Splintering of Poly(3-hydroxybutyrate) Single Crystals by PHB-Depolymerase A from *Pseudomonas lemoignei*

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Received August 13, 1996; Revised Manuscript Received October 1, 1996[⊗]

ABSTRACT: With the aim of improved understanding of the mechanism of depolymerase action, folded chain lamellar single crystals were partially degraded with PHB-depolymerase A from *Pseudomonas lemoignei*. Enzymatically degraded single crystals of bacterial poly(3-hydroxybutyrate), PHB, were observed by transmission electron microscopy and were found to be splintered parallel to their long axes. Prior to degradation, the crystals were lamellar with macroscopic dimensions of approximately 2.5 μm by 20 μm and the classical baseplane single crystal diffraction pattern corresponding to bacterial PHB was recorded. When observed by TEM, the partially degraded crystals had been splintered longitudinally to a needlelike morphology. The needlelike fragments of PHB still yielded the same crystalline baseplane diffraction pattern. These results support an "edge attack" model for the degradation of PHB single crystals and explain, at a molecular level, the lack of decrease in molecular weight during the degradation since the direction of chain folding is parallel to the long axis of the crystals. The proposed mechanism explains the conversion of PHB spherulite lamellae into a needlelike morphology and suggests that PHB-depolymerase A has both *endo* and *exo* activity.

Introduction

In spite of its pronounced crystallinity, bacterial poly(3-hydroxybutyrate), PHB, has always displayed a high level of biodegradability by a number of depolymerases.[1−6] Crystallinity is often cited as a hindrance to biodegradability,[7] although some incongruities relating crystallinity and morphology remain.[8] In the search for a better understanding of the mechanism of depolymerase action on bacterial PHB, model substrates, such as discrete oligomers[6,9] and synthetic PHB of controlled stereoregularity[10] are being used as substrates. In addition, folded chain lamellar single crystals of PHB have recently been used because their colloidal nature permits turbidimetric detection of the degradation.[11] Furthermore, their well-known molecular architecture allows a dual surface texture to be presented to the enzyme: a crystalline core at the edge of the lamellae and a less ordered chain-folded surface. The interest in examining this kind of substrate as a model of naturally occurring crystalline morphology was appreciated some years ago when studies of the texture of lamellar xylan[12] and nigeran[13] single crystals after degradation were reported. It was concluded that these two polysaccharides were degraded according to different modes of attack by the enzyme; xylan was attacked from the crystal edge, whereas in nigeran it was the chain folds that were attacked. Since that time, considerable progress in our understanding of the preparation and characterization of lamellar single crystals has occurred; hence their use as model substrates for PHB is being developed.[9,14−19]

We wish to report a recent electron microscope examination of bacterial PHB single crystals degraded by a purified PHB-depolymerase. In this case, the interpretive benefits of using the lamellar single crystal substrate were especially valuable. From a recently reported study[11] of PHB-depolymerase action on PHB single crystals, it was concluded that the degradation was at the crystalline core edge, which prompted us to examine the partially degraded single crystals themselves. In the previous study,[11] it was found that the degree of polymerization of the bacterial PHB single crystals was unchanged in spite of nearly complete weight loss. From this it was concluded that single layers of adjacent reentry folded chains were being removed sequentially from the crystal edge. The present study complements the previous experiment by examining the crystalline lamellae after enzymatic attack.

Experimental Section

Materials. Bacterial poly(3-hydroxybutyrate), PHB, was supplied by Zeneca Inc. It was analyzed by ^{1}H NMR[20] and found to contain only 3-hydroxybutyrate repeats. The number average molecular weight was determined[20] to be 174 000. Poly(ethylene glycol) and methanol were obtained from Fluka (Ronkonoma, NY). Tris−HCl and $CaCl_2$ were obtained from Sigma (St. Louis, MO).

Single Crystal Preparation. Single crystals of poly(3-hydroxybutyrate), PHB, were grown by a method modified from that of Revol *et al.*[17] Dilute solutions (0.02%) of poly(3-hydroxybutyrate) of bacterial origin, which had been previously dissolved in dimethylformamide and reprecipitated in diethyl ether, were prepared in poly(ethylene glycol) (m.w. 400). The solution was heated to 170 °C to completely dissolve the polymer. After dissolution, the temperature was lowered to 130 °C. After several hours the crystals had formed and the solution was allowed to cool slowly to room temperature. A 5-fold excess of methanol was then added to dilute the poly(ethylene glycol) and allow for the isolation of the crystals.

The crystals were then prepared for enzymatic degradation by either sedimentation or centrifugation. In the case of those prepared by sedimentation, the crystals were allowed to sediment, after which time the supernatant was removed and

[⊗] Abstract published in *Advance ACS Abstracts,* November 15, 1996.

S0024-9297(96)01219-3 CCC: $12.00

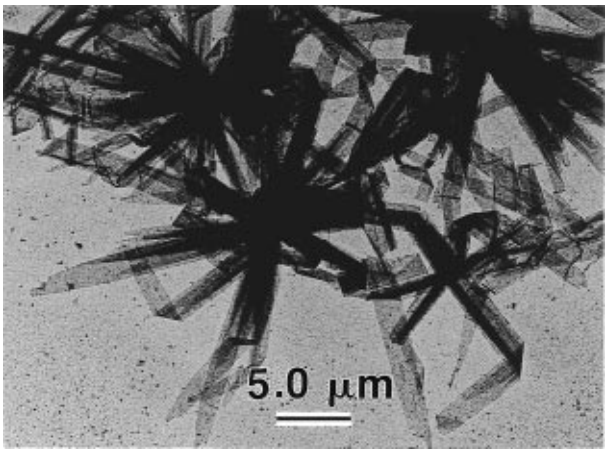

Figure 1. Typical preparation of the PHB single crystals before enzymatic attack.

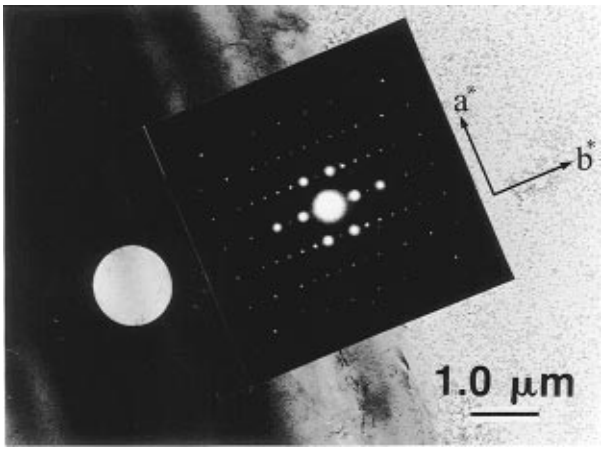

Figure 2. As in Figure 1, but enlarged, showing a domain of one single crystal of PHB. Insert: electron diffractogram with proper orientation to the circled area. Due to its orthorhombic character, the unit cell axes *a* and *b* of PHB are along *a** and *b**, respectively.

an excess of methanol was then added. The suspension was then gently agitated and allowed to resediment. This process was repeated at least ten times in order to ensure complete removal of the poly(ethylene glycol). The crystals were then further washed three times with Tris−HCl buffer (50 mM, pH 8.0, 1 mM $CaCl_2$) in preparation for enzymatic degradation. In the case of those prepared by centrifugation, the crystals were centrifuged and washed with methanol seven times, followed by three washings with Tris−HCl buffer in preparation for enzymatic degradation.

Enzymatic Degradation of Single Crystals. The enzyme used for the study of the degradation of single crystals of poly(3-hydroxybutyrate) was a purified, extracellular depolymerase from *Pseudomonas lemoignei*, PHB-depolymerase A. The isolation and purification of the enzyme is described elsewhere.[21] For the present work, the concentration of depolymerase A used was the same as that reported by Tomasi *et al*.[21] to provide the maximum degradation of PHB films (i.e. 3 μg/mL). PHB-depolymerase A was added to the suspension of PHB single crystals, prepared as described above. The crystal suspension was then incubated, without stirring, for 24 h at 37 °C. The suspension was centrifuged and the crystals washed twice with Tris−HCl buffer, followed by two washings with distilled water and finally once with methanol to terminate the enzyme reaction and prepare the crystals for examination. The crystals were then examined by transmission electron microscopy.

Transmission Electron Microscopy. Drops of PHB single crystals suspended in methanol were deposited on carbon-coated grids and allowed to dry. These grids were examined under low-dose (low intensity current) conditions with a Philips CM 200 CRYO transmission electron microscope operating at 200 kV for electron diffraction and 80 kV for imaging. Imaging was performed without staining or shadowing, using only mass (density) contrast. Photomicrographs were recorded on Agfa Scientia plates.

Results and Discussion

An electron micrograph of the poly(3-hydroxybutyrate) single crystals prior to enzymatic degradation is shown in Figure 1. The slightly aggregated crystals are lath-shaped, with the *a*-axis of the orthorhombic unit cell (0.576 nm) parallel to the long direction of the crystal and the *b*-axis (1.32 nm) perpendicular. The macroscopic dimensions are approximately 20 μm and 2.5 μm, respectively. Many of the characteristic pointed ends of the single crystals[14,17] are broken, probably as a result of the isolation by centrifugation. The electron diffractogram shown in Figure 2 has the *d* spacings and relative intensities of bacterial PHB and the previously reported unit cell orientation in these single crystals.[14,17,19] Due to crystal thickness and secondary scattering some weak reflections which should be systematically absent are observed in the diffractogram.

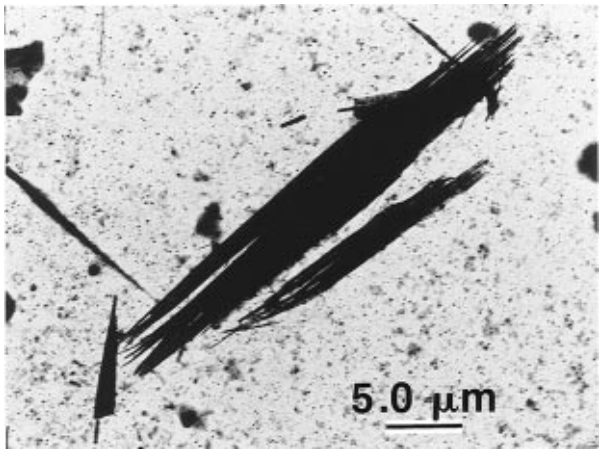

Figure 3. PHB single crystals after 24 h enzymatic attack with PHB-depolymerase A from *P. lemoignei* showing the similarity of both ends of the crystal.

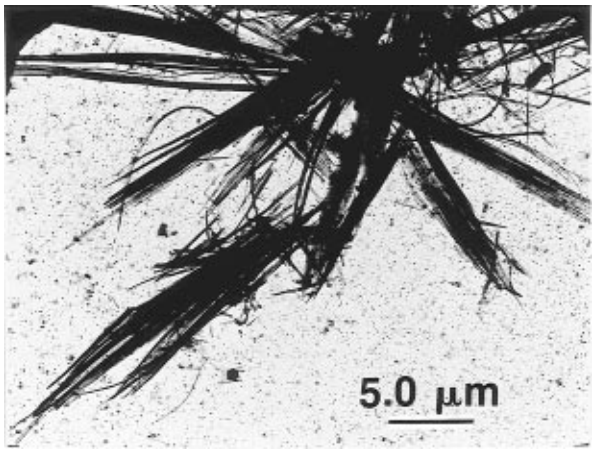

Figure 4. PHB single crystals after 24 h enzymatic attack with PHB-depolymerase A from *P. lemoignei* showing the curved, needlelike crystals.

After 24 h of enzymatic degradation, the crystals were splintered parallel to their long axes (Figures 3 and 4). In addition, it can be concluded that PHB depolymerase A does not have a preferred directionality when attacking the bacterial PHB single crystals since both ends of the crystals are similarly splintered. However, the

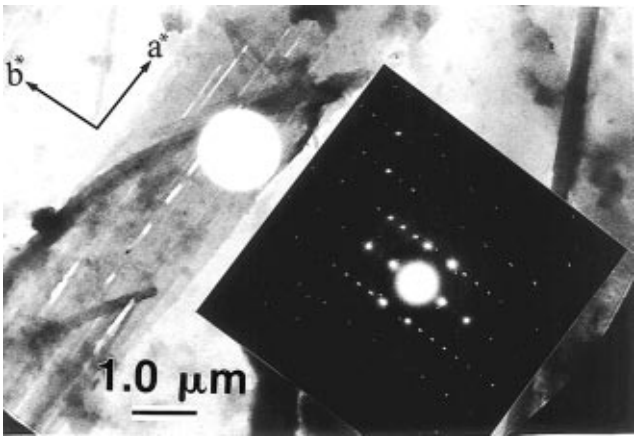

Figure 5. PHB single crystals after 24 h enzymatic attack with PHB-depolymerase A from *P. lemoignei*, showing the slitlike openings in the lamellae. Insert: electron diffractogram with proper orientation to the circled area. As in Figure 2, the unit cell axes *a* and *b* of PHB are along *a** and *b**, respectively.

fragmentation into needlelike entities was unexpected for folded chain lamellar single crystals. Previous studies showed either edge disruption (in the case of xylan[12]) or surface attack (in the case of nigeran[13]).

Figure 4 is noteworthy for showing the distribution in width of the splintered fragments which are created by the enzymatic degradation. It is difficult to avoid the conclusion that the degradation has proceeded topotactically, that is, parallel to the crystallographic *a*-axis which Barham *et al.*[19] concluded corresponds to the direction of chain folding. When the single crystal splinters have widths of 100 nm or less, they are sometimes seen to bend into curved loops, a morphology for PHB first reported by Ellar *et al.*[22] Nevertheless, Figure 5 shows that the crystal splinters retain the single crystal organization of the original material, as demonstrated by the electron diffraction pattern. Furthermore, the electron diffraction spots show asymmetric line broadening along the crystallographic layer line as would happen for diffraction from oriented, narrow lath-shaped crystals.

Slitlike openings in the single crystal are seen in Figure 5 (about 30 unit cells wide) extending parallel to the long axis of the crystal which suggest that topotactic degradation can commence in the middle of the single crystal as well as at the ends. Since it is unlikely that the chain ends are ideally oriented on the surface to promote *exo* attack, this observation may imply that both *endo* and *exo* mechanisms are involved in the initiation according to a "processing mechanism" by a single enzyme.[23] In the field of cellulases, "processing" means that the enzyme forms a complex with the cellulose segment and binds to it throughout the degradation. In a highly processing system such as CBH1 from *Trichoderma*, the enzyme degrades a given chain all the way to its end without desorption from the cellulase cleft. Similarly, for PHB-depolymerases, this would explain why there is no decrease in molecular weight of the polymer during degradation. In less processing systems, there is interruption of the binding of the polymer chain within the enzyme's active site. The concept of "processing" also allows a given enzyme to act, to varying degrees, in an *endo* or *exo* fashion.

The observation of longitudinal splintering suggests that PHB-depolymerase A reacts in a predominantly *exo* mode, behaving like a wedge, along the length of the polymer chain (parallel to the *a*-axis) to produce the

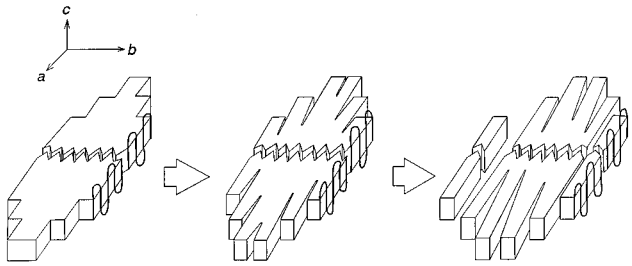

Figure 6. Schematic of PHB lamellar single crystals at various stages of attack by PHB-depolymerase A from *P. lemoignei*. The orthogonal *a*, *b*, and *c* arrows are the unit cell directions for PHB and show that the *a*-axis is parallel to the long axis of the single crystal. The average direction of chain folding is parallel to the *a*-axis. The developing pattern of parallel needles is discussed in the text. For clarity, only the ends of the crystal are shown, with the sawtooth pattern in the center representing the continuum of the crystal.

splinters which are clearly observed in the micrographs (Figures 3 and 4). The wedgelike action implies that the depolymerase degrades stepwise not only along the external crystal edge, as proposed previously,[11] but also along the *de novo* faces of developing splinters. Despite the high crystallinity of the substrate, the depolymerase is able to degrade the crystal in a systematic fashion.

In addition, the observation of splintering is related to an experiment reported by Barham *et al.*[19] who showed that a lamellar single crystal of PHB, when placed on a Mylar film and stretched perpendicular to its long axis, splits longitudinally into a series of near parallel fracture surfaces. When the single crystal is stretched parallel to its long axis, periodic cracks intersecting the long axis develop which are bridged by microfibrils typical of partially extended chains. Since selected area electron diffraction has shown that the crystallographic *a*-axis is parallel to the long direction of the lamellar crystal, it was concluded that the "average direction of chain folding"[24] is parallel to the *a*-axis (as shown in Figure 6). Thus, it seems that PHB-depolymerase A is able to exploit the inherent weakness in the structure of the single crystal, to accomplish the splintering action.

Figure 6 is a schematic of the stages of splintering and breakup of the lamellar single crystal. At the original lamellar edge, the depolymerase acts in an *exo* mode and shaves off complete chains, thus causing degradation with minimum change in degree of polymerization. Near simultaneous action at the steplike ends of the crystal produces new surface edges corresponding to evolving splinters. The V-shaped cuts must have blunt ends since the enzymes have a finite size, e.g., about 3−5 nm.

The visible splintering of the lamellar single crystals is pertinent to the frequently reported[21,25] scanning electron microscopy appearance of depolymerase-etched films of PHB which reveal the spherulitic texture. This degraded texture is no longer lamellar but appears to be a parallel array of needlelike crystals, the origin of which must be the splintering of the radial lamellar ribbons of the spherulite, according to the mechanism proposed in this study. The apparent preference for attack of the depolymerase on the noncrystalline regions is a matter of accessibility governed by the relative lamellar orientation with respect to the film surface. For example, the central region of the spherulites is always more deeply pitted than the rest of the spherulite because the lamellar sheaflike bundles present a more accessible topology to the enzymes. Conceivably, the

endo process may be the more effective mechanism for degradation of this material initially. However, once a small amount of disordered material is removed, the *exo* process predominates for degrading the crystalline lamellae, as proposed previously[11,26,27] and expanded by this TEM observation of single crystal splintering.

This model for enzymatic PHB degradation explains, at the molecular level, the lack of decrease in molecular weight since the enzyme attack is concentrated on only a small fraction of the chain population in a given time interval. However, PHB-depolymerase A, acting as a wedge, creates multiple needlelike surfaces for degradation, as already discussed. This aspect of the crystal "edge attack" mechanism was not anticipated from the results of the molecular weight study.[11]

Although previous studies[11,26,27] used a depolymerase extract from *P. lemoignei*, we used a purified extracellular PHB-depolymerase isolated from this organism from which at least five PHA depolymerases have been identified.[28,29] Thus our mechanistic proposals assume parallel behavior of the two enzyme preparations. Nevertheless these results indicate that PHB-depolymerase A from *P. lemoignei* degrades single crystals of bacterial PHB by a processive attack that does not destroy the overall crystalline order, indicating that the degradation is not accompanied by a swelling step as in dissolution.

By using a direct TEM observation of PHB single crystals degraded by PHB-depolymerase A, this study has shown that an "edge attack" mechanism explains, at the molecular level, the lack of decrease in molecular weight previously observed for enzymatic degradation of PHB single crystals[11] and surface-eroded films.[21,25] The model also explains the conversion of crystalline lamellae into a needlelike morphology which is visible in SEM micrographs of enzymatically degraded PHB spherulitic films.[21,25] Finally, the model integrates both *endo* and *exo* activity into the mechanism of PHB-depolymerase A action on crystalline PHB.

Acknowledgment. The authors wish to thank Zeneca Inc. for the sample of PHB used in this study, Xerox Corp. and Programme d'Échange de France-Québec for financial support, B. Henrissat of CERMAV-CNRS for useful discussions relating to the structure and action of cellulases, and Prof. M. Scandola of Università di Bologna for helpful discussions concerning the surface etching of spherulitic films.

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MA961219U