Chapter 5

Ionic Liquid-Mediated Activation of Lipase-Catalysed Reaction

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Additional information is available at the end of the chapter

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1. Introduction

Ionic liquids (ILs) have very good properties as reaction media in chemical reactions: they are non-volatile, non-flammable, have low toxicity and good solubility for many organic and inorganic materials. [1] It has long been recognized that an enzymatic reaction proceeds in a buffer aqueous solution under appropriate pH conditions, and an enzyme quickly loses its activity in a highly concentrated aqueous salt solution. [2, 3] Therefore, it seems foolish to suggest that enzymatic reaction occurs in a salt medium from the standpoint of biology. However, the use of ILs to replace traditional organic solvents in chemical reactions has recently gained much attention, and it has now been established that ILs could also be used as reaction media for biotransformation: lipase-catalysed reactions in an ionic liquid solvent system have been investigated extensively, and several types of non-lipase enzymatic reactions have even been reported too. [3, 4] This chapter describes recent progress in this area, focusing on "ionic liquid-mediated activation of lipase-catalysed reactions".

2. Ionic liquids as a reaction medium for biotransformation

The first example of a lipase-catalysed reaction in a pure ionic liquid solvent system was reported by the Sheldon group at the end of 2000. [5, 6] The authors demonstrated two types of *Candida antarctica* lipase (CAL-B)-catalysed reaction in a pure IL: CAL-B catalysed amidation of octanoic acid with ammonia and also catalysed formation of octanoic peracid by the reaction of octanoic acid with hydrogen peroxide (Figure 1).

However, these reactions were not enentioselective ones, and the most important aspect of the biocatalysis reactions should be in the enantioselective reaction. Itoh [7] and Kragl [8] inde-

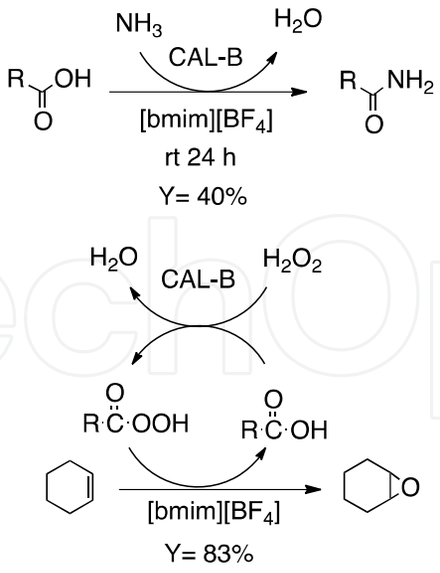

Figure 1. The first enzymatic reaction conducted in a pure ionic liquid solvent system.

pendently reported the first enantioselective enzymatic reactions in early 2001. Itoh demonstrated that lipase was anchored by the ionic liquid solvent, and remained in it after the extraction work-up of the product; they also succeeded in demonstrating that recyclable use of the lipase in the imidazolium type ionic liquid solvent system was possible (Figure 2). [7]

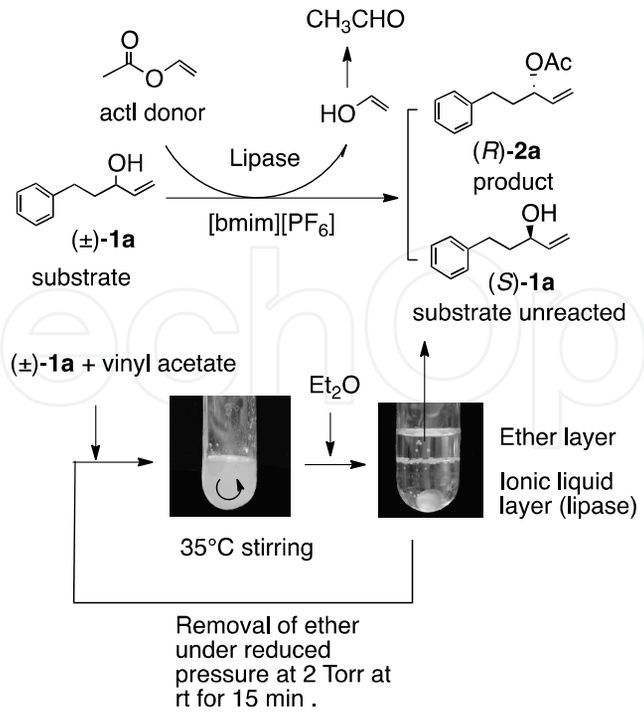

Figure 2. The first example of lipase-catalysed enantioselective transesterification in a pure ionic liquid.

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Itoh mentioned an interesting history of the early days of their study in his review. [4a] Scientists encountered a serious problem, in that the results of lipase-catalysed transesterification in ILs as reaction media were significantly dependent on the ionic liquids that they prepared themselves. They found that the quality of ILs influenced the results strongly; it took more than half a year to establish a preparation method for clean ILs and to obtain reproducible results prior to submitting their paper. This highlights that clean ILs should be required for biocatalysis systems compared to chemical reactions. I imagine that all research groups encountered the same problem in the early days of this field. Fortunately, we are free from such trouble, because many types of ILs with high purity are now commercially available. I give the list of ILs that have been applied as reaction media for lipase-catalysed reactions (Figure 3). Hydrophobic ionic liquids generally act as good reaction media for lipase-catalysed reaction; by contrast, hydrophilic ILs give poor or no reaction, though several ILs are exceptions to this.

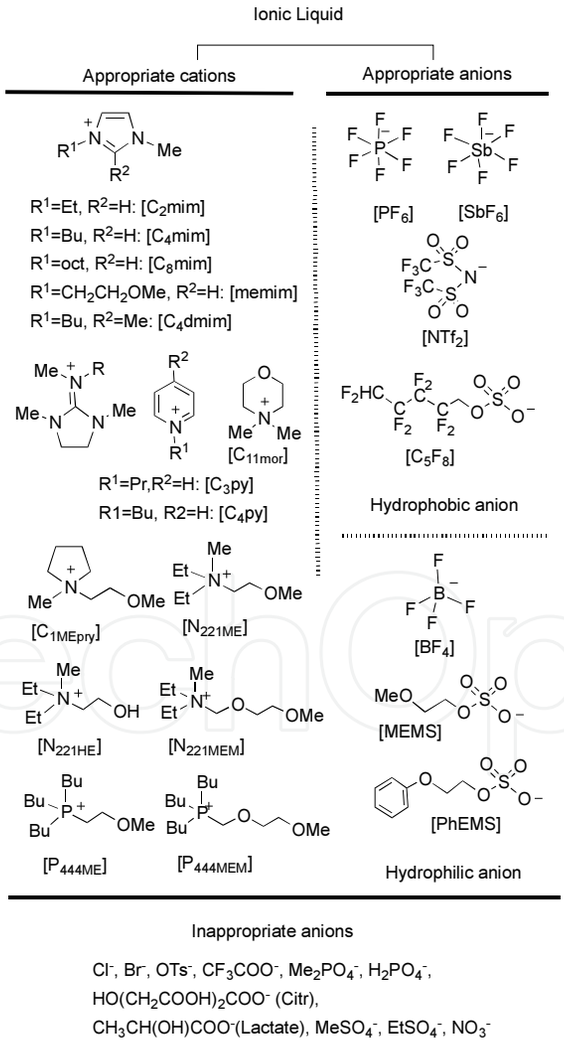

Figure 3. List of ILs for lipase-catalysed reactions.

3. Activation of lipase-catalysed reaction in an ionic liquid solvent system

Vinyl acetate is commonly used as an acyl donor of lipase-catalysed transesterification in organic solvents, because vinyl alcohol produced by the transesterification immediately tautomerizes to acetaldehyde, which easily escapes from the reaction mixture due to its very volatile nature. Thus, no reverse reaction takes place. Then, the reaction equilibrium goes on to produce the desired acetate. Because of this reason, acetaldehyde usually shows no inhibitory action on the lipase, though acetaldehyde acts as an inhibitor of enzymes when it forms a Schiff base with amino residue in the enzyme. [3]

Itoh and colleagues found that the reaction rate of lipase-catalysed transesterification gradually dropped with repetition of the reaction process in 1-butyl-3-methylimidazolium hexafluorophosphate ([C_4mim][PF_6]), while enantioselectvity is perfect in all reactions (E^9 >200); this drop in reactivity was caused by the inhibitory action of acetaldehyde oligomer which had accumulated in the IL solvent system. [10] Itoh hypothesized that oligomerization of acetaldehyde might be caused by the proton derived from the water molecule trapped by the hydrogen bonding at 2-position of the imidazolium ring, due to the high acidity of the 2-position of imidazolium cation (Figure 4). [11] They solved this problem using two methods.

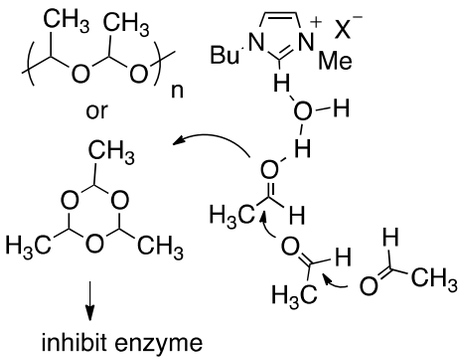

Figure 4. Plausible mechanism for formation of acetaldehyde oligomer in [C_4mim][PF_6].

One solution is the lipase-catalysed transesterification under reduced pressure conditions using methyl ester as an acyl donor. [10] Methyl esters are generally not suitable for lipase-catalysed transesterification as acyl donors, because reverse reaction with produced methanol takes place. [3] However, such a difficulty can be avoided when the reaction is carried out under reduced pressure even if methyl esters are used as the acyl donor, because the methanol produced is removed immediately from the reaction mixture, and thus the reaction equilibrium goes on to produce the ester. [12] The most important characteristics of ionic liquid are its wide temperature range for the liquid phase and its having a very low vapour pressure. [1] The transesterification indeed took place smoothly under reduced pressure at 10 Torr at 40 °C when methyl phenylthioacetate was used as acyl donor in [C_4mim][PF_6] solvent system. Using the system, a completely recyclable use of lipase (Novozym435) was realized (Figure 5): five repetitions of this process showed no drop in the reaction rate while maintaining perfect

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enantioselectivity. [10] The same reaction system was applied to esterification and amidation of carboxylic acids by Irimescu and Kato. [13]

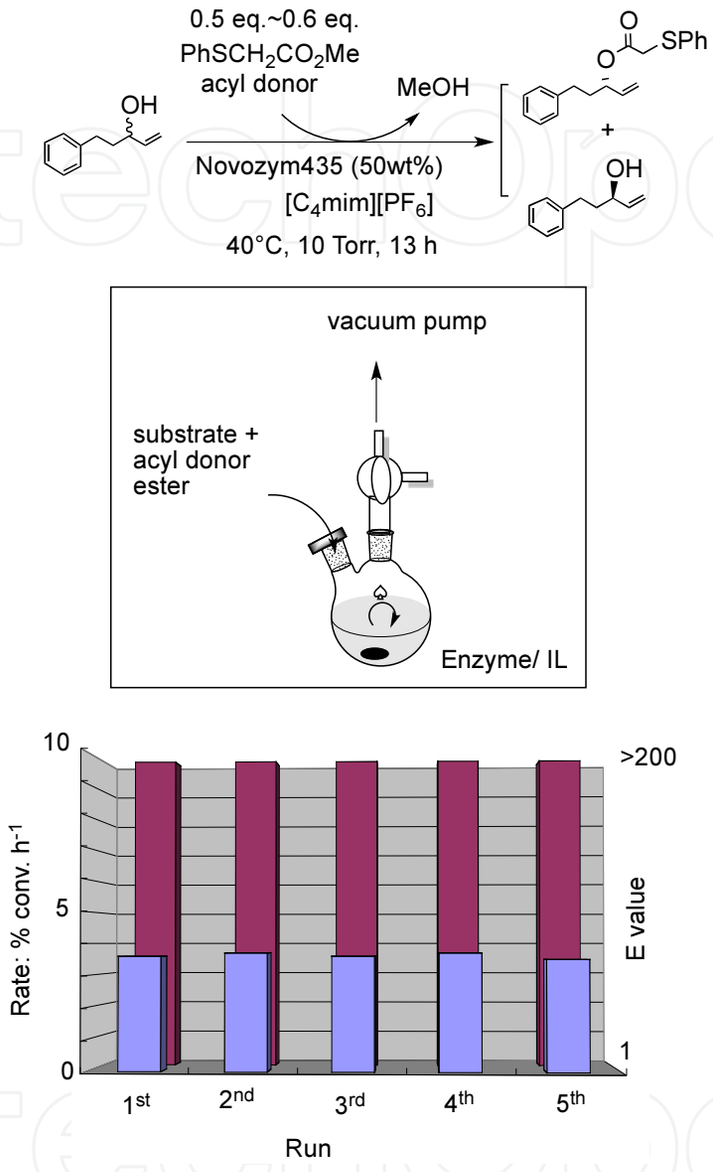

Figure 5. Recyclable use of lipase under reduced pressure conditions in the IL solvent system.

The second method to solve the problem is the use of 1-butyl-2, 3-dimethylimidazolium (C_4dmim) salts, which lacked a hydrogen atom at 2-position on the imidazolium ring:[14] lipase-catalysed transesterification using vinyl acetate was carried out using1-butyl-2, 3-dimethylimidazolium (C_4dmim) salts as solvent. As expected, no accumulation of an acetaldehyde oligomer was in fact observed in this solvent system since [C_4dmim] cation has no acidic proton. The reaction proceeded very smoothly in [C_4dmim][BF_4] and recyclable use of the enzyme was realized while maintaining perfect enantioselectivity, as shown in Figure 6. [14]

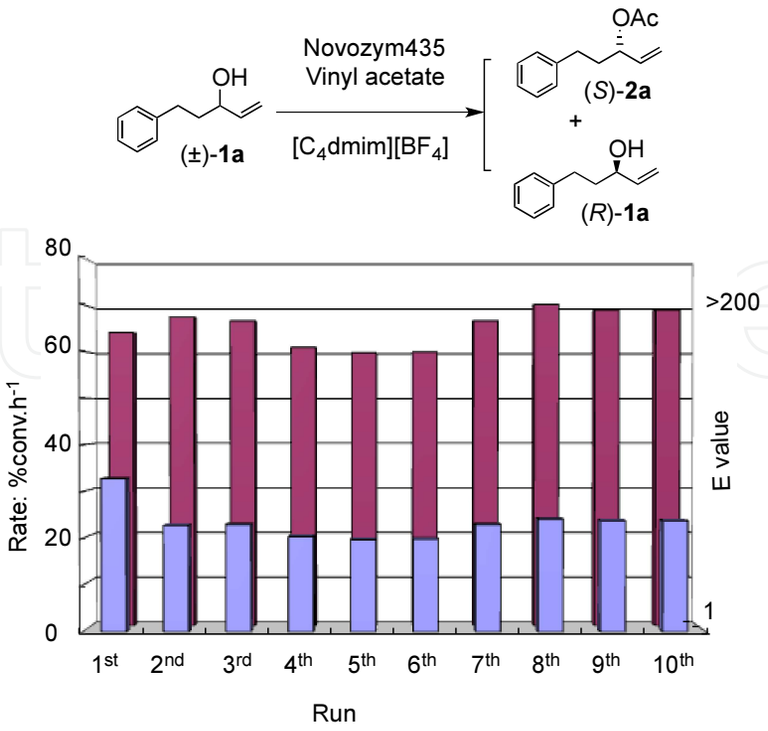

Figure 6. Recyclable use of enzyme in [C_4dmim][BF_4] solvent system.

When performing the reactions, Han found that the lipase was very stable in [C_4dmim][BF_4]. Lipases showed perfect reactivity after two months when lipases were kept in this ionic liquid, although enzymes are generally unstable in organic solvent and even lipases lose its activity quickly in the absence of substrates; both *Burkholderia cepacia* PS (lipase PS) and *Candida antarctica* lipase, Novozym, showed good reactivity after two months in this ionic liquid. [15]

De Diego and colleagues reported a stabilization effect of ionic liquid for lipase-catalysed reaction: the presence of an appropriate substrate was essential for stabilization of enzyme in an ionic liquid solvent. [16] The half-lifetime of native CAL was only 3.2 h in [C_2mim][PF_6] solvent, though it lengthened remarkably to 7, 500 h in the presence of the substrate. Furthermore, Lozano and colleagues demonstrated CAL-B catalysed transesterification of 1-phenylethanol using vinyl butyrate and revealed that ILs solvents gave better reactivity and stability compare to those in hexane: CAL-B maintained activity higher than 75 % after four days of incubation in [C_2mim][NTf_2] solvent. On the contrary, only 25 % of activity was obtained when the lipase was incubated in water or hexane medium. [16] Comparing the ratio of α-helix and β-sheet by CD spectra, the activity was closely related with α-helix content; the content of α-helix reduced to 31 % immediately after lipase was added to hexane and had reached only 2 % after four days in hexane. On the other hand, no significant reduction of α-helix content was obtained in [C_2mim][NTf_2] solvent. Based on these results, the authors concluded that α-helix contents might play an important role in maintaining the enzymatic activity.[16]

Polyethyleneglycol (PEG) treatment is known to cause stabilization of an enzyme. Goto reported that PEG-coated lipase worked well as catalysis for transesterification of vinyl

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cinnamate with butanol in [C_8mim][PF_6] as solvent. [17] Russell and colleagues reported that improved activity of lipase by PEG treatment in IL solvent was mainly dependent on the anionic part of the imidazolium salt ionic liquids; high activity was obtained for [PF_6] salt, while no activity was observed for [NO_3], [OAc], [CH_3SO_3], [OTf], or [NTf_2] salt. [18] The authors proposed that nitric anion or acetate anion might have a strong interaction with some parts of the enzyme protein due to the highly nucleophilic nature of these anions, and caused deactivation of the enzyme activity. [18]

Inspired by these results, Itoh and colleagues prepared two types of alkyl PEG sulphate imidazolium ionic liquids ([C_4dmim][cetyl-$(OCH_2CH_2)_{10}$-OSO_3] (IL1) and [C_4mim][cetyl-$(OCH_2CH_2)_{10}$-OSO_3] (IL2)) and used as an additive for lipase-catalysed acylation of 1-phenylethanol. Enhanced enantioselectivity was obtained when IL1 or IL2 was added at 3 mol% vs. substrate in the *Burkholderia cepacia* lipase (lipase PS) catalysed transesterification using vinyl acetate in diisopropyl ether or a hexane solvent system (see Figure 7 and Table 1). [15a]

On the other hand, Lee and Kim reported an IL-mediated activation of a lipase: they prepared "ionic liquid-coated lipase PS" by mixing lipase PS with 1-(3-phenyl)propyl-3-methylimidazolium hexafluorophosphate ([PhC_3mim][PF_6]) and found that the resulting lipase showed more enhanced enantioselectivity than that of commercial lipase PS in toluene, though no modification of the reaction rate was obtained (Figure 7 and Table 1). [19] However, as shown in Table 1, enhanced enantioselectivity was not significant and reaction speed dropped when [PhC_3mim][PF_6]-PS was used as catalysis.

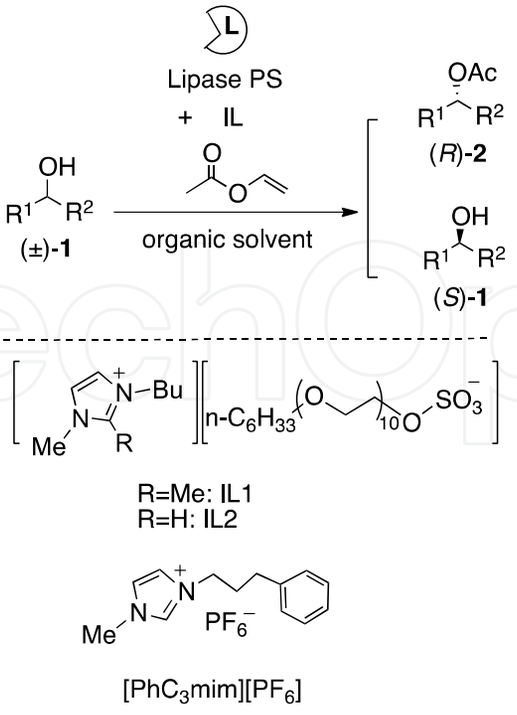
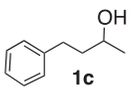
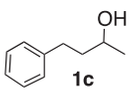


Figure 7. Attempt to activate lipase PS by an ionic liquid.

Substrate	IL	Solvent	E value	% conv. / h	Ref.
 1b	Native PS	i-Pr_2O	10	1.4	[15]
 1b	+ IL1 (3 mol%)[a]	i-Pr_2O	>200 (1057)	1.8	[15]
 1b	+ IL2 (3 mol%)[a]	i-Pr_2O	40	1.6	[15]
 1c	Native PS	toluene	>200 (265)	1.5	[19]
 1c	[PhC_3mim][PF_6]-PS [b]	toluene	>200 (532)	0.4	[19]

[a] added as an additive to the reaction mixture. [b] [PhC_3mim][PF_6]-PS was prepared as follows [19]: 1.0 g of [PhC_3mim][PF_6] was heated at 53 °C, then mixed with lipase PS (0.1 g) and the homogeneous solution was stirred for 1 min. then cooled to rt.

Table 1. Attempt to activate lipase-catalysed transesterification by an ionic liquid treatment.

Itoh and colleagues prepared [C_4dmim][cetyl-PEG10-sulphate](IL1) and established that IL1 worked as an excellent activating agent of lipases. The ionic liquid-coated lipase PS (IL1-PS) by the lyophilisation process gave excellent results for enantioselective transesterification of various types of secondary alcohols using vinyl acetate as acyl donor in i-Pr_2O solvent (Figure 8 and Table 2). [15, 20] The results were dependent on the substrates, and more than 500-to 1000-fold acceleration was accomplished for some substrates. [20]

It should be emphasized that IL1-coating made it possible to accelerate the reaction rate and enhanced enantioselectivity, while simple PEG coating of a lipase could accelerate the reaction but caused no significantly enhanced enantioselectivity. [20] MALD-TOF Mass experiments suggested that IL1 binds with the enzyme protein; therefore, it was assumed that the modified activity of lipase might be due to flexibility or conformation change of the lipase protein caused by the IL1-binding. [21] Furthermore, it was established that imidazolium cation affected the enantioselectivity of the lipase; IL1-coated lipase PS gave better enantioselectivity than an IL2-coated one. [20]

Amino acids have been used as a stabilizer of an enzyme during the purification process: commercial lipase PS involves ca. 20 wt% of glycine as an essential stabilizer during prepara-

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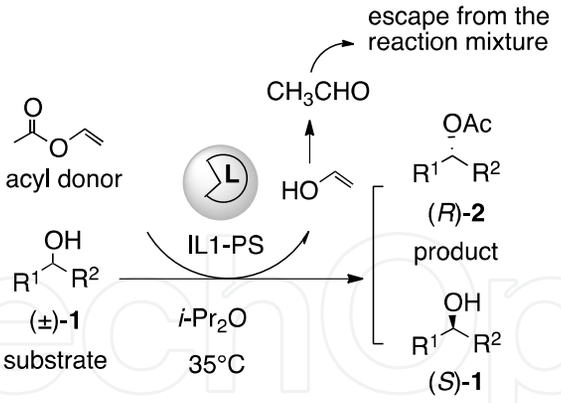


Figure 8. Activation of lipase by coating with alkyl PEG sulphate ionic liquid (IL1).

tion of the lipase protein by the lyophilisation process; Itoh and colleagues found that glycine worked only as a stabilizer of the enzyme and had no influence on the reactivity of lipase PS. [20] Hence, they prepared amino acid-coated lipase PS and investigated its properties in transesterification of (±)-1-phenylethanol (1b) as a model substrate in the presence of vinyl acetate as an acyl donor in the *i*-Pr_2O solvent system. [21] As shown in Figure 9, coating of lipase PS by amino acids neither accelerated nor modified enantioselectivity, though coating of lipase by L-aspartic acid (Asp) and L-cysteine (Cys) caused its significant reduction (see $Rate^1$ in Figure 9). They finally found very interesting synergetic activation of an enzyme for amino acid with IL1.[21] Great acceleration was obtained by the coating of lipase PS with a combination of amino acid and IL1, which was prepared by treating glycine-free PS with 100 mol eq. of L-amino acid and IL1: 100 to 300-fold acceleration compared to the native or amino acid-coated lipase PS (see $Rate^2$ in Figure 9). It was found that the combination of IL1 and L-proline (Pro) was particularly effective for realizing activation of the lipase PS: 330-fold acceleration was accomplished using L-proline and IL1-coated PS (see $Rate^2$ in Figure 9). [21]

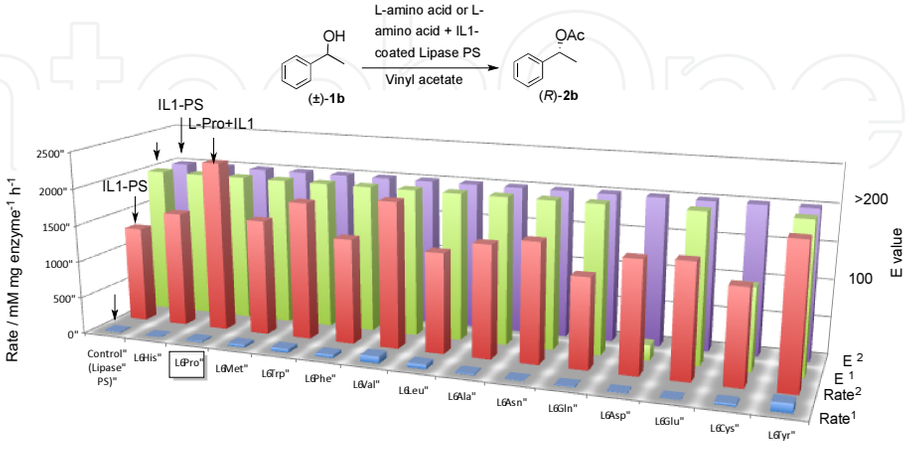


Figure 9. [21] Effect of coating on lipase PS with only an amino acid ($Rate^1$ and E^1) or with both an amino acid and IL1 ($Rate^2$ and E^2) on transesterification of (±)-1-phenylethanol (1b) using vinyl acetate as acyl donor.

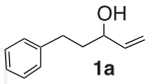
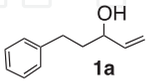
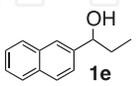
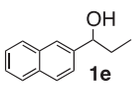
Substrate	IL	E value	% conv. / h
	Native PS	>200	2.6
	IL1-PS	>200	31
	Native PS	>200	65
	IL1-PS	>200	1000
	Native PS	40	100
	IL1-PS	40	9600
	Native PS	199	$10x10^{-2}$
	IL1-PS	>200	10.5

Table 2. Typical results of transesterification mediated by lipase PS and IL1-PS.

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They compared the coating effect between L-amino acids and D-amino acids for lipase PS using (±)-(*E*)-4-phenylbut-3-en-2-ol (1f) as a model substrate, because the reaction rate of lipase PS for this alcohol was not satisfactory, while enantioselectivity was perfect. Some different activation effect levels were found among cysteine, proline, tyrosine, and methionine-coated enzymes. Interestingly, coating with non-natural D-amino acids generally showed a slightly larger acceleration than the natural L-amino acids coating. [21]

Itoh and colleagues attempted to design an ionic liquid-type activating agent for lipase by modification of the cationic part of the IL because it was established that imidazolium cation affected the enantioselectivity of the lipase PS in their previous work. [15, 20] Since it was anticipated that introduction of an appropriate chiral functional group on the imidazolium group may create a more efficient activation of an enzyme, because D-amino acids generally showed a slightly larger acceleration than the natural L-amino acids coating. [21] Cheng and colleagues reported the synthesis of imidazolium ionic liquid derived from L-proline and used it as an efficient asymmetric organocatalyst. [22] Following their method, Itoh's group prepared pyrrolidine-substituted imidazolium bromide and converted it to cetyl-PEG10-sulphate by the metal exchange reaction. [23] Chiral pyrrolidine-substituted imidazolium cetyl-PEG10-sulphate (D-ProMe) derived from D-proline worked as an excellent activating agent of lipase PS; it is particularly interesting that D-isomer of the imidazolium salt worked better than L-isomer. This suggests that the imidazolium cation group directly interacts with the enzyme protein and causes preferable modification of the reactivity. Figure 10 summarizes the results of transesterification of (±)-1-phenylethanol (1b) using commercial lipase PS and four types of chiral imidazolium salt-coated lipase PS using vinyl acetate or 2-trifluoroethyl acetate (CF_3CH_2OAc) (Figure 10). It was established that chiral pyrrolidine-substituted imidazolium salt worked as an excellent activator of lipase PS. In particular, (*R*)-pyrrolidine-substituted salt (D-ProMe), which was derived from unnatural D-proline, was found to be the best agent: an extraordinary acceleration was accomplished with perfect enantioselectivity for D-ProMe-PS-catalysed reaction and a reaction 58 times faster (vs. lipase PS) was recorded (column 6 in Figure 10). [23]

A kinetic experiment showed that K_{cat} value of the IL1-coated lipase PS-catalysed reactions was increased compared to those of native lipase PS. [24] Modified Km values were also observed between enantiomers of the substrate alcohol when lipase PS was coated by chiral imidazolium salts. [23] On the other hand, the K_m value of (S)-isomer was reduced from that of PS when D-ProMe-PS was used as catalyst, while the value was increased for (R)-isomer (two-fold increase over native PS). [23] These results indicate that the cationic part of the ionic liquid might bind with the lipase protein, causing conformational change of the enzyme and contributing to the difference of K_m between enantiomers. Chiral imidazolium cation might strongly affect the enzyme reactivity compared to amino acids when it binds with the protein, mainly on the protein surface, thus contributing to increased flexibility of the enzyme protein. Since these ionic liquids have amphiphilic properties, this also contributes to concentration of the hydrophobic substrate on the enzyme protein, so that an initial acceleration of the rate might be realized.

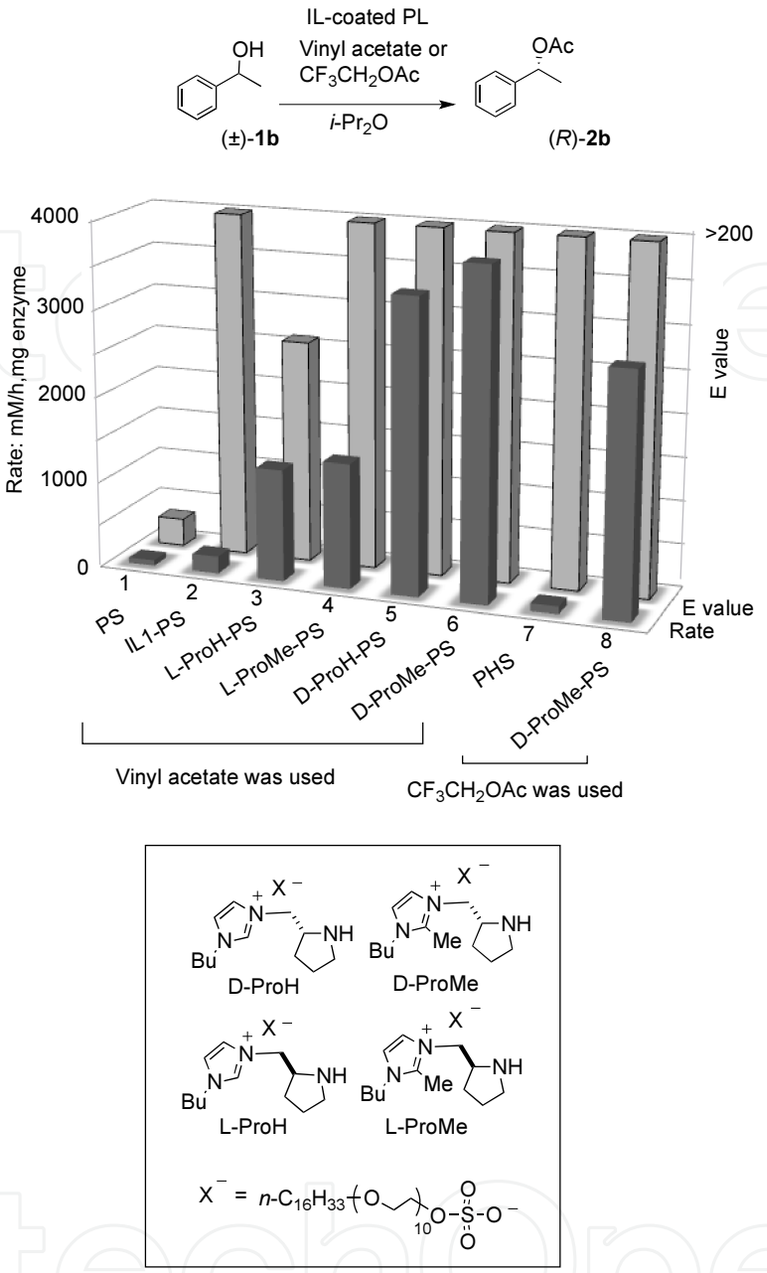

Figure 10. Activation of lipase PS by the chiral imidazolium IL coating

4. Ionic liquid-coated lipase-catalysed reaction in an ionic liquid solvent system

ILs are now established as solvents for use in lipase-catalysed transesterification with excellent enantioselectivity and the IL solvent system makes possible the recylable use of enzymes. However, there still remains a serious drawback, in that the rate of reaction in an ionic liquid is slower than that in a conventional organic solvent such as i-Pr_2O. As mentioned before, a

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powerful method of activating lipase protein by coating it with imidazolium alkyl PEG sulphate ionic liquid IL1 has now been established: the ionic liquid-coated *Burkholderia cepacia* lipase (IL1-PS) displayed excellent reactivity for many substrates in conventional organic solvents. [15, 20, 23, 25]

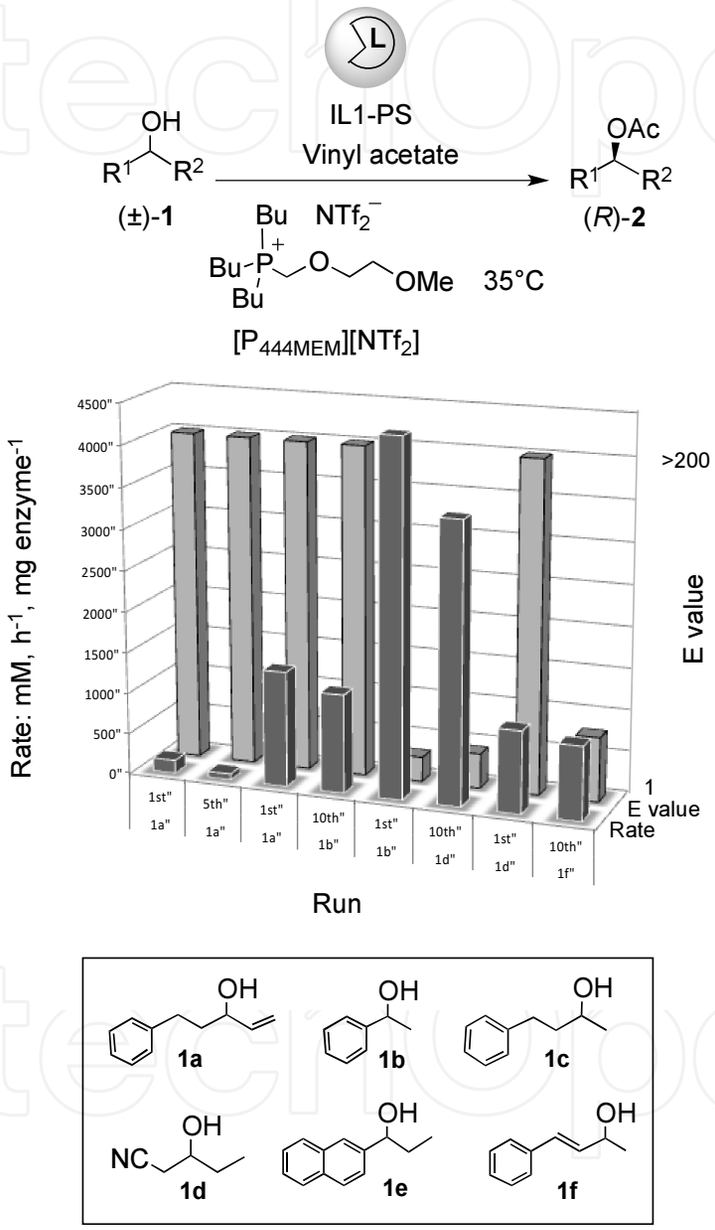

Figure 11. Results of IL1-PS-catalysed transesterification in an IL solvent system.

Itoh and colleagues reported that hydrophilic imidazolium salts ILs, which have alkyl ether functionalized sulphate salts, were appropriate for lipase-catalysed reaction. [26] Dreyer, [27] Guo, [28] Zhao [29] and De Diego [30] have reported that ILs that have an alkyl ether moiety as a cationic part acted as good solvents for these reactions. Zhao and colleagues recently

reported that dissolution and stabilization of a lipase protein took place in ILs that have a long alkyloxyalkyl chain in an ammonium cation, and suggested that this might provide improved catalytic efficiency of the corresponding biochemical reactions. [29] Hence, evaluation of ILs which have alkyl ether moieties was conducted and tributyl((2-methoxyethyl)phosphonium bis(trifluoromethanesulphonyl)amide ($[P_{444ME}][NTf_2]$) [24, 31] and tributyl((2-methoxyethoxy)methyl)phosphonium bis(trifluoromethanesulphonyl)amide ($[P_{444MEM}][NTf_2]$) [32] were developed as useful solvents for IL1-PS-catalysed reactions using 1-phenylethanol (1b) as substrate and realized recyclable use of the enzyme in the IL solvent system. [24, 32]

Phosphonium ionic liquids and ammonium ionic liquids which have alkylether moieties were shown to become excellent reaction media for lipase-catalysed transesterification, especially for ionic liquid-coated lipase PS (IL1-PS). In particular, very rapid acetylation of 1-phenylethanol (1b) has been accomplished using the combination of IL1-PS and $[P_{444MEM}][NTf_2]$ as solvent while maintaining perfect enantioselectivity (Figure 11). This is a record for the most rapid lipase-catalysed transesterification of 1-phenylethanol (1b). [32] It was also established that recyclable use of IL1-PS was possible for various substrates, as shown in Figure 11. Interestingly, $[N_{221MEM}]$ $[NTf_2]$ was always superior to $[N_{221ME}][NTf_2]$ in all substrates and that $[P_{444MEM}][NTf_2]$ was especially suitable for the reaction of 1b. [33]

5. Conclusion

In this chapter, I focus on reviewing the activating method of lipase-catalysed transesterification using ionic liquid technology. Ionic liquid has a certain advantage over conventional organic solvents, because the solvent makes it possible to use the enzyme repeatedly and has less volatile and less flammable properties. As shown in this chapter, phosphonium ionic liquid and ammonium ionic liquids which have alkylether moieties become excellent reaction media for lipase-catalysed transesterification, especially for ionic liquid-coated lipase PS (IL1-PS). It has now been disclosed that introduction of alkyl ether moiety in the cationic part of ILs might be a sure way to design ionic liquids suited for enzymatic reaction. After the reaction, we recovered the ionic liquids and used them repeatedly after simple purification. To meet the challenge in chemistry of developing practical processes, the proper choice of a reaction medium is very important. Breakthroughs have sometimes come through innovation of a reaction medium in chemical reactions, and this is true even in enzymatic reactions. I hope this paper may provide some suggestions for the reader's research.

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