Further Evidence of Crystallinity-Induced Biodegradation of Synthetic Atactic Poly(3-hydroxybutyrate) by PHB-Depolymerase A from *Pseudomonas lemoignei*. Blends of Atactic Poly(3-hydroxybutyrate) with Crystalline Polyesters

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Received July 15, 1998; Revised Manuscript Received September 24, 1998

ABSTRACT: Blends of atactic poly[(*R*,*S*)-3-hydroxybutyrate], a-PHB, with poly(ϵ-caprolactone), PCL, and with poly(L-lactic acid), PLLA, were obtained in the form of compression-molded films. The phase behavior of the blends was different: a-PHB and PCL were totally immiscible, whereas a-PHB/PLLA blends were miscible over the whole composition range. Biodegradation experiments were carried out on the blends and on the plain polymers in a buffered solution of PHB-depolymerase A from *Pseudomonas lemoignei* (Tris−HCl, pH = 8, T = 37 °C). None of the pure blend components (a-PHB, PCL, PLLA) showed any weight loss upon enzyme exposure. Conversely, both a-PHB/PCL and a-PHB/PLLA blends biodegraded. Analysis of the biodegradation products and of blend composition changes during biodegradation demonstrated that in both blends only the a-PHB component undergoes enzymatic hydrolysis. The results support the hypothesis that the crystalline polyester blended with a-PHB promotes a-PHB enzymatic hydrolysis by providing stable binding sites to the enzyme. The dependence of biodegradation rate on blend composition is different in (immiscible) a-PHB/PCL and (miscible) a-PHB/PLLA blends and can be explained in terms of different phase behavior and morphology.

Introduction

In earlier studies it was demonstrated that synthetic atactic high molecular weight poly[(*R*,*S*)-3-hydroxybutyrate], a-PHB, prepared using supramolecular complexes of alkali metal alkoxides,[1,2] does not biodegrade in the pure state[3,4] but undergoes enzymatic attack in the presence of a second crystalline component. Such a crystalline polymer was either a natural poly-3-hydroxyalkanoate, PHA, blended with a-PHB,[3] or a polypivalolactone block in poly[(*R*,*S*)-3-butyrolactone-*b*-pivalolactone] copolymers.[4] It was shown that hydrolysis of a-PHB by PHB-depolymerase A from *Pseudomonas lemoignei* occurred both when the crystalline component was itself susceptible to enzymatic attack (bacterial PHA) and when, conversely, it was intrinsically nonbiodegradable (polypivalolactone).

On the basis of biodegradation results on blends of natural poly(3-hydroxybutyrate), PHB, with synthetic atactic PHB using PHB-depolymerase from *Pseudomonas pickettii*, Abe et al.[5] first suggested that enzymatic hydrolysis of synthetic a-PHB, which is "rubbery" at room temperature, might require the presence of stable enzyme binding sites (provided by the crystals of bacterial PHB in their blends). The present authors confirmed this hypothesis[3] and showed that in order to induce hydrolysis of atactic PHB the required crystalline phase might well be provided by a nonbiodegradable polyester.[4]

An important point that still needs to be clarified is whether there are any specific structural requirements that the crystalline phase of the "supporting polymer" must meet in order to promote enzyme binding. Recently, Kasuya et al.[6] found that PHB-depolymerase from *Alcaligenes faecalis* was able to bind to an aliphatic polyester surface even when it was incapable of hydrolyzing the polymer chain, as in the case of poly(ϵ-caprolactone). Consequently, they suggested that while the active site of PHB-depolymerase from *A. faecalis* was specific for the hydrolysis of selected poly(3-hydroxyalkanoate)s, the binding site was nonspecific.

The aim of this paper is to contribute to the understanding of the enzymatic hydrolysis of synthetic atactic PHB and in particular of the "inducing role" played by a crystalline polymer blended with it. In this study two linear polyesters were selected as the second blend component: poly(L-lactic acid), PLLA, and poly(ϵ-caprolactone), PCL. Both synthetic polyesters have been previously used by other authors[7−10] in blends with natural crystalline PHB.

According to earlier investigations, neither PCL nor PLLA can be hydrolyzed by PHB-depolymerases: PCL does not biodegrade in the presence of PHB-depolymerase from *A. faecalis*,[6,7,11] *Comamonas* sp.,[12] *P. lemoignei*,[12] and *Pseudomonas pickettii*,[13] neither did PLLA using PHB-depolymerase from *Comamonas* sp.,[12] *P. lemoignei*,[12] *Pseudomonas fluorescens*,[12] and *A. faecalis*.[6,14] Consequently, when blended with a-PHB, both PLLA and PCL are expected to behave as non-hydrolyzable supports for the enzyme (PHB-depolymerase A from *P. lemoignei*), allowing comparison of the results of this work with those previously obtained on block copolymers of a-PHB and polypivalolactone.[4]

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10.1021/ma981115e CCC: $15.00
Published on Web 11/07/1998

Experimental Part

Materials. Poly[(*R*,*S*)-3-hydroxybutyrate)] (a-PHB; M_n = 31 000; M_w/M_n = 1.1), synthesized as described previously,[1,2] was found to be atactic and amorphous, as revealed by NMR spectroscopy[15] and DSC analysis, respectively. Poly(L-lactic acid) (PLLA; M_v = 140 000) was kindly supplied by Istituto Donegani (Novara, Italy). Poly(ϵ-caprolactone) (PCL; M_n = 105 000; M_w/M_n = 1.6) was a Union Carbide product.

Blend Preparation. The two blend components in appropriate weight ratios spanning the whole composition range were solubilized in CH_2Cl_2 (5% w/v). The solution was cast on polypropylene plates, and solvent was evaporated at room temperature. All obtained films were placed in an oven at 50 °C under vacuum overnight to minimize residual solvent. The blends were subsequently compression molded between Teflon plates with an appropriate spacer for 1 min under a pressure of 0.3 Ton/m^2, using a Carver C12 laboratory press. For a-PHB/PLLA blends the press was kept at a temperature between 100 and 190 °C depending on blend composition (higher temperature with increasing PLLA content). The press temperature was 95 °C for all a-PHB/PCL blends.

Films of the plain polymers were also prepared. PCL and PLLA were directly compression molded from the original powder material in the same conditions as the respective blends with a-PHB (see above). Since a-PHB does not yield self-supporting films, the polymer was solvent cast on a thin polypropylene sheet as earlier described[3] and the supported a-PHB film obtained was used for biodegradation experiments (in this case only one side of the film was exposed to the enzymatic solution). The thickness of the compression-molded blends and pure polymers was 0.17−0.21 mm. All samples were aged for 3 weeks at room temperature before use.

Experimental Techniques. ^{1}H NMR spectra were recorded by using a Varian Gemini 300 MHz spectrometer in $CDCl_3$. Electrospray ionization mass spectrometry (ESI-MS) was carried out with a Finningan TSQ 700 spectrometer. The samples diluted (1:1000) in methanol were introduced into the electrospray source at 10 μL/min using the syringe pump of the instrument. The ESI source was operated at 5 kV, and the capillary heater was set at 220 °C. The experiments were performed in the positive-ion mode. Differential scanning calorimetry (DSC) was performed with a 2010 TA thermal analyzer at a heating rate of 20 °C/min, in the temperature range from −80 to +220 °C (a-PHB/PLLA) and from −120 to +120 °C (a-PHB/PCL). The temperature scale was calibrated with high-purity standards over the whole temperature range explored. The melting temperature (T_m) was taken as the peak temperature of the melting endotherm, and the glass transition temperature (T_g) was taken as the midpoint of the stepwise increase of the specific heat associated with the transition. Isothermal crystallization experiments were performed using a Zeiss Axioscop optical polarizing microscope, equipped with a Linkham TH600 hot stage. The samples were melted in the hot stage and rapidly quenched (cooling rate >250 °C/min) to the crystallization temperature where they were allowed to crystallize isothermally.

Enzymatic Degradation. Film samples (12 × 8 mm^2; initial weight 15−25 mg) were incubated in duplicate at 37 ± 0.1 °C in separate vials containing 1.0 mL of 50 mM Tris−HCl buffer, pH = 8.0, in the presence of PHB-depolymerase A from *P. lemoignei* (1 mM $CaCl_2$ as enzyme stabilizer) isolated and purified as previously described.[16] The enzyme concentration used (C_e = 4.2 μg/mL) was within the C_e range yielding the maximum enzymatic hydrolysis rate ("plateau" value) with isotactic natural PHB.[16,17] Films were removed from the enzymatic solution after 6 h, washed with distilled water, and dried under vacuum over P_2O_5 at room temperature to constant weight (Sartorius RC210D electronic balance; reproducibility ±0.02 mg). This procedure was repeated using fresh enzymatic solution for each incubation interval.

The extent of biodegradation was quantified as weight loss divided by initial sample surface area ($\Delta m/S$). At the end of the experiments the films were integral and only slightly thinner than before enzyme exposure. In the only case where integrity loss was observed, no quantification of biodegradation is given (see Results). Control tests, carried out in buffer without enzyme addition, showed no appreciable weight losses over the time scale of the biodegradation experiments.

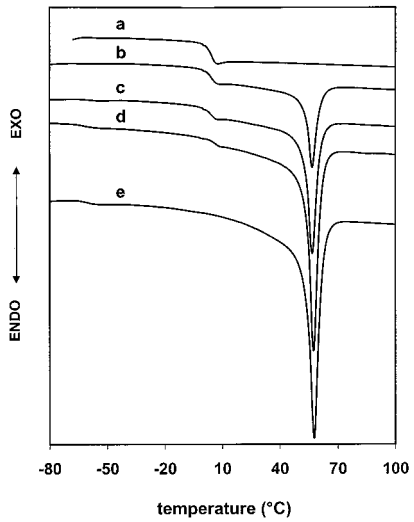

Figure 1. Calorimetric curves after melt quenching of (a) a-PHB, (e) PCL, and a-PHB/PCL blends: (b) 75/25; (c) 50/50; (d) 25/75.

Results

a-PHB/PCL Blends. *Thermal Characterization.* The DSC curves after melt quenching of a-PHB/PCL blends with different compositions and of the pure components are shown in Figure 1.

A well-defined glass transition is observed in all blends at a constant temperature, comparable to that of pure a-PHB. The specific heat increment (ΔC_p) associated with this transition increases proportionally to the content of a-PHB in the blend. An additional low-temperature glass transition is observed in the DSC curve of the blend richer in PCL (75%) in the vicinity of −60 °C, where the T_g of plain PCL is located (compare curves d and e). All a-PHB/PCL blends exhibit a melting endotherm with the same T_m as plain PCL, whose associated enthalpy changes proportionally to PCL content in the blend.

The DSC results of Figure 1 show that a-PHB/PCL blends exhibit the typical thermal transitions of a-PHB and of PCL—independent of the presence of the other component in the blend—clearly indicating that atactic PHB and PCL are immiscible. This result is in line with earlier evidence of immiscibility of blends of PCL with natural (isotactic) PHB.[7,8]

Enzymatic Degradation. The results of enzymatic degradation of a-PHB/PCL blends using PHB-depolymerase A from *P. lemoignei* are plotted in Figure 2 as normalized weight loss (($\Delta m/S$) vs time of exposure. The extent of biodegradation of plain PCL and a-PHB after 20 h of incubation is also shown in Figure 2 for the sake of comparison. Neither of the pure components show any appreciable weight loss in the time scale of the experiments. This result agrees with earlier evidence that (1) synthetic atactic PHB does not undergo biodegradation in the presence of PHB-depolymerases from *P. pickettii*,[5] *Penicillium funiculosum*,[18] and *P. lemoignei*[3,4] (PHB-depolymerase A) and that (2) PCL is not biodegraded by PHB-depolymerases,[6,7,11−13] although it is susceptible to attack by several commercial lipases.[11,12,19]

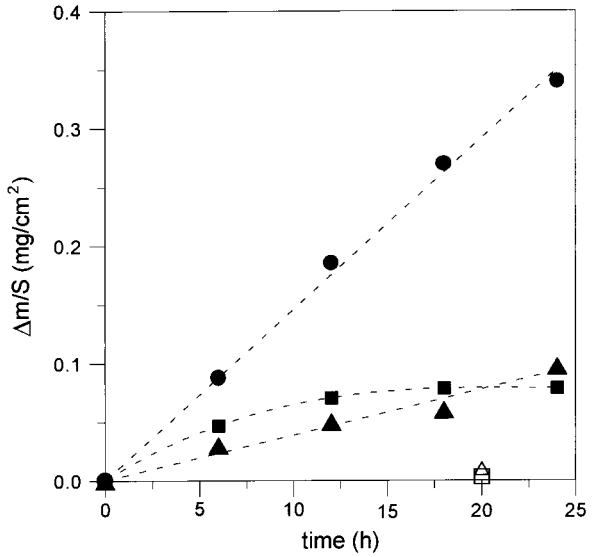

Figure 2. Normalized weight loss of a-PHB/PCL blends as a function of exposure time to the enzymatic solution: 75/25 (▲); 50/50 (●); 25/75 (■). Biodegradation results on atactic PHB (□) and PCL (△) are shown for comparison.

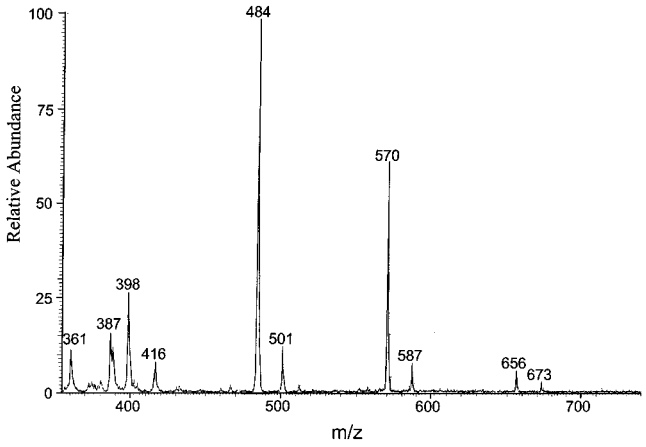

Figure 3. ESI-MS spectrum of the solution collected after 40 h of enzyme exposure of a-PHB/PCL blend (50/50).

The biodegradation results of Figure 2 show that, although the plain components do not biodegrade, all a-PHB/PCL blends investigated lose weight upon exposure to PHB-depolymerase. The dependence of weight loss on exposure time changes significantly with blend composition. The blends containing 25% and 50% PCL show a linear time dependence of weight loss over the experimental time scale. The biodegradation rate increases with PCL content from 0.4×10^{-2} mg cm^{-2} h^{-1} (75/25 blend) to 1.5×10^{-2} mg cm^{-2} h^{-1} (50/50 blend). The blend containing 75% PCL behaves differently: the weight loss does not increase linearly with time but tends to reach a constant value after about 12 h of exposure. The extent of biodegradation after 12 h of the 75/25 blend is much lower than that of the 50/50 blend.

The positive-ion ESI-MS spectrum of the solution collected after 40 h of enzyme exposure of blend 50/50 is shown in Figure 3. The signals at 398, 484, 570, and 656 (m/z) can be attributed to the protonated molecular ions of the Tris salt of the trimer, tetramer, pentamer, and hexamer of hydroxybutyric acid, respectively. Analogous a-PHB oligomers were previously found as biodegradation products of natural-PHA/a-PHB blends.[3] Since

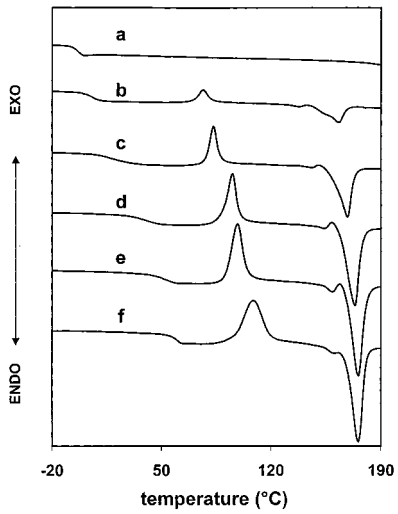

Figure 4. Calorimetric curves after melt quenching of (a) a-PHB, (f) PLLA, and a-PHB/PLLA blends: (b) 75/25; (c) 50/50; (d) 25/75; (e) 10/90.

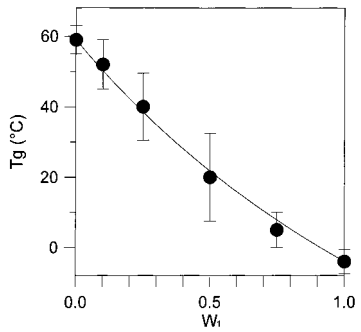

Figure 5. Glass transition temperature of a-PHB/PLLA blends as a function of blend composition. Bars represent the width of the glass transition on the temperature scale (ΔT_g).

no signals related to degradation products of PCL are found in the ESI-MS spectrum of Figure 3, it is concluded that the weight losses of a-PHB/PCL blends observed in Figure 2 are due to enzymatic hydrolysis of the a-PHB component.

a-PHB/PLLA Blends. *Thermal Characterization.* The DSC curves after melt quenching of a-PHB/PLLA blends with different composition are shown in Figure 4 together with the curves of plain a-PHB and PLLA. All blends show a glass transition intermediate to those of the pure polymers, whose T_g is plotted in Figure 5 as a function of blend composition. A vertical bar is associated with each T_g in Figure 5, representing the width of the glass transition on the temperature scale (ΔT_g, taken as the temperature interval between the intercepts of the baselines below and above T_g and the tangent to the specific heat increment). The regular change of T_g with a-PHB content indicates an extensive degree of mixing of the blend components, although the observed transition broadening at intermediate compositions—a common feature in polymer blends—may suggest local composition fluctuations. The curve drawn in Figure 5 represents Wood's equation:[20]

$$T_g = (w_1 T_{g1} + k w_2 T_{g2})/(w_1 + k w_2) \qquad (1)$$

where T_g is the glass transition temperature of the blend, w_1 and w_2 are the weight fractions and T_{g1} and T_{g2} are the T_g's of a-PHB and PLLA, respectively, and k is an adjustable parameter ($k = 0.69$ in this case).

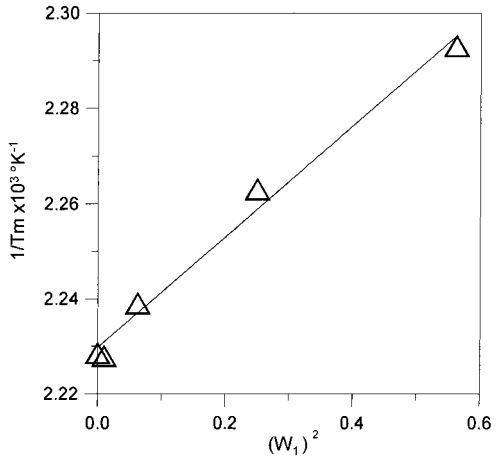

Figure 6. Melting temperature of a-PHB/PLLA blends plotted according to eq 1 (see text).

Equation 1 is one of the well-known relationships used to describe the T_g−composition dependence in plasticized polymers and in miscible polymer blends. The very good fit to the experimental results confirm that PLLA and a-PHB form miscible blends.

Cold-crystallization and melting phenomena are observed in the DSC curves of Figure 4 in all a-PHB/PLLA blends. The enthalpy associated with the thermal transitions (ΔH_c and ΔH_m for crystallization and melting, respectively) increases with PLLA content, whereas the characteristic temperature (T_c and T_m) decreases as the content of a-PHB in the blend increases. The observed T_c change is clearly related to the concomitant significant decrease of T_g with increasing a-PHB content, which widens the crystallization range of PLLA toward lower temperatures.

As regards PLLA melting point depression observed in Figure 4, it is known that the Nishi and Wang[21] equation applies to miscible blends containing a crystallizable polymer:

$$1/T_m = 1/T_m° - (R/(\Delta H_u)(V_2/V_1)\chi_{12}(\phi_1)^2 \qquad (2)$$

where subscripts 1 and 2 refer to the amorphous and crystalline components, respectively, T_m and $T_m°$ are the melting temperatures of blend and pure crystallizable polymer, respectively, V is the repeating unit molar volume, ΔH_u is the repeating unit melting enthalpy, ϕ is the volume fraction, and χ_{12} is the Flory−Huggins (polymer−polymer) interaction parameter. The plot of $1/T_m$ as a function of ϕ_1^2 should be linear in miscible blends. An approximation commonly introduced in eq 2 is substitution of volume (ϕ_1) by weight fraction (w_1). The approximation is reasonable in the present case since the densities of amorphous PHB[22] and PLLA[23] at room temperature are similar, and amorphous polymers commonly have analogous volume expansion coefficients (about 6×10^{-4} K^{-1}).[24] Figure 6 shows the melting temperatures of a-PHB/PLLA blends (from the curves of Figure 4) plotted according to eq 2. From the slope of the linear regression through the experimental data (with $\Delta H_u = 6.69$ kJ/mol,[23] $V_2 = 57.74$ cm^3/mol for PLLA[23] and $V_1 = 73.14$ cm^3/mol for PHB[22]) the interaction parameter $\chi_{12} = -0.12$ is obtained. The negative value of χ_{12} suggests that efficient polymer−polymer interactions promote mixing of PLLA with a-PHB.

The overall thermal behavior of melt-quenched a-PHB/PLLA blends demonstrates that a-PHB and PLLA are miscible in the melt. It is worth recalling that earlier studies on blends of PLLA with natural PHB showed that the blends were miscible when rather low molecular weight PLLA was used ($M_n = 1759$,[9] $M_n < 18\,000$[10]) and were immiscible when PLLA had a molecular weight comparable to that used in this work.[9,10] The different miscibility with high molecular weight PLLA can be explained taking into account that the molecular weight of natural PHB is higher than that of the a-PHB used in this work ($M_n = 31\,000$) and that it is known that decreasing the molecular weight improves miscibility.

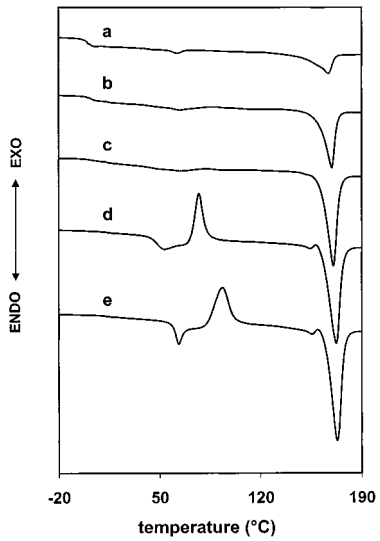

Figure 7. Calorimetric curves after 6 h in Tris−HCl buffer at $T = 37$ °C of (e) PLLA and a-PHB/PLLA blends: (a) 75/25; (b) 50/50; (c) 25/75; (d) 10/90.

In miscible blends containing a crystallizable polymer, phase separation through crystallization above the blend T_g may take place. The present a-PHB/PLLA blends clearly show this phenomenon in the DSC curves of Figure 4. Since in this work the enzymatic degradation experiments were carried out at 37 °C (a temperature that was either below or above T_g, depending on blend composition), a-PHB/PLLA blends were aged at 37 °C and their thermal properties were investigated in order to know the phase behavior of the blends in the experimental conditions of the biodegradation tests. Aging was carried out in Tris−HCl buffer (pH = 8, $T =$ 37 °C) without enzyme addition for different periods of time, and then the samples were carefully dried. Figure 7 reports the DSC curves of the blends after 6 h at 37 °C, an aging time equal to the shortest enzyme exposure in the biodegradation experiments. The DSC curve of plain PLLA (also shown for the sake of comparison) shows an enthalpy relaxation endotherm at the glass transition caused by physical aging, a phenomenon thoroughly investigated in an earlier paper.[25] By comparing the results of Figure 7 with the DSC curves of Figure 4 (melt quenched blends) it is observed that during permanence in buffer at 37 °C a considerable amount of PLLA has crystallized in the a-PHB/PLLA blends whose T_g laid below (75/25, 50/50) or spanned (25/75) the aging temperature (see Figure 5). On the contrary, the blend with the lowest amount of a-PHB (10%), which is characterized by a T_g higher than the aging temperature, shows a large crystallization exotherm in the DSC curve of Figure 7, indicating that most of the crystallinity that melts in this blend is formed

Table 1. Thermal Properties of a-PHB/PLLA Blends after 6 h in Tris−HCl Buffer (pH = 8, T = 37 °C)

a-PHB/ PLLA	T_m (°C)	$(\Delta H_m)^a$ (J/g)	$(\Delta H_m - \Delta H_c)^a$ (J/g)	$(\Delta H_m - \Delta H_c)^b$ (J/g)	$X_c{}^c$ (%)
75/25	167	16	9	36	39
50/50	169	28	18	36	39
25/75	170	44	32	42	45
10/90	172	49	8	9	10
0/100	173	53	14	14	15

[a] Per gram of whole sample. [b] Per gram of PLLA. [c] PLLA crystallinity degree, from $(\Delta H_m - \Delta H_c)^b$.

during the DSC scan (and not upon aging at 37 °C). It is worth pointing out that, by plotting the curves of Figure 7 in an enlarged scale, a small and broad crystallization exotherm can be observed in curves a, b, and c, indicating that also in these blends a small amount of PLLA crystallizes above the aging temperature.

Table 1 reports the relevant melting data for a-PHB/PLLA blends after 6 h at 37 °C. The difference between melting and crystallization enthalpy gives an indication of the amount of crystalline phase present in the blends after aging at 37 °C. By normalizing the obtained $(\Delta H_m - \Delta H_c)$ value with respect to the PLLA content of each blend, the degree of crystallinity (X_c) of PLLA after 6 h at 37 °C can be calculated, using for the melting enthalpy of 100% crystalline PLLA the literature[23] value $\Delta H_m° = 93$ J/g. The X_c values reported in Table 1 show that after aging at 37 °C a-PHB/PLLA blends with 25, 50, and 75% a-PHB contain a significant crystalline fraction. On the contrary, in the blend with 10% a-PHB at 37 °C most of the PLLA remains mixed with a-PHB in the glassy state. It is quite obvious that in a-PHB/PLLA blends segregation of PLLA as a pure crystalline phase leads to changes of the mixed amorphous phase composition, namely a-PHB enrichment and consequent T_g lowering.

Since for aging times longer than 6 h (up to18 h, viz. time-scale of biodegradation experiments) no significant changes of thermal properties of a-PHB/PLLA blends were found apart from increasing enthalpy relaxation at the glass transition of blend 10/90, it can be assumed that the results of Figure 6 correctly represent the phase situation of the blends in the experimental conditions of the enzymatic degradation tests.

In addition to the thermal characterization, a morphological investigation of a-PHB/PLLA blends was carried using polarized optical microscopy. Small fragments of the blend films were melted between two microscope cover glasses and allowed to isothermally crystallize on the microscope hot stage. Figure 8 shows optical micrographs of the blends containing 50 and 75% a-PHB crystallized at T_c =120 °C. Impinging PLLA spherulites are observed in both blends, even when PLLA constitutes a mere 25% (Figure 8b). The micrographs give clear evidence of sharp spherulite impinging fronts (marked by arrows in Figure 8). The amorphous component of the blend (a-PHB) neither accumulates at the spherulite boundary nor segregates as macroscopic inclusions, but remains trapped within the spherulite (either interlamellary or between bundles of lamellae), as normally happens when crystallizable and amorphous blend components are miscible in the melt. In Figure 8 the spherulites of both a-PHB/PLLA blends show concentric extinction bands, a feature commonly attributed to periodic twisting of the crystalline lamellae during growth. Plain PLLA isothermally crystallized

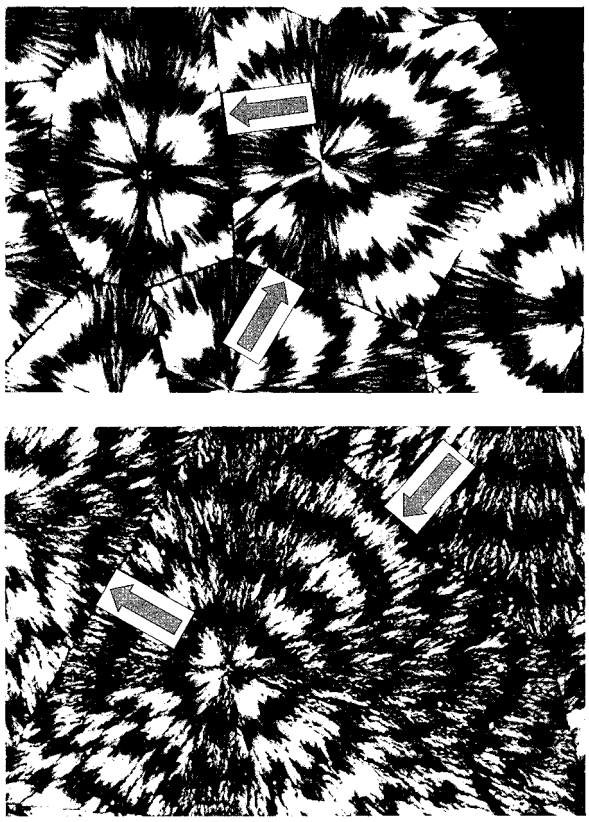

Figure 8. Optical micrographs between crossed polars of a-PHB/PLLA blends isothermally crystallized at T_c = 120 °C: (a) 50/50; (b) 75/25. Same magnification.

at the same undercooling (T_c = 125 °C) yielded non-banded spherulites. It is suggested that in a-PHB/PLLA blends the presence of a-PHB in the melt induces periodic torsion of growing PLLA lamellae, producing banded spherulites.

Enzymatic Degradation. Blends of a-PHB and PLLA were exposed to PHB-depolymerase A from *P. lemoignei*. Figure 9 reports the normalized weight loss (Δm/S) as a function of exposure time, together with results for plain a-PHB and PLLA after 20 h of incubation. The enzyme used in this work hydrolyzed neither plain a-PHB (as already shown in Figure 2) nor pure PLLA.

After each exposure to the enzymatic solution, the weight loss of a-PHB/PLLA blends increases with a-PHB content in the blend. Figure 9a shows that the blend containing 50% a-PHB linearly loses weight with increasing exposure time, with a rather high degradation rate (14.1×10^{-2} mg cm^{-2} h^{-1}). Also the blend containing 25% a-PHB (see Figure 9b) undergoes a continuous weight loss during the course of the whole experiment, although the degradation rate (0.7×10^{-2} mg cm^{-2} h^{-1}) is much lower than that of blend 50/50. Conversely, the blend containing only 10% a-PHB shows no appreciable weight loss in Figure 9b. The fastest biodegradation rate was observed in the blend with the highest a-PHB content (75%). No normalized weight loss data are available for this blend because the degradation rate is so fast that the films lose their integrity soon after enzyme exposure and the observed weight loss cannot be reasonably normalized toward the initial surface area.

ESI-MS analysis of the degradation products of a-PHB/PLLA blend 50/50 after exposure to the enzyme solution for two subsequent 20 h intervals showed the

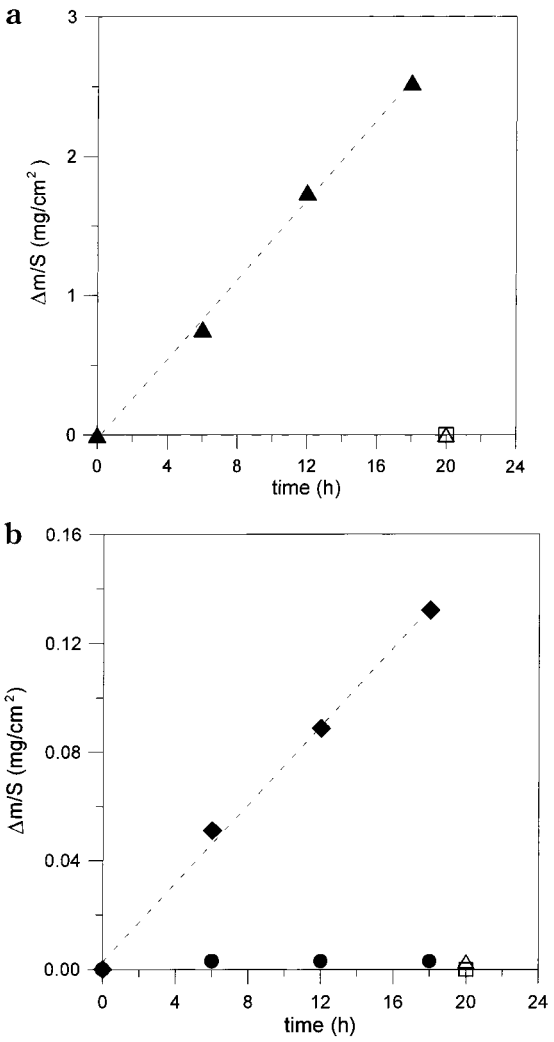

Figure 9. Normalized weight loss of a-PHB/PLLA blends as a function of exposure time to the enzymatic solution: (a) 50/50 (▲); (b) 25/75 (◆) and 10/90 (●). Biodegradation results on atactic PHB (□) and PLLA (△) are shown for comparison.

presence of only a-PHB hydrolysis products in the form of oligomers up to heptamer, without any evidence of PLLA fragments. To further confirm that in the blends only the a-PHB component is attacked by the enzyme, after each exposure time the amount of biodegradation was quantified as weight loss and blend composition was determined by ^{1}H NMR. Table 2 compares the experimental composition obtained from NMR with that calculated on the assumption that the observed weight loss in the blends is only due to a-PHB degradation. The excellent agreement between experimental and calculated composition confirms that in a-PHB/PLLA only a-PHB undergoes enzymatic hydrolysis.

Table 2. Biodegradation Results of a-PHB/PLLA (50/50) Blend

time of exposure to enzymatic solution (h)	weight loss after exposure (%)	wt % a-PHB content after exposure (from ^{1}H NMR)	wt % a-PHB content after exposure (calcd)[a]
20	22	33	35
40	36	20	20

[a] From weight loss, assuming biodegradation of the a-PHB component only.

Discussion

The phase behavior of the two blends investigated in this work strongly differs: a-PHB/PCL blends are completely immiscible, whereas a-PHB and PLLA form miscible blends over the whole composition range. Interestingly, the effect of composition on biodegradation is quite different in the two systems.

The blends of a-PHB with PCL behave similarly to the a-PHB-*b*-PPVL block copolymers investigated earlier,[2] where the two blocks were totally immiscible and, in the presence of crystalline PPVL, atactic PHB underwent enzymatic hydrolysis. Both in the present blend with PCL and in the mentioned block copolymers the rate of enzymatic hydrolysis increases with increasing content of the crystalline component [in the range 0−50 wt % in a-PHB/PCL and 0−23 mol % (0−26 wt %) in a-PHB-*b*-PPVL block copolymers]. In the composition range considered, a-PHB is the major component in both nonmiscible systems and is expected to form the continuous phase (matrix) containing the crystalline polymer as the dispersed phase. On the hypothesis that the crystalline polymer promotes biodegradation of rubbery a-PHB by offering stable binding sites to the enzyme, the observed biodegradation rate acceleration with increasing crystalline polymer content can be easily explained. In fact, if the number of "enzyme-supporting" crystalline domains increases (on the assumption that it is their number rather than their size that increases with concentration in the range considered), a more even distribution of bound PHB-depolymerase on the sample surface is obtained, the enzyme gains access to a higher number of hydrolyzable chains surrounding the crystalline polymer domains, and the overall biodegradation rate increases.

It is clear that the above scheme does not hold in the composition range where phase inversion occurs. The results of Figure 2 show that when the PCL content in a-PHB/PCL blends increases from 50% to 75% the biodegradation trend reverts: after an initial moderate weight loss the PCL-rich blend practically stops biodegrading, showing that the hydrolysis rate is governed by enzyme accessibility to the hydrolyzable component (a-PHB) rather than by the crystalline phase content. In the blend with 75% PCL the crystalline polyester is likely to form the continuous phase and a-PHB the dispersed inclusions. After initial degradation of isolated a-PHB domains available on the surface (promoted by the surrounding PCL phase), the remaining a-PHB buried in the bulk of the sample is inaccessible to the enzyme and biodegradation must stop.

It is interesting to compare the rate of hydrolysis of the two immiscible systems (a-PHB/PCL and a-PHB-*b*-PPVL copolymer[4]) with analogous crystalline polymer contents. In the same experimental conditions the hydrolysis rate of the block copolymer containing 26 wt % PPVL (3.4×10^{-2} mg cm^{-2} h^{-1}) is about 1 order of magnitude higher than that of the blend with 25% PCL (0.4×10^{-2} mg cm^{-2} h^{-1}). The simplest reason for the observed difference is that the chemical bond linking the two blocks in the copolymer promotes a dispersion of crystalline polymer in the a-PHB matrix better than that obtained in the physical blend of a-PHB with PCL, with a more efficient distribution of bound enzyme on the block copolymer surface.

An additional hypothesis for the high biodegradation rate of a-PHB in a-PHB-*b*-PPVL copolymers is that the PHB-depolymerase has a higher "binding affinity" toward PPVL than PCL. This hypothesis is based on earlier evidence[26] that, unlike PCL, PPVL adopts a

chain conformation very similar to that of crystalline bacterial PHB, which is the natural substrate for PHB-depolymerases.

Blends of a-PHB with PLLA have been found to be miscible over the whole range of concentrations and crystallize above T_g in an impinging spherulitic morphology. In the experimental conditions of this study all a-PHB/PLLA blends—except the blend with 10% a-PHB, which was essentially a mixed glass—contain a considerable amount of crystallinity (Table 1) and biodegrade at a rate that increases with a-PHB content to the point that the blend with 75% a-PHB biodegrades so fast that it does not yield measurable normalized weight loss after the first enzyme exposure.

Like in a-PHB/PCL blends discussed above, only the a-PHB component is enzymatically hydrolyzed in the blends with PLLA. The rate of biodegradation is, however, much higher in a-PHB/PLLA than in a-PHB/PCL blends: the hydrolysis rate of the two 50/50 blends, for example, differs by 1 order of magnitude.

In light of the foregoing considerations on the effect of phase distribution on the hydrolysis rate of a-PHB/PCL blends, it seems reasonable to suggest that the morphology of a-PHB/PLLA blends (impinging spherulites of PLLA where the biodegradable a-PHB component is interlamellarly trapped) may represent one of the best possible phase distributions for crystallinity-aided enzymatic hydrolysis of atactic PHB. This kind of "optimal" phase distribution is also present in a-PHB/PLLA blends with rather low PLLA contents, as illustrated by the spherulites of the blend containing 25% PLLA (Figure 8b). As a consequence of this very efficient phase distribution, the biodegradation rate is no more governed by the amount of crystalline phase available for enzyme binding (as found in the immiscible a-PHB/PCL blends up to 50% PCL) but rather by the amount of degradable a-PHB component present in the blend. Noteworthy, in a-PHB/PLLA blends the a-PHB component is accessible to the enzymes also when the PLLA content increases to 75%, as illustrated by the linear weight loss vs concentration plot of Figure 9b. This behavior is different from that shown by the immiscible a-PHB/PCL blend with the same composition (see Figure 2) and is associated with the different morphology of the two blends: PCL matrix with a-PHB inclusions in one case, PLLA spherulites with interlamellar a-PHB in the other.

The a-PHB/PLLA blend containing only 10% a-PHB does not biodegrade (Figure 9b). This blend has a glass transition (Figure 5) higher than the temperature (37 °C) where the biodegradation experiments are carried out. Although the a-PHB concentration is quite low in this blend, it is suggested that the observed nonbiodegradability arises from insufficient mobility of the a-PHB chains mixed with PLLA in the glassy state, rather than to the lack of any a-PHB chains exposed to the enzyme at the sample surface. This suggestion is corroborated by earlier results showing that in miscible blends with high-[27] or low-T_g polymers[28] natural PHB is attacked by PHB-depolymerases only when the mixed amorphous phase formed by PHB and the other polymer component is rubbery[28] and not glassy,[27,29] indicating that a degree of mobility of the hydrolyzable polymer chains is required for enzymatic action.

The results of the present investigation demonstrate that atactic PHB, which does not biodegrade in the pure state, can undergo enzymatic hydrolysis in the presence of a synthetic nonbiodegradable polyester such as PCL or PLLA. This finding supports the idea[3−5] that enzymatic degradation of a-PHB can be induced by the crystalline phase of another blended polymer, which gives the PHB-depolymerase molecule a chance to bind to a more stable surface than that provided by rubbery a-PHB. However, extrapolation of these considerations to blends of a-PHB with other crystalline polymers requires some caution. In addition to the above-mentioned possible specific crystallographic requirements of the enzyme binding site, it is likely that the chemical nature of the "supporting" polymer must meet a certain hydrophilic/hydrophobic balance to allow enzyme binding. We point out that all crystalline polymers that upon blending with a-PHB have successfully induced a-PHB enzymatic hydrolysis to date were polyesters (natural PHAs,[3,5] PPVL,[4] PCL, PLLA).

In this context, preliminary evidence that crystalline polymers belonging to a totally different chemical family are unable to induce enzymatic hydrolysis of atactic PHB has been obtained by investigating the biodegradation behavior of blends of a-PHB with polyolefins (data not shown).[30] As easily predictable, both a-PHB/PE and a-PHB/PP blends are immiscible but, unlike the immiscible blend with PCL discussed above, neither show weight loss upon exposure to PHB-depolymerase A from *P. lemoignei*. Although phase distribution in a-PHB/PE and a-PHB/PP blends may be coarser than in a-PHB/PCL (hence less favorable to crystallinity-induced biodegradation of a-PHB due to reduced interphase contacts), the total absence of weight loss in both 50/50 polyolefin-containing blends clearly demonstrates that a-PHB biodegradation cannot be induced by any crystalline polymer. Binding experiments using PHB-depolymerase A from *P. lemoignei* and different crystalline polymers are currently under way and will be reported in due time.

Acknowledgment. The authors wish to thank Drs. P. Traldi and A. M. Gioacchini for the ESI-MS spectra. This work was partially supported by the Italian Ministry for University and Research (MURST) and by the Polish State Committee for Scientific Research (Grant 7 T08E 019 11).

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(30) Blends of a-PHB with either polyethylene (PE) or polypropylene (PP) in a 50/50 ratio were prepared by melt mixing and compression molding at 150 °C, and enzymatic degradation experiments were carried out as described in the Experimental Part.

MA981115E