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Enzymatic synthesis of poly(hydroxyalkanoates) in ionic liquids

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Abstract

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.

Keywords: Ionic liquids; Poly(hydroxyalkanoates); PHAs; CALB; Ring-opening polymerization; Lactones

1. Introduction

Poly(hydroxyalkanoates) (PHAs) offer a wide range of physical properties, are produced naturally by bacteria, and are biodegradable and biorenewable (Anderson and Dawes, 1990; Pouton and Akhtar, 1996). 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 (Anderson and Dawes, 1990; Terada and Marchessault, 1999). Current *in vitro* routes are not much better. Lipase-catalyzed polymerizations usually require volatile organic solvents (Knani et al., 1993) because lipase-catalyzed bulk polymerizations create viscous solutions that are difficult to work with. PHA-synthase catalyzed reactions, the major alternative to lipases, require expensive coenzyme-A functionalized precursor (Su et al., 2000).

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 (Wasserscheid and Welton, 2003). Some ionic liquids can readily dissolve cellulose (Swatloski et al., 2002). Ionic liquids have been shown to stabilize enzymes (Lau et al., 2000). Further, enzyme activity and lifespan is often greater in ionic liquids than in common organic solvents (Lozano et al., 2001; Park and Kazlauskas, 2003). 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

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cost. Recycling of the ionic liquid could further reduce the cost. For example, Itoh and coworkers recycled an ionic liquid 10 times in a lipase-catalyzed reaction (Itoh et al., 2003). They extracted the low molecular weight compounds with diethyl ether followed by vacuum to remove traces of diethyl ether.

Although many ionic liquids have toxicities comparable to organic solvents, their lack of vapor pressure makes them safer to use since they are easier to contain (Jastorff et al., 2003; Stepnowski et al., 2004). One exception is the fluorine-containing ionic liquids (those with PF_6^-, BF_4^-, and $N(SO_2CF_3)^-$ anions), which are more cytotoxic than common organic solvents (Ranke et al., 2004; Stolte et al., 2006).

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 (Nobes et al., 1996; Kumar et al., 2000; Mei et al., 2003). 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. Materials and methods

2.1. Enzymes

Lyophilized forms of the enzymes *C. antarctica* lipase A (CALA, Roche Chirazyme L-5, 6.5 U g^{-1}), *C. antarctica* lipase B (CALB, Roche Chirazyme L-2, 443 U g^{-1}), *Candida rugosa* esterase (CRE, 10.4 U g^{-1}), *C. rugosa* lipase (CRL, Meito Sangyo Lipase OF, 370 U g^{-1}), cross-linked enzyme crystal of *C. 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 Lipse PS, 483 U g^{-1}), *Pseudomonas fluorenscens* 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 *C. 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. (2005), where 1 U is defined as 1 μmol pNPAc hydrolyzed per minute.

2.2. 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 dicyanamde OMIM [DCA] by anion exchange with halide-containing ionic liquids according to established literature procedures, as described below (Cammarata et al., 2001; Huddleston et al., 2001; Lau et al., 2004; Liu et al., 2005).

In a typical synthesis, the halide salt was prepared by adding methylimidazole to the desired alkyl chloride (1.05:1 mol/mol) 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 rotatory 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) 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 rotatory 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 h and stored in a dessicator 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 (Park and Kazlauskas, 2001). Water content was measured by ^{1}H NMR or by Karl–Fisher titration (Seddon et al., 2000). 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, 0.5, and 0.1 wt%, respectively.

2.3. 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, $\delta \sim 3.6$ ppm) and the repeat unit (t, $\delta \sim 4.0$ ppm) were compared (Cayuela et al., 2006).