Comb-shaped poly(ethylene glycol)-modified subtilisin Carlsberg is soluble and highly active in ionic liquids

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Subtilisin Carlsberg conjugated with comb-shaped poly(ethylene glycol) was solubilized in common ionic liquids without adding water, and exhibited higher transesterification activity in an ionic liquid, [Emim][Tf_2N], than in organic solvents commonly used for enzymatic biotransformation.

Room temperature ionic liquids (ILs) have received considerable attention as a new class of solvents for many applications. ILs possess many attractive properties such as negligible vapor pressure, high solubility of organic and inorganic compounds and controllable physicochemical properties including hydrophobicity, dipolarity, and hydrogen bond basicity.[1–3] Due to their unique and favorable properties, the application of ILs as alternative solvents for organic synthesis has been extensively explored.[4]

Many researchers are becoming increasingly interested in the application of ILs as reaction media for biotransformation.[5] Recent work has shown that enzymes[6–15] and whole-cell biocatalysts[16] exhibited their catalytic activities in pure ILs or IL/aqueous biphasic systems, providing many advantages such as high conversion rates, high enantioselectivity, and increased stability of enzymes. ILs were also used as a stabilizer for preparing immobilized enzymes.[17] However, one of the most significant limitations in enzymatic reactions in ILs is the relatively low activity of enzymes suspended in ILs. Some ILs are known to dissolve enzymes with[6,13] or without[14] a small amount of water, however, dissolved enzymes show little catalytic activity presumably due to their conformational change in ILs. To overcome this limitation, several studies have been conducted to enhance enzymatic activity in ILs, involving the addition of a small amount of water to ILs[8,9] and immobilization of enzyme with solid supports.[8,11] Poly(ethylene glycol) (PEG) shows high solubility in ILs, and was found to provide high stability and dispersibility of enzymes in ILs.[8,10,12,13] We previously showed the effective activation of lipases in ILs by physical complexation of lipases with PEG 20000.[12] Although some researchers attempted to improve solubility and activity of enzymes in ILs by covalent modification with PEG, chemical modification of enzymes with linear PEG did not offer sufficient solubility and catalytic activity in ILs.[11,15]

Here, we report the use of comb-shaped PEG as an enzyme modifier to solubilize an enzyme in ILs. Comb-shaped PEG, PM_{13}, (commercially, SUNBRIGHT AM-1510K, NOF Co., Ltd., Japan) (Fig. 1) is a copolymer of PEG derivative and maleic anhydride with an approximate molecular weight of 15 000 and has multivalent reactive sites, acid anhydrides, which react preferentially with amino groups in a protein molecule.[18] The potential utility of PM_{13} was validated in enzymatic catalysis in organic solvents.[19] In the present study, we have demonstrated perfect solubilization and marked enhancement of catalytic activity of an enzyme in ILs by modification with PM_{13}.

Subtilisin Carlsberg was chosen as the model enzyme, and PM_{13}-modified subtilisin (PM_{13}-Sub) was prepared using a procedure developed by Inada *et al.*[19] To a subtilisin solution (2 mg ml^{-1}, 2 ml) in 0.1 M sodium borate buffer (pH 8.5), PM_{13} (100 mg) was added, followed by gentle stirring at 4 °C for 1 h. The reaction mixture was ultrafiltered using an Ultrafree-CL membrane (Millipore, Billerica, MA) to remove unreacted PM_{13}. Ultrafiltration was repeated three times with subsequent addition of 1 mM HCl solution (1 ml). The concentrated solution of the modified enzyme was lyophilized for 48 h. Modification degree of amino groups in the subtilisin molecule was determined by measuring the amount of free amino groups with 2,4,6-trinitrobenzenesulfonic acid (TNBS),[20] and protein concentration in PM_{13}-Sub was estimated using the bicinchoninic acid (BCA) assay. These investigations revealed that about half of amino groups of subtilisin were modified, and 10 mg of the conjugate contained 0.5 mg of subtilisin. The modified enzyme was confirmed to retain its original activity in hydrolysis of *p*-nitrophenyl butylate, suggesting that inactivation through the modification process was negligible.

Firstly, we investigated the solubility of PM_{13}-modified enzyme (Fig. 2). Native subtilisin was not dissolved in all ILs tested, even in [C_2OHmim][Tf_2N][21] and [C_2OC_1mim][Tf_2N],[22] which were expected to solubilize native enzymes through hydrogen bonding interaction between protein molecules and ionic liquids, resulting in a suspension. Most interestingly, PM_{13}-Sub could be solubilized clearly in a wide range of ILs including [Bmim][PF_6], [Emim][Tf_2N], [C_2OHmim][Tf_2N], and [C_2OC_1mim][Tf_2N] to give a protein concentration of at least 1 mg/ml. Solubilization of the enzyme in these ILs would be due to the high density of PEG chains sufficiently covering the protein surface. We demonstrated

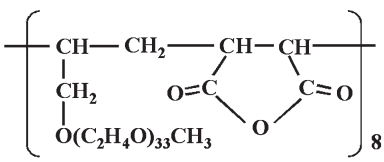

Fig. 1 Molecular structure of comb-shaped PEG, PM_{13}, employed for chemical modification of native subtilisin.

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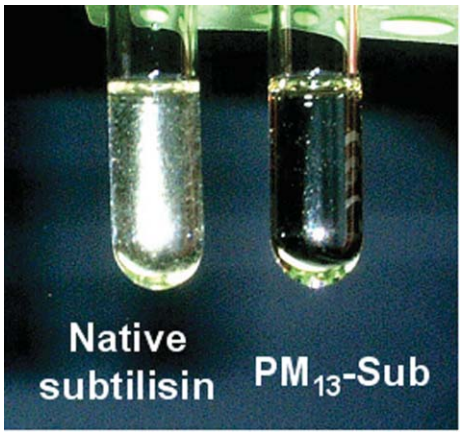

Fig. 2 Photograph showing difference in solubility between native subtilisin and PM_{13}-modified subtilisin (PM_{13}-Sub) in ILs.

that chemical modification of protein with comb-shaped PM_{13} was quite effective for solubilization of enzyme in ILs.

Dissolved enzymes do not always exhibit satisfactory catalytic activity in ILs. Several researchers reported cases of enzymes which were inactivated when dissolved in ILs.[6,13,14] This could be explained by considering the fact that ILs that can solubilize enzymes also interact with protein molecules and cause structural changes, resulting in denaturation of enzymes. Therefore, enzymatic activity of PM_{13}-Sub dissolved in [Emim][Tf_2N] was examined in transesterification of *N*-acetyl-L-phenylalanine ethyl ester (100 mM) with 1-butanol (500 mM). For comparison with the activity of PM_{13}-Sub, the same reaction was carried out with lyophilized native subtilisin or PEG-subtilisin complex, an enzyme physically supported with PEG 20,000.[10,12] Because the latter two biocatalysts were not soluble in [Emim][Tf_2N], the reaction mixture was heterogeneous. As seen in Fig. 3, native subtilisin showed no activity in [Emim][Tf_2N] and reaction rate of the PEG-subtilisin complex was very slow, presumably due to low solubility or salt-induced deactivation.[13] In contrast, PM_{13}-Sub exhibited excellent transesterification activity in [Emim][Tf_2N]. Since the reaction medium was more or less anhydrous, transesterification predominated and the products of hydrolysis were not observed. It was interesting to note that dissolution of subtilisin in ILs did not impair catalytic activity, which differs from what has previously been reported.[14] In the case of PM_{13}-Sub, the substantial PEG chains covering the enzyme surface could protect the enzyme from the injurious ionic solvent. Hydrophilic PEG chains might also contribute to the effective retention of essential water in protein molecules,[23] resulting in suppression of conformational alterations.

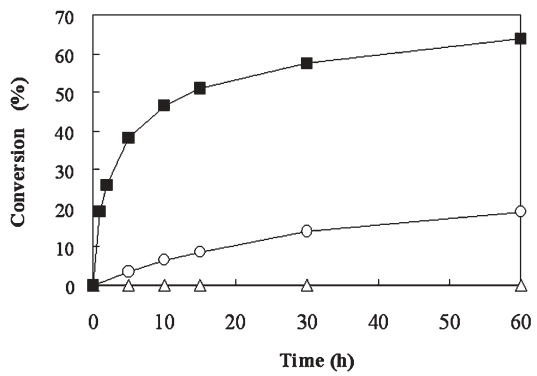

Fig. 3 Transesterification activity of native subtilisin (open triangles), PEG–subtilisin complex (open circles) and PM_{13}-Sub (closed squares) in [Emim][Tf_2N] at 40 °C. The enzyme concentration was 1 mg ml^{-1} in all systems.

One of the major advantages of the use of ILs as reaction media for biotransformation is their high solubility of polar or hydrophilic substrates such as amino acids[6,8,9] and saccharides.[22] Aliphatic organic solvents (*e.g.*, hexane and isooctane) can hardly dissolve amino acids, whereas these solvents are favorable for enzymatic synthesis due to their hydrophobicity, which suppresses the stripping of essential water. Ionic liquids intrinsically possess the dual properties of ionicity and hydrophobicity. One of the ultimate goals in the utilization of ILs as reaction media is to surpass conventional organic solvents. Then, we compared the reaction rate of PM_{13}-Sub in the IL with that in conventional organic solvents. We used toluene, tetrahydrofuran (THF), dimethylsulfoxide (DMSO) and acetonitrile as organic solvents, in which both PM-modified subtilisin and substrates were soluble. We achieved higher enzymatic performance in [Emim][Tf_2N] (306 nmol min^{-1} (mg enzyme)$^{-1}$) than in toluene (93 nmol min^{-1} (mg enzyme)$^{-1}$). In hydrophilic organic solvents such as THF, DMSO and acetonitrile, no enzymatic activity was observed. It was presumed that the polar IL would stabilize the transition state in the reaction, which is relatively polar.[9,24] To our knowledge, this great superiority of enzymatic catalysis in pure ILs compared with organic solvents has not yet been reported.

In summary, we have shown that comb-shaped PEG, PM_{13}, is an excellent modifier to solubilize enzymes in ILs. This approach offers high enzymatic activity in pure ILs without immobilization of enzyme or addition of small amounts of water. We believe that this report will stimulate researchers to develop further applications in the field of enzymology for the use of ILs.

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