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Abiotic Hydrolysis of Poly[(*R*)-3-hydroxybutyrate] in Acidic and Alkaline Media

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Summary: Poly[(*R*)-3-hydroxybutyrate)], P(3HB), is the most common member of polyhydroxyalkanoates, the natural biopolyesters of intrinsic biodegradability and biocompatibility. Abiotic hydrolysis of P(3HB) was investigated in acid and base media by monitoring the formation of two monomer products, 3-hydroxybutyric acid (3HB) and crotonic acid (CA), from three types of P(3HB) samples, amorphous granules, irregular precipitates and solvent cast films. The soluble monomeric products were not detected in acid solutions (0.1 to 4 N H^+), but measured as the major hydrolytic products in base solutions (0.1 to 4 N OH^-). Unlike the protons as catalyst in both hydrolysis and esterification, hydroxyl anions were consumed during formation of carboxylate anions. The amorphous granules of P(3HB) were decomposed 80- to 100-fold faster than the precipitates and solvent cast films. The latter two had around 71% crystallinity. The hydrolysis of amorphous grannules exhibited a quasi 0th-order reaction rate and the activation energy of saponification was 82.2 kJ/mol, silimar to those of the biotic hydrolysis of P(3HB) by enzymes and living cells.

Keywords: abiotic hydrolysis; biopolyesters; degradation kinetics; polyhydroxybutyrate

Introduction

Poly[(*R*)-3-hydroxybutyrate], P(3HB), a biopolyester of high stereo-regularity, is the most common member of short-chain polyhydroxyalkanoates (PHAs) synthesized and accumulated in many bacteria as carbon storage.[1] The thermoplastic biopolymer has the potential for a variety of medical applications in drug delivery and tissue engineering [2,3] because of its nontoxicity,[4] biocompatibility with tissue and blood, and biodegradability.[5] Degradation of P(3HB), like other polyesters, proceeds by one or several mechanisms including chemical hydrolysis,[6] enzymatic catalysis[7] and thermal degradation.[8] The predominant mechanism depends to a great extent on the environmental conditions. Abiotic hydrolysis of P(3HB) occurs during medical applications,[9] and is also an essential phenomenon in P(3HB) analysis,[10,11] polymer purification,[12] processing,[8] and formation of chiral monomers and oligomers.[13,14]

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The in vitro degradation of P(3HB) in physiological conditions (pH 7.4, 37°C) is a slow process.[5] The hydrolytic rate increases with increasing pH and temperature, depending on the sample morphology[15] or specimen preparation technique.[2] The abiotic P(3HB) hydrolysis proceeds through random scissions of the ester bonds and formation of insoluble and soluble oligomers, with monomeric acids as the ultimate degradation products. [16] The alkaline hydrolysis products of P(3HB) and other biopolyesters have been detected, including monomers, oligomers and unsaturated derivatives from dehydration at the OH-terminus. [17-19] The low molecular weight products provide invaluable information for understanding the degradation mechanisms and the potential effects of P(3HB) on living cells. It is usually believed that polyester hydrolysis is catalyzed by both acids and bases. [20, 21] Little is known, however, about the different roles played by protons (H^+) and hydroxyl anions (OH^-) in P(3HB) hydrolysis and their influence on soluble final products. Degradation of a polymer such as polyethylene leads to a complex mixture containing up to hundreds of different products. Polymer degradation, therefore, is usually characterized by weight loss, and to some extent by molecular size decrease.[22-24] As polyesters contain functional groups in their main chains where bond scission initially takes place, the hydrolysis products are more predictable.[16] This work investigated the formation of two monomeric products, 3-hydroxybutyric acid and crotonic acid, from hydrolysis of P(3HB) in acid and base media.

Experimental

Materials. Crotonic acid (2-butenoic acid), 3-hydroxybutyric acid and other chemicals used in P(3HB) biosynthesis were purchased from Aldrich and Sigma (St Paul, MO, USA). Concentrated sulfuric acid was used as 100% acid.

P(3HB) biosynthesis. A *Ralstonia eutropha* strain (laboratory collection) was maintained on nutrient agar containing peptone 5 g/L, meat extract 3 g/L and 1.5 wt% of agar. The strain was cultivated in a 200 mL medium for P(3HB) biosynthesis containing (per liter): yeast 5 g, peptone 5 g, meat extract 2.5 g, glucose 20 g, $(NH_4)_2SO_4$ 5 g, KH_2SO_4 1.2 g, Na_2HPO_4 1.5 g, $MgSO_4.7H_2O$ 0.25 g, and $NaHCO_3$ 0.25 g. The culture, in a 500 mL flask, was incubated in a rotary shaker at 150 rpm and 30 °C for 72 h. The cell mass was harvested with centrifugation at 5,000 g for 15 min, washed two times with distilled water, frozen at -80 °C and lyophilized.

P(3HB) specimens. Three types of P(3HB) specimens were prepared in this work; native granules, irregular precipitates and solvent cast films. The native P(3HB) granules in the freeze-dried cells were directly used to avoid crystallization of the amorphous granules. The irregular precipitates were prepared as follows. The polyester was first extracted from freeze-dried cells with hot chloroform in a Soxhlet extractor, and then precipitated out from the solvent by adding methanol. The precipitates were filtered on filter paper and purified by repeated dissolution and precipitation in chloroform. The purified P(3HB) precipitates were dried in air and then in an oven at 60 °C. The P(3HB) films were prepared by casting a 2 wt% P(3HB)-chloroform solution on the smooth surface of ceramic evaporators. The film was formed under ambient conditions and annealed at 60 °C for 24 h.

Hydrolysis. About 20 mg of P(3HB) samples were immersed in 1 mL aqueous solutions of sulfuric acid or sodium hydroxide at pre-determined concentrations. The abiotic hydrolysis of P(3HB) polymers was performed in sealed glass tubes at predetermined temperatures in a dry bath incubator. At timed intervals, the hydrolytic solutions were neutralized to around pH 7, diluted to 25 mL with distilled and deionized water and centrifuged at 10,000 x g for 15 min. The supernatant was filtered through 0.2 μ membrane filter for chemical analysis with HPLC. The treated P(3HB) films were collected, washed and oven dried at 60 °C for FTIR spectroscopy.

Measurements. Cells containing intracellular P(3HB) granules were examined with a JEM 1011 electron microscope (JOEL, Peabody, MA, USA). Solid-state ^{13}C NMR spectra were recorded for the precipitates and solvent-cast films at 75 MHz in a Varian Inova 300 MHz spectrometer. Differential scanning calorimetry (DSC) experiments were performed using a TA Instruments Q 1000 instrument in a heat-cool-heat mode at heating or cooling rates of 10 °C/min under nitrogen. Thermogravimetric analyses (TGA) were carried out using a TA Instruments Q 500 machine at a heating rate of 10 °C/min under nitrogen. The P(3HB) polymer content in the freeze dried cell mass (FDCM) was determined from the extracted P(3HB) polymer as well as by complete hydrolysis of FDCM in concentrated sulfuric acid to crotonic acid. [10] The crotonic acid and 3-hydroxybutyric acid were measured using a HPLC equipped with a premier C18 column (Shimadzu, Japan). The compounds were eluted at 1.5 mL/min by a water-sulfuric acid solution (pH 2.5) and detected at UV 210 nm. The chemical bonds and hydrolytic residues on P(3HB) films were examined with a Nicolet Avatar 370 FTIR spectrometer (Thermo Electron Co., Madison, WI, USA). The specimens were installed and pressed on a germanium crystal window of a micro-horizontal ATR for measurement of single-reflection and absorption of infrared beams at the depth of 1-3 μm of the P(3HB) films.

Results and Discussion

P(3HB) biosynthesis and characterization. The flask culture of R. eutropha reached a dry cell mass concentration of 11.1 g/L in 72 h, containing 80 wt% P(3HB). Quasi-amorphous P(3HB) granules were prepared by freezing the cell mass quickly to -80 °C and then freeze drying over 24 h.[25] The purified P(3HB) precipitates were brittle aggregates and crystallized during precipitation from a chloroform solution.[26] The DSC

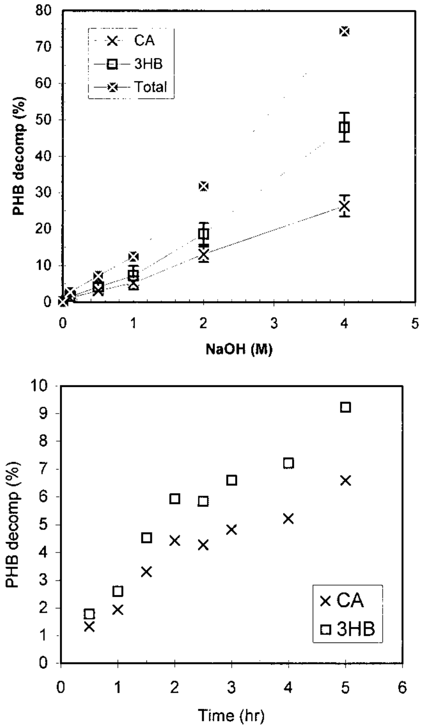
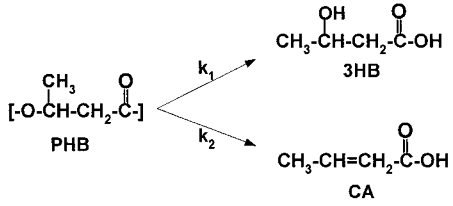


Figure 1. Hydrolysis of P(3HB) precipitate to 3-hydroxybutyrate (3HB) and crotonic acid (CA) in alkaline solutions at 70 °C. Top: the effect of alkaline concentration on hydrolysis in 4 h; Bottom: the time courses at 1 N NaOH.

thermogram for the P(3HB) precipitate shows a large endothermic peak associated with melting in the first and second heating cycles. Its crystallinity (71%) was estimated from the melting enthalpy.[27] According to the TGA thermograms, the temperatures for the onset of thermal degradation and 50% weight loss are 265.5 °C and 289.3 °C, respectively. The ^{13}C solid-state NMR spectrum of the polyester film exhibits the characteristic signals of methyl, methylene, methine and carbonyl carbons at 21.2, 42.7, 68.4 and 169.7 ppm, respectively, confirming that a pure P(3HB) was synthesized and used in this hydrolysis study.

P(3HB) hydrolysis in base media. Fig. 1 shows the percentage of P(3HB) precipitates that was decomposed into the monomeric compounds, 3-hydroxybutyric acid and crotonic acid, in solutions of sodium hydroxide. The alkaline strength was the leading factor in P(3HB) hydrolysis. At 70 °C in 4 h, P(3HB) was decomposed by less than 5% to more than 70% corresponding to the alkaline concentration from 0.1 to 4 N. The time courses in Fig. 1 also reflect the parallel increase of 3-hydroxybutyric acid and crotonic acid with time, implying that crotonic acid might not be formed from dehydration of 3-hydroxybutyric acid. Controlled experiments with 3-hydroxybutyric acid or crotonic acid confirmed no inter-conversion between these two hydrolytic products under the experimental conditions.

The following scheme shows the formation of two monomeric products in P(3HB) hydrolysis.



P(3HB) hydrolysis in acid media. In contrast to the facilitated hydrolysis in an alkaline solution, P(3HB) underwent little hydrolysis in acid media (0.1- 4 N H2SO4) at 70 oC up to 14 h. Neither 3-hydroxybutyric acid nor crotonic acid was detected, implying a low frequency of cleavage of P(3HB) backbones under the experimental conditions. Unlike hydroxy anions (OH-) that generate negatively charged carboxylate from the breakage of ester bonds, the proton (H+) is a catalyst that promotes both the hydrolysis of an ester bond and esterifcation between alcohols and acids. The reversed esterification might be favored because of the closeness of alcohol and acids.groups in the solid P(3HB) matrix. Because of the dynamic balance between scission and formation of the ester bonds, the polymers or oligomers remained in the solid state when the splitting frequency in the backbones of macromolecules was not high enough. This dynamic balance in solid state could be shifted to dissolution of P(3HB) by increasing the splitting frequency in tough conditions. Fig. 2 shows that in concentrated sulfuric acid (90% or above), about 92% of P(3HB) precipitate decomposed into soluble products, 2% in 3-hydrobutyric acid and 90% in crotonic acid. Dehydration of 3-hydrobutyric acid to crotonic acid did occur in tough conditions as indicated by the characteristic time course. A controlled experiment with crotonic acid (CA) showed that little CA was further oxidized in concentrated sulfuric acid.

Effect of P(3HB) sample morphology. Fig. 3 shows the hydrolysis of three different P3HB samples in alkaline media: the amorphous granules, the precipitates, and the solvent cost films. At low alkaline concentrations, the precipitates and films exhibited little difference in hydrolysis degradation, which was attributed to the rate limitation by the concentration of OH- ions.

At high alkaline concentrations, however, the relatively smooth and defined surface area of film samples became the limiting factor in hydrolysis and showed less decomposition than the irregular precipitates. This fact implies that the abiotic hydrolysis of P(3HB) films proceeded via surface erosion.

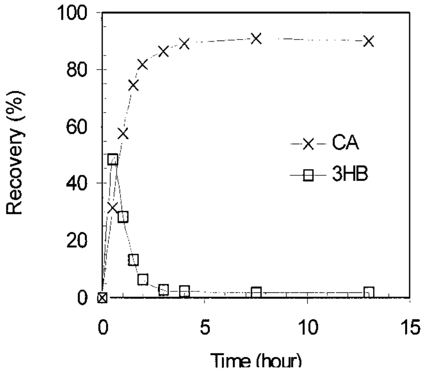


Figure 2. The time course of P3HB hydrolysis to crotonic acid (CA) and 3-hydrobutyric acid (3HB) in 90% sulfuric acid at 70 °C.

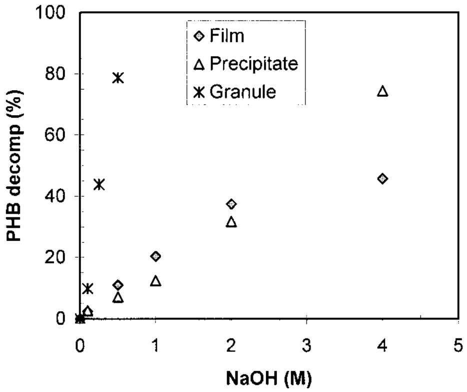


Figure 3. Hydrolysis of three P(3HB) samples in alkaline solution at 70 °C for 4 h. The amorphous P(3HB) in freeze-dried cell mass was completely decomposed in 2 h.

Fig. 4 compares the FTIR spectra of P(3HB) films treated in water and alkaline solution, respectively. Although up to 40 wt% of P(3HB) was decomposed into 3-hydroxybutyric acid and crotonic acid in the base medium, the film surfaces had little difference in the infrared energy absorption. Except the characteristic absorption by C=O stretch at 1720 cm^{-1} and C-O stretch at 1278 cm^{-1} of the ester bonds, the spectrum does not show the characteristic absorptions of hydrolytic products on the alkaline-treated surface such as the asymmetric carboxylate anion (COO^-) stretch at 1444 cm^{-1} or the C=C stretch at 1654 cm^{-1} [28]. These two peaks, however, were observed with crotonic acid in Fig. 4. This fact provides some hints for the hydrolysis mechanism on the P(3HB) film. The first random scission of ester bonds by the OH^- ion generated the groups of $-COO^-$ or $-C=C-CH_3$, making the neighboring ester bonds vulnerable to attack by OH^-. The hydrophilic small products formed from the continuing chain scission migrated and dissolved into aqueous solution, leaving few COO^- and C=C in the solid phase. Actually, when the mass loss of P(3HB) film was determined by measuring the total amount of 3HB and CA in solution and the residual film mass, the two results were very close, with a deviation error of ±3%. Fig. 5 further compares the decomposition of P(3HB) precipitates and amorphous granules. Since the freeze dried cell mass contained about 80% P(3HB), the P(3HB) granules were almost completely decomposed into 3-hydroxybutyric acid and crotonic acid at 1 N NaOH in 1 h. The precipitates with 71% crystallinity, however, lost less than 3% mass as the two hydrolysis products.

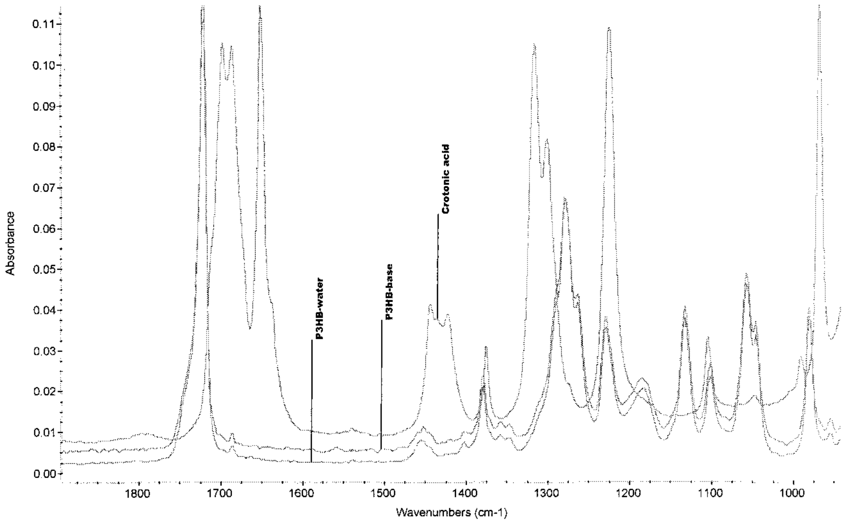


Figure 4. FTIR spectra of crotonic acid and P(3HB) films pretreated in water and base media (2N) at 70 °C for 2 h; -C=O stretching at 1723 cm^{-1}, -CH_3 bending at 1380 cm^{-1} and C-O stretching at 1282 cm^{-1}.

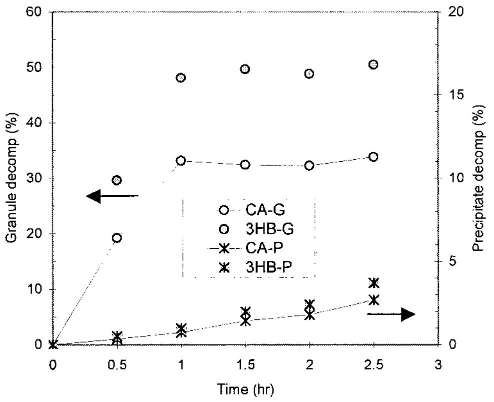


Figure 5. Time courses of P(3HB) hydrolysis to crotonic acid (CA) and 3-hydroxybutyrate (3HB) in 1N NaOH at 70 °C; amorphous granules (G) versus crystallized precipitate (P).

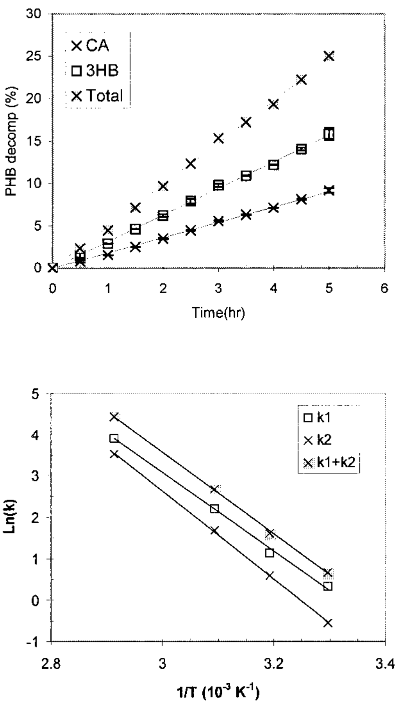


Figure 6. Hydrolysis kinetics of amorphous P(3HB) granules. Top: typical time courses in 1N NaOH at 40 °C; Bottom: Arrhenius equation correlation over a temperature range from 30 to 70 °C.

Temperature effect and kinetics. Fig. 6 shows the typical time courses of the degradation of amorphous P(3HB) granules into 3-hydroxybutyric acid and crotonic acid at 40 °C in 1 N NaOH solution. As discussed above, the two products were formed in parallel. The straight lines of the time course in Fig. 6 imply that the formation of both 3-hydroxyburatye (3HB) and crotonic acid (CA) followed a 0th-order reaction.

$$R_1 = d(W_{3HB}/W_0)/dt = k_1 \qquad (1)$$

and,

$$R_2 = d(W_{CA}/W_0)/dt = k_2 \qquad (2)$$

and,

$$R = -d(W/W_0)/dt = k = k_1 + k_2 \qquad (3)$$

where R_1, R_2 and R are the rates of 3HB formation, CA formation and P(3HB) degradation (h^{-1}), respectively. W_{3HB}, W_{CA}, W and W_0 refer to the mass of 3HB, CA, residual P(3HB) and initial P(3HB) in a hydrolysis solution, respectively. The mass of 3HB and CA has been converted to the equivalent amount of P(3HB) polymer for the convenience of data processing. The three rates in P(3HB) hydrolysis at different temperatures were found from the slopes of time courses and listed in Table 1. The temperature effect on P(3HB) hydrolysis is determined by the activation energy (E) of individual reactions, and correlated with the Arrhenius equation. The values of activation energy are estimated from the plot of ln(k) against 1/T in a temperature range from 30 to 70 °C (Fig. 6). It is interesting to compare the activation energy (82.2 kJ/mol) of the abiotic saponificaiton of P(3HB) with those of the biodegradation of P(3HB) or P(3HB) copolymers. The activation energy of P(3HB) degrdadation by a depolymerase in 0.1-1.0 M phosphate buffer (pH 6-8) is 82 kJ/mol,[29] and the activity energy of the biodegradation of a P(3HB) copolymer (80% P(3HB)) by microbial cells in a mineral solution (pH 7-8) is 68.9 kJ/mol. [24]

Effect of reagent dosage. Alkaline hydrolysis of P(3HB) involves two phases, the solid phase of the polymer and the liquid phase of the alkaline solution. Increasing or decreasing the amount of the polymer or the alkaline concentration resulted in different hydrolysis performance as shown in Fig. 7.

With a constant amount of P(3HB), a linear decomposition pattern of P(3HB) to 3HB and CA was observed at each alkaline concentration level, and the slope or the rate was increased in proportion to the initial alkaline concentration from 0.1 to 1 N. Higher

Table 1. Effect of temperature on hydrolysis of amorphous P(3HB) granules and the kinetic parameters (r^2 is the correlation factor of the Arrhenius equation).

Temperature (°C)	k_1 (h^{-1})	k_2 (h^{-1})	k (h^{-1})
30	1.381	0.574	1.955
40	3.136	1.804	4.940
50	9.067	5.391	14.458
70	50.326	34.194	84.520
r^2	0.998	0.999	0.999
A (h^{-1})	4.94×10^{13}	1.01×10^{15}	2.73×10^{14}
E (kJ/mol)	78.9	88.4	82.2

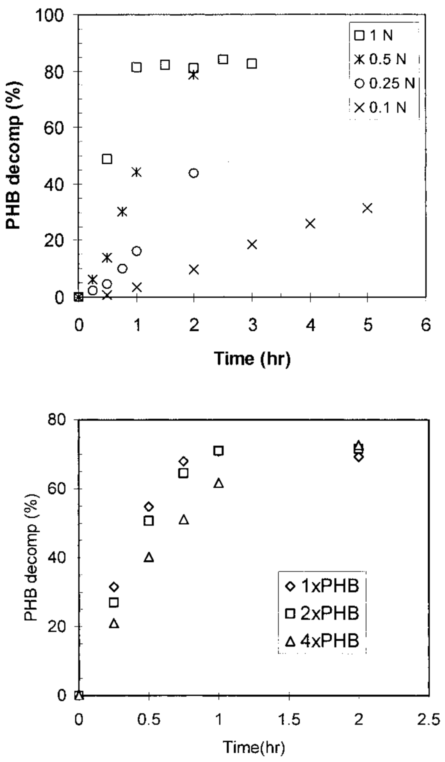


Figure 7. Effects of base strength and P(3HB) loading on hydrolysis at 70 °C. Top: a constant P(3HB) loading (1x FDCM) at different alkaline concentration; Bottom: a constant base concentration (1 N) with different P(3HB) loading.

alkaline concentration means higher attack frequency of OH^- ions to the ester bonds. On the other hand, increasing the amount of P(3HB) by two- and four-fold had little effect on the percentage of polymer decomposed (Fig. 7). This observation indicates that the hydrolysis occured at the interface of solid/liquid and was propostional to the surface area exposed to alkaline medium.

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