Physical Properties and Biodegradability of Microbial Poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate)

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Introduction. An optically active copolymer of (*R*)-3-hydroxybutyric acid (3HB) and (*R*)-3-hydroxyvaleric acid (3HV) has recently been introduced by Imperial Chemical Industries (ICI) as a biodegradable thermoplastic.[1] The copolyester has been produced in a fermentation process using glucose and propionic acid as the carbon sources for *Alcaligenes eutrophus*.[2] The microbial copolyesters have a statistically random distribution of 3HB and 3HV units,[3-5] and they exhibit approximately the same high degree of crystallinity (>50%) throughout a wide range of compositions from 0 to 95 mol % 3HV, since P(3HB-*co*-3HV) copolymers crystallize in the same crystalline lattice as homopolymers of the main component.[4,6,7] The mechanical and physical properties varied with the copolymer composition.[6-12] The rate of enzymatic surface erosion of P(3HB-*co*-3HV) films by P(3HB) depolymerase was slower than the rate of P(3HB) film.[13,14]

Recently, Shiotani and Kobayashi[15] have found that *Aeromonas caviae* produces a copolymer of 3HB and 3-hydroxyhexanoate (3HH) from olive oil. In this paper we investigate the structure, physical properties, and enzymatic degradability of P(3HB-*co*-3HH) copolymers and compare the results with those of P(3HB-*co*-3HV). A terpolymer of 3HB, 3HV, and 3HH was first produced in *Rhodospillium rubrum*.[16]

Experimental Section. Biopolymer Synthesis. *A. caviae* 440 was isolated from soil and used in this study. Polyester synthesis was carried out by a single-stage cultivation of *A. caviae*. The microorganism was grown at 30 °C and pH 7.0 in 2.0 L of a mineral medium containing olive oil (20 g/L) as the carbon source under aerobic conditions in a 3-L jar fermenter. The mineral medium contained 5-20 g of yeast extract, 1.5 g of KH_2PO_4, 1.5 g of K_2HPO_4, 0.25 g of $MgSO_4 \cdot 7H_2O$, and 0.5 g of Tween-85 per liter of distilled water. The olive oil used as the carbon source was purchased from Wako Pure Chemical (Osaka, Japan). The cells were cultivated in the media for 24 h at 30 °C, harvested by centrifugation, and then lyophilized. Polyesters were extracted from the lyophilized cells with hot chloroform in a Soxhlet apparatus and purified by reprecipitation with methanol.

Analytical Procedures. The 125-MHz ^{13}C NMR spectra of polyesters were recorded on a JEOL GX-500 spectrometer at 27 °C in a $CDCl_3$ solution of polymer (25 mg/mL) with a 10-μs pulse width (45° pulse angle), 5-s pulse repetition, 25000-Hz spectra width, and 64K data points. Tetramethylsilane (Me_4Si) was used as an internal chemical shift standard.

Table 1. Molecular Weights and Thermal Properties of P(3HB-*co*-3HH) Samples

sample	composition,[a] mol % 3HB	3HH	mol wt[b] $10^{-3}\bar{M}_n$	$\bar{M}_w/\bar{M}_n$	thermal properties[c] T_g, °C	T_m, °C	ΔH_m, J/g
1	100	0	782	1.8	4	177	84
2	89	11	214	2.2	-1	136	60
3	83	17	662	1.8	-2	130	39

[a] Determined by ^1H NMR. [b] Determined by GPC. [c] Measured by DSC at 10 °C/min.

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 were carried out by using a Shimadzu DSC-50 thermal analysis system in the temperature range -100 to +200 °C at a heating rate of 10 °C/min. The melting temperature and enthalpy of fusion were determined from the DSC endotherms. The glass transition temperature was taken as the midpoint of the heat capacity change.

Wide-angle X-ray diffraction measurements of polyester samples were made on a Rigaku RAV-1VB system. Cu Kα radiation (λ = 0.1542 nm) was used as the source. The X-ray diffraction patterns of polyesters were recorded at 27 °C in the range 2θ = 10-50° 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 3 weeks at room temperature. The percentage of crystallinity was calculated from diffracted intensity data according to Vonk's method.[22]

Enzyme Degradation. The extracellular P(3HB) depolymerase was purified to electrophoretic homogeneity from *Alcaligenes faecalis* T_1.[17] The enzymatic degradation of polyester films by the extracellular P(3HB) depolymerase was carried out for 2 h at 37 °C in a 0.1 M phosphate buffer (pH 7.4). 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 10 μL of an aqueous solution of P(3HB) depolymerase (8 μg). 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.

Results and Discussion. The homopolymer sample 1 of 3-hydroxybutyrate (3HB) was produced by *A. eutrophus* from fructose.[18] The copolymer samples 2 and 3 of 3HB and 3-hydroxyhexanoate (3HH) were produced by *A. caviae* from olive oil at different concentrations (5 and 20 g/L) of yeast extract. The mole fractions of 3HB and 3HH units in samples 2 and 3 were determined from the 500-MHz ^1H NMR spectra and listed in Table 1. The melting temperature (T_m) of P(3HB-*co*-3HH) samples decreased from 177 to 130 °C as the 3HH fraction was increased from 0 to 17 mol %. The enthalpy of fusion (ΔH_m) and glass transition temperature (T_g) were also decreased with increasing the 3HH fraction, as given in Table 1.

Figure 1 shows the 125-MHz ^{13}C NMR spectrum of sample 3, together with the chemical shift assignment for each carbon resonance. The expanded spectrum of carbonyl carbon resonances is shown in Figure 2. The carbonyl resonances are resolved into four peaks, arising

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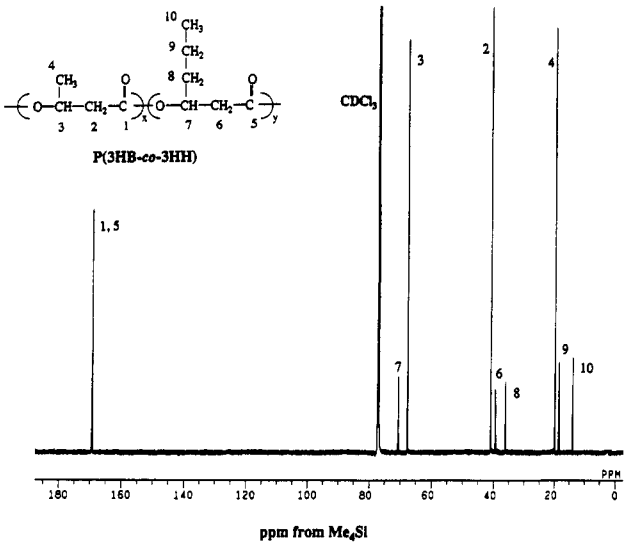

Figure 1. 125-MHz ^{13}C NMR spectrum of P(3HB-*co*-3HH) sample 3 in $CDCl_3$.

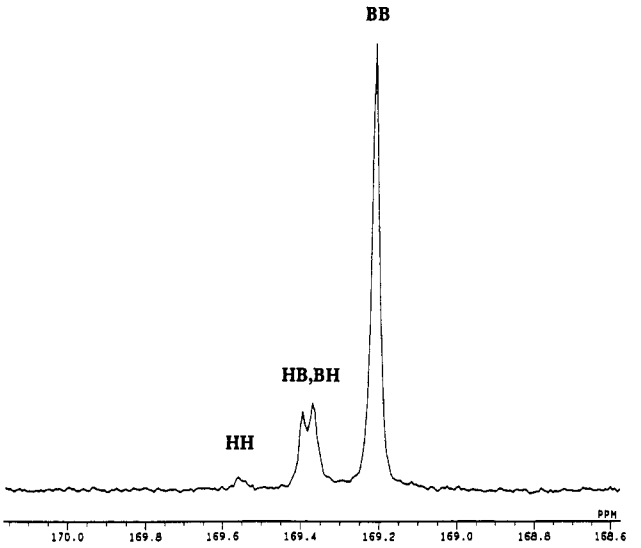

Figure 2. Expanded ^{13}C NMR spectrum for carbonyl resonances of sample 3.

Table 2. Chemical Shifts and Relative Intensities of Carbonyl Resonances in ^{13}C NMR Spectra of P(3HB-*co*-3HH) Samples 2 and 3

		sample 2		sample 3	
		relative intensities			
chemical shift, ppm	sequence	obsd	calcd[a]	obsd	calcd[a]
169.20	B*B	0.81	0.80	0.72	0.71
169.36	B*H	0.18	0.19	0.25	0.26
169.39	H*B				
169.55	H*H	0.1	0.1	0.3	0.3

[a] Calculated by Bernoullian statistics with the mole fraction of the 3HH unit in Table 1.

from the different diad sequences of the 3HB and 3HH units. The ^{13}C chemical shift assignment of four peaks in the carbonyl resonances are given in Table 2, together with the relative areas of the peaks. The diad sequence distribution data for two monomeric units were compared with Bernoullian statistics applicable to a statistically random copolymerization.[3-5] 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 2, the calculated diad fractions (F_{BB}, $F_{BH} + F_{HB}$, F_{HH}) are in good agreement with the observed values for samples 2 and 3. It has been concluded

Table 3. Crystallographic Parameters of X-ray Diffraction Spectra and Enzymatic Degradation Results of P(3HB-*co*-3HH) Samples

	crystallographic parameters				
sample	*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
2. P(3HB-*co*-11% 3HH)	0.569	1.310	0.592	40 ± 5	2.0 ± 0.3
3. P(3HB-*co*-17% 3HH)	0.573	1.314	0.592	29 ± 5	4.2 ± 0.4

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

that the sequence distributions of 3HB and 3HH units in samples 2 and 3 are statistically random.

The X-ray diffraction spectra recorded for solution-cast P(3HB-*co*-3HH) films containing 11 and 17 mol % 3HH showed the presence of only one crystalline phase of P(3HB). 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, $c = 0.596$ nm (fiber repeat).[19,20] Table 3 gives crystallographic parameters of P(3HB-*co*-3HH) samples, together with the degrees of X-ray crystallinity. The crystallographic parameters are little influenced by the presence of the 3HH unit, and the X-ray crystallinities decrease from 60 to 29% as the 3HH fraction is increased from 0 to 17 mol %. This result indicates that 3HH units cannot crystallize in the sequence of 3HB units and act as defects in the P(3HB) crystal lattice.

The enzymatic degradations of three polyester samples 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. The enzymatic degradation occurred on the surface of the film.[13] It was confirmed that no degradation occurred for 2 h at 37 °C in the absence of P(3HB) depolymerase. As given in Table 3, the weight of film erosion by P(3HB) depolymerase for 2 h increased from 0.3 to 4.2 mg as the 3HH fraction was increased from 0 to 17 mol %. Thus the rate of enzymatic degradation of the P(3HB-*co*-17% 3HH) film was 14 times higher than the rate of the P(3HB) film. This result is in contrast to the result of P(3HB-*co*-3HV)[13,14] that the rates of enzymatic degradation on P(3HB-*co*-3HV) films were slower than the rate of the P(3HB) film.

In a previous paper,[21] we reported that the rate of surface erosion of the P(3HB) film by P(3HB) depolymerase was markedly decreased with an increase in 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. It may be concluded that a rapid erosion of the P(3HB-*co*-3HH) film by P(3HB) depolymerase is due to a decrease in crystallinity.

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