Chapter 14

Deep Eutectic Solvents for *Candida antarctica* Lipase B-Catalyzed Reactions

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Many deep eutectic solvents (DES's) are mixtures of ammonium salts and hydrogen-bond donors; e.g., a 1:2 mixture of choline chloride and urea. Like room temperature ionic liquids, DES's are polar, viscous solvents with low vapor pressure and flammability. However, synthesis of DES's is simpler – requiring only mixing of components – and the components are non-toxic and approximately ten-fold less expensive than the components for ionic liquids. Even though components of DES's can include protein denaturants like urea, we found that immobilized *Candida antarctica* lipase B retains activity in a wide range of DES's. Rates of transesterification, aminolysis of esters, perhydrolysis were similar to those in organic solvents and several-fold faster than those in ionic liquids. In a few cases, side reactions with the components of the DES occurred. DES's containing sugars were highly viscous and required temperatures ≥60 °C to permit stirring.

Introduction

Room temperature ionic liquids (RTILs) are liquids composed entirely of cations and anions, e.g. 1-butyl-3-methyl imidazolium tetrafluorborate (*1–3*).

Most ionic liquids are non-volatile, thermally stable, and varying the cation and anion varies their polarity and other physical properties. Ionic liquids may be better than traditional organic solvents as solvents for extractions (*4*, *5*) chemical reactions (*6–8*) and biotransformations (*9–13*). Some limitations of the most common ionic liquids are toxicity similar to or higher than organic solvents (*14–16*), high cost, and the need for high purity, as even trace impurities can affect physical properties (*17*, *18*).

Deep eutectic solvents (DES's) are eutectic mixtures that are liquids at room temperature. Many DES's are 1:1 or 1:2 mixtures of an ammonium or metal salt and a hydrogen-bond donor, e.g. a 1:2 mixture of choline chloride and urea (*19*). DES's are alternatives to ionic liquids as replacements for organic solvents because DES's have low volatility and flammability, high thermal stability and varying the components varies the physical properties of solvent. DES's are not ionic liquids because they contain uncharged components – urea in the example above. Nevertheless, strong hydrogen bonding between the components makes their physical properties similar to those for ionic liquids (*20*).

The advantages of DES's over ionic liquids are lower cost and lower toxicity. The components of common DES's are inexpensive. Most DES's are mixtures of amine chloride salts and urea, glycerol, or ethylene glycol. Scheme 1 lists the components used in this work. Another reason for the low cost is the simple synthesis, which involves only warming and stirring the components for an hour or so. In contrast, synthesis of ionic liquids usually requires removal of salts, which can require multiple precipitations followed by chromatography to remove remaining traces. The components of DES's also are non-toxic; for example, glycerol and choline chloride are used as food additives. It is possible to use expensive or toxic components to make a DES, but the most common ones use inexpensive and nontoxic components. Other possibilities for DES components include: a wide range of organic acids (*21*, *22*) and fluorinated hydrogen bond donors (*23*).

Researchers have already reported numerous applications of DES's. For example, Abbott and coworkers dissolved silver salts in the DES's to dip coat copper surfaces with silver without the need for catalysts (*24*, *25*). Choline chloride-based DES's replaced phosphoric and sulfuric acids for electropolishing stainless steel (*26*, *27*). Adding choline chloride to a biodiesel preparation removed the glycerol side product by forming a choline chloride-glycerol DES as a second phase (*28*). Ma and workers sequestered CO_2 by reacting it with an epoxide to form a cyclic carbonate using immobilized choline chloride-urea as a catalyst (*29*). DES's containing $ZnCl_2$ in place of either the hydrogen bond donor or ammonium salt component are conductive (*30*, *31*) and also dissolve starch (*32*).

Some applications involve reactions of the components of the DES's. Heating a choline-chloride-urea DES caused a breakdown of the urea to an amine, which reacted to form aluminophosphonate materials (*33*). Different urea derivatives yielded different aluminophosphonate structures. To derivatize cellulose with ether links, Abbott and coworkers heated it in a DES containing alkylating agent chlorocholine chloride instead to choline chloride. (*34*).

Our preliminary report of lipase-catalyzed transesterification and aminolysis in deep eutectic solvents (*35*) was surprising for two reasons. First, even though immobilized *Candida antarctica* lipase B (iCALB) denatures in solutions of urea, it did not denature in DES's containing 10 M urea. Second, even though alcohols and amines were reactants in these reactions, the alcohol or amine components of DES's showed up to 200-fold reduced chemical reactivity and usually did not interfere with these reactions. We hypothesized that strong hydrogen bonds between DES components lowered their reactivity. We also reported enzyme-catalyzed hydrolyses in mixtures of water and DES. In one case – hydrolysis of styrene oxide catalyzed by epoxide hydrolase – the reaction rate was 20-fold faster in a water/DES mixture than in water.

In this work we expand these findings to wider range of DES's and reaction types. The new DES components include very strong hydrogen bond donors such as formamide, ammonium salts with more hydrophobic substituents, and sugars. The new reaction types are lipase-catalyzed perhydrolysis and ring-opening polymerization (Scheme 2).

Experimental Section

General

Reagents and enzymes were purchased from Sigma-Aldrich, except where noted otherwise. Ionic liquids were purchased from Solvent Innovation (Cologne, Germany). Blank reactions (no enzyme) gave negligible conversion as compared to the enzyme-containing reactions. Gas chromatography used a flame ionization detector with the detector temperature at 275 °C and injector temperature at 250 °C.

Synthesis of Deep Eutectic Solvents

Ammonium-based DES's were prepared according to Abbott and coworkers method (*19*). For amide and glycerol-based DES's, ammonium salt (0.050 mol) and hydrogen bond donor (0.100 mol) were combined in a 20-mL vial and stirred at 60 to 80 °C until a homogeneous liquid formed, typically one hour. For sugar-based DES's, ammonium salt (0.050 mol) and sugar (0.050 mol) were combined as above, and stirred at 100 °C until a homogeneous liquid formed, typically several hours. The sugar-based DES's are very viscous, so they were warmed to 60 °C before use. The zinc-chloride-based DES was prepared by combining zinc chloride (0.050 mol) and urea (0.175 mol) and stirring at 80 °C.
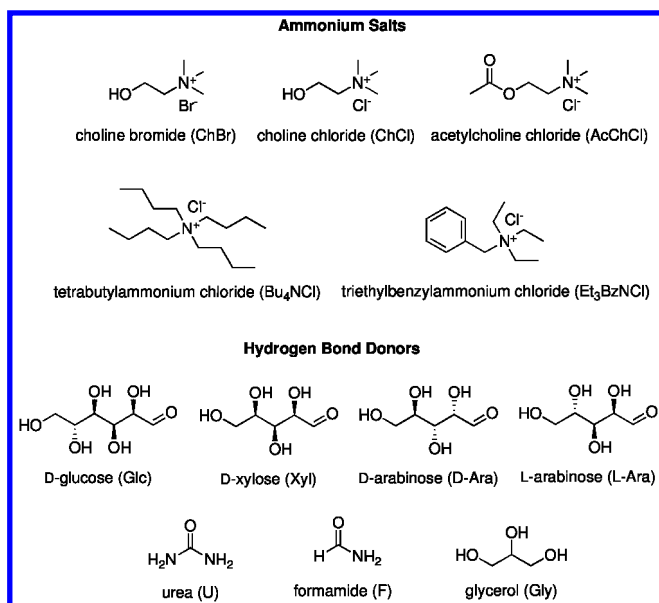
Scheme 1. Many deep eutectic solvents are 1:1 or 1:2 mixtures of an ammonium salts and a hydrogen bond donor. These components were used in this work.

Transesterification

Immobilized CALB (iCALB, 1.0 mg of immobilized enzyme preparation) was suspended in solvent (0.20 mL) in a glass vial. Ethyl valerate (3.0 µL, 100 mM) and 2-butanol (3.7 µL, 200 mM) were added to the suspension and the resulting mixture was stirred at 40 °C for 15 min (60 °C for 30 min for the sugar-based DES's). The reaction products were extracted with toluene (1.0 mL) and analyzed by gas chromatography (Varian CP 7502 column, 25 m x 0.25 mm inner diameter and 0.25 µm film thickness). The initial column temperature of 50 °C was held for 8 min, then increased to 200 °C at 10 °C min^{-1} held at 200 °C for 5 min.

Perhydrolysis

Immobilized CALB (iCALB, 5.0 mg of immobilized enzyme preparation) was suspended in solvent (1.0 mL) in a glass vial. Cyclohexene (0.30 mL, 3.0 M final concentration) and octanoic acid (60 µL, 400 mM) were added to the suspension and the resulting mixture was stirred at room temperature. Hydrogen peroxide (0.18 mL of a 50 wt% solution in water, 440 mM final concentration) was added in six portions over the first five hours of reaction. After 24 h, the reaction products were extracted with toluene (1.0 mL) and analyzed by gas chromatography on an HP-5 column (J&W Scientific, Folsom, CA, 30 m x 0.32 mm inner dia and 0.25 µm film thickness). The initial column temperature of

60 °C was held for 6 min, then increased to 165 °C at 15 °C min^{-1}, then further increased to 200 °C at 25 °C min^{-1} and held at 200 °C for 5 min.

Polymerization

ε-Caprolactone (0.10 mL) was added to a magnetically-stirred suspension of immobilized CALB (iCALB, 3.3 mg of immobilized enzyme preparation) in solvent (0.20 mL) in a glass vial. After 24 h at 70 °C, the reaction was stopped by the addition of methanol (1.0 mL) and the vials were stored at 4 °C for 1 h to precipitate any polymer formed. Control reactions without enzyme or without lactone gave no precipitate.

Results

Wider Range of DES's

We previously reported that iCALB was active in DES's composed of choline chloride or ethylammonium chloride combined with an amide- hydroxyl- or acid-containing hydrogen bond donor (*35*). In this study, we examined a wider range of DES's and found that iCALB was active in all of them (Scheme 1 and Table I). These include several quarternary ammonium salts combined with glycerol or urea; choline chloride combined with sugars or with formamide, and even zinc chloride coupled to urea. Thus, a wide range of DES's are potential solvents for iCALB-catalyzed reactions.

Stability

Previously, we found that iCALB is at least 20 times more stable in ChCl:U than in either a 5 M choline chloride or 10 M urea solution. The enzyme lost < 1% activity in 90 min at 60 °C as compared with 25% loss in the choline chloride solution and 70% loss in the urea solution. Here we tested the long-term stability of iCALB in a glycerol-based DES. We incubated free iCALB in either toluene or ChCl:Gly for 18 h at 60 °C and tested the initial rate of transesterification of ethyl valerate to butyl valerate. In toluene, the activity dropped 12% compared to the rate before incubation, but in ChCl:Gly, it dropped only 5%. Thus, for this glycerol-based DES, iCALB is more stable than in toluene.

Activity in Transesterification

The initial activity of iCALB in the transesterification of ethyl valerate (100 mM) with 2-butanol (200 mM) in DES's was comparable to that in toluene (Table I). Initial rates were calculated from the conversion of ethyl valerate to
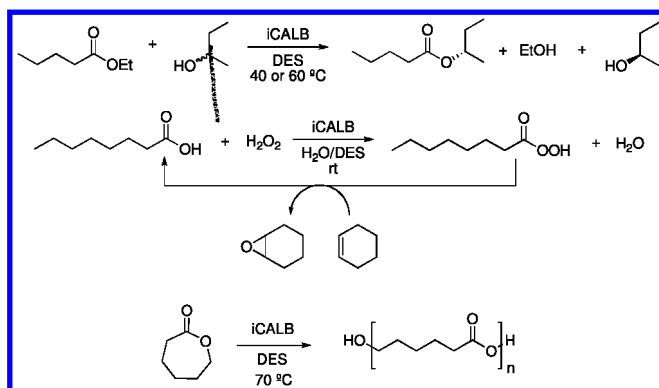
Scheme 2. iCALB-catalyzed reactions in DES's. Transesterification of ethyl valerate with 2-butanol (top), perhydrolysis of octanoic acid with the simultaneous epoxidation of cyclohexene (center), and ring-opening polymerization of ε-caprolactone (bottom).

2-butyl valerate after 15 minutes of reaction at 40 °C. Typical conversions were 10-45%, but the best solvent - ChCl:U – gave a conversion of 74%. Immobilized CALB was most active in ChCl:U, ChCl:Gly, ChBr:Gly, and Et$_3$BzNCl:Gly, with initial activities of 990, 640, 580, and 450 mU mg^{-1}, respectively. Immobilized CALB was less active in AcChCl:Gly (410 mU mg^{-1}), ZnCl$_2$:U (260 mU mg^{-1}), Bu$_4$NCl:Gly (200 mU mg^{-1}), and ChCl:F (150 mU mg^{-1}) than in toluene (430 mU mg^{-1}). These lower activities are all higher than those in the ionic liquid 1-butyl-3-methylimidazolium bis(trifluoromethanesulfonyl)-imide (BMIM[TF$_2$N], 140 mU mg^{-1}).

The activity of iCALB was slightly higher in the more viscous sugar-based DES's, likely due to the higher temperature used for these reactions (60 °C instead of 40 °C). The high viscosity of these sugar-based DES's required the higher temperature. Immobilized CALB had a higher initial activity in each sugar-based DES than toluene (660 mU mg^{-1}). The activity of iCALB in sugar-based DES's decreased as the viscosity increased: ChCl:Glc (750 mU mg^{-1}) was qualitatively the most viscous, ChCl:D-Ara (800 mU mg^{-1}) and ChCl:L-Ara (830 mU mg^{-1}) were less viscous, and ChCl:Xyl (880 mU mg^{-1}) was least viscous. This heterogenous reaction may be limited by slow diffusion in these viscous solvents.

Enantioselectivity

The enantioselectivity of iCALB toward 2-butanol is low ($E = 9.9$ in toluene at 40 °C) likely due to the difficulty in distinguishing the methyl and ethyl substituents at the stereocenter. This enantioselectivity decreased by a factor to two or more in both BMIM[Tf$_2$N] and in DES's. The enantioselectivity in DES's were generally higher than in BMIM[Tf$_2$N] (3.1), with Bu$_4$NCl:Gly (2.8) as the exception. Et$_3$BzNCl:Gly gave the highest enantioselectivity of any DES (5.2),

Table I. Initial Activity and Enantioselectivity of CALB-Catalyzed Transesterification of Ethyl Valerate and Butanol in DES's[a]

Solvent	Type of Solvent	Alcohol	Initial Activity (mU mg^{-1})	Enantio-selectivity
Toluene	Organic	1-butanol	620 (*35*)	N/A
Toluene	Organic	2-butanol	430	9.9
Toluene	Organic	2-butanol	660 [b]	6.2
BMIM[Tf$_2$N]	RTIL	1-butanol	400 (*35*)	N/A
BMIM[Tf$_2$N]	RTIL	2-butanol	140	3.1
AcChCl:Gly	DES	2-butanol	410	5.0
Bu$_4$NCl:Gly	DES	2-butanol	200	2.8
ChBr:Gly	DES	2-butanol	580	4.5
ChCl:F	DES	2-butanol	150	4.4
ChCl:Gly	DES	1-butanol	560 (*35*)	N/A
ChCl:Gly	DES	2-butanol	640	4.9
ChCl:U	DES	1-butanol	340 (*35*)	N/A
ChCl:U	DES	2-butanol	990	3.6
ZnCl$_2$:U	DES	2-butanol	260	7.2
Et$_3$BzNCl:Gly	DES	2-butanol	450	5.2
ChCl:D-Ara	DES	2-butanol	800 [b]	3.7
ChCl:L-Ara	DES	2-butanol	830 [b]	3.2
ChCl:Glc	DES	2-butanol	750 [b]	4.0
ChCl:Xyl	DES	2-butanol	880 [b]	2.8

[a] Conditions: 2-butanol – 15 min, 40 °C, 5 mg mL^{-1} iCALB, 100 mM ethyl valerate, 200 mM 2-butanol.   [b] 30-min reaction, 60 °C. 1-butanol – 15 min, 60 °C, 2.5 mg mL^{-1} iCALB, 40 mM ethyl valerate, 400 mM 1-butanol. 1 U = 1 μmol product formed min^{-1}. N/A = not applicable.

followed by AcChCl:Gly (5.0), ChCl:Gly (4.9), ChBr:Gly (4.5), ChCl:F (4.4), and ChCl:U (3.6).

The enantioselectivity of iCALB was also reduced in the sugar-based DES's compared to toluene ($E = 6.2$ at 60 °C). ChCl:Glc had the highest enantioselectivity of the DES's (4.0), while ChCl:D-Ara (3.7), ChCl:L-Ara (3.2), and ChCl:Xyl (2.8) were marginally lower. The two DES's containing enantiomeric arabinoses as components had similar enantioselectivities, suggesting that the arabinoses do not interact strongly with either the substrate or enzyme.

Perhydrolysis in DES-Water Mixtures

DES's were comparable to an ionic liquid for an iCALB-catalyzed perhydrolysis, but not as good as acetonitrile (Table II). Perhydrolysis of octanoic acid by hydrogen peroxide yielded the peracid, which reacted with cyclohexene to form cyclohexene oxide. The reaction mixture included ~10 vol% water from the added hydrogen peroxide solution. The conversion of cyclohexene to cyclohexene oxide was similar in ChCl:U (8%), ChCl:Gly (22%) and BMIM[BF$_4$] (15%), but substantially higher in acetonitrile (79%). Sheldon and coworkers also found that acetonitrile was the best solvent for this reaction (*9*).

Polymerization

Immobilized CALB also catalyzed ring-opening polymerization of ε-caprolactone in four DES's. We avoided DES's containing hydroxyl groups to prevent side reactions with the DES components. Trace water bound to the immobilized enzyme preparation initiated the polymerization. Similar amounts of polymer precipitate formed in AcChCl:U and Bu$_4$NCl:U as in toluene. A smaller amount of polymer precipitate formed in ChCl:U, and ChCl:F, but none in ZnCl$_2$:U. Further characterization of the polymer is in progress.

Discussion

CALB is active in a wide variety of DES's. We have expanded the range of DES's that may be suitable for enzymatic transformations into the realm of very strong hydrogen bond donors such as formamide, ammonium salts with more hydrophobic substituents, and sugars as hydrogen bond donors. Immobilized CALB was active in all combinations of ammonium salt and amide- or polyol-based hydrogen bond donor that we tested. The requirements for a suitable solvent appear to be: i) the components can form a homogeneous mixture, ii) the hydrogen bonds between DES components are strong enough to reduce the reactivity and hydrogen bond basicity of the two components, iii) the ammonium salt has no potential for proton exchange with desired substrates.

Requirements ii and iii depend on the specific reaction (Scheme 3). For instance, EAC:Gly is a suitable solvent for transesterification between ethyl valerate and butanol, but not for aminolysis of ethyl valerate. Little or no side products form in the transesterification, but the amine reacted with the ethyl ammonium component to make ethyl amine, resulting in significant amounts of aminolysis to ethyl amides instead of the desired amide (*35*). In another example, ChCl:EG was not a suitable solvent for transesterifications. Although the reactivity of the ethylene glycol component was reduced in the DES, it remained significant and the side reaction with ethylene glycol accounted for more than half of total conversion of ester.

Table II. Oxidation of cyclohexene to cyclohexene oxide by peroctanoic acid formed by iCALB-catalyed perhydrolysis of octanoic acid[a]

Solvent	Type of solvent	Conversion (%)
MeCN	Organic	79
BMIM[BF$_4$]	RTIL	15
ChCl:Gly	DES	22
ChCl:U	DES	8

[a] Conditions: 0.18 mL 50 wt% hydrogen peroxide added in six portions over 5 h to a mixture of 1.0 mL solvent, 0.3 mL cyclohexene, 60 μL octanoic acid, and 5 mg iCALB; stirred for 24 h total.

Another consideration for using DES's is viscosity. High viscosity makes handling and filtration difficult and may limit the reaction rates of heterogenous reactions where diffusion is important. The sugar-based DES's had viscosities likely too high for practical use without additives to decrease viscosity.

While iCALB typically had comparable and often higher activity in DES's compared to toluene, its enantioselectivity was lower. We can suggest three possible reasons. First, the molecular volume of a DES may be substantially larger than toluene, so that the substrate will displace different numbers of solvent molecules from active site. The different numbers will yield different entropy contributions to the reaction rate. Further, if the substrate enantiomers displace different numbers of solvent molecules in the two cases, then the different entropy contributions can change the enantioselectivity (*36*).

A second explanation is that some catalysis in DES's occurred outside the active site. Ma and coworkers reported that ChCl:U catalyzed the reaction of CO$_2$ with epoxides (*29*), so it is conceivable that DES's might catalyze transesterification. Control reactions showed that no product formed without enzyme, so this explanation would require the catalyst to be some type of adduct of DES and enzyme.

A third explanation is that the enzyme conformation in the polar DES's differs from that in toluene. Support for this notion comes from recent computer simulations that suggest that CALB changes its conformation in different organic solvents (*37*). Such changes could alter enantioselectivity, especially if the changes involve residues in the alcohol-binding site.

Both BMIM[BF$_4$] and DES's were poorer solvents than acetonitrile for perhydrolysis. This difference may be due to the reaction conditions, especially the ~10 vol% water. Seddon's group reported that the CALB perhydrolysis activity was comparable in both BMIM[BF$_4$] and acetonitrile, but they used less water (a more concentrated hydrogen peroxide solution) (*9*). Another possibility in the case of urea-containing DES's is that the urea acted as a base-catalyst for the hydrolysis of peracid.
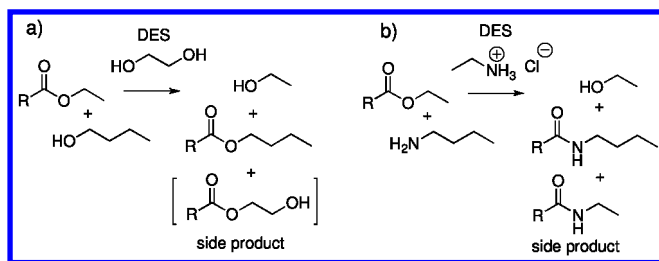
Scheme 3. Two side reactions of DES's in lipase-catalyzed reactions.
a) The ethylene glycol component in DES competed with 1-butanol in
an iCALB-catalyzed transesterification. The brackets indicate that gas
chromato-graphy did not detect this presumed side product. DES's containing
glycerol did not show this side reaction. b) The ethylamine component in DES
competed with butylamine in an iCALB-catalyzed aminolysis. This side reaction
does not occur in transesterifications, presumably because the ethylamine
remains protonated and unreactive.

Conclusion

CALB is active in a variety of ammonium-ion-based DES's, despite the presence of chlorides and strong hydrogen bond donors such as formamide and urea. CALB is also active in DES's containing sugars such as hydrogen bond donors. Generally, the activity of CALB for tranesterifications in most DES's was comparable to toluene, but the enantioselectivity was lower. DES's were comparable to BMIM[BF$_4$] as solvents for perhydrolysis, but were not as good as acetonitrile.

Acknowledgments

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