Visualization of Enzymatic Degradation of Poly[(*R*)-3-hydroxybutyrate] Single Crystals by an Extracellular PHB Depolymerase†

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ABSTRACT: Single crystals of bacterial poly[(*R*)-3-hydroxybutyrate] (P(3HB)) with different molecular weights (M_n = 2500 and M_n = 48 000) were prepared in a mixture of chloroform and ethanol. The enzymatic degradation of P(3HB) single crystals with extracellular PHB depolymerase purified from *Pseudomonas stutzeri* YM1006 was investigated by transmission electron microscopy, atomic force microscopy, and gel-permeation chromatography. Adsorption of PHB depolymerase on P(3HB) single crystals was examined using an immuno-gold labeling technique, which demonstrated a homogeneous distribution of enzymes on the surface of crystals. Unselective adsorption of PHB depolymerase suggests that the enzyme adheres to hydrophobic surfaces of single crystals and contributes to increase in the mobility of P(3HB) chains as a whole. Enzymatic degradation of single crystals progressed from the edge of crystals, or along their long axis as making a small fragment. Both lamellar thicknesses of single crystals and molecular weights of P(3HB) chains remained unchanged during the enzymatic hydrolysis. It has been concluded that the attack by the active site of PHB depolymerase takes place preferentially at the disordered chain-packing regions of crystal edge rather than the chain-folding surfaces of single crystals, in spite of the unselective adsorption of enzyme and that untied or exposed P(3HB) chains are degraded predominantly by *exo*-type behavior of enzyme.

Introduction

Poly[(*R*)-3-hydroxybutyrate] (P(3HB)) and its copolyesters are produced by a wide variety of bacteria[1,2] and have certain superior physical properties comparable to those of chemosynthetic polyesters.[3] In addition, P(3HB) is a biodegradable thermoplastic with a melting temperature around 180 °C. The biodegradability of P(3HB) has been evaluated in various environments such as in soil, sludge, or seawater.[3]

Recently, the extracellular PHB depolymerases have been purified from some bacteria, which were isolated from various environments, such as *Pseudomonas lemoignei*,[4] *Alcaligenes faecalis*,[5] *Comamonas testosteroni*,[6] *Pseudomonas stutzeri*,[7] and *Pseudomonas pickettii*.[8] The characterizations of the structural genes have revealed that PHB depolymerases of 393−488 amino acids are organized with two domains of binding and catalytic functions and the linker domain.[4,6,9,10] Such structures have been already reported in other enzymes which depolymerize water-insoluble materials such as cellulase,[11] xylanase,[11−13] chitinase,[14,15] and arabinofuranosidase.[13]

The enzymatic degradations of P(3HB) and its copolymers have been studied using solution-cast films[16] and melt-crystallized films[17−19] with various degrees of crystallinity. Regarding these films, however, there remain some problems for elucidation of degradation mechanism by atomic level such as distribution of crystal regions, lamellar crystal size, etc. Accordingly, we prepared the single crystals which have clear, uniform, and well-defined structure for carrying out the detailed investigation of adsorption and degradation by enzyme.

The enzymatic degradation of single crystals has been investigated in the field of polysaccharide. Chanzy *et al.*[20] studied the enzymatic degradation of $\beta(1 \rightarrow 4)$xylan single crystals with xylanases, and the observed electron micrographs and diffraction patterns suggested that *endo*-type enzymatic attack took place from the edges of crystals toward their centers. The digestion of lamellar single crystals of nigeran with mycodextranase was reported by Marchessault *et al.*; [21] *endo*-type attack occurred to the disordered surfaces of single crystals which had increasing mobility as temperature was raised.

P(3HB) single crystals, up to this point, have been prepared from many kinds of solvents, chloroform/ethanol,[22,23] propylene carbonate,[17,24−28] polyethylene glycol,[29,30] etc., and the surface morphology and crystal structure were investigated using transmission electron microscopy. However, regarding the enzymatic degradation of P(3HB) single crystals, there exists only one paper: Hocking *et al.*[31] have prepared single crystals of bacterial P(3HB) and synthetic racemic P(3HB) of varying tacticities and elucidated the implications of tacticity on the enzymatic degradation reaction mechanism using two types of PHB depolymerases, the fungus *Aspergillus fumigatus* and the bacterium *Pseudomonas lemoignei* by both turbidimetric and titrimetric assays and molecular weight measurement.[32] On the basis of the observed results, they concluded that preferential degradation occurred from the crystal edges rather than the chain folds of the lamellar surfaces and supported the hypothesis of a combined *endo*−*exo* degradation.

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† This paper is dedicated to the 60th birthday of Professor Dieter Seebach.

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On the other hand, with respect to the adsorption mechanism of enzyme to the substrate, cellulases have been extensively studied. For example, Chanzy *et al.*[33] reported visualization of 1,4-β-D-glucan cellobiohydrolase I adsorbed on cellulose microfibrils using colloidal gold labeling. Gilkes *et al.*[34] also reported the adsorption of both *endo*-β-1,4-glucanase A and its isolated cellulose-binding domain to Valonia cellulose microcrystals which have a well-defined and high crystallinity. In both cases, the adsorption of enzyme occurred preferentially at the crystal edges instead of the crystal surface for the binding. However, the adsorption of PHB depolymerase to single crystals has not previously been reported.

In this paper, we have attempted to obtain more insight on the degradation mechanism of P(3HB) single crystals by an extracellular PHB depolymerase purified from *Pseudomonas stutzeri* YM1006, using transmission electron microscopy, atomic force microscopy, and gel-permeation chromatography. Immuno-gold labeling technique is also adopted to elucidate the adsorption of enzyme to single crystals.

Experimental Section

P(3HB) Samples. The bacterial P(3HB) (number-average molecular weight (M_n) = 358 000 and polydispersity (DPI) = 2.8) sample was purchased from Aldrich Chemical Co., Ltd. The P(3HB) sample was purified by reprecipitation in hexane from chloroform solution and dried *in vacuo* for 1 week. Two kinds of different molecular weight P(3HB)s (LMW-P(3HB), M_n = 2600 and DPI = 1.5, and HMW-P(3HB), M_n = 48 000 and DPI = 2.6) were prepared by 5 N or 1 N aqueous KOH alkali-hydrolysis with 18-crown-6 ether, respectively. Purified P(3HB) (200 mg) was dissolved in chloroform (24 mL). After adding 8 mL of 5N or 1N aqueous KOH and 40 mg of 18-crown-6 ether to the P(3HB) solution, the mixture was placed at 35 °C with stirring. The organic layer was pipetted into a sample vessel and dried over anhydrous magnesium sulfate. After filtering, the organic layer was precipitated into methanol.

Preparation of Single Crystals. The single crystals of P(3HB) were grown from dilute solution according to a method derived from that of Marchessault *et al.*[23] P(3HB) (1 mg) was dissolved into 0.5 mL of chloroform at 30 °C, and then 3.5 mL of ethanol at 60 °C was added. The mixture was then brought quickly to 73 °C and kept there for 10 min, after which slow cooling was applied until 60 °C, and the mixture was kept there for 6 h. Slow cooling was applied by cutting off the heating element of a silicone oil bath. The crystals were partially dissolved by reheating to 73 °C for 10 min. Improved crystals were then obtained by lowering the temperature to 60 °C and waiting for 6 h. After repeating this temperature cycle one or three times, slow cooling to room temperature was then applied. The crystals were collected by centrifugation and washed three times with room temperature ethanol. For enzymatic degradation, the crystals were collected by centrifugation, washed with 50 mM Tris-HCl buffer, and resuspended in the same buffer.

Purification of Extracellular PHB Depolymerase. *Pseudomonas stutzeri* YM1006 isolated from seawater[7] was precultured in a basal mineral medium containing 0.4% sodium succinate at 30 °C for 24 h. Aliquots (4 mL) of the culture were transferred to 200 mL of medium containing 0.2% P(3HB) as a sole carbon source, and the strain was cultivated under aerobic conditions at 30 °C in 500 mL Sakaguchi flasks with occasional shaking. After 48 h the culture was centrifuged at 10000*g* for 30 min to remove the bacterial cells, and the supernatant (3000 mL) was collected for the enzyme preparation. All purification procedures of enzyme were carried out at 0−4 °C.

The supernatant was brought to a final concentration of 0.3 M with solid ammonium sulfate and kept for 1 h. After the precipitate was discarded by centrifugation at 10000*g* for 30 min, the supernatant was applied to a Butyl-Toyopearl column (3 × 30 cm) equilibrated with 10 mM phosphate buffer (pH 7.0) containing 0.3 M ammonium sulfate. The hydrophobic interaction chromatography column was washed with two bed volumes of the same buffer, and the adsorbed enzyme was eluted with a linear gradient of ammonium sulfate from 0.3 to 0 M, and then with a linear gradient of ethanol from 0 to 40%. Fractions with a high activity were pooled and dialyzed to 20 mM Tris-HCl buffer (pH 7.5). These fractions were concentrated by Sumikagel (Sumitomo Chemical Industrial) for 2 days. The concentrated enzyme solution was dialyzed against 20 mM Tris-HCl buffer (pH 7.5) and stored at 4 °C.

Enzymatic Degradation. Degradation of P(3HB) single crystals was monitored using a turbidimetric assay. A 4 μL sample of a 200 μg/mL solution of an extracellular PHB depolymerase purified from *P. stutzeri* YM1006 was added to 1 mL of 50 mM Tris-HCl buffer (pH 7.0) containing 0.435 mg/mL P(3HB) single crystals in a transparent plastic cuvette and then incubated at 37 °C. The turbidity (m_{OD}) at 660 nm (OD_{660}) was measured during the enzymatic hydrolysis up to 120 min.

To observe the time-dependent changes in the morphology of single crystals, 1 μL of diisopropyl fluorophosphate was added into the reaction mixture at various reaction periods. The mixture was then centrifuged at 3000*g* for 3 min, and the supernatant was removed. The crystals were then redispersed in ethanol and washed several times by centrifugation and resuspended in ethanol.

Molecular Weight Measurement. Molecular weights of all P(3HB) single crystals before and after enzymatic degradation were obtained by gel-permeation chromatography (GPC) at 40 °C, using a Shimadzu 10A GPC system and 6A refractive index detector with joint columns of Shodex K-80M and K-802 (each 4.6 × 300 mm). Chloroform was used as an eluent at a flow rate of 0.8 mL/min, and a sample concentration of 1.0 mg/mL was employed. The number-average and weight-average molecular weights (M_n and M_w) were calculated by using a Shimadzu Chromatopac C-R7A plus equipped with a GPC program. Polystyrene standards with a low polydispersity (Shodex Standard SM-105, 1.3×10^3 to 3.1×10^6) were used to generate a calibration curve.

Immuno-gold Labeling. Antiserum against *P. stutzeri* YM1006 extracellular PHB depolymerase was given by injecting 7 mg of PHB depolymerase mixed with the complete Freund's adjuvant into a mature white New Zealand rabbit. A booster injection of 3 mg of PHB depolymerase was applied in the same manner 3 weeks later. The antiserum titer was determined with Western-blotting method.[35] The antiserum, 6 weeks later, was collected and stored at 4 °C. Anti-rabbit IgG (whole molecule) gold conjugate (10 nm nominal particle diameter) was obtained from Sigma Bio Sciences.

A 4 μL sample of a 200 μg/mL solution of *P. stutzeri* YM1006 extracellular PHB depolymerase was mixed with 1 mL of a 0.435 mg/mL suspension of P(3HB) single crystals in 50 mM Tris-HCl buffer (pH 7.0). The mixture was incubated at 37 °C for 30 min to allow adsorption of enzyme to the surface of single crystals and then washed three times by centrifugation with DIG1 buffer containing 1% skim milk and 0.5 M NaCl (DIG1-SM-NaCl buffer). The washed P(3HB) single crystals were resuspended in 1 mL of DIG1-SM-NaCl buffer, mixed with 10 μL antiserum against PHB depolymerase, and incubated at room temperature for 3 h. The P(3HB) single crystals were washed once with DIG1-SM-NaCl buffer, twice with DIG1 buffer containing only 0.5 M NaCl, and once with DIG1 buffer alone to remove unbonded antiserum against PHB depolymerase. The P(3HB) single crystals were resuspended in 200 μL of DIG1 buffer containing 0.1% PEG (nominal M_w = 20 000), mixed with 10 μL of anti-rabbit IgG gold conjugation and incubated at room temperature for 30 min. Then, the gold-labeled preparation was washed once with DIG1 buffer containing 0.05% PEG, once with DIG1 buffer containing 0.02% PEG, and finally resuspended in 200 μL of DIG1 buffer containing 0.02% PEG, for deposition on carbon-coated electron microscope grids.

Table 1. Purification of PHB Depolymerase from *P. stutzeri* YM1006

steps	total activity (units)	total protein (mg)	specific activity (units/mg)	recovery (%)
supernatant	1480	49.3	30.0	100
Butyl-Toyopearl	360	9.6	37.8	24.3

Electron Microscopy. Drops of P(3HB) crystals suspension, before and after enzymatic degradation, were deposited on carbon-coated grids, allowed to dry, and then shadowed with a Pt−Pd alloy. For electron diffraction purposes, the crystals were only allowed to dry. Small drops of P(3HB) crystals suspension, with and without gold labeling, were placed on carbon-coated grids, and then allowed to dry. These grids were observed on a JEM-2000FX II electron microscope operated at an acceleration voltage of 200 kV for electron diffraction and 120 kV for the imaging of shadowed crystals. Images and electron diffraction diagrams were recorded on Kodak SO-163 films developed for 4 min with Kodak D19 developer (diluted in water 1/2, v/v).

Atomic Force Microscopy. The thicknesses of P(3HB) single crystals, before and after enzymatic degradation, were investigated on the basis of Atomic Force Microscopy (AFM). AFM was performed with a SPI3700/SPA300 (Seiko Instruments Inc.). Pyramid-like Si_3N_4 tips, mounted on 100 μm long microcantilevers with spring constants of 0.09 N/m were applied for the contact mode experiments. Simultaneous registration were performed in the contact mode for height and deflection images. Drops of crystal suspension, before and after enzymatic degradation, were given on mica and allowed to dry. All images were recorded at room temperature.

Results and Discussion

Properties of PHB Depolymerase. A P(3HB) degrading bacterium, *Pseudomonas stutzeri* YM1006, was isolated from seawater (Kanagawa, Japan). When succinate, citrate, glucose, fructose, and olive oil were used as sole carbon source, *P. stutzeri* YM1006 grew well, but PHB depolymerase activity was not detected in the culture supernatants. In contrast, the bacterium secreted an extracellular PHB depolymerase on cultivation with several carbon sources such as P(3HB), 3-hydroxybutyric acid, and 4-hydroxybutyric acid. The activity of PHB depolymerase was maximum from the end of logarithmic growth phase to the stationary phase on the cultivation of *P. stutzeri* YM1006 in an aqueous medium containing 0.2% P(3HB) granules as a sole carbon source at 30 °C.

The purification of an extracellular PHB depolymerase was performed by a hydrophobic interaction (Butyl-Toyopearl) column chromatography. The purification steps of the enzyme are summarized in Table 1. Finally, 24.3% of total enzyme was recovered and about 4.1-fold purified enzyme with the specific activity of 37.8 units/mg was obtained. After these procedures, the purified enzyme was homogeneous as judged by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS−PAGE). The molecular weight of PHB depolymerase was about 60 000 as estimated by SDS−PAGE. The N-terminal amino acid sequence of purified enzyme was determined as Gly-Gln-Thr-Phe-Ser-Tyr-Thr-Ser-Pro-Gln-Gln-Ala-Tyr-Ser-Gly-Ser-Arg-Glu-Arg-Ser by Edman degradation. The optimum pH for the hydrolysis of P(3HB) by this enzyme was 7.0, and the enzyme was stable at temperatures below 55 °C. Details on biochemical and molecular properties of the extracellular PHB depolymerase from *P. stutzeri* YM1006 will be published elsewhere.[36]

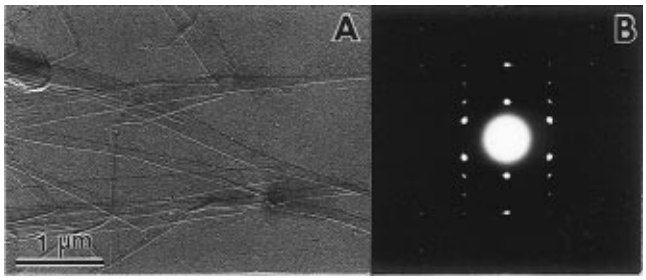

Figure 1. (A) Electron micrograph after shadowing with a Pt−Pd alloy of LMW-P(3HB) single crystals grown from a mixture of chloroform and ethanol (1/7 v/v), and (B) typical electron diffraction pattern corresponding to a single crystal.

P(3HB) Single Crystals. A typical electron micrograph of the LMW-P(3HB) single crystals (M_n = 2500) obtained in this study is shown in Figure 1A. The LMW-P(3HB) single crystals occur as monolamellar lath-shaped crystals with dimensions of around 0.3 μm along the short and of around 5−10 μm along the long axes. Each monolamellar lath-shaped LMW-P(3HB) single crystal yields a sharp electron diffraction pattern as shown in Figure 1B. The diagram contains 25 independent reflections that are mirrored in the four quadrants defined by the two orthogonal axes a^* and b^*. On the basis of P(3HB) having an orthogonal crystal system,[37] the polymer chains align perpendicular to the lamellar base of the crystal.

Figure 2 shows an AFM image of LMW-P(3HB) single crystals and line profile data. The measurements of the thicknesses of crystals yield values of around 5 nm. This result is consistent with that of Sykes *et al.*[27] using the single crystals prepared from an oligomer of 32 HB units, which was revealed to fold once within a crystal, grown in propylene carbonate. Number-average molecular weight (M_n), degree of polymerization (DPn), and polydispersity (DPI) of single crystals measured by GPC, with a calibration curve of polystyrene standards, are 2500, 30, and 1.4, respectively. On the basis of the thickness and DPn of single crystals combined with 0.596 nm of fiber repeat distance,[37] the chain foldings at lamellar surface may not occur in the LMW-P(3HB) single crystals.

A typical preparation of HMW-P(3HB) single crystals (M_n = 48 000) used for this study is illustrated in Figure 3A. It consists of multilamellar lath-shaped crystals grown from aggregation center. The crystals, which consist of stacks of lamellae, have variable thickness: some are transparent to the electron beam, whereas others are completely opaque. When tested by electron diffraction against monolamellar part (Figure 3B), a given crystal displays a well-defined orthogonal pattern consisting of sharp spots. On the basis of both selected-area electron diffraction diagrams of LMW- and HMW-P(3HB) single crystals, it was confirmed that the long axis of a crystal was its crystallographic *a* axis, as previously reported.[17,28,30,38]

The AFM image and line profile data of HMW-P(3HB) single crystals are shown in Figure 4. In spite of a difference in the molecular weight, the monolamellar thicknesses of single crystals are almost 5 nm. This suggests that the chain foldings occur at lamellar surfaces of single crystals as like polyethylene[39,40] and polypropylene.[41] The thicknesses of multilamellar part are the multiple of monolamellar thickness (around 5 nm). This means that crystal starting points from aggregation center are different, whereas chain foldings occur same as monolamellar during the crystallization.

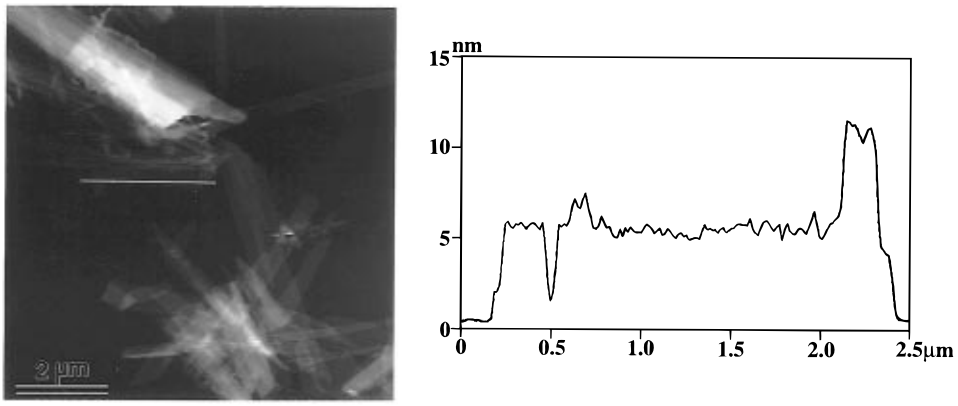

Figure 2. AFM images of LMW-P(3HB) single crystals and line profile data.

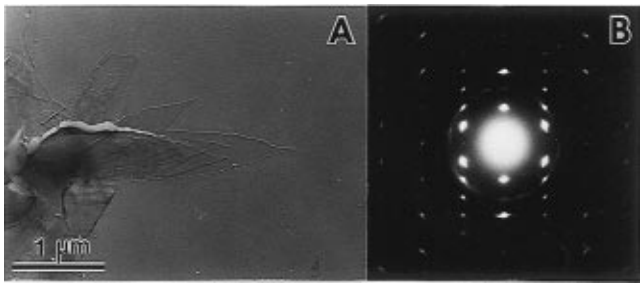

Figure 3. (A) Electron micrograph after shadowing with a Pt−Pd alloy of HMW-P(3HB) single crystals grown from a mixture of chloroform and ethanol (1/7 v/v), and (B) typical electron diffraction pattern corresponding to a single crystal.

The lamellar thicknesses of P(3HB) single crystals and of melt-crystallized and annealed films were reported as a function of crystallization temperature by small-angle X-ray scattering.[17,42] It has been found that lamellar thickness increased with rising the crystallization temperature. HMW-P(3HB) single crystals correspond to one of crystal regions in melt crystallized and annealed films. Compared between two line profile data shown in Figures 2 and 4, the crystal surfaces of LMW-P(3HB) single crystals are rougher than those of HMW-P(3HB) single crystals. This may suggest that the effect of polydispersity is directly reflected in the case of LMW-P(3HB) single crystals, because they have not the chain foldings in themselves.

Enzymatic Degradation of P(3HB) Single Crystals. Figure 5 shows the turbidimetric degradation profiles for the LMW- and HMW-P(3HB) single crystals, hydrolyzed by *P. stutzeri* YM1006 extracellular PHB depolymerase. Each turbidity decreases along two different lines during the enzymatic hydrolysis. This kinetic behavior was confirmed by monitoring the time-dependent change in the absorbance at 210 nm due to the carbonyl groups of water-soluble products (3HB monomer and dimer) on a spectrophotometer (data not shown). This result is slightly different from that reported by Hocking *et al.*[32] They showed that the turbidity of crystal suspension decreased almost linearly until the majority of the P(3HB) crystals had been consumed. These different results may be due to differences in the crystallinity of single crystals. Initial steep and subsequent gradual lines in Figure 5 seem to correspond to the degradations of the amorphous region of the crystal edge and surface and of the tight chain-packing crystal region, respectively. The slopes of two gradual lines (the rate of enzymatic degradation) are almost same, in spite of the difference in molecular weights between LMW- and HMW-P(3HB) single crystals, indicating that the degradation rate of the crystal region is independent of the molecular weight of P(3HB) chain.

Typical electron micrographs of LMW- and HMW-P(3HB) single crystals during the course of enzymatic degradation are shown in Figure 6 A−F. These correspond to the arrows in Figure 5. As degradation time passes, the edges of both crystals become rough and crystal size decreases. Especially, in crystals of HMW-P(3HB) shown in Figure 6F, which have chain folding at the crystal surface, many narrow cracks were detected along the long axis. The narrow fragments kept up the sharp electron diffraction patterns. On the basis of the time-dependent changes in the morphologies of P(3HB) crystals, it seems that the van der Waals energy between molecular chains is weaked by enzyme and that P(3HB) chains are untied from the crystals.

The molecular weight distributions of HMW-P(3HB) single crystals which have chain-folding surfaces, before

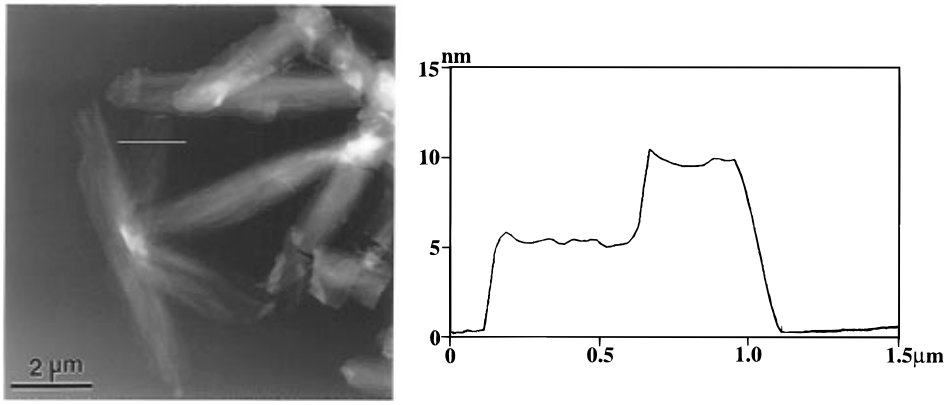

Figure 4. AFM images of HMW-P(3HB) single crystals and line profile data.

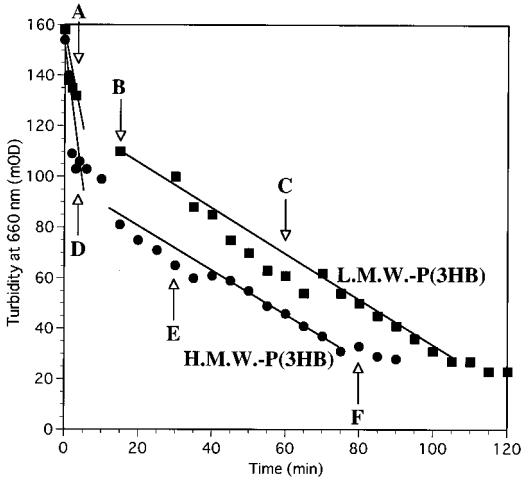

Figure 5. Decrease in turbidity against time during the enzymatic degradations of (■) LMW-P(3HB) single crystals and (●) HMW-P(3HB) single crystals, with 12 U/mL extracellular *P. stutzeri* YM1006 PHB depolymerase at 37 °C.

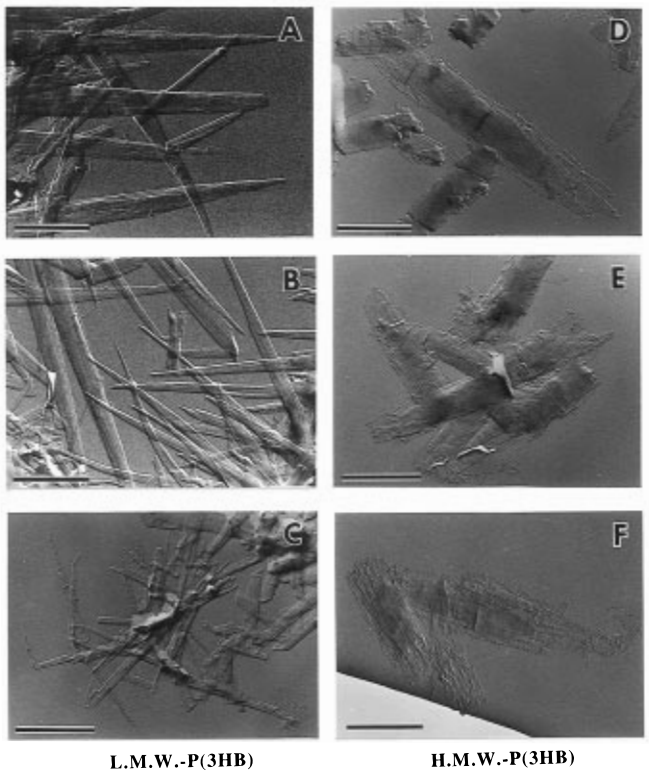

Figure 6. Electron micrographs of P(3HB) single crystals after enzymatic degradation: (A) LMW-P(3HB) single crystals after 3 min of enzymatic hydrolysis; (B) LMW-P(3HB) single crystals after 15 min of enzymatic hydrolysis; (C) LMW-P(3HB) single crystals after 60 min of enzymatic hydrolysis; (D) HMW-P(3HB) single crystals after 5 min of enzymatic hydrolysis; (E) HMW-P(3HB) single crystals after 30 min of enzymatic hydrolysis; and (F) HMW-P(3HB) single crystals after 80 min of enzymatic hydrolysis. The scale bars represent 1 μm.

and after 30 min of enzymatic degradation, are shown in Figure 7. It has been confirmed that 30 min enzymatic attack was enough for degradation, based on the results of turbidity and electron micrographs in Figures 5 and 6. However, the molecular weights and polydispersities of P(3HB) remained unchanged before and after enzymatic degradation, indicating that partial degradation at the chain-folding surface does not take place. Hocking *et al.*[32] reported that the molecular weights of P(3HB) single crystals remained unchanged during the enzymatic degradation with different PHB

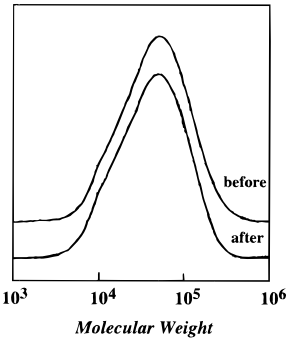

Figure 7. Gel permeation chromatograms of HMW-P(3HB) single crystals before and after partial degradation by extracellular *P. stutzeri* YM1006 PHB depolymerase at 37 °C for 30 min.

depolymerases from the fungus *Aspergillus fumigatus* and the bacterium *Pseudomonas lemoignei*. Our GPC result confirms the previous observation by Hocking *et al.*

AFM image and line profile data of HMW-P(3HB) single crystals after 30 min of enzymatic degradation are shown in Figure 8. The monolamellar parts of crystals remained around 5 nm thick, which was unchanged before and after enzymatic degradation. Both GPC and AFM data showed no evidence for the enzymatic degradation from crystal surface. In contrast, the degradation at the chain-folding surface of P(3HB) single crystals was reported for chemical degradation.[25,26] Sykes *et al.*[27] reported the AFM data of P(3HB) single crystals etched with methylamine and found that a portion of the fold surface was removed and that degradation occurred preferentially in the center of the single crystals with methylamine. However, taking our all results of molecular weight, AFM line-profile data, and electron micrographs into considerations, we reached the conclusion that enzymatic attack occurred preferentially at the edge of crystals rather than at the chain-folding surfaces of single crystals.

Visualizations of the adsorption of *P. stutzeri* YM1006 extracellular PHB depolymerase to two kinds of P(3HB) single crystals with different molecular weights, LMW- and HMW-P(3HB), using anti-rabbit IgG gold conjugation are shown in Figure 9A and 9B, respectively. Homogeneous distributions of extracellular PHB depolymerase on the surface of single crystals were observed in this study, in spite of a difference in the surface morphologies of single crystals: that is, LMW-P(3HB) single crystals have linear chains, whereas HMW-P(3HB) single crystals have the chain-folding surfaces. With respect to the adsorption of PHB depolymerase, the enzyme does not recognize specifically the folding sites in P(3HB) chains.

In spite of unselective adsorption of PHB depolymerase, LMW-P(3HB) single crystals, which do not have the chain-folding surfaces, were degraded from the edge of crystals as shown in Figure 6A–C. This may suggest that the majority of PHB depolymerase adsorbed on the surface contributes to an increase in the mobility of P(3HB) chains as a whole, and facilitates the attacks of active site against the disordered region of crystals. The P(3HB) chains of the crystal edge are more mobile than those in the crystal from the point of view of the chain packing, accordingly crystals are degraded from the edge. The step where P(3HB) chains at the edge of crystals are untied by the adsorption of PHB depoly-

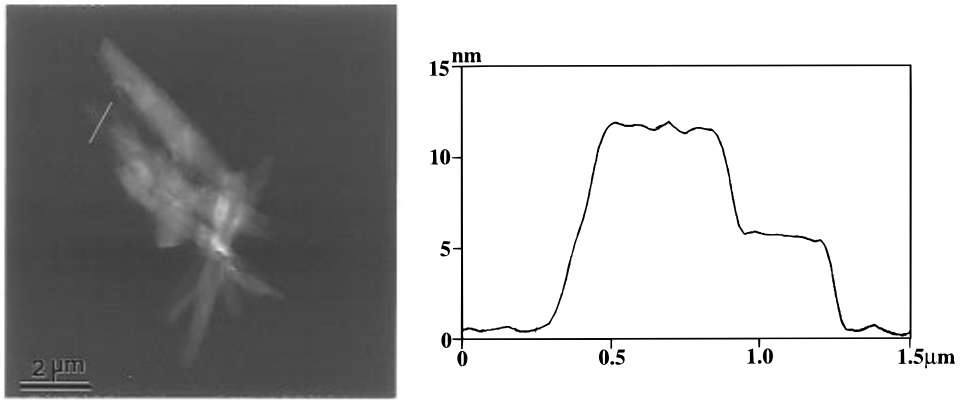

Figure 8. AFM images of HMW-P(3HB) single crystals after partial degradation by extracellular *P. stutzeri* YM1006 PHB depolymerase at 37 °C for 30 min, and line profile data.

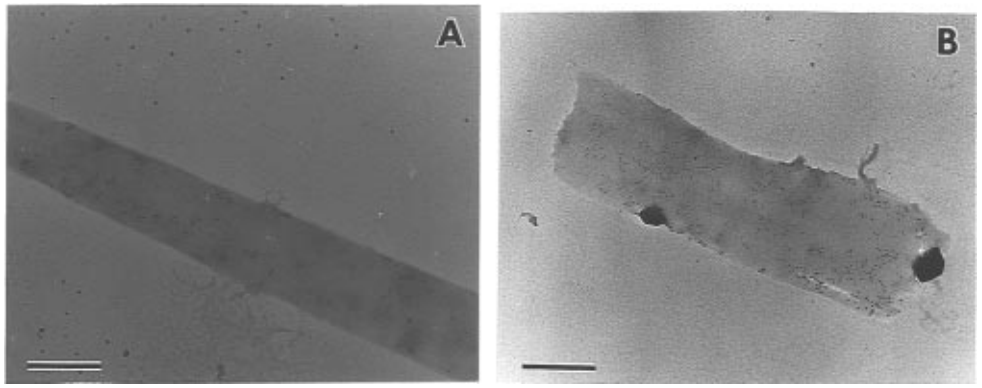

Figure 9. Visualization of the adsorption of extracellular *P. stutzeri* YM1006 PHB depolymerase to P(3HB) single crystals, (A) LMW- and (B) HMW-P(3HB), by gold labeling and transmission electron microscopy. The scale bars represent 500 nm.

merases may be a rate-determining step in this enzymatic degradation.

On the other hand, in the case of HMW-P(3HB) single crystals having chain-folding surfaces, many cracks along the crystal long axis corresponding to the crystallographic *a* axis were observed in Figure 6F. Barham *et al.*[17] have interpreted that the chains fold parallel to this long axis of the crystals, as compared with the single crystal morphology of polypropylene. Furthermore, they suggested the predominant fold model in P(3HB) crystals along the [100] direction with existing successive folds in the [110] and [1$\bar{1}$0] directions, based on the electron micrographs of P(3HB) crystals decorated with polyethylene.[28] If the chain foldings occur along the crystallographic *a* axis as is reported,[28] molecular arrangements in the direction of crystal growth should be generated by hydrogen bonding energy with "head-to-tail" or "head-to-head" packing presented by Marchessault *et al.*[23] Furthermore, the folded chains are packed along the crystal width by weak van der Waals energy. HMW-P(3HB) single crystals were degraded to some narrow fragments without reducing molecular weight of P(3HB) chains by extracellular PHB depolymerase. This result suggests that HMW-P(3HB) crystals are organized with two types of chain foldings: adjacent reentry regular sharp fold, and adjacent reentry sharp/loose loop fold. Extracellular PHB depolymerase may assist to increase the mobility of P(3HB) chains by adsorbing to crystal surfaces by the function of the hydrophobic binding domain of enzyme. Especially, the loose loop fold domain of P(3HB) is expected to be more mobile than the sharp one. As a result, some P(3HB) chains are cropped out from the crystals, and those exposed chains are attacked by the active sites of the enzyme, resulting in the formation of many cracks along the crystal long axis during the enzymatic degradation. The active sites of enzyme might not be able to attack the regular sharp fold; accordingly, hydrophobic binding of PHB depolymerase should be required for making the disordered regions in the crystals. The rate-determination step of this enzymatic degradation may be the step when the P(3HB) chains are untied from the crystal edge or cropped out from the crystal inside.

Conclusions

In this paper we have reported the visualization of enzymatic degradation of P(3HB) single crystals with *P. stutzeri* YM1006 extracellular PHB depolymerase by means of transmission electron microscopy and atomic force microscopy. Enzymatic degradations of single crystals by an extracellular PHB depolymerase progressed from the edge, or along their long axis rather than the chain-folding surfaces of single crystals, and the molecular weights of P(3HB) chains in crystals remained unchanged during the enzymatic degradation. Adsorption of PHB depolymerase to single crystals was studied using an immuno-gold labeling technique, which demonstrated a homogeneous distribution of enzymes on the surface of crystals.

On the basis of these results, it has been concluded that the adsorption of PHB depolymerase to the single crystals has a great effect to increase the mobility of P(3HB) chains in crystals. As the result, the attack by the active site of PHB depolymerase takes place preferentially against the disordered chain-packing region; the chains are untied from the crystal edge or cropped out from the crystals, rather than the chain-folding

surfaces of single crystals, and hydrolyzed by the enzyme with *exo* behavior.

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