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Separation and Structural Characterization of Cyclic and Open Chain Oligomers Produced in the Partial Pyrolysis of Microbial Poly(hydroxybutyrates)

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ABSTRACT: To explore the possibility of using the partial pyrolysis of microbial poly(hydroxybutyrates) (PHBs) for obtaining single 3HB, 4HB, and mixed 3HB/4HB oligomers, the pyrolysis of P(3HB), P(4HB), and P(3HB-*co*-4HB) was performed and the products obtained were analyzed by HPLC and fast atom bombardment mass spectrometry (FAB-MS). Our results show that the partial pyrolysis of P(3HB) and P(4HB), followed by HPLC separation of the products, affords a suitable route for the synthesis of linear 3HB oligomers and of 4HB oligolides, respectively. The thermal degradation of P(3HB), P(4HB), and P(3HB-*co*-4HB) copolymers containing 3−34% of 3HB units was investigated by using several techniques: monitoring the time-dependent changes in molecular weight of melt samples in the range 180−200 °C; direct pyrolysis mass spectrometry; preparative pyrolysis and subsequent IR, NMR, HPLC, and FAB-MS analysis of the pyrolysate. Contrary to the claim that the rate constant (K_d) of chain scission is not influenced by the composition of the copolyesters, our results show that the rate of chain scission is influenced by the composition of copolyesters and that a mixture of cyclic and open chain oligomers is formed in the pyrolysis process. These findings indicate that P(3HB-*co*-4HB) copolymers undergo thermal degradation by two competitive random chain scission mechanisms: a β-CH hydrogen transfer at the 3HB units and an ester exchange cyclization at the 4HB units.

Introduction

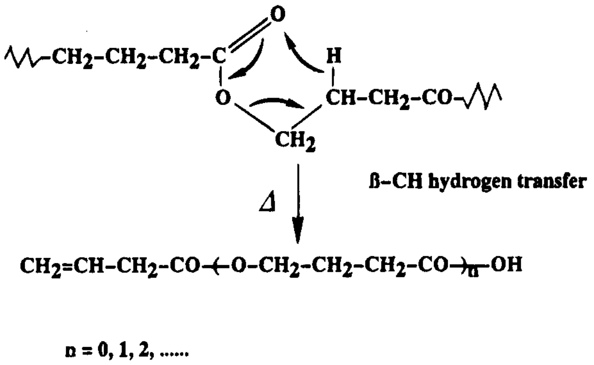
Among the new biomaterials that have been developed during the last decade, poly(3-hydroxyalcanoates) (PHAs) are of particular interest. They are a novel class of polyesters that can be synthesized by a large number of bacteria and bear many attractive properties: they are thermoplastic materials that are obtained from nonoil-based sources; being produced biosynthetically, they possess optical purity and are biodegradable and potentially biocompatible; by careful choice of bacterial species and carbon source feed, a large variety of side chain functionalized polyesters can be obtained.[1−7]

Poly(3-hydroxybutyrate) (P(3HB)), the most widespread and thoroughly characterized PHA, is thermally unstable at temperatures near its melting point (180 °C), and it is, therefore, difficult to process by conventional melt routes. To overcome this problem, a new copolyester poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (P3HB-*co*-3HV) with a lower degree of crystallinity and melting point has been microbially synthesized.

Recently, Doi et al.[6,8,9] developed a fermentation process to produce a new copolyester of 3HB and 4-hydroxybutyrate (4HB) from *Alcaligenes eutrophus*. P(3HB-*co*-4HB) copolyesters generated much interest; in particular, the flexibilty of thin films, even with low 4HB content, is noteworthy and their rate of biodegradation is higher than that of P(3HB) and P(3HB-*co*-3HV).[6]

We have studied[10] the thermal degradation mechanism of P(4HB), comparing it with that of P(3HB). Our evidence showed that 4HB units do not decompose like 3HB units by a β-CH transfer reaction (Scheme 1) and indicated instead an intramolecular ester exchange process (Scheme 2), leading to the formation of cyclic oligomers (oligolides).

Scheme 1



Recently, a number of PHB oligolides have attracted interest for various applications. Mixtures of chiral oligolides of 3-hydroxybutanoic acid and 3-hydroxyvaleric acid were obtained by macrolactonization by the methods of Yamaguchi[11,12] and Shanzer[13] or by acid catalysis of P(3HB).[14] These oligolides were used to study the influence of ring size on biodegradability. They provide a model system for "hairpin loops" which occur on the surface of crystalline regions of the polymer. It is assumed that they provide attachment points for the primary enzymatic degradation.[15] In other studies they served as models for testing the hypothesis that PHB is involved in the formation of ion channels through cell membranes and even in the passage of DNA into genetically competent bacteria, i.e. in cloning.[16]

In order to explore if the partial pyrolysis of PHBs could be used to obtain single 3HB, 4HB, and mixed 3HB/4HB oligomers, we performed the pyrolysis of these

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Scheme 2

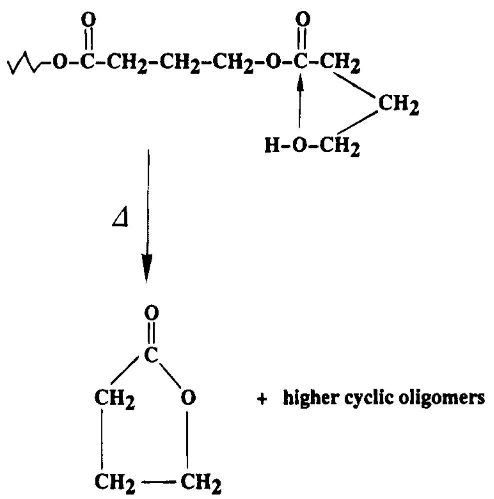


Table 1. Composition and Propoerties of Microbial Polyesters

sample	comp,[a] mol % 3HB	4HB	mol wt[b] $10^{-3}\bar{M}_n$	$\bar{M}_w/\bar{M}_n$	T_m,[c] °C
1	100	0	283	1.8	180
2	34	66	295	2.1	43
3	27	73	270	2.4	43
4	20	80	398	1.7	45
5	10	90	241	2.5	50
6	3	97	251	2.4	55

[a] Determined by 1H-NMR. [b] Determined by GPC. [c] Measured by DSC.

materials and analyzed by HPLC and FAB-MS the products obtained from P(3HB), P(4HB), and P(3HB-*co*-4HB).

Our results show that the partial pyrolysis of P(3HB) and P(4HB), followed by HPLC separation of the products, affords a suitable route for the synthesis of linear 3HB oligomers and of 4HB oligolides, respectively.

The thermal degradation mechanism of the P(3HB-*co*-4HB) copolymers listed in Table 1 was also studied here. Our findings indicate that P(3HB-*co*-4HB) copolymers undergo thermal degradation by a dual route: a β-CH hydrogen transfer at the 3HB units and an ester exchange cyclization at the 4HB units.

Experimental Section

Biopolymers Synthesis. The samples of P(3HB), P(4HB), and P(3HB-*co*-4HB) were obtained by fermentation of *A. eutrophus* H16 (ATCC 17699), according to the Doi[6,8,9] procedure with some modification. Polyester synthesis was carried out by a two-step cultivation of *A. eutrophus. A. eutrophus* cells were grown under aerobic conditions at 30 °C in a nutrient-rich medium (100 mL) containig 1 g of yeast extract, 1 g of polypeptone, 0.5 g of meat extract, and 0.5 g of $(NH_4)_2SO_4$. The cells were harvested by centrifugation after 24 h. To promote polyester synthesis, the centrifugate cells which did not contain polyester were transferred into nitrogen-free mineral media[17] (pH 7.5) containing 4-hydroxybutyric acid sodium salt. The cells were cultivated in these media for 48–168 h at 30 °C, harvested by centrifugation, and finally lyophilized. To obtain P(3HB-*co*-97%4HB), 4-hydroxybutyric acid sodium salt (20 g/L), citrate (5 g/L), and $(NH_4)_2SO_4$ (2 g/L) were added into nitrogen-free mineral medium and the cells were cultivated for 48 h. To obtain P(3HB-*co*-80%4HB), P(3HB-*co*-73%4HB), and P(3HB-*co*-66%4HB), into nitrogen-free mineral medium was added 4-hydroxybutyric acid sodium salt (20 g/L) and the cells were cultivated for 96, 144, and 168 h, respectively. Copolyesters P(3HB-*co*-4HB) were extracted from lyophilized cells with a double extraction. The first extraction was carried out with hot chloroform in a Soxhlet apparatus and purified by precipitation in hexane. The second extraction was carried out with hot acetone in a Soxhlet apparatus and purified by precipitation in hexane.

Kinetics of Isothermal Degradation (180–200 °C). Thermal degradation reactions for the determination of MW changes with time were carried out in Pyrex tubes isothermally for a given time at 180, 190, and 200 °C. Before the experiment, the tubes containing the samples were degassed and sealed. The pyrolysate was taken up with $CHCl_3$ and transferred for GPC analysis. A Waters 6000 A solvent delivery system equipped with a series of four Ultrastyragel columns (in the order 1000-, 500-, 10000-, and 100-Å pore size) was used for gel permeation chromatography (GPC) analyses. A Waters model R401 differential refractometer was used as the detector. Analyses were performed at 25 °C using chloroform as the eluent at a flow rate of 1 mL/min. The columns were calibrated with polystyrene molecular weight standards. Calculations ($\bar{M}_w$, $\bar{M}_n$, $\bar{M}_w/\bar{M}_n$) were done by numerical integration using a Waters 745 Data Module with the GPC Package.

Isothermal Pyrolysis at 200–300 °C. A series of thermal degradation reactions were carried out for the samples in Table 1, placing 5 mg in Pyrex tubes isothermally for a given time at 230 °C. Before the experiment, the tubes containing the samples were degassed and sealed. The pyrolysate was taken up with CH_3CN and transferred to HPLC analysis for the fractionation of pyrolysis products obtained, using a Varian VISTA 5500 HPLC System equipped with a Rheodyne injector with a 50 μL loop, a Varian 2050 UV detector, and a Microbondapak column (C_{18}, Waters) of 30 cm × 4 mm. The acetonitrile solution was injected, using an elution gradient starting with a 5/95 acetonitrile/water composition and ending with 100% acetonitrile in 80 min, with 1 mL/min flow and UV detection at 205 nm. After lyophilization, each fraction collected was analyzed by FAB-MS for structural identification. To obtain FAB mass spectra, a double-focusing Kratos MS 50S equipped with the standard FAB source and with an Eclipse (Data General) data system running with DS90 acquisition software was used. The cesium ion gun was operated at 20 kV. The instrument was scanned from *m/z* 3000 to 60 at a scan rate of 10 s/decade at an acceleration potential of 8 kV. Cesium and rubidium iodides (50:50 by weight) were used for computer calibration.

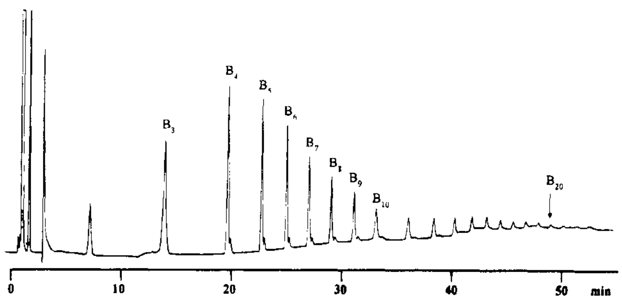
In another set of experiments, 5 mg of sample was placed in a sublimation apparatus under a rotative vacuum pump. Pyrolyses were carried out at 300 °C for 15 min or 200 °C for 70 min. Pyrolysis products condensed at −70 °C were analyzed by IR and NMR.

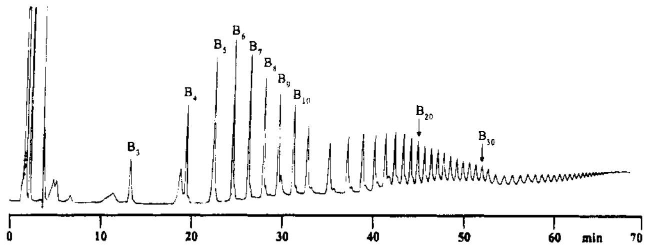
IR and NMR Spectra. Infrared spectra were recorded in $CDCl_3$ solution on a FT-IR Perkin-Elmer System 2000 spectrometer.

The 200 MHz ^{1}H-NMR spectra were recorded at 25 °C in $CDCl_3$ (20 mg/mL) on a Bruker AC 200 spectrometer with a 4 s pulse repetition, a 2000 Hz spectral width, 16K data points, and a 256 scan accumulation. The ^{1}H-decoupled 50 MHz ^{13}C-NMR spectra were recorded on the same samples with a 1.6 s pulse repetition, a 10 000 Hz spectral width, 32K data points, and a 30 000 scan accumulation.

Direct Pyrolysis Mass Spectrometry. The analysis of volatiles evolving from the pyrolysis of P(3HB-*co*-4HB) copolymers was also performed by the direct pyrolysis mass spectrometry technique[18] (DPMS). Direct pyrolysis mass spectra were obtained using a QMD 1000 quadrupole mass spectrometer (Fisons) using the Lab-Base acquisition and processing software. Pyrolyses were carried out using the water-cooled standard direct insertion probe for solid materials heated from 50 to 400 °C at a heating rate of 10 °C/min. Mass spectra were acquired with a 5 s scan time and 1 s interscan delay, using 18 eV ionization energy. The source temperature was maintained at 150 °C.

Thermal Analysis. Thermogravimetry was performed with a Perkin-Elmer TGS/2 apparatus in a nitrogen atmosphere (60 mL/min) at a heating rate of 10 °C/min. Melting points were measured by differential scanning calorimetry


(a) P(3HB)


(b) P(4HB)

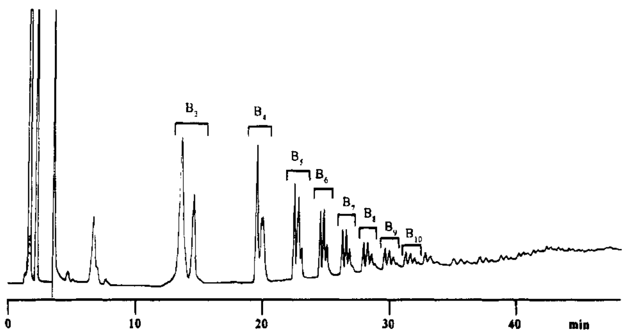

(c) P(3HB-co-73%4HB)

Figure 1. HPLC separation of pyrolysis products obtained by thermal degradation at 230 °C in sealed tubes of (a) P(3HB), (b) P(4HB), and (c) P(3HB-*co*-73%4HB), respectively.

with a Mettler TA 3000 apparatus at a heating rate of 10 °C/min. These measurements refer to first heatings.

Results and Discussion

Our pyrolysis studies were carried out on P(3HB) and P(4HB) homopolymers and on a series of random copolyesters whose composition, molecular weights, and melting temperatures are summarized in Table 1.

HPLC Separation and Structural Characterization of PHB Oligomers. In order to explore if the procedure of partial pyrolysis of PHB polymers and copolymers could be used to obtain single 3HB, 4HB, and mixed 3HB/4HB oligomers, we performed the pyrolysis in sealed tubes at 230 °C (see Experimental Section) and analyzed by HPLC the products obtained from P(3HB), P(4HB), and P(3HB-*co*-73%4HB). The HPLC traces are shown in Figure 1a–c, respectively. Oligomers originating from 3HB and 4HB homopolymers are well separated in the HPLC trace, allowing the collection of each peak in Figure 1a,b.

The HPLC fractions collected were lyophilized and analyzed by FAB-MS. In Figure 2 is shown, as an example, the FAB mass spectrum of peak B_8 in Figure 1a deriving from the pyrolysis products of P(3HB). Three pseudomolecular ions are present: MH^+ at *m/z* 689, MNa^+ at *m/z* 711, and $(M2Na - H)^+$ at *m/z* 733, demonstrating that peak B_8 corresponds to the linear octamer ($n = 7$ in structure I). No relevant fragmentation is observed in the FAB spectrum in Figure 2.

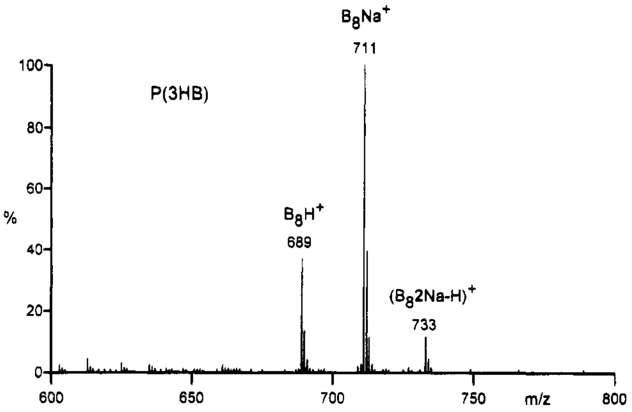
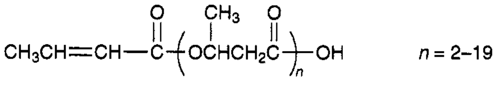


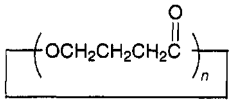
Figure 2. FAB mass spectrum corresponding to peak B_8 in the HPLC trace of Figure 1a.

The oligomers corresponding to peaks B_3–B_{20} in Figure 1a were identified as



$$CH_3CH{=}CH-\overset{O}{\overset{\|}{C}}\left(O\overset{CH_3}{\overset{|}{C}}HCH_2\overset{O}{\overset{\|}{C}}\right)_n OH \qquad n = 2\text{–}19 \qquad \text{(I)}$$

FAB mass spectra of HPLC peaks from the pyrolysis products of P(4HB) yielded the structures and molecular weights of fractions B_3–B_{30} in Figure 1b, corresponding to cyclic oligomers of general formula



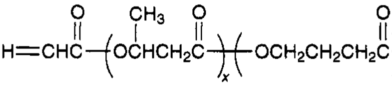
$$\left[\left(OCH_2CH_2CH_2\overset{O}{\overset{\|}{C}}\right)_n\right]_{\text{cyclic}} \qquad n = 3\text{–}30 \qquad \text{(II)}$$

These spectra are similar to those of P(3HB) oligomers in that no fragmentation occurs, but differ in one notable point: only two pseudomolecular ions are present for each product, MH^+ and MNa^+, while the $(M2Na - H)^+$ ion is absent. This fact confirms that the thermal degradation of P(4HB) gives rise selectively to lactones.[10] In fact, the $(M2Na - H)^+$ ions are the sodium adducts of the sodium carboxylate form of the pyrolysis products from P(3HB); in the case of P(4HB) no terminal carboxyl groups are present, and consequently, no $(M2Na - H)^+$ ions are formed.

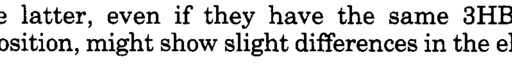
Therefore, the partial pyrolysis of P(3HB) and P(4HB), followed by HPLC separation of the products, affords a suitable route for the synthesis of 3HB and 4HB oligomers.

A question that remains open in the present study, is to identify mixed 3HB/4HB oligomers produced in the pyrolysis of P(3HB-*co*-4HB) copolymers.

The HPLC trace of the partial pyrolysis products from the P(3HB-*co*-73%4HB) copolyester is reported in Figure 1c. Elution conditions were those used for the homopolymers. The chromatogram is quite complex, due to the large variety of products generated: cyclic and open homo-oligomers (I and II) and cyclic and open mixed oligomers (III and IV).



$$\left[\left(O\overset{CH_3}{\overset{|}{C}}HCH_2\overset{O}{\overset{\|}{C}}\right)_x\left(OCH_2CH_2CH_2\overset{O}{\overset{\|}{C}}\right)_y\right]_{\text{cyclic}} \qquad \text{(III)}$$



$$CH_3CH{=}CH\overset{O}{\overset{\|}{C}}\left(O\overset{CH_3}{\overset{|}{C}}HCH_2\overset{O}{\overset{\|}{C}}\right)_x\left(OCH_2CH_2CH_2\overset{O}{\overset{\|}{C}}\right)_y OH \qquad \text{(IV)}$$

The latter, even if they have the same 3HB/4HB composition, might show slight differences in the elution time due to different sequences. Clusters of peaks can be discerned (B_3–B_{14} in Figure 1c), and each single peak within a cluster was found by FAB-MS analysis to have the same molecular mass (i.e. they are isomers). Structural assignment of all the peaks was not possible because it is difficult to collect pure fractions of each peak.

However, we identified homo 4HB oligolides by coinjection of the cyclic oligomers obtained from P(4HB) partial pyrolysis with those obtained from the copolymer. They are the first peaks in each cluster. The presence of $(M2Na - H)^+$ ions in FAB mass spectra of cluster peaks B_3–B_{14} in Figure 1c revealed the presence of open chain compounds bearing carboxyl and crotonyl end groups. Work is in progress to identify other co-oligomers.

Dual Mechanism of Thermal Degradation of P(3HB-*co*-4HB) Copolyesters. Marchessault et al.[19] and Grassie et al.[20] reported that the thermal degradation process of P(3HB) at temperatures greater than 170 °C occurs by a random chain scission due to a β-CH hydrogen transfer involving the cleavage of the ester group.

Doi et al.[9] studied the thermal degradation of microbial P(3HB-*co*-4HB) copolyesters in the temperature range 100–180 °C by monitoring the time dependent change in molecular weight of melt samples. Kunioka and Doi[8] determined the activation energy and the rate constant of the polyester chain thermal cleavage and found that the rate constant K_d of random scission is not influenced by the composition of copolyesters, depending only upon temperature. They concluded that a mechanism of β-CH transfer, is also acting in these copolymers.

If a chain scission process is completely random and little or no volatilization occurs during the thermal degradation of samples, the number average degree of polymerization $\bar{X}_{n,t}$ at the time t is given by[21] where $\bar{X}_{n,0}$

$$(1/\bar{X}_{n,t}) - (1/\bar{X}_{n,0}) = kt \qquad (1)$$

is the initial number average degree of polymerization and k is the rate constant of the thermal degradation reaction.

Parts a and b of Figure 3 show the relationship between $1/\bar{X}_n$ and time t for samples **1**, **4**, and **6** at 180 and 200 °C, respectively.

At 180 °C the relationship is linear and the slope is approximately the same for the three samples examined. At 200 °C the relationship for the three samples is still linear, but with different slopes. In fact, P(4HB) shows lower depolymerization kinetics with respect to P(3HB), while the P(3HB-*co*-4HB) sample has an intermediate rate of depolymerization. This behavior, contrary to earlier reports,[8] clearly shows that the K_d values of random chain scission are influenced by the copolymer composition, also indicating that the 4HB units ought to decompose by a route different from the β-CH transfer.

Furthering our work, the products originated in the pyrolysis of the P(3HB-*co*-4HB) copolymers were identified by DPMS experiments.

In Figure 4a,b are reported the EI mass spectra of the pyrolysis products of P(3HB) and P(4HB), whose structures are identified in Table 2. Parts c and d of Figure 4 show the EI mass spectra of the pyrolysis products of the P(3HB-*co*-80%4HB) copolymer at 260 and 310 °C, respectively. The mass spectrum in Figure 4c at 260 °C reveals mainly the presence of pyrolysis products from 3HB units.[10] In fact, the spectrum is dominated by fragments at m/z 41, 69, 87, 103, 241, and

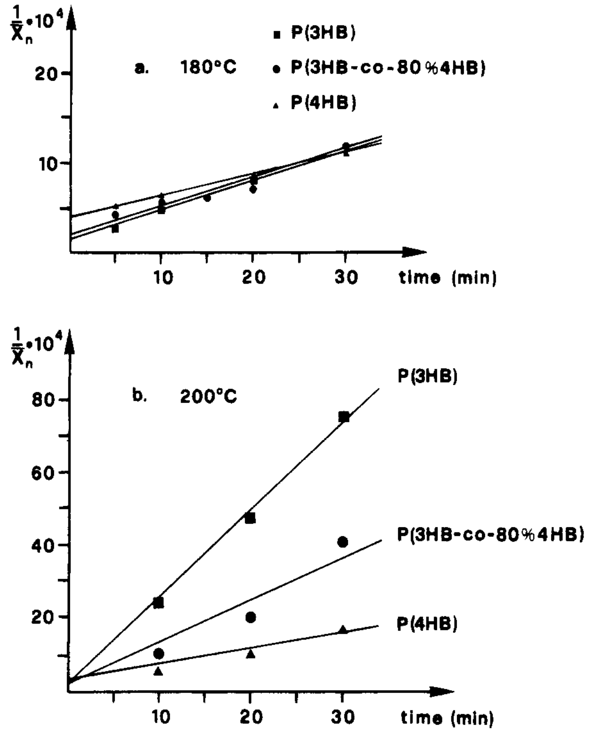


Figure 3. $1/\bar{X}_n$ vs time of heating for the samples **1**, **4**, and **6** at (a) 180 °C and (b) 200 °C. Data relative to P(3HB) in (a) are from ref 7.

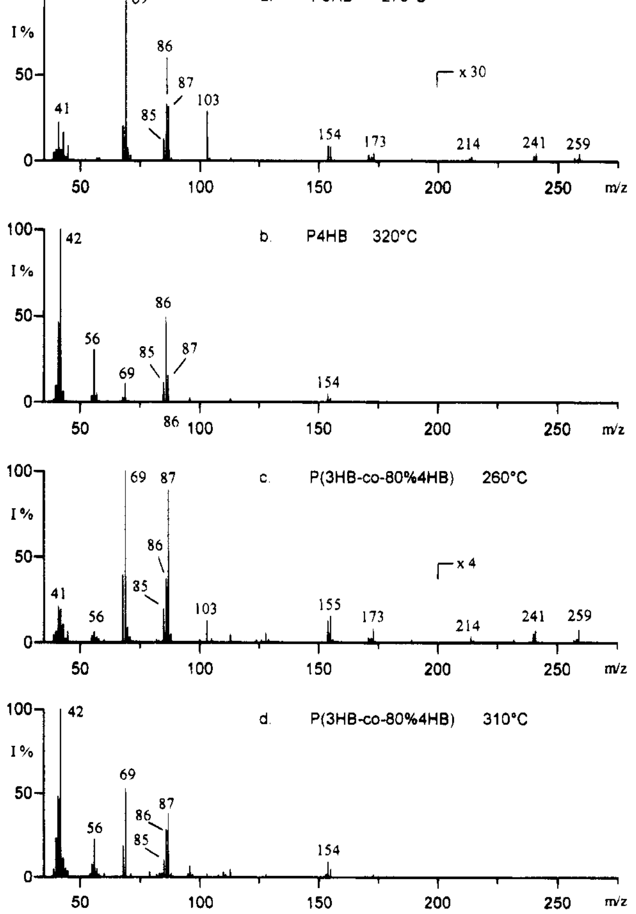
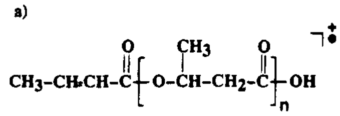
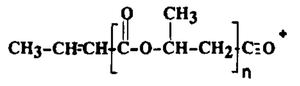
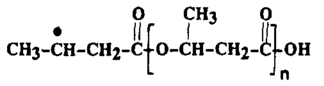
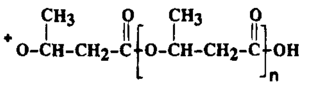
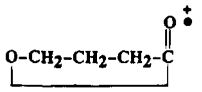
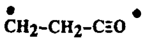
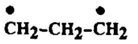


Figure 4. Direct pyrolysis EI mass spectra (18 eV) of (a) P(3HB) at 270 °C, (b) P(4HB) at 320 °C, (c) P(3HB-*co*-80%4HB) at 260 °C and (d) at 310 °C, respectively.

Table 2. EI Fragments of Pyrolysis Products of P(3HB) (a) and P(4HB) (b)

	m/z (n)		
a)			
	86 (0),	172 (1), $\downarrow -18$ 154 (1),	258 (2) $\downarrow -18$ 240 (2)
	69 (0), $\downarrow -28$ 41 (0)	155 (1),	241 (2)
	87 (0),	173 (1),	259 (2)
	103 (0), $\downarrow -18$ 85 (0),	189 (1), $\downarrow -18$ 171 (1),	275 (2) $\downarrow -18$ 257 (2)
b)			
	86		
	56		
	42		

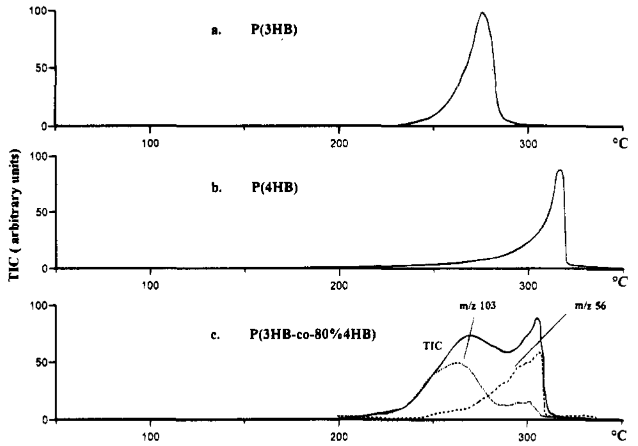


Figure 5. Total ion current (TIC) and single ion curves (SIC) vs temperature of pyrolysis products of (a) P(3HB), (b) P(4HB), and (c) P(3HB-*co*-80%4HB).

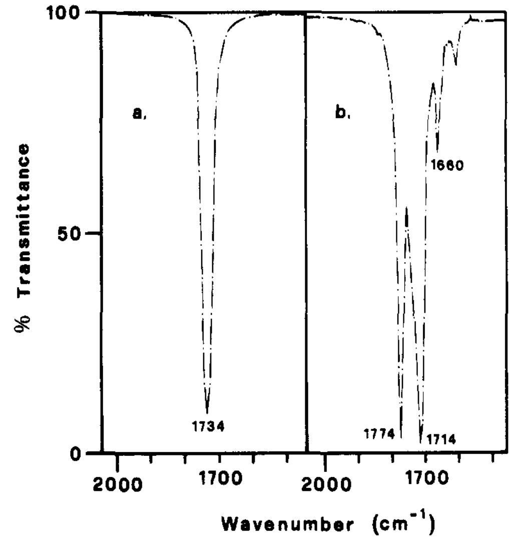


Figure 6. FT-IR spectra of (a) undegraded P(3HB-*co*-66%4HB) and (b) pyrolysis products obtained by thermal degradation in a sublimation apparatus.

259. The mass spectrum in Figure 4d at 310 °C reveals instead the presence of pyrolysis products from P(4HB) units. In fact the spectrum is characterized by fragments at *m/z* 42, 56, 86, and 154 (Table 2).

It can be noted that in the spectrum taken at 260 °C, besides peaks characteristic of 3HB, fragments at *m/z* 42 and 56 are also present, which are instead characteristic of 4HB. The same behavior is found in the spectrum taken at 310 °C, where besides peaks typical of 4HB, an ion at *m/z* 103 is found, which is a "marker" of 3HB units. These effects are partly due to the EI technique, since the "extraneous" ions present in the mass spectra in Figure 4c,d, respectively, may originate from the EI fragmentation of 3HB or 4HB units included in the co-oligomers generated in the thermal cleavage processes of the copolymer chains.

The volatilization profiles against temperature (total ion current, TIC) of P(3HB), P(4HB), and P(3HB-*co*-80%4HB) obtained by DPMS experiments are reported in Figure 5a–c.

The two homopolymers show TIC maxima at 270 and 320 °C, respectively, whereas the P(3HB-*co*-80%4HB) copolymer shows two partially overlapped maxima at 260 and 310 °C. TIC curves of the other PHB copolymers investigated show the same trend, although with a different intensity ratio of the two maxima.

In Figure 5c are also shown the single ion current curves (SIC) at *m/z* 103, characteristic of 3HB units, and the peak at *m/z* 56, characteristic of 4HB units (see Table 2 for structural assignments). These findings indicate that, in the P(3HB-*co*-80%4HB) copolyester, the thermal stability of 3HB units, which degrade yielding oligomers with carboxyl and crotonyl end groups, is lower with respect to that of 4HB units, which instead degrade yielding lactones.[10]

Infrared spectra of the undegraded P(3HB-*co*-66%4HB) and of the volatile pyrolysis products originated from its thermal degradation at 300 °C (see experimental) are compared in Figure 6. The C=O stretch occurs at 1734 cm^{-1} in the case of undegraded copolymer, while in the case of its pyrolysis products we observe that the C=O stretch is shifted to 1714 cm^{-1} and a new band appears at 1774 cm^{-1}, typical of the C=O stretch of the γ-lactone ring. It is well-known that C=O vibrations are shifted to higher frequencies with decreasing ring size in cyclic esters because of increased angle strain. The C=C stretch absorption band, appearing at 1660 cm^{-1} in Figure 6b, indicates the formation of co-oligomers having unsaturated end groups, as predicted by the β-CH transfer cleavage.

Therefore, also this infrared evidence shows that in the case of thermal degradation of P(3HB-*co*-4HB) copolymers, both open chain products and cyclic oligomers are generated.

In order to doublecheck this result, we recorded the 200 MHz ^{1}H-NMR spectra of the pyrolysis products obtained at 300 °C from P(3HB), P(4HB), and P(3HB-*co*-80%4HB). The spectra are shown in Figures 7a–c respectively.

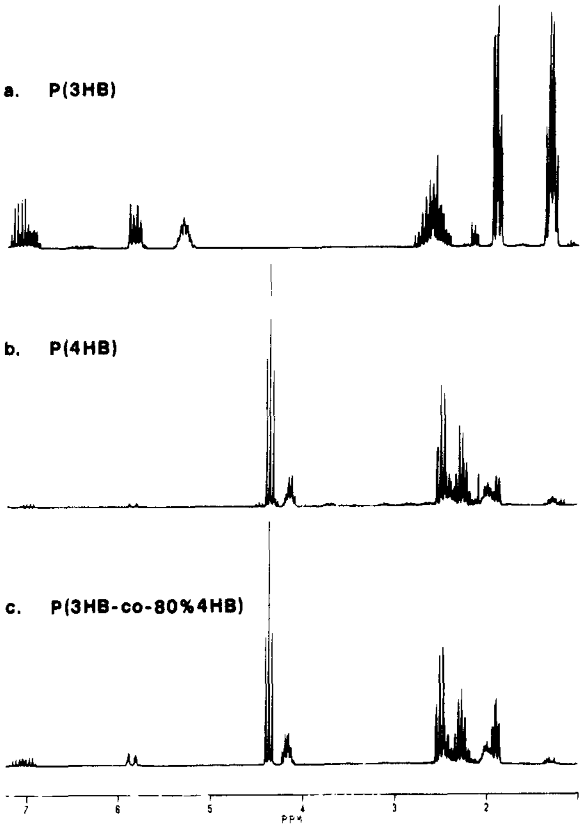


Figure 7. 200 MHz ^{1}H-NMR spectra in $CDCl_3$ of pyrolysis products obtained by thermal degradation in a sublimation apparatus of (a) P(3HB), (b) P(4HB), and (c) P(3HB-*co*-80%4HB), respectively.

The multiplets at 7.0 and 5.8 ppm in Figure 7c correspond to crotonic end groups arising from 3HB units. The multiplets centered at 4.4, 2.5, and 2.3 ppm correspond to the methylene protons of γ-butyrolactone, while the other three multiplets of lower intensity at 4.1, 2.3, and 2.0 ppm may be assigned to 4HB methylene protons of its higher cyclic homologs (III) or of its open chain oligomers (IV).

Noteworthy, in these pyrolysis experiments, we were able to detect (by ^{1}H-NMR) γ-butyrolactone formation at temperatures as low as 200 °C.

The absence in the spectrum of Figure 7c of multiplets at 3.1, 5.1, and 5.8 ppm characteristic of NMR signals of 3-butenoic acid and its higher homologs rules out a β-CH hydrogen transfer reaction for the 4HB units in the copolymer.

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