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Characterization of biosynthesized P(3HB-*co*-3HA)s swellable in organic solvents

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ABSTRACT

Polyhydroxyalkanoate (PHA) copolymers consisting of (*R*)-3-hydroxybutyrate (3HB) and medium-chain-length (*R*)-3-hydroxyalkanoate (3HA), P(3HB-*co*-3HA), are usually solved in chloroform. However, we found that some of the P(3HB-*co*-3HA) aged for more than 1 month under ambient conditions were not solved in chloroform, but instead swelled when the 3HA fraction was over 14 mol%. On the basis of differential scanning calorimetry and wide-angle x-ray diffraction analyses, we predicted that swellable P(3HB-*co*-3HA) contained numerous P(3HB) microcrystals, which may form physical crosslinks between adjacent PHA polymer chains.

1. Introduction

Polyhydroxyalkanoates (PHAs) are bio-based polyesters that can be produced from renewable carbon sources by a wide variety of microorganisms and can be used as intracellular carbon and energy storage materials. They can also be biodegraded in the environment [1]. PHAs have attracted considerable attention as biodegradable and biocompatible thermoplastics in a wide range of agricultural, marine, and medical applications [1].

The most typical bacterial PHA is poly[(*R*)-3-hydroxybutyrate] [P(3HB)], which contains a C4 repeating unit. P(3HB) is a highly crystalline material that has poor elastic quality. Due to its rigidness, P(3HB) is often hardly solved even in chloroform and tetrahydrofuran, which are good solvents for most PHAs. On the other hand, poly[(*R*)-3-hydroxyalkanotate] [P(3HA)] contains medium-chain-length 3HA repeating units (C6–14) that are elastic and less crystalline than the P(3HB). Therefore, P(3HB-*co*-3HA) copolymer has moderate elasticity and crystallinity, which is preferable for practical uses such as manufacturing of plastic materials [2,3].

We have recently established that P(3HB-*co*-3HA) can be produced with a varied 3HA fraction in the range of 4–32 mol% by employing mutants of PHA synthase from *Pseudomonas* sp. 61-3, from soybean oil as the sole carbon source with *Ralstonia eutropha* PHB^{-}4 strain as the production host [4]. During the course of characterization of P(3HB-*co*-3HA), we found that the polymer films aged for 1 month under ambient conditions sometimes swelled in chloroform. To our knowledge, no studies to date have documented the swellable PHAs in organic solvents.

In this study, we investigated the swelling behavior of the aged P(3HB-*co*-3HA) in organic solvents. Further to that investigation, the swellable PHA was characterized by nuclear magnetic resonance (NMR), scanning electron microscope (SEM), differential scanning calorimetry (DSC), and wide-angle x-ray diffraction (WAXD) analyses. This is the first report on the swellability of 3HB-based PHA copolymers.

2. Experimental

P(3HB-*co*-3HA) molecules were synthesized by recombinant *R. eutropha* PHB^{-}4 harboring the mutated PHA synthase gene from *Pseudomonas* sp. 61-3 from soybean oil as the sole carbon source, as previously reported [4]. The copolymer composition of P(3HB-*co*-3HA) varied depending on the substrate specificity of PHA synthase mutants employed for biosynthesis. The PHA composition was determined by gas chromatography (GC) after methanolysis of lyophilized cells in the presence of 15% (v/v) sulfuric acid [5]. The polymers accumulated in the cells were extracted using chloroform for 72 h at room temperature and purified by precipitation with methanol. The precipitates were solved in chloroform again and then subjected to gel permeation chromatography (GPC) to obtain molecular weight data [6]. For NMR analysis, the methanol

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Table 1
Properties of P(3HB-*co*-3HA) used in this study.

Solvent cast film (Synthase mutant used)		P(3HB-*co*-3HA) composition (mol%)					Molecular weight		Appearance of solvent cast film	Swelling ratio[a] (cm^3/g)
		3HB	3HA				Mn ($\times 10^3$)	Mw/Mn		
			3HHx	3HO	3HD	3HDD				
PHB		100	0	0	0	0	240	2.4	Translucent	–[b]
PHBA6	(S477V)	94	3	3	0	0	85	2.2	Translucent	–[b]
PHBA14	(E130L)	86	4	6	4	0	84	1.3	Opaque white	14.0
PHBA17	(E130V)	83	4	7	5	1	91	1.3	Opaque white	17.9
PHBA19	(S477D)	81	4	9	6	0	62	1.5	Opaque white	18.8
PHBA20	(Q481W)	80	4	8	7	1	50	2.0	Opaque white	19.0

3HB: 3-hydroxybutyrate (C4), 3HHx: 3-hydroxyhexanoate (C6), 3HO: 3-hydroxyoctanoate (C8), 3HD: 3-hydroxydecanoate (C10), 3HDD: 3-hydroxydodecanoate (C12).
[a] Swelling ratio when immersed in chloroform.
[b] Solved in chloroform.

precipitates were solved in deuterated chloroform and the molecular structures of P(3HB-*co*-3HA) were determined using a JEOL LA500 spectrometer [7].

The P(3HB-*co*-3HA) films were prepared by solution casting from their chloroform solutions using glass Petri dishes as casting surfaces, allowing the solvent to evaporate and drying at room temperature to remove the residual solvent. The films were aged for 1 month under ambient conditions.

The wide-angle x-ray diffraction patterns of P(3HB-*co*-3HA) films were recorded at 23 °C on a Rigaku RINT2500 system using nickel-filtered Cu Kα radiation operated at 40 kV and 200 mA. The scan was carried out in the 2θ range of 6–60° at a scan speed of 2.0°/min.

For a cross-section observation of P(3HB-*co*-3HA) films, the samples were immersed in liquid nitrogen for a few minutes before cutting to prevent deformation of the cross-section. After coating the samples with Au, imaging was performed with a JEOL JSM-6330F scanning electron microscope operated at an acceleration voltage of 5 kV at room temperature.

3. Results and discussion

3.1. Preparation and appearance of P(3HB-co-3HA) solvent cast films

The P(3HB-*co*-3HA) samples prepared in this study are listed in Table 1. The copolymer composition ranged from 6 to 20 mol% by employing various mutants of *Pseudomonas* sp. 61-3 PHA synthase. After the isolation of the PHAs from the cells, flesh PHAs solved in chloroform were cast on a flat petri dish and air-dried gradually at room temperature to yield solvent cast films of PHA. After that, the films aged for more than 1 month at room temperature. Some of the resultant P(3HB-*co*-3HA) films were not solved in chloroform, but they swelled once they aged.

The appearance of dried solvent cast films were translucent when the 3HA fraction was under 6 mol% and opaque white when it was over 14 mol%. The PHBA14 film (14 mol% 3HA fraction), which resembles cut white paper, is shown in Fig. 1A as an example. SEM analysis was performed to observe the microstructure of PHBA14. Fig. 2 shows micrographs of the surfaces and cross-sections of PHBA14 films at ×500 and ×3000 magnifications. At the surface, there were many voids of approximately 10 μm width. The same voids were observed in the cross-sectional images. These are thought to be formed as vents of evaporating solvents during the formation of the cast film, giving a porous structure and opaque white appearance by a random scattering of light. Elimination of the voids by annealing at 120 °C for 30 s made the PHBA14 film transparent. Thus, the film appearances may be differentiated by the behavior of solvent evaporation in each of the PHA samples, which is mainly dependent on copolymer composition.

3.2. Swellability of P(3HB-co-3HA) films in organic solvents

Fig. 1B shows an aged PHBA14 film after immersion in chloroform for 1 min. The film absorbed chloroform quickly as soon as it was immersed. The swelling ratio (in cm^3/g) was determined according to the formula:

$$\text{Swelling ratio} = (\text{Ws} - \text{Wd})/(\text{Wd} \times 1.48),$$

where Ws and Wd denote the weights of swollen and dry film samples (in g), respectively, and 1.48 is the density of chloroform

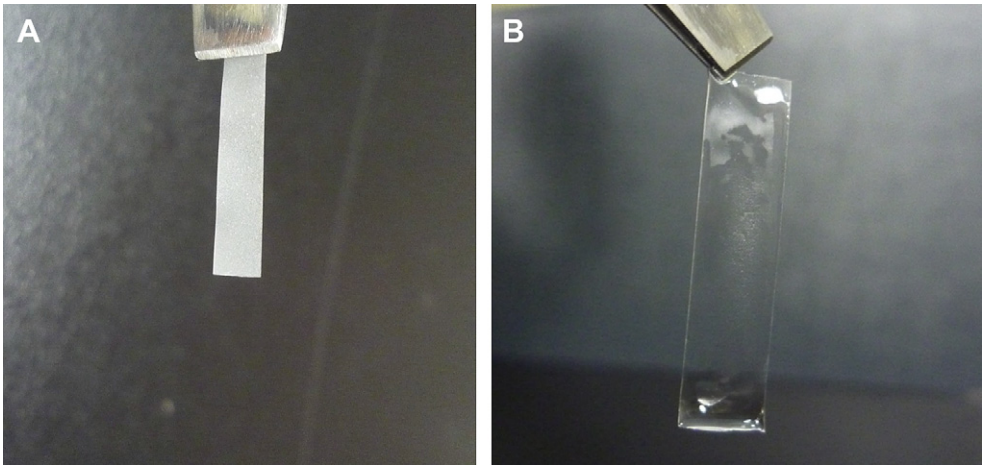

Fig. 1. PHBA14 solvent cast film (A) before and (B) after immersion in chloroform for 1 min.