Enzymatic Degradation of Single Crystals of Bacterial and Synthetic Poly(β-hydroxybutyrate)

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Received September 11, 1995; Revised Manuscript Received December 22, 1995[⊗]

ABSTRACT: Lamellar single crystals of both natural poly[(*R*)-β-hydroxybutyrate], PHB, and synthetic poly[(*R,S*)-β-hydroxybutyrate] of various tacticities were degraded using enzymes isolated from the fungus *Aspergillus fumigatus* and the bacterium *Pseudomonas lemoignei*. Degradation was monitored by both turbidimetric and titrimetric assays. Despite their highly ordered state, single crystals of bacterial PHB were observed to degrade completely; no decrease in molecular weight was observed in the partly degraded polymer. By contrast, the single crystals of synthetic PHB showed only partial degradation, with some decrease in molecular weight; faster and more substantial degradation was observed for the most isotactic material, whereas single crystals from a nearly atactic sample were essentially inert. These results differ from those found for PHB films, implying that tacticity response is linear when crystallinity effects have been normalized. The observed results are consistent with preferential degradation from the crystal edges rather than the chain folds of the lamellar surface and support the hypothesis of a combined *endo*−*exo* degradation mechanism for these two depolymerases.

Introduction

Poly(β-hydroxybutyrate), PHB, is a bacterial polyester of great interest due to its inherent biodegradability.[1,2] Although the natural material contains only repeat units with the *R* configuration [(*R*)-PHB], synthetic PHB containing both *R* and *S* units can be prepared and used to gain additional information about the enzymatic degradation mechanisms. Varying the total stereocomposition of the polymer dramatically affects enzymatic degradation, with the enzymatic preference for (*R*)-PHB evidenced by the fact that PHB with 100% and 96% *S* repeat units was essentially nondegradable;[3,4] at the other extreme, samples with 67−77% *R* content showed higher rates of degradation than the natural polyester, attributed to the combination of adequate *R* content and reduced crystallinity.[4]

Even at constant stereocomposition, as for racemic PHB, wide variation in degradation rates is observed as a function of tacticity. As natural PHB is completely isotactic, PHB depolymerases should be best adapted to degrade isotactic synthetic PHB. However, the increased crystallinity of isotactic PHB relative to atactic PHB is a competing parameter which tends to decrease degradability. Consequently, atactic or slightly isotactic racemic PHB was shown to degrade more rapidly than highly isotactic polymer of the same overall stereocomposition, using depolymerases isolated from *Alcaligenes faecalis*,[3,5] *Pseudomonas lemoignei*,[6−8] or *Aspergillus fumigatus*;[6−8] in all cases, the synthetic polymer degraded more slowly than natural PHB due to the stereochemical effects of only 50% *R* content. Tacticity effects are amplified when both isotacticity and total *R* content increase together; samples ranging from 68% to 92% isotactic diads with 80−96% total *R* content were all degraded more rapidly than natural PHB by both *Pseudomonas pickettii*[9] and *A. faecalis*[9] depolymerases. Syndiotactic PHB, having both unfavorable tacticity and moderately high crystallinity, was not appreciably degraded by *P. pickettii*,[9] *A. faecalis*,[9] *P. lemoignei*,[6−8] or *A. fumigatus*[6−8] depolymerases.

In all the above studies, variations in degradability have resulted from the combination of two or more effects, including crystallinity, tacticity, and stereocomposition. To study the effect of tacticity alone would require that the experiments be conducted under conditions where the crystallinity is held constant for materials of the same stereocomposition but different tacticities. Because isotactic PHB crystallizes readily at temperatures where the enzymes are active,[10] it would be difficult to maintain low crystallinity in these samples throughout a degradation study; further complications arise from the large difference in the crystallization rates for PHB of different tacticities. However, the crystallinity of the less isotactic samples can be increased, for instance, by dilute solution precipitation to yield lamellar single crystals.[11] Single crystals of bacterial PHB are well known;[12−23] similar single crystals can also be prepared from isotactic and atactic synthetic PHB.[24] These single crystals, of both natural and synthetic PHB, formed the substrate for the enzymatic degradation discussed in this paper.

Since single crystals of linear polymers have a unique folded chain lamellar structure, the study of their enzymatic degradation is a new and complementary field of investigation, relative to that of the partially crystalline substrates previously discussed. Research of this sort has been reported in the polysaccharide literature. For example, single crystals of xylan were enzymatically degraded by *endo*-xylanase from *Poria* species and monitored by electron microscopy;[25] the sequence of visual changes and persistence of single-crystal diffraction were attributed to *endo* attack from the edge of the crystal. Single crystals of nigeran[26] were only partly degraded by the *endo*-glucanase mycodextranase, as observed by turbidity decrease; this was again attributed to *endo* attack but on the chain folds

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

0024-9297/96/2229-2472$12.00/0

Table 1. Solvent Compositions and Crystallization Temperatures for Single-Crystal Preparation

sample	EtOH:$CHCl_3$	T_c (°C)
bacterial	2:1	60
isotactic	2.5:1	60
atactic	3:1	40
syndiotactic	5:1	40

only, leaving impenetrable clusters of chain lamellae. Enzymatic degradation of single crystals of PHB has not previously been reported.

The purpose of this paper is 2-fold: (1) to probe the enzymatic degradation of single crystals of bacterial PHB and (2) to compare the enzymatic degradation of single crystals for synthetic racemic PHB of varying tacticities in order to elucidate the implications of tacticity on the enzymatic degradation reaction mechanism. As with a previous study involving solvent-cast films of synthetic racemic PHB,[6−8] the extracellular PHB depolymerase systems of the bacterium *P. lemoignei* and the fungus *A. fumigatus* were used.

Experimental Section

Synthetic PHB Samples. Synthetic polymer samples were obtained by bulk ring-opening polymerization of racemic β-butyrolactone with a preformed methylaluminoxane catalyst, using vacuum techniques. Details of the polymerization, fractionation, and characterization have been previously published.[27]

Bacterial PHB Samples. Bacterial PHB was obtained from Marlborough Biopolymers (Billingham, U.K.) and purified by reprecipitation from *N*,*N*-dimethylformamide/diethyl ether. Additional bacterial PHB was purchased from Aldrich Chemical Co. and used as received. The reprecipitated PHB was used for the single-crystal preparation described in the following section; in addition, reference suspensions of bacterial PHB from both sources were prepared by dispersion in distilled water through sonication. As little difference was observed in the degradation of these reference suspensions, all such suspensions containing bacterial PHB from either source which was not specifically prepared as single crystals are hereafter referred to as PHB reference suspensions.

Single-Crystal Preparation. Single crystals were grown by a method modified from that of Marchessault *et al.*[14] A measured amount (15−30 mg) of polymer was dissolved in hot 1:1 ethanol/chloroform, to which was added warmed ethanol to give an ethanol/chloroform ratio ranging from 2:1 to 5:1, at a fixed polymer concentration of 0.025% w/v. The solvent ratios and crystallization temperatures (T_c's) used for each sample are given in Table 1. The solutions were held at T_c for 7−24 h, slowly cooled to room temperature, and then reheated back to 60−80 °C to encourage self-seeding; crystallization and cooling were then repeated in a similar manner. Crystals were isolated by centrifugation, washed twice with fresh ethanol, and transferred to distilled water, and residual ethanol was removed by rotary evaporation. As the crystals tended to aggregate in water, the suspensions were then sonicated for several hours at 0 °C in an ultrasonic cleaning bath to break up the aggregates. Such crystals from aqueous suspensions retained the morphology and electron diffraction patterns previously observed from organic solvent.[24] Suspension concentrations were determined by weighing the dry solid content of a measured volume of solution, after evaporating to dryness under vacuum.

Depolymerase Preparation. Depolymerase solutions were obtained by growing 1-L cultures of *P. lemoignei* or *A. fumigatus* M2A until depolymerase activity reached a maximum, as determined by the rate of decrease in turbidity exhibited by a suspension of natural PHB granules after mixing with a sample of the supernatant fluid. Once maximum activity was observed, the cultures were centrifuged and/or filtered to remove cells, and then concentrated using ultrafiltration. The *P. lemoignei* depolymerase was purified using variations on published protocols;[28−30] fraction A was used for the following studies. The purification scheme for the *A. fumigatus* depolymerase is about to be published.[31] The purified enzymes were used in 1 mM, pH 8 buffer (Tris-HCl for *P. lemoignei* depolymerase and tricine for *A. fumigatus* depolymerase), at the same temperature as the initial culture had been grown (30 °C for *P. lemoignei* depolymerase and 45 °C for *A. fumigatus* depolymerase).

Table 2. Characteristics of the PHB Samples Used To Prepare Single Crystals

sample	isotactic diads (%)	M_w (g/mol)	T_g (°C)	T_m (°C)	ΔH_f (J/g)
bacterial	100	500 000	4	173	85
isotactic	88	190 000	5	164	65
atactic	60	65 000	8	98	6
syndiotactic	34	2700	4	84	37

Turbidimetric Assay. Degradation of the polymer samples was monitored using both turbidimetric and titrimetric assays, similarly to previous studies.[30,32,33] In the turbidimetric assay,[28,34,35] a measured quantity (1−20 μL) of enzyme solution was dispersed in 1 mL of buffered (pH 8) PHB suspension in a transparent plastic cuvette and then incubated at 30 or 45 °C. The initial optical density at 660 nm (OD_{660}) of such suspensions varied with the initial concentration and particle size of the suspension and ranged from 0.1 to 1.4. As the PHB was degraded by the enzyme, the size of the particles in the suspension, and thus the turbidity, decreased.[36] Initial degradation rates were determined by measuring the slope of the initial linear segment of a plot of OD_{660} versus time (see Figures 2 and 5). This method was also used to define the activities of the two depolymerases, where one unit of activity (U) decreased the turbidity of a 300 μg/mL suspension of unmodified bacterial PHB by 0.001 OD/min under test conditions.

To determine suitable conditions for the degradation experiments, preliminary turbidimetric assays were performed with numerous concentrations of *A. fumigatus* depolymerase and of the various PHB substrates. The goal was to identify conditions of "substrate saturation", where the ratio of substrate surface area to enzyme concentration is so high that variations in the substrate surface area do not affect the degradation rate.[37] It was found that for any of these substrates, the rate of degradation by 2.5 U/mL *A. fumigatus* depolymerase was constant for substrate concentrations in the range of 0.2−1.1 mg/mL. Therefore, direct comparison of the degradation rates of the different substrates within this concentration range was justified, despite the differences in specific surface area of the substrates. Because precise determination of the suitable conditions was not repeated for the *P. lemoignei* depolymerase, the concentration of this depolymerase was reduced to 1.5 U/mL.

Titrimetric Assay. The titrimetric method[30,32,33,38] was used to complement the turbidimetric results by determining the rate at which organic acid degradation products were produced by the degrading polymer. For this assay, a measured quantity (5−20 μL) of purified enzyme solution was added to 1.0 mL of stirred, unbuffered PHB suspension at 30 or 45 °C, and the pH was adjusted to 8.00 using 10 mM NaOH. The free acid released by the PHB degradation was then monitored as a function of time by measuring the rate of NaOH addition required to maintain pH 8.00. The close relationship between titrimetric and turbidimetric assays has been established using depolymerase from *A. faecalis*.[32]

Results and Discussion

Characteristics of the Polymer Samples and Single Crystals. The characteristics of the polymer samples used in this study are as shown in Table 2. These samples represent a subset of those previously used for a study on the enzymatic degradation of PHB films.[6−8] For this study, one highly isotactic, one slightly isotactic/atactic, and one syndiotactic sample were selected. The synthetic isotactic and atactic PHB both formed single crystals of the same structure as bacterial PHB but with decreasing perfection in the material of lower tacticity.[24] A schematic of the "adja-

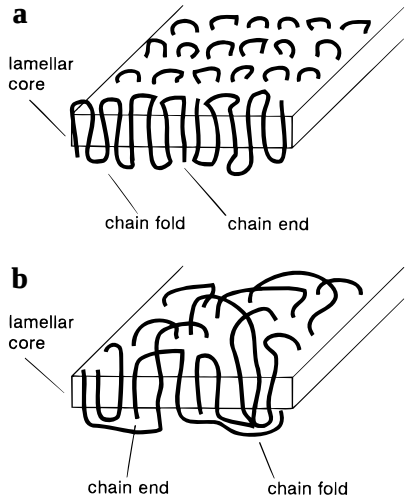

Figure 1. Schematic diagram of the chain fold structure of a polymer single crystal with (a) adjacent re-entry and (b) switchboard re-entry of the polymer chains. The lamellar core, chain folds, and chain ends are indicated.

cent re-entry" model for folded chain structure in polymer single crystals is shown in Figure 1a. Because a high degree of stereoregularity is normally required for the formation of polymer single crystals, it is probable that the more regular parts of the synthetic PHB chains are located in the lamellar core, with the less regular parts of these chains being excluded to the chain folds or lamellar surface.[16,24] The decreased regularity of the chain structure in the less stereoregular samples means that the fold structure existing in single crystals made from these samples is likely to be less regular, as in the "switchboard" model shown in Figure 1b. Syndiotactic PHB did not form syndiotactic single crystals but a precipitate with two morphologies:[24] one similar to the isotactic lamellae and the other in the form of hedrites, presumed to be more syndiotactic.

Degradation by *A. fumigatus* Depolymerase. Turbidimetric degradation profiles for bacterial PHB reference suspensions and bacterial PHB single crystals under saturation conditions are shown in Figure 2a. Initial absorbances differ due to the differing concentrations and particle sizes of the two samples, but initial slopes still show the degradation rate of the single crystals to be approximately one-half that of the reference suspension. The turbidity of the reference suspension decreased almost linearly until most of the PHB had been consumed and then tapered off near zero, as previously described for the degradation of a similar suspension by *P. lemoignei* depolymerase.[39] The single-crystal degradation proceeded in an almost identical fashion; again, the turbidity decreased to near zero.

Degradation profiles for the synthetic single-crystal samples are shown in Figure 2b. In this case, degradation of all samples ceased well before completion. When the initial concentration of the single crystals was changed, the height of this plateau on the OD_{660} scale shifted to a comparable proportion of the initial OD, indicating that a reproducible fraction of the initial concentration was resistant to degradation. Initial degradation rates followed the same order as did the extents of degradation: The isotactic sample showed both the fastest initial degradation rate and the greatest total extent of degradation, the syndiotactic sample was intermediate in both degradation rate and extent of degradation, and the atactic sample showed virtually no degradation. This order is completely different from that observed for solvent-cast films of synthetic racemic

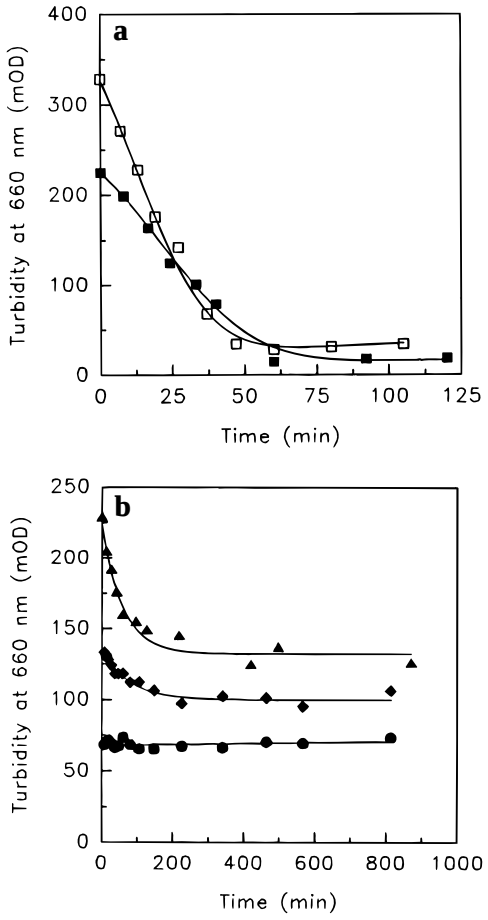

Figure 2. Decrease in turbidity with time for (a) bacterial PHB and (b) synthetic racemic PHB, degraded by 2.5 U/mL *A. fumigatus* PHB depolymerase at 45 °C: (□) bacterial PHB reference suspension (0.19 mg/mL), (■) bacterial single crystals (0.31 mg/mL), (▲) single crystals of synthetic isotactic PHB (0.38 mg/mL), (◆) syndiotactic "single crystals" (0.18 mg/mL), and (●) atactic single crystals (0.28 mg/mL).

PHB,[6–8] where atactic samples degraded essentially to completion and at the fastest rate, isotactic samples degraded significantly more slowly, and syndiotactic samples were the slowest to degrade.

Results from a titrimetric assay using *A. fumigatus* depolymerase (Figure 3) show similar trends; as the assay was performed at a higher enzyme concentration, substrate-saturation conditions could not be assured and surface area effects were likely to be observed. Therefore, the single crystals of bacterial PHB degraded at a faster rate than did the reference suspension. The plateaus in the curves for the bacterial PHB samples after 30−60 min corresponded to the visible clearing of the suspension, with the differences in the heights of these plateaus reflecting the different initial polymer concentrations. Because variations in specific surface area were much less significant among the single crystals of various tacticities than between any of these and the PHB reference suspension, the relative degradation rates for the single crystals were not changed by the increase in enzyme concentration. Thus, as by the turbidity assay, the isotactic single crystals showed a rate of degradation lower than that of bacterial PHB; the isotactic single-crystal degradation then slowed dramatically with significant turbidity remaining. At the higher enzyme concentration, the atactic single crystals showed observable degradation, though still at a much slower rate than for the isotactic crystals.

Degradation by *P. lemoignei* Depolymerase. Results of a titrimetric assay by *P. lemoignei* depolymerase

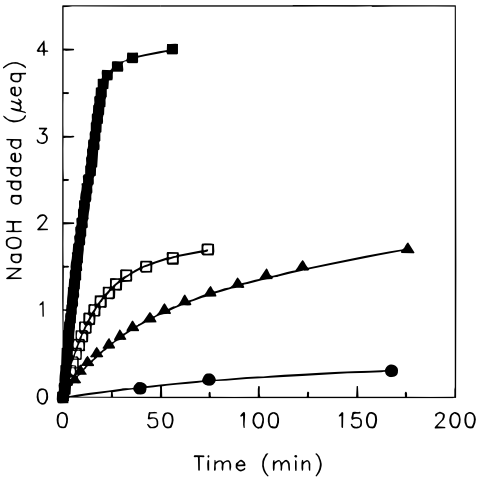

Figure 3. Addition of NaOH as a function of time during degradation with 10 U/mL *A. fumigatus* PHB depolymerase at 45 °C: (□) bacterial PHB reference suspension (0.30 mg/mL), (■) bacterial single crystals (0.62 mg/mL), (▲) isotactic single crystals (0.76 mg/mL), and (●) atactic single crystals (0.44 mg/mL).

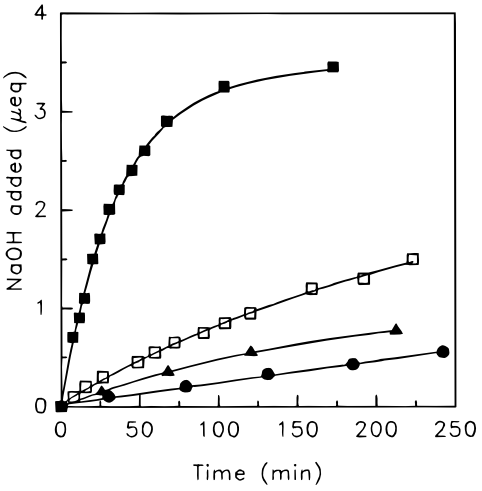

Figure 4. Addition of NaOH as a function of time during degradation with 1.5 U/mL *P. lemoignei* PHB depolymerase at 30 °C: (□) bacterial PHB reference suspension (0.30 mg/mL), (■) bacterial single crystals (0.62 mg/mL), (▲) isotactic single crystals (0.52 mg/mL), and (●) atactic single crystals (0.28 mg/mL).

(Figure 4) are similar to those for *A. fumigatus*: The bacterial single crystals degraded the most quickly followed by the reference suspension and isotactic single crystals, with the atactic single crystals showing the slowest degradation. The same order of degradation rates was observed by turbidimetric assay (Figure 5). As the level of *P. lemoignei* enzyme was reduced relative to that used for *A. fumigatus* depolymerase, degradation rates were decreased such that syndiotactic as well as atactic material showed minimal turbidity decrease. Despite the decrease in *P. lemoignei* enzyme concentration, the single crystals of bacterial PHB showed faster degradation than the reference suspension, which may indicate a substantial difference in the saturation behavior of these two enzymes. Simultaneous determination of the degradation curves by both titrimetry and turbidity indicated that both reached a plateau at about the same time for a given sample, thus confirming the correlation between the two techniques and revealing that further degradation to create new acid end groups did not continue beyond the point when turbidity ceased to fall.

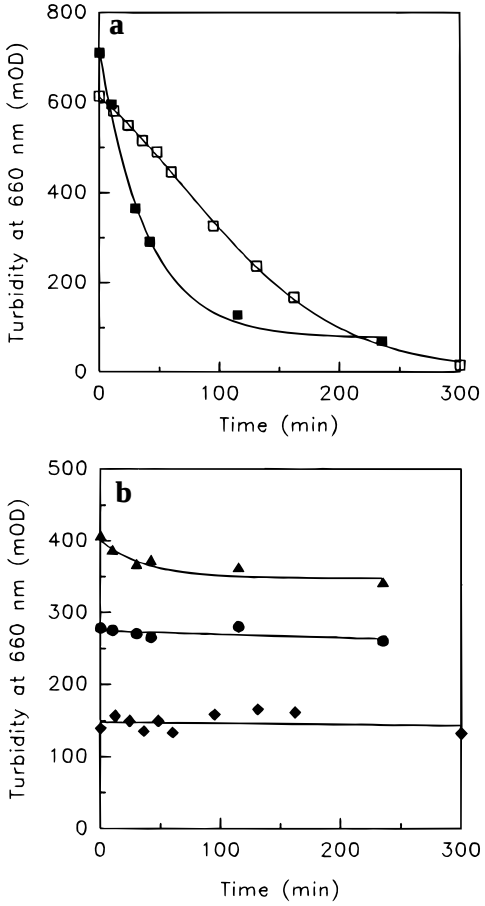

Figure 5. Decrease in turbidity with time for (a) bacterial PHB and (b) synthetic racemic PHB, degraded by 1.5 U/mL *P. lemoignei* PHB depolymerase at 30 °C: (□) bacterial PHB reference suspension (0.15 mg/mL), (■) bacterial single crystals (0.32 mg/mL), (▲) isotactic single crystals (0.32 mg/mL), (◆) syndiotactic "single crystals" (0.18 mg/mL), and (●) atactic single crystals (0.44 mg/mL).

Molecular Weight Changes. The mode of degradation in the single crystals can be examined in more detail by observing the changes in molecular weight distribution during degradation. Partial degradation at the chain fold surface has been observed to cause a reduction in molecular weights, whether by enzymatic[26] or chemical[18,21] means. To determine how the molecular weights of these samples were affected by the enzymatic degradation, each single-crystal sample was examined by gel permeation chromatography (GPC) both before and after degradation by 1.5 U/mL *P. lemoignei* depolymerase. For the synthetic PHB samples, where a clear nonzero plateau was observed in the degradation curve, the "after" GPC trace was obtained for a sample degraded until this plateau was established; for the bacterial PHB single crystals, where no such plateau was observed, the "after" trace was determined on a sample degraded until the turbidity had dropped to 30% of its initial value.

Results for the molecular weight determinations are shown in Figure 6. For the single crystals of bacterial PHB, the molecular weight distribution may narrow slightly, but no observable molecular weight change occurred. As the degradation of this material was shown by turbidity and titrimetry to proceed easily to completion, the lack of molecular weight change is consistent with the idea that the lamellar structure provided little hindrance to this degradation. The isotactic and syndiotactic materials both showed a slight decrease in molecular weight with partial degradation.

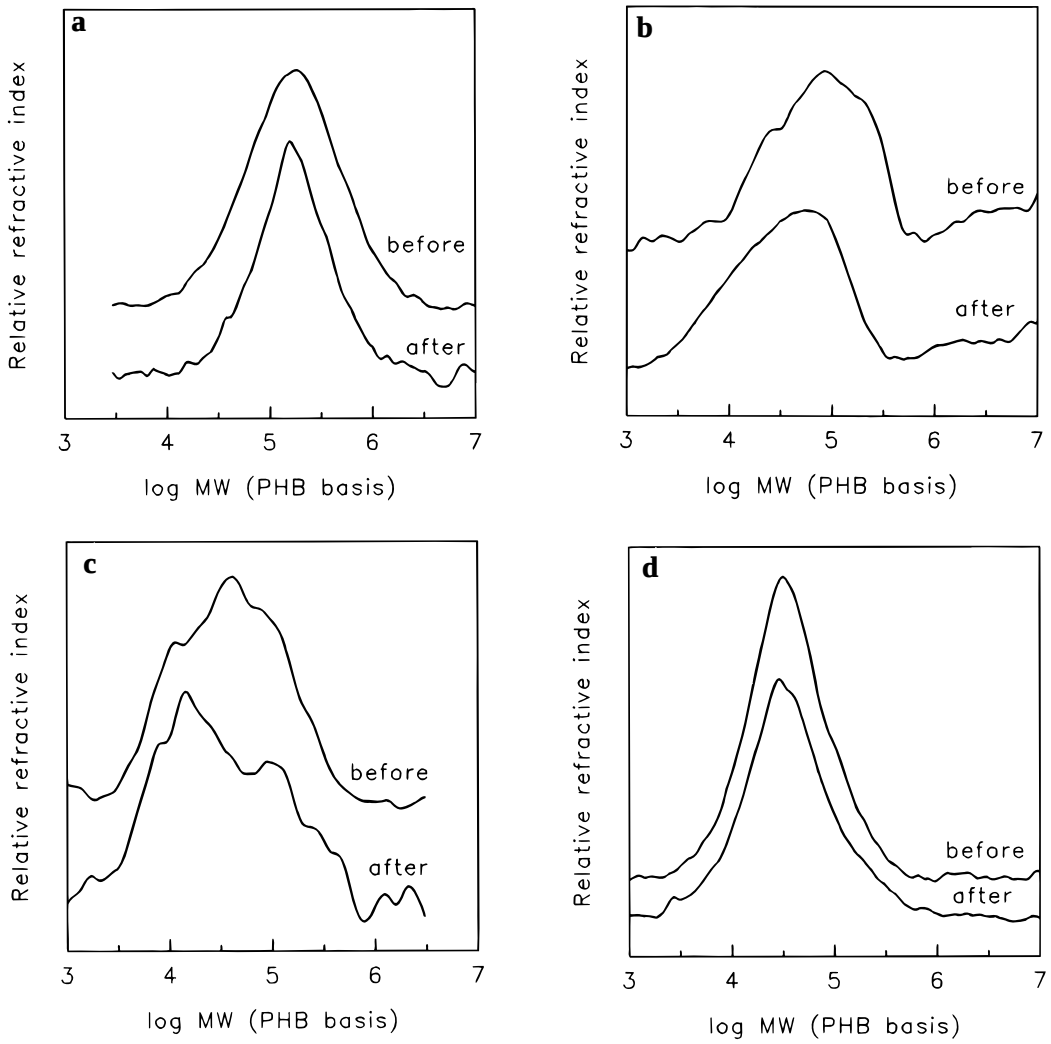

Figure 6. Gel permeation chromatograms of PHB single crystals before and after partial degradation by *P. lemoignei* PHB depolymerase: (a) single crystals of bacterial PHB, (b) single crystals of synthetic isotactic PHB, (c) syndiotactic "single crystals", and (d) atactic single crystals.

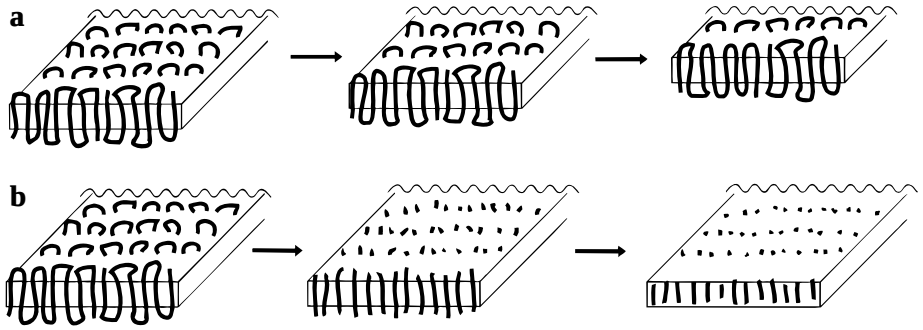

Figure 7. Schematic representation of the effects on single-crystal morphology and polymer molecular weight during degradation by attack from (a) the crystal edge and (b) the chain folds.

No molecular weight change was noticed for the atactic single crystals; the very small extent of degradation of this material observed by titrimetry or turbidity was presumably insufficient to cause observable molecular weight changes. Further interpretation of these results requires more detailed examination of the effects of different modes of enzymatic attack, as discussed below.

Site of Attack on the Single Crystals. Two possible sites of enzymatic attack on single crystals can be envisioned, as shown in Figure 7. For degradation from the crystal edge (Figure 7a), the crystal size is systematically reduced in the lamellar plane, progressing from each crystal edge toward the crystal center. Such a progression has been observed in the enzymatic degradation of xylan single crystals by an *endo*-xylanase isolated from *Poria* species.[25] This mode of attack would also be accessible to *exo* enzymes, which could successively degrade individual chains along the crystal edges, beginning from accessible chain ends at the crystal edge. As only a small proportion of the total number of polymer chains in the crystal is involved in the degradation at any one time, little overall change in the sample molecular weight would be expected throughout most of the degradation.

In contrast, degradation at the chain folds (Figure 7b) relies on *endo*-chain scission: Attack is initiated at the chain folds and then proceeds perpendicular to the lamellar plane until further penetration is halted by the

dense lamellar core. This mechanism was proposed for the degradation of single crystals of nigeran by the *endo*-glucanase mycodextranase;[26] the proportion of the lamellar core which was accessible to the enzyme increased with increasing temperature, presumably due to increasing disorder at the crystal surface as the aqueous dissolution temperature of the crystal was approached. In the mycodextranase study, the base area of the crystals, as observed by electron microscopy, did not significantly change throughout the degradation; however, a sharp molecular weight decrease was observed, corresponding to the simultaneous *endo* scission of virtually every polymer chain.

In the current study, the lack of a molecular weight decrease in the partly degraded single crystals of bacterial PHB suggests that the primary direction of enzymatic attack was from the crystal edge. The complete degradation of this material at an almost constant rate is consistent with this hypothesis, as attack from this direction should not be particularly hindered or prevented by the existence of the lamellar core. The incomplete degradation and slight shift in molecular weight observed for the synthetic PHB single crystals can be rationalized by assuming that the less regular chains of the racemic polymer form single crystals more in accordance with the switchboard model, whereas single crystals of the completely isotactic bacterial polymer contain more adjacent re-entry. The degradation of a polymer chain along the edge of the racemic PHB single crystal would thus be halted when this edge chain folded into a less accessible location in the lamellar core. Degradation of a second edge chain could then commence, but because the polymer is racemic, the point will be reached when all accessible edge chains contain too many nondegradable *S* units to permit enzymatic attack. The degradation will then be halted, with the partial degradation of a number of chains causing a slight drop in the overall molecular weight.

Degradation Mechanisms. Results from the turbidimetric and titrimetric observation of degradation, plus the lack of molecular weight changes, all indicate that single crystals of bacterial PHB are completely accessible to the extracellular depolymerase enzymes of *A. fumigatus* and *P. lemoignei*. The lack of molecular weight change shows an apparent preference toward complete degradation of whole chains, implying that the degradation mechanism involves mainly *exo* attack. However, since *exo* enzymes cleave polymers with directionality from one chain end only,[40] *endo* attack is likely involved in the initiation of the degradation, as it is unlikely that all chains would be conveniently oriented with the most degradable end located on a crystal edge. Once chain ends have been created or exposed, faster *exo* scission would account for the complete degradation of the initial chain prior to further *endo* attack of many more chains. Thus, the combined mechanism of both *endo*- and *exo*-chain scission previously observed[6−8,28−30,41] for both these enzymes appears to fit the single-crystal degradation as well.

The observation of both *endo* and *exo* activity in a single enzyme is not unique to PHB depolymerases. In a recent theoretical treatise classifying enzymatic degradation pathways, *endo* and *exo* were considered as merely representing limiting extremes of behavior along a purported continuum of possible attack pathways;[40,42,43] on this framework, the degradation of PHB by *A. fumigatus* or *P. lemoignei* would appear to have an intermediate position. An alternative classification proposed for glycosyl hydrolases[44] categorizes these enzymes by topology of the active site and replaces the *exo*/*endo* classification by the concept of processivity. According to this model, "*exo*" activity (processivity) is observed when an enzyme with a tunnel-shaped active site progresses along a single polymer chain; an "*endo*" component is added when the enzyme loops creating the tunnel periodically shift to allow random binding followed by progressive action. Such a process could also explain the degradation of PHB by *A. fumigatus* or *P. lemoignei*; examination of the topology of the enzyme active sites should provide more conclusive evidence for this model.

Additional effects were observed in the degradation of the synthetic racemic single crystals, due to the added variable of tacticity. For synthetic racemic PHB films, the atactic material degraded much more rapidly than the isotactic and syndiotactic material due to the combined effects of crystallinity and tacticity;[5−8] by contrast, in single-crystal morphology, the isotactic material degraded much more rapidly than the atactic material, with the syndiotactic "crystals" in between. Assuming that the difference in crystallinity between isotactic and atactic single crystals is smaller than the crystallinity difference between isotactic and atactic films, the relative degradation rates observed in this study give a closer indication of the effect of tacticity alone. Since the syndiotactic "crystals" were shown[24] by TEM not to be true single crystals, but rather a combination of isotactic lamellae and presumed syndiotactic hedrites, it is not fair to compare their degradation to that of isotactic or atactic single crystals of racemic PHB. The intermediate degradation of the syndiotactic material can be assumed to result from the degradation of the lamellar portion only, which, due to segregation, could well be more isotactic than the atactic lamellae.

A schematic model of enzymatic degradation on the surface of microbial and synthetic PHB films[3] proposed that isotactic racemic PHB is only partly degraded before the formation of a surface layer of inactive *S* units while atactic racemic PHB can be completely degraded, presumably as the enzymes tunnel through the amorphous regions. In the case of the single-crystal degradation, the crystalline lamellae contain no amorphous regions for the enzymes to tunnel through, so none of the synthetic samples could be completely degraded. As the isotactic PHB contained longer blocks of both *R* and *S* units,[45] it is more likely that measurable degradation could have occurred before a given crystal was converted to either an undegradable (*S*)-PHB lamellar core or an impenetrable shell of *S* sequences surrounding an otherwise degradable (*R*)-PHB core. Thus, more degradation was observed in these crystals than in the atactic crystals.

Conclusions

This study has shown complete enzymatic degradation of single crystals of bacterial PHB, indicating that the lamellar structure could be penetrated by the *A. fumigatus* and *P. lemoignei* enzymes. The GPC trace of partly degraded bacterial PHB single crystals was virtually indistinguishable from that of the starting polymer, confirming the accessibility of the full length of the polymer chains and implying that the single crystals were attacked from the crystal edge, rather than from the fold surface.

By contrast, single crystals of synthetic racemic PHB were limited to only partial degradation, despite the absence of such a limit in the degradation of solvent-cast films of the same material. The GPC traces of

polymer degraded to the plateau point show a decrease in molecular weight relative to undegraded material, suggesting that the hindrance to complete degradation was caused by a lack of total penetration into the highly ordered lamellar structure. As these synthetic polymers contained 50% undegradable *S* units, the partly degraded crystals can be envisaged as a mixture of undegradable *S* lamellae and crystalline surfaces coated with *S* units, which block entry into an otherwise degradable *R*-lamellar structure.

The relative rates of enzymatic degradation of the synthetic single crystals of varying tacticity differed from those observed in films, due to the minimization of crystallinity differences. Thus, although the slightly isotactic/atactic films degraded most rapidly in the same enzyme systems due to low crystallinity, when crystallinity differences were minimized by single-crystal formation, the enzymatic preference for more isotactic material was clearly revealed. Because the syndiotactic "crystals" used in this study actually contained an isotactic component, they showed an intermediate rate of degradation.

The complete degradation of the bacterial single crystals as well as the partial degradation of the synthetic racemic crystals shows aspects of both *endo*- and *exo*-chain scission. Previous studies[6−8] have suggested that both *endo* and *exo* mechanisms could be involved; this study provides further evidence that the PHB enzymatic degradation process occurs by a combination of slower *endo*- and faster *exo*-chain scissions.

Acknowledgment. Thomas M. Scherer is gratefully acknowledged for providing the *A. fumigatus* depolymerase used in this study. This work was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC), the National Science Foundation of the USA (NSF), and Xerox Corp. P.J.H. thanks NSERC for scholarship support.

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MA951361F