

Poly(3-hydroxybutyrate-co-4-hydroxybutyrate) produced by *Comamonas acidovorans*

H. Mitomo^{a,*}, W.-C. Hsieh^a, K. Nishiwaki^a, K. Kasuya^a, Y. Doi^b

^aDepartment of Biological and Chemical Engineering, Faculty of Engineering, Gunma University, Kiryu, Gunma 376-8515, Japan

^bPolymer Chemistry Laboratory, The Institute of Physical and Chemical Research (RIKEN), Hirosawa, Wako-shi, Saitama 351-0198, Japan

Received 13 July 2000; received in revised form 16 August 2000; accepted 4 September 2000

Abstract

Poly(3-hydroxybutyrate-co-4-hydroxybutyrate) [P(3HB-co-4HB)] was biosynthesized by *Comamonas acidovorans* with mixed carbon sources of *n*-butyric acid and 1,4-butanediol. P(3HB) and P(3HB-co-94 mol% 4HB) were obtained by fermentation with sole carbon source of *n*-butyric acid and 1,4-butanediol, respectively. The content of 4HB component in P(3HB-co-4HB) increased as the composition of 1,4-butanediol in mixed carbon sources increased. These biosynthesized copolymers are not homogeneous, but mixtures of copolymers having different 4HB contents. Fractionation was carried out to obtain fractionated copolymers having a narrower distribution of composition. The change in structural and physical properties of the fractionated copolymers were investigated against the 4HB contents. Crystallinities of P(3HB-co-4HB)s were estimated using density values and the heat of fusion of P(3HB) measured was 125 J g⁻¹ and that of P(4HB) was 76 J g⁻¹. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Poly(3-hydroxybutyrate-co-4-hydroxybutyrate); *Comamonas acidovorans*; Biodegradability

1. Introduction

Poly(3-hydroxybutyrate) [P(3HB)] and its copolymer poly(3-hydroxybutyrate-co-4-hydroxybutyrate) [P(3HB-co-4HB)] are biodegradable thermoplastic polyesters produced by the bacterium *Ralstonia eutropha* or *Alcaligenes latus*. P(3HB-co-4HB) was produced by *R. eutropha* in nitrogen-limited cultures containing 4-hydroxybutyric acid [1], γ -butyrolactone [2] or 1,4-butanediol [3] as the carbon source, whose composition was varied from 0 to 34 mol% 4HB, depending on the composition of carbon substrates supplied in feed. Recently, random copolymers P(3HB-co-4HB) with a wide range of compositions varying from 0 to 83 mol% 4HB were produced by *A. latus* from the mixed carbon substrates of 3-hydroxybutyric and 4-hydroxybutyric acid [4], though these were expensive. The biosynthesis of P(3HB-co-4HB) was also studied by a two-step fermentation of *Comamonas acidovorans* DS-17, *R. eutropha* and *A. latus* from various carbon sources by Saito et al. [5,6]. They reported thermal and physical properties with composition varying from 0 to 100 mol% 4HB.

In a previous paper [7], biosynthesis of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) [P(3HB-co-3HV)] by

Burkholderia cepacia D1 was reported. The production of P(3HB-co-4HB) and terpolyesters by *C. acidovorans* in nitrogen-free solutions of 1,4-butanediol and pentanol was also reported [8]. In the present study, production of P(3HB-co-4HB) by *C. acidovorans* from a mixed carbon sources of *n*-butyric acid and 1,4-butanediol, which are relatively cheaper, is reported and the optimum biosynthesis condition is found experimentally. These polyesters are not homogeneous, but mixtures of several random copolymers having a wide variety of compositions. Fractionation was carried out to obtain the fractionated copolymers having a narrower distribution of composition in the same way as reported in Refs. [9,10]. The variations in molecular weight, melting points, crystallinity and enzymatic degradability of P(3HB-co-4HB) copolymers were investigated against a wide range of compositions. The values of heat of fusion of P(3HB) and P(4HB) crystals were obtained newly from those of enthalpy of melting and crystallinity estimated from the density.

2. Experimental

2.1. Biopolymer synthesis

The strain used in this study was *C. acidovorans* (IFO

* Corresponding author. Tel.: +81-277-30-1494; fax: +81-277-30-1401.
E-mail address: mitomo@bce.gunma-u.ac.jp (H. Mitomo).

13582). P(3HB-*co*-4HB) synthesis was carried out by a two-step cultivation of *C. acidovorans*. The microorganism was first grown under aeration at 26°C in a nutrient-rich medium (1 l) containing 10 g peptone, 5 g meat extract and 5 g (NH₄)₂SO₄. After the cultivation time of 48 h, the cells were harvested by centrifugation. Under these culture conditions, accumulation of polyester in cells was not observed. In the second step to biosynthesize polyester, about 2.5 g (dry cell weight) of microorganism multiplied at the first-stage was transferred into nitrogen-free mineral media (pH 7.0) [5] containing butyric acid and 1,4-butanediol as the carbon sources. The cells were incubated in these media at 26°C for 72 h, harvested by centrifugation, washed with water and finally dried. Polyester was extracted from the dried cells with hot chloroform and purified by precipitation with *n*-heptane.

2.2. Fractionation

The copolymer sample (0.3 g) was dissolved with hot acetone (30 ml) and kept at 8°C for 20 h followed by centrifugation to isolate the precipitate [10]. In the next step, the solution was diluted with distilled water to a concentration of 95 volume % acetone and dissolved completely by warming and then kept at 8°C for 20 h followed by isolation. The same procedure was repeated for samples that are 5% lower in concentration.

2.3. Analytical procedures

The thermal data were recorded on a Perkin–Elmer DSC-7 differential scanning calorimeter (DSC) at a heating rate of 10°C min⁻¹ under nitrogen flow. Melting peak temperature after being corrected for heating rate dependence was defined as the melting point T_m with $\pm 0.1^\circ\text{C}$ accuracy. The temperature scale was calibrated with high-purity standards.

The compositions and sequence distributions of P(3HB-*co*-4HB) copolymers were determined by analysis of ¹H- and ¹³C NMR spectra [11], which were recorded on a JEOL GSX-270 spectrometer. The ¹H NMR spectra were recorded at 80°C for a CDCl₃ solution of P(3HB-*co*-4HB) (10 mg ml⁻¹) with 4.7 μs pulse width, 5 s pulse repetition, 4000 Hz spectral width, 16K data points and 32 accumulations. The 67.5 MHz ¹³C NMR spectra were recorded at 80°C for a CDCl₃ solution of P(3HB-*co*-4HB) (30 mg ml⁻¹) with 4.5 μs pulse width, 3 s pulse repetition, 15000 Hz spectral width, 128K data points and 20000 accumulations. Tetramethylsilane (SiMe₄, $\delta = 0$) was used as an internal chemical shift standard.

The gel permeation chromatography (GPC) was carried out with an HLC-802A high-performance liquid chromatograph (Tosoh Co., Ltd.) at 38°C equipped with a series of four columns of TSK-gel and an RI-8 differential refractometer. Chloroform was used as the eluent at a flow rate of 1.0 ml min⁻¹ and a sample solution of 1 ml with concentration of 1.0 g l⁻¹ was used. Polystyrene standards with the

narrow polydispersity were used to make a calibration curve, and apparent molecular weights were calculated.

The density was measured at 25°C for 24 h using a density gradient column containing mixtures of toluene and butylbromide as the column liquid. The fractionated samples used were films once molten at 185°C and kept at room temperature for several weeks so as to crystallize fully.

The amorphous samples were obtained by melting at 185°C for 2 min and then quenching into liquid nitrogen. The density of amorphous sample was measured at 25°C by flotation in an aqueous KBr solution [10,12]. Once the quenched sample warms up to room temperature, it begins to crystallize easily. Therefore, all measurements of amorphous density were made within 1 min to prevent the onset of crystallization.

2.4. Enzymatic degradation

PHB depolymerase purified from *Ralstonia pickettii* T1 [13] and lipase from *Rhizopus delmer* [14] were used in this study. Enzymatic degradation of P(3HB-*co*-4HB) film was carried out at 37°C in a 0.1 M phosphate buffer (pH 7.4). P(3HB-*co*-4HB) films (initial weight, ca. 10 mg; film dimensions, 10 × 10 mm; film thickness, 0.09 mm) were placed in small test tubes containing 1.0 ml of the buffer. The reactions were started by the addition of an aqueous solution of PHB depolymerase (1.5 μg) or lipase (285 μg). The reaction solutions were incubated with shaking. The film was periodically removed, washed with water, and dried completely. Then weight loss was calculated as the percentage of weight decrease to the original film weight.

3. Results and Discussion

3.1. Microbial synthesis of P(3HB-*co*-4HB)

Table 1 shows the results of production of P(3HB-*co*-4HB) copolymer from *n*-butyric acid and 1,4-butanediol by *C. acidovorans* in the two-step fermentation at 26°C for 72 h. Dry cell weight harvested after the second-step fermentation was hardly increased, i.e. 2.4–2.8 g from about 2.5 g at the first stage. The polymer content was lower than that of *R. eutropha* [1] or *A. latus* [2]. The polymer content seems almost unchanged and independent of the composition of carbon sources (around 13%).

Fig. 1 shows the plot of the composition of 4HB component determined by ¹H NMR of P(3HB-*co*-4HB) against the content of 1,4-butanediol in feed. The 4HB contents linearly increased from 0 to 94% as the content of 1,4-butanediol increased from 0 to 100%, in other words, the 4HB content in copolymer was closely identical to the content of 1,4-butanediol in carbon source feed. A similar result was reported for P(3HB-*co*-4HB) obtained from the mixed carbon sources of 3-hydroxybutyrate and 4-hydroxybutyrate by *A. latus* [15]. In a previous paper [8], P(3HB-*co*-96 mol%

Table 1
Production of P(3HB-co-4HB) from butyric acid and 1,4-butanediol by *Comamonas acidovorans*

Sample no.	Carbon sources (g l ⁻¹)		Dry cell weight (g l ⁻¹)	Polyester content (wt %)	Composition (mol%)	
	Butyric acid	1,4-Butanediol			3HB	4HB
1	10	0	2.6	14	100	0
2	8	2	2.4	8	81	19
3	6	4	2.7	15	62	38
4	4	6	2.5	13	35	65
5	2	8	2.8	11	26	74
6	0	10	2.5	13	6	94

4HB) was obtained from the sole carbon source of 1,4-butanediol by *C. acidovorans* (pH 7.1) for 72 h and P(3HB-co-99 mol% 4HB) was obtained at longer incubation time (96 h).

The fermentation conditions to obtain the highest polymer content at the two-step fermentation by *C. acidovorans* were examined by changing the concentration of carbon source, pH and incubation temperature or time (data are not shown). The result of the most optimum condition decided experimentally was as follows:

pH	7.0
Incubation temperature	26°C
Incubation time	72 h
Concentration of carbon source	10 g l ⁻¹

where the highest polymer content was around 15% per dry cell weight.

Table 2 lists molecular weights M_n and thermal properties of P(3HB-co-4HB) samples measured by GPC and DSC measurements, respectively. Their M_n values ranged from 4×10^4 to 9×10^4 which were slightly lower than those produced by other bacteria [5] and M_w/M_n values were

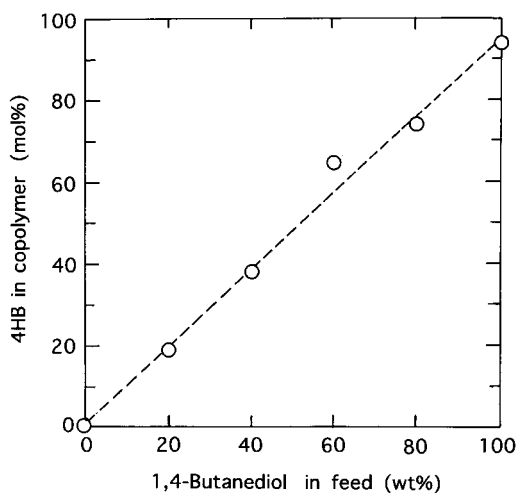


Fig. 1. Relation between compositions of the 4HB component in copolymer and 1,4-butanediol in carbon sources.

around 3.0. Enthalpy of melting, ΔH_m , steeply decreased from 90 to 6 J g⁻¹ as the 4HB content increased from 0 to 38 mol%, then increased to 11 J g⁻¹ at 65 mol% 4HB, and increased more up to 39 J g⁻¹ at 94 mol% 4HB. This suggests that crystallinity of the copolymer is minimum at the composition range of 40–50 mol% 4HB.

Fig. 2 shows DSC thermograms of these copolymer samples. Two series of melting points appeared, the higher ones at 166–158°C and the lower ones at 61–44°C, implying that most of the copolymers were mixtures of at least two copolymers having lower and higher 4HB contents, respectively. In addition, small quantity of other copolymers having intermediate T_m (or having intermediate compositions) were contained in the most of copolymers. To obtain the component copolymers, fractionation was carried out for these samples.

3.2. Characterization of the fractionated P(3HB-co-4HB) samples

Table 3 lists the results of fractionation and characterization of the fractions and Fig. 3 shows the DSC thermograms of these fractions of P(3HB-co-65 mol% 4HB). The unfractionated sample shows three melting peaks and the fraction insoluble or precipitated in 100% acetone at room temperature shows melting peaks corresponding to higher and intermediate temperature sides. These fractions showed extremely low 4HB contents at a range of 15–18 mol%. Whereas the fraction precipitated in 100% acetone at 8°C for 20 h shows a single melting peak appearing at the lower temperature side. The fraction precipitated in aqueous acetone solution with concentration below 95% also shows a single melting peak at the lower temperature side, whose temperature shifts slightly to the lower temperature side with the decrease in acetone concentration. The 4HB contents of these fractions decreased linearly from 84 (100% acetone) to 68 mol% (85%) as the acetone concentration decreased.

Fig. 4 shows the ¹³C NMR spectrum of P(3HB-co-65% 4HB) with its expanded ¹³C NMR spectrum of carbonyl resonance inset. The composition of both components could be also estimated using areas of peak 3 (CH of 3HB) and peak 8 (CH₂ of 4HB), though it was usually estimated from ¹H NMR spectrum. The spectra of carbonyl

Table 2
Molecular weight and thermal properties of P(3HB-co-4HB) samples

Sample no.	4HB fraction (mol%)	Molecular weight		Thermal properties			Crystallinity X (%) ^c
		$\bar{M}_n \times 10^{-4}$	\bar{M}_w/\bar{M}_n	T_g (°C)	T_m (°C) ^a	ΔH_m (J g ⁻¹) ^b	
1	0	5.8	3.4	4	176	90	72
2	19	6.6	3.1	-4	(55) (112)	158	45
3	38	3.7	2.3	-6	54 (118)	(157)	6
4	65	9.1	3.1	-40	52 (115)	(156)	11
5	74	5.2	3.0	-43	53 (156)	22	33
6	94	4.2	2.8	-46	(44) 54	39	54

^a The values in brackets show T_m values of melting peaks having the minor peak area.

^b The whole ΔH_m value calculated from the sum of all the melting peaks.

^c Calculated from the crystalline and amorphous densities.

resonance of both components appear at around 170 ppm. The dyad 3*3 (3HB*3HB), 3*4, 4*3, 4*4 (4HB*4HB) fractions are determined from the well-resolved peaks of carbonyl resonance (1, 5). Assuming that these are statistically random copolymers that can be described by Bernoullian statistics, the dyad fractions, F_{33} , F_{34} , F_{43} , F_{44} can be expressed with mole fraction of the 4HB unit in the polymer, F_4 , as follows [11]:

$$F_{33} = (1 - F_4)^2 \quad F_{34} = F_{43} = F_4(1 - F_4) \quad F_{44} = F_4^2 \quad (1)$$

Fig. 5 shows the dyad fractions obtained from the ¹³C NMR spectrum. The solid line is the line calculated from Eq. (1). The observed fractions of the unfractionated samples (open symbols) largely deviated from the calculated ones. The fractionated samples showed close agreement with the calculated ones, implying that the fractionated samples were typical random copolymers.

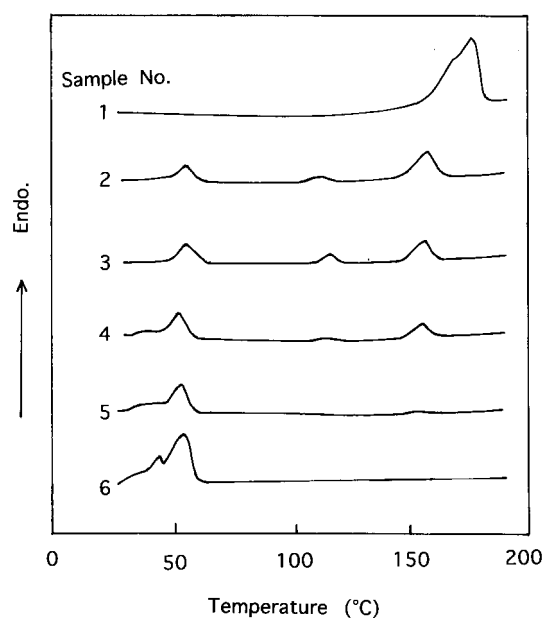


Fig. 2. DSC heating curves of P(3HB-co-4HB) samples listed in Table 1.

The other expression of randomness in sequence distribution is as follows [11]:

$$D = F_{33}F_{44}/F_{34}F_{43} \quad (2)$$

where if D is nearly equal to 1, then the copolymer is a typical copolymer and if D is too larger than 1, then the copolymer is a block copolymer or blend. The D values of the synthesized copolymer samples 4 and 5 (in Table 1) were around 10, while that of sample 6 was 2.7 and those of samples 2 and 3 were larger than 15. This shows that the biosynthesized P(3HB-co-4HB)s are mixture of P(3HB-co-4HB)s having different 4HB contents (especially containing the copolymer having very low composition of the 4HB unit). The D values of the fractionated P(3HB-co-65% 4HB) are listed in Table 3. The D value of the original P(3HB-co-65% 4HB) is 10.7, and that of the copolymer insoluble in hot acetone is 4.6, and that of the precipitated one in acetone at 25°C is 3.6, which all suggest them to be a mixture of P(3HB-co-4HB)s with different 4HB

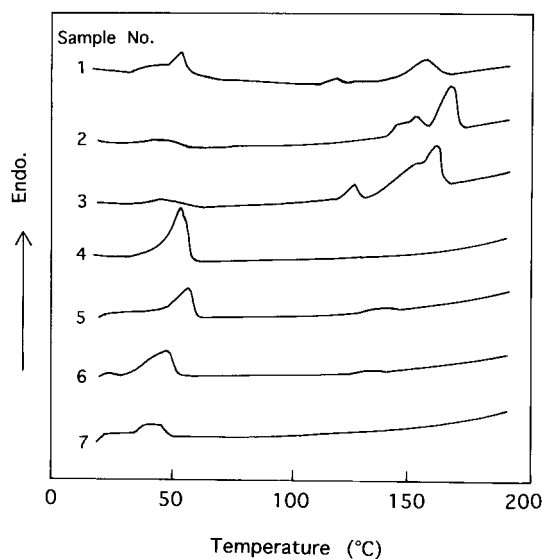


Fig. 3. DSC heating curves of the fractionated samples of P(3HB-co-65 mol% 4HB) listed in Table 3.

Table 3
Fractionation and characterization of P(3HB-co-65 mol% 4HB)

Sample no.	Concentration of aq. acetone (%)	Fraction (wt %)	4HB fraction ^a (mol%)	Melting point ^b			Molecular weight ^c		D^d
				T_m (°C)	(119)	(156)	$\bar{M}_n \times 10^{-4}$	\bar{M}_w/\bar{M}_n	
1	Original	–	65	52	(119)	(156)	9.1	3.1	10.7
2	Insoluble	4	15		(154)	167	8.5	2.7	4.6
3	100 (r.t.)	18	18		(127)	159	14.8	1.2	3.6
4	100 (8°C)	7	84		53		14.6	2.2	1.2
5	95	6	81		54		17.2	2.5	1.9
6	90	5	78		47		17.1	2.0	1.3
7	85	48	68		41		3.9	3.7	0.7

^a Determined by ¹H NMR.

^b The values in brackets show T_m values of melting peaks having the minor peak area.

^c Determined by GPC.

^d $D = F_{33}F_{44}/F_{34}F_{43}$.

compositions. However, the D values of P(3HB-co-4HB) precipitated in cool acetone or diluted aqueous acetone are all less than 1.5, suggesting that these are typical random copolymers.

The melting points of the fractionated samples are plotted against the 4HB content in Fig. 6. The copolymer having the composition lower than 40 mol% 4HB shows multiple melting behavior even after the fractionation. In the preceding paper [16], we reported that the 4HB component could hardly cocrystallize in the 3HB crystal lattice because it distorted the 3HB crystal lattice significantly, showing that the sequence of 4HB component is mostly excluded from the P(3HB) lattice crystal. In this case, the copolymer

mostly shows the less-depressed value of T_m as a result of exclusion of the 4HB unit. Other evidences of exclusion from the major component crystal were no change in the lattice indices of both components over the whole composition range [16].

The solid line in Fig. 6 shows typical T_m –4HB content curve of P(3HB-co-4HB). It is considered that the steep depression of T_m is not caused by occlusion of the second component in the major component crystal, but by introduction of irregularities in crystalline surfaces and low crystallinity as a result of exclusion of the second component.

In rare cases, once the small amount of 4HB unit was partially occluded in the P(3HB) crystal, it is expected that the T_m values decreased significantly because of the introduction of higher irregularity. Hence, the lowest T_m values of each copolymer as shown with a broken line is supposed to be close to real T_m value of the crystal partially occluded with the 4HB component (at a range of 0–10 mol% 4HB). This is also applied to the steep drop at a range of 95–100 mol% 4HB. The drop in T_m of both

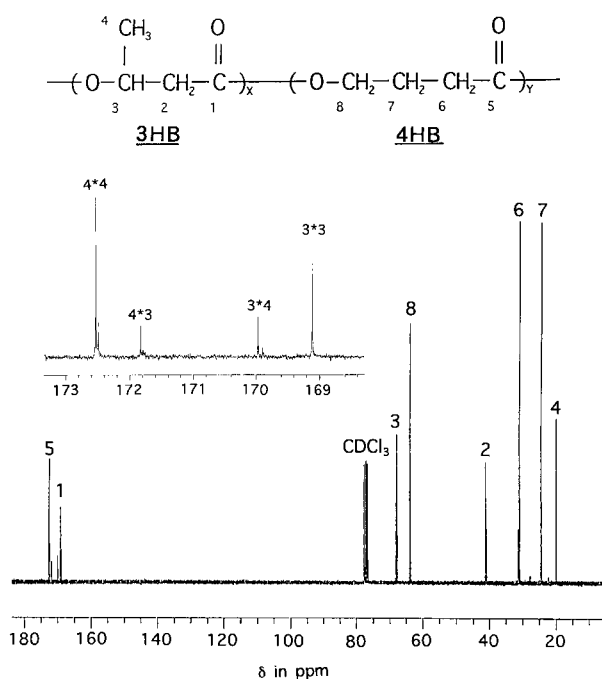


Fig. 4. ¹³C NMR spectrum of P(3HB-co-65 mol% 4HB) with its expanded ¹³C NMR spectrum of carbonyl resonance inset.

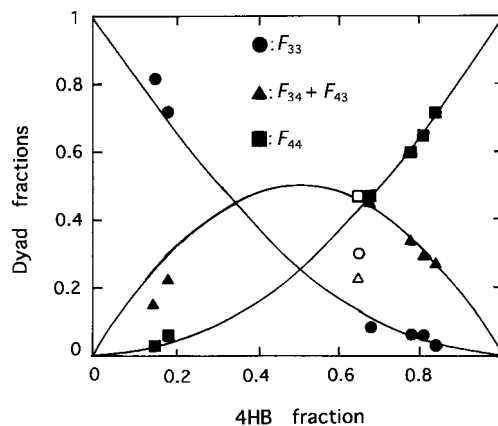


Fig. 5. Dyad fractions obtained from ¹³C NMR spectra of the fractionated and unfractionated P(3HB-co-65 mol% 4HB) samples. (○): F_{33} ; (△): $F_{34} + F_{43}$; (□): F_{44} for the unfractionated sample.

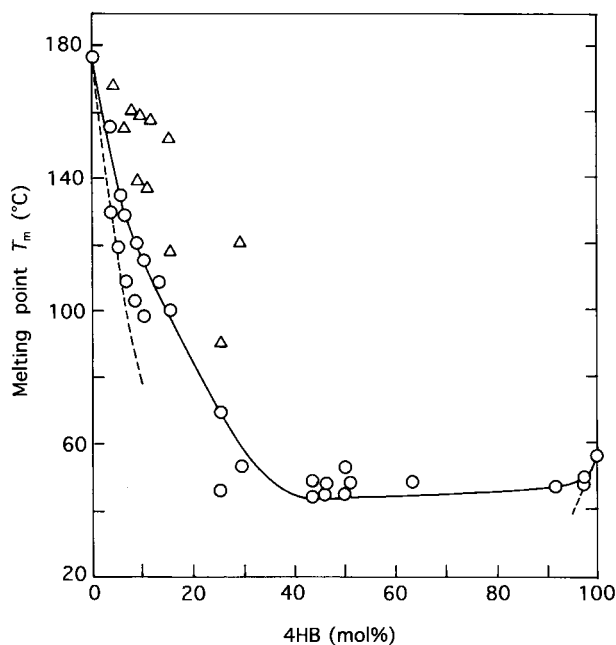


Fig. 6. Melting points of fractionated samples against the 4HB content. (Δ): less-depressed T_m ; (O): T_m series intermediately depressed (solid line) and significantly-depressed (broken line).

broken lines was far steeper than P(3HB-co-3HV) copolymer which showed typical cocrystallization [16].

Fig. 7 shows the crystalline, amorphous and observed densities of P(3HB-co-4HB) plotted against the composition of 4HB unit. The copolymer usually showed the X-ray diffraction patterns of the both crystal components.

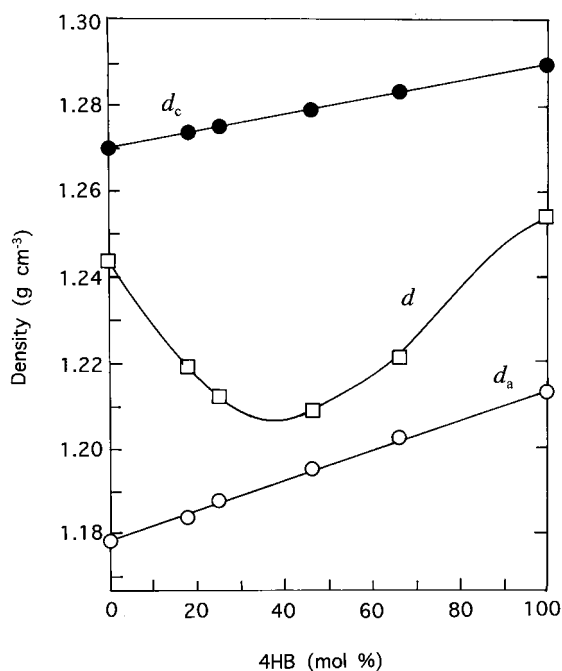


Fig. 7. Observed density with crystalline and amorphous densities plotted against the 4HB content.

The lattice indices of P(3HB) used for calculation of crystalline density d_c were $a = 5.76 \text{ \AA}$, $b = 13.20 \text{ \AA}$, and c (fiber period) $= 5.96 \text{ \AA}$ [17], and those of P(4HB) used were $a = 7.75 \text{ \AA}$, $b = 4.79 \text{ \AA}$, and c (fiber period) $= 11.94 \text{ \AA}$ [18]. Here, the crystalline density d_c of the copolymer was calculated additively. The amorphous density d_a was rapidly measured within 1 min by floating methods in KBr–water solution with the quenched sample after melted completely. The observed density d of the copolymer was also measured using a density gradient column at 25°C . As shown in Fig. 7, observed amorphous densities of P(3HB) and P(4HB) were 1.179 and 1.213, respectively, and those of the copolymers increased linearly with increase of 4HB content. From these value, we can calculate the crystallinity X (%) of the copolymer as follows:

$$X = d_c(d - d_a)/d(d_c - d_a) \times 100 \quad (3)$$

The crystallinity of the copolymer was listed in Table 2. The X of P(3HB) is 72% which steeply decreases to the minimum values of $\sim 18\%$ at the composition of 40–50% 4HB, then gradually increases up to 54% at the 94% 4HB and finally to 55% at P(4HB) sample obtained by the other biosynthesis [5]. Assuming the above values of crystallinity and ΔH_m (42 J g^{-1} from DSC), we can estimate the heat of fusion ΔH_u value to be 76 J g^{-1} , which is almost a half value of ΔH_u (146 J g^{-1}) of P(3HB) [19]. Heat of fusion of poly(ϵ -caprolactone), which has five CH_2 groups along the main chain per monomer unit, was reported as 78.1 J g^{-1} , which is very close to this value [20]. We can also calculate the ΔH_u value of P(3HB) to be 125 J g^{-1} using the value in Table 2, assuming that the recrystallization of P(3HB) sample does not occur during DSC heating process. If the recrystallization has occurred during the heating process, the ΔH_u must be smaller than the above value.

3.3. Enzymatic degradation profiles of P(3HB-co-4HB) samples

Fig. 8 shows enzymatic degradation (erosion) profiles of P(3HB-co-4HB) films in an aqueous solution of PHB depolymerase from *R. pickettii* T1. The copolymers of the lower crystallinity (4HB: 15 or 24%) showed faster degradation rate than P(3HB), implying that degradation preferentially took place in the amorphous regions. The copolymer P(3HB-co-69 mol% 4HB) showed the degradation rate slower than P(3HB). The rate of P(3HB-co-90 mol% 4HB) became slower, which was very similar to that of P(4HB) in the previous paper [5], though both showed weight loss of 25–30% at 24 h. No erosion of films was observed under the same condition without enzyme.

Fig. 9 shows enzymatic degradation profiles in aqueous solution of the lipase from *R. delmer*. This lipase is known to selectively degrade the 4HB component and clearly inverse behavior to Fig. 8 is expected. P(3HB), P(3HB-co-43% 4HB) showed no weight loss even after 24 h treatment. P(3HB-co-92% 4HB) showed fast increase in weight loss

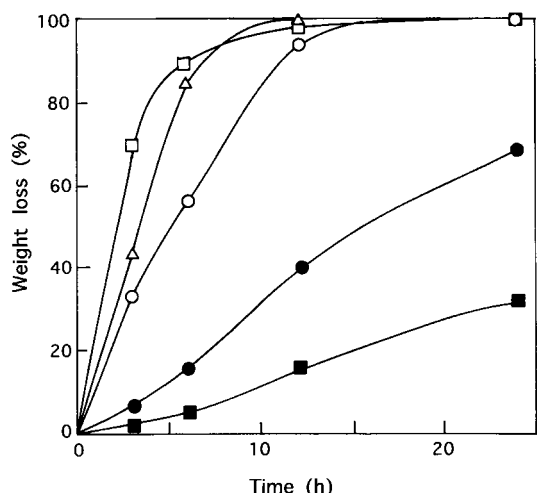


Fig. 8. Enzymatic degradation profiles of P(3HB-co-4HB) films in an aqueous solution of PHB depolymerase from *R. pickettii* T1. (○): P(3HB); (△): 15% 4HB; (□): 24% 4HB; (●): 69% 4HB; (■): 90% 4HB.

which reached to 100% after 12 h treatment. Other samples having intermediate composition showed the lower weight loss by degree as the 4HB content decreased and gradually leveled off at the time longer than 5 h. In contrast to PHB depolymerase (Fig. 8), the erosion rate by lipase hardly depended on the crystallinity of sample. The difference between these two enzymes are caused by their functional structures and biodegradation mechanism. These results are parallel to those reported previously in Ref. [5].

4. Conclusion

P(3HB-co-4HB) was biosynthesized by *C. acidovorans* with the mixed carbon sources of *n*-butyric acid and 1,4-butanediol. The 4HB content increased as the 1,4-butane-

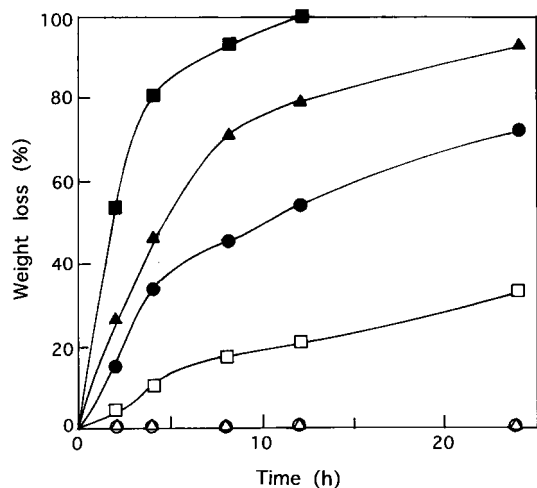


Fig. 9. Enzymatic degradation profiles of P(3HB-co-4HB) films in an aqueous solution of lipase from *R. delmer*. (○): P(3HB); (△): 43% 4HB; (□): 64% 4HB; (●): 78% 4HB; (▲): 83% 4HB; (■): 92% 4HB.

diol in feed increased. The most optimum fermentation condition was found, though M_n of P(3HB-co-4HB) was lower than that obtained in the previous study [5]. The biosynthesized P(3HB-co-4HB) was a mixture of copolymers having different 4HB contents, which was fractionated into random copolymers having homogeneous 4HB composition. P(3HB-co-4HB) fractionated showed multiple melting peaks at lower 4HB composition range, implying the formation of P(3HB) crystal phases excluding or partially occluding 4HB units. Amorphous densities of P(3HB) and P(4HB) were 1.179 and 1.213, respectively, from which the crystallinities of P(3HB-co-4HB)s were calculated and the heat of fusion of both components were obtained, i.e. 125 J g⁻¹ for P(3HB) and 76 J g⁻¹ for P(4HB). The erosion rate of P(3HB-co-4HB) films by PHB depolymerase from *R. pickettii* T1 decreased as the crystallinity and 3HB fraction decreased, while that by lipase from *R. delmer* was roughly proportional to the 4HB composition.

Acknowledgements

This work was supported by CREST (Core Research for Evolutional Science and Technology) of Japan Science and Technology Corporation (JST) and a Grant-in-Aid for Scientific Research on Priority Area, 'Sustainable Biodegradable Plastics', No.1121720(1999) from the Ministry of Education, Science, Sports and Culture (Japan).

References

- [1] Kunioka M, Nakamura Y, Doi Y. *Polym Commun* 1988;29:174.
- [2] Hiramitsu M, Koyama N, Doi Y. *Biotechnol Lett* 1993;15:461.
- [3] Kunioka M, Kawaguchi Y, Doi Y. *Appl Microbiol Biotechnol* 1989;30:569.
- [4] Kang CK, Kusaka S, Doi Y. *Biotechnol Lett* 1995;17:583.
- [5] Saito Y, Doi Y. *Int J Biol Macromol* 1994;16:99.
- [6] Saito Y, Nakamura S, Hiramitsu M, Doi Y. *Polym Int* 1996;39:169.
- [7] Mitomo H, Takahashi T, Ito H, Saito T. *Int J Biol Macromol* 1999;24:311.
- [8] Kimura H, Yoshida Y, Doi Y. *Biotechnol Lett* 1992;14:445.
- [9] Mitomo H, Mirishita N, Doi Y. *Macromolecules* 1993;26:5809.
- [10] Mitomo H, Mirishita N, Doi Y. *Polymer* 1995;36:2573.
- [11] Kamiya N, Yamamoto Y, Inoue Y, Chûjô R, Doi Y. *Macromolecules* 1989;22:1676.
- [12] Barker PA, Mason F, Barham PJ. *J Mater Sci* 1990;25:1952.
- [13] Shirakawa Y, Fukui T, Saito T, Okamoto Y, Narikawa T, Koide K, Tomita K, Takemura T, Masamune S. *Biochem Biophys Acta* 1986;880:46.
- [14] Mukai K, Doi Y, Sema Y, Tomita K. *Biotechnol Lett* 1993;15:601.
- [15] Kang C-K, Kusaka S, Doi Y. *Biotechnol Lett* 1995;17:583.
- [16] Mitomo H, Doi Y. *Int J Biol Macromol* 1999;25:201.
- [17] Yokouchi M, Chatani Y, Tadokoro H, Teranishi K, Tani H. *Polym J* 1973;14:267.
- [18] Mitomo H, Kobayashi S, Morishita N, Doi Y. *Polym Prep Jpn* 1995;44:3156.
- [19] Barham PJ, Keller A, Otun EL, Holmes PA. *J Mater Sci* 1984;19:2781.
- [20] Huarng J, Min K, White JL. *Polym Engng Sci* 1988;28:1590.