Enzymatic and Environmental Degradation of Racemic Poly(3-hydroxybutyric acid)s with Different Stereoregularities

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Received June 24, 1996; Revised Manuscript Received September 25, 1996[⊗]

ABSTRACT: Racemic poly(3-hydroxybutyric acid) (P(3HB)) was prepared by the ring-opening polymerization of racemic β-butyrolactone (β-BL) in the presence of methylaluminoxane and fractionated into five components with various isotactic diad fractions ([*i*]) varying from 0.88 to 0.54. The degree of X-ray crystallinity of isotactic P(3HB) films decreased from 33 to 8% as the [*i*] value was decreased from 0.88 to 0.54. A predominantly syndiotactic P(3HB) ([*i*] = 0.30) was prepared by the polymerization of racemic β-BL in the presence of 1-ethoxy-3-chlorotetrabutyldistannoxane, and its crystallinity was 26%. Enzymatic degradation of P(3HB) films with different stereoregularities was carried out at 37 °C in 0.1 M potassium phosphate buffer (pH 7.4) in the presence of PHB depolymerase purified from *Alcaligenes faecalis*. The highest rate of enzymatic hydrolysis was observed at an [*i*] value of 0.74. Syndiotactic P(3HB) film was hardly hydrolyzed by the enzyme. The enzymatic degradation products of racemic P(3HB) samples ([*i*] = 0.88−0.54) were a mixture of monomer, dimer, trimer, and tetramer of 3-hydroxybutyric acid and showed negative values (−11 ± 1°) of specific optical rotation, which suggests that PHB depolymerase hydrolyzes selectively the ester bond connecting the methine carbon of the (*R*)-3HB unit. The amount of PHB depolymerase adsorbed on the surface of P(3HB) films was measured at 37 °C. PHB depolymerase adsorbed on the surface of predominantly isotactic P(3HB) films, while little adsorption of enzyme took place on the surface of syndiotactic P(3HB) film, which suggests that the binding domain of PHB depolymerase has an affinity toward the isotactic P(3HB) crystalline phase. In addition, biodegradation tests of P(3HB) films were carried out under aerobic conditions in river water. The racemic P(3HB) film with [*i*] = 0.63 was degraded completely within 28 days at 25 °C in the river water, and the decomposed products containing (*R*)- and (*S*)-3HB units were metabolized by microorganisms.

Introduction

A wide variety of bacteria synthesize an optically active polymer of (*R*)-3-hydroxybutyric acid and accumulate it as an intracellular storage material of carbon and energy.[1−3] Poly[(*R*)-3-hydroxybutyric acid] (P[(*R*)-3HB]) isolated from bacteria is a biodegradable thermoplastic with a melting temperature around 180 °C. A remarkable characteristic of P[(*R*)-3HB] is its biodegradability in the environment.[1,2] Aerobic and anaerobic P[(*R*)-3HB]-degrading bacteria and fungi have been isolated from various environments.[4−6] The microorganisms excrete extracellular PHB depolymerases to degrade P[(*R*)-3HB] and utilize the decomposed compounds as nutrients. Several PHB depolymerases have been purified from some microorganisms such as *Pseudomonas lemoignei*,[7] *P. pickettii*,[8] *P. fluoresens*,[9] *Alcaligenes faecalis*,[4] *Comamonas testosteroni*,[10] and *Penicillium pinophilum*.[11] The structural genes of PHB depolymerases of *A. faecalis*,[12] *P. lemoignei*,[13−15] *P. pickettii*,[16] and *Comamonas* sp.[17] have been cloned and sequenced. Analysis of the primary structure revealed that the enzymes of 393−488 amino acids are composed of two functional domains and a region linking the two domains. One is a catalytic domain containing the catalytic triad of serine, aspartate, and histidine, while the other is a substrate-binding domain which adsorbs on the surface of partially crystalline P[(*R*)-3HB] and orients the catalytic domain toward the substrate.[12,18]

Recently, chemosynthetic poly(3-hydroxybutyric acid) (P(3HB)) containing monomeric units of both (*R*)- and (*S*)-3HB has been shown to be hydrolyzed by microbial PHB depolymerases. The chemical synthesis of P(3HB) has been achieved by the ring-opening polymerization of mixtures of (*R*)- and (*S*)-β-butyrolactone (β-BL) in the presence of aluminum-, zinc-, or tin-based catalysts.[19,20] Kemnitzer *et al.*[20] prepared P(3HB) stereocopolymers by the stereorandom copolymerization of (*R*)-β-BL with (*S*)-β-BL at various feed monomer ratios in the presence of $ZnEt_2/H_2O$ catalyst. The fraction of (*R*)-3HB units in P[(*R*,*S*)-3HB] was varied from 6 to 94%, and the stereochemical effects on the rate of enzymatic degradation by the PHB depolymerase from *P. funiculosum* were studied. They demonstrated that the rate of enzymatic degradation of P[77%(*R*)-3HB] was several times higher than the rate of microbial P[(*R*)-3HB], but that P[94%(*S*)-3HB] was hardly hydrolyzed by the enzyme. In a previous paper,[19] we studied the physical properties and enzymatic degradability of P[(*R*,*S*)-3HB] stereocopolymers prepared by the ring-opening copolymerization of (*R*)- and (*S*)-β-BL at various feed ratios (*R*/*S* = 96/4−50/50) in the presence of 1-ethoxy-3-chlorotetrabutyldistannoxane as a catalyst. The isotactic diad fraction [*i*] of P(3HB) stereoisomers decreased from 0.92 to 0.30 as the fraction of (*R*)-β-BL in feed was decreased from 0.96 to 0.50. The physical and thermal properties of P[(*R*,*S*)-3HB] stereoisomers were shown to be strongly dependent on the stereoregularity. It was shown that the rates of enzymatic degradation of P[(*R*,*S*)-3HB] films ranging in [*i*] value from 0.68 to 0.92 were higher than that of microbial P[(*R*)-3HB] film ([*i*] = 1.00). However, the amorphous P[70%(*R*)-3HB] sample ([*i*] = 0.46) and syndiotactic P[50%(*R*)-3HB] sample ([*i*] = 0.30) were hardly hydrolyzed by the PHB depolymerases from *P. pickettii* and *A. faecalis*.

Stereoblock copolymers of racemic P(3HB), with sequences of predominantly (*R*) and predominantly (*S*) units, have been prepared by the polymerization of racemic β-BL with aluminum-based catalysts, and

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⊗ Abstract published in *Advance ACS Abstracts,* December 15, 1996.

S0024-9297(96)00907-2 CCC: $12.00

fractionated to yield a range of tacticities.[31] Doi *et al.*[22] and Jesudason *et al.*[23] demonstrated that low-crystalline P(3HB) fractions were hydrolyzed more readily than more crystalline, predominantly isotactic P(3HB) fractions in the presence of PHB depolymerase from *A. faecalis*. More recently, Hocking *et al.*[24,25] fractionated racemic P(3HB) polymers into several components with a wide range of isotacticities varying from 0.35 to 0.88 and studied the stereochemical effects on the rate of enzymatic degradation with PHB depolymerases from *P. lemoignei* and *A. fumigatus*. Low-crystalline P(3HB) components (55−60% isotactic diads) showed significantly higher rates of enzymatic degradation than those of more crystalline P(3HB) components with either an isotactic or a syndiotactic crystal structure.

In this study, we prepare racemic P(3HB) samples with stereoregularities ranging from isotactic to syndiotactic, and the stereochemical effects on the enzymatic degradability of P(3HB) films in the presence of PHB depolymerase from *A. faecalis* are studied. The water-soluble products produced during the enzymatic degradation of P(3HB) film are characterized by HPLC analysis and optical rotation measurement. The adsorption kinetics of PHB depolymerase on the surface of P(3HB) samples are studied, and the mechanism of enzymatic hydrolysis of racemic P(3HB) films is discussed. In addition, environmental biodegradation tests of racemic P(3HB) films are carried out under aerobic conditions in natural river water.

Experimental Section

Materials. 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] Racemic β-butyrolactone ((*R,S*)-β-BL) (Aldrich Chemical Co.) was dried by CaH_2 and distilled under reduced pressure.

Synthesis of Polymers. Predominantly isotactic poly(3-hydroxybutyric acid) (P(3HB)) samples with different stereoregularities were synthesized by the ring-opening polymerization of (*R,S*)-β-BL in the presence of methylaluminoxane (MAO) (Toso Akuzo Co.) as a catalyst. The monomer (20 mL, 240 mmol), MAO catalyst (5 mL, 15 mmol), and toluene (10 mL) were admitted into a reactor under nitrogen atmosphere. The polymerization of β-BL was carried out at 60 °C for 7 days under a nitrogen atmosphere. The P(3HB) produced was dissolved in chloroform and precipitated in cold methanol at 0 °C. The precipitate was dried in vacuo at room temperature. The whole polymer was fractionated by solubility in acetone at several temperatures in the range from 0 to 56.5 °C for 10 h. Predominantly syndiotactic P(3HB) was prepared by the polymerization of (*R,S*)-β-BL with a distannoxane catalyst without solvent at 100 °C for 4 h under a argon atmosphere.[19]

Preparation of P(3HB) Films. P(3HB) films (about 0.07 mm thickness) were prepared by solvent-casting techniques from chloroform solutions of P(3HB) using glass Petri dishes as casting surfaces. The films were then 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 enzymatic degradation of P(3HB) films (initial weights: about 8 mg, initial film dimensions: 10 × 10 mm in area, about 0.07 mm in thickness) by the purified PHB depolymerase was carried out at 37 °C in 0.1 M potassium phosphate buffer (pH 7.4), as reported in previous papers.[19,34] The weight loss data were averaged on at least three film samples. The pH values of phosphate buffer remained almost unchanged during the enzymatic degradation.

Adsorption of PHB Depolymerase. The adsorption of PHB depolymerase was studied on the surface of P(3HB) films by the method of Kasuya *et al.*[28] A P(3HB) film (initial film dimensions: 10 × 10 mm in area, about 0.07 mm in thickness) was placed in a test tube containing 1.0 mL of 0.1 M potassium phosphate buffer (pH 7.4), and it was preincubated at 37 °C for 5 min. Then, a given amount (0.5−7.0 μg) of PHB depolymerase from *A. faecalis* was added to the test tube. Since the amount of enzyme adsorbed on the surface increased with time to attain a constant value around 2 h,[28] the reaction mixture was incubated at 37 °C for 3.5 h with shaking. After the incubation for 3.5 h, a portion (100 μL) of the supernatant was separated, and the amount of PHB depolymerase in the supernatant was determined by activity measurement for the hydrolysis of *p*-nitrophenylbutyric acid (PNPB) at 37 °C. The relative amount of PHB depolymerase unadsorbed on the surface of P(3HB) film, defined as the amount of PHB depolymerase in the aqueous phase relative to the amount of PHB depolymerase added, was calculated from the esterase activity assay of PNPB hydrolysis. The reaction solution contained 100 μL of 1% solution of PNPB (Sigma) in a mixture of Thesit (Boehringer Mannheim) and dioxane, 400 μL of the dilute supernatant with water, and 500 μL of 0.1 M potassium phosphate buffer (pH 7.4). The formation of *p*-nitrophenol during the hydrolysis of PNPB was monitored on a Hitachi U-2000 spectrophotometer by the time-dependent change in absorbance at 400 nm. It was confirmed that the rate of PNPB hydrolysis increased proportionally with the concentration of PHB depolymerase up to 1.0 μg/mL. The relative amount of unadsorbed enzyme in the aqueous phase was determined from the rate of hydrolysis of PNPB. The adsorption data were averaged on at least three film samples.

Environmental Degradation Test. The biodegradation test was conducted by a modified version of MITI test.[29] All tests were performed under aerobic conditions in a temperature-controlled BOD reactor (Taitec BOD tester) at 25 °C with stirring.[30] A sample film (initial weight about 8 mg; initial thickness about 0.07 mm) was placed in a 300 mL BOD reactor, and 200 mL of natural water from the river Arakawa (Saitama, Japan) was added to the reactor. In addition, 0.2 mL of mineral salts solution was added to the river water. The mineral solution contained the following (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 $FeCl_3 \cdot 6H_2O$. The biodegradation test was carried out at 25 °C for 28 days, and the biochemical oxygen demand (BOD) was measured continuously with a BOD meter. At the end of the test, the dissolved organic carbon concentration (DOC) of the test solution was measured on a Shimadzu TOC-5000. The sample films were removed after the biodegradation test, washed with distilled water, and dried to constant weight in vacuo before analysis. The BOD biodegradability of the P(3HB) film was calculated by subtracting the biochemical oxygen demand of the control blank (BOD_b) from that of the test solution (BOD_t) and by dividing the value ($BOD_t - BOD_b$) by the theoretical oxygen demand (ThOD) of the test sample. The BOD_b of control blank was determined by averaging four blank tests using 200 mL of the river water without test samples. The weight-loss biodegradability of P(3HB) film was calculated by dividing the weight of residual film after testing by its initial weight. The BOD, DOC, and weight loss data were averaged on at least two film samples.

Analytical Procedures. The gel-permeation chromatography (GPC), nuclear magnetic resonance (NMR), differential scanning calorimetry (DSC), X-ray diffraction, and high-performance liquid chromatography (HPLC) analyses were carried out along the literature procedures reported in previous papers.[19,34]

The optical rotation of the water-soluble degradation products was measured at 589 nm (Na lamp) on a JASCO DIP-370 digital polarimeter at 23 °C using water as a solvent. Sample concentrations ranged from 4 to 8 g/L, and a quartz cell (cell dimensions: 3.5 mm diameter, 100 mm length) was used for the analysis.

Results and Discussion

Synthesis and Properties of Racemic P(3HB). Racemic P(3HB) polymers produced were fractionated

Table 1. Molecular Weights and Isotactic Diad Fractions of P(3HB) Samples

sample no.		fractionation condition	molecular weight $M_n \times 10^{-3}$	molecular weight M_w/M_n	isotactic diad fraction, [*i*]
1	100%iso-P[(*R*)-3HB][a]		281	2.3	1.00
2	88%iso-P[(*R*,*S*)-3HB][b]	insoluble in boiling acetone	57.8	8.3	0.88
3	74%iso-P[(*R*,*S*)-3HB]	soluble in boiling acetone (56.5 °C)	21.6	12.8	0.74
4	63%iso-P[(*R*,*S*)-3HB]	soluble in acetone at 40 °C	14.9	11.0	0.63
5	58%iso-P[(R,S)-3HB]	soluble in acetone at 25 °C	2.8	13.7	0.58
6	54%iso-P[(R,S)-3HB]	soluble in acetone at 0 °C	5.6	7.3	0.54
7	70%syn-P[(R,S)-3HB][c]		145	1.8	0.30

[a] Microbial P[(*R*)-3HB] was produced from butyric acid by *Alcaligens eutrophus*. [b] Predominantly isotactic P[(*R*,*S*)-3HB] samples (sample nos. 2−6) were prepared by polymerization of racemic *β*-BL with methylaluminoxane as a catalyst and fractionated with acetone as a solvent. [c] Syndiotactic P[(*R*,*S*)-3HB] was synthesized by ring-opening polymerization of racemic *β*-BL with 1-ethoxy-3-chlorotetrabutyldistannoxane as an initiator.

Table 2. Thermal Properties and X-ray Crystallinities of P(3HB) Samples

sample no.	isotactic diad fraction, [*i*]	T_g,[a] °C	T_m,[a] °C	ΔH_m,[a] J/g	X_c,[b] %
1	1.00	4	177	90	62 ± 5
2	0.88	5	166	42	33 ± 5
3	0.74	2	110	22	18 ± 3
4	0.63	4	64	10	12 ± 3
5	0.58	3	50	5	10 ± 3
6	0.54	0	51	3	8 ± 3
7	0.30	6	52, 62	26	26 ± 5

[a] Determined from DSC measurement. [b] X-ray crystallinity.

by using acetone as a solvent at several temperatures in the range from 0 to 56.5 °C. Table 1 shows the number-average molecular weights (M_n), polydispersities (M_w/M_n), and isotactic diad fractions ([*i*]) of the fractionated P(3HB) samples. The [*i*] values were determined from the relative peak areas of carbonyl carbon resonances in ^{13}C NMR spectra of the polymers.[21] The five P(3HB) samples (2−6) with isotactic diad contents ranging from 0.88 to 0.54 were obtained from the acetone fractionation. In addition, a predominantly syndiotactic P(3HB) sample (7) was obtained by the polymerization of racemic (*R*,*S*)-*β*-BL with 1-ethoxy-3-chlorotetrabutyldistannoxane.[19]

The glass-transition temperatures (T_g), melting temperatures (T_m), and enthalpies of fusion (ΔH_m) of solvent-cast P(3HB) films were determined from DSC thermograms. The results are given in Table 2, together with the values for microbial P[(*R*)-3HB] film. The T_g values of the P(3HB) samples were observed at 3 ± 3 °C, independent of the stereoregularity of P(3HB). In contrast, the T_m value of the P(3HB) sample decreased from 166 to 50 °C as the isotactic diad fraction [*i*] was decreased from 0.88 to 0.54. The value of ΔH_m decreased also with the decrease in the [*i*] value. A 70%syn-P[(*R*,*S*)-3HB] had two endothermic peaks at around 52 and 62 °C.[19] The crystalline structures of racemic P(3HB) samples were characterized by X-ray diffraction. The racemic P(3HB) samples (2−6) with [*i*] values ranging from 0.54 to 0.88 showed the same isotactic P(3HB) crystalline structure as microbial P[(*R*)-3HB] (1). In contrast, the 70%syn-P[(*R*,*S*)-3HB] sample (7) has the crystalline structure of syndiotactic P(3HB).[19] The degrees of X-ray crystallinity (X_c) of solvent-cast P(3HB) films with different stereoregularities are listed in Table 2. The X_c values of isotactic P(3HB) films decreased from 33 to 8% as the [*i*] value was decreased from 0.88 to 0.54.

Enzymatic Degradation of P(3HB) Films. Enzymatic degradation of racemic P(3HB) films with different stereoregularities was carried out at 37 °C in 0.1 M potassium phosphate buffer (pH 7.4) in the presence of PHB depolymerase (1.0 *μ*g) from *A. faecalis*. Figure 1 shows the enzymatic erosion profiles of P(3HB) films. For the P(3HB) films with [*i*] values of 0.54−0.74, the weight loss of film increased proportionally with time over 48 h by the action of PHB depolymerase. The racemic P(3HB) film with [*i*] = 0.88 was eroded only during the initial stage of enzymatic reaction, but no more weight loss of the film occurred after 10 h. Microbial P[(*R*)-3HB] film was eroded proportionally with time by the function of PHB depolymerase, while little erosion took place on the syndiotactic P(3HB) film. In the absence of PHB depolymerase, the P(3HB) films were not hydrolyzed for over 48 h in 0.1 M potassium phosphate buffer at 37 °C. The initial rate of erosion was calculated from the slope of the line obtained by plotting film erosion against time. Figure 2 shows the rates of enzymatic degradation of P(3HB) films by PHB depolymerase. The initial rate of enzymatic erosion of racemic P(3HB) films increased markedly as the [*i*] value was decreased from 0.88 to 0.74 and showed a maximum value at an [*i*] value of 0.74. The highest erosion rate of racemic P(3HB) film was about twice as fast as the rate of microbial 100%iso-P[(*R*)-3HB] film. A very slow rate of enzymatic degradation was observed on the film of 70%syn-P[(*R*,*S*)-3HB]. In this work, we did not study the effect of molecular weight of P(3HB) on the rate of enzymatic erosion.

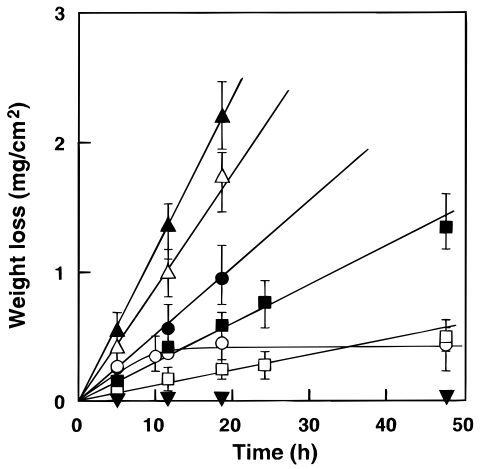

Figure 1. Enzymatic erosion profiles of P(3HB) films at 37 °C in aqueous solution (pH 7.4) containing PHB depolymerase (1.0 *μ*g/mL) from *A. faecalis*: (●) 100%iso-P[(*R*)-3HB]; (○) 88%iso-P[(*R*,*S*)-3HB]; (▲) 74%iso-P[(*R*,*S*)-3HB]; (△) 63%iso-P[(*R*,*S*)-3HB]; (■) 58%iso-P[(*R*,*S*)-3HB]; (□) 54%iso-P[(*R*,*S*)-3HB]; (▼) 70%syn-P[(*R*,*S*)-3HB].

Characterization of Water-Soluble Degradation Products. In a previous paper,[19] we demonstrated that the enzymatic hydrolysis of microbial P[(*R*)-3HB] film

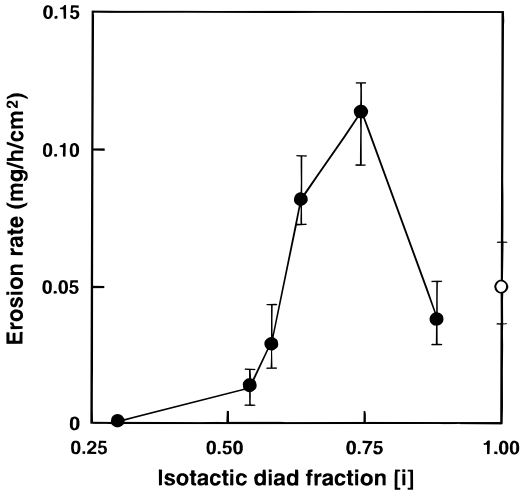

Figure 2. Effect of stereoregularity on the initial rate of erosion of P(3HB) films during the enzymatic degradation with PHB depolymerase (1.0 μg/mL) from *A. faecalis*.

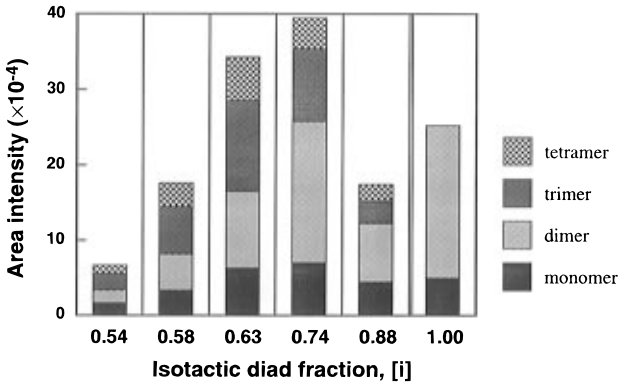

Figure 3. Weight distributions of water-soluble products after enzymatic degradation for 12 h of P(3HB) films with different stereoregularities.

by PHB depolymerases produced a mixture of monomer and dimer of 3-hydroxybutyric acid as water-soluble products. In this study, we measured by HPLC analysis the composition of water-soluble products after enzymatic degradation of racemic P(3HB) films. Figure 3 shows the weight distributions of water-soluble products after the enzymatic degradation of five P(3HB) films with different stereoregularities for 12 h at 37 °C. It has been found that the PHB depolymerase from *A. faecalis* acts as an *endo*-type hydrolase toward the pentamer and higher oligomers of (*R*)-3HB units and that the rate of enzymatic hydrolysis for the dimer of (*R*)-3HB units is much slower than the rates for oligomers and polymers of (*R*)-3HB units.[27] As the result, microbial 100%iso-P[(*R*)-3HB] is hydrolyzed into monomer and dimer of (*R*)-3HB by PHB depolymerase. As Figure 3 shows, racemic P(3HB) samples gave trimer and tetramer as degradation products in addition to monomer and dimer. The compositions of the water-soluble products were determined from the relative peak areas of monomer and oligomers in the HPLC curves. The number-average sequence length of 3HB repeat units in the water-soluble oligomers after enzymatic degradation of racemic P(3HB) films for 12 h ranged from 1.92 to 2.05 (see Table 3).

Table 3 gives the specific rotation values of the water-soluble products of racemic P(3HB) samples after enzymatic degradation for 12 h. The water-soluble oligomers may contain (*R*)-3HB and (*S*)-3HB units at the same amount. The specific rotation of enzymatic degradation products from racemic P(3HB) films showed negative values of $-11 \pm 1°$. The specific rotation value of (*R*)-3-hydroxybutyric acid was −24.5° at 23 °C in water. The optical rotation of the degradation products may arise from the methine carbon of the hydroxyl-terminus 3HB units of the oligomers, which would suggest that the stereochemical configuration of the methine carbon of the hydroxyl-terminus 3HB units in enzymatic degradation products of racemic P(3HB) is of the (*R*) configuration. A PHB depolymerase may hydrolyze selectively with the ester bonds connecting the methine carbons of (*R*)-3HB units, while no reaction may occur with the ester bonds connecting the methine carbons of (*S*)-3HB units, resulting in the formation of oligomers of 3HB units.

Table 3. Specific Rotation Values of Water-Soluble Degradation Products

sample	number-average sequence length[a]	specific rotation $[\alpha]_D^{23}$,[b] deg
58%iso-P[(*R*,*S*)-3HB]	2.05	−11.4
63%iso-P[(*R*,*S*)-3HB]	2.04	−10.2
74%iso-P[(*R*,*S*)-3HB]	1.92	−11.0
100%iso-P[(*R*)-3HB]	1.68	−16.1
(*R*)-3HB[c]	1.00	−24.5

[a] Number-average sequence length of 3HB units for degradation products. [b] Water was used as a solvent, and the concentration ranged from 4 to 8 g/L. The specific rotations were independent of concentration. [c] (*R*)-3-Hydroxybutyric acid.

As reported by Hocking *et al.*,[21] a highly isotactic 88%iso-P[(*R*,*S*)-3HB] sample (2) may be a stereoblock copolymer of racemic P(3HB), with sequences of predominantly (*R*) and predominantly (*S*) units. The sequences of predominantly (*R*)-3HB units are hydrolyzed by PHB depolymerase, while the sequences of predominantly (*S*)-3HB units are not hydrolyzed.[20] After P[(*R*)-3HB] sequences on the surface of highly isotactic P(3HB) sample are hydrolyzed by the enzyme, the enzymatically inactive P[(*S*)-3HB] sequences may cover the surface of film. As a result, the enzymatic degradation of highly isotactic sample would be inhibited after the initial stage of enzymatic reaction.

Adsorption of PHB Depolymerase on P(3HB) Films. Saito *et al.*[12,18,32] have demonstrated that the PHB depolymerase (MW 47 kDa) of *A. faecalis* has a hydrophobic domain (5 kDa) as a binding site to adhere to the hydrophobic surface of P[(*R*)-3HB] film, in addition to a catalytic domain (32 kDa) containing an active site. In a previous paper,[33] the kinetics and mechanism of surface hydrolysis of P[(*R*)-3HB] films were studied using three types of PHB depolymerase from *A. faecalis*, *P. pickettii*, and *C. testosteroni*. The kinetic data were accounted for in terms of a heterogeneous enzymatic hydrolysis reaction, involving two steps of adsorption and hydrolysis where the first step is an adsorption of enzyme on the surface of P[(*R*)-3HB] film by the binding domain and the second step is a hydrolysis of P[(*R*)-3HB] chain by the catalytic domain.

In this study, we measured the amounts of PHB depolymerase adsorbed on the surface of P(3HB) films at 37 °C. The amount of enzyme adsorbed on the surface of P(3HB) films was determined from the difference in the rates of hydrolysis of *p*-nitrophenylbutyric acid (PNPB) in aqueous enzyme solutions with and without P(3HB) film along the method by Kasuya *et al.*[28] Figure 4 shows the relationship between the amount of enzyme added to a solution (1 mL) and the amount of enzyme adsorbed on the surfaces of P(3HB)

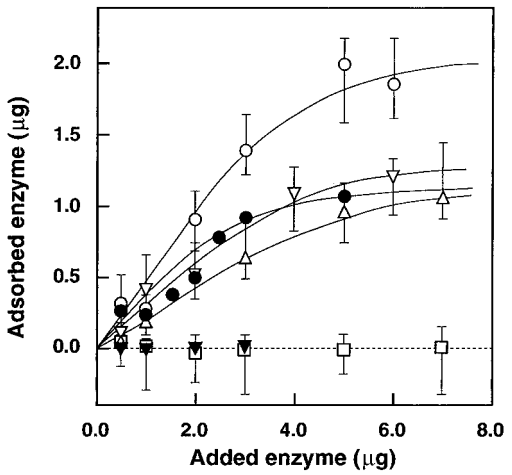

Figure 4. Relation between the amounts of adsorbed and added enzyme during the enzymatic hydrolysis of various P(3HB) films in the presence of PHB depolymerase from *A. faecalis* at 37 °C: (●) 100%iso-P[(*R*)-3HB]; (○) 88%iso-P[(*R*,*S*)-3HB]; (△) 63%iso-P[(*R*,*S*)-3HB]; (□) 54%iso-P[(*R*,*S*)-3HB]; (▼) 70%syn-P[(*R*,*S*)-3HB]; (▽) 100%iso-P[(*R*)-3HB]/70%syn-P[(*R*,*S*)-3HB] (50/50 wt/wt).

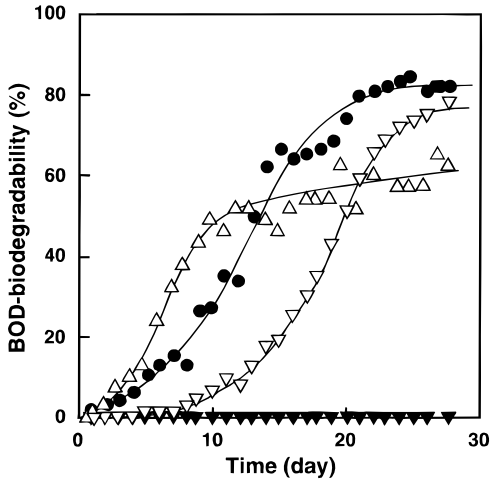

Figure 5. Typical BOD-biodegradation curves of P(3HB) films in the river water at 25 °C: (●) 100%iso-P[(*R*)-3HB]; (△) 63%iso-P[(*R*,*S*)-3HB]; (▼) 70%syn-P[(*R*,*S*)-3HB]; (▽) 100%iso-P[(*R*)-3HB]/70%syn-P[(*R*,*S*)-3HB] (50/50 wt/wt).

films with different isotacticities. The PHB depolymerase adsorbed on the surfaces of films of microbial P[(*R*)-3HB] and predominantly isotactic P[(*R*,*S*)-3HB] with [*i*] values of 0.88 and 0.63, while little amount of enzyme adsorbed on the surfaces of films of lower isotactic 54%iso-P[(*R*,*S*)-3HB] and predominantly syndiotactic 70%syn-P[(*R*,*S*)-3HB].

Kasuya *et al.*[28] investigated the kinetics of adsorption of PHB depolymerase (*A. faecalis*) at 37 °C on the surface of microbial P[(*R*)-3HB] film and found that the adsorption of PHB depolymerase could be expressed by the Langmuir adsorption equation (1).

$$[\mathrm{E}]_a = [\mathrm{E}]_0\left(\frac{K[\mathrm{E}]_e}{1 + K[\mathrm{E}]_e}\right) \quad (1)$$

where $[\mathrm{E}]_0$ and K are the maximum amount of enzyme adsorbed on the surface of the P(3HB) film and the adsorption equilibrium constant of enzyme, respectively, and $[\mathrm{E}]_a$ and $[\mathrm{E}]_e$ are the amount of adsorbed enzyme and the concentration of enzyme in an aqueous solution at the adsorption equilibrium. From Figure 4, the values of $[\mathrm{E}]_0$ and K at 37 °C were determined to be ($[\mathrm{E}]_0 = 1.1 \pm 0.1$ μg, $K = 0.89 \pm 0.05$ mL/μg), (2.9 ± 0.2, 0.38 ± 0.08), and (2.0 ± 0.2, 0.19 ± 0.1) for films of 100%iso-P[(*R*)-3HB], 88%iso-P[(*R*,*S*)-3HB], and 63%iso-P[(*R*,*S*)-3HB], respectively. The adsorption equilibrium constant K is defined by the ratio of rates of adsorption and desorption of enzyme. The rate of adsorption of enzyme may be dependent on the stereochemical structure of P(3HB) chains on the surface of film. Since the PHB depolymerase hardly adsorbs on the surface of atactic and syndiotactic P(3HB) films, the rate of enzymatic erosion is low, as shown in Figures 1 and 2.

As can be seen from Figure 4, the PHB depolymerase also adsorbed on the surface of a blend film of microbial 100%iso-P[(*R*)-3HB] with 70%syn-P[(*R*,*S*)-3HB] (50/50 wt/wt) ($[\mathrm{E}]_0 = 1.5 \pm 0.1$ μg, $K = 0.56 \pm 0.08$ mL/μg). The 100%iso-P[(*R*)-3HB]/70%syn-P[(*R*,*S*)-3HB] blend film was hydrolyzed completely by PHB depolymerase.[34] As reported in a previous paper,[34] the rate of enzymatic erosion on the surface of the blend film was higher than that of P[(*R*)-3HB], and trimer and tetramer of 3-hydroxybutyric acid were produced as enzymatic degradation products with monomer and dimer, which indicated that a portion of ester bonds in the syndiotactic P[(*R*,*S*)-3HB] chain was hydrolyzed by the active site in the catalytic domain of PHB depolymerase. It may be concluded that the binding domain of PHB depolymerases hardly binds to syndiotactic P[(*R*,*S*)-3HB] chains, but the active site hydrolyzes the ester bonds of syndiotactic chain.

It is well known that the rate of enzymatic erosion of microbial P[(*R*)-3HB] film increases with a decrease in the crystallinity and that a PHB depolymerase hydrolyzes predominantly P[(*R*)-3HB] chains in the amorphous state on the surface of film.[35,36] The rate of enzymatic hydrolysis of P(3HB) film may be expressed by equation (2).

$$\text{rate} = k[\mathrm{E}]_a \quad (2)$$

where k is the rate constant of hydrolysis of P(3HB) chains by an adsorbed enzyme molecule, and $[\mathrm{E}]_a$ is the amount of adsorbed enzyme. A decrease in the crystallinity of P(3HB) film may lead to an increase in the value of rate constant k. In contrast, the value of $[\mathrm{E}]_a$ apparently decreases with a decrease in the isotacticity of racemic P(3HB) chains. The rate constant k of hydrolysis for 63%iso-P[(*R*,*S*)-3HB] chains should be larger than that for 88%iso-P[(*R*,*S*)-3HB] chains, while

Table 4. Weight-Loss Biodegradabilities and BOD Biodegradabilities of P(3HB) Films and Changes in DOC Values of Test Solution (River Arakawa Water) for 28 Days at 25 °C

sample no.		weight-loss biodegradability, %	BOD biodegradability, %	DOC, mg of C/L initial	DOC, mg of C/L final
1	100%iso-P[(*R*)-3HB]	100 ± 0	75 ± 8	10.5	5.2 ± 1.2
4	63%iso-P[(*R*,*S*)-3HB]	100 ± 0	61 ± 5	10.4	4.9 ± 0.6
7	70%syn-P[(*R*,*S*)-3HB]	1 ± 1	1 ± 1	10.4	3.9 ± 0.2
8	100%iso-P[(*R*)-3HB]/70%syn-P[(*R*,*S*)-3HB] (50/50 wt/wt)	100 ± 0	75 ± 5	10.4	4.6 ± 0.5

the adsorption equilibrium constant K for highly isotactic 88%iso-P[(*R*,*S*)-3HB] sample was larger than that for the surface of 63%iso-P[(*R*,*S*)-3HB] sample. The film of low isotactic 54%iso-P[(*R*,*S*)-3HB] was apparently hydrolyzed by the enzyme in Figure 2, while little amount of enzyme adsorbed on the film in Figure 4. This result may suggest that the rate of constant k of hydrolysis for 54%iso-P[(*R*,*S*)-3HB] is very large.

Environmental Degradation of P(3HB) Films. The environmental degradations of four films of microbial 100%iso-P[(*R*)-3HB], 63%iso-P[(*R*,*S*)-3HB], 70%syn-P[(*R*,*S*)-3HB], and a binary blend of 100%iso-P[(*R*)-3HB]/70%syn-P[(*R*,*S*)-3HB] (50/50 wt/wt) were performed under aerobic conditions in temperature-controlled reactors containing 200 mL of natural water from the river Arakawa as an inoculum. The biodegradabilities were determined by monitoring the time-dependent changes in the biochemical oxygen demand (BOD), weight loss (erosion) of P(3HB) film, and dissolved organic carbon concentration (DOC) of the test solution.[30]

Figure 5 shows typical BOD-biodegradation curves of P(3HB) films in the river water at 25 °C. The BOD biodegradability of microbial P[(*R*)-3HB] film increased with time to reach around 80% at 28 days. The film of 63%iso-P[(*R*,*S*)-3HB] was degraded in the river water at a rapid rate comparable to that of microbial P[(*R*)-3HB]. The film of 70%syn-P[(*R*,*S*)-3HB] was hardly eroded in the river water at 25 °C. It is noted that the blend film of 100%iso-P[(*R*)-3HB]/70%syn-P[(*R*,*S*)-3HB] (50/50 wt/wt) was eroded in the river water, and the BOD-biodegradability of blend film increased with time to reach around 75% at 28 days. The results (Figure 5) of environmental degradation of P(3HB) films in the river water are almost consistent with the results (Figure 1) of enzymatic degradation with PHB depolymerase.

Table 4 lists the weight-loss and BOD biodegradabilities of P(3HB) films after 28 days of the test and the changes in the dissolved organic carbon concentration (DOC) of the test solution before and after the biodegradation. After 28 days of the test, the films of microbial 100%iso-P[(*R*)-3HB], racemic 63%iso-P[(*R*,*S*)-3HB], and 100%iso-P[(*R*)-3HB]/70%syn-P[(*R*,*S*)-3HB] blend were completely eroded, and the weight-loss biodegradabilities were 100%. The DOC values of test solutions decreased from 10.5 to 4.6−5.2 mg of C/L for 28 days. The difference between DOC values of test solutions and of control blank at 28 days, which is attributed to water-soluble intermediates produced during the biodegradation of samples, was within 1 mg of C/L, which corresponds to about 5% of total carbon weight of added samples. On the other hand, the difference between weight-loss biodegradability and BOD biodegradability for the P(3HB) samples was in the range 25−40%. These results suggest that the films of 63%iso-P[(*R*,*S*)-3HB] and of 100%iso-P[(*R*)-3HB]/70%syn-P[(*R*,*S*)-3HB] blend were completely hydrolyzed into water-soluble products by the function of extracellular PHB depolymerases from PHB-degrading microorganisms and that the majority of the products consisting of oligomers of (*R*)- and (*S*)-3HB units was utilized by microorganisms for energy generation and biomass formation. These results confirm that dimer, trimer, and tetramer containing (*S*)-3HB units are metabolized by microorganisms in the environment.

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MA960907A