Physical Properties and Enzymatic Degradability of Poly(3-hydroxybutyrate) Stereoisomers with Different Stereoregularities

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ABSTRACT: Poly(3-hydroxybutyrate) (P(3HB)) stereoisomers of high molecular weights ($\bar{M}_n > 10^5$) were synthesized by the ring-opening stereocopolymerization of (*R*)-β-butyrolactone with (*S*)-β-butyrolactone at various feed ratios (*R*/*S* = 96/4 to 50/50) in the presence of 1-ethoxy-3-chlorotetrabutyldistannoxane as a catalyst. The isotactic diad fractions [i] of P(3HB) stereoisomer samples were varied from 0.92 to 0.30. A syndiotactic P(3HB) sample ([i] = 0.30) was obtained by polymerizing a racemic mixture of β-butyrolactone. The glass-transition temperatures of P(3HB) samples were 5 ± 2 °C, independent of the stereoregularity. The melting temperature of P(3HB) samples decreased from 177 to 92 °C as the [i] value was decreased from 1.00 to 0.68. The predominantly syndiotactic P(3HB) sample ([i] = 0.30) had two endothermic peaks at around 52 and 62 °C. The degree of X-ray crystallinity of solvent-cast P(3HB) films decreased from 62 to 40% as the [i] value was decreased from 1.00 to 0.68. The X-ray crystallinities of atactic ([i] = 0.46) and syndiotactic ([i] = 0.30) P(3HB) films were 0 and 26%, respectively. Enzymatic degradations of P(3HB) films with different stereoregularities were carried out for 3 h at 37 °C in 0.1 M potassium phosphate buffer (pH 7.4) in the presence of PHB depolymerases purified from *Pseudomonas pickettii* and *Alcaligenes faecalis*. The rates of enzymatic degradation of P(3HB) films ranging in [i] value from 0.68 to 0.92 were higher than that of bacterial P[(*R*)-3HB] film ([i] = 1.00). The highest rate of enzymatic hydrolysis was observed at the [i] value of 0.76. Little erosion of P(3HB) films was observed for the atactic and syndiotactic samples. The water-soluble products liberated during the enzymatic degradation of P(3HB) films were characterized by HPLC analysis. The bacterial P[(*R*)-3HB] film gave a mixture of monomer and dimer of 3-hydroxybutyric acid, but the steroirregular P(3HB) samples of [i] = 0.92–0.68 produced a mixture of monomer, dimer, trimer, and tetramer. In addition, the stereochemical effect on the rate of P(3HB) spherulite growth rate was studied.

Introduction

A wide variety of bacteria synthesize an optically active polymer of (*R*)-3-hydroxybutyric acid and accumulate it as an intracellular storage material of carbon and energy.[1,2] Poly[(*R*)-3-hydroxybutyrate] (P[(*R*)-3HB]) isolated from bacteria is a biodegradable thermoplastic with a melting temperature around 180 °C.[3,4] The bacterial P[(*R*)-3HB] has attracted industrial attention as a possible candidate of the large biotechnological products.[5]

Poly(3-hydroxybutyrate) (P(3HB)) with different stereoregularities has been synthesized by the ring-opening polymerization of racemic β-butyrolactone (β-BL) in the presence of aluminum- or zinc-based catalysts.[6–16] The polymerization of racemic β-BL with the trialkylaluminum/H_2O catalyst leads to the formation of a mixture of predominantly isotactic P(3HB) (with a melting temperature of 160–170 °C) and amorphous atactic P(3HB).[7–10,14,15] A predominantly syndiotactic P(3HB) was isolated as a component of P(3HB) polymers produced with an aluminum-based catalyst.[17] On the other hand, the $ZnEt_2/H_2O$ catalyst produces only amorphous atactic P(3HB).[10] Recently, a predominantly syndiotactic P(3HB) was prepared by the ring-opening polymerization of racemic β-BL in the presence of tin-based catalysts.[18] The syndiotactic P(3HB) showed a crystalline structure different from that of isotactic P(3HB).[17,18] An attempt to prepare optically active P[(*R*)-3HB] has been made by the stereoselective polymerization of racemic β-BL with the chiral initiators $ZnEt_2$/(*R*)-3,3-dimethyl-1,2-butanediol[19] and $AlEt_3$/(*N*,*N*′-disalicylidene-(1*R*,2*R*)-1,2-cyclohexanediaminato)cobalt(II),[20] which gave P(3HB) stereoisomers of only moderate enantiomeric enrichments. Other attempts have been made by the polymerization of optically active (*R*)-β-BL with the achiral initiators $AlEt_3/H_2O$[21,22] or $ZnEt_2/H_2O$.[22–24] The polymerization of (*R*)-β-BL with the $ZnEt_2/H_2O$ catalyst proceeded by acyl cleavage with retention of configuration to give P[(*R*)-3HB]. In the polymerization of an (*R*)- and (*S*)-β-BL mixture with the $ZnEt_2/H_2O$ catalyst, the (*R*)- and (*S*)-3HB units were statistically distributed in the P(3HB) sequence.[23] Recently, Hori et al.[25] found very active distannoxane catalysts for the ring-opening polymerization of (*R*)-β-BL to afford P[(*R*)-3HB] of high molecular weights ($\bar{M}_n > 10^5$), in which the polymerization of (*R*)-β-BL proceeded with retention of configuration.

Bacterial P[(*R*)-3HB] is a relatively stiff and brittle material, and its thermal degradation during processing is significant due to the thermal instability at temperatures above 180 °C.[1,26] A remarkable characteristic of P[(*R*)-3HB] is its biodegradability in the environment. Aerobic and anaerobic P[(*R*)-3HB]-degrading bacteria and fungi have been isolated from various environments.[27–35] The microorganisms excrete extracellular PHB depolymerases to degrade environmental P[(*R*)-3HB] and utilize the decomposed compounds as nutrients. The extracellular PHB depolymerases have been purified from some

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microorganisms such as *Pseudomonas lemoignei*,[36] *P. pickettii*,[34] *P. fluoresens*,[33] *Alcaligenes faecalis*,[29] *Comamonas testosteroni*,[35] and *Penicillium pinophilum*.[37] Recently, Kumagai et al.[38] reported that the rate of enzymatic hydrolysis of P[(*R*)-3HB] film by the PHB depolymerase from *A. faecalis* increased with a decrease in crystallinity. The introduction of (*S*)-3HB units into a P[(*R*)-3HB] sequence may improve the biodegradability and physical properties of P(3HB) materials.

Marchessault et al.[39,40] prepared P(3HB) polymers by the polymerization of racemic β-BL in the presence of $AlMe_3/H_2O$ catalyst and separated them into isotactic, atactic, and syndiotactic fractions. Atactic P(3HB) polymers were found to be hydrolyzed by PHB depolymerases, but isotactic and syndiotactic polymers were hardly degraded. Kemnitzer et al.[24] prepared P(3HB) stereoisomers of relatively low molecular weights ($\bar{M}_n$ = (3.5–7.0) × 10^3) by the polymerization of (*R*)-β-BL with (*S*)-β-BL at various feed ratios in the presence of $ZnEt_2/H_2O$ catalyst. The fraction of (*R*)-3HB units in P(3HB) was varied from 6 to 94%, and the stereochemical effects on the rate of enzymatic degradation were studied with the PHB depolymerase from *P. funiculosum*.[37] They demonstrated that the rate of enzymatic degradation of P[77% (*R*)-3HB] was several times higher than the rate of P[100% (*R*)-3HB], but that P[94% (*S*)-3HB] was hardly hydrolyzed by the enzyme. Those results prompted us to investigate the physical properties and enzymatic degradability of P(3HB) stereoisomers of high molecular weights ($\bar{M}_n$ > 10^5) which can be prepared by the polymerization of (*R*)-β-BL with (*S*)-β-BL at various feed ratios (*R*/*S* = 96/4 to 50/50) with a highly active distannoxane catalyst.[25]

In this paper, we study the thermal properties and crystallization behavior of P(3HB) stereoisomers with different stereoregularities (isotactic diad fractions from 1.00 to 0.30) by means of differential scanning calorimetry, X-ray diffraction, and optical microscopy. The stereochemical effects on the enzymatic degradability of P(3HB) films are studied in the presence of PHB depolymerases from *P. pickettii*[34] and *A. faecalis*.[29] In addition, the water-soluble products during the enzymatic degradation of P(3HB) film are characterized by HPLC analysis.

Experimental Section

Materials. Bacterial poly[(*R*)-3-hydroxybutyrate] (P[(*R*)-3HB]) ($\bar{M}_n$ = 281000, $\bar{M}_w/\bar{M}_n$ = 2.3) was produced from butyric acid by *Alcaligenes eutrophus*.[41] 1-Ethoxy-3-chlorotetrabutyldistannoxane was prepared by the literature method.[42] A mixture of Bu_2SnO (76.1 g, 306 mmol), Bu_2SnCl_2 (31.0 g, 102 mmol), and 95.0% ethanol (1.0 L) was refluxed. After 6 h, the resulting transparent solution was concentrated to give a white powder. The mixture of the powder and 99.5% ethanol was refluxed for 12 h. Recrystallization (hexane, 0 °C) of the crude product afforded 1-ethoxy-3-chlorotetrabutyldistannoxane (72.0 g, 64 mmol): mp 106–107 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.94 (t, *J* = 7.3 Hz, 12H), 0.95 (t, *J* = 7.3 Hz, 12H), 1.13 (t, *J* = 7.0 Hz, 6H), 1.28–1.60 (m, 32H), 1.60–1.90 (m, 16H), 3.62 (q, *J* = 7.0 Hz, 4H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 13.6, 13.6, 19.9, 25.0, 26.8, 27.1, 27.3, 27.5, 58.8. The compound was dried in vacuo at 100 °C for 20 h before use. (*R*)-β-Butyrolactone[25] (ee 92%) ((*R*)-β-BL) and racemic (*R,S*)-β-butyrolactone ((*R,S*)-β-BL) were dried by CaH_2 and distilled under reduced pressure. Racemic β-BL was purchased from Aldrich Chemical Co. The enantiomeric excess of (*R*)-β-BL was determined to be 92% by HPLC analysis. HPLC analysis of this lactone (column, Chiralcel OA; eluent, 9:1 mixture of hexane and 2-propanol; flow rate, 0.5 mL/min; detection, 220 nm light; 40 °C) showed two peaks with t_R = 27.0 and 29.6 min in a 4.2:95.8 ratio assignable to the *S* and *R* enantiomers, respectively.

Synthesis of Polymers. Poly(3-hydroxybutyrate) (P(3HB)) stereoisomer samples with different stereoregularities were synthesized by the ring-opening polymerization of (*R*)-β-BL with (*R,S*)-β-BL at various feed ratios in the presence of 1-ethoxy-3-chlorotetrabutyldistannoxane as a catalyst.[25] The polymerization of β-butyrolactone was carried out without solvent at 100 °C for 4 h under an argon atmosphere. The produced P(3HB) was dissolved in trichloromethane and precipitated in a mixture of diethyl ether and hexane (1/3). The precipitate was dried in vacuo at room temperature.

Preparation of P(3HB) Films. P(3HB) films (0.1 mm thickness) were prepared by solvent-casting techniques from chloroform solutions of P(3HB) using glass Petri dishes as casting surfaces. The films were then aged at least 3 weeks at room temperature to reach equilibrium crystallinity prior to analysis. The amorphous P(3HB) polymer was coated from a chloroform solution of the polymer as a film of 0.1 mm thickness on a Teflon sheet and used for analysis.

Enzymatic Degradation. The extracellular PHB depolymerases from *Pseudomonas pickettii* and *Alcaligenes faecalis* T1 were purified to electrophoretic homogeneity by the methods of Yamada et al.[34] and Shirakura et al.,[43] respectively. The enzymatic degradations of P(3HB) films by the purified PHB depolymerases were carried out at 37 °C in 0.1 M potassium phosphate buffer (pH 7.4). The P(3HB) films (initial weights: 14 mg, initial film dimensions: 10 × 10 × 0.1 mm) were placed in small bottles containing 1.0 mL of the buffer. The reaction was started by the addition of 10 μL of an aqueous solution of PHB depolymerase (2 μg). For the weight loss measurement, the reaction solution was incubated for 3 h at 37 ± 0.1 °C with shaking, and the sample films were removed after the reaction, washed with distilled water, and dried to constant weight in vacuo before analysis. The values of the weight loss of the sample films for 3 h were in the range 0–4 mg, depending on the stereoregularity of the P(3HB) samples. The water-soluble products after enzymatic degradation of the P(3HB) films were examined by HPLC analysis of the reaction solution.

The rate of 3-hydroxybutyrate (3HB) unit liberation from the P(3HB) film during the enzymatic degradation was determined by a UV method of absorbance measurement at 210 nm of phosphate buffer containing water-soluble products as described in a previous paper.[44] In this method, the amounts of monomer and oligomers of 3-hydroxybutyric acid generated as water-soluble products during the course of enzymatic degradation of P(3HB) film were measured by monitoring the increase in absorbance at 210 nm on a spectrophotometer. In this experiment, an enzyme solution (10 μL) of PHB depolymerase (2.0 μg) was added to a reaction cuvette containing 1.0 mL of 0.1 M potassium phosphate buffer (pH 7.4). The cuvette was maintained at 37 °C. The enzymatic degradation was started by the addition of P(3HB) film (initial film dimensions: 10 × 10 × 0.1 mm), and the absorbance at 210 nm *versus* time was monitored continuously on a Hitachi U-2000 spectrometer. The amount of 3HB units in the water-soluble products liberated from P(3HB) film by the enzymatic reaction was calculated using the absorption coefficient of 95 M^{-1} cm^{-1} at 210 nm for 3-hydroxybutyric acid at 37 °C. The absorption coefficients at 210 nm of monomer and dimer of 3-hydroxybutyric acid were found to be 95 and 190 M^{-1} cm^{-1} at 37 °C, respectively. The absorption coefficient (95 M^{-1} cm^{-1}) of (*R*)-3-hydroxybutyric acid (Takasago International Co.) was detrmined at 37 °C in 0.1 M potassium phosphate buffer (pH 7.4), and the absorbance at 210 nm was verified to be proportional to the concentration of (*R*)-3-hydroxybutyric acid in the range 0–10 mM. As will be shown in Figure 8, the enzymatic degradation of bacterial P[(*R*)-3HB] produced a mixture of monomer and dimer of (*R*)-3-hydroxybutyric acid. The degradation product was fractionated into the monomer and dimer by HPLC (column: LiChrospher RP-8). The dimer of (*R*)-3-hydroxybutyric acid (^{1}H NMR at 400 MHz in D_2O: δ 1.21 (d, 3H), 1.30 (d, 3H), 2.45–2.66 (m, 4H), 4.22 (m, 1H), 5.26 (m, 1H)) was collected and used for the determination of the absorption coefficient (190 M^{-1} cm^{-1}) at 37 °C in 0.1 M potassium phosphate buffer. The absorption coefficients of trimer and tetramer were not determined. In this study, the absorption coefficient (95 M^{-1} cm^{-1} at 37 °C) of (*R*)-3-hydroxybutyric acid at 210 nm was used to calculate the amounts of 3-hydroxybutyrate units produced as water-soluble products, on the assumption that the absorption coefficient of 3-hydroxybutyrate units is independent of the chain length.

Table 1. Ring-Opening Stereocopolymerization of (*R*)-β-BL and (*R,S*)-β-BL with Distannoxane Catalyst[a]

sample no.	monomer, mmol		monomer feed ratio, *R*/*S*	yield, %	molecular weight	
	(*R*)-β-BL[b]	(*R,S*)-β-BL[c]			$\bar{M}_n \times 10^{-3}$	$\bar{M}_w/\bar{M}_n$
1	40.0	0	96/4	95	121	1.8
2	34.7	5.2	90/10	97	112	1.8
3	30.4	9.5	85/15	94	115	1.7
4	26.0	13.9	80/20	94	107	1.8
5	17.4	22.5	70/30	90	105	1.8
6	0	40.0	50/50	99	145	1.8

[a] Polymerization conditions of β-BL: mole ratio of catalyst (1-ethoxy-3-chlorotetrabutyldistannoxane)/monomer = 1/4000, reaction temperature = 100 °C, reaction time = 4 h, without solvent. [b] Dried by CaH_2 and distilled under reduced pressure before use (ee 92%). [c] Dried by CaH_2 and distilled under reduced pressure before use (ee 0%).

Analytical Procedures. All molecular weight data were obtained by gel-permeation chromatography at 40 °C, using a Shimadzu 6A GPC system and a 6A refractive index detector with Shodex K-80M and K-802 columns. Chloroform was used as eluent at a flow rate of 0.8 mL/min, and sample concentrations of 1.0 mg/mL were applied. The number-average and weight-average molecular weights ($\bar{M}_n$ and $\bar{M}_w$) were calculated by using a Shimadzu Chromatopac C-R4A equipped with a GPC program. A molecular weight calibration curve of P(3HB) was obtained on the basis of the universal calibration method[47] with polystyrene standards of low polydispersities.

The ^{13}C NMR spectra at 100 MHz of P(3HB) polymers were recorded on a JEOL, Alpha-400 spectrometer at 27 °C in a $CDCl_3$ solution of polymer (20 mg/mL) with a 5.5 ms pulse width (45° pulse angle), 5 s pulse repetition, 25 000 Hz spectral width, 64K data points, and 13 000 accumulations.

Differential scanning calorimetry (DSC) data of P(3HB) samples were recorded in the temperature range −150 to +200 °C on a Shimadzu DSC-50 equipped with a cooling accessory under a nitrogen flow of 30 mL/min. Samples of 10 mg were encapsulated in aluminum pans and heated from 0 to 200 °C at a rate of 10 °C/min. The melting temperature (T_m) and enthalpy of fusion (ΔH_m) were determined from the DSC endotherms. The T_m was taken as the peak temperature. For measurement of the glass-transition temperature (T_g), the samples were maintained at 200 °C for 1 min and then rapidly quenched at −150 °C. They were heated from −150 to +200 °C at a heating rate of 20 °C/min. The T_g was taken as the midpoint of the heating capacity change.

The morphologies of P(3HB) spherulites were observed with a Nikon optical microscope equipped with crossed polarizers and a Linkham hot stage. The films (2 mg) of the P(3HB) samples, as obtained by solvent casting, were first heated on a hot stage from room temperature to 200 °C at a rate of 30 °C/min. Samples were maintained at 200 °C for 30 s, and then the temperature was rapidly lowered to a desired crystallization temperature (T_c). The samples were crystallized isothermally at a given T_c to monitor the growth of the spherulites as a function of time. The radial growth rate of the P(3HB) spherulites was calculated as the slope of the line obtained by plotting the spherulite radius against time with more than ten data points. For each crystallization measurement, three different spherulites were recorded under the same conditions, and the spherulitic growth rates were averaged. During the thermal treatment, the P(3HB) films were kept under a nitrogen flow to limit the degradation of the polymer. To minimize the risk of thermal degradation of the P(3HB) samples, a new sample was used for each crystallization measurement.

The stress–strain curves of solution-cast films (0.1 mm thickness) of P(3HB) were obtained at 23 °C at a strain rate of 20 mm/min on an Imada tensile machine (Model SV-50). Mechanical tensile data were calculated from such curves as an average of three specimens.

The X-ray diffraction patterns of films were recorded at 27 °C on a Rigaku RAD-IIIB system using nickel-filtered Cu Kα radiation (λ = 0.154 nm; 40 kV; 30 mA) in the 2θ range 6–40° at a scan speed of 2.0°/min. Degrees of crystallinity (X_c) of P(3HB) samples were calculated from diffracted intensity data according to Vonk's method.[45]

The surfaces of P(3HB) films were observed with a scanning electron microscope (JEOL JSM-5300) after gold coating of the films using an ion coater.

The water-soluble products after enzymatic degradation of P(3HB) films were analyzed by using a Shimadzu LC-9A HPLC system with a gradient controller and an SPD-10A UV spectrophotometric detector. The stainless steel column (250 × 4 mm) containing LiChrospher RP-8 (5 μM) was used at 40 °C. Sample solutions after the enzymatic degradation were acidified to pH 2.5 with HCl solution, and 50 μL solutions were injected. The gradient of distilled water (pH 2.5, adjusted by the addition of HCl solution) to acetonitrile for 40 min was carried out with a pump speed of 1.0 mL/min. The monomer and oligomers of 3-hydroxybutyric acid were detected at 210 nm. The amounts of monomer and oligomers of 3HB units in the water-soluble products were calculated from the peak areas of the HPLC patterns on the assumption that the absorption coefficients at 210 nm of the oligomers were proportional to the number of 3HB units in the oligomers. The monomer, dimer, and trimer in the degradation product of P(3HB) ([i] = 0.76, 85% (*R*)-3HB) were fractionated by HPLC for ^1H NMR analysis. Each product was collected from the HPLC eluate, and the solvent was evaporated. The 400 MHz ^1H NMR spectra were recorded in D_2O: dimer, δ 1.21 (d, 3H), 1.30 (d, 3H), 2.45–2.66 (m, 4H), 4.22 (m, 1H), 5.26 (m, 1H); trimer, δ 1.21–1.32 (m, 9H), 2.38–2.69 (m, 6H), 4.21 (m, 1H), 5.19 (m, 1H), 5.28 (m, 1H). The tetramer of 3-hydroxybutyric acid in the water-soluble product could not be fractionated due to a low concentration.

Results and Discussion

Synthesis and Stereoregularities of P(3HB). P(3HB) stereoisomer samples with different stereoregularities were synthesized by the ring-opening stereocopolymerization of (*R*)-β-butyrolactone ((*R*)-β-BL) with (*R,S*)-β-butyrolactone ((*R,S*)-β-BL) at various feed ratios from 40.0/0 to 0/40.0 (mol/mol) in the presence of 1-ethoxy-3-chlorotetrabutyldistannoxane as a catalyst. The stereocopolymerization was carried out at 100 °C for 4 h under an argon atmosphere. Table 1 shows the mole ratios of (*R*)- and (*S*)-β-BL monomers used for the stereocopolymerization and the number-average molecular weights ($\bar{M}_n$) and polydispersities ($\bar{M}_w/\bar{M}_n$) of produced P(3HB) polymers. All experiments gave P(3HB) polymers in high yields at different mole ratios of (*R*)-β-BL to (*S*)-β-BL, and the produced polymers had high molecular weights ($\bar{M}_n > 10^5$) as reported in a previous paper.[25]

The stereosequence distributions of the P(3HB) polymers were determined from the 100 MHz ^{13}C NMR spectra. The carbonyl carbon resonance at 169.1–169.3 ppm (from Me_4Si) was resolved into two peaks due to isotactic (i) diad (*R–R* and *S–S*) and syndiotactic (s) diad (*R–S* and *S–R*) sequences of (*R*)- and (*S*)-3HB units. The methylene resonance at 40.2–40.5 ppm was also resolved into four peaks due to isotactic triad (ii), syndiotactic triad (ss), and heterotactic triad (is and si) sequences.[18a] The diad and triad fractions were calculated from the ratios of the peak areas of the carbonyl resonance and the methylene resonance, respectively, by the method of Kemnitzer et al.[18] Table 2 lists the isotactic diad fraction ([i]) and the triad fractions ([ii], [ss], [is], and [si]) of the P(3HB) samples. Hori et al.[25] reported that the distannoxane catalyst initiated ring-opening polymerization of (*R*)-β-

Table 2. Diad and Triad Stereosequence Distributions of P(3HB) Samples

sample no.	isotactic diad fraction,[a] [i] (*RR* + *SS*)	triad fraction[b] [ii] (*RRR* + *SSS*)	[ss] (*RSR* + *SRS*)	[is] (*RRS* + *SSR*)	[si] (*RSS* + *SRR*)
1	0.92	0.95	0.03	0.01	0.01
2	0.84	0.78	0.07	0.07	0.08
3	0.76	0.63	0.15	0.11	0.11
4	0.68	0.56	0.20	0.17	0.07
5	0.46	0.38	0.27	0.20	0.15
6	0.30	0.13	0.42	0.21	0.24

[a] Determined from the carbonyl carbon resonance in the ^{13}C NMR spectra. [b] Determined from the methylene carbon resonance in the ^{13}C NMR spectra.

Table 3. Thermal Properties and X-ray Crystallinities of Solvent-Cast P(3HB) Films

sample no.	isotactic diad fraction, [i]	T_g,[a] °C	T_m,[b] °C	ΔH_m,[c] J/g	X_c,[d] %
1	0.92	3	156	80	56 ± 5
2	0.84	6	132	61	49 ± 5
3	0.76	6	107	56	45 ± 5
4	0.68	5	92	43	40 ± 5
5	0.46	5		0	0
6	0.30	6	52, 62	27	26 ± 5
7[e]	1.00	5	177	90	62 ± 5

[a] Glass-transition temperature; measured by DSC (second scan), from −150 to +200 °C at a rate of 20 °C/min. [b] Melting temperature; measured by DSC (first scan), from 0 to 200 °C at a rate of 10 °C/min. [c] Enthalpy of fusion; measured by DSC (first scan). [d] Degree of crystallinity; determined by X-ray diffraction patterns. [e] Microbial P[(*R*)-3HB]; $\bar{M}_n$ = 281000, $\bar{M}_w/\bar{M}_n$ = 2.3.

BL by breaking the bond between the carbonyl carbon and the oxygen atoms of the (*R*)-β-BL ring (acyl cleavage) with retention of configuration. In this experiment, we obtained six P(3HB) stereoisomer samples with [i] values ranging from 0.92 to 0.30 by varying the (*R*)-β-BL/(*S*)-β-BL monomer feed ratio from 96/4 to 50/50 in the stereocopolymerization. It is worth noting that the P(3HB) sample produced at the monomer feed ratio of (*R*)-β-BL/(*S*)-β-BL = 50/50 has the [i] value of 0.30, that is, a predominantly syndiotactic P(3HB). In this study, we did not compare the stereosequence distribution data (triad distributions) of P(3HB) with the statistics of the stereocopolymerization of (*R*)-β-BL with (*S*)-β-BL, because the P(3HB) stereoisomers were obtained after very high conversions (>90%) of monomers. The (*R*)-β-BL/(*S*)-β-BL monomer ratio may vary during the sterocopolymerization.

Physical Properties of P(3HB) Samples. The glass-transition temperature (T_g), melting temperature (T_m), and enthalpy of fusion (ΔH_m) of solution-cast P(3HB) films were determined from DSC thermograms. The results are given in Table 3, together with the values of the microbial P[(*R*)-3HB] film sample (sample 7). The T_g values of the P(3HB) samples were observed at 5 ± 2 °C, being independent of the stereoregularity of P(3HB). In contrast, the T_m value of the P(3HB) samples decreased from 177 to 92 °C as the isotactic diad fraction [i] was decreased from 1.00 to 0.68. The value of ΔH_m decreased also with a decrease in the [i] value. We could not detect the fusion of P(3HB) in the DSC curve of almost atactic P(3HB) sample 5 ([i] = 0.46). The predominantly syndiotactic P(3HB) sample 6 ([i] = 0.30) had two endothermic peaks around 52 and 62 °C. To see whether the two peaks arise from different crystal populations or rather from a recrystallization process, the DSC curves of syndiotactic P(3HB) sample 6 were recorded at different heating rates of 5–40 °C/min. In all thermograms recorded two melting endotherms were observed, and the proportion of polymer melting endotherms at 52 and 62 °C was almost independent of the heating rate. The result suggests that

Table 4. Mechanical Properties at 23 °C for Solution-Cast Films of P(3HB) with Different Stereoregularities

sample no.	isotactic diad fraction, [i]	Young's modulus, MPa	tensile strength, MPa	elongation at break, %
7	1.00	1560	38	5
2	0.84	1190	15	7
3	0.76	310	11	10
4	0.68	90	11	740
6	0.30	20	13	1020

syndiotactic P(3HB) sample 6 ([i] = 0.30) has different two crystalline structures.

Figure 1 shows the X-ray diffraction patterns of solution-cast P(3HB) films with different stereoregularities. The X-ray diffraction pattern of P(3HB) sample 1 with [i] = 0.92 is the same as that of bacterial P[(*R*)-3HB] sample 7 with [i] = 1.00 (Figure 1a,b). In contrast, the X-ray diffraction pattern of syndiotactic P(3HB) sample 6 with [i] = 0.30 shows a different pattern from that of bacterial P[(*R*)-3HB] (Figure 1a,f). The *d*-spacing values of syndiotactic P(3HB) with [i] = 0.30 were in good agreement with the values reported by Kemnitzer et al.[18a] and Hocking et al.[17] The P(3HB) films with [i] values ranging from 0.84 to 0.68 have both isotactic and syndiotactic P(3HB) cyrstalline structures, as can be seen from Figure 1c–e). The degrees of X-ray crystallinity (X_c) of P(3HB) films with different stereoregularities are listed in Table 3. The X_c values of isotactic P(3HB) films decreased from 62 to 40% as the [i] value was decreased from 1.00 to 0.68. The atactic P(3HB) sample 5 with [i] = 0.46 was amorphous. The syndiotactic P(3HB) film with [i] = 0.30 was a low crystalline polymer, and the X_c value was 26%.

Table 4 lists the mechanical properties of solution-cast films of P(3HB) samples with different stereoregularities. The Young's modulus values of the films decreased from 1560 to 20 MPa as [i] was decreased from 1.00 to 0.30. In contrast, the elongation to break increased from 5 to 1020%. The result indicates that the P(3HB) films become soft and flexible with a decrease in the stereoregularity.

The spherulites of P(3HB) samples with different stereoregularities were observed with a polarized optical microscope. The samples were isothermally crystallized at a given temperature after melting at 200 °C for 30 s. Figure 2 shows typical optical micrographs of P(3HB) spherulites for the isotactic P(3HB) sample with [i] = 0.92 crystallized at 60, 70, and 90 °C. After crystallization, uniform spherulites were well-developed throughout the P(3HB) film. The spherulite radius increased linearly with time. The radial growth rate (*G*) of the P(3HB) spherulites was calculated as the slope of the line obtained by plotting the spherulite radius against time.

Figure 3 shows the rate of spherulite growth (*G*) of isotactic P(3HB) samples ([i] = 1.00, 0.92, 0.84, and 0.76) at different crystallization temperatures. The rates of spherulite growth were dependent on both the stereoregularity of P(3HB) and the crystallization temperature. A maximum value (3.4 μm/s) of *G* was observed around

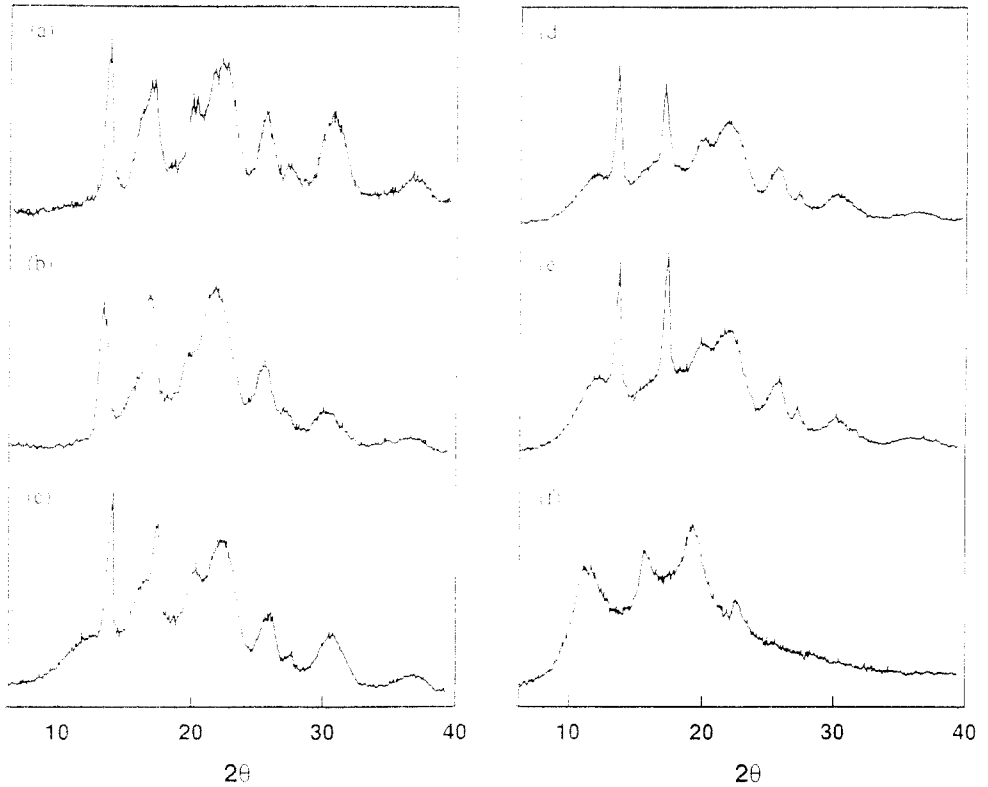

Figure 1. X-ray diffraction patterns of P(3HB) films with different stereoregularities: (a) [i] = 1.00; (b) [i] = 0.92; (c) [i] = 0.84; (d) [i] = 0.76; (e) [i] = 0.68; (f) [i] = 0.30.

90 °C for bacterial P[(*R*)-3HB] ([i] = 1.00). As the [i] value of P(3HB) was decreased, the rates of spherulite growth were markedly reduced, and the crystallization curves were shifted toward lower temperatures. The radial growth rates of spherulites of the P(3HB) sample with [i] = 0.76 were slower by 2 orders of magnitude than the rates of bacterial P[(*R*)-3HB], and the temperature of peak growth rate was observed near 65 °C. The radial growth rate of P(3HB) spherulites may decrease with a decrease in the stereoregularity of P(3HB), because syndiotactic sequences act as defects in the isotactic P(3HB) crystal lattice and the number of crystal defects increases with a decrease in the steroregularity.

Enzymatic Degradation of P(3HB) Films. Enzymatic degradations of P(3HB) films with different stereoregularities were carried out at 37 °C in 0.1 M potassium phosphate buffer (pH 7.4) of PHB depolymerase (2 μg) from *Pseudomonas pickettii*. Sample films were prepared by solvent-casting techniques from chloroform solutions of P(3HB). The amorphous P(3HB) polymer with [i] = 0.46 was coated from a chloroform solution of the polymer on a Teflon sheet and used for enzymatic degradation. The water-soluble products were quantitatively detected by monitoring the absorption at 210 nm due to the carbonyl groups of the 3HB units in products on a spectrophotometer.[44] Figure 4 shows the time-dependent changes in the absorbance of the reaction solution at 210 nm caused by the liberation of 3-hydroxybutyrate units during the enzymatic degradation of P(3HB) films with various stereoregularities ([i] = 0.30–1.00). The amount of 3HB units liberated as water-soluble products was calculated using the absorption coefficient of 95 M^{-1} cm^{-1} at 210 nm of 3-hydroxybutyric acid. The rates of 3HB generation were slow for 30 min at the initial stage of enzymatic degradation, but the amounts of 3HB units liberated from P(3HB) films increased proportionally with time after 30 min. Then the rate of enzymatic degradation was determined from the linear dependence of the amount of liberated 3HB units against time.

Figure 5 shows the effect of the stereoregularity of P(3HB) on the production rate of 3HB units liberated as water-soluble products from P(3HB) films, together with the weight loss data of the P(3HB) films after enzymatic degradation for 3 h. Both the UV kinetic data and the weight loss measurements show the same dependence of isotactic fraction [i] on the rate of enzymatic degradation. The rates of enzymatic degradation of P(3HB) films ranging in [i] value from 0.68 to 0.92 were higher than that of bacterial P[(*R*)-3HB] film ([i] = 1.00). The highest rate of enzymatic degradation was observed at [i] = 0.76 of P(3HB) film, and the rate was about 7 times faster than the rate of bacterial P[(*R*)-3HB]. However, very slow rates of enzymatic degradation were observed on the films of atactic and syndiotactic P(3HB) samples with [i] = 0.46 and 0.30.

Figure 6 shows the weight loss data of P(3HB) films by the enzymatic degradation for 3 h at 37 °C in 0.1 M potassium phosphate solution (pH 7.4) containing 2 μg of the PHB depolymerase from *Alcaligenes faecalis*. Again, the highest rate of enzymatic degradation was observed on the predominantly isotactic P(3HB) films with [i] = 0.76, and little erosion took place on the surface of atactic and syndiotactic P(3HB) films with [i] = 0.46 and 0.30.

Figure 7 shows the scanning electron micrographs (SEMs) of surfaces of P(3HB) films after enzymatic degradation. The surfaces of isotactic P(3HB) films ([i] = 0.84 and 0.76) after enzymatic degradation for 3 h were apparently blemished by the function of depolymerase (Figure 7a,b), suggesting that the enzymatic degradation occurred on the surface of the films. In contrast, the surface of syndiotactic P(3HB) film ([i] = 0.30) remained apparently unchanged even after the film was reacted with depolymerase for 24 h (Figure 7c).

In a previous paper,[38] we reported that the rate of enzymatic degradation of bacterial P[(*R*)-3HB] film increased with a decrease in the crystallinity and that a PHB depolymerase first hydrolyzed the P[(*R*)-3HB]

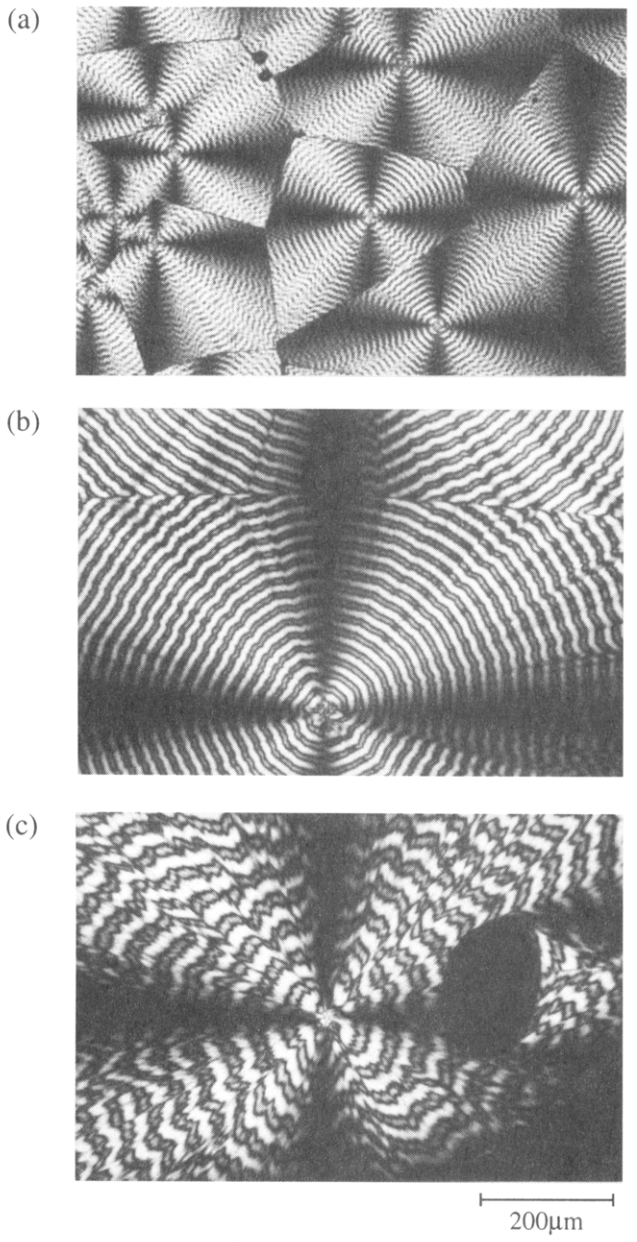

Figure 2. Optical micrographs of P(3HB) spherulites for isotactic P(3HB) sample 1 ([i] = 0.92) grown isothermally at (a) T_c = 60 °C, (b) T_c = 70 °C, and (c) T_c = 90 °C.

chains in the amorphous state on the surface of the film. In addition, it was suggested that the rate of enzymatic degradation for the P[(*R*)-3HB] chains in the amorphous state was about 20 times higher than the rate for the P[(*R*)-3HB] chains in the crystalline state.[46] The acceleration of enzymatic degradation for predominantly isotactic P(3HB) films may be caused by the decrease in crystallinity.

It is interesting to compare the results of enzymatic degradation of P(3HB) stereoisomer samples prepared by a distannoxane catalyst with those of the P(3HB) samples prepared by the $ZnEt_2/H_2O$ catalyst. Kemnitzer et al.[24] prepared P(3HB) stereoisomers by the stereocopolymerization of (*R*)-β-BL with (*S*)-β-BL with the $ZnEt_2/H_2O$ catalyst and studied the enzymatic degradation with the PHB depolymerase from *Penicillium funiculosum*. The P(3HB) stereoisomers had a statistical distribution of (*R*)- and (*S*)-3HB units and low molecular weights ($\bar{M}_n < 10^4$). The initial surface rates of enzymatic degradation of P(3HB) stereoisomers with 81–95% (*R*)-3HB units were lower than the initial surface rate of bacterial P[(*R*)-3HB], but the rates of P(3HB) stereoisomers with 67 and 77% (*R*)-3HB were dramatically larger. In the present study, all of the predominantly isotactic P(3HB) samples ([i] = 0.68–0.92) with 80, 85, 90, and 96% (*R*)-3HB showed larger rates of enzymatic degradation than the rate of bacterial P[(*R*)-3HB]. However, the amorphous P(3HB) sample ([i] = 0.46) with 70% (*R*)-3HB and syndiotactic sample ([i] = 0.30) with 50% (*R*)-3HB were hardly eroded by

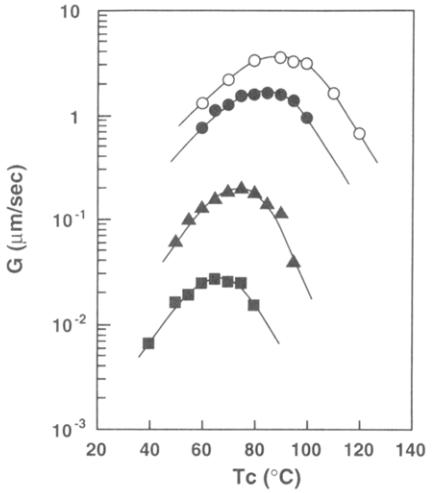

Figure 3. Radial growth rate (G) of P(3HB) spherulites at various crystallization temperatures (T_c) for isotactic P(3HB) samples with [i] = 0.92 (●), [i] = 0.84 (▲), [i] = 0.76 (■), and [i] = 1.00 (○).

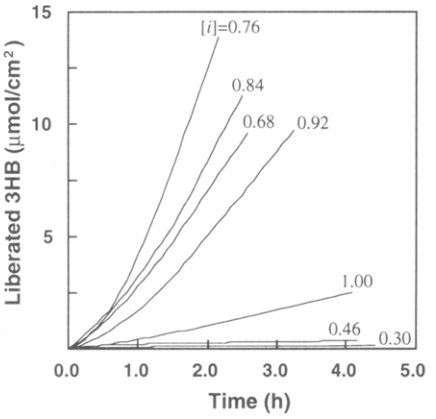

Figure 4. Amounts of 3HB units liberated as water-soluble products during the enzymatic degradation of P(3HB) films with different stereoregularities in 0.1 M potassium phosphate solution (pH 7.4) containing PHB depolymerase (2.0 μg/mL) from *P. pickettii* at 37 °C.

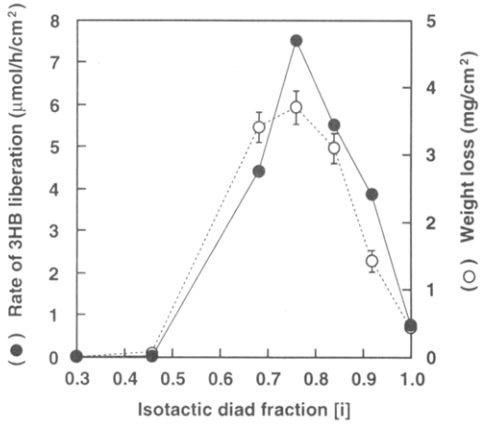

Figure 5. Effect of stereoregularity on the rate of 3HB liberation as water-soluble products (●) and on the weight loss (○) of P(3HB) films after enzymatic degradation for 3 h with PHB depolymerase (2.0 μg/mL) from *P. pickettii* at 37 °C. The weight loss data were averaged on three film samples.

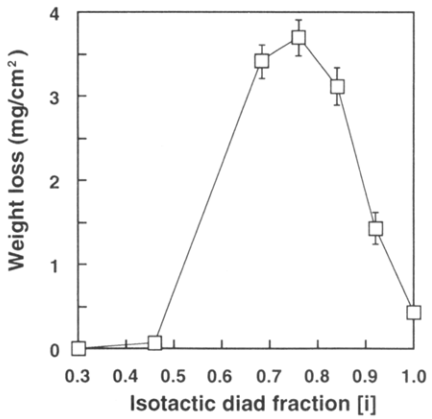

Figure 6. Effect of stereoregularity on the weight loss of P(3HB) films after enzymatic degradation for 3 h with PHB depolymerase (2.0 μg/mL) from *A. faecalis* at 37 °C. The weight loss data were averaged on three film samples.

PHB depolymerases. The stereochemical and morphological effects on the enzymatic degradation of P(3HB) stereoisomers will be discussed in a different paper which reports the morphology and enzymatic degradability of blends of bacterial P[(*R*)-3HB] with atactic or syndiotactic P(3HB).

HPLC Analysis of Water-Soluble Products. The enzymatic hydrolysis of water-insoluble P(3HB) film by PHB depolymerases produces a mixture of monomer and oligomers of 3-hydroxybutyric acid as water-soluble products. In this study, we measured the composition of water-soluble products during the course of enzymatic degradation of P(3HB) films by HPLC analysis. The enzymatic degradations of P(3HB) films with different stereoregularities were carried out at 37 °C in 1 mL of 0.1 M potassium phosphate buffer (pH 7.4) containing a PHB depolymerase (2 μg) from *P. pickettii*. The reaction solutions were collected 1, 2, and 3 h after the enzymatic degradation and analyzed by high-performance liquid chromatography (HPLC).

Figure 8 shows typical HPLC curves of water-soluble products from bacterial P[(*R*)-3HB] and predominantly isotactic P(3HB) ([i] = 0.76) after reaction for 3 h. The component of each peak for 3HB monomer, dimer, and trimer was identified by ^{1}H NMR analysis of each fraction collected from the HPLC eluate using a fraction collector (see Experimental Section). The assignment of the 3HB tetramer peak is tentative. The HPLC curve of the products from bacterial P[(*R*)-3HB] showed only two peaks, arising from the monomer and dimer of the (*R*)-3HB unit. In contrast, the HPLC curve of the products from a predominantly isotactic P(3HB) ([i] = 0.76) with 85% (*R*)-3HB showed five peaks, arising from the monomer, dimer, trimer, and tetramer of the (*R*)- and (*S*)-3HB units. In the HPLC curve (b) the dimer of the (*R*)- and (*S*)-3HB units is resolved into two peaks with t_R = 13.3 and 13.5 min. The peak at 13.5 min was assigned to the (*R*–*R*) dimer, since the retention time was identical with that of the dimer peak from bacterial P[(*R*)-3HB] in the HPLC curve (a). On the other hand, the peak at 13.3 min was assigned to the (*R*–*S*) dimer, since the methyl proton resonances in the ^{1}H NMR spectrum of the fractionated dimer component were resolved into two doublet peaks (δ = 1.294 and 1.297) arising from the stereoisomers of the 3HB dimer. The relative amounts of the 3HB unit in water-soluble products were determined from the peak areas in the HPLC curve.

Figure 9 shows the weight distribution of water-soluble products during the enzymatic degradation of five P(3HB) films with different stereoregularities and (*R*)-3HB fractions. It has been found that the PHB depolymerase from *A. faecalis* acts as an endo-type hydrolase toward the pentamer and high oligomers of (*R*)-3HB units and that the rate of enzymatic hydrolysis for the dimer of the (*R*)-3HB units is much slower than the rates for oligomers and polymers of the (*R*)-3HB units.[43] As a result, bacterial P[(*R*)-3HB] is hydrolyzed into the monomer and dimer of (*R*)-3HB by PHB depolymerases. On the other hand, chemosynthetic P(3HB) stereoisomers containing 4–20% (*S*)-3HB gave trimer and tetramer as products in addition to monomer and dimer, and the fractions of trimer and tetramer increased with an increase in the fraction of (*S*)-3HB units in P(3HB) stereoisomers. As can be seen from Figure 9, the product distributions of monomer, dimer, trimer, and tetramer did not vary during the enzymatic degradation of P(3HB) films for 3 h, but the amounts of products increased proportionally with time. The results suggest that PHB depolymerase is incapable of hydrolyzing the ester bonds of connecting (*S*)-3HB units.

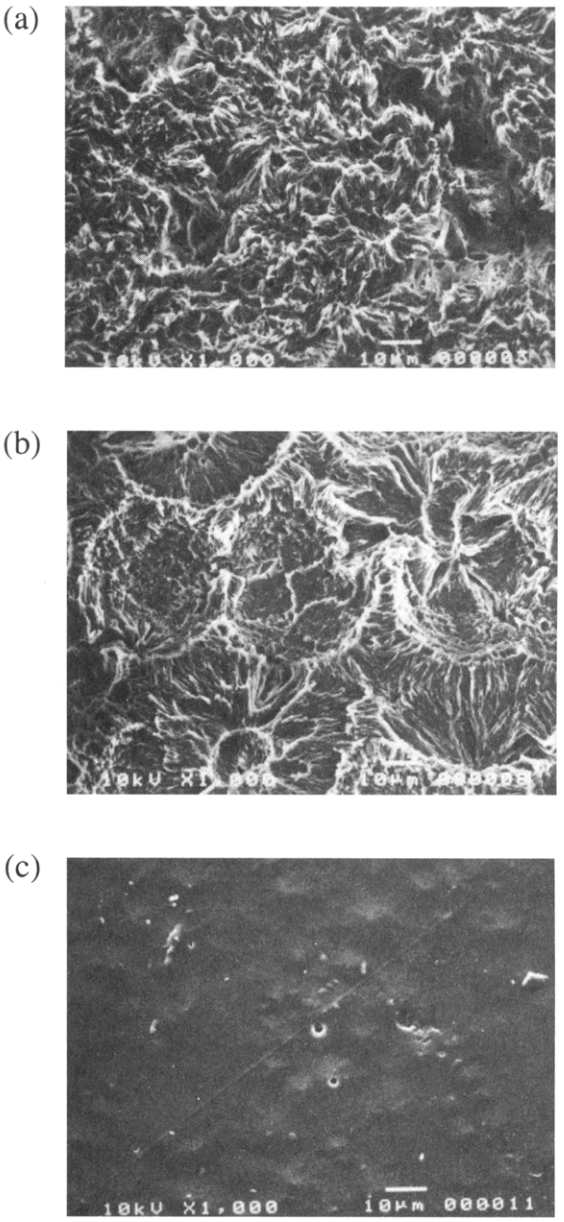

Figure 7. Scanning electron micrographs (SEMs) of the surfaces of P(3HB) films after enzymatic degradation with PHB depolymerase (2.0 μg/mL) from *P. pickettii* at 37 °C: (a) isotactic P(3HB) film with 96% (*R*)-3HB ([i] = 0.92) degraded for 3 h; (b) isotactic P(3HB) film with 85% (*R*)-3HB ([i] = 0.76) degraded for 3 h; (c) syndiotactic P(3HB) film with 50% (*R*)-3HB ([i] = 0.30) reacted for 24 h.

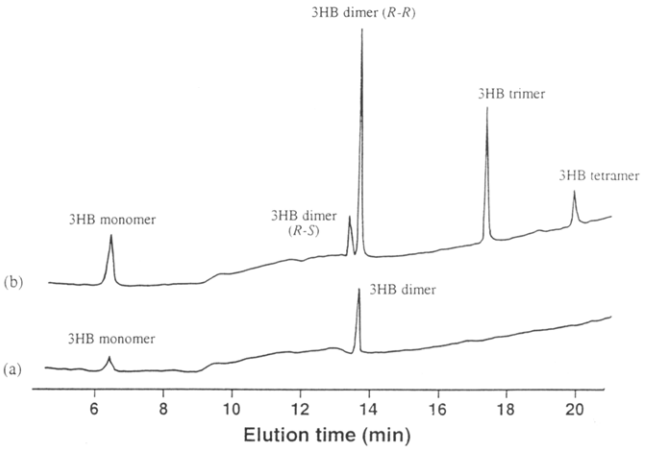

Figure 8. HPLC curves of the water-soluble products from (a) bacterial P[(*R*)-3HB] with 100% (*R*)-3HB ([i] = 1.00) and from (b) chemosynthetic P(3HB) with 85% (*R*)-3HB ([i] = 0.76) after enzymatic degradation for 3 h at 37 °C.

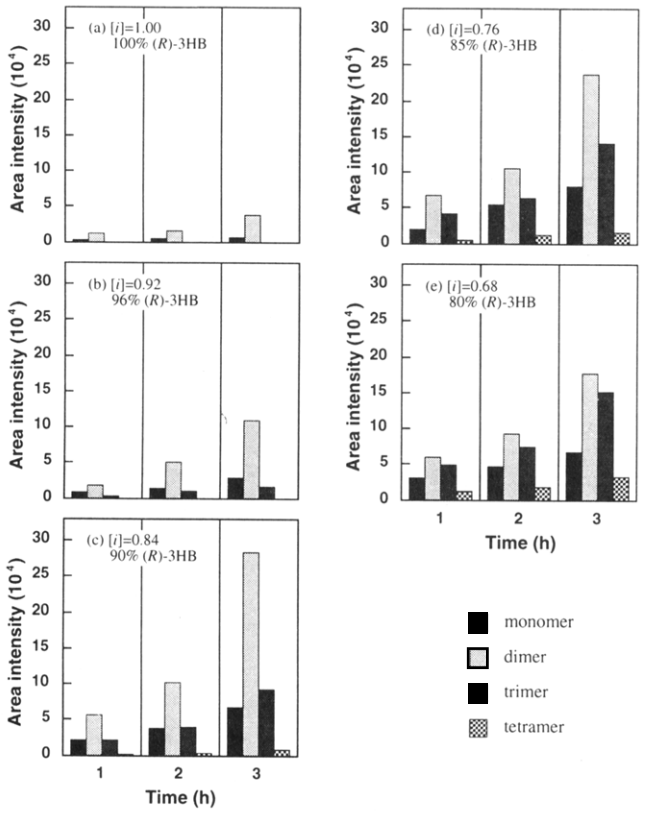

Figure 9. Relative amounts and weight distributions of water-soluble products during the enzymatic degradation of P(3HB) films with different stereoregularities at 37 °C in aqueous solution (pH 7.4) containing PHB depolymerase (2.0 μg/mL) from *P. pickettii*.

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