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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.