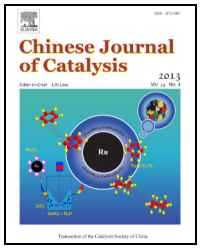
Chinese Journal of Catalysis 34 (2013) 769–780

available at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/chnjc



Article

Effect of imidazolium ionic liquids on the hydrolytic activity of lipase

LI Na [a,*], DU Weiyan [a], HUANG Zhuonan [b], ZHAO Wei [a], WANG Shoujiang [a]

[a] *Key Laboratory of Thermo-Fluid Science and Engineering, Ministry of Education, School of Chemical Engineering and Technology, Xi'an Jiaotong University, Xi'an 710049, Shaanxi, China*

[b] *Department of Chemistry and Engineering, Baoji University of Arts and Science, Baoji 721007, Shaanxi, China*

ARTICLE INFO

Article history:
Received 20 November 2012
Accepted 24 December 2012
Published 20 April 2013

Keywords:
Imidazolium ionic liquid
Lipase
Hydrolysis
Enzyme activity
Critical micellar concentration
Hofmeister

ABSTRACT

The effect of 1-alkyl-3-methylimidazolium ionic liquids (ILs) on the hydrolysis activity of *Candida rugosa* lipase (CRL) toward triacylglycerol was investigated. The critical micelle concentrations (CMC) of ILs with Br^-, Cl^-, and $[BF_4]^-$ anions and the solubility of ILs with $[PF_6]^-$ anions were determined in phosphate buffer. Results suggested that the content of the ILs, not kosmotropicity, highly influenced the effects of anions and cations of ILs on CRL activity. As the length of alkyl chain of the cation $[C_nMIM]^+$ increased, lower IL content was required to achieve high enzyme activity. Once the concentrations of the ILs with Br^-, Cl^-, and $[BF_4]^-$ anions exceeded the CMC value, enzyme activity was suppressed. The positive promotion effect of anions on enzyme activity was in the order of Br^- > Cl^- > $[BF_4]^-$ > $[PF_6]^-$. The effect of ionic liquid on enzyme activity was highly dependent on the pH and temperature of the system, with the optimum pH being 7.000. Under optimal conditions of pH 7.000, 30 °C, and 47.6 mmol/L of $[C_8MIM]Br$, the highest relative activity of CRL (1734%) was achieved, with a specific activity of 54.4 U/mg protein.

1. Introduction

Because of the lack of vapor pressure, thermal stability, non-flammability, and widely tunable physicochemical properties, ionic liquids (ILs) are great reaction media and efficient additives for enzymatic reactions. They provide an alternative reaction media for volatile organic solvents and are advantageous for the use of enzymes as they can obtain higher activity, selectivity, and stability [1–3]. Ionic liquids usually contain a bulky cation and a small anion, and are available in a number of varieties. The structure of the cation and anion both determine the polarity, hydrophobicity or hydrophilicity, and ionization of the ILs. These properties influence the micro structure of the reaction system and the catalytic activity of any enzyme employed. Thus, appropriate ILs need to be screened for each specific enzyme-medium-substrate reaction system [4–6]. As reported, the miscibility of an IL is mainly influenced by the ability of the anions to form hydrogen bonds, and slightly by the length and number of alkyl chains linked to the imidazolium ring. With the same anion, the hydrophobicity of an IL increases as the length of the alkyl chain of the cation increases [7–9]. Some researchers found that enzyme activity was mainly influenced by anions rather than cations [10–12]. Yigitoglu et al. [13] considered that the enzyme activity increased as the length of alkyl chain increased. In contrast, Ulbert et al. [2] and Zhao [4] indicated that enzyme activity and stability increased as the length of the alkyl chain decreased. In addition, the content of the IL affects the ionization and aggregation structure of one or more ILs in the system as well as the properties of ionic strength, pH, viscosity, and interfacial properties of the reaction system as a whole [14]. Zhao et al. [15] found that an appropriate concentration of ILs promoted lipase activity for amino acid

*Corresponding author. Tel: +86-29-82664554; E-mail: lina@mail.xjtu.edu.cn
This work was supported by the National Basic Research Program of China (973 Program, 2009CB2199006).
DOI: 10.1016/S1872-2067(11)60521-4 | http://www.sciencedirect.com/science/journal/18722067 | Chin. J. Catal., Vol. 34, No. 4, April 2013

ester hydrolysis. However, most research has only been carried out with specific concentrations of ILs, thereby limiting a systematic and general study of their influences on enzyme activities. Thus, investigating the effects of structures of different cations and anions on enzyme activity within a wide range of IL content is required.

Lipase has high specific activity, enantioselectivity, and thermal stability. Few studies have been conducted on the influence of IL content and structure on hydrolysis activity in IL-water systems under different pH and temperature conditions. Our previous study [16] showed that lipase hydrolytic activity can be efficiently and accurately analyzed using an automatic pH-stat potentiometric titration method, which was carried out based on changes in micro electrode potential induction. This automatic titration system has advantages of precision, stability, and reproducibility. Thus, using this method to investigate the enzyme activity with various ILs under different conditions can help to understand the influences of the ionic liquid on the mechanism of enzymatic catalytic hydrolysis and determine proper ionic liquid and reaction conditions.

Imidazolium ionic liquid, widely used during biocatalytic processes, can improve enzyme activity, selectivity, and stability [17–19]. In the present study, constant potential automatic titration was applied to investigate the effects of cations / anions and concentrations of 19 ILs with 1-alkyl-3- methylimidazolium on hydrolysis activity. The cations were $[C_n MIM]^+$ (n = 2, 4, 6, 8, 10, 12) with different lengths of the alkyl chain, and the anions were Br^-, Cl^-, $[BF_4]^-$, and $[PF_6]^-$. The hydrolysis activity of *Candida rugosa* lipase (CRL) in different ionic liquid-phosphate buffer solutions was analyzed and the optimum pH and temperature of the systems were determined. Critical micelle concentrations and solubility of ILs were determined using conductance. The relationships among the concentrations of ILs, ionization, and enzyme activity are discussed.

2. Experimental

2.1 Synthesis of ionic liquid

Synthesis of $[C_n MIM]Br$ (n = 2, 4, 6, 8, 10, 12) [20]. To a set quantity of *N*-methylimidazole in a 100 ml round-bottomed flask, alkyl bromide was slowly added with stirring at a molar ratio of 1:1. The following alkyl bromides were used: ethyl bromide, *n*-butyl bromide, 1-bromohexane, bromooctane, 1-bromodecane, and bromododecane. The mixture was put into a microwave synthesis reaction device, and was stirred and refluxed at 70 °C for 6 h. The sticky products were washed three times using ethyl acetate, after which the ethyl acetate was removed under 90 °C using a rotary evaporator to obtain the products of $[C_n MIM]Br$ with yields and purities of 92.1%–94.8% and 98.6%–99.2%, respectively.

Synthesis of $[C_n MIM]BF_4$ (n = 2, 4, 6, 8, 10, 12) [21]: To a set quantity of $NaBF_4$ dissolved in acetone in a 250 ml round-bottomed flask, $[C_n MIM]Br$ dissolved in acetone was slowly added with stirring at a molar ratio of 1:1. The mixture was put into a microwave synthesis reaction device, and was stirred and refluxed under 55 °C for 6 h. The precipitate NaBr and acetone were removed through filtration and rotary evaporation, respectively. The residual was washed 4–6 times using methylene chloride. The ionic liquid was dissolved in deionized water until no white precipitate appeared with the addition of silver nitrate. After methylene chloride removal, $[C_n MIM]BF_4$ was obtained with a yield of 62.5%–73.2%.

Synthesis of $[C_4 MIM]Cl$ [20]. A total of 18.51 g of chlorobutane was added into 16.42 g of *N*-methylimidazole slowly with stirring. The mixture was put into a microwave synthesis reaction device, and was stirred and refluxed under 70 °C for 6 h. The sticky products were washed three times using ethyl acetate, after which the ethyl acetate was removed under 90 °C using a rotary evaporator to obtain the product [BMIM]Cl with a yield and purity of 94.6% and 98.9%, respectively.

Synthesis of $[C_n MIM]PF_6$ (n = 2, 4, 6, 8, 10, 12) [22]. $[C_n MIM]Br$ and KPF_6 at a molar ratio of 1:1.1 were each dissolved in deionized water, and then mixed together and reacted at 80 °C for 3 h in a microwave synthesis reaction device. After removal of the upper aqueous phase, the residue was washed using deionized water repeatedly until no precipitate appeared with the addition of silver nitrate in the upper aqueous phase. $[C_n MIM]PF_6$ was obtained with a yield of 39.2%–66.1% after the removal of water by evaporation under 90 °C.

2.2. Determination of CRL hydrolysis activity

A 7 mg/ml crude enzyme solution was obtained by dissolving CRL powder in 50 mmol/L phosphate buffer solution (pH 7.000), which was centrifuged at 10 °C, 1000 r/min for 15 min. The protein content of the supernatant was measured using the bicinchoninic acid method [23]. A total of 40 ml phosphate buffer (pH 7.000), 2 ml of olive oil, and a certain amount of ionic liquids were mixed together and were preheated in a water bath at 30 °C for 30 min. After pH adjustment to 7.000 with stirring at 1200 r/min, 2 ml of enzyme supernatant solution was added to start the titration. The consumption of the sodium hydroxide standard solution after 15 min of titration was V ml for the systems containing ILs and V_0 ml for the control [16].

One unit of enzyme activity was defined as the production of 1 μmol of fatty acid for every 1 min under the determination conditions, which is calculated by

$$U = \frac{[(V - V_0) \times C_{NaOH} \times 1000]}{15} \quad (1)$$

where C_{NaOH} represents the concentration of sodium hydroxide (mol/L).

The relative enzyme activity is calculated by

$$\text{Relative activity (\%)} = \frac{\text{Enzyme activity in the system with ILs}}{\text{Enzyme activity in system without ILs}} \times 100\% \quad (2)$$

The specific enzyme activity (U/mg protein) is calculated by

$$\text{Specific enzyme activity} = \frac{\text{Enzyme activity}}{\text{Protein content}} \quad (3)$$

Each CRL hydrolysis activity test was carried out 2–3 times with a relative error in the range of 0.19%–3.67%.

2.3 Electrical conductivity measurement of the ILs-water system

Electrical conductivity (EC) of the phosphate buffer solution (pH 7.000) before and after the addition of ILs was measured using a conventional conductometer DDS-11AW (Shanghai Bante) at a constant temperature of 30 °C and 1200 r/min. The value of the sample without ILs was used as the blank.

3. Results and discussion

3.1 Effect of the structure of cations and concentrations of ILs with Br^- and $[BF_4]^-$ anions on CRL hydrolysis activity

Imidazolium ILs with Br^- and $[BF_4]^-$ anions had good miscibility with water. The effect of the cation ($[C_nMIM]^+$(n = 2, 4, 6, 8, 10, 12)) structures and IL concentrations on CRL hydrolysis activity is presented in Fig. 1. When the number of cationic alkyl chain carbon atoms was less than 12, the ILs increased CRL activity. As the concentrations of the ILs increased, the positive effect increased at first and then decreased. Once the concentrations of ILs exceeded a certain value, they actually suppressed CRL activity. If the number of cationic alkyl chain carbon atoms reached 12, both $[C_{12}MIM]Br$ and $[C_{12}MIM]BF_4$ thoroughly suppressed CRL activity. The maximum positive promotion effect of cations on enzyme activity was achieved as the concentrations of ILs reached the optimal values. The maximum positive promotion effect of cations on enzyme activity was in the order of $[C_8MIM]^+$ > $[C_6MIM]^+$ > $[C_4MIM]^+$ > $[C_2MIM]^+$ > $[C_{10}MIM]^+$ for ILs with the Br^- anion, which had the highest relative activities of 1734%, 1729%, 1709%, 1579%, and 505%, respectively. The effect of the ILs with the Br^- anion was higher than that of ILs with $[BF_4]^-$ anion. It was noted that as the carbon atomic number of the alkyl chains of the cations increased, the optimum concentrations of ILs decreased. Once the concentrations of ILs with longer alkyl chains of the cations were over the optimum values, the CRL activity decreased much faster, which led to a narrower concentration range of ILs suitable for promoting CRL activity. The relative enzyme activity would drop to 100% as the increase in IL concentrations reached a certain value (called the maximum concentration hereafter). Thus, the IL content had an important influence on enzyme activity. In the literature, without considering the effect of IL concentrations, conflicting conclusions about the effect of the length of the alkyl chain on enzyme activity have been made. As shown in Fig. 1, only when the number of alkyl chain carbon atoms of the cation was less than 10 and the IL concentrations were less than the optimum values did the influence of ILs on enzyme activity show a Hofmeister effect. This phenomenon was consistent with the conclusion made by Bi et al. [3], who found that only when the concentrations of I^- based imidazolium ILs were low and the number of alkyl chain carbon atoms of the alkyl chain was less than 10 did the promotion effect of ILs on black plum glycosidase (catalyzes the synthesis of salidroside) increase with alkyl chain length, which is consistent with the Hofmeister effect. When the length of the alkyl chain was higher than 10, the promotion effect of ILs on enzyme activity decreased.

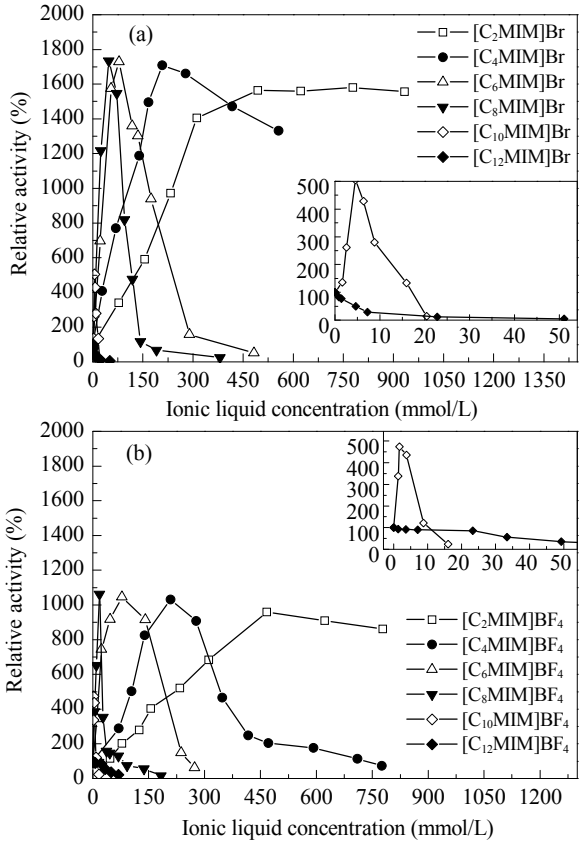


Fig. 1. Effect of concentrations and cation structure of Br^- (a) and [BF4]$^-$ (b) based imidazolium ionic liquids on CRL hydrolytic activity.

As seen in Fig. 1, the effect of the structure of the cations and concentrations of IL on the enzyme hydrolysis activity might be related to IL properties of ionization and aggregation. Within the concentration range presented in Fig. 1, the conductivity of the solution increased as the concentrations of IL increased (Fig. 2). However, the growth rate slowed down gradually. According to the study by Sirieix-Plénet et al. [24], when the concentrations of the IL were less than the critical micelle concentration (CMC), the molar conductivity of the solution increased linearly with the square root value of the concentration. Once the concentration of the IL was higher than the CMC, no linear relationship was observed. Based on this observation, CMC can be determined. Though some researchers have determined the CMC of ILs in pure water and found that the addition of an inorganic salt affected the CMC [25,26], few CMC measurement studies have been conducted in phosphate buffer solution. According to the results illustrated in Fig. 2, the CMC of some of the ILs could be obtained (Table 1). The CMC of the ILs in pure water at 25 °C, as well as the optimum concentrations of ILs and the maximum concentrations of ILs that led to the highest relative enzyme activity and 100% relative enzyme activity, respectively, are all presented in Table 1.

The concentration ranges of $[C_2MIM]Br$ and $[C_4MIM]Br$, which increased enzyme activity, were both less than their reported CMCs. The CMCs of the other ILs with longer alkyl chain of the cation decreased as the carbon atomic number of $[C_nMIM]^+$ decreased, which was coincident with the decreasing ability of the cations to form hydrogen bonds and the increasing hydrophobicity of the ILs, as reported in the literature [9]. It was obvious that the optimum concentrations of the ILs were

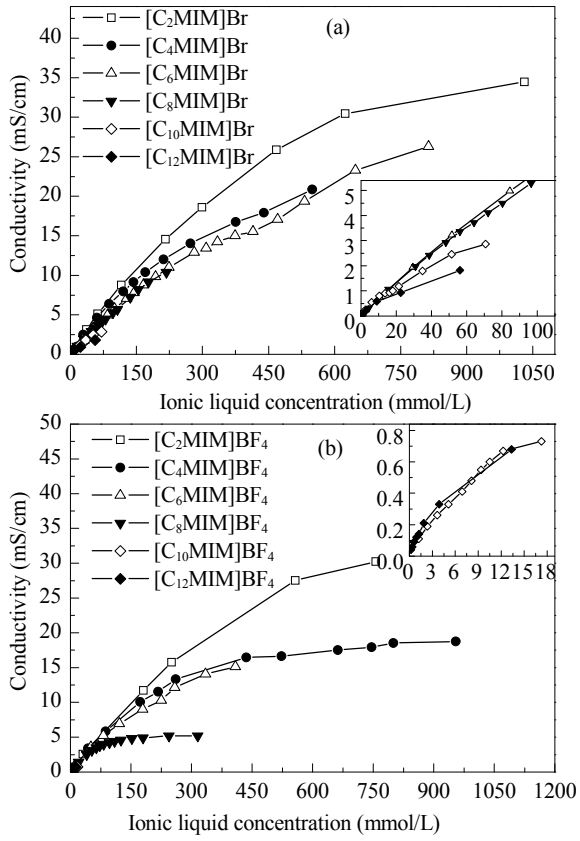


Fig. 2. Effect of concentration and cation structure of Br^- (a) and $[BF4]^-$ (b) based imidazolium ionic liquids on the conductivity of phosphate buffer solution.

all smaller than the CMCs. When the number of alkyl chain carbon atoms reached 6, 8, or 10, their maximum concentrations were almost the same as their CMCs. Moreover, when the concentration of $[C_{12}MIM]Br$ was 0.83 mmol/L (just over the CMC of 0.79 mmol/L), the CRL relative enzyme activity was 83%. When the concentration of $[C_{12}MIM]BF_4$ was 1.3 mmol/L (just over the CMC of 1.2 mmol/L), the CRL relative enzyme activity was 94%. These results suggested that the aggregation behavior of ILs in the system significantly influenced the enzyme activity. When the concentrations of ILs were less than the CMC, the ILs increased the enzyme activity. Once the concentrations were equal to their CMCs, this effect disappeared. If the concentrations were higher than the CMCs, they inhibited enzyme activity. Very few studies on the relationship between the aggregation properties of ILs and enzyme activity have been conducted. Geng et al. [27] systematically studied the secondary structure of bovine serum albumin (BSA) in a $[C_{14}MIM]Br^-$ water system using circular dichroism. When the concentrations of imidazolium ILs were less than their CMCs, the ILs electrostatically interacted with BSA, which stabilized the secondary structure of BSA. However, when the concentrations reached their CMCs, the hydrophobicities of the ILs increased and they strongly adsorbed to BSA, disrupting its secondary structure. Once the concentrations were higher than the CMCs, BSA denaturation occurred. It is known that activity is reduced after enzymes become embedded in surfactant micelles and the amount of enzyme embedded in the micelles increases as the surfactant content increases [28]. Thus, it can be deduced that as the concentrations of ILs increased to their CMCs, the amount of enzymes embedded in the micelles increased, which led to structural disruption of the enzyme and hence a decrease in hydrolysis rate.

Table 1
Optimum concentration and maximum concentration of Br^- and $[BF_4]^-$ based imidazolium ionic liquids and their CMCs in phosphate buffer solution.

ILs	Optimum concentration [a] (mmol/L)	Maximum concentration [b] (mmol/L)	CMC [c] (mmol/L)	Reported CMC [d] (mmol/L)
$[C_2MIM]Br$	778.7	—	—	1900 [29]
$[C_4MIM]Br$	208.6	—	—	700 [29], 970 [30]
$[C_6MIM]Br$	78.4	379.5	385.5	400 [29], 770 [30]
$[C_8MIM]Br$	47.6	81.3	89.4	150 [29], 160 [30]
$[C_{10}MIM]Br$	4.6	17.2	17.5	30 [29], 39 [30]
$[C_{12}MIM]Br$	—	—	0.8	9 [30]
$[C_2MIM]BF_4$	466.9	—	—	—
$[C_4MIM]BF_4$	208.6	732.8	704.5	912 [31], 829 [31], 846 [32]
$[C_6MIM]BF_4$	78.0	244.4	242.5	282 [31], 312 [31]
$[C_8MIM]BF_4$	18.3	75.5	78.2	89 [31], 86 [31]
$[C_{10}MIM]BF_4$	1.7	9.4	7.5	27 [31], 23 [31]
$[C_{12}MIM]BF_4$	—	—	1.2	8.7 [31], 8.3 [31]

[a] Concentrations of ILs with highest CRL activity in phosphate buffer solution (pH 7.000) at 30 °C.
[b] Concentrations of ILs over which the beneficial effect on activity was eliminated.
[c] CMC of ILs in phosphate buffer solution (pH 7.000) at 30 °C.
[d] CMC of ILs in pure water at 25 °C.

3.2 Effect of the structure of cations and concentrations of ILs with $[PF_6]^-$ anion on CRL hydrolysis activity

The ILs with $[PF_6]^-$ anion were strongly hydrophobic. The effect of the concentrations and the length of the cation alkyl chain on CRL activity in the ILs-water system are illustrated in Fig. 3. All the ILs had a beneficial effect on the CRL activity, which reached the highest level under optimum concentrations. When the number of carbon atoms of the alkyl chain was less than 10, the beneficial effect on enzyme activity was in the order of $[C_8MIM]^+ > [C_6MIM]^+ > [C_4MIM]^+ > [C_2MIM]^+$. As the number of carbon atoms of the cation alkyl chain increased, the optimum concentrations of ILs decreased. Once the concentrations of ILs with longer cation alkyl chains were over the optimum values, the CRL activities rapidly decreased. This phenomenon coincided with the ILs containing Br^- and $[BF_4]^-$ anions. However, the length of the alkyl chain had a significant influence on the highest enzyme activity. In addition, ILs with alkyl chains containing 10 and 12 carbon atoms still had strong beneficial effects on enzyme activity. Two reasons can explain this phenomenon: 1) the phase interface formed between the hydrophobic ILs and water favored interfacial lipase catalytic activity, and 2) because of the low solubility of ILs in the buffer solution, the system is less toxic toward the enzyme. Previous research has proven that compared with hydrophilic ILs-water one-phase systems, hydrophobic ILs-water multiphase systems are less toxic for the biocatalyst.

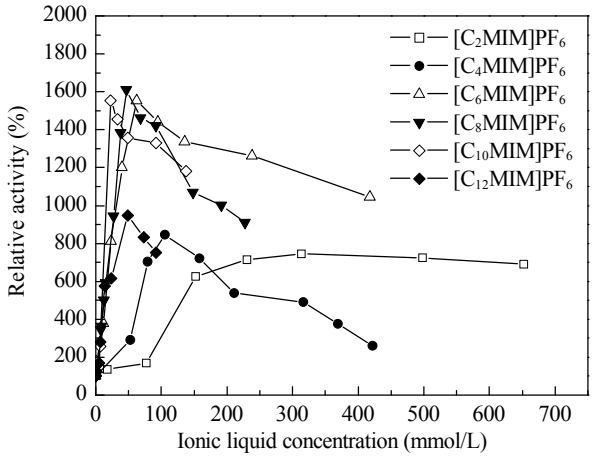


Fig. 3. Effect of concentration and cation structure of $[PF6]^-$ based imidazolium ionic liquids on hydrolytic activity of CRL.

When the content of an IL in the buffer solution was higher than its solubility, phase splitting occurred between the IL and the buffer solution. Consequently, as the concentration of IL with $[PF_6]^-$ anion increased, the EC of the buffer solutions increased, and finally reached a constant high value (Fig. 4). With the same concentration of IL, the EC of the buffer solution increased as the length of the alkyl chain decreased. It has been reported that because of its large size, $[PF_6]^-$ cannot effectively bond to the micelle surface, resulting in phase splitting before the formation of the micelle. Thus, it can be considered that an IL will be fully ionized in water when its concentration is less than its solubility [35]. Based on the relationship between molar conductivity and the square root of concentration, the solubility of the ILs in buffer solution was determined (Table 2). The solubility of $[C_n\text{MIM}]PF_6$ in buffer solution decreased as the length of the alkyl chain of the cation increased, exhibiting the same pattern as IL hydrophobicity. In addition, the optimum concentrations of the ILs were higher than their solubilities in buffer solution, which indicated that when the concentrations of ILs were over their solubility in a certain range, their beneficial effects on enzyme activity still increased as their concentrations increased. The phase interface formed between the ILs and the buffer solution favored interfacial lipase catalytic activity, which might contribute to this phenomenon. However, once the concentrations of the ILs increased to certain values, the enzyme activity decreased. Some researchers support the idea that imidazolium ILs can dissolve CRL to a certain extent, leading to a decrease in enzyme activity [36]. If the concentration of the IL was too high, a larger proportion of the total enzyme amount might have dissolved, further resulting in a lower reaction rate. It was also observed that some oil was transferred into the phase interface between the IL and water. Further, the increase in the amount of IL led to a higher system viscosity. Taken together, this might hinder the mass transfer of the substrate and enzyme. Consequently, the lipase catalytic reaction rate decreased.

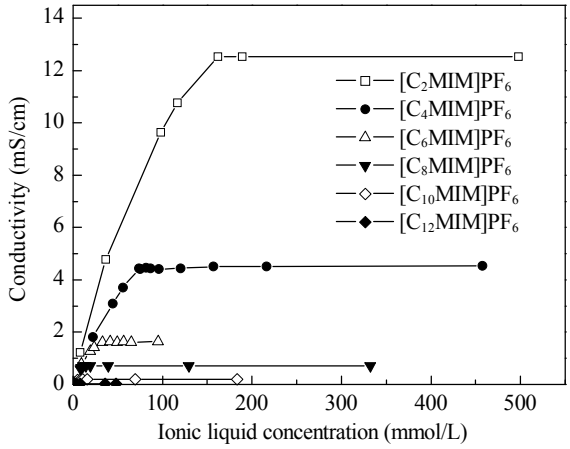


Fig. 4. Effect of concentration and cation structure of $[PF6]^-$ based imidazolium ionic liquids on the conductivity of phosphate buffer solution.

Table 2
Solubility and optimum content of $[PF_6]^-$ based imidazolium ionic liquids in phosphate buffer.

ILs	Optimum concentration [a] (mmol/L)	Solubility [b] (mmol/L)	Reported solubility [c] (mmol/L)
$[C_2\text{MIM}]PF_6$	313.9	133.9	184 [9], 201.2 [37]
$[C_4\text{MIM}]PF_6$	112.0	65.1	71.7 [9], 86.5 [37]
$[C_6\text{MIM}]PF_6$	62.7	28.8	26.0 [37]
$[C_8\text{MIM}]PF_6$	49.1	10.7	8.1 [37]
$[C_{10}\text{MIM}]PF_6$	22.6	6.5	—
$[C_{12}\text{MIM}]PF_6$	47.0	< 1.3	—

[a] Concentrations of ILs with highest CRL activity in phosphate buffer solution (pH 7.000) at 30 °C.
[b] Solubility of ILs in phosphate buffer solution (pH 7.000) at 30 °C.
[c] Solubility of $[C_2\text{MIM}]PF_6$ in pure water at 35 °C; solubility of other ILs in pure water at 30 °C.

3.3 Effect of the structure of anions and concentrations of ILs on CRL hydrolysis activity

The effect of the concentrations of ILs with different anions, such as Br^-, Cl^-, $[BF_4]^-$, and $[PF_6]^-$, and $[C_4\text{MIM}]^+$ cation on CRL activity, is shown in Fig. 5. Obviously, the anions contributed to the enzyme activity in the order of $Br^- > Cl^- > [BF_4]^- > [PF_6]^-$, which was not exactly the same as the previously reported kosmotropicity sequence [4]. According to the Hofmeister effect, the kosmotropicity sequence of anions is $Cl^- > Br^- > [BF_4]^- > [PF_6]^-$. Bi et al. [3] reported that the beneficial effect of $[C_4\text{MIM}]Cl$ was weaker than that of $[C_4\text{MIM}]Br$. In addition, for ILs with $[PF_6]^-$ as an anion, because of the significant influence of the alkyl chain of the cation on the maximum enzyme activity, as the length of alkyl chain increased the effect of $[PF_6]^-$ on the enzyme activity did not fully conform to this order. This also suggests that the effect of hydrophobic ILs on enzyme activity cannot be explained using the Hofmeister effect, which is coincident with prior findings [30].

As shown in Fig. 5, the optimum concentrations of ILs with Br^-, Cl^-, $[BF_4]^-$, and $[PF_6]^-$ anions were 208.6, 209.4, 208.6, and 110.6 mmol/L, respectively. This indicates that the optimum concentrations of hydrophilic ILs are very similar. The ILs (Br^- and $[BF_4]^-$) with the same cation alkyl chain length had similar optimum concentrations (Fig. 1). This indicated that it was the length of the alkyl chain of the cation but not the anion that affected the optimum concentration of hydrophilic ILs. It was

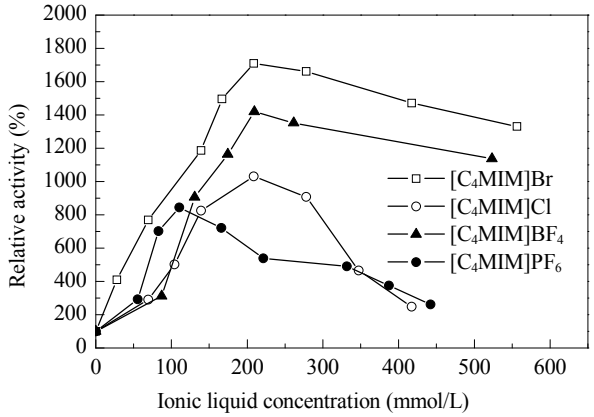


Fig. 5. Effect of concentration and anion type of $[C_4MIM]^+$ based imidazolium ionic liquids on the hydrolytic activity of CRL.

unlikely that it was the anion and not the length of the alkyl chain of the cation that affected the activity of CRL.

The effect of anions on the EC of the buffer solution is shown in Fig. 6. The beneficial effect of the anions was in the order of Cl^- > Br^- > $[BF_4]^-$ > $[PF_6]^-$, coinciding with anion size and mass. It was reported that anions of ILs with larger diameters and masses have lower migration speed, which results in a low EC [38]. According to the relationship between molar conductivity and the square root of IL concentration, as the investigated concentrations of $[C_4MIM]Cl$, $[C_4MIM]Br$, and $[C_4MIM]BF_4$ were less than their CMCs, the enzyme activity should be enhanced. In addition, when the alkyl chain had 2, 6, 8, 10, and 12 carbons, the effect of the anions Br^- and $[BF_4]^-$ on EC was also in the order of Br^- > $[BF_4]^-$.

3.4 Effect of temperature and pH on CRL hydrolysis activity in ILs-water system

High temperature can help achieve a high enzymatic reaction rate but may accelerate enzyme denaturation. With optimum IL concentrations, the effect of temperature and pH on CRL hydrolysis activity in the ILs-water system was investigated. As shown in Fig. 7, as temperature and pH increased, the relative activity first increased and then decreased. The optimum temperature for the systems without an IL, with the addition of a hydrophilic IL with Br^-, Cl^-, and $[BF_4]^-$ anions, and with the addition of a hydrophobic IL with a $[PF_6]^-$ anion, were 37, 30, and 37 °C, respectively (Fig. 7(a)). This indicated that the highest enzyme activity could be reached at a lower temperature in hydrophilic ILs-water systems compared with that without ILs, while the optimum temperature was the same as that of a system without an IL in a heterogeneous system of a hydrophobic $[C_4MIM]PF_6$ IL combination and water.

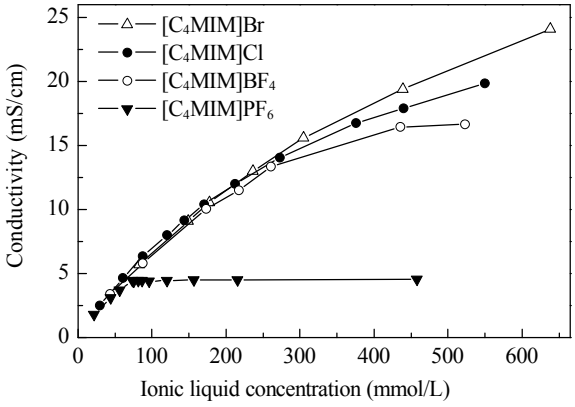


Fig. 6. Effect of concentration and anion type of $[C_4MIM]^+$ based imidazolium ionic liquids on the conductivity of ionic liquid-phosphate buffer solution.

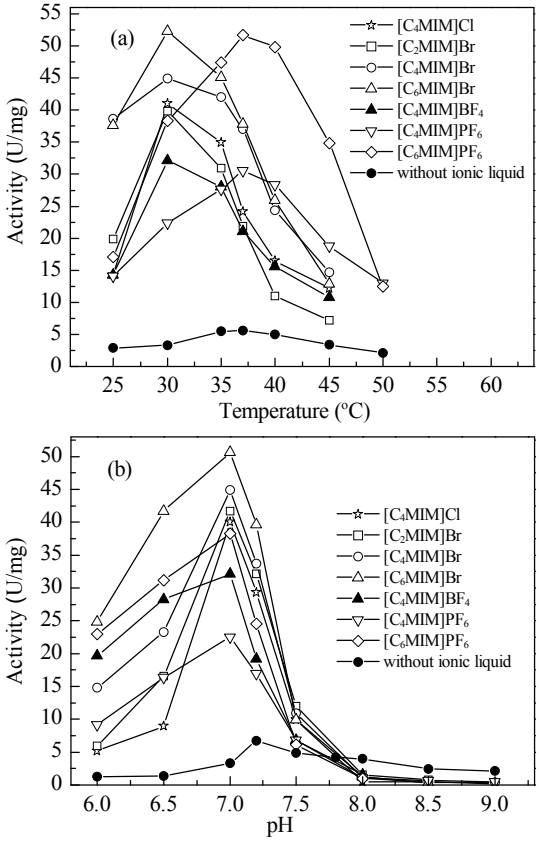


Fig. 7. Effects of temperature (a) and pH (b) of the IL-phosphate buffer solution system on CRL hydrolytic activity.

The pH value of the reaction system may affect the dissociation state and conformational space of an enzyme's active center, thereby affecting enzyme activity. The optimum pH for the reaction decreased from 7.200 to 7.000 after the addition of a variety of ILs at 30 °C (Fig. 7(b)). When the pH value was lower than 6.0, the enzyme activity decreased to 30%–40% of the highest value. When the pH value was higher than 8.0, no enzyme activity was observed. These results suggest that CRL was sensitive to high pH. It might be that the negatively charged enzyme in the alkaline environment interacted with IL cations more severely than in the acid environment as the isoelectric points of CRL are 4.4(A) and 4.9(B).

The beneficial effect of different ILs on enzyme activity changed as the temperature and pH were varied (Fig. 7). Generally speaking, the enzyme activity in systems with the addition of ILs having the same anion but different cations changed similarly with each other as temperature and pH changed. In

contrast, the enzyme activity of systems with the addition of ILs having the same cation but different anions changed quite differently as temperature and pH changed. This suggests that the effect of temperature and pH on CRL hydrolysis activity was highly dependent on the types of anions of the ILs.

As shown in Fig. 7, under the optimum conditions of pH 7.0, 30 °C and 47.6 mmol/L [C_8MIM]Br, the highest relative enzyme activity and specific activity reached 1734% and 54.4 U/mg protein, respectively. Dominguez de Maria et al. [39] measured different CRL activities (Sigma, Roche, and Fluka) using an automatic pH-stat potentiometric titration method. Under the conditions of pH 7.0 and 30 °C, CRL activity was in the range of 3.8–14.0 U/mg protein. The construction of a micro-emulsion system efficiently increased the activity of all the sources of enzymes with the specific enzyme activity reaching 19.8 U/mg protein. By comparison, much higher enzyme activity has been reported herein through the addition of appropriate ILs. In addition, as reported previously [16], the addition of PVA, AOT or lecithin increased the maximum relative activity of CRL to 255%, which was still much lower than the enzyme activity obtained with IL addition.

4. Conclusions

The effect of 1-alkyl-3-methylimidazolium ionic liquids (ILs) on the activity of *Candida rugosa* lipase (CRL) for the hydrolysis of triacylglycerol was investigated. The CMC and solubility of the ILs were determined in phosphate buffer. The results suggested that the effect of the ILs on CRL activity was highly dependent upon their concentration, alkyl chain length of the cation and type of anion employed. The strongest beneficial effect on enzyme activity was achieved at the optimum concentrations of ILs. The maximum beneficial effect of the anions was in the order of Cl^- > Br^- > $[BF_4]^-$ > $[PF_6]^-$. The lengthier the alkyl chain of the cation $[C_n\text{MIM}]^+$, the lower the optimum content of ILs required to achieve high enzyme activity. When the length of the alkyl chain of $[C_n\text{MIM}]^+$ in the ILs with Br^- or $[BF_4]^-$ was less than or equal to 10 and their concentrations in phosphate buffer solution were smaller than their CMCs, the ILs increased enzyme activity. Once the concentrations of the ILs with Br^- or $[BF_4]^-$ anions exceeded their CMC value, enzyme activity was suppressed. Their optimum concentrations were much smaller than their CMCs. Only when the number of carbon atoms of the alkyl chain of $[C_n\text{MIM}]^+$ of ILs with Br^- or $[BF_4]^-$ was in the range of 2–8 and the concentrations were smaller than the optimum values, the beneficial effect of the alkyl chain of the cations on enzyme activity coincident with the Hofmeister effect. The effect of $[C_n\text{MIM}]^+$ from ILs with $[PF_6]^-$ anion showed no relationship with the Hofmeister effect. The effect of ILs on enzyme activity was highly dependent on the pH and temperature of the system and an optimum pH of 7.000 was determined. The optimum temperatures for maximal enzyme activity for hydrophilic and hydrophobic ILs were 30 and 37 °C, respectively. Under the optimal conditions, the highest relative activity of CRL with the addition of [C_8MIM]Br reached 1734%. This study provides insight into the structure-activity relationships of ionic liquids that influence enzyme activity.

Acknowledgments

The authors acknowledge Prof. Bolun Yang's research team at Xi'an Jiaotong University for their provision of materials for the synthesis of ILs.

References

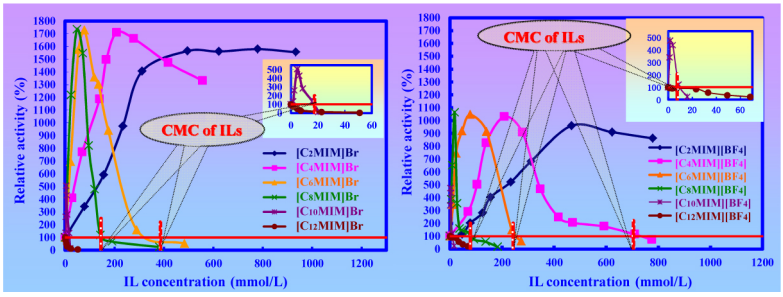
[1] Moniruzzaman M, Nakashima K, Kamiya N. Goto M. *Biochem Eng J*, 2010, 48: 295
[2] Ulbert O, Fráter T, Bélafi-Bakó K, Gubicza L. *J Mol Catal B*, 2004, 31: 39
[3] Bi Y H, Wang Ch Y, Mao Y Y, Zheng Sh Y, Zhang H J, Shi H. *Chin J Catal*, 2012, 33: 1161
[4] Zhao H. *J Mol Catal B*, 2005, 37: 16
[5] Stock F, Hoffmann J, Ranke J, Störmann R, Ondruschka B, Jastorff B. *Green Chem*, 2004, 6: 286
[6] Du W Y, Li N, Wang Sh J. *Chemistry*, 2012, 75: 780
[7] Seddon K R, Stark A, Torres M J. *Pure Appl Chem*, 2000, 72: 2275
[8] Stark A, Seddon K R. Ionic Liquids, in Kirk-Othmer Encyclopedia of Chemical Technology. New York: Wiley, 2007. 859
[9] Freire M G, Neves C M S S, Carvalho P J, Gardas R L, Fernandes A M, Marrucho I M, Santos L M N B F, Coutinho J A P. *J Phys Chem B,* 2007, 111: 13082
[10] Kaar J L, Jesionowski A M, Berberich J A, Moulton R, Russell A J. *J Am Chem Soc*, 2003, 125: 4125
[11] Zhao H, Olubajo O, Song Z Y, Sims A L, Person T E, Lawal R A, Holley L A. *Bioorg Chem*, 2006, 34: 15
[12] Lou W Y, Zong M H, Liu Y Y, Wang J F. *J Biotechnol*, 2006, 125: 64
[13] Yigitoglu M, Temocin Z. *J Mol Catal B*, 2010, 66: 130
[14] Sun Sh, Zhai Y J, Sun Y, Zhang Y P, Liu H, Wang X H, Yu A M, Zhang H Q. *Chem J Chin Univ*, 2010, 31: 468
[15] Zhao H, Luo R G, Malhotra S V. *Biotechnol Progr*, 2003, 19: 1016
[16] Zhao W, Huang Zh N, Li N. *Chin J Anal Chem*, 2011, 39: 351
[17] Shan H X, Lu Y, Li Z J, Li M, Cai Y, Sun X L, Zhang Y Zh. *Chin J Catal*, 2010, 31: 289
[18] Nicole W, Christian W, Vaclav C, Andreas P, Eva H, Rainer R, Frank B. *Chem Biol Chem*, 2008, 9: 1493
[19] Debnath S, Das D, Dutta S, Das P K. *Langmuir*, 2010, 26: 4080
[20] Park S, Kazlauskas R J. *J Org Chem*, 2001, 66: 8395
[21] Lozano P, De Diego T, Guegan J P, Vaultier M, Iborra J L. *Biotechnol Bioeng*, 2001, 75: 563
[22] Huddleston J G, Rogers R D. *Chem Commun*, 1998: 1765
[23] Shang Y, Li N, Qin X H, Lu B W, Fu Q. *Chin J Anal Chem*, 2009, 37: 1173
[24] Sirieix-Plénet J, Gaillon L, Letellier P. *Talanta*, 2004, 63: 979
[25] Dong B, Li N, Zheng L Q, Yu L, Inoue T. *Langmuir*, 2007, 23: 4178
[26] Miskolczy Z, Sebők-Nagy K, Biczók L, Göktürk S. *Chem Phys Lett*, 2004, 400: 296
[27] Geng F, Zheng L Q, Yu L, Li G Z, Tung C H. *Process Biochem,* 2010, 45: 306
[28] Borgstrom B, Donner J. *J Lipid Res*, 1976, 17: 491
[29] Goodchild I, Collier L, Millar S L, Prokeš I, Lord J C D, Butts C P, Bowers J, Webster J R P, Heenan R K. *J Colloid Interf Sci*, 2007, 307: 455
[30] Wang J J, Wang H Y, Zhang Sh L, Zhang H Ch, Zhao Y. *J Phys Chem B*, 2007, 111: 6181
[31] Fu S Zh, Chen Q D, Shen X H. *Acta Phys-Chim Sin*, 2011, 27: 1913
[32] Katayanagi H, Nishikawa K, Shimozaki H, Miki K, Westh P, Koga Y. *J Phys Chem B*, 2004, 108: 19451
[33] Rehmann L, Ivanova E, Ferguson J L, Gunaratne H Q N, Seddon K R,

Graphical Abstract

Chin. J. Catal., 2013, 34: 769–780 doi: 10.1016/S1872-2067(11)60521-4

Effect of imidazolium ionic liquids on the hydrolytic activity of lipase

LI Na*, DU Weiyan, HUANG Zhuonan, ZHAO Wei, WANG Shoujiang
Xi'an Jiaotong University; Baoji University of Arts and Science



Lipase hydrolytic activity was strongly affected by the concentrations, cation structure and anion type of the ILs. The beneficial effect of the IL on lipase activity was eliminated as the IL approached its CMC.

Stephens G M. *Green Chem*, 2012, 14: 725

[34] Gorke J, Srienc F, Kazlauskas R. *Biotechnol Bioprocess Eng*, 2010, 15: 40

[35] Blesic M, Marques M H, Plechkova N V, Seddon K R, Rebelo L P N, Lopes A. *Green Chem*, 2007, 9: 481

[36] Xin J Y, Zhao Y J, Zheng Y, Xia Ch G, Li Sh B. *Chin J Catal*, 2006, 27: 263

[37] Wong D S H, Chen J P, Chang J M, Chou C H. *Fluid Phase Equilibr*, 2002, 194-197: 1089

[38] Wang F H, Li Ch X, Meng H, Wang Z G. *J Beijing Univ Chem Technol* , 2006, 33(6): 17

[39] Dominguez de Maria P, Martinez-Alzamora F, Moreno S P, Valero F, Rua M L, Sánchez-Montero J M, Sinisterra J V, Alcantara A R. *Enzyme Microb Technol*, 2002, 31: 283