Microbial Synthesis of Poly(β-hydroxyalkanoates) Bearing Phenyl Groups from *Pseudomonas putida*: Chemical Structure and Characterization

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New poly(β-hydroxyalkanoates) having aromatics groups (so-called PHPhAs) from a microbial origin have been characterized. These polymers were produced and accumulated as reserve materials when a β-oxidation mutant of *Pseudomonas putida* U, disrupted in the gene that encodes the 3-ketoacyl−CoA thiolase (*fad*A), was cultured in a chemically defined medium containing different aromatic fatty acids (6-phenylhexanoic acid, 7-phenylheptanoic acid, a mixture of them, or 8-phenyloctanoic acid) as carbon sources. The polymers were extracted from the bacteria, purified and characterized by using ^{13}C nuclear magnetic resonance spectroscopy (NMR), gel permeation chromatography (GPC), and differential scanning calorimetry (DSC). Structural studies revealed that when 6-phenylhexanoic acid was added to the cultures, an homopolymer (poly-3-hydroxy-6-phenylhexanoate) was accumulated. The feeding with 8-phenyloctanoic acid and 7-phenylheptanoic acid leads to the formation of copolymers of the corresponding units with the $n - 2$ carbons formed after deacetylation, copoly(3-hydroxy-8-phenyloctanoate−3-hydroxy-6-phenylhexanoate) and copoly(3-hydroxy-7-phenylheptanoate−3-hydroxy-5-phenylvalerate), respectively. The mixture of 6-phenylhexanoic acid and 7-phenylheptanoic acid gave rise to the corresponding terpolymer, copoly(3-hydroxy-7-phenylheptanoate−3-hydroxy-6-phenylhexanoate−3-hydroxy-5-phenylvalerate). Studies on the chemical structure of these three polyesters revealed that they were true copolymers but not a mixture of homopolymers and that the different monomeric units were randomly incorporated in the macromolecular chains. Thermal behavior and molecular weight distribution were also discussed. These compounds had a dual attractive interest in function of (i) their broad use as biodegradable polymers and (ii) their possible biomedical applications.

Introduction

Poly(β-hydroxyalkanoates), PHAs, are polyesters synthesized by a wide variety of organisms (particularly bacteria) that are accumulated as an intracellular energy and carbon source storage material.[1−5] Regarding their composition, the structure of PHAs is quite variable as a function of (i) the bacterial strain used to produce them and (ii) the culture conditions employed for obtaining these polymers.[6]

Although PHAs are commonly stored as copolymers, in some cases only homopolymers are accumulated.[7,8] This difference is caused by (i) the limitation imposed by the substrate specificity of the polymerizing enzymatic system (it is unable to polymerize monomers whose acyl chain is lower than four carbon atoms) and (ii) by the fact that the bacteria which accumulate these polyesters do not use the fatty acids added to the cultures (polymer precursors) to synthesize a longer monomer that, then, could be polymerized.[9]

It has been previously described that *Pseudomonas oleovorans* accumulates PHAs, containing long-chain alkyl pendant groups with more than five carbon atoms, when it is grown on either medium-length *n*-alkanes or *n*-alkanoic acids under essential nutrient limiting conditions.[10−12] However, although the accumulation of some PHAs containing functional groups in the side chain (halogens, olefins, cyano, nitrile, or methyl ester groups) has been described,[13−15] the synthesis of polymers containing unsubstituted phenyl groups (PHPhAs)[11] or phenoxy groups[14,16,17] are rare events. Thus, to our knowledge only two bacterial species, *P. oleovorans* and *Pseudomonas putida*, were able to efficiently synthesize these polymers when cultured in chemically defined media containing as carbon sources different ω-phenylalkanoates.[9,18] The poly(3-hydroxy-5-phenylvalerate) homopolymer (PHPhV), was the first example of PHPhA containing an aromatic group which is synthesized by a microorganism

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(*P. oleovorans*). It was produced when 5-phenylvaleric acid was supplied as a single carbon source.[11] More recently, we have reported that a different *Pseudomonas* species (*P. putida* U) was able to synthesize many different PHPhAs if cultured properly and that the yields of production could be strongly increased by using some mutants disrupted in the β-oxidation pathway.[9]

In this work, we describe the characterization of four different PHPhAs, obtained from these mutants when they were cultured in a chemically defined medium containing 6-phenylhexanoic acid, 7-phenylheptanoic acid, a mixture of them, or 8-phenyloctanoic acid as source of polymers, and 4-hydroxyphenylacetic acid for supporting bacterial growth, respectively. The four polymer samples were extracted, purified and analyzed in detail. Their polymeric compositions, chemical structures, and molecular weights as well as thermal properties were established and discussed.

Experimental Section

Culture Conditions. The experimental procedure followed in the studies related to the biosynthesis of PHAs was identical to that previously reported by García et al.[9] Briefly, the *P. putida* U mutants used in all the experiments were maintained on Trypticase Soy Agar (Difco), and growth slants (8 h at 30 °C) were used to inoculate the liquid medium. Each 2000 mL Erlenmeyer flask containing 500 mL of the required medium[19] was inoculated with 10 mL of a bacterial suspension (10^{10} bacteria). Incubations were carried out in a rotary shaker (250 rpm) at 30 °C for the time required in each set of experiments. When required, the carbon source commonly used in these experiments (phenylacetic acid) was replaced by 6-phenylhexanoic acid (6PhHA), 7-phenylheptanoic acid (7PhHpA), a mixture of 6PhHA and 7PhHpA, and 8-phenyloctanoic acid (8PhOA). When β-oxidation mutants were studied, 4-hydroxyphenylacetic acid (a compound which cannot be used as a polyester precursor) was supplied to the media for supporting bacterial growth. The concentration of the molecules used as carbon sources was in the range between 5 and 15 mM. The resulting PhPHAs were extracted according to the procedure reported by Lageveen et al.[20] The quantity of PhPHAs extracted was higher (54−60% dry weight cells) than that observed in the parental strain, showing that a blockade in the β-oxidation contributed efficiently to improve the synthesis of plastic polymers.

Biosynthetic Pathway. The biosynthetic pathway involved in the production of polyesters containing aromatic side groups in repeating units (PhPHAs) has already been published.[9] This β-oxidation route was integrated in a complex catabolic pathway (*phenylacetyl−CoA catabolon*)[21,22] involved in the degradation of different aromatic compounds.[9,23,24]

P. putida strain U *fad*A$^-$ β-oxidation mutant (blocked in the gene encoding the 3-ketoacyl−CoA thiolase) was obtained by disruption of the desired gene with the p*K*18::*mob* plasmid, as previously reported.[21] All mutants were analyzed by PCR[25,26] to define the insertional position of the disrupting element.

Chemical and Structural Characterization of PhPHAs. ^{13}C nuclear magnetic resonance (NMR) spectra were performed with a Varian Unity 500 NMR spectrometer at 40 °C operating at 125.70 MHz for ^{13}C measurements. The NMR spectra were recorded using a 25% (w/v) $CDCl_3$ solution and a long pulse delay of 4.0 s was inserted between cycles to allow time for full relaxation back to Boltzmann equilibrium of all carbon atoms analyzed. Improved quantitative information was extracted from inverse gated decoupled ^{13}C NMR spectra, where the nuclear Overhauser effect (NOE) was minimized. ^{1}H−^{13}C correlation spectra allowed the specific ^{13}C assignments. The quantitative analysis of the spectra was performed by using a PeakFit v.4 program (Jandel Scientific Software) to fit a baseline and then to create a sum of Lorentzian curves of different peak sites and different peak intensities. The calculated spectra were visually matched to the experimental spectra to obtain an optimal fit. The optimized individual peak intensities were finally estimated by experimental chemical shifts, experimental total peak intensities, and line widths.

Average molecular weights, molecular weight distributions, and the polydispersity index (M_w/M_n) were determined by using a Perkin-Elmer gel permeation chromatograph equipped with a refractive index detector series 200. A set of 10^4, 10^3, and 500 Å PL-gel columns conditioned at 25 °C were used to elute the samples (10 mg/mL) at 1 mL/min HPLC-grade chloroform flow rate. Ten samples of polystyrene standards having molecular weights ranging from 950 000 to 1340 Da were used for calibration.

Differential scanning calorimetry (DSC) experiments were carried out in a Perkin-Elmer DSC 7. Two scans were performed by using a 10 °C/min heating rate and a 320 °C/min cooling rate (quenching) between runs. Thermograms were obtained in the range −50 to +75 °C under nitrogen purge. The glass transition temperature (T_g) values were taken in the onset on the transition of second scans.

Results and Discussion

Structural Characterization and Monomer Composition of the Different PhPHAs. Proton-decoupled ^{13}C NMR experiments were carried out in order to elucidate the composition and chemical structure of the polymers purified from *P. putida fad*A$^-$ mutant. Figure 1 shows the chemical structure of the four polymers reported here, and Table 1 summarizes the types of copolymers obtained as a function of the carbon source used to growth *P. putida* U.

Feeding with a phenylalkanoic acid (with *n* carbons in the aliphatic segment), the bacterial biosynthetic pathway introduced a hydroxyl group in the β-position of the carboxylic groups (which were capped with *Co*A) and thus, this β-hydroxylated residue can be incorporated to the polymeric growing chain (if $n \geq 5$) or it can be deacetylated (by the enzymatic removing of *acetyl−Co*A) to give the corresponding phenylalkanoyl−*Co*A with two carbon atoms less ($n - 2$) (see Figure 1). This new phenylalkanoyl−*Co*A with two carbon atoms less can be again degraded to a $n - 4$ residue, or it can be incorporated to the polymerization process if $n \geq 5$ or $n \geq 6$ (for odd and even number of carbon atoms,

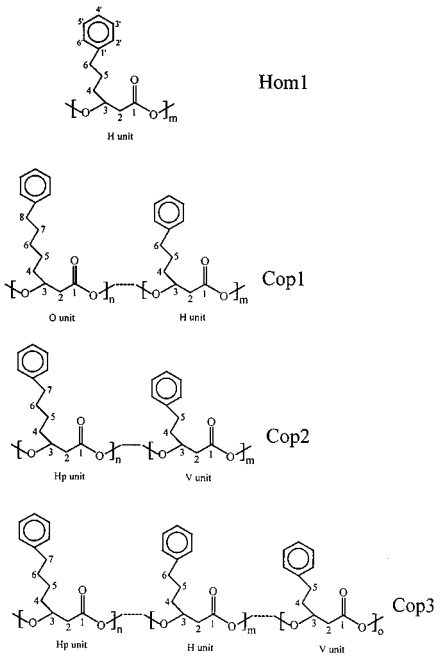

Figure 1. Chemical structure of the four different polyesters accumulated by the *P. putida* U *fad*A$^-$ mutant cultured as reported in the Experimental Section.

Table 1. Carbon Source Used, 6-Phenylhexanoic Acid (6PhHA), 7-Phenylheptanoic Acid (7PhHpA), 8-Phenyloctanoic Acid (8PhOA), and Products Composition[a]

	PHPhA units mole fraction				
carbon source	V	H	Hp	O	sample code
6PhHA		1			**Hom1**
8PhOA		0.27		0.73	**Cop1**
7PhHpA	0.23		0.77		**Cop2**
6PhHA + 7PhHpA	0.12	0.38	0.50		**Cop3**

[a] The units V, H, Hp, and O correspond to 3-hydroxy-5-phenylvalerate, 3-hydroxy-6-phenylhexanoate, 3-hydroxy-7-phenylheptanoate, and 3-hydroxy-8-phenyloctanoate, respectively.

respectively lower carbon sources cannot be polymerized). Finally, the β-oxidation leads to the formation of phenylacetyl−*Co*A or phenylpropionyl−*Co*A (for *n* even or odd respectively).[19]

A homopolymer (**Hom1**) formed by units of 3-hydroxy-6-phenylhexanoate (H units) was obtained when the *fad*A$^-$ mutant (see above) was cultured in a medium containing 6-phenylhexanoic acid (6PhHA) (10 mM) as a polyester precursor and 4-hydroxyphenylacetic acid (10 mM) for supporting bacterial growth, respectively. The ^{13}C NMR spectrum (Figure 2a) exhibited only the nine lines corresponding to H repeating units at δ = 169.21 ppm (H1), 141.74 (H1′), 128.30 (H2′,3′,5′,6′), 125.82 (H4′), 70.51 (H3), 39.01 (H2), 35.37 (H4), 33.35 (H6), and 26.76 (H5).

On the other hand, using 8-phenyloctanoic acid (8PhOA) (10 mM) as carbon source, the ^{13}C NMR analysis showed the formation of the copolymer named **Cop1**, containing 3-hydroxy-8-phenyloctanoate and 3-hydroxy-6-phenylhexanoate units, named O and H units, respectively. This result is in agreement with the proposed biosynthetic mechanism because the first deacetylation step led to the phenylhexanoic formation which can be readily incorporated to the polymerization. The further degradation steps render phenylbutanoic acid and phenylacetic acid which were not appropriate sources for the polymerization as discussed above.

The mole fraction (x_i) of both components in the copolymer **Cop1** was determined by integration of the ^{13}C signals of the carbonyl carbon 1, the tertiary carbon 3, and the aromatic carbon 4′. The relative peak intensities for the corresponding carbon resonances were estimated by the curve resolution program as described in the Experimental Section. The values so obtained were interpreted in terms of comonomer sequence distribution. These mole fractions are quoted in Table 2, being the average values x_O = 0.73 and x_H = 0.27. This means that this strain incorporated approximately four times more phenyloctanoate than the phenylhexanoate produced by the first deacetylation.

On the other hand, the splitting of the carbonyl region can be related to the sensitivity of these carbons to the chemical environment, that is, to the sequence distribution of repeating units along the macromolecular chains. This information is quite relevant because the macroscopic behavior and the material properties will strongly depend on the type of microstructure and, in particular, to elucidate if the macromolecules are block, alternating, random copolymers, or even homopolymer blends, becomes an essential matter. The sequence assignment was made according to the method of Doi et al.[27]

The carbonyl carbons split in four peaks, as shown in Figure 2b. Taking into account the integration of the peaks (quoted in Table 2) and using the spectrum of **Hom1** as reference, the two peaks shifted to lower field (169.31 and 169.34) have been assigned to phenyloctanoic units while the peaks at 169.25 and 169.23 ppm to phenylhexanoic units. The analysis of areas of these four peaks provided a clear result since the ratios 169.31/169.34 = 2.8 and 169.23/169.25 = 2.3 are very close to the compositional data observed in both cases. This result is in agreement with a random distribution of the two types of units and then the following assignment has been done, in term of diads and according to the increasing field: OH, OO, HH, and HO, where OH and HO sequences are not interchangeable because the carbonyl group distinguishes between right and left (−CO−O−alkyl−phenyl and −CO−alkyl−phenyl depending on the reference). The molar fraction of each diad was similar to that calculated by multiplying the compositions (OH = 0.77 × 0.23). This fact means that the strain incorporated hexanoic or octanoic units in a ratio 23/77 without any preferential sequence, following a statistically random sequence.

Thus, the bacteria incorporated approximately 4-fold more 3-hydroxy-8-phenyloctanoic than 3-hydroxy-6-phenylhexanoic in the copolymer. This different incorporation could be explained at least by two different hypothesis: (i) the substrate specificity of the polymerases which would recognize phenyloctanoic acid as a better substrate than phenylhexanoic acid; and (ii) by the existence of a higher intracellular concentration of 3-hydroxy-8-phenyloctanoic acid than 3-hydroxy-6-phenylhexanoic acid. Very recently, some of us have shown that *P. putida* mutants lacking the whole multienzyme system responsible of the β-oxidation of aromatic and aliphatic compounds (*fad*BA genes) are still able to accumulate PHAs (poly-3-hydroxy-*n*-alkanoates) and

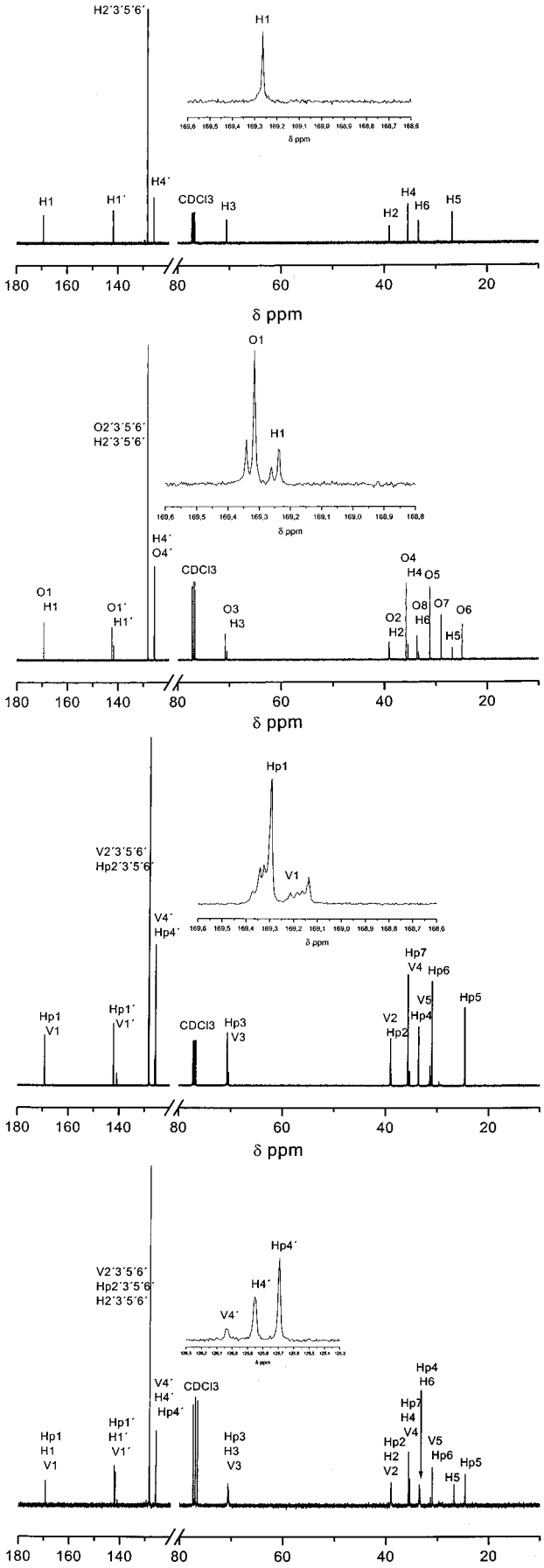

Figure 2. ^{13}C NMR chemical shift: (a) **Hom1**; (b) **Cop1**; (c) **Cop2**; (d) **Cop3**. Expanded view: carbonyl region in parts a−c and aromatic carbon 4′ in part d.

Table 2. Mole Fractions (x_i) Experimentally Determined by ^{13}C NMR for **Cop1**

carbon no.	δ (ppm)	assignment	x_{exp}
1	169.34	OH	0.19
	169.31	OO	0.54
	169.25	HH	0.08
	169.23	HO	0.18
4′	125.86	H	0.27
	125.63	O	0.73
3	70.80	O	0.73
	70.53	H	0.27

PHPhAs (poly-3-hydroxy-*n*-phenylalkanoates), but to a different extent, suggesting the existence of two different β-oxidation pathways in this bacterium. In the experiments reported above, we have used a mutant lacking the 3-ketoacyl−*Co*A thiolase activity. Although this mutation makes the degradation of fatty acids more difficult, bacteria were still able to catabolyse them but much more slowly. This slow in the β-oxidation rate could cause an accumulation of intermediates that were efficiently catabolised by the wild-type but not in the mutant since they cannot be degraded at the same rate. The accumulation of longer β-oxidation derivatives could explain the different percentage of the monomers found in the copolymer, even when the specificity of the polymerases were similar for both compounds (3-hydroxy-8-phenylalkanoyl−*Co*A and 3-hydroxy-6-phenylalkanoyl−*Co*A).

Peaks between 120 and 150 ppm in Figure 2b show clearly the presence of the phenyl pendant group: $\delta = 142.49$ (O1′), 141.78 (H1′), 128.33 (O2′,6′ and H2′,6′), 128.24 (O3′,5′ and H3′,5′), 125.86 (H4′), and 125.63 (O4′). The tertiary carbon 3 also displayed a pattern related to diads, and the peaks at 70.80 ppm, with a shoulder at 70.82, and 70.53 ppm with a shoulder at 70.55 were again attributed to: OO, OH, HO, and HH, respectively. The other peaks were assigned as follows: $\delta = 39.09$ (O2), 39.06 (H2), 35.81 (O4), 35.43 (H3), 33.74 (O8), 33.42 (H6), 31.24 (O5), 29.00 (O7), 26.81 (H5), and 24.92 (O6).

As mentioned above, when 7-phenylheptanoic acid (7PhHpA) (10 mM) was used as carbon source, the copolymer synthesized by the *fad*A$^-$ mutant, **Cop2**, was composed of 3-hydroxy-7-phenylheptanoate and 3-hydroxy-5-phenylvalerate units, named Hp and V units, respectively. This composition was elucidated from ^{13}C NMR analysis, being the spectrum and its assignments shown in Figure 2c. In this case, the calculated mole fractions were also obtained by integration of the carbon signals 1, 3, and 4′ being the average values $x_{Hp} = 0.77$ and $x_V = 0.23$.

The carbonyl carbon signals exhibited a particular pattern. Thus, the carbonyl carbon of Hp units displayed four peaks at $\delta = 169.34$, 169.33, 169.31, and 169.28 ppm, and applying the same strategy as described above, they could be attributed to the following triads: HpVV, HpVHp, HpHpV, and HpHpHp, respectively. For carbonyl carbon of V units, the peaks at $\delta = 169.20$, 169.17, 169.15, and 169.12 were assigned to the triads VVV, VVHp, VHpV, and VHpHp by comparison with the well-resolved Hp signals. However, the assignment of these triads has to be considered as tentative due to the poor resolution of V1 signals.

Table 3. GPC Analysis of the Four Polymers Accumulated by the *P. putida FadA*$^-$ Mutant Culture

sample	M_n	M_w	M_w/M_n
Hom1	216 000	475 000	2.20
Cop1	82 000	165 000	2.01
Cop2	67 000	156 000	2.32
Cop3	138 000	378 000	2.74

The phenyl pendant group was observed at $\delta = 142.18$ (Hp1′), 140.95, 140.93, 140.92, and 140.90 (V1′), 128.43 (V2′,6′), 128.31 (Hp2′,6′), 128.26 (V3′,5′), 128.25 (Hp3′,5′), 126.02 (V4′) and 125.68 (Hp4′). The tertiary carbons 3 displayed two peaks at $\delta = 70.67$ and 70.44 ppm attributed to Hp3 and V3, respectively. The presence of shoulders in those peaks indicated that in this case Hp3 and V3 were also related to the triads above-mentioned. The other peaks were assigned as follows: $\delta = 39.03$ (Hp2 and V2), 35.65 (Hp7), 35.36 (V4), 33.58 (Hp4), 31.40 (V5), 31.01 (Hp6), and 24.64 (Hp5).

Finally, when the source of carbon was a mixture of 7-phenylheptanoic acid (7PhHpA) (5 mM) and 6-phenylhexanoic acid (6PhHA) (5 mM), the copolymer, **Cop3**, had three types of repeating units: Hp, H, and V, as elucidated by ^{13}C NMR spectroscopy. The mole fraction values, calculated in this case only by integration of the well separated ^{13}C signals of the aromatic carbon 4′ showed in Figure 2d, were $x_{Hp} = 0.50$, $x_H = 0.38$, and $x_V = 0.12$. The election of this signal to determine the copolymer composition was due to that peaks of carbons in position 3 and 1 were partially overlapped. ^{13}C NMR chemical shift and assignments were 169.35, 169.32, 169.30 (triads Hp1), 169.29, 169.25, 169.24 (triads H1), 169.14 (V1), 142.21 (Hp1′), 141.77 (H1′), 140.97 (V1′), 128.45 (V2′,3′,5′,6′), 128.32 (Hp2′,3′,5′,6′), 128.27 (H2′,3′,5′,6′, 126.04 (V4′), 125.86 (H4′), 125.70 (Hp4′), 70.68 (Hp3′), 70.53 (H3), 70.45 (V3), 39.02 (Hp2, H2, and V2), 35.66 (Hp7), 35.42 (H4 and V4), 33.60 (Hp4), 33.40 (H6), 31.42 (V5), 31.03 (Hp6), 26.81 (H5), and 24.65 (Hp5). In the same way as for the precedent copolymers, we assumed that this **Cop3** also consisted of a random distribution of Hp, H, and V units.

Molecular Weights and Thermal Properties. The average molecular weight values of PHPhAs samples, determined by gel permeation chromatography (GPC), are shown in Table 3. The polydispersity values of these polyesters were in the range 2.01−2.74. The decrease of the average molecular weight, M_n and M_w, of these three copolymers analyzed with respect to the homopolymer was observed. It indicated that the chemical structure of the feeding medium noticeably affected the production of poly(β-hydroxyphenylalkanoates) from this strain. Although there was not a detectable effect on the stereoselectivity of polymer chains, there was a sensible effect on the macromolecular size of the corresponding copolymer systems. It was probably correlated with the synthetic mechanism described above and the effect of the methylene side chain length on the kinetics of the polymerization process. However, the M_n and M_w values quoted in Table 3 were high enough to consider that the thermal behavior of the copolymer systems do not depend on the molecular weight or the molecular weight distribution.

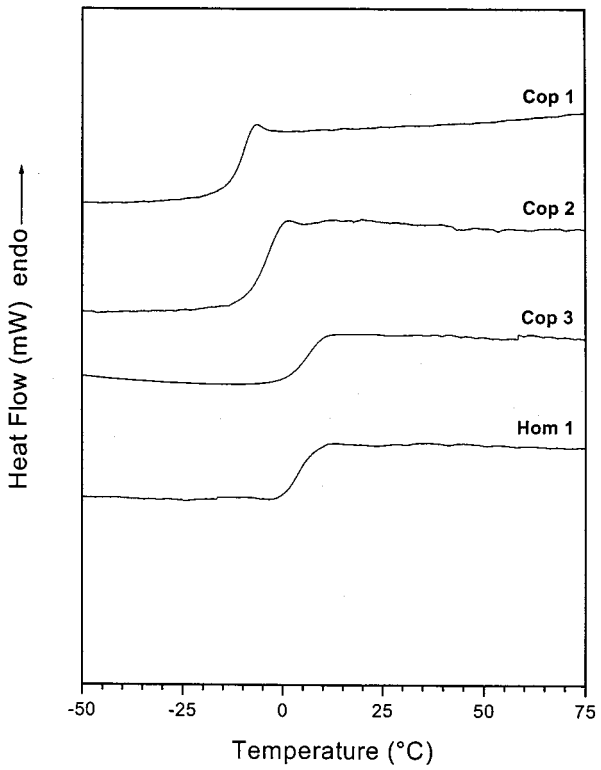

Figure 3. DSC thermograms (second scan) of biosynthesized polymers.

The thermal properties were determined by using differential scanning calorimetry (DSC). The first scan of DSC thermograms did not display any crystalline melting endotherm, and the second one, after quenching (shown in Figure 3) demonstrated that all the studied PhPHAs were amorphous. **Hom1**, poly(hydroxyphenylhexanoate) exhibited a T_g at −1.3 °C, a value lower than the reported for the homopolymer having one methylene unit less, PHPhV, which presented a T_g of 13 °C as described by Fritzsche et al.[11] The copolymer **Cop1** had a T_g at −14.8 °C due to the presence of a fraction of longer side alkyl chains (O units) whereas **Cop2** showed a T_g at −11.2 °C and **Cop3** displayed this transition at −8.2 °C. These T_g values were higher than the corresponding to other PHAs with *n*-alkyl pendant groups reported by Kim et al.,[713] which had T_g values at lower temperatures, in the range of −40 to −25 °C. The T_g values followed the order **Cop3** > **Cop2** > **Cop1**, and it was attributed to the increasing flexibility of macromolecular segments by the introduction of structural irregularities and alkyl side chains longer than three methylene groups.

The essential amorphous character of the copolymer samples seems to be related to the random sequence of units present in all the copolymers studied in this work. The influence of the stereoregularity on the crystallinity was reported to be negligible, even in the homopolymer PHPhV which has a highly ordered, isotactic structure.[28] In this case, the stereoregularity should provide for an ordered packing structure in the solid state, but the very low crystallinity and a small endothermic heat of fusion (about 0.4 J/g) at 58 °C gave clearly unusual results.[16]

Conclusions

The above-reported results evidenced that the use of genetically engineered *P. putida* could have an interest for the preparation of new biodegradable and biocompatible polymers for biomedical applications.[2,3,29]

Four different PHPhAs (one homopolymer and three copolymers) were produced when the *fad*A$^-$ mutant of *P. putida* U was cultured in a chemically defined medium containing several ω-phenylderivatives of fatty acids. The ability of this strain to synthesize a large number of polymers together with the fact that a strong intracellular amount of polymer is accumulated by some of their β-oxidation mutants, could allow the industrial production of large quantities of other polymers in which both the monomer composition and the relative percentage of monomers can be modified.

The microstructural analysis by NMR spectroscopy revealed that there was a random distribution of the ω-phenyl macromolecular chains, independent of the molecular structure and the composition of the feed medium. In all the cases, high-molecular weight polymer systems were obtained. DSC analysis showed that these copolymers were amorphous, which was consistent with the presence of an intrinsic random distribution of monomer units. The measured T_g values were relatively low, and the copolymers can offer interesting opportunities for the design of new controlled delivery systems or for the preparation of composites with biomedical applications.

In addition, these studies open new possibilities for the synthesis of polymers which, as a function of their physicochemical properties, could have many biotechnological applications. Some of these approaches are currently in progress.

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References and Notes

(1) Byron, D. *Trends Biotechnol.* **1987**, *5*, 246.
(2) Steinbüchel, A. Polyhydroxy alkonates. In *Biomaterials*; Byron, D., Ed.; M. Stockton Press: New York,1991.
(3) Steinbüchel, A. In *Biomaterials: Novel Materials from Biological Sources*; Byron, D., Ed., Stockton, New York, 1991; pp 124−213.
(4) Steinbüchel, A. *Curr. Opin. Biotechnol.* **1992**, *3*, 291−297.
(5) Steinbüchel, A.; Hustede, E.; Liebergesell, M.; Pieper, U.; Timm, A.; Valentin, H. *FEMS Microbiol. Rev.* **1992**, *103*, 217−230.
(6) Byron, D. In *Plastics from microtubes: Microbial synthesis of polymers and polymer precursors*; Mobley, D. P., Ed.; Hanser: Munich, Germany, 1994; pp 5−33.
(7) Huisman, G. W.; Wonink, E.; Meima, R.; Kazemier, B.; Terpstra, P.; Witholt, B. *J. Biol. Chem.* **1991**, *266*, 2191−2198.
(8) Schirmer, A.; Jendrossek, D.: Schlegel, H *Appl. Environ. Microbiol.* **1993**, *59*, 1220−1227.
(9) García, B.; Olivera, E. R.; Miñambres, B.; Fernández-Valverde, M.; Cañedo, L. M.; Prieto, M. A.; García, J. L.; Martínez, M.; Luengo, J. M. *J. Biol. Chem.* **1999**, *274*, 29228−29241.
(10) Steinbüchel, A.; Valentin, H. E. *FEMS Microbiol. Lett.* **1995**, *128*, 219−228.
(11) Fritzsche, K.; Lenz, R. W.; Fuller, R. C. *Makromol. Chem.* **1990**, *191*, 1957−1965.
(12) Gross, R. A.; DeMello, C.; Lenz, R. W.; Brandl, H.; Fuller, R. C. *Macromolecules* **1989**, *22*, 1106−1115.
(13) Kim, Y. B.; Lenz, R. W.; Fuller, R. C. *Macromolecules* **1991**, *24*, 5256−5260.
(14) Ritter, H.; Gräfin von Spee, A. *Macromol. Chem. Phys.* **1994**, *195*, 1665−1672.
(15) Kim, Y. B.; Lenz, R. W.; Fuller, R. C. *Macromolecules* **1992**, *25*, 1852−1857.
(16) Kim, Y.; Kim, D. Y.; Rhee, Y. H. *Macromolecules* **1999**, *32*, 6058−6064.
(17) Kim, Y.; Rhree, Y. H.; Han, S.-H.; Heo, G. S.; Kim, J. S. *Macromolecules* **1996**, *29*, 3432−3435.
(18) Hazer, B.; Lenz, R. W.; Fuller, R. C. *Polymer* **1996**, *37*, 5951−5957.
(19) Martínez-Blanco, H.; Reglero, A.; Rodríguez-Aparicio, L. B.; Luengo, J. M.*J. Biol. Chem.* **1990**, *265*, 7084−7090.
(20) Lageveen, R. G.; Huisman, G. W.; Preusting, H.; Ketelaar, P.; Eggink, G.; Witholt, B. *Appl. Environ. Microbiol.* **1988**, *54*, 2924−2932.
(21) Olivera, E. R.; Miñambres, B.; García, B.; Muñiz, C.; Moreno, M. A.; Fernández, A.; Díaz, E.; García, J. L.; Luengo, J. M. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 6419−6424.
(22) Luengo, J. M.; García, J. L.; Olivera, E. R. *Mol. Microbiol.* 2001, in press.
(23) Fernández, A.; Miñambres, B.; García, B.; Olivera, E. R.; Luengo, J. M.; García, J. L.; Díaz, E. *J. Biol. Chem.* **1998**, *273*, 25974−25986.
(24) Luengo, J. M.; García, J. L.; Olivera, E. R. *Mol. Microbiol.* **2001**, in press.
(25) Saiki, R.; Scharf, S.; Faloona, F.; Mullis, K. B.; Horn, G. T.; Ehrlich, H. A.; Arnheim, N. *Science* **1985**, *230*, 1350−1354.
(26) Mullis, K. B.; Faloona, F. A. *Methods Enzymol.* **1987**, *155*, 335−350.
(27) Kamiya, N.; Yamamoto, Y.; Inoue, Y.; Chûjô, R.; Doi, Y. *Macromolecules* **1989**, *22*, 1676−1682.
(28) Curley, J. M.; Hazer, B.; Lenz, R. W.; Fuller, R. C. *Macromolecules* **1996**, *29*, 1762−1766.
(29) Lenz, R. W. *Adv. Polym. Sci.* **1993**, *107*, 1.

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