PHAs Produced by *Pseudomonas putida* and *Pseudomonas oleovorans* Grown with *n*-Alkanoic Acids Containing Aromatic Groups

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ABSTRACT: Poly(3-hydroxyalkanoates), PHAs, bearing aromatic groups were biosynthesized by *Pseudomonas putida* and *Pseudomonas oleovorans* grown with various carbon substrates such as 5-phenylvaleric acid, 5PVA, 8-(*p*-methylphenoxy)octanoic acid, 8pMPO, 6-(*p*-methylphenoxy)hexanoic acid, 6pMPH, 8-(*m*-methylphenoxy)octanoic acid, 8mMPO, 8-(*o*-methylphenoxy)octanoic acid, 8oMPO, and 11-(*p*-methylphenoxy)undecanoic acid, 11pMPU. 11pMPU and 8oMPO did not support PHA production by both *P. oleovorans* and *P. putida* while other carbon substrates supported PHA production. PHAs obtained from 8-(methylphenoxy)octanoic acids contained 3-hydroxy-4-methylphenoxybutyrate and 3-hydroxy-6-methylphenoxyhexanoate units. DSC and X-ray scattering analysis indicated that PHAs biosynthesized from 5PVA and 8mMPO were amorphous while the PHA biosynthesized from 8pMPO was crystalline. PHAs biosynthesized by *P. putida* grown with mixtures of nonanoic acid, NA, and either 5PVA or 8pMPO were fractionated into two fractions on the basis of the solubility in *n*-hexane. The *n*-hexane insoluble fractions were examined to find that these polymers were random copolymers containing repeating units produced from both carbon substrates. Results in this study showed *P. putida* produced random copolymers as long as this microorganism utilized both carbon substrates. The compositions of the copolymers were dependent on the nature of carbon substrates. DSC analysis showed that PHAs from 8pMPO and from 5PVA were miscible in each other. The number-average molecular weight of the PHA from 8pMPO was 25 000 while those of PHAs from NA and 5PVA were 50 000 as determined by gel permeation chromatography. The polydispersity indices were approximately 2.5 regardless of the carbon substrate.

Introduction

Poly(3-hydroxyalkanonates), PHAs, biosynthesized by various microorganisms have attracted interest as biodegradable and biocompatible polymers. Besides biodegradability and biocompatibility, some of these polymers are remarkable as they contain unusual functional groups such as carbon−carbon double bonds and carbon−carbon triple bonds in high fractions.[1,2] Polymers containing such groups in high fractions cannot be easily prepared by conventional synthetic methods.

Most of the PHAs bearing unusual groups have been biosynthesized either by *Pseudomonas oleovorans* (*P. oleovorans*) or by *Pseudomonas putida* (*P. putida*). Of the PHAs containing unusual repeating units, we have been interested in PHAs containing aromatic groups. There have been several reports on PHAs containing aromatic repeating units.[3−11]

The first PHA-containing aromatic group was biosynthesized by *P. oleovorans* grown solely with 5-phenylvaleric acid, 5PVA.[5] The PHA produced from 5PVA was remarkable as this polymer was a homopolymer of 3-hydroxy-5-phenylvalerate (3HPV). Formation of the homopolymer was explained that *P. oleovorans* could not utilize 3-hydroxy-3-phenylpropionyl-*co*-enzyme A for polymerization that would have been produced from 3-hydroxy-5-phenylvaleryl-*co*-enzyme A as a result of deacetylation.[12] The DSC thermogram of poly(3-hydroxy-5-phenylvalerate), PHPV, showed a very small endothermic peak, indicating that this polymer was very low in crystallinity. PHAs biosynthesized from mixtures of 5PVA and nonanoic acid, NA, were interesting as these PHAs were found to be mixtures of PHAs having very different compositions that could be fractionated into two fractions.[4,8] On the other hand, PHAs biosynthesized from *P. putida* grown with mixtures of NA and 11-phenoxyundecanoic acid were reported to be random copolymers containing repeating units produced from both OA and 11-phenoxyundecanoic acid.[10]

In this study, we have biosynthesized PHAs containing phenoxy groups substituted with methyl group in para and meta positions. PHAs were also prepared using mixed carbon substrates of NA and either 5PVA or 8-(*p*-methylphenoxy)octanoic acid, 8pMPO. The resulting PHAs were examined to elucidate whether these PHAs were mixtures of random copolymers or mixtures of PHAs biosynthesized independently from each carbon substrate.

Experimental Section

Preparation of Methylphenoxyalkanoic Acids. Methylphenoxyalkanoic acids were synthesized by the reaction between sodium methylphenoxide and bromoalkanoic acids. Synthesis of 8-(*p*-methylphenoxy)octanoic acid is described below. Other carbon substrates were synthesized following the similar procedures.

Six hundred milliliters of dry toluene was added to a 2 L three-neck flask equipped with a refluxing condenser, magnetic stirring bar, and a dropping funnel. Thirty-six grams (900 mmol) of sodium hydride dispersion in mineral oil (60%) was added, and then 97 g (900 mmol) of *p*-cresol was added in portions. When required, a small amount of absolute ethanol was added to improve the solubility of the mixture. The reaction mixture was refluxed for 1 h, and 50 g (224 mmol) of 8-bromooctanoic acid dissolved in 200 mL of dry toluene was added dropwise approximately for 3 h. The reaction mixture was refluxed overnight and then cooled to room temperature. Approximately 300 mL of distilled water was added, and the reaction mixture was acidified by adding HCl. The organic

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layer was taken and washed with water and dried over anhydrous magnesium sulfate. The solvents were removed using a rotatory evaporator to obtain oily product mixture. The oily product mixture was fractionated into three fractions by vacuum distillation, and the middle fraction distilled out between 120 and 180 °C was taken. The middle fraction solidified when it was allowed to stand overnight at room temperature. The solid product was washed with *n*-hexane until only one spot was observed by thin-layer chromatography. The overall yields were between 40 and 60%.

Biosynthesis of PHAs. *P. putida* KCTC 2407 and *P. oleovorans* ATCC 29347 were cultured as described elsewhere.[2] Seeds were prepared by culturing for 18 h in a rotary shaker using 100 mL of medium containing NA in a concentration of 10 mM if not stated otherwise. Batch fermentation experiments were carried out using 5 L jar fermenter (Korea fermentation Co.) containing 3 L of basal medium. Aeration rate, agitation speed, and the pH of the medium were 0.25 vvm, 250 rpm, and 7.0, respectively. The bacterial growth was monitored by measuring the optical density at 660 nm. When the growth reached stationary phase, cells were harvested by centrifugation and then lyophilized. PHAs were extracted from lyophilized cells by hot chloroform using a Soxhlet extractor, and then the extracted crude PHA was purified by repeated precipitation into vigorously stirred methanol. Determination of the amounts of residual carbon substrates in the growth medium was carried out as described in our previous paper.[8]

PHA Compositions. The total amounts of aromatic repeating units were determined using ^{1}H NMR spectroscopy. The relative amounts of repeating units were determined by either ^{1}H NMR spectra or gas chromatograms. A Bruker 300 NMR spectroscopy and a Hewlett-Packard 6890 gas chromatograph equipped with a flame ionization detector and a HP-1 capillary column (25 m × 0.2 mm, Hewlett-Packard) was used.

Fractionation. PHAs produced from various mixtures of nonanoic acid and alkanoic acid bearing aromatic groups such as 5-phenylvaleric acid and 8-(*p*-methylphenoxy)octanoic acid were fractionated by adding solutions of these polymers in chloroform to *n*-hexane. Approximately 500 mg of the polymer was dissolved into 3 mL of chloroform, and this solution was added dropwise to the vigorously stirred 300 mL of *n*-hexane. The *n*-hexane insoluble fraction was recovered by centrifugation, and the *n*-hexane soluble fraction was recovered after evaporating *n*-hexane. The temperature of *n*-hexane was maintained between 30 and 40 °C.

Miscellaneous. DSC analysis was carried out using a TA 2510 thermal analysis system. The measurement was carried out from −70 and 150 °C at a ramp of 10 °C/min. X-ray diffraction was carried out using a MacScience X-ray image processor model DIP2030K. The distance of the sample from the double-image plate detector was 100 mm, and the samples were exposed to the beam for 10 min. Samples were elongated manually by approximately 5 times their original lengths. GC/MS analysis was carried out using a HP 5988 GC/MS as described in our previous paper.[2] Molecular weights of PHAs were determined using a gel permeation chromatography system equipped with a Waters 6000 solvent delivery system, RI detector, and a Rheodyne injector. Phenogel columns of 500, 10^3, 10^4, and 10^5 Å were used. A standard curve was established with standard polystyrene samples.

Results and Discussion

Biosynthesis of PHAs Using Alkanoic Acid Containing Aromatic Groups. PHA production by *P. putida* and *P. oleovorans* grown with various methylphenoxyalkanoic acids was investigated. Of the carbon substrates used in this study, 11-(*p*-methylphenoxy)-undecanoic acid, 11pMPU, was not soluble in the growth medium and did not support cell growth. PHA production by *P. putida* was mainly investigated in this study as this microorganism utilized aromatic carbon substrates more efficiently than *P. oleovorans* for polymer production as described below. Figure 1 shows the growth curve for *P. putida* grown in a medium that initially contained 8 mmol of 8-(*p*-methylphenoxy)-octanoic acid, 8pMPO, per 1 L. The growth medium prepared in this concentration contained fine particles of undissolved sodium 8-(*p*-methylphenoxy)octanoate that were swollen with water. These particles disappeared a few hours after the medium was inoculated, and they disappeared earlier when the medium was inoculated with *P. putida* than when the medium was inoculated with *P. oleovorans*. These results indicated that *P. putida* started to consume 8pMPO earlier than *P. oleovorans*.

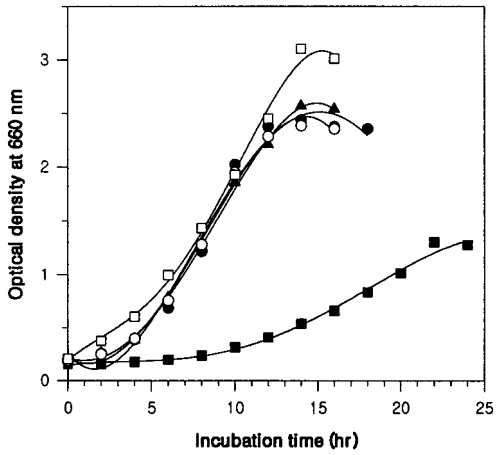

Figure 1. Growth curves for *P. putida* grown with various mixtures of NA and 8pMPO: 10 mM NA (□); 7.5 mM NA + 2.5 mM 8pMPO (▲); 5 mM NA + 5 mM 8pMPO (●); 2.5 mM NA + 7.5 mM 8pMPO (○); 8 mM 8pMPO (■).

The fermentation results for *P. putida* grown with various alkanoic acids are listed in Table 1. The amount of polymer isolated from cells grown with 8oMPO was very low, and this polymer did not contain any aromatic repeating unit as determined by ^{1}H NMR spectroscopy. Polymer yields listed in Table 1 show that the structure of the methylphenoxy group in the carbon substrate was critical for PHA production. Table 1 also shows that the growth of *P. putida* with 6-(*p*-methylphenoxy)hexanoic acid, 6pMPH, was much slower than the growth with 8pMPO. The PHA content and cell yield were also higher when cells were grown with 8pMPO. Considering that 6pMPH was more soluble in the growth medium than 8pMPO, these results indicated that *P. putida* preferred 8pMPO to 6pMPH for both growth and PHA production.

P. putida pregrown with glucose produced almost no PHA from 5PVA while *P. putida* pregrown with NA produced substantial amounts of PHAs from 5PVA. Similar results were obtained when *P. putida* was grown with other unusual carbon substrates such as undecylenic acid and undecynoic acid, indicating that *P. putida* should be induced for PHA production when unusual carbon substrates were used as a sole carbon substrate. No induction was necessary for *P. oleovorans* as *P. oleovorans* pregrown with glucose produced substantial amounts of PHAs from these carbon substrates.[13]

Characterization of PHAs Prepared Using Alkanoic Acid Containing Aromatic Groups. Gas chromatograms of methanolyzed samples of the PHA isolated from *P. putida* grown with 6pMPH and 8pMPO showed two new peaks that had different retention times from those of methyl esters of 3-hydroxy-*n*-alkanoates containing 6−12 carbons. Electron impact mass spectra of these peaks contained an ion fragment

Table 1. Fermentation Results from *P. putida* Grown with Various Mixtures of Carbon Substrates

carbon substrate[a] and concentration (mM)	incubation time (h)	dry cell yield (g/L)	PHA content in dry cell (wt %)	PHA yield (mg/L)	content of repeating units containing methylphenoxy group[b] (mol %)
6pMPH (8)	240	0.22	12.5	30	100
8pMPO (8)	24	0.65	13.0	90	100
8pMPO (15)	44	1.05	13.5	140	100
8mMPO (8)	180	0.3	3	12	100
8oMPO (8)	200	0.23	0.8	2	0
8pMPO (7.5) + NA (2.5)	16	0.88	16.3	140	65
8pMPO (5) + NA (5)	18	0.98	21	210	50
8pMPO (2.5) + NA (7.5)	16	1.01	23.7	240	24
8pMPO (5) + 5PVA (5)	28	0.52	8	40	35
8pMPO (5) + 6PH	28	0.43	11.5	50	40
NA	14	1.06	28.3	310	0

[a] 6pMPH = 6-(*p*-methylphenoxy)hexanoic acid; 8pMPO = 8-(*p*-methylphenoxy)octanoic acid; 8mMPO = 8-(*m*-methylphenoxy)octanoic acid; 8oMPO = 8-(*o*-methylphenoxy)octanoic acid; NA = nonanoic acid; 5PVA = 5-phenylvaleric acid; 6PH = 6-phenoxyhexanoic acid. [b] Determined by ^{1}H NMR spectroscopy.

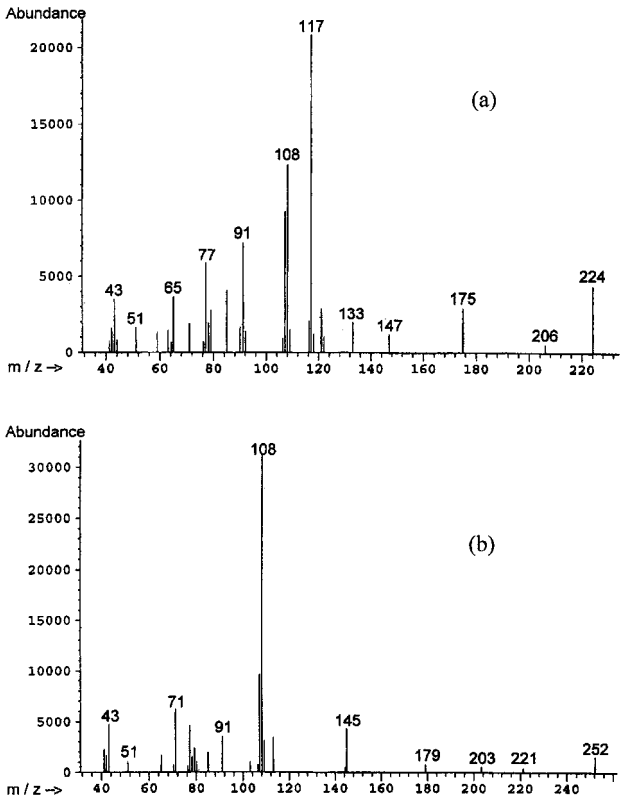

Figure 2. (a) EI mass spectrum of 3H4pMPB. (b) EI mass spectrum of 3H6pMPH.

with a *m*/*z* value of 108 that corresponded to the mass of *p*-methylphenol. The EI mass spectra in Figure 2 indicated the molecular weights of these peaks were 224 and 252, respectively. These molecular weights corresponded to those of methyl esters of 3-hydroxy-4-(*p*-methylphenoxy)butyrate units, 3H4pMPB, and 3-hydroxy-6-(*p*-methylphenoxy)hexanoate unit, 3H6pMPH, respectively. The molecular weights of these peaks were confirmed to be 224 and 252 by gas chromatography/chemical ionization (GC/CI) mass spectrometry. The GC/CI mass spectrum of the methanolyzed sample of a PHA biosynthesized from 8pMPO, PHA(8pMPO), showed a very small peak with a molecular weight of 282. This molecular weight corresponded to that of methyl ester of 3-hydroxy-8-(*p*-methylphenoxy)octanoate unit, 3H8pMPO.

^{1}H NMR and ^{13}C NMR spectra of PHA(8pMPO) are shown in Figures 3 and 4. Figures 3 and 4 correspond to spectra expected from PHAs containing 3H4pMPB and 3H6pMPH.

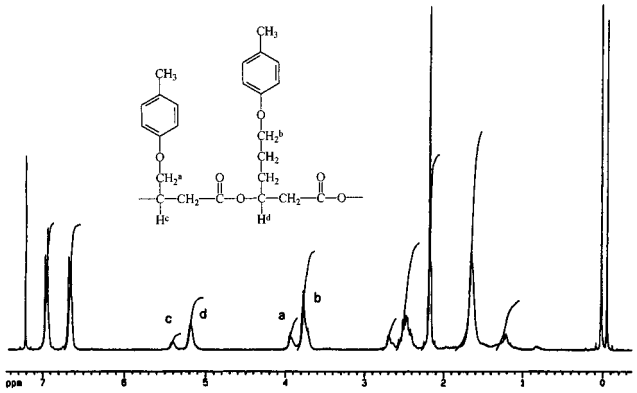

Figure 3. ^{1}H NMR spectrum of PHA(8pMPO).

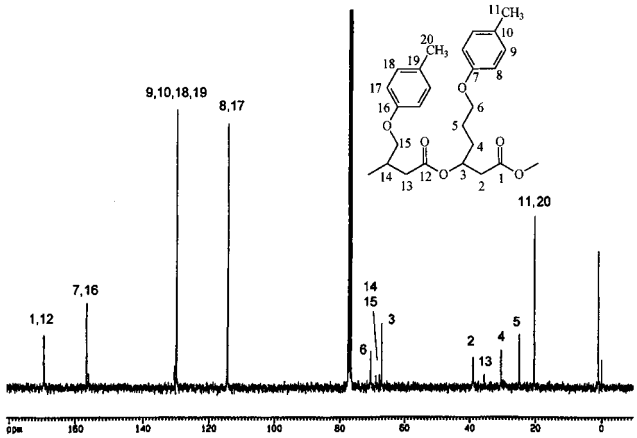

Figure 4. ^{13}C NMR spectrum of PHA(8pMPO).

The gas chromatogram of the methanolyzed sample of the PHA(8mMPO) also showed two new peaks that had different retention times from those of methyl esters of 3-hydroxy-*n*-alkanoates containing 6−12 carbons. Mass spectroscopy analysis showed that these two repeating units were 3-hydroxy-4-(*m*-methylphenoxy)butyrate, 3H4mMPB, and 3-hydroxy-6-(*m*-methylphenoxy)hexanoate, 3H6mMPH. The EI mass spectra of these units were almost identical to those of 3H4pMPB and 3H6pMPH. The amounts of 3H4mMPB and 3H6mMPH were 30 and 70 mol % as determined by ^{1}H NMR spectroscopy, respectively.

Compositions of PHAs produced by *P. putida* grown with methylphenoxyoctanoic acids are listed in Table 2. Table 2 shows that the composition of the PHA biosynthesized from 6pMPH, PHA(6pMPH), was almost identical to that of PHA(8pMPO). Results in Table 2 also

Table 2. Compositions of PHAs Bearing Methylphenoxy Groups Produced by *P. putida*

carbon substrate and concentration (mM)	repeating unit composition in PHA[a]								
	3HHp	3HN	3H4pMPB	3H4mMPB	3H6pMPH	3H6mMPH	3H4PB	3H6PH	3HPV
6pMPH (8)			26.5[b]		73.5[b]				
8pMPO (8)			28.5[b]		71.5[b]				
8pMPO (15)			27.0[b]		73.0[b]				
8mMPO (8)				30		70			
8pMPO (7.5) + NA (2.5)	11.7[c]	23.3[c]	2.9[b]		62.1[b]				
8pMPO (5) + NA (5)	16.2[c]	33.8[c]	4.0[b]		46.0[b]				
8pMPO (2.5) + NA (7.5)	22.0[c]	54.0[c]	1.0[b]		23.0[b]				
8pMPO (5) + 5PVA (5)			6.7[b]		28.3[b]				65.0[b]
8pMPO (5) + 6PH (5)			3.9[b]		36.1[b]		21.9[c]	38.1[c]	
NA	25.3[c]	74.7[c]							

[a] 3HHp = 3-hydroxyheptanoate; 3HN = 3-hydroxynonanoate; 3H4pMPB = 3-hydroxy-4-(*p*-methylphenoxy)butyrate; 3H4mMPB = 3-hydroxy-4-(*m*-methylphenoxy)butyrate; 3H6pMPH = 3-hydroxy-6-(*p*-methylphenoxy)hexanoate; 3H6mMPH = 3-hydroxy-6-(*m*-methylphenoxy)hexanoate); 3H4PB = 3-hydroxy-4-phenoxybutyrate; 3H6PH = 3-hydroxy-6-phenoxyhexanoate; 3HPV = 3-hydroxy-5-phenylvalerate. [b] Mole percent determined by ^{1}H NMR spectroscopy. [c] GC area percent.

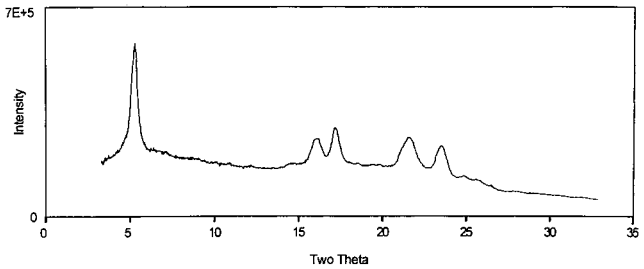

Figure 5. X-ray diffraction profile of PHA(8pMPO).

showed that the composition of PHA(8pMPO) was not affected by the initial concentration of 8pMPO in the growth medium.

The PHA(8pMPO) was white and brittle at room temperature. The X-ray diffraction profile of this PHA presented in Figure 5 showed sharp peaks, indicating that these polymers were crystalline. PHA(8pMPO) precipitated from methanol showed typical spherulitic structure when observed under a polarizing microscope. The number-average molecular weight of PHA(8pMPO) was 25 000 as determined by gel permeation chromatography. The molecular weights of PHPV and PHN determined by the same method were approximately 50 000. These results indicated that the molecular weight of PHA produced by *P. putida* was affected by the carbon substrate. Polydispersity indices of these polymers were approximately 2.5 regardless of the carbon substrate.

Thermal transition temperatures of PHAs biosynthesized by *P. putida* grown with various carbon substrates are listed in Table 3. The thermograms of PHA(8mMPO) and PHPV showed small endothermic peaks, indicating that these polymers might be slightly crystalline. However, the X-ray diffraction profiles of these polymers did not show any sharp peak.

The PHA biosynthesized by *P. putida* grown with an equimolar mixture of 8pMPO and 5PVA, PHA(8pMPO/5PVA), was amorphous as determined by DSC and X-ray scattering analysis. Figure 6 shows the glass transition regions of DSC thermograms of PHA(8pMPO), PHPV, and PHA(8pMPO/5PVA). The glass transition temperature of PHA(8pMPO/5PVA) was a little higher than those of PHA(8pMPO) and PHPV. Considering that PHA(8pMPO/5PVA) contained three repeating units while PHPV and PHA(8pMPO) contained one and two repeating units, this result is rather unusual. The molecular weight of PHA(8pMPO/5PVA) was approximately 33 000, which was slightly higher than that of PHA(8pMPO), and it is likely that this higher molecular weight was responsible for the slightly higher glass transition temperature.

Table 3. Thermal Transition Temperatures of PHAs Biosynthesized from Various Carbon Substrates

description of the sample or carbon substrate	glass transition temp, °C	melting temp, °C	heat of fusion (ΔH_m), J/g
8pMPO	14	97	33.5
8mPMO	6	43	0.2
5PVA	15	58	0.4
PHPV/PHN blend (9:1)	14	44	0.82
PHPV/PHN blend (8:2)	4	43	1.96
PHPV/PHN blend (7:3)	4	43	2.6
n-hexane insoluble fraction containing 88% 3HPV	5		
n-hexane insoluble fraction containing 77% 3HPV	−8		
8pMPO/5PVA (1/1)	17		
PHA(8pMPO)/PHPV blend (8:2)	−1	95	5.2
PHA(8pMPO)/PHPV blend (6:4)	4	92	16.4
PHA(8pMPO)/PHPV blend (4:6)	2	93	8.2
PHA(8pMPO)/PHPV blend (3.5:6.5)	−10	89	7.3
PHA(8pMPO)/PHPV blend (2:8)	3	92	26.5
n-hexane insoluble fraction containing 69% 8pMPO units and 31% NA units[a]	−4	65	12.7
n-hexane insoluble fraction containing 65% 8pMPO units and 35% NA units[b]	−4		
n-hexane insoluble fraction containing 50% 8pMPO units and 50% NA units[c]	−9		
PHA(8pMPO)/PHN blend (9:1)	9	47	0.6
		94	24.5
PHA(8pMPO)/PHN blend (7.5:2.5)	8	47	2
		94	19.5
PHA(8pMPO)/PHN blend (6.5:3.5)	4	48	3.4
		97	11.9
NA	−40	50	20.7

[a] From a PHA biosynthesized from a 7.5:2.5 mixture of 8pMPO and NA. [b] From a PHA biosynthesized from a 5:5 mixture of 8pMPO and NA. [c] From a PHA biosynthesized from a 2.5:7.5 mixture of 8pMPO and NA.

The X-ray profiles obtained from relaxed and uniaxially stretched PHPV samples are shown in Figure 7. Figure 7b shows that the stretched PHPV had a layered structure with a *d* spacing value of 68 Å. The relatively strong scattering observed in the stretched PHPV might be attributed to the high regularity of PHPV as this polymer was a homopolymer of 3HPV. No such peaks were observed in X-ray scattering profiles of elongated PHAs prepared from 8-phenoxyoctanoic acid and NA.

The Nature of PHAs Biosynthesized Using Mixtures of NA with Either 5PVA or 8pMPO. PHAs

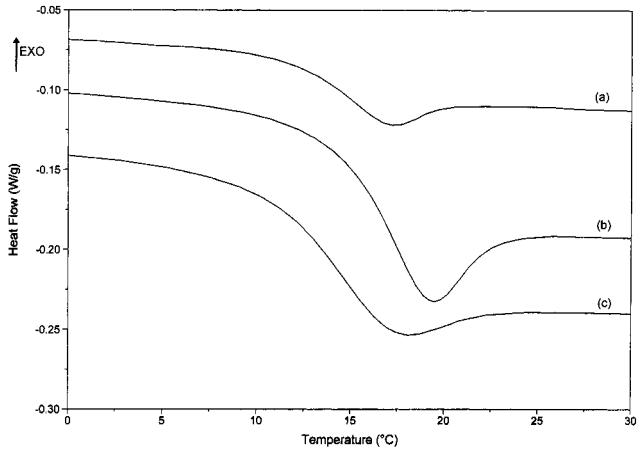

Figure 6. Glass transition of (a) PHPV, (b) PHA(8pMPO/5PVA), and (c) PHA(8pMPO).

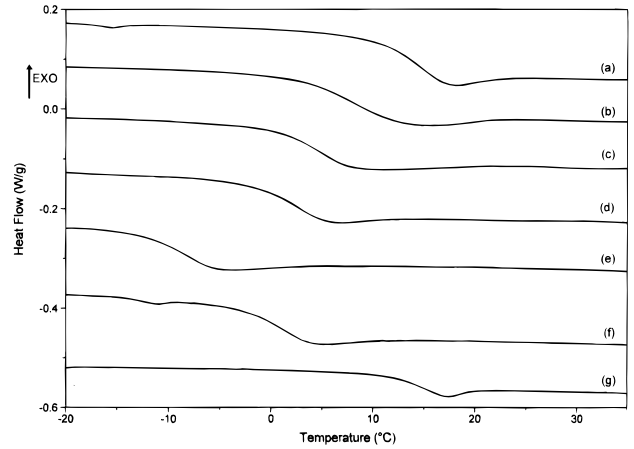

Figure 7. Glass transition of (a) PHA(8pMPO), (b) PHPV/PHA(8pMPO) 2:8 blend, (c) PHPV/PHA(8pMPO) 4:6 blend, (d) PHPV/PHA(8pMPO) 6:4 blend, (e) PHPV/PHA(8pMPO) 6.5:3.5 blend, (f) PHPV/PHA(8pMPO) 8:2 blend, and (g) PHPV.

biosynthesized from mixtures of NA and 5PVA have been reported to be mixtures of polymers having very different compositions. However, the nature of polymers in these mixtures has not been clearly characterized. On the other hand, the PHAs biosynthesized by *P. putida* grown with mixtures of OA and 11-phenoxyundecanoic acid were reported to be random copolymers containing repeating units produced from both carbon substrates.[10]

We have reinvestigated the PHAs biosynthesized by *P. putida* grown with various mixtures of NA and 5PVA. The compositions of PHAs biosynthesized from mixtures of NA and 5PVA are listed in Table 4. The PHAs were fractionated into two fractions depending on the solubility in *n*-hexane. Table 4 shows that the relative amount of *n*-hexane soluble fraction increased significantly as the fraction of NA in the carbon substrate increased. However, the contents of 3HPV in both *n*-hexane soluble and *n*-hexane insoluble fractions did not change significantly according to the composition of the carbon substrate mixtures. The contents of 3HPV in the *n*-hexane soluble fractions were commonly lower than 10%, and the contents of 3HPV in the *n*-hexane insoluble fractions were commonly higher than 70%. Various physical mixtures of PHPV and PHN were prepared, and the thermal properties of these mixtures were compared with those of *n*-hexane insoluble fractions. Results in Table 3 show that the glass transition temperature of the blend decreased significantly as the fraction of PHN increased, indicating that these two polymers were miscible in each other. The DSC thermograms of these blends showed melting endotherms with increasing ΔH_m in proportion to the amount of PHN in the blends. However, the melting temperature of the blend did not change significantly according to the composition. The *n*-hexane insoluble fractions having similar composition showed no melting transition. The glass transition temperatures of *n*-hexane insoluble fractions were lower than those of blends having similar compositions. These results clearly showed that the *n*-hexane insoluble fractions were not physical mixtures of PHPV and PHN but random copolymers containing repeating units produced from 5PVA and NA. The repeating units from NA were 3-hydroxyheptanoate, 3HHp, and 3-hydroxynonanoate, 3HN. The amount of 3HN in the *n*-hexane insoluble polymers was approximately twice of that of 3HHp.

Thermal transition temperatures of blends prepared by mixing PHPV and PHA(8pMPO) in various ratios are listed in Table 3. Results in Table 3 showed that the melting temperatures of blends of PHA(8pMPO) and PHPV did not change significantly according to the composition of the blend. However, the glass transition temperatures of various blends shown in Figure 8 changed significantly according to the composition. It is unusual that the glass transition temperature of the blend was much lower than those of component polymers in the blend. The glass transition temperature of this blend was the lowest when it contained PHPV and PHA(8pMPO) in a ratio of 6.5:3.5. These results clearly showed that the PHA(8pMPO/5PVA) was not a physical mixture of PHA(8pMPO) and PHPV.

The natures of polymers biosynthesized by *P. putida* grown with mixtures of NA and 8pMPO were also investigated. Figure 1 shows that the growth of *P. putida* with mixtures of NA and 8pMPO was identical regardless of the compositions of the carbon substrate mixtures. Fermentation results for *P. putida* grown with mixtures of NA and 8pMPO are listed in Table 1. The PHA(8pMPO) was insoluble in *n*-hexane at room temperature, and the DSC thermograms of physical mixtures of PHN and PHA(8pMPO) showed distinctive melting and glass transition temperatures that corresponded to those of PHN and PHA(8pMPO) as indicated in Table 3.

Fractionation results for PHAs produced by *P. oleovorans* and *P. putida* grown with various mixtures of NA and 8pMPO are listed in Table 5. A physical mixture of PHN and PHA(8pMPO) biosynthesized by *P. oleovorans* and *P. putida*, respectively, was prepared and subjected to fractionation for comparison. Table 5 shows that PHA(8pMPO) and PHN were separated quantitatively by fractionation method used in this study. The content of aromatic repeating units in *n*-hexane soluble fraction was lower than 40 mol % while the content of aromatic repeating units in *n*-hexane insoluble fraction was higher than 40 mol %. Table 5 shows that PHAs biosynthesized by *P. putida* and *P. oleovorans* grown with an equimolar mixture of NA and 8pMPO were significantly different. The contents of aromatic repeating units in the PHA biosynthesized by *P. oleovorans* and *P. putida* grown with this carbon substrate were 30 and 50 mol %, respectively. Almost 80% of the PHA obtained from *P. oleovorans* was soluble in *n*-hexane while only 40% of the PHA biosynthesized by *P. putida*

Table 4. Fractionation Results of PHAs Biosynthesized by *P. putida* with Various Mixtures of 5PVA and NA

description of the carbon substrate	hexane soluble fraction, wt % (A)	3HPV in A, mol %	hexane insoluble fraction, wt % (B)	3HPV in B, mol %
5PVA (7.5 mM) and NA (2.5 mM)	9 ± 0.52		91 ± 0.52	88 ± 1.25
5PVA (5 mM) and NA (5 mM)	48 ± 0.48	6 ± 0.89	52 ± 0.48	78 ± 1.02
5PVA (2.5 mM) and NA (7.5 mM)	86 ± 0.44		14 ± 0.44	75 ± 0.90
5PVA (5 mM) and NA (10 mM)[a]	53 ± 0.55	9 ± 1.35	47 ± 0.55	89 ± 1.08

[a] The fermentation was initiated with an equimolar mixture of 5PVA and NA and NA was added after approximately 13 h.

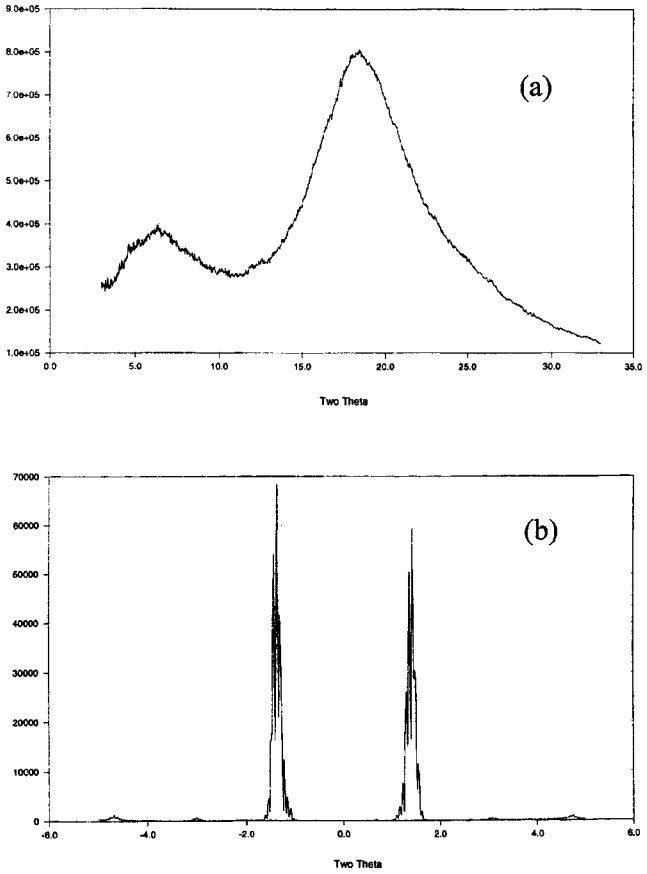

Figure 8. X-ray scattering profile of (a) unoriented PHPV and (b) oriented PHPV.

was soluble in *n*-hexane. The contents of aromatic repeating units in the *n*-hexane soluble fractions obtained from PHAs biosynthesized by both microorganisms were similar. However, the contents of aromatic repeating units in the *n*-hexane insoluble fractions obtained from the PHAs biosynthesized by *P. putida* were higher than in those obtained from PHAs biosynthesized by *P. oleovorans*. These results were most likely caused by the different affinities of these two microorganisms to 8pMPO as described below.

The composition of the *n*-hexane insoluble fraction isolated from the PHA biosynthesized from a mixture of 7.5 mM 8pMPO and 2.5 mM NA, PHA(8pMPO/NA;7.5/2.5), was very similar to that of the *n*-hexane insoluble fraction isolated from the PHA biosynthesized from a mixture of 2.5 mM 8pMPO and 7.5 mM NA, PHA(8pMPO/NA;2.5/7.5), as shown in Table 3. However, the DSC thermogram of the *n*-hexane insoluble fraction from PHA(8pMPO/NA;7.5/2.5) showed melting endotherm with a maximum peak at 65 °C while the DSC thermogram of the *n*-hexane insoluble fraction from PHA(8pMPO/NA;2.5/7.5) did not show any melting endotherm. These results suggested that the microstructures of *n*-hexane insoluble fractions from PHA(8pMPO/NA;7.5/2.5) and PHA(8pMPO/NA;2.5/7.5) were different. The *n*-hexane insoluble fraction isolated from PHA(8pMPO/NA;7.5/2.5) might contain PHA(8pMPO) or block copolymers of repeating units from NA and 8pMPO.

The thermal transitions of *n*-hexane insoluble fractions from PHAs biosynthesized from mixtures of 8pMPO and NA were obviously different from those of blends of PHA(8pMPO) and PHN having similar compositions as shown in Table 3. These results clearly showed that the *n*-hexane insoluble polymers were random copolymers containing repeating units from 8pMPO and NA.

P. putida was grown with an equimolar mixture of NA and 8pMPO, and the concentration of each carbon substrate in the growth medium was determined at different incubation times as shown in Figure 9. Figure 9 shows that the concentration of 8pMPO started to decrease several hours after the concentration of NA started to decrease. Therefore, the PHAs accumulated in *P. putida* during the very early growth stage would be PHN, and the *n*-hexane soluble fractions isolated from the PHA obtained from mixtures of 8pMPO and NA were expected to contain some PHN. However, the amount of PHN produced during this period was expected to be very small as the total amount of cells produced during this period was very small. These results suggested that *P. oleovorans* started to consume 8pMPO later than *P. putida* so that the content of *n*-hexane soluble fraction in the PHA biosynthesized by *P. oleovorans* was higher than that in the PHA biosynthesized by *P. putida* grown with the same carbon substrate mixtures. The higher content of *p*-methylphenoxy unit in the *n*-hexane insoluble fraction in the PHA biosynthesized by *P. putida* showed that *P. putida* utilized 8pMPO for PHA production more efficiently than *P. oleovorans*.

Figure 9 also shows the relative amounts of repeating units, 3HHp, 3HN, 3H4pMPB, and 3H6pMPH, in the PHA accumulated in *P. putida* grown with an equimolar mixture of NA and 8pMPO at different incubation times. Figure 9 shows that the relative amounts of 3HHp and 3HN decreased while those of 3H4pMPB and 3H6pMPH increased rapidly until approximately 12 h. As the PHA produced during the initial stage of growth would be mostly PHN, the rapid increases of the relative amounts of 3H4pMPB and 3H6pMPH indicated that the copolymers produced at early growth stage contained significant amounts of these units. The relatively constant content of each repeating unit after 12 h indicated that the composition of random copolymers produced did not change significantly throughout the growth. Results in Figure 9 indicated that the composition of PHAs biosynthesized at certain incubation time was determined by the amount of remaining carbon substrates at that moment. Therefore, the PHAs biosynthesized at early stage of growth (6−8 h in Figure 9) contained more aromatic repeating units than the PHAs biosynthesized later. However, the ratio of 8pMPO and NA remaining in the growth medium did not change significantly

Table 5. Fractionation Results of PHAs Isolated from *P. putida* and *P. oleovorans* Grown with Various Carbon Substrate Mixtures

microorganism	description of the sample or carbon substrate	wt % of *n*-hexane soluble fraction (A)	aromatic repeating unit in A (mol %)	wt % of *n*-hexane insoluble fraction (B)	aromatic repeating unit in B (mol %)
P. putida and *P. oleovorans*	PHN/PHA(8pMPO) blend (1:1)[a]	47 ± 0.42	2 ± 0.73	53 ± 0.42	98 ± 0.88
P. putida	75 mol % 8pMPO and 25 mol % NA	12 ± 0.53	33 ± 1.02	88 ± 0.53	69 ± 1.22
P. oleovorans	50 mol % 8pMPO and 50 mol % NA	77 ± 0.51	25 ± 0.87	23 ± 0.51	43 ± 0.95
P. putida	50 mol % 8pMPO and 50 mol % NA	38 ± 0.51	23 ± 0.78	62 ± 0.51	65 ± 1.24
P. putida	25 mol % 8pMPO and 75 mol % NA	83 ± 0.48	18 ± 1.11	17 ± 0.48	50 ± 0.94

[a] Mixture of PHN and PHA(8pMPO) biosynthesized by *P. oleovorans* and *P. putida*, respectively.

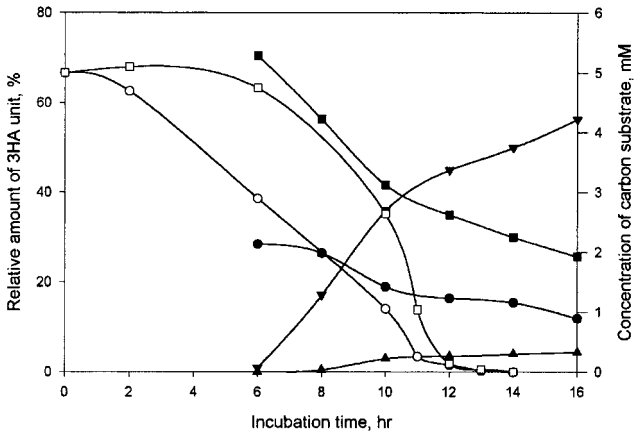

Figure 9. Amount of each repeating unit in the PHAs and the concentration of carbon substrates remaining in the growth medium at different incubation times for a *P. putida* culture grown with an equimolar mixture of 8pMPO and NA (5 mM each): NA (○); 8pMPO (□), 3HHp (●); 3HN (■); 3H4pMPB (▲); 3H6pMPH (▼).

throughout the growth, resulting in production of PHAs having similar compositions.

The fact that the compositions of *n*-hexane insoluble fraction and *n*-hexane soluble fraction obtained from PHAs biosynthesized from mixtures of 5PVA and NA did not change significantly according to the composition of the carbon substrate mixture suggested the PHA production from these mixtures might be different from the PHA production from mixtures of NA and 8pMPO.

The effect of the composition of the carbon substrate mixture on the compositions of *n*-hexane soluble and *n*-hexane insoluble fractions in PHAs obtained from mixtures of NA and 5PVA was examined by adding NA to a culture initially grown with an equimolar mixture of 5PVA and NA. Our preliminary results showed that a significant amount of 5PVA was remaining in the growth medium even when the growth reached the stationary phase. Similar results were reported for the growth of *P. oleovorans* with the same carbon substrate mixture.[8,11] The addition of NA after 13 h altered the composition of the carbon substrate. If the composition of PHA was affected by the composition of the carbon substrate, the amount of *n*-hexane soluble fraction would increase, and if the resulting polymers were still insoluble in *n*-hexane, the content of aromatic repeating unit in the *n*-hexane insoluble fraction would decrease. However, neither of such results was obtained. The relative amounts and compositions of *n*-hexane soluble and *n*-hexane insoluble fractions were similar to those obtained from the PHA biosynthesized from an equimolar mixture of 5PVA and NA. The addition of NA increased only the yields of both *n*-hexane soluble fraction and *n*-hexane insoluble fraction by approximately 1.8 times. These results showed that *P. putida* consumed the remaining 5PVA when additional NA was added, but the compositions of the PHAs were not affected by the composition of the carbon substrate mixtures. These results showed clearly that *P. putida* produced two types of random copolymers from mixtures of 5PVA and NA regardless of the composition of the carbon substrates in the growth medium. It is yet to be investigated how the two random copolymers of constant compositions were produced by this microorganism.

Conclusions

P. oleovorans and *P. putida* grown with 8pMPO produced crystalline polymers consisting of 3-hydroxy-4-(*p*-methylphenoxy)butyrate and 3-hydroxy-6-(*p*-methylphenoxy)hexanoate units. A very small amount of PHA was isolated from cells grown with 8mMPO, and no PHA was produced from cells grown with 8oMPO. PHAs produced from mixtures of 8pMPO and either NA or 5PVA were random copolymers with compositions determined by the composition of carbon substrates in the growth medium when the PHA was biosynthesized. However, the PHAs biosynthesized from microorganisms grown with mixtures of NA and 5PVA were mixtures of two copolymers that had very different compositions regardless of the composition of the carbon substrate; one fraction contained 3HPV unit more than 70%, and the other fraction contained 3HPV unit less than 10%. PHV was miscible with PHN and PHA(8pMPO), and the blends of PHPV and PHA(8pMPO) showed an unusual behavior that the T_g's of blends were lower than the T_g's of PHPV and PHA(8pMPO).

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