

Chain Orientation and Degradation Behavior of Poly[(R)-3-hydroxybutyrate] Lamellar Crystals

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Topological changes caused by the alkaline and enzymatic attacks of solution-grown, chain-folded lamellar crystals (SGCs) of poly[(R)-3-hydroxybutyrate] P(3HB) have been studied in order to investigate the chain-folding structure in P(3HB) crystal regions. NaOH and an extracellular PHB depolymerase purified from *Alcaligenes faecalis* T1 were used for alkaline and enzymatic hydrolysis, respectively. The measurements were performed on crystals attached to a substrate which is inactive to degradation mediums. Both alkaline and enzymatic attacks lead to a breakup of the lamellar crystals along the crystallographic *b*-axis during initial erosion. Since hydrolysis preferentially occurs in amorphous regions, this morphological result reflects relatively loosely packed chains in core parts of lamellar crystals. Additionally, it was supported by the ridge formation along the *b*-axis in the lamellar crystals after thermal treatment at a low temperature because of the thermally sensitive nature of the loosely packed chains in lamellar crystals. However, the alkaline hydrolysis accompanied the chain erosions or scissions in quasi-regular folded lamellar surfaces due to smaller size of alkaline ions in comparison to the enzyme, resulting in the decrease of molecular weight.

Keywords : Poly[(R)-3-hydroxybutyrate], Lamellar crystals, Alkaline and enzymatic attacks, Ridge.

Introduction

Recent trends in polymer research point to an increasing demand for development of a range of environmentally friendly biodegradable polymer products with predetermined lifetime in the areas of biomedical applications and polymer waste management caused by synthetic non-degradable polymers.¹⁻⁹ Among the most studied polymers are biopolymers and blends containing biopolymers. Many microbial systems, especially bacteria, use poly(hydroxyalkanoate)s as energy storage materials, of which poly(3-hydroxybutyrate) (P(3HB)) is the most common for commercial biotechnological products.

Since the extracellular PHB depolymerases were purified from various bacteria isolated from *Pseudomonas pickettii*,¹⁰ *Pseudomonas stutzeri*,¹¹ *Pseudomonas lemoignei*,¹² and *Alcaligenes faecalis*,¹³ the enzymatic degradation of poly[(R)-3-hydroxybutyrate] (P(3HB)) and its copolymers produced from renewable resources by bacterial fermentation has been extensively studied in solution-cast^{14,15} and melt-crystallized films.^{16,17} The results have revealed that the enzymatic degradation of P(3HB) preferentially occurred in the amorphous region and subsequently in the crystalline region. Since the amorphous regions tie crystallites together, catastrophic mechanical failure occurs before weight loss, for example, 1.7% weight loss for 66% strength loss.¹⁸ For this reason, an initial stage in degradation processes is important for applications of disposable materials, although in medical applications, specially drug delivery system, all

degradation processes affect the drug efficiency.

The chain-folded solution-grown lamellar crystals (SGCs) of P(3HB) have been used as a model system for elucidating the mechanism for both enzymatic and hydrolytic degradations in the crystalline region.^{9,10,12,13,19,20} After partial degradations of SGCs by PHB depolymerases, the remaining crystals exhibited needle- or saw-like morphology along the long axis of the crystals; the chain scission predominantly takes place parallel to the average chain-folding direction at the extreme ends of lath-shaped crystals. Thus, it is generally accepted that the behavior of enzymatic hydrolysis of SGCs is governed by the crystal structure and folding. Nobes *et al.*¹² and Iwata *et al.*¹³ suggested that the *endo* and *exo*-type enzyme attack process may be a more effective mechanism for enzymatic hydrolysis when disordered regions exist within the lamellar crystal. Recently, Lee *et al.*²⁰ reported that the preferential enzymatic erosion in P(3HB) lamellar crystals initially occurs at their less ordered regions, parallel to the *b*-axis, resulting in the breakup of lamellar crystals. This was supported by thermal treatment and deformation results of P(3HB) lamellar crystals. However, alkaline hydrolysis showed differences in morphologies and molecular weight changes to enzymatic one.¹⁹ In this article, we attempt to obtain further insight into the alkaline and enzymatic hydrolysis mechanism of SGCs. Additionally, a substrate effect to PHB depolymerases was studied.

Experimental Section

Preparation of solution-grown crystals. The low molecular weight P(3HB) ($M_n = 47,000$ and $M_w/M_n = 1.6$) used in this study was prepared by a basic hydrolysis of bacterial

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P(3HB) ($M_n = 380,000$ and $M_w/M_n = 2.1$) obtained from ICI (trade name: Biopol), as reported previously.¹³ Purified P(3HB) was dissolved in chloroform and 1 N aqueous KOH and 18-crown-6 ether was added. The solution was stirred vigorously at 37 °C. The organic layer was extracted, dried over $MgSO_4$, and filtered. The resulting organic solution was precipitated in an excess of methanol and dried in vacuum for 1 week. SGCs were prepared from an octanol-diluted solution, collected by centrifugation, washed with methanol several times, then resuspended in methanol.

The samples were prepared by dropping crystal suspensions onto cleaned silicon wafers ($3 \times 3 \text{ mm}^2$) for enzymatic hydrolysis and annealing experiments whereas the mica was used for alkaline hydrolysis in order to avoid side reaction of silicon wafer with NaOH.

Thermal treatment. Thermal treatments of SGCs on a silicon wafer were undertaken using DSC (Perkin-Elmer Pyris 1) under nitrogen. A sample was heated to a desired temperature (T_Q) at a rate of 20 °C/min and then immediately quenched to -10 °C (with a cooling rate of at least 350 °C/min which was enough to prevent recrystallization during cooling). The real temperature of samples on a silicon wafer was calibrated to within error of ± 2 °C using a digital surface thermometer (Anritsu meter Co.).

Hydrolysis. The enzymatic hydrolysis of both SGCs and their thermally-treated samples was carried out on clean silicon wafers in 50 mM Tris-HCl buffer (pH 7.5). Samples were placed in a sterilized plastic cuvette containing 600 μL buffer solution. The degradation was started by the injection of 0.5 μL (ca. 0.1 μg) solution (209 $\mu\text{g}/\text{mL}$) of an extracellular PHB depolymerase purified from *Alcaligenes faecalis* T1. On the other hand, alkaline hydrolysis was conducted by SGCs on mica in 1 mL of 0.1 N NaOH solution. The reaction solutions were incubated at 37 °C. Samples were carefully washed with an excess of distilled water and dried in vacuum. To investigate the substrate effect on the enzymatic hydrolysis of P(3HB), 4 μL of 209 $\mu\text{g}/\text{mL}$ solution of an extracellular PHB depolymerase purified from *Alcaligenes faecalis* T1 was added to 1 mL of Tris-HCl buffer containing P(3HB) granule (0.5 mg/mL) in a transparent plastic cuvette. A piece of high oriented pyrolytic graphite (HOPG) (or silicon wafer) ($0.7 \times 0.7 \text{ cm}^2$) was inserted into the solution and then incubated at 37 °C. The turbidity of the solution at 660 nm was measured as a function of hydrolytic time.

AFM studies. AFM measurements were conducted using a scanning force microscope, SPA 300 instrument with SPI 3700 controller (Seiko Instrument Co.) at room temperature. The cantilever used in this study was triangular with a microfabricated Si_3N_4 microtip (Olympus Co.), and a spring constant of 0.022 N/m. The scanning direction was perpendicular to the long axis of the cantilever. Topographic and deflection images were carried out simultaneously in the repulsive force region (ca. 1 nN). Although the deflection image could not give information on the height, it is a useful mode to reveal the changes in the height of sample surface, especially when the sample surface is rough.

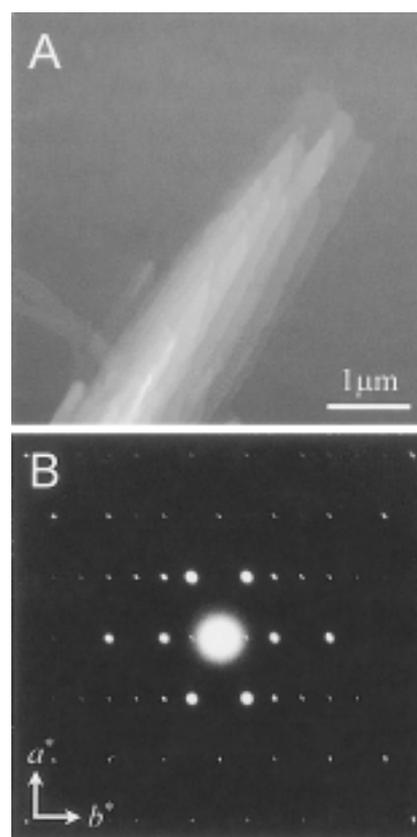


Figure 1. AFM topographic image (A) and typical electron diffraction diagram (B) of solution-grown P(3HB) crystals.

Results and Discussion

Figures 1A and 1B show, respectively, AFM topographic image and typical electron diffraction pattern of P(3HB) SGCs obtained from octanol solution, with multilamellar lath-shaped crystals grown from the aggregation center. The diagram is defined by that the two orthogonal axes a and b where the long axis of lamellar crystals is their crystallographic a axis, as reported elsewhere.^{9,20} The solution-grown lamellar P(3HB) crystals were often aggregated but individual lamellae were available for study at the periphery.

Polymeric materials for surface morphological studies are often cast on very flat substrates, such as silicon wafer, HOPG, and mica, in order to minimize a substrate effect induced by its surface roughness. Before selecting a substrate, the substrate activity to degradation medium was investigated. Since silicon wafer can be reacted with NaOH, mica as a substrate was selected for alkaline hydrolysis. To investigate the activity of PHB depolymerase to silicon wafer and HOPG, turbidity measurements were performed as described in Experimental section. Figure 2 shows the turbidity profile for the degradation of PHB granules by PHB depolymerase purified from *Alcaligenes faecalis* T1 under a piece of substrate. All turbidity curves decreased with time, which indicates that some chains of P(3HB) are broken, leading to a lower molecular weight. However, the extent of the decrease of the turbidity of HOPG-containing

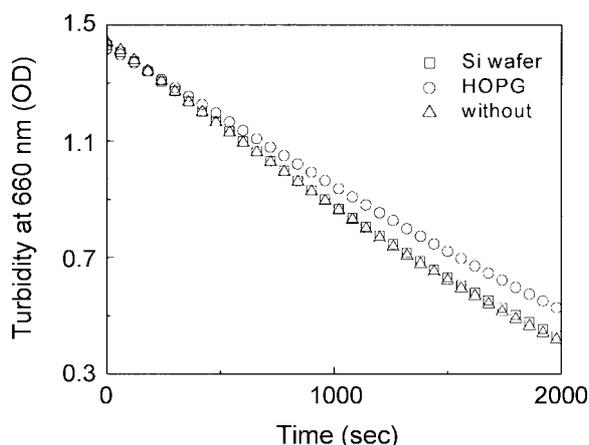


Figure 2. Turbidity profiles of PHB granule-suspended solutions at 37 °C by an extracellular PHB depolymerase purified from *A. faecalis* T1.

PHB granule was not the same as that of PHB granule whereas the turbidity curve of silicon wafer-containing PHB granule was similar to nascent one; the turbidity change of HOPG-containing PHB granule takes place more slowly than the other two. This phenomenon may be attributed to the decreasing numbers of active enzyme due to the adsorption of enzyme to HOPG substrate. The result suggests that a special care on choice of a substrate should be taken in the study of hydrolytic behavior of P(3HB) cast on a substrate.

Single crystals of P(3HB) as a model system have been used for elucidating the crystal structure as well as the mechanism of hydrolytic degradations. Several groups reported that P(3HB) single crystals were degraded at crystal edges as well as at crystal surface, resulting in small crystal fragments with some holes.^{12,13,19,20} However, the thickness of lamellae was almost unchanged after both alkaline (for example, NaOH)¹⁹ and enzymatic¹³ hydrolysis. This indicates that the initial attack of both degradation mediums rapidly occurs at lamellar edges and on chain-folded surface where the chains in lamellae are much loosely packed. However, their GPC results showed that the alkaline hydrolysis decreases the molecular weight of remained crystals whereas there is no difference in molecular weights after enzymatic hydrolysis.^{13,19} The result implies that the chain scission on quasi-chain folded lamellar surface regions is accompanied by alkaline attacks, leading to the decrease of molecular weights. Since Na⁺OH⁻ ions are much smaller than PHB depolymerases of around 18–25 nm,¹⁹ alkaline mediums easily access to on quasi-chain folded lamellar surface regions. Figure 3 shows the topographic images after enzymatic (A) and alkaline (B) hydrolysis. A noticeable feature of lamellae after alkaline hydrolysis is that lamellar interfaces and edges became smooth, even though the interfaces between lamellae after the enzymatic hydrolysis are clear. This alkaline-induced surface morphological change is explained by the erosion of quasi-chain folded lamellar surfaces. Instead, their well-packed core parts in lamellae are hard to be attacked by alkaline ions. The morphological

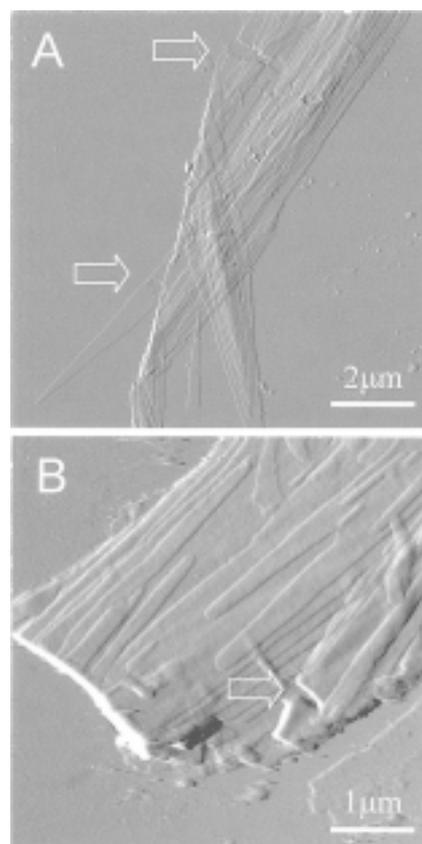


Figure 3. AFM topographic images of solution-grown crystals after hydrolysis at room temperature by an extracellular PHB depolymerase purified from *A. faecalis* T1 (A) and 0.1 N NaOH solution (B). The arrows indicate the breakup of lamellar crystals along the *b* axis.

observations are closely related to the decrease in molecular weight of lamellar crystals after alkaline hydrolysis, as reported by Iwata *et al.*¹⁹

From extensive TEM studies performed on the enzymatic hydrolysis of SGCs, it is known that the hydrolysis by an enzymatic attack progresses from the edges and along their long axes rather than the chain-folding surfaces; this mechanism yields crystal fragments with narrow cracks or splinters.^{12,13,20} The observation of narrow cracks in the enzymatically degraded SGCs was explained in terms of the chain-folding structure and its direction. The deflection image of the SGCs of P(3HB) quenched at 113 °C is shown in Figure 4. After the quenching from 113 °C lower than its melting temperature, small ridges perpendicular to the long axis (*a*-axis) of the lamellar crystal are observed. Considering the *T_m* of SGCs, *i.e.* 136 °C, this is interpreted in terms of the chain extension due to the active molecular motion of loosely packed regions in the lamellar crystals. Our previous result²⁰ showed that the *a*-axis elongation of crystals more easily led to microcracks along the *b*-axis, comparing microcracks along the *a*-axis induced by the *b*-axis elongation and that the ridges on lamellar crystals induced by deformation was preferentially degraded by enzymatic attacks due to their less ordered chain structure.

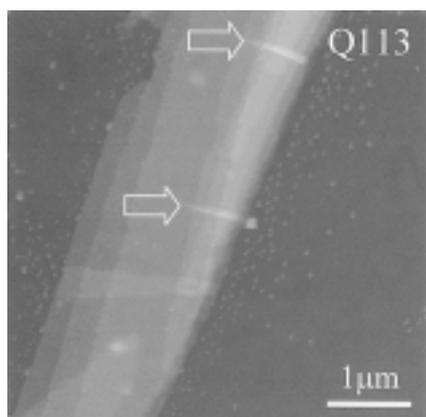


Figure 4. AFM topographic image of solution-grown P(3HB) crystals after quenching at 113 °C. The arrows indicate the ridges.

Since chain lengths of polymeric materials are usually not enough to form single chain lamellar structure, single lamellar crystals are composed with many chains. The irregularity in lamellar crystals will be mainly due to the chain end parts which show high molecular mobility. Once a chain is irregularly packed, the regular packing of the adjacent chains perpendicular to chain folding direction is disturbed and is easily attacked by degradation mediums, *i.e.* neighboring effect. The “neighboring effect” leads to the breakup and ridge formation of lamellae to the *b*-axis by hydrolyses and thermal treatment, respectively. This explanation was supported by the elongation result that the cohesive energy between adjacent chains along the *a*-axis is stronger than that along the *b*-axis.¹⁶

The chain fold structures in lamellar crystals are composed with the core part and chain folded surfaces. Defect regions in the lamellar core can be attacked by both enzymatic and alkaline hydrolytic mediums, leading to the breakup of lamellae along the *b*-axis (see Figure 3). This phenomenon is supported by the morphological change induced by the thermal treatment of lamellae because the chains in the defect regions are thermally susceptible. The thermal treatment of P(3HB) lamellar crystals at a lower temperature leads to the chain extension of defect regions due to the active molecular motion (ridge formation along the *b*-axis, see Figure 4). Since some folded chains with quasi regularity are tied to well-packed core parts and their molecular motion induced by heat is relatively restricted, however, there is no significant change of folding surface after the thermal treatment.

The size of hydrolytic medium has to be considered as an additional factor in the alkaline hydrolysis of the lamellar P(3HB) crystals, since a chemical reagent such as NaOH is much smaller than that of extracellular PHB depolymerase from *A. facalis* (18–25 nm). Some chain folding surfaces with quasi regularity can be attacked only by the alkaline ions, but their core parts are well packed and not to be attacked by ions, resulting in the erosion of chain-folded lamellar surface and the decrease of molecular weight.¹⁹ It should be noted that the lamellar thickness of P(3HB) single

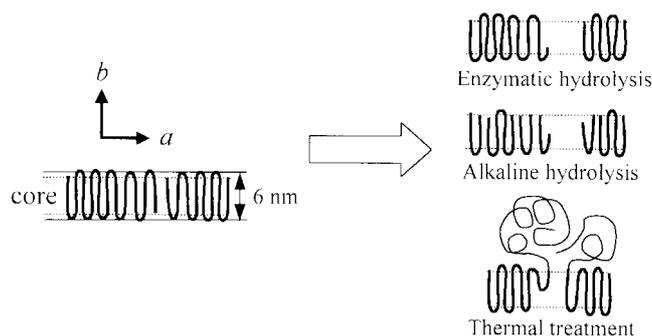


Figure 5. Schematic representation of morphological changes of P(3HB) lamellar crystals caused by hydrolysis and thermal treatment.

crystals is about 6 nm and the thickness of a chain folding surface in lamellae is around 0.5 nm.²¹ This partial change of lamellar thickness (0.5 nm) due to the erosion in some folding surface is negligible in comparison to the thickness of remained parts (5.5 nm). Accordingly, the thickness of lamellae after the alkaline hydrolysis remained unchanged. Such speculation can be schematically summarized in Figure 5.

Conclusions

To elucidate the initial degradation in crystal regions in terms of chain packing structure, the morphological changes of solution-grown crystals P(3HB) multilamellar crystals attached to a substrate during alkaline (NaOH) and enzymatic (an extracellular PHB depolymerase purified from *Alcaligenes faecalis* T1) hydrolysis have been studied using an atomic force microscopy. In order to avoid the activity of degradation mediums to substrates, silicon wafer and mica were used for enzymatic and alkaline hydrolysis, respectively. An initial state of both alkaline and enzymatic attacks leads to a breakup of the lamellar crystals along the crystallographic *b*-axis. This morphological result was supported by the ridge formation along the *b*-axis in the lamellar crystals after thermal treatment at a low quenching temperature because loosely packed chains in lamellar crystals are thermally susceptible. However, the interface between the remained lamellae after alkaline hydrolysis became smooth. This suggests the chain erosion in chain-folded lamellar surfaces where the chain regularity is relatively decreased.

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