Microbial Synthesis and Characterization of Poly(3-hydroxybutyrate-*co*-3-hydroxypropionate)

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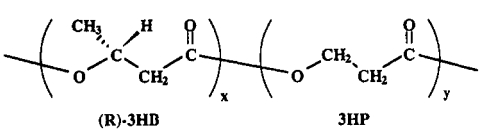
ABSTRACT: Copolyesters of 3-hydroxybutyrate (3HB) and 3-hydroxypropionate (3HP) with a wide range of compositions varying from 0 to 88 mol % 3HP were produced by *Alcaligenes latus* from the mixed carbon substrates of 3-hydroxypropionic acid with sucrose or 3-hydroxybutyric acid. The copolyesters were shown to have a random sequence distribution of 3HB and 3HP monomeric units by analysis of ^{13}C NMR spectra. The number-average molecular weights of the copolymers were in the range (1.1–3.5) × 10^5. The structure and physical properties of P(3HB-*co*-3HP) copolymers were characterized by ^1H and ^{13}C NMR spectroscopy, X-ray diffraction, gel permeation chromatography, differential scanning calorimetry, dynamic mechanical spectroscopy, and dielectric spectroscopy. The crystallographic parameters of copolymers with compositions up to 43 mol % 3HP were little influenced by the presence of the 3HP unit, but the X-ray crystallinities decreased with the 3HP fraction. The isothermal radial growth rates of spherulites of P(3HB-*co*-11% 3HP) were slower by 2 orders of magnitude than the rate of P(3HB). The rate of enzymatic degradation of P(3HB-*co*-3HP) films increased with an increase in the 3HP fraction.

Introduction

Microbial poly(hydroxyalkanoate)s (PHA) are a biodegradable thermoplastic with a wide range of physical properties.[1,2] A number of microorganisms synthesize isotactic homopolymers and copolymers of (*R*)-3-hydroxyalkanoic acids (3HA) with four to fourteen carbon atoms as an intracellular storage material of carbon and energy.[3,4] Saturated,[5–7] unsaturated,[8,9] halogenated,[10–12] branched,[13] and aromatic[14] side chains in 3HA monomeric units have been found as constituents in the sequences of microbial polyesters. In addition, several bacteria have been found to produce copolymers containing hydroxyalkanoate monomeric units without side chains such as 3-hydroxypropionate (3HP),[15] 4-hydroxybutyrate (4HB),[16,17] and 5-hydroxyvalerate (5HV).[18]

In a previous paper,[15] we reported that the copolymer of (*R*)-3-hydroxybutyrate and 3-hydroxypropionate, P(3HB-*co*-3HP), was produced by *Alcaligenes eutrophus* in a nitrogen-free solution containing 3-hydroxypropionic acid, 1,5-pentanediol, or 1,7-heptanediol as the sole carbon source. However, the mole fraction of 3HP unit in the copolymer was lower than 7 mol %. Recently, we reported that P(3HB-*co*-3HP) copolymers with compositions from 0 to 26 mol % 3HP were produced by *Alcaligenes latus* from the mixed carbon substrates of 3-hydroxypropionic acid and sucrose.[19]



(R)-3HB 3HP

In this study, we report that P(3HB-*co*-3HP) copolyesters with a wide range of compositions from 0 to 88 mol % 3HP are produced by *A. latus* from the mixed carbon substrates of 3-hydroxypropionic and 3-hydroxybutyric acids. The structure and properties of P(3HB-*co*-3HP) copolyesters have been characterized by NMR, X-ray diffraction, GPC, DSC, dynamic mechanical spectroscopy, and dielectric spectroscopy.

Experimental Section

Polyester Synthesis. *A. latus* (ATCC 29713) was used in this study. Polyester synthesis was carried out by a single stage fermentation of *A. latus*.[20] The bacterium was grown at 30 °C and pH 7.0 under aerobic conditions on a reciprocal shaker in 500-mL flasks with 100 mL of a mineral medium containing carbon sources of different compositions of sucrose (or 3-hydroxybutyric acid) and 3-hydroxypropionic acid. The mineral medium contained 8.6 g of $Na_2HPO_4{\cdot}12H_2O$, 1.5 g of KH_2PO_4, 1.0 g of $(NH_4)_2SO_4$, 0.2 g of $MgSO_4{\cdot}7H_2O$, 0.06 g of ammonium iron(III) citrate, and 0.01 g of $CaCl_2{\cdot}2H_2O$ per liter of distilled water. In addition, 1 mL of a microelement solution was added to the medium. The microelement solution contained the following (per liter of 1.0 N HCl): 0.3 g of H_3BO_3, 0.2 g of $CoCl_2{\cdot}6H_2O$, 0.1 g of $ZnSO_4{\cdot}7H_2O$, 0.03 g of $MnCl_2{\cdot}4H_2O$, 0.03 g of $NaMoO_4{\cdot}2H_2O$, 0.02 g of $NiCl_2{\cdot}6H_2O$, and 0.01 g of $CuSO_4{\cdot}6H_2O$. The cells were cultivated for 2–8 days as proper times for growth at 30 °C, harvested by centrifugation, washed with distilled water, and finally lyophilized. Polyesters were extracted from the lyophilized cells with hot chloroform in a Soxhlet apparatus and purified by reprecipitation with hexane and methanol. The films of polyester samples were prepared by conventional solvent-casting techniques from chloroform of polyesters using glass Petri dishes as casting surfaces. The solution-cast films were aged for 3 weeks at room temperature to reach equilibrium crystallinity prior to analysis.

Poly(3-hydroxypropionate) homopolymer was prepared by the ring-opening polymerization of β-propiolactone in the presence of methylalumoxane (MAO from Toso Akuzo Co.). β-Propiolactone monomer was dried on CaH_2 and distilled under nitrogen. The propiolactone (19 mL, 300 mmol) and MAO catalyst (15

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Table 1. Production of P(3HB-*co*-3HP) from Sucrose and 3-Hydroxypropionic Acid (3HPA) by *A. latus* at 30 °C for 48 h

run	carbon source, g/L		cell dry wt, g/L	polyester content,[a] wt %	polyester comp,[b] mol %		mol wt[c]	
	sucrose	3HPA			3HB	3HP	$10^{-3}M_n$	M_w/M_n
1	8.0	0	4.0	60	100	0	770	1.9
2	0	2.0	0					
3	10.0	0.3	4.0	53	93	7	320	2.2
4	7.5	0.5	3.3	51	89	11	350	2.1
5	6.5	1.5	2.3	35	80	20	280	2.1
6	4.0	2.0	1.8	26	80	20		
7	4.0	3.0	1.9	24	75	25		
8	4.0	4.0	1.9	26	71	29		
9	4.0	5.0	2.0	30	73	27		

[a] Polyester content in dry cells. [b] Determined by ^{1}H NMR. [c] Determined by GPC.

mmol) were admitted into a reactor under nitrogen. Polymerization was carried out at 60 °C for 5 days, and the mixture was poured into 400 mL of methanol. The precipitated polymers were isolated by filtration and dried at room temperature under vacuum.

Analytical Procedures. The ^{1}H and ^{13}C NMR analyses of polyester samples were carried out on a JEOL ALPHA-400 spectrometer. The 400-MHz ^{1}H NMR spectra were recorded at 23 °C in a $CDCl_3$ solution of polymer (4 mg/mL) with a 5.0-μs pulse width (45° pulse angle), 5-s pulse repetition, 7993-Hz spectral width, 16K data points, and 16 accumulations. The 100-MHz ^{13}C NMR spectra were recorded at 23 °C in a $CDCl_3$ solution of polyester (20 mg/mL) with a 4.5-μs pulse width (45° pulse angle), 1.2-s pulse repetition, 27100-Hz spectral width, 16K data points, and 5000–20000 accumulations. Tetramethylsilane (Me_4Si) was used as an internal chemical shift standard.

Molecular weight data were obtained at 40 °C by using a Shimadzu 6A GPC system and a 6A refractive index detector with a Shodex 80M column. Chloroform was used as eluent at a flow rate of 0.5 mL/min, and a sample concentration of 1.0 mg/mL was used. Polystyrene standards with a low polydispersity were used to make a calibration curve.

Calorimetric measurements (DSC) of polyesters (3 mg) encapsulated in aluminum pans were carried out by using a Shimadzu DSC-50 thermal analysis system equipped with a cooling accessory under a nitrogen flow of 30 mL/min in the temperature range −100 to +200 °C at a heating rate of 10 °C/min. The melting temperatures and enthalpies of fusion were determined from the DSC endotherms. The samples were not annealed prior to recording endotherms. For the measuremennt of the glass transition temperature (T_g), the samples were maintained at 200 °C for 1 min and then rapidly quenched to −100 °C. They were heated from −100 to +200 °C at a heating rate of 20 °C/min. The T_g was taken as the midpoint of the heat capacity change.

Thermal degradation studies of polyesters were performed by using the differential scanning calorimetry (Shimadzu DSC-50) equipment under a nitrogen flow of 30 mL/min. Polyester samples of 3 mg were encapsulated in aluminum pans, heated from ambient to an isothermal temperature at 10 °C/min, and degraded isothermally under nitrogen for a given time. The powders of polyester samples were dried at room temperature under vacuum before the thermal degradation experiment.

Wide-angle X-ray diffraction measurements of polyester samples were made on a Rigaku RAV-1VB system. Cu Kα radiation (λ = 0.1541 nm) was used as the source. The X-ray diffraction patterns of the polyesters were recorded at 27 °C in the range 2θ = 6–40° at a scan speed of 2°/min. X-ray crystallinities were measured for polyester film that had been cast from chloroform solution and allowed to stand for 3 weeks at room temperature. The percentage of crystallinitty was calculated from diffracted intensity data according to Vonk's method.[34]

Measurements of the isothermal radial growth rate of spherulites were carried out on a Zeiss Axioscop polarizing optical microscope equipped with a Linkam TH 600 hot stage. Isothermal crystallization measurements were performed on a small piece of solution-cast film, which was inserted between two microscope cover glasses and subjected to a four-steps thermal program: (1) heating in the microscope hot stage at 20 °C/min to 170 °C; (2) heating at 10 °C/min to 195 °C; (3) quenching at the selected crystallization temperature (T_c) by means of a N_2 gas flow at a cooling rate of 250 °C/min; (4) standing isothermally at T_c. The whole procedure was carried out without removing the sample from the hot stage. A videocamera, attached to the microscope through the Linkam VTO232 interface, allowed real time measurement of the spherulite dimensions after calibration with a micrometric reticle. A new sample was used for each crystallization measurement.

Dynamic mechanical measurements of solution-cast P(3HB-*co*-3HP) films were performed on a dynamic mechanical thermal analysis (Polymer Laboratories) operated in the tensile mode, at a frequency of 3 Hz and a heating rate of 3 °C/min. The temperature range investigated was from −150 to +100 °C. To avoid sample drawing in the softening region, the instrument was equipped with a force-reducing device that automatically changes the pretensioning force when softening occurs.

Dielectric properties of solution-cast P(3HB-*co*-3HP) film were investigated using a dielectric thermal analyzer (Polymer Laboratories) in the frequency range 0.3–50 kHz at a heating rate of 1 °C/min.

Enzymatic Degradation. The extracellular P(3HB) depolymerase was purified to electrophoretic homogeneity from *Alcaligenes faecalis* T1 as described in a previous paper.[35] The enzymatic degradation of polyester films by the extracellular P(3HB) depolymerase was carried out at 37 °C in a 0.1 M phosphate buffer (pH 7.5). Polyester films (initial weights, 6 mg; initial film dimensions, 10 × 10 × 0.05 mm) were placed in small bottles containing 1.0 mL of buffer. The reaction was started by the addition of 3.2 μL of an aqueous solution of P(3HB) depolymerase. (The final concentration of depolymerase was 1.0 μg/mL.) The reaction solution was incubated at 37 °C with shaking. The films were removed after the reaction, washed with water, and dried to constant weight in vacuo before analysis.

The surface appearances of the polyester films were observed with a scanning electron microscope (JEOL JSM-T220) after Au coating of the films using an ion coater.

Results and Discussion

Synthesis and Sequence Structure of P(3HB-*co*-3HP). Table 1 lists the result of P(3HB-*co*-3HP) production by *A. latus* from sucrose and 3-hydroxypropionic acid for 48 h at 30 °C. *A. latus* accumulated P(3HB) homopolymer in the cells up to 60% of the dry weight during the course of growth when sucrose was used as the sole carbon source.[20] However, *A. latus* did not grow in the solution containing 2.0 g/L of 3-hydroxypropionic acid as the sole carbon source. 3-Hydroxypropionic acid may not be a carbon source suitable for growth of *A. latus*. To introduce 3-hydroxypropionate (3HP) units into the P(3HB) sequence, we used mixed carbon sources of sucrose and 3-hydroxypropionic acid. When 3-hydroxypropionic acid was fed with sucrose, P(3HB-*co*-3HP) copolymers were accumulated in *A. latus* cells, suggesting that 3-hydroxypropionic acid was not used for cell growth but for the formation of 3HP units in the copolymers. As shown in Table 1, the cell dry weight and polyester content decreased as the fraction of 3-hydroxypropionic acid in the carbon sources was increased, while the 3HP fraction

Table 2. Production of P(3HB-*co*-3HP) from (*R*)-(−)-3-Hydroxybutyric Acid (3HBA) and 3-Hydroxypropionic Acid (3HPA) by *A. latus* at 30 °C

	carbon source, g/L					polyester comp, mol %		mol wt	
run	3HBA	3HPA	cultiv time, days	cell dry wt, g/L	polyester content, wt %	3HB	3HP	$10^{-3}M_n$	M_w/M_n
10	10.0	0.5	4	2.7	45	75	25		
11	5.0	0.5	4	2.0	48	71	29	210	2.9
12	5.0	0.75	4	2.2	46	63	37	290	2.2
13	5.0	1.0	4	2.1	50	57	43	280	2.4
14	4.0	2.0	4	1.6	45	52	48		
15	3.3	4.0	5	1.5	50	28	72		
16	3.0	4.0	4	1.5	40	33	67	220	3.2
17	2.0	4.0	4	0.9	36	29	71	220	3.2
18	1.7	4.0	3	0.8	30	27	73	302	2.4
19	1.5	4.5	4	0.8	23	24	76		
20	1.0	5.0	6	0.7	19	26	74		
21	0.5	5.5	8	0.4	0				
22	1.7[a]	4.0	5	0.8	16	22	78	140	3.3
23	1.7[b]	5.0	5	0.9	6	12	88	110	2.9

[a] Concentration of (*R,S*)-3-hydroxybutyric acid. [b] Concentration of (*S*)-(+)-3-hydroxybutyric acid.

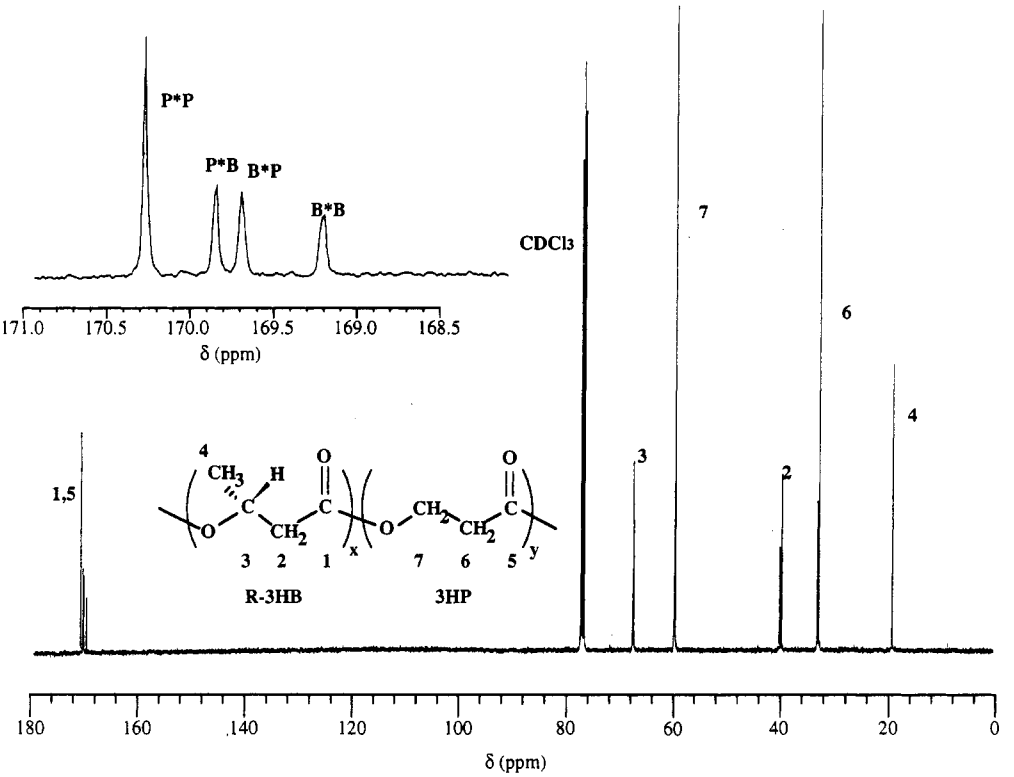

Figure 1. 100-MHz ^{13}C NMR spectrum of P(3HB-*co*-67% 3HP) sample 16 in $CDCl_3$ at 23 °C. Chemical shifts are in ppm from tetramethylsilane.

in the copolymers increased to 29 mol %. The compositions of the copolymers were determined by integration of the proton resonances of the 3HB and 3HP monomeric units in the ^1H NMR spectra. In this study, we did not investigate the quantitative influence of 3-hydroxypropionic acid on the lag time for bacterial cell growth.

To increase the 3HP fraction in the copolymers, we used the mixed carbon sources of 3-hydroxypropionic and 3-hydroxybutyric acids for *A. latus*. Table 2 gives the result of P(3HB-*co*-3HP) production by *A. latus* at 30 °C. When the mixed carbon sources of 3-hydroxypropionic and (*R*)-(−)-3-hydroxybutyric acids were used, the 3HP fraction in the copolymers increased from 25 to 76 mol % with an increase in the proportion of 3-hydroxypropionic acid in the culture solution, though the cell growth times of *A. latus* ranged between 3 and 8 days. When (*S*)-(+)-3-hydroxybutyric acid was used in place of (*R*)-(−)-3-hydroxybutyric acid, the 3HP fraction increased up to 88 mol % (run 23), though the polyester content decreased to 6 wt %. Thus, P(3HB-*co*-3HP) copolymers with a wide range of compositions of 0–88 mol % 3HP were produced by *A. latus*.

Figure 1 shows the 100-MHz ^{13}C NMR spectrum of a representative P(3HB-*co*-3HP) sample (sample 16; 67 mol % 3HP), together with the chemical shift assignment[19] for each carbon resonance. The expanded carbonyl carbon resonances in Figure 1 are resolved into four peaks, arising from different diad sequences of 3HB and 3HP units. The relative intensities of the four peaks in the carbonyl resonances of eight samples are given in Table 3, together with the ^{13}C chemical shift assignment.[19] The diad sequence distribution data for two monomeric units were compared with the Bernoullian statistics applicable to a statistically random copolymerization. In the Bernoullian model, the mole fraction F_{ij} of diad sequence ij can be expressed with the mole fractions F_i and F_j of i and j units as $F_{ij} = F_iF_j$. As shown in Table 3, the calculated diad fractions (F_{BB}, F_{BP}, F_{PB}, F_{PP}) are in good agreement with the observed value for the eight samples. It is concluded that the sequence distributions of the 3HB and 3HP units

Table 3. Chemical Shifts and Relative Intensities of Carbonyl Resonances in ^{13}C NMR Spectra of P(3HB-*co*-3HP) Samples

peak[a]	chem shift, ppm	sequence	3HP mol frac, mol % (sample no.)	relative intensities 7 (3)	11 (4)	20 (5)	29 (11)	27 (12)	43 (13)	67 (16)	71 (17)
a	169.1	B*B	obsd	0.85	0.81	0.65	0.54	0.44	0.38	0.15	0.12
			calcd[b]	0.86	0.80	0.64	0.50	0.40	0.32	0.11	0.09
b	169.6	B*P	obsd	0.08	0.09	0.14	0.18	0.20	0.21	0.20	0.19
			calcd	0.07	0.10	0.16	0.21	0.23	0.25	0.22	0.21
c	169.8	P*B	obsd	0.06	0.09	0.16	0.19	0.22	0.23	0.21	0.21
			calcd	0.07	0.10	0.16	0.21	0.23	0.25	0.22	0.21
d	170.2	P*P	obsd	0.01	0.01	0.05	0.09	0.14	0.18	0.44	0.48
			calcd	0.01	0.01	0.04	0.09	0.13	0.19	0.45	0.50

[a] Peaks in Figure 1. [b] Calculated by Bernoullian statistics with the mole fraction of 3HP unit in Tables 1 and 2.

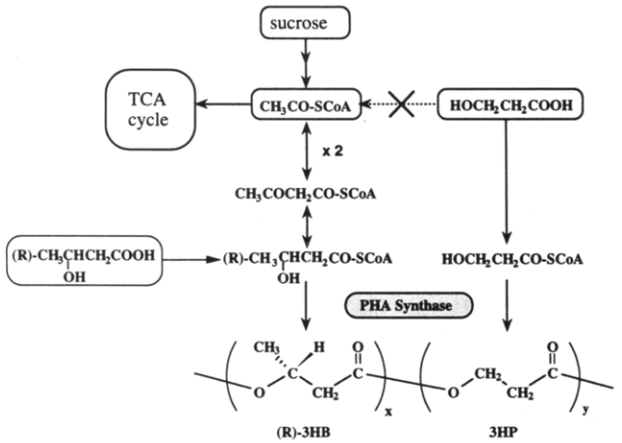

Figure 2. Metabolic pathway of P(3HB-*co*-3HP) biosynthesis by *A. latus*.

Table 4. Molecular Weights and Thermal Properties of P(3HB-*co*-3HP) Samples

sample no.	3HP frac, mol %	mol wt $10^{-3}M_n$	mol wt M_w/M_n	thermal properties T_g, °C	T_m, °C	ΔH, J/g
1	0	768	1.9	4	177	97
3	7	320	2.2	3	160	71
4	11	350	2.1	1	152	36
5	20	280	2.1	−1	143	18
11	29	210	2.9	−2	166	17
12	37	290	2.2	−5	159	8
13	43	280	2.4	−5	155	2
16	67	220	3.2	−10	44	5
17	71	220	3.2	−11	45	7
22	78	140	3.0	−14	61	8
23	88	110	2.9	−15	61	9
24[a]	100	143	1.4	−19	77	74

[a] Sample 24 was synthesized by ring-opening polymerization of β-propiolactone with an Al-based catalyst.

in those samples are statistically random.

Here, we propose a pathway of P(3HB-*co*-3HP) biosynthesis in *A. latus* from the mixed carbon substrates of 3-hydroxypropionic acid with sucrose or (*R*)-(−)-3-hydroxybutyric acid (Figure 2). The schematic pathway in Figure 2 modifies that reported in a previous paper.[19] Sucrose is transported into the cells and metabolized to acetyl coenzyme A (CoA). A portion of acetyl-CoA enters the tricarboxylic acid (TCA) cycle for cell growth, while the remainder is metabolized into (*R*)-(−)-3-hydroxybutyryl-CoA. 3-Hydroxypropionic acid is converted to 3-hydroxypropionyl-CoA in the cells. Then a random copolymer of 3HB and 3HP units may be produced by the copolymerization of (*R*)-(−)-3-hydroxybutyryl-CoA with 3-hydroxypropionyl-CoA under the action of PHA synthase.[21,22] *A. eutrophus* grew on 3-hydroxypropionic acid,[15] while *A. latus* did not grow. When 3-hydroxypropionic acid was used as the sole carbon source for *A. eutrophus*, P(3HB-*co*-3HP) was produced and the 3HP fraction was only 7 mol %.[15] This result suggests that 3-hydroxypropionyl-CoA is metabolized into acettyl-CoA in *A. eutrophus*. In contrast, *A. latus* may be incapable of metabolizing 3-hydroxypropionyl CoA into acetyl-CoA. As a result, P(3HB-*co*-3HP) with a high content of 3HP units may be formed in *A. latus*. In addition, we found that P(3HB-*co*-3HP) copolymers with higher 3HP contents (20–88 mol %) were produced from the mixture of 3-hydroxybutyric and 3-hydroxypropionic acids. Here, 3-hydroxybutyric acid was used for both cell growth and production of 3HB units in copolymers. At present, we have no explanation why a high content of 3HP units in copolymers was obtained by the addition of 3-hydroxybutyric acid in place of sucrose.

Thermal and Physical Properties of P(3HB-*co*-3HP). Table 4 shows the molecular weights and thermal properties of P(3HB-*co*-3HP) samples. The number-average molecular weights (M_n) of P(3HB-*co*-3HP) samples were in the range (1.1–3.2) × 10^5. The glass transition temperature (T_g) decreased from +4 to −19 °C as the 3HP fraction was increased from 0 to 100 mol %. The melting temperature (T_m) decreased from 177 to 44 °C with the 3HP fraction and then increased to 77 °C. The enthalpy of fusion (ΔH_m) showed a trend similar to that of the T_m data.

Thermal degradation studies on samples of P(3HB-*co*-11% 3HP) and P(3HB-*co*-71% 3HP) were carried out for 20 min at different temperatures (150 and 180 °C) above the melting points. Table 5 lists the changes in the number-average molecular weights (M_n) and polydispersities (M_w/M_n) of two samples during the thermal degradation in the temperature range 150–180 °C under nitrogen. At temperatures above 150 °C, the M_n values of the P(3HB-*co*-71% 3HP) sample decreased rapidly with time. In addition, the M_n values of the P(3HB-*co*-11% 3HP) sample also decreased with time at 180 °C. For each sample the molecular weight distributions were unimodal and the polydispersities (M_w/M_n) remained relatively narrow during the thermal degradation. There was no appreciable weight loss of polyester samples for 20 min in the temperature range 150–180 °C.

Grassie *et al.*[23] found that the degradation reaction of the P(3HB) sample in the temperature range 170–200 °C was a random chain scission process. Provided that the chain scission is completely random and no volatilization occurs during the thermal degradation of the copolyester samples, the number-average degree of polymerization $P_{n,t}$ at time t is given by[24]

$$1/P_{n,t} - 1/P_{n,0} = k_d t \quad (1)$$

where $P_{n,0}$ is the initial number-average degree of polymerization and k_d is the rate constant of thermal degradation. Table 5 lists the values of the degradation rate constant

Table 5. Changes in Molecular Weights of P(3HB-*co*-3HP) Samples during Thermal Degradation at Different Temperatures and Rate Constants k_d of Thermal Degradation

degrad temp, °C	sample	$10^{-3}M_n$ (M_w/M_n) 0 min	1 min	2 min	5 min	10 min	20 min	k_d, min^{-1}
150	P(3HB-*co*-71% 3HP)	220 (3.2)	109 (2.8)	102 (2.6)	79 (2.4)	53 (2.6)	43 (2.1)	(6.6 ± 1.5) × 10^{-5}
180	P(3HB-*co*-71% 3HP)	220 (3.2)	43 (2.5)	36 (2.5)	24 (2.3)	17 (2.3)	11 (2.3)	(3.0 ± 1.5) × 10^{-4}
	P(3HB-*co*-11% 3HP)	350 (2.2)	274 (2.1)	227 (2.3)	171 (2.2)	142 (2.1)	91 (2.0)	(3.3 ± 1.5) × 10^{-5}
	P(3HB)							(3.8 ± 1.5) × 10^{-5} [a]

[a] From ref 25.

Table 6. Crystallographic Parameters of X-ray Diffraction Spectra and Enzymatic Degradation Result for P(3HB-*co*-3HP) Samples

sample no.	crystallographic parameters: *a*, nm	*b*, nm	*c*, nm	crystallinity, %	wt loss by depolymerase,[a] mg
1. P(3HB)	0.572	1.318	0.592	60 ± 5	0.3 ± 0.1
3. P(3HB-*co*-7% 3HP)	0.573	1.326	0.591	43 ± 5	1.0 ± 0.2
4. P(3HB-*co*-11% 3HP)	0.573	1.330	0.590	41 ± 5	1.3 ± 0.2
5. P(3HB-*co*-20% 3HP)	0.575	1.332	0.593	28 ± 5	1.9 ± 0.3
11. P(3HB-*co*-29% 3HP)	0.571	1.320	0.593	26 ± 5	
12. P(3HB-*co*-37% 3HP)	0.572	1.320	0.596	19 ± 5	1.6 ± 0.2
13. P(3HB-*co*-43% 3HP)	0.568	1.311	0.590	16 ± 5	1.6 ± 0.2
16. P(3HB-*co*-67% 3HP)				nd	
17. P(3HB-*co*-71% 3HP)				13 ± 5	
24. P(3HP)				37 ± 5	

[a] Enzymatic degradation was carried out for 2 h at 37 °C in an aqueous solution of P(3HB) depolymerase (1.0 μg/mL). The weight loss data were averaged on three film samples.

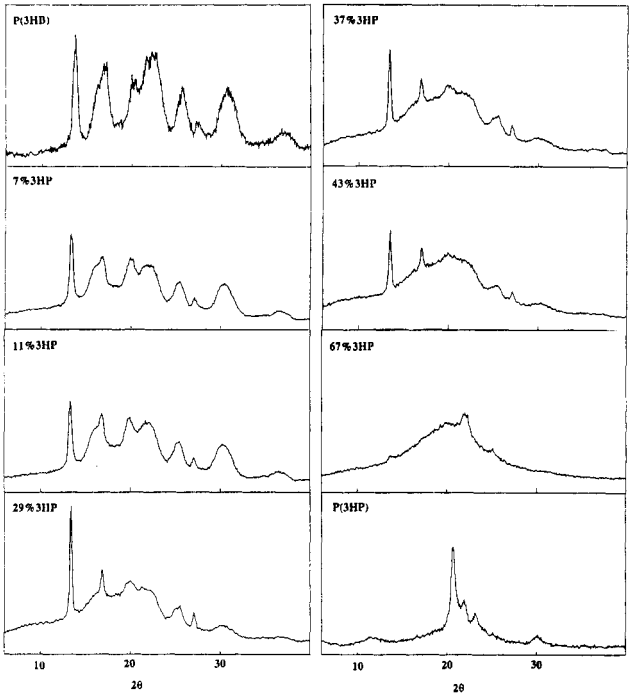

Figure 3. X-ray diffraction patterns of P(3HB-*co*-3HP) films with a range of compositions varying from 0 to 100 mol % 3HP cast from $CHCl_3$ solution. Samples were aged for 3 days at room temperature after evaporation of the solvent.

k_d for the P(3HB-*co*-3HP) and P(3HB) samples in the temperature range 150–180 °C, determined from the slopes of the plots of eq 1. In a previous paper,[25] Kunioka and Doi reported that the rate of random chain scission was not influenced by the compositions of microbial copolyesters of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (P(3HB-*co*-3HV)) and poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) (P(3HB-*co*-4HB)). As shown in Table 5, the degradation rate constant k_d at 180 °C increased with the 3HP fraction. The k_d value of P(3HB-*co*-71% 3HP) was 10 times larger than the value of P(3HB), which indicates that P(3HB-*co*-3HP) chains are more thermally unstable than P(3HB-*co*-3HV) and P(3HB-*co*-4HB) chains.

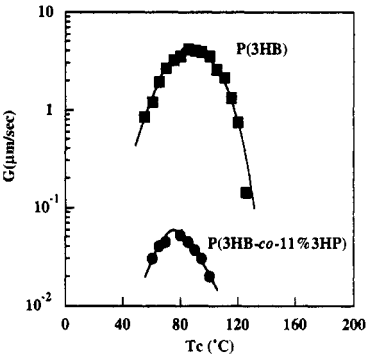

Figure 4. Radial growth rate as a function of T_c for P(3HB) (■) and P(3HB-*co*-11% 3HP) (●).

Figure 3 shows the X-ray diffraction patterns of solution-cast P(3HB-*co*-3HP) films with a wide range of compositions from 0 to 100 mol % 3HP. Only one crystalline form of the P(3HB) lattice is observed for the copolyesters with compositions up to 43 mol % 3HP. The unit cell of P(3HB) homopolymer is orthorhombic, $P2_12_12_1$ (D_2^4) with a = 0.576 nm, b = 1.320 nm, and c = 0.596 nm (fiber repeat).[26,27] Table 6 gives crystallographic parameters of P(3HB-*co*-3HP) samples, together with the degrees of X-ray crystallinity. The crystallographic parameters of the copolyesters with compositions up to 43 mol % 3HP are little influenced by the presence of the 3HP unit, and the X-ray crystallinities decrease from 60 to 7% as the 3HP fraction is increased from 0 to 67 mol %. This result indicates that 3HP units cannot crystallize in the sequence of 3HB units and act as defects in the P(3HB) crystal lattice.[28]

The rates of isothermal radial growth (G) of spherulites of P(3HB-*co*-11% 3HP) at different temperatures (T_c) are shown in Figure 4, together with the G vs T_c behavior of P(3HB). In this study, the P(3HB-*co*-11% 3HP) sample was chosenn as a representative sample of copolymers. The spherulite radial growth rate is much lower in the copolymer than in P(3HB); moreover, the crystallization curve of P(3HB-*co*-11% 3HP) is shifted to lower temperature. In the copolymer it is evident that incorporation of randomly distributed 3HP units into the P(3HB) sequence leads to a remarkable decrease in the rate of

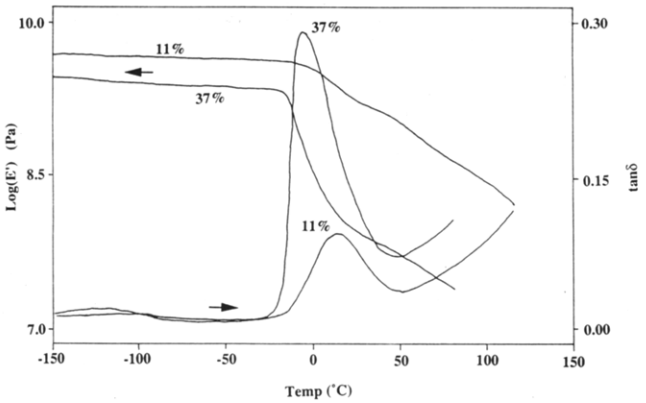

Figure 5. Dynamic mechanical spectra of P(3HB-*co*-11% 3HP) and P(3HB-*co*-37% 3HP) films.

deposition of 3HB segments at the growing front of crystalline lamellae. The observed shift to lower temperature of the crystallization curve of P(3HB-*co*-11% 3HP) with respect to P(3HB) is attributed to the lower T_m value of the copolymer (see Table 4).

Dynamic mechanical measurements were carried out on P(3HB-*co*-11% 3HP) and P(3HB-*co*-37% 3HP) films, and the spectra are reported in Figure 5 as loss factor (tan δ) and dynamic storage modulus (E') as a function of temperature. A relevant relaxation phenomenon is observed in the dynamic mechanical spectrum of the copolymers, evidenced by a peak in tan δ and a drop of the modulus, centered at about 15 and 0 °C in P(3HB-*co*-11% 3HP) and P(3HB-*co*-37% 3HP), respectively. The relaxation is associated with the glass transition and has a different magnitude in the two samples, indicating a different ratio of amorphous to crystalline phase. In agreement with DSC and X-ray results, the larger peak intensity and modulus drop of the P(3HB-*co*-37% 3HP) sample are a consequence of the lower content of crystalline phase of this sample with respect to P(3HB-*co*-11% 3HP).

In the low-temperature range of the dynamic mechanical spectrum, both copolymers show a low-intensity secondary relaxation which is attributed—by analogy with a similar phenomenon present in the spectra of P(3HB),[29] P(3HB-*co*-3HV),[30] and P(3HB-*co*-4HB)[30,31]—to local motions of the poly(hydroxyalkanoate) main chain. In line with previous results on the mentioned PHAs, the water content affects both temperature location and intensity of the local-mode relaxation of P(3HB-*co*-11% 3HP) and P(3HB-*co*-37% 3HP).

Figure 6 shows the multifrequency viscoelastic spectrum of P(3HB-*co*-11% 3HP) where two dielectric relaxation phenomena are observed. In agreement with the dynamic mechanical results, they can be associated with a local motion of the main chain and the glass transition, respectively. As expected, the dielectric peaks are seen to shift to higher temperature with increasing frequency. Although the Arrhenius equation (2)

$$\ln \nu = \ln \nu_0 - E_a/RT \qquad (2)$$

correctly describes only the frequency dependence of the peak temperature of the secondary relaxation, it is found that the plots of ln ν vs $1/T$ are satisfactorily linear for both relaxation processes shown in Figure 6. The values of the apparent activation energy E_a of the secondary and glass transition relaxation have been obtained from the best linear fit to the experimental data and are 38 and 180 kJ/mol, respectively.

Enzymatic Degradation of P(3HB-*co*-3HP). The enzymatic degradations of six samples of P(3HB-*co*-3HP)

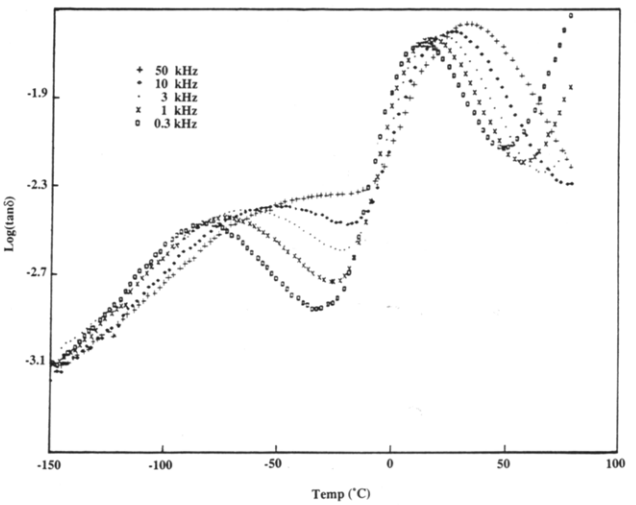

Figure 6. Multifrequency dielectric spectrum of P(3HB-*co*-11% 3HP) film at frequencies of 0.3, 1, 3, 10, and 50 kHz.

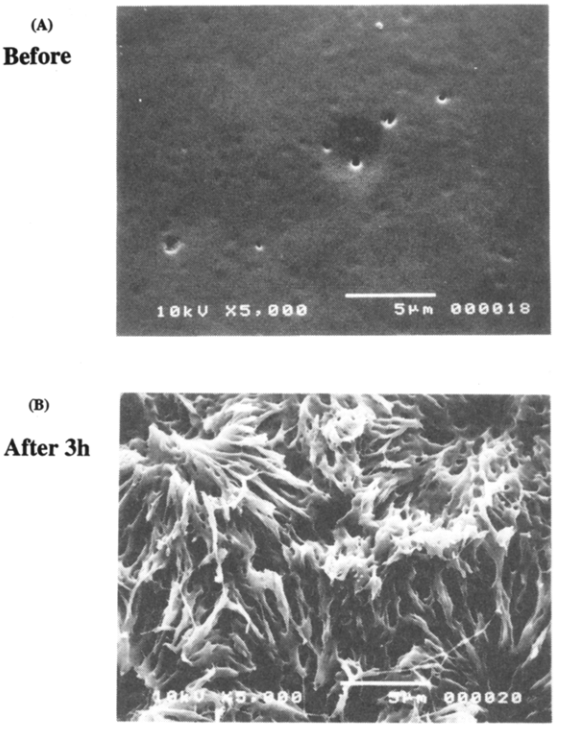

Figure 7. SEM of the P(3HB-*co*-11% 3HP) surface of virgin film (A) and the film after an enzymatic degradation for 3 h (B).

containing 0, 7, 11, 20, 37, and 43 mol % 3HP were carried out on the solution-cast films (initial weights, 6 mg) for 2 h at 37 °C in the aqueous solution of P(3HB) depolymerase from *A. faecalis*. Figure 7 shows the scanning electron micrographs (SEMs) of the surfaces of the P(3HB-*co*-11% 3HP) film before and after enzymatic degradation for 2 h. The surface of degraded P(3HB-*co*-3HP) film is apparently blemished by the function of depolymerase (Figure 7B), suggesting that the enzymatic degradation occurred on the surface of the film. As given in Table 6, the weight of film erosion by P(3HB) depolymerase for 2 h increased from 0.3 to 1.9 mg as the 3HP fraction increased from 0 to 20 mol %. Thus, the rate of enzymatic degradation was accelerated by the incorporation of 3HP units in the P(3HB) sequence. On the other hand, the rates of enzymatic degradation of samples 12 (37% 3HP) and 13 (43% 3HP) were slightly lower than the rate of sample 5 (20% 3HP). This decrease in the rate of enzymatic degradation may be related with the substrate specificity of the depolymerase.

A marked increase in the rate of enzymatic degradation was observed on the films of P(3HB-co-4HB)[31,32] and poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) (P(3HB-co-3HH)).[28] The crystallinities of P(3HB-co-3HP), P(3HB-co-4HB), and P(3HB-co-3HH) copolyesters decreased with an increase in the fraction of the second monomer unit (3HP, 4HB, or 3HH). In contrast, the rate of enzymatic degradation of P(3HB-co-3HV) films was slower than the rate of the P(3HB) film,[32] annd the P(3HB-co-3HV) films showed approximately the same high degree of crystallinity (>50%) throughout a wide range of compositions.[29]

In a previous paper,[33] we reported that the rate of surface erosion of P(3HB) film by P(3HB) depolymerase was markedly decreased with an increase in the crystallinity and that the rate of enzymatic degradation on the amorphous phase of the P(3HB) film was about 20 times higher than the rate on the crystalline phase. A decrease in the crystallinity may result in a rapid erosion of P(3HB-co-3HP) film by P(3HB) depolymerase.

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