Enzymatic Polymerization and Characterization of New Poly(3-hydroxyalkanoate)s by a Bacterial Polymerase

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ABSTRACT: Two new 3-hydroxyalkanoates, 3-hydroxy-3-cyclopropylpropionate (3CyP3HP) and 3-hydroxy-4-chlorobutyrate (4Cl3HB), were prepared, and their coenzyme A (CoA) thioester derivatives were polymerized in aqueous solutions with the polymerase enzyme of a bacterium which can produce poly-3-hydroxyalkanoates (PHAs) as intracellular, reserve polymers. The polymerase used for the in vitro polymerization reactions was that of *E. shaposhnikovii* (EsPHAS), which was produced by a recombinant strain of *E. coli* harboring the appropriate gene. The polymerase of *R. eutropha* (RePHAS) was also evaluated for the in vitro polymerization of the two monomers, but it was inactive with the CoA thioester of 3CyP3HP. The propagation rates of the two monomers with the EsPHAS polymerase were 1.2 and 6.7 mol of monomer reacted per mole of enzyme catalyst per second of reaction time for 3CyP3HPCoA and 4Cl3HBCoA, respectively, and the number-average molecular weights of the PHAs obtained were 371 000 and 189 000, respectively. Both polymers were crystalline, and their crystallinity and NMR spectra indicated that both contained only one chiral center (100% isotactic) even though both of the monomers were racemic mixtures.

Introduction

Many types of bacteria produce biodegradable poly(hydroxyalkanoate) (PHA) polyesters as intracellular energy and carbon storage materials from a variety of carbon sources such as carbohydrates, alcohols, *n*-alkanes, alkanoic acids, and alkenoic acids.[1] The most common type of PHA is poly(3-hydroxybutyrate) (PHB), which has been extensively studied for different bacteria, but more than 90 different types of hydroxyacid units have been identified as constituents of PHAs,[2] including units containing branched alkyl,[3] cyano,[4] halogen,[5−8] phenyl,[9−11] phenoxy,[12,13] and olefin[14−21] groups. In most cases these PHAs are copolymers containing two or more different types of units which are produced by cometabolism within the cell.[22−24] All of these PHAs have three carbon atoms in the main chain, but they can be divided into one of two classes according to the length of the alkyl chain at the 3-position in the repeating unit, that is, either a short chain length PHA, in which the alkyl group has from one to two carbon atoms in the side chain, such as those produced by *Ralstonia eutropha*, or a medium chain length PHA with alkyl chains having more than three carbon atoms in the side chain, such as those produced by *Pseudomonas oleovorans*.

In most cases the PHAs are polymerized within the cell from the coenzyme A (CoA) thioester derivatives of the 3-hydroxyalkanoic acids by a PHA synthase (PHAS), which functions as a polymerase. The polymerization reaction in vivo is initiated and catalyzed by the enzyme to accumulate the PHA within the cells as inclusion bodies, and in vitro studies have shown that the structure and size of the monomer, or substrate, can greatly affect the rate of polymerization by the enzyme. Therefore, these polymerase enzymes may also be referred to as either a short chain length PHAS or a medium chain length PHAS. The polymerase of *R. eutropha* (RePHAS), which belongs to the former category and which has been the most studied for in vitro polymerization reactions, can be readily cloned, overexpressed in recombinant *E. coli*, and purified to homogeneity.[25,26] *R. eutropha* has been used for the industrial production of PHB and poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV) copolymers.

Investigations at the molecular genetic level have provided the necessary tools for developing the genetic engineering approach to producing PHAs in recombinant bacteria, such as *E. coli*,[27−29] and also in yeast[30] and in plants.[31,32] In addition, in vitro polymerization systems in aqueous solutions have been established using purified polymerases from *R. eutropha*, as noted above,[33,34] and from *Chromatium vinosum*[35,36] (EsPHAS) for the synthesis of PHAs. These systems can be used to examine some of the more fundamental aspects of PHA biosynthesis as well as to prepare new types of homopolymers, block polymers, and random copolymers. Such studies indicate that the in vitro polymerization processes are chain growth polymerization reactions which can form living polymers.[37,38] In this report we describe the chemical synthesis of two new monomers with short chain substituent groups and their in vitro polymerization to form two new PHAs: poly(3-hydroxy-3-cyclopropylpropionate) (PHCyPP) and poly(3-hydroxy-4-chlorobutyrate) (PHCB), using the newly purified, recombinant PHAS derived from *Ectothiorhodospira shaposhnikovii*.[39] All of the in vitro polymerization reactions were carried out in aqueous solutions by reacting the polymerase with the CoA thioester monomer.

Experimental Section

A. Monomer Synthesis. Meldrum's acid (2,2-dimethyl-1,3-dioxane-4,6-dione, Aldrich), acetyl chloride (Aldrich), propionyl

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chloride (Aldrich), cyclopropane carbonyl chloride (Aldrich), 2-chloroacetyl chloride (Aldrich), pyridine (Aldrich), anhydrous ethanol (Pharmco), trimethylaluminum (2.0 M in hexane) (Aldrich), thiophenol (Aldrich), coenzyme A sodium salt (Sigma), silica gel 60 for column chromatography (ICN-Biomed) were commercial products used without further purification. Dichloromethane was dried overnight with P_2O_5, filtered, dried overnight with CaH_2, refluxed, distilled, and stored over activated molecular sieves.

^{1}H NMR spectra were recorded in either chloroform-*d*, methanol-d_4, or water-d_2 solutions at 300 MHz (Bruker, DPX-300) referenced to TMS at 0.00 ppm with *J* values given in hertz. ^{13}C NMR spectra were recorded in chloroform-*d* at 75 MHz (Bruker, dPX-300) referenced to chloroform at 77.0 ppm.

3-Hydroxy-3-cyclopropylpropionate CoA (3CyP3HP-CoA). *Ethyl 3-Oxo-3-cyclopropylpropionate,* ***I*** *(R = C_3H_5).* To an ice cold, magnetically stirred solution of Meldrum's acid (7.81 g, 54.19 mmol) in anhydrous dichloromethane (37 mL) in a predried flask was added pyridine (8.57 g, 108.38 mmol) and a solution of cyclopropane carbonyl chloride (5.663 g, 54.17 mmol) in anhydrous dichloromethane (34 mL) at 0 °C under a nitrogen atmosphere. Stirring was continued for 1 h at 0 °C and 2 h at room temperature. The mixture was transferred to a separatory funnel, washed twice with 3% hydrochloric acid and twice with water, and dried over $MgSO_4$, and the solvent was evaporated under reduced pressure to give 10.29 g of crude acylated Meldrum's acid as a dark red oil. The crude acylated Meldrum's acid was refluxed for 3 h in anhydrous ethanol (80 mL), during which time CO_2 evolution was observed. Evaporation of the solvent to dryness afforded 7.70 g of crude ethyl ester as a red oil, 2.51 g of which was purified by flash column chromatography on silica gel 60 (0.032−0.063 mm, 20 × 3 cm diameter, eluent hexane/ethyl acetate = 2:1) to give 1.11 g of pure ethyl ester as a pale yellow oil; 44% yield with respect to the unpurified product. ^{1}H NMR (in $CDCl_3$): δ 4.21 (q, J = 7.1 Hz, 2H), 3.57 (s, 2H), 2.04 (m, 1H), 1.28 (t, J = 7.1 Hz, 3H), 1.12−0.9 (m, 4H). ^{13}C NMR (in $CDCl_3$): δ 203.29, 167.64, 61.67, 50.36, 21.14, 14.47, 12.07.

Ethyl 3-Hydroxy-3-cyclopropylpropionate, ***II*** *(R = C_3H_5).* To a magnetically stirred solution of sodium borohydride (0.756 g, 20.0 mmol) in anhydrous ethanol (10 mL) in a predried flask was added a solution of ethyl 3-oxo-3-cyclopropylpropioate, I (3.12 g, 20 mmol), in anhydrous ethanol (10 mL). Stirring was continued for 2 h at room temperature, and water (40 mL) was added to the solution. The mixture was transferred to a separatory funnel, extracted with dichloromethane twice, dried over $MgSO_4$, and evaporated under reduced pressure to give 1.71 g of 3-hydroxy-3-cyclopropylpropionate as a pale yellow oil. ^{1}H NMR (in $CDCl_3$): δ 4.18 (q, J = 7 Hz, 2H), 3.32 (m, 1H), 2.61 (m, 2H), 1.28 (t, J = 7.1 Hz, 3H), 0.95 (m, 1H), 0.55−0.22 (m, 4H). ^{13}C NMR (in $CDCl_3$): δ 173.14, 73.15, 61.08, 41.79, 17.19, 14.57, 3.56, 2.55.

Thiophenyl 3-Hydroxy-3-cyclopropylpropionate, ***III*** *(R = C_3H_5).* To anhydrous dichloromethane (24 mL) magnetically stirred in a dried flask in an ice bath was added 2 M trimethylaluminum (8 mL, 16 mmol) under a nitrogen atmosphere, and subsequently thiophenol (8 mmol) was added slowly. Stirring was continued for $^1/_2$ h at room temperature, and a solution of ethyl 3-hydroxy-3-cyclopropylpropionate, **II**, in anhydrous dichloromethane (24 mL) was added. The reaction was monitored by TLC (eluent hexane, ethyl acetate 2:1). The mixture was quenched in dichloromethane (80 mL), and a 3% aqueous solution of hydrochloric acid was added until no further bubbling occurred (about 80 mL). The mixture was transferred to a separatory funnel, washed twice with 3% hydrochloric acid solution and twice with saturated brine, dried over $MgSO_4$, and evaporated under reduced pressure to give 2.35 g of crude thiophenyl ester as a dark yellow oil, 2.25 g of which was purified by flash column chromatography on silica gel 60 (20 × 3 cm diameter, eluent hexane/ethyl acetate = 2:1) to give 0.65 g of thiophenyl ester as a clear oil; 28% yield with respect to the unpurified one. ^{1}H NMR (in $CDCl_3$): δ 7.42 (s, 5H), 3.41 (m, 1H), 2.98 (m, 2H), 0.97 (m, 1H), 0.56−0.25 (m, 4H). ^{13}C NMR (in $CDCl_3$): δ 197.82, 134.88, 130.03, 129.68, 127.62, 73.59, 50.66, 17.31, 3.66, 2.76.

3-Hydroxy-3-cyclopropylpropionate Coenzyme A Thioester, ***IV*** *(R = C_3H_5).* To a magnetically stirred solution of sodium coenzyme A (0.593 g, 0.75 mmol) in a 100 mM aqueous potassium phosphate buffer solution (pH 8.0, 15 mL) in a small vial was added thiophenyl 3-hydroxy-3-cyclopropylpropioate, **III** (0.333 g, 1.5 mmol), in acetonitrile (9 mL). Stirring was continued for 3 h at room temperature, and 1 M H_3PO_4 (4 mL) added. The mixture was washed three times with diethyl ether (20 mL) and evaporated under reduced pressure to give 37.6 mM CoA thioester solution. The carbon-to-nitrogen ratio determined by elemental analysis was 3.30, which agrees with the calculated value, but because the sample was contaminated with phosphate buffer, the specific percentages observed were meaningless.

3-Hydroxy-4-chlorobutyrate CoA (4Cl3HBCoA). *Ethyl 3-Oxo-4-chlorobutyrate.* To an ice cold, magnetically stirred solution of Meldrum's acid (15.6 g, 108.4 mmol) in anhydrous dichloromethane (37 mL) in a predried flask was added pyridine (17.1 g, 216.6 mmol) and a solution of 2-chloroacethyl chloride (12.2 g, 108.4 mmol) in anhydrous dichloromethane (37 mL) at 0 °C under a nitrogen atmosphere. The same procedures were followed as in the preparation of I above to yield 14.6 of crude ethyl ester as a red oil, 14.6 g of which was purified by flash column chromatography as described above to give 7.4 g of pure ethyl ester as a pale red oil; 51% yield with respect to the unpurified product. ^{1}H NMR (in $CDCl_3$): δ 4.23 (s, 2H), 4.22 (q, J = 7.1 Hz, 2H), 3.66 (s, 2H), 1.30 (t, J = 7.1 Hz, 3H. ^{13}C NMR (in $CDCl_3$): δ 195.91, 166.84, 62.20, 48.43, 46.57, 14.43.

Ethyl 3-Hydroxy-4-chlorobutyrate. To a magnetically stirred solution of sodium borohydride (0.38 g, 10 mmol) in anhydrous ethanol (10 mL) in a predried flask was added a solution of ethyl 3-oxo-4-chlorobutyrate (3.3 g, 10 mmol) in anhydrous ethanol (10 mL). The same procedure were followed as in the preparation of **II** above to give 1.9 g of ethyl 3-hydroxy-4-chlorobutyrate as a pale yellow oil. ^{1}H NMR (in $CDCl_3$): δ 4.26 (m, 1H), 4.19 (q, J = 7.1 Hz, 2H), 3.62 (dd, J = 5.2 Hz, 2H), 2.63 (m, 2H), 1.28 (t, J = 7.1 Hz, 3H). ^{13}C NMR (in $CDCl_3$): δ 172.18, 68.30, 61.40, 48.54, 38.89, 14.50.

Thiophenyl 3-Hydroxy-4-chlorobutyrate. To anhydrous dichloromethane (30 mL) magnetically stirred in a dried flask in an ice bath was added 2 M trimethylaluminum (10 mL, 20 mmol) under a nitrogen atmosphere, and thiophenol (2 mL, 10 mmol) was added slowly. Stirring was continued for $^1/_2$ h at room temperature, and ethyl-3-hydroxy-4-dichlorobutyrate was added. The same procedures were followed as in the preparation of **III** above to give 0.7 g of thiophenyl ester as a clear oil; 23% yield with respect to the unpurified product. ^{1}H NMR (in $CDCl_3$): δ 7.43(s, 5H), 4.33 (m, 1H), 3.62 (m, 2H), 3.13 (d, J = 7.0 Hz, 2H). ^{13}C NMR (in $CDCl_3$): δ 197.03, 134.90, 130.23, 129.77, 127.20, 68.66, 48.58, 47.46.

3-Hydroxy-4-chlorobutyrate Coenzyme A Thioester. To a magnetically stirred solution of sodium coenzyme A (1185 mg, 1.5 mmol) in a 100 mM aqueous potassium phosphate buffer solution (pH 8.0, 30 mL) in a small vial was added a solution of thiophenyl 3-hydroxy-4-chlorobutyrate (697.5 mg, 3 mmol) in acetonitrile (15 mL). The same procedure was followed as in the preparation of **IV** above to give a 36 mM CoA thioester solution. The carbon-to-nitrogen ratio determined by elemental analysis was 3.16, which is very close to the calculated value of 3.06, but the specific percentages observed were meaningless because of the phosphate buffer contamination mentioned above.

3-Hydroxybutyrate CoA (3HB CoA). *Ethyl 3-Oxybutyrate.* To an ice cold, magnetically stirred solution of Meldrum's acid (3.9 g, 27.1 mmol) in anhydrous dichloromethane (18 mL) in a predried flask was added pyridine (4.3 g, 54.4 mmol) and a solution of acetyl chloride (2.2 g, 28.0 mmol) in anhydrous dichloromethane (18 mL) at 0 °C under a nitrogen atmosphere. The same procedures were followed as in the preparation of I above to give 1.3 g of crude ethyl ester as a red oil, 1.3 g of which was purified as described above to give 0.60 g of pure ethyl ester as a pale yellow oil; 46% yield with respect to the unpurified product. ^{1}H NMR (in $CDCl_3$): δ 4.20 (q, J = 7.1

Hz, 2H), 3.47 (s, 2H), 2.27 (s, 3H), 1.28 (t, $J = 71$ Hz, 3H). ^{13}C NMR (in $CDCl_3$): δ 201.06, 167.44, 61.52, 50.29, 30.32, 14.29.

Ethyl 3-Hydroxybutyrate. To a magnetically stirred solution of sodium borohydride (75.6 mg, 2 mmol) in anhydrous ethanol (2 mL) in a predried flask was added a solution of ethyl 3-oxybutyrate (520 mg, 4 mmol) in anhydrous ethanol (2 mL), and the same procedures were followed as in the preparation of **II** above to give 282 mg of 3-hydroxybutyrate as a pale yellow oil. ^{1}H NMR (in $CDCl_3$): δ 4.17 (q, $J = 7.1$ Hz, 2H), 4.17 (m, 1H), 2.46 (m, 2H), 1.28 (t, $J = 7.1$ Hz, 3H), 1.23 (d, $J = 6.3$ Hz, 3H). ^{13}C NMR (in $CDCl_3$): δ 172.93, 64.28, 60.68, 42.91, 22.49, 14.18.

Thiophenyl 3-Hydroxybutyrate. To anhydrous dichloromethane (6 mL) magnetically stirred in a predried flask on ice was added 2 M trimethylaluminum (2 mL, 4 mmol) under a nitrogen atmosphere, and subsequently thiophenol (2 mmol) was added slowly. Stirring was continued for $^1/_2$ h at room temperature, and subsequently ethyl 3-hydroxybutyrate in anhydrous dichloromethane (6 mL) was added. The same procedures were followed as in the preparation of **III** above to give 532 mg of crude thiophenyl ester as a dark yellow oil, 520 mg of which was purified by flash column chromatography on silica gel 60, as above, to give 125 g of thiophenyl ester as a clear oil; 25% yield with respect to the unpurified product. ^{1}H NMR (in $CDCl_3$): δ 7.38 (s, 5H), 4.33 (m, 1H), 2.83 (m, 2H), 1.25 (d, 3H). ^{13}C NMR (in $CDCl_3$): δ 198.24, 134.90, 130.07, 129.69, 127.61, 65.23, 52.02, 22.85.

3-Hydroxybutyrate Coenzyme A Thioester. To a magnetically stirred solution of sodium coenzyme A (39.5 mg, 0.05 mmol) in a 100 mM aqueous potassium phosphate buffer solution (pH 8.0, 0.5 mL) in a small vial was added a solution of thiophenyl 3-hydroxybutyrate (9.8 mg, 0.05 mmol) in acetonitrile (0.1 mL). The same procedure was followed as in the preparation of **IV** above to give a 30 mM CoA thioester solution.

Enzyme Purification and Activity Assay. The PHA synthase from *E. shaposhnikovii*, EsPHAS, was purified from the recombinant *E. coli* cells according to the method described in Zhang et al.[45] The PHA synthase from *R. eutropha*, RePHAS, was purified from the recombinant *E. coli* cells[25] according to the method previously described.[38] The purified enzymes were stored at −20 °C until use. The synthase activity was assayed by measuring the absorption decrease at 236 nm due to the cleavage of the thioester bond in the monomer.[40] The buffers for the reactions were a 20 mM potassium phosphate solution (pH 7.0) for REPHAS and a 150 mM Tris-Cl solution (pH 8.0) for ESPHAS. The reactions were initiated by the addition of the enzyme.

Poly(3-hydroxy-3-cyclopropylpropionate) (PHCyPP). The polymerization reaction were carried out in 100 mL of a 100 mM aqueous Tris-HCl buffer solution (pH 8.0), containing 100 mg/mL bovine serum albumin (BSA), 8 mM 3CyP3HP CoA, and 0.26 mg/mL EsPHAS for 16 h. The reaction was monitored spectrophotometrically at 412 nm on an aliquot sample (50:l) for the release of CoASH by the addition to a 5% trichloroacetic acid solution (20:l) containing 1 mM 5,5′-dithiobis(2-nitrobenzoid acid) (DTNB) solution (1 mL).[37] The conversion of the substrate was calculated using a molar ϵ 412 of 13 700. After the reaction was completed, the polymer obtained in the aqueous solution was extracted with warm chloroform three times, precipitated in methanol, and collected by centrifugation to give a white pellet. The pellet was redissolved in chloroform, reprecipitated in methanol, and dried under reduced pressure to give 10.3 mg of PHCyPP. Elemental analysis: Observed: 62.41% C, 7.43% H. Calculated: 64.29% C, 7.14% H. ^{1}H NMR (in $CDCl_3$): δ 4.65 (m, 1H), 2.68 (d, $J = 6.5$ Hz, 2H), 1.02 (m, 1H), 0.50−0.32 (m, 4H). ^{13}C NMR (in $CDCl_3$): δ 169.23, 74.79, 39.85, 15.11, 3.86, 2.96.

Poly(3-hydroxy-4-chlorobutyrate) (PHCB). The polymerization reaction was carried out in 100 mL of a 100 mM aqueous Tris-HCl buffer solution (pH 8.0), containing 100 mg/mL BSA, 6.1 mM 4Cl3HBCoA, and 0.2 mg/ms EsPHAS for 16 h. The reaction was monitored, and the polymer obtained was isolated and purified as described above to give 6.6 mg of PHCB. Elemental analysis: Observed: 39.65% C, 4.01% H, 27.95% Cl. Calculated: 39.83% C, 4.15% H, 29.46% Cl. ^{1}H NMR (in DMSO): δ 5.28 (m, 1H), 3.75 (m, 2H), 2.73 (m, 2H). ^{13}C NMR (in DMSO): δ 168.12, 69.60, 45.65, 36.28.

B. Characterization. ^{1}H NMR spectra were recorded in chloroform-*d* or dimethyl-d_6 sulfoxide solutions at 300 MHz (Bruker, DPX-300) referenced to TMS at 0.00 ppm, and *J* values are given in hertz. ^{13}C NMR spectra were recorded in chloroform-*d* or dimethyl-d_6 sulfoxide solutions at 75 MHz (Bruker, DPX-300) referenced to chloroform at 77.0 ppm.

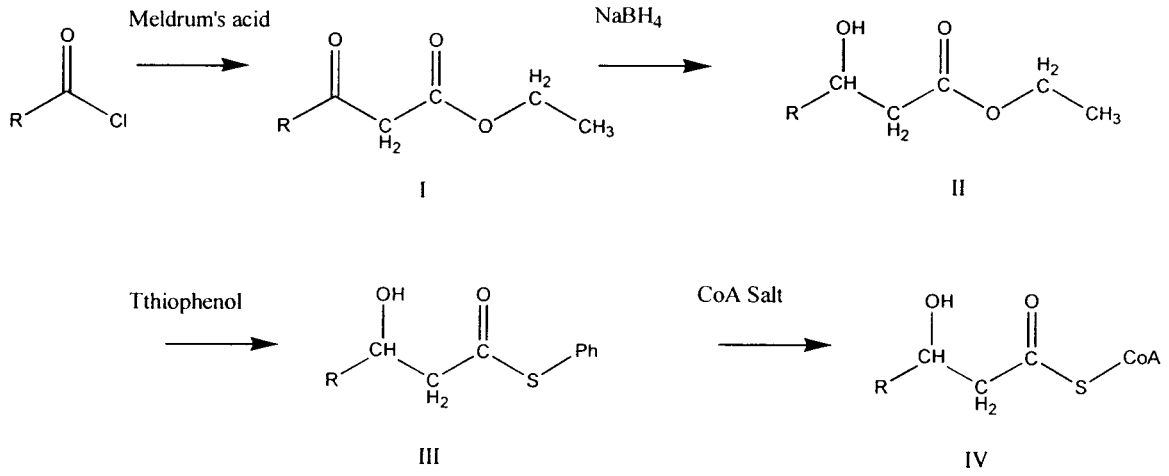
The number-average (M_n) and weight-average (M_w) molecular weights of the polymers were determined by gel permeation chromatography (GPC), either with a Waters 2690 system equipped with a guard column (Shodes KF-G) and three columns in series (Shodex KF802, KF804F, and KF805L), using chloroform as an eluant at flow rate 1.0 mL/min, or with a Tosoh HPLC-8020 system equipped with a guard column (TSK HXL-L) and three consecutive polystyrene gel columns (TSK gels G5000H, G4000H, and G2500H) using *N*,*N*-dimethylformamide (DMF) and 5.8 mM lithium bromide solution as an eluant at a flow rate of 1.0 mL/min. For both methods, polystyrene samples were used for calibration, and refractive index and ultraviolet detectors were used.

The glass transition temperatures (T_g), melt temperatures (T_m), and the heat of fusion (ΔH_m) were measured using a Perkin-Elmer DSC 7. A 3−8 mg sample in an aluminum pan was heated at 20 °C/min from −70 to 200 °C, quickly cooled, and scanned a second time using the same heating rate and temperature range as the first scan. In addition to these scans, a third scan was carried out after annealing the polymer samples at 40 °C for 5 days. The values of T_m and ΔH_m were determined from the thermogram of the third scan, while T_g was determined from the thermogram of the second scan.

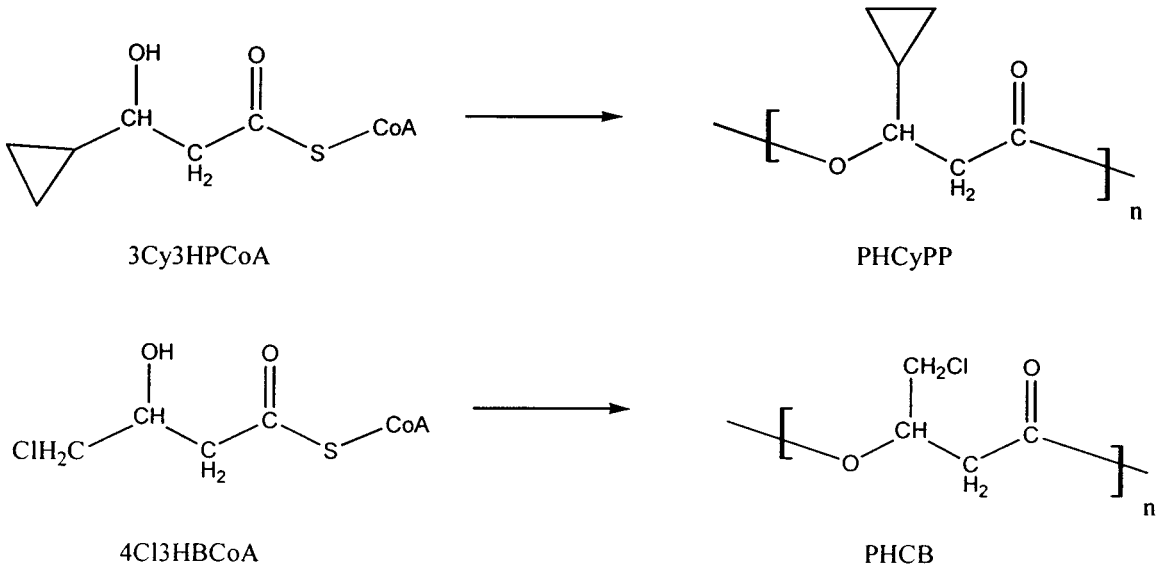
Results and Discussion

The two new 3-hydroxyalkanoate monomers polymerized in this study were prepared by the four-step reaction procedure shown in Scheme 1. Acylation of commercial Meldrum's acid by the Yonemitsu procedure[43] was used for the preparation of the ketoesters, I. The crude acylated Meldrum's acid was prepared in the presence of pyridine in dichloromethane and decomposed in boiling ethanol to form the ethyl 3-ketoester, I, which was converted to the 3-hydroxyester, **II**, by hydrogenation with sodium borohydride. The ethyl ester of **II** was reacted with thiophenol catalyzed by trimethylaluminum under nitrogen, to form the phenyl thioester,[44] **III**. The latter was generally obtained in low yield because some of the ethyl ester remained unreacted. The phenyl thioester was reacted with sodium CoA in an alkaline aqueous solution to form the CoA thioester monomer, **IV**. For that reaction, acetonitrile was used as the solvent to dissolve the hydrophobic thioester and to accelerate the thioester exchange reaction. The thioesters monomers, **IV**, were obtained in high purity as indicated by both their ^{1}H NMR spectra and elemental analysis. The acid chloride containing the cyclopropyl substituent reacted well to give a high yield of **II**, but that with the chloro substituent did not. The polymerase enzymes used, RePHAS and EsPHAS, were obtained from recombinant *E. coli* strains harboring the plasmid with the PHAS genes cloned from either *R. eutropha*, by a modification of the method previously used,[25] or from *E. shaposhnikovii* by the method of Zhang et al.,[39] respectively. The latter polymerase was purified and isolated by using two chromatography columns to improve enzyme separation, and fractionation was performed at a higher salt concentration than usual to maintain the enzyme in its active form, which is a multisubunit structure. The monomeric forms of both polymerases were found to be much less reactive,[39] and the active forms of these

Scheme 1



Scheme 2



enzymes appear to consist of two or more protein molecules. The RePHAS has a molecular weight of 128 000 in the dimeric form, while the ESPHAS enzyme has a much higher molecular weight of 165 000 in the active form, which is believed to have a complex subunit structure of at least four protein molecules.

The polymerization reactivities of a series of new 3-hydroxyalkanoate CoA derivatives were evaluated, but only two of these, the 3-hydroxy-3-cyclopropylpropionate (3CyP3HPCoA) and 3-hydroxy-4-chlorobutyrate (4Cl3HBCoA), reacted to any significant extent, and only the EsPHAS was found to be very active for the in vitro polymerization of both monomers. The other new, potential substrates prepared were the CoA derivatives of 4,4-dichloro-3-hydroxybutyrate, 4,4,4-trichloro-3-hydroxybutyrate, 4-methyl-3-hydroxyhexanoate, and 4-phenyl-3-hydroxybutyrate, but none of these were polymerized by EsPHAS to a measurable extent. EsPHAS polymerized 3CyP3HPCoA and 4Cl3HBCoA at rates of 3% and 14% of the rates of polymerization of 3HBCoA, respectively, but RePHAS showed undetectable activity toward 3CyP3HPCoA, although it had the same relative reactivity with 4Cl3HBCoA as the former polymerase. On the basis of this information, EsPHAS was used for the in vitro polymerization of both 3CyP3HPCoA and 4Cl3HBCoA monomers to prepare sufficiently large amounts of both polymers for structure−property characterization by the polymerization reactions shown in Scheme 2. The rates of the polymerization of these two monomers at a molar ratio of 104/1 of monomer/enzyme (assuming a molecular weight equivalence of 165 000 Da for a single catalytic site of the EsPHAS enzyme complex) are shown in Figures 1 and 2 for reactions catalyzed by EsPHAS. In both figures the conversion has a maximum value of 50% because it represents the

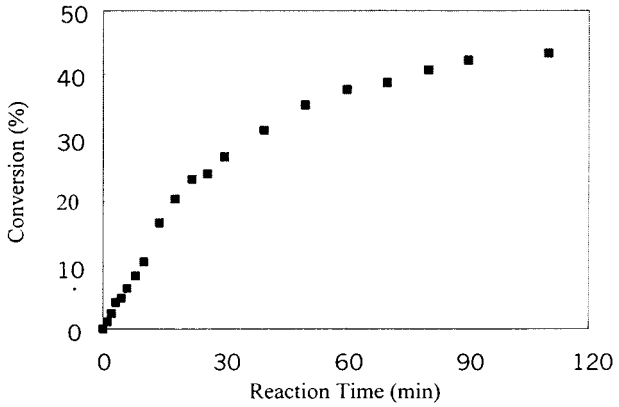


Figure 1. Monomer conversion rate for the polymerization of [R,S]-3H3CyPPCoA by EsPHAS.

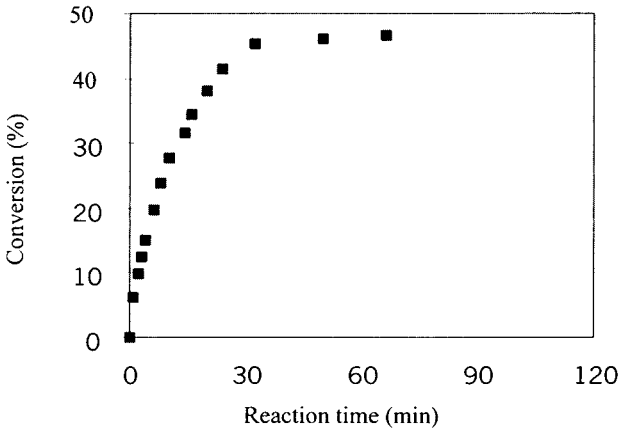


Figure 2. Monomer conversion rate for the polymerization of [R,S]-3H4CBCoA by EsPHAS.

total amount of the racemic monomer that reacted, and only the [R] isomer is polymerized by the enzymes as previously shown.[38,45] Under the conditions used no lag phase was observed for either monomer in the initial stage of the polymerization reaction with this polymerase. The addition of fructose was also previously shown to stabilize the enzyme during storage and to eliminate the lag phase of the polymerization reaction.[38,45] For 3CyP3HPCoA, the reaction took about 2 h to reach 85% conversion, but only 30 min was required for 4Cl3HBCoA to reach 90% conversion. The turnover (or propagation) rate was 1.2 mol monomer/mol enzyme/s for 3CyP3HPcoA and 6.7 mol monomer/mol enzyme/s for 4C13HBCoA. Complete extraction of the polymers from the reaction suspension was possible with warm chloroform, but P(4Cl3HB) was found to have only low solubility in chloroform at room temperature.

Recent studies in our laboratory have shown that free Coenzyme A is a competitive inhibitor for RePHAS, and the polymerization reactions of both monomers with EsPHAS did not reach 100% conversion after 2 h, which suggests that this polymerase was also inhibited by free CoA, but the extent of inhibition was less than that observed for RePHAS.

^{1}H, ^{13}C, and ^{1}H−^{1}H COSY NMR spectra indicated that polymers of the expected structure were obtained. The 300 MHz ^{1}H NMR spectrum of PHCyPP is shown in Figure 3. The chemical shift assignments for each proton resonance in the polymer, as determined by analysis of the two-dimensional ^{1}H−^{1}H COSY NMR spectrum, are given in the Experimental Section. The ^{1}H NMR spectrum of PHCyPP in Figure 3 shows two different multiplet peaks with a 4/1 ratio for the cyclopropane ring, in the range between 0.5 and 0.3 ppm, but the precursor of the CoA monomer, ethyl 3-hydroxy-3-cyclopropylpropionate, **III**, has three different multiplet peaks in a 2/2/1 ratio between 0.55 and 0.25 ppm (data not shown). These results strongly suggest that the repeating units in the polymer have only one configuration at the C3 chiral center, most likely that of the [R] configuration, while the precursor to the monomer, and consequently the monomer, too, was a racemic mixture. That is, as previously observed, the polymerase was selective for only one chiral isomer of the monomer, most likely the monomer with an [R] configuration at the 3-position.[37,38]

Figure 4 shows the 300 MHz ^{1}H NMR spectrum of PHCB in dimethyl-d_6 sulfoxide. Although PHCB has

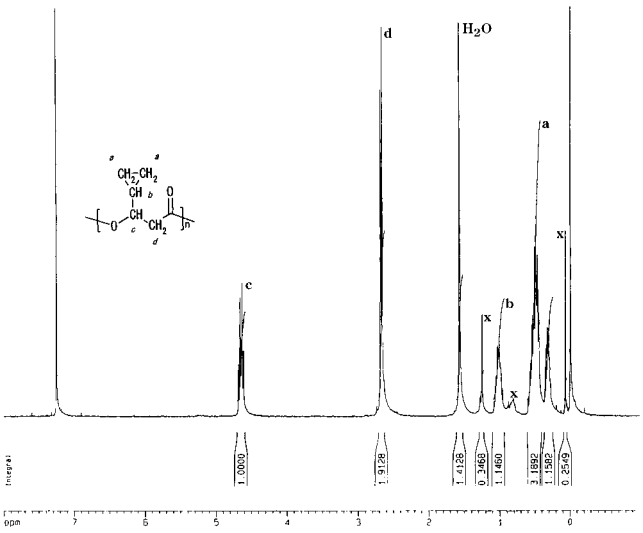


Figure 3. ^{1}H NMR spectrum of PHCyPP in chloroform (peak X is an unknown contaminant.).

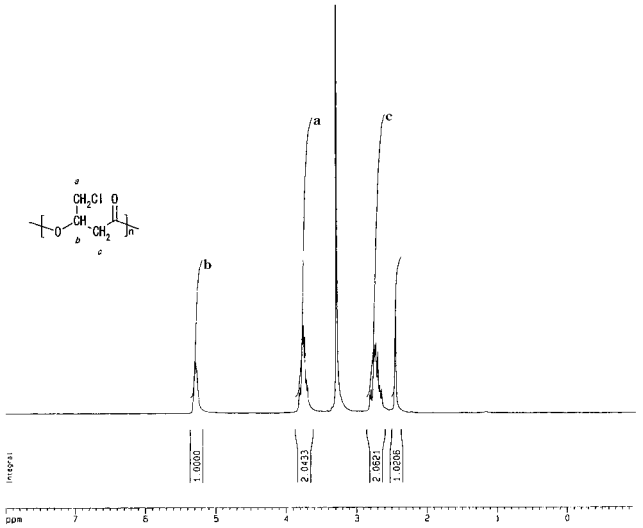


Figure 4. ^{1}H NMR spectrum of PHCB in DMSO.

only a very low solubility in chloroform at room temperature, and it is not completely soluble even at 50 °C, it dissolved completely in dimethyl sulfoxide. The chemical shift assignment for each proton resonance, which were made by analysis of the two-dimensional ^{1}H−^{1}H COSY NMR spectrum, are given in the Experimental Section. The ^{1}H NMR spectrum of PHCB in Figure 4 shows three different multiplet peaks in dimethyl-d_6 sulfoxide for the methine protons of the C-2 position being a doublet. However, the ^{1}H NMR spectrum of this polymer at 50 °C in chloroform-d_3 clearly showed two multiplet peaks at 5.43 and 3.74 ppm and one doublet peak at 2.81 ppm for the methine protons (data not shown), suggesting that PHCB is also an isotactic polymer and that it contains units of only one type of chirality, presumably also that of the [R] configuration.

The number-average (M_n) and weight-average (M_w) molecular weights of the PHAs obtained were determined by GPC, with the results shown in Table 1. The observed values in Table 1 are based on polystyrene standards and are not absolute values. Doi and coworkers[46] have shown that the absolute value of the M_n of PHB, as determined by solution light scattering, is 0.7 of that of the GPC values. The absolute values for

Table 1. Characterization of PHAs Prepared

polymer	M_n obsd[a]	M_n calcd[b]	M_w	M_w/M_n	T_g (°C)	T_m (°C)	ΔH_m (J/g)
PHCyPP	371 000	260 000	539 000	1.45	30	73	28
PHCB	189 000	132 000	241 000	1.28	6	105	46

[a] Determined by GPC relative to polystyrene samples. [b] Calculated on the basis that the absolute value is approximately 0.7 of the observed value.

the samples in Table 1 were calculated on that basis. Previous studies in this laboratory indicated that these in vitro polymerization reactions can form living polymers,[37] but if these polymerization reactions occurred without chain transfer and termination at the monomer conversions obtained and at the molar ratios of monomer-to-enzyme used, the calculated values of M_n would be 485 000 for PHCyPP and 563 000 for PHCB. In contrast, the observed M_n values, converted to absolute values as described above, were 371 000 and 189 000 for PHCyPP and PHCB, respectively, which indicates that these reactions did not form living polymers. Nevertheless, the polydispersity indices (PDI or M_n/M_w) obtained were quite low compared to those of other chain growth polymerization reactions involving covalently bound, growing polymer chains, such as in transition metal catalyzed reactions in general. The PDI values obtained, as shown in Table 1, were 1.45 for PHCyPP and 1.28 for PHCB. These values suggest that the rates of initiation of the polymerization reactions may have been very high compared to the rates of propagation and that the lag phase was completely eliminated in these polymerization reactions by the addition of fructose to the enzyme solution as previously described.[38,39,45]

The DSC thermograms contained very broad melting transitions with multiple endotherms for both of the polymers in the first DSC scan, with the melt temperatures and heats of fusion shown in Table 1. The occurrence of multiple endotherms in polyesters is fairly common and is generally indicative of the presence of crystalline regions of varying size and perfection. The first cycle thermogram of PHCB showed an endothermic peak at 124 °C, which was presumably for a melting transition. After quenching the polymers in liquid nitrogen, the second cycle DSC thermograms of both polymers showed no apparent melting peaks, only a T_g at 30 °C for PHCPP and a T_g at 6 °C for PHCB. Additional DSC analyses, which were carried out after annealing both polymers at 40 °C for 5 days, showed an apparent T_m at 73 °C with a ΔH_m of 28 J/g for PHCyPP and an apparent T_m at 105 °C with a ΔH_m of 46 J/g for PHCB. The higher T_g of PHCyPP suggests that the cyclopropane ring plays an important role restricting the rotation of the main chain.

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