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PII: S0032-3861(97)00240-1

Polymer Vol. 39 No. 1, pp. 59–67, 1997

0032-3861/97/$17.00+0.00

Physical properties and enzymatic degradability of copolymers of (*R*)-3-hydroxybutyric acid and (*S,S*)-lactide

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(Received 24 December 1996)

Copolymers of (*R*)-3-hydroxybutyric acid ((*R*)-3HB) and (*S,S*)-lactide ((*S,S*)-LA) with a wide range of compositions varying from 5 to 92 mol% (*S,S*)-LA were synthesized by the ring-opening copolymerization of (*R*)-β-butyrolactone with (*S,S*)-lactide at various feed ratios in the presence of 1-ethoxy-3-chlorotetrabutyldistannoxane as a catalyst. The structure and physical properties of P[(*R*)-3HB-*co*-(*S,S*)-LA] were characterized by ^{1}H and ^{13}C n.m.r. spectroscopy, X-ray diffraction, differential scanning calorimetry, and optical microscopy. The copolyesters were shown to have a random sequence distribution of (*R*)-3HB and (*S,S*)-LA monomeric units. The glass-transition temperature of P[(*R*)-3HB-*co*-(*S,S*)-LA] increased linearly from 4 to 59°C as the (*S,S*)-LA fraction was increased from 0 to 100 mol%. The degree of X-ray crystallinity of solvent-cast copolyester films decreased from 62% to 18% as the (*S,S*)-LA composition was increased from 0 to 28 mol%, and the copolyesters showed a P[(*R*)-3HB] crystal lattice. In contrast, the X-ray crystallinities of copolymers with (*S,S*)-LA fractions of 70 to 100 mol% increased from 22% to 46% with the (*S,S*)-LA fraction, and those samples showed a P[(*S,S*)-LA] crystal lattice. The P[(*R*)3HB-*co*-(*S,S*)-LA] samples with 47 to 70 mol% (*S,S*)-LA were amorphous polymers. Enzymatic degradations of P[(*R*)-3HB-*co*-(*S,S*)-LA] films were carried out at 37°C in an aqueous solution containing PHB depolymerase purified from *Alcaligenes faecalis* or proteinase K from *Tritirachium album*. The rates of enzymatic degradation by PHB depolymerase of copolymer films ranging from 5 to 18 mol% (*S,S*)-LA were higher than that of microbial P[(*R*)-3HB] film. The highest rate of enzymatic hydrolysis by PHB depolymerase was observed at 5 mol% (*S,S*)-LA. Little erosion was observed for the copolyester films ranging in (*S,S*)-LA fractions from 47 to 100 mol%. In contrast, the weight loss profile of P[(*R*)-3HB-*co*-(*S,S*)-LA] films with a proteinase K showed the opposite trend in the copolymer composition to that of a PHB depolymerase. The highest rate of enzymatic hydrolysis by proteinase K was observed at 92 mol% (*S,S*)-LA. © 1997 Elsevier Science Ltd.

(Keywords: (*R*)-3-hydroxybutyric acid; (*S,S*)-lactide; copolyester; physical properties; enzymatic degradability)

INTRODUCTION

An optically active poly[(*R*)-3-hydroxybutyric acid] (P[(*R*)-3HB]) of pure isotactic structure is synthesized by a variety of bacteria as an intracellular storage material of carbon and energy[1–3]. Recently, many bacteria have been found to produce copolymers (PHA) of hydroxyalkanoic acid units with carbon chains ranging from 3 to 14 carbon atoms[4]. The microbial polyesters are biodegradable and biocompatible thermoplastics and have attracted industrial attention as an environmentally degradable material for a wide range of agricultural, marine and medical applications[1]. A remarkable characteristic of PHA is its biodegradability in the environment[1,2]. Aerobic and anaerobic PHA-degrading bacteria and fungi were isolated from various environments[5–7]. The microorganisms excrete extracellular PHB depolymerases to degrade environmental PHA and utilize the decomposed compounds as nutrients. The extracellular PHB depolymerases were purified from some microorganisms such as *Pseudomonas lemoignei*[8], *Alcaligenes faecalis*[5], *Comamonas testosteroni*[9], and *Penicillium pinophilun*[10]. The structural genes of PHB depolymerases of *A. faecalis*[11], *P. lemoignei*[12–14], *P. pickettii*[15], and *Comamons sp.*[16] were cloned and sequenced.

Poly[(*S,S*)-lactide] (P[(*S,S*)-LA]) was investigated as a material for medical devices such as controlled drug release matrixes, degradable sutures, and implanted for bone fixation[17]. Recently, there is increasing interest in using P[(*S,S*)-LA] for an environmentally degradable plastic materials[18]. Although it was reported that some enzymes hydrolyse P[(*S,S*)-LA][19,20], the degradation of P[(*S,S*)-LA] in an aqueous environment is mainly caused by non-enzymatic hydrolysis of polymer chains catalysed by carboxyl end groups of polymers[21].

In this article, we prepared the copolymers of (*R*)-3-hydroxybutyric acid ((*R*)-3HB)) and (*S,S*)-lactide ((*S,S*)-LA) by the ring-opening copolymerization of (*R*)-β-butyrolactone with (*S,S*)-lactide at various feed ratios in the presence of distannoxane as a catalyst[22]. The structure, thermal properties, and crystallization behaviour of the copolymers were investigated by means of nuclear magnetic resonance (n.m.r.), differential scanning calorimetry (d.s.c.), X-ray diffraction, and optical microscopy. The enzymatic degradabilities of copolymer films are studied in

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the presence of PHB depolymerase from *Alcaligenes faecalis* and of proteinase K from *Tritirachium album*. In addition, the water-soluble products during the enzymatic degradation of copolymer films are characterized by h.p.l.c. analysis.

EXPERIMENTAL

Materials

1-Ethoxy-3-chlorotetrabutyldistannoxane catalyst was prepared according to the method of Okamura and Wada[23], and the synthesis procedures were reported in a previous article[24]. (*R*)-β-Butyrolactone[25] (e.e. 92%) ((*R*)-β-BL) was dried by CaH_2 and distilled under reduced pressure. Microbial poly[(*R*)-3-hydroxybutyric acid] (P[(*R*)-3HB]) ($M_n = 281{,}000$, $M_w/M_n = 2.3$) was produced from butyric acid by Alcaligenes eutrophus[26]. Poly [(*S,S*)-lactide] (P[(*S,S*)-LA]) was prepared by ring-opening polymerization of (*S,S*)-LA in the presence of 1-ethoxy-3-chlorotetrabutyldistannoxane as a catalyst at 100°C for 4 h under argon atmosphere.

Synthesis of P[(R)-3HB-co-(SS)-LA] samples

Copolymers of (*R*)-3HB and (*S,S*)-LA were synthesized by the ring-opening copolymerization of (*R*)-β-BL with (*S,S*)-LA in the presence of 1-ethoxy-3-chlorotetrabutyldistannoxane as a catalyst. The copolymerization of β-butyrolactone and (*S,S*)-LA with 1-ethoxy-3-chlorotetrabutyldistannoxane was carried out at 100°C for 4 h under argon atmosphere. The produced copolymer was dissolved in chloroform and precipitated in a mixture of diethyl ether and hexane(1/3). The precipitate was dried in vacuo at room temperature.

Preparation of copolymer films

Copolymer films (about 0.1 mm thickness) were prepared by solvent-casting techniques from chlorofortn solutions of P[(*R*)-3HB-*co*-(*S,S*)-LA] using glass Petri dishes as casting surfaces. The films were aged at least 3 weeks at room temperature to reach equilibrium crystallinity prior to analysis.

Enzymatic degradation

The extracellular PHB depolymerase from *Alcaligenes faecalis* T1 was purified to electrophoretic homogeneity by the method of Shirakura *et al.*[27]. The proteinase K from *Tritirachium album* were purchased from Beohringer mannheim GMBH Biochemica, and used without further purification. The enzymatic degradation of copolymer films by PHB depolymerase was carried out at 37 ± 0.1°C in 0.1 M potassium phosphate buffer (pH 7.4). The enzymatic degradation of copolymer films by proteinase K was carried out at 37 ± 0.1°C in 0.1 M Tris-HCl buffer (pH 8.5) according to the method of Reeve *et al.*[19]. The copolymer films (initial weights: 14 mg, initial film dimensions: 10 × 10 × 0.1 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 PHB depolymerase (1.0 μg) or proteinase K (200 μg). For the weight loss measurement, the reaction solution was incubated with shaking, and the sample films were removed after reaction for a periodic time, washed with distilled water, and dried to constant weight in vacuo before analysis. The water-soluble products after enzymatic degradation of copolymer films were examined by HPLC analysis of the reaction solution.

Analytical procedures

The gel-permeation chromatography (g.p.c.), nuclear magnetic resonance (n.m.r.), differential scanning calorimetry (d.s.c.), X-ray diffraction, scanning electron microscopy (s.e.m.), and optical microscopy measurements were carried out along the literature procedures reported in previous articles[24,28].

The water-soluble products of enzymatic degradation of copolymer films were analyzed by using a Shimadzu LC-9A HPLC system with a gradient controller and a SPD-10A UV spectrophotometric detector. A stainless steel column (250 × 4 mm) 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 column was eluted with a linear gradient of distilled water (pH 2.5, adjusted by the addition of HCl solution) to acetonitrile over 40 min with a flow rate of 1.0 ml min^{-1}. The monomer and oligomers of 3-hydroxybutyric acid and 2-hydroxypropionic acid were detected spectrophotometrically at a wavelength of 210 nm. The degradation products were fractionated by h.p.l.c. Each product was collected from the h.p.l.c. eluate, and the solvent was evaporated, then used for ^1H n.m.r. analysis. The 400 MHz ^1H n.m.r. spectra were recorded in D_2O: 2HP monomer ($t_R = 4.6$ min), δ 1.24 (d, 3H), δ 4.07 (m, 1H); 3HB monomer ($t_R = 6.5$ min), δ 1.20 (d, 3H), δ 2.42 (m, 2H), δ 4.21 (m, 1H); 2HP-2HP dimer ($t_R = 10.6$ min), δ 1.28 (d, 3H), δ 1.40 (d, 3H), δ 4.20 (m, 1H), δ 4.91 (m, 1H); 3HB-2HP dimer ($t_R = 11.8$ min), δ 1.24 (d, 3H), δ 1.44 (d, 3H), δ 2.60 (m, 2H), δ 4.27 (m, 1H), δ 4.91 (m, 1H); 3HB-3HB dimer ($t_R = 13.3$ min), δ 1.21 (d, 3H), δ 1.30 (d, 3H), δ 2.45-2.66 (m, 4H), δ 4.22 (m, 1H), δ 5.26 (m, 1H); 3HB-2HP-2HP

Table 1 Copolymer compositions and molecular weights of P[(*R*)-3HB-*co*-(*S,S*)-LA] samples.

Sample no.	Monomer feed ratio (mol%)		Composition[a] (mol%)		Molecular weight[b]	
	(*R*)-β-BL	(*S,S*)-LA	(*R*)-3HB	(*S,S*)-LA	$M_n \times 10^{-3}$	M_n/M_w
1	90	10	95	5	91	1.8
2	80	20	82	18	52	2.3
3	70	30	72	28	71	1.7
4	50	50	53	47	50	2.3
5	30	70	30	70	78	2.0
6	10	90	8	92	85	2.1
7[c]			100	0	281	2.3
8[d]	0	100	0	100	258	1.8

[a]Determined from ^1H n.m.r. spectra. [b]Determined by g.p.c. analysis.[c]Microbial poly[(*R*)-3-hydroxybutyric acid]. [d]Poly[(*S,S*)-lactide] was synthesized by ring-opening polymerization of (*S,S*)-LA with distannoxane catalyst.

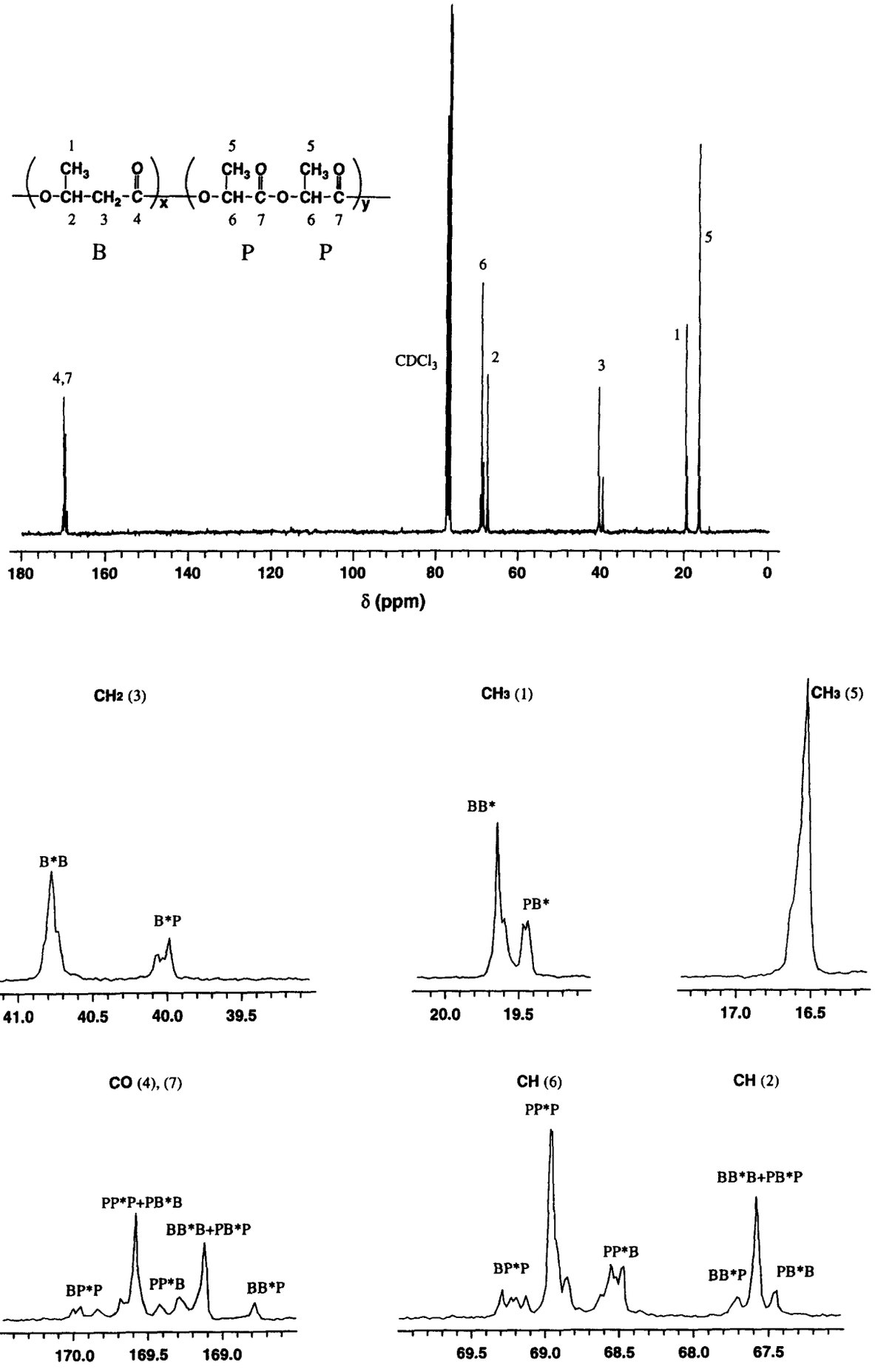

Figure 1 100 MHz ^{13}C n.m.r. spectrum of P[(*R*)-3HB-*co*-47 mol% (*S,S*)-LA] copolymer in $CDCl_3$ at 23°C

Table 2 Chemical shifts and relative intensities of ^{13}C resonances in P[(*R*)-3HB-*co*-(*S,S*)-LA] samples.

Carbon atoms	Chemical shift (ppm)	Sequence	Relative intensities[a] Sample 1 (LA% = 4.7)	Sample 2 (18.3)	Sample 3 (28.2)	Sample 4 (47.1)	Sample 5 (69.5)	Sample 6 (92.3)
CH_3 (5)	16.65	-	-	-	-	-	-	-
CH_3 (1)	19.56	PB*	0.07	0.14	0.18	0.30	0.48	1.00
	19.60		(0.05)	(0.18)	(0.28)	(0.47)	(0.69)	(0.92)
	19.74	BB*	0.93	0.86	0.82	0.70	0.52	0.00
	19.78		(0.95)	(0.82)	(0.72)	(0.53)	(0.31)	(0.08)
CH_2 (3)	40.00	B*P	0.07	0.14	0.18	0.28	0.47	1.00
			(0.05)	(0.18)	(0.28)	(0.47)	(0.69)	(0.92)
	40.81	B*B	0.93	0.86	0.82	0.72	0.53	0.00
			(0.95)	(0.82)	(0.72)	(0.53)	(0.31)	(0.08)
CH (2)	67.50	PB*B	0.07	0.10	0.14	0.16	0.23	n.d.[b]
			(0.05)	(0.15)	(0.20)	(0.25)	(0.21)	(0.07)
	67.63	BB*B + PB*P	0.86	0.78	0.62	0.68	0.54	n.d.
			(0.90)	(0.70)	(0.60)	(0.50)	(0.58)	(0.86)
	67.76	BB*P	0.07	0.12	0.14	0.16	0.23	n.d.
			(0.05)	(0.15)	(0.20)	(0.25)	(0.21)	(0.07)
CH (6)	68.53	PP*B	0.68	0.39	0.36	0.29	0.18	0.05
	68.61		(0.48)	(0.41)	(0.36)	(0.26)	(0.15)	(0.04)
	68.91	PP*P	0.00	0.38	0.39	0.54	0.72	0.90
	69.03		(0.04)	(0.18)	(0.28)	(0.48)	(0.70)	(0.92)
	69.19	BP*P						
	69.26		0.32	0.23	0.25	0.17	0.10	0.05
	69.29		(0.48)	(0.41)	(0.36)	(0.26)	(0.15)	(0.04)
	69.35							
CO (4),	168.82	PB*B	0.04	0.07	0.07	0.06	0.05	0.00
CO (7)			(0.05)	(0.10)	(0.11)	(0.09)	(0.04)	(0.00)
	169.17	BB*B + PB*P	0.78	0.55	0.39	0.24	0.13	0.00
			(0.82)	(0.48)	(0.33)	(0.18)	(0.11)	(0.04)
	169.33	PP*B	0.09	0.13	0.16	0.17	0.13	0.00
	169.46		(0.04)	(0.13)	(0.16)	(0.17)	(0.12)	(0.04)
	169.63	PP*P + BB*P	0.06	0.17	0.28	0.43	0.62	1.00
	169.73		(0.05)	(0.16)	(0.24)	(0.39)	(0.61)	(0.88)
	169.87	BP*P	0.03	0.08	0.10	0.10	0.08	0.00
	169.99		(0.04)	(0.13)	(0.16)	(0.17)	(0.12)	(0.04)

[a] The values in parentheses were calculated by Bernoullian statistics with the mole fraction of (*S,S*)-LA in copolymer samples 1-6. [b] Not detected.

trimer (t_R = 15.4 min), δ 1.24 (d, 3H), δ 1.49 (d, 3H), δ 1.55 (d, 3H), δ 2.63 (m, 2H), δ 4.26 (m, 1H), δ 5.01 (m, 1H), δ 5.19 (m, 1H); 3HB-3HB-3HB trimer (t_R = 17.2 min), δ 1.21-1.32 (m, 9H), δ 2.38-2.69 (m, 6H), 4.21 (m, 1H), 5.19 (m, 1H), 5.28 (m, 1H); 3HB-2HP-2HP-2HP tetramer (t_R = 18.6 min), δ 1.27 (d, 3H), δ 1.44 (d, 3H), δ 1.57 (m, 6H), δ 2.63 (m, 2H), δ 4.25 (m, 1H), δ 4.98 (m, 1H), δ 5.22 (m, 2H).

RESULTS AND DISCUSSION

Synthesis and structure of P[(R)-3HB-co-(S,S)-LA] copolymers

Copolymerization of (*R*)-β-butyrolactone ((*R*)-β-BL) with (*S,S*)-lactide ((*S,S*)-LA) was carried out at various monomer feed ratios in the presence of 1-ethoxy-3-chlorotetrabutyldistannoxane as a catalyst. For all experiments, the yields of copolymers were in the range 85% to 95%. The compositions of the copolymers were determined by integration of the proton resonances of the (*R*)-3HB and (*S,S*)-LA monomeric units in the ^1H n.m.r. spectra. *Table 1* shows the mole ratios of (*R*)-β-BL and (*S,S*)-LA monomers used for the copolymerization and copolymer composition, the number-average molecular weight (M_n), and polydispersities (M_w/M_n) of produced copolymers. The compositions of copolymers produced were good agreement with the monomer feed ratios. The number-average molecular weights and polydispersities of copolymers were in the ranges M_n = (50-91) × 10^3 and M_w/M_n = 1.7-2.3, respectively.

The sequence distributions of P[(*R*)-3HB-*co*-(*S,S*)-LA] copolymers were determined from the 100 MHz ^{13}C n.m.r. spectra. *Figure 1* shows a typical ^{13}C n.m.r. spectrum of P[(*R*)-3HB-*co*-47 mol% (*S,S*)-LA] copolymer, together with the chemical shift assignment for each carbon resonance. The diad and triad fractions were calculated from the ratios of peak areas of resonances. *Table 2* lists the diad and triad fractions of copolymer samples, together with the ^{13}C chemical shift assignments. The each carbon resonance was resolved into several peaks caused by diad or triad sequences of (*R*)-3HB (B) and half-lactide (2-hydroxypropionic acid) (P) units. The diad and triad sequence distribution data for (*R*)-3HB and (*S,S*)-LA units were compared with Bernoullian statistics applicable to a statistically random copolymerization. In the Bernoullian

Table 3 Thermal properties and X-ray crystallinity of P[(*R*)-3HB-*co*-(*S,S*)-LA] samples.

Sample no.	(*S,S*)-LA fraction	Thermal properties[a]			Crystallinity[b]
	(mol%)	T_g (°C)	T_m (°C)	ΔH_m (J/g)	X_c (%)
1	5	8	147	56	50 ± 5
2	18	16	124	31	35 ± 3
3	28	17	105	29	18 ± 3
4	47	30	59	3	0
5	70	45	115	10	0
6	92	57	153	35	22 ± 2
7	0	4	176	96	62 ± 5
8	100	59	176	43	46 ± 3

[a] Measured by d.s.c. [b] Determined from X-ray diffraction patterns.

model, the mole fraction F_{ij} of diad sequence ij can be expressed in terms of the mole fractions F_i and F_j of i and j units as $F_{ij} = F_iF_j$ and the mole fraction F_{iji} of triad sequence iji as $F_{iji} = F_i^2F_j$. In addition, it has been assumed that the transesterification between (*R*)-3HB and (*S,S*)-LA units hardly takes place during the copolymerization. As shown in *Table 2*, the calculated diad and triad fractions are in an agreement with the observed values for all samples, suggesting that the sequence distributions of (*R*)-3HB and (*S,S*)-LA units were statistically random.

Physical properties of P[(R)-3HB-co-(S,S)-LA] samples

The T_g, T_m, and ΔH_m values of solvent-cast copolymer films were determined from d.s.c. thermograms. The results are given in *Table 3*, together with the values of microbial P[(*R*)-3HB] and poly[(*S,S*)-lactide] (P[(*S,S*)-LA]) films. The T_g values for P[(*R*)-3HB-*co*-(*S,S*)-LA] copolymers increased from 4 to 59°C with an increase in the (*S,S*)-LA fraction from 0 to 100 mol%. The T_m values decreased from 176 to 59°C as the (*S,S*)-LA fraction was increased from 0 to 47 mol%. Then, the T_m value increased from 115 to 176°C with an increase in the (*S,S*)-LA fraction from 70 to 100 mol%.

The X-ray diffraction patterns of solvent-cast copolymer films with different (*S,S*)-LA fractions were recorded. For the copolyesters with compositions up to 28 mol% (*S,S*)-LA, only the crystalline form of the P[(*R*)-3HB] lattice was observed. In contrast, the P[(*R*)-3HB-*co*-(*S,S*)-LA] copolymers with compositions of 92 to 100 mol% (*S,S*)-LA showed the P[(*S,S*)-LA] crystal lattice. The degrees of X-ray crystallinity (X_c) of copolymer films are listed in *Table 3*. The X_c values of P[(*R*)-3HB-*co*-(*S,S*)-LA] films decreased from 62% to 18% as the (*S,S*)-LA composition was increased from 0 to 28 mol%. The P[(*R*)-3HB-*co*-(*S,S*)-LA] samples with 47 to 70 mol% (*S,S*)-LA were amorphous. Then, the X_c values increased from 22% to 46% with an increase in (*S,S*)-LA fraction from 92 to 100 mol%.

The crystallization kinetics of P[(*R*)-3HB-*co*-(*S,S*)-LA] were studied in the absence of nucleating agents. The spherulites of P[(*R*)-3HB-*co*-(*S,S*)-LA] copolymers were observed with a polarized optical microscope. *Figure 2* shows typical optical micrographs of spherulites of P[(*R*)-3HB-*co*-(*S,S*)-LA] film. After crystallization, uniform spherulites were well-developed throughout the copolymer film. 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 3* shows the rate of spherulite growth (G) of P[(*R*)-3HB-*co*-(*S,S*)-LA] copolymers at different crystallization temperatures. The rates of spherulite growth were dependent on both the (*S,S*)-LA composition and the crystallization temperature. A maximum value of 3.4 $\mu m\ s^{-1}$ was observed around 90°C for microbial P[(*R*)-3HB], however, as the (*S,S*)-LA component increased, the spherulitic growth rates were remarkedly reduced. The radial growth rates of spherulites for the P[(*R*)-3HB-*co*-18 mol% (*S,S*)-LA] film were slower by two orders of magnitude than the rates determined for microbial P[(*R*)-3HB], while the peak growth rate was observed near 80°C. From this result we concluded that the randomly distributed (*S,S*)-LA units in the P[(*R*)-3HB-*co*-(*S,S*)-LA] were responsible for the remarkable decrease in the rate of 3HB segments at the growth front of crystalline lamellae. For the P[(*S,S*)-LA], a maximum value of 0.8 $\mu m\ s^{-1}$ was observed around 120°C, however, as the incorporation of (*R*)-3HB unit, the spherulitic growth rates were reduced.

Enzymatic degradation of copolyester films

The weight loss of P[(*R*)-3HB-*co*-(*S,S*)-LA] films by PHB depolymerase from *Alcaligenes faecalis* was measured at each 1 h for 5 h and increased proportionally with time. The values of weight loss of the sample films for 5 h were in the range of 0-4 mg, depending on the copolymer composition. The rate of enzymatic erosion was determined from the slope of the line obtained by plotting the weight loss against time. *Figure 4* shows the rates of enzymatic degradation at 37°C of P[(*R*)-3HB-*co*-(*S,S*)-LA] films by PHB depolymerase. No weight loss was observed for the P[(*S,S*)-LA] homopolymer film by the PHB depolymerase. The rates of enzymatic degradation of P[(*R*)-3HB-*co*-(*S,S*)-LA] films with 5 to 18 mol% (*S,S*)-LA were higher than that of the microbial P[(*R*)-3HB] film. The highest rate of enzymatic degradation was observed with the copolymer film with 5 mol% (*S,S*)-LA, and the rate was about 10 times faster than that of microbial P[(*R*)-3HB]. In contrast, very slow rates of enzymatic erosion were observed for the copolyesters with 47 to 92 mol% (*S,S*)-LA. In the absence of PHB depolymerase, P[(*R*)-3HB-*co*-(*S,S*)-LA] films were not hydrolysed for 24 h at 37°C on 0.1 M potassium phosphate buffer.

Figure 5 shows the scanning electron micrographs of surfaces of copolymer films prior to and after enzymatic degradation. The surface of P[(*R*)-3HB-*co*-5 mol% (*S,S*)-LA] film after the enzymatic degradation for 3 h was apparently blemished by the function of depolymerase (*Figure 5b*), suggesting that the enzymatic degradation occurred on the surface of films. In contrast, the surface of P[(*R*)-3HB-*co*-92 mol% (*S,S*)-LA] film with a little enzymatic erosion remained apparently unchanged after the film was incubated with PHB depolymerase for 24 h.

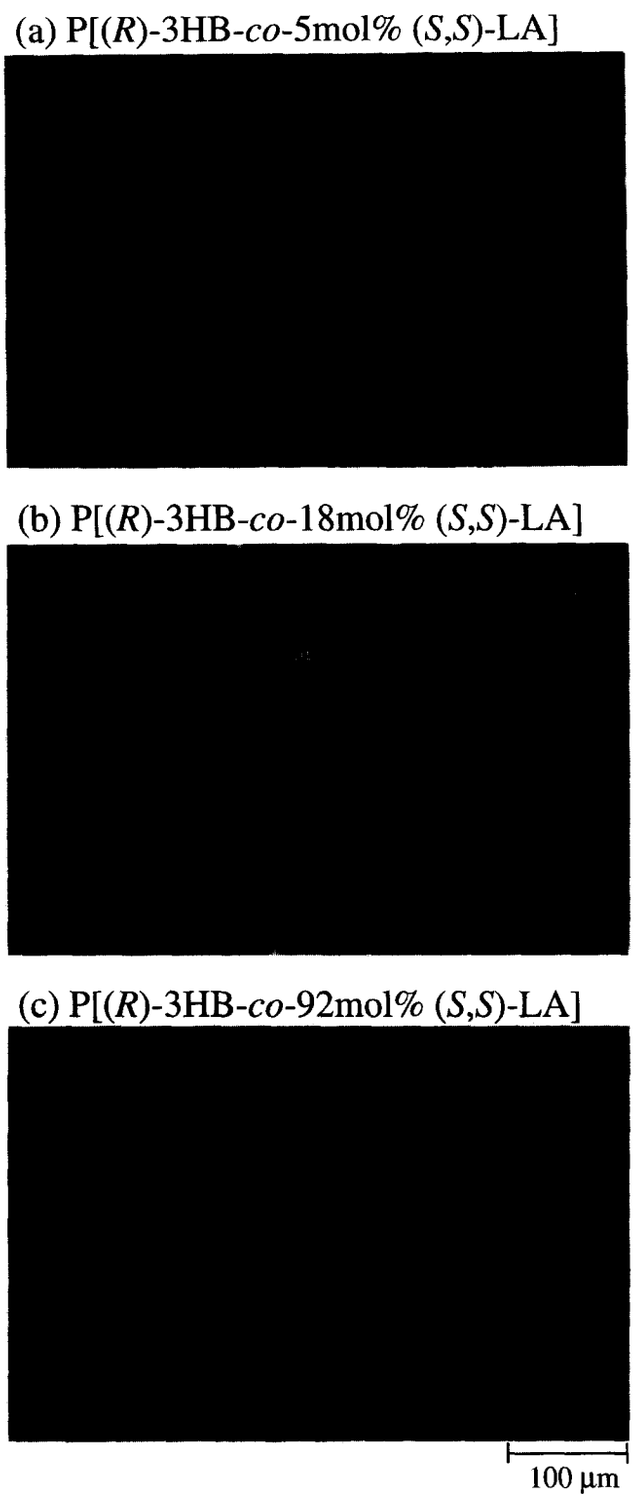

Figure 2 Optical micrographs of P[(*R*)-3HB-*co*-(*S,S*)-LA] spherulites crystallized at 100°C; (a) P[(*R*)-3HB-*co*-5 mol% (*S,S*)-LA], (b) P[(*R*)-3HB-*co*-18 mol% (*S,S*)-LA], and (c) P[(*R*)-3HB-*co*-92 mol% (*S,S*)-LA]

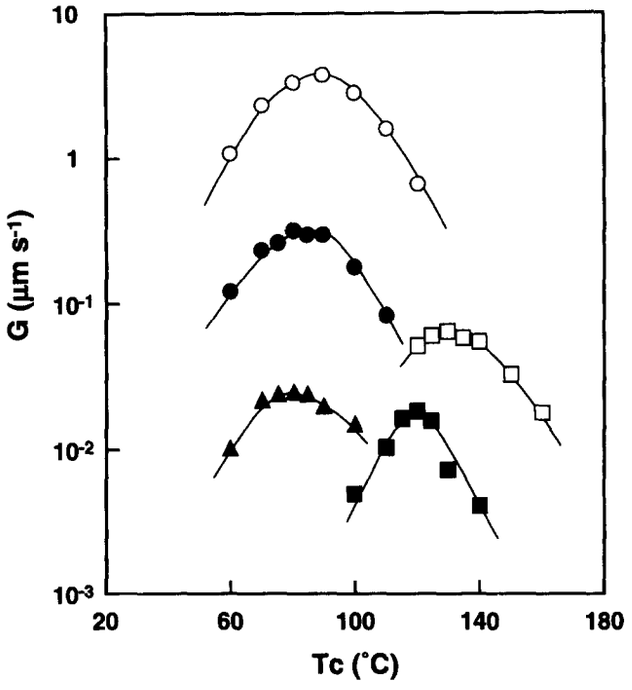

Figure 3 Radial growth rate (G) of spherulites as a function of crystallization temperature (T_c) for P[(*R*)-3HB] (○), P[(*R*)-3HB-*co*-5 mol% (*S,S*)-LA] (●), P[(*R*)-3HB-*co*-18 mol% (*S,S*)-LA] (▲), P[(*R*)-3HB-*co*-92 mol% (*S,S*)-LA] (■), and P[(*S,S*)-LA] (□)

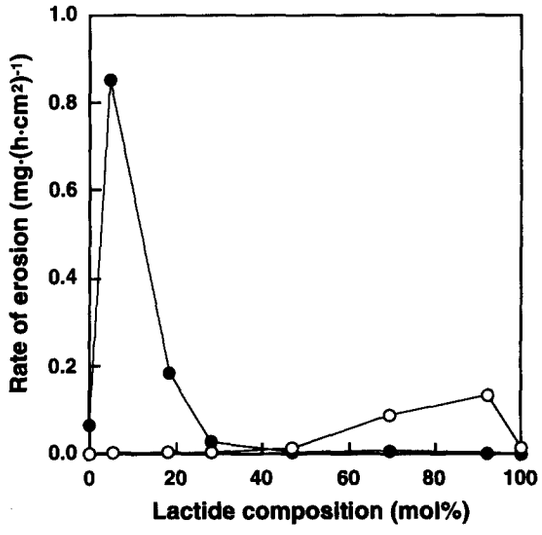

Figure 4 Effect of copolymer composition on the rate of enzymatic erosion of P[(*R*)-3HB-*co*-(S,S)-LA] films by PHB depolymerase from *A. faecalis* in potassium phosphate buffer (pH 7.4) (●) and by proteinase K in Tris-HCl buffer (pH 7.4) (○) at 37°C

In a previous article[29], we reported that the rate of enzymatic degradation of microbial P[(*R*)-3HB] film increased with a decrease in the crystallinity and that a PHB depolymerase from *A. faecalis* hydrolyzed predominantly the P[(*R*)-3HB] chains in the amorphous state on the surface of film. In addition, it was suggested that the rate of enzymatic degradation for the P[(*R*)-3HB] chains in the amorphous state was approximately twenty times higher than the rate for the P[(*R*)-3HB] chains in the crystalline state. The acceleration of enzymatic degradation for P[(*R*)-3HB-*co*-5 mol% (*S,S*)-LA] film may be caused by the decrease in crystallinity. Although the crystallinities of films decreased with (*S,S*)-LA fraction, the rates of enzymatic erosion on P[(*R*)-3HB-*co*-(*S,S*)-LA] films with compositions of 5-18 mol% (*S,S*)-LA decreased markedly with an increase in the (*S,S*)-LA fraction. This result suggests that the rate of enzymatic degradation is regulated not only by the crystallinity of polymer but also by chemical structure of monomeric units and the substrate specificity of PHB depolymerases.

The weight loss of P[(*R*)-3HB-*co*-(*S,S*)-LA] films by a proteinase K was measured at function of time for 24 h and

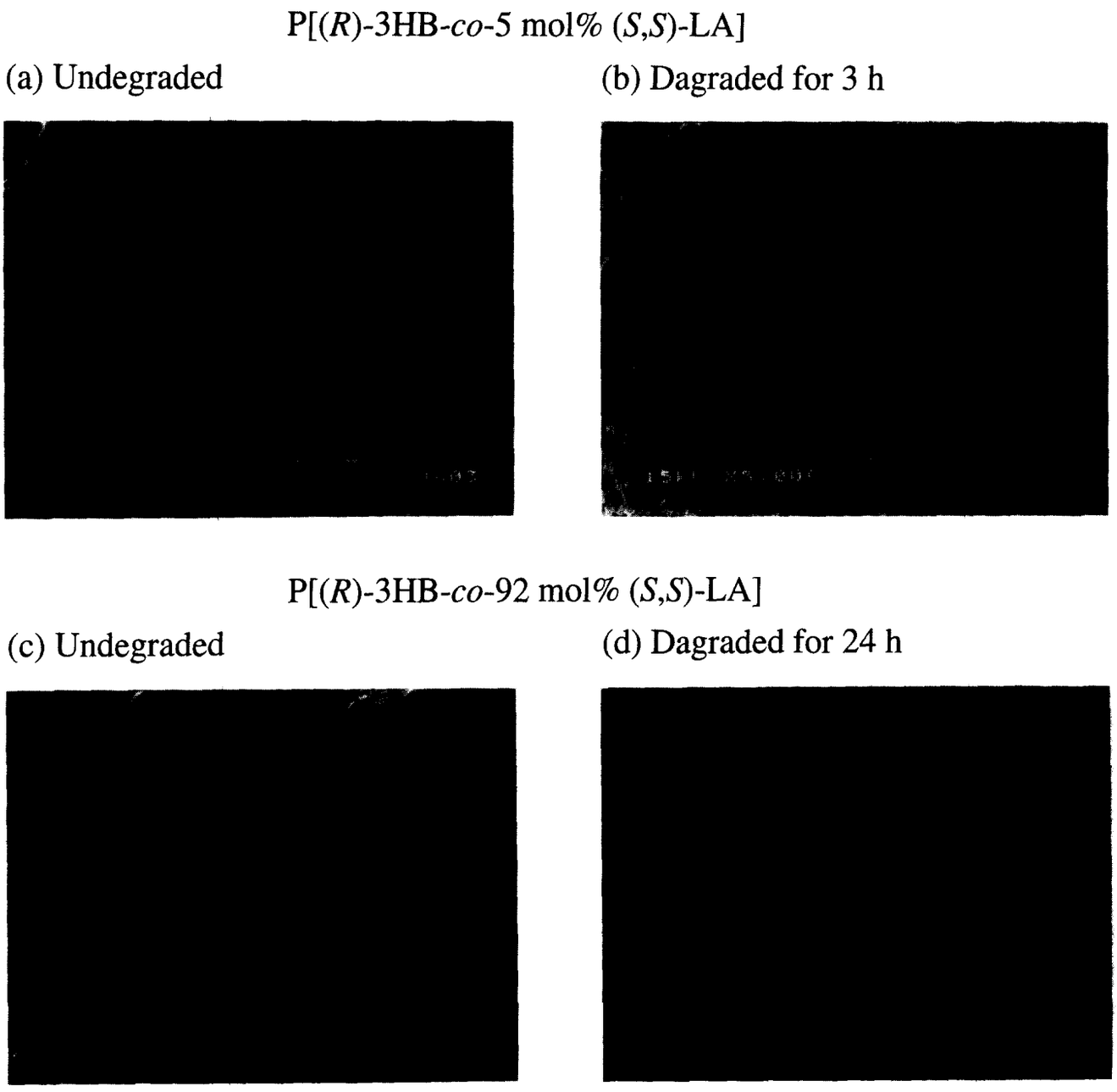

Figure 5 Scanning electron micrographs of surfaces of P[(*R*)-3HB-*co*-(*S,S*)-LA] films before and after the enzymatic degradation at 37°C with PHB depolymerase from *A. faecalis*

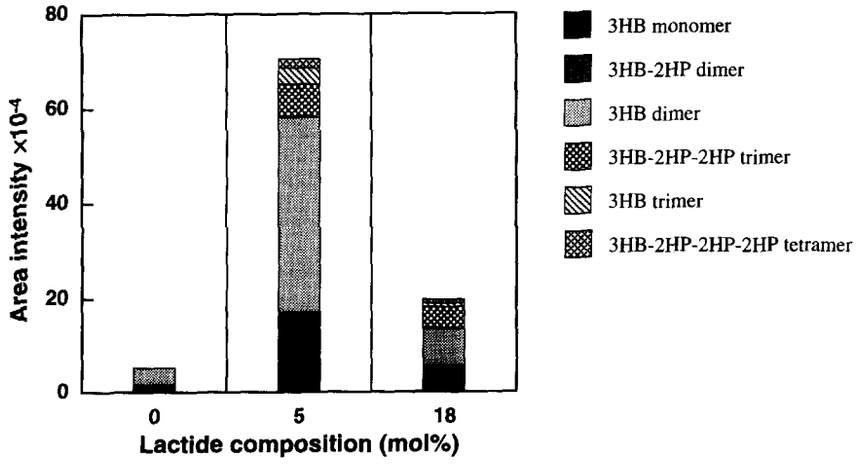

Figure 6 Distributions of water-soluble products after enzymatic degradation of P[(*R*)-3HB-*co*-(*S,S*)-LA] films by PHB depolymerase for 5 h at 37°C

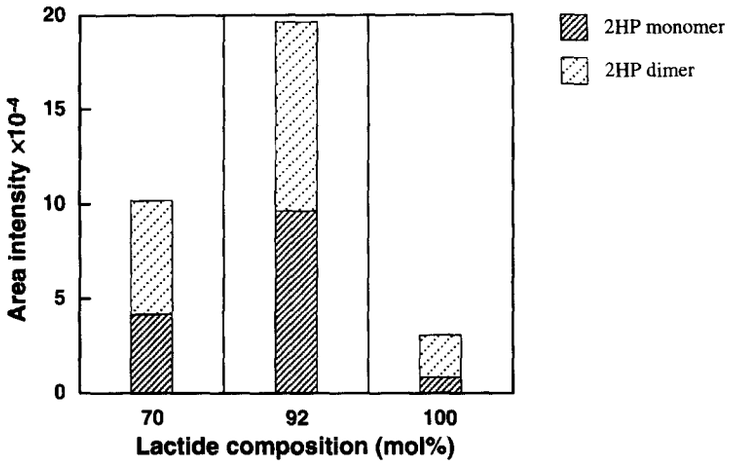

Figure 7 Distributions of water-soluble products after enzymatic degradation of P[(*R*)-3HB-*co*-(*S,S*)-LA] films by proteinase K for 19 h at 37°C.

increased proportionally with time. *Figure 4* shows the rates of enzymatic degradation at 37°C by proteinase K. The film of P[(*R*)-3HB] homopolymer was not eroded by proteinase K. It is of interest to note that the profiles of the degradation rates of P[(*R*)-3HB-*co*-(*S,S*)-LA] films by both PHB depolymerase and proteinase K show symmetric curves against the copolymer composition. The rates of enzymatic degradation by proteinase K of P[(*R*)-3HB-*co*-(*S,S*)-LA] films ranging in (*S,S*)-LA compositions from 70 to 92 mol% were faster than that of P[(*S,S*)-LA] homopolymer. The highest rate was observed on the P[(*R*)-3HB-*co*-92 mol% (*S,S*)-LA], and very slow rates of enzymatic erosion were observed for copolymer films with (*S,S*)-LA compositions ranging from 0 to 47 mol%. In the absence of proteinase K, P[(*R*)-3HB-*co*-(*S,S*)-LA] films were not hydrolysed for 48 h at 37°C in 0.1 M Tris-HCl buffer (pH 8.5).

Characterization of water-soluble degradation products

In a previous article[24], we reported that the enzymatic hydrolysis of microbial P[(*R*)-3HB] film by PHB 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 using h.p.l.c. analysis.

The degradation of microbial P[(*R*)-3HB] by PHB depolymerase yielded only two h.p.l.c. peaks, corresponding to monomer and dimer of (*R*)-3HB unit. In contrast, the h.p.l.c. curves of water-soluble products after the enzymatic degradation of P[(*R*)-3HB-*co*-(*S,S*)-LA] films by PHB depolymerase showed 6 peaks at elution times from 2 to 25 min, corresponding to 3HB monomer, 3HB-3HB dimer, 3HB-2HP dimer, 3HB-3HB-3HB trimer, 3HB-2HP-2HP trimer, and 3HB-2HP-2HP-2HP tertamer. The peaks assignable to 2HP monomer, 2HP-3HB dimer, and 2HP-2HP dimer as water-soluble products from PHB depolymerase action were not detected. This result suggests that the PHB depolymerase from *A. faecalis* is incapable of hydrolysing the ester bonds of 3HB-2HP and 2HP-2HP sequences in dimers and oligomers. *Figure 6* shows the relative amounts of water-soluble products after enzymatic degradation of P[(*R*)-3HB-*co*-(*S,S*)-LA] films for 5 h at 37°C by PHB depolymerase. The fractions corresponding to oligomers containing 2HP units increased with an increase in the fraction of (*S,S*)-LA unit in copolymers.

Figure 7 shows the relative amounts of water-soluble products after enzymatic degradation of P[(*R*)-3HB-*co*-(*S,S*)-LA] films for 19 h at 37°C by proteinase K. The h.p.l.c. curves of the products showed 2 peaks, arising from 2HP monomer and 2HP-2HP dimer, and we could not detect the peaks from monomer and dimers containing 3HB unit. This result suggests that the poteinase K hydrolyses only the ester bond of 2HP-2HP sequence in a polymer chain.

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