Chemoenzymatic Route to Poly(3-hydroxybutyrate) Stereoisomers

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ABSTRACT: Racemic butyrolactone (BL) was selected to determine the feasibility of using enzyme catalysis in organic media for β-substituted β-propiolactone resolutions. Lipases from *Candida cylindracia*, *Pseudomonas fluorescens* (PS-30), and porcine pancreatic lipase Type II (PPL) were investigated. Based on reaction rate and the enantiomeric ratio *E*, PPL was found to be the most effective of these lipases. With respect to the organic media used, BL methanolysis occurred faster to slower as follows: *n*-hexane > diethyl ether > dioxane ≅ ethyl acetate > benzene. PPL-catalyzed BL methanolysis in *n*-hexane and diethyl ether reached ∼50% conversion in 22 h. Irrespective of the solvent used, the slower reacting enantiomer was (*R*)-BL. The *E* values for PPL-catalyzed BL resolutions carried out in diethyl ether, benzene, ethyl acetate, 1,4-dioxane, and hexane were 20.4 ± 2.4, 14.8 ± 2.3, 8.94 ± 1.28, 8.15 ± 1.04, and 5.56 ± 0.37, respectively. Thus, using PPL as the lipase in ether, 90%-(*R*)-BL was obtained in 35% yield. This monomer was polymerized using $Zn(C_2H_5)_2/H_2O$ (1/0.6) as the catalyst to form predominantly (*R*)-poly(3-hydroxybutyrate) (P3HB) which had number-average molecular weight, melting point, and enthalpy of melting values of 41 300, 140 °C, and 64.6 J/g, respectively. Thus, a chemoenzymatic route to high molecular weight enantiomerically enriched P3HB was demonstrated.

Introduction

This study was directed toward developing synthetic routes to enantiomerically pure or enriched substituted microbial polyester synthetic analogues that would provide suitable flexibility in regards to polymer repeat unit structure and stereochemical composition. As a model system, poly(3-hydroxybutyrate) (P3HB) formed by the ring-opening of β-methyl-β-propiolactone (BL) was investigated. P3HB is a member of the microbial polyester family referred to as poly(β-hydroxyalkanoates) (PHA). The chiral centers of PHA repeat units have (*R*) stereochemical configurations[1,2] so that the polymers are isotactic and optically active.[3−5] A number of reviews have been published that describe various aspects of PHA research including the wide range of β-substituents that have been incorporated into these microbial products.[6−11] It is interesting to consider that the invariability of polymer stereochemistry for natural PHA is problematic since the formation of polymer stereoisomers is a powerful tool in modulating the properties of polymeric materials. For example, work by Abe *et al.*[12] showed how by altering the stereochemical composition of P3HB prepared by chemical methods (see below) polymeric materials were formed having elongation to break values ranging from 5% to 1020%. Indeed, by controlled variation of polymer stereochemistry for biodegradable materials, it is envisioned that both polymer properties and biodegradability can be carefully balanced to achieve superior materials. Work in our laboratory[13] as well as elsewhere[12] supports this idea.

Recently, a breakthrough was reported for the rapid polymerization of BL to high molecular weight products which may also prove useful for other β-substituted β-propiolactone. Specifically, Hori *et al.*[14] has shown that distannoxane catalysts can polymerize BL in 4 h reaction times at 100 °C to form products in yields of >95% having M_n values of approximately 150 000. Much effort has also been focused on the use of Al- and Zn-based catalyst systems for the preparation of synthetic PHA analogues.[15] In parallel with the above, research has been carried out to prepare enantioenriched BL. Agostini *et al.*[16] resolved β-bromobutyric acid by reaction with (*R*)-(+)-α-(1-naphthyl)ethylamine and separation of the resulting diastereomers. The (*S*)-β-bromobutyric acid having an enantiomeric excess (ee) of >90% was ring-closed to form (*R*)-BL having an ee of 73%. (*S*)-BL having an optical purity in excess of 97% was prepared in five steps from natural origin (*R*)-P3HB.[17] (*R*)- and (*S*)-BL enantiomers having optical purities >98% were prepared by the asymmetric hydrogenation of methyl acetoacetate, conversion of (*R*)- or (*S*)-methyl-3-hydroxybutyrate to its corresponding free acid, ring-closure by transacetalization to form the cyclic othocarbonate, and then pyrolysis.[13] All of the above synthetic methods either are tedious involving multistep reactions or do not provide BL of high enantiopurity. In a recent report by Takaya *et al.*,[18] (*R*)-BL was prepared with ee values of 92% by the asymmetric hydrogenation of diketene. This method offers an efficient route to optically active BL isomers but is limited in utility since it cannot be used to prepare other enantioenriched β-substituted β-propiolactones. A route to enantioenriched mono- and di-β-substituted β-propi-

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olactones which involves the reaction of aldehydes and ketones with ketene in the presence of the alkaloids quinine and quinidine was reported by Wynberg and Staring.[19] The reaction required the use of aldehydes and ketones which had a high polarization of the carbonyl such that substituents such as $-CCl_3$ and $-CCl_2CH_3$ were used. In addition to chlorinated substituents, monomers with β-(trifluoromethyl)-β-methyl or ethyl have been prepared, and a number of these halogenated optically active monomers have been polymerized.[20−22]

In an alternative strategy, chiral catalysts have been used to form enantioenriched P3HB. Specifically, Spassky *et al.*[23] used a chiral initiator prepared from $Zn(C_2H_5)_2$ and (*R*)-(−)-3,3-dimethyl-1,2-butanediol which when used for racemic BL polymerizations resulted in small enantiomeric enrichment in the final polymer. Takeichi *et al.*[24] used an optically active cobalt/triethylaluminum complex to prepare an (*R*)-enriched product where the extent of stereoselection was not reported. Thus far, this route has not proved useful for the preparation of P3HB or other PHA with high enantiopurity.

Commercially available enzymes have proven to be powerful tools for the resolution of racemic mixtures to obtain products in high enantiopurity.[25−27] Lipases such as porcine pancreatic lipase (PPL), pig liver esterase (PLE), horse liver esterase (HLE), *Pseudomonas fluorescens* lipase PS-30, and *Candida cylindracea* lipase (CCL) have been widely used for the resolution of racemic alcohols and carboxylic acids by catalyzing enantioselective esterification/transesterification reactions. Successful examples of γ, δ, and ϵ-lactone resolutions in aqueous media by using PLE, PPL, and HLE lipases have been reported.[28−30] The use of enzymes in organic media has shown promising substrate conversion efficiency, high enantioselectivity, and advantages relative to reactions in aqueous media such as catalyst recyclability, increased enzyme thermal stability, solubility of a wide range of substrate types in the reaction media, and no requirement for pH adjustment as the reaction proceeds.[31−32] Lipases in organic media have been used successfully for the preparation of enantioenriched γ-butyrolactones, ω-lactones, and δ-lactones by lactonization of racemic γ-hydroxy esters, ω-hydroxy esters, and δ-hydroxy esters, respectively.[33−35]

This paper describes a model study to determine the feasibility of using enzyme catalysis in organic media for the resolution of β-substituted β-propiolactone enantiomers. The resolved lactones could then be polymerized via chemical methods to form substituted poly(β-esters) having the desired stereochemical purity and configuration. For this study, we chose the racemic lactone BL and evaluated various lipases including PPL, CCL, and PS-30. The effect of the lipase-organic solvent pairs used on the rate of BL reactivity and enantioselectivity was determined. Using enantioenriched BL prepared by enzymatic resolution, P3HB having 90% (*R*) repeat units was synthesized.

Experimental Section

Materials. Porcine pancreatic lipase Type II (PPL), *Candida cylindracia* lipase Type VII (CCL), and *p*-nitrophenyl acetate were purchased from Sigma Chemical Co. and *Pseudomonas fluorescens* lipase PS-30 was from Amano Enzyme USA. All the enzymes were used as received. Measurements of esterase activity for these enzymes in organic media are described below.

n-Hexane, diethyl ether, 1,4-dioxane, ethyl acetate, benzene, methanol (Aldrich, >99%), and benzyl alcohol (Eastman Kodak, 98%) were dried by reflux over CaH_2 overnight prior to distillation under argon. BL (Aldrich, 98%) was refluxed over CaH_2 overnight prior to fractional distillation (74−75 °C/25 mmHg). Values for water contents of the dried solvents were determined using a Karl-Fischer automatic titrator as was described elsewhere[36] and were found to be 39 ± 5, 55 ± 4, 160 ± 11, 84 ± 4, 57 ± 5 and 98 ± 9 ppm for *n*-hexane, diethyl ether, 1,4-dioxane, ethyl acetate, benzene, and methanol, respectively.

Assay of PPL, PS-30 and CCL Activity.[37] The enzymes were assayed using *p*-nitrophenyl acetate (pNPA) and methanol in 1,4-dioxane. A 1,4-dioxane solution (5 mL) containing pNPA (1.45 mmol/L) and methanol (2.82 mmol/L) was added to 10 mL vials containing approximately 17 mg of enzyme. The assay reactions were carried out for 2 h in an orbital shaker (2000 rpm, 35 °C) and were terminated by removal of the enzyme by filtration. The concentration of the reaction product *p*-nitrophenol (pNP) was determined by UV-VIS (GBC UV−vis 916) at the λ_{max} (305 nm) of pNP. Enzyme activities for PPL, CCL, and PS-30 are defined herein as the nanomoles of pNPA hydrolyzed in dioxane per unit weight of enzyme per minute (nmol of pNP/min·mg) and have values of 1.2, 1.2, and 3.0, respectively.

Enzymatic Resolution of BL. All glassware was oven-dried at about 150 °C overnight and then cooled in a desiccator (containing Drierite) prior to use. For studies to determine effects of solvent and enzyme on monomer conversion and enantioselectivity, methanol was used as the nucleophile. The enzymes were transferred in a dry bag containing Drierite as desiccant under an argon purge. Liquids were transferred via syringe under an argon atmosphere. BL (1.032 g, 12.0 mmol), anhydrous methanol (0.768 g, 24.0 mmol), and a dried solvent (13.0 mL) were added into 20 mL vials containing 600 mg of enzyme. The reaction vials were capped, magnetically stirred, and maintained at 35 °C for reaction times as specified below. The reactions were terminated by removing the enzymes by filtration using a 0.45 μm Teflon syringe filter, and BL conversions were determined by FTIR as described below. After concentration of solutions by rotoevaporation, the enantioenriched BL was separated from the reaction mixtures by column chromatography (column dimensions of 2.5 cm × 24 cm) using silica gel (purchased from Aldrich, grade 60, Merck, 230−400 mesh, 60 Å) as the stationary phase, methylene chloride as the eluent, a flow rate of ∼2 mL/min, and a ratio of ∼200:1 silica gel to mixture. The major fractions containing BL eluted at volumes between 80 and 120 mL. Larger scale resolution of BL (20.0 g, 0.233 mol) was carried out using PPL, dry diethyl ether as solvent, and dry benzyl alcohol in place of methanol with the same relative molar ratios of reagents and similar precautions to avoid water that were described above. The resolution was carried out in a three-neck 500 mL round-bottom flask at 35 °C for 4 days (∼55% monomer conversion). The workup involved removal of enzyme by filtration, solvent rotoevaporation at 25 °C, and distillation in both the absence and presence of CaH_2 as described above for racemic BL. This gave a colorless liquid (yield 35%) which had $[\alpha]^{25}_D = +23.8°$ (5.0 g/dL, $CHCl_3$) (lit.[41] +30.3° [*c* 4.3, $CHCl_3$] for (*R*)-BL and lit.[42] −30.2° (*c* 3.98, $CHCl_3$) for (*S*)-BL). The ^{1}H NMR spectrum of this product was identical to that published previously[17].

Instrumentation Methods. FTIR (Mattson Instruments Galaxy Series 2020) was used to determine lactone conversions to their corresponding methyl esters. Calibration curves (correlation coefficients of higher than 0.992) were obtained by dilute solution measurements in CCl_4 using a series of BL/methyl 3-hydroxy-3-methylpropionate mixtures of known composition and measuring the ratio of lactone (1835 cm^{-1}) to ester carbonyl group (1735 cm^{-1}) absorptions in a solution cell (6 mm path length). For reactions in hexane, 1,4-dioxane, and diethyl ether, sufficient quantities of the reaction mixtures were added to CCl_4 so that the transmittance of the lactone absorption band was ∼30%. When using ethyl acetate and benzene, solvent removal by rotoevaporation was carried out prior to addition of the reaction mixture to CCl_4.[37]

The resolved BL was purified by either column chromatography or distillation. The enantiomeric excess (ee) of BL was

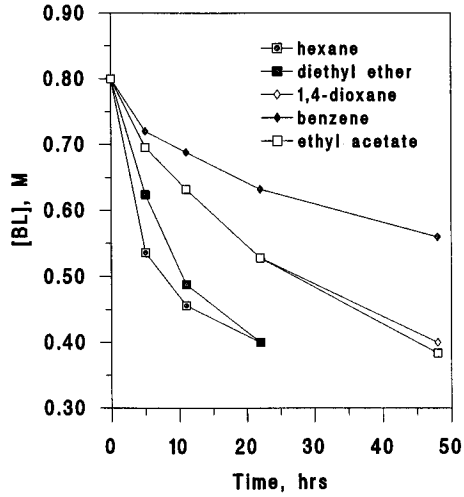

Figure 1. Concentration of BL as a function of reaction time and organic media for PPL-catalyzed BL methanolysis.

determined by ^{1}H NMR (Bruker WP-270 SY or Varian Unity 300 spectrometers) using the chiral shift reagent tris[3-((heptafluoropropyl)hydroxymethylene)-(+)-camphorato]europium(III) (Eu-(+)-[hfc]$_3$)[13] and polarimetry (Perkin-Elmer 241 polarimeter).

Determination of P3HB thermal transitions by differential scanning calorimetry (DSC) and molecular weight by gel permeation chromatography (GPC) was carried out as previously described.[13]

Results and Discussion

PPL-Catalyzed Resolution of BL. The effect of the organic solvent used on the PPL-catalyzed conversion of BL to its corresponding methyl ester was studied. The concentration of BL as a function of time for reactions carried out in hexane, diethyl ether, 1,4-dioxane, benzene, and ethyl acetate is plotted in Figure 1. It is shown that BL methanolysis occurs most rapidly in *n*-hexane followed closely by diethyl ether. For both of these solvents, approximately 50% conversions are reached by 22 h reaction times. Reaction rates in ethyl acetate and 1,4-dioxane are similar (50% conversions in 48 h), while reactions in benzene occur at the relatively slowest rate (29% conversions in 48 h). Thus, the following order of solvents corresponds to media where BL conversions occurred at faster to slower rates: *n*-hexane > diethyl ether > 1,4-dioxane ≅ ethyl acetate > benzene. When these reactions were conducted without enzyme addition, no methyl ester formation was observed (by FTIR) within a 48 h time period; thus the above conversions result from enzyme catalysis.

The enantiomeric ratio E is a measure of the enantioselectivity of an enzymatic transformation. Based on work by Chen *et al.*,[38−39] E can be determined by eq 1.

$$E = \frac{V_{\mathrm{maxA}}/K_{\mathrm{A}}}{V_{\mathrm{maxB}}/K_{\mathrm{B}}} = \frac{\ln[(1-c)(1-\mathrm{ee})]}{\ln[(1-c)(1+\mathrm{ee})]} \qquad (1)$$

In eq 1, (A) and (B) are the fast- and slow-reacting enantiomers, respectively; c is the conversion ($[C_{\mathrm{initial}} - C_{\mathrm{final}}]/C_{\mathrm{initial}}$, C is BL concentration) of the reaction; ee is the enantiomeric excess ($[C_{\mathrm{A}} - C_{\mathrm{B}}]/[C_{\mathrm{A}} + C_{\mathrm{B}}]$) of the unreacted substrate; and V_{max} and K are the maximal reaction rate and Michaelis constant, respectively. The ee values were measured experimentally from ^{1}H NMR spectra recorded in the presence of a chiral shift reagent (see Experimental Section). Monomer conversion as a function of time was conveniently monitored by FTIR, taking advantage of the decrease in the lactone carbonyl absorbance at 1835 cm^{-1} and increase in the absorbance at 1735 cm^{-1} with the formation of the corresponding ring-opened methyl ester (see Experimental Section).

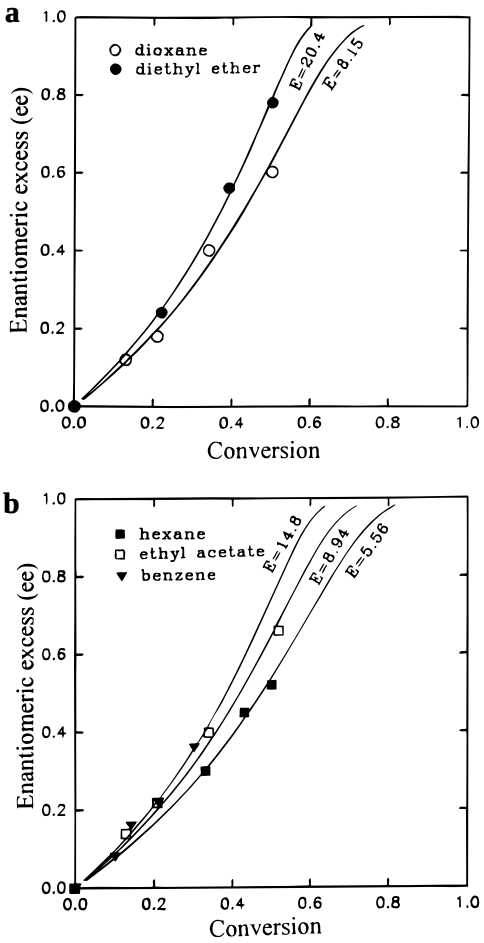

Figure 2. Relationships of enantiomeric excess (ee) of BL and conversion of PPL-catalyzed BL methanolysis: (a) in dioxane and diethyl ether; (b) in hexane, ethyl acetate, and benzene. Dots are experimental data and solid lines are generated by curve fitting using eq 1.

The absolute configuration of the enantioenriched BL stereoisomer (slower reacting enantiomer) in all of the solvents investigated was (*R*) based on the positive optical rotation[13] and the relatively higher intensity of upfield doublets corresponding to the (*R*)-antipode in ^{1}H NMR experiments with chiral shift reagent Eu-(+)-[hfc]$_3$.[17]

Figure 2 shows the relationship between conversion and enantiomeric excess for PPL-catalyzed methanolysis reactions carried out in different solvents. Using eq 1 and a curve-fitting computer program, theoretical curves were generated that fit well with the experimental ee versus conversion results. Since the theoretical curves are in close agreement with the experimental data points, it is reasonable to assume that BL resolution by PPL occurs by a mechanism that agrees with the assumptions used for the derivation of eq 1.[38−39] The E values and corresponding standard deviation from the curve fit of the experimental results for reactions carried out in diethyl ether, benzene, ethyl acetate, 1,4-dioxane, and hexane are 20.4 ± 2.4, 14.8 ± 2.3, 8.94 ± 1.28, 8.15 ± 1.04, and 5.56 ± 0.37, respectively. Recalling from above that the highest rates of BL conversion were

obtained in diethyl ether and hexane, it can be concluded that diethyl ether is the preferred organic media to achieve high BL enantioselectivity as well as conversion rates. It is interesting to note that the *E* values for resolutions of α-methyl-β-propiolactone (MPL) using PPL were much smaller, regardless of the organic media used.[37] For example, the largest *E* value measured for PPL-catalyzed MPL resolutions was 3.93 ± 0.26 in diethyl ether.

In addition to methanol, benzyl alcohol was explored as a nucleophile for the resolution of BL in diethyl ether. The rationale was to form the corresponding benzyl as opposed to methyl β-hydroxybutyrate ester, which would facilitate separation of unreacted lactone and ester by distillation (boiling points of BL and benzyl 3-hydroxybutyrate are 70 °C/30 mmHg and 90 °C/10^{-3} mmHg, respectively[17]). The curve of BL consumption in diethyl ether as a function of reaction time using benzyl alcohol was almost identical to that obtained when methanol was used (not shown). However, the *E* value was relatively lower (8.0 ± 1.0 as compared to 20.4 ± 2.4). Nevertheless, resolutions of BL on a 20 g scale were readily carried out (see Experimental Section) and BL (ee 80%, 90% (*R*)) was obtained.

Investigations carried out with other lipases to resolve BL gave relatively poorer results. In summary, the lipase CCL showed no apparent catalytic activity (by FTIR, see Experimental Section) in diethyl ether at 35 °C for 48 h. It is noteworthy that the catalytic activity of PPL and CCL in nmol pNP/min·mg were identical (1.2). Although investigations of CCL-catalyzed resolutions in other organic media might have shown improved results, the results in diethyl ether were not promising. In contrast, PS-30-catalyzed BL methanolysis, regardless of the organic media used, proceeds slower relative to PPL despite the fact that PPL has an activity for pNPA methanolysis in dioxane which is 2.5 times lower (see Experimental Section). For example, the conversion of BL in both hexane and diethyl ether by PS-30 catalysis was only 38% after 72 h compared to 50% in 22 h when PPL was used. Furthermore, with the exception of hexane, the calculated *E* values based on experimental conversion and ee measurements were considerably higher for PPL- than PS-30-catalyzed BL resolutions. Specifically, *E* values in diethyl ether, benzene, ethyl acetate, 1,4-dioxane, and hexane were 5.2, 3.8, 3.8, 2.7, and 7.0, respectively. Thus, when one considers both rates of monomer conversion and enzyme enantioselectivity for the three lipases studied, PPL is the preferred enzyme for BL resolution. In contrast, for MPL resolution, PS-30 is preferred relative to PPL due to the higher enantioselectivity achieved.[37]

Chemical Polymerization of Enzyme-Resolved Monomers. The (*R*)-enriched BL having 90% (*R*) (90%-(*R*)-BL) was used to prepare enantioenriched P3HB following a literature method.[17] The $Zn(C_2H_5)_2/H_2O$ (1/0.6) catalyst system used is believed to result in ideal random stereocopolymers.[40] Furthermore, ring-opening polymerization of BL catalyzed by $Zn(C_2H_5)_2/H_2O$ (1/0.6) occurs by acyl cleavage (bond breaking between the carbonyl carbon and ring oxygen) with retention of configuration and no apparent racemization.[17] Thus the product formed presumably has a stereochemical composition equivalent to the monomer feed. The P3HB (90%-(*R*)-P3HB, M_n = 41 300 and M_w/M_n = 1.57 by GPC) had a T_m and ΔH_f of 140 °C and 64.6 J/g, respectively. These values are closely consistent with that expected based on previous work which showed that P3HB having 92% (*S*) repeat units (92%-(*S*)-P3HB, M_n = 34 000 g/mol, M_n/M_w = 1.5, obtained from polymerization using $Zn(C_2H_5)_2/H_2O$ (1/0.6) as catalyst) had a T_m and ΔH_f of 136 °C and 64.3 J/g, respectively.[40] Work by us and others has already studied (*R*)-enriched P3HB stereoisomers with respect to microstructure analysis by ^{13}C NMR,[17] physico-mechanical properties,[12] melting behavior,[12,13] crystallization kinetics,[12] and biodegradability.[12,13] Therefore, similar investigations on 90%-(*R*)-P3HB and other P3HB stereoisomers were not undertaken herein.

Summary

A chemoenzymatic route to enantioenriched P3HB which involved resolution of racemic BL using lipase catalysis and subsequent polymerization of predominantly (*R*)-BL was demonstrated. Of the lipases investigated, PPL was found to be preferred for BL resolution when considering both reaction rates and enantioselectivity. Using diethyl ether as the organic medium for PPL-catalyzed resolution, 90%-(*R*)-BL was conveniently prepared in 35% yield. Thus, using the strategy shown herein, it should be possible to extend this work to other β-substituted β-propiolactone monomers so that synthetic analogs of microbial polyesters that have controlled repeat unit structure and stereochemistry can be obtained.

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