What Do We Learn from Enzyme Behaviors in Organic Solvents?
– Structural Functionalization of Ionic Liquids for Enzyme Activation and Stabilization

Hua Zhao*

Department of Chemistry and Biochemistry, University of Northern Colorado, Greeley, CO 80639, United States

Corresponding author: email address: hua.zhao@unco.edu, or huazhao98@gmail.com

Abstract

Enzyme activity in nonaqueous media (e.g. conventional organic solvents) is typically lower than in water by several orders of magnitude. There is a rising interest of developing new nonaqueous solvent systems that are more "water-like" and more biocompatible. Therefore, we need to learn from the current state of nonaqueous biocatalysis to overcome its bottleneck and provide guidance for new solvent design. This review firstly focuses on the discussion of how organic solvent properties (such as polarity and hydrophobicity) influence the enzyme activity and stability, and how these properties impact the enzyme's conformation and dynamics. While hydrophobic organic solvents usually lead to the maintenance of enzyme activity, solvents carrying functional groups like hydroxys and ethers (including crown ethers and cyclodextrins) can lead to enzyme activation. Ionic liquids (ILs) are designable solvents that can conveniently incorporate these functional groups. Therefore, we systematically survey these ether- and/or hydroxy-functionalized ILs, and find most of them are highly compatible with enzymes resulting in high activity and stability. In particular, ILs carrying both ether and *tert*-alcohol groups are among the most enzyme-activating solvents. Future direction is to learn from enzyme behaviors in both water and nonaqueous media to design biocompatible "water-like" solvents.

Keywords: Enzyme, biocatalysis, organic solvent, ionic liquid, nonaqueous solvent

Contents

1. Introduction

The use of biological molecules as catalysts offers many "green" features to the catalytic process such as less hazardous materials, energy efficiency, and catalysis to enhance the reaction rate (Timson, 2019). However, biocatalysis in aqueous solutions has some "non-green" limitations such as the consumption of large volume of diluted aqueous solution (due to poor substrate solubility), poor economic efficiency, and downstream processing difficulties (such as product and enzyme recovery), etc. (Domínguez de María and Hollmann, 2015). On the other hand, nonaqueous enzymatic reactions in conventional organic solvents have paved a unique direction for biocatalysis since 1980s (Dordick, 1989; Klibanov, 1990; Zaks and Klibanov, 1984, 1985, 1988a), leading to over 100 applications in pharmaceutical, agrochemical, and fine chemical industries (Abdelraheem et al., 2019; Gupta, 1992; Klibanov, 2001; Stepankova et al., 2015; Wandrey et al., 2000). Later, nonaqueous media expanded from ordinary organic solvents to supercritical fluids (Cantone et al., 2007; Hobbs and Thomas, 2007), fluorous solvents (Ghaffari-Moghaddam et al., 2015; Hobbs and Thomas, 2007), gas phase (Barzana et al., 1989; Dunn and Daniel, 2004), ionic liquids (ILs) (Moniruzzaman et al., 2010; van Rantwijk and Sheldon, 2007;

Zhao, 2005, 2016), and deep eutectic solvents (DES) (Smith et al., 2014; Xu et al., 2017; Zhao and Baker, 2013). Key advantages of nonaqueous biocatalysis include altered regio-/enantio-selectivity, high thermal stability, easy recovery of enzyme and product, dissolution of water-insoluble substrates in nonaqueous media, reduced microbial contamination, minimized substrate and/or product inhibition, fewer side-reactions involving water, and reversing reaction equilibria (such as turning hydrolase-catalyzed hydrolysis reactions into synthesis), etc. (Brink et al., 1988; Dai and Klibanov, 1999; Dordick, 1992; Gupta, 1992; Klibanov, 1990, 2001; Zaks and Klibanov, 1988b). However, nonaqueous biocatalysis has encountered several major challenges for large-scale applications such as high enzyme cost, protein fragility, and severely depressed enzyme activity. In particular, enzyme activity in nonaqueous media is typically lower by 2–5 orders of magnitude than in aqueous solutions (Klibanov, 1997; Zaks and Klibanov, 1988b). For instance, α-chymotrypsin and subtilisin in octane were 10^4–10^5 times less active than in water (Zaks and Klibanov, 1988b). Likely explanations (Burke et al., 1992; Klibanov, 1997) for activity depression include the limitation of substrate mass transfer to insoluble enzymes in organic solvents, poor accessibility to active sites of lyophilized or cross-linked enzyme particles, structural changes of enzyme molecules [e.g., proteins lyophilization causing drastic (although reversible) changes in secondary structures (Griebenow and Klibanov, 1995), and lyophilization/dehydration and organic solvents inducing active site disruption and protein unfolding (Burke et al., 1992)], unfavorable energetics of substrate desolvation (i.e. enzyme-substrate binding is weaken due to the tendency of substrate staying in organic solvents) and transition state stabilization (i.e. water stabilizes highly polar transition state much better than organic solvents), reduced conformational mobility, decreased molecular dynamics, and poor pH optimization.

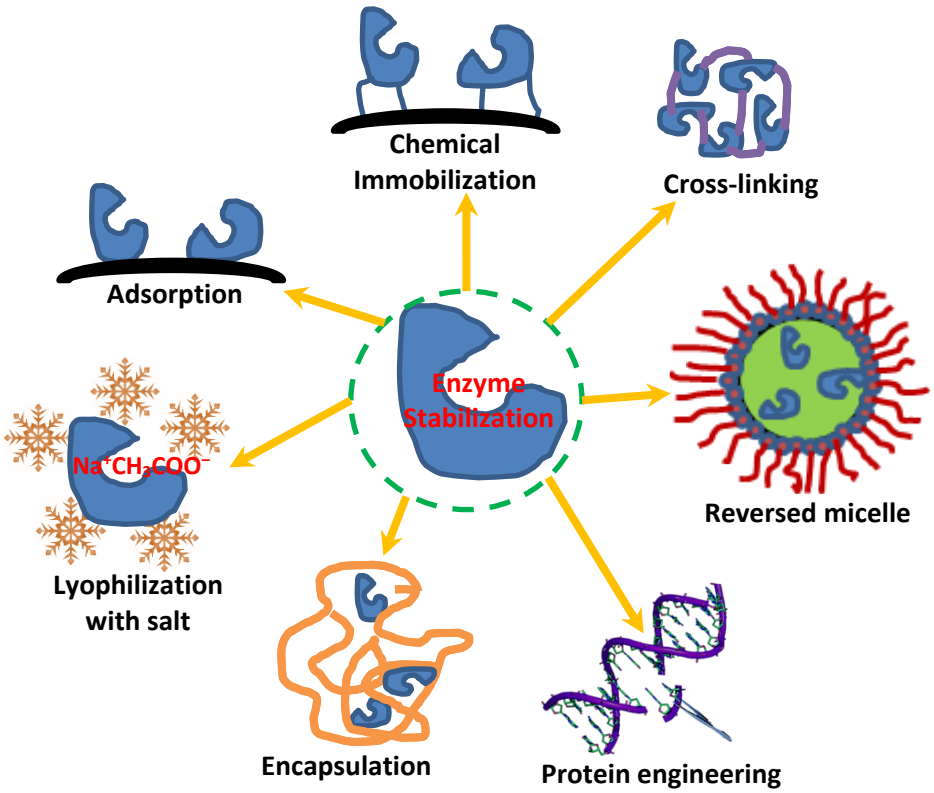


Figure 1 Common methods for enzyme stabilization in nonaqueous solvents.

Extremophilic proteins can withstand extreme conditions (such as high temperature, high pressure, high or low pH, and organic solvents), and are often associated with protein structures with more hydrophobic residues (for stronger hydrophobic interaction), more charged residues (for stronger electrostatic interaction), increased inter- and intramolecular hydrogen bonds (H-bonds), and the formation of disulfide bonds (Liszka et al., 2012). There are a few natural extremophilic enzymes being more tolerant to some molecular and ionic organic solvents, including proteases (Freeman et al., 1993; Gupta et al., 2005; Ogino et al., 1999), lipases (Li et al., 2014; Ogino et al., 2000; ShabtaI and Daya-Mishne, 1992; Shlmada et al., 1993; Sugihara et al., 1992; Yılmaz and Sayar, 2015), and cellulases (Ilmberger et al., 2013; Li and Yu, 2012; Tantayotai et al., 2016; Xu et al., 2016); in addition, thermophilic enzymes usually show high tolerance to organic solvents (Illanes, 1999; Liszka et al., 2012). In general, most native enzymes

are not tolerant to nonaqueous environments. Therefore, many approaches (see Figure 1 for representative examples) have been explored to improve the enzyme activity and stability in nonaqueous media (Stepankova et al., 2013) including:

- Physical or chemical immobilization of enzymes (Cao et al., 2003; Guisan, 2006; Lee and Dordick, 2002; Reslow et al., 1988), such as sol–gel encapsulation (Lee et al., 2007a; Lee et al., 2007b) and cross-linked enzyme aggregates (CLEAs) (Lopez-Serrano et al., 2002; Sheldon, 2007);
- Protein chemical modifications (Inada et al., 1986b) such as PEG modification (Inada et al., 1986a; Maruyama et al., 2004; Nakashima et al., 2005; Woodward and Kaufman, 1996);
- Genetic engineering/gene cloning of enzymes from extremophiles on mesophilic hosts (Gupta et al., 2008; Illanes, 1999; Yan et al., 2017);
- Protein engineering (Arnold, 1990; Liszka et al., 2012; Ogino and Ishikawa, 2001), including rational redesign/site-directed mutagenesis (SDM) (Antikainen and Martin, 2005; Duan et al., 2016; Takwa et al., 2011; Wong et al., 1990), directed evolution (Alvizo et al., 2014; Garcia-Ruiz et al., 2012; Reetz, 2002; Reetz and Carballeira, 2007), semi-rational design (Roth et al., 2017), and *de novo* design (Röthlisberger et al., 2008);
- Lyophilization with excipients/lyoprotectants including salts (for enzyme activation) (Dabulis and Klibanov, 1993; Dai and Klibanov, 1999; Khmelnitsky et al., 1994; Lindsay et al., 2004; Morgan and Clark, 2004; Ru et al., 2000; Ru et al., 2001);
- Molecular imprinting (Lee and Dordick, 2002) and ligand-induced ‘enzyme memory’ (Russell and Klibanov, 1988);

- Salt hydrates for water activity and pH controls (Halling, 1992; Schulze and Klibanov, 1991; Valivety et al., 1992; Yang et al., 1993);
- Enzyme precipitated and rinsed with *n*-propanol (EPRP) (Roy and Gupta, 2004; Shah and Gupta, 2007; Solanki and Gupta, 2008), or propanol-rinsed enzyme preparations (PREP) (Partridge et al., 1998; Theppakorn et al., 2004);
- Enzyme-amphiphile/surfactant complexes/ion-pairing (to solubilize enzymes in organic solvents) (Akbar et al., 2007; Meyer et al., 1996; Okahata and Ijiro, 1988; Paradkar and Dordick, 1994; Wangikar et al., 1997);
- Direct dissolution of hydrophilic enzymes in hydrophobic organic solvents (Mozhaev et al., 1991; Xu et al., 1997);
- Water-in-oil microemulsion or reverse micelles (Luisi, 1985; Oldfield, 1994) and water-in-IL microemulsion (Moniruzzaman et al., 2008; Pavlidis et al., 2009);
- Enzyme coating with ILs (Itoh et al., 2006; Lee and Kim, 2002; Lozano et al., 2007; Zou et al., 2014);
- Addition of organic bases (e.g., Et_3N) or acids (Lee and Dordick, 2002; Parker et al., 1998);
- Using "water-mimicking" or "molecular lubricant" solvents such as formamide, ethylene glycol, glycerol, dimethylformamide (DMF), dimethyl sulfoxide (DMSO), and ethylene glycol dimethyl ether (Almarsson and Klibanov, 1996; Kitaguchi et al., 1990; Kitaguchi and Klibanov, 1989; Riva et al., 1988; Triantafyllou et al., 1993; Xu et al., 1997).

Therefore, a careful design of water-mimicking nonaqueous solvents could lead to transition state stabilization, higher conformational mobility of enzymes, and improved enzyme-substrate binding. This review firstly focuses on the discussion of how organic solvents affect protein

conformation and dynamics, how organic solvent properties (such as polarity and hydrophobicity) influence the enzyme activity/stability, and how solvents with hydroxy and ether groups could induce enzyme activation. Furthermore, we summarize lessons learned from nonaqueous biocatalysis, and then systematically survey how functional groups have been incorporated into ILs to achieve more "water-like" biocompatible solvents.

2. Protein Conformation and Dynamics in Nonaqueous Media

Water associated with enzymes can be defined as two types (Figure 2): water buried inside the protein is known as 'internally bound water' (or 'buried water', or 'structural water') that can act as reactant and/or integral part of protein structure enabling stereospecific interactions; water within surface hydration shell of proteins is known as 'essential water' (or 'free water', or 'hydration water') (Gorman and Dordick, 1992; Lee et al., 1998; Meyer, 1992). Using the ^{18}O-labeling method, Dolman et al. (Dolman et al., 1997) determined residual water molecules per lysozyme and subtilisin Carlsberg molecule as 3–4 and 14–16 respectively after extensive drying; these numbers match well with those of buried or structural water molecules calculated from molecular modeling. The Mitchell-Koch group (Dahanayake et al., 2016) carried out the molecular dynamics (MD) simulations of *Candida antarctica* lipase B (CALB) and horse heart cytochrome *c* with four levels of crystallographic water (i.e. all crystallographic water, buried water, slow-diffusing water, and water within 2.7 Å) in organic solvents (such as acetonitrile, *n*-butanol and *tert*-butanol), and concluded that buried waters make the most contribution to rapid equilibration in nonaqueous media while slow-diffusing waters (diffusion coefficients are lower than 1/3 of the average after 10 ns simulation with all crystal waters) enable similar outcomes; keeping both buried and slow-diffusing waters quickly leads to an equilibrium protein structure and seems ideal for simulating protein dynamics in either aqueous or organic solvents. While one

study (Valivety et al., 1992) suggested that the highest esterification activity of immobilized *Mucor miehei* lipase in each organic solvent (i.e. hexane, toluene, trichloroethylene, diisopropyl ether, or 3-pentanone) was obtained at about the same thermodynamic water activity ($a_w = 0.5$), another group (Corrêa de Sampaio et al., 1996) reported highest transesterification activities of subtilisin Carlsberg in various solvents (hexane, toluene, diisopropyl ether, and their mixtures) being achieved at about 10% protein hydration; in these two cases, different amounts of water were required to reach the optimum enzyme activity in different solvents.

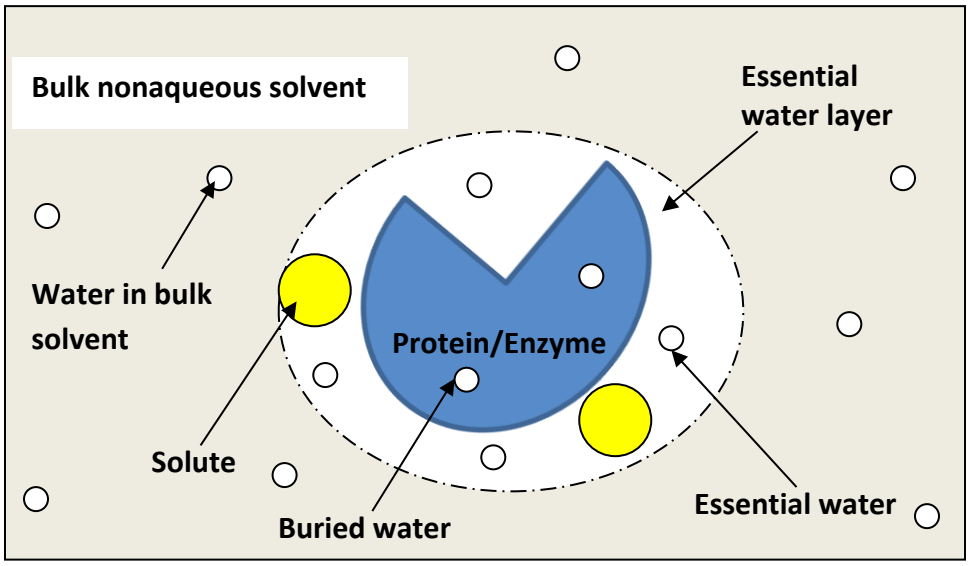


Figure 2 Protein/enzyme suspended in nonaqueous solvent and water distribution (solute could be an additive such as lyoprotectant or inhibitor; essential water layer might disappear due to water stripping by hydrophilic solvents).

The type of nonaqueous organic solvents can have drastic impact on the conformation and dynamics of proteins. Knowledge of conformational and dynamic behaviors of enzymes in organic solvents with low water contents is very important to the understanding of enzyme activity and stability (Guinn et al., 1991). Dry proteins are substantially native and relatively rigid; upon rehydration, protein flexibility increases accompanying with small local conformational changes (Careri et al., 1980; Finney and Poole, 1984; Poole and Finney, 1983).

171 The Klibanov group (Zaks and Klibanov, 1984) pointed out that dry porcine pancreatic lipase
172 was thermally stable at 100 °C for hours; furthermore, this group (Zaks and Klibanov, 1988b)
173 found subtilisin and α-chymotrypsin exhibited high structural rigidity in organic solvents, which
174 led to high kinetic barriers preventing protein unfolding from native-like conformation and thus
175 enabled higher thermal and storage stability of proteins in nonaqueous environment than in water.
176 Affleck et al. (Affleck et al., 1992b) observed a sharp increase (>6-fold) in transesterification
177 activity of subtilisin Carlsberg upon the addition of 0.5% (v/v) water in tetrahydrofuran (THF),
178 followed by a steep decline of the activity with further addition of water. Based on electron
179 paramagnetic resonance (EPR) spectra, they explained the initial increase was caused by an
180 increase in active-site polarity and protein flexibility due to protein hydration, but further
181 hydration led to even higher mobility and lower enzyme activity in nonaqueous environment.
182 They also explained that partially hydrated enzyme molecules have a different conformation
183 from mostly dry protein. Based on fluorescence and EPR spectra, the same group (Ryu and
184 Dordick, 1992) noted that water-miscible solvents (e.g., >30% v/v dioxane, >50% v/v methanol,
185 and >20% v/v acetonitrile) exposed the active site of horseradish peroxidase to the solvent,
186 which reduced the local polarity of active site. Because of the large increase in K_m value, they
187 suggested the major impact of organic solvents being ground-state stabilization of phenolic
188 substrates, which caused catalytic efficiency reduction by four orders of magnitude. The
189 Carpenter group (Dong et al., 1996) determined secondary structures of lyophilized α-
190 chymotrypsin and subtilisin Carlsberg suspended in organic solvents through infrared
191 spectroscopy, and found lyophilization perturbed secondary structures of both proteins. The
192 suspension of lyophilized α-chymotrypsin powder in ethanol, hexane or pyridine exhibited
193 minimum further disturbance of protein structure. However, subtilisin Carlsberg in ethanol

194 suffered further perturbation of secondary structures while suspension in hexane or pyridine
195 showed different degrees of return to native structure. Co-lyophilization of enzymes with
196 trehalose or sorbitol as the lyoprotectant (see Figure 2) retained more native conformations, but
197 their suspension in organic solvents usually led to large structural perturbations. This group
198 found no correlation of enzymatic transesterification activity with its secondary structures. Since
199 infrared spectroscopy is unable to measure the conformational change of active sites, they
200 suggested that solvents could affect the enzymatic process in several ways: polar solvents bind at
201 the active site; the solvent may change the substrate partition into enzyme/water complex; the
202 solvent can drastically modify the thermodynamic activity of a reactant (e.g., the activity
203 coefficient of hexanol in hexane is 10 times of the value in 2-butanone (van Tol et al., 1995));
204 and polar solvents may cause protein dehydration.

205 Following spectroscopic studies using ^{1}H NMR, near-UV and far-UV CD (circular
206 dichroism), the Klibanov group (Knubovets et al., 1999) reported that hen egg-white lysozyme
207 lost most of its tertiary structure after dissolution in ethylene glycol, methanol, DMSO,
208 formamide, and DMF (respectively); additionally, they observed changes in secondary structures:
209 a partially folded protein in ethylene glycol, a molten globule-type in methanol, and a random
210 coil in DMSO, formamide and DMF. Based on multinuclear NMR spectra of water bound to
211 subtilisin Carlsberg in THF, the Clark group (Lee et al., 1998) proposed a three-state model to
212 describe protease hydration: tightly bound, loosely bound, and free water. Tightly bound water
213 preserves the active conformation of lyophilized subtilisin while loosely bound water boosts up
214 the enzyme activity by increasing enzyme flexibility and active site polarity. By adding up to 0.6%
215 (v/v) aqueous solution of sodium dodecyl sulfate (SDS) into diisopropyl ether for lipase-
216 catalyzed esterification of a chiral carboxylic acid, Ueji et al. (Ueji et al., 2001) observed a

drastic increase in reaction rate and enantioselectivity. Further, their EPR spectra suggest this could be attributed to enhanced conformational flexibility of protein by adding the surfactant. The Smith group (Kurkal et al., 2005) conducted picosecond dynamic neutron scattering on pig liver esterase powders at 0%, 3%, 12%, and 50% hydration by mass and at temperatures of 120 to 300 K, and noted the existence of anharmonic and diffusive motion in the protein based on significant quasielastic scattering intensity. They further indicated that hydration-induced dynamical changes may increase the enzyme activity, but this is not a requirement for the enzyme to function. Through examining subtilisin Carlsberg co-lyophilized with inorganic salts and suspended in organic solvents by ^{1}H NMR relaxation experiments, the Clark group (Eppler et al., 2008) found that subtilisin's k_{cat} (apparent unimolecular rate constant or turnover number) was highly correlated with protein motions in the centisecond timescale, weakly related in the millisecond timescale, and unrelated on the piconanosecond timescale. Their ^{19}F NMR chemical shifts and hyperfine tensor measurements reveal enzyme activation being weakly correlated with changes in active-site polarity. In general, faster enzyme dynamics is essential for enzyme activation in nonaqueous media. Circular dichroism (CD) spectroscopy was used by Ogino and co-workers (Ogino et al., 2007) to probe conformational changes of several proteases in methanol. This group discovered that conformational stability and hydrolytic activities of α-chymotrypsin and thermolysin were lower in 25% (v/v) methanol than those in aqueous buffer while subtilisin and organic solvent-tolerant *Pseudomonas aeruginosa* protease (PST-01) showed an opposite order (i.e. higher stability and activity in methanol solution). They also observed less conformational changes for inhibited proteases (vs. proteases without inhibitors); both conformational transitions and autolysis of enzymes contribute to changes in CD spectra without the presence of protease inhibitors. In addition, since poly(amino acids) could form

particular conformations (e.g., α-helix and β-sheets) under certain conditions, their conformational transitions in the presence of methanol implied that proteins with a high content of β-pleated structure tend to be less stable in organic solvents.

Protein solvation is crucial to its structural dynamics as the solvent mobility contributes to protein flexibility. Recent MD simulations have provided valuable insights into protein dynamics. The Mitchell-Koch group (Dahanayake and Mitchell-Koch, 2018a; Dahanayake and Mitchell-Koch, 2018b) evaluated the CALB dynamics in aqueous and organic solvents (e.g., acetonitrile, cyclohexane, *n*-butanol, and *tert*-butanol), pointed out that solvation shell dynamics and protein dynamics are not the same at different regions (i.e. α-helix, β-sheet, and loop/connector region). Therefore, protein flexibility is less correlated with bulk solvent viscosity, but more relevant to local interfacial viscosity or the mobility ratio between organic molecules in a regional solvation layer and hydration dynamics near the same region. This group also compared CALB conformational transition rates (measured by the α5–α10 helix distance) between metastable/long-lived states such as open state, crystal-like state and closed state, and observed fastest transition rates in water and acetonitrile for the change of crystal-like to open conformation and much slower conformational changes in other less polar solvents. Interestingly, three metastable states of CALB in *tert*-butanol were all distributed in crystal-like conformation. Additionally, this group examined the effect of topology and hydrophobicity of protein surface on water structure and dynamics, and found a less dense and more tetrahedral solvation layer near concave and hydrophobic protein surfaces.

3. Effect of Solvent Properties on Enzyme Activity and Stability

Nonaqueous enzymatic reactions can be influenced by many factors including water content/thermodynamic water activity (a_w), pH/pH memory, temperature, biocatalyst forms and

preparations, and the type of nonaqueous media, etc. (Bell et al., 1995; Berberich et al., 2003; Halling, 1992; Ren et al., 2008; Valivety et al., 1992; Wang et al., 2016a; Yang et al., 1993). Most important solvent properties that can be correlated with enzyme activity and stability include polarity and hydrophobicity.

Solvent polarity can be quantified by various parameters such as dielectric constants (ε_r), Hildebrandt solubility (δ), dipole moments (μ), and solvatochromic polarity scales (such as E_T^N and Kamlet-Taft scales) (Reichardt, 1994). Hydrophobicity (or lipophilicity) overlaps with the polarity concept to a great extent, and is considered as a function of solvent polarity and entropic effect on water (Timson, 2019). In general, polar molecules tend to be hydrophilic/lipophobic and non-polar molecules tend to be hydrophobic/lipophilic. But there are some exceptions, for example, hydrophobic perfluorocarbons are non-polar and lipophobic at the same time (Riess, 2005), and some hydrophilic ILs are polar and also lipophilic (Manic and Najdanovic-Visak, 2016). Hydrophobicity is usually quantified by the log *P* scale, which is derived from the partition coefficient (K_{OW} or P) of un-ionized solutes between *n*-octanol and water at the unlimited dilution concentration of solute (Eqn. 1) (Sangster, 1989).

$$\log P = \lim_{c \to 0} K_{OW} = \lim_{c \to 0} \frac{C^o}{C^w} \quad (1)$$

C^o is the solute concentration in *n*-octanol phase and C^w is the solute concentration in aqueous phase.

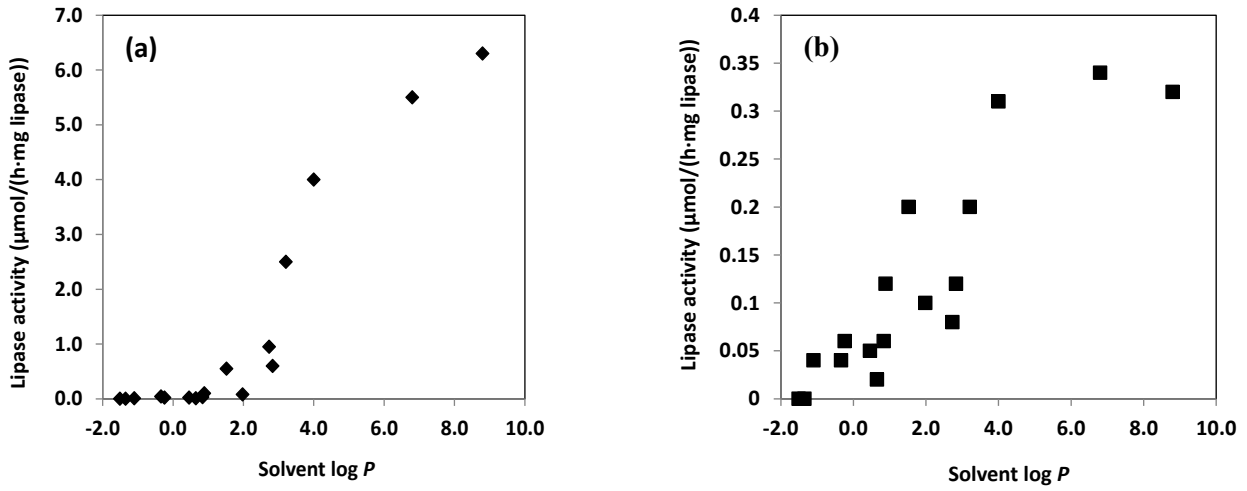


Figure 3 Correlation of transesterification rate of lipases with solvent log *P* values: (a) *Candida cylindracea* lipase and (b) *Mucor* sp. lipase (replotting from literature data: reaction rates of lipase-catalyzed transesterification of tributyrin and heptanol at 20 °C from Ref (Zaks and Klibanov, 1985); log P values from experimental data (Sangster, 1989) except hexadecane and dioxane from calculated data (Laane et al., 1987)).

Solvent polarity in terms of Hildebrandt solubility (δ), dipole moment (μ), and dielectric constant (ε) can be an important factor to enzyme activity (Affleck et al., 1992a; Brink and Tramper, 1985; Fitzpatrick and Klibanov, 1991; Gorman and Dordick, 1992; Halling, 2000; Kim et al., 2000; Schulze and Klibanov, 1991). Hildebrand solubility parameter (δ) can be calculated from the solvent heat of evaporation, which depends on polar interactions between solvent molecules; however, for apolar solvents, δ values are not sensitive to changes in apolarity and thus fall in a narrow range (Laane et al., 1985). Therefore, solvent polarity measured by δ values does not have a strong correlation with enzyme activity in apolar organic media. On the other hand, solvent hydrophobicity in terms of log *P* value has been found a good correlation factor for nonaqueous biocatalysis. The Laane group (Laane et al., 1985; Laane et al., 1987) replotted immobilized-cell activity (Brink and Tramper, 1985) and gas-producing anaerobic cell activity

300 (Playne and Smith, 1983) (respectively) with one of these solvent properties: dielectric constant
301 (ε), Hildebrand solubility parameter (δ), and hydrophobicity (log *P*), and concluded the activities
302 increased with log *P* values in general showing as "S"-shape curves. Similar correlations were
303 observed with yeast lipase and mold lipase activities with log *P* value (Figure 3) although
304 pancreatic lipase failed to follow the trend (lipase activity data from Ref. (Zaks and Klibanov,
305 1985)). Based on these experimental correlations, the Laane group (Laane et al., 1985; Laane et
306 al., 1987) suggested a rule of thumb for biocatalysis in nonaqueous solvents: low activity in polar
307 solvents with log $P < 2$; moderate activity in solvents with log $P = 2$–4, and high activity in
308 apolar solvents with log $P > 4$. Further optimization of biocatalytic activity can be achieved
309 when $|\log P_i - \log P_s|$ and $|\log P_{cph} - \log P_p|$ are at minimal while $|\log P_{cph} - \log P_s|$ and $|\log P_i -$
310 $\log P_p|$ are at maximal, where log P_i is for microenvironment of biocatalyst, log P_{cph} is for
311 continuous organic phase, log P_s is for the substrate, and log P_p is for the product. In case of
312 substrate inhibition, log P_i is optimized with respect to log P_s. For pure enzymes in neat organic
313 solvents where there is no interphase, log P_i is identical to log P_{cph} and thus the medium is
314 optimized with respect to log P_s and log P_p. This group (Hilhorst et al., 1984) further suggested
315 the combination of log *P* and molar mass of solvent as a good indicator of cell activity retention.
316 The log *P* rule was applied to understand the enzymatic reduction of apolar steroids progesterone
317 and prednisone catalyzed by 20β-hydroxysteroid dehydrogenase in reversed micellar media
318 comprising cetyltrimethylammonium bromide, hexanol, another organic solvent (e.g. octane),
319 and Hepes buffer, where $|\log P_i - \log P_s|$ was minimized to reach a high steroid content in
320 interphase and $|\log P_{cph} - \log P_s|$ was maximized to maintain a low concentration of steroid in
321 continuous phase. Reslow et al. (Reslow et al., 1987a) evaluated α-chymotrypsin-catalyzed
322 transesterification reaction in various organic solvents, and noted that the enzymatic reaction

followed Michaelis-Menten kinetics with a slight decrease of K_m with the increasing solvent log *P* and a drastic increase in V_{max} with the log *P* value. They also observed the transesterification activity at 2% (v/v) water increased with the log *P* value to a maximum (when log *P* is about 1.6) and then declined with a further increase in log *P*. The same group (Reslow et al., 1987b) explained that corrected log *P* values taking water into consideration (see Eqn. 2) showed a better correlation with the α-chymotrypsin activity.

$$\log P_{corr} = (1 - x) \log P_{solvent} + xlogP_{water} \quad (2)$$

where *x* is the mole fraction of water (in term of water solubility in organic solvent), organic solvent log *P* is noted as log $P_{solvent}$, and water log *P* is noted as log P_{water} (= –1.396 as calculated from Eqn. (1) using the water solubility in *n*-octanol at 25 °C). Their results suggest that less water is needed for more hydrophobic solvents to reach maximum enzyme activity. A similar 'bell-shape' trend was observed by the Zhao group (Zhao et al., 2009a) in the lipase-catalyzed transesterification reaction in ILs although initial reaction rate reached its maximum at a much lower log *P* value (–0.90). Another study by Lou et al. (Lou et al., 2005) on the lipase-catalyzed ammonolysis of (*R*,*S*)-p-hydroxyphenylglycine methyl ester reported initial rates increasing with hydrophobicity of BF_4^--based ILs to a maximum, and then decreasing with even higher hydrophobicity. The decrease in enzyme activity with log *P* after the maximum is possibly due to substrate ground-state stabilization (e.g., strong substrate solvation (Kim et al., 2000), and up to 10^4-fold reduction in catalytic efficiency (Ryu and Dordick, 1992)) or hydrophobic interactions (moving protein's hydrophobic moieties from interior to exterior (Timson, 2019)) in highly hydrophobic solvents.

The Klibanov group (Zaks and Klibanov, 1988b) compared transesterification activities of subtilisin and α-chymotrypsin in various organic solvents containing <0.02% (v/v) water, and

found the enzymatic reaction followed Michaelis-Menten kinetics with V_{max}/K_m values increasing with high solvent hydrophobicity. Both enzymes were highly active in hexadecane and octane. This study on proteases and another study from the same group (on yeast alcohol oxidase, mushroom polyphenol oxidase, and horse liver alcohol dehydrogenase) (Zaks and Klibanov, 1988a) suggest that hydrophilic solvents could strip off 'essential water' from enzymes (see Figure 2), and influence the enzymatic process directly. Nurok and co-workers (Nurok et al., 1999) developed regression models for transesterification activities of subtilisin Carlsberg and *Candida rugosa* lipase with organic solvent properties, found either log *P* or a descriptor containing log *P* correlates with enzyme activities. Pogorevc et al. (Pogorevc et al., 2002) observed little deactivation of two lipases and hydroxynitrile lyase by organic solvents with log $P \geq 1$; however, the detrimental impact of protic solvents (such as alcohols) caused by their hydrogen-bonding is underestimated by the log *P* scale. Clark and co-workers (Guinn et al., 1991) concluded that the activity of horse liver alcohol dehydrogenase for the oxidation of cinnamyl alcohol increased in a more hydrophobic (a lower dielectric constant ε_r) organic solvent such as hexane (ε_r = 1.9) containing ~0.02–10% water, which corresponded with more rigid protein structures based on EPR spectra. Even in aqueous solutions of water-miscible organic solvents (mole fraction of water 0.8–0.998), the Nagamune group (Hirakawaa et al., 2005) found k_{cat} of 1-pentanol oxidation catalyzed by alcohol dehydrogenase from *Aeropyrum pernix* increased almost linearly with log *P* values of mixed solvents (using the mixing rule in literature (Hilhorst et al., 1984)) and k_{cat} in aqueous acetonitrile (its mole fraction 0.1) was 10 times higher than that in aqueous buffer. They suggested that enzyme activation depended on log *P* value of solvent mixture but was independent of solvent composition. And they further indicated that with an increase in log *P*, both activation enthalpy and entropy decreased but overall free energy of

activation decreased. In aqueous organic solvents (25–50%, v/v), protease and lipases usually stayed catalytically active and stable in C_7–C_{10} alkanes, cyclohexane, and DMSO, but much less active in most mono-alcohols (Ogino et al., 2000; Ogino et al., 1999; Shimada et al., 1993).

The mechanism of water stripping from enzymes by hydrophilic solvents (see Figure 2) was confirmed by MD simulations. Yang et al. (Yang et al., 2004) studied surfactant-solubilized subtilisin BPN' in water and three organic solvents (i.e. octane, THF, and acetonitrile), and found no significant difference in terms of overall enzyme structure and flexibility in these solvents over the timescale of several nanoseconds. The major difference is the partition of hydration water between enzyme molecules and bulk solvent. With the increasing of solvent polarity (octane → THF → acetonitrile), hydration water is more stripped from enzyme's surface, and polar solvent molecules begin to penetrate into crevices on enzyme's surface and into the active site to replace mobile and weakly bound water molecules. Another study of molecular dynamics/molecular mechanics (MD/MM) simulations of a serine protease cutinase in water and five organic solvents (i.e. hexane, diisopropyl ether, 3-pentanone, ethanol and acetonitrile) demonstrated that the nature of organic solvents determines their ability in stripping off water from enzyme's surface (Micaêlo and Soares, 2007). The simulations results picture that enzyme's surface is surround by clusters of water molecules in organic solvents, preferentially near charged/polar residues. In nonpolar solvents (e.g., hexane, diisopropyl ether, and 3-pentanone), large clusters of water molecules are seen around enzyme's surface while smaller aggregates of water exist in polar solvents (e.g. ethanol and acetonitrile). In addition, polar solvents may replace some water molecules at enzyme's surface and affect the structure and dynamics of protein molecules. At low hydration state, ions could preferentially bind to the protein.

On the contrary, a number of studies suggest that enzyme performance is not correlated with solvent polarity and hydrophobicity. The Klibanov group (Narayan and Klibanov, 1993) examined transesterification activities of three lipases and one protease in anhydrous organic solvents, and concluded water-immiscibility and apolarity of the solvent could not be correlated with enzyme activity. The solvent hydrophobicity (usually measured by log *P* value) could indicate general enzyme activity trend in nonaqueous media, but may not correlate with enzyme activity in a selected narrow range of log *P* values. The Wandrey group (Villela Filho et al., 2003) pointed out that the stability of three alcohol dehydrogenases (ADH) in biphasic systems failed to follow the log *P* rule for organic solvent selection. All three alcohol dehydrogenases showed the highest stability in the biphasic system of 50/50 (v/v) aqueous phase/ *tert*-butyl methyl ether.

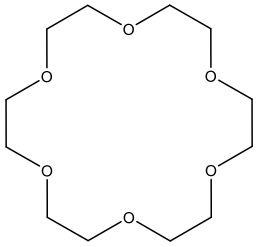


Figure 4 Structure of 18-crown-6.

4. Enzyme Activation by Crown Ethers and Cyclodextrins

Crown ethers can activate enzymes by two methods: as reaction additives, or as co-lyophilizing agents. The most commonly used crown ether is 18-crown-6 (Figure 4). Reinhoudt and co-workers (Reinhoudt et al., 1989) studied the addition of several crown ethers (0.75 mM) in protease-catalyzed transesterification of *N*-acetyl-L-phenylalanine ethyl ester with 1-propanol in *n*-octane, and observed the highest enzyme activation by 18-crown-6 (up to 4.1-fold activation for α-chymotrypsin and 2.0-fold activation for subtilisin). Later, this group (Broos et al., 1992; Engbersen et al., 1996) reported higher α-chymotrypsin activation by using the same transesterification reaction in various organic solvents, and obtained 31-fold protease activation

in dibutyl ether, 29-fold in octane, and 19-fold in cyclohexane via adding 2.0 mM 18-crown-6. The same group (van Unen et al., 2002) further indicated that α-chymotrypsin activity increased with the concentration of 18-crown-6, and reached 13-fold enzyme activation in cyclohexane at 4 mM crown ether. Itoh et al. (Itoh et al., 1993; Itoh et al., 1996) also employed crown ethers as additives (>250 molar equiv. based on the enzyme) in the lipase-catalyzed hydrolysis of 2-cyano-1-methylethyl acetate, and observed faster reactions and higher enantioselectivity in the presence of crown ethers (in particular benzo-crown, armed azacrown, and thiacrown). They confirmed that accelerated reaction rates were due to the binding of reaction product with crown ether, and enhanced enantioselectivity was due to crown ether interaction with active sites modifying the lipase local conformation. When studying the peptide formation in acetonitrile catalyzed by cross-linked crystals of thermolysin and subtilisin Carlsberg, van Unen et al. (van Unen et al., 1998a; van Unen et al., 1998c) found that direct addition of 18-crown-6 into the reaction mixture showed no impact on enzyme activity, but observed 13 times of enzyme activation after soaking enzyme crystals in acetonitrile containing crown ether followed by overnight evaporation of the solvent at room temperature. Chang et al. (Chang et al., 2016) observed that 18-crown-6 was able to improve the thermal stability of cellulase. An early study by Odell and Earlam (Odell and Earlam, 1985) indicated that some proteins (e.g., cytochrome *c*, bovine serum, lysozyme and myoglobin) could form complexes with crown ethers, resulting in protein dissolution in nonaqueous media such as methanol. Following this study, the Tsukube group (Paul et al., 2003) formed cytochrome *c* complexes with 18-crown-6 in methanol to convert biologically inactive heme structure to catalytically cold-active synzymes. They observed non-biological six-coordinate heme in methanol for each cytochrome *c*, and the degradation of hemes with H_2O_2 was considerably depressed at cold temperatures (e.g., –20 to –60 °C). At these low temperatures,

436 cytochrome *c* complexes were able to promote a faster oxidation of (*S*)-naphthyl methyl
437 sulfoxide than its (*R*)-isomer (up to 49% ee and 79% conversion at –40 °C by pigeon breast
438 cytochrome *c*).

439 The second method of activation is the co-lyophilization of crown ether with the enzyme.
440 The Reinhoudt group (Broos et al., 1995; Engbersen et al., 1996) co-lyophilized serine proteases
441 with crown ethers, and reported that α-chymotrypsin co-lyophilized with 250 molar equiv. of 18-
442 crown-6 resulted in 640 times increase in its transesterification activity (only 50 times lowers
443 than the hydrolytic activity in water); co-lyophilization activation was 30 times more than the
444 direct addition of crown ether in the solvent (i.e. cyclohexane). Following the same process using
445 500 equiv. 18-crown-6, subtilisin Carlsberg was more active by 28 times and trypsin was more
446 active by 216 times while acetyltrypsin was not activated. In a later communication (van Unen et
447 al., 2002), this group re-optimized 18-crown-6 concentration to 50 equiv. for co-lyophilization to
448 achieve 470-fold α-chymotrypsin activation for the same reaction; they explained higher crown
449 ether concentrations could lead to enzyme dehydration to become less active (van Unen et al.,
450 2001). Furthermore, the same group (van Unen et al., 1998a, b) found that α-chymotrypsin co-
451 lyophilized with 50 molar equiv. of 18-crown-6 exhibited 425-fold improvement in activity
452 during the enzymatic formation of dipeptide in acetonitrile. They also observed more
453 pronounced activation by crown ether in hydrophilic solvents (i.e. acetonitrile, dioxane, and 2-
454 butanone) than in hydrophobic solvents (i.e. 3-pentanone, *tert*-amyl alcohol, and toluene). On the
455 contrary, this group noted that for the transesterification reaction catalyzed by α-chymotrypsin
456 co-lyophilized with 50 equiv. of 18-crown-6, the crown ether activation decreased with a higher
457 solvent polarity (a lower log *P* value); for example, 470-fold activation in cyclohexane dropped
458 to 93-fold in acetonitrile. The explanation is that enzyme molecules in hydrophobic solvents

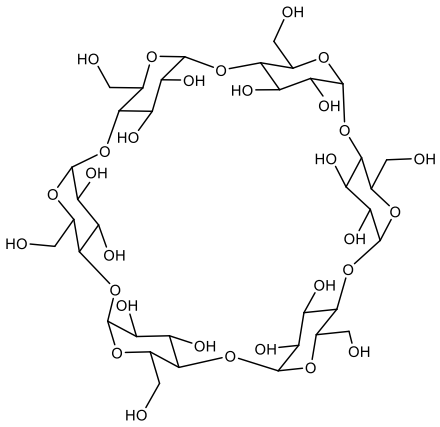
459 have more salt bridges that needs to be disrupted by crown ether; salt bridges tend to lock in
460 inactive enzyme conformations.

461 In terms of activation mechanism, this group (Broos et al., 1995) initially suspected that
462 crown ethers act as amphiphiles between enzyme's polar surface and nonpolar organic solvents
463 to move water molecules from active site upon substrate binding (de Jong et al., 1976). Later, the
464 Reinhoudt group (van Unen et al., 2001) measured Michaelis-Menten kinetics of α-chymotrypsin
465 in toluene, and found significantly higher V_{max} value but slightly lower K_m value in the presence
466 of 18-crown-6, implying noncompetitive interactions and no direct interaction of crown ether
467 with active sites to induce enzyme activation. Odell and Earlam (Odell and Earlam, 1985)
468 suggested that some proteins could form complexes with crown ethers possibly through
469 interactions with alkylammonium ions of lysine residues. Through electrospray ionization (ESI)
470 quadrupole ion-trap mass spectrometry, Julian and Beauchamp (Julian and Beauchamp, 2001)
471 suggested that peptides form stable supramolecular adducts with 18-crown-6 preferentially with
472 protonated amine on the side chain of lysine and further form multi-charged complexes through
473 crown ether interacting with adjacent lysines, while the complexation with side chains of
474 histidine and arginine is less competitive. Therefore, enzyme activation by crown ethers could be
475 due to interactions of 18-crown-6 with lysine ammonium and/or tyrosine hydroxy groups (so
476 called "macrocyclic interactions") (Broos et al., 1995; van Unen et al., 2001, 2002): there are
477 more lysine residues in α-chymotrypsin (14) and trypsin (14) than in subtilisin Carlsberg (7-8);
478 however, in acetyltrypsin, lysine ammonium and tyrosine hydroxys are acetylated (Labouesse
479 and Gervais, 1967). This explains the earlier data (Broos et al., 1995) that α-chymotrypsin and
480 trypsin could be more activated by 18-crown-6 than subtilisin Carlsberg while acetyltrypsin was
481 not activated. Such macrocyclic interactions minimize the formation of inter-and intramolecular

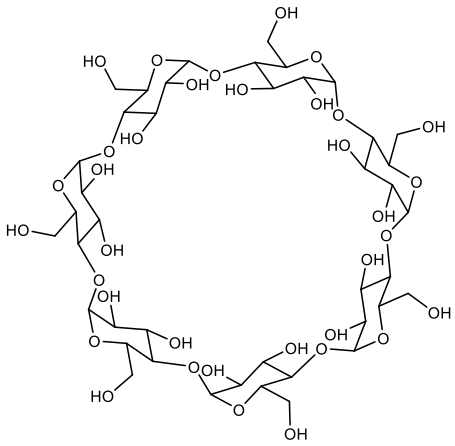
salt bridges, and reduce kinetic conformational barrier allowing the protein to refold back to thermodynamically stable and catalytically active conformations. Enzyme activation due to the direct addition of crown ethers into reaction mixture can be primarily attributed to these macrocyclic interactions. However, for enzymes co-lyophilized with crown ether, the Griebenow group (Santos et al., 2001) pointed out the primary contribution to enzyme activation being "molecular imprinting effect". This means that enzyme's active site structure is locally preserved by crown ethers during lyophilization, and even after exposure to organic solvents when crown ethers leach out. The preserved enzyme structure can be understood as kinetically but not thermodynamically stable in organic solvents. This group (Santos et al., 2001) indicated that subtilisin co-lyophilized with crown ethers showed poor storage stability (at 4 or 25 °C), losing its activities in days. Furthermore, the Griebenow group (Griebenow et al., 2001) determined secondary structures of subtilisin co-dried with 18-crown-6 in 1,4-dioxane and its thermal denaturation temperature (T_d) by infrared spectroscopy, and suggested that crown ether-to-subtilisin preparation at 0.7 mass ratio showed similar secondary structures and rigidity as the protease in water, corresponding to the highest transesterification activity obtained in 1,4-dioxane. On a similar note, the Reinhoudt group (van Unen et al., 2002) concluded that enzyme activation resulted from co-lyophilization with crown ethers is only partially due to specific macrocyclic complexation, but largely due to nonmacrocyclic lyoprotection.

Cyclodextrins are macrocyclic oligosaccharides produced by the enzymatic conversion of starch catalyzed by cyclodextrin glycosyltransferase. Three common naturally-occurring cyclodextrins (α-, β-, and γ-) comprise 6, 7, and 8 α-D-glucopyranosyl units respectively, via α-1,4-glycosidic linkages (Figure 5). Cyclodextrins are generally soluble in water but insoluble in most organic solvents (except DMSO, MDF, and *N*-methylpyrrolidone) (Hedges, 2009).

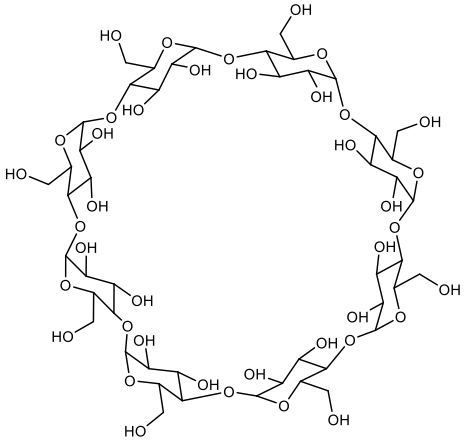
505 Cyclodextrins and their modified forms have wide industrial applications in food, chemical,
506 pharmaceutical, drug delivery, agriculture, and environmental engineering sectors. The review is
507 interested in the use of cyclodextrins as additives or lyoprotectants to activate the enzyme.
508



(a) α-cyclodextrin



(b) β-cyclodextrin



(c) γ-cyclodextrin

509

510 **Figure 5** Structures of α-, β-, and γ-cyclodextrins.

511 The Kise group (Ooe et al., 1999) co-lyophilized α-chymotrypsin with various
512 cyclodextrins, and found 2,3,6-tri-*O*-methyl β-cyclodextrin was able to increase the enzymatic
513 transesterification activity by 40-fold in acetonitrile containing 3% (v/v) water. In addition,
514 hydroxypropylated β- or γ-cyclodextrin was able to maintain >98% of α-chymotrypsin initial
515 activity after 6 h of incubation at 30 °C in acetonitrile (with 3% v/v water). The Griebenow

group (Santos et al., 1999) co-lyophilized subtilisin Carlsberg with methyl β-cyclodextrin (1:6 mass ratio), and reported that this enzyme preparation drastically improved the protease activity and/or enantioselectivity in two transesterification reactions performed in dry THF and acetonitrile: the initial rate of *N*-acetyl-L-phenylalanine ethyl ether with 1-propanol increased by 53 folds in THF; the initial rate (*S*-enantiomer) of vinylbutyrate with 1-phenylethanol increased by 164 times and its enantioselectivity increased by 1.8 times. However, a small amount of water (as low as 0.1%, v/v) in nonaqueous media began to diminish the enhancement in activity and enantioselectivity induced by methyl β-cyclodextrin co-lyophilization. The activation mechanism was explained as methyl β-cyclodextrin increasing enzyme flexibility in organic media, and minimizing structural changes of the protein during lyophilization. Secondary structures of this subtilisin preparation obtained from FTIR by the same group (Griebenow et al., 1999) suggested that the enantioselectivity correlated well with protein's structural integrity: subtilisin enantioselectivity increased with a higher α-helix content (i.e. less perturbed structure). For *Candida rugosa* lipase co-lyophilized with methyl β-cyclodextrin, a similar enzyme improvement (16.8-fold increase in initial rate for *R*-enantiomer and 2.7-fold increase in enantioselectivity) was seen in the transesterification between 1-phenylethanol and vinyl butyrate (Griebenow et al., 1999). The Barletta group (Montañez-Clemente et al., 2002) further expanded the subtilisin-catalyzed transesterification of vinyl butyrate with 1-phenylethanol to several racemic alcohols as substrates in organic solvents (THF, 1,4-dioxane, acetonitrile, dichloromethane, toluene, and octane), and confirmed that subtilisin co-lyophilized with methyl-β-cyclodextrin enhanced its activity and enantioselectivity due to structural preservation during lyophilization. THF and 1,4-dioxane were identified as the best solvents for these enzymatic reactions while acetonitrile was the worst. Watanabe and co-workers (Watanabe et al., 2006)

observed that a small amount (5 mM) of α-, β-, or γ-cyclodextrin could activate 4-α-glucanotransferase by 6.5, 6.6, and 4.9 folds respectively to cleave maltotriosyl residue from the maltotetraosyl branch. Other mechanisms of enzyme activation by cyclodextrins include complexing with the inhibitor (Li et al., 2013; López-Nicolás et al., 2007; Orenes-Piñero et al., 2007), increasing the substrate solubility (Cui et al., 2013), delivering negatively charged substrate to the enzyme (Davis et al., 2004), and enzyme complexing with cyclodextrin through higher secondary interactions (i.e. hydrophobic interactions, hydrogen-bonding, and van der Waals forces) (Canbolat et al., 2017; Denadai et al., 2006). As these mechanisms are primarily explored for aqueous enzymatic processes, they are not discussed in detail by this review.

On the other hand, cyclodextrins and derivatives might inhibit enzyme activities due to the sequestration of substrate to reduce its free concentration as illustrated by several examples, such as cyclodextrins complexes with chlorogenic acid or 4-methyl catechol inhibiting the activity of apple polyphenol oxidase (causing juice browning) (Irwin et al., 1994; Peralta-Altier et al., 2018), the inhibition of hydroperoxidase activity of lipoxygenase due to the formation of xenobiotics complex in the cavity of cyclodextrins (Núñez-Delicado et al., 1999), the complexation of *tert*-butylcathechol in the cavity of hydroxypropyl-β-cyclodextrin and γ-cyclodextrin causing the substrate sequestrant effect and the inhibition of *Streptomyces antibioticus* tyrosinase (Orenes-Piñero et al., 2007). Another inhibition mechanism was reported by Sule et al. (Sule et al., 2015) when they observed *Escherichia coli* methionine aminopeptidase (MetAP) was inhibited by 2-hydroxypropyl-β-cyclodextrin (HP-β-CD). The inhibition reason was described as the formation of non-productive ternary complex by bridging two ends of the substrate (methionyl-7-amino-4-methylcoumarin) with MetAP and HP-β-CD, respectively.

5. Enzyme-Compatible Organic Solvents for Nonaqueous Biocatalysis

Long-chain alkanes and halogenated alkanes are hydrophobic and compatible with many enzymes such as subtilisin and α-chymotrypsin in hexadecane and octane (Zaks and Klibanov, 1988b), α-chymotrypsin in 1,2-dichloroethane, chloroform and toluene (Reslow et al., 1987a), α-chymotrypsin in isooctane containing 2 mM Aerosol OT (AOT) as the surfactant (Paradkar and Dordick, 1994), alcohol dehydrogenase in heptane (Guinn et al., 1991), and lipases and hydroxynitrile lyase in hexane and dodecane (Pogorevc et al., 2002), etc. Some representative examples are illustrated in Table 1.

Table 1 Enzyme-compatible organic solvents for nonaqueous biocatalysis

Enzyme	Compatible organic solvents	Ref
subtilisin (protease)	hexadecane and octane	(Zaks and Klibanov, 1988b)
	DMF	(Riva et al., 1988)
	1,4-dioxane, benzene, Et_3N and THF (enantioselectivity)	(Fitzpatrick and Klibanov, 1991)
	hexane, diisopropyl ether, and THF	(Corrêa de Sampaio et al., 1996; Khmelnitsky et al., 1994)
	tert-amyl alcohol	(Kim et al., 2000)
α-chymotrypsin (protease)	hexadecane and octane	(Zaks and Klibanov, 1988b)
	isooctane	(Paradkar and Dordick, 1994)
	diisopropyl ether, 1,2-dichloroethane, chloroform and toluene	(Reslow et al., 1987a)
Porcine pancreatic lipase	hexane, diethyl ether, diisopropyl ether, dibutyl ether and dodecane	(Zaks and Klibanov, 1985)
	nitromethane, DMF, Et_3N and *tert*-amyl alcohol (enantioselectivity)	(Fitzpatrick and Klibanov, 1991)
Candida cylindracea lipase	hexadecane, dodecane and hexane	(Zaks and Klibanov, 1985)
Lipases from *Pseudomonas* sp. and *Candida rugosa*	hexane and dodecane	(Pogorevc et al., 2002)
Candida rugosa lipase	Diisopropyl ether	(Ueji et al., 2001)
alcohol dehydrogenase	heptane	(Guinn et al., 1991)
hydroxynitrile lyase	hexane and dodecane	(Pogorevc et al., 2002)

570 Polyols and sugars (e.g. sorbitol and trehalose) are so called 'compensatory solutes' that
571 provide stabilization to proteins in aqueous media (Kaushik and Bhat, 1998, 2003). For instance,
572 the thermal stability of glucose dehydrogenase in aqueous solutions can be correlated with the
573 number of hydroxy groups in polyols in an increasing order of glycerol (3) < erythritol (4) <
574 xylitol (5) < sorbitol (6) (Obón et al., 1996). A likely explanation is that polyols increase the
575 surface tension of water leading to preferential hydration of proteins in aqueous media (Kaushik
576 and Bhat, 1998). Protease and lipases could be more stable in 25–50% (v/v) aqueous solutions of
577 ethylene glycol or 1-hexanol than in buffer alone, but not always stable in aqueous alcohols such
578 as methanol, ethanol, isopropanol, 1-butanol, and *tert*-butanol (Ogino et al., 2000; Ogino et al.,
579 1999; Shimada et al., 1993).

580 However, some alcohols and ethers at (nearly) dried state are highly compatible with
581 enzymes. *tert*-Butanol could afford high enzyme activities in many enzymatic processes, such as
582 peptide preparation catalyzed by immobilized papain (Theppakorn et al., 2004), and lipase-
583 catalyzed transesterification and ammoniolysis reactions (Degn et al., 1999; Madeira Lau et al.,
584 2004; Madeira Lau et al., 2000; Royon et al., 2007; Toral et al., 2007; van Rantwijk et al., 2006;
585 Zhang et al., 2011). When carrying out the Novozym 435-catalyzed transesterification
586 between ethyl sorbate and 1-propanol, the Zhao group (Zhao et al., 2019b) observed that the
587 lipase (CALB) was more active in *tert*-butanol than in several ILs including the ether-
588 functionalized type. Compared with primary alcohols (such as 1-butanol), *tert*-butanol is less
589 inhibitory to the enzyme, and less reactive as a substrate (Madeira Lau et al., 2000). MD
590 simulations of CALB suggest a high similarity of CALB structures in *tert*-butanol and in
591 three-site model (TIP3P) water; high compatibility of CALB in *tert*-butanol is due to several
592 reasons (Park et al., 2013) such as high protein flexibility in *tert*-butanol, well-maintained

593 substrate's entrance size and enzyme's binding pocket size, and preservation of hydrogen-
594 bonding of Ser105 with His 224 [Ser105–His224–Asp187 is known as the 'catalytic triad' in
595 the active site of CALB (Uppenberg et al., 1994; Uppenberg et al., 1995)]. Some ethers are also
596 highly compatible with enzymes in nonaqueous biocatalysis. Diisopropyl ether has enabled high
597 transesterification activities when catalyzed by lipases (Itoh et al., 2001; Itoh et al., 2004; Itoh et
598 al., 2006; Itoh et al., 2003; van Tol et al., 1995) or α-chymotrypsin (Reslow et al., 1987a). Other
599 ethers (such as 2,2-dimethoxypropane and 2-ethoxyethyl ether) led to high conversions in
600 enzymatic acylation of 6-aminopenicillanic acid and D-phenylglycine amide catalyzed by cross-
601 linked enzyme aggregates (CLEAs) of Penicillin G acylase (Cao et al., 2001).

602 Ou et al. (Ou et al., 2011a) suggested that enzyme-compatible and enzyme-soluble
603 solvents should have high dielectric constants (ε_r) and high electron pair donating and accepting
604 ability just like water (ε_r = 78.30). Following this rationale, they selected glycerol carbonate (ε_r =
605 82.66) as enzyme-soluble solvent for the transesterification of ethyl butyrate and 1-butanol at
606 40 °C catalyzed by CALB and *Candida rugosa* lipase (CRL) respectively. Glycerol carbonate
607 enabled a much higher ethyl butyrate conversion than acetonitrile and DMF; although
608 comparable conversions were reported in glycerol carbonate and water, the substrate conversion
609 reported in water by this group probably referred to enzymatic hydrolysis of ethyl butyrate
610 instead of transesterification. This group (Ou et al., 2012) further conducted the same enzymatic
611 transesterification reaction in glycerol carbonate or *N*-hydroxymethyl formamide catalyzed by
612 CALB and *Pseudomonas cepacia* lipase respectively, and observed comparable initial rates
613 (based on ethyl butyrate conversion) as that in water. It is important to point out that the substrate
614 conversion includes both enzymatic transesterification and hydrolysis, and does not truly reflect
615 the transesterification activity. Especially in water, the hydrolysis is expected to predominate

over transesterification [although in the presence of surfactant, enzymatic esterification could proceed in aqueous solutions containing miniemulsions (Aschenbrenner et al., 2009)].

6. Lessons Learned from Enzyme Behaviors in Organic Solvents

Despite some exceptions (Fitzpatrick and Klibanov, 1991; Narayan and Klibanov, 1993), many enzymatic reactions in nonaqueous media tend to follow the 'bell-shape' rule of thumb: the enzyme activity and stability increase with the solvent hydrophobicity (i.e. enzymes tend to be more active and stable in hydrophobic solvents than in hydrophilic ones), but may begin to decline when the solvent is too hydrophobic. Very hydrophobic solvents may cause strong substrate solvation and substrate's ground-state stabilization with up to four orders of magnitude in catalytic efficiency reduction (Kim et al., 2000; Ryu and Dordick, 1992).

Although enzymes and their various preparations insoluble in nonaqueous media are advantageous to the recycle and reuse of biocatalysts and to product separation, their suspension in reaction mixture as a heterogeneous system causes the limitation of substrate mass transfer and depression of enzyme activity (Burke et al., 1992; Klibanov, 1997). Efforts have been put forward to solubilize enzymes in nonaqueous environments. Some molecular and ionic organic solvents are capable of dissolving enzymes. Hen egg-white lysozyme could be dissolved at >10 mg/mL solubility in polar, protic, and hydrophilic organic solvents (such as alcohols including glycerol, amides and DMSO), and more than 50 mg/mL in diols (e.g., ethylene glycol and 1,3-propanediol) as demonstrated by the Klibanov group (Chin et al., 1994). The protein solubility shows weak correlations with dielectric constants or Hildebrand solubility parameters of organic solvents. The same group (Rariy and Klibanov, 1999) reported that the addition of 1.0 M salts like LiCl into 60% (v/v) protein-dissolving diols could considerably (up to >100 times) improve the folding of unfolded hen egg-white lysozyme. The underlying cause is that salts increase the

639 protein solubility and thus suppress nonspecific protein aggregation during refolding.
640 Hydrophilic ILs with hydrogen-bonding basic anions (e.g., NO_3^-, lactate, $EtSO_4^-$, and CH_3COO^-,
641 etc.) could dissolve enzymes [while other hydrophilic ILs carrying anions like BF_4^- do not
642 dissolve enzymes (Madeira Lau et al., 2004)], but these ionic solvents strongly interact with the
643 protein (mainly through hydrogen-bonding) causing enzyme deactivation (Bermejo et al., 2008;
644 de los Ríos et al., 2007; Madeira Lau et al., 2004; Toral et al., 2007; Turner et al., 2003; Zhao et
645 al., 2008; Zhao et al., 2009a). On the other hand, several IL systems are capable of dissolving
646 enzymes and maintaining their activities. Cholinium dihydrogen phosphate ([Ch][H_2PO_4], m.p.
647 119˚C) containing 20% (wt) water could dissolve and stabilize cytochrome *c* (Fujita et al., 2006;
648 Fujita et al., 2005; Fujita et al., 2007). This same group (Fujita and Ohno, 2010) further pointed
649 out that [Ch][H_2PO_4] (with 30 wt% water) was able to dissolve various metallo proteins
650 (cytochrome *c*, peroxidase, ascorbate oxidase, azurin, pseudoazurin, and D-fructose
651 dehydrogenase) and also maintained their active sites and secondary structures, leading to the
652 findings that some proteins retained their activities and D-fructose dehydrogenase exhibited
653 much improved thermal stability. Bisht et al. (Bisht et al., 2017) found aqueous cholinium
654 glutarate ([Ch][Glu]:H_2O, 1:1 mass ratio) lead to over 50-time increase in peroxidase activity of
655 cytochrome *c* than buffer, and aqueous [Ch][H_2PO_4] (salt:H_2O, 1:2 mass ratio) led over 25-fold
656 increase in enzyme activity. They further indicated that aqueous cholinium dicarboxylates could
657 also improve the stability of cytochrome *c* in terms of offsetting denaturing factors such as H_2O_2,
658 guanidinium chloride, pH, and temperature, and thus increasing the long-term storage of
659 cytochrome *c* at room temperature (for 21 weeks). Another study (Zhang et al., 2018) suggested
660 that aqueous 20% cholinium L-glutamate could improve the reaction yield by 3.5 times for multi-
661 dehydrogenase-catalyzed conversion of carbon dioxide to methanol. MD simulations hint that

662 the presence of cholinium L-glutamate enables the conformation of formate dehydrogenase to
663 keep carbon dioxide near its active site for a longer time. The Santos-Ebinuma group
664 (Nascimento et al., 2019) examined the hydrolytic activity of *Aspergillus niger* lipase in aqueous
665 solutions (0.05–1.00 M) of several cholinium carboxylates, and observed that the lipase activity
666 was preserved or improved at low IL concentrations (< 0.1 M) while at >0.1M concentrations,
667 anions with longer alkyl chains such as cholinium pentanoate and cholinium hexanoate induced
668 complete enzyme inhibition. Interestingly, the lipase maintained its activity well at all
669 concentrations of cholinium acetate even after 24 h incubation in ionic solutions at 35 °C.
670 Triethylmethylammonium methyl sulfate ([Et_3MeN][$MeSO_4$]) could solubilize >1.2 mg/mL
671 *Candida antarctica* lipase B (CALB) and maintain its catalytic capability (Madeira Lau et al.,
672 2004; van Rantwijk et al., 2006). The Zhao group (Zhao et al., 2008; Zhao et al., 2009c)
673 synthesized several medium-ether-chained ILs carrying acetate anions, and found that these ionic
674 media could dissolve >5 mg/mL CALB (at 50 ˚C) but still maintained reasonable lipase activity
675 (see detailed discussion in Section 7). The Bruce group (Falcioni et al., 2010) examined protic
676 hydroxyalkylammonium-based ILs (containing ~1–2 wt% water) for dissolving proteases (i.e.
677 chymotrypsin and subtilisin), and noted that subtilisin maintained its activity in
678 diethanolammonium chloride while chymotrypsin was inactive in these protic ILs. Furthermore,
679 they employed far and near UV CD spectra to confirm the preservation of secondary and tertiary
680 structures of subtilisin in diethanolammonium chloride. In addition to the manipulation of
681 solvent systems to dissolve enzymes, the modification of enzymes is an alternative route to
682 solubilize them in nonaqueous media, which includes many well-established methods such as
683 PEG modification (Inada et al., 1995; Inada et al., 1986a; Maruyama et al., 2004; Nakashima et
684 al., 2005; Woodward and Kaufman, 1996), graft polymerization to poly(*N*-vinylpyrrolidone),

685 polystyrene or poly (methyl methacrylate) (Ito et al., 1994), and complexing/ion-pairing enzymes
686 with amphiphile/surfactant (Akbar et al., 2007; Meyer et al., 1996; Okahata and Ijiro, 1988;
687 Paradkar and Dordick, 1994; Wangikar et al., 1997). Drastically improved enzyme activities (i.e.
688 an increase by several orders of magnitude) have not been seen for most homogeneous
689 biocatalytic processes, therefore, future endeavors should enable a better solubilization and
690 activation of enzymes.

691 Another two future major efforts in nonaqueous biocatalysis include engineering
692 enzymes to become more tolerant to organic solvent and ILs, and engineering solvents to provide
693 "water-like" environments for enzymes to maintain their high dynamics and flexibility. As
694 pointed out by the Reinhoudt group (van Unen et al., 2001), unlike in aqueous media, charge
695 separation in nonpolar media becomes undesirable process. During enzymatic reactions, anionic
696 tetrahedral intermediates (such as acyl-enzyme intermediates formed in lipase- or protease-
697 catalyzed transesterifications) are less likely to be stabilized by organic solvents (especially
698 nonpolar solvents; but polar solvents may strip water off enzyme molecules) than by water,
699 which causes the intermediate formation to be a rate-limiting step and a dramatic decrease of
700 enzyme activity in nonaqueous media. Very often, the tetrahedral intermediate is formed for the
701 second time during the reaction, such as the nucleophilic attack of acyl-enzyme intermediate by
702 an alcohol during the transesterification; one of these intermediate formations becomes the
703 reaction bottleneck. Typically, the addition of water into organic media can stabilize the
704 transition state and improve catalytic efficiency. Therefore, it is crucial to design 'task-specific'
705 solvents (such as functionalized-ILs) that can interact with the transition state favorably.
706

707 **Table 2** Representative biocatalytic reactions in enzyme-compatible ILs

Enzyme	IL	Reaction/Outcome	Ref.
cytochrome *c*	Aqueous [Ch][Glu] (50 wt%), Aqueous [Ch][H_2PO_4] (33 wt%)	Peroxidase activity measured by using ABTS[1] as a substrate in the presence of H_2O_2. Enzyme activity increased by 25–50 times.	(Bisht et al., 2017)
CALB[2]	[$CH_3OCH_2CH_2$-MIM][BF_4]	Acylation of D-glucose with vinyl acetate. A faster reaction (99% conversion) than in non-functionalized ILs and a high regioselectivity (93% monoacylation).	(Park and Kazlauskas, 2001)
Pseudomonas cepacia lipase	Imidazolium cations paired with polyoxyethylene(10) cetyl sulfate anion (Figure 7) as additives or lipase-coating-agents	Transesterifications of secondary alcohols (e.g. 1-phenylethanol) and vinyl acetate. Enhanced enantioselectivity and/or improved reaction rate.	(Itoh et al., 2004; Itoh et al., 2006)
morphine dehydrogenase	Both cation and anion functionalized by hydroxy groups (Figure 8)	Oxidize of codeine to codeinone. Dissolving morphine dehydrogenase and its cofactor nicotinamide, and a high enzymatic activity.	(Walker and Bruce, 2004a, b)
CALB	Ammoeng type of ILs (Figure 9)	Enzymatic glycerolysis reactions. High lipase activities in Ammoeng 100 and 102.	(Guo et al., 2006; Guo and Xu, 2006)
CALA,[3] CALB	[CPMA][$MeSO_4$]	Transesterification of vinyl esters with alcohols. Higher lipase activities than in hexane and other ILs.	(De Diego et al., 2009)
alcohol dehydrogenase	[$(HOCH_2CH_2)_3MeN$][$MeSO_4$]	Reduction of ketones. Maintain high activity in up to 90% (v/v) IL.	(de Gonzalo et al., 2007)
horseradish peroxidase	[$(HOCH_2CH_2)_4N$][CF_3SO_3]	Oxidation of guaiacol with H_2O_2. 10-Fold more active than in methanol and 30–240 times more active than in conventional ILs.	(Das et al., 2007)
feruloyl esterase	[$HOCH_2CH_2$-MIM][PF_6], [$CH_3(OCH_2CH_2)_2$-MIM][PF_6]	Esterification of glycerol with sinapic acid. Conversion yields up to 72.5% and 76.7% (respectively).	(Vafiadi et al., 2009)
lipase PS from *Burkholderia cepacia*	[$CH_3OCH_2CH_2$-Bu_3P][Tf_2N]	Transesterification of (*E*)-4-phenylbut-3-en-2-ol or 1-phenylethanol with vinyl acetate. Higher activity than in diisopropyl ether.	(Abe et al., 2008)
CALB	[$CH_3(OCH_2CH_2)_n$-Et-Im][OAc],	Transesterification of ethyl butyrate and 1-butanol.	(Zhao et al., 2009c)

	$[CH_3(OCH_2CH_2)_n$-$Et_3N][OAc]$ (n = 2, 3, or 7)	High activities that are comparable to *tert*-butanol.	
CALB and other lipases	$[CH_3(OCH_2CH_2)_3$-Et-Im][OAc], $[CH_3(OCH_2CH_2)_3$-$Et_3N][OAc]$	Transesterification of Miglyol oil with methanol. Dissolving Miglyol oil and enabling high conversions.	(Zhao et al., 2010c)
proteases (subtilisin and α-chymotrypsin)	$[CH_3(OCH_2CH_2)_3$-Et-Im][Tf_2N], $[CH_3(OCH_2CH_2)_3$-$Et_3N][Tf_2N]$	Transesterification of *N*-acetyl-L-phenylalanine ethyl ester with 1-propanol. High synthetic activity (1–3 μmol/min g) and selectivity (97–99%).	(Zhao et al., 2010b)
CALB	$[EtOCH_2CH_2CH_2$-MIM][Tf_2N], $[EtOCH_2CH_2CH_2$-BIM][Tf_2N], $[EtOCH_2CH_2CH_2$-MMIM][Tf_2N]	Enantioselective transesterification of *rac*-1-phenylethanol and vinyl acetate. Enantioselectivity higher than 99% and 50% conversion.	(Zhou et al., 2011)
CALB and *Pseudomonas cepacia* lipase	Imidazolium- and ammonium- ILs containing hydroxy groups on both cations and anions (Figure 12)	Transesterification of ethyl butyrate and 1-butanol. High lipase activities.	(Ou et al., 2016)
CALB	Mono-ether-functionalized phosphonium, imidazolium, pyridinium, alkylammonium, piperidinium, and sulfonium (Figure 13)	Transesterification of ethyl sorbate with 1-propanol; enzymatic ring-opening polymerization (ROP) of L-lactide and ε-caprolactone. High lipase activities, producing polyesters with high molecular mass (M_w ~20–25 kDa) and moderate yields (30–65%).	(Zhao et al., 2018; Zhao et al., 2019b)
CALB	Dual-functionalized imidazolium-based ILs incorporating both *tert*-alcohol and ether groups (e.g. ILs **10**–**12** in Figure 14)	Transesterification of ethyl sorbate with 1-propanol. Up to 40–100% higher than activities in *tert*-butanol and diisopropyl ether.	(Zhao et al., 2019a)
CALB	dual-functionalized ammonium-based IL (ILs **13**–**15** in Figure 14)	Transesterification of ethyl sorbate with 1-propanol; enzymatic ROP of ε-caprolactone. Lipase activity 1.5-fold higher than that in *tert*-butanol, and slightly higher than that in diisopropyl ether. Producing polyesters with high molecular mass M_w (up to 18,000 Da) and high yields (up to 74%).	(Zhao and Toe, 2020)

Note: [1]ABTS = 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid; [2]CALB = *Candida antarctica* lipase B; [3]CALA = *Candida antarctica* lipase A.

7. Designing Enzyme-Compatible Functionalized Ionic Liquids

As discussed earlier, some solvents containing alcohol and ether groups can lead to high enzyme activity and stability possibly due to the favorable hydrogen-bond donating and accepting environment created by these functional groups. Therefore, many studies have incorporated hydroxy- and/or ether-functionality into their IL structures to yield enzyme-compatible ILs. Representative examples are summarized in Table 2 and discussed in detail below.

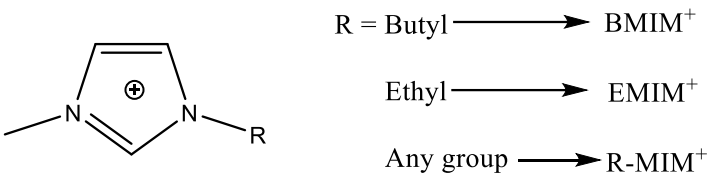


Figure 6 Structure of imidazolium (IM) cations.

The Kazlauskas (Park and Kazlauskas, 2001) dissolved ~5 mg/mL D-glucose in an ether-functionalized IL [$CH_3OCH_2CH_2$-MIM][BF_4] (see general illustration of imidazolium cations in Figure 6) at 55 °C, followed by CALB-catalyzed acylation of D-glucose with vinyl acetate resulting in a faster reaction (99% conversion) than those in non-functionalized imidazolium and pyridinium ILs accompanying with a high regioselectivity (93% monoacylation). Similarly, Kim et al. (Kim et al., 2003) performed *Candida rugosa* lipase-promoted acylation of monoprotected glycosides with vinyl acetate, achieving faster and more selective reactions in ILs ([BMIM][PF_6] and [$CH_3OCH_2CH_2$-MIM][PF_6], see the illustration of cations in Figure 6) than in organic solvents (THF and chloroform). Possible reasons are high substrate dissolution in ILs and more favorable structural adaptation of lipase in polar ILs. Instead of typical cation functionalization, Itoh and co-workers (Itoh et al., 2004; Itoh et al., 2006) paired polyoxyethylene(10) cetyl sulfate anion (Figure 7) with imidazolium cations to form new ILs, which served as additives or lipase-coating-agents in lipase-catalyzed transesterifications of secondary alcohols (e.g. 1-

phenylethanol) and vinyl acetate in diisopropyl ether or hexane. This group reported that both methods using functionalized ILs enhanced the enantioselectivity whereas the lipase-coating method further improved the reaction rate. For IL-coated lipase PS, the binding of 1-butyl-2,3-dimethylimidazolium poly[oxyethylene(10)] cetyl sulfate with the protein was confirmed by MALDI-TOF mass spectrometry (Itoh et al., 2006). The Stephens group (Rehmann et al., 2012) observed that laccase was active in aqueous 20% (v/v) water-miscible ILs containing alkyl sulfate anions with long alkyl chains or alkoxy chains, and also in 20% (v/v) hydrophobic ILs with anions of Tf_2N^-, AOT^- (= 1,4-bis(2-ethylhexyl)sulfosuccinate), or PF_6^-. They further pointed out that water-immiscibility is not always associated with high enzyme activity; for instance, laccase was not active in 20% (v/v) [C_{10}MIM][SCN] and [C_{10}MIM][saccharin]. Walker and Bruce (Walker and Bruce, 2004a, b) synthesized a hydrophilic IL with both cation and anion functionalized by hydroxy groups, 1-(3-hydroxypropyl)-3-methylimidazolium glycolate (Figure 8). The dual-functionalized IL could solubilize morphine dehydrogenase and its cofactor nicotinamide, and enabled a high enzymatic activity for oxidizing codeine to codeinone.

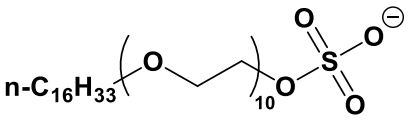


Figure 7 Structure of polyoxyethylene(10) cetyl sulfate.

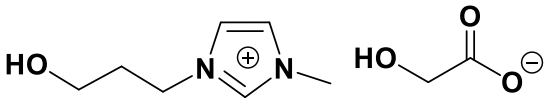
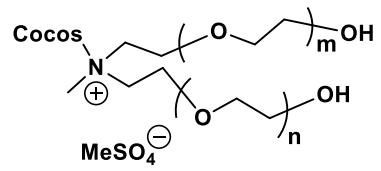


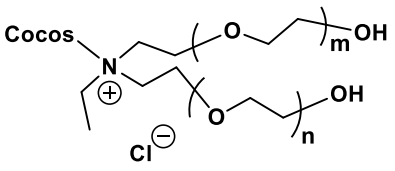
Figure 8 Structure of 1-(3-hydroxypropyl)-3-methylimidazolium glycolate.

Figure 9 illustrates a group of Ammoeng type of ILs, which are ionic mixtures carrying multiple alkoxy and/or hydroxy groups. These ionic solvents resemble polyglycols, having both hydrophilic and hydrophobic properties. The Xu group (Chen et al., 2008; Guo et al., 2006; Guo

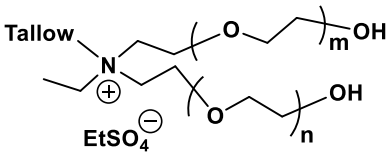
et al., 2009; Guo and Xu, 2005, 2006; Kahveci et al., 2009) systematically evaluated enzymatic glycerolysis performed in these tetraammonium-based ILs, and reported that both Ammoeng 100 (also known as [CPMA][$MeSO_4$], CPMA = cocosalkyl pentaethoxy methylammonium methylsulfate) and Ammoeng 102 could solubilize triglycerides and enabled high lipase activities in glycerolysis reaction (Guo et al., 2006; Guo and Xu, 2006). Another IL [TOMA][Tf_2N] (trioctylmethylammonium bis(trifluoromethylsulfonyl)imide) along with its mixture with Ammoeng 102 were also found suitable solvents for enzymatic glycerolysis (Guo et al., 2009; Kahveci et al., 2009, 2010). De Diego et al. (De Diego et al., 2009) obtained high CALB transesterification activities in [CPMA][$MeSO_4$] although two other lipases from *Thermomyces lanuginosus* (TLL) and *Rhizomuncor miehei* (RML) exhibited lower activities in [CPMA][$MeSO_4$] than in ILs carrying PF_6^- and BF_4^- anions. The Kroutil group (de Gonzalo et al., 2007) reported that hydroxy-functionalized ILs at 50–90% (v/v) concentrations allowed higher alcohol dehydrogenase activities than non-functionalized ILs, and established a decreasing trend of enzyme activity with the type of ILs as [$(HOCH_2CH_2)_3MeN$][$MeSO_4$] > Ammoeng 101 > Ammoeng 100 > Ammoeng 102. Dreyer and Kragl (Dreyer and Kragl, 2008) formed aqueous two-phase (ATP) using Ammoeng 110 (Figure 9) to purify two different alcohol dehydrogenases, and observed this ionic solvent was able to stabilize enzymes and increase the solubility of hydrophobic substrates. Wallert et al. (Wallert et al., 2005) deployed ether-/hydroxy-functionalized ILs (e.g. Ammoeng 100, 101 and 112, and [$HOCH_2CH_2$-MIM]Cl) as additives (1%) in pig liver esterase (PLE)-catalyzed enantioselective hydrolysis of diester malonates in 10% aqueous isopropanol, and achieved higher enzyme activities (up to 4-fold increase) and enantioselectivities (up to 97% ee).



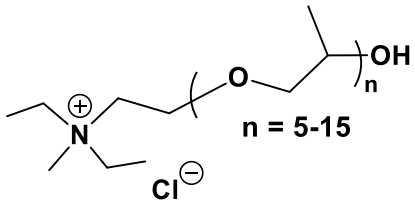
(a) Ammoeng 100
Cocos = C_{14} alkyl group; m + n = 4-14



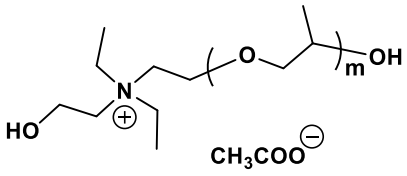
(b) Ammoeng 101
m + n = 14-25



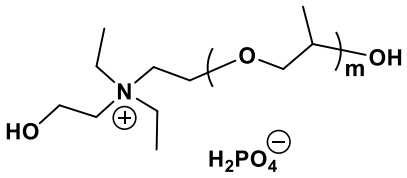
(c) Ammoeng 102
Tallow = C_{18} acyl group; m + n = 14-25



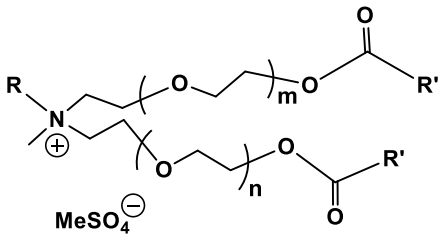
(d) Ammoeng 110



(e) Ammoeng 111
m = 50-60



(f) Ammoeng 112
m = 50-60



(g) Ammoeng 120
R, R' = C_{18} acyl group; m, n, unavailable

775

776 **Figure 9** Structures of functionalized ammonium-based ILs (Ammoeng series).

777 Tris(hydroxymethyl)aminomethane (Tris) is a common component of buffer solutions
778 (pH usually in the range of 7–9). Tris was also used as an excipient to provide lyoprotectant
779 effect for horseradish peroxidase during lyophilization (Dai and Klibanov, 1999). Based on the
780 Tris structure, Das et al (Das et al., 2007) synthesized tetrakis(2-hydroxyethyl)ammonium

triflouromethanesulfonate ([$(HOCH_2CH_2)_4N$][CF_3SO_3], Figure 10), and discovered that horseradish peroxidase in this new ionic solvent was 10-fold more active than in methanol and 30–240 times more active than in non-functionalized ILs. Vafiadi et al. (Vafiadi et al., 2009) carried out feruloyl esterase-catalyzed esterification of glycerol with sinapic acid in two functionalized ILs [$HOCH_2CH_2$-MIM][PF_6] and [$CH_3(OCH_2CH_2)_2$-MIM][PF_6], achieving conversion yields up to 72.5% and 76.7% (respectively) under optimum conditions. Itoh and co-workers (Abe et al., 2008) prepared an ether-functionalized IL known as [$CH_3OCH_2CH_2$-Bu_3P][Tf_2N], and conducted lipase PS-catalyzed transesterification of secondary alcohols in this new medium resulting in a faster reaction rate in diisopropyl ether.

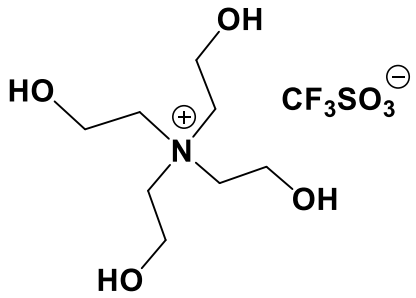


Figure 10 Structure of tetrakis(2-hydroxyethyl)ammonium triflouromethanesulfonate.

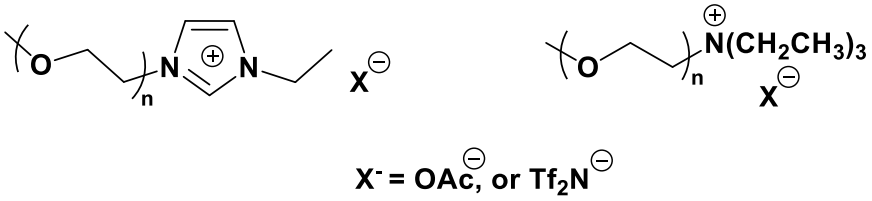


Figure 11 Ether-functionalized imidazolium (IM) and ammonium ILs ([$CH_3(OCH_2CH_2)_n$-Et-IM]X and [$CH_3(OCH_2CH_2)_n$-Et_3N]X, respectively) (n = 1, 2, 3,…).

The Zhao group (Zhao et al., 2010a; Zhao et al., 2008; Zhao et al., 2009c; Zhao and Song, 2010; Zhao et al., 2010c) synthesized a series of glycol-functionalized imidazolium and alkylammonium-based ILs carrying acetate anions (Figure 11), and found these ionic solvents could dissolve a variety of 'unusual' substrates such as cellulose, xylan, lignin, D-glucose, 3,4-

dihydroxy-DL-phenylalanine (DOPA), betulinic acid, and Miglyol oil (a mixture of triglycerides of caprylic acid and capric acid). The high dissolution power is mainly attributed to high hydrogen-bond basicity of anions (e.g. acetate and formate) of these ILs, but this is also the reason that causes enzyme inactivation. However, with a longer glycol chain grafted to ILs, the molar concentration of denaturing anions is reduced; therefore, the deactivating effect of these ILs is minimized (Zhao et al., 2009c). This concept was also demonstrated by another study (Lourenço et al., 2007) where Novozym 435 showed little activity in denaturing [BMIM][dca] (dca^- = dicyanamide), but a relatively high activity and enantioselectivity in [aliq][dca] [$aliq^+$ = trioctylmethylammonium (Aliquat 336® is a mixture of C_8 and C_{10} chains with C_8 predominating)]. Due to the bulky size and high molar mass of $aliq^+$ (*verse* $BMIM^+$), the molar concentration of denaturing dca^- in [aliq][dca] is much lower than in [BMIM][dca]. As a result, synthetic activities of free and immobilized CALB in most acetate- and formate-based ILs are higher than or comparable with those in *tert*-butanol and [BMIM][Tf_2N] as determined by the transesterification of ethyl butyrate and 1-butanol. Due to their strong dissolution ability toward D-glucose and cellulose, these ILs were also successful for achieving the regioselective transesterification of these substrates catalyzed by Novozym 435 (Zhao et al., 2008). Enzymatic transesterification of Miglyol oil with methanol was conducted in these ILs to prepare biodiesel, resulting in up to 70% triglyceride conversion in 1 h and 85% conversion in 48 h (Zhao et al., 2010c). Additionally, since these ILs could dissolve a significant portion of lignocelluloses, these solvents were employed to effectively pretreat biomass prior to their enzymatic saccharification (Tang et al., 2012a; Zhao et al., 2010a; Zhao et al., 2009b). Proteases are not active in these acetate- or formate-based ILs, thus the Zhao group (Zhao et al., 2010b) synthesized the hydrophobic version of ionic solvents carrying Tf_2N^- anions (e.g. [$CH_3(OCH_2CH_2)_3$-Et_3N][Tf_2N],

822 [$CH_3(OCH_2CH_2)_3$-Et-IM][Tf_2N] and [$CH_3(OCH_2CH_2)_3$-Me-Et-IM][Tf_2N] in Figure 11) and
823 found that immobilized subtilisin and α-chymotrypsin showed high synthetic activities (1–3
824 μmol min^{-1} g^{-1}) and selectivity (97–99%, transesterification *verse* hydrolysis) in these
825 hydrophobic ILs containing 10–15% (v/v) water for the transesterification of *N*-acetyl-L-
826 phenylalanine ethyl ester with 1-propanol. The same enzymatic reaction in *tert*-butanol or
827 [BMIM][Tf_2N] displayed low synthetic activities (0.2–1 μmol min^{-1} g^{-1} in *tert*-butanol) and/or
828 poor selectivity (40%) when the water content was higher than 2% (v/v). These hydrophobic
829 glycol-functionalized ILs also showed comparable or higher activities than [BMIM][Tf_2N] and
830 *tert*-butanol in Novozym 435-catalyzed transesterification of ethyl sorbate and 1-propanol (Zhao
831 et al., 2011).

832 Zhou and co-workers (Zhou et al., 2011) prepared six monoether-functionalized
833 imidazolium ILs containing Tf_2N^- or PF_6^- anions, and attained higher enantioselectivities (95-99%
834 *ee*) in some functionalized ILs than in diisopropyl ether or non-functionalized ILs ([BMIM][BF_4]
835 and [BMIM][PF_6]) during lipase-catalyzed kinetic resolution of secondary alcohols. It was
836 argued that ether groups could adjust the hydrophilic environment of media and interact
837 favorably with the enzyme and/or substrates. Ståhlberg et al. (Ståhlberg et al., 2012) conducted
838 enzymatic isomerization of glucose to fructose and reported high glucose isomerase activity in
839 *N*,*N*-dibutylethanolammonium octanoate with 20 wt% water, but no activity in other ILs
840 carrying smaller/denaturing alkanoate anions (e.g. formate, acetate, and propionate). Vila-Real et
841 al. (Vila-Real et al., 2011) prepared sol-gel immobilization of α-L-rhamnosidase and β-D-
842 glucosidase expressed by naringinase using ILs as additives, and observed that a more
843 hydrophobic IL additive led to a higher α-rhamnosidase efficiency; the inclusion of
844 [OMIM][Tf_2N] and [($HOCH_2CH_2$)-MIM][PF_6] in sol-gel matrices could minimize enzyme

deactivation in TMOS/Glycerol matrices (TMOS = tetramethoxysilane). Ou et al. (Ou et al., 2011b) detected no transesterification activity for CALB in denaturing [BMIM][NO_3], but some activities in hydroxy-functionalized ILs (e.g. [$HOCH_2CH_2$-MIM][NO_3] and [$HOCH_2CH_2$-MIM][BF_4], especially in the latter IL) and these activities were further enhanced by 2–5 times by adding 'IL buffer' ([BMIM][H_2PO_4]). The preservation of native compact structure of CALB in hydroxy-functionalized ILs was confirmed by fluorescence spectra. The same group (Ou et al., 2016) suggested that enzyme-compatible ILs should behave like water molecules having high dielectric constants and both solvent donor and acceptor properties to properly ionize protein's ionizable groups so that enzyme molecules can be dissolved and stabilized. Following this rationale, they designed imidazolium- and ammonium-based ILs containing hydroxy groups on both cations and anions (Figure 12), and confirmed that these ILs enabled high transesterification activities for two lipases (CALB and *Pseudomonas cepacia* lipase). One obvious disadvantage of (multi-)hydroxy-functionalized ILs is their relatively high viscosities due to hydrogen-bonding (Ou et al., 2016; Tang et al., 2012b). Kundu et al. (Kundu et al., 2018) constructed a surface-active protic IL, L-proline propyl ester lauryl sulfate ([$ProC_3$][LS]), for the formation of reverse micelles along with cyclohexane and water. The encapsulation of bovine serum albumin (BSA) in these micelles led to a higher content of secondary structures without any buffer than the native protein in the droplet core with higher hydration.

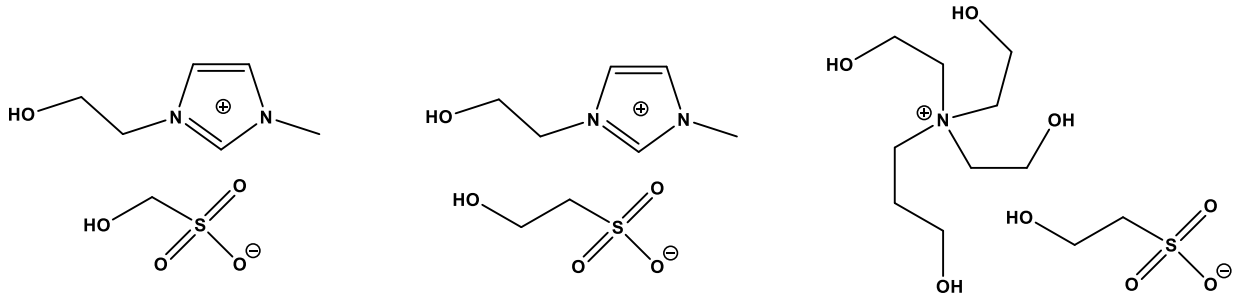


Figure 12 Structures of hydroxy-grafted imidazolium and ammonium ILs.

865 The Zhao group (Zhao et al., 2018; Zhao et al., 2019b) further expanded monoether-
866 functionalized ILs to a series of glycol-functionalized ILs with various chain lengths grafted to
867 different cations cores (i.e. phosphonium, imidazolium, pyridinium, alkylammonium,
868 piperidinium, and sulfonium; see Figure 13); these glycol-functionalized ILs usually have low
869 dynamic viscosities (33–123 mPa s at 30 °C), and high decomposition temperatures (T_{dcp}) in the
870 range of 318–403 °C (except sulfoniums such as IL **9** in Figure 13 with T_{dcp} = 254 °C – data not
871 published). In Novozym 435-catalyzed transesterification of ethyl sorbate with 1-propanol at
872 50 °C (0.02 wt% water in all solvents), [$CH_3OCH_2CH_2$-Et_3N][Tf_2N] (**7**) and [$CH_3OCH_2CH_2$-
873 Py][Tf_2N] (**6**) enabled highest enzyme activities (6.57 and 6.08 μmol min^{-1} g^{-1} CALB,
874 respectively) among all functionalized ILs; these activities are higher than that in [BMIM][Tf_2N]
875 (5.12 μmol min^{-1} g^{-1} CALB) and comparable with that in *tert*-butanol (7.38 μmol min^{-1} g^{-1}
876 CALB); in addition, the thermal stability of Novozym 435 in [$CH_3OCH_2CH_2$-Et_3N][Tf_2N] (**7**)
877 was much higher than that in *tert*-butanol at 50 and 70 °C. Furthermore, these glycol-
878 functionalized ionic solvents were used as co-solvents in enzymatic ring-opening polymerization
879 (ROP) of L-lactide (130 °C for 7 days) and ε-caprolactone (70 °C for 2 days), producing
880 polyesters with high molecular mass (M_w ~20–25 kDa) and moderate yields (30–65%). The Shi
881 group (Yang et al., 2020) prepared several glycol-functionalized ammonium ILs such as
882 [$Me_2N(Et)(CH_2CH_2O)_2H$][PF_6], which can be mixed with 1,2-dimethoxyethane at the 5:18 (v/v)
883 ratio to form a homogeneous solution under 33 °C and a two-layer system above this temperature.
884 This temperature-sensitive phase system was applied to conduct the enantioselective reduction of
885 ethyl 2-oxo-4-phenylbutyrate to an alcohol catalyzed by baker's yeast at 30 °C, and at the
886 completion of reaction, temperature was raised to form the IL layer and organic layer for easy

separation. This unique reaction process improved the product *ee* by 25–30% and yield by 35% compared with the same reduction performed in 1,2-dimethoxyethane.

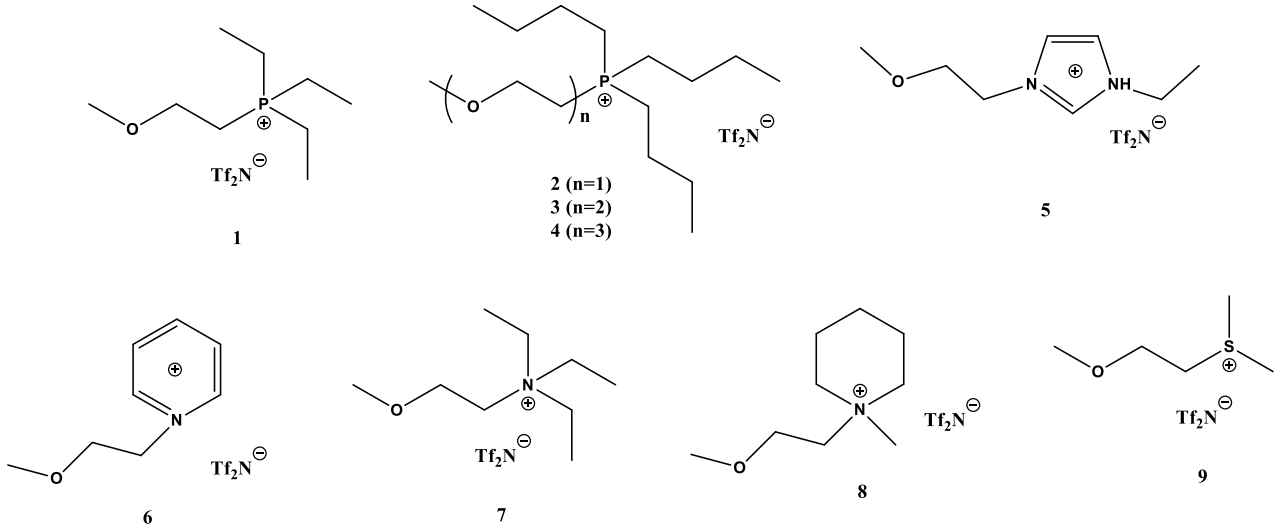


Figure 13 Structures of ether-functionalized ILs.

Earlier functionalized ILs are mostly mono-functionalization with either ether or alcohol groups. Ammoeng series of ILs (Figure 9) contain both ether and hydroxy groups, but their drawbacks include ionic mixtures lacking of absolute structure/composition and well-defined properties, high viscosities, and primary or secondary alcohols being potentially reactive. As mentioned earlier, *tert*-alcohols are less inhibitory to enzymes and much less reactive as substrates than primary and secondary alcohols in nonaqueous enzymatic reactions (Madeira Lau et al., 2000; Zaks and Klibanov, 1984). Therefore, the Zhao group (Zhao et al., 2019a) designed dual-functionalized imidazolium-based ILs incorporating both *tert*-alcohol and ether groups (e.g. ILs **10**–**12** in Figure 14) to resemble the water structure by having both hydrogen-bond donating (-OH) and accepting (R-O-R) properties. As a result, they observed a drastic increase of Novozym 435's transesterification activity in these "water-like" ionic solvents: up to 2–4 times higher than in ordinary ILs such as [BMIM][Tf_2N], and up to 40–100% higher than in *tert*-butanol and diisopropyl ether. The lipase in these dual-functionalized ILs also exhibited much

higher thermal stability than in *tert*-butanol, and comparable thermal stability with that in diisopropyl ether. One disadvantage of these imidazolium-ILs is their slightly high dynamic viscosities (~300 mPa s at 30 °C). The same group (Zhao and Toe, 2020) further prepared dual-functionalized ammonium-based IL (**13**–**15** in Figure 14) with lower viscosities (as low as 129 mPa s at 30 °C), and reported high transesterification activities for Novozym 435 in these new solvents (1.5-fold higher than in *tert*-butanol, and slightly higher than in diisopropyl ether). Enzymatic ROP reactions of ε-caprolactone in these "water-like" ILs as co-solvents produced polyesters with high molecular mass M_w (up to 18,000 Da) and high yields (up to 74%).

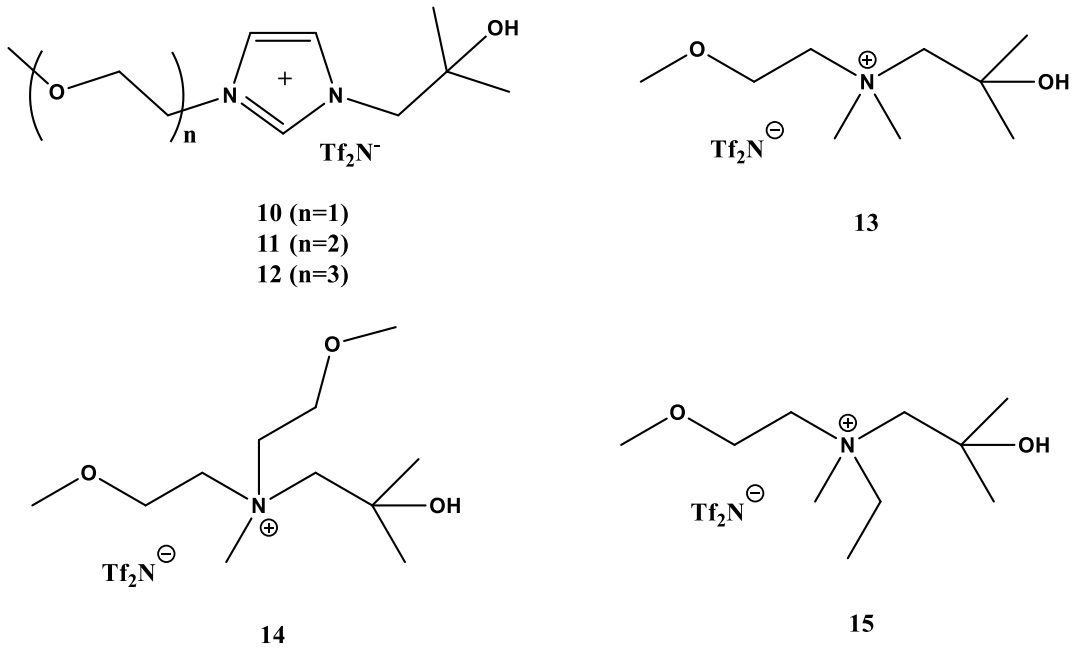


Figure 14 Structures of "water-like" dual-functionalized imidazolium and ammonium ILs.

In limited cases, functionalized ILs did not perform better than regular ILs. For example, in enantioselective transesterification of 1-phenylethanol and vinyl propionate, Lozano et al. (Lozano et al., 2004) observed a higher selectivity but lower CALB activity and stability in $[HOCH_2CH_2CH_2\text{-}Me_3N][Tf_2N]$ than in non-functionalized ammonium ILs. The Goto group grafted an unusual comb-shaped PEG (so called PM_{13}) onto *Candida rugosa* lipase (Nakashima

et al., 2006b) and subtilisin Carlsberg (Nakashima et al., 2005, 2006a) to form covalently immobilized PM_{13}-lipase (Nakashima et al., 2006b) and PM_{13}-subtilisin (Nakashima et al., 2006a). These enzyme preparations are soluble in Tf_2N^- based ILs with high enzyme activity and stability, and are more active in a more hydrophobic IL ([EMIM][Tf_2N], see Figure 6 for the cation structure) than in functionalized ones ([$CH_3OCH_2CH_2$-MIM][Tf_2N] and [$HOCH_2CH_2$-MIM][Tf_2N]).

There have been some mechanistic discussions about why oxygenated chains in ILs are enzyme-compatible. The incorporation of hydroxy, ether or nitrile group to the side chain of ILs usually reduced the solvent lipophilicity, and thus lowers the inhibition of acetylcholinesterase (as a broad toxicity screening assay) by ILs (Arning et al., 2008; Ranke et al., 2007; Siopa et al., 2018; Yan et al., 2012). Luo et al. (Luo et al., 2010) reported the formation of stable complexes between imidazolium and pyridinium cations with PEG-800 or PEG-1000 through ion-dipole interaction; literally, cations were wrapped by PEG molecules. Etherated chains in ether-functionalized ILs are known more flexible than rigid alkyl chains (Siqueira and Ribeiro, 2009), and the grafting of ether chain minimizes intermolecular correlation (especially tail-tail segregation) and cation-anion specific interactions (Ganapatibhotla et al., 2010; Smith et al., 2008). For these reasons, ether-functionalized cations preferentially interact with ether chains (intramolecular and/or intermolecular), decreasing the cation-protein interaction. On the other hand, IL cations with long alkyl chains (e.g. $P_{666(14)}^+$) tend to interact with the Leu17 residue of *Burkholderia cepacia* lipase via hydrophobic interactions based on molecular docking study, resulting in lower enzymatic/hydrolytic activities (Barbosa et al., 2019a; Barbosa et al., 2019b). Spectroscopic and computational tools provide valuable insights into interactions between proteins and functionalized ILs. By using fluorescence and CD spectroscopy, Turner et al.

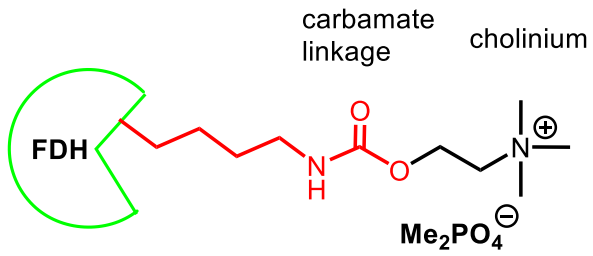
942 (Turner et al., 2005) reported that human serum albumin (HSA) was less denatured by aqueous
943 solutions of hydroxy/ether-functionalized imidazolium ILs than [BMIM]Cl. This is possibly due
944 to some favorable hydrogen-bond interactions between functionalized ILs and HSA. Mann et al.
945 (Mann et al., 2009) determined the thermal stability and refolding patterns of lysozyme in
946 aqueous ILs by near-UV CD, and observed that ethanolammonium formate could stabilize
947 lysozyme against unfolding at high temperature, as well as nearly complete renaturation upon
948 cooling. Additionly, they found that lysozyme in aqueous ethanolammonium formate was six
949 times more active than in aqueous buffer itself. This can be attributed to hydroxy-functionality
950 enabling additional hydrogen-bonding donor/acceptor sites to minimize hydrophobic interactions
951 between IL and the protein. On the other hand, the thermal stability of lysozyme in 2-
952 methoxyethylammonium formate solutions showed no improvement, which was rationalized that
953 ether group only acts as hydrogen-bond acceptor and an additional hydrogen-bond donor is
954 needed for protein stabilization. After subtilisin was dissolved in diethanolammonium chloride,
955 the Bruce group (Falcioni et al., 2010) found the protease remained active, but no activity was
956 detected for subtilisin in other protic hydroxyalkylammonium ILs nor for chymotrypsin in any of
957 these ILs. Fluorescence spectroscopy could not explain the differences, but far and near UV
958 spectra for subtilisin in diethanolammonium chloride agreed well with that in water, implying
959 secondary and tertiary structures were better preserved in this hydroxy-functionalized ionic
960 solvent. The Greaves group (Wijaya et al., 2016) studied the activity and conformation of
961 lysozyme in molecular solvents and protic ILs, and concluded that non-ionic solvents containing
962 hydroxy group and bulky group could maintain enzyme's conformation and activity at high
963 solvent concentrations, but solvents with amine group could only improve the enzyme activity at
964 low concentrations. On the other hand, lysozyme activity increased with the type of protic ILs in

965 the order of (2-hydroxy)ethylammonium nitrate < ethylammonium formate < (2-
966 hydroxy)ethylammonium formate < ethylammonium nitrate, which was explained by the similar
967 water affinity between two kosmotropes or between two chaotropes. Based on fluorescence
968 spectroscopy and DSC measurements, Bose et al. (Bose et al., 2010) reported high stability of
969 *Tricoderma reesei* cellulase in tris(2-hydroxyethyl)methylammonium methylsulfate (HEMA)
970 even at 115 °C, but this enzyme became irreversibly denatured in pH 4.8 citrate buffer at 50 °C.
971 In contrast to imidazolium ILs, this ammonium-based IL does not seem to quench fluorescence
972 signal of tryptophan. That study also confirms that fluorescence quenching of enzymes is not
973 necessarily correlated with protein denaturation, as identified by other groups (Falcioni et al.,
974 2010; Zhao et al., 2009c). The same group (Bose et al., 2012) further examined endo-1,4-β-D-
975 glucanase (EG) in various solvents by fluorescence spectroscopy, and observed the enzyme
976 denaturation at ~55 °C in buffer but a higher transition temperature of ~75 °C in HEMA, which
977 agreed with a high enzyme activity at this temperature. In contrast to enzyme-destabilizing effect
978 of imidazolium cations, HEMA cations tend to stabilize the enzyme. By following the
979 Conductor-like Screening Model for Real Solvents (COSMS-RS), Xu and co-workers (Chen et
980 al., 2008; Guo et al., 2006) obtained various parameters (such as misfit, H-bonding, and van der
981 Waals interaction energy) to understand multiple interactions in ILs; this model enabled a
982 rationale design of particular structures of cations and anions (Guo et al., 2007). The Torkzadeh-
983 Mahani group (Ghanbari-Ardestani et al., 2019) reported that urate oxidase showed an increased
984 activity in 1% (v/v) aqueous triethanolammonium butyrate, but lower activities in 5% and 10%
985 IL solutions. Their MD simulations results suggest that 1% IL was able to increase
986 intramolecular hydrogen bonds of the enzyme, which led to a small decrease in random coil and
987 increase in α-helix and β-sheet, and a more compact enzyme structure. A further molecular

988 docking study reveals that IL cations and anions interact with urate oxidase primarily through
989 hydrogen bond, electrostatic, and hydrophobic interactions. On the other hand, based on MD
990 simulations, Klähn et al. (Klähn et al., 2011) indicated that CALB is more destabilized by polar
991 methoxyethyl group and decyl side chain on IL cations than by non-functionalized and short
992 methyl groups, respectively. However, the destabilization follows different mechanisms:
993 methoxyethyl group destabilizes the protein surface through strong Coulomb interactions with
994 CALB while long alkyl chain interacts directly with protein's hydrophobic core.

995 At microscopic scale, ILs consist of nanostructured segregates/microphases of polar
996 domain (high-charge density region) and nonpolar domain (low-charge density region); with the
997 increase in alkyl-side chains, the nonpolar domain can grow from dispersed/isolated islands to a
998 continuous phase (Brehm et al., 2015; Canongia Lopes and Pádua, 2006; Shimizu et al., 2010).
999 Depending on the similarity of solute molecules to each domain, different solutes could be
1000 solvated in different IL microphases; dipolar or quadrupolar solutes tend to interact strongly with
1001 the polar nanophase and reorganize the polar network in their neighborhood (Shimizu et al.,
1002 2010). Water molecules can modify the local microscopic structure of ILs through the competing
1003 hydrogen-bonding and electrostatic interaction: a) at low water content, water molecules are
1004 dispersed and inserted cavities forming solvent-shared ion pairs via cation–water–anion triple
1005 complexes; b) with the increase in water content, water molecules begin to aggregate forming
1006 small clusters, chain-like structures, large aggregates and finally a water network; c) in diluted
1007 aqueous solutions of ILs, self-organized micelles-like aggregates are formed in a highly
1008 branched water network (Wang et al., 2015). These microscopic structure changes result in
1009 thermodynamically nonideal volumetric properties and unique dynamic properties (Wang et al.,
1010 2016b). Generally, the interaction between water and ILs increases with higher hydrogen-bond

1011 basicity of anions (Khan et al., 2014). The Dupont group (Dupont, 2004) suggested the enclosure
1012 of other molecules and macromolecules in IL network; when aqueous droplets of enzyme are
1013 dissolved or dispersed into the polar domain of ILs, favorable solvophobic interactions could
1014 preserve the enzyme's active conformation (Fehér et al., 2007). The nanostructured
1015 supramolecular IL network allows enzyme molecules to maintain their native structures,
1016 avoiding thermal unfolding (Lozano et al., 2005). Compared with conventional ILs,
1017 functionalized ILs have different physical properties including viscosity, structural flexibility,
1018 phase transition, density, polarity, hydrophilicity, and hydrogen-bonding capability (Tang et al.,
1019 2012b). These structural and property changes likely lead to the modification of IL
1020 nanostructures, resulting in different IL-enzyme interactions; a further study in this area should
1021 be explored in the future.



1022

1023 **Figure 15** Covalent attachment of a cholinium salt to the lysine residue of formate
1024 dehydrogenase (FDH).

1025 Instead of focusing on solvent functionalization, an alternative approach is to modify the
1026 enzyme with functionalized ILs. Itoh and co-workers (Abe et al., 2010) coated lipase PS with
1027 $[CH_3OCH_2CH_2OCH_2PBu_4][Tf_2N]$ to catalyze the acylation of secondary alcohols with vinyl
1028 acetate obtaining high enzymatic activities and selectivities. Bekhouche et al. (Bekhouche et al.,
1029 2010) grafted hydroxy groups of three ILs to lysine residues of formate dehydrogenase (FDH)
1030 from *Candida boidinii* via carbamate linkages (Figure 15). IL-modified FDH maintained about

30–45% of its activity in 70% (v/v) [MMIM][Me_2PO_4] whereas the native FDH was not active in this ionic solution. IL-modification of FDH further enhanced the enzyme stability in aqueous buffer solution by 3–6 folds; in addition, unmodified and one modified FDH showed increased half-time $t_{1/2}$ values in 37.5% (v/v) [MMIM][Me_2PO_4] than in carbonate buffer. Li et al. (Li et al., 2015) grafted ether-functionalized ILs (carrying carboxylic acid group) through covalent linkage onto *Candida rugosa* lipase (CRL) using *N*,*N*′-carbodiimide as the coupling reagent (see Figure 16). The lipase modified with ILs exhibited improvements in catalytic activity, thermal stability, organic solvent tolerance, and adaptability to temperature and pH changes during the enzymatic hydrolysis of olive oil. Furthermore, CD spectra indicate the lipase modification leading to changes in secondary structures: an increase in β-sheet and a decrease in α-helix contents. A similar approach linked CALB and porcine pancreatic lipase (PPL) with carboxylic acid-functionalized imidazolium and cholinium cation respectively, leading to enhanced lipase thermal stability and/or enantioselectivity (Jia et al., 2013a; Jia et al., 2013b). In addition, various functionalized ILs have been used as coupling agents to covalently link enzymes with solid supports such as chitosan-mesoporous silica hybrid nanomaterials (Xiang et al., 2018) and multiwalled carbon nanotubes (MWNTs) (Wan et al., 2017a; Wan et al., 2017b), affording higher enzyme's thermal/storage stability and better reusability.

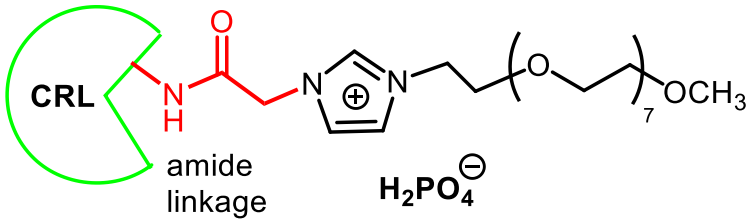


Figure 16 Covalent modification of *Candida rugosa* lipase (CRL) by a glycol-functionalized IL.

8. Lessons Learned from Enzyme Behaviors in Ionic Liquids

To develop functionalized ILs for biocatalytic applications, there are competing factors that often need to be addressed. (1) *Substrate dissolution or enzyme stabilization*. For example, to dissolve carbohydrates, ILs typically carry anions with high hydrogen-bond basicity (such as Cl^-, OAc^-, and dca^-); however, these ILs are expected to interact with enzymes strongly for the same reason interacting with carbohydrates, leading to enzyme inactivation. Therefore, appending a long glycol-chain to the cation could reduce the molar concentration of these anions, minimizing the destabilizing effect of anions (Zhao et al., 2009c). In this case, it is a compromise between substrate dissolution and enzyme stabilization. (2) *Functionalization or low viscosity*. Hydroxy-functionalized ILs could stabilize enzymes while amine-/amino-functionalized ILs could dissolve more substrates (e.g. carbon dioxide), but these functionalizations often lead to high IL viscosities due to their capability of forming hydrogen bonds (Goodrich et al., 2011; Tang et al., 2012b; Zhang et al., 2009). To mitigate the hydrogen-bonding effect, incorporating another functional group (such as ether) or pairing with different anions (e.g. Tf_2N^-, $C(CN)_3^-$, or $B(CN)_4^-$) could alleviate the increase in viscosity (Tang et al., 2012b). (3) *Functionalization or low cost*. Functionalization of ILs potentially increases the cost of already expensive solvents. Therefore, the use of functionalized ILs could be restricted by their applications; for example, these costly solvents could be ideal for developing expensive medicinal molecules but may not be suitable for the production of less expensive biofuel. On another dimension, ILs should be recycled and reused to decrease the overall operation cost. Various methods have been explored to recover ILs including extraction, distillation, adsorption, membrane separation, crystallization, and external force field separation (Zhou *et al*., 2018).

9. Summary and Prospects

There are many viable ways to improve enzyme activity and stability in nonaqueous media (particularly organic solvents and ILs), such as protein engineering to produce enzymes that are tolerant to organic solvents (Ogino and Ishikawa, 2001) and ILs (Pramanik et al., 2019), enzyme immobilization, chemical modification of enzymes, and solvent engineering. One interesting development in chemical modification of enzymes is liquefaction of proteins through cationization of protein surface by coupling glutamic and aspartic acid residues to *N*,*N*'-dimethyl-1,3-propanediamine (DMPA), followed by the protein conjugation with anionic PEG-type surfactant, resulting in liquid proteins/enzymes (Gallat et al., 2012; Perriman et al., 2010; Perriman et al., 2009; Sharma et al., 2014). In terms of solvent engineering, hydrophobic organic solvents generally tend to maintain reasonable enzyme activities whereas solvents with functional groups, especially hydroxys and ethers (including crown ethers and cyclodextrins), could lead to enzyme activation. There are limited options to design organic solvents carrying these functional groups (e.g. glycerol carbonate and *N*-hydroxymethyl formamide). In contrast, ILs offer greater flexibility for structural manipulation. Based on current studies, enzyme-compatible ILs tend to have these desirable properties and structural features: (1) Hydrophilic ILs (especially with denaturing anions such as halides, dca^- and OAc^-) typically have relatively large molecular structures (i.e. large molar mass), or other functional groups (e.g. 1-(3-hydroxypropyl)-3-methylimidazolium glycolate) to minimize their hydrogen-bond basicity and nucleophilicity of anions; (2) Most enzyme-compatible/activating ILs are the hydrophobic type (containing Tf_2N^- and PF_6^- anions). Their structures usually incorporate ether and/or hydroxy groups to provide desirable hydrogen-bond donating and accepting environments for enzymes. The incorporation of these functional groups also results in favorable solvent properties (such as

desirable IL viscosity, hydrogen-bond basicity, and water affinity). Dual-functionalized ILs containing both ether and *tert*-alcohol groups are amongst nonaqueous solvents that lead to the greatest enzyme activation.

In contrast to nonaqueous biocatalysis, aqueous enzymatic reactions have also gained some new developments. For example, in the presence of a nonionic surfactant Lutensol AT50 (poly(ethyleneoxide)hexadecyl ether), lipase-catalyzed esterification could proceed (~90% conversions) in aqueous solutions as reactants being dispersed with the surfactant to form miniemulsions (Aschenbrenner et al., 2009). In aqueous solutions containing a nonionic surfactant TPGS-750-M, micelles were formed to promote alcohol dehydrogenase-catalyzed enantioselective reduction of ketones to chiral alcohols (Cortes-Clerget et al., 2019). Perhaps the future direction is to learn from both aqueous and nonaqueous biocatalysis to develop a new generation of "water-like" solvent systems.

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