Fractionation and Characterization of Microbial Polyesters Containing 3-Hydroxybutyrate and 4-Hydroxybutyrate Repeat Units

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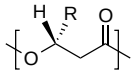
Introduction. Poly(hydroxyalkanoic acids) (PHAs) are a family of polyesters produced by a wide variety of microorganisms.[1−6] Most naturally occurring PHAs have β-linked repeat units that are enantiopure ([R]-stereochemical configuration) and possess the general structure shown below where R is the pendant group.[2]



The variability in both the number of carbons between ester group linkages and the side group structure for β-linked PHAs provides a wide variety of microbial polyesters with diverse physical properties.[5] Research pioneered by Doi and co-workers reported that the bacteria *Alcaligenes eutrophus*, *Alcaligenes latus*, and *Comamonas acidovorans* produce copolymers of 3-hydroxybutyrate (3HB) and 4-hydroxybutyrate (4HB, γ-linked) when presented with suitable carbon sources such as 4-hydroxybutyric acid, 4-chlorobutyric acid, γ-butyrolactone, and even-chain diols such as 1,4-butanediol and 1,6-hexanediol.[7−16] These copolymers, P(3HB-*co*-4HB), have been shown to have rather extraoridinary properties. For example, the product with 90 mol % 4HB had % elongation at break and tensile strength values of 1080% and 65 MPa, respectively.[15]

It has generally been reported that P(3HB-*co*-4HB) copolyesters formed by both *A. eutrophus* (ATCC 17699) and *A. latus* (ATCC 29713) have statistically random sequence distributions of 3HB and 4HB units.[7−14] Abate *et al.*[17] cultured *A. eutrophus* (ATCC 17699) following biosynthetic methods described by Kunioka and Doi; however, a product fraction consisting of 97 mol % 4HB was isolated from the unfractionated product composed of 61 mol % 3HB and 39 mol % 4HB. Furthermore, Doi *et al.*[12] reported that, by feeding γ-butyrolactone and butyric acid as cosubstrates, *A. eutrophus* (ATCC 17699) produced a mixture of P(3HB-*co*-4HB) random copolyesters. Moreover, Saito and Doi[15] used the bacterium *Comamonas acidovorans* DS-17 to produce mixtures of compositionally different random P(3HB-*co*-4HB) copolymers as well as products that deviated significantly from random Bernoullian statistics. These later 3HB/4HB products were not fractionated for further analysis.

Since (i) polymer microstructure will strongly influence the physicomechanical and biological properties of 3HB/4HB containing polymeric materials and (ii) previous work either disregarded or only partially addressed the formation and characterization of P(3HB-*co*-4HB) mixtures, we performed a detailed investigation of 3HB/4HB product microstructure and heterogeneity. For this purpose, *A. eutrophus* (ATCC 17699) was used to form a 3HB/4HB-containing product using 4-hydroxybutyric acid as the sole carbon source.

Experimental Section. Fermentations for Polyester Production. Polyester production by *A. eutrophus* was by two-stage fermentation conditions following a method by Doi and co-workers.[7,8] This involved growth (first stage) and polymer accumulation (second stage) in a nutrient-rich medium and a nitrogen-free defined medium, respectively. Details of the culture conditions and media compositions were described elsewhere (cultivation condition B was followed).[18] The carbon source used in the polymer-producing medium was the sodium salt of 4-hydroxybutyric acid (15.0 g/L). The cells were harvested by centrifugation and lyophilized.[7,8]

Polymer Purification. The intracellular PHAs were isolated by chloroform extraction at room temperature followed by precipitation into methanol, as was described previously.[18] For cells at the end of the second stage of cultivations, the % PHA in lyophilized cells and the volumetric PHA yield were 28% and 1.1 g/L, respectively. Isolation of polymer from cells at the end of the first stage showed that 3% of the cell dry weight contained P3HB (0.1 g/L).

Fractionation of Polyesters. Acetone (10 volumes) was added slowly to a chloroform solution (0.1 g/mL) of the purified PHA, the mixture was stored at −10 °C overnight, and the resulting white precipitate formed was isolated by filtration and termed the acetone-insoluble (AIS) fraction. The solvent (acetone/chloroform) was then removed by rotoevaporation, giving the acetone-soluble (AS) fraction. Further fractionation of the AS fraction was carried out by the addition of methanol (1.25 volumes) to an acetone solution (0.02 g/mL). The precipitate isolated by filtration was termed the AS methanol/acetone insoluble fraction (AS-MAIS); the solvent of the solution was removed by rotoevaporation and gave the corresponding AS methanol/acetone soluble fraction (AS-MAS). Residual solvents were removed from product fractions at room temperature in a vacuum desiccator (10 mmHg, 24 h). The samples were then allowed to age under ambient conditions for at least 1 week prior to thermal analysis (see below).

Structural Analysis by ^{1}H and ^{13}C NMR. Proton (^{1}H) NMR spectra were recorded on a UNITY-250 NMR spectrometer at 250 MHz. Carbon (^{13}C) NMR spectra were recorded on a UNITY-250 NMR spectrometer at 63 MHz. Details on experimental parameters used were given elsewhere.[18]

Molecular Weight Determinations. Molecular weight averages were measured by gel permeation chromatography (GPC). Polystyrene standards (Aldrich) with low polydispersities were used to generate a calibration curve from which product molecular weights were determined with no further corrections. Additional details for the method used are described elsewhere.[18,19]

Thermal Analysis by Differential Scanning Calorimetry. All thermal characterizations were carried out using a DuPont 2910 differential scanning calorimeter (DSC) equipped with a TA 2000 data station at a heating rate of 10 °C/min and with a dry-nitrogen purge. Polyesters obtained either from solution precipitation or rotoevaporation of solvent (see the section on polyester fractionation, above) were dried in vacuo (50 °C, 24 h), sealed in aluminum pans, heated from 25 to 185 °C (first heat), rapidly quenched to −80 °C, and then

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Table 1. Composition, Thermal, and Molecular Weight Analyses of Products Formed by *A. eutrophus* Incubated on 4-Hydroxybutyric Acid as the Sole Carbon Source

		composition (mol %)[c]		thermal properties				
fractions[a]	wt %[b]	3HB	4HB	T_g (°C)[d]	T_m (°C)[e]	ΔH (cal/g)[e]	mol wt M_n	M_w/M_n
unfractionated		72	28	−43, −15, 5	59, 168	0.4, 9.3	260 000	2.8
AIS	46	99	1	4	168	16.8	320 000	1.9
AS	54	48	52	−45, −17	53	3.8	144 000	2.7
AS-MAS	86	72	28	−17	47, 82	4.3	190 000	1.5
AS-MAIS	14	18	82	−43	44	4.3	99 000	1.7

[a] The method of fractionation and fraction abbreviations were described in the Experimental Section. [b] Fraction wt/unfractionated PHA wt for AIS and AS; fraction wt/AS wt for AS-MAS and AS-MAIS. [c] Calculated by ^{1}H NMR analysis. [d] Obtained from the second DSC heating scan. [e] Obtained from the first DSC heating scan.

heated (second heating scan) at 10 °C/min to 60 °C. Data reported for the peak melting temperature(s), T_m, and enthalpy of fusion(s), ΔH_f, were taken from the first heating scan. The reported glass transition temperature(s), T_g, was (were) the midpoint value(s) measured during the second heating scans.

Results and Discussion. The microstructure and homogeneity of a 4HB-containing product accumulated by *A. eutrophus* were studied. The repeat unit composition of the nonfractionated product as well as product fractions was determined by ^{1}H NMR spectral integration.[9] The nonfractionated product consists (in mol %) of 72% 3HB and 28% 4HB. Fractionation was then carried out based on product solubility in acetone, which gave AIS and AS fractions. The AS fraction was then further fractionated by the addition of methanol to an acetone solution, which resulted in AS-MAIS and AS-MAS fractions (see the Experimental Section). The weight percents, molar compositions, thermal analysis results, and molecular weights for the product fractions are listed in Table 1. The AIS, AS, AS-MAS, and AS-MAIS fractions are compositionally dissimilar. Thus, unlike previous reports,[9,10] the conversion of 4-hydroxybutyric acid into polyester by *A. eutrophus* resulted in a complex mixture of products.

DSC thermograms of the nonfractionated product as well as the product fractions were recorded during the first and second heating scans (see parts a and b of Figure 1, respectively). Values of T_g, T_m, and ΔH_f are listed in Table 1. A study of Table 1 shows that the nonfractionated product has three distinct T_g transitions at −43, −15, and +5 °C. Furthermore, this sample showed two melting transitions with T_m values at 59 and 168 °C. Thus, these results for the unfractionated PHA are consistent with it containing a mixture of compositionally different products. Previous studies have shown that P3HB has a T_m of ∼175 °C[20] and a T_g of 4 °C.[21−24] As expected, these values were similar to the thermal transitions of the AIS fraction (99 mol % 3HB; see Table 1). Since the AIS fraction must closely approximate to a compositionally homogeneous sample, it was not further fractionated. In contrast, based on the observation that the AS fraction had two T_g transitions (see Figure 1b and Table 1), work was undertaken to determine whether this fraction could be further separated into compositionally dissimilar components. The alternative was that the AS fraction was composed of block copolymers having different chain segment compositions.

At the methanol/acetone ratio of 5:4, the AS fraction was successfully separated into two samples (AS-MAS, AS-MAIS) which had different T_g, T_m, and 3HB/4HB compositions (see Table 1). These fractions each showed only one T_g, which may be because they each contain a narrow distribution of chain compositions. Alternatively, it is possible that the AS-MAS and AS-MAIS fractions contain miscible blends of different copolyesters. Work was not carried out to further investigate whether AS-MAS or AS-MAIS could be further fractionated into compositionally different products. It seems unlikely because of the very low M_w/M_n values. The two T_g transitions of the AS fraction (−45 and −17 °C) are similar in temperature to the T_g transitions of its component fractions. This suggests that the AS-MAS and AS-MAIS components that have very different 3HB/4HB compositions are immiscible. The results of molecular weight analyses are also given in Table 1. The polydispersities (M_w/M_n) of the unfractionated product and fraction AS were larger than those for fractions AIS, AS-MAS, and AS-MAIS. The high polydispersity values reflect not only that the unfractionated product and AS fractions contain mixtures of compositionally different polyesters but also that the components of these mixtures differ greatly in molecular weight. Indeed, we observed that the AIS and AS fractions had very different M_n values. Similarly, the AS-MAS and AS-MAIS fractions also showed large differences in M_n. Interestingly, fractions containing a relatively higher 3HB content had larger M_n values (see Table 1). One explanation for these results is that the *A. eutrophus* synthase produces chains with M_n values that varied inversely with 4HB content. Alternatively, the depoly-

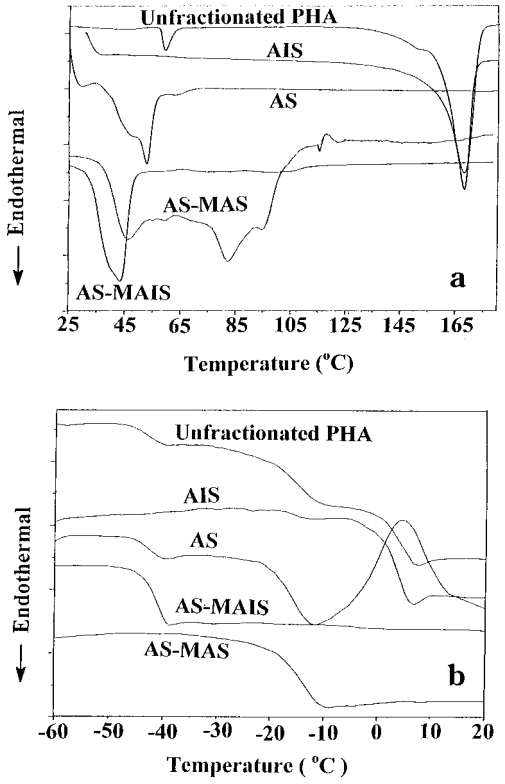

Figure 1. DSC thermograms for products formed by *A. eutrophus* incubated on 4-hydroxybutyric acid: (a) first heating scans and (b) second heating scans after rapid quenching from the melt.

Table 2. Diad Sequence Analysis for Products Formed by *A. eutrophus* Incubated on 4-Hydroxybutyric Acid as the Sole Carbon Source

	3HB*−3HB		3HB*−4HB		4HB*−3HB		4HB*−4HB	
fraction	exp[a]	calc[b]	expt	calc	expt	calc	expt	calc
unfractionated	0.61	0.52	0.11	0.20	0.12	0.20	0.16	0.08
AS-MAS	0.54	0.52	0.20	0.20	0.20	0.20	0.06	0.08
AS-MAIS	0.04	0.03	0.14	0.15	0.14	0.15	0.68	0.67

[a] Determined by measuring the relative peak areas for the carbonyl carbon ^{13}C NMR signals assigned to the four diads. [b] Calculated values[25] using equations for a Bernoullian or random statistical process.

merase may have higher activity for chains with increasing 4HB content.

^{13}C NMR spectra were recorded of the unfractionated PHA and product fractions to determine the corresponding diad sequence distributions (spectra not shown). Expansions of the carbonyl region of these spectra were used to determine the relative fractions of 3HB*−3HB, 3HB*−4HB, 4HB*−3HB, and 4HB*−4HB diad sequences (asterisks designate the observed carbonyl).[9] Experimental values were compared to those calculated assuming a Bernoullian or random statistical process for microbial-catalyzed copolymerizations,[16] and the results are shown in Table 2. The experimental and calculated diad fraction values for the unfractionated PHA accumulated by *A. eutrophus* differed significantly. Furthermore, the D value[26] for the unfractionated product is 7.39, which is well above 1.0 for a random copolymer. This is consistent with the above results, which showed that this product can be separated into compositionally different components. Analyses for both the AS-MAS and AS-MAIS fractions showed that there was excellent agreement between the calculated and experimental diad fractions. Also, the D values for AS-MAS and AS-MAIS closely approached 1.0 (0.81 and 1.39, respectively). Considering the results of NMR and thermal analyses, it appears that the AS fraction (54 wt % of the unfractionated PHA) is composed of an immiscible blend of poly(3HB-*co*-28 mol % 4HB) and poly(3HB-*co*-82 mol % 4HB) random copolyesters. Furthermore, since 46 wt % of the unfractionated product contained 99 mol % 3HB, it was concluded that the PHA accumulated by *A. eutrophus* contains three compositionally different polyesters. To consider the repeatability of the results presented herein, the fermentation experiment for the accumulation of PHA by *A. eutrophus* was repeated three times. These replicate experiments gave PHAs with 46 ± 5 wt % AIS and 54 ± 5 wt % AS fractions. Furthermore, comparison of DSC and ^{13}C NMR analyses for these products gave almost identical results.

It is noteworthy to consider that 9% of the total PHA volumetric yield was accumulated as P3HB in the nutrient-rich medium during the first stage of the cultivation (see the Experimental Section). This is consistent with a previous report by Song *et al.*[27] Thus, the predominant carbon source during polymer accumulation in the second stage of cultivations was 4-hydroxybutyric acid (15 g/L), but low levels of P3HB might also degrade and supply 3-hudroxybutyryl-CoA to cells. The presence of mixed substrates that are metabolized to PHA by different pathways at different rates may contribute to the observed heterogeneity of the PHA formed.[28] Furthermore, repeat unit exchange reactions such as PHA depolymerization, metabolism of 4HB to 3HB, and subsequent polymerization of recycled monomer may also play a role in chain structural heterogeneity.

In summary, careful fractionation of the polyester produced by *A. eutrophus* from 4-hydroxybutyric acid gave at least three compositionally different products, each with one T_g. Specifically, the product formed by *A. eutrophus* on 4-hydroxybutyric acid consisted of the following components: (1) P3HB, (2) P(3HB-*co*-82 mol % 4HB), and (3) P(3HB-*co*-28 mol % 4HB). This result is in contrast to previous work with the same bacterium and culture conditions which claimed that a simple random P(3HB-*co*-4HB) was obtained.[8] Since changes in the microstructure of copolymers will result in different thermal, crystalline, and physicomechanical properties of corresponding materials, this work emphasizes that microbial polyesters may have complex structures that reuqire careful fractionation and subsequent analysis.

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