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

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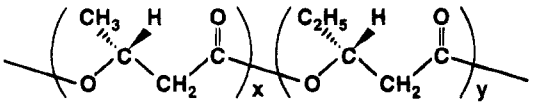
ABSTRACT: *Aeromonas caviae* produced a random copolymer of 3-hydroxybutyric acid (3HB) and 3-hydroxyhexanoic acid (3HH) under aerobic conditions when sodium salts of alkanoic acids of even carbon numbers ranging from C_{12} to C_{18} and olive oil were fed as the sole carbon source. On the other hand, a copolymer of 3HB and 3-hydroxyvaleric acid (3HV) was produced by *A. caviae* from alkanoic acids of odd carbon numbers from C_{11} to C_{17}. The weight-average molecular weights of P(3HB-*co*-3HH) were in the range $(2-11) \times 10^5$. The structure and physical properties of P(3HB-*co*-3HH) with compositions of 5–25 mol % 3HH were characterized by ^1H and ^{13}C NMR spectroscopy, X-ray diffraction, differential scanning calorimetry, mechanical tensile measurement, and optical microscopy. The degree of X-ray crystallinity of solvent-cast P(3HB-*co*-3HH) films decreased from 60 to 18% as the 3HH fraction was increased from 0 to 25 mol %, suggesting that 3HH units are excluded from the P(3HB) crystalline phase. The isothermal radial growth rates of spherulites of P(3HB-*co*-3HH) were markedly reduced with an increase in the 3HH fraction. Enzymatic degradations of P(3HB-*co*-3HH) films were carried out at 37 °C in an aqueous solution of P(3HB) depolymerase from *Alcaligenes faecalis*. The rates of enzymatic erosion increased markedly with an increase in the 3HH fraction to reach a maximum value at 15 mol % 3HH, followed by a decrease in the erosion rate. The above results were compared with the solid-state properties of two other microbial copolymers, P(3HB-*co*-3HV) and P(3HB-*co*-3HP) (3HP: 3-hydroxypropionic acid).

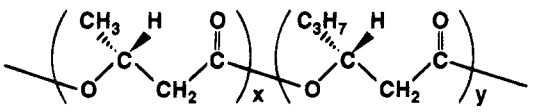
Introduction

A wide variety of bacteria accumulate polyesters of hydroxyalkanoic acids as intracellular storage materials of energy and carbon source.[1–4] At present about 40 different monomeric units as constituents of bacterial poly(hydroxyalkanoates) (PHA) have been found.[3] Since these bacterial polyesters are biodegradable thermoplastics, they have received much attention as a new environmentally compatible material. A random copolymer of (*R*)-3-hydroxybutyric acid (3HB) and (*R*)-3-hydroxyvaleric acid (3HV) has been commercially produced by ZENECA BioProducts, UK, in a large scale fed-batch fermentation of *Alcaligenes eutrophus*, feeding glucose and propionic acid as carbon substrates.[5,6] The copolymers have a statistically random distribution of 3HB and 3HV units,[7–9] and they exhibit a high degree of crystallinity (>50%) throughout a wide range of compositions from 0 to 95 mol % 3HV.[8,10–12] This phenomenon is known as isodimorphism,[8,13] i.e., cocrystallization of the two monomer units in either of the homopolymer crystal lattices of P(3HB) and P(3HV), depending on whether the 3HV composition is above or below ~40 mol %. Cocrystallization of the random copolymers has been interpreted theoretically with a thermodynamic treatment.[14–16] The mechanical and physical properties varied with the copolymer composition.[17–20] The rates of enzymatic surface erosion of P(3HB-*co*-3HV) films by microbial P(3HB) depolymerases were slower than the rate of P(3HB) homopolymer film.[21–23]



(*R*)-3HB (*R*)-3HV

A phototrophic bacterium, *Rhodospirillum rubrum*, was found to produce a terpolymer of 3HB, 3HV, and (*R*)-3-hydroxyhexanoate (3HH) from hexanoic or 3-hydroxyhexanoic acid as the sole carbon source.[24] Recently, a copolymer of 3HB and 3HH was produced from octanoic acid by the genetically engineered bacterium of PHA-negative *Pseudomonas putida* which harbored and expressed PHA-biosynthetic genes of the phototrophic bacteria *Thiocapsa pfennigii* and *Chromatium vinosum*.[25] We found that a random copolymer of 3HB and 3HH was produced from olive oil by the wild-type bacterium *Aeromonas caviae* which was isolated from soil.[26,27]



(*R*)-3HB (*R*)-3HH

In this study we report that *A. caviae* produces P(3HB-*co*-3HH) from alkanoic acids of even carbon numbers ranging from C_{12} to C_{18} and P(3HB-*co*-3HV) from those (C_{11} to C_{19}) of odd carbon numbers. In addition, we discuss the biosynthetic pathway of P(3HB-*co*-3HH) in *A. aviae*. The physical properties and biodegradability of P(3HB-*co*-3HH) films are studied in comparison with those of P(3HB-*co*-3HV).

Experimental Section

Polyester Synthesis. *A. caviae* 440 was isolated from soil[27] and used in this study. Polyester synthesis was carried out by a two-stage batch fermentation of *A. caviae*. The bacterium was first grown for 14 h at 30 °C under aerobic conditions (agitation at 130 rpm) on a reciprocal shaker in 500-mL flasks with 100 mL of a nutrient-rich medium containing 1.0 g of meat extract, 1.0 g of polypeptone, and 0.2 g of yeast extract in distilled water. The cells were harvested by centrifugation at 5000*g* for 15 min. Under these culture conditions, accumulation of polyester in the cells was not observed. To promote polyester synthesis, 0.3–0.4 g (dry

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weight) of centrifuged cells was resuspended in a nutrient-free mineral medium (100 mL) containing different carbon substrates (1.0 g) as the sole carbon source. The olive oil and sodium salts of alkanoic acids as the carbon sources were purchased from Wako Pure Chemicals (Osaka, Japan). The mineral medium contained 2.8 g of KH_2PO_4, 3.32 g of Na_2HPO_4, 0.25 g of $MgSO_4 \cdot 7H_2O$, and 1 mL of trace element solution per liter of distilled water. The microelement solution contained the following (per liter of 0.5 N HCl): 20 g of $FeCl_3 \cdot 6H_2O$, 10 g of $CaCl_2 \cdot H_2O$, 0.03 g of $CuSO_4 \cdot 5H_2O$, 0.05 g of $MnCl_2 \cdot 4H_2O$, and 0.1 g of $ZnSO_4 \cdot 7H_2O$. The cells were incubated in the nitrogen-free media (pH 7.0) for a given time at 30 °C under aeration, harvested by centrifugation, and finally lyophilized. Polyesters were extracted from the lyophilized cells with hot chloroform in a Soxhlet apparatus and purified by reprecipitation with hexane.

Analytical Procedures. The ^1H and ^{13}C NMR analyses of polyester samples were carried out on a JEOL ALPHA-400 spectrometer. The 400-MHz ^1H NMR spectra were recorded at 23 °C in a $CDCl_3$ solution of polyester (5 mg/mL) with a 5.0-μs pulse width (45° pulse angle), 5-s pulse repetition, 8000-Hz spectral width, and 16K data points. The 100-MHz ^{13}C NMR spectra were recorded at 23 °C in a $CDCl_3$ solution of polyester (20 mg/mL) with a 10-μs pulse width (45° pulse angle), 5-s pulse repetition, 25000-Hz spectral width, and 64K data points. Tetramethylsilane was used as an internal chemical shift standard.

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 the eluent at a flow rate of 0.8 mL/min, and sample concentrations of 1.0 mg/mL were applied. Polystyrene standards with a low polydispersity were used to make a calibration curve.

Differential scanning calorimetry (DSC) data of polyesters (3 mg) encapsulated in aluminum pans were recorded at a heating rate of 10 °C/min in the temperature range −100 to +200 °C on a Shimadzu DSC-50 system equipped with a cooling accessory under a nitrogen flow of 30 mL/min. The melting temperature (T_m) and enthalpy of fusion (ΔH_m) were determined from the DSC endotherms. For the measurement 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.

Wide-angle X-ray diffraction measurements of polyester samples were made on a Rigaku RAD-IIIB system using nickel-filtered Cu Kα radiation (λ = 0.154 nm; 40 kV; 30 mA). The X-ray diffraction patterns of polyesters were recorded at 27 °C in the range of 2θ = 6−60° at a scan speed of 2°/min. X-ray crystallinities were measured for the polyester films that had been cast from a chloroform solution and allowed to stand for 4 weeks at room temperature. The percentage of crystallinity was calculated from diffracted intensity data according to Vonk's method.[28]

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

Measurements of the isothermal radial growth rate of polyester spherulites were carried out on a Nikon optical microscope equipped with crossed polarizers and a Linkham TH600 hot stage. Isothermal crystallization measurements were performed on a small piece (2 mg) of solution-cast film, which was inserted between two microscope cover glasses and subjected to a four-step thermal program: (1) heating in the microscope hot stage at 30 °C/min to 200 °C; (2) standing at 200 °C for 30 s; (3) quenching at a 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 to monitor the growth of the spherulites as a function of time. The whole procedure was carried out without removing the sample from the hot stage. A video camera, attached to the microscope, allowed real time measurement of the spherulite dimensions. The radial growth rate of spherulites was calculated as the slope of the line obtained by plotting the spherulite radius against time with more than ten data points. A new sample was used for each crystallization measurement.

Enzymatic Degradation. The extracellular P(3HB) depolymerase was purified to electrophoretic homogeneity from *Alcaligenes faecalis* as described in a previous paper.[29] The enzymatic degradation of solution-cast films of polyester samples by the extracellular P(3HB) depolymerase was carried out at 37 °C in a 0.1 M potassium phosphate buffer (pH 7.4). Polyester films (initial weight, 10 mg; initial film dimensions, 10 × 10 × 0.08 mm) were placed in small bottles containing 1.0 mL of buffer. The reaction was started by the addition of 3.8 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-5300) after Au coating of the films using an ion coater.

The water-soluble products after enzymatic degradation of P(3HB-*co*-3HH) 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 × 4mm) 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 were detected at 210 nm. The relative amounts of products were caluculated from the peak areas of the HPLC curves. The monomer, dimers, and trimers in the degradation products of P(3HB-*co*-3HH) were fractionated by HPLC for ^1H NMR analysis. Each product was collected from the HPLC eluate, and the soluvent was evaporated. The 400-MHz ^1H NMR spectra were recorded in D_2O: 3HB monomer (t_R = 6.5 min), δ 1.20 (d, 3H), 2.42 (m, 2H), 4.21 (m, 1H); 3HB−3HB dimer (t_R = 13.3 min), δ 1.21 (d, 3H), 1.30 (d, 3H), 2.45−2.66 (m, 4H), 4.22 (m, 1H), 5.26 (m, 1H); 3HB−3HH dimer (t_R = 18.2 min), δ 0.89 (t, 3H), 1.21 (d, 3H), 1.33 (m, 2H), 1.62 (m, 2H), 2.53 (m, 2H), 2.61 (m, 2H), 4.23 (m, 1H), 5.25 (m, 1H); 3HB−3HB−3HH trimer (t_R = 21.3 min), δ 0.88 (t, 3H), 1.21 (d, 3H), 1.27 (d, 3H), 1.32 (m, 2H), 1.61 (m, 2H), 2.47−2.75 (m, 6H), 4.22 (m, 1H), 5.25 (m, 2H).

Results and Discussion

Synthesis and Structure of P(3HB-*co*-3HH). *Aeromonas caviae* is a Gram-negative, rod-shaped, and facultatively anaerobic bacterium which produces oxidase, catalase, and lipase. The bacterium grew on glucose and sucrose but did not accumulate polyester within cells. In contrast, the bacterium grew on oleic acid and olive oil and accumulated polyester within cells. In this study, polyester syntheses were carried out by a two-stage batch fermentation of *A. caviae* under aerobic conditions at 30 °C. The bacterium was first grown for 14 h in a nutrient-rich medium in which no polyester was accumulated within cells. The collected cells were incubated in a nitrogen-free mineral medium containing sodium salts of different alkanoic acids and olive oil to accumulate polyester.

Table 1 lists the results of copolyester production by *A. caviae* from various carbon substrates. The compositions of copolyesters were determined by integration of the proton resonances of 3-hydroxybutyrate (3HB), 3-hydroxyvalerate (3HV), and 3-hydroxyhexanoate (3HH) units in the ^1H NMR spectra. *A. caviae* produced polyesters within cells from alkanoic acids of carbon chain length 11−18 (C_{11}−C_{18}). P(3HB-*co*-3HV) was produced from the alkanoic acids of odd carbon numbers, and the mole fractions of 3HB and 3HV were

Table 1. Production of Copolyesters of 3HB, 3HV, and 3HH by *A. caviae* from Sodium Salts of Alkanoic Acids of Carbon Chain Lengths C_{11}–C_{18} and Olive Oil at 30 °C

carbon source (g/L)	incubation time, h	cell dry weight, g/L	polyester content,[a] wt %	polyester composition,[b] mol %		
				3HB	3HV	3HH
$CH_3(CH_2)_9COOH$ (10)	48	3.0	1	5	95	
$CH_3(CH_2)_{10}COOH$ (10)	48	4.3	29	81		19
$CH_3(CH_2)_{11}COOH$ (10)	48	4.3	12	6	94	
$CH_3(CH_2)_{12}COOH$ (10)	48	4.7	11	88		12
$CH_3(CH_2)_{13}COOH$ (10)	48	4.8	4	4	96	
$CH_3(CH_2)_{14}COOH$ (10)	48	4.9	8	89		11
$CH_3(CH_2)_{15}COOH$ (10)	48	4.6	6	3	97	
$CH_3(CH_2)_{16}COOH$ (10)	48	4.8	7	87		13
$CH_3(CH_2)_{10}COOH$ (10)	6	3.8	4	89		11
$CH_3(CH_2)_{10}COOH$ (10)	12	3.8	11	87		13
$CH_3(CH_2)_{10}COOH$ (10)	19	4.4	21	82		18
$CH_3(CH_2)_{10}COOH$ (10)	24	4.6	29	79		21
$CH_3(CH_2)_{10}COOH$ (10)	72	4.2	32	79		21
$CH_3(CH_2)_{10}COOH$ (10)	96	4.5	33	80		20
$CH_3(CH_2)_{10}COOH$ (5)	48	3.7	6	91		9
$CH_3(CH_2)_{10}COOH$ (15)	48	3.8	36	80		20
$CH_3(CH_2)_{10}COOH$ (20)	48	3.8	27	77		23
$CH_3(CH_2)_{10}COOH$ (30)	48	3.7	27	75		25
olive oil (5)	48	3.7	6	91		9
olive oil (10)	48	3.6	12	87		13
olive oil (20)	48	3.5	10	90		10

[a] Polyester content in dry cells. [b] Determined from ^1H NMR spectra.

Table 2. Copolymer Compositions and Crystallographic Parameters of P(3HB-*co*-3HH) Samples

no.	composition,[a] mol %		diad fraction,[b] mol %			mol wt[c]	crystallographic parameters[d]			
	3HB	3HH	BB	BH + HB	HH	10^{-3} M_w (M_w/M_n)	a, nm	b, nm	c, nm	crystallinity, %
1	100	0	100	0	0	1020 (2.0)	0.572	1.318	0.592	60 ± 5
2	95	5	90	10	0	190 (1.9)	0.571	1.317	0.594	42 ± 5
			(90)	(10)	(0)					
3	90	10	80	20	0	304 (2.6)	0.584	1.310	0.593	34 ± 5
			(81)	(18)	(1)					
4	85	15	73	27	0	792 (3.7)	0.573	1.313	0.592	26 ± 5
			(72)	(26)	(2)					
5	83	17	65	34	1	1122 (2.2)	0.575	1.322	0.594	26 ± 5
			(69)	(28)	(3)					
6	81	19	64	33	3	332 (8.3)	0.578	1.328	0.596	22 ± 5
			(65)	(31)	(4)					
7	75	25	53	40	7	742 (3.5)	0.575	1.326	0.596	18 ± 5
			(56)	(38)	(6)					

[a] Determined from ^1H spectra. [b] Determined from relative intensities of carbonyl resonances in ^{13}C NMR spectra. The values in parentheses were calculated by Bernoullian statistics with the mole fraction of the 3HH unit. [c] Determined by GPC. [d] Determined from X-ray diffraction spectra.

respectively 5 ± 2 and 95 ± 2 mol %, almost independent of the chain length of the alkanoic acid. In contrast, *A. caviae* produced P(3HB-*co*-3HH) from the alkanoic acids of even carbon numbers, and the highest content of polyester within cells was obtained on lauric acid (C_{12}). Then, time-dependent changes in the content and composition of P(3HB-*co*-3HH) were studied during the incubation of *A. caviae* in the presence of lauric acid. The polyester content in dried cells increased to 33 wt % during incubation for 96 h, and the 3HH fractions in copolyesters increased from 11 to 20 mol % during the course of incubation from 6 to 96 h. The 3HH fraction in copolyesters obtained for 48 h increased from 9 to 25 mol % as the concentration of lauric acid was increased from 5 to 30 g/L. P(3HB-*co*-3HH) was also produced from olive oil (a mixture of triglycerides of saturated and unsaturated alkanoic acids of even carbon numbers).

Table 2 gives the molecular weights and monomer sequence distributions of P(3HB-*co*-3HH) samples used for further characterization. The diad sequence distribution data for two monomeric units were determined from the relative peak areas of three peaks in the carbonyl resonances (169.2–169.6 ppm) in the ^{13}C NMR spectra,[26] and the data 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 fraction F_i and F_j of i and j units as $F_{ij} = F_iF_j$. As shown in Table 2, the calculated diad fractions (F_{BB}, $F_{BH} + F_{HB}$, and F_{HH}) are in agreement with the observed values for all samples. It is concluded that the sequence distributions of 3HB and 3HH units in those samples are statistically random.

Here, we propose a schematic pathway (Figure 1) of P(3HB-*co*-3HH) and P(3HB-*co*-3HV) syntheses in *A. caviae* from alkanoic acids. The pathway of polyester synthesis is linked to intermediates in the cyclic β-oxidation and thiolytic cleavage of acyl-coenzyme A (CoA) derived from alkanoic acids. The PHA synthase in *A. caviae* may be active only for (*R*)-3-hydroxyacyl-CoA of C_4, C_5, and C_6. As a result, P(3HB-*co*-3HH) is synthesized from alkanoic acids of even carbon numbers of C_{12} to C_{18}. The 3-hydroxyacyl-CoA in the β-oxidation cycle is an (*S*) stereoisomer. The (*R*) stereoisomer of 3-hydroxyacyl-CoA as the substrate for PHA synthase may

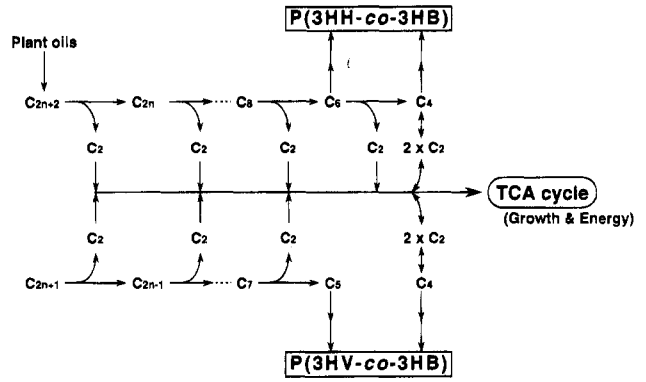

Figure 1. Biosynthetic pathway of P(3HB-*co*-3HH) and P(3HB-*co*-3HV) from alkanoic acid of even carbon numbers (C_{2n+2}) and odd carbon numbers (C_{2n+1}), respectively. C_{2n} ($n \geq 5$) and C_2 denote acyl-coenzyme A, and TCA cycle represents tricarboxylic acid cycle.

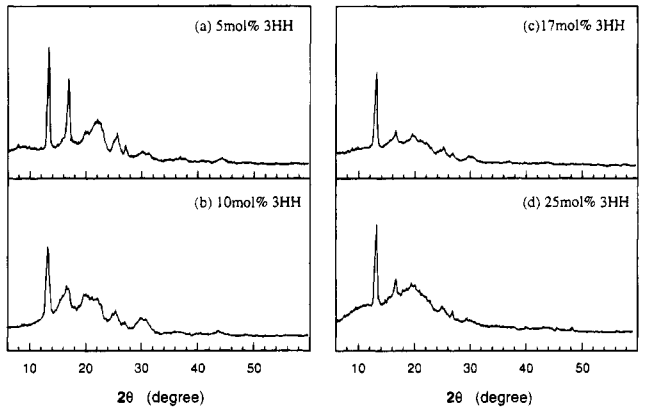

Figure 2. X-ray diffraction patterns of P(3HB-*co*-3HH) films with different 3HH fractions.

be formed via a crotonyl-CoA hydration by enoyl-CoA hydratase or via a 3-ketoacyl-CoA reduction by 3-ketoacyl-CoA reductase. Small amounts (5 ± 2 mol %) of the 3HB unit were formed with the 3HV unit from the alkanoic acids of odd carbon numbers of C_{11} to C_{17}. (*R*)-3-Hydroxybutyryl-CoA may be derived via a condensation reaction of two acetyl-CoA molecules by 3-ketothiolase, resulting in the random copolymerization with (*R*)-3-hydroxyvaleryl-CoA by PHA synthase to give P(3HB-*co*-3HV).

Solid-State Properties of P(3HB-*co*-3HH). Figure 2 shows typical X-ray diffraction patterns of solution-cast P(3HB-*co*-3HH) films. Only one crystalline form of the P(3HB) lattice is observed for the copolymers with compositions to 25 mol % 3HH. 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).[30,31] The crystallographic parameters of P(3HB-*co*-3HH) films with compositions of 0−25 mol % 3HH are given in Table 2, together with the degrees of X-ray crystallinity. The X-ray crystallinities decrease from 60 to 18% as the 3HH fraction is increased from 0 to 25 mol %, but the crystallographic parameters of the copolyesters are little influenced by the presence of the 3HH unit.

Figure 3 shows the effect of copolymer composition on the degrees of X-ray crystallinity of the solution-cast films of P(3HB-*co*-3HH), P(3HB-*co*-3HV),[10] and random copolymers of (*R*)-3-hydroxybutyric acid and 3-hydroxypropionic acid, P(3HB-*co*-3HP),[32] for comparison. As described in the Introduction, P(3HB-*co*-3HV) samples show high degrees of crystallinity (>50%) over the entire

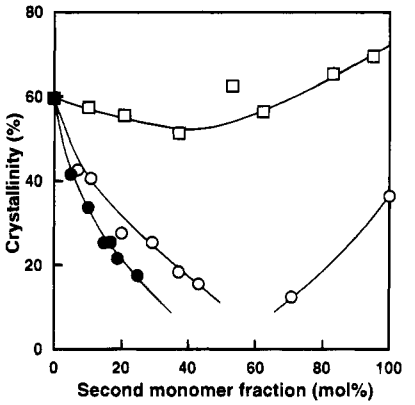

Figure 3. Effects of copolyester composition on the degrees of crystallinity of different microbial copolyesters: (●) P(3HB-*co*-3HH); (○) P(3HB-*co*-3HP) (ref 32); (□) P(3HB-*co*-3HV) (ref 10). P(3HB-*co*-3HP) is a random copolymer of (*R*)-3-hydroxybutyric and 3-hydroxypropionic acids.

Table 3. Thermal and Mechanical Properties of Solution-Cast Films of P(3HB-*co*-3HH) Samples

sample	T_g,[a] °C	T_m,[a] °C	ΔH_m,[a] J/g	tensile strength,[b] MPa	elongation[b] to break, %
1. P(3HB)	4	177	97	43	5
2. P(3HB-*co*-5% 3HH)	0	151	69		
3. P(3HB-*co*-10% 3HH)	−1	127	77	21	400
4. P(3HB-*co*-15% 3HH)	0	115	54	23	760
5. P(3HB-*co*-17% 3HH)	−2	120	34	20	850
6. P(3HB-*co*-19% 3HH)	−4	111	34		
7. P(3HB-*co*-25% 3HH)	−4	52	19		

[a] Measured by DSC. [b] Measured at 23 °C.

range of copolymer compositions due to cocrystallization, and this phenomenon is known as isodimorphism.[8,13] In contrast, the crystallinities of P(3HB-*co*-3HH) and P(3HB-*co*-3HP)[32] decrease steeply with increasing fraction of second monomer, indicating that the 3HH or 3HP units are excluded from the P(3HB) crystalline phase at low compositions up to 25 mol %.

Table 3 lists the thermal and mechanical properties of solution-cast P(3HB-*co*-3HH) films. The tensile strength of the films decreased from 43 to 20 MPa as the 3HH fraction was increased from 0 to 17 mol %. In contrast, the elongation to break increased from 6 to 850%. This result indicates that the P(3HB-*co*-3HH) films become soft and flexible with an increase in the 3HH fraction.

The melting temperature (T_m) of P(3HB-*co*-3HH) samples decreased from 177 to 52 °C as the 3HH fraction was increased from 0 to 25 mol %. The enthalpy of fusion (ΔH_m) decreased from 97 to 19 J/g also with an increase in the 3HH fraction. The glass-transition temperature (T_g) decreased from 4 to −4 °C.

The crystallization kinetics of P(3HB-*co*-3HH) were studied without adding nucleating agents. The spherulites of P(3HB-*co*-3HH) were observed with a polarized optical microscope as a function of time at a given temperature. The samples were isothermally crystallized at a temperature between 50 and 120 °C after melting at 200 °C for 30 s. After crystallization, uniform spherulites were developed throughout the films. The spherulite radius increased linearly with time. The radial growth rate (G) of spherulites was calculated as the slope of the line obtained by plotting the spherulite radius against time. Figure 4 shows the rate of spherulite growth (G) of six P(3HB-*co*-3HH) samples (3HH = 0, 5, 10, 15, 17, and 19 mol %) at different crystallization

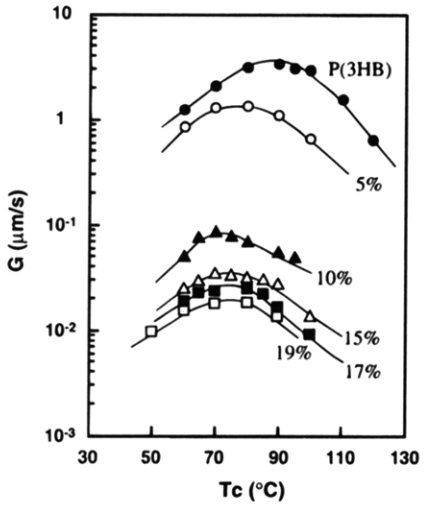

Figure 4. Radial growth rate (G) of spherulites as a function of crystallization temperature (T_c) for P(3HB) and P(3HB-*co*-3HH) with different 3HH fractions (numbers on curves in 3HH mole percent).

temperatures. The rates of spherulite growth were dependent on both the 3HH fraction in the samples and the crystallization temperature. As the 3HH fraction was increased, the rates of spherulite growth were markedly reduced, and the crystallization curves were shifted toward lower temperatures. The radial growth rate of spherulites of P(3HB-*co*-15% 3HH) was slower by 2 orders of magnitude than the rate of P(3HB) homopolymer, and the peak temperature was shifted from 90 to 75 °C. This result indicates that the randomly distributed 3HH units in the P(3HB-*co*-3HH) lead to a remarkable decrease in the rate of deposition of 3HB segments at the growing front of the crystalline lamellae. The observed shift to lower temperature of the crystalline curves of P(3HB-*co*-3HH) with respect to P(3HB) is attributed to the lower T_m values of the copolymers.

Enzymatic Degradation of P(3HB-*co*-3HH). Enzymatic degradations of seven samples of P(3HB-*co*-3HH) containing 0, 5, 10, 15, 17, 19, and 25 mol % 3HH were carried out on the solution-cast films at 37 °C in 0.1 M potassium phosphate buffer (pH 7.4) of P(3HB) depolymerase (1.0 μg/mL) from *A. faecalis*. Figure 5 shows typical scanning electron micrographs (SEMs) of the surfaces of P(3HB-*co*-3HH) film before and after enzymatic degradation. The surfaces of degraded P(3HB-*co*-3HH) films were apparently blemished by the function of depolymerase, which suggests that the enzymatic degradation takes place on the surface of the film. In the absence of P(3HB) depolymerase, P(3HB-*co*-3HH) films were not hydrolyzed for 12 h at 37 °C on 0.1 M potassium phosphate buffer.

The weight loss of P(3HB-*co*-3HH) films by P(3HB) depolymerase increased proportionally with time for 12 h. The rate of enzymatic surface erosion was determined from the slope of the line obtained by plotting the weight loss against time. The result is given in Figure 6, together with the rates of enzymatic surface erosion on the solution-cast films of P(3HB-*co*-3HV) and P(3HB-co-3HP) by P(3HB) depolymerase from *A. faecalis*, which were reported in previous papers.[22,23] Figure 6 shows remarkable effects of copolymer composition on the rate of enzymatic surface erosion. The rates of enzymatic erosion on the films of P(3HB-*co*-3HH) and P(3HB-*co*-3HP) increased markedly with an increase in the fraction of second monomer units (3HH or 3HP) to

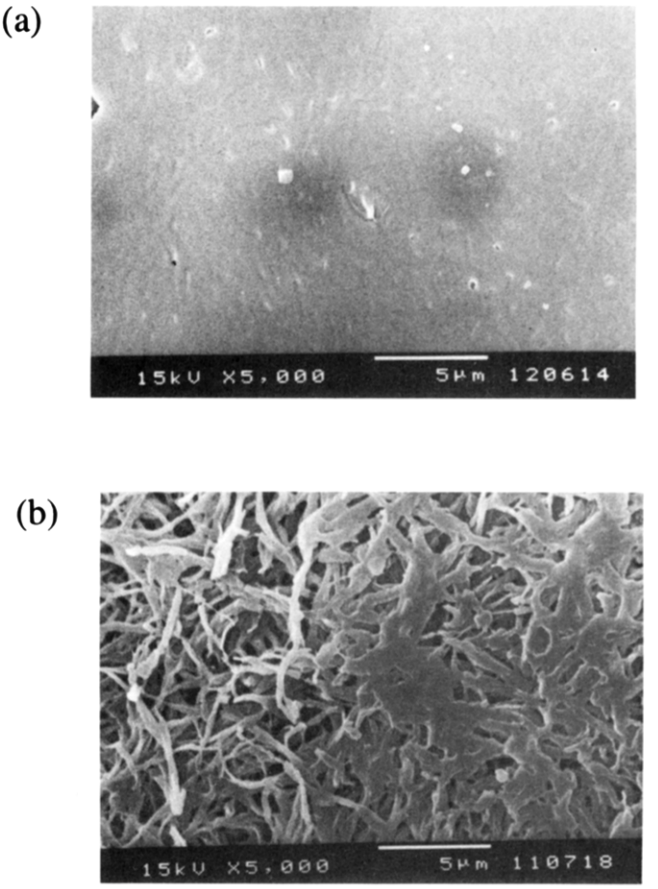

Figure 5. SEMs of surfaces of P(3HB-*co*-15% 3HH) films (a) before and (b) after enzymatic degradation for 2 h.

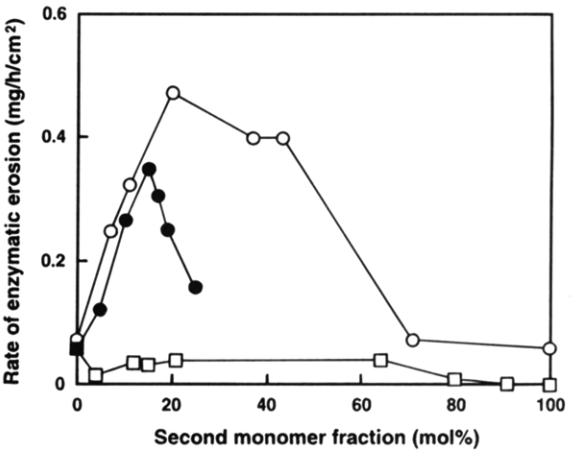

Figure 6. Effect of copolymer composition on the rate of enzymatic erosion of solution-cast copolyester films in the aqueous solution of P(3HB) depolymerase (from *A. faecalis*) at 37 °C and pH 7.4: (●) P(3HB-*co*-3HH); (○) P(3HB-*co*-3HP) (ref 32); (□) P(3HB-*co*-3HV) (ref 22). P(3HB-*co*-3HP) is a random copolymer of (*R*)-3-hydroxybutyric and 3-hydroxypropionic acids.

reach a maximum value followed by a decrease in the erosion rate. The highest rates of enzymatic erosion were observed at 15 mol % 3HH and 20 mol % 3HP on the P(3HB-*co*-3HH) and P(3HB-*co*-3HP) films, respectively, and the values were 5−6 times larger than the rate on P(3HB) film. In contrast, the rate of enzymatic surface erosion on the P(3HB-*co*-3HV) film decreased gradually with an increase in the fraction of 3HV unit.

It is of interest to compare the result (Figure 6) of enzymatic degradation with the result (Figure 3) of

X-ray crystallinities of three different copolyesters. It is noted that the rates of enzymatic degradation on the films of P(3HB-*co*-3HH) and P(3HB-*co*-3HP) increase with a decrease in the crystallinity in the copolymer compositions with low 3HH and 3HP fractions. In a previous paper,[33] we reported that the rate of enzymatic erosion on P(3HB) film was markedly decreased with an increase in the crystallinity and that the rate on the amorphous phase of P(3HB) film was about 20 times higher than the rate on the crystalline phase. This result suggests that a rapid erosion of P(3HB-*co*-3HH) or P(3HB-*co*-3HP) films with compositions of 0−15 mol % 3HH or 0−20 mol % 3HP arises from a decrease in the crystallinity. However, the rates of enzymatic erosion of the P(3HB-*co*-3HH) films with compositions of 15−25 mol % 3HH decreased markedly with an increase in the 3HH fraction, though the crystallinities of films decreased with the 3HH fraction. Similarly, the rates of enzymatic erosion of the P(3HB-*co*-3HP) films with compositions of 20−100 mol % 3HP decreased with the 3HP fraction. These results suggest that the rate of enzymatic erosion is regulated not only by the crystallinity of the polymer but also by the chemical structure of the monomeric unit because of the substrate specificity of P(3HB) depolymerase.[34] The rate of enzymatic surface erosion of P(3HP) film was almost identical with the rate on P(3HB) film.[34] The P(3HV) film was hardly eroded by P(3HB) depolymerase, and a 3HV−3HV dimer was also hardly hydrolyzed by the enzyme,[23] which suggests that enzyme is incapable of hydrolyzing a sequence of 3HH units.

Characterization of Water-Soluble Products. In a previous paper,[35] we demonstrated that the enzymatic hydrolysis of bacterial P(3HB) film by P(3HB) depolymerase produced a mixture of monomer and dimer of 3-hydroxybutyric acid as water-soluble products. In this study, we measured the composition of water-soluble products by HPLC analysis after the enzymatic degradation of P(3HB-*co*-3HH) films. The enzymatic degradations of films were carried out for 5 h at 37 °C in the aqueous solution (1 mL) containing P(3HB) depolymerase from *A. faecalis*, and the reaction solutions were analyzed by high-performance liquid chromatography (HPLC).

Figure 7 shows a typical HPLC curve of water-soluble products from P(3HB-*co*-3HH) (3HH = 15 mol %) film. The component of each peak for the 3HB monomer, 3HB−3HB dimer, 3HB−3HH dimer, and 3HB−3HB−3HH trimer was identified by ^{1}H NMR analysis of each fraction collected from the HPLC eluate using a fraction collector (see Experimental Section). The relative amounts of water-soluble products were determined from the peak areas in the HPLC curves.

Figure 8 shows the distribution of water-soluble products after enzymatic degradation of P(3HB-*co*-3HH) films for 5 h. In all HPLC curves studied, we could not detect the peaks assignable to the 3HH monomer, 3HH−3HB dimer, and 3HH−3HH dimer. The result suggests that the P(3HB) depolymerase from *A. faecalis* is incapable of hydrolyzing the ester bond of 3HB−3HH in dimer and oligomers.

Conclusion

A. caviea produced a random copolymer of 3HB and 3HH units from alkanoates of even carbon numbers, while a copolymer of 3HB and 3HV units was produced from alkanoates of odd carbon numbers. The randomly distributed 3HH units in the P(3HB) sequence were excluded from the P(3HB) crystalline phase to reduce the degree of crystallinity of P(3HB-*co*-3HH) films, resulting in a remarkable increase in the rate of surface erosion on the films by a P(3HB) depolymerase from *A. faecalis*. The distributions of water-soluble products after the enzymatic degradation of P(3HB-*co*-3HH) films have suggested that the enzyme is incapable of hydrolyzing the ester bond of 3HB−3HH in dimer and oligomers.

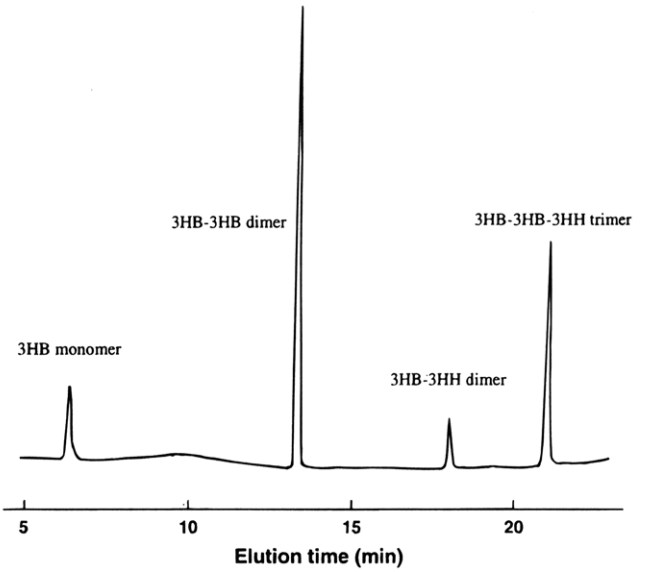

Figure 7. HPLC separation of water-soluble products after enzymatic degradation of P(3HB-*co*-15 mol % 3HH) film by P(3HB) depolymerase from *A. faecalis*.

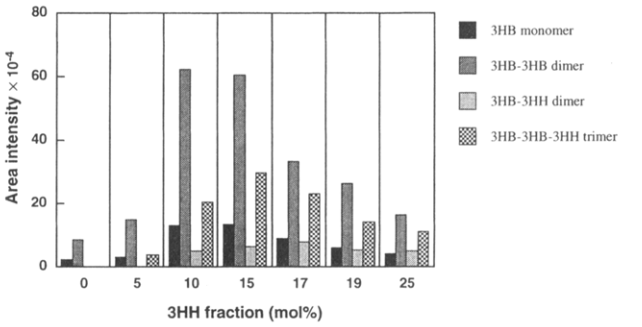

Figure 8. Distributions of water-soluble products after enzymatic degradation of P(3HB-*co*-3HH) films for 5 h at 37 °C.

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