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Solid structure and biodegradation of the compositionally fractionated poly(3-hydroxybutyric acid-co-3-hydroxypropionic acid)s

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Abstract

The solid structure and biodegradation behavior of the compositionally fractionated bacterial poly(3-hydroxybutyric acid-co-3-hydroxypropionic acid)s [P(3HB-co-3HP)] were examined. Utilizing the extracellular poly(3-hydroxybutyric acid) [P(3HB)]depolymerase purified from the bacterium strain *Alcaligenes faecalis* T1, enzymatic degradation behavior was investigated for P(3HB), poly(3-hydroxypropionic acid) [P(3HP)]and the fractionated P(3HB-co-3HP)s. It was revealed that P(3HB) and P(3HP) showed the distinguishable depolymerase concentration dependence of enzymatic degradation feature. For the fractionated copolyesters, the results indicated that P(3HB-co-3HP)s with higher 3HB contents which form P(3HB)-type of crystallites exhibited much higher enzymatic degradation rates than those of copolyesters with higher 3HP contents which form P(3HP)-type of crystallites, and that the amorphous copolyester could hardly be degraded. The ^{1}H NMR analyses of the enzymatic degradation products revealed that P(3HB) was first degraded into dimers and then into monomers. In contrast, the presence of oligomers higher than dimers was suggested in the degradation products of P(3HP), and the 3HP content dependence of degradation products was confirmed for the fractionated P(3HB-co-3HP)s. In addition, environmental degradabilities were assessed by BOD method in the river water. The results confirmed the environmental degradabilities of P(3HB) and copolyesters except for P(3HP), indicating the inducing effect of 3HB comonomers on the biodegradation of 3HP units in copolyesters.

Keywords: Poly(3-hydroxybutyric acid-co-3-hydroxypropionic acid)s; Poly(3-hydroxybutyric acid) (P(3HB)); Poly(3-hydroxypropionic acid)

1. Introduction

Poly(hydroxyalkanoic acid)s (PHA), naturally accumulated in the widely distributed microorganisms as the energy storage compounds, are biodegradable thermoplastics with diverse physical properties [1–2]. This class of bacterial polyesters can be degraded into water soluble oligomers, then be re-utilized as nutrients by a large number of environmental bacteria with various origins. Until now, several extracellular poly(3-hydroxybutyric acid) (P(3HB)) depolymerases have already been purified from the bacterial strains such as *Alcaligenes faecalis* [3–5], *Pseudomonas lemoignei* [6–8], *Pseudomonas pickettii* [9], *Pseudomonas stutzeri* [10], *Comamonas testosteroni* [11–12], *Comamonas acidovorans* [13–14] and some *Bacillus* genera [15]. On the basis of characterization of the structural genes associated with expressing the extracellular P(3HB) depolymerases, it has been known that the purified extracellular depolymerases containing 393–488 amino acid residues commonly involve two important functional domains, a catalytic and a substrate-binding domain (SBD), between which a linker region is present [12–14,16,17].

Up to date, enzymatic degradation behavior of a wide spectrum of bacterial polyesters and copolyesters has been extensively studied by many investigators [1,18–21]. For the aliphatic copolyesters such as poly(3-hydroxybutyric acid-co-3-hydroxyvaleric acid)[P(3HB-co-3HV)], poly(3-hydroxybutyric acid-co-4-hydroxybutyric acid)[P(3HB-co-4HB)], poly(3-hydroxybutyric acid-co-3-hydroxyhexanoic acid) [P(3HB-co-3HH)]and poly(3-hydroxybutyric acid-co-lactide) [P(3HB-co-LA)][22–24], the highest rates of enzymatic erosion were revealed at 10–20 mol% contents of the minor 3HV, 4HB, 3HH and LA comonomers. Meanwhile, the biodegradation features were found to be strongly dependent on the factors such as monomer structure, degree of crystallinity, lamellar size and the detailed solid structure. On the contrary, the recent reports revealed that the bacterially synthesized copolyester P(3HB-co-3HV)s had the

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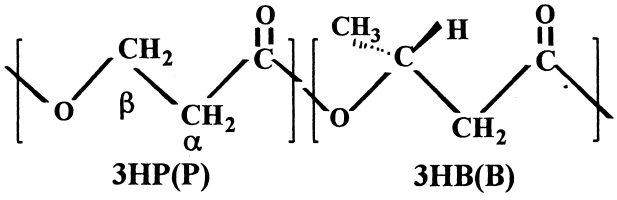
Table 1
Characteristics of the fractionated P(3HB-co-3HP)s[a], P(3HB) and P(3HP)

Sample code	3HP mol%[b]	M_n[c] ($\times 10^{-5}$)	M_w/M_n	Crystallinity (%)[d]	Lattice type[e]	Remarks
P(3HB)	0	2.13	1.52	62.1	P(3HB)	Bacterial origin
AF9	13.2	1.76	1.86	50.9	P(3HB)	Fractionated
AF7	23.8	2.40	2.51	39.1	P(3HB)	Fractionated
AF5	38.3	2.17	2.97	16.1	P(3HB)	Fractionated
AF4	48.0	1.65	3.43	n.d.	n.d.[f]	Fractionated
AF2	60.1	3.33	2.12	0	Amorphous	Fractionated
BF2	89.6	1.83	2.22	44.0	P(3HP)	Fractionated
BF1	95.9	2.50	1.76	57.6	P(3HP)	Fractionated
P(3HP)	100	1.54	2.32	61.7	P(3HP)	Chemosynthetic

[a] Fractionation details were described in Ref. [28].
[b] Determined by ^{1}H NMR in chloroform solution.
[c] Measured by gel permeation chromatography.
[d] Estimated by wide angle X-ray diffraction [28].
[e] Determined by solid state ^{13}C NMR [29].
[f] Not determined.

extremely broad comonomer compositional distributions [25,26]. Hence, even if two original bacterial copolyesters bear the similar average comonomer contents, the detailed solid structures can, however, be discriminated because of their different comonomer compositional distribution features. To exclusively clarify the comonomer content-dependence of biodegradation behavior of bacterial copolyesters, samples bearing no or much narrower comonomer compositional distributions are needed.

Poly(3-hydroxybutyric acid-co-3-hydroxypropionic acid) [P(3HB-co-3HP)]



P(3HB-co-3HP)

is a category of the bacterial copolyester. When compared to P(3HB), P(3HB-co-3HP) possesses the superior physical properties such as lower melting point, higher elongation rate and biodegradability [19]. Further, the variation in 3HP comonomer content can provide the bacterial copolyesters with desirable physical properties and biodegradation features. In the previous studies [27–29], the as-bacterially synthesized copolyester P(3HB-co-3HP)s were fractionated by the difference in the 3HP contents of copolyester chains in the chloroform/*n*-heptane mixed solvent. ^{13}C NMR analyses demonstrated the much narrower comonomer compositional distributions of the fractionated P(3HB-co-3HP)s.

On the basis of the fractionated copolyesters with 3HP contents spanning the whole range of comonomer composition, this article will present an investigation on the comonomer content dependence of the biodegradation behavior. Enzymatic degradation behavior of P(3HB), poly(3-hydroxypropionic acid) [P(3HP)]and the fractionated copolyesters are examined by using the extracellular P(3HB) depolymerase purified from the bacterial strain *Alcaligenes faecalis* T1. After the enzymatic degradation, the water-soluble products are analyzed by means of NMR spectrometer. In addition, the environmental biodegradabilities of homopolymers and the fractionated bacterial copolyester P(3HB-co-3HP)s in the river water are assessed by the biochemical oxygen demand (BOD) method.

2. Experimental

2.1. Materials and purification of extracellular P(3HB) depolymerase

Table 1 describes the detailed characteristics of bacterial P(3HB), chemosynthetic P(3HP) and the fractionated copolyester P(3HB-co-3HP)s [28,29]. Film samples applied to enzymatic degradation were prepared as follows: films of P(3HB), P(3HP) and copolyesters [AF9(13.2 mol% 3HP), AF7(23.8 mol% 3HP), BF2(89.6 mol% 3HP), BF1(95.9 mol% 3HP)] with high and intermediate degrees of crystallinity were prepared using the glass Petri dishes as the casting surfaces. While those of the copolyesters [AF5(38.3 mol% 3HP), AF4(48.0 mol% 3HP), AF2(60.1 mol% 3HP)] with lower or no crystallinity were prepared by repetitively dipping the Teflon sheets (dimension: 1.0 cm × 1.0 cm × 50 μm) into their chloroform solution. The amounts of adhered polymers were manually controlled. Both the pure and Teflon-supported film samples were dried in vacuo, then maintained at ambient temperature for more than one month to prompt the crystallization approaching the equilibrium state prior to characterization.

The extracellular P(3HB) depolymerase from the

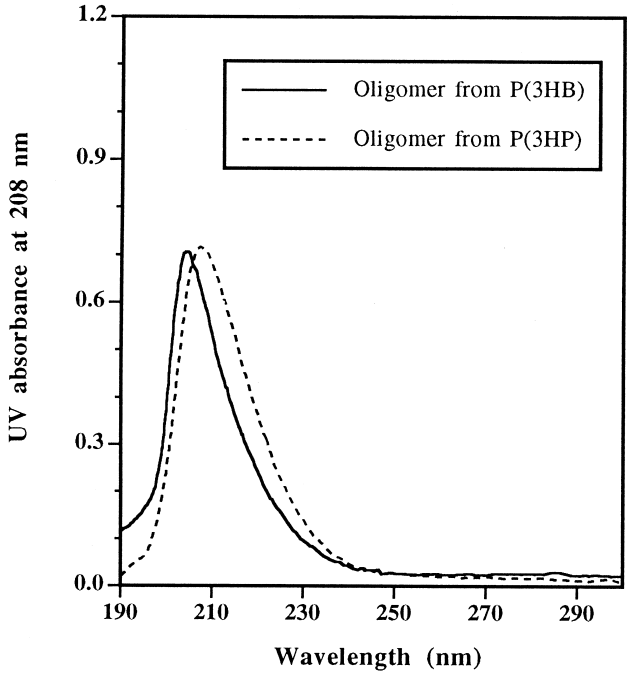


Fig. 1. UV spectra of the water soluble degradation products released from the P(3HB) and P(3HP) films by a catalytic action of *Alcaligenes faecalis* T1 depolymerase.

bacterial strain *Alcaligenes faecalis* T1 was prepared and purified according to the methods as reported in [3–5].

2.2. Analytical procedures

Degradation activity of the purified depolymerase was measured on a UV spectrophotometer (Hitachi U-2000) at 650 nm and 37 ± 0.1°C [13]. The unit of degradation activity was defined as the required amount of enzyme for decreasing the UV absorbance by 1.0 per min. Enzyme concentration was assayed with a Bio-Red protein assay kit using the bovine serum albumin (BSA) as the standard according to the method reported by Bradford [30], and the protein was silver stained. After purification, the final protein concentration and degradation activity were estimated to be 508 μg/ml and 314 units/mg, respectively.

Enzymatic degradation behavior of the fractionated P(3HB-co-3HP)s along with P(3HB) and P(3HP) were monitored at 37 ± 0.1°C and 208 nm on a Shimadzu UV-2100 spectrophotometer. 3.0 ml of 0.1 M potassium phosphate buffer (pH = 7.5) was added into a quartz UV cuvette (light path = 1.0 cm) with a polymer cap, and a required amount of depolymerase was also added. Enzymatic degradation was started by placing a film sample into the reaction solution. The biodegradation rate was derived from the UV absorbance versus degradation time plot (UV method).

Measurements of enzymatic degradation rates were also performed at 37 ± 0.1°C by another conventional method of weight-loss measurement (weight-loss method). Either a polymer or Teflon sheet-supported film was placed into a small bottle containing 1.0 ml of 0.1 M potassium phosphate buffer (pH = 7.5) and a given aliquot of depolymerase solution. After enzymatic degradation, the investigated film was washed with distilled water, and dried to the constant weight in vacuo. The enzymatic degradation rates were estimated in triplicate as the averaged weight loss of the film samples per hour.

Analyses of the water-soluble products of enzymatic degradation were carried out on a JEOL-270 MHz NMR spectrometer in D_2O solution at 30°C. After enzymatic reaction, the reaction solution was filtered with a micro filter (pore size: 0.45 μm), then was lyophilized. The dried water soluble products were dissolved in 1.0 ml of D_2O, and a small amount of sodium 2,2-dimethyl-2-silapentane-5-sulfate (DSS) was added as the internal reference of proton chemical shift.

Measurements of aerobic biodegradation in the river water were carried out on a TAITEC BOD 200F tester at 25°C under gentle stirring [31–32]. A film sample with the same dimension as applied to the enzymatic degradation was placed into a BOD reactor. The reaction solution included 200 ml river water taken from the Tamagawa river (Tokyo, Japan) after the filtration (pore size of filter: 120–160 μm) and 0.2 ml of mineral salts solution. The mineral salts solution contained the inorganic compounds as follows (per liter): 8.50 g of KH_2PO_4, 21.75 g of K_2HPO_4, 33.30 g of $Na_2HPO_4{\cdot}2H_2O$, 1.70 g of NH_4Cl, 22.50 g of $MgSO_4{\cdot}7H_2O$, 27.50 g of $CaCl_2$ and 0.25 g of $FeCl_3{\cdot}6H_2O$. Environmental biodegradability was evaluated in triplicate as the value of the recorded biochemical oxygen demand subtracted by the average value of the controlled-blanks over the theoretical biochemical oxygen demand on an assumption that the total amount of carbon atoms in polymer chains was entirely converted into carbon dioxide. Total dissolved organic compounds (TOC) in the river water before and after BOD experiments were measured on a Shimadzu TOC-5000A total organic carbon analyzer.

3. Results and discussion

3.1. Enzymatic degradation of P(3HB), P(3HP) and the fractionated P(3HB-co-3HP)s

Fig. 1 depicts the UV spectra of water-soluble enzymatic degradation products for P(3HB) and P(3HP). The wavelengths at which the maximum UV absorbance values are observed seem different for the respective degradation products. In this study, the UV absorbance at 208 nm is tentatively utilized to monitor the enzymatic degradation behavior. As it has been reported that the molar extinction coefficients ϵ of the Lambert–Beer's law are proportional to the number of carbonyl functional groups involved in the water-soluble oligomers for P(3HB) [33–34], therefore measuring the UV absorbance can give a quantitative estimation of the total amount of the liberated 3HB units. Here,

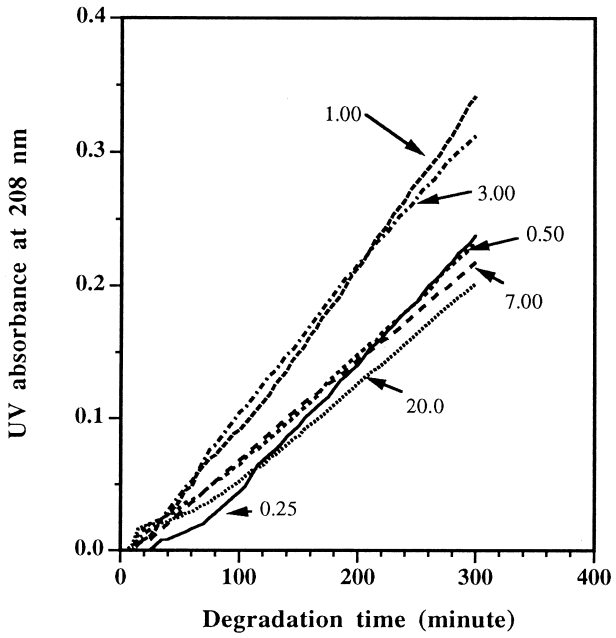


Fig. 2. The degradation time dependence of UV absorbance (208 nm, 37°C) at various depolymerase concentrations for P(3HB). (The values indicated in the figure denote the enzyme concentrations in μg/ml.)

the molar extinction coefficients ϵ_{208} of the 3HB and 3HP units at 37°C in the 0.1 M potassium phosphate buffer (pH = 7.5) are determined to be about 85 and 105 $M^{-1}\,cm^{-1}$ by utilizing (*R*)-hydroxybutyric acid [3HBA] and 3-hydroxypropionic acid [3HPA] as standards.

Fig. 2 shows the degradation time dependence of UV absorbance at various depolymerase concentrations for P(3HB). After a short period of induction, a linear increase

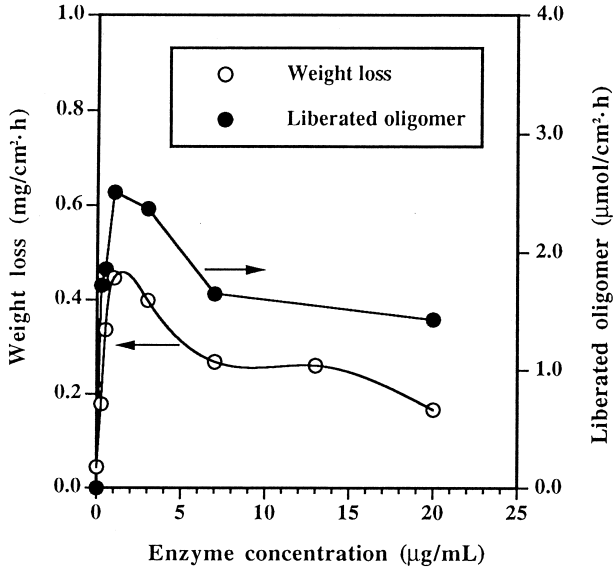


Fig. 3. The effect of concentration of *Alcaligenes faecalis* T1 depolymerase on the biodegradation rates measured by the weight-loss and the UV methods for P(3HB).

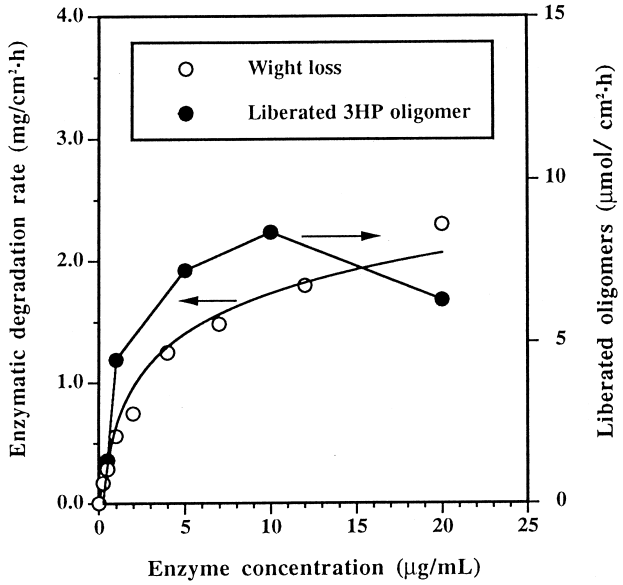


Fig. 4. The effect of concentration of *Alcaligenes faecalis* T1 depolymerase on the biodegradation rates measured by the weight-loss and the UV methods for P(3HP).

of UV absorbance with degradation time is observed. With the above-mentioned values of ϵ_{208}, enzymatic degradation rates which are expressed as the amount of liberated 3HB units per hour were estimated from the inclinations of UV absorbance versus degradation time plot. The results are shown in Fig. 3. Increasing the protein concentration in the reaction buffer, enzymatic degradation rate increases rapidly and approaches a maximum point at about 1.0 μg/ml of protein, then decreases in a gradual manner. This is explainable with the two-step reaction process model [33,35]. i.e. first, the substrate-binding domain (SBD) of depolymerase molecule is absorbed toward the hydrophobic film surface, then the ester bonds in polyester chain are hydrolyzed by the active site in the catalytic domain with substrate specificity. In the case of lower concentration of depolymerase, the increase in protein concentration will lead to the increased amount of the absorbed depolymerase molecules, resulting in the increased degradation rate as a result of the presence of more active sites. However, if the depolymerase concentration further increases to a certain extent, the film surface will be entirely occupied by the SBD of depolymerase molecules, thus prohibiting the active sites to attack the ester bonds. As a result, the enzymatic degradation is ceased. In Fig. 3, the tendency of enzymatic degradation behavior of P(3HB) estimated by the weight-loss measurement is well consistent with that by the UV method.

Enzymatic degradation behavior of chemosynthetic P(3HP) is also studied in the same way as applied to P(3HB). Fig. 4 depicts the depolymerase concentration dependence of enzymatic degradation rate estimated by both the UV and weight-loss methods, indicating a biodegradation feature distinguishable from that of the bacterial

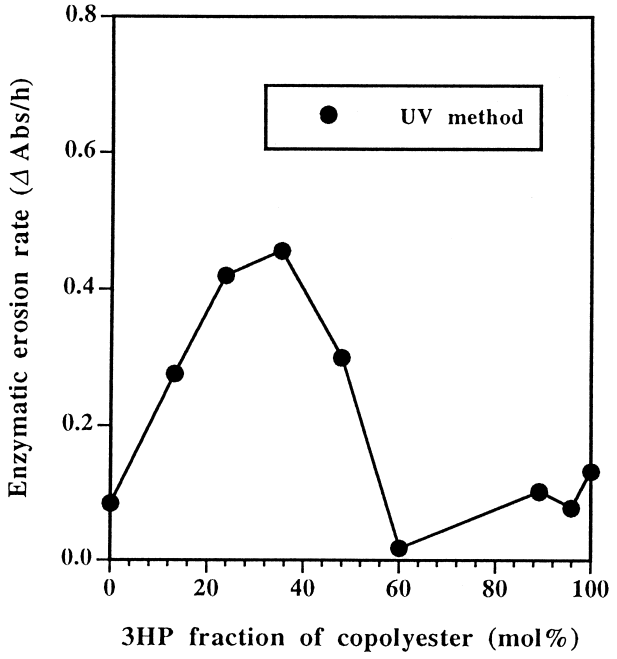


Fig. 5. The 3HP comonomer content-dependence of enzymatic degradation rate estimated by the UV method at a depolymerase concentration of 1.0 μg/ml and 37°C.

P(3HB). P(3HP) shows a much higher enzymatic degradation. The difference in the enzymatic degradation behavior between P(3HB) and P(3HP) can be rationalized with respect to the substrate specificity of the catalytic domain of the extracellular P(3HB) depolymerase from *Alcaligenes faecalis* T1 and the morphology of the polymer film.

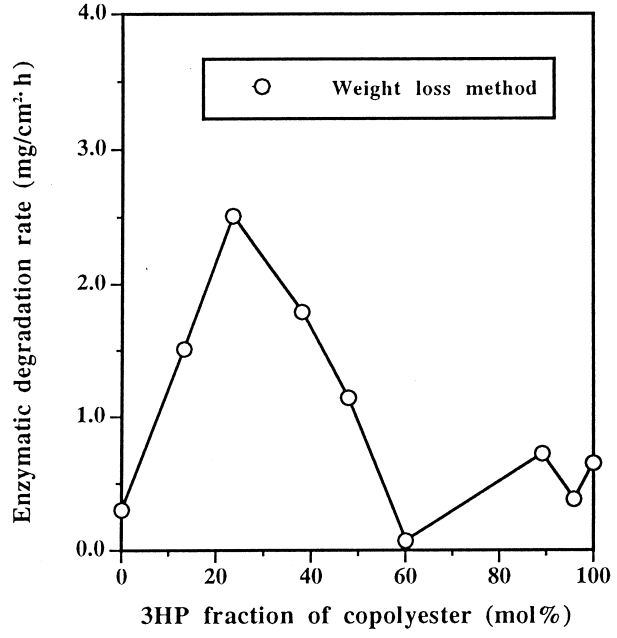


Fig. 6. The 3HP comonomer content-dependence of enzymatic degradation rate estimated by the weight-loss method at a depolymerase concentration of 1.0 μg/ml and 37°C.

On the basis of preliminary investigation on P(3HB) and P(3HP), the enzymatic degradation behavior of the fractionated P(3HB-co-3HP)s are studied. Because of the difference in molar extinction coefficients ϵ_{208} for the 3HB and 3HP units, the biodegradation rates are practically assessed as the increased UV absorbance values at 208 nm per hour (ΔAbs/h), and the results are depicted in Fig. 5. As seen in Fig. 5, the semicrystalline 3HB-rich copolyesters which form P(3HB)-type lattice structures [28–29] exhibit higher degradation rates than those of the semi-crystalline 3HP-rich samples which form P(3HP)-type lattice structures [28–29]. For the semicrystalline 3HB-rich copolyesters, the enzymatic degradation rate increases rapidly with the increase in the 3HP unit content, exhibiting a maximum degradation rate at the 3HP unit content of about 30–40 mol%. It has been reported that the active sites of bacterial depolymerases more facilely attack the ester bonds in the amorphous regions [22,31,36], therefore, a much higher degradation rate in the amorphous regions can be observed in comparison to those in the well-ordered crystalline regions. As seen in Table 1, increasing the 3HP content of copolyester with 3HB-rich comonomer will result in a significant decrease in the degrees of crystallinity. Hence, the apparent enzymatic degradation rate is increased. However, another crucial process of surface adsorption of enzyme as proposed in the two-step reaction model should also be taken into consideration. It has been reported that the amorphous atactic P(3HB) film cannot be degraded by the extracellular depolymerase purified from either the bacterial strain *Alcaligenes faecalis* T1 [31] or *Pseudomonas lemoignei* [37]. This is accounted for the fact that the SBD cannot be adsorbed onto the surface of the amorphous film. Hence, for the P(3HB-co-3HP)s, a further decrease in the crystallinity of copolyester will give rise to a significant decrease in the overall amount of the adsorbed depolymerase molecules, consequently lowering the apparent enzymatic degradation rate. Therefore, it can be concluded that the biodegradation behavior of P(3HB-co-3HP) is regulated by a delicate balance between the above-mentioned two processes, that is, crystal-specific adsorption and amorphous-preferential degradation. In view of Fig. 5, the film of fully amorphous copolyester AF2 with 60.1 mol% 3HP can hardly be eroded by the extracellular depolymerase. This result suggests the crystallinity-dependence of enzymatic degradability [37]. As shown in Fig. 6, the result measured by the weight-loss method indicates a similar tendency of enzymatic degradation behavior as stated above.

3.2. ^{1}H NMR analyses of the enzymatic degradation products

Fig. 7 (top) depicts the ^{1}H NMR spectrum of water-soluble degradation products for P(3HB) after 72 h enzymatic degradation. Because of the disappearance of the methine proton resonance of higher oligomers at about 5.2 ppm [38],

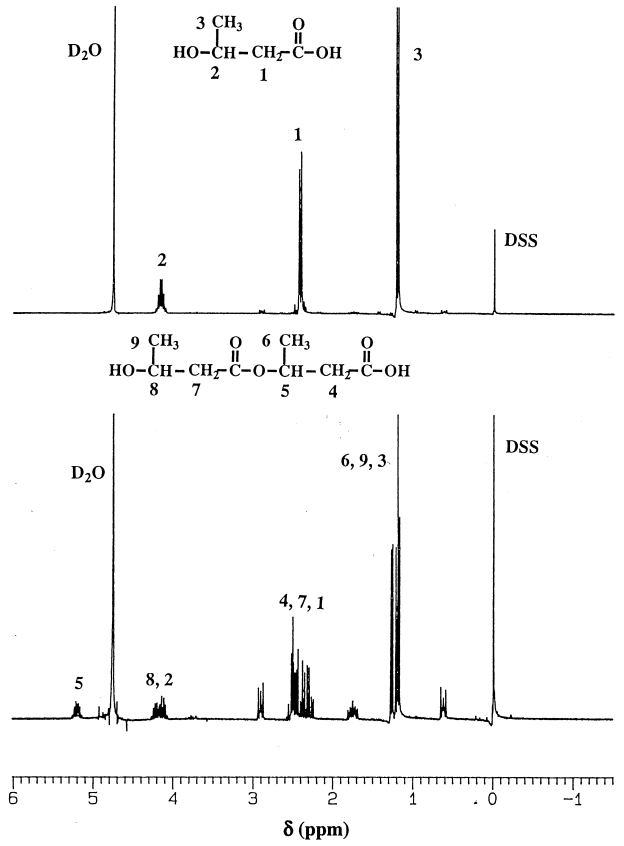


Fig. 7. 270 MHz ^{1}H NMR spectra of the water-soluble products after 72 h (top) and 5 h (bottom) enzymatic degradation at a depolymerase concentration of 1.0 μg/ml and 37°C for P(3HB).

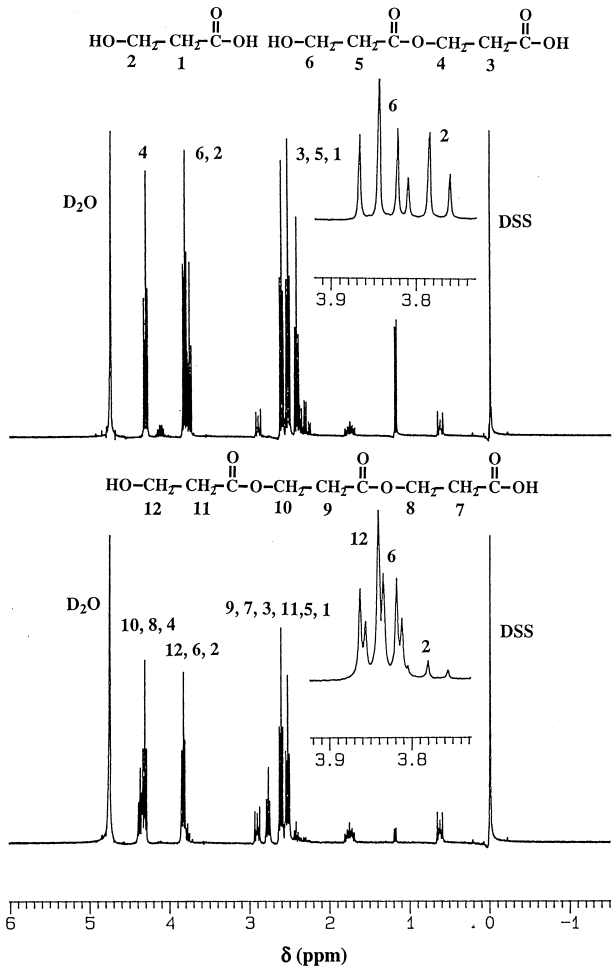


Fig. 8. 270 MHz ^{1}H NMR spectra of the water-soluble products after 72 h (top) and 5 h (bottom) enzymatic degradation at a depolymerase concentration of 1.0 μg/ml and 37°C for P(3HP).

only the presence of monomer in the water-soluble product is confirmed, indicating the complete enzymatic degradation of P(3HB). In contrast, the resultant ^{1}H NMR spectrum of the water-soluble oligomers after 5 h enzymatic erosion of P(3HB) is also presented in Fig. 7 (bottom). It has been reported that the bacterium strain *Alcaligenes faecalis* T1 can simultaneously excrete extracellular depolymerase and oligomer hydrolase when it is cultivated with P(3HB) granules as the sole carbon sources, and that the water-soluble dimers can be more efficiently hydrolyzed into the final products of monomers under the action of oligomer hydrolase [4]. Referring to Refs. [34,38], the proton resonances are assignable to the protons at the individual sites of dimer and monomer structures, and the molar ratio of dimer to monomer is estimated to be about 46:54 after 5 h enzymatic degradation.

On the contrary, the biodegradation feature of P(3HP) revealed by ^{1}H NMR is quite different from that of P(3HB). Fig. 8 (top) depicts the ^{1}H NMR spectra of the water-soluble products for P(3HP) after 72 h enzymatic degradation. The well-resolved resonances are assignable to the protons at the different sites, suggesting the presence of 3HP dimers and monomers (molar ratio dimer:monomer = 60:40). In addition, the results for the water-soluble products after 5 h biodegradation are shown in Fig. 8 (bottom). The resonances are tentatively assigned to the protons attributable to the specific sites of 3HP trimer, dimer and monomer structures. The results indicate that majorities of the enzymatic degradation products for a short period are trimer and dimer, only less amount of 3HP monomer is detected. From these evidences, it can be suggested that the water-soluble oligomers of trimer and dimer were first produced, then the trimers were further degraded into dimers and monomers with the increase in the degradation time, indicating a distinguishable enzymatic degradation behavior from that for P(3HB).

Fig. 9 depicts the ^{1}H NMR spectra of the water-soluble products after 5 h enzymatic degradation for the copolyester P(3HB-co-48.0 mol% 3HP). According to the results of P(3HP), the 3HP methylene proton resonances appeared around 3.8 ppm suggesting the presence of the water-soluble oligomers higher than the dimers. Abe et al. [38] reported that the methine ^{1}H resonances of 3HB trimer split around 5.2 ppm, and that the central 3HB methine proton of the 3HB trimer showed the ^{1}H resonance on the

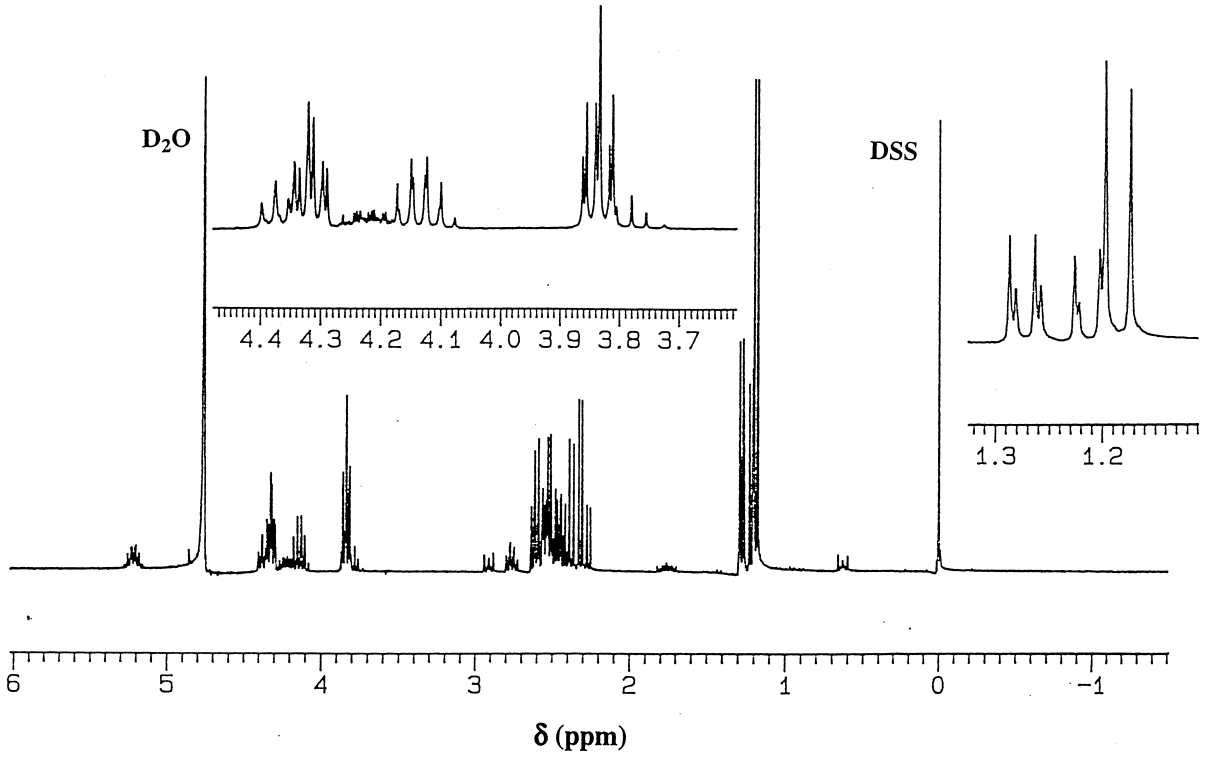


Fig. 9. 270 MHz ^{1}H NMR spectra of the water-soluble products after 5 h enzymatic degradation at a depolymerase concentration of 1.0 μg/ml and 37°C for P(3HB-co-48.0 mol% 3HP).

upfield side of that of the dimer (5.2 ppm). As seen in Fig. 9, no split is found for the 3HB methine ^{1}H resonances appeared around 5.2 ppm. Therefore, it can be suggested that the 3HB units involved in the water-soluble oligomers higher than the dimers are only presented as terminal units. Further, in view of the relatively higher intensities of the ^{1}H resonances at about 1.19 ppm for the methyl protons of the 3HB monomer and the weaker intensities of the ^{1}H resonances at about 3.78 ppm for the methylene protons at the β-site of the 3HP monomer, it can be suggested that less

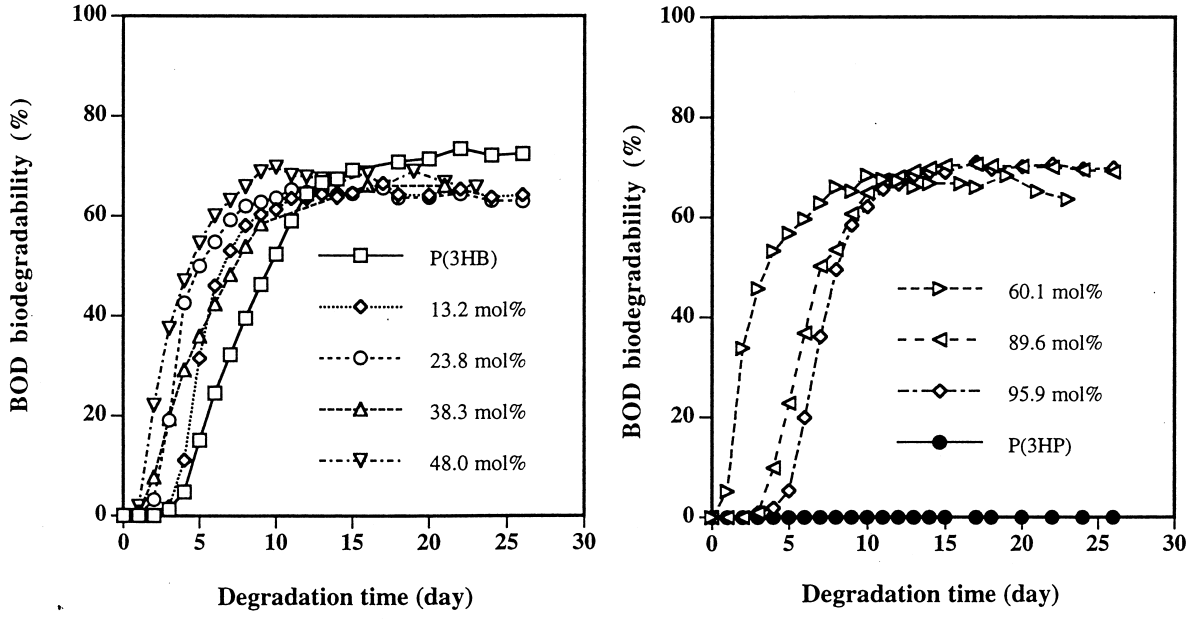


Fig. 10. BOD biodegradabilities of P(3HB), P(3HP) and copolyester P(3HB-co-3HP)s in the river water.

Table 2
BOD biodegradabilities and changes in the total organic carbon (TOC) values of the BOD test solution at 25°C for copolyester P(3HB-co-3HP)s, P(3HB) and P(3HP)

Sample code	3HP mol%[a]	Crystallinity (%)[b]	BOD biodegradability (%)[c]	TOC (ppm)	
				Initial	Final
P(3HB)	0	62.1	72.5	4.40	1.08
AF9	13.2	50.9	64.2	4.40	0.98
AF7	23.8	39.1	63.1	4.40	1.12
AF5	38.3	16.1	66.1	4.40	1.08
AF4	48.0	n.d.[d]	65.8	4.40	1.25
AF2	60.1	0	65.3	4.40	1.23
BF2	89.6	44.0	69.2	4.40	0.96
BF1	95.9	57.6	69.8	4.40	0.85
P(3HP)	100	61.7	0	4.40	1.16

[a] Determined by ^{1}H NMR [28].
[b] Estimated by wide angle X-ray diffraction [28].
[c] Indicates the final BOD biodegradabilities.
[d] Not determined.

amount of 3HP monomer was released, whereas the 3HB-rich sequences were mainly degraded into the final products, that is, the monomers. In addition, the ^{1}H NMR analyses of the fractionated copolyesters with various 3HP unit contents indicate the 3HP-content dependence of enzymatic degradation products (data not shown here). However, strictly, a direct evidence provided by HPLC–^{1}H NMR or HPLC–MS should be needed, and the detailed analysis is in progress in our laboratory.

3.3. Environmental degradation of the fractionated P(3HB-co-3HP)s

Fig. 10 describes the environmental degradation behavior for the P(3HB), P(3HP) and copolyesters P(3HB-co-3HP) assessed by the BOD method in the river water. The BOD biodegradabilities as shown in Fig. 10 were estimated according to the equation

$$\text{Biodegradability (\%)} = \frac{\text{BOD (exp)} - \text{BOD (blank)}}{\text{ThOD}} \times 100\% \quad (1)$$

where BOD(exp), BOD(blank) and ThOD represent the experimental, the control and the theoretical biochemical oxygen demands, respectively. In the case of copolyesters, ThOD values were approximately estimated as

$$\text{ThOD (total)} = \text{ThOD (3HB units)} + \text{ThOD (3HP units)} \quad (2)$$

in which ThOD(3HB units) and ThOD(3HP units) were calculated on the initial comonomer compositions and an assumption that the reactions proceed as follows

$$C_4H_6O_2 + \frac{9}{2}O_2 \rightarrow 4CO_2 + 3H_2O \qquad \text{3HB unit} \quad (3)$$

$$C_3H_4O_2 + 3O_2 \rightarrow 3CO_2 + 2H_2O \qquad \text{3HP unit} \quad (4)$$

As seen in Fig. 10, bacterial copolyester P(3HB-co-3HP)s as well as P(3HB) can be environmentally degraded regardless of their different 3HP contents, degrees of crystallinity and crystalline structures. It is worthwhile to point out that although the copolyester P(3HB-co-95.9 mol% 3HP) contains only about 4 mol% 3HB comonomer, its environmental degradability has been unambiguously confirmed. In contrast, the BOD experimental result explicitly demonstrates that pure P(3HP), that is without the 3HB unit structure, cannot be degraded by the environmental microorganisms in the river water. From these evidences, it can be proposed that the presence of the 3HB unit structure can induce the environmental biodegradation of the P(3HB-co-3HP) copolyesters, i.e. if the 3HB unit structures, are present, the degraders can excrete the extracellular P(3HB)-depolymerase into the environment, then hydrolyze the PHA polymer chains into the water-soluble oligomers. Finally, the environmental microorganisms re-utilize the water-soluble products as nutrients.

On the contrary, the environmental biodegradation behavior of copolyesters with different 3HP contents indicates that the environmental biodegradation behavior are independent of their crystalline structures, i.e. copolyester AF9(13.2 mol% 3HP) and BF1(95.9 mol% 3HP) forming P(3HB)-type and P(3HP)-type crystallites indeed share the similar degradation behavior except for a longer induction period of BF1 as seen in Fig. 10. Interestingly, the amorphous copolyester AF2(60.1 mol% 3HP) exhibits the shortest induction period of 1 day, indicating a fastest degradation rate. As mentioned above, this amorphous copolyester cannot be degraded by the extracellular depolymerase from *Alcaligenes faecalis* T1 because the substrate-binding domains of depolymerase cannot be adsorbed onto the surface of an amorphous copolyester film. Recently, Nakajima-Kambe et al. [39,40] reported a polyester polyurethane depolymerase purified from the soil bacterium strain *Comamonas acidovorans* TB-35, and this

enzyme was bound to the bacterial cell surface. Regarding this fact, it can reasonably suppose the presence of a kind of depolymerase which is bound to the surfaces of microorganisms in the river water. It is this kind of depolymerase that degrades the amorphous copolyesters. i.e. the propagated microorganisms adsorbed onto the film surface can provide the opportunities for active sites of the bound depolymerase molecules to attack the amorphous copolyesters. Further, the fast environmental degradation rates for the amorphous copolyester and the copolyesters with lower crystallinities are accounted for the more facile attack occurring in the amorphous regions.

From Fig. 10, it can also be seen that both copolyesters and P(3HB) exhibit approximately the similar final BOD degradability of about 70%. In order to clarify this result, TOC analyses of the BOD reaction solution before and after the environmental degradation were implemented, and the results are shown in Table 2. It indicates no appreciable reduction in the values of total dissolved organic carbon after the BOD experiment, suggesting that the remaining 30% organic carbon may be converted into biomass and energy [31,41].

4. Conclusions

To sum up, in this article, the solid structure and biodegradation behavior of the fractionated copolyester P(3HB-co-3HP)s, P(3HB) and P(3HP) have been investigated. Through monitoring the UV absorbance at 208 nm, it was revealed that the dependences of enzymatic degradation rate on the protein concentration of the extracellular depolymerase from *Alcaligenes faecalis* T1 showed different features for the bacterial P(3HB) and the chemosynthetic P(3HP). For P(3HB), a maximum degradation rate was observed at a protein concentration of about 1.0 μg/ml. Although the P(3HP) exhibited a higher degradation rate than that of P(3HB) at a given depolymerase concentration of 1.0 μg/ml, the fractionated copolyester P(3HB-co-3HP)s which form P(3HB)-type crystallites showed faster enzymatic degradation rates than those of the copolyesters which can organize P(3HP)-type lattice structures. It was found that the amorphous copolyester could hardly be degraded. The probable reason for this result may be that the substrate-binding domains of depolymerase cannot be adsorbed onto the film surface of an amorphous copolyester. ^{1}H NMR analyses of the water-soluble degradation products indicated that the P(3HB) was first degraded into dimer and monomer structures, then hydrolyzed into the final products, i.e. the monomers. In contrast, the presence of the trimer structure was suggested in the enzymatic degradation products of P(3HP) in addition to the dimer and monomer structures. For the copolyester P(3HB-co-3HP)s, it was revealed that the fractions of constituents of degradation products strongly depended on their 3HP comonomer compositions, and that the liberated 3HB comonomers were only presented as the termini of oligomers higher than the dimers. Moreover, the environmental degradation behavior of the above-mentioned polymers were measured by the BOD method. It was demonstrated that the copolyesters possessing various 3HP contents exhibited the environmental biodegradabilities up to about 70% similar to that of the bacterial P(3HB), while the environmental degradability of the pure P(3HP) was not detected. From this evidence, it can be suggested that the presence of 3HB unit structure can induce the environmental degradation of copolyesters regardless of their different 3HB comonomer contents. The fast environmental degradation rate observed for an amorphous copolyester may be accounted for the presence of a kind of extracellular depolymerase which is bound to the surface of the environmental microorganisms.

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