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Enzymatic Degradation and Aminolysis of Microbial Poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) Single Crystals

Wen-chuan Hsieh,[1,3,4] Hiroshi Mitomo,[1] Ken-ichi Kasuya,[1] and Tadashi Komoto[2]

Solution-grown single crystals of poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) [P(3HB-*co*-4HB)] were hydrolyzed by polyhydroxybutyrate (PHB) depolymerase from *Ralstonia pickettii* T1. Enzymatic degradation proceeded from the edges of lamellar crystals, yielding serrated contour and small crystal fragments. Gel permeation chromatography analysis revealed that the molecular weights of the crystals decreased during enzymatic degradation, suggesting that the enzymatic hydrolysis of chain-folding regions at the crystal surfaces occurred in addition to the enzymatic degradation at crystal laterals or edges. After P(3HB-*co*-4HB) single crystals were aminolysed in 20% aqueous methylamine solution to remove the folded-chain regions and enzymatic degradation by lipase from *Rhizopus oryzae* to remove 4HB components at crystal surfaces of single crystal aminolyzed, it was found that a small amount (up to *ca.* 2 mol%) of 4HB component can be incorporated into the P(3HB) mother crystal lattice irrespective of the 4HB content.

KEY WORDS: Microbial poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate); single crystal; enzymatic degradation; aminolysis.

INTRODUCTION

Biodegradability of microbial polymers has been evaluated from enzymatic degradation by enzymes such as polyhydroxybutyrate (PHB) depolymerase from *Ralstonia pickettii* T1 or lipase from *Rhizopus oryzae* (formerly named as *Rhizopus delemer*) in laboratory and composting in natural environments. It has been reported that physical properties of biodegradable polymers are significantly influenced by their chemical structures and crystalline morphologies. In particular, the chemical structure of the polymer influences not only the crystal growth mechanism, but also its degradation mechanism [1–4]. The mechanism of enzymatic hydrolysis of poly(hydroxyalkanoate)s [(PHA)s] with PHB depolymerase has been investigated using films and single crystals of PHA [5–11]. Marchessault et al. [12,13] and Iwata et al. [14] reported that molecular weights of P(3HB) single crystals remained unchanged during enzymatic degradation with extracellular PHB depolymerases. They also reported that enzymatic degradation of P(3HB) and poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) [P(3HB-*co*-3HV)] single crystals preferentially occurred at the less ordered chain packing regions of the single crystal laterals or edges rather than the chain-folding surfaces of single crystals. However, the suspended and/or dissolved polymer components in the enzymatic solution after degradation have been rarely investigated.

Recently, it has been reported that the cocrystallization of the 3HB and 4HB components in

[1] Department of Biological and Chemical Engineering, Faculty of Engineering, Gunma University, Kiryu, Gunma, 376-8515, Japan.
[2] Department of Chemistry, Faculty of Engineering, Gunma University, Kiryu, Gunma, 376-8515, Japan.
[3] Department of Biological Science and Technology, I-Shou University, No. 1, Sec. 1, Syuecheng Rd., Dashu Township, Kaohsiung 84008, Taiwan, Republic of China.
[4] To whom all correspondence should be addressed. E-mail: wenchuan@isu.edu.tw

P(3HB-*co*-4HB) is difficult due to the difference in lattice indexes of respective unit cell [15–18]. However, the irregular regions in the copolymer, which are excluded from the crystallites, are still unknown. We have reported the crystallization and physical properties of P(3HB-*co*-4HB) using a polarizing microscope, a differential scanning calorimeter, and wide- and small-angle X-ray diffraction apparatus [18], in order to evaluate the influence of mutual disturbance for 3HB and 4HB comonomer units of the copolymer. Under isothermal crystallization, the P(3HB) crystals were assumed to exclude the 4HB units of the copolymer chains because of its different fiber repeat distance and conformation.

In this study, we report the enzymatic and aminolytic degradation of P(3HB) and P(3HB-*co*-4HB) single crystals. The hydrolysis of these single crystals using extracellular PHB depolymerase from *Ralstonia pickettii* T1 [19,20] or lipase from *Rhizopus oryzae* [20,21] were examined. Treating the single crystals with PHB depolymerase, both samples remained as solid film and suspended or dissolved in the enzymatic solution were recovered and investigated whether melting points and molecular weights changed after exposure. Moreover, aminolysis of P(3HB) and P(3HB-*co*-4HB) was demonstrated to remove amorphous regions and crystalline surface layers and isolate the crystal core, then the content of 4HB unit included in the P(3HB) crystal core was evaluated by ^{1}H-NMR spectroscopy.

EXPERIMENTAL

Materials

The strain used was *Delftia acidovorans* (IFO13582). P(3HB-*co*-4HB) synthesis was carried out by a two step cultivation. *D. acidovorans* was grown under aeration at 26°C in a nutrient-rich medium (100 mL) containing 1 g yeast extract, 1 g polypeptone, 0.5 g meat extract and 0.5 g $(NH_4)_2SO_4$ [22]. To promote the polyester synthesis, the bacterial cells were transferred into a nitrogen-free medium containing *n*-butyric acid and 1,4-butanediol as the carbon sources shown in Table I [20]. P(3HB-*co*-4HB) copolymers of different composition were synthesized by *D. acidovorans* grown in different ratio of the carbon sources. Since most of copolymers were a mixture of the copolymers having a wide variety of compositions, the copolymers were fractionated using solvent systems of acetone–water [20,23] or chloroform–heptane [24,25] to isolate the fractionated copolymers having a narrower composition distribution.

Table I. Composition of the Nitrogen-free Medium (1 L)

Medium		Microelement solution	
$Na_2HPO_4{\cdot}12H_2O$	7.16 g	$CoCl_2{\cdot}6H_2O$	217 mg
KH_2PO_4	2.65 g	$FeCl_3{\cdot}6H_2O$	16.2 g
$MgSO_4{\cdot}7H_2O$	0.2 g	$CaCl_2{\cdot}2H_2O$	7.8 g
$(NH_4)_2SO_4$	0.1 g	$NiCl_2{\cdot}6H_2O$	118 mg
$CaCl_2{\cdot}2H_2O$	1.0 g	$CrCl_3{\cdot}6H_2O$	135 mg
Citrate	5.0 g	$CuSO_4{\cdot}5H_2O$	156 mg
Microelement solution	1 mL	0.1 N HCl	1 L

Sample Preparation

Single crystals of P(3HB), P(3HB-*co*-4 mol% 4HB), and P(3HB-*co*-7 mol% 4HB) were obtained from a 0.01% (w/v) polymer concentration of propylene carbonate. The polymer was dissolved completely at 120°C, then cooled to room temperature and kept for one night. The solution was slowly heated up and self-seeded to 110°C followed by cooling rapidly to 60°C. It was kept at 60°C for 24 hours to crystallize isothermally. After crystallization, the single crystals was isolated by filtering, washing with ethanol several times, suspended in ethanol and then kept at room temperature before the measurements. For the amine treatment, the single crystal material (50 mg) was treated at 25°C in 20% aqueous methylamine solutions (10 mL) for various time intervals. Biodegradability was expressed as weight loss, i.e., the percentage of weight decreased relative to the initial weight of the sample.

Thermal Analysis

The thermal property of the polyesters (3 mg) was measured by a Shimadzu differential scanning calorimeter (DSC-60) under a N_2 gas flow of 30 mL minute^{-1} at a heating rate of 10°C minute^{-1}. Melting peak temperature after being corrected for heating rate dependence was defined as the melting point T_m within ±0.1°C accuracy. The temperature calibration was performed using high-purity standards.

Molecular Weight Determination

Gel permeation chromatography (GPC) was carried out at 38°C using an HLC-802A high-performance

liquid chromatograph (Tosoh) equipped with a series of four columns of TSK-gel and an RI-8 differential refractometer. Chloroform was used as the eluent at a flow rate of 0.1 mL minute^{-1}. Concentration of the polymer sample was 1.0 g L^{-1}. Polystyrene and polyethylene glycol standards were used to create a calibration curve, by which the apparent molecular weights were calculated. The count of 6000 corresponded to an elution volume of 34 mL.

Morphology and NMR Analysis

Morphologies of samples before and after hydrolytic degradation were observed with a JEOL transmission electron microscope (TEM; JEM-1200XS) at room temperature. A P(3HB) or P(3HB-*co*-7 mol% 4HB) crystal suspension, before and after enzymatic degradation, dripped on carbon-coated grids, allowed to dry, and then shadowed with a Pt–Pd alloy. The electron microscope was operated at an acceleration voltage of 80 kV for imaging of the crystals.

The compositions of the P(3HB-*co*-4HB) samples before and after hydrolytic degradation of enzyme or amine treatment were determined using a JEOL α-500 spectrometer. The 500 MHz ^{1}H-NMR spectra were recorded at room temperature for a $CDCl_3$ solution of P(3HB-*co*-4HB) with a 4.7 μs pulse width, 5-s pulse repetition, 5000 Hz spectral width, 16 K data points and 32 accumulations. Tetramethylsilane (Me_4Si, $\delta = 0$) was used as an internal chemical shift standard.

Enzymatic Degradation

PHB depolymerase purified from *Ralstonia pickettii* T1 [19,20] (degrades mainly P(3HB) component) and lipase from *Rhizopus oryzae* [20,21] (degrades the P(4HB) component) were used in this study. Enzymatic degradation of P(3HB-*co*-4HB) single crystals were carried out at 37°C. The reaction mixture was composed of 1 mL of 0.1 M phosphate buffer (pH 7.4), 2 μg of PHB depolymerase or 285 μg of lipase, and 15 mg of single crystal samples. The reaction solutions were incubated with shaking. The sample was removed after a constant period, washed with methanol, and dried completely. In this study, enzymatic degradation was expressed as weight loss, i.e., the percentage of weight decreased relative to the initial weight of the sample.

RESULTS

Degradation of P(3HB-*co*-4HB) Single Crystals

Figure 1 shows the typical electron micrographs of P(3HB) and P(3HB-*co*-7 mol% 4HB) single crystals before and after degradation by the enzyme purified from *R. pickettii* T1. These crystals always appear to have an elongated arrow-like or lath shape [10]. P(3HB-*co*-7% 4HB) single crystals (b) closely resemble those of P(3HB) homopolymer (a). Typical electron micrographs of P(3HB) and P(3HB-*co*-7% 4HB) single crystals after enzymatic degradation are shown in (c) and (d), respectively. Both single crystals were degraded along the long axes of the crystals, forming narrow cracks and small crystal fragments like the teeth of a comb as reported by Iwata et al. [14].

Figure 2 shows the DSC thermograms of P(3HB) single crystals before and after enzymatic degradation by PHB depolymerase purified from *R. pickettii* T1 at 37°C for 1 hour. Original single crystal samples showed two peaks, the main peak and the sub peak appearing at the foot of the main peak at the lower temperature. Curve (a) shows the DSC thermogram of water-insoluble fraction of P(3HB) single crystals degraded by the enzyme, whereas curve (b) shows that of both water-soluble and -insoluble fractions, i.e., solvent-evaporated samples of the dissolved and suspended components. This enzymatic solution was made with distilled water instead of phosphate buffer, because the phosphate showed several melting peaks at the temperature range of 60–150°C. It was assured that small changes of pH value hardly affected enzymatic degradation of P(HA)s single crystals using distilled water. Curve (a) shows the melting peak of the water-insoluble residues remained unchanged compared with that of the original P(3HB) single crystals, but the ΔHm is smaller than that of the original. This implied that the water-insoluble residues is composed of the P(3HB) single crystals degraded slightly by the enzyme, which hold a crystalline structure similar to the original. On the other hand, curve (b) shows multiple melting peaks of all recovered fractions shifted to a lower temperature side, i.e., melting peak temperatures at ca. 120°C and 135°C, after enzymatic degradation of P(3HB) single crystals. The peak of a lower temperature side is caused by typically degraded single crystal, while the peak at around 135°C was caused by partial degradation of single crystal. Furthermore, the melting peaks gradually shifted to the

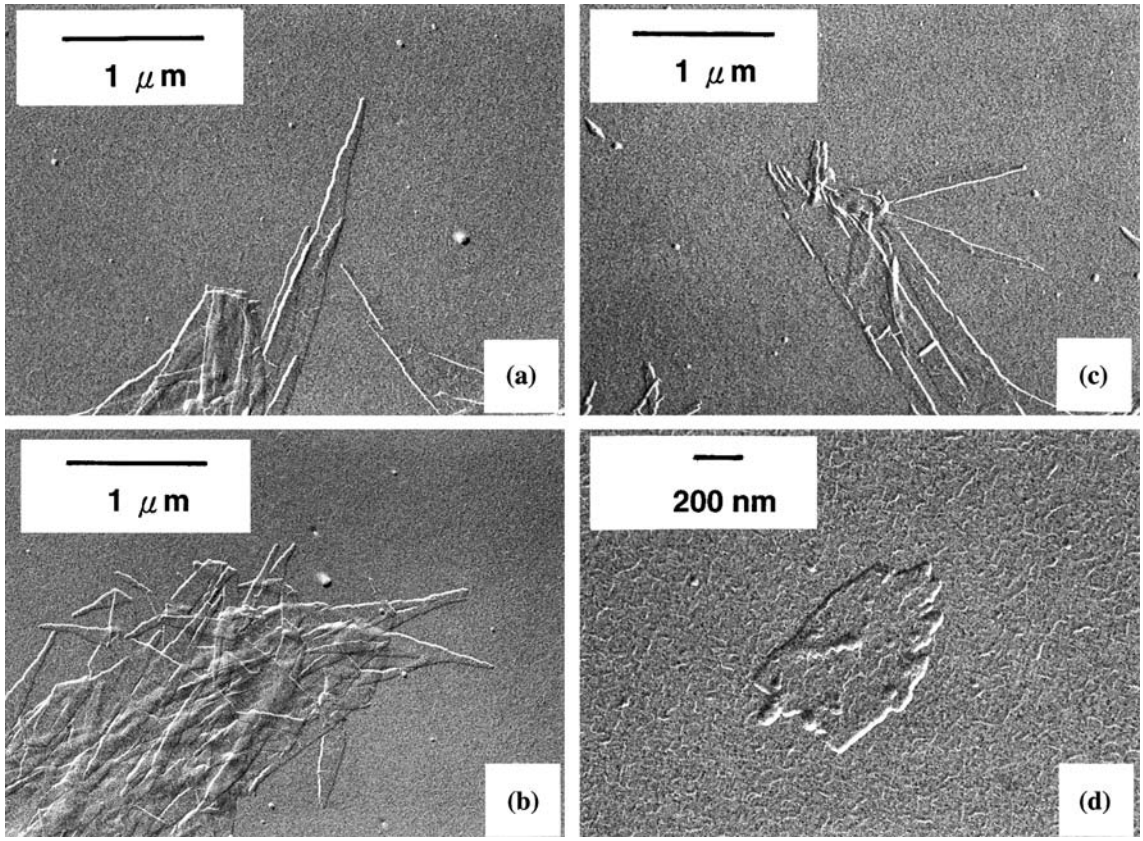

Fig. 1. Transmission electron micrographs of P(3HB) and P(3HB-*co*-7mol% 4HB) single crystals before and ager enzymatic degradation purified from *Ralstonia pickettii* T1. P (3HB) (a) and 7 mol% 4HB (b) before enzymatic degradation; P(3HB) (c) and 7 mol% 4HB (d) after enzymatic degradation.

lower temperature side with increasing the degradation time. These suggest that the sample (b) were composed of the mixture of reduced molecular fragments suspended and the oligomers dissolved in the solution, whose molecular weights and crystal thicknesses decreased with increasing the treatment time. It is reported that enzymatic degradation of P(3HB) single crystals preferentially occurred at crystalline laterals or edges instead of folded chain regions at crystal surfaces [4]. However, the decrease in melting points reflects the decrease in thickness and regularity of crystals occurred for the water-soluble and -insoluble sample of filtrate.

The degradation behavior of single crystals of copolymer P(3HB-*co*-7% 4HB) was found very similar to that of P(3HB). Figure 3 shows the DSC thermograms of P(3HB-*co*-7% 4HB) single crystals before and after enzymatic degradation by PHB depolymerase purified from *R. pickettii* T1 at 37°C for 1 hour. Endothermic melting peaks of P(3HB-*co*-7% 4HB) single crystals after enzymatic degradation shifted to lower temperature side than that of the original sample. Lamellar thickness of P(3HB-*co*-7% 4HB) single crystals was thinner than that of the P(3HB) homopolymer [18], reflecting the introduction of irregularity in crystalline regions, which caused decrease in the melting point or crystallinity, and promotion of the enzymatic degradation rate [20].

Figure 4 shows the molecular weight distributions of P(3HB) single crystals obtained by gel permeation chromatography before and after the degradation process of P(3HB) crystals by PHB depolymerase purified from *R. pickettii* T1. Taking the fiber identity period of 0.596 nm with two fold screw symmetry along the molecular axis into consideration, the molecular weight corresponding to single traverse (or stem length) of P(3HB) single crystals was approximately 1440. The molecular weights of the water-insoluble fraction decreased slightly after the enzymatic degradation (Mn = $1\sim5\times10^4$), however, the low molecular weight

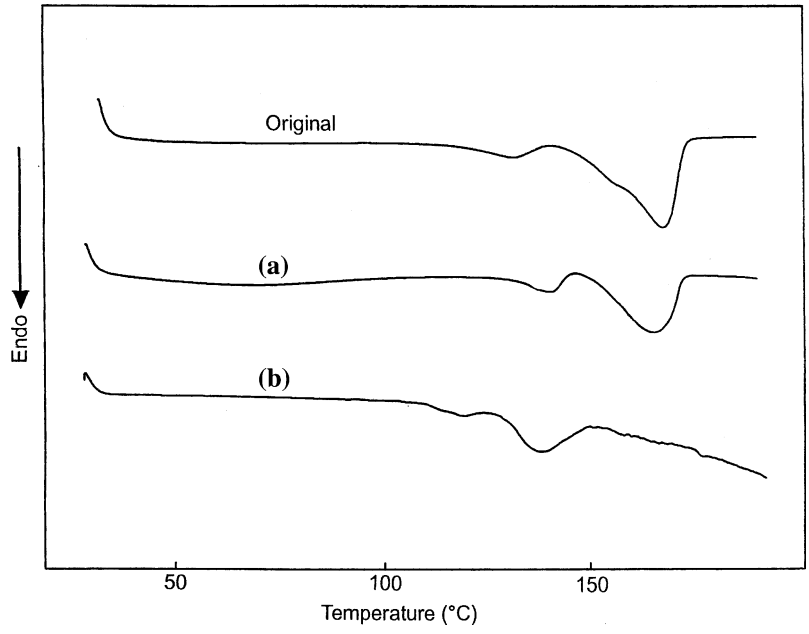

Fig. 2. DSC heating curves of P(3HB) single crystals before (original) and after degradation by PHB depolymerase purified from *Ralstonia pickettii* T1 at 37°C for 1 hour. (a): Solid mass component collected by filtration. (b): Solvent-evaporated component of filtrate after degradation.

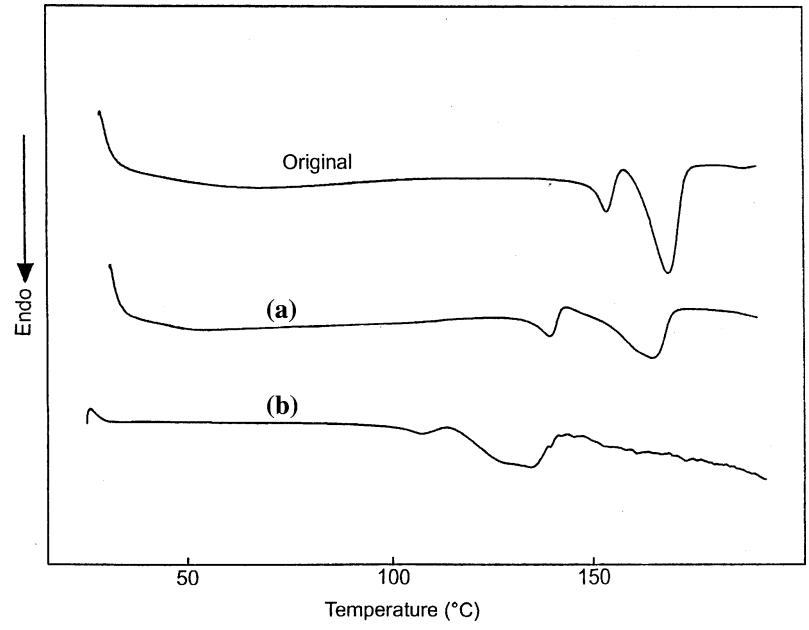

Fig. 3. DSC heating curves of P(3HB-*co*-7 mol% 4HB) single crystals before (original) and after degradation by PHB depolymerase purified from *Ralstonia pickettii* T1 at 37°C for 1 hour. (a): Solid mass component collected by filtration. (b): Solvent-evaporated component of filtrate after degradation.

component was hardly observed at higher count above 5000 (a). On the other hand, the molecular weights of the whole collected residues including both water-soluble and -insoluble fractions after degradation for 1 hour (or 1.5 hour) were observed at high molecular weight side close to the water-insoluble

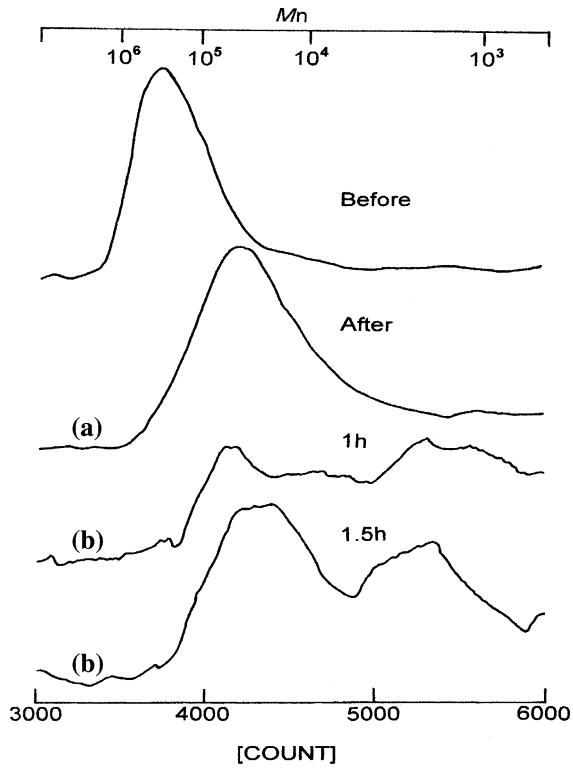

Fig. 4. Gel permeation chromatograms of P(3HB) single crystals before and after partial degradation by PHB depolymerase purified from *Ralstonia pickettii* T1 at 37°C. (a): solid component (1 hour). (b): Solvent-evaporated component (1 hour and 1.5 hour).

fraction (a) and the water soluble one having very low molecular weight close to the lamellae thickness length at a range of 5000~6000 count ($Mn = 1\sim3\times10^3$ (b)), respectively. This implies that the degradation or chain-scission of chain-folding regions at the crystal surfaces occurred simultaneously with the partial degradation by fragmentation from crystal edges. In practice, the tight chain-packing regions of single crystals are hardly affected by enzymatic degradation, however, since the turbidity of P(3HB) single crystal emulsion was turned into transparent solution if the degradation carried out for a long time, it can be said that all solid mass of single crystals was degraded into water-soluble oligomers of low molecular weight at last. Therefore, in the aqueous enzymatic solution after degradation, there remained the water-soluble oligomers in addition to the residual single crystal fragments. Enzymatic degradation behavior of P(3HB-*co*-4% 4HB) or P(3HB-*co*-7% 4HB) single crystals was very similar to that of P(3HB). Consequently, the melting points and molecular weights decreased during enzymatic degradation, indicating that the enzymatic degradation also occurs at the chain-folding region on the crystalline surface as much as lateral degradation of the crystal edges.

Amine Treatment of P(3HB-*co*-4HB) Single Crystals

All samples of P(3HB) and P(3HB-*co*-4HB) single crystals were treated with 20% aqueous methylamine solution at 25°C for various times in order to remove the amorphous part at the crystal surface. This method has been widely applied to synthetic polyesters such as poly(ethylene terephthalate) [26]. P(3HB) also has been degraded by aqueous methylamine [27], yielding the P(3HB) crystal fragment with once and twice the lamellar thickness under optimum treating conditions.

Table II lists the 4HB unit content and melting point of P(3HB-*co*-4HB) after aminolysis. The composition of samples, P(3HB-*co*-4 mol% 4HB) and P(3HB-*co*-7 mol% 4HB) were 4.1 mol% and 7.1 mol% 4HB, after the fractionation using acetone–water system, respectively. The weight loss of aminolysed P(3HB-*co*-4HB) single crystals decreased from ca. 2% to 89% as the treating time increased from 6 to 96 hours, respectively.

Table II. 4HB Unit Content and Melting Points of P(3HB-*co*-4 mol% 4HB) and P(3HB-*co*-7 mol% 4HB) Treated with Methylamine for Various Time

Sample	Treated time (hour)	Weight loss (%)	4HB unit content[a]	Melting point[b] (T_m) (°C)
4mol% 4HB	0	0	4.1	144, 173
	6	2	3.4	143, 172
	12	14	2.3	138, 161
	24	34	2.1	133, 154
	48	57	1.7	121, 139
	72	72	1.9	121, 135
	96	89	2.0	116, 130
7mol% 4HB	0	0	7.1	142, 172
	6	4	5.6	142, 168
	12	12	4.1	140, 163
	24	31	2.1	135, 153
	48	47	1.7	123, 137
	72	81	2.2	121, 134

[a] Determined by ^{1}H-NMR.
[b] Determined by DSC.

The DSC thermograms of P(3HB-*co*-7 mol% 4HB) single crystals before and after partial degradation by 20% aqueous methylamine are shown in Fig. 5. The melting peaks of aminolysed P(3HB-*co*-7 mol% 4HB) single crystals were depressed with increasing treating time from 24 to 72 hours. The melting peaks at higher temperature gradually reduced with increasing time, suggesting that the crystal thicknesses were reduced from surfaces by the amine treatment. On the other hand, the lower

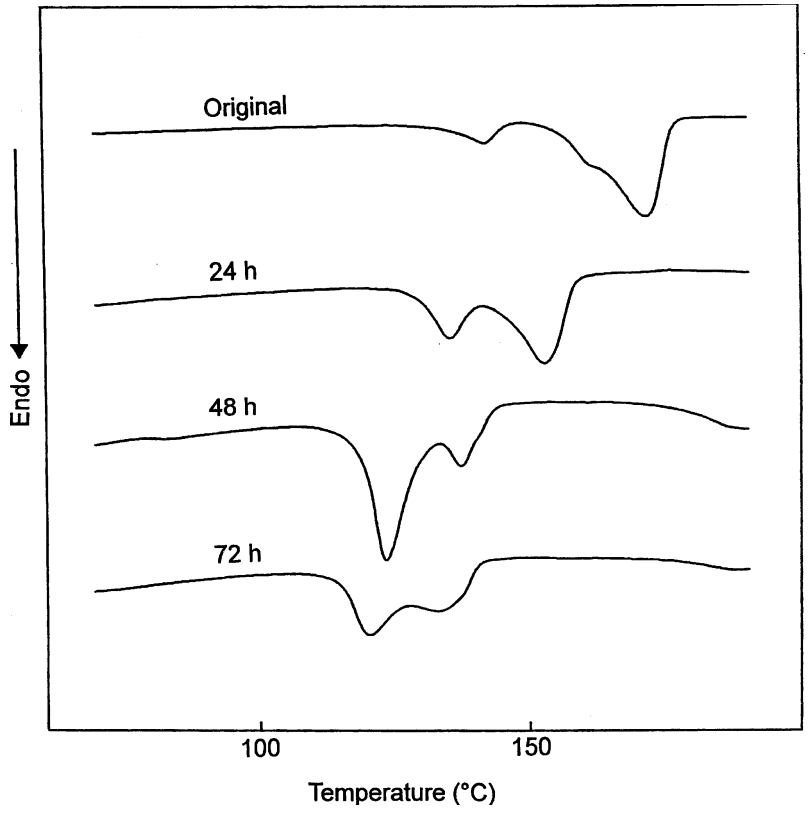

Fig. 5. DSC heating curves of P(3HB-*co*-7 mol% 4HB) single crystals treated with aqueous methylamine for various period of time at 25°C.

melting points (ca. 121°C) of P(3HB-*co*-4 mol% 4HB) (see Table II) and P(3HB-*co*-7 mol% 4HB) treated with times of 48 and 72 hours were almost unchanged, suggesting that the lower melting points of the samples were corresponding to the lamellar crystal thicknesses. Compared with Fig. 2 (b) of the melting point of P(3HB) single crystals degraded by PHB depolymerase, both samples treated with amine and enzyme showed similar melting point at ca. 121°C.

The compositions of P(3HB-*co*-4HB) single crystals before and after aminolysis were evaluated by 500 MHz ^{1}H-NMR spectroscopy are shown in Table II. The P(3HB-*co*-4 mol% 4HB) and P(3HB-*co*-7 mol% 4HB) single crystals aminolysed longer for 24 hours show almost a constant content of ca. 2 mol% 4HB. Moreover, to remove completely the remnant 4HB component at the folded-chain region or the crystal surface layers, all samples were degraded by lipase from *R. oryzae* at 37°C for 24 hours after aminolysis. The composition of P(3HB-*co*-4HB) after enzymatic degradation was evaluated by ^{1}H-NMR spectroscopy. The compositions of all samples remained unchanged after enzymatic degradation. One of the authors [15] have reported the amine treatment of the annealed P(3HB-*co*-3HV) samples for various times. In spite of the structural characteristic of P(3HB-*co*-3HV) being isodimorphic, the composition of the 3HV unit appeared almost constant (ca. 2/3 of the whole 3HV content) in the P(3HB) crystal after aminolysis over 24 hours, which was far smaller than the whole composition of the copolymer sample. In this case, the composition of P(3HB-*co*-4HB) copolymer after aminolysis was much smaller than that of the P(3HB-*co*-3HV) copolymer. This suggests the difficulty of cocrystallization in the P(3HB-*co*-4HB) copolymer, therefore, the composition of the 4HB component included was only ca. 2 mol% in the P(3HB) crystals.

Figure 6 shows the GPC chromatogram of P(3HB-*co*-7 mol% 4HB) single crystals obtained by gel permeation chromatography before and after aminolysis of the single crystals for various period of times. The molecular weight of the aminolysed P(3HB-*co*-4HB) single crystals steeply decreased to a value close to a single traverse of the crystal thickness with increasing treatment time. After degradation for 48 hours, two main peaks remained at different molecular weights of ca. 1500 and 3000, implying that these two molecular weights correspond to a single or double traverse of the lamellae thickness. This result showed very similar tendency

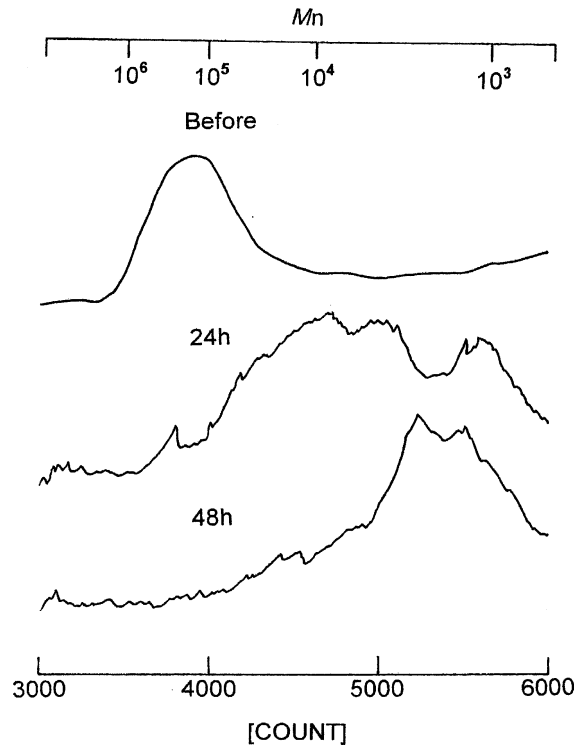

Fig. 6. Gel permeation chromatograms of P(3HB-*co*-7 mol% 4HB) single crystals before and after aminolysis for 24 hours and 48 hours at 25°C.

as previous discussion on Fig. 4, suggesting that aminolysis of P(3HB-*co*-4HB) single crystals was very similar effect to the enzymatic degradation and yielding oligomers whose molecular weights were close to a single and double traverse of the crystal thickness.

DISCUSSION

A typical transmission electron micrograph of P(3HB-*co*-7 mol% 4HB) single crystals closely resembles that of P(3HB). Enzymatic degradation of both single crystals occurred along the longer axes of the crystals to yield narrow cracks as the teeth of a comb. The T_m of the sample collected fragmented single crystals (solid mass only) remained unchanged, while that of solvent-evaporated sample of filtrate of the enzyme solution shifted to a lower temperature side. The molecular weights of the former sample remained almost unchanged, however, those of the latter sample showed the value close to a single or double traverse of the crystal thickness in addition to the former value. These results indicated that the enzymatic degradation of the P(3HB) single crystals had also occurred at the folded chain regions of

P(3HB) crystal surfaces in addition to less ordered crystal laterals or edges.

Under the optimum conditions, T_m and molecular weight of the amine treated P(3HB-*co*-4HB) sample showed the values corresponding to a single or double traverse of the crystal thickness. The aminolysed single crystals of P(3HB-*co*-4 mol% 4HB) and P(3HB-*co*-7 mol% 4HB) determined by ^{1}H-NMR showed an almost constant content of ca. 2 mol% 4HB component. These facts indicate the difficulty of cocrystallization in the P(3HB-*co*-4HB) copolymer. After the aminolysis and enzymatic degradation with lipase to remove the remnant 4HB component at P(3HB-*co*-4HB) single crystal surfaces, the composition of the samples remained unchanged, indicating that a small constant amount of the 4HB component (ca. 2 mol%) was closely occluded in the P(3HB) mother crystal lattices.

CONCLUSION

This paper reports the P(3HB-*co*-4HB) single crystals before and after enzymatic degradation with an extracellular PHB depolymerase purified from *R. pickettii* T1 and aminolysed in 20% aqueous methylamine solution. The mechanism of enzymatic degradation at the P(3HB-*co*-4HB) single crystals were significantly improved by enzymatic and chemical hydrolysis from observed result of TEM, DSC, and GPC. It was revealed that the chain-folding region on crystal surface changed after the partial degradation of P(3HB-*co*-4HB) single crystals by enzymatic degradation. This result shows evidence that enzymatic degradation of single crystals had also occurred at folded chain region of single crystal surfaces.

REFERENCES

1. H. Abe, I. Matsubara, and Y. Doi (1995) *Macromolecules* **28,** 844–853.
2. M. Mochizuki, K. Mukai, K. Yamada, N. Ichise, S. Murase, and Y. Iwaya (1997) *Macromolecules* **30,** 7403–7407
3. H. Abe, Y. Doi, H. Aoki, and T. Akehata (1998) *Macromoecules* **31,** 1791–1797
4. T. Iwata, Y. Doi, S. I. Nakayama, H. Sasatsuki, and S. Teramachi (1999) *Int. J. Biol. Macromol* **25,** 169–176.
5. M. Kunioka, Y. Kawaguchi, and Y. Doi (1989) *Appl. Microbiol. Biotechnol* **30,** 569–573.
6. Y. Doi, Y. Kanesawa, M. Kunioka, and T. Saito (1990) *Macromolecules* **23,** 26–31.
7. K. Mukai, Y. Doi, Y. Sema, and K. Tomita (1993) *Kobunshi Ronbunshu (Japan)* **50**(10), 715–722.
8. K. Kasuya, K. Takagi, S. Ishiwatari, Y. Yoshida, and Y. Doi (1998) *Polym. Degrad. Stab* **59,** 327–332.
9. H. Abe, Y. Kikkawa, T. Iwata, H. Aoki, T. Akehata, and Y. Doi (2000) *Polymer* **41,** 867–874.
10. H. Mitomo, P. J. Barham, and A. Keller (1987) *Polymer J* **19,** 1241–1253.
11. R. H. Marchessault, T. L. Bluhm, Y. Deslandes, G. K. Hamer, W. J. Orts, P. R. Sundararajan, M. G. Taylor, S. Bloembergen, and D. A. Holden (1988) *Makromol. Chem. Macromol. Symp* **19,** 235–254.
12. P. J. Hocking, R. H. Marchessault, M. R. Timmins, R. W. Lenz, and R. C. Fuller (1996) *Macromolecules* **29,** 2472–2478.
13. G. A. R. Nobes, R. H. Marchessault, B. H. Briese, and D. J. Jendrossek (1998) *J. Environ. Polym. Degrad* **6,** 99–107.
14. T. Iwata, Y. Doi, K. Kasuya, and Y. Inoue (1997) *Macromolecules* **30,** 833–839.
15. H. Mitomo and Y. Doi (1999) *Int. J. Biol. Macromol* **25,** 201–205.
16. H. Mitomo, S. Kobayashi, N. Morishita, and Y. Doi (1995) *Polym. Prepr. Jpn* **44,** 3156.
17. W.-C. Hsieh, K. Nishiwaki, N. Tanaka, and H. Mitomo (2002) *Sen-i Gakkaishi (Japan)* **58**(3), 98–105.
18. W.-C. Hsieh, K. Kasuya, and H. Mitomo (2002) *Sen-i Gakkaishi (Japan)* **58**(11), 428–431.
19. Y. Shirakawa, T. Fukui, T. Saito, Y. Okamoto, T. Narikawa, K. Koide, K. Tomita, T. Takemura, and S. Masamune (1986) *Biochem. Biophys. Acta* **880,** 46–53.
20. H. Mitomo, W.-C. Hsieh, K. Nishiwaki, K. Kasuya, and Y. Doi (2001) *Polymer* **42,** 3455–3461
21. K. Mukai, Y. Doi, Y. Sema, and K. Tomita (1993) *Biotechnol. Lett* **15,** 601–604.
22. H. Kimura, Y. Yoshida, and Y. Doi (1992) *Biotechnol. Lett* **14,** 445–450.
23. H. Mitomo, N. Morishita, and Y. Doi (1995) *Polymer* **36,** 2573–2578.
24. A. Cao, K. Kasuya, H. Abe, Y. Doi, and Y. Inoue (1998) *Polymer* **39,** 4801–4816
25. Y. Arai, A. Cao, N. Yoshie, and Y. Inoue (1999) *Polym. Int* **48,** 1219–1228.
26. T. Kurita (1969) *Kobunshi Kagaku (Japan)* **26,** 511–521.
27. H. Mitomo (1992) *Sen-i Gakkaishi (Japan)* **48,** 595–601.