Morphology and Enzymatic Degradation of Poly(L-lactic acid) Single Crystals

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ABSTRACT: Lamellar single crystals of poly(L-lactic acid) (PLLA) were grown from a 0.05% solution of *p*-xylene at 90 °C. The hexagonal, truncated-lozenge and lozenge-shaped single crystals with spiral growth were prepared simultaneously, and these crystals were relatively stable under conditions of electron bombardment. The hexagonal and lozenge-shaped crystals gave well-resolved electron diffractograms from which the reciprocal lattice parameters $a^* = 0.935$ nm^{-1}, $b^* = 1.626$ nm^{-1}, and $\gamma^* = 90°$ could be determined, and these lattice parameters corresponded with those of the α structure of PLLA fibers. Accordingly, the hexagonal crystal was considered as pseudo-hexagonal symmetry, but actually with orthorhombic packing of the PLLA chains; i.e., the ratio of the *a* and *b* cell parameters is quite equal to $\sqrt{3}$. The enzymatic degradation of PLLA single crystals with a proteinase-K from the mold *Tritirachium album* in 50 mM Tris−HCl buffer at pH 8.5 and 37 °C was investigated by means of transmission electron microscopy, atomic force microscopy, high-performance liquid chromatography, and gel permeation chromatography. Enzymatic degradation progressed from the edges of lamellar crystals to yield a rounded shape without decreasing the molecular weights and lamellar thicknesses. It has been concluded that the single crystals were enzymatically hydrolyzed at the disordered chain-packing region of crystal edges rather than chain-folding surfaces of single crystals. As the degradation time increased, in a rare case the diamond holes were observed on the crystal surfaces of lozenge-shaped single crystals.

Introduction

Poly(L-lactic acid), PLLA, produced by some chemical synthetic methods,[1−5] is a biodegradable and biocompatible thermoplastic, and its biodegradability has been extensively studied. Over the past quarter of a century, there has been considerable interest in PLLA as biodegradable materials in dental[6,7] and drug delivery systems[8,9] and in applications for sutures and surgical implants.[7,10,11]

The degradability of PLLA, until now, has been investigated mainly regarding the chemical hydrolysis in water, while the enzymatic degradability of PLLA has been reported by only a few. Williams[12] reported the enzymatic hydrolysis of PLLA in the presence of enzymes such as pronase, proteinase-K, and bromelain. Regarding poly(lactic acid) (PLA) stereocopolymers, Fukuzaki *et al.*[13] reported that the hydrolysis of PLA was accelerated in the presence of specific enzymes and that the most rapid enzymatic degradation of stereocopolymers was observed on the D,L-PLA sample containing 50% L-lactic acid. Furthermore, Makino *et al.*[14] showed that the addition of a carboxylic esterase accelerated a decrease in the weight-average molecular weight of D,L-PLA. Recently, Reeve *et al.*[15] reported the effect of stereochemical composition on enzymatic degradability of PLA films by proteinase-K from *T. album*. The above results in the enzymatic hydrolysis of PLA have been reported with films and fibers of PLA.

It has been demonstrated that PLLA fibers have two crystal modifications depending on the spinning and drawing conditions, on the basis of the study on X-ray fiber diagrams. De Santis and Kovacs[16] reported that the crystal structure of the α modification for the solution-spun fibers consisted of a system of left-handed 10/3 helices packed in an antiparallel fashion with two molecules passing through the unit cell, which was defined as pseudo-orthorhombic with parameters $a = 1.07$ nm, $b = 0.645$ nm, and c (fiber axis) $= 2.78$ nm. On the other hand, Eling *et al.*[17] found that the X-ray diffraction diagrams for the solution-spun fibers were different from those for the melt-extruded fibers, and they revealed the β structure. Recently, the orthorhombic unit cell $a = 1.031$ nm, $b = 1.821$ nm, and c (fiber axis) $= 0.900$ nm, containing six chains for the β structure was proposed by Hoogsteen *et al.*[18] Furthermore, as a result of the conformational energy calculation, they concluded that the chain conformation of the β structure was left-handed 3/1 helices. More recently, the crystal structure (the positions of two helical chains) of the α modification was modified by Kobayashi *et al.*[19] using the X-ray diffraction experiments and energetic computer simulation.

Despite the detailed investigations of the crystal structures and conformations of PLLA fibers, a morphological study on solution-grown crystals using electron microscopy has not been carried out aggressively. Fischer *et al.*[20] prepared the single crystals of the PLA copolymer containing 15% D-units. On the other hand, Kalb and Pennings[21] reported two types of PLLA lamellar crystals grown from the dilute solution in toluene and *p*-xylene. On the basis of the electron diffraction against the hexagonal-like lamellar crystals, they revealed a hexagonal molecular structure with dimensions smaller than reported earlier. More recently, the other morphology (lozenge-shaped crystal) of PLLA solution-grown crystals was prepared from an acetonitrile solution by an isothermal crystallization method by Miyata and Masuko,[22] who determined the orthorhombic unit cell parameters, $a = 1.078$ nm, $b = 0.604$ nm, and c (fiber axis) $= 2.87$ nm, on the basis of

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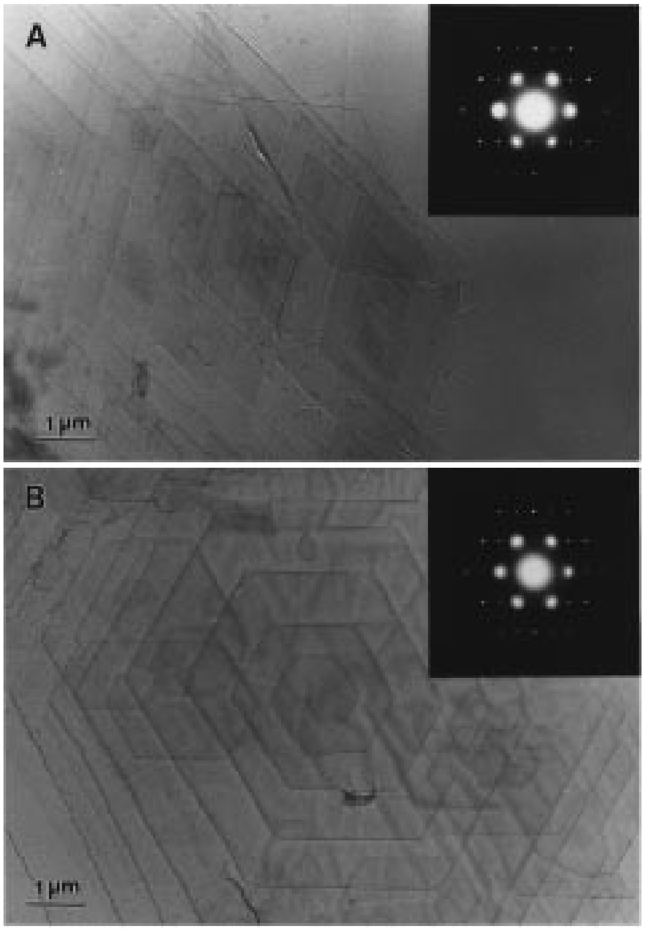

Figure 1. Electron micrographs after shadowing with a Pt–Pd alloy of PLLA lamellar single crystals with screw dislocations grown from solution in *p*-xylene, and typical electron diffraction diagrams: (A) lozenge-shaped and (B) hexagonal-shaped single crystals.

electron diffraction patterns of lozenge-like single crystals and the X-ray fiber diagram of the oriented film analysis.

In the chemical or enzymatic hydrolysis of biomaterials, the distribution of crystal regions, lamellar crystal size, and crystal morphology and structure may play a decisive role in the rate of hydrolysis. To elucidate the mechanism of enzymatic degradation on the crystal region, single crystals were prepared as the model substrate, and their enzymatic degradation was studied. For example, enzymatic degradations using single crystals have been performed on $\beta(1\rightarrow4)$xylan single crystals with xylanases[23] and lamellar single crystals of nigeran with mycodextranase.[24] Recently, these techniques were applied to the enzymatic degradation of poly[(*R*)-3-hydroxybutyrate] single crystals, which are a biodegradable and biocompatible thermoplastic.[25–28]

In this paper, we have attempted to obtain more insight on the morphologies of PLLA crystals grown from the dilute solution and on the mechanism for enzymatic degradation of PLLA lamellar single crystals by a proteinase-K from the mold *T. album*, using transmission electron microscopy, atomic force microscopy, high-performance liquid chromatography, and gel permeation chromatography.

Experimental Section

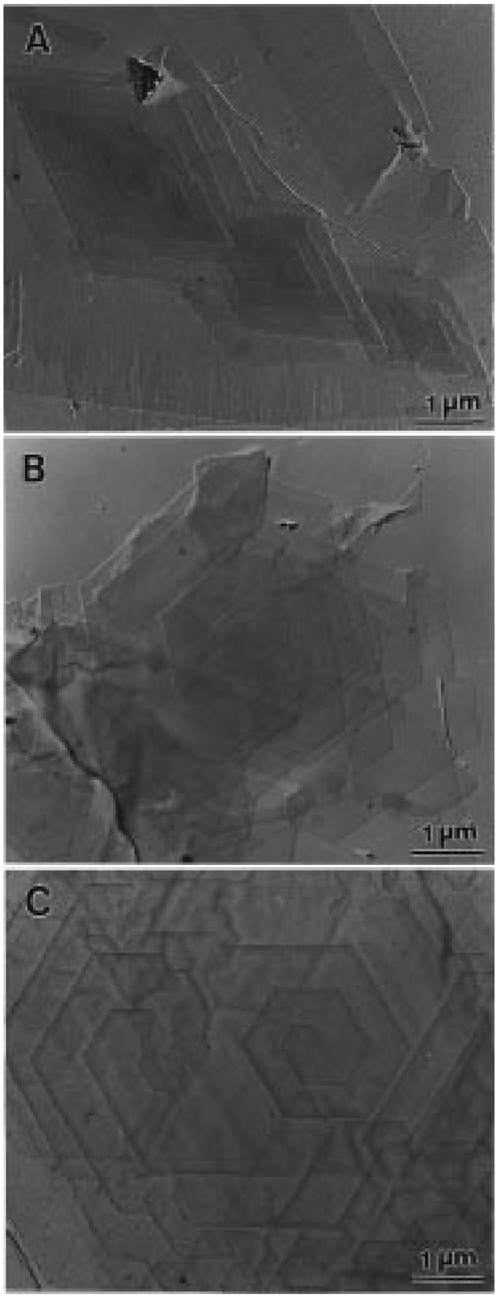

Figure 2. Electron micrographs after shadowing with a Pt–Pd alloy of PLLA lamellar single crystals: (A) successive lozenge-like; (B) truncated lozenge-like; (C) twin screw dislocation hexagonal-like.

Preparation of Lamellar Single Crystals. The poly(L-lactic acid) (PLLA, number-average molecular weight (M_n) = 83 000 and polydispersity (DPI) = 1.4) sample was purchased from Polysciences, Inc. (Warrington, PA). The PLLA sample was purified by the reprecipitation in methanol from chloroform solution and dried *in vacuo* for 1 week. A 5 mg sample of PLLA was dissolved into 10 mL of *p*-xylene at 130 °C. The solution was kept there for 15 min, after which slow cooling was applied until 90 °C, and the solution was kept there for 24 h. Slow cooling was applied by cutting off the heating element of a silicone oil bath. The crystals were collected by centrifugation and washed three times with room-temperature ethanol. For enzymatic degradation, the crystals were collected by centrifugation, washed with 50 mM Tris–HCl buffer at pH 8.5, and resuspended in the same buffer.

Enzymatic Degradation of Single Crystals. A 0.1 mL aliquot of a 2 mg/mL solution of a proteinase-K from the mold *T. album* obtained from Sigma Chemical Co. (St. Louis, MO) was added to 0.9 mL of 50 mM Tris–HCl buffer at pH 8.5 containing PLLA single crystals (0.5 mg/mL) and then incu-

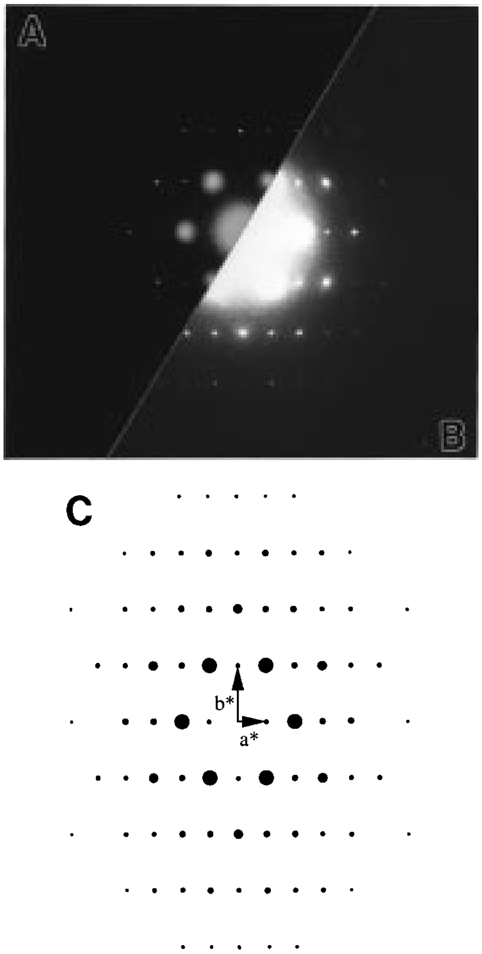

Figure 3. Electron diffraction diagram of a hexagonal-like PLLA lamellar single crystal: (A) underexposed, showing the strong diffraction spots; (B) overexposed, showing the weak diffraction spots; (C) summary of the observed diffraction spots with their relative intensities.

bated at 37 °C. To observe the time-dependent changes in the morphology of single crystals, 1 μL of diisopropyl fluorophosphate was added into the reaction mixture at various reaction periods to stop the enzymatic degradation. The mixture was then centrifuged, and the supernatant solution was used to monitor the adsorption at 210 nm due to the carbonyl group of degraded products on a spectrophotometer. The crystals were washed twice with distilled water to remove the buffer. The crystals were then redispersed in ethanol, washed several times by centrifugation, and resuspended in ethanol.

HPLC Analysis. The water-soluble products produced during the enzymatic degradation of PLLA single crystals by proteinase-K were analyzed by using a Shimadzu LC-9A high-performance liquid chromatography (HPLC) system with a gradient controller and a SPD-10A UV spectrophotometric detector. A stainless steel column (250 × 4 mm) containing LiChrospher RP-8 (5 μm) was used at 40 °C. Sample solutions after enzymatic degradation were acidified to pH 2.5 with HCl, and 50 μL solutions were injected. The column was eluted with a linear gradient of distilled water (pH 2.5, adjusted by the addition of HCl solution) to acetonitrile over 40 min with a flow rate of 1.0 mL/min. The monomer and oligomers of lactic acid were detected spectrophotometrically at a wavelength of 210 nm.

Table 1. Electron Diffraction Data of Hexagonal Shaped PLLA Lamellar Single Crystal

index[a] (*hkl*)	*d* spacing (obs, nm)	*d* spacing (calc, nm)	index[a] (*hkl*)	*d* spacing (obs, nm)	*d* spacing (calc, nm)
100	1.070	1.070	410	0.245	0.245
010	0.614	0.615	320	0.233	0.233
200	0.535	0.535	030	0.205	0.205
110	0.528	0.533	510	0.202	0.202
210	0.399	0.403	420	0.202	0.202
300	0.354	0.356	130	0.201	0.201
310	0.302	0.308	230	0.192	0.191
020	0.307	0.307	600	0.178	0.178
120	0.296	0.295	330	0.178	0.178
400	0.268	0.267	430	0.162	0.163
220	0.268	0.266	620	0.154	0.154

[a] Indexed in terms of the reciprocal lattice parameters: $a^* = 0.935$ nm^{-1}, $b^* = 1.626$ nm^{-1}, and $\gamma^* = 90°$.

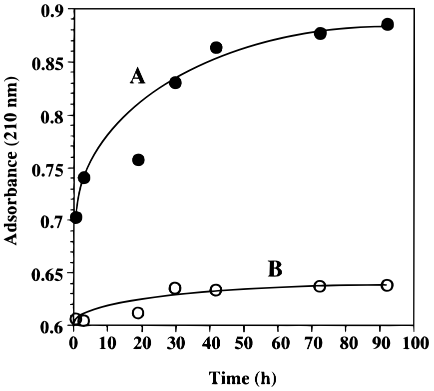

Figure 4. Time-dependent changes in the absorbance at 210 nm of the reaction solutions of PLLA single crystals with (curve A: ●) and without (curve B: ○) 200 μg/mL proteinase-K at 37 °C.

Molecular Weight Measurement. All molecular weight data of PLLA single crystals before and after enzymatic degradation 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-average 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. A molecular weight calibration curve of PLLA was obtained with polystyrene standards of low polydispersities.

Transmission Electron Microscopy. Drops of PLLA crystal suspension, before and after enzymatic degradation, 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. For calibration purposes, some crystals were mounted on carbon-coated grids sputtered with gold. All calibration was achieved at room temperature. These grids were observed with a JEM-2000FX II electron microscope operated at an acceleration voltage of 200 kV for the electron diffraction and 120 kV for 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 D19 developer (diluted in water 1/2, v/v).

Atomic Force Microscopy. The lamellar thicknesses of PLLA single crystals, before and after enzymatic degradation, 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 microcantilevers 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. The crystal suspension, before and after enzymatic degradation, was dropped on mica and allowed to dry. All images were recorded at room temperature.

Results and Discussion

Lamellar Single Crystals. The lozenge- and hexagonal-shaped single crystals of PLLA with spiral

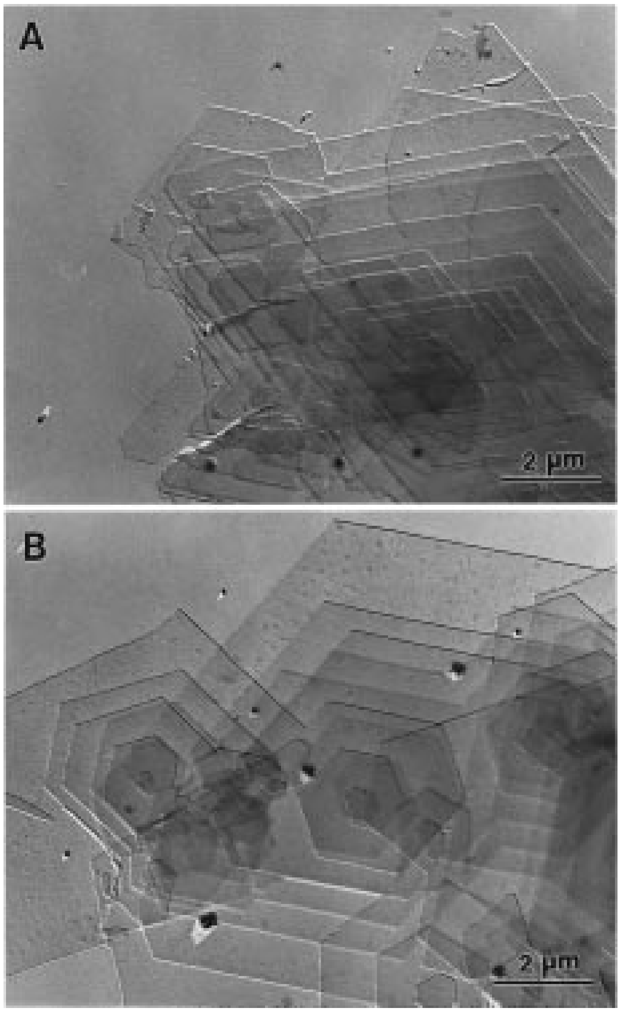

Figure 5. Electron micrographs of PLLA single crystals suspended in 50 mM Tris−HCl buffer (pH 8.5) for 70 h at 37 °C: (A) lozenge-like and (B) hexagonal-like single crystals.

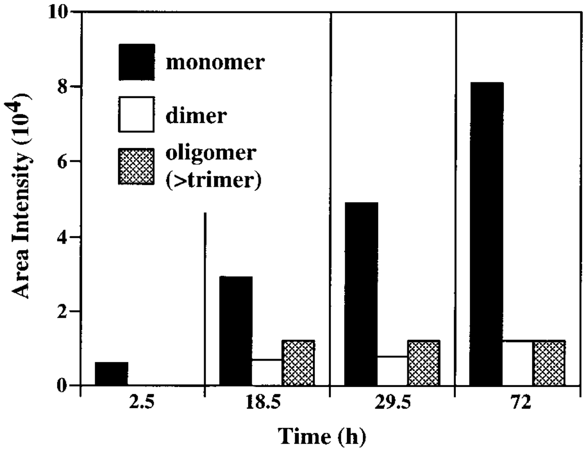

Figure 6. Relative amounts and weight distributions of water-soluble products during the enzymatic degradation of PLLA single crystals by proteinase-K at 37 °C.

growth were prepared simultaneously in *p*-xylene at 90 °C, and the morphologies are shown in Figure 1, together with typical electron diffraction patterns. Monolayer and bilayer platelets in both crystals are observed rarely, and a majority of the crystals contain many layers emanating from screw dislocations. The angles between growth faces of a lozenge-shaped crystal with spiral growth are perfect 60° and 120° (Figure 1A). On the other hand, those of a hexagonal-shaped crystal are almost 120° (Figure 1B). The intensities of electron reflections against lozenge-shaped crystals are stronger than those against hexagonal-shaped crystals. These observations suggest that the molecular packing in a lozenge-shaped crystal is tighter than that in a hexagonal one. On the basis of the orthogonal unit cell in PLLA crystals reported previously,[16,18,19,21,22] the lozenge-shaped crystals could be considered to occur with {110} as growth planes, like a flat lamellar polyethylene crystal.[29] Compared with the smooth faces well-defined by {110} of a lozenge-shaped crystal, the growth faces of a hexagonal-shaped crystal, except {110} planes, are slightly rough. If PLLA crystallized as perfect hexagons, the growth rate should be the same in all planes. This contradiction suggests that the hexagonal crystal has pseudo-hexagonal symmetry but actual orthogonal packing of the PLLA molecules.

The rare observations, spiral growth and truncated-lozenge shaped, of PLLA crystals grown from the dilute solution of *p*-xylene at 90 °C are shown in Figure 2. The successive lamellae with spiral growth shown in Figure 2A have been observed frequently in polyethylene crystals grown with lozenge-shaped terraces in {110} reflection twin orientation[30] and in poly(oxymethylene) crystals with star-shaped lamellae of the spiral growth.[29] Furthermore, these PLLA lozenge-shaped terraces have developed on the basal terrace with two screw dislocations of similar hand, as in the case of polyethylene.[30] On the other hand, the hexagonal crystals with spiral growth shown in Figure 2C demonstrated the stunted growth of the lower turns of the double spiral, as observed in a single crystal of isotactic polystyrene reported by Keith *et al.*[31] and the terrace growth around two screw dislocations of similar hand observed in polyethylene reported by Khoury and Padden.[30] It is well-known that the polymer molecules gather according to their molecular weight during the crystallization.[32] The GPC trace of PLLA single crystals was trimodal (Figure 8) with the reduction of molecular weights, despite the unimodal trace of the original sample. This decrease in the molecular weight during the crystallization of PLLA at 90 °C may be due to the thermal hydrolysis, and the trimodal molecular weight distribution seems to be one reason for growing the crystal morphology.

The thickness by AFM of the monolamellar part of lozenge-shaped crystals yielded the values of 9−10 nm, while hexagonal-shaped crystals had a thickness of 11−12 nm. These results corresponded to those discerned from the length of the shadow in electron micrographs reported by Kalb and Pennings.[21] Taking into consideration the fiber repeat distance and molecular weights, the chain foldings occur at lamellar surfaces of single crystals as with polyethylene,[29,30] polypropylene,[33] and poly((*R*)-3-hydroxybutyrate).[34]

The crystals, which consist of stacks of lamellae, have variable thickness: some are transparent to the electron beam, whereas others are completely opaque. Each monolamellar part at the edge of all PLLA crystals yields the well-resolved electron diffractogram, and all electron diffractograms are identical. Figure 3 shows the selected-area electron diffraction diagram of the hexagonal-shaped lamellar single crystals. The diagram contains 25 independent diffraction spots mirrored in the four quadrants, defined by the two orthogonal axes *a** and *b**. In comparing the observed *d* spacings of the X-ray fiber pattern of the α structure reported

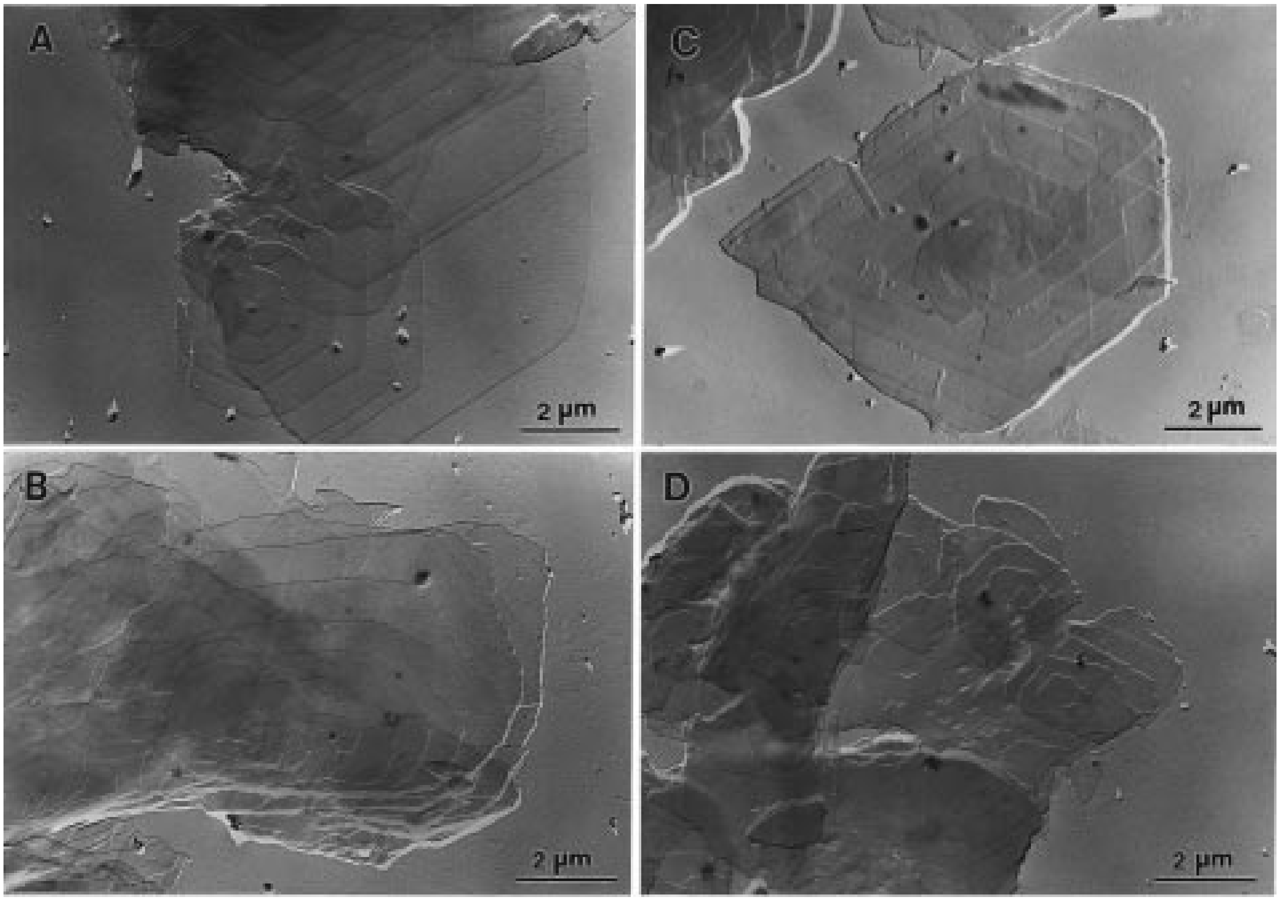

Figure 7. Electron micrographs of PLLA single crystals after enzymatic degradation by proteinase-K at 37 °C: (A) hexagonal-like PLLA single crystals after 20 h of enzymatic hydrolysis; (B) hexagonal-like PLLA single crystals after 50 h of enzymatic hydrolysis; (C) lozenge-like PLLA single crystals after 20 h of enzymatic hydrolysis; (D) lozenge-like PLLA single crystals after 50 h of enzymatic hydrolysis.

previously,[22] one sees that all the $hk0$ reflections of the fiber diagram are also observed in the electron diffraction pattern, which confirms that the electron diffraction pattern is a projection along the c-axis; i.e., the polymer chains are aligned perpendicular to the lamellar base of the crystal. The X-ray pattern has a lower resolution than the electron diffraction pattern, which displays diffraction spots down to 0.15 nm as opposed to only 0.27 nm. Upon calibration of the electron diffraction pattern, all the electron diffraction spots can be indexed in terms of orthogonal reciprocal lattice parameters: $a^* = 0.935$ nm^{-1}, $b^* = 1.626$ nm^{-1}, and $\gamma^* = 90°$. The calculated obtuse angle, 120.2°, between (110) and $(1\bar{1}0)$ planes corresponded perfectly to the observed one. Accordingly, the hexagonal shape could be considered to crystallize with {110} and {100} as growth planes, having the chain-folding along the [110] direction. A comparison of the observed and calculated d spacings in the electron diffraction diagram is given in Table 1.

Enzymatic Degradation. The time-dependent changes in the absorbances at 210 nm, caused by the liberation of lactic acid, of the reaction solution of PLLA single crystals in 50 mM Tris−HCl buffer (pH 8.5) with and without 200 μg/mL proteinase-K at 37 °C are shown in Figure 4. The difference in the starting absorbances with and without enzyme is due to the absorption of proteinase-K. In the case of the chemical (alkali) hydrolysis in 50 mM Tris−HCl buffer at pH 8.5 without proteinase-K, the absorbance (curve B) slightly increased with time. This slight increase in the absorbance seems to result from the chemical hydrolysis of the amorphous region on the crystal surfaces. However, the rate of chemical hydrolysis was negligibly small in comparison with that of enzymatic hydrolysis by proteinase-K (curve A). The electron micrographs of PLLA single crystals after 70 h chemical hydrolysis are shown in Figure 5. The morphologies and sizes of PLLA single crystals remained unchanged during the course of chemical hydrolysis in the absence of proteinase-K. This result indicates that the crystal region of PLLA is hardly

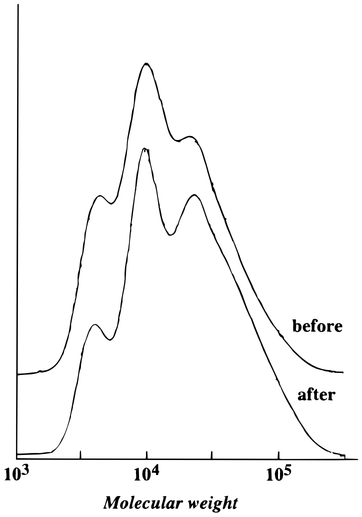

Figure 8. Gel permeation chromatograms of PLLA single crystals before and after partial enzymatic degradation by proteinase-K at 37 °C for 50 h.

hydrolyzed under the conditions of the present experiment. Accordingly, a marked increase in the absorbance of the reaction solution of PLLA single crystals in the presence of proteinase-K suggests the enzymatic degradation of the crystal region.

The enzymatic degradation of PLLA single crystals in 50 mM Tris−HCl buffer (pH 8.5) by proteinase-K at 37 °C produced a mixture of monomer and oligomers of lactic acid (LA) as water-soluble products. The composition of water-soluble products was measured by high-performance liquid chromatography (HPLC). The HPLC curves of water-soluble products after enzymatic degradation of PLLA single crystals showed three main peaks at elution times from 4 to 13 min (data not shown). These peaks were identified as LA monomer, LA−LA dimer, and LA−LA−LA trimer, on the basis of a comparison with the elution times reported previously by Abe *et al.*[35] The relative amounts of LA units in water-soluble products were determined from the peak areas in the HPLC curve. Relative amounts and weight distributions of water-soluble products during the enzymatic degradation of PLLA single crystals are shown in Figure 6. The amounts of dimer and trimer were almost constant during the enzymatic degradation, while the amount of monomer increased proportionally with time.

Typical electron micrographs of hexagonal- and lozenge-shaped PLLA crystals with spiral growth after 20 and 50 h of enzymatic degradation by proteinase-K are shown in parts A, B and C, D of Figure 7, respectively. Despite differences in the crystal morphology, both crystals were degraded from crystal edges to form a rounded shape. Furthermore, as the degradation proceeded, the rounded shape became coarse and the crystal size decreased. The remaining crystals maintained the sharp electron diffraction patterns.

The molecular weight distributions of PLLA single crystals containing several morphologies, before and after 50 h of enzymatic degradation, are shown in Figure 8. It is noted that 50 h of enzymatic attack resulted in a marked degradation of crystals, on the basis of both results of absorbance in Figure 4 and electron micrographs in Figure 7. However, the molecular weights of PLLA crystals remained unchanged before and after enzymatic degradation, indicating that partial degradation at the chain-folding surfaces does not take place. In addition, AFM measurements of PLLA crystals showed that the thickness of monolamellar parts also remained unchanged before and after enzymatic degradation, further showing no evidence for the enzymatic degradation from crystal surfaces (data not shown). The same observations have been reported in the enzymatic degradation of poly((*R*)-3-hydroxybutyrate) single crystals by some extracellular PHB depolymerases.[25−28] Thus, it has been concluded that the active site of proteinase-K hydrolyzed preferentially at the disordered chain-packing regions of crystal edges rather than the chain-folding surfaces of single crystals.

In a few cases the diamond-shaped portions of the crystal separated from the remainder were detected, as shown in Figure 7D, when the enzymatic degradation was applied against lozenge-shaped single crystals. This diamond-shaped hole has been observed in polyethylene crystals involving preparations in which the solvent was removed at an elevated temperature by Reneker and Geil.[29] However, in our study, these diamond-shaped holes could not be observed in the single crystals before enzymatic degradation, indicating that those holes resulted from enzymatic attacks. Further investigations are needed for the elucidation of this phenomenon.

Conclusions

This paper has reported the crystal morphologies and the visualization of enzymatic degradation of PLLA single crystals with proteinase-K from the mold *T. album* by transmission electron microscopy. PLLA single crystals prepared from a dilute solution of *p*-xylene showed the lozenge- and hexagonal-shaped crystals with spiral growth, and these crystals were indexed with the same orthogonal unit cell parameters. Accordingly, the hexagonal crystal was concluded to be pseudohexagonal packing, but actually with orthorhombic packing of the PLLA molecules.

Enzymatic degradation of single crystals by proteinase-K progressed from the edges rather than the chain-folding surfaces of single crystals, and both the molecular weights of PLLA chains in crystals and the thickness of monolamellar parts remained unchanged during the enzymatic degradation. It has been concluded that the attack by a specific biochemical function for the depolymerization of polymers takes place preferentially against the disordered chain-packing region rather than the chain-folding surfaces of single crystals and that the chains are untied from the crystal edges or cropped out from the crystals. The diamond-shaped holes observed after enzymatic degradation might be due to the hydrolysis of polymer chains cropped out from the crystals.

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