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Polyhydroxyalkanoate copolyesters produced by *Ralstonia eutropha* PHB^{-}4 harboring a low-substrate-specificity PHA synthase PhaC2$_{Ps}$ from *Pseudomonas stutzeri* 1317

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Abstract

Polyhydroxyalkanoate (PHA) copolymers consisting of short-chain-length (SCL) and medium-chain-length (MCL) 3-hydroxyalkanoates (3HA) were produced by recombinant *Ralstonia eutropha* PHB^{-}4 harboring a low-substrate-specificity PHA synthase PhaC2$_{Ps}$ from *Pseudomonas stutzeri* 1317. These polyesters, containing a wide range of chain length, were purified and characterized by acetone fractionation, nuclear magnetic resonance (NMR), gel-permeation chromatography (GPC), differential scanning calorimetry (DSC), Fourier transform infrared spectroscopy (FTIR), thermogravimetric analysis (TGA) and mechanical property studies. The physical properties of the copolymers are dependent largely with 3-hydroxyoctanoate (3HO) content in all PHA.

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1. Introduction

Polyhydroxyalkanoates (PHA) are biopolyesters produced by various bacteria as intracellular carbon and energy storage materials [1]. PHA have received increasing attention to replace conventional petrochemical-based plastics due to the shortage of petroleum [2–5]. Generally, PHA are classified into three groups based on the number of carbon atoms in the monomer units incorporated into the polymer chain [6,7]. Short-chain-length (SCL) PHA consists of 3-hydroxyalkanoate (3HA) monomers with 3–5 carbon atoms in length. Medium-chain-length (MCL) PHA consists of 3HA monomers with 6–14 carbon atoms in length. The SCL–MCL PHA copolymer consists of both SCL and MCL-3HA monomers, they have better application properties compared with other PHA [5].

The monomer structures and contents of PHA has considerable effects on their physical properties [3,5,8]. As a typical SCL PHA, polyhydroxybutyrate (PHB) is a stiff crystalline material and has a high melting temperature. PHB is too brittle to be processed, hence, limiting its industrial applications [3,5,9]. MCL PHA has a much lower crystallinity and higher elasticity than that of PHB while their tensile strength is low and elongation to break is high [7]. A number of studies demonstrated that a random copolymer of 3-hydroxybutyrate (3HB) and 3-hydroxyhexanoate (3HHx), abbreviated as P(3HB-*co*-3HHx) or PHBHHx, is a flexible material, and becomes softer with increasing 3HHx fraction [10–13]. A copolymer of 3HB and longer chain 3HA with very high 3HB fraction, P(94 mol% 3HB-*co*-3HA), has mechanical properties similar to low-density polyethylene (LDPE) [9]. It seems that SCL–MCL PHA have preferable mechanical properties ranging from hard crystalline to elastic, depending on the molar percentage of different monomers incorporated into the copolymers [6,8,9,12,14–18]. These superior properties of SCL–MCL PHA can extend the applications of PHA. Thus, regulation of the monomer composition in SCL–MCL PHA during the microbial production process is very important for the application.

Since PHA synthases are the key enzymes for PHA biosynthesis, to a considerable extent, the substrate specificity of the

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PHA synthases determines the composition of the accumulated PHA *in vivo*. We recently reported a PHA synthase $PhaC2_{Ps}$ from *Pseudomonas stutzeri* strain 1317 exhibiting extraordinarily low-substrate-specificity, and being capable of synthesizing SCL–MCL PHA copolymers consisting of monomers with 4–12 carbon atoms in length [19,20]. The monomer composition and content of the synthesized PHA can be effectively regulated by controlling the supply of 3-hydroxyacyl-CoA (3HA-CoA) for PHA synthase $PhaC2_{Ps}$.

In this study, a PHB negative strain of *Ralstonia eutropha* PHB^{-}4 harboring plasmid pCJY08 containing PHA synthase gene $phaC2_{Ps}$ of *P. stutzeri* 1317 accumulated SCL–MCL PHA copolymers when grown on mixed carbon sources. The PHA were purified and investigated for their physical properties.

2. Materials and methods

2.1. Bacterial strain and growth condition

R. eutropha PHB^{-}4 is a PHA-negative mutant of *R. eutropha* HI6, which was kindly provided by Professor A. Steinbüchel of Münster University (Germany). The recombinant *R. eutropha* PHB^{-}4 was prepared using plasmid pCJY08 harboring PHA synthase gene $phaC2_{Ps}$ of *P. stutzeri* 1317. Construction of this plasmid was described before [20]. The recombinant *R. eutropha* PHB^{-}4 was grown at 30 °C in Luria–Bertani (LB) medium. To maintain the stability of the plasmid, kanamycin was added to the medium at a final concentration of 50 mg l^{-1}. All liquid cultures were incubated in conical flasks at 200 rpm (NBS, Series 25D, New Brunswick, USA).

2.2. Production of PHA

The biosynthesis of PHA was carried out using a two-stage cultivation process on a rotary shaker (NBS, Series 25D, New Brunswick, USA) at 200 rpm and 30 °C in 500-ml conical flasks containing 100 ml medium. When the cells were grown on LB medium for 24 h, no PHA was detected. The biomass were harvested and transferred into a mineral salt (MS) medium [2] for another 48 h of incubation to promote PHA accumulation. Sterilized octanoate was added to the MS medium together with 20 g l^{-1} gluconate. For maintenance of plasmid pCJY08 in *R. eutropha* PHB^{-}4, kanamycin was added to the medium at a final concentration of 50 mg l^{-1}.

2.3. Gas chromatography (GC) analysis of intracellular PHA

Liquid cultures were harvested by centrifugation, the biomass were washed twice with distilled water, followed by lyophilization overnight. The lyophilized biomass was subjected to methanolysis in the presence of 15% (v/v) sulfuric acid. The resulting methyl esters of the constituent 3HAs were assayed by GC (Hewlett-Packard model 6890, Palo Alto, CA, USA) as described previously to determine the intracellular PHA content and PHA composition [15]. Gas chromatography–mass spectrometry (GC–MS) (Perkin Elmer AutoSystem XL GC-TurboMass, USA) was used to confirm the compositions of the PHA polymers.

2.4. PHA isolation and acetone fractionation

Intracellular PHA polymers were isolated from lyophilized cells by hot chloroform extraction at 100 °C for 4 h, followed by filtration through a Whitman number 1 paper filter to remove the cellular debris, the PHA dissolved in chloroform was then purified by precipitation with 10 volumes of ice-cold hexane. The purified polyester was fractionated in hot acetone to determine whether the material was a blend or a copolymer, as described by Kato et al. [15].

2.5. Chemical structure analysis of PHA polymers

^{13}C nuclear magnetic resonance (NMR) analysis was performed on PHA samples. Thirty-five milligrams of each polymer was dissolved in 1 ml of $CDCl_3$ and subjected to 125-MHz ^{13}C NMR analysis as described by Kato et al. [15]. The spectra were recorded on a Varian INOVA 500NB spectrometer.

2.6. Gel-permeation chromatography (GPC) analysis of PHA polymers

The molecular mass data for polyesters were obtained by GPC at 40 °C using a Spectra System P2000 equipped with Shimadzu HSG 60 column [21]. Chloroform was used as eluent at a flow rate of 1 ml min^{-1}, and sample concentrations of 1 mg ml^{-1} were applied. Polystyrene standards with low polydispersity were used to construct a calibration curve.

2.7. Physical property studies of PHA polymers

Differential scanning calorimetry (DSC) data were recorded in the temperature range of −100 to 200 °C under a nitrogen flow rate of 50 ml min^{-1} on a TA instruments DSC-2910 Differential Scanning Calorimeter according to the method of Doi et al. [11]. Samples (2–5 mg) were encapsulated in aluminum pans and heated from room temperature to 200 °C at a heating rate of 10 °C min^{-1}, followed by rapid quenching at −100 °C. They were then heated from −100 to 200 °C at a heating rate of 10 °C min^{-1}, during which the heat flow curves were recorded. The glass-transition temperature (T_g) was taken as the mid point of the heat capacity change. The melting temperature (T_m) and the enthalpy of fusion (ΔH_m) were determined from the DSC endotherm.

Thermal stability of PHA copolymers was determined with a TA-Q50 thermogravimetric analyzer (TGA). The temperature range was 40–400 °C in a nitrogen atmosphere. Four to five milligram samples were loaded and the heating rate was 10 °C min^{-1} each time.

Crystallinity was determined using FTIR spectroscope (Nicolet IR 200, Thermo Electron Corporation, USA). A total of 32 scans at a resolution of 4 cm^{-1} were recorded for each sample.

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