Hydrolase-catalyzed biotransformations in deep eutectic solvents†

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Hydrolases show good catalytic activity in deep eutectic solvents, despite the presence of urea, which can denature enzymes, or alcohols, which can interfere with hydrolase-catalyzed reactions.

Room-temperature ionic liquids are potential green alternatives to organic solvents[1] for extractions,[2] chemical reactions[3] and biotransformations.[4] Ionic liquids are non-volatile, thermally stable and their solvation properties vary by changing the cation and anion. The limitations of ionic liquids are cost ($\sim$240 \$US kg^{-1} for 95% pure 1-butyl-3-methylimidazolium chloride *vs.* $\sim$30 \$US kg^{-1} for 99% pure acetone or toluene), toxicity similar to or higher than organic solvents,[5] and the need for high purity, as even trace impurities affect physical properties.[6]

Deep eutectic solvents (DESs)—eutectic mixtures of an ammonium salt and a hydrogen-bond donor such as choline chloride and urea—are alternatives to ionic liquids.[7]‡ Like ionic liquids, DESs often have melting points below room temperature, low volatility, and high thermal stability; but unlike ionic liquids, they include uncharged components—urea in the example above. No one has yet reported biotransformations in DESs, likely because strong hydrogen-bond donors like urea denature proteins. For example, 10 M urea or 5 M choline chloride inactivate lipase B from *Candida antarctica* (70% or 25% loss in activity after 90 min at 60 °C, respectively). DESs also contain halides, which inactivate or inhibit proteins when present in ionic liquids.[8] In spite of these possible problems, we found that many hydrolases retain excellent activity in DESs.

As a test reaction, we used the lipase-catalyzed transesterification of ethyl valerate with 1-butanol, Table 1. CALB§ and the immobilized form—*i*CALB—catalyzed transesterification in all eight DESs tested and showed conversions comparable to that in toluene for five of the DESs. CALA also showed activity in all eight DESs, but showed conversions comparable to toluene only in ChCl:Gly (76 *vs.* 70%). PCL showed lower conversions than the other enzymes, but the conversion in one DES—ChCl:Gly—was higher than that in toluene (22 *vs.* 5%). The transesterification activity of CALB did not decrease in ChCl:U at 60 °C over 90 min, making it at least 20- to 35-fold more stable in the mixture than in aqueous solution of the components: 10 M urea or 5 M choline chloride (see ESI†).

Some DESs contain an alcohol component—ethylene glycol or glycerol, which competes with 1-butanol in transesterification. Indeed, ethylene glycol monoester was the major product for reaction in the two DESs containing ethylene glycol. For example, the CALB-catalyzed reaction in EAC:EG showed 54% of starting material consumed resulting in 31% ethylene glycol monovalerate and 23% ethyl butyrate. This nearly equal amount of the two product esters is surprising because the concentration of ethylene glycol (10 M) was 25 times higher than the concentration of 1-butanol (400 mM). In a competition between ethylene glycol and 1-butanol in *tert*-butanol, 1-butanol reacted three times faster. Thus, ethylene glycol was 9-fold less reactive in transesterification when it was present as a component of a DES.

Even more surprising were the transesterification reactions in glycerol-containing DESs (8 M glycerol) which showed $>$90% conversion and $<$0.5% glyceryl ester formation (see ESI†). In a competition between glycerol and 1-butanol in *tert*-butanol, 1-butanol reacted six times faster. Thus, glycerol was $>$600-fold less reactive in transesterification when it was present as a component of a DES.

The initial specific activity for *i*CALB-catalyzed transesterification was comparable or higher in DESs as compared to typical ionic liquids, Table 2. Previous work showed that *i*CALB had good transesterification activity in 1-butyl-3-methylimidazolium bis(trifluoromethane)sulfonimide (BMIM

Table 1 Percentage conversion of ethyl valerate to butyl valerate at 60 °C

	*i*CALB	CALB	CALA	PCL	No enzyme
ChCl:Acet	23[a]	96	0.5	0.0	0.0
ChCl:EG	11 (99)[b]	32 (93)[b]	3.0	0.2	0.0
ChCl:Gly	96	96	70	22	0.0
ChCl:MA	30	58	0.7	0.0	0.7
ChCl:U	93	99	1.6	0.8	0.0
EAC:Acet	63	92	2.7	0.0	0.0
EAC:EG	23 (54)[b]	33 (79)[b]	20	0.0	0.0
EAC:Gly	93	91	2.1	0.5	0.0
Toluene	92	92	76	5.0	0.0

[a] 40 mM ethyl valerate, 400 mM 1-butanol, 10 mg ml^{-1} enzyme, 24 h. [b] Number in parentheses is the percentage conversion including the side reaction with the ethylene glycol component of the deep eutectic solvent. No side reaction was detected in the other reactions.

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Table 2 Initial specific activity of *i*CALB after 15 min of reaction

	Transesterification activity[a]	Aminolysis activity[a]
Toluene	37 (100%)	46 (100%)
ChCl:Gly	33 (89%)	52 (113%)
ChCl:U	20 (54%)	22 (48%)
EAC:Gly	50 (135%)	Not determined[b]
BMIM[Tf_2N]	24 (65%)	11 (24%)
BMIM[BF_4]	7 (19%)	9 (19%)

[a] μmol ethyl valerate h^{-1} mg solid^{-1}, activity relative to toluene in parentheses. *i*CALB contains ~10 wt% protein. [b] Aminolysis with ethylamine from the DES was the dominant reaction.

[Tf_2N]) compared to other ionic liquids.[9] 1-Butyl-3-methylimidazolium tetrafluoroborate (BMIM[BF_4]) is water miscible like the DESs and preserves enzyme activity.[10] *i*CALB had the highest initial specific transesterification activity in EAC:Gly (50 μmol ethyl valerate h^{-1} mg^{-1}), which was twice as high as for BMIM[Tf_2N] (24 μmol h^{-1} mg^{-1}) and seven times higher than the activity we found in BMIM[BF_4] (7 μmol h^{-1} mg^{-1}). The initial specific activity in toluene, ChCl:Gly, and ChCl:U ranged from 20 to 37 μmol h^{-1} mg^{-1} and were comparable to that in BMIM[Tf_2N].

*i*CALB also catalyzed another test reaction in DESs—aminolysis of ethyl valerate with 1-butylamine, Fig. 1. The reaction rates and final conversion (>90%) were similar in ChCl:Gly, ChCl:U or toluene. Aminolysis was slower in ChCl:Acet and gave only 39% conversion. In EAC:Gly aminolysis with ethylamine from the DES (4.5 M amine, 80% conversion) predominated over aminolysis with butylamine (11% conversion), likely due to proton exchange between butylamine with the ethylammonium cation. The relative amount of ethylamide formed is seven-fold less than the relative concentration of ethylamine. In contrast, the transesterification reactions above did not show competing aminolysis when using ethylammonium-chloride-containing DESs. A possible reason is that the ethylammonium ion remained protonated and unreactive because the transesterification reactions lacked a base.

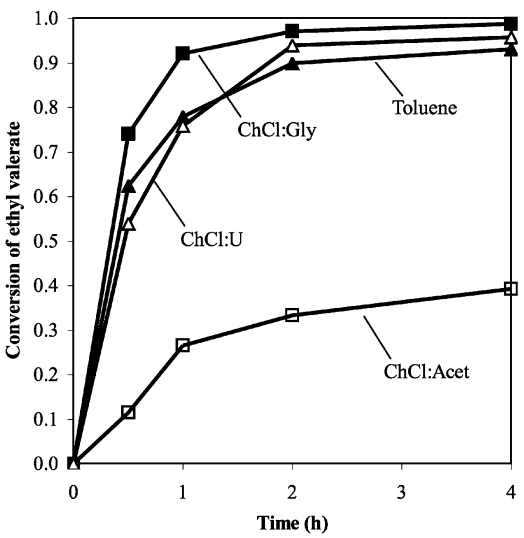

Fig. 1 Aminolysis of ethyl valerate (100 mM) with 1-butylamine (110 mM) at 60 °C with 10 mg ml^{-1} *i*CALB.

Table 3 Percentage conversion of styrene oxide by EHAD1 in ChCl:Gly/buffer mixtures at 37 °C after 2 h

Cosolvent	90%[a]	75%	50%	25%	10%	0%
ChCl:Gly	0	0	2.0	92	36	4.6
DMSO[b]	N.D.	N.D.	N.D.	0.7	1.9	4.6
Acetonitrile	N.D.	N.D.	N.D.	0.1	0.7	4.6

[a] Cosolvent volume faction in solution containing 0.05 mg ml^{-1} enzyme, 100 mM styrene oxide at 5 mM BES at pH 7.2 used as buffer. [b] DMSO or acetonitrile added in place of DES.

The initial specific activity for aminolysis in DESs was also higher than in ionic liquids, Table 2. The aminolysis activity in ChCl:Gly (52) was five times higher than in BMIM[BF_4] or BMIM[Tf_2N] (9–11) and similar to the activity in toluene (46 μmol h^{-1} mg^{-1}).

DESs were also suitable as cosolvents for reactions in aqueous solution, where they enhanced hydrolase-catalyzed reactions up to 20-fold. The rates of esterase-catalyzed hydrolysis of *p*-nitrophenyl acetate increased moderately upon addition of 10 vol% ChCl:Gly: three-fold increase for PLE and ROE and a 25% increase for PFE and CALB (see ESI†) The rate of epoxide hydrolase[11] catalyzed hydrolysis of styrene oxide increased dramatically: a 20-fold increase in conversion, Table 3. The conversion was only 4.6% in buffer, but increased to 92% upon addition of 25 vol% ChCl:Gly, with no change in the enantioselectivity (E = 16; Janssen's group has created a mutant with higher enantioselectivity, but we have not tested whether it shows a similar increase in conversion.[12]) Similar additions of 10 or 25% DMSO or acetonitrile did not increase the conversion for epoxide hydrolase-catalyzed hydrolysis of styrene oxide, suggesting that the effect is not a simple increase in substrate solubility. Adding more than 25 vol% DES decreased conversions. For example, the conversion of the EHAD1-catalyzed reaction decreased from 92% conversion in 25 vol% DES to 2% in 50 vol% DES. Similarly the rates of esterase-catalyzed hydrolysis of *p*-nitrophenyl acetate decreased in solutions containing more than 25 vol% DES (Fig. S2 in ESI†).

The polarity of these DESs is higher than typical imidazolium-based ionic liquids according to the Reichardt's dye method, Table 4. The E_T^N of the DESs ranged from 0.77 to 0.93 as compared to 0.53 to 0.75 for imidazolium-based ionic liquids.[13] The polarity of DESs containing the quaternary salt choline chloride was 0.08 to 0.09 units lower than the

Table 4 Solvent polarity according to color of dissolved Reichardt's dye

Solvent	E_T^N	Solvent	E_T^N
Toluene	0.10[14a]	ChCl:EG	0.80
DMSO	0.44[14]	Glycerol	0.81[14]
Acetonitrile	0.46[14]	ChCl:Gly	0.84
BMIM[Tf_2N]	0.64[13]	ChCl:U	0.84
BMIM[BF_4]	0.68[13]	EAC:Acet	0.85
Methanol	0.76[14]	EAC:EG	0.88
ChCl:Acet	0.77	EAC:Gly	0.93

[a] Solvent polarity according to Reichardt's normalized polarity scale, where water has a polarity of 1.0 and trimethylsilane has a polarity of 0.0.

corresponding primary ethylammonium chloride DESs, which is consistent with the lower polarity of quaternary ammonium-based ionic liquids as compared to primary ammonium-based ionic liquids.[13]

The components within the DESs are 20- to >600-fold less reactive than expected based on their concentration in denaturation and in transesterification reactions. This lowered reactivity corresponds to 2–4 kcal mol^{-1}, similar to the energy associated with the formation of hydrogen bonds. We propose that the hydrogen-bond network in DESs lowers the chemical potential of the components of DESs and thereby makes them suitable as solvents for a much wider range of reactions than one would predict based only on the components. The DES components are inexpensive (*e.g.*, 65 \$US kg^{-1} for choline chloride, 20 \$US kg^{-1} for urea, 35 \$US kg^{-1} for glycerol) and biodegradable, making DESs attractive as green replacements for volatile organic solvents.

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Notes and references

‡ Synthesis of deep eutectic solvents: ammonium salt (0.05 mol) and hydrogen-bond donor (0.1 mol for choline chloride mixtures, 0.075 mol for ethylammonium chloride mixtures) were added to a 20-ml vial and heated at 80 °C until a clear, homogenous liquid formed, typically 1 h.
§ *Abbreviations* of deep eutectic solvents: ChCl:Acet = choline chloride–acetamide; ChCl:EG = choline chloride–ethylene glycol; ChCl:Gly = choline chloride–glycerol; ChCl:U = choline chloride–urea; ChCl:MA = choline chloride–malonic acid; EAC:Acet = ethylammonium chloride–acetamide; EAC:EG = ethylammonium chloride–ethylene glycol; EAC:Gly = ethylammonium chloride–glycerol.
Abbreviations of enzymes: CALA = Roche. Chirazyme L-5 (lyophilized *Candida antarctica* lipase A); *i*CALB = Novozyme 435 (*Candida antarctica* lipase B immobilized on acrylic resin); CALB = Roche Chirazyme L-2 (lyophilized *Candida antarctica* lipase B); CRL = *Candida rugosa* lipase; EHAD1 = epoxide hydrolase AD1 from *Agrobacterium radiobacter*; PCL = Amano PS (lyophilized *Burkholderia* (formerly *Pseudomonas*) *cepacia* lipase); PFE = *Psuedomonas fluorescens* esterase; PLE = pig liver esterase; ROE = *Rhizopus oryzae* esterase; SABP2 = salicylic acid binding protein 2 from tobacco, an esterase.

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