Alkaline Hydrolysis of Solution-Grown Poly[(*R*)-3-hydroxybutyrate] Single Crystals

Tadahisa Iwata* and Yoshiharu Doi

Polymer Chemistry Laboratory, RIKEN Institute, Hirosawa, Wako-shi, Saitama 351-0198, Japan

Fumiaki Kokubu and Shinya Teramachi

Department of Applied Chemistry, Kogakuin University, Hachiouji, Tokyo 192-0015, Japan

Received July 27, 1999; Revised Manuscript Received October 15, 1999

ABSTRACT: The changes in morphology and crystalline state of solution-grown single crystals of bacterial poly[(*R*)-3-hydroxybutyrate] (P(3HB)) in NaOH solution of various strengths were investigated by means of transmission electron microscopy, atomic force microscopy, and gel permeation chromatography. Lath-shaped P(3HB) single crystals, which had widths of 2−3 μm, were split to narrow ribbonlike crystals of around 0.1 μm at the initial stage of hydrolysis without a decrease in lamellar thickness. As hydrolysis time increased, ribbonlike crystals were degraded from the lateral side of the crystal to generate the notched morphology. Furthermore, the molecular weight of degraded P(3HB) crystals corresponded to equal the value calculated from lamellar thickness measured by atomic force microscopy, suggesting that the tight chain-folding region of P(3HB) molecules on the crystal surface was degraded by alkaline hydrolysis. This surface degradation was further supported by the disappearance in the electron micrograph of straight lines on the surface of the degraded crystal decorated with polyethylene. Despite the unselective degradation of alkaline hydrolysis from both crystal edges and crystal surfaces, narrow ribbonlike crystals were generated at the initial stage of hydrolysis. This observation suggests that single crystals with chain-folding have two regions of tight chain-packing region with adjacent reentry folds and a loosely chain-packing region with random reentry folds with interval of 100 nm or less. The model developed in this paper suggests that alkaline hydrolysis with NaOH solution first occurs at the loosely chain-packing region on crystal surface and then gradually progresses from both crystal edges and tight chain-packing region with chain-folding on the crystal surface.

Introduction

Poly[(*R*)-3-hydroxybutyrate] (P(3HB)), which is produced by a wide variety of bacteria, is widespread of interest as a biodegradable thermoplastic,[1,2] and its biodegradability has been evaluated in various environments such as in soil, sludge, or seawater.[3] Enzymatic degradation by several extracellular PHB depolymerases purified from some bacteria[4−9] have been extensively studied using solution-cast films and melt-crystallized films with various degrees of crystallinity, which demonstrated that the enzymatic hydrolysis occurred first at amorphous regions and subsequently at crystal regions and that the crystallinity and lamellar crystal sizes play a decisive role in the degradation process.[10−14]

Recently, for elucidating the mechanism of enzymatic degradation in the crystal region, single crystals were used as the model substrate.[15−20] P(3HB) single crystals have been prepared from many kinds of solvents by an isothermal crystallization, and the crystal structure has been investigated using transmission electron microscopy.[21−25] P(3HB) single crystals occur as monolamellar or multilamellar lath-shaped crystals, with chain-folding along their long axes which was confirmed by the method of polyethylene decoration.[26] Microscopic observation of enzymatic degradation of P(3HB) single crystals demonstrated that the partially degraded crystalline lamellae had been splintered along the long axis of the crystals with the lack of decrease in molecular weight.[15,16,18,19] Furthermore, on the basis of the results of lamellar thickness, it was confirmed that enzymatic degradation of P(3HB) single crystals progressed from crystals edges and ends rather than the chain-folding surfaces of crystals, and an edge attack model for enzymatic degradation of P(3HB) single crystals was proposed.[16,19]

An edge attack model for enzymatic degradation of single crystals was also suggested in the other aliphatic polyester single crystals of P(3HB) copolymers with different second monomer units[17,20] and poly(L-lactic acid).[27] In contrast, regarding the chemical degradation, completely different results have been reported. P(3HB) single crystals were degraded selectively from crystal surfaces with gaseous methylamine, followed by analysis of gel permeation chromatography of their degradation products.[28] This selective degradation was subsequently confirmed on the degradation of P(3HB) single crystals by methylamine and cyclohexylamine in solution, which was investigated by means of transmission electron microscopy and atomic force microscopy.[29,30] This observation was further reported on in the morphological study on hydrolytic degradation of poly(tetramethylene succinate) single crystals.[31]

The present work was undertaken in order to obtain more insight into the degradation mechanism of P(3HB) single crystals by alkaline hydrolysis with a dilute NaOH solution, using transmission electron microscopy, atomic force microscopy, and gel permeation chromatography. The alkaline hydrolyses of single crystals are compared with enzymatic degradation of them, combined with the crystal and surface structures of lamellar single crystals.

* To whom all correspondence should be addressed. Tel +81-48-467-9586, Fax +81-48-462-4667.

10.1021/ma991248f CCC: $18.00
Published on Web 11/24/1999

Experimental Section

Preparation of Single Crystals. Single crystals of P(3HB) were prepared from a mixture of chloroform and ethanol under isothermal crystallization conditions. The P(3HB) sample (M_w = 23 000, M_n = 17 000, and DPI = 1.3) was dissolved into chloroform, recrystallized by the addition of an excess of warm ethanol, and kept at crystallization temperature for several hours, according to the method described previously.[17,18] The crystals were collected by centrifugation and washed three times with room-temperature methanol.

Alkaline Hydrolysis. For alkaline hydrolysis, the crystals were collected by centrifugation, washed once with distilled water, and resuspended in the given concentration of NaOH solution. Single crystals of P(3HB) were hydrolyzed in NaOH solution at 37 °C for various hours, which was not shaken in order to prevent the single crystals from breakdown by trembling. The degraded single crystals were washed three times with distilled water to remove the NaOH solution. The crystals were then redispersed in methanol, washed twice by centrifugation, and resuspended in methanol.

Transmission Electron Microscopy. Drops of the crystal suspension, before and after alkaline hydrolysis, 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. The decoration of single crystals with polyethylene was performed by evaporating polyethylene on the crystals under vacuum according to the method of Wittmann and Lotz[32] and then shadowed with a Pt−Pd alloy. These grids were observed with a JEM-2000FX II electron microscope operated at an acceleration voltage of 120 kV for both electron diffraction and imaging of shadowed crystals. Electron diffraction diagrams and images were recorded on Kodak SO-163 and 4489 films, respectively, developed for 4 min with Kodak D-19 developer (diluted in water 1/2, v/v).

Atomic Force Microscopy. The thicknesses and surface morphology of single crystals, before and after alkaline hydrolysis, 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 micro-cantilevers with spring constants of 0.09 N/m, were applied for the contact mode experiments. Simultaneous registration was performed in the contact mode for height and deflection images. Drops of the crystal suspension, before and after alkaline hydrolysis, were given on mica and allowed to dry. All images were recorded at room temperature.

Molecular Weight Measurement. All molecular weight data of single crystals before and after alkaline hydrolysis 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- 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. Molecular weight was obtained with polystyrene standards of low polydispersities.

Results and Discussion

Morphological Changes of Single Crystals. A typical electron micrograph of P(3HB) single crystals grown from a mixture of chloroform and ethanol and its electron diffractogram are shown in Figure 1. Single crystals of P(3HB) occur as monolamellar or multilamellar lath-shaped crystals with dimensions of around 2−3 μm wide and of around 10−20 μm long. During their handling and especially during the centrifugation step, many ends of single crystals get broken. The

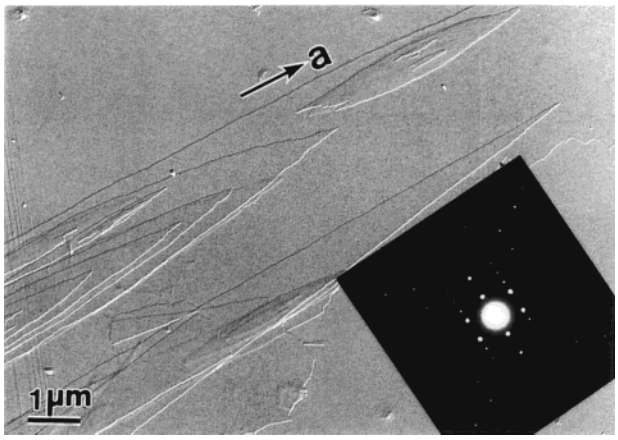

Figure 1. Electron micrograph of P(3HB) single crystals, grown from a mixture of chloroform and ethanol, shadowed with a Pt−Pd alloy. Inset: typical electron diffraction pattern corresponding to a single crystal.

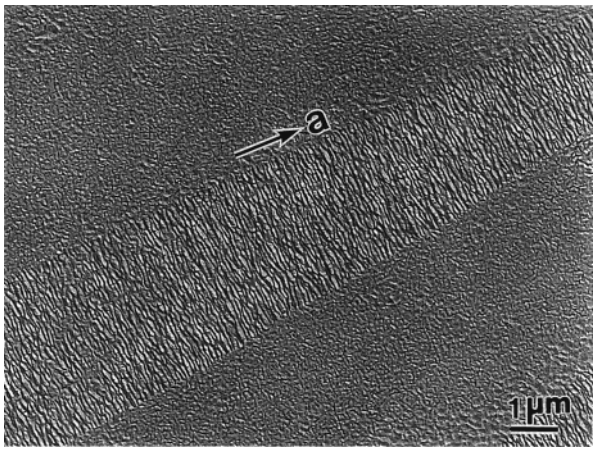

Figure 2. Typical electron micrograph of P(3HB) single crystal decorated with polyethylene and shadowed with a Pt−Pd alloy.

electron diffractogram is defined by two orthogonal axes, with the *a* axis parallel to the long direction of the crystal and the *b* axis perpendicular.

Figure 2 shows a typical electron micrograph of a P(3HB) single crystal decorated with polyethylene. The polyethylene decorated on the surfaces of single crystals appears as crystals that have their long axes perpendicular to the long axes of P(3HB) crystals, and the polyethylene crystals run more or less continuously across the P(3HB) crystals. Therefore, it was deduced that the average direction of molecular chain-folding is along the long axis of single crystals, that is, along the crystallographic *a* axis, as reported by Birley et al.[26] Thus, the predominant fold model in P(3HB) crystals is proposed along the [100] direction with existing successive folds in the [110] and [1$\bar{1}$0] directions.

The thickness by AFM of the monolamellar part of single crystals yielded values of around 5 nm. Sykes et al. reported that the single crystals prepared from an oligomer of 32 HB units had 5 nm lamellar thickness, and this oligomer folded once within a crystal.[29] On the basis of these results, it is concluded that P(3HB) single crystals are intramolecular single crystals that have chain-folding surfaces such as polyethylene[33,34] and polypropylene.[35]

Figure 3 shows two transmission electron micrographs of P(3HB) single crystals degraded in NaOH

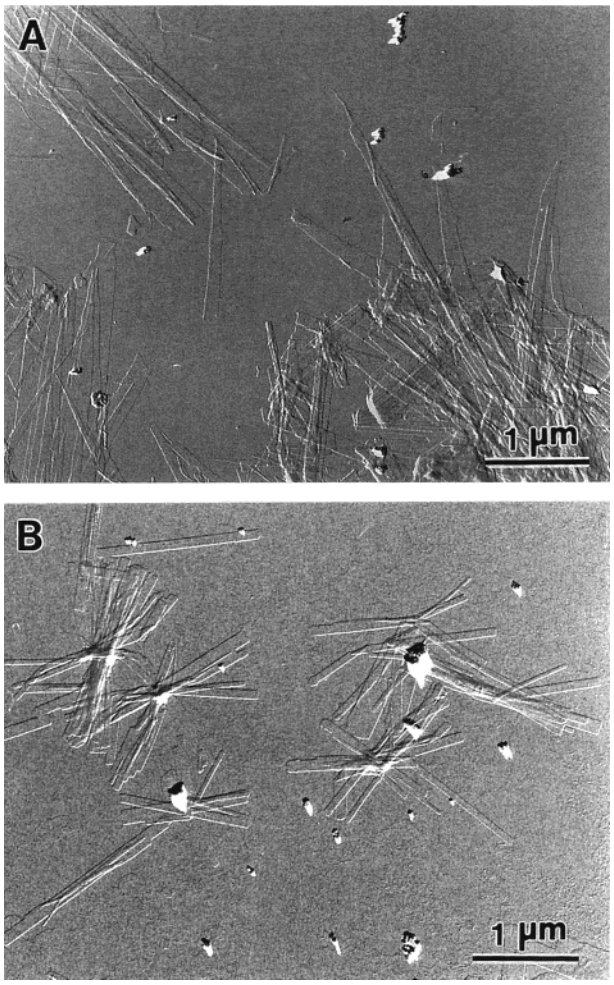

Figure 3. Electron micrographs of P(3HB) single crystals after alkaline hydrolysis: (A) after 1 h in 0.1 N NaOH solution; (B) after 1 h in 0.5 N NaOH solution.

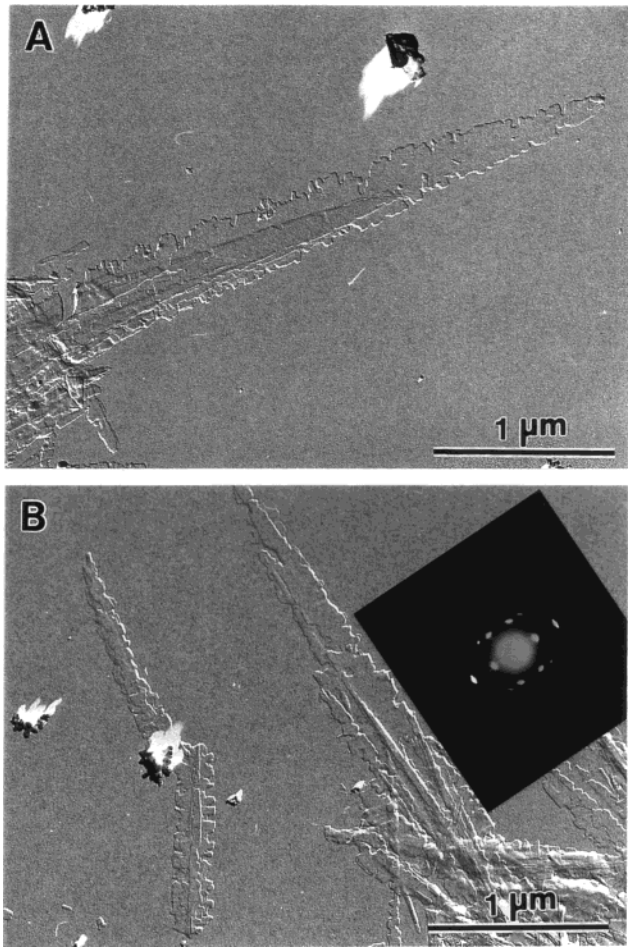

Figure 4. Electron micrographs of P(3HB) single crystals after alkaline hydrolysis: (A) after 16 h in 0.1 N NaOH solution; (B) after 2 h in 0.5 N NaOH solution. Inset: typical electron diffraction pattern corresponding to a degraded single crystal.

solutions of 0.1 N and 0.5 N for 1 h. The crystals were longitudinally split to narrow ribbonlike crystals, which had widths of 100 nm or less, and these ribbonlike crystals were sometimes radially oriented as appeared to be the case in Figure 3B. This split phenomenon occurred parallel to the crystallographic *a* axis which corresponds to the direction of chain-folding of P(3HB) chains. The narrow ribbonlike crystals retained their high crystallinity and gave the same electron diffraction pattern as that of undegraded crystals. This result indicates that the crystal structure was not broken on the molecular level during the initial stage of alkaline hydrolysis.

The ribbonlike morphology of P(3HB) was first reported by Ellar et al.,[36] when native granules were treated with 25% acetone. They found two morphologies in P(3HB) native granules treated with acetone; one was a 10−15 nm fibril structure which had extended polymer chains, and the other was a 5 nm thick ribbonlike structure formed by the reorganization of fibrils in aqueous acetone. Furthermore, this ribbonlike structure was transformed to lamellar single crystals with molecular chain-folding. These ribbonlike crystals had widths of 100 nm or less. These values correspond to those of split crystals degraded by alkaline hydrolysis observed in our present study. Recently, the longitudinal splintering of single crystals have been observed in micrographs, when single crystals of P(3HB) were degraded by some kinds of extracellular PHB depolymerases.[16,18] Single crystals were splintered from crystal ends for generating the de novo faces of developing splinters. These splinters of single crystals had also widths of 100 nm or less, and the crystal splinters retained the single-crystal organization of the original material.

The most prominent changes of single-crystal morphologies were observed with the progress of alkaline hydrolysis. Figure 4 shows P(3HB) single crystals degraded by NaOH solutions of 0.1 N for 16 h and of 0.5 N for 2 h. The lateral sides of both crystals were cut into notched shapes, despite the whole outline retaining the lath shapes. Furthermore, some small fragments that seemed to be separated from the lateral sides of lamellar crystals were observed. An electron diffractogram of a notched-shaped crystal (inset in Figure 4B) showed arc reflections and lower resolution than that of undegraded crystals, suggesting that molecular packing into the crystal lattice became slightly disordered due to the chain rotation around the fiber axis.

Atomic force microscopy images of P(3HB) single crystals before and after alkaline hydrolysis by a 0.1 N NaOH solution for 5 h and line profile data are shown in Figure 5. The lamellar thickness of a narrow ribbonlike crystal split from initial crystals remained unchanged at the initial stage of hydrolysis. On the other hand, the cross section shows the curvature at the crystal edges that was generated by the splitting step

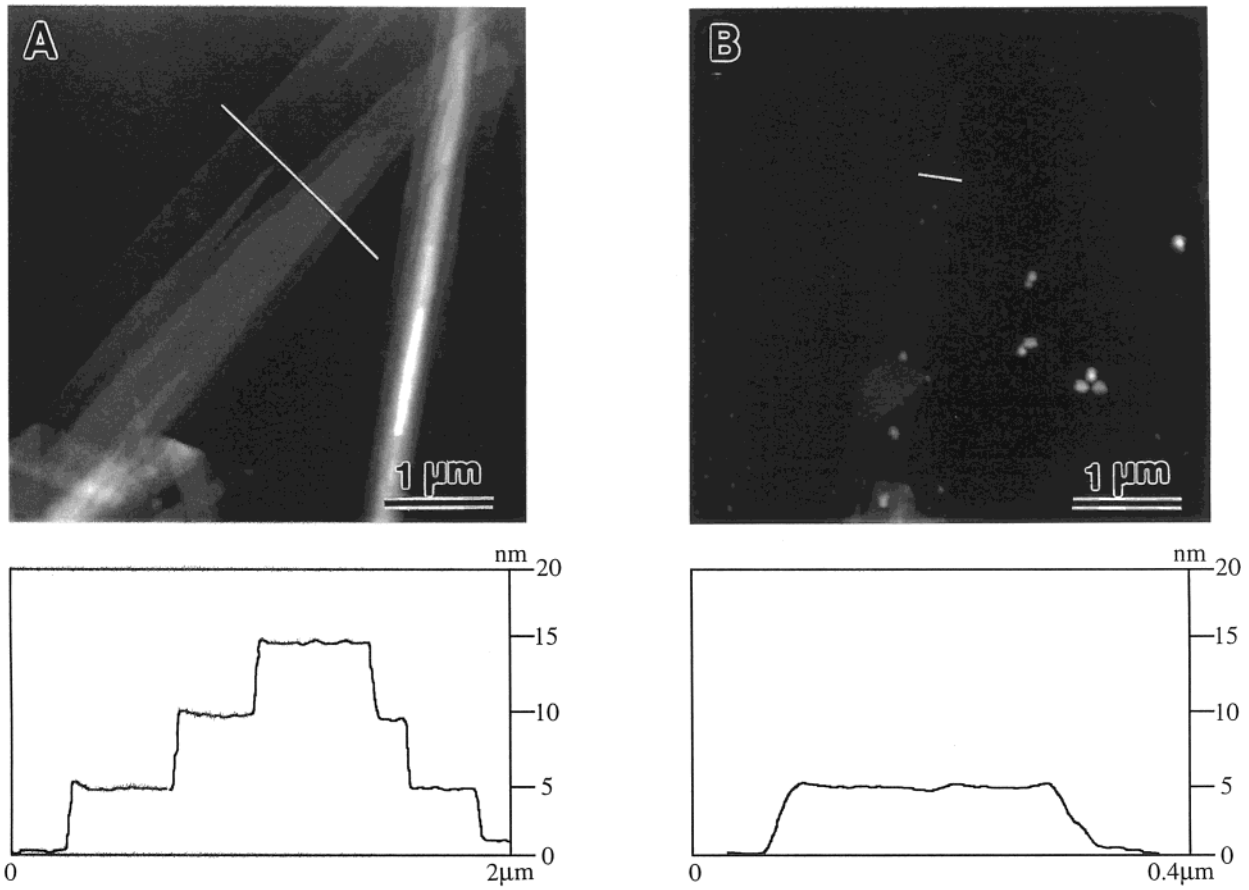

Figure 5. AFM images of P(3HB) single crystals (A) before and (B) after alkaline hydrolysis of 5 h in 0.1 N NaOH solution and line profile data of white line.

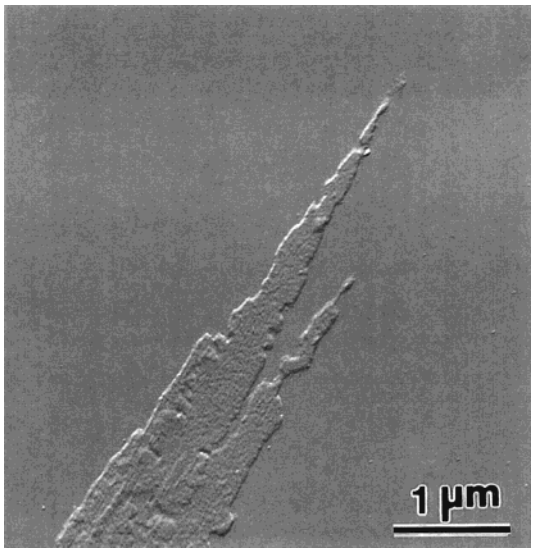

Figure 6. AFM deflection image of P(3HB) single crystals after alkaline hydrolysis of 16 h in 0.1 N NaOH solution.

of single crystals in the initial stage of hydrolysis. However, in the case of the more degraded crystals shown in Figure 6, one sees that the crystal surface of the single crystal was degraded into a bumpy surface with very small holes. This result indicates that the single crystals are degraded from the crystal surface in addition to lateral sides of crystals, as the hydrolysis time increases. This surface degradation was further supported by the disappearance of straight lines on the surface of the degraded crystal decorated with polyethylene in the electron micrograph (data not shown). These results suggested that the hydrolysis occurred at the chain-folding on the crystal surface.

Sykes et al. reported that P(3HB) single crystals were degraded at the center of the crystal surface and also at the crystal edges by the degradants of methylamine and cyclohexylamine.[30] Especially, the methylamine-degraded crystal had a bumpy surface, and in some cases small pits along the long axis could be seen on the surface close to the crystal center. These suggested that chain-folding in crystal centers is looser than at the edges, and the central folds might be easier for degradants to attack. This observation of small pits on the degraded crystal surface seems to be preferentially correlated with the formation of the narrow ribbonlike crystal morphology presented in Figure 4B. Thus, if degradation occurred strongly at the center of crystal surface in the initial stage of hydrolysis, the crystals should be split to ribbonlike crystals with widths of 100 nm or less.

Molecular Weight Changes. The measurement of molecular weight during the hydrolysis of single crystals can aid in elucidating the degradation mechanism of the molecular level. Partial degradation at the chain-folding surface of single crystals has been confirmed to cause a molecular weight change. Figure 7 shows the molecular weight distributions of P(3HB) obtained by gel permeation chromatography before and after degradation of P(3HB) single crystals by a NaOH solution for various lengths of time. At early stages of degradation, the molecular weight decreased and the distribution increased by a heterogeneous hydrolysis correlating to the degradation of crystal edges and the split of crystals. These phenomena exhibit discrete peaks which become

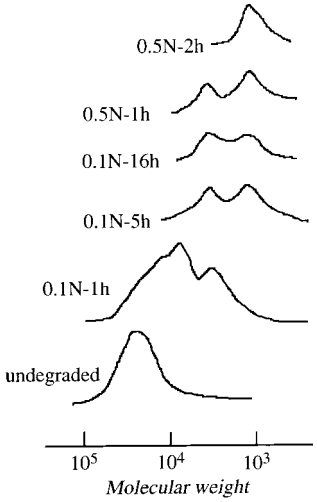

Figure 7. Gel permeation chromatograms of P(3HB) single crystals before and after partial degradation in NaOH solution.

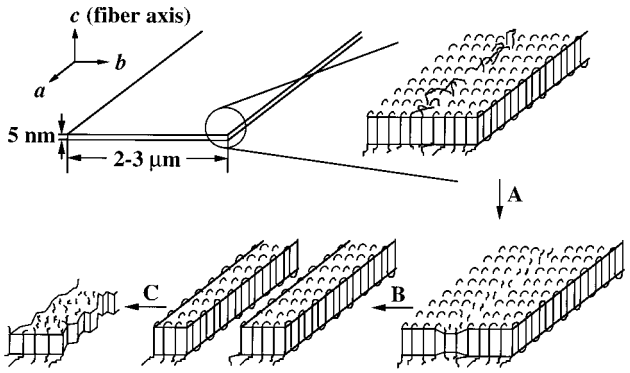

Figure 8. Schematic representation on the alkaline hydrolysis of P(3HB) single crystals in NaOH solution: (A) degradation for switchboard model region with random reentry fold, (B) splitting of crystals, and (C) surface and edge degradation. P(3HB) single crystals have adjacent reentry sharp chain-folding and switchboard reentry loose chain-folding on crystal surface. The average direction of chain-folding is parallel to the crystallographic *a* axis.

more prominent as the degradation proceeds. After the longer degradation time, two main peaks remained at different molecular weights of 1500 and 3000. Taking the fiber repeat distance of 0.596 nm with 2-fold screw symmetry along the molecular axis into consideration,[37,38] these two molecular weights correspond to single and double traverses of crystal lamellae. Thus, the results indicate that alkaline hydrolysis with NaOH solution occurred at the chain-folding surfaces and that the chain-folding of P(3HB) molecules is buried under the crystal surface in some cases.

The surface degradation of P(3HB) single crystals, with gaseous methylamine, methylamine in solution, and cyclohexylamine in solution, has been already reported by Welland et al.[28] and Sykes et al.[29,30] In the cases of methylamine in gaseous phase or in solution, the degradation yielded fragments with lengths corresponding to integer multiples of lamellar thickness. On the other hand, since cyclohexylamine is a bigger molecule than methylamine, the reduction of molecular weight by cyclohexylamine was more gradual, and it was harder to attack the tight chain-folding. In this present study, NaOH is a quite small molecule and is probably able to attack tight chain-folding regions at the crystal surfaces.

Degradation Mechanism. Results from the transmission electron microscopic and atomic force microscopic observations combined with molecular weight measurement showed that solution-grown P(3HB) single crystals were completely degraded from both crystal edges and crystal surfaces by alkaline hydrolysis. Single crystals of aliphatic polyesters, grown from a dilute solution by isothermal crystallization, normally crystallize with chain-folding on the crystal surface. Chain-folding structure in polymer single crystals is mainly classified as two types: an adjacent reentry model with regular sharp fold and a switchboard model with random reentry fold.

P(3HB) single crystals have chain-folding along the crystallographic *a* axis with existing successive folds in the [110] and [1$\bar{1}$0] directions, as confirmed by the method of polyethylene decoration.[26] At the first stage of hydrolysis, single crystals are split along the long axis of the crystal to narrow ribbonlike crystals with widths of 100 nm or less, and this split direction corresponds to the chain-folding one. The chemical reagent can first attack the region of random reentry fold on crystal surface. Accordingly, in P(3HB) single crystals, switchboard model regions with random reentry folds exist at a certain interval between tight chain-packing regions with widths of 100 nm or less, as shown in Figure 8. If the region of random reentry folds is predominantly degraded, one sees the small pits on the crystal surface along the long axis of crystal reported by Sykes et al.[29,30] Subsequently, narrow ribbonlike crystals and curvature of crystal edges should be generated as observed in this paper (Figures 3 and 5). At the longer degradation, the crystal edges are systematically serrated with a certain interval, as degradation at the chain-folding surfaces progressed (Figures 4 and 6). The decrease of molecular weight measured by GPC and surface erosion confirmed by AFM of degraded single crystals supported that the alkaline hydrolysis occurred from crystal surfaces.

P(3HB) single crystals were degraded only from crystal edges without changes in molecular weight by extracellular PHB depolymerases as reported by Hocking et al.,[15] Nobes et al.,[16] and Iwata et al.[17,18] However, during the enzymatic degradation of single crystals, the splintering of single crystals was also observed in electron micrographs as well as during the alkaline hydrolysis. This phenomenon was explained in terms of a selective hydrolysis of P(3HB) chains by active site of enzyme with *endo* type. Thus, the enzyme requires de novo face at the lateral face of the crystal, and enzymatic attack occurs at the disordered chain-packing region of crystals. P(3HB) crystals were first split along the chain-folding direction of crystal long axis during the initial stage of both alkali and enzymatic hydrolyses. This might be linked to the crystal growth of P(3HB) single crystals. In P(3HB) single crystals, loosely chain-packing region between tight chain-packing regions exists with a certain interval. These regions might be generated when the crystals are prepared. From the native granules, first some molecules aggregate as needlelike morphology and then recrystallize to ribbonlike morphology such as the lamellar crystal. Accordingly, in the crystals, original core crystals should exist, and these crystals are linked by the switchboard-type molecules.

The molecular size of extracellular PHB depolymerase is estimated as 18−25 nm on the basis of the results of adsorption kinetics on the film surface of P(3HB),[39] while the size of a chemical reagent such as NaOH and

methylamine is quite small. Accordingly, an enzyme molecule cannot attack tight chain-folding at the crystal surfaces and the reaction of degradation progresses by an edge attack mechanism. This edge attack mechanism was confirmed by the fact that the degradation rate of single crystals without chain-folding molecules on the crystal surface was faster than that of single crystals with chain-folding.[17,18] If single crystals after alkaline hydrolysis are applied to the enzymatic reaction, the rate of degradation should be markedly enhanced. The degradation of biodegradable materials in the environment might be accelerated by the synergistic effect of both enzymatic and chemical hydrolyses.

Conclusions

In this paper we have reported the visualization of alkaline hydrolysis with NaOH solution of P(3HB) single crystals grown from a mixture of chloroform and ethanol by means of transmission electron microscopy and atomic force microscopy. The degradation of lath-shaped single crystals progressed from a loosely chain-folding region on the crystal surface, and as a result the single crystals were split to narrow ribbonlike crystals with 100 nm wide or less. On the hydrolysis time increasing, the ribbonlike crystals were further hydrolyzed from both crystal edges and crystal surfaces to yield notched crystals with bumpy surfaces. The molecular weight of the remaining crystals decreased with time, and the values of stable molecular weight corresponded to single and double traverses of crystal lamellae. This result indicates that alkaline hydrolysis in NaOH solution occurs at the tight chain-folding on the crystal surface and in the lamellar interior.

Chemical degradation of crystal regions of biodegradable materials occurs unselectively from both crystal edges and crystal surface. Chemical degradation leading to an increase in the surface area of crystals should accelerate the degradation of biomaterial by enzyme. The surface erosion of single crystals by a chemical reagent generates a new surface without chain-folding. Taking the crystal structure with space group of $P2_12_12_1$ into consideration, on the crystal surface of degraded crystals there exist two functional groups, hydroxyl and carbonyl groups, alternatively. These systematic surfaces with double functions might be quite useful for generating the new biodegradable material.

Acknowledgment. This work has been supported by CREST (Core Research for Evolutional Science and Technology) of Japan Science and Technology Corporation (JST).

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MA991248F