Impact of Ionic Liquid Physical Properties on Lipase Activity and Stability

Joel L. Kaar,[†,‡] Anita M. Jesionowski,[‡] Jason A. Berberich,[†,§] Roger Moulton,[ǁ] and Alan J. Russell*,[†,‡]

Contribution from the McGowan Institute for Regenerative Medicine, Suite 200, 100 Technology Drive, University of Pittsburgh, Pittsburgh, Pennsylvania 15219, Department of Chemical and Petroleum Engineering, 1249 Benedum Hall, University of Pittsburgh, Pittsburgh, Pennsylvania 15261, Department of Bioengineering, 749 Benedum Hall, University of Pittsburgh, Pittsburgh, Pennsylvania 15261, and SACHEM Inc., 821 East Woodward Street, Austin, Texas 78704

Received September 16, 2002; E-mail: russellaj@msx.upmc.edu

Abstract: Lipase activity and stability was investigated in dialkylimidazolium and pyrrolidinium-based ionic liquids with a variety of anions including hexafluorophosphate, acetate, nitrate, methanesulfonate, trifluoroacetate, and trifluoromethylsulfonate. The initial rate of lipase-catalyzed transesterification of methyl methacrylate in these ionic liquids and several organic solvents was examined as well as the polytrans-esterification of divinyl adipate and 1,4-butanediol. Free lipase (*Candida rugosa*) catalyzed the trans-esterification of methyl methacrylate in 1-butyl-3-methylimidazolium hexafluorophosphate at a rate 1.5 times greater than in hexane. However, no detectable activity was observed in all the "hydrophilic" ionic liquids studied. Methods of enzyme stabilization including adsorption, PEG-modification, and immobilization in polyurethane foam were ineffective in improving enzymatic activity in the hydrophilic ionic liquids. Polytransesterifications performed in 1-butyl-3-methylimidazolium hexafluorophosphate using Novozym 435 produced polyesters with weight average molecular weights limited to 2900 Da due to precipitation of the polymer. Solvatochromic studies and partition coefficient measurements suggest that ionic liquids are more polar and hydrophilic than organic solvents such as hexane, acetonitrile, and tetrahydrofuran. Stability studies indicate that lipases exhibit greater stability in ionic liquids than in organic solvents including hexane.

Introduction

Thermal stability and the nonexistence of vapor pressure, which eliminates volatile organic compound (VOC) emissions, make ionic liquids an environmentally attractive alternative to conventional organic solvents. Recoverability and recyclability also make ionic liquids a practical solution to industrial environmental concerns.[1,2] That said, little is known about the biological consequences of increased concentrations of ionic liquids in the environment.

As solvents for chemical processing, ionic liquids exhibit excellent physical characteristics including the ability to dissolve polar and nonpolar organic, inorganic, and polymeric compounds.[1,3] The chemical and physical properties of ionic liquids may be modified by altering the cation, anion, and attached substituents. This feature plays a key role in manipulating the solvent properties of an ionic liquid, which allows ionic liquids to be designed for specific reaction systems.[3]

The use of ionic liquids to replace organic solvents in biocatalytic processes has recently gained much attention. Cull
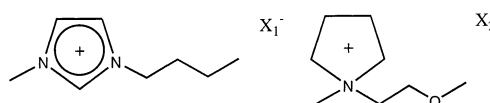

Figure 1. Anion X_1^- is available as hexafluorophosphate [PF_6^-], acetate [$CH_3CO_2^-$], nitrate [NO_3^-], and trifluoroacetate [$CF_3CO_2^-$]. Anion X_2^- is available as hexafluorophosphate [PF_6^-], acetate [$CH_3CO_2^-$], nitrate [NO_3^-], trifluoroacetate [$CF_3CO_2^-$], trifluoromethylsulfonate [$CF_3SO_3^-$], and methanesulfonate [$CH_3SO_3^-$].

et al.[4] used the ionic liquid 1-butyl-3-methylimidazolium hexafluorophosphate, [bmim][PF_6], for the two-phase biotransformation of 1,3-dicyanobenzene to 3-cyanobenzamide and 3-cyanobenzoic acid using the nitrile hydratase from *Rhodococcus* R312. This work established ionic liquids as a potential alternative to organic solvents for multiphase biotransformations. The first reported enzyme-catalyzed reaction in an ionic liquid, performed in our laboratory, involved the thermolysin-catalyzed synthesis of *Z*-aspartame in [bmim][PF_6].[5] This work showed enzyme activity approached that seen in conventional organic solvents and that the enzyme maintained very high stability in the ionic liquid. Since this work, there have been many reports of enzymatic catalysis in ionic liquids, including the anhydrous

[†] McGowan Institute for Regenerative Medicine.
[‡] Department of Chemical and Petroleum Engineering, University of Pittsburgh.
[§] Department of Bioengineering, University of Pittsburgh.
[ǁ] SACHEM Inc.
(1) Gordon, C. M. *Applied Catalysis A: General* **2001**, *222*, 101−117.
(2) Holbrey, J. D.; Seddon, K. R. *Clean Products Process.* **1999**, *1*, 223−236.

(3) Brennecke, J. F.; Maginn, E. J. *AIChE J.* **2001**, *47*, 2384−2389.
(4) Cull, S. G.; Holbrey, J. D.; Vargas-Mora, V.; Seddon, K. R.; Lye, G. J. *Biotechnol. Bioeng.* **2000**, *69*, 227−233.
(5) Erbeldinger, M.; Mesiano, A. J.; Russell, A. J. *Biotechnol. Prog.* **2000**, *16*, 1129−1131.
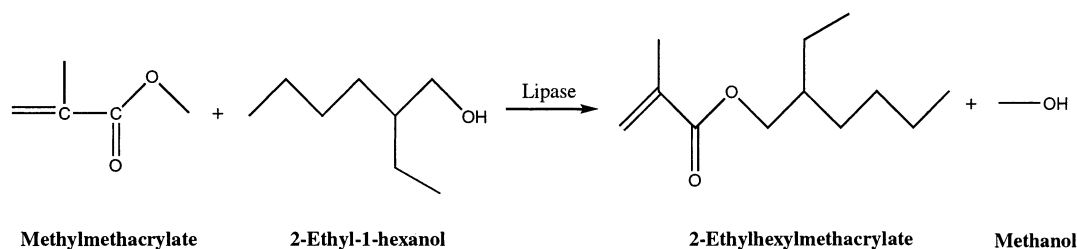
Figure 2. Lipase-catalyzed transesterification of methyl methacrylate with 2-ethylhexanol to 2-ethylhexyl methacrylate.
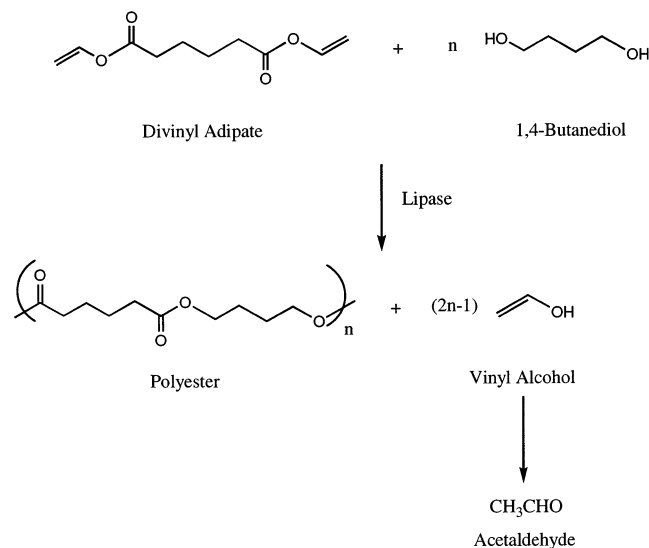
Figure 3. Lipase-catalyzed polytransesterification of divinyl adipate and 1,4-butanediol.

transesterification of ethyl butanoate and butanol catalyzed by *Candida antarctica* lipase type B (CaLB) in 1-butyl-3-methylimidazolium with hexafluorophosphate and tetrafluoroborate anions[6] and the α-chymotrypsin catalyzed transesterification reaction of *N*-acetyl-L-phenylalanine ethyl ester with 1-propanol in 1-butyl-3-methylimidazolium and 1-octyl-3-methylimidazolium cations in conjunction with the hexafluorophosphate anion.[7]

In this paper, we discuss the practicality of replacing conventional biocatalytic solvents with ionic liquids consisting of dialkylimidazolium and pyrrolidinium cations with a wide range of anions including hexafluorophosphate, acetate, nitrate, methanesulfonate, trifluoroacetate, and trifluoromethylsulfonate (Figure 1).

Herein, we describe the first attempt of enzyme catalysis in ionic liquids containing pyrrolidinium cations. We also compare the polarity and hydrophibicity, via solvatochromic and octanol−water partition coefficient analysis respectively, of ionic liquids to those of organic solvents used in biocatalytic reactions. The lipase-catalyzed transesterification of methyl methacrylate and 2-ethylhexanol (Figure 2) and the polytransesterification of divinyl adipate (DVA) and 1,4-butanediol (BD) (Figure 3) were selected as model reactions to determine the activity of enzymes as a function of ionic liquid physical properties.

Materials and Methods

Materials. Lipase L-1754 (*Candida rugosa*) and porcine pancreatic lipase were purchased from Sigma Chemicals (St. Louis, MO). Chirazyme L-2, lyophilizate (*Candida antarctica*) type B, Chirazyme

L-8, lyophilizate (*Thermomyces lanuginosus*), and Chirazyme L-9, lyophilizate (*Mucor miehei*) were purchased from BioCatalytics (Pasadena, CA). Novozym 435 (Candida antartica, type B) was a gift from Novo Nordisk (Denmark). All enzymes were used without further purification. Hypol 3000 prepolymer was purchased from Hampshire Chemical (Lexington, MA). 3,3,4,4,5,5,6,6-octafluorooctan-1,8-diol (OFOD) was purchased from Oakwood Products (West Columbia, SC) whereas all other fluorinated diols were purchased from Lancaster Synthesis (Windham, NH). DVA was a gift from Union Carbide (Dandbury, CT). L-62 Pluronic surfactant was supplied as a gift by BASF (Parsippany, NJ). The [bmim][PF$_6$] used in all polytransesterification reactions was synthesized following the procedure described by Huddleston and co-workers.[8] For all transesterification reactions, ionic liquids were synthesized and purified by SACHEM Inc. (Austin, TX) and used as supplied. All other reagents were purchased from Aldrich Chemicals (St. Louis, MO) and were of the highest purity available.

Characterization of Ionic Liquids. Determination of Octanol−Water Partition Coefficients (log P). Partition coefficients for ionic liquids were determined as a ratio of ionic liquid concentration in the octanol phase to the ionic liquid concentration in the aqueous phase (eq 1). The logarithm of the partition coefficient is referred to as the log P value

$$\text{Partition Coefficient } (P) = \frac{[\text{Solute}]_{\text{Octanol Phase}}}{[\text{Solute}]_{\text{Aqueous Phase}}} \quad (1)$$

The "shake flask" method was used for the determination of all log P values.[9] The imidazolium ring present in the dialkylimidazolium based ionic liquids absorbs strongly at approximately 211 nm and can be quantified using UV spectrometry.[10] Water saturated with octanol and octanol saturated with water were used in all experiments. For each ionic liquid, the maximum wavelength (λ_{max}) due to the absorbance of the imidazolium ring was verified in both phases. The volume ratio of the octanol phase to aqueous phase was varied in an attempt to determine the necessary ratio to force the solute into the hydrophobic octanol phase to a detectable level. A 100:1 volume ratio was used for the partitioning of all ionic liquids. In a 200-mL separatory funnel, ionic liquid was added to the saturated phases so that its concentration did not exceed 10 mM in either phase. Inversion of the separatory funnel was carried out for 5 min at an approximate rate of 20 inversions/min. The phases were then recovered and centrifuged to eliminate emulsions formed during partitioning. The ionic liquid concentration was determined in each phase using standard curves. A Perkin-Elmer Lambda 45 UV/VIS Spectrophotometer was used for the log P analysis.

Solvatochromic Characterization of Ionic Liquids. The protocol used for measuring E^N_T polarity values is described by Fletcher and co-workers.[11] Reichardt's dye, (2,6-diphenyl-4-(2,4,6-triphenylpyridinio)phenolate), was dissolved in the ionic liquid and centrifuged to

(6) Lau, R. M.; van Rantwijk, F.; Seddon, K. R.; Sheldon, R. A. *Org. Lett.* **2000**, *2*, 4189−4191.
(7) Laszlo, J. A.; Compton, D. L. *Biotechnol. Bioeng.* **2001**, *75*, 181−186.
(8) Huddleston, J. G.; Willauer, H. D.; Swatloski, R. P.; Visser, A. E.; Rogers, R. D. *Chem. Comm.* **1998**, *16*, 1756−1766.
(9) Leo, A.; Hansch, C.; Elkins, D. *Chem. Rev.* **1971**, *71*, 525−616.
(10) Anthony, J. L.; Maginn, E. J.; Brennecke, J. F. *J. Phys. Chem. B* **2001**, *105*, 10 942−10 949.
(11) Fletcher, F. A.; Storey, I. A.; Hendricks, A. E.; Pandey, Sh.; Pandey, Si. *Green Chem.* **2001**, *3*, 210−215.

remove any residual dye particulates. Solvents were then analyzed via a scan of the visible spectrum using a Perkin-Elmer Lambda 45 UV–vis Spectrophotometer. The position of the absorption band was correlated to an E_T^N value (eqs 2 and 3)

$$E_T(\text{solvent})[\text{kcal/mol}] = \frac{28591}{\lambda_{\max}[\text{nm}]} \qquad (2)$$

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

The normalized scale ranges from water being the most polar ($\lambda_{\max} = 453$ nm, $E_T^N = 1.0$) to tetramethylsilane being the least polar ($\lambda_{\max} = 925$ nm, $E_T^N = 0.0$). All ionic liquids were dried in a vacuum oven (26 in Hg vac.) at 70 °C for several days prior to solvatochromic analysis.

Enzyme Activity in Ionic Liquids. Lipase-Catalyzed Transesterification of Methyl Methacrylate and 2-Ethylhexanol. The substrates methyl methacrylate and 2-ethylhexanol were dissolved in the solvent at a concentration of 200 mM. Reactions were performed through the addition of solvent containing methyl methacrylate (1 mL, 200 mM) and of 2-ethylhexanol (1 mL, 200 mM) to lipase in a 5-mL Kimble glass vial. The enzyme suspension was sonicated for approximately 15 s. The reaction vials were then immediately placed in a constant temperature shaker set at 30 °C and 300 rpm. When performing reactions in organic solvents, aliquots (1.0 μL) were removed from the reaction mixture using a 1-μL Hamilton syringe. When performing reactions in ionic liquids, 2-ethylhexyl methacrylate was recovered via liquid extraction using hexane. In both cases, samples were withdrawn at specified time intervals for measurement of initial reaction rate. Formation of 2-ethylhexyl methacrylate was analyzed using a Perkin-Elmer Autosystem gas chromatograph (GC). The GC was fitted with an Alltech EC 1000 capillary column (30 m × 0.53 mm × 1.0 μm) and was operated with a 1:4 split ratio using helium as the carrier gas. The injector and detector temperatures were set to 300 °C. The oven program consisted of an initial temperature of 100 °C that was maintained for 2 min after which the temperature was increased at a rate of 25 °C/min to a final temperature of 160 °C and maintained for 3 min. The retention time of 2-ethylhexyl methacrylate was 5.1 min. A calibration curve of peak area versus the concentration of 2-ethylhexyl methacrylate in the presence of the substrates was created for each organic solvent and ionic liquid studied.[12,13] No internal standard was used; however, the GC was calibrated daily.

Lipase-Catalyzed Polytransesterification of DVA and BD. Typically, [bmim][PF₆] was placed in a Wheaton vial followed by the addition of DVA and BD monomers. The vials were then placed in a New Brunswick Scientific Series 25 incubator/shaker set at 50 °C and 250 rpm for 30 min to allow for solubilization of the monomers in the ionic liquid. Once the monomers were solubilized, polymerization was initiated by the addition of enzyme. The polyester product precipitated from the ionic liquid and was easily separated. The polyester product was then dissolved in tetrahydrofuran for molecular weight analysis via gel permeation chromatography (GPC). A Waters 150CV GPC equipped with a refractive index detector was used for the molecular weight analysis. Tetrahydrofuran was used as the mobile phase at a flow rate of 1.0 mL/min and 35 °C. The GPC contains three columns in series to achieve sufficient separation in the molecular weight range of 500–30 000 Da. The first two columns in the series, PL-gel Mixed-E Polymer Laboratories, contained mixed porosity. The third column, a Waters Ultrastyragel column, had a uniform pore size of 500 Å. A calibration curve of the log of molecular weight versus retention time was constructed using 11 polystyrene standards within the molecular

weight range of 580–66 000 Da. Due to error in GPC measurements, all molecular weight results were rounded to the nearest hundred.

Attempts to Improve Enzyme Activity in Ionic Liquids. Synthesis of Polyethylene Glycol (PEG)–Modified Lipase. Free lipase (*Candida rugosa*) was dissolved in an aqueous buffer (10 mM Tris, 5 mM CaCl₂, pH 7.5) at a concentration of 1 mg/mL. PEG-NCO was added in excess to the lipase solution at a molar ratio of 1:100 lipase to PEG-NCO to ensure complete lipase modification. The reaction mixture was mixed for 30 min followed by lyophilization.[14] No further purification to remove the excess PEG was attempted. The freeze-dried enzyme was used in its crude form.

Polyurethane-Lipase Foam Synthesis. The synthesis of lipase-containing (*Candida rugosa*) polyurethane foams was accomplished using the procedure described by LeJeune and Russell.[15] The polyurethane foams were prepared with an aqueous buffer (50 mM Bis-Tris Propane, 5 mM CaCl₂, pH 7.5) containing 1% (w/w) L-62 Pluronic surfactant. Lipase (1 g) was then added to the aqueous buffer (2.5 mL). Hypol 3000 (2.5 g), a toluene diisocyanate based pre-polymer, was added to the aqueous lipase solution and vigorously mixed for 30 s using a 2500 rpm hand drill for sufficient mixing. The lipase-immobilized foam was allowed to dry overnight at ambient temperature and pressure and stored at 4 °C when not being used. In preparation for use in transesterification reactions, the foams were ground into small particle size pieces using a handmade drill bit wrapped in sandpaper (grit no. 180). The drill was operated at low rpm during the grinding process in attempt to maintain constant particle size.

Enzyme Stability in Ionic Liquids. Lipase was incubated in the organic solvent or ionic liquid (1 mL) at a given temperature (30 °C or 50 °C). The solvent was then extracted and the lipase was assayed for activity using a Lipase Diagnostic kit (Sigma 800-B). Typically, assays were performed with lipase, in the presences of substrate (3 mL), incubated at 50 °C and shaken at 300 rpm for 2 h. Titration with sodium hydroxide (0.05 N) was performed to determine the amount of acid produced.

Results and Discussion

Solvent selection for enzymatic reactions can be severely constrained due to the inherent ability of solvents to deactivate enzymes, as is the case with hydrophilic solvents. Hildebrandt solubility parameters (δ), dielectric constants (ϵ), and dipole moments (μ) are all methods of predicting enzyme activity. However, the most common method of predicting enzyme activity is through the use of the octanol–water partition coefficient.[16,17] Solvatochromic parameters represent a simple and common means for assessing solvent polarity.[16]

To understand how the physical properties of ionic liquids influence enzyme activity and stability, one must first understand how the physical properties of the ionic liquids vary with structure. We therefore investigated the polarity and hydrophobicity of a variety of ionic liquids.

Physical Characterization of Ionic Liquids. A partition coefficient is a measure of how a solvent partitions between an aqueous and organic phase, thus serving as an indication of solvent hydrophobicity. Laane reported that solvents with a log P less than two are considered hydrophilic in nature and tend to be unfavorable for enzymatic reactions. In contrast, solvents with a log P of greater than four generally support enzymatic activity. Although log P values are a common method of

(12) Kamat, S.; Critchley, G.; Beckman, E. J.; Russell, A. J. *Biotechnol. Bioeng.* **1995**, *46*, 610–620.

(13) Kamat, S.; Barrera, J.; Beckman, E. J. *Biotechnol. Bioeng.* **1992**, *40*, 158–166.

(14) Drevon, G. F.; Hartleib, J.; Scharff, E.; Rüterjans, H.; Russell, A. J. *Biomacromolecules* **2001**, *2*, 664–671.

(15) LeJeune, K. E.; Russell, A. J. *Biotechnol. Bioeng.* **1996**, *51*, 450–457.

(16) Laane, C.; Boeren, S.; Vos, K.; Veeger, C. *Biotechnol. Bioeng.* **1987**, *30*, 81–87.

(17) Pogorevc, M.; Stecher, H.; Faber, K. *Biotechnol. Lett.* **2002**, *24*, 857–860.

Table 1. Log *P* and Reichardt's Dye Polarity (E^N_T) Values for Ionic Liquids and Select Organic Solvents

solvent	E^N_T	log *P*
1-butyl-3-methylimidazolium acetate	0.57	−2.77 ± 0.11
1-butyl-3-methylimidazolium nitrate	0.65	−2.90 ± 0.01
1-butyl-3-methylimidazolium trifluoroacetate	0.63	
1-butyl-3-methylimidazolium hexafluorophosphate	0.67	−2.39 ± 0.27
1-methyl-1-(−2-methoxyethyl)pyrrolidinium acetate	0.52	
1-methyl-1-(−2-methoxyethyl)pyrrolidinium nitrate	0.84	
1-methyl-1-(−2-methoxyethyl)pyrrolidinium trifluoroacetate	0.37	
1-methyl-1-(−2-methoxyethyl)pyrrolidinium trifluoromethylsulfonate	0.91	
1-methyl-1-(−2-methoxyethyl)pyrrolidinium methanesulfonate	0.78	
hexane	0.009[19]	3.5[16]
tetrahydrofuran (THF)	0.207[19]	0.49[16]
acetonitrile	0.47[19]	−0.33[16]

biocompatibility assessment of organic solvents, it is important to note that this method does not always accurately predict enzyme activity.[16,17]

To our knowledge, these results are the first determinations of log *P* for ionic liquids. Huddleston and co-workers recently reported that there is a distinct correlation between the partitioning of several substituted-benzene derivatives in a 1-butly-3-methylimidizolium hexafluorophosphate/water system and their respective log *P* values.[8] However, they do not explicitly measure, calculate, or approximate the log *P* values of the ionic liquid 1-butly-3-methylimidizolium hexafluorophosphate in a 1-octanol/water system. The experimentally measured values of log *P* for the ionic liquids tested ranged between −2.39 and −2.90 (Table 1). For comparison, the log *P* values for hexane, acetonitrile, and tetrahydrofuran are 3.5, −0.33, and 0.49 respectively.[16] Using the guidelines detailed by Laane and co-workers,[16] this would indicate that the ionic liquids are highly hydrophilic in nature and would likely inactivate enzymes. Because the pyrrolidinium-based ionic liquids did not have any characteristic peaks in the UV spectra, the log *P* values could not be determined for these ionic liquids.

Solvatochromic parameters provide a simple method to determine the polarity of a solvent. The E^N_T empirical polarity scale is based on the shift of the charge-transfer absorption band of a solvatochromic probe in the presence of a solvent. Changes in the position of the charge-transfer absorption band within the visible spectrum are due to hydrogen bonding between the solvent and the phenoxide oxygen atom present in Reichardt's dye. An E^N_T value is then determined as a function of the position of the charge-transfer absorption band.[18,19]

There are only a few reported cases of the solvatochromic characterization of ionic liquids.[11,18,20−23] Results of solvatochromic analysis of [bmim][PF$_6$] using Reichardt's dye reported in the literature indicate that dry [bmim][PF$_6$] has an E^N_T value very similar to that of ethanol (E^N_T = 0.67) and other short chain primary alcohols.[11,18,20,23,24] This value is sensitive to the presence of water in the ionic liquid.[20,21] Muldoon and co-workers[18] suggest E^N_T's for ionic liquids are dependent upon the strength of hydrogen bonding between the cation of the ionic liquid and the phenoxide group present in Reichardt's dye. High E^N_T indicates that ionic liquids exhibit strong hydrogen bonding

forces. Solvents that demonstrate the ability to hydrogen bonding present the potential to interfere with enzyme structure.

Results of our solvatochromic analysis of [bmim][PF$_6$] agree with the reported E^N_T (Table 1). Our results indicate that replacement of the [PF$_6$] anion with the [CH$_3$CO$_2$], [NO$_3$], and [CF$_3$CO$_2$] anions resulted in E^N_T's of 0.57, 0.65 and 0.63 respectively (Table 1). This small change in the polarity series is consistent with previous work.[18] The ionic liquids [mmep]-[CH$_3$CO$_2$], [mmep][NO$_3$], [mmep][CF$_3$CO$_2$], [mmep][CF$_3$SO$_3$], and [mmep][CH$_3$SO$_3$] have E^N_T's of 0.52, 0.84, 0.37, 0.91, and 0.78 respectively (Table 1). The polarity of the ionic liquids containing the pyrrolidinium-based cation appear to be strongly dependent on the anion. For comparison with common organic solvents, the reported E^N_T for hexane, acetonitrile, and tetrahydrofuran are 0.009, 0.47, and 0.207 respectively.[19] With the exception of [mmep][CF$_3$CO$_2$], the E^N_T's for all ionic liquids studied were greater than those of acetonitrile and conventional organic solvents used in biocatalysis. The polar nature of ionic liquids presents an ideal reaction media for performing biotransformations involving highly polar substrates such as carbohydrates, which contain multiple hydroxyl functionalities.

Enzyme Activity in Ionic Liquids. Almost all of the enzymatic reactions performed to date are in dialkylimidazolium based ionic liquids with the hexafluorophosphate and tetrafluoroborate anions. Only recently have other cations and anions began to be studied.[24−26] Our interest is in expanding the portfolio of potential biocatalytic solvents by studying a variety of dialkylimidazoium and *N*-methyl-*N*−(−2-methoxyethyl) pyrrolidinium-based ionic liquids.

Initial reaction rates of the lipase-catalyzed transesterification of methyl methacrylate and 2-ethylhexanol were measured in several ionic liquids and organic solvents to determine the effect of the ionic liquid on enzyme activity. Our experience with this reaction system, combined with an extensive data set, makes this an ideal model system. The selection of lipase for these experiments was based on prior work in which several lipases including Amano lipases G (*Penicillium camembertii*), AY (*Candida rugosa*), AK (*Pseudomonas fluorescens*), MAP (*Mucor javanicus*), FAP (*Rhizopus oryzae*), PS (*Pseudomonas cepacia*), and Sigma lipases L1754 (*Candida rugosa*), L0763 (*Chromobacterium viscosum*), L3001 (wheat germ) were screened for activity in hexane via the lipase-catalyzed transesterification

(18) Muldoon, M. J.; Gordon, C. M.; Dunkin, I. R. *J. Chem. Soc., Perkin Trans.* **2001**, 433−435.
(19) Reichardt, C. *Chem. Rev.* **1994**, *94*, 2319−2358.
(20) Baker, S. N.; Baker, G. A.; Bright, F. V. *Green Chem.* **2002**, *4*, 165−169.
(21) Aki, S. N. V. K.; Brennecke, J. F.; Samanta, A. *Chem. Commun.* **2001**, 413−414.
(22) Dzyuba, S. V.; Bartsch, R. A. *Tetrahedron Lett.* **2002**, *43*, 4657−4659.
(23) Carmichael, A. J.; Seddon, K. R. *J. Phys. Org. Chem.* **2000**, *13*, 591−595.

(24) Kaftzik, N.; Wasserscheid, P.; Kragl, U. *Org. Process Res. Dev. A* **2002**, *6*, 553−557.
(25) Eckstein, M.; Sesing, M.; Kragl, U.; Adlercreutz, P. *Biotechnol. Lett.* **2002**, *24*, 867−872.
(26) Lozano, P.; De Diego, T.; Carrie, D.; Vaultier, M.; Iborra, J. L. *Chem. Commun.* **2001**, *23*, 1529−1533.

of methyl methacrylate with 2-ethylhexanol. The transesterifications were performed under the same conditions, 30 °C and 300 rpm, as were used in this study eliminating any temperature or mixing effects. Results of the lipase screen indicated that *Candida rugosa* lipase exhibited the highest activity and was therefore chosen as the model enzyme for this study.[12,13]

In [bmim][PF$_6$], free lipase (*Candida rugosa*) catalyzed the transesterification at an initial rate of 6.75 μM/hr/mg-enzyme, 1.5 times faster than the reaction in hexane (3.90 μM/hr/mg-enzyme). Although [bmim][PF$_6$] showed promise as an effective solvent for the transesterification, free *Candida rugosa* lipase was unfortunately inactive in all the other ionic liquids investigated ([bmim][CH$_3$CO$_2$], [bmim][NO$_3$], [bmim][CF$_3$-CO$_2$], [mmep][CH$_3$CO$_2$], [mmep][NO$_3$], [mmep][CH$_3$SO$_3$], [mmep][CF$_3$CO$_2$], and [mmep][CF$_3$SO$_3$]). The reaction also did not proceed in the more polar organic solvents THF or acetonitrile.

Water was added to ionic liquids, 25 to 100 μL H$_2$O/mL solvent, to activate the free lipase in [bmim][NO$_3$] and [mmep][CH$_3$CO$_2$]. Water stripping from an enzyme by polar solvents is a common cause of deactivation. However, hydration of the enzyme failed to improve activity in either of the ionic liquids making water stripping an unlikely mechanism of deactivation. The addition of the hydrophilic ionic liquids [bmim][NO$_3$] and [bmim][CH$_3$CO$_2$] as cosolvents with [bmim]-[PF$_6$] was also investigated as a means of maintaining enzyme activity while enhancing the ability to dissolve polar substrates. Transesterification reactions were performed using free lipase (*Candida rugosa*) in mixtures of [bmim][NO$_3$]/[bmim][PF$_6$] and [bmim][CH$_3$CO$_2$]/[bmim][PF$_6$]. Concentrations of [bmim][PF$_6$] in the cosolvent mixtures were varied at 25, 50, and 75 (v/v %). No lipase activity was observed in any of the mixed ionic liquid mixtures indicating the use of [bmim][PF$_6$] as a cosolvent with more hydrophilic ionic liquids did not prevent the inactivation of free lipase.

Our results suggest that enzyme activity in ionic liquids is anion dependent. Anions such as [NO$_3$], [CH$_3$CO$_2$], and [CF$_3$-CO$_2$] are more nucleophilic than [PF$_6$] and may coordinate more strongly to positively charged sites in the lipase's structure causing conformation changes in the enzyme's structure.[27] Similar results observed with [mmep][NO$_3$], [mmep][CH$_3$CO$_2$], [mmep][CF$_3$CO$_2$], [mmep][CF$_3$SO$_3$], and [mmep][CH$_3$SO$_3$] in which all anions are strong nucleophiles further suggest anion nucleophilicity is essential to enzyme activity. However, due to little work with pyrrolidinium-based ionic liquids, the possibility that the pyrrolidinium cation inhibits enzyme activity must not be ruled out. This new data calls into question the broad utility of ionic liquids with enzymes unless the deactivating mechanism can be understood and reversed. Naturally, we are now exploring such mechanisms.

It would appear that although there is much enthusiasm about enzymatic catalysis in ionic liquids, only one such liquids supports significant activity with lipases. Having demonstrated that lipase maintains a high level of activity in [bmim][PF$_6$], a natural extension was to perform an enzymatic polymerization in the ionic liquid. The lipase-catalyzed polytransesterification of DVA and BD in [bmim][PF$_6$] was studied to determine if the polymerizations would proceed in this environment. Initially,

Table 2. Monomer Solubilities at 50 °C in [bmim][PF$_6$]

monomer	maximum solubility in [bmim][PF$_6$]
divinyl adipate (DVA)	3.45M
1,4-butanediol (BD)	0.98M
2,2,3,3-tetrafluoro-1,4-butanediol (TFBD)	not soluble
2,2,3,3,4,4-hexafluoro-1,5-pentanediol (HFPD)	0.20M
3,3,4,4,5,5,6,6-octafluorooctan-1,8-diol (OFOD)	2.23M

the solubility of several monomers was assessed in [bmim][PF$_6$], including several fluorinated diols (Table 2).

Polymerizations catalyzed by Novozym 435 were performed using DVA and each of the soluble diols. Polytransesterifications were performed for 24 h after which time precipitation of the polymer from the ionic liquid was observed. The reaction between DVA and BD provided the highest molecular weight oligomers (M_W = 2900 Da, PDI = 1.20). An enzyme screen was also performed for the DVA/BD reaction. The use of lipases from *Thermomyces lanuginosus* and *Mucor miehei* produced oligomers with lower molecular weights. All other lipases investigated failed to produce polymer in this reaction system. Polytransesterifications between DVA and 2,2,3,3,4,4-hexafluoro-1,5-pentanediol (HFPD) and DVA and 3,3,4,4,5,5,6,6-octafluorooctan-1,8-diol (OFOD) produced oligomers with similar molecular weights, 1300 and 1000 Da respectively. It is possible in all of the described polymerizations that low molecular weight oligomers remained dissolved in the reaction medium and therefore resulted in fractionation of the polymer.

After our initial report,[28] Uyama and co-workers recently described a polycondensations of dicarboxylic acid diesters with 1,4-butanediol in [bmim][PF$_6$] and [bmim][BF$_4$] catalyzed with free lipase (*Candida antarctica*). Polycondensation reactions performed at ambient conditions in [bmim][PF$_6$] generated oligomers (M_n = 350 Da), although oligomers with increased molecular weights (M_n = 1500 Da) were formed at reduced pressure and increased polymerization times.[29]

Attempts to Improve Enzyme Activity in Ionic Liquids. Having shown free lipase to be inactive in the presence of all ionic liquids with the exception of [bmim][PF$_6$], several methods of enzyme stabilization were investigated to determine if conventional activating techniques would be useful. Adsorption onto an acrylic resin, PEG-modification, and immobilization via multipoint attachment in polyurethane foam are three methods that have been shown to prevent deactivating conformational changes in harsh environments and enhance enzyme activity.[15,30,31] Transesterifications catalyzed by the modified enzymes were performed to determine the effectiveness of the activating methods in ionic liquids.

Candida antarctica lipase, type B adsorbed onto a macroporous acrylic support (Novozym 435)[30] was used to catalyze transesterification in several ionic liquids and organic solvents. In hexane and acetonitrile, initial rates of 3.68 and 8.85 μM/hr/mg-enzyme were observed while in THF no lipase activity was observed. In [bmim][PF$_6$],[bmim][CH$_3$CO$_2$], [bmim][NO$_3$],

(27) Sheldon, R. A.; Lau, R. M.; Sorgedrager, M. J.; van Rantwijk, F.; Seddon, K. R. *Green Chem.* **2002**, *4*, 147−151.

(28) Mesiano, A. J.; Beckman, E. J.; Russell, A. J. *Abstract #279k, presented at the National Meeting of the American Institute of Chemical Engineers* **November 2000**.
(29) Uyama, H.; Tetsufumi, T.; Kobayashi, S. *Polymer J.* **2002**, *34*, 94−96.
(30) Arroyo, M.; Sanchez-Montero, J. M.; Sinisterra, J. V. *Enzyme Microb. Technol.* **1999**, *24*, 3−12.
(31) Inada, Y.; Takahashi, K.; Yoshimoto, T.; Ajima, A.; Matsushima, A.; Saito, Y. *Trends Biotechnol.* **1986**, *4*, 190−194.

[mmep][CH₃CO₂], [mmep][NO₃], [mmep][CH₃SO₃] no activity was detected.

PEG-modification of enzymes is another common technique for stabilizing enzymes in denaturing environments. Drevon and co-workers reported the ability to prepare a PEG-diisopropyl-fluorophosphatase (DFPase) conjugate. Their results indicate that a 100% extent of PEG-modification was achieved meaning that all of the native DFPase was modified. Matrix-assisted laser desorption/ionization mass spectrometry (MALDI MS) analysis confirmed that two to seven PEG chains attached per molecule of DFPase.[14] It has also been reported that in some organic solvents, PEG-modified enzymes exhibit increased stability when compared to their untreated form.[31] PEG-modification of *Candida rugosa* lipase was used to stabilize the enzyme in ionic liquids. Although PEG-modified lipase catalyzed the trans-esterification in hexane (0.75 μM/hr/mg-enzyme), no improved lipase activity was observed in [bmim][PF₆], [bmim][NO₃], and [mmep][CH₃CO₂] compared to the use of free *Candida rugosa* lipase.
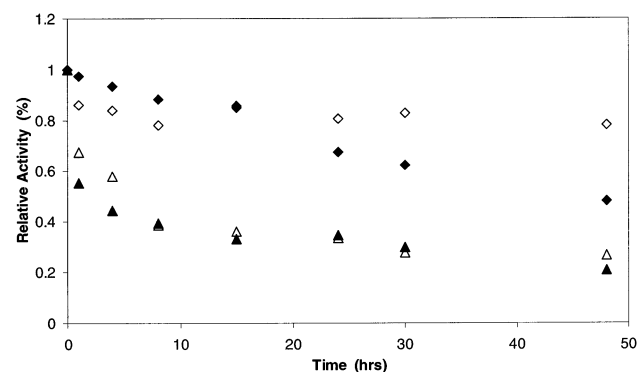
With adsorption and PEG-modification being unsuccessful in stabilizing lipase in ionic liquids, covalent immobilization was considered as another technique for enzyme stabilization. Immobilization of free lipase (*Candida rugosa*) within poly-urethane foam offers multi-point covalent attachment of the enzyme throughout the polymer matrix.[14] Drevon and Russell reported the ability to immobilized DFPase in polyurethane foam. After thorough rinsing with distilled water, only 1% (w/w) DFPase leached from the enzyme-containing polyurethane foams. This suggests that approximately 100% of DFPase is irreversibly immobilized in the polyurethane foams during synthesis. Additionally, the enzyme-containing polyurethane foams retained 67% of the relative activity of native DFPase when assayed against diisopropylfluorophosphate (DFP) in buffer.[32] Polyurethane immobilized lipase catalyzed the trans-esterification in [bmim][PF₆] at an initial rate of 0.93 μM/hr/mg-enzyme, however, no detectable activity was observed in [bmim][CH₃CO₂], [bmim][NO₃], [mmep][CH₃CO₂], and [mmep][CH₃SO₃] using polyurethane immobilized lipase thus demonstrating that this activating technique was ineffective.

Sheldon and co-workers reported similar results using immobilized forms of *Candida antarctica* lipase type B. Conversions of less than 5% were measured in a 24 h period in [bmim][NO₃] using cross-linked enzyme crystals and cross-linked enzyme aggregates.[27] The lack of immobilized lipase activity using a different lipase than was used in this work supports our conclusion that the described activation techniques would most likely prove ineffective in enhancing activity of other lipases in ionic liquids. Clearly, for the broad range of ionic liquids tested, ionic liquids are not the panacea for nonaqueous biocatalysis that we had hoped.

Enzyme Stability in Ionic Liquids. The stability of Novozym 435 and porcine pancreatic lipase in [bmim][PF₆] was investigated. The goal of the stability studies was to determine permanent, or irreversible, inactivation effects on lipase when in ionic liquids. This was achieved by incubating the lipase in the ionic liquid for a specified period of time after which the enzyme was isolated via extraction of ionic liquid, diluted with water, and assayed. Extraction of the ionic liquid was performed using a pipet with care taken to minimize enzyme loss. The

(32) Drevon, G. F.; Russell, A. J. *Biomacromolecules* **2000**, *1*, 571−576.
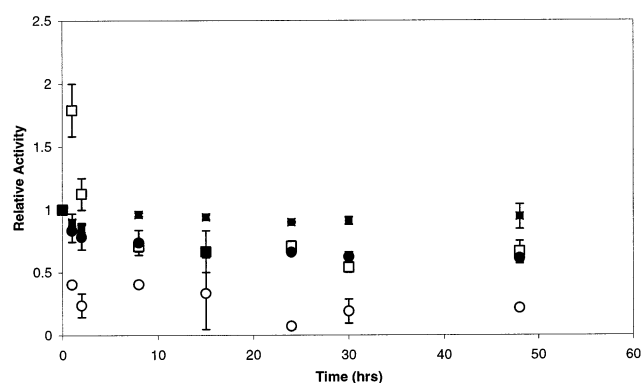
Figure 4. Stability of Novozym 435 in tetrahydrofuran (○) vs [bmim]-[PF6] (□) and procine pancreatic lipase in tetrahydrofuran (●) vs [bmim]-[PF₆] (■) at 50 °C.

Figure 5. Stability of Novozym 435 intetrahydrofuran (▲) vs [bmim][PF₆] (◆) and porcine pancreatic lipase intetrahydrofuran (△) vs [bmim][PF₆] (◇) at 50 °C in the presence of divinyl adipate and 1,4-butanediol.

relative activity measurements indicate the portion of lipase irreversibly inactivated as a result of incubation in ionic liquid. However, the stability studies do not demonstrate the stability of lipase in ionic liquids to catalyze a reaction. After 48 h suspended in [bmim][PF₆] at 50 °C, Novozym 435 and porcine pancreatic lipase retained approximately 67% and 94% of their original activities respectively (Figure 4). This represents an improvement over activity retentions of Novozym 435 (21%) and porcine pancreatic lipase (61%) in tetrahydrofuran. Interestingly, Novozym 435 lost no activity during the initial 50 min of incubation in [bmim][PF₆]. Incubation of Novozym 435 and porcine pancreatic lipase in [bmim][PF₆] over a two week period demonstrate that both lipases retained appreciable activity. After 48 h incubation in [bmim][PF₆] at 50 °C with DVA and BD substrates present, Novozym 435 and porcine pancreatic lipase retained 50% and 80% activity respectively (Figure 5).

High levels of activity retention upon incubation in [bmim]-[PF₆] for 120 h at 70 °C indicated both Novozym 435 and porcine pancreatic lipase (20% and 48% respectively) have increased thermal stability in the ionic liquid in comparison to octane (3% and 35%).

The stability of Novozym 435 was further investigated in the other ionic liquids via the same method (Figures 6 and 7). Incubation in [mmep][NO₃] and [bmim][NO₃] for 24 h at 30 °C resulted in 59% and 0% activity retention. Conversely, results of incubation in [mmep][CH₃SO₃], [bmim][CH₃CO₂], and [mmep][CH₃CO₂] under the same conditions demonstrate increases in enzyme activity once re-suspended in water. More specifically, [mmep][CH₃CO₂], [bmim][CH₃CO₂], and [mmep]-
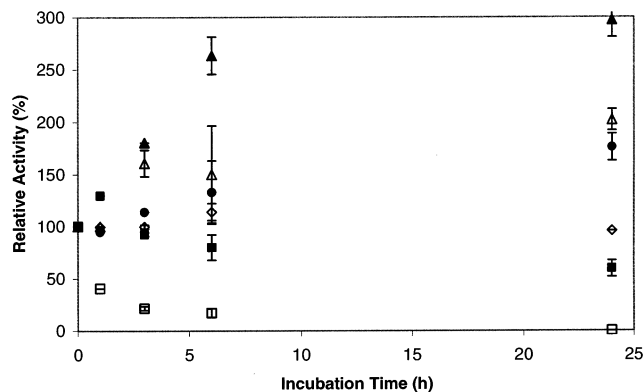
Figure 6. Stability of Novozym 435 at 30 °C in various ionic liquids ([bmim][PF$_6$] (◇), [bmim][NO$_3$] (□), [bmim][CH$_3$CO$_2$] (△), [mmep][NO$_3$] (■), [mmep][CH$_3$CO$_2$] (▲), and [mmep][CH$_3$SO$_3$] (●)). Error bars represent deviation from the mean for two separate experiments.
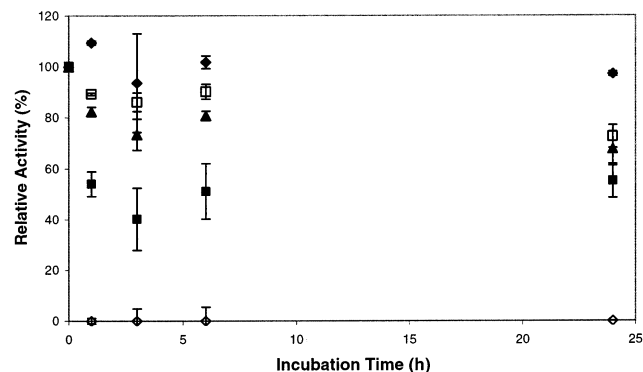
Figure 7. Stability of Novozym 435 at 30 °C in various organic solvents (butanol (◇), tetrahydrofuran (□), acetonitrile (▲), dimethyl sulfoxide (■), and hexane (◆)). Error bars represent deviation from the mean for two separate experiments.

[CH$_3$SO$_3$] suspended − Novozym 435 exhibited 297%, 202%, and 176% relative activity when returned to water, respectively. For comparison, under the same conditions, no activity loss was observed in hexane while Novozym 435 retained 73% of its activity in tetrahydrofuran. Activity retentions of 68% and 55%, respectively, were observed for incubation in acetonitrile and dimethyl sulfoxide, whereas in butanol the enzyme was completely deactivated. Interestingly, although these liquids support no enzyme activity, they cause irreversible activation of the immobilized enzyme. One plausible explanation is that these liquids swell the acrylic resin causing previously inaccessible enzyme to be accessed once returned to water. Another possibility is that the ionic liquid exposed enzyme has a modified tertiary structure with increased activity in water. Although this seems less likely we cannot rule out that possibility.

Sheldon and co-workers recently studied the stability of free lipase (*Candida antarctica*), Novozym 435, cross-linked lipase crystals, and cross-linked lipase aggregates in several ionic liquids. They reported that after 100 hrs of incubation in [bmim]-[PF$_6$] at 80 °C, an increase in free lipase activity (120%) was observed. Incubation of Novozym 435 also resulted in increased activity (350%) after 40 h of incubation in [bmim][PF$_6$] at 80 °C in comparison to untreated free lipase. They suggest that the ionic liquid coated and thus protected the layer of essential water surrounding the lipase,[27] but it is hard to explain increases in activity of the free lipase through a merely protective mechanism. We believe that one must invoke a permanent activating conformational change or an increase in active site concentration to explain this unusual data.

Conclusions

Our previous work has already demonstrated the feasibility of ionic liquids as media for biocatalysis. Solvatochromic analysis and partition coefficients suggest ionic liquids are highly polar and hydrophilic in nature in comparison to organic solvents such as hexane, acetonitrile, and tetrahydrofuran. In this study, we examined the lipase-catalyzed transesterification of methyl methacrylate and the polytransesterification of divinyl adipate and 1,4-butanediol in a variety of hydrophobic and hydrophilic ionic liquids. Although the activity of free lipase in [bmim]-[PF$_6$] was greater than in hexane, no reaction occurred in the hydrophilic ionic liquids investigated. Conventional methods of enzyme stabilization including adsorption, PEG-modification, and covalent immobilization in polyurethane foams proved ineffective in the hydrophilic ionic liquids. Polytransesterifications performed in [bmim][PF$_6$] produced polyesters of limited molecular weight due to precipitation of the polymer. Stability studies indicate that reversible inactivation of lipase is observed when the enzyme is suspended in [bmim][CH$_3$CO$_2$], [mmep][CH$_3$-CO$_2$], and [mmep][CH$_3$SO$_3$], whereas incubation in [bmim]-[NO$_3$] and [mmep][NO$_3$] irreversibly inactivates lipase. To use hydrophilic ionic liquids as solvents for biocatalytic transformations, the mechanism of inactivation must be more clearly understood.