILs were used as the reaction media for the resolution of (*R,S*)-NEA for the first time. The effects of the structure of ILs based on 1-alkyl-3-methylimidazolium ($[C_n mim]^+$) cations and ($[Tf_2N]^-$), $[BF_4]^-$, and $[PF_6]^-$ anions on the enzymatic catalytic activity were investigated. Moreover, the effects of the reaction conditions on the enzymatic activity were also investigated. Circular dichroism (CD) spectroscopy was used to measure changes in the secondary structure of CALB in different solvents, and the resolution kinetics of (*R,S*)-NEA by CALB in $[C_6mim]$

Abbreviations: (R,S)-NEA, (R,S)-1-(1-naphthyl)ethylamine; CALB, Candida antarctica lipase B; ILs, ionic liquids; $[Tf_2N]^-$, bis[(trifluoromethyl)sulfonyl]imide; $[BF_4]^-$, tetrafluoroborate anion; $[PF_6]^-$, hexafluorophosphoric acid anion; $[Cnmim]^+$, 1-alkyl-3-methylimidazolium cations; CD, circular dichroism

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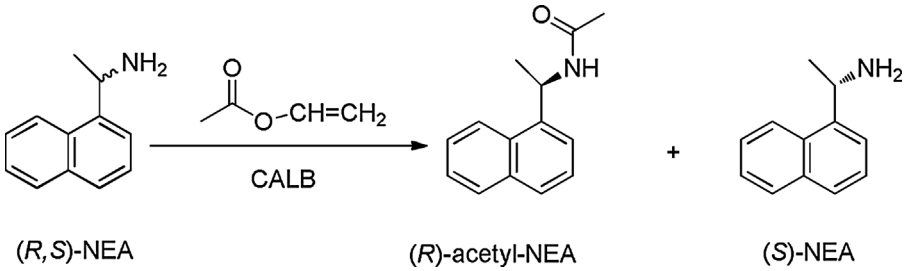
Scheme 1. Route of resolution of (*R,S*)-NEA by CALB.



[Tf_2N] was investigated.

2. Experimental

2.1. Materials

(*R,S*)-NEA (98%), ionic liquids[C_4mim][BF_4], [C_4mim][PF_6], [$C_n$mim][Tf_2N] (n = 2,4,6,8,10) were purchased from Alfa Aesar chemical Co. Ltd (Shanghai, China). The purity of IL was 99%. Recombinant lipase B from *Candida antarctica* from *Aspergillus oryzae* (9 U/mg) was obtained from Sigma-Aldrich(Shanghai, China). All other reagents were of analytical grade, and were obtained from Sinopharm Chemical Reagent Co. Ltd (Shanghai, China).

2.2. (R,S)-NEA kinetic resolution study

Unless otherwise indicated, in a typical resolution of (*R,S*)-NEA by CALB (Scheme 1), reactions were carried out in a 10 mL flask with a stopper; 200 mmol/L(*R,S*)-NEA and 120 mmol/Lvinyl acetate were added to 5 mL IL (or 5 mL toluene). The reaction was catalyzed in the presence of 100 mg CALB at 40 °C with shaking at 180 rpm. The water activities of substrates, solvents and enzyme were not specially controlled. The pH of CALB was also not specially controlled.

2.3. Analysis conditions

Aliquots were withdrawn from the reaction mixture at various intervals and the samples were analyzed using HPLC equipped with a Chiralcel® OJ-H chiral column (250 mm × 4.6 mm, 5 μm) and detected using UV at 222 nm at 25 °C. The mobile phase was composed of a mixture of hexane, isopropanol, and ethanol (300:50:0.8, V/V) with a flow rate of 1 mL/min. The retention times of (*R*)-NEA and (*S*)-NEA were 6.5 and 7.7 min, respectively; the retention time of (*R*)-*n*-octyl acyl-NEA and its enantiomer were 11.4 (*R*) and 14.6 (*S*) min, respectively.

The enantiomeric excess (*ee*) formulas for (*R*)-*n*-octyl acyl-NEA and (*S*)-NEA were $ee_P = \frac{c_{PR} - c_{PS}}{c_{PR} + c_{PS}} \times 100\%$ and $ee_S = \frac{c_S - c_R}{c_S + c_R} \times 100\%$, respectively. The conversion of *c* was calculated according to the changes in the number of substrates. C_{PR} and c_{PS} were the contents of *R* and *Sn*-octyl acyl-NEA, and c_R and c_S were the contents of (*R*)- and (*S*)-NEA, respectively. The enantioselectivity was determined using the following equation: $E = \frac{\ln[1-c(1+ee_p)]}{\ln[1-c(1-ee_p)]}$.

2.4. CD spectroscopy

CALB powder was incubated in [C_6mim][Tf_2N] (or toluene) at 4 °C for 2 h, washed with cooled ethyl acetate to remove the IL, and subsequently washed with cooled ether to remove ethyl acetate; the ether was volatilized at room temperature. The treated CALB powders were dissolved in phosphate buffered solution (50 mM) to a concentration of 0.1 mmol/L, and the enzyme solution was used for CD measurements. Spectra were recorded at 4 °C from 260 to 190 nm (0.5 nm increments) with a 0.1 cm path-length cell, 20 nm/min scan rate, 4 s response time, and 2 nm bandwidth. The mean residue ellipticity, $[\Theta]_{MR}$, was expressed in deg·cm^2·dmol^{-1}. A buffer sample containing no protein was subtracted from all spectra to account for any background signal. Spectra in all the region were averaged after three accumulations.

2.5. Water activity

The substrates and ILs were separately pre-equilibrated at different water activities (a_w) prior to the resolution of (*R,S*)-NEA. All samples were placed in a closed vessel above saturated salts, and equilibrium was reached overnight at room temperature. The salts used in this test included $Na_2HPO_4{\cdot}2H_2O/Na_2HPO_4$, $a_w = 0.17$; $NaAc{\cdot}3H_2O/NaAc$, $a_w = 0.28$; $CuSO_4{\cdot}5H_2O/CuSO_4{\cdot}3H_2O$, $a_w = 0.42$; $Na_2HPO_4{\cdot}7H_2O/Na_2HPO_4{\cdot}2H_2O$, $a_w = 0.63$; and $Na_2HPO_4{\cdot}12H_2O/Na_2HPO_4{\cdot}7H_2O$, $a_w = 0.78$.

2.6. Dynamic parameter fitting

The initial enzymatic reaction rates were determined at different substrate concentrations with certain amounts of (*R,S*)-NEA (50 ~ 400 mmol/L) and vinyl *n*-octanoate (50 ~ 240 mmol/L) respectively, according to the method described in Section 2.2. The dynamic parameters were fitted according to the dynamic model, substrate concentration, and initial reaction rate using Origin 11.0 software.

3. Results and discussion

3.1. Effect of solvent on resolution of (R,S)-NEA catalyzed by CALB

As (*R,S*)-NEA has poor solubility in hydrophilic ILs, several hydrophobic ILs with various anions and cations were used. The initial reaction rates of the resolution of (*R,S*)-NEA catalyzed by CALB in ILs and toluene are shown in Table 1. The initial reaction rates of the resolution by CALB in ILs were lower than that in toluene. Organic solvents, like toluene, have lower viscosities than ILs [12], thus the enzyme and substrates have higher mass transfer coefficients in toluene than in ILs. However, the conversion of (*R,S*)-NEA was not dominant in toluene with prolonged reaction times, and the conversion of (*R,S*)-NEA in [C_6mim][Tf_2N] gradually caught up and exceeded that in toluene (Fig. 1). The *ee* value of *R*-*n*-octyl acyl-NEA remained relatively stable (95.1%–99.5%) in [C_6mim][Tf_2N]; however, the *ee* value of *R*-*n*-octyl acyl-NEA rapidly decreased as the resolution time increased in toluene.

Table 1
Effect of solvents on resolution of (*R,S*)-NEA catalyzed by CALB.

Entry	Solvent	Conversion/%	ee_p/%	*E*	v_0/mmol mg^{-1} min^{-1}
1	[C_4mim][BF_4]	0.347	78.1	12	0.025
2	[C_4mim][PF_6]	0.377	79.8	14	0.027
3	[C_2mim][Tf_2N]	0.426	92.0	49	0.034
4	[C_4mim][Tf_2N]	0.455	88.6	37	0.045
5	[C_6mim][Tf_2N]	0.494	95.1	137	0.052
6	[C_8mim][Tf_2N]	0.439	84.1	23	0.038
7	[C_{10}mim][Tf_2N]	0.401	86.5	24	0.031
8	Toluene	0.465	71.2	11	0.056

E = Enantiomeric ratio, v_0 = Initial reaction rate, reaction time 6 h.

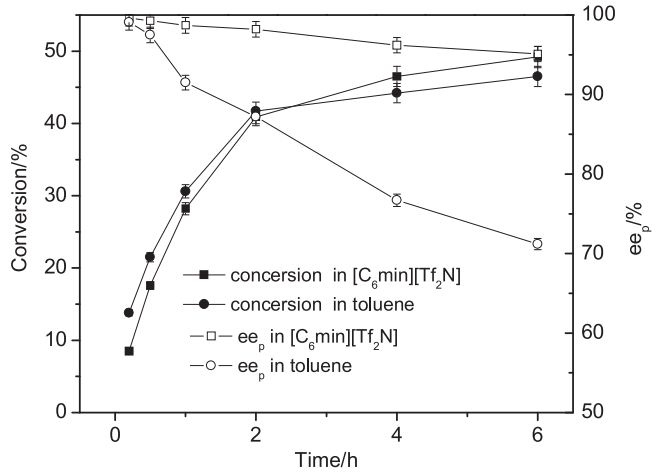


Fig. 1. Time-course curves of resolution of (*R,S*)-NEA catalyzed by CALB in [C_6mim][Tf_2N] and toluene.

[C_6mim][Tf_2N] may provide a more moderate catalytic environment for CALB. [C_6mim][Tf_2N] is a polar substance; it enhanced the electrostatic interactions between the protein molecules, and thus the protein was more flexible [16], which was conducive to the catalytic reaction. However, organic solvents could form hydrogen bonds with the protein and facilitate weak electrostatic interactions between them; thus, the protein structure was more rigid [17,18], which explained why the late conversion in [C_6mim][Tf_2N] was higher than that in toluene.

The effects of alkyl chain length on the cation of the IL on the conversion of (*R,S*)-NEA are illustrated in Table 1 (entries 3–7). As the number of carbon atoms on the cation alkyl chain increased, the hydrophobicity of the IL increased accordingly. However, the conversion decreased in the order of $[C_6mim]^+ > [C_4mim]^+ > [C_8mim]^+ > [C_2mim]^+ > [C_{10}mim]^+$, which was not consistent with the hydrophobicity of the ILs. This phenomenon might be explained as follows: (1) In the microenvironment of hydrophobic IL, a small amount of water was needed to maintain the conformation of the enzyme by hydrogen bonds [19]. The layer of water around the enzyme had a higher dielectric constant than that of the ILs and could not be easily separated from the enzyme. This, in turn, will stabilize the hydrogen bond responsible for the ordered structures of CALB to display its biological function in ILs [20,21]. (2) The IL with shorter alkyl chain on the cation has a lower viscosity and the slight increase of alkyl chain length might raises the viscosity largely [22], and the reaction rates may be affected by the significant viscosity change. In general, the less viscous ILs lead to more active enzymes, and this trend is different from the alkyl chain length in the cation.

As shown in Table 1 (entries 1–2, 4), the anions of the ILs also influenced the CALB activity , and the activity decreased in the order of $[Tf_2N]^- > [PF_6]^- > [BF_4]^-$. Bearing in mind the nucleophilicity order is $[Tf_2N]^- > [BF_4]^- > [PF_6]^-$, the increase in activity was not in agreement with the decrease in this parameter. The nucleophilicity of anions on the activity of CALB for acylation of amines was investigated, the reaction rate order was found to be following the order of the nucleophilicity of anions [23]. The reason might be the anion with strong nucleophilicity can interact with the amino acid residues and steady the structure of enzymes. However, the half-life times of the stability of CALB in different ILs showed that the half-life times were not only influenced by the nucleophilicity of anions, but also influenced by the hydrophobicity of cation [24]. The activity of CALB might be influenced by multiple factors of ILs, including nucleophilicity, hydrophobicity, viscosity and so forth.

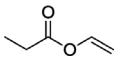
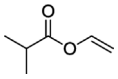
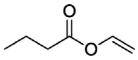
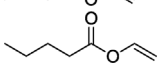
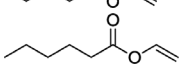
The enantioselectivity of CALB in acylation of (*R,S*)-NEA was also shown in Table 1. The value for enantioselectivity in ionic liquids was actually higher than that in toluene, and [C_6mim] [Tf_2N] was the best reaction media (Table 1, entry 5). The reasons for the improvement of enantioselectivity of enzymes in ionic liquids are multiaspect and complex. The evaluation of enzyme enantioselectivity in ionic liquids was carried out with alcohols as the substrates, and the enantioselectivities of lipases, in general, were higher in hydrophobic ionic liquids than in hydrophilic ionic liquids [25]. The enzyme enantioselectivity was affected by the hydrophobicity of ILs. However, the lipase-catalyzed enantioselective acylation of 1-phenylethylamine and 2-phenyl-1-propylamine were investigated in ionic liquids, the enantioselectivity of the lipase in acylation of 1-phenylethylamine was almost perfect in all ionic liquids, and the enantioselectivity for acylation of 2-phenyl-1-propylamine was very low and did not vary much with the nature of the ionic liquids [26]. The structural difference of substrates accounts for the enantioselectivity of their enzymatic acylation in ionic liquids. The resolution of (*R,S*)-1-phenylethanol catalyzed by a lipase from *Pseudomonas* sp. in [BMIm][Tf_2N] remained highly enantioselective with the E-value of 200 at 55 °C; while in MTBE, the E-value dropped dramatically to 4 [27]. The improvement of enantioselectivity in IL was due to the high enzyme thermostability in IL. Now the question is why enzyme shows such high enantioselectivity in ionic liquids. At this time, no satisfactory answers are available since the enzyme enantioselectivity could be affected by several factors, including the nature of medium, the structure of substrate, the enzyme thermostability and so on.

3.2. Effect of acyl donors on the enzymatic resolution of (R,S)-NEA

The (*R,S*)-NEA can catalyzed itself amidation reaction with acyl donor. The self-catalyzed reaction is non-selective, and non-enzymatic reactions could lead to a decrease in the ee_p. However, an acyl donor with a specific structure might suppress the self-catalyzed reaction [28]. As such, acyl donors with varying levels of structural complexity and steric hindrance were tested, as shown in Table 2. Accordingly, the conversions of the self-catalyzed amination reaction was reduced from 6.5% to 0%.

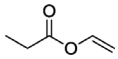
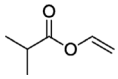
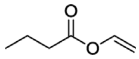
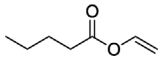
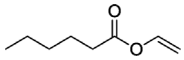
The above acyl donors were used for the resolution of (*R,S*)-NEA, and the results are shown in Table 3. Higher conversion of the self-catalyzed amination reaction led to lower ee_p values of *R*-*n*-octyl acyl-NEA. When vinyl *n*-octanoate was the acyl donor, the conversion was 49.3%, and the ee_p was 99.2%. This result suggested that the non-enzymatic amidation reaction was one of main factors affecting the resolution; structurally complex acyl donors led to lower conversions of the self-catalyzed amidation reaction.

Table 2
Non-enzymatic amidation reaction of acyl donors in [C_6mim][Tf_2N].

Entry	Acyl donor	ee_p/%	Conversion/%
1		0	6.5
2		0	4.1
3		0	1.5
4		0	2.7
5		0	1.2
6		0	0

Reaction condition: 5 mL of [C_6mim][Tf_2N], 200 mmol/L(*R,S*)-NEA, 120 mmol/Lacyl donor, reaction time 6 h.

Table 3
Effect of acyl donors on resolution of (*R,S*)-NEA catalyzed by CALB in [C_6mim][Tf_2N].

Entry	Acyl donor	Conversion/%	ee_p/%	*E*
1		49.4	95.1	137
2		49.0	96.3	180
3		49.1	98.4	> 400
4		49.4	97.2	259
5		48.5	98.4	> 400
6		49.3	99.2	> 400

Reaction condition: 5 mL of [C_6mim][Tf_2N], 200 mmol/L(*R,S*)-NEA, 120 mmol/L acyl donor, reaction time 6 h.

3.3. Effect of pH on the enzymatic resolution of (R,S)-NEA in [C_6mim][Tf_2N]

In non-aqueous enzymatic catalysis, pH plays an important role on lipase activity by regulating the amino acid residues in the active site of the enzyme. The effect of pH on the catalytic activity of the lipase in [C_6mim][Tf_2N] was evaluated by varying the pH of the enzymatic solution before lyophilization (Fig. 2). At pH 7, the conversion of (*R,S*)-NEA was the highest. Rios et al. [29] found that the synthesis of ethylene glycol esters by CALB was maximized when the pH was 7.0 in ILs. Salis et al. [30] found that decreased pH values led to the protonation of Glu^{341} and His^{449} residues of *Candida rugosa* lipase, which did not facilitate the nucleophilic attack on carbon in the substrate by oxygen in Ser^{209}, thereby reducing the enzymatic activity.

3.4. Effect of temperature on the enzymatic resolution of (R,S)-NEA in [C_6mim][Tf_2N]

Next, the effect of temperature on the resolution of (*R,S*)-NEA in [C_6mim][Tf_2N] with CALB was evaluated (Table 4). The initial reaction rate (v_0)increased significantly with increased temperatures and was the highest at 50 °C. However, the conversion was the highest at 40 °C, and higher enantioselectivity (> 400) could be achieved at lower

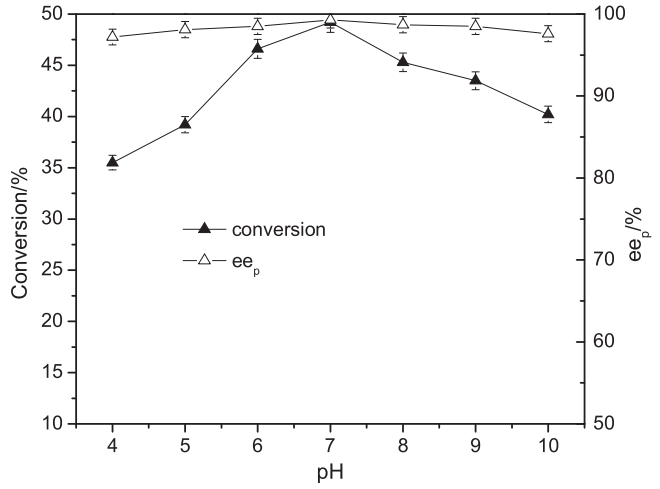


Fig. 2. Effect of pH on resolution of (*R,S*)-NEA catalyzed by CALB in [C_6mim][Tf_2N]. Reaction condition: 5 mL of [C_6mim][Tf_2N], 200 mmol/L(*R,S*)-NEA, 120 mmol/Lvinyl *n*-octanoate, reaction time 5 h.

Table 4
Effect of temperature on resolution of (*R,S*)-NEA catalyzed by CALB in [C_6mim][Tf_2N].

T/°C	v_0/mmol mg^{-1} min^{-1}	Conversion/%	ee_p/%	*E*
30	0.046	32.5	99.3	> 400
35	0.054	44.0	99.1	> 400
40	0.068	49.3	99.2	> 400
45	0.074	46.2	97.0	170
50	0.078	42.1	95.7	95

Reaction condition:5 mL of [C_6mim][Tf_2N], 200 mmol/L(*R,S*)-NEA, 120 mmol/Lvinyl *n*-octanoate, reation time 5 h.

temperatures (30–40 °C). At higher temperatures, the enzyme was deactivated, resulting decreased activity and enantioselectivity. The self-catalyzed reaction rate of (*R,S*)-NEA with vinyl *n*-octanoate was also accelerated at higher temperatures, which led to decreased enantioselectivity. At a temperature above 60 °C, the difference between IL and *n*-hexane could be explained by the strong protective effect of ionic liquid against enzyme thermal deactivation described already in literature [27].

3.5. Effect of water activity (a_w) on CALB activity in [C_6mim][Tf_2N]

The water activities of[C_6mim][Tf_2N], (*R,S*)-NEA, and vinyl *n*-octanoate were controlled by the salts showed in Section 2.5; the effects of water activity are shown in Fig. 3. Under nearly anhydrous conditions (a_w = 0.17), the initial activity was lower in [C_6mim][Tf_2N], and the enzyme activity was the highest when a_w = 0.28; the enzyme activity decreased with further increase in the water activity. Catalytic activity is related to the water molecules adsorbed on the surface of the protein; however, under high water activities, the enzyme is easy to gather and the hydrolysis of vinyl *n*-octanoate affected the catalytic activity of the enzyme [10].

3.6. Substrate concentration on the enzymatic resolution of (R,S)-NEA in [C_6mim][Tf_2N]

Next, the effect of substrate concentration on the enzymatic resolution of (*R,S*)-NEA was studied (Fig. 4) and the formation rate of (*R*)-*n*-octyl acyl-NEA was calculated (Fig. 4, inset). As the substrate concentration increased, the time at which the reaction reached the end point was prolonged. When the substrate concentration was 400 mmol/L, the reaction time was about 12 h and the conversion was 49.1%. However, when the substrate concentration was 300 mmol/L, the formation rate reached the highest 24.7 mmol/(L h).

Mutti [8] applied four (R)-ω-transaminases for the asymmetric

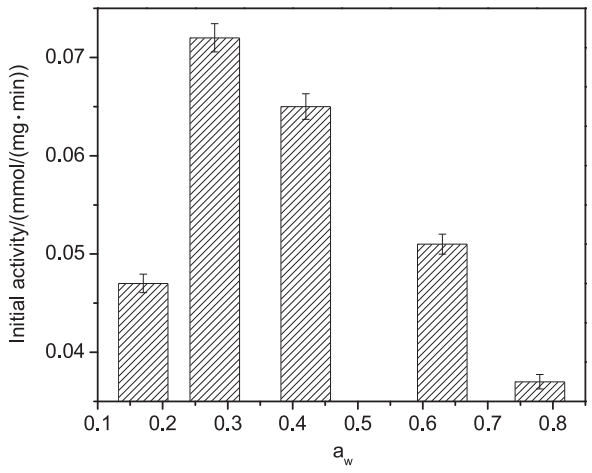


Fig. 3. Initial activities at different water activity in [C_6mim][Tf_2N]. Reaction condition: 5 mL of [C_6mim][Tf_2N], 200 mmol/L(*R,S*)-NEA, 120 mmol/Lvinyl *n*-octanoate.

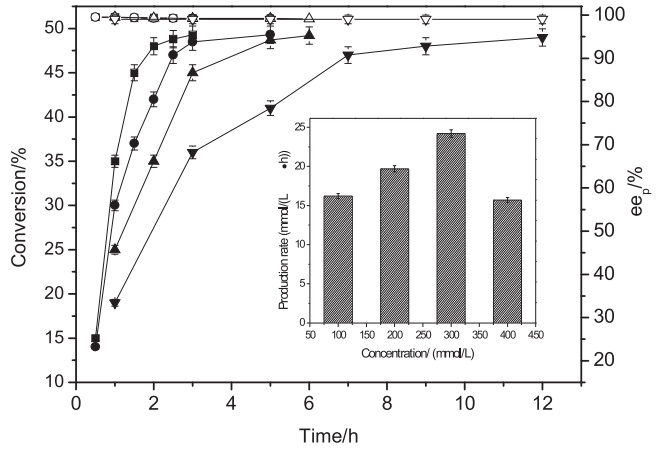


Fig. 4. Effect of substrate concentration on resolution of (*R,S*)-NEA catalyzed by CALB in [C_6mim][Tf_2N]. Experimental results of conversion at: (■) = 100 mmol/L; (●) = 200 mmol/L; (▲) = 300mmol/L; (▼) = 400 mmol/L, experimental results of ee_p at: (□) = 100 mmol/L^{-1}; (○) = 200 mmol/L; (△) = 300 mmol/L; (▽) = 400 mmol/L.

amination of ketones leading to optically pure (*R*)-amines, including the (*R*) -NEA. However, the concentration substrate was 1 mmol/L. Ditrich [9] applied lipase B from *Candida antarctica* for the resolution of NEA, and the concentration substrate was 100 mmol/L. In this paper, the resolution of NEA was carried out in 300 mmol/L with ee_p of 49.3% and yield of 99.2%.

3.7. CD spectroscopy

CD spectroscopy is one of the most convenient and efficient means of detecting the secondary structure of a protein, and is a convenient, sensitive, and time-saving method to explore the conformation stability of a protein in various solvents [31]. As shown in Fig. 5, all permuted proteins maintained a mixed α-helix/β-sheet spectral signature, and the native enzyme showed two negative peaks at 208 and 222 nm, and a positive peak at 192 nm. Upon treatment with [C_6mim][Tf_2N] and toluene, the mean residue ellipticity (Θ) of CALB at 192 nm was consistently lower, and the intensity of the minima at 208 and 222 nm decreased as compared to that of the native enzyme. The interpretation of the data was confirmed using secondary-structure models of the MRE curves byDICHROWEB (Table 5).

The minima at 208 and 222 nm and maximum at 192 nm were thought to correspond to helical structures, and the absolute values of these points were closely related to the percentage of helical residues in the protein [32]. As shown in Table 4, upon warming the CLAB solution in [C_6min][Tf_2N] and toluene, the percentage of α-helix decreased and

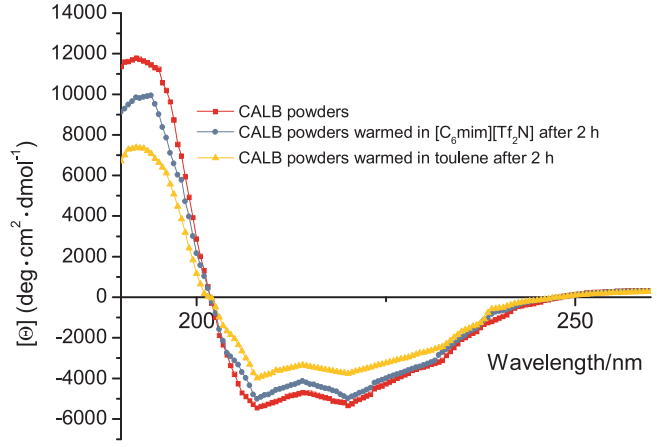


Fig. 5. Far-UV circular dichroism spectra of CALB in different solvents.

Table 5
Percentages of secondary structural elements in CALB at solvents calculated from CD spectra.

Solvent	Secondary structure			
	α-helix/%	β-sheet/%	β-Turn/%	Random coil/%
None	39.6	9.4	17.7	33.3
[C_6mim][Tf_2N]	38.2	10.1	17.2	34.5
Toluene	32.7	15.2	18.1	34.0

that of the β-sheet structure increased compared to the native enzyme, while the other structures remained unchanged. Therefore, it could be concluded that certain solvents induced the formation of β-sheets from α-helices.The increase in β-sheet content could be attributed to the more rigid structure of the treated enzyme [33], which was caused by the loss of water molecules on the surface of the protein in the solvents. In other words, enzymes with high α-helical contents might have a softer structure, which would be responsible for the high enzyme activity [34]. Lipase araujiain hI exhibited a significant decrease in enzyme activity in organic media, which was accompanied by a significant decrease in α-helix content [35]. The deactivation of *Burkholderia cepacia* lipase in methanol was also reported to be related to conformational changes [36].

3.8. Dynamic parameter fitting

The resolution reaction kinetics of (*R,S*)-NEA by CALB in [C_6mim][Tf_2N] were evaluated. Due to the transesterification reaction catalyzed by lipase with a ping-pong mechanism, a two substrate inhibition model was selected and the kinetic parameters were fitted; the kinetic model was as follows [37]:

$$r = \frac{r_{max} c_{VI} c_{NEA}}{c_{VI} c_{NEA} + K_{mb} c_{NEA}(1 + \frac{c_{NEA}}{K_{ia}}) + K_{ma} c_{VI}(1 + \frac{c_{VI}}{K_{ib}})}$$

where r is the reaction rate, r_{max} is the maximum reaction rate; K_{ma} and K_{mb} are Michaelis constants for (*R,S*)-NEA and vinyl *n*-octanoate, respectively; K_{ia} and K_{ib} are inhibition constants of (*R,S*)-NEA and vinyl *n*-octanoate respectively; and c_{NEA} and c_{VI} are the substrate concentrations of (*R,S*)-NEA and vinyl *n*-octanoate, respectively.

The kinetic parameters of the fitting results were as follows: K_{ma} = 461.8 mmol/L, K_{mb} = 262.1 mmol/L, K_{ia} = 8737.2 mmol/L, K_{ib} = 62336.8 mmol/L, and r_{max} = 0.352 mmol/(mg min). These results indicated that the enzymatic reaction in [C_6mim][Tf_2N] was mainly inhibited by the substrate (*R,S*)-HMPC and substrate inhibition by vinyl *n*-octanoate was relatively small, suggesting that increasing the concentration of vinyl *n*-octanoate could promote the lipase-catalyzed reaction rate.

4. Conclusion

The effect of ILs on the activity of CALB in the resolution of (*R,S*)-NEA was investigated. The alkyl chain length on the cation and type of anion influenced the activity of CALB. The enzyme activity as a function of cation decreased as follows: $[C_6MIM]^+$ > $[C_4MIM]^+$ > $[C_8MIM]^+$ > $[C_2MIM]^+$ > $[C_{10}MIM]^+$, which was not completely in accord with the hydrophobicity of the ILs, and the best anion was $[Tf_2N]^-$. The resolution of NEA by CALB in [C_6mim][Tf_2N] was also influenced by the acyl donor, pH, temperature, water activity, and substrate concentration; the optimal conditions were as follows: vinyl *n*-octanoate, pH 7.0, 40 °C, water activity = 0.28, and substrate concentration = 300 mmol/L. Under the optimal conditions, the conversion was 49.3% and ee value of *R*-*n*-octyl acyl-NEA was 99.2%. The resolution reaction kinetics of (*R,S*)-NEA by CALB in [C_6mim][Tf_2N] were determined using a ping-pong mechanism with a two substrate inhibition model. The kinetic parameters of the fitting results were

K_{ma} = 461.8 mmol/L, K_{mb} = 262.1 mmol/L, K_{ia} = 8737.2 mmol/L, K_{ib} = 62336.8 mmol/L, and r_{max} = 0.352 mmol/(mg·min). CD spectroscopy revealed that the secondary structure of CALB in [C_6mim][Tf_2N] was slightly different than that of native lipase.

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