

# Homogeneous enzymatic reactions in ionic liquids with poly(ethylene glycol)-modified subtilisin

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Subtilisin Carlsberg was covalently modified with comb-shaped poly(ethylene glycol) (PM<sub>13</sub>). PM<sub>13</sub>-modified subtilisin (PM<sub>13</sub>-Sub) was readily solubilized in three different ionic liquids (ILs), *i.e.*, [Emim][Tf<sub>2</sub>N], [C<sub>2</sub>OC<sub>1</sub>mim][Tf<sub>2</sub>N] and [C<sub>2</sub>OHmim][Tf<sub>2</sub>N]. Analysis of homogeneous enzymatic reactions in the ILs revealed that PM<sub>13</sub>-Sub exhibited excellent catalytic performance while the native enzyme suspended in ILs showed no activity. Hydrophobicity of ILs slightly affected enzyme activity, and the relatively hydrophobic IL [Emim][Tf<sub>2</sub>N] was the preferred medium for enzymatic reactions, similar to enzymatic reactions in conventional organic solvents. Enzyme activity was much higher in [Emim][Tf<sub>2</sub>N] than in conventional organic solvents, and excellent activity was associated with unique properties of ILs such as hydrophobicity and high polarity. Furthermore, PM<sub>13</sub>-Sub showed good stability in [Emim][Tf<sub>2</sub>N], and maintained 80% of its initial activity after 60 h.

## Introduction

Ionic liquids (ILs) are novel and functional solvents with unique properties such as negligible volatility, thermal stability, and relatively high polarity.<sup>1–3</sup> One of the most attractive features of ILs is their solvent property which can be controlled by changing combinations of cations and anions or by appending functional groups to constituent ions.<sup>4,5</sup> Thus, a “tailor-made solvent” can be chosen for a specific reaction. For example, ILs composed of hydrophobic ions and that are immiscible with water would offer a dual nature, *i.e.*, polarity and hydrophobicity.<sup>6,7</sup>

Some researchers have recently become interested in using ILs as non-aqueous media for biotransformation.<sup>8–10</sup> Using hydrophobic ILs as reaction media in biocatalysis often enhances the efficiency of synthetic reactions by suppressing hydrolytic side reactions. Moreover, ILs can dissolve a variety of polar compounds which are not usually dissolved in non-polar organic solvents. Following the pioneering studies by some groups,<sup>11–13</sup> many works have been actively conducted on biocatalytic reactions in ILs with lipases,<sup>14–17</sup> proteases,<sup>18–20</sup> oxidoreductases,<sup>21–23</sup> and whole-cell biocatalysts.<sup>24,25</sup> Compared to conventional organic solvents, ILs provide a comfortable environment for enzymes,<sup>19,26,27</sup> and some ILs are less harmful to cell membranes,<sup>25</sup> resulting in high biocatalytic performance. The possible use of ILs as novel reaction media for biocatalysis has been demonstrated over the past 5 years, showing relatively high enzyme activity and good regio- and enantioselectivity.<sup>16,28,29</sup>

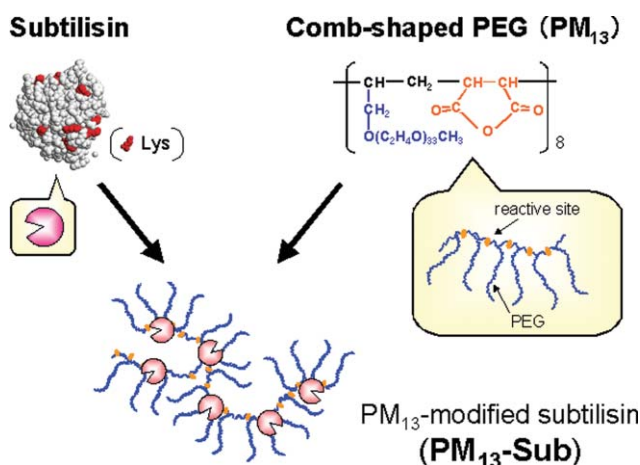
Since enzymes are biomacromolecules composed of polypeptide chains with a hydrophilic nature, native enzymes are usually insoluble in ILs. Therefore, enzymatic reactions in ILs are basically limited to the use of suspended enzymes. Because catalytic activity

of suspended enzymes in ILs is usually modest, several attempts have been made to enhance activity of enzymes dispersed in ILs, including addition of water to ILs<sup>18,20</sup> or immobilization of the enzyme on a solid support.<sup>30</sup> When considering the industrial use of enzymes as heterogeneous catalysts in ILs, low solubility of enzymes is not so serious. However, insolubility of enzymes limits further applications for ILs. Development of universal strategies for solubilization of biocatalysts in ILs would break new ground.

There are at least two strategies for solubilizing enzymes in ILs. One involves the introduction of functional groups into ILs that show high affinity for protein molecules such as hydroxyl, ether, and amide groups. Some ILs are known to solubilize enzymes through hydrogen bonding interactions; however, these ILs often induce conformational changes in proteins, resulting in inactivation of enzymes.<sup>31,32</sup> Another strategy for solubilization of enzymes is to modify them with a compound which has an affinity to ILs, and shows good solubility in ILs. Crown ether and poly(ethylene glycol) (PEG), both of which are composed of ethylene glycol units, dissolve well in ILs. By using the inherent properties of this family of compounds, we successfully demonstrated activation of lipases in ILs by physical complexation with PEG,<sup>33</sup> and quantitative extraction of cytochrome c from an aqueous phase into ILs using crown ether.<sup>34</sup> These results prompted us to conduct chemical modification of enzymes with PEG for solubilization in ILs. However, modification with only a few PEG chains was insufficient to achieve high solubility, and to activate enzymes. Most recently, we succeeded in solubilizing subtilisin Carlsberg in ILs by chemical modification with comb-shaped PEG (PM<sub>13</sub>), which is a promising modifier for introducing a large number of PEG chains into a protein molecule.<sup>35</sup>

In the present study, subtilisin was modified with comb-shaped PM<sub>13</sub> (Fig. 1), and its catalytic behavior in ILs was investigated. We discussed effects of molecular structures of PEG derivatives on catalytic activity, and investigated correlations between enzyme activities and solvent properties of reaction media.

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**Fig. 1** Schematic illustration of modification of subtilisin with comb-shaped PEG (PM<sub>13</sub>).

## Results and discussion

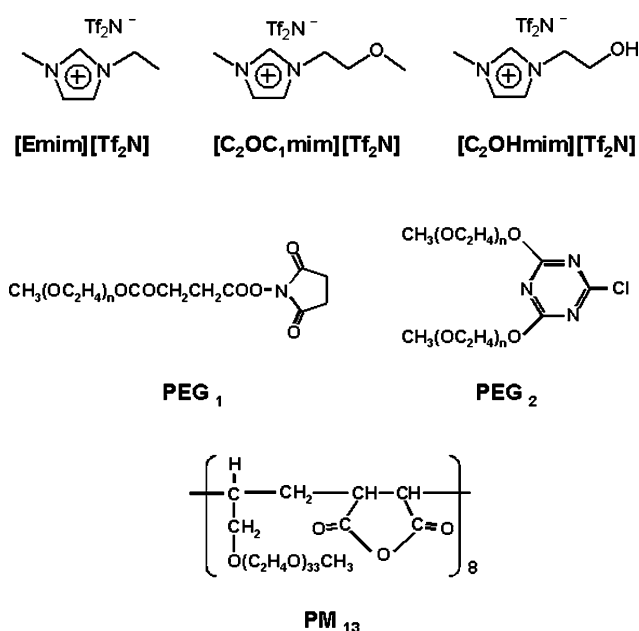
### Characterization and solubility of PM<sub>13</sub>-modified subtilisin

Proteins dissolve in aqueous solutions by significant contributions from hydrogen bonding of water molecules. However, in ILs, there are little interactions between protein molecules and IL molecules, resulting in extremely low solubility of enzymes. Alterations of chemical structures of ILs would be effective for solubilizing enzymes; however, inactivation of enzymes due to denaturation of proteins by surrounding ionic solvents has been reported.<sup>31,32</sup>

Alternatively, modification of enzymes with PEG is a rational strategy for solubilization of enzymes in ILs because PEG dissolves well in ILs. Some researchers attempted to improve catalytic performance of enzymes by covalent modification with PEG;<sup>17,36</sup> however, chemical modification with single-chained PEG did not result in sufficient solubility and activity of enzymes. We assume that this was due to insufficient numbers of PEG chains per enzyme. To test this hypothesis, we used a highly-branched PEG derivative, called comb-shaped PEG, as the enzyme modifier to introduce sufficient quantities of PEG chains onto the surface of a model enzyme, subtilisin Carlsberg.

Comb-shaped PEG, PM<sub>13</sub>, is a branched PEG derivative, and has multivalent reactive sites (carboxylic acid anhydride) in its backbone which can react with amino groups in a protein molecule (Fig. 2). Degree of modification of amino groups in the protein molecule and protein content of PM<sub>13</sub>-Sub were determined by the TNBS method and the BCA assay, respectively. Based on these examinations, we found that about 50% of amino groups in subtilisin were modified with carboxyl groups of PM<sub>13</sub>, and that the resultant PM<sub>13</sub>-Sub conjugate contained 5 wt% of subtilisin.

Since modification of enzymes often induces loss of activity, we first examined enzyme activity of PM<sub>13</sub>-Sub by hydrolysis of *p*-nitrophenyl butyrate in water. The specific activity of PM<sub>13</sub>-Sub was comparable to that of native subtilisin, suggesting that decline of enzyme activity during the modification process was negligible. Secondly, we investigated the size of PM<sub>13</sub>-Sub using size-exclusion chromatography equipped with multi-angle light scattering (SEC-MALS). Since PM<sub>13</sub> has multivalent reactive sites for protein molecules, it is possible to crosslink enzymes *via* PM<sub>13</sub> as a crosslinker. The SEC-MALS measurements revealed that PM<sub>13</sub>-

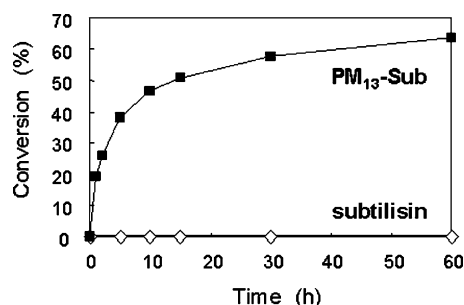


**Fig. 2** Chemical structures and abbreviations of ILs and PEG derivatives.

Sub was a highly crosslinked enzyme with a molecular weight ranging from 20 000 to around 1 000 000 Da. Thirdly, we examined solubility of subtilisin in ILs. As expected, native subtilisin did not dissolve in the typical IL [Emim][Tf<sub>2</sub>N]. Functionalized ILs [C<sub>2</sub>OC<sub>1</sub>mim][Tf<sub>2</sub>N] and [C<sub>2</sub>OHmim][Tf<sub>2</sub>N], which have hydrogen bonding abilities are expected to solubilize enzymes. Nevertheless, native subtilisin did not dissolve in either of these ILs. In contrast, PM<sub>13</sub>-Sub was readily solubilized in all ILs tested to the concentration of at least 40 mg mL<sup>-1</sup> (containing 2 mg mL<sup>-1</sup> of subtilisin). It is noteworthy that PM<sub>13</sub>-Sub was dissolved in pure ILs with no addition of water. Solubilization of the enzyme in these ILs is attributed to the high density of PEG chains on the protein surface, although we could not estimate the exact number of PEG chains attached per enzyme.

### Enzymatic reactions in ILs

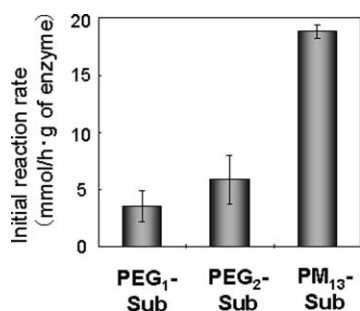
Several groups have reported that enzymes dissolved in ILs significantly lose their original activities.<sup>12,31,37</sup> It is likely that ILs that can solubilize enzymes also interact with protein molecules, and cause structural changes, resulting in inactivation of enzymes. Although we succeeded in solubilization of subtilisin in ILs, the solubilized enzyme may not exhibit maximal activity. Enzyme activity of PM<sub>13</sub>-Sub solubilized in [Emim][Tf<sub>2</sub>N] was thus investigated by transesterification of *N*-acetyl-L-phenylalanine ethyl ester with 1-butanol as a model reaction. Subtilisin Carlsberg has been widely studied in enzymatic reactions in non-aqueous media owing to its comparatively high tolerance for organic solvents and its high catalytic activity.<sup>38,39</sup> However, in ILs, native subtilisin showed no activity (Fig. 3). On the other hand, PM<sub>13</sub>-Sub exhibited remarkably high catalytic performance in ILs. It is worth mentioning that PM<sub>13</sub>-Sub allowed homogeneous enzymatic reactions in ILs. A large quantity of PEG chains covering an enzyme surface could enhance solubility of the enzyme, and protect the enzyme in ILs. Hydrophilic PEG chains may also contribute to retention



**Fig. 3** Time-courses of transesterifications of *N*-Ac-L-Phe-OEt with 1-butanol catalyzed by native subtilisin and PM<sub>13</sub>-Sub in [Emim][Tf<sub>2</sub>N]. Reaction conditions: *N*-Ac-L-Phe-OEt (100 mM) and 1-butanol (500 mM), and subtilisin or PM<sub>13</sub>-Sub were stirred in 1 mL of [Emim][Tf<sub>2</sub>N] at 40 °C. Each reaction was performed under these conditions unless stated otherwise.

of essential water, which is required for enzyme activity.<sup>40</sup> We found that modification with PM<sub>13</sub> was quite effective not only for solubilization, but also for enhancing enzyme activity in ILs.

Next, we examined the effects of the molecular structure of a modifier on enzyme activity in ILs using three PEG derivatives, *i.e.*, single-chained PEG<sub>1</sub>, double-chained PEG<sub>2</sub> and comb-shaped PM<sub>13</sub> (Fig. 2). In terms of solubility, while PEG<sub>1</sub>-Sub was insoluble in [Emim][Tf<sub>2</sub>N], PEG<sub>2</sub>-Sub was solubilized in [Emim][Tf<sub>2</sub>N] as was observed with PM<sub>13</sub>-Sub. Amounts of PEG chains introduced into the enzymes were almost equal in PEG<sub>1</sub>-Sub and PEG<sub>2</sub>-Sub (about 4 to 5 chains). This indicated that solubility of the modified enzymes might be affected by the shape of PEG rather than by the amount of PEG attached to the enzyme. With regard to enzyme activity, the higher enzyme activity was obtained with PM<sub>13</sub>-Sub although PEG<sub>2</sub>-Sub was also solubilized in [Emim][Tf<sub>2</sub>N] (Fig. 4). We suppose that coverage of the protein surface with abundant PEG chains is necessary for achievement of high enzyme activity in ILs.



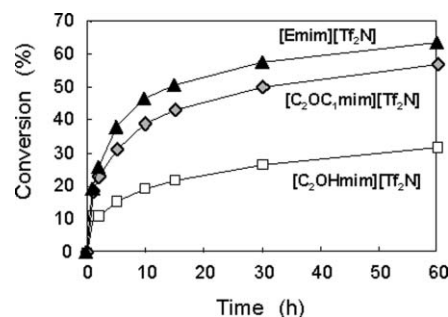
**Fig. 4** Effects of molecular structures of PEG derivatives on catalytic activity with modified subtilisin.

#### Effects of solvents on catalytic activity

We prepared three different ILs with the same Tf<sub>2</sub>N<sup>-</sup> anion, *i.e.*, [Emim][Tf<sub>2</sub>N], [C<sub>2</sub>OC<sub>1</sub>mim][Tf<sub>2</sub>N], and [C<sub>2</sub>OHmim][Tf<sub>2</sub>N] (Fig. 2), all of which were water-immiscible. Table 1 shows the water content of these ILs in dried and water-saturated conditions. [Emim][Tf<sub>2</sub>N] is the most hydrophobic IL among the three types of ILs. Transesterification activity of PM<sub>13</sub>-Sub was examined in the three ILs in the absence of water (*i.e.* dry ILs). Fig. 5 depicts

**Table 1** Water content of ILs in dried and water saturated conditions (20 °C)

	Water content (ppm)	
	Dried	Water saturated
[Emim][Tf <sub>2</sub> N]	495	18 200
[C <sub>2</sub> OC <sub>1</sub> mim][Tf <sub>2</sub> N]	830	24 700
[C <sub>2</sub> OHmim][Tf <sub>2</sub> N]	1640	84 500



**Fig. 5** Transesterification activity of PM<sub>13</sub>-Sub in three ILs with different structures of the imidazolium cation.

time-courses of transesterification catalyzed by PM<sub>13</sub>-Sub in the three ILs. As the ILs became more hydrophilic, activity of PM<sub>13</sub>-Sub decreased. This tendency might be associated with stripping of essential water from the protein microenvironment to the bulk solvent, leading to protein denaturation. Since all reactions were performed in dry ILs, water molecules adsorbed to the protein surface would partition more easily into hydrophilic ILs than into hydrophobic ones. PM<sub>13</sub>-Sub would show a lower activity in the more hydrophilic IL [C<sub>2</sub>OHmim][Tf<sub>2</sub>N]. This tendency was consistent with enzymatic reactions in organic media. In addition, [C<sub>2</sub>OC<sub>1</sub>mim][Tf<sub>2</sub>N] and [C<sub>2</sub>OHmim][Tf<sub>2</sub>N], which possess coordinating and hydrogen bonding abilities, may have directly affected enzymes. The results suggested that hydrophobic IL appeared to be suitable for homogeneous enzymatic reaction in ILs using PEG-modified enzymes. Thus [Emim][Tf<sub>2</sub>N] was used in the following experiments.

We compared enzyme activities in ILs with those in organic solvents commonly used in non-aqueous enzymatic reactions. We selected toluene, dichloromethane, methyl *tert*-butyl ether (MTBE), tetrahydrofuran (THF), dimethylsulfoxide (DMSO), and acetonitrile as organic solvents. Since PM<sub>13</sub>-Sub did not show sufficient solubility in MTBE, the reaction was conducted heterogeneously in MTBE.

Transesterification efficiencies in [Emim][Tf<sub>2</sub>N] and organic solvents, and polarities of reaction media are shown in Table 2. Markedly high activity was observed in [Emim][Tf<sub>2</sub>N], while no enzyme activity was shown in hydrophilic organic solvents such as THF, DMSO, and acetonitrile. Toluene seemed to be a relatively suitable medium for PM<sub>13</sub>-Sub. Absence of activity in MTBE might be due to insolubility of PM<sub>13</sub>-Sub in the reaction medium. We hypothesized that the excellent catalytic activity in [Emim][Tf<sub>2</sub>N] was ascribed to the high polarity and the hydrophobicity of the IL. Based on solvatochromism of Reichardt's dye, polarity of [Emim][Tf<sub>2</sub>N] was higher than that of the organic solvents tested, and was very close to those of short-chain alcohols such as ethanol. The transition state of this

**Table 2** Reichardt's dye polarity ( $E^N_T$ ) values for ILs and organic solvents, and transesterification efficiency of PM<sub>13</sub>-Sub in these media

	Polarity <sup>a</sup> / $E^N_T$	Initial reaction rate [mmol/(h·g of enzyme)]
[Emim][Tf <sub>2</sub> N]	0.66	18.8
Acetonitrile	0.46	0
DMSO	0.44	0
Dichloromethane	0.31	0
THF	0.21	0
MTBE <sup>b</sup>	0.12	0
Toluene	0.10	5.6

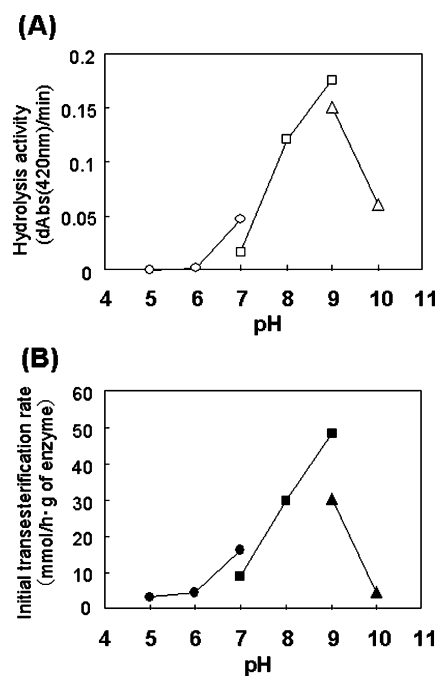
<sup>a</sup> Measurements of  $E^N_T$  values for ILs were conducted according to the literature.<sup>16</sup> The values for organic solvents were taken from a recent review.<sup>44</sup> <sup>b</sup> The reaction mixture was heterogeneous.

transesterification reaction is supposed to be relatively polar,<sup>39</sup> and it would be stabilized more effectively in a highly polar environment provided by the IL, leading to acceleration of the reaction. However, polar organic solvents often cause protein denaturation by stripping essential water as observed in DMSO and acetonitrile. On the other hand, [Emim][Tf<sub>2</sub>N] is highly polar, yet hydrophobic, and thus would strip hardly any water molecules bound to proteins, resulting in retention of catalytically active conformations of enzymes. Possession of these two opposite properties (*i.e.*, high polarity and hydrophobicity) cannot be attained in conventional organic solvents, but is possible in ILs whose physico-chemical properties can be controlled by changing molecular structures of ILs. The present study also demonstrated that ILs had great potential as reaction media for enzyme-catalyzed reactions. Details of high catalytic efficiency in ILs have not yet been clarified. Further investigations including kinetics studies and spectroscopic analyses are required to elucidate the mechanisms of enzymatic activation in ILs.

### pH Memory and stability of subtilisin in ILs

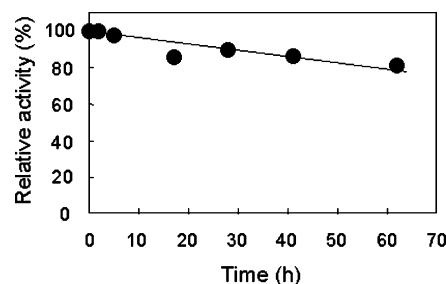
In organic solvents, catalytic activity of enzymes depends on the pH of the last aqueous solution to which the enzyme was exposed because ionizable groups in the enzyme acquire corresponding ionization states, which remain in organic solvents. This phenomenon is known as "pH memory".<sup>41,42</sup> The pH memory of enzymes in ILs is of great interest, although it has been scarcely discussed. Therefore, we examined influence of pH on catalytic activities of subtilisin in water, and PM<sub>13</sub>-Sub in [Emim][Tf<sub>2</sub>N] (Fig. 6). The maximum activity of native subtilisin around pH 8 in water coincided with that in the literature.<sup>42</sup> In [Emim][Tf<sub>2</sub>N], the catalytic activity of PM<sub>13</sub>-Sub was remarkably influenced by the pH of the aqueous solution from which the enzyme was prepared. The highest activity was found at the same pH in the case of the aqueous system. These results suggested that pH memory was also evident in ILs as well as in organic solvents.

For practical use of ILs as reaction media for biotransformations, long-term stability of a biocatalyst in ILs is desirable. Some groups demonstrated increased stability of enzymes in ILs compared to organic media, and further enhancement of stability by addition of a substrate.<sup>19,26</sup> Nonetheless, native enzymes were inactivated within a few hours in ILs. Here, we studied the stability of PM<sub>13</sub>-Sub solubilized in [Emim][Tf<sub>2</sub>N]. We found that PM<sub>13</sub>-Sub showed good stability for a long period in [Emim][Tf<sub>2</sub>N], and



**Fig. 6** Effects of pH on enzyme activity in water and in IL: (A) hydrolysis of *p*-nitrophenyl butyrate in water with different pHs catalyzed by native subtilisin (B) transesterification of *N*-Ac-L-Phe-OEt with 1-butanol in [Emim][Tf<sub>2</sub>N] catalyzed by PM<sub>13</sub>-Sub prepared from aqueous solutions with different pHs. Buffer solutions: 10 mM phosphate (circles), 10 mM tris-HCl (squares), and 10 mM borate (triangles).

maintained 80% of its initial activity after 60 h (Fig. 7). The half-life time ( $t_{1/2}$ ) of PM<sub>13</sub>-Sub in [Emim][Tf<sub>2</sub>N] was about 194 h, which was much higher than those of native enzymes in [Emim][Tf<sub>2</sub>N] without corresponding substrates ( $\alpha$ -chymotrypsin:  $t_{1/2}$  = 1.08 h,<sup>19</sup> *C. antarctica* lipase B:  $t_{1/2}$  = 3.7 h<sup>26</sup>). Enzymes sometimes exhibit improved thermal and chemical stabilities by immobilization or crosslinking. Subtilisin was assumed to acquire improved stability by crosslinking *via* comb-shaped PM<sub>13</sub>. In addition, substantial coverage of the protein surface by PEG chains may play an important role in stabilization.



**Fig. 7** Stability of PM<sub>13</sub>-Sub in [Emim][Tf<sub>2</sub>N] at 25 °C.

### Conclusions

We demonstrated solubilization of subtilisin in three types of ILs in the absence of water by chemical modification with a comb-shaped PEG, PM<sub>13</sub>. The PM<sub>13</sub>-modified subtilisin, PM<sub>13</sub>-Sub, solubilized in [Emim][Tf<sub>2</sub>N], exhibited remarkably high activity and good stability in the IL. A higher activity of PM<sub>13</sub>-Sub was observed



in a more hydrophobic IL, and this activity was much greater than those seen in conventional organic solvents. Since enzymes are homogeneously dissolved in ILs in the present system, it is possible to analyze microscopic protein structures and to evaluate catalytic activities of enzymes in ILs by spectroscopic methods. The solubilization strategy demonstrated in this work could be applied to other enzymes.

## Experimental

### Materials

Subtilisin Carlsberg was purchased from Sigma (St. Louis, MO), and was used without further purification. Comb-shaped PEG (PM<sub>13</sub>; SUNBRIGHT AM-1510 K, Mw: 15 000 to 20 000) and *o*-methoxypoly(ethylene glycol)-succinate *N*-succinimidyl ester (PEG<sub>1</sub>; SUNBRIGHT ME-050CS, Mw: 5000) were obtained from NOF Co. (Tokyo, Japan); and 2,4-bis(*o*-methoxypolyethylene glycol)-6-chloro-*s*-triazine (PEG<sub>2</sub>, Mw: 10,000) was from Seikagaku Co. (Tokyo, Japan). Ionic liquids 1-ethyl-3-methylimidazolium bis(trifluoromethanesulfonyl)imide ([Emim][Tf<sub>2</sub>N]), and its ether ([C<sub>2</sub>OC<sub>1</sub>mim][Tf<sub>2</sub>N]) and hydroxyl ([C<sub>2</sub>OHmim][Tf<sub>2</sub>N]) analogues were prepared as previously described,<sup>6,43</sup> and were dried for 48 h under vacuum. Molecular structures and abbreviations of PEG derivatives and ILs used in this study are shown in Fig. 2. All other chemicals were of reagent grade.

### Water content and polarity evaluation of ILs

Each dried IL was diluted with anhydrous methanol to reduce viscosity, then its water content was measured using a Karl Fischer moisture titrator (Mitubishi, CA-07 Moisturemeter). Water contents of the dry ILs were calculated by subtracting that of anhydrous methanol. Those of water saturated ILs were also measured in the same manner after equilibration of ILs with water.

Polarity of ILs was evaluated by solvatochromism of Reichardt's dye.<sup>44</sup> Reichardt's dye (2,6-diphenyl-4-(2,4,6-triphenylpyridinio)phenolate, 0.8 mg) was dissolved in each IL (1 mL), and was centrifuged to remove insoluble particles. Wavelength of the absorption maximum was measured using a UV-vis spectrophotometer (JASCO V-570) at 25 °C, and was converted to normalized polarity scales ( $E_T^N$ ) ranging from 0.0 for tetramethylsilane to 1.0 for water, using the following equations.

$$E_T(\text{solvent}) [\text{kcal/mol}] = \frac{28591}{\lambda_{\text{max}} [\text{nm}]}$$

$$E_T^N(\text{solvent}) = \frac{E_T(\text{solvent}) - 30.7}{32.4}$$

### Modification of subtilisin with PEG derivatives

Modification of subtilisin with comb-shaped PEG (PM<sub>13</sub>) was performed according to Inada *et al.*<sup>45</sup> To a subtilisin solution (2 mg mL<sup>-1</sup>, 4 mL) in 0.1 M sodium borate buffer (pH 8.5), crystalline PM<sub>13</sub> (200 mg) was added, followed by stirring at 4 °C in an ice-bath for 1 h. The reaction mixture was filtered using an Ultra-4 membrane (MWCO: 30 000, Millipore), and was washed three times with deionized water to remove unreacted PM<sub>13</sub>. Following lyophilization for 48 h, PM<sub>13</sub>-modified subtilisin

(PM<sub>13</sub>-Sub) was obtained. Degree of modification of amino groups in a subtilisin molecule was determined using 2,4,6-trinitrobenzenesulfonic acid (TNBS),<sup>46</sup> and protein content of PM<sub>13</sub>-Sub was estimated by the bicinchoninic acid (BCA) assay.<sup>47</sup> Subtilisin modified with two different PEG derivatives, *i.e.*, single-chained PEG<sub>1</sub> and double-chained PEG<sub>2</sub>, was prepared in a similar manner to PM<sub>13</sub>-Sub except for reaction temperature (25 °C) for PEG<sub>1</sub> modification.

### Size-exclusion chromatography with multi-angle light scattering (SEC-MALS)

To investigate the molecular weight of PM<sub>13</sub>-Sub, we used the SEC-MALS system. Weight-averaged molecular weight ( $M_w$ ) can be determined by light scattering responses of eluted compounds in various fractions separated by SEC. The SEC-MALS analysis was performed using the JASCO HPLC system equipped with a multi-angle light-scattering detector (DAWN EOS, Wyatt Technology Co.), an RI detector (RI-2031 Plus, JASCO) and a UV detector (UV-970, JASCO). A Superdex 200 10/300 GL column (Amersham Biosciences) was used for SEC separation with 10 mM sodium phosphate buffer containing 0.1 M NaCl as eluent, at a flow rate of 0.8 mL min<sup>-1</sup>. PM<sub>13</sub>-Sub was dissolved in the elution buffer, and was injected into HPLC for analysis.

### Preparation of the pH-adjusted enzyme

PM<sub>13</sub>-Sub (70 mg) was dissolved in deionized water (10 mL), and was dialyzed twice against 2 L 10 mM phosphate buffer (pH 7.0), followed by lyophilization for 48 h. A 15 mg portion of the dialyzed PM<sub>13</sub>-Sub was dissolved again in 2 mL of each buffer solution (phosphate, Tris-HCl or borate buffer) with different pHs, and then lyophilized for 48 h to obtain pH-adjusted PM<sub>13</sub>-Sub.

### Enzyme activity assay in water, organic solvents, and ILs

The catalytic activity of subtilisin in water was examined by hydrolysis of *p*-nitrophenyl butyrate in each buffer at 37 °C, monitoring *p*-nitrophenol liberated during hydrolysis by subtilisin with a UV-vis spectrophotometer at 420 nm. In ILs and organic solvents, enzyme activities were evaluated in transesterification of 100 mM *N*-acetyl-L-phenylalanine ethylester (*N*-Ac-L-Phe-OEt) with 500 mM 1-butanol by 1 mg mL<sup>-1</sup> of enzyme at 40 °C. The reaction was started by adding the enzyme into the reaction media containing the substrates. Samples were periodically removed from reaction mixtures, diluted 10-fold with acetonitrile, and injected into a HPLC system (LC-10AT VP, Shimadzu) equipped with an Inertsil ODS-3 column (4 × 250 mm, GL Science). A solution of acetonitrile–5% acetic acid (80 : 20 v/v) was used as eluent, at a flow rate of 1.0 mL min<sup>-1</sup>. Eluted compounds were monitored by a UV-vis detector (SPD-M10A VP) at 257 nm.

In the enzyme stability test, PM<sub>13</sub>-Sub was solubilized in [Emim][Tf<sub>2</sub>N], and was settled at 25 °C. A portion of the solution was periodically taken out, and was mixed with the IL containing substrates to initiate the reaction. The 100% activity corresponded to initial activity at 0 h.

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