Direct Observation of Enzymatic Degradation Behavior of Poly[(*R*)-3-hydroxybutyrate] Lamellar Single Crystals by Atomic Force Microscopy

Tomohide Murase, Tadahisa Iwata, and Yoshiharu Doi*

Polymer Chemistry Laboratory and the RIKEN Group of Japan Science and Technology Corporation, RIKEN Institute, Hirosawa, Wako-shi, Saitama 351-0198, Japan

Received August 29, 2000; Revised Manuscript Received April 30, 2001

ABSTRACT: The enzymatic degradation behavior of solution-grown single crystals of poly[(*R*)-3-hydroxybutyrate] (P(3HB)), which were adsorbed on the surface of highly ordered pyrolytic graphite, with an extracellular PHB depolymerase from *Alcaligenes faecalis* T1 was directly observed by atomic force microscopy. At the initial stage of degradation, enzymatic action of both adsorption and hydrolysis produced smaller crystal blocks by predominant degradation of distorted regions of a single crystal. Subsequently, the hydrolysis progressed from a generated end of the crystal block, resulting in the formation of cracks. The degradation also took place at a middle part of the crystal, yielding slits. Several cracks and slits stood in a line along the long axis of crystal, and further enzymatic hydrolysis combined the cracks and the slits into longer cracks. With these processes, the enzymatic degradation converted a lath-shaped lamellar single crystal into lathlike fingers and crystal fragments. It has been proposed from the degradation texture of one lamellar single crystal that inherent straight degradation pathways exist parallel to the average folding direction of the single crystal of P(3HB). The straight degradation pathway may be a mismatched region of molecular packing of adjacent polymer chains in a lamellar single crystal and may be generated with a crystal growth process as a history line.

Introduction

Recently, poly[(*R*)-3-hydroxybutyrate] (P(3HB)), which is produced by a large number of bacteria, has attracted considerable attention as biodegradable thermoplastic,[1,2] accompanied by growing interest in environmental degradable thermoplastics used for a wide variety of applications.[3−5] The biodegradability of P(3HB) has been evaluated in various natural environments.[6] The enzymatic degradation of P(3HB) and its copolymers by several types of extracellular PHB depolymerases from some bacteria have been extensively studied for solution cast films[7−9] and melt crystallized films[10−13] in order to investigate the mechanism of the enzymatic hydrolysis of the material. These studies reported that the crystalline region played an important role for the degradation behavior of the film, that degradation occurred fast in the amorphous region and subsequently in the crystalline region, and that the degradation rate was strongly affected by a degree of crystallinity. Several researchers have studied the enzymatic degradation of single crystals of P(3HB) and its copolymers, which were employed as an ideal model with a uniform and a well-defined structure, for the degradation of crystalline region in such films.[14−19] Structural investigation of P(3HB) single crystal before and after enzymatic degradation in suspension was carried out mainly by transmission electron microscopy (TEM). A crystalline lamella was splintered longitudinally along their crystallographic *a*-axis, and splintered crystals kept high crystallinity. It was also revealed that the enzymatic degradation occurred preferentially at edges and ends of the single crystal rather than the chain-folding crystalline surface without changing the molecular weight and lamellar thickness.

For understanding the degradation mechanism of P(3HB) single crystals in detail, it is of significant importance to investigate the enzymatic degradation behavior not of multilamellar but of one lamellar single crystal of P(3HB). To elucidate the enzymatic degradation behavior of one lamellar single crystal without an influence of breakdown of the crystal, solution-grown single crystals of P(3HB), which are adsorbed onto surface of a solid substrate, are applied for enzymatic degradation, and their morphologies before and after degradation are directly observed by atomic force microscopy (AFM) in this study. This approach could allow us to visualize not only one degraded lamellar single crystal but also delicate degraded pieces without destruction after enzymatic treatment. The visualization gives us more information for the degradation mechanism of the P(3HB) single crystal and, in addition, for enzymatic action at the surface−solution interface and a structure of the P(3HB) single crystal.

In this paper, we look first for an appropriate substrate for deposition of solution-grown single crystals of bacterial P(3HB). Subsequently, we investigate the morphological changes of the single crystals of P(3HB), which are adsorbed on a surface of the substrate, during the enzymatic degradation by an extracellular PHB depolymerase purified from *Alcaligenes faecalis* T1 by atomic force microscopy, to obtain further insight for enzymatic degradation mechanism of one lamellar single crystal of P(3HB).

Experimental Section

Preparation of Single Crystals of P(3HB). Bacterial P(3HB) was purchased from Aldrich Chemical Co., Ltd. The P(3HB) sample was prepared from the bacterial P(3HB) by alkaline hydrolysis with 18-crown-6 ether according to a

* To whom all correspondence should be addressed. Tel +81-48-467-9402; Fax +81-48-462-4667; E-mail ydoi@postman.riken.go.jp.

10.1021/ma001505c CCC: $20.00
Published on Web 07/11/2001

Table 1. Adsorption of P(3HB) Single Crystals onto Various Substrates

	substrate				
	mica	cover glass	hydrophilic glass[a]	silicon wafer	HOPG[b]
treatment before immersion	cleaved	washed[c]	washed	washed	cleaved
adsorption of single crystals[d]	no	no	no	no	yes

[a] Cover glass with a maximum hydroxyl group density on the surface.[21] [b] HOPG = highly ordered pyrolytic graphite. [c] Washed with methanol several times. [d] After immersion, substrate was rinsed with methanol to remove weakly adsorbed crystals and observed by optical microscopy.

method reported previously.[16] The number-average molecular weight (M_n) and polydispersity (DPI) were determined to be 45 000 and 1.6, respectively. Single crystals of P(3HB) sample were grown from dilute solution (0.025 wt %) of 1-octanol under isothermal crystallization conditions.[20] Single crystals were collected by centrifugation and washed three times with methanol at room temperature.

Deposition of Single Crystals onto Substrate. Mica (Okenshoji Co., Ltd., Japan), cover glass (Matsunami glass Ind., Ltd., Japan), hydrophilic glass prepared from cover glass,[21] silicon wafer (Furuuchi Chemical Co., Japan), and highly ordered pyrolytic graphite (HOPG; Nihon Veeco K.K., Japan) were employed as solid substrates. Deposition of P(3HB) single crystals onto the surface of a substrate was carried out by immersing the substrate, which was washed with methanol or fleshly cleaved just before the experiment, into methanolic suspension of P(3HB) single crystals for several minutes. The substrate was then rinsed thoroughly with methanol to remove weakly adsorbed crystals and allowed to dry. The sample was described as P(3HB)/substrate below.

Enzymatic Degradation of Single Crystals on Substrate. An extracellular PHB depolymerase from *Alcaligenes faecalis* T1 was purified to electrophoretic homogeneity by the method described previously.[22] Before enzymatic degradation, P(3HB)/substrate was kept for at least 24 h in 50 mM Tris-HCl buffer solution (pH 7.5) for adapting the crystal surface to the buffer solution. A 2 μL aliquot of a 200 μg/mL solution of an extracellular PHB depolymerase purified from *A. faecalis* T1 was added to 1 mL of 50 mM Tris-HCl buffer solution in a transparent plastic cuvette containing the P(3HB)/substrate, and then it was incubated at 37 °C without stirring for various reaction periods. After incubation, the P(3HB)/substrate was rinsed with distilled water and methanol vigorously and then allowed to dry for AFM observation.

Atomic Force Microscopy. Atomic force microscopy (AFM) observations were performed with an SPA400/SPI3800N (Seiko Instruments Inc., Japan) with operating in a dynamic force microscope (DFM) mode. A 20 μm scanner (maximum scan range: ca. 24 μm) and rectangular silicon cantilever (Si-DF20, Seiko Instruments Inc.; 200 μm in length, resonant frequency of ca. 150 kHz, stiffness of ca. 15 N/m) were applied in all experiments. All images were captured under ambient conditions at room temperature. The resulting images were flattened and plane-fit using Seiko Instruments software.

Results and Discussion

P(3HB) Single Crystals on Substrate. The results for adsorption of P(3HB) single crystals onto five kinds of substrates are summarized in Table 1. Many single crystals were observed on the surface of highly ordered pyrolytic graphite (HOPG) by optical microscopy, while no crystal was detected on the surface of mica, cover glass, hydrophilic glass, and silicon wafer. On the analogy of specific interaction of hydrocarbon chain with a basal plane of HOPG,[23,24] the surface of P(3HB) single crystal may be dominated by methyl and methylene groups of the polymer chains. Single crystals may be adsorbed onto HOPG surface with a hydrophobic interaction between crystal surface and HOPG surface.

A typical AFM image of P(3HB) single crystals adsorbed on HOPG is shown in Figure 1. P(3HB) single crystals have multilamellar lath-shaped morphology

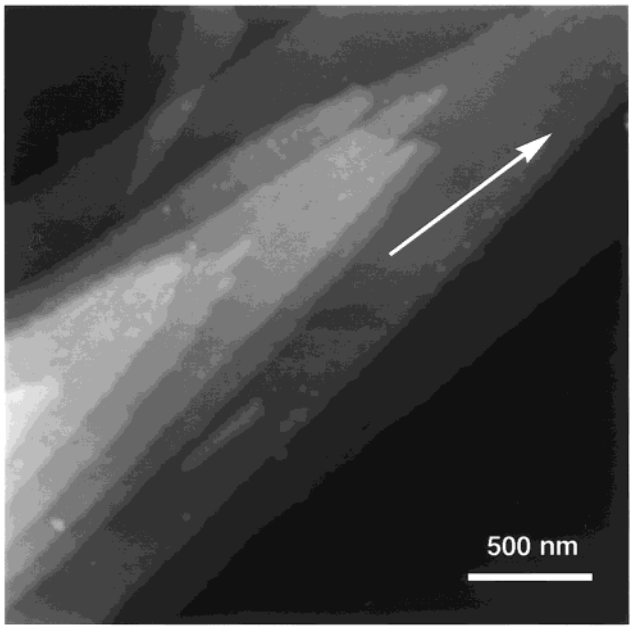

Figure 1. AFM image of solution-grown P(3HB) single crystals adsorbed on the surface of HOPG. Arrow indicates the crystallographic *a*-axis and the average chain-folding direction.

with dimensions of 0.3−2 μm wide and 5−15 μm long. The thickness of the monolamellar part of the crystal is around 5 nm. It has been reported that both the crystallographic *a*-axis and average direction of molecular chain folding are along the long axis of crystal and that the lamellar single crystal has a chain-folding surface.[25,26]

Enzymatic Degradation of P(3HB) Single Crystals on HOPG. An AFM image of P(3HB) single crystals on HOPG after enzymatic degradation at 37 °C for 60 min is shown in Figure 2. Single crystals eroded by enzymatic hydrolysis were observed with keeping lath-shaped morphology, indicating that the degradation of the single crystals progressed as adsorbing on HOPG. The single crystals were chopped into smaller crystal blocks by the formation of crevices across the lamellar crystals. Small cracks along the long axis of crystal were observed at a generated end (*bc*-plane) of crystal blocks, while a primary end and a primary lateral side (*ac*-plane) of a single crystal were not yet degraded.

When examining more than 100 P(3HB) single crystals after enzymatic degradation, the morphological changes were reproducible. On the other hand, no morphological change was observed after incubating P(3HB) single crystals on HOPG without the PHB depolymerase in the buffer solution at 37 °C even for 24 h. Therefore, it was confirmed that the AFM image showed the results of the enzymatic degradation. The use of HOPG substrate for the enzymatic degradation of P(3HB) single crystals enables us to observe the degradation behavior more clearly and in detail, which

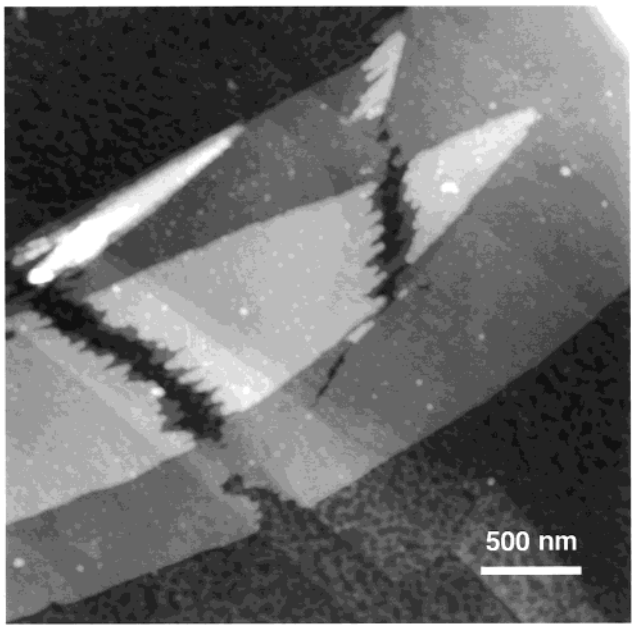

Figure 2. AFM image of P(3HB) single crystals adsorbed on the surface of HOPG after enzymatic hydrolysis at 37 °C for 60 min by PHB depolymerase from *A. faecalis* T1.

is mainly due to both adsorption of the enzymes upon one side of a single crystal and a mild treatment of single crystals on HOPG through the degradation process.

The formation of crystal blocks has never been reported for the enzymatic treatment of P(3HB) single crystals in suspension,[14−17] because generated crystal blocks were not distinguishable from extremely degraded single crystals. A fact, however, that the size of the single crystals drastically decreased after enzymatic treatment even for a short period suggests that crystal blocks were formed in suspension at the initial stage of the enzymatic degradation. Long and thin lamellar single crystals of P(3HB) may be easily distorted with a weak strength such as a dense adsorption of a PHB depolymerase on the surface.[27] Local distortion of a lamellar single crystal may result in slight fissure at the crystal surface, and facile hydrolysis by adsorbed enzymes is initiated to afford a crevice. After crevice formation, enzymatic hydrolysis takes place preferentially from the generated end because chain packing becomes loose. The generation of crystal blocks promotes enzymatic hydrolysis to progress efficiently because the total number of generated ends, where hydrolysis preferentially occurs, increases.

AFM images of P(3HB) single crystals on another lamella onto HOPG after enzymatic degradation at 37 °C for 90 min are shown in Figures 3 and 4. To minimize the influence of crystal adsorption onto HOPG over the degradation behavior, the enzymatic degradation of second lamellar single crystals, which are adsorbed not directly onto HOPG but on another lamella (first lamella) on HOPG, will be discussed below. In Figure 3a, a lamellar single crystal was split parallel to the long axis during enzymatic degradation, resulting in small laths divided by cracks. The enzymatic degradation progressed from the primary or generated end, while the degradation from a generated lateral side hardly occurred. An interval of the cracks in the direction of the short axis of crystal (i.e., the width of the small lath), depending on crystals, was ca. 50 nm,

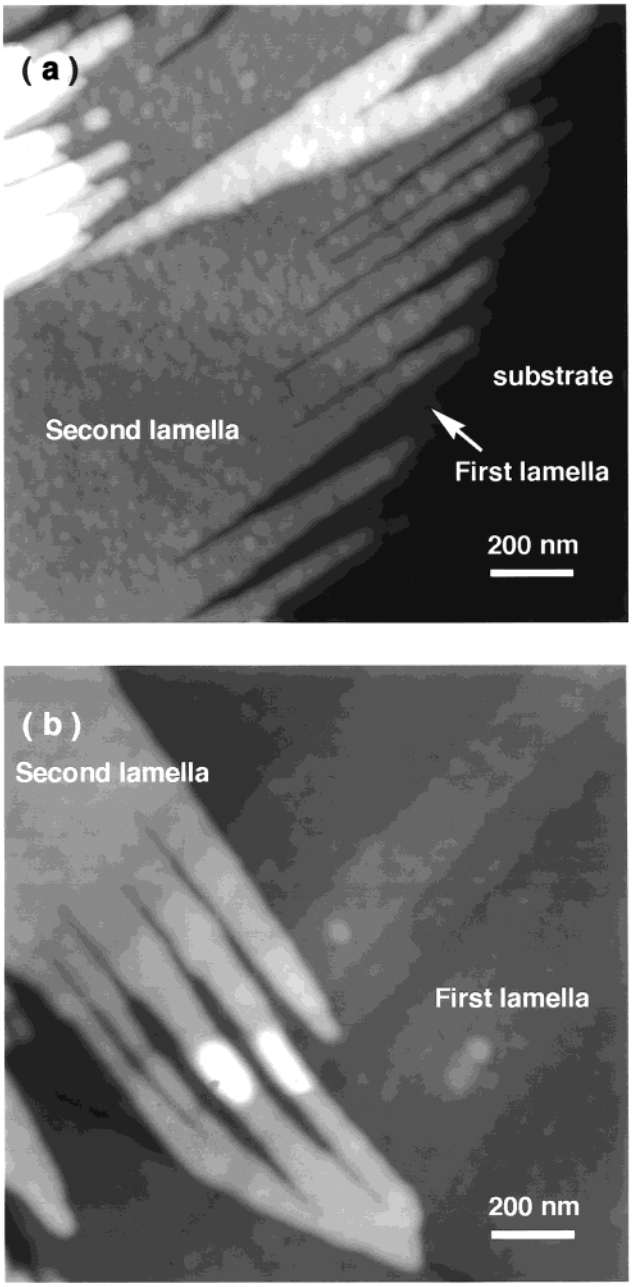

Figure 3. AFM images of P(3HB) single crystals on another lamellar single crystal adsorbed on the surface of HOPG after enzymatic hydrolysis at 37 °C for 90 min by PHB depolymerase from *A. faecalis* T1. The images show (a) several cracks from the end and (b) three slits at the middle of the crystal, all of which were parallel to the long axis of crystal. The first lamella was directly adsorbed to the surface of HOPG, and the second lamella stayed on the first lamella.

and the thickness of the crystal remained unchanged (around 5 nm) during enzymatic treatment. Here, this degradation manner for one lamellar single crystal is named as lathlike fingers. The lathlike fingers were never observed before enzymatic degradation. However, one sometimes sees small laths at the edge of P(3HB) single crystal before degradation as shown in Figure 1. This seems to be due to the handling of the P(3HB) single crystal, especially during the centrifugation step in the preparation. Reasonably, the lathlike fingers from one lamellar single crystal has the same structural origin for the splintering of P(3HB) single crystals as reported by Nobes et al.[15] and Iwata et al.[16,17]

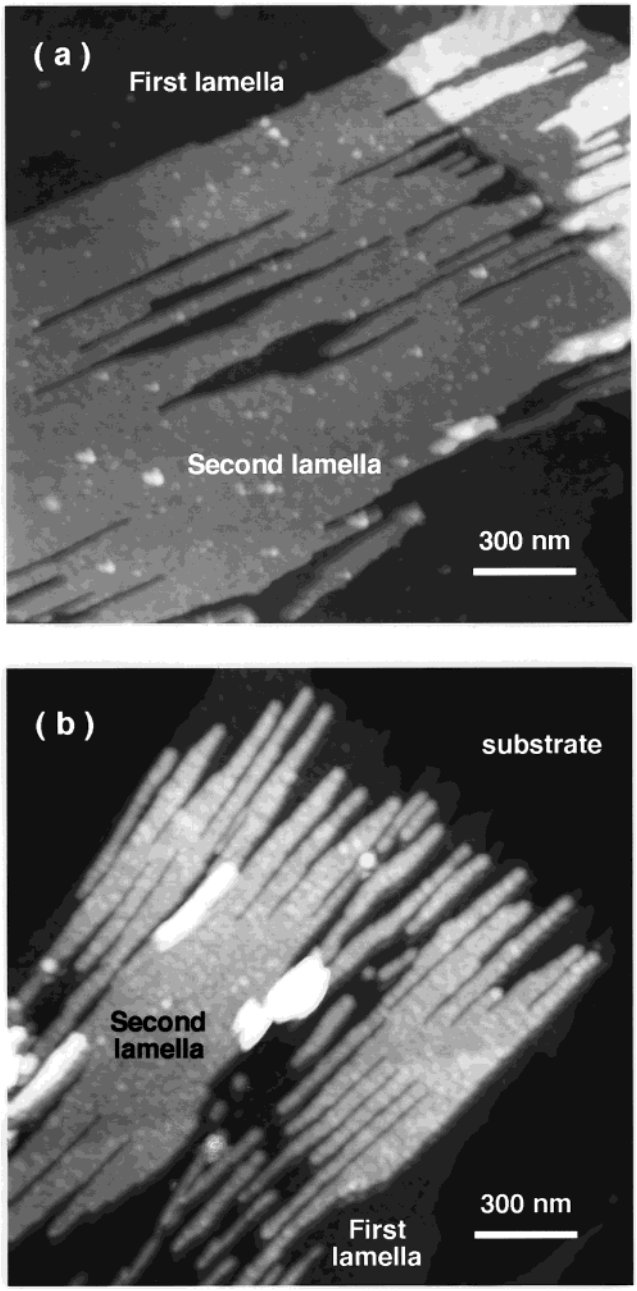

Figure 4. AFM images of P(3HB) single crystals on another lamellar single crystal adsorbed on the surface of HOPG after enzymatic hydrolysis at 37 °C for 90 min by PHB depolymerase from *A. faecalis* T1. The images show (a) both cracks from the end and slits at the middle of the crystal and (b) both lathlike fingers and crystal fragments. All cracks and slits in the images are parallel to the long axis of crystal.

In Figure 3b, several slits were observed at a middle part of the lamellar single crystal. The slit passed through the lamellar crystal of which thickness was ∼5 nm. An interval of the slits in the direction of the short axis of crystal, depending on crystals, was ca. 50 nm. The slit is probably an initiation of slitlike opening reported by Nobes et al.[15] Taking no degradation from the chain-folding surface (*ab*-plane) of the P(3HB) single crystal into consideration,[14−17] the slit may be created by enzymatic action against a defect or disordered region of the lamellar crystalline surface. It should be emphasized that enzymatic degradation also takes place at a middle part of a lamellar single crystal of P(3HB) if there is a defect or disordered region.

In Figure 4a, the characteristic degradation texture of one lamellar single crystal of P(3HB) was observed, where cracks from an end and slits at a middle part of a lamellar single crystal coexisted. All slits and cracks were parallel to one another along the long axis of crystal, and all pairs of a slit at the middle part and a crack from the end of the lamellar single crystal stood in a line. The slits and cracks were located at intervals of ca. 50 nm in the direction of the short axis of crystal. In addition, one long crack was observed at the middle part of the single crystal, which was probably originated from combination of slit and crack in a line. From these points of view, it is suggested that each pair of a slit and a crack in Figure 4a would combine together into one longer crack with further enzymatic treatment. The degradation texture has demonstrated that the location of the slit and the crack along the long axis of crystal correlates to each other.

The degradation texture can be rationalized not by random enzymatic degradation of the single crystal but by predominant degradation of an inherent straight degradation pathway along the long axis of crystal. The enzymatic degradation manner of single crystals of P(3HB) has never been explained by random enzymatic attack to the crystal surface, and the inherent weakness in the structure of the single crystal[15,18] and disordered region in the single crystal[16,17] were proposed for the degradation. On the other hand, adsorption of an extracellular PHB depolymerase from *A. faecalis* T1 onto the surface of the P(3HB) single crystal was visualized by Iwata et al. with immuno-gold labeling technique,[17] reporting a homogeneous distribution of extracellular PHB depolymerases on the surface of P(3HB) single crystals. It was also reported that degraded P(3HB) single crystals kept high crystallinity and that lamellar thickness and molecular weight remained unchanged during the enzymatic degradation.[14−17] Accordingly, we have concluded that only straight degradation pathways are predominantly hydrolyzed by enzymatic action although enzymes can adsorb on the whole surface of a single crystal without any site specificity.

In Figure 4b, remarkably degraded P(3HB) single crystals were observed. Crystal blocks were hydrolyzed from the both ends. The enzymatic hydrolysis generates a small crystal fragment, of which width was ca. 50 nm, located beside the crystal block. The enzymatic hydrolysis also progressed from the end of the crystal fragments to shorten it, whereas the hydrolysis hardly occurred from generated lateral sides of the crystal fragments. It is considered that the small crystal fragments were cut off from parent lathlike fingers by combination of a pair of cracks from both ends. Although enzymatic hydrolysis of the P(3HB) single crystals directly from primary and generated lateral sides is hardly recognized, the formation of crystal fragments reduces the size of the parent crystal block stepwise in the direction of the short axis of crystal. The degradation manner of a lamellar single crystal of P(3HB) in Figure 4b, where almost all pairs of cracks from the both ends of the crystal blocks stood in a line and crystal fragments were finally cut off from parent crystal blocks, fully supports the existence of the straight degradation pathways proposed above.

Iwata et al. reported that the P(3HB) single crystal was split into narrow ribbonlike crystals with width of 100 nm or less after alkaline hydrolysis and that the

split direction corresponded to the long axis of crystal.[26] The shape and the width of generated crystal fragments after the alkaline hydrolysis and the enzymatic hydrolysis in Figure 4b are quite similar to each other, and both results can be interpreted identically in terms of the predominant hydrolysis of straight degradation pathways along the long axis of crystal. For the P(3HB) single crystal, the average folding direction of polymer chains and the crystal growth direction are consistent along the long axis of crystal, and a lamellar single crystal seems to grow like parallel fingers at the growth front.[26,28] Each finger grows independently, and the space between fingers is left as an inherent mismatched region of molecular packing between adjacent polymer chains. According to the crystal growth, the mismatched region is continuously recorded as a straight history line in the lamellar single crystal. Thus, the straight history lines are parallel to each other along the long axis of crystal. The history line could be preferentially degraded by enzymatic action because polymer chains are not tightly packed in such region. Therefore, we have concluded that the history line is essential to the straight degradation pathway and that degradation behavior of P(3HB) single crystal correlates closely with the crystal growth process in solution.

Enzymatic Degradation Behavior of One Lamellar Single Crystal of P(3HB). Two types of enzymatic activities of PHB depolymerase from *A. faecalis* T1 were reported to hydrolyze P(3HB) polymer chains into monomer and dimer.[22,29−31] One is *exo* type activity, which hydrolyzes the polymer chain from the end, and the other is *endo* type activity, which hydrolyzes the polymer chain at any point along the chain. When an enzyme molecule that adsorbs around the straight degradation pathway finds out either an end of polymer chain exposed outside the surface or an excessive loose loop of chain near the surface, the enzyme could hydrolyze it with *exo* or *endo* type activity, respectively. Subsequently, the enzyme may hydrolyze the chain as processive degradation,[15,17] affording water-soluble monomers, dimers, and oligomers, which can be released from single crystal into solution. The degradation may lead to disturbance of the chain packing around the degradation pathway. Successively, the same or another enzyme molecule could attack the disturbed area readily, resulting in a crack or a slit. However, enzyme molecules with *endo* activity hardly hydrolyze the generated lateral sides because folding polymer chains of P(3HB) may pack tightly. An enzyme molecule adsorbing not around the pathway but on regular sharp fold at the surface could not hydrolyze folding polymer chains with little mobility. As a consequence, lamellar thickness and molecular weight of P(3HB) single crystal remain unchanged through these enzymatic degradation processes.

Figure 5 shows a schematic representation of the enzymatic degradation behavior of one lamellar single crystal of P(3HB) in summarizing each stage discussed in the previous section. (a) At the initial stage, enzymatic degradation starts from a distorted region of a lamellar single crystal, which is located almost perpendicular to the long axis of crystal, by both enzymatic functions of adsorption and hydrolysis. (b) The degradation of the distorted region generates a crevice and a crystal block. The formation of the crystal block reduces the crystal size extremely in the direction of the long axis of crystal. The enzymatic hydrolysis progresses

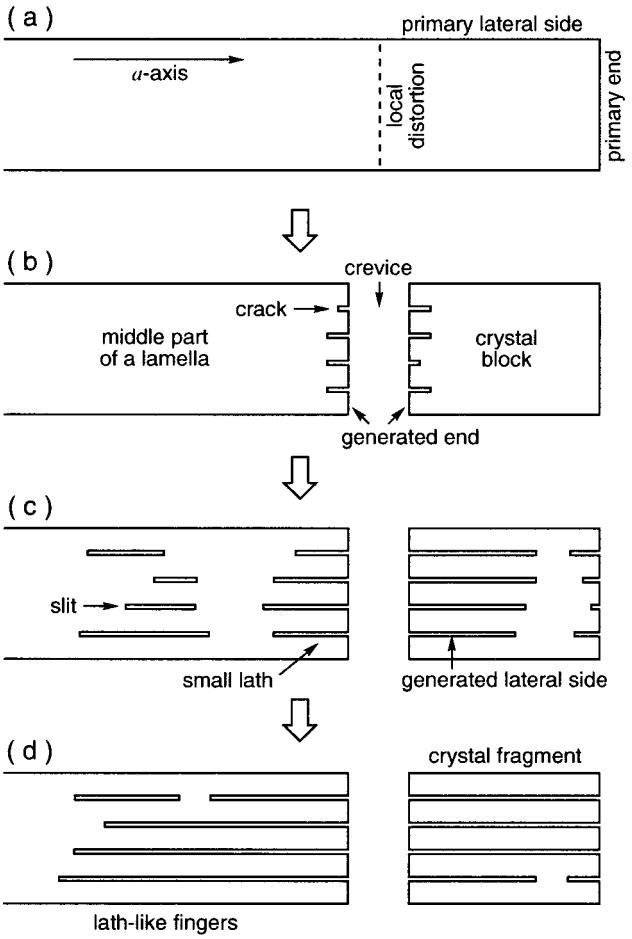

Figure 5. Schematic representation of enzymatic degradation behavior of P(3HB) single crystal with an extracellular PHB depolymerase from *A. faecalis* T1 (top view).

from generated ends to produce small cracks along the long axis of crystal, while the hydrolysis does not occur from a primary end on this stage. (c) Enzymatic hydrolysis makes the crack longer, whereas enzyme molecules hardly hydrolyze the single crystal from primary and generated lateral sides. The degradation also takes place at the middle part of the lamellar single crystal to yield slits. Each pair of a crack and a slit stands in a line because only straight degradation pathway of the lamellar single crystal is predominantly hydrolyzed by the enzymatic action. (d) Further enzymatic hydrolysis of the straight degradation pathway makes a pair of a crack and a slit combine to form one longer crack. The hydrolysis also cuts off crystal fragments from a crystal block by combination of cracks from both ends of the crystal block. The formation of the crystal fragments reduces the crystal size stepwise in the direction of the short axis of crystal. As a result of all these processes, enzymatic action converts the lath-shaped lamellar single crystal into lathlike fingers and crystal fragments.

Conclusions

In this paper, we have demonstrated the visualization of the enzymatic degradation behavior of solution-grown single crystals of P(3HB), which were adsorbed on the surface of highly ordered pyrolytic graphite, with an extracellular PHB depolymerase purified from *Alcaligenes faecalis* T1 by atomic force microscopy. The results revealed the enzymatic degradation mechanism of one

lamellar single crystal in detail. At the initial stage of degradation, the enzymatic action involving adsorption and hydrolysis creates a crystal block by predominant degradation of distorted region of a lamellar single crystal. Subsequently, the hydrolysis progresses from a generated end, resulting in the formation of cracks along the long axis of crystal. Degradation also starts at a middle part of a single crystal to form slits along the long axis of crystal. Several cracks and slits in a lamellar single crystal or cracks from both ends of a crystal block are located in one line, and further hydrolysis of the cracks and the slits in a line have combined them afterward. The whole degradation processes convert a lath-shaped lamellar single crystal into lathlike fingers and crystal fragments efficiently. The crystal size is reduced both by the formation of a crystal block in the direction of the long axis of crystal and by the formation of a crystal fragment in the direction of the short axis of crystal. It has been proposed from degradation texture of one lamellar single crystal that straight degradation pathways exist parallel to one another along the long axis of crystal. The straight degradation pathway may be mismatched region of molecular packing of adjacent polymer chains in a lamellar single crystal and may be generated with a crystal growth process as a history line.

Acknowledgment. This work has been supported in part by CREST (Core Research for Evolutional Science and Technology) of Japan Science and Technology Corporation (JST) and a Grant-in-Aid for Scientific Research on Priority Area, "Sustainable Biodegradable Plastics", No. 11217216 (2000), from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

References and Notes

(1) Dawes, E. A.; Senior, P. J. *Adv. Microb. Physiol.* **1973**, *10*, 135.
(2) Anderson, A. J.; Dawes, E. A. *Microbiol. Rev.* **1990**, *54*, 450.
(3) Holmes, P. A. In *Developments in Crystalline Polymers*; Bassett, D. C., Ed.; Elsevier Applied Science: London, 1988; Vol. 2, pp 1−65.
(4) Nakamura, S.; Doi, Y.; Scandola, M. *Macromolecules* **1992**, *25*, 4237.
(5) Pizzoli, M.; Scandola, M.; Ceccorulli, G. *Macromolecules* **1994**, *27*, 4755.
(6) Doi, Y. *Microbial Polyesters*; VCH Publishers: New York, 1990.
(7) Doi, Y.; Kanesawa, Y.; Kunioka, M.; Saito, T. *Macromolecules* **1990**, *23*, 26.
(8) Shimomura, E.; Scandola, M.; Doi, Y. *Macromolecules* **1994**, *27*, 4429.
(9) Doi, Y.; Kitamura, S.; Abe, H. *Macromolecules* **1995**, *28*, 4822.
(10) Kumagai, Y.; Kanesawa, Y.; Doi, Y. *Makromol. Chem.* **1992**, *193*, 53.
(11) Tomasi, G.; Scandola, M.; Briese, B. H.; Jendrossek, D. *Macromolecules* **1996**, *29*, 507.
(12) Koyama, N.; Doi, Y. *Macromolecules* **1997**, *30*, 826.
(13) Abe, H.; Doi, Y.; Aoki, H.; Akehata, T. *Macromolecules* **1998**, *31*, 1791.
(14) Hocking, P. J.; Marchessault, R. H.; Timmins, M. R.; Lenz, R. W.; Fuller, R. C. *Macromolecules* **1996**, *29*, 2472.
(15) Nobes, G. A. R.; Marchessault, R. H.; Chanzy, H.; Briese, B. H.; Jendrossek, D. *Macromolecules* **1996**, *29*, 8330.
(16) Iwata, T.; Doi, Y.; Kasuya, K.; Inoue, Y. *Macromolecules* **1997**, *30*, 833.
(17) Iwata, T.; Doi, Y.; Tanaka, T.; Akehata, T.; Shiromo, M.; Teramachi, S. *Macromolecules* **1997**, *30*, 5290.
(18) Nobes, G. A. R.; Marchessault, R. H.; Chanzy, H.; Briese, B. H.; Jendrossek, D. *J. Environ. Polym. Degrad.* **1998**, *6*, 99.
(19) Iwata, T.; Doi, Y.; Nakayama, S.; Sasatsuki, H.; Teramachi, S. *Int. J. Biol. Macromol.* **1999**, *25*, 169.
(20) Revol, J.-F.; Chanzy, H. D.; Deslandes, Y.; Marchessault, R. H. *Polymer* **1989**, *30*, 1973.
(21) Yang, Z.; Galloway, J. A.; Yu, H. *Langmuir* **1999**, *15*, 8405.
(22) Shirakura, Y.; Fukui, T.; Saito, T.; Okamoto, Y.; Narikawa, T.; Koide, K.; Tomita, K.; Takemasa, T.; Masamune, S. *Biochim. Biophys. Acta* **1986**, *880*, 46.
(23) Groszec, A. J. *Proc. R. Soc. London A* **1970**, *314*, 473.
(24) Everett, D. H.; Findenegg, G. H. *Nature* **1969**, *223*, 52.
(25) Birley, C.; Briddon, J.; Sykes, K. E.; Barker, P. A.; Organ, S. J.; Barham, P. J. *J. Mater. Sci.* **1995**, *30*, 633.
(26) Iwata, T.; Doi, Y.; Kokubu, F.; Teramachi, S. *Macromolecules* **1999**, *32*, 8325.
(27) Kasuya, K.; Ohura, T.; Masuda, K.; Doi, Y. *Int. J. Biol. Macromol.* **1999**, *24*, 329.
(28) Abe, H.; Kikkawa, Y.; Iwata, T.; Aoki, H.; Akehata, T.; Doi, Y. *Polymer* **2000**, *41*, 867.
(29) Tanio, T.; Fukui, T.; Shirakura, Y.; Saito, T.; Tomita, K.; Kaiho, T.; Masamune, S. *Eur. J. Biochem.* **1982**, *124*, 71.
(30) Bachmann, B. M.; Seebach, D. *Macromolecules* **1999**, *32*, 1777.
(31) Scherer, T. M.; Fuller, R. C.; Goodwin, S.; Lenz, R. W. *Biomacromolecules* **2000**, *1*, 577.

MA001505C