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%. 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 enantiomeric substrate (*S*)-PHB. The *P. funicolusum* 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 $[H^+]/(mm^2 \cdot min)$ were determined by measuring the pH change as a function of time for polymer/enzyme incubations. It was shown that for *R* stereocopolymer compositions of 95, 90, 85, and 81% the degradation rate values (between 0.57×10^{-8} and 0.92×10^{-8} $[H^+]/(mm^2 \cdot min)$) were lower than those measured for a 100% (*R*)-PHB sample (1.41×10^{-8} $[H^+]/(mm^2 \cdot min)$) of similar molecular weight. Therefore, the preference for (*R*)-HB repeat units appears to dominate over crystalline morphology 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 2.85×10^{-8} and 7.51×10^{-8} $[H^+]/(mm^2 \cdot min)$, 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. 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 stereochemical sequences in the degradation of the PHB by *P. funiculosum* and other PHB depolymerases.

Introduction

Poly((*R*)-β-hydroxyalkanoates), (*R*)-PHA's, are synthesized by a wide variety of bacteria[1-7] 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((*R*)-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-workers[11] (*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)[15-18] 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 homogeniety a PHB depolymerase exoenzyme from the fungus *Penicillium funicolusum*.[21,22] It has been determined that the protein has an M_r 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

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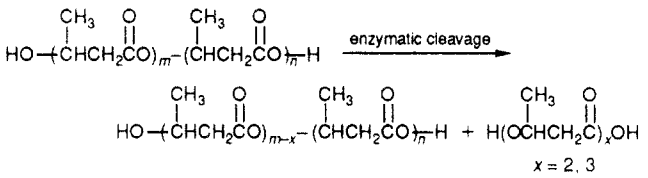
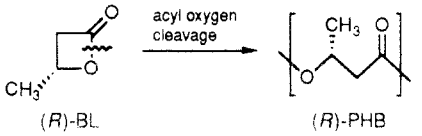
events are easily measured in the absence of appreciable chemical hydrolysis of PHB.

The ester functionalities of PHB, as well as other polyesters, 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 polyesters 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*[18] to involve the formation of dimer and trimer, and for *A. faecalis*[19] to involve the formation of dimer, from the free hydroxyl termini of the polymer chains.



It has been established that the ring-opening polymerization of β-butyrolactone, BL, with the $ZnEt_2/H_2O$ (1.0/0.6) catalyst system proceeds primarily with retention of configuration and, therefore, by an acyl oxygen ring-opening mechanism (see below).[26] It is expected from previous studies[27-29] and work in our laboratory[30] 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.[31-34]



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 stereocopolymer compositions,[29] undergoes subtle as well as extreme modulation of crystalline morphology as a function of the comonomer stereochemical composition[29] and annealing conditions,[35,36] and is readily available by both biosynthetic[1-7] as well as chemical synthetic[26] preparative routes. Specifically, we have prepared a series of PHB stereocopolymers using a $ZnEt_2/H_2O$ (1.0/0.6) catalyst where ideal random copolymerization of the (*R*)- and (*S*)-BL monomers is expected.[29] BL with an optical purity in excess of 98% was used to obtain a wide range of PHB stereocopolymer compositions with the desired relative amounts of the polymeric repeat units (*R*)- and (*S*)-HB. The polymer stereocopolymers were melt cast into thin films. The rate of surface degradation per unit surface area catalyzed by the extracellular esterase isolated from *P. funiculosum* 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 Brucker 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 $CDCl_3$ 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 $CDCl_3$; 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*)-3-hydroxybutyrate and methyl (*S*)-3-hydroxybutyrate and (*R*)- and (*S*)-BL, respectively, were carried out as 0.5% wt/wt solutions in CCl_4/benzene-d_6 (9/1, wt/wt) containing 30 mol % of europium-(III) tris[3-[(heptafluoropropyl)hydroxymethylene]-(+)-camphorato], 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 (^{13}C) NMR spectra were recorded at 67.9 MHz on a Brucker WP-270 SY in 5-mm tubes as $CDCl_3$ solutions, with chemical shifts in parts per million referenced relative to chloroform ($CHCl_3$) as an internal reference at 77.00 ppm. Polymer spectral acquisitions were conducted on 3.5% wt/wt solutions of polymer in $CDCl_3$ 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 Brucker IFS 113v 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: $[\alpha]^{\circ}C_{\lambda(nm)}$ = specific rotation (concentration in grams per 100 mL of solvent).

All molecular weights reported were determined by gel permeation chromatography (GPC) using a Waters Model 510 pump, Model 410 refractive index detector, and Model 730 data module with 10^3-, 10^4-, 10^5-, and 10^6-Å 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 (Polysciences) 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 8.0 and 10.0 mg of sample, a heating rate of 10 °C/min, and a nitrogen purge.

$Ru_2Cl_4[(S)$-(-)-BINAP$]_2NEt_3$ Catalyst Preparation.[37-39] The procedure followed was almost identical to that described in the literature.[38] 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.[38] 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 Na° and distilled over CaH_2 under Ar), 20 mL of dry diethyl ether (distilled over Na° 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 via cannulation as a solution in 10 mL of dry tetrahydrofuran (distilled over Na° under Ar). Methyl acetoacetate (110 g, 0.947 mol) obtained from Aldrich (99+%) was then added to the reactor as a solution in dry methanol (100 mL). Then, hydrogen gas (600 psi) was added to the reactor after which the internal vessel temperature was maintained at 80 °C 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 system 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)

to give 101.5 g (91% yield) of a clear colorless liquid: $[\alpha]^{20}_{578}$ = +51.4° (c 1.0, cyclohexane) (lit.[26] $[\alpha]^{20}_{578}$ = −49.6° (c 1.3, cyclohexane) for the (R) enantiomer); ^{1}H NMR 1.23 (d, 3 H), 2.45 (m, 2 H), 3.24 (br s, 1 H), 3.70 (s, 3 H), 4.20 ppm (m, 1 H); ^{13}C NMR 22.4, 42.7, 51.4, 64.1, 172.9 ppm; IR (neat) 3447, 2974, 1738, 1439, 1410, 1298, 1258, 1196, 1124, 1009, 947, 885 cm^{-1}.

(S)-β-Hydroxybutyric Acid (2a).[40] Into a 1-L flask was added 5 N KOH (200 mL) which was cooled to approximately 0 °C. Compound **1a** (100 g, 0.850 mol) was then added with stirring over a 1.5-h time period, and the temperature was maintained at 0 °C for 24 h. The reaction was terminated by the slow addition of 6 N HCl (166 mL) with stirring at approximately 5 °C. The resultant aqueous solution was then saturated with solid NaCl and extracted continuously for 24 h with diethyl ether using a liquid–liquid extractor. The organic extract was dried over anhydrous magnesium sulfate and the ether removed by rotary evaporation, leaving a viscous pale yellow oil. The crude product was fractionally distilled on a short-path apparatus (78 °C, 0.02 mmHg) to give 62 g (71% yield) of a white crystalline solid: mp 47–48.5 °C; $[\alpha]^{25}_{589}$ = +47.5° (c 5.0, $CHCl_3$) and +22.1° (c 5.0, H_2O) (lit.[40] $[\alpha]^{25}_{589}$ = +24.4° (c 4.2, H_2O)); ^{1}H NMR 1.25 (d, 3 H), 2.50 (m, 2 H), 4.25 (m, 1 H), 7.38 ppm (br s, 2 H); ^{13}C NMR 22.2, 42.6, 64.4, 176.3 ppm.

(R)-β-Butyrolactone ((R)-BL, 3a).[40] The conversion of **2a** to form **3a** followed a procedure that was previously described by Seebach and co-workers. The crude product was fractionally distilled on a 2.5-cm by 15-cm column packed with Raschig rings (52–55 °C, 11 mmHg) to give the product (19% yield) as a clear colorless liquid. Prior to polymerization, the (R)-BL was stirred over CaH_2 for 24 h at room temperature and redistilled: $[\alpha]^{25}_{589}$ = +24.0° (c 5.0, $CHCl_3$) (lit.[26] $[\alpha]^{25}_{589}$ = −26.1° (c 5.0, $CHCl_3$) for the S enantiomer); ^{1}H NMR 1.57 (d, 3 H), 3.06 (dd, 1 H), 3.57 (dd, 1 H), 4.70 ppm (m, 1 H); ^{13}C NMR 20.4, 44.1, 67.7, 167.9 ppm; IR 2984, 1827, 1456, 1389, 1290, 1139, 1022, 964, 827 cm^{-1}.

Methyl (R)-β-Hydroxybutyrate (1b).[26,41] The procedure used to obtain **1b** was the acidic methanolysis of natural origin (R)-PHB which followed directly that which has been previously described in the literature.[26,41] The product obtained had $[\alpha]^{20}_{578}$ = −52.6° (c 1.0, cyclohexane). ^{1}H and IR spectra were identical to those for **1a** above.

(R)-β-Hydroxybutyric Acid (2b). The procedure followed for the saponification of **1b** to form **2b** is the same as that described above for the saponification of the corresponding enantiomer **1a** to give **2a**. The purified product is a white crystalline solid: mp 47–48.5 °C; $[\alpha]^{25}_{589}$ = −49.8° (c 5.0, $CHCl_3$) and −23.0° (c 5.0, H_2O) (lit.[41,42] $[\alpha]^{25}_{589}$ = −24.7° (c 5.0, H_2O) and −24.5° (c 5.0, H_2O), respectively). ^{1}H and ^{13}C spectra were identical to those for **2a** above.

(S)-β-Butyrolactone ((S)-BL, 3b).[40] The procedure for the conversion of **2b** to **3b** follows the methodology previously described.[40] The purified product showed $[\alpha]^{25}_{589}$ = −26.4° (c 5.0, $CHCl_3$) (lit.[26] $[\alpha]^{25}_{589}$ = −26.1° (c 5.0, $CHCl_3$)). ^{1}H, ^{13}C, and IR spectra were identical to those for **3a** above.

Catalyst Preparation for Polymerization. The $ZnEt_2/H_2O$ (1.0/0.6) catalyst preparation is a modification of that previously described in the literature.[26] All of the operations described in the catalyst preparation or for solvent purification were carried out under an inert argon atmosphere or in vacuo vented with argon gas. Into a 50-mL Schlenck tube which had been silanized, flame-dried under vacuum, and purged with argon were transferred dry 1,4-dioxane (30 mL), distilled from Na°) by cannulation and diethylzinc (7.0 mL, 6.83 × 10^{-2} mol; obtained neat from Aldrich and used as received) via syringe. To this solution was added 0.74 mL of distilled, deoxygenated H_2O dropwise over a 30-min time period with stirring, giving a yellow solution with a large amount of precipitated solids. The stirred mixture was then transferred by cannulation to a series of storage tubes, and the volatiles were removed in vacuo (50 °C, 0.2 mmHg) to give a dark yellow-gold solid. The ampules were stored at −15 °C in a desiccator containing Drierite as desiccant for approximately 75 days until their use. For the polymerizations described below, dry toluene (2.5 mL, distilled over Na°) was added to an ampule containing 226.7 mg of the catalyst to give a clear yellow solution above an insoluble powder. The clear yellow toluene solution thus formed was used for the polymer preparations described below.

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 syringe 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 (R)-BL was combined with enantiomerically pure BL to obtain the desired stereoisomeric monomer purities. To each polymerization tube containing BL (1.0 g, 1.16 g × 10^{-2} mol) was added 130 μL 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/hexanes mixture. The insolubles were separated from the supernatant by centrifugation. To each of the individual polymer stereocopolymers was added 7.5 mL of acetylacetone (AcAc) per gram of polymer. The resultant AcAc solutions (for polymers obtained from either 100% (R)-*BL* or 100% (S)-BL the samples swelled but did not dissolve in AcAc) were reprecipitated into 1/1 ether/hexanes (60 mL) followed by centrifugation to isolate the polymers. The isolated polymeric materials were washed two times with 1/1 ether/hexanes (10 mL each) and then dried in vacuo (55 °C, 35 μmHg) for 24 h. The resultant white solids were characterized by ^{1}H and ^{13}C 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. funiculosum* (ATCC 9644).[21,22,44] *P. funiculosum* (ATCC 9644) was used to produce an exoenzyme depolymerase which was highly specific for the degradation of (R)-PHB. The details for the production and isolation of the partially purified enzyme follow exactly those which have been previously described in the literature.[45]

***P. funicolusum* PHB Depolymerase Enzyme Solution Activity Determination.** PHB homopolymer (natural origin) obtained from Marlborough Biopolymers (technical grade powder) was purified by dissolving in $CHCl_3$, filtering through cotton, and precipitating into methanol, followed by washing with acetone and finally with diethyl ether. The resulting powder has a M_n = 121 000 g/mol and a M_w/M_n = 3.06 as determined by GPC (see above).

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 mm^2 rectangles were exposed to the *P. funiculosum* enzyme as described below, giving a standard rate of 1.06 × 10^{-9} [H$^+$]/(mm^2·min).

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. funicolusum* exoenzyme esterase.

Enzyme Degradation Kinetic Measurements. Into a reaction vessel containing 2 mL of doubled-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.[46] A 5.0-μL 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$^+$]/mm^2 versus time profiles (see Figure 3). The rate results 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,[21,22] and as such the tests were conducted at a starting pH of between 6.2 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 ± 0.3 °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 enzyme/polymer incubations performed in an acetate 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 so 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 tempeature 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 to a pH of 1.5 with 12 M HCl, and 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 hydroxy acid degradation products. The average oligomer chain lengths for the methylated degradation products were determined by ^{1}H NMR spectroscopy (see above) from the comparative integration intensities of the end group methyl ester hydrogens and the methylene and methyl hydrogens in the oligomer repeat units.

Polymer Methanolysis. The procedure followed exactly that which was previously described.[26,41]

Results and Discussion

The method developed by Seebach[40] 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 previously reported.[40] The ee value for both (*R*)- and (*S*)-BL was measured by ^{1}H NMR (see Experimental Section) using the chiral shift reagent europium(III) tris[3-[(heptafluoropropyl)hydroxymethylene]-(+)-camphorato], Eu[(+)-(hfc)$_3$], to resolve the NMR signals corresponding to the enantiomeric lactones.[26]

To study the effects of PHB stereochemistry and crystalline morphology on enzymatic degradability, a series of PHB stereocopolymers were synthesized using different enantiomeric monomer compositions and a $ZnEt_2/H_2O$ (1/0.6) catalyst system (see Table I). The polymers obtained using this methodology, with the exception of the 50% *R* monomer feed $ZnEt_2/H_2O$ (1/0.6) catalyzed polymerization, had M_n values of approximately 4500 g/mol and M_w/M_n values of approximately 1.1.

As a result of racemization, the stereochemical 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 methanolysis 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.[26,41] Optical rotation measurements performed on these methyl β-hydroxybutyrate samples as well as analysis of the stereochemical composition by ^{1}H NMR[26] 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 $ZnEt_2/H_2O$ (1.0/0.6) catalyst used herein (see Table I).

Table I
Ring-Opening Stereocopolymerization of β-Butyrolactone: Percent Yield and Polymer Stereochemical and Molecular Weight Analysis

monomer feed % *R*	% yield	M_n[b,c] (g/mol)	M_w/M_n	% *R*[d] polarimetry	% *R*[e] NMR
f		3700	1.5	100	100
100[a]	74	3500	1.2	94	95
95[a]	73	4000	1.1	90	90
91[a]	62	4100	1.1	86[g]	ND[h]
87[a]	62	4900	1.1	83[g]	85
84[a]	62	4900	1.1	80[g]	81
80[a]	63	5300	1.1	76	77
70[a]	68	7000	1.1	68[g]	67
50, At[a,i]	100	50000	1.4	50	50
50, Syn[j]	69	9200	1.1		
0[a]	87	3500	1.1	6	4

[a] Polymerized using a $ZnEt_2/H_2O$ (1.0/0.6) catalyst (see Experimental Section). [b] Molecular weight values before and after melt casting of PHB samples into films at 160 °C for 10 min were identical. [c] Determined by GPC relative to polystyrene in $CHCl_3$ using 10^3-, 10^4-, 10^5-, and 10^6-Å ultrastyragel columns. [d] Determined by optical rotation of the corresponding methyl 3-hydroxybutyrate from the acidic methanolysis of the PHB stereoisomer (see Experimental Section). [e] Determined by analysis of the methyl ester protons of the corresponding methyl 3-hydroxybutyrate (obtained from the acidic methanolysis of the stereoisomers) using Eu[(+)-(hfc)$_3$] as a chiral shift reagent (see Experimental Section). [f] Natural origin (*R*)-PHB degraded to M_n = 3700 by controlled methanolysis.[48] [g] Calculated from the percent racemization determined by degradation of PHB stereoisomers to form methyl 3-hydroxybutyrate and subsequent optical rotation analyses (see Experimental Section). [h] Not determined. [i] Noncrystalline atactic polymer sample. [j] Predominately syndiotactic PHB with the dyad fractions as follows: racemic, 0.66; meso, 0.34

The effect of PHB stereochemistry 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 temperatures and enthalpy of fusion values for this series (see Figures 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.[29] 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) stereochemical chain sequence, we have included Syn-PHB in the current study. The preference for syndiotactic placement was demonstrated by ^{13}C NMR spectroscopy and the observation of the carbonyl resonances corresponding to meso and racemic dyads.[32,43] 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

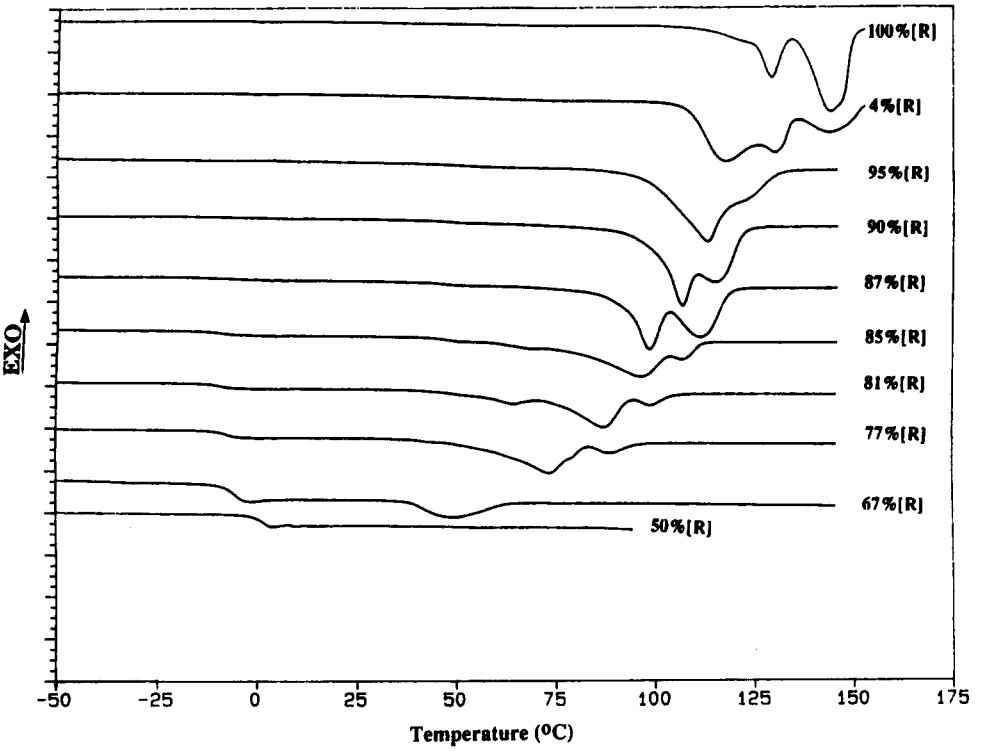


Figure 1. DSC thermograms of bacterial PHB (M_n = 3700) (top thermogram) and synthetic PHB stereocopolymers ranging from 4 to 95% (*R*)-PHB, recorded for melt-cast samples during the first heating scan at a heating rate of 10 °C/min.

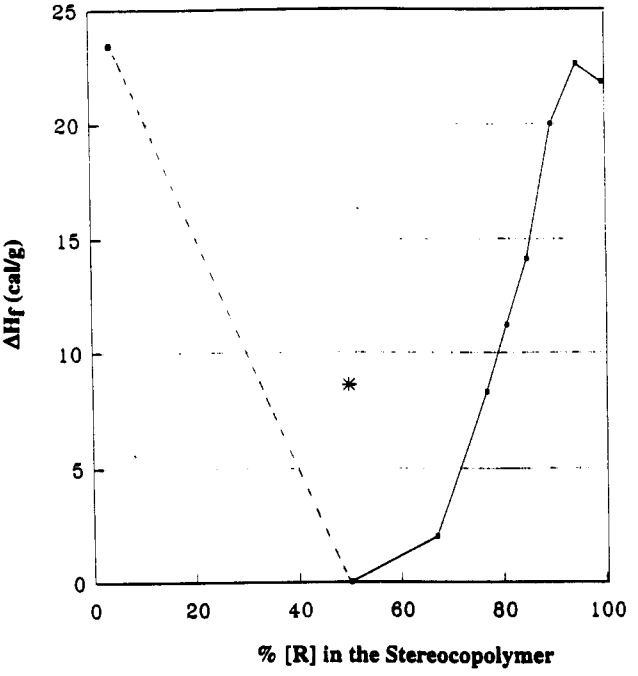


Figure 2. Dependence of the DSC melting enthalpy of fusion on the percent *R* for the PHB stereocopolymers. The ΔH_f values reported are for the first heating scan at a heating rate of 10 °C/min. The asterisk designates the 50% (*R*)-Syn-PHB sample.

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.[47]

The initial surface degradation kinetics for the PHB stereoisomeric samples exposed to the exoenzyme from *P. funiculosum* 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 $[H^+]$.[49] The values obtained for changes in the $[H^+]$ 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 $[H^+]/(mm^2 \cdot min)$ 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-

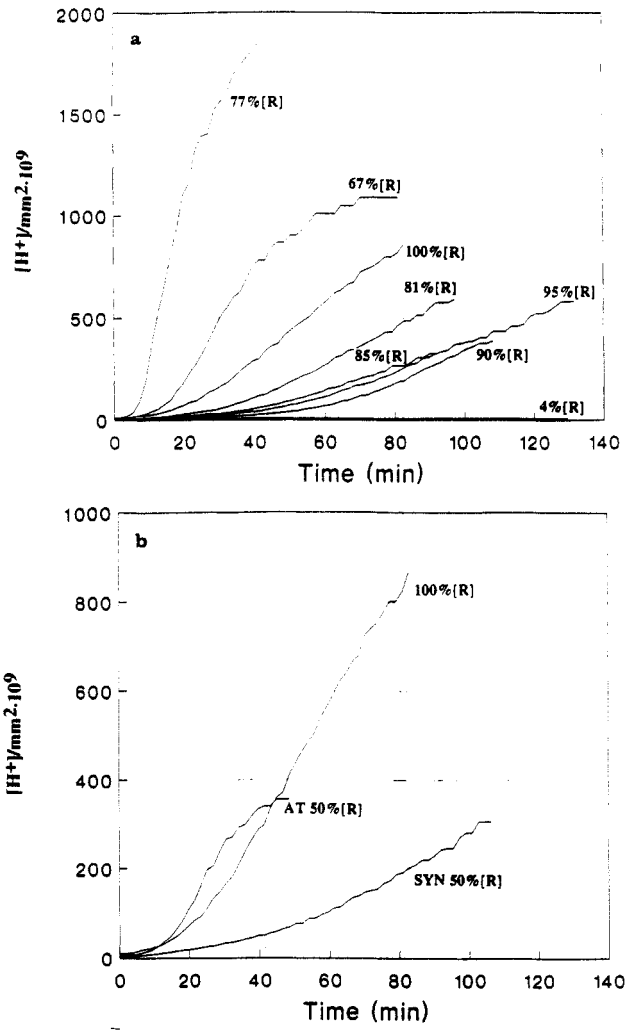


Figure 3. $[H^+]/mm^2$ as a function of time for the PHB stereoisomer/enzyme incubation studies: (a) 4% *R* through 100% *R* stereocopolymer compositions of comparable molecular weights, (b) comparison of the atactic 50% *R*, predominately syndiotactic 50% *R*, and the 100% *R* (natural origin). The curves were generated from data points collected every 2 min.

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* stereochemical compositions 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

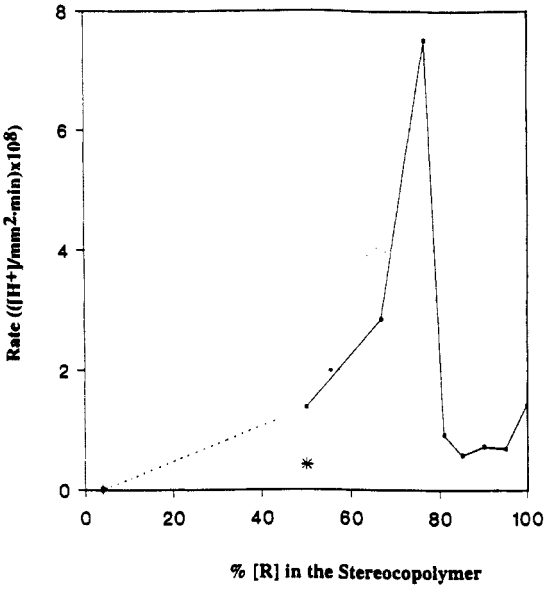


Figure 4. Enzyme degradation rates in $[H^+]/(mm^2 \cdot min)$ as a function of stereochemistry for the PHB stereoisomer/enzyme incubation studies. The asterisk designates the 50% (*R*)-Syn-PHB sample. The rate values were obtained from measuring the slope for the linear region of the curves in Figure 3.

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 (1.4×10^{-8} [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 $[H^+]/mm^2$ 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

Table II
Results from the Long-Term PHB Stereoisomer/Enzyme Incubation Studies[a] Carried out at 30 °C in a $CH_3CO_2H/CH_3CO_2^-Na^+$ Buffer of pH 5.9

% R content[b]	sample form	incubatn time (days)	% wt loss	M_n[c] after incubatn (g/mol)	M_w/M_n[c]	av olig chain length for degradn products
77	powder	4	44	5100	1.1	3.4 ± 0.9
	film	13	83	5200	1.1	3.2 ± 1.3
control[e]	powder	13	<1	5200	1.1	*d*
50, At	film	21	<1	47000	1.4	*d*
control[e]	film	21	<1	49000	1.4	*d*
50, Syn	powder	6	20	7500	1.1	2.7 ± 0.7
control[e]	powder	6	<3	7500	1.1	*d*

[a] 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_n 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/exoenzyme 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*)-atactic-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_n or M_w/M_n 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*)-atactic-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(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), 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

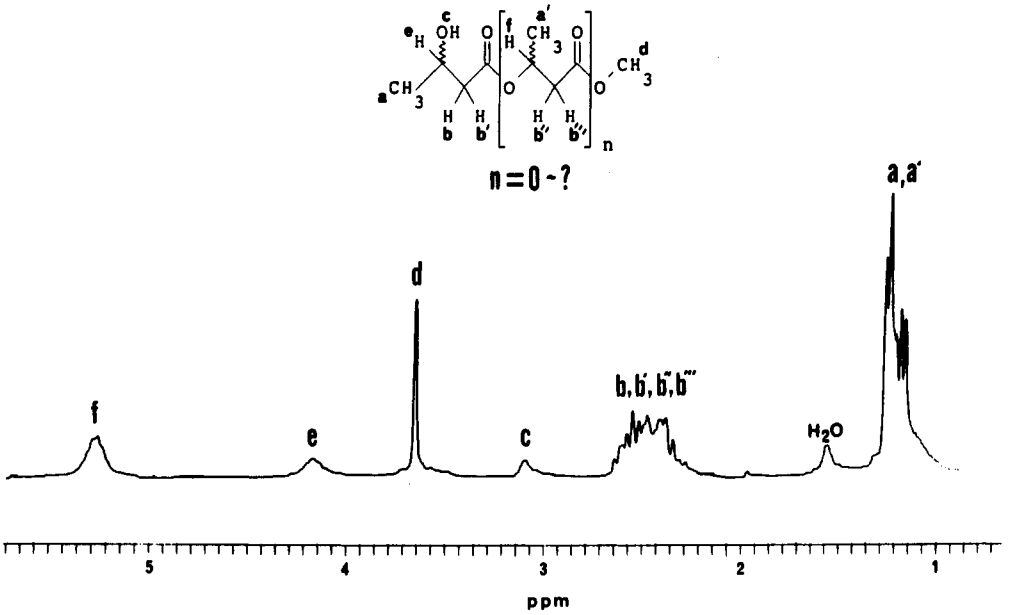


Figure 5. ^1H NMR spectrum (270 MHz) in $CDCl_3$ of the diazomethane-derivatized water-soluble degradation products isolated after a 13-day incubation of the *P. funiculosum* exoenzyme and the 77% (*R*)-PHB sample (see Table II).

initial surface degradation rates in $[H^+]/(mm^2 \cdot min)$ were determined by measuring the pH change as a function of time for polymer/enzyme incubations. It was shown for *R* stereocopolymer 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 time 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 stereochemical sequences in the biodegradation of PHB. Work is currently in progress to carefully define the relative quantities and stereochemical composition of the degradation products as a function of the PHB stereoisomer substrate and the depolymerase enzyme source.

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(49) Assuming that the pK_a for the degradation product mixture is between 4.41 (i.e., pK_a for (*R*)-3-hydroxybutyric acid) and 4.74 (i.e., pK_a for acetic acid) (pK_a's taken from *The Merck Index*, 9th ed.; Windholz, M., Ed.; Merck and Co., Inc.: Rahway, NJ, 1976; pp 636 and 48, respectively, it can be shown that within the pH range of approximately 5.6–4.9 (i.e., the linear pH region used for the rate determinations, see Figure 3) the degree of ionization ranges from 94 to 76% (for $pK_a = 4.41$) and from 88% to 59% (for $pK_a = 4.74$). From this, the actual number of enzymatic cleavage events per unit time can be determined. One could consider the measurement of the $[H^+]/mm^2$ as the apparent measure of enzymatic cleavage events occurring. The results presented herein of degradation kinetics are reasonable when one considers that only the linear region (correlation coefficient greater than 0.99) of the curve is used for the initial surface rate determinations.

Registry No. **1a**, 73349-08-3; **1b**, 3976-69-0; **2a**, 6168-83-8; **2b**, 625-72-9; **2b** (isotactic homopolymer), 141455-97-2; **2b** (SRU), 31759-58-7; **3a**, 32082-74-9; **3a** (homopolymer), 31305-69-8; **3b**, 65058-82-4; **3b** (homopolymer), 110670-57-0; **3b** (SRU), 110715-66-7; (**3a**)(**3b**) (copolymer), 65058-83-5; (**3a**)(**3b**) (SRU), 26744-04-7; $ZnEt_2$, 557-20-0; H_2O, 7732-18-5; methyl acetoacetate, 105-45-3; depolymerase, 9030-73-3.