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Poly(hydroxyalkanoate) biosynthesis from triglyceride substrates

Abstract The biosynthesis of poly(hydroxyalkanoates) (PHA) by *Pseudomonas resinovorans* from triglyceride substrates was investigated. Each triglyceride, whether animal fat or vegetable oil, supported cellular growth to relatively high average cell yields (3.3 ± 0.2 g/l). PHA yields ranged from 1.1 g/l to 2.1 g/l, representing approximately 45% of the bacterial cell dry weight. The repeat-unit composition of the polymers was determined by gas chromatography (GC) and GC/mass spectrometry of the β-hydroxyalkanoate methyl esters from the hydrolyzed polymers. With the exception of PHA from soybean oil (PHA-soy), each polyester was composed of β-hydroxyacyl moieties with chain lengths ranging from C_4 to C_{14}, with C_8 and C_{10} being the predominant species. PHA-soy contained an additional fraction (2%) of C_{16} monomers. The alkyl side-chains of the PHA contained varying degrees of unsaturation. PHA from coconut oil was composed entirely of saturated side-chains, whereas PHA-soy contained 4.2 mol% olefinic groups in its side-chains. The increase in the degree of side-chain unsaturation caused decreased melting temperatures, enthalpies of fusion, and glass transition temperatures. The molar masses of the polymers were relatively constant and ranged from 6.5×10^4 to 10.1×10^4 g/mol.

Introduction

Poly(hydroxyalkanoates) (PHA) are naturally occurring, optically active polyesters that accumulate in numerous bacteria as carbon and energy storage materials (Anderson and Dawes 1990; Brandl et al. 1990; Doi 1990). In most cases the polymers contain β-linked repeat units and are compositionally distinct on the basis of side-chain structure (Steinbuchel and Valentin 1995). Recently there has been significant interest in the use of PHA as biodegradable thermoplastics. Because they are viewed as "environmentally friendly," they are being studied as potential replacements for synthetic plastics in several applications. One major drawback to the use of these polymers is the production cost. Generally, the cost to produce a given PHA polymer on an industrial scale is greater than that for a comparable synthetic polymer. To make PHA production more economical, two avenues can be pursued: (1) produce PHA with properties that allow for use in unique applications, or (2) lower the production costs either by increasing polymer yields or by the use of less expensive substrates. The latter possibility (and to some extent the former) can be achieved by using agricultural triglycerides as carbon substrates.

It is known that several bacteria (primarily pseudomonads) produce medium-chain-length PHA (MCL-PHA) from fatty acids (Brandl et al. 1988; Cromwick et al. 1996; Eggink et al. 1993); however, only recently have intact triglycerides been considered as substrates for PHA production. To date, only three bacterial species have been shown to produce MCL-PHA from triglycerides. These include *Aeromonas caviae* (Shimamura et al. 1994; Shiotani and Kobayashi 1993), *Pseudomonas aeruginosa* (Eggink et al. 1995), and *Pseudomonas resinovorans*, which produced an MCL-PHA from tallow (Cromwick et al. 1996). On the basis of these results, a number of unique PHA were synthesized from other triglyceride substrates using *P. resinovorans* as the biocatalyst. These polymers were characterized by gas

chromatography mass spectrometry (GC/MS), nuclear magnetic resonance spectroscopy (NMR), gel-permeation chromatography (GPC), and differential scanning calorimetry.

Materials and methods

Strain information and preservation

A lyophilized cell culture of *Pseudomonas resinovorans* NRRL B-2649 was obtained from NCAUR, ARS, United States Department of Agriculture, Peoria, Ill. Stock cultures were produced in 250-ml shake flasks by growing the organism in 100 ml nutrient broth (Difco Laboratories, Detroit Mich.) (pH 7.0) at 30 °C with shaking at 250 rpm. At late log phase (approx. 16 h) the culture was diluted 1:2 with sterile 20% glycerol and the organisms aseptically transferred to sterile cryogenic vials. The vials were placed in a solid CO_2/ethanol bath for 15 minutes and stored at −70 °C until use.

Fermentations

Experiments were conducted under two-stage fermentation conditions. Nutrient broth was prepared in 500-ml volumes in 1-l conical flasks and autoclaved to sterilize. The flasks were inoculated with a 0.1% inoculum from a thawed cryovial. Bacterial growth was carried out at 30 °C and 250 rpm, for 24 h in an Innova 4300 orbital shaker with a throw of 2.5 cm (New Brunswick Scientific, Edison N.J.). The cells were harvested by centrifugation (8000 × *g*, 20 min) and washed with sterile deionized water. The cell dry weight of these first-stage cultivations was approximately 1.0 g/l.

PHA production was carried out in 500 ml Medium E* (for medium composition see Brandl et al. 1988; Cromwick et al. 1996). The triglyceride substrates were heated to 60 °C for 30 min and added to sterile Medium E* at a concentration of 1% (w/v). The washed stage-1 cells were then aseptically transferred into the medium. Polymer production was carried out by cultivation of *P. resinovorans* at 30 °C and 250 rpm for 48 h. Aliquots of each culture were periodically removed to monitor the cell viability by the pour-plate method using plate-count agar (Difco Scientific, Detroit, Mich.), and to inspect the cells by phase-contrast microscopy for polymer inclusion bodies. After 48 h the cultures were terminated. Cell dry weights were determined in triplicate by removing 30-ml aliquots, pelleting the cells by centrifugation at 4 °C (8000 × *g*, 20 min), and washing the cell pellets with deionized water. The washed cells were then transferred to preweighed aluminum pans, dried in a 100 °C oven overnight and reweighed. In addition, the remaining culture was placed into preweighed centrifuge bottles and the cells pelleted by centrifugation as above. After the cell pellets had been washed twice with water, the cells were lyophilized to a constant weight (24–36 h) and the bottles reweighed. Unused fats (solids) were removed from the cultures prior to centrifugation while unused oils concentrated at the air/liquid interface upon centrifugation and were removed either with the supernatant or by wiping the walls of the bottles with paper towels.

PHA isolation and purification

PHA isolation was by Soxhlet extraction in chloroform according to the method of Cromwick et al. (1996). After isolation, each PHA was purified by solid-phase extraction using Sep-Pak Plus cartridges (Waters Corporation, Milford, Mass.) containing 690 mg silica gel. The cartridges were washed with 10 ml dichloromethane, after which approximately 20 mg PHA was added as a 2% solution (w/v) in dichloromethane. The impurities were eluted with dichloromethane (five fractions of 2 ml). The PHA polymer was then eluted with ethyl acetate (seven fractions of 2 ml). Each fraction was analyzed by TLC to determine the presence (or absence) of triglyceride, and/or free fatty acids. Each fraction was spotted (approximately 2 µl) onto silica gel G TLC plates (Analtech Inc., Newark, Del.), and developed in hexane/ether/formic acid (80:20:2, v/v/v). After air drying, the plates were sprayed with 50% sulfuric acid and heated.

Polymer characterization

PHA repeat unit compositions were determined by gas chromatography (GC) and GC/mass spectrometry (GC/MS) of the β-hydroxymethyl esters. Samples were prepared according to the procedure of Brandl et al. (1988) and analyzed by GC and GC/MS using conditions reported previously (Cromwick et al. 1996).

Molar mass averages were determined by gel permeation chromatography (GPC) after the procedure of Cromwick et al. (1996). Polystyrene standards (Polyscience Corporation, Warrington, Pa.) with molar masses ranging from 1.28×10^3 g/mol to 3.15×10^6 g/mol and low polydispersities, were used to generate a calibration curve from which molar masses were determined with no further corrections. Chloroform was used as the eluent at a flow rate of 1 ml/min. The sample concentration and injection volume were 0.5% (w/v) and 50 µl respectively.

Carbon (^{13}C) nuclear magnetic resonance (NMR) spectra of the PHA samples were recorded on a Varian 200-MHz Gemini instrument operating at 50 MHz. Chemical shifts are reported in parts per million (ppm) relative to chloroform as an internal reference at 77.00 ppm. Spectral acquisitions were conducted on 5% (w/w) polymer solutions in $CDCl_3$ using the following parameters: temperature 35 °C, pulse width 15 µs, 8k data points, 2.8 s relaxation delay, 48k transients, and 5.0 line broadening.

The glass transition (T_g), melting temperature (T_m), and the enthalpy of fusion (ΔH_m) were measured for each PHA sample using a Perkin-Elmer DSC-7 (Norwalk, Conn.) differential scanning calorimeter at a heating rate of 10 °C/min with a dry-nitrogen purge. The instrument was calibrated using an indium standard with a melting temperature of 156 °C. The pure PHA (approximately 5 mg) were placed into open aluminum pans and heated from −60 °C to 80 °C. The T_g was taken as the midpoint temperature and the T_m as the peak of the melting endotherm.

Results

It was reported that *P. resinovorans* had extracellular esterase activity when tallow was used as the substrate (Cromwick et al. 1996). The inability of other PHA-producing pseudomonads to grow and produce PHA from triglycerides suggested that the esterase (lipase) activity was necessary for PHA biosynthesis by *P. resinovorans*. In this study, six additional triglycerides (lard, butter oil, olive oil, high-oleic-acid sunflower oil, coconut oil, and soybean oil) were screened as substrates for PHA production. Each triglyceride supported cell growth to between 1.1×10^{10} and 1.4×10^{10} colony-forming units (cfu)/ml after 48 h (data not shown). In each case the cells were viewed under a phase-contrast microscope for the presence of phase-bright inclusions, evidence of polymer production. *P. resinovorans* produced an MCL-PHA from each triglyceride. This was made evident by the presence of one or more PHA granules per bacterium that appeared to constitute approximately 50% of the cell volume. The cells were harvested by centrifugation, and the cellular biomass, polymer yield (determined gravimetrically), and polymer production (as a percentage of the cell dry weight)

Table 1 Cell dry weights and poly(hydroxyalkanoate) (*PHA*) polymer content of *Pseudomonas resinovorans* grown on triglyceride substrates. All values are shown ±SD ($n = 3$). Crude cell yield is given as cell dry weight; the crude PHA yield is calculated by multiplying the cell yield (g/l) by the PHA content (% dry weight) of the cells. Averages do not include oleic acid values

Substrate	Crude cell yield (g/l)	PHA content (% dry weight)	Crude PHA yield (g/l)
Control			
Oleic acid	3.8 (±0.3)	48.9 (±2.8)	1.9 (±0.2)
Animal fats			
Tallow	3.0 (±0.2)	39.8 (±2.0)	1.2 (±0.1)
Lard	3.6 (±0.3)	47.4 (±3.0)	1.7 (±0.2)
Butter oil	3.6 (±0.1)	47.0 (±2.3)	1.7 (±0.1)
Vegetable oils			
Olive	3.4 (±0.2)	43.1 (±2.2)	1.5 (±0.2)
Sunflower (high oleic)	3.1 (±0.2)	41.2 (±1.8)	1.3 (±0.2)
Coconut	3.8 (±0.3)	51.0 (±3.2)	1.9 (±0.2)
Soybean	2.9 (±0.2)	44.5 (±3.4)	1.3 (±0.2)
Averages	3.3 (±0.2)	44.9 (±2.6)	1.5 (±0.2)

calculated (Table 1). Because triacylglycerol substrates have solubilities similar to MCL-PHA, they must be physically removed prior to cell drying and weighing (see Materials and methods). In this way average cell dry weights of 3.3 ± 0.2 g/l were achieved. In general, polymer yields were between 40% and 50% of the cell dry weight. This was a 200% increase from the values previously reported for tallow (Cromwick et al. 1996), and resulted in average PHA yields of 1.5 ± 0.2 g/l (Table 1).

Prior to characterization, each PHA polymer was purified by passage through a Sep-Pak cartridge (see Materials and methods). Thin-layer chromatography (TLC) of the dichloromethane fractions revealed the presence of organic material with R_f values of 0.50 and 0.68, corresponding to free fatty acids and triglycerides respectively. The ethyl acetate fractions gave a single spot at the origin corresponding to the PHA polymer. The GPC chromatograms of both crude and pure PHA from lard (PHA-lard) are shown in Fig. 1. The crude polymer contained an impurity with an elution volume of 37.2 ml. A solution of lard injected into the GPC columns also gave a peak at 37.2 ml, supporting the idea that the extraneous peak in the crude PHA polymers was unused lard and not lower-molecular-mass polymer. This peak was absent in the pure PHA polymer. GC analysis of crude PHA polymer showed the presence of normal fatty acid methyl esters (components of the triglyceride substrates). These peaks were absent in the pure PHA. Finally, molecular mass analyses of the crude and purified PHA were similar, indicating that polymer fractionation had not occurred.

The repeat-unit compositions of the polymers were determined by GC and confirmed by GC/MS of the methyl esters obtained by acid hydrolysis of the PHA (Table 2). The electron-impact mass spectrum of β-hydroxydecanoate methyl ester is given in Fig 2. Each β-hydroxyalkanoate methyl ester contained an ion fragment at $m/z = 103$ due to ions formed by 3, 4 cleavage. The chain length of each methyl ester was based on GC retention time and confirmed by mass spectrometry. Lack of silylation results in a loss of water, and methanol in these molecules. Therefore, molecular ions (M^+) were determined on the basis of $m/z = M^+ - 18$ (loss of water) and $m/z = M^+ - (18 + 32)$ (loss of water + methanol) for each methyl ester. Regardless of the substrate, the predominant repeat units in each MCL-PHA were β-hydroxyoctanoate and β-hydroxydecanoate. With the exception of PHA from coconut and soybean oils (PHA-coco and PHA-soy), the compositions of the polymers were relatively similar, with repeat units that ranged in carbon chain length from C_4 to C_{14} with some mono- and di-unsaturation in the alkyl side-chains. PHA-coco was composed of saturated side-chains. This was undoubtedly due to the low levels of unsaturation in the substrate itself (oleic acid, 4.6%; linoleic acid, 0.9%). The PHA-soy repeat unit composition was more complex than that of the other PHAS. It contained additional fractions of tri-unsaturated C_{14}, as well as di-unsaturated C_{16} monomers and

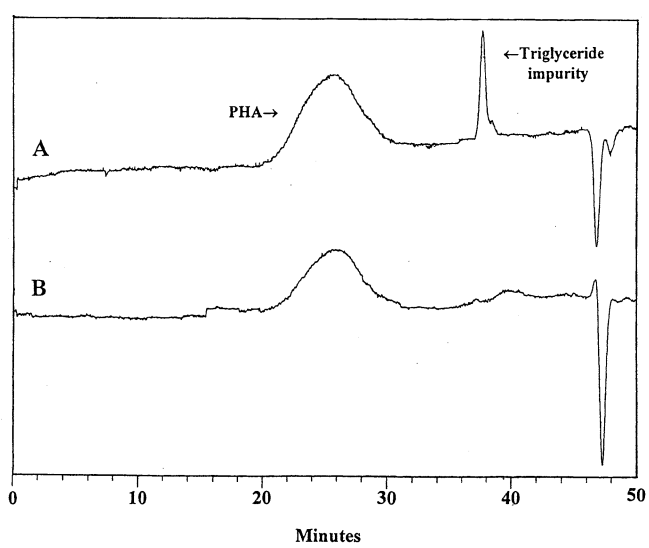

Fig. 1 Gel-permeation chromatograms of poly(hydroxyalkanoate) (*PHA*) from lard: **A** crude polymer, **B** purified polymer

Table 2 Repeat-unit composition of poly(hydroxyalkanoate) polymers isolated from *P. resinovorans* cultures grown on triglyceride substrates. Results show the average percentage ($n=2$) as determined by gas chromatography and GC/mass spectrometry of the β-hydroxymethyl esters obtained by acid hydrolysis of the PHA polymers. *Tr* trace, less than 1%. PHA from soybean oil also contained $C_{14:3}$ (2%), and $C_{16:2}$ (2%)

Substrate	β-Hydroxymethyl ester (%)											
	$C_{4:0}$	$C_{6:0}$	$C_{8:0}$	$C_{8:1}$	$C_{10:0}$	$C_{10:1}$	$C_{12:0}$	$C_{12:1}$	$C_{12:2}$	$C_{14:0}$	$C_{14:1}$	$C_{14:2}$
Control												
Oleic acid	2	10	29		32		13	Tr		2	13	
Animal fats												
Tallow	Tr	7	31		41		11	3		3	5	
Lard	Tr	7	26		34		14	4		4	8	3
Butter oil	Tr	9	31		35		15	Tr		4	5	
Vegetable oils												
Olive	1	8	29		33		14	1		3	10	1
Sunflower (high oleic)	1	9	31		32		12	2		2	10	2
Coconut	Tr	8	37		35		17			3		
Soybean	Tr	8	29	Tr	30	2	5	9	2	2	2	8

had approximately twice as many unsaturated side-chains as the other PHAS.

The PHA were also studied by ^{13}C-NMR (50 MHz). Figure 3 shows a typical spectrum of MCL-PHA, in particular PHA-coco, PHA-tallow, and PHA-soy. The chemical-shift assignments are based on those reported previously (Abe et al. 1994; De Waard et al. 1993; Huijberts et al. 1994). With the exception of the region between 120 ppm and 140 ppm, the chemical shifts of the peaks were identical apart from slight differences in their relative intensities. From the chemical shift intensities between 120 ppm and 140 ppm (olefinic carbons) it was confirmed that PHA-coco contained only saturated side-chains, whereas the side-chains from PHA-soy were highly unsaturated.

The degree of unsaturation within the PHA side-chains affected the thermal properties of the polymers. The thermal properties of each MCL-PHA are given in Table 3. As the degree of side-chain unsaturation increased, the melting temperature (T_m), enthalpy of fusion (ΔH_m), and glass transition temperature (T_g) decreased. With the exception of PHA-soy, which was amorphous, T_m values decreased from 48 °C to 39 °C (ΔH_m values between 9.5 J/g and 12.3 J/g), while T_g values decreased from −38 °C to −46 °C. Molar masses, determined by GPC, are listed in Table 3. Number-average molar masses (M_n) were relatively constant and ranged from 6.5×10^4 to 10.1×10^4 g/mol with polydispersities ranging from 1.6 to 1.8.

Discussion

At present, a number of pseudomonads, including *P. oleovorans* (Brandl et al. 1988; Gross et al. 1989), and *P. putida* (Eggink et al. 1993; Huijberts et al. 1994; Tan et al. 1997), have been reported to produce MCL-PHA from free fatty acids. Relatively few organisms, however,

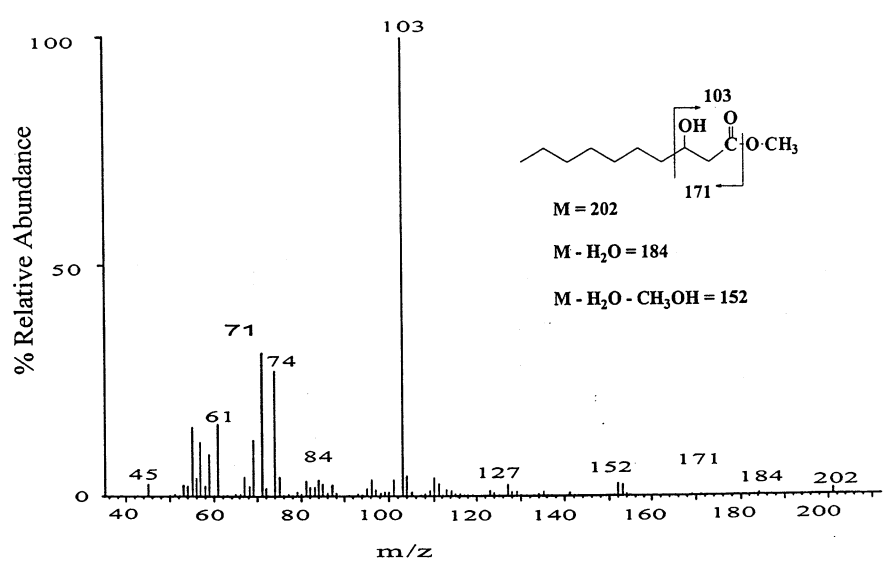

Fig. 2 Electron-impact mass spectrum of β-hydroxydecanoate methyl ester. The presence of an ion fragment with $m/z = 103$ is indicative of a β-hydroxyalkanoate methyl ester

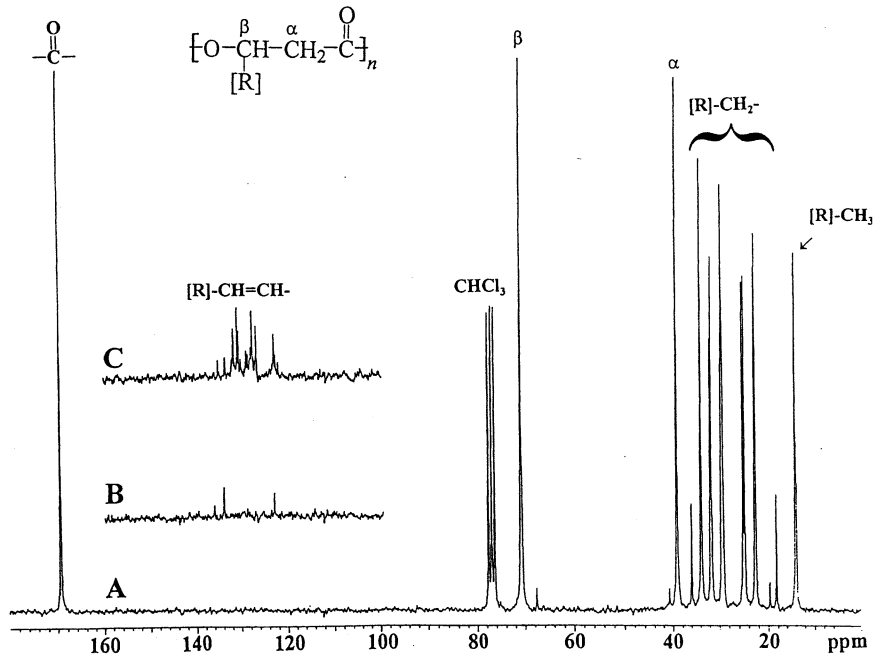

Fig. 3 The ^{13}C-NMR spectrum of poly(hydroxyalkanoate) polymers isolated from *P. resinovorans* grown on **A** coconut oil, **B** tallow, **C** soybean oil

produce PHA from intact triglycerides. Recently, we reported the biosynthesis of a MCL-PHA by *P. resinovorans* from tallow (Cromwick et al. 1996). In that report, extracellular esterase (lipase) activity was established and linked to PHA biosynthesis. With that in mind, the production and characterization of additional MCL-PHA by *P. resinovorans* on other triglycerides was explored.

In addition to triglycerides, most animal fats and vegetable oils contain small concentrations of sterols (approx. 0.1%–0.5%), present both as free sterols and as esters, and tocopherols (primarily α and γ). While utilization of these compounds for growth cannot be ruled out, high cell and polymer yields indicated that the triglycerides present in each fat and oil were the primary carbon sources used by the bacterium. In addition, it was determined, on the basis of relatively equal cell and polymer yields, that the bacterium exhibited no preference between solid (fats) and liquid (oils) substrates. This was further suggested by the similar molecular masses of each PHA. It has been shown that the molecular masses of microbially produced polymers vary as a function of the stage of growth when the cells are harvested (Birrer et al. 1994). Because the polymerization efficiency of a bacterial system is based on enzyme activity or number of enzymes present, it is likely that relatively equal molecular masses are the result of a similar polymerization process.

The composition of MCL-PHA from long-chain fatty acids is controlled by the specificity of the PHA-syn-

Table 3 Molar masses (M_n) and thermal properties of poly(hydroxyalkanoate) polymers produced by *P. resinovorans* cultures grown on triglyceride substrates. All molar masses were determined by gel-permeation chromatography. The PHA from soybean oil was amorphous and showed no melting transition

Substrate	Molecular properties			Thermal properties		
	$10^{-4} \times M_n$ (g/mol)	M_w/M_n	Olefinic groups (mol%)	T_m (°C)	ΔH_m (J/g)	T_g (°C)
Control						
Oleic acid	7.3	2.00	1.3	42	10.7	−44
Animal fats						
Tallow	8.2	1.73	0.8	44	11.4	−45
Lard	8.4	1.66	1.7	39	9.5	−46
Butter oil	8.2	1.65	0.5	44	11.0	−43
Vegetable oils						
Olive	7.2	1.65	1.4	41	10.7	−45
Sunflower (high oleic)	6.5	1.72	1.6	41	10.0	−46
Coconut	10.1	1.63	0	48	12.3	−38
Soybean	7.0	1.81	4.2	–	–	−45

thesizing system, the structures of the fatty acids, and the degradation pathway for long-chain fatty acids (Eggink et al. 1993). Because each triglyceride contains differing ratios of saturated and unsaturated fatty acids, the composition of the MCL-PHA should vary to reflect the substrates and the enzymatic make-up of the organism (particularly those involved in β-oxidation of unsaturated fatty acids). Previous reports have shown that 3-hydroxyacyl-CoA intermediates from the β-oxidation of free fatty acids serve as precursors for PHA biosynthesis by *P. putida* (Eggink et al. 1993; De Waard et al. 1993; Lageveen et al. 1988). Because of the enhanced enzymatic specificity of the PHA synthase for 3-hydroxyoctanoyl-CoA and 3-hydroxydecanoyl-CoA, MCL-PHA generally contain a high concentration of C_8 and C_{10} moieties. Oleic acid ($C_{18:1}$-Δ^9) was used as the standard for PHA biosynthesis because, with the exception of coconut oil, each triglyceride was composed of a relatively large amount (between 21% and 77%) of oleic acid. As expected, the composition of the MCL-PHA made by *P. resinovorans* from oleic acid (PHA-OA) contained a high concentration of 3-hydroxyoctanoate and 3-hydroxydecanoate monomers and closely approximated the reported values from *P. putida* (De Waard et al. 1993). This suggested that the predominant β-oxidation pathway in *P. resinovorans* is similar to that present in *P. putida* and involves the isomerization of the *cis*-Δ^3 double bonds of 3-*cis*-dodecanoyl CoA to *trans*-Δ^2 double bonds by *cis*-Δ^3-enoyl-CoA isomerase (EC 5.3.3.8). This chemical reaction is widespread in both eukaryotes and prokaryotes; however, by itself, it does not explain the presence of β-hydroxytetradecanoate ($C_{14:0}$) in PHA-OA. Recently, it was reported that the presence of 5-*cis* double bonds in fatty acids inhibits further oxidation because of an unfavorable equilibrium. This results in a reduced conversion of 5-*cis*-enoyl-CoA to 3-*cis*-enoyl-CoA, thereby interrupting the conventional oxidation pathway (Tserng and Jin 1990). The presence of a small fraction (2%) of β-hydroxytetradecanoate in PHA-OA implied the presence of an NADPH-dependent 5-enoyl-CoA reductase (at least at basal levels), which catalyzes the removal of the 5-*cis* double bond from 3-hydroxy-5-*cis*-tetradecanoyl-CoA, resulting in the incorporation of a $C_{14:0}$ moiety into the polymer.

The composition of PHA from tallow and butter oil (PHA-tallow and PHA-butter) resembled the composition of PHA-OA. Unlike PHA-OA, the incorporation of β-hydroxytetradecanoate into PHA-tallow and PHA-butter was probably the result of β-oxidation of the long-chain saturated fatty acids that make up the triglycerides (including palmitic acid, and stearic acid). The composition of PHA-lard, PHA-olive, and PHA-sunflower also approximated PHA-OA; however, these polymers each had an additional β-hydroxytetradecadienoate ($C_{14:2}$) fraction present in their side-chains. The substrates lard, olive oil, and sunflower oil (high oleic acid) contained an appreciable concentration (between 8% and 11%) of linoleic acid ($C_{18:2}$-$\Delta^{9,12}$). The β-oxidation of unsaturated fatty acids with a double bond extending from an even-numbered carbon atom requires the involvement of a NADPH-dependent 2,4-dienoyl-CoA reductase (EC 1.3.1.34). This enzyme catalyzes the reduction of 2-*trans*-4-*cis*-decadienoyl-CoA, an intermediate in the β-oxidation of linoleic acid, to 3-*trans*-decenoyl-CoA, which is then isomerized to 2-*trans*-decenoyl CoA. The presence of this enzyme has been demonstrated in *P. putida* (Schulz and Kunau 1987) and explains the incorporation of β-hydroxytetradecadienoate and β-hydroxydodecenoate into the PHA polymers. The presence of these two repeat units, along with the absence of β-hydroxydecenoate, suggests that linoleic acid in *P. resinovorans* is oxidized via the reductase pathway.

PHA-coco contained saturated side-chains ranging from C_6 to C_{14}. This is a result of the low concentration (below 6% total) of unsaturated fatty acids in coconut oil. PHA-soy, however, contained a large percentage of unsaturated side-chains. Soybean oil is composed of 86% unsaturated fatty acids. These include, oleic acid (21%), linoleic acid (57%), and linolenic acid ($C_{18:3}$-$\Delta^{9,12,15}$) (8%). The high degree of polyunsaturation resulted in the additional incorporation of β-hydroxydecenoate, β-hydroxydodecadienoate, β-hydroxytetradecatrienoate, and β-hydroxyhexadecadienoate repeat units into PHA-soy. The presence of these monomers implied the concurrent activity of both the *cis*-Δ^3-enoyl-CoA isomerase as well as the NADPH-dependent 2,4-dienoyl-CoA reductase.

The thermal properties of the MCL-PHA from *P. resinovorans* compared favorably to published values obtained from MCL-PHA derived from *P. oleovorans* (Doi 1990; Gross et al. 1989) and *P. putida* (Tan et al. 1997) when grown on alkanoates and saponified palm kernal oil respectively. With the exception of PHA-soy, each MCL-PHA exhibited some degree of melting transition, indicating that each of the PHA was at least semi-crystalline. In contrast, the thermal properties of PHA-soy showed no melting transition, indicating that it was completely amorphous. This implied that the high degree of unsaturation in the side-chains of PHA-soy inhibited crystallization. It has been suggested that saturated MCL-PHA crystallize with the participation of both the backbone and side-chains in a layer-like order similar to other classes of polymers bearing long side-chains (Gross et al. 1989). The incorporation of *cis* double bonds into MCL-PHA results in the formation of "kinks" within the side-chains. These distortions disrupt the normal planar zig-zag pattern and allow a greater degree of conformational freedom, in much the same way that bacteria control membrane fluidity. This results in decreasing crystallinity as side-chain unsaturation increases.

In conclusion, it has been demonstrated that *P. resinovorans* produces MCL-PHA from triglyceride substrates. Polymer compositions can be manipulated on the basis of the substrate used, and can range from completely saturated (PHA-coco) to highly unsaturated

(PHA-soy). In addition, the data suggest that the bacterium has no preference for solid (fats) or liquid (oils) substrates. Because these polymers are tacky at room temperature, there are some limited potential applications for these polymers as adhesives. We are currently evaluating methods to increase the crystallinity of the polymers through blending with a more crystalline PHA (poly-3-hydroxybutyrate) to increase their potential applications.

Acknowledgements The authors thank Rob DiCiccio for his technical assistance throughout this study, as well as Dr. Alberto Nunez for performing the GC/MS analyses.

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