Effects of Solid-State Structures on the Enzymatic Degradability of Bacterial Poly(hydroxyalkanoic acids)

Naoyuki Koyama and Yoshiharu Doi*

Polymer Chemistry Laboratory and the RIKEN Group of the Research Institute of Innovative Technology for the Earth (RITE), The Institute of Physical and Chemical Research (RIKEN), Hirosawa 2-1, Wako-shi, Saitama 351-01, Japan

Received August 8, 1996; Revised Manuscript Received November 15, 1996[⊗]

ABSTRACT: The effects of solid-state structures on the rate of enzymatic erosion for melt-crystallized films of bacterial poly(hydroxyalkanoic acids) (PHAs) have been studied at 37 °C and pH 7.4 in the aqueous solution of an extracellular PHB depolymerase from *Alcaligenes faecalis*. In this study, the following PHA homopolymer and copolymers were used; poly(3-hydroxybutyric acid) (P(3HB)), poly(3-hydroxybutyric acid-*co*-7 mol % 3-hydroxyvaleric acid), poly(3-hydroxybutyric acid-*co*-22 mol % 3-hydroxyvaleric acid), poly(3-hydroxybutyric acid-*co*-10 mol % 4-hydroxybutyric acid), poly(3-hydroxybutyric acid-*co*-4 mol % 3-hydroxyhexanoic acid), and poly(3-hydroxybutyric acid-*co*-11 mol % 3-hydroxyhexanoic acid). All PHA films were crystallized isothermally at temperatures of 60−140 °C for different periods from the melt. The crystallinity of the films of P(3HB) homopolymer ranged from 53 to 80%, while those of PHA copolymers ranged from 39 to 71%, determined by X-ray crystallography and depending on the crystallization conditions. The rates of enzymatic erosion decreased with an increase in crystallinity. The erosion rates for PHA copolymer films were several times higher than the rates of P(3HB) homopolymer films with the same degree of crystallinity. It has been suggested that the rate of enzymatic erosion on PHA films is influenced not only by the degree of crystallinity but also by the structure of thin PHA lamellar crystals.

Introduction

A wide variety of bacteria synthesize an optically active polymer of (*R*)-3-hydroxybutyric acid and accumulate it as a reserve energy source.[1,2] Poly[(*R*)-3-hydroxybutyric acid] (P(3HB)) isolated from bacteria is a biodegradable and biocompatible thermoplastic with a melting temperature at ∼180 °C.[3,4] Recently, many bacteria have been found to produce copolymers of (*R*)-3-hydroxyalkanoic acids with a chain length ranging from 4 to 14 carbon atoms.[5] In addition, 3-hydroxypropionic acid and 4-hydroxybutyric acid have been found as new constituents of bacterial poly(hydroxyalkanoic acids) (PHAs).[2] The thermal and mechanical properties of PHA can be regulated by varying the compositions of copolymers.[6,7]

A remarkable characteristic of PHAs is their biodegradability in various environments. Aerobic and anaerobic PHA-degrading microorganisms have been isolated from various ecosystems, and the properties of their extracellular PHB depolymerases have been studied. The purified PHB depolymerases consist of a single polypeptide chain, and their molecular weights are in the range 37 000−59 000, having a serine residue at the active site.[8−12]

The films of P(3HB) crystallized isothermally from the melt exhibit large spherulites of diameter 50−500 μm, and the spherulite morphology and the degree of crystallinity depend strongly on the crystallization conditions.[4,13] Kumagai *et al.*[14] reported that the rate of enzymatic hydrolysis of P(3HB) film by the PHB depolymerase from *Alcaligenes faecalis* is strongly dependent on the degree of crystallinity of P(3HB) film but independent of the size of spherulites. In addition, they showed that the rate of enzymatic erosion for P(3HB) chains in an amorphous state is much faster than the rate for those in a crystalline state. Recently, Tomasi *et al.*[15] reported that the rate of enzymatic erosion of P(3HB) film decreases with an increase in the average size of P(3HB) crystals. In previous papers,[16−18] we reported on the enzymatic hydrolysis of the solution-cast films of various PHA copolymers with a PHB depolymerase from *A. faecalis* and reported the effect of copolymer compositions on the rate of enzymatic erosion.

In this paper, we report our results concerning the effects of their solid-state structures on the rate of enzymatic erosion of PHA films. Melt-crystallized PHA films were prepared from P(3HB) homopolymer and PHA copolymers as poly(3-hydroxybutyric acid-*co*-3-hydroxyvaleric acid), poly(3-hydroxybutyric acid-*co*-3-hydroxyhexanoic acid), and poly(3-hydroxybutyric acid-*co*-4-hydroxybutyric acid) under various crystallization conditions.

Experimental Section

Materials. Poly[(*R*)-3-hydroxybutyric acid] (P(3HB)) and poly(3-hydroxybutyric acid-*co*-3-hydroxyvaleric acid) containing 7 and 22 mol % of 3-hydroxyvaleric acid (P(3HB-*co*-7%3HV) and P(3HB-*co*-22%3HV), respectively) were purchased from Aldrich Chemicals. The P(3HB), P(3HB-*co*-7%3HV), and P(3HB-*co*-22%3HV) samples were purified by precipitation in hexane from chloroform solution at room temperature and dried *in vacuo* for 2 days. Poly(3-hydroxybutyric acid-*co*-10 mol % 4-hydroxybutyric acid) (P(3HB-*co*-10%4HB)) was produced by *Alcaligenes eutrophus* from γ-butyrolactone and fructose as carbon sources in the two-stage batch cultivation,[19] and poly(3-hydroxybutyric acid-*co*-3-hydroxyhexanoic acid) containing 4 and 11 mol % of 3-hydroxyhexanoic acid (P(3HB-*co*-4%3HH) and P(3HB-*co*-11%3HH), respectively) were produced by *Aeromonas caviae* from lauric acid as a carbon source in the two-stage batch culture.[18] After cultivation, cells were harvested by centrifugation and lyophilized. Copolyesters were extracted from the lyophilized cells with hot chloroform in a Soxhlet apparatus and purified by reprecipitation with hexane. Thermal properties and molecular weights of the bacterial PHA samples used in this study are listed in Table 1.

Preparation of Melt-Crystallized Films. All films were initially prepared by conventional solvent-cast techniques from

* Author to whom all correspondence should be addressed.
⊗ Abstract published in *Advance ACS Abstracts,* January 15, 1997.

S0024-9297(96)01195-3 CCC: $14.00

Table 1. Thermal Properties and Molecular Weights of Bacterial Poly(hydroxyalkanoic acids)

sample	T_g[a] (°C)	T_{cc}[b] (°C)	T_m[c] (°C)	ΔH_m[d] (J/g)	$M_n \times 10^{-3}$	M_w/M_n
P(3HB)	4	51	177	110	300	2.3
P(3HB-*co*-7%3HV)	2	61	152, 163	85	185	2.2
P(3HB-*co*-22%3HV)	−3	nd[e]	134, 154	62	130	2.0
P(3HB-*co*-10%4HB)	1	89	144, 157	76	113	3.3
P(3HB-*co*-4%3HH)	4	68	144, 157	86	366	3.3
P(3HB-*co*-11%3HH)	−3	59	112, 124	52	419	2.1

[a] Glass transition temperature; measured by DSC (second scan) from −100 to +200 °C at a rate of 20 °C/min. [b] Cold crystallization peak temperature; measured by DSC (second scan). [c] Melting temperature; measured by DSC (first scan), from 0 to 200 °C at a rate of 20 °C/min. [d] Enthalpy of fusion per gram of polymer. [e] Not detected.

chloroform solutions (20 mg/mL) of PHA samples using glass Petri dishes as casting surfaces. The films were dried to constant weight *in vacuo* at room temperature. Solvent-cast PHA films were inserted between two Teflon sheets (0.1 mm thickness) with a Teflon sheet (0.05 mm thickness) as a spacer and were compression-molded on a Mini Test Press (Toyoseiki) by heating at 200 °C for 1 min under a pressure of 75 kg/cm^2. After melting, samples were kept at a given crystallization temperature (T_c) and isothermally crystallized for given periods from 8 to 3504 h. Since the crystallization rates of PHA copolymers were much slower that of P(3HB) homopolymer, the crystallization of PHA copolymers was performed for periods up to 3504 h. The thickness of melt-crystallized films were ∼0.05 mm. All samples were stored at −20 °C until needed.

Enzymatic Degradation. The extracellular PHB depolymerase was purified to electrophoretic homogeneity from *A. faecalis*.[9] The enzymatic degradation of melt-crystallized PHA films was carried out at 37 °C in 0.1 M potassium phosphate buffer (pH 7.4). Films (initial weights, ∼6 mg; initial film dimensions, 10 × 10 × 0.05 mm) were placed in small glass bottles containing 1.0 mL of phosphate buffer. The reaction was started by the addition of 10 μL of an aqueous solution of PHB depolymerase (1.5 μg). The reaction solution was incubated at 37 ± 0.1 °C with shaking at 90 rpm. Sample films were periodically removed, washed with distilled water, and dried to constant weight *in vacuo*.

Analytical Procedures. All 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 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 generate a molecular weight calibration curve.

The differential scanning calorimetry (DSC) data of samples were recorded in the temperature range −100 to +200 °C under a nitrogen flow of 30 mL/min on a Shimadzu DSC-50Q instrument equipped with a cooling accessory. The sample films (3 mg) were encapsulated in aluminum pans and heated from 0 to 200 °C at a rate of 20 °C/min (first scan). The peak melting temperatures (T_m) and enthalpy of fusion (ΔH_m) were determined from the DSC endotherms. For the measurement of glass transition temperature (T_g), the samples were maintained at 200 °C for 1 min and then rapidly quenched at −100 °C. They were then heated from −100 to +200 °C at a heating rate of 20 °C/min (second scan). The T_g was taken as the midpoint of the heat capacity change. The cold crystallization peak temperature (T_{cc}) was determined from the DSC exotherm.

The X-ray diffraction patterns of melt-crystallized PHA films were recorded at 27 °C on a Rigaku RAD-IIIB system using nickel-filtered Cu Kα radiation ($\lambda = 0.154$ nm; 40 kV; 30 mA) in the 2θ range 6−40° at a scan speed of 2.0 deg/min. Degrees of crystallinity of melt-crystallized PHA films were calculated from diffracted intensity data according to Vonk's method.[20] The average size of crystals was determined by the Sherrer equation from the 020 reflection.[21]

Table 2. Degrees of Crystallinity and Rates of Enzymatic Erosion of P(3HB) Films Crystallized Isothermally for Different Periods at Temperatures of 60−140 °C

sample no.	crystallization temp (°C)	crystallization time (h)	X-ray crystallin (%)	cryst size[a] (nm)	erosion rate (μg cm^{-2} h^{-1})
1	60	168	65 ± 5	31 ± 2	49 ± 2
2	90	24	53 ± 5	30 ± 2	102 ± 2
3	90	72	56 ± 5	23 ± 1	92 ± 2
4	90	144	63 ± 5	34 ± 2	66 ± 1
5	90	168	56 ± 5	35 ± 2	79 ± 3
6	90	336	57 ± 5	29 ± 2	86 ± 3
7	110	168	78 ± 5	35 ± 2	33 ± 3
8	120	168	70 ± 5	30 ± 2	37 ± 1
9	130	8	64 ± 5	35 ± 2	54 ± 3
10	130	12	79 ± 5	40 ± 2	47 ± 2
11	130	48	67 ± 5	42 ± 2	35 ± 2
12	130	50	70 ± 5	45 ± 2	27 ± 1
13	140	8	73 ± 5	42 ± 2	23 ± 1
14	140	10	72 ± 5	43 ± 2	16 ± 1
15	140	12	65 ± 5	50 ± 3	28 ± 1
16	140	48	80 ± 5	41 ± 2	11 ± 1
17	140	50	78 ± 5	39 ± 2	15 ± 1

[a] Calculated from X-ray diffraction using the (020) reflection.

The surface of sample films were observed with a scanning electron microscope (JEOL-JSM5300) after gold coating of samples using an ion sputtering device (JEOL JFC-1100E).

Results and Discussion

Enzymatic Erosion of P(3HB) Films. Melt-crystallized P(3HB) films were prepared by isothermal crystallization at a given temperature for different periods from the melt at 200 °C for 1 min. Table 2 lists the crystallization temperature, crystallization time, degree of crystallinity, average size of crystals, and rate of erosion by PHB depolymerase for the melt-crystallized P(3HB) films (samples 1−17). The crystallization temperature and periods varied from 60 to 140 °C and from 8 to 336 h, respectively. All P(3HB) films showed well-developed and volume-filled spherulites, and the mean spherulite size increased from 50 to 250 μm with increasing crystallization temperature (data not shown). The degrees of X-ray crystallinity of P(3HB) films crystallized at temperatures below 100 °C ranged from 53 to 65%, while those above 100 °C ranged from 64 to 80%. The crystallinity of the P(3HB) films tended to increase with both crystallization temperature and time. The average size of the P(3HB) crystals was determined from X-ray diffraction using the (020) reflection. The values of P(3HB) films crystallized at temperatures below 100 °C ranged from 29 to 35 nm, while those above 100 °C ranged from 30 to 50 nm.

The enzymatic hydrolysis of melt-crystallized P(3HB) films (samples 1−17) were carried out in an aqueous solution of PHB depolymerase from *A. faecalis* at 37 °C and pH 7.4. Figure 1 shows typical weight loss profiles of P(3HB) films during the enzymatic hydrolysis. The weight loss of film increased proportionally with time. The rate of enzymatic erosion was determined from the slope of the line obtained by the weight loss against time, and the data are listed in Table 2. The rate of enzymatic erosion decreases markedly with an increase in crystallinity, as shown in Figure 1 and Table 2.[14] Appreciable changes in the molecular weight of P(3HB) films were not detected before and after the enzymatic erosion. In addition, no weight loss of P(3HB) films was observed for 24 h at 37 °C in an aqueous solution without PHB depolymerase in a blank experiment.

Figure 2 shows the scanning electron micrographs (SEMs) of time-dependent changes on the surfaces of

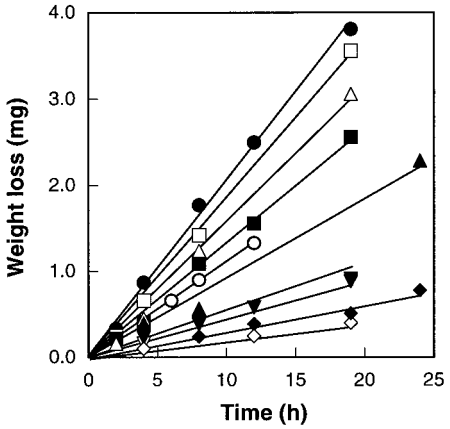
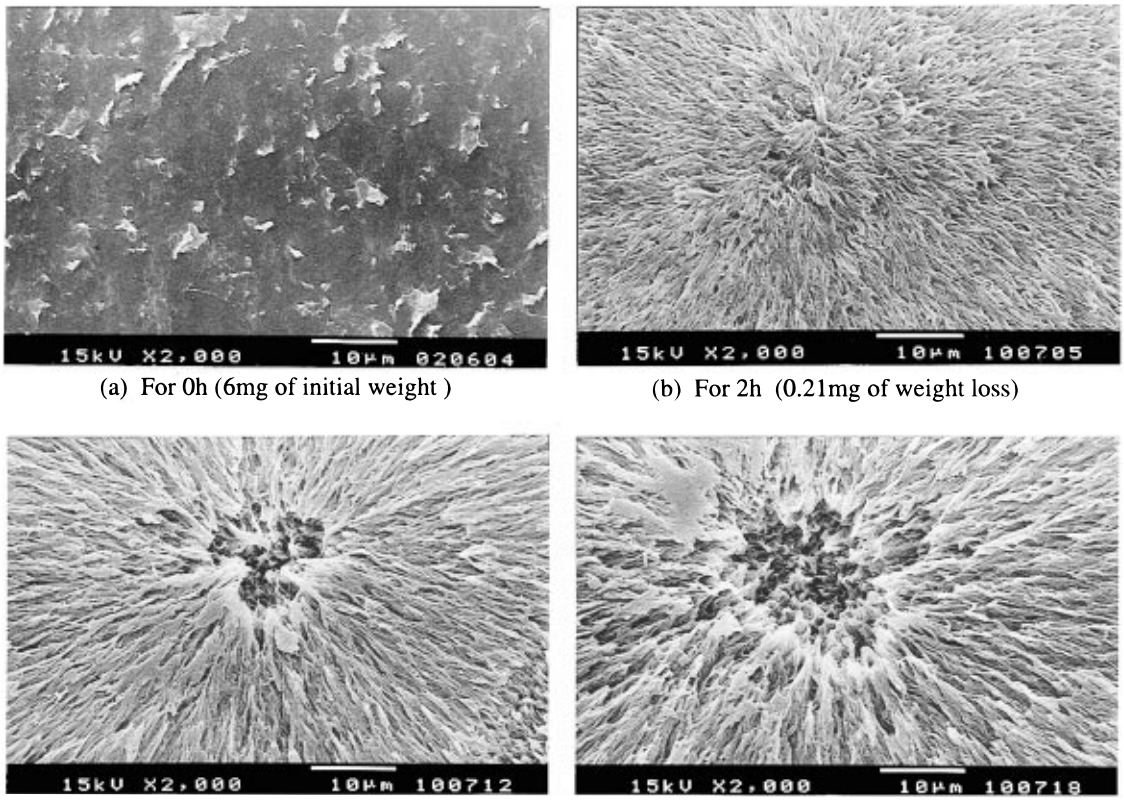


Figure 1. Enzymatic degradation (erosion) profiles of melt-crystallized P(3HB) films in an aqueous solution of *A. faecalis* PHB depolymerase at pH 7.4 and 37 °C. Samples: (○) 1, (●) 2, (□) 3, (■) 4, (△) 5, (▲) 10, (▽) 12, (▼) 14, (◇) 15, and (◆) 16.

P(3HB) film (sample 8) during the enzymatic hydrolysis. Before enzymatic hydrolysis, the surface of the P(3HB) film was almost flat except for unevenness caused by the Teflon sheet (Figure 2a). After 2 h of enzymatic hydrolysis, the surface was apparently blemished by the action of PHB depolymerase, and the ringed texture of the P(3HB) spherulite was visible (Figure 2b). In Figure 2c of the P(3HB) film with 0.97 mg of weight loss after 8 h of enzymatic degradation, the texture of the P(3HB) spherulite became obvious, and fine whiskers (fibrils) were radiately located on the surface. Thus, the PHB depolymerase initially hydrolyzes the amorphous interfibrillar P(3HB) chains on the surface and subsequently erodes the P(3HB) chains in the crystalline state of fibrils (bundle of lamellae). In addition, circular holes were observed in the centers of P(3HB) spherulites, and the size of the holes increased with degradation time (Figure 2d). It is suggested that the center of the spherulite consists of less ordered P(3HB) lamellae which are more susceptible to enzymatic attack.

Figure 3 shows the SEMs of enzymatically eroded surfaces of P(3HB) films crystallized at 60, 120, and 140 °C (samples 1, 8, and 17) with ∼1 mg (∼17%) of weight loss. The mean radius of P(3HB) spherulites increased from 50 to 250 μm as crystallization temperature was raised from 60 to 140 °C (Figure 3a,c,e). The P(3HB) films crystallized at 60 and 120 °C were composed of fine fibrils (bundle of lamellae) with size below 0.5 μm (Figure 3b,d). In contrast, the size of fibrils (1−3 μm) in the P(3HB) film crystallized at 140 °C were much larger than those crystallized at 60 and 120 °C (Figure 3f). The basic feature in polymer crystallization is the formation of thin lamellar crystals. These lamellae are arranged in stacks, with layers of amorphous material being inserted between the crystalline lamellae. Hoffman[22] proposed that there are three distinct regimes in the growth of polymer crystals, depending on the relative rates of formation of new secondary nuclei on the growth front and on the rate at which the nuclei once formed spread along the growth front. At low supercoolings, the rate of spreading is so large compared with the rate of nucleation that a nucleus once formed spreads right across the growth front (regime I). At higher supercoolings, several nuclei form and spread across the growth front together, the separation between them decreasing as the supercooling increases (regime II). At a sufficiently high supercooling, the separation is of the order of the molecular width, when no more spreading takes place (regime III). Barham *et al.*[4] reported that the transition of regime III to regime II took place at 135 °C for P(3HB) crystallization. The difference in the fibrillar morphologies between P(3HB)



(a) For 0h (6mg of initial weight)
(b) For 2h (0.21mg of weight loss)
(c) For 8h (0.97mg of weight loss)
(d) For 19h (1.41mg of weight loss)

Figure 2. SEMs of the surfaces of P(3HB) films crystallized at 120 °C for 168 h from the melt (sample 8) before and after enzymatic erosion.

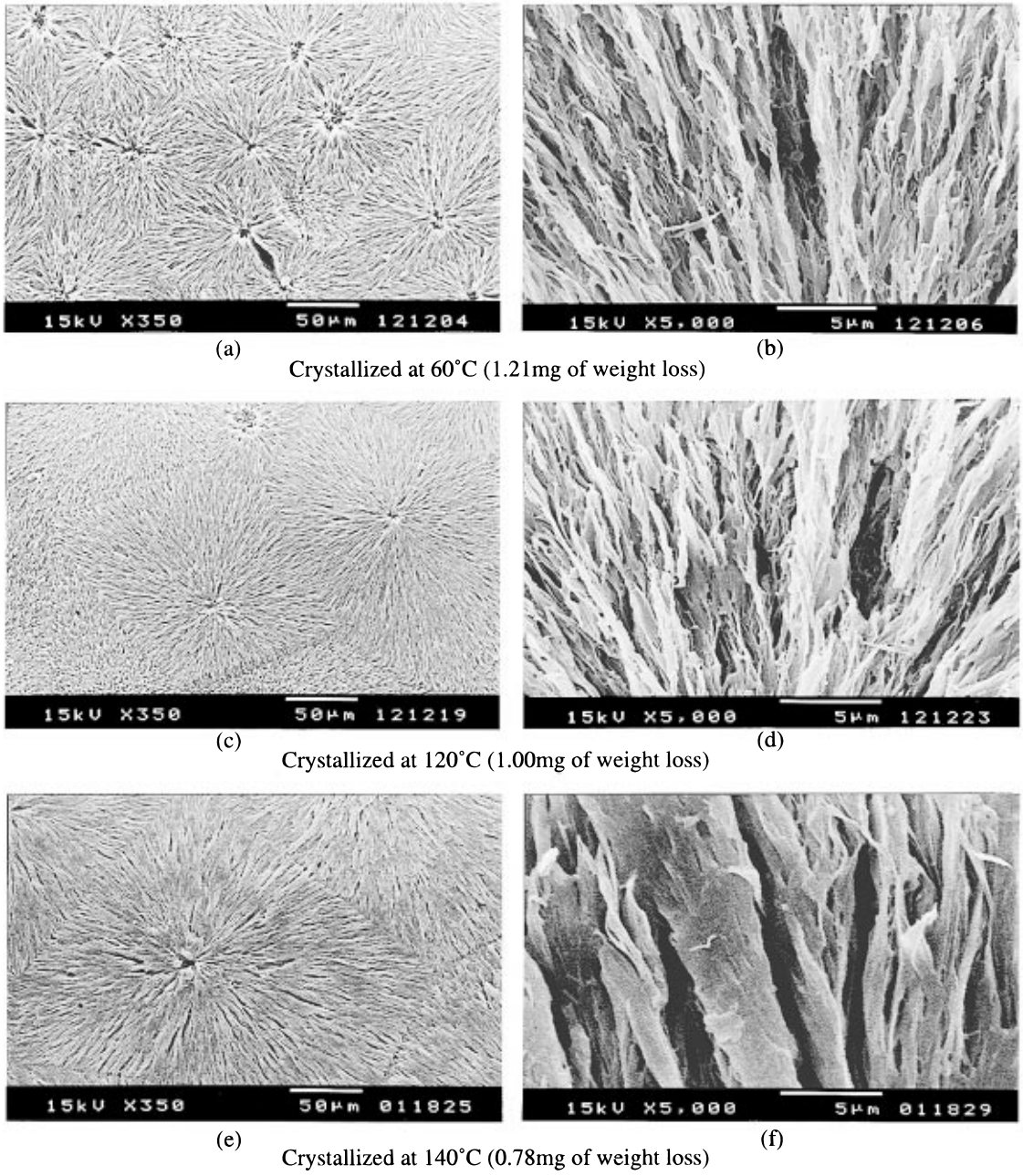


Figure 3. SEMs of enzymatically eroded surfaces of the P(3HB) film crystallized at (a, b) 60 °C for 168 h (sample 1), (c, d) at 120 °C for 168 h (sample 8), and (e, f) at 140 °C for 50 h (sample 17).

spherulites crystallized below 120 °C and those crystallized at 140 °C may be related to the transition of the growth mechanism of P(3HB) crystals.

Enzymatic Erosion of Films of PHA Copolyesters. Melt-crystallized films of five PHA copolymers P(3HB-*co*-7%3HV), P(3HB-*co*-22%3HV), P(3HB-*co*-10%4HB), P(3HB-*co*-4%3HH), and P(3HB-*co*-11%3HH), were prepared by isothermal crystallization at 60 °C for different periods from the melt at 200 °C for 1 min. Table 3 lists the sample of PHA copolymers, crystallization time, degree of crystallinity, average size of crystals, and rate of erosion by PHB depolymerase of the melt-crystallized PHA films (samples 18−30). The crystallization temperature of PHA film was 60 °C, and crystallization time varied from 24 to 3504 h. After crystallization, all PHA films showed well-developed and volume-filled spherulites (data not shown). The degrees of X-ray crystallinity of P(3HB-*co*-7%3HV), P(3HB-*co*-22%3HV), P(3HB-*co*-10%4HB), P(3HB-*co*-4%3HH), and P(3HB-*co*-11%3HH) films were 63−71, 45−51, 53−59, 52, and 39−43%, respectively. It is of interest to note that P(3HB-*co*-7%3HV) films show a high crystallinity (63−71%), while P(3HB-*co*-4%3HH) and P(3HB-*co*-11%3HH) films exhibit relatively low crystallinities of 52 and 39−43%, respectively. Thus, the incorporation of a small amount of 3HH units in the P(3HB) sequences reduces the crystallinity of melt-crystallized P(3HB-*co*-3HH) films. It is well-known that P(3HB-*co*-3HV) copolymers show relatively high crystallinity due to the cocrystallization of 3HB and 3HV units.[23,24] It has been shown that a portion of 3HV units is included in P(3HB) crystals in melt-crystallized P(3HB-*co*-3HV) films.[25−27] The data in Table 3 suggest that a majority of the 3HH monomeric units in the P(3HB) sequences are excluded from the P(3HB) crystals.

The enzymatic hydrolysis of melt-crystallized PHA films (samples 18−30) was carried out at 37 °C in an aqueous solution of *A. faecalis* PHB depolymerase. Figure 4 shows typical weight loss profiles of different

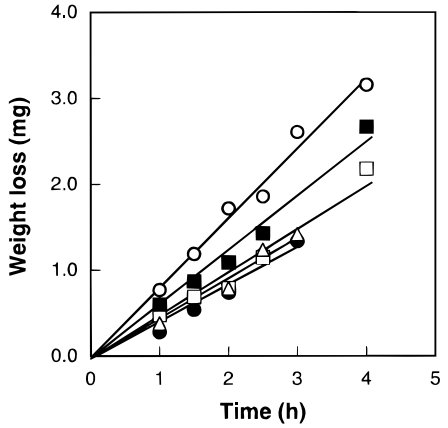


Figure 4. Enzymatic degradation (erosion) profiles of PHA films crystallized at 60 °C in an aqueous solution of *A. faecalis* PHB depolymerase at pH 7.4 and 37 °C. Symbols: (●) P(3HB-*co*-7%3HV) (sample 20), (○) P(3HB-*co*-22%3HV) (sample 23), (□) P(3HB-*co*-10%4HB) (sample 25), (△) P(3HB-*co*-4%3HH) (sample 27), and (■) P(3HB-*co*-11%3HH) (sample 29).

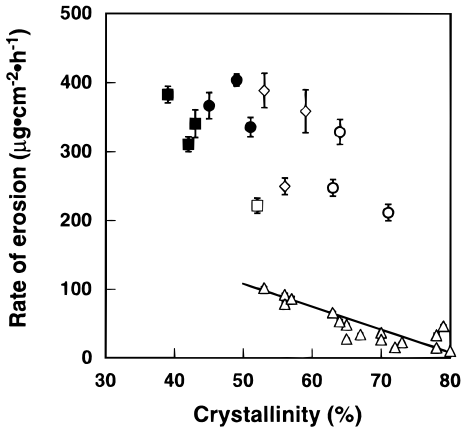


Figure 5. Relation between the rate of enzymatic erosion and the degree of crystallinity for the melt-crystallized PHA films. Symbols: (△) P(3HB), (○) P(3HB-*co*-7%3HV), (●) P(3HB-*co*-22%3HV), (◇) P(3HB-*co*-10%4HB), (□) P(3HB-*co*-4%3HH), and (■) P(3HB-*co*-11%3HH).

Table 3. Degrees of Crystallinity and Rates of Enzymatic Erosion of Poly(hydroxyalkanoic acids) Films Crystallized for Different Periods at 60 °C

sample no.	PHA	time (h)	X-ray crystall (%)	cryst size[a] (nm)	erosion rate (μg cm^{-2} h^{-1})
18	P(3HB-*co*-7%3HV)	24	64 ± 5	32 ± 2	329 ± 18
19		288	63 ± 5	25 ± 1	248 ± 12
20		3504	71 ± 5	44 ± 2	212 ± 12
21	P(3HB-*co*-22%3HV)	24	45 ± 5	34 ± 2	367 ± 19
22		288	51 ± 5	25 ± 2	336 ± 14
23		3504	49 ± 5	30 ± 2	404 ± 9
24	P(3HB-*co*-10%4HB)	24	53 ± 5	32 ± 2	389 ± 25
25		288	56 ± 5	30 ± 2	250 ± 12
26		3504	59 ± 5	28 ± 2	359 ± 31
27	P(3HB-*co*-4%3HH)	240	52 ± 5	27 ± 2	222 ± 11
28	P(3HB-*co*-11%3HH)	24	39 ± 5	27 ± 2	383 ± 12
29		288	42 ± 5	26 ± 2	311 ± 11
30		3504	43 ± 5	32 ± 2	341 ± 20

[a] Calculated from X-ray diffraction using the (020) reflection.

PHA films. The weight loss of PHA films increased proportionally with time. The rates of enzymatic erosion of PHA films are given in Table 3. The relation between the rate of erosion and crystallinity of films is shown in Figure 5 for both melt-crystallized films of P(3HB) homopolymer and PHA copolymers for comparison. The rate of enzymatic erosion of PHA films decreased apparently with an increase in crystallinity

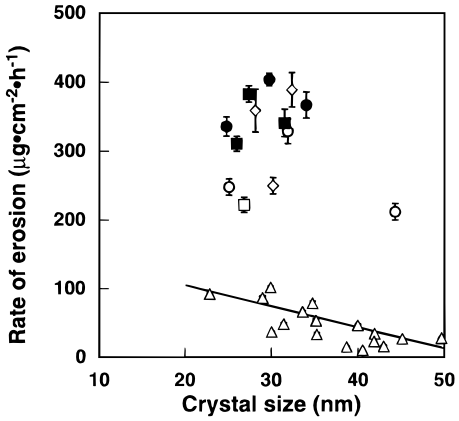


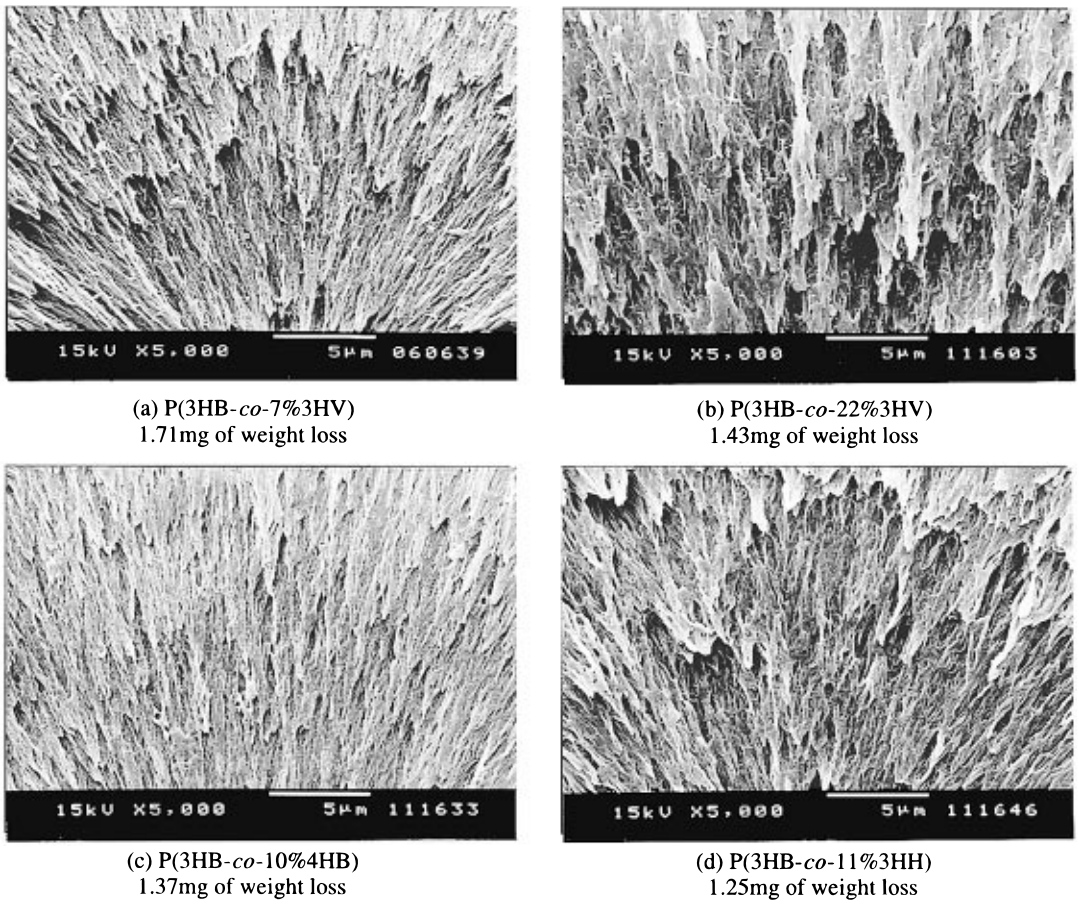
Figure 6. Relation between the rate of enzymatic erosion and the average size of crystal for melt-crystallized PHA films. Symbols: (△) P(3HB), (○) P(3HB-*co*-7%3HV), (●) P(3HB-*co*-22%3HV), (◇) P(3HB-*co*-10%4HB), (□) P(3HB-*co*-4%3HH), and (■) P(3HB-*co*-11%3HH).

as well as the result in the erosion of P(3HB) films. However, it is noted that the rates of enzymatic erosion of the films of PHA copolymers are several times higher than those of P(3HB) films, when the rates are compared with films of an identical crystallinity. For example, the erosion rates of P(3HB-*co*-7%3HV) films with 63−71% degrees of crystallinity are in the range of 210−330 μg cm^{-2} h^{-1}, while the rates of P(3HB) films with 63−70% degrees of crystallinity are in the range 28−66 μg cm^{-2} h^{-1}. These results suggest that the rate of enzymatic erosion depends not only on the degrees of crystallinity but also on the sequence structure of PHA polymers.

Recently, the rate of enzymatic erosion of melt-crystallized P(3HB) films with a PHB depolymerase from *Pseudomonas lemoignei* has been found to be affected by the P(3HB) crystal size.[15] Figure 6 shows the relation between the rate of enzymatic erosion and average size of P(3HB) crystals calculated from X-ray diffraction using the 020 reflection. The rates of enzymatic erosion decreased with an increase in P(3HB) crystal size for both the films of P(3HB) homopolymer and PHA copolymers. However, the rates of enzymatic erosion of PHA copolymer films were faster than those of P(3HB) films. A distinct difference in the erosion rates for melt-crystallized films of P(3HB) homopolymer and PHA copolymers could not be explained only in terms of degree of crystallinity and size of P(3HB) crystals.

Figure 7 shows SEMs of the surfaces of melt-crystallized films of PHA copolymers with 1.5 ± 0.2 mg (25 ± 3%) of weight loss after enzymatic erosion. The surfaces of PHA films after enzymatic erosion were blemished by the function of PHB depolymerase, and bundle of lamellae appeared on all films of PHA copolymers. The morphologies of fibrils in the films of PHA copolymers crystallized at 60 °C are almost identical with the morphology of fibrils in the P(3HB) film (Figure 3b) crystallized at 60 °C.

In earlier studies,[14,15] it was reported that a microbial PHB depolymerase hydrolyzes predominantly P(3HB) chains in the interfibrillar amorphous phase and subsequently erodes highly ordered P(3HB) chains in the crystalline phase. The results reported here confirm the conclusion that the rate of enzymatic hydrolysis for polyester chains in the amorphous state is faster than for those in the crystalline state in the melt-crystallized films of PHA copolymers as well as of P(3HB) ho-



(a) P(3HB-*co*-7%3HV)
1.71mg of weight loss

(b) P(3HB-*co*-22%3HV)
1.43mg of weight loss

(c) P(3HB-*co*-10%4HB)
1.37mg of weight loss

(d) P(3HB-*co*-11%3HH)
1.25mg of weight loss

Figure 7. SEMs of the surfaces of (a) P(3HB-*co*-7%3HV) film (sample 19), (b) P(3HB-*co*-22%3HV) film (sample 22), (c) P(3HB-*co*-10%4HB) film (sample 25), and (d) P(3HB-*co*-11%3HH) film (sample 29). Weight losses after enzymatic erosion are listed.

mopolymer. In addition, this work has demonstrated that the enzymatic erosion rate of PHA copolymers in the crystalline state is several times faster than that of the P(3HB) homopolymer in the crystalline state. The difference in the erosion rates of the crystalline phase in melt-crystallized films of P(3HB) homopolymer and PHA copolymers may arise from the difference in the structure of thin lamellar crystals.

Recently, Hocking *et al.*[28] prepared single crystals of the P(3HB) homopolymer and studied the enzymatic hydrolysis of P(3HB) single crystals with microbial PHB depolymerases from *Aspergillus fumigatus* and *P. lemoignei*. They revealed that the enzymatic hydrolysis takes place from the edge of a P(3HB) crystal, rather than from the fold surface.[28] The P(3HB) chains exposed on the surface of the crystal edge may be partially mobile relative to those in the crystal bulk at a reaction temperature of 37 °C. We propose here that the initial step of enzymatic hydrolysis of a P(3HB) crystal is the formation of disordered P(3HB) chains from the crystal edge by the function of PHB depolymerase. It is well-known that PHB depolymerase have a hydrophobic domain (~5 kDa) as a binding site to adhere to hydrophobic substrates such as the surface of P(3HB), in addition to a catalytic domain as an active site.[29−31] In previous papers,[32,33] we reported that the enzymatic surface erosion of P(3HB) film takes place via two steps, namely, adsorption and hydrolysis; the first step is adsorption of the enzyme on the surface of the P(3HB) film by the binding site (domain) of the enzyme, and the second step is a hydrolysis of the polymer chain by the active site of the enzyme. The binding of PHB depolymerase on the edge of P(3HB) lamellar crystal may cause an increase in the mobility of polymer chains along the crystal edge, resulting in the formation of disordered P(3HB) chains which are facilely attacked by the enzyme active site.

The chains of PHA copolymers exposed on the edge of PHA lamellar crystal may be more mobile than the chains of P(3HB) homopolymer on the crystal edge, which reflects the melting temperature depression of PHA copolymers. As a result, the rate of formation of disordered PHA chains from the crystal edge by the function of PHB depolymerase is larger for a PHA copolymer than that for P(3HB) homopolymer, and then the rate of enzymatic erosion on the lamellar crystal of the PHA copolymer is higher than that on the lamellar crystal of the P(3HB) homopolymer. Thus, more facile erosion by PHB depolymerase was observed in the crystals of PHA copolymers than in the crystals of P(3HB) homopolymer.

Acknowledgment. We thank Ms. M. Shono for her skillful analysis measurements. This study was performed as a part of the development of Biodegradable Plastics supported by the New Energy and Industrial Technology Development Organization (NEDO). This paper is dedicated to the 60th birthday of Professor Dieter Seebach.

References and Notes

(1) Dawes, E. A.; P. J. Senior *Adv. Microb. Physiol.* **1973**, *10*, 135.
(2) Doi, Y. *Microbial Polyesters*; VCH Publishers: New York, 1990.
(3) Marchessault, R. H.; Coulombe, S.; Morikawa, H.; Okamura, K.; Revol, J. F. *Can. J. Chem.* **1981**, *59*, 38.
(4) Barham, P. J.; Keller, A.; Otun, E. L.; Holmes, P. A. *J. Mater. Sci.* **1984**, *19*, 2781.

(5) Steinbüchel, A.; Valentin, H. E. *FEMS Microbiol. Lett.* **1995**, *128*, 219.
(6) Holmes, P. A. In *Developments in Crystalline Polymers-2*; Bassett, D. C., Ed.; Elsevier: London, 1988; p 1.
(7) Inoue, Y.; Yoshie, N. *Prog. Polym. Sci.* **1992**, *17*, 571.
(8) Lusty, C. J.; Doudoroff, M. *Proc. Natl. Acad. Sci. U.S.A.* **1966**, *56*, 960.
(9) Tanio, T.; Fukui, T.; Shirakura, Y.; Saito, T.; Tomita, K.; Kaiho, T.; Masamune, S. *Eur. J. Biochem.* **1982**, *124*, 71.
(10) Brucato, C. L.; Wong, S. S. *Arch. Biochem. Biophys.* **1991**, *290*, 497.
(11) Schrimer, A.; Jendrossek, D.; Schlegel, H. G. *Appl. Environ. Microbiol.* **1993**, *59*, 1220.
(12) Mukai, K.; Yamada, K.; Doi, Y. *Polym. Degrad. Stab.* **1993**, *41*, 85.
(13) de Koning, G. J. M.; Scheeren, A. H. C.; Lemstra, P. J.; Peeters, M.; Renaers, H. *Polymer* **1994**, *35*, 4598.
(14) Kumagai, Y.; Kanesawa, Y.; Doi, Y. *Makromol. Chem.* **1992**, *193*, 53.
(15) Tomasi, G.; Scandola, M.; Briese, B. H.; Jendrossek, D. *Macromolecules* **1996**, *29*, 507.
(16) Doi, Y.; Kanesawa, Y.; Kunioka, M.; Saito, T. *Macromolecules* **1990**, *23*, 26.
(17) Shimamura, E.; Scandola, M.; Doi, Y. *Macromolecules* **1994**, *27*, 4429.
(18) Doi, Y.; Kitamura, S.; Abe, H. *Macromolecules* **1995**, *28*, 4822.
(19) Doi, Y.; Segawa, A.; Kunioka, M. *Int. J. Biol. Macromol.* **1990**, *12*, 106.
(20) Vonk, C. G. *J. Appl. Crystallogr.* **1973**, *6*, 148.
(21) Tadokoro, H. *Koubunshi no Kouzou*; Kagaku Doujin: Kyoto, Japan, 1976; p 148.
(22) Hoffman, J. D. *Polymer* **1983**, *24*, 3.
(23) Bloembergen, S.; Holden, D. A.; Hamer, G. K.; Bluhm, T. L.; Marchessault, R. H. *Macromolecules* **1986**, *19*, 2865.
(24) Kunioka, M.; Tamaki, A.; Doi, Y. *Macromolecules* **1989**, *22*, 694.
(25) Cuesta, M. S.; Martinez-Salazar, J.; Barker, P. A.; Barham, P. J. *J. Mater. Sci.* **1992**, *27*, 5335.
(26) Orts, W. J.; Marchessault, R. H.; Bluhm, T. L. *Macromolecules* **1991**, *24*, 6435.
(27) Kamiya, N.; Yamamoto, Y.; Inoue, Y.; Chujo, R. *Macromolecules* **1991**, *24*, 2178.
(28) Hocking, P. J.; Marchessault, R. H.; Timmins, M. R.; Lenz, R. W.; Fuller, R. C. *Macromolecules* **1996**, *26*, 2472.
(29) Saito, T.; Suzuki, K.; Yamato, J.; Fukui, T.; Miwa, K.; Tomita, K.; Nakanishi, S.; Odani, S.; Suzuki, J.; Ishikawa, K. *J. Bacteriol.* **1989**, *171*, 184.
(30) Jendrossek, D.; Müller, B.; Schlegel, H. G. *Eur. J. Biochem.* **1993**, *218*, 701.
(31) Jendrossek, D.; Frisse, A.; Behrends, A.; Andermann, M.; Kratzin, H. D.; Stanislawski, T.; Schlegel, H. G. *J. Bacteriol.* **1995**, *177*, 596.
(32) Mukai, K.; Yamada, K.; Doi, Y. *Int. J. Biol. Macromol.* **1993**, *15*, 361.
(33) Kasuya, K.; Inoue, Y.; Doi, Y. *Int. J. Biol. Macromol.* **1996**, *19*, 35.

MA961195R