APPLICATION OF DEEP EUTECTIC SOLVENTS AND IONIC LIQUIDS TO HYDROLASE-CATALYZED REACTIONS

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DEDICATION

This thesis is dedicated to my wife Yongling, without whom I would undoubtedly have had no social interaction during its writing. She has been a spring of support and understanding throughout the process of earning a Ph.D., and has taught me that there is a bit more to life than chemical engineering.

ABSTRACT

Hydrolases are important enzymes for stereoselective and environmentally benign synthesis. In nature, hydrolases cleave bonds with water. When used in organic solvents, these enzymes can make synthetically useful bonds through condensation and the release of a small molecule, usually water or an alcohol. Many organic solvents that preserve enzyme activity, such as toluene, can be environmentally damaging or toxic. Room temperature ionic liquids, poorly coordinating salts that are liquid at temperatures below 100 ˚C, are a potential alternative to organic solvents for hydrolase-catalyzed reactions because of their low volatility, moderate polarity, and recyclability. However, many commonly used ionic liquids are orders of magnitude more expensive than conventional organic solvents and may also cause adverse environmental effects if released into aquatic environments. We demonstrate that ionic liquids are effective solvents for the lipase-catalyzed polymerization of ε-caprolactone and other poly(hydroxyalkanoates) and are effective in enhancing the electrical conductivity of carotenoid-containing polymers produced enzymatically. How-ever, we found that they were not as effective as toluene for enzyme catalysis, and strove to find better alternative solvents for biotransformations.

We discovered that deep eutectic solvents, mixtures of ammonium or metal salts such as choline chloride and hydrogen bond donors such as urea or glycerol, were exceptional low-cost, biodegradable alternatives to organic solvents for hydrolase-catalyzed reactions. These physical mixtures may be thought of as ionic liquids, because they share similar physical properties to those solvents. Though they are composed of potential denaturants such as urea or halide anions, deep eutectic solvents stabilize enzymes. This stabilization is likely due to a preference for intra-solvent hydrogen bonding compared to enzyme-solvent hydrogen bonding. Deep eutectic solvents enhanced enzyme activity for

a number of lipases either as pure solvents for reactions such as transesterification or polyesterification; or as additives in aqueous reactions such as epoxide ring opening or ester hydrolysis. We have preliminary evidence that deep eutectic solvents may induce a conformational change in enzymes that can alter reaction rates. These changes appear to be distinct from those caused by denaturing solvents.

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LIST OF ABBREVIATIONS

ABTS – 2,2-*O*-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt

Aliquat – cation of Aliquat 336, a commercial phase transfer catalyst composed of a mixture of methyl tri(octyl or decyl) ammonium chlorides

Ammoeng – a series of commercial quaternary ammonium ionic liquids with one methyl group, two short chains (4-25 units total) of poly(ethylene glycol), and a cocos (100 and 101), tallow (102), or other natural group; and a methylsulfate (100), chloride (101), ethylsulfate (102) or similar anion

BCL or PCL – *Burkholderia* (formerly *Pseudomonas*) *cepacia* lipase

BSE – *Bacillus subtilis* esterase

BSteE – *Bacillus stearothermophilus* esterase

BMIM – 1-butyl-3-methylimidazolium

CALA – *Candida antarctica* lipase A

CALB – *Candida antarctica* lipase B

ChCl – choline chloride

ChCl:Acet – choline chloride/acetamide deep eutectic solvent

ChCl:EG – choline chloride/ethylene glycol deep eutectic solvent

ChCl:Gly – choline chloride/ glycerol deep eutectic solvent

ChCl:MA – choline chloride/ malonic acid deep eutectic solvent

ChCl:U – choline chloride/ urea deep eutectic solvent

CLEA – cross-linked enzyme aggregate

CLEC – cross-linked enzyme crystal

CRL – *Candida rugosa* lipase

DAAO – D-amino acid oxygenase

DCA – dicyanamide

DES – deep eutectic solvent

E – enantioselectivity, the relative rate of reaction of the fast-reacting enantiomer as compared to the slow-reacting enantiomer

EAC – ethylammonium chloride

EAC:Acet – ethylammonium chloride/ acetamide deep eutectic solvent

EAC:EG – ethylammonium chloride/ ethylene glycol deep eutectic solvent

EAC:Gly – ethylammonium chloride/ glycerol deep eutectic solvent

EHAD1 – epoxide hydrolase AD1 from *Agrobacterium radiobacter*

EMIM – 1-ethyl-3-methylimidazolium

HEMIM – 1-(2-hydroxyethyl)-3-methylimidazolium

HPMIM – 1-(3-hydroxypropyl)-3-methylimidazolium

HPO – horseradish peroxidase

MMIM – 1,3-dimethylimidazolium

MML – *Mucor miehei* lipase

MOEMIM – 1-methoxyethyl-3-methylimidazolium

MTEOA – methyltri(2-hydroxyethyl)ammonium

NAD(H) – (reduced) nicotinamide adenine dinucleotide

OMA – methyltrioctylammonium

OMIM – 1-methyl-3-octylimidazolium

OTf – trifluoromethanesulfonic acid

$P_{6,6,6,14}$ – trihexyltetradecylphosphonium

PEG – poly(ethylene glycol)

PFE – *Pseudomonas fluorescens* esterase

PLE – pig liver esterase

ROE – *Rhizopus oryzae* esterase

RMIM – 1-alkyl-3-methylimidazolium

RTIL or IL – (room temperature) ionic liquid

SABP2 – salicylic acid binding protein 2 from tobacco, an esterase.

Tf_2N – bis(trifluoromethanesulfonyl)imide

TLL – *Thermomyces languinosus* lipase

CHAPTER 1. Introduction: enzymatic transformations in ionic liquids and deep eutectic solvents

1.1 Chapter Overview

This chapter serves to introduce the elements of my thesis (section 1.2) and acts as a concise introduction to the large and growing field of biocatalysis in ionic liquids, with an emphasis on potential advantages that ionic liquids offer. Sections 1.3 and higher will appear in the Industrial Encyclopedia of Biotechnology in 2010.

Ionic liquids, also called molten salts, are mixtures of cations and anions that melt below 100 ˚C. Typical ionic liquids are dialkylimidazolium cations with weakly coordinating anions such as $[BF_4]$ or $[PF_6]$. Deep eutectic solvents are also included in this review because their properties are similar. Deep eutectic solvents are mixtures of salts such as choline chloride and hydrogen bond donors such as urea, oxalic acid, or glycerol.

The central advantage of ionic liquids over organic solvents is enhanced solubility of substrates or products without inactivating the enzymes. Ionic liquids can be cosolvents to dissolve nonpolar substrates in water mixtures. Water miscible organic solvents are not suitable alternatives because they inactivate enzymes. The enhanced solubility of substrates usually increases the rate of reaction and often increases the regio- or enantioselectivity. Ionic liquids can also serve as solvents for non-aqueous reactions. In these cases, they are especially suited to dissolve polar substrates. Polar organic solvent alternatives inactivate enzymes, but ionic liquids do not, even when they have similar polarities. Hydrolases and oxidoreductases are particularly well studied in ionic liquids.

Besides their solubility properties, ionic liquids and deep eutectic solvents may be more environmentally friendly than organic solvents because ionic liquids are non-volatile, and non-toxic ionic liquids can be made from non-toxic starting materials.

1.2 Thesis Outline

My primary contribution to the field is the development of enzyme catalysis in deep eutectic solvents, physical mixtures of hydrogen bond donors and acceptors that are inexpensive potential replacements for organic solvents. This dissertation primarily documents the work I've done in establishing these solvents as viable media for enzyme catalysis.

In the first chapter, I review the widespread use of room temperature ionic liquids, also called molten salts, in enzyme catalysis. Though they are physical mixtures and not strictly composed of cation-anion pairs, deep eutectic solvents have similar properties as ionic liquids and may be considered "untraditional" ionic liquids in that sense. Much of the review focuses on hydrolases, as does my research.

The second chapter details my initial work on producing poly(hydroxyalkanoates) in ionic liquids. The work focused on lipase-catalyzed ring-opening polymerization as the primary route to the polymers. I primarily worked with *Candida antarctica* lipase B as a catalyst. I was initially interested in the project because of the prospect of working on a green alternative to organic solvents. However, as the work progressed, it became clear after a visit to Solvent Innovation GmbH that ionic liquids were going to be at a severe cost disadvantage compared to organic solvents. The literature at the time also began pointing out that commonly used ionic liquids offered very few advantages over organic

solvents from an ecotoxicity standpoint.

These facts led me to shift my focus to an initial investigation on whether deep eutectic solvents could be useful media for enzyme catalysis, detailed in the third chapter. Since there hadn't yet been reports of enzyme catalysis in these solvents, I assayed which types of reactions and enzymes could be useful in the solvents. My primary goal was to determine if these solvents could eventually be generally applied to enzyme catalysis or restricted to niche applications with few enzymes. The experimental evidence seems to point to the former.

I return my focus on *Candida antarctica* lipase B in Chapter 4 and go into more detail on reactions with that enzyme in deep eutectic solvents. I explore a variety of reactions with that lipase in a number of interesting solvents.

The fifth chapter concerns the initial synthesis of carotenoid-polyester block copolymers. While the synthesis of the polymers does not directly involve ionic liquids or deep eutectic solvents, I found that doping these polymers with ionic liquids gave a measurable increase in the conductivity of the material. The synthesis of the carotenoid polymers extends some of the knowledge I gained from the work in Chapter 2, specifically regarding lipase-catalyzed ring-opening polymerization. The carotenoid precursors are quite soluble in ionic liquids and deep eutectic solvents compared to toluene or THF. As a result, polymerization and synthesis in these solvents may make an interesting future study.

In the final chapter, I present some ideas and preliminary data for future research directions. I examine some indications that deep eutectic solvents can cause

conformational changes in enzymes, which may explain some of the results I present in Chapters 3 and 4. I also present preliminary data that show that deep eutectic solvent formation could be a driving force for enzymatic biodiesel synthesis, and that deep eutectic solvents could be carbon- and nitrogen sources for cell growth.

1.3 Introduction

Ionic liquids, also called molten salts, are mixtures of cations and anions that melt below 100 ˚C. These fluids may be greener replacements for organic solvents because they have negligibly low vapor pressure. Ionic liquids are typically more viscous than organic solvents (~100 cP, similar to glycerol or honey), and their current cost is too high for industrial scale use. Ionic liquids have been used in electrochemistry *(1)* organic and inorganic synthesis *(2, 3)* and in separations *(4, 5)*, but this article focuses on ionic liquids as media for enzyme-catalyzed reactions. Here the main advantages are increased solubility of substrates or simpler separations of products. In some cases, ionic liquids increase the stability or selectivity of enzymes, but these effects are not predictable. Most applications of ionic liquids use isolated enzymes, but a few examples use whole-cell biocatalysis *(reviews: 2, 3, 6-12)*.

Ionic liquid technology

Ionic liquids have been known since the early 1900's *(13)*, but attracted little interest until the last few decades. The first generation of synthetically useful ionic liquids, discovered in 1982 by Wilkes *(14)*, were sensitive to air and/or water, and thus not suited for biotransformations. These ionic liquids were mainly imidazolium cations with chloroaluminate or other metal halide anions, Figure 1.

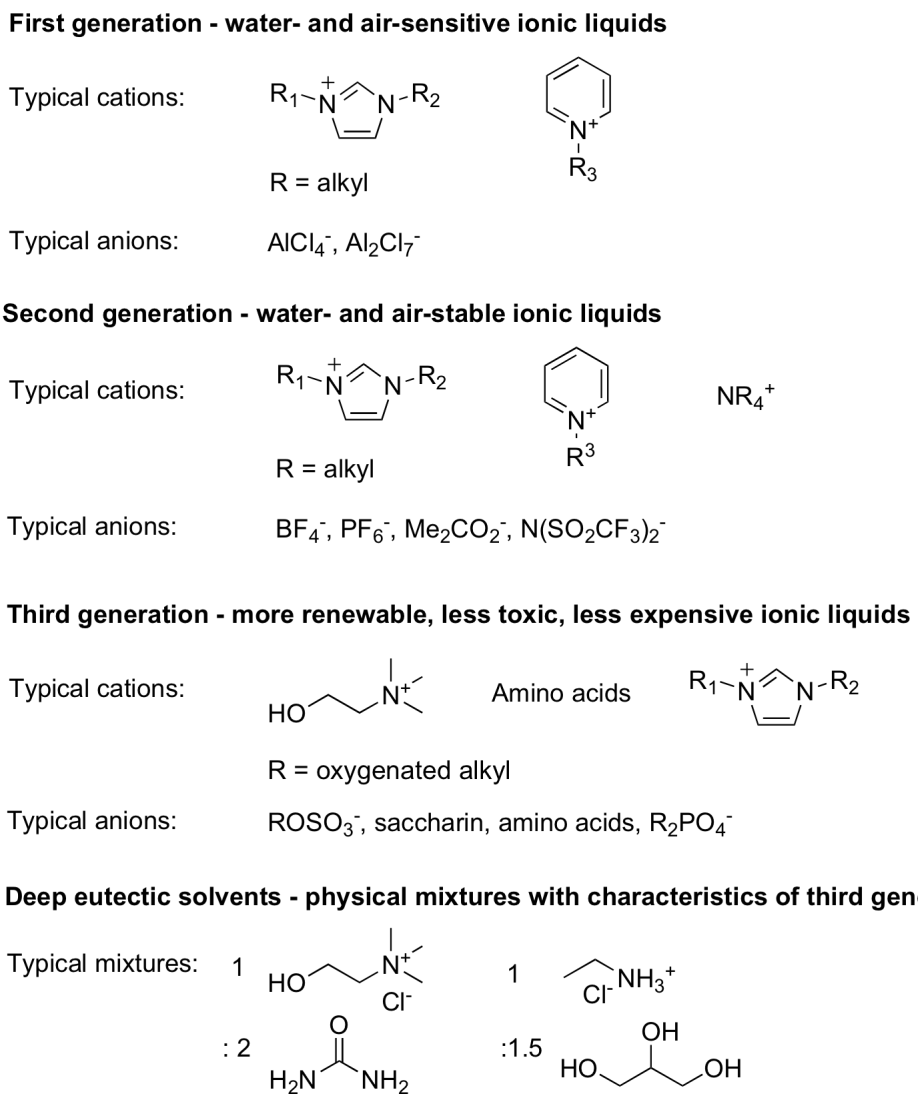


Figure 1. Example ionic liquids and deep eutectic solvents. Ionic liquids are salts that melt below 100°C likely due to a mismatch in the size of the anion and cation. Deep eutectic solvents are physical mixtures of salts and hydrogen bond donors that melt at low temperature.

The second generation of ionic liquids, discovered a decade later by Wilkes and Zaworotko *(15)*, were dialkylimidazolium cations with halides or weakly coordinating

anions such as [BF_4] or [PF_6]. These ionic liquids are stable to water and air and are the best-studied ionic liquids *(2, 3, 6-12)*. The first reports of enzyme-catalyzed reactions in ionic liquids *(16-20)* and most of the subsequent work used these liquids. Some key characteristics of these IL's are moderate polarity (similar to ethanol), air and water tolerance, and distribution of the anion's charge over multiple atoms, which weakens any hydrogen bonds between protein and solvent. Most are hydrophobic so they are immiscible with water. The disadvantages of these dialkylimidazolium-based ionic liquids are high cost, lack of large-scale production, the need for high purity, and toxicity similar to chlorinated and aromatic solvents *(21)*.

The third generation of ionic liquids retains the moderate polarity, stability, and distributed negative charge of the second generation, but use biodegradable, readily available, and lower toxicity cations and/or anions. For example, the cation may be choline and the anions may be sugars or sugar analogs, amino or organic acids, alkylsulfates, or alkylphosphates. These ionic liquids tend to be more hydrophilic than second generation ionic liquids, and are often water-miscible. Research on this new generation of ionic liquids has expanded considerably over the last few years, and many of these solvents are now commercially available. These third generation ionic liquids are still typically more expensive than organic solvents, mainly due to a need for high purity, as even trace impurities of halides or synthesis reagents can affect physical properties *(22)*. Current price targets from Solvent Innovation GmbH (a subsidiary of Merck) for ionic liquids are approximately 10 – 20 USD/kg on the ton scale, which is at least ten fold higher than many organic solvents.

Also included in this third generation are deep eutectic solvents, which are physical mixtures of salts such as choline chloride and hydrogen bond donors such as urea, oxalic acid, or glycerol *(23-25)*. Deep eutectic solvents contain an uncharged component so they are not strictly composed of only anions and cations, though the uncharged hydrogen bond donor likely hydrogen bonds to the anion of the salt *(24)*. At the eutectic ratio, typically 1-4 molecules of hydrogen donor to salt, the mixture forms a liquid at room temperature. These eutectics are similarly stable and have low vapor pressure like cation/anion pair ionic liquids and are usually water-miscible. Ionic liquids are typically prepared by mixing two salts followed by removal of the unwanted salt, typically sodium chloride, from the ionic liquid salt. This removal can be complex and increases the cost of ionic liquids. In contrast, synthesis of deep eutectic solvents requires only stirring the components with gentle warming. No purification is needed and the purity of the starting materials determines the final purity. As a result, deep eutectic solvents are less expensive and the cost is similar to organic solvents.

Ionic liquids typically replace polar organic solvents like acetone, methanol or DMSO in enzyme-catalyzed reaction mixtures. Polar organic solvents usually denature enzymes, but ionic liquids do not, even when their polarity is similar to the polar organic solvents. Replacing the polar organic solvents with ionic liquids allows substrates to dissolve without deactivating enzymes.

The role of polar solvents, or their ionic liquid replacements, differs in aqueous and non-aqueous media. In aqueous media they help dissolve non-polar substrates. Adding a water-miscible organic solvent or ionic liquid to water increases the solubility of non-

polar substrates. In non-aqueous media, polar solvents, or their ionic liquid replacements, are needed to dissolve polar substrates like sugars. They can also dissolve non-polar substrates, but there are alternative solutions in non-aqueous media. Many enzymes tolerate non-polar organic solvents like toluene, which dissolve non-polar substrates, so the ionic liquids are not needed to dissolve these substrates. However, even in these cases, they may provide other advantages like increased selectivity or a greener replacement for organic solvents.

1.4 Hydrolase-catalyzed reactions

Hydrolases are the most commonly used enzymes in biocatalysis *(26)* and most examples with ionic liquids also use hydrolases. The first reports of enzyme activity in ionic liquids involved lipases *(17-19, 27)* or proteases *(20)*.

Non-aqueous reaction conditions for hydrolase-catalyzed reactions can reverse a hydrolysis making it a condensation reaction, or allow reaction intermediates to react with added nucleophiles such as alcohols or amines instead of water. Typical non-aqueous reaction conditions are a suspension of enzyme powder in a non-polar organic solvent such a toluene. Polar organic solvents such as ethanol or DMSO cannot be used because they usually denature enzymes, likely by disrupting the intramolecular hydrogen bonds in the protein.

In contrast to polar organic solvents, many enzymes tolerate ionic liquids even when their polarity is similar to the non-tolerated organic solvents. As a rule of thumb anions that spread their negative charge over multiple atoms are more stabilizing than those that have the negative charge on a single atom. For example, stabilizing ionic liquids often

contain anions like [Tf_2N], [PF_6], and [BF_4]. Ionic liquids with halide or acetate anions usually denature enzymes and even traces of halide in an ionic liquid can inactivate an enzyme. Presumably strong hydrogen bonds between anion and enzyme promote unfolding and denaturation. If the solution contains water, fluoride-containing anions like [PF_6] or [BF_4] should also be avoided. These anions can hydrolyze to hydrofluoric acid, which inactivates or denatures enzymes.

Enzyme powders usually do not dissolve in ionic liquids, but remain as suspensions, as they do in organic solvents. In one case, *Candida antarctica* lipase B (CALB) powders dissolved in an ionic liquid, but this dissolution eliminated catalytic activity *(28)*. Linking enzymes to poly(ethylene glycol) allows them to dissolve and remain active in organic solvents like toluene. The same approach allowed proteases to dissolve and remain active in ionic liquids *(29, 30)*.

Enzyme cross-linking can increase enzyme activity or stability in many conditions, including in ionic liquids. Although BMIM[NO_3] and BMIM[OAc] denatured CALB, cross-linked enzyme aggregates (CLEAs) of CALB retained 40% and 16% of their activity, respectively, as compared to the activity in *tert*-butanol, *(31)*. *Burkholderia cepacia* lipase (BCL) immobilized as a CLEA had up to 40 times more initial activity in BMIM[BF_4] as compared to a sol-gel preparation, but lost activity over 48 h *(32)*. Feruloyl esterase lost activity after one use in HEMIM[PF_6], but retained activity after five 24 h reaction cycles when immobilized as a CLEA *(33)*. In one example, CALB was 1000-fold more stable in EMIM[Tf_2N] and BMIM[PF_6] in the presence of its substrate vinyl butyrate as compared to without substrate *(34)*. This stabilization did not occur in

hexane or 1-butanol.

Many enzymes, such as α-chymotrypsin, require a small amount of water (typically 1%) added to the ionic liquid for activity *(35, 36)*, likely to maintain a hydration shell around the enzyme. One exception is CALB, which tolerates essentially anhydrous ionic liquids *(28, 37)*. This enzyme either does not require a hydration shell or holds it so tightly that solvents cannot remove it. An alternative to adding water is adding supercritical carbon dioxide. α-Chymotrypsin-catalyzed transesterifications and kinetic resolutions proceeded in ionic liquid-supercritical CO_2 mixtures with or without water present *(35, 38, 39)*.

<u>*Lipases and proteases*</u>

Range of active lipases and proteases

A variety of lipases are active in ionic liquids, but the most important lipase for organic chemists is CALB. This lipase is the first one reported active in the ionic liquids BMIM[PF_6] and BMIM[BF_4], Figure 2 *(17)*. CALB is stable in a wide variety of ionic liquids, including those based on the [BF_4], [PF_6], and [Tf_2N] anions, but it has less activity in ionic liquids containing [SbF_6] or [CF_3SO_2] *(19)*. The activity of CALB is often comparable to that in organic solvent, but can be even higher. For example, its activity for the synthesis of butyl butyrate from butanol and vinyl butyrate was 2-4-fold higher in BMIM[PF_6] than in butanol and hexane *(34)*. CALB, BCL, and CALA are also active in deep eutectic solvents *(40)*, Figure 3. Porcine pancreatic lipase (PPL), the only mammalian lipase tested in ionic liquids, was active in aqueous solutions containing up to 2 M EMIM[OAc] *(41)* or 15% *N*-ethylpryidinium[CF_3OO] (*42)*, as well as in nearly

anhydrous BMIM[Tf_2N] and BMIM[PF_6] *(43)*.

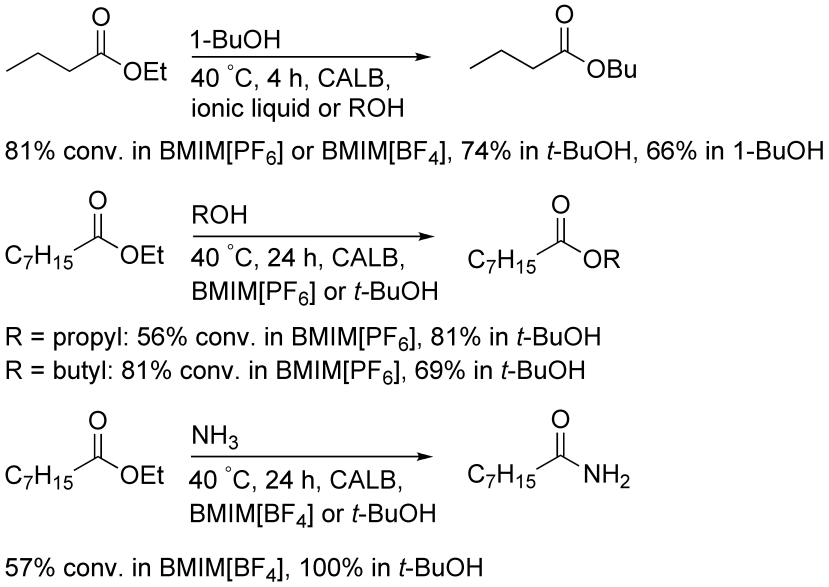
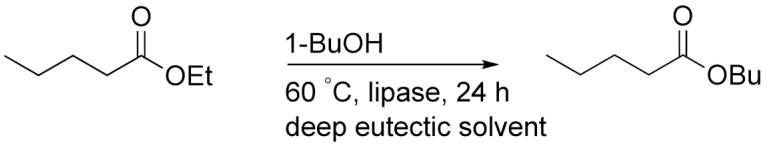


Figure 2. CALB-catalyzed test reactions in BMIM[BF_4] and BMIM[PF_6]. Transformations in the ionic liquid gave comparable conversion compared to those in organic solvents.

Many lipase-catalyzed reactions use vinyl esters as acyl donors because they are effectively irreversible donors. The vinyl alcohol released tautomerizes to acetaldehyde. One problem with vinyl acyl donors in ionic liquids is the accumulation of inhibitory acetaldehyde oligomers. Even mildly acid protons, such as the one in the 2-position of the dialkylimidazolium cation, can catalyze formation of these oligomers. Replacing that hydrogen in BMIM[BF_4] with a methyl group avoided this problem *(44)*. BCL catalyzed the acetylation of 5-phenyl-1-penten-3-ol with vinyl acetate in resultant ionic liquid with rate and enantioselectivity comparable to in organic solvents and without the formation

any oligomeric byproducts. BCL could be reused 10 times without loss of activity in the



Solvent	Conversion (%)		
	CALB	CALA	BCL
Toluene	92	76	5
ChCl[a]:Acetamide (1:2)	96	0.5	0.0
ChCl:Glycerol (1:2)	96	70	22
ChCl:Malonate (1:1)	58	0.7	0.0
ChCl:Urea (1:2)	99	1.6	0.8
EAC[b]:Acetamide (2:3)	92	2.7	0.0
EAC:Glycerol (2:3)	91	2.1	0.5

a: ChCl – choline chloride; b: EAC – ethylammonium chloride

Figure 3. Lipase-catalyzed test reactions in deep eutectic solvents. CALB was active in a variety of deep eutectic solvents with activity comparable to in toluene. CALA and BCL were most active in the solvent composed of choline chloride and glycerol.

new ionic liquid, whereas it lost most of its activity after one run in BMIM[BF_4]. Coating this ionic liquid onto an immobilized enzyme also stabilized the acetaldehyde in organic solvents like hexane or isopropanol. To create the coating, the researchers combined the improved cation with a new anion, poly(ethylene oxy)(10) cetyl sulfate (*45)*. Both the conversion and the enantioselectivity increased using the coated BCL: 50% conversion with coating in 28 h, 25-37% without; *E* >200 with coating, 10-40 without.

Proteases are also active in ionic liquids. The first report used thermolysin, a zinc metalloprotease to make aspartame in BMIM[PF_6] *(20)*. Papain, a cysteine protease, catalyzed hydrolysis of hydroxyphenylglycine methyl ester *(46-48)* in 1-alkyl-3-

methylimidazolium[BF_4] ionic liquids containing >20 vol% water, Figure 4. With a more hydrophobic alkyl group in the 1-alkyl-3-methylimidazolium cation, the enantio-selectivity of papain increased, but the initial rate decreased *(48)*. α-Chymotrypsin, a serine protease, catalyzed formation of an amide link in Leu-enkephalin peptide fragment in *(49)* and the transesterification of *N*-acetylamino acid esters in MOEMIM[PF_6] *(35)*. In both reports, the enzyme required >0.5% water for activity, but supercritical carbon dioxide could be used instead of water *(35)*, Figure 5. Proteases catalyzed the synthesis of cleavage-sensitive peptide and protein fragments in up to 30% MMIM[$(MeO)_2PO_2$] in MOPS buffer *(50)*. The yields were >78% in 30% ionic liquid for trypsin, α-chymotrypsin, and V8-protease as compared to 18, 45, and 0%, respectively, in buffer alone. The ionic liquid suppressed both hydrolysis of the peptide product and proteolytic side reactions on the fragments by reducing available water to the protease. These unwanted activities decreased non-linearly, so the authors speculated that a conformation change of the enzyme may also have occurred.

Subtilisin usually has low activity in ionic liquids with low water content and the activity depend strongly on the enzyme preparation. Changing the purification procedure *(51)* or modifying subtilisin by covalent attachment of comb-shaped PEG *(29, 30)* enhanced activity up to 4 orders of magnitude over free enzyme. The PEG-modified subtilisin dissolved in the ionic liquids, which may also contribute to the higher activity. Subtilisin was active in water organic solvent mixtures. For example, it catalyzed the esterification of *N*-acetylphenylalanine in 2 M EMIM[OAc] and showed 14-fold increased enantioselectivity compared to in 2 M acetonitrile *(41)*.

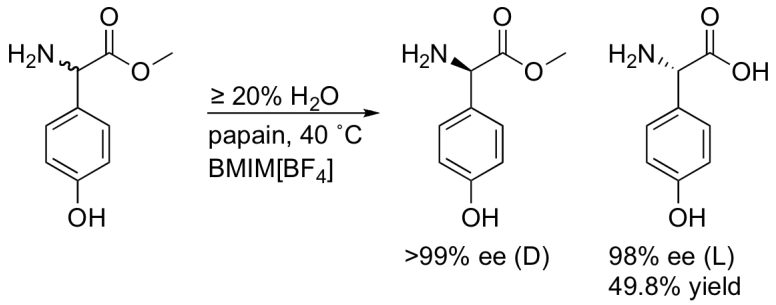


Figure 4. Papain-catalyzed kinetic resolution of D,L-(*p*-hydroxyphenyl)glycine methyl ester. The reaction rate increased by up to 10-fold and the enzyme stability increased up to 3-fold in solutions containing ionic liquid compared to without.

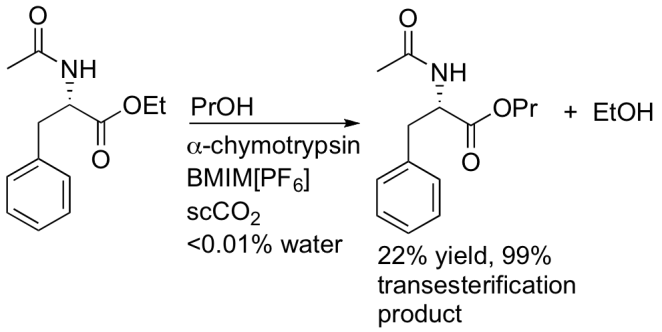
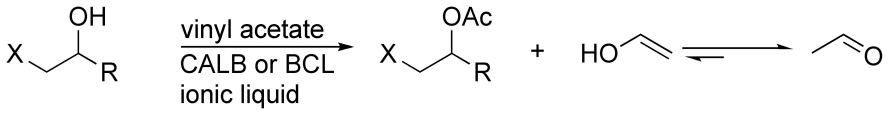


Figure 5. α-Chymotrypsin-catalyzed transesterification of an *N*-acetylphenylalanine ethyl ester in a mixture of ionic liquid and supercritical CO_2 ($scCO_2$). This yield is low, but twice as high as the 9% yield in pure supercritical CO_2.

Kinetic resolutions

Lipases CALB and BCL are already highly enantioselective in the acetylation of secondary alcohols with vinyl acetate in organic solvent, but this enantioselectivity increases further in ionic liquids. The enantioselectivity of the BCL-catalyzed resolution of 1-phenylethanol and several similar reactions was up to ten fold higher in 1-alkyl-3-

methylimidazolium [BF_4]- and [PF_6]-based ionic liquids (*E* ~100 to *E*~1000), Figure 6 *(27, 52, 53)*. Enantioselectivities higher than one hundred require special care to measure since both the conversion and enantiomeric purity must be measured very accurately. For



Enzyme	Substrate		Enantioselectivity			
	X	R	THF	Toluene	EMIM[BF_4]	BMIM[PF_6]
CALB	H	Bn	140	200	>600	>900
CALB	H	-$CH_2C(O)OBn$	26	200	>600	150
BCL	Cl	Ph	56	160	180	>400
BCL	Cl	OPh	150	85	170	>1000

Figure 6. Kinetic resolution of secondary alcohols in ionic liquids. The enantioselectivity of the resolution is comparable to organic solvents or improved in the ionic liquid.

these secondary alcohols there is little practical advantage of the higher enantioselectivity in ionic liquids since the enantioselectivity in organic solvents is already high enough to cleanly separate the enantiomers.

In other cases the enantioselectivity is low in organic solvents, so any increased enantioselectivity in ionic liquids offers a practical advantage. CRL shows only low to moderate enantioselectivity in the resolution of 2-aryl propanoic acids, a class of non-steroidal anti-inflammatory drugs. The enantioselectivity for hydrolysis of the methyl ester of ibuprofen or methyl ester of naproxen increased from an *E* of 7.2 and 33, respectively, in water-saturated isooctane to 24 and >200 in ionic liquids (*54, 55)*. The

enantioselectivity of BCL increased from 10–40 to >200 after coating the lipase with the ionic liquid mentioned above (*45)*. The coated BCL also resolved *(E)*-4-phenylbut-3-en-2-ol with vinyl acetate 10-fold faster in diisopropyl ether compared to the uncoated lipase (*56*). The rate increased an additional 11% higher in the ionic liquid $MeEtBu_3P[Tf_2N]$ compared to in the ether without sacrificing enantioselectivity (*E* >200 in all cases). The enantioselectivity of PPL in the hydrolysis of methyl or ethyl esters of *N*-acetyl amino acids increased up to ten-fold in 15% *N*-ethylpryidinium$[CF_3OO]$ as compared to acetonitrile: from $E = 2.3$ to $E = 23$ for threonine methyl ester *(42)*.

The increased solubility of substrate in ionic liquids can increase the reaction rate of kinetic resolutions. Previous researchers had optimized the kinetic resolution of an intermediate for synthesis of Lotrafiban, platelet aggregation inhibitor, Figure 7. This resolution involved the CALB-catalyzed hydrolysis of the methyl ester in an 88:12 mixture of *tert*-butanol and water. The solubility of this intermediate increased five fold in a BMIM$[PF_6]$-water mixture *(57)*. This solvent switch also allowed the researchers to increase the reaction temperature from 50 °C to 75 °C. These changes increased the reaction rate 4-fold without sacrificing yield or selectivity. Further, the ionic liquid could be reused ten times.

In other cases increased solubility did not lead to an overall improvement of the reaction. Although ionic liquids dissolved adrenaline-type *N*-acyl aminoethanols (*N*-acyl-2-amino-1-phenylethanol and *N*-acyl norphenylephrine) better than organic solvents like *tert*-butyl methyl ether, reactions were unexpectedly slower in the ionic liquids *(58)*. In another example, researchers resolved *cis*-benzyl *N*-(1-hydroxyindan-2-yl)carbamate,

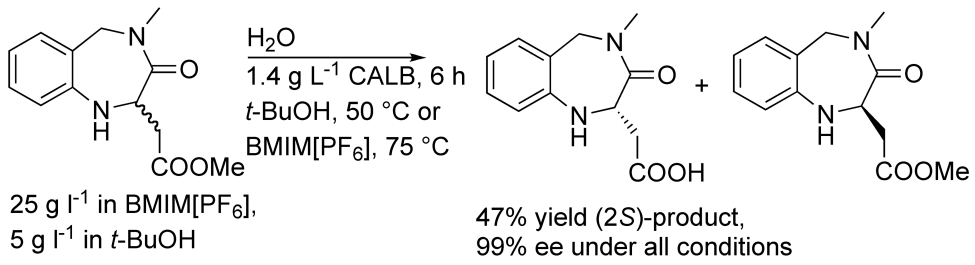


Figure 7. Enhanced production of Lotrafiban intermediate in BMIM[PF_6]. The substrate is more soluble in the ionic liquid allowing for a four-fold faster transformation with identical selectivity and yield. The authors did not specify the purity of the remaining substrate.

Figure 8, which is a precursor to indinavir (Crixivan), an HIV protease inhibitor. A CALB-catalyzed resolution of similar precursor (diacetate derivative) in organic solvents was highly enantioselective, but required approximately three days reaction time (*59*). As an alternative, Lourenço and coworkers (*60*) used an ionic liquid as solvent. The reaction was different (acetylation with vinyl acetate) and the reaction mixture contained more enzyme. It was slightly faster, but less enantioselective (E = 39 versus >100 for the previous resolution), Figure 8. Two novel aspects of this work were the use of an unusual ionic liquid (dicyanamide salt of Aliquat 336, which is a commercial phase transfer catalyst and consists of a mixture of methyl-tri(octyl or decyl) ammonium chlorides) and the recovery of the product from the ionic liquid by sublimation.

Regioselective acylation of sugars

Increased substrate solubility in ionic liquids is a big advantage for the regioselective acylation of sugars. Sugar esters are potentially useful as green, biodegradable

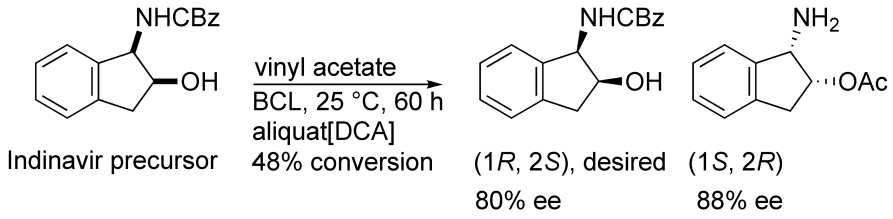


Figure 8. BCL-catalyzed resolution of an indinavir precursor. The product was recovered by sublimation and the catalyst could be reused. The enantioselectivity was lower than in organic solvents.

surfactants. Sugars dissolve poorly in conventional, non-polar organic solvents, but upon acylation the product acyl sugar is more soluble. The high ratio of sugar monoester to sugar in solution makes it likely that the sugar monoester will undergo subsequent undesired acylations. Polar organic solvents dissolve sugars better, but usually inactivate enzymes. Ionic liquids are even better than polar organic solvents at dissolving sugars *(61, 62)*, but maintain the activity of hydrolases. The higher sugar concentration makes the initial acylation faster and the subsequent acylations less likely, Figure 9. Optimizing the structure of the ionic liquid can further increase sugar solubility, Table 1.

The regioselectivity of CALB-catalyzed glucose acetylation by vinyl acetate improved in EMIM[BF_4] and MOEMIM[BF_4] as compared to organic solvent. The acetylation yielded 99% monoacyl product in EMIM[BF_4] (50% conversion in 36 h) and 93% monoacyl in MOEMIM[BF_4] (99% conversion) compared to only 53% in THF (99% conversion) and 76% in acetone (72% conversion). The diacetyl product was the major side product. Glucose was several times more soluble in EMIM[BF_4] *(61)* and approximately one hundred times more soluble in MOEMIM[BF_4] than in the organic

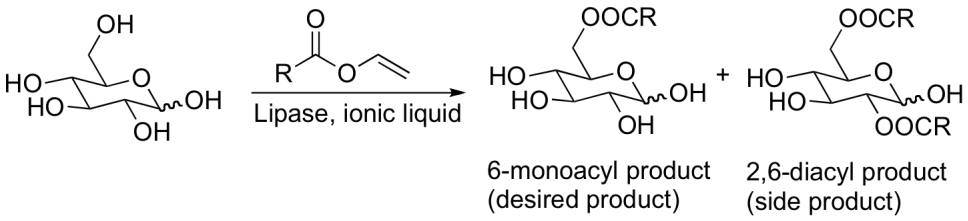


Figure 9. Acylation of glucose by vinyl esters. Generally, the monoacyl product is desired. Use of an ionic liquid that dissolves glucose well minimizes formation of the diacyl side product.

Table 1. Solubility of glucose in dialkylimidazolium ionic liquids. The anion affects solubility more than the cation. The DCA and $MeOSO_3$ anions give the highest solubility.

Ionic liquid	Glucose solubility (g l^{-1}) at 25 ˚C	Reference
tert-butanol	0.3	*(61)*
BMIM[DCA]	145	*(61)*
EMIM[$MeOSO_3$]	89.6	*(62)*
EMIM[OTf]	6.1	*(62)*
BMIM[OTf]	4.8	*(62)*
EMIM[BF_4]	1.1	*(62)*
BMIM[BF_4]	0.9	*(62)*
OMIM[BF_4]	0.7	*(62)*
BMIM[PF_6]	< 0.5	*(62)*

solvents. The increased solubility in EMIM[BF_4] increased the regioselectivity and the even higher solubility in MOEMIM[BF_4] gave both high regioselectivity and high conversion. CALB also catalyzed the acylation of glucose with vinyl myristate to the 6-*O*-myristic acid ester in 89% yield in 60% BMIM[BF_4]-40% *t*-butanol and with vinyl laurate in BMIM[PF_6] *(63)*. Irradiation with ultrasound increased the rate of the last reaction increased 2.6 fold. CALB also catalyzed the acylation of glucose with palmitic acid to the 6-*O*-palmitic acid ester in 48% yield. Replacing the expensive vinyl ester with

the free acid reduces the cost. BMIM[DCA] dissolved >200 g l^{-1} sucrose, enabling CALB to catalyze the acylation of sucrose with dodecanoic acid. The authors did not report the regioselectivity *(61),* but it likely favors the primary alcohol positions (6-*O* and 6'-*O*).

Although ionic liquids such as BMIM[Cl] and EMIM[OAc] can dissolve cellulose, hydrolases are not active in these solvents. Conversely, while hydrolases may be active in apolar or semipolar organic solvents, these solvents cannot dissolve cellulose. Zhao and coworkers designed ionic liquids that dissolve cellulose, but do not inactivate hydrolases *(64)*. On example is $Me(OCH_2CH_2)_3$-NEt_3[OAc], where the acetate anion dissolves the cellulose while the polyether moiety in the cation stabilizes the enzyme. In the CALB-catalyzed acylation of cellulose by methyl methacrylate at 60 °C, CALB lost approximately 60% of its activity in the first 20 minutes, but retained its activity thereafter. FTIR showed an 89% conversion to the 6-*O*-esters after 72 h with no side reactions.

The solubility behavior of acyl nucleosides is similar to the acyl sugars, but acyl nucleosides are used as antiviral or antitumor agents. CALB catalyzed the acylation of 1-β-D-arabinofuranosylcytosine with vinyl propionate 20% faster in an IL-containing solvent (70% THF-10% BMIM[PF_6]-20% pyridine) than in a conventional solvent (28% hexane in pyridine) *(65)*. The regioselectivity was high in both solvents, likely because both contained pyridine to dissolve the nucleoside substrate.

Ionic liquids that dissolved glucose best also gave the highest regioselectivity for the 6-position in the CALB-catalyzed acetylation of glucose *(18)*. Similarly, ionic liquids that best dissolved polyhydroxylated flavanoid glucosides (e.g., BMIM[BF_4]) gave the best

regioselectivity for the 6" position in a CALB-catalyzed acylation with vinyl butyrate *(66)*. The poor solubility of succinic acid accounted for higher relative amounts of diester in a CALB-catalyzed acylation of *rac*-2-octanol with succinic anhydride *(67)*. The initial product is the monoester and a subsequent condensation with octanol forms the diester. Hydrolysis of the monoester forms succinic acid, which, if it remains in solution, competes in condensation to reform the monoester.

Polyesterification

Kobayashi's group *(68)* was the first to make polyesters in ionic liquids either by condensation or by ring-opening polymerization, Figure 10. Condensation polymerization of dicarboxylic acid diesters and 1,4-butanediol catalyzed by CALB produced polymers of M_n up to ~1500, while ring-opening polymerization of ε-caprolactone produced polymers of M_n up to ~4200.

The lower molecular weight of polymers formed by condensation polymerizations is typical. One reason for the lower molecular weight is the less favorable driving force. Condensation polymerizations are usually transesterifications reactions with an equilibrium constant near one. Transesterification with vinyl esters as donors is an exception, see below. In some cases the polymerization is the unfavorable condensation of acids with alcohols that is driven by removal water. In contrast, ring-opening polymerizations are favored by the release of ring strain in the lactone. A second, related, reason the greater likelihood of reverse reaction in condensation polymerization. Both types of polymerization contain small amounts of water associated with enzyme and this water can cleave existing polymer chains *(69, 70)*. However, condensation

polymerizations release equimolar amounts of alcohol or water, which makes cleavage of existing polymer chains more likely *(69)*.

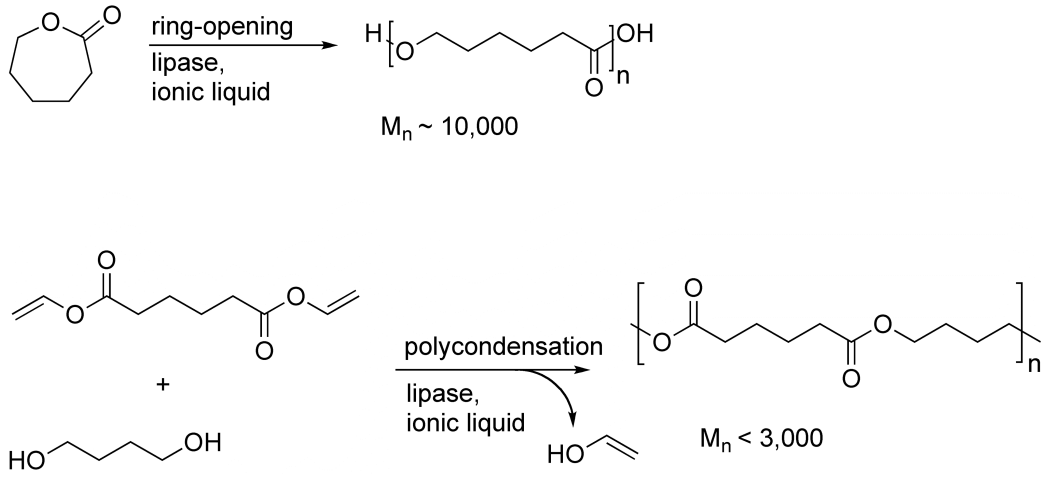


Figure 10. Examples of lipase-catalyzed polyesterifications in ionic liquids. Typically lipases are CALB for ring-opening polymerization and CALB or BCL for condensation polymerization.

Condensation polymerizations using vinyl esters avoid these limitation because the release of vinyl alcohol and its subsequent tautomerization to acetaldehyde provides a strong thermodynamic driving force for the condensation. It also removes alcohol that could cleave the polymer chains.

In both types of polymerizations, solubility of the polymer can limit molecular weight. For example, the BCL-catalyzed condensation polymerization of diethyl adipate and 1,4-butanediol in BMIM[PF_6] *(71)* gave polyester with a molecular weight of only ~2000 at room temperature with a polydispersity of ~1.05. At 60 °C, where the polymer was more soluble, the molecular weight was more than twice as high: ~5000 with a

polydispersity of ~1.25. These values are after precipitation of the polymer with methanol, which leaves lower molecular weight oligomers in solution. The polydispersity in the reaction mixture will be broader. Using a better acyl donor - divinyladipate - did not give higher molecular weights. CALB catalyzed condensation of divinyl adipate and three diols in BMIM[PF_6] at room temperature to give molecular weights (M_n) between 1000 and 2900 *(43)*. The similarity of these molecular weights to those using the diethyl ester suggests that polymer solubility limits the molecular weight. Lipases TLL and MML also catalyzed the polymerizations, but the molecular weights were lower, likely because of lower activity of these lipases in the ionic liquid compared to CALB.

Heise's group *(72)* compared poly(ε-caprolactone) produced in three ionic liquids by CALB-catalyzed polymerization either by condensation of the hydroxy acid or by ring-opening of ε-caprolactone. Condensation polymerization yielded polymer with a molecular weight of 5,500 with polydispersities up to 1.7; ring-opening polymerization yielded polymer with molecular weights approaching 9,000 with polydispersities of 2.3-2.4 before fractionation. For comparison, the ring-opening polymerization in toluene yielded molecular weights of 13,000 with a polydispersity of 2.4. The lower molecular weight in ionic liquid may be due to the lower solubility of polymer or due to more water in the ionic liquid. Although the authors dried the ionic liquids extensively, they may be more difficult to dry than organic solvents. Ring-opening polymerization of β-propiolactone and ε-caprolactone in BMIM[Tf_2N] catalyzed by CALB gave molecular weight ~10,000, but that other lactones gave only low molecular weight oligomers *(73)* similar to the result in organic solvents. CALB also catalyzed the polymerization of ε-

caprolactone in deep eutectic solvents *(74)*.

Esterases

Esterases catalyze the hydrolysis of water-soluble esters in contrast to the water insoluble esters favored by lipases. Both enzymes classes are serine esterases and follow similar mechanisms. Esterases do not tolerate anhydrous organic solvents as well as lipases, and this generalization extends to ionic liquids. Nevertheless, several esterases catalyzed reactions in ionic liquids, Figure 11. PFE catalyzed the transesterification of ethyl valerate to methyl valerate in BMIM[Tf_2N] and BMIM[PF_6] at 60 ˚C, although activity in the latter was diminished several fold *(73)*. Esterases from *B. subtilis* and *B. stearothermophilus* catalyzed the acetylation of 1-phenylethanol in BMIM[Tf_2N], BMIM[PF_6], and BMIM[BF_4], but only when immobilized on Celite *(75)*. The lyophilized enzymes showed no activity. In a special case, many pyridinium and imidazolium-based ionic liquids inhibited acetylcholine esterase at less than millimolar concentrations in water *(76)*. The substrate acetylcholine contains a quaternary ammonium moiety as do known inhibitors of this esterase, so this inhibition by ionic liquids with quaternary ammonium cations is likely specific for this esterase.

The more common use of ionic liquids and deep eutectic solvents with esterases is as a cosolvent with water to increase substrate solubility. Addition of 10-25 vol% 1:2 choline chloride:glycerol cosolvent increased the rate of hydrolysis of *p*-nitrophenyl acetate up to

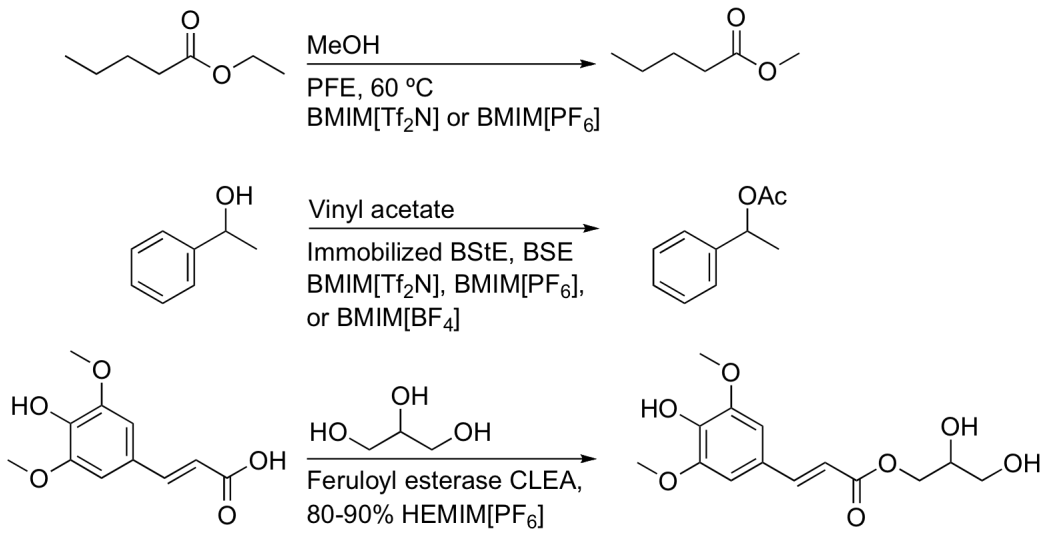


Figure 11. Example esterase-catalyzed reactions in ionic liquids. Esterases typically are not active in organic solvents or ionic liquids. These reactions are exceptions.

3-fold for pig liver esterase and *Rhizopus oryzae* esterase *(40)*. Pig liver esterase catalyzed the enantioselective hydrolysis of diethyl malonate derivatives with several fold faster with 1% Ammoeng 100 (a mixture quaternary ammonium dimethylphosphate ionic liquids based on alkyl and hydroxylated ether side chains) than in buffer only or with 10% isopropanol added. However, with 10 vol% BMIM[PF_6] as the ionic liquid the reaction rate was slower than in buffer *(77)*. CLEAs of feruloyl esterase catalyzed the condensation of glycerol and sinapic acid to produce glycerol sinapate, an antioxidant. The enzyme was most active (~30 mmol product formed l^{-1} h^{-1} mg $protein^{-1}$ at 50 mM sinapic acid: 200 mM glycerol) in 80-90% HEMIM[PF_6] and 10-20% water, but lost activity after ~150 h *(32)*.

Glycosidases

Glycosidases catalyze hydrolysis, but synthetic applications use them in reverse for the

synthesis of saccharides. This synthesis is either a condensation (reverse of hydrolysis) or a transglycoslyation (transfer of a glycosyl group from a donor to an alcohol acceptor). β-Galactosidase from *B. circulans* did not denature after 60 h at room temperature in MMIM[$MeOSO_3$], but it was not catalytically active in this ionic liquid *(78)*. It was active in 20% MMIM[$MeOSO_3$]-80% water where it catalyzed the transfer of the galactosyl group from lactose to *N*-acetylglucosamine. The yield of *N*-acetyllactosamine increased from 30% in buffer to 60%, Figure 12, because the ionic liquid suppressed hydrolysis of product. Similarly, using another glycosidase - β-glycosylhydrolase CelB from *Pyroccocus furiosus* - 45 vol% of MMIM[$MeOSO_3$] increased the yield of galactosyl transfer from lactose to glycerol by 10% *(79)*.

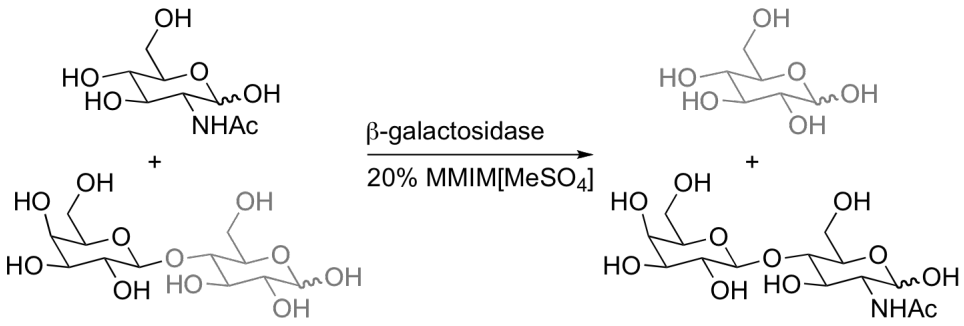


Figure 12. β-galactosidase-catalyzed synthesis of L-*N*-acetyllactosamine by transglycosylation. The ionic liquid cosolvent increased the yield from 30 to 60% by minimizing competing hydrolysis of the product.

One condensation reaction was the synthesis of lactose by condensation of glucose and galactose. The sugars dissolved in MMIM[$MeOSO_3$] with just 0.6% water to stabilize the enzyme, galactosidase from *B. circulans*. Dissolving these sugars usually requires a polar organic solvent such as DMF, which would denature the glycoside. The yield of lactose

was only 18% *(80)*, but this low yield is largely due to the less favorable equilibrium for formation of glycosides as compared to the ester examples above.

Epoxide Hydrolases

Epoxide hydrolases catalyze the enantioselective hydrolysis of epoxides to diols, which can be used as synthetic intermediates. Ionic liquids as cosolvents help dissolve hydrophobic substrates and suppress spontaneous hydrolysis. Further, organic solvents sometime reduce the selectivity or activity or epoxide hydrolases (*81, 82).* For example, epoxide hydrolase from *Rhodococcus* sp. NCIMB 11216 lost 47% of its activity in 10% ethanol in buffer and 33% of its activity in 10% DMF in buffer *(81)*. However, some epoxide hydrolases remain active in ionic liquids *(83, 84)*, but there are no direct comparisons between organic solvent and ionic liquid for the same epoxide hydrolase. The rate of hydrolysis of β-methylstyrene oxide catalyzed by a mammalian epoxide hydrolase-catalyzed was similar in both buffer and in 90 vol% BMIM[PF_6], BMIM[Tf_2N], and BMIM[BF_4] *(83)*. The ionic liquids enhanced not only the solubility of the epoxide substrates, but also suppressed spontaneous hydrolysis of water sensitive epoxides *(84)*, leading to higher apparent enantioselectivity ($E_{apparent}$ = 1 in buffer, 1.6-9.8 in 90% ionic liquid), Figure 13.

Deep eutectic solvents as additives also enhance the activity of epoxide hydrolases. Addition of 25 vol% choline chloride:glycerol enhanced the conversion of styrene oxide to styrene glycol by epoxide hydrolase AD1 from *Agrobacterium radiobacter* by 20-fold compared to in buffer alone *(40)*. In contrast, adding 25 vol% DMSO or acetonitrile as the cosolvent decreased activity 2–6-fold.

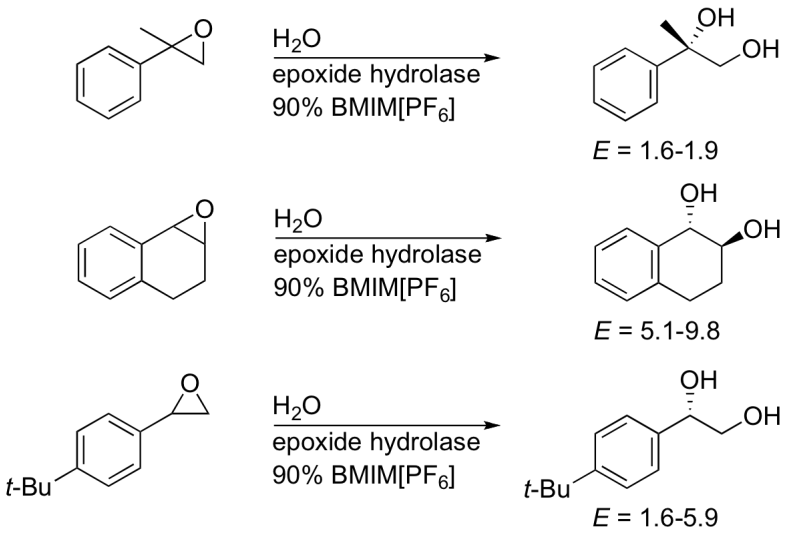


Figure 13. Epoxide hydrolase-catalyzed ring-opening in BMIM[PF_6]. Spontaneous hydrolysis predominates without ionic liquid present, resulting in racemic product ($E_{apparent}$ = 1).

1.5 Oxidoreductase-catalyzed reactions

After the hydrolases, the oxidoreductases are the next most widely studied. The need to regenerate cofactors complicates the use of many oxidoreductases. Many cofactor-dependent reactions use whole cells rather than purified proteins to exploit innate cofactor recycling ability in cells. Unlike hydrolases, most oxidoreductases must dissolve in the reaction medium to function properly. This requirement limits the use of ionic liquids to the water-miscible ones; or as part of an aqueous-ionic liquid biphasic system. The first report *(85)* of oxidoreductase activity in ionic liquids focused on cofactor-independent oxidoreductases - laccase, soybean peroxidase, and horseradish peroxidase in BMPyr[BF_4] and BMIM[PF_6]. The enzymes were 10-fold less active in 25% ionic liquid as compared to in 20% *tert*-butanol and the product yields were

similarly lower. One exception was laccase C from *Trametes* species, which gave up to 30-fold higher conversion in 25% ionic liquid BMPyr[BF_4] compared to in 20% *tert*-butanol. The reaction was a mediator-assisted oxidation of anthracene or vertryl alcohol. These reactions required the use of poorly soluble substrates or mediators, and the reactions in the ionic liquid typically gave higher conversions than those in the organic solvent due to increased solubility.

Dehydrogenases

Ionic liquids as cosolvent for aqueous phase reactions

Ionic liquids can improve dehydrogenase-catalyzed reactions at high water concentrations by enhancing substrate solubility or enzyme activity. Unfortunately, one of these enhancements often comes at the expense of the other. Ionic liquids added to the aqueous phase of an octane-water system enhanced the solubility of androstandione *(86)*, Figure 14. 3-α-Hydrosteroid dehydrogenase (HSDH) catalyzed its reduction and formate dehydrogenase (FDH) and formate regenerated the NADH. While HSDH tolerated 10 vol% of a number of ionic liquids, FDH denatured in solutions containing EMIM[OTf], BMIM[OTf], and BMIM[BF_4]. Both enzymes were active in with BMIM[lactate], which the authors suggested was due to its ability to form the strongest hydrogen bonds. Using 5 vol% BMIM[lactate] gave a higher yield after 8 h: 80% as compared to 60% in buffer alone.

Dehydrogenases tend to lose activity as ionic liquid content increases. Yeast ADH showed 25% of its water activity in 50 vol% 1:2 choline chloride: glycerol/50% buffer (100 mM CHES, pH 9), but was not active a higher concentrations of deep eutectic

solvent (unpublished results). Horse liver alcohol dehydrogenase (ADH) was slightly more active 15 wt to vol% BMIM[Cl] than in buffer alone, but activity decreased with higher amounts of ionic liquid *(87)*.

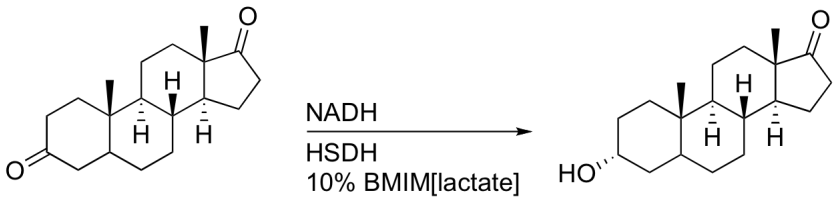


Figure 14. Reduction of androstandione to androsterone. Adding 10 vol% ionic liquid to the aqueous phase increased the yield from 60 to 80%, likely due to increase solubility of the substrate. NADH: reduced nicotinamide adenine dinucleotide, HSDH: 3-α-hydroxy-steroid dehydrogenase.

Cellobiose dehydrogenase (CDH) catalyzed the oxidation of cellobiose, a major disaccharide product of cellulose hydrolysis, to cellobiolactone in 65% choline[H_2PO_4] *(88)*, Figure 15. The electron acceptor was an either cytochrome *c* or 2,6-dichloroindophenolate. Although high salt-content aqueous systems, such as those used in certain electrodes, inhibited the electron transfer reaction, the ionic liquid did not. The authors attributed the slower reaction in the ionic liquid to the higher viscosity (25 cP versus 1 cP for pure water) and speculated that the reaction rates would be comparable at elevated temperature.

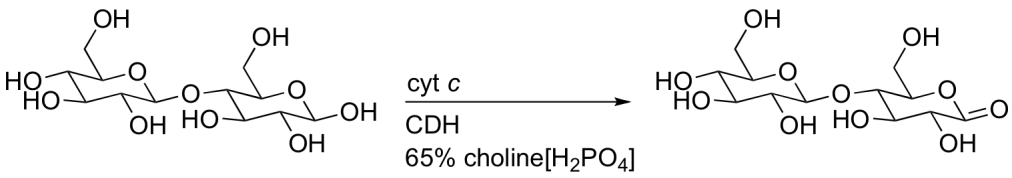


Figure 15. Oxidation of cellobiose to cellobiolactone for use in an electrode. The ionic liquid did not inhibit enzyme activity, unlike typical high-salt electrolyte solutions. CDH: choline dehydrogenase; cyt *c*: cytochrome *c*.

Dehydrogenases in pure ionic liquids

Dehydrogenases tolerate high concentrations of hydroxylated ionic liquids. Walker and Bruce *(89)* used HPMIM[glycolate] containing as little as <100 ppm water for the morphine dehydrogenase-catalyzed oxidation of codeine. The product codienone can be subsequently converted to oxycodone, an important opiod, Figure 16. The hydroxyalkyl moiety on the cation stabilized the dehydrogenase at these low water contents while still dissolving the protein. Codienone reacts with water, so a one-pot synthesis is not possible in buffer. The ionic liquid suppressed this side reaction, allowing the final step to continue with an overall 14% yield based on the starting material. In a subsequent report *(90)*, the researchers added glucose dehydrogenase and gluconolactone to regenerate the cofactor $NADP^+$. The first step, oxidation of codeine to codienone, gave 20% yield as compared to 10% yield in water.

De Gonzalo and coworkers *(91)* also used hydroxyl-functionalized ionic liquids as cosolvents for the asymmetric reduction of ketones catalyzed by crude ADH A from *R. ruber*. The dehydrogenase was active in 90 vol% tris-(2-hydroxyethyl)-methylammonium $[MeOSO_3]$/10% buffer and in Ammoeng 100, 101, 102 (quaternary ammonium salts

containing polyethyleneglycol substituents). The enantioselectivity remained high as all eight ketones tested yielded products with 99% ee. At more than 90 vol% ionic liquid, the activity of the dehydrogenase decreased. Taken with the work of Walker and Bruce, these results support the hypothesis that hydroxyl groups on ionic liquid cations help dehydrogenases maintain their activity at low water contents.

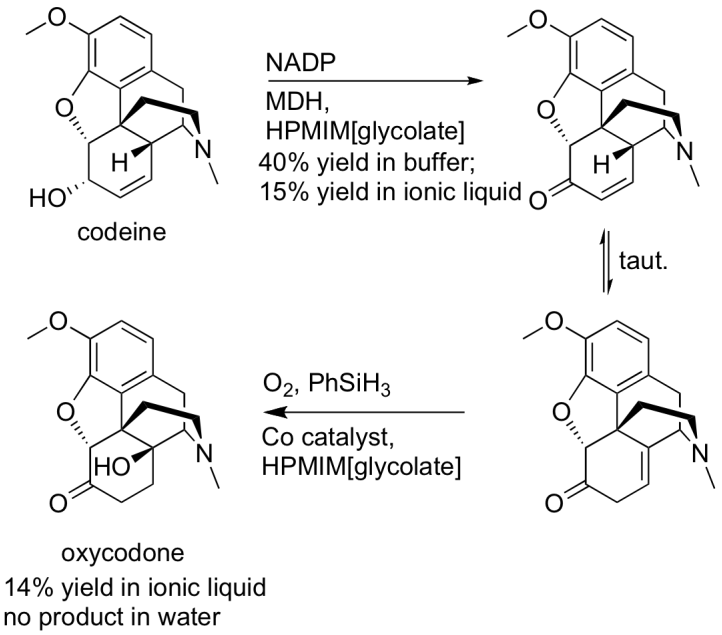


Figure 16. One-pot synthesis of oxycodone via combined enzymatic and chemical catalysis. The intermediate is not soluble in buffer, so a one-pot synthesis is not possible. NADP: nicotinamide adenine dinucleotide phosphate, MDH: morphine dehydrogenase.

Peroxidases

Peroxidases catalyze the oxidation of substrate with hydrogen peroxide. Peroxides contain an iron porphyrin prosthetic group, but do not require a cofactor, which simplifies their use.

Horseradish peroxidase

Horseradish peroxidase (HPO) is a broad specificity enzymes useful in chemical synthesis. HPO functions in a wide range of water contents in ionic liquids. HPO was three fold more stable at 80 °C in 5-10% BMIM[BF_4] as compared to phosphate buffer *(92).* In BMIM[PF_6]/~10% water, HPO could be reused five times as compared to only twice in water for the oxidation of veratryl alcohol, a model lignin compound, to veratryl aldehyde *(93).* Microemulsions of Aerosol OT/water/OMIM[Tf_2N] enhanced the rate of oxidation of pyrogallol to purpurogallin more than ten fold as compared to only the ionic liquid or 1-hexanol in water *(94)*. HPO dissolved in a hydrophobic ionic liquid BMIM[Tf_2N], remained in this liquid and catalyzed the synthesis of polyaniline from a water/aniline solution *(95)*, Figure 17. The resulting polyaniline had conductivities

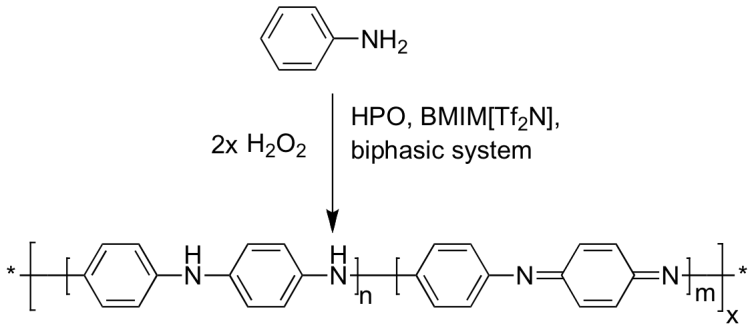


Figure 17. HPO-catalyzed synthesis of polyaniline with buffer. The HPO remained active and dissolved in the ionic liquid phase throughout the reaction. The polyaniline product showed conductivity (~10^{-3} S cm^{-1}) similar to that prepared by HPO without ionic liquids.

comparable to polyaniline produced from solid-immobilized HPO (~10^{-3} S cm^{-1}) in organic solvents or aqueous condition where typical molecular weights were 10,000-30,000 g mol^{-1} *(96)*.

Chloroperoxidases

Chloroperoxidases (CPO) are useful biocatalysts for the epoxidation of olefins. Sanfilippo and colleagues *(97)* investigated MMIM[$MeOSO_3$] and BMIM[$MeOSO_3$] as solvents for the enantioselective oxidation of 1,2-dihydronaphthalene using chloroperoxidase from *Caldariomyces fumago*, Figure 18. While CPO showed activity comparable activity in 10-30% ionic liquid to acetone/water and *tert*-butanol/water mixtures, it was less active and had lower selectivity than in pure citrate buffer. Chiappe and coworkers *(98)* used the same CPO for the enantioselective sulfoxidation of thioanisole in up to 70% choline[citrate], choline[acetate] or MMIM[$(MeO)_2PO_2$] and found less over oxidation to the sulfone (from 89% to >99% sulfoxide) compared to buffer and up to twofold higher conversion at 30-50% ionic liquid.

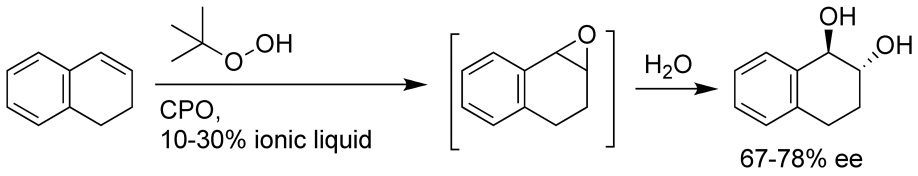


Figure 18. CPO-catalyzed oxidation of 1,2-dihydronaphthalene. Ionic liquids as additives gave either improved enantioselectivity or conversion as compared to added organic solvents.

The substrate hydrogen peroxide can inactivate peroxidases. To minimize the

inactivation of peroxidase from *Coprinus cinereus* (mushroom), hydrogen peroxide was generated continuously in situ using glucose oxidase. The peroxidase catalyzed the asymmetric oxidation of phenyl methyl- and 2-naphthyl methyl sulfides to sulfoxides in BMIM[PF_6] with 10% water *(99)*. Although the enantioselectivity (63-92% ee) and yields (<32%) were similar to those in water, the reaction workup was easier because ionic liquids and the extraction solvent did not form emulsions. Hydrogen peroxide formed twice as fast as the peroxidase-catalyze oxidation of sulfides, so the authors hypothesized that the peroxidase was still inactivated. Reducing the amount of glucose oxidase should reduce the inactivation.

Oxidases

Laccases, blue copper metalloenzymes, catalyze oxidations of substrates with hydrogen peroxide in bioremediation, decolorization of dyes and in pulp processing. Laccases typically do not tolerate organic solvent additives, but they do tolerate ionic liquids. The commercial laccase DeniLite base II had activity comparable to in buffer in a standard dye oxidation assay (ABTS assay) in 10-50% of three different ionic liquids at pH 5-9 *(100)*. Although the laccase tolerated similar amounts of DMSO or acetonitrile at pH 7, it did not tolerate these solvents at pH 5 or pH 9. Adding 10-20% BMIM[Br] or 50-60% BMIM[DCA] increased the laccase-catalyzed oxidation of catechol to benzoquinone *(101)*.

D-amino acid oxidase (DAAO) is used industrially as one enzyme in the production of 7-aminocephalosporanic acid from cephalosporin As a model reaction Lutz-Wahl and coworkers *(102)* used the resolution of *rac*-phenylalanine to the L-phenylalanine and

phenylpyruvic acid. The ionic liquid did not give any clear advantage. The used immobilized DAAO and free catalase (to destroy hydrogen peroxide) in 20% MMIM[$(MeO)_2PO_2$] or 40% BMIM[BF_4]. Higher proportions of ionic liquid inactivated the DAAO. The time needed for complete reaction was the same in the BMIM[BF_4] mixture as in water, but it was 25% faster in the MMIM[$(MeO)_2PO_2$]. The stability of the oxidases depended both on the oxidase and on the ionic liquid. DAAO lost 21-58% of its original activity in the MMIM[$(MeO)_2PO_2$] solution compared to only a loss of only 10% in buffer, but the catalase retained all of its activity in the MMIM[$(MeO)_2PO_2$] solution. In contrast catalase lost 23% of its activity in the BMIM[BF_4] solution compared to 14% loss in water. A reaction analogous to the first step of industrial cephalosporin C production in was 25% slower in 20% MMIM[$(MeO)_2PO_2$] than in buffer.

Whole-cell-catalyzed reactions

Ionic liquids are useful as second phases or cosolvents for whole-cell biocatalysis to increase substrate or product solubility, mitigate toxic effects of reactants or products. As a second phase, ionic liquids can also simplify product separation. Whole-cell biocatalysis in ionic liquids was first reported in 2000 *(16)*, but received less attention than reaction catalyzed by isolated enzymes. Most applications are nicotinamide cofactor-dependent reductions where the whole cell already contains the cofactor and other enzymes for its regeneration. Experiments on whole-cell catalysis in ionic liquids typically involve tens of milliliters in contrast to the tenths of milliliters scale for isolated enzyme reactions. One even larger scale reaction used a 2 L bioreactor containing 1.25 L of an *E. coli* MG1655 culture and 23 vol% OMA[Tf_2N] or $P_{6,6,6,14}$[Tf_2N] for the

production of toluene *cis*-diol from toluene *(103)*. The highest specific yield was 11.7 mmol g cell dry weight^{-1}, which is 2.5-fold higher than in buffer. The higher yield may be due to reduced toxicity of the substrate toluene. Adding the ionic liquid increased the tolerance of the cells for toluene eight-fold.

Reactions involving nitrile hydratase usually use whole cells because the isolated enzyme is unstable. Using a second phase of ionic liquid further stabilize the nitrile hydratase, while adding toluene did not. *Rhodococcus* R312 containing a nitrile hydratase catalyzed the hydration of 1,3-dicyanobenzene to 3-cyanobenzamide in a biphasic system of water and 20 vol% BMIM[PF_6] or toluene *(16)*. The initial rate of reaction was slower in ionic liquid than in toluene, likely due to slower mass transfer in the viscous ionic liquid. The final yield was similar for both ionic liquid and toluene. The nitrile hydratase activity remained constant in the ionic liquid mixture, but decreased 50-90% in the toluene mixture.

Whole cells of *Lactobacillus kefir* with a second phase of ionic liquid catalyzed the asymmetric reduction of chloroacetophenone to (*R*)-1-(4-chlorophenyl)ethanol *(104)*. The yield and enantioselectivity improved BMIM[PF_6], BMIM[Tf_2N], and OMA[Tf_2N] as a second phase instead of MTBE. The membrane integrity was nearly ten fold higher with the ionic liquids than with several organic solvents, suggesting that the ionic liquids do not partition into membranes like organic solvents. Similar experiments with *Saccharomyces cerevisiae* FasB His6 and *Escherichia coli* K12 also showed higher membrane integrity in biphasic ionic liquid systems compared to organic solvents *(105)*.

Microorganisms often tolerate water-immiscible ionic liquids, but growth may

inhibited or completely stopped depending on the microorganism and the ionic liquid. Growing *E. coli* MG1655 in the presence of a second phase of OMA[Tf_2N] and $P_{6,6,6,14}$[Tf_2N] inhibited growth by 39% and 26%, respectively, while EMIM[Tf_2N], BMIM[Tf_2N], and NDecPy[Tf_2N] completely inhibited growth *(103)*. A second phase of BMIM[PF_6] did not affect the growth of *Pichia pastoris*, inhibited *Bacillus cereus* by about 50% and completely inhibited growth of *E. coli (106)*.

Water-miscible ionic liquids are usually toxic to microorganisms, but the effect varies with ionic liquid and microorganism. Adding even 1% BMIM[BF_4] to the culture medium completely inhibited the growth of *P. pastoris*, *B. cereus*, and *E. coli (106)*. Various ionic liquids containing chloride anions completely inhibited the growth of *E. coli* MG1655 *(102)*, which is not surprising, as chloride-containing ionic liquids tend to denature proteins. However, some microorganisms tolerate small amounts of water miscible ionic liquids. *P. membranaefaciens* tolerated 2.5% BMIM[BF_4] while improving the yield and selectivity of ethyl-(*R*)-3-hydroxybutyate from acetoacetate compared to in buffer *(107)*. Lou and colleagues *(108)* used immobilized baker's yeast in 10 vol% BMIM[BF_4] for the synthesis of (*S*) -1-trimethylsilylethanol from acetyltrimethylsilane, though amounts of BMIM[BF_4] greater than 10 vol% greatly reduced both yield and reaction rate until the reaction essentially stopped at 50 vol%. The tolerance for miscible ionic liquids depends on the organism, but is lower than for immiscible ionic liquids.

1.6 Conclusion

Ionic liquids are versatile solvents for enzyme-catalyzed reactions. The central advantage of ionic liquids is enhanced solubility of substrates without inactivating the

enzymes. Ionic liquids can dissolve polar substrates to allow reactions in nonaqueous conditions. Organic solvent alternatives inactivate enzymes, but ionic liquids do not. Ionic liquids also serve as cosolvents to dissolve non polar substrates in water mixtures. Water miscible organic solvent alternatives are not suitable because they inactivate enzymes. The enhanced solubility usually increases the rate of reaction and often increases the regio- or enantioselectivity. Hydrolases and oxidoreductases are particularly well-studied in ionic liquids. Precisely why enzymes tolerate ionic liquids, but not organic solvents of comparable polarity, is still unknown.

Ionic liquids can also enhance stability and activity of enzymes. When combined with the recyclability of ionic liquids, this advantage can reduce the costs of both enzyme and solvent by recycling both together. Despite this advantage, ionic liquids are typically expensive and require a large initial capital investment for a process, even with recycling.

While biotransformations in ionic liquids are still confined to the laboratory scale, ionic liquids are promising for industrial enzymatic applications. As the field further matures, it is inevitable that research will leave the benchtop and move to the preparative and ultimately the industrial scale. With the availability of greener, inexpensive, biodegradable third generation ionic liquids including deep eutectic solvents, many initial concerns about the true “greenness” of ionic liquids are beginning to fade.

CHAPTER 2. Application of ionic liquids to poly(hydroxyalkanoate) synthesis

2.1 Chapter Overview

This chapter details our initial interest in ionic liquids: replacements for volatile organic solvents that could be used in biopolymer synthesis. The chapter also exhibits some of the shortcomings of ionic liquids in this area, namely low molecular weights from ring-opening polymerization. This chapter originally appeared as: J. T. Gorke, K. Okrasa, A. Louwagie, R. J. Kazlauskas, F. Srienc, *J. Biotechnol.* **132**, 306 (2007). Andrew Louwagie and Krzysztof Okrasa synthesized some of the ionic liquids, determined some of the PHA solubility, and aided in the initial experimental design.

Ring-opening polymerization of five lactones catalyzed by *Candida antarctica* lipase B in ionic liquids yielded poly(hydroxyalkanoates) of moderate molecular weights up to *M*n = 13,000. In the ionic liquid 1-butyl-3-methylimidazolium bis(trifluoromethane)-sulfonimide and with a low weight ratio of enzyme to lactone (1:100) we obtained polymers from β-propiolactone, δ-valerolactone, and ε-caprolactone with degrees of polymerization as high as 170, 25, and 85, respectively; oligomers from β-butyrolactone and γ-butyrolactone with degrees of polymerization of 5; and a copolymer of β-propiolactone and β-butyrolactone with a degree of polymerization of 180. Water-immiscible ionic liquids were superior to water-miscible ionic liquids. Reducing the water content of the enzyme improved the degree of polymerization by as much as 50% for β-propiolactone and ε-caprolactone.

2.2 Introduction

Poly(hydroxyalkanoates) (PHAs offer a wide range of physical properties, are produced naturally by bacteria, and are biodegradable and biorenewable (*1,2*). PHAs are currently expensive, mainly due to complex purification required after *in vivo* synthesis. Further, the poor solubility of PHAs in environmentally benign extraction solvents such as water and ethanol makes PHA extraction from cells unattractive from an environmental standpoint (*1,3*). Current *in vitro* routes are not much better. Volatile organic solvents are common solvents for lipase-catalyzed polymerizations (*4*). Though bulk polymerizations are possible, the reaction mixtures often have higher viscosities at high conversions in the absence of a solvent. PHA synthases, the major alternative to lipases, require expensive coenzyme-A functionalized precursors to carry out the polymerization. (*5*).

It is desirable, therefore, to use a solvent that is more environmentally friendly than volatile organic solvents and that support a cofactor-free polymerization reaction. Additionally, the solvent should dissolve PHAs to prevent premature precipitation of the polymer, which could lead to low molecular weights. While, no such solvents are currently being employed, ionic liquids may possess the qualities required for an efficient, environmentally benign synthesis of PHAs.

Ionic liquids are an emerging alternative to volatile organic solvents. Ionic liquids are involatile, highly thermally stable, and have tuneable miscibility, viscosity, and solubility (*6*). Some ionic liquids can readily dissolve cellulose (*7*). Ionic liquids have been shown to stabilize enzymes (*8*). Further, enzyme activity and lifespan is often greater in ionic

liquids than in common organic solvents (*9,10*). Therefore, using ionic liquids as solvents for enzymatic synthesis of polymers could substantially reduce the cost of the process by reducing the amount of enzyme required. Further, recycling of the ionic liquids could greatly reduce the cost of the solvent required in a production process. One necessary step for a commercialized version of this process would be the recycling of the ionic liquid. Such recycling would likely either require precipitation of the polymers followed by removal of the precipitation solvent, byproducts, and unreacted monomers by distillation or removal of the byproducts by liquid-liquid extraction with an immiscible cosolvent.

One potential cause for concern of the use of fluorinated ionic liquids is their toxicity. Jastorff and coworkers measured cytotoxicity of rat cells for ionic liquids containing a variety of anions and found that ionic liquids containing fluorinated anions such as PF_6^-, BF_4^-, and $N(SO_2CF_3)^-$ were among the most cytotoxic, and were more cytotoxic than common organic solvents (*11,12*). However, in a separate study, the group found that ionic liquids containing the BF_4 anion posed a low risk of environmental release, owing to their negligible vapor pressure (*13*). Stepnowski and coworkers found that the same ionic liquids were more toxic than dichloromethane or xylene (*14*). The authors caution that comparisons of cytotoxicity between ionic liquids and other organic solvents should take into consideration the overall bioavailability of ionic liquids, which would depend to some degree on the vapor pressure of the ionic liquids relative to organic solvents (*14*).

In this study, we use ionic liquids to synthesize PHAs *in vitro* from 4-, 5-, 6- and 7-membered lactones. We first determined the ability of several ionic liquids to dissolve

PHAs. We then compared the stability of lipases and esterases in ionic liquids using a model transesterification reaction. We next carried out *in vitro* polymer synthesis reactions in ionic liquids with *Candida antarctica* lipase B as a catalyst. We focused on a ring-opening polymerization because of the potential advantages of having ring strain as a driving force toward polymerization and the lack of water formation during the reaction (*15-17*). This synthesis does not require expensive cofactors, and the high solubility of PHAs in ionic liquids may prevent premature precipitation, leading to molecular weights high enough to be useful in biomedical applications.

2.3 Experimental

Enzymes

Lyophilized forms of the enzymes *Candida antarctica* lipase A (CALA, Roche Chirazyme L-5, 6.5 U g^{-1}), *Candida antarctica* lipase B (CALB, Roche Chirazyme L-2, 443 U g^{-1}), *Candida rugosa* esterase (CRE, 10.4 U g^{-1}), *Candida rugosa* lipase (CRL, Meito Sangyo Lipase OF, 370, U g^{-1}), cross-linked enzyme crystal of *Candida rugosa* lipase (CRL CLEC, 9.5 U g^{-1}), *Humicola languinosa* lipase (HLL, Roche Chirazyme L-8, 488 U g^{-1}), *Pseudomonas cepacia* lipase (PCL, Amano Lipase PS, 483 U g^{-1}), *Pseudomonas fluorescens* esterase (PFE, 30,000 U g^{-1}), and porcine pancreatic lipase (PPL, Sigma, 0.4 U g^{-1}) were a gift from Altus Biologics, Inc. (Cambridge, MA). Lyophilized *Bacillus lentus* protease, α-chymotrypsin, pepsin, subtilisin Carlsberg, and immobilized *Candida antarctica* lipase B (iCALB, Novozyme 435 immobilized on acrylic resin) were purchased from Sigma-Aldrich (Allentown, PA). The reported activities of the Altus enzymes were based on a standard assay for hydrolysis activity

toward *p*-nitrophenyl acetate (pNPAc) at pH 7.2 as described by Bernhardt, et al. (*18*), where one U is defined as 1 μmol pNPAc hydrolyzed min^{-1}.

Ionic Liquids

1-Butyl-3-methylimidazolium tetrafluoroborate (BMIM[BF_4]), 1-butyl-3-methylimidazolium bromide (BMIM[Cl]), 1-butyl-3-methylimidazolium chloride (BMIM[Cl]), 1-butyl-3-methylimidazolium octyl sulfate (BMIM[$OcOSO_3$]), 1-butyl-3-methylimidazolium hexafluorophosphate (BMIM[PF_6]), 1-ethyl-3-methylimidazolium tosylate (EMIM[OTos]), and trihexydodecylphosphonate bis(trifluoromethane) sulfonimide (HHHDP[Tf_2N]) were purchased from Solvent Innovation GmBH (Cologne, Germany) or Sigma-Aldrich. All other chemicals were purchased from Sigma-Aldrich and were of the highest available purity.

We synthesized the ionic liquids 1-butyl-3-methylimizaolium perchlorate (BMIM[ClO_4]), 1-butyl-3-methylimizaolium dicyanamide (BMIM[DCA]), 1-butyl-3-methylimizaolium tetrachloroferrate (BMIM[$FeCl_4$]), 1-butyl-3-methylimizaolium bis(trifluoromethane) sulfonimide (BMIM[Tf_2N]), 1-butyl-3-methylpyridinum dicyanamide (BMPYR[DCA]), 1-methoxyethyl-3-methylimidazolium dicyanamide, MOEMIM[DCA], and 1-methyl-3-octylimidazolium dicyanamide OMIM[DCA] by anion exchange with halide-containing ionic liquids according to established literature procedures, as described below (*19-21*).

In a typical synthesis, the halide salt was prepared by adding methylimidazole to the desired alkyl chloride (1.05:1 mol mol^{-1}) in toluene. The mixture was refluxed for 24 h with magnetic stirring, resulting in a biphasic mixture. The toluene phase was decanted, and the ionic liquid phase was washed repeatedly with toluene or ethyl acetate and then

concentrated by rotary evaporation at 60 °C. Non-halide containing ionic liquids were then prepared by adding the sodium or lithium salt of the desired anion to the halide-containing ionic liquid (1.05:1 mol mol^{-1}) in acetone. The materials were stirred for 48 h at room temperature, after which dichloromethane was added to precipitate out the sodium salts in the mixture. The salts were removed by filtration and the resulting ionic liquid was concentrated by rotary evaporation. Additional dichloromethane additions and filtrations were performed until the solution was free of precipitate. Ionic liquids for the enzymatic synthesis were dried over $CaSO_4$ under vacuum for 48 hours and stored in a desiccator until use. Residual halide content was determined by inductively coupled plasma-atomic emission spectroscopy (ICP-AES), and ionic liquids with a halide content greater than 0.1 wt% were purified by chromatography (*22*). Water content was measured by ^{1}H NMR or by Karl-Fisher titration (*23*). Values of chloride content for ionic liquids used extensively were 0.005 wt% for BMIM[DCA], 0.001 wt% for OMIM[DCA], and 0.001 wt% for BMIM[Tf_2N]. Water contents for those ionic liquids were 0.75 wt %, 0.5 wt%, and 0.1 wt%, respectively.

Molecular weight determination

The average degree of polymerization and number-average molecular weight of polymers were determined by ^{1}H NMR endgroup analysis on a Varian Unity 300 MHz instrument (Varian, Inc., Palo Alto, CA) with deuterated chloroform as a solvent. The areas of the –OCH_2 peaks of the end group (t, δ~3.6 ppm) and the repeat unit (t, δ~4.0 ppm) were compared (*24*).

Gas chromatography

Achiral gas chromatography (GC) analysis was performed using a 30 m HP-5 column (J&W Scientific, Folsom, CA) with 0.32 mm inner diameter and 0.25 μm film size. Chiral products were analyzed on a 25 m CP7502 column (Varian) with 0.25 mm inner diameter and 0.25 μm film size. The temperatures of the sample injector and detector were 250 °C and 275 °C, respectively. The column temperature was set at 70 °C for eight minutes followed by an increase to 200 °C at a rate of 10 °C min^{-1} and then held constant at 200 °C for 20 minutes.

Solubility determination

To determine PHA solubility, ionic liquid (0.5 ml) was added to freeze-dried *Ralstonia eutropha* H16 cells (30 mg) in a 1.5 ml vial stirred with a magnetic stir bar. The cells were grown as described previously (*25*). The cell material contained 65% cell dry weight of random copolymer, poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV), with 29 wt% 3-hydroxyvalerate and a molecular weight of approximately 160,000. The slurries were stirred in an oil bath at 100 °C and samples were taken over time. Samples were removed with a 1.0 ml syringe and filtered through cotton. 100 μl aliquots were withdrawn and propanolyzed at 100 °C according to literature procedures to convert the polymers into propyl esters of the monomers (*26*). These samples were analyzed by GC on the achiral column and quantified by comparison with calibration curves.

Gel permeation chromatography (GPC) was used to investigate post-dissolution PHBV molecular weights. A Polymer Laboratories PL GPC 220 High Temperature

Chromatograph with refractive index detector was used. 1,2,4-Trichlorobenzene (TCB) was used as the column solvent at a temperature of 140 °C and a flow rate of 1.0 ml min^{-1}. Molecular weights were determined from polystyrene standards. PHBV extracted from cells were precipitated from IL with methanol (20:1 v/v), washed again three times with methanol, dried, and dissolved in TCB to a concentration of 1.0 g l^{-1}

Transesterification assay

Enzyme (5.0 mg) was suspended in ionic liquid (0.5 ml) in a glass vial. For the transesterification reaction, ethyl valerate (40 mM) and butanol (400 mM) were added to the suspension and the resulting mixture was stirred at 60 °C for 24 h. The reaction products were extracted with hexanes (1.0 ml) and analyzed by GC on an achiral column.

Ring-opening polymerization of PHAs

Lactone or lactone mixtures (250 mg) were added to suspensions of enzyme (2.5 mg) in ionic liquid (0.5 ml) in a 2 ml vial containing a magnetic stir bar. The vial was sealed and the mixtures were stirred at 25 ºC, 60 ºC, or 90 ºC for 24 to 72 h to yield the corresponding PHA (Figure 1). The reaction mixture was then added to methanol (20:1 v/v methanol: ionic liquid) and centrifuged at 4°C and a relative centrifugal force of 4000 x g for 30 minutes. The supernatant was removed and the resulting precipitate was then washed twice with methanol (20:1 v/v methanol: original ionic liquid volume) to remove ionic liquid traces. The precipitated polymer was dried under vacuum and allowed to sit at room temperature for 24 hours, resulting in a white solid. A sample of this solid was dissolved in deuterated chloroform (5 g l^{-1}) and analyzed by ^{1}H NMR. Some reactions with β-butyrolactone were run as above, but instead of being added to methanol, the

reaction mixture was derivatized, extracted, and analyzed as described for the transesterification reaction.

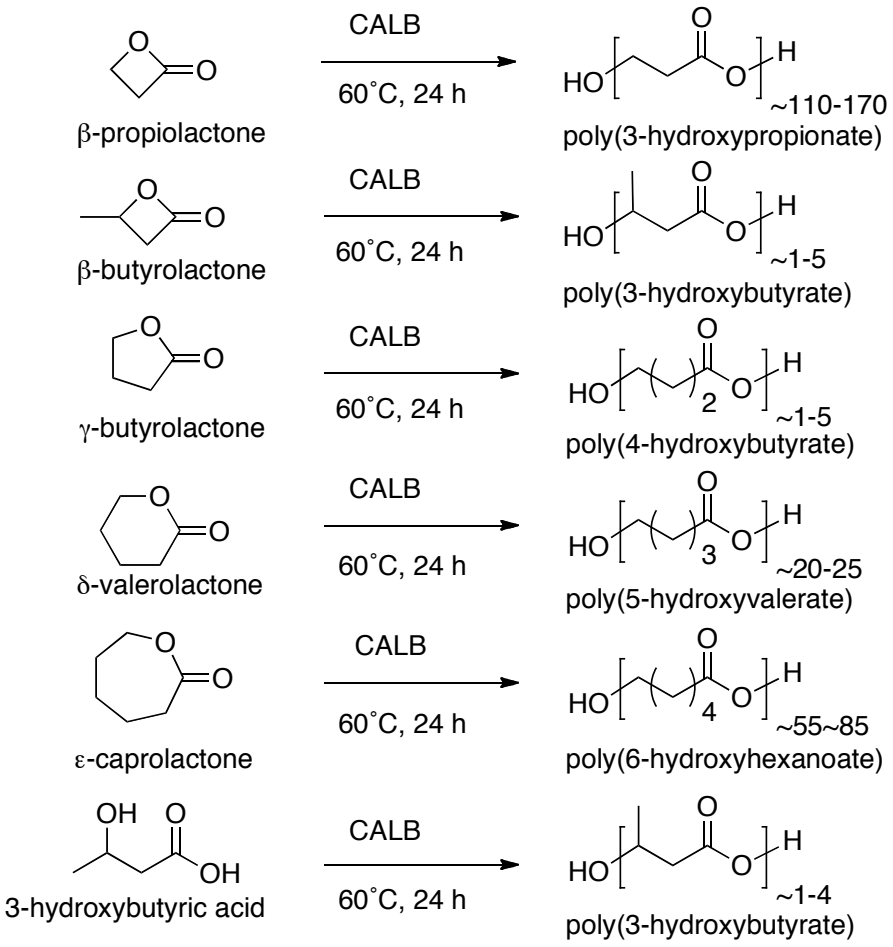


Figure 1. Polymerization reaction carried out in BMIM[Tf_2N].

Reactions with 3-hydroxybutyric acid

Suspensions of iCALB (2 mg) in ionic liquid (0.25 ml) were prepared. Either racemic or (*R*)-3-hydroxybutryate (100 mM) were added to 1-butanol, 2-butanol, or butyric acid (100 mM or 1 M) and the resulting mixture was added to the enzyme-ionic liquid suspension. The reaction mixture was incubated at 60 °C for 10 minutes or 6 hours with

stirring by magnetic stir bar. Bis(trimethylsilyl)acetamide (50 μL) was then added to the mixture as a derivatizing agent, which was then stirred for an additional 30 minutes at 60 °C. The starting materials and products were extracted with toluene (1.5 ml) and analyzed by chiral GC. Kinetic curves were constructed for each substrate combination, and the time of 10 minutes chosen earlier to determine the initial rate was found to be in the linear region of the curve.

2.4 Results

Dissolution of PHBV in ionic liquids

Ionic liquids dissolved up to 21 g l^{-1} of copolymer PHBV (M_n = 160,000) at 100 °C, Table 1. Ionic liquids containing the 1-butyl-3-methyl imidazolium cation and large anions dissolved the most PHBV: 21 g l^{-1} for BMIM[Tf_2N], 17 g l^{-1} for BMIM[$FeCl_4$], and 12 g l^{-1} for BMIM[DCA]. When the 1-butyl-3-methyl imidazolium cation in BMIM[DCA] was replaced by the more hydrophobic 1-octyl-3-methyl imidazolium (OMIM[DCA]), only 8 g l^{-1} of PHBV dissolved. When the 1-butyl-3-methyl imidazolium cation in BMIM[DCA] was replaced by 1-butyl-4-methylpyridinium (BMPYR[DCA]) only 1 g l^{-1} of PHBV dissolved. Ionic liquids containing1-butyl-3-methyl imidazolium cation and small anions – bromide or hexafluorophosphate – (BMIM[Br] or BMIM[PF_6]) dissolved less than 0.01 g l^{-1} PHBV. In all cases, the solubility was higher at higher temperatures. For example, 1 g l^{-1} of PHBV dissolve in BMIM[DCA] at 60 °C, 7 g l^{-1} at 80 °C, and 12 g l^{-1} at 100 °C.

Table 1. Observed equilibrium solubility of PHBV in ionic liquids.

Ionic Liquid	Miscibility with water	Equilibrium Solubility[a]
BMIM[Tf_2N]	immiscible	21 g l^{-1}
BMIM[$FeCl_4$]	immiscible	17 g l^{-1}
BMIM[DCA]	miscible	12 g l^{-1}
BMPYR[DCA]	miscible	1 g l^{-1}
OMIM[DCA]	miscible	8 g l^{-1}
BMIM[PF_6]	immiscible	< 0.01 g l^{-1}
BMIM[Br]	miscible	< 0.01 g l^{-1}

PHBV was extracted at 100°C from freeze-dried *R. eutropha* H16 cells (Pederson and Srienc, 2004).

This dissolution required approximately one hour at 100 °C and was accompanied by a decrease in the molecular weight of PHBV, likely due to hydrolysis despite low water content in the ionic liquids. Dissolution of PHBV with M_n = 160,000 in BMIM[Tf_2N] with 0.1 wt% water for one hour at 100 °C yielded M_n = 109,000 for the dissolved PHBV. The PHBV dissolved completely, so the lower molecular weight was not due to selective dissolution of lower molecular weight fractions in the ionic liquid. More water in the ionic liquid caused larger decreases in the molecular weight. For example, dissolution of the same PHBV in OMIM[DCA] with 0.5 wt% water decreased the molecular weight to M_n = 59,000, and with 1.5 wt% water the molecular weight decreased drastically to M_n = 4,000.

Preliminary enzyme activity screening

We screened hydrolases for their ability to catalyze a test reaction – transesterification of ethyl valerate to methyl valerate, Figure 2. CALB and iCALB had the highest conversions in all ionic liquids tested: 94% and 91% in BMIM[Tf_2N], 82% and 52% in BMIM[DCA], and 84% each in BMIM[PF_6]. In BMIM[Tf_2N] and BMIM[PF_6] the enzymes with the next highest conversion were CALA (63% and 36%), CRL CLEC (55% and 13%), PFE (35% and 3%), and CRL (9% and 6%). In BMIM[DCA] the conversions for CALA, CRL CLEC, PFE and CRL were markedly lower: 2%, 6%, 0.6%, and 0.7%, respectively. Hydrolases CRE, HLL, and PPL gave <1.5% conversion in all ionic liquids, while proteases – pepsin, subtilisin Carlsberg, α-chymotrypsin, and *Bacillus lentus* protease – showed <0.1 % conversion.

Reactions in the water-immiscible ionic liquids like BMIM[Tf_2N] or BMIM[PF_6] usually gave higher conversions than those in water-miscible ionic liquids like BMIM[DCA], Table 2. Transesterifications with CALB, iCALB CALA, and CRL CLEC in BMIM[Tf_2N] gave 1.1-, 1.7-, 21-, and 9-fold higher conversion, respectively, compared with BMIM[DCA]. Transesterification with CALB in BMIM[PF_6] was comparable to that in BMIM[DCA] (84% vs., 82% conversion in BMIM[PF_6]), but transesterifications with iCALB, CALA, and CRL CLEC were 1.6-, 12-, and 2.2-fold higher in BMIM[DCA]. Conversions in BMIM[Tf_2N] and BMIM[PF_6] were comparable for many of the enzymes tested. None of the hydrolases showed appreciable activity in BMIM[$FeCl_4$], possibly due to the acidic nature of the anion. The most promising

enzyme, iCALB, was also tested with several other water-immiscible and water-miscible ionic liquids. Two water-immiscible ionic liquids –BMIM[ClO_4] and HHHDP[Tf_2N] – gave higher conversions for transesterification (75% and 84%, respectively) than two water-miscible ionic liquids – BMIM[BF_4], and EMIM[OTos] (48% and 36%, respectively), but one water-miscible ionic liquid -BMIM[$OcOSO_3$] - gave a high a conversion of 84%.

The most promising ionic liquid was BMIM[Tf_2N] because it both dissolved PHBV and maintained enzyme activity. BMIM[DCA] is an alternative, but dissolves less PHBV and showed lower conversion in the model transesterification assay above. Both of these ionic liquids possess an amide-based anion and an imidazolium-based cation.

Table 2. iCALB-catalyzed transesterification of ethyl valerate to methyl valerate in water miscible and immiscible ionic liquids.

Ionic Liquid	Miscibility with water	Conversion (%)
BMIM[Tf_2N]	immiscible	91
BMIM[PF_6]	immiscible	84
HHHDP[Tf_2N]	immiscible	84
BMIM[ClO_4]	immiscible	75
BMIM[$OcOSO_3$]	miscible	84
BMIM[DCA]	miscible	52
BMIM[BF_4]	miscible	48
EMIM[OTos]	miscible	36

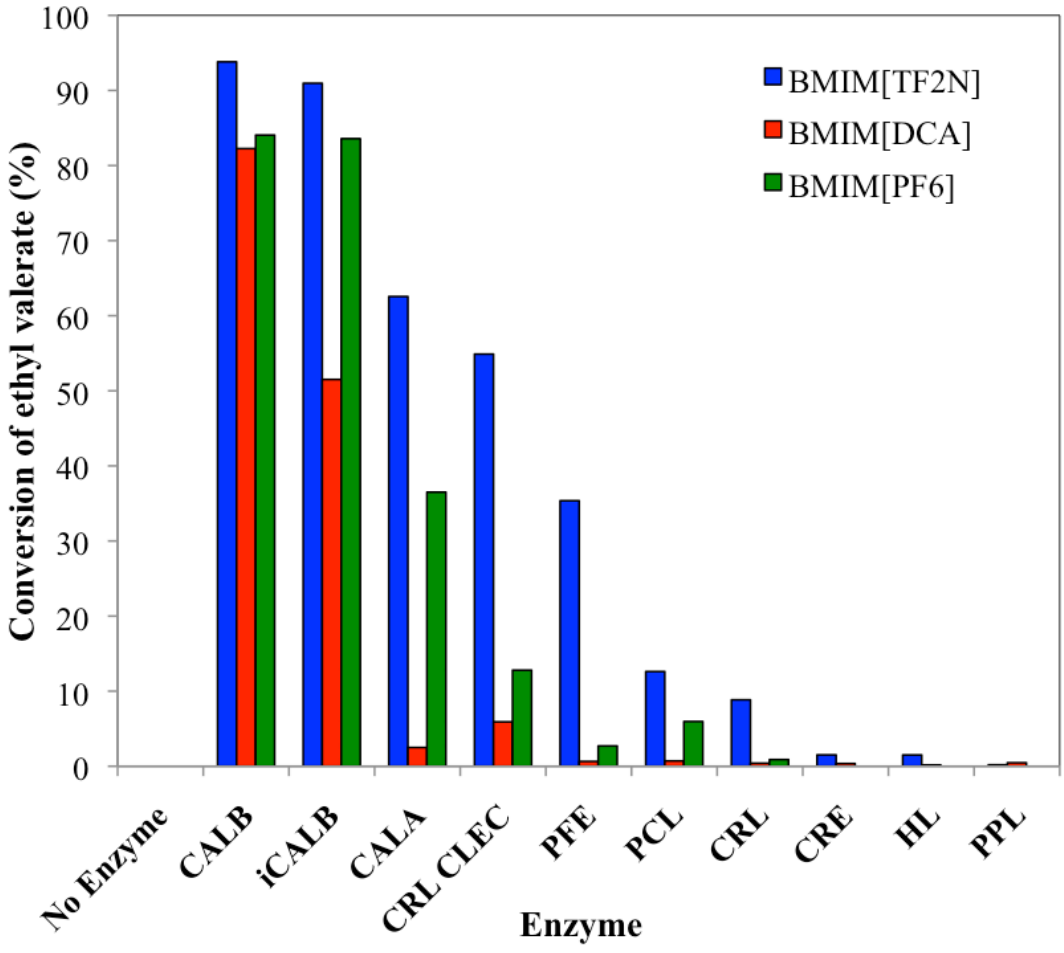


Figure 2. Conversion of ethyl valerate to methyl valerate in enzyme-catalyzed transesterification reaction with three ionic liquids.

Ring-Opening Polymerizations

The polymerization that gave the highest molecular weight with free CALB as a catalyst was the ring-opening of ε-caprolactone in BMIM[Tf_2N] at 90°C, Table 3. This reaction yielded 80-90% poly(6-hydroxyhexanoate) (P6HH) with M_n= 8100, after 24 h. The molecular weight did not increase with longer reaction time (M_n = 8000 at 72 h),

suggesting that the heat had inactivated the enzyme or that the reaction attained equilibrium. At 25 and 60 °C, the molecular weight continued to increase from 100 and 3100 at 24 h to 1200 and 6300 at 72 h. No polymerization occurred in the absence of enzyme. Above 90 °C the reaction mixture yellowed after several hours and yielded lower molecular weight polymer (M_n = 5000 at 100 °C and M_n = 2700 at 110 °C after 24 h), perhaps due to rapid inactivation of CALB or the reaction reaching equilibrium.

Drying iCALB in a desiccators before polymerization increased the molecular weight of the product P6HH from 6300 to 9700. Evaporation of water may also contribute to the higher molecular weight at 90 °C (8100 at 24 h, 8000 at 72 h) as compared to 60 °C (3100 at 24 h, 6300 at 72 h).

The iCALB-catalyzed ring-opening polymerization of the β-propiolactone and ε-caprolactone gave the highest molecular weight polymers: 11,900 and 9,700, Figure 3. These polymerizations used dried iCALB, a moderate temperature of 60 °C and a low 1:100 weight ratio of iCALB to lactone. Similar polymerizations in organic solvents used about ten-fold more iCALB (*17,27*). After polymerization, the product polymer remained dissolved in the ionic liquid at 60 °C. Addition of methanol (20:1 methanol volume to ionic liquid volume) precipitated the polymer, Figure 4. Similar polymerizations of γ-butyrolactone and δ-valerolactone yielded low molecular weight polymer (M_n = 500 and 2500, respectively).

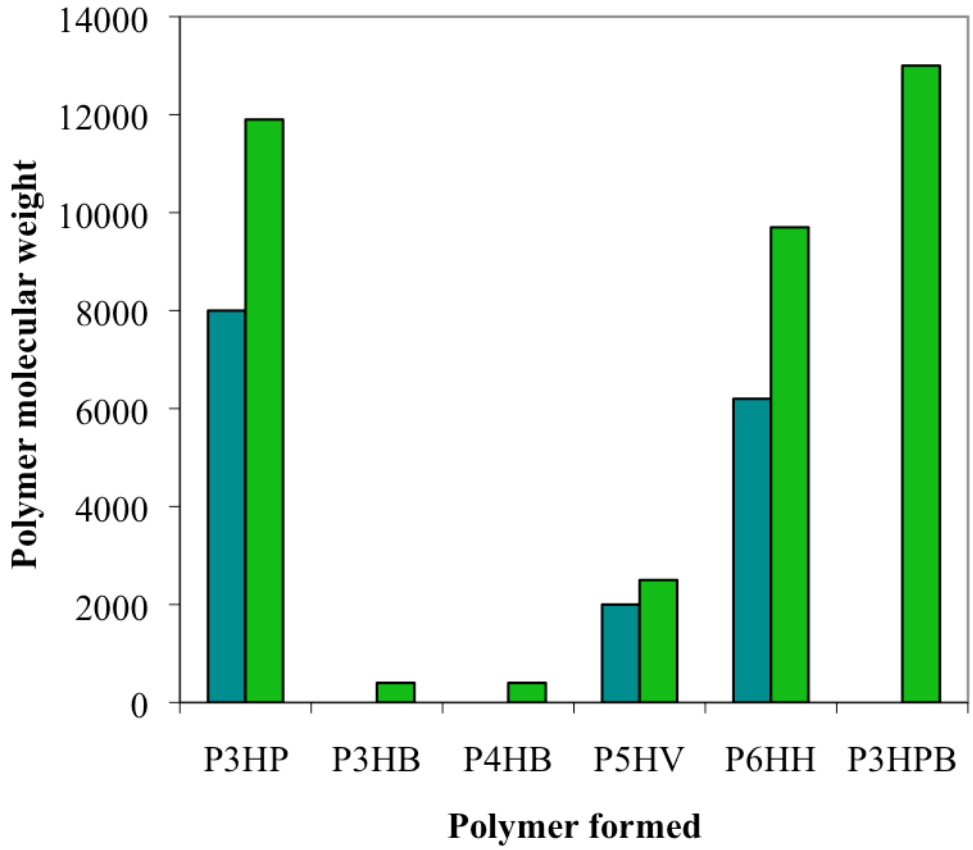


Figure 3. Number-average molecular weight of polymers synthesized by iCALB-catalyzed ring-opening polymerization of lactones in BMIM[Tf_2N] at 60ºC for 24 h before drying (blue) and after drying (green). P3HPB is a random copolymer of P3HP and P3HB containing 6% P3HB. No polymer was obtained after methanol precipitation for P3HB, P4HB prior to drying the enzyme. No P3HB incorporation was detected in the copolymerization reaction prior to drying the enzyme.

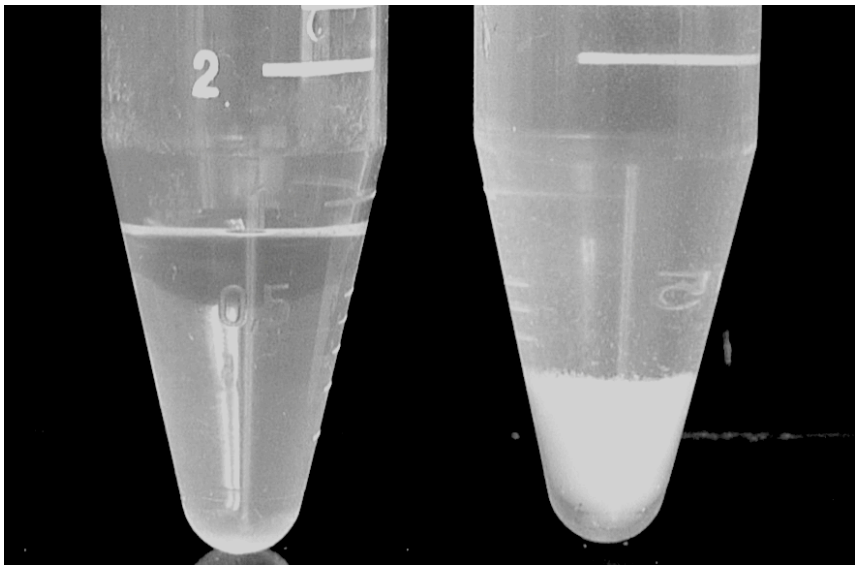


Figure 4. BMIM[Tf_2N]/P3HP reaction mixture before (left tube) and after (right tube) methanol precipitation of P3HP. CALB (1 mg) and β-propiolactone (100 mg) were added to ionic liquid (0.5 mL) and incubated for 24 h at 60°C. After the addition of 10 ml methanol, polymer precipitated and settled as a white precipitate (right tube).

Table 3. Molecular weight (M_n) of P6HH obtained from CALB-catalyzed ring-opening polymerization of ε-caprolactone in BMIM[Tf_2N] at 1:100 w/w enzyme:lactone.

Temperature (°C)	Time (h)	Yield (wt %)	Molecular weight (M_n)
25	24	0	No Polymerization
25	72	15	1200
60	24	50	3100
60	72	75	6300
90	24	85	8100
100	24	55	5200
110	24	15	3400

Polymerization of β-butyrolactone yielded short oligomers as compared to the polymer (M_n = 11,900) formed from β-propiolactone. GC analysis (not shown) of a

polymerization of methyl-substituted β-butyrolactone after 24 h revealed hydrolysis of the lactone to acid (likely due to traces of water in the ionic liquid and iCALB), a smaller amount of dimer and even smaller amounts of trimer and tetramer. The dimer was enriched in the *RR* diastereomer (~2:1 *RR* to other stereoisomers) indicating that the dimerization was enzyme-catalyzed. This result suggests that the rate-limiting step in the polymerization of methyl-substituted β-butyrolactone is not lactone ring opening, but addition of the units to the polymer chain. A copolymerization of the methyl-substituted β-butyrolactone and the unsubstituted β-propiolactone yielded poly(3-hydroxybutyrate-*co*-3-hydroxypropionate) (P3HBP) with 6 mol% poly(3-hydroxybutyrate) and a molecular weight of 13,000, Figure 3.

To study the apparently slow addition of monomer step during polymerization of methyl-substituted β-butyrolactone, we investigated CALB-catalyzed esterification of corresponding 3-hydroxybutyric acid, Figure 1. Polymerization of this hydroxy acid monomer requires that it fit both in the acyl-binding site (the acid part of the ester to be formed) and in the alcohol-binding site (the alcohol part of the ester to be formed). To identify which fit caused the slow reaction, we added a ten-fold excess of either unhindered alcohol (1-butanol or 2-butanol) or unhindered acid (butyric acid). If 3-hydroxybutyric acid fit poorly when it acted as the acyl donor, then addition of excess butyric acid should give a faster ester formation. Conversely, if 3-hydroxybutyric acid fit poorly when it acted as the alcohol, then excess 1-butanol or 2-butanol should give a faster ester formation. We observed six times faster esterification of 3-hydroxybutyric

acid with excess 1-butanol and two times faster esterification of 3-hydroxybutyric acid with excess 2-butanol than with excess butyric acid, Table 4. This result suggests that 3-hydroxybutyric acid fits in the CALB active site better as an acid than as an alcohol. We also observed an 11% faster rate of reaction for butyric acid than for 3-hydroxybutyric acid with both 1- and 2-butanol, which suggests that 3-hydroxybutyric acid may fit poorly in the acid binding site compared to butyric acid.

A similar experiment measured the direct competition for the acid moiety of 3-hydroxybutyric acid by different alcohols: the unhindered 1-butanol or 2-butanol to form butyl esters and the hydroxyl group of 3-hydroxybutyrate to form 3-hydroxybutyrate dimers. CALB favored formation of the 1-butyl esters 34-fold and the 2-butyl ester 12-fold over dimers, Figure 5. These results confirm the poor fit of 3-hydroxybutyric acid in CALB's active site when it acts as an alcohol.

Table 4. Initial rates of esterification of 3-hydroxybutyric acid (Rac-3HB), and butyric acid with 8 g l^{-1} iCALB in BMIM[Tf_2N] at 60°C after 10 minutes.

	Initial reaction rate (mmol h^{-1} mg $enzyme^{-1}$)	
Excess substrate (1 M)	100 mM Rac-3HB	100 mM Butyric acid
1-Butanol	5.2	5.8
2-Butanol	1.4	1.6
Butyric acid	0.8	-

Another competition experiment showed that 3-hydroxybutyric acid also fits poorly when it acts as an acid. We measured a direct competition for the alcohol moiety of 3-hydroxybutyric acid by two different acids: butyric acid and the acid group of 3-hydroxybutyric acid. This reaction was slow, consistent with the poor fit of 3-

hydroxybutyric acid acting as an alcohol. It showed two to four times lower conversion (17%) than the reactions with 1- or 2-butanol (75% or 38%) in the paragraph above. CALB favored formation of the butyrate 49-fold over the dimer. These results suggest that 3-hydroxybutyric acid fits poorly as an alcohol because of the much lower conversion observed, and that it may fit more poorly as an acid as butyric acid because of the large conversion difference.

2.5 Discussion

PHAs dissolve in a wide range of ionic liquids making them potential alternatives to conventional organic solvents for the synthesis and extraction of PHAs. We hypothesize ionic liquids may dissolve PHAs by forming hydrogen bonds to them. Consistent with this notion we found that PHBV dissolves in many ionic liquids containing imidazolium-based cation such as BMIM[DCA], which may form a hydrogen bond via the C-2 hydrogen (*28*), and not at all in similar pyridinium-based ionic liquid BMPYR[DCA]. Previously Benton and Brazel tested the solubility of polyolefins - poly(styrene), poly(methyl methacrylate), and poly(2-hydroxyethyl methacrylate) – in ionic liquids and found little solubility. None of the polyolefins dissolved in BMIM[PF_6] and only poly(2-hydroxyethyl methacrylate) was soluble in BMIM[Cl] (*29*). The solubility of PHBV in BMIM[Tf_2N], BMIM[$FeCl_4$], and BMIM[DCA] at 100 °C compares favorably to those found recently for PHB in another alternative solvent, supercritical carbon dioxide: the maximum reported solubility was 8 g l^{-1} at 75 °C and 355 bar (*30*). We recognize that the PHA solubilities we have obtained are still too low for commercial viability, but we

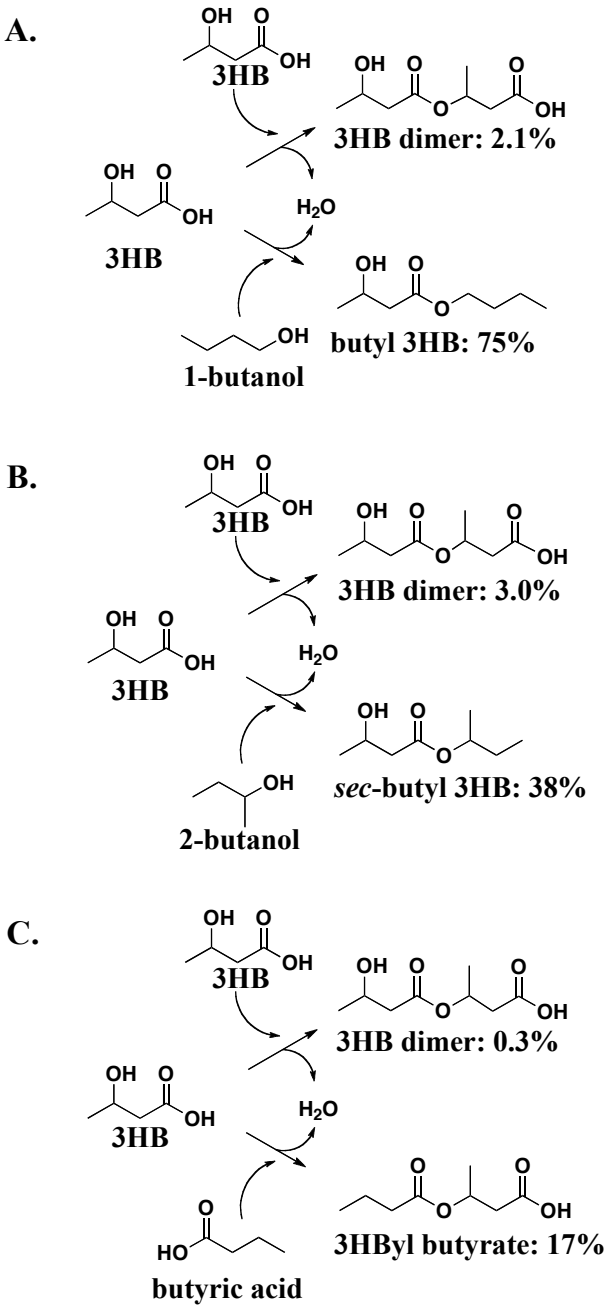


Figure 5. Evaluation of esterification competition of 100 mM racemic 3-hydroxybutyric acid (Rac-3HB) in the presence of equimolar concentrations of A. 1-butanol, B. 2-butanol, and C. butyric acid with 8 g l^{-1} iCALB in BMIM[Tf_2N] at 60°C after 6 h. Values represent conversion of racemic 3-hydroxybutyric acid (3HB) to dimers of 3HB or butyl 3-hydroxybutyrate (butyl 3HB), *sec*-butyl 3-hydroxybutyrate (*sec*-butyl 3HB) or 3-hydroxybutyryl butyrate (3HByl butyrate).

believe that better ionic liquids for dissolving PHAs may be synthesized, and that these initial tests serve as a good basis for future design and experimentation.

Several groups previously reported that esterases and lipases retain their ability to catalyze transesterification reactions in ionic liquids with large anions like Tf_2N. Our results are mostly consistent with previous reports (*10*). For example, the lower of enzyme activity in BMIM[DCA], but good activity in BMIM[Tf_2N] may be explained by the more delocalized negative charge in Tf_2N (charge spread over five atoms) as compared to DCA (charge spread over three atoms). The more basic DCA anion may interfere with the enzyme's internal hydrogen bonds. While our observation of low conversion in BMIM[DCA] appears to be consistent with the observation of van Rantwijk and coworkers (*31*) that BMIM[DCA] deactivates enzymes, it is also possible that the reaction merely reached equilibrium at a lower conversion because of higher water content in the water-miscible ionic liquid. One difference between our results and previous work involve Lau et al.'s report (*32*) is that CALB is inactive in ionic liquids (BMIM[NO_3], BMIM[lactate], EMIM[$EtSO_4$], and $EtNH_3[NO_3]$) in which it dissolves. We found that CALB retains activity in BMIM[DCA] despite the fact that it dissolved. It has been proposed that water-miscible ionic liquids may strip most enzymes, with the notable exception of CALB, of residual water in an otherwise anhydrous environment, thus inactivating the enzymes (*31*). This reasoning could explain the lower enzyme activity in BMIM[DCA] relative to the others, with the exception of CALB, for which denaturation in anhydrous medium is not known to occur.

Our highest molecular weight of poly(6-hydroxyhexanoate) obtained from ring-opening polymerization of ε-caprolactone (10,000 g mol^{-1}) is in the lower end of the range reported by Gross and coworkers (*17*) when they used toluene as a solvent (~10,000-30,000 g mol^{-1}), though our molecular weight is likely higher than what was in our solution because we had to fractionate the polymers with methanol to remove the ionic liquid prior to using NMR. However, we used only a 1:100 weight ratio of catalyst to substrate to achieve this molecular weight, compared to that group's use of a 1:10 ratio. They used toluene as the solvent and obtained polymer molecular weights ranging from 8,000 to 20,000 without fractionation. The critical parameter was water content. A water content of 1.95% yielded polymer with a molecular weight of 8,000, while a lower water content of 0.60% yielded polymer with a molecular weight of 20,000. We expect that further drying of the enzyme and the ionic liquid in our experiment would increase the molecular weight of the polymer.

The low molecular weights obtained in the ring opening polymerization of β-butyrolactone to yield poly(3-hydroxybutyrate) is likely due to the poor fit of the 3-hydroxybutanoate moiety in the active site of CALB. Another enzyme that better accommodates this moiety may be a more suitable catalyst for this important polymer.

CHAPTER 3. Discovery of hydrolase catalysis in deep eutectic solvents.

3.1 Chapter Overview

This chapter concerns the discovery of hydrolase catalysis in deep eutectic solvents. These solvents are very interesting choices because their components can be made petrochemically or renewably, making them a potentially good bridge technology between traditional and emerging solvents. 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. This chapter and supporting information first appeared in: J. T. Gorke, F. Srienc, R. J. Kazlauskas, *Chem. Commun.* 1235 (2008).

3.2 Introduction

Room temperature ionic liquids are potential green alternatives to organic solvents (*1-3*) for extractions (*4,5*), chemical reactions (*6-8*) and biotransformations (*9-13*). 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 (~240 $US/kg for 95% pure 1-butyl-3-methylimidazolium chloride vs. ~30 $US/kg for 99% pure acetone or toluene), toxicity similar to or higher than organic solvents (*14-16*), and the need for high purity, as even trace impurities affect physical properties (*17,18*).

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 (*19-23*). 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 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 (*24*). In spite of these possible problems, we found that many hydrolases retain excellent activity in DESs.

3.3 Results and discussion

As a test reaction, we used the lipase-catalyzed transesterification of ethyl valeratewith 1-butanol, Table 1. CALB[§] and the immobilized form – iCALB – 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 (data in electronic supplemental material).

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 CAL-B catalyzed reaction in EAC:EG showed 54% of starting material

consumed resulting in 31% ethylene glycol monovalerate and and 23% ethyl butyrate. This nearly equal amount of the two product esters is surprising because the concentration of ethylene glycol (10 M) was twenty-five 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.

Table 1. Percent conversion of ethyl valerate to butyl valerate at 60 ºC.

	iCALB	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 percent conversion including the side reaction with the ethylene glycol component of the deep eutectic solvent. No side reaction was detected in the other reactions.

Even more surprising were the transesterification reactions in glycerol-containing DESs (8 M glycerol) which showed >90% conversion and <0.5% glyceryl ester formation (data in electronic supplementary information). 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 iCALB-catalyzed transesterification was comparable or higher in DESs as compared to typical ionic liquids, Table 2. Previous work showed that iCALB had good transesterification activity in 1-butyl-3-methylimidazolium bis(trifluoromethane)sulfonimide (BMIM [Tf_2N]) compared to other ionic liquids (*25*). 1-Butyl-3-methylimidazolium tetrafluoroborate (BMIM[BF_4]) is water miscible like the DESs and preserves enzyme activity (*26*). iCALB had the highest initial specific transesterification activity in EAC:Gly (50 μmol ethyl valerate hr^{-1} mg^{-1}), which was twice as high as for BMIM[Tf_2N] (24 μmol hr^{-1} mg^{-1}), seven times higher than the activity we found in BMIM[BF_4] (7 μmol hr^{-1} mg^{-1}). The initial specific activity in toluene, ChCl:Gly, and ChCl:U ranged from 20 to 37 μmol hr^{-1} mg^{-1} and were comparable to that in BMIM[Tf_2N].

iCALB also catalyzed another test reaction in DESs – aminolysis of ethyl valerate with 1-butylamine, Figure 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 amminolysis with butylamine (11% conversion), likely due to proton exchange between butylamine with the ethylammonium cation. The relative amount of ethylamide formed is 7-fold less than the relative concentration of ethylamine. In contrast, the transesterification reactions above did not show competing aminolysis when using ethylammonium-chloide-

containing DESs. A possible reason is that the ethylammonium ion remained protonated and unreactive because the transesterification reactions lacked a base.

Table 2. Initial specific activity of iCALB in DES after 15 minutes of reaction.

	Tranesterification 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 hr^{-1} mg $solid^{-1}$, activity relative to toluene in parentheses. iCALB contains ~10 wt% protein. [b]Aminolysis with ethylamine from the DES was the dominant reaction.

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 hr^{-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 (Data in supporting information.) The rate of epoxide hydrolase (*27,28*)

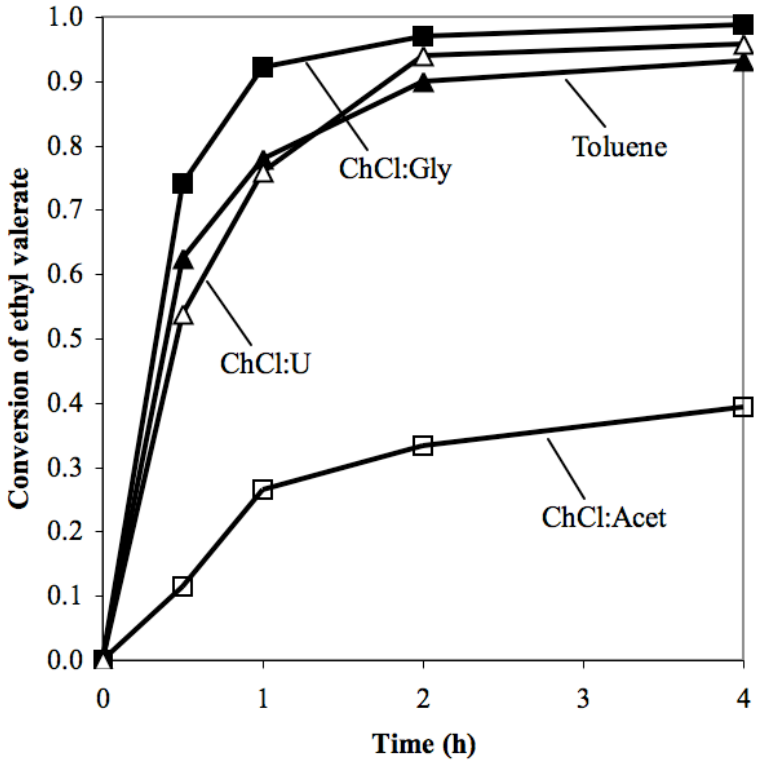


Figure 1. Aminolysis of ethyl valerate (100 mM) with 1-butylamine (110 mM) at 60 ºC with 10 mg ml^{-1} iCALB.

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 (*29,30*)). Similar additions of 10% or 25% DMSO or acetonitrile did not increase the conversion for epoxide hydrolase-catalyzed hydrolysis of styrene oxidc, 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 (Figure 3 in Supporting data).

Table 3. Conversion of styrene oxide by EHAD1 in ChCl:Gly/buffer mixtures at 37 ºC 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 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 two imidazolium based ionic liquids (*31*) The polarity of DESs containing the quaternary salt choline chloride was 0.08 to 0.09 units lower than the corresponding primary ethylammonium chloride DESs, which is consistent with the lower polarity of quarternary ammonium-based ionic liquids as compared to primary ammonium-based ionic liquids (*31*).

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

Solvent	E_T^N	Solvent	E_T^N
Toluene	0.10[*,a]	ChCl:EG	0.80
DMSO	0.44[*]	Glycerol	0.81[*]
Acetonitrile	0.46[*]	ChCl:Gly	0.84
BMIM[Tf_2N]	0.64[**]	ChCl:U	0.84
BMIM[BF_4]	0.68[**]	EAC:Acet	0.85
Methanol	0.76[*]	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. *: data from (*14*), **: data from (*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, 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 for choline chloride, 20 $US/kg for urea, 35 $US/kg for glycerol) and biodegradable, making DESs attractive as green replacements for volatile organic solvents.

3.4 Experimental

General

Reagents and enzymes were purchased from Sigma-Aldrich, except where described below. Ionic liquids were purchased from Solvent Innovation (Köln, Germany). SABP2

was expressed and purified according to Kumar and coworkers (*33*). PFE was expressed and purified according to Cheeseman and colleagues (*34*). In all cases, blank reactions were run alongside the enzymatic reactions and gave negligible conversion, with the exception of ChCl:MA, as shown in Table 1 of the text.

Synthesis of DESs

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 one hour.

Gas chromatography

The detector and injector temperatures were 275 ºC and 250 ºC, respectively. Analysis of transesterification and aminolysis products was performed using a 30 m HP-5 column (J&W Scientific, Folsom, CA) with 0.32 mm inner diameter and 0.25 μm film size. The initial column temperature of 60 ºC was held for 6 min, then increased to 165 ºC at 15 ºC min^{-1}, then further increased to 200 ºC at 25 ºC min^{-1} and held at 200 ºC for 5 min. Products of styrene oxide hydrolysis were analyzed on a 25 m CP 7502 column (Varian) with 0.25 mm inner diameter and 0.25 μm film size. The initial column temperature of 50 ºC was held for 5 min, then increased to 200 ºC at 10 ºC min^{-1} held at 200 ºC for 5 min.

Transesterification reaction

Enzyme (2.0 mg solid; note that iCALB contains only ~10 wt% protein) was suspended in solvent (0.2 ml) in a glass vial. Ethyl valerate (40 mM) and butanol (400

mM) were added to the suspension and the resulting mixture was stirred at 60 ºC for up to 24 h. The reaction products were extracted with toluene (1.0 ml) and analyzed by GC.

Aminolysis reaction

Enzyme (2.0 mg solid; note that iCALB contains only ~10 wt% protein) was suspended in solvent (0.2 ml) in a glass vial. Ethyl valerate (100 mM) and butylamine (110 mM) were added to the suspension and the resulting mixture was stirred at 60 ºC for up to 24 h. The reaction products were extracted with toluene (1.0 ml) and analyzed by GC.

Hydrolysis of p-nitrophenyl acetate

A 0.5 mg protein ml^{-1} solution in 5 mM BES at pH 7.2 (10 μl) was added to a *p*-nitrophenyl acetate solution (30 mM, 90 μl), acetonitrile (7.4 % v/v), and a mixture of 5 mM BES at pH 7.2 and ChCl:Gly. The final DES volume fraction was 0, 0.1, 0.25, 0.5, 0.75, or 0.9. The absorbance was monitored at 404 nm at 6-second intervals with a microplate reader (SpectraMax 384 Plus, Molecular Devices) at room temperature for up to 40 minutes.

Hydrolysis of styrene oxide

A mixture (0.2 ml) of styrene oxide (100 mM), 5 mM BES at pH 7.2, and sufficient ChCl:Gly to give DES volume fractions of 0, 0.1, 0.25, 0.5, 0.75, and 0.9 was prepared. A 10 mg ml^{-1} protein solution in 5 mM BES at pH 7.2 (2 μl) was added to the suspension to start the reaction. The reaction mixture was incubated at 37 ºC for 2 h, after which the mixture was extracted with ethyl acetate (1.0 ml) and analyzed by GC.

Polarity estimation

Reichardt's dye (2,6-diphenyl-4-(2,4,6-triphenylpyridinio)phenolate, 0.4 mg) was dissolved in DES (0.5 ml). An aliquot was transferred to a 96-well microplate. The wavelength of the absorption maximum of the long-wavelength transition (λ_{max}) was measured at room temperature using a Spectra Max 384 Plus. Normalized polarity values (E_T^N) were calculated from the equation (*32*)

$$E_T^N = \frac{E_T(\text{solvent}) - E_T(\text{TMS})}{E_T(\text{water}) - E_T(\text{TMS})} = \frac{E_T(\text{solvent}) - 30.7}{32.4} \qquad (3\text{-}1)$$

where E_T (solvent) is the energy (kcal mol^{-1}) of the maximum of the long wavelength transition and is given by

$$E_T(\text{solvent})\ (\text{kcal mol}^{-1}) = \frac{28591}{\lambda_{max}(\text{nm})} \qquad (3\text{-}2)$$

3.5 Supporting data

Stability of CALB in ChCl:U

We preincubated CALB (2 mg ml^{-1}) in either 10 M urea or 5 M choline chloride in deionized water (0.3 ml) for 30, 60, and 90 min at 60 °C. After the allotted time, we examined the hydrolysis activity of *p*-nitrophenyl acetate and compared it a mixture with no preincubation. After 90 min in 10 M urea, CALB lost 70% of its initial activity, and the first-order rate constant for the degradation had a value of 7 x 10^{-3} min^{-1}. For 5 M choline chloride, the value of the degradation rate constant was 4 x 10^{-3} min^{-1}, corresponding to an activity loss of 25% after 90 min. We then preincubated CALB (0.2 mg ml^{-1}) in ChCl:U (0.3 ml) for up to 90 min. We then examined the transesterification activity of the enzyme, and found a change of < 1% in activity over the course of the

experiment, corresponding to a rate constant of less than 2 x 10^{-4} min^{-1}. Based on these results, CALB is at least 20- and 35-fold more stable in ChCl:U than in 5 M choline chloride or 10 M urea, respectively.

Transesterification in glycerol-containing DES: ChCl:Gly

An iCALB-catalyzed transesterification of pure glycerol with ethyl valerate in *tert*-butanol (glycerol and its esters of valeric acid are poorly soluble in toluene.) showed a peak in the GC trace consistent with glyceryl monoester of valeric acid, panel A in Figure 2. A similar reaction with 1-butanol as the nucleophile and toluene as the solvent showed the expected 1-butyl valerate, panel B in Figure 2. The same reaction in ChCl:Gly after extraction with *tert*-butanol (1.0 ml) instead of toluene, Panels C and D, showed 95% conversion to 1-butyl valerate and <0.5% conversion to the glyceryl monoester of valeric acid.

Hydrolysis of p-*nitrophenyl acetate by lipases and esterases*

We ran hydrolysis assays in 0% to 90% ChCl:Gly with three lipases and four esterases, Figure 3. Four enzymes showed higher activity in 10% DES as compared to buffer only: CALB (125%), PFE (125%), PLE (284%), and ROE (328%). At 25% DES, three enzymes still had higher activity as compared to buffer only: CALB (130%), PLE (215%), and ROE (299%). At 50%, only CALB retained higher activity as compared to buffer only: 112%. At 25% to 90% DES, all enzymes showed lower activity as compared to buffer only. At 90% DES PLE and PCL showed the lowest activity as compared to buffer only: 1% and 6%, respectively.

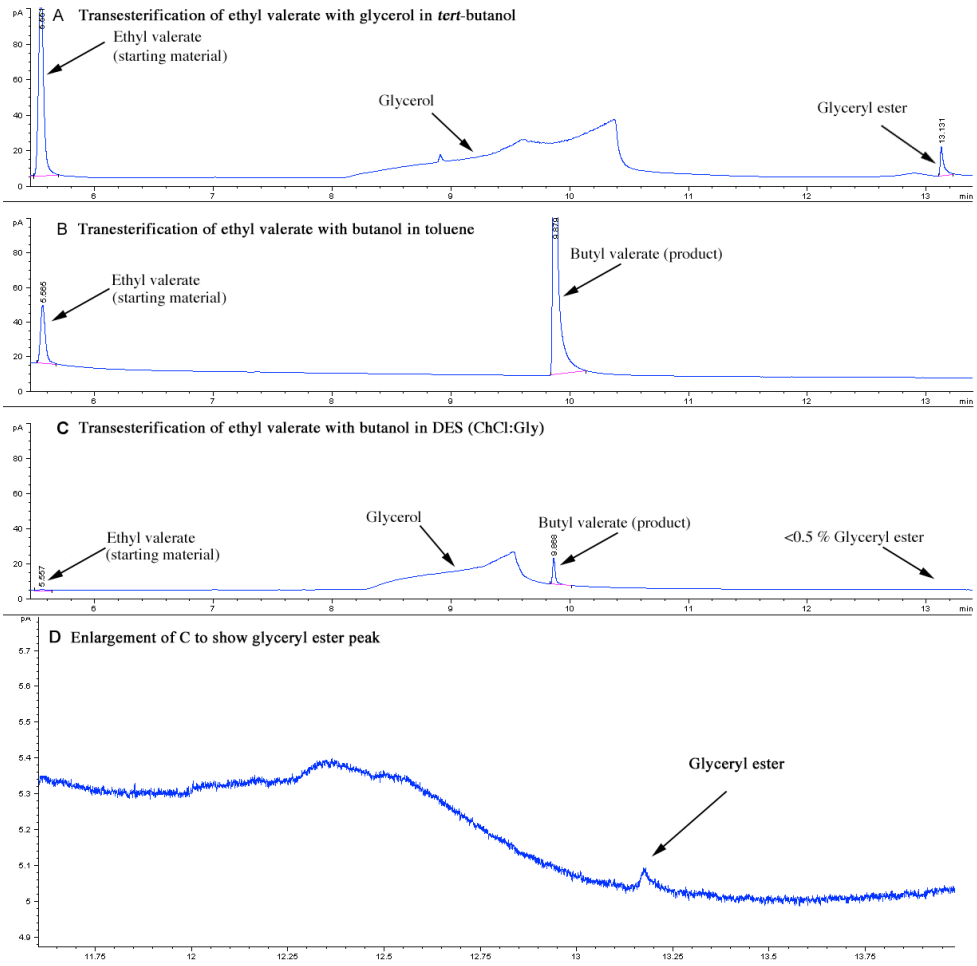


Figure 2. GC traces of iCALB-catalyzed tranesterifications extracted with *tert*-butanol (1.0 ml) instead of toluene. A: transesterification of glycerol (broad peak beginning at 8.2 min) and ethyl valerate (5.6 min) in *tert*-butanol to give a glyceryl ester of valeric acid (13.1 min). B: transesterification of ethyl valerate (5.6 min) with butanol (not shown) to form butyl valerate (9.9 min) in toluene. C, D: the same transesterification as B done in ChCl:Gly; the glyceryl ester peak accounts for <0.5% of conversion (peak shown in D). No other products were observed at longer times in any of the reactions. The broadness and differences in the glycerol peak were typical - depending on the glycerol concentration, the broad peak would begin around 8.2 min and continue up to 10.5 min.

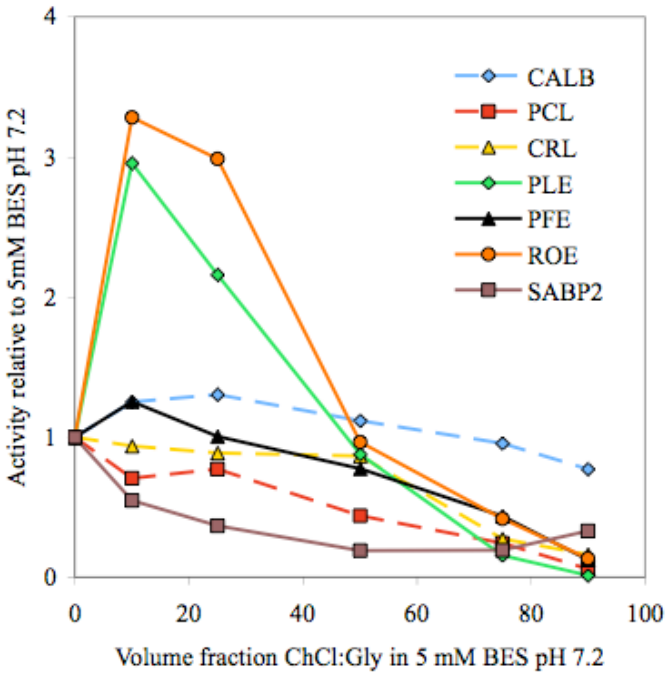


Figure 3. Hydrolytic activity of *p*-nitrophenyl acetate (330 μM) by lipases and esterases (0.05 mg ml^{-1}) at room temperature at different concentrations of ChCl:Gly in 5 mM BES buffer at pH 7.2. Activity is relative to the initial rate in the buffer only. Points are averages of quadruplicate runs and represent activity above the background reaction. Error was typically less than five percent.

CHAPTER 4. Deep eutectic solvents as media for *Candida antarctica* lipase B-catalyzed reactions

4.1 Chapter Overview

Many deep eutectic solvents (DESs) 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, DESs are polar, viscous solvents with low vapor pressure and flammability. However, synthesis of DESs 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 DESs can include protein denaturants like urea, we found that immobilized *Candida antarctica* lipase B retains activity in a wide range of DESs. 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. DESs containing sugars were highly viscous and required temperatures >60 °C to permit stirring. This chapter is in press and will appear as: J. T. Gorke, F. Srienc, R. J. Kazlauskas, in ACS Symposium Series (American Chemical Society Press, Washington, D.C.), in press (2010).

4.2 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 (DESs) are eutectic mixtures that are liquids at room temperature. Many DESs 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*). DESs are alternatives to ionic liquids as replacements for organic solvents because DESs have low volatility and flammability, high thermal stability and varying the components varies the physical properties of solvent. DESs 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 DESs over ionic liquids are lower cost and lower toxicity. The components of common DESs are inexpensive. Most DESs are mixtures of amine chloride salts and urea, glycerol, or ethylene glycol. Figure 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 DESs 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 DESs. For example, Abbott and coworkers dissolved silver salts the DESs to dip coat copper surfaces with silver without the need for catalysts (*24,25*). Choline chloride-based DESs 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*). DESs containing $ZnCl_2$ in place of the 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 DESs. 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 not choline chloride, but the alkylating agent chlorocholine 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 DESs containing 10 M urea. Second, even though alcohols and amines were reactants in these reactions, the alcohol or amine components of DESs 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.

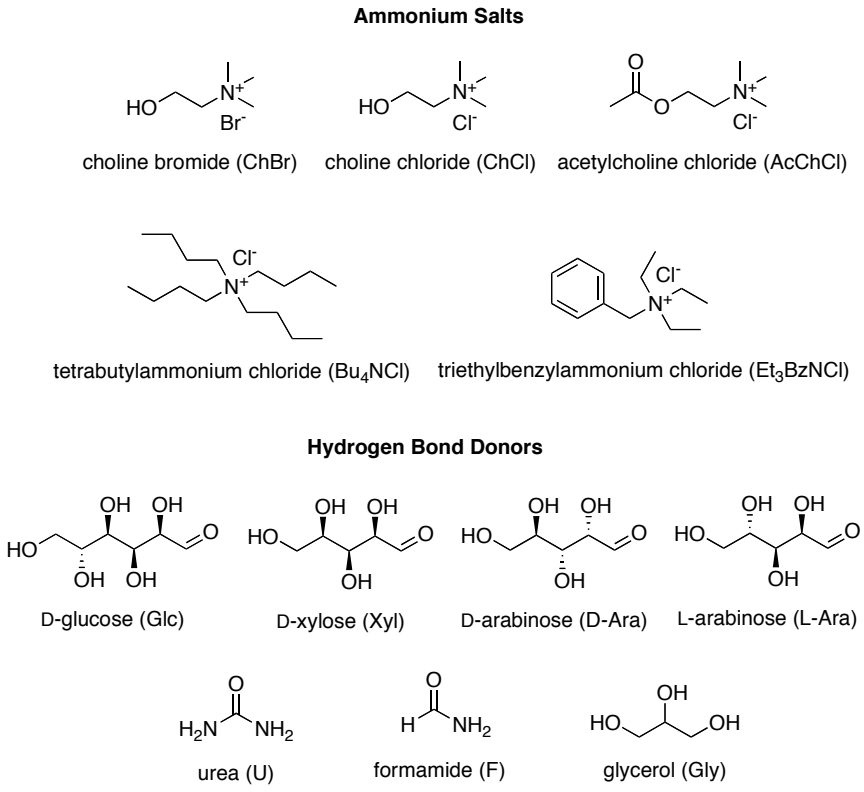


Figure 1. Deep eutectic solvents components in this chapter. Many deep eutectic solvents are 1:1 or 1:2 mixtures of an ammonium salts and a hydrogen bond donor.

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

4.3 Experimental

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 ºC and injector temperature at 250 ºC.

Synthesis of deep eutectic solvents

Ammonium-based DESs were prepared according to Abbott and coworkers (*19*). For amide and glycerol-based DESs, 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 DESs, 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 DESs are very viscous, so they were warmed to 60 °C before use. The zinc-chloride-based DES was prepared by combing zinc chloride (0.050 mol) and urea (0.175 mol).

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 ºC for 15 min (60 °C for 30 min for the sugar-based DESs). 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 ºC was held for 8 min, then increased to 200 ºC at 10 ºC min^{-1} held at 200 ºC for 5 min.

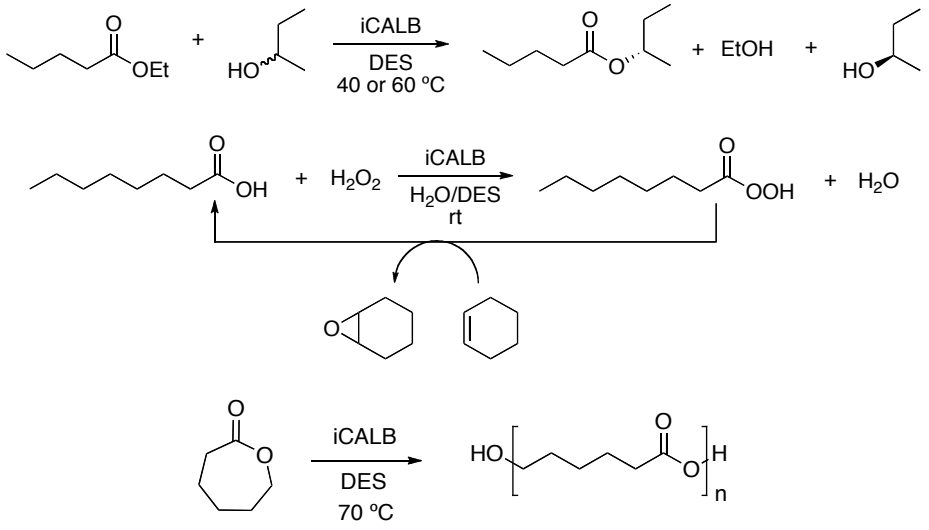


Figure 2. iCALB-catalyzed reactions in DESs. Transesterification of ethyl valerate with 2-butanol in DES (top), perhydrolysis of octanoic acid in water/DES mixtures with the simultaneous epoxidation of cyclohexene (center), and ring-opening polymerization of ε-caprolactone in DES (bottom).

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 diameter and 0.25 μm film thickness). The initial column temperature of 60 ºC was held for 6 min, then increased to 165 ºC at 15 ºC min^{-1}, then further increased to 200 ºC at 25 ºC min^{-1} and held at 200 ºC for 5 min.

Polymerization

ε-Caprolactone (0.10 mL) was added to a suspension of immobilized CALB (iCALB, 3.3 mg of immobilized enzyme preparation) in solvent (0.20 mL) in a glass vial. The vial was incubated at 70 °C for 24 h and the suspension stirred with a magnetic stirrer. To stop the reaction, methanol (1.0 mL) was added 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.

4.4 Results

Wider range of DESs

We previously reported that iCALB was active in DESs 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 DESs and found that iCALB was active in all of them, Figure 1 above, Table 1 below. 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 DESs 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 at 60 °C and 70% loss in the urea solution at 60 °C. 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 DESs was comparable to that in toluene, Table 1. Initial rates were calculated from the conversion of ethyl valerate to 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_3BzNCl:Gly, with initial activities of 990, 640, 580, and 450

mU mg^{-1}, respectively. DESs in which iCALB was less active than in toluene were AcChCl:Gly (410 mU mg^{-1}), $ZnCl_2$:U (260 mU mg^{-1}), Bu_4NCl:Gly (200 mU mg^{-1}), and ChCl:F (150 mU mg^{-1}). These activities are all higher than those in the ionic liquid 1-

Table 1. Initial activity and enantioselectivity of CALB-catalyzed transesterification of ethyl valerate and butanols in DESs.

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 [a]	6.2
BMIM[Tf_2N]	RTIL	1-butanol	400 (*35*)	N/A
BMIM[Tf_2N]	RTIL	2-butanol	140	3.1
AcChCl:Gly	DES	2-butanol	410	5.0
Bu_4NCl: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_3BzNCl:Gly	DES	2-butanol	450	5.2
ChCl:D-Ara	DES	2-butanol	800 [a]	3.7
ChCl:L-Ara	DES	2-butanol	830 [a]	3.2
ChCl:Glc	DES	2-butanol	750 [a]	4.0
ChCl:Xyl	DES	2-butanol	880 [a]	2.8

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

butyl-3-methylimidazolium bis(trifluoromethanesulfonyl)imide (BMIM[TF_2N], 140 mU mg^{-1}), and comparable to those in toluene (430 mU mg^{-1}).

The activity of iCALB was slightly higher in the more viscous sugar-based DESs, likely due to the higher temperature used for these reactions (60 °C instead of 40 °C). The high viscosity of these sugar-based DESs required the higher temperature. iCALB had a higher initial activity in each sugar-based DES than toluene (660 mU mg^{-1}). The activity of iCALB in sugar-based DESs 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 heterogeneous 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_2N] and in DESs. The enantioselectivity in DESs were generally higher than in BMIM[Tf_2N] (3.1), the with Bu_4NCl:Gly (2.8) as the exception. Et_3BzNCl:Gly gave the highest enantioselectivity of any DES (5.2), 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 DESs compared to toluene (E = 6.2 at 60 °C). ChCl:Glc had the highest enantioselectivity of the DESs (4.0), while ChCl:D-Ara (3.7), ChCl:L-Ara (3.2), and ChCl:Xyl (2.8) were marginally lower. The two DESs 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

DESs were comparable to an ionic liquid for an iCALB-catalyzed perhydrolysis, but not as good as acetonitrile, Table 2. 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*).

Table 2. Conversion of cyclohexene to cyclohexene oxide by peroctanoic acid formed by iCALB-catalyzed perhydrolysis of octanoic acid.

Solvent	*Type of solvent*	*Conversion (%)*
MeCN	Organic	79
BMIM[BF_4]	RTIL	15
ChCl:Gly	DES	22
ChCl:U	DES	8

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.

Polymerization

iCALB also catalyzed ring-opening polymerization of ε-caprolactone in four DESs. We avoided DESs containing hydroxyl groups to prevent side reactions. Trace water

bound to the immobilized enzyme preparation initiated the polymerization. Similar amounts of polymer precipitate formed in AcChCl:U and Bu_4NCl:U as in toluene. A smaller amount of polymer precipitate formed in ChCl:U, and ChCl:F, but none in $ZnCl_2$:U.

4.5 Discussion

CALB is active in a wide variety of DESs. We have expanded the range of DESs 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. iCALB 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, Figure 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 reacts with the ethyl ammonium component to make ethyl amine, resulting in significant amounts of ethyl amides instead of the desired amide (*35*).

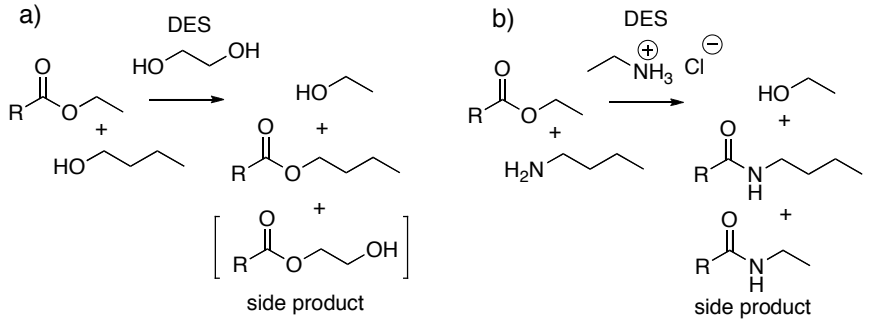


Figure 3. Two side reactions of deep eutectic solvents in lipase-catalyzed reactions. a) The ethylene glycol component in DES competes with 1-butanol in a CALB-catalyzed transesterification. The brackets indicate that gas chromatography did not detect this presumed side product. DES containing glycerol did not show this side reaction. b) The ethylamine component in DES competes with butylamine in a CALB-catalyzed aminolysis. This side reaction does not occur in transesterification reactions, presumably because the ethylamine remains protonated and unreactive.

In another example, ChCl:EG is not a suitable solvent for transesterifications. Although the reactivity of the ethylene glycol component is reduced in the DES, it remains significant and the side reaction with ethylene glycol accounts for more than half of total conversion of ester.

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

While iCALB typically had comparable and often higher activity in DESs compared to toluene, its enantioselectivity was substantially lower. Although we do not know why,

we can suggest three possibilities. 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 in DES as compared to toluene. 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 possible explanation is that the some catalysis in DESs 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 DESs might catalyze transesterification. However, no product formed without enzyme, so this explanation would require the catalyst to be some type of adduct of DES and enzyme. A third possible explanation is a change in the enzyme conformation in the polar DESs that is distinct from that in a non-polar solvent such as toluene. Support for this second argument 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.

DESs do not appear to be suitable for perhydrolysis reactions as compared to acetonitrile. It is possible that there was too much water present in the reaction for the DESs or the ionic liquid to be effective. In previous work, Seddon's group found CALB activity in BMIM[BF_4] to comparable to in acetonitrile when using a higher weight fraction hydrogen peroxide solution compared to what we used in our study (*9*). The additional water in our study coupled with the very polar ionic liquid and DESs more

readily absorbing the water could have led to a faster rate of hydrolysis of the peracid due to increased presence of water in the vicinity of CALB. In the case of low conversion of ChCl:U, it is also possible that the urea acted as a base-catalyst for the hydrolysis of peracid and further decreased the amount of available peracid for epoxidation.

4.6 Conclusion

CALB is active in a variety of ammonium-based DESs, despite the presence of chlorides and strong hydrogen bond donors such as formamide and urea. CALB is also active in DESs containing sugars such as hydrogen bond donors. Generally, the activity of CALB for tranesterifications in most DESs was comparable to toluene, but the enantioselectivity was lower. DESs appear to be comparable to BMIM[BF_4] as solvents for perhydrolysis, but are inferior to acetonitrile.

CHAPTER 5. Enzymatic synthesis of conducting carotenoid-poly(hydroxy-alkanoate) block copolymers

5.1 Chapter Overview

This chapter involves a chemo-enzymatic synthesis of carotenoid-polyester copolymers. Ionic liquids play a lesser role, but important, than in other chapter: doping with an ionic liquid can cause these polymers to increase their electrical conductivity. This work is mainly a preliminary investigation into these interesting materials, and will serve as a foundation for direct synthesis of the materials in ionic liquids. This synthesis would be advantageous because early results show that our carotenoid materials are more soluble in ionic liquids than in toluene, the solvent of choice in this chapter. Yun Jiang performed reactions on bixin and prepared samples for mass spectrometry. The carotenoid precursors are quite soluble in ionic liquids and deep eutectic solvents compared to in toluene or THF. As a result, polymerization and synthesis in these solvents may make an interesting future study.

Carotenoids are naturally occurring anti-oxidants ubiquitous in nature. They are renowned for their anti-oxidant and medicinal properties, but are often unstable and poorly soluble in various solvents. In an effort to increase the solubility and stability of these carotenoids, we demonstrate the synthesis of poly(caprolactone)-carotenoid-poly(caprolactone) triblock copolymers by enzymatic ring-opening polymerization of ε-caprolactone initiated with a 1,3-propandiol diester of crocetin ranging from M_n ~ 2,000 – 35,000 with a polydispersity index of 1.5-1.8 and yields of 80-99%. The initial polymerization rate was linear with enzyme concentration, and average molecular

weights were close to theoretically expected values. The use of a primary diol as a linker molecule was essential to the polymerization of crocetin. We achieved at least a 100-fold reduction in the resistivity of these polymers when doped with an ionic liquid compared to pure poly(caprolactone), suggesting that carotenoid-containing block copolymers can be conducting. In addition to synthesizing crocetin-based polymers, we have also synthesized bixin- and astaxanthin-based polymers, though their reaction rates were several times slower than for polymerizations with crocetin derivatives.

5.2 Introduction

Carotenoids are a group of natural pigments consisting primarily of unsaturated isoprenoid repeat units (*1-5*). These pigments are abundant in nature and are responsible for the coloration of numerous organisms and have many commercial uses in the food and medical supplement industry. For example, farmed salmon are fed astaxanthin to give them a natural pink color and bixin from annatto seeds gives processed cheese a desirable orange color. Medicinally, β-carotene and other pro-vitamin A carotenoids can aid vision. Crocin from saffron and gardenia fruits has long been known in folk medicine to treat depression and may have anti-cancer indications (review: *6*). Despite these advantages, carotenoids can be unstable and sensitive to light and heat, making chemical modifications or synthesis difficult. Until recently, plants were the only reliable source of carotenoids, which made them expensive. However, Schimdt-Dannert and coworkers (*7*) recently engineered *E. coli* to produce functionalized carotenoids. Our group and others (*8, 9, 10*) have used further metabolic engineering techniques to improve the yield of these pigments, putting lower cost carotenoids within the realm of possibility. With lower

cost carotenoids, it may be possible to use these natural products in a variety of coloring or medicinal applications. However, the stability, solubility , or mechanical properties of these materials may not be optimal. It would be desirable to incorporate these materials into polymers to alter the physical properties and solubility of carotenoids and improve their stability through covalent bonds with materials that have desirable properties.

We synthesized block copolymers consisting of blocks of carotenoids and poly(hydroxyalkanoates) (PHAs). Our choice of materials comes from an interest in creating materials that not only could be conducting (polyenic carotenoids) but are also stable, biodegradable, and biocompatible (PHAs). Additionally, linking PHA blocks to carotenoids should enhance the stability of the carotenoids, many of which have poor heat, light, and solvent tolerance. These stabilized materials could be useful as bright color additive in certain commercial plastics and replace the need for multiple paint coatings. Chemical differences between the repeat unit structures of each block could allow phase separation between blocks, leading to a polyester phase and a carotenoid phase (*11*). Bonnet and coworkers showed that a block copolymer consisting of blocks of poly(isoprene) and poly(caprolactone), similar to what a carotenoid-poly(caprolactone) block copolymer could look like, could have ordered microstructure (*12*). To keep reaction conditions as mild as possible, we used a lipase from *Candida antarctica* lipase B (CALB) as the catalyst. This lipase is well known to catalyze the ring-opening of lactones (*13-15*).

In this work, we focused our synthesis on crocetin, a 20-carbon carotenoid diacid commercially available as the gentiobiose diester crocin, Figure 1. This molecule served

as a model for other carotenoids, including one synthesized by our group, 4,4'-diapolycopenediacid (*10*). We produced and characterized copolymers from 1,3-propanediol-functionalized crocetin and ε-caprolactone, Figure 2. We also produced small amounts of polyesters using modified bixin to test the steric effects of the carotenoid, and using unmodified astaxanthin to evaluate the use of alcohol-based carotenoids in the polymerization.

Results

Determination of carotenoid target

One key challenge in achieving this polymerization was the difficulty in obtaining a carotenoid precursor that could initiate a CALB-catalyzed polymerization. We determined that a chemical synthesis of crocetin derivatives from crocin would be best suited for enzymatic polymerization. Free and immobilized BCL, free and immobilized CALB, CALA, and immobilized PAL, common commercial enzymes, did not catalyze the acylation of the gentiobiose moieties of crocin with ethyl succinate or divinyl adipate, nor were these enzymes able to hydrolyze crocin directly to crocetin with water, effectively eliminating the possibility of doing a direct polymerization on the commercial available molecule. Two enzymes, wheat germ lipase and feruloyl esterase, catalyzed the hydrolysis of crocin to crocetin in water, but these enzymes require significant water activity in organic solvents (*16,17*). Further, none of the enzymes were produced polymers when crocetin was used as the initiator. Because potassium hydroxide-catalyzed transesterification of crocin to crocetin esters is known (*18*), we investigated the synthesis of hydroxyl-functionalized crocetin diesters that lipases could more easily

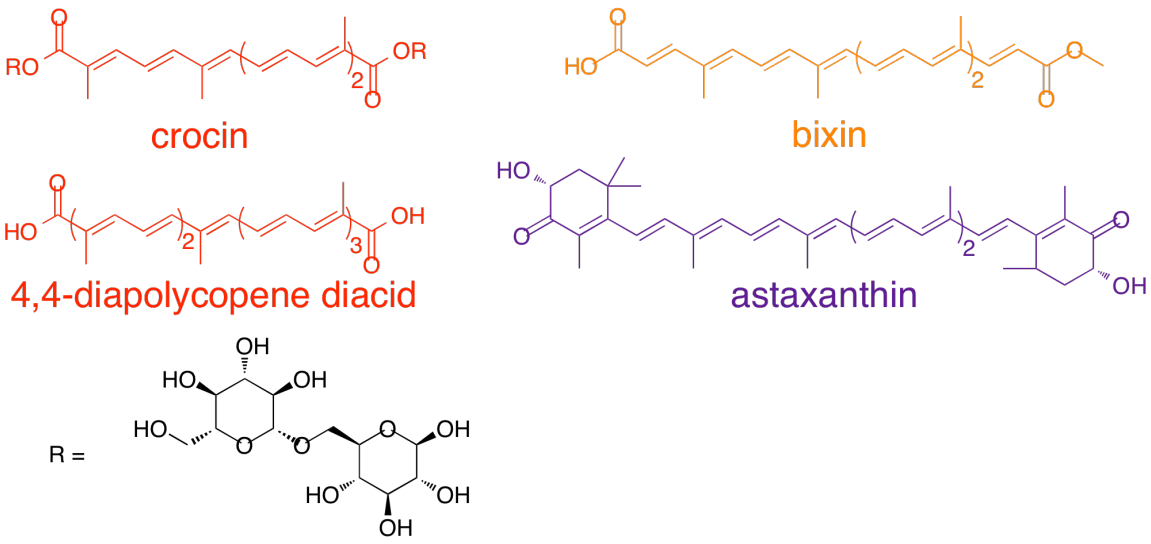


Figure 1. Carotenoids of interest in this chapter. Crocin, a gentiobiose diester (R = gentiobiose) of crocetin (R=H), was the model carotenoid on which the work was based. The other carotenoids have either hydroxyl, acid, or ester moieties on which we can perform similar chemistry. Colors represent the approximate color of the carotenoid in pure form.

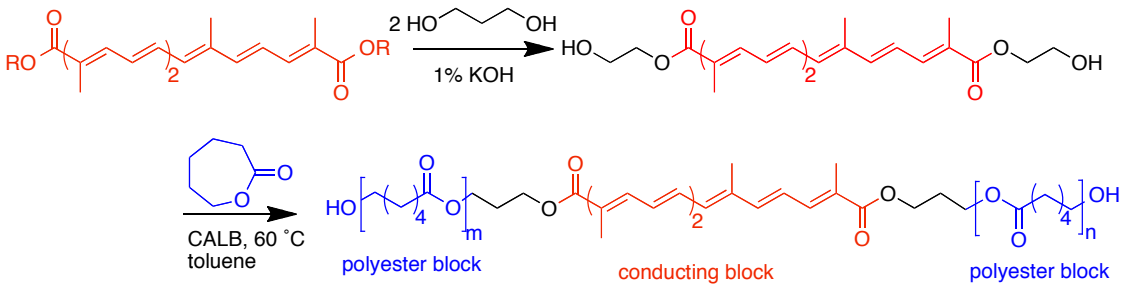


Figure 2. Synthesis of diblock polymer from a carotenoid ester using 1,3-propandiol-linked crocetin and poly(caprolactone). CALB easily catalyzed production of these polymers, but did not accept other modifications of crocetin as a substrate.

accommodate as initiators for ring-opening polymerization. To validate the esterification, we first synthesized dimethyl esters of crocetin from commercially available crocin, a gentiobiose diester using the procedure of Tarantilis and colleagues (*18*). After verifying the dimethyl ester as the sole product after extraction by mass spectrometry (m^+/z = 357), we attempted to use the molecule to initiate ring-opening polymerization, but observed no reaction with iCALB as the catalyst.

Polymerization attempt with ethylene glycol esters

We next attempted to find an appropriate molecule that would both initiate the polymerization and fit inside the active site of CALB. We synthesized ethylene glycol mono- and diesters of crocetin, though they did initiate polymer production. The synthesis of the esters resulted in a substantial amount of crocetin and mono-ester formation with the diester as the minor product (~10% total yield of mono- and diester), likely due to the presence of water in the stock ethylene glycol. After several extractions of the products with toluene and dichloromethane to eliminate any remaining water, we used a toluene solution of the mono- and diesters (200 μl of 0.4 mg ml^{-1} solution) as the solvent for ring-opening polymerization of ε-caprolactone (100 μl). Using immobilized *Candida antarctica* lipase B (iCALB, 4 mg) as the catalyst, we did not find any polymer products by HPLC and GPC. Further, the viscosity of the reaction mixture and the ratio of crocetin to the mono- and diester were essentially unchanged from the starting material.

Substrate selection by molecular modeling

We determined by molecular modeling of CALB that 1,3-propanediol would serve

as a better linker molecule than ethylene glycol. To find an improved initiator substrate, we modeled a tetrahedral intermediate of ring-opened ε-caprolactone (6-hydroxyhexanoic acid) bond to crocetin diol esters of varying lengths in the active site of CALB. In particular, we examined the estimated hydrogen bond distances between the catalytic histidine (H224) and both the catalytic serine (S105) and the ester bond between the diol and the 6-hydroxyhexanoic acid, as well as the hydrogen bonds between the oxyanion hole residues (Q106 and T40) and the oxyanion of the tetrahedral intermediate, Table 1.

Table 1. Estimated hydrogen bond lengths of tetrahedral intermediates of crocetin diol diesters reacting with 6-hydroxyhexanoate in *Candida antarctica* lipase B.

	Estimated Hydrogen Bond Distances (Å)				
Diol	S105-H224	Ester O-H224	Q106-Oxyanion	T40 OH-Oxyanion	T40 N-Oxyanion
Ethylene glycol	**3.09**	2.7	**3.06**	2.61	2.75
1,3-propanediol	2.91	2.77	2.91	2.59	2.83
1,4-butanediol	2.67	**3.03**	2.97	2.59	2.8
cis-1,4-but-2-enediol	2.97	2.66	**3.07**	2.59	2.79
trans-1,4-but-2-enediol	2.92	2.71	**3.01**	2.6	2.8

Values in bold indicate calculated hydrogen bond lengths that may be too long to be favorable. Longer hydrogen bonds are weaker.

The hydrogen bond distance between S105 and H224 appeared distorted (> 3.0 Å) when ethylene glycol was used as a diol. This distortion is possibly due to steric hindrance between the carotenoid ester and the alcohol binding site of the enzyme and could cause the enzyme to assume a less favorable conformation to accommodate the substrate. When longer diols were modeled, the hydrogen bond distance decreased to below 3.0 Å. In the

case of the hydrogen bond between the ester oxygen and H224, only 1,4-butanediol gave a potentially distorted hydrogen bond distance (3.03 Å). With all diols, both hydrogen bonds from T40 to the oxyanion appeared to be favorable, but ethylene glycol and both 1,4-butenediols appeared to distort the hydrogen bond between Q106 and the oxyanion when they were used as linkers. Linkers with even- or odd- numbers of carbons had opposite orientations in the active site, shown for ethylene glycol- and 1,3-propanediol linked crocetin in Figure 3. The 1,3-propanediol appeared to have only favorable

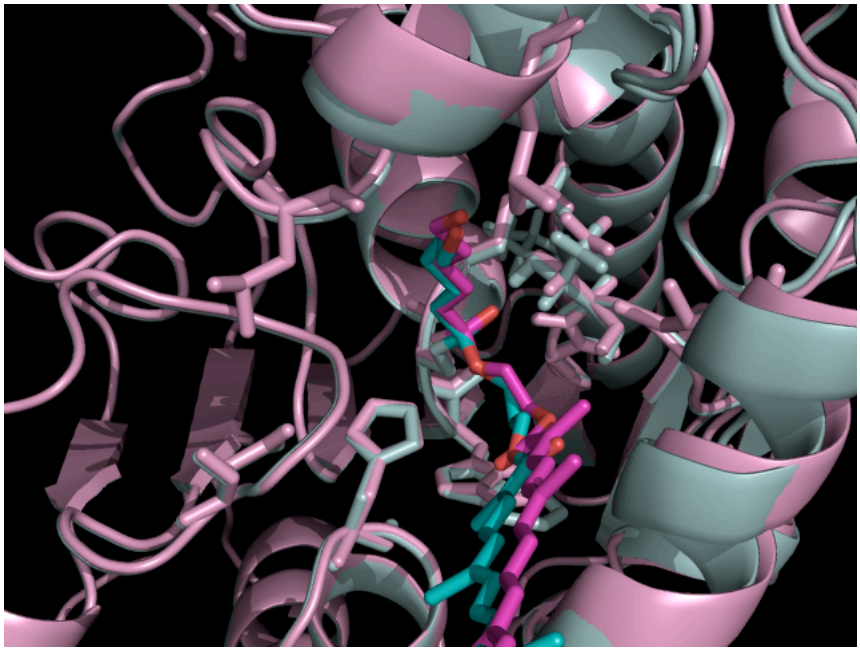


Figure 3. Overlaid calculated active site configurations of CALB with tetrahedral intermediates of ethylene glycol- (magenta) and 1,3-propanediol-linked crocetin (cyan). The orientations of carotenoids functionalized with 2- and 3-carbon diols are rotated 180 degrees from each other.

hydrogen bond distances and we selected it for further study as a linker.

Initial polymerization with 1,3-propanediol esters

We obtained substantial polymer yields (80-99%) with 1,3-propanediol esters of crocetin present. We next attempted to synthesize 1,3-propanediol diesters of crocetin. The diester synthesis resulted in since HPLC peak at 450 nm after extraction (60% yield of diester, no monoester or crocetin detected in final extract) as determined by HPLC. We verified the mass of the diester by ESI LC/MS ($m/z^+ = 380$). We then used a solution of the diesters in toluene (0.4 mg/ml, 500 μl) for ring-opening polymerization of ε-caprolactone (10-250 μl) using iCALB as a catalyst at 60 °C for 20 h, Table 2. In contrast to the ethylene glycol diester-initiated polymerization, we obtained a viscous solution

Table 2. Molecular weight and polymerization rate of trial polymerizations, prior to additional purification of carotenoid diesters.

Monomer concentration (v:v)	Enzyme concentration (mg ml^{-1})	M_n, theoretical (g mol^{-1})†	M_n, observed (g mol^{-1})	Initial rate† (g mol^{-1} hr^{-1})
1:2	5	299,000	25,600	1500
1:3	5	200,000	33,400	1650
1:5	5	119,900	35,000	2200
1:10	2	60,100	22,300	1100
1:10	5	60,100	22,700	2700
1:10	10	60,100	20,800	5300
1:10	20	60,100	19,800	9400
1:20	5	30,300	8,600	2300
1:50	5	12,400	2,400	1200

*: Conditions: 60 ˚C, 18 h, enzyme (*C. antarctica* lipase B), 0.5 ml toluene/carotenoid diester solution (0.4 mg initiator ml^{-1}). Molecular weights are from UV-Vis GPC based on polystyrene standards.

†: Rate of monomer added in first hour of reaction.

after polymerization. After precipitation of the most concentrated reaction mixture with methanol and subsequent drying, we obtained yellow polymers that did not show any trace (detection limit ~ 1 μg ml^{-1}) of starting diester at 450 nm upon HPLC analysis. We then analyzed samples of the reaction mixtures prior to precipitation by UV/Vis-GPC and found single peaks at 450 nm corresponding to M_n (polystyrene equivalent) = 2400 (1:50 lactone:toluene), 8600 (1:20), 22,700 (1:10), 35,000 (1:5), 33,400 (1:3) and 25,600 (1:2). The trial polymers had polydispersities of 1.7, 1.67, 1.62, 1.64, 1.61, and 1.8, respectively. The carotenoid initiators are covalently attached to the polyesters as expected. We compared the GPC traces of a sample of carotenoid-containing poly(caprolactone) with the initiator solution and a blend of the initiator and commercial poly(caprolactone), Figure 4.

Following our successful trial polymerization, we produced and cast films of 1 g polymer samples with ω-pentadecalactone, ε-caprolactone, and a 50:50 mixture of the two lactones under similar conditions of polymer using a 72 h reaction time and only 10 mg of enzyme, Figure 5. The molecular weights determined by GPC were 12,000, 20,000, and 24,000, respectively. Yields were quantitative and the polymers had a PDI of 1.8 or less without fractionation.

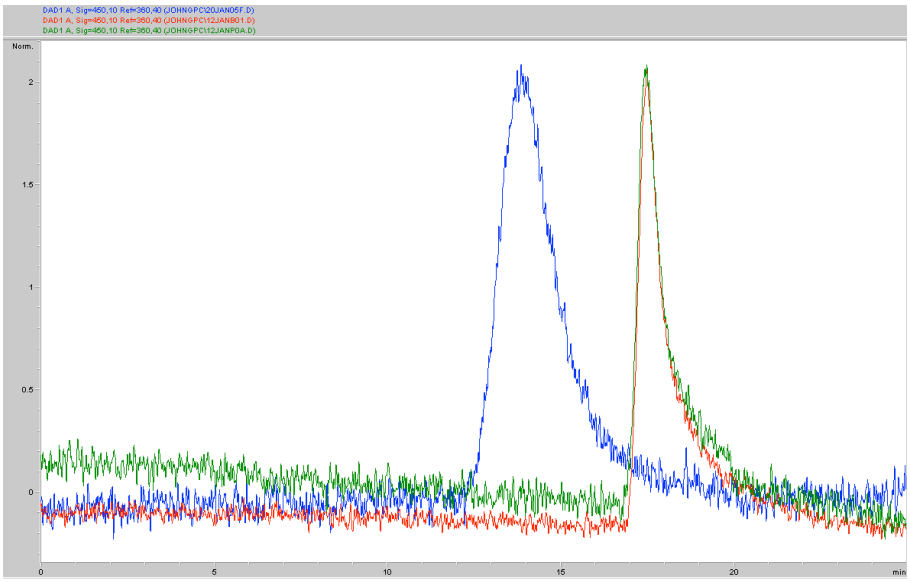


Figure 4. GPC traces indicating covalent attachment of carotenoid and poly(capro-lactone). Blue trace: M_n ~ 20,000 copolymer; red trace: blend of commercial M_n ~ 14,000 poly(caprolactone) and initiator solution; green trace: initiator solution only.

Figure 5. Samples of PHA-crocetin block copolymers. The carotenoid was combined with a long-chain polyester (left), a short-chain polyester (right) and a mixture of the two polyesters (center) via an enzymatic reaction. Even in very low concentrations, the carotenoid gives the polymers a yellow color.

Determination of the presence of impurities

The molecular weights for the initial polymers were lower than theoretically expected for sole initiation by the carotenoid, likely due to the presence of impurities in the carotenoid solution. We determined by HPLC detection at 210 nm that our initial procedure, which required only one wash of the extracted diesters with water, did not completely remove unreacted propanediol from the reaction mixture. To test the impurities' affect on the polymerization, we performed a polymerization at 1:20 ε-caprolactone:toluene using the same initiator and enzyme concentration as in the trial polymerizations. After four hours of reaction (M_n = 7,300), we added an additional equivalent of lactone. The rate of polymerization after this addition was 33% higher (3,100 compared to 2,300 g mol^{-1} hr^{-1}) and the final molecular weight after 20 additional hours of reaction was 30,700, a 3.5-fold increase instead of the anticipated doubling of molecular weight. The added molecular weight accounted (23,400) was 80% of the expected molecular we expected to add, and confirmed that impurities such as 1,3-propanediol could severely affect molecular weight. We prepared subsequent batches of initiator and add two additional washes of the extract with water. These batches did not contain detectable traces of 1,3-propanediol as detected by HPLC.

Kinetics of the crocetin diester-initiated polymerization

The molecular weight of polymers obtained using a purified batch of initiator (1.4 mg ml^{-1} in toluene) closely matched theoretical expectations, Table 3. In calculating a theoretical average molecular weight for the polymers, we assumed the carotenoid-based initiator would be the sole initiator of polymerization, *i.e.*, the solution was essentially

free of water, propanediol, and other impurities. We also assumed complete conversion of the initiator and that polymerization would occur at both ends of the carotenoid. We obtained polymers of M_n = 27,700, 14,000, 6,800, 2,100 for volumetric ratios of monomer to toluene of 1:5, 1:10, 1:20, and 1:50, respectively. The PDIs of these polymers were narrower than for the less purified monomer, all approximately 1.5 to 1.6. With the exception of the polymerizations using the highest concentration and lowest concentration of ε-caprolactone, observed number average molecular weights matched well with expected predictions. Any differences are likely due to chain initiation by trace water on the enzyme. Because poly(caprolactone) initiated by water does not absorb at 450 nm, we did not include these chains in our analysis.

Table 3. Molecular weights from polymerization of ε-caprolactone in toluene/crocetin 1,3-propanediol diester solution after removal of diol impurities.

Monomer concentration (v:v)	M_n, theoretical (g mol^{-1})†	M_n, observed (g mol^{-1})	Percent difference
1:5	29,860	27,700	7.2%
1:10	14,930	14,000	6.2%
1:20	7,465	6,800	8.9%
1:50	2,985	2,100	29.6%

*: Conditions: 60 ˚C, 18 h, 4 mg enzyme (*C. antarctica* lipase B), 1 ml toluene/carotenoid diester solution (1.6 mg initiator ml^{-1}). Molecular weights are from UV-Vis GPC based on polystyrene standards.

†: Average molecular weight expected if all carotenoid initiator reacts and carotenoid is the sole initiator present.

When we examined the kinetics of the polymerization at an initiator concentration (2.0 mg ml^{-1}) that was near the solubility limit in toluene (we could not completely dissolve more than 2 mg ml^{-1} of purified material in toluene). We found that higher enzyme concentration slightly reduced molecular weights, Figure 6. For example, at 1:5 v:v monomer:toluene the final polystyrene equivalent M_n of 21,000, 20,800, and 18,200 for 4, 10, and 20 mg ml^{-1} of iCALB catalyst, respectively. The theoretical M_n is 23,900 under those conditions. We found that the initial rate of polymerization increased approximately linearly with enzyme concentration, though the polymerization went too quickly at the lowest monomer concentrations to differentiate them. At higher enzyme concentrations, the molecular weights tended to be slightly lower than at the lowest enzyme concentration, likely due to more available trace water on the enzymes.

Properties of block copolymers

The bulk properties of the block copolymers were similar to commercial poly(caprolactone). The melting temperature T_m of a M_n ~ 20,000 carotenoid-poly(caprolactone) copolymer was 56 ˚C as determined by differential scanning calorimetry. The glass transition temperature T_g was -63 ˚C. These values were similar to accepted values for commercial caprolactone (T_m ~ 56 to 65 ˚C and T_g ~ -65 to -60 ˚C) (*19*). Qualitatively, the polymer films were as brittle as films cast from the homopolymer, breaking almost immediately when squeezed between two fingers. However, crocetin diester-containing films, which were bright yellow when cast, remained bright yellow after being exposed to laboratory lighting for over 18 months. By contrast, crocin has a

half-life of color strength in aqueous solution of 31 days at 4 ˚C without exposure to light (*20*). When exposed to light at room temperature, the half-life drops to 10 h (*21*).

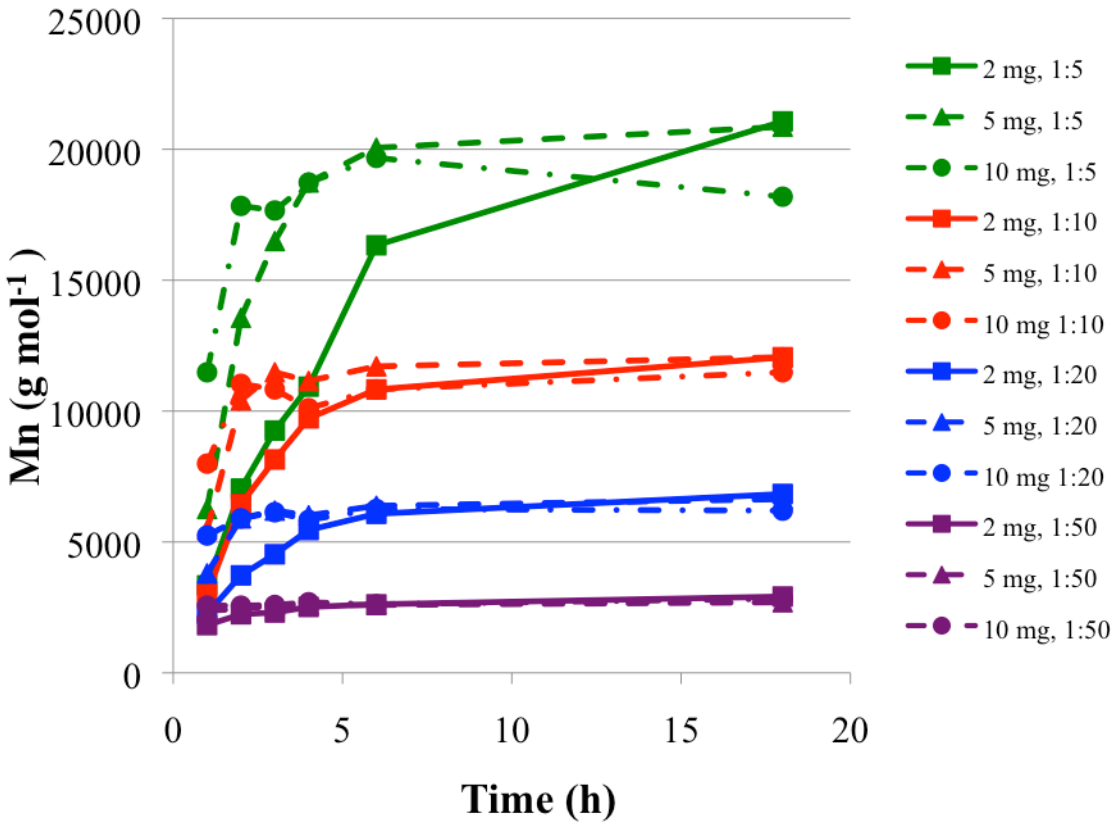


Figure 6. Time course of polymerization with high concentration of carotenoid initiator. Conditions: 60 ˚C, 18 h, 2-10 mg enzyme (*C. antarctica* lipase B), 0.5 ml toluene/carotenoid diester solution (2.0 mg initiator ml^{-1}), 1:5 to 1:50 v:v ε-caprolactone to initiator solution. Molecular weights are from UV-Vis GPC based on polystyrene standards. Varying monomer content predictably controls the molecular weight. Higher concentrations of enzymes can slightly lower molecular weights.

Doping the carotenoid-poly(caprolactone) copolymer with the ionic liquid BMIM[Tf_2N] resulting in at least a 300-fold decrease in the resistivity of the material compared to undoped copolymer and doped poly(caprolactone) homopolymer. We measured the resistivity of chloroform-cast films of the carotenoid-containing copolymers using a multimeter. By itself, the copolymer had resistivity above the detectable threshold of the meter (>3 x 10^6 Ω·m), as did commercial poly(caprolactone). As a dopant, we added BMIM[Tf_2N] (1 μl 100 mg polymer^{-1}) to the chloroform during casting. We subsequently washed the film repeatedly with methanol, and after, drying the film under air at room temperature overnight, we measured a resistivity of 1 x 10^4 Ω·m. Repeating the same procedure for poly(caprolactone) homopolymer resulting in no change in the resistivity of the material.

Synthesis of other copolymers

We also synthesized poly(caprolactone)-carotenoid copolymers based on bixin (*22*), but the reactions were considerably slower than for the crocetin diesters, Table 4. We prepared dipropyl, PEG(400), and 1,10-decanediol esters of bixin enzymatically and confirmed the masses of the diesters by mass spectrometry. We obtained poly(caprolactone) polymers using these bixin derivatives as initiators, though the molecular weights were relatively low (M_n 10,600-12,900) compared to the crocetin diester reactions and took up to 6 days to obtain these molecular weights. In contrast to crocin, CALB catalyzed hydrolysis of bixin (*22*).

Table 4. Formation of bixin-based copolymers.

Initiator	Yield (%)	M_n, observed (g mol^{-1})	Reaction time (days)
Bixin dipropyl ester	82	10,600	6
Bixin-PEG400	70	12,900	3
Bixin-1,10-decanediol	70	11,100	6

*: Conditions: 60 ˚C, 0.9 mg bixin basis of initiator, 10 mg iCALB, 2 ml toluene, 0.4 ml ε-caprolactone.

Astaxanthin, a dihydroxyl-functionalized carotenoid and known substrate for CALB (*23*), was also an initiator for the polymerization. The rate of polymerization was considerably less than for the crocetin derivatives. After 3 days of reaction at 60 ˚C in 1 ml of toluene with 500 μl ε-caprolactone and 4 mg iCALB, only 80% of the astaxanthin (2 mg ml^{-1}) reacted and the resulting polymers were only M_n ~ 10,000. The yield was approximately 20% after precipitation with methanol.

Immobilized BCL (iBCL) catalyzed the oligomerization of crocetin diester and divinyl adipate, Figure 7. We attempted to lengthen the carotenoid block in the copolymer system by synthesizing condensation polymers of the carotenoid diesters and divinyl adipate. We obtained oligomers of approximately M_n = 1,600 (polystyrene equivalent) from condensing a 1.1 : 1 molar ratio of the carotenoid and divinyl adipate (1.2 mg carotenoid basis in 1 ml toluene) using iBCL or iPAL (30 mg ml^{-1}) as a catalyst at 60 ˚C for 24 h. The excess carotenoid was intended to ensure that the oligomers would possess primarily hydroxyl end groups. We removed any condensation products (vinyl alcohol in equilibrium with acetaldehyde) by rotary evaporation before molecular weight analysis. The average molecular weight corresponds to an average 3 carotenoids per

oligomer. CALA and CRL gave oligomers with M_n ~ 1000 (~2 carotenoids per oligomer on average), and CALB did not appear to catalyze the reaction.

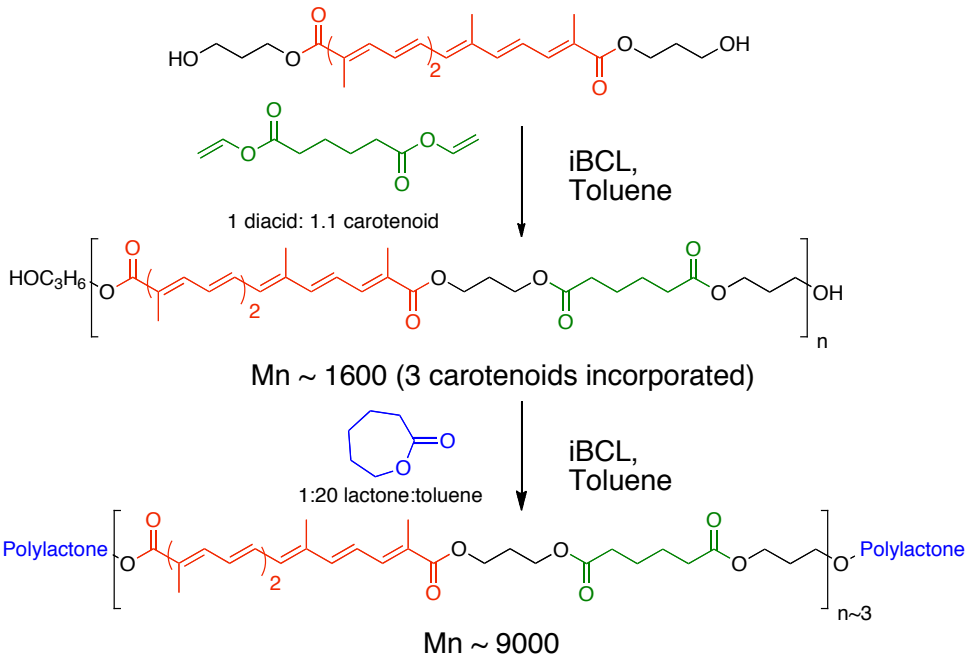


Figure 7. Synthesis of oligomeric carotenoid initiators. The synthesis of the copolymers was in two steps, each requiring new iBCL (30 mg ml^{-1}).

The oligomers did not easily redissolve in toluene, leaving us to seek an alternate route to investigate the oligomers as polymerization initiators. After the oligomerization reaction, we took a sample of the product/toluene mixture (250 μl), placed it in a vial, and exposed it to an air stream for 15 min at 60 ˚C to evaporate most of the volatile products and then added ε-caprolactone (12.5 μl) and new iBCL (7.5 mg) to the mixture and resealed the vial. After 5 days at 60 ˚C for 5 days we obtained polymers of M_n = 9,000 with a yield of 50%. This yield was insufficient to cast a film substantial enough for resistivity testing.

Discussion

Necessity of a linker molecule

Direct acylation of crocin was not a viable reaction, possibly because crocin may inhibit most of the lipases we tested for activity. CALB catalyzes the acylation of gentiobiose moieties of ginsenosides (*24, 25*) with vinyl acetate at the primary hydroxyl of the sugar. However, the enzyme and other lipases did catalyze acylation of those moieties when we used crocin as the substrate. Further, crocin is a known competitive inhibitor of rat pancreatic lipase, and also inhibits gastric lipase (*26*). The molecule could play a similar role in inhibiting many of the lipases we examined during hydrolysis and acetylation studies.

Steric effects of the carotenoid in the enzyme active site likely prevent CALB from catalyzing polymerization with crocetin or dimethylcrocetin. While CALB can, albeit slowly, use bixin as an initiator for polymerization, it did not accept dimethylcrocetin as a substrate. This difference suggests that structural differences between bixin and crocetin are critical for activity. In particular, the methyl groups closest to the carbonyl in crocetin are in the α-position, much closer to the carboxyl group than in bixin (γ-position). When Wahlberg and coworkers examined the polymerization of ε-caprolactone and D,L-lactide in varying proportions, they observed lower initial rates of polymerization and lower molecular weights compared to production of a poly(caprolactone) homopolymer (*27*), even at a 10:1 lactone:lactide ratio. After one week at the 10:1 ratio, only half of the lactone had reacted compared to a complete reaction in the homopolymer-forming system. Further, the authors detected negligible mono-lactate incorporation into the

polymers after 1 week of reaction, meaning that transesterification of the lactide dimer with ε-caprolactone was a very slow reaction. Taken together, these observations suggest it is possible that the α-methyl group of the lactide played a role in slowing the polymerization, and that the α-methyl group of crocin could play a similar role in hindering hydrolysis.

Adding a 1,3-propanediol linker to crocetin was the only viable solution for producing copolymers. We based our use of a linker on the work of Dai and Li (*28*), who used an ethylene glycol-linked poly(β-hydroxybutyrate) (PHB) to initiate ring-opening polymerization of ε-caprolactone. However, the steric effects of the carotenoid α-methyl group combined with a rigid conjugated structure likely prevented CALB from using ethylene glycol-linked crocetin as a polymerization initiator. In the case of the PHB reactions, the linked polymer contains β-methyl groups, and the linker places them on the sixth backbone atom from the hydroxyl. In the case of our crocetin ethylene glycol esters, this methyl group was on the fifth carbon from the reacting hydroxyl group, which was less favorable for hydrogen bonding based on our *in silico* measurements than the 1,3-propanediol, which put the methyl group six carbons from the hydroxyl as with the PHB esters. The 180-degree flip in orientation of the carotenoid substituent could also have a profound impact on the accommodation of the substrate in the active site.

Limitations of polymerization

In the case of the highest monomer concentration, we observed a ceiling between ~20,000 and ~25,000 molecular weight regardless of initiator concentration. This upper limit is very likely due to viscosity limiting the reaction rate, as at lower concentrations of

lactone we were able to attain number-average molecular weights of up to 35,000. At every concentration, we observed a lower than expected molecular weight despite purification efforts, which is likely due to the presence of water bound to the enzyme catalysts (*29*). This bound water, as well as alcohol impurities such as 1,3-propanediol, can initiate and randomly cleave chains, resulting in lower molecular weights and potentially higher polydispersities. It is likely that removing any trace 1,3-propanediol from the system moderately reduced the polydispersity of the polymers by limiting random chain scission.

Crocetin-based polymer properties

The copolymer properties are within the accepted ranges of the polyester homopolymer, but may possess sufficient continuous-phase microstructure to account for the decreased electrical resistivity of the polymers. Carotenoids are structurally similar to isoprene oligomers, albeit the carotenoids have conjugated backbones. Bonnet and coworkers recently designed and described a microstructured poly(isoprene)-*block*-poly(caprolactone) system (*12*). Their system is evidence that the blocks in carotenoid-polyester could be sufficiently different chemically to promote a polymer microstructure. Some experimental evidence of this structure is in the reduction in resistivity of the doped carotenoid block copolymers compared to the homopolymer. Despite a drop in resistivity caused by ionic liquid doping, the resistivity of the polymers is still likely too high for the polymers to be useful as conducting polymers. However, it is likely that increasing the carotenoid or ionic liquid concentration in the system would enhance conductivity.

The role of the ionic liquid as a dopant is to enhance electron transport and improve the electronic properties of the π-conjugated system (*30, 31*). Rodriques and coworkers determined that interaction between the π-system of the polymer and the imidazolium cation were the primary mechanism of this enhancement (*32*). In the case of polyaniline, the ionic liquid acted as a Lewis acid (*32*). Forsyth and coworkers speculated that in polypyrrole, oxidation and reduction of the polymer occurred from intercalation and deintercalation of the ions with the π-system (*30*). It is likely that the ionic liquid in our system plays a similar role, interacting with the conjugated backbone of the carotenoid.

Another interesting property of the copolymers was lasting coloration. This color requires only a small proportion of carotenoid (<1 wt%), providing for potential synthesis of stable, colored materials that would not require as many coatings to provide a stable color. Because the polymer bulk properties are largely dictated by the added polyester, these colored materials could be useful in areas such fibers, thermoplastics, and commercial plastics that require color. While our materials primarily consist of poly(caprolactone), CALB and other lipases can produce a wide variety of polyesters (review: *33*). The range of colors we have produced is currently limited to some natural colors, but synthetic or modified carotenoids could provide a range of possible colors, such as those already engineered by Schmidt-Dannert's group (*34*).

Conclusion

It is remarkable that even with less than 1 wt% carotenoid, the polymers were able to obtain a noticeable increase in conductivity. We must note that despite the improvement, the resistivity of our material is quite high and would require several more orders of

magnitude increase in conductivity to compare to more common conducting polymers (undoped silicon has a resistivity of ~1 x 10^2 Ω·m and polyaniline has a resistivity of ~3 x 10^2 Ω·m).

Experimental

Reagents

Crocin from gardenia fruits was purchased from TCI America (Portland, OR) and potassium hydroxide was purchased from Fisher Scientific (Pittsburgh, PA). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO) and were used as received.

Enzymes

Immobilized *Candida antarctica* lipase B (Novozyme 435, iCALB), immobilized *Burkholderia cepacia* lipase (Lipase PSC-II, iBCL), wheat germ lipase, and *Candida rugosa* lipase (CRL) were purchased from Sigma-Aldrich. Immobilized *Pseudomonas aeroginosa* lipase (Toyobo LIP-300, iPAL), free *B. cepacia* lipase (Amano Lipase PS, BCL), *Candida antarctica* lipase A (Roche Chirazyme L-5, CALA), and free *Candida antarctica* lipase B (Roche Chirazyme L-2, CALB) were gifts from Altus Biologics (Cambridge, MA). Feruloyl esterase (Depol 740 L) was gift from Biocatalysts (Cardiff, UK).

Hydrolysis and acetylation of crocin

Crocin (1 mg) was dissolved in BES buffer (1 ml of pH 7.2, 10 mM) to form an orange solution. Enzyme (1 mg) was placed in a 2 ml vial and the crocin solution (0.2 ml) was added to begin the reaction. Vials were shaken at 600 RPM at 45 ˚C for 1 h. Following the reaction, acetonitrile (1.2 ml) was added to vial and the vial was shaken

vigorously to ensure good mixing. An aliquot (10 μl) was taken and added to a 1:1 v:v mixture of water and acetonitrile (0.5 ml). The resulting solution was used for HPLC analysis to detect the presence of crocetin.

For the acetylation reaction, crocin (1 mg) and enzyme (1 mg) were added to a 2 ml vial. Divinyl adipate or ethyl succinate (0.2 ml) was added to start the reaction, which was shaken at 600 RPM at 60 ˚C for 24 h. An aliquot of the final reaction mixture (10 μl) was added to a 1:1 v:v mixture of water and acetonitrile (0.5 ml). The resulting solution was used for HPLC analysis to detect the presence of acylation products.

Synthesis of crocetin dimethyl diester

Crocin (50 mg, 0.51 mmol) was added to methanol containing 1 w/v% potassium hydroxide in a 2 ml amber glass vial. The mixture was stirred for 90 minutes at 40 °C in the dark. The reaction mixture was transferred to a 20-ml amber vial and extracted with dichloromethane (2 washes of 5 ml). The combined extract was dried by rotary evaporation at room temperature in an aluminum-foil covered round-bottom flask. The resulting mixture (12.5 mg, 50% yield) was dissolved in toluene (10 ml) and stored in an amber vial at 4 °C. The identity of the product was confirmed by ESI-LC/MS.

Synthesis of crocetin ethylene glycol diester

Ethylene glycol was dried over sodium sulfate prior to use. Crocin (50 mg, 0.51 mmol) was added to ethylene glycol containing 1 w/v% potassium hydroxide in a 2 ml amber glass vial. The mixture was stirred for 90 minutes at 40 °C in the dark. The reaction mixture was transferred to a 20-ml amber vial and extracted with toluene (2 washes of 5 ml). The combined extract was dried by rotary evaporation at room

temperature in an aluminum-foil covered round-bottom flask. The resulting mixture (2.4 mg, 10% yield) was dissolved in toluene (10 ml) stored in an amber vial at 4 °C.

Synthesis of crocetin 1,3-propanediol diester

In a typical synthesis, crocin (500 mg, 0.51 mmol) was added to 1,3-propanediol (5 ml) containing 1 w/v% potassium hydroxide in a 10 ml amber glass vial. The mixture was stirred for 90 minutes at 40 °C in the dark. The reaction mixture was transferred to a 30-ml amber vial and extracted with dichloromethane (5 washes of 10 ml). The combined extract was added to a 100-ml round bottom flask and washed with distilled water (3 washes of 15 ml), with separation by separatory funnel between each wash. The final organic phase was obtained and dried by rotary evaporation at room temperature in an aluminum foil-covered round-bottom flask. The resulting orange solid (140 mg, 0.31 mmol, 60% yield) was then dissolved in toluene (70 ml) and stored at 4 °C in the dark in a glass media bottle for subsequent analysis. The identity of the product was confirmed by ESI-LC/MS (m+/z = 445).

HPLC analysis of initiator synthesis and hydrolysis products

Toluene solutions of esterification products (10 μl) were diluted into acetonitrile (90 μl) in a 200 μl glass insert and a 20 μl injection of the mixture of was analyzed on an Agilent 1100 Series HPLC with diode array detector at 210 and 450 nm and an ACE 5 C18 column (250 x 4.6 mm plus guard column) to identify starting materials and products. The mobile phase had a flow rate of 1 ml min^{-1} and consisted of a combination of two eluents, water with 10% methanol and 0.5% acetic acid (A) and acetonitrile. The 30 min elution program was as follows: 65% A from 0 to 3 min, gradient from 65% A to

0% A from 3 to 18 min, hold at 0% A from 18 to 21 min, gradient back to 65% A from 21 to 23 min, hold at 65% A from 23 to 30 min. The retention times of important reagents were: 1,3-propandeiol, 6.2 min; crocetin, 13.2, toluene, 13.6 min, min; mono-ethylene glycol crocetin, 14.1 min; di-ethylene glycol crocetin, 15.5 min; di-1,3-propanediol crocetin, 15.7 min. Polymers were dissolved directly in acetonitrile. The polymer formed when di-1,3-propanediol crocetin was used as initiator had a retention time of 16.3 min. For hydrolysis product analysis, a 20 μl injection of the solution described above was used using the same method.

Synthesis of oligomeric carotenoids

In a typical synthesis, carotenoid 1,3-propanediol diester (1.2 mg, 2.7 μmol) and divinyl adipic acid (2.5 μmol) were dissolved in toluene (1 ml) in a 2-ml amber vial. Enzyme (30 mg ml^{-1}) was added to begin the reaction. Vials were incubated with magnetic stirring at 60 ˚C for 24 h, after which the enzyme was filtered and volatile products were removed by rotary evaporation. A small sample (0.1 mg) of the resulting orange solid was added to THF (500 μl) for molecular weight analysis. When the products were to be used for polymer synthesis, a sample of the reaction mixture (250 μl) was taken after enzyme filtration, kept at 60 ˚C, and exposed to a dry air stream for 15 min to remove any volatile products.

Synthesis of bixin derivatives

In a typical synthesis, bixin (1 mg), CALB (20 mg), and alcohol or diol (20 μl) were added to a 2 ml reaction vial containing 0.5 ml toluene. The mixture was shaken at 800 RPM for 6 days at 60 ˚C to afford diester products. The reaction mixture was cooled to

room temperature and enzyme was filtered through cotton. Excess PEG(400) was removed by mixing with deionized water saturated with potassium chloride and extracting the organic layer. Propanol was removed by evaporating with an air stream. 1,10-Decanediol was removed by along with enzyme during filtration, as it is a solid and practically insoluble in toluene at room temperature. Samples of the reaction mixture were added to acetonitrile for HPLC analysis as described above, or used as part of the initiator solution for bixin polymerization.

Polymer synthesis

Toluene (200-1000 μl) containing crocetin diester (0.4-2.0 mg ml^{-1}) was added to iCALB (4-20 mg ml^{-1}) in a 2 ml reaction vial. ε-caprolactone, ω-pentadecalactone, or a 1:1 mixture thereof (4-100 μl) was then added to start the reaction and vial was sealed and incubated at 60 °C for 18 h in a shaker at 1000 rpm. As needed, aliquots of the reaction mixture (5 μl) was taken. After the final desired time point (typically 18 h) the enzyme was removed by filtration. The aliquot was diluted into HPLC grade THF (500 μl) for GPC analysis. Methanol (1 ml) was added to the remainder to quench the reaction and precipitate the polymer. The vials were then stored at 4 °C for one hour to complete precipitation, after which the polymer was removed by filtration and air dried at room temperature. In the case of polymerization using astaxanthin, bixin derivatives, or oligomeric carotenoids, the reactions were followed for as much as 6 days. For synthesis with oligomer carotenoids, we used a sample of the oligomerization reaction product in toluene (250 μl), ε-caprolactone (12.5 μl), and iBCL (30 mg ml^{-1}) for the reaction.

Film casting and resistivity measurement

We dissolved the precipitated, dry polymers in chloroform (10 ml g polymer^{-1}) and incubated them at 60˚ C for 1 h to fully dissolve the polymer. We then poured the chloroform-polymer mixtures into glass petri dishes on a hot plate at 60 degrees and allowed the chloroform to evaporate for 4 h, during which time the polymers remained molten and evenly distributed in the dishes. We then shut off the hot plate and allowed the polymers to solidify for 24 h. The films were then removed and used for subsequent analysis. In the case of doped films used for resistivity measurements, the ionic liquid 1-butyl-3-methylimidazolium bis(trifluoromethane)sulfonimide (BMIM[Tf_2N], 10 μl g polymer^{-1}) was added to the chloroform prior to dissolving the polymers. After casting, these polymers were washed at least 5 times with methanol to remove any ionic liquid on the surface of the polymer and air dried for an additional 24 h.

To test the resistivity of the films, several samples of the film (~5 mm x 5 mm x 2 mm) were taken and were measured with a caliper. The resistance of the film between several points was measured with a Radioshack Micronta digital multimeter (Fort Worth, TX) and the resistivity was calculated based on the dimensions of the film and the average resistance measurement.

UV/Vis-GPC Analysis

Analysis was carried out using the HPLC system described earlier with a Polymer Labs PLgel MIXED-C 5 μm column (300 x 7.5 mm) with guard column and THF as the eluent. Colored polymers were detected at 450 nm and standards were detected at 278

nm. Polystyrene Calibration Kit S-M-10 was used to estimate polystyrene-equivalent molecular weights.

Differential scanning calorimetry

Differential scanning calorimetry (DSC) was performed on a Thermal Analysis (New Castle, DE) Q1000 instrument with autosampler. Samples (5-10 mg film) were placed in a hermetic aluminum DSC pan, heated to 100 ˚C at a rate of 10 ˚C min^{-1}, cooled to -100 ˚C, then heated again at a rate of 10 ˚C min^{-1} to 200 ˚C. The reported transition temperatures were acquired during the final heating step.

Molecular modeling

The crystal structure of *Candida antarctica* lipase B (1TCA, *35*) was downloaded from the Protein Databank (www.pdb.org) and entered into the Maestro molecular modeling package of Schrödinger (www.schrodinger.com). All minimization was done using the OPLS 2005 force field with a constant dielectric of 1.0 and the PCGR minimization algorithm. Hydrogens were first added to the structure and minimized with other elements fixed. Next, a tetrahedral intermediate of butyl butyrate bound to Ser105 was built and minimized to ensure appropriate active site hydrogen bond lengths. The butyrate was extended to 6-hydroxyhexanoate and the structure was again minimized. Then, the butanol was converted to either ethylene glycol, 1,3-propanediol, 1,4-butanediol, or either isomer of 1,4-but-2-enediol and each structure was minimized. Finally, crocetin was attached to the diol in increments of four backbone atoms at a time with minimization after each addition. After the final minimization, the hydrogen bond distances were estimated using the software and tabulated using Pymol software (*36*).

CHAPTER 6. Future Directions

6.1 Chapter overview

In this chapter, I outline preliminary work on potential future directions of research. The primary purpose is to provide a starting point for future experiments or projects. In the first section, I explore potential evidence for conformation changes in enzymes based on changes the UV spectra of the enzymes in varying concentration DESs in buffer, as well as examine the activity of CALB at varying water concentrations. The next section concerns using the formation of deep eutectic solvents as a driving force for producing biodiesel by inducing a phase separation. Both processes involve the release of a small molecule (glycerol or ethylene glycol) that can readily hydrogen bond with choline- or ethylammonium chloride. The final section is an assessment of the potential of using DESs as a feed medium for cell culture in which the solvent is the primary nitrogen and carbon source. As an added benefit, the cells in the assessment naturally produce poly(hydroxybutyrate). Dan Rouse of the Srienc group performed the growth studies and microscopy of section 6.4 and I performed the analysis.

6.2 Investigation of hydrolysis activity changes of CALB in DES

Introduction

Deep eutectic solvents (DESs) (*1,2*) are inexpensive, physical mixtures of hydrogen bond donors (e.g., urea or glycerol), and hydrogen bond acceptors (e.g. choline chloride or zinc chloride) at their eutectic point that melt near or below room temperatures. DESs can be considered ionic liquids due to the complexation between hydrogen bond donors and salt ions (*3*), though they are not strictly composed of discreet cation-anion pairs.

Some major advantages of using DESs over traditional ionic liquids are the wide available of DES components in bulk; the low cost of those components; and facile, solvent free, waste free synthesis. Despite being composed of strong hydrogen bond donors or other denaturants, DESs are suitable solvents for biotransformations with hydrolases, and offer comparable stability and activity for lipases compared to apolar organic solvents (*4,5,6*).

Results

One interesting finding in Chapter 3 was that DES concentration could greatly affect enzyme activity in DES-water mixtures. We speculated in Chapter 4 that these changes could be the result of conformational changes to the enzyme. To test this theory, we preformed some preliminary experiments examining changes to the UV spectrum of *Candida antarctica* lipase B (CALB) using several representative additives or cosolvents, Figure 1. There are several clear features that occur among the various plots: a high baseline that corresponds to higher absorbance at low wavelengths (<220 nm), an absorbance peak around 230 nm, and an absorbance valley around 240-260 nm. The initial baseline appears in the spectra for methanol (panel A), guanidine hydrochloride (panel C), and BMIM[DCA] (panel E). The peak appears in the spectra for urea (panel B) and ChCl:Gly (panel D), though smaller relative peaks are present in the other three spectra. The valley occurs in the spectra of guanidine hydrochloride and BMIM[DCA]. In the latter spectrum, the valley appears to shift with concentration. This shift was evident in the spectra of other ionic liquids (not show).

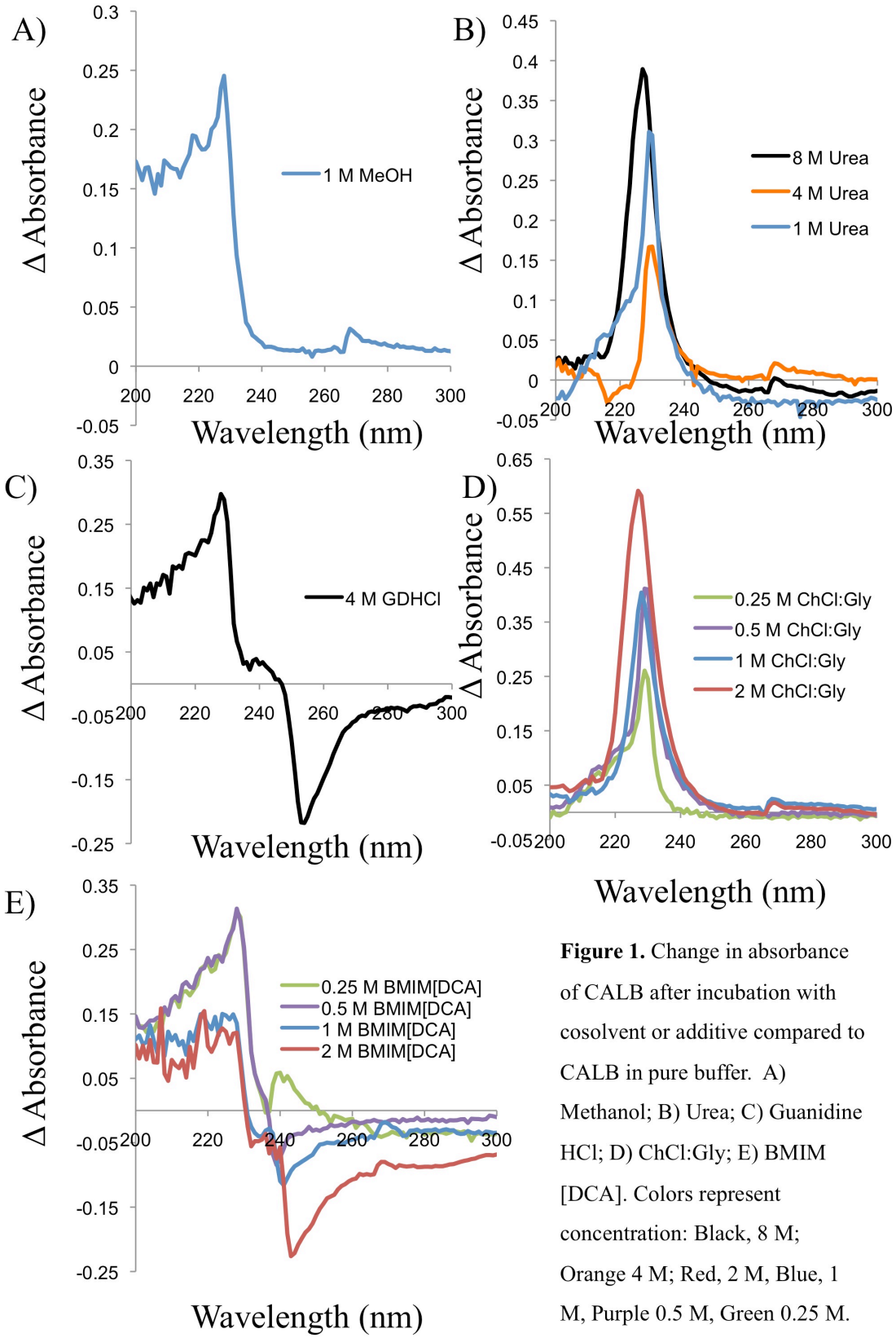


Figure 1. Change in absorbance of CALB after incubation with cosolvent or additive compared to CALB in pure buffer. A) Methanol; B) Urea; C) Guanidine HCl; D) ChCl:Gly; E) BMIM [DCA]. Colors represent concentration: Black, 8 M; Orange 4 M; Red, 2 M, Blue, 1 M, Purple 0.5 M, Green 0.25 M.

The different cosolvents had differing effects on the activity of CALB, Figure 2. One goal of the study was to determine whether the spectral changes could correspond to the activity of the enzyme, so we performed a simple activity assay after incubating the enzyme in cosolvent solutions. We calculated the slope of a best-fit line of relative

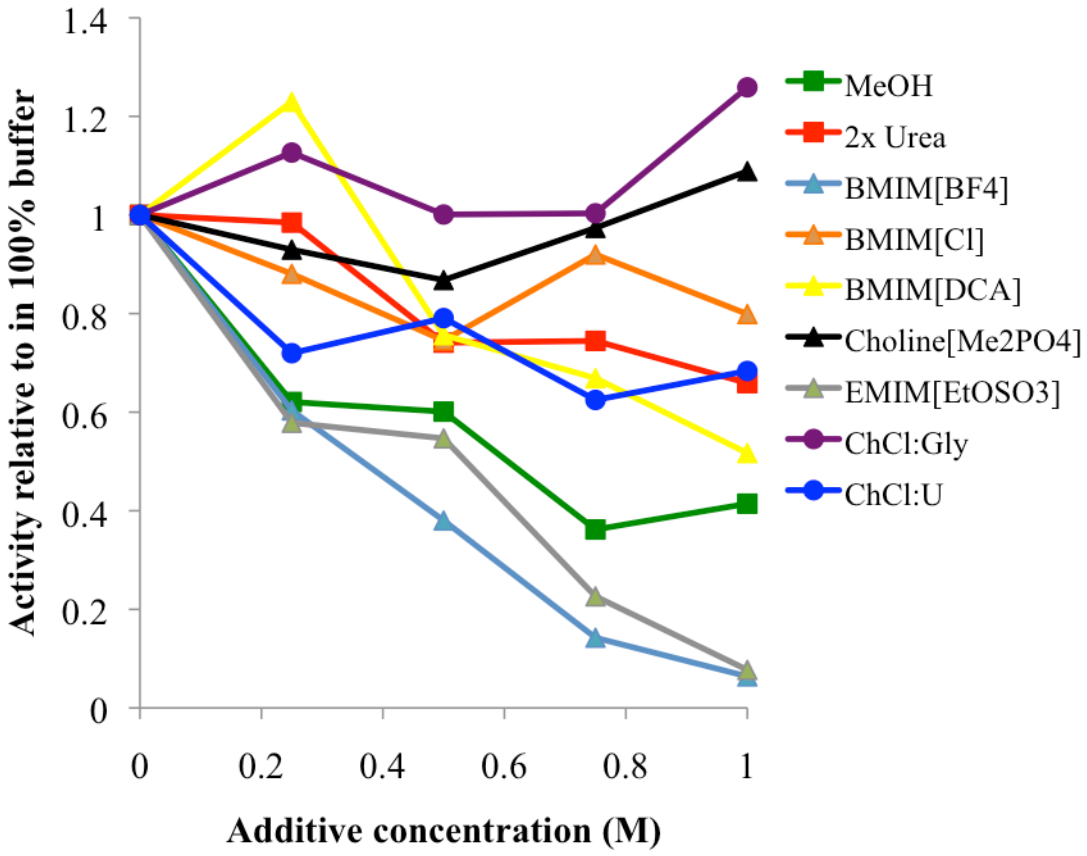


Figure 2. Relative activity of CALB with varying additive concentration. Data points are averages of quadruplicate runs. Errors were typically less than 10%. Traditionally denaturing additives are represented by squares, ILs by triangles, and DESs by circles. Lines are added to aid the eye in following each series. The urea concentrations (red squares) are twice as high as indicated on the abscissa.

activity versus concentration for each cosolvent. In each case, the line had an intercept of 1.0, corresponding to the activity of the enzyme in 100% BES buffer. We compared the deactivation slopes for each additive to the qualitative spectral changes we observed for CALB, Table 1. Spectra of additives that were very clearly denaturing (deactivation slope less than -0.5 M^{-1}), namely BMIM[$EtOSO_3$], BMIM[DCA], and BMIM[BF_4] all had both higher absorbance at low wavelengths and the valley between 240 and 260 nm, which shifted to higher wavelengths as concentration increased. It is likely that these features correspond with unfolding or denaturation of the protein. The peak at 230 nm appeared in most of the spectra, and may correspond with a non-denaturing change in fold, possibly from protein interactions with a less polar environment than water.

Table 1. Fitted slope of CALB deactivation for various additives.

Additive	**Type**	**Deactivation Slope (M^{-1})**	**Increased baseline**	**Peak at ~230 nm**	**Valley at ~245 nm**
GDHCl	Solid	N.D.	Y	N	Y
Urea	Solid	-0.39	N	Y	N
MeOH	Organic	-0.34	Y	N	N
BMIM[BF_4]	IL	-0.74	Y	Y	Y
BMIM[Cl]	IL	-0.03	N	Y	Y
BMIM[DCA]	IL	-0.89	Y	Y	Y
Choline[Me_2PO_4]	IL	0.23	N	Y	N
EMIM[$EtOSO_3$]	IL	-0.73	Y	Y	Y
ChCl:Gly	DES	0.16	N	Y	N
ChCl:U	DES	-0.11	N	Y	N

N.D.: not determined; Y: yes, spectrum contains the feature; N: no, spectrum does not contain the feature.

CALB seems to retain its total activity in varying concentration in ChCl:Gly, but its preference for hydrolysis versus acyl transfer shifts depending on how much DES is

present, Figure 3. The selectivity was calculated based on the formation of hexyl acetate relative to the conversion of phenyl acetate. The immobilized enzyme is considerably more selective toward acyl transfer with the DES present compared to *t*-butanol present

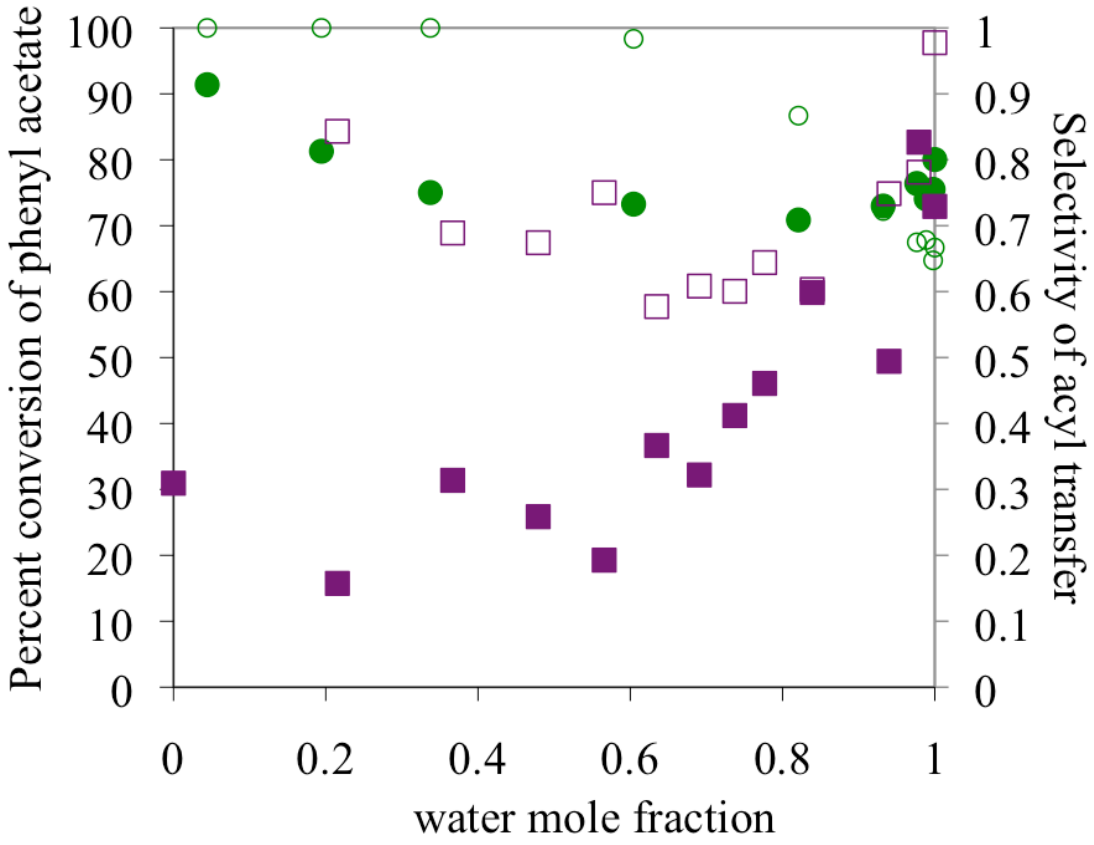


Figure 3. Assessment of total activity and selectivity of acyl transfer activity in CALB toward phenyl acetate. The solvents were ChCl:Gly and *t*-butanol with hexanol as the acyl acceptor. Squares: *t*-butanol as solvent; circles: ChCl:Gly as solvent; closed symbols: total conversion of phenyl acetate in 1 h at 35 ˚C; open symbols: fraction of conversion due to acyl transfer (selectivity).

over a large range of water contents. The concentrations where iCALB had a higher selectivity in *t*-butanol compared to in DES resulted in a biphasic reaction mixture, meaning that the substrates were likely exclusively dissolved in the organic phase and exposed to the hydrophobic beads at the interface, resulting in a low effective water activity.

In general, CALB preferred acyl transfer to hydrolysis in the DES, and there was effectively no acyl transfer above a water mole fraction of 0.4, corresponding to 90 % DES by volume. The total activity (sum of acyl transfer and hydrolysis) of the enzyme was largely unchanged in the DES, and was usually in the range of 70-90%. However, iCALB activity dropped with *t*-butanol concentration, from about 85% in 99% buffer to about 30% in 1% buffer.

Without the hydrophobic support, free CALB also prefers acyl transfer to hydrolysis in a number of DESs, and more so than in water-miscible organic solvents, Table 2. CALB had high acyl transfer preference in DESs (0.66 -1.0 fractional acyl transfer) and the ionic liquid BMIM[BF_4] (0.83) at 10% water content. By contrast, adding the organic solvents acetonitrile, *t*-butanol, or THF (0.32, 0.26, 0.20) actually increased CALB's hydrolysis preference compared to in buffer alone (0.39). It is possible that DES (and IL) could be deactivating water through hydrogen bonding and preventing it from orienting correctly in the enzyme active site for nucleophilic attack.

Table 2. Ratio of hydrolysis to acyl transfer activity of CALB toward hexyl acetate in 90% cosolvent.

Solvent	Type	Acyl transfer selectivity
ChBr:Gly	DES	0.84
ChCl:Gly	DES	0.90
AcChCl:Gly	DES	0.91
AcChCl:U	DES	1.0
$BzEt_3NCl$:Gly	DES	0.66
Acetonitrile	Organic solvent	0.32
t-Butanol	Organic solvent	0.26
THF	Organic solvent	0.20
BMIM[BF_4]	IL	0.83
5 mM BES, pH 7.2	Water	0.39

Conditions: 50 mM hexyl acetate, 100 mM octanol (acyl acceptor), 0.2 ml solution containing 10% of a 5 mg ml^{-1} CALB solution, 40 ˚C, 1 h.

6.3 DES formation as a driving force for reactions

The suitability of DESs for enzyme catalysis combined with low solvent cost makes formation of DESs an attractive means of driving enzyme-catalyzed reactions forward. Biodiesel synthesis could benefit from enhanced catalysis or separability related to deep eutectic solvent formation. Enzyme-catalyzed biodiesel formation is typically slower than the base-catalyzed reaction, in part because of the large quantities of short chain, denaturing alcohols present in the reaction mixture. DES formation is already known to be an effective, low-cost way of glycerol removal from fatty acid esters. Abbott and coworkers (*7*) added a number of different DES-forming salts, most notably choline chloride, to a final biodiesel reaction mixture and removed up to 99% of glycerol from the ester phase. However, the group did not add the salts during the reaction for *in situ* formation of a DES, likely because of the potential for side reactions with the hydroxyl moiety of choline chloride and the lipids. A CALB-catalyzed reaction is unlikely to have this limitation because of the regioselectivity of the enzyme. In this work, we

demonstrate markedly enhanced activity of CALB for fatty acid ethyl ester formation compared to the traditional enzymatic method. Further, the time-scale of the transesterification becomes more competitive with base-catalyzed reactions.

We can exploit this stability for enhanced production of biodiesel driven by *in situ* formation of a deep eutectic solvent. A preliminary experiment suggested that we are able to enhance the initial rate of *Candida antarctica* lipase B-catalyzed ethanolysis of soybean oil by about 3-fold at room temperature when an ammonium salt-glycerol eutectic is formed compared to the traditional process. At higher temperatures, the rates of reaction are nearly identical, however. Elucidating the reason for this difference would be of great interest, as it could allow for better design of deep eutectic solvents that would drive the process forward. The DES-producing process has reaction times that are comparable to base-catalyzed reactions at similar loadings of protein (~1 wt% of iCALB is protein) and potassium hydroxide. Further, only the enzyme-catalyzed reactions with ammonium salt present phase separated during the reaction, Figure 4. Without salt, the final product was a homogeneous mixture, which is undesirable from a processing standpoint.

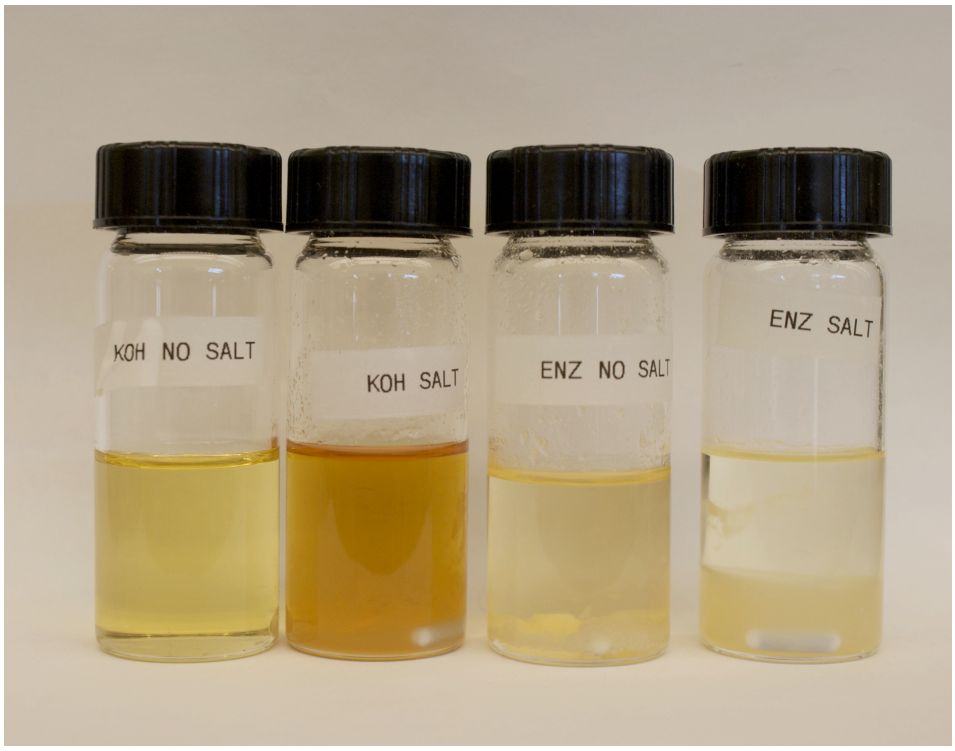


Figure 4. Reaction mixtures following synthesis of fatty acid ethyl esters from soy oil in the presence of choline chloride. Only the enzyme-catalyzed reaction with choline chloride added (far right) gave produced a biphasic reaction mixture. The cloudiness of the enzymatic reactions is a result of pulverization of iCALB beads.

6.4: DES as feedstock for polymer-producing cells

The DESs ChCl:U and ChCl:Gly biodegrade and can be a feedstock for producing value-added products. We fed up to 100 g l^{-1} of either DES to *Cupravidus necator* (formerly *Ralstonia eutropha*) in an otherwise minimal medium. In some cases we supplemented the medium with ammonium chloride, but it was unnecessary for growth. Thus, the bacteria utilized the DESs as the sole carbon and nitrogen source. Further, the organisms are able to produce poly(hydroxyalkanoates) during growth on these media,

Figure 5. *C. necator* can accumulate more than 80% of cell dry weight as polymer under nitrogen-limiting conditions, and in two days on the DESs, the cells produced up to 50% of cell dry weight as polymer. The bacteria produced higher cell dry weights of polymer on ChCl:Gly (max. 52% of cell dry weight on 50 g l^{-1}) than ChCl:U (max. 21% of cell dry weight on 50 g l^{-1}). It is likely that these values could increase after longer times, as the cells likely grow more slowly on higher concentrations due to osmotic stress. These experiments were important for confirming that DESs can be biodegradable if their components are also biodegradable. The consumption of ChCl:Gly is especially interesting, because it may be possible to produce ChCl:Gly from biodiesel production as a waste stream and then feed the DES to bacteria to produce additional triglyceride, polymers, or another value-added produce from a relatively inexpensive feedstock.

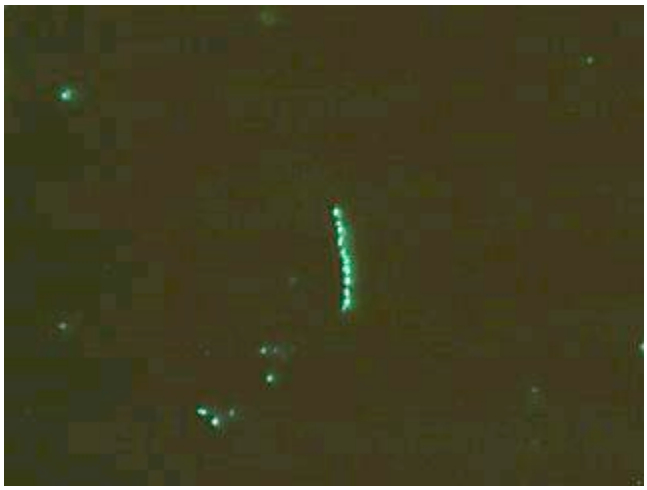


Figure 5. Fluorescent imaging of BODIPY-stained *C. necator* cells after 48 h growth on 10 g l^{-1} ChCl:Gly. Bright green spots indicate poly(hydroxybutyrate) granules.

6.5 Experimental

UV spectrum determination

Enzyme (5 mg) was dissolved in BES buffer (1 ml, 100 mM BES, pH 7.2). An aliquot of enzyme solution (11 μl) was added to a 96-well UV transparent microtiter plate. Denaturant solution (20 ml, 2.25 M in 100 mM BES) The desired IL, DES, or denaturant solution (88 μl, diluted with 100 mM BES as needed) was added to give 0 to 2 M solutions. The solutions were incubated at room temperature in a shaker at 300 RPM for 90 min. After incubation, the UV spectra (absorbance values) were obtained on a (SpectraMax 384 Plus, Molecular Devices). Each experiment was run in triplicate, and incubations without enzyme were run for each concentration (solvent spectra). To determine differences in spectra, the spectrum of the enzyme in each concentration was determined by subtracting the solvent absorbance spectrum at each concentration from the solvent/enzyme spectrum. We then subtracted the spectrum of the enzyme in 100% BES buffer from this spectrum to determine differences in enzyme absorbance.

Hydrolysis of p-nitrophenyl acetate

A 0.5 mg protein ml^{-1} solution in 100 mM BES at pH 7.2 (10 μl) was added to a 96-well microtiter plate. To this solution the desired IL, DES, or denaturant solution (80 μl) was added. The combined solution was incubated as described for the UV assay. After incubation, a *p*-nitrophenyl acetate solution (300 mM in acetonitrile 10 μl). The absorbance was monitored at 404 nm at 6-second intervals with the microplate reader at room temperature for up to 30 minutes. The concentration dependence on deactivation

was calculated by fitting the data for 0 to 1 M additive to a best fit line with an intercept at a fractional activity of 1.0 and calculating the slope of the line.

Hydrolysis/acyl transfer competition reaction with iCALB

BES buffer (5 mM, pH 7.2) and cosolvent (0-100%, 0.5 ml total) were added to a 2 ml vial. Phenyl acetate (2.7 mg), *n*-hexanol (20.4 mg), and iCALB (1 mg) were added to start the reaction. The vials were incubated for 1 h at 35 ˚C in a shaker at 900 RPM, after which the products were extracted with toluene (1 ml). Reactions involving *t*-butanol were biphasic at 50% water or more and were mixed vigorously to form an emulsion before incubation. If any emulsions were present after the reaction, they were broken by centrifugation. The toluene extracts were then subjected to GC analysis. A repeatable impurity in the hexanol was used as the internal standard.

The reaction was analyzed using gas chromatography using a 30 m HP-5 column (J&W Scientific, Folsom, CA) with 0.32 mm inner diameter and 0.25 μm film size. The detector and injector temperatures were 275 ºC and 250 ºC, respectively. The initial column temperature of 60 ºC was held for 6 min, then increased to 165 ºC at 15 ºC min^{-1}, then further increased to 200 ºC at 25 ºC min^{-1} and held at 200 ºC for 5 min.

Hydrolysis/acyl transfer competition reaction with free CALB

CALB in BES buffer (20 μl of 5 mg ml^{-1} solution in 5 mM buffer), and cosolvent (180 μl) were added to a 2 ml vial. Hexyl acetate (2.9 mg) and hexanol (5.1 mg) were added to start the reaction. The vials were incubated for 1 h at 40 ˚C in a shaker at 900 RPM, after which the products were extracted with toluene (1 ml). Any emulsions were broken and the reaction was analyzed as described for the iCALB-catalyzed reaction.

Biodiesel reaction

Cyclohexanone was added to soy oil (0.1% v/v) as an internal standard in a soy oil stock. In a typical reaction, the oil stock (0.5 ml) was added to a 2 ml vial containing immobilized *C. antarctica* lipase B (iCALB, 5-20 mg). Choline chloride (33 mg, approx. 0.5 equiv. compared to oil) was also added as needed. To begin the reaction, ethanol (0.1 ml) was added and the vials were placed in an incubator at room temperature or 60 ˚C shaken at 900 RPM. In the case of KOH-catalyzed reactions, KOH (1 mg) was dissolved in the ethanol.

The reaction was analyzed using gas chromatography using the 30 m HP-5 column above. The detector and injector temperatures were 325 ºC and 300 ºC, respectively. The initial column temperature of 100 ºC was held for 6 min, then increased to 300 ºC at 10 ºC min^{-1} and held at 300 ºC for 5 min.

Cell growth on DESs

C. necator cells were grown on minimal medium according as described by Ramsay and coworkers (*8*) in shake tubes, with DES as the sole carbon and nitrogen source in some cases, and supplemented with ammonium chloride as an additional nitrogen source in others. 48 h after inoculation, some of the cells were prepared for polymer analysis according to Riis and Mai (*9*) and others were stained with BODIPY dye as described previously by our group (*10*).

Gas chromatography analysis was performed using the 30 m HP-5 column above. The temperatures of the sample injector and detector were 250 °C and 275 °C, respectively. The column temperature was set at 120 °C for eight minutes followed by an

increase to 200 °C at a rate of 10 °C min^{-1} and then held constant at 200 °C for 20 min.

6.5 Concluding remarks and suggestions for future study

Deep eutectic solvents are especially promising reaction media for enzymatic reactions. These solvents could be an important bridging technology between petrochemically-derived and biologically-derived solvents because of the nature of their components. Expanding the scope of DESs that are useful in enzyme catalysis as well as which classes of enzymes are suitable for use in DESs is one area I would like to see studied further. Finding more applications for DESs in addition to biodiesel synthesis would make another interesting project. I think determining what other value-added products that cells can make from DESs would also be beneficial.

One potential disadvantage of the DESs I've studied is the presence of halides. These halides could cause corrosion problems and require more expensive materials of construction. A thorough investigation of any corrosive effects of DESs would be most beneficial. Further, studying enzyme catalysis in DESs with anions other than halides would be useful to broaden the scope of these enzymes. In ionic liquids, the anion plays a large role in determining properties especially solubility. DESs based on non-halide anions could have unique advantages for biocatalysis or chemical synthesis.

The behavior of enzymes in deep eutectic solvents and DES-water mixtures merits further study. Circular dichroism would be a very useful tool to better understand exactly what is occurring when the UV spectrum of the enzyme changes when exposed to cosolvents. Those measurements could allow a structure-function correlation for the DES-enzyme interactions, and allow for the design of better enzymes for catalysis in

DES or for better medium design. Investigating the acyl transfer/ hydrolysis relationship for enzymes in these solvents could also be beneficial, and enable better selective "wet" transesterifications or resolutions.

Finally, traditional ionic liquids also merit further study in niche applications, especially if their cost and biodegradability begin to match deep eutectic solvents. Synthesizing the carotenoid-containing polymers in ionic liquids could eliminate the film casting step for doping of the polymers. Based on very preliminary studies, the carotenoid diols are considerably more soluble in ILs such as BMIM[Tf_2N] than in toluene. When I dissolved the carotenoids in toluene and added a second phase of IL, the carotenoid quickly and, to the naked eye, completely partitioned into the ionic liquid phase. The higher solubility of carotenoid would be very useful in synthesizing condensation polymers of oligomers with carotenoid diols and other diesters, such as those I made in Chapter 5 using BCL and divinyl adipate, in higher yields per volume.

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