Poly(3-hydroxybutyrate)-depolymerase from *Pseudomonas lemoignei*: Catalysis of Esterifications in Organic Media

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Lipase catalysis in nonaqueous media is recognized as a powerful tool in organic and more recently polymer synthesis. Even though none of the currently known polyhydroxyalkanoate (PHA) depolymerases have lipase activity, they do have a catalytic center that resembles that of lipases. Motivated by the above, the potential of using the poly(3-hydroxybutyrate), PHB, depolymerase from *Psuedomonas lemoignei* in organic media to catalyze ester-forming reactions was investigated. The effect of different organic solvents (benzene-d_6, cyclohexane-d_{12}, and acetonitrile-d_3) on the activity of the PHB-depolymerase toward propylation of L-lactide was studied. A significant difference in the catalytic rate was observed as a function of solvent polarity. The selectivity of the PHB-depolymerase (*P. lemoignei)* to catalyze the propylation of a series of different lactones including ϵ-caprolactone, δ-butyrolactone, γ-butyrolactone, and D, L, meso, and racemic lactides has been studied with the PHB-depolymerase (*P. lemoignei)* in organic solvents. Important differences in the reactivity of these lactones, as well as selective hydrolysis of stereochemically different linear lactic acid dimers, were observed. Moreover, the ability of the PHB-depolymerase to catalyze the solventless polymerization of ϵ-caprolactone and trimethylene carbonate was investigated.

Introduction

Motivations to minimize the environmental impact of chemical processes have spawned impressive innovations in biocatalytic reactions using both whole cell and in vitro catalysis.[1] An interesting example has been the microbial synthesis and enzymatic degradation of polyhydroxyalkanoates (PHAs).[2] The ability to degrade polyesters in the PHA family is widely distributed among different microorganisms and depends on the secretion of specific hydrolases, which degrade the polymer to water-soluble products. Many PHA-depolymerases from bacteria and fungi have been characterized.[3,4]

PHA-depolymerases have structures that consist of (1) a large catalytic domain, (2) a linking domain, and (3) a C-terminal substrate-binding domain. Three strictly conserved amino acids, namely, serine, aspartate, and histidine, constitute the catalytic triad at the active center of the catalytic domain. The conserved serine is part of the so-called lipase-box pentapeptide (Gly-Xaal-Ser-Xaa$_2$-Gly) that has been found in all known serine hydrolases such as lipases, esterases, and serine-proteases. The hydroxyl of this residue that attacks the ester bond of an enzyme substrate is increased in nucleophilicity through general-base catalysis by the imidazole ring of histidine. The positive charge on the imidazole ring is stabilized by the carboxylate group of the aspartate, and the resulting negatively charged tetrahedral carbon atom of the transient state is stabilized by a hydrogen bond to a main chain peptide bond (oxyanion hole). Thus, the catalytic centers of PHA-depolymerases resemble those of lipases. However, none of the currently known PHA-depolymerases have lipase activity. In contrast, some lipases have PHA-depolymerase activity when the substrates are ω-hydroxyacids, e.g., poly(4-hydroxybutyrate) (PHB) or poly(6-hydroxyhexanoate).[5] Apparently, lipases and PHA-depolymerases differ in the spatial arrangement of their active sites. Also, lipases generally do not accept polymeric substrates having side chains along the carbon backbone.[3]

The utility of hydrolases, most notably lipases and proteases, for both ester and amide bond formation in nonaqueous media, continues to increase in technological importance. This is reflected in the wide participation of many research groups in this area and the resulting large

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number of publications.[1] The rationale for employing enzyme-catalyzed reactions in organic media, as well as the manipulation of the solvent character to control these reactions, has been discussed at length elsewhere.[6] Since lipases and proteases are such powerful catalysts for organic transformations, it seems critical to extend our knowledge of how other ester hydrolase families may contribute new attributes to enzyme catalysis in nonaqueous media. Hence, in this paper, we prepared and purified a PHB-depolymerase from *Pseudomonas lemoignei* and studied its activity and specificity for the catalysis of esterification reactions of cyclic esters and carbonates in organic media. To the best of our knowledge, the use of PHB-depolymerases in nonaqueous media for bond formation has not yet been investigated.

Material and Methods

General Chemicals and Procedures. All chemicals and solvents were of analytical grade and were used as received unless otherwise noted. ϵ-Caprolactone donated by Union Carbide was distilled at 97−98 °C over CaH_2 at 10 mmHg. Lactides donated by Cargill were recrystallized from ethyl acetate before use. β-Butyrolactone, *n*-propanol, benzene-d_6, cyclohexane-d_{12}, and acetonitrile-d_3 were procured from Aldrich Chemical Co. and were dried over 4 Å molecular sieves before use. ^{1}H nuclear magnetic resonance (NMR) spectra were recorded on a Bruker ARX-250 and -200 spectrometers at 250 and 200 MHz. ^{1}H NMR chemical shifts (in parts per million or ppm) are reported downfield from 0.00 ppm using tetramethylsilane (TMS) as the internal reference. The instrumental parameters were as follows: temperature 300 K, pulse width 7.8 μs (30°), 32 K data points, 3.178-s acquisition time, 1-s relaxation delay, and 16 transients. ^{13}C NMR spectra were recorded at 62.9 and 50 MHz on a Bruker ARX-250 spectrometer with chemical shifts in ppm referenced relative to TMS at 0.00 ppm. Infrared (IR) spectra were recorded by using a Perkin-Elmer FTIR model 1720X spectrometer. Optical rotation values were measured by using a Perkin-Elmer model 241 polarimeter and are reported as follows: $[\alpha]_{589}^{25}$ = specific rotation (concentration in g/100 mL of solvent). Gas chromatography (GC) experiments were performed on a Hewlett-Packard instrument fitted with a γ-cyclodextrin chiral column.

Preparation and Assay of PHB-depolymerase. *P. lemoignei* was grown in 10 L of Stinson and Merrick's mineral medium[7] with 50 mM sodium succinate in a Braun Biostat fermentor (model ES 10) at 30 °C for 24 h. The conditions of maximal PHB-depolymerse production by *P. lemoignei* have recently been described.[8] The cell-free culture fluid was obtained by centrifugation, and the proteins were concentrated 10-fold by ultrafiltration (exclusion size 30 kDa). The concentrate was subjected to ammonium sulfate precipitation (20−60% saturation), and the pellet of the 60% step was dialyzed against 5 mM MES-TRIS buffer. The supernatant of the centrifuged dialysate was lyophylized and stored at −20 °C. After solubilization of the lyophylized enzyme in water or 2-propanol, the activity of the enzyme was assayed by the clearing zone formation of opaque PHB granules-containing agar (100 mM Tris-HCl, pH 8.0, 1 mM $CaCl_2$, 1.5% [wt/vol] agar) as was described in detail elsewhere.[9] The PHB granules were isolated from sodium gluconate grown *Ralstonia eutropha* H16 by digestion with sodium hypochlorite and subsequent purification with acetone ether.[10] The weight-average (M_w) and the polydispersity index (M_w/M_n) of the isolated polyesters determined by gel permeation chromatography using polystyrene standards were 2.3×10^6 g/mol and 2.1, respectively. The activity of the enzyme was also measured by determination of the decrease in the optical density (660 nm) of natural PHB suspensions in TRIS-HCl buffer (50 mM), $CaCl_2$ (1 mM), pH 8.0.[10] Activity of the lyophylysate after incubation for 3 h at 70 °C in benzene was measured. The assay was performed by taking 2 μL of the PHB-depolymerase solution (stock solution 1 mg/0.02 mL) in water and dropping it onto the surface of the plate. After the drop had diffused into the agar, the Petri disk was incubated in an oven at about 37 °C. Clearing zone formation was visible after 1 h, indicating strong activity. For comparison, the activity of the lyophylysate was determined after incubating at room temperature for 3 h. It was found that the clearing zones for both the lyophylysates after incubating for 3 h at room temperature and 70 °C in benzene were identical. Hence, thermal inactivation under these conditions was not observed.

Preparation of (*R*)-Propyl 3-Hydroxybutrate (14). The propanolysis of natural PHB under acid conditions was performed exactly as described elsewhere.[11,12] The product was a colorless liquid: $[\alpha]_{589}^{25} = -39.98$ (1.33, $CH_3C_6H_5$); IR (neat) 3465, 1753, cm^{-1}; ^{1}H NMR (250 MHz, C_6D_6) δ 4.21 (q, 1 H, J = 6.3 Hz), 3.97 (t, 2 H, J = 6.7 Hz), 2.31 (ddd, 2 H, J = 8.2 and 16.0 Hz, 4.1 and 16.0 Hz), 1.45 (m, 2 H), 1.14 (d, 3 H, J = 6.3 Hz), 0.81 (t, 3 H, J = 6.7 Hz).

Propyl (3*R*/3*S*)-3-hydroxybutanoate (14/15) was obtained as a colorless oil: $[\alpha]_{589}^{25} = +9.81$ (0.153, $CH_3C_6H_5$); IR (neat) 3465, 1751 cm^{-1}; ^{1}H NMR (250 MHz, C_6D_6) δ 4.21 (q, 1 H, J = 6.3 Hz), 3.97 (t, 2 H, J = 6.7 Hz), 2.31 (ddd, 2 H, J = 8.2 and16.0 Hz, 4.1 and 16.0 Hz), 1.45 (m, 2 H, J = 6.7 Hz), 1.14 (d, 3 H, J = 6.3 Hz), 0.81 (t, 3 H, J = 6.7 Hz); ^{13}C NMR (50 MHz, $CDCl_3$) δ 172.86, 66.18, 64.58, 45.71, 22.81, 22.34, 9.71.

Preparation of Propyl (2*R*)-2-Hydroxypropanoate (10). In dry chloroform D-lactide (**2**) was mixed with $NaOC_3H_7$ at room temperature for 30 min. This mixture was then washed with water, the chloroform layer was evaporated, and compound **10** was isolated as a colorless oil: $[\alpha]_{589}^{25} = +10.8$ (0.25, $CH_3C_6H_5$); IR (neat) 3465, 1756 cm^{-1}; ^{1}H NMR (250 MHz, $CDCl_3$) δ 4.25 (q, 1 H, J = 6.9 Hz), 4.12 (t, 2 H, J = 6.8 Hz), 1.66 (m, 2 H), 1.39 (d, 3 H, J = 6.9 Hz), 0.93 (t, 3 H, J = 6.8 Hz); ^{13}C NMR (50 MHz, $CDCl_3$) δ 176.20, 67.46, 67.12, 22.62, 20.75, 10.58.

General Procedure for PHB-depolymerase Catalyzed Esterification. The PHB-depolymerase catalyzed esterifications of ϵ-caprolactone, β- and γ-butyrolactone, and D-, L-, *dl*-, and *meso*-lactides were performed as follows: A glovebox and dry argon were used to maintain an inert atmosphere during transfers. The cyclic ester substrate (44 mg) and the PHB-depolymerase lyophylysate (2 mg), both dried in a vacuum desiccator (0.1 mmHg, 25 °C, 24 h), were transferred to a dry NMR tube. The NMR tube was immediately stoppered (rubber septum) and purged with argon; deuterated solvent (0.8 mL dried over 4 Å molecular sieve) and 25 μL of *n*-propanol (dried and distilled over CaH_2) were added via syringe under argon. The NMR tube was then placed in a constant temperature oil bath maintained at 70 °C with agitation. The enzyme lyophylysate was dispersed throughout the reaction medium although it does settle to the bottom of the NMR tube while spectra are recorded. A control reaction was set up as described above except PHB-depolymerase was not added. Also, another control reaction was performed in which the thermally deactivated PHB-depolymerase (depolymerase boiled in water for 24 h, dried) was added in place of the active form. All the above

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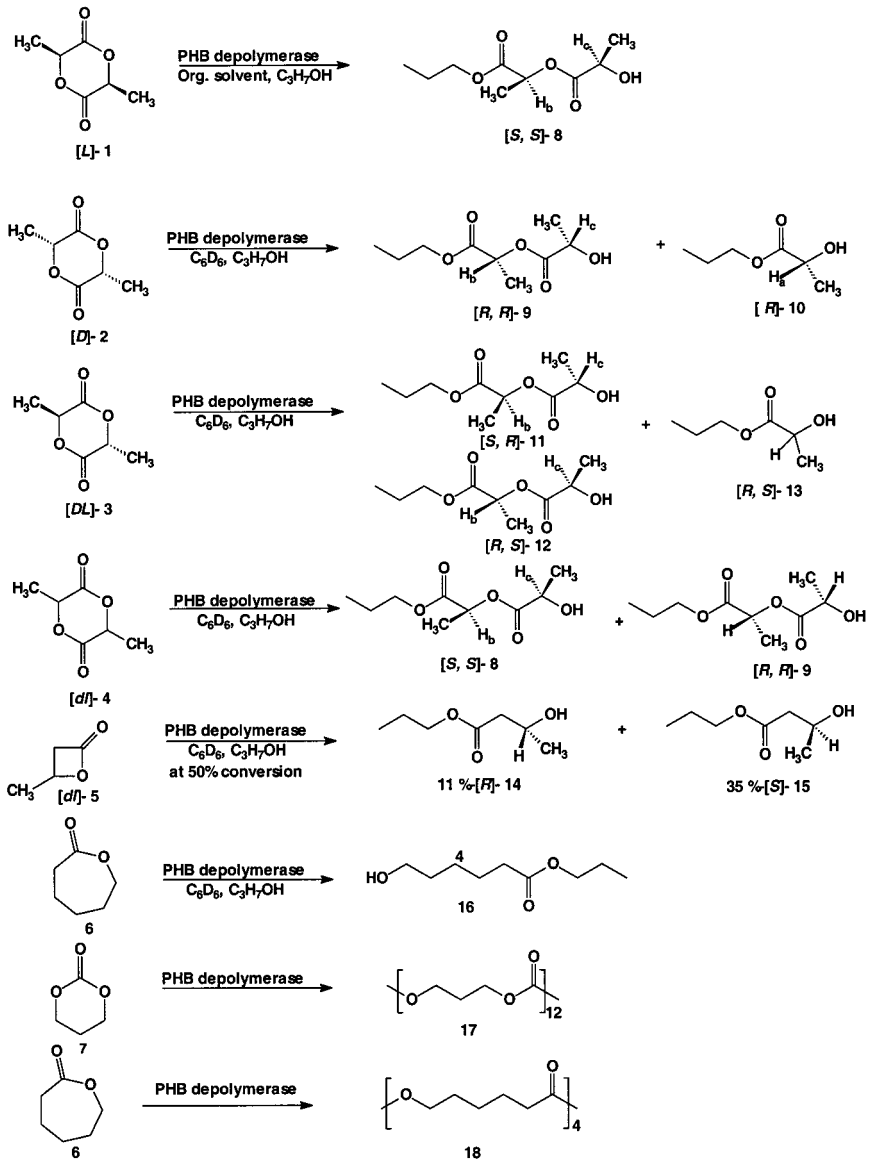
Scheme 1



reactions were monitored from time to time by ^{1}H NMR. The reactions were terminated by removal of the enzyme by filtration under vacuum using a fritted-glass filter (medium-pore porosity, 4.5−5 μm). The enzyme was washed on the fritted-glass filter by 1 to 2 washings with chloroform (0.5 mL portions), the filtrates were combined, and the solvent was removed in vacuo to give the crude products. When needed, the crude products were purified by silica gel column chromatography.

Preparation of (2*S*)-2-hydroxypropanoic acid, 2-propyloxy-(1*S*)-1-methyl-2-oxoethylester (8): obtained as a colorless oil; $[\alpha]_{589}^{25} = -25.87$ (0.143, $CH_3C_6H_5$); IR (neat) 3495, 1761 cm^{-1}; ^{1}H NMR (200 MHz, $CDCl_3$) δ 5.18 (q, 1 H, J = 7.1 Hz), 4.37 (q, 1 H, J = 6.9 Hz), 4.13 (t, 2 H, J = 6.8 Hz), 1.70 (m, 2 H), 1.54 (d, 3 H, J = 7.1 Hz), 1.50 (d, 3 H, J = 6.9 Hz), 0.95 (t, 3 H, J = 6.8 Hz); ^{13}C NMR (50 MHz, $CDCl_3$) δ 175.57, 170.67, 69.87, 67.55, 67.12, 22.28, 20.91, 17.33, 10.66.

Preparation of (2*R*)-2-hydroxypropanoic acid, 2-propyloxy-(1*R*)-1-methyl-2-oxoethyl ester (9): obtained as a colorless oil; $[\alpha]_{589}^{25} = +30$ (0.323, $CH_3C_6H_5$); IR (neat) 3505, 1761 cm^{-1}; ^{1}H NMR (250 MHz, $CDCl_3$) δ 5.18 (q, 1 H, J = 7.1 Hz), 4.36 (q, 1 H, J = 6.9 Hz), 4.12 (t, 2 H, J = 6.8 Hz), 1.67 (m, 2 H), 1.53 (d, 3 H, J = 7.1 Hz), 1.49 (d, 3 H, J = 6.9 Hz), 0.95 (t, 3 H, J = 6.8 Hz); ^{13}C NMR (50 MHz, $CDCl_3$) δ 175.0, 170.65, 69.76, 67.46, 67.10, 22.24, 20.78, 17.25, 10.56.

Preparation of (2*R*/2*S*)-2-hydroxypropanoic acid, 2-propyloxy-(1*S*/1*R*)-1-methyl-2-oxoethyl ester (11/12): obtained as a colorless oil; $[\alpha]_{589}^{25} = +9.2$ (0.403, $CH_3C_6H_5$); IR (neat) 3515, 1756 cm^{-1}; ^{1}H NMR (200 MHz, $CDCl_3$) δ 5.19 (q, 1 H, J = 7.1 Hz), 4.36 (q, 1H, J = 6.9 Hz), 4.12 (t, 2 H, J = 6.8 Hz), 1.68 (m, 2 H), 1.54 (d, 3 H, J = 7.1 Hz), 1.50 (d, 3 H, J = 6.9 Hz), 0.95 (t, 3 H, J = 6.8 Hz); ^{13}C NMR (50 MHz, $CDCl_3$) δ 175.48), 170.72, 69.77, 67.50, 67.11, 22.31, 20.82, 17.29, 10.62.

Propyl hexanoate (15) was obtained as a colorless oil: IR (neat) 3465, 1751 cm^{-1}; ^{1}H NMR (250 MHz, C_6D_6) δ 4.01 (t, 2 H, J = 6.7 Hz), 3.62 (t, 2 H, J = 6.4 Hz), 2.31 (t, 2 H, J = 7.2 Hz), 1.68−1.57 (m, 6 H), 1.44−1.35 (m, 2 H), 0.93 (t, 3 H, J = 6.7 Hz); ^{13}C NMR (50 MHz, $CDCl_3$) δ 174.34, 66.33, 62.87, 34.63, 32.67, 25.79, 25.07, 22.36, 10.77.

Results and Discussions

Studies were performed to determine the catalytic activity and specificity in organic media of the PHB-depolymerase from *P. lemoignei*. The natural substrate for this enzyme is enantiopure ([*R*]-antipode) PHB or structurally related polyhydroxyalkanoates.[3,16] This work

(13) Names of structures in Table 1 and Scheme 1 are given as **1**, (3*S*-*cis*)-3,6-dimethyl-1,4-dioxane-2,5-dione; **2**, (3*R*-*cis*)-3,6-dimethyl-1,4-dioxane-2,5-dione; **3**, (3*R*,6*S*)-3,6-dimethyl-1,4-dioxane-2,5-dione; **4**, (±)-3,6-dimethyl-1,4-dioxane-2,5-dione; **5**, 4-methyl-2-oxetanone; **6**,

Table PHB- depolymerase Catalyzed Esterifications between *n*-Propanol and Cyclic Esters in Organic Solvents[a]

substrate	reactn time (h)	solvent	product	% convn
1	35	benzene-d_6	**8**	98
1	65	cyclohexane-d_{12}	**8**	90
1	65	acetonitrile-d_3	**8**	4
2	70	benzene-d_6	**9**	38
			10	22[b]
3	52	benzene-d_6	**11**, **12**	59
			13	8[b]
4	48	benzene-d_6	**8**	major
			9	minor
5[c]	140	benzene-d_6	**14**	35
			15	11
6	72	benzene-d_6	**16**	21
6	48		**17**	18
7	48		**18**	30

[a] Names of structures (**1**−**18**) in Table 1 and Scheme 1 are given in ref 13. [b] Lactic acid ester unit/2. [c] The enzyme concentration was increased by 10-fold for this reaction.

focused on the catalytic activity of the PHB-depolymerase on a series of nonnatural substrates including lactones, lactides, and a cyclic carbonate (Scheme 1, 1−7). Lactides (**1**−**4**, Scheme 1) differing in stereochemical configuration were included. Similar to PHB, lactides have secondary hydroxyl groups attached to the chiral center that participate in formation of the ester bonds. Furthermore, the substituents attached to the chiral centers of both lactide and PHB are methyl groups. In contrast to PHB which has hydroxyl groups that are β to the carbonyl, lactides have α-hydroxyl groups and are low molecular weight cyclic analogues. The β-methyl-substituted butyrolactone (Scheme 1, **5**) is a small molecule lactone analogue of PHB. Structures **1**−**5** all have chiral centers that permitted the interrogation of PHB-depolymerase enantiospecificity in organic media. ϵ-Caprolactone (Scheme 1, **6**) is an analogue of structures **1**−**5** that is achiral and does not have a methyl substituent. Six-membered trimethylene carbonate (Scheme 1, **7**) was studied in bulk as a monomer for PHB-depolymerase catalyzed ring-opening polymerization.

The PHB-depolymerase catalyzed esterification of lactone and lactide stereoisomers by *n*-propanol (see Scheme 1) was investigated in dry deuterated benzene (C_6D_6), cyclohexane (C_6D_{12}), and acetonitrile (CD_3CN) (Table 1). Although far from comprehensive, these solvents represent a broad range of solvent polarity. The extent of nonenzyme-catalyzed reactions was found to be negligible based on controls that were conducted without adding PHB-depolymerase. The plot in Figure 1 shows that increasing the solvent polarity from benzene-d_6 or cyclohexane-d_{12} to acetonitrile-d_3 resulted in a large decrease in reactivity for L-lactide propylation. An increase in the medium polarity for lipase-catalyzed esterifications also generally results in decreased enzyme activity.[14]

hexahydro-2*H*-oxepin-2-one; **7,** trimethylene carbonate; **8**, (2*S*)-2-hydroxypropanoic acid, 2-propyloxy-(1*S*)-1-methyl-2-oxoethyl ester; **9**, (2*R*)-2-hydroxypropanoic acid, 2-propyloxy-(1*R*)-1-methyl-2-oxoethyl ester; **10**, propyl (2*R*)-2-hydroxypropanoate; **11**/**12**, (2*R*/2*S*)-2-hydroxypropanoic acid, 2-propyloxy-(1*R*/1*S*)-1-methyl-2-oxoethyl ester; **13**, propyl 2-hydroxypropanoate; **14**, propyl (3*R*)-3-hydroxybutanoate; **15**, propyl (3*S*)-3-hydroxybutanoate; **16**, propylhexanoate; **17**, polytrimethylene carbonate; **18**, polycaprolactone.

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(15) DP-TFA-γ-Cyclodextrin column 0.12 μM (40 m × 0.24 mm), oven temperature 135 °C, injection temperature 160 °C, detection temperature 170 °C and at 10 psi.

(16) Fitting by two Gaussian peaks in Microcal Origin, Version 5.

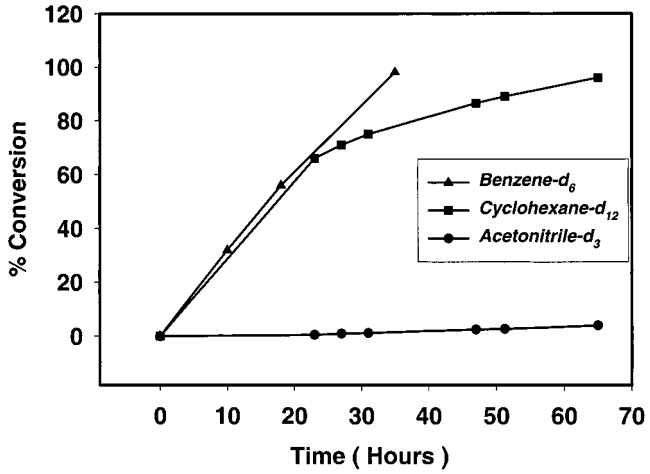

Figure 1. Time versus % conversion for the PHB-depolymerase catalyzed propylation of L-lactide (**1**) in different solvents. (benzene-d_6, cyclohexane-d_{12}, acetonitrile-d_3).

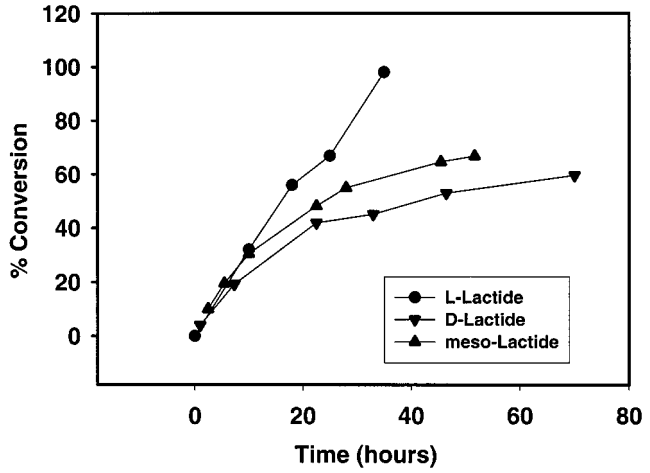

Figure 2. Time versus % conversion for the PHB-depolymerase catalyzed propylation of L-, D-, and *meso*-lactide.

The effect of lactide stereochemistry on lactide propylation catalyzed by the PHB-depolymerase is shown in Figure 2. For % conversions above ~30%, the % conversion increased in the following order: D-lactide < *meso*-lactide < L-lactide. When this reaction was conducted using racemic or *dl* lactide (a 50/50 mixture of D- and L-lactide) to 50%-conversion, a preferential ring-opening of the L-lactide was observed. This resulted in predominantly L-propyl-lactide that had a 26.6% optical purity. The extent of nonenzyme catalyzed reactions, based on controls conducted without adding PHB-depolymerase, resulted in <3% products yields for L- and D-lactides, and <9% for *meso*-lactide (see Figures 4 and 5).

An important question in lactide ring-opening is whether propyl lactide reacts further to form two molecules of propyl lactate. The ^{1}H NMR of L-lactide has the methine protons 2 (**2**) at δ 4.15 in benzene-d_6. As the PHB-depolymerase catalyzed propylation of L-lactide proceeds, the ^{1}H NMR spectra showed a decrease in the intensity of the signal at 4.15 with a concomitant increase in the intensities of the two quartets at δ 5.18 and 4.37 due to the methine protons H_b (**8**) and H_c (**8**), respectively. However, the methine signals due to propyl lactate were not observed for reactions conducted for 35 h to 98%

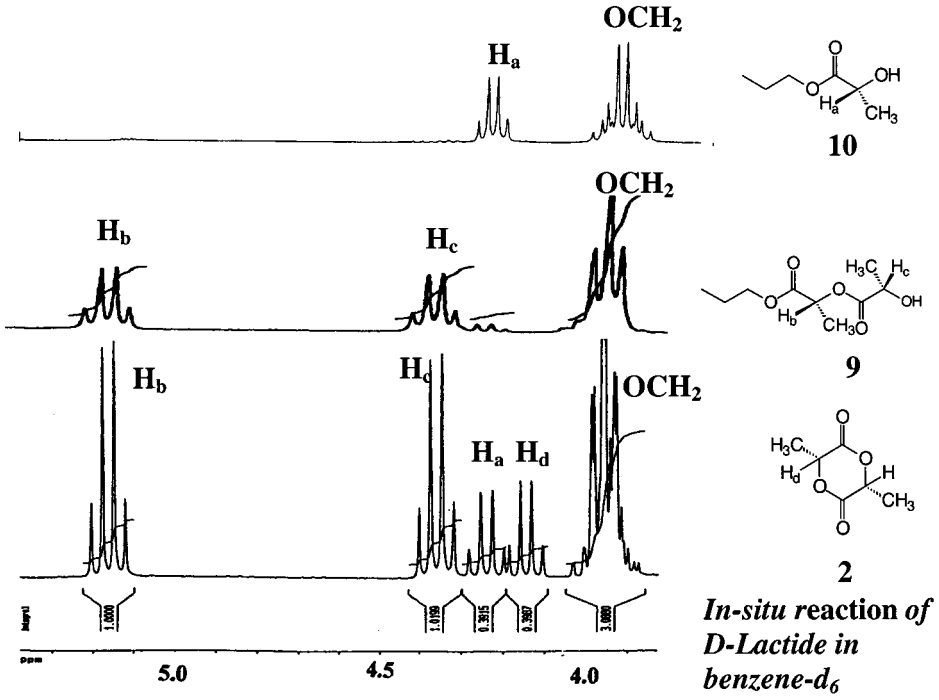

Figure 3. ^{1}H NMR profile of the C*H* and OCH_2 region of compounds **8**, **9**, and **10** in benzene-d_6.

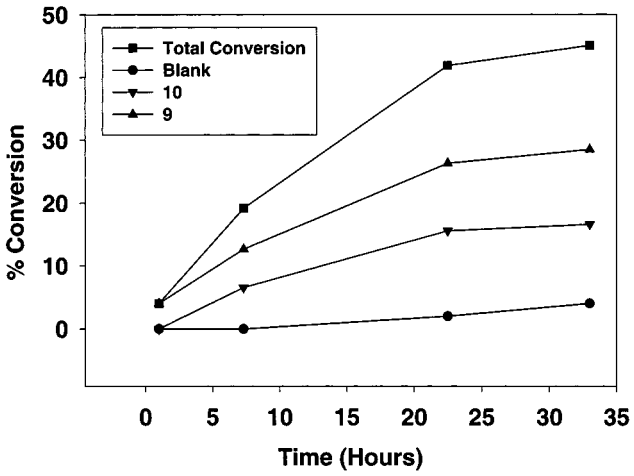

Figure 4. Time versus % conversion for the PHB-depolymerase/without depolymerase catalyzed propylation of D-lactide (**2**).

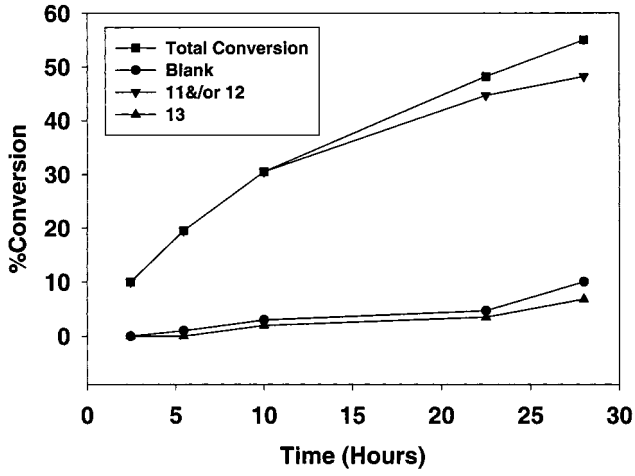

Figure 5. Time versus ester formation for the PHB-depolymerase/without depolymerase catalyzed propylation of *meso*-lactide (**3**).

conversion. Furthermore, the only product of the reaction at 98% conversion had a ^{13}C NMR spectrum that was consistent with the formation of L-propyl lactide (see experimental), with signals at 69.87, 67.55, and 67.12 and 175.57 and 170.67 ppm. Thus, although the *n*-propylation reaction with L-lactide was rapid compared to similar reactions performed with *meso* and D-lactide, L-propyl lactide was a poor substrate for further reaction with *n*-propanol at the internal ester bond.

The *n*-propylation of D- and L-lactide proceeded differently (see Figure 4). As the propylation of D-lactide progressed, in addition to the ^{1}H NMR quartets centered at δ 5.16 and 4.38 due to the methine protons H_b (**9**) and H_c (**9**), quartets at δ 4.28 and 4.15 were also observed (see above and Figure 3). The presence of these signals (δ 4.28 and 4.15) suggests that in addition to D-propyl lactide D-propyl lactate was also formed. After a 35 h reaction, *n*-propylation of D-lactide yielded 28% of **9** and 17% of **10**. In contrast, under similar conditions, *n*-propylation of L-lactide gave 98% of **8**. To confirm that *n*-propylation of D-lactide yielded **9** and **10**, the reaction was repeated for 140 h to 80% total conversion. The products formed were separated by silica gel chromatography (eluent: hexane/ethyl acetate in increasing polarity). Two compounds were isolated in the pure form. Compound **9** showed ^{1}H NMR signals identical to those of **8** but had an optical rotation of equal magnitude but in the opposite direction (see experimental). Thus, **9** is *n*-propyl D-lactide or (2*R*)-2-hydroxypropanoic acid, 2-propyloxy-(1*R*)-1-methyl-2-oxoethyl ester. The ^{1}H NMR in benzene-d_6 of the coproduct had a quartet centered at δ 4.25 (CH_a), a triplet at 3.95 (OCH_2), a doublet at 1.40, and a triplet at 0.75. Further analysis of this product by ^{13}C NMR further confirmed that the coproduct was D-propyl lactate **10** (propyl (2*R*)-2-hydroxypropanoate). The specific rotation of **10** is $[\alpha]_{589}^{25} = +10.5$ (0.25,

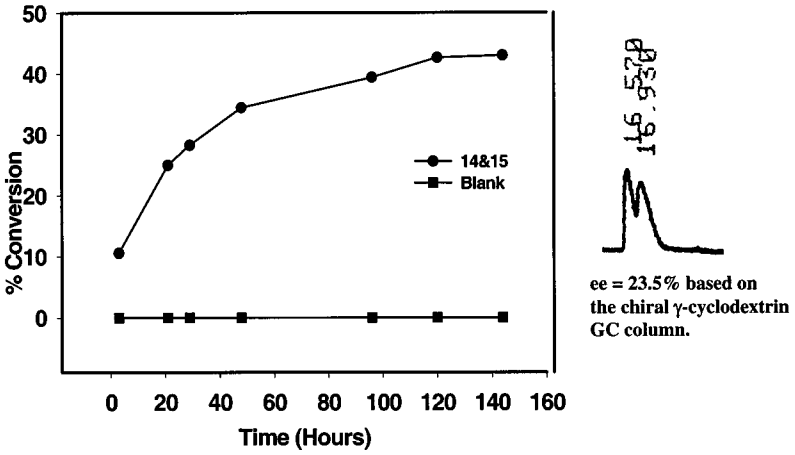

Figure 6. Time versus ester formation for the PHB-depolymerase/without depolymerase catalyzed propylation of 4-methyl-2-oxetanone (**5**).

$CH_3C_6H_5$). This value is consistent with that of D-propyl lactate ($[\alpha]_{589}^{25} = +10.8$ (0.25, $CH_3C_6H_5$) synthesized by us using another route (see Materials and Methods section). Thus, the PHB-depolymerase is able to convert L-lactide to *n*-propyl L-lactide without further cleavage of the internal ester. In contrast, the PHB-depolymerase catalyzes the conversion of D-lactide to *n*-propyl D-lactide, but this product is further converted to 2 equiv of *n*-propyl D-lactate.

The potential exists that the reaction of *n*-propanol with *meso*-lactide (D,L-isomer) will occur with preferential attack at the D- or L-lactide stereocenters. In other words, depending on the enantioselectivity of the PHB-depolymerase, this reaction could give an unequal proportion of the diastereomers *n*-propyl-[L-D]-**11**, or *n*-propyl-[D-L]-**12** (see Scheme 1). In addition, the rates that **11** and **12** react with *n*-propanol to form racemic *n*-propyl lactate **13** may differ. By analysis of the ^{1}H and ^{13}C NMR spectra, the major reaction products formed by 28 h were **13** (7 mol %) and 48 mol % of *n*-propyl lactide that, presumably, consists of some mixture of **11** and **12**. The product mixture of **11**, **12**, and **13** had a specific optical rotation of $[\alpha]_{589}^{25} = +9.2$ (0.403, $CH_3C_6H_5$). The product **13** was then removed from the reaction mixture under vacuum. When the presumed mixture of **11** and **12** was analyzed by GC chromatography using a chiral γ-cyclodextrin column,[15] a single peak at 14.76 min was found. Variation of the GC chromatographic conditions (e.g., temperature and pressure) or derivatization of the ω-hydroxyl groups by trifluoroacetylation did not assist in the GC resolution of the presumed mixture. The chiral shift reagent tris-[3-(heptafluoropropylhydroxymethylene)-(+)camphorato]-europium(III) was used in varying concentrations relative to **11** and/or **12** and ^{1}H NMR spectra were recorded. Once again, a resolution of the corresponding NMR signals was not achieved. The inability of the shift reagent to resolve such a mixture was not surprising after the identical experiments conducted with the [D-D]- and [L-L]-lactide propyl esters did not differentiate these enantiomers.

Generally, for lipase-catalyzed reactions of ester substrates with multiple stereocenters, the stereochemical configuration of the ester unit in closest proximity to the serine or cysteine of the active lipase box determines the stereoselectivity of the reaction.[15] One possibility is that, based on the enantioselectivity of the PHB-depolymerase for the L-lactide enantiomer, the *n*-propyl-(L-D) diastereomer is formed selectively. High selectivity would result in the formation of a product that has high stereochemical purity. If this were the case, it would offer an explanation for our inability to separate the L-D and D-L stereoisomers.

β-Butyrolactone is the single repeat unit cyclic analogue of natural PHB. Hence, the activity of the PHB-depolymerase for the enantioselective *n*-propylation of [*R*]- and [*S*]-β-BL seems particularly relevant to the enzymes natural substrate preferences under aqueous conditions. The propylation of racemic-β-BL proceeded so slowly that, for comparison to lactide substrates, it was necessary to increase the relative enzyme concentration by a factor of 10 times. Even with this disparity in enzyme concentrations, *n*-propylation of racemic-β-BL still proceeded slower than that observed for any of the lactide stereoisomers. The reaction was terminated at ~50% monomer conversion. The enantioselectivity of β-BL *n*-propylation was assessed by analyzing the trifluoroacetyl derivatives of **14** and **15** using a chiral γ-cyclodextrin GC column.[15] The resulting GC trace shows partial resolution of **14** and **15**, giving two peaks at 16.57 and 16.93 min (see Figure 6). On the basis of the relative peak areas, analyzed by using Peak Fit software,[16] the enantiomeric excess was 23.5%. The *n*-propyl-β-HB stereoisomers from the reaction mixture were purified by column chromatography (silica gel column, hexane/ethyl acetate), and the resulting product had a specific rotation, $[\alpha]_{589}^{25}$, of +9.81 (0.153, $CH_3C_6H_5$). To determine the stereochemical configuration of this product, (*R*)-propyl $\beta-$hydroxybutrate was synthesized from natural *[R]*-PHB as described in the Material and Methods section. The optical rotation of this enantiopure *R*-isomer is $[\alpha]_{589}^{25} = -39.98$ (1.33, $CH_3C_6H_5$). Thus, the PHB-depolymerase reacts preferentially with the (*S*)-antipode, and the enantiomeric excess calculated by the optical rotation measurements was 24.5%. Since the PHB-depolymerase from *P. lemoignei* selectively hydrolyzes (*R*)-PHB in aqueous media,[17] it appears that a switch in the stereoselectivity in the organic medium was observed. A change in enzyme enantioselectivity as a function of the reaction medium used is not uncommon.[6c,d]

Since both lactide and β-BL have methyl branches, the

(17) Bachman, B. M.; Seebach, D. *Macromolecules* **1999**, *32*, 1777−1784.

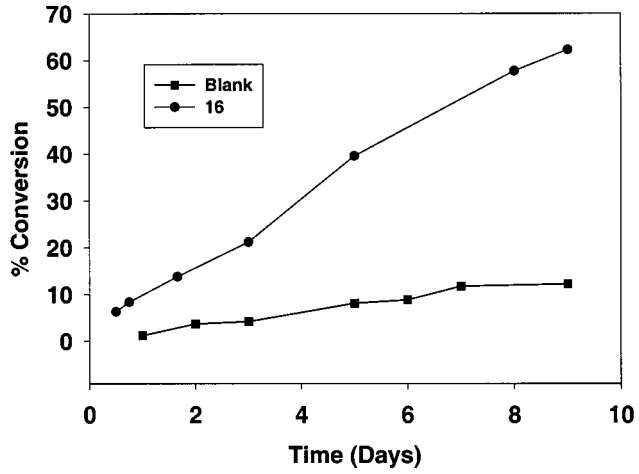

Figure 7. Time versus ester formation for the PHB-depolymerase/without depolymerase catalyzed propylation of hexahydro-*2H*-oxepin-2-one (**6**).

activity of the PHB-depolymerase for the nonbranched, achiral, seven-membered ring ϵ-caprolactone (ϵ-CL) was studied (see Scheme 1, compound **6**). After a 3-day reaction time, the % conversion to the corresponding *n*-propyl ester **16** reached only 21 mol % and proceeded very slowly to 63 mol % in 10 days (Figure 7, control % conversion was 4% and 12%, respectively). Under the identical reaction conditions, the smaller five-membered γ-butyrolactone showed no significant PHB-depolymerase catalyzed *n*-propylation after a 5-day reaction. Review of the above shows that the relative rates of PHB-depolymerase catalyzed *n*-propylation, from fastest to slowest, was as follows: L-lactide, *meso*-lactide, D-lactide, δ-butyrolactone, ϵ-caprolactone, γ-butyrolactone.

Recently, there has been increasing interest in using enzymes as catalysts for polymer synthesis.[18] Studies on lipase-catalyzed polymerizations of lactones[18] are particularly relevant to this work. Therefore, the potential of using the *P. lemoignei* PHB-depolymerase for lactone ring-opening polymerizations was evaluated. The PHB-depolymerase catalyzed polymerization of ϵ-CL resulted in 18% conversion of monomer in 48 h (70 °C) to oligomers (**18**, Scheme 1) with an average DP of 4 (determined by ^{1}H NMR).[19] Under the identical solventless polymerization conditions, a 30% conversion of trimethylene carbonate to oligomers (**17**, Scheme 1) with an average DP of 12 (determined by ^{1}H NMR)[20] was found. Evaluation of control reactions of ϵ-CL and trimethylene carbonate carried out for 48 h at 70 °C without enzyme showed negligible levels of monomer conversion. Hence, based on this early study, the PHB-depolymerase from *P. lemoignei* appears promising for use in ring-opening polymerizations.

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(19) MacDonald, R. T.; Pulapura, S.; Svirkin, Y. Y.; Gross, R. A.; Kaplan, D. L.; Akkara, J.; Swift, G. *Macromolecules* **1995**, *28*, 73−78.

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Summary of Results

For the first time, a member of the family of PHB depolymerase enzymes was studied to evaluate its ability to catalyze bond-forming reactions in organic media. *P. lemoignei* was cultured using conditions developed for high PHB-depolymerase production. The PHB depolymerase from *P. lemoignei* was purified from the cell-free culture liquid and lyophilized. The lyophilized enzyme was then studied for its ability to catalyze transesterification reactions in benzene-d_6. The relative rates of PHB-depolymerase catalyzed *n*-propylation, from fastest to slowest, was as follows: L-lactide (*SS*), *meso*-lactide (*RS*) D-lactide (*RR*), δ-butyrolactone (*dl*), ϵ-caprolactone, γ-butyrolactone. It was observed that the PHB-depolymerase from *P. lemoignei* reacted differently with L- and D-lactides. We were able to get exclusive formation of L-propyl lactide (*SS* configuration) from L-lactide. In contrast, the production of D-propyl lactide (*RR* configuration) from D-lactide underwent further reaction with *n*-propanol to give propyl (2*R*)-2-hydroxypropanoate. Similarly, the selectivity of the PHB-depolymerase resulted in the formation of the *S* enriched *n*-propyl-β-HB from racemic β-butyrolactone. In conclusion, this report demonstrates the potential of using various PHB-depolymerase enzymes as novel catalysts in organic media. The introduction of new families of enzymes for bond formation in organic media provides an opportunity to explore reactions that thus far were not successfully carried out with existing enzymes. It is noteworthy that no attempts were made in this study to improve the enzyme activity of the PHB-depolymerase in organic media by, for example, using immobilization or surfactant pairing techniques. Such work is expected to result in substantial increases in activity of this and other PHB-depolymerases in organic media.

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Supporting Information Available: ^{1}H and ^{13}C NMR spectra of compounds **8**, **9**, **10**, (**11** and **12**), **14**, (**14** and **15**), and **16**. This material is available free of charge via Internet at http://pubs.acs.org.

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