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Using ionic liquids to stabilize lipase within sol–gel derived silica

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Abstract

Ionic liquids (ILs) were used as additives to protect the inactivation of enzymes by released alcohol and shrinking of gel during the sol–gel process. The *Candida rugosa* lipases immobilized by using ILs in sol–gel process showed higher activity and stability than lipase immobilized without ILs. The hydrolytic and esterification activities of lipase coimmobilized with ILs were 5-fold and 16-fold greater than in silica gel without ILs. After 5 days incubation of lipase coimmobilized with 1-octyl-3-methylimidazolium bis[(trifluoromethyl)sulfonyl]amide at 50 °C, residual activity of lipase was about 80% of initial activity, while the lipase immobilized without ILs was completely inactivated. ILs may act as a template during gelation and reduce shrinkage of gel by pore filling. They can also behave as a stabilizer to protect the enzyme from the inactivation.

Keywords: Ionic liquid; Lipase; Sol–gel; Coimmobilization

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 showing that they usually retain their catalytic activity and can be protected against degradation. Sol–gel immobilized enzymes usually exhibit better activity and stability than free enzymes [2–4]. However, there are some drawbacks in the sol–gel immobilization process. There is always some shrinkage of gel during condensation and drying process and the shrinking may cause partial denaturation of enzymes. In addition, the released alcohols during the hydrolysis of silicon alkoxide can 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, crown ethers, and surfactants have been used to increase activity and stability of various proteins [7–9]. These additives can increase thermal stability and activity of immobilized proteins by altering hydration of protein and reducing shrinkage via a "pore filling" effect.

Room temperature ionic liquids (ILs) are organic salts that do not crystallize at room temperature. 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, a few groups have reported that ILs have great potential as alternative reaction media for biocatalysis and biotransformation [12]. It was observed that their use enhanced the reactivity, selectivity, and stability of enzyme [13,14]. The interesting property of IL as an additive in sol–gel immobilization process is their insolubility in hydrophobic organic solvents, and ILs coimmobilized with enzyme in silica can increase the activity and stability of enzyme. A recent paper reported on the sol–gel immobilization of horseradish peroxidase using [Bmim][BF_4] as an additive. It showed that the activity is 30-fold greater than in silica gels without IL [1,15]. In this study, we used various ILs as additives in the sol–gel immobilization of lipase and investigated the influence of IL properties on the activity and stability of immobilized lipase.

Abbreviations: [Emim][BF_4], 1-ethyl-3-methylimidazolium tetrafluoroborate; [Bmim][BF_4], 1-butyl-3-methylimidazolium tetrafluoroborate; [Omim][BF_4], 1-octyl-3-methylimidazolium tetrafluoroborate; [Bmim][PF_6], 1-butyl-3-methylimidazolium hexafluorophosphate; [Omim][PF_6], 1-octyl-3-methylimidazolium hexafluorophosphate; [Emim][Tf_2N], 1-ethyl-3-methylimidazolium bis[(trifluoromethyl)sulfonyl]amide; [Omim][Tf_2N], 1-octyl-3-methylimidazolium bis[(trifluoromethyl)sulfonyl]amide

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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). Tetraethyl orthosilicate (TEOS), 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 diluted 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 and determination of protein content. Protein content of lipase solution was determined with Lowry protein Assay Kit and measured value was usually about 14 mg/ml. In a 5 ml glass vial, the mixture of TEOS (1 ml), deionized water (0.5 ml), and 0.1 M HCl (26 μl) was vigorously stirred for 3 h. After the mixture became homogenous, 0.2 ml of IL was slowly added. When a clear solution containing IL was formed, lipase solution was quickly 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 opened and the bulk gel was air-dried at room temperature for 1 day. The bulk gel was crushed in a mortar, and then it was dried in vacuum oven at 30 °C for 1 day.

2.3. Determination of hydrolytic activity

The 5 mg of immobilized lipase was placed in a Falcon 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 [16].

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 (30 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 by using HPLC. The activity was expressed as μmol of product (benzyl acetate) formed per minute per gram of 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 (300 mM), and *n*-hexane after incubation of immobilized lipase (20 mg) in *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 determination at 250 nm. The mobile phase was acetonitrile/water (50/50, v/v) containing 100 μl phosphoric acid per liter at 1 ml/min [17].

3. Results and discussion

3.1. Hydrolytic activity of immobilized lipase

The immobilized lipases produced by sol–gel process using only TEOS 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]. In this work, various ILs as additives were used to increase the activity and stability of immobilized lipase. Table 1 shows the hydrolytic activity of lipases immobilized by using ILs as an additive in sol–gel process. It is worth noting that the specific activity of lipase coimmobilized with ILs except [Emim][Tf_2N] was higher than that of lipase immobilized without IL. When the hydrophobic and water-immiscible ILs containing [PF_6] and

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

Additives	Activity (mmol/min)	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)	19.0	2.03	187.4	1.0	55.6
[Emim][BF_4]	23.8	1.22	391.1	2.1	20.6
[Omim][BF_4]	13.6	1.27	215.3	1.2	29.1
[Bmim][PF_6]	17.3	1.22	282.7	1.5	20.8
[Omim][PF_6]	52.8	1.21	874.1	4.7	38.9
[Emim][Tf_2N]	9.0	1.22	147.7	0.8	19.0
[Omim][Tf_2N]	58.1	1.22	950.7	5.1	58.9

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

Table 2
Solvent properties of ionic liquids studied [21]

Ionic liquids	δ_H (MPa$^{1/2}$)	log P	E_T^N
[Emim][BF_4]	32.9[a]	−3.53[a]	0.697
[Omim][BF_4]	28.9	−1.14	0.638
[Bmim][PF_6]	29.8	−2.06	0.670
[Omim][PF_6]	27.9	−0.35	0.637
[Emim][Tf_2N]	27.6	−1.18	0.661
[Omim][Tf_2N]	25.0	0.79	0.629

[a] Estimated by extrapolating the data.

[Tf_2N] anions or long alkyl chain cation were used as additives, the specific activity of immobilized lipase in hydrolytic reaction was significantly increased.

There are some possible explanations for enhanced specific activity of enzymes immobilized by using ILs as additives. Firstly, there is always some shrinkage of gel during condensation and drying process and the shrinking may cause partial denaturation of enzymes. ILs can protect the shrinking of gel structure by pore filling and can be employed to template well-ordered mesoporous silica. Since most ILs possess both a hydrophilic ionic head and hydrophobic organic chain, they are also one category of surfactants, which were used as templates to prepare microporous and mesoporous materials in some recent publications [18]. In recent, Liu et al. [1] showed that the activity of horseradish peroxidase is 30-fold greater than in silica gel without IL. Authors suggested that IL behave both as a template for the formation of mesoporous and a stabilizer to protect the enzyme during immobilization. Secondly, the hydrolytic and esterification activity of *C. rugosa* lipase can be increased by pretreatment of suitable organic solvents. The opening of the lid covering active site of lipase is proposed as the reason for explaining the activity enhancement, both in aqueous and anhydrous organic media [19]. In the hydrolytic activity of pretreated lipase, a continuous enhancement in the activity was obtained as the log P was increased [20]. When the log P values of $[Omim]^+$ ILs were compared, log P values increased with the following order $[BF_4]^- < [PF_6]^- < [Tf_2N]^-$ (Table 2). With increasing cation alkyl chain length, the log P values increase [21]. Therefore, the higher hydrolytic activity of lipase coimmobilized with more hydrophobic ILs can be explained with the rearrangement of lid structure of lipase in sol–gel process. We have recently investigated pretreatment of *Mucor javanicus* lipase with various ILs. The activity and stability of lipase pretreated with ILs were higher than those of untreated lipase for the hydrolysis reaction in an aqueous medium [16]. Thirdly, the release of alcohol during the hydrolysis-condensation of silcon alkoxides in the sol–gel process has been considered an obstacle, due to its potential denaturing activity on the entrapped biological moiety [5]. Tetramethyl orthosilicate (TMOS) is therefore currently used instead of TEOS, as methanol is less harmful than ethanol. Although lipase can retain activity at high concentration of ethanol, partial denaturation of lipase can be occurred in sol–gel process. ILs can protect the enzyme from the inactivation by alcohols. ILs may be useful as a stabilizer in the sol–gel process, because some enzymes are very sensitive to traces of alcohol.

The hydrolytic activity of lipase coimmobilized with ILs was decreased after reusing, because the ILs confined in the silica-gel was released to aqueous medium and hydrophilic organic solvents such as acetonitrile, acetone, and methanol. The leaching of lipase and ILs was severely occurred in hydrophilic ILs containing short chain cation. Unfortunately these immobilized lipases are not suitable for reusing in the hydrolytic reaction. However, Deng and co-workers [22] recently 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 [Emim][BF_4] and [Bmim][BF_4] could be completely washed out from the silica-gel matrix. Therefore, ILs with long alkyl chain length can be used as suitable additives to immobilize lipase for the reaction in aqueous medium. Preliminary experiments showed that [C_{16}mim][Tf_2N] and [C_{16}mim][Cl] could be still confined in the silica gel after washing with water. Residual activities of lipase coimmobilized with [C_{16}mim][Tf_2N] and and [C_{16}mim][Cl] after washing were 96 and 89% of initial activity, respectively.

3.2. Esterification activity of immobilized lipase

Table 3 shows the esterification activity of lipases immobilized by using ILs as an additive of sol–gel process. When the hydrophobic ILs were used as additives, specific activity of immobilized lipase in esterification reaction was generally

Table 3
Influence of ionic liquids on the esterification activity of immobilized lipase

Additives	Activity (mmol/min)	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)	3.0	2.03	7.4	1.0	ND
[Emim][BF_4]	28.0	1.22	114.8	15.5	21.9
[Omim][BF_4]	3.2	1.27	13.1	1.8	47.2
[Bmim][PF_6]	6.0	1.22	24.5	3.3	40.9
[Omim][PF_6]	7.4	1.21	30.6	4.1	39.0
[Emim][Tf_2N]	2.6	1.22	10.3	1.4	43.5
[Omim][Tf_2N]	7.6	1.22	31.1	4.2	82.1

[a] After 5 days incubation in *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.

increased. Because these results are similar to the hydrolytic activity of immobilized lipase, enhanced activity can be understood by the protection against gel shrinking and rearrangement of lid structure. In addition, Ru et al. [23] reported that the activity of *C. rugosa* lipase colyophilized with salts such as LiCl, NaCl, and KCl drastically increased by improved diffusion of the enzyme in organic solvent. Similarly, ILs can provide more favorable environment for immobilized lipase in *n*-hexane. However, it is difficult to understand the exceptionally increased esterification activity of lipase coimmobilized with [Emim][BF_4]. It is thought that the increased activity by using [Emim][BF_4] as an additive may be induced by highly well ordered porous structure. Antonierri and co-workers [24] reported that ILs containing [BF_4] anion can be used as template to monolithic mesoporous silica with wormhole framework. Although [BF_4] ILs as template can make well ordered mesoporous matrix, the use of the IL with [Tf_2N] anion as a template resulted in silica aerogels with wide pore size distribution only. Therefore, [Emim][BF_4] mediated high surface area mesoporous silica may induce the high activity of immobilized lipase. The [Emim][BF_4] is the most hydrophilic IL among all ILs studied in this work and fully miscible with water. This hydrophilic nature of [Emim][BF_4] may be effective to make uniform mesoporous structure of silica.

3.3. Stability of immobilized lipase

Fig. 1 shows the thermal stability of immobilized lipase in *n*-hexane at 50 °C. The lipase coimmobilized with [Omim][Tf_2N] has extremely high stability at high temperature. After 5 days incubation, residual activity of lipase coimmobilized with [Omim][Tf_2N] was 80% of initial activity, while the lipase immobilized without ILs was fully inactivated. Although lipase coimmobilized with [Emim][BF_4] showed very high esterification activity, half-life time of that is similar to the lipase immobilized without IL. It has previously been suggested that coating an enzyme in a hydrophobic IL can improve its stability in organic reaction [12]. Rogers and co-workers precoated laccase with hydrophobic [Bmim][Tf_2N] before it was dispersed in hydrophilic [Bmim][Cl]/cellulose solution to provide a stabilizing microenvironment for the enzyme [25]. To understand the relationship between stability of lipase and properties of ILs, various solvent parameters such as Hildebrand solubility parameters, log *P*, and polarity scale (Table 2) of ILs were correlated with residual activities of immobilized lipases after 5 days incubation at 50 °C. The log *P* value was well correlated with residual activity (Fig. 2). It means that hydrophobic ILs can stabilize lipase in organic solvents at high temperature. It is believed that hydrophilic organic solvents strip essential water from the enzyme and lead to the unfolding of the molecule with exposure of the inner hydrophobic residues, while hydrophobic organic solvents keep it flexible and active conformation [26]. This explanation can be applied to the stability of lipase coimmobilized with ILs. In addition, the leaching of enzyme and ILs is not detected during esterification in *n*-hexane or hydrophobic organic solvents. The residual activities of lipase coimmobilized with ILs were over 90% after reuse. After fifth reuse, lipase coimmobilized with [Omim][Tf_2N] retained 90% of the initial activity.

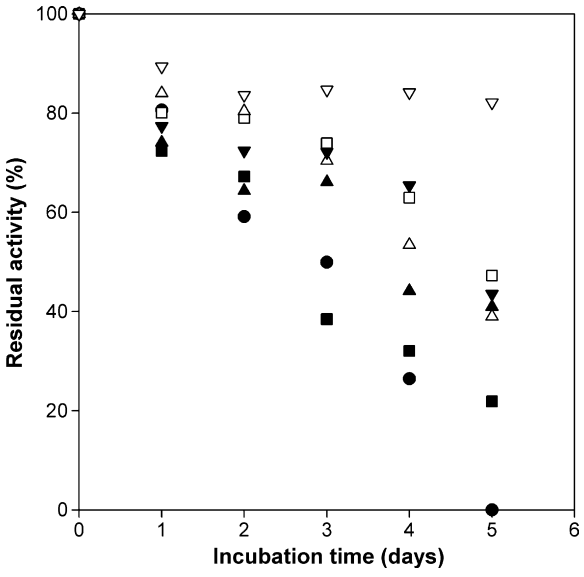

Fig. 1. Effect of ILs and incubation time in *n*-hexane at 50 °C. Esterification conditions: 10 mM benzyl alcohol, 30 mM vinyl acetate, 20 mg immobilized lipase, 1 ml *n*-hexane, 40 °C. (●) control (without IL), (■) [Emim][BF_4], (□) [Omim][BF_4], (▲) [Bmim][PF_6], (△) [Omim][PF_6], (▼) [Emim][Tf_2N], (▽) [Omim][Tf_2N].

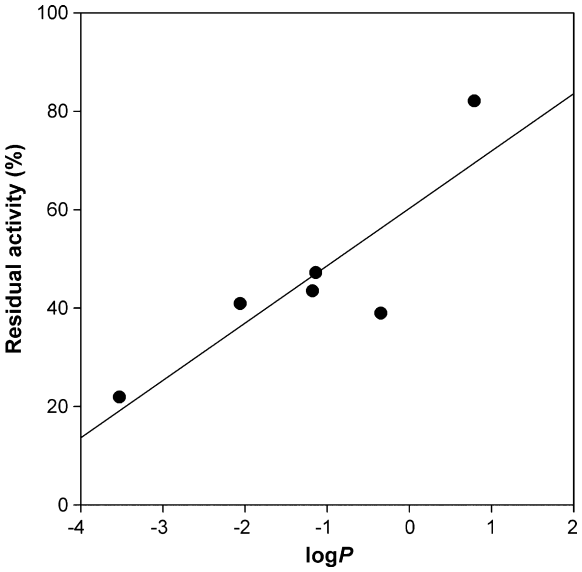

Fig. 2. Correlation between log *P* value of IL and residual activity of immobilized lipase after 5 days incubation in *n*-hexane at 50 °C.

4. Conclusions

The lipases coimmobilized with ILs were very useful for the reaction in organic solvents, although the leaching of ILs caused the decrease of activity in aqueous medium. Most of ILs could increase the activity of immobilized lipase in hydrolytic and esterification reaction. The hydrophobic ILs was effective to

increase the stability of immobilized lipase. It was considered that ILs in the sol–gel process can act as a template during gelation and behave as a stabilizer to protect the enzyme from the inactivation by alcohol or heat.

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