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Influence of ionic liquids as additives on sol–gel immobilized lipase

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Abstract

The immobilization of lipase from *Candida rugosa*, using ionic liquids as additives to protect the inactivation of lipase by released alcohol and shrinking of gel during sol–gel process, was investigated. The influence of various factors, such as structure of ionic liquids, content of ionic liquids and types of precursor in the sol–gel process on the activity and stability of immobilized lipase was also studied. The highest hydrolytic activity of immobilized lipase was obtained when the hydrophilic ionic liquid, $[C_2mim][BF_4]$, was used as an additive, while the highest stability of immobilized lipase was obtained by using hydrophobic ionic liquid, $[C_{16}mim][Tf_2N]$. Therefore, the binary mixtures of these ionic liquids as additives were used to obtain the optimal immobilized lipase, which shows both high activity and stability. The hydrolysis and esterification activities of lipase co-immobilized with the mixture of 1:1 at molar ratio of $[C_2mim][BF_4]$ and $[C_{16}mim][Tf_2N]$ were 10-fold and 14-fold greater than in silica gel without ionic liquids (ILs), respectively. After 5 days incubation of this immobilized lipase in *n*-hexane at 50 °C, 84% of initial activity was remained, while the residual activity of the lipase immobilized without ILs was 28%.

Keywords: Binary mixture; Ionic liquid; Lipase; Sol–gel; Co-immobilization

1. Introduction

Sol–gel-derived silica glasses are most popularly used for the immobilization of biomolecules due to their porosity, transparency, chemical stability and convenient preparation [1]. A very large number of enzymes have been immobilized within sol–gel glasses. Although sol–gel immobilized enzymes usually exhibit better activity and stability than free enzymes [2–4], there are some drawbacks in the sol–gel immobilization process. One is the shrinkage of gel during condensation and drying process, which may cause denaturation of enzymes. The released alcohols during the hydrolysis of silicon alkoxide can also inactivate enzymes [5]. The slow diffusion rate of substrate in silica matrices can lower activity of the immobilized enzymes [6]. One way to overcome these drawbacks would be the use of additives to stabilize enzymes within sol–gel matrices. Sugars, amino acids, polyols and surfactants have been used to increase activity and stability of various enzymes. These additives can increase activity and stability of immobilized enzymes by altering hydration of enzyme and reducing shrinkage of gel. They can also influence gel properties by participating in condensation reactions with free silanol groups [7–9].

Ionic liquids (ILs) are organic salts that melt below 100 °C. Unlike traditional solvents, ILs are comprised entirely of ions [10]. The interest in ILs stems from their potential as 'green solvents' [11] because of their non-volatile character and thermal stability, which makes them potentially attractive alternatives for volatile organic solvents. Recently, many groups have reported that ILs have great potential as alternative reaction media for biocatalysis and their use enhanced the reactivity, selectivity and stability of enzyme [12–14]. Specifically, the interesting property of IL as a stabilizer of lipase is their insolubility in hydrophobic organic solvents, because lipases are usually used in organic solvents to carry out various synthetic reactions. Moreover, ILs-coated lipases showed significantly enhanced activity in organic solvents [12,15,16]. ILs co-immobilized with enzyme in silica can also increase the activity and stability of enzyme [1,17,18]. In this study, we used various ILs and binary

Abbreviations: $[C_nmim]^+$, 1-alkyl-3-methylimidazolium; $[BF_4]^-$, tetrafluoroborate; $[PF_6]^-$, hexafluorophosphate; $[Tf_2N]^-$, bis[(trifluoromethyl)sulfonyl] amide

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mixtures of ILs as additives in the sol–gel immobilization to protect the inactivation of lipase during sol–gel immobilization process. The influence of various factors, such as structure of ionic liquids, content of ionic liquids and types of precursor in the sol–gel process on the activity and stability of immobilized lipase was also investigated.

2. Experimental procedures

2.1. Materials

All ILs were synthesized and purified by C-TRI (Suwon, Korea) and had a residual chloride content of less than 30 ppm. ILs were dried in vacuum oven at 60 °C for several days before use. Commercial *Candida rugosa* lipase (Type VII) was purchased from Sigma (St. Louis, USA). Tetramethyl orthosilicate (TMOS), tetraethyl orthosilicate (TEOS), methyltrimethoxysilane (MTMS), benzyl alcohol, benzyl acetate and vinyl acetate were provided by Aldrich (Steinheim, Germany). All other chemicals used in this work were of analytical grade and were used without further purification.

2.2. Procedure for sol–gel immobilization of lipase

For the preparation of lipase solution, 1 g of enzyme was added in 10 ml of 0.1 M phosphate buffer (pH 7.0) and shaken for 10 min. After centrifugation, the supernatant was used for immobilization experiments. Protein content of lipase solution was determined with Lowry protein assay kit and its concentration was usually about 14 mg/ml. In a 5 ml glass vial, the mixture of precursor (1 ml), IL (20%, w/v related to the precursor), deionized water (0.5 ml) and 0.1 M HCl (50 μl) was vigorously stirred for 3 h. After a clear solution was formed, lipase solution (1 ml) was added. The solution was vigorously shaken for 30 s on a vortex mixer and then gently shaken until gelation. The reaction vessel was left to stand open and the bulk gel was air-dried at room temperature for 1 day, which was followed by drying in vacuum oven at 30 °C for 12 h. The bulk gel was crushed in a mortar and then the powder was dried in vacuum oven for 12 h again.

2.3. Determination of hydrolytic activity

The 5 mg of immobilized lipase was placed in a conical tube together with 10 ml of 20 mM phosphate buffer (pH 7.0). The reaction was started by adding 0.1 ml of substrate solution prepared by dissolving 50 mM *p*-nitrophenyl butyrate in DMF and carried out at 25 °C in water bath with shaking at 200 rpm. Periodically, 300 μl aliquots were taken and diluted with 300 μl of acetonitrile, and then centrifuged to obtain supernatant. The activity was determined by measuring the increase in absorbance at 400 nm by the *p*-nitrophenol produced during the hydrolysis of *p*-nitrophenyl butyrate [18].

2.4. Determination of esterification activity

The sol–gel immobilized lipase (20 mg) was added to a small magnetically stirred glass vial containing benzyl alcohol (10 mM), vinyl acetate (10 mM) and water saturated *n*-hexane (1 ml) at 40 °C with continuous shaking. Periodically, 20 μl aliquots were taken and diluted with 40 μl of *n*-hexane to analyze. The activity was expressed as micromole of product (benzyl acetate) formed per minute per gram of dry support or protein. To measure the thermal stability of immobilized lipase, esterification was started by adding 0.1 ml of substrate solution containing benzyl alcohol (100 mM), vinyl acetate (100 mM) and *n*-hexane after incubation of immobilized lipase (20 mg) in water saturated *n*-hexane (0.9 ml) at 50 °C.

2.5. HPLC analysis

Benzyl alcohol and benzyl acetate were quantified by HPLC equipped with a reverse-phase C18 column (SYMMETRY®, Waters, USA) with UV detector at 250 nm. The mobile phase was acetonitrile/water (50/50, v/v) containing 100 μl phosphoric acid/l at 1 ml/min [18].

3. Results and discussion

3.1. Sol–gel immobilization procedure using ionic liquids as additives

Recently, we reported that ILs could be used as a stabilizer to protect lipase from the inactivation during sol–gel immobilization process [18]. In the previous protocol, ILs were added to the hydrolyzed solution of TEOS and then lipase was added. By the way, it was found that higher activity of immobilized lipase could be obtained when the mixture of ILs and TEOS was hydrolyzed and then lipase was added. For example, lipase co-immobilized with $[C_2mim][BF_4]$ by the above protocol showed about 10 times higher activity than immobilized lipase prepared by previous protocol (Table 1). The lipases co-immobilized with ILs containing $[BF_4]^-$ showed significantly enhanced activities, while lipases co-immobilized with other ILs showed similar activities (data not shown). Particularly, $[BF_4]$ ILs have been reported as good templates to make mesoporous silica. Zhou et al. proposed that the formation of hydrogen bonds between $[BF_4]^-$ and silanol group plays a crucial role in order to make mesoporous silica by using $[C_4mim][BF_4]$ as template [19]. The sufficient formation of hydrogen bonds after long-term incubation of TEOS and ILs may induce the highly well-ordered silica, which is beneficial to the activity of lipase. Therefore, the influence of various factors in the sol–gel immobilization process on the activity and stability of lipase was studied with modified preparation method for the following study.

3.2. Influence of ionic liquids structure on immobilized lipases

Table 1 shows the hydrolytic activities of lipases immobilized by using ILs as additives in sol–gel process. In these experiments, the 20% (w/v) ILs related to the TEOS were used to prepare the sol. It is worth noting that the specific activities of lipases co-immobilized with ILs were higher than that of lipase immobilized without ILs. Specifically, lipase co-immobilized

Table 1
Influence of ionic liquids structure on the hydrolytic activity of immobilized lipase

Additives	Activity (μmol/min/g gel)	Protein content (%, g protein/g gel)	Specific activity (μmol/min/g protein)	Relative activity to control	Residual activity (%) after reuse
Free lipase[a]			13720.0		
Control (without IL)	3.5	2.5	141.8	1.0	47.2
$[C_2mim][BF_4]$[b]	7.8	1.7	459.9	3.2	27.1
$[C_2mim][BF_4]$	75.8	1.7	4456.2	31.4	57.1
$[C_{16}mim][BF_4]$	46.3	1.7	2723.1	19.2	76.4
$[C_{16}mim][Cl]$	20.7	1.7	1217.0	8.6	62.3
$[C_2mim][PF_6]$	1.7	1.7	99.2	0.7	80.0
$[C_8mim][PF_6]$	4.8	1.7	283.4	2.0	71.4
$[C_2mim][Tf_2N]$	2.2	1.7	127.6	0.9	80.0
$[C_{16}mim][Tf_2N]$	11.2	1.7	657.5	4.6	96.7

The sol–gel matrices were obtained by acid hydrolysis of the mixtures of ILs (20%, w/v related to precursor) and TEOS to form the aqueous sol, followed by addition of enzyme solution.

[a] The hydrolytic activity was measured with 1 mg free lipase.

[b] The immobilized lipase was prepared by the protocol described in ref. [18].

with $[C_2mim][BF_4]$ showed the highest hydrolytic activity. The enhanced specific activity of lipase co-immobilized with ILs can be explained by the protection from shrinking of gel and inactivation of lipase during sol–gel process [1,17,18]. However, the activity of lipase co-immobilized with ILs containing cation of short alkyl chain decreased after reuse, while the lipases co-immobilized with ILs containing long alkyl chain showed higher residual activity. Shi et al. [20] also reported that ILs with large molecular size, for example, $[C_{10}mim][BF_4]$ or $[C_{16}mim][BF_4]$, could be confined into the silica-gel nanopores relatively firmly, while smaller ones, such as $[C_2mim][BF_4]$ and $[C_4mim][BF_4]$ could be completely washed out from the silica-gel matrix.

The esterification activity and stability of the immobilized lipases which show high hydrolytic activities were measured in *n*-hexane (Table 2). The lipases co-immobilized with $[C_2mim][BF_4]$ and $[C_{16}mim][Tf_2N]$ showed the highest specific activity and stability, respectively. Highly well-ordered porous structure by $[C_2mim][BF_4]$ may induce the increase of lipase activity. Zhou et al. [19] reported that $[BF_4]$ ILs could make well-ordered mesoporous matrix, while the use of the IL with $[Tf_2N]$ anion as a template resulted in silica aerogels with wide pore size distribution only. On the other hand, the high stability of lipase immobilized with $[C_{16}mim][Tf_2N]$ can be understood by the hydrophobicity of IL [18]. It is believed that hydrophobic ILs containing $[Tf_2N]$ keep lipase flexible and active conformation, while hydrophilic ILs containing $[BF_4]$ strip essential water from the enzyme and lead to the unfolding of lipase. Although lipase co-immobilized with $[C_{16}mim][Cl]$ showed high hydrolytic activity, its activity and stability in *n*-hexane were very low. It may be caused by the intrinsically high Cl^- ion concentration, high hydrogen-bond basicity value and hydrophilic nature of $[C_{16}mim][Cl]$ [21].

3.3. Influence of ionic liquid contents and precursors on immobilized lipases

The contents of ILs can influence the specific activity of immobilized lipase. Fig. 1 shows the influence of IL contents on the hydrolytic activity of immobilized lipases. The specific activities of lipases co-immobilized with $[C_{16}mim][BF_4]$ and $[C_{16}mim][Cl]$ decreased with increasing the content of ILs, while the lipase co-immobilized with $[C_{16}mim][Tf_2N]$ showed the highest activity at 30% (w/v) content.

The immobilized lipases produced by sol–gel process using only TMOS or TEOS without additives usually have displayed extremely low activities. For example, relative activities of less than 5% were obtained in the esterification of lauric acid with octanol in isooctane [2]. Therefore, alkyl-substituted silane precursors have been used to prepare the hydrophobic matrices, which could enhance the activity of immobilized lipase. Fig. 2 shows the influence of precursors on the lipase co-immobilized with ILs. When $[C_{16}mim][Tf_2N]$ was used as

Table 2
Influence of ionic liquids structure on the esterification activity of immobilized lipase

Additives	Activity (μmol/min/g gel)	Protein content (%, g protein/g gel)	Specific activity (μmol/min/g protein)	Relative activity to control	Residual activity (%) after 5 days incubation[a]
Free lipase[b]			1582.0		ND[c]
Control (without IL)	1.7	3.5	47.7	1.0	27.5
$[C_2mim][BF_4]$	25.1	2.4	1045.4	21.9	27.5
$[C_{16}mim][BF_4]$	13.6	2.4	565.7	11.9	61.2
$[C_{16}mim][Cl]$	1.6	2.4	68.3	1.4	ND
$[C_{16}mim][Tf_2N]$	3.8	2.4	156.9	3.3	84.4

[a] After 5 days incubation in water saturated *n*-hexane at 50 °C, the residual activity was measured by esterification of benzyl alcohol with vinyl acetate.

[b] The esterification activity was measured with 10 mg free lipase.

[c] Not determined by low activity.

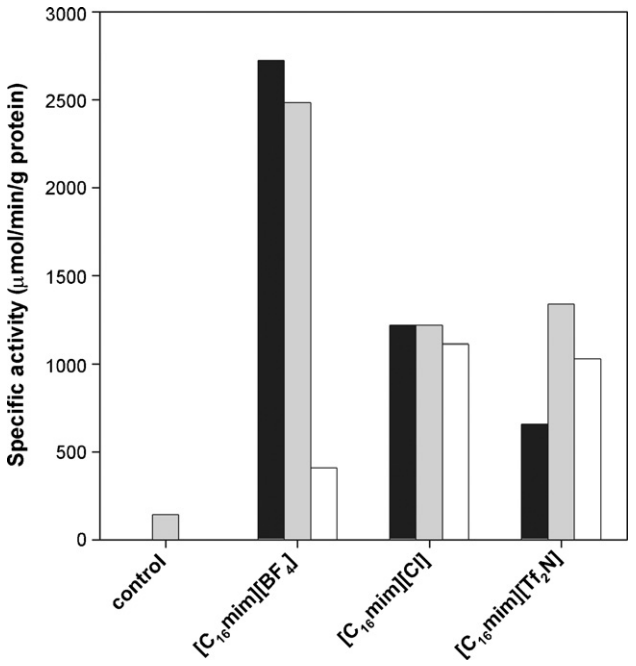

Fig. 1. Influence of IL contents on immobilized lipases. The protein loadings were 2.4% (g protein/g gel). The black, gray and white colors represent 20, 30 and 40% (w/v, related to TEOS) ILs, respectively.

an additive, hydrolytic activity of immobilized lipase drastically increased by using mixture of TMOS and MTMS, compared with the lipase immobilized by using only TMOS. However, the hydrolytic activity of lipase co-immobilized with $[C_{16}mim][BF_4]$ decreased by the addition of MTMS. Although it is difficult to understand the influence of precursor on the lipase co-immobilized with ILs, the effect of $[BF_4]$ ILs as a template may be decreased by the addition of hydrophobic precursor. The lipases immobilized with TEOS precursor generally showed the highest activities among the immobilized lipases prepared by using three types of precursor. The TMOS is usually used instead of TEOS in the sol–gel immobilization of proteins, because methanol as a by-product is less harmful than ethanol [5]. However, it was shown that TEOS is a more suitable precursor to immobilize *C. rugosa* lipase.

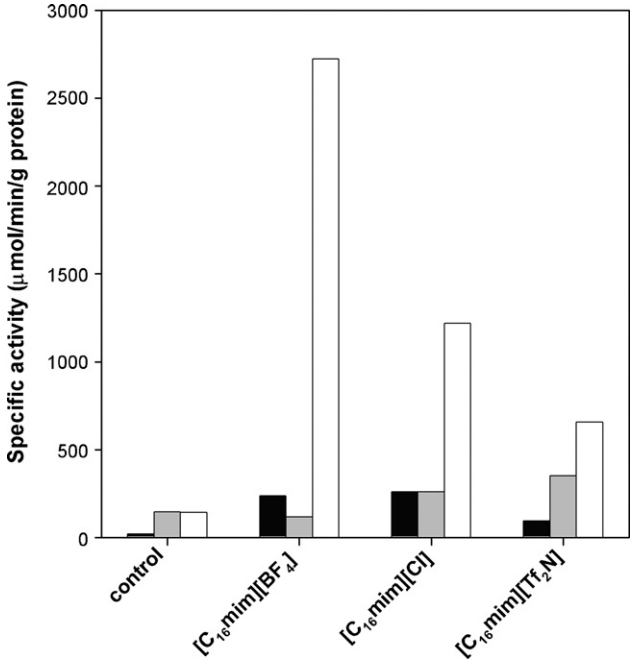

Fig. 2. Influence of precursors on immobilized lipases. The contents of ILs were 20% (w/v) related to TEOS and protein loadings were 2.4% (g protein/g gel). The black, gray and white colors represent TMOS, TMOS/MTMS (1:1 molar ratio) and TEOS, respectively.

3.4. Use of binary mixtures of ionic liquids as additives in the sol–gel process

When the most hydrophilic IL among studied ILs, $[C_2mim][BF_4]$, was used as an additive to stabilize immobilized lipase, the highest hydrolysis and esterification activity of lipase were obtained. However, the highest stability of lipase was acquired by using the most hydrophobic IL among studied ILs, $[C_{16}mim][Tf_2N]$, as an additive. Therefore, the binary mixtures of these ILs as additives were used to obtain the optimal immobilized lipase, which shows both high activity and stability. Mixing of two different ILs, which show different physicochemical properties, can easily make new ILs because hydrophobic ILs and hydrophilic ILs are generally miscible. For example, water-miscible $[C_2mim][BF_4]$ and water-immiscible $[C_8mim][Tf_2N]$ are mutually miscible, although the estimated $\log P$ values for $[C_2mim][BF_4]$ and $[C_8mim][Tf_2N]$ are -3.53 and 0.79, respectively [18]. The mixing of different ILs may be very useful method to make new ILs.

The same content of ILs (20%, w/v related to TEOS) and protein loading (2.4%, g protein/g gel) were used to study the influence of mixtures of ILs on immobilized lipase. Figs. 3 and 4 show the influence of binary mixtures of $[C_2mim][BF_4]$ and $[C_{16}mim][Tf_2N]$ on the hydrolysis and esterification activity

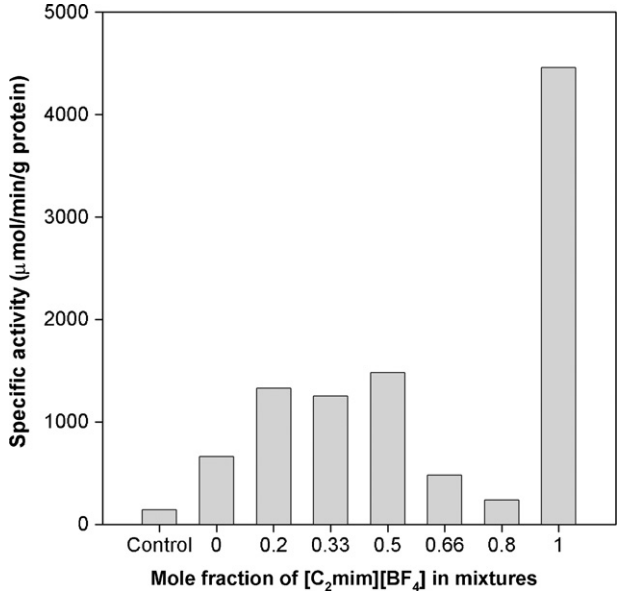

Fig. 3. Influence of the mixtures of $[C_2mim][BF_4]$ and $[C_{16}mim][Tf_2N]$ on the hydrolytic activity of immobilized lipases.

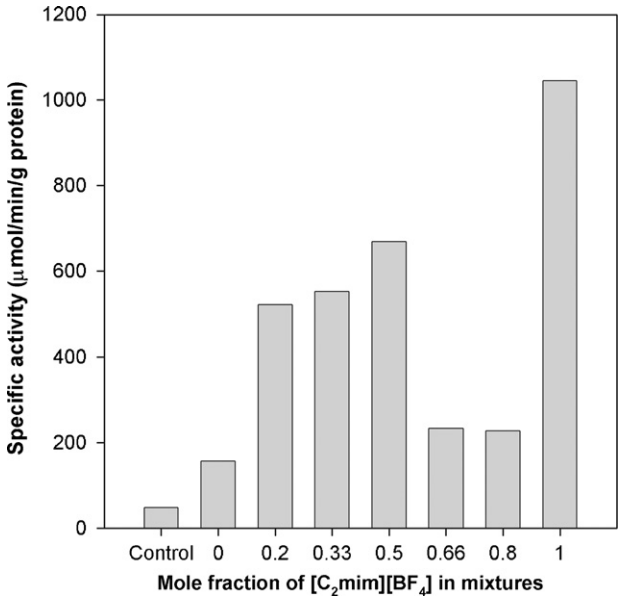

Fig. 4. Influence of the mixtures of $[C_2mim][BF_4]$ and $[C_{16}mim][Tf_2N]$ on the esterification activity of immobilized lipases.

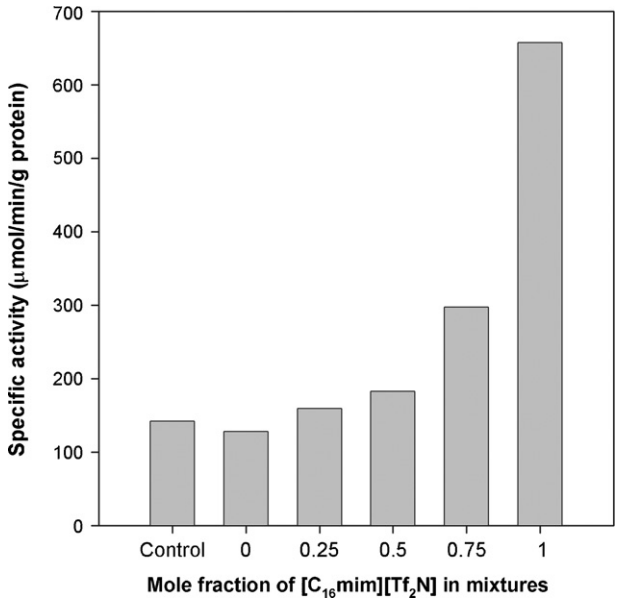

Fig. 6. Influence of the mixtures of $[C_2mim][Tf_2N]$ and $[C_{16}mim][Tf_2N]$ on the hydrolytic activity of immobilized lipases.

of immobilized lipase, respectively. The optimal activities of immobilized lipase in the hydrolysis and esterification reaction were obtained by using the mixture of 1:1 at molar ratio. The hydrolysis and esterification activities of lipase co-immobilized with the mixture of 1:1 at molar ratio were 10-fold and 14-fold greater than in silica gel without ILs, respectively. However, the activities of optimal immobilized lipase were less than those of lipase co-immobilized with only $[C_2mim][BF_4]$. Although it was predicted that the increase of $[C_2mim][BF_4]$ content might monotonously increase the activity of immobilized lipase, so much content of $[C_2mim][BF_4]$ in the binary mixture reduced the activity of immobilized lipase. Fig. 5 shows the stability of lipase co-immobilized with the mixtures of $[C_2mim][BF_4]$ and $[C_{16}mim][Tf_2N]$. Among the tested mixtures, the molar ratio of 1:1 gave the highest stability of immobilized lipase. After 5 days incubation of this immobilized lipase in *n*-hexane at 50 °C, 84% of initial activity was remained, while the residual activity of the lipase immobilized without ILs was 28%. Although the activity of this immobilized lipase was lower than that of lipase co-immobilized with only $[C_2mim][BF_4]$, its stability was similar to that of lipase co-immobilized with only $[C_{16}mim][Tf_2N]$. It means that the immobilized lipase, which shows optimal activity and stability, can be prepared by using the mixture of ILs. The various mixtures of ILs by changing cation and anion were also used as additives in the sol–gel process. When the mixtures of $[C_2mim][Tf_2N]$ and $[C_{16}mim][Tf_2N]$ were added in

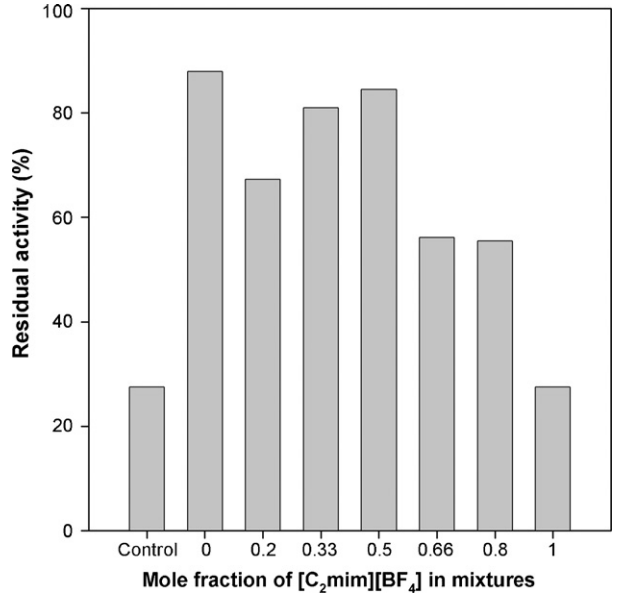

Fig. 5. Influence of the mixtures of $[C_2mim][BF_4]$ and $[C_{16}mim][Tf_2N]$ on the stability of immobilized lipases in *n*-hexane at 50 °C.

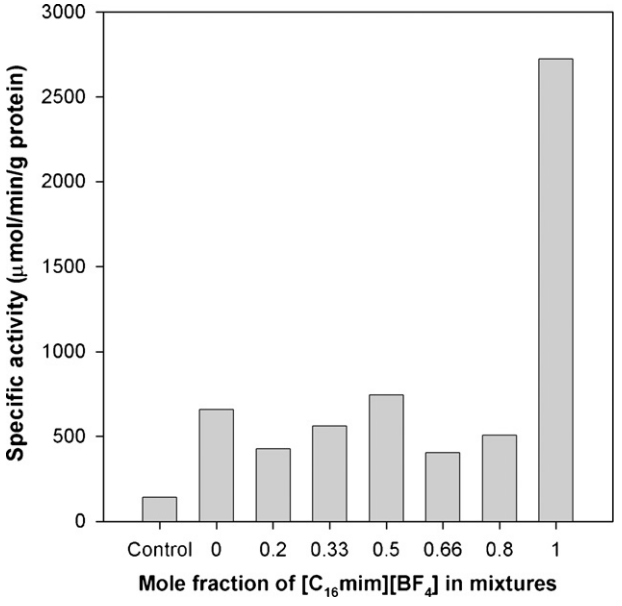

Fig. 7. Influence of the mixtures of $[C_{16}mim][BF_4]$ and $[C_{16}mim][Tf_2N]$ on the hydrolytic activity of immobilized lipases.

the sol–gel process, hydrolytic activity of immobilized lipase monotonously increased with increasing $[C_{16}mim][Tf_2N]$ content (Fig. 6). It means that the increase of content of long alkyl cation, which shows hydrophobic nature positively increased the hydrolytic activity of immobilized lipase. On the other hand, when the mixtures of $[C_{16}mim][BF_4]$ and $[C_{16}mim][Tf_2N]$ were used, the highest hydrolytic activity of immobilized lipase was obtained by using the mixture of 1:1 molar ratio (Fig. 7). These results are similar to the influence of mixtures of $[C_2mim][BF_4]$ and $[C_{16}mim][Tf_2N]$ on the immobilized lipase. The existence of $[Tf_2N]^-$ may negatively influence the interaction of $[BF_4]^-$ with silanol group to form mesoporous silica. From these results, it is difficult to understand the effect of binary mixture of ILs on the immobilized lipase, because there are few reports on the properties of binary mixtures of ILs. However, the binary mixtures of ILs could be successfully used as stabilizers of lipase in the sol–gel process.

4. Conclusions

The immobilized lipase in hydrolysis and esterification reaction can be increased by using most ILs as additives. The hydrophobic ILs containing cation of long alkyl chain can increase the stability of immobilized lipase. Therefore, the lipases co-immobilized with ILs are very useful for the various reactions in hydrophobic organic solvents, although the activity of immobilized lipase decreased after reuse in aqueous solution. Specifically, the binary mixture of ILs can be used to make optimal immobilized lipase, which show both high activity and stability.

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