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Biosynthesis and thermal characterization of polyhydroxyalkanoates bearing phenyl and phenylalkyl side groups

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ABSTRACT

Polyhydroxyalkanoate (PHA) synthase 1 ($PhaC1_{Ps}$) from *Pseudomonas* sp. 61-3 is a broad-substrate-specific enzyme. In this study, by employing *Ralstonia eutropha* PHB$^-$4 recombinant expressing $PhaC1_{Ps}$, a novel type of 3-hydroxybutyrate (3HB)-based PHA copolymer, poly(3HB-*co*-3-hydroxy-3-phenylpropionate) [P(3HB-*co*-3H3PhP)], bearing phenyl side groups, was synthesized. The 3H3PhP fraction was increased up to 8.9 mol% by feeding 3H3PhP precursors. As the phenyl side group was introduced into P(3HB), the melting temperature and the enthalpy of fusion decreased, while the glass transition temperature (T_g) increased, demonstrating a distinct thermal behavior as a result of the phenyl side group. Furthermore, PHAs bearing phenylalkyl side groups were synthesized from ω-phenylalkanoates by *Pseudomonas putida* KT2440, and their thermal properties were characterized. Based on the measured and predicted T_g values, the effect of the alkyl chain length in the aromatic side group on T_g was investigated. This study demonstrates the role that aromatic side groups play in the thermal properties of PHAs.

1. Introduction

Polyhydroxyalkanoates (PHAs) are bacterial polyesters that are synthesized as intracellular storage materials of carbon and energy and are necessary for the survival of bacteria. PHAs can be used as a raw material for manufacturing biodegradable plastics [1]. The most typical PHA produced by bacteria is poly[(*R*)-3-hydroxybutyrate] [P(3HB)]; P(3HB) is a highly crystalline material with poor elasticity. Therefore, 3HB-based PHA copolymers with appropriate flexibility and ductility as a result of reduced crystallinity are preferred for practical use [1]. As a second monomer for 3HB-based PHA copolymers, a variety of side chain structures such as ethyl, propyl, and longer *n*-alkyl groups have been reported [1,2]. When these comonomers are incorporated into the P(3HB) sequence, both the melting temperature (T_m) and glass transition temperature (T_g) of the polymer tend to decrease. P(3HB) has T_m of approximately 175 °C and T_g of approximately 5 °C, whereas P(3HB-*co*-5 mol% 3-hydroxyhexanoate) [P(3HB-*co*-5 mol% 3HHx)], an example of a 3HB-based copolymer, has T_m of 138–147 °C and T_g of 0 °C [3].

It is recognized that the mechanical properties of P(3HB) and its copolymers gradually deteriorate with aging due to embrittlement by a process of secondary crystallization [4]. Crystal growth of polymers proceeds at a temperature above T_g but below T_m. Thus, PHAs with T_g higher than ambient temperature can avoid deterioration by secondary crystallization. Some reports describe the biosynthesis of PHAs having relatively high T_g (up to 49 °C) [5–11]; however, a 3HB-based copolymer with increased T_g has not been reported.

In this study, our efforts were focused on the biosynthesis of 3HB-based copolymers that exhibit increased T_g as a result of the incorporated comonomer. Generally, an increase in the size of the side group in polymers causes steric hindrance and results in an increase in T_g. However, T_g depends not only on the size of the side group, but also on its rigidity. Based on the current knowledge of structure-T_g relationships, we attempted to copolymerize an aromatic monomer, 3-hydroxy-3-phenylpropionate (3H3PhP), which has a large and rigid side group, with 3HB (Fig. 1). In addition, *Pseudomonas putida* KT2440 strains were cultured to synthesize two types of poly(3-hydroxy-ω-phenylalkanoate)s [P(3HPhA)s], poly(3-hydroxy-5-phenylvalerate) [P(3H5PhV)] and poly(3-hydroxy-4-phenylbutyrate-*co*-3-hydroxy-6-phenylhexanoate) [P(3H4PhB-*co*-3H6PhHx)] (Fig. 1), and the effect of the alkyl chain length in the aromatic side group on T_g was investigated.

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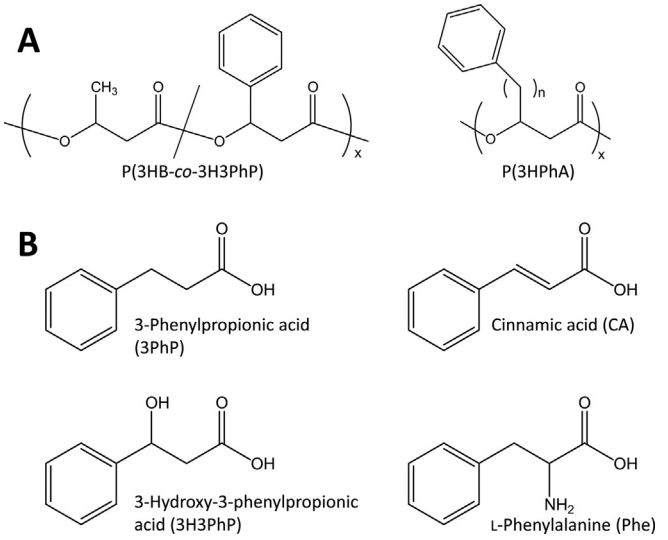

Fig. 1. Structures of the PHAs synthesized in this study (A) and the 3H3PhP precursors used in the synthesis of P(3HB-*co*-3H3PhP) (B). Methylene number (n) in poly(3-hydroxy-ω-phenylalkanoate) [P(3HPhA)] is as follows: $n = 2$, poly(3H5PhV); $n = 1$ and 3, poly(3H4PhB-*co*-3H6PhHx).

2. Materials and methods

2.1. Materials

Racemic 3-hydroxy-3-phenylpropionic acid (3H3PhP), 3-phenylpropionic acid (3PhP), cinnamic acid (CA), 5-phenylvaleric acid (5PhV), and 6-phenylhexanoic acid (6PhHx) were purchased from Tokyo Kasei, Co. Ltd. (Tokyo, Japan). L-Phenylalanine (Phe) and kanamycin were purchased from Wako Pure Chemical Industries (Osaka, Japan). Other chemicals were purchased from Kanto Chemical Co., Ltd. (Tokyo, Japan).

2.2. Bacterial strains and gene knockout

For the synthesis of P(3HB-*co*-3H3PhP), *Ralstonia eutropha* PHB$^-$4 (DSM541), which is a PHA negative mutant of *R. eutropha* H16 and is commonly used as a PHA production host, was transformed by the plasmid pBBR1″C1$_{Ps}$AB$_{Re}$ [12] carrying PHA synthase 1 gene (*phaC1*$_{Ps}$) from *Pseudomonas* sp. 61-3 and 3HB monomer supplier genes (*phaAB*$_{Re}$) from *R. eutropha* H16. Also, *P. putida* KT2440 (ATCC47054) and *P. putida* KT2440Δ*phaG* were used as host strains for P(3HPhA) production in this study. The 3-hydroxyacyl-acyl carrier protein-coenzyme A (CoA) transferase gene (*phaG*) knockout in *P. putida* KT2440 was performed by a method described by Schäfer et al. [13]. The plasmid pNS2X-*phaG*$_{Pp}$-UD carrying a knockout cassette consisting of *phaG*$_{Pp}$ upstream and downstream regions was constructed as follows: *phaG*$_{Pp}$ upstream and downstream regions were amplified by PCR using the genome DNA of *P. putida* KT2440 as a template and the primers phaG-UF (5′-CGG GCA TTT CAA CAA CAA GGT GAT C-3′), phaG-UR (5′-TGG CTA CAA GGC GCC GAG CCG CGT CAT CGA CTC CTG GCG C-3′), phaG-DF (5′-GCG CCA GGA GTC GAT GAC GCG GCT CGG CGC CTT GTA GCC A-3′), and phaG-DR (5′-GAG AGA AAC ATG CGT TGT CC-3′). By using the two obtained fragments [upstream (0.5 kb) and downstream (0.4 kb)] as templates, an upstream-downstream fragment was amplified by overlap extension PCR. The PCR product (0.9 kb) was inserted into pNS2X-sacBR1, modified pMT5071 [14], at the *Swa*I site, and the resultant plasmid was named pNS2X-*phaG*$_{Pp}$-UD. The knockout plasmid pNS2X-*phaG*$_{Pp}$-UD was introduced into *P. putida* KT2440 by conjugational transfer [15], and the *phaG*$_{Pp}$ gene was deleted by homologous recombination as described previously [14], yielding *P. putida* KT2440Δ*phaG*.

2.3. PHA biosynthesis

PHA production was performed in 100 mL or 1 L of a mineral salt (MS) medium [16] with carbon sources in a shaking flask at 30 °C for 40 h (*P. putida*) or 72 h (*R. eutropha*). For P(3HB-*co*-3H3PhP) synthesis by *R. eutropha*, fructose (20 g/L) and racemic 3H3PhP or its analogs were used (Fig. 1). Kanamycin (50 mg/L) was added to the medium to maintain the expression plasmid. For P(3HPhA) synthesis by *P. putida*, butyric acid (3.5 g/L) was used as a carbon source for cell growth, and 5PhV and 6PhHx (3 g/L) were used as 3-hydroxy-ω-phenylalkanoate precursors [9]. After cultivation, the collected cells were washed with water or a water/hexane mixture to remove the remaining carbon source and then lyophilized.

2.4. HPLC analysis

The PHA content of the dried cells and the monomer composition were determined by high-performance liquid chromatography (HPLC). Prior to HPLC analysis, the dried cells were hydrolyzed under alkaline conditions as follows: approximately 10 mg of the dried cells were hydrolyzed by 2 mL of 1 N KOH at 100 °C for 3 h, followed by neutralization with 2 mL of 1 N HCl. By this alkaline hydrolysis, PHA was converted to monomeric 2-alkenoic acids [17]. The cell hydrolyzates were filtered through a 0.45 μm syringe filter and subjected to HPLC analysis using a Shimadzu LC-10Avp system (Kyoto, Japan) with an ion-exclusion column Aminex HPX-87H (Bio-Rad, CA, USA) at 60 °C using 0.014 N H_2SO_4 with 8% CH_3CN as a mobile phase. The chromatograms were recorded at 210 nm using a UV detector.

2.5. PHA characterization

The PHA polymers in cells were extracted by stirring with chloroform for 72 h at room temperature and purified by precipitation into methanol. After recovery of the PHA polymers, they were further purified by successive reprecipitation into hexane and methanol.

The number average molecular weight (M_n) and the weight average molecular weight (M_w) were determined by gel permeation chromatography (GPC) using a Shimadzu 10A GPC system. Chloroform was used as the eluent at a flow rate of 0.8 mL/min, and each sample (1 mg/mL) was applied. Polystyrene standards with a low polydispersity were used to plot a molecular weight calibration curve.

PHA films for thermal analysis were prepared by a solvent casting method. The extracted and purified PHA was dissolved in chloroform, and the polymer solution was poured into Petri dishes. The solvent was evaporated at room temperature, and the films were aged for at least three weeks to reach equilibrium crystallinity prior to analysis. For differential scanning calorimetry (DSC) analysis, 2–3 mg of the PHA film was encapsulated in an aluminum pan and analyzed with a Perkin–Elmer Pyris 1 DSC (Perkin–Elmer, Waltham, MA, USA) in the temperature range of −50 to 200 °C at a heating rate of 20 °C/min under a nitrogen atmosphere.

The PHAs bearing phenyl and phenyl alkyl groups were analyzed by NMR spectroscopy. Each polymer (40 mg) was dissolved in $CDCl_3$ (0.7 mL) and subjected to 500 MHz ^{1}H NMR and 125 MHz ^{13}C NMR analyses. The NMR spectra were recorded using a JEOL JNM-LA500 spectrometer as previously described [12].