Adsorption and Hydrolysis Reactions of Poly(hydroxybutyric acid) Depolymerases Secreted from *Ralstonia pickettii* T1 and *Penicillium funiculosum* onto Poly[(*R*)-3-hydroxybutyric acid]

Keiji Numata,[†] Koichi Yamashita,[‡,§] Masahiro Fujita,[§] Takeharu Tsuge,[†] Ken-ichi Kasuya,[∥] Tadahisa Iwata,[§] Yoshiharu Doi,[§] and Hideki Abe*[,†,§]

Department of Innovative and Engineered Materials, Tokyo Institute of Technology, 4259 Nagatsuta, Midori-ku, Yokohama 226-8502, Japan, Chemical Analysis Team, RIKEN Institute, 2-1 Hirosawa, Wako-shi, Saitama 351-0198, Japan, Polymer Chemistry Laboratory, RIKEN Institute, 2-1 Hirosawa, Wako-shi, Saitama 351-0198, Japan, and Department of Biological and Chemical Engineering, Faculty of Engineering, Gunma University, Tenjin-cho, 1-5-1 Kiryu, Gunma 376-8515, Japan

Received February 25, 2007; Revised Manuscript Received April 13, 2007

Reaction processes of poly[(*R*)-3-hydroxybutyric acid] (P(3HB)) with two types of poly(hydroxybutyric acid) (PHB) depolymerases secreted from *Ralstonia pickettii* T1 and *Penicillium funiculosum* were characterized by means of atomic force microscopy (AFM) and quartz crystal microbalance (QCM). The PHB depolymerase from *R. pickettii* T1 consists of catalytic, linker, and substrate-binding domains, whereas the one from *P. funiculosum* lacks a substrate-binding domain. We succeeded in observing the adsorption of single molecules of the PHB depolymerase from *R. pickettii* T1 onto P(3HB) single crystals and the degradation of the single crystals in a phosphate buffer solution at 37 °C by real-time AFM. On the contrary, the enzyme molecule from *P. funiculosum* was hardly observed at the surface of P(3HB) single crystals by real-time AFM, even though the enzymatic degradation of the single crystals was surely progressed. On the basis of the AFM observations in air of the P(3HB) single crystals after the enzymatic treatments, however, not only the PHB depolymerase from *R. pickettii* T1 but also that from *P. funiculosum* adsorbed onto the surface of P(3HB) crystals, and both concentrations of the enzymes on the surface were nearly identical. This means both enzymes were adsorbed onto the surface of P(3HB) single crystals. Moreover, QCM measurements clarified quantitatively the differences in detachment behavior between two types of PHB depolymerases, namely the enzyme from *R. pickettii* T1 was hardly detached but the enzyme from *P. funiculosum* was released easily from the surface of P(3HB) crystals under an aqueous condition.

Introduction

A wide variety of bacteria synthesize the optically active polymer of (*R*)-3-hydroxybutyric acid from renewable carbon sources and accumulate it as intracellular carbon and energy storage material. Poly[(*R*)-3-hydroxybutyric acid] (P(3HB)), isolated from bacteria, is a crystalline, biocompatible, and biodegradable thermoplastic.[1−4] In the last two decades, the biodegradability of P(3HB) has been investigated by many groups, and P(3HB) has been known to be hydrolyzed by various extracellular poly(hydroxybutyric acid) (PHB) depolymerases secreted from many microorganisms.[5−8] Extracellular PHB depolymerases are typically comprised of three functional domains: a catalytic (320−400 aa), a linker (50−100 aa), and a substrate-binding (40−60 aa) domain. The PHB depolymerase with a binding domain has been reported to adsorb onto polymer surfaces by the substrate-binding domain and to hydrolyze P(3HB) molecules by the catalytic domain in the degradation process.[9] The catalytic domain contains a lipase-like catalytic triad [Ser, Asp, and His] as well as a signature sequence of a pentapeptide [Gly-Xaa-Ser-Xaa-Gly] (where Xaa denotes any amino acid residue), known as the lipase box.[10] The substrate-binding domain is responsible for the adsorption of the enzyme to the surface of water-insoluble polymer, which permits the catalytic domain to interact with polymer chains, thus catalyzing their efficient hydrolysis.[11,12] Recently, it has been proposed that the substrate-binding domain has an additional function to disrupt the surface structure of polymer crystals.[13,14]

The PHB depolymerase from *Ralstonia pickettii* T1 (formally *Alcaligenes faecalis* T1) used in this study has been investigated widely by many groups.[5−15] The PHB depolymerase from *R. pickettii* T1 is the typical one that consists of three characteristic domains, i.e. catalytic, substrate-binding, and linker domains. The molecular weight of the PHB depolymerase is 47 kDa, and the optimum pH for enzyme reaction is 7.5.[5,7] Saito and co-workers reported that an active serine is a part of the highly conserved pentapeptide [Gly-Leu-Ser[139]-Ser-Gly], the lipase box.[5] Recently, Yamashita et al. studied the adsorption of the PHB depolymerase from *R. pickettii* T1 by a quartz crystal microbalance (QCM) and suggested the irreversible adsorption of the PHB depolymerase onto P(3HB) thin films.[15] Kikkawa et al. observed the PHB depolymerase from *R. pickettii* T1 adsorbed onto amorphous poly(l-lactide) thin films by an atomic force microscopy (AFM) and reported that the size of the enzyme molecule is 4.4 ± 1.1 nm high and 24 ± 3 nm wide.[14]

The PHB depolymerase from *Penicillium funiculosum* has been investigated by a couple of groups.[16−18] Brucato and Wong

* To whom correspondence should be addressed. Phone: +81-48-467-9404. Fax: +81-48-462-4667. E-mail: habe@riken.jp.
[†] Tokyo Institute of Technology.
[‡] Chemical Analysis Team, RIKEN Institute.
[§] Polymer Chemistry Laboratory, RIKEN Institute.
[∥] Gunma University.

10.1021/bm070231z CCC: $37.00
Published on Web 06/05/2007

purified and characterized the PHB depolymerase from a strain of *P. funiculosum* and reported that the enzyme has a molecular mass of 37 kDa and the optimum pH is 6.0.[16] Kasuya and coworkers reported that the PHB depolymerase from *P. funiculosum* degrades P(3HB) and a trimer of (*R*)-3HB, though the degradation of P(3HB) is less efficient by 2 orders of magnitude in comparison with the multidomain enzyme from *R. pickettii* T1.[17] Moreover, they speculated that the enzyme from *P. funiculosum* lacks a substrate-binding domain, because the molecular weight of the PHB depolymerase from *P. funiculosum* was found to be much smaller than typical PHB depolymerase (around 50 kDa).[17] Recently, the crystal structures of the PHB depolymerase from *P. funiculosum* and the mutant-(*R*)-3HB trimer methyl ester substrate complex were revealed at resolutions of 1.71 Å and 1.66 Å, respectively.[18] The enzyme is shown to be a globular molecule with approximate dimensions of 5.2 nm × 4.8 nm × 4.1 nm and with a single domain.[18] In addition, it was suggested that 13 hydrophobic residues around the active site induce the enzyme to bind onto polymer chains without a substrate-binding domain.[18] Furthermore, a cDNA encoding PHB depolymerase from *P. funiculosum* was cloned, and the homology analysis showed that the enzyme lacks linker and substrate-binding domains.[19]

In our previous studies, we observed the enzymatic degradation process of melt-crystallized thin films and single crystals of P(3HB) in the presence of the PHB depolymerase from *R. pickettii* T1 by real-time AFM, and we proposed a degradation mechanism of lamellar crystal by the PHB depolymerase.[20,21] We also investigated the adsorption effect of the PHB depolymerase on the folding surface of P(3HB) single crystals by means of AFM and a frictional force microscopy.[22,23] The adsorption of the PHB depolymerase from *R. pickettii* T1 was found to change the molecular state of P(3HB) and to generate concaves at the surface of P(3HB) crystal at the initial stage of enzymatic reaction.[22,23]

In this study, we investigate the single molecular reactions of the PHB depolymerases with P(3HB) single crystals using real-time AFM, and we also perform QCM measurements complementarily to reveal the detachment process of the enzymes. Furthermore, we use two types of PHB depolymerases with and without the substrate-binding domain, which will reveal the effects of the substrate-binding domain on the reaction process of the enzymes.

Materials and Methods

Preparation of Poly[(*R*)-3-hydroxybutyric acid] Single Crystals. P(3HB) was prepared by microbial synthetic methods.[1,2] Alkaline hydrolysis of P(3HB) (weight-averaged molecular weight: $M_w = 52 \times 10^4$; polydispersity: $M_w/M_n = 2.4$) was performed according to the method reported previously.[24] P(3HB) ($M_w = 1.1 \times 10^4$, $M_w/M_n = 1.4$) after the alkaline hydrolysis was used to prepare single crystals. M_w and M_w/M_n were measured by a gel permeation chromatography (GPC) system with polystyrene standards (Shodex Standard SM-105, 1.3×10^3 to 3.1×10^6). GPC measurement was performed at 40 °C, using a Shimadzu 10A GPC system with joint columns of Shodex K-806 and K-802. Chloroform was used as the mobile phase at a flow rate of 0.8 mL/min, and a sample concentration was set to be 1.0 mg/mL. Shimadzu CLASS-VP software was used to process the data. P(3HB) single crystals were grown isothermally from a dilute solution of a mixture of chloroform and methanol at 60 °C for 12 h, as reported previously.[21] The solvent ratio of chloroform/methanol (v/v) was 1/7, and the concentration of P(3HB) dilute solution was 0.025 (w/v)%. After the crystallization, P(3HB) single crystals were collected by decantation at 25 °C. P(3HB) single crystals were observed with a JEM-2000FXII electron microscope which was operated at an acceleration voltage of 120 kV for electron diffraction to ensure the crystal structure.[21]

Purification of PHB Depolymerases. The PHB depolymerases from *R. pickettii* T1 and *P. funiculosum* were purified to electrophoretic homogeneity according to the methods reported previously.[6,17] Esterase assays of both enzymes were performed as follows: the reaction mixture containing 0.1% *p*-nitrophenylbutyrate (Sigma) was prepared in 1.0 mL of 0.1 M phosphate buffer (pH 7.0). The reaction was started by the addition of an enzyme solution and monitored at 400 nm using a HITACHI U-2000 spectrophotometer. One unit of enzyme was defined as the amount of protein required to increase the value of absorbance at 400 nm by 1 per min at 37 °C. As a result, the purified PHB depolymerases from *R. pickettii* T1 and *P. funiculosum* had the enzyme activities of 132 and 6 U/mg, respectively.

Enzymatic Reaction Experiment. P(3HB) single crystals were deposited on a square silicon wafer (10 × 10 mm^2) and dried in air. Subsequently, the P(3HB) single crystals on the silicon substrate were immersed in 0.1 M phosphate buffer containing 0.5 μg/mL of PHB depolymerase for 1 h at 37 °C. The samples were washed lightly with Milli-Q water after the enzymatic treatment in order to remove salt of the buffer solution, and they were dried at 25 °C for 1 day.

Surfactant Experiment. Triton X-100 was purchased from ICN Biomedicals Inc. The solution containing Triton X-100 was diluted into 0.1 or 0.2% with 0.1 M phosphate buffer solution (pH 6.0 or 7.5). Then, 0.5 μg/mL of enzyme solution containing Triton X-100 was prepared. The P(3HB) single crystals on a silicon wafer were immersed in the enzyme and surfactant solution for 1 h at 37 °C. The samples were washed lightly with Milli-Q water after the immersion.

AFM Observations. The P(3HB) single crystal after the enzymatic treatment for 1 h was observed by AFM (Seiko Instruments Inc. SPI3800/SPA 300HV) in air at 25 °C. A 400 μm long silicon cantilever with a spring constant of 1.5 N/m was adopted in dynamic force mode (tapping mode) AFM. A scan rate was set to 0.8 Hz. A light tapping force (set-point values = 0.85) was applied for all AFM observations in this study, to avoid damage to both the PHB depolymerase molecules and the P(3HB) single crystals by the cantilever tip. Height and phase images were simultaneously obtained. The calibration of cantilever tip convolution effect was carried out to obtain the true dimensions of objects according to the method described previously.[20,22] The height and width of the enzyme on each single crystal were determined from AFM height images (scan area: 2 × 2 μm^2). The average value of 50 measurements was adopted as the size of enzyme in this study. The number of adsorbed enzymes was calculated from five typical AFM phase images (scan area: 2 × 2 μm^2), and the average value in 1 μm^2 of the surface of the single crystals was used in this study.

The reaction process of the PHB depolymerase onto P(3HB) single crystals was observed by real-time AFM in 0.1 M phosphate buffer solution at 37 °C. P(3HB) single crystals were deposited on the surface of a silicon wafer. A 10 × 10 mm splitting of the silicon wafer was fixed to the bottom of the reaction vessel (diameter: 25 mm; height: 5 mm) with double-sided carbon adhesive sheets. The vessel with sample was set on AFM scanner, and 1.5 mL of phosphate buffer solution was poured into the vessel. P(3HB) single crystals before enzymatic reaction were observed in the phosphate buffer solution at 37 °C. Then, the enzymatic reaction was initiated by the addition of concentrated enzyme solution into the phosphate buffer solution in the vessel, resulting in a final concentration of enzyme solution of 0.5 μg/mL.

QCM Measurements. A commercially available QCM setup of QCA-922 (SEIKO EG&G) was equipped with a cell of an inner volume of ca. 0.2 mL.[15,25] The temperature of cell was controlled at 37 °C by circulating water. The oscillator was a polished 9 MHz AT-cut quartz crystal, on both sides of which Au electrodes were deposited (area size: 0.196 cm^2 × 2). A frequency shift of 1.0 Hz corresponds to a mass change of 1.0 ng on the electrode (0.196 cm^2) according to the Sauerbrey equation.[26] In our system, the resonant frequency and the

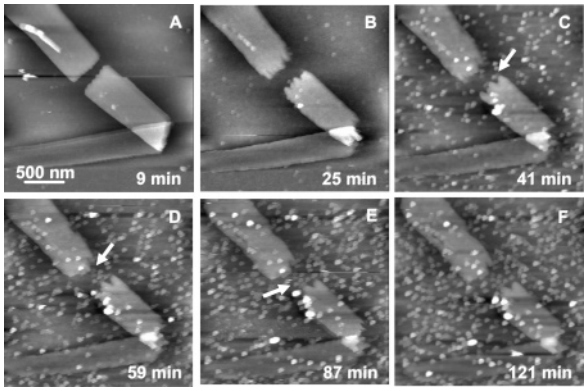

Figure 1. Real-time AFM height images of the typical enzymatic reaction process onto P(3HB) single crystals by the PHB depolymerase from *R. pickettii* T1. The frames were recorded during enzymatic reaction for 9 (A), 25 (B), 41 (C), 59 (D), 87 (E), and 121 min (F). The concentration of the enzyme was 0.5 μg/mL. These images were obtained in a phosphate buffer solution (pH 7.5) at 37 °C. White arrows indicate the enzyme molecules degrading the P(3HB) single crystals.

resonant resistance were monitored simultaneously to evaluate the influence of frictional changes. The chloroform solution (1.5 wt %) of P(3HB) was cast on the one side of the oscillator placed on a spin coater (ME-300MOC, Co. Ltd.) at 4000 rpm under dry air. The film was melted at 210 °C for 30 s and then kept at 110 °C for 24 h. After the stabilization of QCM in 0.1 M phosphate buffer solution (pH 7.5 or 6.0), the enzymatic reaction was initiated by replacing the buffer solution with the enzyme solution containing the PHB depolymerase from *R. pickettii* T1 or *P. funiculosum*, respectively. The total volume used for replacement was 1.25 mL. The frequency change during the reaction was recorded by a microcomputer system.

Results

Real-Time AFM Observations of Enzymatic Reaction Processes. Real-time AFM observation of the enzymatic reaction process was performed in a 0.1 M phosphate buffer solution (pH 7.5) containing 0.5 μg/mL of the PHB depolymerase at 37 °C. Figure 1 shows the real-time AFM images of the reaction process of the PHB depolymerase secreted from *R. pickettii* T1 with P(3HB) single crystals. As shown in Figure 1, the presence of homogeneous white dots was clearly observed. The height and width of the dots were approximately 2.6 and 54 nm, respectively, and they were identical to those of the PHB depolymerase reported previously.[21] Therefore, the dots were considered to be the enzyme molecules. Each enzyme molecule that adsorbed on the P(3HB) single crystals remained at the same position during the observation for 121 min. The number of the enzymes adsorbed onto the surface of P(3HB) single crystals increased with time. White arrows in Figure 1 show PHB depolymerase molecules adsorbed onto the surface of both the single crystals as well as the substrate. In addition, degradation was observed in the area surrounding the enzyme molecule located at the edge of the P(3HB) single crystal. Thus, we have succeeded in observing the adsorption of PHB depolymerase from *R. pickettii* T1 and the degradation of single crystals at the same time.

Figure 2 shows the typical real-time AFM height images of the enzymatic degradation process of P(3HB) single crystals in a phosphate buffer solution (pH 6.0) containing 0.5 μg/mL of the PHB depolymerase secreted from *P. funiculosum* at 37 °C. Figure 2A exhibits the AFM height image of P(3HB) single crystals in the buffer solution before the enzymatic degradation.

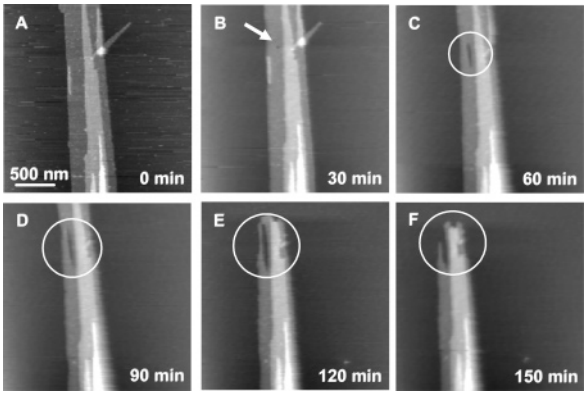

Figure 2. Real-time AFM height images of the typical enzymatic reaction process onto P(3HB) single crystals by the PHB depolymerase from *P. funiculosum*. The first frame (A) was recorded before the enzymatic reaction, and the following images were obtained during enzymatic reaction for 30 (B), 60 (C), 90 (D), 120 (E), and 150 min (F). The concentration of the enzyme was 0.5 μg/mL. These images were obtained in a phosphate buffer solution (pH 6.0) at 37 °C. White arrow and circles denote the hole and cleft which were generated by the enzymatic degradation.

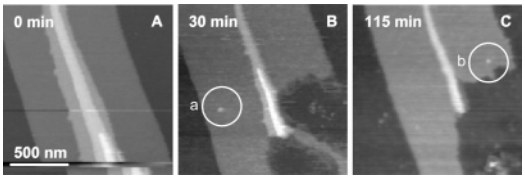

Figure 3. Real-time AFM height images of the typical enzymatic reaction process onto P(3HB) single crystals by the PHB depolymerase from *P. funiculosum*. The concentration of the enzyme was 0.5 μg/mL. These images were obtained in a phosphate buffer solution (pH 6.0) at 37 °C. Each circle indicates the enzyme molecule on the P(3HB) single crystal.

In Figure 2B, the white arrow indicates the position that the enzyme degraded first, and then a hole was generated at the same place. As the reaction progressed, the P(3HB) single crystal was split from the hole along the crystallographic *a*-axis. Namely, the enzymatic degradation of the P(3HB) single crystal proceeded. However, no enzyme molecule was observed around the hole and cleft by AFM.

With repetitive observation by real-time AFM, we rarely succeeded visualizing the enzyme molecules from *P. funiculosum* in the image. Figure 3 shows the real-time AFM images of the PHB depolymerase from *P. funiculosum* onto P(3HB) single crystals in a buffer solution. Two white circles, "a" and "b" in Figure 3B,C, indicate that the enzyme molecule existed on the P(3HB) single crystal. The average size of the enzymes in Figure 3 was around 3.8 nm high and 51 nm wide. In addition, the enzyme molecule at "a" in Figure 3B disappeared from the surface of single crystal, even though the crystal as a scaffold was hardly degraded.

AFM Observations in Air of P(3HB) Single Crystals after Enzymatic Treatment. Adsorption behaviors of two types of PHB depolymerases onto P(3HB) single crystals were also studied by AFM in air. P(3HB) single crystals were immersed in the phosphate buffer solution containing 0.5 μg/mL of PHB depolymerase from *R. pickettii* T1 or *P. funiculosum* for 1 h at 37 °C. After the enzymatic reaction, the P(3HB) single crystals were washed by Milli-Q water in order to remove the enzyme molecules that did not adsorb onto the crystals, and they were subsequently dried for 12 h. After that, the enzyme molecules adsorbed on the P(3HB) single crystals were observed by AFM in air at 25 °C. Figure 4 shows the AFM phase images of the

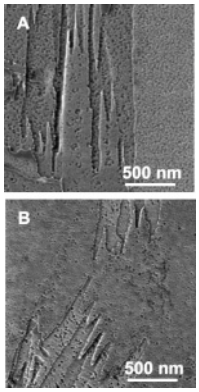

Figure 4. AFM phase images of P(3HB) single crystals after the enzymatic reaction for 1 h at 37 °C by PHB depolymerases from *R. pickettii* T1 (A) and *P. funiculosum* (B). The concentration of the enzyme was 0.5 μg/mL. The images were obtained in air.

Table 1. Sizes and Concentrations of Two Types of PHB Depolymerases at the Surface of P(3HB) Single Crystals

PHB depolymerase (source)	size of PHB depolymerase height (nm)	size of PHB depolymerase width (nm)	concn of adsorbed PHB depolymerase (enzymes/μm^2)
R. pickettii T1	1.4 ± 0.6	29 ± 5	207 ± 24
P. funiculosum	1.5 ± 0.5	27 ± 7	172 ± 51

P(3HB) single crystals after the enzymatic treatment containing PHB depolymerase either from *R. pickettii* T1 (A) or from *P. funiculosum* (B). In the AFM phase images, enzyme molecules were recognized as black dots at the surface of P(3HB) single crystals. Table 1 lists the sizes and concentrations of two types of PHB depolymerases adsorbed onto the surface, which were obtained from the AFM height images of Figure 4. The size of PHB depolymerase from *R. pickettii* T1 was 1.4 ± 0.6 nm high and 29 ± 5 nm wide and was nearly identical to the size of single molecule reported previously.[22] The dimensions of enzyme from *P. funiculosum* was 1.5 ± 0.5 nm high and 27 ± 7 nm wide, which were almost the same as those from *R. pickettii* T1. The shape of enzyme from *P. funiculosum* obtained by AFM was much lower and wider than that obtained by X-ray which was reported previously.[18] This may be due to the denaturation of the enzyme molecule to a hemispheric shape after the adsorption and drying on the surface of the single crystals. Moreover, it should be noted that two types of PHB depolymerases were observed at the surface of P(3HB) single crystals and that the concentration of the enzyme molecules adsorbed onto the surface was nearly identical for each enzyme in air.

In addition, we carried out a surfactant experiment to investigate the affinities between PHB depolymerase and the surface of the P(3HB) crystal. Triton X-100 was used as a surfactant to inhibit the effect of hydrophobic interaction.

The P(3HB) single crystals immersed in a phosphate buffer solution containing Triton X-100 and 0.5 μg/mL of PHB depolymerase either from *P. funiculosum* or from *R. pickettii* T1 were observed by AFM in air, as shown in Figure 5. The AFM images in Figure 5A,B demonstrate that the PHB depolymerase from *R. pickettii* T1 remained on the P(3HB) single crystals in the presence of 0.1% or 0.2% of Triton X-100. In contrast, the enzyme from *P. funiculosum* adsorbed onto the surface of the P(3HB) single crystals in 0.1% of Triton X-100, whereas the enzyme hardly remained on the surface in 0.2% of Triton X-100 (see Figure 5C,D).

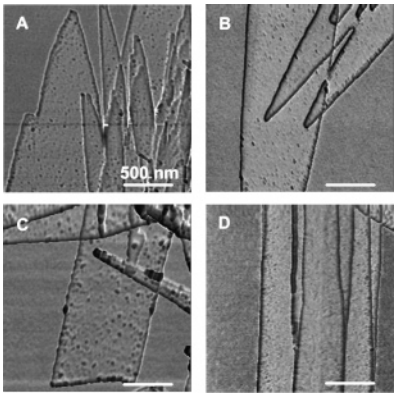

Figure 5. AFM phase images of P(3HB) single crystals after immersion in an enzyme solution containing 0.5 μg/mL of PHB depolymerases from *R. pickettii* T1 (A, B) or *P. funiculosum* (C, D) and 0.1% (A, C) or 0.2% (B, D) of Triton X-100 for 1 h at 37 °C. The images were obtained in air. Each scale bar shows 500 nm.

QCM Analysis To Clear Detachment Behavior of PHB Depolymerase. We analyzed the detachment behavior of the PHB depolymerases from the P(3HB) thin films by QCM. It was reported that the enzymatic degradation of the film is quantitatively followed by QCM as a positive frequency shift, and the rate of frequency change reflects the erosion rate of P(3HB) film.[15] QCM was stabilized in a phosphate buffer solution at 37 °C. The buffer solution (pH 7.5 or 6.0) was then replaced with 1.0 or 5.0 μg/mL of enzyme solution to start the enzymatic reaction by PHB depolymerases from *R. pickettii* T1 or *P. funiculosum*, respectively. Since, under an enzyme concentration similar to that in the AFM experiment (0.5 μg/mL), the enzymatic degradation rates were too slow to detect significant weight changes for the short-term reaction, the concentration of enzyme was applied at higher values for the QCM measurements.

Figure 6 demonstrates the time course of frequency changes (ΔF) observed by QCM during the enzymatic degradation of P(3HB) thin films by PHB depolymerase from *R. pickettii* T1 (A) and from *P. funiculosum* (B). The enzymatic reaction was started by adding the enzyme solution into the P(3HB) thin films. After the fast 2 h of enzymatic degradation, the added enzyme solution was replaced with a pure buffer solution in order to remove the enzyme molecules remaining in the buffer phase. As a separate control experiment, the enzymatic degradation was allowed to continue for 18 h without the replacement of the enzyme solution with a pure buffer solution. The frequency changes for the control experiments showed an almost linear relationship with time in both cases of the two enzymes (see curves labeled "a"). The curve b in Figure 6A shows that the replacement of the enzyme solution from *R. pickettii* T1 with a phosphate buffer solution hardly affects the degradation rate in comparison with the control (a). In contrast, the replacement of the enzyme solution from *P. funiculosum* (b) resulted in the apparent retardation of the degradation rate compared with the control experiment (a), as shown in Figure 6B.

Discussion

The focus of this study is to clarify the single molecular reactions of two types of PHB depolymerases with different domain structures by means of AFM and QCM. The PHB depolymerase from *R. pickettii* T1 has a multifunctional domain structure, consisting of catalytic, substrate-binding, and linker

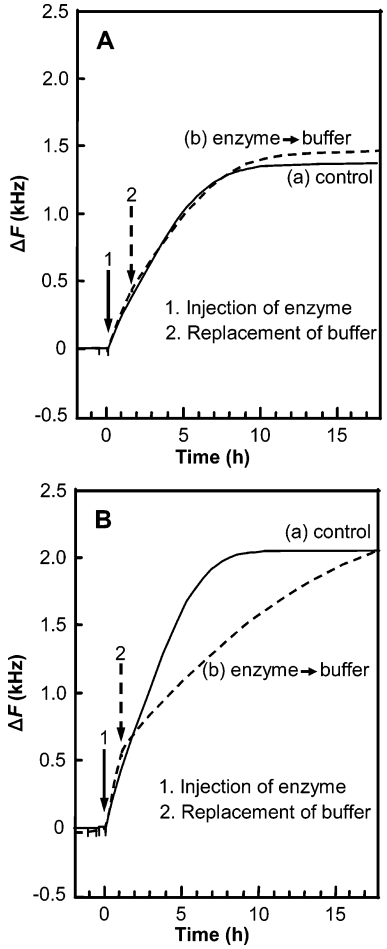

Figure 6. Time courses of frequency changes (ΔF) observed during enzymatic degradation of P(3HB) thin films by two types of PHB depolymerases from *R. pickettii* T1 (A) and *P. funiculosum* (B) at 37 °C. The concentrations of the enzyme were 1.0 and 5.0 μg/mL, and pH values of the buffer were 7.5 and 6.0, respectively. As indicated by arrow 1, the degradation reaction was started by the injection of enzyme solution at time 0 h. After 2 h of reaction, the enzyme solution was replaced with a pure buffer solution (at the point denoted by arrow 2). The solid line is the frequency changes for the enzymatic degradation without replacement of enzyme solution with a pure buffer (control).

domains.[11] The depolymerase from *P. funiculosum* is a single domain hydrolase without a characteristic substrate-binding domain.[17,18] Here, we discuss the reaction of single enzyme molecules with P(3HB) crystals and the difference in reaction behavior between two types of PHB depolymerases.

Both the real-time and static AFM observations (Figures 1 and 4A) revealed that the PHB depolymerase from *R. pickettii* T1 adsorbs homogeneously onto the surface of P(3HB) single crystals. The enzyme molecules once adsorbed on the P(3HB) single crystals stayed at the same position during the real-time AFM observation (see Figure 1). The P(3HB) film was degraded completely without substantial retardation when the enzyme solution was replaced with a buffer solution during QCM measurement, suggesting that the PHB depolymerase from *R. pickettii* T1 hardly desorbed from the surface of P(3HB) film (Figure 6A). These results indicate that the enzyme molecule of *R. pickettii* T1 strongly interacts with the surface of P(3HB) films, so that the adsorption of enzyme is apparently irreversible.

In contrast to the PHB depolymerase from *R. pickettii* T1, the enzyme molecule from *P. funiculosum* was rarely detectable on the surface of P(3HB) single crystals in the real-time AFM images (Figures 2 and 3). The enzyme molecules attached on the P(3HB) single crystal disappeared at the observed position during continuous scanning under the aqueous condition, as shown in Figure 3. This might be caused by the facilitation of detachments of the enzyme adsorbed on the crystals by contact with the scanning cantilever tip. On the other hand, the enzyme molecules attached on the surface of P(3HB) single crystals were detected by AFM in air after enzymatic treatment containing PHB depolymerase from *P. funiculosum* (Figure 4B). The enzyme molecules adsorbed on the single crystals were reported to be squashed in air, resulting that the AFM observation of the enzyme molecules without the detachment of the molecules is easier in air, as reported previously.[22] It is indicated that the PHB depolymerase from *P. funiculosum* surely has an ability to adsorb onto the surface of P(3HB) single crystals. The adsorption of PHB depolymerase from *P. funiculosum* can also be confirmed by the QCM measurement. Although the replacement of enzyme solution from *P. funiculosum* with a buffer solution retarded the degradation rate of the film, the enzymatic erosion of the film continued (Figure 6B). These results suggest that PHB depolymerase from *P. funiculosum* adsorbs onto the P(3HB) surface to degrade P(3HB) molecules, while the adsorbed enzyme is easily released from the P(3HB) under an aqueous condition. The occurrence of reversible adsorption of enzyme molecule makes it difficult to capture the adsorbed enzyme molecules on the P(3HB) single crystals in the real-time AFM images.

Recently, Hisano et al. succeeded in obtaining the three-dimensional structure of the PHB depolymerase from *P. funiculosum*.[18] They reported that the enzyme has a globular appearance, and they proposed that 13 hydrophobic residues around the active site take part in binding of the enzyme onto polymer substrate.[18]

Previously, we investigated the adsorption behavior of PHB depolymerase from *R. pickettii* T1 on a silicon wafer and P(3HB) single crystals by AFM.[22] It was found that the PHB depolymerase from *R. pickettii* T1 adsorbs onto the surfaces of both the silicon wafer and the P(3HB) single crystals. The PHB depolymerase adsorbed onto the silicon wafer was easily detached from the substrate surface by the addition of a small amount (0.01%) of Triton X-100 as a surfactant, whereas the enzyme adsorbed onto the P(3HB) single crystals was not easily removed by the addition of the same amount of the surfactant. Therefore, we have concluded that the enzyme molecule binds to the P(3HB) single crystals via a specific affinity with P(3HB) molecules in addition to hydrophobic interactions, while the greatest interaction on the silicon wafer was the nonspecific one. In the case of the enzyme from *P. funiculosum*, the kinetic analysis of the enzymatic hydrolysis was performed to show the lowest value of the adsorption equilibrium constant.[17] Thus, the binding affinities for the surface of P(3HB) granules were the weakest among all of the PHB depolymerases.[17]

In this study, we performed the enzymatic adsorption experiments containing a surfactant for two types of PHB depolymerases. As shown in Figure 5, the dependence of surfactant concentration on enzyme adsorption apparently differed for the two enzymes. By the addition of 0.2% of surfactant, the enzyme from *P. funiculosum* hardly adsorbed on the substrate surface, while the adsorbing enzyme from *R. pickettii* T1 was still detectable on the P(3HB) single crystals. These results suggest that a specific affinity is present between the enzymes and P(3HB) crystals in addition to hydrophobic interaction and that the specific interaction for the enzyme of *R. pickettii* T1 is stronger than the enzyme of *P. funiculosum*.

Such difference in binding affinity between two types of enzymes may be caused by the difference of domain structures, and this is reflected in the adsorption manner of enzymes. The PHB depolymerase from *R. pickettii* T1 is anchored to the substrate surface with the substrate-binding domain by strong affinity with P(3HB) molecules.[22] The absence of a substrate-binding domain leads to the decrease in binding affinity of the enzyme from *P. funiculosum* compared with that of the enzyme from *R. pickettii* T1. Because of the difference in adsorption manner of enzymes, the degradation rate of P(3HB) by the enzyme of *P. funiculosum* is much lower than that by the *R. pickettii* T1 at an identical enzyme concentration.

Conclusions

This is the first report on the visualization of the difference in single molecular reactions between two types of PHB depolymerases with different domain structures. The reaction processes with P(3HB) of the enzymes secreted from *R. pickettii* T1 and *P. funiculosum* were characterized by means of AFM and QCM. We succeeded in observing the adsorption of single molecular PHB depolymerase from *R. pickettii* T1 onto the surface of P(3HB) single crystals and the degradation of P(3HB) single crystals at the same time. The AFM and QCM measurements showed that the PHB depolymerase from *R. pickettii* T1 adsorbs onto the surface of P(3HB) single crystals and hardly desorbs from the surface of P(3HB) film. On the other hand, it was found that the enzyme molecule from *P. funiculosum* adsorbs onto the P(3HB) surface, but the adsorbed enzyme is easily released from the P(3HB) under an aqueous condition. The result indicated that the adsorption process is apparently reversible in the enzymatic reaction by the PHB depolymerase from *P. funiculosum* at the surface of P(3HB). Furthermore, the result of the surfactant experiment clarified that the two types of enzyme differ in binding affinity. The difference in the binding affinity may be caused by the difference of domain structure, namely, the substrate-binding domain for *R. pickettii* T1 enhances the degrading activity in addition to the binding affinity in comparison with the enzyme from *P. funiculosum*.

Acknowledgment. This work has been supported by a grant for Ecomolecular Science Research from RIKEN Institute and was completed while K.N. was a JSPS Research Fellow. We appreciate the assistance provided by Dr. Sudesh Kumar for correcting the English of our manuscript.

References and Notes

(1) Doi, Y. *Microbial Polyesters*; VCH publishers: New York, 1990.
(2) Doi, Y., Steinbüchel, A., Eds.; In *Polyesters I and II*; Biopolymers, Vol. 3; Wiley-VCH: Weinheim, Germany, 2001.
(3) Sudesh, K.; Abe, H.; Doi, Y. *Prog. Polym. Sci.* **2000**, *25*, 1503.
(4) Lenz, R. W.; Marchessault, R. H. *Biomacromolecules* **2005**, *6*, 1.
(5) Tanio, T.; Fukui, T.; Shirakura, Y.; Saito, T.; Tomita, K.; Kaiho, T.; Masamune, S. *Eur. J. Biochem.* **1982**, *124*, 71.
(6) Shirakura, Y.; Fukui, T.; Saito, T.; Okamoto, Y.; Narikawa, T.; Koide, K.; Tomita, K.; Takemasa, T.; Masamune, S. *Biochem. Biophys. Acta.* **1986**, *880*, 46.
(7) Jendrossek, D.; Schirmer, A.; Schlegel, H. G. *Appl. Microbiol. Biotechnol.* **1996**, *46*, 451.
(8) Hocking, P. J.; Marchessault, R. H.; Timmins, M. R.; Lenz, R. W.; Fuller, R. C. *Macromolecules* **1996**, *29*, 2472.
(9) Mukai, K.; Yamada, K.; Doi, Y. *Int. Biol. Macromol.* **1993**, *15*, 361.
(10) Jendrossek, D.; Handrick, R. *Annu. Rev. Microbiol.* **1996**, *56*, 403.
(11) Fukui, T.; Narikawa, T.; Miwa, K.; Shirakawa, Y.; Saito, T.; Tomita, K. *Biochem. Biophys. Acta.* **1988**, *952*, 164.
(12) Kasuya, K.; Ohura, T.; Masuda, K.; Doi, Y. *Int. J. Biol. Macromol.* **1999**, *24*, 329.
(13) Murase, T.; Suzuki, Y.; Doi, Y.; Iwata, T. *Biomacromolecules* **2002**, *3*, 312.
(14) Kikkawa, Y.; Fujita, M.; Hiraishi, T.; Yoshimoto, M.; Doi, Y. *Biomacromolecules* **2004**, *5*, 1642.
(15) Yamashita, K.; Funato, T.; Suzuki, Y.; Teramachi, S.; Doi, Y. *Macromol. Biosci.* **2003**, *3*, 694.
(16) Brucato, C. L.; Wong, S. S. *Arch. Biochem. Biophys.* **1991**, *290*, 497.
(17) Miyazaki, S.; Takahashi, K.; Shiraki, M.; Saito, T.; Tezuka, Y.; Kasuya, K. *J. Polym. Environ.* **2000**, *8*, 175.
(18) Hisano, T.; Kasuya, K.; Tezuka, Y.; Ishii, N.; Kobayashi, T.; Shiraki, M.; Oroudjev, E.; Hansma, H.; Iwata, T.; Doi, Y.; Saito, T.; Miki, K. *J. Mol. Biol.* **2006**, *356*, 993.
(19) Kasuya, K.; Tezuka, Y.; Ishii, N.; Yamagata, Y.; Shiraki, M.; Saito, T.; Hisano, T; Iwata, T.; Doi, Y. *Macromol. Symp.* **2007**, *249*−*259*, 540.
(20) Numata, K.; Hirota, T.; Kikkawa, Y.; Tsuge, T.; Iwata, T.; Abe, H.; Doi, Y. *Biomacromolecules* **2004**, *5*, 2186.
(21) Numata, K.; Kikkawa, Y.; Tsuge, T.; Iwata, T.; Doi, Y.; Abe, H. *Biomacromolecules* **2005**, *6*, 2008.
(22) Numata, K.; Kikkawa, Y.; Tsuge, T.; Iwata, T.; Doi, Y.; Abe, H. *Macromol. Biosci.* **2006**, *6*, 41.
(23) Numata, K.; Sato, S.; Fujita, M.; Tsuge, T.; Iwata, T.; Doi, Y.; Abe, H. *Polym. Degrad. Stab.* **2007**, *92*, 176.
(24) Iwata, T.; Doi, Y.; Kasuya, K.; Inoue, Y. *Macromolecules* **1997**, *30*, 833.
(25) Yamashita, K; Yamada, M; Numata, K.; Taguchi, S. *Biomacromolecules* **2006**, *7*, 2449.
(26) Sauerbrey, G. *Z. Phys.* **1959**, *155*, 206.

BM070231Z