Activation of lipase in ionic liquids by modification with comb-shaped poly(ethylene glycol)

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Received 4 April 2006; received in revised form 29 April 2006; accepted 14 June 2006
Available online 27 October 2006

Abstract

Outstanding activation of an enzyme in ionic liquids (ILs) has been demonstrated by covalent modification with comb-shaped poly(ethylene glycol) (PEG) (PM13). Candida rugosa lipase modified with PM13 (PM13–CRL) was readily solubilized in all the ILs tested ([Emim][Tf2N], [C2OC1mim][Tf2N] and [C2OHmim][Tf2N]) containing 0.5% (v/v) of water, whereas native lipase did not dissolve in any of the ILs. The results for transesterification of 2-phenyl-1-propanol with vinyl acetate using lipase in ILs revealed that the PM13–CRL conjugate exhibits a high catalytic activity while suspended native lipase shows little activity. The hydrophobicity of ILs somewhat affected the enzyme activity and a more hydrophobic IL such as [Emim][Tf2N] was preferable for the lipase reaction, as was also observed in enzymatic reaction in conventional organic solvents. The enzyme activities in ILs were much higher than those in organic solvents, the excellent activity being associated with unique properties such as the hydrophobicity and the high polarity of ILs. Furthermore, the PM13–CRL conjugate exhibited a high storage stability in [Emim][Tf2N].

Keywords: Ionic liquids; Biocatalysis; Enzyme reaction; Chemical modification; Transesterification

1. Introduction

Ionic liquids (ILs) are novel and functional solvents that have unique properties such as non-volatility, thermal stability and relatively high polarity [1,2]. One of the most attractive features of ILs is that their solvent properties are controllable by changing the combination of cations and anions or by appending functional groups in the constituent ions [3]. Thus, a “tailor-made solvent” can readily be chosen for a given reaction. For example, ILs which are composed of hydrophobic ions and are immiscible with water appear to have a dual nature, i.e., polarity and hydrophobicity [4,5].

Recent researchers have been interested in using ILs as non-aqueous media for biotransformation [6,7]. Utilizing hydrophobic ILs as reaction media in biocatalysis often improves the efficiency in synthetic reactions using polar substrates with suppression of hydrolytic side reactions. Since the pioneering studies by some groups [8–10], many biocatalytic reactions in ILs have been actively conducted with lipases [11–14], proteases [15,16], oxidoreductases [17,18], and even with a whole-cell biocatalyst [19]. When compared to reactions performed in conventional organic solvents, ILs provide a more suitable environment for enzymes [16,20] and are obviously less harmful to cell membranes [19], resulting in high catalytic performance. The feasibility of ILs as novel reaction media for biocatalysis has been demonstrated over the past 5 years with relatively high enzyme activity and selectivity.

Enzymes, however, are biomacromolecules which are composed of polypeptide chains and are hydrophilic, thus native enzymes are usually insoluble in ILs. Furthermore, the catalytic activities of suspended enzymes in pure ILs were modest, except for relatively high activities of some lipases such as Candida antarctica lipase B (CALB) and Pseudomonas cepacia lipase (PCL). Several attempts have been made to enhance the activity of enzymes dispersed in ILs, including the addition of water to ILs or immobilization of the enzymes on a solid support. When industrial use
of enzymes is limited to heterogeneous catalysis, low enzyme solubility is not a serious obstacle. However, insolubility of enzymes in ILs restricts the expansion of these applications, thus solubilization of versatile biocatalysts in ILs will break new ground (Scheme 1).

There are two strategies for solubilizing enzymes in ILs. One is the introduction of functional groups that show high affinity for protein molecules into ILs, such as hydroxyl, ether and amide groups. Some ILs are known to solubilize enzymes through hydrogen bonding interactions, however, these ILs often cause conformational changes in proteins, resulting in catalytic inactivation [21]. The other strategy is modification of the enzymes with a compound which has a high affinity for ILs and dissolves well in them. It is well known that crown ethers and poly(ethylene glycol) (PEG), both of which are composed of ethylene glycol units, dissolve in ILs. By taking advantage of the inherent properties of these compounds, high activation of lipase in ILs by physical complexing with PEG [22] and extraction of cytochrome c from an aqueous phase into ILs with crown ether [23] have been successfully demonstrated. These results prompted us to attempt chemical modification of enzymes with PEG for solubilization in ILs. However, modification with single-chained PEG was insufficient for achieving high solubility and activating enzymes [14,24]. Most recently, we have succeeded in solubilizing subtilisin Carlsberg in ILs by chemical modification with comb-shaped PEG (PM13) (Fig. 1), which is ideal for introducing a large number of PEG chains into a protein molecule [25].

In the present study, the feasibility of the comb-shaped PEG, PM13, for solubilization and activation of lipase was investigated. Candida rugosa lipase (CRL) was selected as a model enzyme and was modified with PM13. We then examined lipase-catalyzed transesterification in ILs and elucidated the effect of the modification on the catalytic activity. Correlations between the enzyme activity and solvent properties of the reaction media are also discussed.

2. Materials and methods

2.1. Materials

CRL was purchased from Sigma-Aldrich (Milwaukee, WI). PM13 (SUNBRIGHT AM-1510 K, Mw: 15,000 to 20,000), was obtained from NOF Co. (Tokyo, Japan) and used without further purification. All other chemicals were reagent grade.

2.2. Synthesis of ILs

ILs used in this study were prepared in our laboratory. The molecular structures and abbreviations of ILs are shown in Fig. 1. Three different ILs, 1-ethyl-3-methylimidazolium bis(trifluoromethanesulfonyl)imide ([Emim][Tf2N]), and its ether ([C2OC1mim][Tf2N]) and hydroxyl ([C2OHmim][Tf2N]) analogues were prepared according to the literature methods [4,26] and dried over 48 h under vacuum.

2.3. Water content and polarity evaluation of ILs

Each dried IL was diluted with anhydrous methanol to reduce its viscosity, then its water content was measured using a Karl Fischer moisture titrator. The water content in

![OH](AcO)CH2CH2OAc + CH3CHO

lipase

lipase-catalyzed transesterification between 2-phenyl-1-propanol and vinyl acetate.

![Chemical structures and abbreviations of comb-shaped poly(ethylene glycol) (PEG) and ILs used in this study.](image)
the dry ILs was calculated by subtracting that of anhydrous methanol. The water contents of water saturated ILs were measured in the same manner after equilibration of the ILs with water.

The polarity of ILs was evaluated by the solvatochromic effect of Reichardt’s dye [27]. The measurement was conducted by the following procedure. Reichardt’s dye (2,6-diphenyl-4-(2,4,6-triphenylpyridinio)phenolate, 0.8 mg) was dissolved in the ILs (1 ml) and centrifuged to remove insolubles. The wavelength of the absorption maximum was measured by a JASCO V-570 UV–Vis spectrophotometer at 25 °C and was converted to normalized polarity values \( E_T^N \), which range from 0.0 for tetramethylsilane to 1.0 for water, using the following equations.

\[
E_T^N = \frac{28591}{\lambda_{max}} \times \frac{E_T}{32.4}
\]

2.4. Modification of enzyme with comb-shaped PEG.

For pretreatment of lipase, the crude lipase powder (5 g) was suspended in 50 ml of 25 mM Tris-HCl buffer (pH 7.5) and stirred for 30 min at 4 °C. The solution was centrifuged at 8000 g for 20 min and the clear supernatant was collected, followed by freeze drying for 24 h. The pre-treated lipase was used as a native CRL. Modification of the lipase with comb-shaped PM13 was performed according to the literature procedure [28]. To 4 ml of the lipase solution (2 mg/ml) in 0.1 M sodium borate buffer (pH 8.5), crystalline PM13 (200 mg) was added, followed by stirring at 4 °C in an ice bath for 1 h. The reaction mixture was ultrafiltered using an Ultra-4 membrane (MWCO: 100,000, Millipore, Billerica, MA) and washed three times with deionized water to remove unreacted PM13. After lyophilization for 48 h, PM13-modified lipase (PM13–CRL) was obtained. The degree of modification of amino groups in the lipase molecule was determined with 2,4,6-trinitrobenzenesulfonic acid (TNBS) [29], and the protein content in the PM13–CRL conjugate was estimated using the bicinchoninic acid (BCA) assay [30].

2.5. Enzyme activity in water, organic solvents or ILs

The enzyme concentration was adjusted to 1 mg/ml considering the protein content of each biocatalyst from the BCA assay. The catalytic activity of lipase in the aqueous solution was determined by hydrolysis of p-nitrophenyl butyrate in tris-HCl buffer at 37 °C, monitoring p-nitrophenol liberated with a UV–Vis spectrophotometer at 420 nm. In ILs, the activity of enzymes was evaluated by transesterification of 2-phenyl-1-propanol (100 mM) with vinyl acetate (100 mM) in ILs containing 1.5% water at 40 °C. Samples were periodically removed from the reaction mixture and diluted 10-fold with acetonitrile, then injected into an HPLC system (LC-10AT VP, Shimadzu) fitted with an Inertsil ODS-3 column (4 × 250 mm, GL Science) using 70:30 (v/v) acetonitrile : 0.5% v/v acetate buffer as an eluent at a flow rate of 1.0 ml min⁻¹. The eluted compounds were monitored by a UV–Vis detector (SPD-M10A VP) at 257 nm. The enzyme activity in organic solvents was measured in a similar manner.

3. Results and discussion

3.1. Solvent properties of ILs

Physicochemical properties of reaction media significantly affect enzyme activity. We used hydrophobic ILs as the reaction media because hydrophobic solvents would be preferable in non-aqueous enzymatic reactions. The hydrophobicity of the ILs was estimated as a measure of the water content (Table 1). The water content of the ILs increased in the order [Emin][Tf2N] < [C2OC1mim][Tf2N] < [C2OHmim][Tf2N] in both dried and water saturated conditions, which indicates that [Emin][Tf2N] is the most hydrophobic. This result is predictable from the chemical structures of the ILs.

The \( E_T \) value is one of the most widely used empirical polarity scales, and is based on the charge-transfer absorption band of Reichardt’s dye. Since the ground state of the dye is highly polarized, highly polar solvents stabilize the ground state, increasing the absorption energy. Thus the position of the absorption is strongly influenced by the ability of the solvent to act as a hydrogen bond donor to the phenoxide oxygen in the dye. For convenience, \( E_T \) values are often normalized to \( E_T^N \), where \( E_T^N = 0.0 \) for tetramethylsilane and 1.0 for water. The \( E_T^N \) polarity scales of ILs are shown in Table 1, along with comparative \( E_T^N \) values of organic solvents. The polarities of the ILs were generally high, similar to that of ethanol (\( E_T^N = 0.67 \)). The highest polarity was observed for

| Water content (ppm) | Polarity | \( E_T^N \) |
|---------------------|----------|------------|
| [Emin][Tf2N]        | 495      | 0.66       |
| [C2OC1mim][Tf2N]    | 830      | 0.68       |
| [C2OHmim][Tf2N]     | 1640     | 0.88       |
| Acetonitrile        | 0.46a    |            |
| DMSO                | 0.44a    |            |
| THF                 | 0.21a    |            |
| MTBE                | 0.12a    |            |
| Toluene             | 0.10a    |            |
| Hexane              | 0.01a    |            |

*The values for organic solvents were taken from a recent review [27].
[C$_2$OHmim][Tf$_2$N], due to its hydrogen bond donor ability as seen in alcohols. It is noted that as these ILs are water immiscible, they have contradictory hydrophobicity and polarity properties.

3.2. Characterization and solubility of PM$_{13}$-modified enzyme

Protein is generally dissolved in aqueous solution with a significant contribution by hydrogen bonding with water molecules, while in ILs there should be little interaction between protein molecules and ILs, resulting in extremely low solubility of enzymes in ILs. Although adding a small amount of water improves the solubility of enzymes, excessive addition of water would lead to a decline in enzyme activity [15]. Altering the chemical structure of ILs is a promising and effective way of solubilizing enzymes, however, this often causes inactivation of the enzyme [21]. That is, ILs which interact strongly with a protein could also induce conformational changes in a protein molecule.

Alternatively, modification of enzymes with PEG that dissolves well in ILs appears to be a rational strategy for solubilization of enzymes in ILs without reducing their activities. Some researchers have tried to improve enzymatic performance by covalent modification with PEG. However, chemical modification of enzymes with single-chained PEG did not offer sufficient solubility and activity of enzymes.

We used a highly-branched PEG derivative, called comb-shaped PEG, as an enzyme modifier to attach sufficient quantities of PEG chains to the enzyme. The comb-shaped PEG, PM$_{13}$, has several branched PEG chains and multivalent reactive sites in its backbone which can react with amino groups in a protein molecule. In the present study, CRL was chemically modified with PM$_{13}$. The degree of modification of amino groups in the protein molecule and the protein content in the PM$_{13}$–CRL conjugate were determined by the TNBS method and the BCA assay, respectively. Based on these examinations, it was determined that about 35% of amino groups in lipase were modified with the carboxyl groups of PM$_{13}$, and that PM$_{13}$–CRL conjugate contained 2.3 wt% of lipase. The protein content in PM$_{13}$–CRL was considered in adjusting the protein concentration in enzyme activity evaluations.

In general, modification of enzymes often induces loss of activity, thus we examined the enzyme activity of the PM$_{13}$–CRL in a hydrolytic reaction of p-nitrophenyl butyrate in water, comparing it with that of native lipase. The activities were comparable, suggesting that inactivation of enzymes during the modification process did not take place.

First, we examined the solubility of enzymes in ILs. It was found that native lipase did not dissolve in typical [Emim][Tf$_2$N], even when a small amount of water (0.5% v/v) was added. Although the functionalized ILs [C$_2$OC$_1$, mim][Tf$_2$N] and [C$_2$OHmim][Tf$_2$N], which have hydrogen bonding ability, were expected to solubilize enzymes, native lipase was not dissolved in either of these ILs. In contrast, whereas, the PM$_{13}$–CRL conjugate did not dissolve entirely in dry [Emim][Tf$_2$N] (containing ca. 500 ppm of water), resulting in a slightly cloudy solution and it was clearly solubilized in all the ILs tested when they contained a small amount of water (0.5% v/v), up to a concentration of 85 mg/ml of PM$_{13}$–CRL (containing 2 mg/ml of original lipase) (Fig. 2). Solubilization of the enzyme in these ILs is attributed to the high density of PEG chains that cover the protein surface, although we could not estimate the quantity of PEG chain attached to the enzymes since PM$_{13}$ has several reactive sites and can connect with proteins at more than one point. Based on this result, a very small amount of water was essential for solubilizing lipase, in contrast to our previous report in which subtilisin was solubilized in dry ILs [25].

3.3. Enzymatic reaction in ILs

We succeeded in solubilizing lipase in ILs. However, solubilized enzymes do not always possess satisfactory activity. Several groups have reported that enzymes dissolved in ILs lose significant amounts of their original activities [9,21]. It seems likely that ILs that can solubilize enzymes also interact with protein molecules and cause structural changes, resulting in inactivation of enzymes. Therefore, the enzymatic activity of PM$_{13}$–CRL solubilized in [Emim][Tf$_2$N] was studied using the transesterification of 2-phenyl-1-propanol with vinyl acetate.

Lipases have been widely studied in enzymatic reactions in non-aqueous media, owing to their comparatively high tolerance to organic solvents and high catalytic activity. In IL, however, native CRL showed negligible activity as shown in Fig. 3. This result is in agreement with those from previous reports [11,12]. This loss of activity would be caused by the insolubility of the biocatalyst or by salt-induced inactivation of the lipase. On the other hand, the PM$_{13}$–CRL conjugate exhibited remarkably high catalytic performance in [Emim][Tf$_2$N]. It should be pointed out...
that the dissolved enzyme maintained its catalytic activity in spite of complete solubilization, which is contradictory to what has been reported previously. A large quantity of PEG chains covering the enzyme surface could afford enzyme dispersibility in the reaction media and protect the enzyme from the IL. Hydrophilic PEG chains might also contribute to the preservation of essential water, which is required for retention of the catalytically active conformation. It was found that the modification with PM13 is effective not only for solubilization but also for activation of the enzyme in ILs.

As described above, the solubility of PM13–CRL was significantly dependent on the water content in ILs and the addition of water to at least 0.5% v/v was required to entirely solubilize PM13–CRL in the ILs. The water content of the reaction media also plays an important role in enzyme catalysis because it affects the flexibility of proteins, which is responsible for enzymatic activity. We investigated the influence of water content on the catalytic activity of the PM13–CRL conjugate in [Emim][Tf2N] (Fig. 4). In the absence of added water (<0.05 wt% water), PM13–CRL was insoluble and showed little activity in the ILs. The activity gradually increased on addition of water, up to 2% v/v of water. Above a water content of 1.5% v/v, the reaction mixture formed two phases due to the oversaturation of water in the IL (see Table 1). At a water content above 2% the activity declined. This result can be explained as follows: Until the optimum water content, water addition enhances the flexibility of the enzyme, which has a positive effect on its catalytic activity, leading to an increase in the reaction rate. However, excessive water addition would be accompanied by a further increase in the enzyme flexibility, resulting in a conformational change that reduces catalytic activity [31]. At a high water content, competing hydrolysis of the substrate might also occur. In the present work, we used ILs containing 1.5% v/v of water to investigate the enzyme performance in homogeneous reaction.

A number of cases of improved thermal stability of enzymes in non-aqueous media have been reported. The effect of the reaction temperature on the catalytic activity of PM13–CRL in [Emim][Tf2N] was examined (Fig. 5). The highest activity was observed at 40 °C, which is in good agreement with the optimum temperature in water. In anhydrous organic solvents, enzymes are extremely thermostable due to their conformational rigidity in the dehydrated state. Further improvement of thermostability was not observed in the IL because PM13–CRL was sufficiently flexible to allow inactivation in the IL at a high temperature.

3.4. Effect of solvents on catalytic activity

Enzymatic activity in non-aqueous media is significantly influenced by the properties of solvents, such as hydrophobicity, dielectric constant and binding ability. In this study we prepared three ILs containing the Tf2N anion with different side chains in the cation, [Emim][Tf2N], [C2OC1mim][Tf2N] and [C2OHmim][Tf2N]. Therefore, transesterification activity of PM13–CRL was examined in
the three different ILs, and compared to that in organic solvents commonly used in non-aqueous enzymatic reactions. We selected hexane, toluene, methyl tert-butyl ether (MTBE), tetrahydrofuran (THF), dimethylsulfoxide (DMSO) and acetonitrile as organic solvents. PM13-CRL was solubilized in all the ILs and the organic solvents, except for hexane and MTBE, where the reaction proceeded heterogeneously.

Transesterification rates in the ILs and in the organic solvents are summarized in Fig. 6. The enzyme activities were quite low in the hydrophilic solvents such as THF, DMSO and acetonitrile due to the stripping of essential water from the protein to the bulk solvent [32,33]. The hydrophobic organic solvents including hexane, toluene and MTBE appeared to be somewhat preferable for PM13-CRL. These solvents hardly strip any essential water from the protein molecule, resulting in adequate activity. In contrast, markedly higher activity was observed in all the ILs than in the conventional organic solvents. The excellent activity in the ILs could be partly ascribed to the high polarity and the hydrophobicity of the ILs. As shown in Table 1, the polarity of the ILs was much higher than that of organic solvents. Since the transition state of the intermediate complex in this reaction is supposed to be relatively polar, it would be stabilized more effectively in such highly polar ILs, leading to the acceleration of the reaction. A highly polar organic solvent, however, often causes protein denaturation as observed in DMSO and acetonitrile. The ILs, however, are hydrophobic and strip hardly any water molecules bound to protein. Possession of these two opposing properties, high polarity and hydrophobicity, is not possible in ordinary organic solvents but is possible in ILs whose physicochemical properties are tunable by suitable design of the IL structure. It was demonstrated that ILs are desirable media for enzyme-catalyzed reactions. Although the details of the high catalytic efficiency in ILs have not yet been clarified, further investigations including kinetics studies and spectroscopic analysis are now underway, to elucidate the mechanism of enzyme activation in ILs. The possibility that the imidazolium cation in ILs might directly stabilize charged groups of the active site in a protein is also interesting.

In the three different ILs, the activity of PM13-CRL was increased with increasing hydrophobicity of the ILs. This tendency would be associated with the stripping of essential water. That is, the partitioning of essential water from the enzyme surface into ILs would occur more easily in hydrophilic ILs than in hydrophobic ones. Consequently, PM13-CRL exhibited the highest activity in the most hydrophobic IL, [Emim][Tf2N]. This is consistent with the enzymatic reaction in conventional organic solvents. Moreover, the enzyme might be directly damaged by coordinating ILs, [C2OC1mim][Tf2N] and [C2OHmim][Tf2N], which still retain abundant hydrogen bonding ability or nucleophilicity.

3.5. Stability of enzyme in ILs

In practical utilization of ILs as reaction media for biotransformation, the stability of biocatalysts in ILs is crucial. Some groups reported increased enzyme stability in ILs as compared with that in organic media [9,16] and further enhancement by addition of a substrate [16]. The stability of enzymes depended strikingly on the species of enzymes and ILs used. It was also reported that native lipase and α-chymotrypsin were deactivated within a few hours. Here, we examined the stability of PM13-CRL solubilized in [Emim][Tf2N]. PM13-CRL was incubated in [Emim][Tf2N] at 20 °C, and portions of the solution were removed periodically to perform the reaction with a substrate solution. It is apparent from Fig. 7 that PM13-CRL is exceedingly stable in the IL, maintaining its original activity after 144 h (six days). Although we could not make a comparison between the stability of PM13-CRL and that of native CRL because the native CRL showed no activity in the IL, the enzyme stability must be improved by modification of PM13. Substantial coverage of the protein surface by PEG chains could play an important role in the stabilization of enzyme.

![Graph](image1)

**Fig. 6.** Transesterification efficiency of PM13-CRL in ILs and in organic solvents. Each IL contained 1.5% (v/v) water. “The reaction was performed heterogeneously.

![Graph](image2)

**Fig. 7.** Stability of PM13-CRL in [Emim][Tf2N] at 20 °C.
4. Conclusions

We have demonstrated solubilization of an enzyme in a variety of ILs by chemical modification of the enzyme with comb-shaped PEG, PM\textsubscript{13}. The PM\textsubscript{13}-modified lipase, PM\textsubscript{13}–CRL, dissolved in ILs exhibited remarkably high activity in all the ILs, whereas unmodified native lipase showed little activity. Comb-shaped PM\textsubscript{13} was found to be a promising modifier. Higher activity of the PM\textsubscript{13}–CRL conjugate was observed in the more hydrophobic IL, and the activities in these ILs were all much greater than those in conventional organic solvents. Moreover, PM\textsubscript{13}–CRL showed high storage stability in ILs for a long period. Since ILs can dissolve various organic compounds, they can be regarded as “non-volatile organic solvents”. To date, many studies have been conducted on the utilization of ILs as alternative solvents to conventional organic solvents in a wide range of areas. These results are greatly beneficial for the use of ILs as novel reaction media in non-aqueous enzymatic reactions. Further studies to improve enzyme activity in ILs are currently underway in our laboratory.

Furthermore, as enzymes can be solubilized homogeneously in ILs, it is possible to analyze the microscopic protein structure and evaluate the catalytic activity of enzymes in ILs by spectroscopic methods. We hope that the methodology demonstrated in the present study will make a substantial contribution for further development in the new field of ILs combined with versatile biomaterials.

Acknowledgments

This work is supported by a Grant-in-Aid for the 21st Century COE Program, “Functional Innovation of Molecular Informatics” from the Ministry of Education, Culture, Sports, Science and Technology of Japan. K.N. was supported by Research Fellowships from the Japan Society for the Promotion of Science (JSPS) for Young Scientists. The authors would like to thank Prof. Atsushi Maruyama (Kyushu University) for his helpful discussions.

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