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Microbial Synthesis and Characterization of Poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate)

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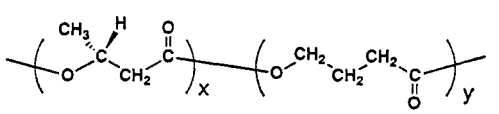
ABSTRACT: Copolyesters of 3-hydroxybutyrate (3HB) and 4-hydroxybutyrate (4HB) with a wide range of compositions from 0 to 100 mol % 4HB were microbially synthesized at 30 °C by *Alcaligenes eutrophus* from 4-hydroxybutyric acid in the presence of some additives. When 4-hydroxybutyric acid, citrate, and ammonium sulfate were fed as mixed substrates in the aqueous culture of *A. eutrophus*, P(3HB-*co*-4HB) copolymers with compositions of 70–100 mol % 4HB were produced. The structure and physical properties of P(3HB-*co*-4HB) copolymers were characterized by ^1H and ^{13}C NMR spectroscopy, X-ray diffraction, gel-permeation chromatography, differential scanning calorimetry, tensile test, and dynamic mechanical spectroscopy. In addition, the effects of 4HB units on the rate of enzymatic degradation of P(3HB-*co*-4HB) films were studied in the aqueous solution of an extracellular P(3HB) depolymerase from *A. faecalis*.

Introduction

A wide variety of microorganisms synthesize an optically active polymer of (*R*)-3-hydroxybutyric acid (3HB) and accumulate it as an intracellular storage material of carbon and energy.[1-3] *Alcaligenes eutrophus* used in this study accumulates P(3HB) in the cells in amounts up to 70% of the dry weight, when growth is limited by the depletion of an essential nutrient but the cells have an excess of carbon.[4] Recently, *A. eutrophus* has been found to synthesize various copolymers containing several hydroxyalkanoate monomeric units such as (*R*)-3-hydroxyvalerate (3HV),[5-7] 4-hydroxybutyrate (4HB),[8-11] 5-hydroxyvalerate (5HV),[12] and 3-hydroxypropionate (3HP)[13] other than 3HB, when different carbon substrates are fed as the carbon sources. These microbial polyesters are thermoplastics with biodegradable properties,[2,3] and the physical properties can be regulated by varying the compositions of the copolymers.[14-22]

In previous papers,[8-11] we reported that the copolyesters of (*R*)-3-hydroxybutyrate and 4-hydroxybutyrate, P(3HB-*co*-4HB), were produced by *A. eutrophus* in nitrogen-free culture solutions containing 4-hydroxybutyric acid, 1,4-butanediol, or γ-butyrolactone. The copolymer compositions varied from 0 to 49 mol % 4HB, depending on the carbon source supplied. The copolymers have been shown to have a statistically random distribution of 3HB and 4HB units.[23,24] The thermal, mechanical, and biodegradable properties of P(3HB-*co*-4HB) copolymers with compositions ranging from 0 to 49 mol % 4HB have been reported.[19-22]

In this paper we report that the copolyesters with a wide range of compositions (0–100 mol % 4HB) are produced by *A. eutrophus* from 4-hydroxybutyric acid in the presence of additives such as citrate and ammonium sulfate. The physical and biodegradable properties of P(3HB-*co*-4HB) copolymers with 85–100 mol % of 4HB units are reported.



(R) - 3HB 4HB

Experimental Section

Biopolymer Synthesis. *A. eutrophus* H16 (ATCC 17699) was used in this study. Polyester synthesis was carried out by a two-step cultivation of *A. eutrophus*. *A. eutrophus* cells were first grown under aerobic conditions at 30 °C in a nutrient-rich medium (100 mL) containing 1 g of yeast extract, 1 g of polypeptone, 0.5 g of meat extract, and 0.5 g of $(NH_4)_2SO_4$. The cells were harvested by centrifugation after 24 h. Under these culture conditions accumulation of polyester in the cells was not observed. To promote polyester synthesis, about 0.4-g (dry weight) quantities of the centrifuged cells were transferred into nitrogen-free mineral media[25] (100 mL, pH 7.5) containing 4-hydroxybutyric acid as carbon source. When required, prescribed amounts of potassium dihydrogen citrate (1K-citrate), butyric acid, and $(NH_4)_2SO_4$ were added to the media. The cells were cultivated

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Table I
Production of P(3HB-*co*-4HB) from 4-Hydroxybutyric Acid in the Presence of Additives Such as Citrate and $(NH_4)_2SO_4$ by *Alcaligenes eutrophus* at 30 °C

run	substrate, g/L			incub time, h	cell dry wt, g/L	polyester content,[a] wt %	polyester comp,[b] mol %		mol wt[c]	
	$HOCH_2CH_2CH_2COOH$	1K-citrate	$(NH_4)_2SO_4$				3HB	4HB	$10^{-3}\bar{M}_n$	$\bar{M}_w/\bar{M}_n$
1	20	0	0	48	5.8	16	66	34	392	2.5
2	20	0	0	72	6.7	27	73	27	544	2.1
3	20	0	0	96	7.0	28	76	24	375	2.6
4	20	0	0	120	6.0	20	74	26	175	4.2
5	16	0	0	48	4.5	19	73	27		
6	20	0	2	24	3.8	1	42	58	60	2.9
7	20	0	2	48	4.5	7	38	62	443	2.4
8	20	0	2	72	5.6	10	50	50	357	2.8
9	20	0	2	96	5.1	9	48	52	155	4.5
10	20	0	3	48	4.3	7	37	63	362	4.7
11	0	15	0	48	3.7	0				
12	15	10	0	48	4.6	19	53	47	121	2.2
13	15	15	0	48	5.2	16	64	36	360	2.8
14	15	5	1	48	4.3	8	30	70	510	2.0
15	15	15	1	48	4.6	1	0	100		
16	15	15	2	48	5.5	2	10	90	231	2.1
17	15	15	4	48	5.9	1	14	86	388	2.1
18	20	5	2	48	3.0	6	17	83	273	2.8
19	20	10	2	48	4.3	2	0	100	274	2.8
20	20	15	2	24	3.5	2	10	90	315	3.8
21	20	15	2	48	3.5	4	12	88	211	5.3
22	20	15	2	72	3.5	5	6	94	315	3.0
23	20	15	2	96	4.5	5	9	91	442	2.5
24	25	15	2	48	5.2	2	0	100	93	2.4
25	30	15	2	48	3.2	1	8	92		

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

in these media for 24–120 h at 30 °C, 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 NMR analysis of polyester samples was carried out on a JEOL FX-100 spectrometer. The 100-MHz ^1H NMR spectra were recorded at 27 °C in a $CDCl_3$ solution of polyester (5 mg/mL) with a 15-μs pulse with (45° pulse angle), 5-s pulse repetition, 1000-Hz spectral width, 8K data points, and 200 accumulations. The ^{13}C NMR analysis of polyester samples was performed on a JEOL GX-500 spectrometer. The 125-MHz ^{13}C NMR spectra were recorded at 27 °C in a $CDCl_3$ solution of polyester (25 mg/mL) with a 10-μs pulse width (45° pulse angle), 5-s pulse repetition, 25 000-Hz spectral width, 64K data points, and 15 000 accumulations.

All molecular weight data were obtained at 40 °C by using a Shimadzu 6A GPC system and a 6A refractive index detector with a Shodex 80 M 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.

The glass transition and melting temperatures of polyesters were recorded on a Shimadzu DSC-50 equipped with a cooling accessory under a nitrogen flow of 30 mL/min. Polyester samples of 3 mg were encapsulated in aluminum pans and heated at 10 °C/min from 0 to 200 °C. The melting temperature and enthalpy of fusion 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 at −100 °C. They were heated from −100 to +200 °C at 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-1VB system. Cu Kα radiation (λ = 0.1542 nm) was used as the source. The X-ray diffraction patterns of polyester samples were recorded at 27 °C in the range 2θ = 6–40° at a scan speed of 1–3°/min. X-ray crystallinities were measured for the polyester films that had been cast from chloroform solution and allowed to stand for 2 weeks at room temperature. Crystallization kinetics of the P(3HB-*co*-94% 4HB) sample were measured for the melt-quenched sample that had been heated for 5 min at 100 °C and immediately quenched at room temperature. Crystallization was allowed to proceed at room temperature (23 °C). Time zero was approximated as the time at which the sample was quenched. The percentage of crystallinity was calculated from diffracted intensity data according to Vonk's method.[26]

The stress–strain curves of cast films of polyester samples were obtained at 23 °C with a strain rate of 20 mm/min on a Imada SV-50 tensile machine. The mechanical tensile data were calculated from such curves on an average of three specimens.

Dynamic mechanical measurements were performed with a dynamic mechanical thermal analyzer (Polymer Laboratories Ltd.) operated in the tensile mode at a frequency of 3 Hz and a heating rate of 3 °C/min. Strips cut from solvent-cast films (thickness 0.08 mm) were investigated in the temperature range −150 to +30 °C.

Enzymatic Degradation. The extracellular P(3HB) depolymerase was purified to electrophoretic homogeneity from *A. faecalis* T_1 as described in a previous paper.[27] 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, 7 mg; initial film dimensions, 10 × 10 × 0.06 mm) were placed in small bottles containing 1.0 mL of buffer. The films were prepared by conventional solvent-casting techniques from chloroform solutions of the polyesters. The reaction was started by the addition of 5 μL of an aqueous solution of P(3HB) depolymerase (3 μg). The reaction solution was incubated at 37 °C with shaking. The films were periodically removed, washed with water, and dried to constant weight in vacuo before analysis.

Results and Discussion

Microbial Synthesis of P(3HB-*co*-4HB). Table I lists the results of P(3HB-*co*-4HB) production by *A. eutrophus* from 4-hydroxybutyric acid in the presence of additives such as potassium dihydrogen citrate (1K-citrate) and ammonium sulfate for 24–120 h at 30 °C. The productivity and composition of the copolyesters varied depending on the combination and concentration of substrates supplied. When 4-hydroxybutyric acid was added as the sole carbon source in the nitrogen-free culture solution (runs 1–5), the polyester content in dried cells increased to 28 wt % during incubation, while the 4HB fractions in copolyesters were as low as 30 ± 5 mol % during the course of incubation from 48 to 120 h. When

Table II
Production of P(3HB-co-4HB) from 4-Hydroxybutyric Acid and Butyric Acid in the Presence of Citrate (15 g/L) and $(NH_4)_2SO_4$ (2 g/L) at 30 °C

run	carbon source, g/L		incub time, h	cell dry wt, g/L	polyester content, wt %	polyester comp, mol %		mol wt	
	$HOCH_2CH_2CH_2COOH$	$CH_3CH_2CH_2COOH$				3HB	4HB	$10^{-3}\bar{M}_n$	$\bar{M}_w/\bar{M}_n$
26	15	5	24	7.0	33	77	23	437	2.4
27	15	5	48	6.7	32	55	45	484	2.3
28	15	5	72	6.3	27	30	70	396	2.5
29	15	5	96	5.4	26	25	75	367	2.4
30	20	2	24	4.7	20	70	30	436	2.4
31	20	2	48	4.6	19	49	51	417	2.6
32	20	2	72	5.3	22	32	68	497	2.3
33	20	2	96	4.8	16	14	86	371	2.7

Table III
Compositions, Molecular Weights, and Properties of P(3HB-co-4HB) Samples

compn,[a] mol %		mol wt[b]		thermal properties[c]			
3HB	4HB	$10^{-3}\bar{M}_n$	$\bar{M}_w/\bar{M}_n$	T_m, °C	ΔH_m, cal/g	T_g, °C	crystallinity,[d] %
100	0	768	1.9	177	20.8	4	59 ± 5
94	6	494	2.1	162	13.5	-1	56 ± 5
90	10	395	3.0	159	13.0	-3	46 ± 5
72	28	252	2.6			-15	23 ● 5
15	85	168	3.7	48	8.6	-41	29 ± 5
10	90	327	2.1	50	10.2	-44	
6	94	315	3.0	51	11.0	-46	42 ± 5
0	100	93	2.4	54	11.0	-50	

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

ammonium sulfate of 2–3 g/L was added to the nitrogen-free culture solution of 4-hydroxybutyric acid (runs 6–10), the productivity of P(3HB-co-4HB) copolymers was markedly suppressed, and a maximum content of polyesters in dried cells was 10 wt % at 72 h. However, the 4HB repeating units in the copolyesters increased by the addition of ammonium sulfate and became as high as 56 ± 6 mol %, independent of incubation period. Further addition of citrate of 5–15 g/L to the culture solution containing 4-hydroxybutyric acid and ammonium sulfate resulted in the formation of P(3HB-co-4HB) copolymers with extremely high 4HB compositions of 80–100 mol % (runs 14–25), though the polyester contents were further decreased. It is noted that no polyester was produced by *A. eutrophus* from citrate alone, which is an intermediary metabolite in the tricarboxylic acid (TCA) cycle (run 11).

The sequence distribution of monomeric units in the P(3HB-co-91% 4HB) copolymer obtained in run 23 was studied by analysis of the 125-MHz ^{13}C NMR spectrum. The carbonyl resonances (δ 169–173) were clearly resolved into four peaks, arising from the different diad sequences of the 3HB and 4HB units.[23] The diad sequence distribution in the copolymer was F_{33} 0.03, F_{34} 0.07, F_{43} 0.06, and F_{44} 0.84, which indicates that the copolymer has a statistically random distribution of 3HB and 4HB units.

In a previous paper,[10] we proposed a pathway of P(3HB-co-4HB) biosynthesis in *A. eutrophus* from 4-hydroxybutyric acid. (4-Hydroxybutyryl)-coenzyme A (CoA) is first formed from 4-hydroxybutyric acid in the cells. However, a portion of (4-hydroxybutyryl)-CoA is then metabolized into [(*R*)-3-hydroxybutyryl]-CoA from intermediates of the β-oxidation of 4-hydroxybutyric acid.[10] A random copolyester of 3HB and 4HB units is synthesized by the copolymerization of [(*R*)-3-hydroxybutyryl]-CoA with (4-hydroxybutyryl)-CoA under the action of PHA polymerase. When $(NH_4)_2SO_4$ and citrate are added to *A. eutrophus*, (acetoacetyl)-CoA from (4-hydroxybutyryl)-CoA is metabolized into acetyl-CoA rather than into [(*R*)-3-hydroxybutyryl]-CoA under growth conditions, resulting in an increase in the 4HB fraction and in a decrease in the polyester content in the cells.

To promote the formation of P(3HB-co-4HB) copolymer with a high 4HB fraction, butyric acid of 2–5 g/L was added into the culture solution containing 4-hydroxybutyric acid, citrate, and ammonium sulfate. The result is given in Table II. The polyester content in dried cells was as high as 30 ± 5 wt % in runs 26–29 and 19 ± 3 wt % in runs 30–33, independent of incubation period from 24 to 96 h. The 4HB fraction in the copolyester increased with time to about 80 mol % after 96 h. Thus, the copolyesters with high 4HB fractions were produced in *A. eutrophus* cells in a relatively high yield when 4-hydroxybutyric and butyric acids were used as carbon sources in the presence of citrate and ammonium sulfate.

Butyric acid is well-known to be metabolized to (acetoacetyl)-CoA in the β-oxidation cycle. An intermediate produced in the β-oxidation cycle from butyric acid in cells may inhibit the transformation of (4-hydroxybutyryl)-CoA into (acetoacetyl)-CoA, resulting in an effective formation of P(3HB-co-4HB) copolymer with a high 4HB fraction.

Physical and Thermal Properties of P(3HB-co-4HB). In previous papers,[19,21,22] the physical and thermal properties were reported for the P(3HB-co-4HB) copolymers with compositions ranging from 0 to 49 mol % 4HB. Here, we report the properties of P(3HB-co-4HB) copolymers with compositions of 85–100 mol % 4HB.

Table III summarizes compositions, molecular weights, thermal properties, and X-ray crystallinities of P(3HB-co-4HB) samples used in this study. The thermal properties of P(4HB) homopolymer were characterized as the glass transition temperature (T_g) of −50 °C, the crystallization temperature (T_c) of −15 °C, the melting temperature (T_m) of 54 °C, and the enthalpy of fusion (ΔH_m) of 11.0 cal/g. The T_g value of P(3HB-co-4HB) decreased from +4 to −50 °C as the 4HB fraction increased from 0 to 100 mol %.

Figure 1 shows the X-ray diffraction patterns of P(3HB-co-4HB) films varying from 0 to 94 mol % 4HB. The X-ray crystallinity of P(3HB) homopolymer was 59%, and the crystallinity of P(3HB-co-94% 4HB) was 42%. A low crystallinity of 23–29% was observed for P(3HB-co-28%

Table IV
Changes in Molecular Weights of the P(3HB-*co*-94% 4HB) Sample during Thermal Degradation at Different Temperatures

degrad temp, °C	$10^{-3}\bar{M}_n$ ($\bar{M}_w/\bar{M}_n$) 1 min	2 min	5 min	10 min	20 min
100	268 (2.8)	244 (2.9)	261 (2.8)	294 (2.5)	259 (2.6)
150	242 (2.4)	223 (2.5)	210 (2.4)	184 (2.3)	152 (2.2)
180	181 (2.3)	167 (2.2)	131 (2.2)	98 (2.1)	

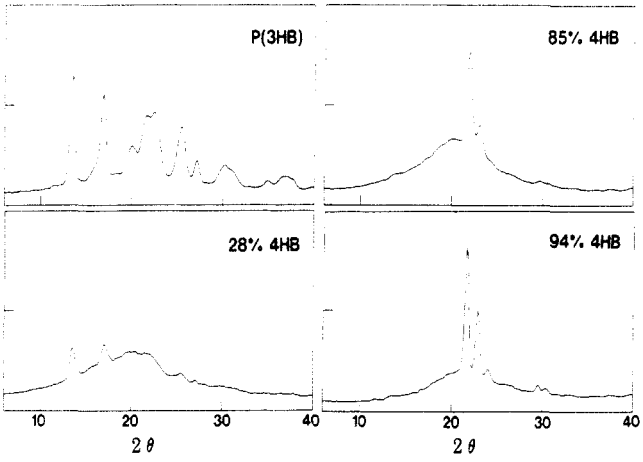

Figure 1. X-ray diffraction patterns of P(3HB) and P(3HB-*co*-4HB) films cast from $CHCl_3$ solution. Films had been aged for 2 weeks at room temperature after evaporation of the solvent.

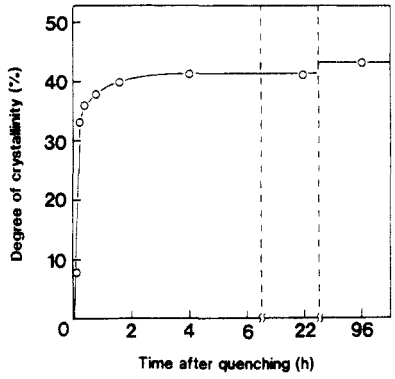

Figure 2. Time dependence of X-ray crystallinities for melt-quenched sample of P(3HB-*co*-94% 4HB). The deviations in crystallinity are ±5%.

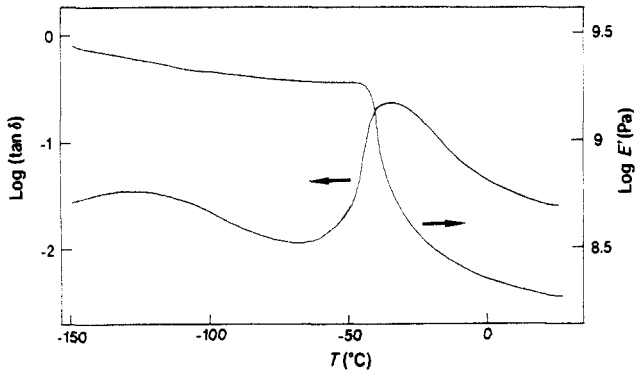

Figure 3. Dynamic mechanical spectrum of P(3HB-*co*-90% 4HB) film.

4HB) or P(3HB-*co*-85% 4HB).

The rate of crystallization was measured by X-ray diffraction for the sample of P(3HB-*co*-94% 4HB) quenched at 23 °C from the melt. Figure 2 shows the time dependence of crystallinity of the melt-quenched sample. The crystallization is almost complete within 0.5 h. The rate of crystallization of P(3HB-*co*-94% 4HB) was almost identical with that of P(3HB) homopolymer.[19]

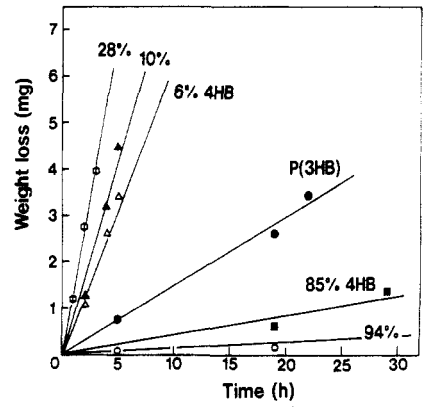

Figure 4. Enzymatic degradation (erosion) profiles of P(3HB-*co*-4HB) films in an aqueous solution of P(3HB) depolymerase at 37 °C and pH 7.5: (●) P(3HB); (△) P(3HB-*co*-6% 4HB); (▲) P(3HB-*co*-10% 4HB); (○) P(3HB-*co*-28% 4HB); (■) P(3HB-*co*-85% 4HB); (○) P(3HB-*co*-94% 4HB).

To evaluate the thermal stability of P(3HB-*co*-94% 4HB), a thermal degradation study was carried out for 20 min at temperatures above the T_m (51 °C). Table IV lists the changes in the number-average molecular weights ($\bar{M}_n$) and polydispersities ($\bar{M}_w/\bar{M}_n$) of P(3HB-*co*-94% 4HB) during thermal degradation in the temperature range 100–180 °C under nitrogen. At 100 °C, the $\bar{M}_n$ values were almost unchanged for 20 min. At temperatures above 150 °C, the $\bar{M}_n$ values decreased with time, but the polydispersities remained relatively narrow. There was no appreciable weight loss during the thermal degradation. The time-dependent changes in $\bar{P}_n$ values were found to be represented by the equation

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

$\bar{P}_{n,0}$ and $\bar{P}_{n,t}$ are the number-average degrees of polymerization at time 0 and t, respectively, and k_d is the rate constant of thermal degradation. The values of k_d were 1.1×10^{-5} min^{-1} at 150 °C and 4.5×10^{-5} min^{-1} at 180 °C. This result indicates that the thermal degradation of P(3HB-*co*-94% 4HB) follows the kinetic model of random chain scission at the ester groups.[21]

The stress–strain curves of P(3HB-*co*-94% 4HB) film were obtained at 23 °C on a tensile testing machine. The Young's modulus, tensile strength, and elongation to break were 55 MPa, 39 MPa, and 500%, respectively.

In addition, the dynamic mechanical measurement was carried out on the P(3HB-*co*-90% 4HB) film which had been treated for 30 min at 30 °C under a dry nitrogen flow to exclude humidity. Figure 3 shows the loss factor tan δ and dynamic storage modulus E' as a function of temperature. Two relaxation processes are apparent in the viscoelastic spectrum. A very intense tan δ peak (main relaxation) at about −40 °C arises from the glass-to-rubber transition, and a weak tan δ peak (secondary relaxation) at about −120 °C may arise from local motions of the methylene sequence in the 4HB unit.[22]

Biodegradable Properties of P(3HB-*co*-4HB). The enzymatic degradations of P(3HB-*co*-4HB) films were carried out at 37 °C in the 0.1 M phosphate buffer of the extracellular P(3HB) depolymerase purified from *A. faeca-*

lis. In this study six P(3HB-co-4HB) samples containing 0, 6, 10, 28, 85, and 94 mol % of 4HB fractions were used. Figure 4 shows the weight loss (erosion) profiles of the sample films as a function of time. The weight of film erosion increased proportionally to time for all the samples. The rate of film erosion by P(3HB) depolymerase was strongly dependent upon the copolymer composition. The rate of enzymatic degradation remarkably increased with an increase in the 4HB fraction, and the highest rate was observed at 28 mol % 4HB. Thus, the presence of 4HB units of 6–28 mol % in the P(3HB) sequence accelerated the enzymatic degradation. However, the rates of enzymatic degradation on the films of P(3HB-co-85% 4HB) and P(3HB-co-94% 4HB) were much slower than that of the P(3HB) film.

In a previous paper,[28] we reported that the rate of enzymatic degradation of P(3HB) film increased with a decrease in the crystallinity and that the P(3HB) depolymerase first hydrolyzed the P(3HB) chains in the amorphous state on the surface of the film. The acceleration of enzymatic degradation for the P(3HB-co-4HB) films with compositions of 6-28 mol % 4HB may be caused by the decrease in crystallinity. Since P(3HB) depolymerase is hardly susceptible to the sequence of 4HB units, the presence of 4HB units over 85 mol % in the copolyester suppresses the enzymatic degradation.

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Registry No. 3HB/4HB (copolymer), 117068-64-1; 3HB (homopolymer), 26063-00-3; 4HB (homopolymer), 114959-05-6; 3HB (homopolymer)(SRU), 26744-04-7; 4HB (homopolymer)(SRU), 28728-97-4; P(3HB) depolymerase, 9014-11-3.