**ABSTRACT:** In this study, we have prepared poly(3-hydroxybutyrate), PHB, random stereocomplexes from (R)-3-hydroxybutyrate, (S)-3-hydroxybutyrate, and (R)-3-hydroxybutyrate and (S)-3-hydroxybutyrate. Synthesis was carried out through the use of a Claisen-Schmidt condensation. The initial degradation rates were determined by measuring the increase in weight loss as a function of time. The relative degradability of the stereocomplexes was compared to that of (S)-PHB and (R)-PHB. It was found that (S)-PHB was the most resistant to degradation, followed by (R)-PHB and then the stereocomplexes. The stereocomplexes with a higher content of (R)-PHB showed a lower degradation rate than those with a higher content of (S)-PHB. These results suggest that the degradation rate of the stereocomplexes is determined by the relative content of (R)-PHB and (S)-PHB in the stereocomplexes.

**Keywords:** Poly(3-hydroxybutyrate), PHB, stereocomplexes, morphology.
Poly(β-hydroxybutyrate) Stereoisomers: A Model Study of the Effects of Stereochemical and Morphological Variables on Polymer Biological Degradability

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ABSTRACT: In this study, we have prepared poly(β-hydroxybutyrate), PHB, random stereocopolymers from β-butyrolactone, BL, using a diethylzinc/water (1.0/0.6) catalyst system. (R)- and (S)-BL were synthesized in high enantiomeric purity (>98% ee (enantiomeric excess)), and approximately 5% racemization occurred at the methine stereocenters upon polymerization. The PHB stereoisomers produced had R repeat unit compositions of 95, 90, 85, 81, 77, 67, and 50% R. In addition, a 50% (R)-PHB stereoisomer with a predominantly syndiotactic repeat unit placement was prepared in our laboratory. The relative degradability of these PHB stereoisomers was studied with a PHB depolymerase enzyme isolated from Penicillium funiculosum. This enzyme has been shown to catalyze the hydrolysis of (R)-PHB, but does not show activity for the enantiomERICally substrate (S)-PHB. The P. funiculosum depolymerase/PHB stereocopolymer system, therefore, allowed the study of two opposing effects on the degradation rate: the increase due to the disruption of the crystalline phase, and the decrease due to a stereochemical enzyme impediment, as the (S)-HB content of PHB is increased from 0 to 50%. The initial surface degradation rates in [H+]/(mm²-min) were determined by measuring the pH change as a function of time for polymer/enzyme incubations. It was shown that for stereocopolymer compositions of 95, 90, 85, and 81% R the degradation rate values (between 0.57 × 10⁻⁴ and 0.92 × 10⁻⁴ [H⁺]/(mm²-min)) were lower than those measured for a 100% (R)-PHB sample (1.41 × 10⁻⁴ [H⁺]/(mm²-min)) of similar molecular weight. Therefore, the preference for (R)-HB repeat units appears to dominate over crystalline morphology effects for the compositional range 81-100% (R)-HB. However, the initial surface degradation rates for the 67 and 77% (R)-PHB samples were 2.85 × 10⁻⁴ and 7.51 × 10⁻⁴ [H⁺]/(mm²-min), respectively, showing dramatically larger rate values compared to that for 100% (R)-PHB. This result suggests that, at a critical degree of disruption of the crystalline order which occurred for compositions between 77 and 81% (R)-HB, effects of crystalline morphology dominate. The noncrystalline 50% (R)-atactic-PHB sample displayed an initial degradation rate which was slightly higher than that observed for crystalline bacterial 100% (R)-PHB. However, this initial observed rate was followed by an abrupt decrease in the rate which was probably due to depletion of (R)-HB-rich segments on the polymer surface. Results from substrate/exoenzyme incubations up to 21 days further confirmed that the 50% (R)-atactic-PHB sample was a poor substrate for the enzyme after the rapid initial degradation of (R)-HB-rich polymer chain segments at the film surface. In contrast, a significant portion of the polymeric chains for both the predominantly syndiotactic 50% (R)-PHB and the 77% (R)-PHB were degraded by the P. funicolusum esterase to products containing on average 3 ± 1 HB repeat units. The relative degradability of these PHB stereoisomers has interesting implications on the acceptability of specific stereochmical sequences in the degradation of the PHB by P. funiculosum and other PHB depolymerases.

Introduction

Poly(β-hydroxyalkanoates), (R)-PHA's, are synthesized by a wide variety of bacteria1-7 and serve as intracellular carbon and energy reserves.1 The most thoroughly investigated member of this family of natural origin polyesters is the homopolymer poly(β-hydroxybutyrate), (R)-PHB, which is a highly crystalline biodegradable thermoplastic.8

Several researchers have studied the depolymerization of (R)-PHB and (R)-P(HB-co-HV) (where HV is β-hydroxyvalerate) by nonbiologically mediated chemical hydrolysis.9-13 It is apparent from these studies that these natural polyesters degrade rather slowly by simple chemical hydrolytic mechanisms. For example, according to Doi and co-workers14 (R)-PHB and (R)-P(HB-co-68% HV) in a 0.01 M phosphate buffer (pH 7.4) at 37 °C showed no weight loss and a number-average molecular weight change of less than 15% over a period of 180 days.

The susceptibility of bacterial polyesters to microbial degradation has been demonstrated by various methods and by different laboratories. Researchers at Imperial Chemical Industries showed that natural origin PHB was biodegradable in the soil, anaerobic and aerobic sewage, seawater, and estuarine sediment.14 Delafield et al.15 carried out the isolation of 16 strains of soil microorganisms which were capable of growing under aerobic conditions with PHB as the sole source of carbon. The bacterial strains Pseudomonas lemoignei (ATCC 17989)16-18 and Alcaligenes faecalis T1,19,20 isolated from soil and activated sewage sludge, respectively, have been identified as being capable of using PHB as an exogenous source of carbon by excreting extracellular enzymes that depolymerize it. In our laboratory, we have successfully isolated and purified to electrophoretic homogeneity a PHB depolymerase exoenzyme from the fungus Penicillium funiculosum.21,22 It has been determined that the protein has an Mₐ value of 38 000. The enzyme has an isoelectric point of 5.8, a pH optimum range of 5.5-6.2, and a temperature optimum range of 30-35 °C.21,22 In-laboratory simulations of natural environments have also been utilized in our laboratories to demonstrate the degradability of bacterial copolyesters.23-25 Since the kinetics of PHB enzyme degradation with isolated enzymes such as that from P. funiculosum may be much more rapid than nonbiologically mediated chemical hydrolysis (see above and corresponding references), enzyme-mediated hydrolytic degradation...
events are easily measured in the absence of appreciable chemical hydrolysis of PHB.

The ester functionalities of PHB, as well as other polymers, presents an opportunity to study the enzyme-catalyzed surface degradation phenomena with great sensitivity by simply monitoring pH change. The rationale of determining degradation kinetics by this method for polymers lies in the fact that for every chain cleavage event that takes place a free carboxylic end group is produced, thus causing a decrease in the pH value. The mechanism for PHB enzyme cleavage has been shown for Ps. lemoignei to involve the formation of dimer and trimer, and for A. faecalis to involve the formation of dimer, from the free hydroxyl termini of the polymer chains.

\begin{align*}
\text{CH}_3 & \quad \text{CH}_3 \\
\text{HO-O-CC(CH_2)_COO-} & \quad \text{CH}_2\text{CH}_2\text{COO-} \\
\text{HO-O-CC(CH_2)_COO-} & \quad \text{CH}_2\text{CH}_2\text{COO-} \\
\text{CH}_3 & \quad \text{CH}_3 \\
\text{HO-O-CH_2COO-} & \quad \text{CH_2CH_2COO-} \\
\text{HO-O-CH_2COO-} & \quad \text{CH_2CH_2COO-} + \text{H}_2\text{O}(CH_2CH_2)_nOH \\
x &= 2, 3
\end{align*}

It has been established that the ring-opening polymerization of β-butyrolactone, BL, with the ZnEt₂/H₂O (1.0/0.6) catalyst system proceeds primarily with retention of configuration and, therefore, by an acyl oxygen ring-opening mechanism (see below). It is expected from previous studies and work in our laboratory that this initiator system provides random stereochemical placement during the polymerization of β-butyrolactone enantiomeric monomer mixtures. Aluminum-based catalyst systems have been shown to demonstrate stereoregulation during the polymerization of racemic β-butyrolactone, giving rise to isotactic block sequences and, therefore, crystalline PHB.

In this study, we have explored the complex interplay between the effects of material crystalline morphology and enzyme stereospecificity on polymer degradability. We have chosen for this model system PHB since it is semicrystalline in a range of stereopolymer compositions, undergoes subtle as well as extreme modulation of crystalline morphology as a function of the comonomer stereochemical composition and annealing conditions, and is readily available by both biosynthetic and chemical synthetic preparative routes. Specifically, we have prepared a series of PHB stereopolymermers using a ZnEt₂/H₂O (1.0/0.6) catalyst where ideal random copolymerization of the (R)- and (S)-BL monomers is expected. BL with an optical purity in excess of 98% was used to obtain a wide range of PHB stereopolymer compositions with the desired relative amounts of the polymeric repeat units (R)- and (S)-HB. The polymer stereopolymers were melt cast into thin films. The rate of surface degradation per unit surface area catalyzed by the extracellular esterase isolated from P. funiculosa was then determined by monitoring the pH change over the pH optimum range for this enzyme.

**Experimental Section**

**Instrumental Methods.** Proton (1H) nuclear magnetic resonance (NMR) spectra were recorded on a Bruker WP-270 SY spectrometer at 270 MHz. 1H NMR chemical shifts in parts per million (ppm) are reported downfield from 0.00 ppm using tetramethylsilane (TMS) as an internal reference. The synthetic intermediates for the synthesis of polymer samples were run in 5-mm tubes as CDC₁₃ solutions, with the following parameters: temperature, 298 K; pulse width, 2 μs; relaxation delay, 0.50 s; 16K data points; and 64-128 transients. The parameters for the polymer spectra are as follows: 3.5% wt/wt polymer in CDC₁₃; temperature, 308 K; pulse width, 4.9 μs; 32K data points; relaxation delay, 0.50 s; and 100-200 transients. The determination of the enantiomeric excess values for methyl (R)- and (S)-hydroxybutyrate and methyl (S)-3-hydroxybutyrate and (R)-BL, respectively, were carried out as 0.5% wt/wt solutions in CDC₁₃ containing 30 mol % of europium-(III) tris[(heptafluoropropyl)hydroxymethylene]-(+)-camphoratol. Eu(+)-(hfc)₃ (Aldrich Chemical Co.), at 25°C. Peak areas were determined by spectrometer integration and are reported as relative intensities representing a given number of hydrogens. The following abbreviations are used: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, and br = broad.

Carbon (13C) NMR spectra were recorded at 67.9 MHz on a Bruker WP-270 SY in 5-mm tubes as CDC₁₃ solutions, with chemical shifts in parts per million referenced relative to chloroform (CHCl₃) as an internal reference at 77.00 ppm. Polymer spectral acquisitions were conducted on 3.5% wt/wt solutions of polymer in CDC₁₃ using the following: temperature, 308 K; pulse width, 10 μs; 16K data points; relaxation delay, 1.0 s; and 10000-20000 transients.

Infrared spectra (IR) were recorded neat between NaCl plates on a Perkin-Elmer 1320 FT-IR at 25°C. The spectral positions of sample IR absorbances are given in units of reciprocal centimeters.

Optical rotation data were determined on a Perkin-Elmer 241 polarimeter attached to a refrigerated constant-temperature circulator, and are reported as follows: [α]D, where [α]D = specific rotation (concentration in grams per 100 mL of solvent).

All molecular weights were determined by gel permeation chromatography (GPC) using a Waters Model 510 pump, Model 410 refractive index detector, and Model 730 data module with 105-, 104-, 103-, and 102-A ultrastyragel columns in series. Chloroform was used as the eluent at a flow rate of 1.0 mL/min. Sample concentrations of 0.3% wt/v and injection volumes of 125 μL were used. Polystyrene standards with a low polydispersity (Polydise) were used to generate a calibration curve.

Differential scanning calorimetry (DSC) was conducted on a DuPont DSC 2910 equipped with a TA 2000 data station, using between 5.0 and 100 mg of sample, a heating rate of 10 °C/min, and a nitrogen purge.

RuCl₃[(S)-(-)-BINAP]NEt₃ Catalyst Preparation. The procedure followed was almost identical to that described in the literature. All manipulations were carried out in a 30-mL Schlenk tube, and an orange-brown solid was obtained.

Methyl (S)-β-Hydroxybutyrate (1a). The following procedure represents a modification of that previously described in the literature. Into an oven-baked (at greater than 115 °C) 500-mL glass-lined Parr pressure reactor (rated at 2500 psi) was added 1.75 g of Dowex 50 W-X8 resin (H⁺ form, 20-50 mesh, obtained from Baker) which had been washed twice with 40 mL of distilled water, 20 mL of dry methanol (initial drying with Na₂O₃ and distilled over CaH₂ under Ar), 20 mL of dry diethyl ether (distilled over Na₂O₃ under Ar), followed by a final rinse with 10 mL of dry methanol. The reactor was purged with argon for several hours after which the above catalyst system was charged with stirring. Additional hydrogen gas was added after 3-h time intervals to raise the reactor pressure back to approximately 600 psi. This was continued until negligible hydrogen uptake was observed and the total reaction time was 24 h. The reactor was then allowed to cool to room temperature before opening. The reaction contents were filtered through a cotton plug, and the solvent was removed by rotary evaporation (40 °C, approximately 125 mmHg). The remaining liquid was fractionally distilled (58.5-60 °C, 7.5 mmHg).
Poly(3-hydroxybutyrate) Stereosomers

Polymer Preparations. The polymerization reactions were carried out in 3-ml polymerization tubes previously silanized, flame-dried under vacuum, and argon gas purged, with all transfers taking place via syringes through rubber septum caps under an argon atmosphere. Racemic BL obtained from Aldrich, distilled, and dried in the same manner as was described above for the pure polymerization mixture was combined with the pure BL to obtain the desired stereoisomeric monomer purities. To each polymerization tube containing BL (1.0 g, 1.16 x 10^2 mol) was added 130 mL of the clear yellow toluene catalyst solution. The ampules were sealed under vacuum (150 mmHg, argon gas bleed) with cooling. The polymerization reactions were carried out at 60 °C for 7 days.

The purification procedure involved the dissolution of the ampule contents in chloroform (3 mL) and precipitation of this solution into 35 mL of a 1:1 diethyl ether/hexane mixture. The precipitated polymers were washed two times with 1:1 ether/hexane (10 mL each) and then dried in vacuo (50 °C, 35 mmHg) for 24 h. The resulting solid was characterized by 1H and 13C NMR which agreed with previously published spectral data,26,27,43 and showed that the polymers were obtained in greater than 98% purity. Molecular weight and stereochemical analysis of these polymeric materials is provided in the Results and Discussion.

Enzyme Isolation from *P. fucatus* (ATCC 9644).2,14 *P. fucatus* (ATCC 9644) was used to produce an exoenzyme polymerase which was highly specific for the degradation of (R)-PHB. Details for the purification and isolation of the partially purified exoenzyme follow exactly those which have been previously described in the literature.4,15

*P. fucatus* PHB Depolymerase Enzyme Solution Activity Determination. PHB homopolymer (natural origin) obtained from Marlborough Biopolymers (technical grade powder) was purified by dissolving in CHCl3, filtering through cotton, and precipitating into methanol, followed by washing with acetone and finally with diethyl ether. The resulting powder has a M9 = 121,900 g/mol and a Mw/Mn = 3.06 as determined by GPC (see table).

The polymer was melt cast between Teflon-coated glass plates at 195 °C, and immediately placed into a 37 °C oven and annealed for 10 days before use. Films of 30 mm2 rectangles were exposed to the *P. fucatus* enzyme as described below, giving a standard rate of 1.06 x 10^{-4} (H^+)-1/mm^2 min^-1.

Preparation of Polymer Films for Enzymatic Exposures. The polymer samples were melt cast into thin films between Teflon-coated glass plates at 160 °C for 10 min, and allowed to anneal at room temperature for at least 30 days prior to their use. These samples were cut into 30 mm^2 rectangles for the determination of their degradability by the *P. fucatus* exoenzyme esterase.

Enzyme Degradation Kinetic Measurements. Into a reaction vessel containing 2 mL of doubly-distilled/deionized water deoxygenated with argon was placed a polymer film sample. The vessel was equilibrated at 30.0 ± 0.3 °C while magnetic stirring of the polymer film suspension was initiated under an argon atmosphere.4,15 A 5.0-pL volume of the above enzyme solution was added, and the pH as a function of time was monitored on a calibrated strip chart recorder at a chart speed of 10 cm/h, from which data points were taken every 2 min for (H^+)-1/mm^2 versus time profiles (see Figure 3). The result reported represent an average of a minimum of four trials per test sample. The optimum pH range for this enzyme was determined to be between approximately pH 5.5 and pH 6.2 at 30 °C,4,15 as and such the tests were conducted at a starting pH of between 5.2 and 6.4. Since the pH is equal to log (H^+), measured values of the pH as a function of the enzyme/polymer film incubation time were used to calculate the kinetics of H^+ formation and, therefore, enzyme cleavage events.

For extended-time enzyme/polymer incubation studies, 30 mg of a selected polymer sample in the form of either a film or powder...
was added to 15 mL of a 0.1 M acetic acid/sodium acetate buffer at pH 5.9. The powdered polymer samples in the buffer suspension were then sonicated to obtain relatively smaller particle sizes so as to increase the available sample surface area. To this was added 30 μL of the above enzyme solution, and the system was placed into a 30 ± 0.5 °C bath while maintaining magnetic stirring and an argon purge for the duration of the incubation period (from 4 to 21 days).

Control experiments for both short-term kinetic studies where pH changes were determined as a function of time as well as long-term enzymatic degradation performed in an acetic acid buffer system at pH 5.9 were carried out by simply omitting the addition of the enzyme solution. Short-term kinetic control experiments were carried out by starting at the reduced pH value of 4.0 as to simulate extremes in the pH range observed where events of chemically mediated hydrolytic degradation would be accelerated.

Analysis of Long-Term Degradation Studies. The mixture resulting from the long-term exposure of a PHB polymer sample and the PHB depolymerase was centrifuged to remove the remaining water-insoluble polymer, and the supernatant was decanted. The isolated water-insoluble polymers were triturated two times with distilled water (2-mL volumes), and the polymer and wash supernatant solutions were separated by centrifugation. The resultant insoluble polymer was then dried at room temperature in vacuo and further analyzed (see Results and Discussion).

The water-soluble degradation products remaining in the original incubation supernatant combined with the supernatant solutions from trituration were isolated by saturation of this aqueous phase with sodium chloride, acidification at 3 °C, extraction with diethyl ether at 3 °C for 48 h using a continuous liquid-liquid extractor. Rotary evaporation of the diethyl ether extract gave the isolated water-soluble hydroxy acid degradation products. This was then reacted with excess diazomethane in diethyl ether to form the corresponding methyl 0-hydroxybutyrate from the end group hydrogen and the methylene and methyl hydrogens from the oligomer repeat units.

Polymer Methanalysis. The procedure followed exactly that which was previously described.28,41

Results and Discussion

The method developed by Seebach40 for the preparation of (R)- and (S)-BL from (S)- and (R)-β-hydroxybutyric acid, respectively, proceeded with inversion of configuration and gave BL with an enantiomeric excess (ee) of greater than 98%. This result is in agreement with that reported.4 This ee value for both (R)- and (S)-BL was measured by 1H NMR (see Experimental Section) using a chiral shift reagent europium(III) triis-[3-[((heptafluoropropyl)hydroxymethylene]-(+)-camphorato], Eu[(+)-(hfc)]3, to resolve the NMR signals corresponding to the enantiomeric lactones.26

To study the effects of PHB stereochirality and crystalline morphology on enzymatic degradability, a series of PHB stereocopolymers were synthesized using different enantiomeric monomer compositions and a ZnEt2/H2O (1/0.6) catalyst system (see Table I). The polymers obtained using this methodology, with the exception of the 50% R monomer feed ZnEt2/H2O (1/0.6) catalyzed polymerization, had M0 values of approximately 4500 g/mol and Mw/M0 values of approximately 1.1.

As a result of racemization, the stereochirality composition of the polymers formed was found to vary by 2–6% from that of the monomer feed (see Table I). For the measurement of the stereoisomeric purity of these polymer samples, isomerically pure natural origin PHB as well as the chemically synthesized PHB stereocopolymers was subjected to acid-catalyzed methanalysis to form the corresponding methyl β-hydroxybutyrate stereoisomer(s). It is well known from earlier published work that this chemical transformation took place with complete configurational retention.28,41 Optical rotation measurements performed on these methyl β-hydroxybutyrate samples as well as analysis of the stereochemical composition by 1H NMR26 using the chiral shift reagent Eu[(+)-(hfc)]3 (see Experimental Section) both showed that approximately 5% racemization at the methine stereocenter takes place upon polymerization of BL with the ZnEt2/H2O (1/0.6) catalyst used herein (see Table I).

The effect of PHB stereochirality on the crystalline morphology was studied by DSC. Observation of Figures 1 and 2 shows, as was anticipated, that the increase in S repeat unit content from 0 to 50% results in a dramatic disruption of the crystalline order. This disruption is manifested in decreased melting temperature and enthalpy of fusion values for this series (see Figure 1 and 2). These results are in agreement with those obtained by Doi and co-workers for PHB stereocopolymers synthesized in up to 79% (R)-HB repeat unit composition.22 Indeed, the synthesis of a series of PHB stereocopolymer samples creates an interesting opportunity to determine whether the change in enzyme substrate specificity due to stereochemical preferences will dominate over the creation of materials with less ordered crystalline domains and, probably, lowered percent crystallinity.

Since we have recently discovered a methodology for the synthesis of crystalline PHB from racemic monomer which has a predominantly syndiotactic (Syn) stereochirality chain sequence, we have included Syn-PHB in the current study. The preference for syndiotactic placement was demonstrated by 13C NMR spectroscopy and the observation of the carbonyl resonances corresponding to meso and racemic dyads.22,23 Since Syn-PHB has a predominantly alternating stereochemical sequence along the polymer chain, it provides an interesting opportunity to investigate the enzymatic degradability of a PHB

<p>| Table I |
|---|---|---|---|</p>
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<tr>
<th>monomer feed % R</th>
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* Polymersized using a ZnEt2/H2O (1/0.6) catalyst (see Experimental Section). * Molecular weight values before and after melt casting of PHB samples into films at 160 °C for 10 min were identical. * Determined by GPC relative to polystyrene in CHCl3 using 10-, 10-, and 10-Å ultrastyragel columns. * Determined by optical rotation of the corresponding methyl 3-hydroxybutyrate from the acidic methanalysis of the PHB stereoisomer (see Experimental Section). * Determined by analysis of the methyl ester protons of the corresponding methyl 3-hydroxybutyrate (obtained from the acidic methanalysis of the stereoisomer) using Eu[ (+)-(hfc)]3 as a chiral shift reagent (see Experimental Section). * Natural origin (R)-PHB degraded to M0 = 3700 by controlled methanalysis. * Calculated from the percent racemization determined by degradation of PHB stereoisomers to form methyl 3-hydroxybutyrate and subsequent optical rotation analyses (see Experimental Section). * Not determined. * Noncrystalline tactic polymer sample. * Predominately syndiotactic PHB with the dyad fractions as follows: racemic, 0.68; meso, 0.34.
Figure 1. DSC thermograms of bacterial PHB ($M_w = 3700$) (top thermogram) and synthetic PHB stereocopolymers ranging from 4 to 95% (R)-PHB, recorded for melt-cast samples during the first heating scan at a heating rate of 10 °C/min.

Figure 2. Dependence of the DSC melting enthalpy of fusion on the percent R for the PHB stereocopolymers. The $\Delta H_f$ values reported are for the first heating scan at a heating rate of 10 °C/min. The asterisk designates the 50% (R)-Syn-PHB sample.

The substrate with 50% (R)-HB composition where the statistical probability of chain segments with relatively long (S)-HB comonomer sequences (greater than three repeat units) is significantly decreased. The details for the synthesis and characterization of this new PHB stereoisomer is the subject of a separate publication.

The initial surface degradation kinetics for the PHB stereoisomeric samples exposed to the exoenzyme from P. funiculosus were measured by following the pH change as a function of time for film samples of uniform dimensions (see Experimental Section). The change in pH allows the calculation of the $[\text{H}^+]$. The values obtained for changes in the $[\text{H}^+]$ as a function of time were divided by the film surface area (mm$^2$) to normalize for effects due to the film thickness. Indeed, for a film or solid powder substrate where the enzyme cannot diffuse into the solid matrix, the substrate concentration can be defined as the measured surface area of the solid substrate.

The relative rates of the initial surface degradation kinetics as a function of the percent (R)-HB comonomer content (determined by NMR, see Table I) are shown in Figure 4. The rate values were obtained by measuring the slopes from the linear portion of the curves in Figure 3. These linear regions of the curves were within the pH range of 4.9–5.6 where the enzyme shows greater than 90% of its optimum activity.$^{21,22}$ In all cases, the data points (taken every 2 min) used to measure the rate values (plotted in Figure 4) have correlation coefficients of greater than 0.99 and standard deviations of between 2 and 5%. The linear portion of the curve was preceded by an induction period of variable duration (depending on the polymer stereochemistry) where a significantly reduced value for $[\text{H}^+]$/(mm$^2$-min) was observed. The reason for an induction period is currently unknown but may be due to the time needed for saturation of the film surface with the exoenzyme. This will be verified in later work by the use of a radiolabeled exoenzyme. Controls for the PHB stereoisomer samples were carried out by monitoring the pH in the absence of enzyme (see Experimental Section). These control experiments showed negligible change in the pH as a function of time, indicating that for this analysis contributions by nonbiologically mediated chemical hydrolysis can be ignored.

In discussing the results presented in Figure 4, it is important to first state that the 4% (R)-PHB sample showed an initial surface enzyme degradation rate which was nondetectable by the method used herein (see Figures 3 and 4). The introduction of increasing relative amounts of the S stereochemical repeat unit into (R)-PHB profoundly alters the crystalline morphology so as to decrease the crystalline order and, most likely, the degree of crystallinity (see Figures 1 and 2). This effect considered alone would be expected to result in increased rates of degradation as we increase the (S)-HB content. Con-
versely, the enzyme has a stereochemical preference for (R)-HB repeat units since a 95% (R)-PHB sample is degradable while the corresponding 4% (R)-PHB sample showed no apparent degradation. Indeed, we therefore have a very interesting series of polymers where variables have been introduced which create a complex interplay between opposing effects due to crystalline morphology and enzyme stereochemical specificity.

Observation of Figures 3a and 4 shows that there is a rather large increase in the degradation kinetics for the 67 and 77% (R)-PHB samples relative to the other PHB stereoisomers studied. In contrast, introduction of lower quantities of (S)-HB repeat units into the stereocopolymer (81, 85, 90, and 95% R) showed a significant decrease in the degradation kinetics relative to the higher percent R sample (natural origin, 100% (R)-PHB) and the lower percent R samples (67 and 77% (R)-PHB). Therefore, it appears that the decreased crystalline order of the PHB samples containing 81, 85, 90, and 95% R was not the dominant factor, and therefore, the effects of decreased enzyme specificity in the degradation of (S)-HB repeat units is causing the observed depression in the rate values for these stereoisomers. However, the dramatic increase in the rate values observed in Figure 4 for R stereocomposition of 67 and 77% indicate that, below a certain threshold value of crystalline order in the PHB samples, crystalline morphology effects dominate the enzyme’s preference for (R)-HB repeat units. In this way, we have demonstrated how polymer stereochemical effects can be used to decrease as well as dramatically increase the degradation kinetics for this model system. Further work is currently in progress to verify the generality of these observations for other PHB depolymerase enzyme systems isolated from various mixed microbial communities.

The 50% R atactic sample (At-PHB) is not crystalline and showed a large initial degradation rate ($1.4 \times 10^{-3}$ [H+]/(mm²·min)) followed by an abrupt decrease in the rate value (see Figures 3b and 4). This decrease in the rate occurred even though the pH values in the study remained well within the optimum values for the exoenzyme during the relatively short (approximately 50 min) exposure period. The above may be due to a depletion of (R)-HB-rich segments on the polymer surface and indicates that the 50% (R)-At-PHB sample may not be completely degradable by this enzyme system. Conversely, the 50% (R)-Syn-PHB sample (see above) should have a distribution of R and S repeat units in which the occurrence of (R)-HB- or (S)-HB-rich segments is less probable. Indeed, Syn-PHB may be expected to meet the stereochemical requirements for the enzyme to completely degrade this polymer stereoisomer to low molecular weight oligomeric species defined for the present discussion as having a degree of polymerization less than 5. Observation of Figure 3b shows that the change in [H+]/(mm²) for the 50% (R)-Syn-PHB sample as a function of time does not abruptly decrease at later time points during the study as was observed for the 50% (R)-At-PHB sample. Unfortunately, consideration of only the rate information derived from Figures 3 and 4 does not allow one to conclude whether the PHB depolymerase from P. funiculosum can completely degrade polymeric chains of the PHB stereoisomers studied to oligomeric species. The complete degradability of polymeric chains is not only of great importance in clarifying the discussion above on the 50% (R)-Syn- and (R)-At-PHB samples, but also should be demonstrated for the 77% (R)-PHB sample which showed the largest degradation rate (see Figure 4). Therefore, we carried out long-term degradation studies on the 50% (R)-At- and (R)-Syn-PHB samples as well as the 77% (R)-PHB sample (see Experimental Section). The average oligomer length of the soluble products as well as the sample weight loss.
Results from the Long-Term PHB Stereoisomer/Enzyme Incubation Studies Carried out at 30 °C in a CH$_3$CO$_2$H/CH$_3$CO$_2$Na* Buffer of pH 5.9

<table>
<thead>
<tr>
<th>% R content</th>
<th>incubation time (days)</th>
<th>% wt loss</th>
<th>$M_w$ after incubation (g/mol)</th>
<th>$M_w/M_r$</th>
<th>av olig chain length for degrad products</th>
</tr>
</thead>
<tbody>
<tr>
<td>77 powder</td>
<td>4</td>
<td>44</td>
<td>5100</td>
<td>1.1</td>
<td>3.4 ± 0.9</td>
</tr>
<tr>
<td>50, At film</td>
<td>21</td>
<td>&lt;1</td>
<td>47000</td>
<td>1.4</td>
<td>d</td>
</tr>
<tr>
<td>50, Syn powder</td>
<td>6</td>
<td>20</td>
<td>7500</td>
<td>1.1</td>
<td>2.7 ± 0.7</td>
</tr>
<tr>
<td>control film</td>
<td>13</td>
<td>6</td>
<td>5200</td>
<td>1.1</td>
<td>d</td>
</tr>
</tbody>
</table>

* See Experimental Section. b Percent R content by NMR (see Table I). c GPC measurements on the remaining water-insoluble polymer (see Table I for $M_w$ values before incubation). d No isolated degradation products. e Controls were carried out under identical conditions to the experimental samples without the addition of the P. funiculosum exoenzyme.

and molecular weight change was determined for these longer exposure time period studies.

The results for the longer polymer substrate/enzyme incubation time periods as well as appropriate controls are shown in Table II. For the 77% (R)-PHB sample, there was an 83% weight loss after a 13-day exposure period whereas the control experiment where no enzyme was added showed less than 3% weight loss over the same time period. Interestingly, the 50% (R)-At- and (R)-Syn-PHB samples exhibited completely different long-term degradation profiles. The 50% R atactic material showed essentially no weight loss in the presence or absence of the enzyme after 21 days, while the predominately syndiotactic material had lost approximately 20% of its original weight after 6 days in the presence of the enzyme and less than 3% in the absence of the enzyme. For all of the above incubations including the 21-day exposure of the 50% (R)-atatic-PHB sample, the molecular weights before and after the exposures, whether in the presence or the absence of the enzyme, showed no significant change in the measured $M_w$ or $M_w/M_r$ values obtained. In addition, the water-soluble oligomeric degradation products recovered from the long-term incubation experiments (see Experimental Section) had on average three repeat units (see Table II). This was determined by reaction of the respective oligomer sample with diazomethane to form the corresponding methyl ester followed by analysis of the relative peak intensities by NMR (see Experimental Section and Figure 5). In no case were soluble degradation products found for the control experiments.

The above results considered in combination strongly suggest that the exoenzyme from P. funiculosum is capable of degrading the polymeric chains for both the 77% (R)-PHB sample and the predominantly syndiotactic 50% (R)-PHB sample to low molecular weight oligomeric species. Longer term biodegradability studies are in progress to confirm whether these samples are indeed 100% biodegradable. However, it appears that the 50% (R)-atatic-PHB sample is a very poor substrate for the depolymerase after the initial degradation of R-rich chain segments on the polymer sample surface. This can be concluded even when considering the relatively higher molecular weight of the 50% R atactic (by a factor of approximately 10, see Tables I and II) since, on the basis of the initial measured rate of degradation (Figures 3b and 4), film weight loss would have been expected over the 21-day incubation period but was not observed.

We were rather fortunate in this study that nonbiologically mediated chemical hydrolysis of the PHB stereoisomer samples was negligible for both the short-term initial surface degradation rate measurements as well as the long-term incubation studies. Indeed, effects of hydrolytic degradation would surely have greatly complicated interpretation of the rate measurements obtained for all of the above studies. The slow rates for chemical hydrolysis of PHB make it ideal for model studies of enzyme-mediated hydrolytic degradation.

Summary of Results

In this study, we have prepared poly$(\beta$-hydroxybutyrate), PHB, random stereocopolymers from $\beta$-butyrolactone, BL, using a diethylzinc/water (1.0/0.5) catalyst system. (R)- and (S)-BL were synthesized in high enantiomeric purity (>98% ee), and approximately 5% racemization occurred at the methine stereocenters upon polymerization. The relative degradability of these PHB stereoisomers was studied with a PHB depolymerase enzyme isolated from P. funiculosum. This enzyme was shown to catalyze the hydrolysis of (R)-PHB, but did not show activity for the enantiomeric substrate (S)-PHB. The

![Figure 5](image-url)
initial surface degradation rates in [H+]/(mm^2-min) were determined by measuring the pH change as a function of time for polymer/enzyme incubations. It was shown for R stereoblock copolymer compositions of 95, 90, 85, and 81% that the degradation rates were lower than those measured for a 100% (R)-PHB sample where the samples were of comparable molecular weights. Therefore, the preference for (R)-HB repeat units by the P. funiculosum esterase appears to dominate over crystallinity effects for the compositional range of 81-100% (R)-HB. However, the initial surface degradation rates for the 67 and 77% (R)-PHB samples were dramatically larger relative to that measured for the 100% (R)-PHB sample. This result suggests that the critical degree of disruption of the crystalline order which occurred for compositions between 77 and 81% (R)-HB dominates the degradation rate. Results from extended short period polymer substrate/exoenzyme incubations in combination with the above kinetic studies suggested that the 50% (R)-atactic-PHB sample was a poor substrate for the enzyme after the rapid initial degradation of (R)-HB-rich polymer chain segments at the film surface. In contrast, predominantly syndiotactic 50% (R)-PHB and the 77% (R)-PHB polymeric chains appeared completely degraded by the P. funiculosum esterase to products containing on average 3 ± 1 HB repeat units. The relative degradability of these PHB stereoisomers has interesting implications on the acceptability of specific stereochanical sequences in the biodegradation of PHB. Work is currently in progress to carefully define the relative quantities and stereochomical composition of the degradation products as a function of the PHB stereoisomer substrate and the depolymerase enzyme source.

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References and Notes

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(43) Assuming that the pH value for the degradation product mixture is between 4.41 (i.e., pKₐ for (R)-3-hydroxybutyric acid) and 4.74 (i.e., pKₐ for acetic acid) (pKₐ values taken from The Merck Index, 9th ed.; Windholz, M., Ed.; Merck and Co. Inc.: Rahway, N.J., 1976; pp 636 and 48, respectively, it can be shown that within the pH range of approximately 5.6-6.9 (i.e., the linear pH region used for the rate determinations, see Figure 3) the degree of ionization ranges from 94 to 76% (for pKₐ = 4.41) and from 88% to 59% (for pKₐ = 4.74), the actual number of enzymatic cleavage events per unit time can be determined. One could consider the measurement of the [H⁺]/[mM²]* as the apparent measure of enzymatic cleavage events occurring. The results presented herein of degradation kinetics are reasonable when one considers the enzyme substrate ratio and the linear pH region (correlation coefficient greater than 0.99) of the curve is used for the initial surface rate determinations.